NHS

Screening Programmes

Sickle Cell and Thalassaemia

Tandem Mass Spectrometry for Sickle Cell and Thalassaemia Newborn Screening Pilot Study

Version 1.0

21 January 2015

Authors: Dr Yvonne Daniel and Joan Henthorn

About Public Health England

Public Health England exists to protect and improve the nation's health and wellbeing, and reduce health inequalities. It does this through world-class science, knowledge and intelligence, advocacy, partnerships and the delivery of specialist public health services. PHE is an operationally autonomous executive agency of the Department of Health.

Public Health England
Wellington House
133-155 Waterloo Road
London SE1 8UG
Tel: 020 7654 8000
www.gov.uk/phe
Twitter: @PHE_uk
Facebook: www.facebook.com/PublicHealthEngland

Published December 2015
PHE publications gateway number: 2015549

Contents

Executive Summary

The results from sites A, B and C (AB Sciex 4,000, Waters Micromass Zevo) demonstrate the method has detected all of the target haemoglobins with no false negatives. Site D (Waters Micromass Premier) demonstrated a lower sensitivity and some carriers were not detected. This instrument is no longer available for purchase. High numbers of false positive results which would require second line confirmation were a problem with Hb D^{Punjab} Carriers. The results suggest the use of Cliquid^{R} Chemoview^{TM} software (AB Sciex) may resolve this for AB Sciex users, however a different strategy will be required for Water Micromass users.

The results demonstrate that this is an acceptable method for newborn screening of haemoglobinopathies., however instruments of sufficient specificity must be used and the action values for Hb D^{Punjab} in particular require further refinement.

This report is designed to be read in conjunction with the earlier report from October 2012, (Review: Current Progress and Issues Requiring Consideration in the Use of Tandem Mass Spectrometry for Newborn Sickle Cell Screening). Please note the financial document referred to in this earlier report has been superceded.

Introduction

Newborn sickle cell screening is offered to all babies born in England following specific guidelines which designate haemoglobin variants that must be detected along with recommended screening procedures (NHS Sickle Cell and Thalassaemia Screening Programme, 2012). The aim of screening is the detection of sickling disorders where there is clear evidence that early intervention is likely to be beneficial. Thus haemoglobin variants which must be detected are Hb S, C, D^{Punjab}, O^{Arab} and E. In addition it has been agreed that other clinically significant conditions where detected, such as beta thalassaemia major, are reported to clinicians to facilitate management of the condition (NHS Sickle Cell and Thalassaemia Screening Programme, 2012). Methods currently in use for this screening are isoelectric focusing (IEF), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

Electrospray mass spectrometry-mass spectrometry (ESI-MSMS) also known as tandem mass spectrometry is currently adopted in England as the only screening technology used for detection of the inherited metabolic disorders phenylketonuria (PKU) and medium chain acyl-CoA dehydrogenase deficiency (MCADD). The procedure is automated, highly sensitive, quantitative, accurate, and suited to high volume testing. Given that MSMS is available in all newborn screening laboratories, if newborn screening for sickle cell disorders using MSMS is technically feasible, usage of this equipment could be optimised.

Following the earlier report of October 2012, (Review: Current Progress and Issues Requiring Consideration in the Use of Tandem Mass Spectrometry for Newborn Sickle Cell Screening), a pilot screening project was introduced in four newborn screening laboratories in England. The laboratories were selected on the basis of suitability of equipment and diversity of haemoglobin variants and thalassaemia cases expected. The four laboratories that participated in the pilot study were: Birmingham, Leeds, Oxford and Guy's & St Thomas', (Viapath, known as GSTS at the time of the study). The sites have been anonymised; sites A and B utilised AB Sciex (Warrington, UK) AP4000 mass spectrometers and sites C and D Waters Micromass (Manchester, UK) instruments, the Zevo TQMS and the Premier respectively. Implementation at each site was over two days with both the kit manufacturers (SpOtOn Diagnostics, day one only) and the programme laboratory advisors present. Day one involved instrument setup and optimisation, preparation and analysis of samples, data review and interpretation of results. The later continued continued on the second day. Samples were analysed as per the manufacturer's instructions at all sites. The

method incorporates a series of experiments performed simultaneously to detect: Hb S, Hb C, Hb D^{Punjab}, Hb O^{Arab}, and Hb E. The signal for the wild type beta and gamma globin is also assessed, acting as a surrogate for Hb A and Hb F. The gamma/beta wild type ratio represents the relative proportion of Hb F to Hb A. Therefore as with current procedures a high ratio suggests low Hb A and prematurity or beta thalassaemia whilst a low ratio indicates a low Hb F which may be due to age, transfusion or gamma thalassaemia. An internal standard is also included to act as a methodological control. The material is a synthetic, stable isotope labelled, sickle sequence peptide with an extension peptide, which requires tryptic activity to generate the stable isotope labelled peptide to be detected in the analysis. This is to check for the addition of reagents and activity of trypsin in each well, in addition to the MSMS performance within and between runs. Results are expressed as a ratio of variant haemoglobin divided by corresponding wild type signal. Following interpretation and comparison with existing methods the data was submitted to the laboratory advisors for statistical analysis.

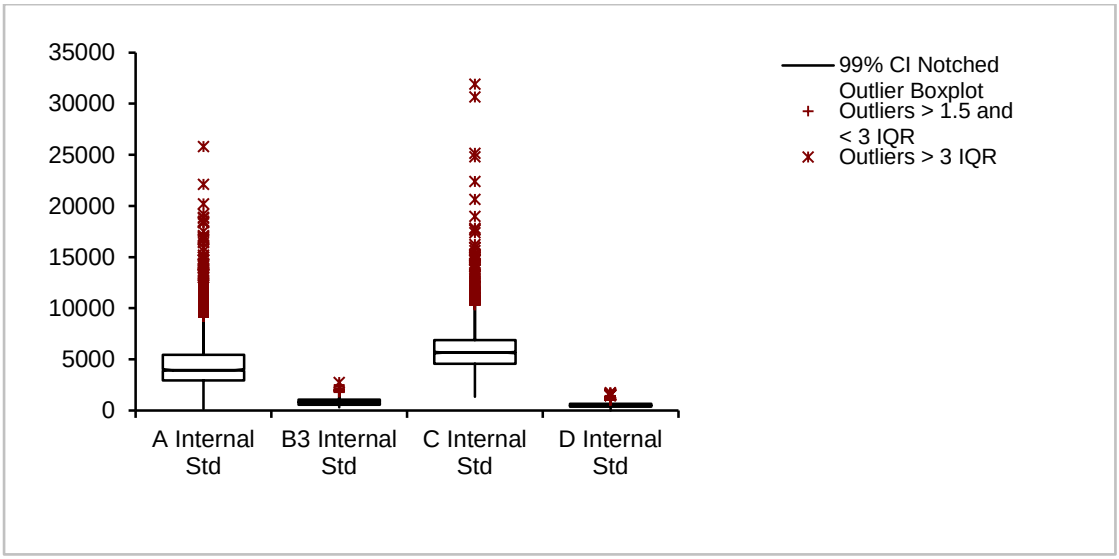
Each site has prepared a short report on the evaluation these are included in the appendix. With the exception of site B the evaluation proceeded without significant issues. At site B there were technical issues, which were initially difficult to resolve. Progress was made when Cliquid^{R} Chemoview^{TM} software (AB Sciex) was installed, resolving issues with peak integration and improving the quality of the results, but not resolving all sensitivity issues. Trouble shooting procedures continued and although the autosampler injection volume had originally been set appropriately, it was found that increasing the sample volume improved results, suggesting that the autosampler injection volume was not correctly calibrated. The results from this site are therefore divided into three categories, allowing for all three stages of the resolution process, these are shown in the general results section however in the summary slides only the results with the Chemoview^{TM} software and increased volume are shown.

One of the study aims was to set action values and acceptable criteria and these were adjusted as the study progressed therefore it was difficult to assess wastage and repeat numbers. This is one of the objectives of an extended pilot being carried out at the Leeds site.

Results

Internal standard results – all sites

Site	Number	Min/Max	Mean	99% CI	SD
A	6251	166/25800	4454.4	4379.2 – 4529.7	2308.88
B (final protocol)	2364	305/2729	841.4	824.0 – 858.3	323.21
C	7951	1350/31900	5879	5821.7 – 5936.8	1991.4
D	7332	4/1747	526.1	519.7 – 532.4	210.71



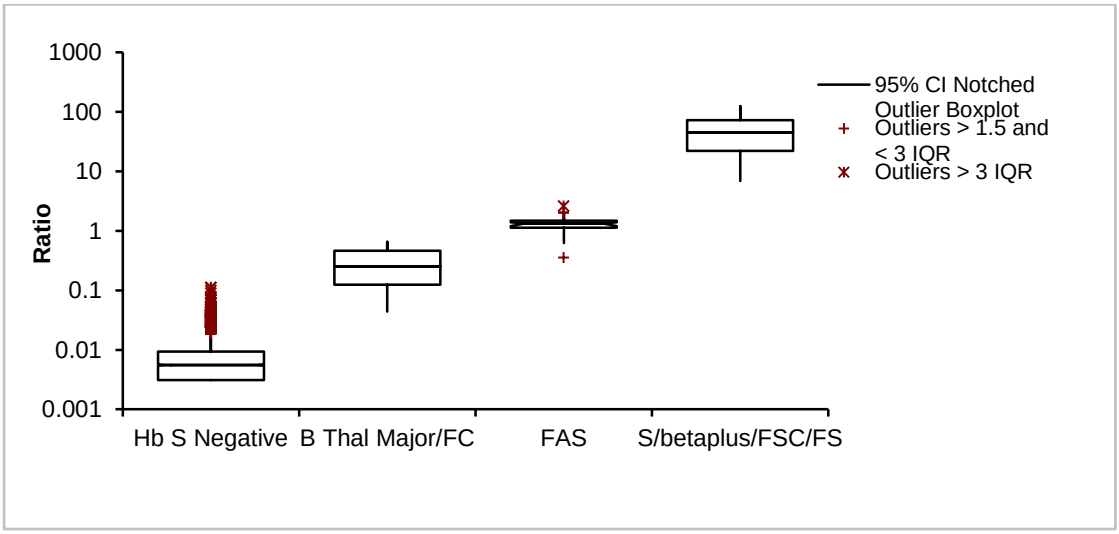
The table and graph show the results obtained for the internal standard at each of the sites. All results are calculated as area under peak with the exception of site B. Site B utilized the Cliquid Chemoview™ software (AB Sciex) which derives an average ion count. Site B data is therefore not comparable to the other three sites. Site D (Waters Premier) shows a significantly reduced signal (mean = 526) compared to the other two sites (AB Sciex 4000, Waters Xevo TQS).

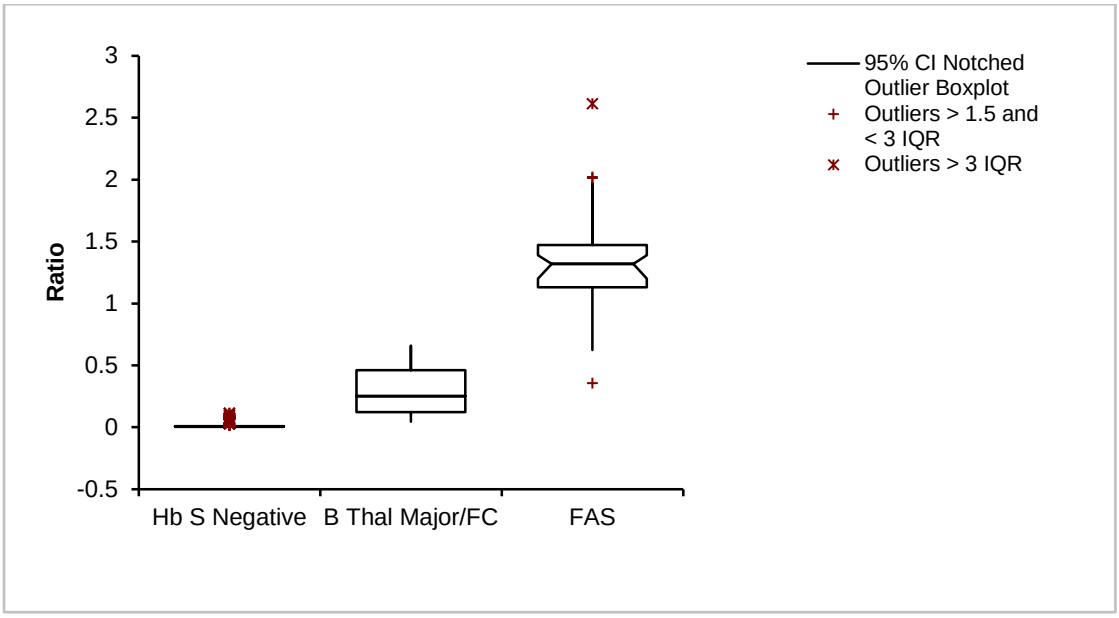
Site A results

This section shows the results obtained for site A operating an AB Sciex 4000 without Cliquid^{R} Chemoview^{TM} software (AB Sciex). A total of 6,251 samples were reported for evaluation.

Site A: Hb S

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	6188	0.00/0.11	0.007	0.0071 – 0.0075	0.007
B Thal Major/FC	6	0.04/0.66	0.30	0.063 – 0.53	0.22
FAS	52	0.35/2.61	1.33	1.23 – 1.44	0.37
Sbeta plus/FSC/FS	5	6.87/124	50.19	-4.64 - 105	44.17



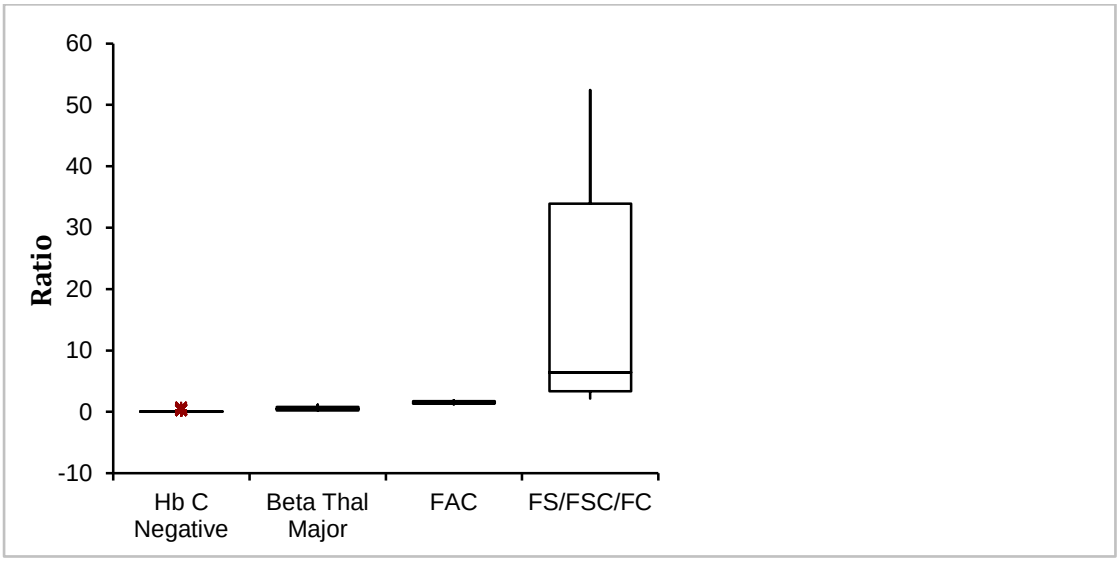


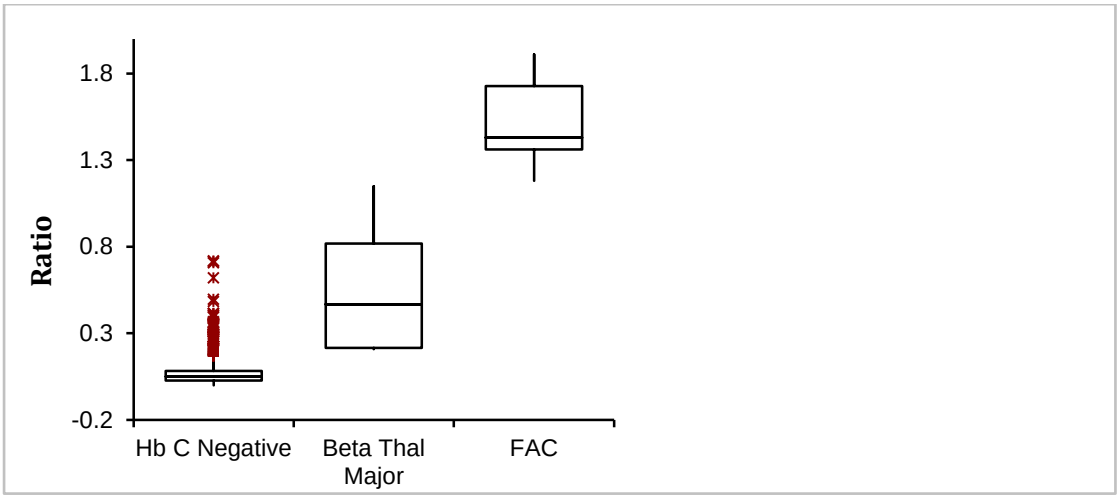
The results shown indicate the excellent discrimination between samples negative for Hb S and those with sickle cell disease. Six samples (five beta

thalassaemia major, one FC) lacked wild type beta T1 and therefore some gave ratios that fell into the FAS range. There were no false negatives.

