



# ESGLI

ESCMID STUDY GROUP  
FOR LEGIONELLA INFECTIONS

European Society of Clinical Microbiology and Infectious Diseases

## **Analysis of the *N*-Acylneuraminate Cytidyltransferase homologue (*neuAh*) found in some non-serogroup 1 strains of *Legionella pneumophila***

**VERSION 1.0**

### **SUMMARY**

This procedure describes the European Legionnaires' Disease Surveillance Network (ELDSNet) method for the analysis of the *N*-Acylneuraminate Cytidyltransferase homolog (*neuAh*) found in some non-serogroup 1 (non-sg1) strains of *Legionella pneumophila*. This homologue has been described by Farhat *et al.* (2011) and is present in non-sg1 strains when *neuA* is not amplified with the standard *neuA* primers in the Sequence-based Typing protocol. New primers were designed (unpublished data) in order to improve the quality of the sequences obtained from that of Farhat *et al.* (2011). Some strains belonging to *L. pneumophila* sg11 remain refractory to amplification with these new and the original primers.

Genomic DNA is extracted and then amplified using primers targeting *neuAh*. Following purification, amplicons are sequenced and the resulting consensus sequences trimmed and compared to previously assigned *neuAh* allele numbers using the *L. pneumophila* Sequence Quality Tool (SQT) hosted by the HPA server ([www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq\\_assemble\\_legionella1.cgi](http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi)).

The *neuAh* allele result can be used in place of the *neuA* result according to the pre-determined SBT order (i.e., *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, *neuAh*), the combination of alleles is defined as 7-digit allelic profile (e.g. 11,14,16,18,15,13,201) and a Sequence Type (ST) represented by a number (e.g., ST1282). In order to quickly differentiate between *neuA* and *neuAh* alleles, the *neuAh* nomenclature starts from 201. Putative new allele types can be submitted via the "New Allele Submission" link from the EWGLI SBT database ([www.hpa-bioinfotools.org.uk/legionella/php/sbt\\_query1.php](http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1.php)). Putative new sequence types can be submitted via the "Submit a new profile" link from the EWGLI SBT database ([www.hpabioinfotools.org.uk/legionella/php/sbt\\_query1.php](http://www.hpabioinfotools.org.uk/legionella/php/sbt_query1.php)).

This method can be used in the epidemiological typing of *L. pneumophila* isolates.

## Legionella cultures

Procedures which involve handling of live microorganisms in liquid suspensions (likely to create aerosols) must be carried out in a Microbiological Safety Cabinet wearing latex gloves. Prior to SBT, *Legionella pneumophila* growth must be harvested from plates or slopes using a sterile loop and gently transferring 1-2 loopfuls into a labelled 1.5 ml microcentrifuge tube containing ca. 1 ml sterile water. Cap tube. Using a centrifuge with sealed rotor, centrifuge the tube at 15,000 ×g for ca. 10 minutes to pellet cells. In the Class 1 Safety Cabinet, open tube, remove and discard supernatant. Cell pellets may be processed directly or stored at -70 to -80°C.

## DNA EXTRACTION

The following methods have been found to be reliable and the first two are rapid:

1. BIO-RAD Instagene Matrix (Catalogue n. 732-6030), using 1-5 µl of supernatant as template DNA in the primary PCR amplification.
2. Heating emulsified colonies in 0.5 ml of sterile water at 100°C for 8 min, using 1-5 µl of supernatant as template DNA in the primary PCR amplification.
3. Nucleon BACC2 DNA extraction kit (Gen-Probe), using 10-100 ng DNA as template DNA in the primary PCR amplification.

### neuAh primers:

Primer name	Primer sequence (5'-3')	Expected Fragment size (bp)
neuAh_L	ATCCAGCAGTTTTTAMAAATTTAGG	791-794*
neuAh_R	TGGCTGCATAAAYTAATTCTTTAGCCA	

\*due to the INDEL of a GAA triplet.

The above primers are used for both amplification and sequencing reactions.

### M13-modified neuAh primers

Primer name	Primer sequence (5'-3')	Expected Fragment size (bp)
neuAh_L (M13F)	TGTA AACGACGGCCAGTATCCAGCAGTTTTTAMAAATTTAGG	827-830*
neuAh_R (M13R)	CAGGAAACAGCTATGACCTGGCTGCATAAAYTAATTCTTTAGCCA	

\*due to the INDEL of a GAA triplet.

