Molecular Culture kit

CE Complies with the Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices

IVD **For In Vitro Diagnostic Use Only**

Test Instructions

Amplification DNA assay for the relative quantitative in vitro detection of the bacterial phyla *Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia, Bacteroidetes* and *Proteobacteria* in pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue, and water.

Catalogue Number: MolCul 15000, 24 tests

Store at -20°C upon receipt

Contents

1. Intended use

Molecular Culture is intended to be used for detection and identification of bacteria isolated from pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue, and water. Bacteria can be relatively quantified, based on their proportion of the total bacterial population within a specimen. Total peak height of a sample is correlated to absolute quantification.
The intended user will be a specialized molecular diagnostic laboratory. The test will be carried out by trained laboratory personnel. No special training will be required for professional routine diagnostic laboratories performing semi-quantitative PCR.

2. Summary and explanation of the test

Molecular Culture is based on IS-pro™. IS-pro™ is a bacterial profiling technique based on species-specific length polymorphisms of the IS region and phylum-specific sequence polymorphisms of 16S rDNA. IS-pro™ consists of two multiplex PCRs. The first PCR is specific for *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Bacteroidetes*, the second PCR is specific for *Proteobacteria*. An internal PCR amplification control is used to control for PCR inhibition and efficiency. A marker is added to the IS-pro™ eMix as a length reference to determine lengths of the PCR amplicons.

3. Principle of the test procedure

IS-pro™ is a bacterial profiling technique based on species-specific length polymorphisms of the IS region and phylum-specific sequence polymorphisms of 16S rDNA. IS-pro™ consists of two multiplex PCRs. The first PCR reaction sorts species into the groups *Firmicutes* / *Actinobacteria* / *Fusobacteria* / *Verrucomicrobia* (FAFV) or *Bacteroidetes*, based on forward primer label, the second PCR is specific for *Proteobacteria*.

The IS region is amplified with fluorescently labeled phylum specific primers targeting the 16S rDNA and unlabeled primers targeting the 23S rDNA. The IS-pro™ reaction yields peak profiles that provide two levels of information: color of fragments sorts species into phyla and fragment lengths can be used to further identify bacteria to the genus, species, or subspecies level.

4. Reagents

PRECAUTIONS

CAUTION: Handle patient samples as Biohazardous material.
Handle samples as if capable of transmitting an infectious agent.

All clinical samples should be regarded as infectious. These samples should be handled at the Biosafety Level 2 as recommended for any potentially infectious specimen in the Centre for Disease Control/National Institutes of Health Manual "Biosafety in Microbiological and Biomedical Laboratories," 1984.

Use sterile, DNase-RNase free, aerosol resistant pipette tips.

A uni-directional workflow must be adhered to in the laboratory with different rooms for sample preparation, pre-amplification area and post-amplification.

Protect kit contents or generated PCR product from direct sunlight.

Wear protective gloves.

For handling IS-pro™ eMix (MolCul 15007) work in a well-ventilated area, wear safety goggles with side protection and protective gloves which meet the specification of standard norm EC directive 89/686 / EEC and the resultant standard EN374. Disposal considerations: Do not let product enter drains. Keep away from surface and ground water. Dispose of contents/containers must be in accordance with local, regional, national and international regulations.

NOTE: IS-pro™ eMix (MolCul 15007) contains Formamide. See MSDS and handle product accordingly!

Hazard statement code	Hazard statement	Hazard Class (GHS chapter)	Hazard Category
H351	Suspected of causing cancer	Carcinogenicity (chapter 3.6)	2
H360FD	May damage fertility. May damage the unborn child	Reproductive toxicity (chapter 3.7)	1A, 1B
H373	May cause damage to organs (blood, cardiovascular system) through prolonged or repeated exposure	Specific target organ toxicity, repeated exposure (chapter 3.9)	2

4.1 Components in each Molecular Culture kit

Mastermix FIRBAC (MolCul 15001)
3 vials with a blue colour insert, labelled “Mastermix Firbac” containing 132 µl ready to use PCR mix. This mixture contains five primers for amplification of the bacterial DNA of *Firmicutes/Actinobacteria/ Fusobacteria/Verrucomicrobia* and *Bacteroidetes*. The mastermix contains all ingredients for PCR amplification including DNA polymerase.

Mastermix PROTEO (MolCul 15002)
3 vials with a yellow colour insert, labelled “Mastermix Proteo” containing 132 µl ready to use PCR mix. This mixture contains eight primers for amplification of the bacterial DNA of *Proteobacteria*. The mastermix contains all ingredients for PCR amplification including DNA polymerase.