Site A: Hb C

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	6230	0.00/0.718	0.062	0.061 – 0.063	0.052
Beta Thal Major	5	0.21/1.15	0.54	0.057 – 1.02	0.39
FAC	11	1.18/1.91	1.51	1.36 – 1.66	0.22
FS/FSC/FC	5	2.15/52.4	17.93	-8.48 – 44.34	21.27

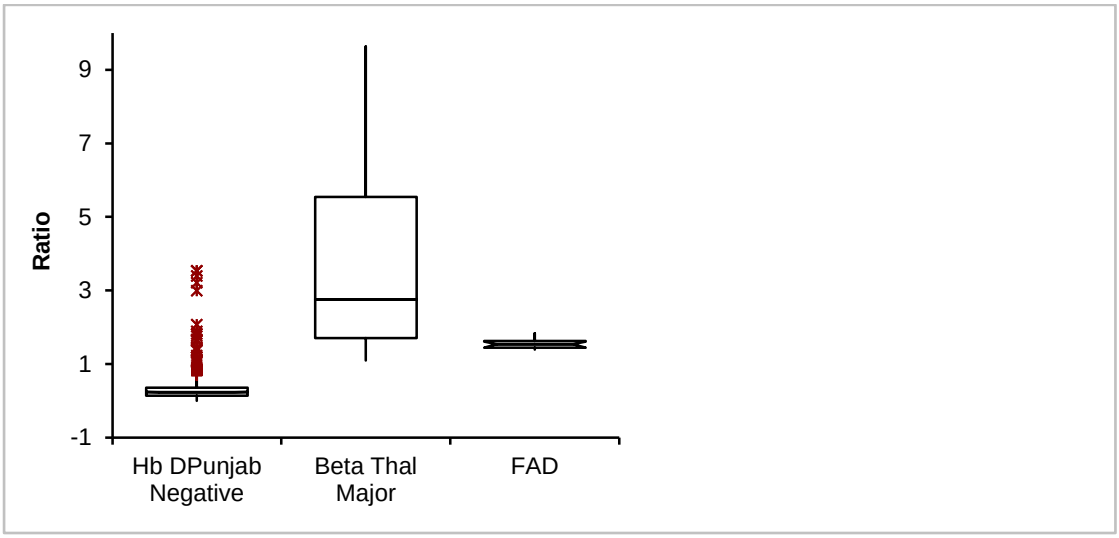


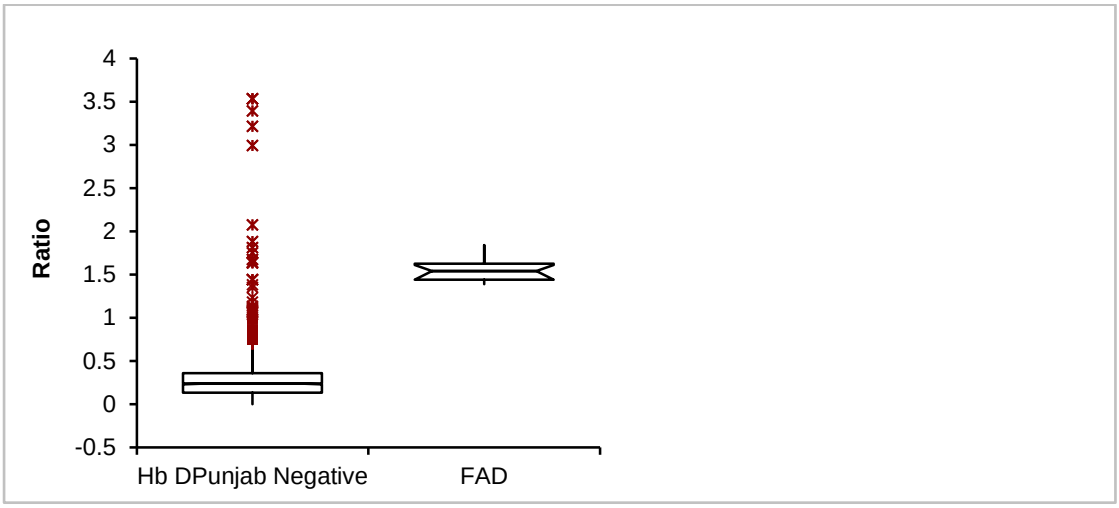


The results shown indicate the good discrimination between samples negative for Hb C and positive for Hb C. Samples lacking wild type beta T1, again generated some ratios that just fell into the FAC range. There were no false negatives.

Site A: Hb D^{Punjab}

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb D^{Punjab} Negative	6229	0.00/3.53	0.27	0.263 – 0.273	0.20
Beta Thal Major	5	1.10/9.63	3.79	-0.39 – 7.99	3.38
FAD^{Punjab}	17	1.39/1.84	1.55	1.49 – 1.62	0.13



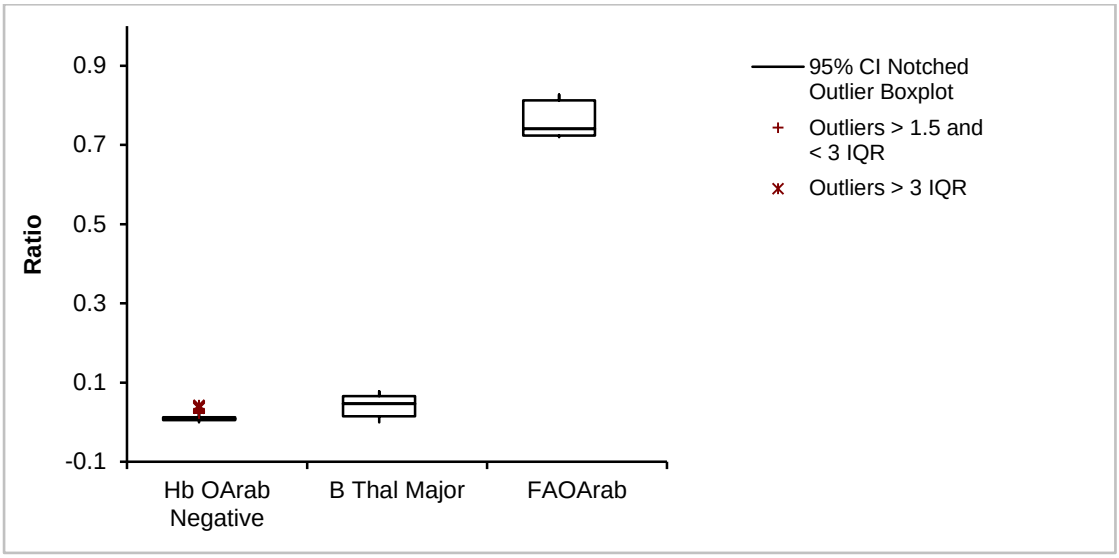


The results shown indicate that the discrimination between samples negative for Hb

D^{Punjab} and positive for Hb D^{Punjab} was not complete. As previously samples lacking the corresponding wild type beta, generated some ratios that fell into the FAD^{Punjab} range. There were no false negatives. Please note that FAD in the graph refers to FAD^{Punjab}.

Site A: Hb O^{Arab}

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	6243	0.00/0.04	0.009	0.008 – 0.009	0.005
Beta Thal Major	5	0.00/0.08	0.041	0.034 – 0.079	0.031
FAO^{Arab}	3	0.72/0.83	0.76	0.62 – 0.90	0.056

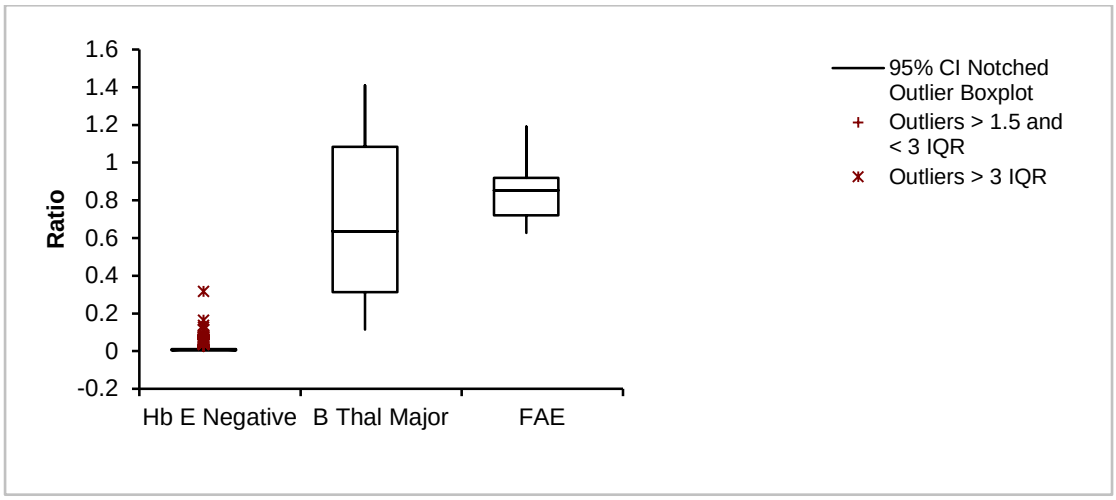


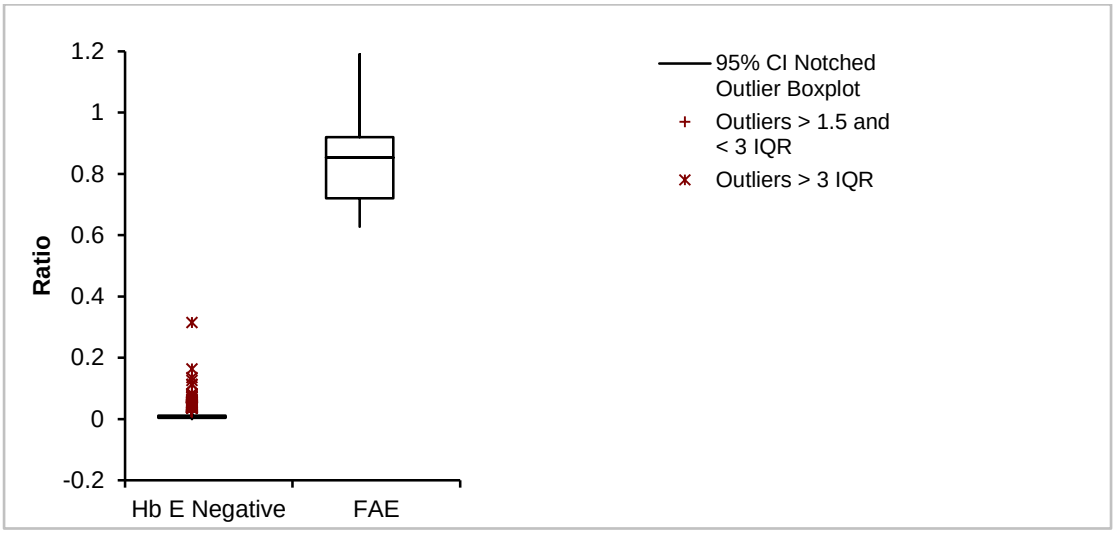
The results shown indicate the excellent discrimination between samples negative for Hb

O^{Arab} and positive for Hb O^{Arab}. Unlike the previous experiments samples lacking the corresponding wild type beta, did not generate ratios that fell into the FAO^{Arab} range. There were no false negatives.

Site A: Hb E

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	6234	0.00/0.32	0.009	0.0089 – 0.0094	0.0098
Beta Thal Major	7	0.11/1.41	0.70	0.25 – 1.14	0.48
FAE	10	0.63/1.19	0.85	0.73 – 0.97	0.17

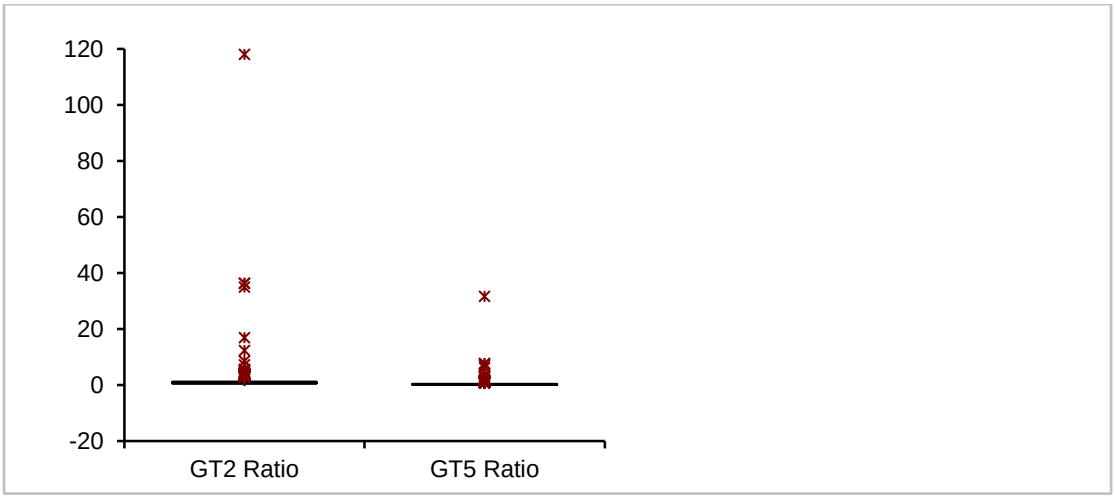




The results shown indicate the excellent discrimination between samples negative for Hb E and positive for Hb E. As previously samples lacking the corresponding wild type beta, generated some ratios that fell into the FAE range. Note the beta thalassaemia major category includes two results with concomitant Lepore/beta thalassaemia. There were no false negatives.

Site A: Gamma

Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	6251	0.00/118.0	0.96	0.916 – 1.00	1.77
Gamma T5	6251	0.00/31.6	0.25	0.241 – 0.264	0.47



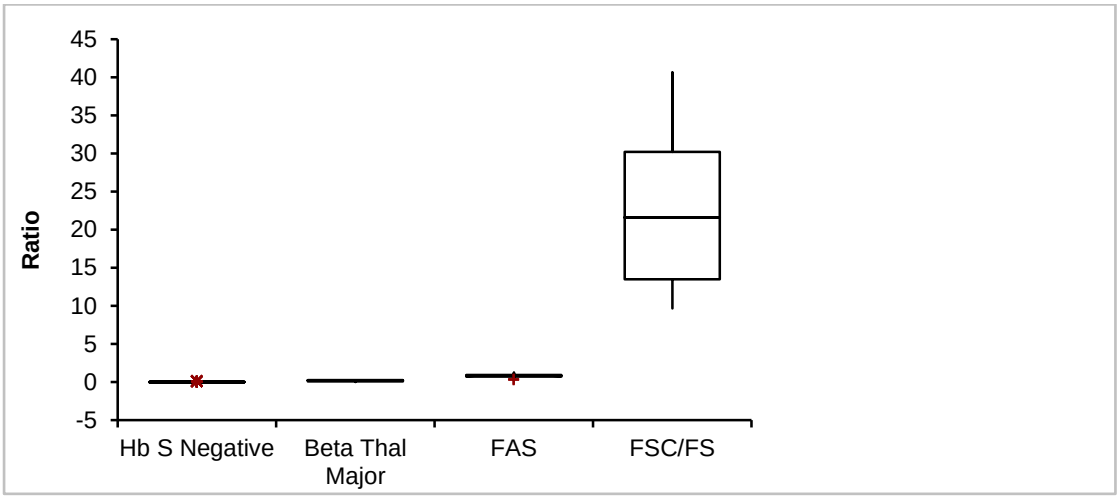
Gamma/beta wild type ratios for the T2 and T5 peptides beta thalassaemia major results were detected using the ratios however as with existing methods some premature babies also gave high ratios. Range for beta thalassaemia major T2; 7.15 – 118, T5; 2.98 – 31.60

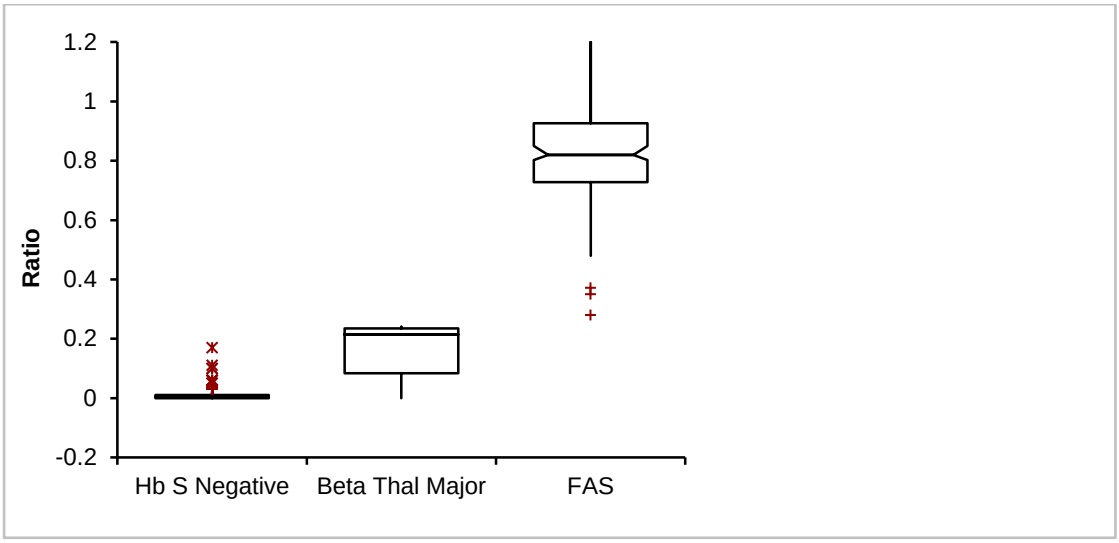
Site B results

This section shows the results obtained for site B operating an AB Sciex 4000, the results are divided into three sections, without Cliquid^{R} Chemoview^{TM} software, with Cliquid^{R} Chemoview^{TM} software and original sample injection volume and with Cliquid^{R} Chemoview^{TM} software (AB Sciex) and increased sample injection volume. A total of 5,411 samples were reported for evaluation.

