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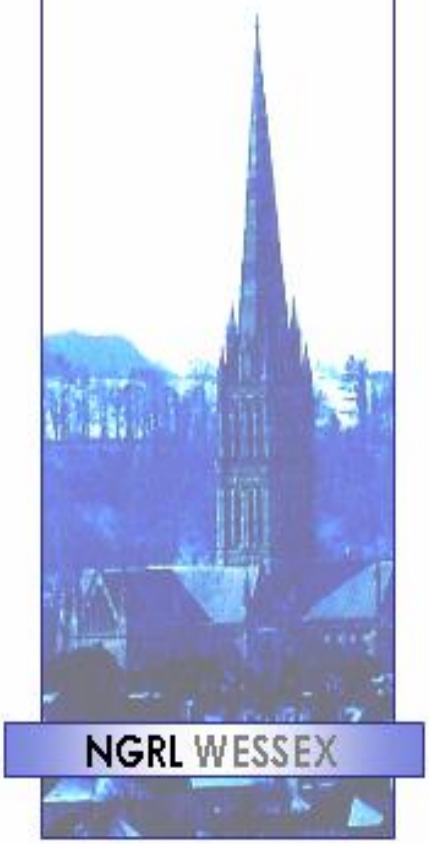
NHS

Reference Reagents



Armored RNA as reference material for standardisation of BCR-ABL RQ-PCR methods: report of field trial evaluation

June 2008

Title	Armored RNA as reference materials for standardisation of *BCR-ABL* RQ-PCR methods: report of field trial evaluation
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Contributors to report writing

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Peer Review and Approval

This document has been reviewed by the field trial participants.

Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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SUMMARY

- An international scale (IS) for quantitative measurement of *BCR-ABL* mRNA has been established that is anchored to two key points defined in the IRIS trial: a common baseline (100% *BCR-ABL*^{IS}) and major molecular response (0.1% *BCR-ABL*^{IS}). Definition of the IS currently relies on relating results directly or indirectly to the Adelaide international reference laboratory. A more robust definition of the IS requires the development of internationally accredited reference reagents.

- The aim of this collaborative study was to produce and assess the use of Armored RNA® (aRNA) as a candidate reference material for the standardisation of *BCR-ABL* RQ-PCR protocols.

- Nine aRNA reference standards were prepared containing estimated numbers of molecules as follows: $3x10^4$ (Level 1), $3x10^3$ (Level 2), $3x10^2$ (Level 3) and $3x10^1$ (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 (October - December 2007) that involved 29 laboratories (22 EU, 3 USA, 4 Asia/Australasia) analysing 3 different control genes on 14 different RQ-PCR platforms. The aRNA samples were tested following RNA extraction or direct heat lysis.

- The median number of copies/ul obtained for *ABL*, *BCR* and *GUSB* for the b3a2 aRNA samples following RNA extraction were $8.13x10^3$, $3.46x10^2$ and $4.09x10^2$ respectively and following direct heat lysis were $1.25x10^5$, $1.25x10^4$ and $7.72x10^3$ respectively. The expected copy number for the control genes was $3x10^4$ copies/ul

- The median number of copies/ul obtained for *ABL*, *BCR* and *GUSB* for the b2a2 aRNA samples following RNA extraction were $6.32x10^3$, $4.86x10^2$ and $3.80x10^2$ respectively and following direct heat lysis were $1.16x10^5$, $1.57x10^4$ and $7.9x10^3$ respectively. The expected copy number for the control genes was $3x10^4$ copies/ul

- Overall, the aRNA samples tested after RNA extraction showed a 12 fold extraction loss when compared to the samples analysed after direct heat lysis. Labs using Trizol demonstrated a median 28 fold loss and those using QIAGEN columns showed a median 2 fold loss.

- 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 failed to detect level 3 (n=2) and level 4 (n=6) b3a2 transcripts respectively and 6/22 labs failed to detect level 3 (n=1) and level 4 (n=6) b2a2 transcripts respectively. For the heat lysed aRNA samples level 4 b3a2 and b2a2 could not be detected by one lab.

- The coefficient of variance for the %*BCR-ABL* / control gene values for the extracted and heat lysed samples were statistically different (90% confidence; i.e. $p<0.1$) for 7 analyses (b3a2: *ABL* Level 4, *BCR* Levels 1 & 4, *GUSB* Level 1; b2a2: *BCR* Level 2, *GUSB* Levels 2 & 3).

- Linear regression plots were produced for log transformed lab data plotted against the log transformation of the reference standard values for the b3a2 and b2a2 a RNA samples. The r^2 values for the linear regression of the b3a2 samples were >98% in 53% and 18% of labs for the heat lysed and extracted samples respectively. The r^2 values for the linear regression of the b2a2 samples were >98% in 63% and 34% of labs for the heat lysed and extracted samples respectively.

- We conclude that the pilot aRNA reference standards worked well when directly heat lysed prior to cDNA synthesis but further protocol optimisation is required to ensure adequate recovery of low aRNA mass input during RNA extraction. The aRNA samples will undergo a further round of field trial evaluation with the aim of establishing them as secondary reference reagents for *BCR-ABL* measurement.

1. INTRODUCTION

Reverse-transcription real-time quantitative PCR (RQ-PCR) is routinely used to quantify levels of *BCR-ABL* mRNA transcripts in peripheral blood and bone marrow samples from chronic myeloid leukaemia (CML) patients. The technique can determine accurately the response to treatment and is particularly valuable for patients who have achieved complete chromosomal remission. Despite efforts to establish standardised protocols for *BCR-ABL* fusion transcript quantitation[1] there is still substantial variation in the way in which RQ-PCR for *BCR-ABL* is carried out and how results are reported in different laboratories worldwide[2]. In particular, the use of different control genes for normalisation of results means that there are several different units of measurement worldwide, e.g. *BCR-ABL/ABL*; *BCR-ABL/BCR*; *BCR-ABL/GUSB, BCR-ABL/G6PD, BCR-ABL/β2M* etc.

The CML meeting at the National Institutes of Health in Bethesda in October 2005 made several recommendations for the harmonisation of RQ-PCR for *BCR-ABL* including the use of one of three control genes (*ABL*, *BCR* or *GUSB*) [3,4]. Most importantly, a new international scale (IS) for *BCR-ABL* RQ-PCR measurements was proposed which is anchored to two key levels used in the IRIS study [5], namely a standardised baseline defined as 100% *BCR-ABL*^{IS}, and major molecular response (3 log reduction relative to the standardised baseline) defined as 0.1% *BCR-ABL*^{IS}. Laboratories interested in using the IS should derive a laboratory-specific conversion factor to relate values obtained in their laboratory to IS values. The converted value from a given laboratory should then be equivalent to an analogous converted value obtained in any other collaborating laboratory. The strength of this approach is that (i) laboratories can continue to use their existing assay conditions (provided their assay is linear on analysis of the reference samples), and (ii) that they can continue to express results according to local preferences in addition to expressing results on the international scale. The concept of the international scale is analogous to established procedures for other quantitative assays, for example the International Normalised Ratio (INR) for prothrombin time.

