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 a 3130 Genetic Analyzer.

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DNA Sequencing analysis of DNA product

NB. For optimal results purify DNA product with spin columns before use in Sequencing

# Cycle sequencing reaction setup with BigDye v3.1

## Create sample # to corresponding well # key

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 (x1.05 for spillage)

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Reagent | Amount in µL | Amount in µL | Amount in µL | Amount in µL | Amount in µL | 2XBD Amount in µL |
| Big Dye RR Mix | 2.1 | 4.2 | 8.4 | 12.6 | 25.2 | 8.4 |
| 5x Sequencing Buffer | 15.8 | 31.5 | 63 | 94.5 | 189 | 31.5 |
| Water (Ultra-pure) | 24.2 | 48.3 | 96.6 | 144.9 | 289.8 | 44.1 |
| Total volume | 42 | 84 | 168 | 252 | 504 | 84 |
| # of Samples | **4** | **8** | **16** | **24** | **48** | **8** |

* If using individual bubble cap tubes label them on the upper half of the outside
* Dispense 10µl of master mix to each 200µl clean bubble cap PCR tube/well

## Add sample to be tested

Add in the other reagents as listed below to the Big Dye mix close caps after each tube is completed to prevent cross contamination, use pipette/vortex to mix.

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | PCR Fragment 20-50ng | Plasmid 300-500ng | Positive Control |
| Primer (10µM) | 1.5µL | 1.5 µL | 1.5 µL |
| Product (DNA) | 1.5 µL | 5 µL | 1.5 µL |
| Water (Ultra-pure) | 7 µL | 3.5 µL | 7 µL |
| Total | **10 µL** | **10 µL** | **10 µL** |

Centrifuge spin up to 1000rpm

# Cycle sequencing reaction-Method

Place tubes/plate in a thermal cycler (If using plate be sure to cover and seal with thermal sealing film)

Set volume to 20uL, run program on thermal cycler as listed below.

|  |  |  |  |
| --- | --- | --- | --- |
| Stage | Temperature (c) | Time |  |
| Initial denature | 96 | 1min |  |
| Cycle denature | 96 | 10sec | **X 25 Cycles** |
| Cycle anneal | 50 | 5sec |
| Cycle extension/incorporation | 60 | 2min |
| Hold until ready | 4 | ∞ |  |

*(Adapted from; Ref. Applied Biosystems Big Dye version 3.1 protocol, Applied Biosystems, part # 4337035 Rev. A, 09/2002)*

# Purification/Cleanup of sequencing reactions

\*Please note only perform stage 1 if having major difficulty with leftover di-deoxynucleotides\*

## SDS Cleanup (Not required for most samples)

* Remove tubes from thermal cycler and add 2µL of 2.2% SDS solution
* Seal tubes and mix thoroughly with vortex
* Spin down contents @ 1000rpm
* Load onto a thermal cycler and run program below

|  |  |  |
| --- | --- | --- |
| Stage | Temperature (c) | Time |
| Heat | 98 | 5min |
| Cool | 25 | ∞ |

*Table 4, DNA sequencing by Capillary electrophoresis Chemistry guide, Applied Biosystems, Part Number 4305080 Rev. C 05/2009, p.116*

## Column cleanup using EdgeBio Performa® DTR gel filtration cartridges

1. Remove 0.2µL tubes from the thermal cycler and spin @ 1000rpm
2. Remove spin columns from foil bag and layout according to samples (Table 3)
3. Centrifuge tube containing spin column for 3mins @800g/rcf
4. Transfer only the insert column to a clean 1.5ml tube and close lid
5. Label top of tube with well (Column and row) number (Table 3)
6. Pipette entire cycle sequencing reaction into the corresponding cartridge/tube (Ensure fluid drops onto center of gel column)
7. Close cap and continue for each samples
8. Centrifuge for 3min @ 800g/rcf
9. Discard cartridge and retain filtered liquid in bottom of 1.5ml tube
10. Pipette entire elute (Set pipettor to 22µL) into microplate using the corresponding well#
11. Cover microplate with film and centrifuge to spin down contents
12. Remove film and place grey ‘septa’ over the microplate
13. Insert into black ‘retainer bottom’ and snap on white ‘retainer top’ by lining up 96 holes
14. Set aside, preferably in a dark area (Inside a drawer)

# Setting up and running the Genetic Analyzer (Applied Biosystems 3130)

## 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 1hour per run