DEVELOPMENT OF NUCLEIC ACID BASED LATERAL FLOW IMMUNOCHROMATOGRAPHIC TEST PLATFORM FOR *SALMONELLA* DETECTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ONUR BULUT

IN PARTIAL FULLFILLMENT OF THE REQUIRMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

SEPTEMBER 2014

Approval of the thesis:

DEVELOPMENT OF NUCLEIC ACID BASED LATERAL FLOW IMMUNOCHROMATOGRAPHIC TEST PLATFORM FOR *SALMONELLA* DETECTION

submitted by **ONUR BULUT** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by**,**

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan Adalı _____
Head of Department, **Biology**

Prof. Dr. Hüseyin Avni Öktem _____
Supervisor, **Biology Department, METU**

Examining Committee Members:

Prof. Dr. Meral Yücel _____
Biology Department, METU

Prof. Dr. Hüseyin Avni Öktem _____
Biology Department, METU

Prof. Dr. Füsun İnci Eyidoğan _____
Education Faculty, Başkent University

Assoc. Dr. Remziye Yılmaz _____
Molecular Biology and Biotechnology R&D Center, METU

Dr. Fuat Yiğit Aksoy _____
NANObiz R&D Ltd.

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Onur Bulut

Signature :

ABSTRACT

DEVELOPMENT OF NUCLEIC ACID BASED LATERAL FLOW IMMUNOCHROMATOGRAPHIC TEST PLATFORM FOR *SALMONELLA* DETECTION

BULUT, Onur
M.S., Department of Biology
Supervisor: Prof. Dr. Hüseyin Avni ÖKTEM

September 2014, 77 pages

Foodborne diseases have been a crucial problem for the public health. Various agents transmitted by food cause these diseases. However, *Salmonella* accounts for most of the cases leading to most of the hospitalization and even death. Therefore, rapid detection of *Salmonella* is a considerable step in order to improve food safety and minimize outbreaks.

Nucleic acid based biosensors are fast, simple, economic, easy-to-use and do not require trained personnel or high-cost equipments when compared to the current detection methods such as culture and molecular methods. In this study, a nucleic acid based biosensor called lateral flow immunochromatographic assay was developed as a rapid, sensitive, easy-to-use and economic detection system for *Salmonella*.

Accordingly, a synthetic oligonucleotide was designed from *invA* gene which is found in most *Salmonella* serotypes. The synthetic oligonucleotide was used as the target in order to establish assay procedure and optimize the parameters including time, concentration and temperature. After optimization studies, real targets which were two different PCR products were applied to the assay as targets.

The real target samples were 105 bp and 284 bp amplicons of *invA* gene. In order to form signal probes, AuNPs were functionalized with thiol-modified ssDNAs which were complementary to one part of the target. Biotinylated ssDNAs which were complementary to other part of the target were immobilized onto nitrocellulose membrane to create the capture probe on the test line.

When the target was applied to assay, it firstly hybridized with signal probe and then migrated among the test strip. In the test zone, it was capture by the immobilized probe by forming sandwich-type hybridization which resulted in formation of naked eye detectable dark-red colored band.

Lateral flow immunochromatographic assay developed in this study achieved detection of different *Salmonella* specific DNA sequences and it is a promising device for rapid, accurate, easy-to-use and low-cost detection systems.

Keywords: Foodborne Diseases, Pathogen Detection, *Salmonella*, Biosensor, Lateral Flow Assay, Sandwich Hybridization, Gold Nanoparticles (AuNPs)

ÖZ

SALMONELLA TANISI İÇİN NÜKLEİK ASİT TABANLI YATAY AKIŞ İMMÜNOKROMATOGRAFİK TEST PLATFORMUNUN GELİŞTİRİLMESİ

BULUT, Onur

Yüksek Lisans, Biyoloji Bölümü

Tez Yöneticisi: Prof. Dr. Hüseyin Avni ÖKTEM

Eylül 2014, 77 sayfa

Gıda ile bulaşan hastalıklar kamu sağlığı için ciddi bir sorun oluşturmaktadır. Gıda ile bulaşan çeşitli ajanlar bu tür hastalıklara sebep olmaktadır. Fakat, *Salmonella* uzun süreli tedaviye hatta ölüme yol açan vakaların çoğundan sorumludur. Bundan dolayı, *Salmonella*'nın hızlı ve erken teşhisi gıda güvenliğini artırma ve salgınları asgariye indirmede oldukça önemli bir adımdır.

Kültür metotları veya moleküler metotlar gibi günümüzde kullanılan metotlarla kıyaslandığında, nükleik asit tabanlı biyosensörler hızlı, basit, ekonomik ve kullanımı kolay sistemler olarak göze çarpmaktadır ve yüksek maliyetli ekipman veya eğitimli personel gerektirmemektedir. Bu çalışmada, *Salmonella* tanısı için hızlı, hassas, kullanımı kolay ve ekonomik bir tanı sistemi olarak yatay akış immünokromatografik testi denilen nükleik asit tabanlı biyosensör geliştirilmiştir.

Bu sebeple, çoğu *Salmonella* serotipinde bulunan *invA* geninden sentetik bir oligonükleotid dizayn edilmiştir. Bu sentetik oligonükleotid, test prosedürünü kurmak ve zaman, konsantrasyon, sıcaklık gibi parametreleri optimize etmek için hedef molekül olarak kullanılmıştır. Optimizasyon çalışmalarından sonra, iki farklı PZR ürünü olan gerçek örnekler hedef molekül olarak teste uygulanmıştır.

Gerçek hedef moleküller olarak, *invA* geninden PZR ile çoğaltılan 105 ve 284 baz çifti uzunluğundaki DNA'lar kullanılmıştır. Sinyal problarını oluşturmak için, hedef molekülün bir kısmına tamamlayıcı özellikteki tiyol-modifiye edilmiş DNA, altın nanoparçacıklar ile fonksiyonize edilmiştir. Hedef molekülün diğer kısmına tamamlayıcı özellikteki biyotinlenmiş DNA ise yüzey probunu oluşturmak için nitroselüloz membran üzerine sabitlenmiştir.

Hedef molekül teste uygulandığında, ilk olarak sinyal probu ile hibridize olmuştur ve sonra test çubuğu boyunca hareket etmiştir. Sinyal probu test bölgesinde sandviç tipi hibridizasyon oluşturarak sabitlenmiş yüzey probu probu tarafından tutulmuştur. Bu reaksiyon sonucu çıplak gözle tespit edilebilen koyu kırmızı renkli bir bant oluşmuştur.

Bu çalışmada geliştirilen yatay akış testi Salmonella'ya özgü farklı DNA sekanslarının tanısını gerçekleştirmiştir ve hızlı, doğru, kullanımı kolay ve düşük maliyetli tanı sistemleri için umut verici bir cihazdır.

Anahtar Kelimeler: Gıda ile Bulaşan Hastalıklar, Patojen Tanısı, *Salmonella*, Biyosensör, Yatay Akış Testi, Sandviç-tipi Hibridizasyon, Altın Nanoparçacıklar

To My Family...

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Hüseyin Avni ÖKTEM for his endless support and encouragement, valuable guidance and inspiration and mentoring throughout this study.

I wish to express my thanks to Prof. Dr. Meral Yücel for her encouragement and valuable support. I have always felt lucky and been proud of being a member of YÜCEL&ÖKTEM Lab.

I am also thankful to members of the examining committee, Prof. Dr. Füsun İnci Eyidoğan, Assoc. Dr. Remziye Yılmaz and Dr. Fuat Yiğit Aksoy for their participation, criticism and comments during my thesis defense.

I am greatly indebted to Oya Ercan Akça for teaching me so many valuable things and answering all of my questions with patience. Without her devotion, endless support and helps during each step of this study, I would not have been able to finish this study on time.

I am grateful to Dilek Çam, Dr. Çağla Sönmez and Ceren Bayraç for their both technical and moral supports, comments and helps.

My lab mates Buse İşbilir, Dilan Akın, Evrim Aksu, Seren Baygün and Sinem Demirkaya have always been extremely helpful and supportive. I would like to thank them all one by one for their friendships.

My special thanks and gratitude go to my parents Kayhan & Ayfer Bulut and my brother Okan Bulut for their constant support and love throughout my life. Without them, this thesis would not have existed.

This work was supported by METU, BAP-07-02-2014-007-026 project and partially supported by TEYDEB 1120192 project granted to Nanobiz R&D Limited.

TABLE OF CONTENTS

ABSTRACT v

ÖZ vii

ACKNOWLEDGEMENTS x

TABLE OF CONTENTS xi

LIST OF TABLES xv

LIST OF FIGURES xvi

LIST OF ABBREVIATIONS xviii

CHAPTERS 1

1. INTRODUCTION 1

1.1. Foodborne Diseases 1

1.2. Foodborne Pathogens 3

1.2.1. *Salmonella* 6

1.2.1.1. *invA* gene 10

1.3. Detection Methods 11

1.3.1. Conventional Culture Methods 11

1.3.2. Molecular Methods 12

1.3.2.1. Enzyme-Linked Immunosorbent Assay (ELISA) 12

1.3.2.2. Fluorescent *In Situ* Hybridization (FISH) 14

1.3.2.3. Polymerase Chain Reaction (PCR) 15

1.3.2.3.1. Conventional PCR 15

1.3.2.3.2. Real-Time PCR 16

1.3.3. Biosensors 18

1.3.3.1. Lateral Flow Immunochromatographic Assay (LFIA) 20
1.3.3.1.2. Labels for Lateral Flow Immunochromatographic Assay 23
1.4. Aim of the Study 23
2. MATERIALS & METHODS 25
2.1. Materials 25
2.1.1. Chemicals 25
2.1.2. Buffers and Solutions 25
2.1.3. Test Strip Components 25
2.1.4. Synthetic Oligonucleotides 25
2.1.5. PCR Primers and pUC19 Plasmid 27
2.1.6. Bacterial Strains 27
2.1.7. Gold Nanoparticles 27
2.2. Methods 28
2.2.1. Target Preparation 28
2.2.1.1. Genomic DNA Isolation from *Salmonella* and *E.coli* Cells 28
2.2.1.2. Quantification of Genomic DNA Concentration 28
2.2.1.3. Polymerase Chain Reaction (PCR) 28
2.2.1.3.1. Primer Selection and Design 28
2.2.1.3.2. PCR Conditions for 284 bp Product 29
2.2.1.3.3. PCR Conditions for 105 bp Amplicon 30
2.2.1.3.4. PCR Conditions for MCS 31
2.2.3.4. Agarose Gel Electrophoresis of PCR Products 32
2.2.2. Synthesis and Characterization of Gold Nanoparticles 32
2.2.3. Functionalization of AuNPs with Thiolated DNA Probes 33
2.2.4. Preparation of Test Strip Components 33
2.2.4.1. Sample Pad 33
2.2.4.2. Conjugate Pad 33

2.2.4.3. Nitrocellulose Membrane 34
2.2.3.4. Absorbent Pad 34
2.2.5. Assembly of Lateral Flow Immunochromatographic Test Strip 34
2.2.6. Assay Procedure 34
2.2.6.1. Assay Procedure for the Synthetic Target 34
2.2.6.2. Assay Procedure for PCR Products 35
3. RESULTS & DISCUSSION 37
3.1. Target Preparation 37
3.1.1. Bacterial Growth 37
3.1.2. Isolation and Quantification of Genomic DNAs 38
3.1.3. Agarose Gel Electrophoresis of PCR Amplicons 41
3.2. Synthesis and Characterization of Gold Nanoparticles 44
3.2.1. UV-Visible Spectroscopy of AuNPs 44
3.2.2. Dynamic Light Scattering Analysis of AuNPs 45
3.2.3. Zeta Potential of AuNPs 46
3.3. Functionalization of AuNPs with Thiolated DNA probes 47
3.3.1. UV-Visible Spectroscopy of Signal Probes 48
3.3.2. Dynamic Light Scattering Analyses of Signal Probes 49
3.3.3. Zeta Potentials of Signal Probes 51
3.4. Fabrication of Lateral Flow Test Strips 52
3.5. Assay Results 53
3.5.1. Assay Results of Synthetic Target 54
3.5.2. Assay Results of 105 bp amplicon 55
3.5.3. Assay Results of 284 bp amplicon 60
4. CONCLUSION 65
REFERENCES 67
APPENDICES 73

A. BUFFERS AND SOLUTIONS 73

B. SEQUENCES OF PCR PRIMERS 75

C. MAP OF pUC19 PLASMID DNA 77

LIST OF TABLES

TABLES

Table 1.1 Number of culture-confirmed cases and estimated actual cases in US 2
Table 1.2 Some pathogens involved in foodborne diseases 4
Table 1.3 List of some diseases related to foods 5
Table 1.4 Foodborne infections and intoxications in Turkey in 1999-2000 9
Table 1.5 Detection of Salmonella using real-time PCR assay 17

Table 2.1 Sequences and modifications of synthetic probes 26
Table 2.2 Optimized PCR conditions for 284 bp amplicon 29
Table 2.3 PCR cycling conditions for 284 bp amplicon 29
Table 2.4 Optimized PCR conditions for 105 bp amplicon 30
Table 2.5 PCR cycling conditions for 105 bp amplicon 30
Table 2.6 Optimized PCR conditions for MCS 31
Table 2.7 PCR cycling conditions for 284 bp amplicon 31

Table 3.1 Starting concentrations of cultures 37
Table 3.2 Concentrations and purity of genomic DNA samples 38

Table B.2 Sequences and locations of primers for 284 bp amplicon 75
Table B.3 Sequences and locations of primers for 105 bp amplicon 75
Table B.4 Sequences and locations of primers for MCS of pUC19 plasmid 75

LIST OF FIGURES

FIGURES

Figure 1.1 Forms of foodborne diseases 3
Figure 1.2 Scanning electron microscope image of Salmonella typhimurium 6
Figure 1.3 *Salmonella* typhi systemic infection 7
Figure 1.4 Virulence factors in *Salmonella* typhimurium and *Salmonella* typhi 8
Figure 1.5 Flow diagram for *Salmonella* detection 11
Figure 1.6 Schematic representations of 3 main types of ELISA 13
Figure 1.7 Flow chart of FISH procedure 14
Figure 1.8 Stages of polymerase chain reaction 16
Figure 1.9 Comparison of culture based method and real-time PCR method 18
Figure 1.10 Schematic representation of a typical biosensor 19
Figure 1.11 Classification of biosensors 20
Figure 1.12 Representation of a typical LFIA 21
Figure 1.13 Schematic representation of a nucleic acid based LFIA 22

Figure 3.1 Single colonies on agar plates. 38
Figure 3.2 Spectrum result of *S.* infantis genomic DNA sample 39
Figure 3.3 Spectrum result of *S.* enteritidis genomic DNA sample 40
Figure 3.4 Spectrum result of *S.* enteritidis genomic DNA sample 40
Figure 3.5 Spectrum result of *E. coli* DH5α genomic DNA sample 41
Figure 3.6 1.2 % agarose gel electrophoresis. 42
Figure 3.7 2.5 % agarose gel electrophoresis. 43
Figure 3.8 2.5 % agarose gel electrophoresis of MCS with different dilutions 44
Figure 3.9 Absorption speactrum and visual appearance of AuNPs 45
Figure 3.10 DLS histogram of the synthesized AuNPs 46
Figure 3.11 Zeta potential distribution of 25 nm AuNPs at neutral pH 46
Figure 3.12 Signal probe 1 and signal probe 2, respectively 48
Figure 3.13 Comparison of absorption spectra AuNPs, SP1 and SP2 49

Figure 3.14 DLS histogram of signal probe 1 50
Figure 3.15 DLS histogram of signal probe 2 50
Figure 3.16 Zeta potential distribution of signal probe 1 51
Figure 3.17 Zeta potential distribution of signal probe 2 51
Figure 3.18 Components of lateral flow immunochromatographic test strip 52
Figure 3.19 Illustration of detection of nucleic acids by sandwich hybridization 54
Figure 3.20 Assay results of synthetic target. 55
Figure 3.21 Assay result of 105 bp amplicon of *S.* infantis. 56
Figure 3.22 Results of the assay, before washing. 57
Figure 3.23 Results of the assay, after washing 58
Figure 3.24 Results of 284 bp amplicon application to the assay. 59
Figure 3.25 Results of the assay, before washing. 61
Figure 3.26 Results of assay, after washing 62
Figure 3.27 Results of 105 bp amplicon target application to the assay 63

Figure C.1 pUC19 plasmid DNA map 77

LIST OF ABBREVIATIONS

AuNP	Gold Nanoparticle
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dsDNA	Double-stranded Deoxyribonucleic Acid
LB	Luria-Bertani
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SSC	Saline-Sodium Citrate
ssDNA	Single-stranded Deoxyribonucleic Acid
TCEP	Tris(2-Carboxyethyl)phosphine
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

CHAPTER 1

INTRODUCTION

1.1. Foodborne Diseases

Foodborne diseases can be defined as diseases caused by consumption of contaminated food and food products. Major causes of contamination are typically microbial pathogens such as bacterial, viral, fungal and parasitic agents which might occur at the different steps of food production and preparation processes (Tauxe *et al*., 2010). Foodborne diseases most often manifest themselves through gastrointestinal system. Diarrhea caused by contaminated food is responsible for the deaths of two million children in the developing countries (Pigot, 2008). However, they can also lead to severe and long-term sequelaes such as respiratory, hepatic, immunological, neurological disorders, cancer and in some cases they can be lethal. The general symptoms of foodborne diseases comprise diarrhea, bloody diarrhea, abdominal pains, vomiting, nausea, headache and fever. As many factors play critical role in severity of illness, the most significant factor is susceptibility to foodborne disease. Especially infants, children, pregnant women, elderly people and people with immunodeficiency are in higher risk (WHO, 2007).

Foodborne disease causing agents are classified in different manners. Commonly, they are classified according to taxonomy and mechanism of action. In addition to this, there are some other classifications such as according to the source of the agent and clinical symptoms of the disease.

Although chemical, physical and biological agents transmitted by foods cause the foodborne diseases, primary and the most important cause is biological agents. In addition, these agents can be transmitted not only by food or water but also other ways including the fecal-oral route and the respiratory route (Kass PH and Riemann HP, 2006).

33 % of the world is affected by foodborne pathogens each year. According to the study of the Centers for Disease Control and Prevention (CDC) in 2011, each year foodborne diseases result in nearly 48 million illnesses, 320000 hospitalizations, and 3000 deaths in the United States. However, only a small portion of foodborne diseases are diagnosed and reported. Therefore, it is estimated that actual number of foodborne disease cases is much higher. 9.4 million illnesses, 56000 hospitalizations, and 1400 deaths are caused by 31 identified foodborne pathogens. Eight of those 31 identified pathogens accounts for 91% of illnesses, 88% of hospitalizations and 88% of deaths (Table 1.1) (Scallan *et al*., 2011).

Table 1.1 Number of culture-confirmed cases and estimated actual cases in the United States (* Data not available) (Branden and Tauxe, 2013)

Pathogen	Culture-confirmed Cases per 100 000 Population	Estimated Actual Cases per 100 000 Population	Percent Foodborne (%)
Norovirus	n/a*	7000	26
Campylobacter spp	14.3	442	80
Salmonella	16.7	411	94
Cryptosporidium spp	2.8	408	8
Clostridium perfringens	n/a*	324	100
Shigella	3.2	165	31
STEC	2.1	88.7	77[a]
Staphylococcus aureus (foodborne)	n/a*	80.9	100
Yersinia enterocolitica	0.3	39	90
Toxoplasma gondii	n/a*	58	50
Vibrio	0.3	27	74[a]

1.2. Foodborne Pathogens

Foodborne pathogens lead to many severe illnesses resulting from food intoxication, toxicoinfection or food infection (Figure 1.1). In food intoxication, toxins of pathogen are preformed during growth of the pathogen in the food and ingestion of these toxins cause the foodborne disease. On the other hand, toxicoinfection occurs when pathogen contaminated food is ingested and accordingly toxins are produced in the host. In addition, the last type of foodborne disease is the food infection which is induced by ingestion of infective and live pathogen (Singh *et al.*, 2012).

Food intoxication is generally caused by toxins produced by *Bacillus cereus*, *Staphylcoccus aureus* and *Clostridium botulinum*. Toxicoinfection results from toxins of *Clostridium perfringers*, enterotoxigenic *Escherichia coli* and *Vibrio cholerae.* Primary reason of the foodborne infection is viruses, parasites and bacteria including *Salmonella enterica, Escherichia coli* O157:H7, *Compyloacter jejuni*, *Listeria monocytogenes* and *Yersinia enterocolitica* (Bhunia, 2008).

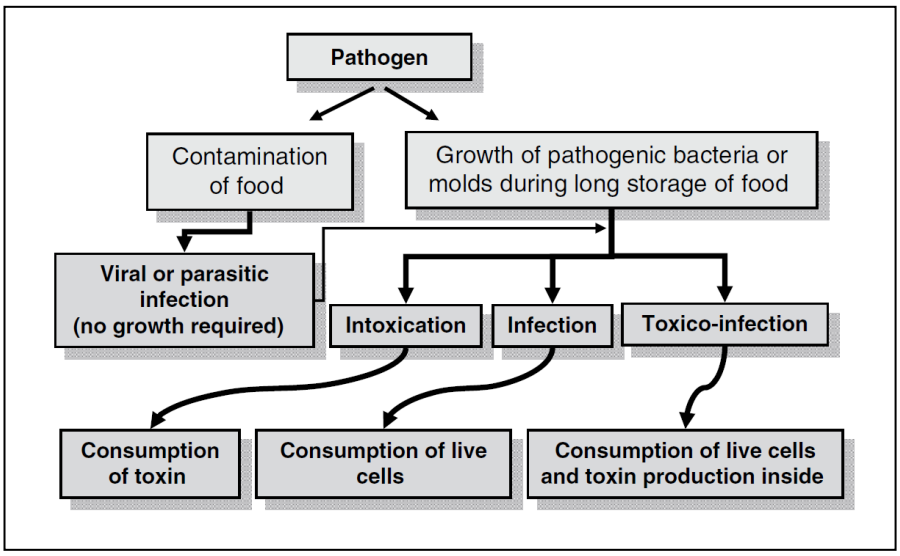

Figure 1.1 Forms of foodborne diseases (Bhunia, 2008)

Pathogens involved in food contamination are classed into bacterial, viral, fungal, protozoan and parasitic agents (Table 1.2). However among those, eight pathogens which are Norovirus, *Campylobacter* spp, *Salmonella*, *Cryptosporidium* spp, *Clostridium perfringens*, *Shigella*, Shiga toxin producing *Escherichia coli*, and

Staphylcoccus aureus are responsible for the most of foodborne diseases (Branden and Tauxe, 2013).

Table 1.2 Some pathogens involved in foodborne diseases (Labbe and Garcia, 2013)

Gram Negative	**Gram Positive**	**Virus**	**Parasite**	**Fungus**
Salmonella	*Staphylococcus*	Norovirus	Giardia	*Aspergillus*
Escherichia	*Clostridium*	Rotavirus	Entamoeba	*Penicillium*
Yersinia	*Listeria*	Hepatitis A	Toxoplasma	*Fusarium*
Shigella	*Bacillus*	Hepatitis E	Cryptosporidium	*Claviceps purpura*
Campylobacter	*Enterobacter*	Astrovirus	Taenia	
Yersinia			Ascaris	
Vibrio			Trichinella	
Aeromonas				
Arcobacter				

Most of foodborne pathogens are naturally found in soil, water, plants and animals. Pathogens get involved in different types of raw materials and subsequently contaminate various types of foods. Foodborne diseases are also classified according to role of foods, and can be two types. In the first group, food only hosts the pathogen and does not provide growth and reproduction of the pathogen. But in the second group, food does not only host the pathogen but also provide a medium for the pathogen (Singh *et al.*, 2012). Therefore, attribution of foodborne diseases to specific foods might be useful in terms of food safety and food surveillance. However, this process is challenging due to fact that most of foods are internationally traded and pathogens are transmitted in different steps of production, processing and supply chains.