The original standards used for the IRIS trial are no longer available, however traceability to the IRIS scale is provided by the extensive quality control data generated by the Adelaide laboratory over a period of several years. Establishment of the IS therefore requires the alignment of local test results either directly or indirectly with those obtained in Adelaide. Currently, this can be achieved by exchange of a series of patient samples with either the Adelaide or Mannheim international reference laboratories. Although this system works well, it is very laborious and consequently only open to a limited number of laboratories at any given time. The availability of internationally accredited reference reagents should in principle help to make the IS more accessible, as well providing a more robust framework for the scale itself.

Ideally, the formulation for reference reagents should be as close as possible to the usual analyte, should cover the entire analytical process, i.e. from RNA extraction to result and should be applicable to methods in use throughout the world. However it is essential that the formulation is stable over a period of several years and that it is physically possible to produce batches of sufficient size to satisfy demand over a similar period of time.

The aim of this collaborative study was to produce and perform a preliminary assessment of the use of Armored RNA® (aRNA) mixtures as prototype reference materials. aRNA is a proprietary technology invented and developed by Asuragen Inc. (www.asuragen.com) and Cenetron Diagnostics (Austin, Texas) for protecting RNA from degradation by ribonucleases. aRNA is based on bacteriophage coat protein encapsulation of specific RNA targets to form pseudo-viral particles [6]. The bacteriophage coat protein protects RNA transcripts from nuclease degradation and can stabilize RNA sequences. aRNA is designed for use as standards and controls in assays, in particular for use as positive controls or quantitative internal spiked controls for amplification and detection using RT-PCR and are produced in a non-infectious configuration that enables safe handling and shipping. This report details the preparation of nine pilot aRNA reference materials and their evaluation in an international field trial that involved 29 laboratories (22 EU, 3 USA, 4 Asia/Australasia) analysing 3 different control genes on 14 different RQ-PCR platforms.

2. MATERIALS AND METHODS

2.1 Cloning of target sequences

ABL, *BCR*, *GUSB* and b3a2 target sequences were amplified from K562 cDNA using the primers listed in table 1. The b2a2 target sequence was amplified from the cDNA of a patient expressing the b2a2 fusion transcript (informed consent obtained). The five amplicons were cloned into pCR2.1 using the TA Cloning kit (Invitrogen). The plasmid constructs were transferred to Asuragen for synthesis of the aRNA constructs.

Target	Forward Primer	Reverse Primer	Ref seq start	Ref seq finish	Amplicon (bp)
ABL	CGTTGGAACTCCAAGGAAAA	GAAGGCGCTCATCTTCATTC	142	1774	1633
BCR	GTCCACTCAGCCACTGGATT	CAAGGACCAGCTGTCAGTCA	3345	4307	963
GUSB	TTTCCGTACCAGCCACTACC	GTAAACGGGCTGTTTTCCAA	1166	1978	813
b3a2	TCTCTGCACCAAGCTCAAGA	CTGCACCAGGTTAGGGTGTT	-	-	1399
b2a2	TCTCTGCACCAAGCTCAAGA	CTGCACCAGGTTAGGGTGTT	-	-	1324

Table 1: Primer sequences used to amplify ABL, *BCR*, *GUSB*, b3a2 and b2a2. Reference sequences for *ABL*, *BCR* and *GUSB* are NM005157.3, NM_004327.3 and NM_000181.1 respectively

2.2 aRNA production

The aRNA constructs were produced by Asuragen Inc and were supplied at the concentrations given in table 2.

Construct	Product name	Concentration (copies / µl)	Quantification Method	Volume	Trace DNA detected
GUSB	USH-1	2.28×10^{11}	PA	25 µl	No
ABL	USH-2	8.93×10^{9}	OD_{260}	112 µl	No
BCR	USH-3	4.63×10^{10}	PA	25 µl	Yes
b2a2	USH-4	6.67×10^{10}	PA	25 µl	No
b3a2	USH-5	4.58×10^{10}	PA	25 µl	No

Table 2: Concentration and volume of aRNA constructs supplied by Asuragen. PA = NIST traceable phosphate assay, One unit of OD_{260} is roughly equivalent to 1.9×10^{14} copies.

Using the stock concentrations provided by Asuragen, nine aRNA reference standards were prepared containing $3x10^4$ (Level 1), $3x10^3$ (Level 2), $3x10^2$ (Level 3) and $3x10^1$ (Level 4) copies/ul of b3a2 (or b2a2) aRNA with each control gene (*ABL*, *BCR*, *GUSB*) at $3x10^4$ copies/ul. aRNA constructs were diluted in 1X TSM (10mM Tris (pH 7.0), 100mM NaCl, 1mM $MgCl_2$) supplemented with 0.1% gelatin using a CAS-1200™ Precision Liquid Handling Instrument (Corbett Life Sciences). Note that the amount of aRNA produced for *ABL* was insufficient to measure by the NIST traceable phosphate assay and therefore an estimation of copy number by OD_{260} measurement was used

2.3 aRNA field trial (October – December 2007)

2.3.1 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.

Samples were shipped to labs by express courier at ambient temperature. The samples were supplied in three bags labelled batch 1, batch 2 and batch 3. Each bag contained either 9 tubes (labs testing b3a2 and b2a2) or 5 tubes (labs testing b3a2 only) containing 60µl aRNA mix labelled as follows:

a) Labs testing b3a2 and b2a2:

Level 1 b3a2 aRNA
Level 2 b3a2 aRNA
Level 3 b3a2 aRNA
Level 4 b3a2 aRNA
Level 1 b2a2 aRNA
Level 2 b2a2 aRNA
Level 3 b2a2 aRNA
Level 4 b2a2 aRNA
Control genes only aRNA

b) Labs testing b3a2 only:

Level 1 b3a2 aRNA
Level 2 b3a2 aRNA
Level 3 b3a2 aRNA
Level 4 b3a2 aRNA
Control genes only aRNA

Field trial participants were asked to analyse the aRNA samples from each batch on a different day using the following protocol and schedule given in figure x:

1. Add 20µl of aRNA sample to 1ml Trizol or 600µl RLT Buffer (QIAGEN), mix well (eg vortex) and extract aRNA following your usual RNA extraction protocol. Please record the volume of elution buffer or RNase free water used for resuspension.

2. Set up **two** cDNA reactions (using your established method). Please use the same cDNA reagents for both reactions and perform both of the cDNA reactions at the same time:

cDNA reaction 1: add **extracted** aRNA sample (from step 1) to the cDNA reaction to give a final cDNA reaction volume of 40µl. Please use as much of the extracted sample that you can and if possible all of it in a single reaction (this will depend on your elution/resuspension volume). Please record the volume of aRNA added and the final volume of the cDNA reaction. There is no need to measure spectrophotometrically how much RNA was recovered.

cDNA reaction 2: heat 20µl of **unextracted** aRNA sample (i.e. sample supplied) at 80ºC for 3 minutes. Add this directly to the cDNA reaction to give a final cDNA reaction volume of 40µl.

3. Using **2ul of cDNA per reaction** perform quantitative PCR for *BCR-ABL* (b3a2 and b2a2 as appropriate) and the control gene(s) on each set of cDNA samples. Please process samples from each batch in separate quantitative runs. Analyse the samples for *BCR-ABL* and the control gene(s) in the usual manner (established lab method).

