High resolution typing of *Trichinella spiralis* and *T. britovi* by multi locus analysis of polymorphic microsatellites

STANDARD OPERATING PROCEDURE

SUMMARY

1. SCOPE

To provide a Standard Operating Procedure (SOP) for performing microsatellites (MSAT) analysis of single larvae of *Trichinella spiralis* and *T. britovi* and define multilocus genotypes (MLGs) of individuals to inform on the genetic structure of parasite isolates circulating in farmed (e.g., pigs) or wild animals. The SOP shall be applied to single larvae collected from both infected muscles and meat-derived products (e.g., sausages), either fresh or fixed in ethanol. It is recommended to use this SOP when a detailed genetic characterization of the parasite is required, as in outbreak investigations and/or when assessing a possible source of infection [Bilska-Zajac et al., 2021; La Rosa et al., 2018a].

2. INTRODUCTION

Microsatellites are composed by tandem repeats of short nucleotide sequences (1-6 base pairs, bp), and are often characterized by large genetic variability caused by expansion/contraction of the repeats. The larval cohorts of *Trichinella* spp. that infect a new host undergo a bottleneck event that increases their inbreeding (consanguinity) and decreases their genetic variability, then multiple passages may result in lineages of larval cohorts that could differ greatly from each other [La Rosa et al., 2018a; La Rosa et al., 2012; Rosenthal et al., 2008]. Since loci containing MSATs are inherited according to Mendelian laws, the type and frequency of MSATs alleles can be used to distinguish alternative parental linages. The evolutionary history of two larval cohorts could be assessed by characterizing their MLGs if no outcrossing (such as it occurs after double infection) has occurred since their separation from the last common ancestor.

The analysis of MLGs defined by multiple polymorphic microsatellites proved to be a very useful tool to understand if two independent larval cohorts belong to the same lineage, thus allowing to trace back the source of infection in human and pig outbreaks [Bilska-Zajac et al., 2021; La Rosa et al., 2018b].

This SOP describes the laboratory procedures used to define *T. spiralis* and *T. britovi* MLGs by MSAT analyses, and resulted from a multi-year experience at the International Trichinella Reference Centre (ITRC), during which many isolates collected across Europe and elsewhere have been studied [Bilska-Zajac et al., 2021; La Rosa et al., 2018a, La Rosa et al., 2018b; La Rosa et al., 2012; Rosenthal et al., 2008]. The procedure includes 1) amplification of multiple MSATs; 2) fragment typing by capillary electrophoresis (CE); 3) definition of the MLG in single larvae; and 4) analysis of MLGs from single larvae and/or isolate to infer their relationships.

This protocol describes the procedures in use at the EURLP, which are based on specific instruments, including an automated DNA extraction station (Biosprint96 robotic station, Qiagen) and a capillary electrophoresis system (Qiaxcel, Qiagen). Adjustments to the protocol might be required if different devices are used for capillary gel electrophoresis (CE).

Single larvae analysis is mandatory. For the identification of *Trichinella* larvae at the species level, please refer to the official method of the EURLP (MI-02, see https://www.iss.it/web/iss-en/-/diagnostic-3).

3. REFERENCES

- Bilska-Zajac E., Tonanzi D., Pozio, E. et al. Genetic evidence substantiates transmission of *Trichinella spiralis* from one swine farm to another. Parasites Vectors 14, 359 (2021)
- La Rosa G., Calero-Bernal R., Pérez-Martín J.E., Tonanzi D., Galati F., Serrano-Aguilera F.J., et al. Rare but evolutionarily consequential outcrossing in a highly inbred zoonotic parasite. Int J Parasitol. 48:543–53.4 (2018a)

- La Rosa G., Vallée I., Marucci, G. et al. Multilocus genotype analysis outlines distinct histories for *Trichinella britovi* in the neighboring Mediterranean islands of Corsica and Sardinia. Parasites Vectors 11, 353 (2018b).
- La Rosa G, Marucci G, Rosenthal BM, Pozio E. Development of a single larva microsatellite analysis to investigate the population structure of *Trichinella spiralis*. Infect Genet Evol. 12:369–76.2 (2012)
- Rosenthal B., La Rosa G., Zarlenga D., Dunams D., et al. Human dispersal of *Trichinella spiralis* in domesticated pigs. Infection, Genetics and Evolution 8, 799–805 (2008)

4. DEFINITIONS

- **MLG:** Multi Locus Genotype
- **CE:** Capillary Electrophoresis
- **MSAT:** Microsatellites
- **DNA-plate:** PCR plate containing the DNA purified from single larvae
- **MSAT-PCR-plate:** PCR plate assembled for MSATs amplification
- **Larval cohort:** a random set of larvae collected from an isolate
- **Isolate:** the larvae harboured by infected host

