

European Working Group for *Legionella* Infections

M13 Variant of the NESTED Sequence-Based Typing (SBT) protocol for epidemiological typing of *Legionella pneumophila* directly from clinical samples.

Version 1.0

SUMMARY

This procedure describes the use of nested Sequence-Based Typing (SBT) PCR to obtain typing data from *Legionella pneumophila* in samples with low genomic DNA content. Typically DNA extracts from clinical samples previously demonstrated to be positive for the presence of *L. pneumophila* DNA by PCR are used as the starting material. This protocol is a modification to the one recently published by Ginevra C *et al* (JCM 2009). The first round of PCR is carried out with 7 primer pairs (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) which bind externally to those used in the second round. The M13 forward and reverse primers are attached respectively to the second round forward and reverse primers. Approximately five microlitres from each of the resulting PCR mixes from the first round is used as the starting template for a second round of PCR with the internal primers. Following purification, the resulting amplicons are then sequenced with the M13 forward and reverse primers, and assigned allele numbers and sequence types (ST) in the standard way.

DNA Template:

Previously extracted genomic DNA from clinical or environmental samples using standard DNA extraction methods: e.g. the Instagene Matrix (BIO-RAD), MagNaPure Compact, Qiagen.

SBT targets:

We recommend that all 7 loci (i.e. *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*) are determined if possible.

First round nested SBT PCR amplification primers:

Gene	Primer name	Primer sequence (5'-3')	Annealing temperature
<i>flaA</i>	flaA-L-N ¹	TAT GCG TGA GCT TTC CGT TC	50 °C
	flaA-960R ²	CCA TTA ATC GTT AAG TTG TAG G	
<i>pilE</i>	pilE-L-N ¹	CGT TGG AAT CGG CTT GTC	50 °C
	pilE-R-N ¹	CGC ATT GGC AGA GGA ATC TA	
<i>asd</i>	asd-1-N ¹	CCC TGG AAG TGA ATC CTC AT	50 °C
	asd-2-N ¹	TTG CAG TAT TTC AGC GAT CTG T	
<i>mip</i>	mip-1-N ¹	TGA AGA TGA AAT TGG TGA CTG C	50 °C
	mip-2-N ¹	AAT AGG TCC GCC AAC GCT AC	
<i>mompS</i>	mompS-450F ²	TTG ACC ATG AGT GGG ATT GG	50 °C
	mompS-R-N ¹	TGG ATA AAT TAT CCA GCC GGA CTT C	
<i>proA</i>	proA-L-N ¹	CCG CTT CTC CAA CCA ATg A	50 °C
	proA-R-N ¹	CAC TCA ACA TAC CGC AAC CA	
<i>neuA</i>	neuA-F-N ¹	CCT TGC AGT CGT CTT GTT GT	50 °C
	neuA-R-N ¹	TTT CTG TTA GAG CCC AAT CG	

¹ Ginevra C *et al* JCM 2009

² The number in the primer name is the position in the reference sequence where the 3'-terminus of the oligonucleotide binds

Second round SBT PCR amplification primers:

Gene	Primer name	Primer sequence (5'-3')	Annealing temperature
<i>flaA</i>	<u>flaA-587F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>GCG TAT TGC TCA AAA TAC TG</u>	55 °C
	<u>flaA-R-N</u> ¹ (M13R)	CAGGAAACAGCTATGACC <u>GGT ATC ACC TGC GGT TCC A</u>	
<i>pilE</i>	<u>pilE-35F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>CAC AAT CGG ATG GAA CAC AAA CTA</u>	55 °C
	<u>pilE-453R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>GCT GGC GCA CTC GGT ATC T</u>	
<i>asd</i>	<u>asd-511F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>CCC TAA TTG CTC TAC CAT TCA GAT G</u>	62 °C
	<u>asd-1039R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>CGA ATG TTA TCT GCG ACT ATC CAC</u>	
<i>mip</i>	<u>mip-74F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>GCT GCA ACC GAT GCC AC</u>	60 °C
	<u>mip-595R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>CAT ATG CAA GAC CTG AGG GAA C</u>	
<i>mompS</i>	<u>mompS-509F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>GAC ATC AAT GTG AAC TGG</u>	55 °C
	<u>mompS-1015R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>CAG AAG CTG CGA AAT CAG</u>	
<i>proA</i>	<u>proA-1107F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>GAT CGC CAA TGC AAT TAG</u>	55 °C
	<u>proA-1553R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>ACC ATA ACA TCA AAA GCC</u>	
<i>neuA</i>	<u>neuA-196F</u> ² (M13F)	TGAAAACGACGGCCAGT <u>CCG TTC AAT ATG GGG CTT CAG</u>	55 °C
	<u>neuA-634R</u> ² (M13R)	CAGGAAACAGCTATGACC <u>CGA TGT CGA TGG ATT CAC TAA TAC</u>	

