

ESTABLISHMENT OF FIRST TRIMESTER PRENATAL DIAGNOSIS THROUGH CHORIONIC VILLUS SAMPLING

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OBJECTIVES OF STUDY

- Identification of families with genetic disorders from various regions of Pakistan
- To investigate molecular basis of common and rare genetic diseases
- To establish programs for prevention/control of these disorders through:
 - Carrier screening
 - Genetic Counseling
 - Mutation detection
 - Prenatal diagnosis
- Better treatment strategies and gene therapy in future

INTRODUCTION

What are Genetic Disorders?

**A genetic disorder is a disease caused by ABNORMALITIES
In an individual's genetic material (genome).**

- There are more than 6,000 single gene disorders
- No satisfactory treatment is available for majority of the genetic disorders

Types of Genetic Disorders

1: Single gene disorders (Mendelian or Monogenic)

Mutations in one gene produce the disease. Sickle Cell Anemia, Thalassemias, Microcephaly, Skin disorders, Cystic Fibrosis, Huntington Disease etc.

2: Multifactorial disorders (Complex or Polygenic)

Caused by a combination of environmental factors and mutations in multiple genes. For example, different genes that influence breast cancer susceptibility found on chromosomes 6, 11, 13, 14, 15, 17, and 22. e.g. Heart disease, high blood pressure, Alzheimer's disease, Arthritis, Diabetes, Cancer and Obesity etc.

3: Chromosomal disorders

Abnormalities in chromosome number or structure. e.g. Down syndrome or trisomy 21 a person has three copies of chromosome 21. Turner syndrome (45,X), Klinefelter syndrome (47, XXY).

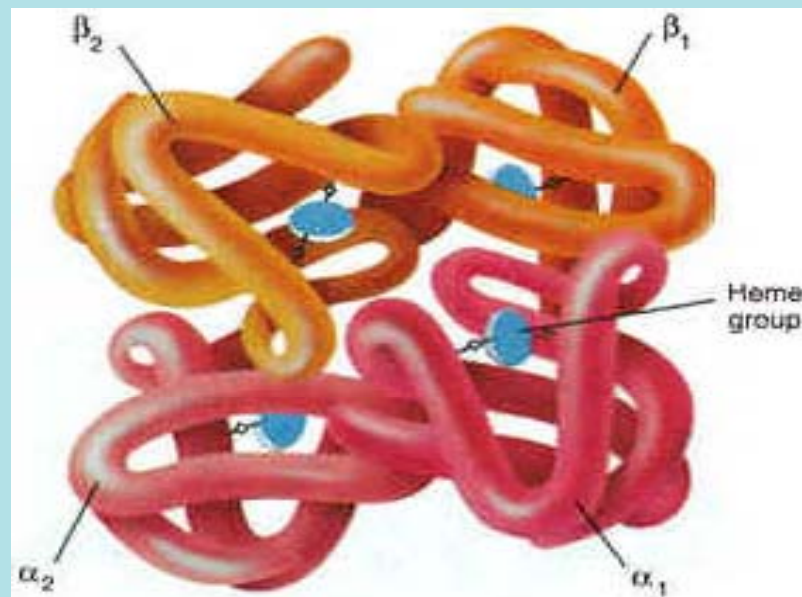
HEMOGLOBINOPATHIES

Inherited disorders of Hemoglobin

The most common single gene disorders in the world

Hemoglobin

Oxygen carrying tetra-meric molecule consisting of two alpha and two beta subunit ($\alpha_2\beta_2$)



Hemoglobinopathies

1. Thalassemias

- Alpha Thalassemia: Mutations in the α globin chain
Homozygous state: not compatible with postnatal life
Fetal Hemoglobin HbF($\alpha_2\gamma_2$)
- Beta Thalassemia: Mutations in the β globin chain
Homozygous state: Transfusion dependent
Adult Hemoglobin HbA($\alpha_2\beta_2$)

2. Hemoglobin Variants

change in amino acid
Hb S, Hb E, Hb C, HbD, etc

β -Thalassemia

Autosomal recessive disorder

- Absent or reduced synthesis of β -chains of hemoglobin
- The affected children require regular blood transfusion and iron chelation therapy to sustain life
- The most common genetic disorder in Pakistan, >5.6% allele frequency (9 million carriers)
- >50,000 registered cases of transfusion dependent thalassemia in Pakistan
- >5,000 Transfusion dependent children are born each year in Pakistan

Treatment

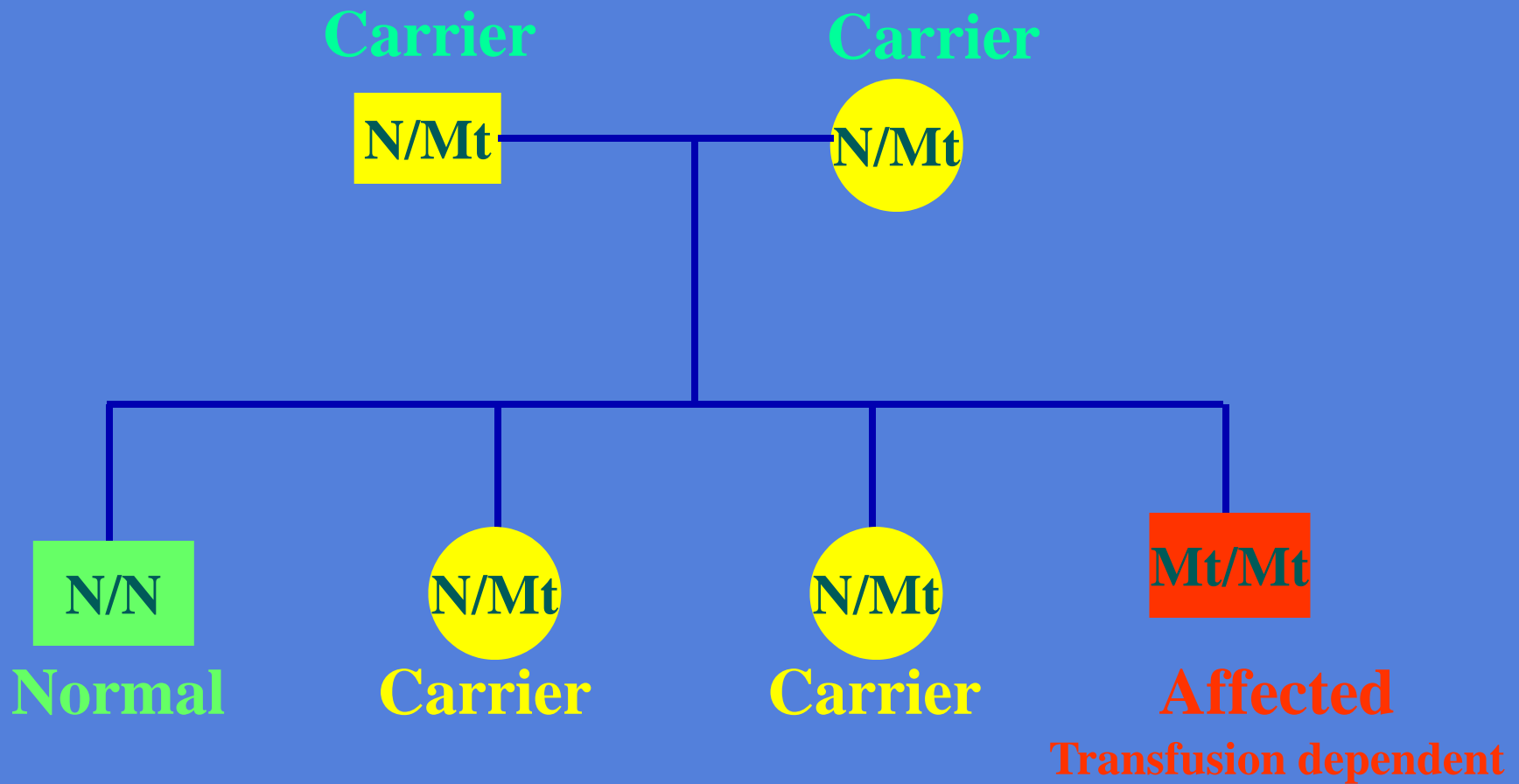
- **Regular Blood Transfusion** required to sustain life
- **Iron Chelation Therapy** (Annual cost US\$4,400/patient, Annual average income in Pakistan US\$420)
- **Bone Marrow Transplant** (Not affordable)
Associated with HVGD (Host Versus Graft Disease)
- **Gene Therapy** (Still under animal trials; Not affordable)

