

TEMPLATE VALIDATION PROTOCOL MUTATIONAL PROFILING GROUP

Production Author: Jerry Reed

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Version 1.1

PURPOSE: This protocol describes the barcoded template validation steps for the Mutational Profiling Group. Steps include amplification, gel loading, PCR clean-up, sequencing, and sequence clean-up using tailed PCR and sequencing primers.

MATERIALS AND EQUIPMENT:

AmpliTaq Gold ready reaction 2x mix	70% ethanol
ddH ₂ O	Eppendorf ep5075 robot
50% Glycerol	Genetix Q-fill or Multidrop
TE Buffer (10mM Tris., 0.1mM EDTA)	384 well MJ hard shell trays
Tailed PCR primers @ 1.2uM in 1.7mL tubes	384 well source tray
Patient DNA in Costar trays or 1.7mL tubes, 1:10 wga	384 well dental dams
Genomic control DNA, 1:10 wga	15mL or 50mL conical tubes
1.5% agarose gel	1.7mL microcentrifuge tubes
Xylene Cyanol	Tape plate seals
Marker VI	Paper towels
1X TAE buffer	Pipettes of appropriate size
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	Disposable mask
100% ethanol	Ethanol wipes
	Gloves
	10% bleach

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 for both pipettes and robots.

2. Starting Sherlock

- 2.1. To initialize Sherlock, type the following command in an open terminal window:

```
sherlock --group franklin
```
- 2.2. Hit return.

3. Assign Amplicon Group

- 3.1. In the **Amplification** heading, navigate to the **Assign Amplicon Group** subdirectory. Click to highlight.
- 3.2. Right click and drag to **Execute Process Step: assign amplicon group**.
- 3.3. A new interface will appear containing two windows.

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- 3.4. In the window on the left, locate the four following validation primers and highlight:
 - PIK3CA – 28
 - FLT3 – 40
 - FGFR2 – 6
 - EGFR – 23
- 3.5. Locate the **DNA barcodes** in the window on the right. Click to highlight.
- 3.6. The **Assign** button can now be selected.
- 3.7. The database will calculate the number of needed reactions based on the amplicon and dna tube selections. A display of this number will appear in the lower left-hand corner of the interface. Make sure the calculation is correct by multiplying the number of amplicons with the number of dna tubes. If the numbers are the same, proceed to step 3.9.
- 3.8. In some situations, the database calculation may not match the calculation done by the technician OR an error may occur after the Assign button is pressed stating that the group has already been assigned. In either of these scenarios, check the gene status in the **SIFU** program for more information.
 - 3.8.1. In an open terminal, type **sifu --access rw**.
 - 3.8.2. On the left side of the interface, locate the **Gene** tab and click to open.
 - 3.8.3. Enter the **Gene Name** in the appropriate field.
 - 3.8.4. Enter the **Locus Link**.
 - 3.8.5. Leave the **Gene Description** line blank.
 - 3.8.6. Click **Search**. **Note:** *The field in the lower portion of the interface will fill with information. It may take several minutes for this process to complete.*
 - 3.8.7. Sort by clicking on the either the **#Completed** or the **#Incomplete** column headings. After the column sorts, the information will indicate if the amplicon group has been assigned, completed, partially completed, or not completed.
 - 3.8.8. It is possible to “unassign” an amplicon group, however, it is imperative to check with other technicians in the group to ensure there are not reactions in progress using the same amplicon. **Note:** *Unassigning an amplicon group while reactions are in progress will cause the barcode process to fail confirmation at the touchscreen.*
 - 3.8.9. To unassign an amplicon group, highlight incomplete rows using shift-click or control-click.
 - 3.8.10. Select **Unassign** at the top of the interface.
 - 3.8.11. A dialogue box will appear asking, “**Do you want to resolve duplicate amplicons with PSE ID xxxxxxxx?**” (For example PSE ID 57031703.)
 - 3.8.12. Choose **Yes** to unassign. Choose **No** to cancel the process.
 - 3.8.13. Attempt to Assign the amplicon group once again by following steps 3.1. to 3.7.
 - 3.8.14. If calculations match, proceed to step 3.9. If calculations do not match or an error appears, contact the appropriate person.
- 3.9. Highlight all the calculated pcr reactions in the bottom of the screen.
- 3.10. Confirm by clicking **OK**. (This will assign amplicon group and exit the application, which brings the user back to the original Sherlock screen.) **OR**

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Click **Apply** to assign amplicon and remain in the Assign Amplicon Group interface. **Note:** Both confirmation options will successfully assign amplicon group.

