PCR BASED DIAGNOSIS OF THALASSAEMIA

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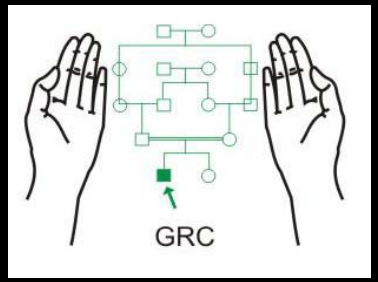
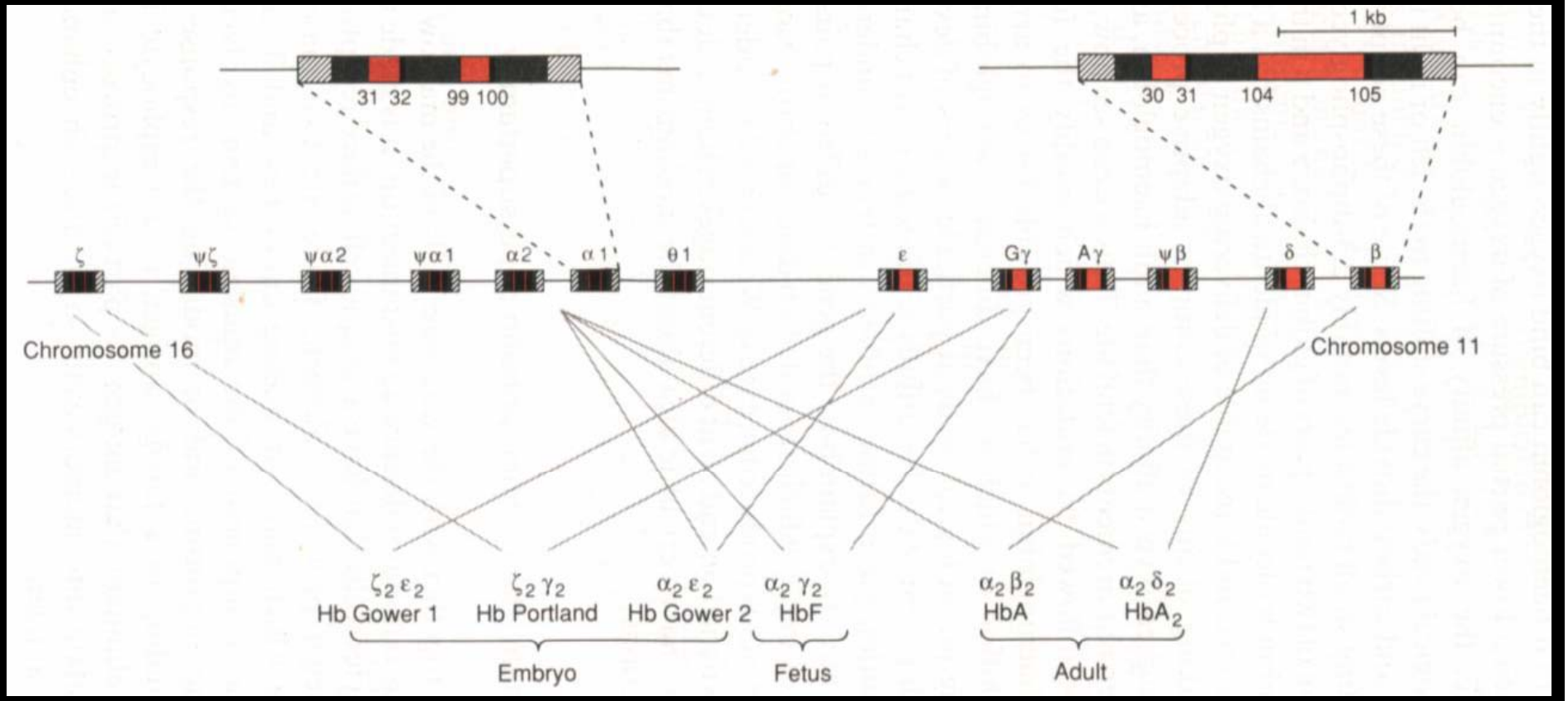
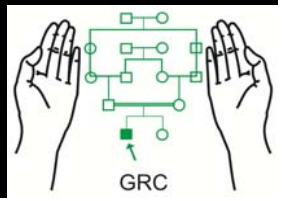
Genetics Resource Centre (GRC)



www.grcpk.com


1 kb
31 32 99 100
30 31 104 105
ζ
ψζ
ψα2
ψα1
α2
α1
θ1
ε
Gγ
Aγ
ψβ
δ
β
Chromosome 16
Chromosome 11
$\zeta_2\varepsilon_2$
Hb Gower 1
$\zeta_2\gamma_2$
Hb Portland
$\alpha_2\varepsilon_2$
Hb Gower 2
$\alpha_2\gamma_2$
HbF
$\alpha_2\beta_2$
HbA
$\alpha_2\delta_2$
HbA_2
Embryo
Fetus
Adult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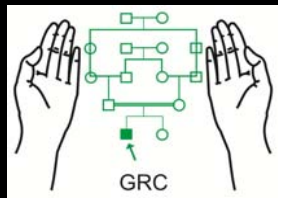
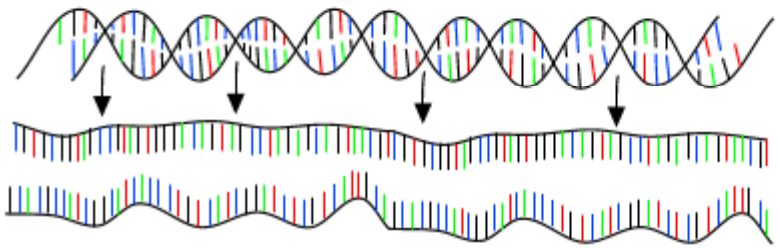
Molecular basis of Thalassaemia

- α-thalassaemia
  - Gene deletions
  - Point mutations (non-deletional)
- β-thalassaemia
  - Point mutations
  - Gene deletions
- Rare forms of thalassaemia (mostly gene deletions)
  - δβ –thalassaemia
  - δ-thalassaemia
  - γ-thalassaemia
- HPFH



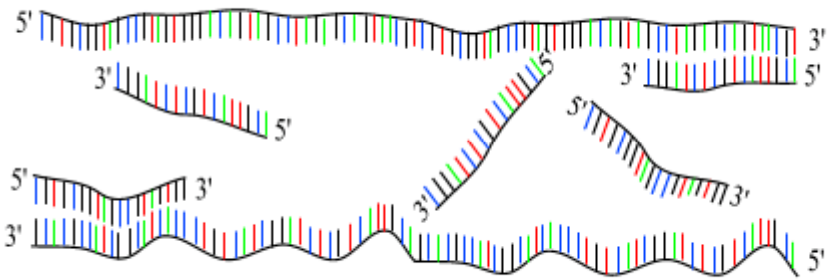
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

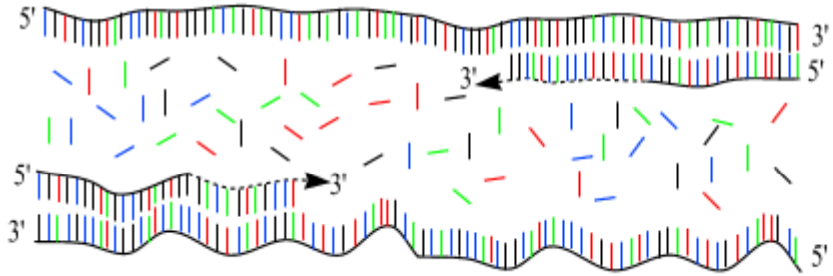
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