No satisfactory treatment available for β -thalassemia

THE ONLY AFFORDABLE SOLUTION IS Prevention through

- Carrier Screening
- Genetic Counseling
- Characterization of Mutations
- First Trimester Prenatal Diagnosis by CVS
- Retrospective inductive screening in extended families

Genetics of Thalassemia



1 : 2 : 1

25 : 50 : 25

Subjects

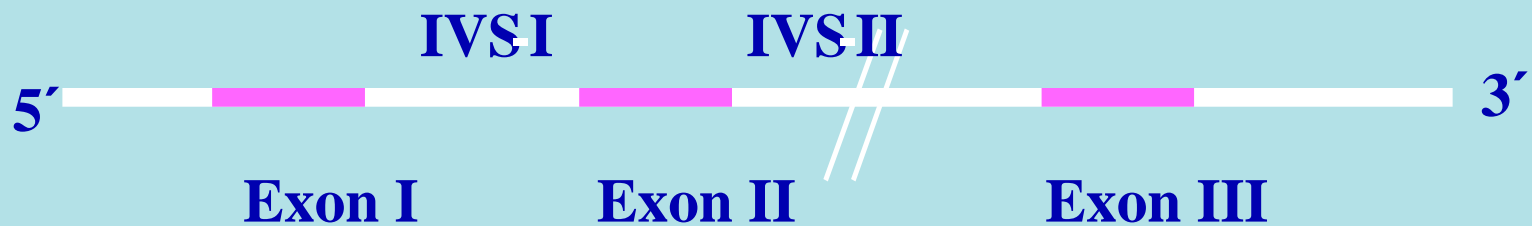
- Analyzed 600 β -thalassemia carrier families with at least one affected child
- More than 1200 β -thalassemia alleles from various areas of Pakistan

β -GLOBIN GENE

Location: 11q13.1

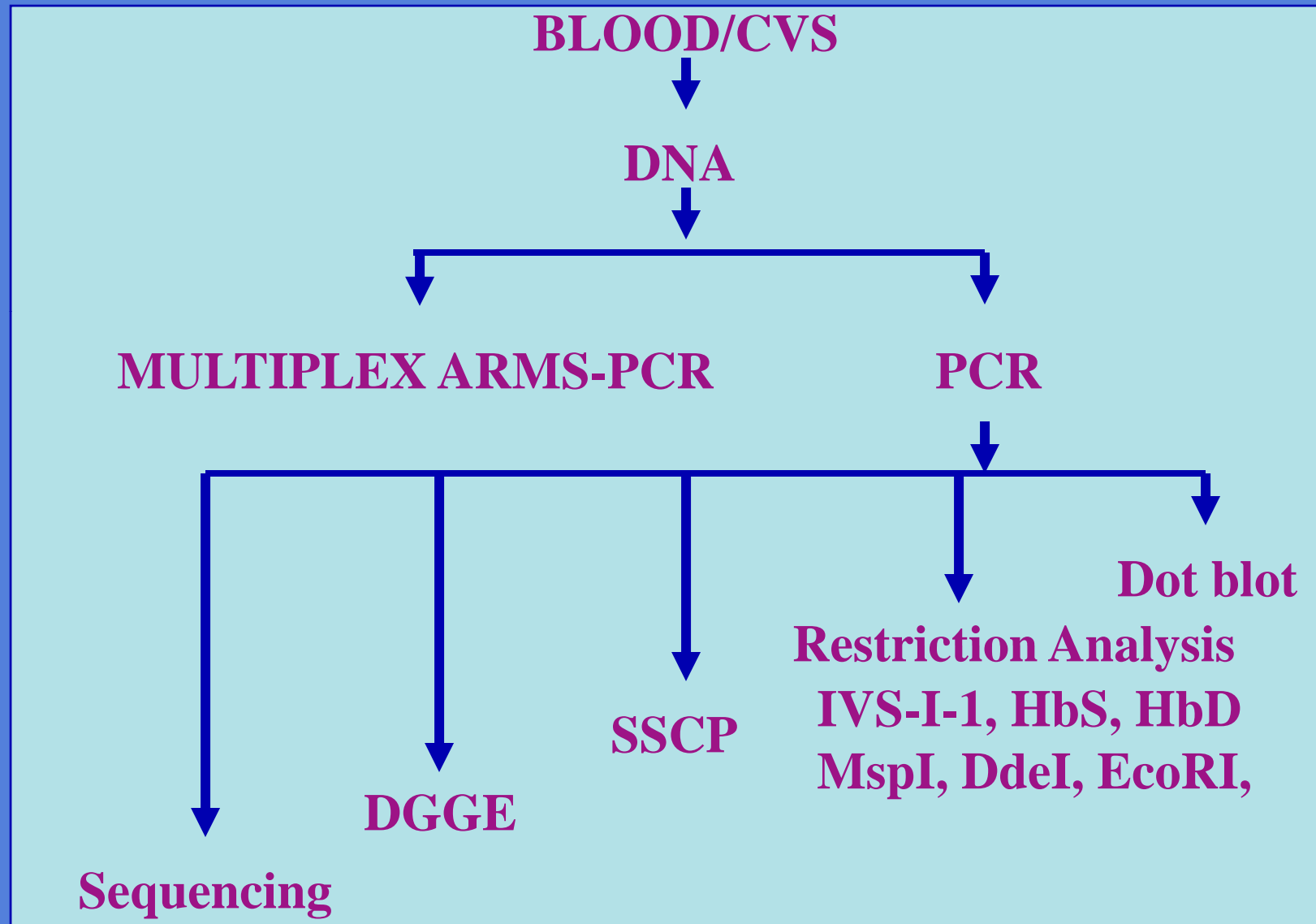
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Mutations Reported: >380



**DIAGNOSTIC STRETEGY
AND
TECHNIQUES USED**

Diagnostic Strategy



Techniques used

- ARMS PCR
- Allele specific oligonucleotide hybridization
 - Regular PCR(for deletions)
 - Restriction endonuclease test

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ARMS

(Amplification Refractory Mutation System)

Principle of the Technique

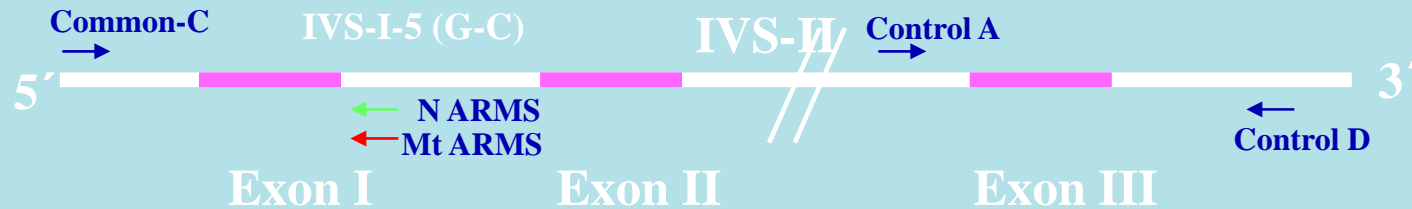
A PCR primer is designed so that it can discriminate between templates, which differ at a specific single nucleotide residue; an ARMS primer can specifically amplify one allele of a multiallelic system.

Taq polymerase has no 3' to 5' exonuclease activity; therefore a mismatch between the 3' end of the primer and the template will result in its inability to function as a primer under appropriate conditions.

