TECHNICAL DOCUMENT

Laboratory standard operating procedure for MLVA of *Salmonella enterica* serotype Typhimurium

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Abbreviations

DNA	Deoxyribonucleic acid
RNase	Ribonuclease
dNTP	Deoxyribonucleotide triphosphate
ECDC	European Centre for Disease Prevention and Control
MLVA	Multiple-locus variable-number tandem repeat analysis
PCR	Polymerase chain reaction
SOP	Standard operating procedure
VNTR	Variable-number tandem repeat
CE	Capillary electrophoresis

1 Scope

To provide laboratories with a single protocol for performing MLVA of *S.* Typhimurium and thereby ensure inter-laboratory comparability of the generated results using capillary electrophoresis (CE).

2 Principle

This protocol describes the standardised laboratory protocol for molecular subtyping of *Salmonella enterica* serotype Typhimurium. It is based on the 5-locus multiple-locus variable-number tandem repeat analysis (MLVA) method published by Lindstedt et al [1].

Recently a suggested new nomenclature for standardised reporting of MLVA-profiles has been widely adopted in Europe [2]. This nomenclature is based on the actual number of repeat units in each locus and is, therefore, independent of the equipment used for fragment analysis. To normalise raw data obtained in each laboratory to the actual fragment size, a set of 33 standardisation strains has been selected and the five MLVA loci of these strains have been sequenced. Further details on standardisation strains, calculation of the number of repeat units, and the nomenclature are described in Larsson et al [2].

MLVA measures the variable number of tandem repeat units (VNTRs) in specific regions of the genome. The MLVA procedure includes:

- amplification of the VNTRs with fluorescent labelled primers;
- detection of fragment length by capillary electrophoresis; and
- calculation of the actual number of repeat units (Figure 1).

This protocol describes the MLVA procedure using 96-well PCR plates and the described dyes are suitable for ABI CE machines. It should be emphasised that each laboratory should use the type of tubes, plates and other equipment appropriate for the instruments available. Regarding set-up, maintenance and reagents for CE, it is recommended to follow the recommendations provided by the instrument supplier.

Figure 1 Flow chart of MLVA analysis
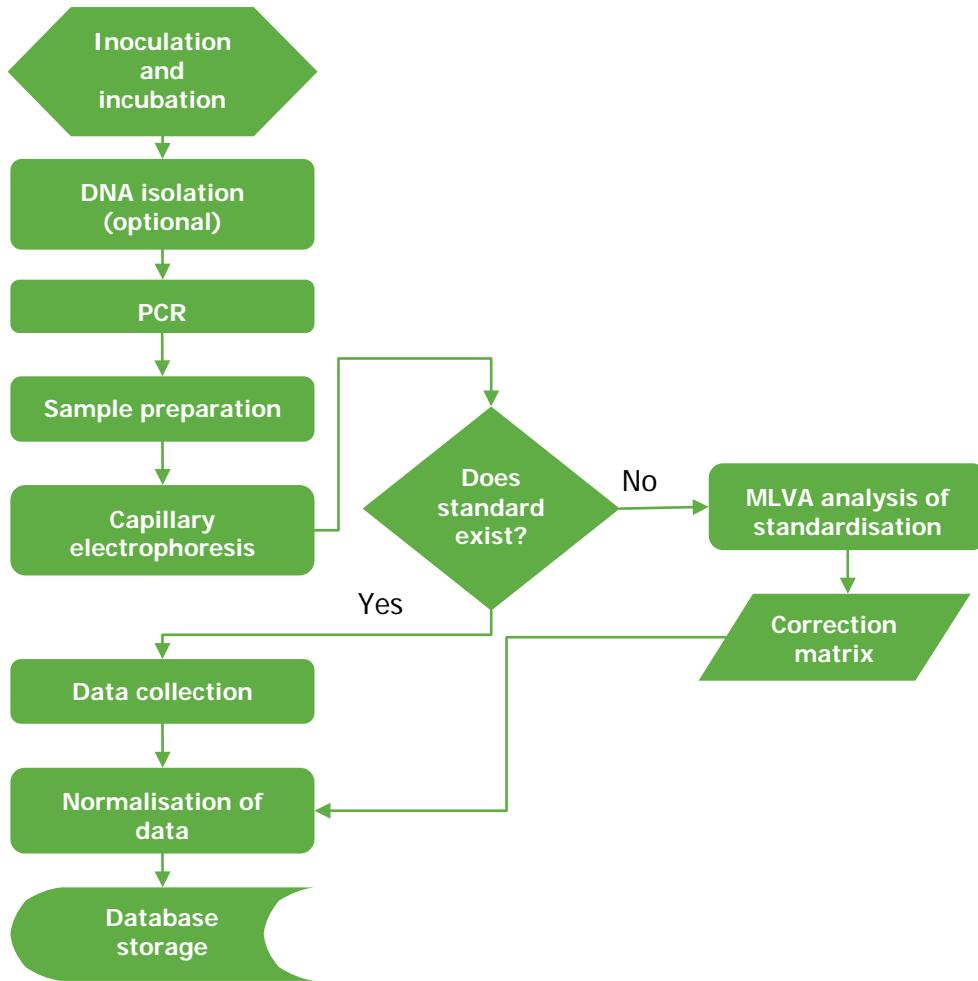
3 Reagents and supplies