Table 1.3 List of some diseases related to foods (McEntire, 2013)

Illness	Notes
Botulism	Foodborne disease is associated with unprocessed or underprocessed canned or jarred low acid foods
Brucellosis	Associated with consumption of unpasteurized milk or cheese, and wild game meat; virtually eradiated in animals in the United States but still problematic internationally
Cryptosporiasis	Generally caused by exposure to contaminated water; produce treated with contaminated water can transmit the parasite
Giardiasis	Generally caused by exposure to contaminated water; produce treated with contaminated water can transmit the parasite
Hepatitis (A)	Infected food handlers with poor personal hygiene can contaminate food
Listeriosis	Associated with ready-to-eat foods, and unpasteurized milk and cheese
Salmonellosis	Historically associated with poultry products, but increasingly associated with a diversity of products
Shiga toxin–producing *Escherichia coli*	Historically associated with ground beef products, but increasingly associated with a diversity of products, including produce
Shigellosis	Mostly associated with person-to-person contact, but food handlers with poor personal hygiene can contaminate food
Trichinellosis	Associated with pork products; generally controlled in the United States but still problematic internationally
Typhoid fever	Most cases are associated with international travel; the most recent US outbreak was associated with imported frozen fruit
Vibriosis	Associated with seafood, particularly shellfish harvested from contaminated water

Increasing human population has increased need and consumption of many types of foods including fruits, vegetables and animal products like meat, egg, dairy products, poultry and sea foods (Broglia and Kapel, 2011). Based on the analysis of 1565 foodborne disease outbreaks by the CDC, poultry, leafy greens, dairy products and meat were responsible for most of the outbreaks.

In 2011, *Salmonella* infections also called salmonellosis accounted for most of the infections (16.4 cases per 100000). Primary reason of *Salmonella* infections is consumption of food and water contaminated with animal feces. Poultry and poultry products including shell eggs were the source of *Salmonella* in most of the cases (CDC, 2013).

1.2.1. *Salmonella*

Salmonella is gram negative, facultative aerobic, and non-spore forming genus of Enterobacteriaceae family, living as commensals and pathogens by causing a number of diseases in humans and animals (Mandell *et al*., 2010). According to completely sequenced fifteen *Salmonella* serotype genome, their genomes are composed of approximately 4.8-4.9 million base pairs, which include 4400-5600 coding sequence. In the genomes, pseudo genes are also found, for example in *S.* typhi, there are more than 200 genes which are functionally inactive or disrupted (Jong *et al*., 2012). Except *S.* gallinarum – *S.* pullorum, they are motile by their peritrichous flagella.

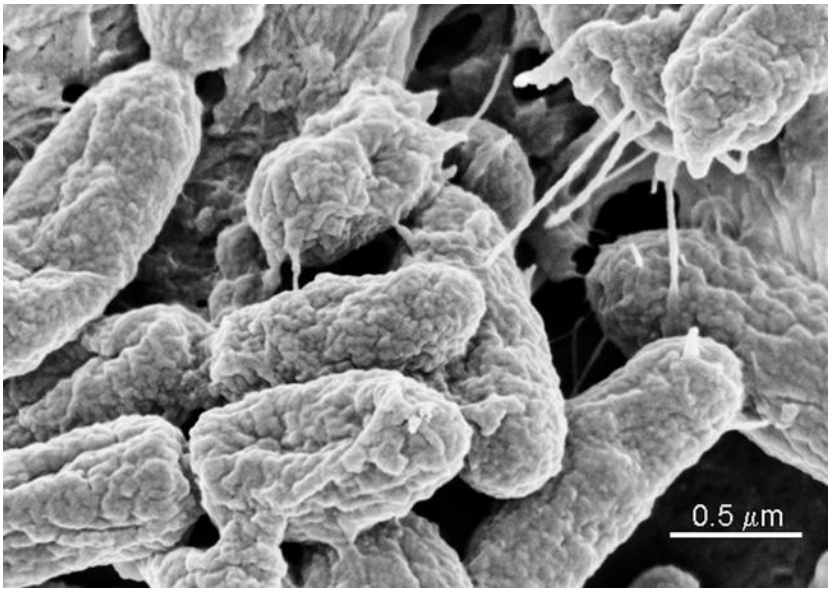

Figure 1.2 Scanning electron microscope image of *Salmonella* typhimurium (Sanchez-Vargas et al., 2011)

The *Salmonella* genus is divided into two main species according to DNA sequence similarity: *Salmonella enterica* and *Salmonella bongori*. While *S. enterica* have 6 identified subspecies which are enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI), *S. bongori* doesn't have any subspecies. (Sa´nchez-Vargas *et al*., 2011). *Salmonella enterica* subspecies are also classified according to antigens that they belong; according to O antigen, which is a somatic antigen, there can be more than 50 serotypes can be identified, on the other hand,

according to H antigen, which is a flagellar antigen, more than 2.500 serotypes of it can be classified (Miliotis, 2003).

According to the syndromes that they create during human infections, *Salmonella* spp. is divided into two main types as typhoid *Salmonella* and non-typhoid *Salmonella* (NTS). Typhoid *Salmonella*, including *S. enterica* subspecies enteric, serotypes Typhi and Paratyphi, cause enteric fever. Remaining strains are in the NTS group, which cause gastrointestinal diseases. *S. enterica* serotypes, *S.* typhi and *S.* paratyphi, infects only human hosts and cause typhoid and paratyphoid fever respectively (Mastroeni and Grant, 2011).

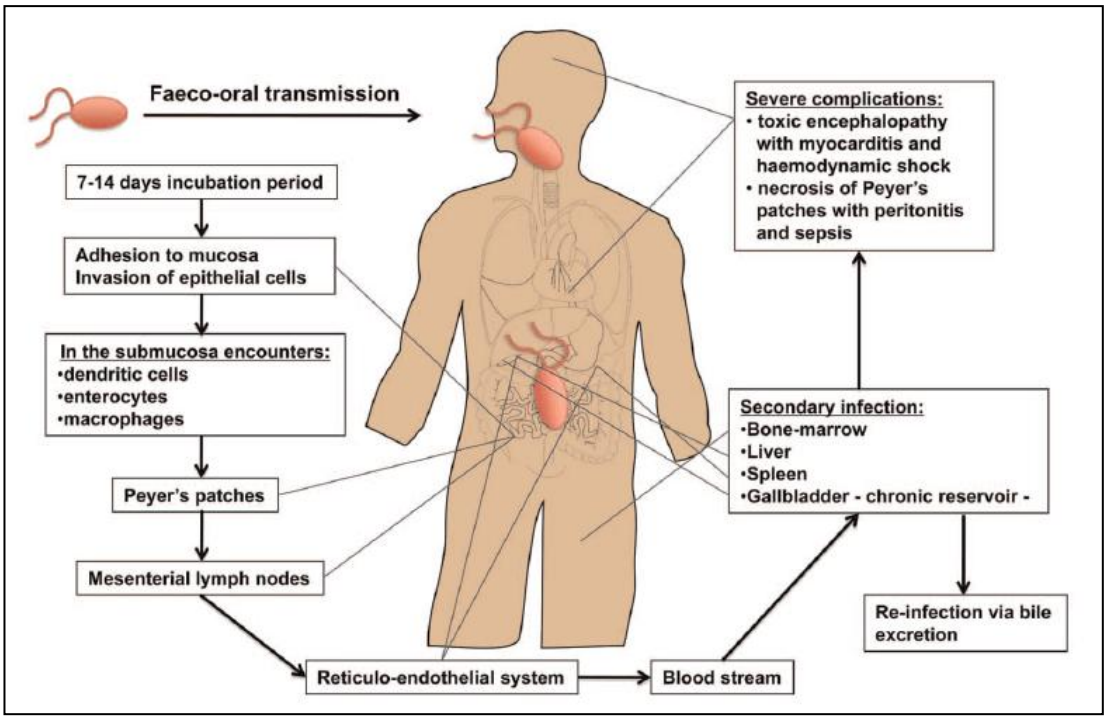

Figure 1.3 *Salmonella* typhi systemic infection (Jong *et al*., 2012)

S. typhi and *S.* paratyphi have very similar genomes with 90 % similarity rate. 10 % of their genome is responsible from their pathogenicity so those genomic parts are called as *Salmonella* Pathogenicity Island (SPI). According to SPIs, different virulence factors such as Type III secretion system, flagella, fimbriae and polysaccharides, are produced in different serotypes (Jong *et al*., 2012).

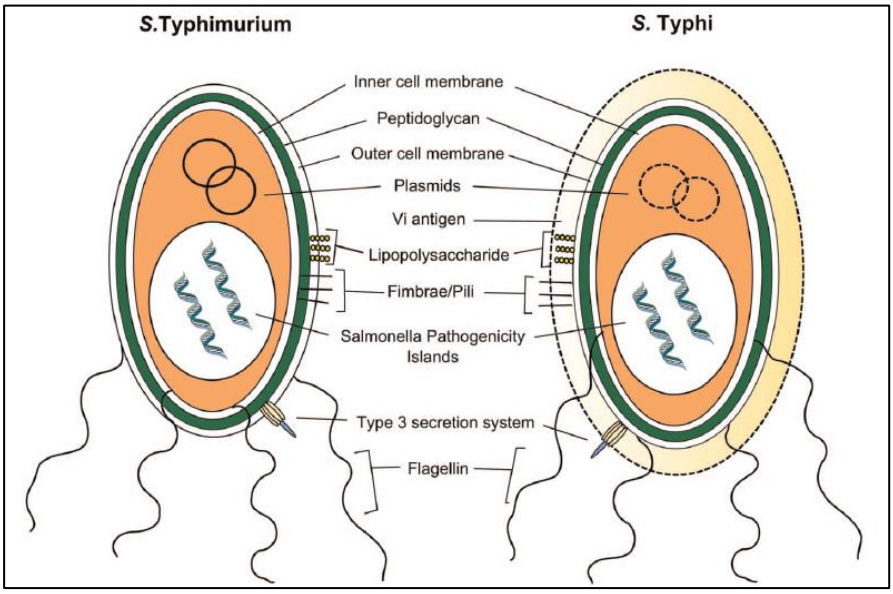

Figure 1.4 Virulence factors in *Salmonella* typhimurium and *Salmonella* typhi (Jong et al., 2012)

S. enterica is a foodborne pathogen, so its invasion occurs in gastrointestinal tract**.** In gastrointestinal tract, around distal ileum and caecum, by using type III secretion system, bacteria invade epithelial cells and M cells. Type III secretion system, by which effector proteins of *Salmonella* can be transferred to membranes of host cells, carries crucial importance for invasion of cells. Highly replicative and invasive bacteria become very much in number in those cells and when cells move through epithelial monolayer, all bacteria is released which will result in gut invasion later on (Mastroeni and Grant, 2011).

Other serotypes of *S.* enterica; *S.* Typhimurium and *S.* Enteritidis, which are in typhoid *Salmonella* group, can infect wide host range human to animals, by causing gastrointestinal diseases.

S. enterica can be transmitted via contaminated food or water, contact with contaminated animals and with contaminated environment, such as by fecal matter. The level of the disease after infection depends on both the serotype of *Salmonella* and the host species. Epidemiology of *Salmonella* also highly depends on the species that they infect. The typhoid *Salmonella* group, *S.* typhi and *S.* paratyphi, have severe and lethal symptoms on hosts by causing enteric fever. On the other

hand, non-typhoid *Salmonella* infections without any lethal results, is seen worldwide (Sa´nchez-Vargas *et al*., 2011).

Table 1.4 Foodborne infections and intoxications in Turkey in 1999-2000 (WHO)

Foodborne infections and intoxications notified

TURKEY 1999-2000

Disease	1999		2000	
	No. of cases	Incidence rate	No. of cases	Incidence rate
Salmonellosis	28884	43.9	26498	39.2
Staphylococcosis	0	0.0	0	0.0
Botulism	96	0.1	18	0.0
Campylobacteriosis	0	0.0	0	0.0
Shigellosis	1120	1.7	1093	1.6
E. coli enteritis	0	0.0	0	0.0
Listeriosis	0	0.0	0	0.0
Cholera	0	0.0	0	0.0
Brucellosis	11462	17.4	10742	15.9
Other bacterial foodborne infections and intoxications	5146	7.8	4672	6.9
Hepatitis A	14323	21.8	10435	15.4
Other viral enteritis	0	0.0	0	0.0
Echinococcosis	0	0.0	0	0.0
Trichinellosis	0	0.0	0	0.0
Giardiasis	0	0.0	0	0.0
Amoebiasis	22980	34.9	23723	35.1
Mushroom poisoning	329	0.5	334	0.5
Infectious Enteritis of unknown origin	0	0.0	0	0.0
Total	84340	128.1	77515	114.6

According to WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe 8th Report 1999-2000, among foodborne infections and intoxications, *Salmonella* infections come first in statistics, in Turkey.

Because of incidence and severeness of *Salmonella* infections, detection and identification of the pathogen are key factors in minimization, prevention and treatment of foodborne outbreaks.

1.2.1.1. *invA* gene

Salmonella spp. does the pathogenic cycle by invading the cells of intestinal epithelium. *Inv* gene locus was identified previously and it was understood that it lets *Salmonella* spp. to enter epithelial cells. Intestinal mucosa invasion is accredited as common property of all pathogenic *Salmonella* (Galan and Curtiss, 1991). However, i*nv* genes are found in most but not all *Salmonella* serotypes. There are four types of *inv* locus namely; *invA, invB, invC* and *invD*. Alignment of *invA, invB* and *invC* genes are on the same transcriptional unit, however, *invD* gene is settled on a different transcriptional unit. It was reported that *invA* is the first gene on the *invABC* operon. Location of this gene was determined on *Salmonella* genome and role of the *invA* gene on the invasion mechanism was understood by doing some mutations on this gene. Insertions in *invA* gene remove the invasive property of organism (Galan *et al*., 1992).

InvA is found in the inner membrane part of *Salmonella* type III secretion system (T3SS) and its role is organizing exportation of virulence proteins termed as "effectors" which are utilized by many Gram-negative pathogenic bacteria (Worrall *et al*., 2010). These effectors react with host cell proteins and leads to pathogenesis. That is, T3SS is used for injection of virulence inducing proteins into cells. By this way, *Salmonella* can make systemic infection and gastroenteritis (Lostroh and Lee, 2001).

invA gene is found in most of *Salmonella* serotypes and responsible for their pathogenicity. Therefore, *invA* is considered as a *Salmonella* specific gene and can be used for specific detection of *Salmonella* spp. In 1992, Rhan *et al*. developed a set of PCR primers for amplification of nucleotide sequences within the *invA* gene and evaluated the PCR product as a means of detection. With slight exceptions, most of *Salmonella* serotypes yielded the specific PCR product while any of tested non-*Salmonella* did not result in a product. Therefore, this unique region of *invA* gene is usually preferred and used for specific detection of *Salmonella*.

1.3. Detection Methods

1.3.1. Conventional Culture Methods

Conventional culture methods for detection of pathogens in food are based on incorporation of the different types of samples such as food, blood and feces into a specific nutrient medium in which pathogens reproduce, thus allowing enumeration and isolation of viable cells. These conventional test methods are generally sensitive, inexpensive and give quantitative and qualitative information about the sample. The conventional culture analysis of pathogens involves four main steps: (i) pre-enrichment in a non-selective medium, (ii) selective enrichment, (iii) isolation on selective agar plates and (iv) identification of the pathogen by biochemical testing and serological confirmation (Mandal *et al.*, 2011).

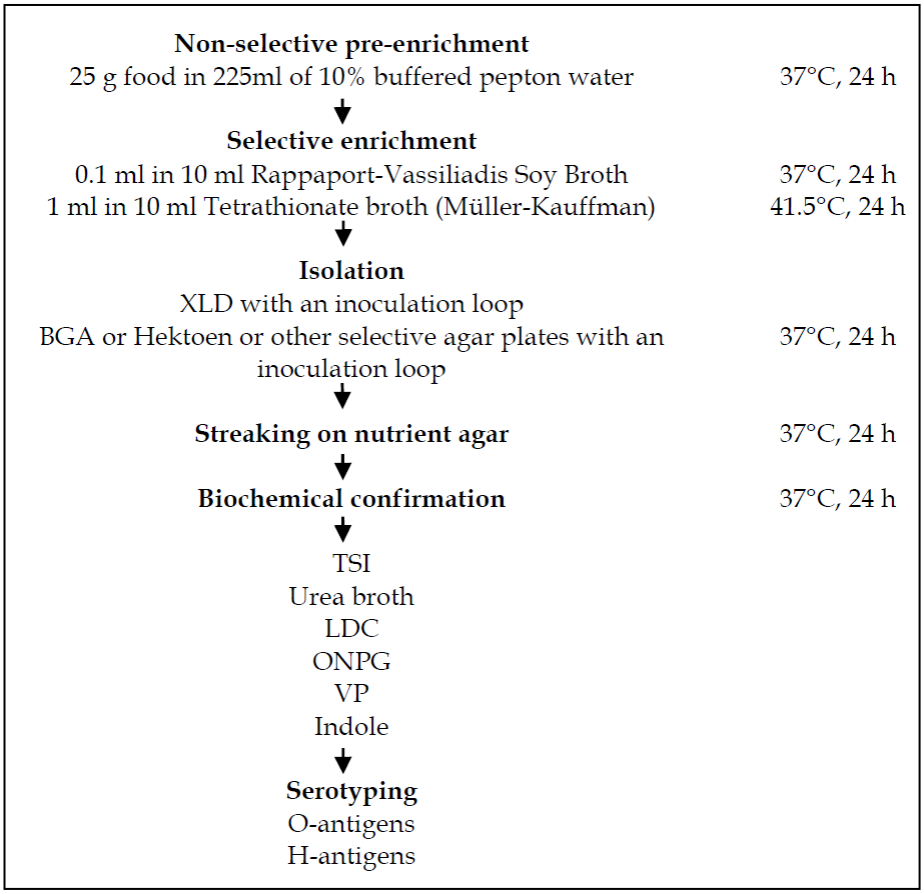

Figure 1.5 Flow diagram for *Salmonella* detection (Zadernowska and Chajecka, 2011)

Pre-enrichment in a non-selective media is applied to recover sublethal *Salmonella* in processed samples such as food and feed. However, non-selective media also induce growth of competing microorganisms which might cause to disguise the presence of *Salmonella*. In selective medium, it is aimed to inhibit the growth of other microorganisms and increase levels of *Salmonella* among others. In the third step, selective enrichment broths are streak onto selective agar plates in order to obtain single colonies of isolated organism. Although other microorganism are tried to be inhibited by addition of some chemicals, atypical colonies including *Salmonella*-like false positives might occur and interfere the further analyses. In the final step of culture method, some biochemical confirmation tests are done and following colonies are serologically verified by determining antigenic compositions. Determination of antigens (flagellar (H) or somatic (O)) is carried out by tube agglutination test.

Despite the conventional culture methods are considered as the golden standard for pathogen detection, the whole procedure takes 3-7 days. Thereby, they are time-consuming and labor-intensive. So there has been extensive research for alternative methods to overcome these issues (Löfström *et al.*, 2010).

1.3.2. Molecular Methods

Because of a significant increase in the foodborne disease incidence, there has been a rising demand for development and improvement of detection methods. These methods should be rapid, accurate, simple, low-cost and sensitive. However, the conventional culture methods are not good enough to meet these demands. Thus, rapid methods which give quicker results than the conventional culture methods have been developed for detection of macromolecules of pathogens including DNA, RNA, and proteins (Foley and Grant, 2007).

1.3.2.1. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an immunological method that is based on specific binding reaction of antibodies and antigens. For detection of foodborne pathogens, generally used ELISA is the sandwich type which is composed of different steps. Antigen specific antibodies are immobilized to surface of wells of a 96-well plate. Then the target analyte is applied to the plate and allowed to bind to the immobilized antibodies.

After the washing step for removal of unbound antigens, enzyme-labeled secondary antibodies are added and bound to the target. In the final step, a chemical substrate which is converted to a detectable signal by the enzyme is added. Although ELISA takes 2-3 hours itself, it requires pre-enrichment for production of target antigens in high quantities.

ELISA provides higher sensitivity and shorter time when compared to traditional culture methods. However, it still has some disadvantages including false negative results, cross-reactivity with closely related bacteria and requirement for pre-enrichment step (Jasson *et al.*, 2010).

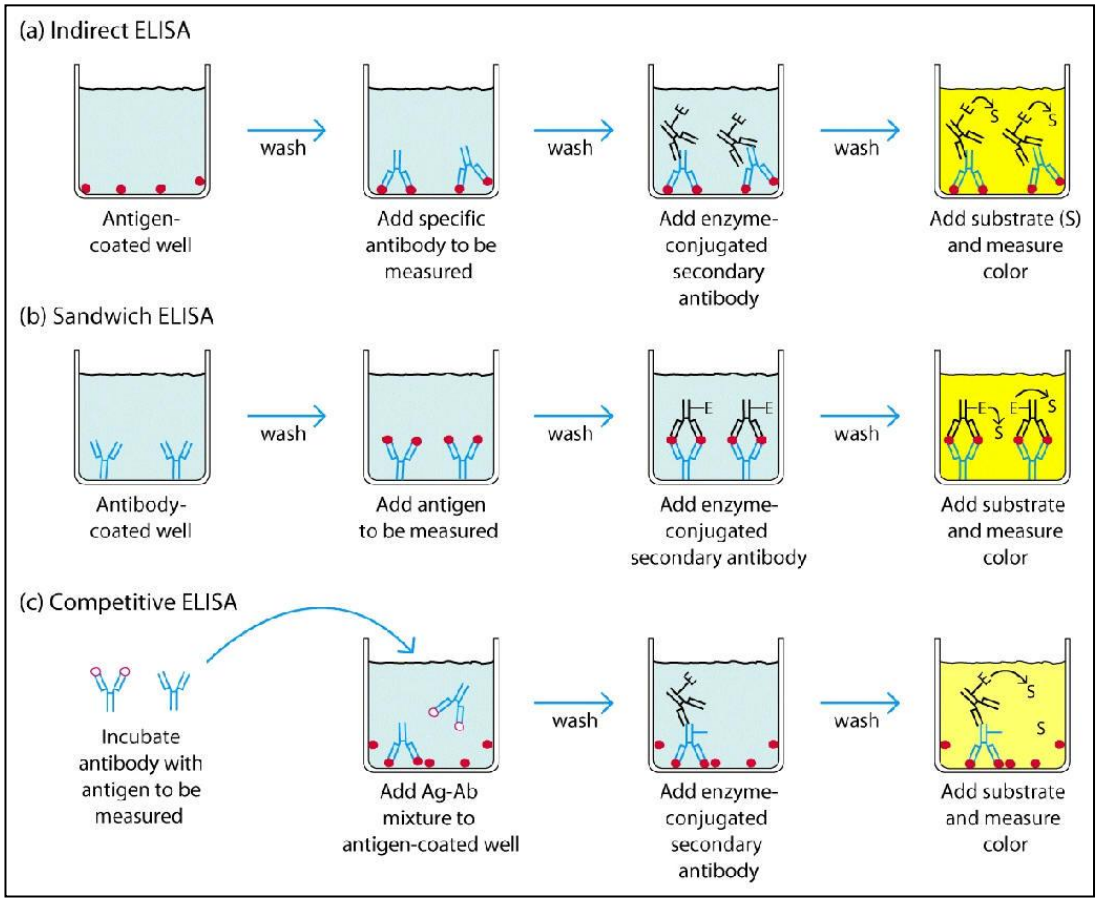

Figure 1.6 Schematic representations of 3 main types of ELISA (UTexas)

1.3.2.2. Fluorescent *In Situ* Hybridization (FISH)

FISH is one of the mostly applied hybridization based molecular technique for detection of foodborne pathogens. Instead of DNA, usually ribosomal RNA molecules (rRNA) of the pathogen are targeted and hybridized with labeled complementary oligonucleotides because of their high copy numbers.