4. Prepare the PCR Primers and Controls

- 4.1. Locate the four validation primers. If new dilutions are necessary follow the steps below, beginning with step 4.3.
- 4.2. If primer stock tubes are already dissolved, skip to step 4.5.
- 4.3. Currently, primers should be dissolved in tubes to a concentration of 100uM. Do this by adding XuL of TE buffer equal to the nanomoles of primer in the tube multiplied by 10. For example, if there are 24.3 nmol, add 243uL of TE buffer.
- 4.4. Let primer tubes sit for at least thirty minutes.
- 4.5. Vortex the primer tubes for one full minute and quick spin in the microfuge.
- 4.6. Pool primers to appropriate volume and pooled concentration of 1.2µM.
- 4.7. If necessary, print pooled primer barcodes from mp_grande.
 - 4.7.1. At an open terminal, open the **mp_grande** program.
 - 4.7.2. Select the needed gene.
 - 4.7.3. Choose the appropriate amplicons. For multiple selections, use shift-click or control-click.
 - 4.7.4. Right click and choose **Print Amplicon Barcode**.
 - 4.7.5. Choose **pooled** or **not pooled** appropriately.
 - 4.7.6. Choose the desired printer.
 - 4.7.7. Select **Print All**.
 - 4.7.8. If the primers are not yet pooled, a dialogue box will appear asking “**Pool now?**” Click **Yes**.

5. Prepare PCR brew

- 5.1. If necessary, select the **Add DNA Control** step to associate positive and negative controls with the DNA plate or tubes.
- 5.2. Determine the amount of reagents needed for the number of reactions to be done using the following recipe:
 - 5.0uL Amplitaq Gold reaction 2x mix X number of reactions
 - 2.0uL DNA (1:10 wga if possible) X number of reactions
 - 2.0uL PCR primer mix (0.6uM each primer) X number of reactions
 - 1.6uL 50% glycerol
 - 0.4uL ddH2O

Note: Add roughly 20 percent onto the number of reactions to be done to account for robot use or pipetting errors.
- 5.3. Depending on the amount of reactions to be completed, add the reagents listed above in a 15mL or 50mL conical tube. Add the ddH2O first. Mix gently by inverting the tube. Place tube on ice or in the four degree until needed.

Note: PCR brew must be used the same day it is made.
- 5.4. Using an electronic repeating pipette, aliquot PCR brew into needed wells of a new MJ hard shell tray. **Note:** If the sample size is large, use the ep5075 to add brew. Programs can be found in the MP folder labeled “Brew Addition.”
- 5.5. Quick spin trays before adding DNA or primers on the ep5075.

