TECHNICAL REPORT

Laboratory procedures for diagnosis and typing of human *Clostridium difficile* infection

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This document was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Pete Kinross and Olivia Aya Nakitanda and produced by the consortium that provides the ECDC project *Microbiological Support to European Surveillance of* Clostridium difficile *infections:*

Prof. Dr. E.J. Kuijper, Leiden University Medical Centre (LUMC), Leiden, the Netherlands; Dr. D.W. Notermans, Center for Infectious Diseases Control (Cib), RIVM, Bilthoven, the Netherlands; Prof. M.H. Wilcox, University of Leeds, Microbiology, Leeds, United Kingdom; Univ. Prof. Dr. F. Allerberger, Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien, Austria; and Prof. Dr. F. Barbut, UHLIN National Reference Laboratory for *Clostridium difficile*, Groupe Hospitalier de l'Est Parisien (HUEP), Paris, France.

The project was initiated and funded by ECDC through a framework service contract (ECDC/2016/016) to Leiden University Medical Centre (LUMC), Leiden, the Netherlands, following an open call for tender (OJ/05/11/2015-PROC/2015/029). The project adopted the name 'European *C. difficile* Infection Surveillance Network 2' (ECDIS-Net-2).

Authors
Chapter 1
Frédéric Barbut, Groupe Hospitalier de l'Est Parisien (HUEP), Paris, France;
Daan W. Notermans, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands;
Mark H. Wilcox, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom;
Ed J. Kuijper, Leiden University Medical Centre (LUMC), Leiden, the Netherlands.

Chapter 2
Daan W. Notermans, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands;
Elisabeth M. Terveer and Ed J. Kuijper, Leiden University Medical Centre (LUMC), Leiden, the Netherlands;
Frédéric Barbut, Groupe Hospitalier de l'Est Parisien (HUEP), Paris, France;
Warren N. Fawley and Mark H. Wilcox, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom;
Franz Allerberger, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria.

Chapter 3
Ingrid M. J. G. Sanders, Céline Harmanus and Ed J. Kuijper, Leiden University Medical Centre (LUMC), Leiden, the Netherlands.

Chapter 4
Céline Harmanus, Ingrid M. J. G. Sanders and Ed J. Kuijper, Leiden University Medical Centre (LUMC), the Netherlands;
Warren N. Fawley and Mark H. Wilcox, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom;
Marion Blaschitz and Alexander Indra, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria.

Chapter 5
Marion Blaschitz and Alexander Indra, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria.

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Suggested citation: European Centre for Disease Prevention and Control. Laboratory procedures for diagnosis and typing of human *Clostridium difficile* infection. Stockholm: ECDC; 2018.

Stockholm, October 2018

ISBN 978-92-9498-273-5
doi: 10.2900/04291
Catalogue number TQ-03-18-332-EN-N

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Abbreviations

bp	Base pair
C. difficile	*Clostridium difficile*
CCEY	Cycloserine cefoxitin egg yolk agar (Brazier's CCEY agar)
CCFA	Cycloserine cefoxitin fructose agar
CCNA	Cell cytotoxicity neutralization assay
CDI	*Clostridium difficile* infection
cdtA	Binary toxin A
cdtB	Binary toxin B
CLO medium	*Clostridium difficile* medium
CNA	Colistin-nalidixic acid agar
CT	Cycle threshold
DNA	Deoxyribonucleic Acid
EIA	Enzyme immune assay
ELISA	Enzyme-linked immune-sorbent assay
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESGCD	ESCMID Study Group on *Clostridium Difficile*
EtBr	Ethidium bromide
GA	Genetic analyser
GDH	Glutamase dehydrogenase
GluD/GDH	Glutamase dehydrogenase
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
NAAT	Nucleic acid amplification tests
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RT	Ribotype
SOP	Standard operating procedure
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TCCA	Taurocholate cycloserine cefoxitin agar
TcdA	Toxin A
TcdB	Toxin B
TE	Tris EDTA pH=8.0
TPI	Triosephosphate isomerase
UV	Ultraviolet

Introduction

In 2016, a consortium of national microbiological reference laboratories for *Clostridium difficile* infections (CDI) was established to support the European surveillance of CDI (ECDIS-Net-2). The consortium consists of participating laboratories from France, Austria, United Kingdom and the Netherlands, and is led by the reference laboratory at Leiden University Medical Center (LUMC), in close collaboration with the Center for Infectious Disease Control (Cib) at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

The aim of this document is to support CDI surveillance in Europe with appropriate microbiological standardised procedures, and can be used by microbiologists, infectious diseases specialists and infection prevention control specialists.

The official new genus name to which *C. difficile* belongs is 'Clostridioides', based on 16S rRNA gene sequences. *C. difficile* belongs to cluster XI, a large distance from the species *Clostridium butyricum* in cluster I (Lawson, 2016). Therefore, the novel genus Clostridioides gen. nov. is now used for *Clostridium difficile*.

Chapter 1 summarises the recommendations for CDI diagnostics based on recently published data and discussions with experts of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and ECDC. It advises against using molecular tests as stand-alone tests, since NAATs are not capable of differentiating between asymptomatic colonization of *C. difficile* and infection by *C. difficile*. Recent publications indicate that the bacterial load of *C. difficile* is generally higher in patients with CDI compared to carriers, resulting in a lower cycle threshold (CT) in the PCRs (Crobach et al., 2018). The use of a quantitative NAAT has been implemented in many laboratories and its precise role will be discussed in 2019 when the guidelines will be revised. A worthwhile document to read is the recently published 'Pitfalls of laboratory diagnostics of CDI' (Krutova et al., 2018).

Chapters 2 and 3 provide recommendations for culturing and identification of *C. difficile* from faeces samples, advising on the storage of faeces samples of patients tested positive for CDI to have the possibility of culturing *C. difficile* for further characterisation, typing and antimicrobial susceptibility testing later. Faeces samples can be unreservedly stored at -20 °C or lower, since the spores of *C. difficile* remain viable for a long period.

Chapters 4 and 5 contain information on PCR ribotyping of *C. difficile* using microchip electrophoresis or capillary electrophoresis. An option for analysis of PCR products using 'Webribo' is included in Chapter 5, enabling laboratories to type *C. difficile* using a free online database. Agarose gel electrophoresis is not recommended for PCR ribotyping, as the method provides insufficient inter-laboratory reproducibility, especially compared to capillary electrophoresis (Fawley et al., 2015). Although PCR ribotyping is the standard typing technique for *C. difficile*, new molecular approaches with data obtained by whole genome sequencing should be further explored, as has already been done for many other bacterial species. The first attempts to use a core genome multilocus sequence typing (cgMLST) scheme are promising and is expected to better standardise *C. difficile* typing in the near future (Bletz et al., 2018).

We hope that this document will contribute to a microbiological supported CDI surveillance in Europe. We welcome suggestions to further improve this document and appreciate any feedback on its usefulness.

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1. Summary list of ECDC HAI-Net standard operating procedures for in vitro diagnostic of *Clostridium difficile* infection

Scope

Recommendations for diagnosing CDI, as approved by ESGCD and ESCMID

Introduction

The recently published European guideline by Crobach et al. [1] has been approved by ESGCD and ESCMID and should be considered as the standard. The conclusion of the guideline is that no single currently available, commercial test can serve as a standalone test for diagnosing CDI, and that the use of a two-step algorithm is strongly recommended, including a test to detect free toxins in faeces. Faeces samples without free toxins but with positive glutamate dehydrogenase EIA, nucleic acid amplification test or toxigenic culture results need clinical evaluation to discern CDI from asymptomatic carriage. Recent publications suggest that the bacterial load of *C. difficile* is generally higher in patients with CDI compared to carriers, resulting in a lower cycle threshold (CT) of NAAT. The precise place of the application of quantitative NAAT with CT-values as threshold needs to be further assessed.

Table 1.1. Recommended two-step algorithm by ESGCD and ESCMID

Categorization of CDI diagnostics	CDI diagnostic algorithm		
	First test	Second test	Optional third test
ESCMID-recommended	NAAT	Toxin A/B EIA	N/A
	GDH EIA	Toxin A/B EIA	NAAT or toxigenic culture
	GDH and Tox A/B EIA	NAAT or toxigenic culture*	N/A
Not recommended	All other algorithms		

N/A: not applicable
** In this testing strategy, NAAT or toxigenic culture is an optional second test (there is no third test option).*

- Toxin A/B EIA: Enzyme immunoassays, including enzyme-linked immune sorbent assays (ELISA) that test for both toxins A and B.
- GDH EIA: Enzyme immunoassays, including enzyme-linked immune sorbent assays (ELISA), that test for glutamate dehydrogenase.
- GDH and Tox A/B EIA: Enzyme immunoassay that combines detection of both GDH and Tox A and B
- NAAT: Nucleic acid amplification tests.
- Toxigenic culture: Demonstration that a *C. difficile* culture is able to produce toxins *in vitro*, e.g. by cytotoxicity assays or Toxin A/B EIA from colonies. NAAT may be an alternative, but only demonstrates the presence of toxin genes.

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2. Standard operating procedure for best practice procedures for culture and identification of *Clostridium difficile*

Safety/caution

- Laboratory-acquired infections with *C. difficile* have been described in the literature [Bouza, 2008]. The laboratory methods should be carried out under containment-level-2 conditions, using the principle of good laboratory practice, OR containment level 3 if hazard-group-3 organisms are also suspected in the specimen. Standard laboratory precautions must be adhered to.
- Methyl alcohol and ethanol are highly flammable. Store at ambient temperature in a suitable flammable liquid storage container. When using this reagent avoid proximity to ignition sources e.g. naked flame.
- Long wave (365–366 nm) ultraviolet light is less hazardous than short wave UV. However, do not expose eyes or skin to it. Spectacles are protective. If not wearing spectacles, wear protective goggles. Do not look directly into the UV light source or point it towards other people.
- Although *C. difficile* cultures may have a characteristic odour, sniffing culture plates is not recommended.

Introduction

C. difficile is an anaerobe, spore forming, gram-positive bacterium. It is an environmental organism and the spores can survive in the environment for a prolonged time and are resilient to drying, alcohol and many common detergents. It may be carried asymptomatically in the intestine and is known to cause antibiotic-associated diarrhoea in susceptible persons. Symptoms in infected persons range from mild diarrhoea to pseudo-membranous colitis, toxic megacolon or death. Susceptible persons are those with disturbed intestinal flora (commonly due to usage of broad-spectrum antibiotics) and/or immunosuppressed.

Principle and method of the procedure used for examinations

To isolate *C. difficile* from the multitude of bacteria in a faecal sample, both selective media are used, and the resistance of bacterial spores is exploited. 'Alcohol shock' or 'heat shock' selectively decontaminates faeces by killing vegetative organisms, but spores of *Clostridium* species and aerobic-spore bearers can survive this exposure. Most *C. difficile* selective agars contain the sodium salt taurocholate or cholic acid to promote germination of spores, p-hydroxyphenylacetic acid to enhance colony odour and cycloserine/cefoxitin to suppress most other clostridia and many other organisms. Egg yolk may be included to aid differentiation of *C. difficile* from certain other clostridia. Horse blood (minimum 0.5%) enhances the demonstration of UV fluorescence.

Scope

This 'best practice procedure' is for the isolation by culture and presumptive identification of *C. difficile* from stool samples. Isolated strains can be used for further characterisation and typing. It is based upon the procedure previously developed for the European Centre for Disease Prevention and Control (ECDC) funded project to enhance laboratory capacity for CDI detection and surveillance in Europe (2010–2014), ECDIS-Net. This procedure is not to be used as a standalone test for patients suspected of CDI, although culture may be part of these diagnostic algorithms. For guidance on patient diagnostics, see Crobach et al., 2016.

This procedure will provide several different alternatives; for example the choice of culture media (as there is not just one single way to perform a culture), or for identification, and to allow laboratories in different countries, with differences in techniques, equipment and resources, to adequately obtain strains for further characterisation, in accordance with national certification/accreditation rules.

This procedure is intended for use by suitably qualified personnel in routine and/or reference medical microbiological laboratories.

Reagents

- Methyl alcohol diluted to 70% in sterile water or ethanol 96%.
- *C. difficile* culture media
  - selective media: such as TCCA, CCEY, CLO medium or chromID *C. difficile* agar
  - Non-selective media, such as CNA, depending on method(s) used for identification.

Biological materials

- Faecal sample received in leak-proof container. Store specimens at +4 to -20 °C prior to testing
- *C. difficile* control strain(s).

Equipment

- Anaerobic jar and aerobic incubator or anaerobic workstation at 37 °C.
- If used for identification: long-wave (365–366 nm) UV lamp (e.g. from UV products Ltd) in a dark box or dark room. UV protective goggles.
- Depending on method(s) used for identification:
  - Gram-staining equipment
  - Agglutination with *C. difficile* latex reagent
  - Proline-specific aminopeptidase kit
  - MALDI-TOF MS
  - Thermal cycler for PCR.

Procedures

- Faeces can be directly transferred to a selective medium (TCCA, CLO medium, CCEY, chromID *C. difficile* agar). To enhance the selectivity, it is also advised to culture heat- or alcohol shock pre-treated samples onto a (non)-selective medium.
- A lower cost option is to plate the faeces onto a medium not specifically selective for *C. difficile* (CNA) subsequently after heat or alcohol shock.

Sample preparation

For alcohol shock:

- Use ~1 mL or a small pea-sized portion of faeces to make an approximate 1:1 suspension in 70% methyl alcohol or 96% ethanol in a sterile test tube.
- Mix by vortexing and leave to settle at room temperature for 30–60min.
- With a sterile disposable Pasteur pipette, inoculate 2 drops (approximately 50–75 uL) of deposit on a selective agar and spread to obtain single colonies.
- Transfer promptly to anaerobic jar or workstation, incubate at 37 °C for 24–48 hrs (preferably 48 hrs).

For heat shock:

- Use ~1 mL or a small pea-sized portion of faeces, add to 1 mL PBS, homogenise and heat at 80 °C for 10 min.
- Centrifuge (4 000 x g for 1 min) and add 100 mL supernatant to a selective agar and spread to obtain single colonies.
- Transfer promptly to anaerobic jar or workstation, incubate at 37 °C for 24–48 hrs (preferably 48 hrs).

Culture interpretation/identification of *C. difficile*

Supplements, such as taurocholate in *C. difficile* selective agars inhibit spore formation and stimulate germination. The (vegetative) colonies on selective agar are oxygen sensitive. Do not leave selective plates exposed to air for longer than 30 mins. Conversely, mature (sporulating) colonies on non-selective agar are very oxygen-tolerant and may remain viable after storage on the bench for several days.

The isolation and presumptive identification of *C. difficile* colonies can be confirmed by a number of different criteria:

Characteristic colony morphology, colonies of varying sizes may appear on the same plate, diameter<1–5 mm (see figure):

- TCCA: grey/whitish, flattish, irregular-edged, non-haemolytic colonies.
- CLO medium: grey/whitish, flattish, irregular-edged, non-haemolytic colonies.

