PATHOGENOMIX

PROTOCOL FOR 16S DNA SEQUENCING DIRECTLY FROM HUMAN CLINICAL SAMPLES USING DUAL PRIMING OLIGONUCLEOTIDES (DPOS)

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

This protocol uses the DPO primer designing principle (1) in order to avoid cross-reactivity with human DNA when the bacterial 16S rRNA gene is amplified directly from clinical samples. Co-amplification of human DNA can result in chromatogram interpretation difficulties and can also affect Ct values in SYBR-green based real-time PCR assays.

MATERIAL

Example of relevant sample types:

- Abscesses in internal organs (brain, lung, spleen, liver, pancreas, kidney, ovaries, aorta)
- Retroperitoneal abscesses
- Deep soft-tissue or muscular abscesses
- Aspirate/biopsies from spondylodiscitis and other bone related infections
- Sterile body fluids (pleural, synovial, bile, CSF, pericardial)

Samples should be transported in sterile containers without additives. Sterile water might be contaminated with DNA from pseudomonas and pseudomonas like bacteria. The amount of material must be adequate to maintain maximum sensitivity. In general we recommend ≥200 µl for liquid material and a "finger nail" for solid samples.

Non-relevant samples:

- Samples from areas in direct contact with mucus membranes or skin (BAL, pus from perianal abscesses, vaginal swabs, superficial wounds etc.)
- Very small biopsies or heavily diluted aspirates
- Abscesses/samples from locations in direct connection to intestine

BACTERIAL LYSIS

1. Add 400 µl MagNA Pure Bacterial Lysis Buffer (Roche) to a bead tube (SeptiFast Lys Kit MGRADE, Roche).
2. Add 200-700 µl sample material.
3. Run for 2 x 45 seconds at maximum speed in a MagnaNA Lyser (Roche) or FastPrep (Q-BIOgene) instrument.
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 -80°C.

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 -80°C.

PCR

Primers:

Universal_DPO-F	5'-AGAgTTTgATCMTGGCTCA-I-I-I-I-I-AACGCT-3'
Universal_DPO-R	5'-CGCGGCTGCTGGCA-I-I-I-A-I-TTRGC-3'

I = Deoxyinosine; Lower case indicates a locked nucleic acid (LNA)
Note: *Please observe the alternative design of the poly-I linker of the reverse primer*

The resulting product has a size of approximately 510 bp, covering the variable areas V1, V2 and V3 of the *16S rRNA* gene (2).

Examples on PCR mixtures and PCR conditions (Please see comments and recommendations below):
We use a SmartCycler real-time PCR machine (Cepheid) with 25 µl reaction tubes. After initial enzyme activation (Step 0), the real-time PCR reaction is run for 40 cycles (Steps 1-3).

Note: We have tested two different polymerases (Roche and TaKaRa), mixture A and B respectively, and have slight optimizations for the cycling conditions as noted below:

Mixture A (Roche):

LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche)	5.0 µl
F-primer (10 µM)	1.0 µl
R-primer (10 µM)	1.0 µl
H_2O (PCR grade)	13.0 µl
Template	5.0 µl
Total volume	25.0 µl

PCR Conditions for mixture alternative A (Roche):

0	Initial enzyme activation	95°	600 sec
1	Melting	95°	10 sec
2	Annealing	62°	15 sec
3	Extension	72°	25 sec

Mixture B (TaKaRa):

SYBR Premix Ex Taq (TaKaRa)	12.5 µl
F-primer (10 µM)	1.0 µl
R-primer (10 µM)	1.0 µl
H2O (PCR grade)	8.5 µl
template	2.0 µl
Total volume	25.0 µl

PCR Conditions for mixture alternative B (TaKaRa):

0	Initial enzyme activation	95°	30 sec
1	Melting	95°	10 sec
2	Annealing	62°	15 sec
3	Extension	72°	20 sec

Interpretation of PCR results:

- A positive sample is detected if the fluorescence intensity reaches threshold value ≥3 cycles before the negative control.
- 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.
- Samples reaching threshold less than 3 cycles before the negative control are sequenced if the melt point analysis shows a tall distinct peak significantly different from the pattern seen in the negative control. The result should be interpreted with caution and taken into consideration only if a single well known relevant human pathogen is detected.

CYCLE SEQUENCING

Primers:

Universal_DPO-F	5'-AGAgTTTgATCMTGGCTCA-I-I-I-I-I-AACGCT-3'
Universal_DPO-R	5'-CGCGGCTGCTGGCA-I-I-I-A-I-TTRGC-3'

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 for alternative A (Roche): 1:5 (1

part product, 4 parts PCR-grade water) or for alternative B (TaKaRa): 1:3. The diluted product is used as template in the following cycle sequencing mixture:

Mixture cycle sequencing:

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 62 °C/extension 60 °C in the cycle sequencing reaction, and run it for 28 cycles (increased number of cycles because mixed chromatograms normally benefit from higher raw-signals).

(In our lab Sequencing is performed off site, using the ABI PRISM Big-dye sequencing kit and a 3730 DNA Analyzer; Applied Biosystems, now part of Life Technologies).

COMMENTS AND RECOMMENDATIONS

1. Cross-reactivity against human DNA has been extensively validated using the TaKaRa enzyme. No cross reactivity has been observed. Occasionally primer-dimer formation can be seen in the negative control using this enzyme. The tendency is weak and will occur after cycle 30. For the Roche enzyme we have neither observed primer-dimer formation nor cross-reactivity with human DNA. Roche also gives more reproducible CT values for the negative controls. However, it has not been as extensively validated in regards to cross-reactivity as the TaKaRa enzyme. We consider the DPO principle to represent a robust way to prevent cross-reactivity and do NOT expect cross-reactivity to become a problem with the Roche enzyme either (or other alternative enzymes).
   Mastermixes are examples only and other mastermixes can of course be used. Both we and some of our customers have experienced contamination of some Mastermix lots resulting in lower Ct values for the negative extraction controls and consequently lower sensitivity. In our opinion a mastermix should consistently deliver Ct values of 30 or higher in the negative extraction controls.

