**Campbell Lab Genotyping (R403Q Mice)**

**Agarose Gel PCR**

**Tail snips**

1.  Cut a 2-4 mm section from tip of tail and place in 0.2 uL microcentrifuge tube

2.  Add **100 µL** of 50 mM NaOH (make sure tail snip is completely submerged)

3.  Cook tails at 95°C for 1 hour or 98C for 30 min. (I’ve had success with both) and hold at 15-22 C.

**\*Note**: Can place tails in the 4°C following cooking until ready for use.

4.  Vortex (to break up tail pieces)

5.  Add **100 µL** of DNAse/RNAse free water

6.  Add **10 µL** of 1 M Tris-HCl (pH 8.0)

**\*Note**: I do this to neutralize the NaOH

7.  Centrifuge at 3,000-4,000 rpm for 5 min.

**\*Note**: I like to remove the supernatant and place the supernatant in a new microcentrifuge tube. Discard the hair pellet after the centrifugation because I vortex samples prior to adding them to the PCR reaction tube but this is not necessary.

Again, you can either place in the 4°C or go straight into making your PCR reactions from here.

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**PCR Master Mix** (per reaction/tail snip) – the online protocol says to make a 25 µL reaction.  I make a 12.5 µL reaction to save on reagent and have had good results in doing so.  I have also compared the 25 µL to the 12.5 µL reaction and the results are the same.

**6.25 µL** of Green GoTaq® Master Mix

**5.15** **µL** of RNAse free H2O

**0.05** **µL** of F. Primer (stock: 100 uM)

**0.05** **µL** of R. Primer (stock: 100 uM)

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**1.00** **µL** of tail DNA sample

Total Reaction: **12.5** **µL** (11.5 µL of Master Mix + 1 µL of tail DNA sample)

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**PCR Reaction Protocol**

**TnI-G203S** (Undigested/Unspliced product: bp)

1. 95°C for 2 min

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1. 95°C for 30 sec
2. 58°C for 30 sec
3. 72°C for 1 min

---------------------35 cycles (steps 2-4)

1. 72°C for 5 min
2. 4°C Hold

Proceed to endonuclease digestion step

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**AvaI Restriction Enzyme Digestion**

To the PCR reaction (post-thermocycler) add the following to each reaction:

**1 µL** (10 Units) of AvaI Restriction Enzyme (comes as 10,000 units per mL)

**5 µL** of CutSmart 1X Buffer (comes as 10X)

**1.5 µL** of DNAse/RNAse free water

Total Reaction: **12.5 µL** (original PCR reaction) + **7.5 µL** (restriction enzyme cocktail)

Place the **20 µL** reaction in the thermocycler and incubate at 37°C for 5 hours followed by an 80°C inactivation step for 20 minutes, hold at 12°C.

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**Make and Load Agarose Gel**

Make a 2% agarose gel with 5% EtBr.

-Large gel: 2.2 g of agarose into 110 mL of 1x TAE buffer.

-Small gel: 1.1 g of agarose into 55 mL of 1x TAE buffer.

Cook in an Erlenmeyer flask in the microwave for 2:45 min covered with a paper towel. Be sure to stop the microwave every 30 sec or so to swirl the mixture so that all the agarose melts and is in solution.

You will want to let the agarose cool for about 5-10 min by placing it on the work bench OR run cool water from the faucet over the base of the flask for ~45 seconds.

**Under a fume hood:**

Add **6 µL** of EtBr for a large gel or **3 µL** of EtBr for a small gel into the agarose mixture and swirl and mix well.

**\*Note**: I then let sit for 15-30 sec and then add it to the gel casting unit.

**\*Note**: If you allow the gel to be poured and formed under the fume hood, it reduces the time that you have to wait on it to set and harden (~15-20 min rather than +1 hr).

**\*Note:** Alternatives to EtBr that I would recommend are SYBR Safe DNA gel stain (10,000x) from Invitrogen Thermo Fisher or GelRed Nucleic Acid Gel Stain from Biotium. We use EtBr and I do all my gel pouring and EtBR mixing under a fume hood.

Once gel has set, place gel in running apparatus filled with 1X TAE Buffer, load **5** **µL** of DNA loading ladder (500 bp), then load **18-19 µL** into the gel for genotyping because you normally lose **0.5-1** **µL** of volume while making the PCR reaction.

Run gel at 90V for 60 minutes.