3.1 Inoculation and incubation

3.1.1   Blood agar plate 5% or comparable media.

3.2 DNA isolation (optional)

3.2.1   Sterile disposable 1µl inoculation loops.

3.2.2   0.5 ml microcentrifuge tubes with safe caps.

3.2.3   1.5 ml microcentrifuge tubes.

3.2.4   RNase-free, molecular biology grade water.

3.3 Preparation of primer mix solutions

3.3.1   0.5ml amber-coloured tubes (to protect primer mix from light).

3.4 PCR reagents

Note: PCR can be performed with colony mass (alternative I) or DNA lysate (alternative II).

Alternative I: PCR with colony mass

3.4.1   Blood agar plate 5% or comparable media with pure *Salmonella* Typhimurium cultures.

3.4.2   RNase-free, molecular biology grade water.

3.4.3   Qiagen Multiplex PCR kit (comes with Q-solution and RNase-free water). This PCR kit contains a unique PCR buffer and one should take this into account if different PCR reagents are used.

3.4.4   96-well v-bottom PCR plate or 0.2ml PCR tubes with caps.

3.4.5   Sealing tape for 96-well plate.

3.4.6   1.5 ml microcentrifuge tubes.

3.4.7   One strain to be used as positive control (STm-SSI13).

3.4.8   PCR primers.

3.4.9   Fluorescent-labelled forward PCR primers (see Annex 1).

3.4.10   Unlabeled reverse PCR primers (see Annex 1).

3.4.11   Applied Biosystems are currently the supplier of all primers.

3.4.12   Dilute the concentrated stocks so the primer concentration is 100 pmol/µl stock solution.

Alternative II: PCR with DNA lysate (optional)

3.4.13   All items listed in Alternative I.

3.4.14   DNA lysates from isolates instead of colony mass.

3.5 Dilution of PCR products

3.5.1   96-well v-bottom PCR plate or 0.2 ml PCR tubes with caps.

3.5.2   RNase-free, molecular biology grade water.

3.6 Preparation of samples for fragment analysis on ABI

3.6.1   Diluted PCR product.

3.6.2   RNase-free, molecular biology grade water.

3.6.3   Labelled size standard marker for CE. GeneScan 600 LIZ or GeneFlo 625 ROX for Filterset G5 and D, respectively.

3.6.4   2.0 ml or 15 ml sterile tube.

3.6.5   96-well v-botton PCR plate.

3.6.6   Sealing tape for 96-well plate.

3.6.7   96-well plate retainer.

3.6.8   Rubber septa for 96-well reaction plate.

4 Apparatus

4.1 Pipettes and other equipment

4.1.1   PCR plate cooling block or ice.

4.1.2   Complete 'clean set' (1 000 µl, 200 µl, 100 µl, 20 µl and 10 µl) of single channel pipettes for PCR master mix set-up.

4.1.3   10 µl single channel pipette for transferring DNA to PCR tubes.

4.1.4   Optional: 8-channel pipettes (200 µl and 10 µl) for dilution PCR products and preparation of samples for fragment analysis, respectively.

