Protocol: Bisulfite treatment of DNA, cloning & sequencing to verify specific gene region

DNA Samples:

- Fresh tissue patient samples:
  - ⇒ **Precancerous lesion**
  - ⇒ **Normal colon mucosa**

Gene region:

- *SFRP4*

Amount of DNA for Bisulfite conversion:

Recommended amount: **200-500ng in 20µl** for optimal results.

required concentration: 500ng/20µl = **25ng/µl**
required volume: **20µl**

Amount of DNA for PCR:

Template bisulfite converted DNA **~50ng/µl**

1. Bisulfite Treatment of DNA

1.1. Ensure DNA samples have thawed completely
1.2. Spin down samples and reagents
1.3. Add **130µl** of ***Lightning Conversion Reagent*** to 20µl of DNA sample in a PCR tube. **Vortex** & **centrifuge** briefly to ensure there are **no** droplets in the cap or sides of tube
1.4. Place the tube in a thermal cycler and perform the following steps:

I. **98°C** for **8 minutes**
II. **54°C** for **60 minutes**
III. Hold at **4°C** for 3-4 minutes and then proceed immediately with purification

1.5. Add **600µl** of ***M-Binding Buffer*** to a *Spin Column* and place the column into a *Collection Tube*
1.6. Load the sample from step 2 into the *Spin Column* containing the M-Binding Buffer. Close the cap and mix by inverting several times.
1.7. Centrifuge at full speed for *30 seconds*. Discard the flow-through.
1.8. Add **100µl** of ***M-Wash Buffer*** to the column. Centrifuge at full speed for *30 seconds*.
1.9. Add **200µl** of ***L-Desulphonation Buffer*** to the column and let stand at room temperature for *20 minutes*.
1.10. After the incubation centrifuge at full speed for *30 seconds*.
1.11. Add **200µl** of ***M-Wash Buffer*** to the column. Centrifuge at full speed for *30 seconds*
1.12. Repeat this wash step
1.13. Place the column into 1.5ml microcentrifuge tube and add **10µl** of ***M-Elution Buffer*** directly to the column matrix.
1.14. Centrifuge for *30 seconds* at full speed to elute the DNA.
1.15. **NanoDrop: Measure Bisulfite Converted DNA concentration to make the dilutions for the PCR reaction - from these calculate the amount of DNA required.**

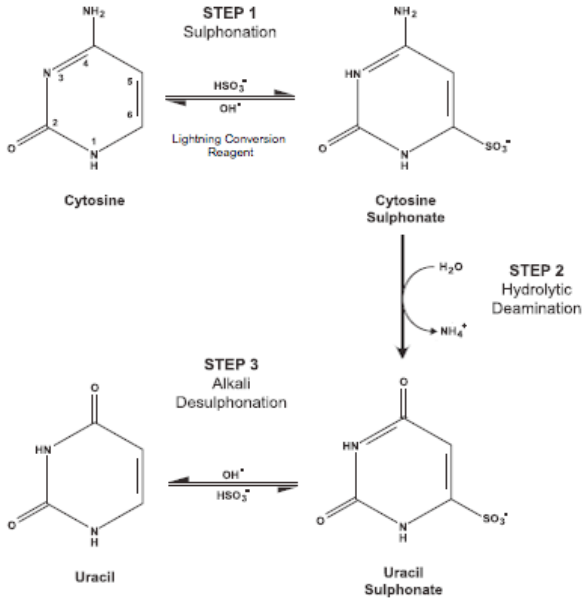

Figure: Bisulfite conversion of Cytosine to Uracil

2. PCR

2.1. Set up PCR machine with conditions (see below)
2.2. Calculate amount of each PCR component (see below) for the number of samples
2.3. Label the required number of PCR tubes and add the required amount of ddH_20 to each tube
2.4. Prepare the master mix of the PCR components
2.5. Mix gently by pipetting up and down. **Do not vortex**
2.6. Aliquot master mix into sample tubes.
2.7. Add **1µl DNA** to the appropriate sample tubes
2.8. Briefly spin samples down

Reagent	Volume
PCR master mix	12.5µl
Coral Load	2.5µl
Forward Primer (10µM)	0.5µl
Reverse Primer (10µM)	0.5µl
ddH_20 NEG	9µl
ddH_20 Sample	8µl

Conditions:

Initial Denaturation: **95°C - 15 min**

Denaturation: **94°C - 30 sec**
Annealing: ..... - **30 sec**
Extension: **72°C - 30 sec**
] **45** cycles

Final Extension: **72°C - 7minutes**

4. Gel Electrophoresis

Prepare a **2% gel** in **60mL** volume

4.1. Weigh out the required amount of Agarose powder into a conical flask
4.2. Fill up to **60ml** with TAE buffer and heat in the microwave until completely melted
4.3. Whilst melting, set-up the chamber with the 8 well-comb
4.4. Allow to cool by running cold water over the flask
4.5. Add Gel Red to the conical flask containing the melted gel
4.6. Pour into the chamber and remove any bubbles from the gel
4.7. Leave to set
4.8. Set up the chamber for the electrodes and fill up with TAE Buffer
4.9. Once set, place the gel into the chamber containing the TAE buffer and pour more buffer over the top to ensure the wells are completely submerged
4.10. Load the gel with PCR products
4.11. Attach the electrodes and allow the gel to run
4.12. Visualise gel (picture)

