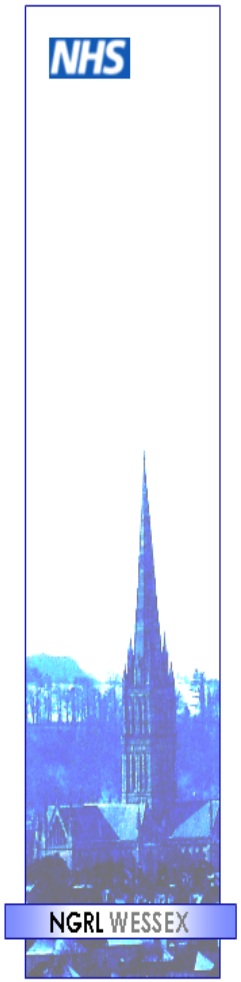
Salisbury NHS
NHS Foundation Trust



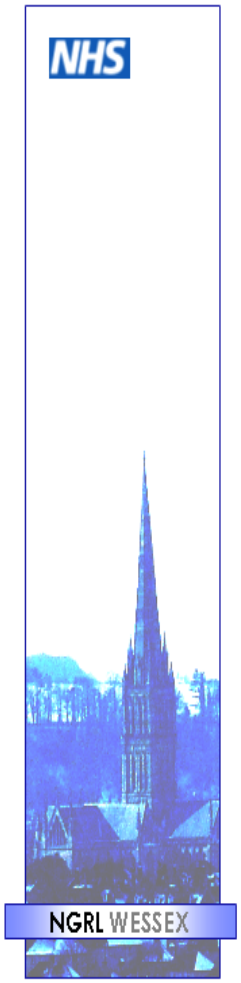
Progress towards establishing *BCR-ABL* reference reagents

Helen White

National Genetics Reference Lab (Wessex)

DH Department of Health

Reference materials



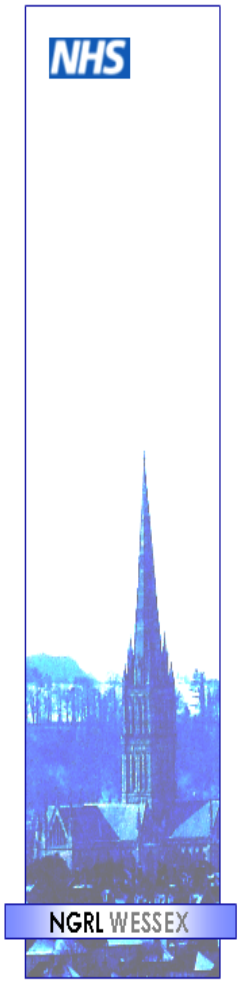
1) Primary reference material

- Ideally be as close as possible to real samples.
- Must be stable over several years (=freeze dried)
- Batches must last several years
- Must cover all or most existing methods (incl. RNA extraction).
- Prepared centrally and WHO accredited by NIBSC
- May be very limited in supply
- Used by reference labs or companies to calibrate 2º reference material

2) Secondary reference material

- Easily available: used by testing labs on every run? Every week?
- Prepared locally/nationally? Or by companies? e.g. Asuragen, Molecular MD, Ipsogen
- Calibrated to primary reference reagents
- Similar to real samples: function to monitor efficiency of RNA extraction and/or RT and assay drift but may also provide conversion factor

Formulation for primary and/or secondary reagents reagents



- [CML cells (primary or K562) diluted in normal leucocytes]

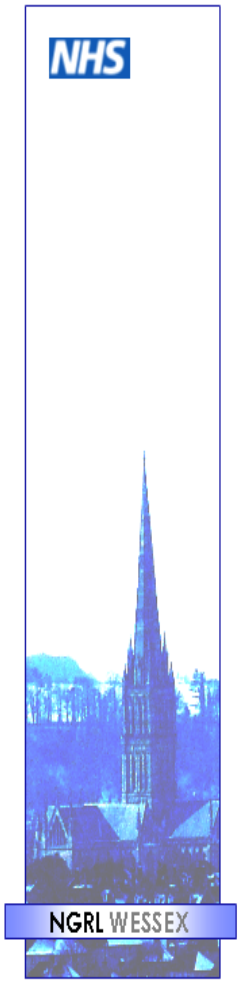
  Hard to obtain large batches
  vCJD concerns
  Viral screening

- Freeze dried cell line mixtures

- Armored RNA

Field trial studies of freeze dried cell lines and armored RNA:
June 2007 – Dec 2007

Field trial of freeze dried cell line mixes (June – Oct ’07)



- Cell lines used:

HL60	BCR-ABL negative
KG1	BCR-ABL negative
K562	BCR-ABL (b3a2) positive

- Prepared cell mixtures of HL60 and KG-1 spiked with K562 at 4 dilutions spanning c.10% - 0.01% BCR/ABL (Levels 1 – 4)
- HL60 mix
  - 9×10^8 HL60 grown up in total
  - Level 1 required 1:20 dilution of K562 ~ 10% BCR-ABL (ABL as control gene)
- KG1 mix
  - 9×10^8 KG-1 grown up in total
  - Level 1 required 1:10 dilution of K562 ~ 10% BCR-ABL
- Transferred *c.* 2.1×10^8 cells per dilution (6×10^6 cells / ml in 2x PBS) to NIBSC for freeze drying

Field Trial: Freeze Dried material available

3 x 10^6 cells / vial

HL60 / K562 Cell mixtures

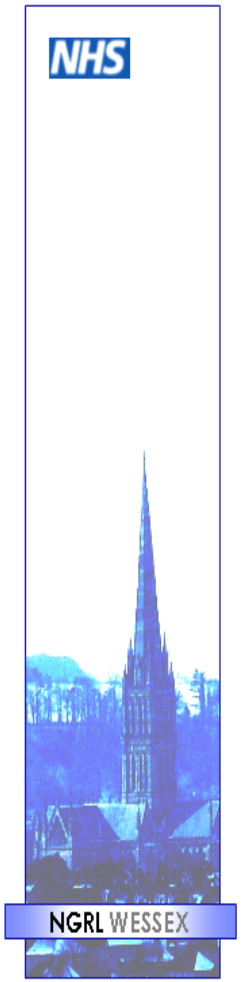
Level 1	(~10% BCR-ABL)	PM-07-011-A	45 vials
Level 2	(~1% BCR-ABL)	PM-07-011-B	43 vials
Level 3	(~0.1% BCR-ABL)	PM-07-011-C	45 vials
Level 4	(~0.01% BCR-ABL)	PM-07-011-D	45 vials

