Bloodspot Sample Quality Project
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Background

Newborn screening for phenylketonuria (PKU), congenital hypothyroidism (CHT), sickle cell disease (SCD), cystic fibrosis (CF) and medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is recommended for all babies in the UK by the National Screening Committee. Newborn bloodspot screening is offered to all newborn babies up to one year of age and aims to detect and provide treatment for those children who have the potential to develop disease. There are currently nine standards that are to ensure that all aspects of the screening process are performed at an acceptable level of quality.

In 2008, new standards were introduced in addition to the original six standards. One of the standards that were introduced was Standard 5 – Quality of blood spot sample. This standard sets an avoidable repeat rate and has the aim of reducing the number of repeat samples due to avoidable reasons such as:

- Baby too young (sample taken on or before day 4)
- Sample taken too soon after a transfusion (less than 72 hours after transfusion)
- Unsuitable sample (e.g. bloodspot card past the expiry date, contamination, sample received more than 14 days after being taken, anti-coagulated sample, no NHS number)
- Insufficient sample (e.g. blood not soaked all the way through the card)
- Unsatisfactory sample (e.g. multilayered, multispotted).

Repeat samples requested for any of these 'avoidable' reasons may cause delay in the identification and treatment of screen positive babies, anxiety to parents, distress to babies and places a strain on the laboratory and midwifery in terms of both workload and resources.

The core standard for avoidable repeat rate is less than or equal to 2.0% with the developmental standard being less than or equal to 0.5%. The avoidable repeat rate for samples analysed in the Manchester Newborn Screening Laboratory is currently 4.0% (Q3 2012 figures), most of which is due to poor sample quality. There have been various initiatives both in the community and laboratory to address sample quality including laboratory visits for midwives and targeted training for midwives who repeatedly submit poor quality samples. In order to determine whether there is an effect on newborn bloodspot screening results from poor quality bloodspots, it was decided to investigate the actual effect of producing spots with excess blood (i.e. multilayered or multispotted), or those that have been compressed.

Blood from a laboratory volunteer was obtained so that these experiments could be performed. As the volunteer had low (normal) levels of some of the analytes in their blood, it was necessary to 'spike' the blood with exogenous materials. An increased Thyroid Stimulating Hormone (TSH) level (used to screen for Congenital Hypothyroidism) was produced by the addition of quality assurance material containing TSH. A measurable Immuno Reactive Trypsin (IRT) level (used to screen for Cystic Fibrosis) was produced by the addition of a small amount of faecal material (known to have high IRT concentrations).

Method

Sample preparation

25ml whole blood (heparinised) was collected from a laboratory volunteer and a faeces sample was obtained from specialist biochemistry (discarded during the routine processing of faecal sugar analysis).

Spiked TSH samples

The TSH concentration of the volunteers' blood was a normal value of approximately 0.9mU/L. In order to determine the effect of multilayering around the critical concentrations such as the cut-off for requesting a repeat sample due to a borderline TSH result, a higher TSH concentration was required. The laboratory has quality control material which contains a high concentration of TSH of approximately 37mU/L (BioRad Lyphochek Immunoassay plus level 3 – LY3). This material was used to spike the whole blood in order to produce a TSH concentration of approximately 9mU/L (500µl LY3 was added to 1500µl whole blood and mixed thoroughly prior to being spotted onto bloodspot cards).

IRT

A small amount of faecal material was mixed with de-ionised water to produce an IRT rich liquid. Two samples were prepared with the addition of different amounts of faecal material (sample 1: 50µl faecal material added to 1ml whole blood and sample 2: 100µl faecal material added to 1ml whole blood) which produced blood with 74.6ng/mL and 127ng/mL IRT respectively (on preliminary testing). Sample 1 had the lower concentration which allowed the affect of multilayering around the cut-off for DNA testing to be investigated, and so sample 1 was used for the study. The sample was thoroughly mixed prior to being spotted onto bloodspot cards.

Bloodspots

A set of 6 cards was produced for each of the analytes investigated. Within this set was a card with single spots to replicate the ideal/reference sample type. There was also a card with spots that had been compressed after the blood had been applied to the card. Four other cards were produced within this set which simulated different types of multilayering. These were 2 spots overlaid whilst the original spot was still wet (**m/l 1**), 1 spot applied to a spot that had dried (**m/l 2**), 1 spot applied to the top of the card and re-applied on the bottom of the card (**m/l 3**) and application of 4 small spots to make one larger spot (**m/l 4**). Photographs of these cards can be seen in figure 1.

