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Development of 11-plex assay for the rapid screening of samples for detection of Shiga toxin-producing E. coli

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**DEVELOPMENT OF 11-PLEX ASSAY FOR THE RAPID
SCREENING OF SAMPLES FOR DETECTION OF SHIGA
TOXIN-PRODUCING *E. COLI***

by

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THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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Biomedical Engineering**

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Dedication

I would like to dedicate this thesis to my father who instilled in me a curiosity and strong held desire to figure out how the world works, even though he was not able to see it through until today. I would also like to dedicate this to my mother, who gave me the love and support that every child deserves. And to my husband, Ryan for being the support and push that I needed to finish.

ACKNOWLEDGEMENTS

DEVELOPMENT OF 11-PLEX ASSAY FOR THE RAPID SCREENING OF SAMPLES FOR DETECTION OF SHIGA TOXIN-PRODUCING *E. COLI*

By

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) have been identified by the USDA as a serious threat to the nation's health stemming from contaminations in the food supply, specifically, the beef chain. I have developed an assay that is able to screen samples for STEC in a rapid and multiplexed format. Multiplex oligonucleotide ligation-PCR (MOL-PCR) is a nucleic acid based assay patented at Los Alamos National Laboratory (LANL) that uses flow cytometry and multiplex microsphere arrays for detection of nucleic acid based signatures. By using MOL-PCR for detecting unique STEC DNA signatures in samples from the beef supply chain (farm to table) this assay will provide a multiplex and high throughput complement to the multiplex PCR assays currently in use. This research is focused on DNA detection of 8 STEC serotypes (STEC-8): O26, O45, O103, O104, O111, O121, O145, and O157:H7 as well as the virulence genes: *stx1*, *stx2*, and *eae*. The goal is to produce a multiplex panel of MOL-PCR probes for identifying DNA signatures corresponding to each of the STEC-8 serotypes and ultimately strain specific identification.

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Chapter 1: Introduction

1.1 Shiga Toxin-Producing E. coli as a Pathogen of Interest

In recent years it has become obvious from recent foodborne disease outbreaks that early detection of such outbreaks could lessen their impacts and/or prevent them from happening. A 2011 study showed that there were 48 million food related illnesses in the United States and 9.4 million were the result of known and detectable pathogens [1]. In 2013 there were 818 recognized foodborne disease outbreaks reported leading to 13,360 illnesses and 14 food recalls as outlined in the CDC's 2013 Surveillance for Foodborne Disease Outbreaks, United States. On a global scale, Shiga toxin-producing *E. coli* (STEC), one of the known pathogens causing foodborne illness, accounted for an estimated 2.8 million illnesses, 3,890 cases of hemolytic uremic syndrome (HUS), and 230 deaths [2]. The major STEC outbreak serotype in the United States is O157:H7, which results in 4,928 human infections per year; the majority of those infections stemming from beef products [3].

It has also come to light that STEC O157:H7 is not the only major concern for foodborne illness, but there are also a set of Top-6 serogroups O26, O45, O103, O111, O121, O145 with the addition of the German outbreak serotype O104:H4 [4]. The presence of any of these serogroups does not constitute a STEC outbreak as not all strains of these serogroups are toxin producing. It is not enough to be able to detect the serotype alone of the bacteria that is present, but, at a minimum, also detect the presence of Shiga toxin and intimin genes to elucidate a picture of the possible health threat present. The intimin gene (*eae*) is responsible for expressing an outer membrane protein needed for binding and effacing [5,6].

Shiga toxin (*stx*), also known as Shiga-like toxin or verotoxin, is a group of bacterial toxins. It is a diverse group of toxins comprised of two major groups: *stx1* and *stx2*. Within the main groups of *stx* are subgroups that occur in various combinations across Top-6 [7, 8, 9] and the main U.S. outbreak serotype O157:H7 as well as the German outbreak strain of O104:H4. Shiga toxin is a complex molecule that is moleculary comprised of two subunits: A-subunit and B-subunit. (Figure 1.1) The A-subunit for *stx1* and *stx2* share only 55% homology in amino acid sequence [10]. Additional to the *stx* genes are several other genes found from previous nucleic acid studies as virulence factors associated with STEC or more appropriately enterohemorrhagic *E. coli* (EHEC): intimin (*eae*), intimin receptor (*tir*), hemolysin (*hylA or ehxA*), as well as others [11, 12]. The distinction of EHEC is made here as not all toxin producing *E. coli* are infectious as they lack the appropriate helper proteins [6], but as this work aims to detect a broader pool of *E. coli* the STEC nomenclature will be used.

1.2 Detection of STEC

There are several forms of pathogen detection in use today, which can range from antibodies, sensing technologies, sequencing, and nucleic acid amplification based technologies [13]. As with all technologies and assays, each of these detection techniques has a set of strengths and weaknesses that must be addressed and understood before being utilized in the field or laboratory. Of particular interest here were the ability to use these pathogen detection systems for identifying Shiga toxin-producing *Escherichia coli* (STEC) and its associated components. STEC monitoring and detection can be accomplished using any number of methods: ELISA, culture, direct immunofluorescent staining, etc. [14]. It was not within the scope of this work to examine each method able to

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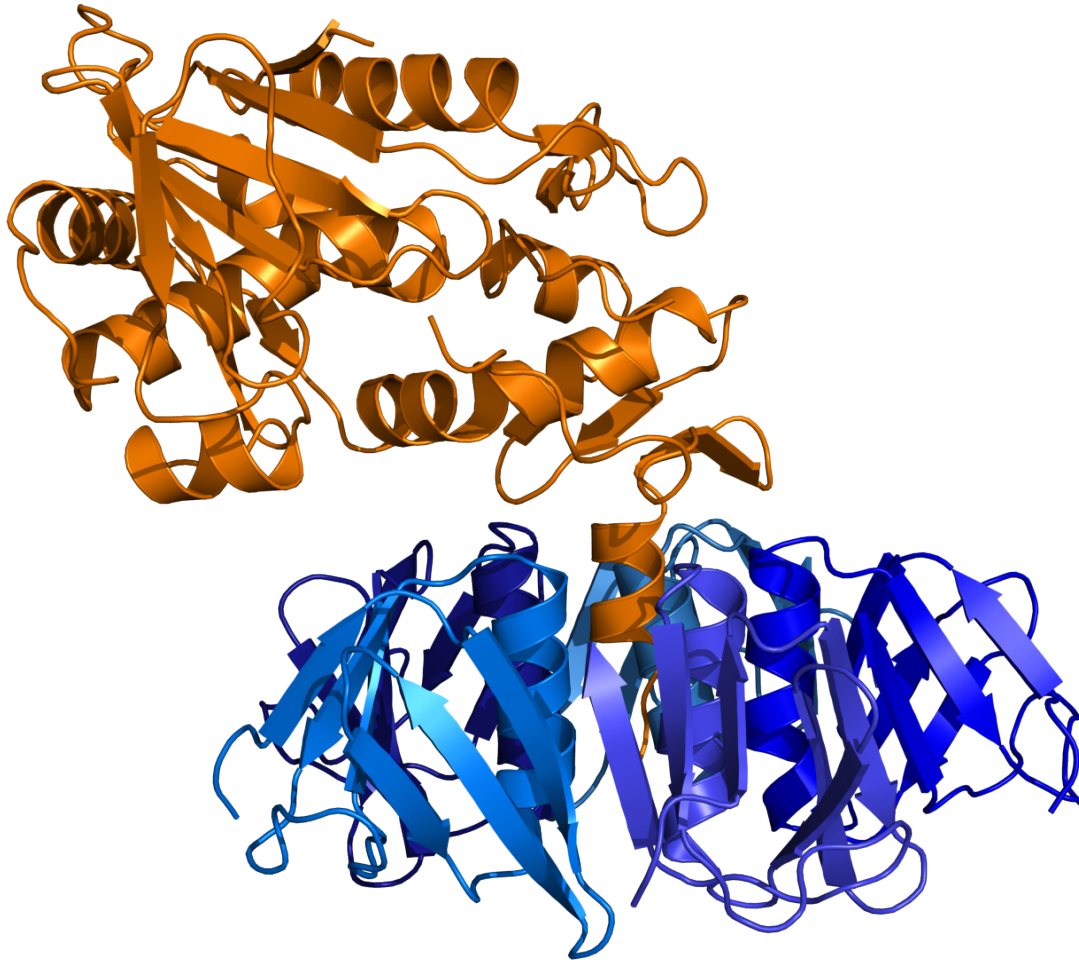
Figure 1.1: Ribbon diagram of Shiga toxin (stx) from O157:H7. *Stx1* and *stx2* are made of two units an A-subunit and a B-subunit. The A-subunit is in orange and the B-subunits are in shades of blue. The A subunit is a single molecule and the B-subunit is pentamer of 5 subunits. Fraser et al., 2004 © 2004 The American Society for Biochemistry and Molecular Biology

be employed, but instead to limit it to a set of widely used nucleic acid based assays because of their high level of sensitivity to detect specific deoxyribonucleic acid (DNA) sequences by using polymerase chain reaction (PCR) amplification of any assay detection product. Even when limiting the scope of this work to what appears to be a narrow subset, nucleic acid assays, STEC detection work still spans across several assisting technologies each with its own set of limitations and advancements. Trying to describe all possible methods for pathogen detection would require much more detail than was

appropriate and was not relevant to the type of work that was being performed here. Nucleic acid based detection assays were chosen, in this work and elsewhere, because of their high level of sensitivity and capability of being multiplexed. In order to show the work done here was of importance a description of the current state of the art nucleic acids assays for STEC detection will be explained in the following sections.

1.3 Multiplex PCR

Multiplex PCR has historically been an effective way to detect several specific genetic sequences of interest. In multiplex PCR the target DNA is amplified using a set of forward and reverse primers for each probe being detected in the multiplex [15]. The process requires that each target probe have its own set of primers, which will be used during the amplification. It is required that these primers hybridize to the target template DNA that they were designed for, but do not cross react with each other (primer-dimer) or with non-target DNA. There are several versions of multiplex PCR, but all rely on the use of polymerase-mediated amplification of either the target DNA or a detection section of DNA in order to be visualized at a later point. In particular, STEC nucleic acid based detection assays rely on serotyping strains of STEC by concentrating on particular genes expressed in the O-antigen region of the DNA [11, 16, 17]. Multiplex detection assays are able to detect a multitude of genes (serotyping and virulence) on a single sample, where it would take multiple single reactions to do this with single-plex assays. These additional assays would greatly increase time to screen before a STEC identification could be made, which could impact timeliness of alert for potential outbreaks.

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1.3.1 End-Point Multiplexed PCR

Early multiplexed PCR assays used traditional amplification techniques and relied on visualization of STEC amplicons by gel electrophoresis, as the hardware required to perform it are easily acquired and are used in most molecular biology laboratories. The principles of electrophoresis rely on the fact that DNA of different sizes moves at different speeds through a gel slab when an electric field is applied across the gel, hence separating in physical space on the gel. The negatively charged DNA moves through the gel with the smaller molecules progressing quicker than the larger molecules due to molecular sieving. This size based movement then requires that each resulting target-specific amplicon in the multiplex assay must be of a significantly different molecular weight or number of base pairs to be able to be visualized as separate bands [18]. Gel electrophoresis, while being a well-tested tool, suffers from several problems: time consuming (1-4 hours per gel), very low throughput (8-12 samples) [19], user biased analysis. There are several STEC multiplex PCR detection assays that still use this very accessible method for visualization despite its limitations [11, 20, 21, 22, 23].

Capillary electrophoresis (CE) was developed as a way to get away from some difficulties with gel electrophoresis. Briefly, CE utilizes a small micrometer wide capillary in place of an agarose or polyacrylamide gel as the medium for separation [24]. With capillary electrophoresis run times were reduced from hours to less than 30 minutes while also gaining a semi-quantitative analysis from the incorporation of dye into the DNA via end labeled primers or intercalating dye [18]. These systems also benefit from the ability to have arrays of 16 to 96 capillaries similar to 96 well PCR plates.

1.3.2 Real-Time (Quantitative) PCR with Multiplexing

Real-Time PCR (qPCR), also called quantitative-PCR, is a closed PCR system where the increasing fluorescence over PCR cycles is measured to determine the presence and amplification of target DNA. In addition to the pairs of forward and reverse primers an additional probe sequence is used with a fluorescent reporter and a quencher molecule that exhibit fluorescence resonance energy transfer (FRET). When these two molecules are close in proximity no fluorescence is produced. Similar to end-point PCR, the forward and reverse primers hybridize to the target template DNA, but in qPCR the third FRET labeled probe binds downstream of the forward primer. As the polymerase progresses down the growing amplicon from 5' end to 3' end it removes nucleotides in its path. The loss of the reporter molecule bound to the probe sequence on the 5' end during the amplification process removes the distance required for FRET causing fluorescence increase. Multiplex Real-Time PCR (mqPCR) is accomplished by adding fluorescent reporter molecules to the multiplex FRET probe sets [25]. mqPCR being a single tube, closed reaction provides the benefit of reduced amplicon contamination, while the ability to record increase in fluorescence over time makes the process quantitative [19]. As with all PCR assays, it is also quite sensitive and because the analysis is done during the PCR step it can be done in 96-well or higher plates. In mqPCR each primer set is assigned a specific fluorescence reporter in a wavelength range. These wavelength ranges produce an upper limit to the number of multiplexes typically capable of by commercial instruments: 4-6 wavelength ranges (ThermoFisher). STEC detection assay have been varied using qPCR. There have been studies using Intimin Gene (eae) subtype expression profiles for doing serotype specific STEC detection [26]. Harada *et al.* required 3 separate

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mixtures using serotype and subtype specific sequences to perform a mqPCR with a multiplexing of 3, 2, and 2 to accomplish *stx*-type, O26, O157, and O111 [27]. There is a commercial system available from Pall Corporation using their GeneDisc® Rapid Microbiology System, which utilizes a duplex fluorescence for detecting VTEC screens and a separate disc for doing serotyping, but only on 6 samples at a time [28]. Most recently there was research done with a mqPCR assay by Noll *et al.* looking at O157 specific gene rfbE along with the 3 major virulence genes: *stx1*, *stx2*, and *eae* [29].

1.3.3 Novel PCR Based Multiplex Assays

As with all facets of research, once a technique becomes well accepted various novel approaches are applied to broaden the technique. End-Point PCR and multiplex PCR is no different. Multiplex PCR has been adapted to various new readout platforms. One such platform was Luminex microsphere based assay technology, which will be discussed in greater detail later in this chapter. In one approach, Taniuchi *et al.,* adapted their multiplexed PCR assay to a custom set of fluorescent highly multiplexed microspheres, where they covalently attached a unique set of anti-tags that are specific to unique sequences internal to the STEC amplicons [30,31]. The benefit of this adaptation is that once the multiplex PCR amplification is complete, the visualization is no longer limited by the resolution and dynamic range of the gel or the capillary, but instead by the array of the microspheres: 50-500 analytes.

Briefly, there was also work done by Li, F. *et al.*, where Ramification amplification was utilized to detect *stx1* and *stx2* in various STEC [32]. In this assay the lysed bacteria is captured to a magnetic bead for separation, where a specially designed C-probe binds to the target DNA in a way that when the 5' and 3' ends of the DNA are

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ligated they produce circular DNA. The circular probe is then amplified in long repeating sequences by excess forward and reverse primers. The benefits of this assay approach are that it is an isothermal reaction removing the requirement for thermocycling conditions, which could be difficult to produce in remote locations. This work was not inherently multiplex capable as there was no way to distinguish the long ramified product of one target, *stx1*, from the second target, *stx2*.

1.4 Genome sequencing as detection

With recent advances in technology it has become easier and cheaper to do sequencing on samples. Whole genome sequencing (WGS) is approaching the area where it can be deployed as a detection method for real samples in the field for STEC outbreaks [33]. In sequencing analysis the entire or major portions of the STEC DNA sequence are then known and can be compared to databases of STEC genomes, which removes uncertainty generated by PCR based assays, which look at small amounts, hundreds of base pairs, of sequence data [7]. In order to do this the genomic DNA (gDNA) from samples is first fragmented and tagged for multiplexing before being mapped in smaller, hundreds of base pair reads [34]. The gDNA sequence data that is generated is in the billions of base pairs, which creates a computational component that is non-trivial. To make sense of the sequence information it must be processed and returned with virulence context and alignments applied, which can be done through high-speed computers and/or web-based tools like VirulenceFinder (Center for Genomic Epidemiology) [33, 7]. Sequencing of the bacterial DNA allows not only determination of the virulence profile: Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), *eae,* but also identifies the many subtypes of these genes and all other genes of interest, while maintaining serotype identifiaction[33]. It

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cannot be overstated that the amount of data obtained from WGS provides a more all-encompassing look at the organism being identified. In the exploratory research from Joensen *et al.* the processing time for WGS took a combined 19.5+ hours (spanning 4 days), with 3.5 hours devoted to computational analysis alone, with the ability to run 4 isolates in parallel [33].

Pyrosequencing has been used as a subset of WGS, where gene sequencing was performed on samples as a way to reduce the amount of data that was generated, while maintaining subtyping information [7]. In Goji *et al.* they utilized a combination of multiplex PCR to generate the amplicons for *stx1*, *stx2*, *eae*, and *rfbE*, which were then sequenced to provide the full subtyping of the samples [7]. In an effort to reduce time and complexity of analysis, pyrosequencing also decreased the amount of unique data generated when compared to whole genome sequencing.

When examining the various STEC detection assays that utilize nucleic acid signatures or sequence information it becomes clear that there is not yet described a truly multiplexed, rapid, and high throughput method. Table 1.1 is a representative look at the various STEC detection methods outlined here. While there are methods available that are quite comprehensive in their scope of detection, they are limited in their throughput (mean time between samples) and expandability.

Table 1.1
Summary of nucleic acid based STEC detection methods

STEC Assay	Detection Technique[1]	Reference	Serotype/Genes Detected	Comments[2]	Limitations
End-Point PCR	Gel Elect.	Li, Y., et al., 2005	O157, *Salmonella, Shigella flexneri*	3-plex assay looking only at O157	Only O157, Low throughput
	Gel Elect.	Bai, J., et al., 2012	O26, O45, O103, O111, O121, O145, O157, *stx1, stx2, eae, ehxA*	Comprehensive 11-plex, 5-17 samples	Not high throughput
	Gel Elect.	Paddock, Z., et al., 2012	O26, O45, O103, O111, O121, O145, O157	7-plex serotype, 8-17 samples	Low throughput
	Luminex	Taniuchi, M., et al., 2012	*stx1, stx2, eae,* unrelated genes	3-plex STEC and 9-plex over all	No serotype
	Gel Elect.	Conrad, C., et al., 2014	O157, O145, O121, O111, O103, O45, O26, *repA, ehxA, eae, stx1, stx2*	7-plex serotype and 5-plex virulence gene, 6 samples	Low throughput
Real-Time PCR	FRET	Beutin, L., et al., 2009	*stx1, stx2, eae,* O157, O26, O103, O111, O145	GeneDisc 2-disc system VTEC and EHEC, 6 sample	Low throughput, High Cost
	FRET	Delannoy, S., et al., 2012	O26, O45, O103, O111, O121, O145, O157	Single plex serotype, unknown throughput	Low throughput, Low multiplex
	FRET/ Chromogenic agar	Tzschoppe, M., et al., 2012	O26, O103, O111, O118, O121, O157, O104, *st x1, stx2, eae, ehxA, terB, aggR*	Comprehensive assay 2-plex, unknown samples	Low multiplex
	FRET	Harada, T., et al., 2015	*Stx1, stx2, stx2f,* O26, O157, O111,	Single 3-plex and 2x 2-plex, plate based assay	Low multiplex
Sequencing	WGS	Joensen, K., et al., 2014	Complete comprehensive panel database	Complete serotype and genotype analysis, 4 sample	High Cost, Low throughput
	WGS	Dallman, T., et al., 2015	O157, Comprehensive gene panel database	Internally O157 limited, but comprehensive inside O157	High Cost, Low throughput
	Pyrosequencing	Goji, N., et al., 2015	*stx1, stx2, eae, rfbE*	Full genotyping for major STEC genes, duplex mPCR	No serotype, Low throughput

[1]Gel Elect is any variant of gel electrophoresis, FRET is multiplex or single plex fluorescence resonance energy transfer, WGS is whole genome sequencing
[2] STEC is Shiga toxin-producing *E. coli,* VTEC is verotoxin producing *E. coli,* mPCR is multiplex PCR

1.5 Multiplex Oligonucleotide Ligation-PCR for detection of STEC

Multiplex oligonucleotide ligation polymerase chain reaction was first outlined and used by Dr. Alina Deshpande as a rapid screening assay that: 1) would not require multiple rounds of analysis 2) was of low cost to setup and operate continually 3) capable of being able to both, identify pathogen and provide additional characterization [35]. The assay was originally used to detect a panel of 3 pathogens (*B. anthracis, Y pestis, and F. tularensis)*, with additional characterization probes, in a 13-plex. The technology has since been used to characterize *Salmonella* [36] and *Bacillus anthracis* [37], as well as others. These assays, as the other assays described above, are all nucleic acid based assay that have a multiplex capable component. The MOL-PCR assay has yet to be adapted for detection and characterization of Shiga toxin-producing *E. coli*, but that work will be represented here.

It is the goal of the work described here to show the ability to generate a functional, adaptable, and robust assay able to detect Shiga toxin-producing *E. coli* DNA signatures relating to the 8 STEC serogroups of national interest along with important virulence genes (*stx1*, *stx2*, *eae*) using the high throughput multiplexed oligonucleotide ligation-PCR (MOL-PCR). Of concern to the worldwide community is the identification of Shiga toxin being produced by STEC originating from contaminated foodstuffs and of particular interest here, in the beef food chain.

Current work utilizing nucleic acid assays, as outlined earlier in Chapter 1, do not offer all of the flexibility that is provided by MOL-PCR. It is the benefit of the MOL-PCR assay that it uses a multiplexed microsphere based system commercially available from Luminex Corporation that allows for upwards of 100 unique possible targets. In the

work that is to be discussed here only a small number of target sequences are examined, but with little additional work it would be possible to add in additional interesting targets. Given the detection methods and assays developed previously of great benefit would be a minimum of an 11-plex assay: O26, O45, O103, O104, O111, O121, O145, O157, *stx1*, *stx2*, and *eae*. This level of complexity is approachable for a nucleic acid assay as it is possible to predict and control for DNA probe interactions; a hallmark design parameter for the detection system discussed above. Multiplexed oligonucleotide polymerase chain reaction is particularly well suited for this undertaking as it is highly multiplex capable, is a nucleic acid based assay, and approaches the sensitivity limits of multiplex-PCR [35].

