

VALIDATION OF TAILED PRIMERS FOR MUTATIONAL PROFILING

Production Author: Ginger Fewell

Revision Date: 10/05/05

Version 2.5

PURPOSE: This protocol describes the PCR amplification, gel loading, PCR clean up, sequencing and sequence clean up steps for the validation of tailed PCR primers used in Mutational Profiling. All Mutational Profiling validation PCR reactions will be set up with the ep5070 robot.

MATERIALS AND EQUIPMENT:

Tailed PCR primers @ 1.2uM

- 1.2uM fwd + 1.2uM rev mixed in tubes or plates

Genomic template @ 5ng/uL

- 3 individual DNA samples from Watson lab
- 2 commercial DNA samples

2X AmpliTaq Gold Master Mix

50% glycerol

ddH₂O

10:0.1mM TE buffer

1.7mL microfuge tube

384 well MJ hard shell trays

ep5070 or ep5075 liquid handling robot

ep5070 or ep5075 accessories

30mL ep5070 reservoirs

384 well source tray

1.5% agarose gel

Xylene cyanol

Marker VI

Gel rig

1X TAE buffer

SAP

Exonuclease I

Sigma Water

384 V-groove reservoir

MP forward sequencing brew

MP reverse sequencing brew

Biomek FX with 384 well head

3M-sodium acetate

100% ethanol

70% ethanol

Pipettes of appropriate volume

Centrifuge

Thermal cycler

Multidrop or Q-fill

Paper towels

Speed Vac

384 well cap mats

Disposable mask

Ethanol wipes

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

2. Pull primers to be validated

- 2.1. If primers are at -20° C pull primers from freezer and thaw on ice.
- 2.2. Primers will be either from ABI in Matrix tubes or from Illumina in 96 well Costar trays.
- 2.3. If Illumina primers are dried down, resuspend in 100uL of 10:0.1mM TE buffer and let sit for 2 hours. Primers will be @ 30uM when suspended in 100uL
 - Make a 1.2uM stocks from the 30uM source plates if the forward and reverse primers are not mixed in the source plate and combine the forward and reverse stocks together. When the stocks are pooled there will be 0.6pmol of each primer in the plate.
 - Make a 1.2uM stock from the 30uM source if the forward and reverse

VALIDATION OF TAILED PRIMERS FOR MUTATIONAL PROFILING

Production Author: Ginger Fewell

Revision Date: 10/05/05

Version 2.5

primers are already mixed in the source plate.

3. Prepare PCR brew

- 3.1. Determine amount of reagents needed for number of reactions to be done using the following recipe.
 - 0.4uL – ddH₂O x number of reactions
 - 1.6uL – 50% glycerol x number of reactions
 - 5.0uL – 2X AmpliTaq Gold Master Mix x number of reactions
- 3.2. In a 50mL conical tube, add the reagents listed above in that order and mix gently by inverting the tube.
- 3.3. Pour the PCR brew into a 30mL ep5070 reservoir and cap tightly. Place the reservoirs on ice or in the 4° C until ready to use.

4. Prepare DNA

- 4.1. Start with original DNA tubes from the Watson lab and the commercial DNA and check the concentration with the NanoDrop.
- 4.2. Determine how much volume will be needed from each sample and make a 5ng/uL stock in a 1.7mL microfuge tube.

5. Add PCR brew to 384 well PCR plates

- 5.1. Find the appropriate program in the MP folder on the control panel, highlight and select START.
- 5.2. Confirm the deck layout with what is displayed on the control panel and hit OK.
- 5.3. A prompt will appear to enter the minimum volume. *NOTE: The volume to be filled in must be greater than or equal to the volume prompted by the program.
- 5.4. Watch the robot scan the tip box and begin the method to ensure that it calibrated correctly.

6. Add primers to 384 well PCR plates

- 6.1. Find the appropriate program in the MP folder on the control panel, highlight and select START.
- 6.2. Place the primer tubes or plates in the appropriate order/position in the deck position shown by the control module.
- 6.3. Confirm the deck layout with what is displayed on the control panel and hit OK.
- 6.4. A prompt will appear to enter the minimum volume. *NOTE: The volume to be filled in must be greater than or equal to the volume prompted by the program.
- 6.5. Watch the robot scan the tip box and begin the method to ensure that it calibrated correctly.

7. Add DNA to 384 well PCR plates

- 7.1. Find the appropriate program in the MP folder on the control panel, highlight and select START.
- 7.2. Place the DNA tubes or plates in the appropriate order/position in the deck position shown by the control module.
- 7.3. Confirm the deck layout with what is displayed on the control panel and hit OK.
- 7.4. A prompt will appear to enter the minimum volume. *NOTE: The volume to be filled in must greater than or equal to the volume prompted by the program.
- 7.5. Watch the robot scan the tip box and begin the method to ensure that it calibrated correctly.
- 7.6. When the program finishes, quick spin the PCR plate and prepare for cycling.

VALIDATION OF TAILED PRIMERS FOR MUTATIONAL PROFILING

Production Author: Ginger Fewell

Revision Date: 10/05/05

Version 2.5

8. PCR cycling

- 8.1. Seal PCR plates with 384 well cap mats and quick spin.
- 8.2. Place in the thermal cycler. Run the ABI-PCR program being sure to change the volume to 10uL. Cycling conditions are as follows:
 - 95° C for 5 minutes
 - 94° C for 30 seconds
 - 60° C for 45 seconds
 - 72° C for 45 seconds
 - Repeat from step 2 40 times
 - 72° C for 10 minutes
 - 10° C forever
- 8.3. When PCR cycles are complete pull the plates from the thermal cycler and quick spin the trays and prepare to run gels. If gels cannot be run right away place the trays in the 4° C refrigerator until needed.