Types of ARMS

- 1. Monoplex ARMS**
- 2. Multiplex ARMS**
- 3. Fluorescent ARMS**

ARMS



IVS-I-5 Mt 5'-CTC CTT AAA CCT GTC TTG TAA CCT TGA TAG-3'

IVS-I-5 N 5'-CTC CTT AAA CCT GTC TTG TAA CCT TGA TAC-3'

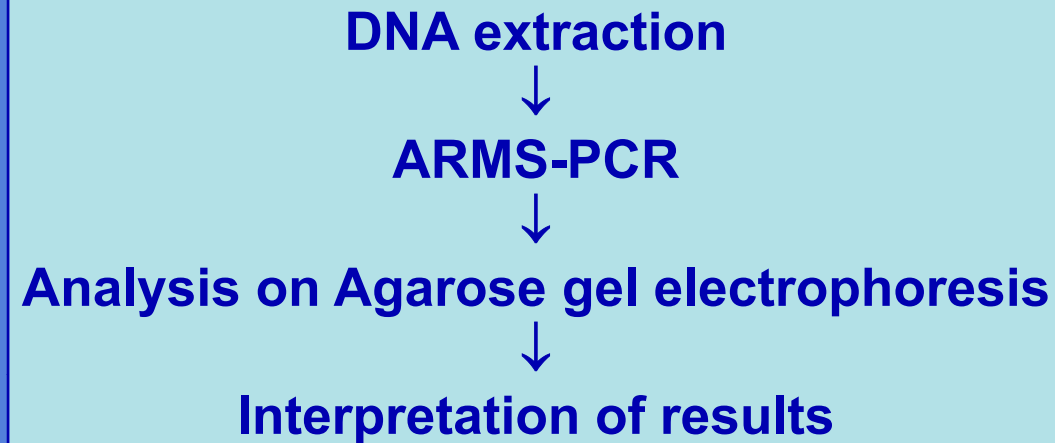
Mt ARMS PCR

DNA template
 PCR Buffer
 dNTPs
 F Primer Common C
 R Mt ARMS Primer
 F Control A Primer
 R Control D Primer
 Taq Pol
 Water

N ARMS PCR

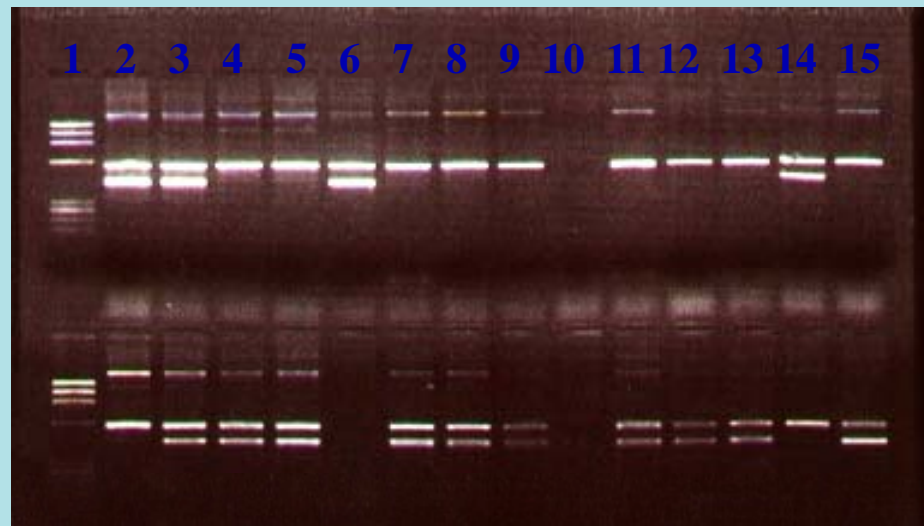
DNA template
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ARMS-PCR steps



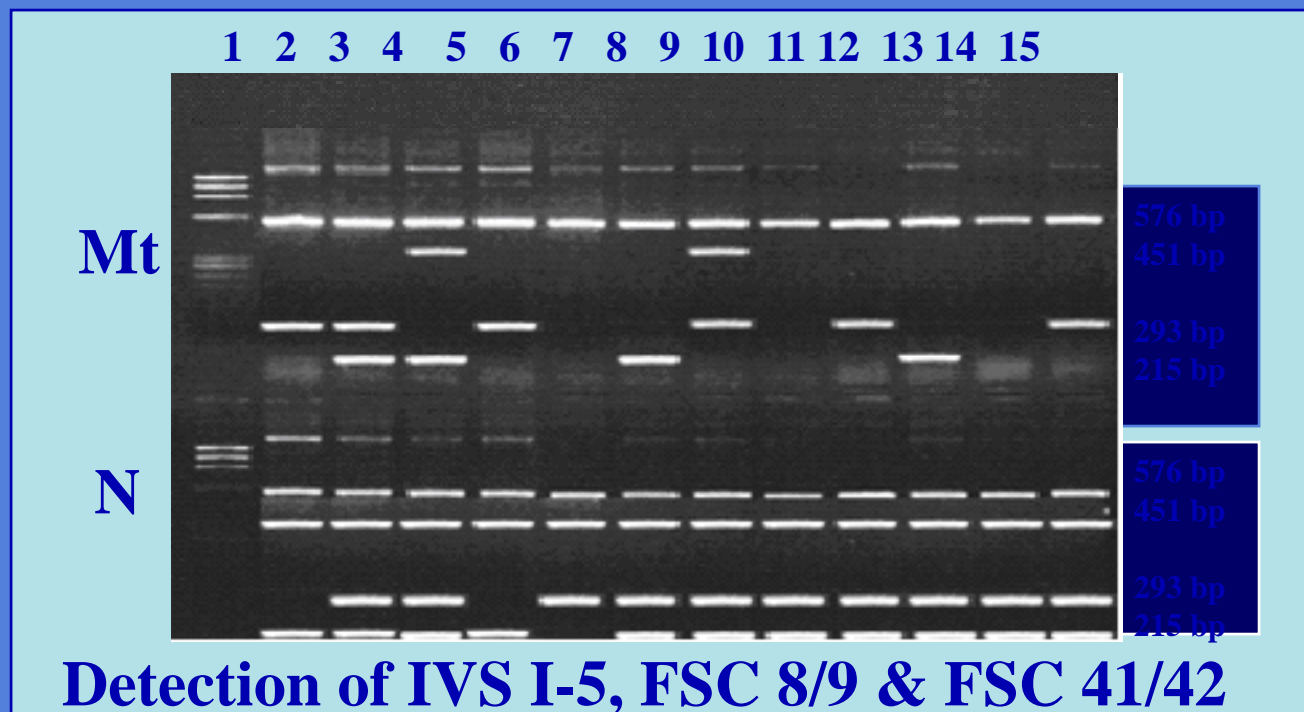
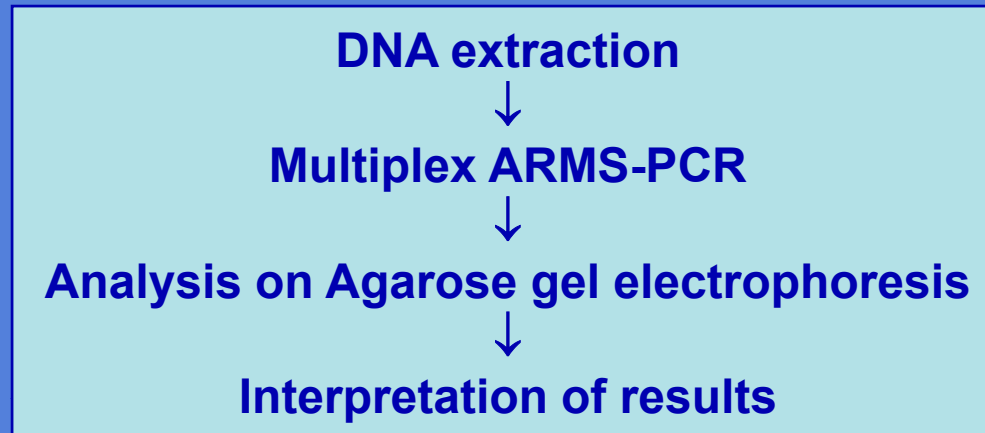
Mt

N



Detection of FSC-41/42 (-CTTT) by ARMS-PCR

MULTIPLEX ARMS-PCR STEPS



Requirements for Multiplex ARMS-PCR

- Knowledge of the mutations (known sequence)
- DNA Synthesizer (Gene Assembler) for primer synthesis
- Thermal cycler
- PCR reagents (Buffer, dNTPs, Taq polymerase etc)
- Gel Electrophoresis system
- No labeling and detection system required
(in conventional PCR system)
- Labeling of primers required in the real time PCR
(No post PCR processing)