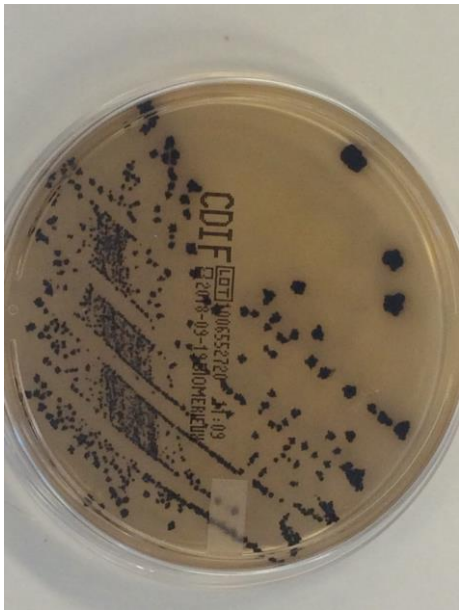
- chromID *C. difficile*: grey to black, irregular-edged colonies.
- CCEY: non-coloured, flattish, irregular-edged. There is no opacity around the colonies (lecithinase not produced, unlike *C. bifermentans*/*sordellii* or *C. perfringens*). A sample can be seen here: www.eolabs.com/product/pp4070-braziers-ccey-agar/#iLightbox

For the chromID *C. difficile* agar it is important to keep in mind that certain *C. difficile* strains (RT 023, 056, 058, 059 & 248) are non-pigmented.

Growth of *C. difficile* on different agars

Figure 2.1 *C. difficile* on blood-based agar

Figure 2.2. *C. difficile* on chromID *C. difficile* agar



Confirm characteristic colonies

- Gram-staining of colony from non-selective medium revealing gram-positive rods with subterminal spores.
- Optional: green-yellow fluorescence under long-wave UV light.

Confirm isolate as C. difficile

Use one of the following methods:

- Agglutination with *C. difficile* latex slide agglutination method (various companies).
- MALDI-TOF MS using Bruker or Shimadzu (Biomerieux) platform (Kim et al.). Building a specific library will also enable the labs to detect certain PCR ribotypes (Reil et al.).
- Proline-aminopeptidase disk as described by Fedorko et al.

- PCR with glutamate dehydrogenase gene (GluD) or triose phosphate isomerase gene (TPI) as target (as described by Paltansing et al. or Lemee et al.).

Confirm toxin production

Isolation of *C. difficile* is not sufficient to diagnose CDI (Diagnostic guidance document of Crobach et al.). Not all *C. difficile* strains produce toxin. Non-toxigenic isolates can be considered as non-pathogenic. The toxin production status of individual *C. difficile* isolates can be determined by a multiplex PCR with *tcdA*, *tcdB* and binary toxin as target (Paltansing et al.). Second best alternatives are to use a cell cytotoxicity neutralization assay (CCNA) or a *C. difficile* toxin A/B EIA using in-house protocols.

Quality control

Check each new batch of agar for growth and typical characteristics with a *C. difficile* reference strain.

Use known positive and negative controls for Gram stain, UV fluorescence, the proline-specific aminopeptidase kit, latex agglutination, MALDI-TOF and the PCR reaction and record results of these controls.

Interpretation of results

Fluorescence is dependent on the presence of blood in the medium and may fade if plates are exposed to air (>1/2h), and in old cultures. Some other bacteria may fluoresce yellow/green or red. This test must be used in conjunction with other confirmatory tests. The Microscreen *C. difficile* latex agglutination test has been shown to cross-react with certain other clostridia, including *C. sordellii* and *C. bifermentans*. Therefore it is essential to perform this test in conjunction with UV fluorescence to avoid false identifications. The manufacturer's procedure uses 1 drop (~50 µL) of each reagent from a dropper pipette/screw cap of reagent. However, in the interests of economy, efficacy of using reduced volumes has been evaluated. It has been found that reagent volumes may be reduced to10 µL without compromise to readability, accuracy or reproducibility of results. Apart from this modification, follow the manufacturer's instructions for storage and performance of this test.

Reporting results

Report through usual laboratory management system as a *Clostridium difficile* or no *Clostridium difficile* isolated.

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3. Standard operating procedure for multiplex PCR for characterisation of *Clostridium difficile*

Safety issues

When working at the laboratory with living microorganisms, there are operations involved which can cause infection in laboratory workers. It is necessary to apply general safety requirements for microbiological laboratories.

The multiplex PCR should be carried out under containment level 2 conditions using the principle of good laboratory practice, OR containment level 3 if hazard-group-3 organisms are also suspected in the specimen. Standard laboratory precautions must be adhered to.

Clostridium difficile is an environmental organism (Advisory Committee on Dangerous Pathogens (ACDP) hazard group 2) that may be carried asymptomatically in the intestine and is known to cause antibiotic-associated diarrhoea in susceptible persons. Symptoms range from mild diarrhoea to pseudo-membranous colitis, toxic mega colon or death. Susceptible persons are those with disturbed intestinal flora (commonly due to usage of broad-spectrum antibiotics) and/or immunosuppressed. Risk is increased in elderly (> 65 years) patients but is extremely low in children (< 2 years).

A successful use of PCR technology is only possible if the established rules are followed to avoid contamination. PCR is a 'one-way' process carried out in the direction of PCR 1 -> PCR 2 -> 3 PCR.

PCR 1: clean room for storage and aliquot of stock reagents, primers and probes. To create primer-probe mix and make reaction mixes.

PCR 2: this space serves to extract the nucleic acids from clinical material and to put in the PCR reaction mixes. Remember that every sample can in principle be a high positive sample and therefore a source of contamination.

Amplification of nucleic acids takes place in a closed system PCR.

PCR 3: post-PCR area, this is the "dirty space". It contains electrophoresis equipment, and this is the only area where you can work with PCR products.

Always use gloves when working with PCR products.

SYBR-based dyes: Be careful when working with SYBR-based dyes as they are a possible carcinogen.

Precautions

Micro centrifuge: Check tubes are equally weighted and placed in a balanced position. Ensure that the lid is sealed and all fittings secured. Deal with any spillage promptly as per apparatus SOP. If equipment creates unusual noise or movement, switch off immediately, stand clear, alert senior personnel and do not open lid. Refer to apparatus SOP for further action.

Introduction

Clostridium difficile consist of different PCR ribotypes which can be determined with PCR-ribotyping. Since 2002, a so called 'hypervirulent' PCR ribotype 027 has been circulating in the United States, Canada and Europe. In order to detect this hypervirulent type several confirmation tests can be performed. These tests examine the presence of the genes coding for toxins A and B and the gene for the binary toxin.

The two major toxins of *C. difficile* are the enterotoxin A and the cytotoxin B.

The *Tcd*A gene, encoding the 308 Kd enterotoxin A, is present in approximately 90% of all toxin-producing *C. difficile* strains. In PCR ribotype 017, a deletion of 1.8 kb is present in the *TcdA* gene as well as a mutation which introduces a stop codon, thus producing a non-functional toxin A. There are also a number of strains with other deletions occurring in the *Tcd*A gene. All these deletions are not detected with this multiplex PCR.

The *TcdB* gene, encoding for cytotoxin B, is present in all toxin-producing *C. difficile* strains and occurs in approximately 5% of the strains that do not produce *TcdA*. All toxicogenic PCR ribotypes contain *TcdB*.

A third toxin is a binary toxin encoded by *cdtA* and *cdtB* which encode for the two enzymatic parts.

The presence or absence of these three toxins are used to characterise pathogenic *C. difficile* isolates. Two controls are always included in the multiplex PCR; a first control to test appropriate DNA isolation using a 16S primer. A

second control is directed to the presence of the gene for glutamate dehydrogenase, confirming identification of *C. difficile*.

The *gluD* PCR is based on the detection of the *gluD* gene coding for glutamase dehydrogenase (GDH) that is only present in *C. difficile*. GDH is a metabolic enzyme that is produced in a high degree by all *C. difficile* strains. Following isolation of total bacterial DNA from suspected colonies and/or patient materials for *C. difficile* (if present), the specific target DNA is amplified with support of a polymerase chain reaction (PCR).

This instruction describes the operating procedure for identification of C. difficile and to determine the presence of toxic genes. This is performed by multiplex PCR and read by agarose gel or capillary electrophoresis system

Primers

Table 3.1. Information about the primers for multiplex PCR

Name	Target	Sequence	Amplicon size
tcdA-F	*TcdA* gene	5'-GCATGATAAGGCAACTTCAGTGGTA-3'	629
tcdA-R		5'-AGTTCCTCCTGCTCCATCAAATG-3'	
tcdB-F	*TcdB* gene	5'-CCAAARTGGAGTGTTACAAACAGGTG-3'	410
tcdB-RA		5'-GCATTTCTCCATTCTCAGCAAAGTA-3'	
tcdB-RB		5'-GCATTTCTCCGTTTTCAGCAAAGTA-3'	
cdtA-FA	*cdtA* gene	5'-GGGAAGCACTATATTAAAGCAGAAGC-3'	221
cdtA-FB		5'-GGGAAACATTATATTAAAGCAGAAGC-3'	
cdtA-R		5'-CTGGGTTAGGATTATTTACTGGACCA-3'	
ctdB-F	*cdtB* gene	5'-TTGACCCAAAGTTGATGTCTGATTG-3'	262
cdtB-R		5'-CGGATCTCTTGCTTCAGTCTTTATAG-3'	
PS-F	16S-rDNA	5'-GGAGGCAGCAGTGGGGAATA-3'	1062
PS-R		5'-TGACGGGCGGTGTGTACAAG-3'	
GluD-F	*gluD* gene	5'-GTCTTGGATGGTTGATGAGTAC-3'	158
GluD-R		5'-TTCCTAATTTAGCAGCAGCTTC-3'	

Scope

To detect the presence of the genes coding for toxins A and B, and the gene for the binary toxins in C. difficile strains by agarose gel or capillary electrophoresis system.

Reagents

PCR

- Hotstar taq mastermix (203446, Qiagen Benelux BV).
- RNAse free water (203446, Qiagen Benelux BV).

Biological materials

DNA isolated from colonies of pure growth of *C. difficile* grown on blood agar incubated anaerobically at 37 °C for 24–48 hrs.

Equipment

- Bio-Rad Molecular Imager GelDoc XR (or equivalent)
- Adjustable pipettes 1–1000 µL and tips – one set each for DNA extraction 'dirty', PCR set-up 'clean' and post-PCR areas.
- Sterile 0.5 mL and 1.5 mL Eppendorf tubes
- Sterile thin-walled 0.5 mL Eppendorf tubes (for PCR)
- Vortex mixer
- Micro centrifuge
- -20 °C freezer
- Laminar flow cabinet
- PCR machine
- 60 °C water bath
- GelCompar/BioNumerics software

- A gel-electrophoresis or automated high-resolution capillary electrophoresis such as MultiNA (Shimadzu) or QIAxcel (Qiagen)

Procedures

To minimise the risk of DNA contamination it is essential that the various stages of this procedure are performed in the designated areas/laboratories using reagents, racks, pipettes and other equipment assigned to that area only (PCR 1: laboratory for preparing PCR mix, PCR 2: laboratory for DNA isolation, pipetting DNA to the PCR mix, PCR 3: running gel electrophoresis or capillary electrophoresis system).

Do not transfer laboratory coats, reagents, equipment or amplified DNA from the PCR-1 room to other PCR areas. Use sterile filter tips and pipettes designated 'clean' for non-DNA reagents or 'dirty' for DNA-containing solutions. Clean benches and racks etc. regularly with 1% sodium hypochlorite solution, and always after any spillage. Then rinse with water.

- Isolate the DNA with your preferred protocol.
- The multiplex PCR form calculates the PCR mix, take for 'number' the number of reactions +3 (for a positive and negative control), see Addendum below.

Multiplex PCR

The PCR mix for one reaction consists of:

Hot star mastermix	12.5 µl
tcdA-F	1 µl
tcdA-R	1 µl
tcdB-F	0.4 µl
tcdB-RA	0.2 µl
tcdB-RB	0.2 µl
cdtA-FA	0.05 µl
cdtA-FB	0.05 µl
cdtA-R	0.01 µl
cdtB-F	0.01 µl
cdtB-R	0.01 µl
PS-F	0.05 µl
PS-R	0.05 µl
GluD-F	0.1 µl
GluD-R	0.1 µl
H_2O	6.7 µl
Total	22.5 µl

Primer conc: 50 pmol/µl

PCR 1

- prepare the PCR mix in accordance with the multiplex PCR form and enter the mix in sterile thin-walled 0.5 mL Eppendorf tube strips (for PCR). Code the strips and store in the refrigerator. See addendum below.

PCR 2

- remove the positive control from -20 °C. (The UK027)
- pipette 2.5 µl of the isolated DNA in the PCR mix including the positive control. The total volume is 25 µl
- close the strip.

PCR 3

- perform the PCR on your PCR system in accordance with the programme (this protocol was optimised for a Bio-Rad MyCycler PCR system).

PCR programme

15 min	94 °C	
45 sec	94 °C	
45 sec	50 °C	35X
1 min	72 °C	

30 min	72 °C	
Hold	15 °C	

When the PCR is done, the products are transferred to an agarose gel or to an automated high-resolution capillary electrophoresis system, such as MultiNA (Shimadzu) or QIAxcel (Qiagen).

Quality control

Adherence to this SOP will help to maintain quality of the procedure.

Positive and negative controls should be processed with every batch and yield expected results.

Results are invalid if positive or negative controls give unexpected results. In this case, the entire batch must be repeated.

Gels or capillary electrophoresis patterns cannot be analysed unless the correct molecular size ladder is used and in the case of the gel, ladder lanes are in place.

Bands of test isolates must be sufficiently clear and dense for reliable analysis.

Interpretation of results

On the basis of the specific banding pattern, it can be determined if the strains are positive or negative for the various toxin genes. When the strain is positive, bands/peaks are visible at:

TcdA	629 bp
TcdB	410 bp
cdtA	221 bp
cdtB	262 bp
16S	1062 bp
GDH	158 bp

The positive control 027 is positive for all toxins, so all the peaks should be screened at the right size whether the strain may be read.

If there is no peak, the current strain is negative for that toxin. The peaks for the 16S and GDH should always be visible. The 16S determines whether there is DNA present and the GDH is decisive for a *Clostridium difficile*.

Important note: This PCR does not detect the deletions in the TcdA gene. A separate PCR should be performed to detect these [3,4].

Report of results

Report through usual laboratory management system as '*Clostridium difficile* isolated'.

TcdA	When there is a product of 629 bp	*TcdA* positive
	When there is not a product of 629 bp	*TcdA* negative
TcdB	When there is a product of 410 bp	*TcdB* positive
	When there is not a product of 410 bp	*TcdB* negative
cdtA	When there is a product of 221 bp	*cdtA* positive
	When there is not a product of 221 bp	*cdtA* negative
cdtB	When there is a product of 262 bp	*cdtB* positive
	When there is not a product of 262 bp	*cdtB* negative
16S	When there is a product of 1062 bp	DNA present
	When there is not a product of 1062 bp	No DNA present
GDH	When there is a product of 158 bp	*Clostridium difficile* positive
	When there is not a product of 158 bp	No *Clostridium difficile*

Bibliography

1. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A and toxin B and the binary toxin genes applied to a Danish strain collection. Clin Microbiol Infect. 2008;14: 1057-1064.
2. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuijper EJ. Characteristics and incidence of *Clostridium difficile*-associated disease in the Netherlands, 2005. Clin Microbiol Infect. 2007;13:1058-64.
3. Kato H, Kato N, Katow S, Maegawa T, Nakamura S, Lyerly DM. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol Lett. 1999 Jun 15;175(2):197-203.
4. Drudy D, Fanning S, Kyne L. Toxin A-negative, toxin B-positive *Clostridium difficile*. Int J Infect Dis. 2007 Jan;11(1):5-10. 2007;11:5-10.
5. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, et al. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol. 2008 Feb; 46(2): 431–437.