Site B: Pre-Chemoview Hb S

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	2283	0.00/0.17	0.006	0.005 – 0.006	0.008
B Thal Major	4	0.00/0.24	0.17	-0.012 – 0.35	0.11
FAS	195	0.28/1.20	0.83	0.80 – 0.85	0.16
FSC/FS	13	9.65/40.60	22.54	16.64 -28.44	9.76

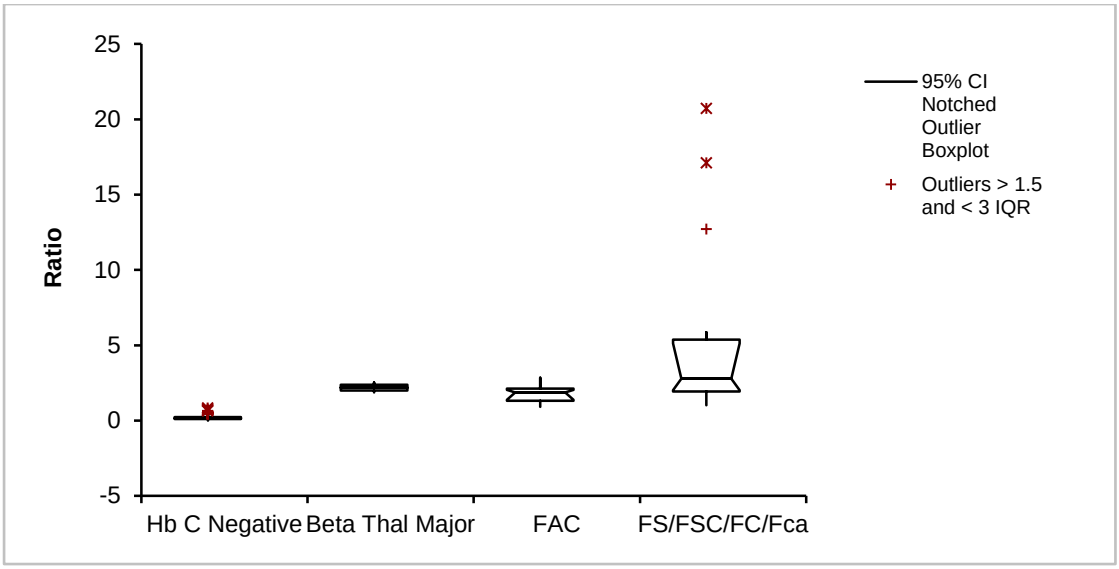


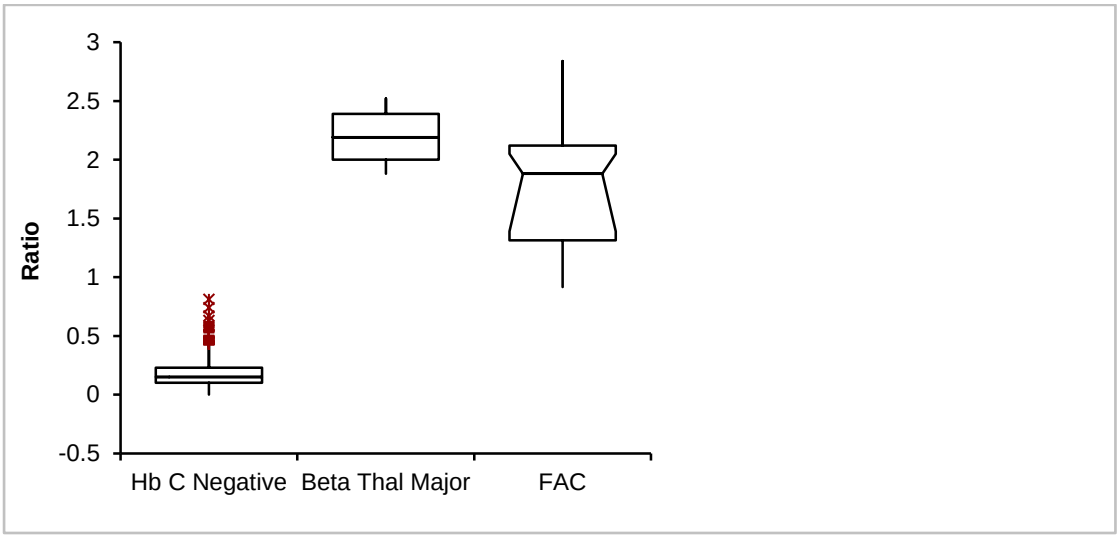


The results shown indicate the good discrimination between samples negative for Hb S and those with sickle cell disease, however the discrimination between samples negative for Hb S and sickle cell carriers was not as good as site A. As previously samples lacking the corresponding wild type beta, generated some ratios that fell into the FAS range. There were no false negatives.

Site B: Pre-Chemoview Hb C

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	2442	0.00/0.81	0.18	0.17 – 0.18	0.10
Beta Thal Major	4	1.88/2.52	2.20	1.78 – 2.61	0.26
FAC	32	0.92/2.84	1.76	1.58 – 1.94	0.51
FS/FSC/FC/FCa	17	1.02/20.70	5.27	2.27 – 8.28	5.84



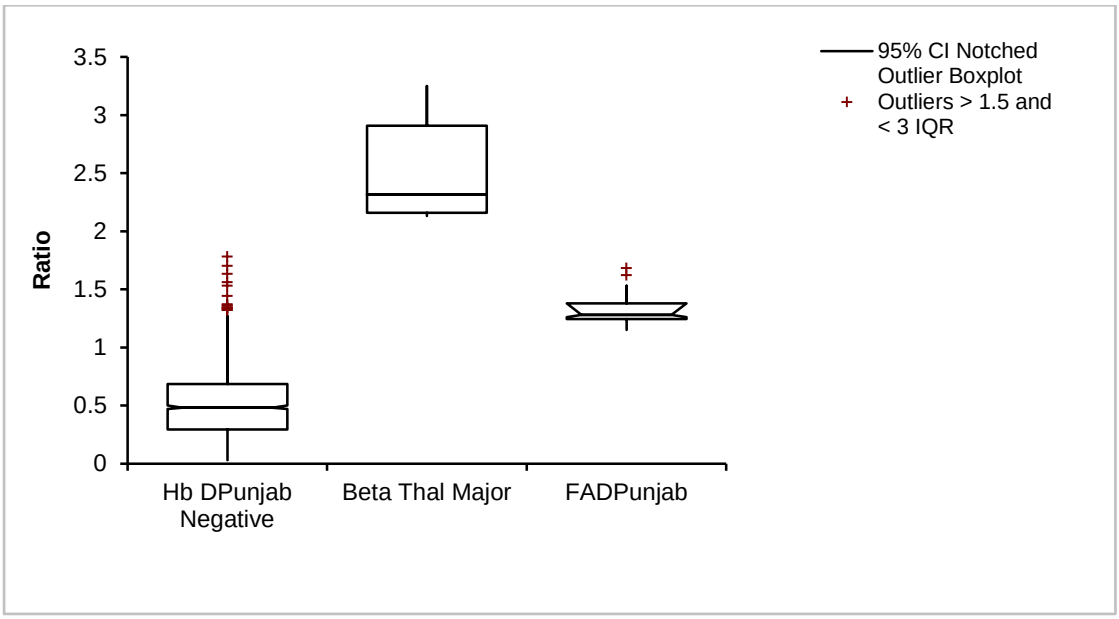


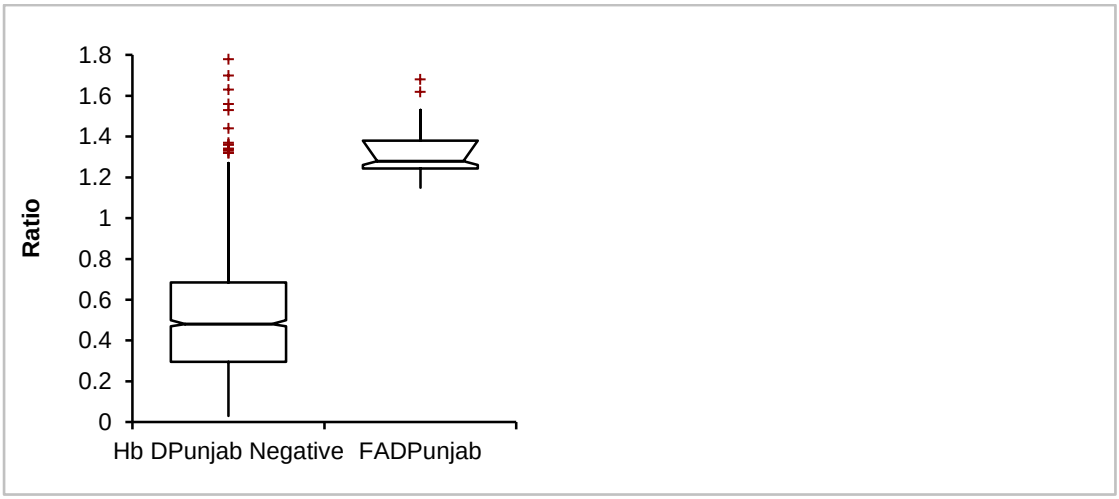
The results shown indicate the good discrimination between samples negative for Hb C and positive for Hb C. As previously samples lacking the

corresponding wild type beta, generated ratios that fell into the FAS range. There were no false negatives.

Site B: Pre-Chemoview Hb D^{Punjab}

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb D^{Punjab} Negative	2462	0.03/1.78	0.51	0.50 – 0.52	0.27
Beta Thal Major	4	2.13/3.25	2.50	1.68 – 3.32	0.51
FAD^{Punjab}	29	1.15/1.68	1.32	1.27 – 1.37	0.12



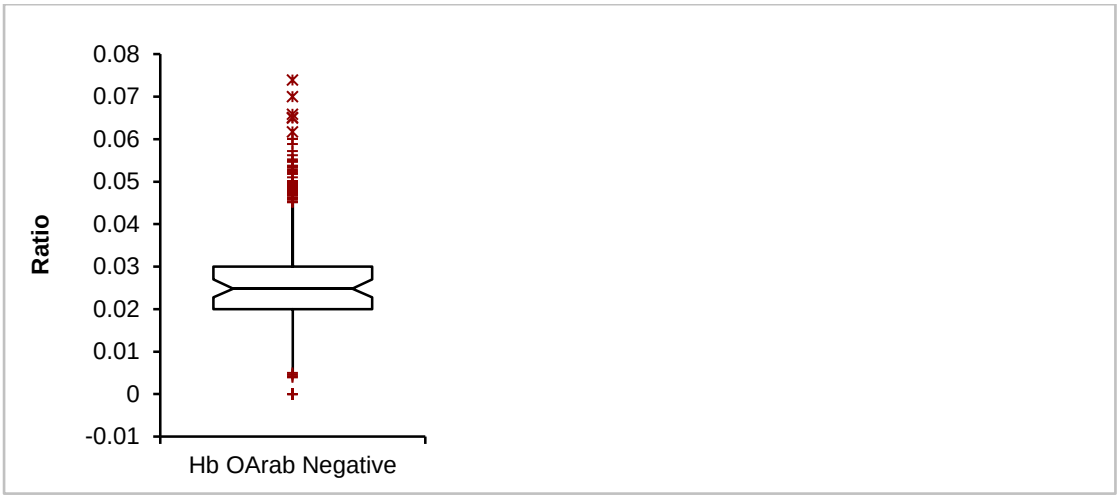


The results shown indicate that the discrimination between samples negative for Hb

D^{Punjab} and positive for Hb D^{Punjab} was not complete. As previously samples lacking the corresponding wild type beta, generated ratios that fell into the FAD^{Punjab} range. There were no false negatives.

Site B: Pre-Chemoview Hb O^{Arab}

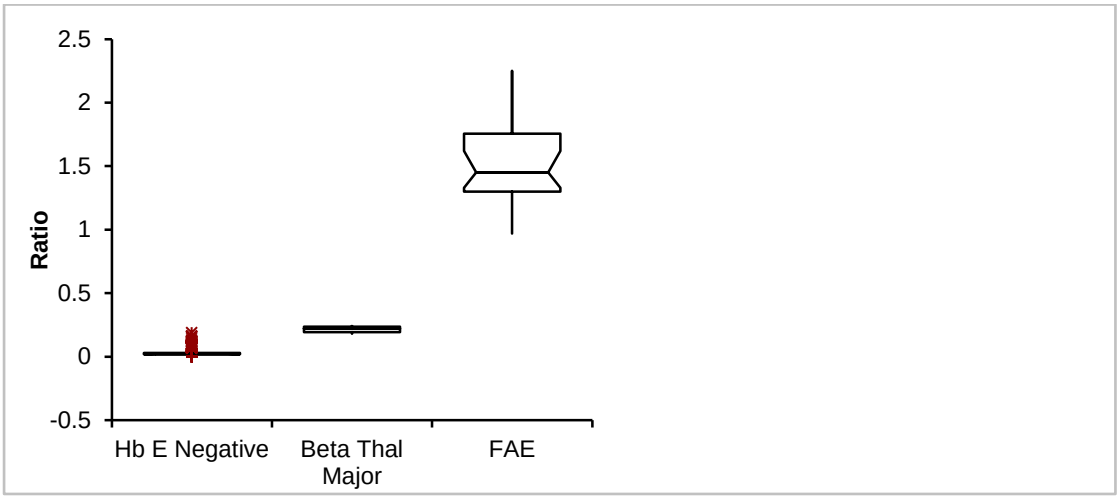
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	2495	0.00/0.07	0.026	0.025 – 0.026	0.010
FAO^{Arab}	0	NA	NA	NA	NA



No positive samples processed, however all results fell within proposed 'action values'.

Site B: Pre-Chemoview Hb E

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	2462	0.00/0.19	0.026	0.026 – 0.027	0.017
Beta Thal Major	4	0.18/0.24	0.22	0.17 – 0.26	0.027
FAE	29	0.97/2.25	1.52	1.41 – 1.64	0.31

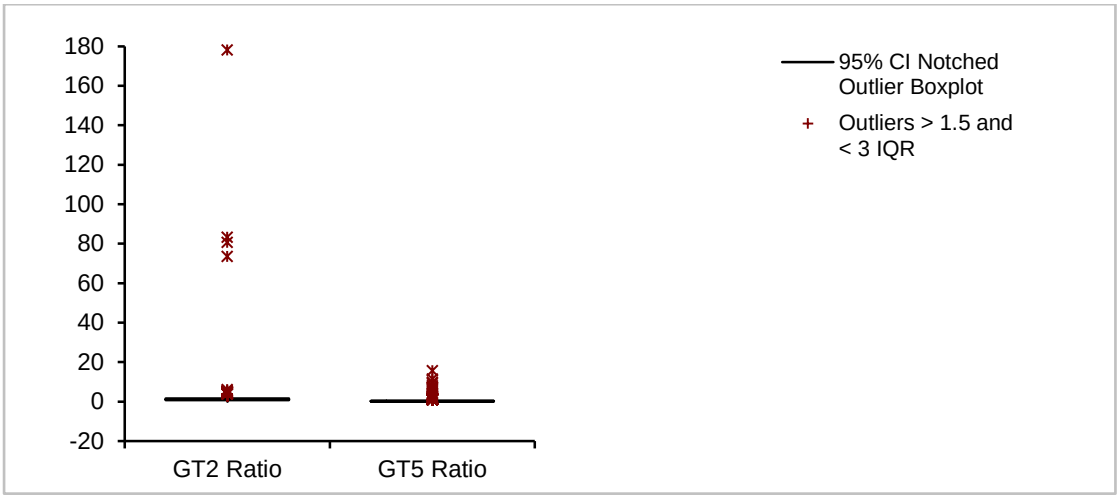


The results shown indicate the excellent discrimination between samples negative for Hb E and positive for Hb E. Samples lacking the corresponding

wild type beta did not generate ratios that fell into the FAE range. There were no false negatives.

Site B: Pre-Chemoview Gamma

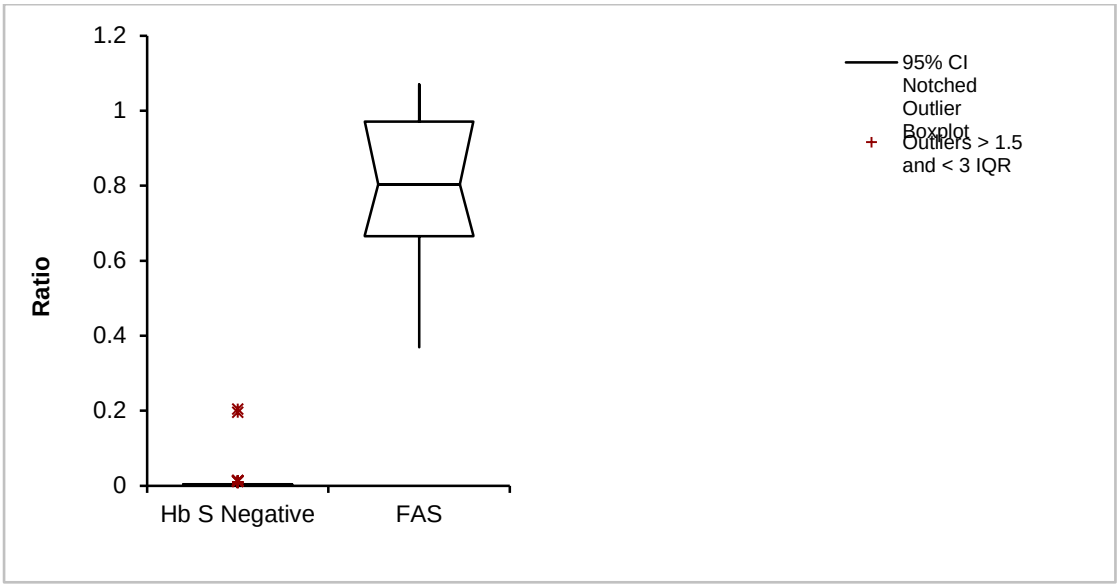
Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	2495	0.01/178.0	1.35	1.17 – 1.53	4.50
Gamma T5	2495	0.00/15.6	0.28	0.26 – 0.31	0.61



Gamma/beta wild type ratios for the T2 and T5 peptides beta thalassaemia major results were detected using the ratios however as with existing methods some premature babies also gave high ratios. Range for beta thalassaemia major T2; 73.4 – 178, T5; 7.24 – 15.60.

Site B: Post-Chemoview with Original Volume Hb S

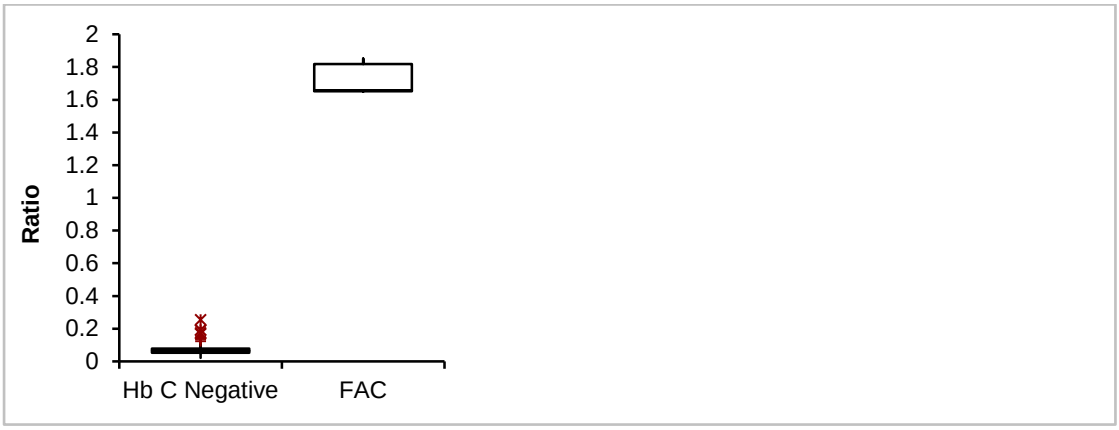
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	534	0.001/0.20	0.004	0.003 – 0.005	0.012
FAS	18	0.37/1.07	0.81	0.71 – 0.90	0.19



The results shown indicate the improved discrimination between samples negative for Hb S and positive for Hb S. There were no false negatives.