In FISH procedure, pathogen cells are treated with fixative agents and hybridized with fluorescent labeled oligonucleotide probes on a glass slide. Then unbound probes are removed by washing the glass slide. Remaining stained cells are visualized and detected by fluorescence microscopy.

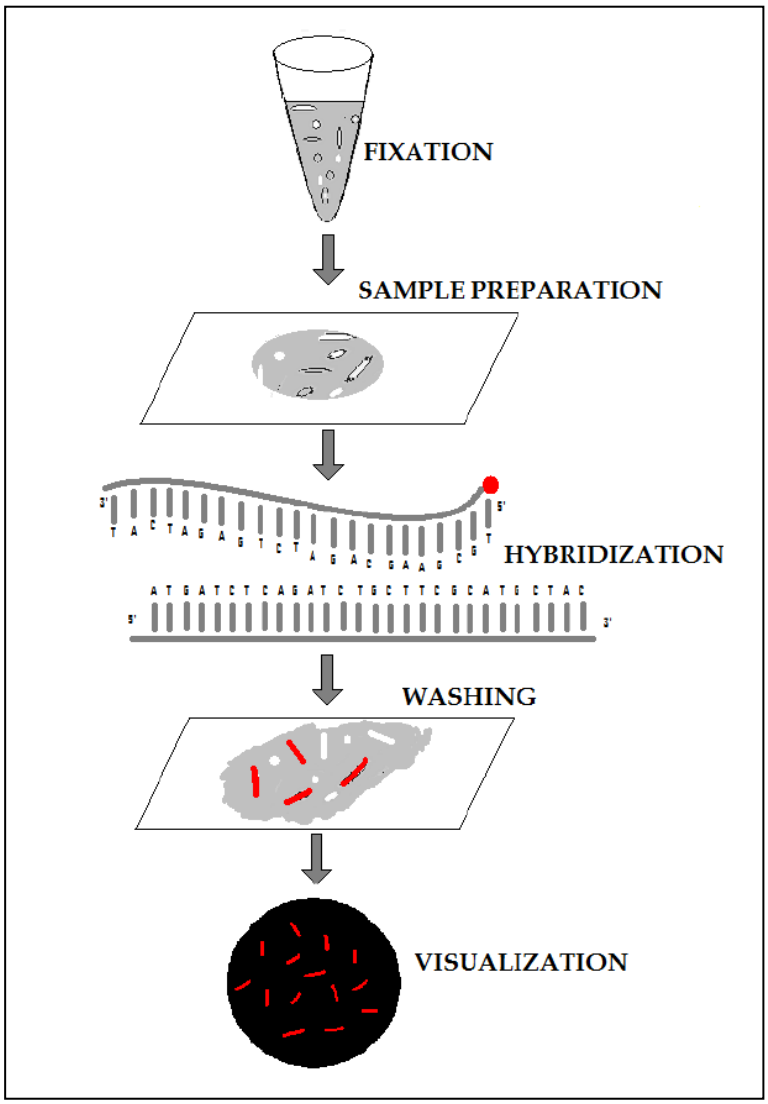

Figure 1.7 Flow chart of FISH procedure (Zadernowska and Chajecka, 2011)

After overnight enrichment of microbial cell, FISH procedure takes 3-4 hours. Even though FISH is considered a rapid detection method, it is labor-intensive and requires advanced and expensive equipments (Jasson *et al.,* 2010).

1.3.2.3. Polymerase Chain Reaction (PCR)

PCR is an efficient and rapid molecular method for detection and characterization of foodborne pathogens due to its sensitivity, specificity, accuracy and speed when compared to the traditional culture based methods. Its potential has encouraged advances and improvements for detection of *Salmonella* spp. in various food, water and environmental samples. In addition, an enrichment step is generally applied prior to PCR procedure in order to maximize detection sensitivity (Goldenberg, 2013).

1.3.2.3.1. Conventional PCR

PCR is an enzymatic reaction that allows *in vitro* amplification of nucleic acid molecules. In a typical PCR procedure, desired region in nucleic acid of the target organism is exponentially amplified during three step cycling process.

Prior to PCR, cell is lysed to release the nucleic acid from the target organism. After denaturation of the nucleic acid molecule, synthetic starters (forward and reverse primers) attach to both ends of target sequence and they are extended via Taq polymerase by adding complementary bases. Amount of nucleic acid is doubled at the end of each three step cycling (denaturation, annealing and extension). 35-40 cycles of PCR result in billions of copies of specific region (Harris and Griffiths, 1992).

In conventional PCR, amplified nucleic acid molecules are separated and visualized under agarose gel electrophoresis in which amplified molecules can be only characterized according to their size. For the further characterizations to confirm specific region, usually hybridization methods or sequencing are used (Foley and Grant, 2007).

There are many PCR assays for detection of foodborne pathogens especially for *Salmonella*. The PCR assay developed by Rhan *et al*. in 1992 has the highest specificity and sensitivity among the others. They chose *invA* gene which is found in

not all but most of *Salmonella* serotypes and visualized specific 284 bp PCR amplicon on agarose gel electrophoresis.

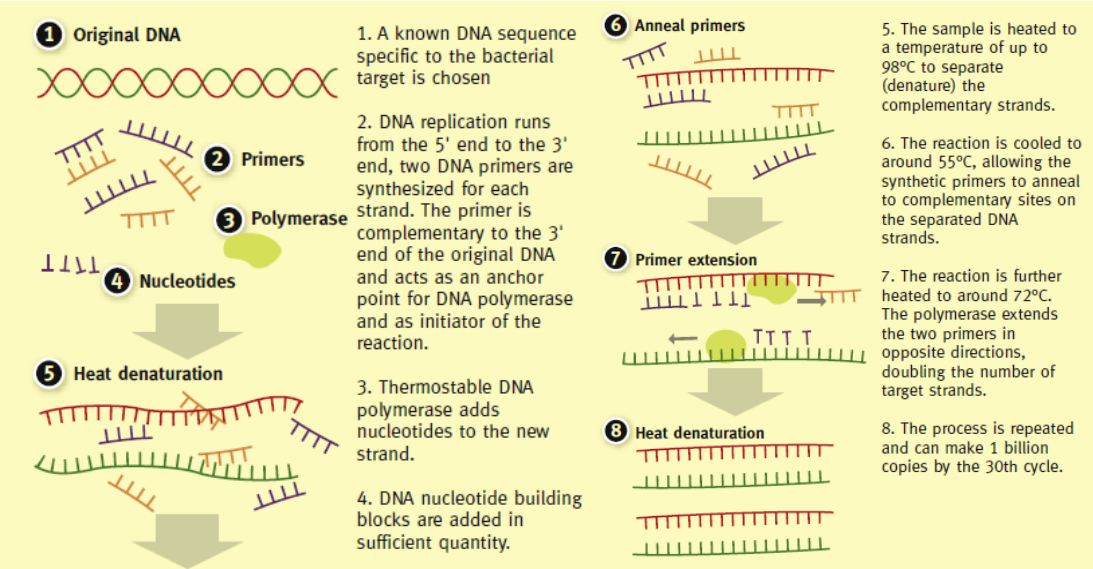

Figure 1.8 Stages of polymerase chain reaction (Goldenberg, 2013)

PCR assay has some advantages over other methods such as increased sensitivity and specificity by allowing detection of small concentrations of target DNA. However, agarose gel electrophoresis is not sufficient, singly. Therefore, a system which enables specific detection of amplified target is required and should be combined with the PCR assay. The main disadvantage of the PCR assay is that many food samples contain substances that inhibit the polymerase. But this issue can be overcome by an effective nucleic acid extraction method (Harris and Griffiths, 1992).

1.3.2.3.2. Real-Time PCR

Real-time is one of the rapid methods for foodborne pathogen screening. It is an improvement of conventional PCR methods and allows observation of the reaction in real time. Amplified nucleic acid sequences are monitored by using fluorescently labeled probes which eliminates the post-PCR procedures such as agarose gel electrophoresis and hybridization methods. The most important advantage of the method is that it allows quantification of produced amplicons cycle by cycle which can give an idea about the initial concentration of pathogen DNA.

For *Salmonella* detection and quantification from various samples including dairy and poultry products and egg, TaqMan probe based real-time PCR platforms have been employed. Fluorescently labeled TaqMan probes specifically bind to a sequence of interest which is characteristic for *Salmonella* (Cheung and Kam, 2012).

Table 1.5 Detection of Salmonella using real-time PCR assay (Zadernowska and Chajecka, 2011)

Target Gene	Matrices	Enrichment	Limit of Detection
ttrBCA	Chicken Minced meat Fish	20 h	<3 CFU/ml
fimC	Ice cream	without	10^3 CFU/ml
invA	Salmone Chicken Milk	16 h	2.5-5 CFU/ml
invA	Chili powder Shrimp	35°C-24 h (pre-) 41°C-24 h (selective)	0.04 CFU/g
oriC	Cheddar Turkey Meat	48 h (selective)	$6.1x10^1$ CFU/ml

A real-time PCR procedure consisted of 40 cycles takes approximately 1.5 hours. When the time required for extraction of target DNA is added up, whole process of detection and quantification takes hours. In addition, sample should be subjected to a pre-enrichment step which lasts 18 hours at 37°C. Thereby, complete procedure for detection and quantification does not take longer than 24 hours (Zadernowska and Chajecka, 2011).

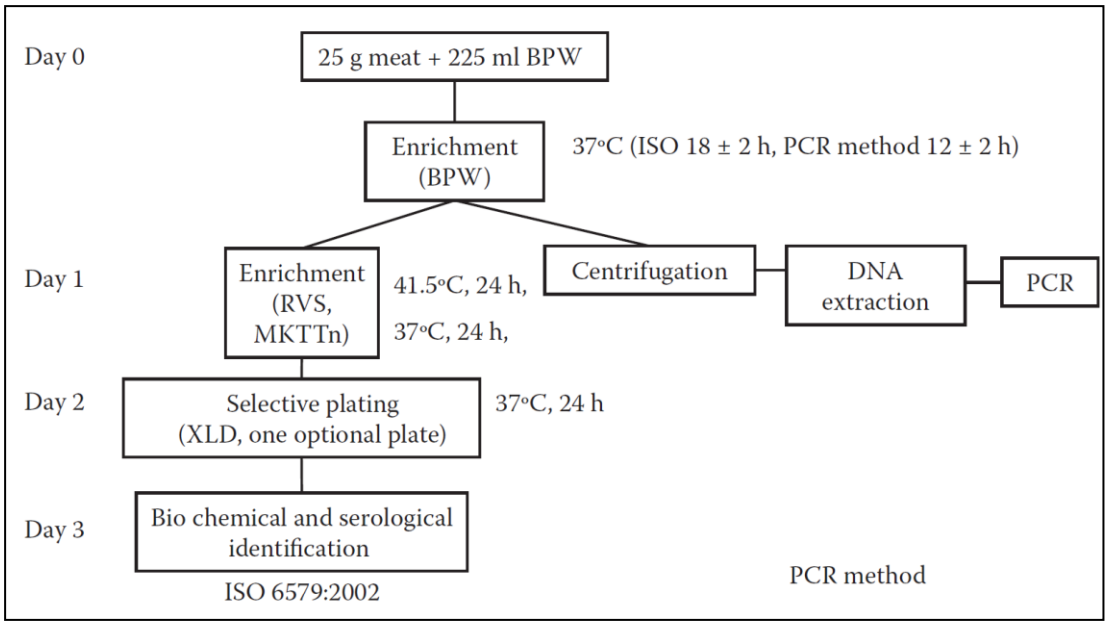

Figure 1.9 Comparison of culture based ISO 6579:2002 method and real-time PCR method for *Salmonella* detection in meat (Löfström *et al.*, 2010)

When compared to the conventional PCR, real-time PCR provides higher degree of sensitivity, reproducibility and quantification. Although its advantages over other methods, real-time PCR requires expensive, complicated equipments and trained personnel which makes it not-applicable to the field (Park *et al.*, 2014).

1.3.3. Biosensors

Although both antibody-based and nucleic-acid based detection methods have greater potentials than the traditional culture based methods, they still are not low-cost, reliable, accurate, user-friendly and field-applicable systems. Biosensors are emerging technologies that can fully or partially meet these standards. A biosensor is defined as an independently integrated receptor transducer which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element (Thevenot *et al.*, 1999).

Biosensors are composed of two compartments: a biological receptor which recognizes specific biological target and a transducer which converts the biological signal into a detectible signal such as electrical, electrochemical and physical signal. They allow rapid, real-time, accurate and reliable detection of different types of

target molecules in complex sample matrices and have a great potential for some fields including medicine, food industry, environmental monitoring and agriculture (Leonard *et al.,* 2003).

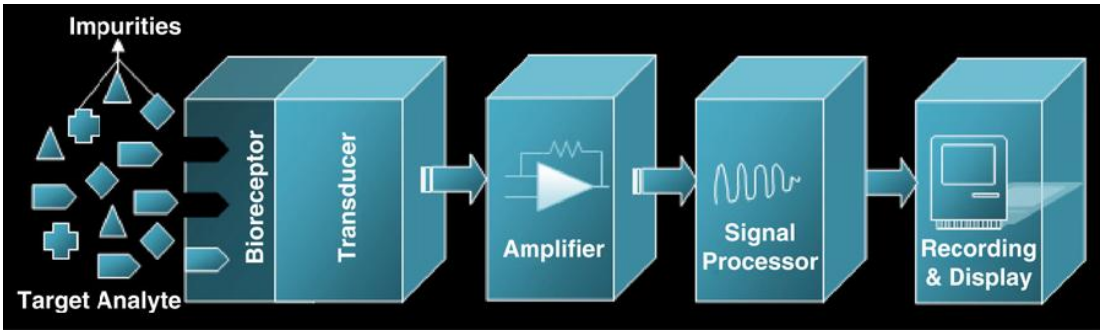

Figure 1.10 Schematic representation of a typical biosensor (Velusamy *et al.,* 2010)

A biological receptor or biological recognition element is composed of nucleic acid probes, antibodies, cells, enzymes or aptamers. Reactions between the biological receptor and the target analyte result in a biological or biochemical signal such as heat output, electrical output, light output, redox reaction and changes in pH or mass. The created biological or biochemical signal is converted to a electrical signal by the transducer. However, the converted signal is generally weak for electronic readout, therefore it is amplified and sent to the data processor (Thakur and Ragavan, 2013).

Biosensors are classified by their type of biological receptor or transduction system. Currently, there are five main types of biological receptors: enzyme based, antibody based, DNA based, cell based and biomimetic or aptamer based mechanisms. In addition, there are four major transduction methods: electrochemical, piezoelectric, calorimetric and optical. First type of biosensors, electrochemical, measure the changes resulting from oxidation and reduction reactions. Piezoelectric biosensors are based on changes in mass. On the other hand, calorimetric biosensors are based on detection of output or absorption of heat resulting from biochemical reaction between the target and the biological receptor. Finally, optical biosensors relatively have higher sensitivity and specificity for detection of foodborne pathogens and use different types of strategies including absorption, refraction, reflection, dispersion of light (Perumal and Hashim, 2014).

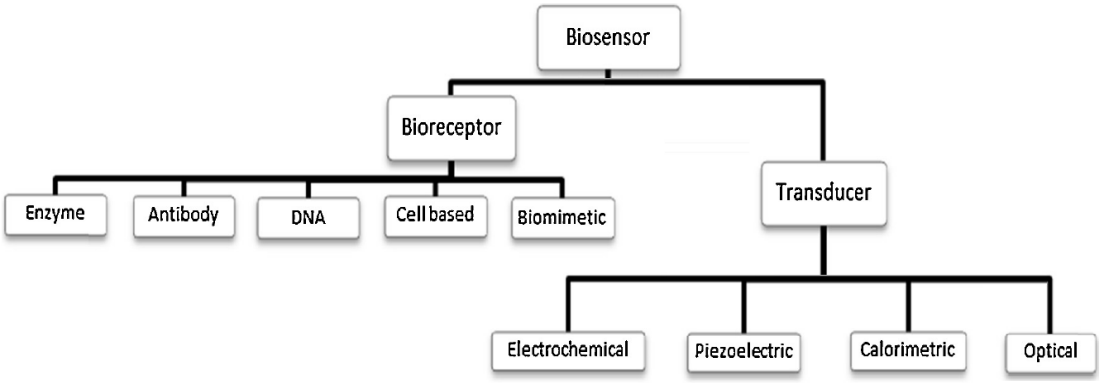

Figure 1.11 Classification of biosensors (Perumal and Hashim, 2014)

Nucleic acid based biosensors have been prominent detection methods due to their sensitivity, low-cost and speed when compared to other types of biosensors. Currently, there are numerous nucleic acid biosensors for different purposes including gene and drug discovery and detection of pathogens. However, most of them are still not applicable to the field because they require expensive instrumentation and complex procedures. In order to become extensively used and to be applicable at the near patient (point-of-care), complexity and instrumentation should be eliminated (He *et al.*, 2011).

Paper-based biosensors are emerging trends for both diagnostic and research applications. They are considered as good candidates to meet demands for inexpensive, sensitive, specific, rapid and equipment-free detection systems. Lateral flow immunochromatographic assay is a remarkable type of paper-based biosensors.

1.3.3.1. Lateral Flow Immunochromatographic Assay (LFIA)

Emerging needs for developing new-generation diagnostic devices brought about rapid point-of-care tests such as LFIAs. This new technology provides a large number of advantages including rapid, robust, simple and cost-effective over other detection methods. The earliest application of LFIAs was the detection of human chorionic acid as a pregnancy test. Observing the result by naked-eyes and use of inexpensive membrane strips enabled an efficient detection platform (Ngom *et al.*, 2010).

Lateral flow test strips consist of four components: a nitrocellulose membrane on a backing pad, sample or sample application pad, conjugated pad and absorbent pad. All components of test strip are placed in an overlapping sequence in order to organize the fluid flow among the strip. Absorbent pad also called wicking pad is the driving force of the strip. Porous nitrocellulose membrane is the essential component of strip due to providing a backdrop for the reaction. Capture probes (either antibodies or nucleic acids) are immobilized onto nitrocellulose membrane to form test and control lines. Conjugate pad contains conjugated particle also known as labeled signal probe which is specific to the target analyte.

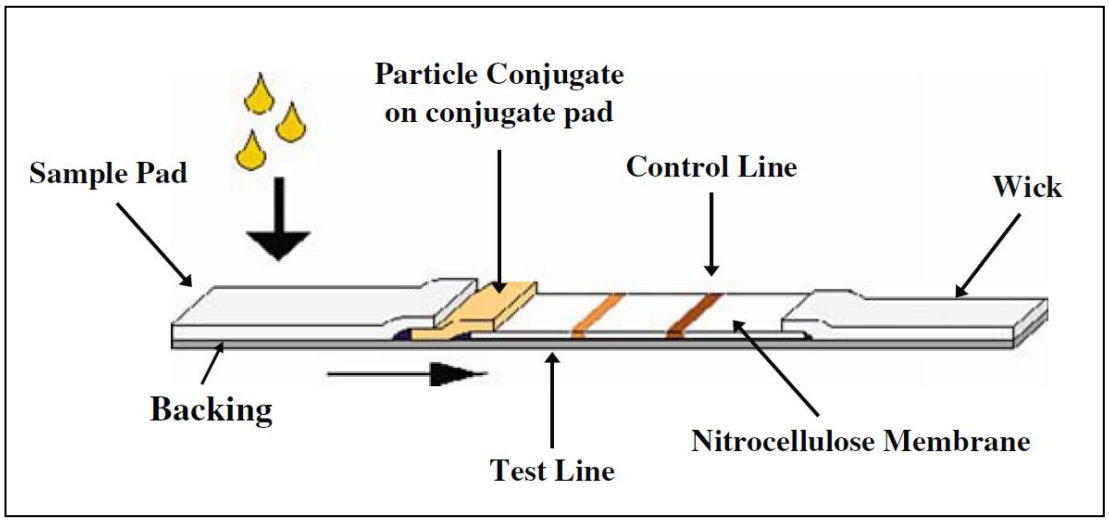

Figure 1.12 Representation of a typical lateral flow immunochromatographic assay (Wong and Tse, 2009)

When the assay is performed, a small volume of liquid sample is applied to sample pad which is pre-treated with a buffer to adjust pH and improve the assay performance. If the target (antibodies, nucleic acids or cell) is present in the analyte, it binds to labeled signal probe. The target-signal probe mixture migrates along the strip and then it is captured in the test line by reaction with capture probe and the target molecule. Formation of a colored line on the test line that is detectable by naked-eyes indicates a positive result (Posthuma-Trumpie *et al.*, 2009).

Currently, LFIA is the easiest method to use, and it does not require any instrumentation or trained personnel. Whole test procedure takes ten minutes.

Although first applications of LFIA were based on recognition of a specific antibody, nucleic acid based LFIAs have been also developed because of some issues of antibodies such as false negative results, production and modification processes and stability. Therefore, nucleic acids are more convenient than antibodies for biosensors and lateral flow assays especially when combined with PCR. Because, recognition of complementary ssDNAs each other by base-pairing reaction is more specific and potent. In order to achieve this, two main hybridization strategies are performed with nucleic acid based LFIAs: direct hybridization and sandwich hybridization. In the direct hybridization strategy, pre-labeled target sequence is applied to the assay and hybridizes with the complementary capture probe which have been deposited on the test line. On the other hand, sandwich hybridization is the most preferred strategy because it does not require chemical labeling of the target sequence. Three different sandwiched nucleic acid sequences are used in this format: capture probe, target and signal probe. Firstly, the conjugated signal probe is partially hybridized with the target sequence. Then the target-signal probe complex move among the fluid flow and finally it is captured by the complementary immobilized probe (Figure 9) (Hu *et al.*, 2014).

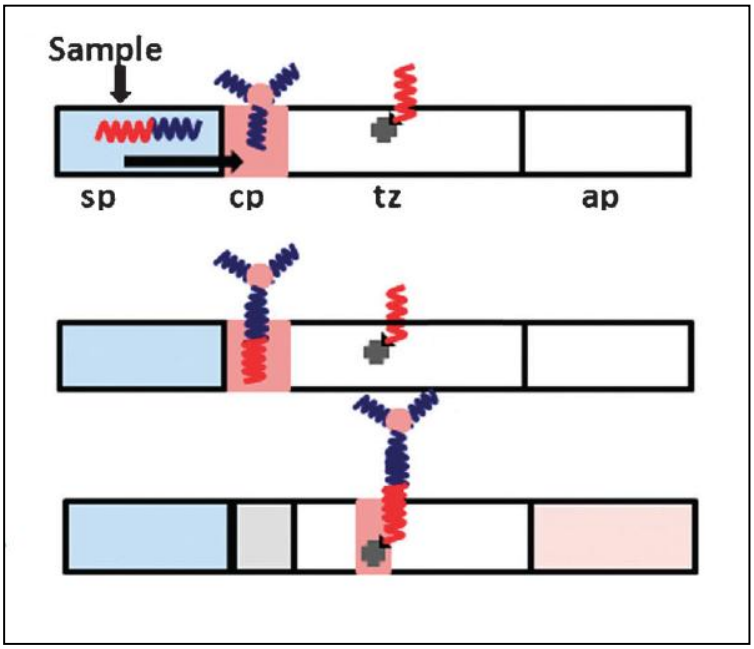

Figure 1.13 Schematic representation of a nucleic acid based LFIA employing sandwich hybridization. Above, three steps of the assay are described in detail (Rastogi et al., 2012)

1.3.3.1.2. Labels for Lateral Flow Immunochromatographic Assay

LFIAs employ various types of labels including metallic nanoparticles (gold or silver), silica particles, carbon particles, magnetic particles, up-converting phosphors, quantum dots and enzymes in order to improve sensitivity. However, nanoparticles are favorable for LFIAs due to their significant advantages over other labels. Their characteristics such as size, shape, composition and surface modification can be easily manipulated.

