DIAGNOSIS OF THE THALASSAEMIA SYNDROMES:

MEASUREMENT OF HAEMOGLOBIN A_2

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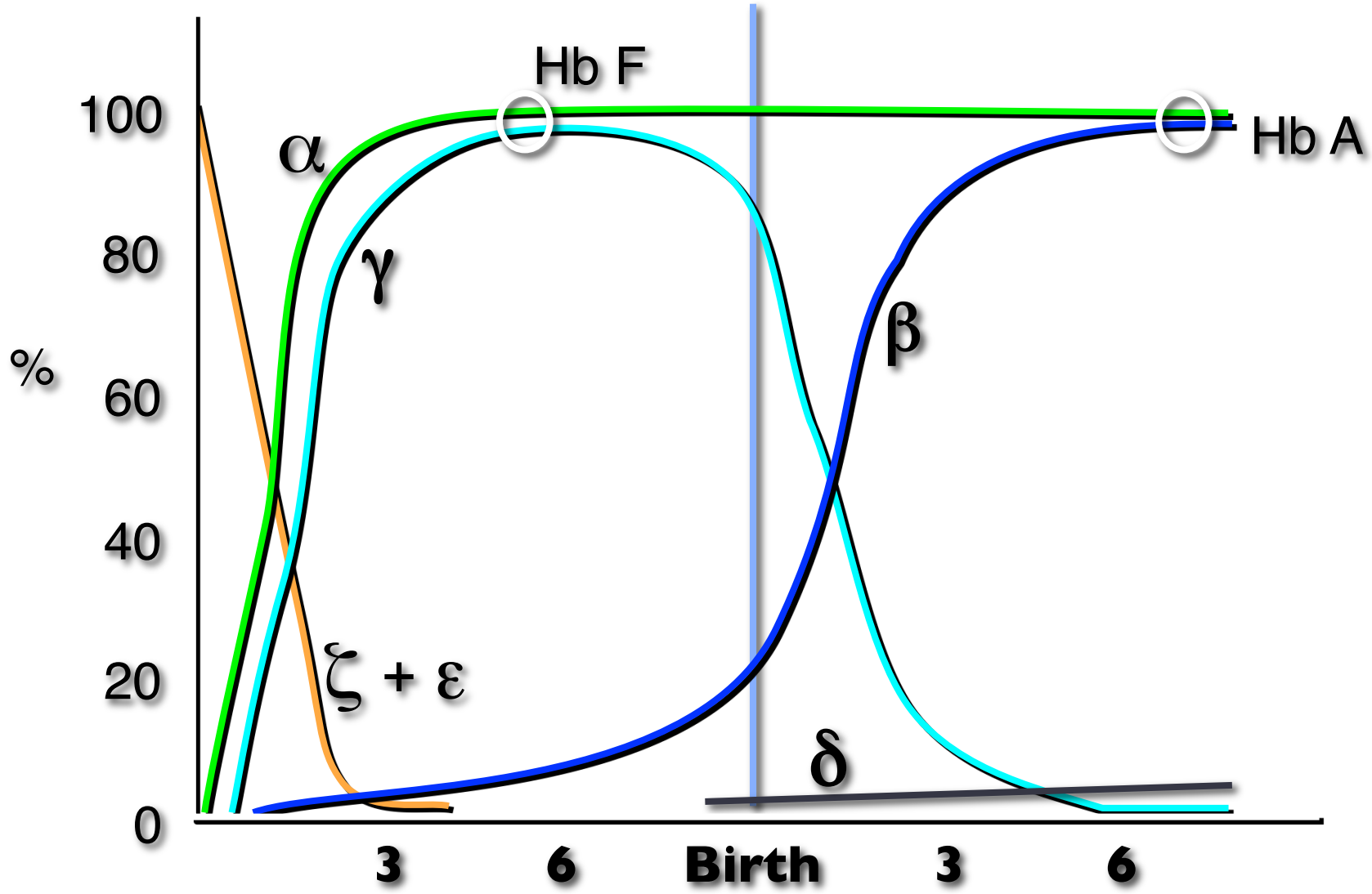
UK National External Quality Assessment Scheme
London

Globin biosynthesis



The importance of Hb A_2 measurement

Hb A_2 is measured as a ***proportion*** of the ***total*** haemoglobins present, not as an absolute amount

Hb A_2 measurement is used as a marker for beta thalassaemia trait. Carrier detection is important because:

Beta thalassaemia carriers are asymptomatic but

homozygous beta thalassaemia is a life-threatening disorder

The importance of Hb A_2 measurement

- Accurate and reliable measurement of Hb A_2 is essential for the diagnosis of beta thalassaemia trait
- Small difference (if any) between normal & abnormal levels

- Antenatal women should be screened for beta thalassaemia trait
- Carriers: recommend partner testing
- prediction of genetic risk

- Failure to detect condition may result in newborn with a medically significant condition

Screening for beta thalassaemia trait

- Full blood count with red cell indices:

  RBC, Mean Cell Volume and Mean Cell Haemoglobin
- Hb A_2 %
- Hb F %
- Screen for haemoglobin variants
- Iron status - ferritin, zinc protoporphyrin

- Family history

Measurement of Hb A_2

Automated methods

- High Performance Liquid Chromatography
- Capillary electrophoresis
- Mass spectrometry

Manual methods

- Hb electrophoresis with elution
- Microcolumn chromatography

Interpretation

Normal: 2.2-3.5% (usually<3.3%)

Beta thalassaemia trait: >3.5%

High performance liquid chromatography

General principle

- Utilises a weak cation-exchange column
- Hb molecules adsorb onto the column saurated with low ionic strength buffer
- Buffer with increased ionic strength used to elute haemoglobins from column
- Haemoglobins will elute when ionic strength of eluting solution exceeds that of the haemoglobins

- Retention time of a particular haemoglobin is characteristic and reproducible, ***but not unique***

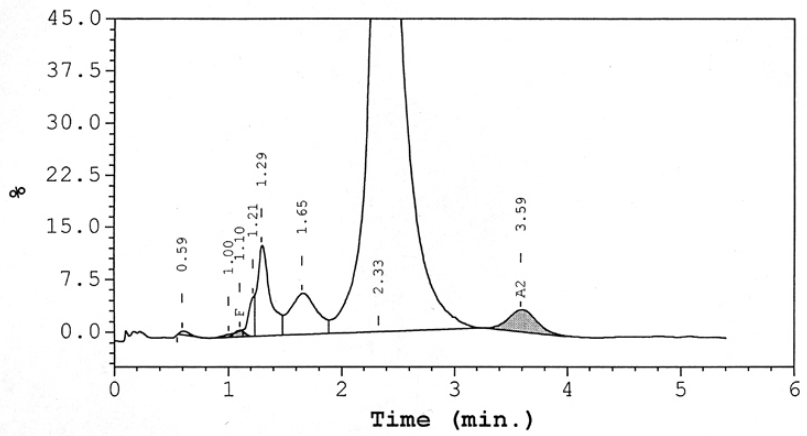
HPLC analysis - normal adult

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.59	3217
Unknown	---	0.1	1.00	3321
F	0.3	---	1.10	7753
Unknown	---	1.1	1.21	29205
P2	---	4.8	1.29	125635
P3	---	4.7	1.65	121998
Ao	---	86.0	2.33	2232443
A2	3.1	---	3.59	70883

Total Area: 2594456

F Concentration = 0.3 %
A2 Concentration = 3.1 %

Analysis comments:



Beta thalassaemia trait

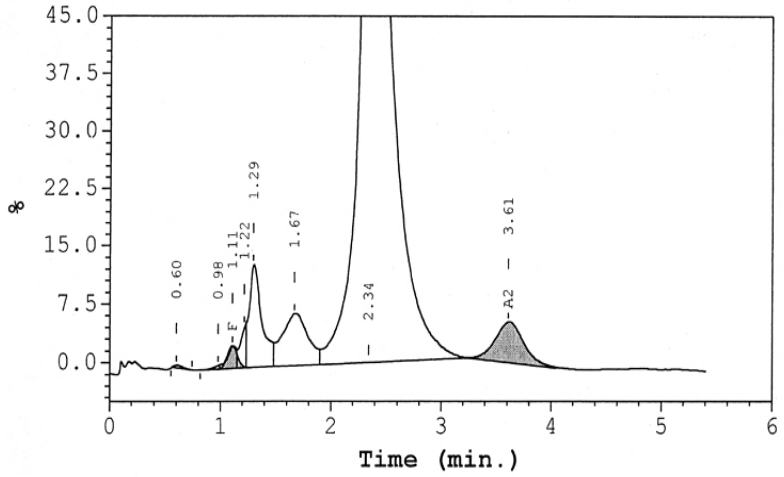
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.60	2439
Unknown	---	0.1	0.98	2463
F	1.0	---	1.11	23374
Unknown	---	1.0	1.22	26601
P2	---	5.0	1.29	128590
P3	---	5.2	1.67	133229
Ao	---	83.0	2.34	2115341
A2	5.2*	---	3.61	115816

Total Area: 2547853

F Concentration = 1.0 %
A2 Concentration = 5.2* %

*Values outside of expected ranges

Analysis comments:



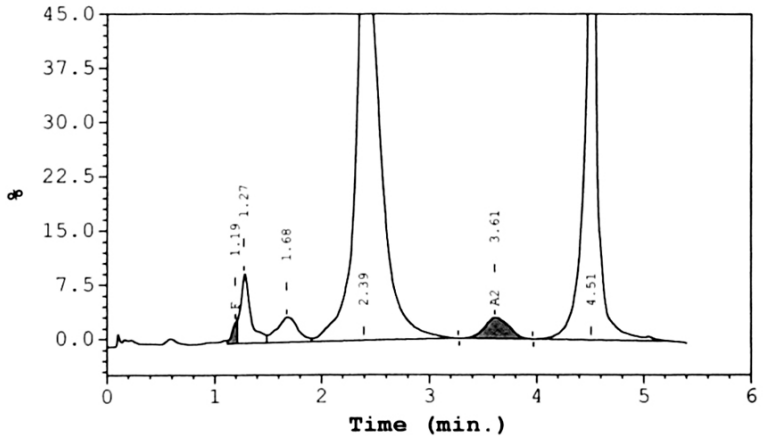
Sickle cell trait

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.6	---	1.19	16136
P2	---	3.8	1.27	106506
P3	---	2.8	1.68	78920
Ao	---	54.8	2.39	1539681
A2	2.9	---	3.61	75289
S-window	---	35.3	4.51	991769