Site B: Post-Chemoview with Original Volume Hb C

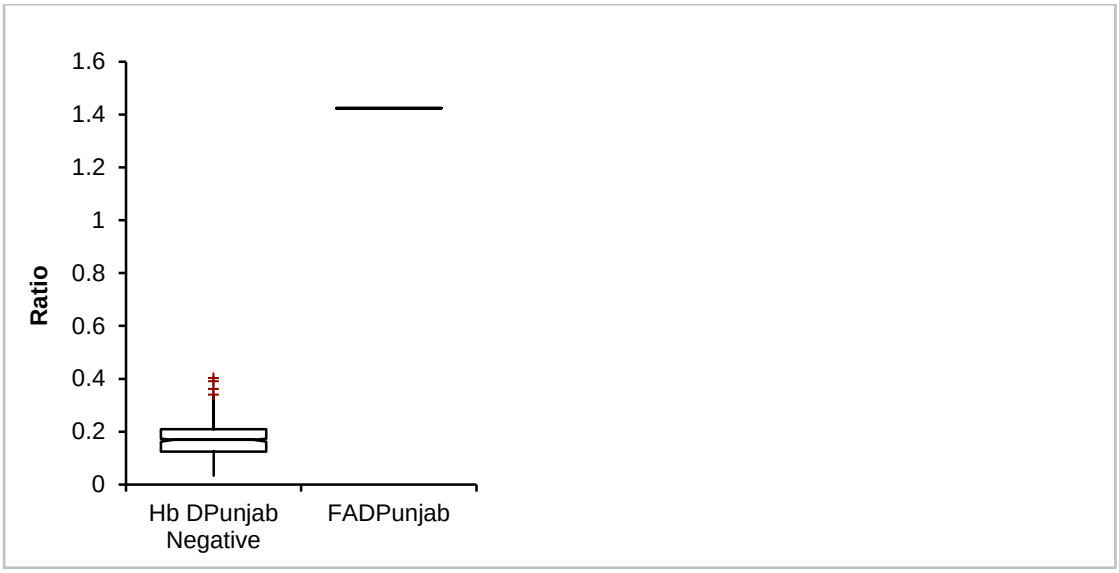
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	549	0.022/0.25	0.068	0.066 – 0.067	0.023
FAC	3	1.6/1.85	1.72	1.43 – 2.00	0.11



The results shown indicate the significantly improved discrimination between samples negative for Hb C and positive for Hb C. There were no false negatives.

Site B: Post-Chemoview with Original Volume Hb D^{Punjab}

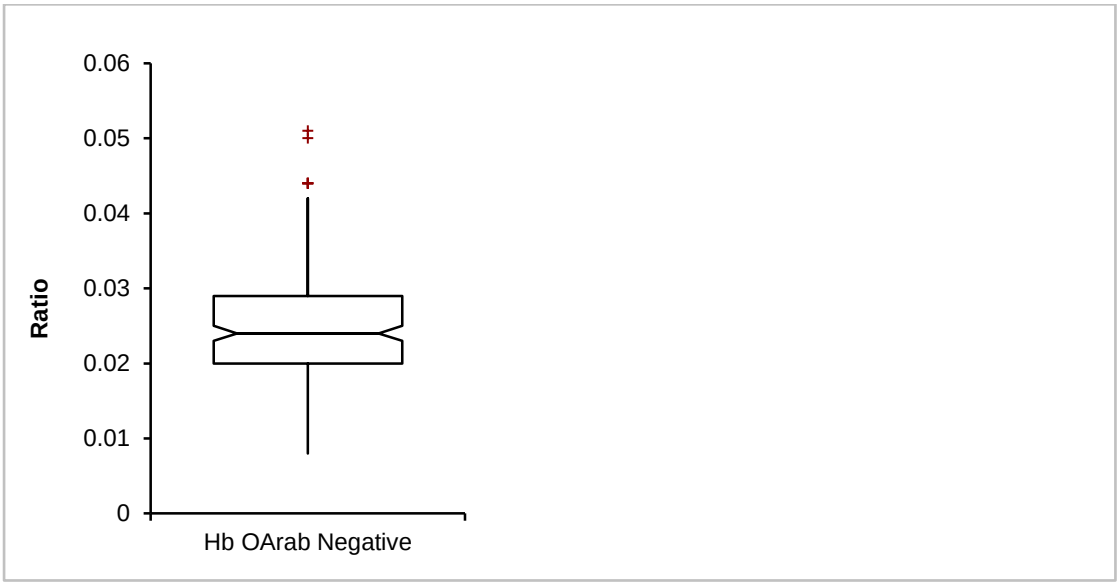
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb DPunjab Negative	551	0.033/0.40	0.17	0.17 – 0.18	0.062
FADPunjab	1	NA	NA	NA	NA



The results shown suggest improved discrimination between samples negative for Hb D^{Punjab} and positive for Hb D^{Punjab}, although numbers are low. There were no false negatives.

Site B: Post-Chemoview with Original Volume Hb O^{Arab}

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	552	0.008/0.051	0.025	0.024 – 0.025	0.007
FAO^{Arab}	NA	NA	NA	NA	NA



No positive samples processed however all results fell within proposed ‘action values’.

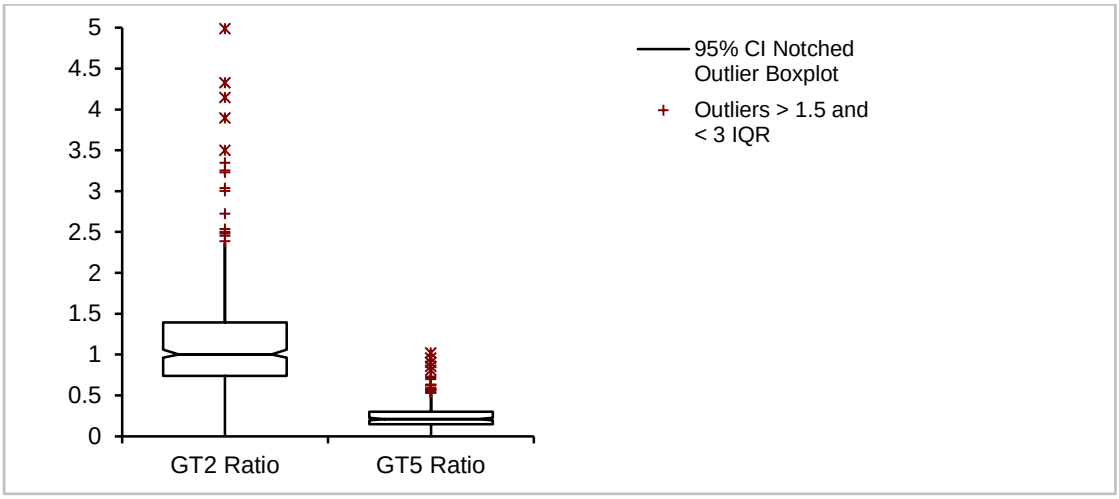
Site B: Post-Chemoview with Original Volume Hb E

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	550	0.004/0.095	0.021	0.020 – 0.022	0.010
FAE	2	NA	NA	NA	NA

The results shown indicate the excellent discrimination between samples negative for Hb E and positive for Hb E , although the numbers are low. There were no false negatives.

Site B: Post-Chemoview with Original Volume Gamma

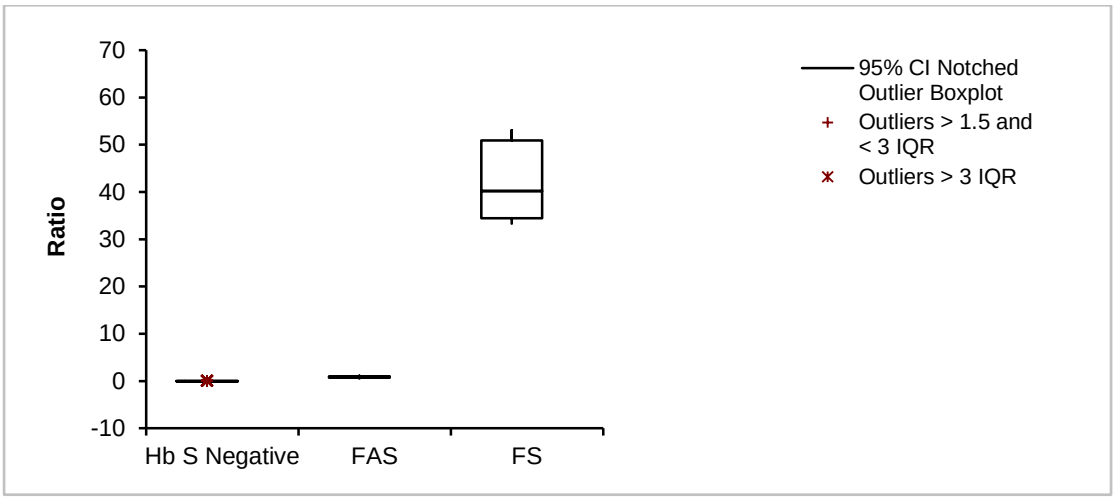
Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	552	0.011/4.99	1.12	1.08 – 1.17	0.58
Gamma T5	552	0.003/1.02	0.24	0.23 – 0.26	0.14

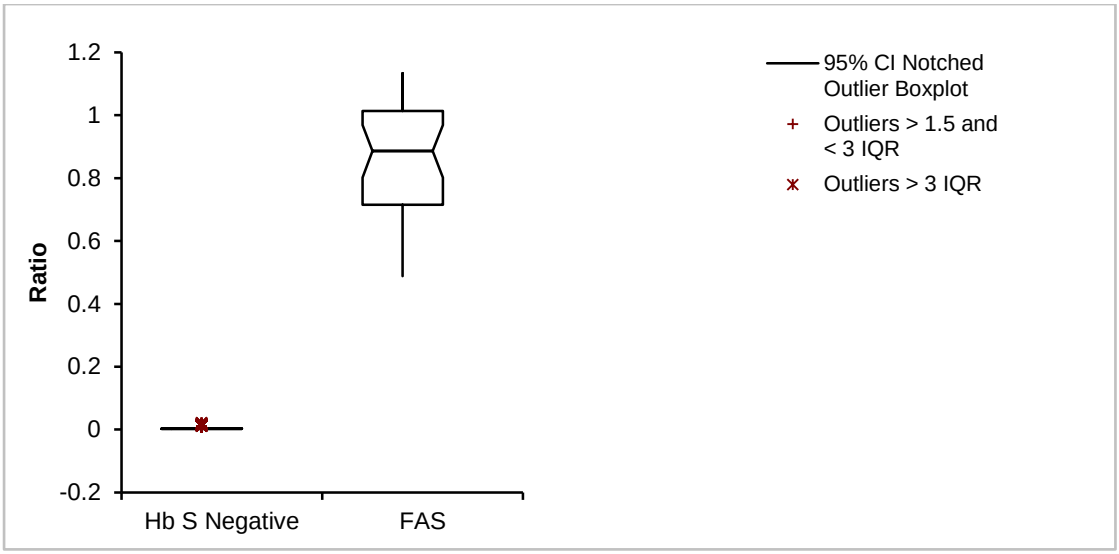


Gamma/beta wild type ratios for the T2 and T5 peptides no beta thalassaemia major samples in this cohort.

Site B: Post-Chemoview with Increased Volume Hb S

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	2325	0.00/0.021	0.003	0.003 – 0.004	0.002
FAS	36	0.49/1.13	0.86	0.80 – 0.93	0.18
FS	3	33.30/53.06	42.18	17.25 – 67.10	10.03

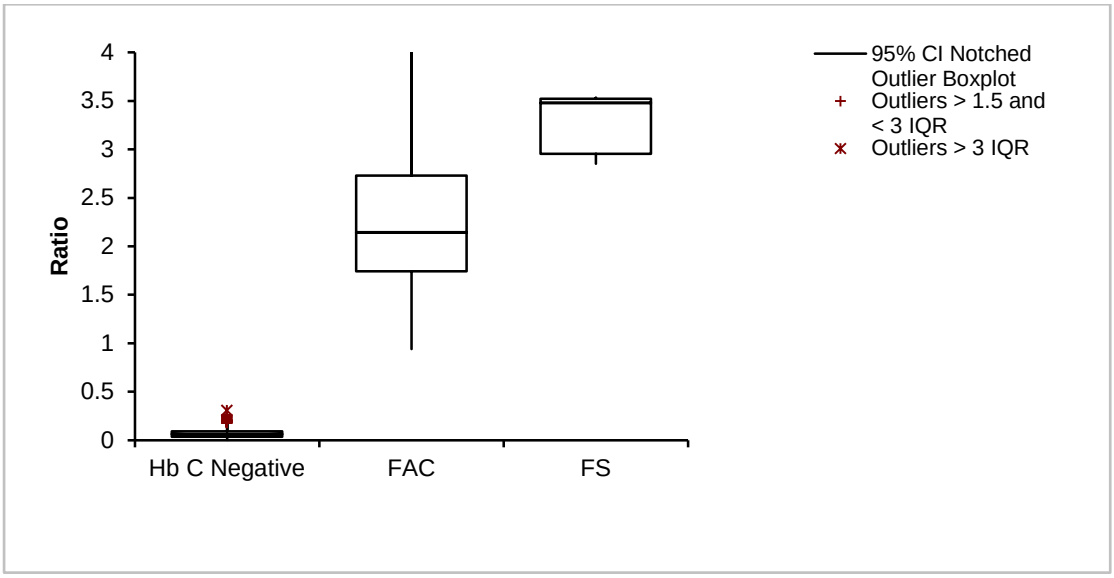


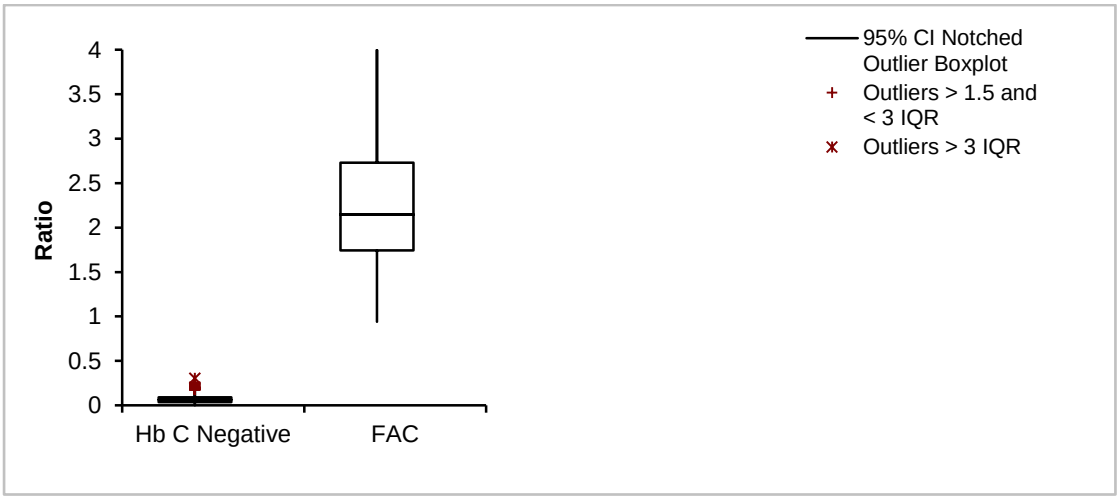


The results shown indicate the excellent discrimination between samples negative for Hb S and those positive for Hb S. There were no false negatives.

Site B: Post-Chemoview with Increased Volume Hb C

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	2349	0.003/0.030	0.068	0.066 – 0.070	0.041
FAC	12	0.94/4.00	2.29	1.75 – 2.84	0.86
FS	3	2.85/3.53	3.29	2.35 – 4.23	0.38

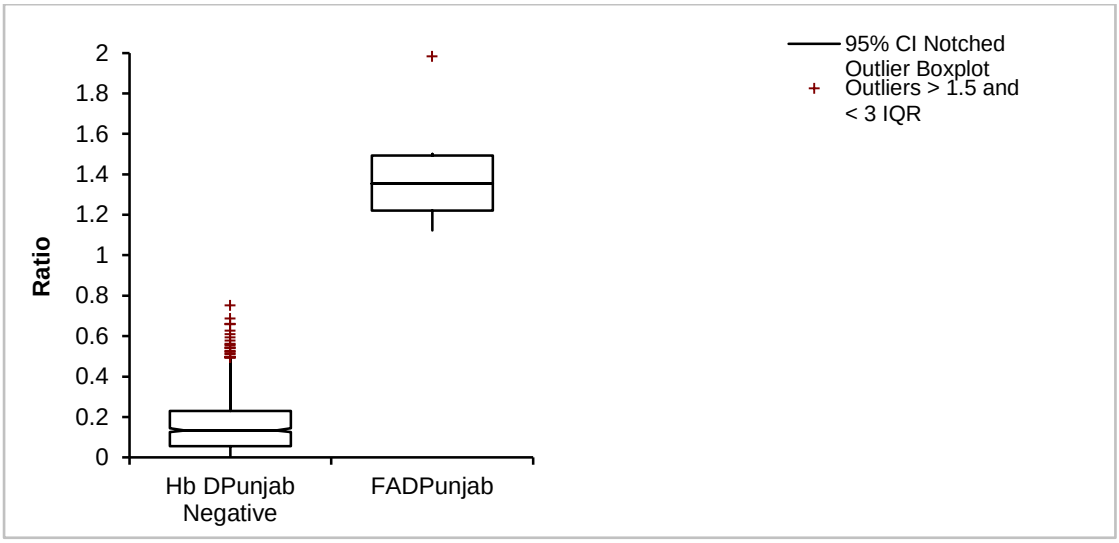




The results shown indicate the good discrimination between samples negative for Hb C and positive for Hb C. Samples lacking wild type beta T1, again generated ratios that fell into the FAC range. There were no false negatives.

