Breeding Protocol

Chose mating combination:

- Mating pair - one male, one female; common for strains with good fecundity. Leave pair together continuously, whenever possible, to maximize production.
- Mating trio - one male, two females; maximizes cage space for breeding but Moffitt will require you separate pregnant female or male before pups arrive.
- Male rotation - single male placed with 1-2 female(s) per week; useful to quickly establish a colony. Once male is removed, do not return to original cage until pups are weaned, but can be placed with a different 1-2 female(s) while original cage of pups is waiting to be weaned.

Reproductive characteristics:

Mating age: 6-8 weeks of age
Gestation: ~19-21 days
Wean age: ~21 days; up to 28 days
Litter size: 2-12 pups; highly strain-dependent
Replace breeders: ~7-8 months of age

Select appropriate breeding scheme:

Homozygous mutant (-/-) x homozygous mutant (-/-)

- Can be used if both genders are viable and fertile as homozygotes
- Offspring: 100% homozygous; genotyping not required

Heterozygous mutant (-/+) x homozygous mutant (-/-)

- Can be used if only one gender is viable and fertile as a homozygote
- Offspring: 50% homozygous; 50% heterozygous; genotyping required

Heterozygous mutant (-/+) x heterozygous mutant (-/+)

- Must be used if neither gender is viable or fertile as homozygotes
- Offspring: 25% homozygous; 50% heterozygous; 25% wild-type; genotyping required

Marking cage card (Moffitt/USF Comparative Medicine)

Species: mouse
Strain: LSL-Braf;PtenFL;TyrCre
Cage#: optional
Investigator: Karreth
IACUC#: 2017M (for breeding)
ID Male: 101, ID Female: 102, DOB Male: 1/1/11, DOB Female: 1/1/11
Mated: 3/1/11
DOB: will be filled out by you or ACT who discovers pups

Timed Pregnant Females

- House stud males (individually) for 2 weeks prior to mating
- Use 8 to 15 week old females
- Add 1 or 2 female(s) into each stud male's cage in the late afternoon/evening, prior to the lights going off (the dark cycle)
- Check for vaginal plugs early the next morning (plug will dissolve over time)
- The date a plug is observed is gestational day 0 to 0.5

NOTE: Not all females will plug and not all plugged females will become pregnant; success rate varies by strain, male's experience, conditions, etc.

DNA Lysis

Proteinase K method

1) Add 500 µl of tail lysis buffer plus proteinase K (add 100 µl of proK/10ml of lysis buffer) and heat overnight at 50 – 60 °C.
   - Can add 5 µl of proK per Eppendorf individually if preferred.
2) Add 1000 µl of 96 – 100 % of ethanol to precipitate the DNA and mix until clear.
3) Spin at 4 °C for 5 - 10 min at max speed.
4) Carefully pour off supernatant to waste.
5) Wash the pellet with ~500 µl 70 % ethanol.
6) Carefully pour off supernatant to waste.
7) Invert tubes over paper towel or kimwipe to dry and leave standing for ~20 – 30 minutes to allow ethanol to fully evaporate.
8) Re-suspend pellet in 100 µl of sterile water. Expected DNA concentrations of ~100 ng/µl.

Lysis Buffer (1X) Stock:
For 100ml total volume:
5 ml of 2M Tris, pH7.5
2 ml of 5M NaCl
1 ml of 0.5M EDTA
2 ml of 10% SDS
90ml ddH_2O

NaOH method

1) Add 200 µl of 50 mM NaOH to the ear/tail if they're in a plate. If in an Eppendorf tube, then add 500 µl.
2) Place in 95 °C for 15 - 30 minutes. If using Eppendorf tubes, find a way to prevent the caps from opening due to steam pressure or manually open and close the caps to let out pressure after the first 10 - 15 minutes.
3) Then add 10 % volume of 1 M Tris at 8.0 pH.
4) Mix and it can be used immediately for PCR.

NaOH tail buffer (50mM NaOH)
5 ml of 10 N NaOH
1 L of distilled or ddH_2O

Genotyping PCRs

GoTaq green (M7122/M7123) and colorless (M7132/M7133) master mixes from Promega have been successfully used for all genotyping. The colorless is better used for the Qiaxcel machine as the green loading dye causes weaker bands and/or increased fuzziness in the results over time on the lifespan of a single cartridge.
Primers are diluted to 25μM and are used at 0.5 μl per reaction. Primer ratios are represented in the order the primers are presented (typically common first, then mutant/KO, then wildtype).

