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Development and Performance Evaluation of an Antibody-Based Technology for Detection of E. coli O157 in Meat Samples and Its Potential Evolution Using Antibody Engineering

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Abstract

Escherichia coli O157 is a persistent pathogen linked to food and waterborne infectious outbreaks with severe health consequences such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Because it is considered one of the major pathogens that contributes to the global burden of foodborne disease, its early detection within the food chain is an important milestone towards reducing foodborne diseases and economic losses due to contaminated food. Herein, the development and validation of a lateral flow immunoassay (LFIA) point-of-care (POC) device is described. Application of the LFIA test kit was focused on detection of *E. coli* O157 in raw meat products due to the fact that ground beef has been one of the major food items implicated in *E. coli* outbreaks and recalls within Canada. Moreover, the LFIA Test Kit was subjected to an independent validation study based upon Health Canada's guidelines for the validation of alternative microbiological methods as established in the Compendium of Analytical Methods. The protocol comprised a pre-collaborative study, where the LFIA Test Kit was compared against the reference culture method, MFHPB-10, using eight different raw meat products following an unpaired samples experimental layout. The results demonstrated that the newly developed LFIA Test Kit exceeds the performance parameters criteria established by the Microbiological Methods Committee (MMC), thus suggesting that the LFIA Test Kit represents a reliable alternative for meat producers in order to obtain presumptive presence/absence results in less than one day. The design and expression of a single-chain variable fragment (scFv) targeting *E. coli* O157 is also presented. Recombinant antibody fragments such as scFv have not been extensively exploited within food safety diagnostics, especially for pathogen detection. Thus, in this project the anti-O157 mouse monoclonal antibody (mAb) used as the detection reagent in the LFIA Test Kit was genetically sequenced prior to bioengineering a scFv that could potentially be used to improve the performance of the LFIA Test Kit.

Keywords

Escherichia coli O157, pathogen detection, food safety, food microbiology, pre-collaborative study, rapid method, lateral flow immunoassay, monoclonal antibody, single-chain variable fragment, recombinant antibody

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List of Abbreviation

°C	degrees Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
µTAS	micro total analysis systems
×g	times gravity
AC	alternative confirmation result
A/E	attachment and effacing
AF	alternative final result
AIDS	acquired immunodeficiency syndrome
AM	alternative method
AOAC	Association of Analytical Communities
AP	alternative presumptive result
APC	aerobic plate count
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual, FDA
BHI	brain heart infusion
bp	base pair
BPW	buffered peptone water
BSA	bovine serum albumin
C_H	constant region of the heavy chain
C_L	constant region of the light kappa chain
CDC	Centers for Disease Control and Prevention, USA
cDNA	complementary DNA
CDR	complementarity determining region
CFIA	Canadian Food Inspection Agency
CFU	colony forming unit
CGC	colloidal gold conjugate secondary antibody
cm	centimeter

CO_2	carbon dioxide
COBALT	constraint-based multiple alignment tool
CR-SMAC	cefixime rhamnose sorbitol MacConkey
CT-SMAC	cefixime tellurite sorbitol MacConkey
d	day
Da	dalton
DAEC	diffusely adherent *Escherichia coli*
DALY	disability-adjusted life year
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
eae	*E. coli* attaching and effacing gene
EAEC	enteroaggregative *Escherichia coli*
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *Escherichia coli*
EtBr	ethidium bromide
ETEC	enterotoxigenic *Escherichia coli*
Fab	antigen binding site
FBS	fetal bovine serum
Fc	crystallizable fragment
FDA	Food and Drug Administration, USA
FERG	Foodborne Disease Burden Epidemiology Reference Group
FITC	fluorescein isothiocyanate
FN	false negative
FP	false positive
FR	framework region
FSIS	Food Safety and Inspection Service
g	gram
GMPs	Good Agricultural Practices

GMQE	global model quality estimation
h	hour
HACCP	Hazard Analysis and Critical Control Point
HCl	chlorhydric acid
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HUS	hemolytic uremic syndrome
IEF	isoelectric focusing
Ig	immunoglobulin
IgG	immunoglobulin G
IMS	immunomagnetic separation
IPTG	isopropyl-D-thiogalactopyranoside
ISO	International Standardization Organization
kb	kilo-base
kDa	kilodalton
kg	kilogram
L	liter
LAMP	loop-mediated isothermal amplification
LB	luria broth
LCL	lower confidence limit
LEE	locus of enterocyte effacement
LFIA	lateral flow immunoassay
LOD	limit of detection
LPS	lipopolysaccharide
M	molar
MFHPB	Microbiology Food Health Protection Branch
MFLP	Laboratory Procedure for the microbiological analysis of food
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter

MLG	Microbiology Laboratory Guidebook, USDA FSIS
mm	millimeter
mm^2	square millimeter
mM	millimolar
mAb	monoclonal antibody
MMC	Microbiological Methods Committee
MPN	most probable number
MSA	multiple sequence alignment
mTSB	modified tryptic soy broth
mTSBN	modified tryptic soy broth with novobiocin
MWCO	molecular weight cut-off
N	novobiocin
NaCl	sodium chloride
NAAT	nucleic acid amplification test
NALF(IA)	nucleic acid lateral flow (immunoassay)
NASBA	nucleic acid sequence based amplification
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometer
NMWL	nominal molecular weight limit
OD	optical density
O/N	overnight
pAb	polyclonal antibody
PHAC	Public Health Agency of Canada
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
PDB	Protein Database
PEG	polyethylene glycol
pI	isoelectric point
PIGS	prediction of immunoglobulin structure
POC	point-of-care

POD	probability of detection
PVA	polyvinyl acetate
PVP	polyvinylpyrrolidone
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
RBSS	rehydration buffer stock solution
RM	reference method
RMSD	root-mean-square deviations
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RU	reflective units
s	second
scFv	single-chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SERS	surface enhanced Raman spectroscopy
SFM	serum free medium
SIB	Swiss Institute of Bioinformatics
SPR	surface plasmon resonance
Stx	Shiga toxin
STEC	Shiga toxin-producing *Escherichia coli*
TBE	tris/borate/EDTA buffer
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TCA	trichloroacetic acid
TE	Tris-EDTA
TEV	tobacco etch virus
TMB	3,3',5,5'-tetramethylbenzidine

TN	true negative
TP	true positive
TrxA	thioredoxin
TSA	tryptic soy agar
TSAYE	tryptic soy agar with yeast extract
TSB	tryptic soy broth
TSBYE	tryptic soy broth with yeast extract
TSBN	tryptic soy broth with novobiocin
TVC	total viable count
U	units
UCL	upper confidence limit
USDA	United States Department of Agriculture
UV	ultraviolet light
V	volts
V_H	variable heavy chain
V_L	variable light chain
Vh	volt hours
VTEC	verotoxigenic *Escherichia coli*
v/v	volume/volume
WB	western blotting
WHO	World Health Organization
w/v	weight volume

CHAPTER 1 INTRODUCTION

1.1. General Overview of Food Safety

1.1.1 Food Safety

The World Health Organization (WHO) defines food safety as a set of actions necessary to ensure that all food is as safe as possible throughout the production chain (1). Moreover, it is considered a multidisciplinary activity that requires full integration of a broad spectrum of disciplines, from technological to legal, while requiring the engagement of the different stakeholders within the food supply chain in order to create a successful food safety management system. Borchers *et al.* defined food safety as a "reasonable certainty of no harm" because it is impossible, from a feasible and economic perspective, to ensure with absolute certainty that all food will be safe (2). In addition, food safety can be considered as an intrinsic attribute that refers to the absence of hazards with an acceptable risk (3).

In the last few decades, food safety has been gaining more attention as a global health issue due to the huge impact that foodborne illness is having on public health and socio-economic development (4). Main concerns involve the emergence and/or redistribution of microbial and chemical hazards (e.g. mycotoxins), especially due to extreme weather conditions (5–7) and the increase in global food trade (7,8). Safe food and water supplies are relevant components of a healthy environment; therefore any new threats caused by the world's evolution and dynamics can alter the agro-food production chain. The latest estimations from WHO indicate that >200 different diseases, including diarrhea and cancer, are linked to consumption of unsafe food (9), causing approximately 1 in 10 people worldwide to become ill and 420,000 to die annually, representing 33 million disability-adjusted life years (DALYs)[1] lost due to consumption of unsafe food (10). Specifically within Canada, approximately 3,000 food safety investigations are carried out each year, resulting in almost 250 recalls with an estimate of 4 million cases of food-related illness reported annually (11).

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[1] The disability-adjusted life year (DALY) measure was developed by the WHO to summarize the years of healthy life lost due to an acute illness and sequelae because it includes data on premature mortality and morbidity (58).

In fact, in 2000 food safety was recognized as an essential public health function by the Member States of the WHO (1). More recently, in April 2015, the WHO official World Health Day was dedicated to raising awareness about food safety and to highlight the impact of food safety on public health (9). At the national level, governments have implemented new programs and initiatives with the aim of increasing food safety awareness among all parties involved in food production, from farm-to-table and from governments-to-consumers. As an example, in 2010 the Healthy People 2020 initiative was launched in the USA. Included as one of its main goals is the reduction of foodborne diseases by improving food safety measures (12). Meanwhile in Canada, the Safe Food for Canadians Action Plan came into force in 2015. Among the activities included in this Action Plan are strengthening and developing food safety rules to update the Canadian food safety system and to better protect Canadians from food safety risks as a result (13).

1.1.1.1 Microbiological food safety

The WHO recently published the latest burden of foodborne illnesses, where a total of 600 million cases and 418,000 deaths worldwide were estimated in 2010 (4). Interestingly, more than 50% of these estimates (360 million cases and 273,000 deaths) were linked to bacterial agents (4), emphasizing their prominent role as food safety hazards. In addition, the latest data from the Centers for Disease Control and Prevention (CDC), estimated that 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths occurred annually in the USA due to known foodborne pathogens (2). The CDC identified seven bacterial pathogens and two parasites as the major causes of foodborne illnesses with an incidence (in cases per 100,000 people, in 2008) of 12.68 for *Campylobacter*, 16.2 for *Salmonella*, 6.59 for *Shigella*, 2.25 for *Cryptosporidium*, 1.12 for *Escherichia coli* O157, and <1 for the other 4 pathogens (*Listeria*, *Vibrio*, *Yersinia*, *Cyclospora*) (2). In Canada, 1.6 million cases of domestically acquired foodborne disease happen annually caused by 30 known pathogens (14). Based on these data, the first Canadian report of hospitalizations and deaths due to foodborne diseases estimated that 3,943 hospitalizations and 105 deaths are caused every year, from which 66% and 76%, respectively, are due to bacterial pathogens (15). Of note, the top bacterial pathogens found to cause most of the foodborne illness cases are *C. perfringens*, *Campylobacter* spp. and nontyphoidal *Salmonella* spp., in this order (14).

These differ from those considered to be major contributors to the number of hospitalizations (nontyphoidal *Salmonella* spp., *Campylobacter* spp. and verotoxigenic *E. coli* O157 (VTEC O157), in order) and deaths (*L. monocytogenes*, nontyphoidal *Salmonella* spp., and VTEC O157, in order), suggesting that the latter tend to have more severe outcomes (15). The statistics presented showed that foodborne pathogens result in considerable morbidity and mortality. Unlike toxic agents, foodborne pathogens can enter at almost any stage during the food production chain. In addition, bacterial pathogens can easily reach a new host using food as a vehicle because they can adapt, survive and/or grow within the food chain (8,16). Moreover, foodborne pathogens are dynamic and their effects are difficult to predict; they are constantly evolving into new, resistant strains and emerging in unusual food commodities (8). For this reason, microbiological food safety requires different approaches and strategies than food toxicity, in order to counteract the challenges that microorganisms represent to food safety (16). Specifically within Canada, the Safe Food for Canadians Framework is expected to establish effective prevention and control measures targeting the pathogens responsible for the greatest burden of disease and most severe illness. Focusing on microbial food safety, such measures include more stringent controls and testing requirements for pathogens like *E. coli* and *Listeria*, better tracing systems and compliance verification (11).

1.2. *Escherichia coli* O157 and Its Role in Food Safety

1.2.1 General Overview of Pathogenic *E. coli*

Through the years, *Escherichia coli* spp., a Gram negative facultative anaerobe, has been one of the most studied microorganisms. Although it is a typical resident of the human and animal intestinal tracts (17–19), several strains have acquired specific virulence factors that are known to cause diseases to either humans or animals (20–22). Based on the virulence mechanism(s) they use to interact with eukaryotic cells, these pathogenic strains have been classified into pathotypes, six of which have been identified as diarrheagenic: enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (20,22–24). EPEC and ETEC most commonly cause diarrhea in infants and mostly frequently in developing countries (20,24). On the other hand, EIEC,

which causes dysentery and bloody diarrhea, is an intracellular pathotype that invades and replicates within colonic epithelial cells (20,23). In fact, EIEC is the only pathotype that does not adhere to the epithelial cells using their fimbriae or pili, as the rest of the pathogenic *E. coli* do (22). EAEC and ETEC are known to be major causes of traveler's diarrhea. In addition, the EAEC mechanism of pathogenicity relies on bacterial cell stacking attachment to enterocytes from either the large or the small bowel, forming "bricked wall" biofilms on the cell surface (20,22,25). DAEC is a relatively new pathotype, which requires attachment to eukaryotic cells, but through the formation of finger-like cellular projections that engulf the bacterial cell (20,23,26). All five of these pathotypes are relevant to public health due to their potential to cause disease through consumption of contaminated food and water. Much research has focused on understanding the EHEC pathotype that has been in the spotlight since the first serotype, *E. coli* O157:H7 was discovered in 1982 during an outbreak related to contaminated ground beef hamburgers (21,27,28). Since then, *E. coli* O157 has been persistently linked to food and waterborne outbreaks with severe health consequences including hemorrhagic colitis and hemolytic-uremic syndrome (HUS). In addition, several studies have reported that *E. coli* O157 has an extremely low infectious dose (<100 cells) (20,26,29,30), thermal resistance above normal ground beef cooking temperature (71°C) (29,31,32) and acid resistance that allows survival in environments with low pH (33–36). These features, together with the production of one or both of the potent Shiga toxins, Stx1 and Stx2, (18,37,38) have made *E. coli* O157 a major foodborne pathogen that requires sensitive and precise surveillance coupled with control measures to counteract its public health and economic effects.

1.2.2 Main Reservoirs and Transmission

Through the years, the primary reservoir of *E. coli* O157 was shown to be the gut of ruminants (39,40), most frequently cattle, sheep, and goats (40,41). However, cattle are considered the major source of *E. coli* O157 (31,39,40) mainly due to the consumption of beef, which can become contaminated during slaughtering through contact of the carcass with hides contaminated with feces (27). In fact, from all food categories, ground beef has been consistently considered as the main cause of human EHEC infections (27,42,43). Interestingly, cattle remain asymptomatic while carrying and shedding the pathogen in

their feces (43–45) at a typical range of 10 to 100 CFU/g of feces (44). Normally, *E. coli* O157 is found at the terminal end of the colon, although colonization has been shown to be more prominent at the recto-anal junction (44,46). This has led to the hypothesis that some cattle, called "supershedders", can excrete higher levels, $>10^4$ CFU/g of feces, of *E. coli* O157 (18,44,46,47). Although they represent less than 10% of the total cattle, studies have shown that "supershedders" might be responsible for 99% of the *E. coli* O157 environmental contamination (44). This represents a major risk factor for humans because *E. coli* O157 is known to remain viable in feces, soil, and water for extended periods of time (40,46). For example, contamination of produce fields directly with feces or indirectly through contaminated water has been traced as the potential cause of the increasing number of *E. coli* O157 outbreaks linked to leafy green vegetables (45,48).

Focusing on North America, two recent studies estimated that 65-68% of *E. coli* O157 infections in the USA are transmitted by food products (27,43). In fact, many of the recent outbreaks are linked to leafy vegetables, which, together with beef, caused >25% of the *E. coli* O157 outbreaks and >40% of the illnesses reported in the 2003-2012 period in the USA (27). In agreement with USA findings, the most recent source attribution of enteric illness data in Canada estimated that foodborne transmission is still considered the main route (49). However, these estimations were obtained through an expert elicitation rather than an outbreak data analysis and were based on the analysis of a broad range of transmission routes, including water, person-to-person and animal contact in addition to food. Concerning this, *E. coli* O157 outbreaks are also linked to additional minor transmission routes besides contaminated food (2,27,43,44), such as water (2,27,43,44), direct contact with infected animals (27,43,44) or their environment (27,43) (**Figure 1A**). Moreover, person-to-person contact and fomite are also possible sources (27,43), although from 1982-2002 it only accounted for 14% of *E. coli* O157 outbreaks, mainly in child daycare centers (43); while from 2003-2012 it represented only 10% of the outbreaks identified in the USA (27).

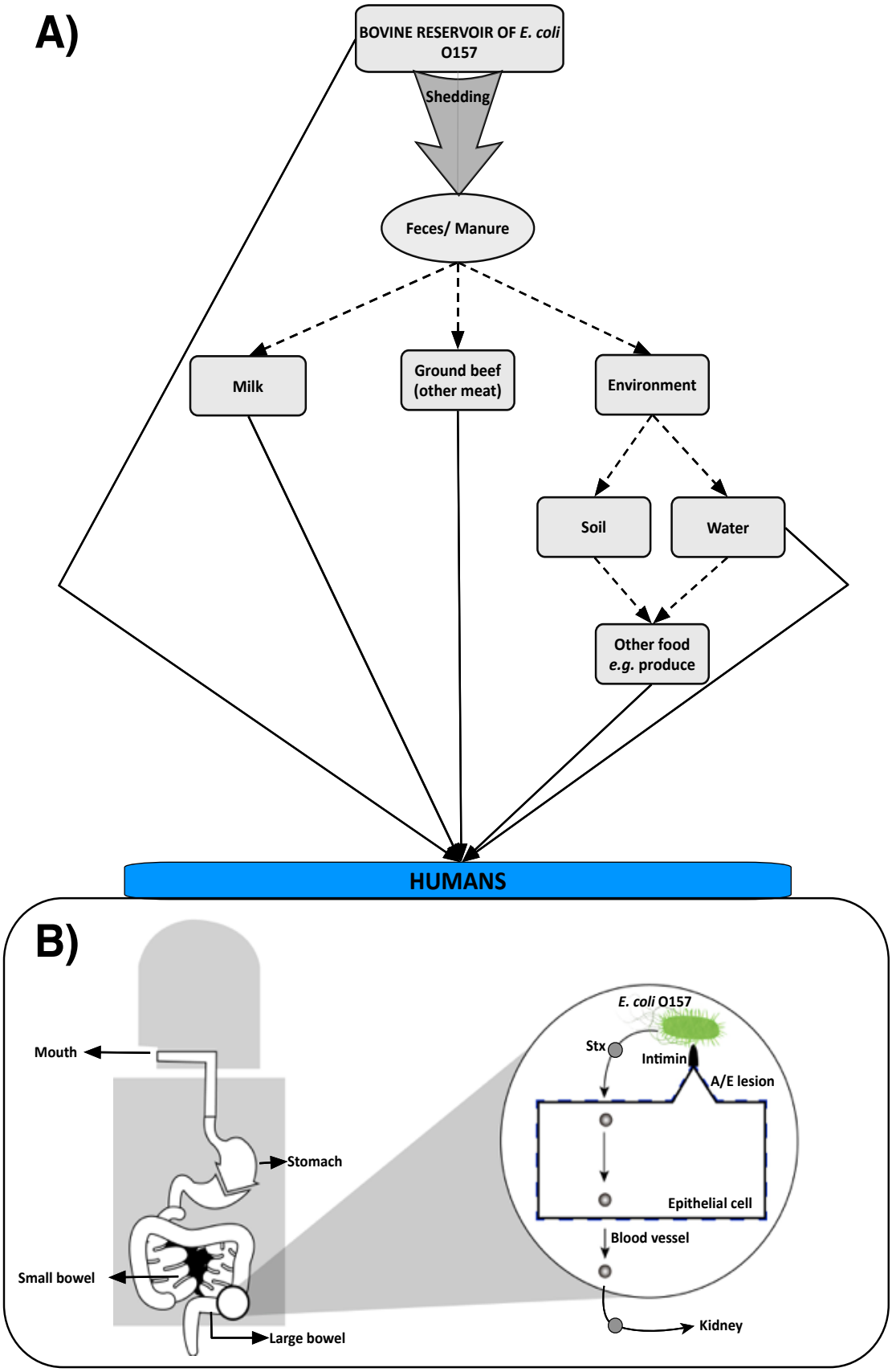
A different study, using a historical analysis of outbreaks from 1976 through 2005 estimated that 37% of the *E. coli* infections were linked to beef while 23% and 11% were related to cooked multi-ingredient dishes and other types of meat except beef, respectively

(50). This study provided the first extensive analysis of illness attributions from Canadian foodborne outbreaks however, it did not reflect the emerging impact of fresh produce. In light of the high consumption rate of fresh fruits and vegetables in Canada, a recent report gathered data from 27 produce-related outbreaks occurring from 2001 through 2009 and estimated that 66% of these were caused by bacteria, of which 33% were attributed to *E. coli* O157 (51). These data show that, together with beef, fresh produce is becoming a major source of *E. coli* O157 infections (51).

1.2.3 Mechanisms of Pathogenesis

E. coli O157 is considered a zoonotic foodborne pathogen of major public health concern and is one of the predominant pathogens in the etiology of gastrointestinal diseases. Infection of humans occurs after ingestion of contaminated food or water. Due to its acid resistance, *E. coli* O157 is able to overcome the acidic environment of the stomach and colonize the intestine, the early stage of the infection (18). It is believed that attachment to epithelial cells occurs in the colon and distal small intestine (22,52) through the formation of an attachment and effacing (A/E) lesion, which is frequently related to the development of bloody diarrhea and HUS (18). EHEC possesses a pathogenicity island called the locus of enterocyte effacement (LEE), which contains the genetic information that encodes for the synthesis of all proteins necessary for the A/E lesion (18,20). Intimin, which is a product of the *eae* (*E. coli* attaching and effacing) gene, is the outer membrane protein involved in the A/E lesion and is responsible for the adherence of *E. coli* O157 to the surface of the epithelial cells, triggering structural changes, such as loss of microvilli and pedestal formation (18,21,42) (**Figure 1B**). Therefore, the A/E lesion is a localized effect, whereas systemic complications arise due to the release of Stxs (18,37). The presence of the LEE virulence factor distinguishes EHEC strains from other Shiga toxin producing *E. coli* (STEC) strains. Therefore, EHEC, including *E. coli* O157, is considered a subset of the STEC group, which is known to produce hemorrhagic colitis and HUS due to the presence of the LEE and expression of Stxs (21,52).

Figure 1. Overview of the routes of transmission and human disease due to *E. coli* O157. A) The main source of *E. coli* O157 (cattle) is represented together with the major routes of transmission to humans: a) direct contact with cattle, b) consumption of contaminated food from bovine origin like milk and/or meat or c) through environmental contamination by feces that can be passed on to water or soil and thus, spread contamination to other food products. **B)** Once *E. coli* O157 is ingested by humans, it survives passage through the gastrointestinal tract until it reaches the colon, where it adheres to the epithelial cells through the membrane protein intimin. The latter is required for the formation of the characteristic A/E pedestal lesion and triggers the release of Stxs. The Stxs are then absorbed into blood vessels where it can travel to the kidneys, a major organ site for *E. coli* O157 infection. Images were modified from Gyles (18) and Croxen *et al.* (22).


A)
BOVINE RESERVOIR OF E. coli O157
Shedding
Feces/ Manure
Milk
Ground beef (other meat)
Environment
Soil
Water
Other food e.g. produce
HUMANS
B)
Mouth
Stomach
Small bowel
Large bowel
E. coli O157
Stx
Intimin
A/E lesion
Epithelial cell
Blood vessel
Kidney

Stxs are the other major virulence factor that *E. coli* O157 possesses. They are classified into two main groups, Stx1 and Stx2, based on their antigenicity (53). *E. coli* O157 can produce both or only one type of Stx (21,42,54). Although Stx1 is structurally identical to the toxin produced by *S. dysenteriae* type 1, some studies have shown that Stx2 is 1,000 times more toxic for renal cells thus Stx2 is commonly associated with the pathogenesis of HUS (18,21,42,54). Once the A/E lesion is formed, Stxs are released and bound to their receptor within epithelial cells (18), triggering the uptake of the toxin by endocytosis (18,21). It is after the onset of hemorrhagic colitis that Stxs enter the blood, leading to renal failure associated with HUS (53) (**Figure 1B**). Stxs will produce damage to the vascular endothelial cells in the glomeruli and arterioles of the kidney, triggering clotting and clogging resulting in accumulation of waste in the blood (20,21), Meanwhile in the intestine, Stx is also involved in the development of bloody diarrhea, hemorrhagic colitis, and induction of apoptosis and necrosis of the epithelial intestinal cells resulting in perforation (20,22).

Signs and symptoms may appear within an incubation period of 3-8 days (2,21), progressing from stomach cramps (2,27,54), watery diarrhea (52,54,55), bloody diarrhea (2,27,52,54) and vomit (2,27) to hemorrhagic colitis (52), renal failure (56) and/or HUS (27,52,56). However, the severity of the disease depends on complex mechanisms that interplay between regulation of the expression of virulence features and host factors (57). In fact, the role of other virulence factors can be as relevant as the LEE island, because atypical EHEC strains lacking the LEE have occasionally been shown to produce HUS (20,42,54). Additional virulence factors include hemolysin, a toxin that can contribute to the disruption of erythrocytes (18,21,57), O157 lipopolysaccharide that has a proinflammatory effect (53), and secreted proteins that aid in the formation of the A/E lesion (21,42), among many others.

Recovery from the disease can take 8-10 days (2). Generally, infants, young children, the elderly and the immunocompromised are more susceptible. In fact, 5-15% of patients may develop HUS, especially young children (53–55). HUS involves acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (20,21,54) with a fatality rate

of 3-5% (2,21). Thus far, there is no treatment for *E. coli* O157 infections, other than monitoring the illness, providing comfort, and preventing dehydration through proper hydration and nutrition. In addition, patients who develop complications may need further treatment, such as dialysis, to treat kidney failure (54). Although treatments have been developed, it has been shown that some *E. coli* O157 strains can increase the production of Stxs when exposed to antibiotics such as ampicillin, tetracycline or norfloxacin (55). On the other hand, some approaches have focused on preventing the release of Stxs during the diarrheal phase to decrease further damage, however results lacked efficacy (37). Therefore, current research is focused on improving the prevention of infections by adopting measures throughout the food chain to reduce the risk of transmission of *E. coli* O157 to humans.

1.2.4 Epidemiology and Economic Burden of *E. coli* O157 Infections

In 2015, several published national, regional and global assessments of the burden of foodborne disease described *E. coli* O157 as one of the foodborne pathogens contributing to the overall burden of foodborne disease (4,15,27,58,59). The global initiative launched by WHO, namely the Foodborne Disease Burden Epidemiology Reference Group (FERG), estimated that STEC infections accounted for 1 million incidents, causing 128 deaths and loss of 13,000 DALYs in 2010, worldwide (4). A 2014 study of the global incidence of STEC infections estimated 2,801,000 acute illnesses, 3890 cases of HUS, 270 cases of permanent end-stage renal disease and 230 deaths annually (56). Of note, global estimates grouped STEC O157 and non-O157 together, however findings suggested that sequelae and fatalities were higher with STEC O157 cases (56). On the contrary, the number of STEC cases was below those attributed to other foodborne pathogens including typhoid fever and nontyphoidal salmonellosis nevertheless, the relevance of STEC infections relies on the severe sequelae that follow infection (56). Frenzen *et al.* reported that in the USA, medical care and loss of productivity due to STEC O157 cost approximately $30 million and $5 million in the USA annually, respectively (60). Moreover, an average of $635 million US was estimated to be the total economic cost of 63,000 annual cases of STEC O157 caused foodborne illness, according to Scharff (61). On the other hand, in Canada, Sockett *et al.* estimated that long-term outcomes cost $377 million CAD annually for

approximately 37,800 on-going cases, while new primary cases had a cost of illness of $26.7 million CAD (22,300 cases annually), adding up to a total annual cost of illness of $403 million CAD (62). Despite these estimates being dramatic, they do not represent the true total cost of *E. coli* O157 illness because they mainly focus on medical and productivity losses, which ignore industrial and/or government costs (i.e. cost due to recalls) (61,62).

1.2.5 *E. coli* O157 Outbreaks, Recalls and Regulatory Aspects

The first *E. coli* O157 outbreak ever reported was in 1982 during an investigation of hemorrhagic colitis linked to contaminated hamburgers (28,63). However, in 1992 the Jack in the Box *E. coli* O157 outbreak, the largest outbreak ever recorded up to that time, caused 502 illness cases from which 31% were hospitalized, 9% developed HUS and 0.6% died (64,65). Further investigations found that improper processing and cooking of hamburgers were the main causes, which stimulated the establishment of regulatory measures to reduce meat contamination and public health actions to educate consumers about proper meat handling (64,65). In fact, this outbreak is considered a breakthrough in the evolution of food safety, especially for the control of *E. coli* O157 in food products. Indeed, in 1994 the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) established that *E. coli* O157 was to be considered an adulterant in beef, prohibiting the sale of contaminated meat and, subsequently, starting programs for its detection (66–68). Other measures that were taken to prevent outbreaks were as follows: 1) establishment of new cooking temperature guidelines in the Model Food Code for restaurants by the US Food and Drug Administration (FDA) (63,67); and 2) development of objective measures of meat doneness suggested by the National Livestock and Meat Board's Blue Ribbon Task Force in the USA (63,67). More recently, the USDA-FSIS determined that *E. coli* O157 was more prevalent than initially thought, and thus stricter regulations that included testing of hides and pre-eviscerated carcasses were recommended (69). These modifications to the testing programs influenced members of the beef industry positively, which was reflected in declining numbers of positive *E. coli* O157 beef samples and thus in the incidence of infection cases in 2002 (43).

Meanwhile in Canada, in 1990, STEC (including *E. coli* O157) became a notifiable disease, requiring the Public Health Agency of Canada (PHAC) to report any cases (66). Recently, the Canadian "Guidance Document on *Escherichia coli* O157:H7 and *E. coli* O157:NM in Raw Beef" was released. This document provides better recommendations, focusing on Good Agricultural Practices (GMPs) and Hazard Analysis and Critical Control Point (HACCP) programs, aiming to enhance the verification and control to minimize the prevalence of *E. coli* O157 in raw beef products (70). Due to the nature of raw beef, it possesses a high risk for contamination with *E. coli* O157 either during slaughtering or further processing and/or packaging. In addition, according to Statistics Canada and the Canadian Meat Council, beef continues to be the meat with the highest per capita consumption of 12 kg (boneless weight) annually (71). Therefore, in pursuit of minimizing the risk that this highly-consumed product represents to Canadians, Health Canada established that both precursor materials and finished raw ground beef products and beef products processed for raw consumption should not contain detectable levels of *E. coli* O157 (70).

Despite the improvement of food safety practices, including creation and implementation of more stringent regulations, outbreaks are still occurring sporadically around the world. Of note, outbreaks not only have a huge health impact, but they also represent an economic burden for food manufacturers. In 2007, Topps Meat Company recalled approximately 21.7 million pounds of frozen ground beef patties (72), which caused the company to leave the market shortly afterward (68). Moreover, global food trade means greater responsibility towards ensuring food safety across national borders because international distribution scales up the effect of potential outbreaks. Indeed, cases such as the XL Foods Inc. recall in 2012 proved the tremendous impact not only to the company, but also to our country's beef industry. XL Foods Inc. exported products to over 20 countries; thus the safety of Canadian beef was questioned after the recall, not only within Canada but worldwide. This lead to an estimated cost to the Canadian beef industry between $16 million and $27 million CAD (73). Not surprising, this recall is considered the biggest recall in Canada. Some of the major outbreaks in North America are presented in **Table 1**. Although beef products continue to be the main source of *E. coli* O157 outbreaks and recalls in Canada and the USA (27,43,50,74), it is evident that fresh produce is becoming just as relevant in the

Table 1. Major *E. coli* O157 foodborne outbreaks in the USA and Canada, 2006-2016.

Year	Implicated food	No. of cases				Country	Product Recall (lbs product)	Reference
		Total	Hospitalizations	HUS	Deaths			
2016	Jack & The Green Sprout's alfalfa sprouts	11	2	0	0	USA	NR	(72)
2015	Not identified	31	7	NR	0	Canada	No	(75)
2015	Leafy greens	12	NR	NR	NR	Canada	NR	(75)
2015	Taylor Farm Pacific, Inc. celery and onion diced blend used in Costco rotisserie chicken salad)	19	5	2	0	USA	Yes	(72)
2014	Wolverine Packing Company's ground beef	12	7	0	0	USA	(1.8 million)	(72)
2013	Gort's Gouda Cheese Farm	28	4	1	1	Canada	Yes	(75)
2013	Cardinal Meat Specialists Limited's frozen beef burgers	8	2	0	0	Canada	Yes	(75)
2013	FreshPoint Inc.'s shredded lettuce distributed to some KFC and KFC-Taco Bell restaurants	30	13	1	0	Canada	Yes	(75)
2013	Glass Onion Catering's ready-to-eat salads	33	7	2	0	USA	(181,620)	(72)
2012	State Garden's pre-packaged leafy greens	33	13	2	0	USA	(31,000)	(72)
2012	XL Foods' beef products	18	6	0	0	Canada	(12 million)	(75)
2011	Romaine lettuce	58	34	3	0	USA	NR	(48,72)
2011	Palmyra Bologna Company's Lebanon bologna	14	3	0	0	USA	(23,000)	(72)
2011	DeFranco & Sons's in-shell hazelnuts	16[1]	12	0	0	USA and Canada[#]	(20,000)	(72,76)
2010	Bravo Farms' cheese	38	15	1	0	USA	(105,000)	(72)
2010	National Steak and Poultry's beef	21	9	1	0	USA	(248,000)	(72)
2009	Chicken (suspected)	69	5	NR	NR	Canada	NR	(77)
2009	Fairbanks Farm's beef	26	19	5	2	USA	(545,700)	(72)

Year	Implicated food	No. of cases				Country	Product Recall (lbs product)	Reference
		Total	Hospitalizations	HUS	Deaths			
2009	JBS Swift Beef Company's beef	23	12	2	0	USA	(421,280)	(72)
2009	Nestle Toll House raw refrigerated cookie dough	72	34	10	0	USA	3.6 million packages	(72,78)
2008	Aunt Mid's Produce Company's iceberg lettuce	74[2]	21	NR	0	USA and Canada	No	(51,77)
2008	Romaine lettuce	29	NR	1	NR	Canada	No	(51,77)
2008	Harvey's Restaurant (Spanish onions (suspect))	235	26	NR	0	Canada	No	(51,77)
2008	Kroger/Nebraska Ltd. ground beef	49	27	1	0	USA	(5.3 million)	(72)
2007	Totino's and Jeno's frozen pizza (pepperoni)	21	8	4	0	USA	5 million pizzas	(72,79)
2007	Topp's ground beef patties	40	21	2	0	USA	(21.7 million)	(72)
2006	Lettuce (suspect)	7	NR	NR	NR	Canada	NR	(51)
2006	Natural Selection Foods, LLC's fresh bagged spinach	199[3]	102	31	3	USA and Canada[#]	NR	(51,72,80,81)
2006	Taco Bell	71	53	8	NR	USA	NR	(72)

NR: not reported; Yes: there was a product recall but no information regarding quantities; No: there was no product recall linked to the outbreak.

[1]*In Canada, 8 cases were found, all of them hospitalized*

[2]*Three cases were in Canada*

[3]*One case was reported in Ontario, Canada.*

[#]*The recall comprised both countries.*

increasing number of *E. coli* O157 outbreaks in Canada and the USA (43,48,51,80). In fact, after the 2006 outbreaks caused by fresh spinach in the USA, the FDA announced the implementation of the "Leafy Greens" initiative with the aim of identifying the potential public health concerns inherent to these products (82). Finally, outbreaks and recalls represent a relevant source of information and provide an opportunity to learn while prompting governments to improve guidelines and regulations to strengthen the food safety systems throughout the whole food chain.

1.3. Current State-of-the-Art in Detection of Food Pathogens

It is evident that food pathogen detection is an important environmental health milestone towards reducing the burden of foodborne diseases and economic losses due to contaminated food. The major objective of testing plans is to verify the adequacy of control manufacturing processes during food production (83). Focusing on *E. coli* O157, the impact of testing strategies on reducing risk has been proved since 2002, when the USDA-FSIS reassessed the HACCP plans and extended the testing programs, after determining that *E. coli* O157 is likely to occur at all stages of raw beef production (69). Further implementation of these measures, resulted in a reduction of the prevalence of *E. coli* O157 in ground beef from 0.73% in 2002 to 0.17% in 2006 (70), while the number of recalls also decreased from 21 in 2002 to 5 in 2005 (84). In Canada, data from 2009 have been encouraging, showing that testing of beef trim, potentially used for the production of ground beef, has prevented contaminated product to be further processed (70). Currently, as part of the new guidance document on *E. coli* O157, it is recommended that all precursor material used for the production of raw ground beef and beef products should be tested and only lots below detectable levels should be accepted (70). Therefore, testing programs, combined with proper sampling protocols and process controls, have played a key role not only in reducing the risk of *E. coli* O157 contamination, but also in determining the efficacy of the control measures established to prevent *E. coli* O157 contamination throughout the manufacturing process.

In order to implement successful testing programs, effective detection methods must be available. Ideally, pathogen detection methods used for food testing should be rapid and

easy-to-use for early identification of potential hazards. Conversely, however, the "gold standard" conventional testing methods are known to be time-consuming and lengthy because they rely on culture, isolation, and biochemical identification. Food pathogens are commonly found in lower concentrations than food microbiota (85); thus a selective enrichment step is required to enable the recovery of the target microorganism while suppressing non-target organisms (86,87). The enrichment step involves transferring the food sample into a selective nutrient medium and incubation to allow the multiplication of the target pathogen to a detectable level (86,88,89). Furthermore, an isolation step using selective and differential agar plating helps to identify presumptive colonies of the target pathogen. Lastly, typical colonies are screened using biochemical and/or serological analysis. Presumptive results from this procedure can take up to four days, while confirmation may require up to one week (86,90,91). Therefore, one of the main challenges that food producers normally face when implementing testing protocols is that lengthy methods can delay the release of minimally processed products with short shelf life such as raw meat until they are screened and considered to be microbiologically safe (88,92). Consequently, a major research field has focused on the refinement of current methods and the development of more efficient technologies designed improve testing programs. Based on the needs of the food sector, three main characteristics of such tests are crucial: speed, sensitivity, and ease-of-use (16,91,92). Interestingly, the food pathogen testing market was valued at 7.42 billion USD as of 2015 and is expected to continue growing due to the establishment of ever more stringent regulations (93). Moreover, in a report from 2003, it was estimated that the beef and poultry industry in the USA performed approximately 369 tests per processing plant per week, representing 22% of the total microbial tests within the USA food industry (91). Therefore, due to the establishment of new testing plans and recent enhancement of regulations applicable to the meat industry, it is expected that the food pathogen rapid testing market will continue growing.

1.3.1 Trends in Rapid Point-of-Care (POC) Methods

The combination of scientific and technological approaches to improve global health care has been the basis of the exponential growth of novel POC diagnostic techniques. The main application of POC tests has been in the clinical diagnostics field to screen for infectious

diseases such as dengue, hepatitis B, HIV/AIDS, malaria and syphilis in patients from developing countries (94–97). The success achieved by on-site disease diagnosis and the rise in the market availability of POC tests helped to establish the optimal characteristics that a POC test should have. Indeed, in 2004 WHO's Sexually Transmitted Diseases Diagnostics Initiative determined that a POC test should comply with the "ASSURED" principle, which means: Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free and Delivered (95–98). Based on this, a POC test can be defined as a simple and affordable assay for food producers that is performed at the location where the sample is found, and will provide a rapid outcome, which is crucial for taking appropriate immediate action (95,98,99).

Even though most of the progress has been achieved in developing medical diagnostic tools, affordable and rapid technologies are also necessary to improve other fields such as environmental and food safety (99–101). As noted above, there is a dire need in food safety diagnostics for more rapid and sensitive methods that can replace traditional techniques that require more than two days to determine the presence of pathogens in food samples (91). However, due to recent advances in biotechnology, chemistry, and molecular biology, it has been possible to address some of the challenges that conventional pathogen detection possesses. In fact, significant advances towards developing rapid state-of-the-art microbiological methods that do not require laboratory facilities or special training so that they can be used throughout the food chain, but maintain high specificity and sensitivity, have occurred (101). The upcoming sections will provide an overview of the most relevant methodologies that are influencing the development of rapid POC tests for food safety.

1.3.1.1 Molecular methods

This research field has significantly grown in the last few decades with an increasing number of commercial diagnostic tools available not only in the clinical market but also in the food safety market. Application of nucleic acid-amplification tests (NAATs) such as polymerase chain reaction (PCR), quantitative reverse transcription polymerase chain reaction (RT-qPCR) (90,102–107), and nucleic acid sequence-based amplification (NASBA) (68,86,90,106–109) have been commonly reported in the literature for pathogen detection. Moreover, a recent approach has focused on developing NAATs based on loop-

mediated isothermal amplification (LAMP), with the advantage that it does not require thermocycling instruments or purified DNA (90,102,106), an important advantage for a POC test model. LAMP is known to be more specific and sensitive than standard PCR methods (106). Conversely, multiplex quantitative PCR (qPCR) has focused on the detection of several pathogens using only one sample, markedly reducing the labor intensive limitation of culture methods and traditional single PCR techniques (86,90,106,107).

Regardless of the advantages that molecular diagnostic methods have such as specificity and sensitivity (95), one of their major shortcomings is they can detect DNA/RNA from dead microorganisms, resulting in misinterpretation of results in complex samples such as food (107). Moreover, most of these methods still require complex equipment, special training, several steps for sample preparation, and are considered to be expensive processes for routine food analysis when compared to a conventional culture method (95,107,110).

1.3.1.2 Optical methods

Although some optical techniques have been used for more than a decade, the main challenge has been to transfer them into portable POC tests. The best example is surface plasmon resonance (SPR), which has the advantage of detecting molecular interactions without the need for labeled reagents (95,96). Thus, it can be used with nucleic acids or immunoassays (96). SPR has been used to detect *C. jejuni*, *S. enterica* ser. Typhimurium, *Y. enterocolitica* and *E. coli* O157, as well as other pathogens (108). Another optical technology that has been exploited for potential POC test development is surface enhanced Raman spectroscopy (SERS). Contrary to SPR, this technique requires a SERS tag component, normally gold or silver nanoparticles coated with a SERS dye encapsulated with silica and conjugated with the detection antibodies, which is combined with traditional Raman spectroscopy (111,112). This technique has been used to monitor, in real time, the growth of *E. coli* O157 during enrichment, showing high sensitivity and specificity with food samples (111). Some of the advantages of Raman spectroscopy are speed and minimal manipulation of samples (94).

1.3.1.3 Nanotechnology

Some of the advances in nanotechnology in the diagnostics field are focused on labeling with metal or magnetic nanoparticles for immunoassays (96,111,113). The range of reagents for labeling goes from antibodies (106,114–116) to nucleic acids (99), the basis for biosensors (nanosensors) for real-time detection (108,117). Quantum dots are also a novel alternative for fluorescent labeling because they possess higher brightness, photostability and are more resistant to chemical degradation that traditional fluorescent dyes (96,99,118). In the food safety field, quantum dots have been used for detection of *E. coli* O157, *Salmonella* and *L. monocytogenes* (115). Working with nanomaterials has the advantage of increasing the number of reactive sites, resulting in higher sensitivity and specificity (94). The development of nanosensors represents a portable alternative with a shorter time-to-result at a low cost (117). Nanocantilevers, which are made of silicon-based materials and can detect biological binding interactions with high sensitivity and short time-to-result, exemplify this novel class of label-free nanosensors (101,117).

1.3.1.4 Microfluidics

Microfluidics represent a breakthrough in the POC research field with the development of the lab-on-a-chip or micro total analysis systems (μTAS) technology, recently applied to the analysis of a variety of biological samples (96,100,119). The introduction of this type of nanosensors has the advantage that sample processing, reactions, and reading of results happens in a miniature flow-through format requiring only small sample volumes (95,96,117). In addition, engineering efforts have resulted in platforms that are accessory-free but contain complete analysis systems including fluid handling, and sample separation (119,120). Interestingly, low-cost devices have been produced using paper instead of plastic molds or nitrocellulose, with the extra benefit that paper allows multiplex analysis using microchannels (100,121,122). Moreover, paper is easily modifiable to bind proteins, DNA or other small molecules and allows for the development of colorimetric assays due to its inherent white color (122). Microfluidics have been combined with other techniques such as immunoassays, capillary electrophoresis, and DNA-analysis to design efficient microreactors (119). In fact, it has been possible to create DNA microarrays, which have been used to better understand the interactions between host and pathogens as well as the

mechanisms of microbial drug resistance (95). It is evident that microfluidics represents one of the most promising research areas in POC test development, with the potential to optimize sensitivity and specificity without requiring laboratory equipment.

1.3.1.5 Immunoassays

Enzyme immunoassays are one of the first described immunochemical techniques still widely used for diagnostics due to its ability to produce a colorimetric reaction that can be quantitated and visualized at a macroscopic scale (95,123). The most well-known immunoassay is the enzyme-linked immunosorbent assay (ELISA), which has become a notable tool for *in vitro* diagnostics, regulatory and quality assessments. Immunoassays are a versatile method that has been combined with several of the technologies described above. Recently, Weidemaier *et al.* reported a nanotechnology-based immunoassay using antibody-conjugated magnetic SERS nanoparticles added directly into the food sample, which capture the target pathogens as they grow and produces a real-time detectable signal read through the sample vessel (111). Moreover, Ho *et al.* developed a colorimetric immunoassay based POC test using immunoliposomes with an encapsulated visible dye to detect *E. coli* O157 (124). However, in light of developing POC tests, lateral flow immunoassays (LFIA) or immunochromatographic test strips are considered the most popular POC technology in clinical diagnostics (95,96). In fact, they are defined as an ASSURED technology (125,126). Thus, it is not surprising that it has also gained popularity within other fields such as food safety and has become a target technology for research (127). LFIAs are similar to an ELISA because they rely on the principle of antigen-antibody binding (128). Therefore, their sensitivity and specificity will be completely dependent on the concentration and accessibility of the target analyte in the sample, as well as of the binding strength and affinity between the antibody and the antigen (106). Contrary to ELISA, LFIAs have the advantage that results can be obtained relatively fast and do not require intensive training (95,96). Similar to other immunoassays, LFIAs have been combined with other techniques such as the prototype developed by Mondesire *et al*. This device consists of a POC LFIA, which relies on a solid-phase extraction merged with a NASBA or PCR technique for analysis of clinical samples and detection of pathogenic bacteria (129). Lateral flow assays that are combined with nucleic acids are

commonly known as NALF or NALFIA (nucleic acid lateral flow or nucleic acid lateral flow immunoassay). The former does not require antibodies because it uses amplicons or probes as capture and detection reagents, while the latter uses antibody recognition against a labeled amplicon (128). By combining LFIA not only with DNA/RNA techniques and also with nanoparticles labeling or miniaturized thin-layer chromatography, the sensitivity and selectivity of the assays has been improved (130).

1.4. Overview of Lateral Flow Immunoassays (LFIAs)

The first lateral flow or dipstick assay was developed in the 1960s for quantifying glucose in urine (131). Its principle relied on an enzymatic reaction that caused a color change, which was compared to a color chart to obtain a semi-quantitative result (131). However, it was two decades later, in the mid-1980s, when LFIAs were introduced to the clinical diagnostic field with the development of the pregnancy test (100,127). Shortly after, their application gradually expanded to other fields, where on/off signals were sufficient, such as drug screening (100), detection of cardiac markers (121), food (130,132,133) and environmental applications (100,130,133), particularly in resource-limited countries.

LFIAs possess a long history within medical diagnostics. Several publications have focused on compiling state-of-the-art of LFIAs (130), which includes the development of quantitative or semi-quantitative LFIAs by using photometric readers (96,132) or signal amplification enhancement (95,96). However, LFIAs are relatively new in the food safety industry, where they have gained attention for detection of toxic compounds such as mycotoxins and pathogenic bacteria (90,133). Of note, detection of mycotoxins has been the major focus for developing quantitative LFIAs complemented with a mobile scanner (134–136). However, for pathogen detection, LFIAs have been unable to detect concentrations as low as 10 cells per g or ml, the infectious dose of some pathogens, such as *E. coli* O157 (21). Therefore, concentration techniques such as magnetic and paramagnetic beads (95,137,138) and/or pairing with enrichment steps (23,68,86) have been developed for maximizing sensitivity and improving the limit of detection. Following the recent implementation of stringent food safety regulations, the scientific and industrial desire to develop LFIAs to detect the major pathogens has increased due to their ease-of-use, rapid outcome, and lack of training needed to perform the test. As such, development

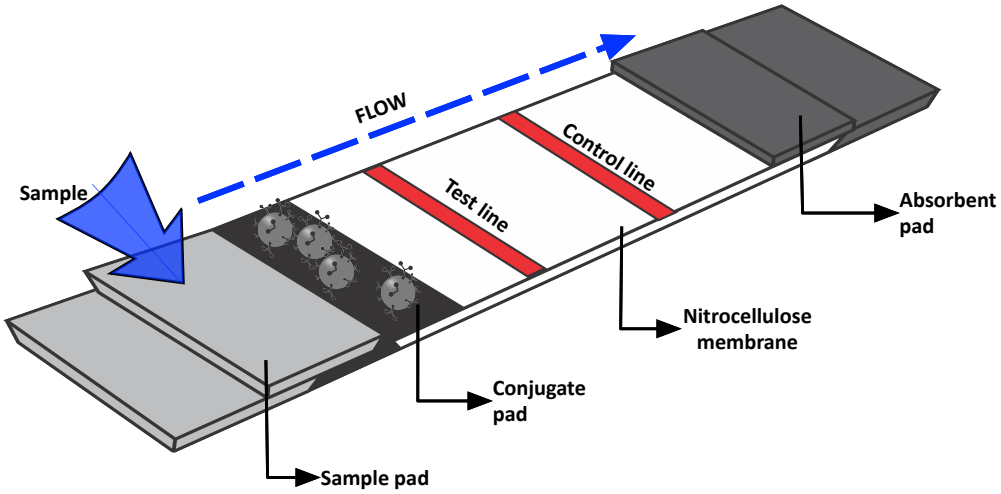
of LFIAs has mainly focused on presence/absence formats for rapid and easy screening of food samples (90,106,133). Indeed, progress has been such that Dzantiev *et al.* compiled the recent advances on immunochromatographic assays developed for food analysis between 2007 and 2013 (132). These authors estimated that 14% of the scientific publications on immunochromatographic test systems were focused on the development of LFIAs for pathogen detection whereas 30% focused on mycotoxins detection. Furthermore, low development costs and facile production (127) give LFIAs an extra advantage over other technologies. From an economic perspective, a publication from 2009 estimated that the food and beverage sector are the largest producer of rapid tests while being the third highest consumer of LFIA-based tests with $30 million USD generated in 2007, just below the clinical and veterinary sectors (139). LFIAs combine the selectivity and sensitivity of immunoassays, such as ELISA, with the simplicity and speed of operation of a POC device. LFIAs are designed to provide results in less than 15 min after loading the sample; thus they represent an excellent alternative for the routine testing of food products.

1.4.1 LFIAs Principle and Main Components

LFIAs rely on the movement, through capillarity, of a liquid sample that is initially loaded onto a sample pad found at one extreme of the nitrocellulose membrane, towards the other extreme where an absorbent pad will capture the remainder of the liquid. Capillary movement through the membrane allows the sample to pass through different zones, where immobilized recognition reagents will interact with the target analyte to form complexes that will attach within the test zone, while unreacted reagents will continue flowing to the control line, where they will attach (130,140). The target analyte complexes captured in the test zone will produce a visible line, similar to the one produced by unreacted reagents captured within the control line. However, the control line will develop whether or not the sample contains antigen, thereby ensuring that the test system is functioning properly (**Figure 2**).

A typical LFIA format, besides the sample and absorbent pad, will include a conjugate release pad right after the sample pad, where recognition reagents are kept ready to interact with the target analyte as the sample starts flowing (130,140). Recognition reagents can be

Figure 2. Overview of the main components of a typical LFIA. Once the sample is applied in the sample pad, it will move through capillarity along the conjugation pad and nitrocellulose membrane until it reaches the absorbent pad. In the conjugation pad, the target antigen will react with some of the detection reagents, which will vary depending the type of assay (e.g. colloidal gold nanoparticles). In a direct assay, the detection reagent-antigen complex will continue traveling until it reaches the test line, where an immobilized capture reagent will trap the complexes and produce a positive signal. The rest of the unbound reagents bind to the control line.


FLOW
Sample
Test line
Control line
Absorbent pad
Nitrocellulose membrane
Conjugate pad
Sample pad

formed by the primary detection antibody or a secondary antibody against the primary, which are conjugated to a label or marker (141). Although new alternatives for preparing recognition reagents, such as quantum dots (141,142) and liposomes with colored dyes (124,141,143), are being introduced, colloidal gold nanoparticles remain the most frequently used label alternative for LFIAs mainly due to their availability and low cost for large scale production (114,130,140,141). In addition, two main types of reactions are commonly used, which are based on the analyte to be determined. The direct assay normally is selected when the target has more than one epitope. In this case, the recognition reagent will bind to one epitope, while the capture reagent, normally attached to the test line, will bind an alternative epitope (130,133,140). In this case, the development of a test line is directly linked to the presence of the target analyte. On the other hand, the competitive assay is frequently selected when the target analyte is a small molecule, which is the case for mycotoxins (134,144). Two alternatives can be used, either the capture reagent is attached to the test line and labeled analyte competes with the sample analyte, or a protein-analyte is attached to the test line while the labeled antibody is initially mixed with the sample. In both cases, a colored test line will be indirectly linked to the presence of the target analyte in the sample (114,130,133). Therefore, because bacteria possess several surface antigens, the sandwich assay is preferred for development of LFIA for pathogen detection.

Since the development of the LFIA technology, its main structural components have remained the same, while major improvements have focused on the analytical reagents and reaction conditions to enhance the performance of the assay according to its final application. In fact, the proper functionality of a LFIA depends on the selection of the capture and detection reagents, which are often antibodies. Generally, two types of antibodies are included in a sandwich assay, a polyclonal and a monoclonal antibody (pAb and mAb). The former is often immobilized in the test line zone acting as a capture antibody, while the latter will act as detection antibody either by direct labeling or by using a secondary labeled antibody against the mAb. Generally, the detection reagent will be dried in the conjugation pad, waiting for the sample to start running through the device for interaction with the target analyte. However, an alternative consists of the dried detection

reagents being stored in a tube, to which the sample is added, acting as a reconstituent, before inserting the LFIA test strip (145). Although both capture and detection antibodies are responsible for the analytical characteristics of the LFIA based on their interactions with the target analyte (146), mAbs are key in determining the specificity of the method. Therefore, efforts are continuously being made to maintain and improve the affinity of the mAbs. This includes using labeled secondary antibodies instead of direct labeling of the mAb because it has been shown that conjugation can decrease the affinity of the antibody while interfering in the antibody-antigen binding due to steric hindrance (130). In addition, research has focused on developing more specific antibodies with the adequate binding characteristics for their application in immunoassays. This has included optimizing hybridoma technologies as well as the development of antibody derivatives through genetic engineering (147). As a result of these approaches, LFIAs have evolved to comply with the needs demanded by the recent diagnostics markets.