Site B: Post-Chemoview with Increased Volume Hb D^{Punjab}

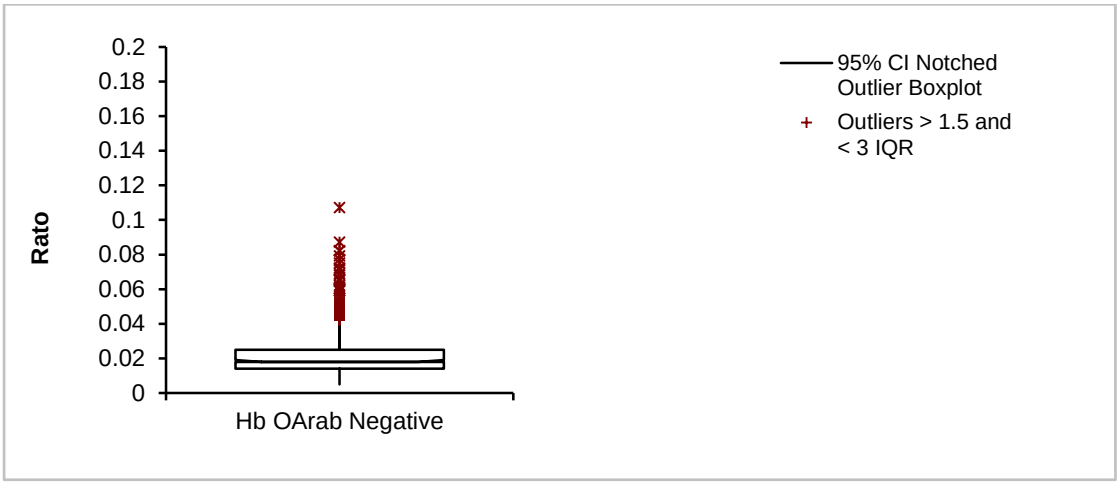
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb D^{Punjab} Negative	2351	0.010/0.75	0.16	0.15 – 0.16	0.12
FAD^{Punjab}	13	1.12/1.98	1.38	1.24 – 1.51	0.23



The results shown indicate acceptable discrimination between samples negative for Hb D^{Punjab} and positive for Hb D^{Punjab}. There were no false negatives.

Site B: Post-Chemoview with Increased Volume Hb O^{Arab}

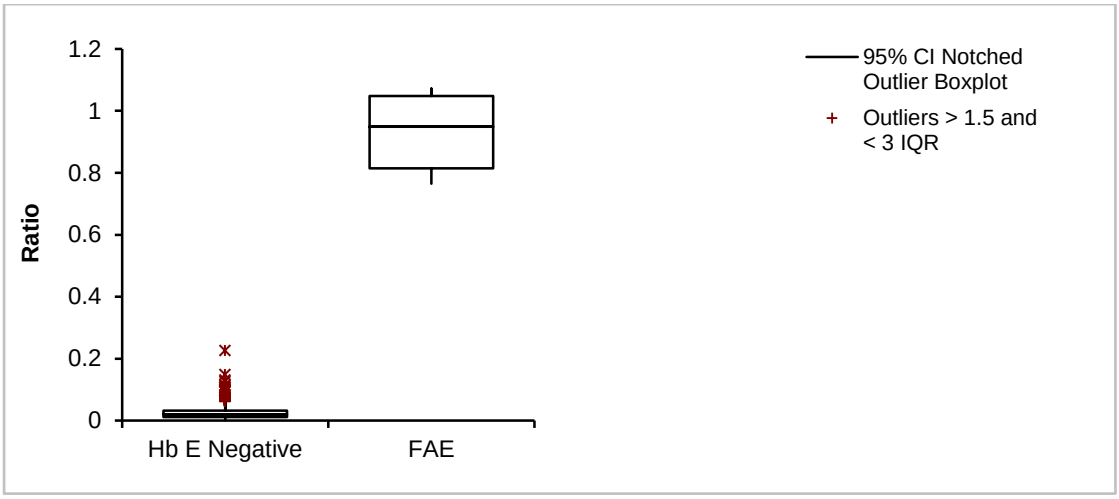
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	2364	0.010/0.11	0.021	0.020 – 0.021	0.010
FAO^{Arab}	NA	NA	NA	NA	NA



No positive samples processed however all results fell within proposed ‘action values’.

Site B: Post-Chemoview with Increased Volume Hb E

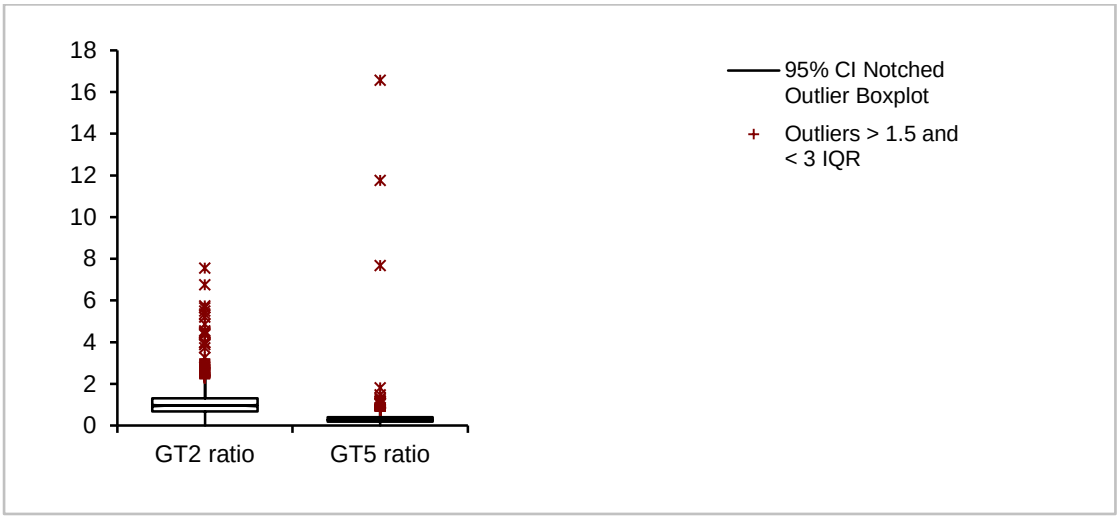
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	2360	0.001/0.23	0.025	0.024 – 0.025	0.018
FAE	4	0.77/1.07	0.93	0.72 – 1.15	0.14



The results shown indicate the excellent discrimination between samples negative for Hb E and those positive for Hb E. There were no false negatives.

Site B: Post-Chemoview with Increased Volume Gamma

Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	2364	0.003/7.54	1.07	1.05 – 1.10	0.61
Gamma T5	2364	0.001/16.56	0.33	0.31 – 0.34	0.47



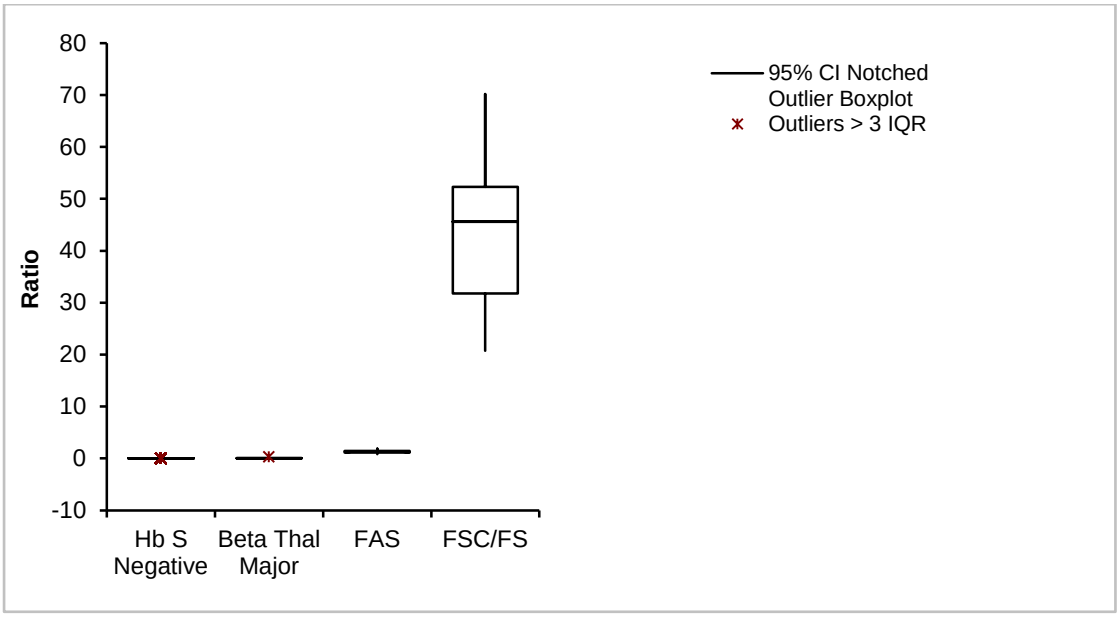
Gamma/beta wild type ratios for the T2 and T5 peptides no beta thalassaemia major samples in this cohort.

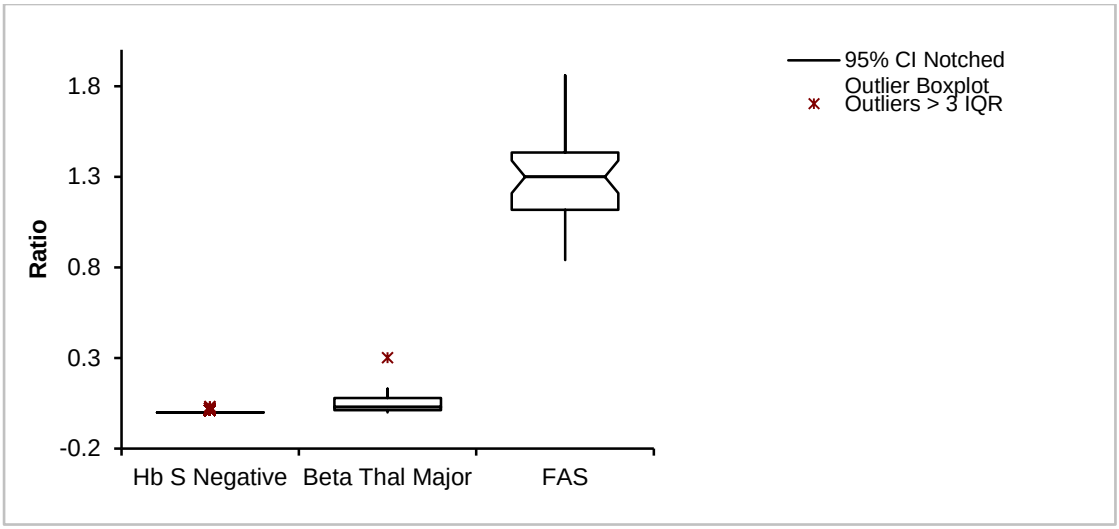
Site C results

This section shows the results obtained for site C operating Waters Micromass Zevo TQMS (Manchester, UK). A total of 7,951 samples were reported for evaluation.

Site C: Hb S

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	7877	0.00/0.03	0.001	0.001 – 0.001	0.003
B Thal Major	11	0.00/0.30	0.066	0.008 – 0.12	0.086
FAS	53	0.84/1.86	1.30	1.23 – 1.37	0.25
FSC/FS	10	20.73/9906	1029	-1201 – 3260	3119
FSC/FS (outlier removed)	9	20.73/70.20	43.54	31.55 – 55.53	15.60

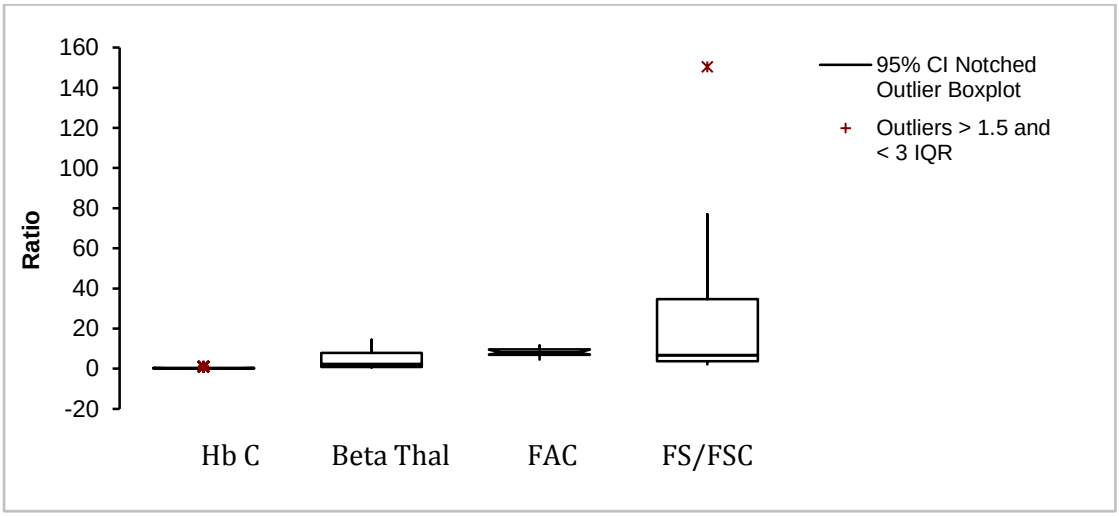


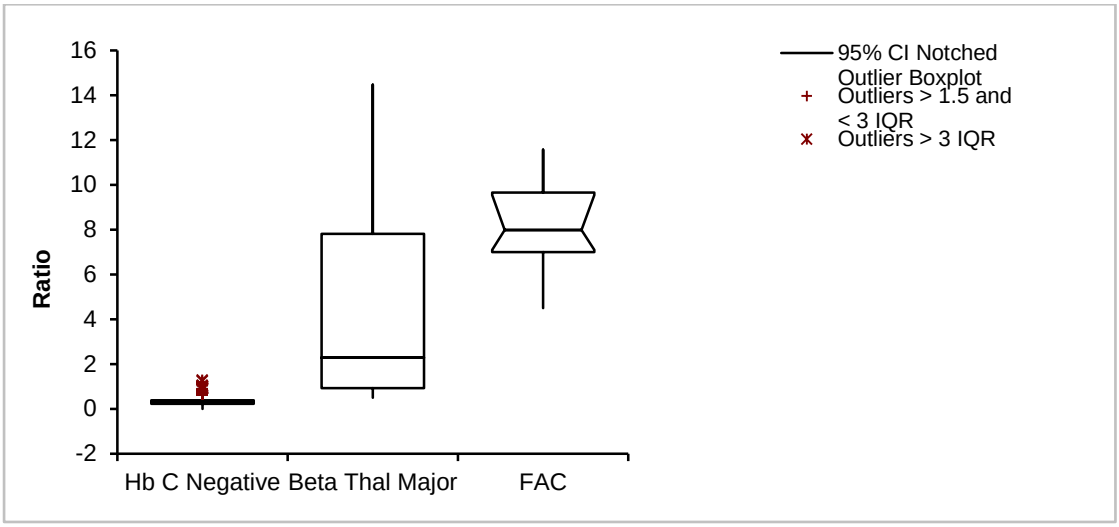


The results shown indicate the excellent discrimination between samples negative for Hb S and those positive for Hb S. Samples lacking the corresponding wild type beta did not generate ratios that fell into the FAS range. There were no false negatives.

Site C: Hb C

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	7914	0.00/1.28	0.33	0.32 – 0.33	0.13
B Thal Major	11	0.50/14.47	4.82	1.25 – 8.38	5.30
FAC	17	4.50/11.56	8.12	7.14 – 9.10	1.90
FSC/FS (outlier removed)	9	2.14/150.31	30.37	-8.66 – 69.40	50.77



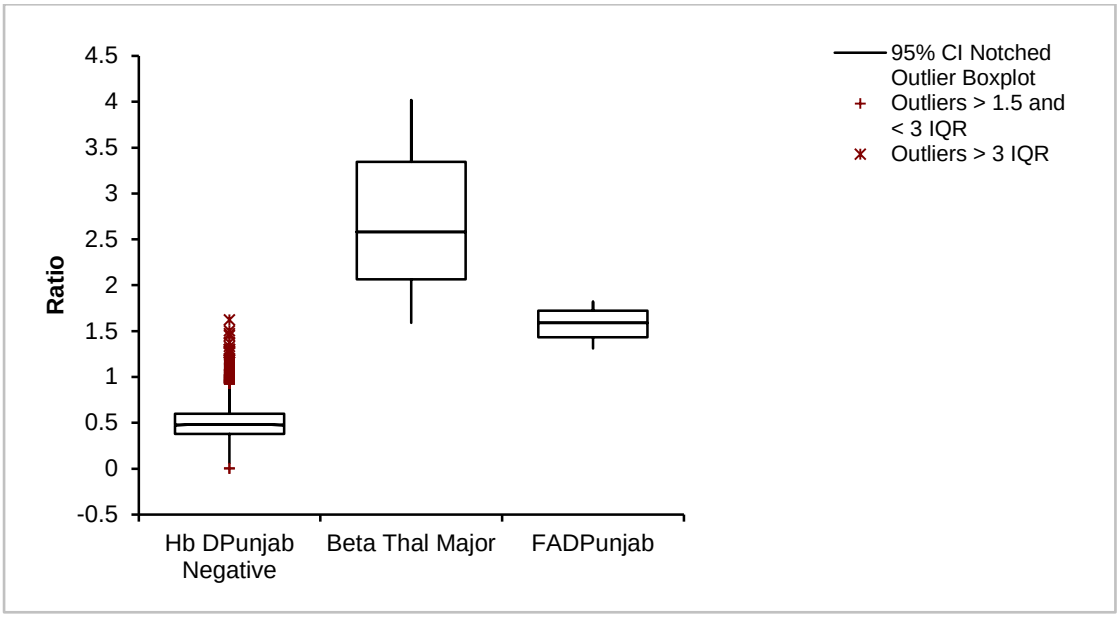


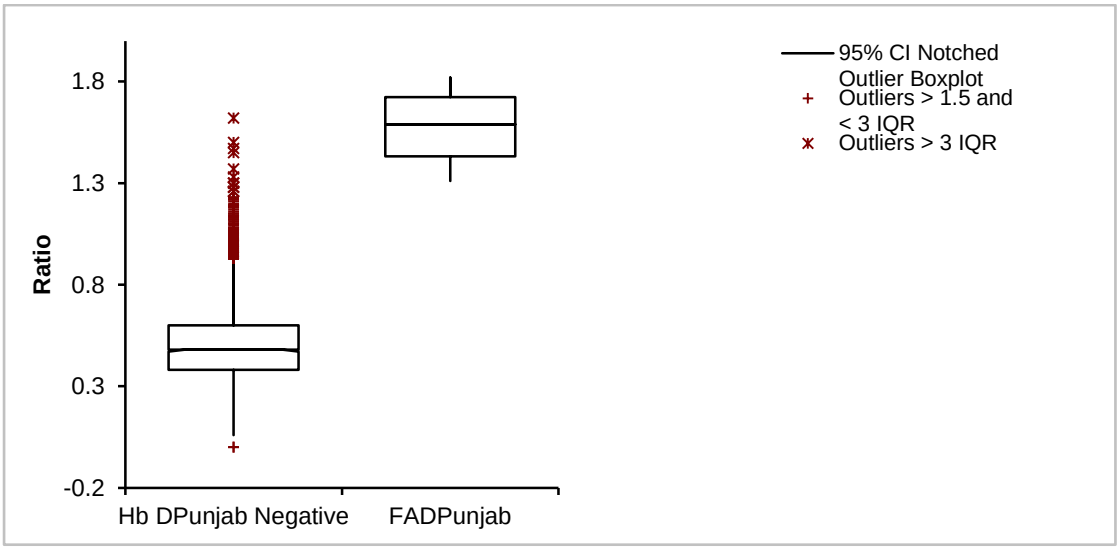
The results shown indicate the good discrimination between samples negative for Hb C and positive for Hb C. Samples lacking wild type beta T1, again generated some ratios that fell into the FAC range. There were no false

negatives. The outlier removed to facilitate graphical presentation had a ratio of 1324.