4.1.5   Sterile pipette tips.

4.2 Apparatus

4.2.1   Incubator, capable of operating at 37°C ± 1°C.

4.2.2   Thermo cycler with heated lid option.

4.2.3   Heat block or thermo cycler, capable of operating at 95°C ± 1°C and 100°C ± 1°C.

4.2.4   Centrifuge, accommodating 0.2 ml PCR tubes, 1.5 ml microcentrifuge tubes, and 96-well v-bottom PCR plates.

4.2.5   Whirl mixer.

4.2.6   CE machine set up for fragment analysis in the range 140 to 615 bp. For ABI CE machines the standard fragment analysis protocols should work well.

5 Procedure

Note: Preparation of primer mix solution can be performed in advance.

5.1 Inoculation and incubation

5.1.1   Streak an isolated colony from test cultures to a 5% blood agar plate (or comparable media). Incubate cultures over night (14–20 hours) at 37°C.

5.2 DNA isolation (optional)

5.2.1   Check that plates contains pure cultures of *Salmonella* Typhimurium.

5.2.2   For each isolate to be typed, aliquot 200 µl of sterile water into a 1.5 ml microcentrifuge tube.

5.2.3   Use a sterile, disposable 1 µl loop to pick 1 colony (about ¼ of a loopfull); rotate the loop in the tube to release the bacteria into the water.

5.2.4   Cap and vortex for 10–15 seconds to disperse any clumps.

5.2.5   Place the tubes in a 100°C water bath or heat block for 10 minutes.

5.2.6   Cool briefly on ice or in thermal cycler at 4°C for 1 minute and centrifuge for 10 minutes at 10 000 rpm.

5.2.7   Transfer the supernatant containing the DNA into a new 1.5 ml microcentrifuge tube and discard the tube containing the pellet.

5.2.8   The DNA lysate can be stored at -4°C if PCR is performed no later than the next day.

5.2.9   These DNA lysates can be stored at -20°C or -80°C for years.

5.3 Preparation of primer mix solutions

Note: This should be prepared in a clean LAF bench where no DNA is handled. Keep primers protected from excessive exposure to light.

Note: Avoid repetitive freeze-thaw cycles of concentrated primer stocks. It is an advantage to prepare a batch of new primer mix solutions every second month and also if a significant drop in fluorescence level is observed.

5.3.1   Use the 'primer mix scheme' in Annex 2. RNase-free water is provided with the Qiagen Multiplex PCR kit.

5.3.2   Thaw all reagents and place on ice.

5.3.3   Prepare two separate primer mixes (forward and reverse) in 0.5 ml amber-coloured tubes or comparable. Mix by pipetting slowly up and down 5–6 times.

5.3.4   One vial contains 105 µl, which is enough for preparing 11 PCR reactions.

5.3.5   When protected from light (e.g. by the amber-coloured tube or covered in tin foil) and stored at minimum -20°C this primer mix can last three months.

5.4 PCR assay

Note: PCR master mix should be prepared in a clean area where no DNA or cultures are handled.

Note: PCR can be performed with colony mass (alternative I) or DNA lysate (alternative II).

5.4.1   Check that plates contain pure cultures of *Salmonella* Typhimurium.

5.4.2   Calculate volumes for PCR master mix using the volumes in Table 1 multiplied by the number of isolates to be tested plus 4 extra (for one negative control and two samples of the positive control strain (placed as the first and the last sample), and one extra).

Table 1 PCR master mix for one sample

Primer mix and reagents	Volume (µl)
Qiagen Master mix	12.5
Q-solution	2.5
Sterile water	8.25
Primer mix Fw	0.9
Primer mix Rv	0.9
Total volume	25.0
Volume per sample	25

5.4.3   Thaw all reagents and supplies needed for PCR reaction and keep these on ice during the whole PCR procedure. Briefly spin down the primer mixes and keep them protected from light.

5.4.4   Place a 96-well v-bottom PCR plate or required number of 0.2 ml PCR tubes in a PCR cooling block or on ice.

5.4.5   Prepare the master mix in 1.5 ml microcentrifuge tubes using the volumes shown in Table 1.

5.4.6   Add the components in the following order: water, Q-solution, primers, and then Qiagen Mastermix and mix in the whirlmixer for 1–2 seconds.

5.4.7   Dispense 25 µl of master mix into each tube/well. Keep tubes/plate on ice.

5.4.8   Add colony mass (alternative I) or DNA lysate (alternative II). If using 96-well plates, place one positive control in the first well and the second last well.