Positive Control FIRBAC (MolCul 15003)
1 vial with a red colour insert, labelled “Positive Control Firbac” containing 30 µl positive control. The positive control contains bacterial DNA of *Firmicutes/Bacteroidetes*.

Positive Control PROTEO (MolCul 15004)
1 vial with a violet colour insert, labelled “Positive Control Proteo” containing 30 µl positive control. The positive control contains bacterial DNA of *Proteobacteria*.

Negative Control FIRBAC (MolCul 15005)
3 vials with a white colour insert, labelled “Negative Control Firbac” containing 132 µl negative control mastermix.

Negative Control PROTEO (MolCul 15006)
3 vials with a transparant colour insert, labelled “Negative Control Proteo” containing 132 µl negative control mastermix.

IS-pro™ eMix (MolCul 15007)
2 vials with a black colour insert, labelled “IS-pro™ eMix” containing 1078 µl IS-pro™ eMix.

Note: Use all components of the same kit lot number.

4.2 Additional materials and instruments required

Use sterile DNase-free polypropylene disposables for all steps in the procedure.

Molecular Culture can be used with standard guanidine iso-thiocyanate based lysis reagents and magnetic beads capture devices for DNA isolation. Molecular Culture is validated for Roche MagNA Pure 96 Instrument and BioMérieux NucliSENS® easyMAG™. Validated sample types NucliSENS® easyMAG™: Pus, Punctate, Drain fluid, Ascites fluid, Cerebrospinal fluid, Joint aspirate, tissue and water; MagNA Pure 96 Instrument: Pus, Punctate, Drain fluid, Ascites fluid, Cerebrospinal fluid, Joint aspirate, tissue and water.

PCR amplification can be carried out with GeneAmp® PCR system 9700 version 3.11 by Applied Biosystems. For use of other PCR systems, please contact IS-Diagnostics Ltd. for further instructions. For separation and detection of the PCR amplicons an ABI Genetic Analyzer 3500 is needed.

NOTE: The separation and detection process should be initiated within 12 hours after the PCR reaction is completed.

4.3 Reagent preparation and storage

Store all components at -20 °C.

Thaw the tubes prior to opening.
Mix and spin down briefly (3 seconds) before use.

All components are temperature sensitive.
Thaw only the components that are going to be used.
It is necessary to keep kit reagents at 2 – 8 °C (on ice or in cooling block) when in use.
Refreeze within half an hour after thawing.
Components can be refrozen and thawed again only twice.

4.4 Chemical or physical indications of instability

Alteration in the physical appearance of test kit materials may indicate instability or deterioration. Expiry dates shown on component labels indicate the date beyond which components should not be used.

5. Sample collection and preparation

The Molecular Culture kit is intended to be used for detection and identification of bacteria isolated from pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue and water. General collection devices can be used for standard DNA isolation procedures according to the manufacturer's protocols.

CAUTION: Handle patient samples as Biohazardous material. Handle samples as if capable of transmitting an infectious agent.

5.1 Sample Preparation

This procedure must be performed in the Sample Preparation Area in a class 2 Biological Safety Cabinet (protection to user and material).
Include a blank solution for every isolation run to control for contamination

5.1.1 Sample preparation validated for BioMérieux NucliSENS® easyMAG™:

Reagents

- BioMérieux NucliSENS® easyMAG™ reagents
- Qiagen AL buffer (article nr: 19075)

- Qiagen ATL buffer (article nr: 939011)
- Qiagen Proteinase K solution (article nr: 19133)
- Gibco PCR grade water (Invitrogen article nr: 10977035)

Sample preparation

Biopsies

- Cut into small pieces with scalpel.
- Add biopsy (~2 mm diameter) to a sterile vial
- Add to the vial:
  - ✓ 360 µl ATL buffer
  - ✓ 40 µl proteinase K
- Put the vial into a Mixer Incubator and shake and incubate at 56°C and 1400 rpm until all tissue is lysed. The sample is lysed when no particles are visible anymore. This takes approximately 1 hour.
- Centrifuge the vial for 30 seconds at 3500 rcf. This prevents aerosol formation when opening the container.
- Add 400 µl AL buffer to the vial and vortex 15 seconds
- Incubate vial for 10 minutes at 70 °C
- Centrifuge vial for 2 minutes at 18.000 rcf
- Use complete volume for DNA isolation

Pus sample

- Vortex the sample
- Add to vial:
- 200 µl of AL buffer
- 20 µl of proteinase K
- Add 50 µl of material to the vial.
- Vortex vial for 15 seconds
- Centrifuge the vial for 30 seconds at 6000 rpm
- Put the vial into a Mixer Incubator and shake and incubate at 56 °C and 1400 rpm until all material is lysed. The sample is lysed when no lumps are visible anymore. This takes approximately 1 hour.
- Centrifuge vial for 2 minutes at 18.000 rcf
- Use complete volume for DNA isolation