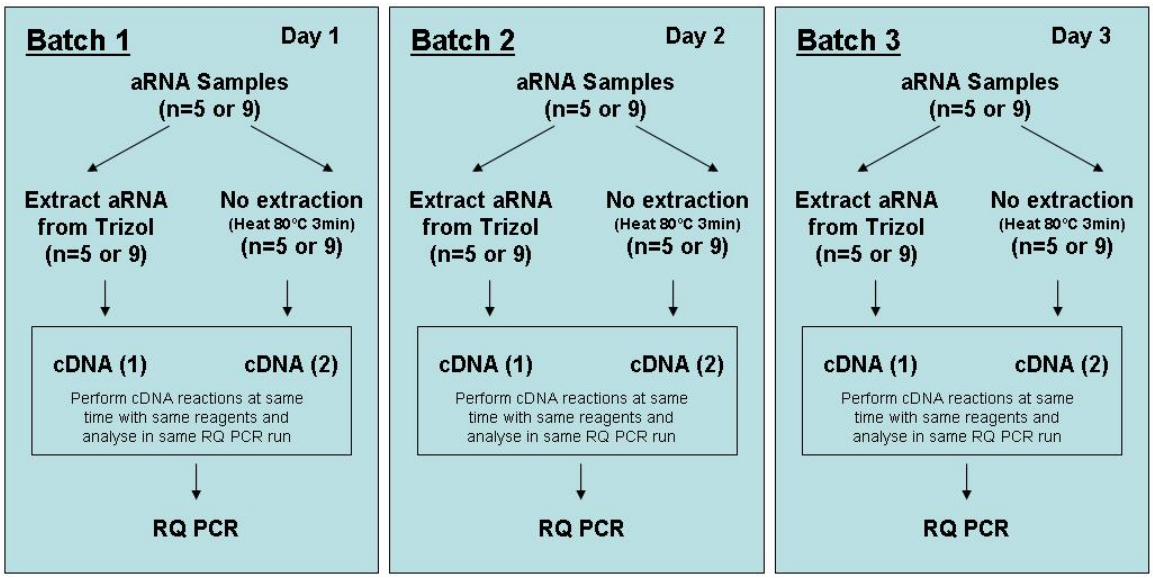


Figure 1: Schematic flow diagram showing suggested order of analysis for the minimal number of reverse transcription reactions and RQ-PCR runs.

The following information was requested: date of 'RNA extraction' or heat lysis, resuspension volume, volume used for cDNA synthesis, final volume of cDNA reaction, date of cDNA synthesis, date of qPCR, *BCR-ABL* transcript value (Ct value and copy number), control gene transcript value(s) (Ct value and copy number), *BCR-ABL* / control gene(s) (%) before conversion to IS, *BCR-ABL* / control gene(s) (%) converted to IS.

Participants were advised that since the aRNA control genes have been supplied at different relative levels compared to normal leucocytes the results on conversion to the international scale were expected to differ depending on the control gene analysed.

3. RESULTS OF aRNA FIELD TRIAL

3.1 Field trial participants

29 laboratories participated in the field trial and all labs returned data; 22 participants from Europe, 3 from the USA, 3 from Asia and 1 from Australasia (Appendix A). Results were obtained from all laboratories and 28 labs returned completed methodology forms.

3.2 Methodologies used

3.2.1 Transcripts analysed

Three control genes were analysed: *ABL* (25 labs), *BCR* (7 labs), *GUSB* (18 labs). 18 labs analysed the samples using more than one control gene. One lab failed to amplify *ABL* as the region cloned into the *ABL* aRNA construct was not compatible with the location of their primer sequences. 22 labs analysed both the b3a2 and b2a2 aRNA samples and 6 labs analysed the b3a2 samples only.

3.2.2 RQ-PCR machines used

14 different type of RQ-PCR machines were used: ABI 7000 (n=4), ABI 7500 (n=2), ABI 7300 (n=1), ABI 7700 (n=5), ABI 5700 (n=1), ABI 7900HT (n=4), ABI 7900 (n=1), Corbett RotorGene 3000 (n=1), Corbett RotorGene 6000 (n=1), Roche LightCycler 1.0 (n=1), Roche LightCycler 1.5 (n=1), Roche LightCycler 2.0 (n=2), Roche LightCycler 480 (n=3), Stratagene MX3000P (n=1).

3.2.3 RQ-PCR methods

3.2.3.1 RNA Extraction methods

7 RNA extraction protocols were used; Trizol (Invitrogen) (n=15), Trizol LS (Invitrogen) (n=1), RNeasy kits (QIAGEN) (n=8), Trizol followed by an RNeasy kit (n=1), TriReagent (Sigma) (n=1), RNAzol (n=1), Roche MagNa pure LC HP protocol (n=1).

3.2.3.2 RT-PCR method cited

22 of the participants cited Gabert et al. (2003)[1] as one of the references used as their RQ-PCR protocol and 2 labs cited Emig et al (1999)[7]. Other references cited are listed in the reference section [8-10]. One lab used a 'home brew' method and another laboratory's method was proprietary.

3.2.3.3 RT-PCR method and primers used

27 labs reported the use of random hexamers for reverse transcription. The most common final concentration was 25µM. One lab used a proprietary method and another lab did not provide information.

3.2.3.4 Standard type and source

25 labs used plasmid DNA as standards and these were either made in house or obtained from another lab (n=11) or purchased from Ipsogen (n=15) and Nanogen (n=1). Other standards used were cDNA standards derived from the K562 cell line (n=1), RNA purchased from *In vivo* Scribe (n=1) and Armored RNA Quant reagents (Asuragen) (n=1).

3.3 Field trial data analysis

3.3.1 Control gene copy numbers from extracted and heat lysed (unextracted) aRNA samples.

Box plots showing the copy numbers (log transformed) obtained from the aRNA samples following direct heat lysis (unextracted, pink boxes) and following an RNA extraction procedure (extracted, blue boxes) are shown below. The bottom of the box corresponds to the first quartile (Q1), and the top to the third quartile (Q3) value. The whiskers extend from the lowest and highest observations inside the region defined by the following limits: Lower Limit: Q1 - 1.5 (Q3 - Q1), Upper Limit: Q3 + 1.5 (Q3 - Q1). Outliers are indicated by asterisks and the median value is indicated by the line running through the box. The dotted horizontal line corresponds to the expected copy number ($\log_{10}$ $3x10^4$ = 4.4771). Copy number values have been corrected for variation in the volume of aRNA extracted and used in

the cDNA reaction and the amount of cDNA added to the RQ-PCR so that the data between labs are comparable.

3.3.1.1 ABL copy number loss following RNA extraction of b3a2 samples

Box plots showing the number of *ABL* copies obtained by labs using different RNA extraction protocols are shown in figure 2 (Trizol extraction) and figure 3 (QIAGEN and other RNA extraction protocols). Tables 3 and 4 shows the median number of *ABL* copies (Trizol and other methods of RNA extraction respectively) obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol and QIAGEN protocols were 18.4 and 2.1 respectively.