2. 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.

3. 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.

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).

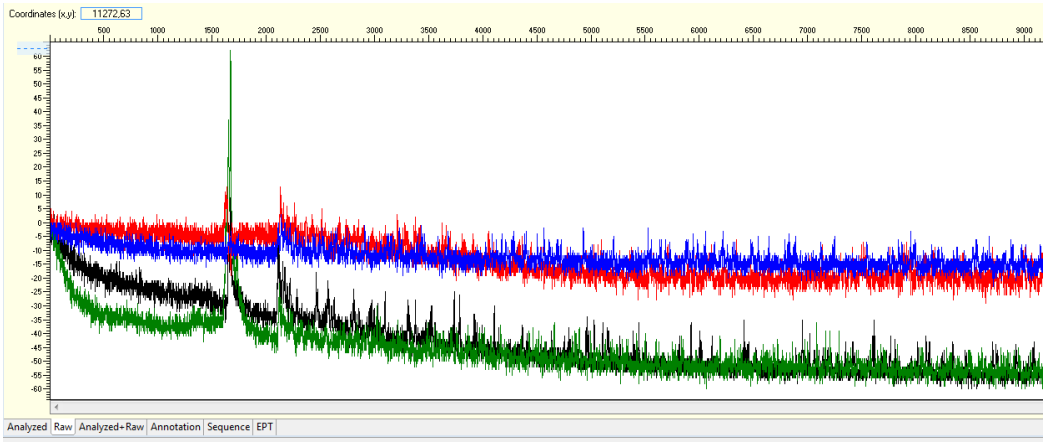

Fig 1. Not successfully sequenced. Only base-line.

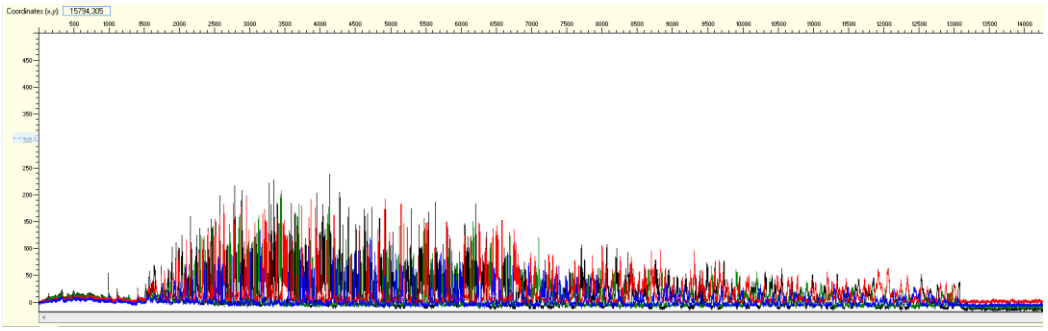

Fig 2. Sequenced, but with very low raw-signal intensity towards the 3'-end (<100), which results in a poor chromatogram quality in this part.

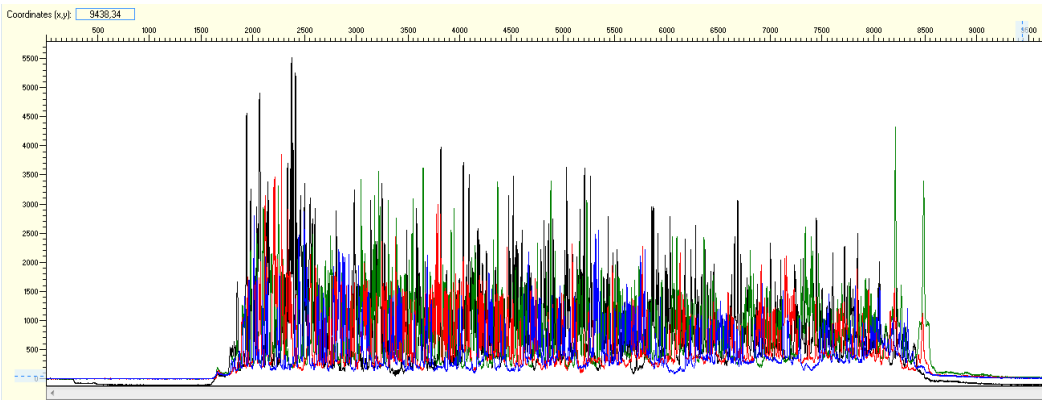

Fig 3. Good intensity throughout the sequence.

5. If you experience too high raw-signals in your chromatograms, dilution of PCR products before cycle sequencing should be increased (1:10 for Roche, 1:5 for TaKaRa). With an ABI 3730 sequencer the best chromatogram quality is normally seen with raw signals in the range of 1000-5000. For the 3100 series raw signals should be below 1000.

   For further troubleshooting of too low or too high raw signals please see: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041003.pdf

REFERENCES

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2. **Baker, G. C., J. J. Smith, and D. A. Cowan.** 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods **55:**541.

3. **Kommedal, O., Simmons K., Karaca D., Langeland N., Wiker HG.** 2012. Dual-priming oligonucleotides for broad-range amplification of the bacterial 16S rRNA gene directly from human clinical specimens. J. Clin. Microbiol. Jan 2012 available ahead of print.

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