IFU Molecular Culture Starter Kit

Procedure validation machinery dedicated for use of Molecular Culture

Catalogue Number: MolCul 22000, 9 tests
Plus 8 tests Molecular Culture ID kit (MolCul 15000)

Store at -20°C upon receipt

Content

1. Intended use

1a. Before use of 'Molecular Culture ID', the dedicated Capillary Electrophoresis machine (ABI/SeqStudio) and dedicated thermocycler need to be validated to ensure optimal results with Molecular Culture.

1b. After the fragment analyser and thermocycler are tested, reagents are provided for 8 samples of own choice. For intended use of Molecular Culture ID please see instructions for use Molecular Culture ID kit.

2. Molecular Culture Starter kit components

2.1 Included in Molecular Culture Starter kit

Mix for ABI 1
One vial with prepared ABI mixture containing 250 µl ready to use ABI mix.
This mix contains Molecular culture amplicons and eMix

Mix for ABI 2
One vial with prepared ABI mixture containing 250 µl ready to use ABI mix.
This mix contains Molecular culture amplicons in low concentration and eMix

Mix PCR 1 FIRBAC
One vial with prepared PCR reaction containing 250µl ready to use PCR mix.
Mastermix FIRBAC (*Firmicutes/Actinobacteria/ Fusobacteria/Verrucomicrobia* and *Bacteroidetes)* with bacterial DNA. The PCR mix is ready to use for transfer to a thermocycler plate or strip.

Mix PCR 2 FIRBAC
One vial with prepared PCR reaction containing 250µl ready to use PCR mix.
Mastermix FIRBAC (*Firmicutes/Actinobacteria/ Fusobacteria/Verrucomicrobia* and *Bacteroidetes)* with bacterial DNA in lower concentration then Mix PCR 1 FIRBAC. The PCR mix is ready to use for transfer to a thermocycler plate or strip.

Mix PCR 1 PROTEO
One vial with prepared PCR reaction containing 250µl ready to use PCR mix.
Mastermix PROTEO (Proteobacteria and internal control) with bacterial DNA. The PCR mix is ready to use for transfer to a thermocycler plate or strip.

Mix PCR 2 PROTEO
One vial with prepared PCR reaction containing 250µl ready to use PCR mix.
Mastermix PROTEO (Proteobacteria and internal control) with bacterial DNA in lower concentration then Mix PCR 1 PROTEO. The PCR mix is ready to use for transfer to a thermocycler plate or strip.

eMix
1 vial labeled "eMix" containing 560 µl eMix.

The vials labelled with **Mastermix FIRBAC** (120 µl), **Mastermix PROTEO** (120 µl**), Positive Control FIRBAC** (30 µl), and **Positive Control PROTEO** (30 µl), and part of the eMix are intended to be used with the Molecular Culture ID kit (For test instructions read ' Instructions for use Molecular Culture ID kit')

2.2 Not included in Molecular Culture Starter kit

- PCR machine
- Capillary Electrophoresis machine
- Computer and web browser, connected to the internet
- PCR plate or tubes
- ABI plate

3. Storage

- Molecular Culture Validation set is shipped on dry ice. The components of the kit should arrive frozen at least at -20°C. If one or more components are not frozen upon receipt, or if tubes have been compromised during shipment, please contact inbiome.
- All components should be stored at -20°C upon arrival. Repeated thawing and freezing of Mix for ABI1, Mix for ABI2, Mix PCR 1 FIRBAC, Mix PCR2 FIRBAC, Mix PCR1 PROTEO, Mix PCR2 PROTEO more than 2 times should be avoided, as this might affect the performance of the assay.
- Repeated thawing and freezing of Mastermixes, positive control, and eMix more than nine times should be avoided, as this might affect the performance of the assay.
- Refreeze components within half an hour after thawing
- All components are temperature sensitive.
- It is necessary to keep kit reagents at 2 – 8 °C (on ice or in cooling block) when in use.
- Protect all components from light.
- 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.

4. Warnings and Precautions

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

Use sterile, DNase-RNase-free, aerosol resistant pipette tips and wear protective gloves. Protect kit contents or generated PCR product from direct sunlight.

