



TECHNICAL DOCUMENT

**Laboratory standard operating
procedure for multiple-locus
variable-number tandem
repeat analysis of
Salmonella enterica
serotype Enteritidis**

ECDC TECHNICAL DOCUMENT

**Laboratory standard operating procedure
for multiple-locus variable-number
tandem repeat analysis
of *Salmonella enterica* serotype Enteritidis**



This report was commissioned by the European Centre for Disease Prevention and Control (ECDC) and coordinated by Saara Kotila and Karin Johansson.

Authors

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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 European Union/European Economic Area (EU/EEA) data providers with a single protocol for performing multiple-locus variable-number tandem repeat analysis (MLVA) of *Salmonella enterica* serotype Enteritidis (*S. Enteritidis*) and thereby ensure interlaboratory comparability of the generated results using capillary electrophoresis (CE). The described process is applicable to Applied Biosystems (ABI) CE genetic analysers (intralaboratory validation performed on an ABI 3730 instrument)¹. Adjustments to the protocol might be required if different CE genetic analysers available on the market for fluorescent dye-labelled DNA fragment size analysis are used.

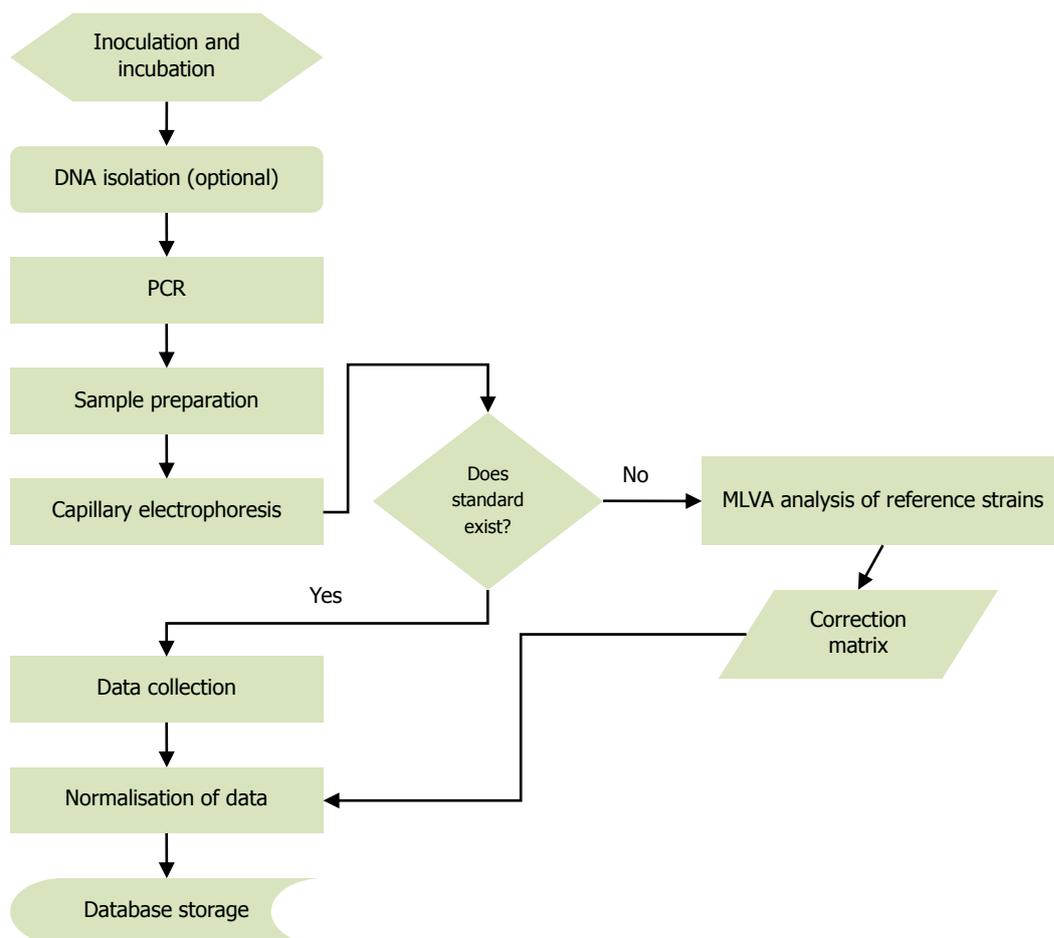
2 Principle

This protocol describes the standardised laboratory protocol for molecular subtyping of *S. Enteritidis*. It is based on the 5-locus MLVA method published by Hopkins et al. 2011 [1].