1.5. LFIAs for Detection of *E. coli* O157 in Food

LFIAs, similar to most of the novel technologies that have been developed for pathogen detection, rely on biorecognition of surface antigens in order to detect whole bacterial cells (101). Some of the surface antigens found in *E. coli* O157 are commonly used as detection biomarkers, while also serving to classify *E. coli* isolates by serotyping. The latter is a technique based on three main antigens: “O”, “H” and “K”, which are frequently identified by serology (52,148). The former is the outermost variable part of the polysaccharide in the outer membrane (somatic antigen), the second is a flagellar protein, and the third is a capsular antigen (18,52,149,150). However, the K-antigen is rarely used by laboratories; only the O:H combination is considered the standard for classifying or serotyping *E. coli* strains (150–152). Of interest is a recent publication from the *Escherichia coli* O-antigen database (ECODAB) which suggested the existence of 180 O-antigens and >60 H-antigens (151). The O-antigen structure is extremely variable within *E. coli* spp. It is hence considered antigen-specific and therefore, particular to each serogroup (52,151). Although it is not considered a virulence factor, it has shown to play a relevant role in overall bacterial virulence, and hence it is closely associated with the pathogenicity of certain serotypes (149,153). Due to the fact that it is expressed in the outer membrane and is extremely

immunogenic, it is considered a target for both immune cells and bacteriophages (149). Furthermore, contrary to other surface antigens, it is heat-stable (149). Therefore, the O-antigen is of particular interest for epidemiological studies, classification during outbreak investigations and as a diagnostic target (150). Indeed, through surveillance and epidemiological data, different O-serogroups have been associated more often with outbreaks than others, despite their H-antigen. This has been the case of *E. coli* O157:non-H7 strains, which have been isolated from humans and patients with diarrhea including *E. coli* O157:H45, O157:H39 and O157:H16, among others (154). In light of these findings and considering *E. coli* O157 as one of the most implicated serotypes involved in human illness, antibodies against the O157-antigen have been constantly produced and used in the development of LFIAs for routine screening of *E. coli* O157 in food (155,156) or clinical samples (157,158).

While there have been numerous LFIAs described for detecting *E. coli* O157 in food samples, only a few of them have been marketed. Nine gold nanoparticle-based LFIAs and one using magnetic nanoparticles, were commercially available at the time of writing (**Table 2).** Six of these were previously reported by Farrokh *et al.* and Jasson *et al.* (42,159) as validated methods for the detection of *E. coli* O157 in different types of food. Contrary to this, the remaining four methods, MaxSignal®, Quicking, SAS™, and SMART™-II, did not possess supporting information regarding their validation status (160–163). Besides commercialized LFIAs, Singh *et al.* summarized the information of six non-commercial LFIAs for the detection of *E. coli* O157, which have been developed and published by different research groups (133). Overall, the LFIAs described thus far, require an enrichment step in order to achieve detectable levels that range between 10^4 and 10^5 CFU/ml (**Table 2**) (133,159). Despite the fact that the enrichment step can be as short as 6 h, it is still necessary and a major feature to be considered when selecting a rapid POC method.

1.5.1 Validation of Alternative Microbiological Methods

It is evident that through the years, national and international food legislation is becoming more stringent with respect to food safety, emphasizing surveillance and monitoring along the production chain as major components of programs such as HACCP and GMPs.

Table 2. Commercial LFIAs for detection of *E. coli* O157 in food samples (as of June, 2016).

Brand	Manufacturer	Food Type	Enrichment (h) Enrichment broth	Sensitivity	Validation	Reference
Dupont™ Lateral Flow System	DuPont	Ground beef, boneless beef trim	Yes (8-18) P/E	1 CFU/ 25g	AOAC-RI PTM	(42,164)
FoodChek™*	FoodChek™ Systems Inc.	Raw ground beef, beef trim	Yes (6-8) mTSB	1 CFU/ 25g	AOAC-RI PTM	(42,165,166)
MaxSignal® *E. coli* O157 Strip Test Kit	Bioo Scientific Corporation	Meat and meat products, dairy products	Yes (18-24) mEC (meat)/ mTSB-n (dairy)	1×10^4 CFU/ml post-enrichment	N/S	(160)
Quicking	Quicking Biotech Co., Ltd.	N/S	Yes	1×10^5 CFU/ml post-enrichment	N/S	(161)
RapidChek® *E. coli* O157 (incl. H7)	Romer Labs	Raw ground beef, raw boneless beef, apple cider	Yes (8-18) P/E, mEC or EEB	N/S	AOAC-RI PTM USDA FSIS MLG	(42,167)
Reveal 2.0	NEOGEN Corporation	Raw ground beef, raw beef trim	Yes (8-20) P/E	1 CFU/ 25 or 375g 10^4 CFU/ml post enrichment	AOAC-RI PTM	(42,168)
SAS™ *E. coli* O157 and O157:H7 Test	SA Scientific	N/S	Yes (16-24) mEC	N/S	N/S	(162)
Singlepath® *E. coli* O157	EMD Millipore Corporation	Raw ground beef, pasteurized milk	Yes (24) mTSB-n and/or EEB	1 CFU/ml or 25g	AOAC-RI PTM Health Canada	(42,169)

Brand	Manufacturer	Food Type	Enrichment (h) Enrichment broth	Sensitivity	Validation	Reference
SMART™-II Rapid *E. coli* O157 Strip Test	New Horizon Diagnostics	N/S	Yes	1 CFU/25g 3.3×10^4 CFU/ml post-enrichment	N/S	(133,163)
VIP® Gold- EHEC	BioControl Systems, Inc.	N/S	Yes	N/S	AOAC OMA	(42,170,171)

The information provided in this table is based on the latest version of the manufacturer's web pages, product data sheets and/or validation certificates available at the time of writing.
**FoodChek™ test is a magnetic nanoparticle LFIA.*
AOAC OMA: AOAC INTERNATIONAL Official Method of Analysis℠
AOAC-RI PTM: AOAC Research Institute Performance Tested Method℠
N/S: not specified
P/E: proprietary enrichment

Consequently, the development and commercialization of rapid methods have advanced because they represent an alternative to maintaining processing efficiency while complying with screening requirements. However, due to the critical role rapid methods play in ensuring the safety of food, evidence regarding their performance and fit for purpose is required before they can be considered reliable alternatives for pathogen screening (159,172,173). This process is known as validation and normally involves two main phases: 1) comparison of the alternative rapid method against a reference method, and 2) an interlaboratory study (105,159,174). The latter requires the participation of different laboratories in order to assess the reproducibility and repeatability of the results obtained with the alternative method (105). During the validation process, the alternative method is represented by the method that has been designed and is intended to be used instead of a gold standard, also known as the reference method (172). Most often an alternative method will be a system that intends to reduce the time necessary for getting a reliable result (159). Therefore, a rapid alternative method will be any new technique or system that can be used instead of a traditional culture method and provide results in a shorter time with a high degree of reliability. On the other hand, a reference method will comprise of any widely accepted method, normally internationally recognized with a well-established protocol, such as traditional culture methods (159,172).

Something to highlight regarding the validation process is that it has to be recognized by all the parties involved; hence several private and regulatory standards have been developed worldwide that provide protocols for the validation of new microbiological methods (159,172). In North America, regulatory bodies such as Health Canada, FSIS Microbiology Laboratory Guidebook (MLG), and FDA Bacteriological Analytical Manual (BAM), have established validation protocols for microbiological detection methods in Canada and the USA, respectively (175), while in Europe the main standard for validation is the International Standardization Organization (ISO) 16140 (105,159). On the other hand, independent validation bodies include the Association of Analytical Communities (AOAC), mainly in North America, while NordVal, AFNOR or MicroVal are European organizations, which adopted ISO 16140 (127,159,172). Although these protocols agree with the main aim of ensuring the proper performance of the alternative methods, they are

specifically designed to comply with the needs of their particular market. They may not be fully recognized worldwide or by all stakeholders. In addition, validation by any of these bodies is not considered mandatory, however, the use of alternative methods may be restricted by the needs of certain stakeholders that require a specific validation scheme (e.g. official control by government agencies) (159). For this reason, method developers need to select carefully the type of validation protocol they will follow.

1.5.1.1 Health Canada's procedure for the validation of alternative microbiological methods

In Canada, the Microbiological Methods Committee (MMC) is responsible for supplying the appropriate methods for ensuring food safety throughout the supply chain. To do that, the MMC reviews all submitted methodologies to guarantee that they are fit for purpose and that sound science was used along the validation procedures (174). Once a method is approved, it is included in the Compendium of Analytical Methods, which contains all methodologies that are used by Health Canada, the Canadian Food Inspection Agency (CFIA), and other organizations, to determine compliance with standards and regulations (174).

In order for an alternative method to request inclusion in the Compendium, it must follow the "guidelines for the relative validation of indirect qualitative food microbiological methods", which are found within the Compendium as well. Briefly, the method developer has to submit a package containing a pre-collaborative study, inclusivity/exclusivity studies, determination of the limit of detection (LOD) and a transfer study (174). The pre-collaborative study involves comparison of the alternative method with a cultural reference method using a paired or unpaired protocol for further determination of the performance parameters (sensitivity, specificity, false negative and false positive rates, and efficacy) (174). Overall, the results obtained should show that the alternative method meets or exceeds the performance criteria established by the MMC: sensitivity ≥98%, specificity ≥90.4%, false negative rate <2%, false positive rate <9.6%, efficacy ≥94%, and LOD 3-5 CFU/25g (174). Once a complete submission package is received by the MMC, a Technical Group is formed and is responsible for making recommendations to the MMC after assessing all the data from the alternative method against the MMC criteria (174).

Furthermore, a final decision is made by the Bureau of Microbial Hazards Director, who then notifies the MMC and relays the decision to the developer. (174). Finally, an accepted method gains a Laboratory Procedure (MFLP) status, which means that the method complies with the minimum requirements of the Compendium of Analytical Methods (174). Interestingly, at the time of writing, the latest summary of methods for *E. coli* O157 published in the Compendium contained 13 MFLPs and one MFHPB, represented by the gold standard for isolation of *E. coli* O157 from foods and environmental samples (174). Of note, an MFHPB is a fully validated and documented method (174). From the 13 MFLPs alternative methods, only one (MFLP-82: Merck Singlepath® Kit) involves an immunological approach whereas seven are genetic-based methods and one is based on phage technology, three are focused on detection of verotoxins, and the final one is for isolation of VTECs (174). Although rapid alternative methods, in addition to the ones listed in the Compendium, can be used by food manufacturers for their routine analysis, it is evident that methods accepted by Health Canada present a greater advantage, especially considering that CFIA-mandated testing requires use of methods included in the Compendium (174). In fact, this request is clearly stated in the Guidance Document on *E. coli* O157:H7 and *E. coli* O157:NM in Raw Beef and the Meat Hygiene Manual of Procedures, where only an approved rapid method should be used for screening of *E. coli* O157 in precursor material or ground beef or beef products (70,176).

Overall, validation of alternative methods is highly recommended regardless of the protocol selected, as long as it fulfills the current needs of the different stakeholders involved in the food chain. Regarding detection of *E. coli* O157 in Canada, it is evident that stricter policies can indirectly restrict the options of alternative methods available for meat producers. However, for method developers, this also represents an opportunity to provide meat producers with new alternatives that can satisfy the criteria established by competent authorities.

1.6. Development of Single-Chain Variable Fragments (scFv) for Pathogen Detection

LFIAs have become an inexpensive, rapid and easy-to-use screening method with the extra advantage that they can be performed on-site. As noted above, the proper performance of this type of assay relies on high-affinity and specificity, attributed to the antibodies used

against the target antigen. Despite the fact that antibodies are currently widely used in research and commercial applications, their study started with the basis of immunology. The field of immunology as we know it can be traced back to the late 19th century, when Paul Ehrlich laid the groundwork by introducing the concepts of "antibody" and presented the "lock-and-key" principle, giving rise to the "magic bullet" theory (177,178). The latter explains the idea of a chemical substance that should target, with high affinity, any pathogen or toxin entering the body, while remaining harmless to the host (177,179). In 1975, Köhler and Milstein expanded Ehrlich's work by developing hybridoma technology, which allows production of limitless amounts of a single specific antibody, also known as monoclonal antibody (mAb) (179–181). Hybridoma technology represented a breakthrough in medical science because it provided endless opportunities to produce mAbs with precise specificities for clinical treatments and diagnostics (180). However, despite the advantages of being considered a standardized *in vitro* technique, it has limitations that led to the further application of genetic engineering techniques. The most well-known limitation is the human anti-mouse antibody reaction developed when mouse mAbs are used as human therapeutics (147,182). In addition, sometimes hybridomas are unstable and low yielding resulting in increased production costs whenever high amounts of mAbs are necessary (182,183). In light of this, researchers have focused their efforts on increasing knowledge on structure and function of antibodies by applying molecular biology techniques in order to overcome some of the limitations of conventional hybridoma technology through advanced antibody bioengineering.

1.6.1 Structure of Immunoglobulins (Ig)

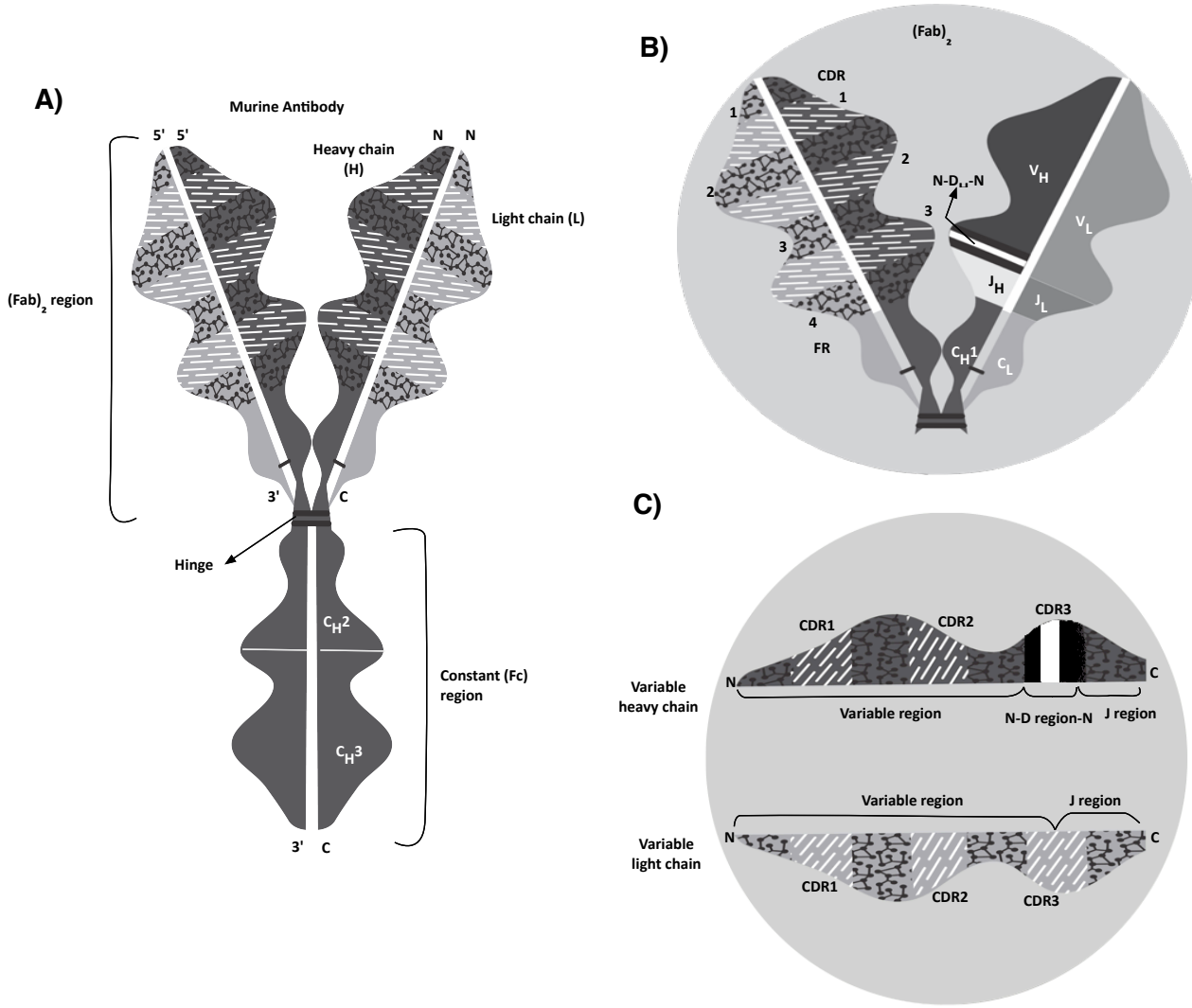
Antibodies are Y-shaped molecules belonging to the glycoproteins family, formed by four polypeptides: two identical heavy chains and two identical light chains (123,184). Each heavy and light chain possesses a molecular weight of approximately 50-75 and 25 kDa, respectively, which contributes to the total IgG molecular weight estimated of 150 kDa (123,184,185). There are five types of heavy chains that define the different isotypes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM (123,184). In addition, two types of light chains are commonly found, κ and λ, which only differ structurally and not functionally (184). IgGs are the isotype most frequently exploited in research for therapeutics and as a

diagnostics tool. Interestingly, mice IgGs are primarily formed by κ light chains, while human IgGs can be 40:60 λ:κ (184). Despite the species, IgGs share a similar structure, consisting of the two heavy chains connected to each other by two to four inter-disulfide bonds at the carboxy-terminal and each light chain joined to the amino–terminal region of one of the heavy chains, respectively, by one disulfide bond (123,185). The inter-chain bonds are found close to the flexible hinge region, which separates the IgG into two important fragments: the crystallizable fragment (Fc) and the dimeric-antigen binding fragment [$F(ab)_2$] (123,184). The former is the effector region and corresponds to the base of the "Y" (183,184), while the $F(ab)_2$ region is responsible for the specificity that each IgG will have because it is involved in antigen recognition (123) (**Figure 3A**).

The $F(ab)_2$ is a dimeric structure that entails two Fab sites, each one containing a complete light chain and a fraction of the heavy chain corresponding to the first constant region (C_H1) and the entire heavy variable region (V_H) (186). Contrary to the heavy chain that has three C_H, two of which form the Fc, light chains consist only of one constant region (C_L) belonging entirely to the Fab together with its variable region (V_L) (187). The Fab can be divided into a variable fragment (V_H and V_L) and a constant fragment (C_H1 and C_L) (186). Although the Fab region is involved in the interaction of the IgG with an antigen, the biggest impact relies on the variable fragment, which is suggested to be responsible for the great diversity of IgGs that exist. However, within the variable region, researchers have identified hypervariable segments, which have been proposed to be the actual sites where the antigen interacts with the IgG (123,187). Three hypervariable segments or complementarity determining regions (CDRs) are known in each V_H and V_L. When the first X-ray crystal structure of a Fab region was obtained, these CDRs were found to form six loops that were separated by relatively conserved regions that act as support; thus they are known as framework regions (FRs) (123,187). As noted, the six hypervariable CDR loops, derived from both heavy and light chains, form the binding site that will interact with its antigen through specific regions known as epitopes (123) (**Figure 3B**).

The mechanism through which the variable domains are assembled is known as VDJ recombination for V_H and VJ for V_L (188). Through this process, three (or two for the V_L) separate gene segments - variable (V), diversity (D), and the joining (J) - suffer a somatic

Figure 3. Overview of the structure of an IgG antibody. Schematic representation of **A)** a complete IgG molecule consisting of two heavy (H) and two light (L) chains. In general, an IgG molecule is divided into $(Fab)_2$ and the Fc regions, which are joined by the hinge region, **B)** the $(Fab)_2$ region, which is responsible for binding to the antigen, mainly through the six CDR loops found in the variable region of the heavy and light chain (V_H and V_L) (left chains). The right chains show the different segments based on the encoding germlines V, D, J and the constant regions found within the Fab. The composition of the diversity segment (D) is highlighted with the nontemplated (N) nucleotides represented by black lines on each side of the D segment, and **C)** V_H and V_L chains structure based on the somatic recombination process of the V, D, and J gene segments. The difference in the CDR 3 from the V_H and V_L is emphasized. Images are based on Georgiou *et al.* (189), Sarantopoulos (322), and Ahmad *et al.* (205).

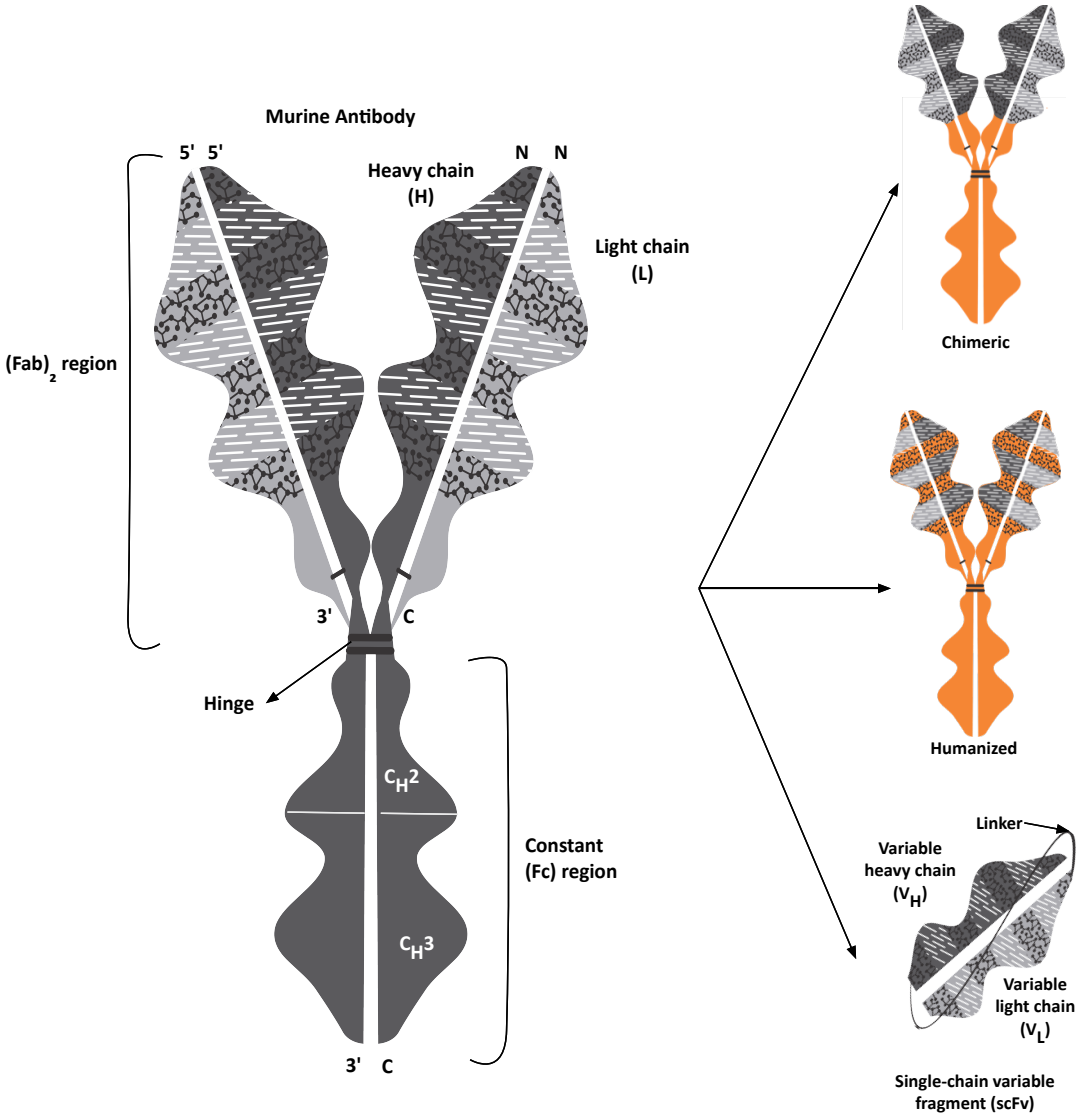

A)
Murine Antibody
5' 5'
N N
Heavy chain (H)
Light chain (L)
(Fab)2 region
3' C
Hinge
CH2
CH3
Constant (Fc) region
3' C
B)
(Fab)2
CDR
1
2
3
4
FR
N-DH-N
VH
VL
JH
JL
CH1
CL
C)
CDR1
CDR2
CDR3
N
C
Variable heavy chain
Variable region
N-D region-N
J region
Variable region
J region
N
C
Variable light chain
CDR1
CDR2
CDR3

rearrangement that is accompanied by other mechanisms such as imprecise joining of any of the three segments, trimming and/or random addition of nucleotides (N nucleotides) (188,189). The V gene encodes the first three FRs, CDR1, CDR2, and the amino-terminal of the CDR3 in both V_H and V_L (186,190). Moreover, the J gene, which is found between the V and the constant region of either the heavy or light chain, contains the carboxy-terminal of the CDR3 and the last FR4 (186,190). Finally, the D gene, found between the V and the J regions within the V_H, complements the CDR3 (123). The latter is considered the hallmark of any IgG because it is highly variable due to the random insertion of N-nucleotides at any of the extremes of the D gene (186). Thus, CDR3 can vary not only in sequence, but also in length (**Figure 3C**). These whole variable region assembly mechanisms, together with the numerous possibilities of association between different heavy and light chains, are responsible for creating an infinite repertoire of IgGs from a finite number of genes (123,186,189).

1.6.2 Antibody Engineering

Traditionally, mouse hybridomas have been the main source of monoclonal antibodies for medical applications. However, they possess a major limitation when used as therapeutics because they trigger the human anti-mouse antibody reaction (147,191). Thus, it is not surprising that most of the focus on engineering antibodies has been towards reducing their immunogenicity through techniques such as humanization or chimerization (147,191). Humanization involves grafting the murine CDRs into a human FR, while a chimeric antibody is constructed by replacing the murine constant regions (192) (**Figure 4**). Through these genetic engineering advancements, the market of recombinant antibodies has rapidly grown with a global value estimated at $20 billion per year in 2007 (193). Yet, recombinant antibodies have not been intensely exploited in other fields besides therapeutics (194). This can be attributed to the fact that great variability implies higher complexity, which hinders the use of simple techniques such as PCR because two primers are not sufficient to amplify the sequences of all V_H and V_L chains (194). Therefore, more complex approaches have been necessary in order to discern their specific sequence for further recombination. Techniques such as degenerate primers have been developed in order to overcome antibody variability during PCR (194–196). In addition, other novel PCR techniques such as

Figure 4. Examples of antibody engineering. Schematic representation of some recombinant antibodies using a murine IgG as template. On the left, an intact murine IgG is shown. On the right, three different structures are depicted: first, a chimeric antibody, which comprises the variable regions of the murine antibody joined to a constant human region. The second example is a humanized antibody consisting of the murine CDRs grafted into a human backbone. The last one, is a scFv, which is the most common antibody fragment that only contains the variable regions joined by a peptide linker.


Murine Antibody
5' 5'
N N
Heavy chain
(H)
Light chain
(L)
(Fab)2 region
3'
C
Hinge
CH2
CH3
Constant
(Fc) region
3' C
Chimeric
Humanized
Linker
Variable
heavy chain
(VH)
Variable
light chain
(VL)
Single-chain variable
fragment (scFv)

5'-rapid amplification of cDNA ends (5'-RACE) (189,197) have been complemented with methods already applied for the development of therapeutic antibodies including phage display (198,199), leading to the production of newly improved antibodies. Furthermore, the advantages of recombinant over murine hybridoma antibodies surpass their production process, because properties such as their binding and specificity towards a particular antigen can be easily enhanced (147,194).

1.6.2.1 Single-chain variable fragments (scFv)

Several formats of recombinant antibodies have been developed, from chimeric antibodies to smaller fragments such as Fab, $F(ab)_2$, or single-chain variable fragments (scFv) as monomers, dimers (diabodies), or trimers (triabodies) (200). However, one of the most frequently used formats used for production of recombinant antibodies is the expression of scFv, which are composed of the V_H and V_L domains of an antibody with a molecular weight between 27-30 kDa (201). Both chains are covalently bound by a flexible peptide linker forming a single polypeptide (197,202) (**Figure 4**). scFv are becoming extremely useful as drug therapies when complete antibodies struggle to reach their target due to their size, as proven for some cancer treatments (202). In some cases, the Fc is not crucial for functionality; in fact, it can increase the chances of non-specific binding due to interactions with other cells present in the samples. Thus, scFv, which are considered the smallest antibody fragment that contains the complete antigen binding site, can have better penetration and biodistribution within their target organs and cells (197,203). Besides the development of scFv for treatment purposes, the study of these antibody fragments has led to a better understanding of the stability and roles that each variable chain plays in antibody functionality; thus scFv are also a model system for antibody research (204,205). Moreover, scFv represent potential immunodiagnostics reagents because they can bind to a series of antigens such as proteins, haptens, chemicals and even whole bacterial cells using different immunoassay formats (205). **Table 3** summarizes some of the most recent technologies that have been combined with the development and application of scFv as immunodiagnostics reagents.

Table 3. Summary of applications of scFv as immunodiagnostics reagents (as of June, 2016).

Target Antigen	Organism	Technique	Research Group	Reference
Aflatoxin B_1	N/A	SPR	Dunne *et al.* (2005)	(206)
Deoxynivalenol (DON)	N/A	Competitive ELISA	Wang *et al.* (2007)	(207)
Diverse antigens	*Entamoeba hystolytica*	SERS microspectroscopy combined with nanoyeast-scFv	Wang *et al.* (2014)	(208)
Fluoroquinolone	N/A	ELISA	Wen *et al.* (2012)	(209)
Fumonisin B_1	N/A	Competitive ELISA	Zou *et al.* (2014)	(210)
Heat-labile and heat-stable toxins	ETEC	Immunoblotting/ ELISA	Ozaki *et al.* (2015)	(201)
Heat shock protein 6o (HSP6o)	*Strongyloides sp.*	ELISA	Levenhagen *et al.* (2015)	(211)
Protective antigen (PA)	*Bacillus anthracis*	Protein chip (immunoassay)	Wang *et al.* (2006)	(212)
Protein D (OmpD)	*Salmonella enterica* serovar Typhimurium	Competitive ELISA	Meyer *et al.* (2011)	(213)
N/S	*Brucella melitensis*	ELISA	Hayhurst *et al.* (2003)	(214)

N/A: not applicable; N/S: not specified

Production of scFv requires the proper folding of the V_H-linker-V_L peptide and formation of disulfide bonds (215), which has been accomplished through bacterial expression systems, especially using *E. coli* (203,216). Moreover, other expression systems have also been used including, but not limited to yeast (204,205,217) and plants (204,205) with encouraging results. Wörn *et al.* summarized several studies that have reported the production of scFv with similar monomeric binding affinity as their mAb counterpart (204). Even though several scFv have been effectively produced as alternatives to whole antibodies, some others have proven to be challenging due to protein solubility and stability issues after expression (202,218). Some of these limitations are due to the great variability of the primary amino acid sequences that resulted in the development of two different approaches to solve these issues. The first group, involves modifications to the primary sequence of the scFv, including loop grafting by transplanting the CDRs into an acceptor framework to improve biophysical properties (218,219). Site-directed mutagenesis is based on the rational modification of specific amino acid residues (218,220). The second approach focuses on modifying protein expression conditions to favor the proper folding, such as expression hosts, isolation and purification (203,217). Together, these techniques have increased the success rate for obtaining functional scFv, promoting their applications beyond therapeutics and clinical diagnosis.

1.6.2.2 Application of scFv to food pathogen detection

As discussed above, intensive efforts have been made to improve the production of scFv has resulted in an increasing scientific and commercial interest to progressively introduce scFv into other fields such as immunodiagnostics (200,221). Within the food safety field, antibodies have been widely used for detection of toxic compounds and pathogenic bacteria using an extensive variety of immunological techniques. Even though there have been few reports of the application of scFv as alternatives to whole antibodies, scFv have already shown to be valuable reagents when used in biosensors due to their smaller size and high specificity (101,206,221). Thus far, most of the studies have focused on detection of mycotoxins for food analysis (**Table 3**). However, production of scFv for detection of food pathogenic bacteria has been recently reported, mainly using phage display technology for detection of *Salmonella enterica* serovar Typhimurium infections using competitive

ELISA (213). This scFv developed by Meyer *et al.* targeted protein D (OmpD) found in the outer membrane of *S. enterica* ser. Typhimurium, which is considered highly immunogenic (213). To our knowledge at the time of writing, this is the only study focused on the application of scFv for food safety purposes. It is thus evident that the use of scFvs for food safety can be considered a rather new research field, which could greatly benefit from the application of scFv as detection reagents. This knowledge provides the foundation for the antibody engineering work described in this thesis.

CHAPTER 2 RATIONALE, HYPOTHESES AND OBJECTIVES

2.1. Rationale

It is clearly evident that *E. coli* O157 remains one of the major foodborne pathogens with huge implications for public health. Foodborne infection with *E. coli* O157 was first identified in 1982. Due to the severity of the disease and its long-term negative outcomes, *E. coli* O157 was designated as a food adulterant in 1994 by the USDA-FSIS, leading to the establishment of a detection program in beef (66).

Meanwhile in Canada, mandatory testing programs, with a zero tolerance were established for *E. coli* O157 initially in ground meat (222). Since then, control and detection of *E. coli* O157 have become active research fields in food safety. The current standard detection method requires up to a week to confirm the presence of *E. coli* O157 in food samples, an obstacle for some food producers to promptly deliver safe food. On the other hand, alternative methods, approved by Health Canada, are mostly genetic-based and thus require special equipment, multiple steps or specialized training for their performance. In the work to be described, it was sought to develop and validate a POC test, based on the LFIA principle. This is intended to provide a reliable alternative for meat producers to enable easy and rapid detection of *E. coli* O157. Early detection of this pathogen is vital to prevent contaminated food from reaching consumers, attenuating or preventing the effects arising from a recall or an outbreak. Although the expansion of LFIA into the food safety field is relatively new, an increase in the development of new tests has followed the establishment of more stringent regulations mainly for food pathogen detection (139). Recently, advances in molecular biology have been combined with the LFIA principle to develop more sensitive, specific and faster LFIAs. The synthesis of single-chain variable fragments (scFv) can offer significant advantages over monoclonal antibodies as detection reagents. These advantages include enhancement of the specificity and sensitivity of the immunoassays (108). Previous to the work described in this thesis, synthesis and application of scFv as detection reagents has been mainly applied for the detection of mycotoxins using ELISA or SPR (206,210). The second part of this thesis focuses on the design and expression of a scFv that can be used to detect *E. coli* O157 in an immunoassay.

2.2. Hypotheses

1) The LFIA Test Kit developed in this study will have similar or better performance, measured by the determination of the performance parameters and Probability of Detection when compared to the traditional culture method using a relative validation study.

2) Proper pairing of the enrichment step and the LFIA device will result in shorter time-to-result, when compared to the time required for detection of *E. coli* O157 using the traditional culture method and other immunoassays found in the current Compendium of Analytical Methods.

3) Using the anti-O157 mAb as a starting point, a scFv will be derived by expression in *E. coli* cells, which will retain the binding properties of the parental anti-O157 mAb when used in a functional immunoassay such as ELISA.

2.3. Objectives

1) To develop a rapid POC test based on the LFIA principle that can be used to visually demonstrate the presence or absence of *E. coli* O157 in meat samples.

2) To compare the performance parameters of the newly developed LFIA Test Kit *versus* the criteria established by the MMC as stated in the current Compendium of Analytical Methods.

3) To design, clone, express, and characterize the functionality of a scFv based upon the structure of the anti-O157 mAb used as the detection reagent in the LFIA Test Kit.

CHAPTER 3 MATERIALS AND METHOD

3.1. Bacterial Strains, Sample Preparation and Culture Conditions

3.1.1 Inclusivity and Exclusivity Strains

The objective of this study was to ensure that the LFIA test kit had the ability to selectively detect a wide range of *E. coli* O157 strains (including H7 and non-motile), while excluding non-target organisms closely related to *E. coli* O157 and/or commonly found in raw meat products. The inclusivity panel was assembled considering the variability within *E. coli* O157 strains and the relevance of other O157:non-H7 *E. coli* as causes of human illness (27,154,223). A total of 50 *E. coli* O157 strains (including H7 and non-motile) was selected from different sources. Only 39 were O157:H7 serotype, 5 were O157:NM, and 6 of them were O157:non-H7 serotypes (H45, H42, H29, H25, H19, H12). In addition, 27 strains were from bovine origin isolated from different sources such as feces and meat (ground and salami). Around 18 strains were from human origin; 16 mainly from clinical isolation and 2 from feces. However, four strains did not have an origin specified, but their original depositor was stated by the contributor. Only one strain did not have any information regarding its history. All strains were biochemically characterized and confirmed as *E. coli* O157 prior to testing (**Appendix A**).

For exclusivity, 37 non-*E. coli* O157 strains were selected from different sources that reflected the variability of organisms that can be present in the food matrices chosen for this study. Most of these (33) were Gram negative and belonged to the *Enterobacteriaceae* family, with the exception of *Aeromonas hydrophila*. From those Enterobacteria, 18 were *E. coli* non-O157, while the rest were bacteria frequently found in meat and closely related to *E. coli* O157. In addition, four Gram positive strains that can be found in meat were included in the panel (*Bacillus subtilis, Enterococcus faecalis,* and *Listeria monocytogenes* 4a and 1/2c). Strains obtained from the Public Health Agency of Canada (PHAC) in Guelph, ON, Canada, were verified using the Vitek® microbial identification system before they were provided (**Appendix A**).

Upon receipt, all 87 strains were initially grown in 5 ml of BBL™ Trypticase™ Soy Broth [TSB; Becton, Dickinson and Company (BD), Sparks, MD, USA] or Bacto™ Brain Heart Infusion (BHI) medium (BD, USA) incubated overnight at 37°C to reach late-exponential

phase (10^8–10^9 CFU/ml). A sub-culture was prepared by transferring 50 µl of the initial culture into 5 ml of TSB or BHI and incubated as previously stated. Cells were harvested by centrifugation (Eppendorf Centrifuge 5430, Rotor FA-45-30-11, Eppendorf AG, Hamburg, Germany) at 5000 ×g and re-suspended using TSB or BHI with 20% (v/v) glycerol as cryoprotectant and stored at -80°C for long-term storage. Furthermore, working cultures were prepared from frozen stocks by loop inoculation of 5 ml of TSB incubated overnight at 37°C to reach late-exponential phase (10^8–10^9 CFU/ml).

3.1.2 Bacterial Culture Enumeration

Bacterial enumerations or plate counts were performed by plating 50 µl on Trypticase™ Soy Agar (TSA; BD, USA) and/or the *E. coli* O157:H7 selective agar, cefixime rhamnose sorbitol MacConkey (CR-SMAC; Oxoid Limited, Basingstoke, Hampshire, UK) using the Spiral Plate Method (224) (Eddy Jet Spiral Plater, E Mode; Neu-tec Group Inc., Farmingdale, NY, USA) then incubating the plates at 37°C for 18-24 h. Two serial dilutions were normally plated per sample. Colonies on the agar plates were counted using the spiral plate counting grid and following the "rule of 20 colonies". In brief, a wedge from the grid was selected and colonies were counted from the outer edge towards the center until more than 20 colonies had been reached. A similar area in the opposite side to the wedge selected was also counted, added up and divided by the sample volume deposited in those two areas as shown in the formula below:

$$\frac{Count\ 1 + Count\ 2}{Volume\ deposited} = \frac{CFU}{ml}$$

Results were reported as CFU/ml of sample plated and recorded for further use in dilution calculations or estimation of cell concentration in samples (CFU/g or ml).

3.1.3 Preparation of Stressed *E. coli* O157 Cells.

The protocol selected was adopted from a previous study done by Jasson *et al.* (225), which consisted of mimicking inherent factors normally present in food. Working cultures of *E. coli* O157 DSM17076, EC20060233, EC20001018, EC19970515, and EC20040339 were prepared as described in **Section 3.1.1**. In addition, a "food" broth was formulated using

TSB supplemented with 0.6% yeast extract (Sigma-Aldrich, St. Louis, MO, USA) (TSBYE) and acidified with HCl until a pH of 4.9 was reached. In addition, 130 g/L NaCl was added to simulate the salt concentration of a fermented sausage. The broth was filter sterilized and kept at 4°C. To determine the initial cell concentration, cultures were plated on TSA supplemented with 0.6% yeast extract (TSAYE) and CR-SMAC agar plates, which were considered non-selective and selective respectively. Serial dilutions were performed using the "food" broth up to a level of 10^6 CFU/ml. The inoculated broths were kept at 4°C for 10 d after which aliquots were plated on TSAYE and CR-SMAC plates for enumeration. The percentage of sub-lethal injury was determined by comparing the number of colonies in non-selective and selective agars using the formula below:

$$\%\ sub-lethal\ injury = \frac{nonselective - selective}{nonselective} x\ 100$$

For each strain, at least three different experiments were undertaken and the percentage of sub-lethal injury was expressed as the average ± standard error of the mean. The % of sub-lethal injury caused solely by the stress treatment was confirmed by determining if significant difference between selective and non-selective media performance existed using a two-sided *t*-test with α= 0.05.

3.1.4 Artificial Inoculation of Food Samples

Fresh retail lean ground beef or trimmed beef was obtained from local supermarkets one day before starting the experiments (Loblaws, London, ON, Canada) and aseptically divided into 25 g samples using Stomacher® bags. In order to mimic real scenarios, samples were inoculated either with healthy or stressed *E. coli* O157 cells prepared as described in **Section 3.1.3**. Both cultures were serially 10-fold diluted using Difco™ Buffered Peptone Water (BPW; BD, USA) in order to achieve three inoculation levels: low (1-10 CFU/25 g), medium (10^2 CFU/25 g) and/or high (10^3 CFU/25 g), as per the objective of each experiment. Cell concentrations of inocula were confirmed by plate counts on TSA and CR-SMAC. In addition, an uninoculated 25 g sample was included as a negative control. After manual homogenization, samples were stored at 4°C for 48 h for equilibration before further use.

3.1.5 Determination of Aerobic Plate Count (APC) of Food Samples

APC, also known as total viable count (TVC), was also determined to establish the initial level of contamination in the food samples. A 25 g sample, from the same batch used in each experiment, was weighed into a Stomacher® bag and mixed with 225 ml (1:10) of BPW. The bag was loosely closed and stomached using a lab blender (BagMixer® 400P, Interscience Laboratories Inc., Woburn, MA, USA) for 1 min. A 10-fold dilution was prepared using BPW and an aliquot of both was plated on TSA. Plates were incubated at 37°C for 48 h for enumeration. Cell concentrations were expressed as CFU/g of food sample.

3.1.6 Evaluation of Enrichment Conditions

Table 4 summarizes the commercially available enrichment broths used throughout the development process. The first screening involved using pure cultures. Briefly, a working culture of *E. coli* O157 DSM 17076 was prepared as previously described and serially diluted in TSB to a final concentration of 10^2 CFU/ml. Initial inoculation levels were confirmed by plate counts on TSA and CR-SMAC. Cultures were incubated at 42°C for a maximum of 24 h. When necessary, bacterial growth was monitored by plating 50 µl every 2 h on TSA and CR-SMAC.

The final selection of the enrichment broth was based on the following: 1) the ability to recover *E. coli* O157 from artificially inoculated food samples prepared as per **Section 3.1.4**; and 2) its interaction with the LFIA Test Kit. Therefore, after equilibration, Stomacher® bags containing the inoculated samples were filled with 225 ml of the appropriate enrichment broth, stomached for 1 min and regenerated 40 min at RT (room temperature). Food samples were incubated at 42°C for a maximum of 24 h. When necessary, a time-course study was performed with sampling every 2 h starting at 16 h. For enumeration, samples were plated on TSA and CR-SMAC and, if needed, they were previously serially diluted using BPW.

3.1.7 Cloning and Expression Bacterial Strains

E. coli XL1-Blue competent cells (Stratagene) were used for plasmid expression and *E. coli* BL21 (DE3) competent cells (Novagen) were used to express the recombinant protein.

Table 4. Enrichment broths and their selective agents.

Broth	Selectivity	Selective Agents	Supplier
Modified Tryptone Soya Broth (mTSB)	Selective	Bile salts No. 3	Oxoid Limited (Basingstoke, Hampshire, UK)
RapidCult™ *E. coli*	Selective	Sodium thioglycolate	EMD Chemicals (Darmstadt, Germany)
BBL™ Tripticase™ Soy Broth+ Novobiocin (TSBN)	Selective	Novobiocin	Becton, Dickinson and Company (Sparks, MD, USA)/ Sigma-Aldrich Co. LLC (St. Louis, MO, USA)
BBL™ Tripticase™ Soy Broth (TSB)	Non-selective	N/A	Becton, Dickinson and Company (Sparks, MD, USA)

Both strains were grown aerobically at either 37°C or RT in Luria Broth (LB) (BD, USA) or on LB with 1.5% agar (BioShop, Burlington, ON, Canada). To maintain plasmid selectivity, the LB broth or agar was supplemented with ampicillin (20 µg/ml) and/or chloramphenicol (10 µg/ml), both from Sigma-Aldrich, USA. For long-term storage, bacterial strains were frozen in LB broth with 20% glycerol and kept at -80°C. **Table 5** summarizes the main characteristics of the two strains used within this part of the study.

3.2. Development of a Lateral Flow Immunoassay (LFIA) for Detection of *E. coli* O157

3.2.1 Assembling of the LFIA

The LFIA devices used during this project were manufactured at the International Point of Care Inc. (IPOC) facilities in Toronto, ON, Canada following their internal specifications and protocols (Garth Styba, president IPOC, 2016, pers. comm.). Standard LFIA devices were stored at RT, while bovine serum albumin (BSA) blocked devices were stored at 4°C upon arrival at our facilities.

The standard LFIA device consists of one or two (tandem) plastic backed nitrocellulose membrane strips coated with streptavidin on the test line and a polyclonal goat anti-mouse antibody on the control line. A 28 mm polyester sample pad is adhered at one extreme of the membrane, while a cellulose membrane is attached as an absorption pad on the opposite side. The assembled LFIA strip(s) is (are) encased on a plastic cartridge, either a single or tandem unit, as shown in **Figure 5**.

3.2.2 In-Tube Sandwich Immunoassay

An in-tube sandwich immunoassay was developed using three antibodies: a biotinylated capture polyclonal antibody (pAb-b), a primary detection monoclonal antibody (mAb) and a secondary antibody conjugated with colloidal gold (CGC) (**Table 6**). Briefly, the three antibodies were mixed, either in solution or lyophilized, in an Eppendorf tube with 200 µl of the sample assessed. Tubes were incubated for 30 min at RT. Furthermore, 150 µl of the suspension was then pipetted onto the sample window of the LFIA device and allowed to

Table 5. Cloning and expression *E. coli* competent cells.

Bacterial Strain	Characteristic	Purpose	Source
E. coli BL21 (DE3)	*FompT hsdSB(rB -, mB -) gal dcm (DE3)*	Protein Expression	Novagen
E. coli XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (TetR)]*	Cloning	Stratagene

Figure 5. Schematic representation of the LFIA. A) The LFIA device with the nitrocellulose strip(s) encased in either a single or tandem plastic cartridge. **B)** Diagram of the assembled nitrocellulose strip with the sample and absorbent pad on each extreme. The control line is formed by an immobilized polyclonal goat anti-mouse antibody, while the test line contains streptavidin.

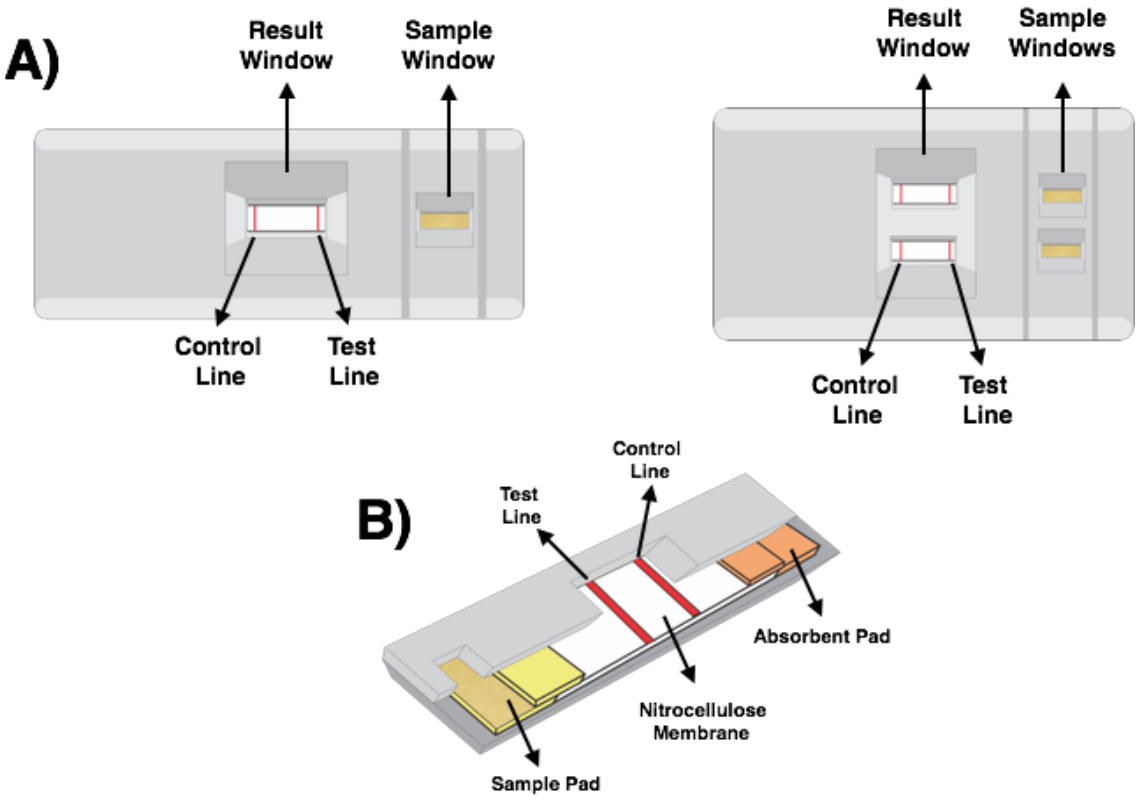

A)
Result Window
Sample Window
Control Line
Test Line
Result Window
Sample Windows
Control Line
Test Line
B)
Control Line
Test Line
Absorbent Pad
Nitrocellulose Membrane
Sample Pad

Table 6. Antibodies used for the development of the LFIA Test Kit.

Antibody		Clonality	Host/ Isotype	Application	Specificity	Supplier
Primary, detection	13B3 anti-*E. coli* O157 (mAb)[1]	Monoclonal	Mouse/ IgG3	ELISA, IF, Agglutina-tion	O157 LPS antigen	USDA Agricultural Research Service, Meat Safety and Quality Research Unit, NE
Capture	biotin conjugate (pAb-b)	Polyclonal	Goat	ELISA	*E. coli*	Pierce Biotechnology, IL, USA
Secondary	DGMG-B001, colloidal gold conjugate (CGC)[1]	Polyclonal	Goat	Lateral Flow Assay	mouse IgG (heavy and light chains)	BioAssay Works®, MD, USA

[1]The mAb ascites and colloidal gold conjugate were further produced by International Point of Care (IPOC), Toronto, ON, Canada.

flow through the membrane for 15 min before reading. **Table 6** summarizes the main characteristics of each antibody used.

3.2.3 Optimization of Reagents and Blocking Conditions

Optimization of the LFIA Test Kit was focused on membrane blocking, antibody concentrations and enhancement of the positive signals. The standard LFIA and the in-tube sandwich immunoassay described earlier were used throughout this process. Elimination of non-specific binding, involved common strategies using a wide variety of proteins or polymers, such as BSA, casein, fish serum, polyvinyl acetate (PVA), polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP), which can be adsorbed by the nitrocellulose (114,133,134). Their main effect relies on interfering or competing with binding of the target reagents (226). Three sequential approaches were undertaken. The first approach involved using only a working culture of *E. coli* O157 DSM 17076 as positive control. Cells were harvested by centrifuging at 5,000 ×g for 10 min at RT and re-suspending the pellet in a blocking buffer consisting of 1% BSA (BioShop, Canada) and 0.05% Tween 20 (Sigma-Aldrich, USA) in 1×PBS. As a negative control, blocking buffer was used. For the in-tube sandwich immunoassay, an aliquot of each sample was mixed with the capture and detection antibodies at a final concentration of 3 μg/ml respectively, while the CGC was used at 0.5 μg/ml throughout these experiments. The second approach consisted of pre-treating the LFIA membrane with 50 μl of blocking buffer, allowing it to flow through the device before loading the sample. The in-tube sandwich immunoassay composition and procedure were kept the same. The final approach substituted the pre-treatment with the incorporation of the blocking agents during the manufacture of the LFIA device. For this purpose, various ratios of BSA and Tween 20 were sprayed onto the nitrocellulose membrane and/or the sample pad during assembly. In addition, different volumes of blocking solution per mm^2 of nitrocellulose membrane were tested and compared. An overnight *E. coli* O157:H7 DSM17076 working culture was always used as positive control, while the blocking buffer or culture broth wwas used as a negative control, depending on the experiment. At a later stage, working cultures of other non-*E. coli* O157 strains were tested for cross-reaction evaluation. Once potential prototypes of the LFIA device were selected, their performance was assessed not only with pure cultures, but also

using food samples prepared as per **Section 3.1.4** and enriched using the selected enrichment protocols.

The antibody concentrations were kept constant to compare the performance of all the different LFIA device prototypes. However, after the potential prototypes were chosen, antibody concentrations were fine-tuned for signal enhancement. Adjustments were made by preparing antibody suspensions using either 1×PBS or blocking buffer as the diluent. In order to extend the shelf life of the antibodies and facilitate their handling, potential working concentrations for the different antibodies were used to produced lyophilized beads at IPOC facilities following their standard manufacturing process (Garth Styba, president IPOC, 2016, pers. comm.). Although antibody beads eventually replaced the antibody suspension during the in-tube sandwich immunoassay, the procedure did not change. Because the beads were sensitive to humidity, they were stored in Eppendorf tubes inside a sealed metallic pouch with a desiccant and kept at 4°C until used.

Biochemical and microbiological sample composition played a key role in signal intensity. Thus, to counterbalance the effect of such variations, it was necessary to standardize the LFIA Test Kit procedure. Two alternatives were evaluated, pH adjustment and dilution of sample matrix after enrichment. The final LFIA prototype and antibody beads for the in-tube sandwich immunoassay were used throughout these experiments. Pure working cultures and food samples were prepared as before and enriched according to the final protocol. Before enrichment, the pH of the homogenized sample was measured. After enrichment, the sample was cooled to RT and 200 µl aliquots were pipetted into different Eppendorf tubes containing the three lyophilized beads used for the in-tube sandwich immunoassay. Different volumes of 1M HEPES buffer were added to each tube to adjust the pH, except for one that was used as a control. After 30 min incubation at RT, the sample was loaded into the LFIA device and the pH was measured using the remaining sample. pH values were compared before and after adjustment and correlated to the results obtained in the LFIA devices. All pH measurements were taken using a pHTestr®20 (Oakton® Instruments, Il, USA) and/or pH strips (VWR International, Radnor, PA, USA).

