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NHS
University Hospitals Birmingham
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Laboratory Investigations of Haemoglobinopathies

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Background

- Haemoglobinopathies are a heterogeneous group of more than 1,000 mutations.

- Characterised into 2 main groups

1. Haemoglobin variants, arise from an alteration in the globin protein structure e.g Hb S, HbC

2. Thalassaemias, arise from inadequate production of structurally normal globin e.g alpha thalassaemia, beta thalassaemia

There are also thalassaemic haemoglobinopathies that are produced when a structurally abnormal haemoglobin is synthesised at a reduced rate e.g. HbE

Haemoglobin

- **haemoglobin** is a tetramer
- **haemoglobin** is the oxygen binding protein of red blood cells and is a globular protein.
- **haemoglobin** consists of four polypeptide subunits; 2 α chains and 2 non α

Adult Hb (Hb A) 2 α and 2 β subunits

- **HbA_1** is the major form of Hb in adults and in children over 7 months.
- **HbA_2** (2 α, 2 δ) is a minor form of Hb in adults. It forms only 2 – 3% of a total Hb A.

Fetal Hb (Hb F) = 2 α and 2 γ subunits in fetus and newborn infant,

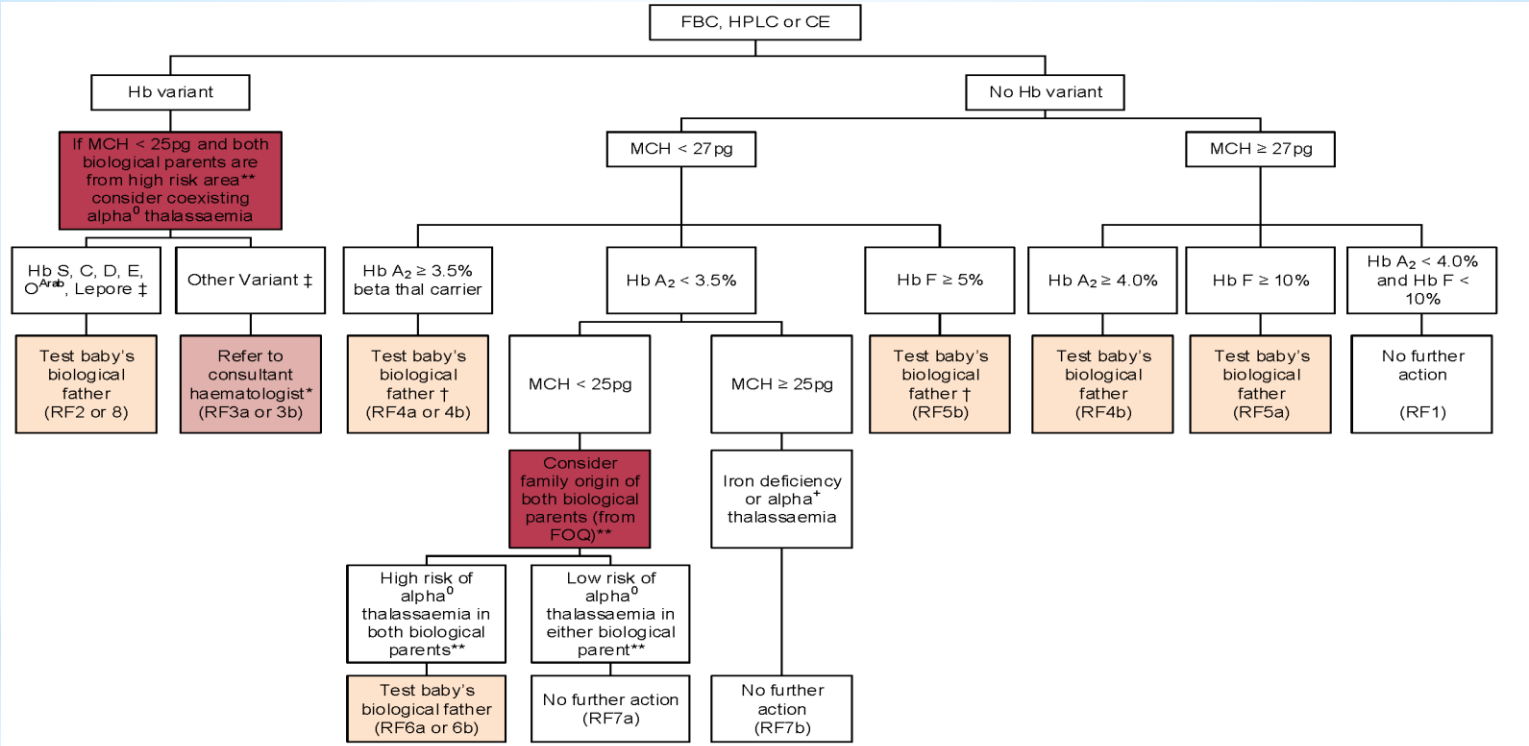
After birth, Hb F is replaced by Hb A during the first few months of life.

Workload at UHB NHS Foundation Trust

- Perform total of 18,000 haemoglobinopathy screens per year
  - 11,000 antenatal haemoglobinopathy screens (HGS)
  - 7,000 non antenatal screens i.e. GP's, OPD, In Patients (HGS and QEH)
    - Pre op
    - Family history of haemoglobinopathy
    - Unexplained anaemia
    - FBC results suggestive of a haemoglobinopathy – no reflex testing.

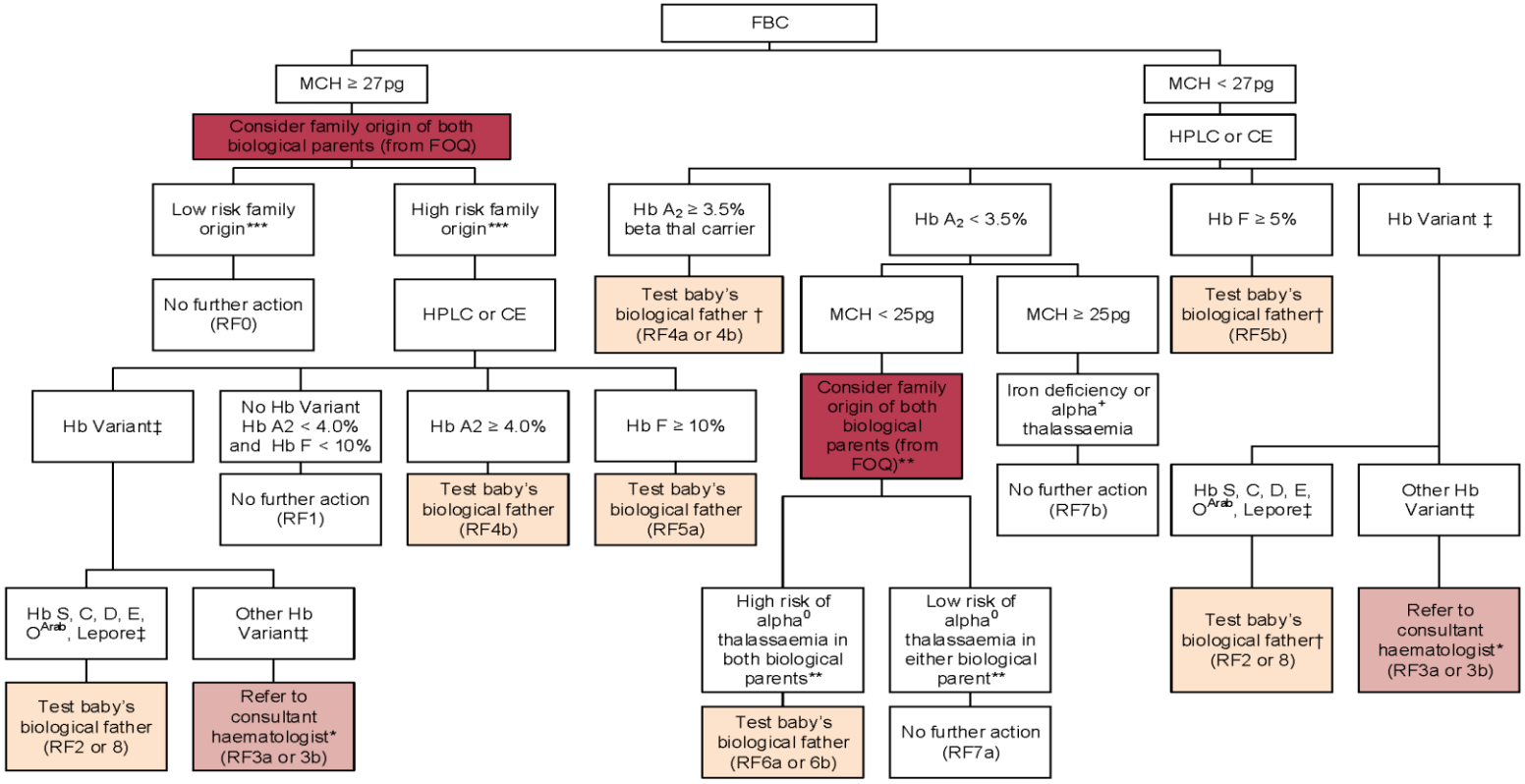
Antenatal screening

- **High prevalence** – Trusts are considered high prevalence if 2% or more of the booking bloods are screen positive. The high prevalence algorithm is used for interpretation.



* Refer analytical results to consultant for an opinion on the need for a clinical referral or consult the laboratory support service helpline.
** Consider at high risk if any ethnic origins in China (including Hong Kong), Taiwan, Thailand, Cambodia, Laos, Vietnam, Indonesia, Burma, Malaysia, Singapore, Philippines, Cyprus, Greece, Sardinia, Turkey, or if ethnic/family origin is uncertain or unknown.
Reconsider low risk couples if fetal anaemia/hydrops seen on ultrasound scanning or if family history of hydrops fetalis.
† In all cases consider coexisting α^0 thalassaemia if both parents are from a high risk area and MCH <25pg.
‡ Consider coexisting beta thalassaemia

- **Low prevalence** - Trusts are considered low prevalence where <1% of the booking bloods received by the laboratory are screen positive. The low prevalence algorithm is used for interpretation.



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*** Low risk or high risk as determined by the family origin questionnaire. **Note: If baby's father is in high risk group, test the mother's sample regardless of her family origins.**
† In all cases consider coexisting α^0 thalassaemia if both parents are from a high risk area and MCH <25pg.
‡ Consider co-existing beta thalassaemia

Laboratory Investigations.

