**Protocol for Preparing sample for Sanger Sequencing (To check mutation)**

1. Take out the reagent (DreamTaq, buffer), Oligo (primer F&R), sample@template (ML2 cell line)
2. The template Cell line (seed 100uL) enough -just need a small pellet
3. Centrifuge and withdraw the supernatant.
4. Put in 1x Lysis Buffer (500uM EDTA NaOH). Cell line no need do Nanodrop but primary sample need to do nanodrop.
5. Neutralised DNA or ML2 with 50uL (same amount as lysis buffer) filtered sterile 40mM Tris HCL.
6. Prepare diluted oligo primer F&R (10uM) – Withdraw 10uL oligo (blue cap) and add 90uL Ultrapure water.
7. Prepare the small PCR tubes row. Usually prepare extra one reaction for master mix. (If 3 reaction, then prepare 4 reaction). Label
8. Do 50uL reaction for primary sample. Cell line 25uL per reaction. Follow the list of items Thomas bench.

Pcr small tubes (row)

|  |  |  |  |
| --- | --- | --- | --- |
| 1  ML2 DNA  F&R PLK1 | 2  ML2 DNA  F&R MDM2 336 | 3  ML2 DNA  F&R MDM2 523 | 4  Control (without DNA) THP-1 DNA  - |

**DreamTaq Master Mix (green colour solution)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sequence add in** | **Items** | **1 reaction (uL)** | **4 reactions (uL)** |
| **2** | **10x Buffer** | 2.5 | 10 |
| **5** | **F & R primer** | 0.5 each | 2uL of F & 2uL of R |
| **4** | **dNTP** | 0.5 | 2 |
| **3** | **DreamTaq** | 0.1 | 0.4 |
| **6** | **DNA/Cell line (after lysed & neutralized)** | 1 | 1 uL for 3 gene |
| **1** | **Ultrapure H2O** | 19.9 | 79.6 |

1. Add in the 24uL reaction (without the 1uL DNA) of the master mix into the small PCR tubes. 1uL DNA last to put into all small PCR (strip of tubes). (MUST HAVE CONTROL-no DNA inside)
2. Fingertap and spin down (use the small circle spinner behind)
3. Set the PCR machine (DreamTAq program)
4. Put in the samples and close it.
5. Clean the rack and comb thoroughly with ethanol.
6. Weigh the Agarose 0.8g (1%) and pour into 80mL TAE solution (20x bottle)- for 1 small and 1 big combed rack
7. Boil it till no bubble using the heater.
8. Add GelRed 10000x 3uL. Pour the final mixture solution to the combed gel rack and let it solidify.
9. Pipette in 5uL of the boiled PCR product into the gel that immersed in the TAE solution.
10. Setting for the Gel 120V 30 mins
11. Image gel to locate the band and print out the paper (Acquisition).
12. Do PCR Purification. (Monarch PCR DNA Cleanup Kit) (5ug) #T1030L
    1. Dilute sample with DNA Cleanup Binding Buffer according to the table given.

-Sample type: dsDNA<2kb (some amplicons, fragments) Ratio 5:1

- Do not vortex

* 1. Insert column into collection tube and load sample onto column.
  2. The rest of the steps follow the protocol card in the kit.
  3. **No.6 Add 20uL** od DNA Elution Buffer to the center of the matrix. Wait for 1 min and spin for 1 min at 13000rpm

1. Take 1uL of sample and do Nanodrop. Write down the concentration (ng/uL) on the eppendorf.
2. Check website how much sample concentration needed and withdraw appropriate amount of sample DNA.
3. Add in ultra pure H2O and also oligo primer (F@R) into the PCR strip. (if 3 genes then 6 pcr tubes -1 for F and 1 for R)
4. Eg 0.5uL Sample +0.5uL Oligo (either F @R) +14ul pure H2O (Total 15uL)
5. ***DNA Template and Sequencing Primer Pre-mix Guidelines***

* DNA template and sequencing primer must be mixed (pre-mixed) before submitting for sequencing.
* Prepare 15 µL of pre-mixed for submission.
* Aliquot appropriate amount of template DNA using below table as a guideline:

|  |  |  |
| --- | --- | --- |
| **Sample Type** | | **DNA amount (ng)** |
| PCR Product | 100-200bp | 1-3 |
|  | 200-500bp | 3-10 |
|  | 500-1000bp | 5-20 |
|  | 1000-2000bp | 10-40 |
|  | >2000bp | 20-50 |
| ssDNA | | 25-50 |
| dsDNA, Plasmid | | 150-300 |
| Cosmid, BAC | | 500-1000 |
| Bacterial genomic DNA | | 2000-3000 |

* Add 1 µL of 5 µM sequencing primer.
* Top up to 14 µL with DNase-free water.

*# It is NOT recommended to use TE or other EDTA-containing buffer in the pre-mix, also AVOID adding any divalent cations, such as Mg, Ca, and Mn, etc.*

Check out the sample submission format - <http://gn.cpos.hku.hk/portal/index.php/dna-sequencing/sample-submission>

1. Submit the sample and the hardcopy form. Submit the e-form as well before 3pm collection time.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **PLk1 F** *(Label in the excel form)* | **MDM2 336-F** | **MDM2 523-F** | **PLK1-R** | **MDM2 336-R** | **MDM2 523-R** |
| **A1** (Label on the pcr tube) | **B1** | **C1** | **D1** | **E1** | **F1** |

**\*Elute Buffer has:**

**TRIS-stabilize the pH**

**EDTA- prevent hydrolysis**