5. EQUIPMENT AND SUPPLIES

- Stereo microscope, magnification from 20 to 100x
- Thermomixer allocating full skirted and unskirted PCR plates and operating from 37° to 90°C (±1°C)
- Set of micropipettes covering volumes from 1 to 1000 µL and suitable tips with aerosol filter
- Dispenser pipette covering the volume from 5 to 200 µL and suitable tips
- Thermocycler 96 wells; ±0.25°C accuracy and <0.5°C uniformity
- Centrifuge, accommodating 96-well PCR plates
- Biosprint 96 (Qiagen) robotic station for automated DNA purification, or equivalent
- CE apparatus (Qiaxcel, Qiagen) and dedicated supplies, or equivalent
  - QIAxcel DNA High Resolution Kit (1200) (Qiagen, Cat. no. 929002)
  - 15-500 bp alignment marker (Qiagen, cat. no. 929520)
  - GelPilot 100 bp ladder (Qiagen, cat. no. 239035)
  - DNA QuantLadders (Lonza, cat. no. 50334)
- 5 cm Petri dishes
- Full skirted PCR plate 0.2 mL wells
- Cap strips or adhesive film
- Unskirted PCR plate 0.15 mL wells N. (Eppendorf, cat. no. 0030133307)
- Masterclear Cap Strips (Eppendorf, cat. no. 0030132874)
- Optional: 8-12 channels micropipettes (10 µL and 200 µL)

6. KITS, REAGENTS AND SOLUTIONS

- DNA purification system using magnetic beads technology
  - DNAIQ kit (Promega, code DC6700)
    - Lysis buffer (LB)
    - 2x washing buffer (WB)

- Elution buffer (EB)
- MagneSphere resin (MAG)
    - Tissue and Hair Extraction Kit (Promega, code DC6740)
        - Incubation buffer (IB)
        - Proteinase k buffer (PK)
        - DTT solution (DTT)
- RNase-free molecular biology-grade water
- 96% ethanol
- *T. spiralis* and *T. britovi* reference larvae fixed in ethanol 80-90%
- Purified *T. spiralis* and *T. britovi* reference DNA
- Type-it, 2x master mix (Qiagen)
- Primers for amplification of MSAT loci

7. PROCEDURE

The following procedure describes the genotyping of 96 individual larvae using PCR plates. Users have to consider to include one or more negative samples (i.e., no larva added into the well) in each experiment, typically at positions D12 and H12 of the PCR plate. The procedure is scalable to the desired number of isolates and larvae. Please refer to the MI-02 official method of the EURLP for identification of *Trichinella* larvae at species level by multiplex PCR, (https://www.iss.it/web/iss-en/-/diagnostic-3).

7.1 Single larvae collection in a PCR plate

- Transfer the fresh or ethanol-fixed larvae to be analysed into a small petri dish
- Under the stereo microscope select a well conserved larva[1] and aspirate it by a micropipette set at 5-10 µL
- Transfer one larva into each well of the plate using a micropipette set at 5-10 µL. During the procedure: keep the micropipette in vertical position; avoid the tip edge to contact the bottom of the well; do not release the piston until the tip is out of the well
- Repeat the procedure until the plate is complete[2]
- Check each well under the stereo microscope to confirm the presence[3] of a single larva
- Cover the plate using Parafilm
- Centrifuge the plate at maximum speed for 3-5 min
- Remove the Parafilm and let the ethanol to evaporate (10-15 minutes)
- Continue the procedure or store the plate at 4°C or -20°C until use[4]

Note1: prefer coiled larvae and avoid "comma shaped" diaphanous larvae
Note2: to reliably define the genetic structure of an isolate, consider to analyse 24-36 larvae (corresponding to 2-3 lanes of the PCR plate); consider a percentage of PCR failures depending on the conservation status of the larvae; using well preserved larvae this percentage is likely <10%
Note3: larvae are quite sticky and could remain attached to the well wall. If that happens, use 5 µL of ethanol to flush them down to the bottom, then wait for ethanol evaporation
Note4: use 4°C for short term storage; -20°C for long-term conservation

7.2 DNA purification
- Keep the PCR plate containing larvae at room temperature for few minutes

- Prepare IB+, LB+, LBW+ buffers from the "DNA IQ" and "Tissue and Hair Extraction" kits according to Table 1

Table 1. Mix solutions needed for DNA extraction using the "DNA IQ" and "Tissue and Hair Extraction" kits. The IB, DTT and PK are included in the Tissue and Hair Extraction kit, while LB, WB, MAG (paramagnetic resin) and EB are included in the DNA IQ kit.