Finally, to evaluate the effect of sample composition and bacterial cell concentration, a tandem test was developed. Right after enrichment, a 200 µl aliquot was pipetted directly to an Eppendorf tube containing the antibody beads, named sample (A). A second aliquot was taken from the enriched sample and diluted 1:100 using BPW, which was called sample (B). An aliquot of 200 µl of this dilution was pipetted into a second tube containing beads. Both tubes were incubated in parallel according to the in-tube sandwich immunoassay protocol before 150 µl were loaded into the appropriate window in the tandem LFIA device. All samples were run through the nitrocellulose membrane for 15 min before the signal intensity was assessed.

3.2.4 Assessment of LFIA Device Signal

All LFIA devices were assessed 15 min after sample loading. During the initial development stage, devices were only visually examined using the following criteria: 1) well-defined control lines in both positive and negative controls, 2) clear presence of test line in positive controls and total absence in negative controls, and 3) absence of red smear across the nitrocellulose stripe in both positive and negative controls (**Figure 6**). Pictures were taken using an 8-megapixel iSight camera (Apple Inc., Ca, USA) and representative images were evaluated by an independent team of at least four people. Selection of potential prototypes was determined by consensus.

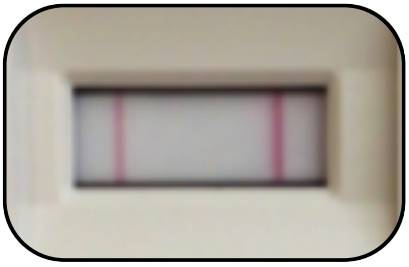
During fine-tuning of the LFIA Test Kit, the intensity of the control and test line was measured using an optical reader, the i-Lynx™ system (Spectral Diagnostics Inc., Toronto, ON, Canada), for comparison to the results obtained during visual evaluation. Measurements were mostly performed in duplicate or triplicate and reported as the mean ± standard error of the mean (SEM).

3.2.5 Determination of the Limit of Detection (LOD)

During the development phase, the LOD was determined to establish the sensitivity of the potential LFIA device prototypes. Therefore, only a working culture of *E. coli* O157 DSM17076 was used. Serial 10-fold dilutions were prepared using the appropriate broth up to a concentration of 10 CFU/ml. Aliquots of 200 µl of each dilution were incubated with the antibodies following the in-tube sandwich immunoassay protocol and 150 µl were

Figure 6. LFIA standard results. Positive Standard: result obtained with an in-tube sandwich immunoassay using a 10^7 CFU/ml *E. coli* O157 DSM 17076 working culture. Negative Standard: result obtained using TSB. Both samples were incubated 30 min at RT before they were loaded onto the device. Pictures were taken 15 min after loading. Both devices complied with the three criteria established for visual evaluation: clear control lines, defined test line with the positive control and complete absence on the negative, and no red smear along the nitrocellulose membrane.

Positive Standard



Negative Standard

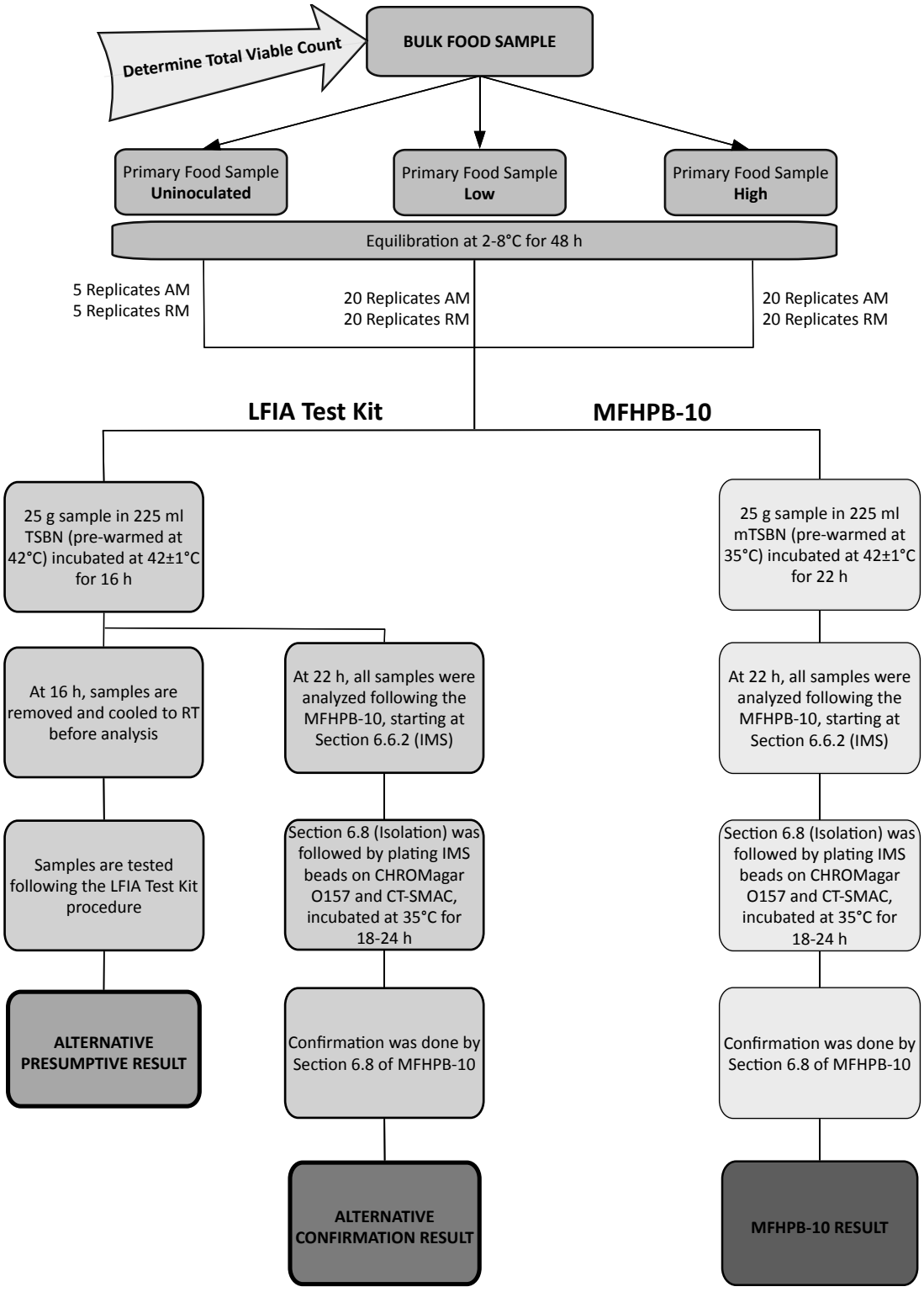
loaded into the LFIA devices. After 15 min, results were evaluated as described in **Section 3.2.4**.

3.3. Relative Validation of a LFIA Test Kit for Detection of *E. coli* O157 in Raw Meat Products

This section focuses on the methodologies performed to generate the data necessary for requesting the inclusion of the ADx™ Decision Point™ test kit for *E. coli* O157 (also referred as LFIA Test Kit in this document) in the Health Products and Food Branch Compendium of Analytical Methods from Health Canada. For this purpose, the guidelines outlined in the Procedure for the Development and Management of Food Microbiological Methods, Part 4 (March, 2011) from the Compendium of Analytical Methods were followed (174). The initial submission required a pre-collaborative study, also known as preliminary study, which consisted of comparing the alternative method (AM), in this case the LFIA Test Kit, to an approved reference method (RM). For this study, the reference method followed was the MFHPB-10 Isolation of *E. coli* O157:H7/NM from foods and environmental surface samples (October, 2014), which can also be found in the Compendium of Analytical Methods and is included in **Appendix B**. Furthermore, the relative validation not only involved the comparison of the AM to the RM, but also considered the results obtained with the RM as the true values, which were the basis for estimating the performance parameters of the LFIA Test Kit. Because the AM was developed using a different enrichment procedure than that suggested in the RM, the validation study followed an experimental layout for unpaired samples. This approach requires that each sample evaluated with the AM must be diverted to follow the RM path at the earliest stage possible to confirm the AM results. **Figure 7** summarizes the protocol for the relative validation using unpaired samples that was followed to assess the performance of the LFIA Test Kit at Laboratory Services Division, University of Guelph, ON, Canada.

Experiments in **Section 3.3.1** were performed at Robarts Research Institute, London, ON, Canada; while the submission report was prepared in collaboration with IPOC, Canada. The relative validation had to be performed by an accredited laboratory for Test Method

Figure 7. Workflow diagram for the relative validation of unpaired samples for the LFIA Test Kit. Sample preparation, inoculation levels and distribution between the alternative method (AM) and the reference method (RM) are depicted as the top of the workflow. The left path shows the alternative method procedure, which includes the divergence of samples to the reference method for confirmation of results. The right path describes the procedure of the 45 samples allocated to the reference method for comparison.


Determine Total Viable Count
BULK FOOD SAMPLE
Primary Food Sample Uninoculated
Primary Food Sample Low
Primary Food Sample High
Equilibration at 2-8°C for 48 h
5 Replicates AM
5 Replicates RM
20 Replicates AM
20 Replicates RM
20 Replicates AM
20 Replicates RM
LFIA Test Kit
MFHPB-10
25 g sample in 225 ml TSBN (pre-warmed at 42°C) incubated at 42±1°C for 16 h
25 g sample in 225 ml mTSBN (pre-warmed at 35°C) incubated at 42±1°C for 22 h
At 16 h, samples are removed and cooled to RT before analysis
At 22 h, all samples were analyzed following the MFHPB-10, starting at Section 6.6.2 (IMS)
At 22 h, all samples were analyzed following the MFHPB-10, starting at Section 6.6.2 (IMS)
Samples are tested following the LFIA Test Kit procedure
Section 6.8 (Isolation) was followed by plating IMS beads on CHROMagar O157 and CT-SMAC, incubated at 35°C for 18-24 h
Section 6.8 (Isolation) was followed by plating IMS beads on CHROMagar O157 and CT-SMAC, incubated at 35°C for 18-24 h
ALTERNATIVE PRESUMPTIVE RESULT
Confirmation was done by Section 6.8 of MFHPB-10
Confirmation was done by Section 6.8 of MFHPB-10
ALTERNATIVE CONFIRMATION RESULT
MFHPB-10 RESULT

Development and Evaluation and Non-Routine Testing under ISO/IEC 17025. Thus, the experiments explained in **Sections 3.3.2** and **3.3.3** were done by the Laboratory Services Division, University of Guelph, ON, Canada, who also provided an extensive report. Hence, information contained in both reports (Garth Styba, president IPOC, 2016, pers. comm.) was used in preparation of this section.

3.3.1 Inclusivity and Exclusivity Study

The 50 *E. coli* O157 strains and the 37 non-*E. coli* O157 strains listed in **Appendix A** were used. In both cases, working cultures were prepared as described in **Section 3.1.1** and coded using random numbering. Cultures were blindly run by an analyst using the LFIA Test Kit instructions found in **Appendix C**. All samples were run in triplicate to assess the consistency and reliability of the results.

If false positives were found during the exclusivity study, the bacterial strains were retested by streaking a loop of the overnight culture on TSA plates and incubating at 37°C for 24 h. A single isolated colony was selected and inoculated using TSBN at 42°C for 22 h, which is the enrichment protocol suggested in the LFIA Test Kit. Then, the culture was evaluated using the LFIA device. In addition, these strains were biochemically characterized to rule out any cross-contamination, following the methodology described in **Appendix A**.

3.3.2 Relative Validation of the LFIA Test Kit Using a Protocol for Unpaired Samples

3.3.2.1 Sample preparation

The validation study comprised two food types within the Raw Meat Food Category, which were Raw Meat (unprocessed) and Raw Meat (processed), as suggested in the Compendium of Analytical Methods. The main aim of performing a relative validation study was to demonstrate that the LFIA Test Kit could detect *E. coli* O157 equally or better than the cultural reference method (RM), MFHPB-10. Four food items per food type were evaluated; each one with twenty replicates that were equally split between the AM and the RM. In addition, ten more samples from each food type were included as negative controls (uninoculated) and were similarly split between AM and RM. A total of 180 samples, both unprocessed and processed raw meat products, were used. For each food type, Health

Canada's guidelines require that food items chosen for evaluation must be as different as possible (174). Thus, raw ground beef, raw ground veal, raw beef trim and carpaccio were chosen as representatives of unprocessed raw meat products. Raw sausage, raw hamburger patty, raw meatballs and raw kebabs were selected for representing processed raw meat products. Each food item had 10 high (H), 10 low (L) and 2 uninoculated (U) samples, with the exception of ground beef and raw sausage, which had 4 uninoculated samples each one.

All food samples used in this study were obtained by Laboratory Services Division, University of Guelph, from local retail stores in Guelph, ON, Canada. **Figure 8** depicts the distribution of samples evaluated.

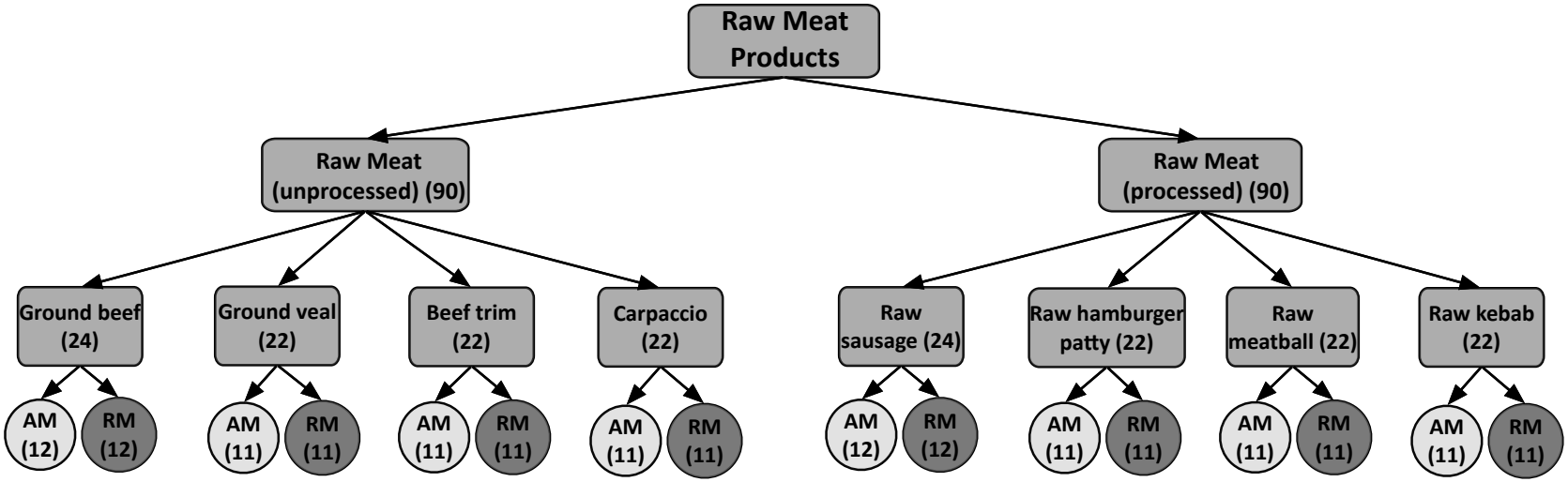
3.3.2.2 Preparation of inocula for processed and unprocessed food samples

The *E. coli* O157:H7 strains were procured from the Agriculture and Food Laboratory from the University of Guelph culture collection. For inoculation of Raw Meat (unprocessed) food items, strain *E. coli* O157:H7 ATCC 43889 from human origin was used. Briefly, an isolated colony, taken from a blood agar plate incubated overnight at 37°C, was used to inoculate 10 ml of BHI broth and further incubated 18-22 h with shaking at 35°C. Cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C. Moreover, the pellet was re-suspended and washed once using 10 ml of 0.1% peptone water for further use.

Raw Meat (processed) food items must be inoculated with a stressed culture, which was prepared using *E. coli* O157:H7 strain 380-94 from unknown origin. The culture was prepared as above and further incubated in a water bath at 50°C for 1 h (heat stress) before proceeding with sample inoculation. The % of sub-lethal injury was calculated as described in **Section 3.1.3** by plating in triplicate using non-selective (TSA) and selective (CHROMagar O157) agars, which were incubated at 35°C for 18-24 h. The % of sub-lethal injury achieved was reported as the average of the triplicates.

Once both inocula were prepared and equilibrated for 18-24 h at 4°C, they were serially diluted 10-fold using 0.1% peptone water. Two inoculation levels were needed: a "low inoculum level (L)", based on the likelihood of having a fractional recovery (a target inoculum level of <5 CFU/25g), and a "high inoculum level (H)", calculated at

Figure 8. Scheme of the distribution of samples for the unpaired validation study. A total of 90 samples were evaluated for each food type (Raw Meat Unprocessed and Processed) distributed among four different Food Items respectively. Each Food Item had 24 or 22 replicates, which were equally split between the alternative method (AM) and the reference method (RM).


Raw Meat Products
Raw Meat (unprocessed) (90)
Raw Meat (processed) (90)
Ground beef (24)
Ground veal (22)
Beef trim (22)
Carpaccio (22)
Raw sausage (24)
Raw hamburger patty (22)
Raw meatball (22)
Raw kebab (22)
AM (12)
RM (12)
AM (11)
RM (11)
AM (11)
RM (11)
AM (11)
RM (11)
AM (12)
RM (12)
AM (11)
RM (11)
AM (11)
RM (11)
AM (11)
RM (11)

approximately 1 log higher than (L). The definite culture titers were estimated using standard plate counts on TSA incubated at 35°C for 18-24 h.

3.3.2.3 Food sample inoculation

Firstly, TVC for each food item was estimated according to the method MFHPB-33 Enumeration of Total Aerobic Bacteria in Food Products and Food Ingredients Using 3M™ Petrifilm™ Aerobic Count Plates from the Compendium of Analytical Methods (**Appendix D**). To ensure sample homogeneity, all food items were initially kept in bulk before splitting them. The equivalent of 20 replicates of each food item was split into 2 primary groups for low (L) and high (H) inoculation levels. The rest of the replicates were assigned to the uninoculated group (U). Each primary group was inoculated in bulk with the correspondent *E. coli* O157 strain, either (L) or (H) inocula prepared as previously described.

Inoculated bulk samples were mixed and equilibrated at 4°C for 48 h before splitting into 25 g portions and randomly assigned to either of the methods described in **Figure 8**. This procedure assured that the true inoculation status of the samples is unknown and thus the number of true positives within each method could be considered as statistically equal. In addition, an unpaired samples protocol is performed whenever an alternative method has a different enrichment than the reference method used for comparison. Therefore, the final sample distribution consisted of 20 (L), 20 (H) and 5 (U) per food type and method of analysis respectively.

3.3.2.4 Most Probable Number (MPN) determination of inoculated bulk samples

To determine *E. coli* O157 concentration after the 48 h equilibration at 4°C before enrichment, the MPN was determined using a remaining portion of the primary inoculated bulk samples of each food item evaluated. Portions of 50 g were taken in triplicate from each high and low inoculation level. Each replicate was mixed with 450 ml of mTSBN (1:10 dilution). Then, 10% (50 ml) was taken and mixed with another 450 ml of mTSBN (1:100 dilution). A third dilution was performed in the same way to yield a 1:1000 dilution. Finally, 50 ml were removed from this last sample to maintain the same volume as the

previous samples. The final result was a 3×3 (three replicates, three dilution-levels) MPN preparations, equivalent to having 45 g, 4.5 g and 0.45 g sample sizes. Samples were incubated at 42°C for 22-24 h before they were further processed using immunomagnetic separation (IMS) as per MFHPB-10. Furthermore, suspect colonies grown on CHROMagar O157 and cefixime tellurite sorbitol MacConkey (CT-SMAC) were confirmed as described in Section 6.8 of the MFHPB-10 method. Finally, the MPN calculator (http://members.ync.net/mcuriale/mpn/index.html) was used to estimate the MPN, based on the number of confirmed positives and reported as MPN/25g.

3.3.2.5 LFIA Test Kit alternative method

The 90 inoculated test portions of 25 g that were randomly assigned to the AM were placed in Stomacher® bags and homogenized in 225 ml of TSBN for 60 s using a Stomacher®. Sample bags were loosely closed to allow air exchange for *E. coli* O157 growth and incubated at 42±1°C for 16-24 h. After 16 h of enrichment, samples were analyzed using the LFIA Test Kit Method. Briefly, 200 µl of the sample were pipetted directly into tube A, while another aliquot of 10 µl of the enriched sample was diluted 1:100 with sample diluent before pipetting 200 µl into Vial B. Furthermore, 10 µl of the sample buffer were added to each vial, which were mixed thoroughly and incubated 30 min at RT. Subsequently, 150 µl from each vial were loaded into their correspondent sample port in the LFIA device. Results were visually assessed after 15 min according to the following pattern: a positive result would show control lines in both A and B result windows and test lines in either A and/or B. On the other hand, negative test results would show control lines in both A and B result windows, but no test lines in neither A nor B. These results were recorded as the Alternative Presumptive Result (AP). The complete LFIA Test Kit methodology and schemes for the proper interpretation of results are shown in **Appendix C**.

Because the validation study required an unpaired sample protocol due to different enrichments used for the AM and RM, the AM enriched samples were re-incubated until completing 22 h at 42°C. Afterwards, samples were analyzed as described in the MFHPB-10 using concentration by IMS followed by selective isolation and ending with confirmation of suspected colonies (**Appendix B**). The results obtained were recorded as

the Alternative Confirmation Result (AC). **Figure 9** summarizes the process path followed to analyze the samples assigned to the alternative method.

3.3.2.6 Reference method MFHPB-10

The 90 samples assigned to the RM were tested following the RM MFHPB-10, which is found in **Appendix B**. Briefly, 225 ml of mTSB with 20 µg/ml Novobiocin (mTSBN) were mixed with each 25 g sample and homogenized for 2 min in a Stomacher®. Samples were incubated at 42±1°C for 22 h, before they were analyzed as described above, starting with IMS and finishing with confirmation of suspected colonies following the MFHPB-10 method (**Appendix B**). The results obtained were recorded as the MFHPB-10 reference method result (RM).

3.3.2.7 LOD

For the determination of the LOD, only one food item was sufficient; thus ground beef was chosen. The methodology described in Annex 4.5, Supplement to the Procedure for the Development and Management of Food Microbiological Methods, Determination of the Limit of Detection found in the Compendium of Analytical Methods (174) was followed. Briefly, five spiking levels with six replicates each of artificially inoculated samples at a range of known inoculum, were analyzed using the AM. In order to determine the inoculum levels, a 3×3 MPN was performed using the highest inoculation level and following the methodology described in **Section 3.3.2.4**. The lowest cell concentrations were extrapolated from the results obtained from the MPN, while the LOD was estimated within the two levels that gave respectively more and less than 50% (3/6) positives. A scheme summarizing the LOD determination is found in **Figure 10**.

3.3.3 Evaluation of Probability of Detection (POD) and Performance Parameters

For calculating the performance parameters of an AM using a relative validation procedure, the Compendium of Analytical Methods contains the statistical analyses for assessing the data generated experimentally. Annex 4.1 Performance Parameters of Microbiological Methods - Note on Sensitivity and Specificity and Annex 4.4 Supplement to the Procedure for the Development and Management of Food Microbiological Methods, Procedure for the Statistical Evaluation and Calculation of Performance Parameters of a New Alternative

Figure 9. Flow chart of the LFIA Test Kit alternative method. The enriched sample was evaluated at 16 h of enrichment using the alternative method. The results obtained were considered the Alternative Presumptive Results (AP) (right). Furthermore, samples were re-incubated up to 22 h and analyzed following the reference method starting at the immunomagnetic separation (IMS) procedure. These results were considered the Alternative Confirmation Results (AC) (left).

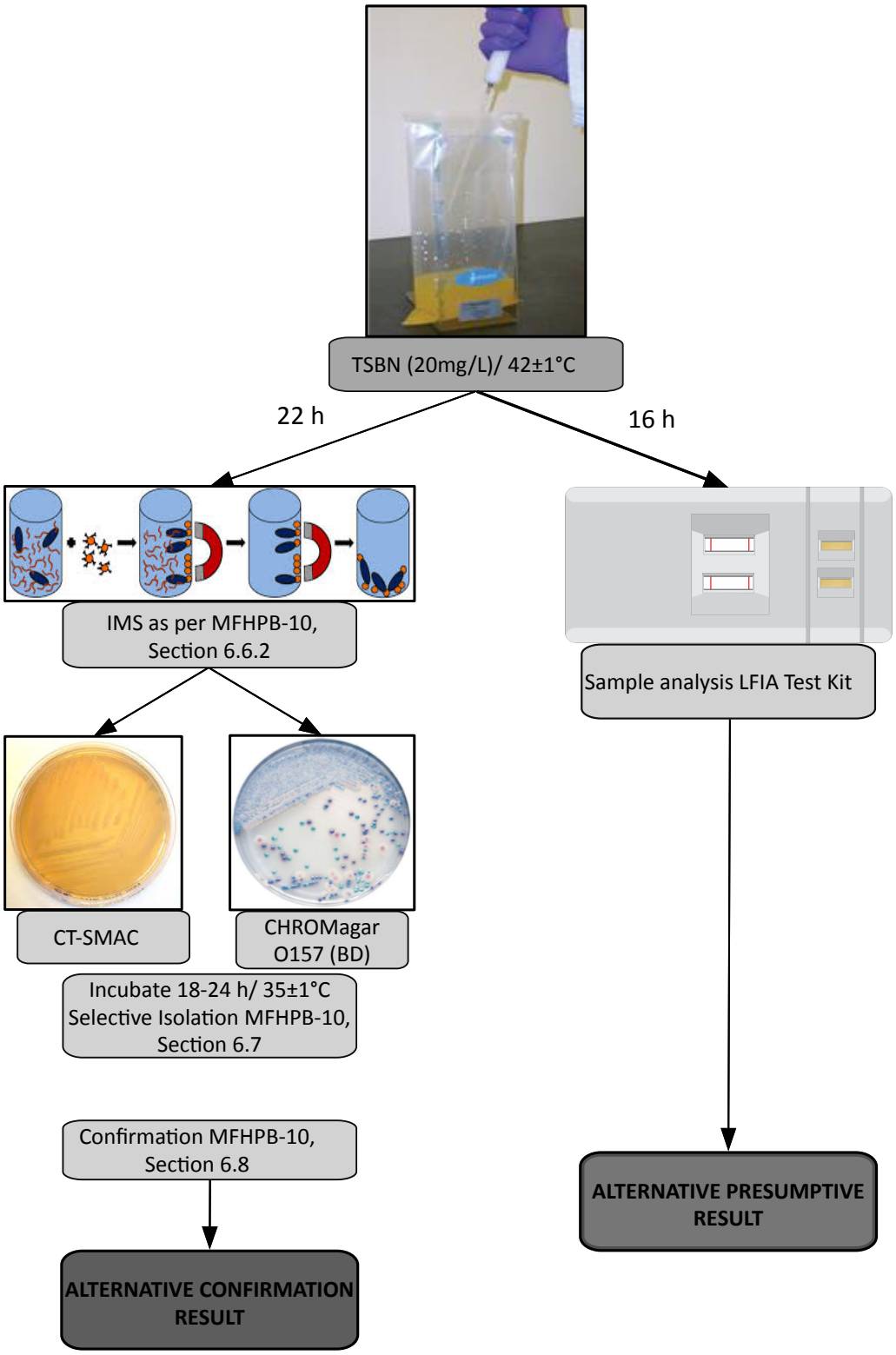
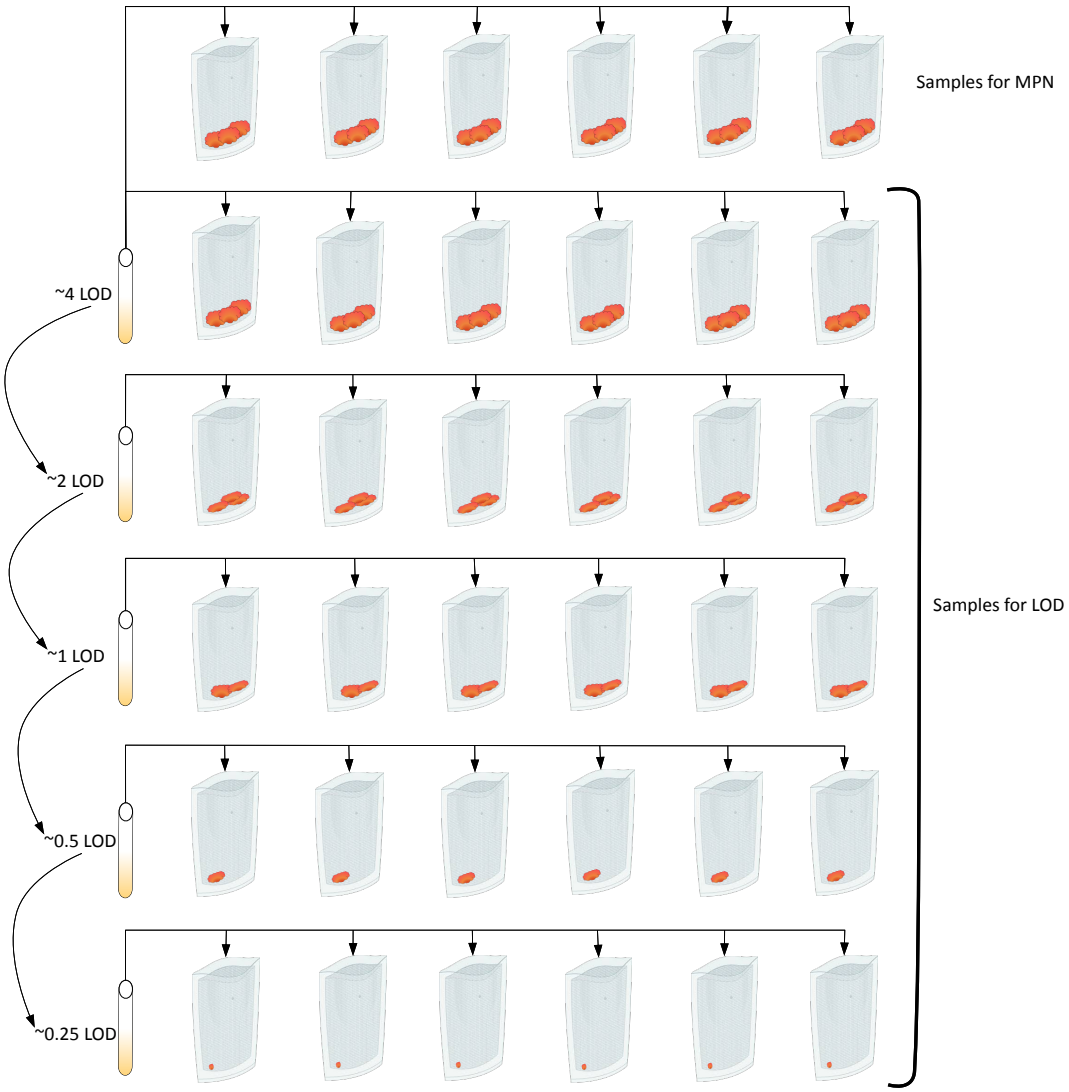

TSBN (20mg/L)/ 42±1°C
22 h
16 h
IMS as per MFHPB-10, Section 6.6.2
Sample analysis LFIA Test Kit
CT-SMAC
CHROMagar O157 (BD)
Incubate 18-24 h/ 35±1°C Selective Isolation MFHPB-10, Section 6.7
Confirmation MFHPB-10, Section 6.8
ALTERNATIVE CONFIRMATION RESULT
ALTERNATIVE PRESUMPTIVE RESULT

Figure 10. LOD sample preparation scheme. The first row represents the number of samples and inoculation level needed for calculating the MPN. Second to sixth rows show the sequence of dilutions needed to estimate the LOD of the LFIA Test Kit.


Samples for MPN
~4 LOD
~2 LOD
~1 LOD
~0.5 LOD
~0.25 LOD
Samples for LOD

Qualitative Method Compared to a Reference Cultural Method (174), were followed accordingly for presentation and analysis of data. Based on these guidelines, raw data were summarized in separate tables comprising the AM results and the RM results for each food type, due to the fact that an unpaired sample study was required. For the AM the Presumptive Result (AP) and the Confirmation Result (AC) were combined to obtain the Alternative Final Result (AF). Moreover, the Reference Method was considered to be 100% sensitive and specific because it was based on traditional culturing techniques. Thus, it could not produce false positives nor false negatives. These raw data were used to conduct a Probability of Detection (POD) analysis, which included estimating the POD for the alternative method presumptive results (POD_{AP}), the POD for the alternative method final results (POD_{AF}) and the POD for the reference method results (POD_{R}) with a 95% confidence interval. The POD is calculated by dividing the number of positives (x) by the number of samples analyzed (N). Thus, the POD is calculated for each spiking level per food type evaluated. Moreover, the comparative performance of the two methods was estimated by the differences in the POD values according to the following formulas:

$$dPOD_{(AF,R)} = POD_{AF} - POD_{R}$$

$$dPOD_{(AP,AF)} = POD_{AP} - POD_{AF}$$

If the 95% confidence interval associated with each difference included the value zero (0), the results of the AM were considered equivalent to those of the RM, and the range of false positive for that specific inoculation level and food item were considered acceptable, respectively. Finally, data from the AP and AF were used to calculate the performance parameters of the AM. When the AP result was confirmed by the RM, the AF was considered a true positive (TP); however, if the confirmation resulted as negative, then the final result was considered a false positive (FP). On the other hand, if the AP was negative and confirmed by the AF, it was considered a true negative (TN), but if the confirmation was positive, the result was a false negative (FN). This final evaluation was used:

$$Sensitivity = \frac{True\ Positives}{True\ Positives + False\ Negatives} x100$$

$$Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positives} x100$$

$$False\ Negative\ Rate = \frac{False\ Negatives}{True\ Positives + False\ Negatives} x100$$

$$False\ Positive\ Rate = \frac{False\ Positives}{True\ Negatives + False\ Positives} x100$$

3.4. Development of a Humanized Single-Chain Variable Fragment (scFv) Against the O-antigen of *E. coli* O157

3.4.1 Hybridoma Cell Line Growth Conditions, Screening and Propagation

The 13B3 hybridoma cell line used in this study was kindly provided by Dr. James Bono from the Agricultural Research Service, U.S. Meat Animal Research Center, USDA, in Nebraska, USA (227). Cryopreserved cells were thawed in a water bath at 37°C and quickly diluted 1:10 in pre-warmed Gibco® Hybridoma-serum free medium (SFM) (Gibco®, Life Technologies Inc., Carlsbad, CA, USA). Cells were harvested by centrifugation at 200 ×g for 5 min at 4°C, and the supernatant removed. The pellet was re-suspended in 5 ml of Hybridoma-SFM supplemented with 10% fetal bovine serum (FBS; Gibco®, Life Technologies Inc., USA) and placed in T25-flasks and incubated at 37°C in 5% CO_2. After 24 h, 5 ml of Hybridoma-SFM with 10% FBS were added to the T25-flasks and returned to the incubator. Cell viability was checked using an haemocytometer and the Trypan Blue Exclusion Method (228,229). When the cell density reached 10^5–10^6 cells/ml and the viability was above 90%, cells were subcloned following the 96-well plate Limiting Dilution Method, using a theoretical final concentration of 1 cell/well (228,230–233). After one week, 100 µl of Hybridoma-SFM were added to those wells with single colonies. The 96-well plates were re-incubated until single colony wells reached 30–50% confluence and the medium turned yellow (228,231). At this point, the supernatants of single colony wells were screened for antibody production using a sandwich ELISA against *E. coli* O157:H7. To ensure the stability and monoclonality of the hybridoma cell-line, one of the positive clones was randomly selected and subcloned for a second time, as described above. After this second subcloning, the three clones with the highest antibody titre were sequentially

expanded to a 12-well plate, then a 6-well plate, and finally to a T75-flask. When cells reached >90% viability and a density of 10^6 cells/ml, they were harvested for cryopreservation by centrifugation at 200 ×g for 5 min at 4°C, and the supernatant discarded. The pellet was re-suspended in freshly made FBS with 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, USA) and quickly transferred into cryopreservation vials for storage at -20°C overnight. Subsequently, cryovials were moved to a -80°C freezer for a maximum of one week before they were placed in a liquid nitrogen tank for long-term storage.

For antibody production and RNA extraction, cells were harvested from a cryopreserved sample as previously described. This time, the pellet was re-suspended in 2 ml of Hybridoma-SFM with 10% FBS and transferred to the first well (#1) of a 24-well plate, with 7 more wells filled with 1 ml of Hybridoma-SFM with 10% FBS. A serial dilution was performed by transferring 1 ml from well #1 to well #2. Then, 1 ml of well #2 was transferred to well #3, and followed the same sequence until well #7 was reached. Cultures were mixed thoroughly in the well before proceeding with each dilution. The plate was incubated at 37°C in 5% CO_2 for 1–2 d until the initial wells reached 70% confluence. At that point, cells were collected and placed in 15 ml conical tubes with 10 ml of fresh Hybridoma-SFM. Cells were harvested by centrifugation at 200 ×g for 10 min at RT and the supernatant was replaced with 2 ml of Hybridoma-SFM for pellet resuspension. The cells were transferred to a 6-well plate containing 4 ml of the same medium, and incubated at 37°C in 5% CO_2. Plates were checked daily for cell viability and overcrowding. When wells in the 6-well plate reached 60–70% confluence, cells were transferred to a T75-flask containing 14 ml of Hybridoma-SFM. Cells were subcultured in T75-flasks in a 1:10 ratio for a maximum of three passages. When cell viability was >90%, an aliquot was taken for RNA extraction. The rest of the culture was re-incubated for antibody production until the medium turned yellow and the cell viability decreased to approximately 50%. Finally, the supernatant was collected by centrifugation at 200 ×g for 10 min at 4°C for antibody purification.

3.4.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting (WB) for Protein Visualization

A 10 or 12% polyacrylamide resolving gel with 5% stacking gel was prepared using Tris-Glycine Running Buffer, then 1D vertical SDS-PAGE was performed for 1.7 h at 110 V using a Mini-PROTEAN System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were mixed in a 3:1 ratio with Laemmli buffer (4×) and boiled for 10 min before loading into the gel. The gel was fixed and stained with Coomassie Blue staining solution for 10 min followed by destaining until the background of the gel was clear. For size determination, the BLUeye Prestained Protein Ladder (FroggaBio Inc., Toronto, ON, Canada) was used for comparison.

For Western Blotting analysis, protein samples were separated on a 12% polyacrylamide resolving gel prepared as described above, without end-stage staining. Instead, proteins were immobilized for detection by transferring the samples from the gel matrix to a nitrocellulose membrane, 0.45 μm (Bio-Rad Laboratories Inc., USA) using electroblotting with a Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories Inc, USA) for 1.2 h at 16 V, 0.26 limit for one gel. Prior to transfer, both the resolving gel and the membrane were equilibrated with Towbin Semi-Dry Transfer Buffer for 20 min with gentle shaking. After transfer, the membrane was blocked using 5% (w/v) non-fat dry milk in 1×TBST overnight at RT with shaking. For protein detection the biotinylated tag was probed for 2 h at RT with conjugated streptavidin-IRDye800 (1:10,000 in 1×TBST with 0.5% (w/v) non-fat dry milk). The membrane was washed 3 times with 1×TBST for 5, 7 and 10 min, respectively, and a last wash of 5 min with 1×TBS prior to viewing using a LI-COR Odyssey® Infrared Imaging System (LI-COR, Lincoln, NE, USA). The composition of all buffers and reagents is described in **Appendix E**.

3.4.3 Enzyme-Linked Immunosorbent Assay (ELISA) and Fluorescent-Antibody Microscopy for Antibody Functionality Assessment

An ELISA was performed for hybridoma screening and to determine the functionality of both the purified murine monoclonal anti-O157 and its derived single-chain variable fragment (scFvO157). The procedure was adapted from Westerman *et al.* (227). Briefly, microtiter plates were coated with 0.1 ml of an overnight working culture diluted to

approximately 5×10^8 CFU/ml in Coating Buffer. The plates were sealed with Saran wrap and incubated overnight at 4°C. *E. coli* O157 DSM 17076 was used as a positive control, while *E. coli* ATCC 25922, *S. enterica* ser. Typhimurium and *L. monocytogenes* were used as negative controls. Plates were washed 3 times with 1×PBST before adding 200 µl of Blocking Buffer and incubated for 1 h at RT. Washing was repeated as before and the appropriate sample (hybridoma supernatant, purified mAb or scFvO157) or controls (Hybridoma-SFM or protein diluent) was added to the wells. This time, plates were incubated for 2 h at RT. For hybridoma screening, the supernatant was undiluted, however, the mAb and scFvO157 samples were diluted in Blocking Buffer and assessed at different concentrations. Furthermore, the plates were washed 6 times as described above, blocked again with 200 µl of Blocking Buffer and incubated for 17 min or 1 h at RT then washed again. When evaluating the mAb functionality, plates were incubated 1 h at RT with 100 µl of peroxidase-conjugated anti-mouse IgG heavy and light chains (Jackson ImmunoResearch Inc., PA, USA) diluted 1:5,000 in Blocking Buffer. On the other hand, horseradish peroxidase conjugated streptavidin (Streptavidin-HRP; Moss, Inc., Maryland, USA) was used for scFvO157. Finally, plates were washed 6 times and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) ELISA peroxidase substrate (Rockland Immunochemicals Inc., USA) were added to each well. After 20 min at RT, plates were read using a VMax Kinetic ELISA Microplate Reader (Molecular Devices, LLC., CA, USA) at a wavelength of 650 nm. The absorbance of control samples was used to determine the cut-off value as established by the formula:

$$Cut-off\ Value = Average\ Blank + 3(SD)$$

Absorbance values above the cut-off were considered positive, while values below it were considered negative.

For fluorescent microscopy, a loop from an overnight working culture was streaked onto a TSA plate and incubated at 37°C for 24 h. A colony was picked and spread onto a glass slide using a drop of water. Slides were allowed to air dry before they were fixed in cold (-20°C) acetone (Sigma-Aldrich, USA) for 10 min and air dried again. Two circles were drawn on each slide using a wax pencil to keep the reagents from spreading and drying out during incubation. As a negative control, 100 µl of 1×PBS were added to one circle, while

100 µl of either scFvO157 or mAb dilution were added to the other circles. After incubation at 37°C for 30 min in a moisture chamber, the slides were washed 3 times in 1×PBS and dried at RT before 100 µl of Streptavidin-fluorescein isothiocyanate conjugate (Streptavidin-FITC; BioLegend, San Diego, CA, USA) or anti-mouse IgG (whole molecule)-FITC (Sigma-Aldrich, USA) diluted 1:40 in 1×PBS with 5% BSA were added to each circle. The slides were re-incubated for 30 min at 37°C and washed as before with a final 2 min wash in distilled water. Slides were air dried and coverslips were mounted by using a drop of AquaPerm mounting medium (Life Technologies Inc., Carlsbad, CA, USA). The slides were then examined with an Olympus IX71 Inverted Micropscope (Olympus Canada Inc., Richmond Hill, ON, Canada).

3.4.4 Murine mAb Characterization and Purification

The supernatant collected from the T75-flasks was initially isotyped using the Mouse Immunoglobulin Isotyping ELISA Kit from BD Pharmigen™ (BD Biosciences, San Jose, CA, USA). Eight different mouse immunoglobulin isotype-specific rat monoclonal antibodies (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, Ig κ, and Ig λ) were coated in each row of a 96-well plate. The positive control was an antigen mixture provided with the kit, while the negative control was the Hybridoma-SFM medium. As the detection antibody, a HRP-labeled rat anti-mouse Ig monoclonal antibody was used. Preparation of reagents and assay conditions were according to the manufacturer's instructions.

The supernatant was concentrated 10 times using the Amicon® Ultra-15 Centrifugal Filter Units NMWL 30 kDa (EMD Millipore, Germany) at 4000 ×g before purification using the Nab™ Spin Kit for Antibody Purification (Thermo Scientific, IL, USA). A gravity-flow procedure was followed as per the manufacturer's instructions. In brief, the sample was equilibrated with binding buffer in a 1:1 ratio and applied to the column. The column was washed using 15 ml of binding buffer, then the elution buffer (12 ml) was applied. The eluted antibody was collected in 3 ml fractions in tubes containing the appropriate volume of neutralization buffer. Purified fractions were analyzed by SDS-PAGE; those that stained positive pooled together, concentrated and dialyzed against 1×PBS pH 7.4 using the Amicon® Ultra-15 Centrifugal Filter Units NMWL 30 kDa. The antibody was assessed for homogeneity and purity by SDS-PAGE, and quantified using a NanoDrop 1000

Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Finally, aliquots were prepared and stored at 4°C for short-term use and at -20°C for long-term (up to 6 months).

The isoelectric point (pI) of the mAb was also determined with Ettan IPGphor II Isoelectric Focusing (IEF; Amersham Bioscience AB, Uppsala, Sweden) equipment. An aliquot of the purified mAb in 1×PBS, pH 7.4 was buffer exchanged using only water and the Amicon® Ultra-15 Centrifugal Filter Units NMWL 30 kDa. The sample was washed three times to remove 99% of the buffer. The appropriate sample volume was mixed with rehydration buffer stock solution (RBSS) and IPG buffer (GE Healthcare Life Sciences, Piscataway, NJ, USA). The mixture was spread evenly along the groove of each sample boat. Furthermore, the 13 cm gel strip (Immobiline DryStrip, GE Healthcare Life Sciences, USA) was placed with the gel facing down into each boat. For this assay, linear pH 3-10 range strips were used. The strips were covered with Dry Strip Cover Fluid (GE Healthcare Life Sciences, USA) and then covered with the lid. The following step-n-hold protocol was used to run the samples: 20 V for 12 h, 100 V for 20 min, 500 V for 500 Vh, 1000 V for 1000 Vh, 2000 V for 4000 Vh, 4000 V for 8000 Vh, 6000 V for 12000 Vh, and 8000 V for 30000 Vh, for a total running time of 24 h. The gels were taken out of the boat, fixed with 20% trichloroacetic acid (TCA; BDH Chemicals, VWR International, LLC) for 30 min, then washed with 40% methanol (Caledon Laboratories, ON, Canada) and 7% acetic acid (Sigma-Aldrich, USA) for 3 min, and used for direct staining with Gelcode™ Blue (Thermo Fisher Scientific Inc., USA) for 1 h. Strips were destained twice for 15 min with distilled water before distinct bands were visualized. The approximate distance for the bands with respect to the beginning of the gel strip was measured in cm and the pI was determined using the DryStrip gradient data provided by the gel strip supplier.

3.4.5 Hybridoma RNA Isolation

Between 1×10^6 and 5×10^6 hybridoma cells from an expanded ELISA-positive clone were harvested for RNA extraction. Hybridoma cells were centrifuged for 5 min at 200 ×g at 4°C, the supernatant was discarded and the cells were washed with 1×PBS. The PureLink® RNA Mini Kit (Life Technologies, USA) was used following the manufacturer's protocol for RNA purification from animal and plant cells. To ensure that total RNA was DNA-free,

an on-column DNase digestion was performed using a RNase-Free DNase Set (Qiagen Gmbh, Hilden, Germany) before washing the column. Total RNA elution was achieved with 50 µl of Dnase/RNase/Protease-free water (BioShop, Canada) and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA).

3.4.6 mAb Variable Regions Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

The primers used for the RT-PCR were synthesized by Invitrogen® (Life Technologies, USA) based on the sequences published by Wang *et al*. (196), shown in **Table 7**. The highly degenerate 5' primers for both variable heavy (V_H) and light (V_L) chains were designed to start at the first nucleotide of the framework region 1 (FR1). For the V_H, a combination of two high degeneracy primers was used to cover the majority of possible sequences, ensuring amplification of the V_H gene. On the other hand, the 3' primers were complementary to the first constant region of each of the heavy and light kappa chains (C_H1 and $C_{\kappa L}$). In addition, to be chain specific, the C_H1 primer used in this study was isotype-specific for IgG3. All primers were rehydrated with the appropriate volume of Tris-EDTA (TE) buffer (Thermo Fisher Scientific Inc., USA) to obtain 100 µM stock solutions that were stored at -20°C. They were diluted 1:10 in TE buffer for preparation of working solutions that were stored at 4°C for short-term use.

The QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used to synthesize cDNA from 2 µg of total RNA. Two reactions were carried out, each one containing a final concentration of 0.7 µM of either the C_H1 or $C_{\kappa L}$ gene-specific primer. The rest of the procedure was followed as per the manufacturer's protocol. The resultant cDNA was amplified by PCR (T100™ Thermal Cycler, Bio-Rad Laboratories, USA) using 1.25 U of Taq Polymerase (Life Technologies Inc., USA), 0.2 mM deoxyribonucleotide triphosphates (dNTP; Life Technologies Inc., USA), 1.5 mM $MgCl_2$, 0.2 µM of each primer (C_H1 and M_H1/M_H2 for V_H reaction; $C_{\kappa L}$ and M_κ for V_L), 1×PCR buffer and 2 µl of the respective cDNA. The thermal cycling conditions for the amplification of the V_H were as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 95°C for 45 s, 45°C for 45 s, and 72°C for 1 min. The V_L amplification was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 10 cycles of 95°C for 45 s,

Table 7. Sequences of primers for mAb V_H and V_L chains RT-PCR.

Primer Name	Sequence (5'-3')[1]	Description
C_H1	gga aga tct AGG GAC CAA GGG ATA GAC AGA TGG	Mouse heavy chain first constant region primer. IgG3 isotype specific (196)
$C_{\kappa L}$	ggt gca tgc GGA TAC AGT TGG TGC AGC ATC	Mouse kappa chain constant region primer (196)
M_H1	ctt ccg gaa ttc SAR GTN MAG CTG SAG SAG TC	Mouse heavy chain FR1 high degeneracy primers (196)
M_H2	ctt ccg gaa ttc SAR GTN MAG CTG SAG SAG TCW GG	
M_κ	gg gag ctc GAY ATT GTG MTS ACM CAR WCT MCA	Mouse kappa chain FR1 universal degenerate primer (196)

[1]*Underlined nucleotides represent restriction enzyme recognition site.*

45°C for 45 s, and 72°C for 1 min. Then, 10 cycles of 95°C for 45 s, 47°C for 45 s, and 72°C for 1 min. Finally, 10 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 1 min. In both cases, the final extension was achieved at 72°C for 7 min and the reaction cooled to 4°C. Both of these amplification thermal cycling conditions were previously published by Koren *et al.* (195).

3.4.7 Plasmid DNA Isolation

Plasmid DNA was isolated and purified from *E. coli* XL1 Blue or BL21 (DE3) cells in stationary phase (O/N aerobic growth at 37°C) with a QIAprep Spin Miniprep Kit (Qiagen, Venlo, Limburg, Netherlands) as per the manufacturer's microcentrifuge protocol. Plasmid DNA was eluted with 50 µl of EB buffer (Qiagen, Netherlands) and stored no longer than one month at -20°C for further use.

3.4.8 DNA Visualization and Gel Extraction

DNA was resolved by electrophoresis on 1%, 1.5% or 2% agarose DNA grade high-melt gels (Thermo Fisher Scientific Inc, USA) in 1×TBE buffer at 85 V using a Mini-Sub Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories Inc., USA). Samples were mixed 2:1 with gel loading buffer for nucleic acids (Sigma-Aldrich, USA), stained with ethidium bromide (EtBr; MO BIO Laboratories, Inc., Qiagen, USA) and visualized under ultraviolet light (UV; EpiChemi3 with 3UVTM Transilluminator, UVP, LLC., CA, USA). For DNA fragment size determination, bands were compared against a 1 Kb Plus DNA ladder (Invitrogen, Life Technologies Inc., USA). The DNA bands of interest were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Netherlands) following the manufacturer's protocol. Purified DNA was eluted with 30 µl of EB buffer (Qiagen, Netherlands) and the final concentration measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA).

3.4.9 DNA Sequencing

Sequencing of the V_H, V_L chains and plasmid DNA was performed at Robarts Research Institute Sequencing Facility (Western University, London, ON, Canada) using either the specific primers in **Table 7** or the standard primers provided by the Sequencing Facility,

which are described in **Table 8**. All samples were prepared following the standard operating procedures of the Sequencing Facility (www.robarts.ca/sample-preparation).

3.4.10 Construction of the Humanized scFvO157

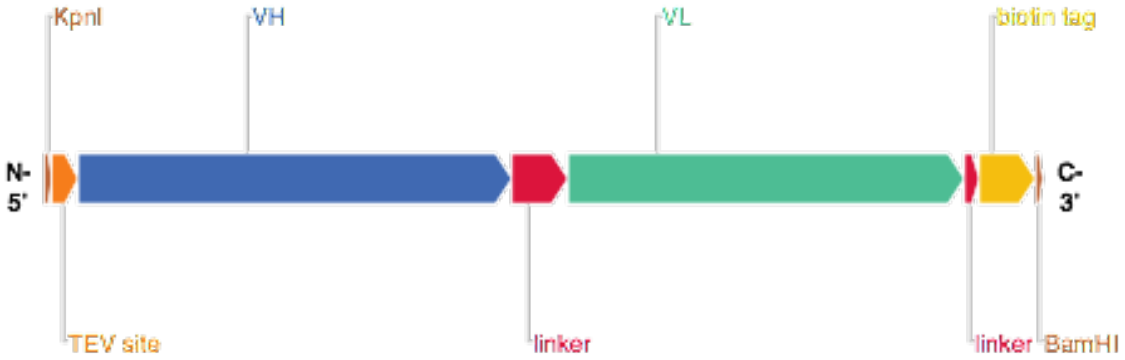
The V_H and V_L nucleotide sequences were translated into amino acids using the ExPASy online translation tool from the Swiss Institute of Bioinformatics (SIB; http://web.expasy.org/translate/). To confirm that both sequences were new and corresponded to V_H and V_L murine domains, each one was submitted to the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), where the non-redundant protein database was selected to perform a multiple sequence alignment (MSA) using the Constraint-Based Multiple Alignment Tool (COBALT). This helped to determine the degree of conservation of each residue based on the Identity Conservation Setting. Moreover, the specific CDR boundaries were defined based on predicted topological 3D modeling. Briefly, the V_H and V_L sequences were submitted to SWISS-MODEL (www.swissmodel.expasy.org) to obtain a structure homology model based on the template with the highest sequence identity found in the Protein Database (PDB). Using the COBALT results, the regions with more variability were used to define the complementarity determining region (CDR) loops in the 3D model. Predicted CDR loops and the immediate amino acids flanking them were finally grafted into a consensus humanized backbone previously described by Patterson *et al.* (234). Finally, the humanized scFvO157 construct was designed to have a Tobacco Etch Virus (TEV) cleavable site at the N-terminal and a C-terminal biotin tag, as well as KpnI and BamHI restriction enzyme sites, respectively, for strategic cloning purposes (**Figure 11**). The final humanized scFvO157 was codon optimized for cloning and expression in *E. coli* cells, synthesized and cloned into pUC57 plasmid by GenScript Corp (Piscataway, NJ, USA).