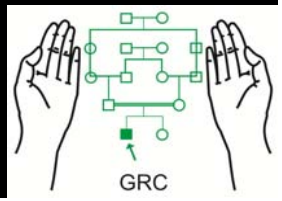


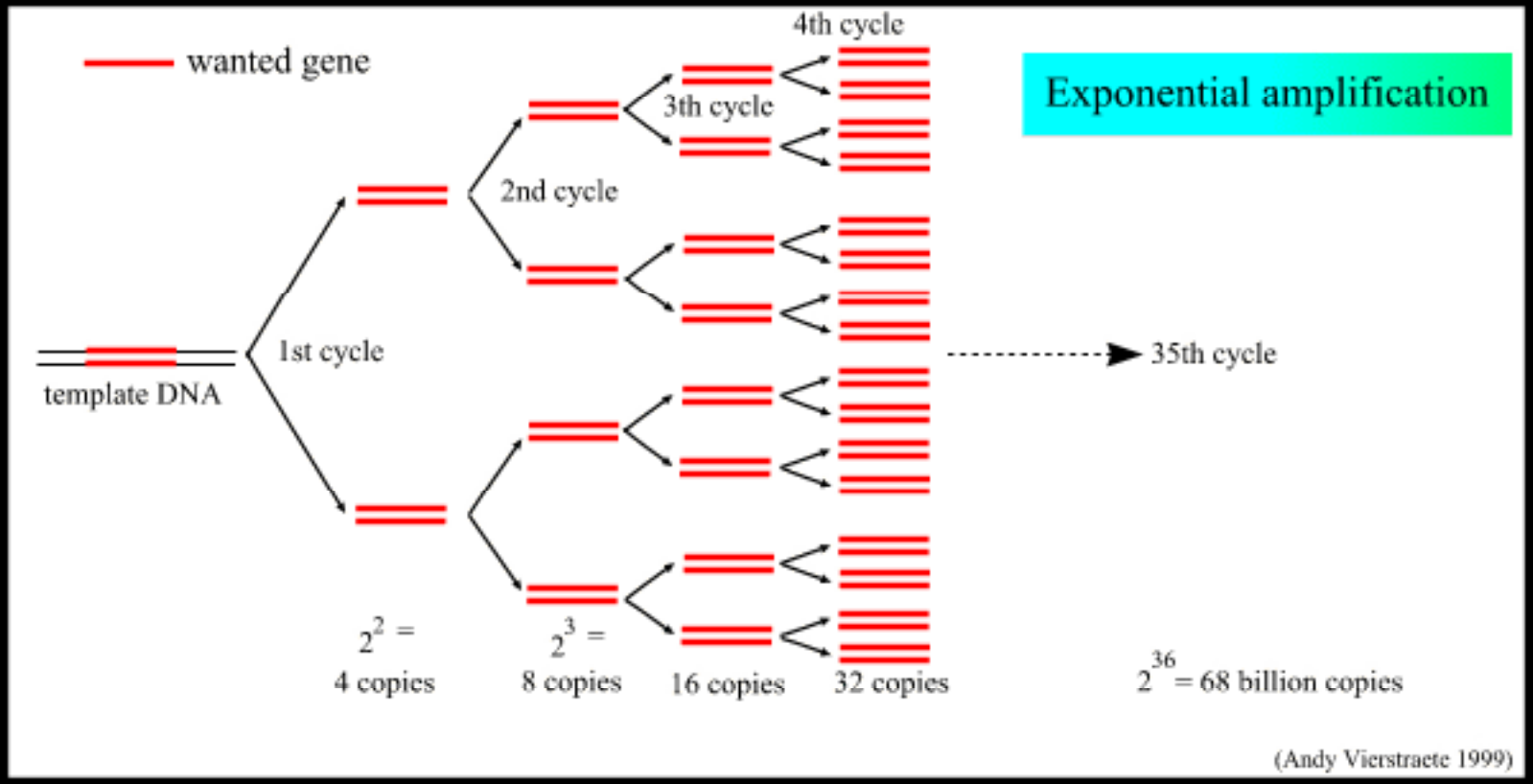
Step 3 : extension

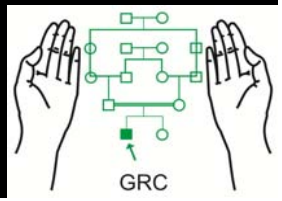
2 minutes 72 °C

only dNTP's

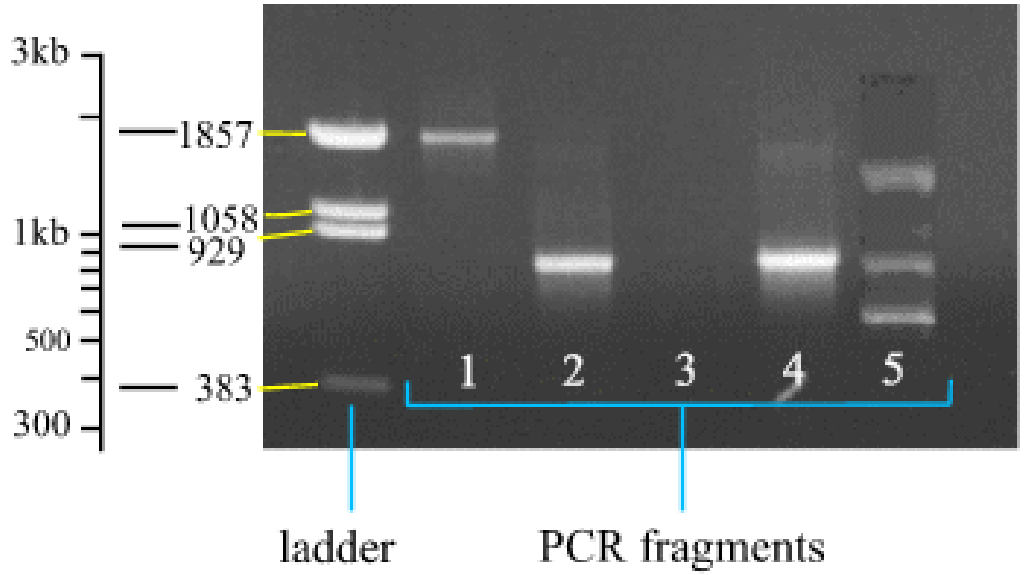
(Andy Vierstraete 1999)

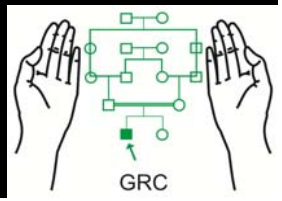



wanted gene
Exponential amplification
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^2 =
4 copies
2^3 =
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)


GRC

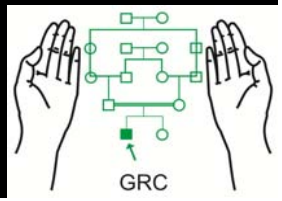
Verification of PCR product on agarose or separide gel





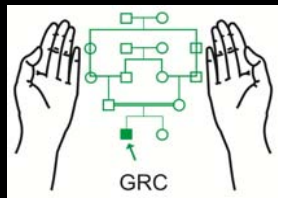
Molecular Methods in Diagnosis

- Mutation Analysis
  - Specific methods
    - ARMS
    - Dot Blot & Reverse Dot Blot
    - Sequencing
  - Non specific methods
    - DGGE
    - SSCP
- Linkage analysis



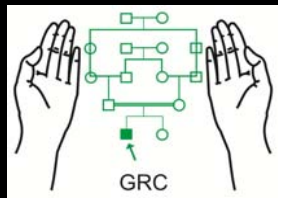
Applications in Thalassaemia

- Prenatal Diagnosis
- Diagnosis in previously transfused patients
- Silent thalassaemia alleles
- Distinction between structural variants
- Thalassaemia intermedia
- α-thalassaemia
- β-Thalassaemia carriers in certain situations
- Rare thalassaemias