	Volume (µl) x100 samples (96 wells PCR plate)							Volume (µl) x1 sample
	IB	LB	DTT[1]	PK[2]	MAG	tot		
IB+	1,600	--	200	200	--	2,000		20
LB+	--	3,800	40	--	400	4,000		40
LBW+	--	7,920	80	--	--	8,000		80
1x WB1[3]	--	--	--	--	--	8,000		80
1x WB2[3]	--	--	--	--	--	8,000		80
EB	--	--	--	--	--	10,000		100

- Dispense 20 µl of IB+ solution (Table 1) into each well of the PCR plate containing larvae.
- Incubate at 42°C x 20 min/1250 rpm on Thermomixer
- Dispense 40 µl of LB+ solution (Table 1) into each well.
- Incubate at 25°C x 5 min/1250 rpm on Thermomixer
- Meanwhile, dispense volumes (as in Table 1) of washing and elution solutions (LBW+, WBs and EB buffers) into four separate full skirted PCR plates and place[4] them into the Biosprint96 robotic station in the position assigned by the software (see Annex 1)
- Place the plate containing the digested larvae into the Biosprint 96 robotic station
- Start the Biosprint program "DNAIQ" (see Annex 1)
- At the end of program, close the plate containing DNA with strip caps or adhesive film. Proceed with the amplification or store the plate at -20°C. Afterwards we refer to the PCR plate containing purified DNA as "**DNA-plate**"

Note1: prepare as recommended by the manufacturer. In brief: dissolve 5g of DTT in 32.4 mL nuclease-free (final concentration is 1M). Dispense the DTT solution into small aliquots and store at -20°C.
Note2: prepare as recommended by the manufacturer. In brief: gently dissolve the provided lyophilized proteinase K in 5.5 mL of Incubation Buffer (final concentration is 18 mg/mL). Dispense the proteinase K solution into small aliquots and store at -20°C.
Note3: prepare as recommended by the manufacturer and store at room temperature
Note4: make sure that the A1 position of the plate matches the A1 position of the Biosprint 96 robotic station

7.3 Species identification of single larvae

This step is mandatory to verify that all the larvae analysed belong to the same species as double infections due to different species are known to occur. Follow the official method MI-02 of the EURLP for identification of *Trichinella* larvae at species level by multiplex PCR. [https://www.iss.it/web/iss-en/-/diagnostic-3].

7.4 PCR for MSAT genotyping

The following procedure describes the amplification of a single MSAT locus. The procedure is to be repeated to amplify each selected locus containing MSAT (see Table 2 for the list of loci). PCR master mixes should be prepared in a clean area where no *Trichinella* DNAs are handled, or using a dedicated hood.

- Select a primer pair to be tested from Table 2

Table 2. PCR primers used for MSAT amplification

MSAT locus	Forward primer (5'–3')	Reverse primer (5'–3')	Alignment marker
TS103	ATTAAGAGGGGAGGGGGTAA	GAATAGCTGCTAGAAGTGCCG	GelPilot 100 bp ladder, Qiagen cat. 239035
TS128	TGTTGTCCAGTTCGGATGAA	GCTTGCACTGGACTTCAACA	GelPilot 100 bp ladder, Qiagen cat. 239035
TS130	ACAATTTCAACCCACCCT	TCCTCATTATCGTCTTCGTC	GelPilot 100 bp ladder, Qiagen cat. 239035
TS1122	GTCCCGTGGTTTAGCTTTGA	CCAATAACAGCAGCAGCATC	DNA QuantLadders, Lonza cat. 50334
TS1131	ACACCACCTTCACCACCA	TAATCACGACAAAAGATCTCCC	GelPilot 100 bp ladder, Qiagen cat. 239035
TS1380	CGGACAGATTCAGCGGA	ATGGGCCAACAACTACCACTA	GelPilot 100 bp ladder, Qiagen cat. 239035
TS1444	CAAACGGATACACAATAGGAAG	AAAACAAAGGCAAGGCAC	GelPilot 100 bp ladder, Qiagen cat. 239035
TS10.07	ACCAATCGAATAGAGATATTTGAAT	TGATTAAATTGCTTTCTACTTCTTT	GelPilot 100 bp ladder, Qiagen cat. 239035
TS10.10B	CATTAACGATGTGCTATTTAACGCT	TCAATTCATTTCATTTCAATCTGCG	GelPilot 100 bp ladder, Qiagen cat. 239035
TB1019	CCAACAACATCCTCAACA	CACCTTACAATCAAGTAACC	GelPilot 100 bp ladder, Qiagen cat. 239035
TB922	AGCCAACTCAAGTCCCAAA	AAACGGCAATGCAACAAC	GelPilot 100 bp ladder, Qiagen cat. 239035
TB2880	CCGCCTGAAAATGAATAAC	CAGAGGAAAACAACACAATC	GelPilot 100 bp ladder, Qiagen cat. 239035
TB1837	ATGGCCAGTATGAAGGTG	TTTTTGCTTGGGTTGCTG	GelPilot 100 bp ladder, Qiagen cat. 239035

