**PCR procedures**

1. **Converting whole (unspun) animal blood to purified dna**
   1. Nucleated (ie. Bird)
      1. Pipet 20 µl proteinase K into a 1.5 ml microcentrifuge tube
      2. Add 5-10 µl anticoagulated blood
      3. Adjust the volume to 220 µl with PBS (not provided in DNEasy kit)
         1. PBS: pH 7.2 (50 mM potassium phosphate and 150 mM NaCl)
      4. Add 200 µl Buffer AL (without added ethanol). Mix by vortexing and incubate at 56deg C for 10min.
      5. Add 200 µl ethanol (96-100%) to the sample, and mi x by vortexing
      6. Pipet the mixture from step 3 into the DNeasy Mini spin column. Centrifuge at ≤ 6000 x g (8000rpm) for 1 min. Discard flow-through and collection tube
      7. Place the DNeasy Mini spin column in a new 2ml collection tube, add 500 µl Buffer AW1, and centrifuge for 3 min at 6,000 x g (8000 rpm). Discard flow-through and collection tube.
      8. Place the DNeasy Mini spin column in a new 2ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
      9. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube,a nd pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≤6000 x g (8000rpm) to elute.
      10. For max yield repeat the elution step again. (use new microcentrifuge tube)
      11. Store: in freezer. It is best to remove the final product from the ‘mini spin column’ and place in new microtube.
2. **Nano Drop (to determine concentration of blood)**
   1. Initial volume = 320 🡪 10 µl blood and 90 µl dH20
      * 1. 104 🡪 5 µl blood and 20 dH20
        2. 550 🡪 6 µl blood and 93 dH20
   2. Procedure:
      1. Open program on desktop ‘Nanodrop 1000’
      2. Select ‘Nucleic Acid’
      3. Take 1µl water and add to sensor
      4. Close the lid
      5. Hit okay (this should be prompted by computer)
      6. Hit ‘blank’ (wait for computer to respond)
      7. Wipe sensor and lid with kemwipe (after each trial)
      8. Type the name of your sample. And add 1µl of sample to sensor
      9. Hit ‘Measure’
      10. 260/280 = around 1.8
      11. ng/µl should be from 20-50
      12. If sample is 100 ng/µl dilute with dH20 by ½ (ie. Add 100 µl sample with 100µl dH20)
3. **Make ‘Primers Forward/Reverse’ working stock**
   1. Add 98 µl dH20 to 2µl rehydrated primer
   2. Make sure to label each one
   3. Keep in freezer

**Primers**

HAEMF: ATGGTGCTTTCGATATA-TGCATG

HAEMR2: GCATTATCTGGATGTGATAATGGT

HAEMNF: CATATATTAAGAGAATTATGGAG

HAEMNR2: AGAGGTGTAGCATATCTATCTAC

1. **Make ‘working stock’ (with Green master mix🡪 to place in PCR machine)**
   1. (to make this easier you can times this by the # of samples you have)
   2. You should have a ‘working stock’ for each set of the primers you have
   3. Vortex green master mix and primers before adding
   4. This should be for HaemNF/HaemNR2
   5. For one sample:
      1. Add 8.5 µl dH20
      2. Add 12.5 µl Master mix
      3. Add 1.0 µl Primer Forward & Reverse
      4. Add 4.0 µl DNA (after it is converted with DNEasy kit)
   6. Make sure to calculate ~2 extra ‘sample worth’🡪 ie. If you are running 5 samples, make 7 samples worth
   7. **Vortex final product**
   8. Store these at: --shouldn’t be any extra (maybe one sample worth)
2. **Prepare for PCR**
   1. Add 4µl of DNA and 23µl PCR ‘working stock’ (Step V) to PCR tubes
   2. MAKE SURE TO LABEL EACH TUBE – or put in a multi-well plate
3. **Running PCR** (20 cycles-“Nested”)
   1. Select the “avmal1”
   2. HaemNF/HaemNR2 primers should be used in this pcr cycle
   3. “tube” setting
   4. Place pcr tubes in machine, close lid, hit start
      1. WILL TAKE 1 HOUR
4. **Gel Setup** (immediately after putting in first set of pcr)
   1. Make the gel:
      1. Big gel (more than 20 samples): 250mL 1X TAE + 5g agarose
      2. Small gel: 100mL 1X TAE + 2g agarose
   2. Stir until you get GelRed ready
      1. Big gel: 10mL Syber Safe
      2. Small gel: 5mL Syber Safe
   3. Microwave for about 40sec (boiling slightly)
   4. Slide the ‘holder’ into place (Gel runs from black to red)
   5. Pour in agarose mixture
   6. Place comb in ‘holder’
   7. Should set in about 30min – make sure the gel has set

**REPEAT STEP IV: green master mix ‘working stock’**

* 1. Do this about 45 minutes into the first cycle of pcr
  2. This should be for HaemF/HaemR2
  3. For one sample:
     1. Add 8.5 µl dH20
     2. Add 12.5 µl Master mix
     3. Add 1.0 µl Primer Forward & Reverse
     4. Add 4.0 µl DNA (after it is converted with DNEasy kit)

1. **Prepare for 2nd round of PCR**
   1. Add 23µl of PCR ‘working stock’ and 4µl of PCR product
   2. MAKE SURE TO LABEL EACH TUBE
   3. Vortex primers and green master mix
2. **Running PCR** (35 cycles- “par”)
   1. HaemF/HaemR2 primers should be used in this pcr cycle
   2. Select the “avmal2” cycle
   3. “tube” setting
   4. Place pcr tubes in machine, close lid, hit start
      1. WILL TAKE 1 hour 43 min
3. **Run the Gel**
   1. Cover the gel completely with .5 TBE buffer
   2. Load 3ul of Easy ladder into the first column
   3. Do not load ‘into’ gel, but rather into little square…
   4. Load 3ul of each sample into the other columns
   5. Make sure to write down which is which
   6. Put the corresponding colors on the matching electrodes
   7. Turn on machine (110volts, ~300amps, 30 mins)
4. **Completing the gel**
   1. Pour used TBE buffer back into container
   2. Place gel on light box to take picture
5. **If sending for Sanger…**
   1. Use the Zymo Gel DNA cleanup kit (4007) according to manufacturer’s instructions
6. **Save and send for Sanger… more later on this.**
   1. Send at least 1ul of the primers per reaction they are running.