5. TOPO TA Cloning reaction

Reagent	Volume
PCR product	2µl
Salt Solution	1µl
H20	2µl
TOPO vector	1µl
Final Volume	**6µl**

5.1. Remove Salt solution, H20 and vector from the -20C to thaw on ice
5.2. Add the reagents in the order above. Mix gently and incubate for *5 minute* at **RT**
5.3. Leave the reaction on ice

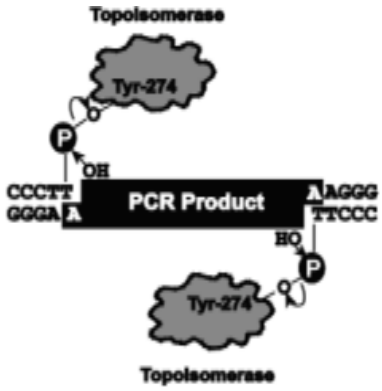

Figure: TOPO TA cloning reaction

6. Preparation for Transformation

Components
S.O.C medium
TOPO Cloning reaction from step 4.1
LB plates containing 50 µg/mL ampicillin
42°C water bath
37°C shaking and non-shaking incubator
General microbiology supplies (e.g. plates, spreaders)

6.1. Equilibrate water bath or heat block to **42°C**
6.2. Warm the vial of S.O.C medium to **RT**
6.3. Warm selective plates at **37°C** for *30 minutes*
6.4. Thaw on ice 1 vial of E-coli TOP10 chemically competent cells for each transformation

7. Transformation

7.1. Add **2µl** of the TOPO Cloning reaction from ***step 4.1*** into a eppendorf tube of the chemically competent E.coli and mix gently. (Do not mix by pipetting up and down, instead gentle flicking of the tube)
7.2. Incubate on ice for *30 minutes*
7.3. Heat shock the cells for *30 seconds* at **42˚C** without shaking
7.4. Immediately transfer the tubes to ice
7.5. Add **250µl** of room temperature S.O.C medium
7.6. Cap the tube tightly and shake the eppendorf tube horizontally (200rpm) at **37°C** for *1 hour*
7.7. Spread **10 - 50µl** from each transformation on a pre-warmed selective plate and incubate overnight at **37°C**

8. Analysis of Transformants

8.1. Prepare a 14ml tube (with a vent cap) for each colony with 4ml of LB medium containing 100µg/mL ampicillin
8.2. Pick single colonies with a pipette tip and put into tubes
8.3. Incubate overnight at **37°C, 250rpm**

9. Isolation of the plasmid DNA using Plasmid Mini-Prep kit

9.1. Centrifuge (at **RT, 5000rpm, for *10 minutes***) the 14ml tubes containing **4ml** of the overnight LB-culture
9.2. Remove all of the medium: Pour the majority into a flask for autoclaving and remove the rest with a pipette.
9.3. Pre-heat an aliquot of TE buffer to **65-70°C**

9.4. Add **250µl** *Resuspension Buffer* (R3) with RNase A to the cell pellet and re-suspend the pellet using a pipette until it is homogenous. Transfer to eppendorf tubes.

9.5. Add **250µl** *Lysis Buffer* (L7). Mix gently by inverting the capped tube until the mixture is homogenous. DO NOT vortex. Incubate the tube at **RT** for *5 minutes*.

9.6. Add **350µl** *Precipitation Buffer (*N4). Mix immediately by inverting the tube, or for large pellets, vigorously shaking the tube, until the mixture is homogenous. DO NOT vortex. Centrifuge the lysate at >12,000 x g for *10 minutes.*

9.7. Load the supernatant onto a spin column in a 2mL wash tube. Centrifuge the column at 12,000 x g for 1 minute. Discard the flow-through and place the column back into the wash tube.

9.8. Add **700µl** *Wash Buffer* (W9) with ethanol to the column. Centrifuge the column at 12,000 x g for *1 minute*. Discard the flow-through and place the column into the wash tube. Centrifuge the column at 12,000 x g for *1 minute*. Discard the wash tube with the flow-through.

9.9. Place the spin column in a clean 1.5ml eppendorf. Add **75µl** of preheated *TE buffer* (TE) to the centre of the column. Incubate the column for 1 minute at RT.

9.10. Centrifuge the column at 12,000 x g for *2 minutes*. The Eppendorf contains the purified plasmid DNA. Discard the column. Store the plasmid DNA at 4**°**C (short-term) -20°C (long term)

9.11. Measure concentrations on the NanoDrop

10. <u>Analyse the transformants by restriction enzyme digest</u>

10.1. Make a master mix for the following for the number of samples required:

- 1.5µl of NEBuffer 2.1
- 0.2µl of EcoR1
- 12.3µl of H_20

10.2. Add 14µl of the master mix for the number of samples

10.3. Add 200ng of DNA to the samples with master mix

10.4. Incubate for at least 1 hour at 37°C

10.5. Prepare gel

10.6. Run transformants on gel and visualise

11. Sequencing

11.1. Prepare samples to be sent to Microsynth for sequencing