Techniques used

- ARMS PCR

Allele specific oligonucleotide hybridization

- Regular PCR(for deletions)
- Restriction endonuclease test

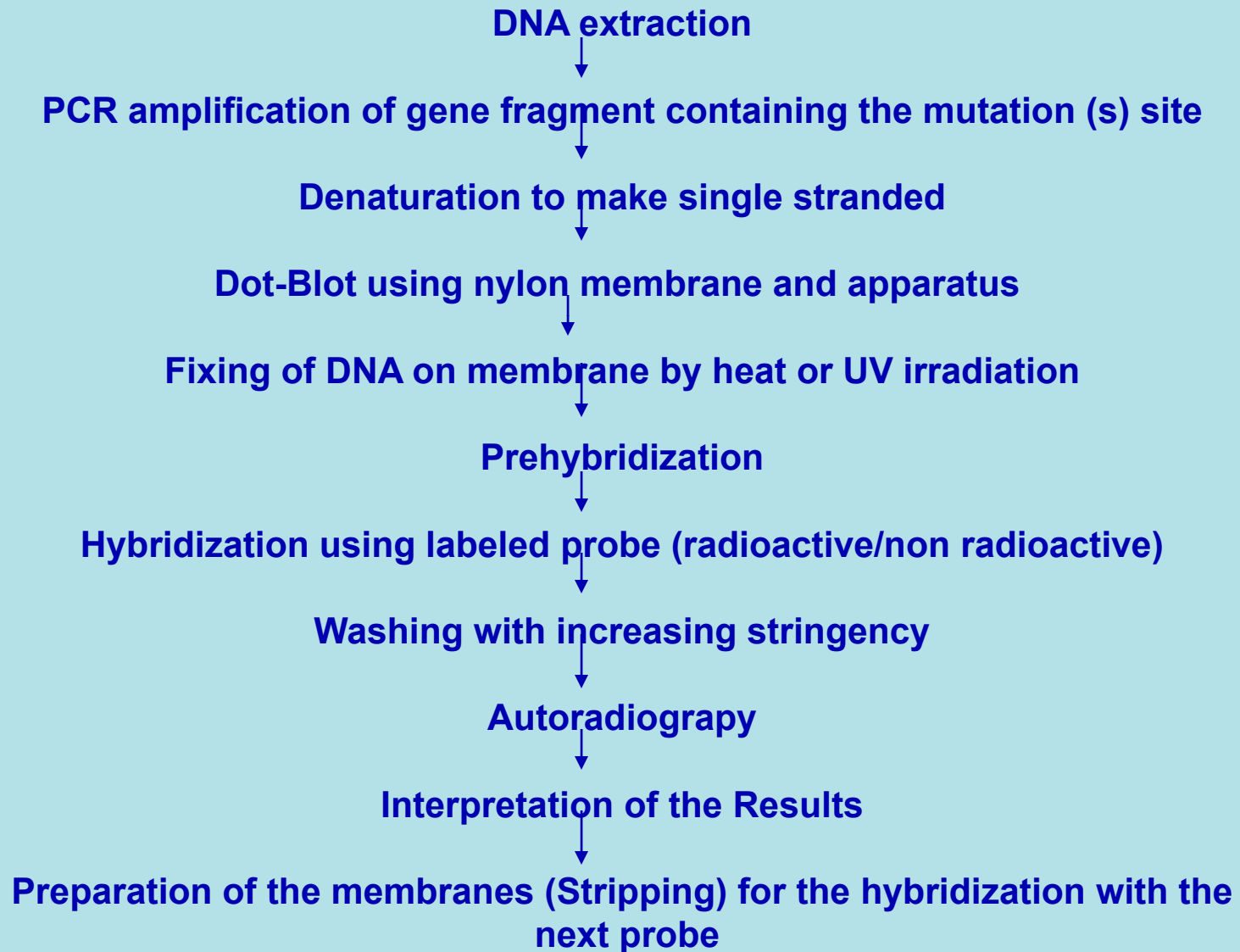
Allele Specific Oligo (ASO) Hybridization or Dot-Blot Hybridization

- Hybridization with a pair of oligos corresponding to alleles of a known mutation is used to detect that mutation.
- A region of genomic DNA or cDNA containing the mutation is amplified by PCR and transferred onto duplicate membranes.
- This can be by dot/ slot blotting using appropriate apparatus, spotting by hand, or digestion and Southern blotting

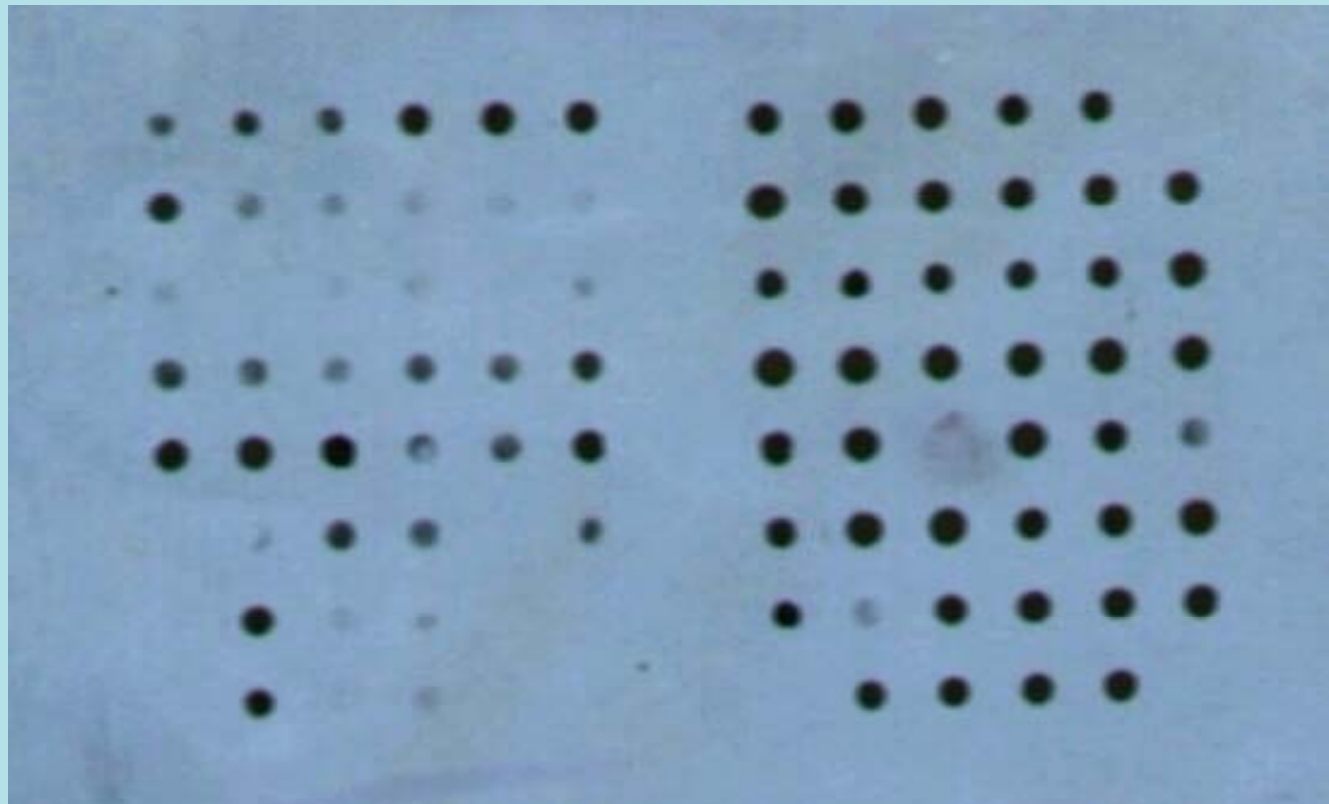
ASO Probes

IVS-I-5 N ASO Probe 5'-GCA GGT TGG TAT CAG GT-3'
IVS-I-5 Mt ASO Probe 5'-GCA GGT TGC TAT CAG GT-3'

Allele Specific Oligo (ASO) Hybridization



Dot Blot Hybridization for IVS-I-5 (G-C)



Mt

N

Reverse Dot Blot Hybridization (ASO)

