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National Food Institute

European Union Reference Laboratory Antimicrobial Resistance

PROTOCOL FOR PCR AMPLIFICATION OF *E. FAECIUM AND E. FAECALIS*

RECOMMENDED BY THE EURL-AR

3RD VERSION - JANUARY 2014

Changes from previous version: We have added a third pair of primers to amplify the 16S rDNA region and to serve as a positive control for the amplification of the DNA sample in the PCR. Please note the change in volumes of primers mixes by adding the third primer pair to the set-up and the compensating change in the volume of water. As a consequence of this change, the results should be validated in function of the amplification of the specific fragments and of the internal control that should always be amplified. To be able to better distinguish the amplified fragments, the electrophoresis conditions have been changed to 2% agarose gel run at 110V for 90 min.

Background:

The speciation of Enterococci is important for the strain characterization and also for the choice of the right breakpoints to determine the antimicrobial susceptibility. The multiplex PCR recommended in this protocol has been reduced to the identification of *Enterococcus faecalis* and *E. faecium* which can be used in combination with the EQAS and monitoring programs. In this PCR protocol, the identification of these two enterococci species is based on the amplification of $ddl_{E\ feacalis}$ and $ddl_{E\ faecium}$ (D-Ala:D-Ala ligases) specific for each of the species. In addition, the initial description by Dutka-Malen et al. 1995 contains additional primers for vancomycin resistance determinant detection which have not been included in this simplified protocol. In addition, a 16S primer set has been included as quality assurance of the DNA-preparation and analysis (internal control).

Protocol

DNA extraction (boiling lysates):

- Dissolve a single colony into 100 µl TE 10:1
- Boil the tube for 10 minutes.
- Centrifuge at 20.000 g (4 °C) for 5min.
- Dilute the sample 1:10 in TE 10:1
- Store DNA samples at -20°C.

PCR Controls:

E. faecalis - ATCC 29212

E. faecium - BM4147

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Preparation of primers:
At the EURL-AR we use a working concentration of primers of 130mg/ml from which we use 0.25 µl of each of the primers per reaction (it corresponds to circa 10 pmol per reaction).

Reaction mix:

Prepare the following mix in a microcentrifuge tube (for a 25 µl reaction). Prepare additionally one blank reaction without template DNA as negative control. This PCR at our laboratory is optimized for the VWR taq (containing 5 Units per µl) and the Extra buffer containing 15 mM $MgCl_2$ (1.5 mM in the final reaction) which can be replaced by another polymerase, although the protocol might need some optimization to adjust for the particular conditions at your laboratories. If you need assistance with the optimization, you are welcome to contact us.

- PCR-buffer (Extra Buffer)
- 10 mM dNTP (2.5mM each)
- Primer 1 (forward primers)
- Primer 2 (reverse primers)
- VWR Taq-polymerase (0.5 units)
- Destillled mili-Q water up to 25 µl

Template:

As template for the PCR we recommend to use 5 µl of the above prepared DNA in a 25µl PCR reaction.

Primers used in this PCR:

Target gene	Primer name (# EURL)	Sequence	Fragment amplified
ddl$_{E.\ faecalis}$ (98-116)	E1 (1551)	5'–ATCAAGTACAGTTAGTCTT-3'	941 bp
ddl$_{E.\ faecalis}$ (1038-1021)	E2 (1552)	5'-ACGATTCAAAGCTAACTG-3'	
ddl$_{E.\ faecium}$	F1 (1553)	5'-GCAAGGCTTCTTAGAGA-3'	550 bp
ddl$_{E.\ faecium}$	F2 (1554)	5'-CATCGTGTAAGCTAACTTC-3'	
16S	16S primer 804 RX (442)	5'-GACTACCNGGGTATCTAATCC-3'	800 bp
16S	16S primer 10FX (444)	5'-AGAGTTTGATCCTGGCTNAG-3'	

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Electrophoresis:

Run 5-8µl of the PCR with 2 µl loading buffer. Run in parallel with a 100bp ladder molecular weight marker on a 2% agarose gel in TBE 1X. Run for 90 min at about 110V.

Stain the gel in ethidium bromide circa 20-30min.

De-stain briefly in milliQ water.

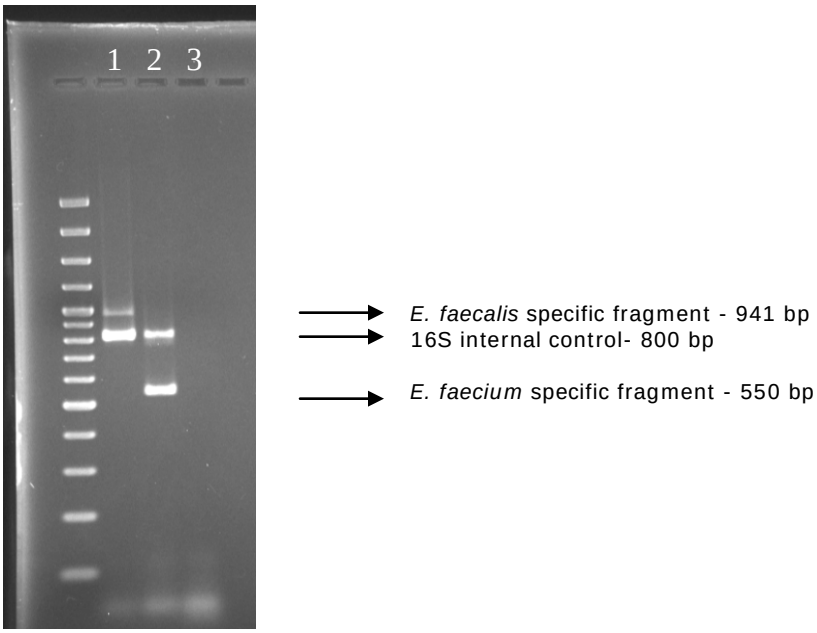
Take a picture in the transilluminator under UV light. Observe the bands and interpret the results according to the description below and Figure 1:

Figure 1. Multiplex PCR for detection of *E. faecalis and E. faecium*.

Lane 1: *E. faecalis ATCC 29212*

Lane 2: *E. faecium* BM4147

Lane 3: Negative control (Mastermix)



Reference:

Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995 Jan;33(1):24-7.

(with the above mentioned modifications to the original protocol)

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PCR SAMPLE SHEET **Enterococci**

Primer 1 (forward): 1551+1553+442
Primer 2 (reverse): 1552+1554+444
DNA polymerase: VWR taq
PCR products: *EF* (941 bp); *EFM* (550bp); 16S (800bp)
Remarks: 5 µl of the DNA template. Run: 2% agarose gel run at 110V for 90 min. **Reference:** Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995 Jan;33(1):24-7.

No. of reactions	1	**4**
PCR H_2O	15,625	**62,5**
10X PCR Extra Buffer	2,5	**10**
dNTP	0,25	**1**
25 mM $MgCl_2$	0	**0**
Primer1 (0,25 µl of each)	0,75	**3**
Primer2 (0,25 µl of each)	0,75	**3**
Taq polymerase	0,125	**0,5**
Total volume	20	**80**

1. 5 min at 94 °C
2. 30 Cycles
   - 30 sec at 94 °C
   - 90 sec at 50 °C
   - 60 sec at 72 °C
3. 10 min at 72 °C
4. hold at 4 °C

M:	100 bp Plus				
1	E. faecalis ATCC 29212				
2	E. faecium BM 4147				
3	Mastermix				
4					
5					
6					
7					
8					
9					
10					
11					
12					