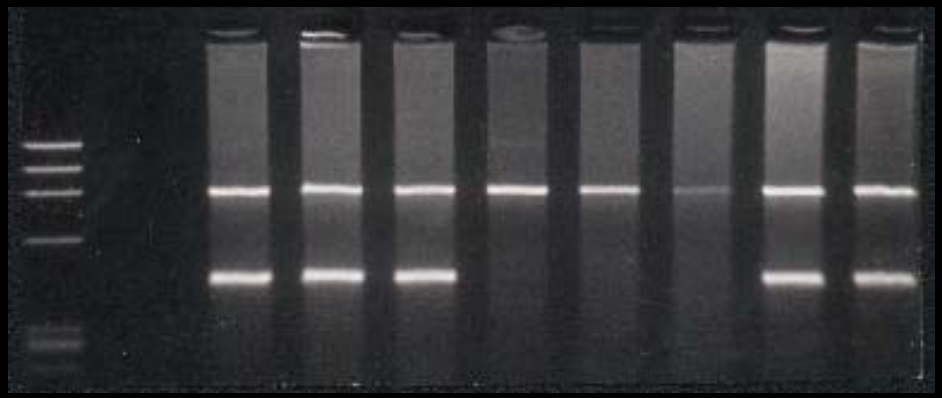


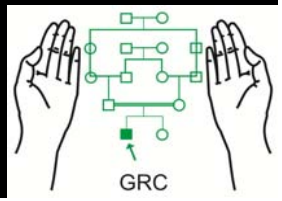
Amplification Refractory Mutation System (ARMS)

- Allele specific primer
- Amplification dependent on a match/mismatch at the 3'-end of the primer
- Separate primers for the mutant and the normal sequence
- Stringent PCR conditions
- False positives and negatives

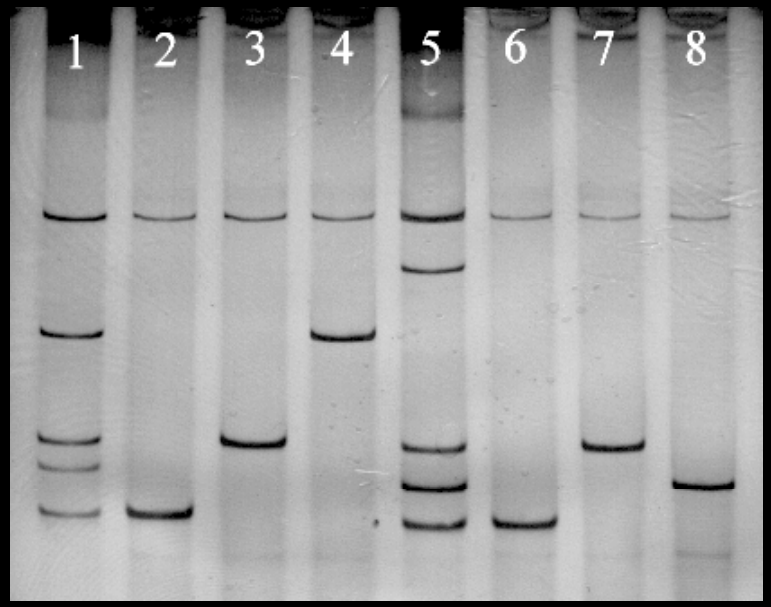


ARMS PCR For Thalassaemia



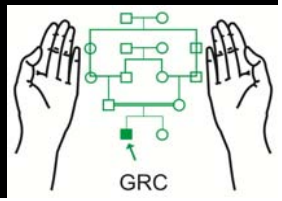


Multiplex ARMS PCR

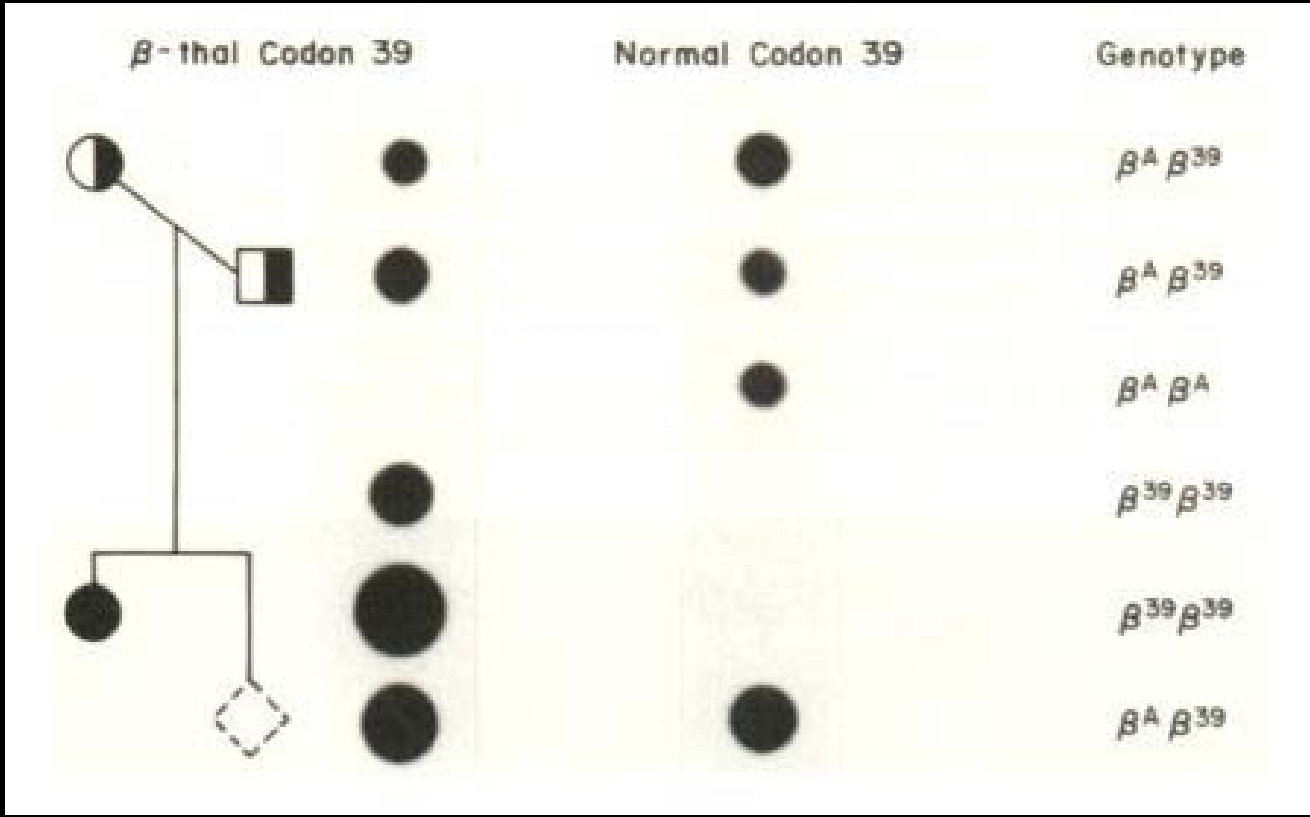


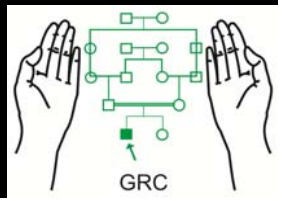
Primer ID:	Mutations Pooled:	Amplified Product size:
AD-1	Fr 8-9 (+G)	215 bp
	IVSI-5 (G-C)	285 bp
	Fr 41-42 (-TTCT)	439 bp
	IVSI-1 (G-T)	280 bp
	Del 619bp	242 bp
AD-2	Cd 5 (-CT)	205 bp
	Fr 16 (-C)	238 bp
	IVSI-1 (G-T)	280 bp
	Cd 30 (G-C)	280 bp
	Cd 30 (G-A)	280 bp
	IVSII-1 (G-A)	634 bp
AD-3	Cd 15 (G-A)	500 bp
	Cap+1 (A-C)	567 bp