Addendum 3-1

Multiplex PCR form.

Preview:

A44 PCR

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	**Multiplex PCR form**												
2													
3	**Date:**												
4	**Technician:**												
5													
6													
7		**Mix**											
8		Primer conc: **50 pmol/ul**											
9				1	10	← **fill in number of samples**							
10		Hotstar mastermix		12.5	125								
11		tcdA-F		1	10								
12		tcdA-R		1	10								
13		tcdB-F		0.4	4								
14		tcdB-RA		0.2	2								
15		tcdB-RB		0.2	2								
16		cdtA-FA		0.05	0.5								
17		cdtA-FB		0.05	0.5								
18		cdtA-R		0.1	1								
19		cdtB		0.1	1								
20		cdtB-R		0.1	1								
21		PS-F		0.05	0.5								
22		PS-R		0.05	0.5								
23		GluD-F		0.1	1								
24		GluD-R		0.1	1								
25		H_2O		6.7	67								
26		Total		22.5	225								
27													
28	**Samples**												
29		1	2	3	4	5	6	7	8	9	10	11	12
30													
31													
32													

Addendum files are available for download (Excel format) from the ECDC website. Addenda can be found on the same

4. Standard operating procedure for capillary ribotyping of *Clostridium difficile*

Safety issues

When working with microorganisms, operations are involved which can cause infection. Always work under the general safety requirements for microbiological laboratories.

This method should be carried out under containment-level-2 conditions using the principle of good laboratory practice, OR containment level 3 if hazard-group-3 organisms are also suspected in the specimen. Standard laboratory precautions must be adhered to.

Clostridium difficile is an environmental organism (Advisory Committee on Dangerous Pathogens (ACDP) hazard group 2) that may be carried asymptomatically in the intestine and is known to cause antibiotic-associated diarrhoea in susceptible persons. Symptoms range from mild diarrhoea to pseudo-membranous colitis, toxic mega colon or death. Susceptible persons are those with disturbed intestinal flora (commonly due to usage of broad-spectrum antibiotics) and/or immunosuppressed. Risk is increased in elderly (> 65 years) patients but is extremely low in children (< 2 years).

A successful use of PCR technology is only possible if the established rules are followed to avoid contamination. PCR is a one-way process carried out in the direction of PCR 1 -> PCR 2 -> 3 PCR.

PCR 1: Clean room for storage and aliquot of stock reagents, primers and probes. To create primer-probe mix and make reaction mixes.

PCR 2: This space serves to extract the nucleic acids from clinical material and to put in the PCR reaction mixes. Remember that every sample can in principle be a high positive sample and therefore a source of contamination.

Amplification of nucleic acids takes place in a closed system PCR.

PCR 3: Post-PCR area, this is the 'dirty space'. It contains electrophoresis equipment, and this is the only area where you can work with PCR products.

- Always use gloves when working with PCR products.
- Mop up any spillage with paper towels, wash area with water, mop with towels and dispose of these in designated bin or bag.
- Risks are minimised by the small volumes used, by use of ready-prepared solution, and by adherence to the above methodology.
- Wear clean disposable gloves for all steps of this procedure to prevent contamination of reagents with bacterial DNA from shed skin particles.

Precautions

Heating block: Care must be taken to avoid burns when placing/removing tubes from heating block. Switch off after use.

Micro centrifuge: Check tubes are equally weighted and placed in a balanced position. Ensure that the lid is sealed and all fittings secured. Deal with any spillage promptly as per apparatus SOP. If equipment creates unusual noise or movement, switch off immediately, stand clear, alert senior personnel and do not open lid. Refer to apparatus SOP for further action.

Hi-Di Formamide is a highly deionised formamide, formulated with a stabiliser which can cause eye, skin, and respiratory tract irritation and is a possible developmental and birth defect hazard.

Introduction

Clostridium difficile consist of different PCR ribotypes which can be determined by PCR ribotyping. The basis for PCR ribotyping relies on the presence of 11 RNA operons at different locations in the DNA. The number and the location of these operons varies between *C. difficile* strains. As a result, the number and size of the fragments that lie between these operons (intergenic spacer region, ISR) also varies. Each PCR ribotype has a specific band pattern that can be determined through capillary gel electrophoresis after amplification with specific primers.

These primers are complementary to the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene, with this, the variable ISR is amplified. The addition of a fluorescent label (e.g. 6FAM or TET, on the forward primer) allows the forward strand of each PCR fragment product to be detectable on an automated fragment analysis

system. The simultaneous inclusion of a fluorescent molecular marker allows highly accurate sizing of labelled fragments.

Target: ribosomal DNA operons

Table 4.1. Information on the three different primer sets that can be used for capillary ribotyping

Primer set	Label	Sequence (5' -> 3')	Comment
Stubbs	6FAM or TET	CTG GGG TGA AGT CGT AAC AAG G	
		GCG CCC TTT GTA GCT TGA CC	
Bidet	6FAM or TET	GTGCGGCTGGATCACCTCCT	Preferred primer set
		CCCTGCACCCTTAATAACTTGACC	
Janežič	6FAM or TET	GCTGGATCACCTCCTTTCTAAG	
		TGACCAGTTAAAAAGGTTTGATAGATT	

Fragment size: 200–600 bp

Bidet primers are the preferred primer sets to be used, especially if you want to send your results to Leiden or Leeds. Their databases are built on strains processed with Bidet primers and for comparison reasons those strains need to be performed with Bidet primers also.

If you would like to make use of the online database Webribo (webribo.ages.at) you can use any of the three primer sets stated in Table 5.1.

Scope

Typing of *C. difficile* strains by capillary ribotyping.

Reagents

PCR

- Hotstar taq mastermix (203446, Qiagen Benelux BV)
- RNAse free water (203446, Qiagen Benelux BV).

Capillary electrophoresis/fragment analysis

- Hi-Di Formamide (Applied Biosystems, PN: 4311320)
- GeneScan 1200 LIZ size standard (Applied Biosystems, PN: 4379950)
- POP-7 Polymer (Applied Biosystems, PN: 4352759)
- 10 x Genetic Analyser Buffer with EDTA, (Applied Biosystems, PN: 402824).

Biological materials

- DNA isolated from colonies of pure growth of *C. difficile* grown on blood agar incubated anaerobically at 37 °C for 24–48 hrs.

Equipment

- Adjustable pipettes 1–1000 µL and tips – one set each for DNA extraction 'dirty', PCR set-up 'clean' and post-PCR areas.
- Sterile 0.5 mL and 1.5 mL Eppendorf tubes
- Sterile thin-walled 0.5 mL Eppendorf tubes (for PCR)
- Vortex mixer
- Micro centrifuge
- -20 °C freezer
- Laminar flow cabinet
- PCR machine
- GelCompar/BioNumerics software (created by Applied Maths NV. Available from http://www.applied-maths.com).
- MultiNA MCE-202, Microchip Electrophoresis System for DNA/RNA Analysis (created by Shimadzu. Available from: http://www.shimadzu.com).
- Automated Sequence and Fragment Analysis System, like;
  - Applied Biosystems 3500xl (ThermoFisher Scientific)
  - ABI-PRISM 3130*xl* (Applied Biosystems)
- 96-well PCR plate (Thistle, PN: 107344 or Thermo Fisher Schientific)
- Capillary array, for your system

- Plate base, for your system
- Plate retainer, for your system
- Plate septa for a 96-wells plate, Thermo Fisher Scientific
- Septa for buffer plates.

Procedures

To minimise the risk of DNA contamination it is essential that the various stages of this procedure are performed in the designated areas/laboratories using reagents, racks, pipettes and other equipment assigned to that area only. (PCR 1: laboratory for preparing PCR mix, PCR 2: laboratory for DNA isolation, pipetting DNA to the PCR mix, PCR 3: running PCRs).

Do not transfer laboratory coats, reagents, equipment or amplified DNA from the PCR-1 room to other PCR areas. Use sterile filter tips and pipettes designated 'clean' for non-DNA reagents or 'dirty' for DNA-containing solutions. Clean benches and racks etc. regularly with 1% sodium hypochlorite solution, and always after any spillage. Then rinse with water.

Principles of CE ribotyping

Capillary electrophoresis (under denaturing conditions) allows single-stranded DNA fragments to be resolved, based on their relative size.

The single-stranded DNA fragments are injected from sample wells into individual capillaries and a potential difference is applied across the capillary. The DNA (which is negatively charged) migrates through the capillary towards the anode. The relative migration distance of each DNA fragment through the POP polymer (contained in the capillaries) is dependent on molecular size and total charge.

Before they reach the anode, the labelled fragments pass a laser, which excites the fluorescent dye. Subsequent fluorescent patterns are converted into digital data and visualised/interpreted using analysis software. The inclusion of a fluorescently-labelled marker (DNA fragments of known size) with each sample allows an accurate size estimation of all fragments to be made.

Capillary ribotyping PCR

Note: This protocol is optimised for a Bio-Rad MyCycler PCR systems.

- CE PCR ribotyping is performed on suspected *C. difficile* strain.
- Isolate the DNA with any DNA isolation method that produces high-quality DNA (e.g. any commercial kit available).
- The PCR ribotype form (see Addendum below) calculates the PCR mix; as 'number' use the number of reactions +3 (for a positive and negative control).

The PCR mix for 1 reaction for CE PCR ribotyping PCR consists of:

Hotstar taq mastermix	12.5 µl
16S primer (6FAM/TET)	0.25 µl
23S primer	0.25 µl
H_2O	10 µl
Total:	23 µl

Primer conc: 10 pmol/µl

PCR 1

- Prepare the PCR mix and aliquot the mix in sterile thin-walled 0.5 mL Eppendorf tube strips (for PCR). Code the strips and store in the refrigerator. See Addendum below.

PCR 2

- Remove the positive control from -20 °C. (e.g. DNA from the UK027 strain).
- Pipette 2 µl of the isolated DNA in the PCR mix (including the positive control). The total volume is 25 µl.
- Close the strip.

PCR 3

- Perform the PCR on your PCR system in accordance with the programme.

PCR programme

15 min	95 °C	24X
1 min	95 °C	
1 min	57 °C	
1 min	72 °C	
30 min	72 °C	
Hold	15 °C	

- When the PCR is complete, the products need to be prepared for analysis on the automated fragment analyser.
- Make a mix containing 8.5 µL Hi-Di, 0.5 µL 1200LIZ marker and 1 µL PCR-product per sample.
- Pipet 9 µL in each well of an ABI 96-well plate. (Fill all the wells that won't be used in the injection with 10 µL Hi-Di. On the ABI 3500XL instrument 1 injection is 3 lanes. This instrument reads the plate from top to bottom (example: one injection will consist of A1-H1; A2-H2; A3-H3).
- Heat the plate for 5 minutes at 95 °C, then set on ice to stop the reaction.
- Keep the plate on ice until analysis on the ABI.

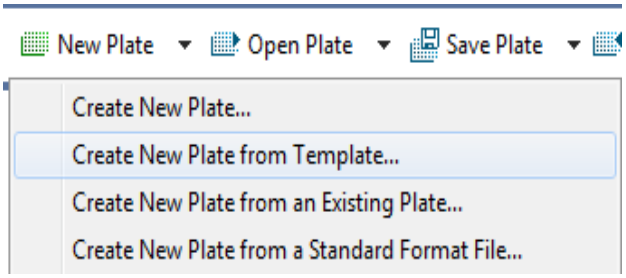
ABI 3500XL

Note: This protocol is optimised for the Applied Biosystems 3500xl (ThermoFisher Scientific) and assumes that the user was trained on the automated sequencer and is familiar with: 1) daily/weekly maintenance requirements and 2) data collection software associated with this instrument.

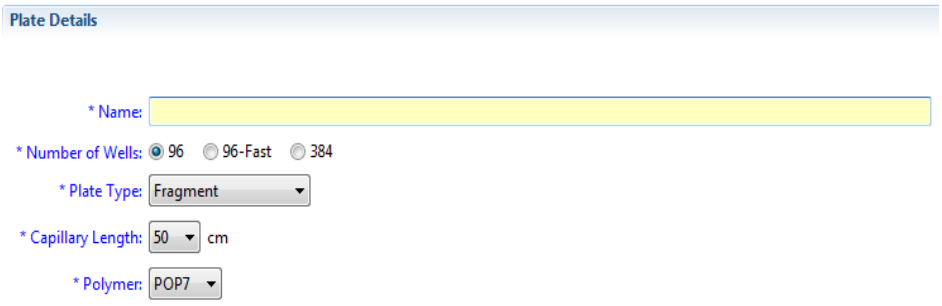
- Press the tray button on the front of the ABI3500.
- Open the door when the tray stopped moving.
- Place the plate in the blue holder and put the white top on.
- Place the plate with the cut-off right corner down directly into the ABI.
- Close the door.

Make a new plate in the ABI:

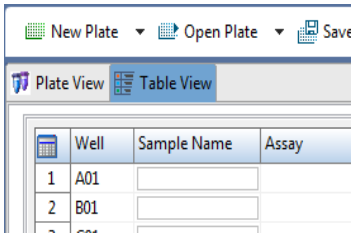
- Open the 3500 series data collection software 2.
- Under the dashboard icon choose -> *Create New Plate.*



- Fill in the *runname*.



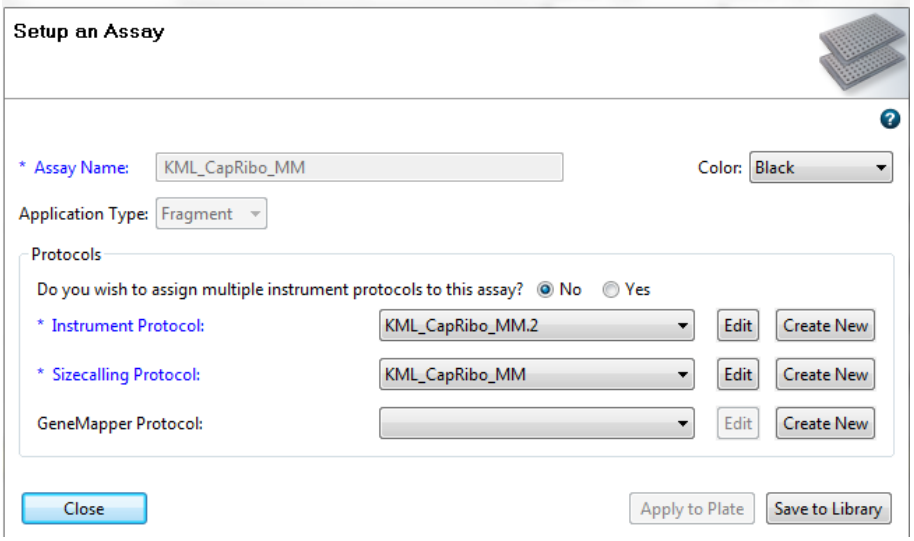
- Under secondary analysis, choose *Assign Plate Contents.*
- A new screen appears, choose *Table View.*



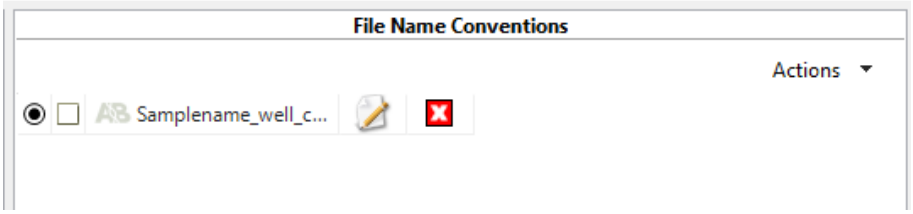
- Fill in all the samples in the right positions.
- Important: one injection is 24 samples; if the number of samples is less than 24, fill in the remaining wells with HI-DI.