Total Area: 2808300

F Concentration = 0.6 %
A2 Concentration = 2.9 %

Analysis comments:



Hb Sβ+thalassaemia

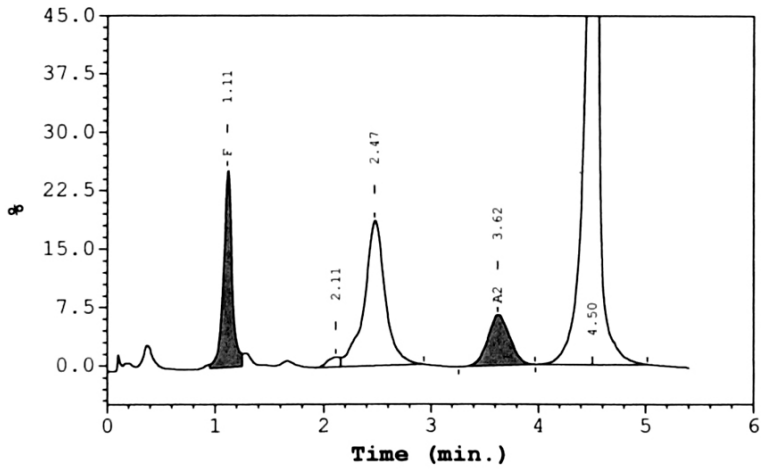
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	9.0*	---	1.11	153554
Unknown	---	0.7	2.11	11443
Ao	---	16.2	2.47	273375
A2	6.5*	---	3.62	99298
S-window	---	68.1	4.50	1149258

Total Area: 1686929

F Concentration = 9.0* %
A2 Concentration = 6.5* %

*Values outside of expected ranges

Analysis comments:



δ chain variant

Consider total Hb A_2

and

review red cell indices

Note:

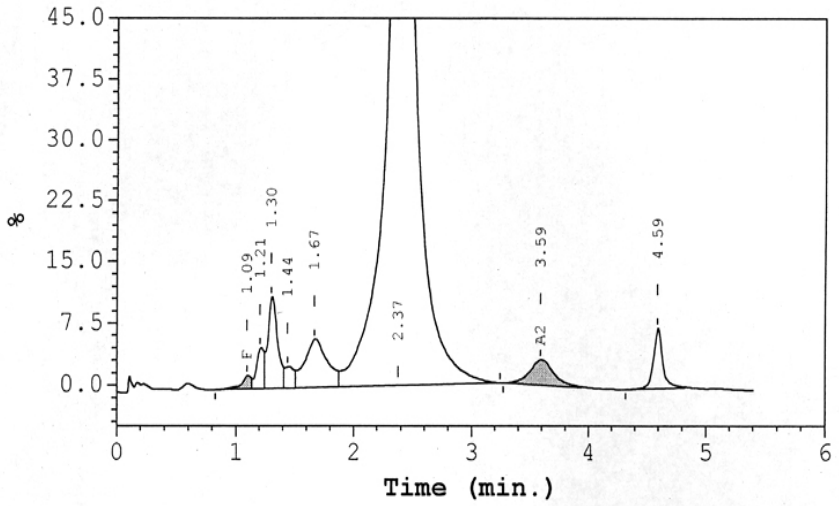
also check for carry-over

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.5	---	1.09	9567
Unknown	---	1.3	1.21	22251
P2	---	3.7	1.30	64598
Unknown	---	0.8	1.44	14465
P3	---	4.5	1.67	78283
Ao	---	84.2	2.37	1465577
A2	3.1	---	3.59	44847
S-window	---	2.4	4.59	41401

Total Area: 1740990

F Concentration = 0.5 %
A2 Concentration = 3.1 %

Analysis comments:



Hb Lepore trait

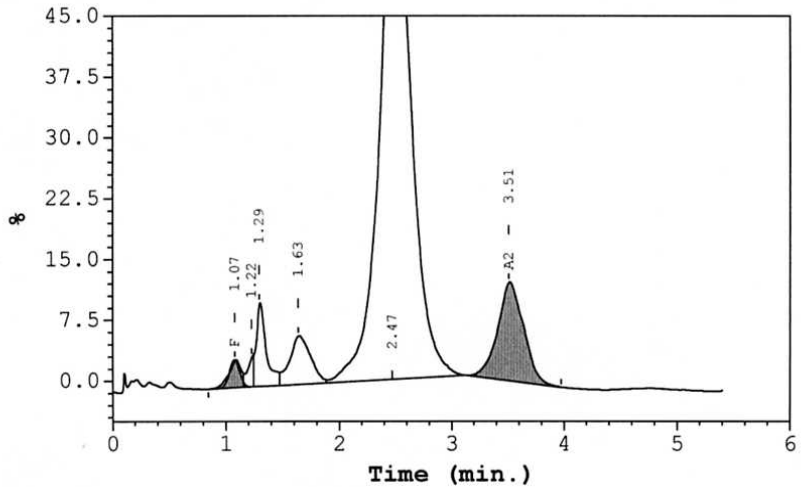
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.5	---	1.07	25436
Unknown	---	0.9	1.22	16792
P2	---	3.7	1.29	71547
P3	---	4.6	1.63	88564
Ao	---	78.0	2.47	1518633
A2	12.2*	---	3.51	225141

Total Area: 1946114

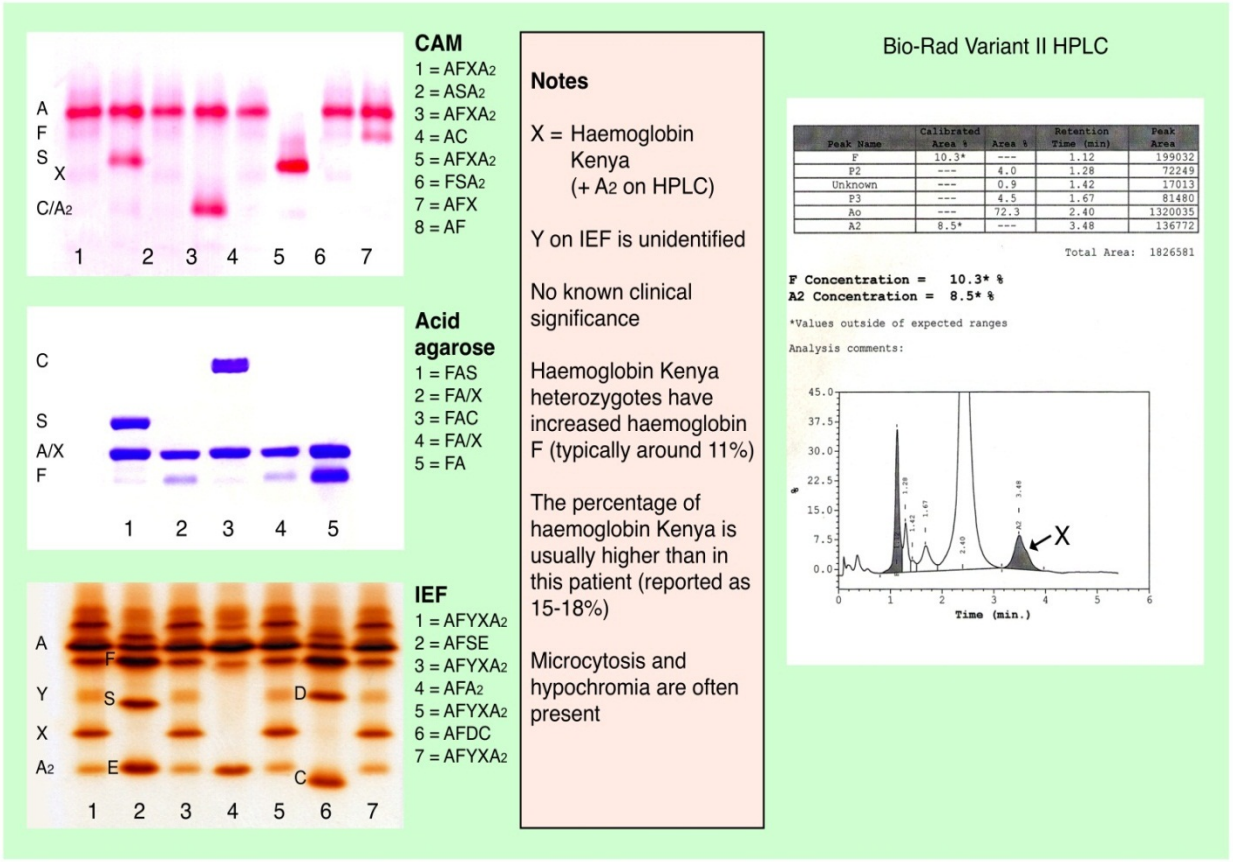
F Concentration = 1.5 %
A2 Concentration = 12.2* %

*Values outside of expected ranges

Analysis comments:

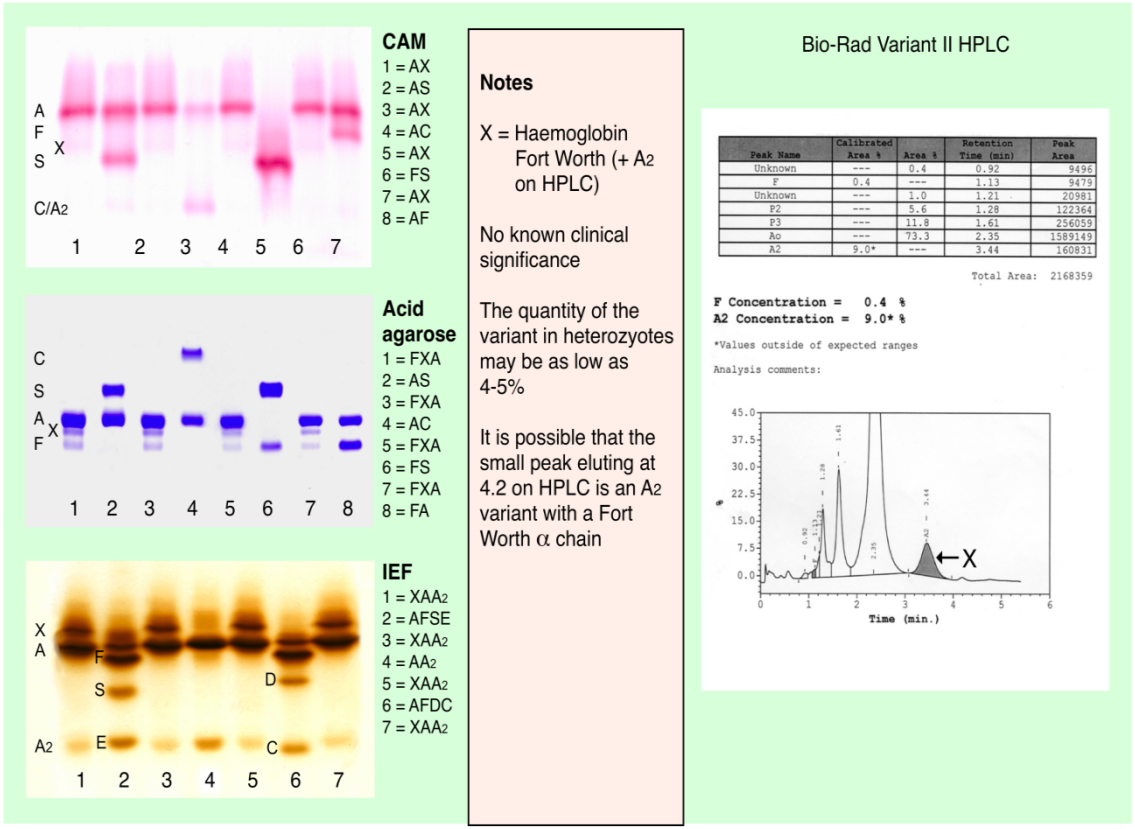


Hb Kenya trait



Haemoglobin Kenya (γβ fusion) heterozygote

Hb Fort Worth trait



Haemoglobin Fort Worth - α27 (Glu → Gly) heterozygote

Capillary electrophoresis

- Utilises a thin capillary of silica, diameter approx 50-75υm
- Inner surface of the capillary has a negative charge
- High voltage applied (10-30kv) – capillary generates endo-osmotic flow (EOF) towards cathode
- Hbs separated because of different charges-fractions move towards the cathode because of EOF

- Electropherograms of peaks of a particular haemoglobin is characteristic and reproducible, ***but not unique***

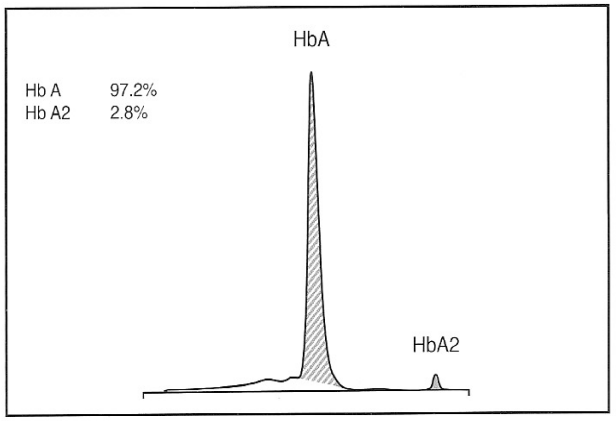


Figure 1: Normal sample

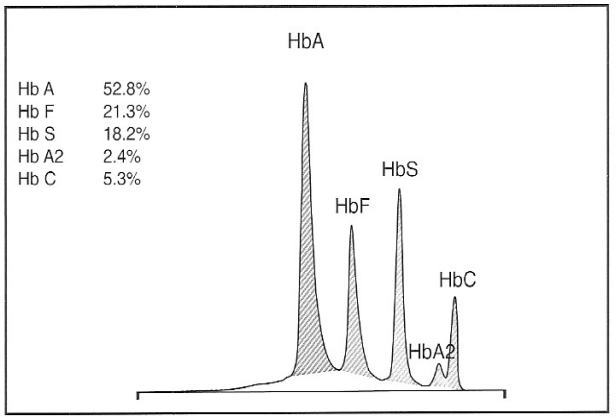


Figure 2: AFSC control

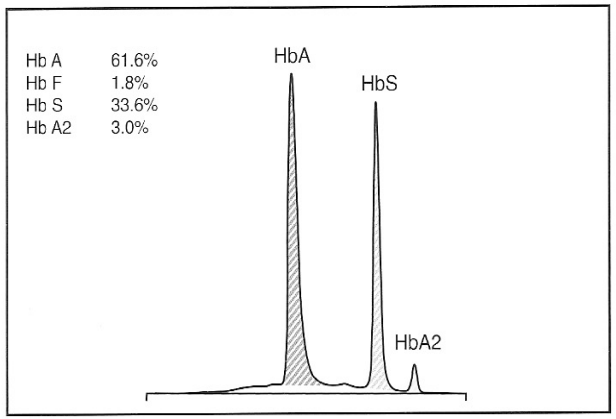


Figure 3: Heterozygous A/S

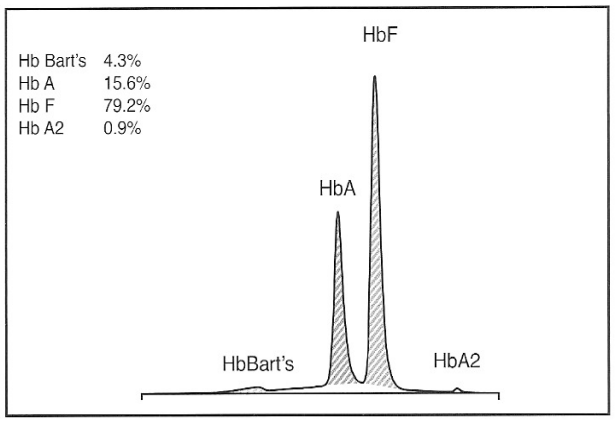


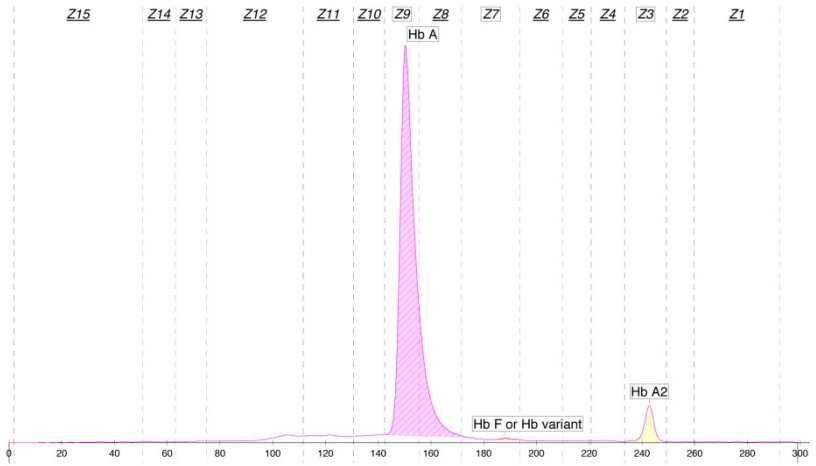
Figure 4: Alpha thalassemia with Hb Bart's

Capillary electrophoresis

portoir: SEBIA Pos.: 1

Sample # : **145** Date : **28/02/2011** ID : **A2 NIBSC**

Depart. : Birth. :



Haemoglobin Electrophoresis

Name	%	Normal Values %
Hb A	**94,4**	<
Hb F or Hb variant	**0,4**	
Hb A2	**5,2**	>

Guy's and St Thomas' NHS
NHS Foundation Trust

WellChild

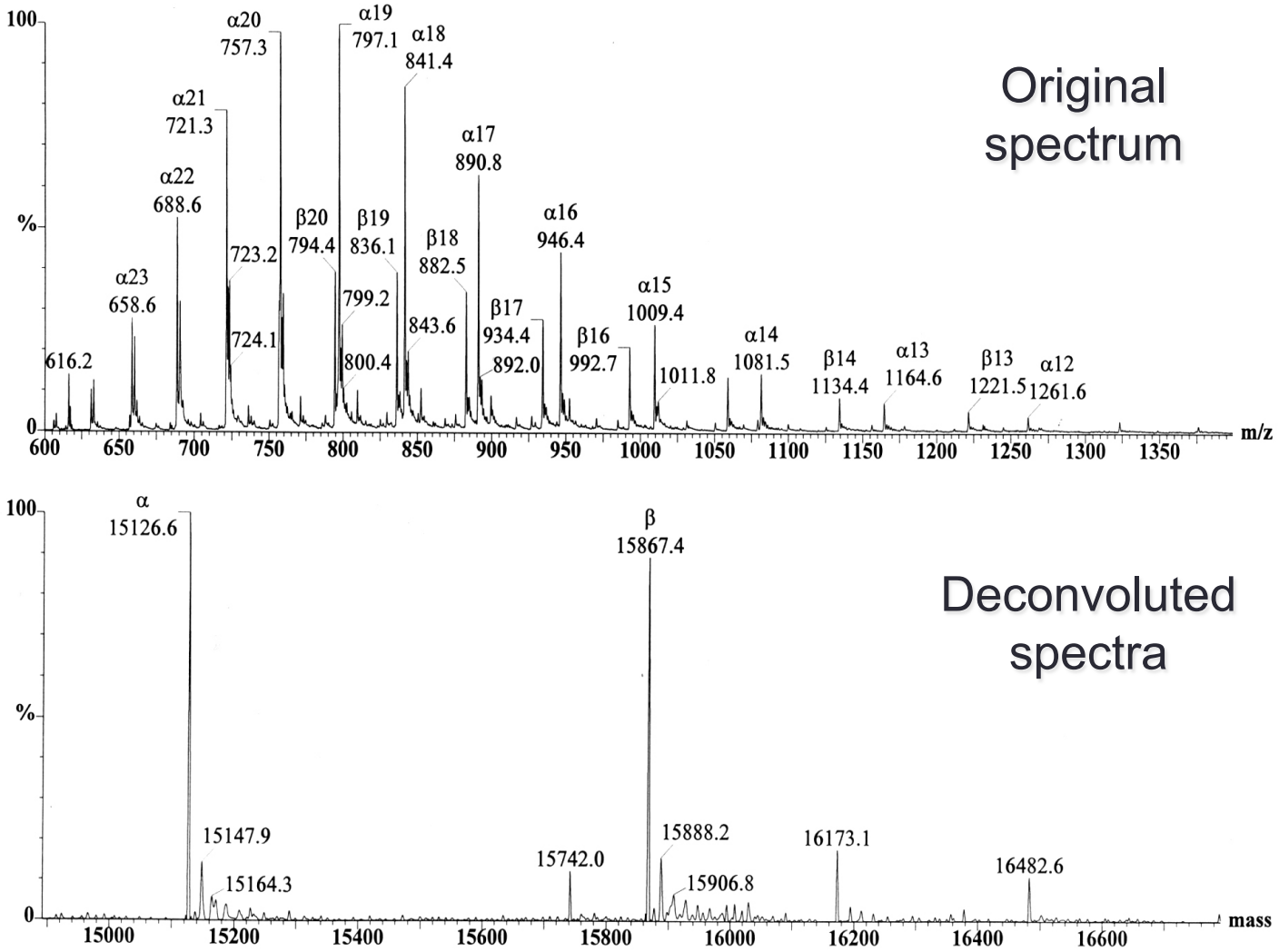
High throughput haemoglobin variant mutation analysis and protein biomarker quantitation using dried blood spots