**Genotyping explained**

A screenshot of a computer

Description automatically generated with low confidence

380 bp

260 bp

120 bp

Left side: **PCR Reaction only**

Right side: **PCR Reaction + AvaI Restriction Enzyme Digestion**

Samples 1 & 2 are loading control mice (Null)

Lanes 3 & 4 are R403Q Heterozygous mice of the 129S6 strain

Lanes 5,6,7, & 8 are Null mice of the 129S6 strain

**PCR: what’s happening?**

* Green GoTaq® Master Mix: amplifies target DNA that the primers attach to
* F. Primer: amplifying Exon 13 with specificity to 5'-GCTGGACAAAGGAATGGAGGTA-3'
* R. Primer: amplifying Exon 13 with specificity to 5'-CTGATGGTCTGAGTGGGTAGGTGAG-3'
* The forward primer attaches to the start codon of the template DNA (the anti-sense strand), while the reverse primer attaches to the stop codon of the complementary strand of DNA (the sense strand). The 5' ends of both primers bind to the 3' end of each DNA strand. In this case it is targeting exon 13 of the mouse α cardiac MHC gene. In between this start codon of the template DNA and the stop codon of the complementary strand of DNA is the AvaI binding site. The entire stand is 380 bp

**AvaI Restriction Enzyme Digestion: what’s happening?**

* AvaI Restriction Enzyme: is a restriction enzyme that targets/cleaves at the AvaI site.
* CutSmart Buffer: has over 215 restriction enzymes are 100% active
* Incubate at 37°C for 5 hours: to allow restriction enzymes to cleave DNA sequence (see below)
* 80°C inactivation step for 20 minutes: inactivates the AvaI restriction endonuclease

AvaI targets the C/YCGRG sequence that is located ~260/120 bp

5’… C∨**Y**CG**R** G …3’

3’… C **R**CG**Y**∧G …5’

**R** = A or G (purine) - **Y** = C or T (pyrimidine)

The R403Q heterozygous mouse lacks the AvaI site on one of its MYH6 alleles but contains it on the other. This explains why the heterozygous mouse has an non-cleaved band (380bp) and the set of two bands (260/120 bp). While the Null mice control un-mutated contains only the set of two bands (260/120 bp) as both of their MYH6 alleles contain the AvaI site.

**Analysis of R403Q mouse line:**

Exon 13 of mouse DNA was amplified, digested with Ava I, and fractionated on an agarose gel. (R403Q Heterozygous mice contain one mutated (lacking) and one normal allele of the Ava I site)

**Further Notes:**

The final concentration of glycerol in any reaction should be less than 5% to minimize the possibility of **star activity**. For example, in a 50 µl reaction, the total amount of enzyme added should not exceed 5 µl. Under non-standard reaction conditions, some restriction enzymes are capable of cleaving sequences which are similar, but not identical, to their defined recognition sequence. This altered specificity has been termed “**star activity**". It has been suggested that star activity is a general property of restriction endonucleases and that any restriction endonuclease will cleave noncanonical sites under certain extreme conditions

By definition, 1 unit of restriction enzyme will completely digest 1 μg of substrate DNA in a 50 μl reaction in 60 minutes. This enzyme : DNA : reaction volume ratio can be used as a guide when designing reactions. However, most researchers follow the "typical" reaction conditions listed, where a 5–10 fold over digestion is recommended to overcome variability in DNA source, quantity and purity. NEB offers the following tips to help you to achieve maximal success in your restriction endonuclease reactions.

**AvaI Restriction Enzyme:**

* AvaI is purified from a recombinant source.
* Cleavage with AvaI restriction enzyme may be blocked or impaired when the substrate DNA is methylated by CpG methylase.
* AvaI enzyme will digest unit substrate in 5-15 minutes under recommended reaction conditions, and can also be used safely in overnight digestions.
* AvaI Targets sequence: C/YCGRG
* AvaI has 100% activity in rCutSmart NE Buffer
* AvaI has a heat incubation of 37°C for 5 hours
* AvaI has a heat inactivation: 80°C for 20 minutes

What is the R403Q Mutation?

The R403Q mutation consists of a point mutation (arginine replaced with glutamine) at position 403 on the actin binding pocket of the myosin heavy chain molecule. This model consisted of transgenic mice that contained the R403Q point mutation with an additional amino acid deletion and insertion of non-myosin amino acids in the myosin heavy chain. This model was constructed to produce a mutant myosin that would have a strongly altered interaction with actin. The development of this model was based on the hypothesis that genetically linked hypertrophic cardiomyopathy is a disease of the sarcomere, whereby alterations in crossbridge interactions can lead to further downstream effects that ultimately generate a hypertrophic phenotype. Only a small percent of hypertrophic cardiomyopathy contained the mutation (1-12%) but a similar histopathology to human patients is observed. By 12-14 weeks common pathologic features are apparent including hypertrophied cells, myocellular disarray and LV hypertrophy.

Another mouse model was developed that included only the R403Q point mutation. Mice homozygous for the mutation died at day 7; however heterozygous mice survived for 1 year. After ~15 weeks of age, the R403Q mice show similar pathology to R403Q human patients including myocyte disarray and interstitial fibrosis. In addition, these pathologies increased with age. However, the major difference in this model was the presentation of left atrial enlargement rather than LV hypertrophy observed in humans.