For handling **eMix** 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 groundwater. Disposal of contents/containers must be following local, regional, national, and international regulations.

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

Precautionary Statements
P308 + P313 - IF exposed or concerned: Get medical advice/attention
P202 - Do not handle until all safety precautions have been read and understood
P260 - Do not breathe dust/fume/gas/mist/vapours/spray
P201 - Obtain special instructions before use
P281 - Use personal protective equipment as required
P314 - Get medical advice/attention if you feel unwell

NOTICE: any serious incident that has occurred in relation to the device must be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Classification eMix according to regulation (EC) No 1272/2008 (CLP)					
Section	**Hazard Class**	**Category**	**Hazard class and category**	**Hazard statement code**	**Hazard statement**
3.6	Carcinogenicity	2	Carc. 2	H351	Suspected of causing cancer
3.7	Reproductive toxicity	1B	Repr. 1B	H360F	May damage fertility, may damage the unborn child
3.9	Specific target organ toxicity, repeated exposure	2	STOT RE 2	H373	May cause damage to organs (blood, cardiovascular system) through prolonged or repeated exposure

5. Procedure

5.1 Capillary Electrophoresis test procedure

This procedure aims to validate the Capllary Electrophoresis (CE) machine to ensure CE machine settings are optimal for use with Molecular Culture.
Previously validated CE machines are: ABI3500, ABI3500XL, SeqStudio and SeqStudio flex

1. Install the instrument with the settings as described in attachment A.
2. Read 'MSDS eMix' before use and handle products containing eMix according to the MSDS
3. Thaw the 'Mix for ABI 1' and 'Mix for ABI 2'
4. Homogenize content by vertexing and centrifuge shortly to ensure contents are at the bottom of the tube.
5. Per tube transfer 20 µl in triplicate to an ABI plate.
6. Cover the ABI plate, so no evaporation of product can take place.
7. Centrifuge shortly so contents are at the bottom of the plate
8. Heat the ABI plate in a thermocycler at 94°C for 3 minutes followed by a cooling step to 4 °C
9. Place ABI plate with septa in the CE machine
10. Name the fsa files according to the Mix (1 or 2) and the replicate (a,b,c).

    CE1a
    CE1b
    CE1c
    CE2a
    CE2b
    CE2c

11. Run the CE-machine with the settings as described in Appendix A
12. Upload your data to Antoni via https://antoni.inbiome.com with your login and password.
13. Click on 'Onboarding' and follow the online instructions.
14. Contact techsupport@inbiome.com if you have problems with analyzing the data.
15. Save the settings on the dedicated CE machine when criteria are met.

5.2 PCR test procedure

The PCR validation is done to ensure PCR settings are optimal for use with Molecular Culture.. It is possible to optimize the PCR reaction by finding the best ramp rate of the machine.

Please note that this procedure must be performed in the Pre-Amplification area. Use aerosol barrier tips during the whole test procedure. Thaw only the components that are going to be used. Mix and spin down reaction tubes briefly (three seconds) before use.

This validation is done for only one PCR machine. When this PCR machine is validated, this will be the dedicated PCR machine to use with Molecular Culture.

1. Thaw 'Mix PCR FIRBAC 1', 'Mix PCR FIRBAC 2', 'Mix PCR PROTEO 1' and 'Mix PCR PROTEO 2'

   25 µl up and down for 3 times.

2. Per tube pipet 25 µl in triplicate in a PCR plate or PCR strip. As an example, see the 'example PCR pipetting scheme'.

example PCR pipetting scheme			
Plate location	Tube	Amount	Replicate
A01	Mix PCR FIRBAC **1**	25 µl	a
B01	Mix PCR FIRBAC **1**	25 µl	b
C01	Mix PCR FIRBAC **1**	25 µl	c
D01	Mix PCR FIRBAC **2**	25 µl	a
E01	Mix PCR FIRBAC **2**	25 µl	b
F01	Mix PCR FIRBAC **2**	25 µl	c
A02	Mix PCR PROTEO **1**	25 µl	a
B02	Mix PCR PROTEO **1**	25 µl	b
C02	Mix PCR PROTEO **1**	25 µl	c
D02	Mix PCR PROTEO **2**	25 µl	a
E02	Mix PCR PROTEO **2**	25 µl	b
F02	Mix PCR PROTEO **2**	25 µl	c