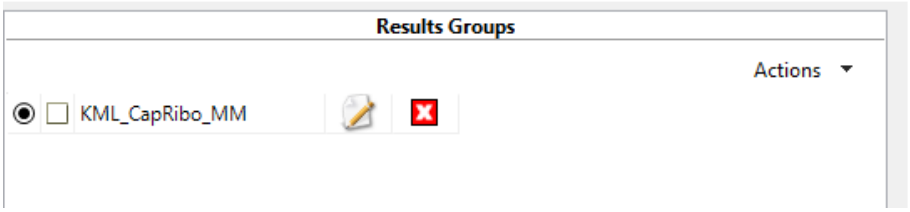
For assays:

- Choose protocols:
  Instrument protocol, perform capillary electrophoresis in accordance with the following parameters:
  - Application type: Fragment
  - Capillary length: 50 cm
  - Polymer: POP7
  - Run module: FragmentAnalysis50_POP7xl
  - Dyeset: G5
  - Oven temperature: 60^0C
  - Electrophoresis run voltage: 15.0 kV
  - PreRun voltage: 15.0 kV
  - Sample injection voltage: 5 kV
  - Electrophoresis run time: 3000 sec.
  - PreRun time: 180 sec.
  - Sample injection time: 5 sec.
  - Date delay: 80 sec.
- Sizecalling protocol: default for LIZ1200



- Do the same for *File Name Conventions* and *Results Groups*.



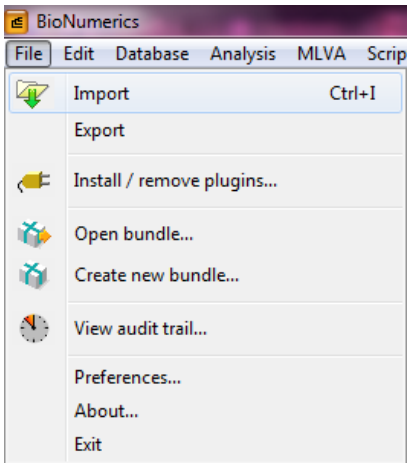


- The *File Name Conventions* will state in which order your sample is named. For example *Samplename_well_capillarynumber*.
- The *Results Group* will place data collection results for the run (.fsa files) in your designated folder on the PC that assists the sequencer.
- Select the sample box: *Assays, File Name Conventions* and *Results Groups*.
- Choose *Link Plate for Run*.
- Click on *run* when the plate is linked correctly.
- 1 injection is 1 hour and 17 minutes. (24 samples).

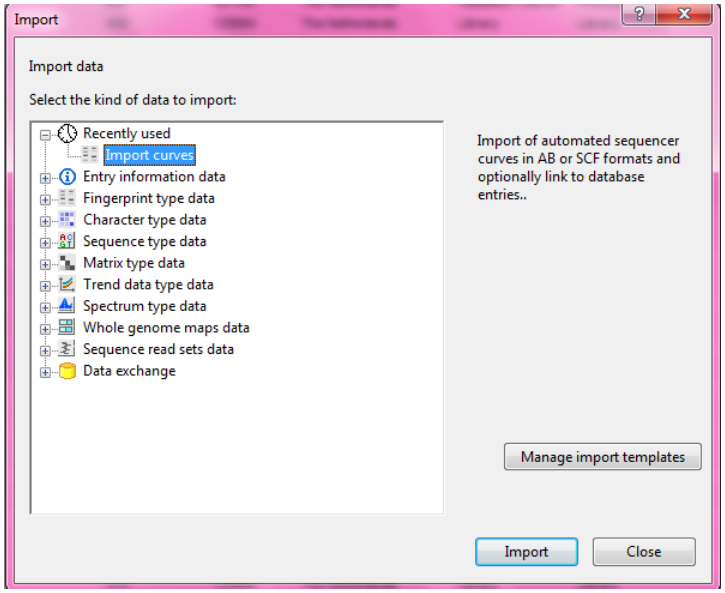
Analyse the FSA files in BioNumerics 7.6

Note: This protocol assumes that the user was trained on, and is familiar with, BioNumerics (Applied Maths) software.

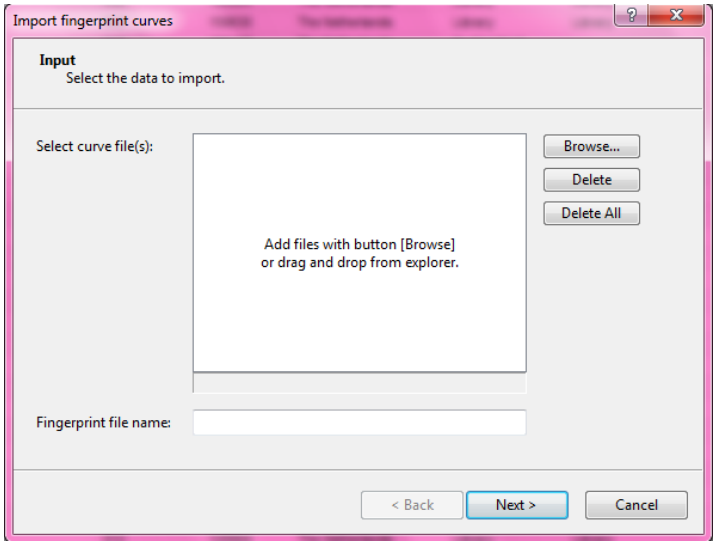
- Open BioNumerics 7.6
- Import the FSA files by selecting *Import* under *File* (upper left) or pressing *Ctrl+I* 

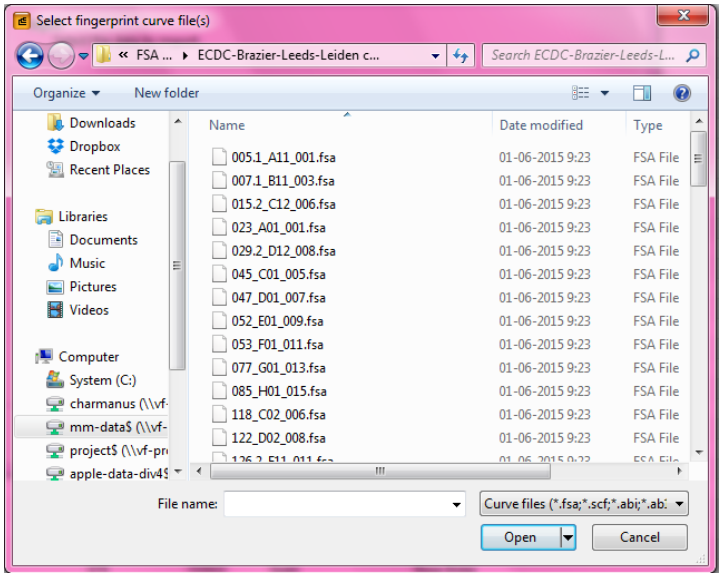


- Choose *Import curves* and click *Import*.

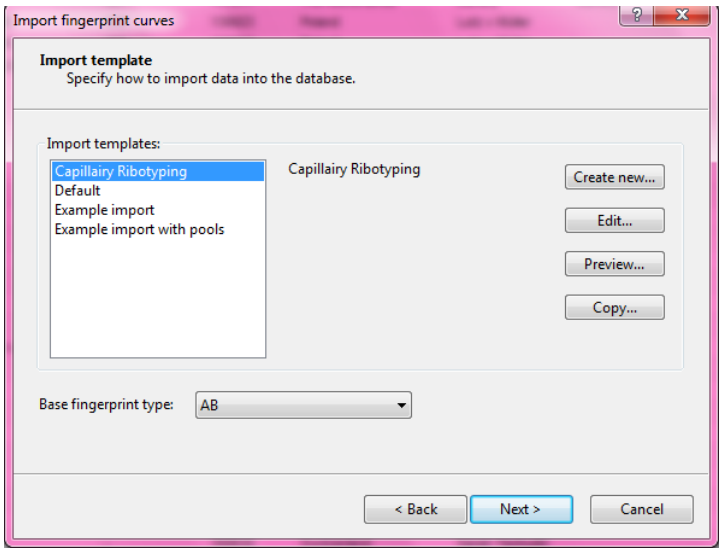


- Select the FSA files you want to import by clicking *Browse*; select the files you want to import from where you saved them.

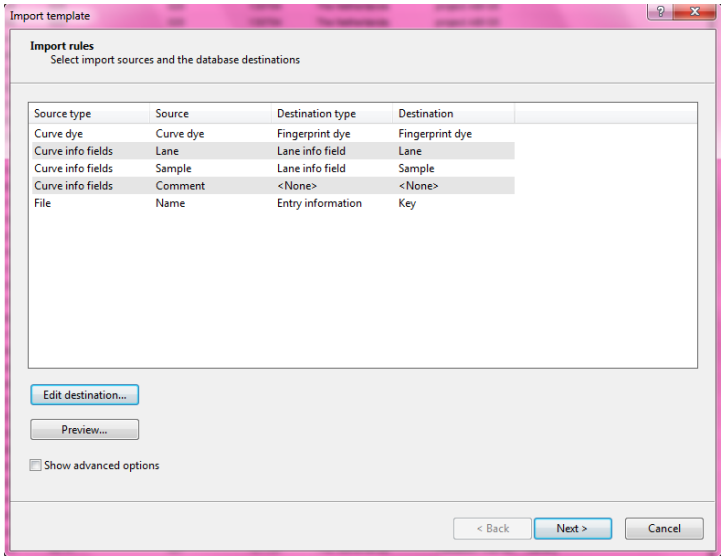


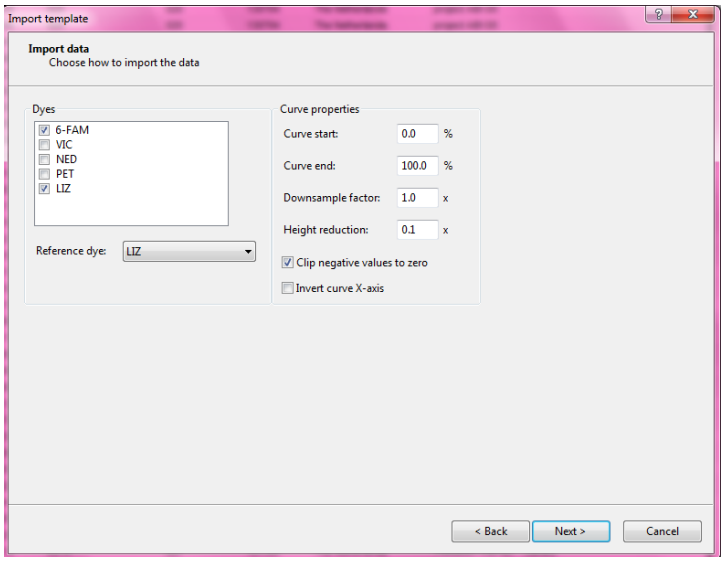


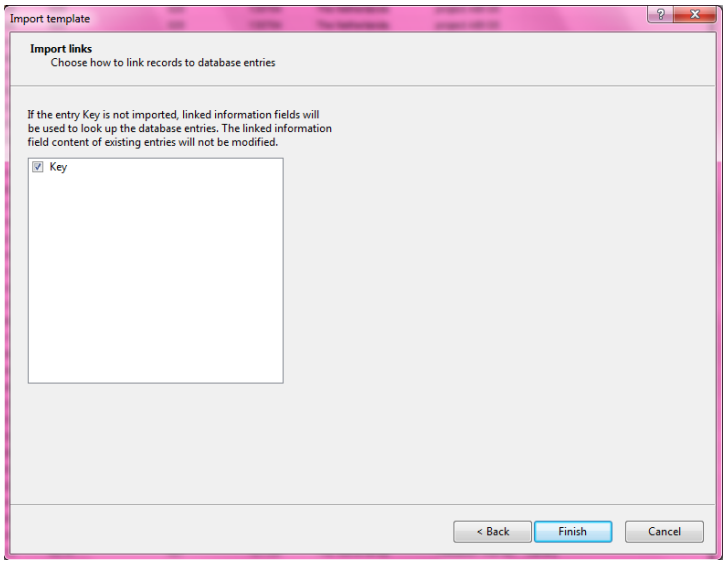
- Select the files you want to view and click *Open.*
- Click *Next.*



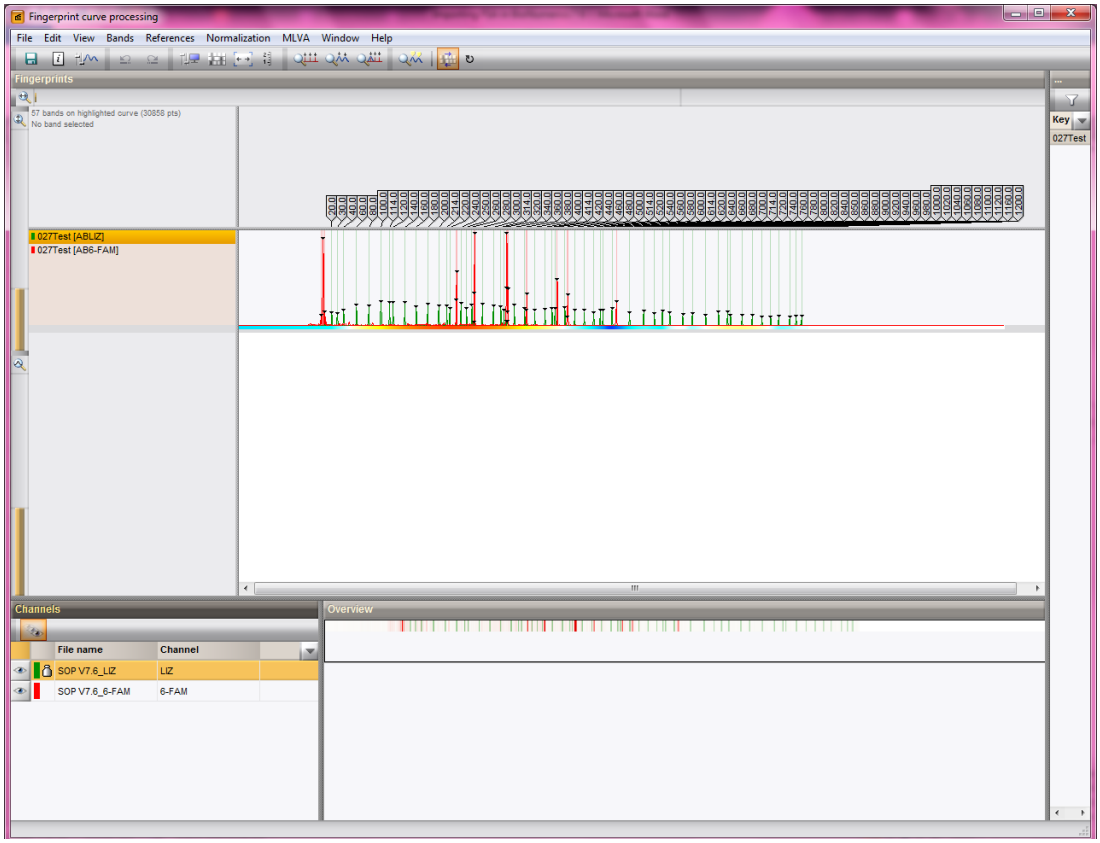
- Select which template you want to import and click *Next* twice and then *Finish*.
- This is the template used at LUMC.