It is easy to see that the samples where 2 drops of blood have been applied to the same area (m/l 1 and m/l 3) are a lot bigger than the single spot produced from individual drops of blood. The sample produced to show a second spot being applied to a dry spot (m/l 2) produces spots that are a similar size to single spots, sometimes these are a bit bigger than in this photograph, but smaller than those seen in m/l 1 and m/l 3. There is usually evidence of the second spot visible on the bloodspot (either as dried blood or a smaller spot within the larger original spot). The spots that were produced by applying several small spots to the same area (m/l 4) show how varied a sample this can produce. There are some spots which obviously show 4 smaller spots, but some of them are less clear. The last set of spots is where the single drop of blood has been compressed and this is

less easy to detect. The centre of the spots tend to be more pale than normal (compare with single spots) and tend to be larger but this is not always very obvious.

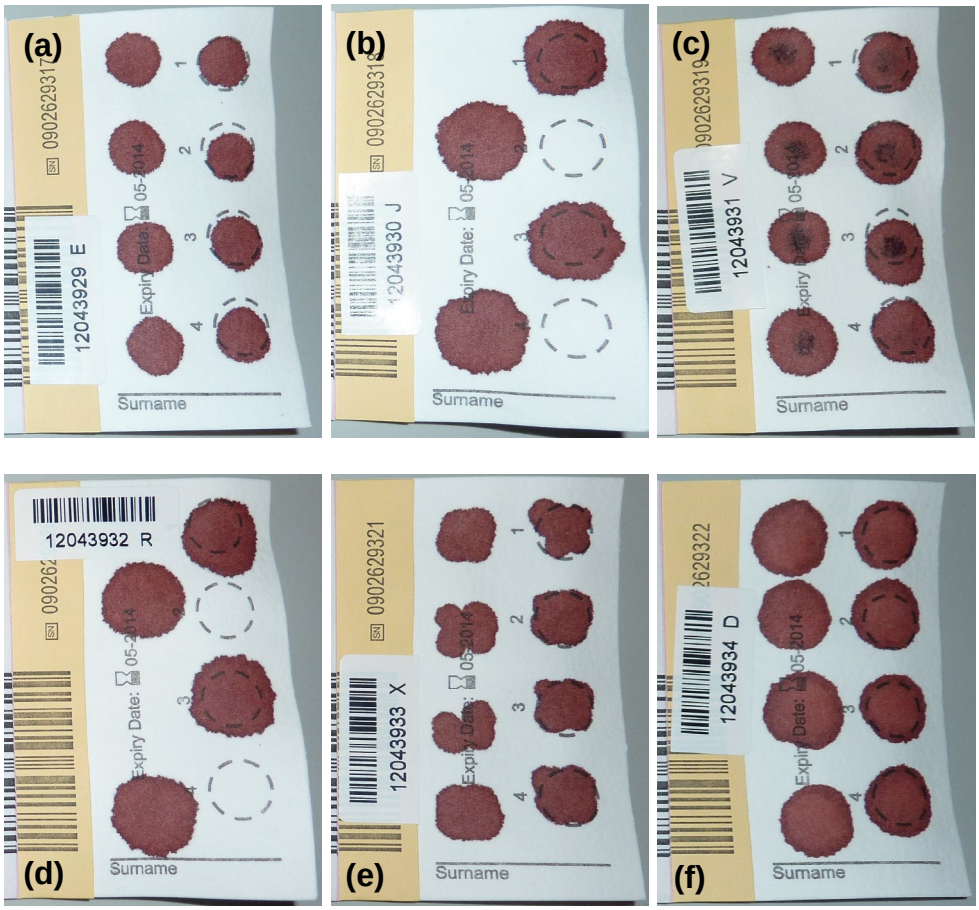


Figure 1: Bloodspot samples prepared from fresh blood.

Top row (from left to right): single spot (a), m/l 1 (b), m/l 2 (c).

Bottom row (from left to right): m/l 3 (d), m/l 4 (e), compressed (f).