KG-1 / K562 Cell mixtures

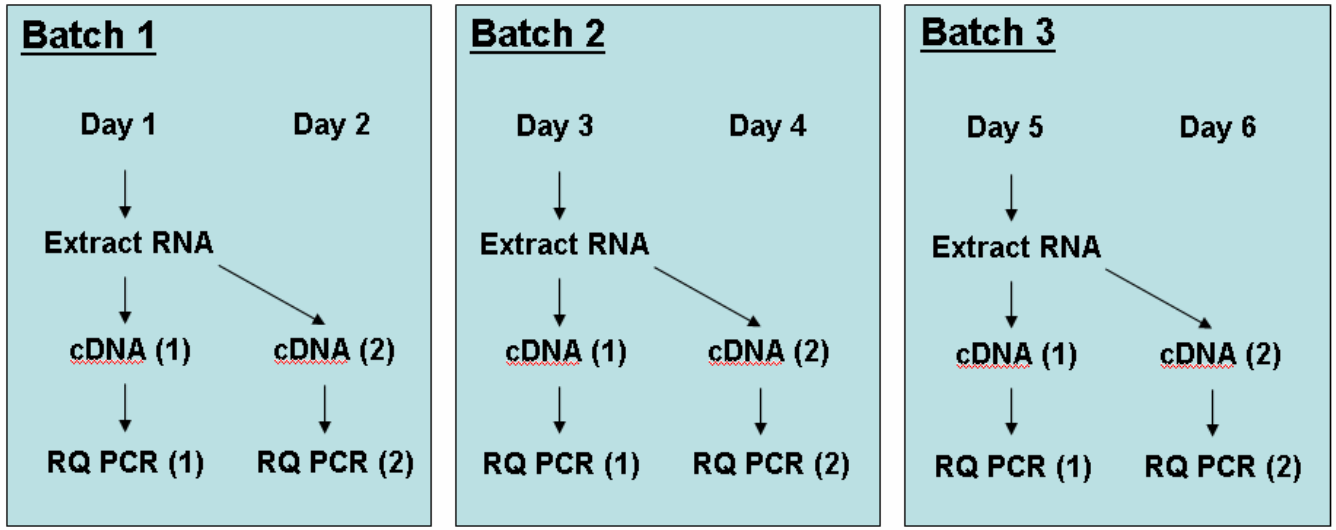
Level 1	(~10% BCR-ABL)	PM-07-012-A	42 vials
Level 2	(~1% BCR-ABL)	PM-07-012-B	45 vials
Level 3	(~0.1% BCR-ABL)	PM-07-012-C	45 vials
Level 4	(~0.01% BCR-ABL)	PM-07-012-D	45 vials

*** 10 Vials retained for each level for accelerated degradation / stability testing. Duplicates at 5 temperatures**

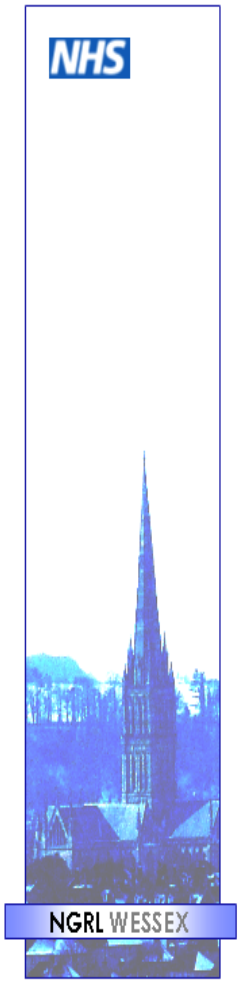
Field Trial Protocol



- Freeze Dried Cells sent to 14 labs
- Each lab sent 24 vials packaged into 3 batches
- Each batch contained 8 vials: HL60/K562 Levels 1 – 4
  KG1/K562 Levels 1 - 4
- Cells lysed directly in 1ml Trizol or 600µl RLT Buffer
- RNA Extracted following usual lab protocol
- cDNA synthesis and RQ-PCR was performed:



Number of labs, methods & equipment used



Labs

14 participants: Europe (7), USA (4), Australia (1), Hong Kong (1), Korea (1)

Protocols

7 different protocols (50% use EAC, Gabert et al. *Leukemia* 17, 2318, 2003)

Control Genes Analysed

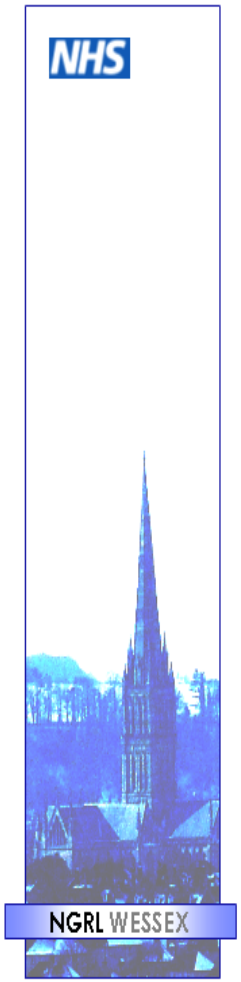
ABL (12), BCR (5), GUS (7). G6PD (1)

RQ-PCR Machines

9 different platforms

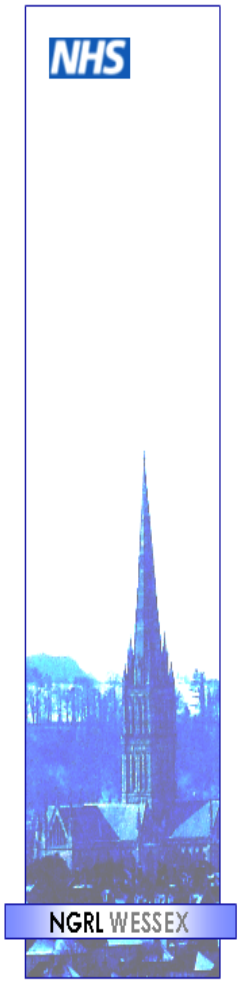
Roche:	Lightcycler 2 (3), Lightcycler 1.5 (1), Lightcycler 480 (1)
ABI:	ABI 7000 (2), ABI 7500 (2), ABI 7700 (1), ABI 5700 (1)
Other:	Stratagene MX3000P (1), Corbett RotorGene 6000 (1)

RNA Yield



Reagent	Mean µg RNA	Median µg RNA
HL60 Level 1	30.30	28.21
HL60 Level 2	31.03	31.39
HL60 Level 3	31.90	31.17
HL60 Level 4	28.87	27.74
HL60 Combined	**30.53**	**29.61**
KG1 Level 1	33.52	33.09
KG1 Level 2	29.84	30.31
KG1 Level 3	30.61	31.26
KG1 Level 4	29.92	30.89
KG1 Combined	**30.92**	**31.37**