Neil Dalton, Charles Turner & Yvonne Daniel

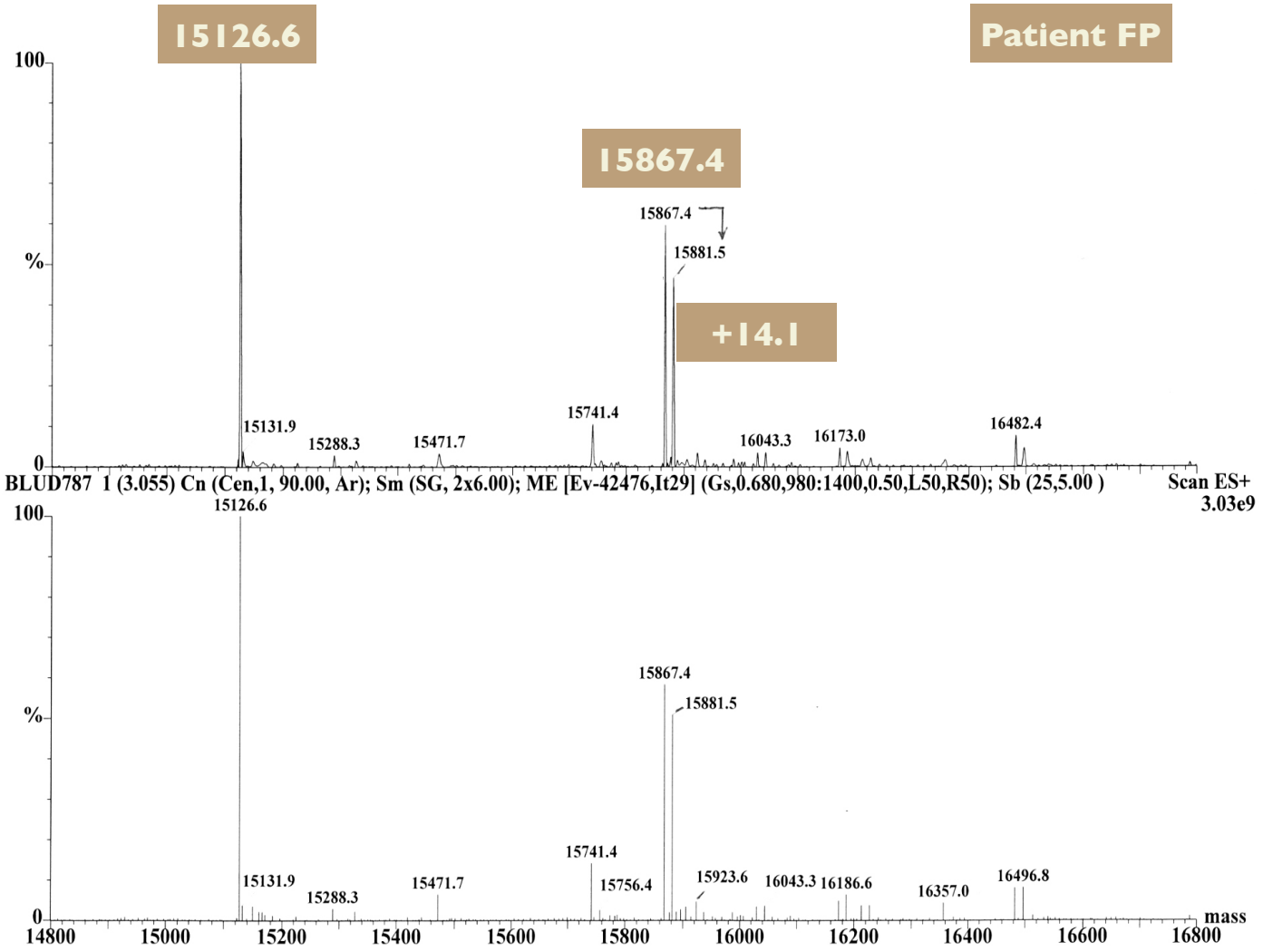
The use of Mass Spectrometry for screening and identification of the haemoglobinopathies

- MS technique based on mass differences in globin chains
- Initially used for **identification** of variants detected on screening
- Being developed as potential approach for haemoglobinopathy **screening**

ESI-MS: normal whole blood



Electrospray ionisation mass spectrometry
Hb Johnstown



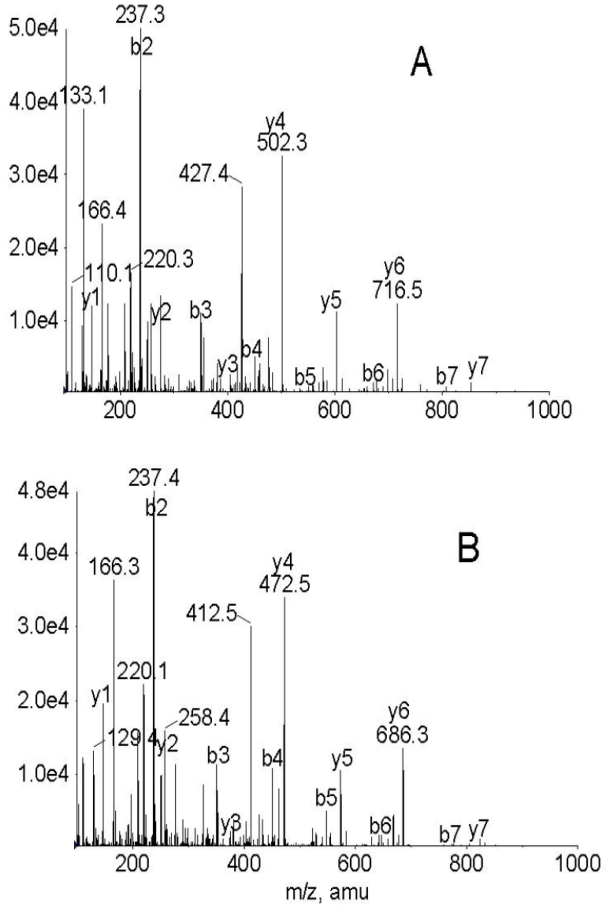
High throughput haemoglobin variant mutation analysis and protein biomarker quantitation using dried blood spots

Wild-type T1 VHLTPEEK
MW 951.5

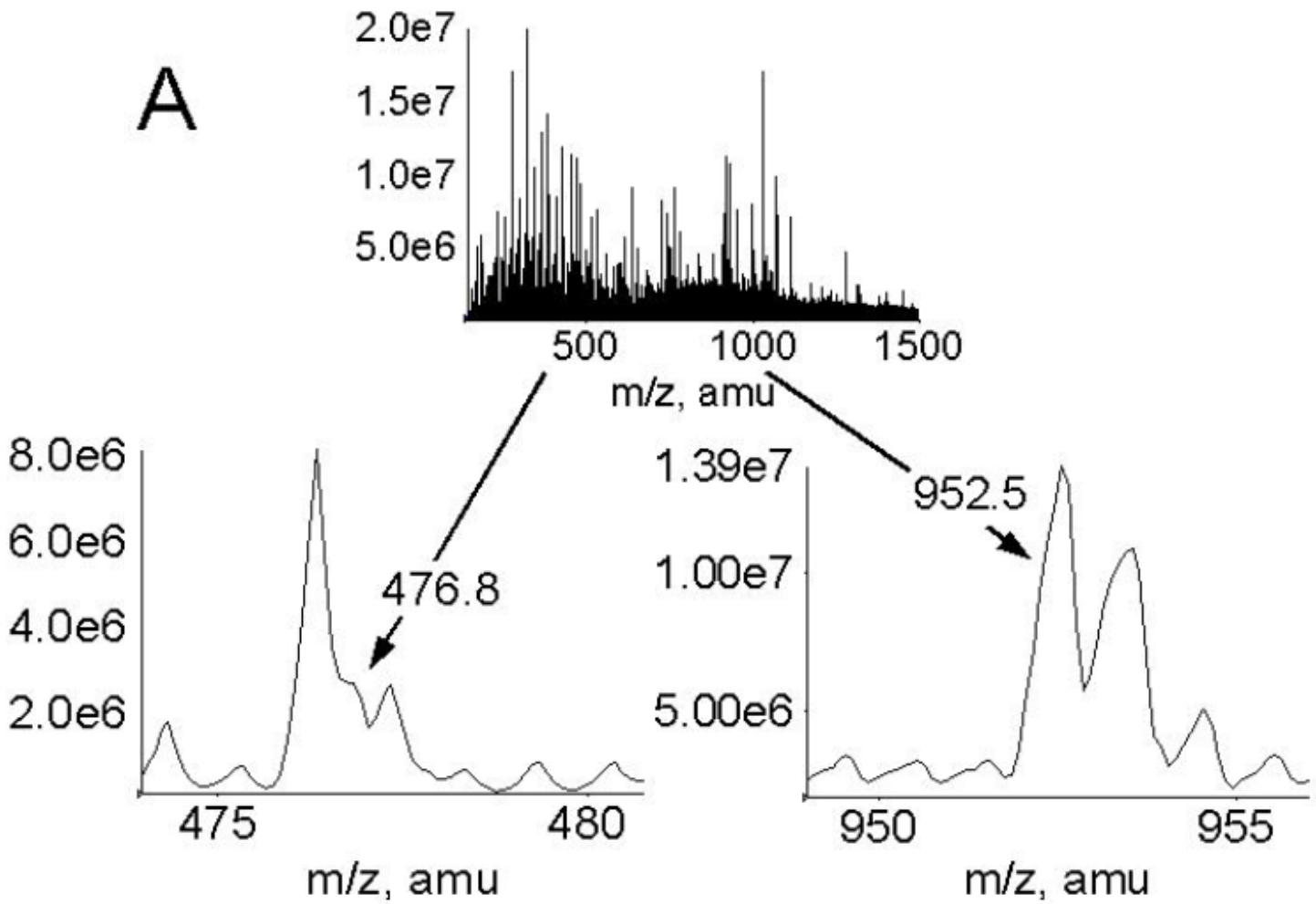
Doubly charged peptide, m/z 476.8
Product ion (y4), m/z 502.3