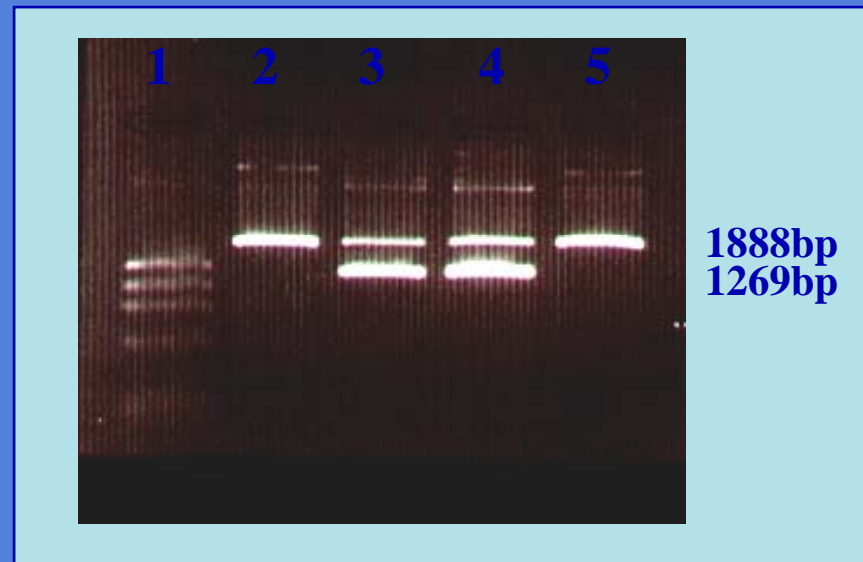
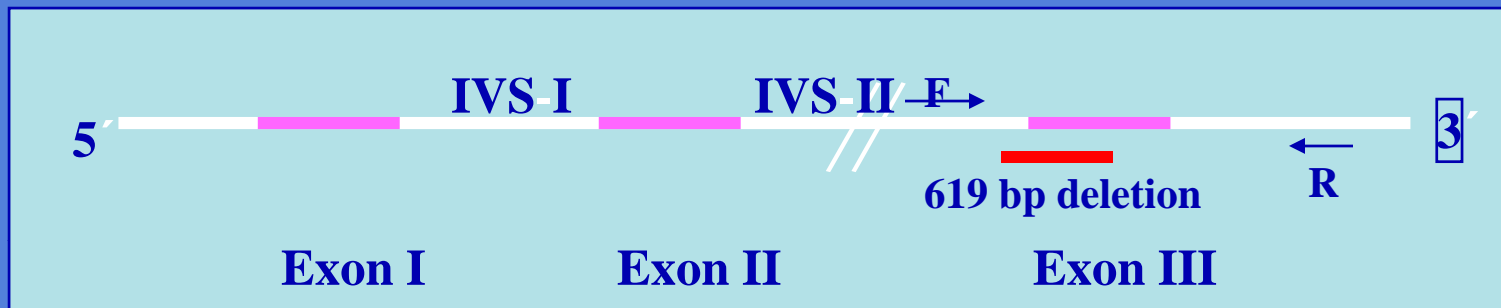
- Commercially available population specific strips
- The mutation specific probes are fixed on the membrane
- One DNA samples is hybridized with one Strip
- For use in clinical diagnosis set up
- Ideal for prenatal diagnosis (In cases where no prior knowledge of parental genotype)
- Not recommended for large series of samples (Research)

Techniques used

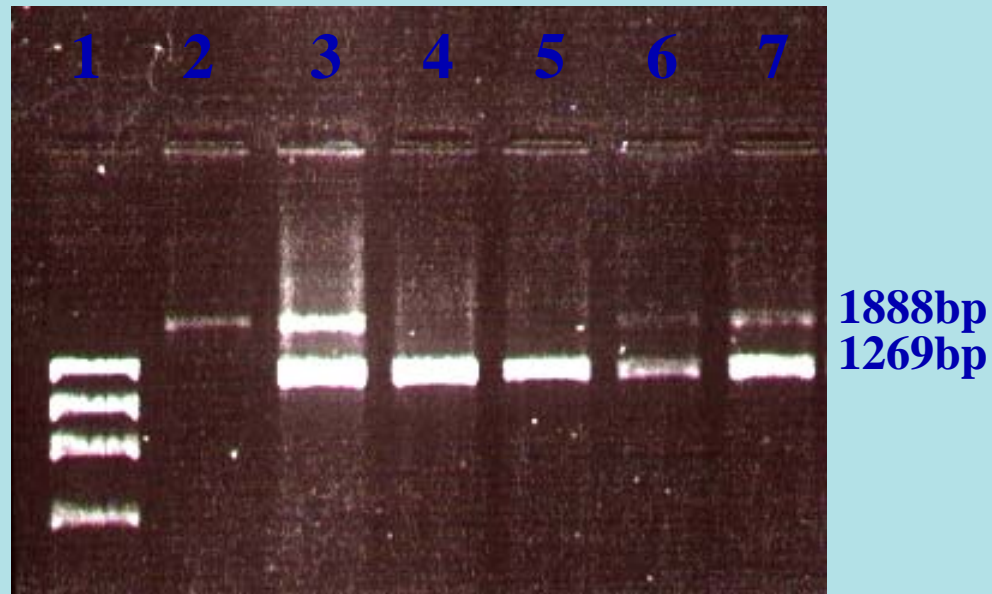
- ARMS PCR
- Allele specific oligonucleotide hybridization
- **Regular PCR(for deletions)**
- Restriction endonuclease test

Direct Detection of deletion mutation by PCR

619 bp deletion mutation in β -thalassemia



Detection of 619bp deletion mutation by PCR



Lane 1: ϕ X174 M; Lane 2: negative for 619 deletion mutation; Lane 3, 6 and 7: Heterozygote; Lane 4 and 5: homozygote for 619 bp deletion mutation.

Techniques used

- ARMS PCR
 - Allele specific oligonucleotide hybridization
 - Regular PCR(for deletions)
- Restriction endonuclease test

Restriction Endonuclease Analysis

Some mutations may create a new restriction site or abolish an existing one in the gene.

- e.g. HbS (A-T) abolishes the restriction site of DdeI (C↓TNAG),

Restriction Endonuclease Analysis for HbS (Steps)