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
2	Trizol	3.65E+04	2.69E+05	7.4
3	Trizol	2.79E+04	1.19E+05	4.2
5	Trizol	1.33E+03	2.35E+04	17.6
8	Trizol	7.43E+03	1.59E+05	21.4
9	Trizol	2.86E+03	7.56E+05	264.3
11	Trizol	1.32E+04	1.32E+05	10.0
12	Trizol	9.66E+02	2.73E+04	28.2
13	Trizol	5.36E+03	2.60E+05	48.5
16	Trizol	2.31E+03	1.75E+05	75.8
18	Trizol	4.52E+03	8.33E+04	18.4
21	Trizol	2.12E+00	9.97E+01	47.0
22	Trizol	6.27E+04	3.23E+05	5.1
25	Trizol	5.28E+03	7.07E+05	133.9
28	Trizol	7.54E+03	6.68E+04	8.9
29	Trizol	3.65E+04	2.69E+05	7.4
Median Fold extraction loss	**Trizol**	**-**	**-**	**18.4**

Table 3: Median *ABL* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol.

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
4	QIAGEN	2.44E+03	1.96E+04	8.0
6	QIAGEN	4.29E+04	8.85E+04	2.1
7	QIAGEN	3.86E+05	7.38E+05	1.9
10	QIAGEN	6.31E+03	9.70E+04	15.4
15*	QIAGEN	1.74E-01	3.67E-01	2.1
17	QIAGEN	1.40E+05	2.93E+05	2.1
19	QIAGEN	1.65E+05	2.97E+05	1.8
24	QIAGEN	1.33E+05	2.28E+05	1.7
14	Roche	2.22E+03	1.52E+04	6.8
20	RNAzol	1.75E+05	2.15E+05	1.2
26	TriReagent	1.72E+03	9.86E+04	57.4
Median Fold extraction loss	**QIAGEN**	**-**	**-**	**2.1**

Table 4: Median *ABL* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using QIAGEN, Roche, RNAzol and TriReagent RNA extraction protocols. * relative values compared to K562 expression where the level of K562 *ABL* expression is defined as 1.

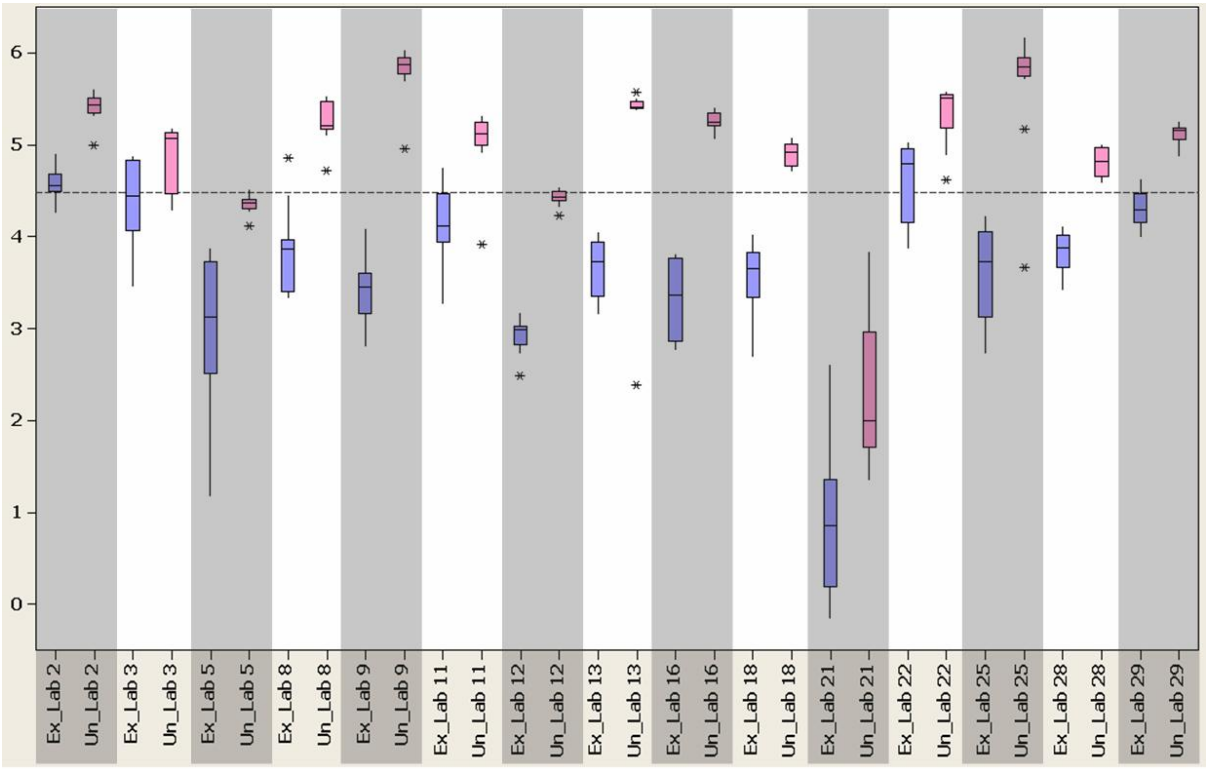


Figure 2: Box plot of *ABL* copy numbers (log transformed) obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

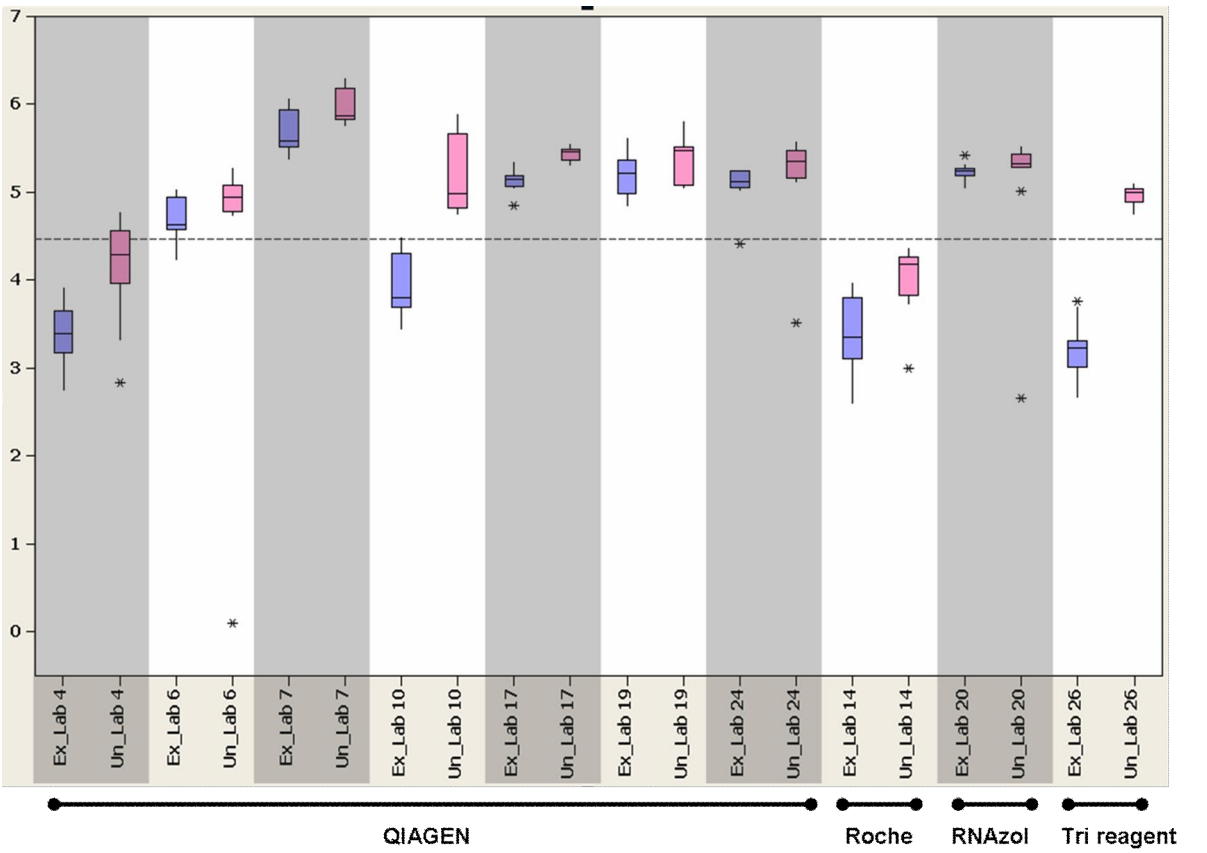


Figure 3: Box plot of *ABL* copy numbers (log transformed) obtained from the b3a2 aRNA samples analysed by labs using QIAGEN, Roche, RNAzol and TriReagent RNA extraction protocols. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

