Bacterial DNA assay

Introduction

"Universal" bacterial primers targeting a highly conserved region of the ribosomal RNA gene can be used to measure the bacterial content in Oragene®/saliva samples†. The purpose of this assay is to determine the bacterial DNA content of purified DNA samples. Pure bacterial DNA is used to produce a standard curve from which the amount of bacterial DNA in Oragene/saliva samples can be calculated.

Equipment and reagents

- Real-time thermal cycler (Excitation: 470 nm, Emmission: 510 nm)
- Taq polymerase and buffers (Invitrogen – Cat. No. 18038-042) Taq DNA polymerase, Native 5 U/µL, 500 units. Taq DNA polymerase is supplied with 10× PCR buffer (200 mM Tris, 500 mM KCl, without $MgCl_2$) and 50 mM $MgCl_2$.
- Bacterial gDNA (Sigma-Aldrich – Cat. No. D4889) Deoxyribonucleic Acid Genomic from Escherichia coli strain B.
- 1 mg/mL Albumin, Bovine (Sigma-Aldrich – Cat. No. A8806) Albumin, Bovine Fraction V powder (fatty acid free, low endotoxin).
  - Store in 250 µL aliquots at -20°C. Discard unused portion.
- Syto 9 dye (Invitrogen – Cat. No. S34854)
  - Prepare a 50 µM solution by diluting 1:100 in TE buffer.
  - Store in 20 µL aliquots at -20°C. Discard unused portion.
- dNTP (Invitrogen – Cat. No. 10297-018)
  - Prepare a 10 mM dNTP solution by mixing 10 µL each of dATP, dCTP, dGTP, dTTP and 60 µL of TE buffer.
  - Store in 100 µL aliquots at -20°C.
- Reduced TE (rTE) 10 mM Tris, 0.1 mM EDTA
- Primers BacrRNA173-F 5' ATTACCGCGGCTGCTGG 3'
  BacrRNA173-R 5' CCTACGGGAGGCAGCAG 3'
  - Prepare as a 10 pmol/µL stock in water.
  - Store in 100 µL aliquots at -20°C.

† Saliva samples were collected with Oragene®•DNA or Oragene®•DISCOVER.

Procedure

1. Preparation of master mix

a) Prepare a master mix solution, sufficient for all tubes to be assayed.
b) Master mix, add components in the order shown.

Master mix components (A)	Amount per reaction (B)	Total Amount needed $B \times (19 + 2n)$ n = number of unknown samples
ddH_2O	12.1 µL	
10 × PCR buffer	2.5 µL	
50 mM $MgCl_2$	0.75 µL	
10 mM dNTPs	0.5 µL	
1 mg/mL albumin	2.5 µL	
10 pmol/µL Bac Forward primer	0.5 µL	
10 pmol/µL Bac Reverse primer	0.5 µL	
50 µM Syto 9 dye	0.5 µL	
Taq DNA polymerase	0.2 µL	
Total	20 µL	

2. Preparation of standard curve

a) Dilute stock bacterial gDNA with rTE to 4 ng/µL.
Serially dilute DNA with rTE to:
– 2 ng/µL
– 1 ng/µL
– 0.5 ng/µL
– 0.25 ng/µL
– 0.125 ng/µL
– 0.0625 ng/µL

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b) Label 0.2 mL PCR tubes (in duplicate) for each standard.
c) Add 5 µL of DNA standard to the corresponding tubes.
d) Add 5 µL of rTE to PCR tubes (no template control).
e) Add 20 µL of master mix to each tube, mix by vortexing.

Start standard (ng/µL)	Volume (µL)	Total DNA (ng)
4	5	20
2	5	10
1	5	5
0.5	5	2.5
0.25	5	1.25
0.125	5	0.625
0.0625	5	0.3125
0	5	0

3. Preparation of unknown Oragene/saliva samples (unspiked)

a) Add 3 µL of rTE to each tube.
b) Add 2 µL (~15 ng) of purified DNA to each tube. [Amount of DNA added to each tube can be verified by fluorescence (not absorbance)].
c) Add 20 µL of master mix to each tube. Mix by vortexing.

4. Preparation of internal standard

a) Prepare an internal standard using bacterial DNA by preparing a 1.67 ng/µL dilution in rTE.
b) Add 2 µL of rTE to PCR tubes (in triplicate).
c) Add 3 µL of the internal standard DNA to each PCR tubes for a total of 5 ng DNA per tube.
d) Add 20 µL of master mix to each tube. Mix by vortexing.

5. Preparation of unknown Oragene/saliva samples with the internal standard (spiked)

a) Add 3 µL of the internal standard to each tube (5 ng).
b) Add 2 µL (~15 ng) of Oragene/saliva DNA to each tube (as above).
c) Add 20 µL of master mix to each tube. Mix by vortexing.

6. PCR conditions

1 cycle 95°C 2 minutes

35 cycles 95°C 30 seconds
55°C 20 seconds
72°C 20 seconds (acquire at end of extension)

Melt Pre-condition: 72°C for 90 seconds
Range: 72°C to 95°C
Ramp rate: 0.2°C/s

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7. Calculation of results

a) Analyze results using the "quantitation" analysis tool.
b) Analyze the channel where the raw data has the maximum fluorescence but is not saturated.
c) Copy and paste results into an Excel sheet.
d) All results produced by the rotorgene have a unit of "ng/rxn".
e) Calculate the % bacteria using the following method:
f) Difference [D] = subtract the "Unspiked" [U] values from the "Spiked" [S] values
    [D] = [S] – [U]
g) % Bacteria [%B] = divide the "Unspiked" [U] values by the Total DNA [TD] added to each tube (~15 ng)
    [%B] = [U]/[TD]
h) Quantify Internal Standard [IS] = take the average of the internal standard triplicates
i) Correction Factor‡ [CF] = Internal Standard [IS] divided by the difference [D] multiplied by the "Unspiked" [U] values
    [CF] = ([IS]/[D])×[U]
j) Corrected % Bacteria [%CB] = Correction Factor [CF] divided by the Total DNA [TD] added to each tube
    [%CB] = [CF]/[TD]

‡ Correction factor is used to control for efficiency of PCR reaction

Technical support is available Monday to Friday (9h00 to 17h00 EST):

- Toll-free (North America): 1.866.813.6354, option 6
- All other countries: 613.723.5757, option 6
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