Site C: Hb D^{Punjab}

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb D^{Punjab} Negative	7924	0.00/1.62	0.50	0.50 – 0.51	0.18
Beta Thal Major	11	1.59/4.02	2.76	2.23 – 3.28	0.78
FAD^{Punjab}	16	1.31/1.82	1.59	1.50 – 1.68	0.16



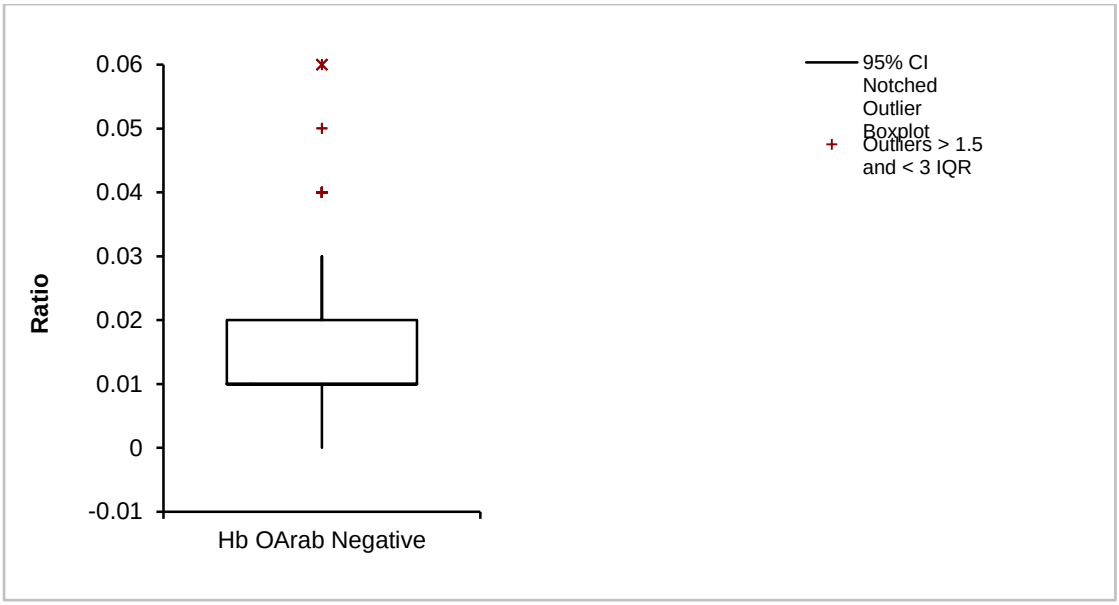


The results shown indicate that the discrimination between samples negative for Hb

D^{Punjab} and positive for Hb D^{Punjab} was not complete. There were no false negatives.

Site C: Hb O^{Arab}

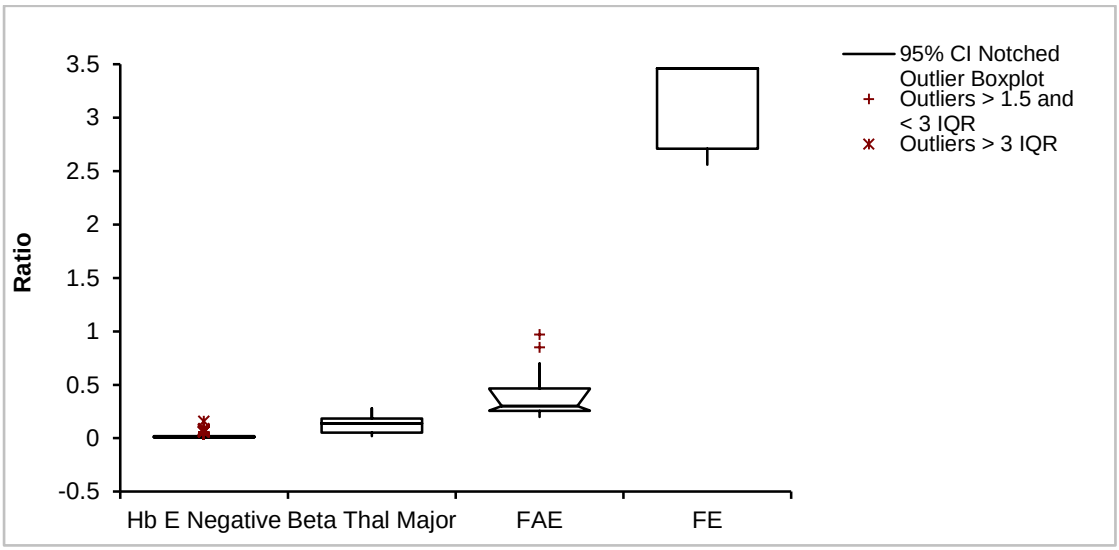
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	7951	0.00/0.060	0.013	0.013 – 0.013	0.005
FO^{Arab}	NA	NA	NA	NA	NA

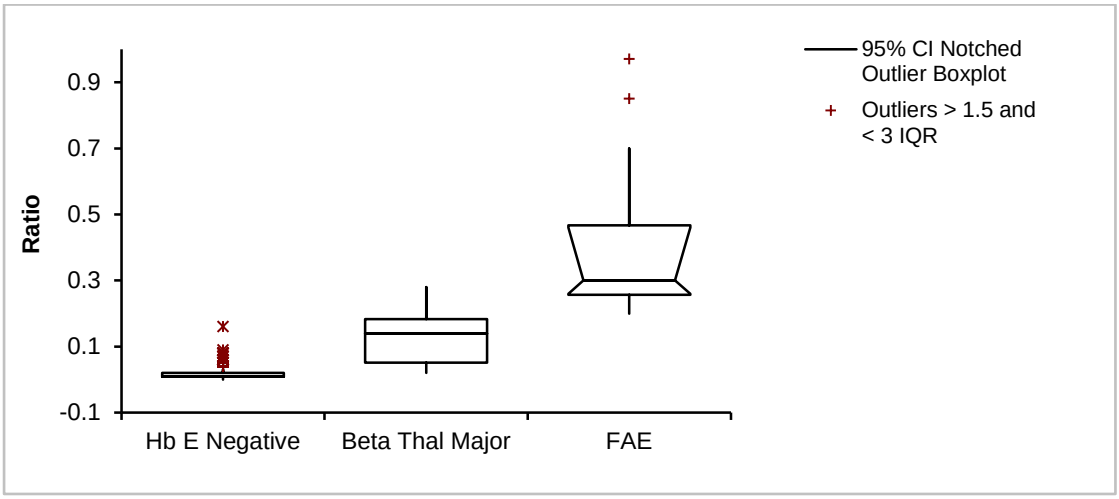


No positive samples processed however all results fell within proposed 'action values'.

Site C: Hb E

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	7921	0.00/0.16	0.015	0.015 – 0.015	0.008
Beta Thal Major	11	0.02/0.28	0.12	0.069 – 0.18	0.081
FAE	17	0.20/0.97	0.40	0.26 – 0.52	0.23
FE	2	N/A	N/A	N/A	N/A

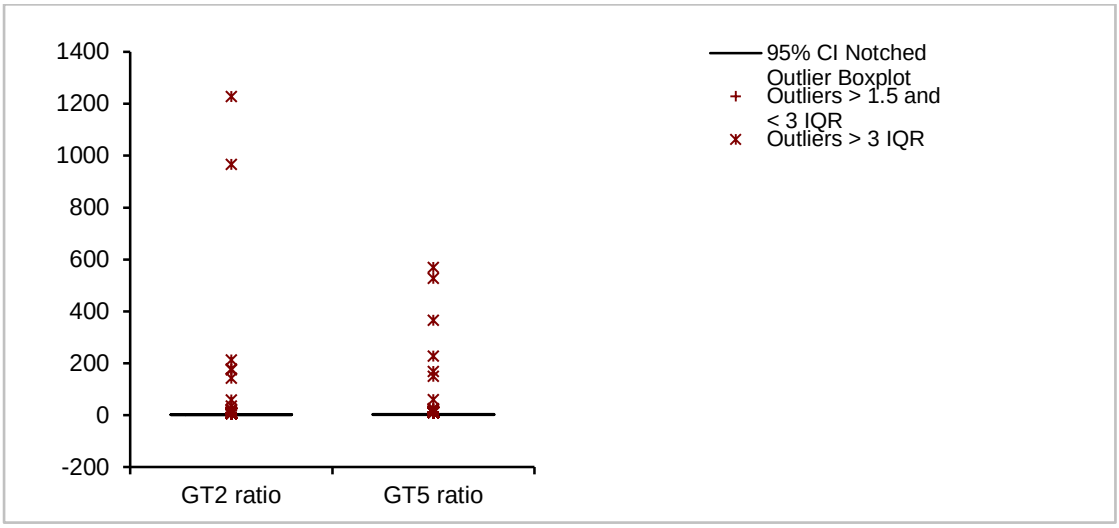




The results shown indicate the acceptable discrimination between samples negative for Hb E and positive for Hb E. As previously samples lacking the corresponding wild type beta, generated some ratios that fell into the FAE range. There were no false negatives.

Site C: Gamma

Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	7951	0.00/1227.26	2.03	1.63 – 2.42	17.97
Gamma T5	7951	0.00/568.41	2.94	2.71 – 3.16	10.32



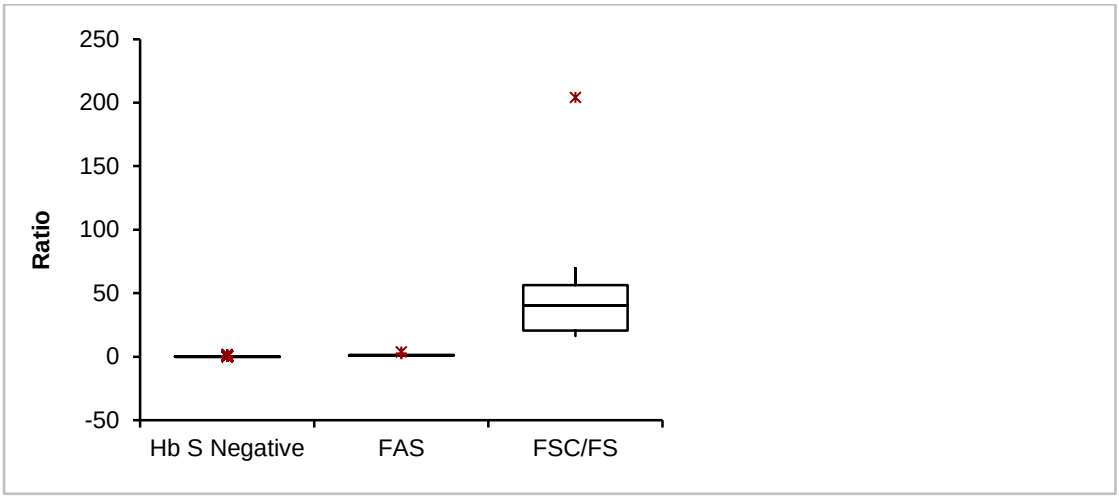
Gamma/beta wild type ratios for the T2 and T5 peptides beta thalassaemia major results were detected using the ratios, however as with existing methods some premature babies also gave high ratios. Range for beta thalassaemia major T2; 17.15 – 1227.26, T5; 16.2 – 568.41.

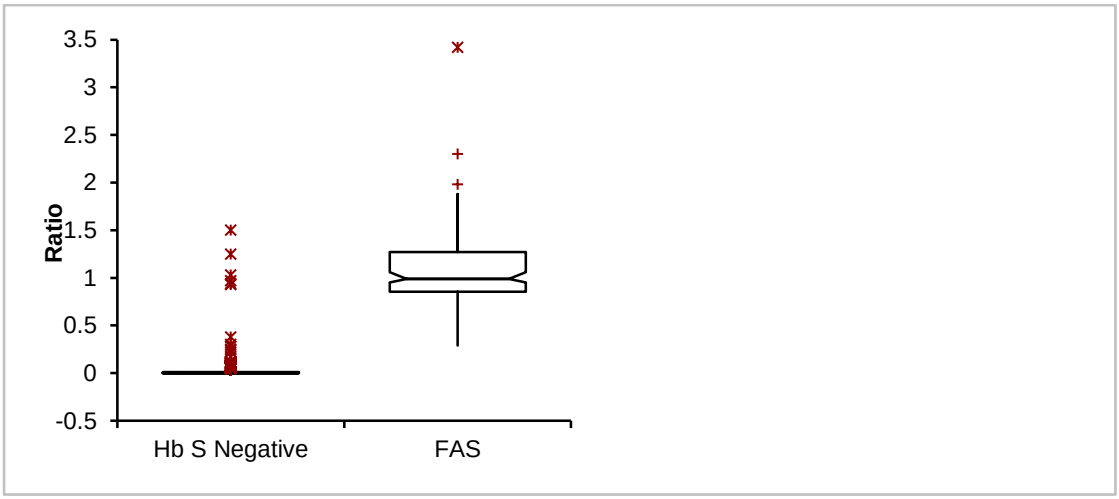
Site D results

This section shows the results obtained for site D operating Waters Micromass Premier (Manchester, UK). A total of 7,332 samples were reported for evaluation.

Hb S

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb S Negative	7145	0.00/1.50	0.009	0.008 – 0.009	0.036
FAS	176	0.29/3.42	1.09	1.03 – 1.14	0.37
FSC/FS	11	16.30/204.00	51.67	15.70 – 87.63	53.53

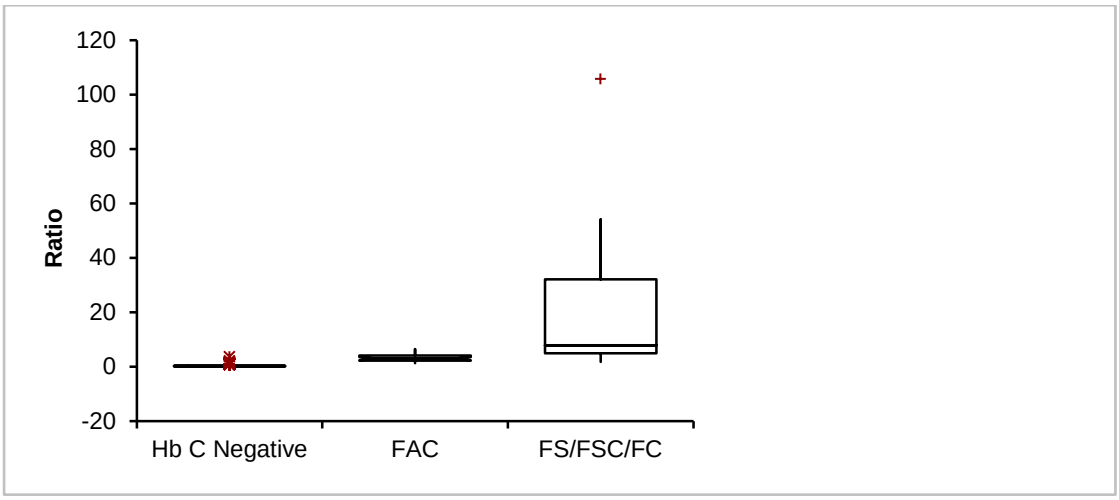


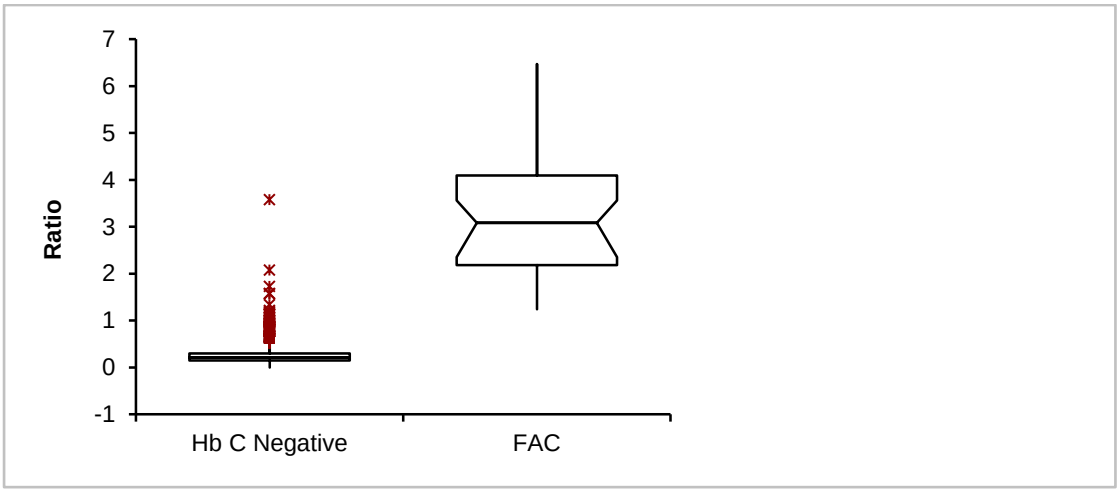


The results shown indicate the excellent discrimination between samples negative for Hb S and those with sickle cell disease. However the discrimination between samples negative for Hb S and sickle cell carriers was not complete. There were no false negatives.

