

Supplementary Methods

Additional in silico primer design

Additional primers targeting the *gtrIV*, *gtrV*, *wzx6*, and *opt* genes (Supplementary Table 6) were designed and tested in singleplex reactions to resolve ambiguous and inconsistent classifications. The *opt* gene was included due its known association with serotype switching events giving rise to variant *S. flexneri* serotypes such as 4av, Xv, and Yv¹⁻⁴. While not incorporated into the final multiplex assay, these additional primers were used in the same initial singleplex reactions for Batch 1 DNA samples (Supplementary Figure 1).

10 Supplementary Table 1. Full list of DNA samples used in Batch 1 testing with results of mPCR and *in silico* methods.

Sample number	Lab ID	Year	Species/serotype	Accession	Lane in Figure 1	mPCR	ARIBA	ShigaPass	ShigaTyper
S002	FC0388	-	<i>S. flexneri</i> 1	-	1	<i>S. flexneri</i> 1b	-	-	-
S003	FC1124	-	<i>S. flexneri</i> 1	-	2	<i>S. flexneri</i> 1b	-	-	-
S004	FC1409	-	<i>S. flexneri</i> 1	-	3	<i>S. flexneri</i> 1b	-	<i>S. flexneri</i> 1b	EIEC
S017	FC0756	2016	<i>S. flexneri</i> 1	-	4	<i>S. flexneri</i> 1b	-	-	-
S018	FC1090	2016	<i>S. flexneri</i> 1	-	5	<i>S. flexneri</i> 1b	-	-	-
S019	FC1337	2016	<i>S. flexneri</i> 1	-	6	<i>S. flexneri</i> 1b	-	-	-
S005	FC1773	-	<i>S. flexneri</i> 2	-	7	<i>S. flexneri</i> 2a	-	-	-
S006	FC1730	-	<i>S. flexneri</i> 2	-	8	<i>S. flexneri</i> 2a	-	-	-
S020	FC1813	2016	<i>S. flexneri</i> 2	-	9	<i>S. flexneri</i> 2a	-	-	-
S021	FC2101	2016	<i>S. flexneri</i> 2	PDXU000000000	10	<i>S. flexneri</i> 2a	<i>S. flexneri</i> 2a	<i>S. flexneri</i> 2a	<i>S. flexneri</i> 2a
S022	FC2190	2016	<i>S. flexneri</i> 2	-	11	<i>S. flexneri</i> 2a	-	-	-
S007	FC1633	-	<i>S. flexneri</i> 3	-	12	<i>S. flexneri</i> 3a	-	-	-
S023	FC0164	2016	<i>S. flexneri</i> 3	-	13	<i>S. flexneri</i> 3a	-	-	-
S024	FC0875	2023	<i>S. flexneri</i> 3	-	14	<i>S. flexneri</i> 3a	-	-	-
S008	FC1372	-	<i>S. flexneri</i> 4	-	15	<i>S. flexneri</i> 2a	-	<i>S. flexneri</i> 2a	<i>S. flexneri</i> 2a
S009	FC1220	-	<i>S. flexneri</i> 4	-	16	<i>Shigella</i> /EIEC	-	<i>S. flexneri</i> Yv	<i>S. flexneri</i> Yv
S025	FC2244	2016	<i>S. flexneri</i> 4	-	17	<i>S. flexneri</i> X/Xv	-	<i>S. flexneri</i> Xv	<i>S. flexneri</i> Xv (4c)
S026	FC2254	2016	<i>S. flexneri</i> 4	-	18	<i>S. flexneri</i> X/Xv	-	<i>S. flexneri</i> X	<i>S. flexneri</i> Xv (4c)
S010	FC0052	2016	<i>S. flexneri</i> 6	-	19	<i>Shigella</i> /EIEC	-	-	-
S027	FC0065	2016	<i>S. flexneri</i> 6	-	20	<i>Shigella</i> /EIEC	-	-	-
S028	FC1768	2016	<i>S. flexneri</i> 6	-	21	<i>Shigella</i> /EIEC	-	-	-
S029	FC1996	-	<i>S. flexneri</i> 6	-	22	<i>Shigella</i> /EIEC	-	-	-
S001 (NCTC 12984)	-	-	<i>S. sonnei</i>	-	23	<i>S. sonnei</i>	-	-	-
S015	FC1032	-	<i>S. sonnei</i>	-	24	<i>S. sonnei</i>	-	-	-
S016	FC1208	-	<i>S. sonnei</i>	-	25	<i>S. sonnei</i>	-	-	-
S030	FC1512	2016	<i>S. sonnei</i>	-	26	<i>S. sonnei</i>	-	-	-
S031	FC2144	2016	<i>S. sonnei</i>	-	27	<i>S. sonnei</i>	-	-	-
S032	FC2183	2016	<i>S. sonnei</i>	-	28	<i>S. sonnei</i>	-	-	-
S013	FC0513	-	<i>S. boydii</i>	-	29	<i>Shigella</i> /EIEC	-	-	-
S014	FC1278	-	<i>S. boydii</i>	-	30	<i>Shigella</i> /EIEC	-	-	-
S033	FC0513	2021	<i>S. boydii</i>	-	31	<i>Shigella</i> /EIEC	-	-	-
S034	FC1559	2016	<i>S. boydii</i>	-	32	<i>Shigella</i> /EIEC	-	-	-
S035	FC2045	2021	<i>S. boydii</i>	-	33	<i>Shigella</i> /EIEC	-	-	-
S011	FC1856	2018	<i>S. dysenteriae</i>	-	34	<i>Shigella</i> /EIEC	-	-	-
S012	FC2382	-	<i>S. dysenteriae</i>	-	35	<i>Shigella</i> /EIEC	-	-	-
S036	FC0477	2023	<i>S. dysenteriae</i>	-	36	<i>Shigella</i> /EIEC	-	-	-
S037	FC1856	-	<i>S. dysenteriae</i>	-	37	<i>Shigella</i> /EIEC	-	-	-
S038	FC2358	2018	<i>S. dysenteriae</i>	-	38	<i>Shigella</i> /EIEC	-	-	-