1) Full Blood Count

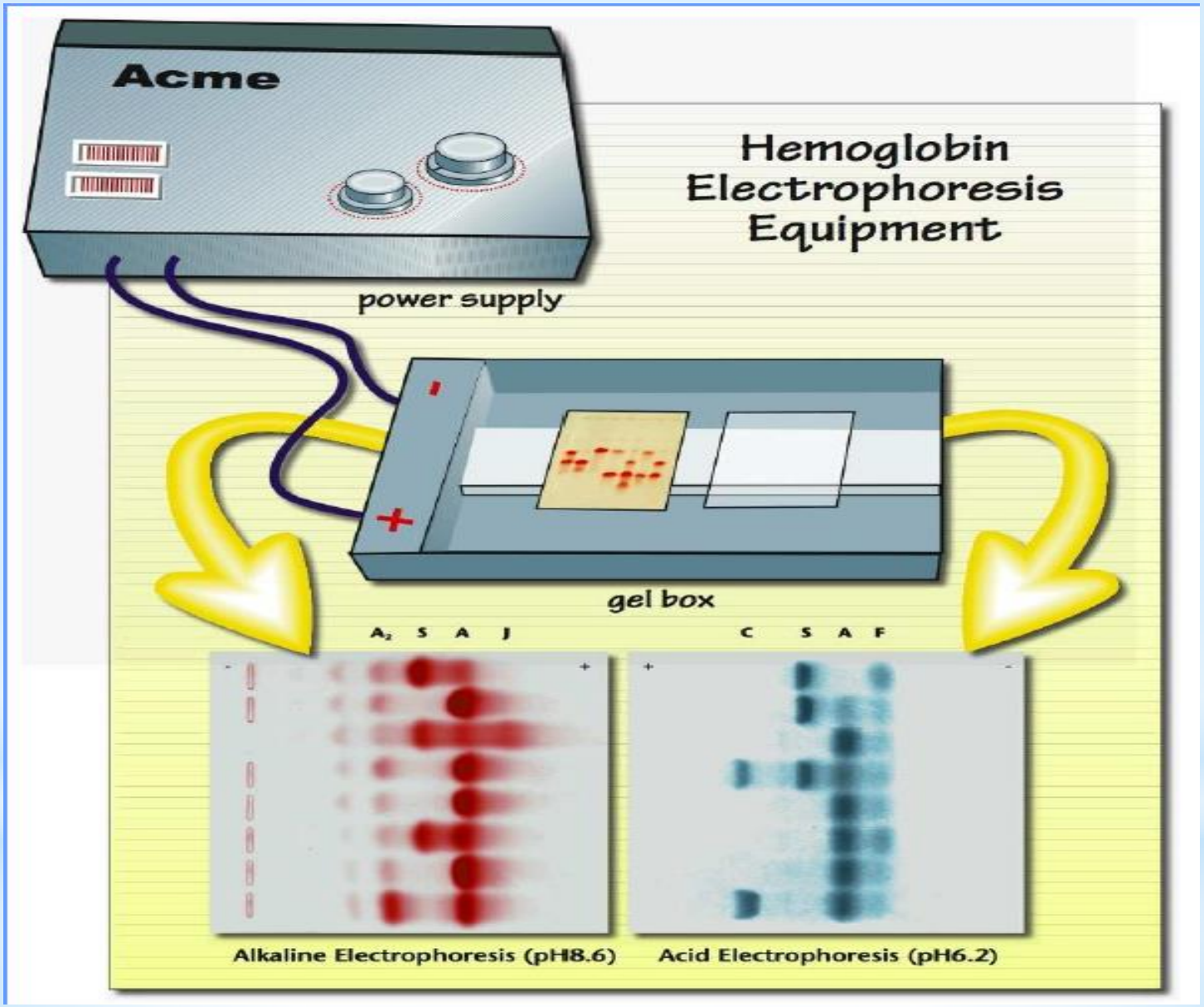
Presumptive HPLC Diag	Hb (g/dl)±SD	MCV (fl)±SD	MCH (pg)±SD	MCHC (%)±SD	RBC count (×106/µl)±SD	RDW±SD
β-Thal trait	9.8±2.4	68.5±6.2	21.3±2.6	28.3±1.8	5.06±0.9	15.4±6.1
β-Thal major	5.6±1.5	54.9±6.5	16.8±3.6	26.3±2.9	2.5±0.8	31.5±5.3
β-ThalIntermedia	8.6±1.7	62.2±4.5	18.2±2.3	27.0±2.2	2.9±0.7	30.7±4.3
Sickle cell trait	11.6±1.8	84.9±3.4	27.3±2.1	31.7±2.3	4.45±0.54	16.2±4.4
HbS/β-thal	7.8±1.5	70.2±5.0	21.5±2.3	30.5±2.1	3.67±0.6	17.1±3.8
Sickle cell disease	8.3±1.7	91.2±0.9	28.9±1.1	32.1±1.7	3.4±1.5	20.4±2.1
HbE-trait	11.8±1.4	84.2±2.6	26.8±2.1	32.5±1.2	4.1±2.2	12.4
HbE/β-thal	7.2±1.1	64.5±5.2	18.9±2.1	29.2±1.5	3.1±0.8	15.5
Homozygous HbE disease	10.5±1.2	70.2±0.8	20.2±1.0	32.1±0.8	4.1±0.9	10.2
HbD-Punjab trait	10.3±2.6	85.5±4.5	22.4±1.4	29.2±2.1	3.8±1.9	15.9
HPFH	11.2±2.1	74.3±3.3	23.8±1.5	32.7±2.2	4.59±2.2	12.1
Hb Q India trait	11.6±1.6	81.3±3.1	26.8±0.6	32.4±0.9	4.6±0.3	13.2

MCV-Mean corpuscular volume; MCH- Mean corpuscular hemoglobin

Indian Journal of Pathology and Microbiology
Year : 2013 | Volume : 56 | Issue : 2 | Page : 109-113

2) **Haemoglobin Electrophoresis (Alkaline/Acid gel Electrophoresis)**

- Haemoglobin electrophoresis is the movement of haemoglobin proteins in an electric field at a fixed pH.
- Since different types of haemoglobin molecules are comprised of different combinations of globin chains (normal or abnormal), they will demonstrate different degrees of mobility.
- Typically, an alkaline electrophoresis is performed which may be confirmed with acid electrophoresis as several Hb variants comigrate together.
- The proteins are visualized by the application of a dye.


Acme
Hemoglobin Electrophoresis Equipment
power supply
gel box
A2 S A J
C S A F
Alkaline Electrophoresis (pH8.6)
Acid Electrophoresis (pH6.2)

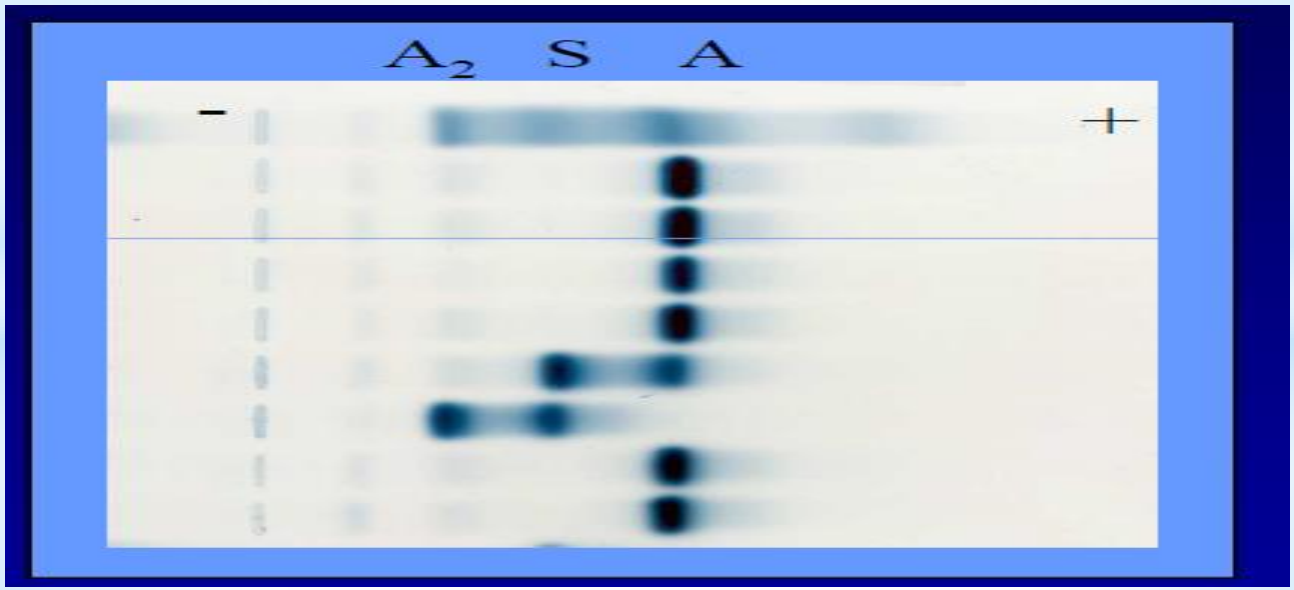
Alkaline Electrophoresis

- pH 8.6

- Cellulose acetate is support medium

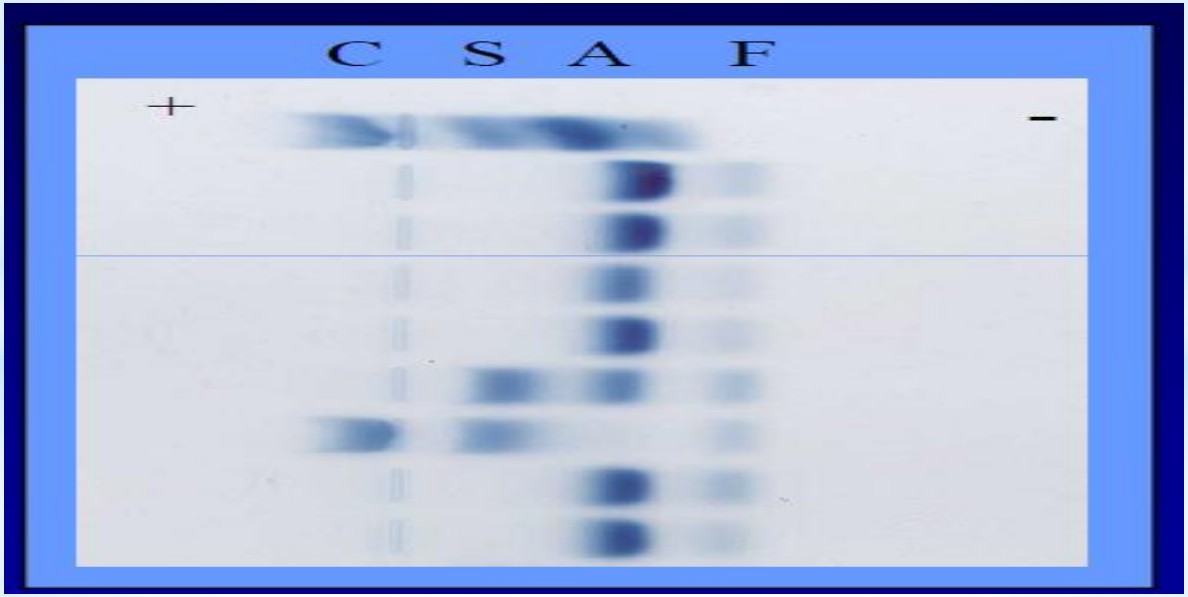
- Hemoglobin molecule is negatively charged and will migrate from the cathode toward anode

- Method is based on charge differences



Acid Electrophoresis

- Citrate buffer- pH 6.2
- Agar is support medium, “Citrate Agar”
- Agaropectin combines with some variants to alter mobility compared to Hb A
- Useful for confirmation of Hb S, C, E



Advantages

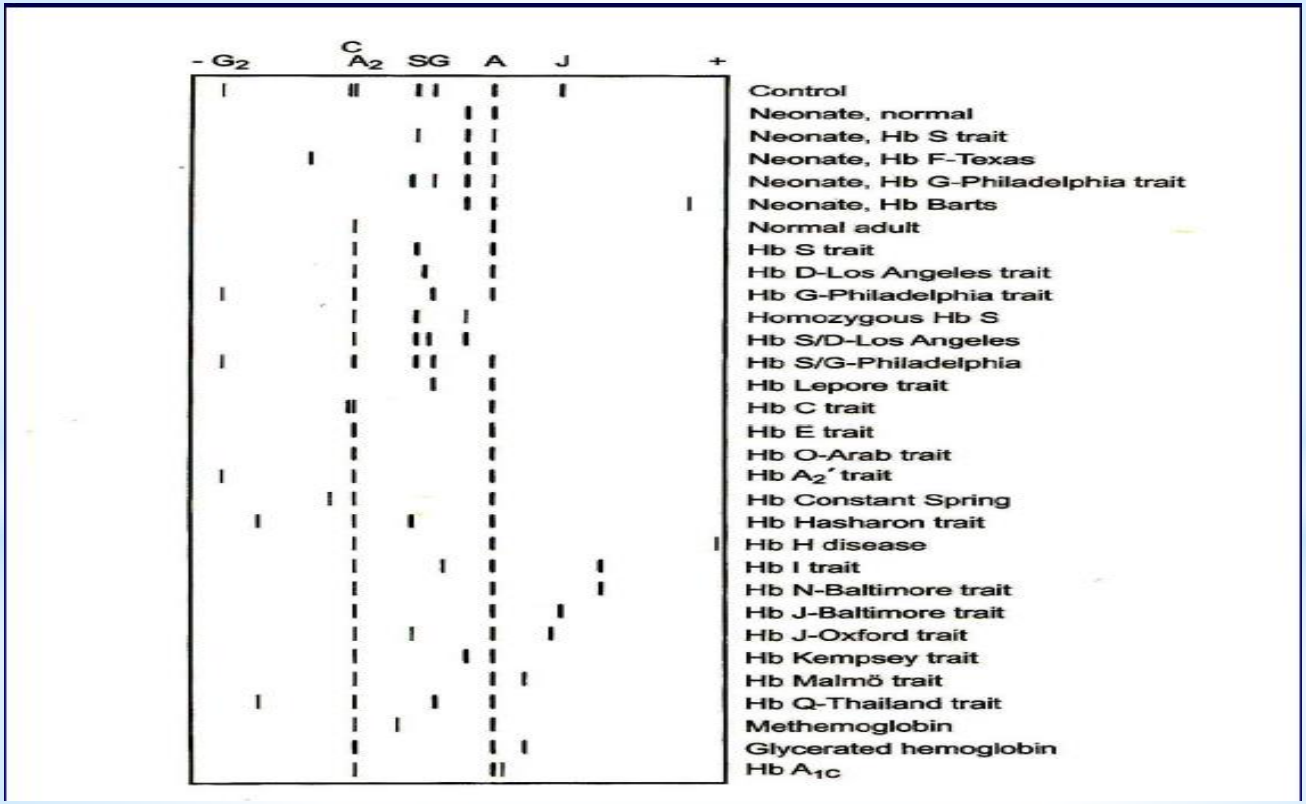
- Inexpensive, user friendly systems available
- Satisfactory to confirm Hb's S, C and E

Disadvantages

- Specimens must be batched, long run times
- Hb A2 quantitation is imprecise
- Many variants show similar mobility, or do not separate from Hb A

3) **Isoelectric Focussing**

- Utilizes carrier ampholytes to establish a pH gradient throughout the medium (agarose)
- pH range usually 6-8
- Hb fractions will travel to their isoelectric point and stop



Advantages

• Better separation of Hb variants that show similar mobility's on alkaline electrophoresis, but still many variants have similar mobility's

• Better separation of rarer variants from Hb A

• Minor bands may be seen more easily (Hb H, Hb Bart's, and delta chain variants)