Sickle T1 VHLTPVEK
MW 921.5

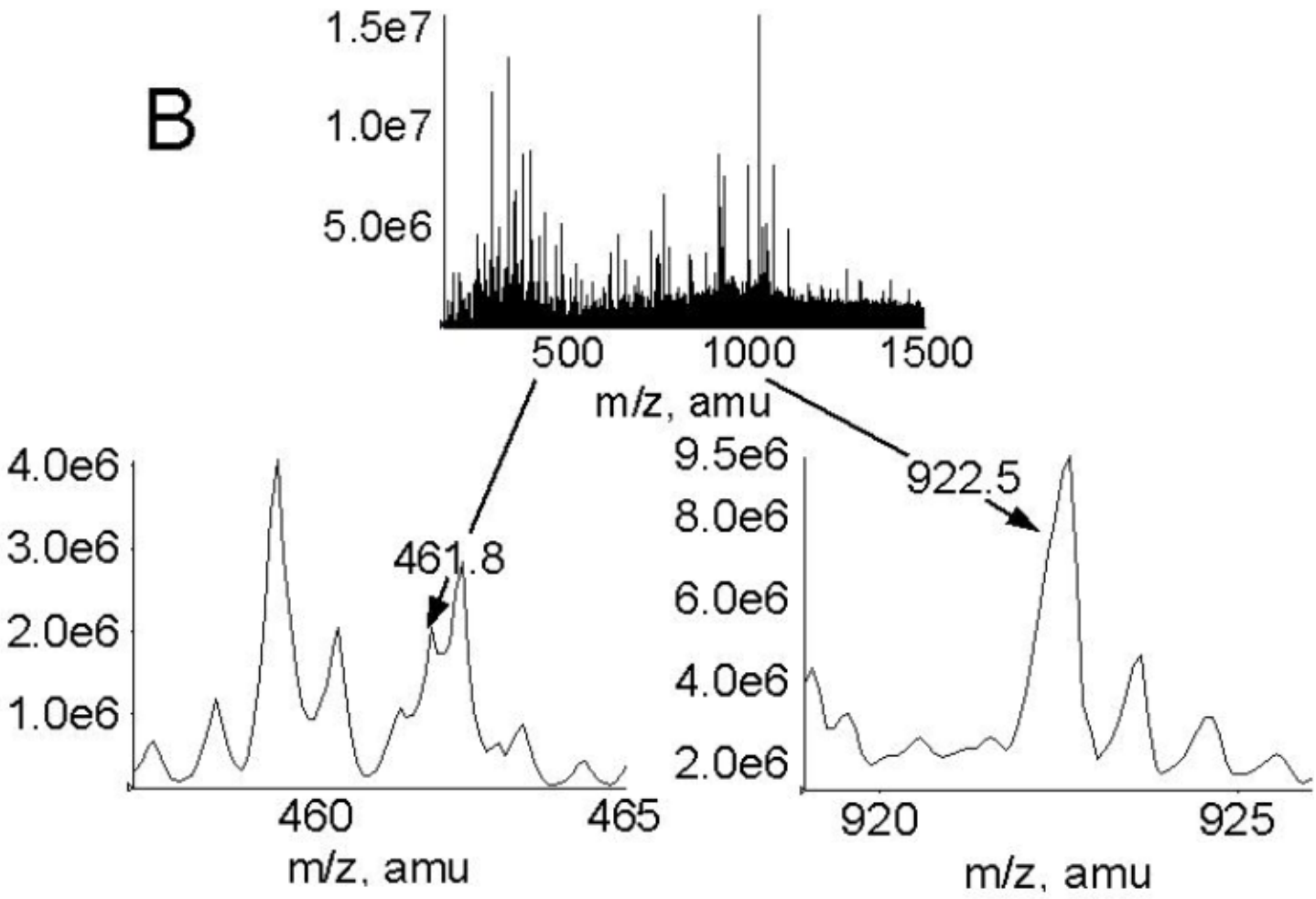
Doubly charged peptide, m/z 461.8
Product ion (y4), m/z 472.5



Wild-type βT1 isolation



Sickle βT1 isolation



High throughput haemoglobin variant mutation analysis and protein biomarker quantitation using dried blood spots

Protein/peptide quantitation: Antenatal screening for β-thalassaemia trait

HbA_2 is about 2% of total haemoglobin

4% in β-thalassaemia trait

HbA is $\alpha_2\beta_2$

HbA_2 is $\alpha_2\delta_2$

Could the δ/β ratio be used as a biomarker for β-thalassaemia trait?

What are the differences in the peptides?

	T1	T2	T3
Beta	Val-His-Leu-Thr-Pro-Glu-Glu-Lys	**Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys**	**Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg**
Delta	Val-His-Leu-Thr-Pro-Glu-Glu-Lys	**Thr-Ala-Val-Asn-Ala-Leu-Trp-Gly-Lys**	**Val-Asn-Val-Asp-Ala-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg**
	T4	**T5**	**T6**
Beta	Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg	**Phe-Phe-Glu-Ser-Phe-Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys**	Val-Lys
Delta	Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg	**Phe-Phe-Glu-Ser-Phe-Gly-Asp-Leu-Ser-Ser-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys**	Val-Lys
	T7	**T8**	**T9**
Beta	Ala-His-Gly-Lys	Lys	Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asp-Leu-Lys
Delta	Ala-His-Gly-Lys	Lys	Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asp-Leu-Lys
	T10	**T11**	**T12**
Beta	**Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys**	Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg	**Leu-Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-Lys**
Delta	**Gly-Thr-Phe-Ser-Thr-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys**	Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg	**Leu-Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-Arg**
	T13	**T14**	**T15**
Beta	**Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys**	Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys	Tyr-His
Delta	**Asn-Phe-Gly-Lys**	**Glu-Phe-Thr-Pro-Gln-Met-Gln-Ala-Ala-Tyr-Gln-Lys**	Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys
	T16		
Delta	Tyr-His		

Measurement of δ:β globin peptide ratio

- Samples subjected to tryptic digestion
- Multiple Reaction Monitoring undertaken for

  δ T2, T3 and T14 peptides

  β T2, T3 and T13 peptides

  δ:β peptide ratios calculated

Study validated the quantitative δ:β globin peptide ratio as a surrogate marker of Hb A_2

Developed within concept of National Screening Programme needs

Daniel et al 2007

Interpretation of Hb A_2 levels

Hb A_2 percentage is ***increased*** in:

- Beta thalassaemia trait
- Presence of an unstable haemoglobin
- Hyperthyroidism
- Some cases of congenital dyserythropoietic anaemia, type I
- HIV infection

- Sickle cell trait or anaemia

HPLC analysis – sickle cell trait

Normal FBC

Hb S% : 35-45

Hb A_2 may be raised

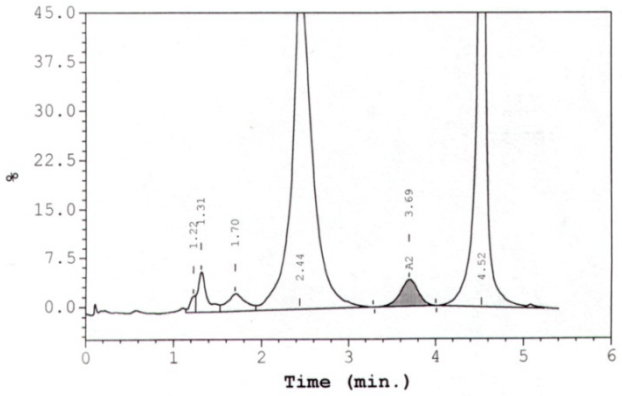
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.7	1.22	16980
P2	---	2.7	1.31	66113
P3	---	2.4	1.70	59077
Ao	---	51.0	2.44	1269933
A2	4.0*	---	3.69	87752
S-window	---	39.8	4.52	990327

Total Area: 2490183

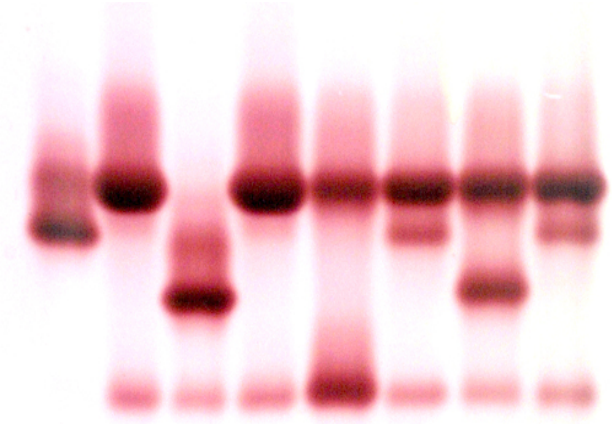
F Concentration = %
A2 Concentration = 4.0* %