MOL-PCR is similar to other assays that rely on a ligation event as the detection signal, which is later amplified during a PCR process to facilitate visualization. Oligo ligation assay (OLA) and multiplex ligation-dependent probe amplification (MLPA) both work in a similar manner to MOL-PCR. In MLPA[38] and OLA[39] pairs of probes that have been designed to anneal adjacent to each other on target DNA and in the presence of a thermostable ligase enzyme are ligated together into a single oligonucleotide (oligo) with known characteristics: fluorescence, size, radio-labels etc. The products are amplified via PCR and visualized as appropriate. MOL-PCR works in the same way as MLPA and OLA with a ligation dependent detection event. It is because of the unique combination of fast thermocycling conditions for detection (ligation step) and amplification, along with the high level of multiplexing and high throughput (96-well plate) on the Luminex instrument (visualization step) that facilitates a near real time characterization of STEC samples in MOL-PCR. The current vision for the MOL-PCR assay is to deploy it as a screening assay on enriched samples from various stages in the beef supply chain.

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Additional goals to be described in this work are the particular measures that were implemented after several starts and stops of assay production and finalization. MOL-PCR relies on a large number of components in order to be successful, but lacks midpoint checks along the protocol. However, with the proper protocol design, step specific controls, and extreme attention paid to a *one directional workflow* with safeguards it is possible to have a highly functional and reproducible assay. The results shown here will demonstrate the usefulness of this assay.

With the careful attention to detail and utilizing best PCR practices along with the screening assay daily run design, which includes a statistical component for rendering *positive* or *negative* calls, it is possible to screen several samples per day. In this screening test 96-well plates were used for all steps, which allowed 24 samples to be screened in triplicate in an 8-hour day. This should prove to be of a great benefit to current screening methods for the detection of STEC-8.

Chapter 2: Material and Methods

2.1 MOL-PCR Assay Description

MOL-PCR is able to perform simultaneous detection of several specific DNA signatures in a single reaction using a multiplex ligation technique (Figure 2.1). A pair of modular probes, MOLigos, defines each signature that is being assayed. In the presence of the target template DNA, the pair of probes will hybridize to the template DNA. In the presence of thermostable ligase, the bound probe pairs will covalently link together forming a full-length oligonucleotide to be later amplified. When incubated in PCR cycling conditions with asymmetrical concentrations of universal forward primer and reverse primer along with polymerase enzyme, a biotin-labeled, capture-TAG containing oligo is generated. This full-length oligo is captured to a fluorescently arrayed set of microspheres with complementary anti-TAG sequence, where the captured complex is labeled with streptavidin-phycoerythrin (SAPE) [35].

2.2 MOL-PCR Assay Design

The work here is designed off of multiplex PCR work for detecting O26, O45, O103, O111, O121, O145, and O157:H7 serogroups done by Dr. T.G. Nagaraja at Kansas State University [11, 21]. In that work Bai *et al.* downloaded gene sequence that code for O-antigen regions of the STEC Top-6 along with O157:H7. Bai *et al.* used this data to design forward and reverse primers that were serotype specific for each serogroup in the multiplex PCR assay. Dr. Jainfa Bai, Kansas State University, provided similar primer sequence data from the wzx gene specific that conferred serotype O104 [22] and a modified version of primers for O111 serotype. The sequences for STEC and EHEC virulence genes were obtained from similar work; where searches were conducted using BLASTN

A.

| 5' — Reverse | Anti-TAG | Target 1A | —3' |

| 3' — Forward | Target 1B | —5' |

B.

C.

D.
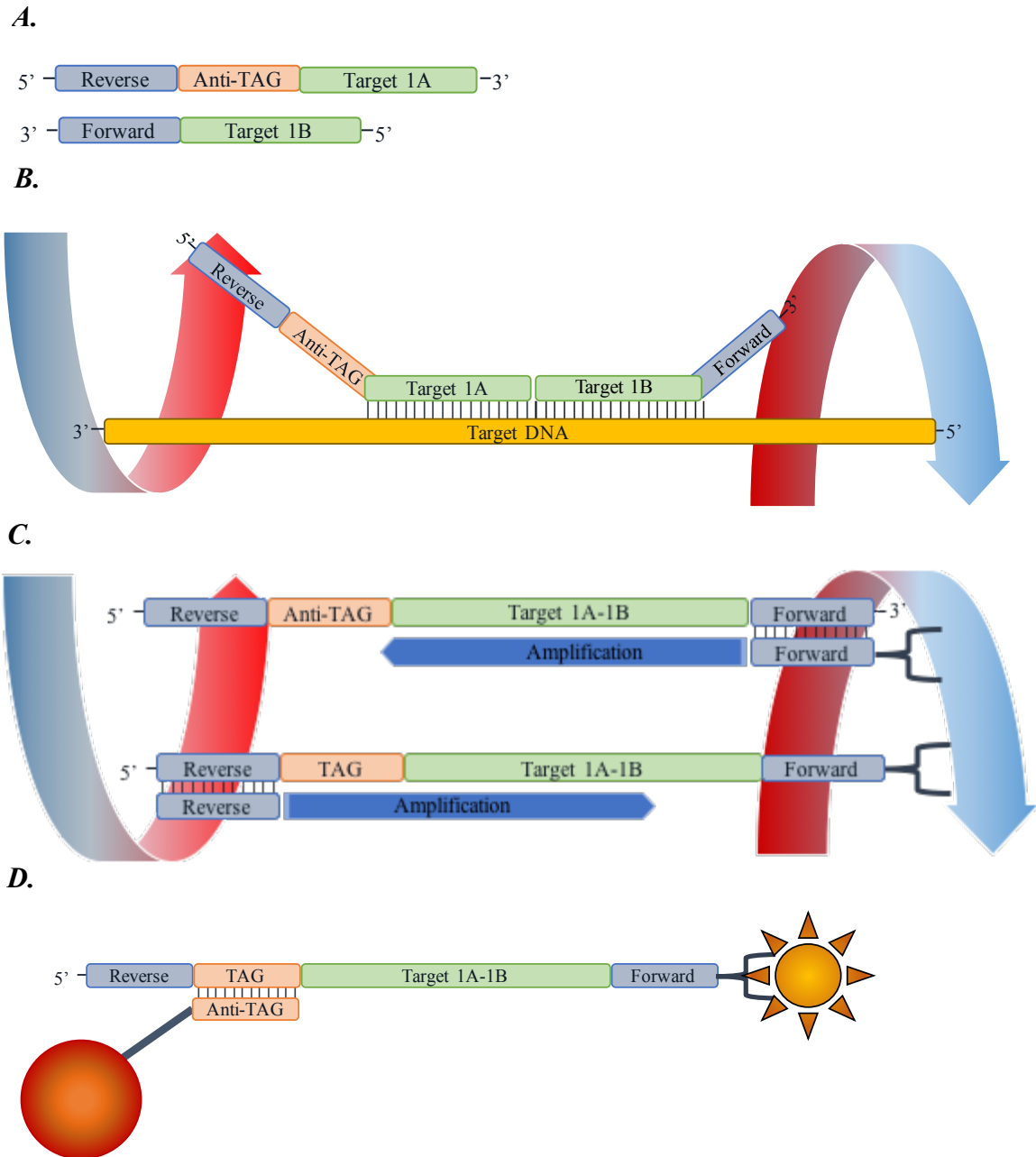
Figure 2.1: *A.* Details the main components of MOL-PCR assay. MOLigo-2 is a synthesized oligonucleotide comprised of universal reverse primer, anti-TAG binding sequence, and a portion of DNA complementary to the target DNA. MOLigo-1 is a synthesized oligonucleotide comprised of universal forward primer and a portion of the DNA complementary to the target DNA. *B.* MOLigo-1 and MOLigo-2 bind adjacent to each other on target DNA if present. Under cycling conditions, DNA ligase recognizes the structure and covalently links MOLigo-1 and MOLigo-2. The full length linked oligonucleotide (oligo) is between 100-127 bases. *C.* In the presence of biotin-universal forward and reverse primer, ampligase enzyme, and appropriate cycling conditions, the full length oligo is amplified. *D.* The biotin labeled full oligo, with TAG sequence, hybridizes to the unique sequence (anti-TAG) on the microsphere. Captured oligos are labeled with streptavidin-phycoerythrin as the fluorescent reporter. This figure is a simplified representation, but can be a multiplex of microspheres.

to generate forward and reverse primers unique to each virulence marker [11]. Used in this work were the primers for *stx1*, *stx2*, and *eae*.

Multiplex PCR primers sequences for all serotypes as well as virulence genes were searched against all available genomes on GenBank using the BLASTN nucleotide collection. This search was done as a first step to verify that this section of sequence was truly unique and able to confer serotype specificity, within reason. Both sets of primers for each sequence did not return homology outside of their expected serotype specific sequences. In most cases the forward primer sequence for each marker from the previous multiplex PCR work, with the exception of a redesign due to contamination issues that developed later in this work, was used as the basis for the MOLigo pair design.

2.2.1 MOLigo Pair Design

The oligonucleotides were designed using Moligo Designer developed at Los Alamos National Laboratory (LANL) [40]. The tool from LANL generates the sequence design for two single stranded MOLigos (MOLigo-1 and MOLigo-2) for each unique sequence being targeted. The MOLigo detection event requires the annealing of two oligos adjacent to each other, one upstream and one down stream on the target DNA. MOLigo-2 contained the universal reverse primer sequence (5'-ACTCGTAGGGAATAAACCGT-3'), followed by the Luminex microsphere specific anti-TAG 24-mer sequence, then the site-specific STEC target DNA sequence on the 3' end with a total nucleotide length varying between 61 and 74 bases. MOLigo-1 was synthesized with at its 5' end a phosphorylation tag followed by the site-specific STEC DNA sequence followed by the universal forward primer sequence (5'-TCTCACTTCTTACTACCGCG-3') with a varying total nucleotide length between 36

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and 55 bases (Table 2.1). The total amplicons (fully ligated MOLigo-1 to MOLigo-2) length varies between 101 and 127 bases with the site-specific region being between 37 and 63 bases on the target sequence.

The MOLigo binding sites were designed to be highly specific to the target STEC DNA using MOLigoDesigner that utilizes the nearest-neighbor model for calculating the melting temperature (T_m). The ligation T_m, 55°C here, must also fall between other limits set in the algorithm: STEC DNA specific sequence between 12-45 base pairs, with a maximum Delta T_m of 5°C, and others. During the design process the various tag sequences and primer sequences are added to either appropriate MOLigo partner as described previously.

After MOLigo pairs have been designed they were computationally tested against all-available genomes in NCBI's nucleotide BLAST program to look for possible interactions of MOLigos with non-target genome sequences. Any interactions with high scores for non-target serotype/gene sequences were then excluded from further development. All MOLigo pairs that returned low non-target interactions were then considered to be unique targets for STEC-8 and associated DNA sequences. The remaining MOLigo pair sequences were then tested using NUPACK (NUPACK.org) for interactions between various MOLigo-1/MOLigo-2 pairings, primer-dimers reactions. Interactions between the fully functional MOLigos (primer sequence-tag sequence-specific sequence) in a multiplex setup would inhibit the ability of the MOLigo to anneal to the STEC target sequence adjacent to its other MOLigo half. No interactions were found that had a free energy lower than -2.5kcal/mol at 50°C and were then considered to be non-interacting.

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Table 2.1
Sequences for MOLigo pairs with all portions defined

Serotype/gene[1]	xTAG[2]	ID[3]	Sequence[4]
O26		rNO26Fwzx958A(+)M1	Phos-ACCCACCCCCCCTAAACTTCTCACTTCTTACTACCGCG
	A034	rNO26Fwzx958A(+)M2	ACTCGTAGGGAATAAACCGTgatatagtagtgaagaaataagtGATACTTTGAACCTTATATCCCAATATAGT
O45		NO45Fwzx377G(+)M1	Phos-TGGACAGCCCACTTGCAGTCTCACTTCTTACTACCGCG
	A065	NO45Fwzx377G(+)M2	ACTCGTAGGGAATAAACCGTgagtaagttgtatgtttaagtaGCCAAACCAACTATGAACTGTC
O103		NO103Fwzx303G(+)M1	Phos-CCCGTACTTATAATAAAACAACAGGCTCTCACTTCTTACTACCGCG
	A038	NO103Fwzx303G(+)M2	ACTCGTAGGGAATAAACCGTagtaagtgttagatagtattgaatTCTGATATTTACTGGAAAAAAGCACC
O104		M1O104-b62-wzx821G	Phos-AATAAAACCTGCGATA1CTGCTTCTCACTTCTTACTACCGCG
	A062	M2O104-b62-wzx821G	ACTCGTAGGGAATAAACCGTgaaatgtgtatttgtatgtttagGTTGAAAATTCTTTGCGCGAC
O111		JBO111Fwzx496C(+)M1	Phos-CACTCTTGTAAATTACTTCAAAAAAACATGATTCTCACTTCTTACTACCGCG
	A046	JBO111Fwzx496C(+)M2	ACTCGTAGGGAATAAACCGTgtgattgaatagtagatgtttaaGCCATATATTACTATAGAAGCCAGAG
O121		NO121Fwzx420T(+)M1	Phos-AATATAATGATGAATCTAAGCGTTGTTATAAAAATTCTCACTTCTTACTACCGCG
	A027	NO121Fwzx420T(+)M2	ACTCGTAGGGAATAAACCGTaagatgatagttaagtgaagttaAGTATAACCTTTACTTTCATGACAGGA
O145		M1O145b35Fwzx98T	Phos-AAAGTCGAGCAAGCAAAACATCTCACTTCTTACTACCGCG
	A035	M2O145b35Fwzx98T	ACTCGTAGGGAATAAACCGTaataagagaattgatatgaagatgCACTCCTAAATCTGTTGATGGTA
O157:H7		NO157FECs2841-578G(+)M1	Phos-CACCTTCACCTGTAGTAATAGTTTATTTCTCACTTCTTACTACCGCG
	A028	NO157FECs2841-578G(+)M2	ACTCGTAGGGAATAAACCGTgatagatttagagatgaattaagtgTGTCATTCGTGACAACCATTC
Stx1		M1stx1-b45-626A	Phos-CATCCAGTGTTGTACGAAATCCTCTCACTTCTTACTACCGCG
	A045	M2stx1-b45-626A	ACTCGTAGGGAATAAACCGTgttagttatgatgaatattgtaATAAGAACGCCCACTGAGAT
Stx2		M1stx2-b19-565C	Phos-GACAGCAGTTATACCACTCTGTCTCACTTCTTACTACCGCG
	A019	M2stx2-b19-565C	ACTCGTAGGGAATAAACCGTgtgtgttatttgtttgtaaagtatCGGTTTCCATGACAACG
eae		eae2120A(+)M1	Phos-TGGTCAGGTCGGGGCGTCTCACTTCTTACTACCGCG
	A056	eae2120A(+)M2	ACTCGTAGGGAATAAACCGTaattagaagtaagtagagtttaagTTCCGAAAACATGCTGGCATT
Universal Forward		DualBiotin Univ Forwd Primer	Dual-Biotin-CGCGGTAGTAAGAAGTGAGA
Universal Reverse		Universal Reverse Primer	ACTCGTAGGGAATAAACCGT

[1]Serotype and virulence marker of MOLigo pair
[2]MagPlex-TAG microsphere with anti-TAG sequence
[3]MOLigo probe identification name
[4]Target hybridizing sequence (underlined and uppercase), universal forward primer sequence (bold and uppercase), universal reverse primer sequence (italic and uppercase), TAG sequence (lowercase). Phos is 5' phosphorylation and Dual-Biotin is a 5' Dual Biotin label.

Finalized MOLigos were synthesized by Integrated DNA Technologies (IDT DNA) (Coralville, IA) on a 25nmole to 100nmole scale (depending on oligo length) with standard desalting as the purification method. IDT DNA also synthesized the universal forward and reverse primers as well as the full-length control, which was used as a positive control for PCR amplification. All custom oligonucleotides were reconstituted in TE buffer (10mM Tris, pH 7.5 and 1mM EDTA) (Affymetrix; Santa Clara, CA) at 200µM where able or 20µM if suspension volume was unmanageable. Reconstituted DNA was stored at -20°C for long-term stability.

2.3 Moligo Ligation to Template DNA

Custom oligonucleotides were designed to be complementary to the desired conserved sequences in the O-antigen region unique to each serotype of the 8 serogroups of Shiga Toxin producing *E. coli* (STEC-8). Each oligonucleotide detection pair (MOLigo pairs or MOLigos) was designed as the reverse complement of a unique contiguous sequence previously identified as serotype-specific for each of the STEC-8. The assay was designed to also detect conserved sequences for *stx1*, *stx2*, and *eae* at the same time as the STEC-8 serogroups.

The assay consisted of the separate steps: 1) target DNA sequence detection by ligation, 2) ligated MOLigo signal amplification by PCR, 3) hybridization to microspheres for readout (Figure 2.1) on a Luminex instrument. It has been shown that these three steps can be combined into a single step, but in this initial work these tasks were carried out independently. Each these steps will be described in more detail here.

Discrimination of the STEC-8 relevant DNA sequences was accomplished during the first step: ligation. The ligation reaction was carried out with either PFU DNA

(Agilent Technologies; Santa Clara, CA) ligase or Ampligase DNA (Epicentre; Madison, USA) ligase in a reaction where the activity level of the DNA ligase enzyme required small adjustments to the quantity of ligase (units/µl) per reaction. The ligase reaction volume was 10µl for capped tube reactions and 20µL for PCR plate (USA Scientific; Orlanodo, FL) reactions sealed with TempPlate sealing film (USA Scientific); to account for evaporation. 10X Ligase buffer (Epicentre or Agilent Technologies) was diluted to 1X with water and reagents in a list to follow. Individual MOLigos were in the reaction at 4nM, sheared UltraPure™ Salmon Sperm DNA (ThermoFisher, Grand Island, NY) at 0.15mg/ml DNA Ligase was used at 0.5 units (U) per reaction to 2.5 U/reaction. Uracil-DNA Glycosylase (ThermoFisher) (UDG) (5U/µl) was added at 2.5U per reaction to control for amplicon carry-over from previous experiments. The sealed tubes or plate were run on a thermo cycler with the following conditions: 37°C for 30 minutes optimal UDG activity followed, by 3 minutes at 95°C and then 30 cycles of 25 seconds at 95°C and 50°C for 2 minutes. The reaction was held at 95°C for 30 minutes at the end to inactivate the UDG before holding at 4°C.

It should be noted that ligation setup, including addition of all non-target DNA, buffers, and enzymes were performed inside a PCR hood to prevent any amplicon or target STEC DNA from contaminating assay stocks. Following the PCR plate setup, the inside of the hood was wiped with 20% bleach followed by a wipe down with water. After this wipe down the UV lamp inside the hood was run for 30 minutes to crosslink any remaining DNA. The addition of the target DNA and any control DNA, purified or synthetic, were added to the PCR plate in a separate hood from the hood where the

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master mix was dispensed. This hood was cleaned in the same manner as stated previously.

2.4 Amplification of Detection Event

PCR amplification of the ligation product was done using a standard PCR amplification protocol with only the forward primer of the two universal primers containing a site for reporter label binding. 2μl of the ligation product was transferred to tubes or a PCR plate containing 10μl or 20μL of PCR master mix. The PCR master mix comprised of 10X Amplitaq Gold buffer (Applied Biosystems; Foster City, CA) diluted to 1X with water, 4mM $MgCl_2$ solution, 0.5μM universal forward primer, 0.1μM universal reverse primer, dUTP mix (ThermoFisher, Grand Island, NY) (2μM A, C, G and 4μM U) to work in conjunction with the UDG in the previous step. The amplification reaction required a varying concentration in day to day reactions of Amplitaq Gold DNA polymerase (Applied Biosystems; Foster City, CA) to account for decreased activity of the enzyme over time using the stock. The final amount in the PCR amplification varied from 0.5U to as high as 2.5U per reaction. The PCR amplification was set in a thermocycler with the following conditions: 95°C for 10 minutes followed by 60 cycles: 95°C for 15s, 58°C for 20s, 72°C for 20s. A 7-minute extension at 72° C was added after the cycles with a final hold at 4°C until further processing.

As with the ligation setup, amplification master mix setup including addition of all non-target DNA, buffers, and enzymes was performed inside a PCR hood to prevent any MOLigo amplicon or target STEC DNA from contaminating assay stocks. Following the PCR plate setup, the inside of the hood was wiped with 20% bleach followed by a wipe down with water. After this wipe down, the UV lamp inside the hood was run for 30

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minutes to crosslink any remaining DNA. The addition of the ligation product and any control DNA, purified or synthetic, were added to the PCR plate in a separate hood from the hood where the master mix was dispensed. This hood was cleaned in the same manner as stated previously.

As an additional control for amplicon contamination, once the amplification thermocycling was started in the post-PCR area the pre-PCR, ligation, room was not re-entered until the next day. In implementing this one-way workflow, contamination from the high quantity of amplified MOLigo amplicons during PCR would be reduced greatly.

2.5 Capture of Amplified Product to Multiplex Array

Hybridization of the amplified product to the anti-tag bearing Luminex MagPlex-Tag™ (Luminex Corp.; Austin, TX) microspheres was done using a mix of 13 different microspheres; one for each MOLigo product (STEC-8 along with *stx1*, *stx2*, and *eae*) as well as an assay positive control and an additional microsphere serving as either a blank or PCR positive control. The microsphere mix was made by combining 36μl of each MagPlex-Tag™ microsphere stock in a microcentrifuge tube, pelleting the microspheres at 7,500 x G for 5 minutes then suspended the pellet in 900μL of 800mM NaCl/50mM MES buffer (Fisher Bioreagents). 10μL of this solution was added to each well of the PCR product plate giving 1,000 microspheres for each region (13,000 microspheres total) per reaction well on the plate. Total volume for hybridization reaction was 30μL; 20μL PCR product and 10μL microsphere mix. Hybridization is done on a thermocycler block with a slow ramp down from 95°C (95°C for 3 minutes (min), 85°C for 1 min, 75°C for 1

min, 65°C for 1 min, 55°C for 1 min, 50°C for 30 min, 45°C for 1 min, 40°C for 1 min, 35°C for 1 min, 30°C for 1 min and hold at 25°C).

2.6 Fluorescent Reporter Labeling of Complex and Analysis

Before analysis on the Luminex 100®/ Luminex MAGPIX® the samples were placed on a magnetic plate (MagPlex® beads contain a magnetic core) for 5 minutes before being inverted sharply to remove supernatant. The microsphere pellets in each well were then suspended in 25μl of 10μg/ml Streptavidin Phycoerythrin (SAPE) (BD Biosciences; San Jose, CA) in TE buffer and incubated at room temperature for 30 minutes, protected from light. Following incubation with the SAPE, the plate was pelleted again on the magnetic plate as stated above and the pellet was suspended in 100μl of TE buffer and transferred to a standard 96-well plate for analysis.