Puncture sample, drain fluid (ascites fluid)

- Vortex the sample
- Add to vial:
- 200 µl of AL buffer
- 20 µl of proteinase K
- Add 200 µl of material to the vial.
- Vortex vial for 15 seconds
- Centrifuge the vial for 30 seconds at 6000 rpm
- Put the vial into a Mixer Incubator and shake and incubate at 56 °C and 1400 rpm until all material is lysed. The sample is lysed when no lumps are visible anymore. This takes approximately 1 hour.
- Centrifuge vial for 2 minutes at 18.000 rcf
- Use complete volume for DNA isolation

Cerebrospinal fluid

- Add up to 1 ml of sample to the vial
- Centrifuge vial for 2 minutes at 18000 rcf
- Remove supernatant until approximately 500 µl is left.
- Add 500 µl of EasyMAG™ lysis buffer to vial
- Put the vial into a Mixer Incubator and shake and incubate at RT and 1400 rpm for 5 minutes
- Centrifuge the vial for 30 seconds at 3500 rcf. This prevents aerosol formation when opening the container
- Use complete volume for DNA isolation

Buffer or Water for QC control or bacterial or bacterial DNA contamination

- Add 1 ml of buffer or water to vial

For general usage of the EasyMAG see instruction manual of the machine.

Use one blank solution per EasyMAG isolation run, i.e. one of the wells is used for a blank solution. A blank solution is treated exactly like a bacterial sample without the actual specimen.

1. For each sample (including blanc solution):
   - For each well of the EasyMAG container:
     - Add 2 ml of EasyMAG lysis buffer
     - Add pretreated material except to the blanc sample
     - Pipet 3 times up and down to mix the contents
     - Incubate at least for 10 minutes at RT. For pus/puncture samples extra incubation of 1 hour in EasyMAG lysis buffer at RT is necessary.
     - Remove visible particles with a sterile toothpick or by pipet
     - Add 70 ul of EasyMAG magnetic silica
     - Mix the contents by pipetting
2. Start the isolation run as described in the EasyMAG manual using the following run conditions:
   - Select the Specific A protocol with off-board lysis incubation
   - Use 70 µl eluate volume.

After isolation is completed, transfer DNA from EasyMAG container wells into the corresponding Eppendorf container for extracted DNA.

Store DNA at 2-8 ºC

5.1.2 Sample preparation validated for Roche MagNA Pure 96 Instrument

Reagents

- Proteinase K (Qiagen, Cat. No. 19133)
- MagNA Pure Lysis/binding buffer (Roche, MagNA Pure LC Total Nucleic Acid Isolation Kit 03 246 779 001)

Sample preparation

Ascites fluid and Pus

- Add 200 µl to a sterile 2 ml vial
- Perform a prot. K pretreatment for MagNA Pure 96 on this vial (see section 5.4)

Biopsies

- Cut into small pieces with scalpel.
- Add biopsy (~2 mm diameter) to a sterile vial with 200 µl PBS.
- Perform a prot. K pretreatment for MagNA Pure 96 on this vial (see section 5.4)

Liquid patient material: Punctate and Cerebrospinal fluid

- Add 0,5 - 1 ml to a sterile vial and centrifuge for 10 minutes at 18.000 rcf
- Remove supernatant, leaving approximately 200 µl and resuspend in this 200 µl material.
- Perform a prot. K pretreatment for MagNA Pure 96 on this vial (see section 5.4)

5.1.3 Prot K pretreatment MagNA Pure96

- Add 20 µl Proteinase K to sterile vial.
- Add 200 µl MagNA Pure Lysis/binding buffer to sterile vial.
- Vortex for 10 seconds.
- Put the vial into a Mixer Incubator and shake and incubate at 56 °C and 1400 rpm until all tissue is lysed. The sample is lysed when no lumps are visible anymore. This takes approximately 1 hour. Incubation in Mixer Incubator can also be performed overnight at 37ºC and 1400 rpm.
- Centrifuge the vial for 30 seconds at 3500 rcf.

- Incubate 10 minutes at 100ºC in heating block
- Remove vials from heating block
- Incubate at RT for 3 minutes
- Centrifuge the vial for 30 seconds at 3500 rcf. This prevents aerosol formation when opening the container.
- Use complete volume for DNA isolation

For usage of the MagNA Pure96 Instrument, see instruction manual of the machine.

Use complete volume from Vials to transfer to the MagNA Pure96 plate.