5.4.9   Alternative I. For each isolate use a sterile, disposable 1 µl inoculation loop to pick approximately 1 µl colony mass directly from a single colony and carefully rotate the loop 5–10 times in the tube/well to dispose the colony mass into the PCR master mix. If using 96-well v-bottom PCR plates take care that the liquid does not splash into the adjacent wells.

5.4.10   Alternative II: Add 1 µl DNA lysate to each tube/well.

5.4.11   Cover all tubes with caps/wells with sealing tape to avoid evaporation during PCR amplification. Spin down tubes/plate shortly to remove air bubbles and master mix attached to the side of the tube.

5.4.12   Use the PCR run conditions in Table 2.

Table 2 PCR run conditions

Temperature and time	Number of cycles
95°C for 15 min	1
94°C for 30 sec	
60°C for 90 sec	25
72°C for 90 sec	
72°C for 10 min	1
10°C forever	

Note: Use heated lid option in order to avoid condensation of liquids during PCR amplification.

5.5 Dilution of PCR products

5.5.1   For each sample dilute the PCR product 1:85 by adding 170 µl RNase free, molecular biology grade water to the required number of PCR tubes or wells in a 96-well plate, and subsequently add 2 µl PCR product. Mix well by pipetting up and down and spin the plate shortly.

5.6 Preparation of samples for fragment analysis on ABI

5.6.1   Prepare a fragment analysis master mix containing RNase free-water and size standard marker.

5.6.2   The fragment analysis master mix for one sample contains 1 µl size standard marker and 12 µl sterile water.

5.6.3   Mix well by pipetting up and down.

5.6.4   For each sample aliquot 13 µl fragment analysis master mix to the required number of wells in a 96-well v-bottom PCR plate.

5.6.5   By using the 8-channel pipette, add 1 µl 1:85 diluted PCR product to the appropriate columns. Place a positive control in the first and in the second last well. In the last well, put the negative control. Cover the plate with sealing tape in order to avoid evaporation.

5.6.6   Denature the sample plate at 95°C for 3 minutes, cool sample to 94°C on ice or ice block for 1 minute, and briefly spin down the sample plate to remove any air bubbles.

5.6.7   Place the sample plate in a plate base. Seal the plate carefully with the rubber septa. Snap the plate retainer onto the plate and the plate base. Place the prepared 96-well plate on the auto sampler tray.

5.7 Capillary electrophoresis

The CE instrument should be prepared according to the procedures specified by Applied Biosystems. Fragment analysis is prone to be affected by expired consumables, old buffer, bad calibrations, etc. An instrument will show a drift in results over longer time periods so it is good practice to keep track of the results from the positive control isolates that are included with each run.

5.7.1   Check that the buffer and POP7 polymer has not expired the recommended dates. ABI recommends changing buffer every day, but our experience is that once per week is adequate during normal circumstances.

5.7.2   Check that you have enough polymer for the number of runs you have scheduled.

5.7.3   Create a run protocol according to the machines' instruction.

5.7.4   Start the machine. Data is automatically saved as .fsa files.

6 Data analysis

6.1 Determining fragment sizes

6.1.1   The .fsa files from section 5.7 are imported into either GeneMapper or the free PeakScanner. In this text, the PeakScanner software will be described.

6.1.2   Add the .fsa files to a new project.

6.1.3   Choose the correct size marker for your project. The very first time you use the size marker you might need to create a new size standard under the <New> menu in PeakScanner.

6.1.4   Choose the analysis method 'Sizing Default – PP'.

6.1.5   Make sure that you have a good quality score indication in the <Score> column before proceeding. Sometimes you will have to tweak the settings or manually correct the size marker, sometimes you will have to perform a re-run. The exact procedure for every specific situation is beyond the scope of this document.

6.1.6   Find the correct sizes of the peaks by marking them. Peak sizes are read from the bottom pane.

6.1.7   The peak sizes are transferred to the analysis excel file 'STm MLVA allele assigning template'. Remember that in order to show correct allele numbers the template has to be calibrated to your own equipment! This procedure is described in section 7.

6.1.8   The Excel file is self explanatory, just enter you data in the yellow field and you will get corrected sizes and allele names to the right.