- Combine 20 µL of forward and reverse primers (stock solutions 100 pmoles/µL) and add 460 µL of DNase-free water to obtain the 10x MSAT primer mix solution for use in PCR reaction. Prepare under a laminar flow hood that is not used to handle the DNA
- Label this solution 10x MSAT primer mix (where MSAT is the target locus selected, see Table 2). Stock this solution frozen for up to 3 months
- Thaw all necessary reagents (see Table 3) and place them on ice

Table 3. PCR recipes for MSAT PCR

Reagents	Volume (μL) single sample/well	Volume (μL) Reaction mix[1] x102 samples/wells
2x Type-it master mix	8	832
10x MSAT primer mix	1.6	163.2
DNA-plate[2,3]	6.4	-
Total volume	**16 μL**	995.2 : 102 = **9.6 μL**

- Keep **DNA-plate** containing the purified DNA of the single larvae on ice
- Spin the **DNA-plate** before removing caps or adhesive film
- Prepare a reaction mix[1] for 102 samples (96 + 6 additional samples to compensate for pipetting errors (see Table 3)
- Dispense 9.6 μL of the reaction mix[1] into each well of a PCR plate where the PCR amplification will be performed; hereinafter referred to as **MSAT-PCR-plate**. Use aerosol filter tips and a laminar flow hood different from that used to handle the DNA
- Move to a different room and, in a dedicated hood, transfer[2,3] 6.4 μL of purified DNA from **DNA-plate** to **MSAT-PCR-plate** PCR plate (see Table 3)
- Mark the plate with the name of the MSAT used, date and any other information useful to trace back the experiment
- Close the **MSAT-PCR-plate** plate using Masterclear Cap Strips[4] (Eppendorf) and spin it
- Load the plate in the thermocycler and start the MSAT PCR program (see Table 4)

Table 4. MSAT PCR amplification program

temperature	n. of cycles	Time
95 °C	1	5 minutes[4]
95 °C		30 seconds
55 °C	35	30 seconds
72 °C		30 seconds
72 °C	1	15 minutes
14 °C	1	∞[5]

- At the end, remove the **MSAT- PCR-plate** from the thermocycler, spin and store the plate at 4°C for immediate capillary electrophoresis analysis, or to -20°C for delayed analysis

Note1: reaction mix does not take into account DNA, which has to be added later in a separate room or under a PCR cabinet
Note2: use a dedicated micropipette to handle DNA
Note3: the use of multichannel micropipette is recommended to avoid cross contaminations and to maintain the correspondence of samples between the 2 plates (**DNA-plate** and **MSAT-PCR-plate**)
Note4: caps have inverted dome to reduce the volume of vapour inside the well, thus permitting stable conditions during amplification reaction
Note5: only when PCR is performed overnight

7.5 Capillary electrophoresis by Qiaxcel instrument

The Qiaxcel (Qiagen) device uses 12 independent capillaries combined into a compact cartridge ready for use. One cartridge allows 100 run sessions (12 samples in each run) for a total of 1200 samples. The independent runs are made comparable by using alignment markers that are run along

with the samples. The MSAT PCR products are analysed using the high resolution kit cartridge[1]. The cartridge, the solutions and the buffers required for electrophoresis are included in the high resolution kit (Qiagen). The Qiaxcel Screengel software application is provided with the Qiaxcel instrument and is used to set the CE runs and to analyse the results. The software allows to visualize results as electropherograms or as a virtual image of the gel, leaving the user to decide for the most appropriate method according to his/her purposes.

- Keep the **MSAT- PCR-plate** containing samples closed and at room temperature
- Spin the plate to collect sample at the bottom
- Move to the room[2] where the Qiaxcel instrument is based
- Start the Screengel application
- Remove the caps or adhesive film and put the plate in the appropriate position into the Qiaxcel device
- Dispense 15 µL in each well of a 0.2 mL 12-tubes PCR strip with the appropriate alignment markers[3] (Table 2). Once prepared, add mineral oil to each tube to prevent evaporation. The strip can be re-used multiple times if stored at 4°C
- Put the alignment marker strip in the appropriate position into the Qiaxcel device
- Follow the "Set the Process Profile" menu as recommended by the manufacturer for the use of high resolution cartridge, select "OH700" as Method[4] in the "Run Parameters" menu, and "No Marker"[5] in the "Sample Selection/Reference Marker" menu
- Start run
- At the end of the run, an approximated analysis of the result is displayed in the main view window based on the absolute run time of the 12 capillaries. This result is automatically saved in a specific directory for subsequent analyses

Note1: recommended for products in the 100 to 400 bp range. Use the appropriate method and an adequate alignment marker, assuming a resolution of 2-3 bp
Note2: it could be the same room where PCR is performed
Note3: since the 12 capillaries work independently, to increase the resolution of the instrument it is recommended to use a pair of alignment markers whose bands are positioned as close as possible to the PCR product (see Table 2 for suggestions).
Note4: the method defines the running conditions, i.e. voltage, sample loading and run time. Follow the user manual to select the method that best fits with the amount of product "usually" obtained by PCR. For PCR products of 100-400 bp with a concentration of 10-50 ng/µL, the OH700 is recommended
Note5: identification of microsatellite alleles is done by visual comparison between products. Size markers are not necessary (see Data analysis section)

In this document the MSAT alleles are coded using their size, expressed in bp.