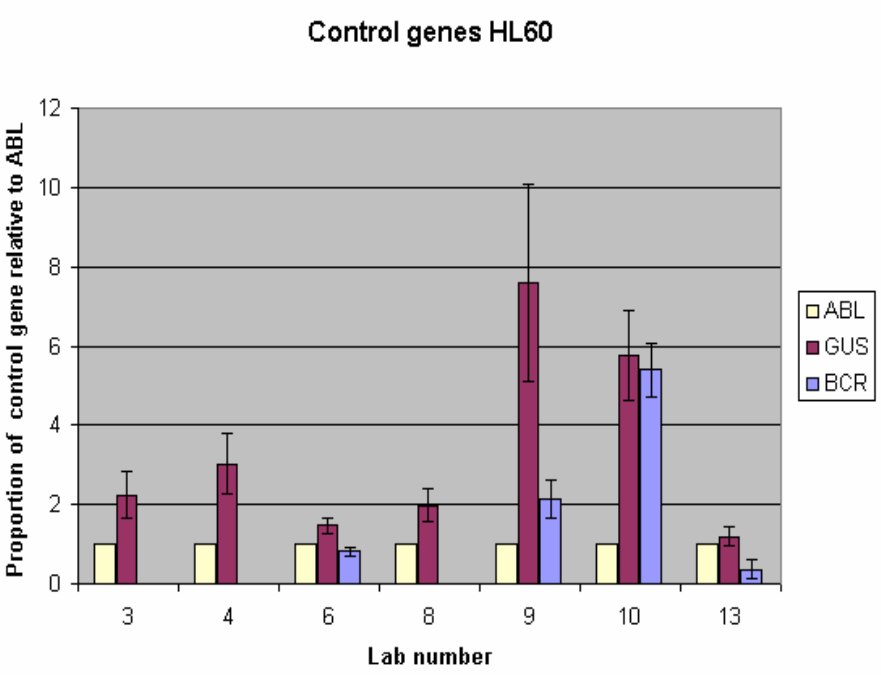
Median copy number for control genes

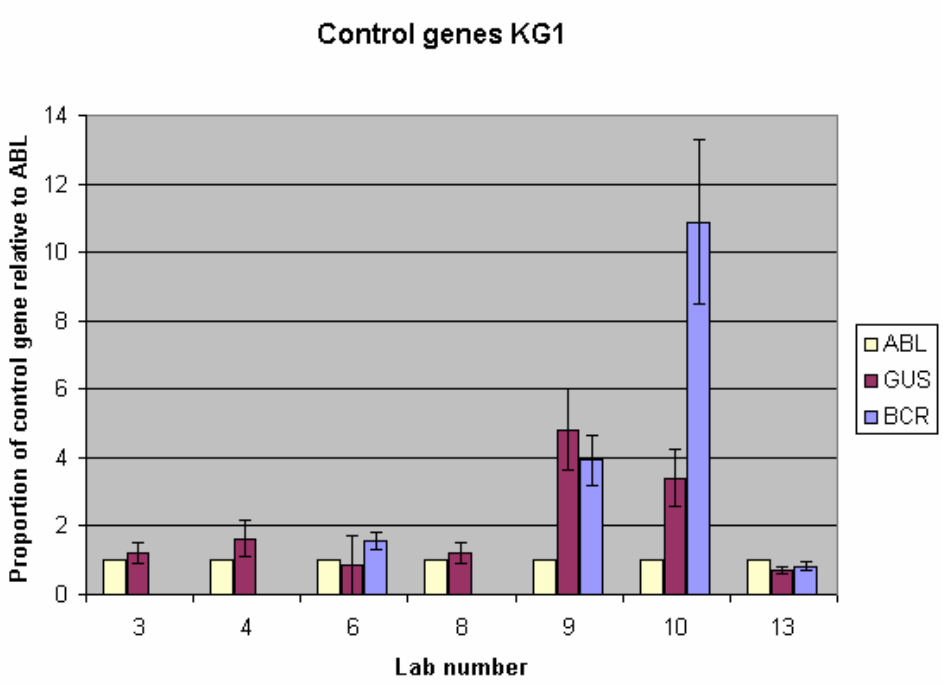


	HL60	KG1
ABL	1.16 x 10^5	1.02 x 10^5
BCR	2.6 x 10^5	5.85 x 10^5
GUS	1.7 x 10^5	1.13 x 10^5

Relative expression of each control gene

Relative amounts of each control gene in relation to *ABL* for laboratories who tested more than one control gene

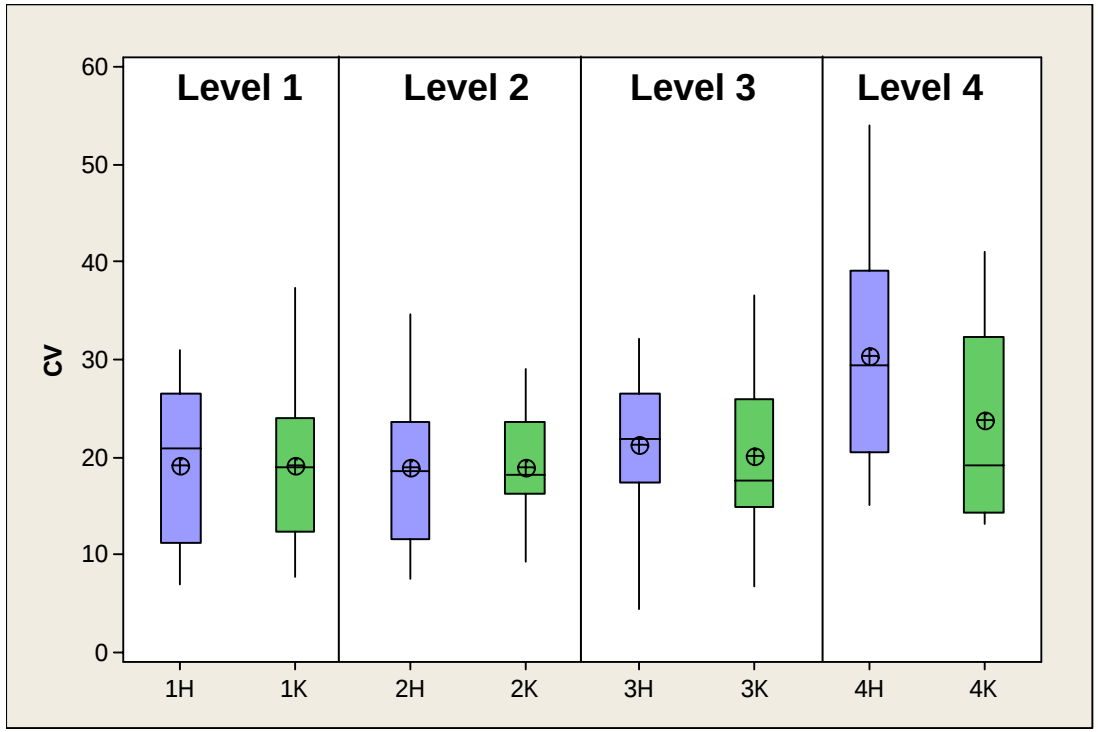




- Ratios varied between labs
- In general: *GUSB* was most highly expressed in HL60