Site D: Hb C

Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb C Negative	7288	0.00/3.58	0.22	0.24 – 0.25	0.15
FAC	31	1.24/6.47	3.23	2.76 – 3.70	1.28
FSC/FS/FC	13	1.76/105.71	22.91	5.04 – 40.78	29.57

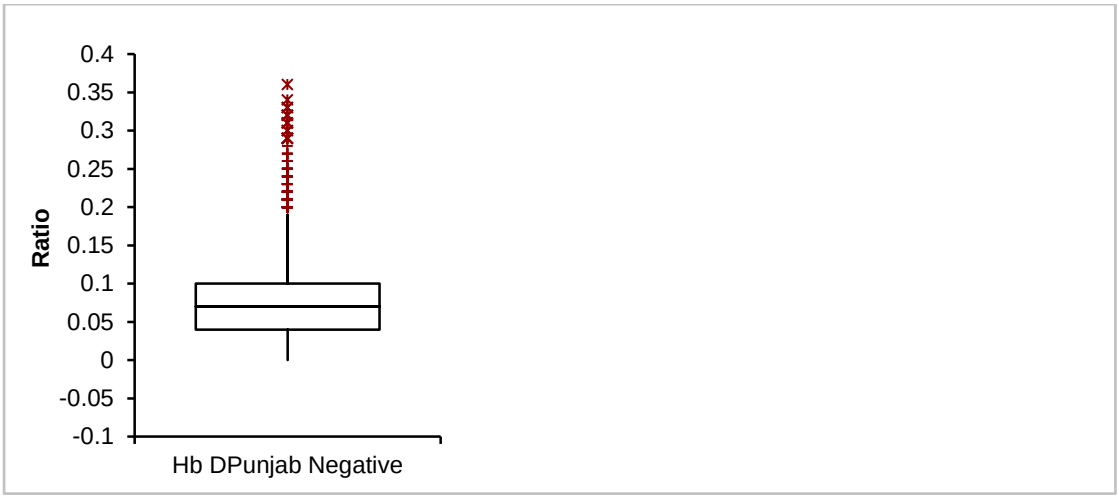




The results shown indicate the good discrimination between samples negative for Hb C and those with Hb C and no corresponding beta wild type, however the discrimination between samples negative for Hb C and Hb C carriers was not complete. There were no false negatives.

Site D: Hb D^{Punjab}

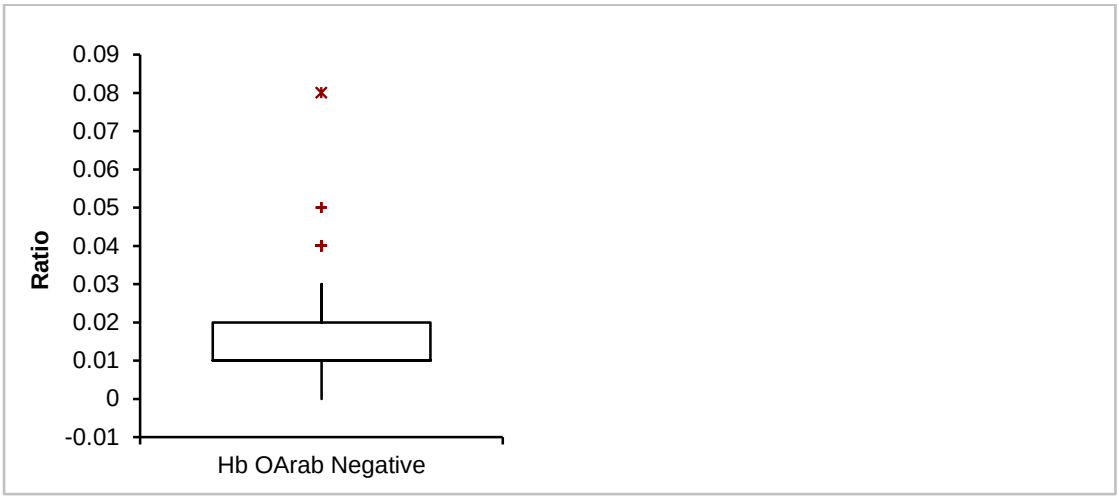
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb D^{Punjab} Negative	7330	0.00/0.36	0.074	0.073 – 0.075	0.044
FAD^{Punjab}	2	NA	NA	NA	NA



Two positive samples processed which were outside the proposed 'action values' and were therefore not detected (values obtained: 0.29 and 0.32).

Site D: Hb O^{Arab}

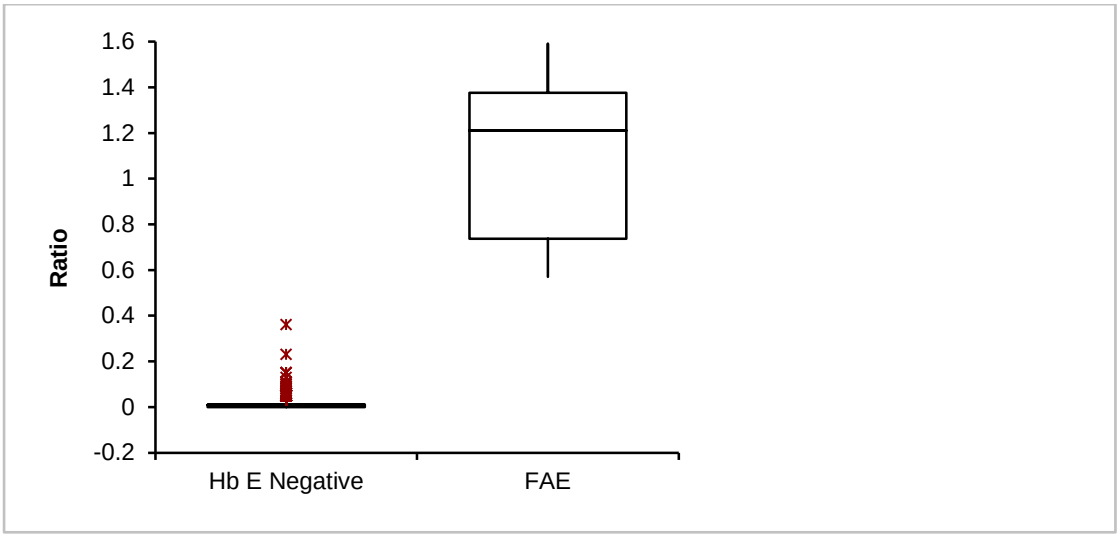
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb O^{Arab} Negative	7332	0.00/0.080	0.011	0.011 – 0.011	0.008
FAO^{Arab}	NA	NA	NA	NA	NA



No positive samples processed, however all results fell within proposed 'action values'.

Site D: Hb E

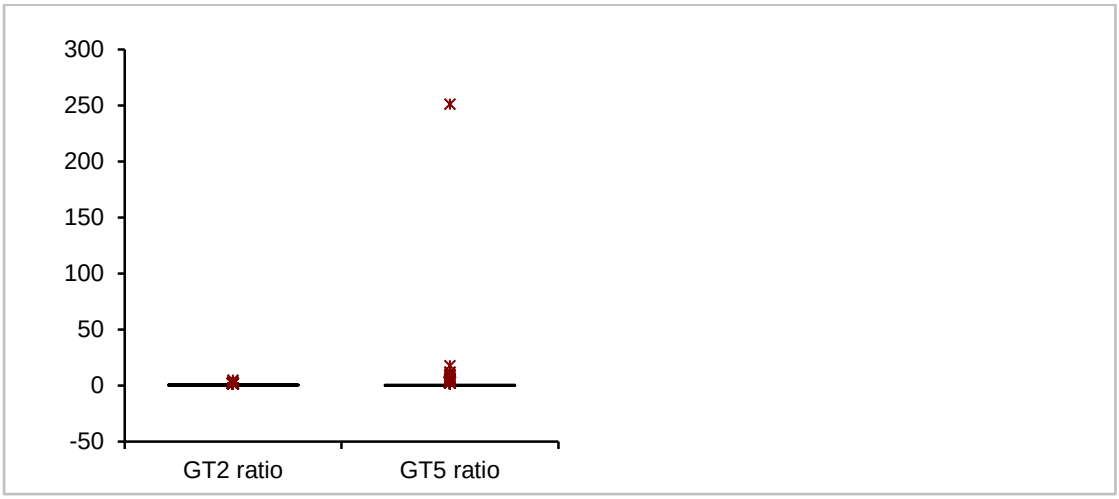
Phenotype	Number	Min/Max	Mean	95% CI	SD
Hb E Negative	7325	0.00/0.36	0.008	0.008 – 0.009	0.012
FAE	7	0.57/1.59	1.10	0.74 – 1.45	0.38



The results shown indicate the good discrimination between samples negative for Hb E and positive for Hb E. There were no false negatives.

Site D: Gamma

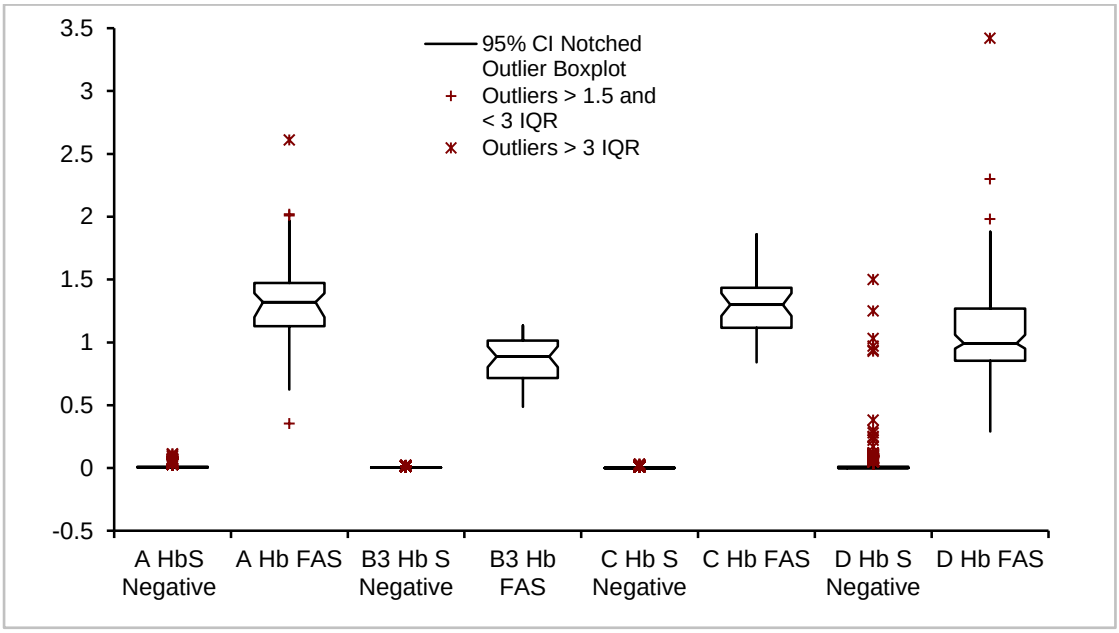
Ratio	Number	Min/Max	Mean	95% CI	SD
Gamma T2	7332	0.00/4.62	0.48	0.48 – 0.49	0.29
Gamma T5	7332	0.00/251.0	0.50	0.43 – 0.57	2.99



Gamma/beta wild type ratios for the T2 and T5 peptides no beta thalassaemia major samples in this cohort.

All sites Hb S

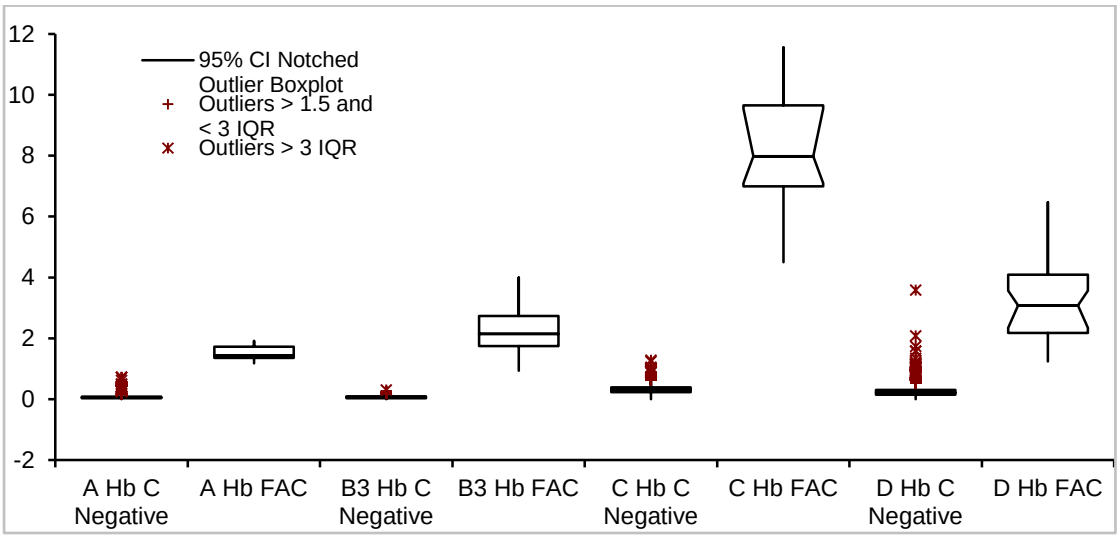
Phenotype	Number	Min/Max	Mean	95% CI	SD
Site A Hb S Negative	6188	0.00/0.11	0.007	0.0071 – 0.0075	0.007
Site A Hb AS	52	0.35/2.61	1.33	1.23 – 1.44	0.37
Site B* Hb S Negative	2325	0.00/0.02	0.003	0.0033 – 0.0035	0.002
Site B* Hb AS	36	0.49/1.13	0.86	0.81 – 0.92	0.18
Site C Hb S Negative	7877	0.00/0.03	0.001	0.0006 – 0.0007	0.003
Site C Hb AS	53	0.84/1.86	1.30	1.23 – 1.37	0.25
Site D Hb S Negative	7145	0.00/1.50	0.009	0.0077 – 0.0093	0.04
Site D Hb AS	176	0.29/3.42	1.09	1.03 – 1.14	0.37



Summary of the discrimination for all sites for Hb S negative and sickle cell carriers, note the lack of discrimination at site D.

All sites Hb C

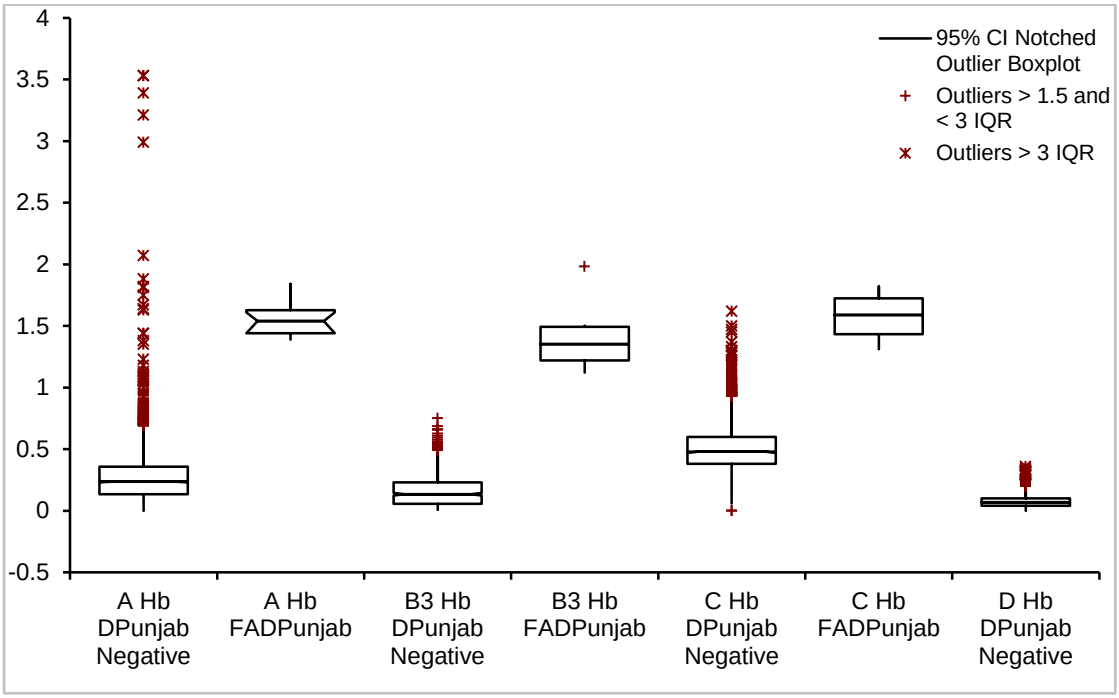
Phenotype	Number	Min/Max	Mean	95% CI	SD
Site A Hb C Negative	6230	0.00/0.72	0.062	0.061 – 0.063	0.052
Site A Hb AC	11	1.18/1.91	1.51	1.36 – 1.66	0.22
Site B* Hb C Negative	2349	0.003/0.30	0.068	0.066 – 0.070	0.041
Site B* Hb AC	12	0.94/3.99	2.29	1.75 – 2.84	0.86
Site C Hb C Negative	7914	0.00/1.28	0.326	0.323 – 0.329	0.131
Site C Hb AC	17	4.5/11.56	8.12	7.14 – 9.10	1.90
Site D Hb C Negative	7288	0.00/3.58	0.244	0.240 – 0.247	0.14
Site D Hb AC	31	1.24/6.47	3.23	2.76 – 3.70	1.28



Summary of the discrimination for all sites for Hb C negative and Hb C Carriers, note the lack of discrimination at site D.