Gold nanoparticles (AuNPs) are the most preferred nanoparticles for LFIA applications. Most importantly, robust affinity of AuNPs towards thiol groups brings about formation of strong covalent bonds between AuNPs and thiol molecules. By this means, surface chemistry of AuNPs can be easily controlled and thiol modified probes such as nucleic acids and antibodies can be conjugated (Kaittanis *et al.*, 2010).

AuNPs have further advantages as following (Parolo *et al.*, 2013):

- ✓ Detectable even at low concentrations due to its strong red color.
- ✓ Low-cost
- ✓ Ease of production
- ✓ Stable
- ✓ Biocompatible

1.4. Aim of the Study

This study aims to develop a detection platform technology based on nucleic acid lateral flow assay coupled with conventional PCR. The detection platform was optimized for detection of *Salmonella* spp.

Although increasing incidence and severeness of *Salmonella* outbreaks, current detection methods both traditional culture based methods and molecular methods are not sufficient. Therefore, advanced detection methods, which are rapid, low-cost, and easy-to-use, are required. With this point of view, a lateral flow assay for *Salmonella* was developed which can fully meet these standards.

CHAPTER 2

MATERIALS & METHODS

2.1. Materials

2.1.1. Chemicals

All chemicals used in this study were analytical grade and purchased from Sigma-Aldrich, Merck, or AppliChem Chemical Companies. All of the solutions were prepared with ultrapure MilliQ water.

2.1.2. Buffers and Solutions

Compositions and preparations of buffers and solutions were given in Appendix A.

2.1.3. Test Strip Components

Hi-Flow Plus (HF240) membrane cards were used as the support material. In addition to the support material, glass fiber pads were used as the conjugate pad and cellulose fiber pads were used as the sample application pad and backing pad. Both nitrocellulose membrane card and pads were purchased from Millipore, Germany.

2.1.4. Synthetic Oligonucleotides

All modified and unmodified synthetic oligonucleotides were purchased from Integrated DNA Technologies with standard desalting or HPLC purification and they were used without further purification. All synthetic oligonucleotides were

resuspended in sterile nuclease free ultrapure water according to the manufacturer's guide and stored at -20°C.

For the optimization studies, a 60 base-long target sequence was selected from *Salmonella*'s *invA* gene and purchased synthetically. 3'-biotin modified capture probe (CP0) and 5'-thiol modified signal probe (SP0) were specifically designed for the synthetic target. Each is probe complementary to half of the target. As a control, another 60 base-long uncomplementary oligonucleotide was designed and used in the optimization experiments.

Upon optimization of the assay with synthetic target and control, two different real targets, which were 105 bp and 284 bp amplicons of *invA*, were experimented. For the real targets one capture probe (CP1) and two different signal probes were designed. 3' biotin modified CP1 is complementary to the 5' ends of both 105 bp and 284 bp amplicons. 5' thiol modified signal probe 1 and probe 2 are complementary to 3' end of 105 bp and 284 bp amplicons, respectively.

Sequences and modifications of synthetic DNA probes are given in Table 2.1.

Table 2.1 Sequences and modifications of synthetic probes

Description	**Sequence**
Synthetic target	5'-TGGCGATAGCCTGGCGGTGGGTTTTGTTGTCTTCT CTATTGTCACCGTGGTCCAGTTTAT-3'
Synthetic control (uncomplementary)	5'-ATCAAGAAACGCGAGATGGGATGATATATTCTGG TTACCTTACCAGACATAGTTATGCAT-3'
Capture Probe 0	5'-AACAAAACCCACCGCCAGGCTATCGCCA/**3Bio**/-3'
Signal Probe 0	5'-/**5ThioMC6-D**/Poly$A_{(10)}$TAAACTGGACCACGGTGACA ATAGAGAAG
Capture Probe 1	5'-CGAATTGCCCGAACGTGGCGATAATTTCAC/**3Bio**/-3'
Signal Probe 1	5'-/**5ThioMC6-D**/Poly$T_{(10)}$CAAAACCCACCGCCAGGCTA TCGCCAATAA-3'
Signal Probe 2	5'-/**5ThioMC6-D**/Poly$T_{(10)}$TCATCGCACCGTCAAAGGAA CCGTAAAGCT

2.1.5. PCR Primers and pUC19 Plasmid

Using one forward primer and two different reverse primers, 105 bp and 284 bp regions of *invA* gene were amplified by PCR and then used as real targets. All of the primers were purchased from Integrated DNA Technologies.

Multiple cloning site (MCS) of pUC19 plasmid was used as the negative control. M13 forward primer and M13 reverse primer were used for amplification of 103 bp MCS by PCR. pUC19 plasmid and primers were purchased Life Technologies. The map of pUC19 is given in Appendix C.

All PCR primers were resuspended as 100 μM stock solutions in sterile and nuclease free ultrapure water according to the manufacturer's guide and stored at -20°C. Sequences of forward and reverse primers were listed in Appendix B.

2.1.6. Bacterial Strains

Three different *Salmonella enterica* serotypes *Salmonella* typhimurium (ATCC 14028), *Salmonella* enteritidis (ATCC 13076) and *Salmonella* infantis and a non-*Salmonella* species, *Escherichia coli* strain DH5α, were used for genomic DNA extractions and polymerase chain reactions.

Salmonella typhimurium (ATCC 14028) and *Salmonella* enteritidis (ATCC 13076) were purchased from American Type Culture Collection (USA). *Salmonella* infantis and *Escherichia coli* strain DH5α were generous gifts from NANObiz Ltd. Co., Ankara, Turkey.

2.1.7. Gold Nanoparticles

Gold(III) chloride hydrate also known as auric chloride was purchased from Sigma-Aldrich Company (USA) and used to synthesize 25 nm AuNPs.

2.2. Methods

2.2.1. Target Preparation

2.2.1.1. Genomic DNA Isolation from *Salmonella* and *E.coli* Cells

For genomic DNA isolation, *Salmonella* cultures were grown for 16 hours in Tryptic soy broth (TSB) by incubating on a rotary shaker (100 rpm) at 37°C. *E. coli* cultures were grown in Luria-Bertani (LB) broth at 37°C, 120 rpm overnight. Compositions of mediums are given in Appendix A. DNA isolation was carried out using NANObiz DNA4U Bacterial Genomic DNA Isolation kit according to the manufacturer's instructions with some modifications. Additionally, samples were incubated at 65°C for 5 minutes to remove extra ethanol at the final step.

2.2.1.2. Quantification of Genomic DNA Concentration

Concentration of isolated genomic DNA samples was determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

2.2.1.3. Polymerase Chain Reaction (PCR)

2.2.1.3.1. Primer Selection and Design

In order to amplify a 284 bp region of *Salmonella invA* gene, forward primer (F 139) and reverse primer (R 141) (Rahn *et al*, 1992) were used. In addition, a second reverse primer which amplifies a 105 bp region of *invA* gene with F 139 was designed by using Primer3 program (Untergrasser *et al*, 2012). Compatibility of homodimer and heterodimer of primers were analyzed by OligoAnalyzer (Integrated DNA Technology). Also, PrimerBLAST (National Center for Biotechnology Information) was used to determine specificity of primers.

All PCR primers were resuspended as 100 μM stock solutions in sterile nuclease free ultrapure water according to the manufacturer's guide and stored at -20°C. Sequences of forward and reverse primers were listed in Appendix B.

2.2.1.3.2. PCR Conditions for 284 bp Product

PCR was performed in a total reaction mixture of 25 µL by using Taq Polymerase (Thermo Scientific). All materials used in PCR were kept on ice prior to use. Components, optimized conditions and optimized cycling program of PCR (F 139 and R 141 primers) were given in Table 2.2 and Table 2.3, respectively.

Table 2.2 Optimized PCR conditions for 284 bp amplicon

Components	Amount (µL)	Final Concentration
dH_2O	16.55	-
10X Reaction Buffer	2.5	1X
25 mM $MgCl_2$	2	2 mM
10 µM Forward Primer	1	0.4 mM
10 µM Reverse Primer1	1	0.4 mM
10 mM dNTP Mix	0.75	0.3 mM
5 U/µL Taq Polymerase	0.2	1U
10 ng/µL DNA	1	-

Table 2.3 PCR cycling conditions for 284 bp amplicon

Steps		Conditions
Initial Denaturation		95°C, 5 minutes
35 Cycles	Denaturation	95°C, 30 seconds
	Annealing	64°C, 30 seconds
	Extension	72°C, 30 seconds
Final Extension		72°C, 4 minutes

2.2.1.3.3. PCR Conditions for 105 bp Amplicon

PCR was performed in a total reaction mixture of 25 µL by using Taq Polymerase (Thermo Scientific, USA). All materials used in PCR were kept on ice prior to use. Components, optimized conditions and optimized cycling program of PCR (F 139 primer and reverse primer 2) were given in Table 2.4 and Table 2.5.

Table 2.4 Optimized PCR conditions for 105 bp amplicon

Components	Amount (µL)	Final Concentration
dH_2O	17.05	-
10X Reaction Buffer	2.5	1X
25 mM $MgCl_2$	1.5	1.5 mM
10 µM Forward Primer	1	0.4 mM
10 µM Reverse primer2	1	0.4 mM
10 mM dNTP Mix	0.75	0.3 mM
5 U/µL Taq Polymerase	0.2	1U
10 ng/µL DNA	1	-

Table 2.5 PCR cycling conditions for 105 bp amplicon

Steps		Conditions
Initial Denaturation		95°C, 5 minutes
35 Cycles	Denaturation	95°C, 30 seconds
	Annealing	58°C, 30 seconds
	Extension	72°C, 30 seconds
Final Extension		72°C, 4 minutes

2.2.1.3.4. PCR Conditions for MCS

PCR was performed in a total reaction mixture of 25 µL by using Taq Polymerase (Thermo Scientific, USA). All materials used in PCR were kept on ice prior to use. Components, optimized conditions and optimized cycling program of PCR (M13 forward and reverse primers) were given in Table 2.6 and Table 2.7.

Table 2.6 Optimized PCR conditions for MCS

Components	**Amount (µL)**	**Final Concentration**
dH_2O	16.05	-
10X Reaction Buffer	2.5	1X
25 mM $MgCl_2$	2	2 mM
10 µM M13 Forward P.	0.625	0.25 mM
10 µM M13 Reverse P.	0.625	0.25 mM
10 mM dNTP Mix	0.75	0.3 mM
5 U/µL Taq Polymerase	0.4	2U
10 ng/µL DNA	1.25	-

Table 2.7 PCR cycling conditions for 284 bp amplicon

Steps		**Conditions**
Initial Denaturation		95°C, 5 minutes
35 Cycles	Denaturation	95°C, 30 seconds
	Annealing	53°C, 30 seconds
	Extension	72°C, 30 seconds
Final Extension		72°C, 4 minutes

2.2.3.4. Agarose Gel Electrophoresis of PCR Products

60 ml of 1.2 % and 2.5 % (w/v) agarose gels prepared with 1X TAE containing 3.5 µL of ethidium bromide (5 mg/ml) were used to visualize 284 bp amplicon, 105 bp amplicon and MCS, respectively. Appropriate amount of agarose was weighed and dissolved in 60 ml of 1X TAE. The solution is boiled in microwave until agarose completely dissolved. After a few minutes of cooling, 3.5 µL of ethidium bromide (5 mg/ml) was added to the gel solution. The gel solution was poured into an electrophoresis gel tray and comb was placed. When the gel solidified, the comb was removed and the gel was placed in an electrophoresis tank filled 1X TAE. PCR samples and molecular weight markers (GeneRuler 1 kb and 50 bp DNA Ladder, Thermo Scientific and 50 bp DNA Step Ladder, Sigma-Aldrich) were mixed with 6X loading dye and loaded into wells. The gel was run at 75V for 45 minutes and visualized by UV gel acquisition system.

2.2.2. Synthesis and Characterization of Gold Nanoparticles

Gold nanoparticles (AuNPs) with average diameter 25 nm were prepared by reduction of $HAuCl_4$ with sodium citrate (Grabar *et al*, 1995). All glassware used in synthesis of AuNPs was cleaned with chromic acid, rinsed with distilled water and then oven-dried overnight. Briefly, 100 ml of 5 mM $HAuCl_4$ solution was boiled with vigorous stirring. When boiling started, 4 ml of 1 % sodium citrate was quickly added. After the color of the solution turned into wine-red, boiling continued for an additional 15 minutes and finally the solution was allowed to cool down to room temperature. AuNP solution was filtered through 0.45 µm acetate membrane filter.

Subsequently, the filtered AuNP solution was concentrated to 5X for oligonucleotide conjugation by centrifuging through a 50000 MWCO concentrator (Millipore) at 45000 rpm for 30 minutes.

Both AuNPs and AuNP-DNA conjugates (signal probes) were characterized with respect to their spectroscopic and morphological properties, sizes and charges by using UV-Visible Spectroscopy (Multiskan GO 1.00.40), Dynamic Light Scattering (Malvern CGS-3) and Zetasizer (MALVERN Nano ZS90).

2.2.3. Functionalization of AuNPs with Thiolated DNA Probes

5'-thiolated synthetic oligonucleotides were used for conjugation with synthesized AuNPs to form signal probes: SP0 for synthetic target, SP1 for the 105 bp amplicon and SP2 for the 284 bp amplicon.

Prior to conjugation, TCEP (*tris*(2-carboxyethyl)phosphine) was used as a reducing agent to reduce the disulfide linkages of the probes and activate oligonucleotides. For this, equal volumes of 100 μM TCEP and 100 μM thiolated oligonucleotide were mixed and incubated for 1 hour at room temperature. Activated oligonucleotide was added to 5-fold concentrated AuNP solution and resulting mixture was incubated again at 4°C for 24 hours.

After incubation, the conjugate was slowly aged by adding 0.1 M PBS until a final concentration of 0.01 M. The solution was subjected to another incubation at 4°C for 24 hours. The excess of reagents were removed by centrifuging at 12000 rpm for 20 minutes and after discarding the supernatant, the pellet was resuspended in 20 mM sodium phosphate buffer containing 5 % BSA, 0.25 % Tween20 and 10 % sucrose (Appendix A) (Xu *et al*, 2009 and Liu *et al*, 2006).

2.2.4. Preparation of Test Strip Components

The lateral flow immunochromatographic test strip consists of four components: sample application pad (17 x 4 mm), conjugated pad (5 x 4 mm), absorbent pad (21 x 4 mm), and nitrocellulose membrane (20 x 4 mm). Each component was prepared separately and then mounted together.

2.2.4.1. Sample Pad

The cellulose fiber sample application pad (Millipore, Germany) was soaked with pH 8.0 0.05M tris buffer containing 0.25 % Triton X-100, 0.15 M NaCl (Appendix A) and dried overnight at room temperature prior to use.

2.2.4.2. Conjugate Pad

The conjugate pad was prepared by dispensing 100 μL of AuNP-DNA conjugates (signal probes) on a glass fiber pad (Millipore, Germany) and dried overnight at room temperature.

2.2.4.3. Nitrocellulose Membrane

In order to create test lines for each strip, capture probes (CP0 for synthetic target and CP1 for 105 bp and 284 bp amplicons) were immobilized onto nitrocellulose membrane by biotin-streptavidin interaction.

Firstly, 2 mg/ml streptavidin, 1X PBS and 1 mM biotinylated probe were mixed. After standing at room temperature for 1 hour, the excess oligonucleotide was removed by centrifugation with Amicon 30K device (Millipore) at 6000 rpm for 30 minutes. The remaining solution in the filter was collected and suspended in 1X PBS. 2.5 µl of the 3' biotin modified oligonucleotide probe which was conjugated to streptavidin was dispensed on the test line by pipetting (Mao *et al*, 2009).

2.2.3.4. Absorbent Pad

The cellulose fiber absorbent pad (Millipore, Germany) was used without any modification. It provides a capillary mechanical force for the assay and also holds the used reagents.

2.2.5. Assembly of Lateral Flow Immunochromatographic Test Strip

For assembly of the test strip, sample application pad, conjugate pad, nitrocellulose membrane, and absorbent pad were cut into the desired dimensions (mentioned in part 2.2.7) and then placed on an overlapping order about 1-2 mm for optimal assay procedure.

2.2.6. Assay Procedure

Three different sets of lateral flow immunochromatographic test strips were prepared for detection of *Salmonella* specific synthetic target, 105 bp and 284 bp amplicons of *invA* gene.

2.2.6.1. Assay Procedure for the Synthetic Target

Both synthetic target oligonucleotide and synthetic control oligonucleotide were diluted with 5X SSC for 1 µM final concentration. 80 µL of each oligonucleotide was applied to the strip. As a second control, 80 µL of 5X SSC was also applied. Applied samples were allowed to migrate through the nitrocellulose membrane and reach the absorbent pad. Following, each strip was washed with 80 µL of 5X SSC.

2.2.6.2. Assay Procedure for PCR Products

105 bp and 284 bp amplicons of *invA* from 3 different *Salmonella* serotypes (*S.* infantis, *S.* enteritidis, *and S.* typhimurium) were separately applied to the assay. Prior to application, both 105 bp and 284 bp amplicons were diluted with 5X SSC in following ratios 1:2, 1:3, 1:5 and 1:10. Diluted targets were incubated at 95°C for 5 minutes and then immediately chilled on ice for denaturation.

As control, 103 bp multiple cloning site of pUC19 plasmid (1:2 and 1:5 diluted), PCR mix containing no DNA (1:2 diluted) and 5X SSC were applied.

90 µL of each sample was applied to the assay and allowed to migrate towards the absorbent pad. Following, each strip was washed with 90 µL of 5X SSC.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Target Preparation

3.1.1. Bacterial Growth

Three *Salmonella* serotypes (*S.* infantis, *S.* enteritidis and *S.* typhimurium) and *Escherichia coli* DH5α were grown on TSA and LB agar plates, respectively. Preparation and composition of medium were given in Appendix A.

Single colonies obtained from agar plates were then subjected to pre-enrichment for 16 hours. Genomic DNA was extracted from 2 ml of each pre-enriched pure culture. Starting concentrations of cultures are given in Table 3.1. *E. coli* DH5α genomic DNA was used as a negative control for PCR.

Table 3.1 Starting concentrations of cultures

Sample	Starting Concentration (cfu/ml)
S. infantis	8×10^7
S. enteritidis	3×10^8
S. typhimurium	7×10^8
E. coli DH5α	7×10^7

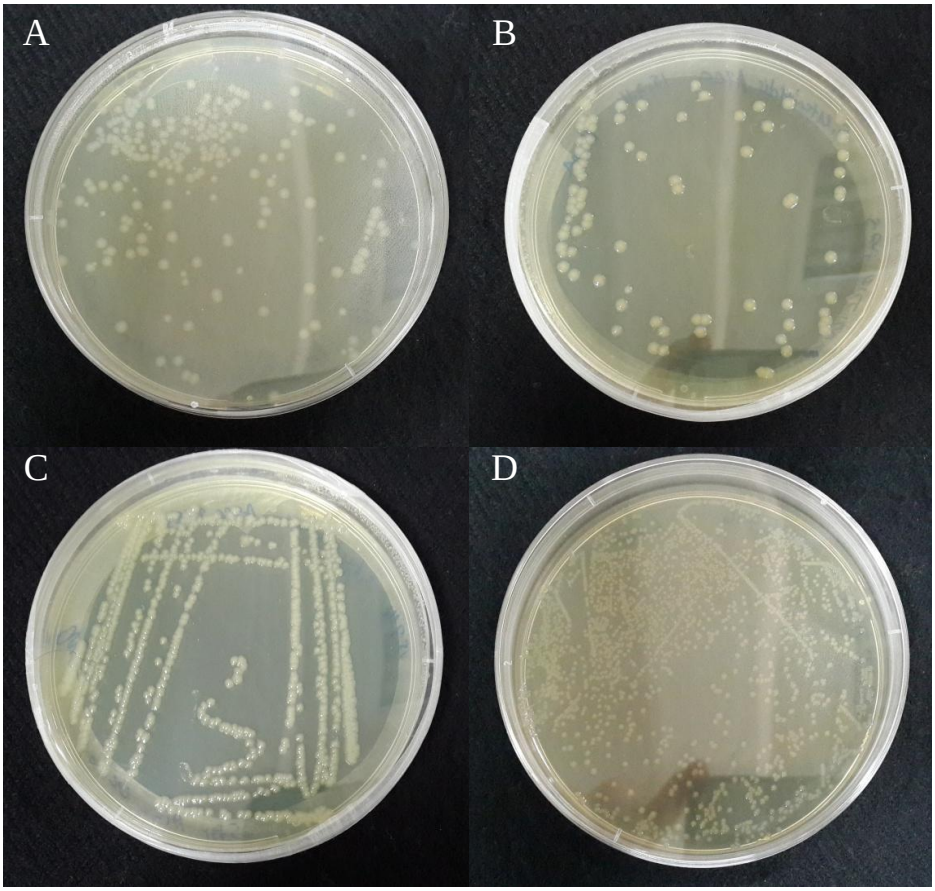

Figure 3.1 Single colonies on agar plates. A, B, C and D illustrate *S.* infantis, *S.* enteritidis, *S.* typhimurium and *E. coli* DH5α

3.1.2. Isolation and Quantification of Genomic DNAs

Genomic DNA isolation was performed using NANObiz DNA4U isolation kit. After the isolation, genomic DNAs were quantified by measuring optical densities by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Table 3.2 Concentrations and purity of genomic DNA samples

Sample	Concentration (ng/µl)	A260/A280	A260/A230
S. infantis	2402.2	2.11	2.14
S. enteritidis	275.6	2.13	2.46
S. typhimurium	1607.7	2.14	2.06
E. coli DH5α	2152.9	2.14	2.09

Nucleic acids and proteins exhibit maximum absorbance at 260 nm and 280 nm, respectively. Thereby, the ratio of these wavelengths (A260/A280) is very useful to determine purity of the sample. ~ 1.8 is considered as an optimum ratio for DNA molecules. A lower ratio than 1.8 is the indicator of contamination such as protein and chemical contaminations. A260/A280 ratios of isolated genomic DNAs in this study are higher than 1.8. These higher ratios might result from pH of the solution, because a basic solution increases the ratio by 0.2-0.3. In spite of higher ratios, each sample was seamlessly used in further procedures.

In addition, absorbance at 230 nm is considered as an indicator of contamination such as phenol. For this reason, the A260/A230 value is also calculated and the preferred value is within the range of 2.0 and 2.2. Only, A260/A230 of *S.* enteritidis sample does not fit in the interval.

However, these ratios are not indicators of purity, alone. Spectrum profile is important for the sample quality, as well. Figures 3.2, 3.3, 3.4 and 3.5 illustrate good spectrum quality in each sample.