  ABL and *GUSB* levels were comparable in KG1

Coefficient of Variation of %BCR-ABL / Control gene



HL60 ABL

KG1 ABL

⊕ Mean

N=12

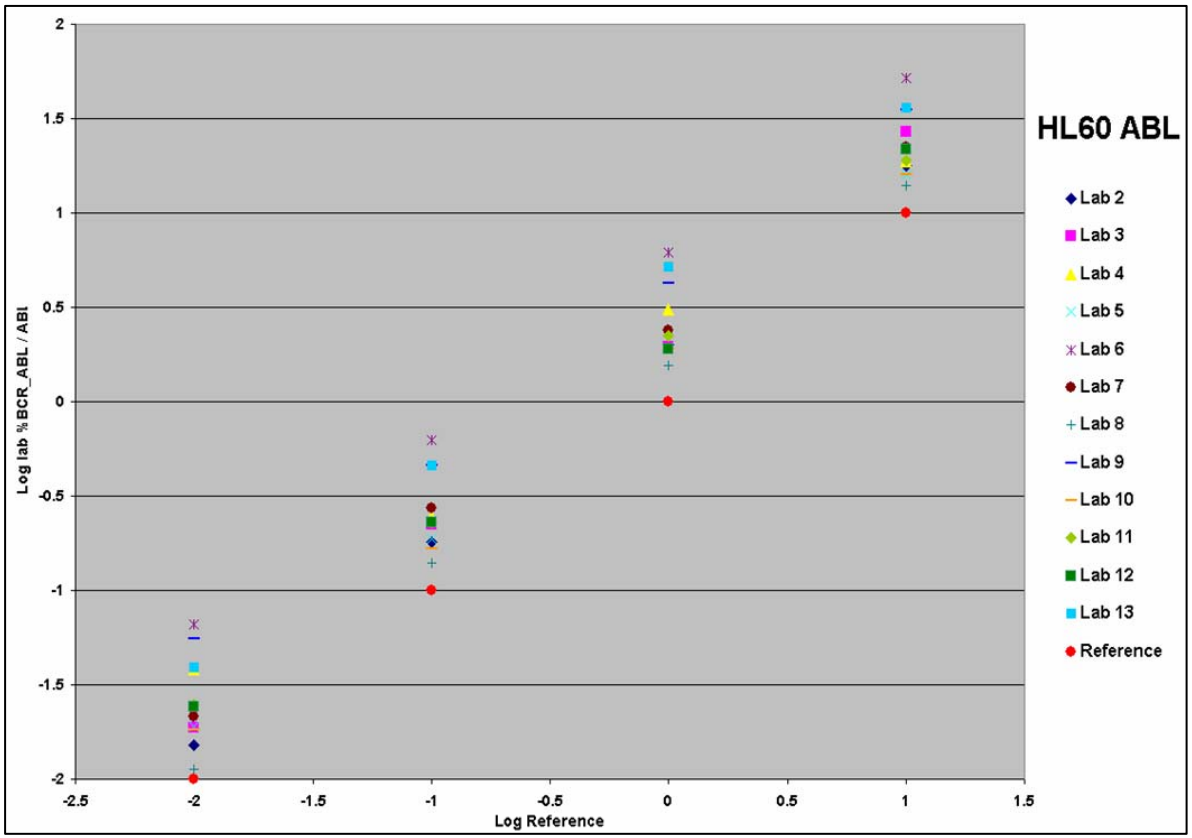
- CVs comparable to those obtained in IS conversion rounds
- No statistical difference between cell lines for any control gene

Linear regression

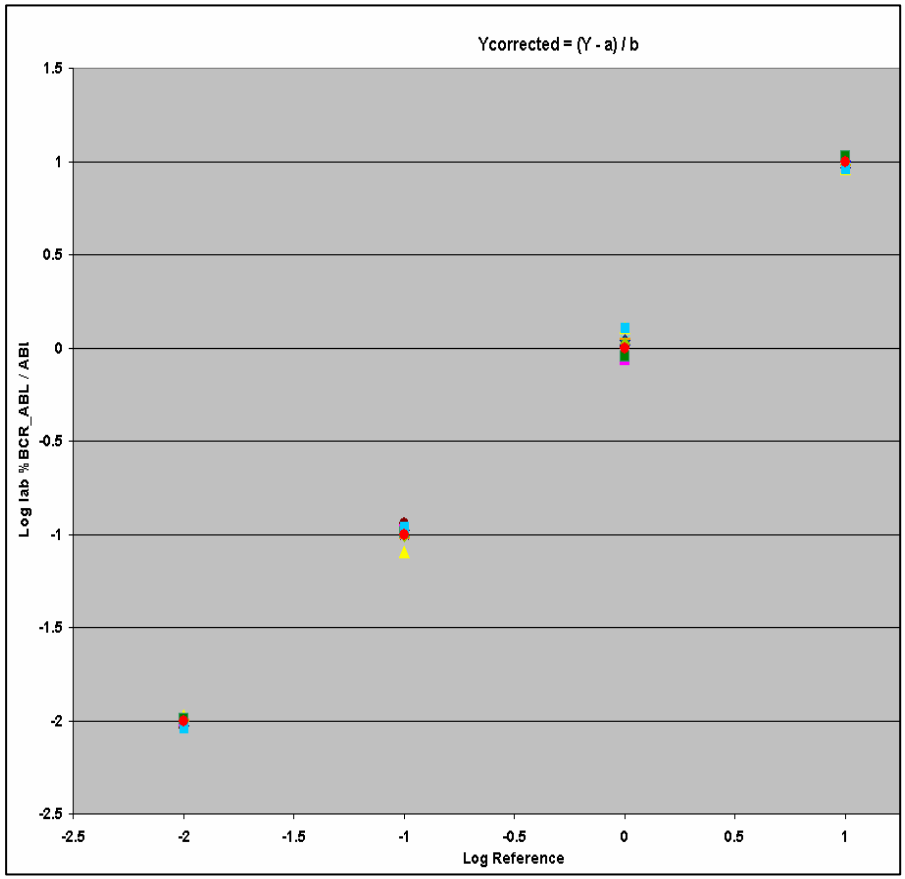
- Values of 10%, 1%, 0.1% and 0.01% *BCR-ABL* / control gene were assigned to levels 1 – 4