Disadvantages

• Long run times, samples must be run in batches

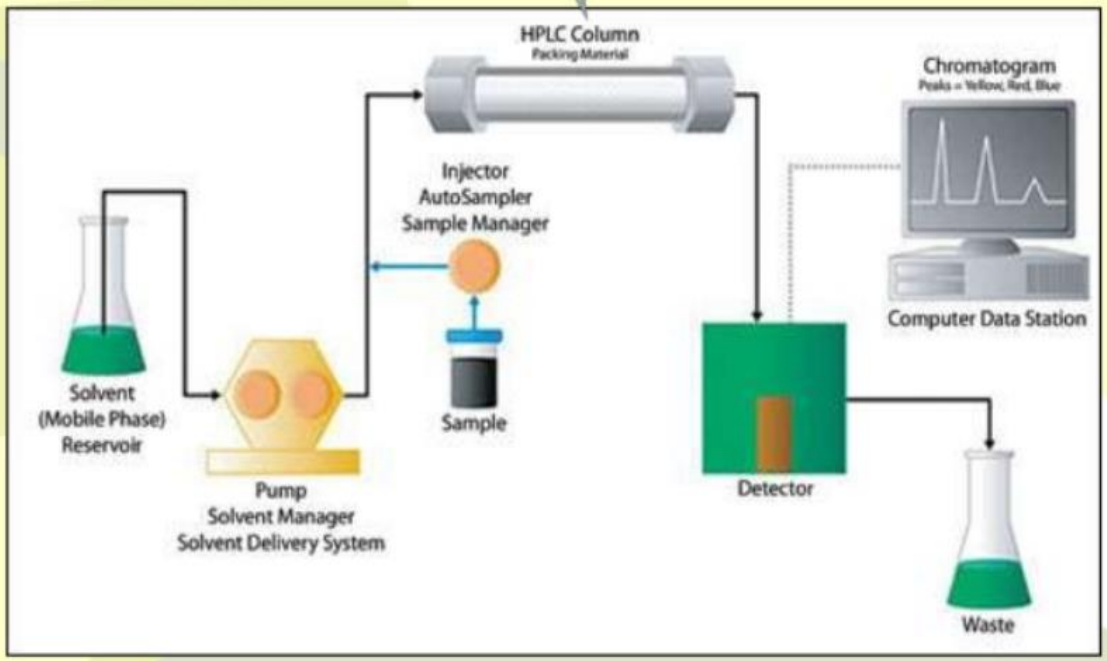
• Many minor bands (degradation, aging, glycosylated)

• Difficult to quantitate off IEF

4) **HPLC (High Performance Liquid Chromatography)**

- Allows Haemoglobin separation using a cation exchange non porous polymer column (stationary phase),
- Hb is +ve charged and adsorbs onto –ve charged column.
- The elution buffers (mobile phase) is a liquid with an increasing concentration of cations flowing through the column that cause the elution of the Hb's off the column at a rate related to their affinity to the stationary phase.
- The eluted Haemoglobins are detected optically and provisionally identified by the retention time and quantitated by computing the area under the corresponding peak.

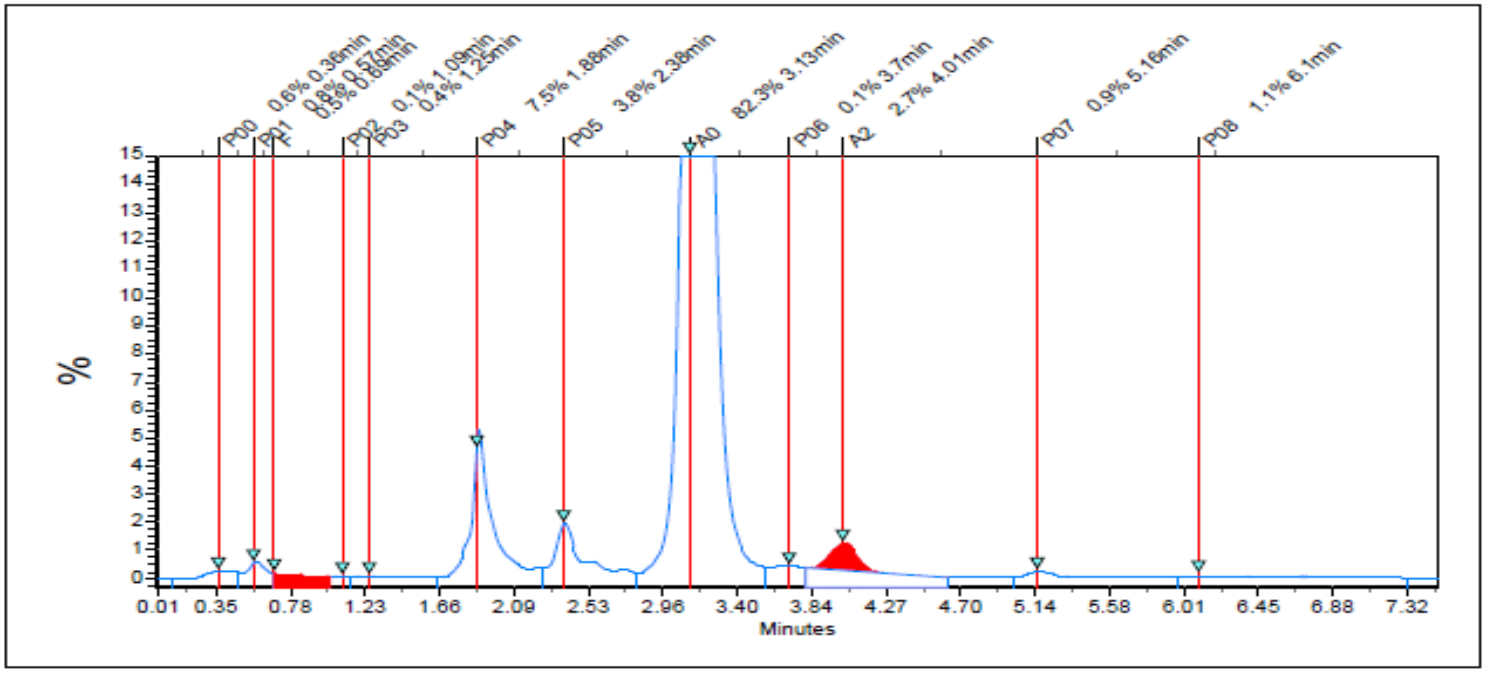
Ion-exchange HPLC



CATION-EXCHANGE HPLC

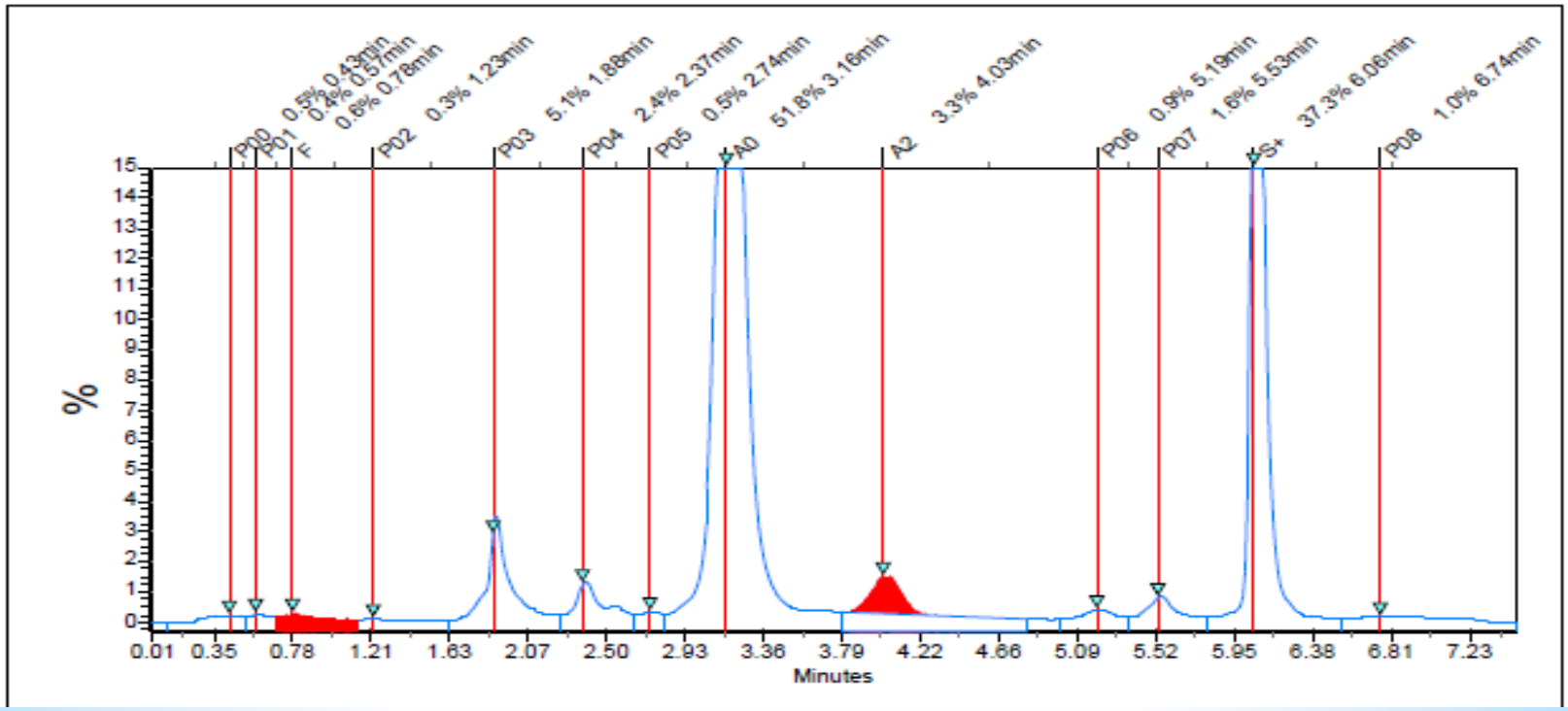
Parameter	Value %	Time min.	Area	Total Area
P00	0.6	0.36	7.3	1,169.6
P01	0.8	0.57	8.8	
F	0.5	0.69	5.7	
P02	0.1	1.09	1.3	
P03	0.4	1.25	4.3	
P04	7.5	1.88	87.3	
P05	3.8	2.38	44.8	
A0	82.3	3.13	962.8	
P06	0.1	3.7	1.7	
A2	2.7	4.01	22.6	
P07	0.9	5.16	10.3	
P08	1.1	6.1	12.6	

Normal Patient
(TOSOH G7)



Parameter	Value %	Time min.	Area	Total Area
P00	0.5	0.43	6.6	1,370.2
P01	0.4	0.57	5.2	
F	0.6	0.78	8.3	
P02	0.3	1.23	4.7	
P03	5.1	1.88	69.4	
P04	2.4	2.37	33.2	
P05	0.5	2.74	6.9	
A0	51.8	3.16	709.7	
A2	3.3	4.03	32.4	
P06	0.9	5.19	12.8	
P07	1.6	5.53	22.5	
S+	37.3	6.06	445	
P08	1.0	6.74	13.7	

Sickle Cell Carrier (TOSOH G7)



Advantages

- high precision
- high throughput
- automation
- short run times (6 mins)
- computer controlled
- small volume of blood required (4µl whole blood)

Disadvantages

- Hb A2 cannot be quantitated in the presence of Hb E
- Many variants show similar retention times

5) **Sickle Solubility Test.**

Based on the relative insolubility of sickling haemoglobin when exposed to a reducing agent ie sodium hydrosulfite.