Use one blank solution per 10 samples of the MagNA Pure96 isolation, at different positions in the plate. i.e. one or more of the wells of the plate is/are used for a blank solution depending on the sample size. A blank solution is treated exactly like a bacterial sample without the actual specimen.

5.2 DNA Isolated samples

This kit was validated with use of Nucleic Acid extracted samples by means of BioMérieux NucliSENS® easyMAG™ and Roche MagNA Pure 96 Instrument. Please follow the manufacturer's instructions for a description of the system features, isolation protocols and operational guidelines. Always isolate a blank solution. NB: The blank solution is a solution that is identical to the solution needed for sample preparation except that the blank solution does not contain analyte. The blank solution is used to identify contamination of samples.

6. Molecular Culture Kit Test Procedure

This procedure must be performed in the Pre-Amplification Preparation Area. Use aerosol barrier tips during the whole test procedure.

6.1 Reagent Preparation

Thaw the mixes needed and keep them at 0-8°C (on ice or a cooling block).

6.2 Procedure PCR

1. Prepare the required number of reaction tubes or wells for the number of samples to be measured (two reactions per sample: Firbac and Proteo), plus two tubes for the negative control. NOTE: The first run of each kit should contain a Positive Control Firbac (MolCul 15003) and Positive Control Proteo (MolCul 15004), to validate machine set-ups. Handle positive controls as DNA.

2. Thaw and vortex Mastermix Firbac (MolCul 15001) and Mastermix Proteo (MolCul 15002). Add 15 µl of Mastermix Firbac (MolCul 15001) to the first reaction tube and 15 µl of Mastermix Proteo (MolCul 15002) to the second reaction tube.

3. Vortex and spin down all DNA extracts. Carefully open sample containers one by one and avoid contamination of gloves and pipette. Using a new aerosol barrier tip for each reaction tube. Add 10 µl DNA (Chapter 5) of each sample to the reaction tube/well containing the Mastermix Firbac (MolCul 15001) and add 10 µl DNA of each sample to the reaction tube/well containing the Proteo Mastermix (MolCul 15002). Replace gloves when contamination is suspected.

4. Using a new aerosol barrier tip, add 15 µl of the Negative Control Firbac (MolCul 15005) and 15 µl of Negative Control Proteo (MolCul 15006) in two different reaction tubes/wells. Add 10 µl of blank sample (isolation blank)

5. Close the reaction tubes or seal the plate, spin down for 30 seconds at approximately 1000 rpm and move the reaction tubes/plate to the Amplification Area.

6. Load the reaction tubes/plate into the PCR instrument: For detailed instructions on how to use the PCR machine refer to the PCR machine manual.

7. Program the PCR System with following settings:

   10 cycles
   - 30 sec 94˚C
   - 45 sec 67˚C, reduce 1˚C per cycle
   - 1 min 72˚C

   25 cycles
   - 30 sec 94˚C
   - 45 sec 57˚C
   - 1 min 72˚C

   Final elongation and cooldown
   - 11 min 72˚C
   - ∞ 4˚C

8. The PCR products can be stored for 12 hours at 2-8˚C.

6.3 Procedure ABI

1. Thaw and vortex IS-pro™ eMix (MolCul 15007).

2. Per sample two PCR reactions were performed previously (Firbac and Proteo). For every sample, fill two wells of the ABI plate with 20 µl IS-pro™ eMix (MolCul 15007). To one well add 5 µl of the Firbac PCR product and to the other well 5 µl of the Proteo PCR product.

3. Place ABI septa on the ABI plate.

4. Heat the ABI plate in the PCR machine at 94^{o}C for 3 minutes followed by a cooling step to 4 ^{o}C.

5. Store plate at 2-8°C until capillary gel electrophoresis on the ABI. The ABI plate can be stored for five days at 2-8°C. The ABI is started using the ABI settings described below:

- Instrument protocol ABI 3500
  - Application type: Fragment
  - Capillary Length: 50 cm
  - Polymer: POP7
  - Dye Set: D (DS-30)
  - Instrument Protocol Properties
    - Run Module: FragmentAnalysis50_POP7
    - Oven Temperature: 60oC
    - Run Time: 4500 sec
    - Run voltage: 11.0 kVolts
    - PreRun Time: 180 sec
    - PreRun Voltage:15 kVolts
    - Injection Time: 15 sec
    - Injection Voltage: 1.6 kVolts
    - Data Delay: 200 sec
  - (Advanced Options: unchanged from default setting)
- Dye Set protocol ABI 3500
  - Dye Set Name: D (DS-30)
  - Chemistry Matrix Standard
  - Dye Selection: Blue Green Yellow Red
  - Reduced Selection: Blue Green Yellow Red
  - Calibration Peak Order: Blue=4, Green=3, Yellow=2, Red=1
  - Parameters
    - Matrix Condition Number Upper Limit: 20.0