3.3.1.2 ABL copy number loss following RNA extraction of b2a2 samples

A box plot showing the number of *ABL* copies obtained from the b2a2 aRNA samples by labs using a Trizol RNA extraction protocol is shown in figure 4. Table 5 shows the median number of *ABL* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol protocol was 12.3

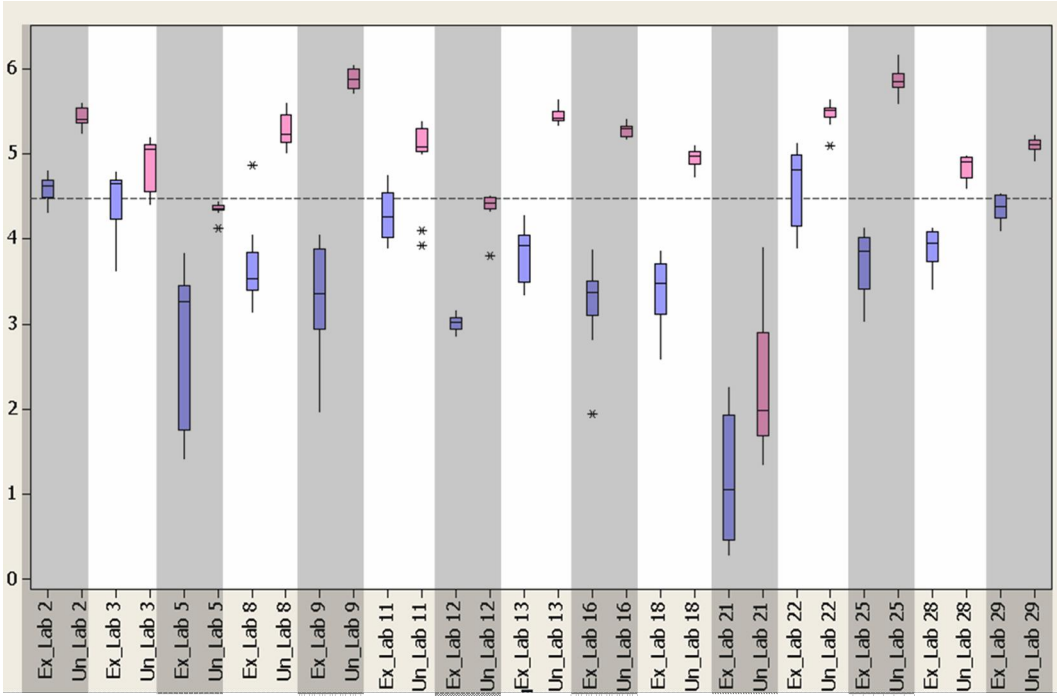


Figure 4: Box plot of *ABL* copy numbers (log transformed) obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
2	Trizol	4.15E+04	2.56E+05	6.2
3	Trizol	4.45E+04	1.13E+05	2.5
5	Trizol	1.85E+03	2.28E+04	12.3
8	Trizol	3.38E+03	1.71E+05	50.7
9	Trizol	2.30E+03	7.56E+05	328.7
11	Trizol	1.82E+04	1.19E+05	6.6
12	Trizol	1.06E+03	2.63E+04	24.8
13	Trizol	8.40E+03	2.59E+05	30.8
16	Trizol	2.38E+03	1.95E+05	82.0
18	Trizol	2.99E+03	9.39E+04	31.4
21	Trizol	1.22E+01	9.51E+01	7.8
22	Trizol	6.48E+04	3.21E+05	5.0
25	Trizol	7.17E+03	7.00E+05	97.6
28	Trizol	8.85E+03	8.13E+04	9.2
29	Trizol	2.43E+04	1.29E+05	5.3
Median Fold extraction loss	**Trizol**	**-**	**-**	**12.3**

Table 5: Median *ABL* copy numbers and the fold loss in copy number obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol.

A box plot showing the number of *ABL* copies obtained from the b2a2 aRNA samples by labs using other RNA extraction protocols is shown in figure 5. Table 6 shows the median number of *ABL* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the QIAGEN protocol was 2.1

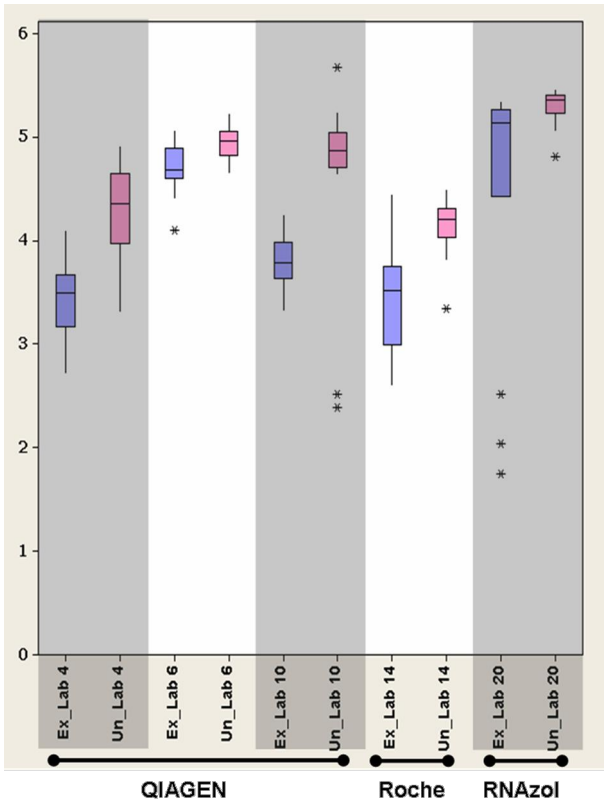


Figure 5: Box plot of *ABL* copy numbers (log transformed) obtained from the b2a2 aRNA samples analysed by labs using QIAGEN, Roche and RNAzol RNA extraction protocols. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
4	QIAGEN	3.10E+03	2.25E+04	7.2
6	QIAGEN	4.76E+04	9.08E+04	1.9
10	QIAGEN	6.13E+03	7.30E+04	11.9
14	Roche	3.27E+03	1.62E+04	4.9
20	RNAzol	1.36E+05	2.25E+05	1.6
Median fold extraction loss	**QIAGEN**	**-**	**-**	**2.1**

Table 6: Median *ABL* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using QIAGEN, Roche and RNAzol RNA extraction protocols.