(Ahmed et al, Prenatal Diagnosis 2000)

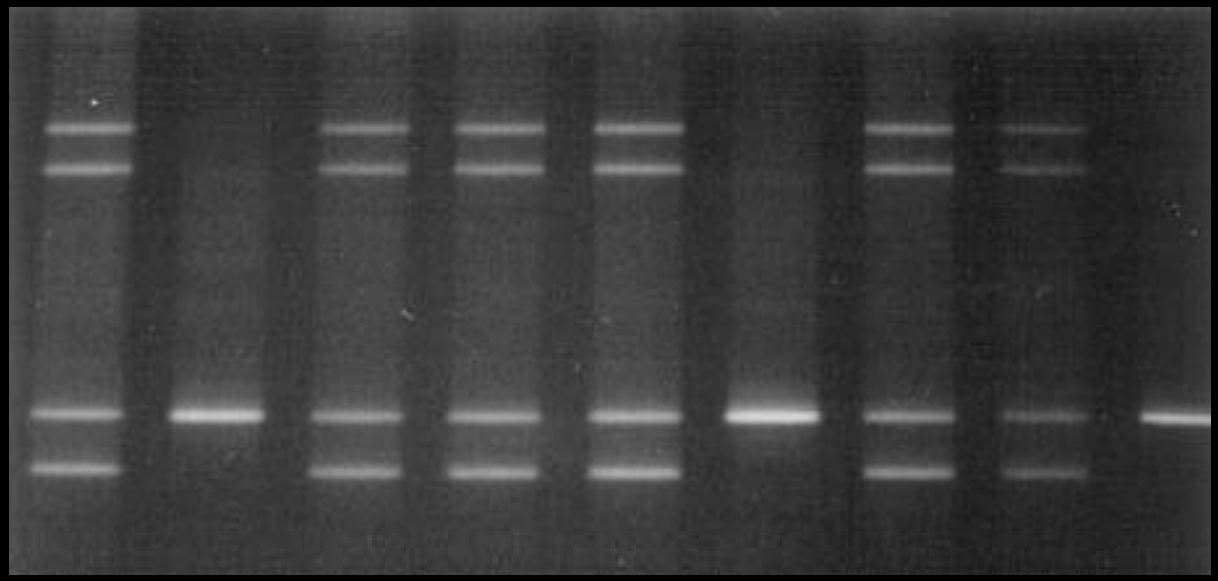


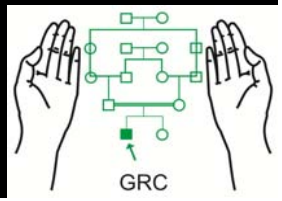
Dot Blot


β-thal Codon 39
Normal Codon 39
Genotype
$\beta^A \beta^{39}$
$\beta^A \beta^{39}$
$\beta^A \beta^A$
$\beta^{39} \beta^{39}$
$\beta^{39} \beta^{39}$
$\beta^A \beta^{39}$