The above primers are used for amplification reactions, while the following primers are used for sequencing reactions:

- M13F (5'-TGTA AACGACGGCCAGT-3');
- M13R (5'-CAGGAAACAGCTATGACC-3').

## Reference sequences and regions used for allele assignment:

Target	Amplicon size (bp)	GenBank accession no.	Target region for allele assignment	Size of target region (bp)
<i>neuAh</i>	794	FN256429	229-582	354
<i>neuAh</i>	791	FR750546	229-579	351

## Oligonucleotide storage and handling recommendations

Appropriate storage of oligonucleotides will extend their shelf life. Most oligonucleotides are supplied dry (lyophilised) and manufacturers 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 “stock solutions” (e.g., 100 pmol/μl) 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 working solutions (e.g., 10 pmol/μl) are made in 1x nuclease-free Tris (10 mM Tris pH 7.5-8.0). Working solutions are frozen in 10-50 μl 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. Working solutions for primers used for sequencing reactions should be made up fresh on the day of use, then discarded.

## Primary amplification

PCR amplification is performed in a final volume of 20 μl. Place PCR plate on a cold block and add to each well 18 μl of the Reaction Mix, then add 2 μl of the relevant template. Pipette solutions in the order of: negative control, test samples, positive control. Gently centrifuge plates briefly in an appropriate centrifuge before placing them in the thermal cycler.

Reaction Mix:

Reagent	Stock concentration	Final concentration	Volume/reaction (μl)
Nuclease-free water			13.2
PCR buffer	10 x	1 x	2.0
MgCl <sub>2</sub>	50 mM	2.5 mM	1.0
Primer F	10 pmol/μl	4 pmoles	0.4
Primer R	10 pmol/μl	4 pmoles	0.4
dNTPs	5 mM	200 μM	0.8
Taq DNA polymerase	(5U/μl)		0.2
Volume/reaction			18.0

## Controls

For each PCR run include a negative (2 µl of nuclease-free water) and a positive (2 µl of known *L. pneumophila* DNA template) control. Pipette solutions in the order of: negative control, test samples, positive control.

### Thermal cycler parameters

Step	Temperature (°C)	Time		No. cycles
1	95	5 min	Initial denaturation	1
2	95	30 sec	Denaturation	35
	55	30 sec	Annealing	
	72	60 sec	Extension	
3	72	10 min	Final Extension	1
4	12	∞	Hold	

### Analysis of PCR products by gel electrophoresis

Run PCR products (2 µl + 18 µl of 1x loading buffer) with a low range quantitative DNA ladder on 1-2% agarose gel, then analyse results using a UV light system. If positive and negative controls as well as the test sample results are satisfactory, proceed with the DNA sequencing procedure.

### DNA Sequencing

PCR products can be purified and sequenced in-house according to the instructions provided by the manufacturer of the Sequencing Instrument or sent to a commercial sequencing provider.

## INTERPRETATION OF RESULTS

### Sequence Quality Tool

Forward and reverse sequence trace files (.scf or .abi) are submitted to the “Sequence Quality Tool” ([www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq\\_assemble\\_legionella1.cgi](http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi)). 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).

### SBT Database

([www.hpa-bioinformatics.org.uk/legionella/legionella\\_sbt/php/sbt\\_homepage.php](http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php))

- 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*, *neuAh*. For example, for strain EULV no. 0194, the allelic profile is 11,14,16,18,15,13,201.
- 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 11,14,16,18,15,0,201 and if the *mompS* allele was not determined, it would be 11,14,16,18,0,13,201.

### 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.

### **New Allele Submission**

A new allele must be submitted via the [New Allele Submission](http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1.php) link from the EWGLI SBT database ([http://www.hpa-bioinfotools.org.uk/legionella/php/sbt\\_query1.php](http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1.php)).

This link allows you to submit forward and reverse sequence trace files to an automated sequence quality tool. If your sequences successfully pass the criteria, you will be asked to submit your data to the curators via the same link.

### **New Profile Submission**

Once a new allele has been submitted the complete profile of the strain must also be submitted via the [Submit a new profile](http://www.hpabiointools.org.uk/legionella/php/sbt_query1.php) link, from the EWGLI SBT database page ([http://www.hpabiointools.org.uk/legionella/php/sbt\\_query1.php](http://www.hpabiointools.org.uk/legionella/php/sbt_query1.php)).

**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

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