GENOTYPING – Bridgewater Laboratory Protocol

Preparations:

- Retrieve the samples from the CAF freezer. Put the samples in a -20°C freezer until ready for genotyping.
- Make sure all reagents and materials are present.
    - ddH_2O
    - 5X Lysis Buffer A (-20°C – box "Genotyping")
    - 10X Lysis Buffer B (-20°C – box "Genotyping")
    - 2X HS-Red TAQ Mix (-20°C – box "Genotyping")
    - Primers (-20°C – box "Genotyping")
    - RedSafe (+4°C box "Genotyping")
    - 1 kb and/or 100 bp DNA ladder(s) (+4°C box "Genotyping")
    - Agarose (chemicals shelf)
    - Small Erlenmeyer flask (bench where electrophoresis is performed)
    - 100 ml cylinder (bench where electrophoresis is performed)
    - Pipettes and pipette tips
- Write down the specific protocol and make the calculations beforehand.
    - Which samples (the written numbers as indicated by the CAF; write down how many samples there are in total and their numbers) need to be processed.
    - Which primer to use for each sample.
    - Count the number of samples for each primer and the total number of samples and plan how to lay out the samples on the agarose gel – the gel has 20 columns and 2 rows.
    - Keep in mind to include at least one column for a ladder for each side of the gel, one column for the positive control, and one column for the negative control for each primer being genotyped for.
- **Make a schedule about when to perform each step.**
    a) Lysis of samples: ~1h
    b) Set up PCR: ~30min.
    c) PCR will run for ~ 2hrs
    d) Make the agarose gel: ~40min
    e) Run gel electrophoresis + take picture of the gel + upload results: ~1h
        - DAY 1: a) + b) = total of ~1.5hrs → then run c) = total of ~2hrs OR overnight (proceed to *DAY 2* if protocol is carried on to the next day)
        - DAY 2: d) + e) = total of ~2hrs

Step 1 – Lysis

1) Get samples out from -20°C freezer and line up on a 1.7 ml tube rack in numerical order.
2) Take out 5X Buffer A (white cap) + 10X Buffer B (red cap) from -20°C freezer & thaw.
3) Get 50ml tube with ddH_2O (from *Milli-Q*).
4) Add **70 µl of ddH_2O** to each sample.
5) Add **20 µl of 5X Buffer A** to each sample.
6) Add **10 µl of 10X Buffer B** to each sample.

**** TIP –instead of pipetting 70ul of ddH_2O, 20ul of 5X Buffer A, and 10ul of 10X Buffer B one at a time, follow this formula:*

$$70\ ul\ ddH_2O \times\ (x) =\ \_ul$$
$$20\ ul\ 5X\ Buffer\ A \times\ (x) =\ \_ul$$
$$10\ ul\ 10X\ Buffer\ B \times\ (x) =\ \_ul$$

Where (x) is the total number of samples. For example, for 20 samples, (x) will equal to 20 samples.

7) Vortex the samples and lysis buffer contents well. Centrifuge on *MBI centrifuge*.
8) Incubate on the *MBI Thermo-Shaker* for 5 minutes at 95°C with 1000 speed (shaking).
    - Open the tubes' caps a few times, close the caps again when using the vortex. Vortex well. Centrifuge samples on *MBI centrifuge*.
9) Put back on the *MBI Thermo-Shaker* at 95°C, with 1000 speed (shaking), and incubate for another 10 minutes.
    - Open the tubes' caps a few times, close the caps again when using the vortex. Vortex well. Centrifuge samples on *MBI centrifuge*.
10) Put the samples back on the rack and add **900 µl of ddH_2O** to each sample.
11) Put samples in *Centrifuge 5424* and spin for 2 minutes at 12500 rcf (or rpm).
12) Put samples back on the rack and store in the 4°C fridge until further use.

Step 2 – Polymerase Chain Reaction (Set up ONE PCR machine for each primer)

13) Get ice.
14) Label PCR tubes for the samples, positive control, and negative control.
15) Label ONE Eppendorf tube for each primer Master Mix (i.e., for each kind of primer) and put on ice.
16) Take out 2X HS-Red TAQ mix and the primers that will be used from the -20°C freezer. Thaw (on ice).
17) Make the primer master mix for each primer (on ice).

- **5 µl 2X HS-Red TAQ mix /sample** → Multiply by number of samples + 1 positive control + 1 negative control
- **3.6 µl ddH_2O / sample** → Multiply by number of samples + 1 positive control + 1 negative control
- **0.4 µl Primer Mix / sample** → Multiply by number of samples + 1 positive control + 1 negative control

18) Set the labeled PCR tubes onto purple *isofreeze* box (in the bottom shelf of -20°C freezer).
19) Add **9 µl of primer master mix** to each PCR tube.
20) Add **1 µl of each sample** to each PCR tube (get a POSITIVE control from +4°C box "Genotyping")
21) Close the lid of the PCR tubes tightly.
22) Vortex the PCR tubes.
23) Spin the PCR tubes down in the *Hoefer mini-centrifuge* to get rid of most of the bubbles and bring the contents back to the bottom of the tube.
24) Put PCR tubes into the *MBI PCR machine(s)* and start the program(s).

