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Real-time PCR bacterial DNA detection

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We use this protocol and it's working

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Abstract

The identification of bacterial species through molecular techniques is faster and more accurate than phenotypic identification. Analysis of the 16S rRNA gene can potentially be applied to identify a large number of bacteria. The use of universal primers designed for the sequence of the 16S rRNA gene, a conserved region among Eubacteria, allows for an initial approach to their identification by amplifying this region and subsequently sequencing the amplified fragment. Most of the time, identification is possible at the species level; however, in some cases, identification is only done at the genus level, thus requiring the use of specific primers for a more comprehensive identification. The primers used in this assay amplify all genera of Eubacteria, except for the genera *Rickettsia*, *Coxiella*, and *Mycoplasma*, as well as genera belonging to the Phyla *Spirochaetes* and *Chlamydiae*.

Materials

LABWARE:
Eppendorf-type microtubes
Glass capillaries for LightCycler 20ul
Tube racks
Calibrated micropipettes (P10 or P20, P200, and P1000)
Sterile micropipette tips with filters
Cold block for capillaries

REAGENTS:
Primer 16s F (5' AGA GTT TGA TCM TGG CTC AG 3') (20 uM)
Primer 16s R (5' GTA AGG TTC TKC GCG TTGC 3') (20 uM) (amplify a 972 bp fragment)
MgCl2 (25 mM stock solution)
LC Fast Start DNA Master SYBR Green (Roche Diagnostics)
Ultra-pure distilled water free of DNAses and RNAses

EQUIPMENT:
Real-time Roche LightCycler thermocycler
Workstation or Class II laminar flow cabinet with Microcentrifuge and UV lamp
Nucleotide-nucleotide BLAST database - National Centre for Biotechnology Information

Troubleshooting

Reagent preparation

1 Rehydrate the primers with ultra-pure water free of DNAses and RNAses to obtain a concentration of 100uM. Make a 1/5 dilution to achieve a working concentration of 20uM. Prepare aliquots according to the working volume and freeze at -20°C.

2 The MgCl2 supplied in the LC Fast Start DNA Master SYBR Green Kit is ready to use and should be stored frozen at -20°C. Upon first use, thaw it, prepare aliquots according to the working volume, and store at -20°C.

3 The kit is stored at -15°C to -25°C and is stable until the expiration date. After preparation, store it at +2°C to +8°C (stable for up to one week).

PCR - Amplification of the 16S rRNA gene

4 The reaction mixture is prepared in a microtube, considering the number of samples to be studied, inhibition controls, positive control, and negative control, based on the following formulation for each reaction:

	Reagent	Final concentration	Volume
	H2O	-	10 µL
	MgCl2	4.0 mM	2.4 µL
	Forward primer (20µM)	0.3 µM	0.3 µL
	Reverse primer (20µM)	0.3 µM	0.3 µL
	SYBR Green master mix	0.2 µM	2.0 µL
	DNA sample	-	5.0 µL
	Total reaction volume	-	**20 µL**

Reagent volumes and concentrations used per qPCR reaction.

5 Place in the adapters of the cold block a number of capillaries equal to twice the number of samples (1 capillary per sample and 1 capillary per inhibition control), plus two (one

capillary for the negative control and one capillary for the positive control).

6 The reaction mixture is prepared in the cleanroom. For each reaction, add 15 ul of the mixture to the top of each 20 ul glass capillary for LightCycler.

7 In the negative control, place 5 ul of sterile distilled water free of DNAses and RNAses in the capillary.

8 The extracted DNAs are manipulated in the workstation or Class II laminar flow cabinet with a UV lamp. Place 5 ul of each extracted sample into their respective capillaries, taking care not to produce aerosols or pass over the remaining capillaries.

9 In the inhibition control, place 4 ul of the sample extract and 1 ul of a known extract.

10 The positive control is obtained by adding 5 ul of a known extract to the 15 ul of the mixture.

11 Cover the capillaries with their respective caps.

12 Place the adapters in the microcentrifuge (maintaining the order) and centrifuge briefly to ensure that the liquid has descended from the top to the interior of the capillary.

13 Place the capillaries in the holes of the LightCycler support strictly vertically. The tubes will fall by gravity or, if necessary, gently rotate them. Finally, give a small tap on the top of the capillaries for a complete adjustment.

14 Place the tubes in the LightCycler and use the following program:

	Reaction Step	Hold time (s)	Slope (ºC/s)	Temperature (ºC)	Acquisition mode	Number of cycles
	Denaturation	600	20	95	None	1
	Amplification	0	20	95	None	35
		5	20	60	None	
		39	20	72	Single	

	Reaction Step	Hold time (s)	Slope (°C/s)	Temperature (°C)	Acquisition mode	Number of cycles
	Melting	0	20	95	None	1
		30	20	65	None	
		0	0.1	95	Continuous	
	Cooling	20	20	40	None	1

qPCR reaction protocol.

15 At the end of the program, analyze the amplification curves and the melting curve, expecting the melting temperature of positive samples to be around 89-90°C.

Protocol references

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