Analysis and statistics

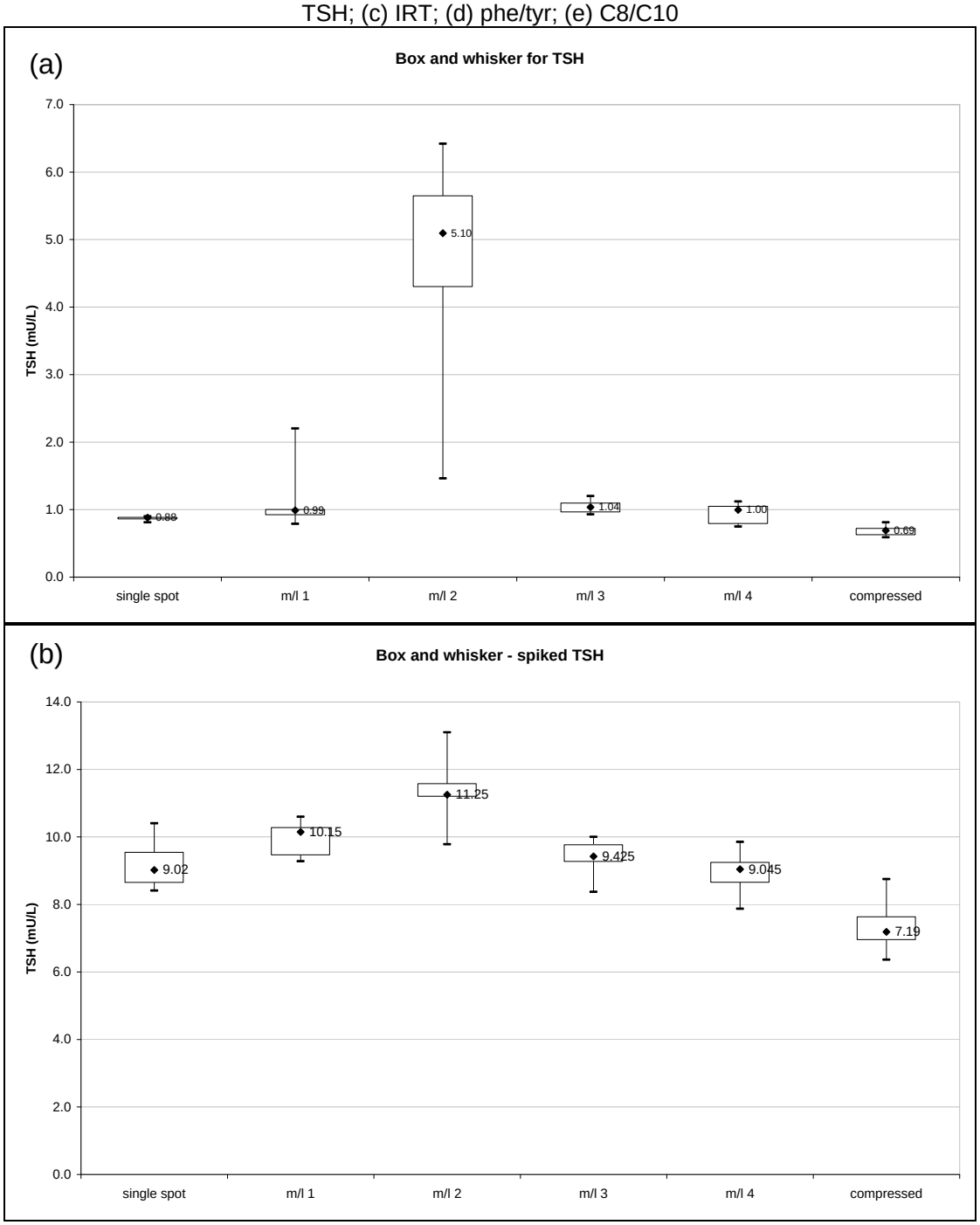
Each of these cards were sampled (punched) randomly 10 times and analysed within the same batch for each of the analytes. This approach was taken to eliminate any of the differences seen being attributed to analytical variation.

The results were analysed statistically using the one sided t-test. Box and whisker plots were generated for all analytes.

