

**PRODUCTION PROTOCOL FOR PREPARING PCR BREW  
MUTATIONAL PROFILING GROUP**

Production Author: Sacha Scott

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Version 1.2

**PURPOSE:** This protocol describes the preparation and quality control check of PCR brew for Mutational Profiling. These steps should be completed each Monday to make brew for the group's use during the week. Also included are the following associated steps: amplification, gel loading, PCR clean-up, sequencing, and sequence clean-up steps using tailed PCR and sequencing primers.

**MATERIALS AND EQUIPMENT:**

AmpliTaq Gold ready reaction 2x mix	70% ethanol
Sigma H <sub>2</sub> O	Genetix Q-fill or Multidrop
50% Glycerol	384 well MJ hard shell trays
TE Buffer (10mM Tris., 0.1mM EDTA)	384 well source tray
4 Tailed PCR primers @ 1.2uM in	384 well dental dams
1.7mL tubes	15mL or 50mL conical tubes
Genomic control DNA @ 5ng/ul	1.7mL microcentrifuge tubes
1.5% agarose gel	Tape plate seals
Xylene cyanol	Paper towels
Marker VI	Pipettes of appropriate size
1X TAE buffer	Electronic repeating pipette
SAP	Centrifuge
Exonuclease I	Microfuge
Sigma Water	Thermal cycler
P1K forward sequencing brew	Gel rig and power source
M13 reverse sequencing brew	Speed Vac
3M Sodium Acetate	10% bleach
100% ethanol	Ethanol wipes

**PROCEDURE:**

**1. General procedures and information**

- 1.1. Wipe all pipettes with ethanol wipes prior to use in this procedure.
- 1.2. Clean bench with 10% bleach before and after use.
- 1.3. Wear a clean lab coat and gloves at all times
- 1.4. Do not allow other personnel to be in the area where reactions are being done without also following clean procedure.
- 1.5. During times when a hood cannot be accessed, a disposable mask must be worn while working with DNA, WGA and PCR reactions.
- 1.6. Filtered tips must be used during ALL medical sequencing activities

**2. Prepare PCR Brew**

- 2.1. Determine the amount of reagents needed for the number of reactions to be done for the week using the following recipe:
  - 5.0uL Amplitaq Gold reaction 2x mix X number of reactions
  - 1.6uL 50% glycerol
  - 0.4uL Sigma H<sub>2</sub>O

**Note:** Add roughly 20 percent onto the number of reactions to be done to account for robot use or pipetting errors.
- 2.2. Add the reagents listed above in a 50mL conical tube covered in foil. Add the ddH<sub>2</sub>O first. Mix gently by inverting the tube. Place tube on ice or in the four degree until needed. **Note:** PCR brew must be stored in a covered tube in the back of the 4°.

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- 2.3. Using an electronic repeating pipette, aliquot PCR brew into the desired wells of a new MJ hard shell tray
- 2.4. Quick spin trays before adding DNA or primers.

**3. Adding Human Genomic DNA and primers to brew**

- 3.1. Retrieve the Human Genomic DNA and primers.
- 3.2. Vortex the DNA for 10 seconds, and quick spin in the microfuge.
- 3.3. Using a P2 pipette, dispense 1ul of Human Genomic DNA into desired wells of a 384 MJ hardshell plate.
- 3.4. Vortex the primer(s) for 10 seconds and quick spin in the microfuge.
- 3.5. Using either a P2 pipette or an electronic repeating pipette, dispense 2ul of primer into the desired wells of a 384 MJ hardshell plate.
- 3.6. After the Human Genomic DNA and primers have been added, quick spin the tray.
- 3.7. Check the fluid level in the wells to ensure that the volume is consistent.
- 3.8. Cover with dental dams, and place in the thermal cycler as soon as possible using the ABI-PCR program. Be certain to change the volume on the program to 10uL when prompted and check that the heated lid prompt is selected as "yes." **Note:** *The lid cover must be heated to ensure the success of a PCR reaction. The heated cover allows for a quicker ramp time for the desired temperature and a constant cycle temperature.*
- 3.9. Cycling conditions for PCR are as follows:
  - 96° for 5 minutes
  - 94° for 30 seconds
  - 60° for 45 seconds
  - 72° for 45 seconds
  - Repeat from step 2, 39 times
  - 72° for 10 minutes
  - 10° forever