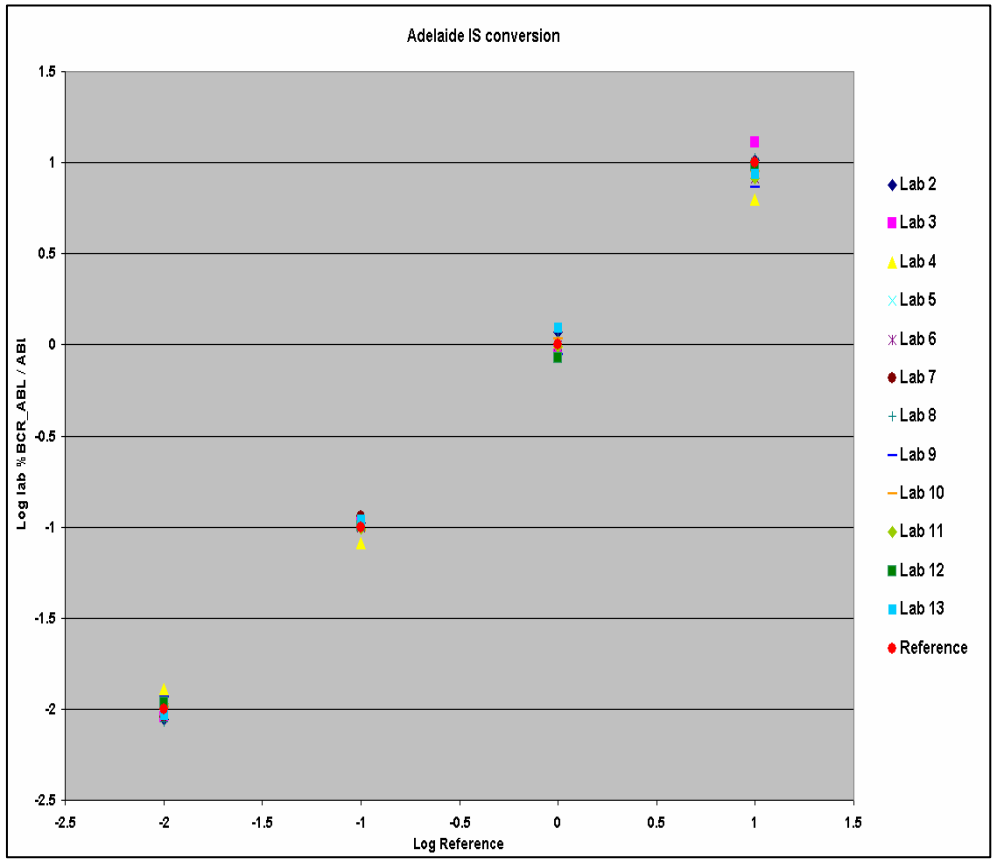
- Linear regression plots were produced for log transformed lab data plotted against the log transformed reference standard values.

- Some linear regression plots showed statistically significant variation in the slope of the line when compared to the reference standard. Most prevalent for KG1 *ABL* (6 labs, 46%).

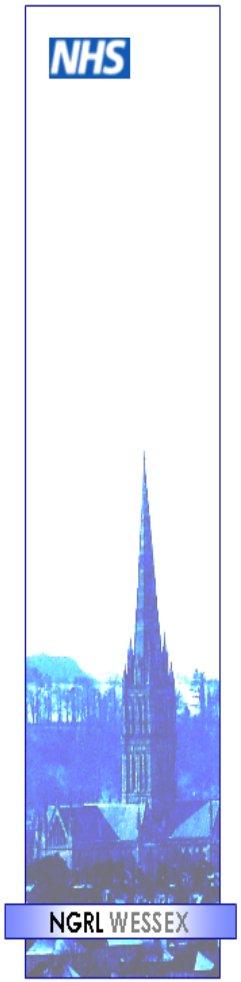


Linear regression: Converted / standardised data





Summary



- Overall, it appears that both freeze dried cell line mixes could be suitable for development of *BCR-ABL* reference reagents

- 14 labs took part in the field trial, 2 RNA extraction protocols were used and over half of the laboratories used the EAC protocol. Random hexamers were used for reverse transcription by all labs

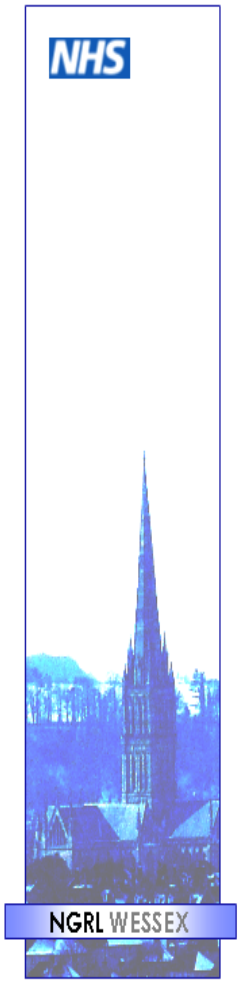
- RNA (median 30µg/vial) was successfully extracted from freeze dried cell mixtures shipped worldwide at ambient temperature

- Median copies obtained for *ABL*, *BCR* and *GUSB* for the HL60 and KG1 material were > 1 x 10^5 for all control genes although the relative ratios for control gene expression varied between labs

- Linear regression plots were produced for log transformed lab data plotted against the log transformation of the reference standard values. The resulting regression equations were used to successfully standardize data to the reference material values

- HL60 / K562 and KG1 / K562 cell mixes performed equally well producing CVs that were comparable to those expected for primary patient samples

Future Work



- Medium scale pilot freeze dry study of HL60 / K562 cell mix in collaboration with ECACC and NIBSC. Testing feasibility of:

  - Growing and processing cells at ECACC
  - Using 1.5 x 10^6 cells / vial vs 3 x 106 cells / vial
  - Use of 3ml glass ampoules vs glass vials

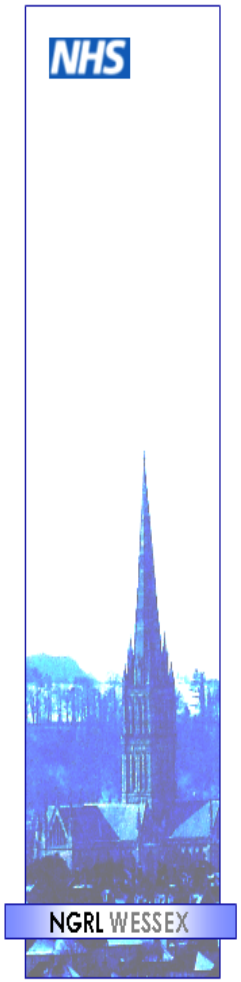
Status:

  - freeze drying complete
  - control gene copy numbers appear acceptable
  - no difference in sample quality using glass ampoules

- Large scale culture of HL60 / K562 will be undertaken by ECACC in the Summer (3 x 10^{10} HL60; 60 litre culture)
- Large scale freeze dry planned at NIBSC in Sept 2008: 3000 ampoules for each of 4 levels of %BCR-ABL (12,000 vials total)

  - vials will not be available for testing labs on demand: risk of depleting stocks
  - essential to promote production and widespread availability of 2º reference material

Armored RNA (aRNA) Field Trial Oct – Dec 2007



- aRNA is based on bacteriophage coat protein encapsulation of specific RNA targets to form pseudo-viral particles tha protect RNA transcripts from nuclease degradation and stabilise aRNA sequences

- aRNA is designed for use as standards and controls in assays, in particular a as positive controls for amplification and detection using RT-PCR

- Survey of primer sets performed July 2006

- Plasmids made (BCR, ABL, GUS, b2a2, b3a2) that cover the regions targeted by all members of the international group

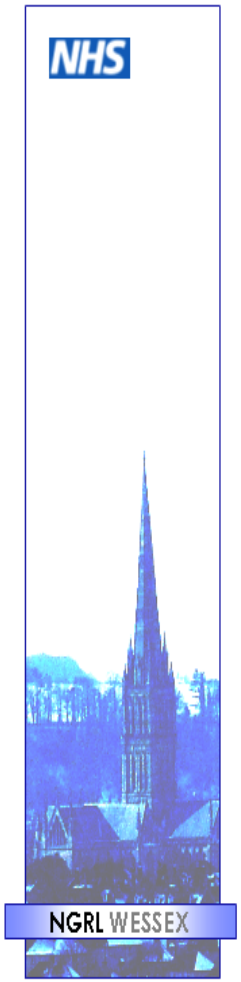
- Sequence verified; sent (essentially gifted) to Asuragen Nov 2006

- Armored RNAs arrived in Salisbury May 2007

- Copy numbers estimated by NIST-traceable phosphate assay
  - –Due to lack of sufficient ABL product yield, target was quantified through a standard OD260 conversion used for estimating copy numbers for aRNA non-quantitative products.

Asuragen

Aims of field trial



The aims of the field trial were to:

i. test if aRNAs could be shipped worldwide successfully at ambient temperature

ii. test four different levels of b3a2 aRNA and / or b2a2 aRNA diluted in a background of GUSB, BCR and ABL aRNA.

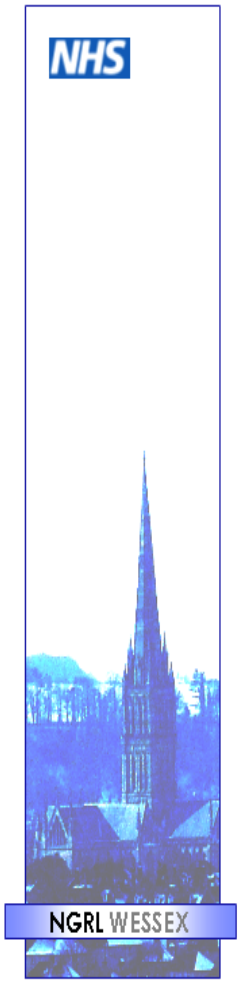
iii. measure absolute copy numbers of BCR-ABL (b3a2 and / or b2a2), absolute copy numbers of control genes (GUSB, BCR and ABL) and the BCR-ABL / control gene ratios.

iv. compare the performance of:

a. aRNA mixes put through an RNA extraction procedure

b. aRNA mixes which were heat lysed and added directly to a cDNA reaction without undergoing an RNA extraction procedure.

aRNA field trial design



- aRNAs were produced for b2a2 and b3a2 *BCR-ABL*, plus *BCR*, *ABL* and *GUSB*

- Nine aRNA prototype reference standards were prepared containing:

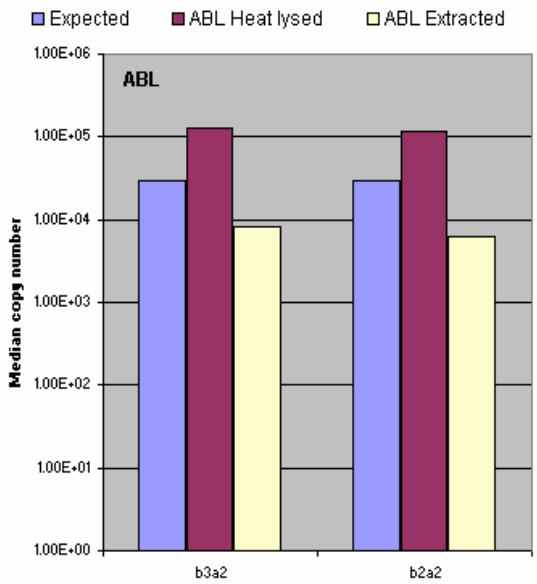
  $3x10^4$ (Level 1)
  $3x10^3$ (Level 2)
  $3x10^2$ (Level 3)
  $3x10^1$ (Level 4) copies/ul of b3a2 or b2a2 aRNA

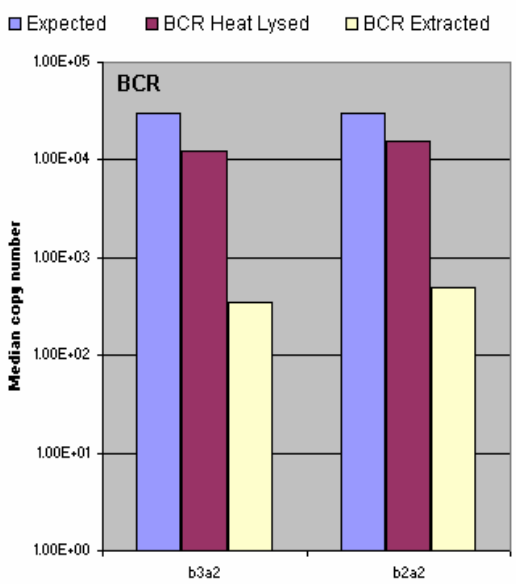
with each control gene (*ABL*, *BCR*, *GUSB*) at $3x10^4$ copies/ul.