The 96-well plate was analyzed on a Luminex 100® or Luminex MAGPIX®, recording the median fluorescence intensity (MFI) values for each region with a lower limit of 100 events per region. Luminex MagPlex® microspheres/beads contain a paramagnetic core and are internally red fluorescent with particular dyes and ranges of concentrations to create an array of uniquely "barcoded" beads. These beads are analyzed on either a Luminex 100®, which is a fluid flow system with single particle laser excitation for detecting fluorescence from bead and reporter molecule, or on a MAGPIX®, which uses magnetic capture of beads and an imager to record fluoresce after excitation from a pair of light emitting diodes (LED) (Figure 2.2). The MAGPIX® is capable of up to a 50-plex assay with the MagPlex-TAG™ beads and the Luminex 100® is capable of up to an 80-plex assay with the same beads.
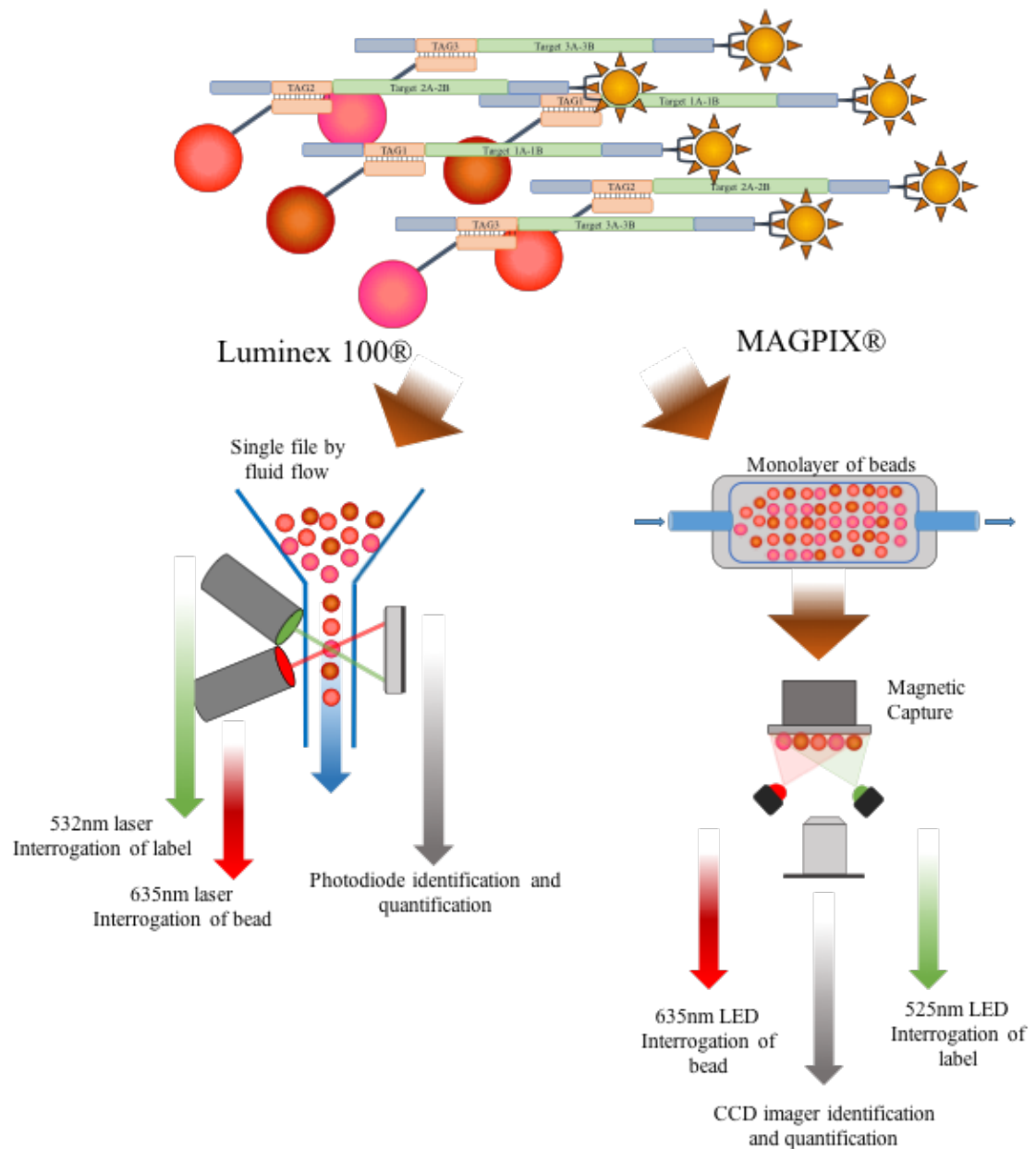
Figure 2.2: Internally fluorescently barcoded beads with unique DNA anti-TAGs attach a single target fluorescent amplicon. The beads and fluorescent amplicon complex are analyzed on a single particle laser excited Luminex 100®. Alternatively, the beads and fluorescent amplicon are analyzed on a static CCD imager system with light emitting diode (LED) excitation system.

Included on the plate is a column of eight no template controls to account for any cross reactivity of the MOLigo pairs in the absence of target DNA. Also included on the plate were 8 wells, one for each STEC-8 target, serving as a positive control for each

target reaction. These MFI values for the no template control and the samples were used as the basis to make a call for "positive" or "negative" for each target DNA sequence. An exact Wilcoxon rank sum analysis was done between the eight independent no template controls and the three replicates of each sample, 11-plex, being tested.

2.7 Exact Wilcoxon Rank Sum Statistical Test

In the assay as it was designed there are small sample sizes upon which decisions will be made about whether a sample was "positive" for a signature or whether there was no evidence to support a statistical difference in populations: "negative". Non-parametric tests are of value in cases where data sets are small, there are non-matched pairs, and little is know of the distribution of the data [41]. In the assay being described here, experimental sets consisted of 11 samples, 8 no-template controls and 3 replicates of single samples. Under all of these considerations it was appropriate to implement a Wilcoxon rank sum test. On small data sets, such as being explored here, it was best to perform an "exact" version of the test; making no comparisons to normally distributed data as is possible on sample sets larger than 10 per pair [42]. Wilcoxon rank sum was performed by 3 steps, which were automated in this assay via an excel spreadsheet. First, all observations were listed in order and assigned a rank where the highest value received the highest rank. If there was an occasion of ties in the data then the tied ranks were averaged and that averaged rank was assigned to each observation involved in the tie. Second, the data sets were separated into their original observations sets and the ranks of the smaller group were summed. This sum (S) was then used as the observation point for the statistical test. Thirdly, the critical value for the statistical test was referenced from a table (Appendix 2) with the appropriate *P* value [43, 44]. (Figure 2.3) In the case here, the

hypothesis was that the treatment observations were greater than the no template observations, which provided that only the critical value from the upper limit table was needed. If S was greater than the upper limit critical value, then the values were considered statistically different and that was considered "positive" for that probe sequence.

| Sample	O26	O45	O103	O104	O111	O121	O145	O157	Stx1	Stx2	Eae
No Template	114	112	104	79	128	125	93	174	125	104	99
No Template	243	526	669	762	1164	1381	413	680	851	1436	482
No Template	189	405	557	628	847	1266	425	800	780	1322	499
No Template	228	416	626	651	1047	1282	403	691	778	1356	490
No Template	192	420	587	660	943	1220	389	665	725	1303	463
No Template	160	376	469	518	724	1025	363	701	638	1062	397
No Template	98	281	378	398	512	716	392	765	592	653	436
No Template	173	375	530	579	957	1006	336	623	654	1201	363
Moxley 46	153	287	283	437	713	2844	491	706	851	5292	2005
Moxley 41	193	431	542	537	956	1385	381	656	790	5159	446
Moxley 17	105	1551	233	342	431	868	379	489	1995	612	1497
Moxley 49	152	353	350	510	725	2822	429	737	777	4967	1990
Moxley 36	61	89	77	100	4346	323	172	191	2453	178	1589
Moxley 56	62	103	100	145	190	402	1002	247	329	4477	1693
Moxley 14	85	1831	175	292	338	726	318	393	2262	511	1579
Moxley 28	74	120	3844	150	210	355	246	333	4683	6082	3036
Moxley 46	135	312	359	486	736	3686	423	617	733	5574	2279
Moxley 41	175	382	441	541	775	1194	451	622	801	5674	613
Moxley 17	96	1907	194	308	400	793	326	444	2383	509	1773
Moxley 49	148	311	413	538	749	2662	399	613	711	4213	1607
Moxley 36	73	131	145	217	3198	605	269	342	1913	421	1399
Moxley 56	63	105	100	124	180	371	906	270	329	3790	1500
Moxley 14	84	1448	178	298	383	702	317	408	1943	503	1352
Moxley 28	81	193	2648	236	394	736	326	402	2600	4558	1787
Moxley 46	146	321	308	490	716	3150	421	590	757	5708	2144
Moxley 41	158	317	446	518	762	1150	412	634	748	4950	478
Moxley 17	84	1744	154	231	322	646	304	389	2374	422	1726
Moxley 49	146	312	393	576	763	2581	386	660	749	4376	1739
Moxley 36	75	117	129	161	3264	539	258	287	2011	312	1347
Moxley 56	52	83	84	113	157	302	890	219	250	3657	1491
Moxley 14	88	1201	206	343	377	712	332	423	1608	577	1163
Moxley 28	80	152	2130	210	328	618	325	405	2220	4277	1517

O45

| 112 | 526 | 405 | 416 | 420 | 376 | 281 | 375 | 1551 | 1907 | 1744 |

O45	Rank
112	1
526	8
405	5
416	6
420	7
376	4
281	2
375	3
1551	9
1907	11
1744	10

No Template Control	Sample Replicates
1	9
8	11
5	10
6	
7	
4	
2	
3	
Sum	
30	

$S(30) > CV_{95\%CI\text{-}upper} \rightarrow$ "Positive"

OR

$S(30) < CV_{95\%CI\text{-}upper} \rightarrow$ "Negative"

Figure 2.3: An example set of data with 8 No Template controls showing MFI for each STEC-8 MOLigo probe along with 8 samples in triplicate, showing MFI for each STEC-8 MOLigo probe. For a particular STEC-8 MOLigo probe (e.g. O45) the 8 No Template controls and 3 replicates of one sample (e.g. Moxley 17) were aligned in a list. This list of 11 values was evaluated assigning a rank of 1 to the lowest value and a rank of 11 to the highest value. The list was then separated back in to the individual sets: No Template controls and Sample Replicates. The rank values for the set with the fewer values were summed together. This sum (S) was compared to a Wilcoxon Rank Sum Critical Value (CV) table for a 8 x 3 sample set. If S was greater than the upper CV then the sample (Moxley 17) was considered "positive" and if S was less than CV then the sample was considered "negative".

Chapter 3: Results of MOL-PCR Analysis

3.1 STEC-8 MOL-PCR Assay Optimization

As described in Chapter 2, a significant amount of care was used to design the probes for detecting STEC-8, which relied initially on work done by Bai *et al*. in their multiplex PCR assay development. In most of that work the *wzx* gene in the O-antigen region of O26, O45, O103, O104, O111, O145 was targeted to look for unique serotype specific sequences and *wbqE* and *wbqF* for O121. O157 was detected using the *rfbE* gene, as it is unique to O157 serotype. Initially each MOLigo pair was tested individually on its respective serotype to develop the appropriate ligation and amplification protocol. Template DNA used in these optimization tests was obtained from American Type Culture Collection (ATCC)(Manassas, VA) (BAA-460D; O157:H7) (BAA-2192D; O145:NM) (BAA-2193D; O45:H2) (BAA-2196D; O26:H11) (BAA-2215D; O103:H11) (BAA-2217D: O146, mislabeled as O111 by ATCC) (BAA-2219D; O121:H19) (BAA-2326D; O104:H4) (BAA-2440D; O111) as the DNA was well characterized, purified, and lyophilized. Each of these strains has an associated virulence profile relative to the specific genes tested in this assay; *stx1*, *stx2*, and *eae*.

During early testing and validation each assay product was split, so that half of the product was visualized on an agarose gel stained with ethidium bromide and the second half was hybridized to Luminex microspheres. MoligoDesigner produces a positive strand MOLigo pair as well as a negative strand (reverse complement) MOLigo pair. When each MOLigo strand was computationally analyzed no interactions with other strands were predicted, so during the protocol optimization step each set (positive or negative) was evaluated against only their complement target DNA from ATCC. STEC

serogroup samples from ATCC were evaluated at 5×10^6 copies of DNA per reaction. This allowed for a very reproducible reaction, which assisted in making any modifications. The reaction was tested on each serotype from ATCC with the MOLigos from the respective set from 1×10^7 copies down to 1×10^4 copies, run on an agarose gel without reaching the failure point of the assay (data not shown). Further limits of detection need to be tested, but expected limits are between 1×10^3 and 1×10^4 copies per reaction [35].

The STEC-8 MOL-PCR assay was tested against two different sets of STEC-8 samples; one set from ATCC (see above) and a second set from KSU. KSU sample strains are H30 (O26), CDC 96-3285 (O45), CDC 90-3128 (O103), ATCC BAA-2326 (O104), JB1-95 (O111), CDC 97-3068 (O121), 83-75 (O145), and 380-94 (O157). Both sets contained all of the STEC-8 serotypes and had slightly different virulence gene profiles. In these tests the full set of all 11 MOLigos pairs were mixed together to perform the fully multiplexed assay.

For easier visualization the data for the 11-plex MOL-PCR assay was displayed as signal to noise ratios calculated from median fluorescence intensity values (sample values divided by the no template control values). A comparison was made between a perfectly performing assay for each set (ATCC or KSU) with the high signal to noise ratios being displayed in red and low signal to noise rations in white in Figure 3.1. There was agreement between the assay data and the predicted data.

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A. *ATCC Predicted Profile*

Target Serogroup	O26 MOLigo	O45 MOLigo	O103 MOLigo	O104 MOLigo	O111 MOLigo	O121 MOLigo	O145 MOLigo	O157 MOLigo	Stx-1 MOLigo	Stx-2 MOLigo	Eae MOLigo
O26 DNA	■								■	■	■
O45 DNA		■							■		■
O103 DNA			■						■		■
O104 DNA				■						■	
O111 DNA					■				■	■	■
O121 DNA						■			■	■	■
O145 DNA							■		■	■	■
O157 DNA								■	■	■	■

B. *ATCC MOL-PCR Assay Signal/Noise Ratio*

Target Serogroup	O26 MOLigo	O45 MOLigo	O103 MOLigo	O104 MOLigo	O111 MOLigo	O121 MOLigo	O145 MOLigo	O157 MOLigo	Stx-1 MOLigo	Stx-2 MOLigo	Eae MOLigo
O26 DNA	29	1	1	1	1	1	1	1	37	90	29
O45 DNA	1	32	1	1	1	1	1	1	40	2	35
O103 DNA	1	1	35	1	1	1	1	1	32	2	24
O104 DNA	1	2	1	12	1	1	1	2	1	41	7
O111 DNA	1	1	1	1	23	1	1	1	36	88	31
O121 DNA	1	1	1	1	1	19	1	1	18	57	15
O145 DNA	1	1	1	1	1	1	5	1	27	80	19
O157 DNA	1	1	1	1	1	1	1	29	20	64	9

C. *KSU Predicted Profile*

Target Serogroup	O26 MOLigo	O45 MOLigo	O103 MOLigo	O104 MOLigo	O111 MOLigo	O121 MOLigo	O145 MOLigo	O157 MOLigo	Stx-1 MOLigo	Stx-2 MOLigo	Eae MOLigo
O26 DNA	■								■	■	■
O45 DNA		■							■		■
O103 DNA			■						■		■
O104 DNA				■						■	
O111 DNA					■				■	■	■
O121 DNA						■			■	■	■
O145 DNA							■		■	■	■
O157 DNA								■	■	■	■

D. *KSU MOL-PCR Signal/Noise*

Target Serogroup	O26 MOLigo	O45 MOLigo	O103 MOLigo	O104 MOLigo	O111 MOLigo	O121 MOLigo	O145 MOLigo	O157 MOLigo	Stx-1 MOLigo	Stx-2 MOLigo	Eae MOLigo
O26 DNA	23	0	0	0	0	0	0	0	18	2	42
O45 DNA	0	24	0	0	0	0	0	0	26	0	81
O103 DNA	0	0	20	0	0	0	0	0	26	1	77
O104 DNA	0	0	0	6	0	0	0	0	0	66	1
O111 DNA	0	0	0	0	28	0	0	0	27	133	78
O121 DNA	0	0	0	0	0	22	0	0	0	115	60
O145 DNA	0	0	0	0	0	0	3	0	0	116	62
O157 DNA	0	0	0	0	0	0	0	27	26	129	84

Figure 3.1: *A.* A perfectly performing assay would return the table as shown, with the high values being deep red and low values being white for ATCC samples. Table is read right to left. Example ATCC O26 DNA would be positive for O26, *stx1*, *stx2*, and *eae*. *B.* shows the actual performance of the 11-plex MOL-PCR assay with agreement of samples to what is predicted. *C.* A perfectly performing assay for the KSU STEC-8 product with high values as deep red and low values in white. *D.* KSU samples tested with the 11-plex MOL-PCR assay with signal to noise intensity values in agreement with what would be expected from the perfectly performing assay.

3.2 Evaluation of Reference Panel

A set of 99 reference purified DNA samples were obtained from Dr. Rodney Moxley, University of Nebraska-Lincoln, which contained O26 (10), O45 (10), O103 (10), O104 (9), O111 (10), O121 (10), O145 (10), O157 (10) across a range of H (flagellar) antigens expressing mixed combinations of virulence genes. Along with these STEC expressing serogroups there were included 6 samples that were O (lipopolysaccharide) antigen classified as O101, O8, O142, O150, O104 (non-STEC), O124. Also included in these samples were the 14 samples that are STEC unrelated used here as an exclusivity panel. For all tests the assay was performed with all MOLigos present in all reactions in order to perform and evaluate the complete 11-plex STEC-8 MOL-PCR assay.

It was the goal of this assay to require as little user interpretation of the data as possible and to that end it was decided that an automated data analysis system should be implemented to prevent any user bias. The reference samples were analyzed with the STEC-8 MOL-PCR assay and used to train the exact Wilcoxon algorithm for making semi-automated calls for serotype and virulence gene profile. It was important to have the ability of the test assay to be able to exclude detection of unrelated genomic DNA from organisms that are present in background of actual farm to table samples (feces, hide, carcass,). In order to test the STEC-8 MOL-PCR assay, an exclusivity panel was evaluated in the same manner as the assay development samples from ATCC and KSU. The exclusivity panel was evaluated against the STEC-8 MOL-PCR assay, but the raw median fluorescent intensity values for the panel were close enough to the no template

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control MFI values that at a 95% confidence interval (CI) many false positives were being identified. The exact Wilcoxon for a small observation set of 11 samples (8 no template controls and 3 sample replicates) was susceptible to false positives. The mean signal to noise ratio for each sample, across all markers, was calculated and used as a basis to adapt the exact Wilcoxon rank sum test. 80.3% of the exclusivity MOL-PCR markers (of 168 possible values) returned a mean signal to noise ratio below 1.5 (Figure 3.2). When the Wilcoxon rank sum statistical test was used to evaluate each of the additional samples in the reference DNA panel, there was an addition condition of a signal to noise ratio above 1.5 as well as a rank sum above the Wilcoxon critical value. Even with this addition, some exclusivity samples still identified as "positive".
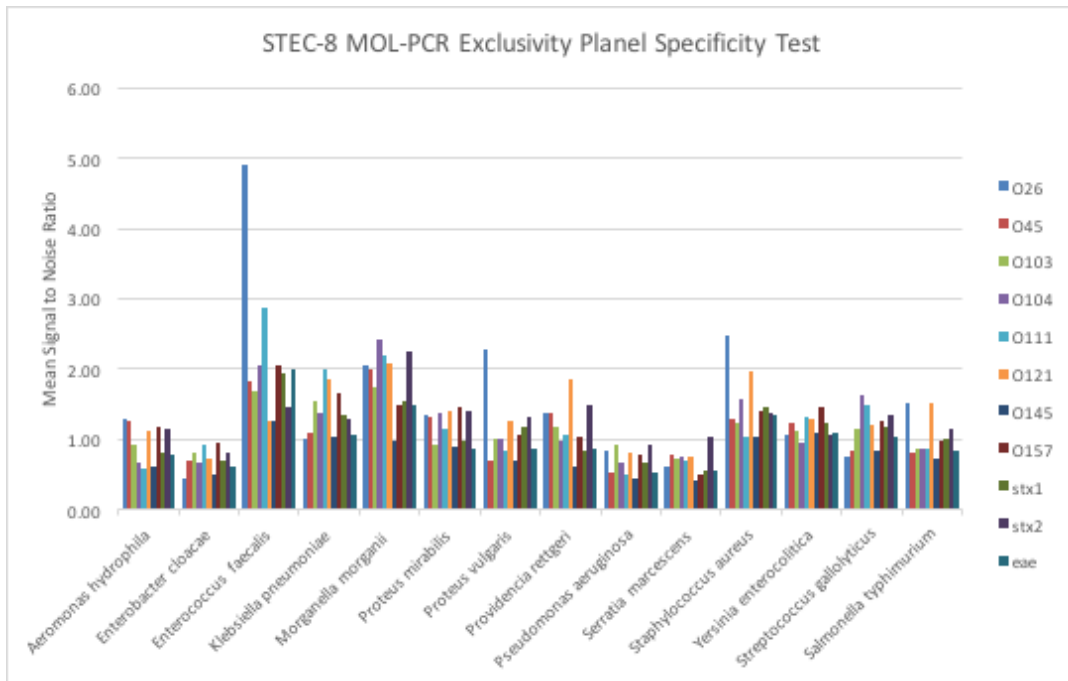
Figure 3.2: Exclusivity panel of STEC unrelated DNA samples from bacterium as noted in figure evaluated with the STEC-8 MOL-PCR assay. The evaluation 11-plex is color coded on the figure with corresponding signal to noise ratio being shown. The majority of the marker responses are below 1.5 signal to noise.

The remaining STEC reference samples were analyzed with the addition of this signal to noise threshold to account for any small amount of interaction with background

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that could be present in the purified samples. In order for a positive call to be made both criteria must be met. Evaluations of the reference purified STEC DNA samples, excluding the exclusivity panel, were tested between 10ng – 30ng STEC DNA per reaction. As with the initial test samples, all reactions were run in triplicate on a single plate to account for variations in reactions. The data show (Table 3.1) that the serotype calls agreed with the known serotypes provided from Dr. Moxley's lab. The virulence profiles for the isolates tested match in most cases. The data provided from Moxley's lab was incomplete and independent searches done in the Michican State University STEC database (www.shigatox.net) were also not complete for virulence gene profiles. There were missing data for *stx1*, *stx2*, and *eae* for the various samples in the set, where missing information is denoted as a blank space in the table and a confirmed "negative" result was displayed as a "-". As a result of this it was difficult to determine the specificity of the assay as it pertains to the virulence gene profile of these samples. When examining the data manually, and even with the algorithm, the MOL-PCR assay identified virulence genes that are present, but not listed in the documentation. JB1-95, 0201 9611, MI01-88, 8266-1, and G5508 isolates all returned more than the predicted serotype, which results in an overall assay specificity (serogroup only as discussed previously) of 93.6%. There are also cases where the assay and algorithm combined returned no serotype signature present, when the test should have detected one of the STEC-8: MDCH-4, KDHE 47, DEC10I (87-1713), 1:361, 1553-1, DA-37, 10C-3114, 2002-3211, KDHE 55, 314-S, 86-24, 403-3. The validity of all of the data that was supplied with these samples was called into question as two of the aforementioned samples were reported incorrectly.

