PROTOCOL FOR 16S DNA SEQUENCING DIRECTLY FROM HUMAN CLINICAL SAMPLES USING GROUP SPECIFIC PRIMERS

Bacterial 16S rRNA gene; variable areas V1, V2, V3

PURPOSE

The group specific primers are intended for infections that frequently are poly-bacterial. Samples from typical mono-bacterial infections can be investigated with a standard universal primer approach (see protocol P1001).
The group-specific PCRs increase the maximum number of detectable species from 3 to 9, using the RipSeq Mixed algorithm, and make the assay more sensitive in situations with unequal species concentrations. The group specific primers were also designed with the consideration of reducing the effects of human DNA cross reactivity, which is seen in other 16S rRNA gene broad range primer sets.

SAMPLES

Relevant samples:

- Abscesses in internal organs (brain, lung, spleen, liver, pancreas, kidney, ovaries, aorta).
- Retroperitoneal abscesses.
- Deep soft-tissue or muscular abscesses.
- Pleural fluid/empyema
- Bile

Non-relevant samples:

- Samples from areas in direct contact with mucus membranes or skin (BAL, pus from perianal abscesses, vaginal swabs, etc.).
- Very small biopsies or heavily diluted aspirates.

TRANSPORT AND SAMPLE CONSIDERATIONS

Samples should be transported in a sterile container without additives. Sterile water might be contaminated with DNA from e.g. *Pseudomonas* and *Pseudomonas*-like bacteria.
The amount of material must be adequate to maintain maximum sensitivity. In general we recommend 200 µl or more for liquid material and a "finger nail" for solid samples.

Eight-hundred µl is the maximum capacity of the bead-tube and can be used for liquid samples with low viscosity. For other samples 400 µl should be used, if available. Two hundred µl is the lowest volume that will still provide 400 µl of supernatant for the subsequent DNA purification.

INSTRUMENTS AND CONSUMABLES

Consumables

MagNA Pure Bacteria Lysis Buffer	Roche	04 659 180 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	Roche	03 730 964 001
SeptiFast Lys Kit M-GRADE	Roche	04 404 432 001
SYBR Premix Ex Taq/Ex Taq II*)	TaKaRa	RR041A/RR081A*
Nuclease-Free Water (10 x 50 ml)	Qiagen	129114

*)The manufacturer recommends "SYBR Premix Ex Taq" for the SmartCycler instrument and "SYBR Premix Ex Taq II" for LightCycler (Roche) and ABI Prism (Life Technologies) instruments.

Instruments

FastPrep instrument (Q-BIOgene)
Centrifuge
MagnaPure Compact machine (Roche)
SmartCycler machine (Cepheid)

These are the instruments that were used for validating the protocol. The protocol can be adapted to other extraction platforms and real-time instruments.

PROTOCOL

BACTERIAL LYSIS

1. Add 400 µl MagNA Pure Bacterial Lysis Buffer (Roche) to a bead tube (SeptiFast Lys Kit MGRADE, Roche).
2. Add 200-800 µl sample material.
3. Run for 2 x 45 seconds at speed 6.5 in the FastPrep instrument (Q-BIOgene) (see comments).
4. Centrifuge at 13.000 rpm for 3 minutes.
5. Transfer the supernatant to a 2 ml tube that fits into the MagnaPure Compact machine (Roche).
6. If not purified immediately, store at -80ºC.

Negative control:
A negative control follows every step of a direct 16S sequencing set-up. The negative control consists of a tube with beads (SeptiFast Lys Kit MGRADE), 400 µl Bacterial lysis buffer and 400 µl of PCR-grade water.

PURIFICATION OF DNA

1. DNA extraction and purification is performed on MagnaPure Compact (Roche) according to the manufacturer's instructions.
2. Choose elution volume 50 µl.
3. If not used directly for PCR, store at -80ºC.

PCR

Primers:
The group specific primers are covered by patents pending.

Forward	
Group A-forward	5′-gt-tTg-atc-mtg-gct-cag-rAc-3′
Group B-forward	5′-gt-tTg-atc-mtg-gct-cag-aKtg-3′
Group C-forward	5′-agt-ttg-atc-mtg-gct-cag-gAt-3′
Reverse	
Groups A,B,C-reverse (pD) **	5′-gta-tta-ccg-cgg-ctg-ctg-3′

**(Edwards *et al.*, 1989); Capital letters indicate Locked Nucleic Acids (LNAs) (Di Giusto & King, 2004, Rupp *et al.*, 2006).