**4. Running a gel on the PCR products**

- 4.1. Retrieve a 1.5% agarose gel from the Mutational Profiling four degree refrigerator.
- 4.2. Prepare gel to be loaded from a 384 well source tray by choosing the appropriate Biomek Gel program. The Biomek will aliquot the following to the source tray:
  - 2.0uL xylene cyanol
  - 2.0uL PCR product
- 4.3. Dispense 5uL of Marker VI on both sides of the gel.
- 4.4. Gel is run at 140 volts for 50 minutes.
- 4.5. Take a picture of the gel. Make sure the marker is exposed consistently from gel to gel using a 0.8 second exposure. If the PCR product is faint or bright, adjust exposure and take a second photo.

**5. PCR clean-up with Exo-SAP**

- 5.1. Make Exo-SAP cocktail using the following recipe:
  - 1.45uL Sigma Water X number of reactions
  - 0.37uL Exonuclease X number of reactions

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- 0.18uL SAP X number of reactions
- 5.2. Using a P2 pipette or an electronic repeating pipette, dispense 2ul of Exo-SAP brew into desired wells of a 384 MJ hardshell plate.
  - 5.3. Quick spin tray.
  - 5.4. Check the fluid level in the wells to ensure the volume is consistent.
  - 5.5. Cover with dental dams, and place in the thermal cycler as soon as possible using the ABI-EXO program. Be certain to change the volume on the program to 10uL when prompted and check that the heated lid prompt is selected as "yes."
  - 5.6. Cycle at the following conditions:
    - 37° for 30 minutes
    - 80° for 15 minutes
    - 10° forever
  - 5.7. After cycling is complete, quick spin sample.
- 6. Adding sequencing brew**
- 6.1. Label new 384 well MJ hard shell trays for the forward and reverse reactions. Include sequencing brew name, date, and batch information on each tray.  
**Note:** Reserve one tray for forward reactions and one tray for reverse reactions. It is not recommended to do both reactions in the same tray.
  - 6.2. Retrieve P1K Forward sequencing brew, M13 Reverse sequencing brew, labeled 384 well MJ hard shell trays, and the PCR trays.
  - 6.3. Dispense 4ul of P1K forward sequencing brew into the desired wells of the forward tray using an electronic repeating pipette.
  - 6.4. Dispense 4ul of M13 reverse sequencing brew into the desired wells of the reverse tray using an electronic repeating pipette.
  - 6.5. Using a twelve-channel pipette, transfer 2ul of PCR product from each row of the PCR tray into the corresponding row of the tray containing forward brew.  
**Note:** It is imperative to change tips between each row that is transferred. This prevents contamination of sequencing brew and pcr samples.
  - 6.6. Transfer 2ul of PCR product from each row of the PCR tray into the corresponding row of the tray containing reverse brew. Again, be sure to change tips between transfers.
  - 6.7. When complete, quick spin and check that the volume in the wells is consistent.
  - 6.8. Cover with dental dams and place in the thermal cycler as soon as possible using the BD25 program. Be certain to change the volume on the program to 6uL when prompted and check that the heated lid prompt is selected as "yes."
  - 6.9. Cycling conditions are as follows:
    - 96° for 1 minutes
    - 96° for 10 seconds
    - 50° for 5 seconds
    - 60° for 4 minutes
    - Repeat from step 2 for 25 cycles
    - 10° forever
  - 6.10. Remove trays from cycler, quick spin, and prepare for sequence clean up.

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**7. Sequence reaction clean-up**