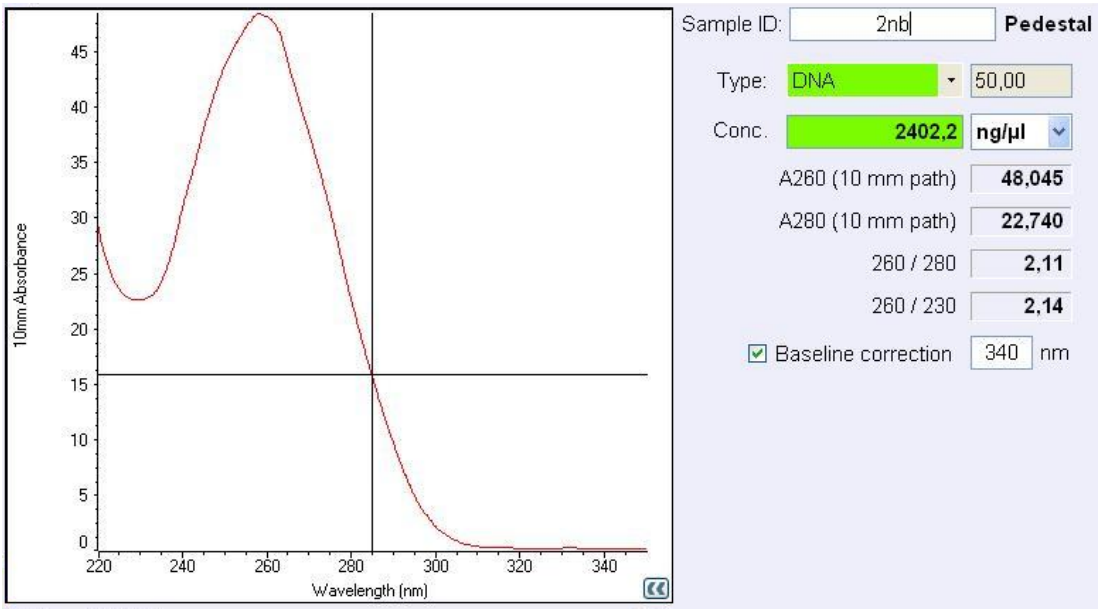

Figure 3.2 Spectrum result of *S.* infantis genomic DNA sample

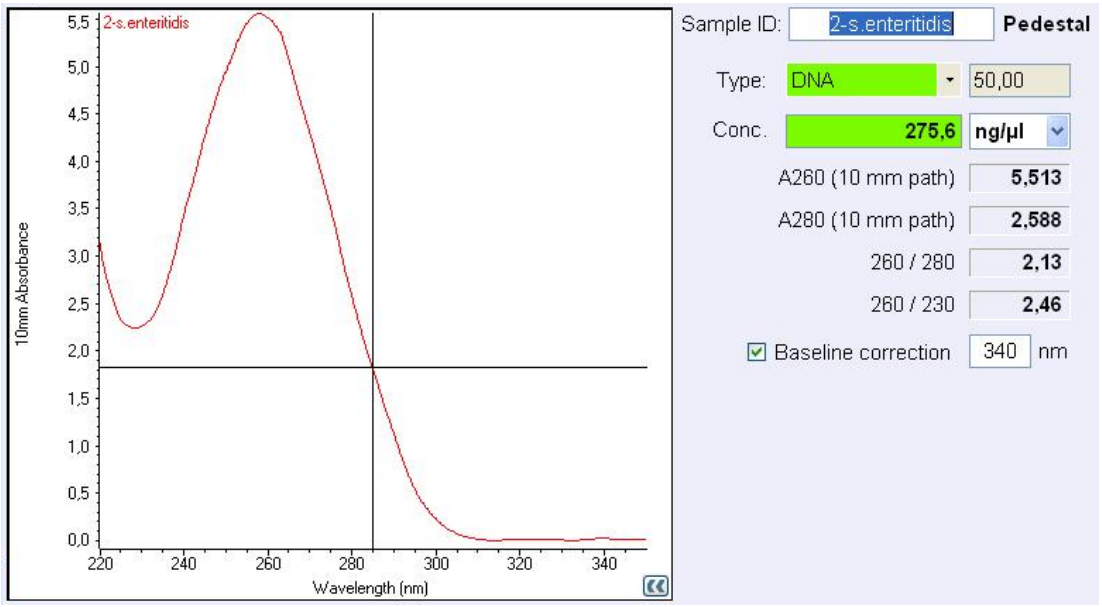

Figure 3.3 Spectrum result of *S.* enteritidis genomic DNA sample

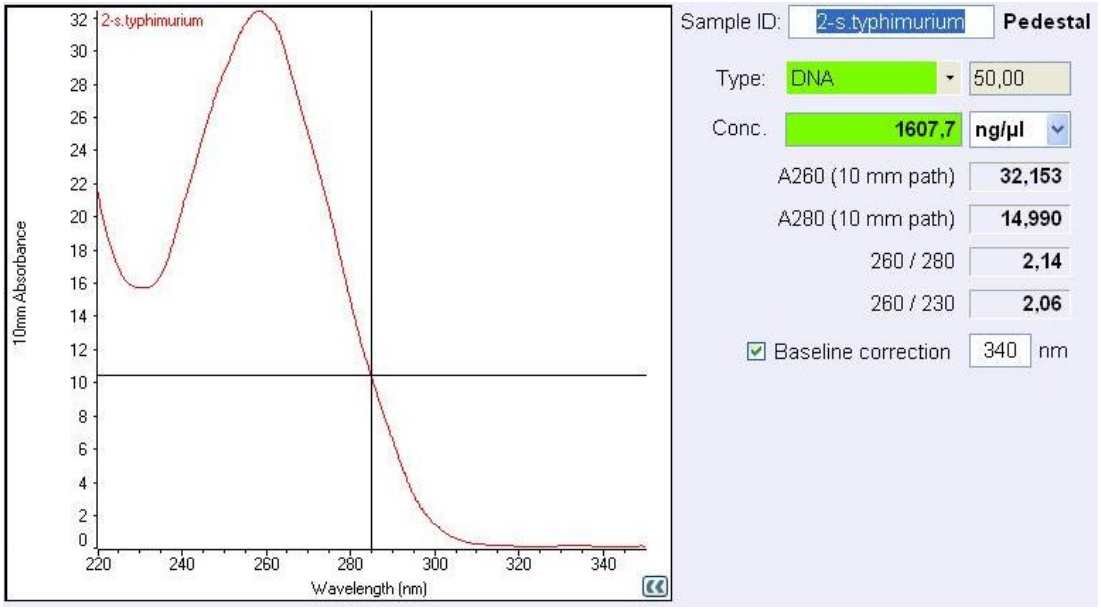

Figure 3.4 Spectrum result of *S.* enteritidis genomic DNA sample

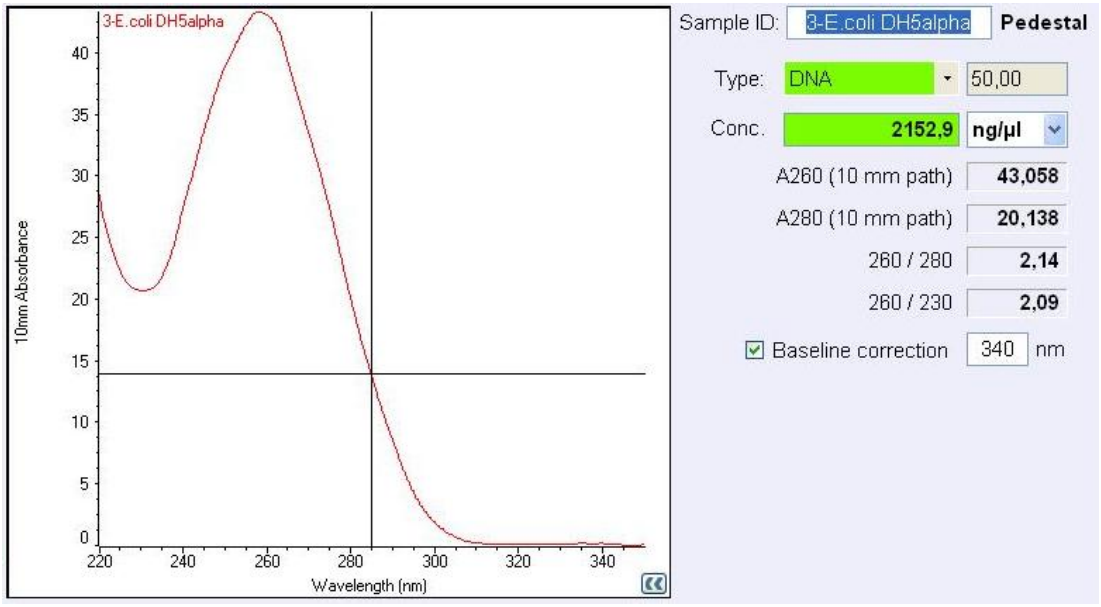

Figure 3.5 Spectrum result of *E. coli* DH5α genomic DNA sample

3.1.3. Agarose Gel Electrophoresis of PCR Amplicons

Two different regions of *invA* gene of *Salmonella* were amplified by PCR and used as targets in the lateral flow assay. Genomic DNAs of *S.* infantis, *S.* enteritidis and *S.* typhimurium were used as templates in PCRs. Additionally, *E. coli* DH5α and ultrapure water were also used as controls.

First amplicon was amplified using F 139 forward primer and R 141 reverse primer as previously described by Rhan *et al.*, 1992. The PCR yielded in a 284 bp fragment which is found in only *Salmonella*.

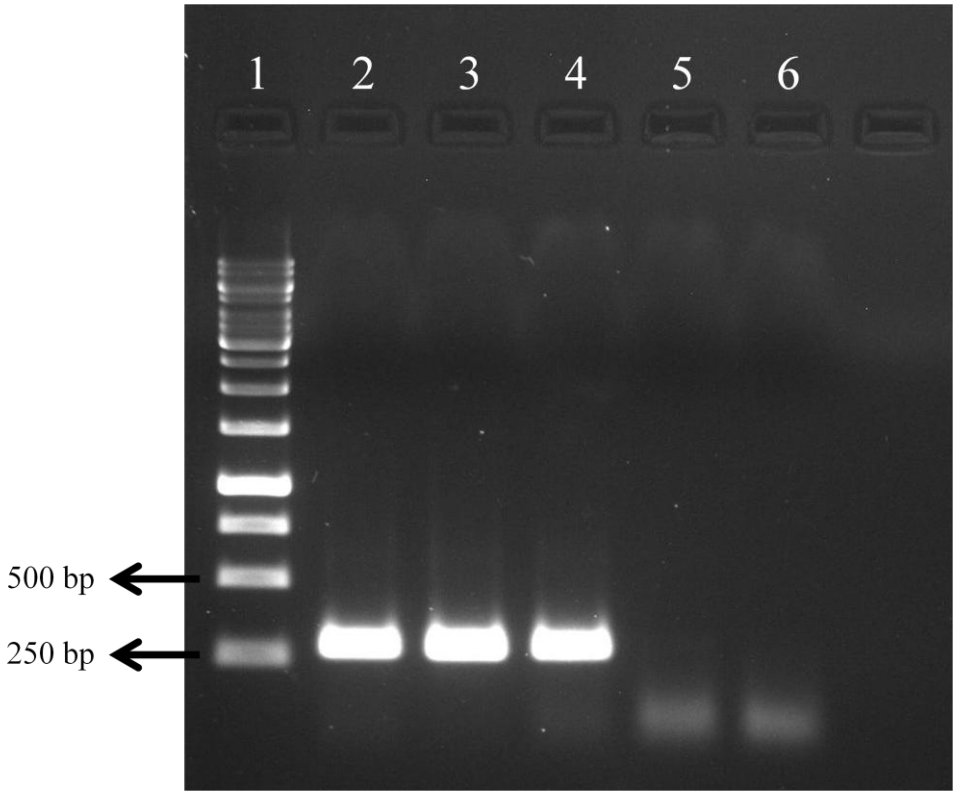

Figure 3.6 1.2 % agarose gel electrophoresis. Lane 1: 1 kb DNA ladder. Lanes 2, 3 and 4: 284 bp amplicons of *S*. infantis, *S*. enteritidis and *S*. typhimurium, respectively. Lane 5: *E. coli* DH5α. Lane 6: Control for PCR

Additionally, a shorter, 105 bp fragment of the same gene was amplified using F 139 forward primer and another reverse primer. Because same forward primers were used in both reactions, sequence of the 105 bp amplicon is completely identical to first 105 bp of the 284 bp amplicon.

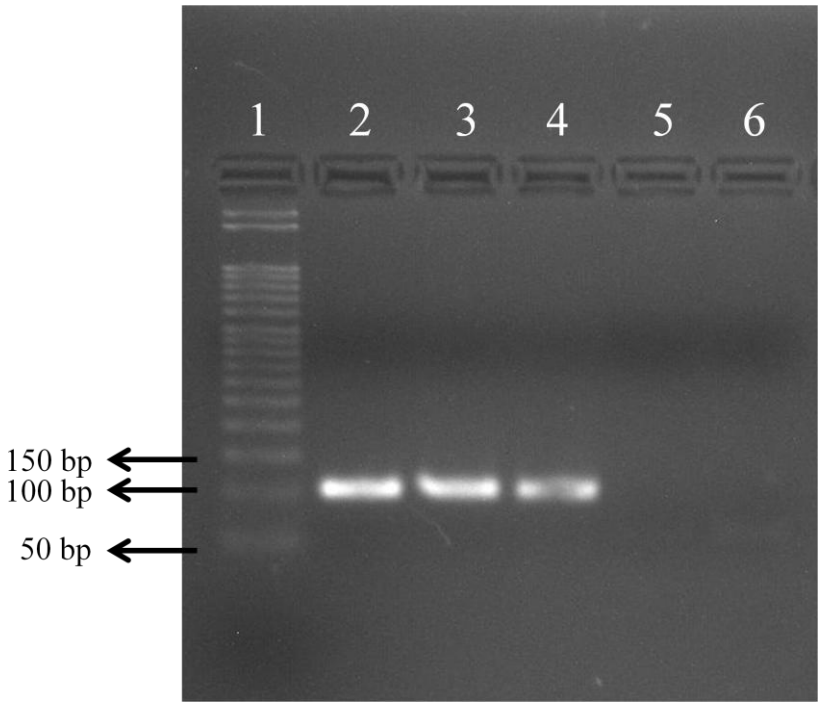

Figure 3.7 2.5 % agarose gel electrophoresis. Lane 1: 50 bp DNA ladder. Lanes 2, 3 and 4: 105 bp amplicons of *S.* infantis, *S.* enteritidis and *S.* typhimurium, respectively. Lane 5: *E. coli* DH5α. Lane 6: Control for PCR

Multiple cloning site (MCS) of pUC19 plasmid was also amplified by PCR in order to apply to lateral flow assays as a control. M13 forward and M13 reverse primers which yielded in 103 bp MCS were used in the amplification reaction.

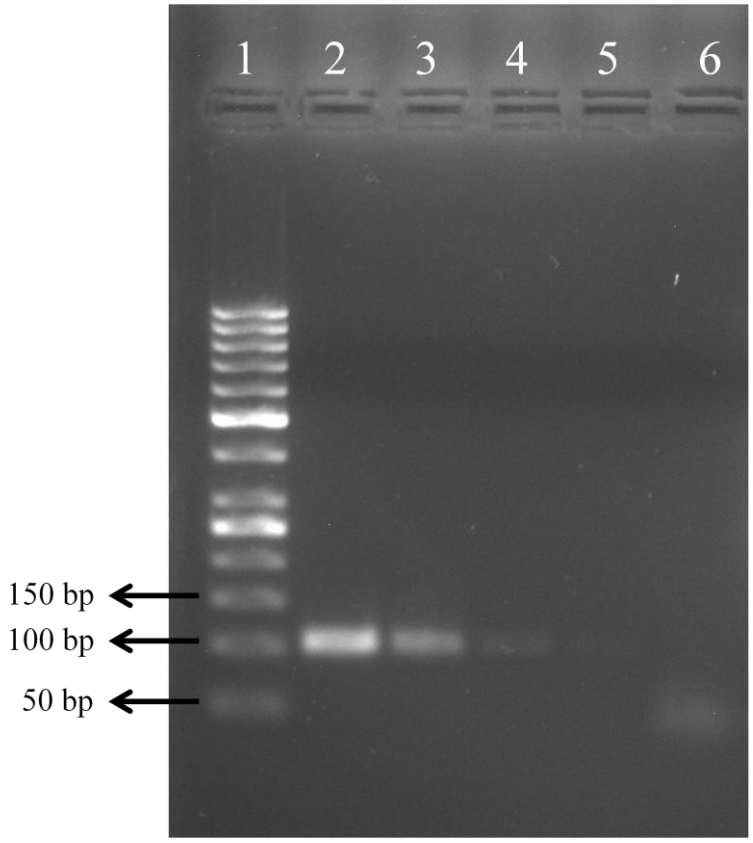

Figure 3.8 2.5 % agarose gel electrophoresis of MCS with different dilutions. Lane 1: 50 bp DNA ladder. Lanes 2, 3, 4 and 5: undiluted, 1:2 diluted, 1:10 diluted and 1:25 diluted MCS, respectively. Lane 6: control for PCR

3.2. Synthesis and Characterization of Gold Nanoparticles

Gold nanoparticles (AuNPs) with an average diameter of 25 nm were synthesized by the $HAuCl_4$ reduction method. After the synthesis, characterizations were done with respect to the optical property, size and charge of AuNPs by UV-visible spectroscopy, DLS and zeta potential analyses.

3.2.1. UV-Visible Spectroscopy of AuNPs

Optical properties of AuNPs are directly correlated with their size and shape. Because of this property, the synthesized AuNPs can be characterized by analyzing the absorption maxima. Spherical colloidal AuNPs generally have absorption maxima between 500 nm and 570 nm. Smaller AuNPs absorb the light and have absorption maxima near 520 nm whereas the larger particles have peaks near longer wavelengths. In addition, the optical properties and absorption patterns of AuNPs

significantly alter when aggregation occurs. In the case of aggregation, absorption peaks become broader and red-shift is observed. Therefore, UV-visible spectroscopy can be performed to monitor stability of particles.

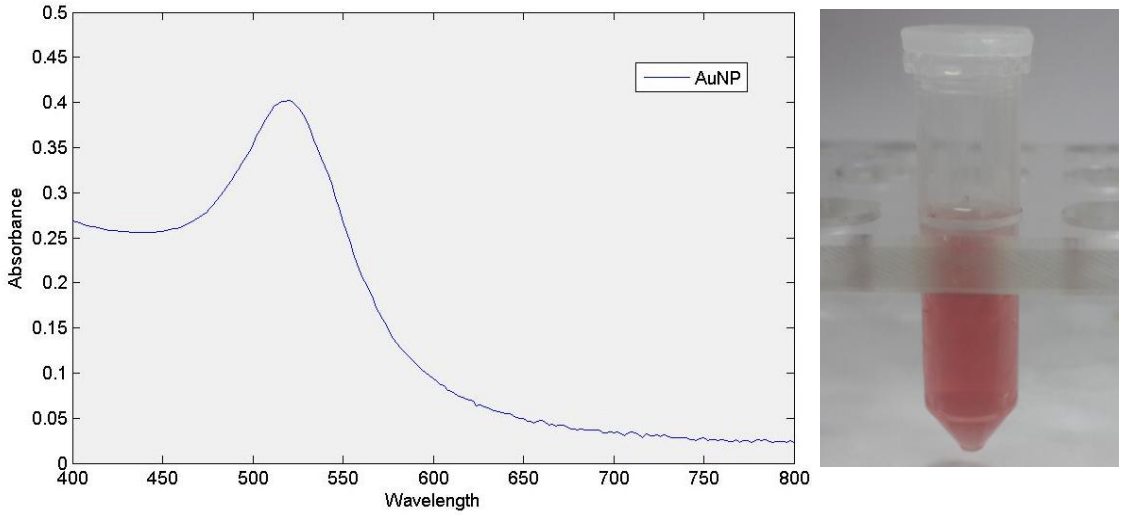

Figure 3.9 Absorption speactrum (left) and visual appearance (right) of synthesized AuNPs

Figure 3.9 illustrates the absorption spectrum of AuNPs. The spectrum pattern and maximum absorption at 522 nm indicate pure, unaggregated and stable solution of AuNPs with average diameter 25 nm. Visual appearance of the solution also points out no aggregation occurred during synthesis, since aggregation changes color of the solution from red to blue.

3.2.2. Dynamic Light Scattering Analysis of AuNPs

Although the sizes of AuNPs were determined and confirmed with UV-Visible spectroscopy, a second analysis was also performed. In this analysis, both particle size and size distribution were measured by Dynamic Light Scattering (DLS) which is one of the most preferred analytical methods for the characterization of AuNPs. In the measurement, particles are exposed to a laser beam and then a scattering pattern of the light is observed to conclude the particle size.

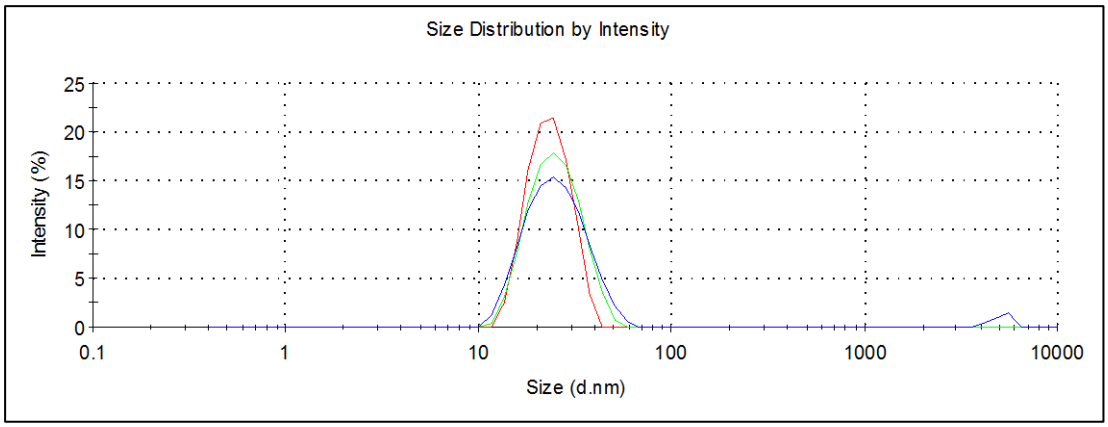

Figure 3.10 DLS histogram of the synthesized AuNPs

Since same sample is subjected to three different measurements, three different lines (red, green and blue) are presented on the histogram. Each line indicates the distribution of particle intensities. All of the curves are accumulated in the same narrow interval which means size distribution of particles is not too variable. In addition, the average diameter of AuNPs in the solution is approximately 25 nm. Overlapping narrow peaks also indicate that aggregation did not occur.

3.2.3. Zeta Potential of AuNPs

The term "zeta potential" describes the electrostatic potential around the particle surface. Nanoparticles bearing a zeta potential less than -10 mV or greater +10 mV are considered as charged and more likely remain stable in the solution.