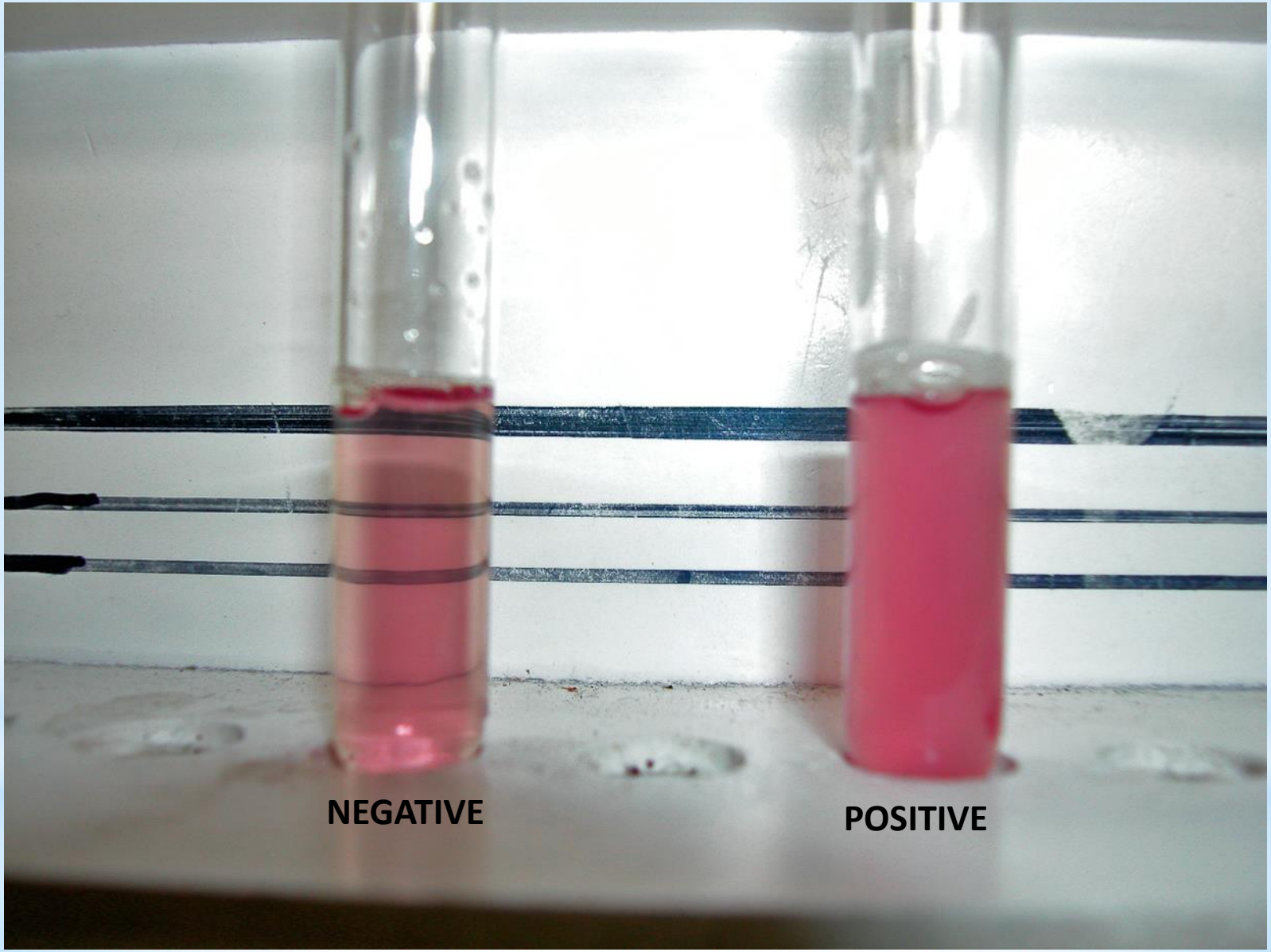
Limitations include

- not able to detect Hb S levels below 10% often seen in neonates

- False negatives seen when reducing agent is not mixed correctly or expired, or low Hb or transfused patients.

- False positives seen when excess paraproteins found in sample **(ALWAYS WASH RED CELLS)**

- Does not distinguish Hb S trait from Homozygous HbS.


NEGATIVE
POSITIVE

6) Capillary Electrophoresis (CE)

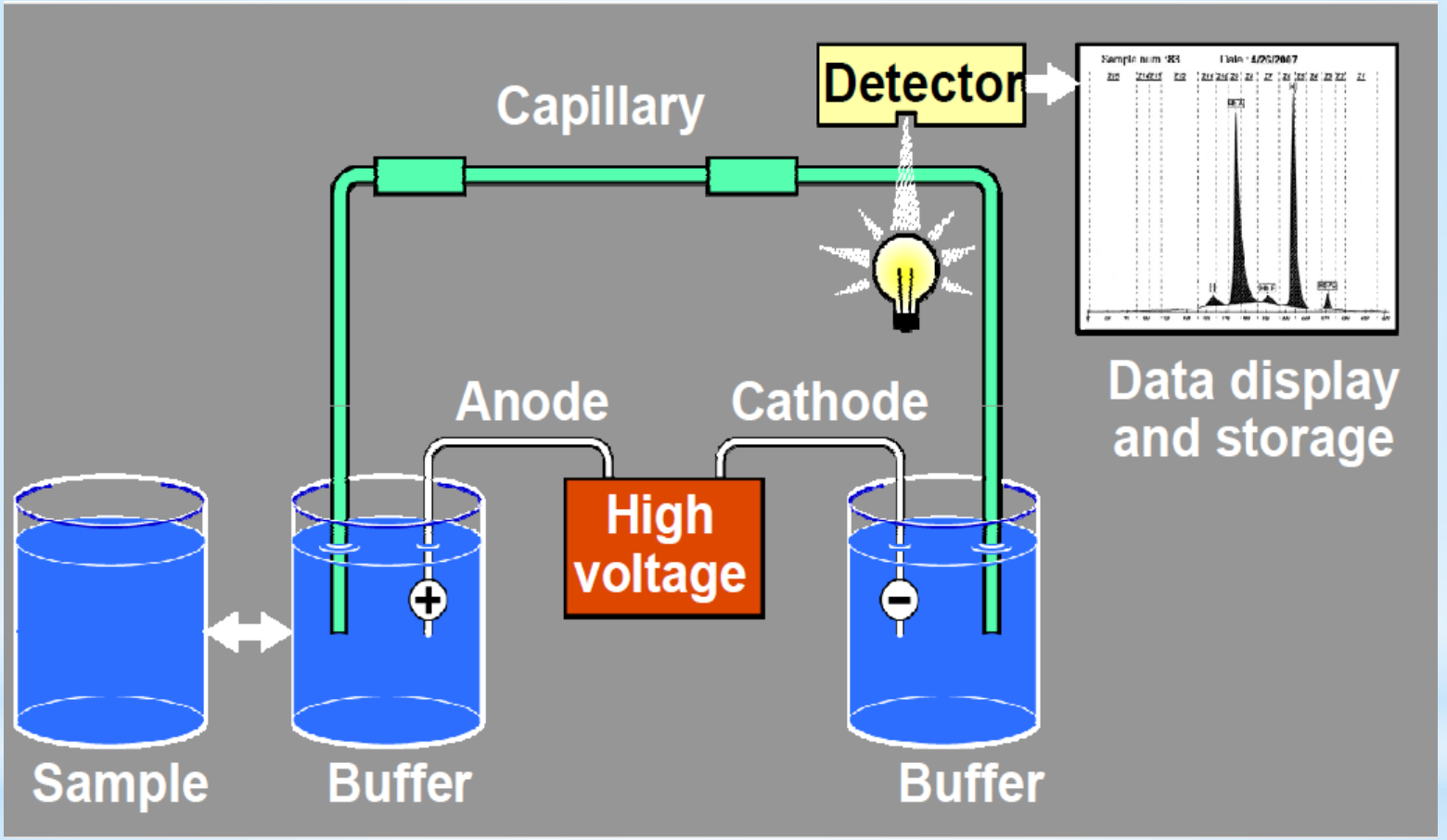
- Instrumentation has been around since early 1990's
- Utilizes very long thin capillary (100 µm diameter)
- Excellent dissipation of heat so can use very high voltages (10,000V), thus greater resolution
- Multiple capillaries can be run in parallel (12), this reduces run times.

- Charged molecules are separated by their electrophoretic mobility at a specific pH in an alkaline buffer. Separation occurs according to the electrolyte pH and electro osmotic flow.

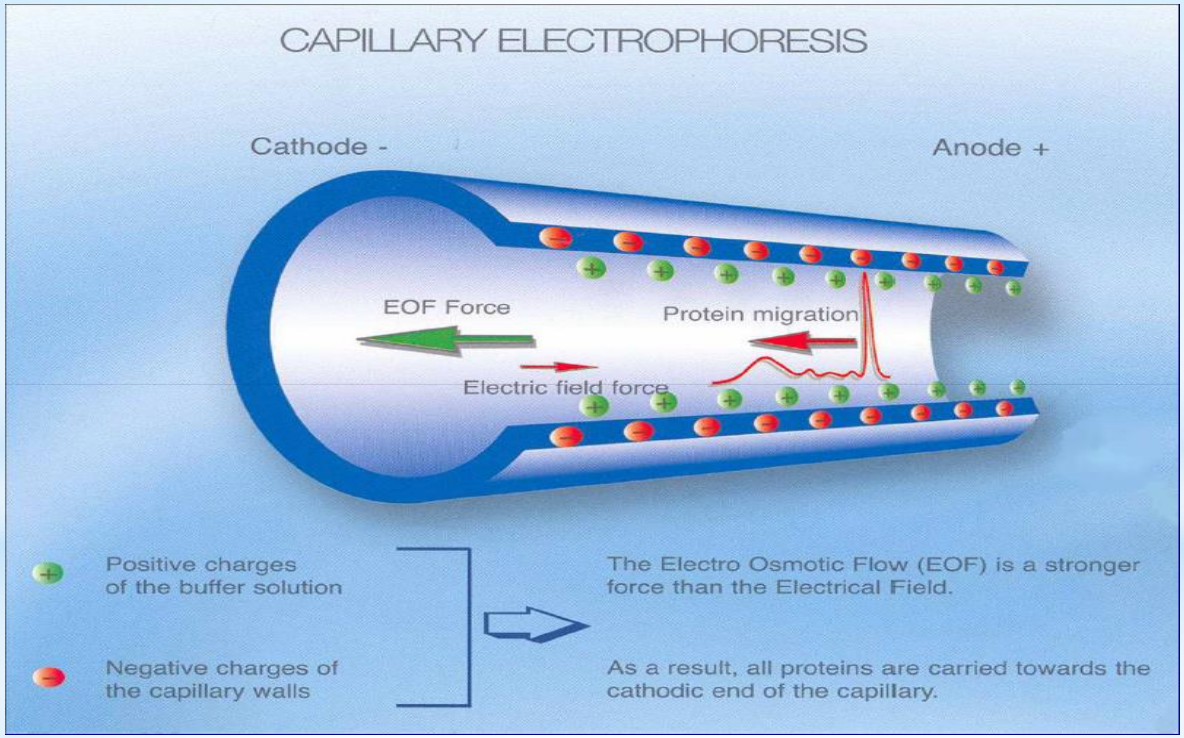
- Each sample is diluted in a dilution buffer and the capillaries are filled with the separation buffer; samples are then injected by aspiration into the anodic end of the capillary.

- A high voltage protein separation is then performed ; direct detection and quantification of the different haemoglobin fractions is performed at a specific wavelength at the cathodic end of the capillary.

- Post analysis, the capillaries are immediately cleaned with a wash solution and then refilled with buffer in preparation for the next samples.

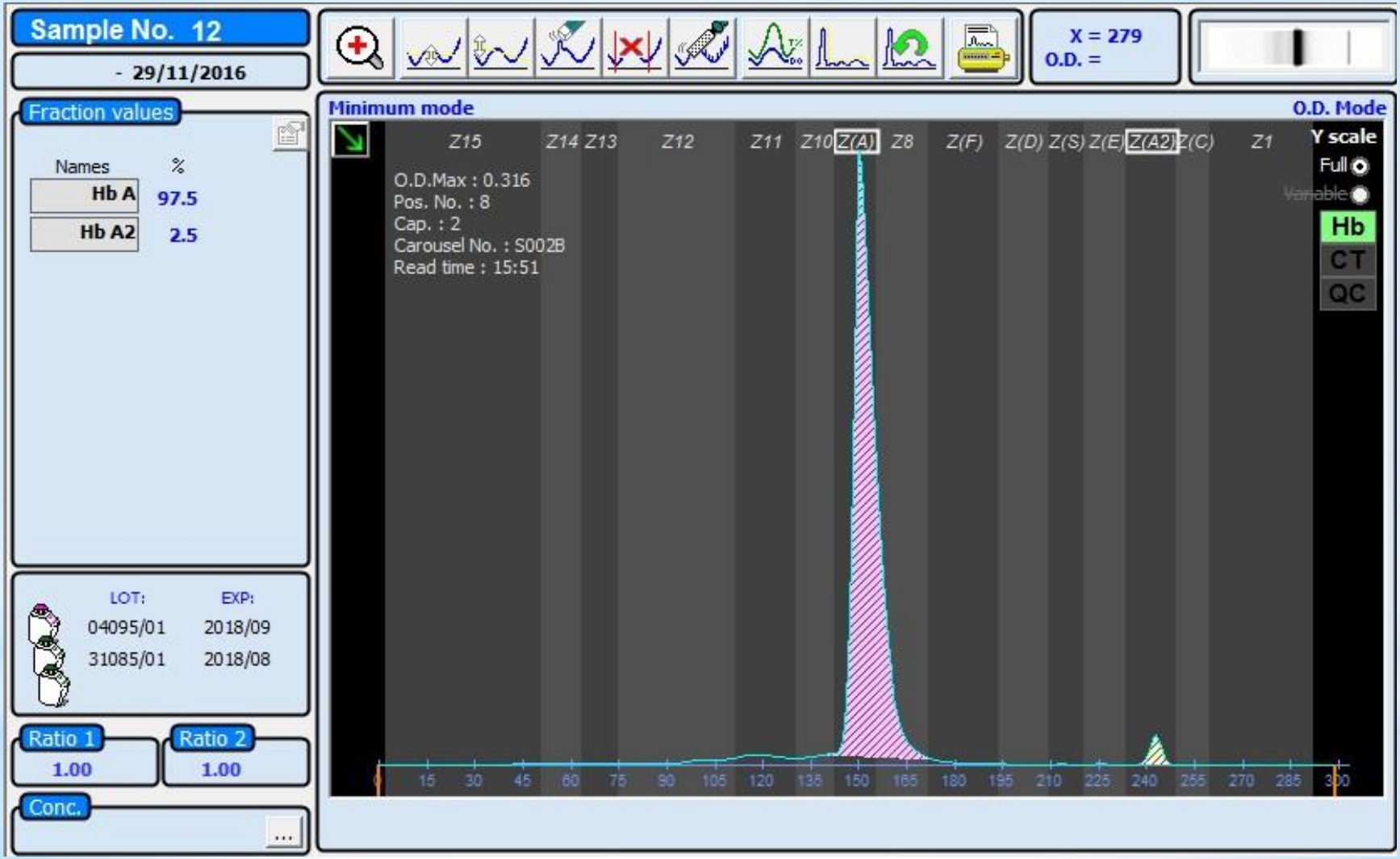

Capillary
Detector
Data display
and storage
Anode
Cathode
High
voltage
Sample
Buffer
Buffer