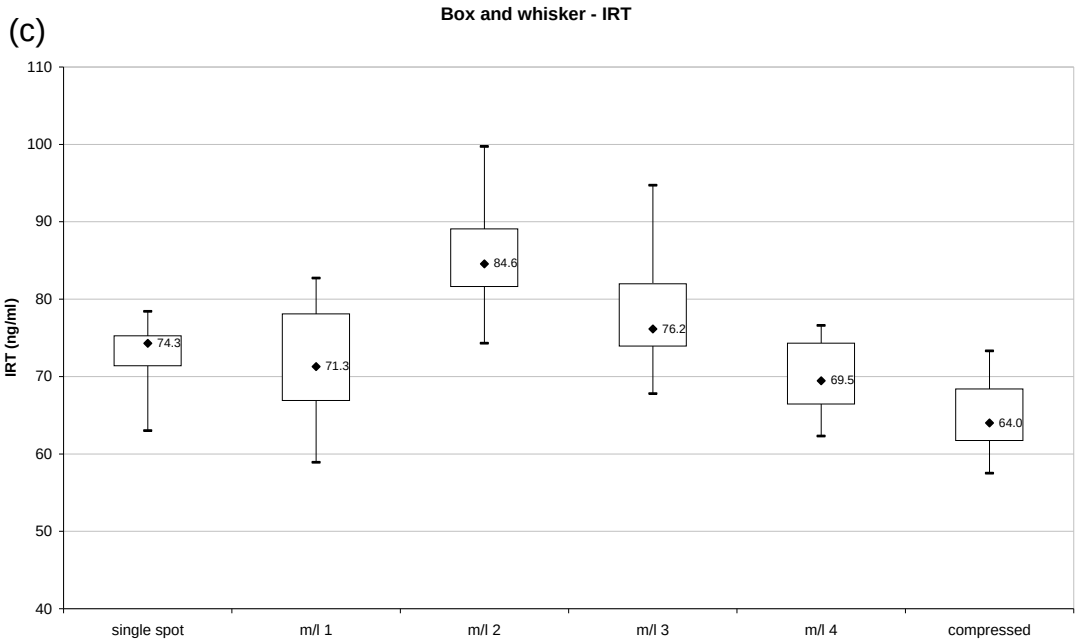
Results

Box and whisker plots clearly demonstrate that results for multilayered (of all types) and compressed samples are different both in average concentration and variation of results when compared to the results obtained in the single spotted sample, and this is the case for all analytes (figure 2). Raw data are displayed in Appendix 1. Data for box and whisker plots are displayed in Appendix 2.

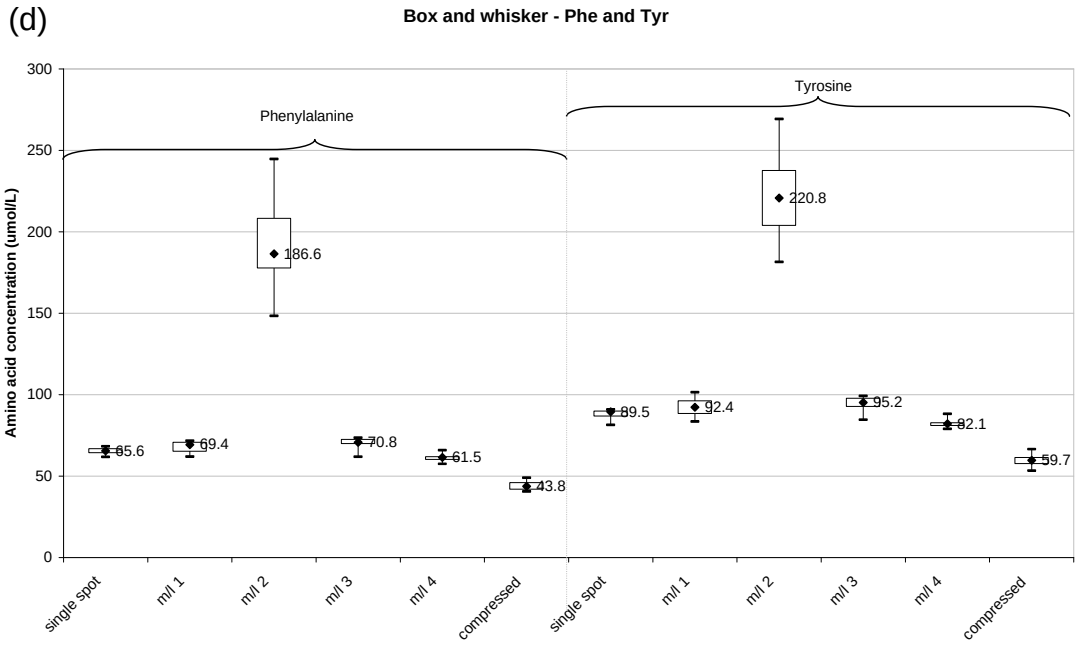
Figure 2: box and whisker plots for all analytes used for standard newborn screening (a) TSH; (b) spiked TSH; (c) IRT; (d) phe/tyr; (e) C8/C10