Further along the process, the humanized scFvO157 sequence was reanalyzed using antibody informatics tools such as the Prediction of ImmunoGlobulin Structure (PIGS) web-based server (http://circe.med.uniroma1.it/pigs/)(235) and Maestro Software (Schrödinger, New York, NY, USA). The former was used to re-design the mAb. Briefly, the single sequence submission was chosen, which allowed for the prediction of a single

Table 8. Sequences of standard primers provided by the Sequencing Facility at Robarts Research Institute, Western University.

Primer Name	Sequence (5' - 3')	Description
M13/pUC forward	CGC CAG GGT TTT CCC AGT CAC GAC	Primers for sequencing inserts into pUC plasmids.
M13/pUC reverse	TCA CAC AGG AAA CAG CTA TGA C	
T7 promoter	TAA TAC GAC TCA CTA TAG GG	Primers for sequencing inserts into pET plasmids.
T7 terminator	GCT AGT TAT TGC TCA GCG G	

Figure 11. Humanized scFvO157 construct. Complete humanized scFvO157 construct used for further cloning and expression. KpnI and BamHI restriction enzyme sites are shown at the N- and C-terminal respectively. TEV cleavable site is found at the N-terminal (orange) followed by the V_H chain (blue) joined by a $(G_4S)_3$ linker (red) to the V_L chain (green). The biotin tag (yellow) found in the extreme C-terminal was attached to the V_L by a SG_3 linker.


KpnI
VH
VL
biotin tag
N-
5'
C-
3'
TEV site
linker
linker
BamHI

antibody by uploading the sequences of the V_H and V_L chains from the mAb. For template selection, the default number of results shown, which is 20, was used. Moreover, the "Best H and L chains" method was chosen and the V_H and V_L chain structure templates with the highest percentage of identity were selected for the prediction step. The Loop Grafting Method and Side Chain Modeling Method were kept as default. The predicted structure in PDB format was visualized with Maestro Software for subsequent structure analysis. On the other hand, the amino acid sequence of the consensus humanized scFv used as backbone for CDR grafting was submitted to SWISS-MODEL to obtain a 3D model and further imported into Maestro Software for analysis. 3D models were superimposed using the Protein Structure Alignment tool and the alignment score and root-mean-square deviations (RMSD) were calculated to quantify the similarity between the two models. Finally, the Multiple Sequence Viewer was used to compare some of the intrinsic properties such as hydrophobicity between both proteins.

3.4.11 Molecular Cloning of the scFvO157

3.4.11.1 Restriction digestion

The gene encoding the TEV::scFvO157::biotin tag was excised from pUC57 using the restriction enzymes KpnI and BamHI (New England BioLabs Inc., Ipswich, MA, USA) following the manufacturer's instructions. Briefly, the double digestion reaction consisted of the target DNA plasmid, 1 U of each enzyme/µg DNA, 1×enzyme specific buffer and if necessary, 1×BSA (New England BioLabs Inc., USA). Then, the reaction was incubated for 1-2 h in a water bath at 37°C. As controls, single digestion reactions were prepared and compared by visualization in a 1% agarose gel. The construct was purified from the gel using a Qiaquick PCR Purification kit (Qiagen, Netherlands) and eluted with 50 µl of EB Buffer as per the manufacturer's instructions. The same procedure was followed for preparing the digested pET32a(+) vector used for insertion of the TEV::scFvO157::biotin tag construct.

3.4.11.2 DNA ligation

Ligation of the TEV::scFvO157::biotin tag and the digested pET32a (+) plasmid was performed for 1 h in a water bath at 16°C. The reaction contained 40 U T4 DNA Ligase

(New England BioLabs Inc., USA), 1×T4 Ligase Buffer (New England BioLabs Inc., USA), and appropriate concentrations of both scFv construct and pET32a(+) digested plasmid, based on a sticky-ends ligation.

All vectors used and obtained in this study are summarized in **Table 9**. In addition, all plasmid inserts were sequenced at the Robarts Research Institute Sequencing Facility (London, ON, Canada).

3.4.11.3 Transformation of chemical competent *E. coli* cells

A 100 µl aliquot of chemical competent *E. coli* cells was thawed and incubated with 10 µl of the appropriate plasmid DNA for 30 min on ice. Heat shock was induced by incubating the cell at 42°C for 45 s and immediately placing it on ice for 2 min. Subsequently, 900 µl of LB broth were added to the cells, which were incubated for 1 h at 37°C with shaking. Finally, the transformed cells were spread plated on LB agar containing the appropriate antibiotics and incubated overnight at 37°C for 18 h.

3.4.11.4 *E. coli* clone selection

Individual colonies taken from the spread plates were picked and inoculated into 4 ml of LB with the appropriate antibiotics. Tubes were incubated overnight for 18 h with shaking. Plasmid DNA was isolated as previously described and visualized using a 1% agarose gel. The insert size was also verified by restriction digestion and by sequencing for final confirmation. Cultures that were confirmed to be successfully transformed with the appropriate plasmid were prepared for storage at -80°C as described in **Section 3.1.7**.

3.4.12 Expression of scFvO157

The *E. coli* BL21 (DE3) transformed with pBirACm (containing the *birA* gene coding for biotin ligase), and the pET32a(+)::TEV::scFvO157:biotin tag plasmids was used to express the recombinant protein of interest (TrxA::His•Tag®::TEV::scFvO157::biotin). Cells were grown aerobically at 37°C in LB medium with the appropriate antibiotics to an OD_{600}= 0.1- 0.5 (early log phase). For optimization of protein expression, induction was performed under different isopropyl-D-thiogalactopyranoside (IPTG; Bio Basic Canada Inc., Markham, ON, Canada) concentrations (1, 0.5, 0.3, 0.2, 0.1 mM), temperatures (4°C, 37°C

Table 9. Plasmids used in this study.

Plasmid Name	Relevant Characteristic	Source
pET32a(+)	Protein expression vector; Ap^r; 109aa Trx•Tag™ thioredoxin protein (TrxA) and His•Tag® sequences	Novagen
pBirACm	pACYC184 with inducible biotin ligase; Cm^r	Avidity
pUC57::TEV:: scFvO157:: biotin tag	Codon optimized scFvO157 sequence with KpnI and BamHI sites; Tobacco Etch Virus (TEV) protease cleavage site; C-terminal biotin tag; Ap^r	GenScript
pET32a(+)::TEV::scFvO157::biotin tag	TEV:: scFvO157:: biotin from pUC57:: TEV:: scFvO157:: biotin inserted into KpnI and BamHI sites of pET32a (+)	This study

Ap^r, Ampicillin resistance; Cm^r, Chloramphenicol resistance

and RT) and incubation times (3, 6, 48 h, and overnight). In addition, protein biotinylation was achieved by adding 50 mM D-biotin (BioBasic Inc., Canada). Cells were harvested by centrifugation at 5,000 ×g for 7 min at 4°C and the pellet re-suspended in cold Native Buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl). For cell lysis, the pellet was treated with 0.25 mg/ml lysozyme (Sigma-Aldrich, USA) and 0.02 mg/ml DNase I (Sigma-Aldrich, USA) and incubated on ice for 1 h prior to sonication (Branson Sonifier 450, Branson Ultrasonics Corporation, USA) with output 5, 3 cycles of 30 pulses each. The soluble and insoluble fractions were separated by centrifugation at 4°C at 4,000 ×g for 15 min and both fractions stored at -20°C for further analysis.

3.4.13 Purification and Refolding of scFvO157

The recombinant protein expressed was purified using nickel-nitrilotriacetic acid (Ni-NTA) His•Bind® resin (Novagen, EMD Millipore, USA) under three different conditions, which were adapted from *Akbari et al.* (203) and are described below.

3.4.13.1 Native conditions

The soluble fraction was applied to a Ni^{2+}-charged Ni-NTA affinity column and incubated at RT for 1 h with agitation using a rotator wheel. The column was washed with Native Buffer and eluted with increasing concentrations of imidazole (5 ml each of 15 mM, 30 mM, 60 mM, and 200 mM imidazole in Native Buffer).

3.4.13.2 Denaturing conditions

The pellet containing the scFvO157 inclusion bodies was re-suspended in Denaturing Buffer pre-warmed at 37°C and gently rocked for 10 min at RT to ensure lysis. Furthermore, the cell lysate was sonicated on ice (3 cycles of 10 pulses using output 5). Cell debris was removed by centrifugation at 4,000 ×g for 15 min. Then, the supernatant was pipetted into a Ni^{2+}-charged Ni-NTA affinity column and incubated at RT for 30 min with gentle agitation. The column was washed twice with Denaturing Binding Buffer and eluted using the Denaturing Elution Buffer. For refolding, the eluted recombinant protein was dialyzed (dialysis membrane, 12,000 Da MWCO; Sigma-Aldrich, USA) against 10 mM Tris, 0.1 % Triton X-100, pH 5.5 buffer at 4°C to eliminate urea.

3.4.13.3 Hybrid conditions

This protocol combined the previous denaturing and native methods. First, a bacterial pellet was lysed following the denaturing protocol. The supernatant was applied to a Ni^{2+}-charged Ni-NTA affinity column and incubated at RT for 30 min with gentle agitation. The column was washed twice with Denaturing Binding Buffer and four times with Native Wash Buffer. Finally, the protein was eluted using Native Elution Buffer.

All purified fractions were dialyzed 3 times, 1 h each, against 200-400 times the volume of Native Buffer at RT. Afterwards, the N-terminal tag (TrxA::His•Tag®::TEV) was cleaved by autoinactivation-resistant His_7::TEV protease (30 µl/ml sample) incubated 2 d at RT. Cleaved proteins were purified using a second Ni-NTA affinity column following the native conditions protocol. The fraction containing the pure scFvO157::biotin was dialyzed against Native Buffer as described above, assessed for homogeneity by SDS-PAGE and quantified using a Pierce™ BCA Protein Assay Kit and/or a NanoDrop 1000 Spectrophotometer, both from Thermo Fisher Scientific Inc., USA.

CHAPTER 4 RESULTS

4.1. Development of a LFIA for Detection of *E. coli* O157

4.1.1 Preparation and Evaluation of Bacterial Cultures

4.1.1.1 Preparation of healthy bacterial cultures

During the LFIA Test Kit development phase, five bacterial strains were frequently used throughout the process: *E. coli* O157 DSM 17076 served as the positive control, while *S. enterica* ser. Typhimurium LT-2, *Shigella flexneri* ATCC 25929*, E. coli* ATCC 25922 and *Listeria monocytogenes* ATCC 19115 were used as cross-reaction controls. *S. enterica* ser. Typhimurium*, S. flexneri* and *E. coli* ATCC 25922 are Gram negative bacteria belonging to the *Enterobacteriaceae* family and thus closely related to *E. coli* O157 (24,236). These bacteria represented ideal candidates for assessment of antibody cross-reactivity as they also possess the O-antigen as part of their outer membrane, although with structural variations that distinguish them from *E. coli* O157 (149). *L. monocytogenes*, a well-described human pathogen, was used as a representative of the Gram positive group. All healthy cultures reached 10^9 CFU/ml after an 18-24 h incubation period, with the exception of *S. enterica* ser. Typhimurium, which was approximately 10^8 CFU/ml, as confirmed by plate counts.

4.1.1.2 Preparation of stressed *E. coli* O157 cells

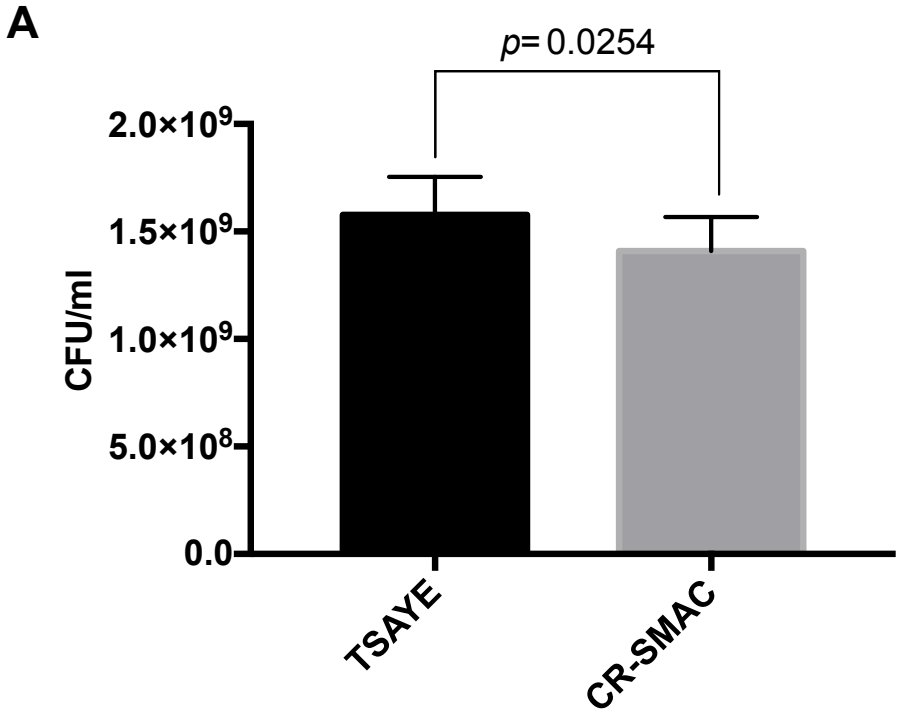
Healthy cells, prepared as previously described, were used when assessing the performance of the LFIA Test Kit under unprocessed raw meat conditions. However, when evaluating processed raw meat conditions, it was necessary to mimic realistic situations, where bacterial replication may be impaired due to exposure of meat to physical or chemical treatments (170,225). Therefore, a "food stress" treatment, which consisted of incubating an *E. coli* O157 culture for 10 d at 4°C with conditions similar to real processed raw meat samples (TSB, 0.6% yeast extract, pH 4.9, and 130 g/L NaCl), was chosen for preparing stressed cells that could be used for artificial inoculation (225). After treatment, *E. coli* O157 cells were plated in parallel on non-selective (TSAYE) and selective (CR-SMAC) media and the difference in growth was further compared to obtain the % of sub-lethal injury. Therefore, to ensure the proper performance of both TSAYE and CR-SMAC media, they were initially evaluated with a healthy *E. coli* O157 control. Plate counts were further compared using a two-sided *t*-test (α=0.05). The results showed that cells plated on

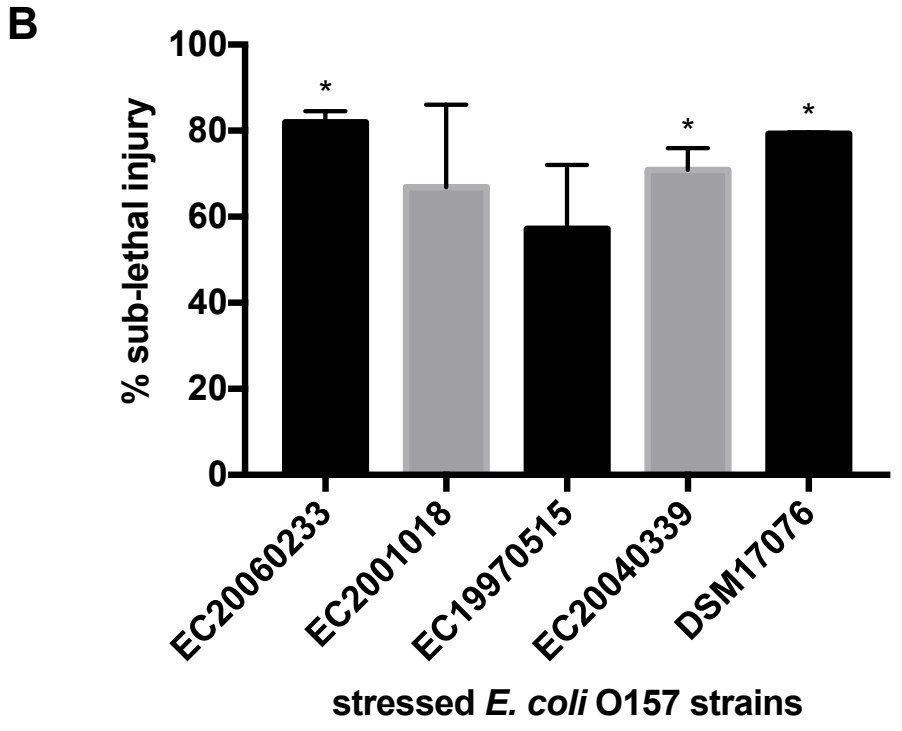
selective media (CR-SMAC) produced significantly lower CFUs compared with non-selective media (TSAYE) (p<0.05, **Figure 12A**). Hence, it was necessary to establish a % of sub-lethal injury, or threshold value, attributed to the media performance in order to distinguish from the true effect of the "food stress" treatment. The threshold value was estimated as one standard deviation above the mean of % of sub-lethal injury calculated for the eight healthy control samples assessed (225), which resulted in 21.7%. This threshold value was further used to assess whether the % of sub-lethal injury was truly caused by the "food stress" treatment or was simply an effect of the media performance. Furthermore, five different *E. coli* O157 strains were treated following the "food stress" protocol and the % of sub-lethal injury was compared with the threshold value previously obtained using a one-sample *t*-test (α=0.05). Two of them, EC20001018 and EC19970515, did not differ from the threshold value estimated (p=0.1426 and p=0.1385, respectively). Therefore, the % of sub-lethal injury for both of them was assumed to be due to the media performance rather than the effect of the "food stress" treatment. On the other hand, three strains showed a difference in % of sub-lethal injury (p<0.05), which was attributed to the effect of the "food stress" treatment (**Figure 12B**). These data demonstrated that although all strains evaluated belong to the same species, *E. coli* O157, there was an inherent cell-to-cell variation that could be reflected in the response to stress. Finally, despite the fact that there was an intrinsic effect caused by the performance of the media, three strains showed an adequate % of sub-lethal injury, which was optimal for their further use in the artificial inoculation of meat samples.

4.1.2 Optimization of the LFIA Device Blocking Conditions

In order to improve the visualization of the test line in the positive control while eliminating background signals, the first phase of the LFIA Test Kit development process focused on reducing the non-specific binding. Although different combinations of proteins and polymers were initially assessed, BSA, which is frequently reported to block non-specific binding in nitrocellulose strips (237–239), was selected as the best alternative for further optimization, based on our evaluation. In addition, Tween 20 was preferred over Triton X-100 as a nonionic detergent for improvements in sample flow through wettability. Using a blocking buffer either as a sample diluent or pre-treatment decreased the red smear along

Figure 12. Preparation and assessment of stressed *E. coli* O157 cells. A) Bar graph shows the difference in CFUs between the non-selective (TSAYE) and selective (CR-SMAC) media performance using an *E. coli* O157 control culture. CFUs were significantly lower on CR-SMAC compared with TSAYE ($1.41\times10^9 \pm 1.57\times10^8$ and $1.57\times10^9 \pm 1.77\times10^8$, respectively). The *p*-value is from a two-sided paired *t*-test (p= 0.0254, n=8). Values represent mean ± SEM. **B)** Bar graph representing the % of sub-lethal injury of 5 different *E. coli* O157 strains subjected to a "food stress" treatment. Each bar represents the mean value of % of sub-lethal injury ± SEM (n=3-5). A one sample *t*-test, using the threshold value (21.7%), was performed to determine whether the % of sub-lethal injury was truly caused by the "food stress" treatment or was mainly due to the performance of the media (*$p \leq 0.05$)


A
p= 0.0254
2.0×10^9
1.5×10^9
1.0×10^9
5.0×10^8
0.0
CFU/ml
TSAYE
CR-SMAC


B
100
80
60
40
20
0
% sub-lethal injury
*
*
*
EC20060233
EC2001018
EC19970515
EC20040339
DSM17076
stressed E. coli O157 strains

the membrane and improved the flow of the sample through the nitrocellulose membrane, as indicated by a well-defined control line when compared with the device without blocking buffer (**Table 10**). In addition, positive samples showed a clearly defined test line within the 15 min window established for reading, while negative samples remained clear (**Table 10**). Despite the improved performance, the two approaches required additional steps, increasing the complexity of the assay. Thus, it was necessary to assess additional blocking strategies.

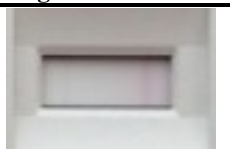
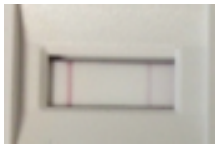
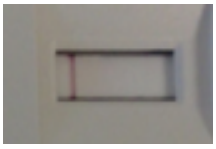
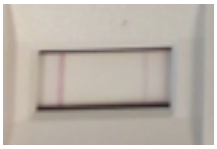
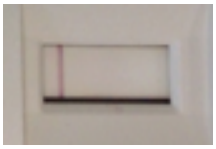
4.1.2.1 Assessment of different nitrocellulose blocking solutions

Prior work indicated that incorporating a blocking step increased the complexity of the assay despite the improvement in the performance of the LFIA. Thus, an alternative approach, where the blocking reagents were incorporated directly into the nitrocellulose membrane during manufacture, was assessed.

The optimal concentration and type of blocking were determined by assessing different ratios of BSA and/or Tween 20. The most representative prototypes of the blocking optimization process are summarized in **Table 11**. The positive control was *E. coli* O157 DSM 17076 diluted to 10^7 CFU/ml using TSB, while the negative control was TSB alone. Based on the criteria previously established for visual evaluation, LFIA devices A, E, and F were ruled out because there was no control line either in the positive or the negative control. Device B had twice the concentration of BSA and Tween 20 compared to A, which slightly improved the development of control lines, however, it did not decrease the formation of red smear along the membrane. This appearance was similar to device D, which contained the same concentration of BSA only. Device B blocking treatment was doubled in device C, causing better control lines and a slight decrease in the red smear. However, the test line in the positive control was still unclear. Finally, device G was blocked with a higher volume per mm^2 of B treatment, resulting in an intense, well-defined control line in the negative control. The positive control had a clear background with the appearance of both the control and test lines, though the signal intensity was weak.

Based on these results, the volume of the B solution was optimized and three more alternatives (1/2G, 1/4G, and 1/6G) were tested. The objective of using blocking post-

Table 10. Initial assessment of nitrocellulose blocking conditions.

Blocking	Positive Control	Negative Control
A) No blocking		
B) Blocking buffer as diluent		
C) Blocking buffer as pre-treatment		

A) *Standard LFIA with no blocking buffer used.* ***B)*** *Blocking buffer was used to re-suspend an overnight culture of E. coli O157 DSM 17076 before mixing the reagents for the in-tube sandwich immunoassay.* ***C)*** *Pre-treatment of the LFIA with 50 µl of blocking buffer before sample loading. The positive control consisted of a 10^7 CFU/ml sample of E. coli O157 DSM 17076 while the negative control contained only blocking buffer. Images of the LFIA devices were taken after 15 min after loading 150 µl of the corresponding sample. The concentration of antibodies was kept constant throughout these experiments.*

Table 11. Screening of blocking conditions for the LFIA devices.

LFIA	Nitrocellulose Treatment	Visual Evaluation[†]	
		Positive Control	Negative Control
A	0.75% BSA and 0.019% Tween 20		
B	2A		
C	2B		
D	2A-BSA only		
E	2A-Tween 20 only		
F	Unblocked control kits		
G[1]	50 µl of B per 5.5 mm strip		
1/2G[1]	25 µl of B per 5.5 mm strip		
1/4G[1]	12.5 µl of B per 5.5 mm strip		
1/6G[1]	9.375 µl of B per 5.5 mm strip		

Performance was evaluated for 9 different blocking solutions sprayed over nitrocellulose strips during manufacture of the LFIA devices. All cassettes had a 28 mm sample polyester pad blocked with Tween 20. [1]G devices were treated with different volumes of B blocking solution. [†]Images and visual evaluation of the cassettes were performed after a prolonged incubation time of 15 min. Positive Control: E. coli O157:H7 DSM 17076 (10^7 CFU/ml). Negative Control: TSB.

treatment was to evenly coat the surface of the membrane, which becomes chemically uneven due to different chemical species found in the capture reagents immobilized on the control and test lines. This can cause irregular flow when the sample is applied (240). However, the results in **Table 11** demonstrate that post-treatment is only effective when a balance between BSA and Tween 20 is reached, allowing for removal of nonspecific adsorption, while maintaining the optimal activity of the capture reagents reflected on well-defined control and test lines with a clear background. Therefore, the optimal blocking composition was achieved with the 1/6G device, which was selected for further studies.

4.1.3 Optimization of the In-Tube Sandwich Immunoassay

Once the optimal LFIA device prototype was selected, other parameters that influenced the sensitivity and specificity of the LFIA Test Kit, such as concentration of antibodies, sample pH and sample concentration, were evaluated. Hence, most of the optimization focused on the in-tube sandwich immunoassay, which was based on noncompetitive indirect detection of the antigen by using a secondary anti-mouse antibody conjugated with colloidal gold. This format had the advantage of keeping intact the detection antibody because it was not directly labeled and thus it was fully immunoreactive. The detection antibody used was a mAb raised against the O-antigen of *E. coli* O157 previously described by Westerman *et al.* (227). Its selection was based on reported ability to react with 47 *E. coli* O157:H7 strains and 17 O157:non-H7 strains. Moreover, this mAb did not cross-react with any of 38 non-*E. coli* enterobacteria tested by its developers (227). On the other hand, the biotinylated polyclonal antibody (pAb-b) acted as the capture antibody, binding to the streptavidin immobilized on the test line. This antibody was commercially available and chosen due to its synergy when combined with the mAb.

The in-tube sandwich immunoassay optimizations were performed using checkerboard titrations to identify the best combinations of the different reagents or conditions assessed. In addition, fine-tuning involved supporting visual evaluation with the measurement of the control and test lines' intensity by using the i-Lynx system. Previous studies performed at IPOC facilities using their standard LFIA commercial products have shown that values below 0.055 reflective units (RU) were not visually detected by an untrained panel (Garth

Styba, president IPOC, 2016, pers. comm.), therefore this value was adopted as the cut-off for discriminating positive *versus* negative results during the optimization studies.

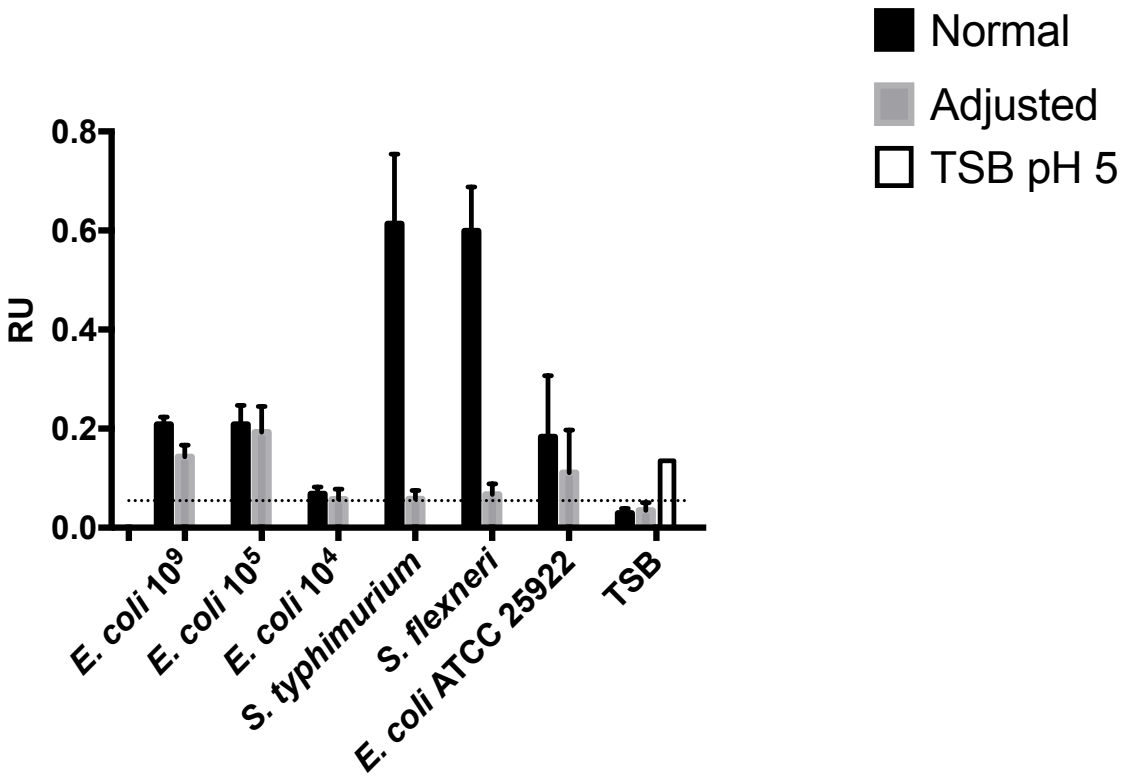
4.1.3.1 Optimization of the pH

Besides antibody pairing, determining the suitable concentration of antibodies used in the in-tube sandwich immunoassay played a key role in the sensitivity and specificity of the LFIA Test Kit. However, even when adequate concentrations are used, their interaction with the antigen can be compromised by sample conditions, such as the pH. In fact, the effect of pH on the LFIA performance was noticed due to the presence of false positives with overnight cultures of non-target organisms, which were included as cross-reaction controls during the optimization stage. Previous screening had shown that none of the antibodies cross-reacted with these strains. Moreover, when the cultures were diluted 10 fold with culture broth, the false-positives were eliminated. A previous study, which focused on the development of an immunochromatographic assay, reported that when the pH of samples was <5, false positives were detected and thus maintaining the pH between 6 and 9 was recommended (241). Therefore, to assess the effect of pH on the LFIA Test Kit, the pH of overnight grown cultures was determined and adjusted to neutral (pH 7) using 1 M HEPES buffer. The normal pH of an *E. coli* O157 culture was estimated to be 6, similar to the non-pathogenic *E. coli* ATCC 25922. Conversely, *S. enterica* ser. Typhimurium and *S. flexneri* cultures had a pH of 5, which resulted in high intensity false-positive signals, as shown in **Figure 13**. However, pH neutralization reduced the appearance of the false-positive signals, especially with *S. enterica* ser. Typhimurium and *S. flexneri*. The effect of low pH on the development of false positives was confirmed when TSB, which has a neutral pH, showed RU values around 0.135 when its pH was adjusted to 5 (**Figure 13**). Overall, these data showed that, to maintain the optimal performance of the LFIA Test Kit, it is necessary to adjust the sample pH during the in-tube sandwich immunoassay.

4.1.3.2 Optimization of the colloidal gold conjugated secondary antibody (CGC)

In order to continue with the in-tube sandwich immunoassay optimization, different concentrations of the colloidal gold conjugate were assessed. This type of secondary

Figure 13. Optimization of the in-tube sandwich immunoassay pH conditions. Effect of pH on the performance of the LFIA device using pure bacterial cultures. Graph represents the intensity of the test lines before and after pH adjustment with 1 M HEPES buffer. Normal pH values were 6 for *E. coli* O157 at a concentration of 10^9 CFU/ml and *E. coli* ATCC 25922, while *S. enterica* ser. Typhimurium and *S. flexneri* cultures had a pH of 5. *E. coli* O157 at a concentration of 10^5 and 10^4 CFU/ml, and TSB had a normal pH of 7 similar to all sample after adjustment with 1 M HEPES. Data corresponds to the mean ± SEM (n=2-5).


Normal
Adjusted
TSB pH 5
RU
0.8
0.6
0.4
0.2
0.0
E. coli 10^9
E. coli 10^5
E. coli 10^4
S. typhimurium
S. flexneri
E. coli ATCC 25922
TSB

labeled antibody is widely used in the development of immunochromatographic test strips due to the visible red color produced by the gold nanoparticles (114,133,140,242,243). CGC targets the mAb detection antibody which binds directly to the O157-antigen. A checkerboard titration of four samples and three concentrations was used to determine a suitable CGC concentration to continue with the optimization of the other two antibodies. Early in the process, it was noticed that 10^9 CFU/ml overnight cultures of *E. coli* O157 presented a drastic decrease in intensity of the control and test lines, which could be mistaken for a false negative. Hence, a 10^5 CFU/ml dilution was included as a true representation of a positive result. In addition, *S. enterica* ser. Typhimurium was included as a false positive control, representative of non-target organisms. Finally, TSB was used as a negative control. Optimal conditions were determined based on the maximum color intensity of the control and test lines that could be achieved without producing background coloration or non-specific binding with the negative controls. **Figure 14** shows a graphic representation of the results obtained for the CGC optimization, where the *E. coli* O157 signal increased proportionally with the increase in CGC. However, there was also an increase in the false positive with the *S. enterica* ser. Typhimurium culture. Therefore, it was concluded that using the lowest CGC concentration assessed was optimal to further optimize the concentrations of the mAb and pAb-b because it gave a clear positive read out with *E. coli* O157 10^5 CFU/ml without producing false positive signals with either *S. enterica* ser. Typhimurium or TSB.

4.1.3.3 Optimization of the antibodies

During CGC optimization it was noted that high levels of *E. coli* O157 produced weak signals that were close to the cut-off value. Hence, different combinations of mAb and pAb-b were assessed to tackle the "hook-effect" or prozone effect, seen with *E. coli* O157 at high concentrations. This effect occurs when the concentration of the target antigen exceeds that of the antibodies, reducing the formation of the sandwich complex and decreasing the intensity of the positive signal (143,244,245). **Figure 15** represents the main antibody combinations assessed, however, the intensity of the test line was not improved when high levels of *E. coli* O157 were present. At this point, other modifications within IPOC's manufacturing protocols, such as the concentration of streptavidin in the test line,

Figure 14. Optimization of the colloidal gold conjugate secondary antibody concentration. Data represent the trend in the test line intensity with four different samples and three main concentrations of CGC assessed during one checkerboard titration. All cultures used in these experiments were grown in TSB and pH adjusted for the in-tube sandwich immunoassay using 1 M HEPES buffer. For *E. coli* O157 10^5 CFU/ml dilution, BPW (pH 7) was used. The cut-off value (0.055 RU) is also represented as a dotted line.

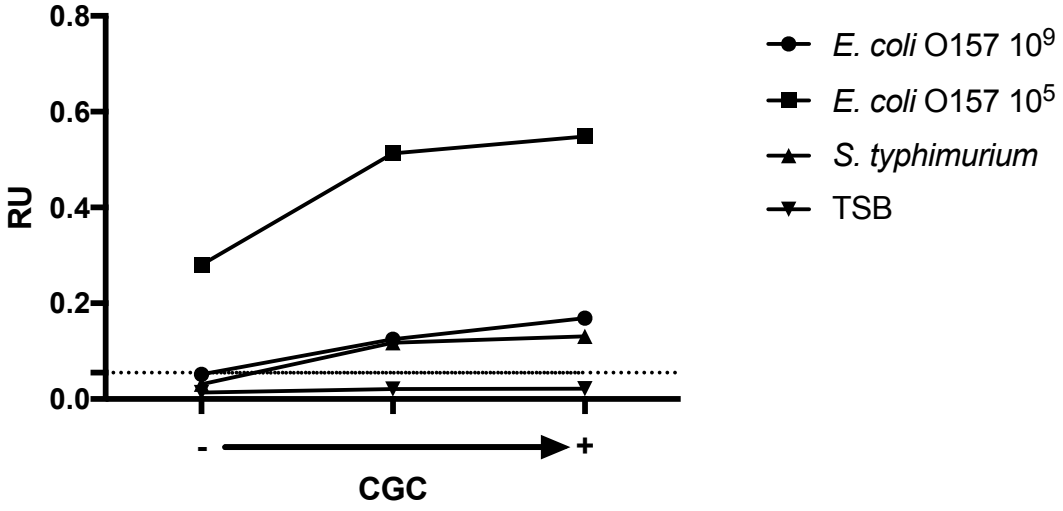
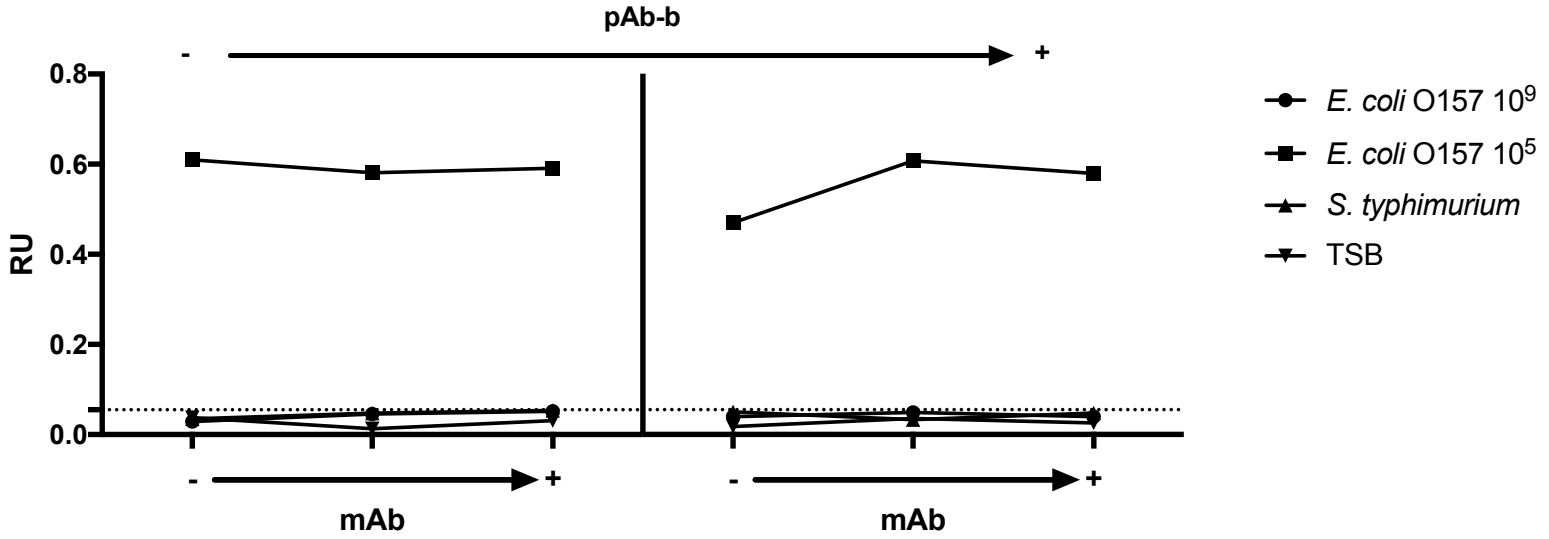

0.8
0.6
0.4
0.2
0.0
RU
-
+
CGC
E. coli O157 10^9
E. coli O157 10^5
S. typhimurium
TSB

Figure 15. Optimization of the mAb and pAb-b concentrations. The data represent the three most relevant concentrations of the mAb in combination with the two main concentrations of the pAb-b using the same four bacterial cultures as before. All cultures used in these experiments were grown in TSB and pH adjusted for the in-tube sandwich immunoassay using 1 M HEPES buffer. For *E. coli* O157 10^5 CFU/ml dilution, BPW (pH 7) was used. The cut-off value (0.055 RU) is also represented as a dotted line.


pAb-b
-
+
0.8
0.6
0.4
0.2
0.0
RU
-
+
mAb
-
+
mAb
E. coli O157 10^9
E. coli O157 10^5
S. typhimurium
TSB

membranes with faster capillary flow rate and increasing the reading time, were evaluated. Nevertheless, none of these approaches decreased the prozone effect without affecting the sensitivity and/or specificity of the LFIA Test Kit; thus other alternatives were further investigated.

4.1.4 Development of a Tandem LFIA Test Kit

Besides the "hook-effect" previously described, it was hypothesized that matrix interference was another factor involved in low signal intensity. Food matrices are complex and contain high levels of contaminants that can hinder the ability of the detection method to produce a reliable result. Therefore, in order to tackle both issues, the approach adopted was to dilute the sample to an extent that matrix effect would be reduced, while the target organisms would be at an optimal concentration to eliminate the "hook-effect" without compromising specificity and sensitivity. In fact, this approach has been widely applied in the development of detection techniques for toxins or pathogens in food (246,247).

A tandem LFIA device, containing two independent membranes was assembled, where the neat or undiluted sample was loaded in one window (A), while a 100-fold dilution was included in a second window (B). **Figure 16** shows the results obtained for a concentration curve for a pure culture of *E. coli* O157 using the tandem LFIA device. The prozone effect was seen at 10^9 CFU/ml, where the RU value for both control and test lines in the (A) window were decreased due to the high concentration of bacteria in the sample. However, the 100-fold dilution in window (B) had an RU value that was almost doubled, allowing the sample to be accurately detected as a positive instead of a false negative. When using pure cultures, 10^4 CFU/ml was established as the limit of detection (LOD) of the LFIA, because it was the lowest cell concentration with readings above the cut-off value and confirmed by a visible test line detected by all members of the panel. **Table 12** shows the proper visual interpretation of results when using the tandem test based on the data from **Figure 16**. Overall, these outcomes demonstrated that, by combining the test line results in both windows of the tandem LFIA device, it was possible to overcome the "hook-effect" obtaining positive results from samples containing 10^4-10^9 CFU/ml, with a maximum signal intensity achieved at 10^6 CFU/ml.

Figure 16. *E. coli* O157 concentration curve using the tandem LFIA device. Control and test line curves for both neat (A) and 100-fold diluted (B) samples obtained using the tandem LFIA device with serial dilutions of an *E. coli* O157 pure culture. Data represent one experiment (mean ± SEM) of two independent experiments (n=2 performed in triplicate) using pure cultures of *E. coli* O157 grown on TSB overnight. The visual cut-off value (0.055 RU) is shown in both graphs. CL: control line; TL: test line.

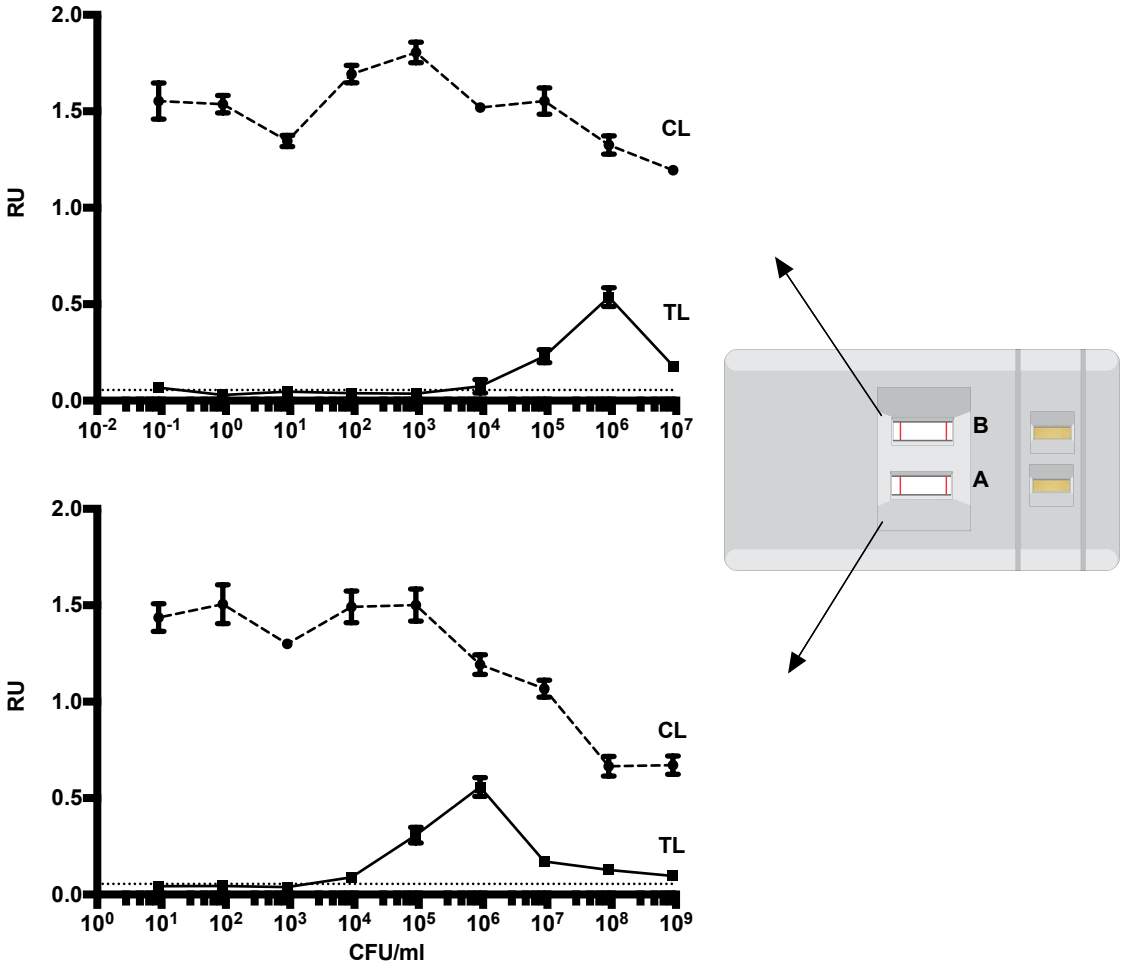

2.0
1.5
1.0
0.5
0.0
RU
CL
TL
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7}
2.0
1.5
1.0
0.5
0.0
RU
CL
TL
10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7} 10^{8} 10^{9}
CFU/ml
B
A

Table 12. Visual interpretation of an *E. coli* O157 concentration curve using the tandem LFIA device.

Sample	Neat (A)	1/100 (B)	Visual Interpretation		
	Mean RU ± SEM	Mean RU ± SEM	Neat (A)	1/100 (B)	Final Result
EC9	0.096±0.007	0.180±0.021	+	+	POS
EC8	0.127±0.013	0.537±0.049	+	+	POS
EC7	0.171±0.004	0.230±0.033	+	+	POS
EC6	0.558±0.048	0.040±0.014	+	-	POS
EC5	0.308±0.041	0.036±0.011	+	-	POS
EC4	0.090±0.009	0.039±0.001	+	-	POS
EC3	0.039±0.004	0.046±0.011	-	-	NEG
EC2	0.044±0.018	0.030±0.010	-	-	NEG
EC1	0.043±0.004	0.055±0.007	-	-	NEG

Data shown correspond to the i-Lynx readings of the test lines from serial dilutions of a pure E. coli O157 culture grown in TSB overnight. Results represent one experiment (mean ± SEM) of two independent experiments (n=2 performed in triplicate). For visual interpretation values >0.055 RU are considered positive (+) while values ≤0.055 are considered negative (-). The combination of (A) and (B) produced the final result of the test. EC9-EC1: E. coli O157 10^9 CFU/ml – 10^1 CFU/ml. POS: positive result; NEG: negative result.

4.1.5 Pairing the Enrichment Procedure with the LFIA Test Kit

Previous data have demonstrated an optimal performance of the tandem LFIA device with pure cultures. Thus, the following stage consisted in proving the effectiveness of sample dilution to overcome matrix interference by combining the tandem test with the selected enrichment broth to detect *E. coli* O157 in artificially inoculated food samples.

Bacterial pathogens are often found in low concentrations in food, hence an enrichment step is often necessary to increase the number of *E. coli* O157 cells to a detectable level, notably when stressed cells must be recovered. Extensive studies, which compared and assessed the performance of a variety of enrichment media and incubation conditions in order to recover *E. coli* O157 as fast and efficiently as possible, have been done by others (170,248–250). Therefore, this information was taken into consideration while selecting potential enrichment broths and conditions that could improve the performance of the LFIA device.

4.1.5.1 Assessment of the RapidCult™ enrichment medium

Although most of the selective enrichment media require the presence of antibiotics to inhibit the growth of competing microbiota, some antibiotics, such as cefixime and cefsulodin, have been reported to affect the growth rate of *E. coli* O157 (249,251,252). For this reason, RapidCult™ enrichment medium, which is a relatively new broth, was evaluated and selected for the LFIA Test Kit. This medium was the only commercially available medium that claimed to recover *E. coli* O157 from meat samples in 8 h at 42°C. Although its specific composition is not publicly available, one of the ingredients reported, sodium thioglycolate, has been used as a selective agent that maintains reducing conditions by lowering the oxygen concentration in the liquid medium and hence inhibiting the growth of most of the organisms found in certain types of food (253). When RapidCult™ was combined with the LFIA device, it became possible to detect 10^6 CFU/g in artificially inoculated ground beef samples and 10^5 CFU/ml when using pure cultures. In both cases, we started the procedure with 10 CFU/25g or ml of either healthy or stressed *E. coli* O157 cells after an 8 h incubation time (data not shown). Regrettably, RapidCult™ had to be replaced during the development process as the manufacturer withdrew it from the market.

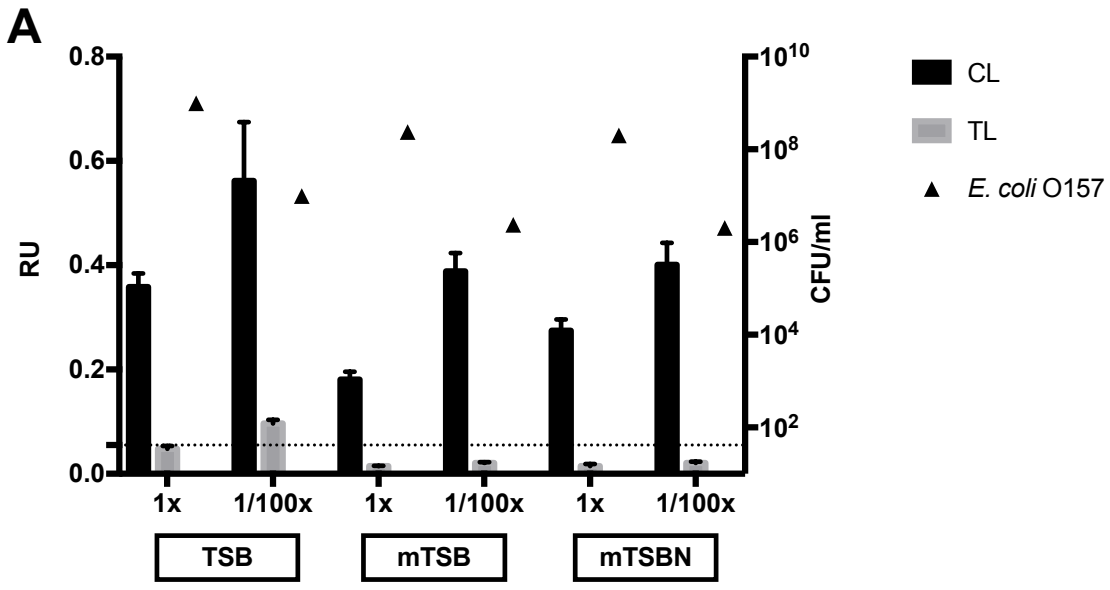
4.1.5.2 Assessment of mTSBN and mTSB enrichment media

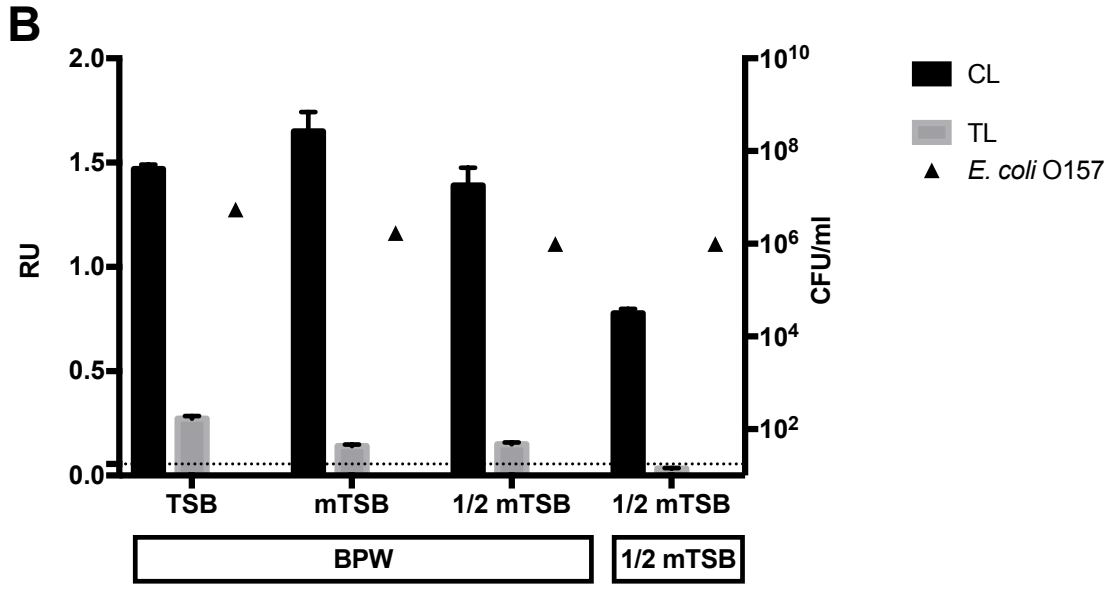
In order to replace the RapidCult™ enrichment medium, mTSBN and TSBN were further evaluated in combination with incubation at 42°C, which was earlier shown to yield higher recovery rates by acting as a selective factor for non-target organisms that commonly grow at 37°C (249,251,254). mTSBN was selected for the kit because it is the recommended enrichment broth in Health Canada's reference method for isolation of *E. coli* O157 (255). The performance of enrichment was assessed in combination with the LFIA device using ground beef samples inoculated with 5 CFU/25g of non-stressed *E. coli* O157 cells. After 12 h enrichment, *E. coli* O157 counts reached 1.6×10^7 CFU/ml[2], however, the LFIA device failed to detect them. In order to investigate further, pure cultures of *E. coli* O157 were used to eliminate the influence of the food matrix. Samples of *E. coli* O157 cultures enriched in TSB, mTSB and mTSBN were tested. For these experiments, it was necessary to increase the cell concentration approximately 100 times because bacterial counts in mTSB and mTSBN only reached 3.63×10^4 and 3.44×10^3 CFU/ml[3], respectively, after 12 h of enrichment. The results with mTSBN were consistent with those obtained with the ground beef samples, where it was not possible to detect the presence of *E. coli* O157 despite reaching $>10^8$ CFU/ml after 12 h enrichment. **Figure 17A** compares the results of 12 h enrichment using TSB, mTSB or mTSBN. After a 100-fold dilution, cells grown in TSB media gave a positive signal, while the other selective enrichments failed, even after dilution. As well as reducing the growth approximately 0.5 $\log_{10}$, with and without novobiocin, the presence of bile salts in mTSB and mTSBN appeared to inhibit the antibody-antigen interaction. mTSB contains 1.5 g/L of bile salts No. 3, which is a mixture of sodium cholate and sodium deoxycholate. The latter, which is considered an anionic salt, has proven to have a concentration-dependent inhibitory effect on the antigen-antibody interaction (256). To test the hypothesis that bile salts inhibited the assay, different enriched samples grown in TSB, mTSB or ½ mTSB, where the concentration of

[2] Average of *E. coli* O157 counts obtained in two experiments.

[3] Average of *E. coli* O157 counts obtained in three experiments

Figure 17. Effect of bile salts on the LFIA Test Kit performance. A) Comparison of TSB, mTSB, or mTSBN enrichment media effect on the intensity of LFIA signals using undiluted samples (1×) and a 100-fold dilutions (1/100×) using each enrichment broths as diluent. **B)** Intensity of LFIA signals using 100-fold dilutions of enriched samples in TSB, mTSB or ½ mTSB, with either BPW or ½ mTSB. Graphs represent the mean ± SEM of one experiment measured in triplicate using 10^2 CFU/ml of non-stressed *E. coli* O157 cells prior to enrichment. The cut-off value of 0.055 is also represented. The CFU/ml calculated for each sample is also included. These experiments were run with the 1/6G single LFIA device. CL: control line; TL: test line.