- 12 Supplementary Table 2 Additional primers tested in singleplex reactions on Batch 1 DNA. ^aReference genome from GenBank used for primer
 13 design. *Primer sequences were taken from a previous study⁵.

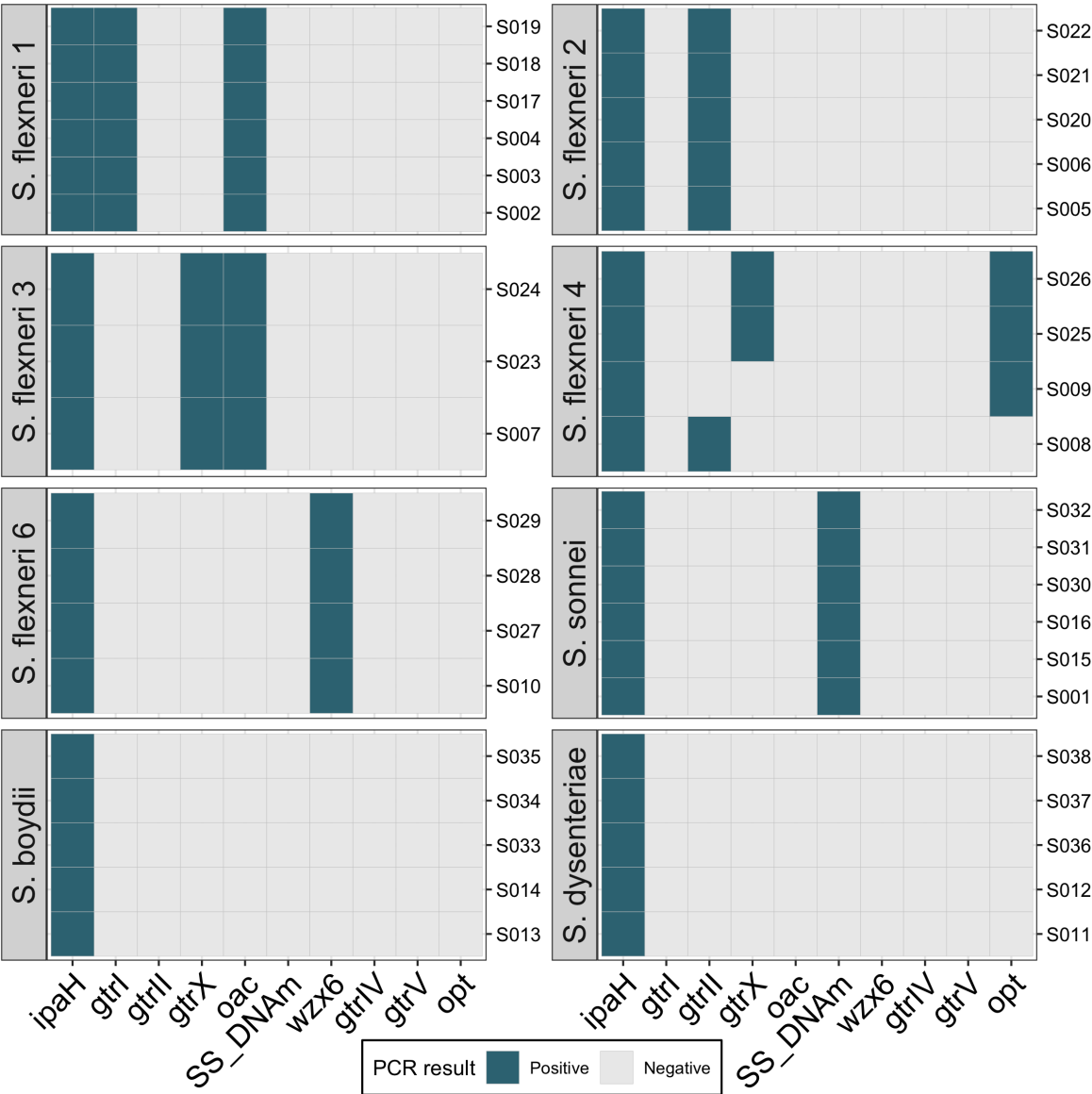
Target	Target gene	Serotype/variant specificity	Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Genome accession number ^a	Source
<i>Shigella flexneri</i>	<i>gtrIV</i>	4a, 4b, 4av(4c)	gtrIV_F	ATG TTCCTCCTTCTTCCTTT*	378	AF288197.1	Sun <i>et al.</i> ⁵
			gtrIV_R	TCCTGATGCTACCTTATCCA*			
	<i>gtrV</i>	5a, 5b	gtrV_F	AATACGATTCTCCTGGTGCTAAAC	920	CP000266.1	This study
			gtrV_R	CTCCGCAATTATTGTTAGGGCA			
	<i>wzx6</i>	6	Sf6_F	GACCGAGCAATTTGGCCTTT	722	EU294165.1	This study
			Sf6_R	CTCTGGACAACGCACTTGGT			
	<i>opt</i>	2av, 4av, Xv, Yv	opt_F	TGTGTGACTCCTTGATCCCA	796	CP001385.1	This study
			opt_R	TCTAAGGAGCTAACCCCGAA			

15 Supplementary Table 3. Accession numbers for long-read sequencing data of nine samples.

Sample name	Sample classification	Accession
S004	<i>S. flexneri</i> 1	ERR15096314