*Values outside of expected ranges

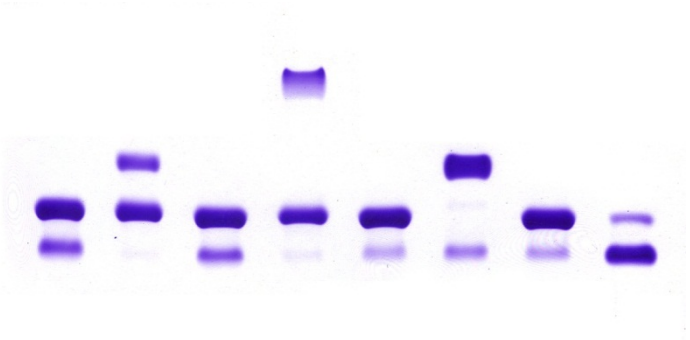
Analysis comments:



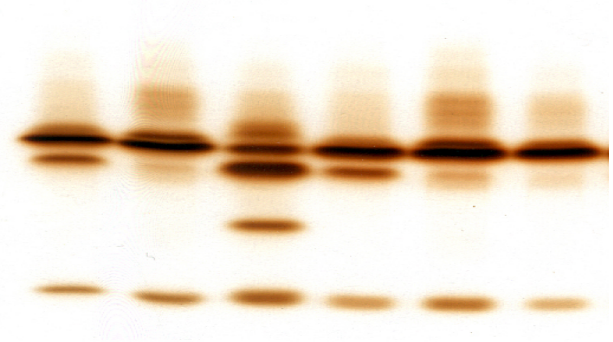
Hb Yokohama trait



FA Dad SF Dad AC RB AS RB



RB AS RB AC Dad SF Dad FA

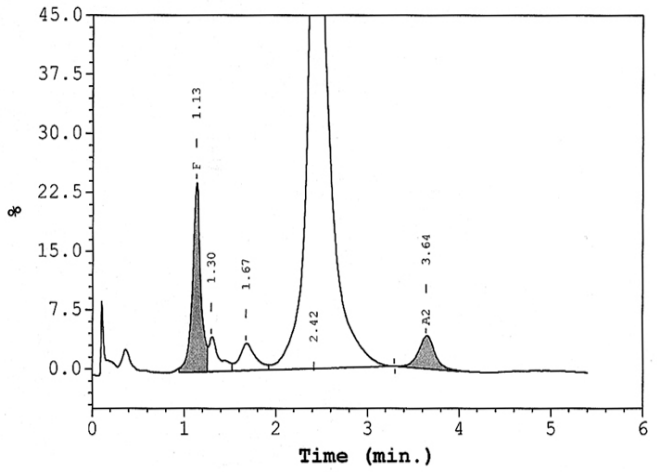


RB Dad AFSE RB Dad AA

F Concentration = 10.3* %
A2 Concentration = 4.2* %

*Values outside of expected ranges

Analysis comments:



Interpretation of Hb A_2 values

Haemoglobin A_2 percentage is decreased in

- δ thalassaemia
- Delta/beta thalassaemia
- α thalassaemia trait or haemoglobin H disease

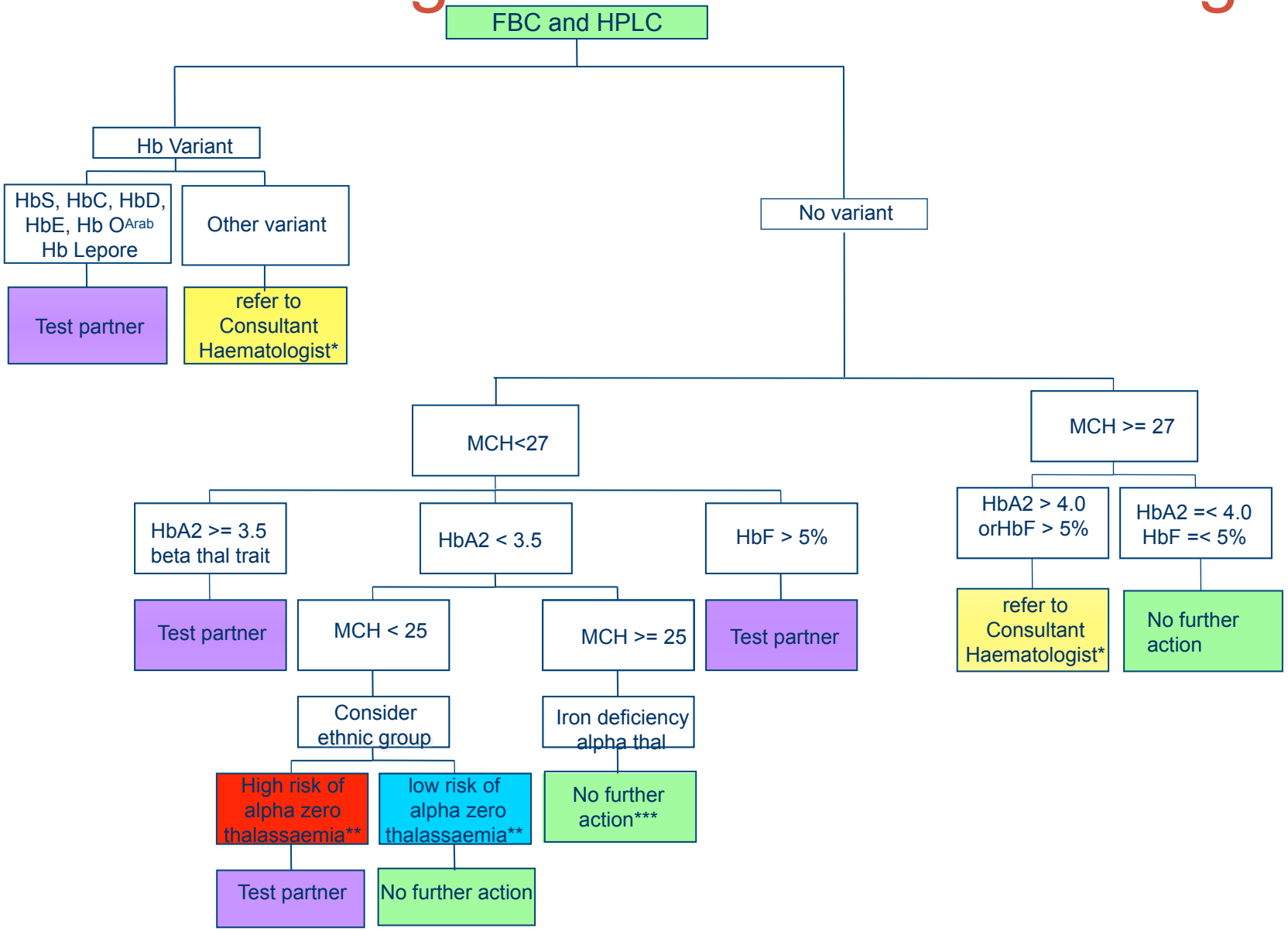
- **Severe** iron deficiency

National Sickle & Thalassaemia Screening Programme

- Established to provide a linked screening programme for antenatal women and newborn
- Universal screening
- Established laboratory standards
- Standardised reporting formats
- Standardised methodology (newborn)

- Decision algorithm (antenatal)

NSC&TSP: High Prevalence Screening



From : Haemoglobinopathy diagnosis, BJ Bain

Mutation	Origin	Usual mean Hb A_2 (%)	Usual mean MCH (pg)	Usual mean MCV (fL)
Silent β thalassaemia trait (normal MCV, MCH, and Hb A2 %)				
−101 (C → T)	Mediterranean	3.3	28	85
−92 (C → T)	Mediterranean	3.5	28	82
IVSII-844 (C → G)	Mediterranean (Italian)	3.5		85
+33 C → G [64]	Mediterranean (Greek Cypriot)	3.0	29	86
+10 (−T) [65]	Mediterranean (Greek, one case)	2.6	32	97
+1480 C → G (termination codon +6 C → G)	Mediterranean (Greek)	2.7 [62] 2.4 [61]	28	88
Almost silent β thalassaemia trait (reduced MCV, MCH, normal Hb A2 %)				
IVSI-6 (T → C)	Mediterranean*	3.5	23	71
Codon 27 (G → T) (haemoglobin Knossos†)	Mediterranean and Middle Eastern	2.1	25	71
IVSI-5 (G → A) Corfu δβ‡	Mediterranean			
IVSI-128 (T → G)	Saudi	3.5	25	70
CAP + 1 (A → C)	South Asian	3.4	25	80
Mutation not linked to β globin gene cluster [43]	Italian	1.6§	23.5§	76§
+22 G → A [66]	Turkish, Bulgarian	3.9	23.5	79
Indices typical of thalassaemia trait but Hb A2 % normal				
β Thalassaemia caused by deletion of the locus control region	Various	Normal	Typical of β thalassaemia	Typical of β thalassaemia
γδβ Thalassaemia	Various	Normal	Typical of β thalassaemia	Typical of β thalassaemia

Risk assessment:

UK National screening programme

- The following conditions will be missed:
- Silent or near silent beta thalassaemia carrier
- Possible beta thalassaemia carrier obscured by severe iron deficiency
- Alpha zero thalassaemia occurring outside of the defined at-risk family origins
- Dominant haemoglobinopathies where the woman has no haemoglobinopathy
- Any significant variant not detected by HPLC

Normal Hb A_2 β thalassaemia in Europe

Aim: To determine the extent of the problem associated with normal Hb A_2 β thalassaemia mutations

