Molecular Culture Starter Kit

For Research Use Only. Not for use in diagnostic procedures

Procedure verification machinery dedicated for use of Molecular Culture

Catalogue Number: MolCul22200, 9 tests

Store at -20°C upon receipt

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1. Intended use

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

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
One vial labeled "eMix" containing 560 µl eMix.

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 Starter kit 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 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.
- 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.

Handle **eMix** in a well-ventilated area and 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 22003) Mix for ABI1 (MolCul 22010) and Mix for ABI2 (MolCul 22011) contain Formamide. See MSDS eMix 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 ID.
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 vortexing 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-research.inbiome.com/#/onboarding with your login and password following the online instructions.
13. Contact techsupport@inbiome.com if you have problems with analyzing the data.
14. Save the settings on the dedicated CE machine when criteria are met.
15. Be aware that changing the settings of the dedicated CE machine, new settings need to be verified again.

5.2 PCR test procedure

The PCR verification is done to ensure PCR settings are optimal for use with Molecular Culture ID. 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 verification 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' and spin down the contents of the tubes.
2. Mix the contents per tube by pipetting 25 µl up and down for 3 times.

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

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

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

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

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

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

16. After the PCR program is finished, thaw the eMix and homogenize content by vortexing and centrifuge shortly to ensure contents are at the bottom of the tube.

8. Pipet the PCR products in the ABI plate according the 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.

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

10. 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.

11. Replace the plate-cover with ABI septa.

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

13. Name the fsa filenames as indicated below

PCR1a
PCR1b
PCR1c
PCR2a
PCR2b
PCR2c

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

15. Upload your data to Antoni via https://antoni-research.inbiome.com/#/onboarding with your login and password following 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.
19. Be aware that changing the settings of the dedicated PCR machine, new settings need to be verified again.

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, vic and ned peak intensity must be above 25000. For the pet channel, first peak must be above 18.000, last peak must be above 4000.

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 and ned peak, intensity must be above 5000. For the vic peak, intensity must be above 3000. For the pet channel, first peak, intensity must be above 18.000 and for the last peak, 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, vic and ned, peak intensity must be above 25000.. For the pet channel, first peak, intensity must be above 18000, last peak, intensity must be above 4000.

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 and ned, peak intensity must be above 4000, for vic intensity must be above 2000. For the pet channel first peak, intensity must be above 18000 and last peak, intensity must be above 4000.

Only visible for techsupport, fam peak 299-301nc, vic peak 534-537nc, ned peak 856-859nc, pet first peak 503-505nc, last peak 1124-1128nc.

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 (sizemarker) only settings are not met, adjust with 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
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

Sizecalling protocol ABI 3500	
Protocol name	Fragment Analysis PA_Protocol
Size Standard	GS600LIZ (60-600) + Normalisation
Sizecaller	SizeCaller v1.1.0

Analysis Settings										
Analysis Range	Sizing Range	Size Calling Method	Primer Peak	Minimum Peak Height	Use Smoothing	Use Baselining (Baseline window (Pts))	Minimum Peak Half Width	Peak Window Size	Polynomial Degree	Slope Treshold Peak Start
Full	Full	Local Southern	Present	All colors 175	None	51	2	15	3	0.0

QC Settings				
Fail if Value	Suspect Range	Pass if Value	Assume Linearity	Pull Up
0.25	0.25-0.75	≥ 0.75	0 bp To 800 bp	Actuate Pull-Up flag if Pull-Up Ratio ≤0.05 and Pull-Up Scan≤ 1

* The online software ‘antoni’ does not require a sizecalling protocol because it uses raw data from the .fsa. Since sizecalling is obliged in the instrument software, please use the sizecalling protocol above.

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
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

Sizecalling protocol ABI 3500XL	
Protocol name	Fragment Analysis PA_Protocol
Size Standard	GS600LIZ (60-600) + Normalisation
Sizecaller	SizeCaller v1.1.0

Analysis Settings										
Analysis Range	Sizing Range	Size Calling Method	Primer Peak	Minimum Peak Height	Use Smoothing	Use Baselining (Baseline window (Pts))	Minimum Peak Half Width	Peak Window Size	Polynomial Degree	Slope Treshold Peak Start
Full	Full	Local Southern	Present	All colors 175	None	51	2	15	3	0.0

QC Settings				
Fail if Value	Suspect Range	Pass if Value	Assume Linearity	Pull Up
0.25	0.25-0.75	≥ 0.75	0 bp to 800 bp	Actuate Pull-Up flag if Pull-Up Ratio ≤0.05 and Pull-Up Scans≤ 1

* The online software ‘antoni’ does not require a sizecalling protocol because it uses raw data from the .fsa. Since sizecalling is obliged in the instrument software, please use the sizecalling protocol above.

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	24 capillary: 45 sec 8 capillary 22 sec	3 kVolts	200 sec

* The online software 'antoni' does not require a sizecalling protocol because it uses raw data from the .fsa. Since sizecalling is obliged in the instrument software, please use a LIZ sizecalling protocol.

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	4.25 kVolts	300 sec	180 sec	13 kVolt	18 sec	1.2 kVolts	350 sec

* The online software 'antoni' does not require a sizecalling protocol because it uses raw data from the .fsa. Since sizecalling is obliged in the instrument software, please use a LIZ sizecalling protocol.