DNA sequencing Standard Operating Protocol (SOP) for Montclair State University

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This document contains Protocol and instructions in order to carry out DNA sequencing using both the 3130 Genetic Analyzer and the SeqStudio

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DNA Sequencing analysis of DNA product

# Cycle sequencing reaction

## Plan sample plate

Use the table below (96 Well plate layout) to show the layout of your samples**. They must be in columns and multiples of 4**, add more negative controls if needed to make up to a multiple of 4. Annotate where samples will be placed

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  |  |  |  |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  |  |  |  |  |  |  |  |  |  |
| D |  |  |  |  |  |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

## Prepare Big Dye Master Mix

Prepare Big Dye Master mix according to how many samples are to be run for genetic analysis

Note-All values other than column 1 are multiplied by 1.20 to account for spillage)

|  |  |
| --- | --- |
|  | Number of Samples |
|  | 1 | 8 | 16 | 24 | 48 |
| **Reagent** | **Volume of reagent required** |
| Big Dye RR Mix | 0.5µL | 4.2µL | 8.4µL | 12.6µL | 25.2µL |
| 5x Sequencing Buffer | 3.75µL | 33.6µL | 67.2µL | 100.8µL | 201.6µL |
| Water (Mol-grade) | 5.5µL | 46.2µL | 92.4µL | 138.6µL | 277.2µL |
| Total volume | **10µL** | **84µL** | **168µL** | **252µL** | **504µL** |

* If using individual bubble cap tubes label them on the upper half of the outside
* If using a 96 well plate, label with relevant experimental details

## Add sample and primer

Into a new labelled, 96 well 0.2mL PCR plate or tube add 10µL of the master mix

Then add the following according to your DNA source

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | PCR Fragment (1:10 dilution\*) | Plasmid 50-500ng | Positive Control |
| Primer (10µM) | 2.0µL | 2.0µL | 1.5µL |
| Product (DNA) | 2.0µL | 5.0µL | 1.5µL |
| Water (Ultra-pure) | 6µL | 3.0µL | 7µL |
| Total | **10µL** | **10µL** | **10µL** |

\*Depending on results of gel electrophoresis

If not using a master mix use the volumes in the table below.

|  |  |
| --- | --- |
| Reagent | Volume |
| Primer (10µM) | 1.5µL |
| PCR Product | 1.5µL |
| BigDye v3.1 RR mix | 0.5µL |
| Seq Buffer 5x | 3.75µL |
| Water | 12.75µL |
| **Total** | **20**µ**L** |

## Run on thermal cycler

Run the final 20µL sequencing reaction on a thermal cycler using the following program

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature | Time | Cycles |
| Initial Denaturation | 96c | 1min | 1 |
| Denaturation | 96c | 10sec | 30 |
| Annealing | 50c | 5sec |
| Extension | 60c | 3mins |
| Hold | 4c | Until removed | ∞ |

After program is completed and reaction has cooled to 4OC remove plate/tubes and keep in the dark whenever possible. For best results proceed immediately to cleanup and then run on instrument.

# Bead purification

Post cycle cleanup (MCLAB`s Dye Sequencing beads Clean Up Kit, BCB-100)

Prepare 40µL of 80% ethanol per sample (include overage)

Prepare 160µL of 70% ethanol per sample (include overage)

For these solutions ideally use 200 proof molecular grade ethanol 100% diluted using the equation

C1 x V1 = C2 x V2

Where C is concentration and V is volume

**Method**

**Note**-Thoroughly vortex beads at room temperature before use

1. On a new 96 well plate add 10µl of beads to each well corresponding to a sample
2. Remove tubes/plate from the thermal cycler and spin @ 1000rpm for 10 seconds
3. Transfer 10µl of sample to new plate with beads and mix using 10x pipette up/down
4. Add 40µl of 80% Ethanol to each sample, mix using 10x pipette up/down. Wait for 2 minutes
5. Transfer the plate to the magnetic plate stand, wait 2 minutes
6. Pipette out all the liquid and discard without disturbing the beads
7. Wash by adding 80µl of 70% Ethanol to each well, hold for 30 seconds then pipette out and discard
8. Wash again by adding 80µl of 70% Ethanol to each well, hold for 30 seconds then pipette out and discard
9. Using 10µl pipette tips remove as much remaining ethanol as possible
10. Remove the plate from the magnetic plate stand, add 40µl of water\* and bring the beads back into solution by pipetting up and down, hold for 2 minutes
11. Place the plate back onto the magnetic plate stand, wait 2 minutes
12. Pipette 20µl from the plate on the magnetic stand into a new labelled plate
13. Cover new plate with film and centrifuge to spin down contents
14. Proceed immediately with sequencing analysis to avoid degradation loss