S008	<i>S. flexneri</i> 2a	ERR15096315
S009	<i>S. flexneri</i> Yv	ERR15096316
S025	<i>S. flexneri</i> Xv	ERR15096317
S026	<i>S. flexneri</i> X	ERR15096318
UKHSA-1	<i>S. flexneri</i> 5b	ERR15096319
UKHSA-2		ERR15096320
UKHSA-3		ERR15096321
UKHSA-4		ERR15096322

16

17 Supplementary Figure 1. Presence/absence of singleplex PCR on Batch 1 DNA samples.



18
19 *Shigella* DNA samples are grouped by original laboratory serotyping classification. Results
20 include additional targets described in Supplementary Methods. Blue indicates presence of
21 the target based on amplification of PCR product.

22 Supplementary Table 4. mPCR and *in silico* serotyping results for samples with discrepant
 23 results.

Sample	Lab serotyping	mPCR	ShigaTyper	ShigaPass
S004	<i>S. flexneri</i> 1	1b	EIEC	1b
S008	<i>S. flexneri</i> 4	2a	2a	2a
S009		<i>Shigella</i> /EIEC	Yv	Yv
S025		X/Xv	Xv (4c)	Xv
S026				X

24

25 Supplementary Table 5. Summary of mPCR and *in silico* classification of Batch 2 DNA
 26 samples compared to laboratory agglutination results.