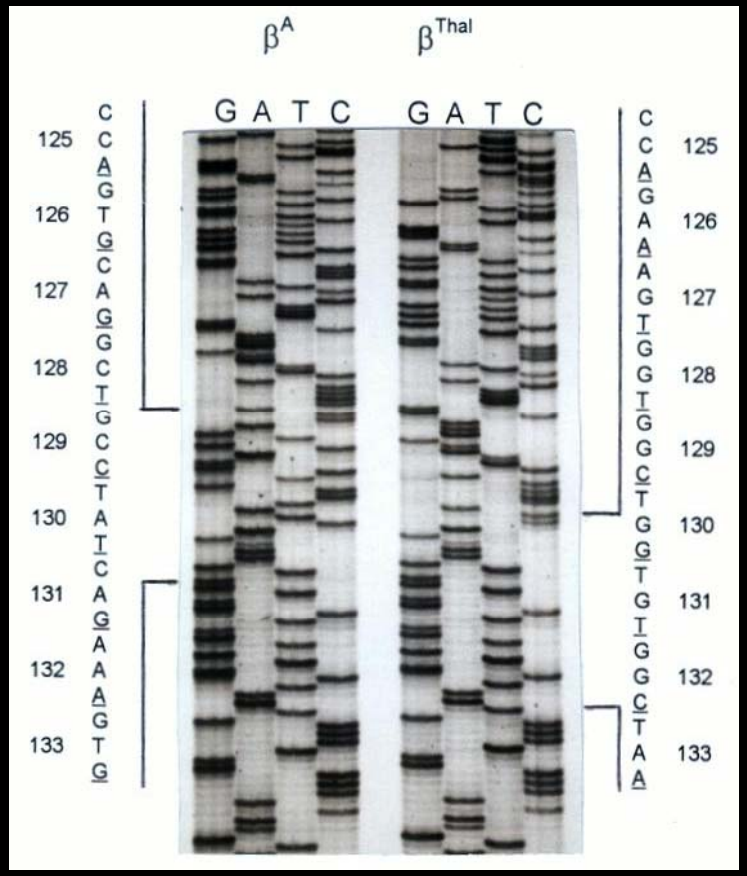

GRC

DGGE

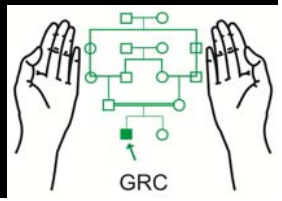




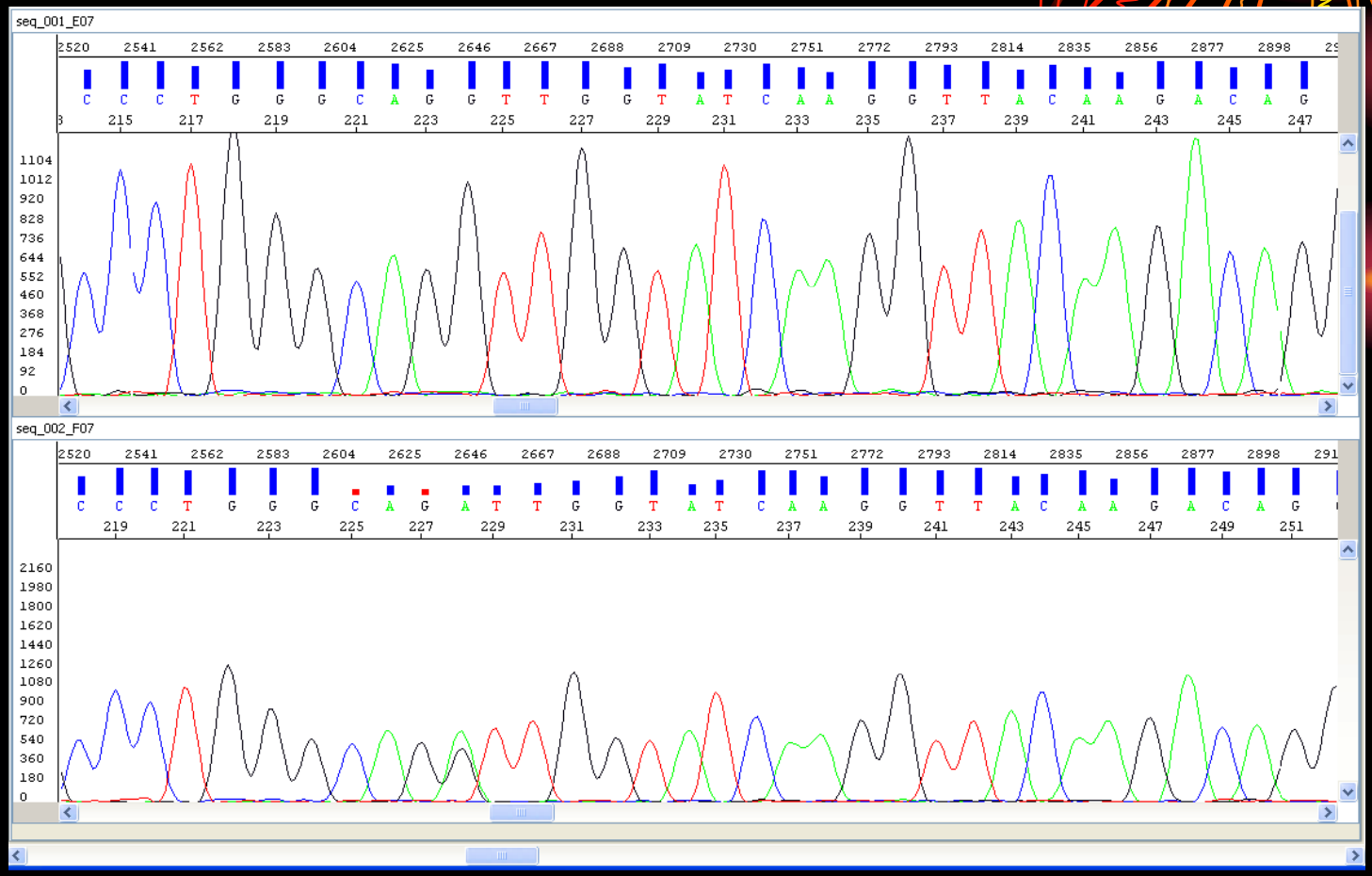
Genomic Sequencing

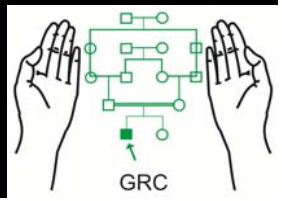


(Ahmed et al, Br J Haematol 1996)

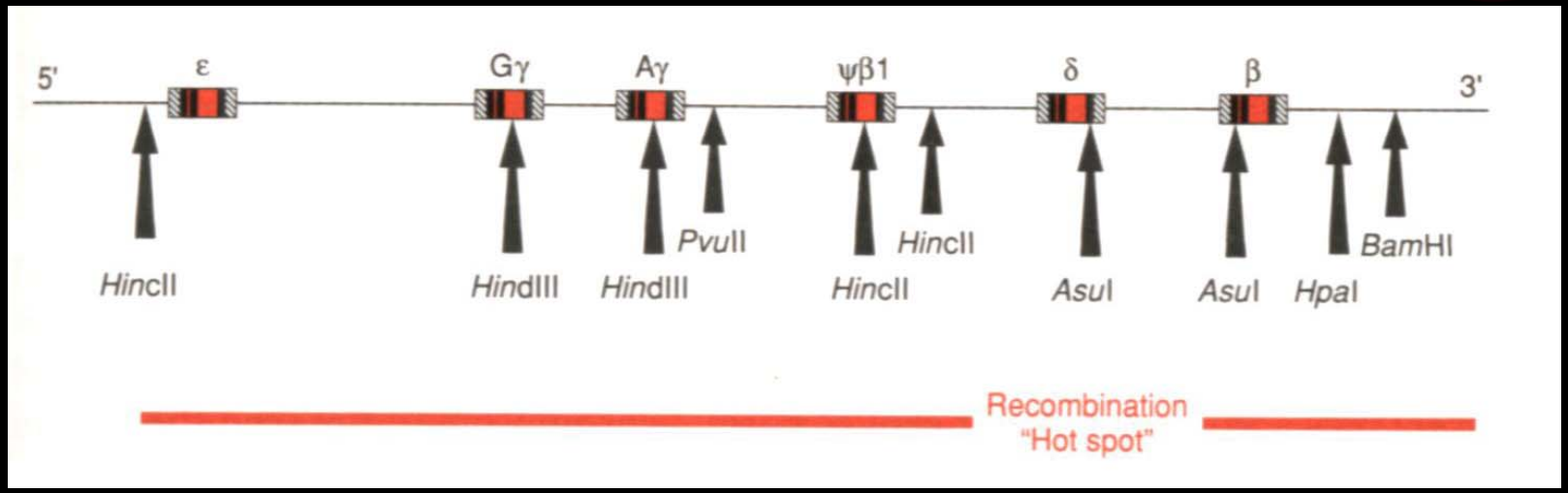


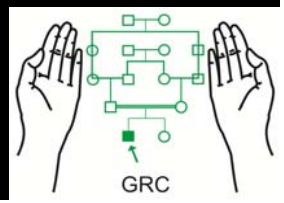
IVSI-1 (G-A)