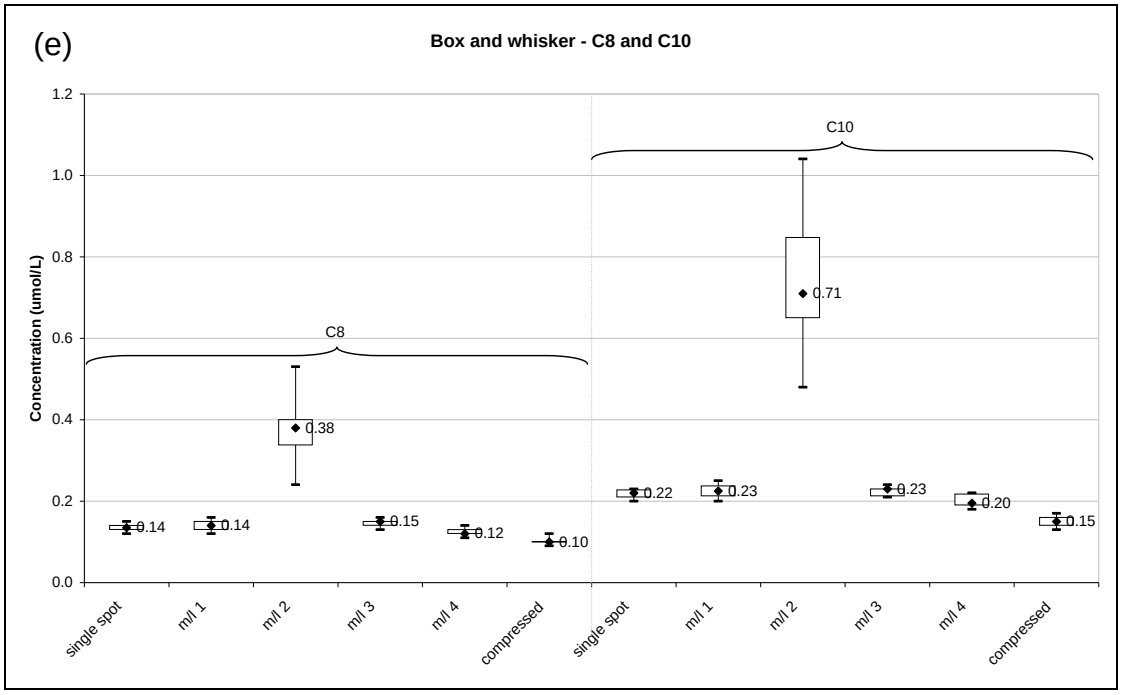


(c)


Box and whisker - IRT
IRT (ng/ml)
110
100
90
80
70
60
50
40
74.3
71.3
84.6
76.2
69.5
64.0
single spot
m/l 1
m/l 2
m/l 3
m/l 4
compressed

(d)


Box and whisker - Phe and Tyr
Amino acid concentration (umol/L)
300
250
200
150
100
50
0
Phenylalanine
Tyrosine
65.6
69.4
186.6
70.8
61.5
43.8
89.5
92.4
220.8
95.2
82.1
59.7
single spot
m/l 1
m/l 2
m/l 3
m/l 4
compressed
single spot
m/l 1
m/l 2
m/l 3
m/l 4
compressed



Statistical analysis on these results showed that for all types of multilayering and compression, the results are significantly different when compared to the single spot for all or some of the analytes (table 1).

Table 1: statistical evidence for all analytes and types of multilayering. Pink = difference in mean compared to single spot **is** significantly different, green = difference is **not** statistically significant [*met and leu/ileu included which are markers for conditions screened for as part of the extended panel*].

	m/l 1			m/l 2		
	mean difference	95% Confidence Interval	p value	average difference	95% Confidence Interval	p value
TSH	-0.211	-0.538 to 0.115	0.188	-3.676	-5.066 to -2.287	<0.0001
TSH (spiked)	-0.779	-1.320 to -0.238	0.0073	-2.125	-2.855 to -1.395	<0.0001
IRT	0.78	-5.13 to 6.69	0.7847	-12.58	-18.06 to -7.10	0.0001
Phe	-2.74	-5.55 to 0.07	0.0556	-126.62	-145.77 to -107.47	<0.0001
C8	-0.004	-0.015 to 0.007	0.443	-0.245	-0.298 to -0.192	<0.0001
Met	-1.209	-2.236 to -0.182	0.0236	-41.474	-48.393 to -34.555	<0.0001
Leu/Ileu	-9.367	-21.562 to 2.828	0.124	-508.298	-602.768 to -413.828	<0.0001

	m/l 3			m/l 4		
	mean difference	95% Confidence Interval	p value	average difference	95% Confidence Interval	p value
TSH	-0.169	-0.243 to -0.098	0.0002	-0.073	-0.190 to 0.044	0.2011
TSH (spiked)	-0.265	-0.810 to 0.280	0.3208	0.22	-0.402 to 0.842	0.467
IRT	-5.25	-10.92 to 0.42	0.0675	2.89	-1.43 to 7.21	0.1771
Phe	-4.66	-7.59 to -1.73	0.0036	4.02	1.96 to 6.08	0.0007
C8	-0.012	-0.020 to -0.004	0.008	0.012	0.004 to 0.020	0.008
Met	-1	-2.141 to 0.141	0.0822	1.473	0.614 to 2.332	0.002
Leu/Ileu	-14.816	-26.377 to -3.255	0.0149	16.098	7.777 to 24.419	0.0007

	compressed		
	mean difference	95% Confidence Interval	p value
TSH	0.187	0.128 to 0.245	<0.0001
TSH (spiked)	1.79	1.143 to 2.437	<0.0001
IRT	8.4	4.07 to 12.73	0.0007
Phe	21.14	18.67 to 23.61	<0.0001
C8	0.034	0.027 to 0.041	<0.0001
Met	6.801	5.760 to 7.842	<0.0001
Leu/Ileu	78.255	68.221 to 88.289	<0.0001

Negative values for mean difference and 95% confidence intervals indicate that the results from the multilayered/compressed samples are higher than from the reference (single spot) sample. Positive values indicate that results from the multilayered/compressed samples are lower than for the reference sample.