- 7.1. Make precipitation cocktail using the following recipe:
  - 25uL 100% ethanol X number of reactions
  - 2.5uL 3M Sodium Acetate X number of reactions
- 7.2. Using a Genetix Q-fill or a multidrop, add 25uL of the precipitation cocktail to the wells of the sequenced tray.
- 7.3. Cover the tray with a clear adhesive plate seal and spin in the centrifuge for **30 minutes** at 3500 rpm. (Program 2)
- 7.4. Decant trays and place upside down on paper towels. Place the trays (still upside down) and paper towels into the centrifuge. Spin at 400 rpm for **30 seconds**. (Program 3)
- 7.5. Retrieve 70% ethanol and decanted trays for the second step of the sequencing reaction clean-up process.
- 7.6. Add 25uL of 70% ethanol to the trays using a Genetix Q-fill or a multidrop.
- 7.7. Cover the tray with a tape plate seal and spin in the centrifuge for **15 minutes** at 3500 rpm. (Program 1)
- 7.8. Decant trays and place upside down on paper towels. Place the trays (still upside down) and paper towels into the centrifuge. Spin at 400 rpm for **30 seconds**. (Program 3)
- 7.9. Retrieve the trays from the centrifuge and place into the speed vac for 15 minutes to dry.
- 7.10. If needed, elute trays with 15uL of ddH<sub>2</sub>O using a Genetix Q-fill or a multidrop.

**8. Create DNA resource Item Order**

- 8.1. Under “**Funded Project Management**” menu, scroll down to **Create DNA Resource Item Order**. Click it to highlight.
- 8.2. Right click and drag over the “**Execute Process Step: create dna resource item order.**” Another interface will appear.
- 8.3. The first box is the **DNA Resource** menu. Click and scroll to find the appropriate project name. **Note:** *If the DNA Resource cannot be found, ask a coordinator for help or create a new DNA Resource.*
- 8.4. The first dialogue box will ask for an estimated insert size. Type in “Unknown”.
- 8.5. The next box asks for Primer information. Use the scroll-down menu to find the appropriate primer information.
- 8.6. The next dialogue box asks for container type, choose the appropriate container for the samples. (For example, “new 384 well plate”)
- 8.7. The final box asks, “How many plates?” Type the desired number of plates. **Note:** *This step will need to be repeated for the reverse direction.*
- 8.8. Click on “**Add Items**” and wait for the samples to show on the left.
- 8.9. Click on “**Execute**” and wait for a pop-up window to appear. It will read: “**Save and Commit or Cancel**”. Click on “**Save and Commit**”
- 8.10. Find the newly created DNA resource item order at the bottom of the screen. Click to highlight. Once highlighted right click and drag down to select “**Print Custom Barcode Sheet**”. **Note:** *Attach any data tracking information such as test variables to this page.*
- 8.11. A barcoded paper will print. Ensure that the barcode description appears. This will be useful for data tracking. If the barcode description does not print, this means the information has yet to be processed by the server.

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- 8.12. Click on “**Accept DNA Resource Item Order**” to highlight the tree-scroll menu on the left of the interface. Once “**Accept DNA Resource Item Order**” is highlighted, right click and drag to “**Execute Process Step: Accept DNA Resource Item Order**”.
- 8.13. A new interface will appear. Click on the “**Show list**” button where another interface will become active.
- 8.14. Click to highlight the newly created DNA resource item order.
- 8.15. Click on the “Accept” button.
- 8.16. Click on the **print barcodes** button. Barcodes will print at the nearest barcode printer.
- 8.17. Click on the “**Accept**” button to enter the information into the database.
- 8.18. Place the barcodes on the plates.
- 8.19. Return to the touchscreen and claim the barcodes using the following pathway: Franklin → Sequence → Claim DNA → sequenced dna from incoming dna
- 8.20. Scan the barcoded plates and wait until the confirm key becomes available. Press **confirm**.
- 8.21. Refer to the “LOADING BARCODED TRAYS ON THE APPLIED BIOSYSTEMS 3730 ANALYZER” protocol for loading information and instruction.

**Revision History:**

09/19/05 Version 1.1

1. Xylene cyanol replaced agarose loading dye throughout protocol.
2. Added the following items to the Materials and Equipment section:
  - 4 Tailed PCR primers @ 1.2uM in 1.7mL tubes
  - 384 well source tray
  - 10% bleach
  - ethanol wipes
3. Removed the following items from the Materials and Equipment section:
  - 4 Tailed PCR primers @ 0.6uM in 1.7mL tubes
4. Added the following sections to the protocol:
  - General Procedures and Information
  - Create DNA Resource Item Order
5. Added the following step to the Running a gel on the PCR products section:
  - 8.22. Prepare gel to be loaded from a 384 well source tray by choosing the appropriate Biomek Gel program. The Biomek will aliquot the following to the source tray:
    - 2.0uL xylene cyanol
    - 2.0uL PCR product