Table 3.1
Comparison of MOL-PCR assay to known database values For STEC reference samples

Isolate	MOL-PCR Assay[1]				Database Values[2]			
	Serotype	stx1	stx2	eae	Serotype	stx1	stx2	eae
CDC 97-3068	O121	-	stx2	eae	O121		stx2	
MDCH-4		-	stx2	-	O113		stx2	-
2000-3039	O45	stx1	-	eae	O45	stx1	-	eae
8-084	O121	-	stx2	eae	O121			
10049	O111	stx1	-	eae	O111	stx1	-	eae
83-75	O145	-	stx2	eae	O145		stx2	
B8026-C1	O45	stx1	-	eae	O45	stx1	-	eae
236-1	O103	stx1	stx2	eae	O103	stx1	stx2	eae
MT#2	O121	-	stx2	eae	O121	stx1		
2011-0-1256	O104	stx1	-	-	O104	stx1	-	-
KDHE 47		-	stx2	eae	O121		stx2	-
TB154A	O103	stx1	-	eae	O103	stx1	-	eae
S2006 #1	O157	stx1	stx2	eae	O157	stx1	stx2	eae
DA-21	O45	stx1	-	eae	O45	stx1	-	eae
2006-3008	O103	stx1		eae	O103	stx1		eae
DEC11C	O45	stx1	-	eae	O45	stx1	-	
IHIT2087	O26	stx1	-	eae	O26	stx1		eae
3215-99 (F6627)	O111	stx1	stx2	eae	O111	stx1	stx2	eae
B2387	O157	-	stx2	eae	O157		stx2	
DEC10I (87-1713)		stx1	-	eae	O145	stx1	-	eae
1234-1	O145	stx1	stx2	eae	O145	stx1	stx2	eae
DEC10E	O26	stx1	-	eae	O26	stx1	-	eae
B8227-C8	O45	stx1	-	eae	O45	stx1	-	eae
7726-1	O111	stx1	stx2	eae	O111	stx1	stx2	
TY-2482	O104	-	stx2	-	O104		stx2	-
B6820-C1	O145	stx1	stx2	eae	O145		stx2	eae
MI-0041B	O104	-	stx2	-	O104			
1.2622	O45	stx1	-	-	O45	stx1	-	-
IH 16	O145	-	stx2	eae	O145	-	stx2	eae
S2006 #4	O157	stx1	stx2	eae	O157	stx1	stx2	eae
15612-1	O103	stx1	-	eae	O103	stx1		eae
236-5	O103	stx1	stx2	eae	O103	stx1	stx2	eae
99-3311	O145	stx1	stx2	eae	O145	stx1	stx2	eae
1:361		-	stx2	-	O157	-	stx2	eae
B8228-C2	O45	stx1	-	eae	O45	stx1	-	
RD8 (7075)	O111	-	stx2	-	O111	-	stx2	-
1553-1		stx1	stx2	-	O121	stx1		-
CDC 1994 3023	O104	-	stx2	-	O104	-	stx2	-
H30	O26	stx1	-	eae	O26	stx1	-	
DA-37		-	stx2	-	O121	-	stx2	eae
2003-3014	O26	stx1	stx2	eae	O26	stx1	stx2	eae
JB1-95	O45, O111	stx1	stx2	eae	O111	stx1	stx2	
IHIT1703	O111	stx1	-	eae	O111	stx1	-	eae
DEC8b	O111	stx1	stx2	eae	O111	stx1	stx2	eae
S2006 #2	O157	stx1	stx2	eae	O157	stx1	stx2	eae
10C-3114		-	stx2	-	O111		stx2	-
IHIT0304	O145	-	stx2	eae	O145	-	stx2	eae
93-111	O157	stx1	stx2	eae	O157	stx1	stx2	eae
MT (CDC 1994 3024)	O104	-	stx2	-	O104	-	stx2	-

Table 3.1 (cont.)

Isolate	Serotype	stx1	stx2	eae	Serotype	stx1	stx2	eae
2002-3211		-	stx2	eae	O121	-	stx2	eae
CDC 96-3285	O45	stx1	-	eae	O45	stx1		
8419	O103	stx1	-	eae	O103	stx1	-	eae
DA-10	O26	stx1	-	eae	O26	stx1	-	
97-3250	O26	stx1	stx2	eae	O26	stx1	stx2	eae
GS G5578620	O145	stx1	-	eae	O145	stx1	-	eae
KDHE 55		-	stx2	eae	O121		stx2	-
7744	O145	stx1	-	eae	O145	stx1		
CDC 90-3128	O103	stx1	-	eae	O103	stx1		
89-118	O103	stx1	-	eae	O103			
933	O157	stx1	stx2	eae	O157	stx1	stx2	eae
314-S		stx1	-	eae	O145	stx1	-	eae
MT#80	O103	stx1	-	eae	O103	stx1	-	eae
M535	O104	stx1	stx2	-	O104	stx1	stx2	
S2006 #3	O157	stx1	stx2	eae	O157	stx1	stx2	eae
413/89-1	O26	stx1	-	eae	O26	stx1	-	eae
86-24		-	stx2	eae	O157		stx2	eae
DEC10B	O26	stx1	-	eae	O26	stx1	-	eae
16272	O26	stx1	-	eae	O26	stx1		
88-1577	O26	-	-	eae	O26	stx1	-	eae
D88-28058	O45	stx1	-	eae	O45			
403-3		-	stx2	-	O121	stx1	-	
9:100	O157	-	stx2	eae	O157	-	stx2	eae
RW1372	O103	stx1	-	eae	O103	stx1	-	eae
0201 9611	O26, O103, O111	stx1	-	eae	O111	stx1	-	eae
MI01-88	O45, O121	stx1	-	eae	O45	stx1	-	
2011-5-383-1	O104	stx1	stx2	-	O104	stx1	-	-
8266-1	O103,O111	stx1	stx2	eae	O111	stx1	stx2	
G5508	O26, O104	-	stx2	-	O104	-	stx2	
MDCH_male_ 069311	O104	-	stx2	-	O104			
G58-1		-	-	-	O101			
2534-86		-	-	-	O8			
11182-2		stx1	-	-	O142			
16118-2		stx1	-	-	O150			
ECOR 26	O104	-	-	-	O104			
43893		-	-	-	O124			

[1]A negative symbol field denotes value not statistically different
from background, value in field denotes value statistically different
from background
[2]Negative symbol denotes isolate known to not express gene, empty
field denotes information not known about gene

MDCH-4 was originally designated as O121 serotype, but it was later discovered
that it was in fact O113, which was corrected in the table. ATCC originally reported 10C-
3114 as O111:H8 and included that strain in its "Big-Six" non-O157 genomic DNA panel
as its reference O111 serotype. ATCC now lists 10C-3114 as O146. With these two
samples removed from calculation the specificity of the entire assay is 87%. These results

demonstrated that the STEC-8 MOL-PCR assay was capable of detecting samples in a large scale and doing so with data analysis automation.

3.3 Evaluation of Blinded Samples

Further testing of the MOL-PCR assay was conducted using a set of more varied samples provided from Kansas State University. These 144, blinded samples were from pure cultures that were boiled, centrifuge separated, and the supernatant lysates were evaluated here. During this testing several problems with contamination arose, which affected our initial results. The background median fluorescence intensity increased for all STEC-8 signatures being evaluated with the assay and approached intensity values that rivaled actual values. Under these conditions the first round of possible solutions for the 144, blinded samples were sent off. The suspected answers were separated into two categories: algorithm based and a manual, human determined, set. The Wilcoxon ranked sum algorithm returned with a correct identification of 55.5%, while the manual evaluation returned 70.8% correct identifications. Identification meant a correct call of only a single serotype and each virulence gene associated with that sample. This required both specificity and sensitivity, which was problematic with increasing backgrounds. The high median fluorescence intensity in the no template controls caused the sensitivity to be reduced. Appendix 1 contains a complete list of all the raw data used to produce this work and work to be discussed. Increased amplicon contamination in the samples caused non-target signatures to appear as if they were "positive" identified for certain signatures, affecting the specificity. The roughly 15% increase in correct identification from human data interpretation shows this. These results provided enough positive matches to continue refining the assay as it was with only little modifications.

As was stated in Chapter 2, the requirements for separation of the reaction steps into well defined compartments and rooms became absolutely necessary. Amplicon carry over was reduced by the substitution of uracil (U) in the place of thymine (T) during the amplification step. Any amplicons generated after this change would contain only U, where there had previously been T. Uracil-DNA Glycosylase was added to the ligase reaction; DNA containing uracil would be released by catalytic hydrolyzis from the UDG [45, 46]. The ligation product, which contained no U, would be unaffected by the UDG. In addition to this, any opening of reaction tubes or plates was done inside of sealed PCR hoods that were segregated by reaction task to prevent carry over. In addition to hood segregation, pre-PCR and post-PCR activities were moved to separate rooms with a unidirectional flow from pre-PCR to post-PCR and never back in the same day [46]. After much time was spent implementing these precautions and multiple reaction tests, re-evaluation of samples from the 144 blinded STEC DNA samples was performed.

In Table 3.2 is a list of all 144 samples with identification, as it is currently known, along with any serotype and virulence gene returned by the algorithm with a comparison to what was provided by the Kansas State University laboratory. In certain cases more than one serotype returned as being present: 48 MSU Red, 8 MSU Red, 7 KSU Green, 5 KSU Green. There are also instances where the STEC-8 MOL-PCR assay did not return any serotype and virulence gene.

Table 3.2
Blinded STEC samples evaluated with MOL-PCR assay as compared to reported values

| Sample ID | MOL-PCR Assay | | | | Reported Values | | | |
	Serotype	stx1	stx2	eae	Serotype	stx1	stx2	eae
51 MSU Red	O26	stx1	-	eae	O26	stx1	-	eae
48 MSU Red	O103, O145	stx1	stx2	eae	O145	-	stx2	eae
47 MSU Red	O145	-	stx2	eae	O145	-	stx2	eae
46 MSU Red	O26	stx1	-	eae	O26	stx1	-	eae
45 MSU Red	O145	-	stx2	eae	O145	-	stx2	eae
44 MSU Red	O145	-	-	eae	O145	-	-	eae

Table 3.2 (cont.)

43 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
42 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
41 MSU Red	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
40 MSU Red	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
39 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
38 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
37 MSU Red	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
36 MSU Red	O26	*stx1*	*stx2*	*eae*	O26	*stx1*	*stx2*	*eae*
35 MSU Red	O26	-	*stx2*	*eae*	O26	-	*stx2*	*eae*
34 MSU Red	O26	-	*stx2*	*eae*	O26	-	*stx2*	*eae*
33 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
32 MSU Red	O145	*stx1*	-	*eae*	O145	*stx1*	-	*eae*
31 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
30 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
29 MSU Red	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
28 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
27 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
26 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
25 MSU Red	O26	*stx1*	*stx2*	*eae*	O26	*stx1*	-	*eae*
24 MSU Red	O145	-	*stx2*	*eae*	O145	-	*stx2*	*eae*
23 MSU Red	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
21 MSU Red	O26	*stx1*	*stx2*	*eae*	O26	*stx1*	*stx2*	*eae*
20 MSU Red	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
19 MSU Red		*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
18 MSU Red	O26	-	-	*eae*	O26	-	-	*eae*
17 MSU Red	O26	-	-	*eae*	O26	-	-	*eae*
16 MSU Red	O26	-	-	*eae*	O26	-	-	*eae*
15 MSU Red	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
14 MSU Red	O111	-	*stx2*	-	O111	-	*stx2*	-
13 MSU Red	O103	*stx1*	*stx2*	*eae*	O111	*stx1*	-	*eae*
12 MSU Red	O103	*stx1*	*stx2*	*eae*	O103	*stx1*	*stx2*	*eae*
11 MSU Red	O103	*stx1*	*stx2*	*eae*	O103	*stx1*	*stx2*	*eae*
10 MSU Red	O145	-	-	*eae*	O145	-	-	*eae*
8 MSU Red	O26, O103	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
7 MSU Red	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
6 MSU Red	O145	-	*stx2*	*eae*	O145	-	*stx2*	*eae*
4 MSU Red	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
3 MSU Red	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
2 MSU Red	O26	-	-	*eae*	O26	-	-	*eae*
25 Blue	O103	*stx1*	-	-	O104	*stx1*	-	-
24 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
23 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
22 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
21 Blue	O104	*stx1*	-	-	O104	-	*stx2*	-
20 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
19 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
18 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
17 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
16 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
15 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
14 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
13 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
12 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
11 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
10 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-

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Table 3.2 (cont.)

Sample	Serogroup				Serogroup			
9 Blue	O45	-	-	*eae*	O45	-	-	-
8 Blue	O45	*stx1*	-	-	O45	*stx1*	-	-
7 Blue	O45	-	-	-	O45	-	-	-
6 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
5 Blue	O104	*stx1*	-	-	O104	*stx1*	-	-
4 Blue		-	*stx2*	-	O104	-	*stx2*	-
3 Blue	157	*stx1*	*stx2*	*eae*	O157	*stx1*	*stx2*	*eae*
2 Blue	157	*stx1*	-	*eae*	O157	*stx1*	-	*eae*
1 Blue	157	-	*stx2*	*eae*	O157	-	*stx2*	*eae*
47 KSU Green	O121	*stx1*	-	-	O121	-	-	-
44 KSU Green		*stx1*	-	*eae*	O145	*stx1*	-	*eae*
43 KSU Green	O145	*stx1*	-	*eae*	O145	*stx1*	-	*eae*
41 KSU Green	O145	*stx1*	*stx2*	*eae*	O145	*stx1*	*stx2*	*eae*
39 KSU Green	O121	*stx1*	-	-	O121	-	-	-
38 KSU Green	O121	*stx1*	-	-	O121	-	-	-
36 KSU Green	O121	*stx1*	-	-	O121	-	-	-
35 KSU Green	O121	*stx1*	-	-	O121	-	-	-
33 KSU Green	O121	*stx1*	-	-	O121	-	-	-
32 KSU Green	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
30 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
28 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
26 KSU Green		-	-	-	O111	*stx1*	*stx2*	*eae*
24 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
22 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
20 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
18 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
17 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
15 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
13 KSU Green	O111	*stx1*	*stx2*	*eae*	O111	*stx1*	*stx2*	*eae*
12 KSU Green	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
11 KSU Green	O103	*stx1*	-	*eae*	O103	*stx1*	-	-
9 KSU Green	O103	*stx1*	-	*eae*	O103	*stx1*	-	-
7 KSU Green	O103, O111	*stx1*	-	*eae*	O103	*stx1*	-	-
5 KSU Green	O45, O103	*stx1*	-	*eae*	O103	*stx1*	-	-
3 KSU Green	O103	*stx1*	-	*eae*	O103	*stx1*	-	-
1 KSU Green	O103	*stx1*	-	*eae*	O103	*stx1*	-	-
60 KDHE Yellow	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
59 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
58 KDHE Yellow	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
57 KDHE Yellow	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
56 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
55 KDHE Yellow		-	*stx2*	*eae*	O121	-	*stx2*	-
54 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	-
53 KDHE Yellow	O145	*stx1*	*stx2*	*eae*	O145	*stx1*	*stx2*	*eae*
51 KDHE Yellow	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
50 KDHE Yellow	O26	*stx1*	-	*eae*	O26	*stx1*	-	*eae*
49 KDHE Yellow	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
48 KDHE Yellow	O121	-	*stx2*	*eae*	O121	-	*stx2*	*eae*
47 KDHE Yellow	O121	-	*stx2*	*eae*	O121	-	*stx2*	*eae*
46 KDHE Yellow	O26	*stx1*	-	*eae*	O26	*stx1*	-	-
44 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
43 KDHE Yellow	O111	*stx1*	-	*eae*	O111	*stx1*	-	*eae*
41 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	*eae*
40 KDHE Yellow		-	-	-	O103	*stx1*	-	*eae*
39 KDHE Yellow	O103	*stx1*	-	*eae*	O103	*stx1*	-	-

Table 3.2 (cont.)								
38 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
35 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
32 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
31 KDHE Yellow	O111	stx1	stx2	eae	O111	stx1	stx2	eae
30 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
29 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
28 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
25 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
23 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
22 KDHE Yellow	O45	stx1	-	eae	O45	stx1	-	eae
21 KDHE Yellow	O111	stx1	-	eae	O111	stx1	-	eae
20 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
19 KDHE Yellow	O111	stx1	-	eae	O111	stx1	-	eae
18 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
16 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
15 KDHE Yellow	O111	stx1	-	eae	O111	stx1	-	eae
13 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
12 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
11 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
10 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
9 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
8 KDHE Yellow	O111	stx1	stx2	eae	O111	stx1	stx2	eae
7 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
6 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
4 KDHE Yellow	O111	stx1	stx2	eae	O111	stx1	stx2	eae
3 KDHE Yellow	O26	stx1	-	eae	O26	stx1	-	eae
2 KDHE Yellow	O103	stx1	-	eae	O103	stx1	-	eae
1 KDHE Yellow	O145	stx1	-	eae	O145	stx1	-	eae

[1]Fields are marked with the values that were statistically different from background, fields marked with negative symbol denote no difference from background
[2]Fields are marked with the known values for the samples as provided from collaborators, fields marked with negative symbols are known to be negative for that gene

Given the information that was provided about these 144 samples it is possible to determine the sensitivity and the specificity of the STEC-8 MOL-PCR assay. The actual strains and isolate IDs for the samples is not known, but the serotype and presence of stx1, stx2, and eae was reported. These values were used to determine the performance of the assay (Table 3.3). Sensitivity was calculated by counting the number of times the assay returned the correct serotype or gene, even if it also identified an additional serotype or gene, out of the number of times that serotype or gene was present.

Specificity was calculated by counting the number of times the assay reported the incorrect serotype or gene from all 144 samples.

Table 3.3
MOL-PCR assay ability to correctly identify STEC DNA

Signature	Sensitivity[1]		Specificity[2]	
O26	96.6%	*(28/29)*	100.0%	*(144/144)*
O45	100.0%	*(4/4)*	99.3%	*(143/144)*
O103	81.1%	*(30/37)*	97.9%	*(141/144)*
O104	89.5%	*(17/19)*	100.0%	*(144/144)*
O111	90.0%	*(27/30)*	99.3%	*(143/144)*
O121	88.9%	*(8/9)*	100.0%	*(144/144)*
O145	84.6%	*(11/13)*	100.0%	*(144/144)*
O157	100.0%	*(3/3)*	100.0%	*(144/144)*
stx1	98.3%	*(114/116)*	95.1%	*(137/144)*
stx2	94.4%	*(34/36)*	98.6%	*(142/144)*
eae	98.1%	*(103/105)*	92.4%	*(133/144)*

[1](m/n) number of returns over the number possible
[2](m/n) number of correct returns over number of samples

In addition to the detailed look at the assay with respect to specificity and sensitivity, it is also important to look at the samples that were incorrectly identified. Table 3.4 takes a look at these samples, with these exceptions: the exclusivity panel was not included, if a particular virulence gene presence was unknown then any value returned by the assay was considered correct. Under these conditions the assay never completely misidentified the serotype and so that was not shown in Table 3.4. The STEC-8 MOL-PCR assay identified an additional serogroup 9 times and completely missed the serotype 14 times. The virulence gene *eae* was the most frequent misidentified gene with 12 occurrences, compared to 6 for *stx1* and 4 times for *stx2*. The STEC-8 MOL-PCR assay missed the complete gene profile or missed one particular gene 4 times.

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Table 3.4
Detailed examination of incorrect identifications

Sample ID	Additional Serogroups[1]	Missed Serotype[2]	Incorrect stx1[3]	Incorrect stx2[4]	Incorrect eae[5]	Missed Gene[6]
STEC Reference Samples						
KDHE 47		X				
DEC10I (87-1713)						
1.2622				X		
1:361						
1553-1		X				
DA-37		X				X
JB1-95	X					
10C-3114		X				
2002-3211		X				
KDHE 55		X			X	
314-S		X				
86-24		X				
403-3		X		X		X
0201 9611	X					
MI01-88	X					
2011-5-383-1				X		
8266-1	X					
G5508	X					
144 Blinded STEC Samples						
48 MSU Red	X					
19 MSU Red		X				
08 MSU Red	X					
Blue 21			X			
Blue 09					X	
Blue 04		X				
47 KSU Green				X		
39 KSU Green			X			
38 KSU Green			X			
36 KSU Green			X			
35 KSU Green			X			
33 KSU Green			X			
26 KSU Green		X				X
11 KSU Green					X	
09 KSU Green					X	
07 KSU Green	X				X	
05 KSU Green	X				X	
03 KSU Green					X	
01 KSU Green					X	
55 KDHE Yellow		X			X	
54 KDHE Yellow					X	
46 KDHE Yellow					X	
40 KDHE Yellow		X				X
39 KDHE Yellow					X	
Sum	9 of 226	14 of 226	6 of 226	4 of 226	12 of 226	4 of 226

[1]Additional serogroups along with the correct serotype identified by the STEC-8 MOL-PCR assay

[2]No serotype identified by the STEC-8 MOL-PCR

[3]*stx1* identified by the STEC-8 MOL-PCR assay when it is known not to be present

[4]*stx2* identified by the STEC-8 MOL-PCR assay when it is known not to be present

[5]*eae* identified by the STEC-8 MOL-PCR assay when it is known not to be present

[6]Any virulence or all virulence genes were not identified by the STEC-8 MOL-PCR assay

Chapter 4: Discussion and Future Directions

4.1 STEC Detection: MOL-PCR Comparison

      The landscape for pathogen detection is vast and ever expanding, with new assays being developed or modified continually as advancements in technology and tools become available or become inexpensive to implement. As discussed in Chapter 1, there are a host of assays available to perform detection of Shiga toxin-producing *Escherichia coli* and many of them are capable of doing multiple tests in a single reaction [11, 28-29]. In many of the assays referenced detection of STEC is limited to a set of virulence related genes as the primary tool for early detection. The trend that was displayed across the multitude of nucleic acid assays detecting STEC was that virulence gene profile is most important, followed by ability to identify STEC serotype. Novel assays like the ramification amplification assay, Li, et al. [32], looked for only *stx* genes, while the other assays expanded upon gene detection like Blanco, et al. [5], Shen, et al. [47], Son, et al. [48]. There STEC virulence gene detection was expanded to include subtypes of important genes: *eae (α1, α2, β1, β2, γ1, γ2/θ, δ/κ, ε, ζ, λ, η, μ), stx1 (1a, 1c, 1d,), stx2 (2a, 2b, 2c, 2dact, 2e, g)*, *ehxA*. There were, of course, nucleic assays that detect virulence genes along with STEC serotypes, but these assays were either done in two separate assays as in Conrad, et al. [12] or detected serotype and gene in a single assay, but approached their limit of multiplexing on gels as in Bai, et al. [11].

      The goal of the multiplex oligonucleotide ligation polymerase chain reaction assay I have developed is to test enriched samples as a screening assay for samples that are suspected to be STEC in a high throughput way with a high amount of meaningful content (multiplex analysis). The STEC-8 MOL-PCR assay that I have described is

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capable of performing this task in a reliable way, which adds to the ability to detect STEC in a large number of samples collected in the entire production process of beef from farm to table. We have shown the ability of the assay to perform an 11-plex assay, screening STEC-8 (O26, O45, O103, O104, O111, O121, O145, O157) along with the 3 major virulence genes (*stx1*, *stx2*, and *eae*) on as many as 24 samples in one day with a high level of confidence.