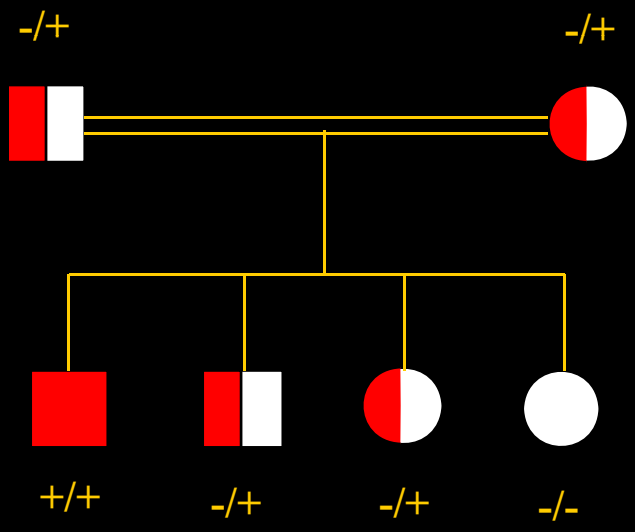


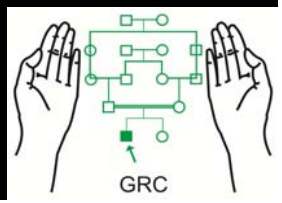
Linkage Analysis



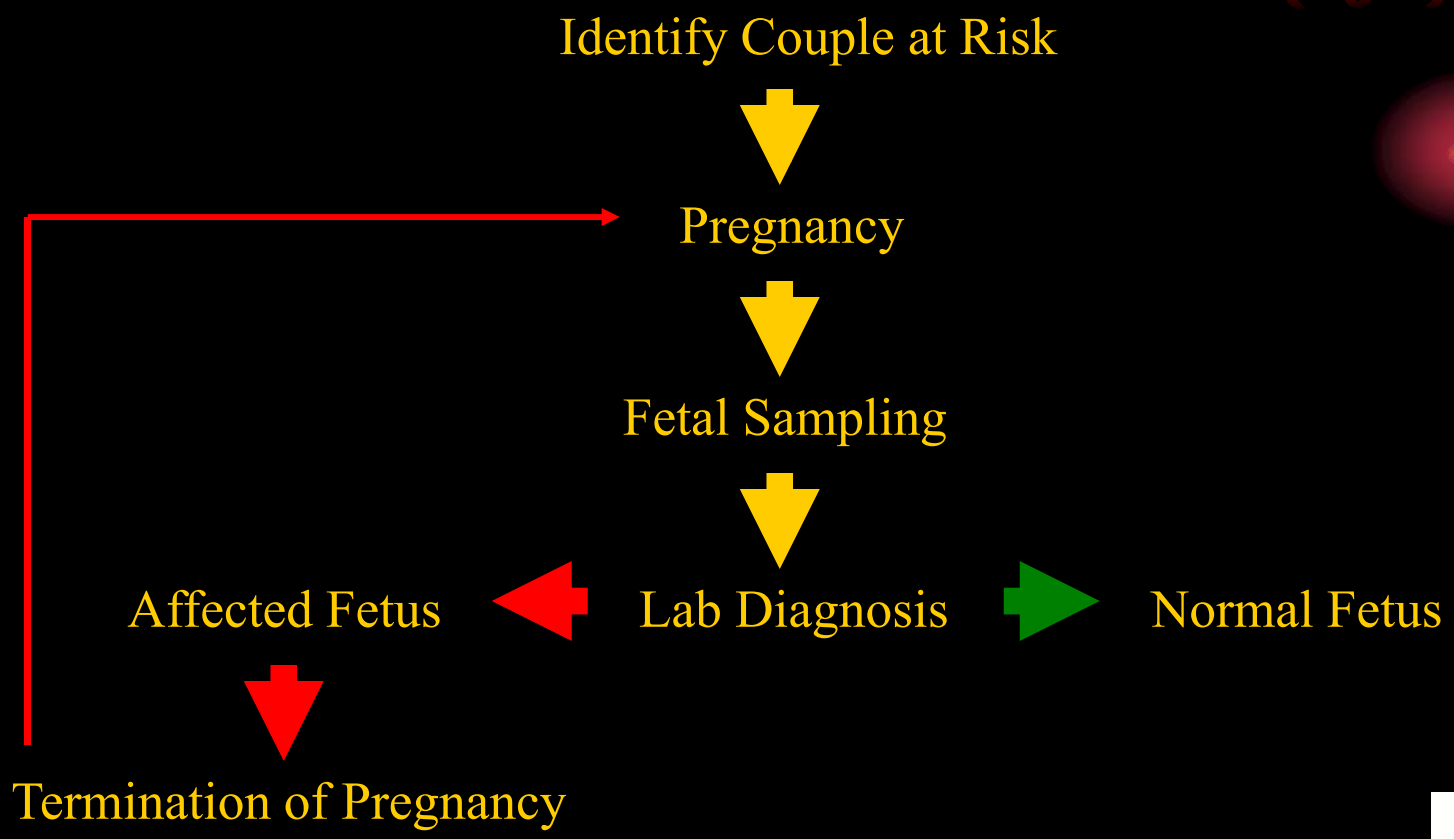


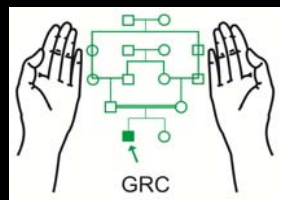
Linkage Analysis





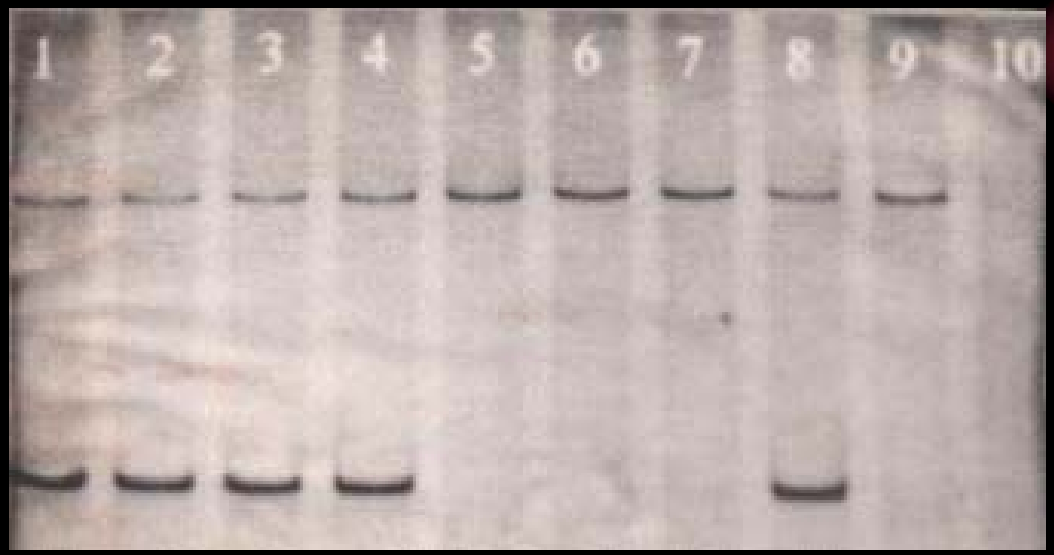
Prenatal Diagnosis

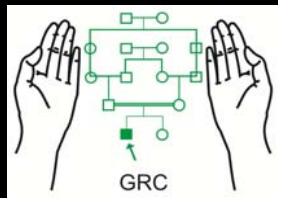




Prenatal Diagnosis by ARMS

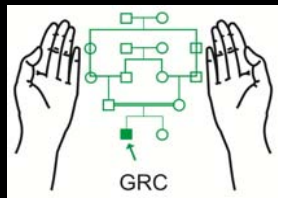
- Mutation
  1. Father
  2. Mother
  3. CVS
  4. CVS
  5. -ve
- Normal
  6. CVS
  7. CVS
  8. +ve
  9. -ve
  10. Blank



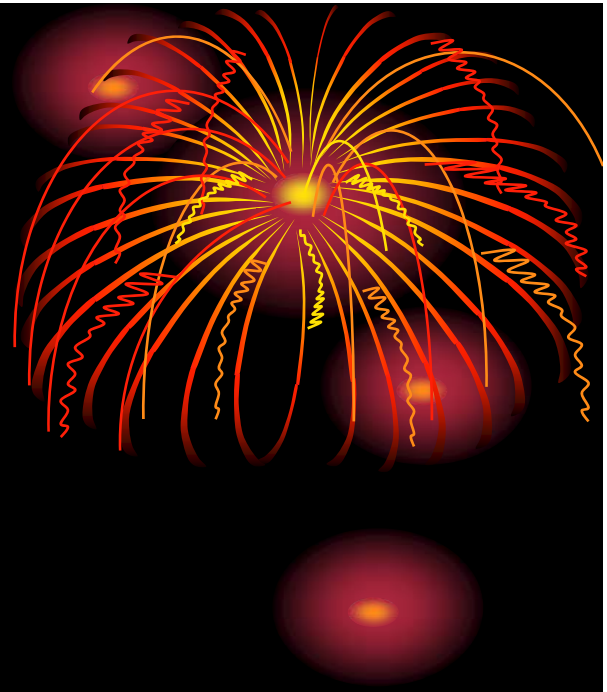


Misdiagnosis in PND (<0.5%)

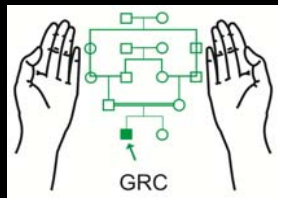
- Maternal Contamination in Fetal Sample
- PCR Failure
- Clerical Mistakes
- Meotic Crossover



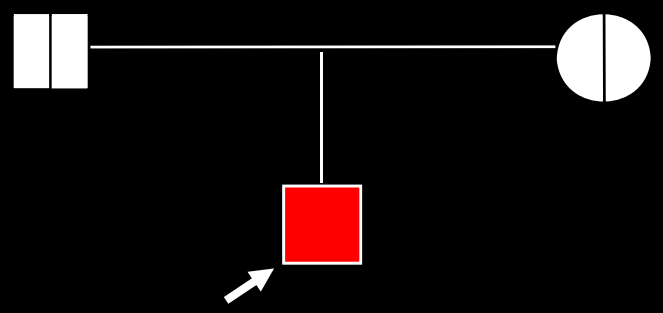
Future Prospects
Single Cell PCR



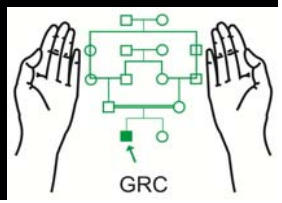
- Pre-Implantation Diagnosis
- Fetal Cells in Maternal Blood
- Fetal Cells in Cervical Mucus