A
RU
CFU/ml
0.8
0.6
0.4
0.2
0.0
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
1x
1/100x
1x
1/100x
1x
1/100x
TSB
mTSB
mTSBN
CL
TL
E. coli O157


B
RU
CFU/ml
2.0
1.5
1.0
0.5
0.0
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
TSB
mTSB
1/2 mTSB
1/2 mTSB
BPW
1/2 mTSB
CL
TL
E. coli O157

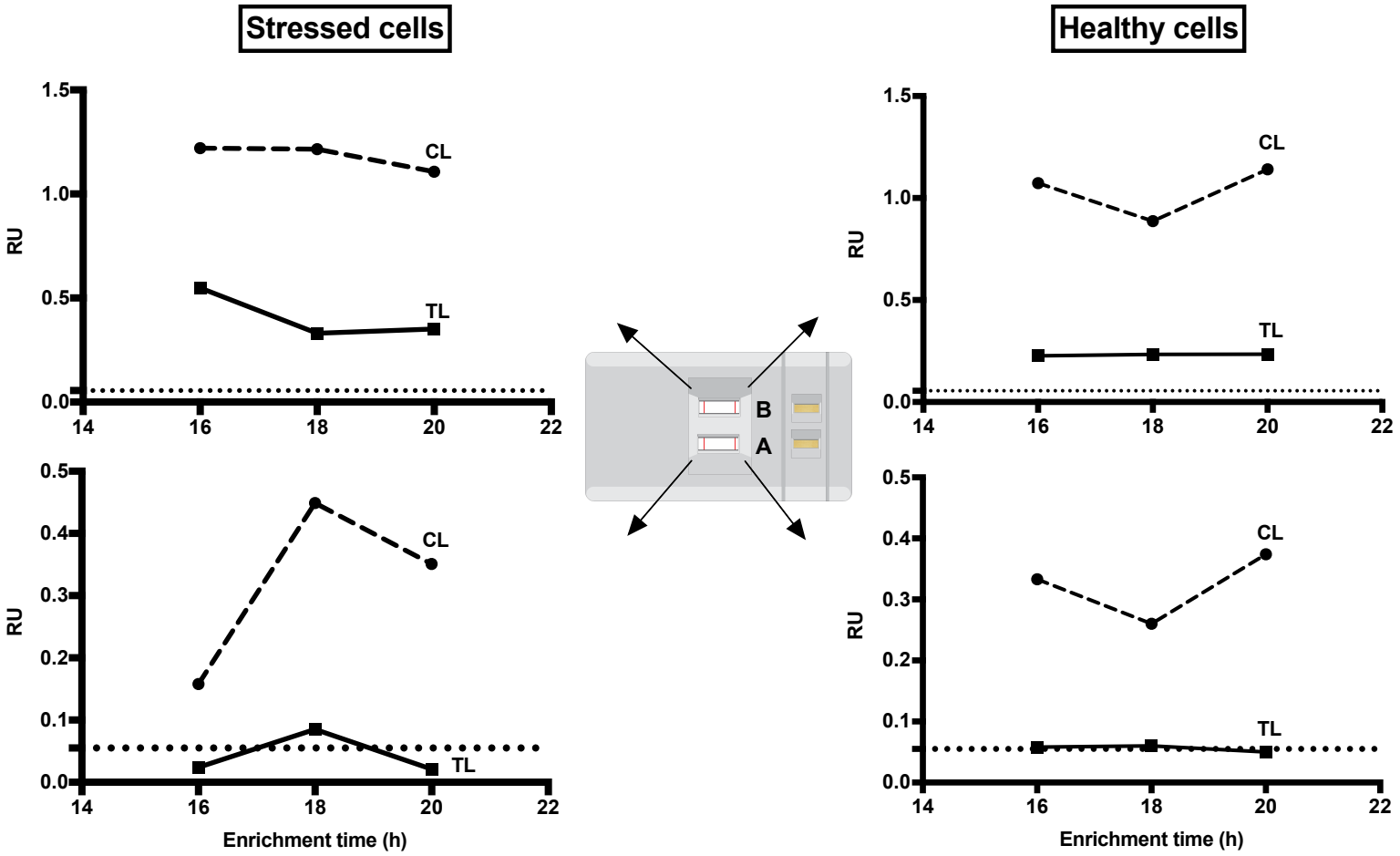
bile salts was diluted with either BPW or ½ mTSB just before the assessment with the LFIA Test Kit, were tested (**Figure 17B**) The intensity of the signals increased when TSB or ½ mTSB enriched samples were diluted with BPW, while the ½ mTSB sample diluted with the same enrichment broth failed to produce a positive signal. Despite the dilution, the intensity was still lower when compared with the TSB sample, perhaps also due to the approximately 0.5 $\log_{10}$ growth reduction in the presence of bile salts. These results are consistent with those from the previous experiment; thus both mTSBN and mTSB were eliminated as potential enrichment media.

4.1.5.3 Assessment of TSBN enrichment medium

Due to the previous findings, where the presence of bile salts interfered with the performance of the LFIA Test Kit, TSBN medium was further evaluated in combination with the LFIA. Novobiocin-containing medium has been reported to have better performance when compared against other antibiotics used as selective reagents for recovery of *E. coli* O157 (251,252). In addition, Novobiocin is described as the most common antibiotic used for selective enrichment (250), making it readily available and affordable from a commercial perspective.

Using TSBN in combination with the tandem LFIA Test Kit failed to detect low concentrations of stressed *E. coli* O157 cells after 8 h of enrichment at 42°C (data not shown). Hence, after performing time-course studies, the enrichment time was increased to 16 h at 42°C to reach detectable levels. Samples inoculated with healthy or stressed, low or high inoculation levels produced visually positive results after 16 h of enrichment, an observation confirmed by further measuring the intensity of the test lines (**Figure 18**). In addition, both the presence of *E. coli* O157 and its concentration were confirmed by plate counts in CR-SMAC and CHROMagar. After 16 h, samples inoculated with healthy cells, regardless of the initial concentration, reached $>7\times10^8$ CFU/ml, while samples inoculated with high levels of stressed cells reached approximately 1×10^8 CFU/ml. However, after 20 h each of the samples had a concentration close to 1×10^9 CFU/ml. On the other hand, stressed cells inoculated at low concentrations reached approximately 2×10^8 CFU/ml and

Figure 18. TSBN enrichment medium time-course combined with the tandem LFIA Test Kit using artificial inoculated ground beef samples. Results from two different sets of ground beef samples inoculated with a low *E. coli* O157 cell concentration (<5 CFU/25g): stressed cells (left) and healthy cells (right). Both control and test lines (CL and TL) are shown in each graph corresponding to either the A or the B window, respectively. Samples were assessed at 16, 18, and 20 h of enrichment using TSBN at 42°C and plated on CR-SMAC and CHROMagar for confirmation and enumeration. Results represent one experiment of 5 different time-courses that were performed under similar conditions.


Stressed cells
Healthy cells
RU
CL
TL
B
A
Enrichment time (h)
1.5
1.0
0.5
0.0
0.4
0.3
0.2
0.1
14
16
18
20
22

plateaued even after 20 h. Nevertheless, the results suggested that TSBN enrichment conditions were adequate for establishing the final LFIA Test Kit instructions for use.

4.1.6 Assessing the LOD Using Meat Samples

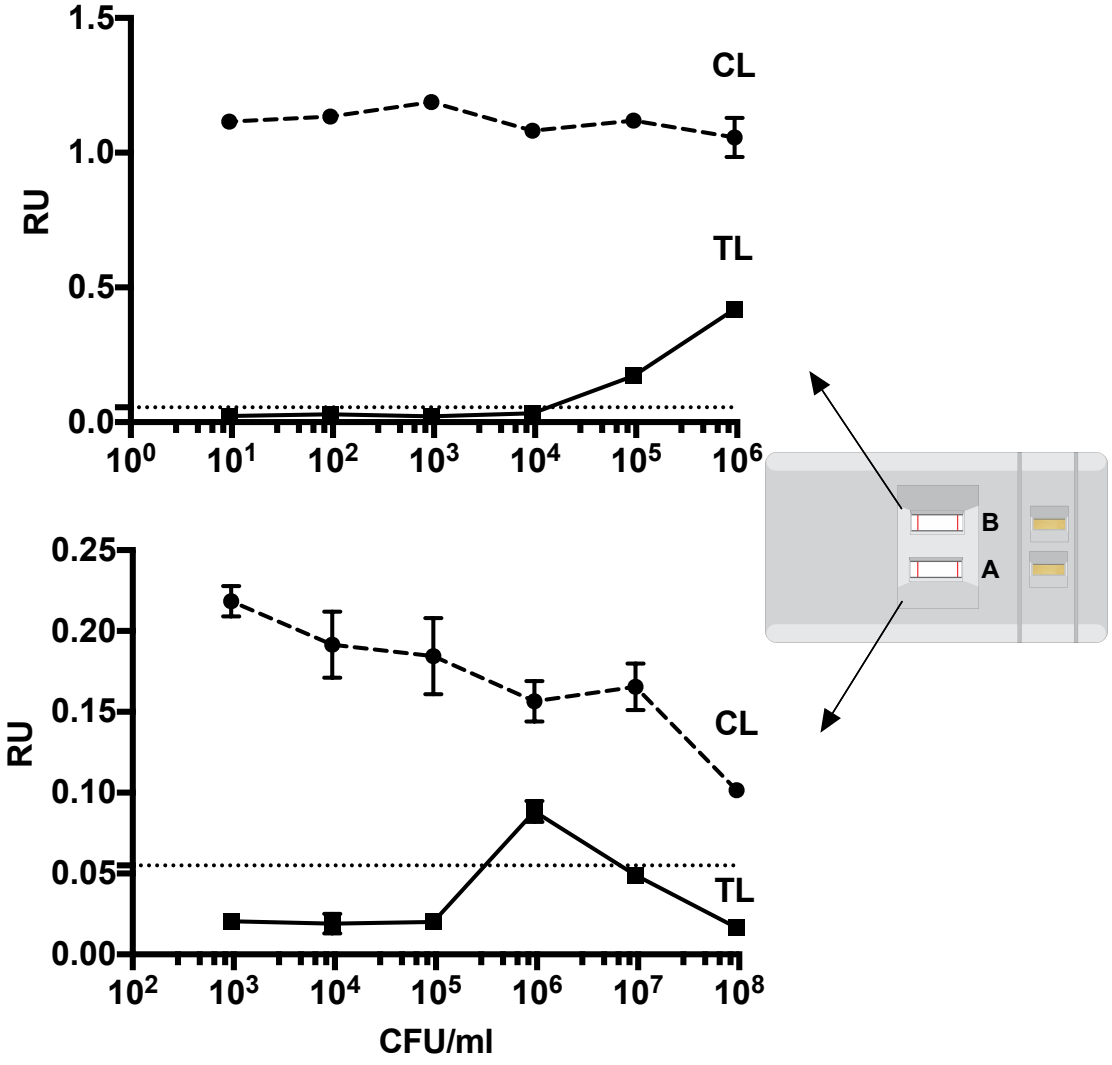
Previously, the LOD of the LFIA Test Kit was determined using pure cultures. However, the food matrix and microbiota are known to interfere with the signal intensity. Thus, the LOD was re-assessed using an uninoculated ground beef sample previously enriched and used as a diluent for preparing serial dilutions of *E. coli* O157 culture. Results showed that the LOD of the LFIA device was ~2×10^5 CFU/ml (**Figure 19**), which is approximately 10 fold higher than the LOD obtained with pure cultures. This increase is attributed to the influence of the food matrix mainly in the binding of the antigen-antibody complex to the test line and of the free mAb to the control line.

Moreover, as shown in **Figure 16**, as the concentration of *E. coli* O157 increased, the intensity of the control line began to decrease, especially with the undiluted sample (A). This is believed to be due to a lower concentration of free mAb as more antibody-antigen sandwich complex is formed, augmenting the intensity of the test line proportionally. However, when the prozone effect starts, at around 10^7-10^8 CFU/ml, the intensity of both lines in the (A) window decreases. Window (B) showed the same effect, although in a delayed manner. This trend in signal intensity is also seen in **Figure 19**. Hence, besides providing an estimated LOD of the tandem LFIA Test Kit when using meat samples, these data also revealed an alternative to further investigate the relation between the concentration of *E. coli* O157 in meat samples and the intensity trends of both the control and test lines of the tandem device.

4.1.7 Estimating *E. coli* O157 Content in Artificially Inoculated Meat Samples Using the Tandem LFIA Test Kit

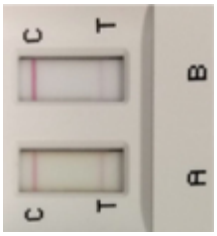
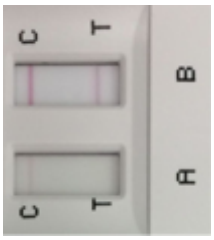
Although the LFIA Test Kit is intended to obtain qualitative information regarding the presence/absence of *E. coli* O157 in meat samples, previous data showed that the combination of control and test line intensities within the tandem LFIA device could be used as an indicator of *E. coli* O157 concentration. Therefore, to investigate this further, six additional artificially inoculated meat samples were evaluated. Samples were

Figure 19. Assessment of the LOD of the LFIA Test Kit using food samples. Concentration curves for both neat (A) and 100-fold diluted (B) samples from a serial dilution of an overnight *E. coli* O157 culture (9.42×10^8 CFU/ml)) using an enriched ground beef sample as diluent. Data represent the mean ± SEM of one experiment measured in duplicate. The visual cut-off value (0.055 RU) is shown in both graphs. CL: control line; TL: test line.


1.5
1.0
0.5
0.0
RU
CL
TL
10^0 10^1 10^2 10^3 10^4 10^5 10^6
0.25
0.20
0.15
0.10
0.05
0.00
RU
CL
TL
10^2 10^3 10^4 10^5 10^6 10^7 10^8
CFU/ml
B
A

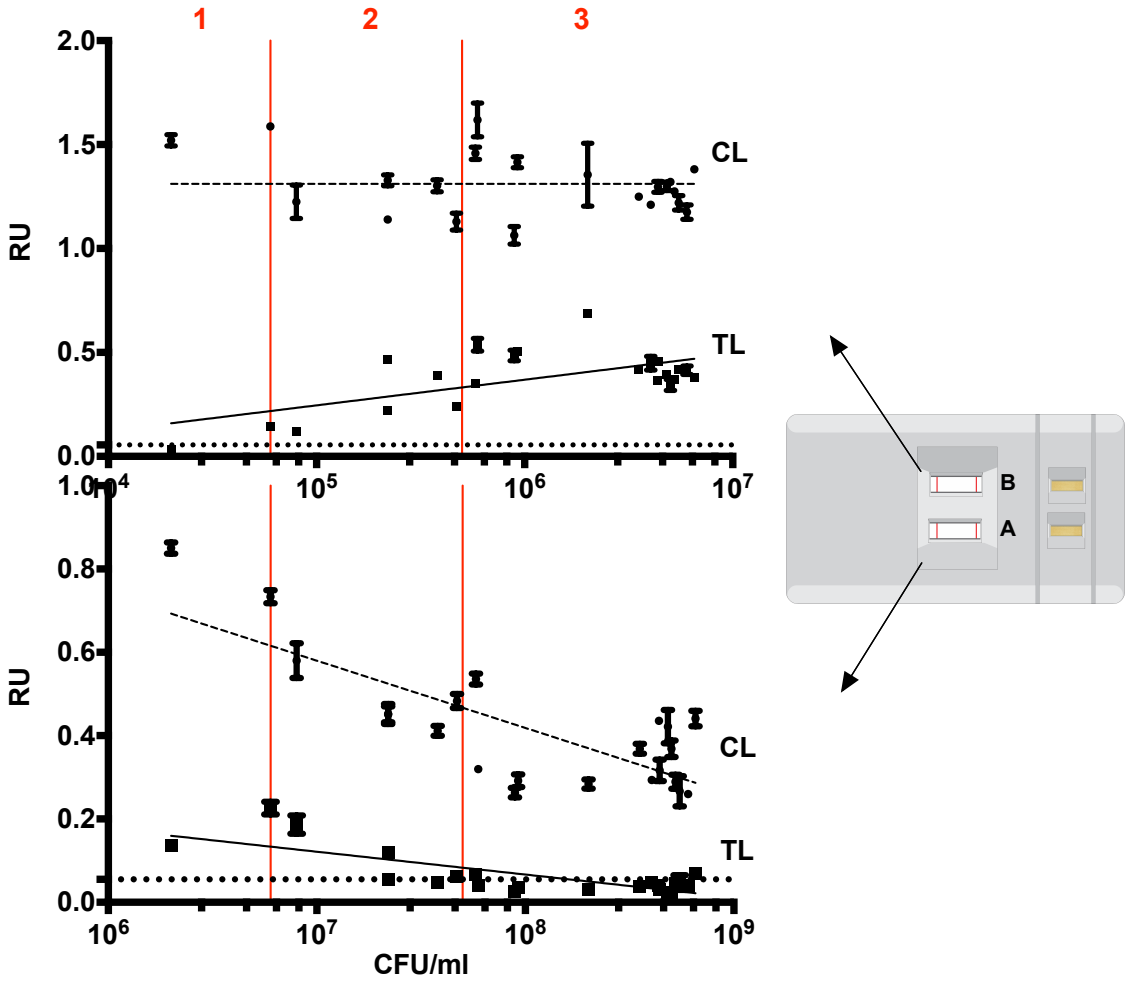
inoculated with 5 CFU/25g and incubated for 12-24 h using TSBN enrichment conditions. All samples were assessed with the tandem LFIA device at different time-points. Tandem LFIA device results and their respective cell counts for each time-point were assessed individually to establish the criteria in **Table 13**. Even though a wide time for incubation range was assessed (12-24 h) to obtain a broad spectrum of *E. coli* O157 concentrations within the detectable levels of the tandem LFIA device, only one sample had its initial time-point enumeration within the lowest range. Six time-points were used to determine the upper and lower limits of the mid-range by calculating the mean value ± SD. Furthermore, 15 time-points were considered within the maximum level (**Figure 20**). In addition, an intensity score was linked to each criterion to account for the intensity of the four lines into one single result. A one-way analysis of variance (ANOVA) using SPSS v.23 (IMB Corporation, Armonk, NY, USA) was performed to examine the effect of the concentration of *E. coli* O157 in meat samples on the intensity score. Results showed that there was an effect of the *E. coli* O157 concentration levels on the intensity scores [$F(2,63)=27.61$, $p<0.001$]. Bonferroni post-hoc tests showed that there was a significant difference in mean concentration values between scores 1 and 3 (2×10^6 *vs* $3.62\times10^8 \pm 2.06\times10^8$; $p= <0.003$), and 2 and 3 ($2.72\times10^7 \pm 1.98\times10^7$ *vs* $3.62\times10^8 \pm 2.06\times10^8$; $p=<0.001$). This suggested that the combination of line intensities established for score 3 is a good indicator of a high concentration of *E. coli* O157 in a sample. Despite the fact that scores 1 and 2 indicated low cell concentrations, with score 1 suggesting a lower mean concentration than score 2, the difference between these was not significant. The latter might have been due to the small number of time-points that had cell concentrations within the low range (5×10^5- 5×10^6), being underrepresented in score 1. Therefore, further studies must be performed to determine whether or not lower concentration ranges can be better differentiated. Overall, the data obtained demonstrated that the prozone effect, which showed an intensity inversely proportional to the concentration of the target antigen (*E. coli* O157), in combination with the relationship between control and test line intensities, can be a good indicator of target antigen concentration whenever a positive sample is obtained using the tandem LFIA Test Kit.

Table 13. *E. coli* O157 concentrations and intensity scores using the tandem LFIA Test Kit with meat samples.

Sample Estimated Concentration (CFU/ml)	Visual Interpretation Food Samples — Tandem LFIA Device Image	Neat (A) CL	Neat (A) TL	1/100 (B) CL	1/100 (B) TL	Intensity Score
~5×10^5- 6×10^6		LI	LI	HI	-	1
~6×10^6- 5×10^7		LI	LI	HI	LI	2
>5×10^7		LI	-	HI	LI	3

Different combinations of control and test line intensities obtained with the tandem LFIA device when analyzing meat samples. These combinations were linked to three different E. coli O157 concentrations, where the lower and upper limits of the mid-range were used to establish the low and high concentration levels, respectively. In addition to visual evaluation, the line intensities were linked to i-Lynx readings as follows: high intensity (HI) >1.000 RU, low intensity (LI) <1.000 RU, and negative (-) <0.055 RU. To perform the statistical analysis, intensity scores were assigned to each set of line combinations. Results were obtained from six different time-courses run independently with 3-5 time-points, each one assessed in triplicate (n=66).

Figure 20. Relationship between the tandem LFIA device control and test line intensities with *E. coli* O157 concentrations in meat samples. Graphs represent the general trend of the relationship between cell concentration and intensity (RU) of the control and test lines obtained by curve fitting using a total of 22 time-points. The limits of the three intensity scores (1, 2, and 3) are also defined by red lines. Data correspond to the mean ± SEM (*n*=3) obtained for each timepoint.


1
2
3
2.0
1.5
1.0
0.5
0.0
RU
CL
TL
10^4
10^5
10^6
10^7
0.8
0.6
0.4
0.2
0.0
RU
CL
TL
10^6
10^7
10^8
10^9
CFU/ml
B
A

4.2. Relative Validation of a LFIA Test Kit for Detection of *E. coli* O157 in Raw Meat Products

Prior results demonstrated that the tandem LFIA Test Kit was optimized to detect *E. coli* O157 in meat samples containing < 5 CFU/25g by using TSBN as the enrichment medium in combination with incubation for 16 h at 42°C. Therefore, the method was considered completely prepared for the relative validation of the pre-collaborative study. In order to be recognized as an alternative method (AM) by Health Canada's Microbiological Methods Committee (MMC), the guidelines for the relative validation of indirect qualitative food microbiological methods found in Part 4 of the Procedure for the Development and Management of Food Microbiological Methods from the Compendium of Analytical Methods had to be followed (174). The LFIA Test Kit was considered to be an indirect qualitative method because it is based on the detection of the O157-antigen, which is a physical characteristic, found in the membrane of the target pathogen. In addition, it is considered qualitative due to the fact that it solely determines the presence or absence of *E. coli* O157.

For the LFIA Test Kit validation study, two food types within the Raw Meat Food Category were considered, based on the classification of food categories found in the Compendium of Analytical Methods. These were Raw Meat (unprocessed) and Raw Meat (processed). All data presented in this section were generated in two facilities: Robarts Research Institute, Western University (**Section 4.2.1**) and Laboratory Services Division, University of Guelph (**Section 4.2.2**) and are adapted from the reports prepared for the submission package sent to Health Canada's Microbiological Methods Committee. Detailed raw data are found in **Appendix F**, which are presented in the format required by the MMC for its evaluation.

4.2.1 Inclusivity and Exclusivity Study

Inclusivity results using 50 *E. coli* O157 strains (including H7 and non-motile) tested positive with the LFIA Test Kit. Five strains had negative (-) A strips, but positive (+) B strips, which occurs when high cell concentrations are present in a sample (>10^7 CFU/ml). Moreover, two samples showed a positive (+) A strip, but a negative (-) B strip, which

occurs when the sample has a lower cell concentration (<10^6 CFU/ml). Six strains had at least one replicate differing from the rest, but without compromising the final result, which was positive (strains 6, 10, 35, 42, 44 and 47). The rest of the samples were consistent within the replicates, showing a positive signal (+) in both strips, suggesting that the cell concentration was between 10^6 and 10^7 CFU/ml. Overall, the inclusivity rate obtained was 100%.

Exclusivity results showed that 31 *E. coli* non-O157 strains included in this study were negative with the LFIA Test Kit. Six strains showed at least one weak positive replicate; thus they were retested by obtaining isolated colonies on TSA plates and then using LFIA Test Kit enrichment (TSBN at 42°C) incubated up to 24h. Four strains (*E. coli O78:H11, E. coli O3:H2, S. flexneri, S. enterica* ser. Typhimurium*)* were negative and only two *E. coli* strains, 044:H18 and O124:NM, remained weakly positive. These results represented an exclusivity rate of 94.6%.

4.2.2 Sample and Inocula Preparation

For each food item used, the APC or TVC was first determined as it is required to be at least 10 times higher than the *E. coli* O157 inoculum. Results presented in **Table 14** confirmed that the APC and *E. coli* O157 ratio complied with the requirements. Moreover, the inoculation levels for both (H) and (L) were initially determined by calculating the culture titers using the plate counts of each culture. These values were confirmed after equilibration by the most probable number (MPN) method, as shown in **Table 14**. In the case of the *E. coli* O157 strain 380-94 inoculum used for raw processed meat, the average percentage of sub-lethal injury achieved after stress treatment was 63.5%, considered sufficient for the purpose of the study. Together, these data demonstrated that samples and inocula were properly prepared in accordance with the requirements established by the Compendium of Analytical Methods for further evaluation.

4.2.3 Artificial Inoculation and Experimental Layout Using an Unpaired Samples Protocol

Using an unpaired samples protocol, two sets of data for each food type were obtained, one corresponding to the presumptive alternative method (AP) with its confirmation and the

Table 14. Estimation of TVC and inoculation levels for each food item.

Food Item	TVC (CFU/g)	Target Level	Estimated Inoculation Level (CFU/25g)	MPN Results Post-spiking	
				MPN Index Value	Estimated Inoculation Level (MPN/25g)
Raw ground beef	3.20×10^{3}	Low	1.26	3-0-0	1.2
Raw ground beef		High	15	3-3-1	25
Raw ground veal	1.05×10^{4}	Low	1.26	2-0-0	0.5
Raw ground veal		High	15	3-3-0	13.3
Raw beef trim	3.4×10^{4}	Low	1.26	3-0-0	1.3
Raw beef trim		High	15	3-3-0	13.3
Carpaccio	7.7×10^{3}	Low	1.26	3-0-0	1.3
Carpaccio		High	15	3-2-1	8.3
Raw sausage*	1.35×10^{4}	Low	0.96	3-0-0	1.3
Raw sausage*		High	9.6	3-3-0	13.3
Raw burger patty*	3.5×10^{6}	Low	0.96	2-0-0	0.5
Raw burger patty*		High	9.6	3-3-0	13.3
Raw meatballs*	4.6×10^{3}	Low	0.96	3-0-0	1.3
Raw meatballs*		High	9.6	3-2-1	8.3
Raw kebabs*	6.2×10^{5}	Low	0.96	3-0-0	1.3
Raw kebabs*		High	9.6	3-3-1	25

**These food items were inoculated with strain 380-94, which was stressed as described in the Inoculum Preparation Section.*

results from the RM. **Table 15** and **Table 16** summarize the data obtained for both food types, unprocessed and processed raw meat. For (H) samples, all 20 were presumptive positives and agreed with the confirmation results, while (L) samples, with a fractional inoculation, showed only 6 and 8 positives, respectively, from the 20 samples. The rest of (L) samples, 14 unprocessed and 12 processed, were neither detected with the AM nor the RM. Finally, all (U) samples were negative with the AM, which fully agreed with the confirmation results. Hence, based on the results presented, it was concluded that there were no discordant results (false positives/false negatives) when confirming the AM with the RM, with a total of 54 true positives and 36 true negatives in the complete study.

4.2.4 Evaluation of Probability of Detection (POD)

Using the previous results, the performance parameters and POD were calculated for each spiking level of each food type. The results for the POD calculation, which is the proportion of positive results for a specific food type and level of inoculation (257) are shown in **Table 17**, which confirmed that all 95% confidence intervals encompass the value zero (0). Thus, the AM was considered equivalent to the RM for all spiking levels of the two food types evaluated. In addition, the 95% confidence interval from the $dPOD_{(AP,AF)}$ also included the value zero (0), suggesting that the rate of false positives obtained was acceptable.

4.2.5 Evaluation of Performance Parameters

Previous results showed that all combinations of spiking level and food type passed the POD analysis. Hence, they were all considered for the calculation of the performance parameters using only the results from the AM, which were the alternative presumptive (AP) and the alternative final (AF) together with the formulas previously described in **Section 3.3.3**. Overall, the AM complies with the criteria established by the MMC for each performance parameter evaluated. It achieved 100% sensitivity, which means that all positives confirmed by the RM were correctly identified by the AM and no false negatives were obtained. In addition, the LFIA Test Kit had 100% specificity with no false positives, suggesting that it only detected the target organism. Finally, it was considered 100% efficient because the presumptive results matched their confirmation (**Table 18**).

Table 15. Summary of the alternative method (AM) and reference method (RM) unpaired samples results for the unprocessed raw meat food type.

Total Samples	Alternative Method								Reference Method	
	AP		AC		AF[1]				RM	
	POS	NEG	POS	NEG	TP	TN	FP	FN	POS	NEG
(H) 20	0	20	0	20	0	0	0	20	0
(L)	6	14	6	14	6	14	0	0	8	12
(U)	0	5	0	5	0	5	0	0	0	5
TOTAL	**26**	**19**	**26**	**19**	**26**	**19**	**0**	**0**	**28**	**17**

[1]Alternative Final Results (AF) are defined as True Positives (TP), True Negatives (TN), false positives (FP) or false negatives (FN).

AP: Alternative Presumptive; AC: Alternative Confirmation; RM: Reference Method; POS: positive result; NEG: negative result.

Table 16. Summary of the alternative method (AM) and reference method (RM) unpaired samples results for the processed raw meat food type.

Total Samples	Alternative Method								Reference Method	
	AP		AC		AF[1]				RM	
	POS	NEG	POS	NEG	TP	TN	FP	FN	POS	NEG
(H)	20	0	20	0	20	0	0	0	20	0
(L)	8	12	8	12	8	12	0	0	9	11
(U)	0	5	0	5	0	5	0	0	0	5
TOTAL	**28**	**17**	**28**	**17**	**28**	**17**	**0**	**0**	**29**	**16**

[1]Alternative Final Results (AF) are defined as True Positives (TP), True Negatives (TN), FP: false positives, or FN: false negatives.
AP: Alternative Presumptive; AC: Alternative Confirmation; RM: Reference Method; POS: positive result; NEG: negative result.

Table 17. POD analysis for unprocessed and processed raw meat food types.

Unprocessed raw meat

Level	Alternative Presumptive (AP)			Alternative Final (AF)			Reference (R)			$dPOD_{AF,R}$			$dPOD_{AP,AF}$			MMC Pass/ Fail
	POD[1]	LCL	UCL	POD	LCL	UCL	POD	LCL	UCL	dPOD	LCL	UCL	dPOD	LCL	UCL	
(L)	1.00	0.88	1.00	1.00	0.88	1.00	1.00	0.88	1.00	0.00	-0.12	0.12	0.00	-0.12	0.12	Pass
(H)	0.30	0.16	0.48	0.30	0.16	0.48	0.40	0.24	0.58	-0.10	-0.33	0.14	0.00	-0.23	0.23	Pass
(U)	0.00	0.00	0.35	0.00	0.00	0.35	0.00	0.00	0.35	0.00	-0.35	0.35	0.00	-0.35	0.35	Pass

Processed raw meat

Level	Alternative Presumptive (AP)			Alternative Final (AF)			Reference (R)			$dPOD_{AF,R}$			$dPOD_{AP,AF}$			MMC Pass / Fail
	POD[1]	LCL	UCL	POD	LCL	UCL	POD	LCL	UCL	dPOD	LCL	UCL	dPOD	LCL	UCL	
(L)	0.40	0.24	0.58	0.40	0.24	0.58	0.45	0.28	0.63	-0.05	-0.29	0.20	0.00	-0.24	0.24	Pass
(H)	1.00	0.88	1.00	1.00	0.88	1.00	1.00	0.88	1.00	0.00	-0.12	0.12	0.00	-0.12	0.12	Pass
(U)	0.00	0.00	0.35	0.00	0.00	0.35	0.00	0.00	0.35	0.00	-0.35	0.35	0.00	-0.35	0.35	Pass

[1]Probability of Detection (POD) was calculated as POD= x/N, where x represents the number of positives and N the number of samples evaluated.
LCL: lower confidence limit; UCL: upper confidence limit; MMC: Microbiological Methods Committee.

Table 18. Performance parameters for the Alternative Method.

Food Type	Performance Parameters				
	Relative Sensitivity	**Relative Specificity**	**FP Rate**	**FN Rate**	**Test Efficacy**
MMC Criteria	>98%	≥90.4%	<9.6%	<2%	≥94%
Raw meat-unprocessed-25g	100%	100%	0%	0%	100%
Ready-to-cook-processed-25g	100%	100%	0%	0%	100%
Total	**100%**	**100%**	**0%**	**0%**	**100%**
Pass/Fail	**Pass**	**Pass**	**Pass**	**Pass**	**Pass**

The results showed were achieved at 16 h of enrichment following the LFIA Test Kit procedure.

4.2.6 Determination of the LOD

The last criterion assessed for the AM was the LOD, which is defined in the Compendium of Analytical Methods as the smallest amount of culturable microorganisms that can be detected by a specific method in 50% of the samples evaluated (174). For this study, five spiking levels were included with six replicates each. All samples were analyzed with the AM, but confirmed with the RM. Moreover, to determine the proper cell concentration used for inoculation, a 3×3 MPN was also performed using a different set of samples inoculated with the highest level used for the LOD. Thus, the highest inoculation was estimated to be 2.35 MPN/25g with a confidence limit between 0.575 and 9.5 MPN/25g, obtained from the MPN Index Value of 3-1-0. Using this result, the lower cell concentrations included in the LOD study were extrapolated and included in **Table 19**. Finally, the LOD was between the two levels that give respectively more and less than 50% positives. Thus, based on the recorded number of positive and negative replicates for each inoculum level, the LOD was 0.588-1.175 MPN/25g (level 2-3).

Based on the results presented in this section, it was concluded that the LFIA Test Kit exceeded the performance parameters criteria established by the MMC after 16 h of enrichment as shown in **Table 18**. In addition, considering the MPN range obtained for the LOD as an estimate of CFUs (258), it was lower than the range settled by the Microbiological Methods Committee, which is 3-5 CFU/25g (174); thus, its performance was considered comparable to that of the RM. Therefore, it represents a faster and simpler alternative for pre-screening of raw meat samples. Moreover, these outcomes resulted in an official submission of the LFIA Test Kit to the MMC requesting its evaluation for inclusion into Health Canada's Compendium of Analytical Methods.

4.3. Development of the scFvO157

4.3.1 Stability of the Hybridoma Cell Line

The hybridoma cell line (13B3) that produced a monoclonal antibody specific for the O157-antigen that was used throughout this study was first reported in 1997 by Westerman *et al*. from the U.S. Meat Animal Research Center, USDA (227). Because stocks were stored for an extended period of time, it was necessary to ensure that proper antibody

Table 19. Determination of the LOD for the raw meat products category using MPN.

Level	No. Positives/ No. Negatives
Level 1, 2.35 MPN/25g[1]	6/6
Level 2, 1.175 MPN/25g	*4/6*
Level 3, 0.588 MPN/25g	*1/6*
Level 4, 0.294 MPN/25g	1/6
Level 5, 0.147 MPN/25g	0/6

[1]The highest inoculation level was estimated using a 3×3 MPN and used to determine the lower inoculation levels.

specificity was retained. Once the hybridoma was recovered from liquid nitrogen, it was cloned twice using the limiting dilution method, resulting in 97.8% of positive clones when screened using ELISA (data not shown). Previous studies have shown that hybridomas, which yield >90% of positive clones after repeated subcloning, can be considered as stable (183,228,231,232,259). Thus, once ensuring that cells were healthy and stable, three clones were further expanded for antibody production and RNA extraction.

4.3.2 Anti-O157 mAb Characterization

Antibody characterization had two main objectives within this study: 1) to guarantee that the hybridoma cell line was producing the mAb of interest before proceeding with RNA extraction and genetic sequencing; and 2) to determine the main features and properties necessary for their optimal performance as a diagnostic reagent. For this purpose, the mAb was produced by incubating the hybridoma cell cultures until the cells reached a saturated density (viability <50%). At this point, the medium turned yellow and the supernatant was collected. The supernatant of three expanded clones was initially assessed for the mAb isotype. This isotyping analysis indicated that the anti-O157 mAb was an IgG3 isotype with kappa light chains (**Figure 21**), thus confirming the outcome reported by Westerman *et al*. for this specific cell line (227).

4.3.2.1 Anti-O157 mAb purification and ELISA

Further characterization of the anti-O157 mAb required the mAb to be pure in order to eliminate potential interference effects of non-Ig components found in the hybridoma supernatant. Thus, the mAb was affinity purified using immobilized protein G resin. The presence of the pure mAb in the eluted fraction was confirmed by SDS-PAGE, where the 50 kDa and 25 kDa bands characteristic of the heavy and light chains were visualized (**Figure 22A**). Furthermore, the functionality of the mAb was determined by ELISA using *E. coli* O157 and three other non-target strains (*E. coli* ATCC 25922, *S. enterica* ser. Typhimurium and *L. monocytogenes*) at a concentration of 10^7 CFU/well. The absorbance obtained with the mAb in the presence of *E. coli* O157 was 0.606 ± 0.058, while all non-target organisms were below the average cut-off value estimated for each individual strain (**Figure 22B**). Based on these results, it was confirmed that the mAb produced by the

Figure 21. Anti-O157 mAb isotyping results. The supernatant of three selected positive clones (3A5, 3C4, and 1B3) was used for antibody isotyping using an isotyping ELISA kit. The positive control was an antigen reference mixture provided with the isotyping kit, while the negative control was the Hybridoma-SFM. The positive reaction wells developed a yellow color after the addition of stop solution demonstrating the IgG3 isotype and kappa light chain for the anti-O157 mAb.

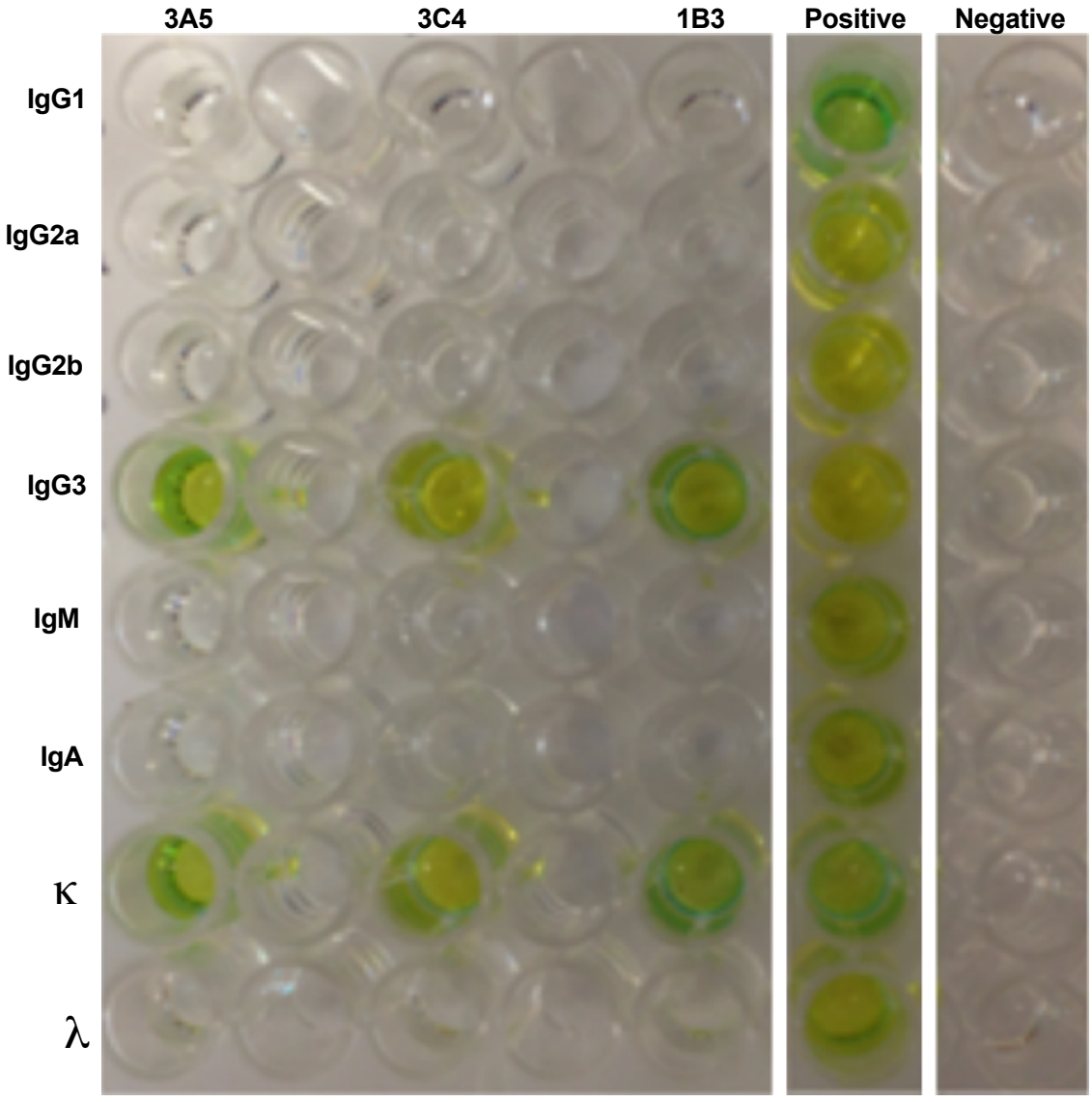
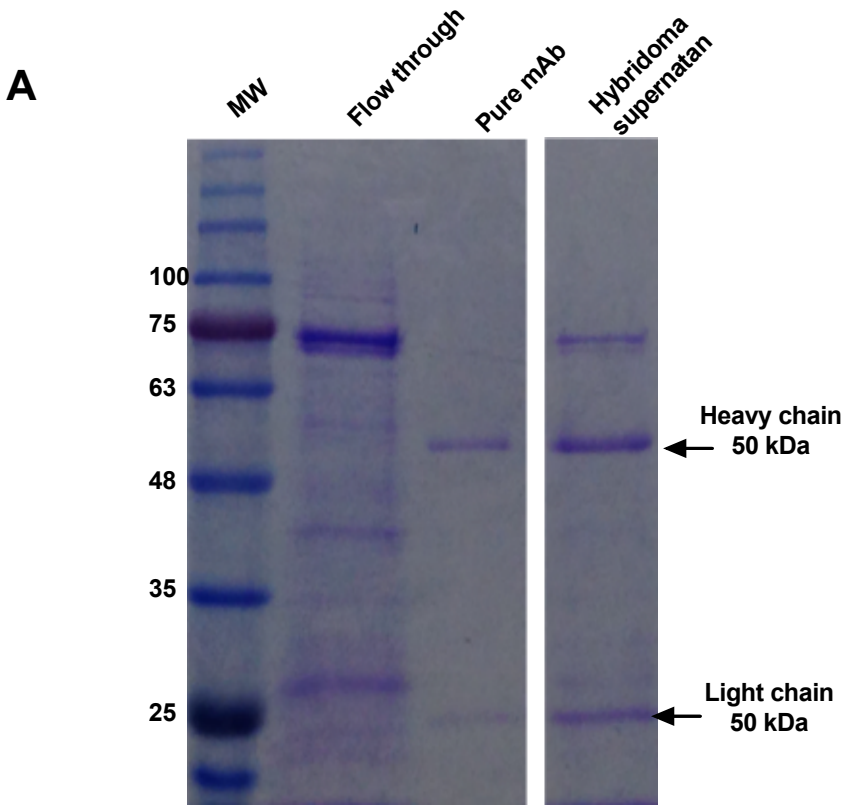
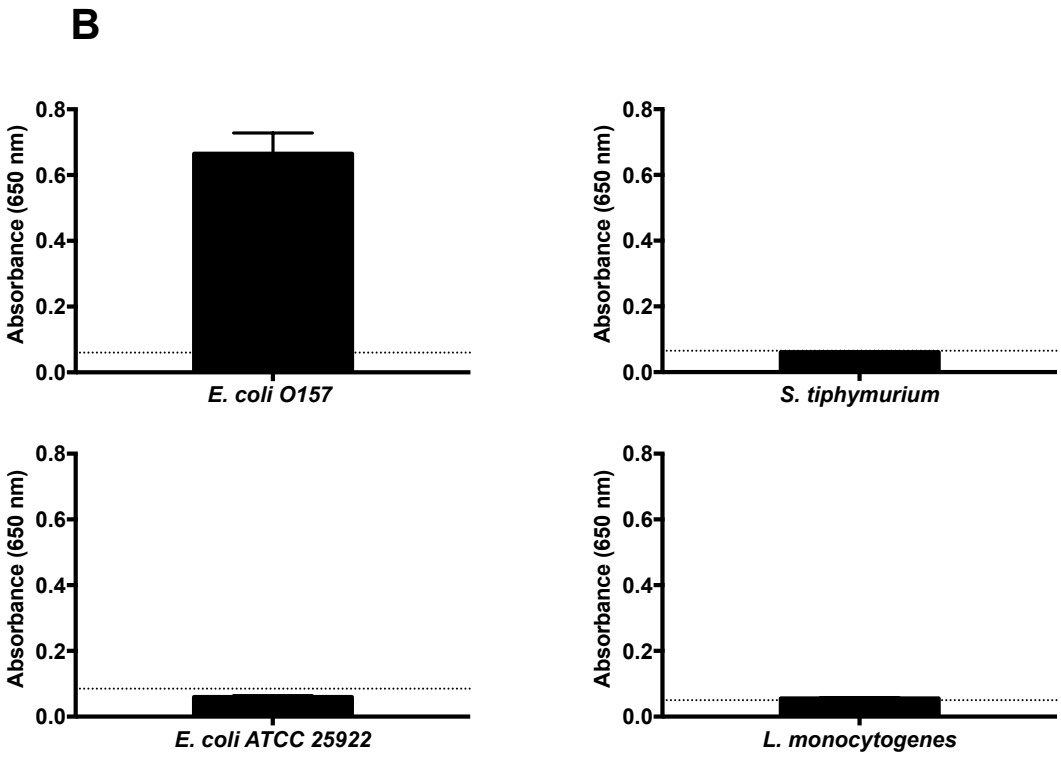

3A5
3C4
1B3
Positive
Negative
IgG1
IgG2a
IgG2b
IgG3
IgM
IgA
κ
λ

Figure 22. Overview of the anti-O157 mAb purification steps and ELISA results. A) Representative image of the affinity purification steps of the mAb using 10% SDS-PAGE gel stained with Coomasie blue. 1) Molecular Weight Marker (kDa); 2) Column flow through; 3) Pure mAb; 4) Crude supernatant before purification. **B)** ELISA results obtained with the positive control (*E. coli* O157) and 3 negative controls (*S. enterica* ser. Typhimurium*, E. coli* ATCC 25922 and *L. monocytogenes*). Data was obtained using 1.25 μg/ml of the mAb and 10^7 CFU/well of each culture. Bars represent the mean ± SEM (*n*=6). The cut-off value was calculated for each sample as the blank mean ± 3SD (*n*=4-6), represented as a dotted line.


A
MW
Flow through
Pure mAb
Hybridoma supernatan
100
75
63
48
35
25
Heavy chain
50 kDa
Light chain
50 kDa


B
Absorbance (650 nm)
0.0
0.2
0.4
0.6
0.8
E. coli O157
S. tiphymurium
E. coli ATCC 25922
L. monocytogenes

hybridoma cell line had the expected specificity towards *E. coli* O157 when used in an ELISA.

4.3.2.2 Determination of the anti-O157 mAb isoelectric point (pI)

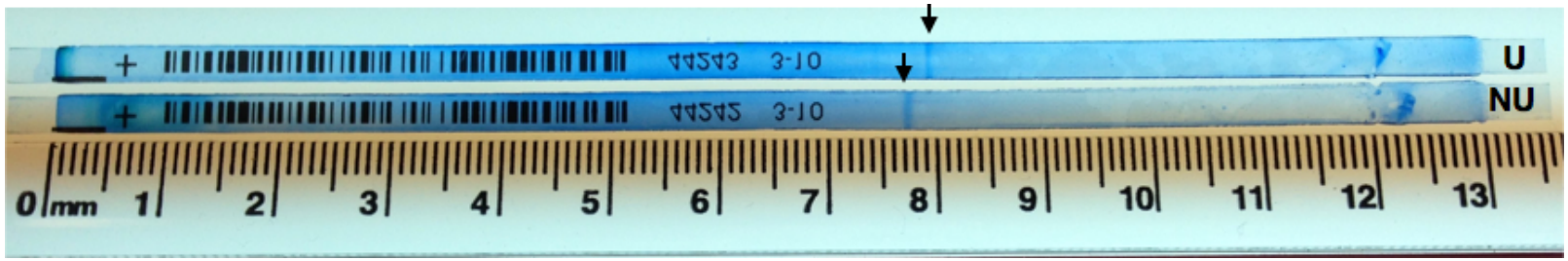
In addition to isotyping and functional ELISA characterization, there are other relevant features that can help to predict the performance of an antibody in a particular assay or technique. As an example, the pI of antibodies is infrequently available despite its effect on proper folding, solubility and thus antigen-antibody interaction. The pI, by definition, is the pH at which the net charge equals zero and thus the antibody may precipitate (260–262). In addition, knowledge of the pI increases the success rate of labelling techniques because most of them depend on the presence of charged amino acids (123). Therefore, knowledge of an antibody pI can help to predict its behavior in an assay and, if necessary, improve the conditions to ensure its proper function. For these reasons, the pI of the anti-O157 mAb was determined by using isoelectric focusing (IEF) and immobilized pH gradient gel strips. **Figure 23A** shows the stained gel strips with a defined band approximately at 7.9 cm when urea was present in the sample mixture and at 7.7 cm when urea was absent (native). The pH gradient data provided by the supplier was graphed in order to interpolate the pI values, which were estimated to be 6.75 and 6.63, respectively (**Figure 23B**). This slight shift between native and denaturing conditions can be explained by the effect of urea, which causes a conformational change and hence alters the total charge of the antibody. This effect has been previously reported by comparing the pI values of denaturing and native proteins using IEF (263). Moreover, a previous study measuring the pI of around 50 different hybridoma mAb, found that >90% were within a pH of 6-8 (262). Therefore, the pI obtained under native conditions, which was approximately 6.63, was considered acceptable for the anti-O157 mAb.

4.3.3 RT-PCR and Sequencing of the Variable Heavy and Light (V_H and V_L) Chains of the Anti-O157 mAb

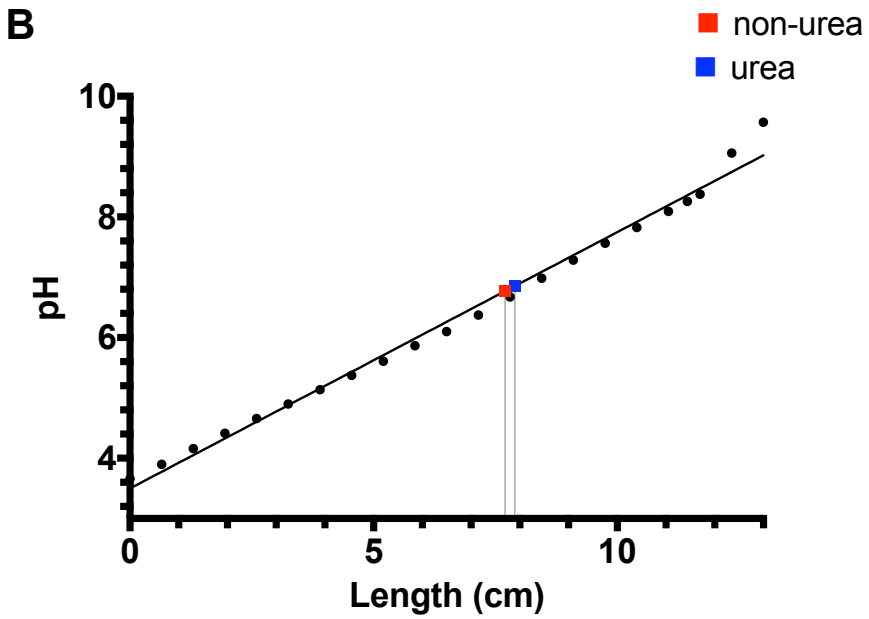
Besides the previous characteristics that were obtained for the anti-O157 mAb, the most important, considered as the “fingerprint” of an antibody, is the amino acid sequence of the complementarity determining regions (CDRs). CDRs represent the specific antigen

Figure 23. pI determination of the anti-O157 mAb using IEF. A) Linear pH 3-10 Immobiline DryStrip gels showing the mAb band after IEF. Sample containing urea (U) showed a band at approximately 7.9 cm, while the sample without urea (NU) has a band at approximately 7.7 cm. Strips were stained with GelCode™ Blue after IEF. **B)** Graph represents the DryStrip gradient data provided by the supplier, which was used to estimate the pI of the anti-O157 mAb by interpolation. Both pIs, with and without urea, are shown.

A


U
NU
0 mm 1 2 3 4 5 6 7 8 9 10 11 12 13

B


non-urea
urea
pH
10
8
6
4
0
5
10
Length (cm)

binding sites; hence knowing these boundaries allows for the understanding of the antibody-antigen interactions while permitting further bioengineering of alternative recombinant antibodies with potentially improved features. The approach adopted for the anti-O157 mAb was to use its V_H and V_L chains amino acid sequences to synthesize a single-chain variable fragment (scFv) that could be used for targeting the O157-antigen. For this purpose, it was necessary to sequence the antigen binding site of the mAb. The PCR products corresponding to both V_H and V_L chains of the mAb were obtained by using the primers described by Wang *et al.* (196) and the thermal cycling conditions stated by Koren *et al.* (195). The length of the amplicons obtained after sequencing corresponded to 409 and 381bp, for the murine V_H and V_L chains, respectively (**Figure 24**). Hence, these data were in agreement with previous studies that have shown that V_H and V_L chains' PCR products can vary between 400-470 and 360-390 bp, respectively, depending on the primers degeneracy and conditions used (192,195).

4.3.4 Characterization of the Anti-O157 mAb V_H and V_L Chains

In order to continue with the development of the scFvO157, the nucleotide sequences of the murine V_H and V_L chains were translated into amino acids. Then, each individual sequence was submitted to the BLASTP program to search for possible identical sequences that had been reported to date. The analysis provided us with the 100 amino acid sequences that produced a significant alignment with the query. **Table 20** summarizes the results into two representative sequences for each variable chain, the one with the highest percentage of identity regardless of the sequence coverage and the one with the highest sequence coverage despite the level of identity. This analysis demonstrated that there was no characterized sequence 100% identical to the anti-O157 mAb in the database. Antibody variable domains are highly conserved, especially in the framework regions (FRs), which was reflected in the high percentages of identity achieved. In particular, V_L chains tend to be more conserved than V_H chains, consistent with the higher percentage of identity we found among the query sequences for the V_L than for the V_H chain. Moreover, the analysis of the conserved domains suggested that both sequences, obtained experimentally, belonged to a murine-heavy chain and a murine kappa-light chain variable region, which were the results expected.