- The silica capillary has a negative charge on its inner surface
- Electro-osmotic force (EOF) – flow of the buffer solution towards the cathode
- Haemoglobin fractions will separate out based on their affinity for the positive or negative pole, but overall are still carried towards the cathode due to the EOF

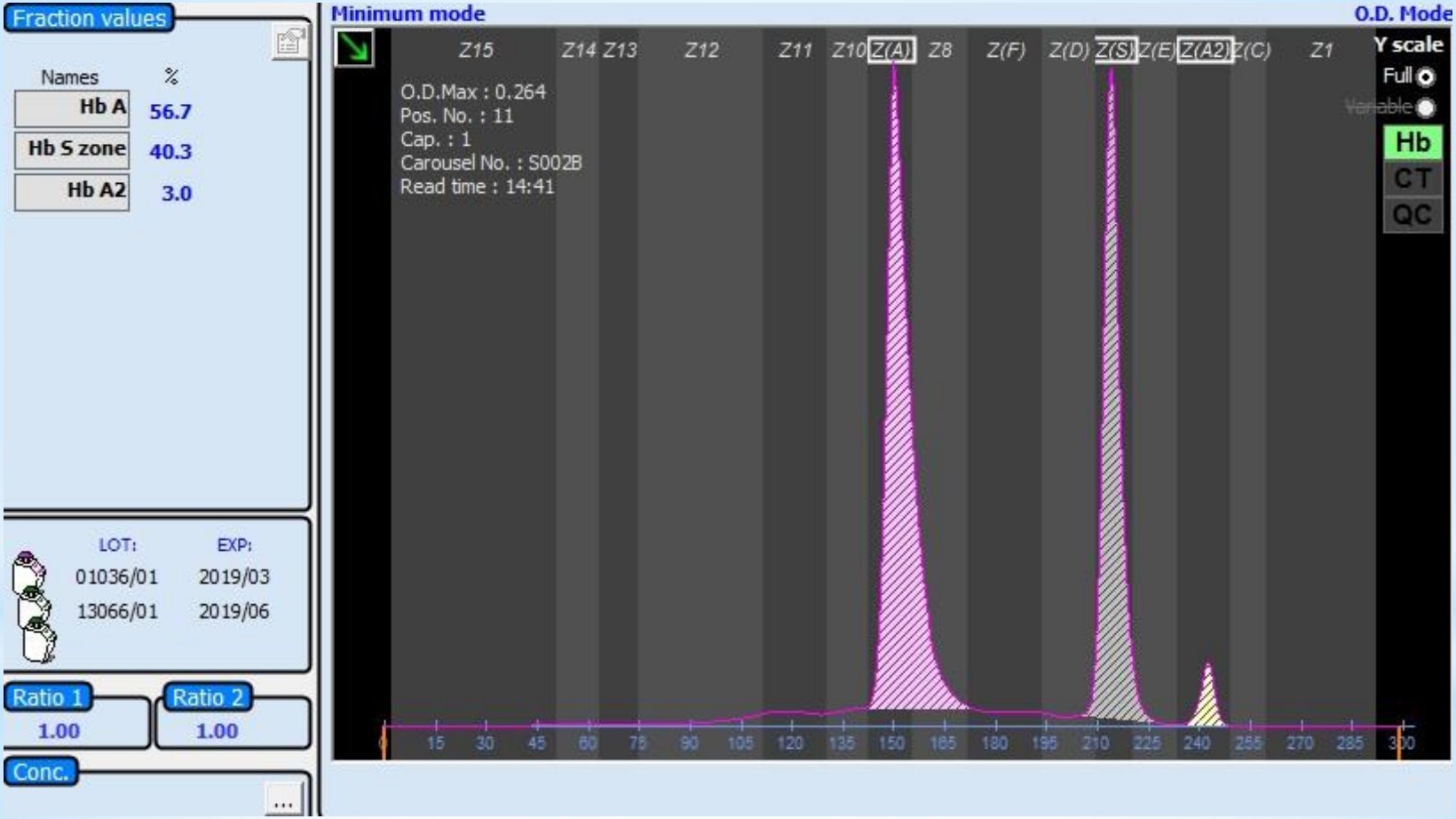


Sebia®

Normal e-gram (Sebia CE)



Hb S carrier (Sebia CE)



Advantages

- Good quantitation of Hb A2 and Hb F
- Hb E separates from Hb A2
- Detects minor variants very well
- Easy to use system, specimens may be run individually or in batches

Disadvantages

- Many variants have similar mobility, but fewer are similar to Hbs S, C and E
- Rare variants may not separate from Hb A
- If Hb A is not present (e.g. Homozygous Hb S, C, or E), no zones will be produced. Must mix specimen with normal in order to see zones

7) Mass Spectrometry (MS)

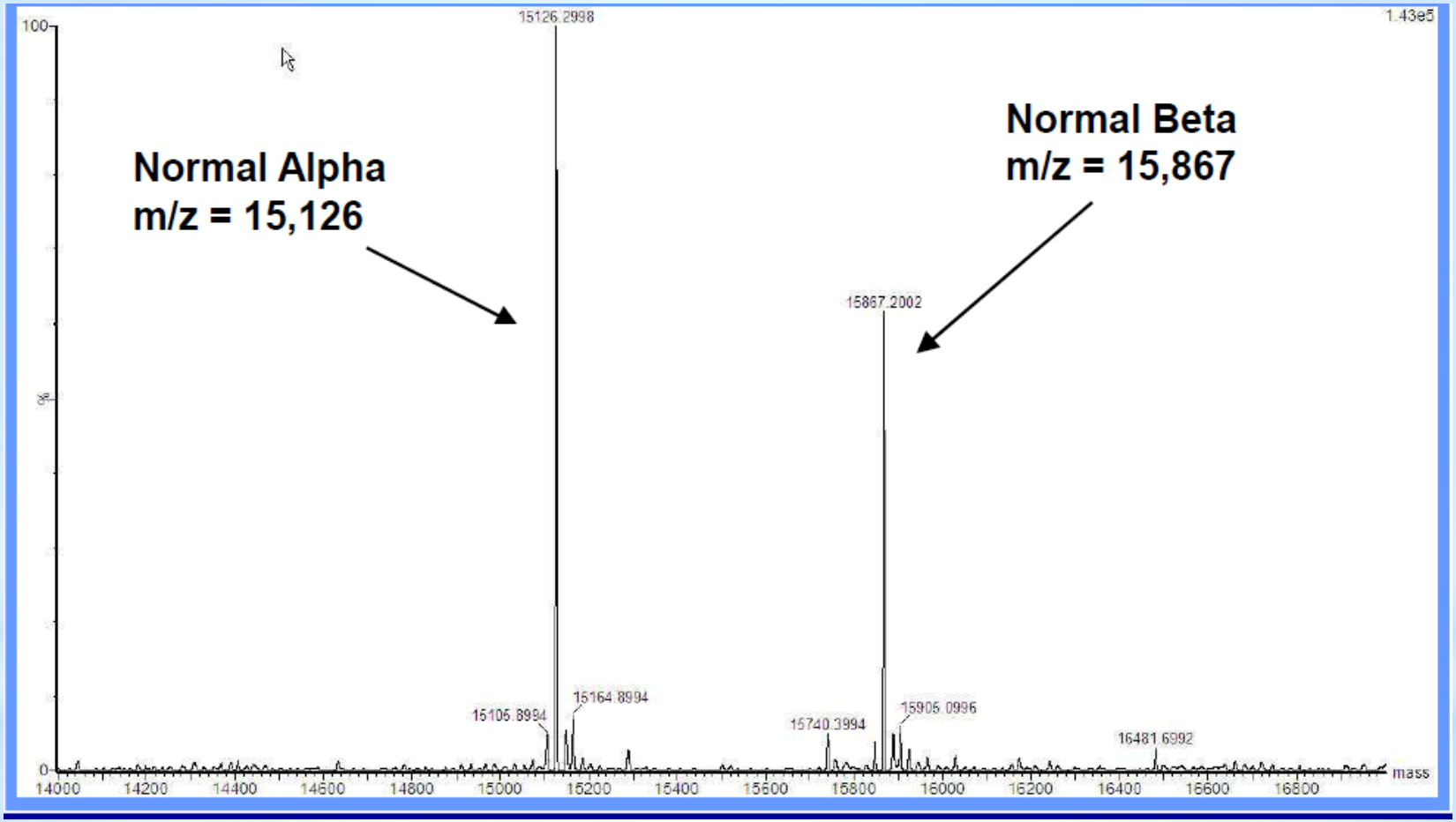
- Around for decades in clinical laboratories
- Drug identification
- Hormones
- Metabolites – inborn errors of metabolism
- Applied to analysis of proteins
- MS detects mass to charge ratios (m/z) of ionized molecules

Three stage process

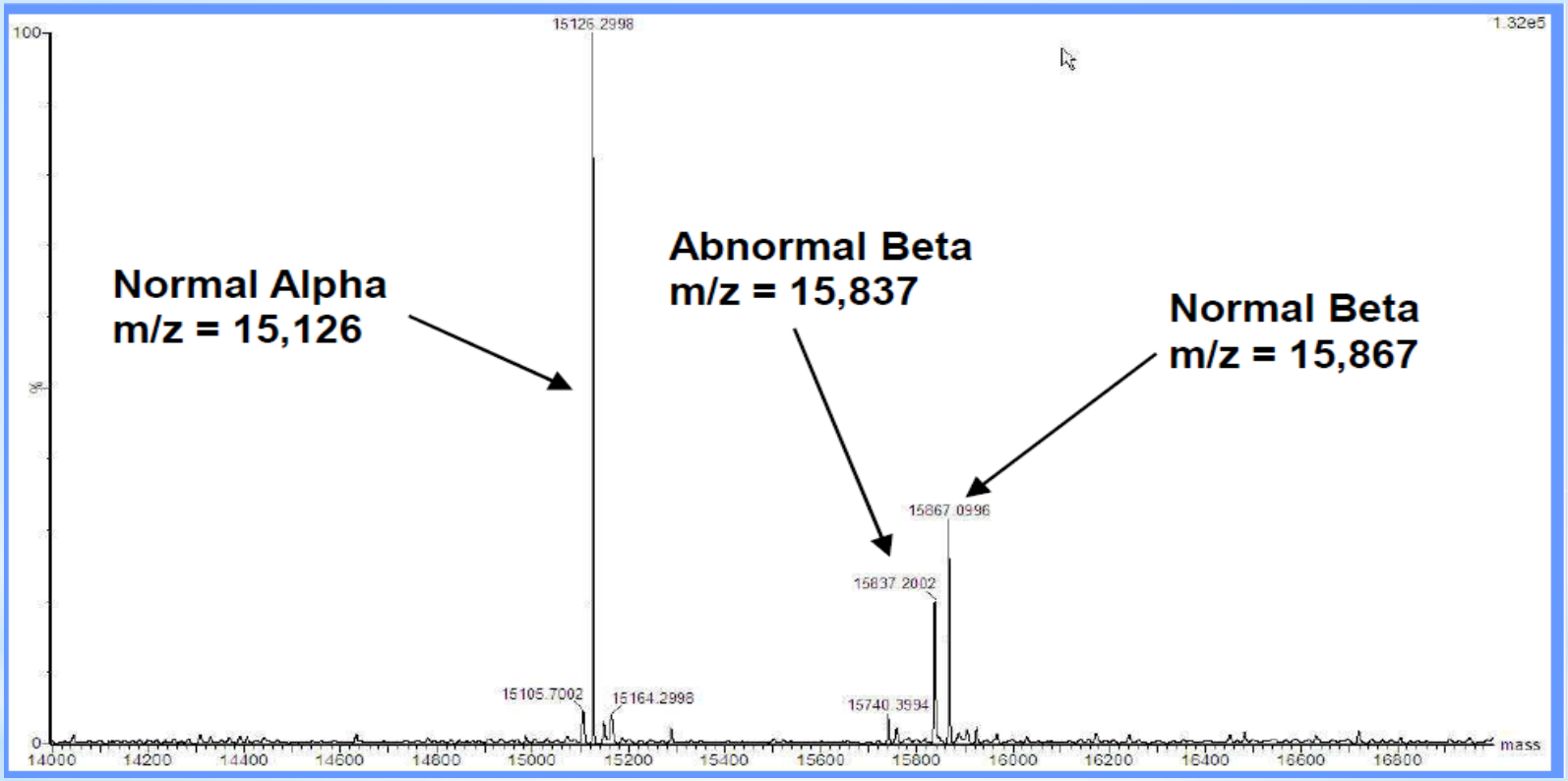
- Intact globin chain analysis – determines the mass change and the globin chain involved.
- Digest analysis – narrows down the area of the globin chain under investigation, by digesting into tryptic fragments
- MSMS – uses ‘collision induced dissociation’ to generate product ions and pinpoint the exact amino acid residue at which the mass change occurs.