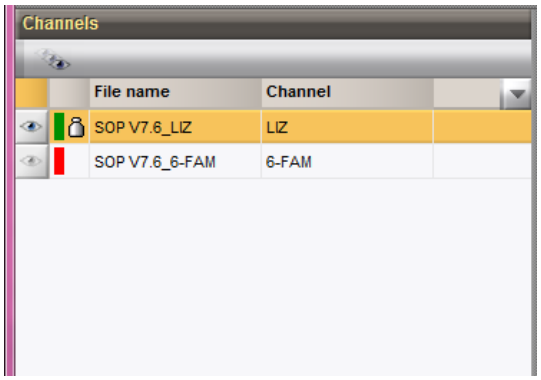




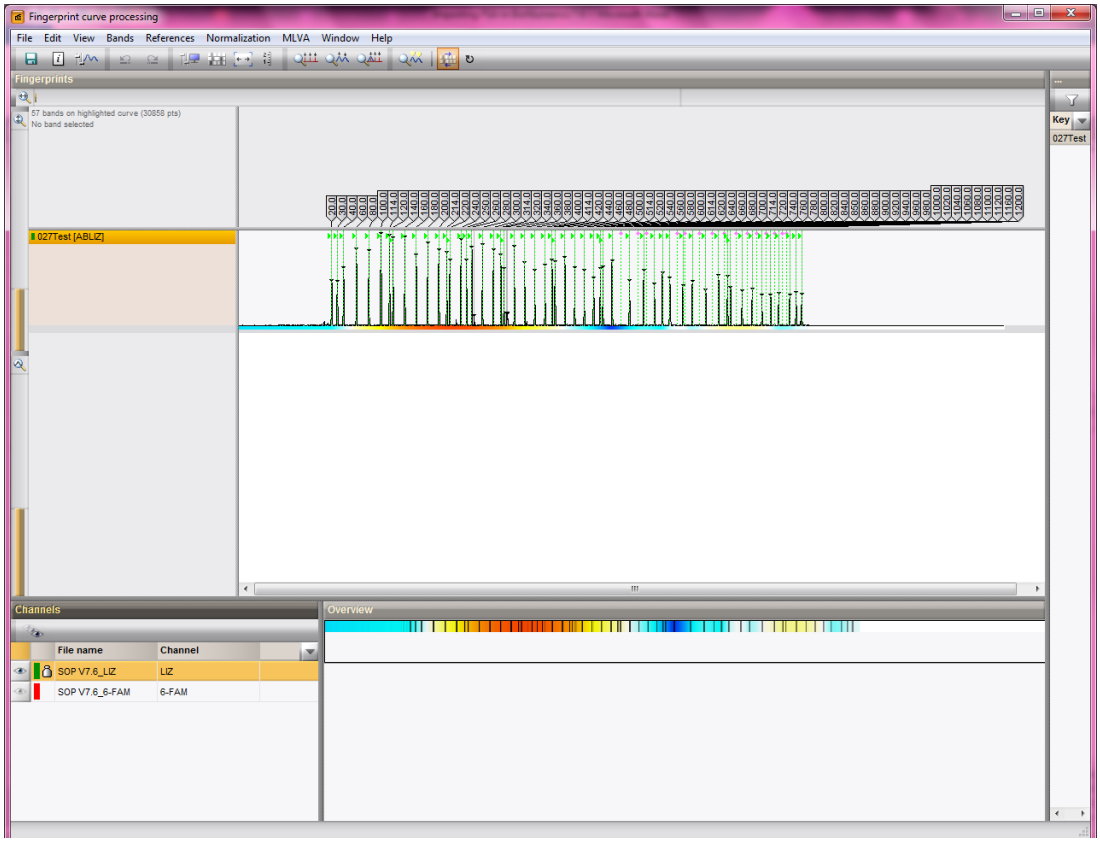
- A window for fingerprint curve processing opens automatically.



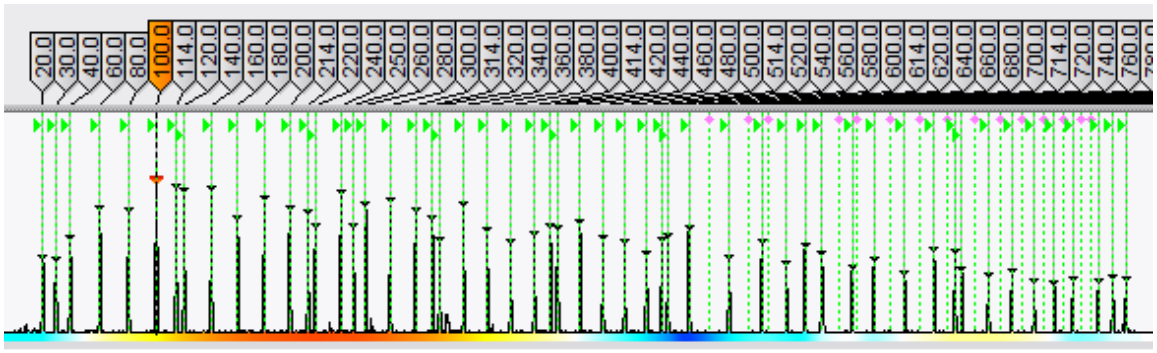
- Switch the 6-FAM channel off by clicking on the eye.



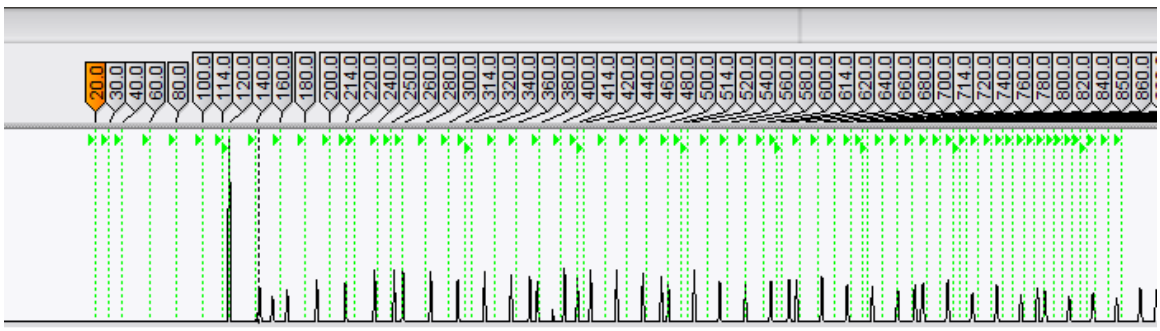
- Only the LIZ1200 marker will be visible now.



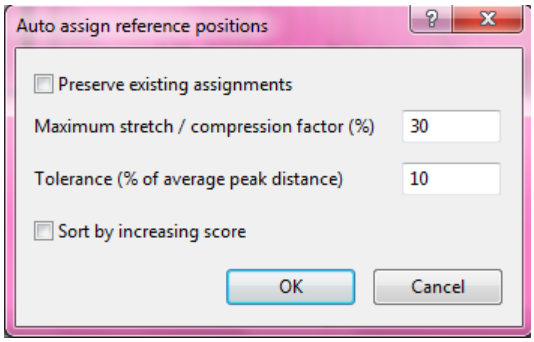
- Check if all the peaks were assigned the right size by clicking on the peak; the corresponding size will turn orange in the reference.



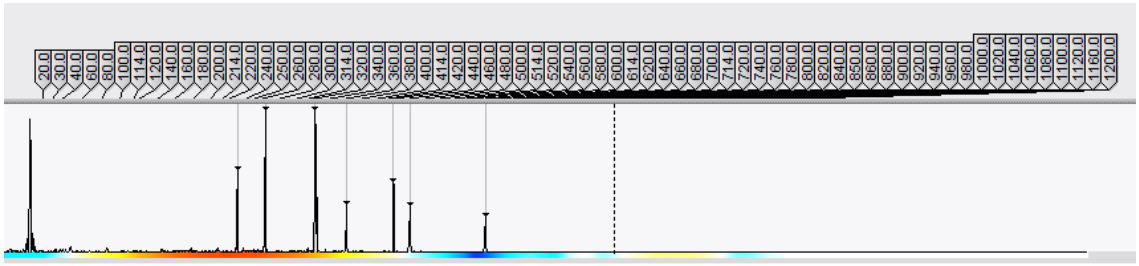
- If not, assign the right size to the peaks by clicking on the peak; then select the right size at the top and *Enter*.



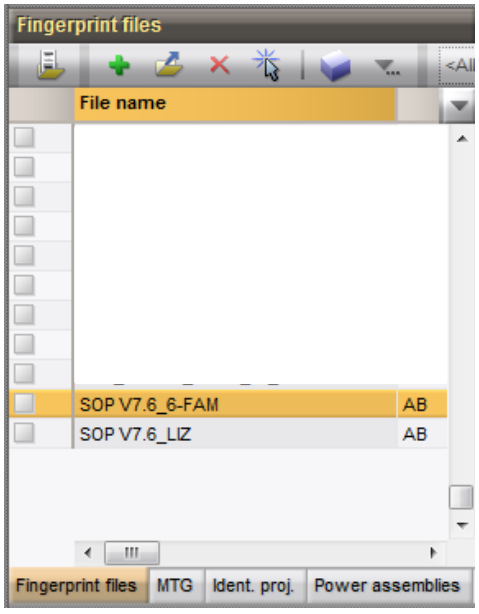
- Click on *Auto assign reference positions*  or *Ctrl+A.*

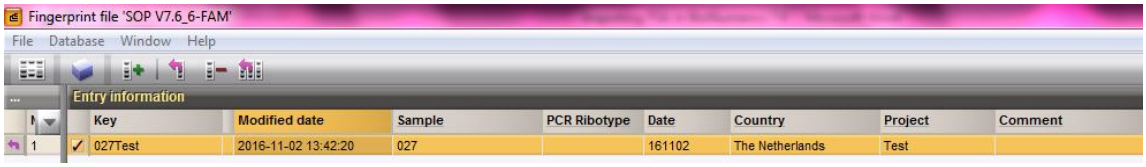


- Select *OK.*
- Make sure that all the bands are assigned correctly and adjust accordingly
- Switch the 6-FAM channel on again. Also switch off the LIZ channel.
- Only the peak pattern of the sample files are visible now.
- Check that all the relevant peaks were assigned.

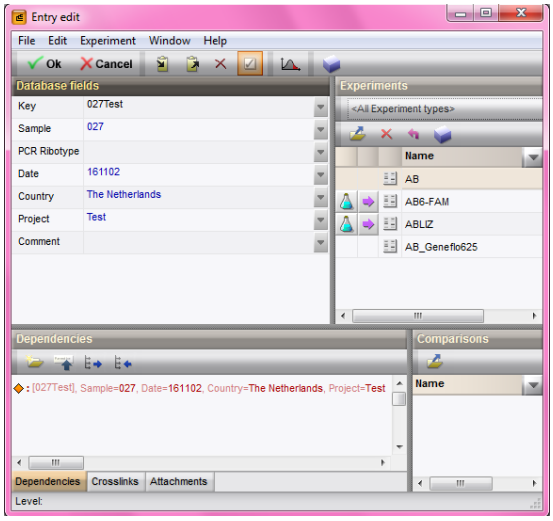


- Save.
- Open the file that you just made in Fingerprint files by double-clicking on the file or selecting *Use*.

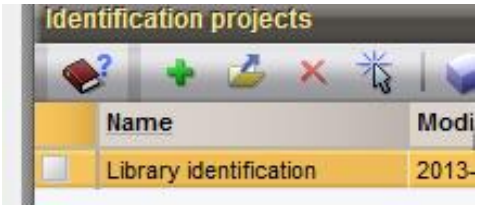




- Fill in all the information by double-clicking on the sample; a pop-up form appears which you can fill in.



- You can now compare the samples with your library by selecting your library and clicking  .



- When finished, close BioNumerics.

Quality control

Adherence to these SOPs will help maintain quality of the procedure.

Positive and negative controls should be processed with every batch and yield the expected results.

Results are invalid if positive or negative controls give unexpected results. In this case, the entire batch must be repeated.

The capillary ribotyping data cannot be analysed unless the correct molecular size ladder has been used with each sample (per run on the Automated Sequence and Fragment Analysis System).

Data quality associated with all test isolates (peak shape and height) must be sufficient for reliable analysis.

Interpretation of results

On the basis of the specific banding pattern, a PCR ribotype will be given to the sample with the use of the programme BioNumerics, GelCompar or an appropriate database. When no match has been found, consider sending the strain to Leiden or Leeds for further characterisation.

Report of results

Report through usual laboratory management system as '*Clostridium difficile* isolated. PCR ribotype'.

Bibliography

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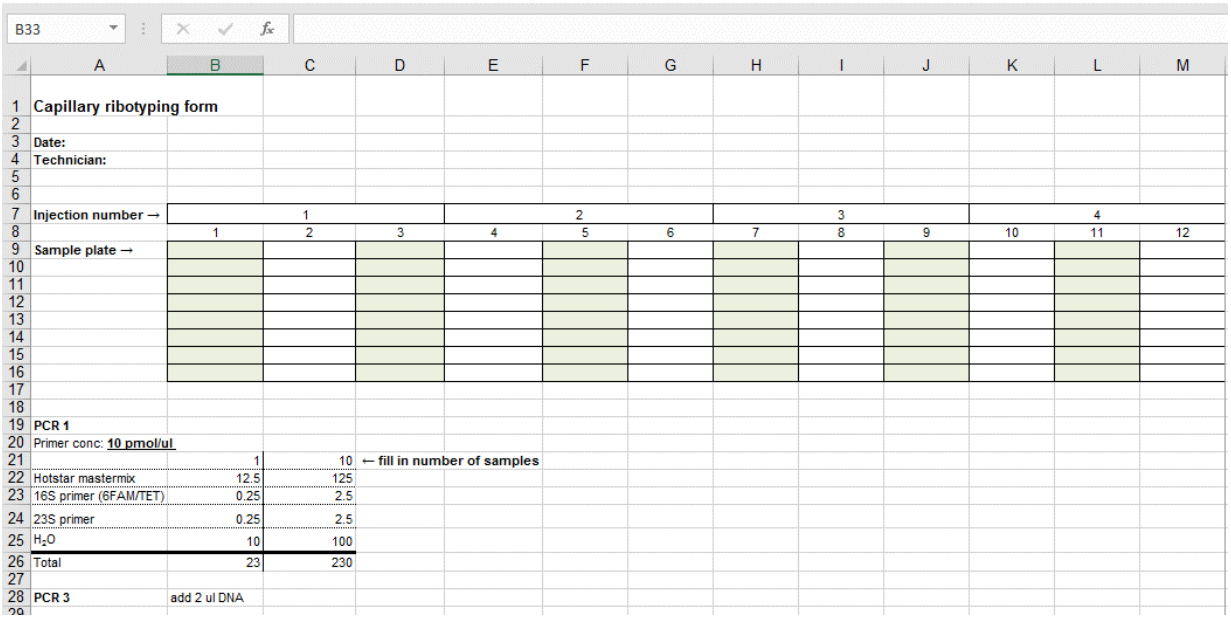
2. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Micribiol. 2008 Nov;57(Pt 11): 1377-82
3. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, Paulick A, Anderson L, Kuijper EJ, Wilcox MH. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. PLoS One. 2015 Feb 13;10(2):e0118150. doi: 10.1371/journal.pone.0118150. eCollection 2015.
4. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR Targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR Ribotypes. J Clin Microbiol. 1999 Feb;37(2):461-3.
5. Janezic S, Strumbelj I, Rupnik M. Use of modified PCR ribotyping for direct detection of *Clostridium difficile* ribotypes in stool samples. J Clin Microbiol. 2011 Aug;49(8):3024-5. doi: 0.1128/JCM.01013-11. Epub 2011 Jun 1.