All sites Hb D^{Punjab}

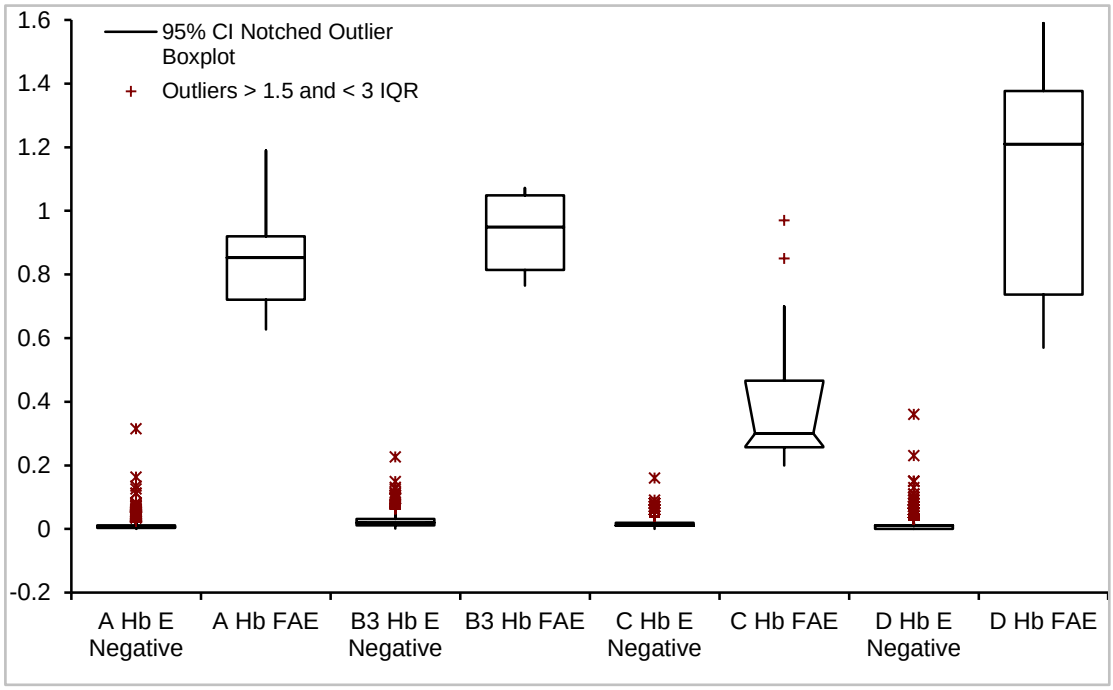
Phenotype	Number	Min/Max	Mean	95% CI	SD
Site A Hb D^{Punjab} Negative	6229	0.00/3.53	0.27	0.263 – 0.273	0.20
Site A Hb AD^{Punjab}	17	1.39/1.84	1.55	1.49 – 1.62	0.13
Site B* Hb D^{Punjab} Negative	2351	0.01/0.75	0.157	0.152 – 0.161	0.12
Site B* Hb AD^{Punjab}	13	1.12/1.98	1.38	1.24 – 1.51	0.22
Site C Hb D^{Punjab} Negative	7924	0.00/1.62	0.503	0.499 – 0.507	0.18
Site C Hb AD^{Punjab}	16	1.31/1.82	1.59	1.50 – 1.68	0.16
Site D Hb D^{Punjab} Negative	7330	0.00/0.36	0.07	0.072 – 0.075	0.04
Site D Hb AD^{Punjab}	2	N/A	N/A	N/A	N/A



Summary of the discrimination for all sites for Hb D^{Punjab} negative and Hb D^{Punjab} Carriers, note the lack of discrimination at sites A and D.

All sites Hb E

Phenotype	Number	Min/Max	Mean	95% CI	SD
Site A Hb E Negative	6234	0.00/0.32	0.009	0.009 – 0.009	0.052
Site A Hb AE	10	0.63/1.19	0.85	0.73 – 0.97	0.22
Site B* Hb C Negative	2359	0.001/0.23	0.025	0.024 – 0.025	0.041
Site B* Hb AE	4	0.77/1.07	0.93	0.71 – 1.15	0.86
Site C Hb E Negative	7921	0.00/0.16	0.015	0.015 – 0.015	0.131
Site C Hb AE	17	0.20/0.97	0.40	0.28 – 0.52	1.90
Site D Hb E Negative	7325	0.00/0.36	0.008	0.008 – 0.009	0.14
Site D Hb AE	7	0.57/1.59	1.10	0.74 – 1.45	1.28

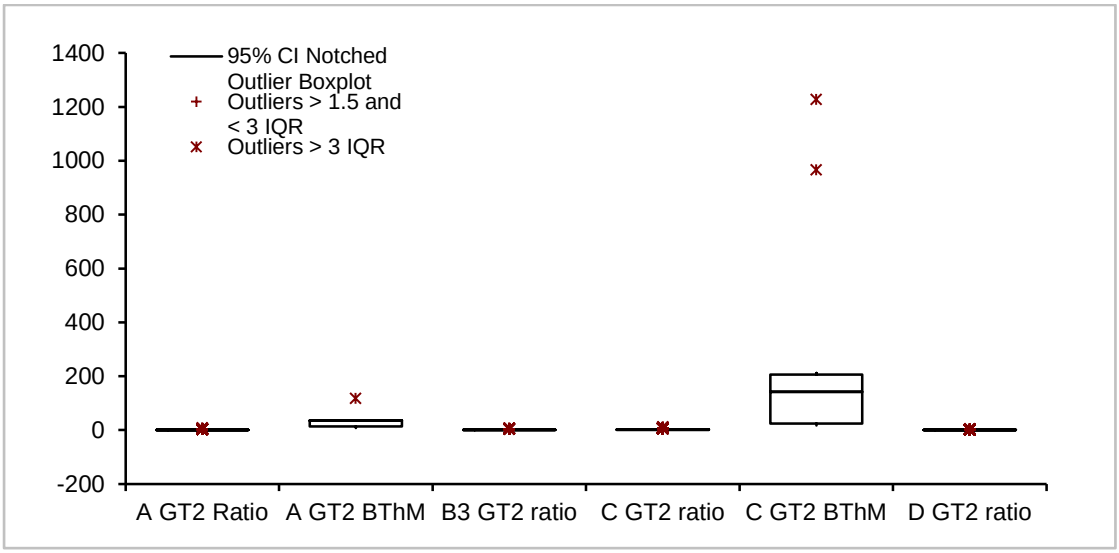


Summary of the discrimination for all sites for Hb E negative and Hb E carriers.

All sites Gamma T2

Phenotype	Number	Min/Max	Mean	95% CI	SD
Site A GT2	6244	0.00/8.46	0.92	0.91 – 0.93	0.052
Site A GT2 BTM	7	7.15/118.0	37.39	2.63 – 72.15	0.22
Site B* GT2	2364	0.003/7.54	1.07	1.05 – 1.10	0.041
Site B* GT2 BTM	0	N/A	N/A	N/A	N/A
Site C GT2	7940	0.00/11.54	1.65	1.63 – 1.67	0.131
Site C GT2 BTM	11	17.15/1227.3	276.90	-2.31– 556.10	1.90
Site D GT2	7332	0.00/4.62	0.48	0.48 – 0.49	0.14
Site D GT2 BTM	0	N/A	N/A	N/A	N/A

*Site B results are only shown for optimized method



Gamma/beta wild type ratios for all sites for the T2 peptide. Where sites have beta thalassaemia major samples these results are also shown (BThM).

Discussion

The results from sites A, B and C demonstrate the method has detected all of the target haemoglobins with no false negatives. Site D will be discussed separately. In order to obtain 100% sensitivity there were some false positives. These occurred at varying rates across the sites. Site A showed good discrimination on all haemoglobins with the exception of Hb D^{Punjab}. There was a total of 38 false positives, 31 of which were Hb D^{Punjab}. At site B false positive rates varied dramatically, before the modifications there were 115 false positives of which 105 were Hb D^{Punjab}. After the final modifications no false positives were detected. Site C had 108 false positive results of which 104 were Hb $D^{Punjab.}$.

Site D had two Hb D^{Punjab} Carriers neither of which were detected with the action values in use. There were a total of 37 false positives, 24 of these were Hb S Carriers.

The results indicate that in comparison to sites A, B and C, site D had significantly less sensitivity, clearly apparent in the internal standard results, which was also reflected in the results obtained for the haemoglobin ratios. Prior to the establishment of action values for the internal standard some results with low values were included in the analysis. The results from site D suggest that instrument sensitivity is a crucial factor in having a robust and reliable method.

The results from sites A, B and C indicate that with the exception of Hb D^{Punjab} the false positive rate is minimal. There is evidence from the site B results that the introduction of the Cliquid^{R} Chemoview^{TM} software (AB Sciex) reduced false positive rates significantly and this is being further assessed in the extended pilot at Leeds. The high false positive rate at site C remains a concern however there is a potential strategy for this, the raw signal in addition to the ratio data, which will require further investigation with this manufacturers equipment. Assessment of the surrogate Hb F and Hb A values using the gamma:beta ratio also requires further investigation to finalise action values which align the predicative value with that of current methods. Hb O^{Arab} has shown no false positives or negatives at any site but due to the low numbers of positive samples this remains under review.

The results from sites A, B and C also indicate that it should be possible to set common action values for all of the haemoglobin variants provided instruments of sufficient sensitivity are used, however it should be noted that a limited number of instruments have been investigated. The internal standard

results indicate that it will not be possible to align internal standard values and laboratories may need to set and monitor these individually.

Although the kit manufacturers are investigating controls specifically for this kit, at the time of evaluation these were not available and this created some problems. Site A used in house controls as did site C, although they did not have controls for all haemoglobins. Both sites B and C investigated the commercially available controls used for HPLC, with limited success, largely due to the high wild type signal which normalised the ratios obtained. Sites which tested NEQAS samples during the evaluation obtained good results.

As can be seen in the appended reports from each site, although the laboratories experienced some issues getting established, in general they found the procedure to be quick and easy. Site A demonstrated that all staff were quickly able to perform the method.

Conclusion

The results demonstrate that this is an acceptable method for newborn screening of haemoglobinopathies, however instruments of sufficient specificity must be used and the action values for Hb D^{Punjab} in particular require further refinement.

Acknowledgments

Thank you to all of the laboratories who participated in the pilot study and Waters Micromass for the loan of an instrument

Appendix

Site A

Evaluation of Screening for Haemoglobinopathies by MS/MS using the Spot-On assay kit

From our laboratories perspective we were looking for a variety of outcomes from the evaluation, staffing in the Antenatal and Newborn laboratory is at a critical level and we needed to determine if the methodology would ease the pressure on the lab and staff. We evaluated its ease of use by all levels and grades of staff and essentially the ease of reporting and integration into our current LIMS newborn system Specimengate. As the section lead I had limited hands on experience of mass spectrometry but for the rest of the haematology staff this was one of their biggest obstacles to overcome and a huge learning curve.

The evaluation commenced on 10 November 2012 with the choice for the mass spectrometer being the AbSciex API4000 processing the samples using analyst software. The staff involved were individuals with no previous mass spectrometry experience, following installation and basic training over the course of a day and a half we were left to proceed with the evaluation. Initial obstacles for haematology from the onset were not problems with the equipment or the assay but with personalities and integrating the haematology staff in with the mass spec biochemistry staff. There was a significant degree of resistance from staff currently using the equipment, they expressed concerns that we were not trained sufficiently, we would break the equipment and that the samples being processed were not pure but dirty and they would cause blockages resulting in downtime of the equipment. These were probably some of the hardest obstacles to overcome, gaining the trust of the mass spec staff and proving our capabilities on the instrument.

The Spot-on assay was extremely easy to use, straightforward, simple instructions and fast processing time an hour max from start to finish of the pre-analytical phase. Our lab currently uses labour intensive IEF (isoelectric focussing), the staff using spot-on found it easy to use, quick with straightforward easy to follow instructions. All levels and grades of staff were asked to use the kits form biomedical support workers through to senior staffing, who were all visibly impressed by ease of use and the short pre-analytical processing times of the samples. While using the kit we also looked at the stability of reagent 1 and reagent 2 once defrosted and prepared for use. We found that any leftover prepared reagent was stable and could be used the following day if kept refrigerated.

For the evaluation the samples were processed through analyst on the API4000 and manually interpreted through an excel spread sheet employing conditional formatting which highlighted positive results (ratios) that would be referred for further work-up. Throughout the evaluation various positive results were identified for haemoglobinopathy carriers and disease states. There were no missed positive results and the false positive obtained were used to fine tune the conditional formatting and action values for HbS,C,D,E,O and gamma chains on the spread sheet and later for use in setting up chemoview.

The primary evaluation finished in February 2014 and we proceeded onto the extended evaluation. As the excel spread sheet was labour intensive and involved manual manipulation it was essential that this procedure was standardised in addition to ensuring positive patient identification throughout the process and integrating it into our newborn LIMS system. For the extended evaluation Chemoview was set up on the API4000 to process the results from analyst with the inclusion of positive patient ID and the cut-offs fixed into the system. The positive patient identification was an essential requirement for the assay, and as there was currently no interface for the analyser to or from the LIMS system Specimengate. Essential elements of positive identification were collated for import and export and the interface was built around our specifications by CSols (laboratory systems integration). Resulting in a fully integrated assay with positive patient ID from start to finish, fully integrated with our lab LIMS system, through to reporting.

There were problems and issues along the way with the evaluation, but all were overcome during the course of the evaluation. One of issues we hit that was a limiting factor for us was the number of plates we were able to process on the API4000 as we could only process two plates at one time, this was an issue if we were to integrate the assay into our current newborn screening system with the biochemistry assays. An essential requirement we identified was that the lab needed a sample handler to handle the full newborn screening workload.

Another issue identified was with the internal standard reduced sensitivity after five months of storage, this however was identified as incorrect storage on our initial receipt of the kits storing them at -20 instead of -80! The API4000 instrument was a problem throughout the evaluation due to our low usage/requirement for the instrument, the machine was used by other areas of the lab and we often came to the instrument to find it blocked, broken or parts being removed for other machines. A positive outcome of the evaluation and us utilising the assay was that the sensitivity of the internal standard was a good indicator of issues on the machine. As the Internal standard was a very sensitive indicator of the API’s performance by monitoring this IS it was

an early indicator of instrument issues, and maintenance requirements and lead to the mass spec staff having more confidence in the haematology staff using the machine as through our persistence that there were issues with the instrument from our assay results and persistence with the engineer a problem was identified and rectified with the machines injector needle. In addition to this it became evident that to maintain the efficiency of the analyser a daily maintenance schedule has been employed to clean the mass spectrometers cone on a daily basis, this has resulted in a visible increase in sensitivity for the IS since being employed.

Site B Tandem mass spec (TMS) project overview

Site B was selected as one of the centres to trial the TMS project. The project was started December 2012 and final data was submitted 7 May 2014. The TMS project equipment specifications were as follows:

1. **Equipment Type**

HPLC/Mass Spectrometry

2. **Version**

ABSciex API 4000 triple quadrupole mass spectrometer

Shimadzu Prominence HPLC system

3. **Software used**

Operational – Analyst 1.5.2

Calculation – ChemoView 2.0.2

4. **How results are analysed**

Spot-On method

Multiple reaction monitoring analysis of tryptic digest.

The TMS project has taken longer than expected to complete and not as many samples were analysed as we would have wanted due the project encountering a few unexpected issues.

1. False Hb D Punjab positives on plates following initial setup
2. Concerns re sensitivity and specificity following initial setup.
3. Analysis of data using analyst was time-consuming due to the checks and the manual gating required.
4. Expertise, experience, time and staff shortages needed to troubleshoot problems.
5. Lack of appropriate commercially available controls containing Hb's S, C, D, E and OArab.
6. Sensitivity issues post annual maintenance - (issues with low HbS IS)
7. Post mass spec upgrade - parameters needing manual inputting (time consuming)

While these above issues were ultimately resolved, troubleshooting these took time and was a huge learning experience for all involved. Initial sample set up using the Spot On technique was quick, simple and easy to perform. Installing Chemoview 2.02 helped vastly in analysing numerous plates quickly and together with Excel 2007 allowed out of limit parameters to be highlighted and easily visualised amongst the vast amount of data available. The low HbS IS sensitivity issues that have plagued this study were finally resolved in February 2014 by increasing the injection volume which would suggest there was a problem with the autosampler. Once this major obstacle was overcome, we were able to proceed quickly and successfully analysed a significant number of plates in a short space of time. The results we obtained showed good correlation to those obtained by Bio-Rad Vnbs HPLC. Analysis by HPLC/mass spec is quick to perform and data analysis using Chemoview makes analysing the results quick and easy.

Sickle cell screening by tandem mass spectrometry pilot study

Site C Newborn Screening Laboratory

The study commenced at the start of November 2013 and finished at the start of March 2014. The manufacturers method was followed without any alterations.

All instrumentation had to be supplied on loan –

- Waters Xevo TQMS with Acquity HPLC and 2 plate autosampler
- Masslynx 4.0 software
- Panthera multipunch

Results were analysed in Neolynx. The report scheme settings were altered in order to export the results to EXCEL. The results were then copied and pasted into the provided template. A macro had to be created to change the order of the tests to enable direct transfer to the template.

Issues with equipment and procedures

- the main initial problem was with the nitrogen supply. Our laboratory had two nitrogen generators (located outside of the laboratory) that supplied two tandem mass specs, two biochrom amino acid analysers and was also used to dry down organic acids on a daily basis. There were concerns about using the line, therefore a free standing unit was supplied to feed into the existing system, however, locating the generator outside of the laboratory resulted in continuous low pressure readings on the instrument, therefore the generator had to be brought into the laboratory and plumbed directly into the instrument – this solved the low pressure problem
- needle blockage on a couple of occasions requiring fitting of new needle
- variation of internal standard counts throughout the run - on one day the count varied from 3500 to 13000 within the same batch.
- LC problem with the XY carriage which required a new part which first had to be delivered and then fitted by an engineer.
- restricted analysis of samples as the autosampler provided as part of the Acquity system only allowed two plates to run at a time.

Site D: Sickle cell screening by tandem mass spectrometry

The Laboratory started the pilot for sickle cell screening by mass spectrometry in Feb 2013 and concluded in June 2013. Approximately 8,000 blood spot samples were analysed during this pilot. Samples were prepared according to manufacturer's instructions and analysed on a Waters Quattro Premier XE with an Acquity UPLC front end, using MassLynx v 4.1 software.

NeoLynx was not optimised to process the data as all data was exported into a spreadsheet provided by the SCT sub group and anlaysed by them. We included gestational age, DOB, DOS and transfusion status as well as the initial sickle screening result (Biorad IEC first line, IEF second line confirmation).

In 2012 during the initial stage of the pilot, it was noted that using the cut off for HbC/HbA ratio required to identify true positives was resulting in a number of false positive results for HbC carriers. Approximately 2,800 patients were analysed in 2012 and 65 true positive and 68 false positives were identified. This inability to discriminate between increased HbC due to carrier status and background noise on the Premier XE lead to the conclusion that the instrument did not have the required sensitivity for newborn sickle cell screening.

In comparison to the BioRad, the sample preparation for the TMS method takes a little longer (two additional pipetting steps are required and a 30 minute incubation), however, the TMS method is more specific and would be likely to reduce the number of confirmatory tests required.

Post analytical time – data processing

Biorad

35 mins band 3 – pre-analytical

80 mins band 7 – post-analytical

10 mins band 3 – post-analytical

TMS

60 mins band 6 – pre-analytical

65 mins band 7 – post-analytical