PCR Reactions [25 μM diluted primers]
10 μl GoTaq MM
1 μl DMSO (optional)
1 μl primer mix
7 μl Ultrapure/DNase-free H_2O
1 μl genomic DNA
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20 μl total volume

Kras
Common: LSL-Kras y116
TCCGAATTCAGTGACTACAGATG
Mutant (323 bp): LSL-Kras y117
CTAGCCACCATGGCTTGAGT
Wildtype (450 bp): LSL-Kras y118
ATGTCTTTCCCCAGCACAGT
Ratio 1:1:1

C2 (used for transgenes inserted into the CHC)
Common: C2 new 1
AATCATCCCAGGTGCACAGCATTGCGG
Transgene (400 bp): C2 new 2
CTTTGAGGGCTCATGAACCTCCCAGG
Wildtype (250 bp): C2 new 6
GACGGCAATTTCGATGATG
Ratio 1:1:1

Cags-LSL-rtTA (rosa26 kock-in)
Common: Rosa A
AAAGTCGCTCTGAGTTGTTAT
Mutant (350 bp): Rosa B
GCGAAGAGTTTGTCCTCAACC
Wildtype (297 bp): Rosa C
CCTCCAATTTTACACCTGTTC
Ratio 2:1:2

Cags-rtTA
Common: Ch8 Rev2
CGAAACTCTGGTTGACATG
Transgene (200 bp): SApA For2
CCTCCTCTCCTGACTACTCC

Wildtype (363 bp): Ch8 For1
TGCCTATCATGTTGTCAAA
Ratio 5:1:4

P53KO
Common: oIMR7778
TATACTCAGAGCCGGCCT
KO (650 bp): oIMR8306
CTATCAGGACATAGCGTTGG
Wildtype (450 bp): oIMR7777
ACAGCGTGGTGGTACCTTAT
Ratio 2:3:1

LSL
Primer #1: LSL geno F
TGCTGTCCATCTGCACGAGA
Primer #2: LSL geno R
GAAAAGGCCGAGATCGCCATA
TG: ~400 bp
Ratio 1:1:1:1 (Using P53KO WT primers)

Tyrosinase-CreERt2
Primer #1: CRE26
CCTGGAAAATGCTTCTGTGCG
Primer #2: CRE36
CAGGGTGTTATAAGCAATCCC
Wildtype #1: GABRA12
CAATGGTAGGCTCACTCTGGGAGATGATA
Wildtype #2: GABRA70
AACACACACTGGCAGGACTGGCTAGG
Ratio 1:1:1:1 (Using P53KO WT primers)
- Alternative: Use p53KO WT primers instead of Gabra primers

LSL-NrasQ61R
Primer #1: Nras - 1
AGACGCGGAGACTTGGCGAGC
Primer #2: Nras - 2
GCTGGATCGTCAAGGCGCTTTTCC
Primer #3: GENO2
GCAAGAGGCCCGGCAGTACCTA
WT: 487bp TG: 371 bp
Ratio 1:1:1

PTEN Flox
Primer #1: PTEN genoF
CAAGCACTCTGCGAACTGAG
Primer #2: PTEN genoR
AAGTTTTTGAAGGCAAGATGC
WT: ~160 bp Mut: ~340 bp

Ratio 1:1

CDKN2A Flox
Primer #1: CDKN2a genoFnew
CCTGACTATGGTAGTAAAGTGG
Primer #2: CDKN2a genoRnew
ACGTGTATGCCACCCTGACC
WT: ~290 bp TG: ~360 bp
Ratio 1:1

LSL-Braf V600E
Common: LSL Braf up
CCAAAGTCCCCATAATCTCG
Mutant (422 bp): LSL Braf dwn
GAATGGAAGGATTGGAGCTAC
Wildtype (241 bp): deltaLSL Braf dwn
GCTGCTAACAGGGAGTGGAA
- Instead of the Wildtype primer we use the p53 KO wildtype primers. Ratio 1:1:1:1