Addendum 4-1

Capillary ribotyping form.

Preview



	A	B	C	D	E	F	G	H	I	J	K	L	M
1	**Capillary ribotyping form**												
2													
3	**Date:**												
4	**Technician:**												
5													
6													
7	**Injection number →**	1			2			3			4		
8		1	2	3	4	5	6	7	8	9	10	11	12
9	**Sample plate →**												
10													
11													
12													
13													
14													
15													
16													
17													
18													
19	**PCR 1**												
20	Primer conc: **10 pmol/ul**												
21		1	10	← **fill in number of samples**									
22	Hotstar mastermix	12.5	125										
23	16S primer (6FAM/TET)	0.25	2.5										
24	23S primer	0.25	2.5										
25	H_2O	10	100										
26	Total	23	230										
27													
28	**PCR 3**	add 2 ul DNA											

Addendum files are available for download from the ECDC website. Addenda can be found on the same download page as the main document.

5. *Clostridium difficile* ribotyping and Webribo data analysis

Methods

Principle

Ribotyping is a typing method based on polymorphism in the intergenic spacer region located between the 16S and the 23S rRNA genes of *C. difficile* (Bidet et al.). Typing is performed by PCR amplification of the 16S-23S intergenic spacer regions; they vary between 200 and 680 bp in length and the fragment lengths are subsequently determined. DNA fragments are PCR amplified using a fluorescently labelled 16S specific primer and a 23S specific primer (1). PCR products are denatured and stabilised using Hi-Di Formamide and a fluorescently labelled size standard is added. Fragment analysis is performed on a 3130 Genetic Analyzer (Applied Biosystems). Fluorescently labelled DNA fragments are separated by size according to their charge by applying voltage. Peaks are analysed using the Webribo database (http://webribo.ages.at) (Indra et al.).

Method shortcut

Clostridium difficile ribotyping and Webribo data analysis is divided into five steps:

- DNA isolation from a pure cultured sample on nutrient agar
- DNA amplification with FAM 16S primer (for) and 23S primer (rev) in a standard thermocycler
- Fragment denaturation
- Fragment analysis using capillary gel electrophoresis
- Assignment of ribotypes using the Webribo database.

Warnings and security measures

Hi-Di Formamide is toxic. Formamide can be absorbed by the skin, is harmful to eyes, skin, respiratory tract and central nervous system. EDTA buffer may cause skin irritation. Always wear gloves.

Instruments and materials

Instruments

- MagNA Pure Compact (Roche)
- Thermocycler
- PCR hood
- ABI 3130 Genetic Analyzer
- Vortex mixer
- Mini spin centrifuge
- Centrifuge for 96-well plates (Eppendorf Centrifuge 5804 or Perfect Spin, Peqlab).

Materials

- Gloves
- Pipettes
- Filter tips
- Sterile 1.5 ml tubes (Eppendorf)
- PCR strips 200 µl with 8 tubes (Bio Products #040320100)
- PCR strip lids (Bio Products #040420100)
- MicroAmp optical 96-well reaction plate (Thermo Fisher Scientific #N8010560)
- Plate septa 96-well (Thermo Fisher Scientific #4315933)
- Plate base 96-well (Thermo Fisher Scientific #4317237)
- Plate retainer 96-well (Thermo Fisher Scientific #4317241)
- Adhesive film for microplates (VWR #89087-690).

Reagents, solutions and organisms to be tested

Positive and negative controls

A positive control, the Standard RT027 from the Leiden strain collection, and a negative control (nuclease-free water) have to be run with every analysis.

Reagents

Table 5.1. Reagents

Reagent	Company	Order #
MagNA Pure Compact Nucleic Acid Isolation Kit I	Roche	03730964001
HotStarTaq Master Mix Kit (1000 units)	Qiagen	203445
FAM-16S for (5´-GTGCGGCTGGATCACCTCCT-3´) 23S rev (5´-CCCTGCACCCTTAATAACTTGACC-3´)	TIB MOLBIOL Syntheselab GmbH	
GeneScan 1200 LIZ Size Standard	Thermo Fisher Scientific	4379950
POP-7 polymer for 3130/3130xl Genetic Analyzers, 7000 µl	Thermo Fisher Scientific	4352759
Buffer (10x) with EDTA	Thermo Fisher Scientific	402824
Hi-Di Formamide	Thermo Fisher Scientific	4311320

Organism to be tested

Clostridium difficile

Solutions/culture media

Double distilled water (aqua bidest, inhouse facility).

Waste disposal

Metal waste containers are lined with autoclavable waste disposal bags for biologically contaminated waste (Semadeni, Sterilin). Waste containers are autoclaved for 15 min at 134 °C and 3.05 bar. Waste that has been thermally disinfected in accordance with regulations is subsequently discarded.

C. difficile DNA isolation – preparations

- Take *C. difficile* cultured on nutrient agar plates from the AGES Department of Medical Microbiology.
- Check for purity of the bacterial culture.
- Pipette 640 µl aqua bidest in a Magna Pure sample tube (Roche). Take some streaks from a *C. difficile* pure culture with a sterile swab, immerge into the sample tube and agitate until a slight turbidity is visible (approx. McFarland turbidity standard 2). After swab removal the sample volume is 400 µl.
- Sample number and date of receipt are to be noted in form F 199 (Table 6.2).

Table 5.2. Form F199 *C. difficile* DNA

Date of receipt	Sample ID	Ribotype	Date	Toxin A/B	cdtA	cdtB	tcdC	Date	Result transferred Ribo Tox	+ Toxin

C. difficile DNA isolation with the MagNA Pure Compact (Roche)

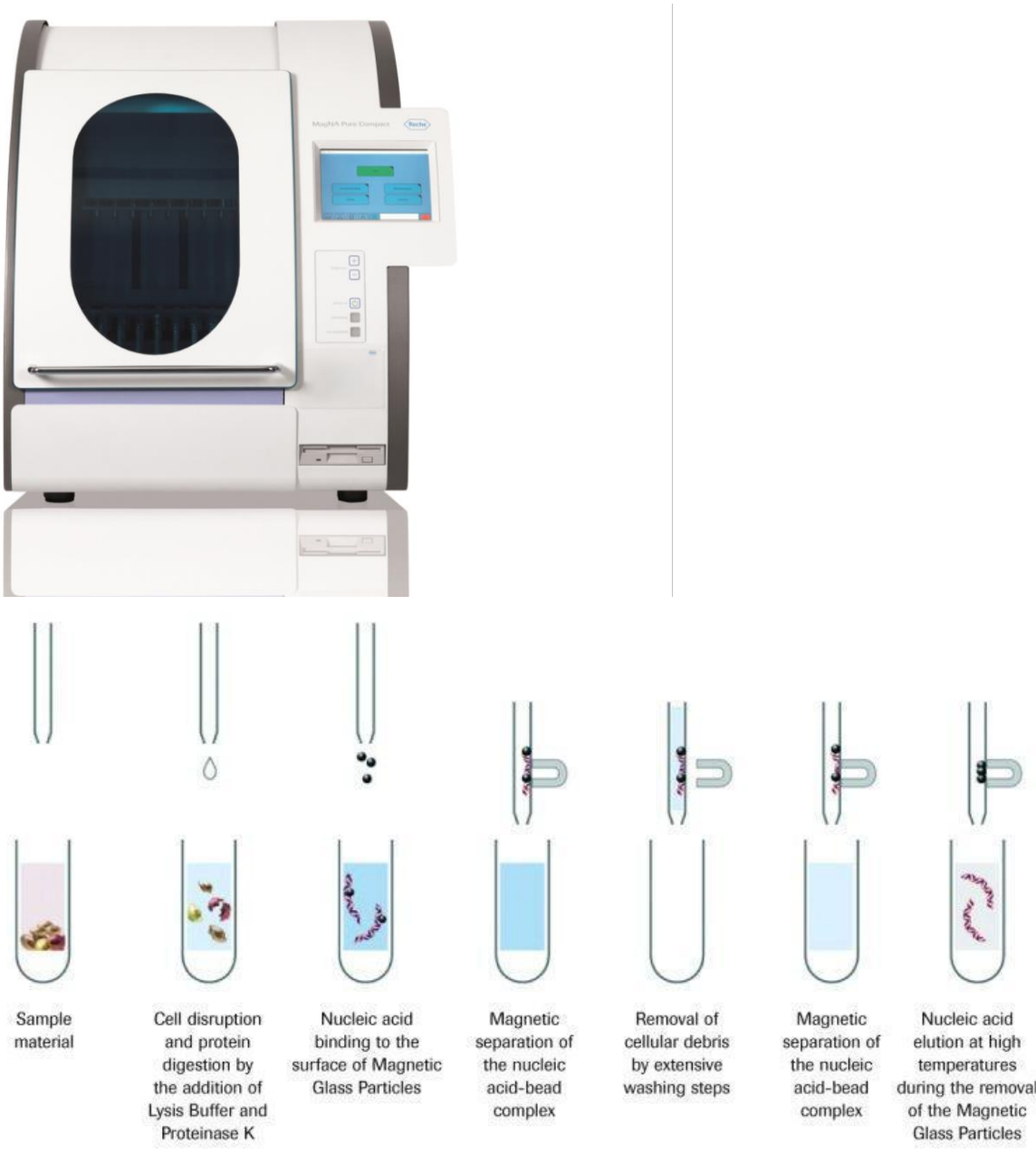
MagNA Pure Compact is an instrument for the automated bead-based isolation of nucleic acids from various starting materials with the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche #03730964001).

Protocol for *C. difficile*: DNA_Bacteria_V3_2, sample volume: 400 µl, elution volume: 100 µl

Working principle: The MagNA Pure Compact automatically performs all isolation steps such as cell lysis, protein digestion, binding of DNA, washing steps and elution of the pure nucleic acid. A reagent cartridge, a tip tray and an elution tube are prepared for each sample. Lysis buffer and proteinase K are added to each sample; cell disruption and protein digestion take place, magnetic glass beads are added, DNA binds to their surface, DNA-magnetic bead-complex is separated from cellular debris by several wash steps. Purified DNA is eluted in a Magna Pure elution tube (Figure 6.1).

After every DNA isolation run the instrument is UV decontaminated for one hour.

Figure 5.1. Magna Pure Compact instrument and working principle



Source: Roche

DNA amplification

C. difficile sample protocol

C. difficile sample IDs are to be inscribed into the sample protocol according to their position in the PCR strip(s) (Table 6.3).

Table 5.3. *C. difficile* sample protocol for DNA isolation

	PCR strip 1		PCR strip 2		PCR strip 3		PCR strip 4		PCR strip 5		PCR strip 6
1		9		17		25		33		41	
2		10		18		26		34		42	
3		11		19		27		35		43	
4		12		20		28		36		44	
5		13		21		29		37		45	
6		14		22		30		38		46	
7		15		23		31		39		47	
8		16		24		32		40		48	

Primer

To be prepared in the Mastermix room (room 109) in a UV3 HEPA PCR-Workstation – no DNA allowed.

- UV light must be off
- Open workbench
- Switch on the HEPA/UV
- Switch on UV/air circulator
- Switch on white light
- Optional: for stronger air circulation turn the fan to high.

Primer stock solution

Briefly spin down lyophilised primers and subsequently dissolve in nuclease-free water in accordance with the manufacturer's instructions (TIB MOLBIOL Syntheselabor GmbH). Rotate the tubes carefully and incubate for 10 minutes at room temperature, protected from light. Briefly spin down again and label stock solution tubes with primer ID, concentration and date.

Primer working solution

For the ribotyping protocol, the primer stock solutions have to be diluted at a working solution of 10 pmol/µl with nuclease-free water. Tubes have to be labelled with primer ID, concentration and date. After usage primers are stored at –20 °C (+/- 5 °C). A small working solution volume should be prepared in order to guarantee quick consumption.

Mastermix

To be prepared in the Mastermix room (room 109) in a UV3 HEPA PCR-Workstation – no DNA allowed.

- Mastermix is prepared in a 1.5 ml Eppendorf tube in accordance with protocol, spun down briefly and dispensed into the PCR strips. PCR strips are closed using PCR lids.

Table 5.4. Mastermix protocol

		µl for n samples (select column 3 and press F9)
Reagent	**1x**	**11**
Hotstar Mastermix (Qiagen)	25 µl	275
FAM-16S for (10 pmol/µl)	0.3 µl	33
23S rev (10 pmol/µl)	0.3 µl	33
Nuclease-free water	22.4 µl	246.4
Total	48 µl	587.4

- When finished, clean workbench with LTK 008 (License To Kill – a detergent that removes DNA, RNA, DNAse, RNAse, bacteria and phages from surfaces, AL-Labortechnik) using a lint-free tissue.
- Close workbench
- Turn all switches off
- Turn on chamber UV timer 30 min.

PCR

Under the PCR hood (room 107), open the PCR strips and add 2 µl of *C. difficile* DNA to each sample. Close the PCR strips with PCR lids. Briefly spin down with the minicentrifuge and put PCR strips in the thermal cycler using the following amplification programme (Figure 6.2):

Figure 5.2. Standard thermal cycler and PCR protocol Bidet primer

Source: Analytik Jena

15min	95 °C	Taq polymerase activation
1 min	94 °C	25 (up to 30) cycles
1 min	60 °C	
1 min	72 °C	
30 min	72 °C	Final extension
Hold at	4 °C	

Depending on DNA concentration, the number of PCR cycles can be increased (or decreased) up to 30 cycles.

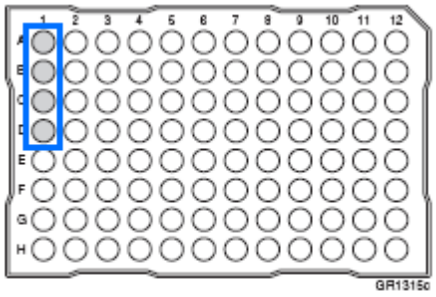
Fragment denaturation

- Under the PCR hood (room 107) prepare a MicroAmp optical 96-well reaction plate using the reagent mix described in Table 5 for each sample to be analysed.
- Add 1 µl of PCR product using a multichannel pipette (0.5–10 µl).
- Mix by pipetting 3-4× up and down.
- The ABI 3130 GA works with four capillaries, always fill groups of four. If you do not have sufficient samples, fill the remaining wells with Hi-Di Formamide only.
- Seal with adhesive film for microplates.
- Briefly spin down the 96-well plate with the Perfect Spin centrifuge (Peqlab, room 107).
- Aliquot Hi-Di Formamide (25 × 1ml) in order to avoid frequent freeze/thaw. Aliquots are stored at -20 °C (± 5 °C). Thaw one tube at a time and store it in the fridge at 4 °C (± 3 °C) up to 1 week.
- Pipette at least 10 µl into the 96-well plate in direction A-H (Figure 6.5).

