Identification of PBP3 mutations
in invasive *Haemophilus influenzae* isolates

I. DNA extraction

Extraction is achieved using a heating block, pre-set to 100°C.

1. Aliquot 200 µl sterile DNAse-free distilled water into a labelled 1.5 ml Eppendorf tube.
2. Resuspend 1-3 well isolated colonies from an overnight culture.
3. Transfer the tube to the heating block and boil for 10 min, then cool down at -20°C for 10 min and centrifuge for 1 min at 11,000 g.
4. Transfer the supernatant into a second appropriately labelled tube. Discard the pellet.
5. This DNA extract can be used immediately or stored a -20°C until required.

II. PCR amplification

PCR tailed-primers

Name	Sequence (5'-3')*	*ftsI* target sequence**
ftsI1F	GTTTCCCAGTCACGACGTTGTAGTTAATGCGTAACCGTGCAATTAC	936-960
ftsI1R	TTGTGAGCGGATAACAATTTCACCACTAATGCATAACGAGGATC	1640-1617

* Includes non-complementary 5'-end adapter tail (green lettering) for further sequencing.
** Nucleotide numbering refers to that of the *ftsI* sequence of *H. influenzae* Rd KW20 (ATCC 51907), accession no. L_{42023}

Primer stock solutions, 100 µM in TE buffer, kept at -20°C
Primer working dilutions, 10 µM in TE buffer

PCR reaction mix:

	per reaction
H_2O	31.2 µl
dNTPs (5 µM of each)	2 µl
$MgCl_2$ (25 mM)	**5*** µl
ftsI1F (10 µM)	2 µl
ftsI1R (10 µM)	2 µl
High-fidelity Taq polymerase**	0.3 µl
10 x corresponding buffer	5 µl
DNA extract	5 µl

* final concentration in the mix, 2.5 µM (*i.e.*, 125 nM per reaction). If PCR buffer already contains $MgCl_2$, adjust concentration to 2.5 µM.
***e.g.* AmpliTaq Gold DNA polymerase (Roche).

PCR cycle:

3 min, 94°C (denaturation), then:
   10 cycles
         30 sec, 94°C (denaturation)
         30sec, **50°C** (annealing)
         40 sec, 72°C (extension)
   30 cycles
         30 sec, 94°C (denaturation)
         30 sec, **60°C** (annealing)
         40 sec, 72°C (extension)
End with 1 cycle of 5 minutes at 72°C
   Once the programme has finished the samples can be run on an electrophoresis
   gel immediately or stored at 2-8°C.

Expected size of the amplified fragment (*fstl*$_{int}$) : **705 bp**.

IV. Purification of PCR products

Use a PCR fragment clean-up kit (*e.g.*, NucleoSpin$^{®}$ gel and PCR clean-up kit,
Macherey-Nagel).
Elution volume of purified PCR product, 15-30 µl.

V. Sequencing

Sequencing primers

Name	Sequence (5'-3')
SeqftsF	GTTTTCCCAGTCACGACGTTGTA
SeqftsR	TTGTGAGCGGATAACAATTTC

Primer working dilutions, 1 µM in water

Sequencing reaction using BigDye® Terminator v3.1 cycle sequencing kit

	per reaction
Water	7.8 µl
SeqftsF *or* SeqftsR (1 µM)	3.2 µl
5X buffer	3 µl
BigDye v3.1	2 µl

Add 4 µl of PCR product purification eluate (minimum DNA amount, 20 ng)

Cycling program
     1 min, 96 °C, then:
          25 cycles
               10 sec, 96°C
               5 sec, 50°C
               4 min, 60°C
     Cool down to 4°C

Purification of sequencing products

Use a clean-up kit (*e.g.*, NucleoSEQ®, Macherey-Nagel).

VI. Sequence analysis and data submission

*fstI*int sequences will be compared to that of reference strain Rd KW20.
Before uploading to the web site, sequences must be trimmed to a 621-bp fragment corresponding to nucleotides 977-1597, as shown:

Complete sequence including the **ftsI1F** and **ftsI1R** primers :

GTTAATGCGTAACCGTGCAATTACCGATACTTTTGAGCCA**GGT**TCTACGGTAAAACCTTTCGTTGTTTTAACC GCACTTCAACGAGGTGTAGTTAAACGAGATGAAATTATTGATACTACGTCCTTTAAATTAAGCGGTAAAGAAAT TGTGGACGTTGCACCACGTGCTCAGCAAACTTTAGACGAGATTTTAATGAACTCTAGTAACCGTGGTGTAAGT CGTCTTGCATTACGTATGCCACCTAGTGCATTAATGGAAACTTATCAAAATGCAGGTTTAAGTAAACCGACAG ATTTAGGCTTGATCGGAGAGCAAGTTGGGATTTTGAATGCAAATCGTAAACGCTGGGCAGATATTGAGCGTG CAACAGTCGCTTATGGTTATGGTATTACTGCGACACCTTTACAAATTGCTCGTGCCTATGCAACCCTTGGTAG TTTCGGTGTTTATCGTCCGCTTTCTATCACTAAAGTTGATCCGCCAGTTATTGGGAAACGGGTTTTCTCTGAAA AAATAACTAAAGATATTGTGGGAATTTTAGAGAAAGTAGCAATTAAAAATAAACGCGCAATGGTGGAAGGCTA CCGTGTCGGCGTAAAAACAGGTACGGCACGTAAGATTGAAAATGGACATTATGTAAATAAGTATGTGGCATTT **ACT**GCGGGTATTGCACCAATTAGTGA**TCCTCGTTATGCATTAGTGGT**

Deduced peptide sequence of the 621-bp fragment (in red, amino acids most frequently substituted in isolates with reduced susceptibility to β-lactams):

326GSTVKPFVVLTALQRGVVKRDEII**D**TTSFKL**S**GKEIVDVAPR**A**QQTLDEIL**M**NSSNRGV**S**RLAL**L**RMP PSALMETYQNAGLSKPTDLGLIGEQVGILNANRKRWADIERATV**A**YGYGITATPLQ**I**ARAYATLGSFGV YRPLSITKVDPPVIGKRVFSEKITKDIV**G**ILEKVAIKNKR**A**MVEGYRVGVKTGTA**R**KIENGHYV**N**KYVAF T**532**

Upload protocol, web access :

Participants will submit their strain information and *fstI* sequence to a PubMLST.org database for *H. influenzae* (to be created soon). Information and upload protocol will be made available on the web site. In order to maintain the quality of the data, the database will be curated and submissions will be checked before entry.

For any questions, please contact Olivier Gaillot, olivier.gaillot@chru-lille.fr