All mixtures:

SYBR Premix Ex Taq (TaKaRa)	12.5 µl
F-primer (10 µM)	2.0 µl
R-primer (10 µM)	1.0 µl
H_2O (PCR grade)	7.5 µl
Template	2.0 µl
Total volume	25.0 µl

***NOTE!* The double concentration of F primer.**
***NOTE!* The polymerase has 3'-5'-exonuclease activity (see Comments).**

Make three separate mixtures (A, B and C) using Forward primer A, B and C respectively. The common reverse primer is used as reverse primer in all mixtures. A negative control is made for each of the three amplification mixtures and will also function as a positive control due to amplification of background bacterial DNA from reagents.

PCR conditions:
The real-time PCR reactions are run on a SmartCycler machine (Cepheid) for 45 cycles.

Initial enzyme activation	95°C	10 s
Melting	95°C	10 s
Annealing	64°C	15 s
Extension	72°C	20 s

Interpretation of PCR results:

- A positive sample is detected if the fluorescence intensity reaches threshold value ≥3 cycles before the earliest negative control (see below) *AND* no later than cycle 30 (see Comments).

- The earliest negative control: The negative control that reaches Ct (Crossing threshold) value first defines the Cut-off for all amplification reactions. E.g. Negative control for Mastermix A reaches Ct at cycle 35, Negative control for Mastermix B reaches Ct at cycle 34 and Negative control for Mastermix C reaches Ct at cycle 39. The cut-off for a positive PCR reaction will then be defined based on Ct value for B and set to 34-3=31 cycles.

- Samples that never reach threshold are likely to contain inhibitory substances or inhibitory amounts of DNA. These samples are diluted 1:10 and re-run.

CYCLE SEQUENCING

PCR clean up:

- The PCR products from the positive samples are purified with ExoSap-IT (Affymetrix).

- After ExoSap-IT treatment, the purified PCR products are diluted 1:3 (1 part product, 2 parts PCR-grade water – see Comments).

- The diluted product is used as template in the following cycle sequencing mixture:

Primers:

(NB! R-primer different from R-primer in broad range PCR. Sequencing only in reverse direction)

Common reverse (534R)***	`5'-tac-cgc-ggc-tgc-tgg-cac-3'`

***(Petti *et al.*, 2008)

Mixture:

BigDye version 1.1	1.0 µl
Seq. buffer	2.0 µl
Primer (10 µM)	1.0 µl
H_2O (PCR grade)	5.0 µl
Template	1.0 µl
Total volume	10.0 µl

Cycling conditions:
We use annealing 64 °C/extension 60 °C in the cycle sequencing reaction, and run it for 28 cycles.

(Sequencing is performed using the ABI PRISM Big-dye sequencing kit and a 3730/3130/3500 DNA Analyzer; Applied Biosystems, now part of Life Technologies).

COMMENTS AND RECOMMENDATIONS

1. Polymerase 3'-5'-exonuclease activity in the PCR-reaction has been reported to be essential for obtaining robust discrimination with the primer design used in this protocol (Di Giusto & King, 2004) If you want to use other polymerases, you are recommended to confirm that the discriminatory potential is conserved. The highest tendency for cross-reactivity is seen between groups A and C:

   - A suspension with a PCR A target (e.g. *Enterococcus faecalis*) that reaches Ct at cycle *n* in PCR A, should reach Ct in PCR C no earlier than cycle *n* + 8.

   - A suspension with a PCR C target (e.g. *Staphylococcus aureus*) that reaches Ct at cycle *n* in PCR C, should reach Ct in PCR A no earlier than cycle *n* + 10.

2. The cut-off for a positive sample consists of one flexible element (>3 cycles before the earliest negative control) and one static element (before cycle 30). The static element will show variation from lab to lab and might need to be established over time.

3. The optimal degree of dilution for the PCR product will need to be established in the lab. Typically somewhere between 1:3 and 1:10. Use the chromatogram raw signal pattern as guidance. Equal intensity throughout the chromatogram indicates an appropriate dilution. Labs that uses gel based detection (not real-time with SYBR-green) and consequently do not know if the PCR reaction has reached plateau level should ideally measure the DNA concentration before (eventual) dilution.

4. Before a chromatogram is uploaded in RipSeq®, you should check that your sample has actually been successfully sequenced, and that your chromatogram is not just representing noise/base-line. This is easily done in "Sequence Scanner" a free software available from ABI (Life Technologies):

   https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600583&tab=Overview

   Upload your chromatograms and choose "Raw" to see the raw-signal intensities of your chromatograms. For an explanation of what you may find, please see examples below (from a 3130 Genetic Analyzer).