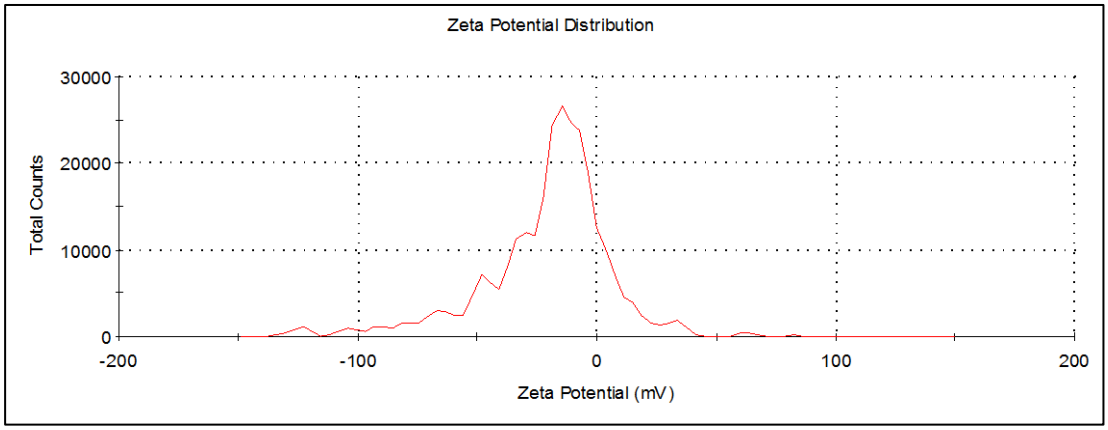

Figure 3.11 Zeta potential distribution of 25 nm AuNPs at neutral pH

As presented in Figure 3.11, the synthesized AuNPs have a zeta potential of -12.5 mV which indicates that they are anionic.

During the synthesis of AuNPs in aqueous solutions, cetyltrimethylammonium bromide (CTAB) is also added. CTAB serves as an antiseptic against bacterial and fungal contaminations. In addition, it functions as a surfactant and undergoes strong interactions with AuNPs by stabilizing them. However, AuNPs synthesized during this study were freshly used. Therefore surfactants like CTAB were not used in the synthesis. Also all the characterizations indicate that AuNPs are anionic, stable and convenient for the functionalization with DNA.

3.3. Functionalization of AuNPs with Thiolated DNA probes

25 nm AuNPs were functionalized with 5' thiol modified DNA probes in order to obtain signal probes for lateral flow assay. Thiol modified DNA probe 0 was complementary to the half of synthetic target. On the other hand, thiol modified DNA probe 1 and 2 were complementary to 3' ends of 105 bp and 284 bp amplicons of *invA*. DNA probes were started to be called signal probes after conjugation with AuNPs.

Thiol modified DNA probes were pre-treated with TCEP before conjugation. TCEP is a reducing agent and activates the thiol modified probe. There is also another reducing agent known as DTT. However, TCEP has some advantages over DTT and other agents such as higher reducing capacity, increased hydrophilicity and stability at high pH and temperatures. Additionally, DTT is very susceptible to oxidation while TCEP is more resistant.

Intermolecular electrostatic repulsions between DNA and AuNP prevent DNA-AuNP conjugation since they are both negatively charged. To overcome this issue and maximize conjugation capacity, the solution containing activated DNA probe and AuNPs is subjected to salt aging with PBS. If DNA cannot adsorb onto AuNPs, salt aging causes aggregation in which color of the solution turns into blue, thereby it also serves as a determiner for adsorption (Figure 3.12).

After salt aging, excess salt and DNA probes were removed by centrifugation and remaining pellet was resuspended in 20 mM phosphate buffer containing 5 % BSA,

0.25 % Tween20 and 10 % sucrose. The resuspension buffer stabilizes AuNPs and also contributes to release of signal probes from conjugate pad during the assay procedure. In addition, it prevents non-specific capturing of signal probes on the test line.

Signal probes 1 and 2 were subjected to some characterizations such as UV-Visible spectroscopy, DLS and zeta potential analysis.

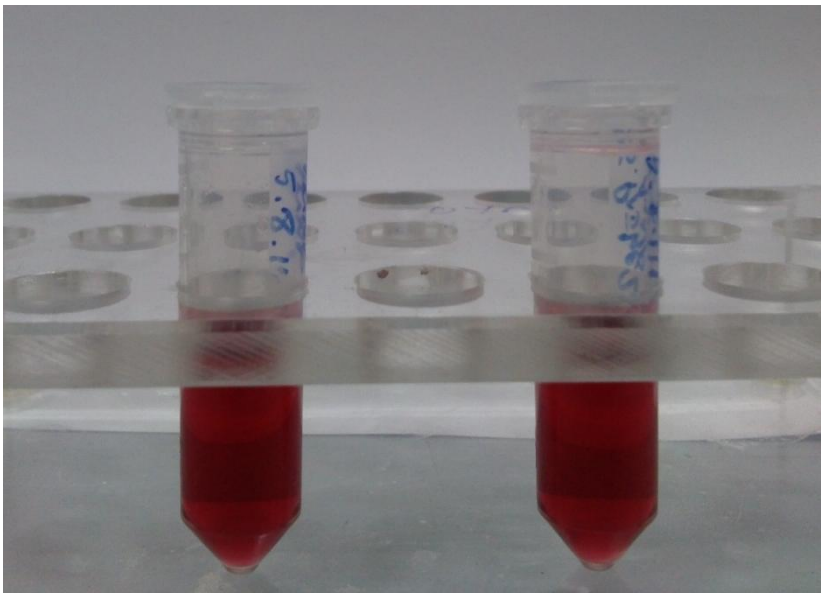

Figure 3.12 Signal probe 1 and signal probe 2, respectively

3.3.1. UV-Visible Spectroscopy of Signal Probes

Red-shift of absorption spectrum by a few nm is observed, after the adsorption of DNA onto AuNP surface. Therefore, UV-Visible spectroscopy was performed for signal probe 1 and signal probe 2 in order to monitor functionalization of AuNPs.

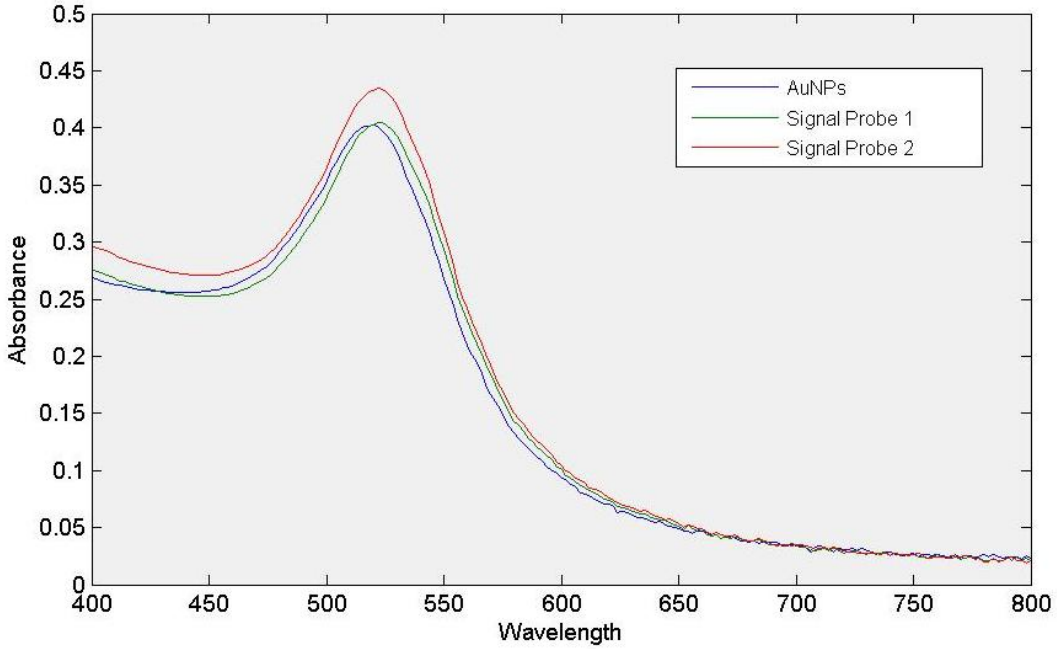

Figure 3.13 Comparison of absorption spectra AuNPs, Signal Probe 1 and Signal Probe 2

Figure 3.13 represents absorbance spectra of AuNPs (blue), signal probe 1 (green) and signal probe 2 (red) from bottom to top. When compared to AuNPs, both signal probe 1 and signal probe 2 red-shifted about 2 nm and had absorption maxima at 524 nm. These changes in absorption maxima point out DNA-AuNP conjugation. Besides, it can be concluded that aggregation did not occur during and after functionalization studies since aggregation causes broader absorption peaks.

3.3.2. Dynamic Light Scattering Analyses of Signal Probes

DLS is able to monitor not only the core of nanoparticle but also the surface modifications. Because adsorption of DNA probes onto AuNPs would cause an increase in the size of particles, DLS can be used to evaluate adsorption by measuring and comparing sizes of the modified particles (Signal probes 1 and 2).

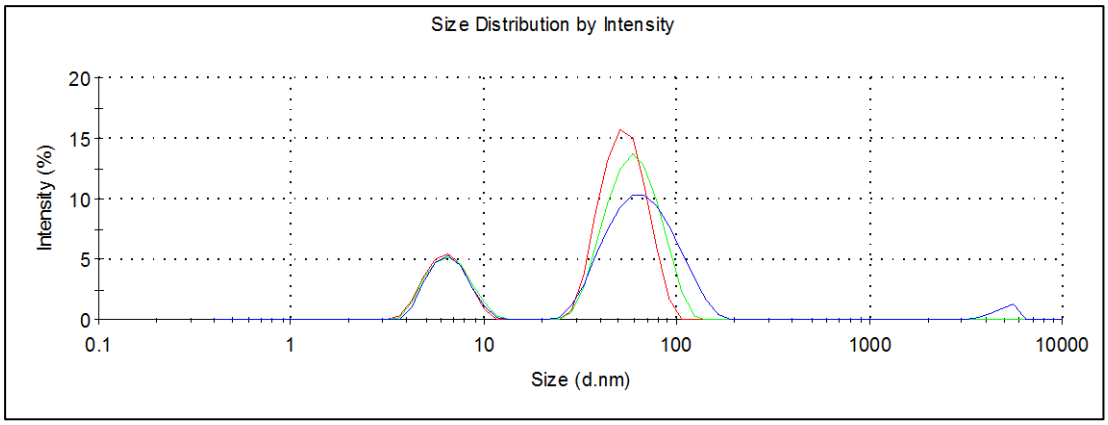

Figure 3.14 DLS histogram of signal probe 1

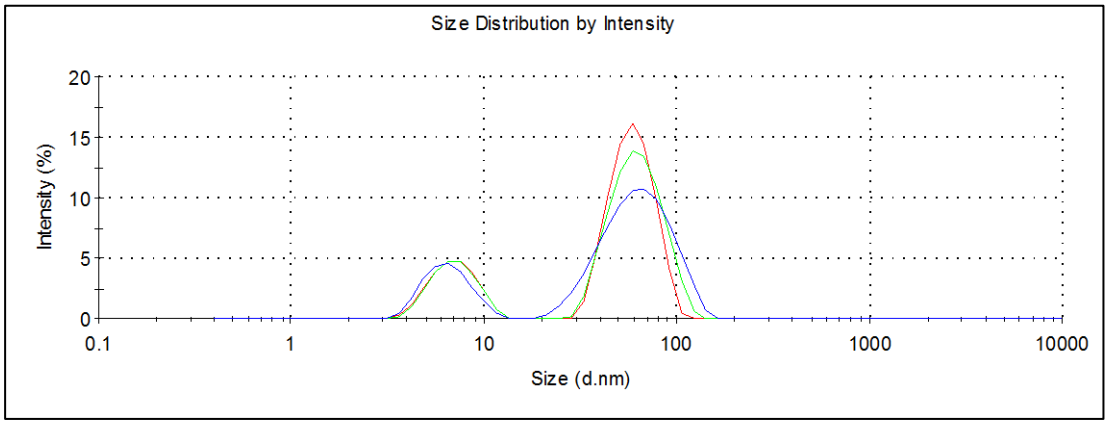

Figure 3.15 DLS histogram of signal probe 2

By considering the peaks on the right side of the histograms (Figures 3.14 and 3.15), it can be inferred that sizes of both signal probe 1 and signal probe 2 are evidently larger than AuNPs because of the DNA probes on the particle surfaces. Despite curves on the right hand side of the both histograms are accumulated in a narrow interval, they do not completely overlap with each other which indicates varying size distribution of signal probes. Varying sizes of signal probes are most likely caused by conformational changes which are a result of bending of DNA molecules on the particle surfaces.

Smaller curves on the left side of the both histograms indicate the presence of unconjugated DNA molecules. Although the final solution was centrifuged and washed to remove excess DNA during the functionalization process, there were still

small fractions in both solutions. Most probably, these fractions can be eliminated by additional washing steps. However, they are present in very low amounts thereby their presence did not affect the any of assay procedures.

3.3.3. Zeta Potentials of Signal Probes

Signal probe 1 and signal probe 2 were also subjected to zeta potential analyses to determine their electrostatic potentials and charges.

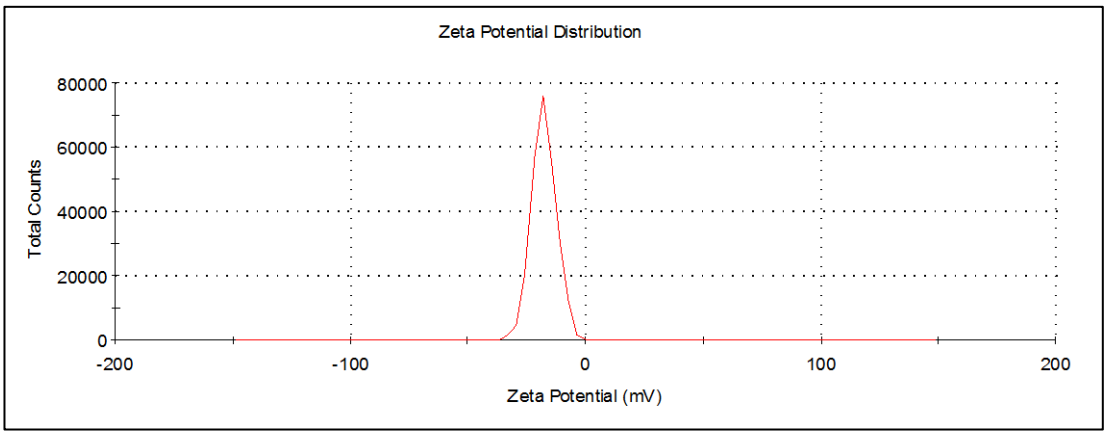

Figure 3.16 Zeta potential distribution of signal probe 1

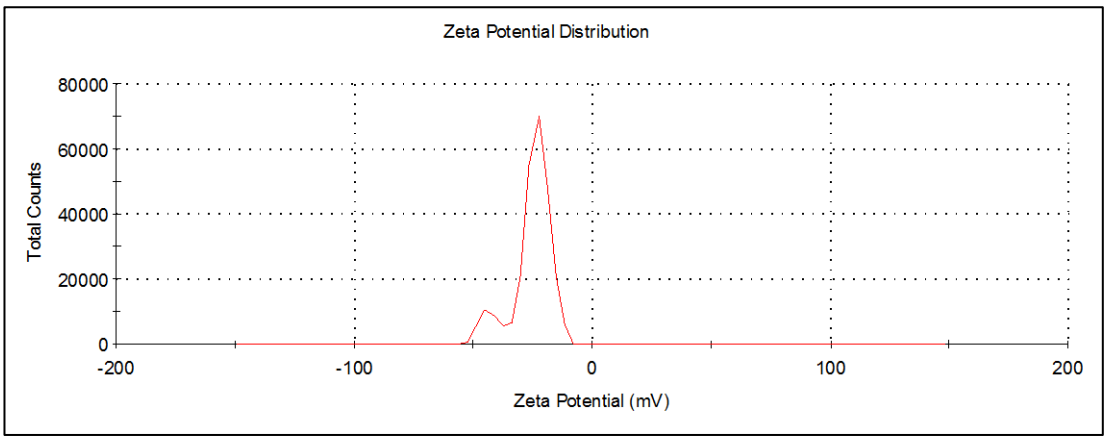

Figure 3.17 Zeta potential distribution of signal probe 2

Figures 3.16 and 3.17 show the zeta potential distribution of signal probes. According to the histograms, signal probes 1 and 2 are more negatively charged than AuNPs, -22 mV and -19 mV, respectively. Decreases in the zeta potentials are caused by functionalization of AuNPs with negatively charged DNA probes.

To sum up, each of the characterization studies including UV-Visible spectroscopy, DLS and zeta potential analyses confirms that 25 nm AuNPs were properly functionalized with DNA probes for the formation of signal probes.

3.4. Fabrication of Lateral Flow Test Strips

LFI test strips used in this study were fabricated by assembling four components on an overlapping sequence. The sample pad was treated with 0.05M tris buffer containing 0.25 % Triton X-100, 0.15 M NaCl prior to assembly. Pre-treatment with this buffer was made to adjust pH of the pad. It also contributes to the assay by facilitating releasing and moving of the target DNA across the strip. The conjugate pad was used as a platform for signal probes to hybridize with the target DNA. The nitrocellulose membrane served an environment for hybridization and capturing of signal probe-target complexes. For this purpose, test lines were created by immobilization biotinylated capture probes onto the surface via a biotin-streptavidin interaction. The absorbent pad was used with any modifications (Figure 3.18).

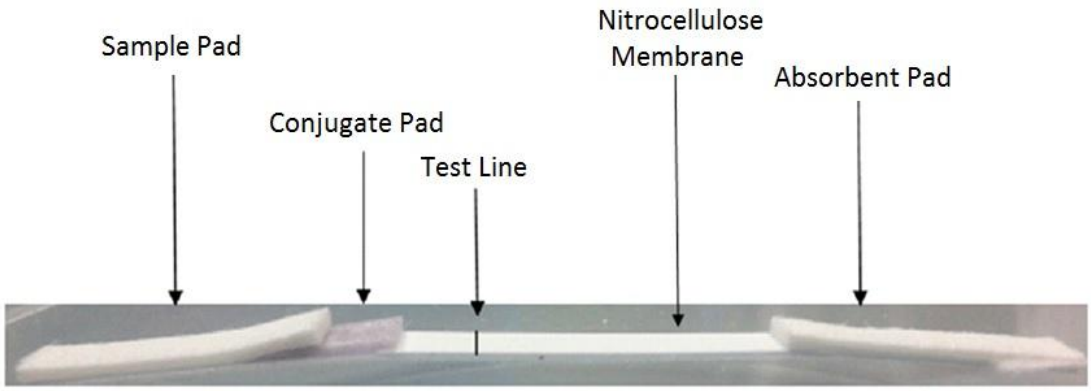

Figure 3.18 Components of lateral flow immunochromatographic test strip

3.5. Assay Results

Three different sets of LFIAs were developed for detection of target nucleic acid molecules. In the first assay that used a 60 base long synthetic *Salmonella* target, signal probe 0 and capture probe 0 were used to establish and optimize assay procedures with respect to time, concentration and temperature. Upon optimization of the assay, second and third assays were generated for detection of 105 bp amplicon and 284 bp amplicon of *invA*, respectively. Combination of signal probe 1 and capture probe 1 were used in the second assay while the third assay used signal probe 2 and capture probe 1 together.

Working principle of the nucleic acid based LFIA is based on capturing the target sequence on the test line by sandwich hybridizing it with capture probe and signal probe (Figure 3.19). When the target solution is applied to the sample pad, it starts to be migrated by the capillary force. Firstly, it moves to the conjugate pad where it hybridizes with the complementary sequence of the signal probe. Then the signal probe-target complex continues to migrate towards the test line where a second hybridization reaction takes place. The capture probe on the test line hybridizes with the remaining part of the target sequence.

Nitrocellulose membrane is one of the important parameters for the assay. Assay time which depends on the migration time of the applied solution directly affect the hybridization time and sensitivity. Therefore, Hi-Flow Plus 240 membrane cards were used in this study due to their relatively longer assay time and increased sensitivity.

As a result of sandwich hybridization, AuNPs accumulate in the test line and form a naked-eye detectable red band. Qualitative analysis can be easily done by visually observing the forming red band.

However, if the applied solution does not contain the target sequence, no color change is observed in the test line.

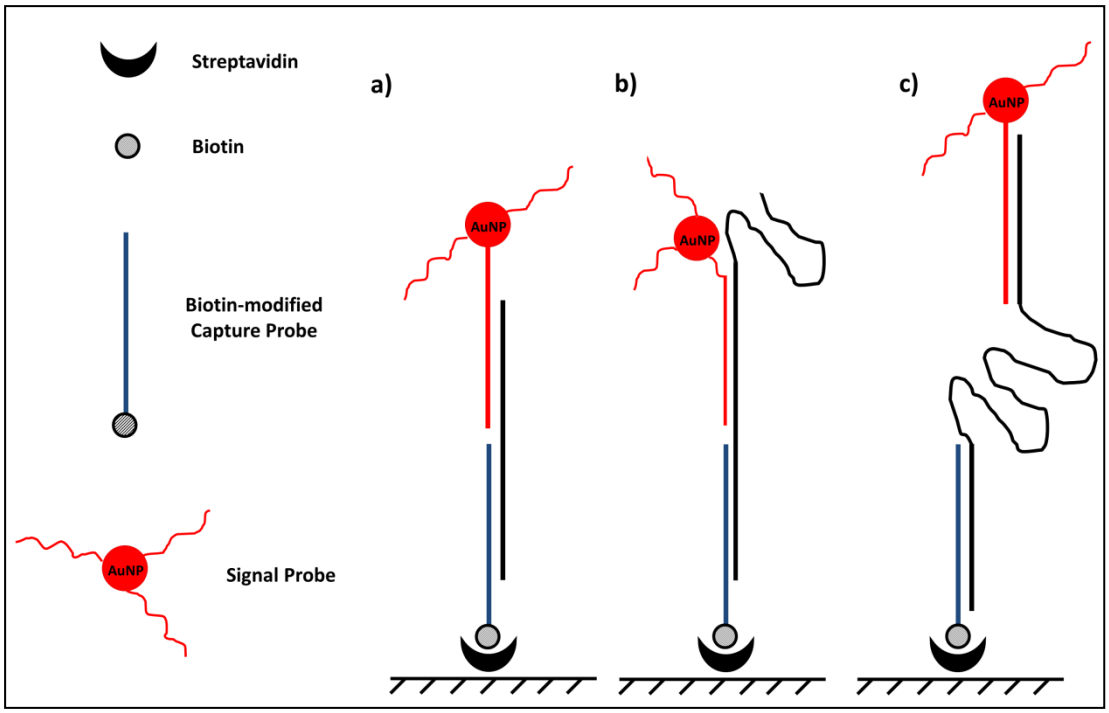

Figure 3.19 Illustration of detection of target nucleic acids by sandwich hybridization. **a**, **b**, and **c** represent the synthetic target, 105 bp amplicon and 284 bp amplicon, respectively

3.5.1. Assay Results of Synthetic Target

As the target, *Salmonella*-specific synthetic oligonucleotide was applied to the assay which is comprised of signal probe 0 and capture probe 0, while an uncomplementary synthetic oligonucleotide was applied as the control. Both target and control oligonucleotides were brought to final concentrations of 1 µM with 5X SSC. As a second control, 5X SSC was also used in the assay. Upon applied solutions reached the absorbent pad in 10 minutes, strips were washed with 5X SSC.