Classification	<i>S. flexneri</i> (n=55)				<i>S. sonnei</i> (n=45)			
	mPCR	ARIBA	ShigaPass	ShigaTyper	mPCR	ARIBA	ShigaPass	ShigaTyper
<i>S. flexneri</i>	52	54	54	36	0	0	0	32
<i>S. sonnei</i>	0	0	0	0	45	45	45	0
<i>Shigella</i> /EIEC	1	1	0	0	0	0	0	0
<i>S. flexneri</i> novel	0	0	0	0	0	0	0	1
EIEC	0	0	0	3	0	0	0	11
Novel	0	0	0	15	0	0	0	0
Non- <i>Shigella</i> /EIEC	2	0	0	0	0	0	0	0
No prediction	0	0	0	1	0	0	0	1
Unknown	0	0	1	0	0	0	0	0
Total	55	55	55	55	45	45	45	45

28 Supplementary Table 6 Discrepancies in classification of *S. flexneri* Serotypes 3a and 5b
 29 samples from GEMS.

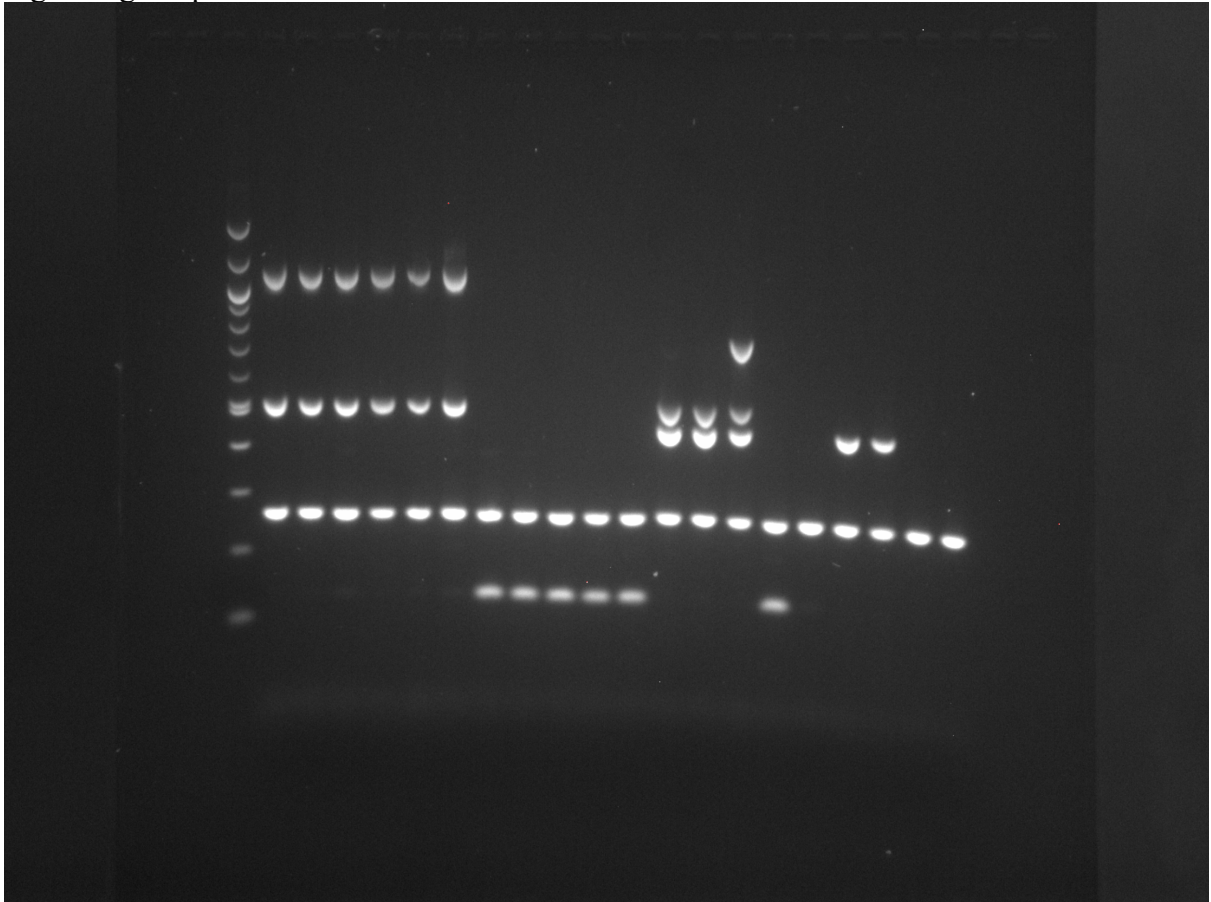
Flex serotype	GEMS	ShigaType r	ShigaPass	ARIBA
3a	109	67	67	0
3a/5b	0	0	0	106
5b	4	32	36	0

