Mitochondrial DNA Lab

Standard Operating Procedure (SOP)

# DNA extraction

**Notes**

Use preferred method, below is an example of saline cheek cell DNA extraction

**Procedure**

Prepare 10ml of 0.9% saline for each extraction in addition to 100uL of 5% Chelex

Turn on 1.5ml block heater and set to 99c, alternatively use water bath

Ensure all tubes/consumables to be used are labelled with unique identifier for each sample (1x cup, 2x Eppendorf 1.5ml tubes and 1 x Chelex tube)

* Mix 10ml of saline solution in mouth for 1 min. (Ensure no food or fluids are consumed before)
* Spit contents into labelled cup
* Swirl contents and pipette out 1ml to put into a **labelled** 1.5ml Eppendorf tube
* Spin at 14,000rpm for 2 min. to make pellet
* Remove supernatant either by carefully tipping tube or pipetting out; ensure pellet is still in place after removal.
* Re-suspend in 100uL water
* Add 30uL of re-suspended pellet mix to 100uL Chelex tube and vortex for 30sec.
* Heat @ 99c for 10mins
* Put in to ice to cool for 1 min. vortex for 30sec after
* Spin at 14,000rpm for 1 min. and transfer DNA supernatant to a clean labelled 1.5ml Eppendorf tube

# PCR

**Notes**

Mastermix must not be colored as sequencing utilizes fluorescence for detection

Primers must be the sequence as shown below and at a concentration of 10uM

F-5’-TTA-ACT-CCA-CCA-TTA-GCA-CC-3’

R-5’-GAG-GAT-GGT-GGT-GGT-CAA-GGG-AC-3’

Due to the small volumes, pipetting must be carried out carefully under supervision

**Procedure**

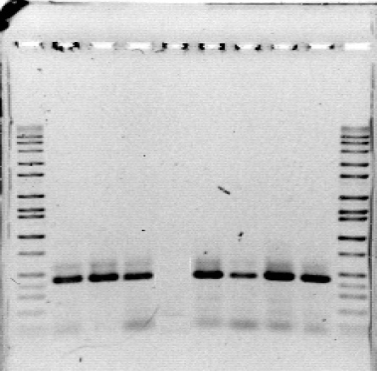
Prepare the master mix for each reaction in a labelled, dome cap, 0.2uL PCR tube

|  |  |
| --- | --- |
| Reagent | Volume |
| Water | 9.0uL |
| Taq Master Mix | 12.5uL |
| Primers | 2.5uL(1.25uL each F/R) |
| **Total** | **24uL** |

Add 1.0uL of extracted DNA to the corresponding PCR tube, also use a known positive control and water for negative control.

Run final 25uL PCR reaction on a thermal cycler with the following conditions;

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature | Time | Cycles |
| Initial Denaturation | 95c | 2min | 1 |
| Denaturation | 95c | 45sec | 30 |
| Annealing | 56c | 30sec |
| Extension | 72c | 90sec |
| Final Extension | 72c | 10min | 1 |
| Hold | 4c | Until removed | ∞ |



Upon completion remove tubes and run an electrophoresis gel to check for amplification. Only samples with definitive bands should be sequenced therefore a gel **must** be run in order to save time and cost of sequencing. See example to left, sample 4 has no band and would therefore not be run. Only continue with samples that have a definitive band.

Finally freeze at -20c until ready to be sequenced.

# Sequencing

**Notes**

Use template ‘Sequencing Protocol EdgeBio Spin columns’ excel file

**Procedure**

Into a labelled, dome capped, 0.2uL PCR tube add the master mix in addition to 1.5uL of Primer (F **or** R only) and 1.5uL PCR product.

|  |  |
| --- | --- |
| Reagent | Volume |
| Primer (10uM) | 1.5uL |
| PCR Product | 1.5uL |
| BigDye v3.1 | 0.5uL |
| Seq Buffer 5x | 3.75uL |
| Water | 12.75uL |
| **Total** | **20uL** |

Run final 20uL sequencing reaction on a thermal cycler with the following conditions;

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature | Time | Cycles |
| Initial Denaturation | 96c | 1min | 1 |
| Denaturation | 96c | 10sec | 30 |
| Annealing | 50c | 5sec |
| Extension | 60c | 4mins |
| Hold | 4c | Until removed | ∞ |

After cycle is completed and reaction has cooled to 4c remove and keep in dark whenever possible.