As was discussed in Chapter 1, other nucleic assays, while sensitive and multiplex capable (Table 1.1), have difficulty when trying to process large sets of samples in as short a period of time as possible. Most of the assays described are able to process between 4-17 samples with a single replicate. Here we have shown that we can perform our assay in triplicate on 24 samples at a time. The STEC-8 MOL-PCR assay was designed to be of a robust design, which when run as described it produced results with limited amounts of false positive and false negatives. If this number were decreased to only a single replicate it would be possible to process 80-88 samples for all 11 unique signatures in single day.

I conducted a large-scale sample analysis with two groups of STEC and STEC associated samples. In the first set I was able to identify STEC samples across the major serogroups of interest in the United States, with a high level of specificity, 93.6%. It was shown that even in cases where serotype or gene identification was initially misrepresented, the assay was still able to make a correct identification utilizing the modified exact Wilcoxon rank sum algorithm and assay design. The MOL-PCR assay was also able to identify several virulence genes as being present that have not been

previously or routinely documented (Table 3.1). It is my hope that this work will help provide useful information to the larger community of STEC research.

As has been stated, the data that was provided to me along with the enriched beef supply chain DNA samples appears to be incomplete and in some cases incorrect. I believe that this gives hope to an increased specificity to the assay greater than what was stated in Table 3.3 for the 144 initially blinded samples provided from KSU. The assay is a robust assay with a specificity ranging from 92% to 100% across the various 11-plex of markers present. With additional strain information the specificity of detection could dramatically increase to near 100% across all markers. It should be noted that some issues with sample contamination from earlier optimization runs of the assay could have also contributed to the reduction of specificity of the MOL-PCR assay. Other concerns affecting the sensitivity are the activity of the reagents and their stability over long periods of time along with proper storage; understanding this is a goal for translation of this technology to real world use in other laboratories and/or field deployment.

4.2 Translation of STEC-8 MOL-PCR into Wide Use

From the beginning I was tasked with developing an assay that I could, not only do reliably and robustly, but that also could transition into the hands of additional laboratories. My experience in designing this assay has provided me with a great deal of knowledge on issues that arise in a complex multicomponent assay such as what I have described. MOL-PCR as implemented it here is a three-step process, with each step having a number of reagents (Table 4.1). This table shows the points of failure that I experienced during the development of the assay that inhibited progress, but also served

as a learning experience. During development I experimented with many types of reagent

handling such as aliquoting, temperature, and cleaning procedures. I have

Table 4.1
Points of process failure during MOL-PCR assay

Process Step	Components	Failure Mechanisms
Ligation		
	Ligase Enzyme	Reduction of specific activity
	MOLigo Pairs	Degradation of DNA
	Buffers	Contamination, Reduction in activity
	UDG Enzyme	Reduction of specific activity
Amplification		
	Polymerase Enzyme	Reduction of specific activity
	dUTP/dNTP Mix	Stability of nucleotides
	Univeral Primers	Degradation of DNA
	Buffers	Contamination
Capture Hybridization and Labeling		
	Luminex MagPlex TAG Beads	Amplicon Contamination
	StreptAvidin-PE	Activity, Contamination
	Buffers	Amplicon Contamination

experienced reduction in assay performance as reagents begin to lose specific activity

over repeated uses and/or freeze thaw cycles and even complete loss of responsiveness of

the assay. As regents are used and the volume in the stock tube or aliquot is reduced, so is

the activity of the enzyme or buffer depending on its components. I believe this to be an

issue with loss of enzyme activity and degradation of DNA from repeated changes in

temperature and relative DNA concentration. When these issues were experienced, replacement of that reagent would return the assay to previous activity levels.

Very briefly, it was also found that amplicon contamination began to be a very serious problem. The addition of special zones and rooms for performing all related tasks made all components of the assay more reliable and easier to troubleshoot. Proper cleaning of all tools, pipettes, pipette-tip boxes, reagent holders, and the like with bleach and water and/or repeated exposure to UV light helped to reduce the amounts of contamination as well [46].

4.3 Additional MOL-PCR Assay

There are many areas of this assay that still need to be explored and have been planned for the very near future. One of the first experiments planned to better understand this assay is to get a reliable determination on the limits of detection of each of the 11-plex unique signatures used in the assay. This particular determination has been outlined in previous work by Paddock, et al.[21], where cultures for each serotype were inoculated and incubated at 37°C until Absorbance$_{600}$ reached 0.4 (~10^8 colony forming units per milliliter, CFU/mL). This cultures stock was diluted by 10 -fold dilutions down 10^2 CFU/mL, and the dilutions were tested by growing on agar plates. Each of these preparations will provide points to test until assay failure. These samples have already been prepared and are frozen with plans to test them in the very immediate future.

In addition to this limit of detection we plan to test the performance of the MOL-PCR assay in cattle feces samples that have been spiked with STEC DNA. These samples were prepared in a similar way to the ones described above. In this test we will determine

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where the assay begins to fail in the presence of the complex background from cattle feces.

One of the benefits to the MOL-PCR assay, which has largely been ignored in this report is the ability for the assay to easily changed. It was not discussed, but this assay has had a few probe redesigns to change MagPlex-TAG™ beads as well as MOLigo redesigns. Throughout these changes the reaction conditions and the non-modified probes did not have to be adjusted to accommodate these desired manipulations. This becomes important when large scale multiplexing is implemented. The Luminex MAGPIX® instrument is capable of looking at 50 unique signatures or markers, of which I used only 11. I have discussed here additional components that are very interesting to add to the assay including: *ehxA* (the gene for enterohaemolysin) and performing allelic *eae* subtyping for further characterization data of beef samples.

There is a second STEC detection assay that is being developed by Heather Mendez at the University of New Mexico that relies on single nucleotide polymorphisms (SNP) for detection of serotypes and is able to distinguish between STEC and Non-STEC samples [49]. In the assay we developed it is not currently possible to determine in a mixed sample of STEC DNA, which of the serogroups are producing *stx1*, *stx2*, or *eae.* This is due to the way this assay was designed to detect only the presence of each DNA signature sequence. The SNP MOL-PCR assay would have the ability to determine STEC virulence and producing serotype in a single test. While these assays were developed separately, they were also designed to have very minimal multiplexing overlap. With changing of a couple TAG/anti-TAG capture sequence associations; it would be possible

to run these two assays simultaneously to achieve single sample screening (STEC-8 MOL-PCR) and characterization (SNP MOL-PCR).

A long-term goal as described in this chapter is to translate this assay to other laboratories, in doing so a prevalence study will be implemented. In this study I would be collecting samples from various beef associated locations (feed lots, slaughterhouses, etc.) to determine the prevalence of the STEC-8 being currently detecting. The hope is to find out if certain serotypes are more prevalent overall or if there are regional pockets of serotype locality. There is a recent study from Dewsbury, et al., [50] that looks at the seasonal prevalence of STEC across 24 different feedlots. I believe that this assay could add an increased throughput to a research study such as this as well as provide more characterization information if the above modification are also implemented.

I believe that movement of this STEC-8 (or greater with future modifications) MOL-PCR assay into the wider research community would be of unique benefit for STEC detection and characterization studies. MOL-PCR has the same technology backbone as multiplex PCR and other PCR based assay that are already present in many laboratories. With the addition of an inexpensive Luminex MAGPIX® instrument to a laboratory, the amount of data and sample processing would be greatly increased to the benefit of STEC detection.

List of Appendices

Appendix 1

Median Fluorescent Intensity Data for Reference STEC and 144 Blinded

STEC Samples

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	0	23	0	0	12	13	5	5	4	36	22
No Template	0	37	21	0	0	6	22	35	15	12	1
No Template	14	15	14	9	0	7	21	0	29	22	0
No Template	2	13	23	36	0	4	8	3	10	24	16
No Template	31	0	0	21	9	0	19	0	16	50	27
No Template	327	963	4	1121	1404	87	630	0	246	36	700
No Template	37	0	0	38	0	7	0	46	0	3	0
No Template	30	67	29	21	17	64	23	59	43	11	58
51 MSU Red	2495	74	22	721	35	19	43	0	2446	0	2522
48 MSU Red	11	7	1637	52	6	40	751	31	2356	1341	2787
47 MSU Red	0	0	0	0	0	0	635	0	0	1564	2273
46 MSU Red	1521	0	9	35	11	0	0	10	2221	17	2122
45 MSU Red	21	0	8	1	21	0	601	8	11	1358	2534
44 MSU Red	12	18	0	0	5	4	623	0	36	28	3088
43 MSU Red	0	14	1129	30	8	12	15	0	2037	5	2642
42 MSU Red	0	0	891	52	1	0	37	12	2171	59	2910
51 MSU Red	1966	15	42	26	32	23	0	0	2062	48	2010
48 MSU Red	3	31	1797	14	23	14	714	13	2662	1504	2784
47 MSU Red	0	41	0	8	29	27	753	18	19	1335	2745
46 MSU Red	4	28	10	0	20	2	26	5	677	41	1816
45 MSU Red	7	0	16	0	0	16	679	1	0	1599	2594
44 MSU Red	23	29	14	0	6	18	622	0	0	29	2172
43 MSU Red	0	35	1079	13	3	26	0	17	2079	26	2405
42 MSU Red	25	8	797	0	30	9	0	15	1932	13	2375
51 MSU Red	2403	2	10	25	0	19	5	0	2325	3	2283
48 MSU Red	31	20	1480	38	12	0	520	16	1879	1180	2072
47 MSU Red	15	197	16	272	62	23	792	0	33	1293	2182
46 MSU Red	1560	0	0	0	0	0	12	5	2080	4	2232
45 MSU Red	3	10	0	3	23	6	585	0	6	1411	2000
44 MSU Red	28	8	0	1	0	33	561	39	0	15	2236
43 MSU Red	14	20	1050	1	17	19	18	6	1878	15	1998
42 MSU Red	29	41	951	0	14	0	42	44	1922	15	1984
Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	0	23	0	0	12	13	5	5	4	36	22
No Template	0	37	21	0	0	6	22	35	15	12	1
No Template	14	15	14	9	0	7	21	0	29	22	0
No Template	2	13	23	36	0	4	8	3	10	24	16
No Template	31	0	0	21	9	0	19	0	16	50	27
No Template	327	963	4	1121	1404	87	630	0	246	36	700
No Template	37	0	0	38	0	7	0	46	0	3	0
No Template	30	67	29	21	17	64	23	59	43	11	58
41 MSU Red	11	43	27	25	1490	11	18	50	1912	8	2237
40 MSU Red	6	16	1	23	1925	0	0	22	2326	0	2102
39 MSU Red	32	44	1061	0	27	15	21	0	2165	11	2114
38 MSU Red	1137	1201	1337	1184	1257	1201	803	27	1921	161	1833
37 MSU Red	2457	966	47	1014	1166	670	609	43	1994	38	2396
36 MSU Red	1816	31	35	221	78	21	31	3	1796	952	2085
35 MSU Red	1909	1199	73	1274	25	201	1103	130	2081	1588	927
34 MSU Red	1788	0	0	42	12	23	62	0	107	1043	1119
41 MSU Red	37	40	7	41	2206	20	52	31	2670	17	2905
40 MSU Red	33	53	24	31	2861	49	0	2	3593	22	3583
39 MSU Red	22	46	1539	552	73	16	63	1	2650	24	2608
38 MSU Red	32	22	2261	262	54	22	67	6	3461	30	3922
37 MSU Red	2668	18	31	2	34	0	54	9	2763	9	3005
36 MSU Red	3120	19	12	3	11	20	0	38	3661	1831	4078
35 MSU Red	1768	19	11	60	36	16	11	7	2	1837	2404
34 MSU Red	2498	0	0	30	0	10	97	31	49	1417	3433
41 MSU Red	2	15	7	44	1495	0	58	46	1891	49	2345
40 MSU Red	5	53	13	40	2000	11	14	24	3083	10	2916
39 MSU Red	70	13	1130	46	6	4	21	36	1795	51	1961
38 MSU Red	33	27	2225	40	60	24	74	6	3044	0	3007
37 MSU Red	2368	27	36	27	16	23	16	25	2794	13	3087
36 MSU Red	1945	15	17	0	35	0	22	0	2211	1463	2772
35 MSU Red	2382	44	31	37	22	0	0	23	8	1849	3306
34 MSU Red	1833	37	0	150	67	13	38	38	42	1698	2539
Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	0	23	0	0	12	13	5	5	4	36	22
No Template	0	37	21	0	0	6	22	35	15	12	1
No Template	14	15	14	9	0	7	21	0	29	22	0
No Template	2	13	23	36	0	4	8	3	10	24	16
No Template	31	0	0	21	9	0	19	0	16	50	27
No Template	327	963	4	1121	1404	87	630	0	246	36	700
No Template	37	0	0	38	0	7	0	46	0	3	0
No Template	30	67	29	21	17	64	23	59	43	11	58
33 MSU Red	0	27	1378	202	7	42	41	14	1747	38	2103

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
32 MSU Red	8	0	7	11	7	0	773	0	2070	0	2681
31 MSU Red	61	338	1240	910	621	56	411	5	2070	28	2305
30 MSU Red	121	21	1510	13	2	4	20	0	2519	1	2374
29 MSU Red	2416	24	3	61	0	5	0	9	2744	2	2912
28 MSU Red	46	30	1538	54	12	21	37	1	2414	12	2466
27 MSU Red	4	28	1597	0	0	3	0	19	2343	28	2830
26 MSU Red	0	28	1367	28	0	21	0	0	2621	7	2904
33 MSU Red	34	43	2057	17	9	8	1	18	2857	16	2969
32 MSU Red	1078	1526	399	1432	1599	940	1158	17	2252	44	2643
31 MSU Red	55	21	1561	112	0	43	86	0	2490	23	2549
30 MSU Red	237	15	1661	40	33	3	37	0	3024	0	2614
29 MSU Red	1946	12	34	22	0	0	16	0	2401	24	2780
28 MSU Red	4	9	1833	2	18	10	1	17	2702	0	3093
27 MSU Red	13	33	2163	34	50	20	8	10	2852	18	3315
26 MSU Red	28	27	0	69	10	0	30	79	0	0	18
33 MSU Red	34	35	1356	16	8	38	24	36	2904	0	2664
32 MSU Red	40	84	0	46	17	17	709	4	1787	46	2485
31 MSU Red	35	0	1441	13	23	35	76	42	1948	33	3015
30 MSU Red	14	6	1151	42	0	0	0	19	2322	38	3489
29 MSU Red	1610	2	13	25	31	5	19	8	2242	10	2132
28 MSU Red	0	16	1405	112	6	6	0	22	2629	2	2608
27 MSU Red	1	19	1616	22	14	53	81	26	2467	47	2778
26 MSU Red	6	0	13	10	0	19	0	17	32	38	15

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	7	10	5	12	13	7	12	9	12	11	12
No Template	7	9	5	7	6	7	16	1	7	8	9
No Template	6	12	3	8	9	4	7	4	10	10	9
No Template	11	11	10	11	11	7	8	10	13	7	8
No Template	10	11	5	1	4	14	11	4	8	4	10
No Template	8	3	8	5	9	5	8	7	5	6	7
No Template	7	7	0	6	5	5	4	5	9	4	7
No Template	11	7	5	9	8	9	7	11	10	8	7
25 MSU Red	646	5	5	7	7	12	6	5	1576	11	5244
24 MSU Red	8	9	10	8	6	7	141	3	13	36	4730
23 MSU Red	1021	8	10	11	9	10	10	9	1784	11	5305
21 MSU Red	1316	0	5	9	7	9	4	13	2641	519	5889
20 MSU Red	6	9	12	7	12	5	9	13	430	10	4487
19 MSU Red	9	9	1	7	9	3	9	8	47	39	3854
18 MSU Red	53	6	7	10	4	8	4	8	10	7	4057
17 MSU Red	51	14	11	9	12	11	9	8	11	8	2658
25 MSU Red	3147	15	11	16	14	12	17	7	3592	13	3126
24 MSU Red	7	8	10	7	6	9	130	9	9	45	4974

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
23 MSU Red	733	7	3	5	0	3	6	8	1354	5	5948
21 MSU Red	828	8	8	13	9	10	8	7	1428	207	4614
20 MSU Red	3	0	10	1	6	0	2	10	834	6	5110
19 MSU Red	6	7	3	11	6	7	5	5	72	28	2812
18 MSU Red	48	9	6	14	11	11	8	11	4	7	3842
17 MSU Red	360	5	9	10	9	14	8	7	7	7	3830
25 MSU Red	496	8	10	10	8	4	5	8	1060	9	4067
24 MSU Red	9	2	7	8	1	7	72	2	8	24	3227
23 MSU Red	209	8	8	4	4	9	9	7	497	10	3791
21 MSU Red	119	11	9	8	8	9	8	8	306	37	3556
20 MSU Red	8	6	9	6	2	9	8	4	337	5	3651
19 MSU Red	8	5	6	10	11	8	9	8	46	14	2097
18 MSU Red	15	7	7	8	6	5	3	7	3	10	2673
17 MSU Red	60	8	9	14	10	5	12	10	7	9	2395
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	7	10	5	12	13	7	12	9	12	11	12
No Template	7	9	5	7	6	7	16	1	7	8	9
No Template	6	12	3	8	9	4	7	4	10	10	9
No Template	11	11	10	11	11	7	8	10	13	7	8
No Template	10	11	5	1	4	14	11	4	8	4	10
No Template	8	3	8	5	9	5	8	7	5	6	7
No Template	7	7	0	6	5	5	4	5	9	4	7
No Template	11	7	5	9	8	9	7	11	10	8	7
16 MSU Red	1166	12	10	10	9	9	9	10	12	9	4074
15 MSU Red	11	9	6	12	12	12	8	11	841	62	4584
14 MSU Red	10	11	4	11	13	10	8	6	11	13	9
13 MSU Red	10	10	10	14	14	11	8	6	2083	16	3982
12 MSU Red	6	6	6	8	6	5	8	2	7	8	41
11 MSU Red	8	6	11	12	7	5	11	10	425	28	4344
10 MSU Red	7	9	7	12	8	6	468	5	9	7	4479
08 MSU Red	145	15	15	13	11	13	13	11	221	14	3280
16 MSU Red	1416	9	7	10	9	8	11	5	10	11	3568
15 MSU Red	7	7	6	11	7	7	8	10	168	20	2983
14 MSU Red	7	5	5	8	6	5	7	8	8	8	6
13 MSU Red	7	9	10	11	12	8	10	7	1576	11	3860
12 MSU Red	6	10	4	7	6	5	7	4	41	8	2367
11 MSU Red	7	4	14	10	5	8	9	4	352	31	3923
10 MSU Red	11	11	9	11	7	10	288	9	8	6	3617
08 MSU Red	147	10	13	13	12	8	10	4	241	10	3548
16 MSU Red	957	7	8	12	7	10	8	7	10	9	3524
15 MSU Red	7	8	6	8	10	6	8	6	199	30	3323
14 MSU Red	4	7	6	7	6	4	9	7	10	4	3

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
13 MSU Red	6	8	8	9	13	7	10	9	1384	10	4087
12 MSU Red	6	7	8	9	6	8	5	5	43	14	2526
11 MSU Red	5	6	10	8	3	7	10	5	166	19	3545
10 MSU Red	10	8	10	11	7	9	435	8	10	12	4497
08 MSU Red	151	9	12	15	12	13	12	13	254	9	3485

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	7	10	5	12	13	7	12	9	12	11	12
No Template	7	9	5	7	6	7	16	1	7	8	9
No Template	6	12	3	8	9	4	7	4	10	10	9
No Template	11	11	10	11	11	7	8	10	13	7	8
No Template	10	11	5	1	4	14	11	4	8	4	10
No Template	8	3	8	5	9	5	8	7	5	6	7
No Template	7	7	0	6	5	5	4	5	9	4	7
No Template	11	7	5	9	8	9	7	11	10	8	7
07 MSU Red	1324	9	11	8	9	11	12	8	1741	8	4013
06 MSU Red	10	8	5	8	9	11	327	11	6	107	4227
04 MSU Red	806	10	8	7	7	8	7	8	1087	10	4084
03 MSU Red	5	5	7	7	8	7	5	4	657	9	4281
02 MSU Red	118	8	7	8	4	7	9	7	6	5	3858
25 Blue	7	6	7	1216	8	9	12	6	1944	9	8
24 Blue	6	6	6	1212	2	6	9	10	2040	5	7
23 Blue	8	9	9	1182	10	10	9	7	1099	11	8
07 MSU Red	1667	8	7	11	10	7	9	9	2055	11	3729
06 MSU Red	7	12	8	11	8	2	63	7	5	49	3733
04 MSU Red	537	9	12	11	6	8	6	5	851	10	3916
03 MSU Red	4	10	4	11	9	11	8	7	421	8	3851
02 MSU Red	57	10	6	12	8	6	8	6	12	12	3290
25 Blue	9	10	9	1187	5	5	7	9	1788	8	5
24 Blue	9	10	7	1180	10	7	8	5	1507	7	4
23 Blue	5	7	10	1066	6	5	11	6	1602	7	9
07 MSU Red	2528	15	15	14	16	8	15	14	2573	13	2417
06 MSU Red	9	10	4	9	8	8	78	11	7	75	3944
04 MSU Red	920	9	10	11	12	10	8	6	1341	7	4165
03 MSU Red	8	5	8	10	8	7	5	9	556	6	4054
02 MSU Red	43	7	3	11	6	6	6	4	9	8	3048
25 Blue	5	8	9	1101	8	11	6	6	1589	6	5
24 Blue	6	7	6	1133	9	6	7	7	2024	6	10
23 Blue	5	6	11	1036	8	4	7	5	1798	8	8

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	440	1650	45	2714	1791	336	432	53	1512	37	2062
No Template	101	1011	29	927	559	79	328	28	388	24	726

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	50	458	21	663	182	35	349	23	169	24	293
No Template	56	460	25	791	126	40	296	24	105	26	224
No Template	38	373	23	230	165	39	311	14	78	20	158
No Template	25	176	19	443	45	22	252	20	50	16	76
No Template	47	559	23	812	137	36	327	20	158	23	277
No Template	146	1250	29	1790	828	90	382	37	900	31	1371
Blue 22	1032	1697	64	3253	2213	689	463	46	4874	28	3107
Blue 21	451	1077	31	3009	1602	180	306	24	4520	20	1959
Blue 20	262	838	25	2786	1175	67	263	27	4312	25	1428
Blue 19	214	788	20	2897	757	64	221	21	4413	20	863
Blue18	114	610	19	2492	557	32	175	16	3615	23	613
Blue 17	188	909	22	3192	931	53	267	35	4867	33	1087
Blue 16	119	659	21	3454	678	49	315	24	5235	28	756
Blue 15	398	1567	45	3526	1817	156	426	43	5181	45	2334
Blue 22	776	1603	52	2728	1623	400	367	45	3816	36	2763
Blue 21	363	1075	24	2664	1349	100	286	25	3699	21	1684
Blue 20	216	845	21	2657	923	62	253	19	3798	26	1366
Blue 19	179	466	19	2948	1048	45	215	21	4388	26	581
Blue18	69	536	21	2736	394	28	171	22	4042	19	313
Blue 17	163	661	31	2872	1007	47	210	24	4180	32	685
Blue 16	136	488	28	3101	928	51	281	25	4789	30	612
Blue 15	235	975	44	3099	1436	91	333	40	4394	43	1505
Blue 22	613	1342	43	2192	1776	239	334	37	2899	35	2677
Blue 21	187	642	20	2003	1156	56	192	21	2582	18	1280
Blue 20	119	742	20	2495	635	43	209	23	3654	22	896
Blue 19	69	289	18	2147	357	29	176	20	3074	19	277
Blue18	50	213	21	2349	210	24	159	18	3458	29	173
Blue 17	62	377	19	2432	296	28	166	25	3508	21	294
Blue 16	145	847	24	3009	618	47	284	28	4527	27	678
Blue 15	152	838	24	1648	1005	56	269	26	3052	23	881