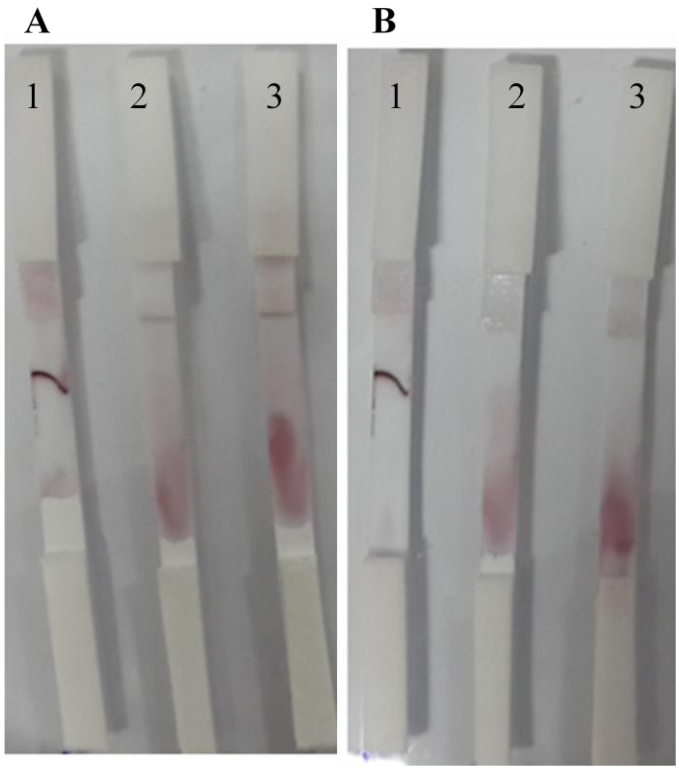

Figure 3.20 Assay results of synthetic target. Figure A shows the strips before washing and figure B shows the strips after washing with 5X SSC. Synthetic target, uncomplementary control oligonucleotide and 5X SSC were applied to the strips 1, 2 and 3, respectively

Figure 3.20 represents the photo images of assay designed for synthetic target before and after washing with 5X SSC. In the sample containing the target, signal probes were capture in the test line by the capture probe by sandwich hybridization. Thus a naked-eye detectable red band in the test line indicating a positive result occurred as expected. Additionally, in the control oligonucleotide applied and 5X SSC applied strips, no band was observed which means a negative result. Only non-specific red regions occurred as a result of sticking of signal probes in the nitrocellulose membrane. These non-specific forms can be easily discerned from a positive result.

3.5.2. Assay Results of 105 bp amplicon

Firstly, 105 bp amplicon of *S.* infantis was used to monitor and evaluate the detection of PCR products. For this, 1:2 and 1:3 diluted target was applied as target. In addition, 5X SSC and 1:5 diluted uncomplementary MCS were also used as control. After 10 minutes of assay procedure, each strip was washed with 5X SSC.

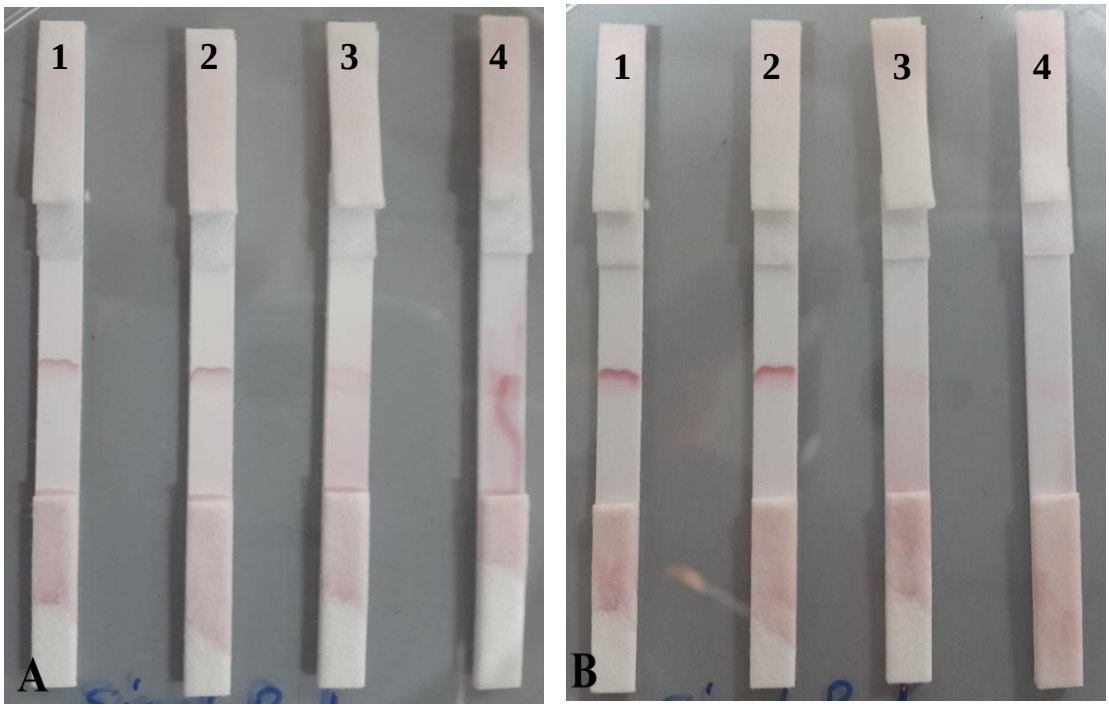

Figure 3.21 Assay result of 105 bp amplicon of *S.* infantis. Figures A and B represent the assay results before washing and after washing, respectively. 1:2 diluted and 1:3 diluted targets are applied to strips 1 and 2, while strips 3 and 4 denote controls, 1:5 diluted MCS and 5X SSC, respectively

Figure 3.21 represents the assay result of 105 bp amplicon. As expected, both 1:2 diluted and 1:3 diluted targets were captured at the test line and naked-eye detectable red bands formed, which is indicating target was sandwich-hybridized. Besides, controls did not lead to formation of such bands. Some non-specific binding occurred in the other regions of the strip. However, these non-specific color changes can be easily differentiated from a positive result. On the hand, washing step did not only remove non-specific bindings but also it increased intensities of the bands.

Upon optimization of the assay for a PCR product, 105 bp amplicons of other *Salmonella* serotypes including *S.* enteritidis, *and S.* typhimurium were separately applied to the assay. Prior to application, targets were diluted with 5X SSC in the following ratios; 1:2, 1:3, 1:5 and 1:10.

Additionally, 1:2 and 1:5 diluted uncomplementary MCS, 1:2 diluted PCR mix containing no DNA and 5X SSC were also applied as controls. After 10 minutes of assay procedure, each strip was washed with 5X SSC.

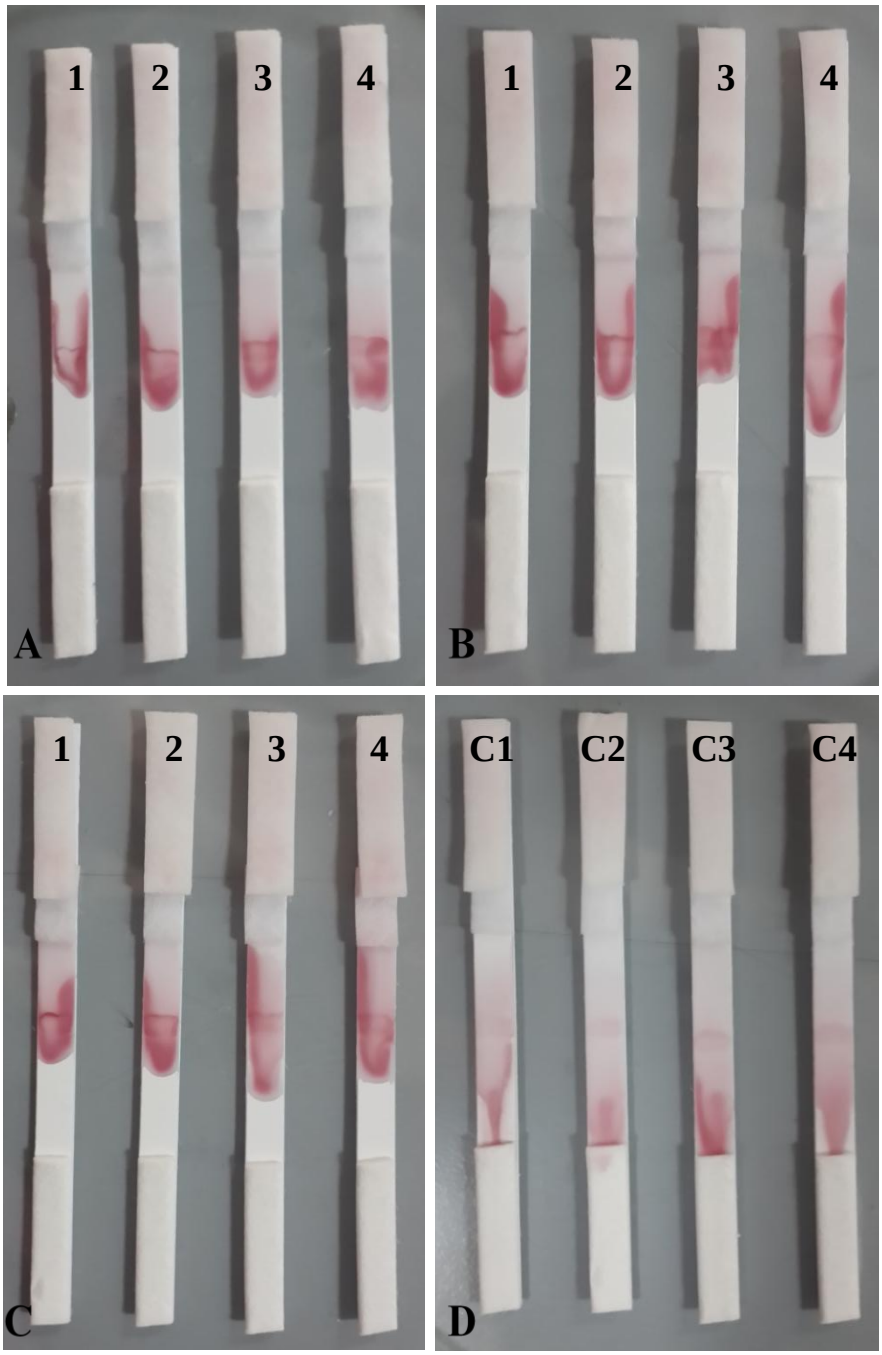

Figure 3.22 Results of the assay, before washing. Figures A, B and C represent sources of the 105 bp target, *S.* infantis, *S.* enteritidis, *and S.* typhimurium, respectively. Numbers 1, 2, 3 and 4 in each figure indicate the dilution ratio of the target; 1:2, 1:3, 1:5 and 1:10, respectively. Controls used in the assay are shown in Figure D; 1:2 diluted MCS, 1:5 diluted MCS, 1:2 diluted PCR mix containing no DNA and 5X SSC, respectively

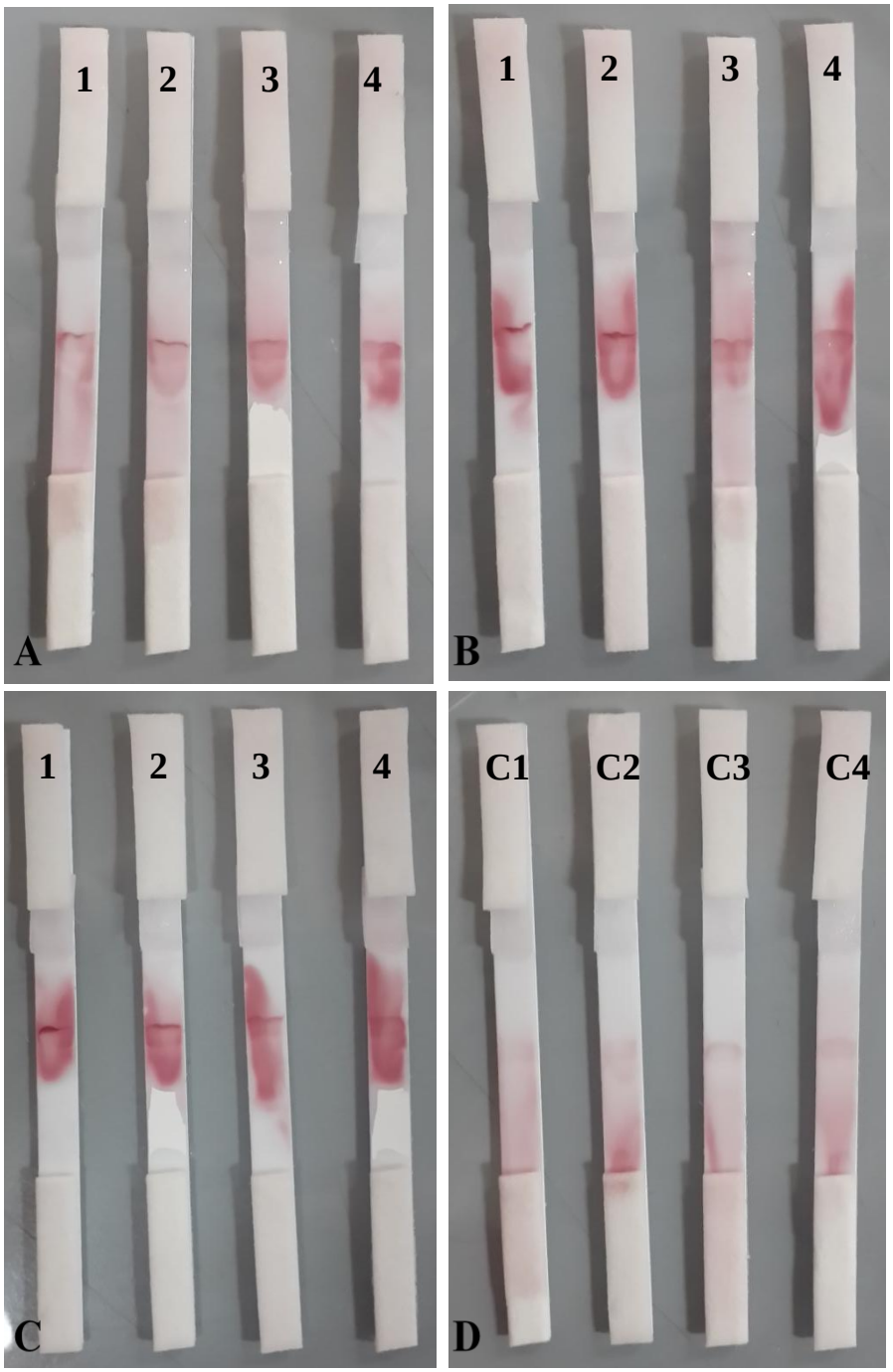

Figure 3.23 Results of the assay, after washing

Figures 3.22 and 3.23 show the assay results of 105 bp amplicon targets without washing and with washing, respectively. All targets caused formation of visible red bands in the test line. However, 1:2 diluted, 1:3 diluted, and 1:5 diluted targets constituted relatively sharper bands which might be a result of higher target concentrations. All controls including MCS, PCR mix, and SSC did not form any bands. Despite, bands can evidently observed, washing with 5X SSC was performed to eliminate non-specific bindings and clogging around the test line. With the washing step, red bands on the test line became sharper and non-bindings in the control assay disappeared.

As an additional control, 284 bp amplicon was also applied to the assay designed for detection of 105 bp amplicon.

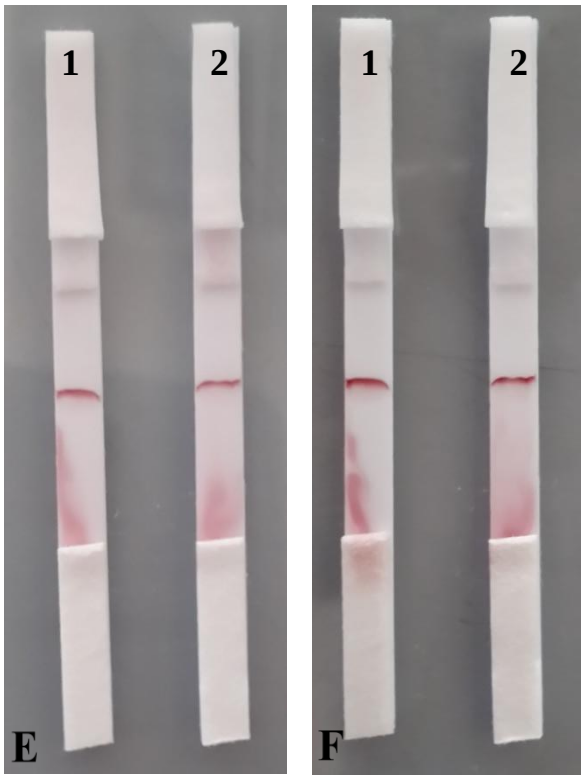

Figure 3.24 Results of 284 bp amplicon application to the assay. E and F denote assay results before washing and after washing, respectively. 1:2 diluted 284 bp amplicon was applied to strip 1 while 1:3 diluted was applied to strip 2

105 bp and 284 bp amplicons were amplified from the same region of *invA* gene by using same forward primer and two different reverse primers. So, PCR resulted in one short and one long fragments of the gene region. In other words, 284 bp amplicon contains entire sequence of 105 bp amplicon.

Therefore, since first 105 base pairs of 284 bp amplicon is completely same with sequence of 105 bp amplicon, the lateral flow assay designed for detection of 105 bp amplicon is also capable of detecting 284 bp amplicon (Figure 3.24)

3.5.3. Assay Results of 284 bp amplicon

284 bp amplicons of *S.* infantis, *S.* enteritidis, *and S.* typhimurium were separately applied to the third assay which is comprised of capture probe 1 and signal probe 2. Prior to application, targets were diluted with 5X SSC in following ratios; 1:2, 1:3, 1:5 and 1:10.

Additionally, 1:2 and 1:5 diluted uncomplementary MCS, 1:2 diluted PCR mix containing no DNA and 5X SSC were also applied as controls. After 10 minutes of assay procedure, each strip was washed with 5X SSC.

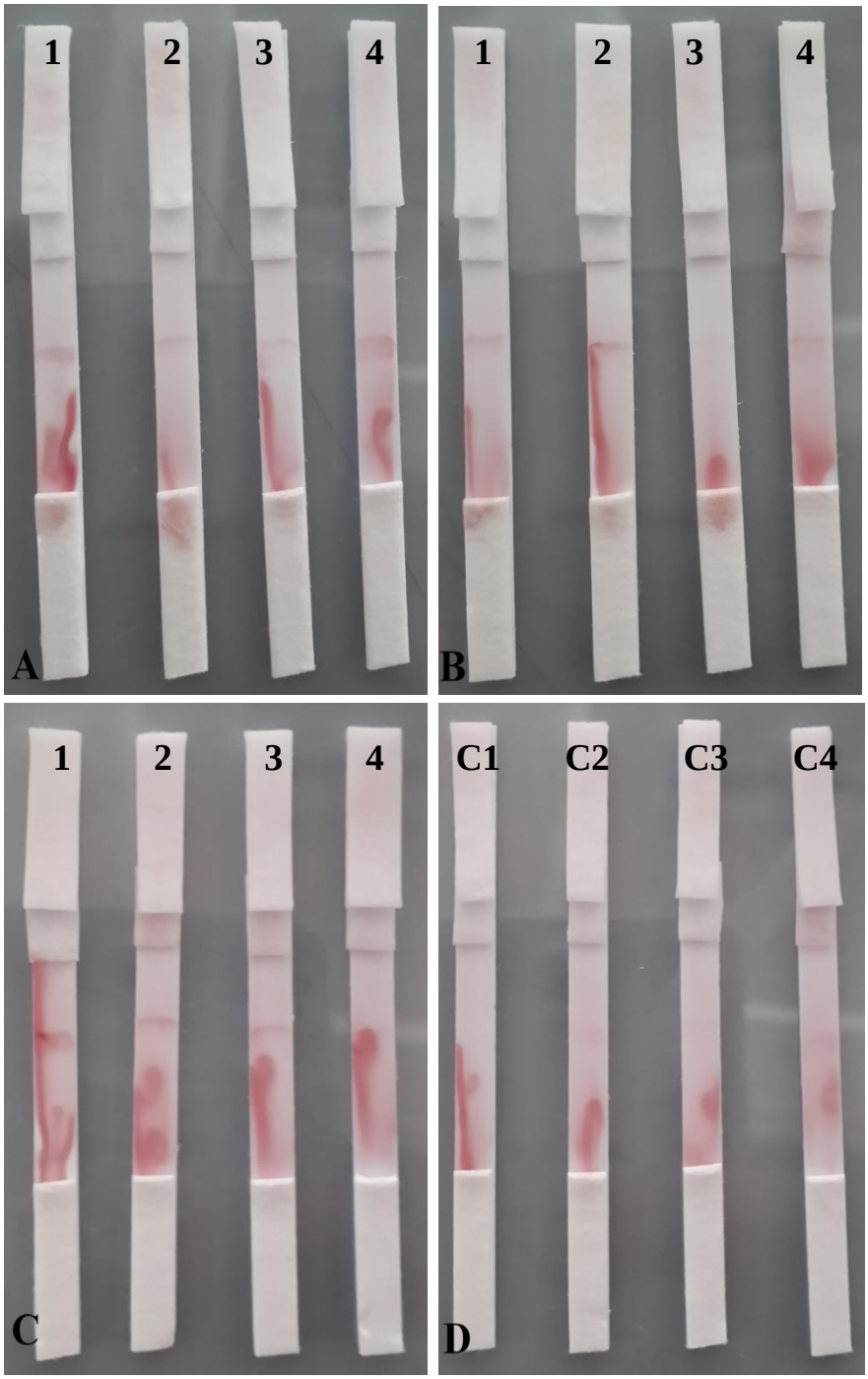

Figure 3.25 Results of the assay, before washing. Figures A, B and C represent sources of the 284 bp target, *S.* infantis, *S.* enteritidis, *and S.* typhimurium, respectively. Numbers 1, 2, 3 and 4 in each figure indicate the dilution ratio of the target; 1:2, 1:3, 1:5 and 1:10, respectively. Controls used in the assay are shown in Figure D; C1, C2, C3 and C4 are 1:2 diluted MCS, 1:5 diluted, 1:2 diluted PCR mix containing no DNA and 5X SSC, respectively

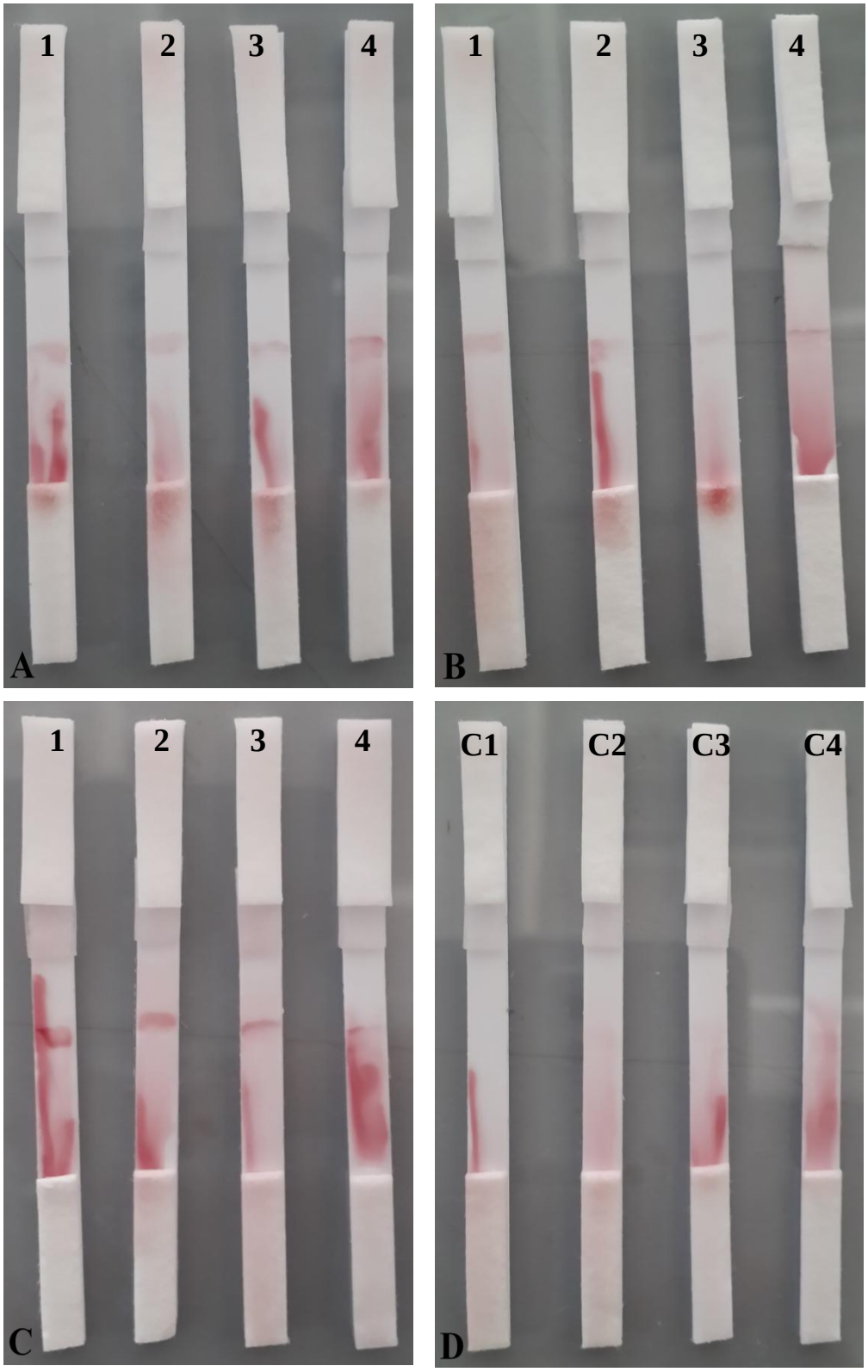

Figure 3.26 Results of assay, after washing

Figures 3.25 and 3.26 represent the assay results of 284 bp amplicon targets without washing and with washing, respectively. All targets expect for B3 resulted in red band in the test line with lower intensity. Any of controls did not form bands, at all. Lower color intensity might be caused by relatively longer length of 284 bp amplicons.