¹ Ginevra C *et al* JCM 2009

² The number in the primer name is the position in the reference sequence where the 3'-terminus of the oligonucleotide binds

Sequencing primers: M13F (5'- TGAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3')

Oligonucleotide storage and handling recommendations:

Appropriate storage of oligonucleotides will extend their shelf life. Most oligonucleotides are supplied dry (lyophilised) and manufacturers (e.g., MWG Biotech AG, Germany) usually supply detailed information on appropriate resuspension and storage conditions. Information on data-sheets and instructions from the manufacturers provided with the oligonucleotides should be read, noted and followed. To maximise the shelf-life of oligonucleotides it is recommended that concentrated "stock solutions" (e.g., 100pmol/ μ l) are made in 1x nuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5-8.0) and stored below -20°C, and dilute "working solutions" (e.g., 10 pmol/ μ l) made in nuclease-free 10 mM Tris pH 7.5-8.0. Working solutions are frozen in 50 μ l aliquots and should **NOT** be subjected to repeat freeze-thaw cycles and must be kept for **no longer than one week at 4°C** before discarding.

First round PCR reaction

PCR amplification is performed in a total volume of 50 μ l. Place PCR plate or tubes on a cold block. Start with adding 10 μ l of Taq DNA polymerase (2.5 units/reaction) to each PCR tube or 96 well plate, then add 30 μ l of the Master Mix, finally add the DNA template (10 μ l /tube or /well). Gently spin tubes/plates briefly in an appropriate centrifuge before placing them in a thermocycler heating block. Include a negative (10 μ l of nuclease-free water) and a positive control (1pg of *Legionella pneumophila* DNA in 10ul of nuclease-free water). Pipette solutions in the order of: negative control, test samples, positive control.

Prepare Master Mix by adding:

Reagent	Stock concentration	Final concentration	Volume/reaction (μ l)
PCR buffer	10 x	1 x	5.0
MgCl ₂	50 Mm	2.5 mM	2.5
Nested Primer 1	10 pmol/ μ l	10 pmoles	1.0
Nested Primer 2	10pmol/ μ l	10 pmoles	1.0
DNTPs	5 mM	200 μ M	2.0
Nuclease-free water			18.5
Volume/reaction			30.0

Prepare Taq DNA polymerase by adding:

Nuclease-free water	9.0 μ l
PCR buffer (x10)	0.5 μ l
Taq DNA polymerase (5U/ μ l)	0.5 μ l
Volume/reaction	10.0 μ l

Thermocycling parameters (1st Round)

Step	Temperature (°C)	Time		No. cycles
1	94	5 min	Initial denaturation	1
2	94	30 sec	Denaturation	35
	50	30 sec	Annealing	
	72	40 sec	Extension	
3	72	10 min	Final extension	1
4	12	∞	Hold	

Second round PCR reaction

PCR amplification is performed in a total volume of 50 µl. Place PCR plate or/tubes on a cold block. Start with adding 10 µl of *Taq* DNA polymerase (2.5 units/reaction) to each PCR tube/or 96 well plate, then add 35 µl of the master mix, finally add 5 µl from each of the completed PCR reaction mixes from the first round as starting template. Gently spin tubes/plates briefly in an appropriate centrifuge before placing them in a thermocycler heating block. Include a negative (5 µl of nuclease-free water). Pipette solutions in the order of: negative control, test samples, positive control.