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6. Setting up the ep5075

- 6.1. Retrieve the appropriate DNA patient tray or tubes and primers. Claim if necessary.
- 6.2. Vortex the tubes of DNA for 10 seconds, and quick spin in the microfuge.
- 6.3. In **Amplification**, print a barcode for the 384 well MJ hard shell plate to be used for PCR by selecting **Create PCR Products** at the touch screen. At this screen, choose **Print Barcodes**. Enter the number of barcodes needed and hit print. Attach this barcode to the 384 well MJ hard shell plate containing the brew.
- 6.4. At the touch screen, select **Create PCR Products**. Choose a transfer pattern from the touchscreen according to the number of samples and oligos to be used.
- 6.5. Have ready a materials core barcode for the enzyme (Which at this time is Amplitaq Gold.), the pooled primer tubes needed in an A1, A2, B1, B2 order (or an "empty" barcode as needed), the patient tubes, positive control (the barcode associated with the tube), negative control barcode, and a new 384 well MJ hard shell plate. **Note:** *These barcodes may need to be scanned twice according to the transfer pattern in use. Scan items in the order listed above. Hit confirm. Note: The confirm step may take up to thirty minutes.*
- 6.6. At the ep5075, find the appropriate program in the MP folder on the control panel, highlight, and select **"START."**
- 6.7. The first screen to appear will be the deck layout. Place the DNA patient tray or tubes, primers, and 384 well MJ hard shell plates containing PCR brew onto the deck using the deck layout as a guide. Place the appropriate tips on the deck.
- 6.8. Check all trays and tubes to ensure that fluid levels are at appropriate volumes and ready for aspiration/dispensing. **Note:** *The ep5075 will install the proper tool onto the head automatically with no manual intervention.*
- 6.9. After the deck layout has been checked, hit **"OK."**
- 6.10. The next series of screens will prompt the user to fill in appropriate volumes for the labware in use. Check that the labware listed in this prompt is in the proper location. This information can be found in the first line of the prompt at the top of the screen. After this has been confirmed, fill in a volume equal or greater to the volume prompted by the program.
- 6.11. Watch the robot scan the tip box and other labware to ensure that it has calibrated properly.
- 6.12. After the robot has completed the program, quick spin the trays. Check fluid levels in the wells to ensure that the robot has pipetted accurately.
- 6.13. Cover with dental dams, and place in the thermal cycler as soon as possible using the appropriate PCR program. **Note:** *All Varmus reactions are cycled with a 60 cycle pcr program. (ABI6060) All other reactions are cycled with the 40 cycle program (ABI6060) listed below. All four validation primers have a 60° annealing temperature. Be certain to change the volume on the program to 11uL 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.*

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6.14. 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

7. Running a gel on the PCR products

- 7.1. Retrieve 1.5% agarose gels from the Mutational Profiling four degree refrigerator.
- 7.2. At the touch screen (in amplification), use the **Load Gel** step to get gel barcodes and generate gel sheets.
- 7.3. 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
- 7.4. Load the samples with a 12-channel pipette. Load 5uL Marker VI on both sides of the gel in the marker lanes.
- 7.5. Run the gel at 140 volts for 50 minutes.
- 7.6. Take a picture of each gel. Make sure the marker is exposed consistently from gel to gel using a 0.8 second exposure. If the PCR product is faint, over-expose the gel and take another picture.
- 7.7. At the touch screen (in amplification), use the **Check Gel** step to fail PCR products. Select wells that have failed and hit **confirm**.

8. PCR clean-up with Exo-SAP

- 8.1. Make Exo-SAP cocktail using the following recipe:
 - 1.45uL Sigma Water X number of reactions
 - 0.37uL Exonuclease I X number of reactions
 - 0.18uL SAP X number of reactions
- 8.2. Follow instructions below for the platform to be used.

8.3. Biomek

- 8.3.1. Open the Biomek FX software by double-clicking on the “Shortcut to Biomek FX” icon on the desktop.
- 8.3.2. Click on the “**File**” tab at the top of the page and highlight “**Open**”.
- 8.3.3. Select the appropriate program for the number of trays to which you will add EXO/SAP mix using the following pathway: C: Program Files/BiomekFX/Methods/Mutational Profiling/Exo_sap/ / Exo_sap_#trays_vgroove_MIX.bmt
- 8.3.4. Quick spin all 384 well MJ plates to be processed at 1500rpm for 30 seconds.
- 8.3.5. Remove dental dams and place the 384 well MJ plates on the deck of the Biomek as indicated in the “**Instrument Set-Up**” window.