3.3.1.3 BCR copy number loss following RNA extraction of b3a2 samples

A box plot showing the number of *BCR* copies obtained from the b3a2 aRNA samples by labs using a Trizol RNA extraction protocol is shown in figure 6. Table 7 shows the median number of *BCR* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol protocol was 36.4

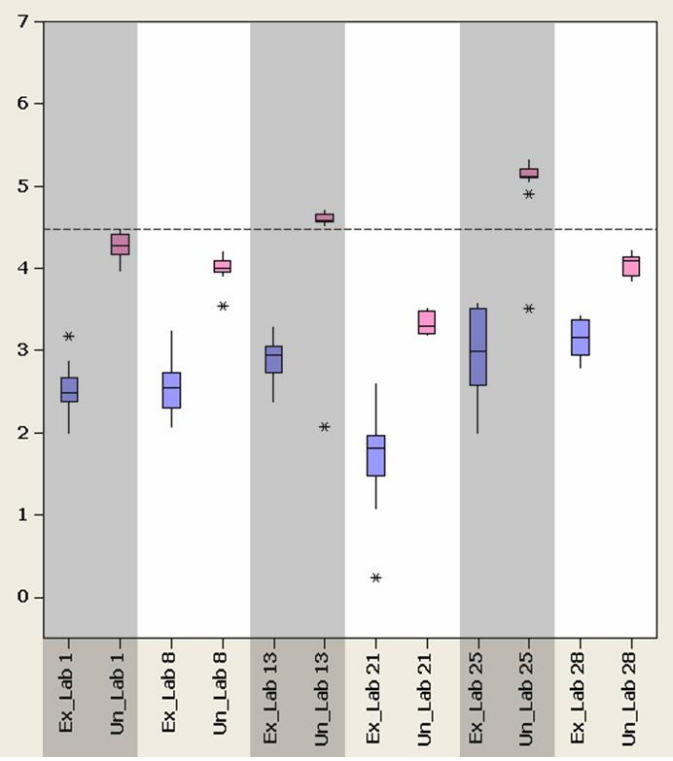


Figure 6: Box plot of *BCR* copy numbers (log transformed) obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
1	Trizol	3.10E+02	1.92E+04	61.8
8	Trizol	3.46E+02	1.01E+04	29.2
13	Trizol	8.75E+02	3.76E+04	43.0
21	Trizol	6.56E+01	1.96E+03	29.9
25	Trizol	7.42E+02	1.33E+05	179.1
28	Trizol	1.45E+03	1.25E+04	8.6
15*	QIAGEN	1.77E-01	3.03E-01	1.7
Median fold extraction loss	**Trizol**	-	-	**36.4**

Table 7: Median *BCR* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. * relative values compared to K562 expression where the level of K562 *BCR* expression is defined as 1.

3.3.1.4 BCR copy number loss following RNA extraction of b2a2 samples

A box plot showing the number of *BCR* copies obtained from the b2a2 aRNA samples by labs using a Trizol RNA extraction protocol is shown in figure 7. Table 8 shows the median number of *BCR* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol protocol was 37.3.

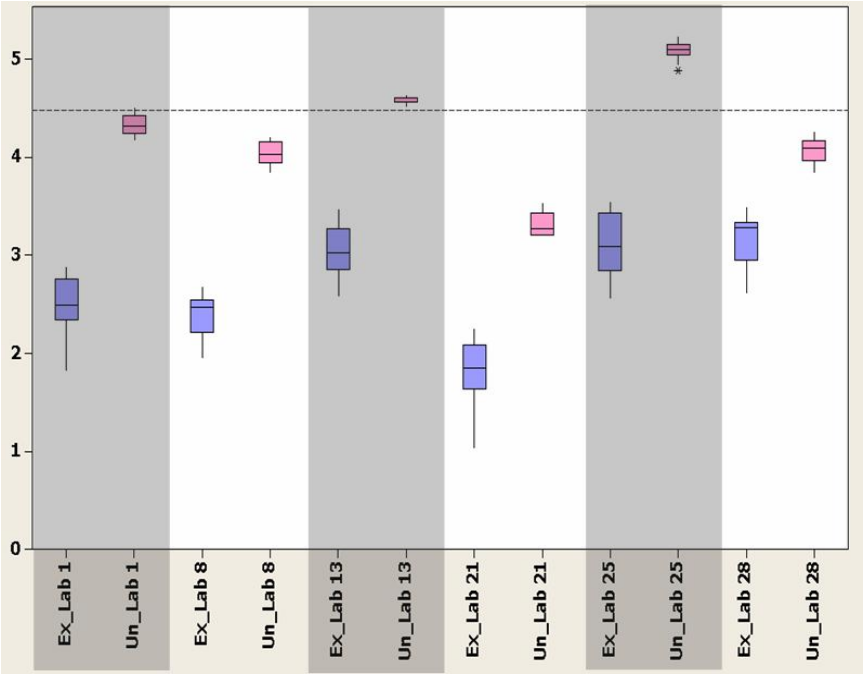


Figure 7: Box plot of *BCR* copy numbers (log transformed) obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss (Unextracted / Extracted)
1	Trizol	3.05E+02	2.06E+04	67.4
8	Trizol	2.94E+02	1.07E+04	36.6
13	Trizol	1.05E+03	3.99E+04	38.1
21	Trizol	7.13E+01	1.85E+03	25.9
25	Trizol	1.21E+03	1.25E+05	103.5
28	Trizol	1.90E+03	1.25E+04	6.6
Median fold extraction loss	**Trizol**	-	-	**37.3**

Table 8: Median *BCR* copy numbers and the fold loss in copy number obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol.

3.3.1.5 GUSB copy number loss following RNA extraction of b3a2 aRNA samples

A box plot showing the number of *GUSB* copies obtained from the b3a2 aRNA samples by labs using a Trizol RNA extraction protocol is shown in figure 8. Table 9 shows the median number of *GUSB* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol protocol was 26.8.