3. Cover with an appropriate cover or strip caps for use in the PCR machine.

4. Centrifuge shortly to make sure contents are at the bottom of the plate/strip.

5. Run the PCR program according to the PCR protocol described below.

6. Make sure the ramp rate of the thermocycler is set to 100% or maximum.

PCR Protocol	
Hotstart	10 min 95˚C
10 cycles	30 sec 95˚C
	45 sec 67˚C, reduce 1˚C per cycle
	1 min 72˚C
25 cycles	20 sec 95˚C
	30 sec 57˚C
	30 sec 72˚C
Final elongation and cooldown	2 min 72˚C
	∞ 4˚C

7. After the PCR program is finished, thaw the eMix and pipet the PCR products in the ABI plate according the ABI pipetting scheme.

ABI pipetting scheme				
FSA Filename	**Well**	**eMix**	**PCR product FIRBAC reaction**	**PCR product PROTEO reaction**
PCR1aRR100	A01	20µl	2,5µl FIRBAC 1 replicate a	2,5µl PROTEO 1 replicate a
PCR1bRR100	B01	20µl	2,5µl FIRBAC 1 replicate b	2,5µl PROTEO 1 replicate b
PCR1cRR100	C01	20µl	2,5µl FIRBAC 1 replicate c	2,5µl PROTEO 1 replicate c
PCR2aRR100	D01	20µl	2,5µl FIRBAC 2 replicate a	2,5µl PROTEO 2 replicate a
PCR2bRR100	E01	20µl	2,5µl FIRBAC 2 replicate b	2,5µl PROTEO 2 replicate b
PCR2cRR100	F01	20µl	2,5µl FIRBAC 2 replicate c	2,5µl PROTEO 2 replicate c

NB: the FIRBAC 1 is the PCR product from 'Mix PCR FIRBAC 1', the FIRBAC 2 the PCR product from 'Mix PCR FIRBAC 2', etc.

8. Centrifuge the plate shortly to ensure contents are at the bottom of the plate.

9. Heat the plate in the thermocycler at 94°C for 3 minutes followed by a cooling step to 4 °C. Using the appropriate cover for the plate so no evaporation can occur.

10. Replace the plate-cover with ABI septa.

11. Store plate at 2-8°C until capillary gel electrophoresis on the fragment analyzer.

12. Name the fsa filenames as indicated below

PCR1a
PCR1b
PCR1c
PCR2a
PCR2b
PCR2c

13. Run the CE machine with the settings as described in Appendix A.

14. Upload your data to Antoni via https://antoni.inbiome.com with your login and password.
15. Click on 'Onboarding' and resume the online instructions.
16. Contact techsupport@inbiome.com if you experience problems with analyzing the data.
17. If the settings of the intensities are not as expected, please repeat the PCR test procedure and lower the ramp rate to 90% and 80%.
18. Save the settings on the dedicated PCR machine when criteria are met.

6. Criteria

ABI mix **1 High load**: SM only graph: first peak is in the most left red zone and the last peak is in the most right red zone. All channels graph: For the channels fam (299-302nc), vic (535-537nc) and ned (856-859nc), intensity must be above 25000. For the pet channel, peak 503-505 must be above 3000, peak 1124-1128 must be above 1500.

ABI mix **2 Low load**: SM only graph: first peak is in the most left red zone and the last peak is in the most right red zone. All channels graph: For the fam (299-302nc) and vic (535-537nc), intensity must be above 10.000, for ned, intensity above 25000. For the pet channel both peaks (peak 503-505nc and peak 1124-1128nc, intensity must be above 4000.

PCR mix **1 High load**: SM only graph: first peak is in the most left red zone and the last peak is in the most right red zone. All channels graph: For the channels fam (299-302nc), vic (535-537nc) and ned (856-859nc), intensity must be above 25000. For the pet channel, peak 503-505nc and peak 1124-1128nc must be above 500.

