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

DNA Samples:

- Fresh tissue patient samples:

  ⇒ **Precancerous lesion** (label "L")
  ⇒ **Normal colon mucosa** (label "N")

Gene region:

- *SFRP4*
- Amplicon: 144bp, 12 CpG sites

Amount of DNA for Bisulfite conversion:

- Required Amount: **500ng in 20µl**

Amount of DNA for PCR:

- Template bisulfite converted DNA **~1ng/µl**

1. **Bisulfite Treatment of DNA** using Zymo's Lightning Conversion Kit

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.**

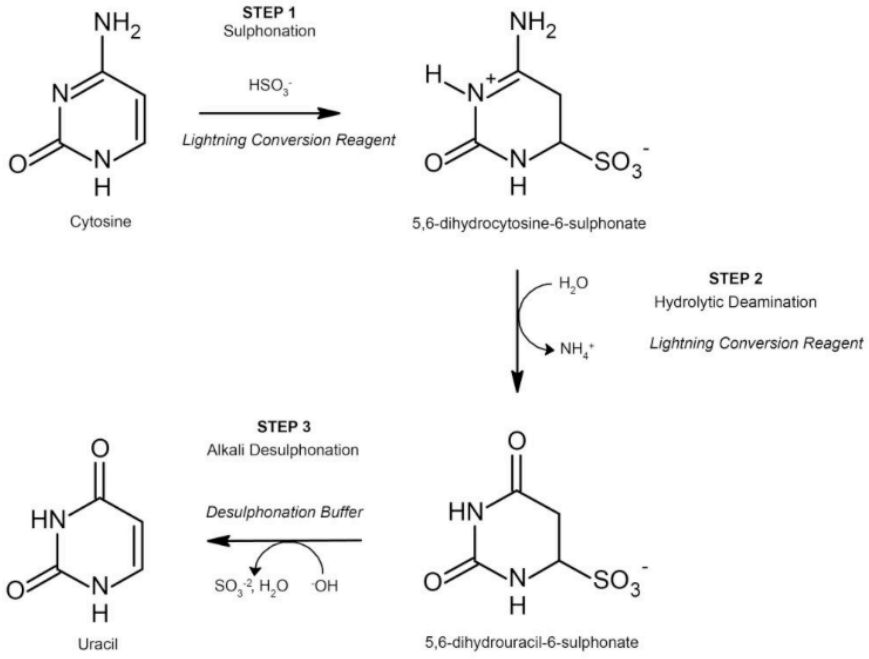


Figure: Bisulfite conversion of Cytosine to Uracil. Steps 1 and 2 occur during bisulfite conversion, while Step 3 is performed as the DNA is bound to the column matrix.

2. PCR

2.1. Set up the thermocycler/PCR machine with the following conditions (see below)

PCR conditions:

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

II. *Denaturation:* **94°C - 30 sec**
III. *Annealing:* **59°C** - **30 sec**
IV. *Extension:* **72°C - 30 sec**

] **45** cycles

V. *Final Extension:* **72°C - 7minutes**

2.2. Calculate amount of each PCR component (see below) for the number of samples

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

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

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/Ethidium Bromide 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, H_20 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

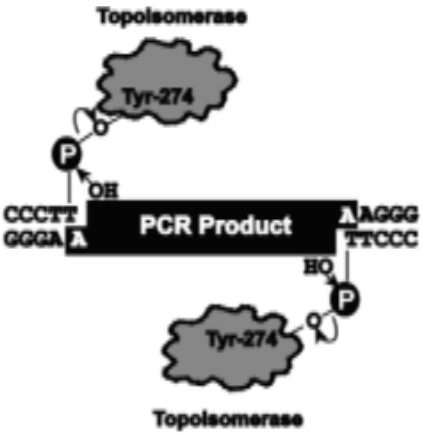
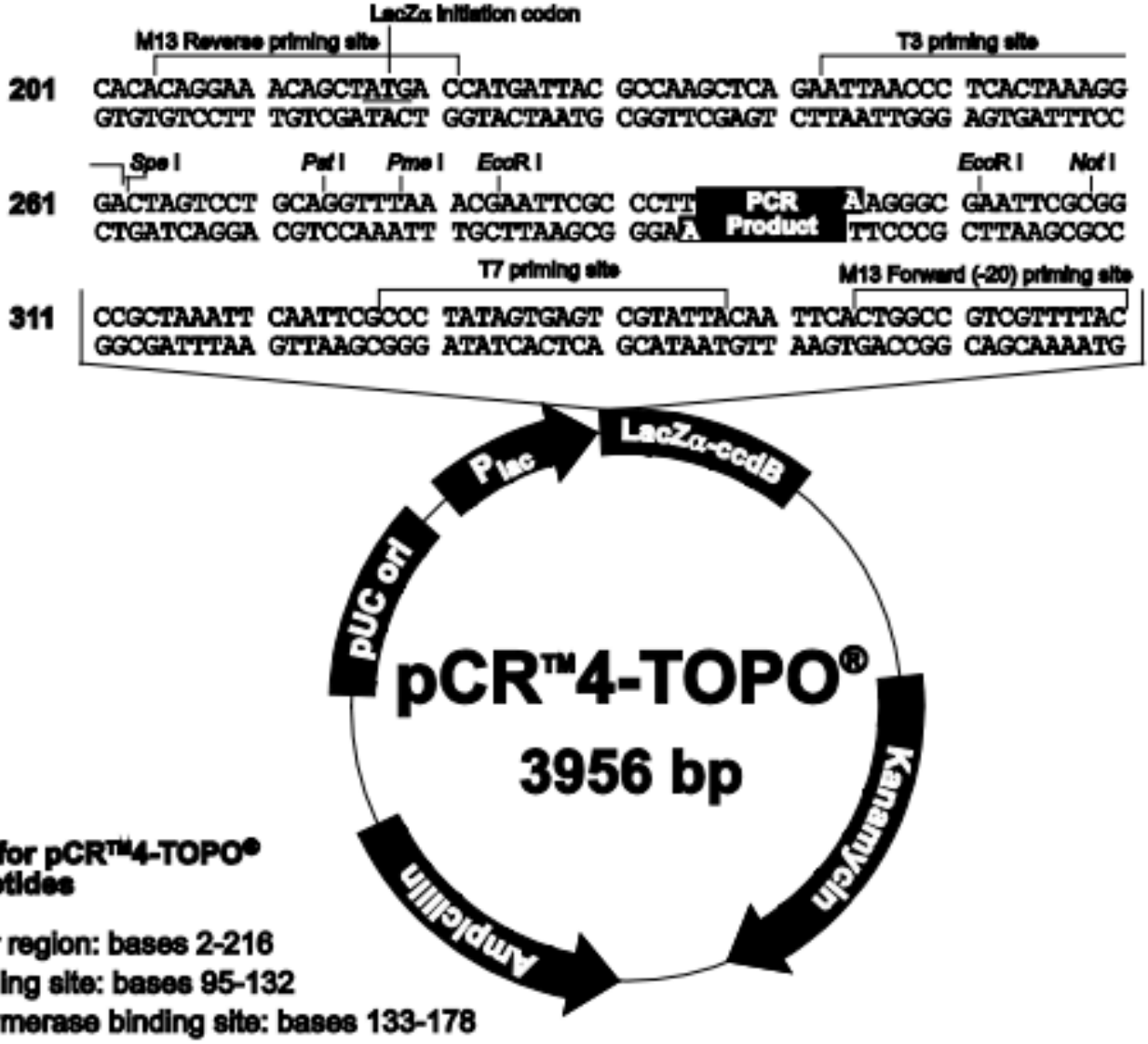