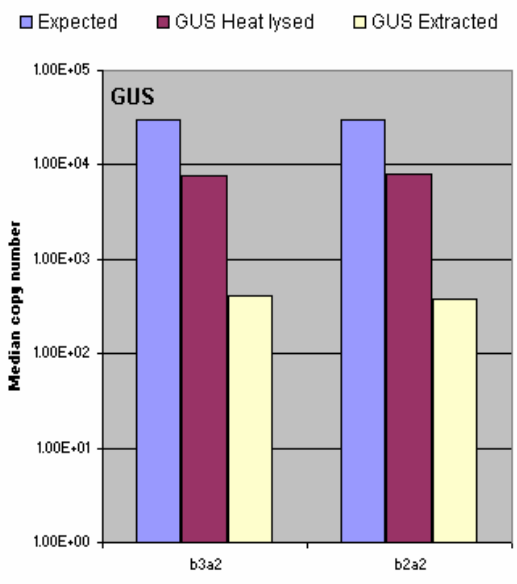
- The performance of the aRNA samples was assessed by an international field trial (Oct - Dec 2007) that involved 29 laboratories. (22 EU, 3 USA, 4 Asia/Australasia) analysing 3 different control genes on 14 different RQ-PCR platforms.

- aRNA samples were tested after RNA extraction or direct heat lysis.

Median Copy Numbers for control genes

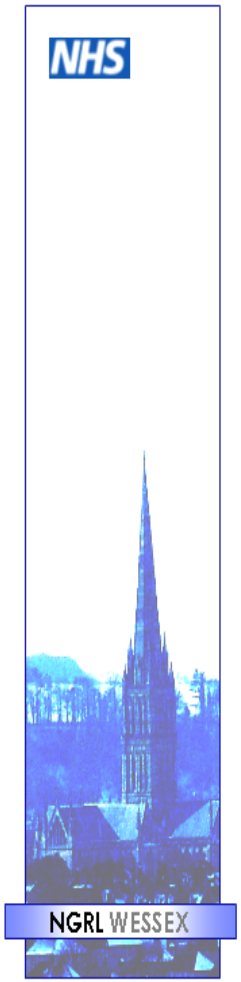






RNA Extraction method	Number of extractions	Median fold loss of control gene copy number following extraction
Trizol	65	**27.8**
QIAGEN	17	**2.1**
Roche	4	5.85
RNAzol	2	1.8
TriReagent	2	60.3
Overall	**90**	**12.1**

%BCR-ABL / Control gene values



- The mean % *BCR-ABL* / control gene values for the extracted and heat lysed aRNA samples were not statistically different (2 sample t-test at 99% confidence; i.e. $p<0.01$).

- **However** for the extracted samples:

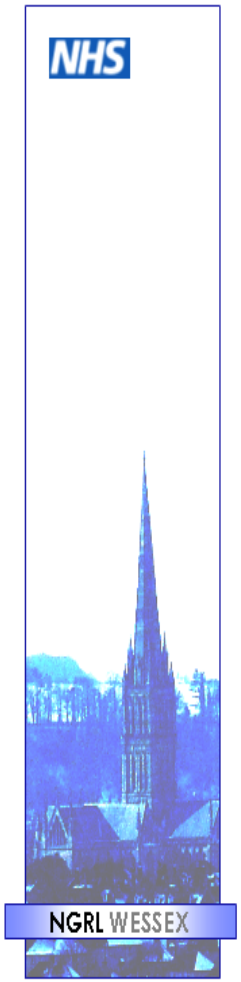
  6/28 labs (21%) failed to detect level 3 and 4 b3a2 transcripts

  6/22 labs (27%) failed to detect level 3 and 4 b2a2 transcripts

- For the heat lysed aRNA samples level 4 b3a2 and b2a2 could not be detected by one lab

- Lack of detection of BCR-ABL transcripts was most likely due to the loss of recovery of the aRNA following RNA extraction using the Trizol protocol.

Coefficient of Variation of %BCR-ABL / Control gene



b3a2

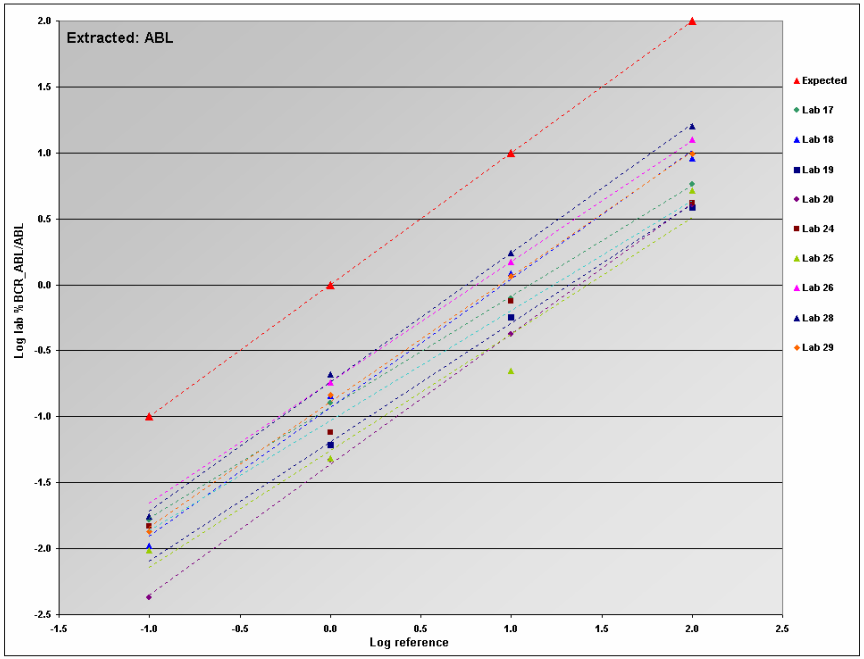
Protocol	Control gene	Level 1	p	Level 2	p	Level 3	p	Level 4	p
Extracted	ABL	24.99	0.405	29.37	0.198	50.43	0.161	68.06	**0.019***
Unextracted	ABL	17.77		24.05		39.08		33.89	
Extracted	BCR	24.97	**0.099***	28.10	0.358	35.37	0.329	84.00	**0.070***
Unextracted	BCR	14.28		22.44		30.29		34.86	
Extracted	GUSB	38.92	**0.058***	36.85	0.163	36.97	0.259	41.27	0.405
Unextracted	GUSB	22.72		21.97		34.13		32.25	