        - Locate Start Point: After Scan 300, Before Scan 5000, Limit Scans To 20000, Sensitivity 0.1, Minimum Quality Score 0.8

- Sizecalling protocol ABI 3500
    - Size Standard GS500ROX
    - SizeCaller v1.1.0
    - Analysis Settings
        - Analysis Range: Full
        - Sizing Range:Full
        - Size Calling Method: Local Southern
        - Primer Peak: Present
        - Minimum Peak Height: All colors 175
        - Use Smoothing: None
        - Use Baselining (Baseline window (Pts)):51
        - Minimum Peak Half Width: 2
        - Peak Window Size: 15
        - Polynomial Degree: 3
        - Slope Treshold Peak Start: 0.0
        - Slope Treshold Peak End: 0.0
    - QC Settings
        - Fail if Value is: < 0.25
        - Suspect Range: 0.25-0.75
        - Pass if Value is: ≥ 0.75
        - Assume Linearity from: 0 bp To 800 bp
        - Pull Up: Actuate Pull-Up flag if Pull-Up Ratio ≤0.05 and Pull-Up Scan≤ 1

- File name convention
    - Filenames, should be created by the 'ABI genetic analyzer software' according special instructions when chosen for IS-Diagnostics Ltd. peak translation software service. See section 6.4.

6.4 Analysing data

Analyzing data should be performed according to the instructions provided in section 7.

Various software packages are available to analyse your data. Examples include Peak Scanner and GeneMapper (Thermo Fisher Scientific) and 'Bionumerics (Applied Maths), or the **free** IS-Diagnostics Ltd. software service.

IS-Diagnostics Ltd. offers a free software service for analysing your data. The software handles all steps from raw data processing to peak calling and species identification. Fragments are assigned to bacterial species according to our database and translation algorithm. If the peaks are not identified by our database, a phylum name and fragment length will be assigned to each peak. Visualizations of profiles and interpretations of positive and negative controls are also available for users.

When users choose to work with the IS-Diagnostics Ltd. software service, then username and password should be requested via info@is-diagnostics.com 10 working days before first usage of the kit.

With IS-Diagnostics Ltd. software service >150 bacterial species from the phyla *Firmicutes*, *Actionbacteria*, *Fusobacteria*, *Verrucomicrobia*, *Bacteroidetes* and *Proteobacteria* can be identified. Amongst others, the species that can be identified derive from the genera *Acinetobacter, Aeromonas, Bacillus, Bacteroides, Bordetella, Campylobacter, Capnocytophaga, Citrobacter, Clostridium, Corynebacterium, Eikenella, Enterobacter, Enterococcus, Escherichia, Finegoldia, Fusobacterium, Gardnerella, Haemophilus, Hafnia, Klebsiella, Lactobacillus, Lactococcus, Legionella, Listeria, Micrococcus, Moraxella, Morganella, Mycobacterium, Neisseria, Pasteurella, Porphyromonas, Prevotella, Propionibacterium, Proteus, Providencia, Pseudomonas, Raoultella, Rothia, Salmonella, Serratia, Staphylococcus, Streptococcus* and *Yersinia*. For an exhaustive list of all currently identifiable species, we refer to the IS-Diagnostics Ltd. software service manual (www.is-diagnostics.com).

6.5 Procedural notes

1. Use a uni-directional workflow in the laboratory.

   Sample Preparation area: Dedicated area to prepare the samples. All materials (equipment, supplies, protection, gloves, etc.) have to be dedicated to this area. Materials from this area may not be moved to the Pre-Amplification area.
   Pre-Amplification area: Dedicated area to prepare the reagents. All materials (equipment, supplies, protection, gloves, etc.) have to be dedicated to this area.
   Amplification area: Dedicated area for amplification. All materials (equipment, supplies, protection, gloves, etc.) have to be dedicated to this area. Materials from this area, may not be moved to the Pre-Amplification Area, and may not be moved to the Specimen Preparation Area.

2. Always use aerosol resistant tips.

3. Be extremely careful when handling materials to prevent contamination. Always mix and spin down reagents and samples before opening. In case of any suspicion of contamination, discard the materials.

4. Discard all consumed reagents upon completion of procedure in compliance with local, regional, national and international waste regulations.

5. Careful analytical techniques and strict adherence to the directions in the test instructions are essential to obtain reliable results.