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	440	1650	45	2714	1791	336	432	53	1512	37	2062
No Template	101	1011	29	927	559	79	328	28	388	24	726
No Template	50	458	21	663	182	35	349	23	169	24	293
No Template	56	460	25	791	126	40	296	24	105	26	224
No Template	38	373	23	230	165	39	311	14	78	20	158
No Template	25	176	19	443	45	22	252	20	50	16	76
No Template	47	559	23	812	137	36	327	20	158	23	277
No Template	146	1250	29	1790	828	90	382	37	900	31	1371
14 Blue	214	1181	35	2423	1076	100	326	36	3422	37	2084
13 Blue	100	596	21	2308	678	41	278	27	3442	27	758
12 Blue	56	302	20	2557	305	28	210	23	3775	18	309

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
11 Blue	33	103	19	2575	90	19	178	21	4059	22	75
10 Blue	37	213	17	2645	64	28	178	18	4147	20	100
09 Blue	22	2622	18	207	24	40	73	19	150	21	3525
08 Blue	59	2879	28	206	35	50	223	24	4135	23	478
07 Blue	139	3134	30	1157	94	166	366	33	1440	31	2244
14 Blue	377	1529	46	2448	1480	167	340	36	3472	39	2405
13 Blue	87	438	18	2731	653	34	267	24	4097	24	662
12 Blue	77	621	24	2372	510	39	244	25	3504	29	577
11 Blue	50	391	20	3128	184	22	252	24	4728	31	193
10 Blue	50	335	21	2682	288	31	246	24	4162	22	228
09 Blue	33	2464	23	775	27	55	134	24	314	25	3285
08 Blue	80	3025	22	493	52	71	264	22	4184	24	783
07 Blue	316	2557	51	1867	169	378	392	44	2143	34	2761
14 Blue	285	1353	35	2271	1064	150	312	36	3228	36	2213
13 Blue	132	677	22	2720	714	43	296	31	4073	25	902
12 Blue	81	523	28	2652	504	36	233	28	3900	31	429
11 Blue	50	319	20	2235	153	27	177	23	3486	22	121
10 Blue	44	289	22	2172	177	26	189	22	3427	22	148
09 Blue	26	2673	20	589	20	60	159	21	291	19	3579
08 Blue	103	2420	23	596	56	101	266	23	3248	28	823
07 Blue	401	3116	66	2176	215	484	451	42	2492	34	2747
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	440	1650	45	2714	1791	336	432	53	1512	37	2062
No Template	101	1011	29	927	559	79	328	28	388	24	726
No Template	50	458	21	663	182	35	349	23	169	24	293
No Template	56	460	25	791	126	40	296	24	105	26	224
No Template	38	373	23	230	165	39	311	14	78	20	158
No Template	25	176	19	443	45	22	252	20	50	16	76
No Template	47	559	23	812	137	36	327	20	158	23	277
No Template	146	1250	29	1790	828	90	382	37	900	31	1371
06 Blue	144	1120	38	3099	717	59	342	36	4490	40	1171
05 Blue	57	374	21	2787	160	29	229	25	4059	26	274
04 Blue	61	519	22	2425	176	30	157	26	59	3292	265
03 Blue	13	62	21	80	38	28	31	3253	4355	4291	4097
02 Blue	26	66	19	194	52	28	45	2809	4047	26	3644
01 Blue	18	113	20	86	46	32	34	2882	41	3745	3690
47 KSU Green	84	457	22	1030	229	2879	239	28	4042	27	417
44 KSU Green	47	920	22	1208	495	115	489	23	3974	22	3798
06 Blue	56	475	25	2486	229	28	223	22	3670	24	303
05 Blue	34	117	17	2118	59	19	138	21	3198	23	82
04 Blue	27	79	17	2158	39	15	104	19	26	3195	56
03 Blue	18	25	16	38	19	23	22	2715	3483	3573	3270

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
02 Blue	15	43	19	65	29	19	23	2513	3417	17	3072
01 Blue	15	43	19	41	24	19	22	2309	17	2821	2741
47 KSU Green	55	225	19	390	163	2498	198	22	3800	21	232
44 KSU Green	21	280	21	1096	211	42	462	19	3868	23	3767
06 Blue	69	469	19	2658	310	35	233	20	3775	24	340
05 Blue	30	99	22	2492	70	20	152	19	3673	25	70
04 Blue	26	85	18	2224	39	15	84	18	23	3295	46
03 Blue	15	21	15	26	16	25	20	2566	3299	3386	2884
02 Blue	24	57	20	63	45	27	36	2348	3394	27	3421
01 Blue	17	28	20	33	29	20	21	2235	24	2867	2724
47 KSU Green	56	270	23	562	115	2527	197	24	3817	21	190
44 KSU Green	24	252	17	610	150	38	482	19	4122	19	3823
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	2277	719	296	2049	1455	688	197	100	793	39	1210
No Template	931	144	97	262	504	393	88	52	129	41	328
No Template	2871	1040	241	2781	1711	821	250	94	1069	55	2147
No Template	2225	734	134	1055	2071	769	227	89	694	48	1468
No Template	2376	1098	197	1855	1551	960	203	93	1197	43	1520
No Template	2264	1216	246	2061	2125	877	208	88	1293	56	1514
No Template	1799	968	141	1595	1374	526	168	60	777	35	1082
No Template	2330	1073	259	1919	2330	1019	203	85	1171	53	1927
Green 43	2854	2477	336	2944	2690	1865	885	117	5723	44	6424
Green 41	1340	618	142	1054	1066	382	785	76	5358	4002	6196
Green 39	2156	1782	256	3091	2528	5732	261	131	5830	47	2639
Green 38	1365	553	267	1064	1431	6515	156	45	5787	38	1319
Green 36	1936	1175	149	2748	2355	5649	224	110	5748	38	2243
Green 35	1105	837	171	1697	1161	5124	187	63	4478	38	1138
Green 33	1156	755	353	1183	1237	5950	140	58	4823	38	1225
Green 32	1385	260	111	1381	6081	490	117	60	4681	39	5834
Green 43	2059	1315	143	1276	2074	868	974	75	6298	35	6371
Green 41	1692	908	108	1583	1287	588	767	57	5260	4165	6489
Green 39	1340	630	185	2212	1736	5209	148	74	5171	33	1246
Green 38	802	336	141	641	1051	5786	100	110	5471	79	853
Green 36	373	129	42	293	390	5237	44	45	4937	45	295
Green 35	1418	515	101	1827	1396	5388	168	94	5731	43	1279
Green 33	1106	581	165	447	1031	5597	82	40	5258	27	852
Green 32	1984	664	208	2002	6153	809	216	73	5440	39	6385
Green 43	1584	735	91	1386	1480	573	835	84	5029	28	5680
Green 41	1392	431	56	992	1060	303	821	57	5407	3326	5600
Green 39	1300	877	109	1489	1471	4970	181	45	4543	44	1403
Green 38	953	778	112	655	1630	5344	123	45	4746	27	1085
Green 36	870	475	76	846	1105	4570	84	33	4189	24	740

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
Green 35	1110	431	80	1131	1228	5089	123	50	4942	27	768
Green 33	703	376	81	403	1146	4832	80	48	4200	26	678
Green 32	1137	347	78	571	4550	470	88	48	3526	24	4703

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	2277	719	296	2049	1455	688	197	100	793	39	1210
No Template	931	144	97	262	504	393	88	52	129	41	328
No Template	2871	1040	241	2781	1711	821	250	94	1069	55	2147
No Template	2225	734	134	1055	2071	769	227	89	694	48	1468
No Template	2376	1098	197	1855	1551	960	203	93	1197	43	1520
No Template	2264	1216	246	2061	2125	877	208	88	1293	56	1514
No Template	1799	968	141	1595	1374	526	168	60	777	35	1082
No Template	2330	1073	259	1919	2330	1019	203	85	1171	53	1927
Green 30	1780	547	59	728	6338	712	163	71	5656	4340	6565
Green 28	1621	847	168	2290	6065	538	123	61	5516	4357	6267
Green 26	1464	363	182	1527	6306	441	92	64	5611	4260	6447
Green 24	1562	549	107	1601	6369	530	142	50	5576	4270	6478
Green 22	1422	524	165	137	6279	489	95	51	5470	4284	6194
Green 20	1602	848	101	1912	6314	521	161	60	5318	4303	6193
Green 18	1610	268	81	1864	5949	487	133	60	5362	3985	5834
Green 17	1966	870	152	1223	6178	915	166	53	5378	4176	6246
Green 30	1869	812	126	2315	6232	740	165	81	5446	4381	6650
Green 28	1792	839	470	2015	6062	600	179	54	5221	4082	6234
Green 26	148	16	160	251	1099	838	67	13	25	7	257
Green 24	1631	688	179	652	5935	534	149	57	5220	4101	6358
Green 22	1468	826	129	506	6135	498	114	51	5365	4252	6329
Green 20	2038	738	101	2058	6298	787	164	76	5949	4517	6539
Green 18	1617	511	90	1490	5813	522	146	61	4711	3785	5703
Green 17	2197	1280	124	1885	6008	1076	245	61	5312	4224	6397
Green 30	1926	718	93	1525	6186	768	156	83	5443	4345	6474
Green 28	1350	348	136	1083	5832	559	123	59	4927	4083	5947
Green 26	10	11	17	11	18	29	8	35	29	13	11
Green 24	1612	808	124	1334	6084	567	140	62	5514	4262	6065
Green 22	1792	1353	144	2902	5993	671	182	63	5585	4204	6376
Green 20	1781	590	137	1935	6024	654	200	64	5487	4252	6212
Green 18	1664	1011	122	1045	6094	540	130	64	5192	4128	5950
Green 17	1838	626	179	1560	6070	722	177	80	5171	4115	6307

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	2277	719	296	2049	1455	688	197	100	793	39	1210
No Template	931	144	97	262	504	393	88	52	129	41	328
No Template	2871	1040	241	2781	1711	821	250	94	1069	55	2147
No Template	2225	734	134	1055	2071	769	227	89	694	48	1468

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	2376	1098	197	1855	1551	960	203	93	1197	43	1520
No Template	2264	1216	246	2061	2125	877	208	88	1293	56	1514
No Template	1799	968	141	1595	1374	526	168	60	777	35	1082
No Template	2330	1073	259	1919	2330	1019	203	85	1171	53	1927
Green 15	1511	478	43	815	5818	446	117	49	5285	4032	6193
Green 13	1276	517	60	835	6027	420	102	64	5279	4059	6036
Green 12	1369	425	89	1799	5736	508	117	48	4919	39	5782
Green 11	1257	397	4612	924	1024	413	122	43	4653	42	5236
Green 09	1296	543	4601	1007	1328	510	106	42	4782	40	4881
Green 07	2056	1068	4581	1834	2866	930	251	58	4886	44	5729
Green 05	2022	1297	5142	1733	1756	767	226	64	5150	43	5721
Green 03	1983	1297	4881	1747	1767	995	233	80	4361	44	5657
Green 15	1582	786	111	1406	5929	648	140	60	5219	4206	5964
Green 13	1032	390	96	382	5760	321	89	65	4861	3963	5666
Green 12	1285	698	108	1120	5492	645	114	72	4747	37	5536
Green 11	1395	346	5091	1447	954	505	140	54	5274	38	4637
Green 09	939	409	3993	363	763	396	79	51	3796	40	4039
Green 07	1910	1106	4816	1650	2484	836	240	56	4960	40	5865
Green 05	1700	1344	4539	767	1324	598	203	39	4027	38	5154
Green 03	2038	967	5304	1593	1640	873	204	77	5533	54	6085
Green 15	2020	1455	179	1051	5774	904	196	70	4931	3840	5929
Green 13	1987	832	161	1734	5833	619	180	96	5032	4153	5899
Green 12	2160	1532	338	2687	5544	886	306	84	4850	35	5671
Green 11	2148	737	5459	1863	1161	767	250	62	5482	51	6029
Green 09	1380	1048	3977	954	908	497	146	50	4445	37	4451
Green 07	1758	1003	5108	1635	2840	925	214	60	5042	46	5904
Green 05	2173	1393	5023	2228	2106	957	239	53	4998	43	5780
Green 03	1803	550	5231	1464	836	945	192	57	5137	53	5090

Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	43	47	45	41	45	40	45	42	71	48	84
No Template	52	69	52	44	44	48	40	60	98	38	98
No Template	52	56	50	49	45	49	52	48	65	46	60
No Template	36	39	39	43	47	36	36	33	59	40	51
No Template	50	44	39	45	45	49	41	40	50	45	67
No Template	40	41	41	35	32	33	37	32	46	35	45
No Template	52	50	41	44	45	48	38	42	61	48	55
No Template	112	199	80	82	67	59	96	67	2598	52	2774
Yellow 30	4457	37	34	33	36	32	29	27	4607	29	4990
Yellow 29	4399	37	35	32	31	38	35	31	4907	34	4826
Yellow 28	26	30	5017	26	32	34	32	29	5112	28	5502
Yellow 25	42	46	6288	40	41	39	48	46	6327	50	6158
Yellow 23	31	36	5451	28	31	32	36	32	5862	36	5789

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
Yellow 22	36	3593	39	36	41	36	35	34	5347	39	5099
Yellow 21	47	46	40	37	5831	47	44	49	6283	38	6066
Yellow 20	67	59	5657	42	49	59	71	44	5583	39	5691
Yellow 30	4254	35	32	34	34	41	36	30	4723	37	4846
Yellow 29	4781	38	42	36	31	34	30	31	5157	36	4978
Yellow 28	32	39	4785	35	33	35	31	31	4660	33	4942
Yellow 25	27	25	4575	38	28	32	34	27	4659	32	4755
Yellow 23	35	32	5051	37	36	36	34	29	5083	37	5126
Yellow 22	38	4521	28	41	40	34	36	37	5275	36	5260
Yellow 21	42	45	48	47	5278	51	43	38	5641	45	4879
Yellow 20	38	45	5228	31	40	41	34	31	5204	34	5382
Yellow 30	4021	30	29	25	29	25	23	27	4197	22	4355
Yellow 29	3935	36	33	28	24	31	30	27	5012	28	4427
Yellow 28	38	38	4895	31	33	33	34	36	5220	39	5034
Yellow 25	39	38	4530	38	35	45	36	36	4176	34	4322
Yellow 23	30	23	4134	26	26	23	24	22	4021	26	4195
Yellow 22	30	3959	29	29	25	27	31	29	4682	30	4692
Yellow 21	32	39	34	30	4198	35	30	33	4045	33	4212
Yellow 20	43	43	5551	41	39	42	43	44	5507	35	5465
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	43	47	45	41	45	40	45	42	71	48	84
No Template	52	69	52	44	44	48	40	60	98	38	98
No Template	52	56	50	49	45	49	52	48	65	46	60
No Template	36	39	39	43	47	36	36	33	59	40	51
No Template	50	44	39	45	45	49	41	40	50	45	67
No Template	40	41	41	35	32	33	37	32	46	35	45
No Template	52	50	41	44	45	48	38	42	61	48	55
No Template	112	199	80	82	67	59	96	67	2598	52	2774
Yellow 19	27	29	28	29	4473	28	31	23	4027	26	3860
Yellow 18	26	34	4138	30	28	31	28	30	4207	31	4226
Yellow 16	35	32	4617	26	28	32	25	25	4546	29	4967
Yellow 15	26	26	23	30	3296	31	30	23	3869	26	3828
Yellow 13	4096	31	28	30	27	30	25	23	4468	29	4285
Yellow 12	4291	35	27	30	32	39	33	32	5028	35	4623
Yellow 11	37	32	4633	33	33	33	31	29	4688	30	4947
Yellow 10	3965	28	24	24	24	29	25	19	4283	24	4548
Yellow 19	34	36	31	34	5289	39	38	35	5354	37	4901
Yellow 18	29	33	4289	27	33	33	32	30	4319	30	4387
Yellow 16	33	34	4758	32	33	36	33	31	4842	36	5121
Yellow 15	33	41	38	32	5188	39	31	33	4985	32	5003
Yellow 13	4101	40	32	37	34	33	31	30	4841	34	4786
Yellow 12	4423	40	31	39	30	41	30	32	5069	36	4812

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
Yellow 11	42	43	5458	43	49	40	40	38	5475	42	5173
Yellow 10	4172	35	34	37	34	29	35	33	4044	33	4820
Yellow 19	32	31	31	32	4290	34	34	27	4521	29	4091
Yellow 18	27	34	3849	30	30	29	26	25	3733	31	3893
Yellow 16	29	28	4248	26	30	31	27	24	4118	31	4135
Yellow 15	29	28	29	33	4225	28	26	24	3829	28	4035
Yellow 13	3450	31	27	27	22	27	27	29	4182	30	4162
Yellow 12	3724	35	33	33	33	33	33	29	4491	32	4478
Yellow 11	33	30	4710	31	34	31	34	31	4627	33	4660
Yellow 10	4157	40	33	35	35	38	36	42	4636	36	4874
No Template	43	47	45	41	45	40	45	42	71	48	84
No Template	52	69	52	44	44	48	40	60	98	38	98
No Template	52	56	50	49	45	49	52	48	65	46	60
No Template	36	39	39	43	47	36	36	33	59	40	51
No Template	50	44	39	45	45	49	41	40	50	45	67
No Template	40	41	41	35	32	33	37	32	46	35	45
No Template	52	50	41	44	45	48	38	42	61	48	55
No Template	112	199	80	82	67	59	96	67	2598	52	2774
Yellow 09	3512	32	30	35	26	33	32	26	4089	30	4177
Yellow 08	31	32	28	30	3716	31	33	26	4272	3613	2941
Yellow 07	3302	36	35	37	31	32	32	31	4448	36	4531
Yellow 06	4037	58	48	49	46	54	49	44	5734	47	5557
Yellow 04	32	35	29	39	4606	33	36	34	4747	4493	4648
Yellow 03	3585	42	39	38	37	38	35	37	4824	39	4681
Yellow 02	37	30	4086	30	34	36	34	34	3517	33	3511
Yellow 01	50	47	44	42	45	49	3049	40	5427	40	5532
Yellow 09	2798	28	31	30	28	30	30	28	3400	35	3679
Yellow 08	32	30	29	26	4001	25	31	28	3531	3223	3016
Yellow 07	3312	34	29	30	31	29	29	32	4558	28	4535
Yellow 06	1892	28	27	24	24	29	24	23	2708	28	2797
Yellow 04	34	35	32	29	4197	32	31	27	3978	3950	4023
Yellow 03	3700	40	38	39	38	38	39	39	4738	44	4667
Yellow 02	43	38	4663	37	39	38	38	43	4640	35	4373
Yellow 01	38	37	35	36	36	41	2152	42	4435	42	4269
Yellow 09	3056	38	33	36	33	36	35	31	4078	34	4217
Yellow 08	35	40	33	35	4261	39	33	32	3953	3659	3700
Yellow 07	3062	25	28	29	29	31	31	27	3993	27	4073
Yellow 06	2624	28	26	30	28	32	25	23	3042	25	3041
Yellow 04	30	32	30	29	4026	32	29	27	3883	3509	3773
Yellow 03	3391	28	26	34	29	33	29	24	3443	34	4076
Yellow 02	36	40	4443	34	35	34	38	31	4096	32	4254

Yellow 01	36	44	31	30	32	36	2191	34	4580	34	4487
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	35	36	36	37	44	40	118	49	41	33	34
No Template	35	35	30	30	37	39	59	48	44	33	32
No Template	31	34	32	39	40	44	224	70	44	31	32
No Template	34	38	34	41	44	51	213	86	52	34	34
No Template	31	31	30	36	43	49	385	128	51	32	29
No Template	39	43	36	41	49	50	179	85	55	38	39
No Template	33	35	30	35	42	42	150	67	44	33	34
No Template	30	33	31	33	33	35	47	39	40	30	27
Green 01	26	31	572	25	31	35	67	41	3987	26	817
Yellow 60	311	25	23	28	29	32	105	57	3416	23	754
Yellow 59	29	36	657	31	34	41	94	60	3400	28	756
Yellow 58	31	34	29	31	617	41	164	75	4225	31	991
Yellow 57	29	31	28	33	438	40	149	68	3654	28	942
Yellow 56	31	30	202	30	37	44	192	82	4117	26	1063
Yellow 55	21	22	17	22	28	37	125	67	39	1168	823
Yellow 54	19	22	354	21	20	23	65	34	4313	19	707
Green 01	23	26	203	22	27	27	63	29	3596	22	760
Yellow 60	328	30	26	27	33	38	104	61	3455	29	708
Yellow 59	22	24	442	28	31	32	77	50	2863	21	618
Yellow 58	27	28	26	29	815	36	120	62	4053	26	872
Yellow 57	28	29	29	32	408	38	111	61	4233	27	764
Yellow 56	26	28	484	33	33	39	127	80	3312	27	672
Yellow 55	16	20	17	22	26	33	128	50	32	518	847
Yellow 54	19	22	745	21	24	23	48	33	3401	19	247
Green 01	13	16	1475	15	14	16	23	19	1680	14	145
Yellow 60	173	25	22	22	33	33	96	36	4389	26	1111
Yellow 59	57	70	408	36	89	82	142	83	4115	68	952
Yellow 58	24	24	23	26	452	31	124	53	4488	21	948
Yellow 57	28	33	27	29	890	39	125	60	4216	30	862
Yellow 56	24	26	1885	30	29	36	93	59	2812	22	504
Yellow 55	14	16	15	21	15	75	60	36	30	2677	220
Yellow 54	14	15	181	18	17	16	66	21	4933	16	1104
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	55	138	140	159	324	425	125	194	253	258	121
No Template	67	148	215	232	440	596	160	212	282	421	142
No Template	57	141	176	166	355	468	130	179	258	358	103
No Template	65	172	187	190	399	553	148	197	286	392	123
No Template	61	153	168	175	348	535	151	192	304	403	128
No Template	57	145	207	162	405	561	138	198	260	410	130