Prepare Master Mix by adding:

Reagent	Stock concentration	Final concentration	Volume/reaction (µl)
PCR buffer	10 x	1 x	5.0
MgCl ₂	50 Mm	2.5 mM	2.5
Std Primer 1	10 pmol/µl	10 pmoles	1.0
Std Primer 2	10pmol/µl	10 pmoles	1.0
dNTPs	5 mM	200µM	2.0
Nuclease-free water			23.5
Volume/reaction			35.0

Prepare *Taq* DNA polymerase by adding:

Nuclease-free water	9.0 µl
PCR buffer (x10)	0.5 µl
<i>Taq</i> DNA polymerase (5U/µl)	0.5 µl
Volume/reaction	10.0 µl

Thermocycling parameters (2nd Round)

Step	Temperature (°C)	Time		No. cycles
1	94	5 min	Initial denaturation	1
2	94	30 sec	Denaturation	35
	55, 60 or 62	30 sec	Annealing	
	72	40 sec	Extension	
3	72	10 min	Final extension	1
4	12	∞	Hold	

Analysis of purified PCR products by E-Gel electrophoresis

Run PCR products (2 µl) + 1x BlueJuice (Invitrogen) (18 µl). Also run E-Gel low range quantitative DNA ladder (Invitrogen) (10 µl) + nuclease free water (10 µl). After 20 minutes of run, the E-Gel is analysed using the UVP gel imaging system. If positive and negative controls as well as the test sample results are satisfactory, proceed with the DNA sequencing reaction.

DNA Sequencing using the CEQ 8000 Genetic Analysis System (Beckman Coulter)

Purification of PCR products

PCR products are purified using the Millipore MultiScreen Separation System.

DNA Sequencing materials

Dye Terminator Cycle Sequencing is undertaken using the DTCS Quick Start Kit (Beckman Coulter). Follow the manufactures instructions carefully. The Sample Loading Solution contains formamide. Formamide waste (i.e., sequencing reactions and plates) should **NOT** be autoclaved and must be disposed for direct incineration.

The CEQ DTCS Quick Start Kit contains the following reagents (sufficient for 100 reactions):

- 1) Quick Start Mix (800 µl)
dATP, dCTP, dTTP, dITP
ddUTP, ddGTP, ddCTP, ddATP (WellRED label)
Tris-HCl, MgCl₂, reaction buffer - pH 8.9
Thermo Sequenase DNA Polymerase
Pyrophosphatase
- 2) 47 Sequencing Primer (240 µl)
- 3) pUC18 Control Template (20 µl)
- 4) Glycogen (110 µl)
- 5) Mineral Oil (5 ml)
- 6) Sample Loading Solution (SLS) 6 ml

DNA Sequencing procedure

DNA sequencing reactions are prepared **following the manufacturers instructions** by combining the following:

- 1) PCR-grade water (e.g., Nuclease-Free Water, W4502, Sigma)
- 2) DNA template
- 3) User supplied sequencing primer or (-) 47 Sequencing Primer (1.6 pmol/µl or 1.6µM)
- 4) DTCS Premix (Beckman Coulter)

N.B. Can run half-volume reactions (total of 10µl/reaction).

Thermocycler parameters (for DNA sequencing):

Step	Temperature (°C)	Time		No. cycles
1	96	20 sec	Denaturation	30
	50	20 sec	Annealing	
	60	4 min	Extension	
2	4	∞	Hold	

N.B. The above parameters are those recommended for the CEQ 8000 Genetic Analysis System (Beckman Coulter). For alternative sequencing platforms the relevant manufacturer's recommendations should be followed.

Ethanol precipitation is carried out as per manufacturer's instructions using a suitable centrifuge. For ethanol plate precipitation in a CEQ sample plate, please refer to the "Dye terminator cycle sequencing chemistry protocol", page 13. For ethanol precipitation in 1.5 ml eppendorf tubes, please refer to the manufacturer's instructions provided with the DTCS Quick Start Kit (Beckman Coulter).

N.B. for half volume reactions add 10 µl of H₂O, before proceeding to ethanol precipitation.

Sample preparation for loading into the CEQ

Add 55 µl of Sample Loading Solution to each well, leave on plate-shaker for 10 minutes. Overlay each re-suspended sample with one-drop of mineral oil.

From this point follow the instructions provided by the manufacturer of the Sequencing Instrument.