6. Samples with equivocal results must be verified by repeat assays or isolation.

7. Do not pool reagents from different lots.

8. If the kit is damaged upon receipt, please contact your local distributor and/or IS-Diagnostics Ltd.

7. Interpretation of results

The following dyes are used for the different targets:

Target	Dye
Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia	FAM
Bacteroidetes	HEX
Proteobacteria	FAM
Internal Control	HEX
Marker	ROX

Marker peaks are found at: 25, 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1400 and 1500nc

Internal control peaks are found at: 192, 308, 502, 651 and 800nc

For valid runs (Valid Positive and Negative Control – See Quality Control), interpret the specimen results as follows:

Inhibitory Specimen
At least three out of five internal control peaks should be present in a sample to be sure no significant inhibition of the PCR is present. In case of inhibition the results are unreliable. Process the original specimen again or process a newly collected specimen.

DNA detection may indicate that a bacteria is present. However, it is always possible that a specimen may contain target DNA, without the presence of any living organisms (e.g. after antibiotic administration).

Quality control
In each kit one positive control and one negative control for each PCR run are provided. Additional controls may be analysed in addition to those provided. Established statistical methods for analysing control values and trends should be employed.

If the controls do not comply with the established limits and repetition excludes a technical issue, check the following areas:

1. Expiration date on reagent package and prepared reagents
2. Temperature of the reagents
3. Settings PCR System
4. Settings ABI System
5. Contamination

If controls are still invalid, please contact IS-Diagnostics Ltd. or your local distributor.

Positive Control Firmicutes
True peak position should be at 299-301 nucleotides and the intensity should be at least 15.000 RFU. If these criteria are not met, the complete run is invalid and the test procedure must be repeated.

Positive Control Bacteroidetes
True peak position should be at 535-537 nucleotides and the intensity should be at least 15.000 RFU. If these criteria are not met, the complete run is invalid and the test procedure must be repeated.

Positive control Proteobacteria
True peak position should be at 920-922 nucleotides and the intensity should be at least 15.000 RFU. If these criteria are not met, the complete run is invalid and the test procedure must be repeated.

Internal control
Internal control peaks should be at positions 192, 308, 502, 651 en 800 nc +/- 1 nc. Intensities should be lower than 30.000 RFU. At least 3 out of 5 peaks should be detected per sample. If these criteria are not met, the sample is invalid and the test procedure must be repeated.

Negative Control Firmicutes
Peaks at nc 188, 189, 192, 282 and 283 should be below 5000 RFU.
All other peaks > 145 nc should be below 100 RFU. If these criteria are not met, all specimens are invalid and the test procedure must be repeated.

Negative Control Bacteroidetes
All peaks >145 nc should be below 10 RFU. If these criteria are not met, all specimens are invalid and the test procedure must be repeated.

Negative Control Proteobacteria
All peaks >145 nc should be below 2000 RFU. If these criteria are not met, all specimens are invalid and the test procedure must be repeated.

8. Limitations of the procedure

1. Use only pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue, and water. Other specimen types have not been validated and may result in false positive or false negative results.

2. Specimen collection, transport and storage may affect the number of organisms and their associated DNA present in the specimen, affecting the outcome of the result. (causing a false positive or a false negative result)

3. Good laboratory practices and strict adherence to these Test Instructions are indispensable to avoid contamination of reagents and/or specimens.

4. Bacteria from other phyla than *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia Bacteroidetes* and *Proteobacteria* are not detected.

5. The user should have a formal training in PCR techniques or have gained appropriate experience in the field of PCR techniques.

9. Performance characteristics

9.1 Analytical specificity

Cross reactivity with non-bacterial organisms

The analytical specificity of the Molecular Culture kit was tested against cultured *Candida albicans, Cryptococcus neoformans/neoformans, Malassezia furfur, Absidia corymbifera, Rhizomucor pusillus, Rhizopus oryzae, Aspergillus fumigatus, Fusarium oxysporum, Saccharomyces cerevisiae, Trichosporon asashii, Blastocystis hominus* and *Cytomegalovirus*. All tested organisms gave negative results.

Cross reactivity between the three different phyla (FAFV group, *Bacteroidetes* and *Proteobacteria*)

376 single cultured bacteria, 8 clinical pus samples which contained a single species and 41 cultured lyophilized bacteria (1 McFarland) dissolved in DNase/RNase free water were tested for cross reactivity. The IS-pro™ primers gave no cross reactivity with DNA of other phyla in clinical material. Cross reactivity between *Firmicutes* (*Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus brevis*) and Proteoprimers could be observed when the DNA concentration was very high (cultured bacteria), which give the primers a chance to bind aspecifically. In practice the risk is very low, but if this happens in clinical samples it may be used as additional information to identify a bacterial species.