Figure 24. Gel electrophoresis of V_H and V_L chains PCR products. The correctly amplified V_H and V_L of the mAb expressed by the hybridoma cell-line were approximately 409 and 381 bp, respectively. The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

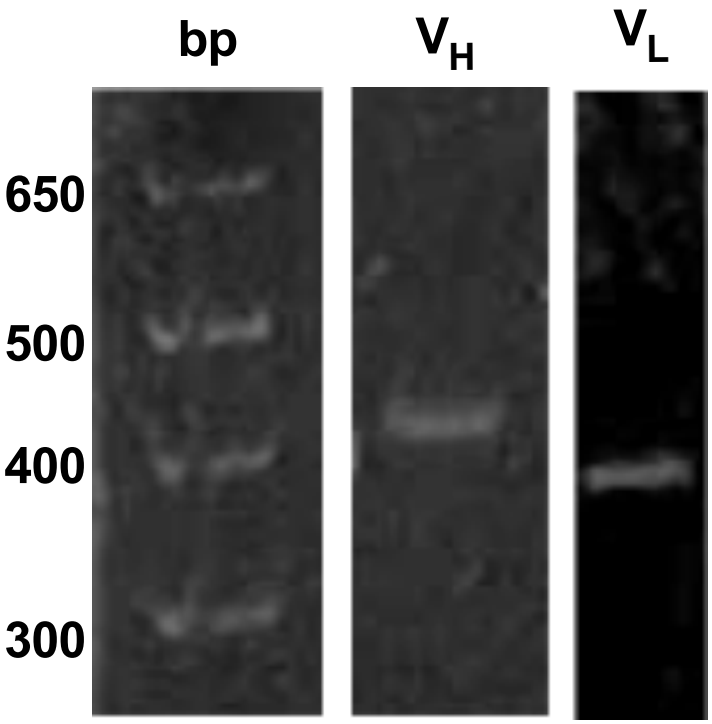

bp
V_H
V_L
650
500
400
300

Table 20. anti-O157 mAb V_H and V_L chains BLASTP results.

Murine Variable Region	BLAST MSA Result			
	Accesion	**Sequence**	**%Query Coverage**	**%Identity**
V_H	AJG06889.1	immunoglobulin heavy chain variable region [*Mus musculus*]	98	77
	AAO18783.1	immunoglobulin heavy chain variable region [*Mus musculus*]	86	92
V_L	ADE80875	anti-botulism toxin B immunoglobulin kappa light chain variable region, partial [*Mus musculus*]	99	90
	AAC13704	Ig kappa light chain variable region, partial [*Mus musculus*]	90	98

For each variable sequence query, the results with the highest sequence coverage and identity, respectively, were selected among the 100 hits.

To supplement the results obtained with BLASTP, a COBALT analysis was performed to pre-screen the V_H and V_L chains for FRs and CDRs. **Figure 25** presents a preliminary overview of the conserved and variable residues, which mainly shaped the FRs and CDRs, respectively. The most noticeable CDR detected was H3, which corresponds to the most diverse loop among the six that formed the variable domain or V-domain. Although it was possible to outline the other five CDRs, the COBALT analysis did not provide concise information to allow us to determine the exact boundaries of the CDR. Therefore, this information was further evaluated using 3D modelling.

4.3.5 Construction of the Humanized scFvO157

After the experimental sequences were confirmed to belong to murine V_H and V_L domains, they were used to create 3D models for estimating the CDR loops based on topological predicting modeling. For this purpose, SWISS-MODEL was used to find the template with the highest identity within the Protein Database (PDB). **Table 21** summarizes the relevant characteristics for each of the models created. Besides the sequence identity and coverage, the Global Model Quality Estimation (GMQE) was also reported. It combines properties from the target and the template alignment reflecting the accuracy of the model built. Values closer to one indicated higher reliability in the result. For the V_H and V_L, the GMQE values were 0.93 and 0.98, respectively, suggesting that models obtained were good enough to determine the CDR loops. **Figure 26A** and **Figure 26B** shows the 3D models with the CDR loops outlined by refining the COBALT results with the topology of the model.

Once CDR loops were defined, both murine V_H and V_L sequences could have been used for constructing the scFv. However, previous studies have shown that recombinant proteins, including scFv, tend to aggregate when expressed in *E. coli* (215,264,265). Therefore, the murine V_H and V_L sequences were aligned with a humanized consensus scFv (234) and adjusted for loop grafting into the humanized backbone. Loop grafting, especially murine loops into humanized backbone, has been shown to improve the stability of scFv synthesized for therapeutic purposes (218,220). The final amino acid sequence of the humanized scFvO157, described in **Figure 26D**, was submitted to SWISS-MODEL to

Figure 25. anti-O157 mAb V_H and V_L chain amino acid sequences and COBALT analysis results. The 121 and 116 amino acid sequences are shown with a color code representing the degree of conservation of each residue based on the alignment with the 100 most significant sequences found in the literature. RED: highly conserved residues within that position. BLUE: less conserved residues, but without gaps in that position when aligned. GREY: residues which position had gaps at least in one sequence aligned. For the latter, upper case residues mean that less than 50% of the analyzed sequences contain gaps and lowercase, greater than 50%. Underlined regions were found to be the most variable within each chain. Heavy chain CDRs: H1, H2, and H3; Light chain CDRs: L1, L2, and L3.

V_H

lpefQVKLEESGGGLVKPGGSLKLSCAASGFAFSSYDMSWVRQTPEKRLEWVAFISSG

H1 H2

GGRTYYPDTVKGRFTISRDNAKNTLYLQMSSLKSEDSGMHYCART---------EWY-----

H3

FDVWGAGTTVTVSSa

V_L

welDIVITQSPSSLAVSAGEKVTMSCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIY

L1

WASTRESGVPDRFTGSGSGTDFTLTINSVQAEDLAVYYCHQYLSS--WTFGGGTKLEI

L2 L3

KR

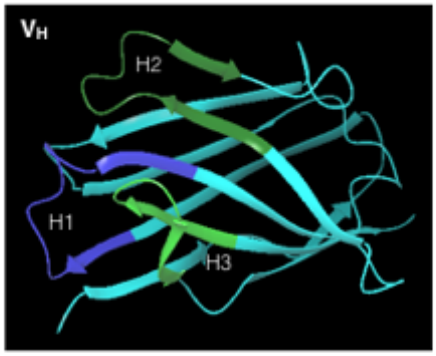
Table 21. SWISS-MODEL homology modeling results used for the construction of the scFvO157.

Sequence	SWISS-MODEL Homology Modeling Results					
	Template (PDB)	**Description**	**Seq. Identity**	**Seq. Similarity**	**Seq. Coverage**	**GMQE[1]**
Murine V_H	2zuq.1.C	Fab fragment heavy chain, X-ray	86.32	0.57	0.97	0.93
Murine V_L	4m61.2.A	Fab A52 light chain, X-ray	95.58	0.60	0.97	0.98
Humanized scFvO157	2ghw.1.B	anti-sars scFv antibody, 80R	78.39	0.54	1.00	0.83

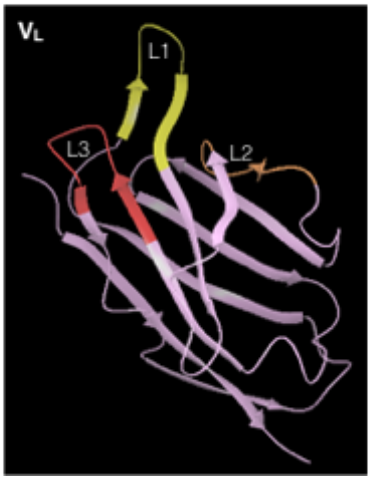
Templates with the highest identity were chosen to create the 3D-model for each variable chain and the final humanized scFvO157. The characteristics that are relevant to assess the quality and reliability of the model created are included for each sequence. [1]GMQE stands for Global Model Quality Estimation and is expressed as a number between zero and one. The highest the value, the higher the reliability in the model.

Figure 26. Construction of the humanized scFvO157 based on 3D modeling. Ribbon representation of 3D models obtained for the **A)** murine V_H, **B)** murine V_L, and **C)** humanized scFvO157. **D)** The final humanized scFvO157 amino acid sequence, with the grafted murine CDRs underlined in their corresponding V_H and V_L human backbone. The polylinker is shown in purple. Murine V_H CDRs are represented as follows: dark green, H1; blue, H1; light green, H3. On the other hand, murine V_L CDRs are as followed: yellow, L1; orange, L2; red, L3.

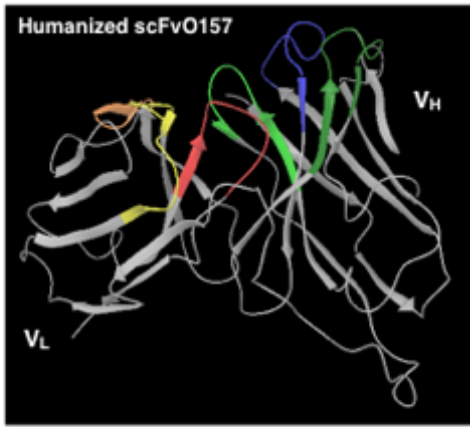
A)



B)



C)



D) Humanized scFvO157

EVQLVESGGGLVQPGGSLRLSCAASGFAFSSY
DMSWVRQAPGKGLEWVAFISSGGGRTYYAD
SVKGRFTISRDNSKNTLYLQMNSLRAEDTAV
YYCARTEWYFDVWGQGTLVTVSS*GGGGSGG*
*GGSGGGGS*DIVMTQSPSTLSASVGDRVTITCL
YSSNQKNYLAWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTLTISSLQPEDFATYY
CHQYLSSWTFGQGTKLEIK

obtain the 3D model, as shown in **Figure 26C**. The humanized scFvO157 was complemented with two more sequences: a tobacco etch virus (TEV) protease cleavable site on the N-terminus and a biotin tag on the C-terminus. Once completed, the amino acid sequence was reverse translated into a DNA sequence, with KpnI and BamHI restriction enzyme sites incorporated onto the 5' and 3' ends, respectively (**Figure 27**). This sequence was codon optimized in order to improve translational efficiency in *E. coli*, synthesized and cloned into the pUC57 plasmid for further processing.

4.3.6 Molecular Cloning of the Humanized scFvO157

Besides using loop grafting as an approach for increasing protein solubility, the humanized scFvO157 was also fused, at the N-terminal, with thioredoxin (TrxA). The latter has the ability to confer translation efficiency and solubility to fused proteins (265,266). TrxA is a protein feature included in pET32a(+) plasmid, which was selected for expression of the scFvO157 construct. In addition, pET32a(+) contains a His_6tag, commonly used for protein purification with Ni-NTA affinity resin columns.

Because the scFvO157 was to be used as a detection reagent, the construct was designed to carry a biotin tag sequence at the C-terminal, which consisted of 15 amino acids (AviTag™ Technology, Avidity). Biotinylation was proved to be successful through the insertion of the pBirAcm plasmid, which contains the gene that codes for the BirA enzyme and is also induced by the presence of IPTG. The AviTag™ sequence was recognized by the BirA enzyme for an *in vivo* addition of biotin, which was combined with the culture medium during induction. Therefore, the final protein expressed in *E. coli* BL21(DE3) was TrxA::His_6 tag::TEV::scFvO157::biotin. **Figure 28C** depicts the pET32a(+) cloned map with the most relevant features.

After the cloning process, the presence of the 801 bp scFvO157 construct was initially assessed by agarose gel electrophoresis. **Figure 28A** shows the shift in gel bands when the scFvO157 construct was successfully cloned into pET32a(+). A restriction enzyme digestion was performed to corroborate the presence of the construct by its size. **Figure 28B** shows a ~800 bp band, which corresponds to the size of the scFvO157 construct, while

Figure 27. Deduced amino acid and DNA sequences used for scFvO157 expression. **A)** scFvO157 amino acid sequence including the TEV cleavable site on the N-terminal (bold) and the biotin tag (red) in the C-terminal. **B)** Reverse translated and codon optimized nucleotide sequence with the KpnI (blue) and BamHI (green) restriction enzyme sites at 5' and 3' ends respectively.

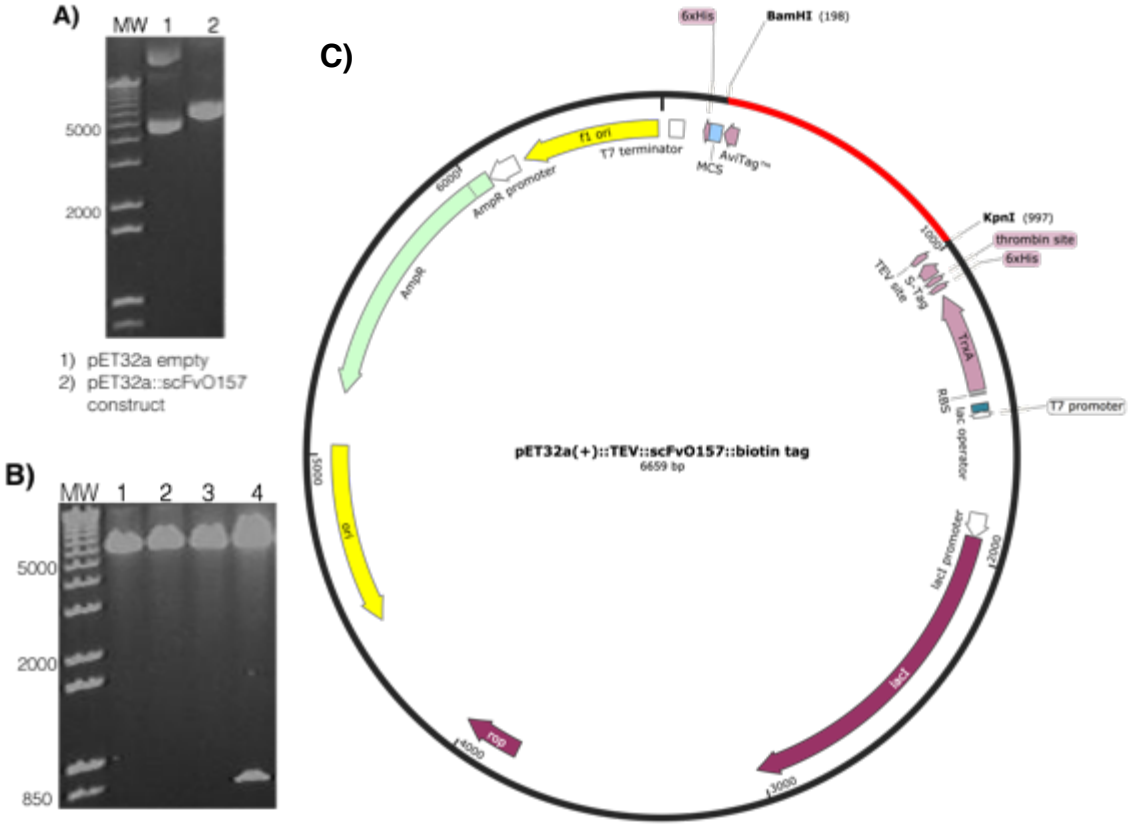
A)

ENLYFQ↓GEVQLVESGGGLVQPGGSLRLSCAASGFAFSSYDMSWVRQAPGKGLE
WVAFISSGGGRTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARTEW
YFDVWGQGTLVTVSSGGGGSGGGGSGGGGSDIVMTQSPSTLSASVGDRVTITCLYS
SNQKNYLAWYQQKPGKAPKLLIYWASTRESGVPSRFSGSGSGTDFTLTISSLQPEDF
ATYYCHQYLSSWTFGQGTKLEIK**SGGGLNDIFEAQKIEWHE**

B)

GGT ACC GAA AAC CTG TAT TTC CAA GGC GAA GTC CAA CTG GTC GAA TCG
GGT GGC GGT CTG GTC CAA CCG GGC GGC TCC CTG CGT CTG TCC TGC GCG
GCC AGC GGC TTT GCA TTC AGC TCT TAT GAT ATG TCC TGG GTT CGT CAG
GCA CCG GGT AAA GGC CTG GAA TGG GTC GCT TTT ATT AGT TCC GGC GGT
GGC CGC ACC TAT TAC GCT GAT TCT GTG AAA GGT CGT TTC ACC ATC TCT CGC
GAC AAC AGT AAA AAT ACG CTG TAT CTG CAG ATG AAC AGC CTG CGT GCA
GAA GAT ACC GCT GTG TAT TAC TGC GCG CGC ACG GAA TGG TAC TTT GAC
GTT TGG GGT CAA GGC ACC CTG GTG ACG GTT TCA TCG GGT GGC GGT GGC
AGC GGT GGC GGT GGC TCT GGT GGC GGT GGC AGT GAT ATT GTC ATG ACC
CAG AGC CCG TCT ACC CTG AGT GCG TCC GTC GGT GAC CGT GTG ACC ATC
ACG TGT CTG TAT AGC TCT AAC CAG AAA AAC TAT CTG GCC TGG TAT CAG
CAA AAA CCG GGC AAA GCG CCG AAA CTG CTG ATT TAC TGG GCC TCC ACC
CGT GAA TCA GGT GTT CCG TCG CGC TTT TCA GGT TCG GGC AGC GGC ACC
GAT TTC ACC CTG ACG ATC AGT TCC CTG CAG CCG GAA GAC TTT GCC ACC
TAT TAC TGC CAT CAG TAT CTG TCA TCG TGG ACC TTC GGC CAG GGT ACG
AAA CTG GAA ATT AAA TCG GGC GGT GGC CTG AAC GAC ATC TTT GAA GCA
CAG AAA ATT GAA TGG CAC GAA TAA **GGA TCC**

Figure 28. Cloning of the scFvO157 construct into pET32a(+) expression plasmid.A) Image comparing the empty pET32a(+) (lane 1) with the pET32a(+)::scFvO157 construct (lane 2). **B)** Image of restriction digests, where lane 1 and 2 include KpnI or BamHI alone resulting in single bands of the full size empty plasmid. Lane 3 corresponds to a double digested KpnI/ BamHI empty plasmid, which produced a single band of slightly lower size. Lane 4 shows the products of a double digested KpnI/ BamHI cloned pET32a(+), matching the size of the backbone (~5858 bp) and the released insert of ~800 bp. Both images correspond to 1% w/v agarose gels stained with EtBr. **C)** pET32a(+) map containing the 801 bp scFvO157 gene (red) cloned using KpnI and BamHI. The TrxA::His_6tag is situated where protein translation starts.


A)
MW 1 2
5000
2000
1) pET32a empty
2) pET32a::scFvO157 construct
B)
MW 1 2 3 4
5000
2000
850
1) pET32a empty-KpnI
2) pET32a empty-BamHI
3) pET32a empty-KpnI/BamHI
4) pET32a::scFvO157 construct KpnI/BamHI
C)
6xHis
BamHI (198)
f1 ori
T7 terminator
MCS
AviTag™
AmpR promoter
AmpR
KpnI (997)
thrombin site
6xHis
S-Tag
TEV site
TrxA
RBS
lac operator
T7 promoter
pET32a(+)::TEV::scFvO157::biotin tag
6659 bp
ori
lacI promoter
lacI
rop

no smaller bands were seen when empty plasmids were also cut. In addition, the plasmid was sequenced to confirm that the construct sequence was correct after the cloning process.

4.3.7 Expression of the Humanized scFvO157

Despite the efforts made during the design of the scFvO157 construct to ensure protein solubility, the initial trials for expression and purification resulted in poor yields of the purified protein (**Figure 29**). Attempts were made to assess its functionality by ELISA using *E. coli* O157 DSM 17076 as a positive control and *E. coli* ATCC25922 as a negative control. However, the results obtained with *E. coli* O157 (0.035 ± 0.001) did not notably surpass the cut-off value, besides, the negative control was also positive although with a lower absorbance (0.013 ± 0.004)[4]. Because these results were considered inconclusive, immunofluorescence microscopy was also attempted to evaluate the scFvO157 specificity, but did not result in an improved outcome that could serve as evidence of the proper function of the scFvO157.

Due to the previous results, efforts to increase the expression of soluble protein were made. It has been shown that overexpression of heterologous genes can promote protein misfolding (217), which can lead to aggregation and consequently formation of intracellular inclusion bodies (264,267). Taking this into consideration, different attempts were made to modulate production rate through the induction step. Modification of conditions, including induction time, temperature, and concentration of the inducer (IPTG), were tried, however the SDS-PAGE analysis after cell disruption showed that none of the variations succeeded in increasing the expression of soluble protein and thus the yield of purified scFvO157 (**Figure 30**). The fact that optimization of process parameters improves protein solubility has been well-established (197,203,267), however, their effects are still unpredictable and dependent on the particular requirements of the protein expressed (264,268).

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[4] Average of the absorbance obtained from duplicates± SEM. The cut-off value average was calculated from a total of four blank replicates for each of the two strains assessed.

Figure 29. Overview of the purification and cleavage of the scFvO157. SDS-PAGE and Western Blot analysis of the purification steps including **A)** separation of the TrxA::His_6tag::TEV::scFvO157::biotin protein (45 kDa) from the crude extract (flow) by increasing imidazole concentrations (mM) using $Ni^{2\pm}$affinity gravity chromatography, **B)** removal of the TrxA::6×His tag using autoinactivation-resistent His_7::TEV cleavage, and **C)** purification of the scFvO157 using an imidazole gradient and $Ni^{2\pm}$affinity gravity chromatography. Schematic representations of the target protein throughout the process are also included. Images correspond to 12% SDS-PAGE gels stained with Coomassie blue or nitrocellulose membranes stained with Pierce™ Reversible Protein Stain Kit. Western Blot images correspond to detection using streptavidin-IRDye800.

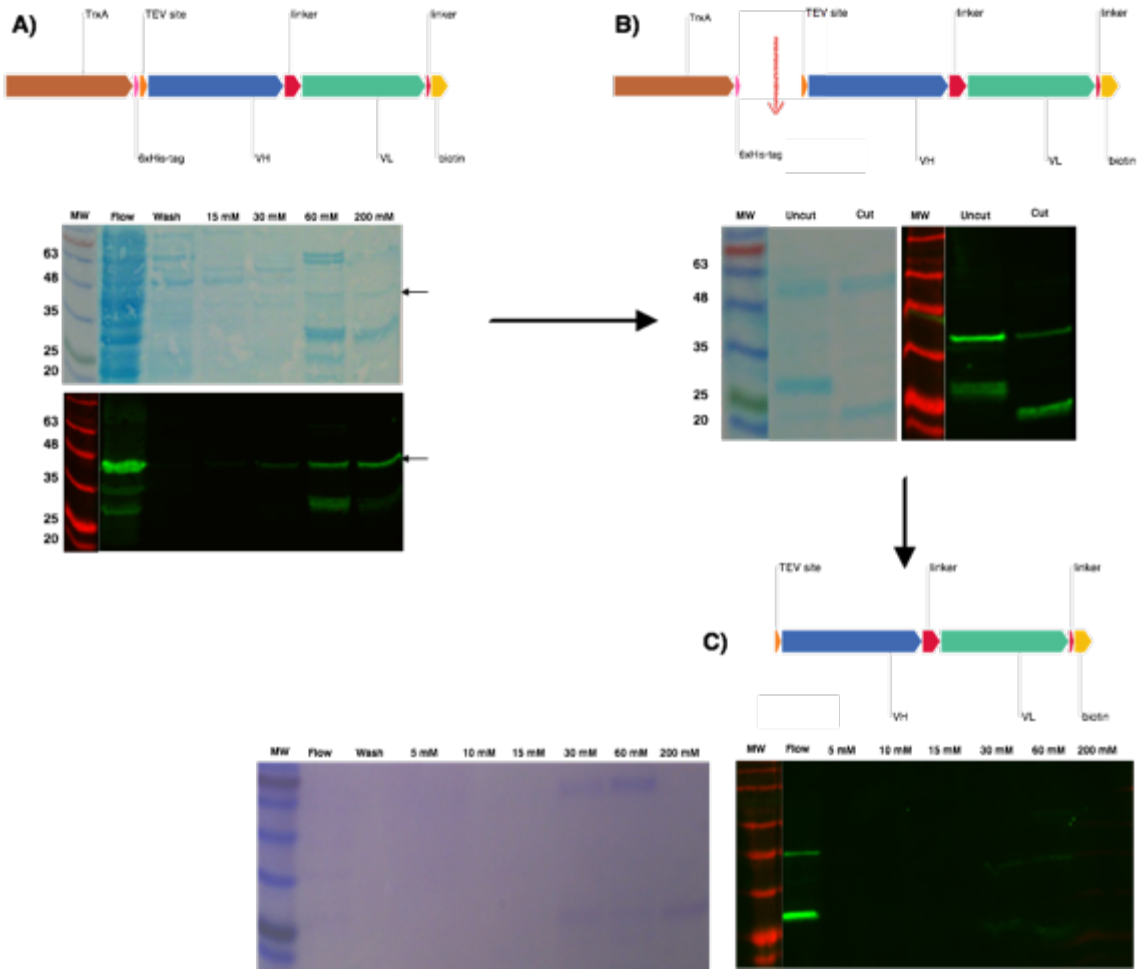
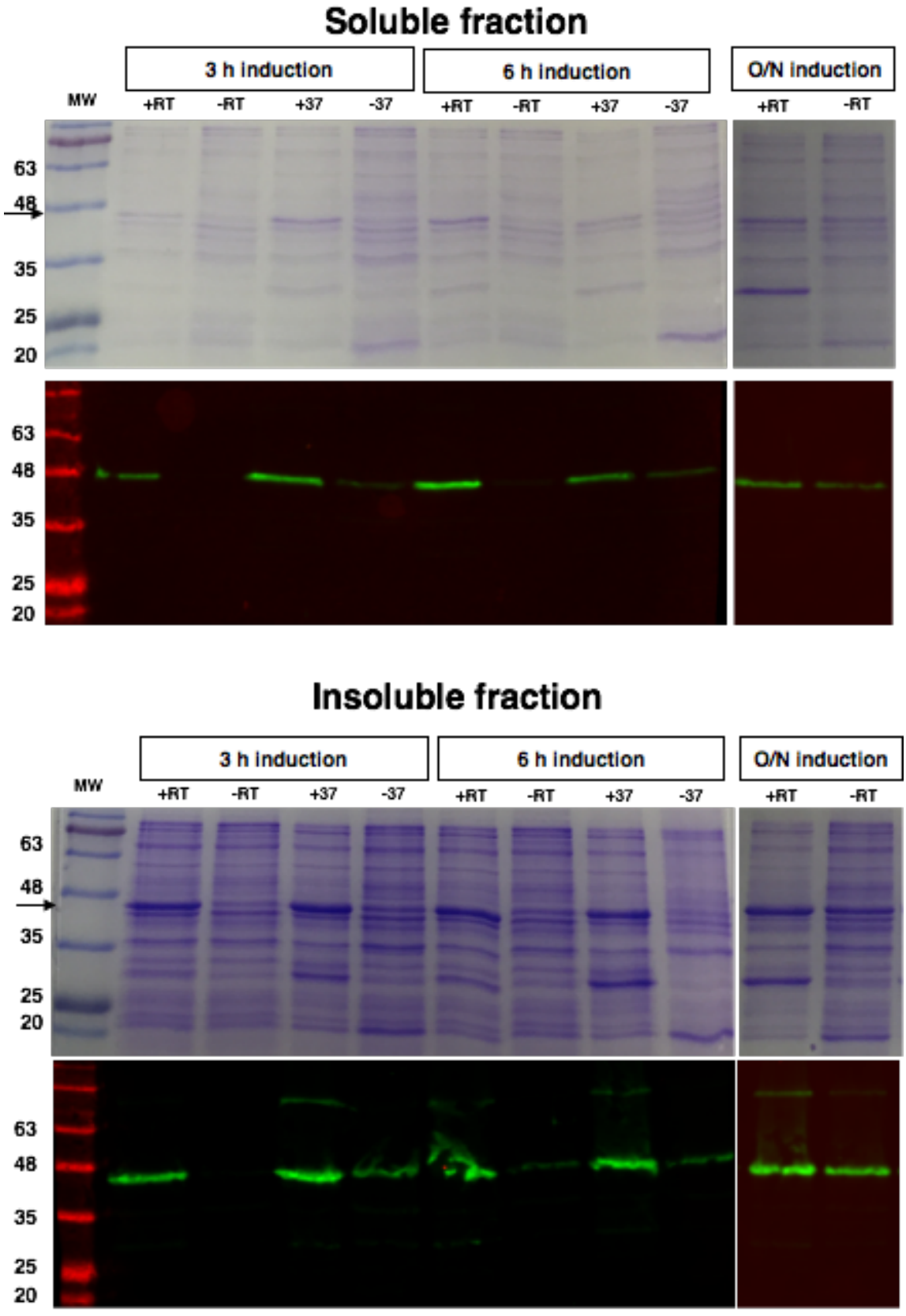

A)
TrxA
TEV site
linker
linker
6xHis-tag
VH
VL
biotin
B)
TrxA
TEV site
linker
linker
6xHis-tag
VH
VL
biotin
MW
Flow
Wash
15 mM
30 mM
60 mM
200 mM
63
48
35
25
20
MW
Uncut
Cut
MW
Uncut
Cut
C)
TEV site
linker
linker
VH
VL
biotin
MW
Flow
Wash
5 mM
10 mM
15 mM
30 mM
60 mM
200 mM
MW
Flow
5 mM
10 mM
15 mM
30 mM
60 mM
200 mM

Figure 30. Effect of temperature and induction time in the expression of soluble scFvO157. SDS-PAGE and Western Blot analysis of soluble and insoluble fractions showing the effect of different induction conditions on protein expression. Inductions were carried out in small scale with (+) and without (-) 0.1 mM IPTG. SDS-PAGE images correspond to 12% SDS-PAGE gels stained with Coomassie blue. Western Blot images correspond to detection using streptavidin-IRDye800.


Soluble fraction
3 h induction
6 h induction
O/N induction
MW
+RT
-RT
+37
-37
+RT
-RT
+37
-37
+RT
-RT
63
48
35
25
20
63
48
35
25
20
Insoluble fraction
3 h induction
6 h induction
O/N induction
MW
+RT
-RT
+37
-37
+RT
-RT
+37
-37
+RT
-RT
63
48
35
25
20
63
48
35
25
20

As a final alternative, attempts were focused on recovering the cytoplasmic inclusion bodies from the cell pellet by using denaturing conditions and solubilization in 8 M urea. Although the advantage of this approach was that inclusion bodies were obtained mostly unadulterated after cell disruption, *in vitro* refolding proved to be difficult due to precipitation of the target protein. Therefore, a second alternative using a combination of denaturing and native buffers was also tried, but it was not advantageous to the overall yield. Both methods proved to increase the protein extraction yield when compared to the previous results obtained from the soluble protein fraction. However, further *in vitro* refolding and/or processing was particularly challenging due to constant precipitation. Through all these efforts, it was concluded that obtaining sufficient yields of the pure scFvO157 was and still is a challenge that requires a thorough study to adapt either the induction or the *in vitro* refolding procedures to the particular characteristics of the expressed protein.

4.3.8 Re-Design of the scFvO157 Structure

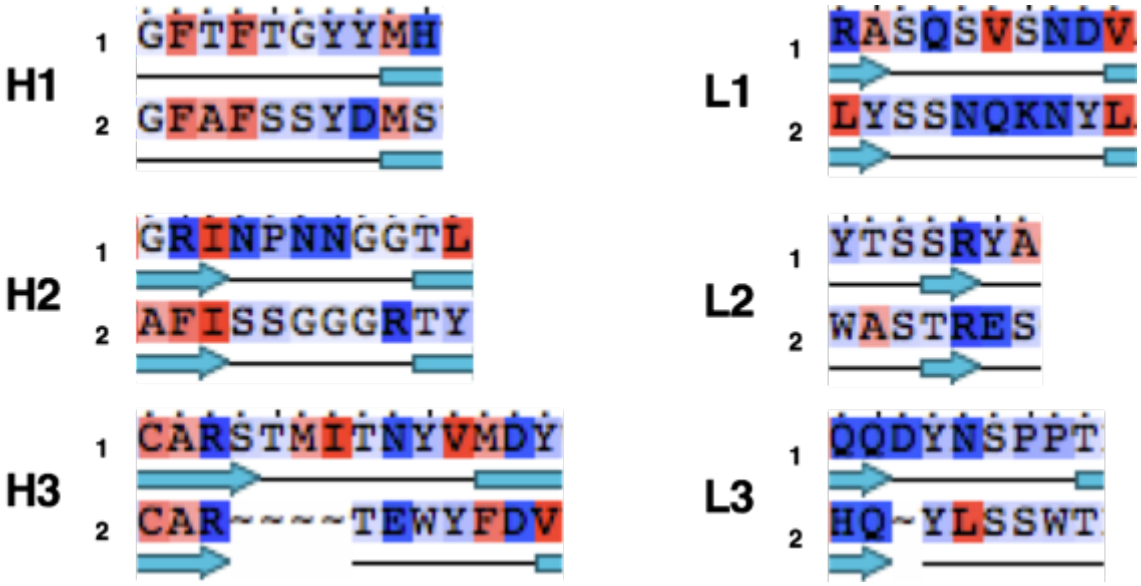
The major constraint in this study was the difficulty in obtaining an adequate yield of purified scFvO157 to perform proper functional assessments. Two main challenges were identified: a) low expression of soluble protein and b) precipitation of purified inclusion bodies during *in vitro* refolding. Even though both limitations can be overcome by adjusting conditions during induction or further refolding, whether a recombinant protein is soluble or forms inclusion bodies relies mostly on its primary structure (197,264,269,270). Protein sequence provides information regarding functional conformation (268), thus minor changes in the primary amino acid sequence (197,268,270,271), or in its length (271) may affect the expression of soluble protein or affect the proper folding. In order to investigate the potential effect of the scFvO157 primary sequence in its soluble expression levels, a retrospective approach was adopted; two of the most relevant intrinsic properties of the scFvO157 were compared against the humanized scFv template used for its construction.

The initial alignment score between the humanized scFv0157 and its template was 0.015 with a RMSD of 0.609 Å as obtained with Maestro Software. The default settings were

used, where all residues within both structures were considered for the alignment. Both values were particularly small, confirming the high structural similarity and thus meaningful alignment of the 3D models. One of the major intrinsic protein properties that plays a key role in promoting aggregation is hydrophobicity, especially when clusters of hydrophobic residues are exposed in the surface of the expressed protein (264,272). For this reason, the primary sequences were aligned and compared using the Kyte-Dolittle hydrophobicity color scheme in the Multiple Sequence Viewer, where minor amino acid distinctions were found between the CDR loops of both sequences (**Figure 31**). To have a better estimation of the effect of those residue variations, the amino acid sequences were used to calculate the hydrophobicity index for each scFv using the GPMAW bioinformatics tool (http://www.alphalyse.com/gpmaw_lite.html). The outcome was -0.28 and -0.34 for the scFvO157 and the humanized scFv template, respectively, suggesting that the former was slightly more hydrophobic. Formation of inclusion bodies has been assumed to be favored by non-specific interactions among hydrophobic residues found in different molecules (216,273). Therefore, the modifications in amino acid residues due to loop grafting may have predisposed the aggregation of the expressed protein, when compared with its humanized template.

To complement the hydrophobicity results, the theoretical pI of both scFv was also obtained, using the GPMAW bioinformatics tool, with the result being 8.95 for the scFvO157 and 9.22 for the humanized scFv template. Because the scFv were not expressed alone, the hydrophobicity index and pI were also calculated for the protein including the fusion partner, TrxA, cleavage sites and tags. The hydrophobicity index was similar to the one obtained for the scFv alone however pI values were 6.05 for the scFvO157 and 5.65 for the humanized scFv template. As noted, there was a remarkable difference in pI values between the humanized scFv template and the scFvO157, regardless of the presence of the fusion partner and tags. These results support the hypothesis that even though the humanized backbone was kept constant the differences in certain amino acid residues found in the CDR loops can significantly change the protein properties. In addition, they reinforce the fact that extraction and processing conditions need to be tailor made for each particular protein taking into consideration such characteristics.

Figure 31. Hydrophobicity comparison of the scFvO157 and scFv humanized template CDR loops. The six CDR loops were aligned and compared using the Kyte-Dolittle hydrophobicity color scheme in the Multiple Sequence Viewer of Maestro Software. Hydrophobic residues are red, hydrophilic residues are blue, and residues without hydrophobicity are white. The first line represents the CDR loops of the scFv humanized template (1) while the second line represents the CDR loops of the scFvO157 (2). Blue arrows represent beta sheet secondary structures.


H1
1 GFTFTGYYMH
2 GFAFSSYDMS
L1
1 RASQSVSNDV
2 LYSSNQKNYL
H2
1 GRINPNNGGTL
2 AFISSGGGRTY
L2
1 YTSSRYA
2 WASTRES
H3
1 CARSTMITNYVMDY
2 CAR~~~~TEWYFDV
L3
1 QQDYNSPPT
2 HQ~YLSSWT

CHAPTER 5 DISCUSSION

Safe food and water are crucial elements of a healthy society. However, given current food processing techniques and practices, it is not reasonable to guarantee that all food will be safe; however, it is possible to ensure, that all food will be free of hazards to an acceptable level of risk (3). In pursuit of this, initiatives around the world have been implemented, where one of the key elements to increase food safety relies on early detection of potential hazards and monitoring of food following a farm-to-fork approach (5,8). Of particular interest has been the development of improved detection tools for targeting bacterial pathogens in food, where research efforts have been focused in order to achieve the following: a) identify microbial hazards (7) and b) decrease the burden of disease caused by foodborne diseases and pathogens (8).

In this work, the focus was on *E. coli* O157, which is of particular interest due to its relevance in food safety since this serotype was first described in 1982. Of importance, in 2015, the first Canadian study that focused on estimates of hospitalizations and deaths due to foodborne illness considered *E. coli* O157 was among the top five foodborne pathogens with the highest number of hospitalizations (246) and among the four pathogens with the highest number of deaths (8) each year (15). Although it was not considered as one of the pathogens that causes the greatest number of illnesses (15), either due to infrequent reporting (62) or to a lower incidence (274), its contribution to the total number of hospitalizations and deaths due to the severity of the illness and long-term negative outcomes, including death, establishes this pathogen as a major public health concern. In addition, a study published in 2014 estimated a significant annual cost of illness due to *E. coli* O157 infections in Canada (62). Based on an estimate of 22,344 annual primary infections and 37,867 on-going long-term cases, extrapolated from the incidence rate of 2008 (2.28 infections/100,000 persons) reported by the Public Health Agency of Canada (PHAC), an annual cost of at least $403.9 million CAD was estimated due to primary cases and long-term outcomes caused by *E. coli* O157 infections (62). Since 1990, verotoxigenic *E. coli* (including *E. coli* O157) became a notifiable disease, meaning that any case has to be reported by PHAC (66). In addition, due to the low infectious dose of *E. coli* O157 and considering that meat, especially raw beef products, has been the most implicated in outbreaks in Canada (31), a no detectable level policy for *E. coli* O157 was established specifically for these food products (70). However, despite all these efforts made to control

foodborne diseases, new challenges, including climate change (5,7), are having a huge impact on microbial ecology with the emergence of foodborne diseases and unusual outbreaks. These all have to be rapidly investigated (5). Together with *Salmonella spp.*, *E. coli* O157 is considered of great concern due to its stress tolerance response (e.g. acid resistance) that could potentially make it a strong survivor in the event of climate change (7). In addition, with the globalization of the food market, the risks of spreading potential foodborne diseases globally have also increased (7,8). For these reasons, it is evident that *E. coli* O157 has been and still is one of the major foodborne pathogens upon which research in Canada should be focused.

One of the tools that food producers use as part of their food safety surveillance system is microbial testing for monitoring and early pathogen detection. Conventional bacterial culture has most commonly been used, which, in practical terms, requires up to a week to confirm the presence of *E. coli* O157 in contaminated food. Therefore, in the last decade, there has been an increase in rapid detection methods (159) that can be used for fast screening instead of the lengthy, laborious traditional culture method. These technologies are allowing food producers to release safe products at an earlier stage instead of waiting for the traditional cultural results that take several days. Numerous reviews have focused on analyzing the advantages and disadvantages of emerging technologies that are being used for developing rapid methods for the detection of foodborne pathogens (106,159,275). In particular, the lateral flow immunoassay (LFIA) format has received significant attention in food safety research (106,132,140) and commercial (106) sectors, as it is considered an inexpensive, easy-to-use, and fast alternative for pre-screening (106). Despite the fact that several LFIA were commercially available at the time of writing, for example, MaxSignal® *E. coli* O157 Strip Test Kit (Bioo Scientific Corporation), DuPont™ Lateral Flow System for *E. coli* O157 (DuPont), FoodChek™ *E. coli* O157 test (FoodChek™ Systems Inc.), Reveal 2.0 for *E. coli* O157:H7 (Neogen Corporation), and VIP® Gold for EHEC (BioControl), not all of them were approved by Health Canada. In fact, in the latest published version of the summary of methods found in Health Canada's Compendium of Analytical Methods for *E. coli* O157:H7, only one immunological method, the Merck Singlepath® *E. coli* O157 Kit, has met the standards required for it to be approved for screening purposes (276). In practice, essentially two methods are widely

used in the Canadian food production system, conventional culture and PCR, thus a reliable and validated point of care test that has regulatory authority would be a major advance in food safety in Canada and potentially beyond.

Given the critical role that an alternative method plays in determining the reasonable level of risk of food products, these tests must be subjected to a thorough evaluation before commercialization and use for food analysis. Several standards have been developed worldwide that provide protocols for the validation of new microbiological methods. The most widely recognized are the AOAC International programs, the ISO 16140 Standard (159), USDA-FSIS Guidance for Evaluating Test Kit Performance (277), and Health Canada's Guidelines for the Development and Management of Food Microbiological Methods (174). As it is critical that the results obtained with the alternative method are reliable and recognized by the government parties (159) involved in a food safety management system, Health Canada's guidelines were selected for the validation of the LFIA Test Kit. In fact, food producers can determine which testing method to use based on the fit for purpose. However, particularly in the case of raw beef products, the CFIA based on the Meat Hygiene Manual of Procedures, which is under the Meat Inspection Act and Meat Inspection Regulations, requires that the testing of products must be done using an approved method listed under Health Canada's Compendium of Analytical Methods (176). Collectively, this information, in combination with the fact that Merck Singlepath® was the only approved immunological method, brought up the possibility to proceed with the validation process to seek the inclusion of the alternative LFIA Test Kit method in the Compendium of Analytical Methods. It should be noted that the LFIA Test Kit developed during this study for the detection of *E. coli* O157 is currently undergoing the assessment by the Technical Group of the Microbiological Methods Committee for its inclusion in the Compendium of Analytical Methods (174).

In general terms, the validation of an alternative method can be defined as the process of confirming its "fitness for purpose" by providing and examining objective evidence that will lead to the establishment of the method's performance parameters (159,172). Regardless of the validation standard used, the initial stage involves the comparison of the alternative method against a reference method (105,159,173). In most cases, the reference

method is represented by the conventional culture method, which is internationally recognized by the standard agencies or governmental authorities.

Focusing on the LFIA Test Kit validation, this procedure is defined in the Compendium as a Relative Validation, where the performance of the Test Kit was assessed in comparison with the reference method MFHPB-10 as shown in **Table 15** and **Table 16**. Although these results showed that the LFIA Test Kit was able to detect the same number of positives as the reference method (true positives), it does not provide evidence of an equivalency between both methods. Therefore, the POD analysis had to be performed, which is a model that helps to determine equivalency between two methods particularly in cases where different sample portions are evaluated with each method due to different enrichments. As shown in **Table 17**, the POD is calculated for each inoculation level, which makes the qualitative data conditional on the concentration of *E. coli* O157 present in the sample (278). In addition, the POD compares the response of the alternative and the reference method by the mathematical difference of their POD values (*d*POD), while the statistical significance is given by the calculation of its confidence interval (278). These final results were further used to determine the performance parameters in **Table 18**. Overall, the relevance of the POD analysis is that it allows one to determine the compliance of an alternative method with established performance parameters, as stated in **Table 17**, based on concentration. It also determines the equivalence between two methods (278). In addition to the POD and performance parameters analysis, an effective alternative method should have a LOD comparable to the standard method or ≤3 CFU/g (174). In the case of alternative methods for detection of *E. coli* O157 in raw meat products, this is critical due to the total absence of established criteria (176), which implies that 1 CFU/25g sample should be detectable. Indeed, the results obtained for the LOD evaluation (**Table 19**) demonstrated that the LFIA Test Kit was able to comply with this requirement. The LFIA Test Kit was able to deliver presumptive results in 17 h of total assessment time compared with the 2 days that it takes to obtain isolated presumptive colonies with the culture reference method (255). Therefore, it proved to be faster but was still sensitive enough to meet the required criteria. In addition, the validation results were similar to the Merck Singlepath® results reported in the AOAC Research Institute certificate for raw ground

beef (169), but with the great advantage of providing results in <25 h, which is the reported total analysis time found in the AOAC® Performance Tested℠ Certificate.

The principle of the LFIA relies on a sandwich immunoassay, where the monoclonal antibody confers the specificity necessary to detect *E coli* O157. In fact, Westerman *et al.* reported that the mAb selected for this assay reacted with 47 *E. coli* O157:H7 strains and 17 O157:non-H7 strains (227). This was further supported by the results obtained with the inclusivity study performed for the validation of the LFIA Test Kit (**Section 4.2.1** and **Appendix A**), where all *E. coli* strains expressing the O157 somatic antigen were detected, regardless of the H antigen they possessed. Although two strains, *E. coli* O44:H18 and *E. coli* O124:NM belonging to the enteroaggregative (EAEC) and enteroinvasive (EIEC) pathotypes, respectively, produced weakly positive results during the exclusivity study, they were not considered as a critical cross-reaction issue due to the nature of the strains and the overall result obtained during the exclusivity study. Further analysis using an ELISA suggested that the mAb did not cross-react with these strains. In addition, no evidence was found that could structurally link the O44 and O124 antigens with the O157. Therefore, this represents an opportunity to further investigate these results and potentially improve the performance of the LFIA Test Kit.

The concept of sandwich immunoassays has been known for some time and has been widely used in pathogen detection because bacteria have many different epitopes that can be targeted by different antibodies without interfering with each other (140). Frequently used formats are based on immobilized captured antibody on a nitrocellulose membrane (140) or by using conjugation pads, where the immune complex is formed (239). However, the former technology has proven to alter the binding site of the antibody by adsorption-induced denaturation, which can cause non-specific interactions (279).

An ideal method for a sandwich immunoassay is one that can maintain the native structure of the antibodies, which involves keeping the antibodies in solution and avoiding immobilization. In addition, allowing a longer incubation time has proven to increase the sensitivity of the assay, without a significant impact on the total assay time (132). To our

knowledge, this is the first attempt to combine an in-tube-sandwich immunoassay with a LFIA device for pathogen detection.

The main challenge that rapid methods, such as the LFIA Test Kit, normally face within food safety, is the complexity and variability of the food samples (132,280) and the low concentration of pathogens, which in combination decrease the sensitivity and specificity of the assay. Therefore, over the course of the development process, several modifications and optimization of reagents and assay conditions were evaluated to obtain an acceptable LOD, while maintaining a clear difference between positive and negative samples distinguishable by the naked eye. We can highlight the relevance of ensuring proper nitrocellulose blocking to prevent non-specific binding (**Table 10**). Particularly, it was noticed that over-blocking the membranes caused weak control and test lines, which might have been due to an excess of BSA and/or Tween 20 that interfered with the binding of complexes with the capture reagents immobilized in either the control or test line (134) (**Table 11**). Importantly, however, it was also demonstrated that low sample pH promotes the development of false positive results (**Figure 13**). Indeed, minor changes in pH can alter the stability of any protein, not only of antibodies, by influencing their net charges and thus their conformation (241,281,282). This can produce non-specific interactions with either the immobilized streptavidin in the test line or with other ligands found in the sample (241,281). In fact, Kim *et al.* reported false positives during the development of a dipstick immunoassay used for detection of *E. coli* O157 in ground beef samples (283). They attributed this effect to the possible denaturation or degradation of the polyclonal antibody, which could then have non-specific interactions with the detection and/or conjugated antibodies.

On the other hand, background microbiota, naturally present in food matrices, has proven to act as a competitor to target pathogens, which normally are found in lower numbers. The food microbiota can potentially inhibit the growth of the target microorganism and thus its detection (248,257,284) when present in high concentrations, making it necessary to use enrichment techniques frequently supplemented with growth inhibitors. However, some inhibitors, such as cefixime, have also been shown to affect *E. coli* O157 growth. In fact, this effect was demonstrated when comparing the performance of non-selective and

selective media for preparation of stressed cells (**Figure 12A**). Ogden *et al.* have reported that cefixime, a supplement found in CR-SMAC, can inhibit the growth of some *E. coli* O157 strains (285), perhaps explaining the difference in counts. These findings agreed with the results reported by Jasson *et al*., where using a combination of cefixime and tellurite with MacConkey medium (CT-SMAC) as selective agar caused a significant difference in performance with TSAYE (225). Therefore, for enrichment purposes, novobiocin was preferred in combination with TSB. This enrichment medium proved to be compatible with the LFIA device by detecting *E. coli* O157 from food samples after 16 h of enrichment at 42°C (**Figure 18**).

Particularly, in LFIA, matrix composition alters the capillary flow and thus the intensity of the control and test lines (134). In addition, the matrix composition can also interfere with the formation of antigen-antibodies complex (132). Indeed, this effect was noted when comparing the results with the undiluted samples (window A) in **Figure 16** and **Figure 19**, where reduction of the control line intensities was approximately 7-fold in the presence of food matrix, but without compromising the final qualitative result. The concept of sample dilution has been reported for dealing with matrix effects in immunochromatographic assays however this approach has the potential disadvantage of decreasing assay sensitivity (132). Therefore, the adoption of a tandem device represented an opportunity to solve the prozone and matrix effect, without altering the sensitivity of the assay, which was approximately 10^4 CFU/ml when using pure cultures and 10^5 CFU/ml with meat samples. The latter was an acceptable LOD, which agreed with data Shan *et al.* reported for six different LFIA (140). These LFIAs were previously developed for detection of foodborne pathogens using colloidal gold, showing LODs between 10^5 and 10^6 CFU/ml (140).

The initial objective of immunochromatographic assays is to deliver qualitative results (132). However, lately, there has been more interest in developing quantitative assays for food safety, especially for determination of toxic compounds such as mycotoxins (134,135,144,286) and chemical contaminants (281), where more than a presence/absence result is needed. Interestingly, few studies were found regarding quantitative applications of LFIA for bacterial analysis (287). In these devices, to determine concentration, the intensity of the test line is normally measured by using a photometric device. However,

this is impractical for a point-of-care rapid test. The tandem LFIA, although focused only on *E. coli* O157, demonstrated that it could be potentially useful in determining semi-quantitatively the concentration of a target bacteria by measuring the intensity of the lines in the two windows of the device (**Figure 20**). It was also possible to link the control and test line intensity measurements with a simple visual evaluation of the tandem LFIA device (**Table 13)**. To our knowledge, the tandem LFIA device represents a new alternative, not only for solving LFIA issues such as prozone and matrix effect, which are frequently faced in food safety due to the complex nature of the samples, but also as a potential alternative for transitioning from qualitative to semi-quantitative results without needing extra equipment.

The application of immunochromatographic systems, such as LFIA, has shown great potential in the food safety sector. Specifically, improvement in sensitivity and specificity has been shown when they are combined with new detection schemes or novel reagents (e.g. gold nanoparticles combined with enzymatic activity or thermal contrast (125,126,246,288). Of importance is the application of single-chain antibody fragment (scFv) in diagnostic tools such as LFIAs. Indeed, recent studies have introduced scFv as novel alternatives (108,200,207,210,289) to monoclonal antibodies. Significant advantages, such as large-scale (207,210) and more cost-effective production methods (120,210,290) with enhanced or similar sensitivity and specificity than the parent monoclonal antibody, have been demonstrated when scFv are used in ELISAs (108,201,210). Within the food safety field, most of the scFv applications have been reported for detection of mycotoxin (207,210). In addition, promising results have also been reported for detection of antibiotics such as fluoroquinolones (209), enterotoxigenic *E. coli* toxins (201), and for detection of *S. enterica* ser. Typhimurium using competitive ELISA (213). Therefore, it is evident that scFv have not been fully exploited for detection of foodborne pathogens. Within this study, a scFv recombinant antibody directed against the O157-antigen expressed in the outer membrane of *E. coli* O157 (scFvO157), was developed.

Although, monoclonal antibodies still represent the main alternative for high-sensitivity reagents for immunoassays, it has been well-documented that cell culture supernatants tend

to have low concentrations of mAb, ranging from 1-100 μg/ml (180,291) depending on the cell line, culture medium, culture conditions and/or cell line stability. Thus, the concentration of large volumes of supernatant before purification has become a common practice (180), which can increase the complexity and cost of the production process. Indeed, the average yield of the anti-O157 mAb obtained through hybridoma technology was 11.58± 2.24[5] μg/ml of supernatant purified. The concentration of mAb was considered sufficient for the purpose of this study, nevertheless, it may not be ideal for the downstream processing requirements of the LFIA Test Kit manufacturing process. Hence, it was of particular interest, not only from a manufacturing perspective, but also for the advancement of food safety, to engineer a scFv that could target one of the major foodborne pathogens, which is *E. coli* O157. The scFvO157 was derived from the anti-O157 mAb (227), which was used as the capture antibody for the development of the LFIA Test Kit. This mAb was confirmed to be an IgG3 isotype (**Figure 21**), which is the preferred immunoglobulin isotype secreted against bacterial antigens (228,291). Moreover, after purification (**Figure 22A**) and functional characterization through ELISA (**Figure 22B**), it was confirmed that the anti-O157 mAb was successfully produced by the 13B3 hybridoma cell line and specific against *E. coli* O157.

The early development of the scFvO157 consisted of the molecular cloning and sequencing of the anti-O157 mAb variable regions, which is formed by the variable heavy (V_H) and light (V_L) chains (**Figure 24**). Monoclonal antibodies are considered unique molecules due to their particular specificity, which, surprisingly, is only attributed to the genetic diversity and length of six loops known as complementarity determining regions (CDRs) found within the V_H and V_L (292,293). Such variability has led to a classification of 15 V_H and 18 V_κ gene families (192), which makes amplification of murine Ig genes challenging. Attempts to successfully amplify V-genes have led to the development of consensus or degenerate primers that target the framework and constant conserved regions of both the V_H and V_L chains (192,196,261,294). The translated amino acid sequences were further

[5] Average obtained from three different purifications± SEM.

characterized using the BLASTP program and COBALT, which is a constraint-based alignment tool (295). In combination, they compared the V_H and V_L chain sequences with protein databases providing as an end-result a multiple sequence alignment where conserved and significant variable residues within the sequences could be identified (**Figure 25**) (296). In fact, it was possible to recognize the conserved residues of the variable chains that mainly represent the framework regions. These conserved regions were responsible for the identity percentage obtained on the sequence comparison (**Table 20**). The difference between the target and the templates was due to the areas within the sequences that outlined the CDR loops (**Figure 25**). Besides confirming that the variable regions belonged to a murine antibody and corresponded to V_H and V_L chains, the analysis demonstrated that at the time of writing no other murine antibody against the O157-antigen was previously published in the database. A search performed in the protein database (PDB) showed 207 results related to "O157", but none of them were antibodies that could target the O157-antigen. Overall, cloning and sequencing of the anti-O157 mAb variable region represents a unique opportunity to preserve its particular specificity, which can be relevant for extensive characterization or in the event of hybridoma loss. To our knowledge, this may represent the first sequenced V-gene of a murine mAb against *E. coli* O157.