Mr Jason Eyre.
Lead BMS, Haemolysis Lab
Sheffield Teaching Hospitals NHS Trust

Normal HbA


Normal Alpha
m/z = 15,126
Normal Beta
m/z = 15,867
15126.2998
15867.2002
15105.8994
15164.8994
15740.3994
15905.0996
16481.6992
1.43e5
100
%
0
mass
14000
14200
14400
14600
14800
15000
15200
15400
15600
15800
16000
16200
16400
16600
16800

HbS - predicted mass change for glu to val substitution is -30 Daltons



Advantages

- In most cases definitive identification of Hb variants by a single method
- Very fast analysis times, can analyse relatively small amounts
- Can screen for silent variants

Disadvantages

- Instruments are expensive, require high degree of expertise
- Variants with small mass shift may be difficult to detect

Case 1

K L-R , White Caucasian female, 28 yrs of age , 7 weeks gestation at testing

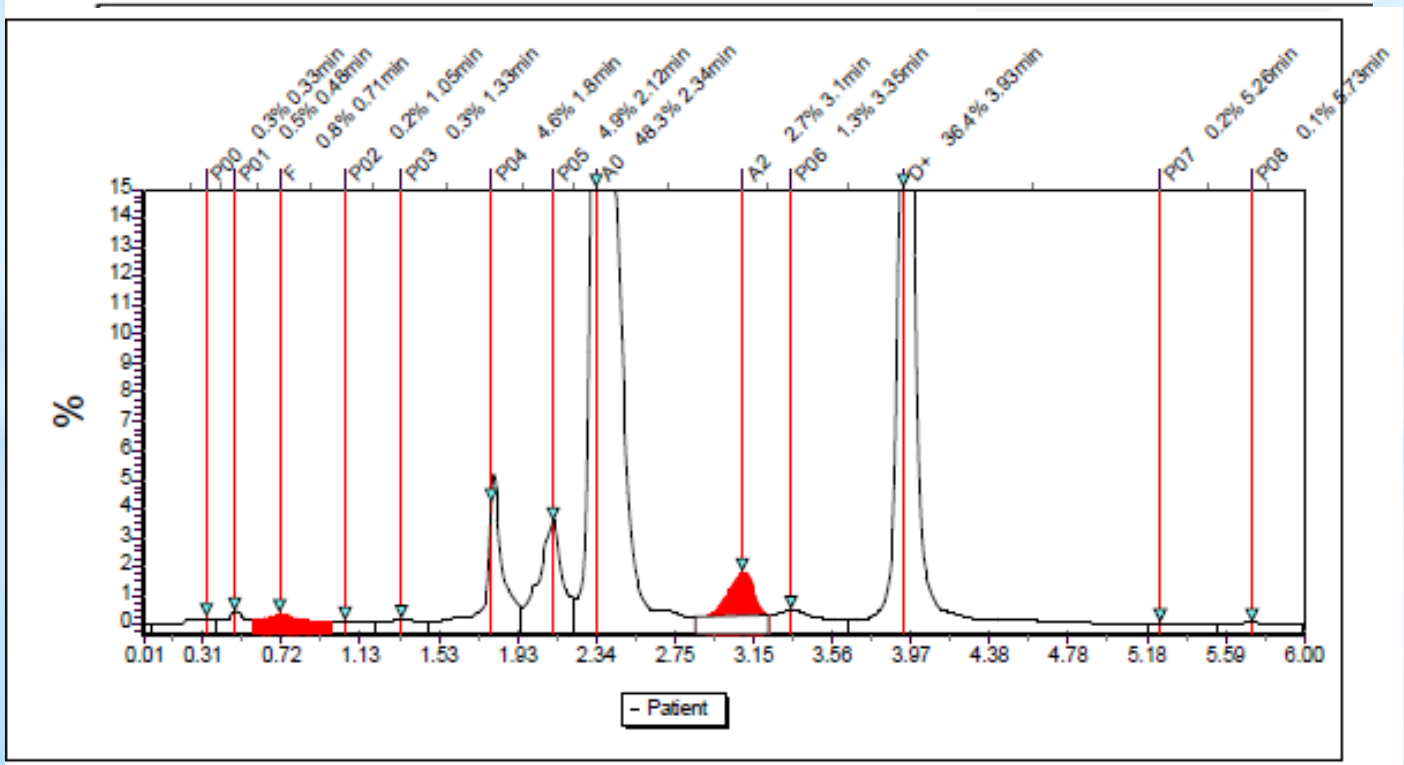
Full Blood count

Rbc 4.59 x10^12/l
Hb 132 g/l
MCV 83 fl
MCH 28.8 pg
MCHC 346 g/l
RDW 13.3%

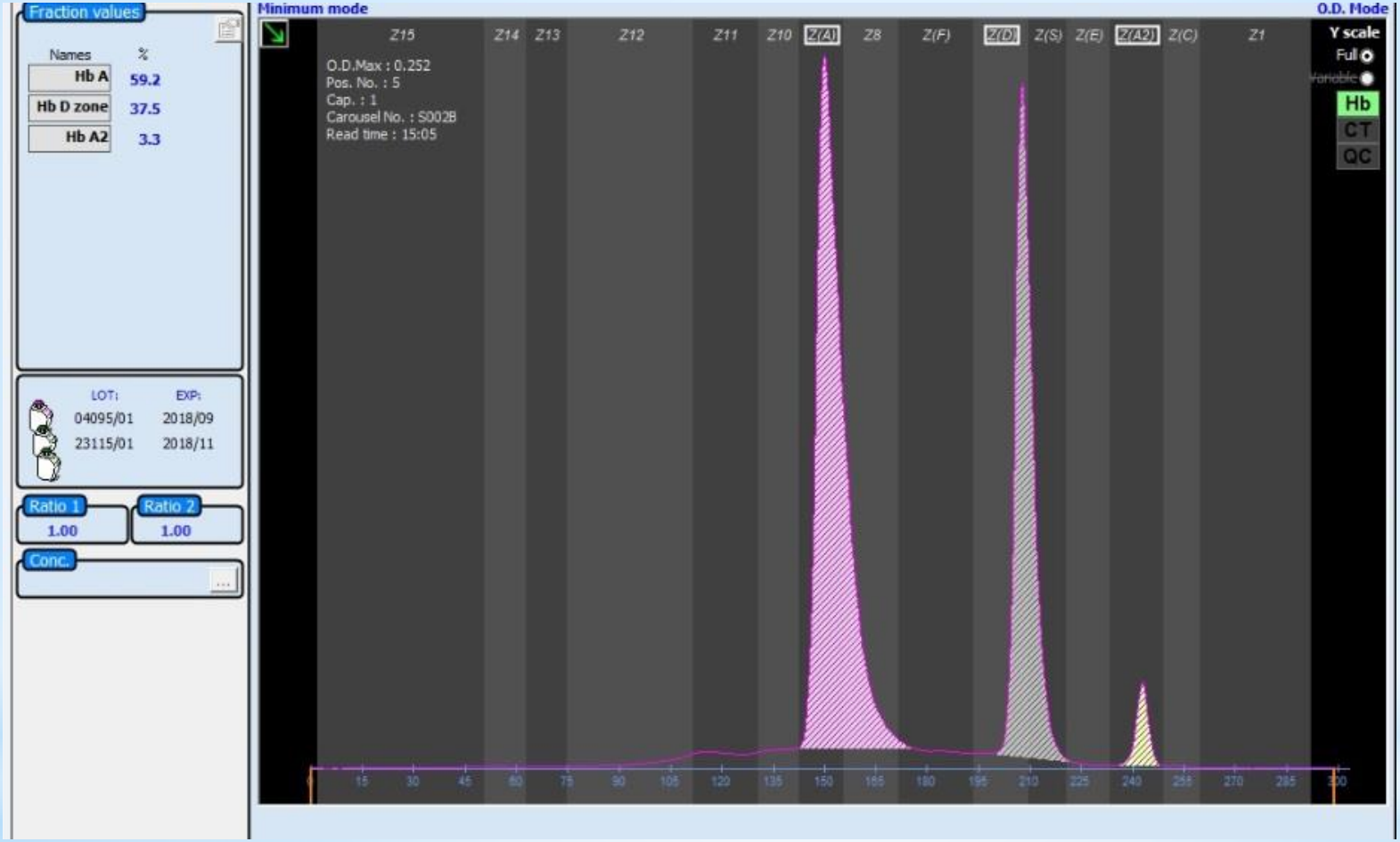
TOSOH G8 HPLC

Parameter	Value %	Time min.	Area	Total Area
P00	0.3%	0.33	16.28	4,853.8
P01	0.5%	0.48	25.39	
F	0.8%	0.71	35.91	
P02	0.2%	1.05	8.07	
P03	0.3%	1.33	14.25	
P04	4.6%	1.8	224.36	
P05	4.9%	2.12	237.63	
A0	48.3%	2.34	2,342.53	
A2	2.7%	3.1	105.45	
P06	1.3%	3.35	63.5	
D+	36.4%	3.93	1,767.44	
P07	0.2%	5.26	7.32	
P08	0.1%	5.73	5.65	

B-Thalassemia



Sebia Capillary Electrophoresis



HbA = 59.2%
HbD = 37.5%
HbA2 = 3.3%

BIORAD beta thal short

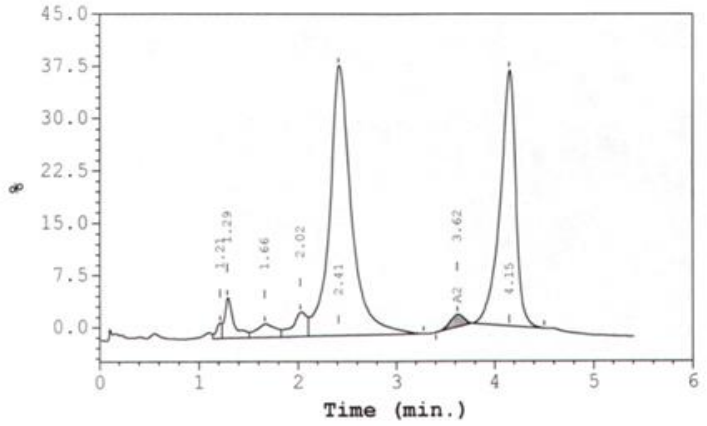
- Hb D = 35.8%
- RT 4.15
- Note A2 compromised by close eluting D peak

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.8	1.21	13401
P2	---	3.3	1.29	57990
P3	---	2.4	1.66	41125
Unknown	---	3.1	2.02	55111
Ao	---	53.1	2.41	929466
A2	1.8	---	3.62	26234
D-window	---	35.8	4.15	626477

Total Area: 1749803

F Concentration = %
A2 Concentration = 1.8 %

Analysis comments:



Sickle solubility test – negative

Results consistent with a Hb D carrier

Testing of babys biological father recommend.

