**Pyromark MD Biotage Assay Setup and Run Notes**

PyroMark Gold Q96 CDT Reagents (6 x 96) Qiagen 972824

Pyromark Q96 HS Capillary Tips (8) Qiagen 979104

PyroMark Q96 HS Reagent Tip (4) 979102

PyroMark Q96 HS Plate (100) Qiagen 979101

GE Healthcare Streptavidin Sepharose 17-5113-01

GE Healthcare Illustra MicroSpin G-50 Columns 27-5330-01

Genesee Zymo Quick-RNA MiniPrep Genesee Cat #: 11-327

**PCR assay design**

Amplicon can be up to 300 bp but <200 is better.

One of the two amplification primers must be biotinylated and HPLC purified.

Sequencing primer can be up to 5 bp away from SNP, but sequence quality drops as a function of length so closer is better. Immediately adjacent to SNP is best. 19bp is enough for seq primer, no concern about annealing temperature, only concern is self-priming. Can sequence a SNP on either strand of amplified product.

1. Primer design for amplicon
	1. 80 to 300bp amplicon
	2. SNP can be read from one direction (f/r)
	3. Ideal SNP is flanked by bases that do not match either allele. Runs of two are acceptable but assay accuracy decreases in doublets from deconvolution of peaks.
	4. Sanger Web-based query tool (or use local database):

http://www.sanger.ac.uk/sanger/Mouse\_SnpViewer/rel-1303

1. PCR test
	1. B6, Cast, and no RT cDNA made with SSIII using 0.5ug of RNA is fine
	2. Test each amplicon under standard conditions first:
		1. Apex Taq, chromataq buffer, 1.5mM MgCl2
		2. 32 cycles:
			1. 94 20sec
			2. 56 30sec
			3. 72 30sec
	3. (For unsuccessful amplicons, just order new primers, or can vary MgCl2 or annealing temp, etc.)
	4. For successful amplicons, column purify each product, sequence B6 and Cast amplicons with both PCR primers
		1. From sequence data, ensure that SNP of interest can be accessed via a short sequencing primer (annealing temp of at least 50 C) and that this primer does not sit over a SNP or indel
		2. Decide which strand is best to sequence based on sequence diversity following SNP
		3. Order primers:
			1. Sequencing primer is on same strand as SNP, ends directly adjacent to SNP or at most one base removed
			2. Biotinylated PCR primer is on opposite strand of sequencing primer
2. For successful assays, find linear range of amplification on pyrosequencing-grade cDNA
	1. DNase treat 10 - 20ug of RNA using Zymo QuickRNA MiniPrep kit and column:
		1. 80ul DNase reaction (off column):
			1. 5ul Zymo DnaseI
			2. 8ul 10x DNaseI reaction buffer
			3. H20 to 80 ul
		2. Incubate 20 minutes at Room temp
		3. Add 4x RNA Lysis Buffer (RLB; 320ul)
		4. Add 400ul 100% Ethanol
		5. Add mixture to Zymo-Spin IIICG column, centrifuge for 30secs
		6. Add 400ul of RNA Prep Bufer, spin 30secs
		7. Add 700ul of RNA Wash Buffer, spin 30secs
		8. Add 400ul of RNA Wash Buffer, spin for **2 minutes**
		9. Add 35ul of millipure h20, wait 5 – 10 minutes, place column in clean elution tube, spin for 30 seconds to elute RNA
	2. pyrosequencing-grade cDNA:
		1. 5ug of RNA RT’d with SSIII in 20ul rxn
		2. After RT, add 30ul of TE to cDNA to allow product to be used for multiple assays
	3. For PCRs that go on pyrosequencer, column purify biotinylated primer to remove trace free biotin leftover from sequencing rxn:
		1. Add 30ul of 100uM primer to G25 or G50 microspin
		2. Measure elution volume, assume 85% recovery, and calculate new [primer]
	4. Under appropriate rxn conditions, find linear range in 25ul PCR:
		1. 24, 26, 28 30 cycles
		2. run 15ul on gel, select cycle number that shows an increase from previous cycle# and an increase after as well
3. Test pyroseq assay:
	1. Run 50ul PCR in linear range on B6, Cast, and no RT cDNA. Include no template as well.
	2. Assay 15 and 10ul of each product.
4. Run pyroseq assay:
	1. Run triplicate, 50ul PCRs starting with 1ul of cDNA
	2. Run triplicate for B6 and Cast cDNA – especially important if assay has background
	3. One no RT and one rxn with no template at all to ensure primer does not self prime
	4. Run optimal amount of product determined in step #4 on Biotoge AQ96

**Pyromark Assay Entry**

Simplex Entry – Assay Design

Right click “new entry”

SNP should be one base removed from Primer in format X/Y

Follow snp by remaining sequence to analyze – like 20 bases or so, instrument seems to select only about 6 bases after SNP

**Setting up pyrosequencing assay**

Get streptavidin beads from 4C.

Separate 15ul of PCR product for quantification, otherwise you will get trailing peaks in pyrosequencer

Add 70ul of 20x streptavidin bead mix. Per well:

 2ul beads

 40ul binding buffer

 28ul diH20

Shake 4 min @1200 rpm in eppendorf MTP. If using a 96 well plate, ensure to tape it down.

Add 42.7ul of 10uM sequencing primer to 1400ul of annealing buffer, in reservoir tray specific to sequencing primer.

 4.3ul of 100uM to 1438 of annealing buffer

Add 12ul of primer annealing buffer to white PSQ HS96 plate/Biotage

Rinse out used primer resevoirs, put avidin beads back at 4C.

Place Al Biotage in di H20 30 seconds to wash vacuum tips.

Hold up tips, make sure all H20 is gone.

Pick up sample from tubes with vacuum line.

Put in EtOH 20 seconds, then lift and make sure EtOH is gone.

Put in NaOH 20 seconds, then lift and make sure NaOH is gone.

Put in wash buffer 20 seconds, then lift and make sure wash buffer is gone.

DISCONNECT PUMP

Stick ceramic in primer mix and jiggle (30sec)

Wash out pump with H20.

Denature primers @ 80 C 2 minutes

Wash ceramic probes with H20 so they will be clean for next use.

Let different plates cool before stacking.

**Running pyrosequencer**

AQ mode

SNP run

Instrument parameter = Gold II

Select cells of interest, click activate

Click setup

Assign cells click activate and close

Click batch tab button at top

Right click to make new batch, add name

Drag SNP run into appropriate plate slot

multiple plates can be used here, again gold II params.

Click off use barcodes

Click analyze AQ and SNP

Plate #1 should be loaded into instrument on top, plate 10 on bottom

Save and wait until hitting run

Now set up reagent resevoirs.

From Batch drop down, get volume of reagents needed by clicking “setup info”

Increase net amount of reagent needed by 20%.

Mix nucleotides in TE in separate eppendorf 1:2

NO AIR BUBBLEs and add to appropriate resevoir

Click Instrument tab

Open center, add reagents.

Take test plate, lock into plate with tab carefully

Click instrument manage, to prime click Test

Run test, make sure you see dot pattern:

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. . . .

If not, you need to clean out reagent dispensers.

Load plate, click run

After run, remove cartridge and clean out with diH20 by filling and gently squeezing.

**Analyzing data**

In completed batch, click “AQ mode”

Select cells of interest

Click “Analyze selected”

Save

Report

Click “Go”

Save, run word file through perl script