Ovgp1-iCreER
Primer #1: Ovgp1-F1
GATGGTGGGAGAATGCTGAT
Primer #2: Ovgp1-R1
AAGGTGGCAGCTCTCATGT
TG: approximately 500 bp
Ratio 1:1

H11-Cas9
Common: 23907H11comR
ATCTGGGGCCATAAATGCT
Mutant (422 bp): 23908H11MutF
CCGCCACTCGACGATGT
Wildtype (241 bp): 23906H11WTF
TGGCAGTTTGACACATCCTG
WT: 410bp Mut: 373bp
Ratio 1:1:1

PCR programs
Standard (STD on PCR machine)
- 95°C for 2:00
- 35 cycles of 95°C for 0:30
58°C for 0:30
72°C for 1:00
- 72°C for 5:00
- 4°C for forever

Cags
- 95°C for 2:00
- 35 cycles of 95°C for 0:30
  54°C for 0:30
  72°C for 1:00
- 72°C for 5:00
- 4°C for forever

Include water (negative) and positive control (typically DNA from a heterozygous mouse)

The Standard program works for all genotyping with the exception of Cags. Other genotypes that I have run using the Cags program were C2, CDKN2A Flox, LSL-NrasQ61R, LSL, and PTEN Flox and there were no issues with the bands. The plates I've been using for both lysing DNA using NaOH and genotyping are the semi-skirted plates from USA Scientific (cat. No. 1402-9700). The film lids used for the plates are the MicroAmp Optical Adhesive Film (cat. No. 4311971).

Tyrosinase-CreERt2 copy number PCR

- Isolate genomic DNA from tail or ear punch (or ES cells), preferably by clean method (lysis buffer plus proteinase K, followed by ethanol precipitation)

- Dilute gDNA to approximately 10ng/μl in H_2O

- Set up master mix (Run each tail sample in triplicates):
  per sample
  5μl Taqman Mastermix
  0.5μl Cre probe
  0.5μl Tfrc control probe

  Include a WT, homozygous, and heterozygous control

- In real-time PCR plate, combine 6μl of Mastermix and 4μl of gDNA (= ~40ng)

- Run RT-PCR:
  - Taqman
  - Quantitation – ddCt
  - Regular (~2hrs) reaction

  - On target/sample set up page, the two targets are Cre (FAM/NFQ-MGB) and Tfrc (VIC/TAMRA)

  - On plate set up page, each sample needs to have 2 targets (Cre AND Tfrc)

  - Set Normalization to Tfrc and a sample that is a known heterozygous

Agarose Gels and QIAxcel

Running on an Agarose Gel

You will need 1x TAE (Tris base, acetic acid and EDTA) buffer, agarose powder, a gel case with combs to create wells in the solidified TAE, and a chemical such as Ethidium Bromide or SYBR Green to add to the TAE to stain the DNA.
Since the bands of all of the genotyping are between 100 and 1000 bp, you should only need to utilize 2% agarose gels to see the bands. For every 100 ml of 1x TAE, add 2 grams of agarose powder and microwave the container until the powder has mixed in with the TAE. Then add 3-4 µL of EtBR per 100 ml of the agarose solution and swirl to mix. Prevent leakage of the gel case with tape or lined rubber and pour in the gel solution (it's recommended to let the solution cool down for some minutes before pouring due to possible warping of the gel case from heat).

No loading dye is required if GoTaq Green is used. Load 10µl of each PCR reaction per well. We use NEB's 2-log marker as the DNA ladder. Run the gel at 120-130V for >30 minutes.

Using the Qiaxcel

The kit being used is the DNA Screening Kit (cat. No. 929004), but initially the kit used when the machine was new was the DNA Fast Analysis Kit (cat. No. 929008). I'm not entirely sure what the real differences between the two kits are besides speed and cost, with the fast analysis kit likely faster and pricier.

Some of the default settings may have been made/modified by Luke before I modified it into running our plates. I'll attempt to detail all of the settings that are related to the plate reading:

Process Profile:
After I modify all of the settings mentioned, I saved them under a process profile so I don't have to keep configuring the same thing over and over.
In the "Included Steps" section Luke's machine had "Analysis" and "Report" both ticked. I'm not sure if we really need the "Analysis" one but the "Report" one is essentially mandatory for logging and for remote printing, assuming the computer isn't already hooked up to a printer.