Cross reactivity with human DNA

Cross-reactivity with human DNA can indeed occur when human DNA load is high and bacterial DNA absent or in very low concentrations relative to human DNA. Human cross-reactivity peaks may occur alone or in various combinations. The freely available IS-Diagnostics Ltd. software service can automatically detect and delete such fragments.

9.2 Analytical sensitivity

The **limit of detection** (LoD) of the Molecular Culture kit was determined using quantified stock cultures (by counting CFU). Five replicates were tested at the concentrations of 0.1, 1, 10, 100 and 1000 CFU. For *Firmicutes* the bacteria *Streptococcus cristatus* (DSM 5017/ATCC 13637), for *Bacteroidetes* the bacteria *Bacteroides fragilis* (NCTC 9343/DSM 2151) and for *Proteobacteria* the bacteria *Stenotrophomas maltophilia* (DSM 5017/ATCC 13637) was used. Subsequently, the appropriate dilution was analysed 55-fold, adding up to 60 separate analyses of the LoD, therefore enabling the determination of the LoD with at least 95% confidence. Table 1 shows the limit of detection per phylum.

Table 1: Limit of Detection

Phylum	Bacteria	LoD	Confidence
Firmicutes	*Streptococcus cristatus* (DSM 5017 / ATCC 13637)	10 CFU	>95%
Bacteroidetes	*Bacteroides fragilis* (NCTC 9343 / DSM 2151)	10 CFU	>95%
Proteobacteria	*Stenotrophomas maltophilia* (DSM 5017 / ATCC 13637)	100 CFU	>95%.

To determine **dynamic range** of a bacterium in the presence of another bacterium from the same phylum, two different bacteria from the same phylum were tested in a dilution series from undiluted to 1:10.000.000 with dilution steps of 1:10. Each dilution was tested with the complete dilution series of the other bacteria and vice versa. This resulted in the following conclusion. The dynamic range of the FAFV group was 1:1.000.000, the dynamic range of the *Bacteroidetes* was 1:100 and the dynamic range of the *Proteobacteria* was 1:1000. For the overall dynamic range the lowest measured dynamic range was taken. This was 1:100.

9.3 Precision

The **repeatability** (within run values) was tested with one bacterium per phylum in 15 fold. Results were within the range shown in table 2 ‘Repeatability per phylum log2 transformed’.

Table 2: Repeatability per phylum log2 transformed

	-2StDev (log2intensity)	Average (log2intensity)	+2StDev (log2intensity)
Firmicutes	14.78	14.94	15.10
Bacteroidetes	14.75	14.89	15.04
Proteobacteria + IC	14.78	14.92	15.05

For the **reproducibility**, the effects of the variables were evaluated for two values (i.e. two different run dates, two different operators, two different PCR machines and two different ABI machines). Different variables were tested simultaneously. The repeatability was tested with one bacterium per phylum group and was evaluated fifteen-fold. Results were within the range of 2SD (1,86 log2RFU) which has been set as a reference for reproducibility as described in ‘IS-pro: high-throughput molecular fingerprinting of the intestinal microbiota’

9.4 Accuracy

The systematic error (trueness of results) is defined as the performance of the Molecular Culture kit relative to culture (golden standard).

In total, 76 species were identified by culture. 72 of these (95%) were also found with IS-pro™. The four cultured strains that were not found by IS-pro™ came from two samples. Overall, for all abscess-derived samples, IS-pro™ detected significantly more species than culture. Results of IS-pro™ were backed up by a next-generation sequencing approach. From this study we concluded that IS-pro™ generally outperforms culture in terms of detection of bacteria in clinical samples.

For the entire published dataset of this study we refer to Budding, AE et al., Automated broad range molecular detection of bacteria in clinical samples, J Clin Microbiol. 2016 Apr;54(4):934-43.

9.5 Interfering Substances

The presence of PCR inhibitors may cause false negative results. To check whether the internal control adequately monitors PCR inhibition, one µl of the following substances was added to the PCR.

- Magnetic Silica
- Metronidazol (5mg/mL)
- DNAse/RNAse free water
- EDTA 0.5M
- Lysis buffer of EasyMAG
- Ethanol 96%
- NaCl 5M

Metronidazole and Magnetic Silica interfered only with the Firbac PCR. The Proteo PCR was not inhibited by these two interfering substances. DNAse/RNAse free water was no interfering substance. EDTA inhibited the Firbac PCR up to a dilution of 1:10.000.000 and the Proteo PCR for up to 1:100. Lysisbuffer of the easyMAG inhibited the Firbac PCR up to a dilution >1:10.000.000 and for Proteo PCR up to dilution 1:100. Ethanol 96% inhibited the Firbac PCR up to a dilution of 1:10.000.000 and for the Proteo PCR up to 1:10. NaCl 5M inhibited the Firbac PCR up to dilution >1:10.000.000 and NaCl inhibited Proteo PCR up to dilution 1:10. The data showed that the internal control is able to monitor inhibition well.