PCR mix **2 Low load**: SM only graph: first peak is in the most left red zone and the last peak is in the most right red zone. All channels graph: For the fam (299-302nc) and vic (535-537nc), intensity must be above 4000, for ned (856-859nc) intensity must be above 25000. For the pet channel both peaks (peak 503-505nc and peak 1124-1128nc), intensity must be above 1000.

7. Tips and tricks

If criteria are not met, first check if settings of the machine are according the settings in the instructions for use. Is maintenance is up to date?

If SM only settings are not met, adjust with run voltage and data delay time.

If intensity settings are not met for the ABI validation, please contact techsupport@inbiome.com

If intensity settings are not met for the PCR validation, adjust with lowering the ramp rate of the PCR machine.

Capillary Electrophoresis settings; attachment A

Settings ABI 3500

Instrument protocol ABI 3500								
Application type:	Capillary Length	Polymer	Dye Set	Advanced Options				
Fragment	50 cm	POP7	G5	unchanged from default settings				
Instrument Protocol Properties								
Run Module	Oven Temperature	Run Time	Run voltage	PreRun Time	PreRun Voltage	Injection Time	Injection Voltage	Data Delay
adjusted from FragmentAnalysis50_POP7	60°C	4500 sec	11.0 kVolts	180 sec	15 kVolt	15 sec	3 kVolts	200 sec

Dye Set protocol ABI 3500				
Dye Set Name	Chemistry	Dye Selection	Reduced Selection	Calibration Peak Order
G5	Matrix Standard	Blue Green Yellow Red Orange	Blue Green Yellow Red Orange	Blue=5, Green=4, Yellow=3, Red=2, Orange=1
Parameters				
Matrix Condition Number Upper Limit			13,5	
Locate Start Point			After Scan 1000, Before Scan 5000, Limit Scans To 3250, Sensitivity 0.4, Minimum Quality Score 0.95	

Settings ABI 3500XL

Instrument protocol ABI 3500XL								
Application type:	Capillary Length	Polymer	Dye Set	Advanced Options				
Fragment	50 cm	POP7	G5	unchanged from default settings				
Instrument Protocol Properties								
Run Module	Oven Temperature	Run Time	Run voltage	PreRun Time	PreRun Voltage	Injection Time	Injection Voltage	Data Delay
adjusted from FragmentAnalysis50_POP7	60°C	4500 sec	11.0 kVolts	180 sec	15 kVolt	45 sec	3 kVolts	200 sec

Dye Set protocol ABI 3500XL				
Dye Set Name	Chemistry	Dye Selection	Reduced Selection	Calibration Peak Order
G5	Matrix Standard	Blue Green Yellow Red Orange	Blue Green Yellow Red Orange	Blue=5, Green=4, Yellow=3, Red=2, Orange=1
Parameters				
Matrix Condition Number Upper Limit			13.5	
Locate Start Point			After Scan 1000, Before Scan 5000, Limit Scans To 3250, Sensitivity 0.4, Minimum Quality Score 0.95	

Settings SeqStudio Flex

Instrument protocol SeqStudio Flex								
Application type:	Capillary Length	Polymer	Dye Set					
Fragment analysis	50 cm	POP7	G5					
Instrument Protocol Properties								
Run Module	Oven Temperature	Run Time	Run voltage	PreRun Time	PreRun Voltage	Injection Time	Injection Voltage	Data Delay
adjusted from FragmentAnalysis50_POP7	60°C	4500 sec	11.0 kVolts	180 sec	15 kVolt	45 sec	3 kVolts	200 sec

Settings SeqStudio

Instrument protocol SeqStudio									
Application type:	Dye Set								
Fragment analysis	G5								
Instrument Protocol Properties									
Run Module	Capillary Temperature	Run Time	Run voltage	Run Ramp Duration	PreRun Time	PreRun Voltage	Injection Time	Injection Voltage	Data Delay
adjusted from LongFragAnalysis	60°C	5960 sec	4 kVolts	300 sec	180 sec	13 kVolt	10 sec	1.2 kVolts	350 sec