Run Parameters:
"Allow marker definition" is optional once you have the size marker reference table set up (details are in the quick-start manual they provide with every kit). However if you want to have a more accurate size reading on your bands, then this should be checked and you should have one of the wells filled with the size marker you wish to use.
The method I usually use is the AL320 one with 20 second injection time. The injection time affects the saturation of the signals/bands, with higher injection time making the signal stronger as well as making the run somewhat longer.
I was informed that L, M, and H refer to the band strength you're aiming to detect, but for genotyping that doesn't seem to really matter so I choose L to try to make the bands visually stronger and to see if there were any other weak bands. According to the user manual, there is an option to run multiple methods on the same row or separate methods on different rows but I have not used this option.

Analysis
I have not changed anything in this section. The method used is called "Default DNA v2.0" which is what Luke uses in his runs.

Marker

I've used the same reference marker table for a year based on Luke's experience on his having used the same reference table at his old lab for a minimum 1.5 – 2 years. Running the size marker is where the machine will try to make an accurate guess at the size of the bands by using predetermined sized bands. I've run it a handful of times, the last of which was to make the reference marker table.

Report/Export

Most of the options here should be self-explanatory and customized to what you're looking for. The key options I use when exporting to pdf for printing is to have the "Gel Image overview" checked, scale the y-axis to size (it will default to relative time if the alignment marker is not detected properly), have the gel layout read from A1 -> A12 rather than from A1 -> H1, and to zoom to the alignment marker. The gel layout allows for you to make your plate data be read vertically with 12 rows of 8 samples rather than the 8 rows of 12. The cartridge can only be used horizontally however, so this option should be reserved for full plate readings. The report options section is likely referring to the popup of results that can appear after the runs have all finished, but I set it to exactly the same as the export options.

Sample Selection

Here you can change the name of the file(s) that will be saved to the directory of your choice based on your report/export settings. We've only really used the 15 bp – 3 kb alignment marker but Qiaxcel has several other options for smaller or bigger ranges. The alignment marker is where the machine will take the lowest and highest bands and read those as the range that is set (in this case as 15 bp and 3 kb), normalizing all of the other bands based on that information. The sample band size should not exceed the alignment marker size or else it will end up being labeled incorrectly as an alignment marker band.
You can only input sample information if the "Provide Sample Information" box is ticked.

Run Check

This section will prevent the program from being run if the confirmations, errors, and warnings are not checked. Because I use an old reference table (and because it was made for another cartridge) it notifies me of that and forces me to check an extra box. The rest of the window is a summary of what you've told the machine you're about to run.

Notes

- All wells in the rows that are being read should have at minimum 10 µl of solution inside to prevent air bubbles from getting into the cartridge tips and messing with the readings.
- If the nitrogen runs out in the middle of the run or the window cover on the machine is opened, the results will be discarded and you will have to run the program again.
- The machine's buffer tray should be set to the parked position after you're finished with the runs so that the cartridge tips sit inside of the wash buffer when not in use.
- The alignment marker has only a few days lifespan in room temperature, so unless the machine is in constant use the tube strip with the marker should be placed in a 4 °C fridge to extend its usability.
- You should preferably clean out the buffer tray when changing to a new cartridge.
- The machine uses a very small amount of the sample (~0.1 µl), so if necessary you can run the samples on a regular agarose gel after the Qiaxcel runs finish.

- The runs take roughly 8 - 9 minutes to finish per row, with a full 8 row plate taking 68 minutes on a regular screening kit running with the AL320 setting with a 20 second injection time.

If the alignment marker will not align properly

If redoing the plate with a new alignment marker or the newer alignment marker will not function correctly due to a problem with the cartridge itself, then you will have to export the results using absolute migration time instead of relative migration time. This, along with other useful sample selection and editing functions, can be done in the analysis tab by re-exporting the results with changed parameters. This should now produce results similar to when it is run on an agarose gel where not all the bands are aligned but an educated guess can be made based on the position of the bands relative to the rest of the samples and the alignment marker.