8. DATA ANALYSIS

At the end of the CE run, the results are displayed without any alignment correction among capillaries, since the Screengel software is unable to recognize automatically which bands have to be used to align the 12 runs. As such, the results are inadequate for the purpose of the experiment.

The following steps describe the standard procedures to be applied to align MSAT PCR amplified products. The reader has to keep in mind that it is not possible to foresee all possible situations, and from time to time suitable adjustments are needed to achieve the correct

result/alignment. Working on virtual gel or electropherogram visualization do not affect the following procedure. This document refers to a visualization of the results as a virtual gel.

8.1 Alignment of CE results for genotype identification

- Using the "Analysis" menu of the Screengel application, open the experiment to be analysed
- Drag and drop all lanes in the main view window to display the results as virtual gel
- Select the option "Absolute migration time" in the "Image Option …" menu, press "select all" or select the lanes of interest, then press the "Start analysis" button
- Recognize "visually" the PCR products in the run/gel and limit the Screengel analysis to the area of the run that includes: i) the PCR products, ii) the alignment marker immediately below and iii) the alignment marker immediately above
- Restriction of the zone analysis is done acting on the software parameter indicated as "Suspend Integration" (refer to the manual)
- In the "Image Option …" menu select the "Relative migration time" and "Highlight alignment markers" options
- The alignment markers will be aligned and visualized in green and the PCR products become comparable
- Check for each channel that the proper alignment bands are highlighted. If needed, exclude or add bands/peaks acting on the table at the bottom
- Each sample has to show one (homozygote) or two (heterozygote) bands[1,2]

8.2 Genotype identification

- Identify homozygotes present in the gel and purify those showing a clear and abundant PCR product. A pair of each type of the homozygotes observed should be sufficient
- Sequence these samples in both directions (forward and reverse) using the same primers used for PCR. Trim the consensus sequence properly
- Use data in Annex 2 to confirm the correctness of the PCR product amplified
- The sequence size in bp will be used in this procedure to encode the individual alleles
- Go back to the virtual gel and define the genotype of each individual using as size reference the sequences; follow the rule, same size same allele
- If an allele is displayed only as heterozygous among all individuals of the same population, it is arbitrarily named[3] by comparing its size with the homozygotes present in the same gel
- Record the genotype of each individual present in the plate
- Repeat the procedure "7" for each MSAT that you want to analyse
- At the end, for each individual[4] the MLG will result from the combination of all genotypes defined by all MSATs identified
- Arrange all genotype results for each individual as a text table[5] to be used as input for downstream software application

Note1: faint additional bands could be present but they do not affect the final analysis
Note2: if more than two bands are displayed, and unspecific product could not be suspected, probably two or more larvae were used in the procedure, and for this reason the individual have to be excluded from the analysis
Note3: the way alleles are defined is not important, and could be defined by numbers (e.g., 100/100 or 100100), letters (e.g. AA/AA or AAAA), or any other code of choice

Note4: individuals for which more than one MSAT did not produce positive amplification should be excluded from the analysis or treated with caution
Nota5: software usually requires the data file in a specific format, and the user should adapt the input file accordingly

8.3 Software for population genetics data analysis

Different software are available to calculate the main statistical indices of population genetics (see the appended list for suggestions). They can be used to describe the biological traits of a population, including 1) number and frequencies of alleles; 2) level of polymorphism; 3) level of consanguinity between individuals in a population; and 4) genetic differentiation between populations. Analysis of these indices can help understanding the present and past evolutionary relationships of a population.

List of commonly used software

- **GenAlEx**, basic indices and PCoA analysis. Peakall R. and Smouse P.E. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28, 2537-2539.
- **Fstat**, basic indices. Goudet J. FSTAT (version 1.2): a computer program to calculate F-Statistics. J Hered. 1995;86:485–6.
- **Genpop**, basic indices. Rousset, F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol. Ecol. Resources 8: 103-106.
- **Arlequin**, basic indices Excoffier, L. G. Laval, and S. Schneider (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47-50.

These could be used to summarize the frequencies of alleles and genotypes and to broadly perform population genetics tests. GenAlEx is probably the friendliest, since it is implemented in the Microsoft Excel application. The other software are more appropriate to perform specific tests on the state of consanguinity of the populations, exact tests for the Hardy-Weinberg equilibrium, evaluate the linkage disequilibrium between the markers, calculate and compare values of FST or RST and estimate their significance.