**Clean up of cycle sequencing reactions using spin columns (Edge Bio)**

* Remove spin columns from box in fridge and spin for 3mins at 770g
* Transfer the spin cartridge to the new 1.5ml Eppendorf tube and label with well number (A1 to H12) according to final destination on sequencing run plate
* Pipette entire sequencing reaction contents into the corresponding labelled cartridge & tube,

**NOTE ensure fluid runs into center of gel in column**

* Close caps and centrifuge for 3min @ 770g
* Discard cartridge and retain elute
* Pipette entire elute from 1.5ml Eppendorf into well on microplate corresponding to the label for well (A1 to H12)
* Centrifuge microplate up to @500rpm to spin down contents and eliminate bubbles

|  |  |  |
| --- | --- | --- |
| **Loading plate onto 3130GA Genetic Analyzer** | |  |
|  |  |  |
| * Change buffer and water reservoirs (1%Buffer=5ml (10x buffer) + 45ml sterile water) | | |
| * Place septa over top of plate and transfer to plate retainer | | |
| * Press 'Tray' button on 3130GA and wait for tray to stop at front then open doors | | |
| * CAREFULLY load plate onto tray ensuring correct orientation | | |
| * Ensure there are no large bubbles in array port (Right of top block) | | |
| * Check that there is enough polymer for all samples | |  |
| * Close doors and use protocol below to input plate/sample details | | |

**Inputting Sample data for analysis**

NOTE; Password for computer and software is 3130User

* Use 3130 data collection v3.0 program
* Under 'ga 3130' header click plate manager, click 'new plate'
* Input the information below

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Box input header** | **Input** | **Notes** | | | |
| **Name** | The plate name | e.g. monsen6-11-13 | | | |
| (The plate name) |  |  |  |  |  |
| **Application** | SequencingAnalysis | For sequencing samples | | | |
| (Type of experiment) |  |  |  |  |  |
| **Plate type** | 96 | Always | | | |
| **Owner** | initials | e.g. AP | | | |
| **Operator** | initials | e.g. AP | | | |
|  | **Click OK** |  |  |  |  |
| **Results Group 1** | Seq\_install | sequencing | | | |
| (Location of run data) |  |  |  |  |  |
| **Instrument Protocol 1** | Seq\_BDv3\_install | sequencing | | | |
| (How samples will be run) |  |  |  |  |  |
| **Analysis Protocol 1** | 3130 Pop7\_BDTv3\_KB-Deno | Sequencing | | | |
| (Only for Seq) |  | (Use with Big Dye v3.1) | | | |

* Click OK
* Click 'plate view'
* Search for plate by exact name/find all list
* Highlight plate name and click on orange plate on rht (should turn grn)
* Click on 'plate view' and ensure all runs are correct and in correct places
* Click green arrow to run

# Processing results

**Notes**

There are various ways to accomplish the same goals and objectives and many analysis programs and websites in order to do this as such this is only a brief overview.

**Checking overall sequence**

The first and most important part is to check that the sequence is visible and has good separation in the peaks. Ideally the electropherogram should be around 400bp long and have clean sharp peaks that have a clear separation between them. If any samples deviate from this there can be many reasons the most common being contamination (By salt or leftover product from extract/PCR) or over/under load of DNA.

**General analysis**

There are 2 basic methods of analyzing the data produced by finding a similar sequence for comparison (BLAST) or by finding differences to a reference sequence (rCRS).

**Common single base repeat problem**

In this sequence it is quite common (Approx. 10% students) to encounter a repeating ‘c’ that causes the sequence to fail at that point. The way to compensate for this is to run the reverse sequence, this will stop after repeating ‘g’. Invert and flip this sequence and you will have the other half to the forward sequence, simply copy and paste them together.