National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Protocol for Capsular Sequence Typing of *Streptococcus pneumoniae*

Version: CST-Spn-01-2014

Principle

Capsular Sequence Typing (CST) is based on a part of the sequence of the *wzh* gene of the capsular locus. The 3 forward and 4 reverse primers (Table 1) used in the CST are based on the publicly available sequences of the capsular genes of the 90 known pneumococcal serotypes described by Bentley et al. (*2006 PLoS Genet 2:e31.*). The primers contain an M13-tail, which facilitates sequencing with universal M13-primers. After sequencing of 506 base pairs of the *wzh* gene, a capsular type (CT) can be assigned. The CT is a composite assignment; the first part of the assignment is based on the phenotype assessed by conventional serotyping and the second part of the assignment is the consecutive number of the capsular type belonging to the same serotype. As an example, CT09V-01 designated the first variant in *wzh* sequence of an isolate serotyped as 9V.

Reagents, equipment and software

- Tris-EDTA buffer (TE), 10 mM Tris.HCl, 1 mM EDTA pH 8.0 (store at room temperature)
- Unlabeled oligonucleotide primers (e.g. Eurogentec, Seraing, Belgium, store at 4°C)
- Qiagen Hotstartaq Mastermix PCR kit (Qiagen, Hilden, Germany; Art. No. 203445, store at -20°C)
- MilliQ water (Water purified by the milliQ system, Millipore, Billerica, USA)
- Exo-sap it (GE Healthcare Life Sciences (Catalogus # US78202)
- Nucleic Acid Stain (e.g. Gelred Nucleic Acid Stain, Biotium, Catalogus # 41002-0,5mL)
- Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, CS, USA)
- Sequencebuffer, 200 mM Tris·HCl, 5 mM MgCl2 pH 9 (store at room temperature)
- PCR machine (e.g. Applied Biosystems GeneAmp PCR System 9700)
- Electrophorese unit (e.g. I-MyRun Gel system, Cosmo Bio, Carlsbad, CA, USA)
- Automated DNA sequencer (e.g. Applied Biosystems 3730 DNA analyzer)

Optional: Bionumerics software (v.5.1 or higher, Applied Maths, Sint-Martens-Latem, Belgium)

Source of DNA

CST can be performed using 10 ng purified genomic DNA. However, the procedure has been optimized for use with bacterial lysates. A loop full of colonies from cultures grown overnight on Columbia agar plates with 5% sheep blood at 37°C are suspended in 500 µl TE and heated for 10 min at 95ºC. After the inactivation the lysate is used either directly or stored at -20°C until use in PCR.

PCR

PCR is performed in 20-µl volumes. For a PCR 5 µl DNA (10 ng) or 5 µl *S. pneumoniae* lysate (diluted 1:10 in MilliQ water) is added to a mixture containing Qiagen HotStarTaq Mastermix, and the primermix as indicated in Table 2.

Table 1. CST primer sequences

Primer name	Primer sequence
CST 01-M13F	GTAAAACGACGGCCAGCATTCGCATATCGTTTTTG
CST 02-M13F	GTAAAACGACGGCCAGCATTCTCACATTATTTTTGATGT
CST 03-M13F	GTAAAACGACGGCCAGCATTCGCACATCGTCTTTG
CST 01-M13R	CAGGAAACAGCTATGACCTGAGCTCTTTTTTTCATGA
CST 02-M13R	CAGGAAACAGCTATGACGTGAACTCGTTTCTTCATGA
CST 03-M13R	CAGGAAACAGCTATGACCCGAGCTCTCTTTTTCATAA
CST 04-M13R	CAGGAAACAGCTATGACCCGAGCTCTCTTTTTCATGA

Table 2. The composition of the PCR mixture

Component	Amount (µl)
Primermix*	1.0
HotStar Mastermix	10.0
MilliQ water	4.0
Lysate or DNA	5.0
Total	20.0

* All primers are mixed and have a 10 µM concentration within the primermix. For example, add 1 µl of all primers (7 µl in total) and add 3 µl of MilliQ water.

Table 3. PCR program

Time	Temperature	Cycles
15 min.	95°C	1
20 sec.	95°C	
30 sec.	51°C	35
30 sec.	72°C	
7 min.	72°C	1
Hold	4°C	1

Gel-electrophoresis

PCR product may be checked on agarose gel-eletrophoresis.
Analyse 5 µl of PCR product on a 2% standard agarose gel in ½x TBE using GelRed 1/10000.
Run for 30 minutes on gel system.

Clean-up of the PCR product

5 µl of PCR product is treated with 2 µl ExoSAP-IT reagent.
In a PCR machine, run the following program:
15 min 37°C
15 min 80°C

Sequence reaction

The sequence reaction is performed in 20-µl volumes. For a sequence reaction 5-10 ng PCR product (≈ 1 µl) is added to a mixture containing Big Dye Terminator, sequence buffer and primer as indicated in Table 5.

Table 4. CST sequence primers

Primer	Primer sequence
M13F(-20)	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC

Table 5. The composition of the sequence mixture

Component	Amount (µl)
Primer F or R*	1.0
Big Dye Terminator	1.0
Sequence buffer	7.0
MilliQ water	10.0
Lysate or DNA	1.0
Total	20.0

* Primer concentration is 5 µM

Table 6. Sequence program

Time	Temperature	Cycles
1 min.	96°C	1
10 sec.	96°C	
5 sec.	50°C	25
4 min.	60°C	
Hold	4°C	1

Sequence purification

The sequence reaction is purified using the Big DYe X Terminator kit according to manufacturer's protocol. The sequences are analysed using the AB 3730 DNA analyzer.

Analyses of the sequence

Sequencing on the DNA sequencer results in .AB1 files, which can be imported, stored and analysed in BioNumerics. Trimming sequences are used to make the sequences the correct length.

Trimming sequences are:

Forward	GGBCCMAARTCMAKR	Tolerance : 2	Offset : -12
Reversed	KMHARAWTYATGAAAAA	Tolerance : 0	Offset : 9

The sequences can be uploaded to the online typing tool at www.rivm.nl/mpf/spn/cst to automatic assign a Capsular Type to a sequence. A detailed tutorial can be found at the website.

New alleles

PCR product that do not yield a known sequence are assigned non-typable (NT). A new allele is confirmed by the repeat of the assay and is assigned the consecutive number of capsular type belonging to the same serotype by the curator.

Additional remarks

For high throughput, PCRs can be performed in 96 well PCR trays (e.g. Greiner, Art. No. 652280).