Diagnosis in Previously Transfused Patients



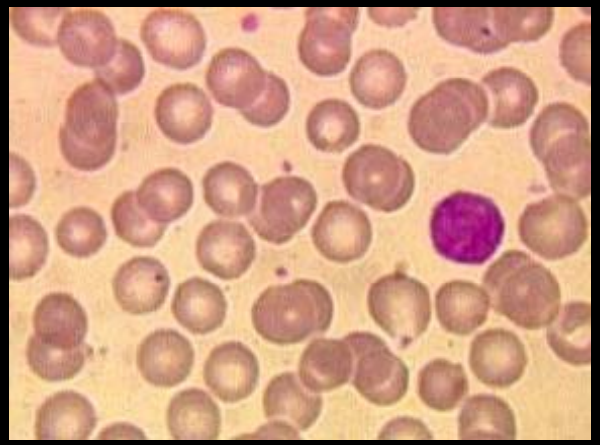
Transfusion Dependent Anaemia ??
Hb: 6.7 g/dl
MCV: 76 fl
MCH: 24 pg
Hb-F: 3.5%

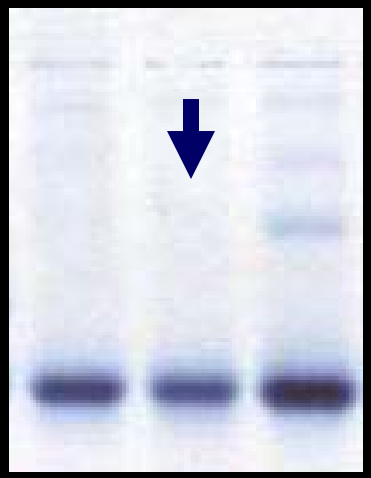


Silent β-Thalassaemia Alleles

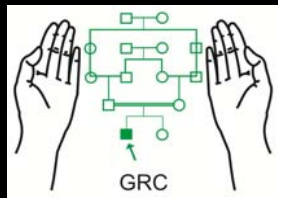
No. 58
DATE: 22/ 3/95
MODE: WHOLE BLOOD

WBC	9.2	$\times 10^3/\mu\ell$
RBC	4.36	$\times 10^6/\mu\ell$
HGB	- 11.7	g/dℓ
HCT	- 35.0	%
MCV	80.3	fℓ
MCH	26.8	pg
MCHC	33.4	g/dℓ
PLT	368	$\times 10^3/\mu\ell$





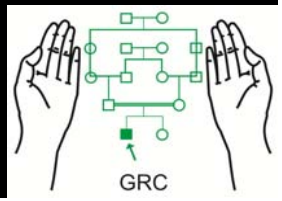
PCR: Cap+1 or –88 mutation

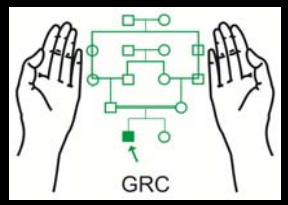


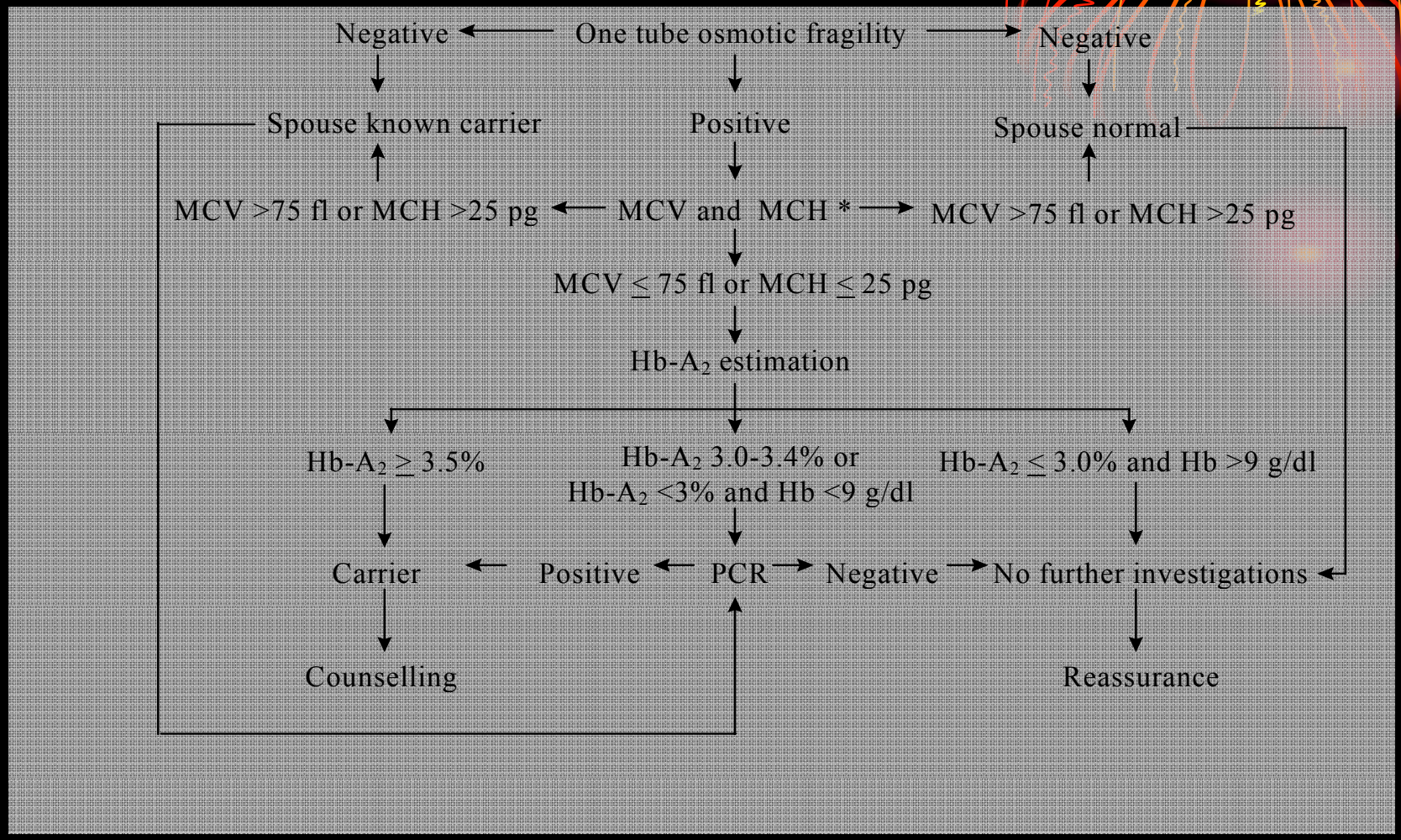
Typical β-Thalassaemia Carriers

- Associated Iron deficiency
- Co-incidental α-thalassaemia
- Cost effective in screening index families
- Only one or two mutant alleles

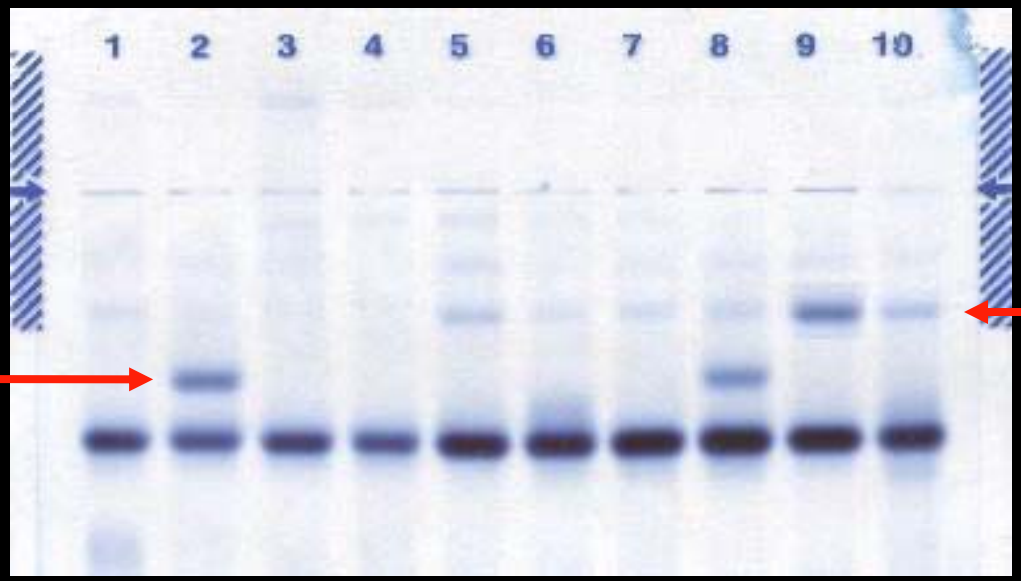
(Ahmed et al, New Engl J Med 2002)