MLVA measures the variable number of tandem repeat units (VNTRs) in specific regions of the genome. The MLVA procedure includes 1) amplification of the VNTRs with fluorescent-labelled primers, 2) detection of fragment length by capillary electrophoresis (CE), and 3) 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 genetic analysers. It should be emphasised that each laboratory should use the type of tubes, plates, etc. appropriate for the instruments available. Regarding set-up, maintenance and reagents for CE, it is suggested to follow the recommendations provided by the instrument supplier.

Figure 1. Flow chart of MLVA analysis



¹ Disclaimer: ECDC does not endorse any particular commercial product or device for this analysis but indicates which products/devices have been standardised in the cited reference.

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

3.4 PCR supplies

Note: PCR can be performed with colony mass or DNA lysate.

- 3.4.1 Blood agar plate 5% or comparable media with pure *S. Enteritidis* cultures or DNA lysates from isolates
- 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 0.2 ml 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 (SEnt-HPA005, see Annex 3)
- 3.4.8 PCR primers
- 3.4.9 Fluorescent-labelled forward PCR primers (see Annex 1)
- 3.4.10 Unlabelled reverse PCR primers (see Annex 1)
- 3.4.11 Stock solutions of the primers (concentration 100 pmol/µl)

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

- 3.6.1 Diluted PCR product
- 3.6.2 RNase-free, molecular biology-grade water
- 3.6.3 Hi-Di formamide
- 3.6.4 Labelled size-standard marker for CE. GeneScan 600 LIZ or GeneFlo 625 ROX for Filterset G5 and D, respectively
- 3.6.5 Sterile tube for the fragment analysis master mix
- 3.6.6 96-well v-bottom PCR plate
- 3.6.7 Sealing tape for 96-well plate
- 3.6.8 96-well plate retainer
- 3.6.9 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' (1000 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 of 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 Thermocycler with heated lid option
- 4.2.3 Heat block or thermocycler, 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 genetic analyser set up for fragment analysis in the range 140 to 615 bp. Applied Biosystems: Filter set D/G5 dyes

For ABI CE genetic analysers, 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 overnight (14–20 hrs.) at 37 °C.

5.2 DNA isolation (optional)

- 5.2.1 Check that the plate contains pure cultures of *Salmonella* Enteritidis.
- 5.2.2 For each isolate to be typed, aliquot 100 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 loopful); 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 a 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 For longer term, the DNA lysates can be stored at -20 °C or -80 °C.

5.3 Preparation of primer mix solutions

Note: This should be prepared in a clean laminar flow hood 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 advised 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 the primer mix as instructed in Annex 2 in 0.5 ml amber-coloured tubes or comparable alternative. Mix by pipetting slowly up and down 5–6 times.
- 5.3.4 When protected from light (by e.g. the amber-coloured tube or covered in tin foil) and stored at minimum -20 °C, this primer mix can be used for three months.

5.4 PCR procedure

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 or DNA lysate.

- 5.4.1 Check that the plate contains pure cultures of *S. Enteritidis* if the PCR is done directly from the colony without DNA isolation.
- 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 four 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	6.25
Q-solution	1.25
Primer mix	2.0
Sterile water	2.0
Total volume	11.5
Volume per sample	11.5

- 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 mix and keep it 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 Mix the components and whirlmix 1–2 seconds.
- 5.4.7 Dispense 11.5 µl master mix into each tube/well. Keep tubes/plate on ice.
- 5.4.8 Add colony mass or DNA lysate. If using 96-well plates, place one positive control in the first well and the second to last well.
- 5.4.9 When using colony mass as a template: 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 disperse 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.
When using DNA lysate as a template: Add 1 µl DNA lysate to each tube/ well and mix carefully by pipetting up and down at least three times.
- 5.4.10 Cover all tubes with caps/wells with sealing tape to avoid evaporation during PCR amplification. Spin down tubes/plate briefly to remove air bubbles and master mix attached to the side of the tube.
- 5.4.11 Use the PCR run conditions in Table 2.

Table 2. PCR run conditions*

Temperature and time	No of cycles
95 °C for 15 min	1
94 °C for 30 s	28 (20–35)
55 °C (55–60)* for 90 s	
72 °C for 90 s	
72 °C for 10 min	1
10 °C forever	

* Annealing temperature and number of cycles might need to be optimised by each laboratory. Range used in 12 European laboratories given in brackets.

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 1:40 (range of dilutions used in 12 European laboratories 1:10–1:800; this variable might need to be optimised by each laboratory) by adding 80 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 briefly.