Figure: TOPO TA cloning reaction.



Comments for pCR™4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
- CAP binding site: bases 95-132
- RNA polymerase binding site: bases 133-178
- Lac repressor binding site: bases 179-199

Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
- LacZα portion of fusion: bases 217-497
- *ccd*B portion of fusion: bases 508-810

T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834

(c) = complementary strand

6. Preparation for Transformation

Components
S.O.C medium
TOPO Cloning reaction from step 5
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 5*** into an 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 **50µl** from each transformation together with **50µl** of S.O.C medium 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. <u>Isolation of the plasmid DNA</u> 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. Analyse the transformants by restriction enzyme digest

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 (as in step 4).

10.6. Run transformants on gel and visualise.

11. Preparation for Sanger sequencing

11.1. Prepare samples to be sent to Microsynth for Sanger sequencing.

- Amount: 18ng per 100bp in 12µl
- m13reverse primer from Microsynth's standard primer list

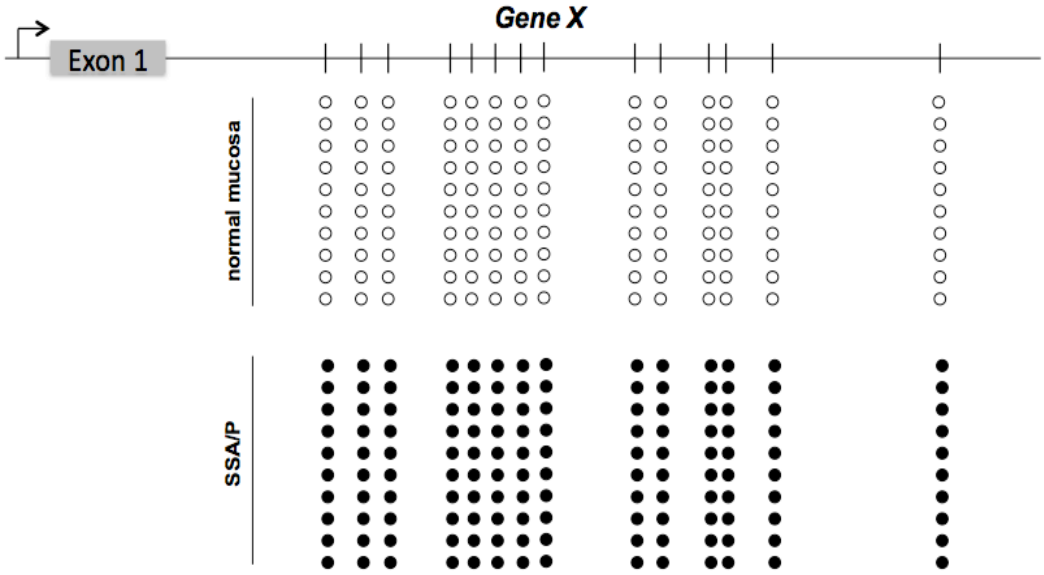


Figure: Template for Sanger Sequencing results. Each circle represents one CpG site and each horizontal line of circles represents one clone. Filled in circles represent methylated CpGs and open circles unmethylated CpG sites. Shown are 10 clones of both normal mucosa and SSA/P (precancerous lesion) samples for Gene X.