7 Standardisation strains for creating the conversion table

In order to get a proper normalisation of your MLVA raw data you will first have to determine the offset from the true fragment lengths that your specific hardware setup produces. This is done via a calibration run of the 33 isolates in the standardisation set (Annex 3). The results from the standardisation set is used to produce a correction table that will show you how much each locus needs to be compensated for every possible fragment length. The correction table approach is favoured compared to the use of equations since the compensation needed does not vary linearly with increasing fragment length.

7.1.1   Analyse the 33 standardisation strains with the protocol detailed in this document.

7.1.2   Insert the results from the 33 strains to the Excel document 'STm MLVA creating compensation table' (Annex 4). Insert your data to the yellow field in the sheet 'Original data'. Take note that you should have all data from all strains. Check the lower right graph so that no value is deviating significantly from their trends. Significant deviation in this case is > 3–5 bases – not from zero, but from the neighbouring values. The sheets are also annotated with help instructions, so read carefully.

7.1.3   Switch to the second sheet 'Allele data' and follow the instructions there. This sheet contains the calculations and uses a floating average. This is one of many ways to calculate the final correction table. It was chosen for simplicity and since a longer trend would be bad in circumstances where the compensation value varies in a non-linear fashion.

7.1.4   The third sheet, 'Correction table' is the generated correction table which shall be copied into the Excel template file 'STm MLVA allele assigning template'. To do this, open the Excel template via right-clicking and choosing 'Open' (not 'New', which is the default). Paste the correction table into the correct position in the third sheet 'Correction table'. Save the template and close it. Now the template is ready to use.

It is important to notice that the read raw value from the CE machine can change due to various reasons. So beyond the initial setup of the MLVA analysis it is advised to re-run the standardisation strains if:

- you have changed dyes or size standard in the analysis;
- you have changed polymer type on your CE machine;
- you have changed capillary length on your CE machine;
- you have had service performed on your CE machine.

Over a longer time span the raw values will show some drift, so re-running the strains 1–2 times a year is also advisable. It is recommended to look at the raw values from the positive control and plot them over time in order to detect changes in your setup.

References

1.   Lindstedt BA, Vardund T, Aas L, Kapperud G. Multiple-locus variable-number tandem-repeats analysis of Salmonella enterica subsp. enterica serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. J Microbiol Methods 2004 Nov; 59(2):163-72.

2.   Larsson JT, Torpdahl M, Petersen RF, Sørensen G, Lindstedt BA, Nielsen EM. Development of a new nomenclature for Salmonella Typhimurium multilocus variable number of tandem repeats analysis (MLVA). Euro Surveill. 2009;14(15):pii=19174. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19174

Annex 1: MLVA PCR Primers

Locus	Filter set D dyes	Filter set G5 dyes	Forward Primer (5'–3')	Reverse Primer (5'–3')	Fragment size (bp) in LT2	Repeat length (bp)
STTR9	6FAM-blue	6FAM-blue	AGAGGCGCTGCGATTGACGATA	CATTTTCCACAGCGGCAGTTTTTC	180	9
STTR5	HEX-green	VIC-green	ATGGCGAGGCGAGCAGCAGT	GGTCAGGCCGAATAGCAGGAT	259	6
STTR6	6FAM-blue	6FAM-blue	TCGGGCATGCGTTGAAA	CTGGTGGGGAGAATGACTGG	342	6
STTR10	NED-yellow	NED-yellow	CGGGCGCGGCTGGAGTATTTG	GAAGGGGCCGGGCAGAGACAGC	371	6
STTR3	HEX-green	PET-red	CCCCCTAAGCCCGATAATGG	TGACGCCGTTGCTGAAGGTAATAA	490	27/33

All primer sequences are from Lindstedt et al. [1].

Annex 2: Preparation of primer mix

Forward primer mix

Primers	Volume (µl)	Final conc. (µM)
STTR-9 F (100pmol/µL)	1.5	1.43
STTR-5 F (100pmol/µL)	1.5	1.43
STTR6-F (100pmol/µL)	3.0	2.86
STTR-10 F (100pmol/µL)	1.5	1.43
STTR3-F (100pmol/µL)	3.0	2.86
RNase-free water	94.5	
Total volume	105.0	

Reverse primer mix

Primers	Volume (µl)	Final conc. (µM)
STTR-9 R (100pmol/µL)	1.5	1.43
STTR-5 R (100pmol/µL)	1.5	1.43
STTR-6 R (100pmol/µL)	3.0	2.86
STTR-10 R (100pmol/µL)	1.5	1.43
STTR-3 R (100pmol/µL)	3.0	2.86
RNase-free water	94.5	
Total volume	105.0	