Table 5.5. Reagent mix protocol for fragment denaturation

µl for n samples (select column 3 and press F9)		
	1x	5
Hi-Di Formamide	10 µl	50
ize standard Liz 1200	0.5 µl	2.5
Reagent mix	10 µl	52.5

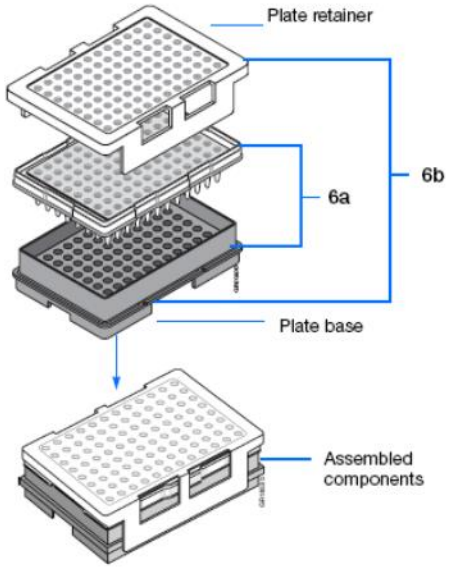
Figure 5.3. MicroAmp optical 96-well reaction plate with working direction of four capillary arrays



Source: Thermo Fisher Scientific

- Denature in a thermal cycler for 2 min at 95 °C, hold at 4 °C.
- Put 96-well plate on a cooling rack in the fridge for 10 min
- Spin down briefly (Perfect Spin, Peqlab)
- Remove the adhesive film and attach the 96-well plate septum
- Assemble the 96-well plate to the 96-well plate base and the 96-well plate retainer and snap them in place (Figure 6.4).

Figure 5.4. Assembly of 96-well plate and septum with plate base and plate retainer



Source: Thermo Fisher Scientific

Fragment analysis using capillary gel electrophoresis

Warnings and security measures

Always wear gloves when working with the ABI 3130 Genetic Analyzer. Always wear gloves and protective glasses when working with polymer.

Operating principle

Capillary gel electrophoresis separates DNA fragments by size, based on their total charge. Labelled DNA fragments reach the capillary detection window, fluorescent dyes are excited by the laser and emit light of a specific wavelength. The emitted light is put in relation to the emitted light of fragments of defined size of the size standard (Liz 1200). The size standard is used to extrapolate the size of the samples product peaks.

Figure 5.5. Fragment analysis using capillary gel electrophoresis

Source: Thermo Fisher Scientific

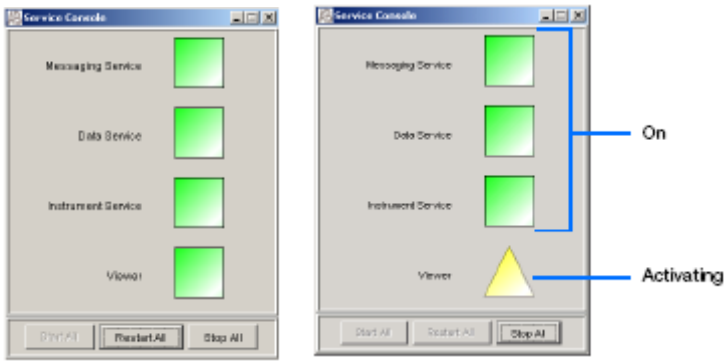
Starting the ABI 3130 Genetic Analyzer

- Turn on monitor
- Turn on computer
- Fill in Windows dialog box:
  - User: 3130User
  - Password: 3130User
- Turn on the 3130 GA by pressing the on/off button at the front of the instrument; wait for green light
- Start data collection software
- Select *Start>All Programs>Applied Biosystems>Data collection>Run 3130 v3.0.*
- Wait for all symbols to turn green (Figure 6.7).

Figure 5.6. ABI 3130 Genetic Analyzer

Source: Thermo Fisher Scientific

Figure 5.7. ABI 3130 GA service console



- Click *Instrument Status*
- Laser power approx. 15 mW
- Oven temperature 60 °C
- Doors must be closed before starting a run
- System status must be *Ready*.

Figure 5.8. Instrument status

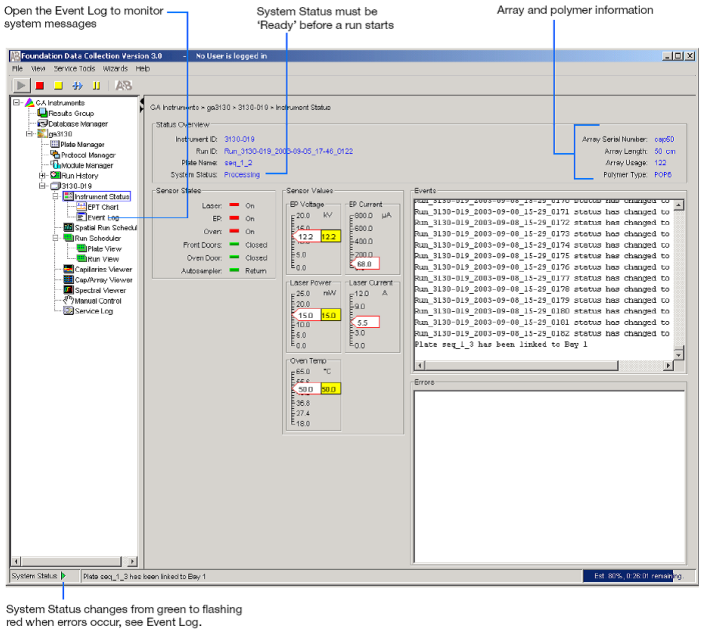
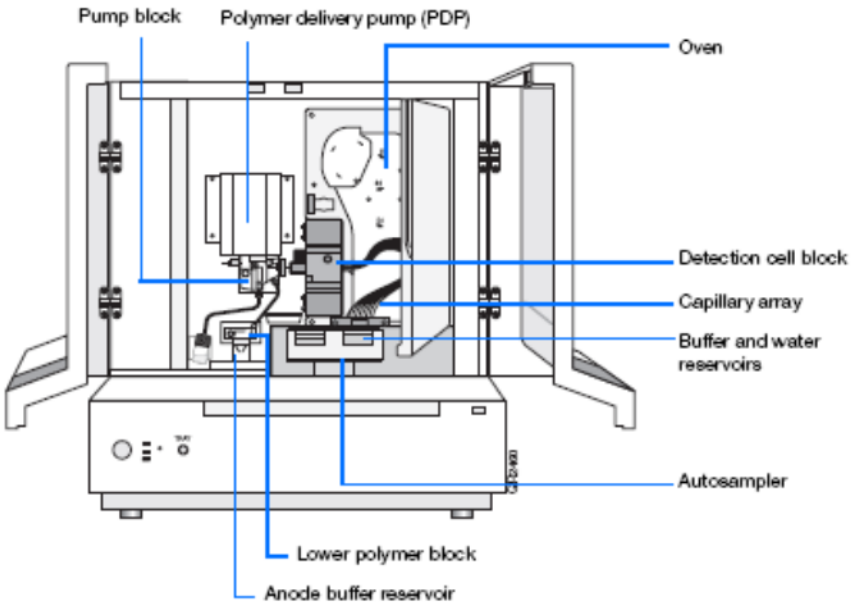


Figure 5.9. ABI 3130 Genetic Analyzer with front doors and oven door opened



Source: Thermo Fisher Scientific

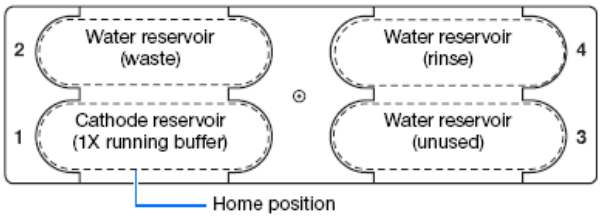
Running conditions

To start a fragment analysis, all reagent reservoirs have to be filled, including polymer. We use a 36cm long 4 capillary array, a running temperature of 60 °C, the POP7 polymer and the GeneScan 1200 LIZ size standard.

- Prepare 1x EDTA buffer
  - Dilute 10× EDTA buffer 1:10 with aqua bidest, fill in sterile containers, label with date
  - 1× EDTA buffer is used as cathode and anode buffer for gel electrophoresis in the 3130 GA.
  - 1× EDTA buffer can be stored up to 1 month at 4 °C (± 3 °C)
  - Change buffer in the 3130 GA weekly.

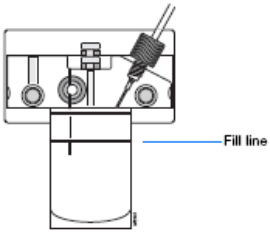
- Fill the buffer reservoirs
  - Cathode buffer reservoir: With instrument doors closed, press the tray button; wait for the autosampler to reach its forward position; open instrument doors; discard 1× EDTA buffer and water; clean reservoirs with double distilled water; fill reservoirs with 1× EDTA buffer and aqua bidest in accordance with the scheme shown in Figure 10 (~ 16 ml); close reservoirs with clean septa.

Figure 6.10. Buffer and water filling scheme of the 3130 GA



Source: Thermo Fisher Scientific

Figure 6.11. Anode buffer reservoir of the 3130 GA



Source: (Thermo Fisher Scientific)

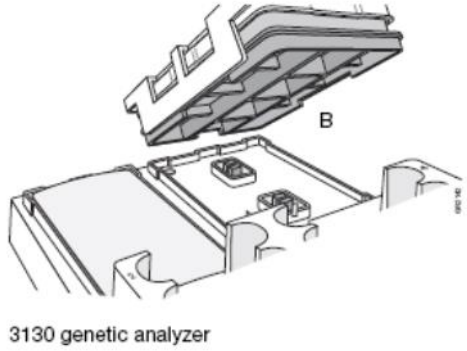
- Anode buffer reservoir: Remove anode buffer reservoir (Figure 6.11) by pulling it down while turning it; remove 1x EDTA buffer; wash reservoir with double distilled water; fill with fresh running buffer up to fill line (~16 ml); reinstall buffer reservoir and close the instrument.

Table 6.5. Storage and expiration dates of reagents used for the 3130 GA

Reagent	Storage	Expiration dates
EDTA buffer (10×)	4 °C	According to manufacturer
EDTA buffer (1×)	4 °C	2 weeks
POP-7 polymer	4 °C	According to manufacturer
Double distilled water (aqua bidest)	4 °C	

- Push the tray button, wait for the tray to reach its forward position and put the sample plate onto the autosampler (Figure 6.12). Close instrument doors, wait for the tray to reach its backward position and for the green light on the front of the 3130 GA to glow continuously.

Figure 6.12. Placing the plate assembly into the instrument

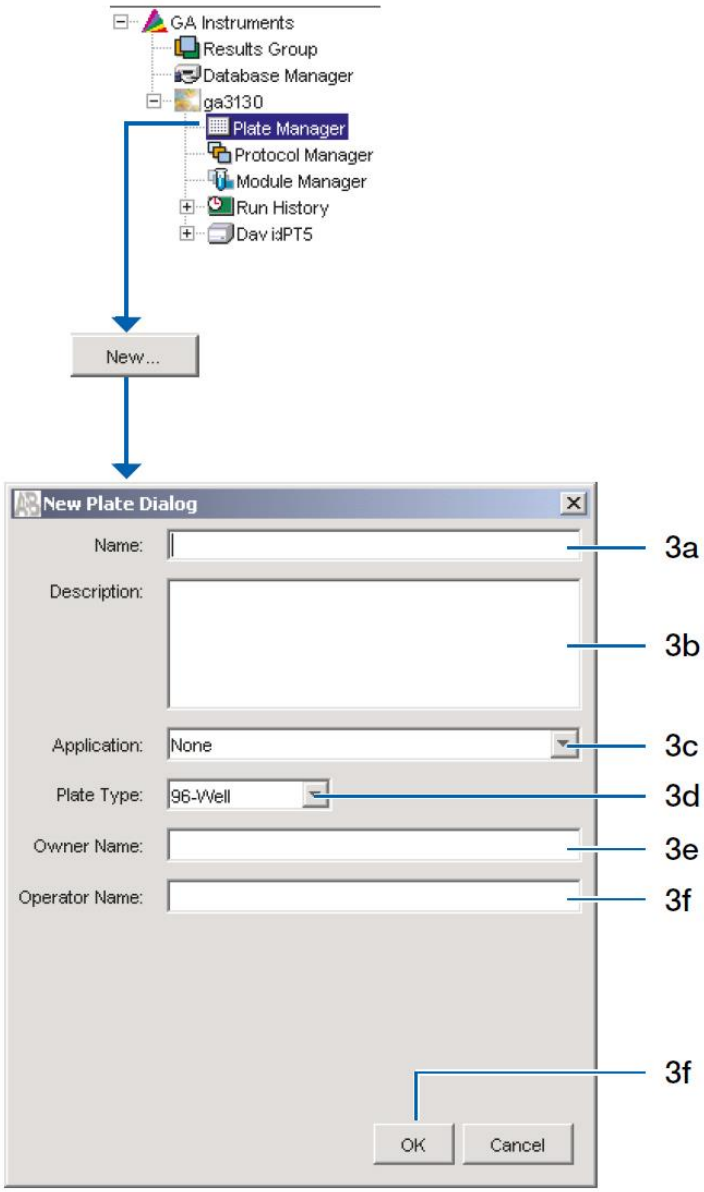


Source: Thermo Fisher Scientific

Plate manager

- In the data collection software click **GA instruments**>**ga3130**>**plate manager**
- Click **new**
- Fill in new plate dialogue
  - Run name
  - description (optional)
  - Application e.g. Sequencing Analysis, Genemapper Generic, Mixed
  - Plate type: 96-well
  - Owner name
  - Operator name
  - Click **OK:** Sequencing Analysis plate editor is opened.