9. Running a gel on the PCR product

- 9.1. Retrieve agarose gels from the Mutational Profiling 4° C refrigerator.
- 9.2. Fill gel rigs with approximately 1L of 1X TAE buffer.
- 9.3. 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
- 9.4. Gel is run at 140 Volts for 50 minutes
- 9.5. Take a picture of each gel. Make sure that the marker is exposed consistently from gel to gel using 0.8 second exposure. If the PCR product is faint or bright, adjust exposure and take a second photo.

10. PCR clean-up with Exo-SAP

- 10.1. Retrieve Exo-SAP cocktail from Materials Core.
- 10.2. At the biomek, open the 384exo-sap.bmt method.
- 10.3. Place the PCR product trays and a clean 384 V-groove reservoir on the deck as shown in the instrument setup.
- 10.4. Pour the Exo-SAP cocktail into the 384 V-groove reservoir.
- 10.5. To start the run click the green start arrow.
- 10.6. When the method is complete, quick spin the trays and cover with 384 well cap mats and cycle at the following conditions. Change volume on the thermal cycler to 10uL before beginning the Exo-SAP program.
 - 37° for 30 minutes
 - 80° for 15 minutes
 - 10° forever
- 10.7. After cycling is complete, quick spin samples and prepare for sequencing.

11. Sequencing on the Biomek

- 10.1. Sequencing brew for tailed primers is made in materials core
- 10.2. Open the 384seq.bmt method
- 10.3. Place the sequencing brew, PCR product tray and MJ hard shell sequence trays on the deck as shown in instrument setup.
- 10.4. Start the method by clicking on the green run arrow

VALIDATION OF TAILED PRIMERS FOR MUTATIONAL PROFILING

Production Author: Ginger Fewell

Revision Date: 10/05/05

Version 2.5

- 10.5. When the method is complete, cover with dental 384 well cap mats, quick spin and put in the thermal cycler as soon as possible.
- 10.6. Run the BD25 program on the thermal cycler and change the volume to 6uL before starting the program. Cycling conditions are as follows:
- 10.7. Remove from cycler, quick spin and prepare for sequence clean-up.
 - 96° for 1 minute
 - 96° for 10 seconds
 - 50° for 5 seconds
 - 60° for 4 minutes
 - Repeat from step 2 for 25 cycles
 - 10° forever

11. Sequence reaction clean-up

- 11.1. Make precipitation cocktail using the following recipe:
 - 25uL 100% ethanol x number of reactions
 - 2.5uL 3M Sodium Acetate x number of reactions
- 11.2. Using a Q-fill or multidrop, dispense 25uL of the precipitation mix into each well of the tray.
- 11.3. Spin trays at 3500 rpm for 30 minutes.
- 11.4. Decant trays and place upside down on paper towels. Place the trays and paper towels upside down in the centrifuge and spin at 400 rpm for 5 seconds.
- 11.5. Add 25uL of 70% ethanol to each well using a Q-fill or multidrop.
- 11.6. Spin trays at 3500 rpm for 15 minutes.
- 11.7. Decant trays and place upside down on paper towels. Place the trays and paper towels upside down in the centrifuge and spin at 400 rpm for 5 seconds.
- 11.8. Dry the samples in the speed vac for about 15 minutes.
- 11.9. Prepare trays for loading by resuspending with 15uL of ddH₂O using the Q-fill or multidrop.

12. Loading and Data handling.

- 12.1. All samples will now be barcoded using the Sherlock application to assign read names.
- 12.2. Fxgasp will put reads into a new directory based on xgasp date.

Revision History:

6/20/05 Version 2.2

1. Added General Procedures and Information section at step 1.
2. Added disposable masks and ethanol wipes to the Materials and Equipment list

6/29/05 Version 2.3

1. Changed sequencing cycling parameters from ABI-SEQ to BD25.
2. Added Sigma Water to Materials and Equipment list.
3. Removed dH2O from EXO/SAP cocktail list.
4. Added Sigma Water to EXO/SAP cocktail list.

09/19/05 Version 2.4

1. Xylene cyanol replaced agarose loading dye throughout protocol.

VALIDATION OF TAILED PRIMERS FOR MUTATIONAL PROFILING

Production Author: Ginger Fewell

Revision Date: 10/05/05

Version 2.5

10/05/05 Version 2.5

1. Added the following items to the Materials and Equipment section:
 - Tailed PCR primers @ 1.2uM
1.2uM fwd + 1.2uM rev mixed in tubes or plates
 - 384 V-groove reservoir
 - 384 well source tray
 - 10% bleach
2. Removed the following item from the Materials and Equipment section:
 - Tailed PCR primers @ 0.6uM
0.6uM fwd + 0.6uM rev mixed in tubes or plates
3. Added the following step to the General procedures and information section:
 - 11.1. Clean bench with 10% bleach before and after use.
4. Changed the following steps in the Running a gel on the PCR product section:
 - 11.2. 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
 - 11.3. Take a picture of each gel. Make sure that the marker is exposed consistently from gel to gel using 0.8 second exposure. If the PCR product is faint or bright, adjust exposure and take a second photo.
5. Changed steps in PCR clean-up with Exo-SAP section to reflect that cocktail is obtained from Materials Core rather than prepared by technician.