GENEMER SICKLE CELL
Background

Sickle cell anemia is an autosomal recessive disease. A single base change (A to T) in the β-globin chain causes the substitution of amino acid glutamine to valine: the cause of the disorder sickle cell anemia. The resulting mutant globin chain is termed as the Hb S. Hemoglobin S is freely soluble when fully oxygenated, under conditions of low oxygen tension the red cells become grossly abnormal assuming a sickle shape leading to aggregation and hemolysis. Homozygous Hb S is a serious hemoglobinopathy found almost exclusively in the black population. About 8% of American Blacks are carriers and about 0.2% are affected. Heterozygotes (sickle cell trait) are clinically normal, although their red cells will sickle when subjected to very low oxygen pressure in vitro.

DNA analysis for the sickle cell mutation is done by specific amplification of the DNA region spanning the mutation using polymerase chain reaction followed by enzymatic cleavage of the amplified product. Sickle cell mutation abolishes a restriction endonuclease site (Dde I). Electrophoretic resolution of the fragment pattern reveals the presence or absence of the mutation. Clear genotyping of normal, carrier and homozygous DNA is achieved.

Protocol for Sickle Cell DNA Genotyping

DNA EXTRACTION FROM BLOOD
1. Collect 0.5mls of whole blood in an eppendorf tube, then add 10µl of saponin.
Saponin is a differential lysing agent. If you lyse blood with only water, both RBC and WBC will be lysed. Saponin perforates only RBC.
Vortex the mixture, leave for 3mins. Spin for 3 mins. Decant supernatant.
2. Wash off RBC debris & Hb with 0.05% saponin in normal saline (NaCl) X2ce
3. Lyse WBC by adding
    - Suspend pellet in 25µl Neucleolysing buffer
    - Add 5µl Proteinase K (25mg/100µl)
4.Incubate at 56^{o}C-2hrs
5. Extract DNA by adding 300µl distilled H2O to the mixture. Vortex. Add equal volume (≈ 500µl) of phenol-chloroform mix (Phenol (25) - chloroform (24) - isoamy1 (1)). Centrifuge at 13,000 – 15,000 RPM for 5mins. Remove lower layer. Repeat extraction twice.
6. Add 500µl of chloroform and vortex. Spin for 5mins and remove lower layer. X2ce
7. Remove the aqueous top layer into a pre-labeled tube and add 2 vols of cold Absolute ethanol (≈ 1ml) mix gently. DNA strand may become visible.
8. Spin and decant supernatant. Wash DNA in 70% EtOH. Spin and decant supernatant.
9. Dissolve DNA in distilled H_2O (DNA can be denatured with time) or TE; which is a chelating agent. it removes ion cofactors like Ca2+, Mg2+ required for DNAses.

PCR REACTION

Material Supplied
Two lyophilized oligonucleotide primers SC2 and SC5 are supplied. Each tube contains 10 nmoles of the primer. The quantity supplied is sufficient for 400 regular 50m1 PCR reaction.

Reconstitution

Stock solution: Add 50m1 sterile water to each tube containing the primer.
The 10 nmoles of primer when dissolved in 50m1 water will give a solution of 200m molar i.e. 200 pmoles/m1.

PCR* reaction (see Appendix for Details)

PCR Reaction mix

	50µl rxn	1mlrxn
10 x PCR Buffer	4.5µl	100µl
dNTP mix (2.5 mM each)	4µl	100µl
Primer Mixz (10 pmal/ml each)	2.5µl	63µl
(25 pmol of each primer/50m /)		
Sterile water	34µl	737µl
Total	**45**µl	**1ml**

ß globin primer

SC5
SC2

PCR profile

Denaturation 94ºC 30 sec.
Annealing 58ºC 30sec.
Elongation 72ºC 1 min.
30 cycles, 7min. 72ºC extension, 4ºC soak.

Restriction enzyme digestion (100m 1 reaction)

DNA 45m1 (45m1 of 50m1 PCR reaction)
10 × buffer 10m1
Dde-I 10-30 units
Sterile water up to 100ml

Precipitate after over night digestion, dissolve pellets in 5m1 1× loading buffer.

A. Electrophoresis

Load samples to 1.5% agarose gel. Run at 90 mAmps for 2.5 hrs.

B. Results

Mutation abolishes restriction site.

Figure 1: PCR gel
PCR product Fragment Size 233 bp
Fragment Sizes After Dde I Digestion

A/A	A/S	S/S
178+55bp	233+178+55bp	233 bp

1 2 3 4 5 6 7

Figure 1, Typical Sickle cell genotype analysis of PCR product digested with Dde I. Lane 1 is molecular weight markers. Lane 2 is undigested PCR product. Lanes 3, 4 and 6 is DNA with A/S genenotype . Lane 5 is A/A genotype DNA and Lane 7 represents DNA with S/S genotype.

References

1. Saiki et al. (1985) Science 230 1350-1354
2. Wu et al. (1989) PNAS 86:2757-2760
3. Conner et al. (1983) PNAS 80:278-282

**The polymerase chain reaction (PCR) process is covered by patents owned by Hoffmarin-La Roche. A license to perform is automatically granted by the use of authorized reagents.

Appendix

PCR Premix preparation

Typical Premix

PCR Reaction mix

	50µl rxn	1mlrxn
10 x PCR Buffer	4.5µl	100µl
dNTP mix (2.5 mM each)	4µl	100µl
Primer Mixz (10 pmal/ml each) (25 pmol of each primer/50m /)	2.5µl	63µl
Sterile water	34µl	737µl
Total	**45**µl	**1ml**

Nucleotide Dilution

Stock---------100 mM: Prepare a final diluted 2.5 mM solution

Preparation	
Each 100 mM dNTP	25 µl (Total 500 µl)
Water	4.5 ml
Total Volume	5.0ml

Taq Premix (per 50µl reaction, scale up as required)

10 x PCR Buffer	0.5µl
Taq polymerase	0.25µl
Sterile water	4.25µl
Total	**5µ1/rxn**

PCR reaction (50µl)	
Diluted DNA (100ng/ml)	1µ1
PCR premix	4.5µl
Taq premix	5µl

PCR products post-processing

1. For all layered PCR only Acid 200ml of CHC13 to each tube, vortex and spin

2. Transfer the upper aqueous layer to a fresh eppendorf tube, add 1/10 volume of 3M NaAc (pH 5.2) and 2 volumes of absolute ethanol, precipitate DNA at -80ºC for 10 minutes.

3. Spin, rinse the DNA pellet with 700ml of 75% ethanol and dry the pellet in the speedvac

4. Dissolve the pellet in adequate amount of TE.