- **STRUCTURE**, Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

The software uses a clustering method based on multiple simulations of a user-defined model to identify the genetic structure of the population, assigning to each individual the probability of belonging to a specific group. No a priori assessment of individuals is required. Very useful for evaluating the relationships of recently differentiated isolates.

Biosprint 96 protocol script

[PROTOCOL PROPERTIES]

Name = DNAIQ
Protocol template version = 2.5.4
Instrument type = BioSprint 96
Creator = LaRosa
Created = 10/1/2013 13:46:21
Description = DNA extraction from Trichinella larvae using "DNA/IQ kit" and "hair extraction kit".
Kit = Promega DNA/IQ
Plate layouts = Bind, Wash LB, Wash 02, Wash 03, Elution, Rod cover

[PLATE LAYOUTS]

Bind
Plate type = PCR plate
Plate change message = Load Digestion

A:
- volume = 20, name = IB+
- volume = 38, name = LB+
- volume = 2, name = MagSil

Wash LB
Plate type = PCR plate
Plate change message = Load WB1

A:
- volume = 80, name = LBW+

Wash 02
Plate type = PCR plate
Plate change message = Load WB 02

A:
- volume = 80, name = WB solution

Wash 03
Plate type = PCR plate
Plate change message = Load WB 03

A:
- volume = 80, name = WB solution

Elution

Plate type = PCR plate
Plate change message = Load elution plate

A:
- volume = 100, name = EB solution

Rod cover
Plate type = PCR plate
Plate change message = Load rod cover

A:
- EMPTY

[STEPS]

HEATING
Step parameters
- Name = Heating
- Plate = Bind

Beginning of step:
- Premix = Yes

Heating parameters:
- Heating time = 5min 0s
- Preheat = No
- Temperature = 25
- During heating = Pause, tip position = Inside well/tube
- Postmix time = 10s, speed = Very slow

End of step:
- Collect beads = No

BIND
Step parameters
- Name = Bind
- Plate = Bind

Beginning of step:
- No Action = Yes

Bind parameters:
- Bind time = 10min 0s, speed = Very slow

End of step:

- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = Wash LB
- Plate = Wash LB

Beginning of step:
- Release = Yes, time = 10s, speed = Slow

Wash parameters:
- Wash time = 30s, speed = Medium

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = Wash 01
- Plate = Wash 02

Beginning of step:
- Release = Yes, time = 10s, speed = Slow

Wash parameters:
- Wash time = 30s, speed = Medium

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = Wash 02
- Plate = Wash 03

Beginning of step:
- Release = Yes, time = 10s, speed = Slow

Wash parameters:
- Wash time = 30s, speed = Medium

End of step:
- Collect beads = Yes, count = 3

DRY
Step parameters
- Name = Dry

- Plate = Wash 03
- Dry time = 10min 0s
- Tip position = Outside well

ELUTION
Step parameters
- Name = Elution
- Plate = Elution

Beginning of step:
- Release = Yes, time = 10s, speed = Slow

Elution parameters:
- Elution time = 1min 0s, speed = Slow

Heating parameters:
- Heating time = 4min 0s
- Preheat = Yes
- Temperature = 65
- During heating = Pause, tip position = Tip angles in liquid
- Postmix time = 10s, speed = Medium

Remove beads:
- Remove beads = Yes, collect count = 3, disposal plate = Wash LB

Annex 2

MSAT alleles identified in *Trichinella spiralis* and *Trichinella britovi* isolates from different geographical regions (Europe, Asia, North and South America). The typical nucleotide sequence of each marker is shown. Forward and reverse primer used for PCR amplification are highlighted in bold. Microsatellite sequences are shown into bracket, and the first repeat is underlined. The number and size range (in base pairs) of alleles recognized in multiple studies is reported. Low frequency substitutions of single nucleotides, occasionally present, are not shown.

Trichinella spiralis:

1. MSAT-TS103, four alleles identified, from 157 to 173 bp
   ATTAAGAGGGGAGGGGGTAAGCA(TATTTATTTATTTATTTATT)TTCTTTTTTTTCCCAGAAATCTAATC
   TTTCCACTTTATATTAAACTGCCAAATTTGATTGTTTTCTTTTCTTTTTCGT(TCGTTCGTTCGTTCGT)TTGT
   TTGTTTGCTCT**CGGCACTTCTAGCAGCTATTC**

2. MSAT-TS1122, two alleles identified, 200 and 212 bp
   GTCCCGTGGTTTAGCTTTGACATTGAAGCCGTACGTCGGGTATGTGGACCACCATCGTGTTCTGTGCTC
   AATCAGCTCGTCATATTTATTCGACACCGATGGTGCCATTGTTCTGCTT(CACCACCACAACCACCACC)A