Discussion

Overlaid samples

The box and whisker plots show that for all multilayered samples that are more than one spot applied on top of each other (m/l 1, m/l 2 and m/l 3) the results are higher than for the results for the single spot. This is particularly strongly demonstrated with m/l 2 (a wet spot applied over a dry spot) where the differences are very strongly statistically significant ($p<0.0001$) for all analytes. For m/l 1 (1 extra spot applied whilst original spot was still wet) and m/l 3 (as for m/l 1 but one applied to top and other to bottom of card), there are still several analytes for which there is a statistical difference, although this is not as strong ($p<0.001$ to $p<0.1$).

This increase in results was expected, and is due to the larger volume of blood on the card. As the results from these types of samples are higher than in the reference sample, it is very unlikely that a positive result will be missed in any baby. In fact, the impact of accepting this type of sample is that false positive results will be more likely. This could mean that there may be added harm due to inappropriate clinical referral, diagnostic test samples being taken and the associated anxiety for the families of these babies.
However, the current laboratory practice is to request repeat samples to be taken on all babies who have had a (detectably) multilayered sample taken. By applying the analytical and clinical cut-offs to overlaid samples, it will be possible to reduce the number of babies who are re-bled unnecessarily and only repeat those that fall above the designated cut-off.

Recommendation: any samples that are multilayered but whose results fall below analytical or clinical cut-offs may be reported.

Multiple small spots to make one larger spot

Samples where multiple small spots are applied to the card in order to make one whole spot (m/l 4) have both areas of overlayering and sparse areas where blood has not soaked through the card adequately. This means that the sample is not homogeneous and results may therefore differ depending on where the spot is punched. This is demonstrated by looking at the box and whisker plots (figure 2). Results for all analytes (except TSH) are lower from these multilayered samples than for the single spotted samples. Table 2 shows the mean concentrations for all analytes for single spot and m/l 4 as well as the % difference between the results.

As the results for m/l 4 are mostly lower than for the reference (single) spot, the impact would be that false negatives may be more likely if this type of sample was accepted. This represents a significant risk of harm to the screened population and the screening programme.

Recommendation: samples made up of several small spots are unacceptable and it is not possible to report results on samples that appear to be multilayered in this way – repeat sample required.

Table 2: difference and % difference between mean concentrations of single spot and m/l 4 samples.

	TSH (mU/L)	Spiked TSH (mU/L)	IRT (ng/ml)	Phe (µmol/L)	Tyr (µmol/L)	C8 (µmol/L)	C10 (µmol/L)	Met (µmol/L)	Leu/Ileu (µmol/L)
Single spot mean	0.87	9.16	73.0	65.3	87.9	0.14	0.22	24.63	242.5
m/l 4 mean	0.94	8.94	70.1	61.3	82.2	0.12	0.20	23.16	226.4
Difference	0.07	-0.22	-2.9	-4.0	-5.7	-0.02	-0.02	-1.47	-16.1
% difference	9%	-2 %	-4%	-7%	-7%	-9%	-7%	-6 %	-7%

Compressed samples

It is very obvious from the box and whisker plots (figure 2) and from the statistical analysis (table 1) that results for compressed samples for all analytes are significantly lower than in single spots ($p<0.0001$). Table 3 shows the mean concentrations for all analytes for single spot and compressed spots as well as the % difference between the results. These results were 25% lower on average and up to 32% lower for some analytes. This would definitely constitute a significant risk to the patient and the screening programme as compressed samples are likely to produce false negative results.

Recommendation: samples that are compressed are unacceptable and it is not possible to report results on these samples – repeat sample required.

Table 3: difference and % difference between mean concentrations of single spot and compressed samples.