DNA extraction



PCR amplification of β -globin gene fragment containing the mutation site

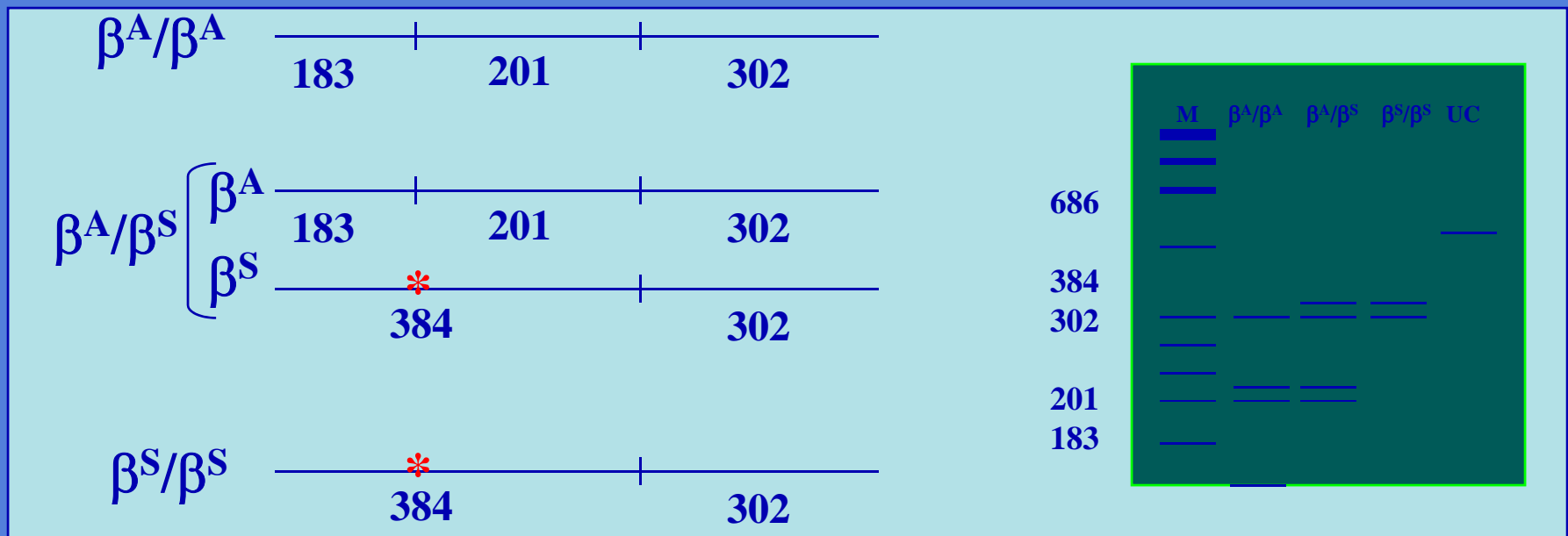
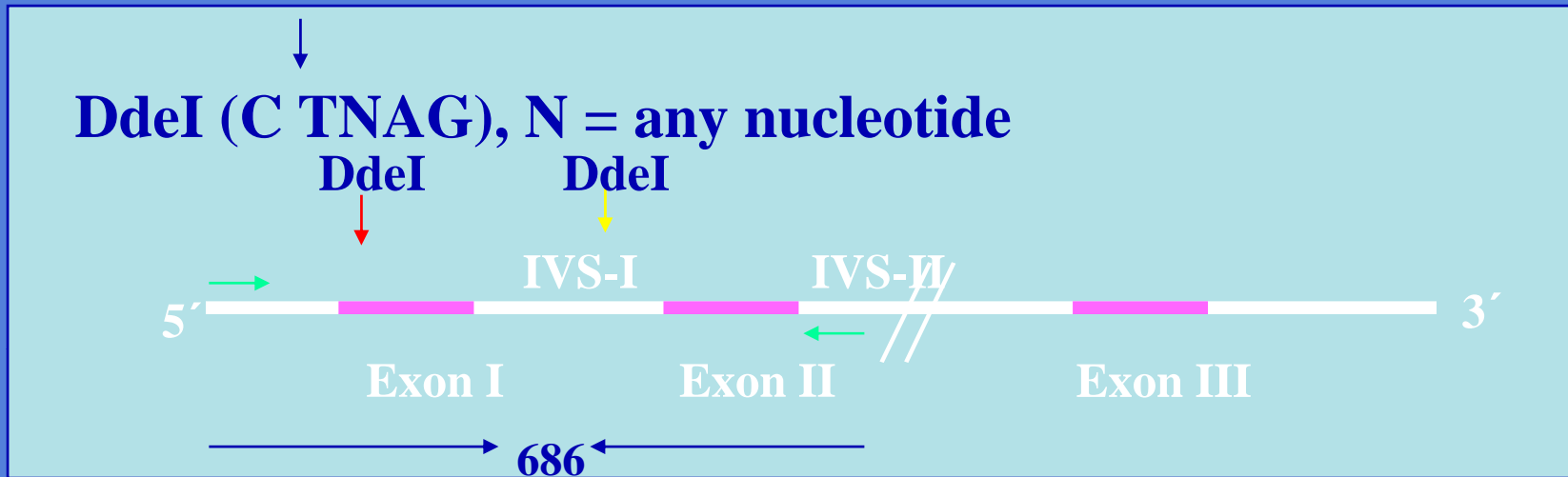


Restriction by DdeI



Analysis on Agarose gel electrophoresis

HbS (A-T) Detection by Restriction Analysis



Serial #	Mutations	Rawalpindi Islamabad	Faisalabad Lahore Sargodha	D.G.Khan Bahawalpur Multan	TOTAL
01	IVS-I-5, G→C	171 (33.8%)	185 (42.1%)	88 (45.0%)	444 (38.9%)
02	Codons 8/9, +G	175 (34.8%)	177 (40.35%)	72 (36.6%)	424 (37.3%)
03	Codons 41/42, -TTCT	76 (15.0%)	33 (7.4%)	12 (5.9%)	122 (10.6%)
04	619 bp deletion	9 (1.8%)	12 (2.5%)	2 (0.7%)	23 (2.0%)
05	IVS-I-1, G→T	9 (1.8%)	3 (0.6%)	10 (5.2%)	22 (1.9%)
06	Codon 15, G→A	9 (1.8%)	6 (1.1%)	6 (3.3%)	21 (1.8%)
07	Codon 5, -CT	8 (1.5%)	5 (1.1%)	3 (1.3%)	15 (1.3%)
08	IVS-I-1, G→A	8 (1.5%)	7 (1.7%)	-	15 (1.3%)
09	Codon 16, -C	10 (2.0%)	-	1 (0.6%)	11 (1.0%)
10	IVS-II-1, G→A	5 (1.0%)	4 (0.9%)	-	9 (0.8%)
11	Codon 30, G→C	7 (1.3%)	-	1 (0.6%)	8 (0.7%)
12	Codon 39, C→T	4 (0.8%)	-	-	4 (0.3%)
13	Codon 26, G→A	3 (0.5%)	-	-	3 (0.2%)
14	Codon 30, G→A	1 (0.2%)	-	-	1 (0.1%)
15	Initiation Codon, T→C	1 (0.2%)	-	-	1 (0.1%)
16	Cap+1, A→C	1 (0.2%)	-	-	1 (0.1%)
17	-88, C→T	1 (0.2%)	-	-	1 (0.1%)
18	IVS-II-848, C→A	-	-	-	-
19	Codons 47/48 +ATCT	-	-	-	-
20	Hb D, G→C	1 (0.2%)	4 (0.9%)	-	5 (0.4%)
21	Hb S, A→T	3 (0.5%)	-	1 (0.6%)	4 (0.3%)
22	Hb E Codon 26, G→A	-	3 (0.6%)	-	3 (0.1%)
	Uncharacterized	3 (0.5%)	3 (0.6%)	-	6 (0.5%)
	Total	505	439	196	1140

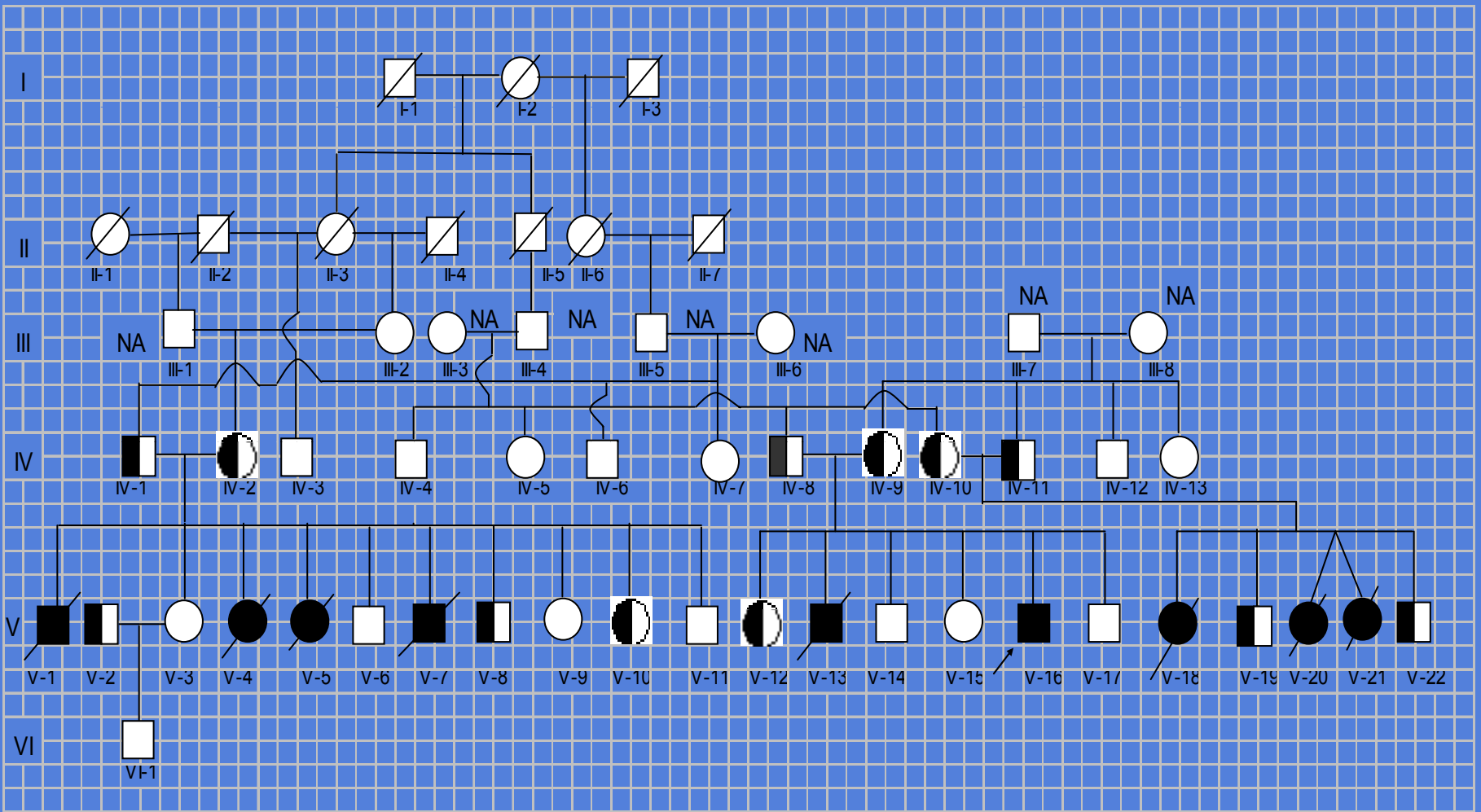
**CASCADE TESTING
AND
GENETIC COUNCELLING**