\*Molecular Biology grade

# Running samples on the Genetic Analyzer

## For 3130xl Instrument only

**Change buffers and check reagents**

* Press tray on the front of the 3130GA, wait for green light
* Open front doors (Right first then left)
* Remove reservoirs located on the autosampler tray (Gliding stage) and carefully remove septa (Grey cover) these can be placed on clean Kimwipe tissue.
* Discard contents, wash (With ultra-pure water) and dry
* Refill all reservoirs located on the autosampler. Reservoir 1= 1x buffer and 2, 3, 4 are for ultra-pure water. (1x Buffer=1:10 dilution from NanoPop buffer 10x)
* Carefully replace septa back onto the top of the reservoir and carefully return reservoirs into the autosampler in corresponding marked locations
* Check all septa are level and placed correctly by checking at eye level
* Remove buffer jar located on the left side marked with a red ring
* Discard contents, wash (With ultra-pure water), dry and refill with 1x buffer
* Check polymer bottle on far left (At a 450 angle) to ensure there is approx. enough polymer for samples (One full syringe = 5 runs (1 run =4 samples, so 1 syringe=5runs=20samples))
* Close front doors (First left then right)

**Input sample data and running conditions**

*Note; Password for computer and all software is ‘3130 User’*

* Use program ‘Foundation data collection Version 3.0’ (Normally already open)
* Under GA instrument>ga3130 click ‘plate manager’
* Click ‘New…’ located at bottom left of window
* Input the plate name in the format ‘facultyMM-DD-YY’ e.g. gaynor10-2-17
* Input application as ‘Sequencing Analysis’
* Plate type should be set to 96
* Owner should be ‘AP’ operator should be your initials
* Click ‘OK’
* Input the sample info. next to the corresponding well location
* Under ‘Results Group 1’ click the drop down and select ‘Seq\_install’
* In order to copy this to all samples click away from active cells (Any grey cells) then click back on the first box and hold the left mouse button and drag to bottom of sample list. This should highlight all cells in blue, then hold ‘Ctrl’ and press ‘D’ this should copy down.
* Under ‘Instrument protocol 1’ click the drop down and select ‘Seq\_BDv3\_install’ follow the above steps to copy down to all cells.
* Under ‘Analysis Protocol 1’ click the drop down and select ‘3130Pop7\_BDTv3\_KB-Deno’ follow the above steps to copy down to all cells.
* Once checked all is correct, click ‘OK’ located at bottom right of window

**Load plate and start running**

* On the instrument computer click on ‘GA Instruments>ga 3130>APPLIED-FEA7177>Plate View’ a figure of the 96 well plate is displayed on the right of the screen
* Press ‘Tray’ button on front of 3130GA and wait for the green light when the autosampler tray (Moving stage) stops
* Open front doors (Right first then left) CAREFULLY load plate/retainer onto autosampler (Can slightly push down autosampler to give more room) orientation is determined by the cutout under plate/retainer facing towards instrument.
* Check on the computer that the plate figure on the right has turned yellow
* Check at eye level that plate is fully on correctly (If incorrect can cause massive damage to the capillary needles) and at the same flat level as reservoirs
* Close instrument doors (Left then right)
* Wait for autosampler to stop moving and solid green light indicator
* On computer in the search box type your plate name e.g. gaynor10-2-17, click ‘search’ your plate should come up in the area below
* Highlight your plate name and click on the yellow figure of the 96 well plate, it will turn green
* Click on ‘GA Instruments>ga 3130>APPLIED-FEA7177>Run View’, click through the runs and ensure they are showing the correct location on the diagram on the right
* Once ready click on the green ‘Start’ arrow located in the far top right on the software window, click ‘Ok’
* The instrument will now run for approximately 1 hour per run

## For SeqStudio Instrument only

Use plate manager to setup your plate

* From the home screen click create a plate file, name your plate using faculty initials and date
* Application type should be sequencing, then click on the ‘plate’ tab at the top
* You can now add your samples by sets of 4 using unique names under the sample name box
* Select the dye set (Z-BigDye Terminator v3.1) and run module (medium or long seq)
* Once you input all of your samples click on the ‘run’ tab at the top leave all settings as default click save
* On computer attached to SeqStudio place this file into the folder desktop>share data>plates
* On the instrument press the eject button on the top right, select plate
* Load your plate with septa onto the instrument close lid until click
* Press retract and close lid once stage is retracted far enough
* Press setup run, network drive, find your file, review one last time
* Start run, an estimated time till completion will be displayed