Figure 6.13. Plate manager



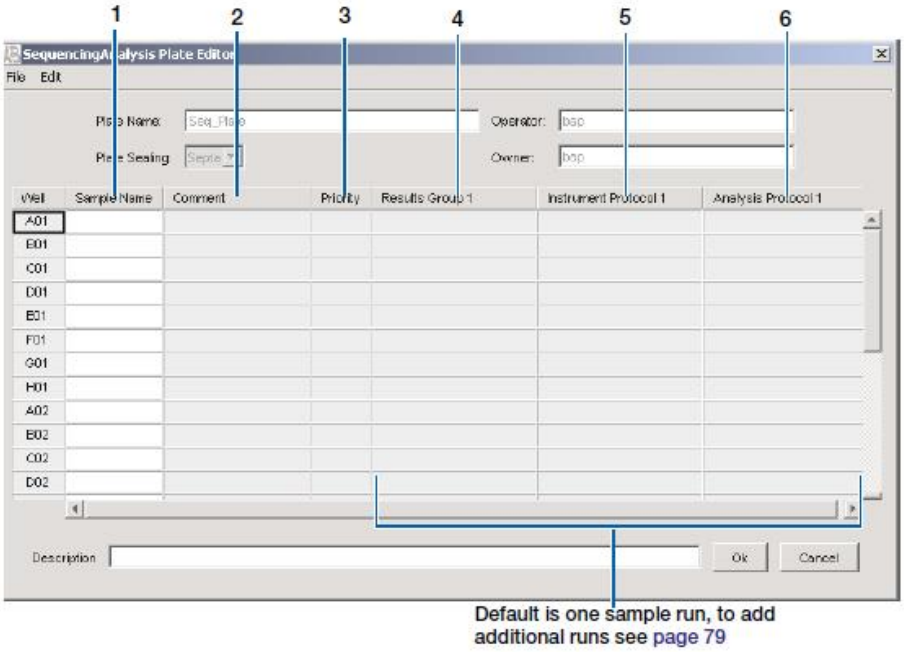
Source: Thermo Fisher Scientific

Sequencing analysis plate editor

- Sample name
- Comment (optional)
- Priority (groups of 4 can be ranked)

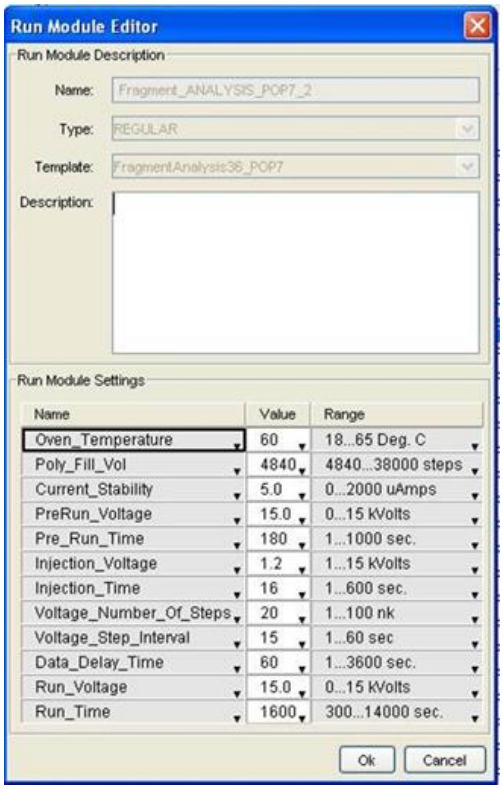
- Results group – choose from pool down menu – **fragm neu 1**
- Instrument protocol – **FA Std_RUN**
- Analysis protocol – not applicable.

Figure 6.14. Plate editor



Source: Thermo Fisher Scientific

Figure 6.15. FA_Std_RUN run conditions for ribotyping fragment analysis

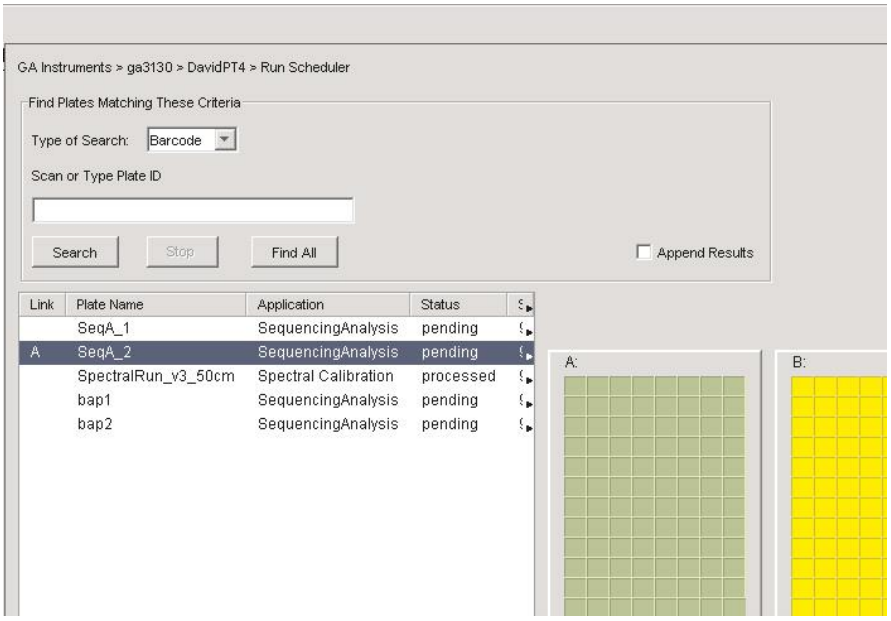


Link a plate

- Click *GA instruments>ga3130>instrument name>run scheduler*
- Click *find all >search plate record list*
- Link the plate.

The plate to be analysed has to be linked with a plate record in the plate manager.

Figure 6.17. Link a plate



Source: Thermo Fisher Scientific

- Select a plate
- Click plate position B, plate turns from yellow to green; plate is linked
- Press the green *play*  button; fragment analysis starts.

Turn off the instrument

- Close data collection software
- Turn 3130 GA off
- Turn computer off
- Turn monitor off.

Assignment of ribotypes using the Webribo database

Optional: For quality assessment, fragment analysis results can be reviewed using Peak Scanner or GeneMapper software.

GA 3130 data collection

- Find FSA files in data collection
- Select FSA files to be uploaded
- Right mouse button, send to *zip*
- Save zip-file on USB flash drive.

Webribo

- Login to your Webribo account at webribo.ages.at
- Click upload, select country and settings, select zip-file, click data file upload
- Click check results
- Check your results
- Logout (a .csv results file will be sent automatically by email).

Figure 6.18. Webribo login

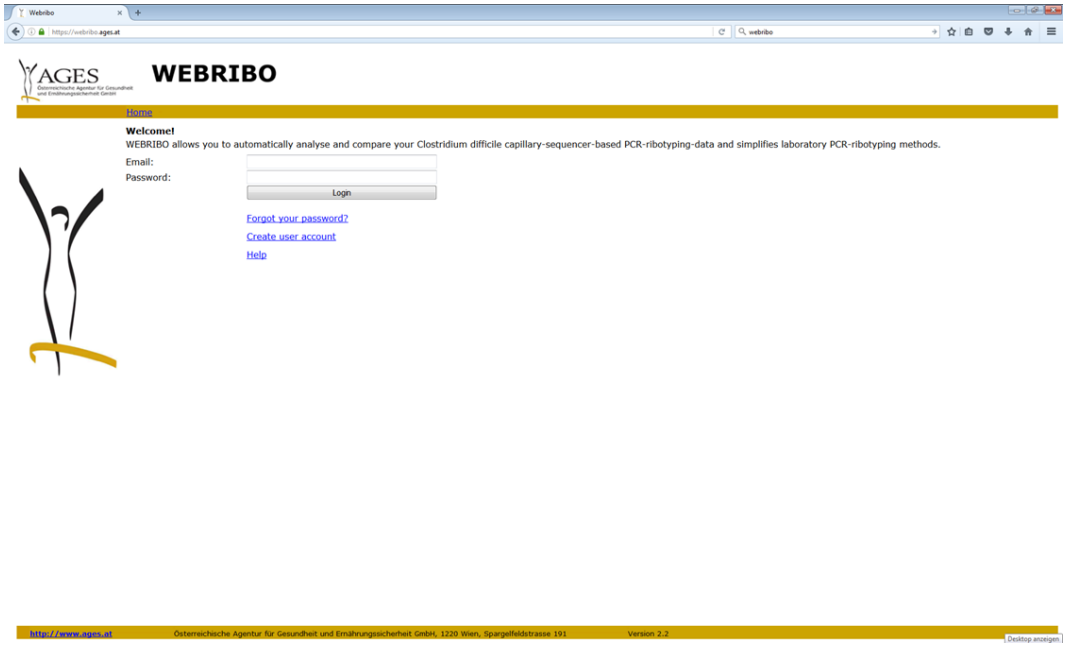
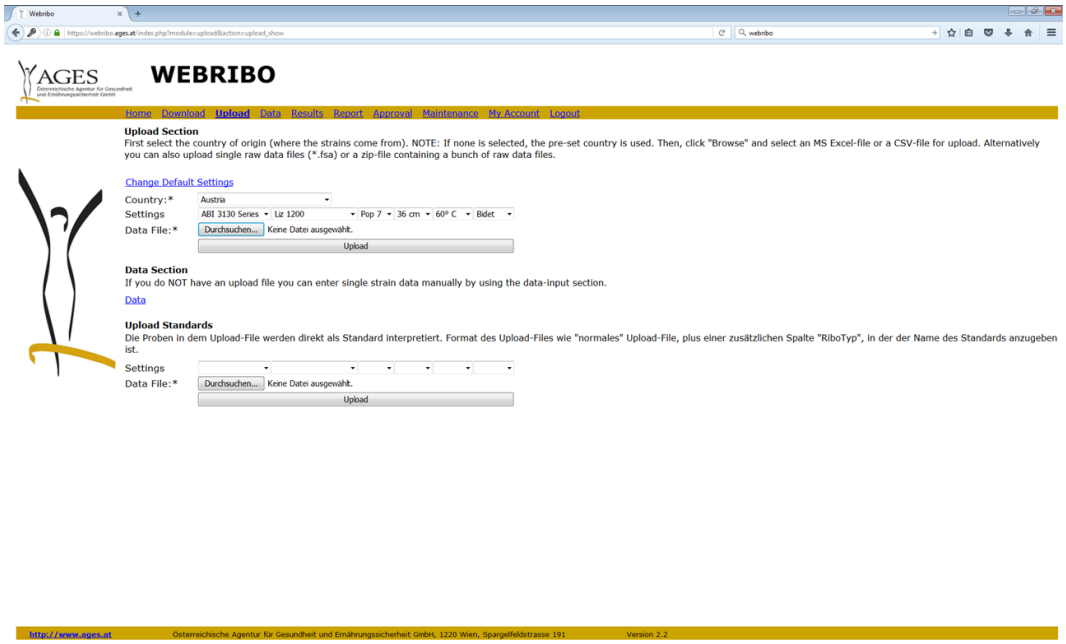


Figure 6.19. Webribo data upload

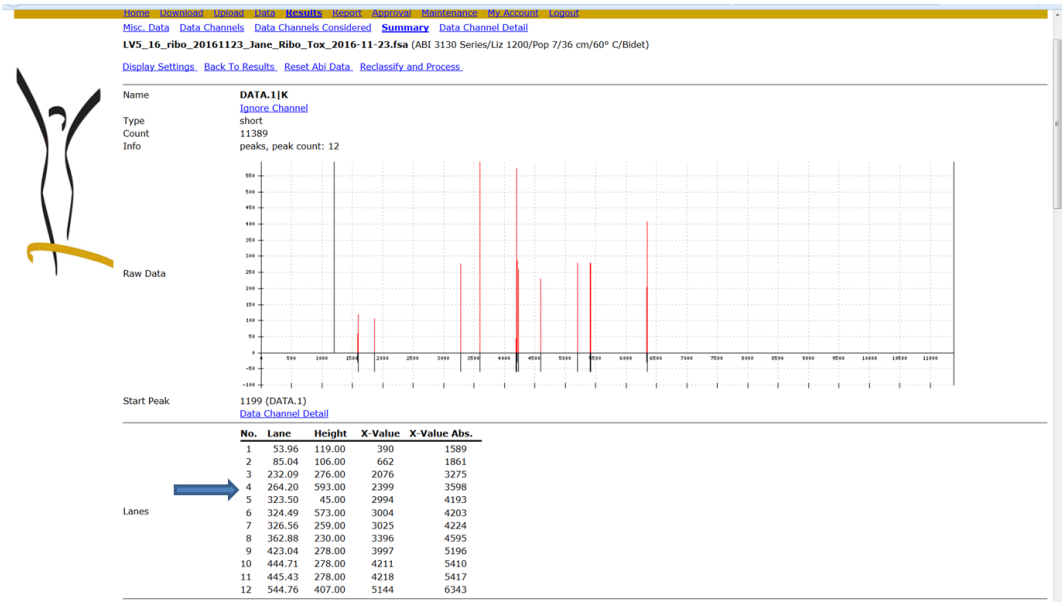


Webribo results

Figure 6.20. Examples of Webribo results

402804_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	PR06460	New Ribo-Type	Raw Data	Reclassify	Delete
910067_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	711	Existing Ribo-Type	Raw Data	Reclassify	Delete
910099_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	106	Existing Ribo-Type	Raw Data	Reclassify	Delete
910109_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	PR14705	Most Likely: 451	Raw Data	Reclassify	Delete
910110_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	-------	The highest height value is too low	Raw Data	Reclassify	Delete
910111_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	014/0	Existing Ribo-Type	Raw Data	Reclassify	Delete
910112_16_ribo_20161124_Ribo_Tox_2016-11-24.fsa	014/0	Existing Ribo-Type	Raw Data	Reclassify	Delete
400433_17_25_Ribo_Jane_20170317_2017-03-17.fsa	020	Existing Ribo-Type	Raw Data	Reclassify	Delete
400496_17_25_Ribo_Jane_20170317_2017-03-17.fsa	448	Existing Ribo-Type	Raw Data	Reclassify	Delete
400515_17_25_Ribo_Jane_20170317_2017-03-17.fsa	-------	The highest height value is too high	Raw Data	Reclassify	Delete
KO027_ribo_Ribo_Tox_20170103_2017-01-03.fsa	027	Existing Ribo-Type	Raw Data	Reclassify	Delete
negKO_ribo_Ribo_Tox_20170103_2017-01-03.fsa	-------	No sample found	Raw Data	Reclassify	Delete
910113_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	446	Existing Ribo-Type	Raw Data	Reclassify	Delete
910114_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	-------	Wrong standard found	Raw Data	Reclassify	Delete
910116_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	002/2	Existing Ribo-Type	Raw Data	Reclassify	Delete
910117_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	018	Existing Ribo-Type	Raw Data	Reclassify	Delete
910119_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	-------	No standard found	Raw Data	Reclassify	Delete
910121_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	AI-8/0	Existing Ribo-Type	Raw Data	Reclassify	Delete
910122_16_ribo_Ribo_Tox_20170103_2017-01-03.fsa	106	Existing Ribo-Type	Raw Data	Reclassify	Delete

Figure 6.21. Webribo troubleshooting example: highest height value too low



Webribo/ribotyping troubleshooting

- The highest height value is too low: peaks are visible but too low for data analysis, click raw data > summary: find highest peak and repeat PCR with more cycles accordingly (Figure 20).
- The highest height value is too high: sample is too high – click *raw data > summary*: find highest peak and repeat PCR with fewer cycles.
- No sample found: no peaks visible
  - repeat PCR with more cycles
  - use more input DNA
  - repeat *C. difficile* culture and DNA isolation.
- No standard found: Liz1200 is too low, run the PCR product again on the 3130 GA with more Liz1200.
- Wrong standard found: Liz1200 is of bad quality, check expiration date of Liz, and either run the PCR product again with more Liz1200 or order new Liz and use the usual amount.

- New ribotype: repeat PCR with Primers FAM-5´GCTGGATCACCTCCTTTCTAAG 3` (Janezicfor) and 5´ TGACCAGTTAAAAAGGTTTGATAGATT 3´ (Janezicrev) (Janezic et al.) and the PCR protocol described in Figure 21.

Figure 6.22. PCR protocol, Janezic primer

15min	95 °C	Taq polymerase activation
1min	95 °C	35 cycles
1min	58 °C	
1min	72 °C	
10min	72 °C	Final extension
Hold at	4 °C	

Ribotyping results

Ribotyping results are archived in form F199 (Table 2). Sample IDs together with ribotypes are transferred to the department of medical microbiology for appraisal.

Validation

Internal quality control: The standard RT027 from the Leiden strain collection is run with every analysis. External quality control: participation in interlaboratory comparison tests.

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European Centre for Disease Prevention and Control (ECDC)

Gustav III:s Boulevard 40
169 73 Solna, Sweden

Tel. +46 858 60 1000
Fax +46 858 60 1001
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