GRC

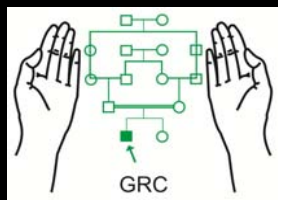

One tube osmotic fragility
Negative
Positive
Negative
Spouse known carrier
Spouse normal
MCV >75 fl or MCH >25 pg
MCV and MCH *
MCV >75 fl or MCH >25 pg
MCV ≤ 75 fl or MCH ≤ 25 pg
Hb-A2 estimation
Hb-A2 ≥ 3.5%
Hb-A2 3.0-3.4% or
Hb-A2 <3% and Hb <9 g/dl
Hb-A2 ≤ 3.0% and Hb >9 g/dl
Carrier
Positive
PCR
Negative
No further investigations
Counselling
Reassurance

Structural Hb Variants



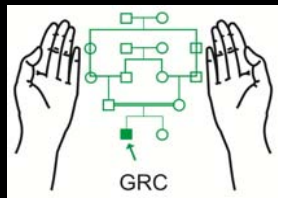
Hb-D/S/Lepore

Hb-A_2/E/C



Thalassaemia Intermedia

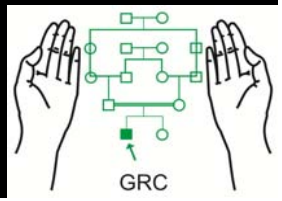
- Mild/Silent Alleles
- Co-incidental α-thalassaemia trait
- Co-incidental structural variants
- Xmn-I polymorphism
- δβ-Thalassaemia etc.



Thalassaemia Intermedia in Pakistan

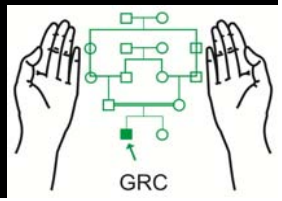
Cause of Thalassaemia Intermedia:	n (%):	Mean age:	
		At 1st transfusion:	At Examination:
Xmn-I +/+ genotype	14 36%)	6 years	13 years
β^+-mutation	6 (15%)	3 years	8 years
β^+-mutation and coincidental α-thalassaemia	6 (15%)	11¼ years	18 years
Unidentified thalassaemia mutation	2 (6%)	7½ years	12½ years
Coincidental α-thalassaemia	11 (28%)	9½ years	13½ years
Total	39	7 years	14 years

(Ahmed 1998)

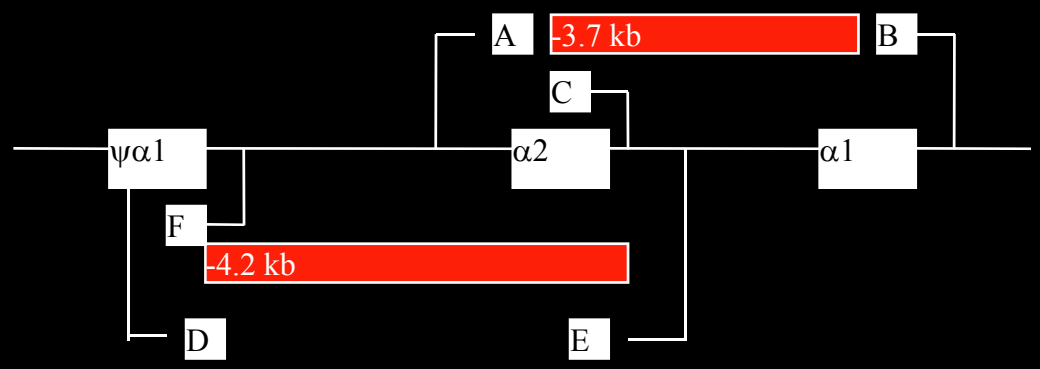


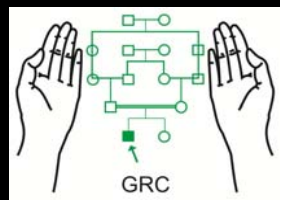
α-Thalassaemia

- Silent carrier: -α/αα
- α-Thalassaemia Trait: -α/-α or --/αα
- Hb-H Disease: --/-α
- Hydrops Fetalis: --/--

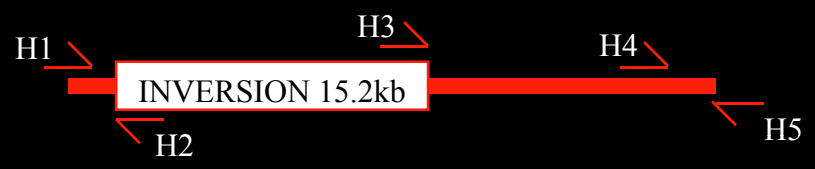


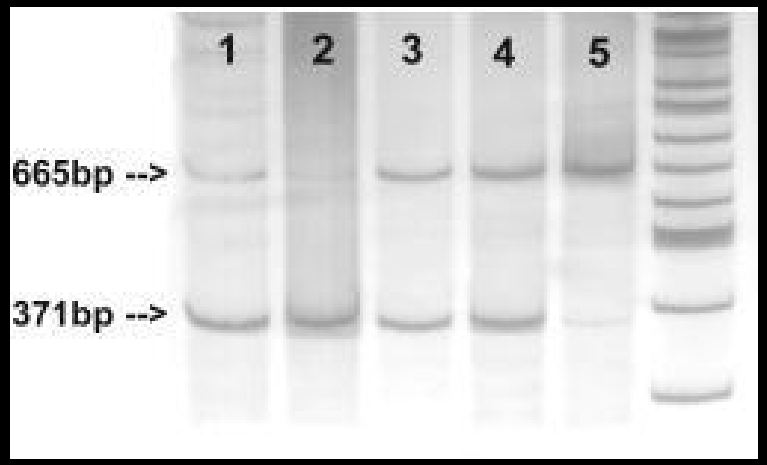
α-Thalassaemia

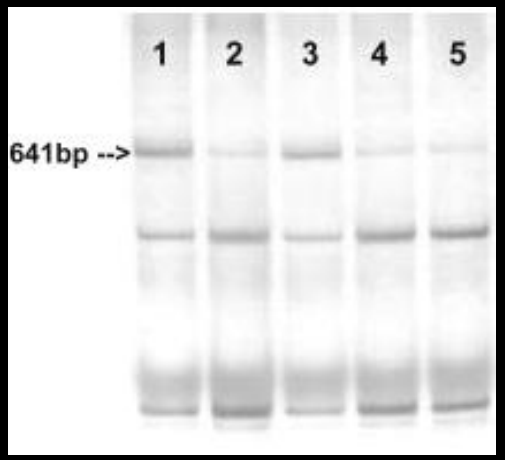




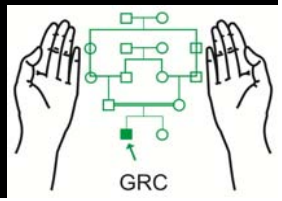
δβ-Thalassaemia Inv/Del $^{G}\gamma(^{A}\gamma\delta\beta)^{o}$







(Ahmed and Anwar, Am J Haematol 2005)



PCR Based Diagnosis of Thalassaemia

Luxury or Utility ?