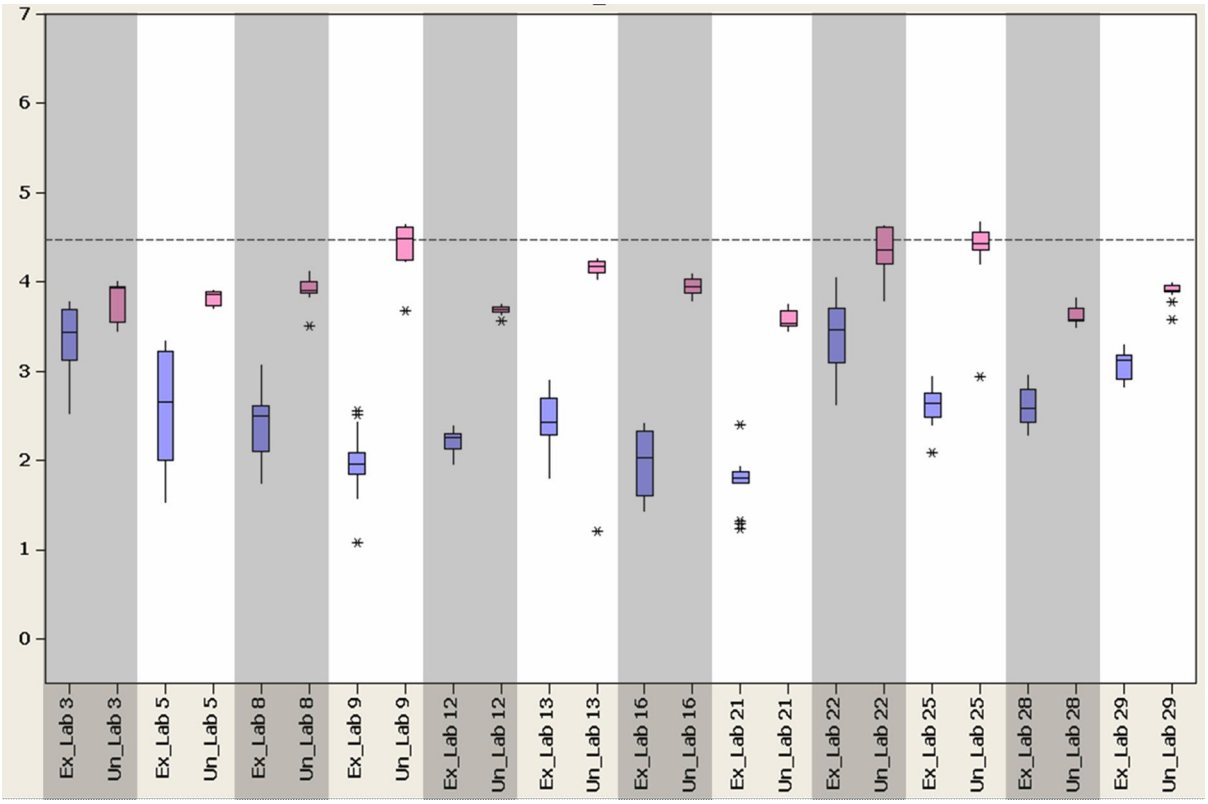


Figure 8: Box plot of *GUSB* copy numbers (log transformed) obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
3	Trizol	2.70E+03	8.44E+03	3.1
5	Trizol	4.45E+02	7.24E+03	16.3
8	Trizol	3.14E+02	8.09E+03	25.8
9	Trizol	9.00E+01	3.07E+04	341.1
12	Trizol	1.78E+02	4.94E+03	27.8
13	Trizol	2.68E+02	1.49E+04	55.5
16	Trizol	1.05E+02	8.86E+03	84.2
21	Trizol	6.40E+01	3.43E+03	53.6
22	Trizol	2.92E+03	2.28E+04	7.8
25	Trizol	4.08E+02	2.69E+04	66.0
28	Trizol	3.80E+02	3.73E+03	9.8
29	Trizol	1.32E+03	8.09E+03	6.1
Median fold extraction loss	**Trizol**	**-**	**-**	**26.8**

Table 9: Median *GUSB* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol.

A box plot showing the number of *GUSB* copies obtained from the b3a2 aRNA samples by labs using other RNA extraction protocols is shown in figure 9. Table 10 shows the median number of *GUSB* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the QIAGEN protocol was 3.1

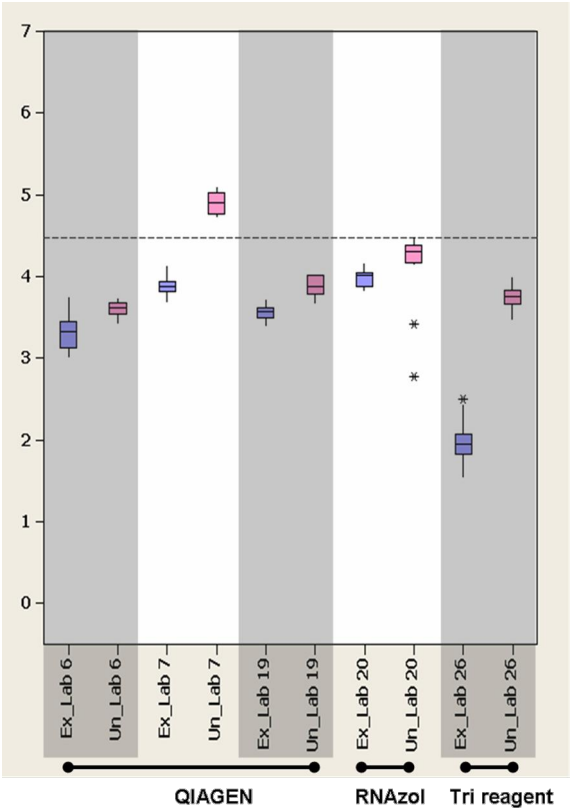


Figure 9: Box plot of *GUSB* copy numbers (log transformed) obtained from the b3a2 aRNA samples analysed by labs using QIAGEN, RNAzol and TriReagent RNA extraction protocols. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
6	QIAGEN	2.15E+03	4.07E+03	1.9
7	QIAGEN	7.63E+03	8.08E+04	10.6
15*	QIAGEN	2.85E-02	1.21E-01	4.2
19	QIAGEN	3.70E+03	7.44E+03	2.0
20	RNAzol	1.02E+04	2.02E+04	2.0
26	TriReagent	9.00E+01	5.69E+03	63.2
Median fold extraction loss	**QIAGEN**	**-**	**-**	**3.1**

Table 10: Median *GUSB* copy numbers and the fold loss in copy number obtained from the b3a2 aRNA samples analysed by labs using QIAGEN, RNAzol and TriReagent RNA extraction protocols. * relative values compared to K562 expression where the level of K562 *GUSB* expression is defined as 1.

3.3.1.6 GUSB copy number loss following RNA extraction of b2a2 aRNA samples

A box plot showing the number of *GUSB* copies obtained from the ba2 aRNA samples by labs using a Trizol RNA extraction protocol is shown in figure 10. Table 11 shows the median number of *GUSB* copies obtained from each lab following extraction and heat lysis. The median fold loss in copy number resulting from RNA extraction using the Trizol protocol was 28.4.