GCAGCGGTAGGTAGTAGTTGCCCGAAAATTCTTGCACAACTAATATGATTCGAT**GATGCTGCTGCTGTT**
ATTGG

3. MSAT-TS1131, four alleles identified, from 224 to 239 bp
ACACCACCTTCACCACCAACAACAGCAACAACATCAATTCACAACTACTCGCACATTGCATACCATGCAC
ACATACACACGCCTGTGTATGTGTGAGATTTGCATGTTTTTATTTTTTAAATTATTATTTCTCTCTGTATGT
CGTCT(<u>TAA</u>TAATAATAATAATAATAA)TTTATAATTTGTTTTCAATCTTAAAGTAATGATTTATTCA**GGGA**
GATCTTTTGTCGTGATTA

4. MSAT-TS128, three alleles identified, from 208 to 220 bp
TGTTGTCCAGTTCGGATGAAGGTGGCGGTTGCCTCTCGTCGCCATCTTTGACGCACGTTTTTGTCGCCG
GACCGGGGCAGACCAACTTCAAGTTGGCTT(<u>TTT</u>GTTTGTTTGTTTGTTTGTTTG)TTTATTTTGCTCATCT
ACTCTCAGGGCAGCTTATTCGTTGTCAGAAGTGTGGAATTCAGTGAACGAAGT**TGTTGAAGTCCAGTG**
CAAGC

5. MSAT-TS130, three alleles identified, from 145 to 151 bp
ACAATTTCAACCCACCCTGCACCACTGCTGCTACTACATATAATATCACCAATGGTCGACAAACATCAGT
TGCTGTTGGTGTTGATGTGAATGATTACAAC(<u>GAT</u>GATGATGAT)GACGAAGACGACAAC**GACGAAGA**
CGATAATGAGGA

6. MSAT-TS1380, nine alleles identified, from 252 to 282bp
CGGACAGATTCAGCGGATAAGGCTTCGGTTTAGCTGGTCGCTTTTTCACCACCGGGCTAGCAGGCTTTT
TCTCGCTT(<u>TGC</u>TGCTGCTGC)TGTTGTTGTCCATGAGAATGATGATGACTGTTGTGATGATGATGC(<u>TGA</u>
TGATGATGATGATGATGATGA)ATACGATTATGATGATGGTGA(<u>CTG</u>CTGCTGCTGCTGC)TGCTATTGC
GATCATCTACC(<u>TGA</u>TGATGATGATGATGA)TCAGCATCGAATACATCATCAGCACTAG**TAGTGGTAGTT**
GTTGGCCCAT

7. MSAT-TS1444, three alleles identified, from 114 to 120 bp
CAAACGGATACACAATAGGAAGTTGATTGGTCAAGAAATTGGAAAAAGTCATT(<u>TTC</u>TTCTTCTTCTTCT
TCTTCTTC)CAATTGTGCATTTCACCATTCCCGC**GTGCCTTGCCTTTGTTTT**

8. MSAT-TS1007, two alleles identified, 174 and 177 bp
ACCAATCGAATAGAGATATTTGAATAGTTTTAAAAAAAATACAAGTTAAATCTGATTTTCAAAGCTGAA
ATTAATTAATTAATTAATTAATTAAAA(<u>AAT</u>AATAATAATAAT)(<u>GATT</u>GATT)TGTAAAGTATTTTATTTGA
AAATAATTTCACAATGA**AAAGAAGTAGAAAGCAATTAATCA**

9. MSAT-TS1010B, eight alleles identified, from 240 to 258 bp
CATTAACGATGTGCTATTTAACGCTATATATTGACCTAAAAAGTGAAAAGTGCTTATTTCTGTTGCAAG
AGTACTTACTCAATTATTTTAAGCACATAATGTTCATGTACAAGTGGTACCAT(<u>AC</u>ACACAC)(<u>AT</u>ATATAT
ATATATATATATAT)TTGTAGAACTGTGAGTTATTAAGTTTATTGATTCAAAATGGAAAT(<u>TTG</u>TTTGT)TT
TATCATATAATCTGACGCTTTG**CGCAGATTGAAATGAAATGAATTGA**

Trichinella britovi:

1. MSAT-TS1122, one allele identified, 212 bp
GTCCCGTGGTTTAGCTTTGACATTGAAGCCGTACGTCGGGTATGTGGACCACCACCGTGTTCTGTGCTC
AATCAGCTCGTCATATTTATTCGACACCGATGGTGCCATTGTTCTGCTT(<u>CAC</u>CACCAC<u>A</u>ACCACCACCA)
GCAGCGGCAGGTAGTAGTTGCCCGAAAATTCTTGCACAACTAATATGATTCGAT**GATGCTGCTGCTGTT
ATTGG**