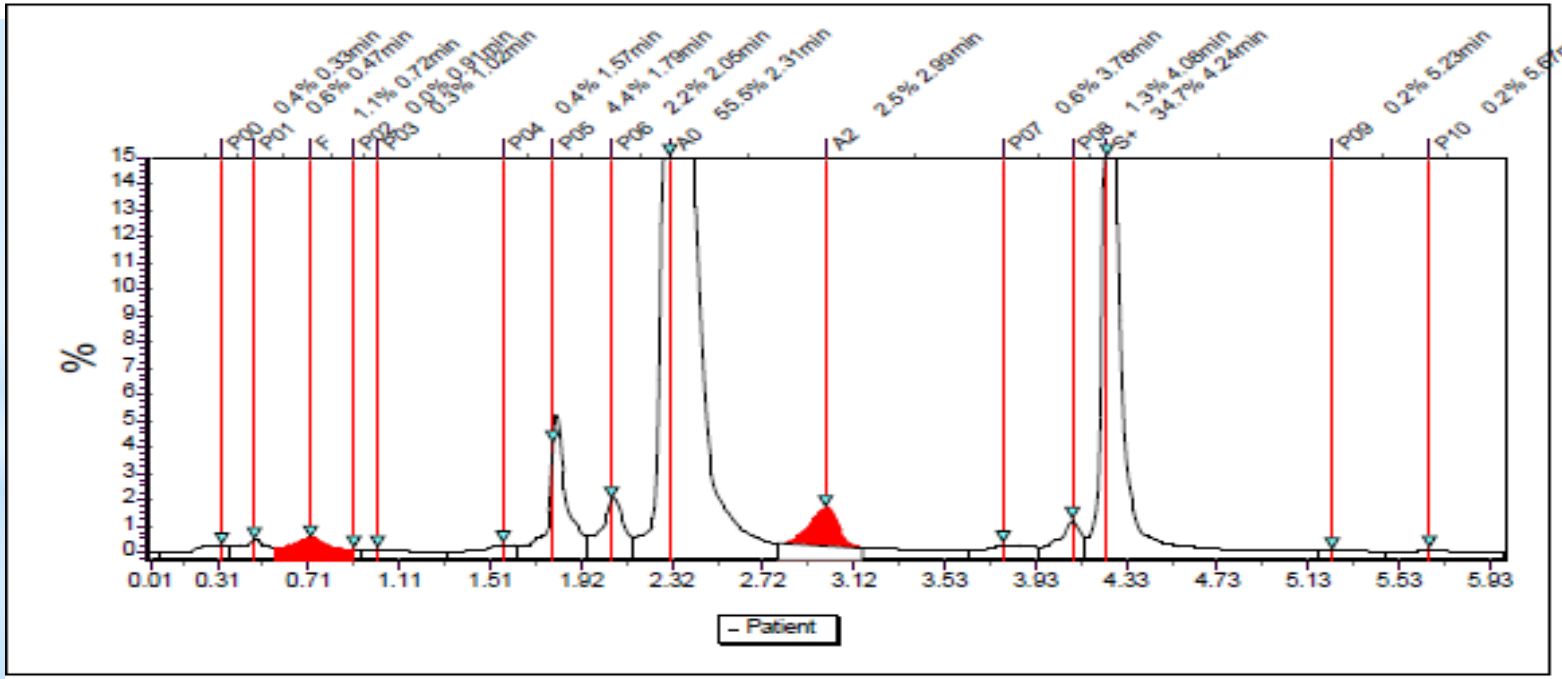
Father of baby

E.R , 27 year old, African-Caribbean

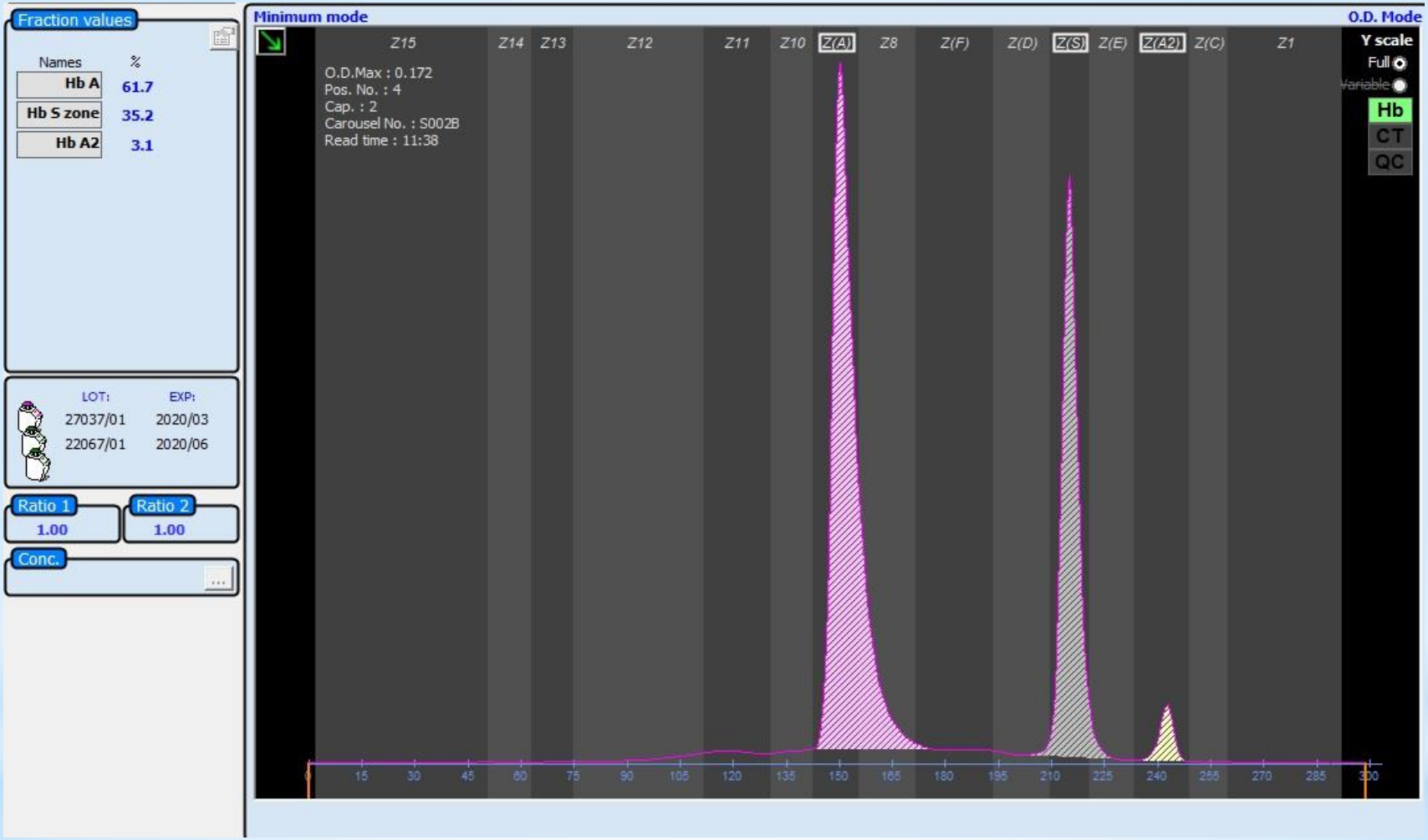
Rbc	5.36 x10^12/l
Hb	148 g/l
MCV	86 fl
MCH	27.6 pg
MCHC	321 g/l
RDW	11.4 %

TOSOH G8 HPLC

Parameter	Value %	Time min.	Area	Total Area
P00	0.4%	0.33	15.91	4,209.5
P01	0.6%	0.47	24.05	
F	1.1%	0.72	39.74	
P02	0.0%	0.91	0.91	
P03	0.3%	1.02	10.84	
P04	0.4%	1.57	18.44	
P05	4.4%	1.79	185.57	
P06	2.2%	2.05	94.46	
A0	55.5%	2.31	2,335.49	
A2	2.5%	2.99	84.85	
P07	0.6%	3.78	25.27	
P08	1.3%	4.08	54.07	
S+	34.7%	4.24	1,304.73	
P09	0.2%	5.23	7.4	
P10	0.2%	5.67	7.72	



Sebia Capillary Electrophoresis



Hb A = 61.7%

Hb S = 35.2%

HbA2 = 3.1%

Sickle Solubility screen – positive.

Results consistent with HbS carrier.

At risk pregnancy – antenatal screening coordinator informed

Both parents counselled and permission obtained for DNA analysis.

DNA report.

K L-R (mother) was found to be heterozygous
for the beta globin gene mutation Codon 121 (GAA>CAA)
[HBB:c.364G>C] which gives rise to the haemoglobin variant Hb D-Punjab.

E R (father) is heterozygous for the mutation in Codon 6
(GAG>GTG) of the beta globin gene [HBB: c.20A>T] which gives rise to the haemoglobin variant Hb S . He is also heterozygous for the 3.7Kb single alpha globin gene deletion (genotype: -a3.7/aa). This deletion results in alpha plus thalassaemia which is a common and benign condition

Both Hb D-Punjab and Hb S are benign in the carrier state but they can interact to produce a severe sickling disorder.

Therefore, this couple are at 1:4 risk of having a child affected with Hb S/D disease.

Prenatal diagnosis is available if required.

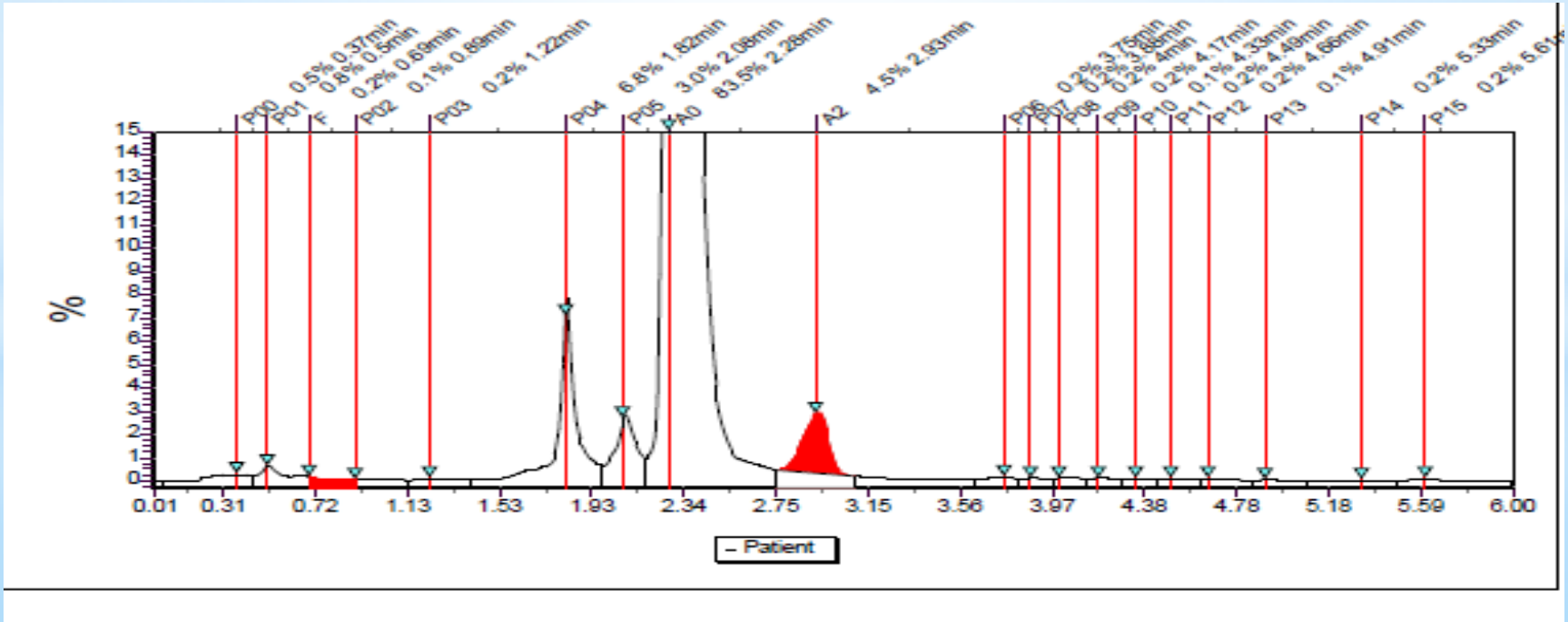
Case 2

C.G , white Caucasian , 9 weeks gestation at testing

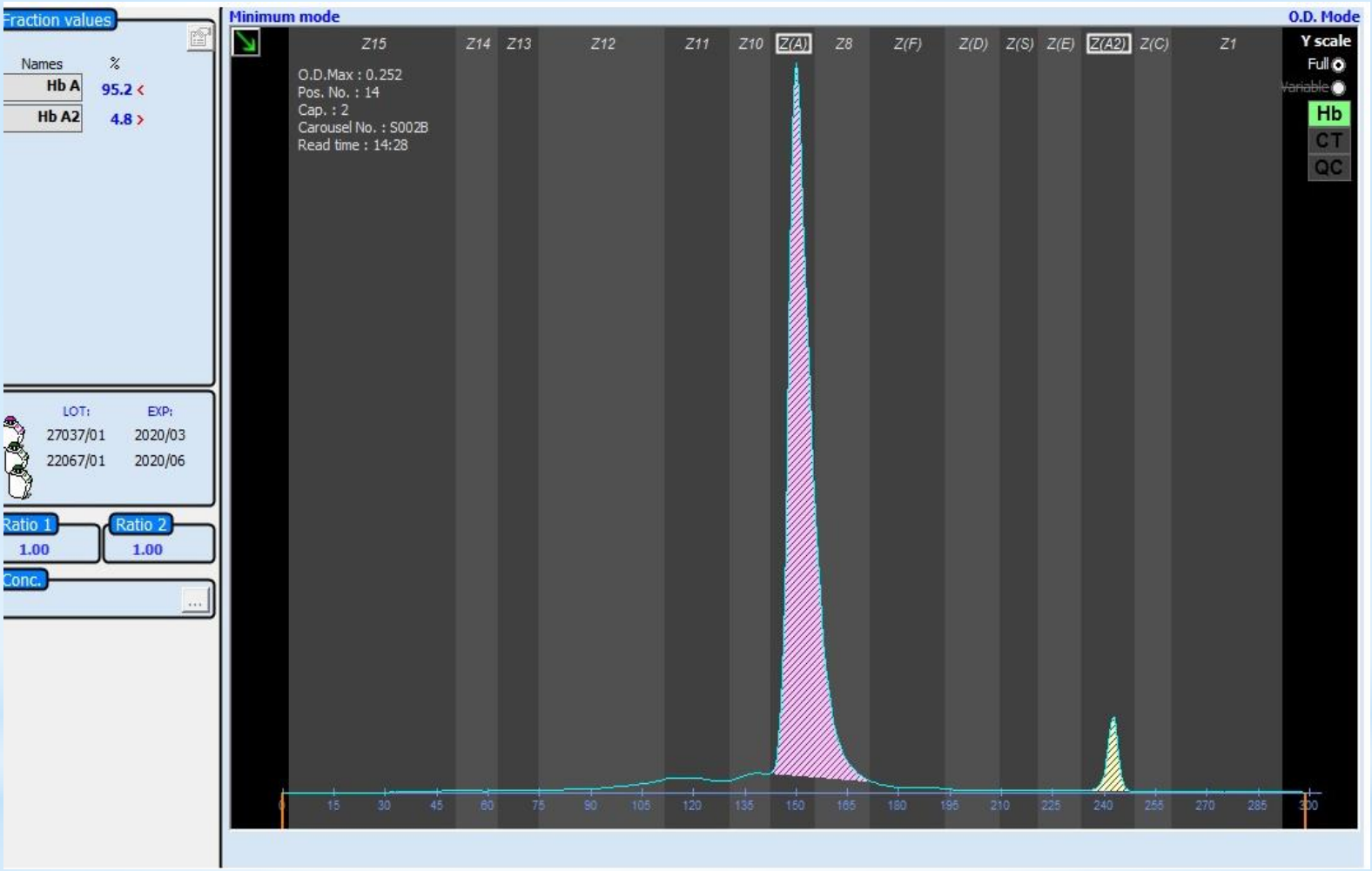
Rbc	5.30 x10^12/l
Hb	139 g/l
MCV	88 fl
MCH	28.6 pg
MCHC	311 g/l
RDW	11.6 %