Subjects: 226 patients from Tunisia, Greece, Cyprus and UK

Criteria for selection: Hb A_2 values of 3.3-3.8%

Methods: Samples analysed by ARMS-PCR & β sequencing

Normal Hb A_2 β thalassaemia in Europe

- 22 cases were outside of the 'average' A_2 and MCH groups

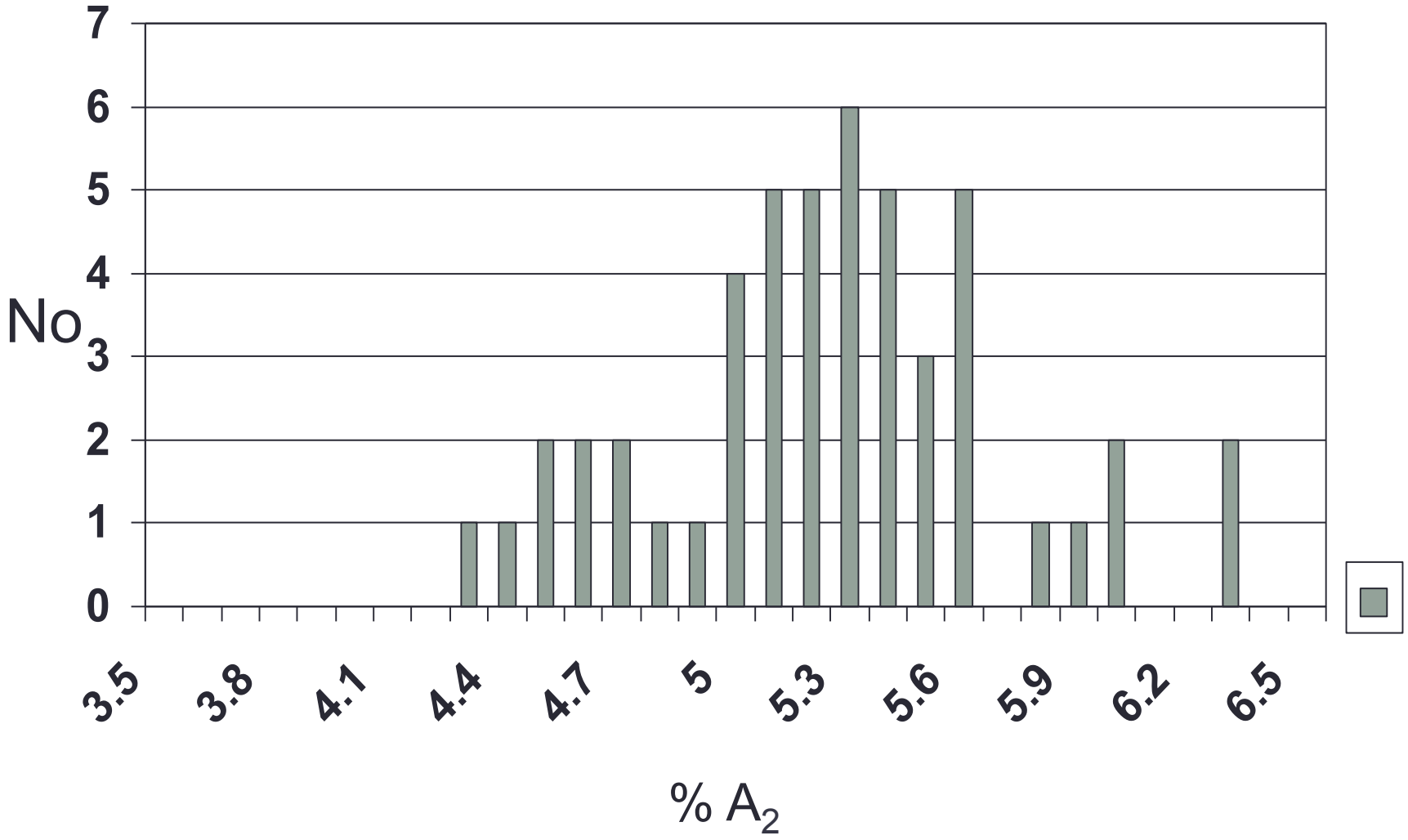
  Of these:

  All of the IVS1-6 patients had a reduced MCH

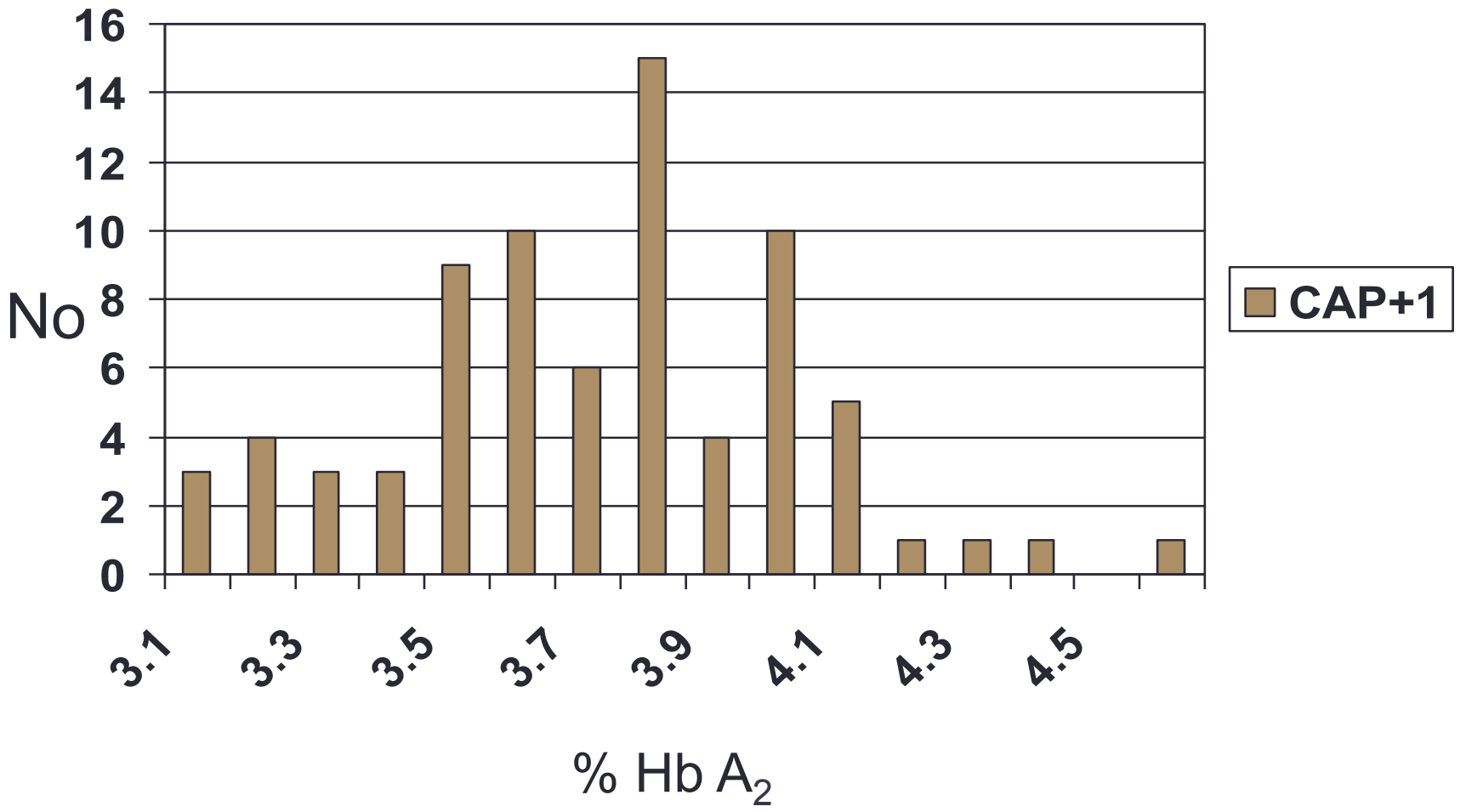
  10/13 of the CAP+1 patients had a reduced MCH

- An additional 35 patients with Hb A_2 values >3.5% gave normal β gene sequencing results

Hb A_2 values of a standard β-thalassaemia mutation (IVSI-5 G→C): 4.5% - 6.5%



Hb A_2 values of an atypical β-thalassaemia mutation (CAP+1 A→C)



Average values

mutation	cases	Hb A_2	MCH	MCV
+1480 (C→G)	18	2.9	28.2	89
-101 (C→T)	42	3.8	29.0	89
CAP+1 (A→C)	75	3.7	25.4	79
IVSI-6 (T→C)	34	4.2	22.7	72
Poly A (A→G)	10	3.9	24.7	76
Poly A (T→C)	5	4.0	22.4	73
Poly A (-AT)	2	3.8	22.7	72
Poly A (-AA)	8	4.0	23.6	73

Patients with a raised Hb A_2 and no β-thalassaemia

25 patients had a normal β-globin gene sequence

average values:	Hb A_2	MCH	MCV
	3.8	28.8	87

- **3** had MCH below 27 pg with normal α-genotype
- Possible causes: mutation in LCR or enhancer sequence

- **21** had a MCH above 27pg: Are these patients normal?
- Possible known causes:
  HIV drug treatment,
  Hyperthyroidism
- Is it the tail end of the range for normal individuals?

UKNEQAS:

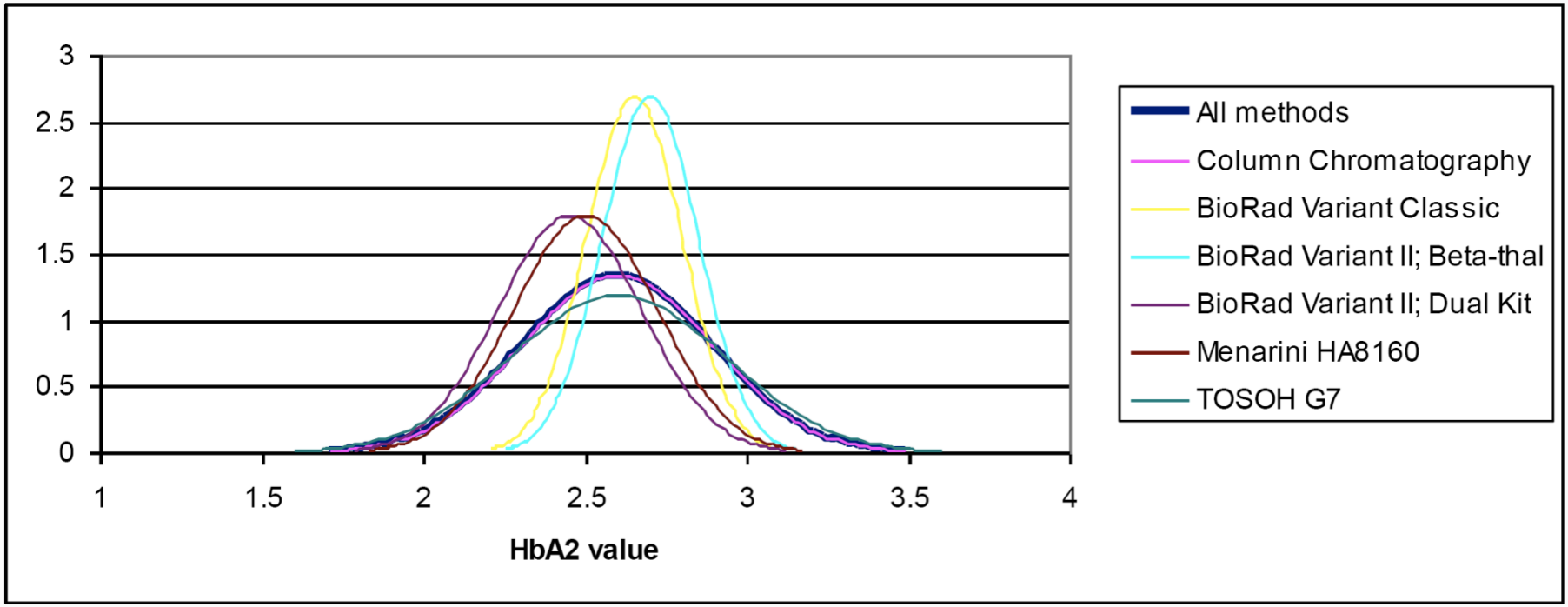
UK National External Quality Assessment Scheme