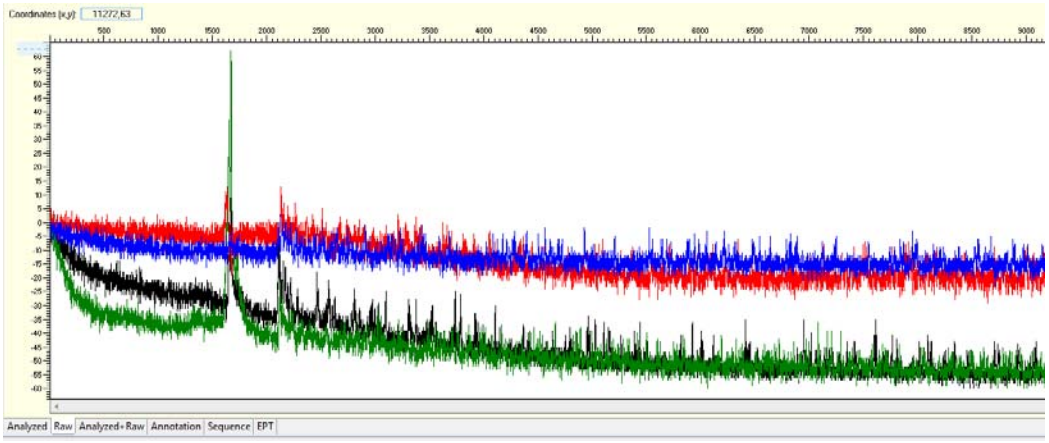

Fig 1. Not successfully sequenced. Only base-line.

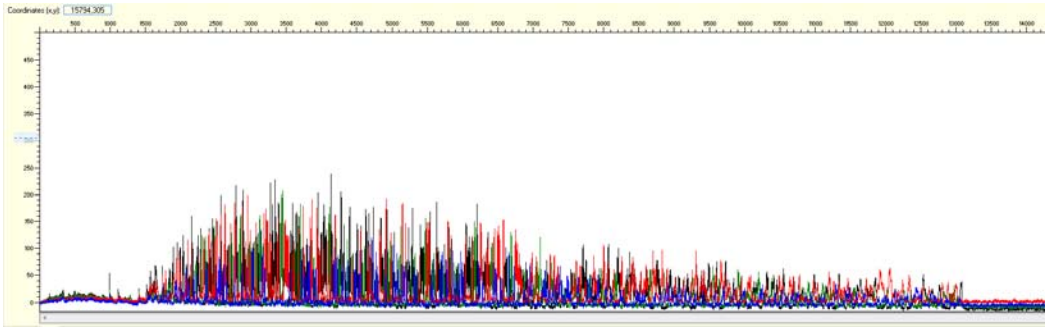

Fig 2. Sequenced, but with very low raw-signal intensity towards the 3'-end (<100), which results in a poor chromatogram quality in this part.

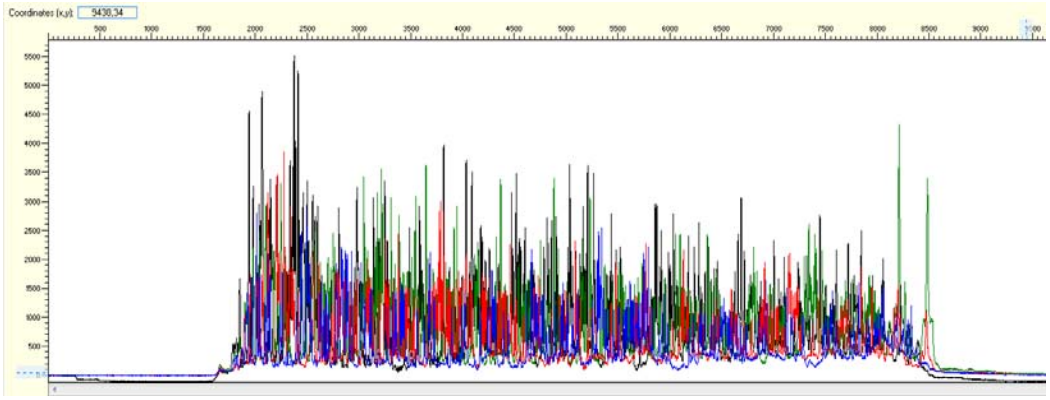

Fig 3. Good intensity throughout the sequence:

5. Earlier, we used the Lysing matrix B (Q-BIOgene) instead of the MGRADE tubes from Roche. We have not found any differences in the lysis efficacy, but Roche guaranties their LysKit tubes to be DNA free.

6. We are using the FastPrep Instrument for lysing. The manufacturer recommends one 45 sec run only, but we have found that 2 x 45 sec gives better results on spiked EDTA blood.
   We have also had the possibility to test a MagnaLyser from Roche and the performance is equal.

REFERENCES

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END NOTES

The Isentio products are intended for research use only.

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