Using the sequence information of the murine V-gene has been helpful in creating molecular models for guiding the *in vitro* engineering of scFv (209). Homology modeling is the most common computational tool used to predict 3D structures of proteins based on the comparison of their primary amino acid sequence with a template protein (209,297,298). For this purpose, SWISS-MODEL, which is considered one of the most frequently used public modeling servers (298,299), was used. Overall, homology modeling is efficient and reliable, however V-gene prediction, particularly CDR loops, may represent a challenging and more laborious procedure. Reliable models generally can be obtained when the target-template sequence identity is >40% (297,298). However, the second requirement for homology modeling involves the correct target-template alignment (297), which was represented by the GMQE score (300). The molecular modeling performed led to the identification of the CDR loops using 3D models with a high GMQE score and sequences with >80% identity (**Figure 26A** and **Figure 26B**, **Table 21**). This step was

critical because minor modifications to the CDR loops sequence structure can lead to loss of functionality.

As most of the development of scFvs has been focused on drug therapy development, humanization of scFvs has become a common practice (301). A second benefit obtained from loop grafting has been the achievement of a higher stability of the scFv structure when compared to the original murine scFv (218). Therefore, the scFvO157 construct was designed by loop grafting of murine CDRs onto a humanized consensus backbone previously described to be successfully expressed in *E. coli* (234) (**Figure 26C** and **Figure 26D**). As a fact, scFvs have frequently been reported to be difficult to express as a soluble protein in *E. coli*. Several strategies, such as codon optimization (203) and fusion with highly soluble proteins (e.g. TrxA) (266), have been developed in order to overcome this issue. In fact, both strategies were adopted during the design of the scFvO157 construct. Expression of the recombinant protein was successful, however despite the efforts made to ensure its solubility, it was mainly expressed as inclusion bodies found in the insoluble fraction of the cell lysate (**Figure 30**). Several factors, such as use of strong promoters (268,302), high inducer concentrations (268,302), or high temperatures during induction (268), have been linked to the formation of these insoluble aggregates, which overall increased overexpression of the recombinant protein (303). Throughout this study, several attempts were made to improve the expression of soluble protein (**Figure 30**). Induction was tried at different temperatures, such as 4°C, however it resulted in low yields of both soluble and insoluble fractions mainly due to a reduced bacterial growth rate. The optimal temperature range frequently reported for induction is 15-25°C (RT) (265,304), which showed a slightly higher amount of soluble protein when induction was performed during 6 h (**Figure 30**). Induction was also started at early log phase because it promotes the production of more soluble protein (265). Different concentrations of inducer (IPTG) were also tried and showed a proportional increase of expressed protein with higher concentrations of inducer, but mainly as inclusion bodies (data not shown).

Inclusion body formation is commonly reported in scFv synthesis; hence, isolation and refolding of insoluble aggregates has become a common practice for recovery of functional proteins (203,210,218,290,303). Within this study, isolation of inclusion bodies was

proved to be successful under denaturing and hybrid conditions, however refolding strategies were not ideal for recovery of a functional protein that could be used for functional assessment. These results demonstrate that synthesis of recombinant proteins using the *E. coli* expression system, in particular scFvs, still represents a challenging process in order to fulfill the requirements of large-scale production, which is the end-stage objective of their application.

Different approaches, such as expression in yeasts (217) or Gram-positive bacteria (e.g. *Bacillus brevis* and *Bacillus subtilis*) (215), can be used in order to prevent inclusion bodies formation. However, importantly, several studies have reported the relevance of protein structure in the proper expression and folding. Fahnert *et al.* highlighted the fact that specific structural characteristics, such as point mutations, can be critical in promoting protein aggregation (268). In addition, Akbari *et al.* have shown that the order of the V_H and V_L chains in scFv can affect the expression and stability (203), while Gu *et al.* have reported that the functional cooperativity of both V_H and V_L chains can be influenced by the length and sequence of the peptide linker (305). Overall, recombinant antibody design is challenging and when loop grafting is performed, proper selection of the framework is critical because it can affect the CDR conformation and also proper pairing of the V_H and V_L chains (184). Potential substitution of specific residues from the murine backbone into the new framework may affect the proper folding and functionality (180). In fact, these backmutations can be recognized by homology modeling of the V-genes or by structure analysis (180). In light of this evidence, it was speculated that expression of soluble scFvO157 may be enhanced by a re-design of the primary scFvO157 structure using computational tools to aid in predicting its properties and to overcome the obstacles faced during the experimental stage.

CHAPTER 6 CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

6.1. Overall Findings and Implications

The results obtained after the relative validation study clearly demonstrated that the LFIA Test Kit was faster and sensitive enough to serve as an alternative detection method for Canadian meat producers to rapidly detect *E. coli* O157 in contaminated samples, while meeting the criteria required by the Microbiological Methods Committee (MMC) described in Health Canada's Compendium of Analytical Methods. In addition, these findings suggested a reduction in total analysis time, requiring only 17 h from enrichment-to-result, as compared with 25 h reported in the Merck Singlepath® AOAC® Performance Tested℠ Certificate (169). The newly developed LFIA Test Kit is capable of detecting *E. coli* O157 in contaminated processed and unprocessed meat samples, with a time-to-results advantage over its direct competitors (Merck Singlepath® and the MFHPB-10), emphasizing its inherent commercial value as a potential *E. coli* O157 detection alternative for meat producers.

Moreover, by assembling a tandem LFIA device, we devised a novel and simple alternative to overcome one of the most common weaknesses of this type of immunoassay, which is the prozone or "hook-effect" (143,244,245). Previous studies suggested using sample dilution as the potential solution to the prozone effect (288). However, this directly decreased assay sensitivity. Therefore, the novel design of the tandem LFIA device, which allows analysis of both undiluted and diluted samples simultaneously, maintains the benefit of sample dilution without compromising the sensitivity of the method. In addition, this new LFIA design provides an opportunity to further develop a visual semi-quantitative technique for estimating bacterial concentrations in complex matrices including food samples. Although we were able to demonstrate the relationship between the intensities of the control and test lines with *E. coli* O157 cell concentration in meat samples, further research should focus on validating this approach using different food items, such as produce. Overall, this tandem device advances LFIA performance by providing a feasible, simple and easy-to-use alternative that effectively overcomes the "hook-effect", while concomitantly offering the possibility to transform a qualitative method into a semi-quantitative LFIA without needing extra equipment for interpreting the results.

Although the LFIA Test Kit successfully passed the relative validation for submission to Health Canada's Microbiological Methods Committee, we elected to try to further improve the detection method by the synthesis of a scFv derived from the anti-O157 mAb used as the immunochemical reagent.

Several advantages were foreseen with this approach, including the following a) potential enhancement of the assay sensitivity, b) easy and cost-effective production of detection reagents in bacterial hosts and c) positive proof of the application of a scFv as a detection reagent for food pathogens. The use of scFv in food pathogen detection has not been well-exploited, with only a few publications focusing on the detection of enterotoxigenic *E. coli* toxins (201) and *S. enterica* ser. Typhimurium membrane protein D (213). Hence, the synthesis of a scFv that could target the antigen of a major food pathogen such as *E. coli* O157 represents an innovative adaptation that could benefit the food sector. Moreover, we can validate that no protein or nucleotide sequences related to antibodies or recombinant proteins against *E. coli* O157 are publicly available at the time of writing. This is interesting because this study might represent the first attempt to genetically characterize a mAb targeting *E. coli* O157. Knowledge of the variable domain genetic sequence opens a wide range of possibilities for in-depth antibody engineering research using bioinformatics that could help to develop novel alternatives for tackling *E. coli* O157 food contamination. Some examples of these approaches are predicting antigen-antibody interactions and analyzing the effects of site-directed mutagenesis for improvement of the binding affinity, among others.

Our results indicated correct sequencing of the target variable regions of the anti-O157 mAb was achieved. We were also able to express this scFvO157 in a cell-based *E. coli* host system. Unfortunately, we were unable to completely evaluate the functionality of the scFvO157 due to the limited yield of bioengineered soluble protein obtained, a result of the formation of inclusion bodies. Many different conditions were explored in an attempt to improve the solubility and yield of the *E. coli* expressed bioengineered protein. These included testing different purification techniques to enhance the recovery of these inclusion bodies.

These experimental problems prompted us to reconsider the scFvO157 gene design because modification of the primary scFv sequence could positively alter the folding and stability properties of the expressed, bioengineered protein. Although we were able to initiate this retrospective analysis by using novel bioinformatics tools for antibody and protein design, this component of the study was not completed within the time available. This last part of the study provided the foundation for improved scFv design with the help of computational tools as the main thrust for future research.

6.2. Research Limitations

As mentioned previously, the LFIA Test Kit was successfully submitted for assessment by the MMC. However, several limitations were noted during the development process. First, that the enrichment broth RapidCult™ was no longer on the commercial market represented a major setback. RapidCult™ broth was initially selected, among other broths assessed, due to its ability to recover healthy and stressed *E. coli* O157 cells from artificially inoculated meat samples in 8 h of incubation. Therefore, new readily available enrichment broths were also evaluated. We evaluated commonly used alternatives such as mTSB, mTSB+novobiocin, and TSB+novobiocin. The latter was the final enrichment broth selected because the presence of bile salts in the other two alternatives interfered in the detection of *E. coli* O157 using the LFIA device. Despite the successful results obtained with the LFIA Test Kit, the presence of novobiocin seemed to delay the recovery of *E. coli* O157 cells, especially when stressed. Thus, it was not feasible to replicate the results obtained with RapidCult in the 8 h incubation time, forcing us to increase the enrichment period to 16 h. Although other potential alternatives could also be evaluated, previous comparison studies performed by others (87,170,248–250) were used to screen for the most appropriate broths to evaluate with the LFIA Test Kit.

During the optimization phase, small lots of LFIA devices and/or reagents were manufactured, limiting the number of duplicates and/or experimental replicates within each experiment. To overcome this issue, the reagents and LFIA devices were strategically allocated using primarily checkerboard titrations. We included experimental controls to ensure that the screening and optimization data are representative, while achieving a robust performance that could be confirmed during the relative validation process. Whereas this

approach allowed us to determine the optimal conditions for a functional LFIA Test Kit, it could present a source of bias due to the lack of sufficient replicates.

Sample preparation was also a main limitation for the performance of the LFIA Test Kit. As noted, physicochemical properties of food samples, including pH and matrix composition, interfered with specificity and the intensity of the control and test lines. As a result, pH optimization was necessary when the pH of samples was below 5. Although meat samples used during the development process demonstrated a consistent pH value of 6 after enrichment, they were less acidic than the *S. enterica* ser. Typhimurium and *S. flexneri* pure cultures evaluated. Hence, it was necessary to create a general methodology to account for the potential diversity of sample pH. Sample dilution was also necessary to counteract the effect of sample composition and the prozone effect caused by an excess of antigen. Consequently, the general procedure using the tandem LFIA Test Kit was validated including a pH adjustment step. Both approaches resulted in a functional LFIA Test Kit, although this compromised the simplicity of the test because it required the incorporation of simple, but extra, pipetting steps. This represents an opportunity to further assess the sample buffer as lyophilized beads included with the antibodies beads which, due to time constraints and manufacturing limitations, it was not possible to continue the optimization with this format.

Finally, during the production of the scFvO157 the major limitation faced was the inability to obtain an appropriate yield of purified scFvO157. Initial attempts to demonstrate the functionality of the scFvO157 through ELISA and immunofluorescence microscopy were made, however, the results were inconclusive due to low signal magnitude. Expression of the recombinant protein was successful as demonstrated by Western Blot analysis (**Figure 29**). However, despite cautionary efforts made during the design of the scFvO157 gene, it was mainly expressed as inclusion bodies found in the insoluble fraction of the cell lysate. It has been well-documented that expression of scFv as soluble protein represents a challenging process (197,205,215). In fact, different approaches to tackling this limitation have been published, including expression in a different host such as yeasts (217), Gram-positive bacteria (e.g. *Bacillus brevis* and *Bacillus subtilis*) (215), or other *E. coli* strains such as *E. coli* Origami B (DE3) (265,304). These approaches are designed to improve

disulfide bond-dependent protein folding and may be more appropriate here. In addition, the influence of induction conditions has been widely assessed (197,267,306). Because the latter are considered to be easily modifiable as they are involved in the final stage of scFvO157 synthesis, they were initially evaluated, as shown in **Figure 30**. The results obtained confirmed only the fact that the scFvO157 was mainly produced as insoluble protein regardless of the induction conditions used. Thus, the next approach used was focused on the purification of the inclusion bodies, which showed promising results in terms of purification but its further refolding step required further optimization. Therefore, despite the fact that we were able to express the bioengineered scFvO157, the overall results were considered inconclusive due to the lack of unambiguous evidence that could support the successful production of the scFvO157 through functional assessment.

6.3. Future Directions

6.3.1 Extending the Application of the LFIA Test Kit

Due to an increasing awareness of healthy eating habits, trends are switching towards higher consumption of sustainable, fresh produce (6,7). Thus, fresh products have been recently recognized as an emerging source of foodborne pathogens because they are mainly eaten raw (7,48,307). In fact, in the last decade, there have been several foodborne outbreaks linked to consumption of fresh vegetables worldwide. These include an outbreak related to iceberg lettuce and the well-known case of contaminated bagged spinach that occurred in 2006 (308). In 2011, a multistate outbreak caused by contaminated romaine lettuce was also reported (48). All three of these outbreaks occurred in the US. Thus, it will be relevant to assess the applicability of the LFIA Test Kit on detection of *E. coli* O157 in fresh produce. It is important to focus on leafy green vegetables, which are considered the commodity with the highest microbiological safety concern because they are prone to contamination with *E. coli* O157 from irrigation water, soil and/or manure used as fertilizer (6). Therefore, demonstrating the suitability of the LFIA Test Kit and further validating its performance against the reference method may represent a new possibility for expanding the applicability of the LFIA Test Kit to fresh produce, a commodity that is emerging as a high-risk carrier for *E. coli* O157.

6.3.2 Collaborative Study

The study presented in this work focused on the first stage of the validation process for alternative detection methods, namely a pre-collaborative study. It will be important to continue with the second stage, which is a collaborative study comprising the comparison of the alternative method against the reference method, but this time pursued in parallel by multiple laboratories (174). The main objectives of this study will be as follow: a) to determine the variability of the outcomes obtained when using the LFIA Test Kit in different laboratories with similar samples, and b) to compare these results with the ones obtained during the pre-collaborative study. The collaborative study will be performed following the guidelines established in Health Canada's Compendium of Analytical Methods. Overall, these guidelines require a minimum of eight accredited laboratories, evaluating at least one relevant food type with three contamination levels (negative control, slightly above the alternative method detection level, and 10 times higher than the alternative detection level). Each level should comprise 8 replicates for a total of 24 samples assessed by the LFIA Test Kit and the reference method (174). Although artificially inoculated samples are accepted, including naturally contaminated samples is preferred. Performing a collaborative study is highly recommended because it will generate robust evidence to evaluate the performance of the LFIA Test Kit, especially if this method is expected to aid regulatory activity as suggested through our submission of the pre-collaborative study to Health Canada's Microbiological Methods Committee.

6.3.3 Tandem LFIA Test Kit as a Semi-Quantitative Assay

To date, most of the commercially available LFIAs have been developed for obtaining qualitative results (presence/absence). However, research has been focusing lately on adapting the LFIA format to provide semi-quantitative or even quantitative results. Although, the focus has been mainly on measuring the intensity of the test line (130), modification in the design of the traditional LFIA format has also been considered as a feasible approach for developing more sensitive and quantitative formats (309,310). In this work, the tandem LFIA device was adopted as a solution to the prozone and matrix effect faced during the development of the alternative detection method. However, results obtained with enriched meat samples suggested that it could potentially be used to estimate

the concentration of *E. coli* O157 in contaminated samples. Future studies should focus on proving the concept of a tandem LFIA as a visual semi-quantitative method. For this purpose, the results obtained during this study should be confirmed by using a larger number of samples with emphasis on the lower concentration ranges so that they are as equally represented as the high concentration range. In addition, the subsequent visual evaluation of the results should also be carried out using an untrained panel to ensure that the combination of lines is easily interpreted and linked to the concentration levels previously stipulated. Overall, the tandem LFIA design offers a technology with significant advantages over the standard LFIA device once it if confirmed to consistently provide an easy-to-interpret visual result linked to a level of contamination with *E. coli O157*.

6.3.4 Alternative Strategies for Improving the Synthesis of Soluble scFvO157

It is important to further study the expression of the humanized scFvO157 using different expression hosts in order to avoid the necessity of isolating inclusion bodies and to obtain a higher yield of soluble protein. Within *E. coli* expression hosts, there is a wide variety of strains that have been developed to improve soluble protein expression. *E. coli* strains have many advantages over other expression systems including fast and easy transformation, and inexpensive culture broths with high cell density easily achievable due to rapid growth rate (265,266,290,306). Hence, for future studies, it is recommended to initially evaluate the expression in strains such as *E. coli* Origami B (DE3) or Novagen AD494, which are designed to promote proper folding of proteins through the formation of disulfide bonds, especially when the recombinant protein is fused to TrxA (216). Different vectors, which aim at expressing soluble recombinant proteins using *E. coli* as an expression host (271,306), have also been described. Other alternatives are represented by co-expression of chaperones (216,306,311), and/or fusion of other protein carriers besides TrxA, such as maltose binding protein (216,311) or the *E. coli* N-utilizing substance A (NusA) (216). Both have shown to substantially increase the solubility of highly insoluble scFv when expressed in *E. coli* (216).

Soluble protein expression in different hosts other than *E. coli* has also been suggested. As an example, the Gram-positive *Bacillus megaterium* has been successfully used for expression of a scFv, with the advantage of secreting the expressed protein directly into

the growth medium, facilitating its further purification with superior yields than expression in *E. coli* (215). Moreover, expression in eukaryotic cells such as yeasts also provides a cost-effective alternative for expression of soluble recombinant proteins, which can be easily purified from the culture supernatant (290). Due to the vast number of combinations that can be assessed, microexpression incubator shakers have also been developed, which allows screening of all possible conditions faster and cost-effectively in small, less expensive reactions (271).

6.3.5 Alternative Strategies for *in vitro* Refolding of Inclusion Bodies

Within this study, purification and refolding of the scFvO157 inclusion bodies represented a challenging process due to constant precipitation. Therefore, alternative protocols must be assessed in order to increase the yield of purified protein. *In vitro* refolding can be facilitated by dialysis (273,303,312), dilution (218,273) or solid phase (273,303). Dialysis and solid-phase were the methods tried for the refolding step of the scFvO157; thus it is strongly advisable to evaluate the dilution method. Something to be considered of the refolding strategy is that buffer composition is strongly dependent on the inherent characteristics of the recombinant protein. Hence, different buffer compositions should be assessed regardless of the methodology chosen for refolding. An ideal screening method for selecting the proper conditions for scFvO157 refolding is described by Vincentelli *et al.* (273), where 96-well plates were used to assess the solubility of a target protein in a selection of refolding conditions by measuring the turbidity of the solution as a result of protein precipitation. Those conditions that showed absorbance values close to the absorbance of the buffer alone were regarded as optimal for protein solubility and further used to determine the proper folding of the protein through techniques such as circular dichroism (204,273,303) and dynamic light scattering (273). Recovery of inclusion body is becoming more common due to an increase in production of recombinant proteins mainly for therapeutics. This has switched the perspective of looking at inclusion body as an advantage rather than a problem needed to be solved. Inclusion body recovery allows for a faster and more efficient purification, however efficient *in vitro* refolding strategies still require careful optimization that can only be accomplished by proper knowledge of the protein of interest.

6.3.6 Re-Evaluation of the scFvO157 Using Bioinformatics

Besides assessing other expression and *in vitro* refolding strategies, re-evaluation of the scFvO157 structure should also be pursued. Characteristics such as folding, solubility, and stability can be predicted and improved once structural information is available and properly analyzed. In recent years, several computational tools have been developed with the main objective of assisting in the successful engineering of recombinant proteins, including scFv. In fact, a field known as "antibody informatics" has evolved to tackle some of the major obstacles faced during antibody drug discovery (313). A recent publication from Shirai *et al.* highlighted many of the obstacles presented during the antibody engineering experimental workflow and linked them to the ideal informatics tools that can be used to overcome these problems (313). Hence, this approach could be easily adopted to identify the major constraints and identify the necessary tools to improve the design of scFv regardless of their future application.

Focusing on scFvO157, the main obstacle is the formation of inclusion body and aggregation during expression, which may be attributed to unsuitable physicochemical properties of the bioengineered protein and may be improved by an adequate *in silico* design. Some of the scFvO157 re-evaluation was started by analyzing and comparing the theoretical hydrophobicity and pI of both the humanized scFvO157 and the human scFv acceptor primary sequences (**Section 4.3.8**). Findings suggested that differences found between the scFvO157 and the human scFv acceptor are due to the loop grafting process and could have influenced the improper folding of the scFvO157. These results support the assertion that proper knowledge of the scFv characteristics could help to determine the optimal conditions for its expression and purification.

During antibody engineering, there are three main structural characteristics that have to be considered: a) the primary amino acid sequence, which is the result of V(D)J gene segment and somatic mutations, b) the structure of the six CDR loops, which form the antigen-binding site, and c) the relative orientation of the V_H and V_L chains, which will partially determine the interaction with the target antigen. Based on these, it is suggested to first analyze the murine V_H and V_L chain sequences and further compare them with the humanized scFvO157 initially designed. Kuroda *et al.* suggested a simple procedure that

starts by using the V_H and V_L chain sequences for antibody modeling, followed by an antibody-antigen docking simulation, which provides a prediction of potential mutations for enhancing the desired antibody properties including solubility (314). For the first step, which is antibody modeling, a web server named PIGS (prediction of immunoglobulin structure) developed by Marcatili *et al.*, is suggested to model the variable domain of the anti-O157 mAb. This server takes into consideration not only the alignment of the primary amino acid sequence, but also the canonical structures of the CDR loops and the final packing (orientation) of the V_H and V_L chain in order to derive the quaternary structure that best predicts the conformation of the antibody structure (315,316). The PIGS murine anti-O157 mAb model can be further superimposed with the scFvO157 to determine if the latter maintains the proper folding, secondary structure and V_H and V_L chain packing of the original mAb. Furthermore, the anti-O157 mAb model can be subjected to a detailed analysis of its primary and secondary structure to identify those residue positions that are crucial for antigen recognition. Interestingly, some residues within the FRs have been identified as crucial for antigen binding, while conserved residues within the CDRs have been regarded as providing structural support rather than contributing directly to active binding (219). Hence, discrimination among functional and structural residues will allow the refinement of the CDR grafting process to potentially enhance biophysical properties such as solubility. Finally, as an additional *in silico* analysis, the PIGS murine anti-O157 mAb model can be used to develop an antibody-antigen docking model using programs such as Glide from Schrödinger Software (317,318). Besides providing supporting information for enhancement of scFvO157 design, attempting to develop an antibody-carbohydrate docking model will further expand the knowledge regarding this type of interaction because most of the research has focused on antibody-protein/ peptide binding (319).

In conclusion, by using a combination of bioinformatics tools it is possible to undertake an *in silico* modeling strategy to achieve the following a) understand the possible structural cause(s) that underlie the formation of inclusion body and aggregation during recombinant protein expression stage, and b) increase the knowledge of the murine anti-O157 mAb sequence and tertiary structure to re-design the scFvO157 and potentially improve the experimental design to enhance its solubility during expression.

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Appendices

Appendix A: Characterization of inclusivity and exclusivity strains

Both inclusivity and exclusivity panels were assembled by collecting strains from multiple reliable contributors including the following: Public Health Agency of Canada, Guelph, ON, Canada (PHAC), Western University, Department of Microbiology and Immunology, London, ON, Canada (UWO), Canadian Research Institute for Food Safety, Guelph, ON, Canada (CRIFS), Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSMZ), University of Manitoba, Department of Food Science, Winnipeg, MN, Canada (UofM), American Type Culture Collection, VA, USA (ATCC), and Health Canada, Listeriosis Reference Service, Ottawa, ON, Canada.

The characterization of the 50 *E. coli* O157 strains was done according to the confirmation assays described in the MFHPB-10 Isolation of *Escherichia coli* O157:H7/NM from foods and environmental surface samples, Section 6.8 (255), except for the presence of verotoxins, which was provided by the respective contributors.

Strains, taken from the frozen stocks, were grown in TSB at 37°C for 24 h. A loop was taken from each culture and streaked into TSA plates to obtain isolated colonies. Plates were incubated at 37° for 18-24 h. A grid was drawn in two selective agars: Oxoid CR-SMAC (Oxoid Limited, UK) and BBL CHROMagar O157 (BD, USA). For each strain, isolated colonies from the TSA plates were picked and inoculated in each grid cell of both agars. Finally, plates were incubated at the appropriate time and temperature suggested by the suppliers. After incubation, colonies were considered positive (+) if they presented the characteristic growth being translucent, colorless or straw-colored edges, with a dark center ranging from grey to pink (non-sorbitol fermenting) for the CR-SMAC and, mauve against white background for the CHROMagar. If they were red in the CR-SMAC and/ or colorless, blue, green, or blue-green on the CHROMagar, they were considered negative (-). For cellobiose fermentation, tubes containing 5ml of Purple Broth (Difco™, BD, USA) with 1% cellobiose (Sigma-Aldrich, USA) were inoculated with one isolated colony of each strain obtained from the TSA plates. Then, they were incubated at 37°C for 18-24 h. After incubation, positive (+) strains showed color change from purple to yellow since they fermented cellobiose, while negative (-) strains showed no color change. Furthermore, the API 20E Test rapid identification system was performed following the manufacturer's instructions (API 20E Identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods; bioMérieux, Inc.; 07584D-GB-2002/10). The numerical profiles obtained were used to confirm the strains as *E. coli* according to their database. The presence of the O157 antigen was confirmed by O157 antisera agglutination using an isolated colony from the TSA plates and re-suspended in a small drop of saline solution on a glass slide. Then, a similar drop of *E. coli* O157 antiserum (BD, USA) was added. Both drops were mixed with the needle. To promote agglutination, the slide was rocked back and forth for 1 min (320). Positive strains (+) showed the presence of white lumps, while negative (+) strains maintained a homogeneous turbid solutions. Finally, H7 serotyping was done by immobilization. Briefly, columns of

semi-solid agar (TSA/ 0.4% agar) were prepared containing H7 antiserum (BD, USA). Then, they were stabbed with a needle containing an isolated colony taken from the TSA agar plates for each strain. Tubes were incubated at 37°C for 18-24 h. After incubation, positive tubes (+) showed growth only along the stab because they were immobilized due to the presence of the flagellar H7 antigen. Negative tubes (-) showed growth (cloudiness) away from the stab line, which meant that *E. coli* cells were motile, but did not have the H7 antigen. Finally, positive strains (+) were inoculated in agar columns without antigen to discriminate from real H7 motile strains and non-motile ones (NM). Tubes were also incubated at 37°C for 18-24 h. *E. coli* H7 strains grew away from the stab line when no H7 antiserum was present (+), while non-motile strains remained along the stab line (-) (320,321).

The results for the characterization of the fifty *E. coli* O157 strains collected are shown below. The O- and H-antigen confirmation assays showed the expected results, where all strains were positive for the O157-antigen agglutination and H7-antigen strains were immobilized with the H7 antiserum. Moreover, all strains were negative for cellobiose fermentation. On the other hand, 84% of the strains were sorbitol negative, while 16% were sorbitol positive according to the results obtained with the CR-SMAC. From the latter, two were H7, while the rest had a different flagellar H-antigen. CHROMagar results showed characteristic mauve colonies for all O157:H7/ NM strains, while strains with different H-antigen did not grow or presented a different color (negative). The API 20E results corresponded to an *Escherichia coli* 1 taxon with a percentage of identity (ID) above 97.7% for all strains but one. The latter, *E. coli* O157:NM 02-1840, showed a mixed taxon, *Escherichia coli* 1 and *Escherichia coli* 2, with an ID of 83.1 and 10.1% respectively.

***E. coli* O157 strains and characterization results**

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Shiga Toxin*		Confirmatory Tests					
						VT1	VT2	CR-SMAC	CHROM Agar	O157	H7	Cellobiose	API Test
1	CRIFS	C480	*Escherichia coli*	O157:H7	N/A	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
2	CRIFS	C654, EC20321	*Escherichia coli*	O157:H7	L.Goodridge	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
3	CRIFS	C670, EC960282	*Escherichia coli*	O157:H25	Bovine	N*	N*	-	-	+	-	-	*Escherichia coli 1*
4	CRIFS	C671, EC940076	*Escherichia coli*	O157:H19	Bovine	N*	N*	-	-	+	-	-	*Escherichia coli 1*
5	CRIFS	C677, EC930195, 32511	*Escherichia coli*	O157:NM	L.Goodridge	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
6	CRIFS	C899, ATCC 43888	*Escherichia coli*	O157:H7	Human , feces	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
7	CRIFS	C901, ATCC 43894	*Escherichia coli*	O157:H7	Human , feces	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
8	CRIFS	C918, 380-94	*Escherichia coli*	O157:H7	Bovine, salami	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
9	CRIFS	C919, 9490	*Escherichia coli*	O157:H7	USDA	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
10	CRIFS	C1264, ATCC 700927	*Escherichia coli*	O157:H7	Derived from ATCC43895	N/I	N/I	+	+	+	+	-	*Escherichia coli 1*
11	DSMZ	DSM 17076, NCTC 12900	*Escherichia coli*	O157:H7	Human, clinical	N	N	+	+	+	+	-	*Escherichia coli 1*
12	PHAC	EC19920333	*Escherichia coli*	O157:H7	Human, clinical	N	Y	+	+	+	+	-	*Escherichia coli 1*

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Shiga Toxin*		Confirmatory Tests					
						VT1	VT2	CR-SMAC	CHROM Agar	O157	H7	Cellobiose	API Test
13	PHAC	EC19930038	*Escherichia coli*	O157:H7	Human, clinical	N	Y	+	+	+	+	-	*Escherichia coli 1*
14	PHAC	EC19930553	*Escherichia coli*	O157:H7	Human, clinical	Y	Y	+	+	+	+	-	*Escherichia coli 1*
15	PHAC	EC19950095	*Escherichia coli*	O157:NM	Bovine, meat	Y	Y	+	+	+	-	-	*Escherichia coli 1*
16	PHAC	EC19960266	*Escherichia coli*	O157:H7	Human, clinical	N	N	+	+	+	+	-	*Escherichia coli 1*
17	PHAC	EC19960274	*Escherichia coli*	O157:H7	Human, clinical	N	N	+	+	+	+	-	*Escherichia coli 1*
18	PHAC	EC19961016	*Escherichia coli*	O157:H7	Human, clinical	Y	N	+	+	+	+	-	*Escherichia coli 1*
19	PHAC	EC19961025	*Escherichia coli*	O157:H7	Human, clinical	N	Y	+	+	+	+	-	*Escherichia coli 1*
20	PHAC	EC19961027	*Escherichia coli*	O157:H7	Human, clinical	Y	Y	+	+	+	+	-	*Escherichia coli 1*
21	PHAC	EC19961090	*Escherichia coli*	O157:H7	Human, clinical	Y	N	+	+	+	+	-	*Escherichia coli 1*
22	PHAC	EC19970409	*Escherichia coli*	O157:H7	Bovine, meat	Y	Y	+	+	+	+	-	*Escherichia coli 1*
23	PHAC	EC19970419	*Escherichia coli*	O157:H7	Bovine, meat	Y	Y	+	+	+	+	-	*Escherichia coli 1*
24	PHAC	EC19970462	*Escherichia coli*	O157:H7	Bovine, meat	Y	N	+	+	+	+	-	*Escherichia coli 1*
25	PHAC	EC19970465-1	*Escherichia coli*	O157:H45	Bovine, meat	N	N	-	-	+	-	-	*Escherichia coli 1*
26	PHAC	EC19970515	*Escherichia coli*	O157:H7	Bovine, meat	Y	Y	+	+	+	+	-	*Escherichia coli 1*

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Shiga Toxin*		Confirmatory Tests					
						VT1	VT2	CR-SMAC	CHROM Agar	O157	H7	Cellobiose	API Test
27	PHAC	EC19970524	*Escherichia coli*	O157:H42	Bovine, meat	N	N	-	-	+	-	-	*Escherichia coli 1*
28	PHAC	EC19990951	*Escherichia coli*	O157:H7	Bovine, salami	Y	Y	+	+	+	+	-	*Escherichia coli 1*
29	PHAC	EC20000671	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
30	PHAC	EC20000673	*Escherichia coli*	O157:H7	Bovine, Meat	N	Y	+	+	+	+	-	*Escherichia coli 1*
31	PHAC	EC20001018	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
32	PHAC	EC20010294	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
33	PHAC	EC20011231	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
34	PHAC	EC20011236	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
35	PHAC	EC20011244	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
36	PHAC	EC20020335	*Escherichia coli*	O157:H7	Human, clinical	Y	Y	+	+	+	+	-	*Escherichia coli 1*
37	PHAC	EC20040339	*Escherichia coli*	O157:H7	Bovine, meat	Y	Y	+	+	+	+	-	*Escherichia coli 1*
38	PHAC	EC20050147	*Escherichia coli*	O157:H12	Bovine, feces	N	N	-	-	+	-	-	*Escherichia coli 1*
39	PHAC	EC20060233	*Escherichia coli*	O157:H7	Bovine, ground	Y	Y	+	+	+	+	-	*Escherichia coli 1*
40	PHAC	EC20060754	*Escherichia coli*	O157:H29	Bovine, feces	N	N	-	-	+	-	-	*Escherichia coli 1*

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Shiga Toxin*		Confirmatory Tests					
						VT1	VT2	CR-SMAC	CHROM Agar	O157	H7	Cellobiose	API Test
41	PHAC	EC20130376	*Escherichia coli*	O157:H7	Bovine, feces	N	Y	+	+	+	+	-	*Escherichia coli 1*
42	PHAC	EC20130378	*Escherichia coli*	O157:H7	Bovine, feces	N	Y	+	+	+	+	-	*Escherichia coli 1*
43	PHAC	EC20130390	*Escherichia coli*	O157:NM	Bovine, feces	Y	Y	+	+	+	-	-	*Escherichia coli 1*
44	PHAC	EC20130395	*Escherichia coli*	O157:NM	Bovine, feces	Y	Y	+	+	+	-	-	*Escherichia coli 1*
45	PHAC	EC20132075	*Escherichia coli*	O157:H7	Bovine, feces	N	Y	+	+	+	+	-	*Escherichia coli 1*
46	UofM	00-3581	*Escherichia coli*	O157:H7	Human, clinical	N	N	-	+	+	+	-	*Escherichia coli 1*
47	UofM	02-0304	*Escherichia coli*	O157:H7	Human, clinical	N	N	-	+	+	+	-	*Escherichia coli 1*
48	UofM	02-0627	*Escherichia coli*	O157:H7	Human, clinical	N	N	+	+	+	+	-	*Escherichia coli 1*
49	UofM	02-0628	*Escherichia coli*	O157:H7	Human, clinical	N	N	+	+	+	+	-	*Escherichia coli 1*
50	UofM	02-1840	*Escherichia coli*	O157:NM	Human, clinical	N	N	+	-	+	-	-	*Escherichia coli***

**Shiga toxin information was provided by the contributors. N:absence of Shiga Toxin; Y: presence of Shiga Toxin; +: positive results; -: negative results; N/I: no information provided; N/A: not available. **This strain showed a percentage of identity of 83.1% corresponding to Escherichia coli 1 and a 10.1% corresponding to Eschericha coli 2.*

On the other hand, only the six strains that initially showed at least one positive replicate during the LFIA Test Kit exclusivity study, were characterized following the methodology below.

Strains, taken from frozen stocks were grown in TSB at 37°C for 24 h. A loop was taken from each culture and streaked into TSA plates to obtain isolated colonies. Plates were incubated at 37° for 18-24 h. The cellobiose, API 20E Test and O157 antisera agglutination tests were performed as described in the inclusivity studies. In addition, two more tests were included in order to characterize *Shigella flexneri*. Briefly, a lactose fermentation test was prepared using tubes containing 5ml of Purple Broth with 1% lactose (J.T.Baker® Chemicals, Avantor Performance Materials, PA, USA) inoculated with one isolated colony obtained from TSA plates. Then they were incubated at 37°C for 18-24 h. After incubation, positive (+) strains showed color change from purple to yellow because they fermented lactose, while negative (-) strains showed no color change. As positive control we used *E. coli* ATCC 25922. Mobility was assessed using columns of semisolid agar (TSA/ 0.4% agar) stabbed with a needle containing an isolated colony taken from TSA agar plates. Tubes were incubated at 37°C for 18-24 h. After incubation, positive tubes showed growth (cloudiness) away from the stab, which meant that the strain was motile. Negative tubes showed a delimited growth along the stab meaning that the strain lack motility.

A summary of the results obtained from the biochemical characterization of the six strains that had at least one replicate positive during the exclusivity study is found below. Strains with a percentage of identity (ID) higher that 90% were no further evaluated. Strain EC20130473 had 85.5% ID corresponding to an *E. coli* 1, however, the 14.4% ID missing was related to an *E. coli* 2 taxon thus, it was not considered either. On the other hand, ATCC 25929 *Shigella flexneri*, had 68.8% ID corresponding to *Shigella spp*., while 17.5% matching the *E. coli* 2 taxon. Therefore, this strain was subjected to two more biochemical tests: lactose fermentation and motility following the methodology described above. The results obtained for this strain showed a lack of ability to ferment lactose and no motility, which are typical from *Shigella spp.* when compared against *E. coli spp.*

Non-*E. coli* O157 strains used within this study

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source
1	ATCC	ATCC 19114	*Listeria monocytogenes*	4a	Animal, tissue
2	ATCC	ATCC 43886, CDC E2539-C1	*Escherichia coli*	O25:K98:NM	Human, feces
3	ATCC	ATCC 43893, CDC EDL 1284 [929-78]	*Escherichia coli*	O124:NM	Human, feces
4	CRIFS	C390	*Salmonella Heidenberg*	N/A	N/A
5	CRIFS	C398	*Salmonella newport*	N/A	N/A

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source
6	CRIFS	C417	*Salmonella Enteritidis*	N/A	N/A
7	CRIFS	C1116	*Salmonella javiana*	N/A	N/A
8	CRIFS	EC 910005	*Escherichia coli*	O111:NM	N/A
9	CRIFS	EC 910040	*Escherichia coli*	O145:NM	N/A
10	CRIFS	EC 910060	*Escherichia coli*	O121:H7	N/A
11	CRIFS	EC 920232	*Escherichia coli*	O2:H5	Bovine
12	CRIFS	EC 930004	*Escherichia coli*	O103:H2	N/A
13	HC	HPB 5949	*Listeria monocytogenes*	1/2C	Food, ready-to-eat
14	PHAC	ATCC 6051	*Bacillus subtilis*	N/A	HJ Conn
15	PHAC	ATCC 7966	*Aeromonas hydrophila*	N/A	From tin of milk with a fishy odor
16	PHAC	ATCC 12014	*Escherichia coli*	O55:NM	CDC
17	PHAC	ATCC 13047	*Enterobacter cloacae*	N/A	Spinal fluid
18	PHAC	ATCC 23715	*Yersinia enterocolitica*	N/A	Human, blood
19	PHAC	ATCC 25922	*Escherichia coli*	O6	Human, clinical
20	PHAC	ATCC 25929	*Shigella flexneri*	N/A	Human, feces
21	PHAC	ATCC 25931	*Shigella sonnei*	N/A	Human, feces
22	PHAC	ATCC 33650	*Escherichia hermanii*	N/A	Human, female toe
23	PHAC	ATCC 43162	*Citrobacter braakii (freundii)*	N/A	Clinical isolate, California
24	PHAC	ATCC 43887	*Escherichia coli*	O111:NM	Human, feces
25	PHAC	ATCC 49149	*Enterococcus faecalis*	N/A	Clinical isolate
26	PHAC	ATCC 700926	*Escherichia coli*	OR:H48:K-	Derived from parent strain W1485
27	PHAC	EC20130462	*Escherichia coli*	O75:H5	N/A
28	PHAC	EC20130463	*Escherichia coli*	O21:H4	N/A
29	PHAC	EC20130466	*Escherichia coli*	O44:H18	N/A
30	PHAC	EC20130467	*Escherichia coli*	O3:H2	N/A
31	PHAC	EC20130469	*Escherichia coli*	O28ac:NM	N/A
32	PHAC	EC20130473	*Escherichia coli*	O78:H11	N/A
33	PHAC	EC20130476	*Escherichia coli*	O25:NM	N/A

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source
34	PHAC	MB1914	*Hafnia alvei*	N/A	N/A
35	PHAC	NO152388	*Enterobacter aerogenes*	N/A	N/A
36	UWO	ATCC 13883, UWO#479	*Klebsiella pneumoniae*	N/A	NCTC
37	UWO	LT-2	*S. enterica* ser. Typhimurium	N/A	N/A

PHAC: Public Health Agency of Canada, Guelph; UWO: Western University, Department of Microbiology and Immunology; CRIFS: Canadian Research Institute for Food Safety, Guelph; ATCC: American Type Culture Collection; HC: Health Canada, Listeriosis Reference Service; N/A: not available.

Characterization of non-target strains

#	Culture Collection	Reference	Strain	Serotype	Confirmatory Tests		
					O157	Cellobiose	API Test (%identity)
1	PHAC	EC20130473	*Escherichia coli*	O78:H11	-	-	*Escherichia coli***
2	PHAC	EC20130467	*Escherichia coli*	O3:H2	-	-	*Escherichia coli* 1 (98.1)
3	PHAC	EC20130466	*Escherichia coli*	O44:H18	-	-	*Escherichia coli* (98.1)
4	ATCC	ATCC43893, CDC EDL 1284 [929-78]	*Escherichia coli*	O124:NM	-	-	*Escherichia coli* 1 (96.3)
5	PHAC	ATCC 25929	*Shigella flexneri*		-	-	*Shigella spp.* (68.8)
6	UWO		*S. enterica* ser. Typhimurium		-	-	*Salmonella spp.* (98.6)

***This strain showed a percentage of identity of 85.5% corresponding to Escherichia coli 1 and a 14.4% corresponding to Escherichia coli 2.*

Appendix B: MFHPB-10 Isolation of *Escherichia coli* O157:H7/NM from foods and environmental surface samples

Government of Canada / Gouvernement du Canada

HPB Method **MFHPB-10**
October 2014

Health Products and Food Branch

Ottawa

Isolation of *Escherichia coli* O157:H7/NM from foods and environmental surface samples

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1. Application

This method is applicable to the isolation of *Escherichia coli* O157:H7/NM in foods to determine compliance with the requirements of Sections 4 and 7 of the *Food and Drugs Act* and/or other relevant federal regulations. This method is Health Canada's standard cultural reference method and can be used for all foods and environmental surfaces. This revised method encompasses the former MFLP-90, dated July 2006, and replaces MFLP-80, dated March 2008.

2. Principle

This method determines the presence of *E. coli* O157:H7/NM in food and environmental samples. Some strains of *E. coli* O157:H7 lose the ability to express the H7 flagellar antigen and become non-motile (NM), but still possess the genes for H7 and the other typical characteristics of *E. coli* O157:H7 – these *E. coli* O157:NM strains are included in the definition of pathogenic *E. coli* O157:H7. A portion of the product (or sample from an environmental surface) is inoculated in a primary enrichment broth, concentrated by immunomagnetic separation using Dynabeads® anti-*E. coli* O157, and then plated onto at least two specified agar media. It is assumed that viable *E. coli* O157:H7 or NM cells will multiply under these conditions and give rise to visible colonies which can be identified. Confirmatory biochemical and serological tests are performed on purified colonies. This method is based on the work of Dr. S. Weagant (7.4, 7.5) with modifications by Szabo and colleagues (7.2), Warburton et al. (7.3), as well as in-house unpublished studies conducted by HC and CFIA Laboratories.

Dynabeads® anti-*E. coli* O157 ImmunoMagnetic Separation (IMS) uses a uniform, superparamagnetic, polystyrene microsphere, which is coated with adsorbed and affinity purified anti-*E. coli* O157 antibodies. The beads are incubated with 1ml of pre-enriched sample, during which time *E. coli* O157 present in the sample bind to the surface of the bead. The bead-bacteria complexes are separated from the sample using a magnetic particle concentrator (Dynal MPC®-S).

3. Definition of Terms

See Appendix A of Volume 1.

4. Collection of Samples

See Appendix B of Volume 1.

5. Materials and Special Equipment

Note: The Laboratory Supervisor must ensure that the analysis described in this method is carried out in accordance with the International Standard referred to as ISO/IEC 17025:2005 (or latest version): General Requirements for the Competence of Testing and Calibration Laboratories".

The media and reagents listed below are commercially available and are to be used, prepared and/or sterilized according to the manufacturer's instructions. See Appendix G of Volume 1 for the formulas of media.

Note: It is the responsibility of the laboratory to ensure equivalency if any variations of the media formulations listed here are used (either product that is commercially available or made from scratch). Please forward equivalency data to the Editor of *Compendium of Analytical Methods* for consideration of modification of this method.

1) Modified Tryptic Soy Broth with 20 mg/L Novobiocin (mTSB-n)

2) Enterohemorrhagic *E. coli* (EHEC) Enrichment Broth (EEB)

3) Group 1 Agars:
   - Modified Hemorrhagic Coli Agar (mHC) with Tellurite and Cefsulodin
   - mHC Agar with CT supplement (Oxoid SR0172)
   - BBL CHROMagar for *E. coli* O157 (BD)

4) Group 2 Agars:
   - Modified Sorbitol MacConkey agar (TCCSMAC) with Tellurite, Cefixime, and Cefsulodin
   - CT-SMAC (Oxoid CR-SMAC agar base CM0813 and CT supplement SR0172)
   - CR-SMAC Agar (Oxoid CR-SMAC Agar Base – CM1005 and Cefixime Supplement - SR0191)

5) Trypticase Soy Agar with Yeast Extract (TSA-YE)

6) Phenol Red Sorbitol agar with MUG

7) Urea Agar Slants

8) Purple broth with cellobiose or carbohydrate agar with cellobiose

9) Potassium sulfite (K_2SO_3)

10) Stomacher, blender, vortex mixer or equivalent

11) Control cultures: positive control *vt1*- and/or *vt2*-gene carrier *E. coli* O157:H7 (e.g., ATCC 35150)
    ATCC negative control: non carrier of *vt1*- and *vt2 genes* non-O157 *E. coli* (e.g., 25922)

12) Dynabeads® anti-*E. coli* O157 cat. nos. 71003 or 71004 (or equivalent immunomagnetic beads or concentration steps). Dynabeads® and Dynal MPC® are trademarks of Dynal A.S Oslo, Norway. Dynal products are available through Invitrogen Canada Inc., 2270 Industrial St., Burlington, ON, 1(800) 263-6236.

13) Dynal MPC®-S (Magnetic Particle Concentrator) unit (or equivalent)

14) Sample mixer capable of tilt, swirl or rotational mixing (e.g., Dynal Sample Mixer)

15) BeadRetriever® automated immunomagnetic separation instrument (or equivalent) (optional)

16) PBS-Tween (Available from Sigma Product # P3563)

17) O157 antisera

18) Latex Agglutination Kits for *E. coli* O157 (e.g., Pro-Lab Developments, Oxoid, Wellcolex)

19) Incubators capable of maintaining 35°C and 42°C

Note: It is the responsibility of each laboratory to ensure that the temperatures of the incubators or water baths are maintained at the recommended temperatures. Where 35°C is recommended in text of the method the incubator may be at 35 ± 1.0°C. Similarly, lower temperatures of 30 or 25 may be ± 1.0°C. However, where higher temperatures are recommended, such as 43 or 45.5°C, it is imperative that the incubators or water baths be maintained within 0.5°C due to potential lethality of the higher temperatures on the microorganism(s) being isolated.

20) Gram stain reagents

21) IMViC Reagents

22) MUG (4-methylumbelliferyl-β-D-glucuronide), LST-MUG broth (Oxoid), or Colicomplete (MUG disks; BioControl)

23) BCIG (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) either -Na or -CHX (cyclohexylammonium) salt

24) Rapid identification kits such as Vitek 2, API or equivalent

25) Verotoxin detection kits (see Appendix K of the Compendium)

26) Ultraviolet light

6. Procedure

Each sample unit may be analyzed individually or the analytical units may be combined up to a maximum of 325 g. Carry out the test in accordance with the following instructions:

6.1 Handling of Sample Units

6.1.1 In the laboratory prior to analysis, except for shelf-stable foods, keep sample units refrigerated or frozen, depending on the nature of the product. Thaw frozen samples in a refrigerator, or under time and temperature conditions which prevent microbial growth or death.

6.1.2 Analyze sample units as soon as possible after their receipt in the laboratory.

6.2 Preparation for Analysis

6.2.1 Have ready sterile mTSB-n (EEB) if required), pre-warmed to 35 ± 2°C.

6.2.2 Clean the surface of the working area with a suitable disinfectant.

6.3 Preparation of Sample

To ensure a truly representative analytical unit, agitate liquids or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit. To reduce the workload, the analytical units may be combined for analysis. Composite up to five analytical units, up to a maximum of 325 g.

6.4 Primary Enrichment

6.4.1 Prepare a 1 in 10 dilution of the food by aseptically adding 25 g or ml into 225 ml of the enrichment broth mTSB-n (and EEB, if applicable). Other analytical unit sizes, such as 65 g for ground beef may be used while maintaining the 1 in 10 dilution. For composite samples, analytical units may be combined up to 325 g or ml (e.g., 325 g or ml of food in 2925 mL of mTSB-n). Do not exceed the maximum composited sample size of 325 g.

OPTIONAL: A second primary enrichment broth started directly in EEB may be done when there is a high bacterial load of competing organisms, during food-borne illness investigations and when otherwise deemed appropriate. (In this case, you would have 2 separate primary enrichments.)

6.4.2 Blend, stomach or vortex as required for thorough mixing.

Note: Some spices, such as onion and garlic powder are antimicrobial in nature. When analyzing spices, add 0.5% K_2SO_3 to mTSB-n before autoclaving. When analyzing products containing large amounts of spices, consider adding 0.5% K_2SO_3 to mTSB-n before autoclaving. Garlic especially affects *E. coli* O157. Therefore garlic, garlic-containing products, and other spices may need to be diluted 1 in 100 for analysis.

6.4.3 A positive and a negative control should be set up at the same time.

6.4.4 Incubate the enrichment mixture and controls for 22-24 h at 42°C.

6.4.5 OPTIONAL: Incubated mTSB-n may be refrigerated for up to 48 h.

6.5 OPTIONAL: Secondary Enrichment in EEB

Note: This step should be used when a rapid kit has identified the presence of *E. coli* O157 but it was not isolated when the primary enrichment broths, mTSB-n (and EEB if applicable), were plated onto the selective agars. *E. coli* O157 may be difficult to isolate from some samples with high ACC levels.

6.5.1 After agitation of the enrichment broth, transfer 1 mL of the mTSB-n and/or EEB to 9 mL of EEB.

6.5.2 Incubate 18-24 h at 35°C.

6.5.3 Do not refrigerate EEB, analysis must be continued at this stage. Optional: To delay incubation of the secondary enrichment in EEB, while waiting for confirmation of initial plating, retain original mTSB-n refrigerated for up to 48 h, only transferring to EEB for incubation when it is determined that this step is necessary.

6.6 Immunomagnetic Separation (IMS)

Note: The following procedure uses Dynabeads® anti-*E. coli* O157. At the discretion of the laboratory, equivalent immunomagnetic beads or concentration steps may be substituted if validated.

6.6.1 Manual IMS

To avoid cross contamination and for safety reasons, it is recommended that immunomagnetic separation be performed using the BeadRetriever®. In the absence of the BeadRetriever®, strict adherence to good laboratory practices and the following instructions are a prerequisite to obtaining valid results.

6.6.1.1 Remove the magnetic plate and load the necessary number of 1.5 mL microcentrifuge tubes into the Dynal MPC®-S.

6.6.1.2 Resuspend Dynabeads® anti-*E. coli* O157 until the pellet in the bottom disappears by using a vortex machine. Pipette 20 µL of Dynabeads® anti-*E. coli* O157 and dispense into each tube.

6.6.1.3 Add 1 mL of the pre-enriched sample aliquot from section 6.4 or 6.5 and close the tube. Change to a new pipette for each new sample.

6.6.1.4 Invert the Dynal MPC®-S rack a few times. Incubate at room temperature for 10 min with gentle continuous agitation to prevent the beads from settling.

6.6.1.5 Insert the magnetic plate into the Dynal MPC®-S. Invert the rack several times to concentrate the beads into a pellet on the side of the tube. Allow 3 min for proper recovery.

6.6.1.6 Open the cap using a tube opener and carefully aspirate and discard the sample supernatant as well as the remaining liquid in the tube's cap.

6.6.1.7 Remove the magnetic plate from the Dynal MPC®-S.

6.6.1.8 Add 1 mL of wash buffer (PBS-Tween). Do not touch the tube with the pipette since this can cross-contaminate the samples as well as the wash buffer. Close the cap and invert the Dynal MPC®-S a few times to resuspend the beads.

6.6.1.9 Repeat steps 6.6.1.5 – 6.6.1.8.

6.6.1.10 Repeat steps 6.6.1.5 – 6.6.1.7. Proceed to 6.6.3.

6.6.2 Automated IMS

Users must aseptically dispense all reagents and samples into the tubes sequentially after the strips of tubes are fitted into the rack.

Place one disposable sample 5-tube strip into a BeadRetriever rack for each sample to be processed and using aseptic technique, dispense reagents into each tube. The tab on the tube strip may be used for labelling samples.

6.6.2.1 Aseptically add 10 µL of properly mixed Dynabeads® anti-*E. coli* O157 into two sample tubes 1 and 2.

6.6.2.2 Aseptically add 500 µL of wash buffer to the sample tubes 1 and 2.

6.6.2.3 Aseptically add 1 mL of wash buffer to tubes 3 and 4 within the strip.

6.6.2.4 Aseptically add 100 µL of wash buffer to the 5th tube.

6.6.2.5 Remove the desired tube from rack A and place in rack B (one meter away, to help reduce possible cross contamination). Add 500 µL of a test sample to tubes 1 and 2 and transfer the inoculated tube to rack A. Repeat for the remaining samples.

6.6.2.6 Aseptically insert the sterile protective tip combs into the instrument.

6.6.2.7 Insert the rack with filled tubes into the instrument to lock it in place.

6.6.2.8 Check that everything is properly aligned and close the instrument door.

6.6.2.9 Select the EPEC/VTEC program sequence by scrolling with the arrow keys and press the START button.

6.6.2.10 While the instrument is in operation, its door must be kept closed. Each processing step and the total time remaining can be followed on the LC display.

6.6.2.11 At the end of the program run, remove the tube's rack from the instrument and use the bead-bacteria complexes from the 5th tube to proceed with resuspension at step 6.6.3.

6.6.2.12 Remove the tip combs and discard into a biohazard waste container together with the tube strips.

6.6.3 Resuspension of the Dynabeads®-bacteria complex

Resuspend the Dynabeads®-bacteria complex in 100 µL wash buffer (PBS tween). Mix briefly by pipetting up and down a few times.