	TSH (mU/L)	Spiked TSH (mU/L)	IRT (ng/ml)	Phe (µmol/L)	Tyr (µmol/L)	C8 (µmol/L)	C10 (µmol/L)	Met (µmol/L)	Leu/ Ileu (µmol/L)
Single spot mean	0.87	9.16	73.0	65.3	87.9	0.14	0.22	24.63	242.5
compressed mean	0.68	8.94	64.6	44.2	59.7	0.10	0.15	17.83	164.2
Difference	-0.19	-1.79	-5.4	-21.1	-28.2	-0.04	-0.07	-6.8	-78.3
% difference	-22%	-20%	-12%	-32%	-32%	-25%	-31%	-28%	-32%

Assessment of sample quality

Currently, the Luminex CardScan is being trialled in the laboratory. This is to remove the subjectivity surrounding the assessment of sample quality which is currently a visual check made by senior scientists. The CardScan measures the transmission of light through the bloodspots and is able to provide information on whether the sample is homogeneous and of adequate volume and can indicate the best spots and areas within the spots to take the best quality samples (punches). As previously mentioned, spot compression in particular is extremely difficult to detect visually and can have a hugely detrimental effect on outcome, so this tool will be invaluable in ensuring that the laboratory is reporting results on valid samples.

Conclusion

From the results of this study, it is clinically safe to report normal results on samples where the spots appear to be overlaid (i.e. 1 spot directly on top of another) as long as the multilayering is not of the type where several spots have been applied to make up a whole spot. Overlaid samples should still be identified in the laboratory so that the results can be reviewed at the reporting stage and also for audit purposes.

Compressed samples must not be reported; a repeat sample should be requested. If compressing is suspected, the person collecting the sample should be contacted and advised that compressing the sample is forbidden for the reasons stated above.

It must be noted that it can be very difficult to detect compressed and multilayered (in the style of m/l 4) samples by eye in the laboratory and this poses a significant risk to the patient outcome if samples of this type are collected. The Luminex CardScan has the potential to improve detection of multilayered and compressed samples and is currently undergoing evaluation in the laboratory.

Glossary

Leu/Ileu	Leucine/Isoleucine
IRT	Immunoreactive Trypsin
MCADD	Medium Chain Acyl Co-A Dehydrogenase Deficiency
Met	Methionine
Phe	Phenylalanine
PKU	Phenylketonuria
TSH	Thyroid Stimulating Hormone
Tyr	Tyrosine

Appendix 1: Raw data

a. TSH

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	0.81	0.79	5.52	0.94	1.01	0.68
2	0.86	1.07	4.17	0.95	0.77	0.73
3	0.88	0.99	6.42	1.00	1.09	0.72
4	0.86	0.90	1.46	1.06	1.06	0.81
5	0.90	0.95	5.69	0.93	0.86	0.70
6	0.89	1.00	4.69	1.11	0.98	0.72
7	0.88	0.99	5.73	1.20	1.12	0.61
8		2.20	1.58	1.04	1.01	0.64
9		0.91	5.07	1.03	0.75	0.59
10		1.00	5.12	1.12	0.77	0.62

b. Spiked TSH

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	10	9.37	11.2	9.25	9.1	7.02
2	8.67	10.3	11.2	9.81	8.99	6.9
3	10.4	10.1	11.2	8.37	9.85	6.93
4	9.61	10.3	11.5	9.95	7.87	7.11
5	8.53	10.6	13.1	9.49	8.03	6.36
6	9.07	9.68	11.6	9.32	8.73	7.27
7	8.64	9.39	11.7	9.09	8.63	7.35
8	8.41	10.2	9.78	9.36	9.12	8.75
9	8.97	9.28	10.3	9.64	9.29	7.73
10	9.33	10.2	11.3	10	9.82	8.31

c. IRT

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	78.4	81.9	99.7	75.8	62.3	58.4
2	74.8	64.8	91.6	76.2	65.6	62.6
3	70.5	58.9	74.3	76.1	74.8	61.4
4	70.9	69.5	81.1	94.7	72.8	57.5
5	63.0	66.0	79.2	82.7	76.6	63.8
6	75.4	73.1	85.5	83.2	76.6	69.1
7	74.6	82.7	83.1	72.7	67.1	69.2
8	72.7	69.5	88.1	67.8	66.2	66.3
9	74.0	78.4	89.4	79.8	68.4	73.3
10	75.5	77.2	83.6	73.3	70.5	64.2

d. Phenylalanine

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	61.7	63.9	193	64.8	62.0	46.5
2	64.1	70.8	213.3	70.0	57.5	49.0
3	67.5	69.3	190.8	72.8	61.1	43.0
4	66.3	71.7	180.9	61.8	65.9	40.5
5	68.2	69.0	164.4	70.0	61.8	48.5
6	67.1	63.0	225.0	69.8	59.2	43.4
7	65.9	70.8	244.6	73.6	59.8	44.4
8	62.3	61.9	182.3	71.8	62.5	40.6
9	65.2	69.4	176.6	71.5	61.2	44.2
10	64.7	70.6	148.3	73.5	61.8	41.5