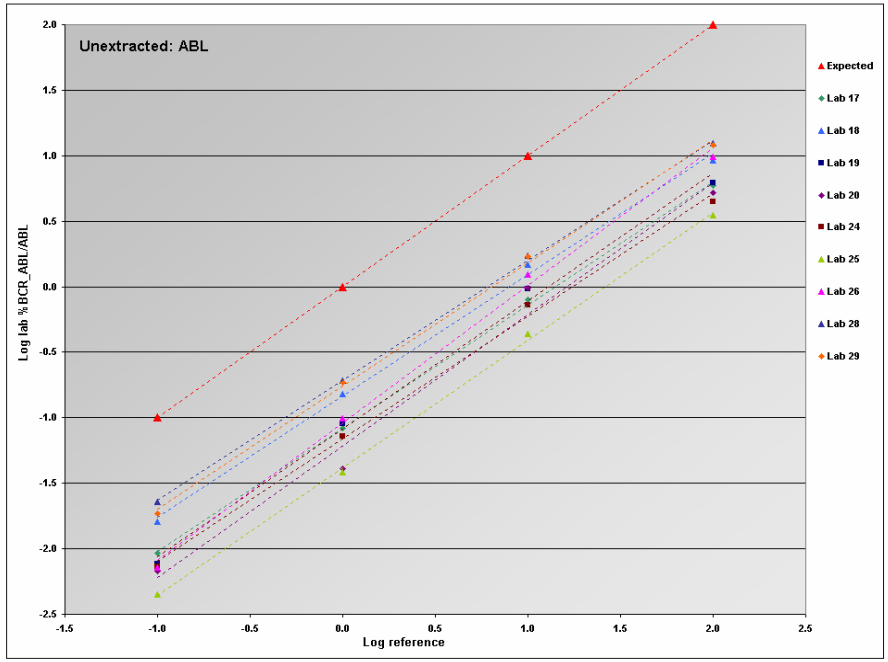
b2a2

Protocol	Control gene	Level 1	p	Level 2	p	Level 3	p	Level 4	p
Extracted	ABL	21.43	0.840	30.35	0.375	36.67	0.507	40.70	0.708
Unextracted	ABL	22.03		21.70		27.70		36.48	
Extracted	BCR	19.91	0.778	60.86	**0.005***	81.51	0.244	30.04	0.873
Unextracted	BCR	15.53		13.20		15.35		39.24	
Extracted	GUSB	30.07	0.438	36.18	**0.075***	80.83	0.043*	30.72	0.986
Unextracted	GUSB	30.30		19.89		28.57		38.67	

CVs for the extracted samples were generally higher than the heat lysed samples.

Linear regression



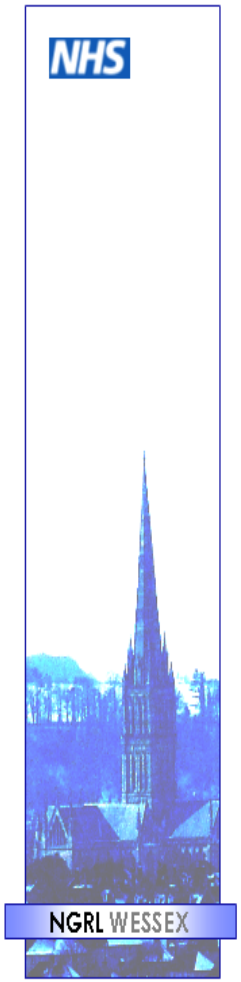


Number of labs where linear regression r^2 values are >98%:

	b3a2 aRNA		b2a2 aRNA	
	Unextracted	**Extracted**	**Unextracted**	**Extracted**
ABL	14/25 (56%)	4/25 (16%)	11/20 (55%)	7/20 (35%)
BCR	4/7 (57%)	1/7 (14%)	5/6 (83%)	1/6 (17%)
GUS	9/19 (47%)	4/19 (21%)	10/15 (67%)	6/15 (40%)

For freeze dried cell line samples all linear regressions had r^2 values of >98%

Summary and Future plans



- aRNA samples worked well when directly heat lysed prior to cDNA synthesis and the aRNA could be shipped at ambient temperature

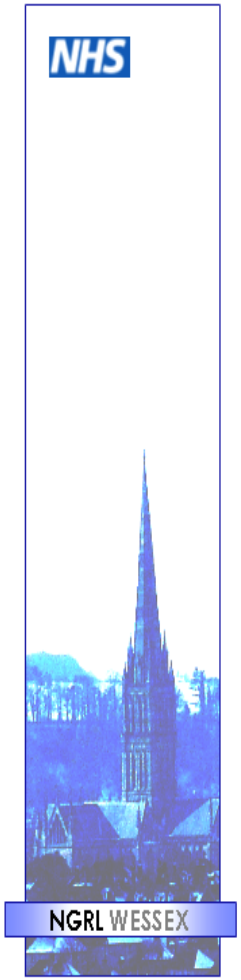
- Further protocol modifications are required to ensure adequate yields following RNA extraction e.g. addition of carrier RNA during extraction or formulation of the aRNA in a carrier-containing biological matrix.

- Samples used in this study contained copies of aRNA corresponding to about 80 fg/µL or less of RNA. At this low RNA input mass, RNA extraction protocols have to be optimized to obtain efficient and reproducible recovery of RNA. Alcohol precipitation problematic (Trizol protocol)

- aRNA samples will undergo a further round of field trial evaluation with the aim of establishing them as secondary reference reagents.

- Higher copy numbers of the control genes will be used and if possible the control gene ratios will be altered to reflect the control gene transcript levels found in normal leucocytes or will be calibrated to the freeze dried cell line samples

Acknowledgements



Field Trial participants

Asia / Australasia:
Adelaide: T Hughes, S Branford, L Fletcher. **Hong Kong:** ESK Ma. **Japan:** K Miyamura. **Korea:** D-W Kim, H Goh, S Park. **Singapore:** G-F How.

Europe:
Austria: D Denk, T Lion, M Neßlböck. **Czech Republic:** D Dvorakova, J Moravcová, J Rulcová. **France:** N Beaufils, J-M Cayuela, X Fund, J Gabert, F Hermitte, E Lippert, F-X Mahon, N Maroc. **Finland:** V Kairisto. **Germany:** A Hochhaus, MC Müller, H Pfeifer, O Ottmann. **Greece:** K Zoi. **Italy:** I Iacobucci, G Martinelli, F Quarantelli, G Saglio. **Spain:** D Colomer, MT Gómez Casares. **Sweden:** G Barbany, A Carpelan. **UK:** A Awan, N Cross, L Foroni, G Gerrard, E Gray, R Hawkins, P Matejtschuk, A Sproul, L Wang, H White.

USA:
Austin: J Hedges, C Walker-Peach. **Boston: J Longtine.** **Houston:** S Hai, D Jones. **New York:** YL Wang. **Portland:** YE Beillard, S Evonuk, C Fuller, R Press.

NGRL (Wessex)
Nick Cross
Gemma Watkins
Andy Chase

NIBSC
Paul Metcalfe
Elaine Gray
Ross Hawkins
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Field trial reports available at www.ngrl.org.uk/Wessex