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	58	127	140	167	295	449	146	231	251	272	154
No Template	57	157	204	203	378	557	163	221	294	396	136
Yellow 53	30	58	63	68	151	206	193	87	657	862	345
Yellow 51	50	107	138	114	1326	456	111	149	782	292	547
Yellow 50	185	87	102	83	213	397	78	109	705	245	473
Yellow 49	38	72	94	73	1274	347	84	108	758	201	511
Yellow 48	31	65	95	86	190	787	77	98	142	1480	509
Yellow 47	41	96	125	104	251	782	95	148	193	1460	543
Yellow 46	146	57	62	60	137	221	61	79	742	138	399
Yellow 44	34	89	923	82	221	333	100	131	877	215	577
Yellow 53	53	120	133	110	300	466	378	177	924	1517	642
Yellow 51	47	94	130	120	1313	396	110	148	777	235	508
Yellow 50	275	101	134	129	316	471	122	156	881	264	614
Yellow 49	49	95	113	106	1480	424	94	136	859	247	625
Yellow 48	41	104	130	98	234	794	97	132	164	1356	573
Yellow 47	42	112	121	106	241	745	89	136	180	1444	516
Yellow 46	236	75	91	81	179	342	90	127	787	204	514
Yellow 44	42	102	732	91	203	355	103	152	761	209	557
Yellow 53	24	68	47	52	159	125	371	232	1168	534	1236
Yellow 51	47	94	138	108	1363	467	110	148	773	300	546
Yellow 50	172	70	93	80	231	324	90	107	694	166	448
Yellow 49	36	67	94	92	1222	332	78	117	743	211	514
Yellow 48	36	93	105	95	227	873	80	114	172	1562	517
Yellow 47	45	115	162	132	354	853	115	149	204	1574	554
Yellow 46	241	77	105	97	227	395	96	131	823	242	535
Yellow 44	41	91	737	101	200	365	106	146	762	197	547

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	55	138	140	159	324	425	125	194	253	258	121
No Template	67	148	215	232	440	596	160	212	282	421	142
No Template	57	141	176	166	355	468	130	179	258	358	103
No Template	65	172	187	190	399	553	148	197	286	392	123
No Template	61	153	168	175	348	535	151	192	304	403	128
No Template	57	145	207	162	405	561	138	198	260	410	130
No Template	58	127	140	167	295	449	146	231	251	272	154
No Template	57	157	204	203	378	557	163	221	294	396	136
Yellow 43	32	78	64	78	1124	245	118	156	852	101	554
Yellow 41	26	51	668	50	127	154	110	149	882	44	625
Yellow 40	35	69	922	70	193	362	91	120	882	175	566
Yellow 39	49	94	819	113	273	455	104	162	813	285	539
Yellow 38	51	81	841	117	247	428	102	136	753	259	541
Yellow 35	31	61	643	69	170	276	68	104	665	164	405
Yellow 32	26	47	664	65	155	252	58	87	659	135	388

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
Yellow 31	32	68	84	74	1056	292	84	108	673	1745	478
Yellow 43	22	72	45	59	882	155	117	182	804	55	567
Yellow 41	33	60	755	74	178	291	85	112	759	146	494
Yellow 40	8	10	9	7	10	9	6	7	10	9	8
Yellow 39	52	105	915	125	291	478	121	160	808	286	583
Yellow 38	45	92	924	108	248	451	103	133	823	246	543
Yellow 35	46	91	893	101	254	431	113	136	785	218	564
Yellow 32	35	84	850	85	221	372	83	122	853	194	527
Yellow 31	26	63	64	57	996	228	85	118	635	1503	453
Yellow 43	19	60	39	50	923	108	111	186	933	48	771
Yellow 41	37	60	797	70	161	287	83	112	756	119	529
Yellow 40	36	68	869	79	208	315	100	135	860	156	551
Yellow 39	50	101	811	119	237	465	121	160	802	271	567
Yellow 38	43	97	825	107	239	384	118	157	801	203	543
Yellow 35	47	93	823	115	250	372	115	149	773	206	524
Yellow 32	43	86	870	96	203	371	108	138	893	202	570
Yellow 31	25	69	55	68	904	185	157	244	1004	1171	826
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	175	489	300	505	363	242	1421	1453	1679	158	539
No Template	1126	1036	1360	1071	1536	869	1561	1610	1605	1344	1037
No Template	956	994	1252	953	1376	788	1398	1523	1499	1249	947
No Template	1073	1041	1293	985	1432	852	1513	1556	1594	1312	1015
No Template	1033	1117	1296	971	1431	846	1591	1593	1586	1287	1000
No Template	1099	1045	1296	1036	1474	842	1543	1588	1606	1269	938
No Template	1025	1096	1296	949	1543	837	1592	1583	1630	1343	1024
No Template	42	109	62	92	68	46	377	386	555	41	93
Red 48	614	565	2249	632	881	633	1858	1212	2319	1668	1873
Red 46	1796	449	569	477	711	540	924	986	2804	670	1984
Red 44	363	358	395	405	618	455	2150	983	908	541	1943
Red 38	412	396	2197	463	654	506	939	994	2880	584	2039
Red 37	1971	483	588	499	708	563	967	1086	2978	693	2179
Red 26	390	370	2188	451	631	499	869	968	2975	607	2162
Red 24	362	367	421	370	605	471	2267	960	934	2030	2102
Red 19	448	495	513	516	2457	526	884	1073	3005	2411	2161
Red 48	622	625	2196	678	918	667	1850	1233	2339	1804	1844
Red 46	1987	548	648	529	826	612	1096	1093	2928	755	2149
Red 44	248	272	304	332	476	413	1974	800	745	387	1688
Red 38	409	431	2107	426	688	516	907	1027	2867	605	2055
Red 37	2042	386	446	434	623	510	880	1027	3088	589	2249
Red 26	414	387	2144	443	644	497	878	997	2966	570	2186
Red 24	396	394	457	513	642	493	2376	1081	970	2097	2210
Red 19	435	444	529	508	2572	528	938	1109	3050	2425	2194

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
Red 48	551	608	2032	593	848	584	1718	1224	2221	1635	1686
Red 46	2075	581	692	597	845	663	1084	1244	3037	864	2253
Red 44	304	338	336	347	585	429	2067	937	791	462	1802
Red 38	390	378	2154	406	615	497	812	925	2887	557	2068
Red 37	1972	402	569	455	700	570	920	1052	2909	720	2146
Red 26	436	429	2244	464	674	544	929	1014	3094	609	2307
Red 24	344	367	377	398	584	443	2262	893	909	1986	2042
Red 19	385	498	498	490	2421	530	941	1062	3002	2399	2080
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	175	489	300	505	363	242	1421	1453	1679	158	539
No Template	1126	1036	1360	1071	1536	869	1561	1610	1605	1344	1037
No Template	956	994	1252	953	1376	788	1398	1523	1499	1249	947
No Template	1073	1041	1293	985	1432	852	1513	1556	1594	1312	1015
No Template	1033	1117	1296	971	1431	846	1591	1593	1586	1287	1000
No Template	1099	1045	1296	1036	1474	842	1543	1588	1606	1269	938
No Template	1025	1096	1296	949	1543	837	1592	1583	1630	1343	1024
No Template	42	109	62	92	68	46	377	386	555	41	93
Red 17	2214	637	723	620	984	631	1189	1355	1207	846	2325
Red 15	581	415	543	478	2579	523	870	1019	2853	2083	2066
Red 14	722	502	669	564	2586	538	909	1040	913	1606	637
Red 12	672	587	1958	668	857	655	1058	1188	2726	2041	1976
Red 08	1950	366	1647	407	604	504	1218	977	2984	598	2157
Red 07	1713	252	289	260	400	378	559	653	2787	354	1927
Red 04	2065	380	502	453	666	532	912	1024	3023	602	2210
Red 03	373	473	433	490	2564	488	868	1003	3021	487	2089
Red 17	2233	655	716	686	1048	672	1166	1369	1218	805	2311
Red 15	624	447	562	540	2615	550	883	1011	2882	2124	2082
Red 14	776	575	777	664	2731	593	1121	1136	984	1735	756
Red 12	662	615	2078	641	938	687	1095	1259	2882	2145	2114
Red 08	2143	418	1730	449	665	528	1349	998	3266	603	2338
Red 07	2121	350	404	407	556	476	825	941	3218	503	2261
Red 04	2141	413	500	457	710	532	949	1049	3173	568	2233
Red 03	456	459	456	554	2728	521	924	1059	3231	507	2238
Red 17	2312	752	773	725	1017	658	1250	1471	1274	875	2461
Red 15	608	399	569	456	2554	543	811	962	2841	2035	2030
Red 14	842	628	797	746	2854	618	1211	1292	1063	1950	798
Red 12	636	592	2033	652	968	692	1089	1249	2724	2052	1972
Red 08	2242	453	1770	518	761	601	1430	1171	3424	615	2451
Red 07	2229	359	433	421	600	482	828	989	3250	484	2318
Red 04	2074	388	470	448	640	509	877	980	3124	544	2269
Red 03	502	549	511	665	2911	591	1101	1258	3493	600	2459

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	175	489	300	505	363	242	1421	1453	1679	158	539
No Template	1126	1036	1360	1071	1536	869	1561	1610	1605	1344	1037
No Template	956	994	1252	953	1376	788	1398	1523	1499	1249	947
No Template	1073	1041	1293	985	1432	852	1513	1556	1594	1312	1015
No Template	1033	1117	1296	971	1431	846	1591	1593	1586	1287	1000
No Template	1099	1045	1296	1036	1474	842	1543	1588	1606	1269	938
No Template	1025	1096	1296	949	1543	837	1592	1583	1630	1343	1024
No Template	42	109	62	92	68	46	377	386	555	41	93
Blue 23	184	272	268	2226	409	310	629	721	2593	285	353
Blue 22	153	243	256	2261	403	279	553	635	2639	283	300
Blue 21	92	145	156	1986	247	199	428	469	2346	155	186
Blue 15	185	263	323	2712	488	372	718	781	3055	378	375
Blue 14	498	507	700	2542	867	614	1082	1174	2696	749	593
Blue 12	407	434	575	2511	790	552	956	1109	2712	616	541
Blue 10	525	562	730	2377	854	582	1087	1078	2569	766	606
Blue 07	466	2892	698	642	941	548	1207	1226	1069	668	699
Blue 23	340	424	477	2462	704	449	904	979	2948	515	552
Blue 22	174	258	311	2312	440	332	680	753	2675	365	413
Blue 21	158	214	267	2265	372	298	562	642	2741	291	272
Blue 15	203	306	325	2503	496	384	810	851	2871	367	414
Blue 14	459	547	721	2527	848	591	1065	1195	2777	791	593
Blue 12	411	465	618	2656	771	558	1074	1164	2827	690	547
Blue 10	559	547	772	2426	898	623	1126	1223	2643	834	595
Blue 07	613	3226	879	835	1169	639	1445	1581	1369	828	885
Blue 23	243	402	443	2420	620	367	897	1005	2824	460	556
Blue 22	179	271	309	2269	391	300	639	745	2717	350	345
Blue 21	102	174	211	2020	272	241	496	574	2469	174	235
Blue 15	152	233	291	2533	410	346	727	754	2983	345	368
Blue 14	507	476	696	2595	794	599	1094	1115	2809	860	587
Blue 12	362	449	569	2368	734	523	944	1009	2634	635	520
Blue 10	494	567	716	2362	964	586	1115	1224	2709	840	716
Blue 07	609	3321	919	1020	1252	632	1507	1814	1582	964	992

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	114	112	104	79	128	125	93	174	125	104	99
No Template	243	526	669	762	1164	1381	413	680	851	1436	482
No Template	189	405	557	628	847	1266	425	800	780	1322	499
No Template	228	416	626	651	1047	1282	403	691	778	1356	490
No Template	192	420	587	660	943	1220	389	665	725	1303	463
No Template	160	376	469	518	724	1025	363	701	638	1062	397
No Template	98	281	378	398	512	716	392	765	592	653	436

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	173	375	530	579	957	1006	336	623	654	1201	363
CDC 97-3068	153	287	283	437	713	2844	491	706	851	5292	2005
MDCH-4	193	431	542	537	956	1385	381	656	790	5159	446
2000-3039	105	1551	233	342	431	868	379	489	1995	612	1497
8-084	152	353	350	510	725	2822	429	737	777	4967	1990
10049	61	89	77	100	4346	323	172	191	2453	178	1589
83-75	62	103	100	145	190	402	1002	247	329	4477	1693
B8026-C1	85	1831	175	292	338	726	318	393	2262	511	1579
236-1	74	120	3844	150	210	355	246	333	4683	6082	3036
CDC 97-3068	135	312	359	486	736	3686	423	617	733	5574	2279
MDCH-4	175	382	441	541	775	1194	451	622	801	5674	613
2000-3039	96	1907	194	308	400	793	326	444	2383	509	1773
8-084	148	311	413	538	749	2662	399	613	711	4213	1607
10049	73	131	145	217	3196	605	269	342	1913	421	1399
83-75	63	105	100	124	180	371	906	270	329	3790	1500
B8026-C1	84	1448	178	298	383	702	317	408	1943	503	1352
236-1	81	193	2648	236	394	736	326	402	2600	4558	1787
CDC 97-3068	146	321	308	490	716	3150	421	590	757	5708	2144
MDCH-4	158	317	446	518	762	1150	412	634	748	4950	478
2000-3039	84	1744	154	231	322	646	304	389	2374	422	1726
8-084	146	312	393	576	763	2581	386	660	749	4376	1739
10049	75	117	129	161	3264	539	258	287	2011	312	1347
83-75	52	83	84	113	157	302	890	219	250	3657	1491
B8026-C1	88	1201	206	343	377	712	332	423	1608	577	1163
236-1	80	152	2130	210	328	618	325	405	2220	4277	1517
No Template	114	112	104	79	128	125	93	174	125	104	99
No Template	243	526	669	762	1164	1381	413	680	851	1436	482
No Template	189	405	557	628	847	1266	425	800	780	1322	499
No Template	228	416	626	651	1047	1282	403	691	778	1356	490
No Template	192	420	587	660	943	1220	389	665	725	1303	463
No Template	160	376	469	518	724	1025	363	701	638	1062	397
No Template	98	281	378	398	512	716	392	765	592	653	436
No Template	173	375	530	579	957	1006	336	623	654	1201	363
MT#2	89	158	178	255	383	2159	276	372	386	3154	1322
2011-0-1256	113	244	261	2944	557	942	449	504	1740	709	449
KDHE 47	115	235	257	367	508	2834	384	491	543	4266	1599
TB154A	53	83	1799	97	155	326	180	206	2098	165	1489
S2006 #1	53	68	66	175	86	186	113	1449	2694	4826	1852
DA-21	100	2423	213	313	412	812	325	429	3343	624	2307
2006-3008	87	157	2134	231	344	721	284	397	2279	485	1457
DEC11C	76	1461	144	194	258	588	265	332	1856	405	1387

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
MT#2	103	159	191	304	447	3482	299	407	437	5451	2118
2011-0-1256	99	216	249	2728	503	912	409	489	1717	630	359
KDHE 47	132	270	327	446	650	2098	363	518	586	3393	1378
TB154A	93	155	1951	237	419	704	333	393	2232	439	1506
S2006 #1	52	81	83	183	145	388	163	1252	2470	4612	1659
DA-21	119	2740	241	379	504	900	351	523	4169	705	2647
2006-3008	103	176	1737	263	444	778	311	416	1935	592	1226
DEC11C	47	1370	127	179	219	511	247	311	1759	377	1320
MT#2	130	261	332	418	644	2327	368	497	627	3885	1529
2011-0-1256	66	149	141	2852	290	555	317	336	1728	321	257
KDHE 47	134	342	438	402	832	904	268	480	577	981	362
TB154A	63	127	1971	194	284	653	312	339	1976	358	1445
S2006 #1	65	110	109	297	198	464	207	1060	1962	3512	1416
DA-21	78	1782	199	310	376	751	312	391	2325	536	1591
2006-3008	90	158	1715	243	402	749	329	423	1817	510	1245
DEC11C	80	1241	148	219	304	622	293	366	1690	371	1258
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	114	112	104	79	128	125	93	174	125	104	99
No Template	243	526	669	762	1164	1381	413	680	851	1436	482
No Template	189	405	557	628	847	1266	425	800	780	1322	499
No Template	228	416	626	651	1047	1282	403	691	778	1356	490
No Template	192	420	587	660	943	1220	389	665	725	1303	463
No Template	160	376	469	518	724	1025	363	701	638	1062	397
No Template	98	281	378	398	512	716	392	765	592	653	436
No Template	173	375	530	579	957	1006	336	623	654	1201	363
IHIT2087	1747	179	159	321	337	666	282	389	4612	427	2683
3215-99 (F6627)	32	49	79	65	3269	184	85	110	2056	4350	1485
B2387	50	84	85	112	152	352	186	1511	169	5124	1887
DEC10I (87-1713)	62	128	126	176	231	503	282	309	2150	319	1563
1234-1	43	65	61	83	112	204	836	144	2140	4137	1410
DEC10E	984	260	243	355	488	890	354	465	3195	783	2136
B8227-C8	64	1349	93	149	170	408	215	238	1874	203	1415
7726-1	49	95	86	120	2332	399	193	217	1594	2889	1198
IHIT2087	688	215	210	271	427	748	348	486	2150	568	1456
3215-99 (F6627)	55	84	108	106	2433	362	179	191	1530	2824	1091
B2387	45	81	73	128	153	338	180	969	211	2636	1258
DEC10I (87-1713)	93	222	205	306	423	758	373	515	1549	612	1255
1234-1	43	81	81	98	153	345	576	208	1338	2609	909
DEC10E	489	180	232	312	390	681	294	414	1703	666	1189
B8227-C8	84	852	169	244	307	605	269	367	1384	421	1023
7726-1	50	70	93	115	1575	327	168	189	1089	1758	840

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
IHIT2087	611	217	199	308	409	719	355	538	2102	519	1487
3215-99 (F6627)	59	95	124	124	2580	415	186	237	1747	3216	1413
B2387	57	120	127	203	286	552	268	897	332	2581	1210
DEC10I (87-1713)	83	175	201	266	399	717	367	495	1567	526	1256
1234-1	42	75	79	87	139	298	636	195	1589	2917	1081
DEC10E	619	176	231	326	436	690	317	485	2319	621	1355
B8227-C8	61	908	122	189	200	450	265	329	1448	305	1137
7726-1	64	118	103	160	1969	371	255	330	1380	2576	1062
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	28	95	104	144	110	157	261	412	295	70	139
No Template	22	44	54	84	96	92	132	197	143	44	67
No Template	23	112	101	135	118	158	249	349	243	62	106
No Template	22	66	92	156	114	153	212	291	213	64	112
No Template	23	69	74	130	96	110	240	255	179	38	75
No Template	24	61	89	94	119	151	225	294	234	56	91
No Template	30	66	84	108	104	129	231	336	218	57	107
No Template	31	100	76	131	135	169	242	355	240	54	107
TY-2482	27	69	74	916	83	93	249	308	224	1303	75
B6820-C1	19	29	33	34	36	43	569	76	266	706	298
MI-0041B	20	59	84	410	95	124	200	274	177	1300	85
1.2622	36	726	77	121	91	123	183	268	1118	77	85
IH 16	23	39	34	44	47	54	672	123	115	1041	354
S2006 #4	24	40	37	41	52	58	66	303	1924	2052	541
15612-1	24	48	291	65	63	70	111	154	1804	39	410
236-5	20	31	241	47	49	48	89	126	1047	1123	219
TY-2482	24	52	55	1195	70	80	160	208	150	1211	55
B6820-C1	16	29	30	41	40	45	485	104	396	946	199
MI-0041B	11	29	34	358	48	62	103	147	91	804	45
1.2622	28	622	77	118	93	126	185	262	986	71	90
IH 16	28	38	43	53	60	73	543	117	114	815	308
S2006 #4	13	14	11	10	15	15	10	12	24	15	40
15612-1	21	47	254	62	58	63	111	176	1477	38	369
236-5	23	40	189	47	47	59	85	134	634	677	149
TY-2482	27	73	51	1887	63	82	164	210	126	1104	65
B6820-C1	20	32	38	46	52	57	574	107	437	1171	199
MI-0041B	20	40	55	584	83	126	173	203	133	801	84
1.2622	28	653	79	124	109	146	207	262	1059	71	80
IH 16	23	42	43	53	55	77	563	125	126	1060	280
S2006 #4	21	34	32	42	43	48	69	283	1542	1855	331
15612-1	23	48	270	60	56	68	99	144	1246	37	355
236-5	21	44	370	64	69	70	120	183	1062	1262	156

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	28	95	104	144	110	157	261	412	295	70	139
No Template	22	44	54	84	96	92	132	197	143	44	67
No Template	23	112	101	135	118	158	249	349	243	62	106
No Template	22	66	92	156	114	153	212	291	213	64	112
No Template	23	69	74	130	96	110	240	255	179	38	75
No Template	24	61	89	94	119	151	225	294	234	56	91
No Template	30	66	84	108	104	129	231	336	218	57	107
No Template	31	100	76	131	135	169	242	355	240	54	107
99-3311	20	27	27	34	31	34	682	66	1260	1616	323
0.292361111	21	38	39	54	52	56	101	416	67	1621	173
B8228-C2	29	1097	50	72	69	62	92	133	2150	52	382
RD8 (7075)	30	63	76	94	147	123	182	210	146	1023	101
1553-1	26	76	81	137	117	200	227	277	1264	66	111
CDC 1994 3023	23	55	46	1925	63	79	137	162	96	1582	56
H30	211	37	40	57	60	75	83	132	1804	41	559
DA-37	24	47	56	70	68	118	125	224	112	1202	90
99-3311	16	33	32	47	50	48	487	120	903	1377	221
0.292361111	21	45	56	69	71	87	129	428	107	1516	116
B8228-C2	20	778	45	80	73	78	106	161	1505	46	264
RD8 (7075)	28	82	80	123	448	177	224	283	179	1096	115
1553-1	29	75	98	148	135	216	251	306	928	86	125
CDC 1994 3023	24	57	55	1604	63	76	170	194	115	1553	55
H30	168	52	53	72	86	93	130	187	1233	52	335
DA-37	23	57	51	85	74	116	149	274	149	1011	68
99-3311	18	30	29	40	41	43	571	101	1104	1690	238
0.292361111	22	46	50	70	68	91	130	412	96	1429	121
B8228-C2	22	871	46	78	68	86	124	166	1677	53	289
RD8 (7075)	28	62	80	116	281	151	186	236	154	1072	102
1553-1	31	86	99	151	146	206	240	331	1069	102	146
CDC 1994 3023	23	63	58	1710	85	106	178	192	147	1569	61
H30	192	58	59	75	96	114	143	210	1356	54	399
DA-37	25	83	75	135	118	173	190	330	188	1091	91
Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	28	95	104	144	110	157	261	412	295	70	139
No Template	22	44	54	84	96	92	132	197	143	44	67
No Template	23	112	101	135	118	158	249	349	243	62	106
No Template	22	66	92	156	114	153	212	291	213	64	112
No Template	23	69	74	130	96	110	240	255	179	38	75
No Template	24	61	89	94	119	151	225	294	234	56	91
No Template	30	66	84	108	104	129	231	336	218	57	107