e. Tyrosine

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	81.4	87.6	225.7	88.1	83.1	61.2
2	86.9	92.3	241.6	92.4	78.9	66.5
3	89.4	90.5	222.8	97.1	82.1	57.7
4	90.5	101.4	202.6	84.6	88.2	53.5
5	90.9	94.2	198.9	93.2	82.1	66.0
6	89.6	83.5	264.2	93.4	80.5	58.6
7	89.7	96.9	269.2	98.0	79.6	60.8
8	83.6	84.0	218.7	98.7	82.9	53.3
9	90.0	92.4	207.4	97.0	82.3	61.5
10	86.7	96.8	181.5	99.2	82.1	57.5

f. C8

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	0.13	0.13	0.38	0.15	0.12	0.1
2	0.14	0.12	0.4	0.14	0.13	0.1
3	0.12	0.15	0.4	0.15	0.12	0.1
4	0.15	0.16	0.38	0.14	0.11	0.09
5	0.14	0.14	0.32	0.16	0.11	0.1
6	0.13	0.13	0.46	0.15	0.12	0.1
7	0.14	0.14	0.53	0.16	0.13	0.12
8	0.13	0.12	0.36	0.15	0.13	0.1
9	0.14	0.15	0.33	0.13	0.12	0.1
10	0.13	0.15	0.24	0.14	0.14	0.1

g. C10

replicate	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
1	0.2	0.21	0.78	0.21	0.2	0.16
2	0.23	0.22	0.87	0.22	0.18	0.16
3	0.22	0.23	0.73	0.24	0.19	0.14
4	0.23	0.25	0.65	0.21	0.22	0.14
5	0.22	0.24	0.58	0.24	0.19	0.16
6	0.22	0.21	1	0.23	0.19	0.15
7	0.23	0.22	1.04	0.21	0.19	0.17
8	0.2	0.2	0.69	0.23	0.22	0.14
9	0.21	0.25	0.65	0.23	0.22	0.15
10	0.21	0.23	0.48	0.23	0.21	0.13

Appendix 2: Data for box and whisker plots

a. TSH

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	0.81	0.79	1.46	0.93	0.75	0.59
1st quartile	0.86	0.92	4.30	0.96	0.79	0.63
median	0.88	0.99	5.10	1.04	1.00	0.69
3rd quartile	0.89	1.00	5.65	1.10	1.05	0.72
max	0.90	2.20	6.42	1.20	1.12	0.81

b. Spiked TSH

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	8.41	9.28	9.78	8.37	7.87	6.36
1st quartile	8.65	9.46	11.20	9.27	8.66	6.95
median	9.02	10.15	11.25	9.43	9.05	7.19
3rd quartile	9.54	10.28	11.58	9.77	9.25	7.64
max	10.40	10.60	13.10	10.00	9.85	8.75

c. IRT

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	63.0	58.9	74.3	67.8	62.3	57.5
1st quartile	71.35	66.875	81.6	73.925	66.425	61.7
median	74.3	71.3	84.6	76.2	69.5	64.0
3rd quartile	75.25	78.1	89.075	81.975	74.3	68.4
max	78.4	82.7	99.7	94.7	76.6	73.3

d. Phenylalanine

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	61.7	61.9	148.3	61.8	57.5	40.5
1st quartile	64.3	65.2	177.7	69.9	60.1	41.9
median	65.6	69.4	186.6	70.8	61.5	43.8
3rd quartile	66.9	70.8	208.2	72.6	62.0	46.0
max	68.2	71.7	244.6	73.6	65.9	49.0

e. Tyrosine

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	81.4	83.5	181.5	84.6	78.9	53.3
1st quartile	86.8	88.3	203.8	92.6	80.9	57.6
median	89.5	92.4	220.8	95.2	82.1	59.7
3rd quartile	89.9	96.2	237.6	97.8	82.8	61.4
max	90.9	101.4	269.2	99.2	88.2	66.5

f. C8

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	0.12	0.12	0.24	0.13	0.11	0.09
1st quartile	0.13	0.13	0.34	0.14	0.12	0.10
median	0.14	0.14	0.38	0.15	0.12	0.10
3rd quartile	0.14	0.15	0.40	0.15	0.13	0.10
max	0.15	0.16	0.53	0.16	0.14	0.12

g. C10

	single spot	m/l 1	m/l 2	m/l 3	m/l 4	compressed
min	0.20	0.20	0.48	0.21	0.18	0.13
1st quartile	0.21	0.21	0.65	0.21	0.19	0.14
median	0.22	0.23	0.71	0.23	0.20	0.15
3rd quartile	0.23	0.24	0.85	0.23	0.22	0.16
max	0.23	0.25	1.04	0.24	0.22	0.17