Annex 3: Standardisation strains

Standardisation strains of *Salmonella* Typhimurium sequenced at the Statens Serum Institut in Denmark (n=33)

Strain	MLVA fragment sizes	MLVA profile
STm-SSI01	198-235-342-371-490	6-9-13-10-211
STm-SSI02	207-271-336-383-517	7-15-12-12-311
STm-SSI03	216-247- NA- NA-490	8-11-NA-NA-211
STm-SSI04	225-265- NA- NA-490	9-14-NA-NA-211
STm-SSI05	171-253-330-437-517	3-12-11-21-311
STm-SSI06	171-277-342-455-517	3-16-13-24-311
STm-SSI07	171-295-324- NA-490	3-19-10-NA-211
STm-SSI08	171-307-330- NA-490	3-21-11-NA-211
STm-SSI09	162-319-396-389-523	2-23-22-13-212
STm-SSI10	162-325- NA- NA-463	2-24-NA-NA-111
STm-SSI11	162-337-306-359-523	2-26-7-8-212
STm-SSI12	162-247-342-365-523	2-11-13-9-212
STm-SSI13	171-271-348-377-517	3-15-14-11-311
STm-SSI14	171-265-354-449-517	3-14-15-23-311
STm-SSI15	162-253-408-359-523	2-12-24-8-212
STm-SSI16	162-241-414-359-550	2-10-25-8-312
STm-SSI17	171-265-438- NA-517	3-14-29-NA-311
STm-SSI18	162-247-342-335-523	2-11-13-4-212
STm-SSI19	162-235-336-341-523	2-9-12-5-212
STm-SSI20	171-277-342-485-517	3-16-13-29-311
STm-SSI21	180-235-300-359-616	4-9-6-8-314
STm-SSI22	162-301-342-377-469	2-20-13-11-12
STm-SSI23	162-277-318-395-484	2-16-9-14-310
STm-SSI24	180-283-312-347-265	4-17-8-6-105
STm-SSI25	162-253-342-347-298	2-12-13-6-106
STm-SSI26	171-283-378-407-517	3-17-19-16-311
STm-SSI27	189-253-312-371-436	5-12-8-10-11
STm-SSI28	189-259-300-353-337	5-13-6-7-8
STm-SSI29	171-223-360-497-517	3-7-16-31-311
STm-SSI30	162-211-288-389-370	2-5-4-13-9
STm-SSI31	171-253-306- NA-571	3-12-7-NA-511
STm-SSI32	171-283-390-419-517	3-17-21-18-311
STm-SSI33	162-259-318-377-496	2-13-9-11-112

'NA' designates a locus not present.

The fragment sizes are the true size according to sequence results.

The MLVA profile is based on the number of repeated units as described in Larsson et al. [2].

Annex 4: Excel files for calculations

The Excel file named 'STm MLVA creating compensation table v3.xls', attached to this PDF file, contains three sheets:

- Sheet 1 'Original data'
- Sheet 2 'Allele data'
- Sheet 3 'Correction table'

The Excel template file named 'STm MLVA allele assigning template v2.XLT', also attached to this PDF file, contains three sheets:

- Sheet 1 'MLVA allele calling'
- Sheet 2 'Lookup table'
- Sheet 3 'Correction table' – which should be populated with your own data from the other file.

Annex 5: Positions and size ranges of MLVA loci

In the table and figure below the ranges and positions of the MLVA loci are annotated. It should be noted that STTR3 loci smaller than ~371 base pairs are very rare. Therefore STTR5 and STTR3 can share a dye colour, but there will sometimes be cases where STTR3 is smaller than STTR5. This is exemplified in strain STm-SSI24. STTR9 can always share dye with either STTR6 or STTR10.

Locus	Genome position	Size range (repeats)	Maximum observed size	Minimum observed size
STTR9	3246595..3246774	1–9	153	225
STTR5	3184503..3184761	4–27	205	343
STTR6	2730723..2731064	3–29	282	438
STTR10	53518..53888 (pSLT plasmid)	3–31	329	497
STTR3	3629458..3629947	27: 0–5 33: 4–14	232	616