Capture probe and signal probe were designed as complementary to 5' end and 3' end of 285 bp amplicon, respectively. In other saying, probes are complementary to two distinct region of the target. So, longer length might have caused change in conformational changes by bending. As a result of bending, hybridization rate of probes with the target might have decreased.

As an additional control, 105 bp amplicon was also applied to the assay designed for detection of 284 bp amplicon.

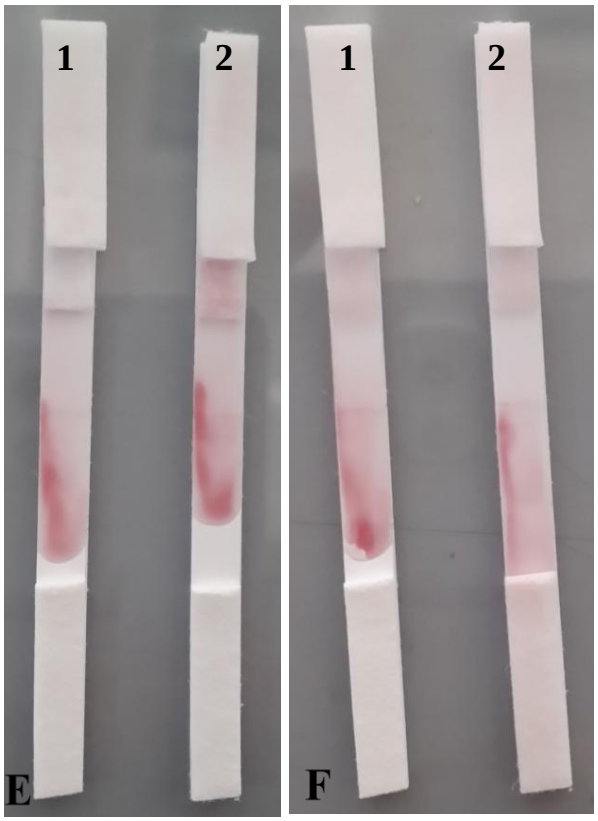

Figure 3.27 Results of 105 bp amplicon target application to the assay. E and F denote assay results before washing and after washing, respectively. 1:2 diluted 105 bp amplicon was applied to strip 1 while 1:3 diluted was applied to strip 2

When 105 bp amplicon was applied to the assay designed for 284 bp amplicon, no visible band occurred, as expected. Because it lacks the sequence required for hybridization with the signal probe 2. Therefore, red bands in the test line formed neither before washing nor after washing.

All the assays were performed with serial dilutions of each target sequence to evaluate limit of the detection. Generally, 1:2 diluted, 1:3 diluted and 1:5 diluted targets yielded better results when compared to the 1:10 diluted targets. Each assay was experimented as at least three replicates to take reproducibility into account. Assay worked out and allowed detection of the specific target, each time. Since every component of the test strip was prepared by hand, an automated fabrication system would increase the reproducibility.

Additionally, different controls were performed in order to assess the assay in a complex background. Along with the targets, uncomplementary MCS was also applied to assay to examine whether a non-specific hybridization would occur. 5X SSC assay buffer was subjected to the assay to check potential effects, as well. As a last control, no DNA containing PCR mix was experimented due to its complex content that could have interfered with the assay. However, none of the controls caused a red band in the test line, and they did not affect the assay procedure, either.

CHAPTER 4

CONCLUSION

In this study, a nucleic acid based lateral flow immunochromatographic test platform coupled with conventional PCR was developed. The platform is based on visually detection of PCR amplified target sequences by sandwich hybridization format. The main advantage of hybridization is no requirement for labeling of the target. Assay was performed on nitrocellulose membrane which contains specific capture probe and AuNP-labeled signal probe for the target.

Before PCR amplified targets, a commercial synthetic target was used for the optimization of the assay. In this step, different parameters affecting the assay sensitivity such as AuNP synthesis, DNA adsorption onto AuNP surface, type of nitrocellulose membrane and immobilization of the capture probe were experimented. As an outcome of the optimization, PCR amplified real products were used as the target.

The assay, which yields the result in minutes, enables detection of *Salmonella* specific PCR products. The result is assessed by visually observing the formation of a red band in the test line. Therefore, the test platform developed in this study can be considered as a rapid, accurate, easy-to-use, and cost-effective detection method.

REFERENCES

Bhunia, A.K. (Ed.) (2008). Foodborne Microbial Pathogens: Mechanisms and Pathogenesis, Springer LLC, New York, NY.

Braden, C.R., and Tauxe, R.V. (2013) Emerging trends in foodborne diseases. Infectious Disease Clinics of North America, **27**(3), 517-533.

Broglia, A., and Kapel, C. (2011). Changing dietary habits in a changing world: emerging drivers for the transmission of foodborne parasitic zoonoses. Vet. Parasitol., 182, 2–13.

Centers for Disease Control and Prevention. (2013). Surveillance for foodborne disease outbreaks—United States, 2009–2010. MMWR Morb Mortal Wkly Rep., 62, 41–7.

Cheung, P.Y., and Kam, K.M., (2012). *Salmonella* in food surveillance: PCR, immunoassays, and other rapid detection and quantification methods. Food Research International, 45, 802-808.

Foley, S.L., and Grant, K.A. (2007). Molecular Techniques of Detection and Discrimination of Foodborne Pathogens and Their Toxins. In Simjee, S., (Ed.), Foodborne Diseases, Humana Press.

Galan, J.E., and Curtis, R. (1991). Distribution of *invA*, *-B*, *-C*, and *-D* genes *Salmonella typhimurium* among other *Salmonella* serovars: *invA* mutants of *Salmonella typhi* are deficient for entry into mammalian cells. Infection and Immunity, 59(9), 2901-2908.

Galan, J.E., Ginocchio, C., and Costeas, P. (1992). Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. Journal of Bacteriology, 174(13), 4338-4349.

Goldenberg, S. (2013). Molecular-based diagnostics, including future trends. Medicine, 41(11), 663-666.

Grabar, K.C., Freeman, R.G., Hommer, M.B., Natan, M.J. (1995). Preparation and characterization of Au colloid monolayers. Anal. Chem, 67, 735–743.

Harris, L.J., and Griffiths, M.W. (1992). The detection of foodborne pathogens by the polymerase chain-reaction (PCR). Food Res. Int., 25, 457-469.

He, Y., Zhang, S., Zhang, X., Balod, M., Gurung, A.S., Xu, H., Zhang, X., Liu, G. (2011). Ultrasensitive nucleic acid biosensor based on enzyme–AuNPs dual label and lateral flow strip biosensor, Biosens. Bioelectron., 26, 2018–2024.

Hu, J., Wang, S.Q., Wang, L., Li, F., Pingguan-Murphy, B., Lu, T.J., Xu, F. (2014). Advances in paper based point-of-care diagnostics. Biosensors and Bioelectronics, 54, 585–597.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., Uyttendaele, M. (2010). Alternative microbial methods: an overview and selection criteria. Food Microbiology 27, 710–730.

Jong, H., Parry, C., Poll, T., Wiersinga, W. (2012). Host-pathogen interaction in invasive salmonellosis. Plos Pathog., 8(10), 1-9.

Kaittanis, C., Santra, S.,Perez, J.M. (2010). Emerging nanotechnology-based strategies for the identification of microbial pathogenesis. Adv. Drug Deliv. Rev., 62(4–5), 408–423.

Kass, P. H., and Rieman, H. P. (2006). Epidemiology of Foodborne Diseases. In H.P. Rieman and D.O. Cliver (Eds.), Foodborne Infections and Intoxications, San Diego Academic Press, 3-26.

Labbe, R.G., and Garcia, S. (2001). Guide to foodborne pathogens. Wiley Publishers, Inc.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T., *et al*. (2003) Advances in biosensors for detection of pathogens in food and water. Enzyme Microb Technol, 32, 3-13.

Liu, J., and Lu, Y. (2006). Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes. Nature Protocols, 1:1, 246-252.

Lostroh, C.P., and Lee, C.A. (2001). The *Salmonella* pathogenicity island-1 type III secretion system. Microbes and Infection, 3(14-15), 1281-1291.

Löfström, C., Hoorfar, J., Schelin, J., Radström, P., Malorny, B. (2010). Salmonella. In Liu, D. (Ed). Molecular Detection of Foodborne Pathogens, CRC Press.

Mandal, P.K., Biswas, A.K., Choi, K., and Pal, U.K. (2011). Methods for rapid detection of foodborne pathogens: An overview. American J Food Tech, 6(2), 87-102.

Mandell, G.L., Bennett, J.E., Dolin, R. (Eds.), (2011) Mandell, Douglas and Bennett's principles and practice of infectious diseases. 5th ed., Philadelphia: Churchill Livingstone.

Mao, X., Ma, Y., Zhang, A., Zhang, L., Zeng, L., Liu, G. (2009) Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip. Anal Chem., 81(4), 1660-8.

Mastroeni, P., Grant, J. (2011). Spread of Salmonella enterica in the body during systemic infection: unravelling host and pathogen determinants. Expert. Rev. Mol. Med., 13, e12.

McEntire, J. (2013). Foodborne disease: The global movement of food and people. Infectious Disease Clinics of North America, **27**(3), 687-693.

Miliotis, M.D., Bier, J.W. (Eds.), (2003). International Handbook of Foodborne Pathogens. Marcel Dekker, New York, NY.

Ngom, B., Guo, Y., Wang, X., Bi, D. (2010). Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. Anal. Bioanal. Chem., 397, 1113-1135.

Park, S.H., Aydin, M., Khatiwara, A., Dolan, M.C., Gilmore, D.F., Bouldin, J.F., Ahn, S., and Ricke, S.C. (2014) Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry product. Food Microbiology, 38, 250-262.

Parolo, C., Escosura-Muniz, A., Merkoci, A. (2013). Enhanced lateral flow immunoassay using gold nanoparticles loaded with enzymes. Biosens. Bioelectron., 40, 412–416.

Perumal, V., and Hashim, U. (2014). Advances in biosensors: principle, architecture and applications. Journal of Applied Biomedicine, 12, 1-15.

Pigot, D.C. (2008). Foodborne Illness. Emerg Med Clin N Am. 26, 475-497.

Posthuma-Trumpie, G.A., Korf, J., Amerongen, A. (2009) Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal. Bioanal. Chem., 392, 569-582.

Rahn. K., De Grandis, S.A., Clarke, R.C., *et al*. (1992). Amplification of an *invA* gene sequence of *Salmonella* typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. Mol Cell Probes, 6, 271-9.

Rastogi, S.K., Gibson, C.M., Branen, J.R., Aston, D.E., Branen, A.L., Hrdlicka, P.J. (2012). DNA detection on lateral flow test strips: enhanced signal sensitivity using LNA-conjugated gold nanoparticles. Chemical Communications, 48, 7714–7716.

Sánchez-Vargas, F.M., Abu-El-Haija, M.A., Gómez-Duarte, O.G. (2011). Salmonella infections: an update on epidemiology, management, and prevention. Travel. Med. Infect. Dis., 9, 263-77.

Scallan, E., Hoekstra, R.M., Angulo, F.J., *et al*. (2011). Foodborne illness acquired in the United States–major pathogens. Emerg Infect Dis 17(1), 7–15.

Singh, S.K., Pandey, V.D., and Verma, V.C. (2012). Bacterial Food Intoxication. In S.K. Pandey and V.D. Panday (Eds.), Microbial Toxins and and Toxigenic Microbes, Studium Press LLC, 215-232.

Tauxe, R.V., Doyle, M.P., Kuchenmuller, T., Schlundt, J., and Stein, C.E. (2010). Evolving public health approaches to the global challenge of foodborne infections. International Journal of Food Microbiology 139 (Suppl. 1), S16–S28.

Thakur, M.S., and Ragavan, K.V. (2013). Biosensors in food processing. J. Food. Sci. Technol., 50(4), 625-641.

Thevenot, D.R., Toth, K., Durst, R.A., Wilson, G.S. (1999). Electrochemical biosensors: recommended definitions and classification. Pure Appl. Chem., 71, 2333–2348.

Untergrasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012). Primer3 - new capabilities and interfaces. Nucleic Acids Research 40(15), e115.

UTexas, The University of Texas at Austin, http://www.sbs.utexas.edu/sanders/Bio347/Lectures/2006/Lecture%208%202006.htm, visited on July 2014.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C. (2010). An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnology Advances, 28 (2), 232–254.

Worrall, L.J., Vuckovic, M., Strynadka, N.C. (2010). Crystal structure of the C-terminal domain of the Salmonella type III secretion system export apparatus protein InvA. Protein Sci., 19(5), 1091-1096.

WHO, Surveillance Program for Control of Foodborne Infections and Intoxications in Europe. http://www.bfr.bund.de/internet/8threport/8threp_fr.htm visited on July 2014.

WHO, The World Health Report 2007, www.who.int/whr/2007/en/index.html, last visited on August 2014.

Wong, R.C., Tse, H.Y. (Eds.), (2009). Lateral Flow Immunoassay. Humana Press, New York.

Xu, H., Mao, X., Zeng, Q., Wang, S., Kawde, A.N., Liu, G. (2009) Aptamer-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for protein analysis. Anal. Chem, 81, 669–675.

Zadernowska, A., and Chajecka, W. (2011). Detection of Salmonella spp. presence in food. In Barakat, S.M.M. (Ed) Salmonella - A Dangerous Foodborne Pathogen. Intech Publishing.

APPENDIX A

BUFFERS AND SOLUTIONS

Tryptic Soy Broth (TSB)

17 g casein peptone, 3 g soya peptone, 5 g NaCl, 2.5 g K_2HPO_4 and 2.5 g glucose were suspended in 1L of distilled H_2O. Sterilization was performed by autoclaving at 121°C for 15 minutes after pH was adjusted to 7.3. The medium was stored at 4°C.

Tryptic Soy Agar (TSA)

17 g casein peptone, 3 g soya peptone, 5 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose and 15 g agar were dissolved in 1L of H_2O and pH was adjusted to 7.3. The medium was autoclaved at 121°C for 15 and freshly used.

Luria-Bertani (LB) Broth

10 g tryptone, 5 g yeast extract and 10 g NaCl were dissolved in 1L of distilled H_2O and pH was adjusted to 7.0. Sterilization was performed by autoclaving at 121°C for 15 and the medium stored at 4°C.

Luria-Bertani (LB) Agar

10 g tryptone, 5 g yeast extract, 10 g NaCl and 10 g agar were dissolved in 1L distilled H_2O and pH was adjusted to 7.0. The medium was autoclaved at 121°C for 15 and freshly used.

5X SSC Buffer

11.03 g sodium citrate and 21.09 g sodium chloride were dissolved in 400 ml of distilled H_2O. After adjusting pH to 7.0, the final volume was completed to 500 ml with distilled H_2O.

10X PBS Buffer

80 g NaCl, 2 g KCl, 7.62 g NaH_2PO_4 and 0.77 g KH_2PO_4 were suspended in 800 ml of distilled H_2O. pH was adjusted to 7.2 and the final volume was brought to 1000 ml with distilled H_2O. The sterilization was done by autoclaving at 121°C for 15 minutes.

50X TAE Buffer

242 g tris base, 57.1 ml acetic acid and 100 ml 0.5M EDTA were dissolved in 1L of distilled H_2O. The buffer was diluted to 1X prior to use.

1 mM TCEP

0.00286 g TCEP was dissolved in 1 ml of distilled H_2O. The solution was diluted with distilled H_2O in 1:10 ration for 1mM TCEP. Then, diluted solution was stored at -20°C as aliquots.

0.05M Tris Buffer for Sample Pad

0.6057 g tris was dissolved in 80 ml of distilled H_2O. 0.25 µl Triton X-100 and 0.876 g NaCl added to the solution. Upon adjusting pH to 8.0, the final volume was brought to 100 ml.

Suspension Buffer for AuNP-DNA Conjugates

0.22396 g NaH_2PO_4 and 0.28392 g Na_2HPO_4 were separately dissolved in 100 ml distilled H_2O to obtain 20 mM NaH_2PO_4 and 20 mM Na_2HPO_4, respectively. Then, 28 ml of 20 mM NaH_2PO_4 and 72 ml of 20 mM Na_2HPO_4 were mixed. Finally, 5 g BSA, 250 µl Tween20 and 10 g sucrose were added to the solution.

APPENDIX B

SEQUENCES OF PCR PRIMERS

Table B.1 Sequences and locations of primers for 284 bp amplicon

Description	Sequence (5'-3')	Location within the gene
F139 Forward Primer	GTGAAATTATCGCCACGTTCGGGCAA	287-312
F141 Reverse Primer	TCATCGCACCGTCAAAGGAACC	571-550

Table B.2 Sequences and locations of primers for 105 bp amplicon

Description	Sequence (5'-3')	Location within the gene
F139 Forward Primer	GTGAAATTATCGCCACGTTCGGGCAA	287-312
Reverse Primer 2	TGGTAATAACGATAAACTGGACCAC	391-367

Table B.3 Sequences and locations of primers for MCS of pUC19 plasmid

Description	Sequence (5'-3')	Location within the plasmid
M13 Forward Primer	GTAAAACGACGGCCAG	379-394
M13 Reverse Primer	CAGGAAACAGCTATGAC	481-465

APPENDIX C

MAP of pUC19 PLASMID DNA

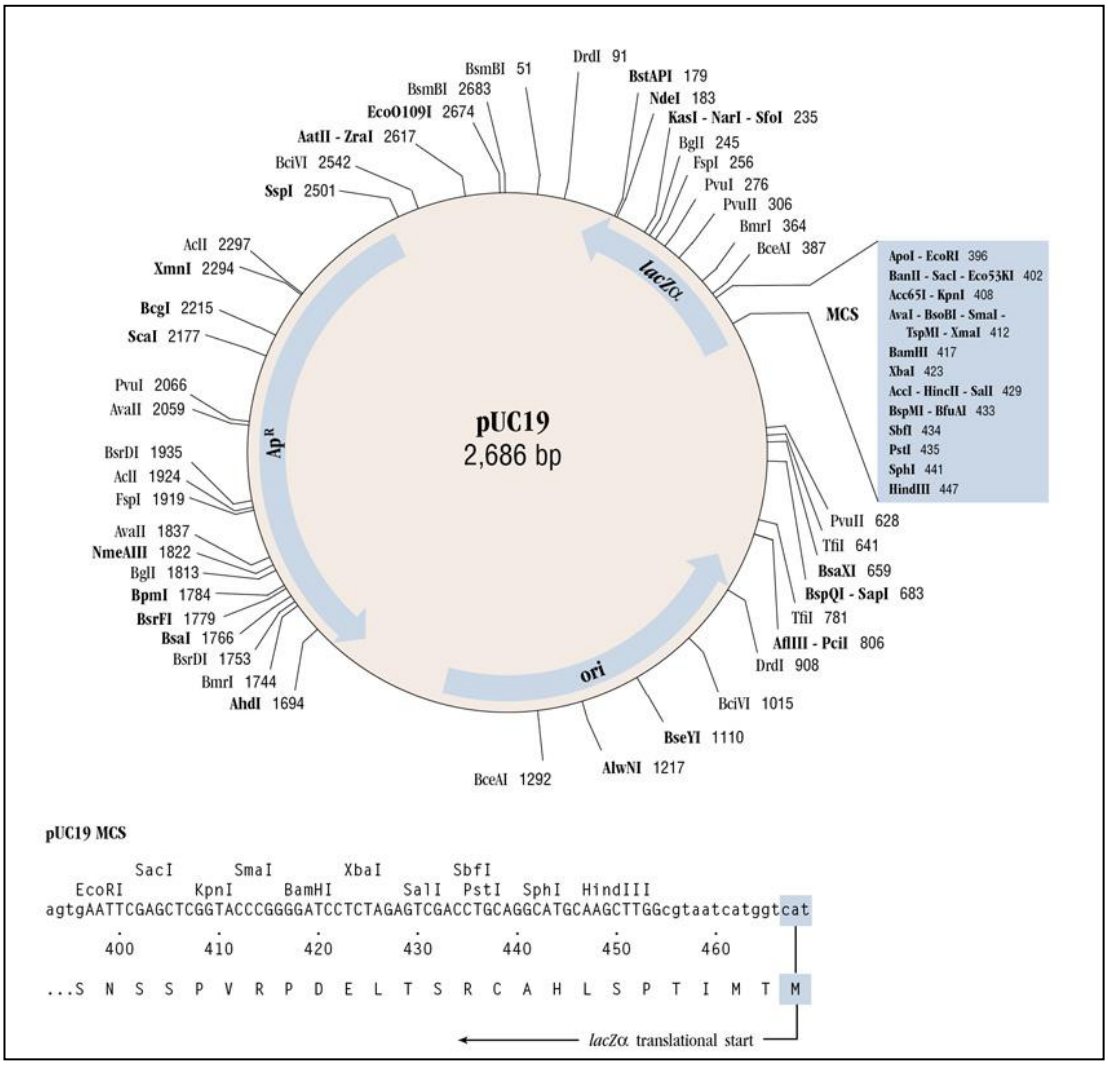

Figure C.1 pUC19 plasmid DNA map