TOSOH G8 HPLC

Parameter	Value %	Time min.	Area	Total Area
P00	0.5%	0.37	24.38	4,522.2
P01	0.8%	0.5	35.64	
F	0.2%	0.69	8.66	
P02	0.1%	0.89	6.42	
P03	0.2%	1.22	8.32	
P04	6.8%	1.82	306.11	
P05	3.0%	2.08	135.32	
A0	83.5%	2.28	3,774.43	
A2	4.5%	2.93	145.22	
P06	0.2%	3.75	9.94	
P07	0.2%	3.88	8.11	
P08	0.2%	4	8.54	
P09	0.2%	4.17	7.58	
P10	0.1%	4.33	6.15	
P11	0.2%	4.49	7.2	
P12	0.2%	4.66	6.85	
P13	0.1%	4.91	5.66	
P14	0.2%	5.33	8.97	*B-Thalassemia*
P15	0.2%	5.61	8.73	



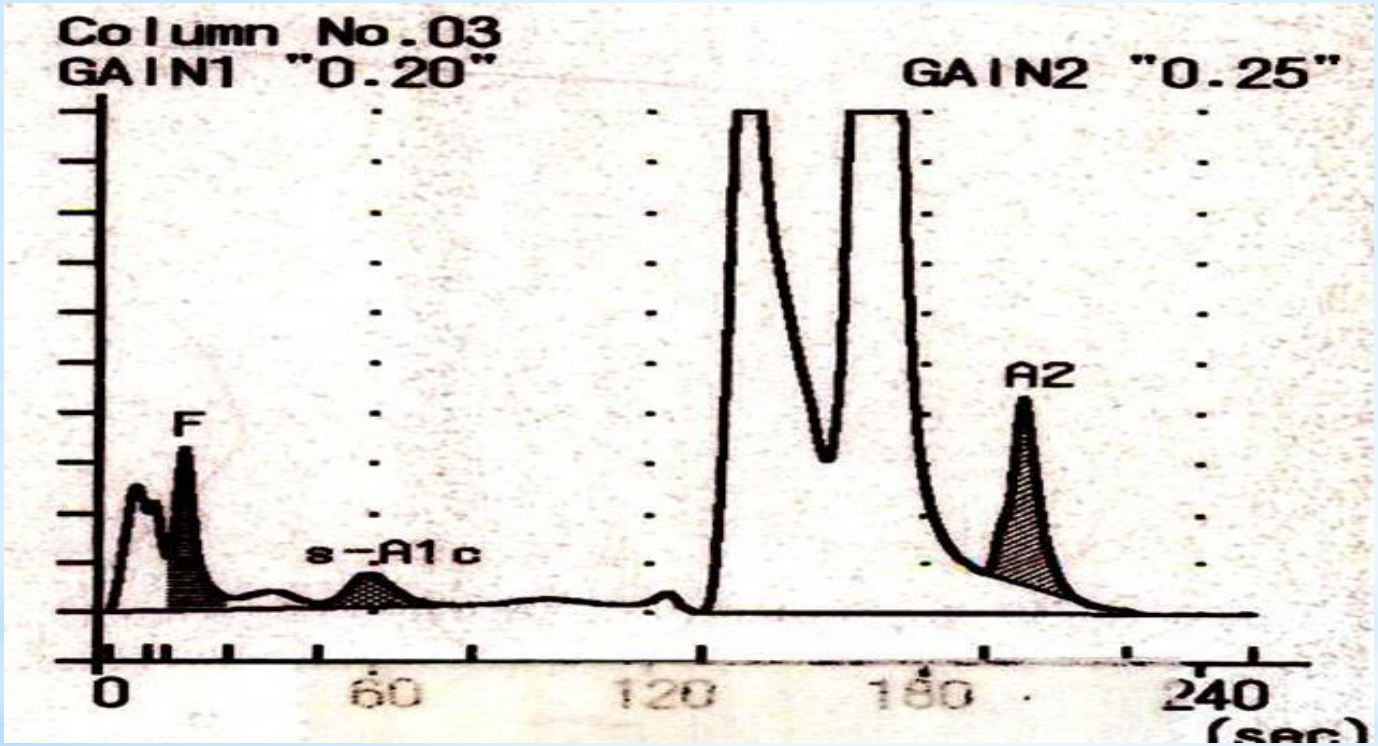
Sebia Capillary Electrophoresis



HbA= 95.2

HbA2 = 4.8

HA 8160



Hb A2 = 4.6

- Results consistent with possible beta thalassaemia carrier
- Testing of baby's biological father recommended.

Acquired causes of an increased percentage of HbA2

- Hyperthyroidism
- HIV treatments
- Megaloblastic anaemia

Case 3

A.B. , 23 year old Bangladeshi lady , 6 weeks gestation at testing

Rbc	5.20 x10^12/l
Hb	104 g/l
MCV	59 fl
MCH	20.0 pg
MCHC	295 g/l
RDW	16.2 %

TOSOH G8 HPLC

Parameter	Value %	Time min.	Area
P00	1.4%	0.21	25.66
P01	6.6%	0.53	116.94
F	36.4%	1.31	563.29
P02	0.1%	1.59	2.21
P03	3.2%	1.92	57.46
P04	1.9%	2.02	33.67
P05	5.6%	2.13	98.38
A0	8.0%	2.49	141.94
A2	51.2%	3.26	655
P06	0.7%	3.78	11.8
P07	0.3%	3.88	5.11
P08	0.5%	3.97	8.07
P09	0.9%	4.25	16.2
P10	0.3%	4.6	4.48
P11	0.2%	4.73	3.9
P12	0.6%	4.94	11.36
P13	0.8%	5.27	14.87

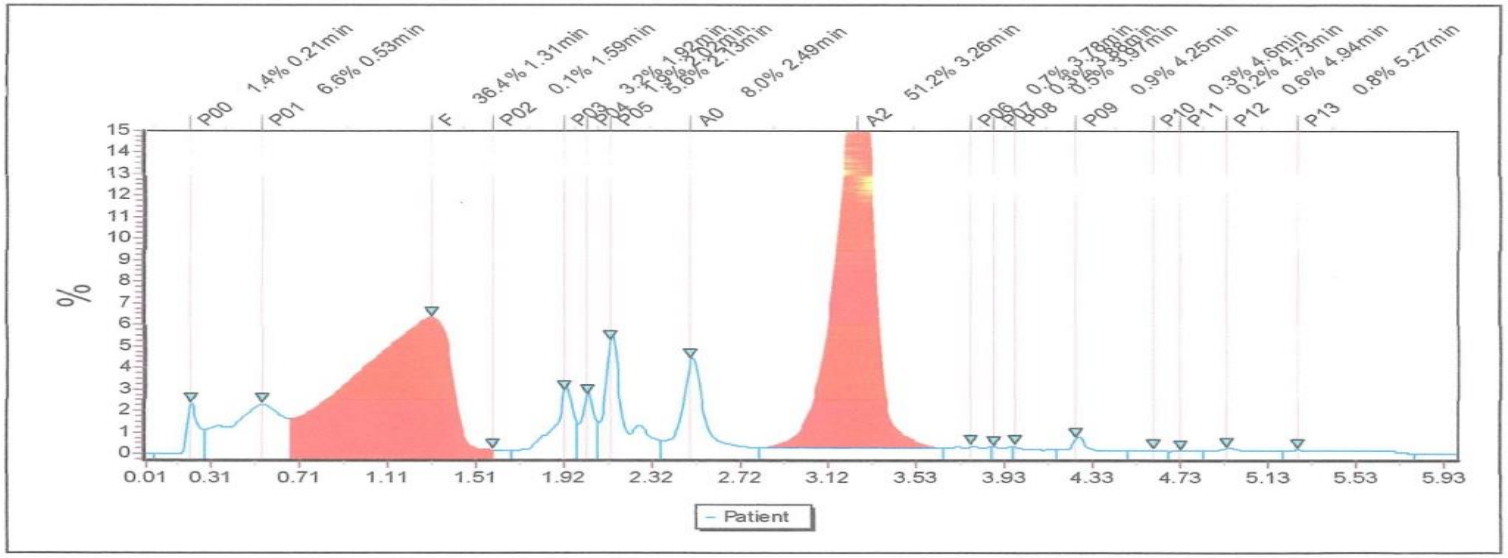
Total Area
1,770.3

Y=(Ax+B)

Element	Factor-A	Factor-B
1	1.1438	0.0000
2	1.2699	0.0000

Analyzer: B Thal
Serial Nb.: 13433704
Soft. Version: 5.24
UIN: 30451

B-Thalassemia

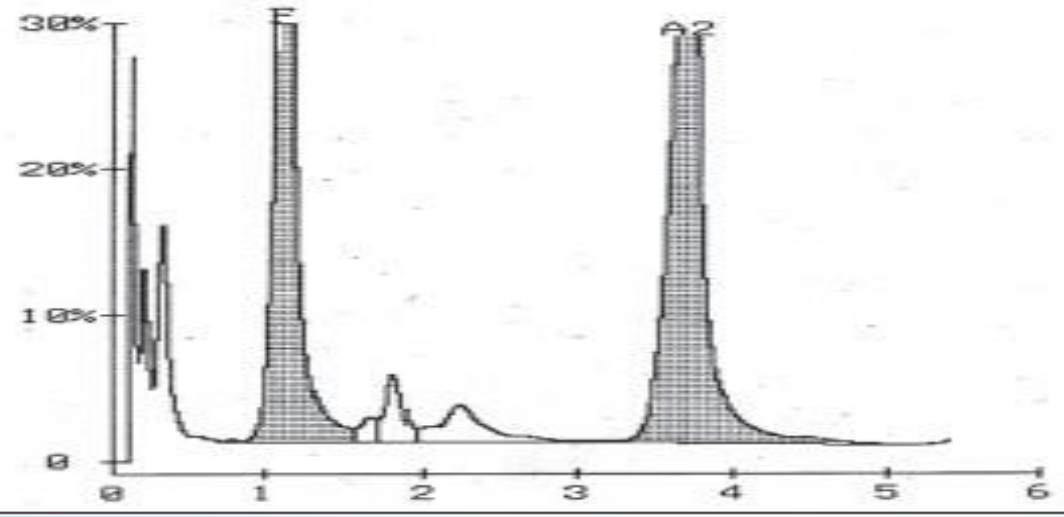


*** Beta Thal Short 90650-A ***
DATE:06/09/12 TIME:02:55:52

TECH ID# 0
VIAL# 15
SAMPLE ID# 00000000000000009893

ANALYTE ID	%	TIME	AREA
F	43.1	1.12	332439
Unknown 1	1.1	1.67	8697
P3	3.1	1.82	23481
Ao	4.7	2.22	35704
A2	51.6	3.71	406477
TOTAL AREA			806798

F 43.1% A2 51.6%



BIO-RAD

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Sickle solubility test – negative

- Results consistent with HbE/beta zero thalassaemia compound heterozygosity
- Testing of baby's biological father recommended.
- Will require DNA analysis for confirmation

	Hb E/ Beta zero thalassaemia	HbE Homozygosity (Hb E Disease)
% HbE	40-60	85-99
% HbF	30-60	<15 (often <5%)

Haemoglobinopathy Diagnosis, Barbara Bain, 2nd edition 2006

Thank You