2. MSAT-TS128, one allele identified, 208 bp
TGTTGTCCAGTTCGGATGAAAGTGGCGGTTGCCTCTTGTCGCCATCTTTGACGCACGTTTTTGTCGCCG
GACCGGGGCAGACCAACTTCAAGTTGGCTT(<u>TTT</u>GTTTG)TTTATTTATTTGTTTATTTTGCTCATCTACTC
TCAGGGCAGCTTATTCGTTGTCAGAAGTGTGGAATTCAGTGAACGAAGT**TGTTGAAGTCCAGTGCAAG
C**

3. MSAT-TS1380, six alleles identified, from 267 to 300 bp
CGGACAAATTCAGCGGATAAGGCTTCGGTTTAGCTGGTCGCTTTTTCACCACCGGACTAGCAGGCTTTT
TCTCGCTTTGCTGTTGCTGCTGCTGTTGTTGTCCA(<u>CTATTGTGATGATGA</u>CTATTGTGATGATGA)CTGT
TG(TGATGATGA)CTGTTGTGATGATGATGATGATGATGTTGATGAATACGATTATGATGGTGGTGATT
GCTGCTGCTATTGCGATCATCTACCTGATGATGATGATGATCAGCATCGAATACATCATCAGCACTAGT
AG**TAGTGGTAGTTGTTGCCCCA**

4. MSAT-TS1444, one allele identified, 117 bp
CAAACGGATACACAATAGGAAGTTGATTGGTCAAGAAATTGGAAAAAGTCATT(<u>TTC</u>TTCTTCTTCTTCT
TCTTC)CAATTGTGCATTTCACCATTCCCGC**GTGCCTTGCCTTTGTTTT**

5. MSAT-TS1010B, five alleles identified, from 230 to 248 bp
CATTAACGATGTGCTATTTAACGCTATATATTGACCTAAAAAGTGAAAAGTGCTTATTTCTGTTGCAAG
AGTACTTACTCAATTATTTTAAGCAGATAATGTTCTTGTACAAGTGGTACCATACA(<u>AT</u>ATATATATATAT
ATATAT)TTGTAGAACTGTGAGTTATTAAGTTTATTGATTCAAAATGGAAATTTCTATTATATAATCTGAC
GCTTTG**CGCAGATTGAAATGAAATGAATTGA**

6. MSAT-TB1019, five alleles identified, from 272 to 292 bp
CCAACAACATCCTCAACATGTCCGCCTGCATACCAGTCGTAGTGATCACCGGTATATCCACAGCAGGCC
ATTTTTCGATCTGAAAAATTAAATTATTAAGA(<u>AAT</u>AATAATAATAATAAT)AAATTTATCAAAATTTATAC
TCTGCAATTTTGCATATCTAAAATGACCGCTTTCGCACCGCTATCGAATGCAAACAAAGCCTTGAAACGA
AGAAAAAACTATTTTAATAATGAAAAATTCATATAAACTGCTTGAAATTTT**GGTTACTTGATTGTAAGGT
G**

7. MSAT-TB922, ten alleles identified, from 239 to 257 bp
AGCCAACTCAAGTCCCAAAGCCGTCAAAGTACAAAGCAAAAATGTTGTCGTCATAAATACAGTGATTG
GTCGATTGCAGATAAACATGGCATATCTGAAAGTATAG(<u>TA</u>TATATATATATATATATATATATATA)CGG

ATGCAGTGTAGATTTAAAATATACAACAACACAAATTGTTCTTACTGTTTCCGAACATTTTCAAGCTGCG
TAACAGCTTTCTGAGAATAACTAACTTCAGAA**GTTGTTGCATTGCCGTTT**

8. MSAT-TB2880, five alleles identified, from 308 to 318 bp
CCGCCTGAAAATGAATAACAATTGGAAAAGATTTT(T)ATTTTAAA(<u>TTG</u>TTTGTTTGTTTGTTTGT)TGTT
TTCACATTATTTGCTTTGCAAGACAAAGATGAATACGGTTTTCTGGCCTGATTGTTACACGTTGCACTTG
TGCAA(<u>TGT</u>TGTTGTTGTTGTTGT)TTTTTGTTGTTTTGTTTGATTTTGTTAATTTGGTTAATTTTTTCGTTA
GCGAGAAAAAGAAAGCATATTTCATTGCTTTCAATGCAAAAGTGAAAGTAAAGATTATCATCATCATT
GGCATTGGTGATAAATATAT**GATTGTGTTGTTTTCCTCTG**

9. MSAT-TB1837, two alleles identified, 211 and 214 bp
ATGGCCAGTATGAAGGTGGAAACTTCACGAACTTATTTTGAAACAAAGCGCTTAATTTCACTCTAGATC
ATTTAAACGTTT(<u>ACA</u>ACA)TTTGTAACAATTGAATCAGAATGAATCACTAATAAATAATATTGATGTTTG
ATAAATTTCATTTACAATTTTCATGGCAAAATTTATGAAACAACACCCCATTAAAATA**CAGCAACCCAAG
CAAAAA**