9.6 Assay list

Assay Performance	
Dynamic Range	**1:100**
Precision - Repeatablility	***Firmicutes*: 14.78-15.10 (log2intensity), *Bacteroidetes*: 14.75-15.04 (log2intensity), *Proteobacteria*: 14.78-15.05 (log2intensity)**
Precision - Reproducibility	**2SD log2RFU(1.86) for 95% of the peaks**
Cross Reactant:	
Bacterial species of other Phyla	**No cross reactivity except for selected species**
Human DNA	**Cross reactivity in high human DNA load samples. Fragments resulting from cross-reactivity have been identified.**
Interfering substances	
Amplification inhibitors	**Preferably monitored by internal amplification control**
Assay Sensitivity	
Analytical	**At least 95% sensitivity compared to culture for species included in assay**
Functional (peak position (nc))	**±1nc**
Functional (peak height (log2RFU))	**1 log2RFU and 3 log2RFU for <1% of peaks**
Limit of Blank (LoB (RFU))	**Intensity < 500**
Limit of Detection (LoD (RFU))	***Firmicutes* 10 CFU, *Bacteroidetes* 10 CFU, *Proteobacteria* 100 CFU (>95% confidence).**
Correlation Studies	
Reference method:	**Culture**
Reference standard	**Clinical samples**
Assay Protocol	
Sample pretreatment	**Lysis + DNA isolation, (Lysis + DNA isolation reagents are not included in the kit)**
Reagent pretreatment	**NA**
Type of sample	**DNA derived from pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue, water.**
Sample size limitations (w/o dead volume)	**NA**

Assay format	**GeneAmp® PCR system 9700 version 3.11 , ABI3500**
Automatic/Manual Dilution	**NA**
Total assay time	**5 hr**
Reagents, Calibrators and Controls	
Tests per kit	**24**
Positive control	
A. Matrix	**Liquid frozen inactivated DNA**
B. Physical form	**Liquid frozen**
C. Stability (shelf life)*	**12 months**
D. Number of freeze/thaw cycles	**2**
E. Stability after defrosting (2-8°C)	**30 minutes between every freeze/thaw step**
Negative control	
A. Matrix	**Liquid frozen with Taq Polymerase and no DNA control**
B. Physical form	**Liquid frozen**
C. Stability (shelf life)	**12 months**
D. Number of freeze/thaw cycles	**2**
E. Stability after defrosting (2-8°C)	**30 minutes between every freeze/thaw step**
Mastermix	
Physical form	**Liquid frozen with Taq polymerase**
Stability (shelf life)	**12 months**
Stability after defrosting (2-8°C)	**30 minutes between every freeze/thaw step**
Shipping conditions	**-78°C**
Storage conditions	**-20°C**
Legal/Regulatory	
Intended use	**Molecular Culture is intended to be used for detection and identification of bacteria isolated from pus, punctate, drain fluid (ascites fluid), cerebrospinal fluid, joint aspirate, tissue and water. Bacteria can be relatively quantified, based on their proportion of the total bacterial population within a specimen. Total peak height of a sample is correlated to absolute quantification. Used by professional user, not for self-testing**

10. References

Budding et al., (2010). IS-pro: high-throughput molecular fingerprinting of the intestinal microbiota, FASEB J. 2010 Nov;24(11):4556-64.
Budding et al., (2016). Automated broad range molecular detection of bacteria in clinical samples, JClin Microbiol. 2016 Apr;54(4):934-43.

List of symbols as used in labelling

Contents (XXX µl)


Complies with the Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices.

For In Vitro Diagnostic Use Only.

List of Abbreviations

ABIq	**ABI Genetic Analyser 3500**
CE	**Conformité Européenne**
DNA	**Deoxiribonucleic acid**
DNAse	**Desoxyribonuclease**
EDTA	**Ethylenediaminetetraacetic acid**
FAFV	**Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia**
IC	**Internal Control**
IS	**Interspace**
IS-pro™	**Interspace Profiling**
IVD	**In Vitro Diagnostics**
MSDS	**Material Safety Data Sheet**
NaCl	**Sodium Chloride**
Nc	**Nucleotide**
PC	**Positive Control**
PCR	**Polymerase Chain Reaction**
rDNA	**ribosomal DNA**
RNAse	**Ribonuclease**

For additional information, please visit www.is-diagnostics.com

For technical assistance please refer to the Catalogue Number:
MolCul 15000

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