5.6 Preparation of samples for fragment analysis

- 5.6.1 Prepare a fragment analysis master mix containing size standard marker and either RNase free water or Hi-Di Formamide.
- 5.6.2 The fragment analysis master mix for one sample contains 0.5 µl of size standard marker and 10 µl Hi-Di Formamide (formamide can be replaced with RNase free water to improve work safety, however in this case the dilution of the PCR product needs to be carefully optimised since the signals are weaker; it might also be necessary to reduce the number of cycles in the PCR).
- 5.6.3 Mix well by pipetting up and down.
- 5.6.4 For each sample aliquot 10.5 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 of the diluted PCR product to the appropriate wells in each column. Place a positive control in the first and in the second-to-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 onto the auto sampler tray

5.7 Capillary electrophoresis

- 5.7.1 The CE genetic analyser should be prepared according to the procedures specified by the supplier. 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. Check that the buffer and POP7 polymer have not expired. Applied Biosystems recommends changing buffer every day, but based on user experience once per week is adequate under 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 CE genetic analyser's instructions
- 5.7.4 Start the CE genetic analyser. Data are automatically saved as .fsa files.

6 Data analysis

6.1 Determining fragment sizes

- 6.1.1 The .fsa files from chapter 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 for each locus are translated into repeat numbers according to the table in Annex 4.
- 6.1.8 For example, for SENTR7 a peak size of 121.74 bp is within range 121–126 and so corresponds to 2 repeat numbers, as indicated in Annex 4. The 'offset' represents the parts of the fragment that do not contain tandem repeats.
- 6.1.9 The repeat number is determined in the same way for each of the five loci to obtain an MLVA profile consisting of five tandem repeat numbers, e.g. '2-11-10-3-2' (order of loci SENTR7 – SENTR5 – SENTR6 – SENTR4 – SE-3).

- 6.1.10 Please note that the values in the tandem repeat table (Annex 4) have been optimised for one particular CE genetic analyser used in only one laboratory (ABI 3730) and might need adjustment before being used in other laboratories. A set of tables developed by Jonas Björkman (Statens Serum Institut, Denmark) that can be used to mitigate interlaboratory variation during result analysis (creating a compensation table and allele assignment) is available upon request from fwd@ecdc.europa.eu.
- 6.1.11 If unexpected results are obtained for the positive and/or negative controls, troubleshooting should be performed to identify and fix the source of the deviation before re-running the samples. The expected MLVA profile for the positive control is 3-10-6-4-1, and there should be no amplification in the negative control.

7 Standardisation strains

The measured fragment lengths might vary depending on the sequencing platform used and differences in laboratory protocols. As a consequence, the results need to be standardised to enable interlaboratory MLVA data comparisons. This is done via a calibration run of the 16 reference strains in the standardisation set (Annex 3).

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

- changed dyes or size standard in the analysis;
- changed polymer type on your CE genetic analyser;
- changed capillary length on your CE genetic analyser;
- had a service performed on your CE genetic analyser.

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

8 References

1. Hopkins KL, Peters TM, de Pinna E, Wain J. Standardisation of multilocus variable-number tandem-repeat analysis (MLVA) for subtyping of *Salmonella enterica* serovar Enteritidis. Euro Surveill. 2011 Aug 11;16(32).
2. Malorny B, Junker E, Helmuth R. Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. BMC Microbiol. 2008 May 30;8:84.

Annex 1. MLVA PCR primers

Locus	Applied Biosystems filter set D dyes	Applied Biosystems filter set G5 dyes	Forward primer (5'–3')	Reverse primer (5'–3')	Fragment size (bp) in SEnt-HPA005	Repeat length (bp)
SENTR7	6FAM-blue	6FAM-blue	ACGATCACCACGGTCACTTC	CGGATAACAACAGGACGCTTC	131.30	9
SENTR5	6FAM-blue	6FAM-blue	CACCGCACAATCAGTGGAAC	GCGTTGAATATCGGCAGCATG	267.23	6
SENTR6	NED-yellow	NED-yellow	ATGGACGGAGGCGATAGAC	AGCTTCACAATTTGCGTATTCG	182.82	7
SENTR4	HEX-green*	VIC-green	GACCAACTCTATGAACCAATG	ACCAGGCAACTATTCGCTATC	118.09	7
SE-3	HEX-green*	VIC-green	CAACAAAACAACAGCAGCAT	GGGAAACGTAATCAGAAAGT	305.14	12**

* In the electropherogram *SENTR4* peak usually ranges from 118 to 190 bp, and *SE-3* peak from 305 to 319 bp, which makes it possible to use the same dye.

** Third tandem repeat 14 bp

All primer sequences as in Malorney et al. 2008 and Hopkins et al. 2011 [1, 2].