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	31	100	76	131	135	169	242	355	240	54	107
2003-3014	116	71	62	102	113	146	185	288	889	1108	269
JB1-95	18	31	29	37	67	37	54	85	1532	1814	112
IHIT1703	18	27	32	41	54	47	71	91	1567	24	146
DEC8b	20	30	31	45	62	47	59	80	1598	1613	150
S2006 #2	15	27	27	33	37	37	67	255	1860	1857	483
10C-3114	28	70	76	142	119	144	257	298	218	803	124
IHIT0304	18	22	20	18	27	22	747	31	34	823	649
93-111	16	27	26	30	33	34	62	366	1681	1923	268
2003-3014	129	53	56	77	67	93	131	230	1293	1509	214
JB1-95	19	36	30	42	79	47	62	100	1539	1802	95
IHIT1703	17	26	28	38	52	50	66	103	1471	23	115
DEC8b	18	37	38	47	70	59	85	128	1430	1481	154
S2006 #2	18	34	32	43	43	61	82	305	1643	1915	306
10C-3114	30	87	85	137	118	155	236	354	252	873	130
IHIT0304	19	25	24	24	31	29	623	54	43	1212	306
93-111	15	28	33	38	41	51	74	317	1792	1931	716
2003-3014	117	60	64	89	82	101	156	276	1055	1405	190
JB1-95	14	29	25	28	108	31	50	82	1464	1646	108
IHIT1703	17	30	32	45	62	53	81	118	1392	25	122
DEC8b	22	41	37	53	67	67	92	129	1450	1500	154
S2006 #2	22	34	35	46	49	59	82	314	1613	1882	321
10C-3114	24	73	80	131	99	156	211	301	204	909	104
IHIT0304	19	26	27	29	33	34	592	78	60	1171	294
93-111	18	32	37	46	39	60	85	313	1700	1892	538

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	37	350	92	133	290	176	419	663	625	84	197
No Template	39	239	104	163	385	213	376	493	431	106	166
No Template	36	195	88	143	256	172	366	455	475	80	126
No Template	40	247	155	173	421	228	449	694	597	113	199
No Template	45	224	133	150	366	264	362	517	390	120	189
No Template	39	190	135	146	376	200	423	543	461	104	174
No Template	29	239	107	146	343	221	419	539	506	72	161
No Template	11	73	52	65	83	45	192	261	294	26	84
MT (CDC 1994 3024)	22	147	63	495	181	67	222	302	296	1180	82
2002-3211	25	164	57	107	259	153	247	418	393	914	923
Mox 16	22	877	55	125	183	99	243	329	1030	65	939
Mox 23	19	127	676	73	184	70	191	319	1046	60	1028
DA-10	270	132	75	105	270	132	260	405	938	70	983
97-3250	228	187	96	161	336	204	291	454	959	666	941
GS G5578620	10	71	32	43	74	46	298	221	768	24	816

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
KDHE 55	16	91	39	54	112	49	153	280	263	695	777
MT (CDC 1994 3024)	20	151	56	547	228	85	222	361	380	901	116
2002-3211	30	184	84	152	262	183	260	432	398	849	775
Mox 16	29	822	81	130	253	182	261	373	952	89	915
Mox 23	24	132	527	122	215	154	218	321	700	71	767
DA-10	280	185	89	172	309	188	275	445	973	87	884
97-3250	246	192	107	170	308	208	260	424	878	697	863
GS G5578620	13	80	39	71	123	60	258	236	594	34	682
KDHE 55	30	170	86	153	273	218	242	426	323	675	645
MT (CDC 1994 3024)	18	146	45	419	173	68	168	316	313	773	68
2002-3211	28	153	75	187	266	160	215	410	348	733	739
Mox 16	29	656	88	141	314	212	254	396	721	103	811
Mox 23	23	153	547	109	200	119	200	331	643	69	665
DA-10	265	195	101	159	332	201	315	442	949	112	892
97-3250	246	237	129	166	357	253	276	467	830	735	862
GS G5578620	19	93	48	96	161	100	309	299	662	44	789
KDHE 55	21	120	43	92	127	76	172	311	287	699	644

Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	37	350	92	133	290	176	419	663	625	84	197
No Template	39	239	104	163	385	213	376	493	431	106	166
No Template	36	195	88	143	256	172	366	455	475	80	126
No Template	40	247	155	173	421	228	449	694	597	113	199
No Template	45	224	133	150	366	264	362	517	390	120	189
No Template	39	190	135	146	376	200	423	543	461	104	174
No Template	29	239	107	146	343	221	419	539	506	72	161
No Template	11	73	52	65	83	45	192	261	294	26	84
7744	11	44	20	32	60	26	278	131	741	18	671
CDC 90-3128	24	163	772	103	234	136	200	299	858	78	990
89-118	36	192	638	154	406	258	287	441	753	124	821
933	15	55	38	50	93	47	163	397	709	713	745
314-S	24	148	62	163	261	157	225	389	1233	79	1006
MT#80	25	142	548	92	214	148	189	270	653	72	773
M535	31	137	82	637	285	108	252	409	1036	1045	123
S2006 #3	20	121	47	94	192	104	175	478	849	901	838
7744	15	139	38	68	171	45	441	251	862	32	678
CDC 90-3128	30	165	758	121	277	157	265	360	823	78	739
89-118	50	214	859	198	454	262	396	455	1001	139	870
933	28	161	83	165	281	177	286	481	652	942	642
314-S	41	218	105	167	374	209	346	475	939	105	816
MT#80	42	219	733	163	362	226	333	501	727	105	658
M535	23	140	66	372	254	146	216	371	700	792	88

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
S2006 #3	22	156	51	99	239	116	281	552	1012	1169	911
7744	35	178	58	78	197	72	895	302	1323	51	695
CDC 90-3128	32	151	657	111	260	177	236	344	879	72	678
89-118	42	184	553	163	394	197	286	406	781	112	659
933	34	158	74	112	281	153	285	476	753	806	664
314-S	32	189	86	130	345	189	283	411	785	92	694
MT#80	31	176	594	138	295	178	281	316	913	89	686
M535	29	164	62	487	299	172	238	346	930	890	100
S2006 #3	15	93	39	68	122	77	206	408	719	780	602
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	37	350	92	133	290	176	419	663	625	84	197
No Template	39	239	104	163	385	213	376	493	431	106	166
No Template	36	195	88	143	256	172	366	455	475	80	126
No Template	40	247	155	173	421	228	449	694	597	113	199
No Template	45	224	133	150	366	264	362	517	390	120	189
No Template	39	190	135	146	376	200	423	543	461	104	174
No Template	29	239	107	146	343	221	419	539	506	72	161
No Template	11	73	52	65	83	45	192	261	294	26	84
413/89-1	414	112	35	71	112	39	196	240	869	33	732
86-24	21	123	54	105	244	100	264	467	293	796	591
DEC10B	281	130	73	111	215	123	270	337	837	60	639
16272	318	157	62	131	207	89	267	361	1158	61	943
88-1577	348	137	55	94	179	108	212	288	690	59	644
D88-28058	11	896	34	64	97	60	152	162	1015	41	887
403-3	22	171	66	108	255	131	262	413	325	864	97
0.444444444	17	116	39	68	156	59	216	433	278	899	751
413/89-1	280	76	28	44	80	39	169	196	641	21	588
86-24	18	116	55	71	158	94	232	498	326	629	701
DEC10B	223	137	63	89	209	136	316	377	923	57	857
16272	229	141	76	96	190	110	291	401	1045	63	820
88-1577	247	118	60	114	182	112	261	343	635	51	610
D88-28058	17	824	38	64	123	69	185	236	853	38	736
403-3	17	117	54	99	190	95	216	343	277	759	77
0.444444444	14	69	34	46	97	34	167	342	251	564	623
413/89-1	182	88	35	44	73	36	186	206	693	25	707
86-24	19	142	82	119	176	124	266	524	318	787	736
DEC10B	199	147	65	93	230	135	293	413	876	59	841
16272	202	173	81	132	244	115	304	417	1062	78	830
88-1577	265	162	81	113	246	141	284	393	694	75	628
D88-28058	16	807	44	70	140	67	232	249	858	44	720
403-3	20	152	57	110	217	130	226	348	343	829	113
0.444444444	10	61	33	42	56	30	120	277	192	447	433

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Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	10	33	18	48	37	17	397	121	92	14	27
No Template	19	43	27	51	63	17	615	159	104	10	26
No Template	15	64	17	67	74	15	526	128	83	12	31
No Template	29	41	19	44	99	21	429	131	97	12	38
No Template	56	75	40	51	108	22	476	177	121	13	66
No Template	13	46	25	48	67	18	507	124	94	10	28
No Template	15	27	19	57	59	13	450	154	107	10	27
No Template	9	41	15	58	42	10	419	123	83	15	24
RW1372	58	50	768	53	41	27	184	105	3186	14	2495
0201 9611	41	44	42	26	1024	27	119	146	2976	17	2367
MI01-88	45	2482	31	54	51	29	90	55	3057	18	2348
2011-5-383-1	18	27	24	2621	36	23	148	50	2238	21	23
8266-1	39	30	35	39	995	27	129	81	2743	1716	2204
G5508	49	47	29	2491	32	24	251	88	80	1691	33
MDCH_male_069311	18	54	23	2608	38	18	189	57	46	2438	31
Aeromonas hydrophila	11	63	16	29	27	18	307	150	59	11	19
RW1372	30	51	230	54	57	18	360	180	2351	15	1693
0201 9611	56	45	28	31	632	19	265	96	2444	17	1875
MI01-88	44	2653	33	54	65	25	87	72	3106	16	2424
2011-5-383-1	21	48	33	2506	46	27	160	93	2353	19	22
8266-1	41	31	33	34	973	19	174	105	2785	1548	2171
G5508	19	54	25	73	96	24	587	196	176	24	37
MDCH_male_069311	15	31	21	2224	80	24	144	167	87	1449	23
Aeromonas hydrophila	18	56	24	36	56	19	295	217	92	14	28
RW1372	27	21	416	29	37	24	98	47	2653	15	2110
0201 9611	88	38	34	27	1337	26	156	73	3131	16	2346
MI01-88	51	2954	33	57	43	22	153	79	3329	14	2552
2011-5-383-1	24	53	26	2606	35	22	260	116	2472	17	20
8266-1	50	43	40	32	1324	28	201	133	3126	2076	2485
G5508	44	52	27	67	125	19	647	112	158	17	38
MDCH_male_069311	34	37	24	2395	43	23	220	129	89	1284	27
Aeromonas hydrophila	51	57	22	43	36	20	297	130	89	16	31
Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	10	33	18	48	37	17	397	121	92	14	27
No Template	19	43	27	51	63	17	615	159	104	10	26
No Template	15	64	17	67	74	15	526	128	83	12	31
No Template	29	41	19	44	99	21	429	131	97	12	38

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	56	75	40	51	108	22	476	177	121	13	66
No Template	13	46	25	48	67	18	507	124	94	10	28
No Template	15	27	19	57	59	13	450	154	107	10	27
No Template	9	41	15	58	42	10	419	123	83	15	24
Enterobacter cloacae	10	62	15	36	56	12	205	110	81	11	18
Enterococcus faecalis	19	125	42	113	219	29	627	360	148	21	94
Klebsiella pneumoniae	39	31	34	78	169	25	423	186	96	16	33
Morganella morganii	58	128	40	188	165	43	458	239	182	30	60
Proteus mirabilis	14	44	22	73	51	38	337	227	94	18	24
Proteus vulgaris	16	39	20	42	51	23	224	105	97	16	24
Providencia rettgeri	31	27	27	60	33	18	293	132	83	17	25
Pseudomonas aeruginosa	11	18	22	32	43	11	248	97	77	11	23
Enterobacter cloacae	9	17	19	34	64	14	242	165	64	9	24
Enterococcus faecalis	242	85	31	142	247	24	637	284	229	18	62
Klebsiella pneumoniae	11	80	41	91	145	36	604	246	151	18	46
Morganella morganii	27	52	41	123	209	33	534	201	136	28	50
Proteus mirabilis	15	58	18	69	73	18	538	253	122	15	29
Proteus vulgaris	21	26	24	71	77	19	438	196	150	12	35
Providencia rettgeri	15	85	33	51	126	42	291	140	100	18	37
Pseudomonas aeruginosa	27	43	29	59	43	16	307	201	81	10	15
Enterobacter cloacae	9	19	21	38	71	10	282	123	58	9	21
Enterococcus faecalis	44	44	41	72	124	11	545	217	189	14	43
Klebsiella pneumoniae	13	41	29	52	96	32	476	261	145	13	29
Morganella morganii	43	99	36	73	77	28	423	182	136	23	40
Proteus mirabilis	56	81	23	79	111	15	432	127	69	18	35
Proteus vulgaris	104	31	23	46	47	22	347	147	98	20	30
Providencia rettgeri	40	80	20	47	64	33	294	168	61	19	26
Pseudomonas aeruginosa	14	14	12	16	19	13	93	35	35	12	14
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	10	33	18	48	37	17	397	121	92	14	27
No Template	19	43	27	51	63	17	615	159	104	10	26
No Template	15	64	17	67	74	15	526	128	83	12	31

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	29	41	19	44	99	21	429	131	97	12	38
No Template	56	75	40	51	108	22	476	177	121	13	66
No Template	13	46	25	48	67	18	507	124	94	10	28
No Template	15	27	19	57	59	13	450	154	107	10	27
No Template	9	41	15	58	42	10	419	123	83	15	24
Serratia marcescens	19	40	22	45	46	15	244	63	72	15	16
Staphylococcus aureus	38	44	30	114	97	33	380	162	146	20	39
Yersinia enterocolitica	21	67	36	56	148	26	532	172	118	14	36
Streptococcus gallolyticus	21	46	36	148	138	30	423	264	156	24	39
Salmonella typhimurium	18	30	21	40	51	25	258	105	57	17	26
G58-1	29	31	17	42	66	23	243	121	66	16	24
2534-86	17	33	43	75	61	20	272	162	117	18	36
11182-2	13	36	30	25	33	20	222	107	3267	13	19
Serratia marcescens	11	59	16	57	79	13	303	87	62	10	24
Staphylococcus aureus	22	82	39	87	70	21	626	293	179	16	62
Yersinia enterocolitica	23	73	21	55	78	21	485	263	115	13	38
Streptococcus gallolyticus	15	43	21	59	59	16	378	169	116	13	35
Salmonella typhimurium	66	50	22	62	96	38	495	236	156	13	41
G58-1	12	34	17	46	75	12	390	111	77	12	18
2534-86	46	42	26	78	191	20	384	174	148	14	40
11182-2	10	23	33	27	24	18	252	79	2861	13	21
Serratia marcescens	9	11	12	19	20	10	73	57	30	12	17
Staphylococcus aureus	94	53	14	48	49	45	491	139	103	14	34
Yersinia enterocolitica	23	30	19	42	47	18	559	174	127	11	35
Streptococcus gallolyticus	12	28	21	52	106	15	384	94	77	12	29
Salmonella typhimurium	11	34	16	34	29	13	300	72	86	11	18
G58-1	19	32	29	57	111	30	599	237	112	11	37
2534-86	14	33	18	56	46	12	360	134	86	11	23
11182-2	9	11	15	9	10	10	64	18	2346	8	10
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	25	21	25	20	31	47	37	39	25	19	20
No Template	19	20	34	19	27	29	82	97	69	14	18
No Template	17	24	18	25	105	54	62	88	35	16	24
No Template	24	17	22	16	19	19	38	89	26	13	20

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	19	20	24	16	38	30	81	68	38	14	19
No Template	25	18	28	18	30	23	55	88	29	14	19
No Template	22	17	18	17	30	17	69	66	27	14	21
No Template	16	16	17	15	17	14	24	27	19	14	15
KDHE 47	24	22	22	22	26	29	24	56	28	63	682
DEC10I (87-1713)	25	26	55	25	34	24	43	71	2095	22	1766
S2006 #4	21	24	33	21	32	22	46	531	2347	1229	2736
JB1-95	23	46	27	21	156	38	59	82	2659	858	2769
IHIT1703	18	20	21	22	118	24	39	47	2476	20	2599
DEC8b	21	24	19	24	136	38	57	46	2249	725	2606
S2006 #2	24	23	24	26	37	31	22	94	1115	99	1497
10C-3114	29	27	27	25	32	28	30	32	31	320	27
KDHE 47	24	23	25	21	31	23	33	66	27	191	1189
DEC10I (87-1713)	27	23	25	23	42	29	37	72	2502	21	2148
S2006 #4	23	25	21	20	28	50	86	531	2309	995	2761
JB1-95	22	35	22	20	231	33	62	101	2687	736	2712
IHIT1703	20	20	20	18	85	22	50	86	2307	18	2153
DEC8b	19	21	22	18	89	20	36	121	2219	700	2311
S2006 #2	19	22	24	18	29	29	24	293	2215	429	2053
10C-3114	23	23	24	19	25	22	29	62	32	257	20
KDHE 47	20	18	24	16	49	17	31	60	19	83	1323
DEC10I (87-1713)	18	18	17	16	27	26	43	48	2137	16	2065
S2006 #4	23	22	20	22	34	30	71	507	2197	938	2558
JB1-95	24	47	25	27	271	118	41	75	2576	697	2821
IHIT1703	27	27	18	17	111	22	76	72	2605	15	3026
DEC8b	40	18	29	23	132	20	96	60	2198	682	2326
S2006 #2	27	23	19	19	42	22	47	307	2492	406	1775
10C-3114	18	16	16	17	18	18	17	29	20	153	15
Sample	**O26**	**O45**	**O103**	**O104H4**	**O111**	**O121**	**O145**	**O157H7**	**Stx1**	**Stx2**	**Eae**
No Template	25	21	25	20	31	47	37	39	25	19	20
No Template	19	20	34	19	27	29	82	97	69	14	18
No Template	17	24	18	25	105	54	62	88	35	16	24
No Template	24	17	22	16	19	19	38	89	26	13	20
No Template	19	20	24	16	38	30	81	68	38	14	19
No Template	25	18	28	18	30	23	55	88	29	14	19
No Template	22	17	18	17	30	17	69	66	27	14	21
No Template	16	16	17	15	17	14	24	27	19	14	15
93-111	18	19	18	20	26	21	22	495	2430	1253	2526
2002-3211	15	22	23	19	31	28	50	49	30	543	2309
GS G5578620	31	23	34	23	34	29	939	81	2231	21	2542

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
KDHE 55	36	20	21	17	28	21	43	98	31	758	2409
7744	21	20	42	18	35	25	962	91	2302	17	2351
933	20	23	29	24	45	29	91	354	2118	633	2432
314-S	19	16	15	17	17	17	27	39	2131	14	2304
S2006 #3	15	15	16	15	17	17	18	221	1279	521	1320
93-111	19	19	18	18	21	20	18	132	1661	385	1217
2002-3211	15	15	23	16	23	19	54	70	27	418	2662
GS G5578620	14	14	15	14	30	22	1056	49	2124	13	1943
KDHE 55	15	17	19	15	16	22	29	77	29	256	2442
7744	14	15	15	14	23	13	1039	36	1841	12	2225
933	14	14	14	14	64	16	50	173	1849	282	1991
314-S	12	12	12	15	18	22	19	28	1805	11	1834
S2006 #3	12	13	13	12	16	14	14	95	1302	147	902
93-111	18	18	17	16	19	18	25	222	1995	497	2040
2002-3211	20	16	18	15	44	18	39	39	26	431	1993
GS G5578620	18	16	23	19	45	20	965	36	1792	14	2228
KDHE 55	13	33	22	14	87	22	69	103	22	307	2484
7744	47	18	17	20	29	23	1026	105	2232	14	2194
933	29	26	41	20	25	30	67	265	2160	445	2420
314-S	14	15	17	14	40	22	23	43	1763	14	1700
S2006 #3	14	13	15	12	16	16	16	196	1628	404	1760

Sample	O26	O45	O103	O104H4	O111	O121	O145	O157H7	Stx1	Stx2	Eae
No Template	25	21	25	20	31	47	37	39	25	19	20
No Template	19	20	34	19	27	29	82	97	69	14	18
No Template	17	24	18	25	105	54	62	88	35	16	24
No Template	24	17	22	16	19	19	38	89	26	13	20
No Template	19	20	24	16	38	30	81	68	38	14	19
No Template	25	18	28	18	30	23	55	88	29	14	19
No Template	22	17	18	17	30	17	69	66	27	14	21
No Template	16	16	17	15	17	14	24	27	19	14	15
86-24	15	15	16	15	18	16	15	170	18	409	1320
403-3	17	15	24	13	28	25	36	53	24	271	16
9:100	12	13	12	13	22	13	25	284	19	519	2261
16118-2	13	13	14	13	15	13	14	16	457	12	12
ECOR 26	15	16	20	1205	23	17	27	35	19	14	15
43893	13	15	16	17	36	20	123	31	20	13	13
86-24	18	16	18	16	19	17	25	118	22	309	937
403-3	17	21	20	15	42	17	44	57	27	212	18
9:100	19	17	17	16	23	16	19	446	20	921	2669
16118-2	17	16	16	14	21	16	14	30	749	15	15

ECOR 26	15	16	20	1375	17	36	29	34	21	13	14
43893	23	15	15	15	18	32	33	43	23	12	13

86-24	20	21	21	20	23	24	20	66	26	117	557
403-3	16	15	16	22	33	40	29	57	24	191	15
9:100	19	17	22	17	21	23	24	229	23	332	2567
16118-2	16	15	15	13	17	17	18	20	632	15	15
ECOR 26	14	21	14	1073	17	16	33	34	18	14	13
43893	26	16	18	14	16	18	20	24	17	14	14

Appendix 2

Wilcoxon Rank Sum Critical Values

N1	3		4		5		6		7		8		9		10	
N2	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi
3	5	16	6	18	6	21	7	23	7	26	8	28	8	31	9	33
4	6	18	11	25	12	28	12	32	13	35	14	38	15	41	16	44
5	6	21	12	28	18	37	19	41	20	45	21	49	22	53	24	56
6	7	23	12	32	19	41	26	52	28	56	29	61	31	65	32	70
7	7	26	13	35	20	45	28	56	37	68	39	73	41	78	43	83
8	8	28	14	38	21	49	29	61	39	73	49	87	51	93	54	98
9	8	31	15	41	22	53	31	65	41	78	51	93	63	108	66	114
10	9	33	16	41	24	56	32	70	43	83	54	98	66	114	79	131

N1	3		4		5		6		7		8		9		10	
N2	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi	Lo	Hi
3	6	15	7	17	7	20	8	22	9	24	9	27	10	**29**	11	31
4	7	17	12	24	13	27	14	20	15	33	16	36	17	**39**	18	42
5	7	20	13	27	19	36	24	40	22	43	24	46	25	**50**	26	54
6	8	22	14	30	20	40	28	50	30	54	32	58	33	**63**	35	67
7	9	24	15	33	22	43	30	54	39	66	41	71	43	**76**	46	80
8	9	27	16	36	24	46	32	58	41	71	52	84	54	**90**	57	95
9	10	29	17	39	25	50	33	63	43	76	54	90	66	**105**	69	11
10	11	31	18	42	26	54	35	67	46	80	57	95	69	**111**	83	127

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