30

Supplementary References

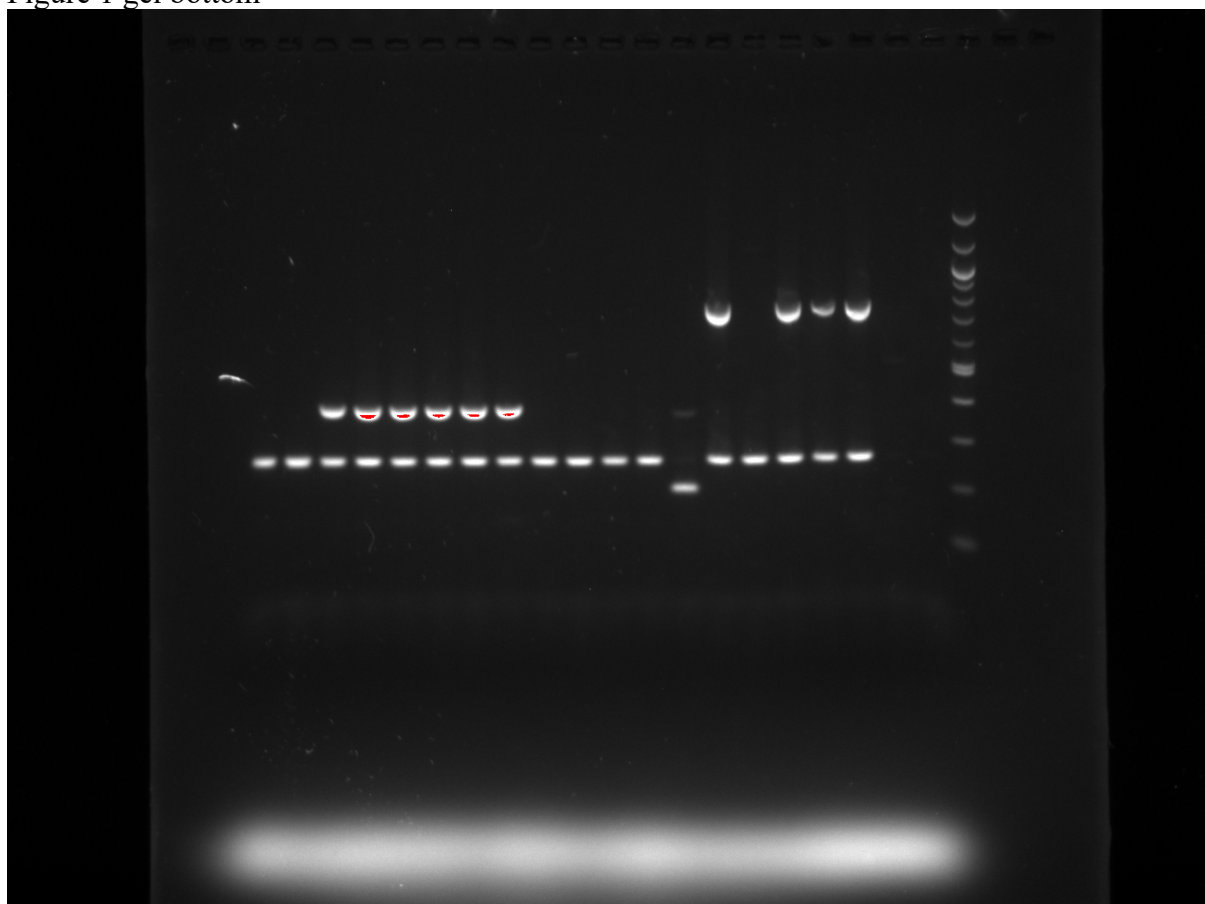
- 1 Knirel, Y. A. *et al.* O-antigen structure of *Shigella flexneri* serotype Yv and effect of the *lpt-O* gene variation on phosphoethanolamine modification of *S. flexneri* O-antigens. *Glycobiology* **23**, 475-485 (2013).
- 2 Perepelov, A. V. *et al.* A new ethanolamine phosphate-containing variant of the O-antigen of *Shigella flexneri* type 4a. *Carbohydr Res* **344**, 1588-1591 (2009).
- 3 Sun, Q. *et al.* A Novel Plasmid-Encoded Serotype Conversion Mechanism through Addition of Phosphoethanolamine to the O-Antigen of *Shigella flexneri*. *PLoS ONE* **7**, e46095 (2012).
- 4 Sun, Q. *et al.* Identification and Characterization of a Novel *Shigella flexneri* Serotype Yv in China. *PLoS ONE* **8**, e70238 (2013).
- 5 Sun, Q. *et al.* Development of a Multiplex PCR Assay Targeting O-Antigen Modification Genes for Molecular Serotyping of *Shigella flexneri*. *Journal of Clinical Microbiology* **49**, 3766-3770 (2011).