Sequence analysis:

EWGLI Sequence Quality Tool

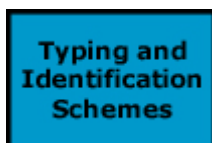
Forward and reverse sequence trace files (.scf or .abi) of all seven targets are submitted to the “**Sequence Quality Tool**” which can be accessed from the EWGLI website (www.ewgli.org). This tool assembles contig(s) from the traces, finds start and end (reference) positions in the contig, trims the contig using these positions and finally matches the trimmed sequence against those in the SBT database. The tool identifies individual alleles as well as an allelic profile and a sequence type (ST). Sequences with <100% match are identified as -1, and the position of mismatches are also indicated. Sequences of poor quality should either be repeated or further analysed using a DNA analysis software e.g., BioNumerics (Applied Maths).

EWGLI SBT Database

- Data from both forward and reverse sequencing reactions are combined and aligned to produce a consensus sequence. Reference sequences trimmed to the correct length can be downloaded from the website in order to aid contig assembly. Consensus sequences trimmed to the correct length are submitted to the SBT database as flat text file. The database returns an allele type eg., 1, when submitted sequence shows a 100% match to a pre-designated allele type. Sequences with <100% match are identified as the closest match to a pre-existing allele type with the number of mismatches specified. The SBT database also returns an alignment with mismatches highlighted.
- For each isolate, the combination of alleles at each of the loci is defined as the allelic profile using a pre-determined order, i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuA*. For example, for strain EUL no. 120, the allelic profile is 4,7,11,3,11,12,9.
- If an individual allele number has not been determined, a zero is entered into the allelic profile, thus maintaining its integrity. For example, if the *proA* allele number was not determined for the examples above, the profile would be 4,7,11,3,11,0,9 and if the *mompS* allele was not determined, it would be 4,7,11,3,0,12,9.

Instructions for accessing the EWGLI SBT Website

- Go to (www.ewgli.org)
- Click the “**Typing and identification schemes**” button:



Follow the link for the EWGLI Sequence-Based Typing (SBT) Database for *Legionella pneumophila*

Instructions for submitting sequence data to the EWGLI Sequence Quality Tool

- Under Query Functions, click on the “**Sequence Quality Tool**”
- Choose the number of sequence trace files to be uploaded.
- Click on the “**Browse**” button to download sequence files.
- Forward and reverse sequences must be downloaded for each allele in standard file format (*.scf) or ABI trace file format (*.abi).
- Click the “**Analyse Trace Files**” button.
- The tool also produces a sequence quality report for each uploaded contig and generates a seven-figure allelic profile (e.g., 3,4,1,1,14,9,1), as well as indicating whether the obtained allelic profile is of a novel combination. The tool also identifies a single designated sequence type (ST) for a pre-existing allelic profile.
- You can print a summary sheet by clicking the link: “**Print Friendly Summary**”
- For further details on the sequence quality tool please click on this link: “**explanation and frequently asked questions**”
- Putative novel alleles must be submitted for verification following the link “**New Allele Submission**”.
- To submit a novel allelic profile click on the link “**Strain Data Submission**”.

Instructions for submitting sequence data to the EWGLI SBT Database

- Under Query Functions, click on the “**Check a sequence for it’s allele number**”
- Before pasting in a query sequence the user must ensure that the consensus sequence is trimmed to the correct length.
- Reference sequences trimmed to the correct position and length can be downloaded to aid contig assembly.
- Consensus sequences of the correct length can be submitted for more than one target, alternatively you can submit consensus sequences one at a time before hitting the “**upload sequence**” button.
- Please note that sequences of incorrect length will be rejected.
- Sequences of correct length and with a 100% match to a sequence in the database will return an allele number e.g., “1”.
- Sequences of correct length and with <100% match to a sequence in the database will return an alignment with the closest matches showing where differences are.
- To enter consensus sequences for another strain, click the “**Reset form**” button and proceed as before.

N.B. The website is under constant development, so please see the website for any changes, new tools and to ensure you are using the latest version of this protocol etc.

References

Ginevra C, Lopez M, Forey F, Reyrolle M, Meugnier H, Vandenesch F, Etienne J, Jarraud S, Molmeret M (2009). Evaluation of a nested-PCR-derived sequence-based typing method applied directly to respiratory samples from patients with Legionnaires' disease. *J Clin Microbiol.* **47**:981-7.

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