Retrospective Inductive/Cascade Screening

An affected child is a marker of a group (family/*biradari*) at higher risk of disease or more affected births in the family in future.

Due to limited national resources it is not possible to screen the whole Pakistani population for β -thalassemia or any other genetic disorder

- Large consanguineous families with at least one transfusion dependent child (index case/proband) screened for β -thalassemia.
- To identify carriers of β -thalassemia in consanguineous extended families to provide genetic counseling for prevention of disease.
- Families identified through an index case show more than 40% allele frequency (5.6% in the general Pakistani population).
- Screening of extended families is a more practical and cost effective way of establishing DNA based genetic testing to control recessive single gene disorders in communities like Pakistan in which consanguineous marriages are common.



Inductive/Cascade Screening through index case V-16

Baig et al., 2008. Community Genetics

Genetic Counseling

The process of providing individuals and families with information on the:

- Nature of disease
- Mode of Inheritance
- Implications of genetic disorders

To help them make informed medical and personal decisions

- How to live with the genetic disease and
- How to prevent the genetic disease

This is how the affected families are helped in the developed countries and Pakistan

PRENATAL DIAGNOSIS

What is Prenatal Diagnosis?

Prenatal Diagnosis is performed during the pregnancy to examine whether or not a fetus is at risk for various genetic disorders.

CAN BE PERFORMED EITHER BY

- Ultrasonography
- Amniocentesis
- Chorionic villus sampling
- Fetal blood cells in maternal blood
- Maternal serum alpha-fetoprotein
- Maternal serum beta-HCG
- Maternal serum estriol

- Amniocentesis and chorionic villus sampling (CVS) are tests that help find genetic disorders before birth.
- Some parents are at increased risk of having a baby with a genetic disorder or other problem and may want to have one of these tests.
- Knowing about problems before the baby is born may help parents make decisions about health care for their infant.
- Certain problems can be treated before the baby is born, while other problems may need special treatment right after delivery.
- In some cases, parents may decide not to continue the pregnancy.

How is CVS performed?

- CVS is performed by removing a small sample of the placenta (nourishment for the baby) from the uterus.
- It is removed with either a catheter (a thin tube) or a needle. Local anesthesia is used for this test.
- The sample of placenta may be obtained through the cervix.
- A catheter is inserted into the vagina and through the cervix and the sample is withdrawn.
- The sample also can be obtained by inserting a needle into the abdomen and withdrawing some of the placenta.

Uterus

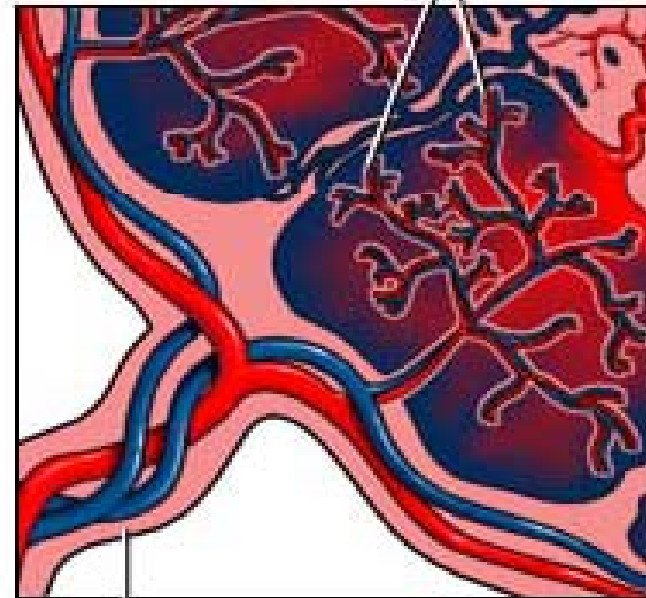
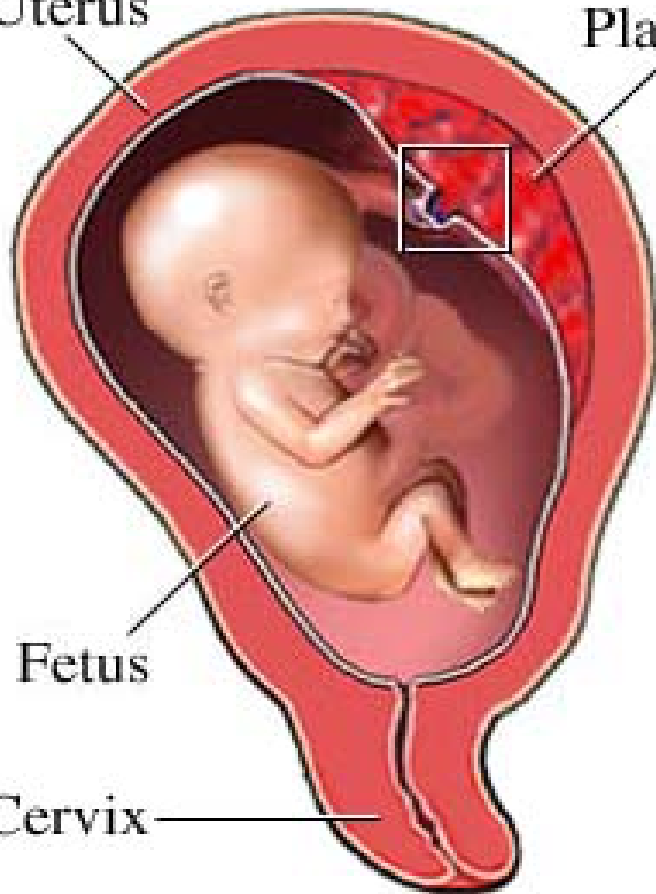
Placenta