6.7 Selective Isolation

Streak 50 µL of the resuspended beads onto each of two different agars; either two Group 1 agars or one Group 1 agar and one Group 2 agar. Spread the bead-bacteria complexes over one half of the plate with a sterile swab. This ensures the break-up of the bead-bacteria complexes. Dilute further by streaking with a loop. Always carry the loop back into the previously streaked quadrant several times to ensure that the beads reach a fresh unstreaked quadrant.

Group 1 Agars

mHC (with tellurite and Cefsulodin): Incubate plates for 18-24 h at 42°C. Typical *E. coli* O157:H7/NM colonies do not ferment sorbitol and appear blue. Sorbitol-fermenting strains of *E. coli* will be yellow on mHC/mHC-CT.

mHC-CT (CT supplement Oxoid SR0172): Incubate plates for 18-24 h at 42°C. Typical *E. coli* O157:H7/NM colonies do not ferment sorbitol and appear blue. Sorbitol-fermenting strains of *E. coli* will be yellow on mHC/mHC-CT.

BD CHROMagar: Incubate plates for 18-24 h at 35°C. Typical *E. coli* O157:H7/NM colonies appear mauve against a white background. Other Gram-negative organisms will appear colourless, blue, green or blue-green.

Group 2 Agars

TCCSMAC: Incubate plates for 18-24 h at 42°C. Typical *E. coli* O157:H7 or NM colonies do not ferment sorbitol and appear translucent, colourless or straw-coloured edges, with a darker centre

ranging from grey to pink (i.e., ferment sorbitol slowly or not at all). Sorbitol-fermenting strains of *E. coli* will be red on TCCSMAC.

CT-SMAC: Incubate plates for 18-24 h at 35°C. Typical *E. coli* O157:H7/NM colonies do not ferment sorbitol and appear translucent, colourless or straw-coloured edges, with a darker centre ranging from grey to pink (i.e., ferment sorbitol slowly or not at all). Sorbitol-fermenting strains of *E. coli* will be red on CT-SMAC.

CR-SMAC: Incubate plates for 18-24 h at 35°C. Typical *E. coli* O157:H7/NM colonies do not ferment sorbitol and appear translucent, colourless or straw-coloured edges, with a darker centre ranging from grey to pink. Sorbitol-fermenting strains of *E. coli* will be red on CR-SMAC.

6.8 Confirmation

Note: Always use a positive and negative culture control for each assay.

6.8.1 Where the suspect colonies are well isolated and large enough, at least 10 colonies (if available) should be carried forward. O157 serology on these colonies can help in the screening process at this point; however, this is optional. If this optional step is used, then continue with the confirmation steps only on those colonies that are O157 positive.

Where not well isolated or the colonies are small (a slow growing serotype), streak suspect colonies onto selective agars and/or TSA-YE for purity, and then continue, as below.

Note: Steps 6.8.2 to 6.8.7 may be done either sequentially or concurrently. Refer to Table 1 for typical biochemical and serological reactions. Proceed only with typical isolates.

6.8.2 Draw a grid on the two agars chosen from group 1 and/or 2. Inoculate at least 10 typical (if available) and isolated colonies onto the grid cells of the two agars. Incubate at the appropriate temperature and for the appropriate time of incubation for the specific agars used.

6.8.3 Inoculate tubes of purple broth containing 1% cellobiose or stab onto carbohydrate agar containing 1% cellobiose. Incubate at 35°C for 18-24 h.

6.8.4 Confirm that the suspect isolates are *E. coli* with a rapid identification system (VITEK 2, API, or equivalent). Analysis of isolates must continue, regardless of the results of this step.

6.8.5 Confirm isolates as O157 using latex agglutination kits or O157 antisera.

6.8.6 Confirm the presence of verotoxins using either:

A) VT toxin test (refer to Appendix K, vol. 1);

or

B) Methods that detect VT genes (refer to Appendix K, vol. 1);

If A) is negative then B) must be performed. If B) is negative then consider the isolate to be negative for *E. coli* O157:H7/NM. If A) or B) is positive, then consider the sample confirmed for the presence of *E. coli* O157:H7/NM.

6.8.7 Optional Confirmation Steps: For difficult identifications, these optional steps may be helpful.

1) Do a Gram stain.

2) MUG test for fluorescence. Do one of the following:

a. BCIG: Streak suspect colonies onto SMAC agar with BCIG. Incubate plates at 35°C for 22-24 h. Typical *E. coli* O157:H7 or NM strains will form colourless colonies. Most other *E. coli* strains will form blue to purple colonies.

or

b. Streak suspect colonies onto Phenol Red Sorbitol agar with MUG (PSRA). Incubate plates at 35°C for 22-24 h.

or

c. Inoculate LST broth with MUG. Incubate at 35°C for 22-24 h. Ensure that the uninoculated tube does not show fluorescence.

or

Inoculate TSA plate to form a lawn of bacteria. Aseptically transfer a MUG disks (Colicomplete) onto the agar plate. Incubate at 35°C for 22 - 24 h.

For b, c, and d, check the MUG reaction using UV Light at 345 nm. Ensure that positive and negative controls give proper reactions.

3) Inoculate IMViC tests (see MFHPB-19) and urea slants. Incubate at 35°C for 22-24 h.

4) H7 Agglutination (complete serological testing using H7 antisera following manufacturer's instructions) or genetic testing of H7 using a validated method (refer to Appendix K).

Note: When using latex agglutination kits and antisera, the isolate may have to be "resuscitated" in M broth or motility agar several times (at least three times). Although some kits do not require this resuscitation, Health Canada has found that this step aids in the serology.

6.9 Interpretation

If a presumptive positive result was obtained using a screening kit and a typical isolate cannot be identified, consider secondary enrichment as in section 6.5.

If an isolate exhibits typical reactions for all of the mandatory confirmation steps, report as a confirmed *E. coli* O157:H7 / NM. If H7 testing has been done, report as *E. coli* O157:H7 or *E. coli* O157:NM, as appropriate.

7. References

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Table 1. Characteristics of *E. coli* O157:H7/NM

	E. coli O157:H7 / NM Reactions	Other *Escherichia* Reactions
Gram Stain	Negative	Negative
IMViCs (see MFHPB-19)		
Indole	+ (Red)[A]	variable
Methyl Red	+ (Red)	+ (Red)
Voges-Proskauer	- (No Colour)	- (No Colour)
Citrate	- (Green or No Growth)	- (Green or No Growth)
Cellobiose	- (Purple)	- (Purple)
Sorbitol	- (Pale or No Colour)	+ (Coloured)
Urea slants	- (Pale)	- (Pale)
Pigment Production on Nutrient Agar	- (No Pigment)	- (No Pigment)
MUG Reaction	- (No fluorescence[B])	+ (Fluorescence)
BCIG Reaction	- (Pale)	+ (Coloured)
Latex Agglutination	+ (Positive)	- (Negative)
O157	+ (Positive)	- (Negative)
H7	+ (Positive)	- (Negative)
Verotoxin production (VT)	VT 1 + and/or VT 2 +	VT 1 and/or VT 2 + or -

[A] Some *E. coli* O157 strains are indole negative.

[B] *E. coli* O157:H16 and H45 fluoresce when MUG is present.

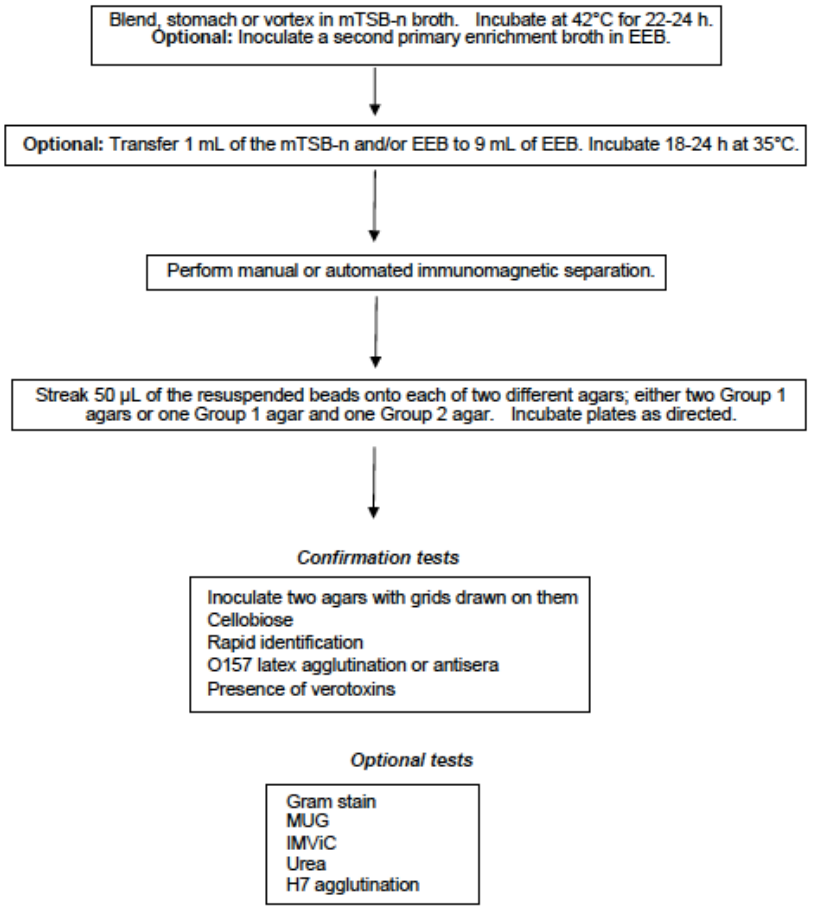


Figure 1. Flow diagram showing the isolation procedure

Appendix C: LFIA Test Kit package insert

ADx™

DECISION *POINT*™
Rapid Food Safety Testing Solutions

E. coli O157

Application
This method is applicable for the recovery and detection of *Escherichia coli* O157 (including H7 and non-motile) in raw meat and ready-to-cook meat products.

Description
DECISION *Point* ™ is a qualitative immunoassay that employs specific antibodies against target antigens found in *E. coli* O157.

Assay Principles
The system uses a selective enrichment medium for the rapid recovery and growth of the target organism. After enrichment, a portion of the sample is placed into a sample vial containing dried antibody reagents. The sample is incubated with the reagents for 30 minutes at room temperature. If *E.coli* O157 is present in the sample, it will form an antibody-antigen-antibody complex in solution. The sample is loaded onto the cassette, where it will flow through the nitrocellulose membrane. Any complex formed will be captured in the test zone of the membrane, thus displaying a visible red line. The rest of the sample will continue to migrate until it reaches the control line. The control line will develop whether or not the sample contains *E.coli* O157, thereby ensuring that the test system is functioning properly.

Intended User
The test is designed to be used by personnel who are familiar with the aseptic techniques required in a microbiological laboratory. Specialized training is not required; however, basic knowledge of food microbiology is recommended.

Materials Provided:
- ***DECISION*** *Point* ™ foiled test cassettes – single use
- ***DECISION*** *Point* ™ foiled test reagent vials – single use
- ***DECISION*** *Point* ™ Sample Diluent
- ***DECISION*** *Point* ™ Sample Buffer
- Eppendorf tubes for dilutions

Materials Required But Not Provided:
- Pipettes and tips for pipettes
- Stomacher®-type filter bags
- Sterile (deionized or distilled) water
- Dried enrichment media: Tryptic Soy Broth + 20 mg/L Novobiocin

Equipment Required:
- Autoclave
- Analytical balance
- Stomacher®
- Timer

- Incubator capable of maintaining 42 ± 1ºC
- Vortex (if available)

Storage and Handling
- Store ***DECISION*** *Point* ***TM*** test components at 2-8°C.
- Inspect the ***DECISION*** *Point* ***TM*** foiled test cassette and foiled test reagent vials for damage prior to use. Do not use if the foil shows evidence of damage or perforation.
- Each ***DECISION*** *Point* ***TM*** test cassette and reagent vials is intended for single use only.
- ***DECISION*** *Point* ***TM*** test cassette and test reagent vials should remain in their sealed foil pouches until ready to perform the test.

Sample Preparation and Cultivation:

1. Sample Addition
- **25 g samples:** Aseptically combine 25 g of sample with 225 mL of enrichment medium (1:10) in a Stomacher® bag. Place the bag in a Stomacher® machine and mix for 60 seconds at normal speed.
- **325 g samples:** Aseptically combine 325 g of sample with 2925 mL of enrichment medium (1:10) in a Stomacher® bag. Place the bag in a Stomacher® machine and mix for 60 seconds at normal speed.

2. Incubation
Close the sample bag loosely to allow air exchange for *E. coli* O157 growth. Incubate at 42 ± 1ºC:
- **25 g samples:** 16-24 hours.
- **325 g samples:** 22–24 hours.

3. Cooling
After the appropriate incubation time has elapsed, allow the enriched sample to cool to room temperature prior to testing.

4. Mixing
Hold the bag so that the bottom is supported and gently mix the contents using a side-to-side motion.

DECISION *Point* ***TM*** **Test Procedure (FIGURE 1):**
1. Remove the required number of foiled test cassettes, foiled test reagent vials, sample diluent bottles and sample buffer bottles from 2-8°C storage.
   Note: Allow the foiled test cassettes to equilibrate to room temperature during the 30 minutes sample incubation.
2. Open up reagent vial pouch and label both vials with the sample number.
3. Pipette 1.0 mL of Sample Diluent into a clean Eppendorf tube, add 10 µL of the sample directly from the Stomacher® bag (yielding 1/100 dilution) and mix thoroughly by vortexing or pipetting up and down several times.
4. Transfer a 200 µL sample directly from the Stomacher® bag into Vial A.
5. Transfer a 200 µL of the diluted sample (from Step 3) into Vial B.
6. Add 10 µL of Sample Buffer into both vials, close caps and mix thoroughly by vortexing or inverting tubes several times.
7. Let the samples incubate on the bench at room temperature for 30 minutes.
8. Open pouches containing the ***DECISION*** *Point* ***TM*** test cassettes 1-2 minutes before the end of the incubation time and label the cassettes with the sample number.
9. When the incubation period is completed, load 150 µL of sample from vial A into sample port A (left) on the test device and 150 µL of sample from vial B into sample port B (right).
10. Read the test results at 15 minutes. Observations after 17 minutes may be inaccurate due to overdevelopment of the reaction. For results interpretation please see below.

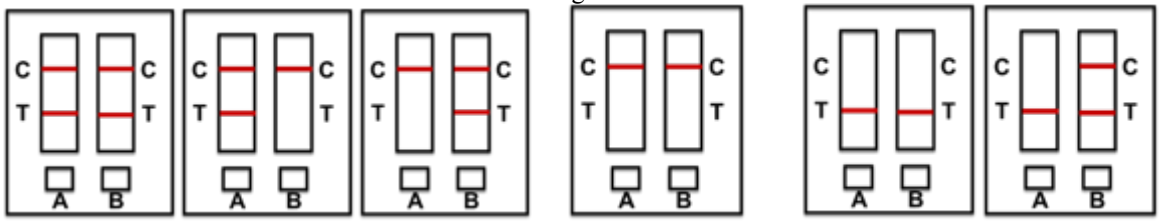
Visual Interpretation of Results

Check for the presence of red lines in the Control (C) and Test (T) areas in result windows A and B at 15 minutes.

- **A positive test result is indicated by:**
  - ✓ Control lines in both A and B result windows.
  - ✓ Test lines in either one or both A and B result windows.
- **A negative test result is indicated by:**
  - ✓ Control lines in both A and B result windows.
  - ✓ Test lines in neither A and B result windows.
- **An invalid test result is indicated by:**
  - ✓ No Control lines in either A and B result windows.
- **Notes:**
  - ✓ Different intensities of Test and Control lines are acceptable.
  - ✓ The test is not quantitative and test line intensity does not reflect target antigen concentration.
  - ✓ If the test interpretation is invalid, the sample should be retested.

Possible Test Results

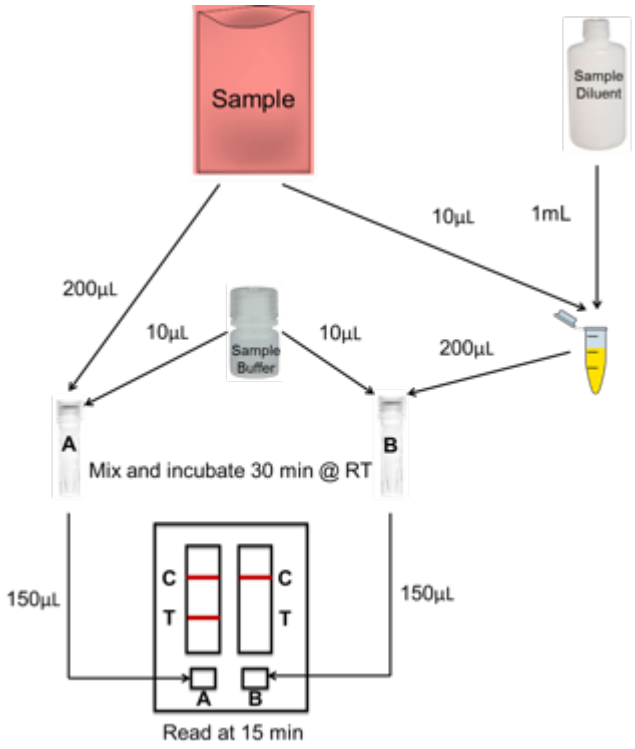
Positive Results Negative Result Invalid Results



Disposal

Decontaminate (autoclave, bleach, *etc.*) and dispose of used ***DECISION*** *Point* ™ test cassettes, reagent vials, media, and pipettes/tips in accordance with good laboratory practices and local and federal regulations.

FIGURE 1: Test Flow Chart


Sample
Sample Diluent
10µL
1mL
200µL
10µL
Sample Buffer
10µL
200µL
A
B
Mix and incubate 30 min @ RT
150µL
C
T
C
T
150µL
A
B
Read at 15 min

Appendix D: MFHPB-33 Enumeration of total aerobic bacteria in food products and food ingredients using 3M™ Petrifilmt™ aerobic count plates

Government of Canada / Gouvernement du Canada

HPB Method **MFHPB-33**
February 2001

HEALTH PRODUCTS AND FOOD BRANCH

OTTAWA

ENUMERATION OF TOTAL AEROBIC BACTERIA IN FOOD PRODUCTS AND FOOD INGREDIENTS USING 3M™ PETRIFILM™ AEROBIC COUNT PLATES

Don Warburton
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Food Directorate, HPB
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Ottawa, Ontario, K1A OL2

Contact the M icrobiology Methods Committee: mmc-cmm@hc-sc.gc.ca

1. APPLICATION

This method is applicable for the enumeration of aerobic bacteria in food products and food ingredients. This product may also be used for environmental sampling (see Laboratory Procedure MFLP-41A and MFLP-41B). This revised method replaces MFHPB-33, dated April 1997.

2. PRINCIPLE

Petrifilm plates are a ready-to-use product developed by the 3M Company, St. Paul, MN. Media are coated onto films, so that traditional media preparation is unnecessary, and both labour and time savings are realized. Aerobic bacteria can be enumerated on the Petrifilm Aerobic Count (AC) plates. The method uses bacterial culture plates containing dry medium and a cold-water-soluble gelling agent. One mL samples are added directly to the plates. Pressure, when applied to the plastic spreader placed on the overlay film, spreads the sample over 20 cm^2. The gelling agent is allowed to solidify, and the plates are then incubated and counted. Validation and collaborative studies have found the Petrifilm AC method to be not significantly different from the traditional method (7.1 - 7.13).

3. DEFINITION OF TERMS

3.1 See Appendix A of Volume 1.

3.2 Petrifilm AC plates contain modified Standard Methods nutrients; a cold-water-soluble gelling agent; and 2, 3, 5-triphenyl tetrazolium chloride (TTC) indicator. Reduction of the TTC will cause the colonies to become red.

3.3 Plastic spreading devices are provided with the Petrifilm plates. The concave side is designed to spread the sample over the growth area.

4. COLLECTION OF SAMPLES

See Appendix B of Volume 1.

5. MATERIALS AND SPECIAL EQUIPMENT

1) Petrifilm Aerobic Count plates 6400/6406 (3M Canada Inc., PO Box 5757, London, ON, N6A 4T1)

   A. Petrifilm AC plates. Store at or below 8 °C.

   B. Plastic spreading device

   C. Package insert, including instructions for use. "Interpretation Guide," available upon request.

2) Appropriate diluents: Butterfield's phosphate buffer, IDFphosphate buffer (KH2PO4 at 0.0425 g/L pH 7.2), peptone salt diluent (ISO method 6887), buffered peptone water (ISO method 6579) 0.1% peptone water, saline solution (0.85 to 0.9%), bisulfate-free letheen broth and distilled water.

Note: Do not use citrate buffer or sodium thiosulfate with the Petrifilm plate method. If citrate buffer is indicated in the standard, substitute warmed (40° - 45°C) Butterfield's phosphate buffer.

3) Stomacher, blender or equivalent.

4) Incubator capable of maintaining 32 or 35°C.

5) pH meter or paper capable of distinguishing 0.3 to 0.5 pH units within a range of 6.0 to 7.5

6) 1N NaOH

7) colony counter and/or magnified illuminator

Note: It is the responsibility of each laboratory to ensure that the temperature of the incubators or water baths are maintained at the recommended temperatures. Where 35°C is recommended in the text of the method, the incubator may be at 35 +/-1.0° C. Similarly, lower temperatures of 30 or 25°C may be +/- 1.0°C. However, where higher temperatures are recommended, such as 43 or 45.5°C, it is imperative that the incubators or water baths be maintained within 0.5°C due to potential lethality of higher temperatures on the microorganism being isolated.

6. PROCEDURE

Carry out the test in accordance with the following instructions:

6.1 Handling of Food Sample

6.1.1 Prior to analysis, except for shelf-stable foods, keep sample refrigerated (2-8 °C) or frozen, depending on the nature of the product. Thaw frozen samples in a refrigerator or under time and temperature conditions which prevent microbial growth or death.

6.1.2 Analyze samples as soon as possible after their receipt in the laboratory.

6.2 Preparation for Analysis

6.2.1 Have sterile diluent ready. Disinfect the surface of the work area.

6.2.2 Place the Petrifilm plate on a flat surface. Mark identifying sample information on the film.

6.3 Preparation of Sample

6.3.1 To ensure a representative analytical unit, agitate liquids until the contents are homogeneous. If the food sample is a solid, take representative portions from several locations within the sample. Prepare a 1:10 dilution in an appropriate (see 5.2) diluent. Mix thoroughly.

6.3.2 For acid products adjust pH of the diluted sample to 6.6 - 7.2 with 1N NaOH.

6.4 Inoculation and Incubation

6.4.1 Lift the top film and carefully inoculate 1 mL of sample or diluted sample to the center of bottom film. A pipette, pipettor or a plate loop continuous pipetting syringe can be used for sample addition.

6.4.2 Drop top film onto sample.

6.4.3 Distribute sample evenly using a downward pressure on the center of the plastic spreader, concave side down. Do not slide the spreader across the film. Leave plate undisturbed for at least 1 minute to permit the gel to solidify.

6.4.4 Return unused plates to foil pouch. Seal pouch by folding and taping the open end. Store resealed foil pouch in a cool dry place. Use plates within one month after opening pouch. Exposure of Petrifilm plates to temperatures above 25 °C and/or humidities >50% RH can affect the performance of the plates. Do not use plates that show orange or brown discoloration. Expiration date and lot number are noted on each package of Petrifilm plates. The lot number is also noted on individual test films.

6.4.5 Incubate plates with the clear side up in stacks not exceeding 20 units. Follow current industry standards for incubation temperature. Incubate plates 48 ± 3 hours. Examine for bacteria.

6.5 Reading Results

6.5.1 Count plates promptly after incubation period. If impossible to count at once, store plates in the freezer. This should be avoided as a routine practice.

6.5.2 Use a standard colony counter for counting purposes. A magnified-illuminator may also be used to facilitate counting.

6.5.3 The circular growth area is approximately 20 cm^2. Estimates can be made on plates containing more than 250 colonies by counting the number of colonies in one or more representative squares and determining the average number per square. Multiply the average number by 20 to determine total count per plate.

6.5.4 Calculate the number of colonies per mL or g of sample from the number of colonies obtained in plates chosen at dilution levels which give a statistically significant result.

6.5.5 When counting colonies on duplicate plates of consecutive dilutions, compute mean number of colonies for each dilution before determining average bacterial count.

6.5.6 To isolate colonies for further identification, lift the top film and pick the colony from the gel.

6.6 Interpretation of Results

6.6.1 Count all red dots regardless of size or intensity as colonies. The presence of very high concentrations of colonies on the plates will cause the entire growth area to become red or pink; record results as "too numerous to count" (TNTC). Occasionally, on overcrowded plates, the center may lack visible colonies but many small colonies will be seen on the edges. When this occurs, record results as TNTC; further dilution of the sample is required.

6.6.2 Some organisms can liquefy the gel, allowing them to spread out and obscure the presence of other colonies. If a liquefier interferes with counting, an estimated count should be made by counting the unaffected areas.

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END OF DOCUMENT

Appendix E: Buffer and reagents prepared for this study.

Buffer/ Reagent	Composition	Application
Blocking Buffer	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4, 10% BSA	ELISA
Coating Buffer	0.1 M sodium carbonate buffer pH 9.5	ELISA
Coomassie Blue Staining Solution	45% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.3% (w/v) Coomassie brilliant blue R-250	Protein Visualization
Denaturing Binding Buffer	8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8	scFvO157 Purification
Denaturing Buffer	6M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8	scFvO157 Purification
Denaturing Elution Buffer	8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4	scFvO157 Purification
Destaining Solution	40% (v/v) methanol, 10% (v/v) glacial acetic acid	Protein Visualization
Laemmli Buffer (4×)	250 mM Tris-HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 8% (v/v) β-mercaptoethanol	Protein Visualization
Native Buffer	20 mM Tris-HCl, pH 7.4, 200 mM NaCl	scFvO157 Purification
Native Elution Buffer	50 mM NaH_2PO_4, 0.5 M NaCl and 250 mM imidazole, pH 8.0	scFvO157 Purification
Native Wash Buffer	50 mM NaH_2PO_4 and 0.5 M NaCl, pH 8.0	scFvO157 Purification
PBS (1×)	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.4	Various
PBST (1×)	137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 0.05% (v/v) Tween-20	ELISA
Polyacrylamide Resolving Gel (10%)	2.0 ml ddH_2O, 1.7 ml 30% acrylamide/bisacrylamide (37.5:1) aqueous solution, 1.3 ml 1.5 M Tris-HCl pH 8.8, 0.05 ml 10% SDS, 0.05 ml 10% APS, 0.002 ml TEMED (5 ml Final Volume)	Protein Visualization

Buffer/ Reagent	Composition	Application
Polyacrylamide Resolving Gel (12%)	1.7 ml ddH_2O, 2.0 ml 30% acrylamide/bisacrylamide (37.5:1) aqueous solution, 1.3 ml 1.5 M Tris-HCl pH 8.8, 0.05 ml 10% SDS, 0.05 ml 10% APS, 0.002 ml TEMED (5 ml Final Volume)	Protein Visualization
Polyacrylamide Stacking Gel (5%)	0.01 ml 10% SDS, 0.13 ml 0.5 M Tris-HCl pH 6.8, 0.17 ml acrylamide/bisacrylamide (37.5:1) aqueous solution, 0.01 ml 10% APS, 0.001 ml TEMED (1 ml Final Volume)	Protein Visualization
Rehydration Buffer Stock Solution (RBSS)	48% urea (w/v), 2% CHAPS (w/v), 0.02% bromophenol blue (w/v)	IEF
TBE (1×)	89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA pH 8.0	DNA Visualization
TBS (1×)	50 mM Tris-HCl pH 7.6, 150 mM NaCl	Protein Visualization
TBST (1×)	10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween-20	Protein Visualization
Towbin Semi-dry Transfer Buffer	191 mM glycine, 50 mM Tris	Protein Visualization
Tris-Glycine Running Buffer (1×)	192 mM glycine, 25 mM Tris, 0.1% (w/v) SDS, pH 8.3–8.6	Protein Visualization

Appendix F: Supporting information for the LFIA Test Kit relative validation study (raw data)

Inclusivity study

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Decision Point™ Test Kit	
						A	**B**
1	PHAC	EC20060233	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	+
						+	+
2	PHAC	EC20040339	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
3	PHAC	EC20011244	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	+
						+	+
4	PHAC	EC20011236	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	+
						+	+
5	PHAC	EC20011231	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	+
						+	+
6	PHAC	EC20010294	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	-
						+	+
7	PHAC	EC20001018	*Escherichia coli*	O157:H7	Bovine, ground	-	+
						-	+
						-	+
8	PHAC	EC20000673	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
9	PHAC	EC20000671	*Escherichia coli*	O157:H7	Bovine, ground	+	+
						+	+
						+	+
10	PHAC	EC19990951	*Escherichia coli*	O157:H7	Bovine, salami	+	+
						+	+
						-	+

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Decision Point™ Test Kit	
						A	B
11	PHAC	EC19970515	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
12	PHAC	EC19970462	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
13	PHAC	EC19970419	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
14	PHAC	EC19970409	*Escherichia coli*	O157:H7	Bovine, meat	+	+
						+	+
						+	+
15	PHAC	EC19950095	*Escherichia coli*	O157:NM	Bovine, meat	+	+
						+	+
						+	+
16	PHAC	EC20130376	*Escherichia coli*	O157:H7	Bovine, feces	+	+
						+	+
						+	+
17	PHAC	EC20130395	*Escherichia coli*	O157:NM	Bovine, feces	+	+
						+	+
						+	+
18	PHAC	EC20132075	*Escherichia coli*	O157:H7	Bovine, feces	+	+
						+	+
						+	+
19	PHAC	EC20130390	*Escherichia coli*	O157:NM	Bovine, feces	+	+
						+	+
						+	+
20	PHAC	EC20130378	*Escherichia coli*	O157:H7	Bovine, feces	+	+
						+	+
						+	+
21	PHAC	EC19961016	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Decision Point™ Test Kit	
						A	B
22	PHAC	EC19961090	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
23	PHAC	EC19961025	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
24	PHAC	EC19961027	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
25	PHAC	EC20020335	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
26	PHAC	EC19930553	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
27	PHAC	EC19930038	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
28	PHAC	EC19920333	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
29	PHAC	EC19960266	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
30	PHAC	EC19960274	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
31	PHAC	EC20050147	*Escherichia coli*	O157:H12	Bovine, feces	-	+
						-	+
						-	+
32	PHAC	EC20060754	*Escherichia coli*	O157:H29	Bovine, feces	+	+
						+	+
						+	+

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Decision Point™ Test Kit	
						A	B
33	PHAC	EC19970524	*Escherichia coli*	O157:H42	Bovine, meat	-	+
						-	+
						-	+
34	PHAC	EC19970465-1	*Escherichia coli*	O157:H45	Bovine, meat	+	-
						+	-
						+	-
35	CRIFS	C480	*Escherichia coli*	O157:H7	N/A	+	+
						+	+
						-	+
36	CRIFS	EC20321	*Escherichia coli*	O157:H7	L.Goodridge	+	+
						+	+
						+	+
37	CRIFS	ATCC 43888	*Escherichia coli*	O157:H7	Human, feces	+	+
						+	+
						+	+
38	CRIFS	ATCC 43894	*Escherichia coli*	O157:H7	Human, feces	+	+
						+	+
						+	+
39	CRIFS	ATCC 700927	*Escherichia coli*	O157:H7	Derived from ATCC43895	+	+
						+	+
						+	+
40	CRIFS	EC960282	*Escherichia coli*	O157:H25	Bovine	+	-
						+	-
						+	-
41	CRIFS	EC940076	*Escherichia coli*	O157:H19	Bovine	-	+
						-	+
						-	+
42	CRIFS	EC930195; 32511	*Escherichia coli*	O157:NM	L.Goodridge	+	+
						-	+
						+	+
43	DSMZ	DSM 17076/ NCTC 12900	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+

#	Culture Collection	Reference	Strain	Serotype	Source/ Origin	Decision Point™ Test Kit	
						A	B
44	UofM	00-3581	*Escherichia coli*	O157:H7	Human, clinical	-	+
						+	+
						-	+
45	UofM	02-0304	*Escherichia coli*	O157:H7	Human, clinical	+	+
						+	+
						+	+
46	UofM	02-0627	*Escherichia coli*	O157:H7	Human, clinical	-	+
						-	+
						-	+
47	UofM	02-0628	*Escherichia coli*	O157:H7	Human, clinical	+	+
						-	+
						+	+
48	UofM	02-1840	*Escherichia coli*	O157:NM	Human, clinical	-	+
						-	+
						-	+
49	CRIFS	380-94	*Escherichia coli*	O157:H7	Bovine, salami	+	+
						+	+
						+	+
50	CRIFS	9490	*Escherichia coli*	O157:H7	USDA	+	+
						+	+
						+	+

PHAC: Public Health Agency of Canada, Guelph; CRIFS: Canadian Research Institute for Food Safety, Guelph; DSMZ: Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures; UofM: University of Manitoba. N/A: not available.

Exclusivity study

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source	DECISION Point™ Test Kit	
						A	B
1	PHAC	ATCC 7966	*Aeromonas hydrophila*		From tin of milk with a fishy odor	-	-
						-	-
						-	-
2	UWO	ATCC 13883 UWO#479	*Klebsiella pneumoniae*		NCTC	-	-
						-	-
						-	-
3	PHAC	ATCC 6051			HJ Conn	-	-

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source	DECISION Point ™ Test Kit A	B
			Bacillus subtilis			-	-
						-	-
4	PHAC	ATCC 43162	*Citrobacter braakii (freundii)*		Clinical isolate, California	-	-
						-	-
						-	-
5	PHAC	NO152388	*Enterobacter aerogenes*		N/A	-	-
						-	-
						-	-
6	PHAC	ATCC 13047	*Enterobacter cloacae*		Spinal fluid	-	-
						-	-
						-	-
7	PHAC	ATCC 49149	*Enterococcus faecalis*		Clinical isolate	-	-
						-	-
						-	-
8	PHAC	ATCC 25922	*Escherichia coli*	O6	Human, clinical	-	-
						-	-
						-	-
9	PHAC	ATCC 700926	*Escherichia coli*	OR:H48:K-	Derived from parent strain W1485	-	-
						-	-
						-	-
10	PHAC	ATCC 12014	*Escherichia coli*	O55:NM	CDC	-	-
						-	-
						-	-
11	PHAC	ATCC 43887	*Escherichia coli*	O111:NM	Human, feces	-	-
						-	-
						-	-
12	PHAC	EC20130476	*Escherichia coli*	O25:NM	N/A	-	-
						-	-
						-	-
13	PHAC	EC20130473	*Escherichia coli*	O78:H11	N/A	-	-
						-	-
						-	-
14	PHAC	EC20130469	*Escherichia coli*	O28ac:NM	N/A	-	-
						-	-
						-	-
15	PHAC	EC20130467	*Escherichia coli*	O3:H2	N/A	-	-
						-	-
						-	-
16	PHAC	EC20130466	*Escherichia coli*	O44:H18	N/A	+	-
						-	-
						+	-
17	PHAC	EC20130463	*Escherichia coli*	O21:H4	N/A	-	-
						-	-
						-	-
18	PHAC	EC20130462	*Escherichia coli*	O75:H5	N/A	-	-
						-	-
						-	-
19	CRIFS	EC 920232	*Escherichia coli*	O2:H5	Bovine	-	-
						-	-
						-	-

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source	DECISION Point ™ Test Kit A	B
20	CRIFS	EC 930004	*Escherichia coli*	O103:H2	N/A	-	-
						-	-
						-	-
21	CRIFS	EC 910040	*Escherichia coli*	O145:NM	N/A	-	-
						-	-
						-	-
22	CRIFS	EC 910005	*Escherichia coli*	O111:NM	N/A	-	-
						-	-
						-	-
23	CRIFS	EC 910060	*Escherichia coli*	O121:H7	N/A	-	-
						-	-
						-	-
24	ATCC	ATCC43893, CDC EDL 1284 [929-78]	*Escherichia coli*	O124:NM	Human, feces	+	-
						+	-
						+	-
25	ATCC	ATCC 43886, CDC E2539-C1	*Escherichia coli*	O25:K98:NM	Human, feces	-	-
						-	-
						-	-
26	PHAC	ATCC 33650	*Escherichia hermanii*		Human, female toe	-	-
						-	-
						-	-
27	PHAC	MB1914	*Hafnia alvei*		N/A	-	-
						-	-
						-	-
28	PHAC	ATCC 49347	*Shigella dysenteriae*		Human, feces	-	-
						-	-
						-	-
29	PHAC	ATCC 25929	*Shigella flexneri*		Human, feces	-	-
						-	-
						-	-
30	PHAC	ATCC 25931	*Shigella sonnei*		Human, feces	-	-
						-	-
						-	-
31	PHAC	ATCC 23715	*Yersinia enterocolitica*		Human, blood	-	-
						-	-
						-	-
32	UWO	LT-2	*Salmonella enterica* ser. Typhimurium		N/A	-	-
						-	-
						-	-
33	CRIFS	C398	*Salmonella newport*		N/A	-	-
						-	-
						-	-
34	CRIFS	C390	*Salmonella Heidenberg*		N/A	-	-
						-	-
						-	-
35	CRIFS	C417	*Salmonella Enteritidis*		N/A	-	-
						-	-
						-	-
36	CRIFS	C1116	*Salmonella javiana*		N/A	-	-
						-	-
						-	-
37	ATCC	ATCC 19114		4a		-	-

#	Culture Collection	Reference	Strain	Serotype	Origin/ Source	DECISION Point ™ Test Kit A	B
			Listeria monocytogenes		Animal, tissue	-	-
						-	-
38	HC	HPB 5949	*Listeria monocytogenes*	1/2C	Food, ready-to-eat	-	-
						-	-
						-	-

PHAC: Public Health Agency of Canada, Guelph; UWO: Western University, Department of Microbiology and Immunology; CRIFS: Canadian Research Institute for Food Safety, Guelph; ATCC: American Type Culture Collection; HC: Health Canada, Listeriosis Reference Service; N/A: not available.

Estimation of the (H) and (L) inoculum levels for each food item evaluated

Food Item	Target Level	Cell Titre (CFU/ml)	10-fold Serial Dilution Selected	Volume Inoculated (ml)[1]	CFU Inoculated[2]	Estimated Inoculation Level (CFU/25g)
Raw ground beef	Low	1.26×10^{8}	1.00×10^{-7}	4.0	50.4	1.26
Raw ground beef	High	1.50×10^{8}	1.00×10^{-6}	4.0	600	15
Raw ground veal	Low	1.26×10^{8}	1.00×10^{-7}	4.0	50.4	1.26
Raw ground veal	High	1.50×10^{8}	1.00×10^{-6}	4.0	600	15
Raw beef trim	Low	1.26×10^{8}	1.00×10^{-7}	4.0	50.4	1.26
Raw beef trim	High	1.50×10^{8}	1.00×10^{-6}	4.0	600	15
Carpaccio	Low	1.26×10^{8}	1.00×10^{-7}	4.0	50.4	1.26
Carpaccio	High	1.50×10^{8}	1.00×10^{-6}	4.0	600	15
Raw sausage*	Low	9.6×10^{7}	1.00×10^{-7}	4.0	38.4	0.96
Raw sausage*	High	9.60×10^{7}	1.00×10^{-6}	4.0	384	9.6
Raw burger patty*	Low	9.6×10^{7}	1.00×10^{-7}	4.0	38.4	0.96
Raw burger patty*	High	9.60×10^{7}	1.00×10^{-6}	4.0	384	9.6
Raw meatballs*	Low	9.6×10^{7}	1.00×10^{-7}	4.0	38.4	0.96
Raw meatballs*	High	9.60×10^{7}	1.00×10^{-6}	4.0	384	9.6

Food Item	Target Level	Cell Titre (CFU/ml)	10-fold Serial Dilution Selected	Volume Inoculated (ml)[1]	CFU Inoculated[2]	Estimated Inoculation Level (CFU/25g)
Raw kebabs*	Low	9.6×10^{7}	1.00×10^{-7}	4.0	38.4	0.96
Raw kebabs*	High	9.60×10^{7}	1.00×10^{-6}	4.0	384	9.6

[1]Volume from the selected dilution that was used to inoculate 1.0kg of each food item.
[2]Total number of CFU that were inoculated into 1.0kg of each food item.
**These food items were inoculated with strain 380-94, which was stressed as described in the Inoculum Preparation Section.*

AM results for the fresh meats (unprocessed) food type.

Sample Item	Inoculation Level	MPN/25g	Alternative Results		
			Presumptive	Confirmation	Final*
AM ground beef H1	H	25	POS	POS	TP
AM ground beef H2	H	25	POS	POS	TP
AM ground beef H3	H	25	POS	POS	TP
AM ground beef H4	H	25	POS	POS	TP
AM ground beef H5	H	25	POS	POS	TP
AM ground veal H1	H	13.3	POS	POS	TP
AM ground veal H2	H	13.3	POS	POS	TP
AM ground veal H3	H	13.3	POS	POS	TP
AM ground veal H4	H	13.3	POS	POS	TP
AM ground veal H5	H	13.3	POS	POS	TP
AM beef trim H1	H	13.3	POS	POS	TP
AM beef trim H2	H	13.3	POS	POS	TP
AM beef trim H3	H	13.3	POS	POS	TP
AM beef trim H4	H	13.3	POS	POS	TP
AM beef trim H5	H	13.3	POS	POS	TP
AM carpaccio H1	H	8.25	POS	POS	TP
AM carpaccio H2	H	8.25	POS	POS	TP
AM carpaccio H3	H	8.25	POS	POS	TP
AM carpaccio H4	H	8.25	POS	POS	TP
AM carpaccio H5	H	8.25	POS	POS	TP
AM ground beef L1	L	1.2	NEG	NEG	TN
AM ground beef L2	L	1.2	NEG	NEG	TN
AM ground beef L3	L	1.2	POS	POS	TP
AM ground beef L4	L	1.2	NEG	NEG	TN

Sample Item	Inoculation Level	MPN/25g	Alternative Results		
			Presumptive	Confirmation	Final*
AM ground beef L5	L	1.2	NEG	NEG	TN
AM ground veal L1	L	0.5	POS	POS	TP
AM ground veal L2	L	0.5	PO	POS	TP
AM ground veal L3	L	0.5	NEG	NEG	TN
AM ground veal L4	L	0.5	NEG	NEG	TN
AM ground veal L5	L	0.5	NEG	NEG	TN
AM beef trim L1	L	1.3	POS	POS	TP
AM beef trim L2	L	1.3	NEG	NEG	TN
AM beef trim L3	L	1.3	NEG	NEG	TN
AM beef trim L4	L	1.3	NEG	NEG	TN
AM beef trim L5	L	1.3	POS	POS	TP
AM carpaccio L1	L	1.3	NEG	NEG	TN
AM carpaccio L2	L	1.3	POS	POS	TP
AM carpaccio L3	L	1.3	NEG	NEG	TN
AM carpaccio L4	L	1.3	NEG	NEG	TN
AM carpaccio L5	L	1.3	NEG	NEG	TN
AM ground beef U1	U	-	NEG	NEG	TN
AM ground beef U2	U	-	NEG	NEG	TN
AM ground veal U	U	-	NEG	NEG	TN
AM beef trim U	U	-	NEG	NEG	TN
AM carpaccio U	U	-	NEG	NEG	TN

**Alternative Final Results are defined as True Positives (TP) or True Negatives (TN).*

AM results for the ready-to cook (processed) food type.

Sample Item	Inoculation Level	MPN/25g	Alternative Results		
			Presumptive	Confirmation	Final*
AM raw sausage H1	H	13.3	POS	POS	TP
AM raw sausage H2	H	13.3	POS	POS	TP
AM raw sausage H3	H	13.3	POS	POS	TP
AM raw sausage H4	H	13.3	POS	POS	TP
AM raw sausage H5	H	13.3	POS	POS	TP
AM raw patty H1	H	13.3	POS	POS	TP
AM raw patty H2	H	13.3	POS	POS	TP
AM raw patty H3	H	13.3	POS	POS	TP
AM raw patty H4	H	13.3	POS	POS	TP

Sample Item	Inoculation Level	MPN/25g	Alternative Results		
			Presumptive	Confirmation	Final*
AM raw patty H5	H	13.3	POS	POS	TP
AM raw meatball H1	H	8.3	POS	POS	TP
AM raw meatball H2	H	8.3	POS	POS	TP
AM raw meatball H3	H	8.3	POS	POS	TP
AM raw meatball H4	H	8.3	POS	POS	TP
AM raw meatball H5	H	8.3	POS	POS	TP
AM raw kebab H1	H	25	POS	POS	TP
AM raw kebab H2	H	25	POS	POS	TP
AM raw kebab H3	H	25	POS	POS	TP
AM raw kebab H4	H	25	POS	POS	TP
AM raw kebab H5	H	25	POS	POS	TP
AM raw sausage L1	L	1.3	POS	POS	TP
AM raw sausage L2	L	1.3	NEG	NEG	TN
AM raw sausage L3	L	1.3	POS	POS	TP
AM raw sausage L4	L	1.3	NEG	NEG	TN
AM raw sausage L5	L	1.3	NEG	NEG	TN
AM raw patty L1	L	0.5	POS	POS	TP
AM raw patty L2	L	0.5	NEG	NEG	TN
AM raw patty L3	L	0.5	POS	POS	TP
AM raw patty L4	L	0.5	NEG	NEG	TN
AM raw patty L5	L	0.5	POS	POS	TP
AM raw meatball L1	L	1.3	POS	POS	TP
AM raw meatball L2	L	1.3	NEG	NEG	TN
AM raw meatball L3	L	1.3	NEG	NEG	TN
AM raw meatball L4	L	1.3	NEG	NEG	TN
AM raw meatball L5	L	1.3	NEG	NEG	TN
AM raw kebab L1	L	1.3	POS	POS	TP
AM raw kebab L2	L	1.3	NEG	NEG	TN
AM raw kebab L3	L	1.3	NEG	NEG	TN
AM raw kebab L4	L	1.3	NEG	NEG	TN
AM raw kebab L5	L	1.3	POS	POS	TP
AM raw sausage U1	U	-	NEG	NEG	TN
AM raw sausage U2	U	-	NEG	NEG	TN
AM raw patty U	U	-	NEG	NEG	TN

Sample Item	Inoculation Level	MPN/25g	Alternative Results		
			Presumptive	Confirmation	Final*
AM raw meatball U	U	-	NEG	NEG	TN
AM raw kebab U	U	-	NEG	NEG	TN

**Alternative Final Results are defined as True Positives (TP) or True Negatives (TN).*

RM results for the fresh meats (unprocessed) food type.

Sample Item	Inoculation Level	MPN/25g	MFHPB-10 Results
RM ground beef H1	H	25	POS
RM ground beef H2	H	25	POS
RM ground beef H3	H	25	POS
RM ground beef H4	H	25	POS
RM ground beef H5	H	25	POS
RM ground veal H1	H	13.3	POS
RM ground veal H2	H	13.3	POS
RM ground veal H3	H	13.3	POS
RM ground veal H4	H	13.3	POS
RM ground veal H5	H	13.3	POS
RM beef trim H1	H	13.3	POS
RM beef trim H2	H	13.3	POS
RM beef trim H3	H	13.3	POS
R beef trim H4	H	13.3	POS
RM beef trim H5	H	13.3	POS
RM carpaccio H1	H	8.25	POS
RM carpaccio H2	H	8.25	POS
RM carpaccio H3	H	8.25	POS
RM carpaccio H4	H	8.25	POS
RM carpaccio H5	H	8.25	POS
RM ground beef L1	L	1.2	NEG
RM ground beef L2	L	1.2	POS
RM ground beef L3	L	1.2	NEG
RM ground beef L4	L	1.2	POS
RM ground beef L5	L	1.2	NEG
RM ground veal L1	L	0.5	NEG
RM ground veal L2	L	0.5	POS
RM ground veal L3	L	0.5	NEG
RM ground veal L4	L	0.5	POS

Sample Item	Inoculation Level	MPN/25g	MFHPB-10 Results
RM ground veal L5	L	0.5	NEG
RM beef trim L1	L	1.3	POS
RM beef trim L2	L	1.3	NEG
RM beef trim L3	L	1.3	NEG
RM beef trim L4	L	1.3	NEG
RM beef trim L5	L	1.3	NEG
RM carpaccio L1	L	1.3	POS
RM carpaccio L2	L	1.3	POS
RM carpaccio L3	L	1.3	POS
RM carpaccio L4	L	1.3	NEG
RM carpaccio L5	L	1.3	NEG
RM ground beef U1	U	-	NEG
RM ground beef U2	U	-	NEG
RM ground veal U	U	-	NEG
RM beef trim U	U	-	NEG
RM carpaccio U	U	-	NEG

RM results for the ready-to-cook (processed) food type.

Sample Item	Inoculation Level	MPN/25g	MFHPB-10Results
RM raw sausage H1	H	13.3	POS
RM raw sausage H2	H	13.3	POS
RM raw sausage H3	H	13.3	POS
RM raw sausage H4	H	13.3	POS
RM raw sausage H5	H	13.3	POS
RM raw patty H1	H	13.3	POS
RM raw patty H2	H	13.3	POS
RM raw patty H3	H	13.3	POS
RM raw patty H4	H	13.3	POS
RM raw patty H5	H	13.3	POS
RM raw meatball H1	H	8.3	POS
RM raw meatball H2	H	8.3	POS
RM raw meatball H3	H	8.3	POS
RM raw meatball H4	H	8.3	POS
RM raw meatball H5	H	8.3	POS
RM raw kebab H1	H	25	POS
RM raw kebab H2	H	25	POS

Sample Item	Inoculation Level	MPN/25g	MFHPB-10Results
RM raw kebab H3	H	25	POS
RM raw kebab H4	H	25	POS
RM raw kebab H5	H	25	POS
RM raw sausage L1	L	1.3	POS
RM raw sausage L2	L	1.3	NEG
RM raw sausage L3	L	1.3	POS
RM raw sausage L4	L	1.3	NEG
RM raw sausage L5	L	1.3	POS
RM raw patty L1	L	0.5	POS
RM raw patty L2	L	0.5	NEG
RM raw patty L3	L	0.5	NEG
RM raw patty L4	L	0.5	NEG
RM raw patty L5	L	0.5	POS
RM raw meatball L1	L	1.3	POS
RM raw meatball L2	L	1.3	NEG
RM raw meatball L3	L	1.3	NEG
RM raw meatball L4	L	1.3	NEG
RM raw meatball L5	L	1.3	NEG
RM raw kebab L1	L	1.3	POS
RM raw kebab L2	L	1.3	NEG
RM raw kebab L3	L	1.3	POS
RM raw kebab L4	L	1.3	NEG
RM raw kebab L5	L	1.3	POS
RM raw sausage U1	U	-	NEG
RM raw sausage U2	U	-	NEG
RM raw patty U	U	-	NEG
RM raw meatball U	U	-	NEG
RM raw kebab U	U	-	NEG

Curriculum Vitae

Yadira Tejeda Saldaña, MSc.

EDUCATION

2011-present	Doctor of Philosophy candidate (PhD) Department of Pathology and Laboratory Medicine, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada
2008-2010	Master's of Science candidate (MSc) Food Safety, Wageningen University and Research Centre, Wageningen, The Netherlands
2001-2005	Bachelor of Science Degree (BSc) Food Chemistry. La Salle University, Mexico City, Mexico

HONOURS AND AWARDS

2011-present	Mexican National Council on Science and Technology Scholarship (CONACyT) ($143,700)
2013-2015	Mitacs Accelerate Internship Program, University of Western Ontario, ON, Canada ($60,000)
2011-2015	Western Graduate Research Scholarship, University of Western Ontario, ON, Canada
2015	Best Basic/ Clinical Science Collaborative Poster Presentation, Pathology Research Day, University of Western Ontario, ON, Canada ($100)
2014	Dutkevich Memorial Foundation Award, Department of Pathology Travel Award, University of Western Ontario, ON, Canada ($500)
2013	BioVision.Nxt Fellowship Programme, Lyon, France ($2000)
2008-2010	HSP Huygens Scholarship, The Dutch Minister of Education, Culture and Science, Wageningen University and Research Centre, Wageningen, The Netherlands ($56,500)
2009	1st place IFTSA Developing Solutions for Developing Countries Competition team project, Anaheim, CA, USA ($7000 total prize)
2006	National Association of Faculties and Schools of Chemistry Award, Mexico City, Mexico

2006	University Student Council Award, La Salle University, Mexico City, Mexico
2005	Brother Miguel Medal, La Salle University, Mexico City, Mexico
2004	University Student Council Award, La Salle University, Mexico City, Mexico

RELATED WORK EXPERIENCE

2013-2015	Teaching Assistant, Department of Pathology and Laboratory Medicine, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada
2011 (Jun-Jul)	MexicoG.A.P Program Coordinator, Mexico Supreme Quality, Mexico City, Mexico
2010 (Sept-Oct)	Teaching Assistant, Laboratory of Microbiology, Wageningen University and Research Centre, Wageningen, The Netherlands
2010 (Jan-Jul)	MSc. Thesis Intern, KWR Watercycle Research Institute, Nieuwegein, The Netherlands
2009 (Aug-Dec)	Intern, Food Safety and Zoonoses Department, World Health Organization (WHO), Geneva, Switzerland
2006-2008	Innovation Best Practices Engineer, R&D Department, Unilever Mexico, Mexico City, Mexico
2006 (Jan-Jun)	Intern, Quality Assurance Department, Bimbo Bakeries Mexico, Mexico City, Mexico

PRESENTATIONS

2016	Y. Tejeda Saldana, J. McCormick, M. J. Rieder. Development of a Single Chain Antibody for Detection of *Escherichia coli* O157. Poster presentation. Annual Pathology Research Day, London, Canada.
2015	Y. Tejeda Saldana, J. McCormick, M. J. Rieder. Variable Region Sequencing of a Monoclonal Antibody Against *Escherichia coli* O157. Poster presentation. Annual Pathology Research Day, London, Canada. ***Best Basic/Clinical Science Collaborative Poster Presentation***
2014	Y. Tejeda Saldana, M. J. Rieder. Validating Inclusivity/ Exclusivity of an Alternative Method for Detecting *E. coli* O157 According to AOAC Guidelines. Poster presentation. Annual Pathology Research Day. London, ON, Canada
2014	Y. Tejeda Saldana, M. J. Rieder. Relative Validation of an Alternative Qualitative Method According to Health Canada's Procedure for the Development and Management of Food

Microbiological Methods. Oral presentation. IUFoST 17th World Congress of Food Science and Technology, Montreal, Canada

2013 Y. Tejeda Saldana, M. J. Rieder. Development of a Point of Care (POC) Platform for Detecting *Escherichia coli* O157:H7 in Food and Water. Poster presentation. Annual Pathology Research Day. London, ON, Canada

2012 Y. Tejeda Saldana, M. J. Rieder. Development of a Rapid Point of Care (POC) Platform for Detecting *Escherichia coli* O157:H7 in Food and Water. Poster presentation, 7th Annual Infection and Immunity Research Forum. London, ON, Canada

2012 Y. Tejeda Saldana, M. J. Rieder, J. R. Bend. Beyond *E. coli*: A Novel Approach for Detecting non-O157 *E. coli* in Food and Water. Poster presentation, Annual Pathology Research Day. London, ON, Canada