- Participants are required to give analytical results and an interpretation

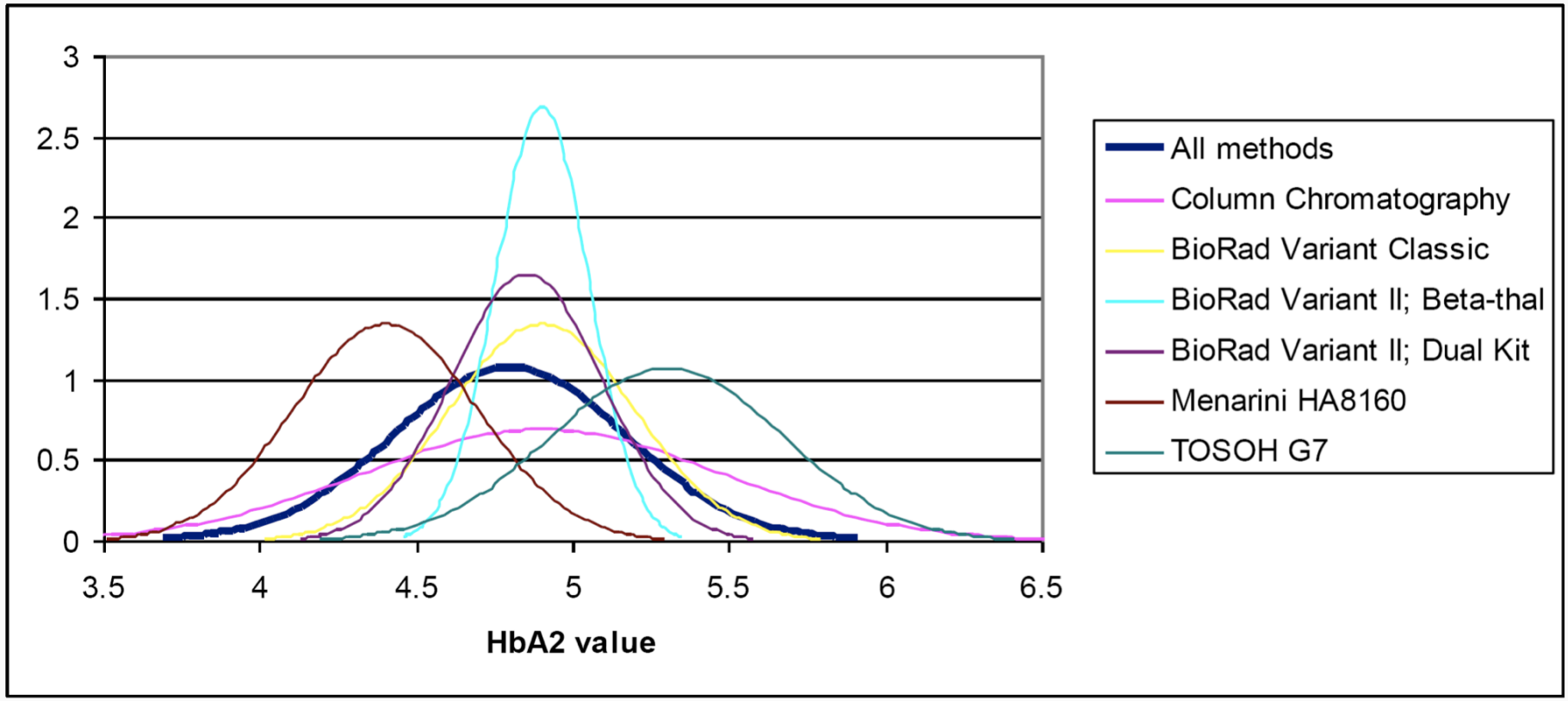
- With increase in technologies:
- Results of Hb A_2 measurement related to methodology used

- Identified differences in values obtained from different technologies and/or kits

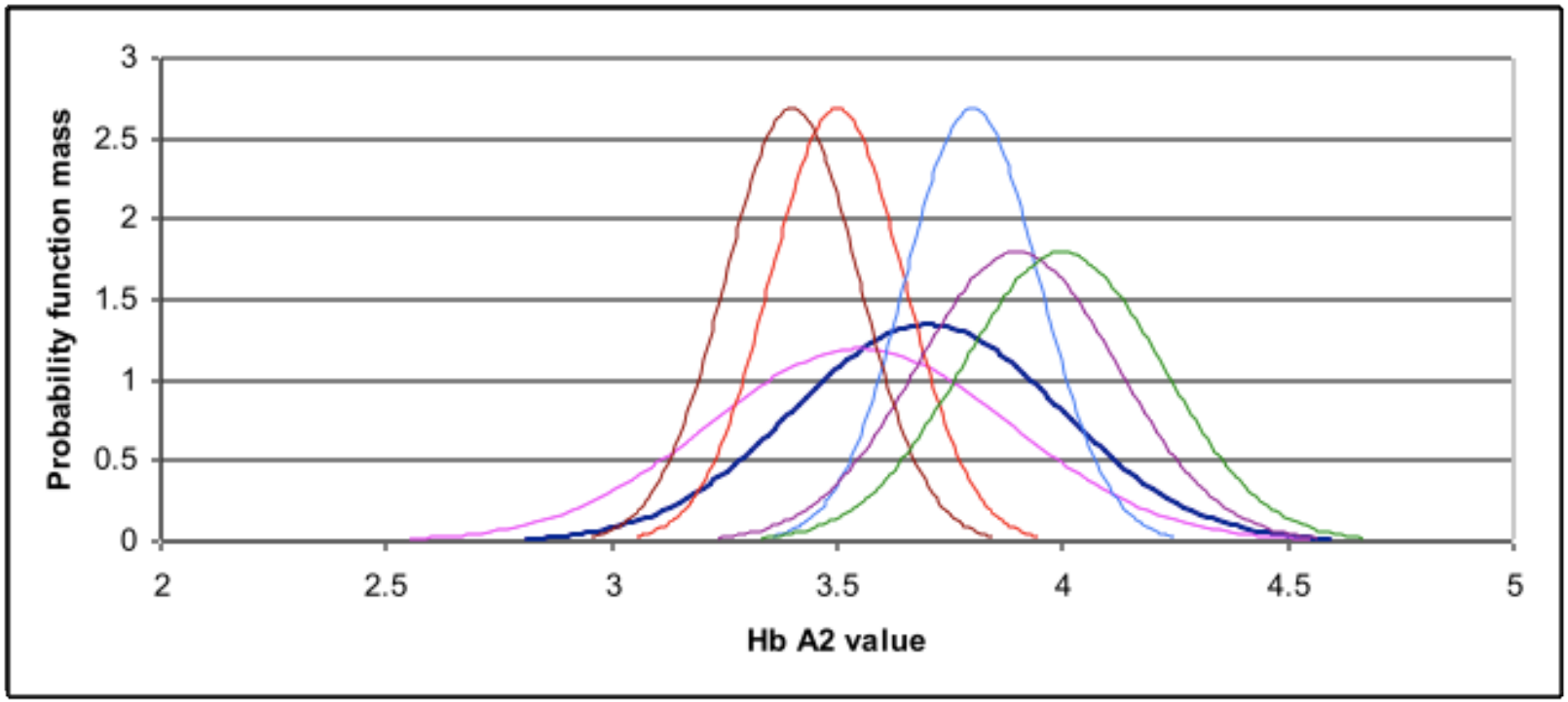
Normal sample:Hb A_2 2.6%



Beta thal trait sample: Hb A_2 4.8%



Borderline sample: Hb A_2 3.7%



Performance scoring for Hb A_2: Considerations for UKNEQAS

- Use of different normal ranges –
  variation even within same instrument group

- Use of a universal cut-off
  Instrument bias – impact on borderline values

Measurement of Hb A_2 ICSH recommendations ISLH Oct 2011

- Previous ICSH recommendations written in 1978
- Hb A_2 is measured as a percentage of haemoglobin present relative to any other haemoglobin present – not an absolute value
- Therefore analytically important to measure the A_2 and any other fractions present – separation, resolution and integration crucial
- In the presence of an Hb A_2 variant, it is the total of the normal and abnormal Hb A_2 which is significant

ICSH recommendations ISLH Oct 2011

- Fraction separation by
- Electrophoresis with elution or microcolumn chromatography
- Quantification by spectrophotometry at 415nm

- HPLC

- Capillary Zone Electrophoresis

- Capillary Isoelectric Focusing

- DNA analysis is required for the characterization of
- beta thalassaemia mutations

ICSH recommendations ISLH Oct 2011

- Measurement of the Hb A_2 alone cannot absolutely confirm or exclude the carrier state as the there may be little difference between A_2 in normals and some beta thalassaemia carriers

- Precision levels should be +/- 0.1% of the final answer (SD 0.05%)

- Common beta thalassaemia trait Hb A_2 = 4.0 - 6.0%
- Beta thalassaemia trait overall usually Hb A_2 = 3.5 - 7.0%
- Normal subjects usually Hb A_2 = 2.2-3.3%

Current developments

- Instrument calibration-use of calibrant(s)

- Target value for performance scoring:
  - all methods mean
  - method-specific mean – current target
  - submethod-specific mean

- Development of new Hb A_2 reference material

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