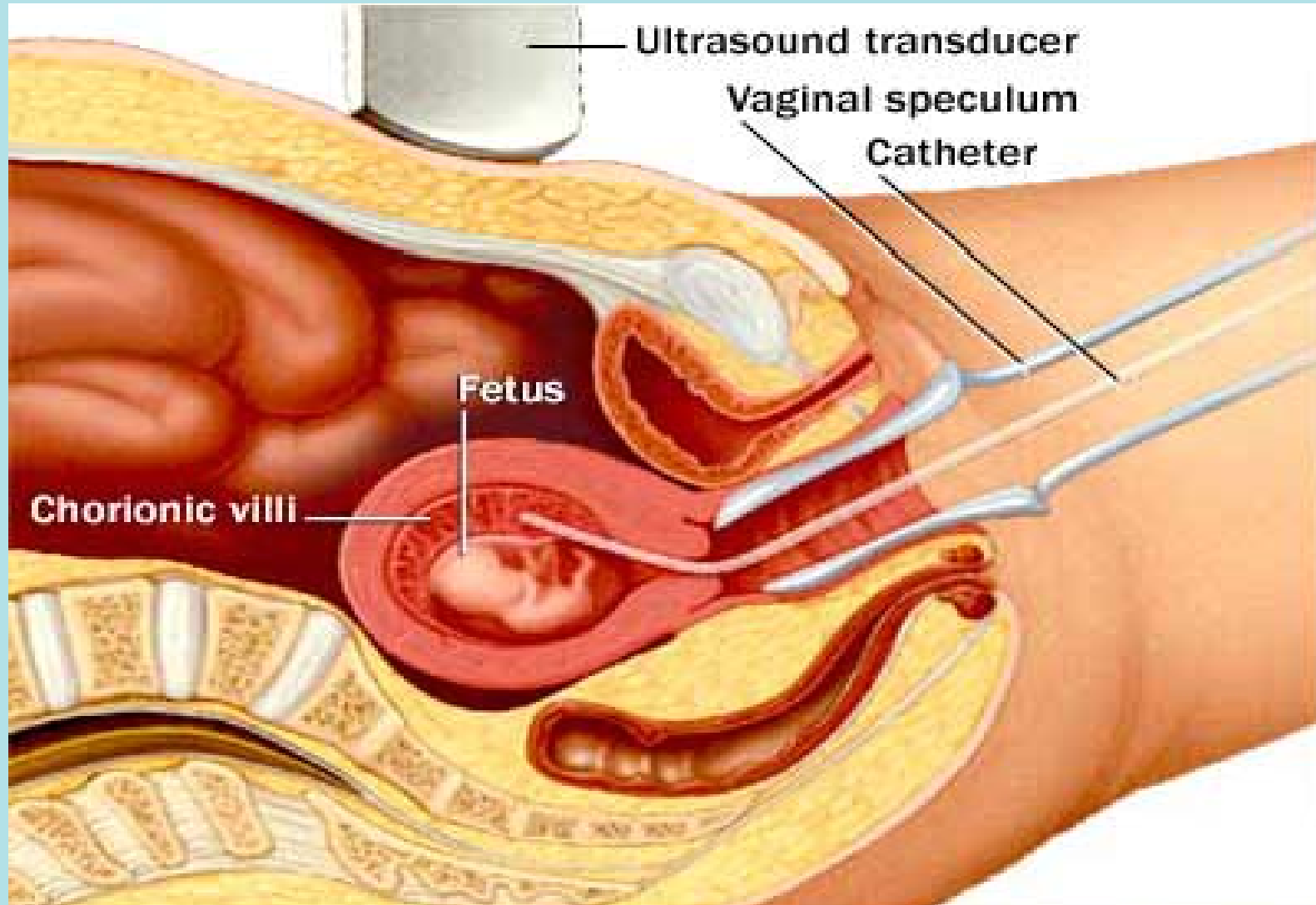
Chorionic villi

Fetus

Cervix

Umbilical cord





Ultrasound guided Chorionic villus sampling (CVS)

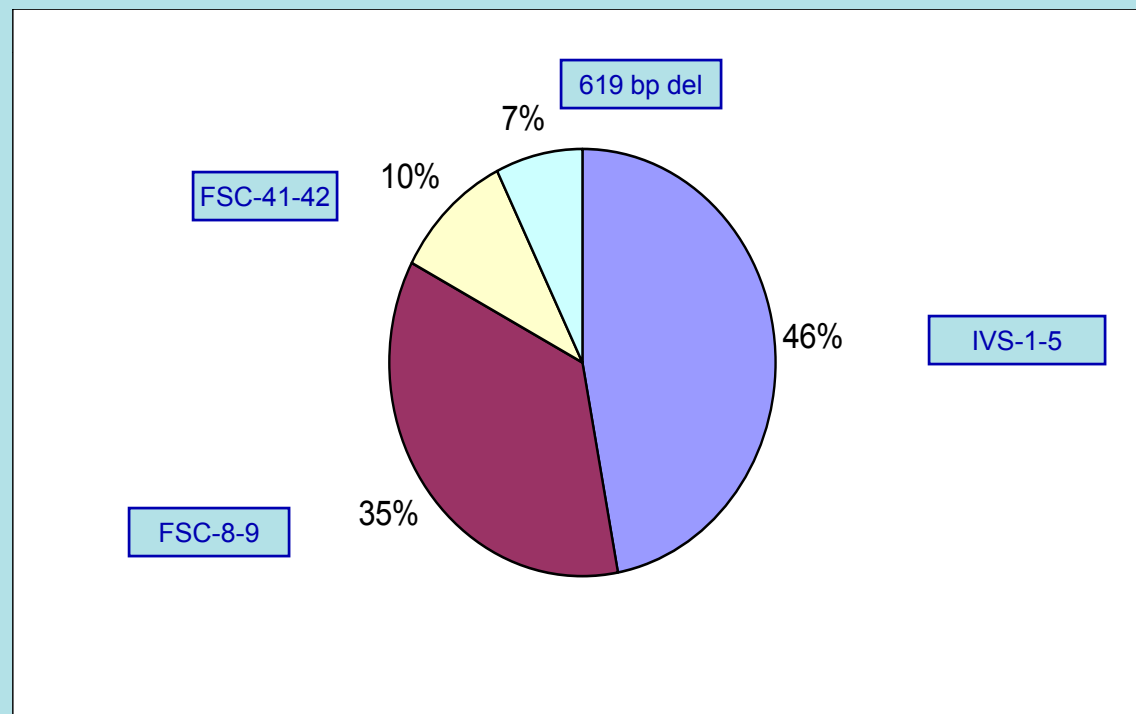
Subjects for prenatal diagnosis

- Limitation for PD: CVS expertise
- 32 Prenatal diagnoses for Fatmid Foundation Multan through MINAR(Multan Institute of Nuclear Medicine and Radiotherapy) in the last two months
 - CVS (Chorionic villus sample) collected by Director MINAR
 - Genotyping for Prenatal Diagnosis at HMG NIBGE
- Prenatal diagnosis reporting within two days of the receipt of the CVS

Representative Prenatal Diagnoses at NIBGE

DIAGNOSTIC APPROACH	PARENTAL GENOTYPE (F/M)	FETAL GENOTYPE	FETAL PHENOTYPE	RESULTS
ARMSPCR	-619bp/-619bp	-619bp/-619bp	Affected	Pregnancy terminated
ARMSPCR	IVS-I-5/IVS-I-5	IVS-I-5/IVS-I-5	Affected	Pregnancy terminated
ARMSPCR	FSC-8-9/FSC-8-9	FSC-8-9/N	Carrier	Pregnancy continued
ARMSPCR	FSC-5(-CT)/ FSC-5(-CT)	N/N	Normal	Pregnancy continued
ARMSPCR	Cd-15/FSC-8-9	FSC-8-9/N	Carrier	Pregnancy continued
ARMSPCR	IVS-I-I(G-T)/IVS-I-5	IVS-I-I(G-T)/IVS-I-5	Affected	Pregnancy terminated
ARMSPCR	FSC-8-9/FSC-8-9	FSC-8-9/N	Carrier	Pregnancy continued
ARMSPCR	Cd-41-42/Cd-41-42	Cd-41-42/N	Carrier	Pregnancy continued
ARMSPCR	FSC-8-9/FSC-8-9	FSC-8-9/FSC-8-9	Affected	Pregnancy terminated
ARMSPCR	Cd-41-42/IVS-I-5	IVS-I-5/Cd-41-42	Affected	Pregnancy terminated
ARMSPCR	IVS-I-5/IVS-I-5	IVS-I-5/N	Carrier	Pregnancy continued
ARMSPCR	Cd-41-42/FSC-8-9	Cd-41-42/FSC-8-9	Affected	Pregnancy terminated
ARMSPCR	IVS-I-5/IVS-I-5	IVS-I-5/N	Carrier	Pregnancy continued

FREQUENCY OF MUTATIONS FOUND IN CVS



Are there risks involved with these tests?

Amniocentesis and CVS carry 1 to 5% risk of miscarriage.

**For establishment of Non-invasive prenatal
Diagnosis of β -thalassemia major through
Maternal blood in compound heterozygote
couples**

Collaborating with:
Prenatal Medicine and Gynaecological
Oncology Laboratory, Women's hospital
Basel University, Switzerland.

THANK YOU