LEFT *MBI PCR* MACHINE (#1)	RIGHT *MBI PCR* MACHINE (#2)
• **Cre** → 'login' under "Antje" → 'enter' → 'program' → "cre genotyping" → 'start' • **β-gal (Shrm3)** → 'login' under "Antje" → 'enter' → 'program' → "shrm3/bgal" → 'start' • **Shroom3-LoxP** → 'login' under "Kristina" → 'enter' → 'program' → "shroom3-loxp" → 'start' • **Jarid F/R** → 'login' under "Kristina" → 'enter' → 'program' → "jarid sexing" → 'start' • **Six2Cre-Insertion** → 'login' under "Kristina" → 'enter' → 'program' → "six2cre-insert" → 'start'	• **LoxP** → 'login' under "tony" → 'enter' → 'program' → "loxp-new" → 'start' • **Cre** → 'login' under "Felix" → 'enter' → 'program' → "genotyping" → 'start' • **Shroom3-LoxP** → 'login' under "Kristina" → 'enter' → 'program' → "shroom3-loxp" → 'start' • **Jarid F/R** → 'login' under "Kristina" → 'enter' → 'program' → "jarid sexing" → 'start' • **Six2Cre-Insertion** → 'login' under "Kristina" → 'enter' → 'program' → "six2cre-insert" → 'start'

25) Wait ~2hrs OR let the PCR run overnight and continue the protocol the next day, i.e., Day 2.

Step 3 – Gel Electrophoresis

26) Measure **100ml of 1X TAE buffer** and add to Erlenmeyer flask.
27) Weigh out **1g of agarose** and add to Erlenmeyer flask. This 1% agarose applies to *all* primers being used in the laboratory *EXCEPT* for the Jarid F/R primer.
    - IF genotyping for sex (using the Jarid F/R primers), weigh out **2g of agarose** (agarose is on the chemicals shelf above the scale) and add to Erlenmeyer flask. This 2% agarose applies to ONLY the Jarid F/R primer.
28) Microwave for 30 seconds → stop and swirl → microwave for 30 seconds → stop and swirl → microwave for 15 to 20 seconds (stop when it starts to boil) – CAUTION: Erlenmeyer flask will be HOT.
    - IF genotyping for sex (using the Jarid F/R primers), add an extra 15 to 20 seconds (stop when it starts to boil). This ONLY applies to the 2% agarose gel.
29) Add **5 µl of RedSafe** to Erlenmeyer flask and swirl until the agarose gel is homogenous.
30) Put the gel cast into the apparatus and make sure it is tight.
31) Pour in the 1g agarose and 100ml TAE buffer solution into the gel cast.
32) Put in the gel combs.
    - Put the thicker side of the 20-well combs down.
33) Wait ~ 30 minutes for the gel to solidify
34) Take out the combs carefully (upwards motion).
35) Transfer the gel cast over to the *OWL* gel chamber. Check that the TAE buffer volume is up to the 'fill line'.
36) Take samples out from the *MBI PCR machine(s).*
    - Press 'STOP' make sure the program has stopped.
    - Select 'EXIT' or 'LOG OUT'
    - Turn the *MBI PCR machine* OFF.
37) Load **10 µl of 1 kb ladder** in well #1 on each side of the gel.
38) Load **10 µl of each sample** from each PCR tube into corresponding well.
    - When loading, be careful NOT to damage or poke the gel. Release the contents from the pipette tip *just above* the well.
39) Close the *OWL* gel chamber lid and turn on *BioRad.* Adjust the settings to 'Run' at 135V for 45 to 60 minutes (depending on the primers being used).
    - IF genotyping for sex (using the Jarid F/R primers), set the time to at least 60 minutes.
    - IF genotyping other primers (NOT the Jarid F/R primers), set the time to 50 minutes.

40) Carefully take out the gel cast with the agarose gel from the *OWL* gel chamber.

41) Take a picture of the gel.

- Put the agarose gel inside the *SYNGENE* machine (NOT including the gel cast).
- Click on the '**GeneSys**' application in the computer.
- Click on '**Ethidium Bromide**'.
  - Only click *after* the interface on the left side of the screen (where it says gels, blots, etc.) goes from black/white to coloured.
- Modify zoom/brightness of the gel as needed.
- 'Invert' the picture to make the samples black and the background white/light gray).
- Save the image and print a copy → click the printer icon in the page that pops out.
- Click "X" to close program
- Take out the agarose gel and gently clean the *SYNGENE* machine with ddH_2O and paper towels.

42) Clean up.

- Throw the agarose gel into biohazardous garbage (yellow bags).
- Close the *OWL* gel chamber and tightly cover with plastic wrap.
- Make sure all the reagents and samples are put back into their respective places, i.e., the fridge or the freezer.
- Wash any used materials (scoops, glassware, graduated cylinders, etc.).

43) Enter the genotyping results in OneDrive for the CAF staff.

- For samples that used Cre and Six2Cre-Insertion primers, upload results as "**Cre Pos**" or "**Cre Neg**"
- For samples that used β-gal (Shroom3), upload results as "**WT**" or "**Het**"
- For samples that used Shroom3-LoxP, upload results as "**LoxP**"
- For samples that used Jarid F/R, upload results as "**MALE**" or "**FEMALE"**

44) Send an e-mail to the CAF technicians to inform them about the uploaded genotyping results on OneDrive. Attach the link to the OneDrive Sheet in the e-mail.

- Include the actual results in the text of the email as well, as this is some of the staff's preference, ex., **261 → CRE POS**