47 Figure 1 gel top



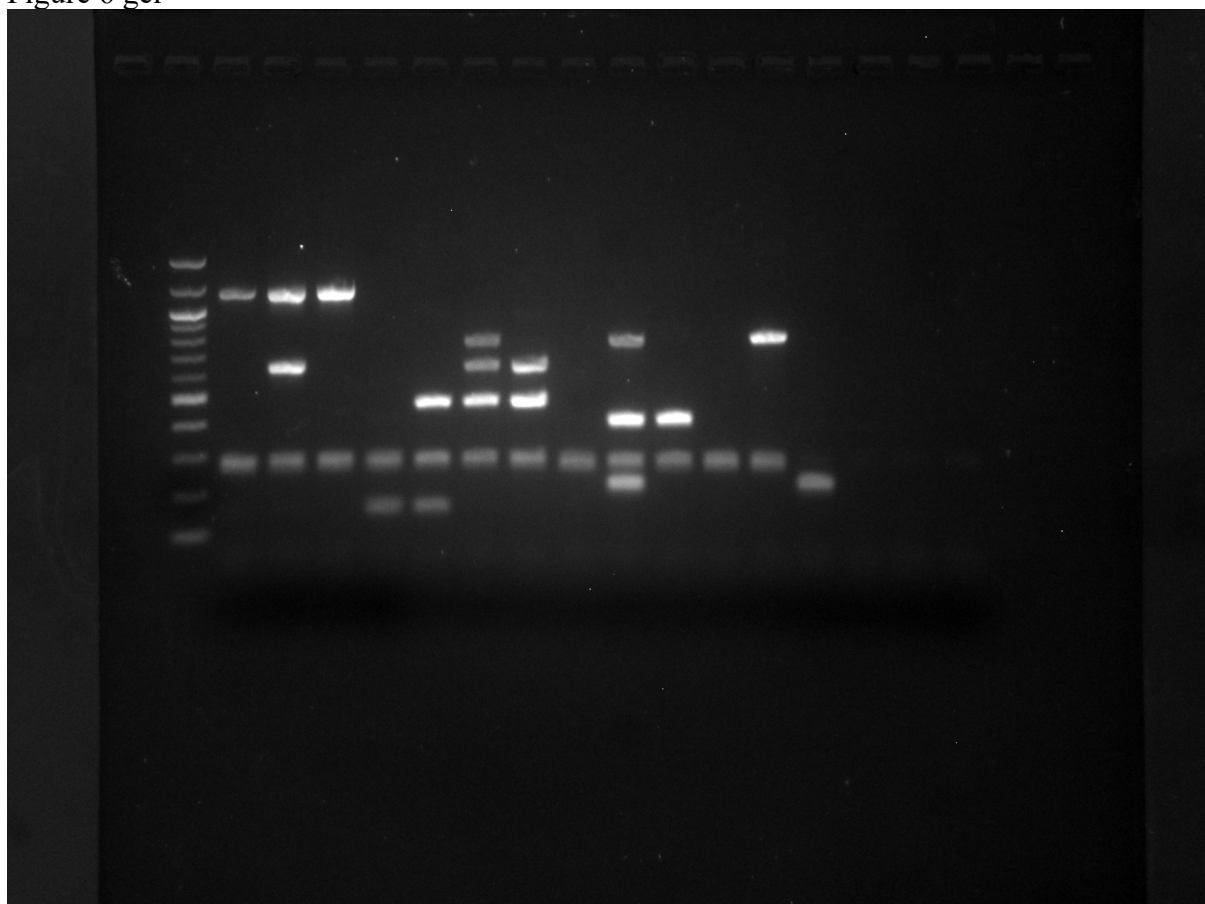
48
49

50 Figure 1 gel bottom



51
52

53 Figure 6 gel



54