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- 8.3.6. Empty the waste bucket and fill the water bucket with fresh dH₂O if necessary.
 - 8.3.7. According to the “**Instrument Set-Up**” window, place a 384 head V-Groove reservoir on the deck in the correct position for the EXO/SAP mix.
 - 8.3.8. Retrieve the EXO/SAP mix from the back of the refrigerator and carefully pour the mixture into the 384 head V-Groove reservoir. Be sure the mix thoroughly covers the bottom of the reservoir.
 - 8.3.9. Double-check the deck set-up and click the green arrow to run the program.
 - 8.3.10. Carefully, watch the aspiration of EXO/SAP mix to be sure there is enough in the reservoir.
 - 8.3.11. When EXO/SAP mix has been added to all trays, immediately pour the remaining EXO/SAP mix back into the foil-wrapped conical tube and place it in the back of the refrigerator.
 - 8.3.12. Quick spin trays at 1500rpm for 30 seconds.
 - 8.3.13. 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 11uL when prompted and check that the heated lid prompt is selected as “yes.”
 - 8.4. Cycle at the following conditions:
 - 37° for 30 minutes
 - 80° for 15 minutes
 - 10° forever
 - 8.5. After cycling is complete, quick spin samples.
 - 8.6. At the touch screen, select **Clean Up PCR Products** to process the trays for the PCR clean up step.
- 9. Sequencing on the Biomek**
- 9.1. At the touch screen, use the **Create Sequenced DNA** step to generate barcodes for the forward and reverse sequence trays. Place the barcode on the side **opposite** of the numbered columns of the MJ 384 well hard shell tray. (This is the only location at which the ABI 3730 will be able to read the barcode for loading.)
 - 9.2. Restart the **Create Sequenced DNA** step at the touch screen.
 - 9.3. Retrieve P1K Forward sequencing brew, M13 Reverse sequencing brew, empty 384 well MJ hard shell trays with newly printed/attached barcodes, and the PCR trays to set up the Biomek for sequencing.
 - 9.4. At the touch screen, use the **Create Sequenced DNA** step. Choose the appropriate transfer pattern by touching the “1-384 to 1-384 with SeqBrew” option.
 - 9.5. Scan the PCR tray, the forward sequencing brew materials core barcode, and the empty forward sequencing tray. Now scan the pcr tray again and then the reverse sequencing brew materials core barcode and the empty reverse sequencing tray. Hit **confirm**. **Note:** *This step may take a long time to confirm.*
 - 9.6. At the Biomek, open the appropriate 384seq_rxn_(X)trays.bmt method.
 - 9.7. Place the sequencing brews, PCR product trays, and the empty 384 well MJ hard shell trays onto the deck as shown in the **Instrument Setup**.

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- 9.8. Check both water and waste before beginning the method.
- 9.9. Start the method by clicking on the **green run arrow**.
- 9.10. When the method is complete, quick spin and check that the volume in the wells is consistent.
- 9.11. 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."
- 9.12. Cycling conditions are as follows:
 - 95° for 15 seconds
 - 50° for 5 seconds
 - 60° for 2 minutes
 - Repeat from step 1 for 24 cycles
 - 10° forever
- 9.13. Remove trays from cycler, quick spin, and prepare for sequence clean up.

10. Sequence reaction clean-up

- 10.1. Make precipitation cocktail using the following recipe:
 - 25uL 100% ethanol X number of reactions
 - 2.5uL 3M Sodium Acetate X number of reactions
- 10.2. Using a Genetix Q-fill or a multidrop, add 25uL of the precipitation cocktail to the wells of the sequenced tray.
- 10.3. Cover the tray with a tape plate seal and spin in the centrifuge for **30 minutes** at 3500 rpm. (Program 2)
- 10.4. 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)
- 10.5. Retrieve 70% ethanol and decanted trays for the second step of the sequencing reaction clean-up process.
- 10.6. Add 25uL of 70% ethanol to the trays using a Genetix Q-fill or a multidrop.
- 10.7. Cover the tray with a tape plate seal and spin in the centrifuge for **15 minutes** at 3500 rpm. (Program 1)
- 10.8. 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)
- 10.9. Retrieve the trays from the centrifuge and place into the speed vac for 15 minutes to dry.
- 10.10. At the touch screen use the **Clean-up Sequenced DNA** step for the ethanol precipitation.
- 10.11. Select **Resuspend Sequenced DNA** at the touch screen to prepare the trays for the loading process.
- 10.12. Place trays in appropriate Loading bin to be loaded. **Note:** *On occasion, trays will need to be eluted by 15uL of ddH2O and sealed for the loading group. Please check with your coordinator or the Load Group coordinator to determine if this is necessary.*