Annex 2. Preparation of primer mix

Primers	Volume (µl)	Final concentration in PCR reaction (µM)*
SENTR7 F (2.5 pmol/µL)	10.0	0.05 (0.04–2.50)
SENTR7 R (2.5 pmol/µL)	10.0	0.05 (0.04–2.50)
SENTR5 F (5 pmol/µL)	10.0	0.09 (0.05–2.00)
SENTR5 R (5 pmol/µL)	10.0	0.09 (0.05–2.00)
SENTR6 F (20 pmol/µL)	10.0	0.35 (0.08–2.00)
SENTR6 R (20 pmol/µL)	10.0	0.35 (0.08–2.00)
SENTR4 F (2.5 pmol/µL)	10.0	0.05 (0.04–2.50)
SENTR4 R (2.5 pmol/µL)	10.0	0.05 (0.04–2.50)
SE-3 F (5 pmol/µL)	10.0	0.09 (0.04–2.00)
SE-3 R (5 pmol/µL)	10.0	0.09 (0.04–2.00)
Total volume	100.0	

* Needs to be optimised by each laboratory. In brackets are the ranges of concentrations used in 12 European laboratories.

Annex 3. Reference strains

Reference strains of *Salmonella* Enteritidis for standardisation (sequenced at and proprietary to Public Health England; n=16).

Strain	MLVA fragment sizes (SENTR7 – SENTR5 – SENTR6 – SENTR4 – SE-3)	MLVA profile
SEnt-HPA001	131.30 – 261.17 – 168.72 – 118.02 – 304.96	3-9-4-4-1
SEnt-HPA002	131.26 – 297.10 – 175.76 – 118.05 – 317.47	3-15-5-4-2
SEnt-HPA003	122.23 – 273.22 – 175.73 – 110.96 – 317.57	2-11-5-3-2
SEnt-HPA004	131.33 – 285.19 – 175.73 – 118.05 – 305.03	3-13-5-4-1
SEnt-HPA005	131.30 – 267.23 – 182.82 – 118.09 – 305.14	3-10-6-4-1
SEnt-HPA006	113.17 – 261.17 – 189.90 – 110.72 – 317.49	1-9-7-3-2
SEnt-HPA007	122.23 – 291.20 – 203.54 – 110.71 – 317.47	2-14-9-3-2
SEnt-HPA008	122.26 – 273.13 – 210.95 – 110.77 – 317.39	2-11-10-3-2
SEnt-HPA009	122.26 – 279.30 – 225.61 – 110.69 – 317.65	2-12-12-3-2
SEnt-HPA010	122.26 – 231.47 – 204.16 – 125.31 – NA	2-4-9-5-NA
SEnt-HPA011	122.32 – 243.24 – 183.03 – 125.28 – 305.05	2-6-6-5-1
SEnt-HPA012	122.22 – 249.20 – 225.51 – 132.72 – 305.18	2-7-12-6-1
SEnt-HPA013	122.25 – 255.29 – 197.17 – 125.27 – 317.72	2-8-8-5-2
SEnt-HPA014	122.27 – 267.29 – 232.58 – 125.28 – 305.03	2-10-13-5-1
SEnt-HPA015	122.20 – 297.18 – 197.10 – 139.81 – NA	2-15-8-7-NA
SEnt-HPA016	122.25 – 249.00 – 189.85 – 147.19 – NA	2-7-7-8-NA

'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 repeat units.

Annex 4. Tandem repeat table

	offset		offset		offset
SENTR7	103bp	SENTR5	207bp	SENTR6	140bp
No. of TR	size	No. of TR	size	No. of TR	size
(= allele no.)	(bp)	(= allele no.)	(bp)	(= allele no.)	(bp)
1	112–113	4	231–232	3*	161–162
2	121–126	5	?	4	167–169
3	130–132	6	243–244	5	175–177
		7	249–250	6	183–184
		8	255–256	7	189–191
		9	261–262	8	197–198
		10	267–268	9	203–205
		11	273–274	10	210–211
		12	279–280	11	?
		13	285–286	12	225–226
		14	290–291	13	232–233
		15	297–298		
				* sequencing identified 5 TRs	
	offset		offset		
SENTR44	~88bp	SE-3	293bp		
No. of TR	size	No. of TR	size		
(= allele no.)	(bp)	(= allele no.)	(bp)		
3	109–111	1	305–306		
4	117–119	2	317–319		
5	125–126				
6	132–133				
7	139–140				
8	145–147				
	Offset has been roughly calculated based on fragment size minus tandem repeat insert, e.g. SENTR7 (9bp TR)				
	= 112 – (9x1) = 103bp and not sequenced amplicon length				
	Repeat sizes				
	SENTR7	9bp	Allele in red not sequenced		
	SENTR5	6bp			
	SENTR6	7bp			
	SENTR4	7bp			
	SE-3	12bp			

?: Allele size has not been defined for the number of repeats

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