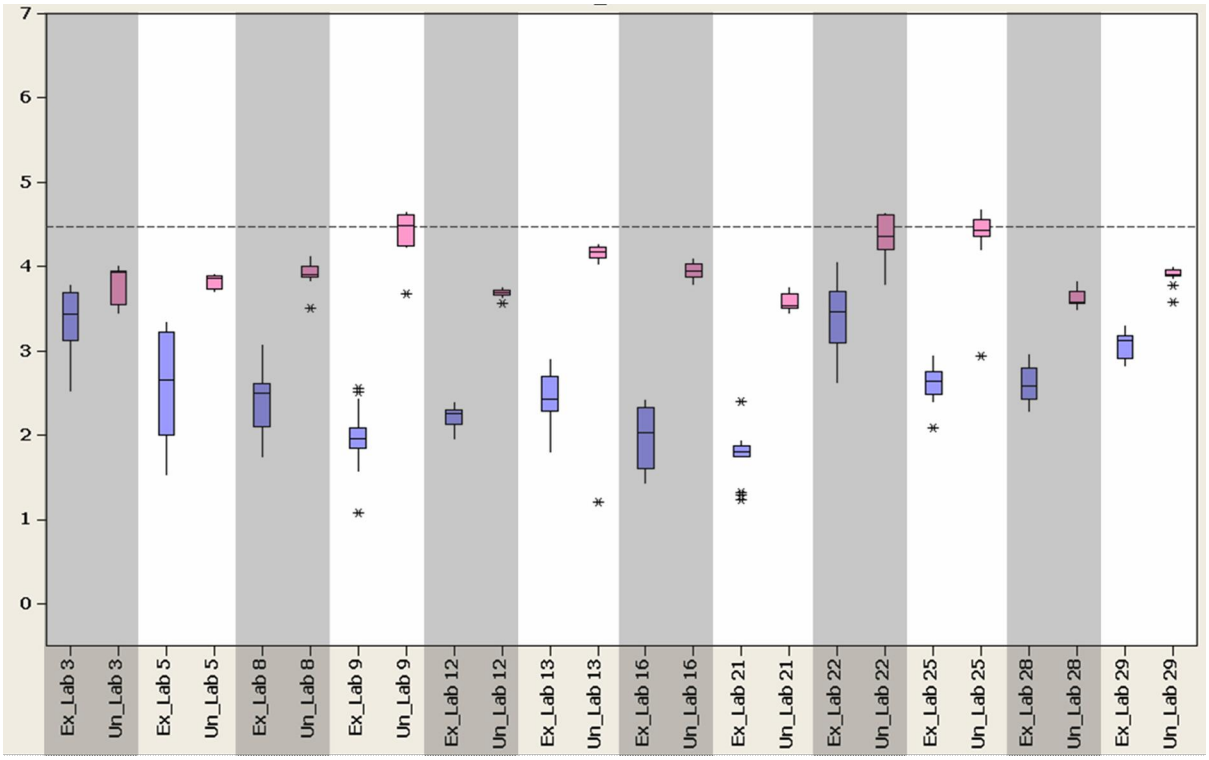


Figure 10: Box plot of *GUSB* copy numbers (log transformed) obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss (Unextracted / Extracted)
3	Trizol	3.46E+03	7.96E+03	2.3
5	Trizol	5.20E+02	7.24E+03	13.9
9	Trizol	6.45E+01	3.12E+04	483.7
12	Trizol	1.78E+02	5.05E+03	28.4
13	Trizol	4.05E+02	1.34E+04	33.2
16	Trizol	1.11E+02	9.20E+03	83.2
21	Trizol	6.02E+01	3.49E+03	58.0
22	Trizol	2.60E+03	2.36E+04	9.1
25	Trizol	3.46E+02	3.13E+04	90.7
28	Trizol	5.17E+02	4.03E+03	7.8
29	Trizol	1.52E+03	8.47E+03	5.6
Median fold extraction loss	**Trizol**	**-**	**-**	**28.4**

Table 11: Median *GUSB* copy numbers and the fold loss in copy number obtained from the b2a2 aRNA samples analysed by labs using a Trizol RNA extraction protocol.

A box plot showing the number of *GUSB* copies obtained from the b2a2 aRNA samples by labs using other RNA extraction protocols is shown in figure 11. Table 12 shows the median number of *GUSB* copies obtained from each lab following extraction and heat lysis.

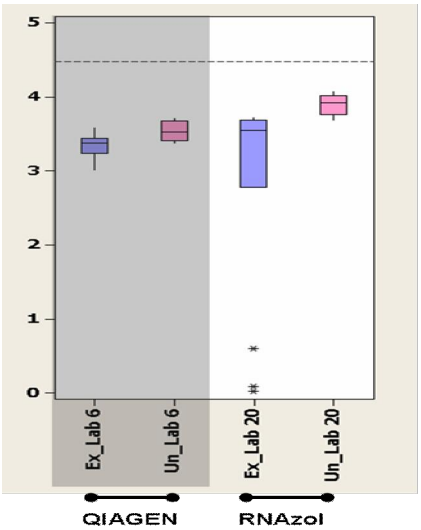


Figure 11: Box plot of *GUSB* copy numbers (log transformed) obtained from the b2a2 aRNA samples analysed by labs using QIAGEN and RNAzol RNA extraction protocols. Ex = extracted (blue bars), Un = unextracted, heat lysis only (pink bars).

Lab ID	RNA extraction method	Median Extracted Copy number	Median Unextracted Copy number	Fold Extraction loss
6	QIAGEN	2.36E+03	3.34E+03	1.4
20	RNAzol	3.53E+03	8.40E+03	2.4

Table 12: Median *GUSB* copy numbers and the fold loss in copy number obtained from the b2a2 aRNA samples analysed by labs using QIAGEN and RNAzol RNA extraction protocols.

3.3.1.7 Control gene copy number loss following RNA extraction

The overall median fold loss of control gene copy number for the five different RNA extraction protocols are shown in table 13.

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**

Table 13: Median fold loss of control gene copy number for the five different RNA extraction protocols.

3.3.2 Mean, standard deviation and coefficient of variation for % BCR-ABL / Control gene

The mean, standard deviation (SD) and coefficient of variation (CV) for the % *BCR-ABL* / control gene values for each reference material in each lab are shown in tables 14, 15 and 18 (*ABL*; b3a2 extracted, b3a2 unextracted and b2a2 respectively), tables 16 and 19 (*BCR*; b3a2 and b2a2 respectively) and tables 17 and 20 (*GUSB*; b3a2 and b2a2 respectively). The expected % BCR-ABL / control gene based on the aRNA copy number prior to dilution and blending of the 9 pilot reference samples is 100%, 10%, 1%, and 0.1% for Level 1, 2, 3, and 4, respectively.

ABL Extracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	Trizol	10.182	2.916	28.636	1.481	0.312	21.086	0.133	0.067	50.106	0.030	0.006	19.666
3	Trizol	10.400	2.385	22.936	1.628	0.652	40.040	0.221	0.116	52.239	0.018	0.011	60.832
4	QIAGEN	7.990	2.767	34.627	0.347	0.402	115.895	0.039	0.024	60.310	0.006	0.011	173.205
5	Trizol	5.267	0.819	15.550	0.645	0.144	22.334	-	-	-	-	-	-
6	QIAGEN	6.873	3.067	44.624	1.197	0.308	25.761	0.183	0.127	69.067	0.017	0.006	34.641
7	QIAGEN	9.421	2.432	25.814	1.247	0.383	30.675	0.160	0.088	54.824	0.015	0.007	46.578
8	Trizol	6.203	2.661	42.899	0.841	0.236	28.074	0.111	0.032	29.354	0.031	0.023	75.281
9	Trizol	6.077	1.439	23.675	0.658	0.230	34.946	0.073	0.037	50.753	-	-	-
10	QIAGEN	2.024	0.318	15.700	0.449	0.124	27.588	0.017	0.015	87.919	0.001	0.001	173.205
11	Trizol	4.249	2.535	59.661	0.665	0.228	34.346	0.057	0.018	31.733	0.009	0.008	92.647
12	Trizol	4.172	1.340	32.130	0.203	0.206	101.496	0.138	0.239	173.205	-	-	-
13	Trizol	4.715	1.140	24.166	0.871	0.366	42.018	0.158	0.234	148.014	0.019	0.018	94.632
14	Roche	23.012	9.415	40.912	3.491	1.965	56.272	0.296	0.263	88.923	0.150	0.243	162.335
15	QIAGEN	10.077	1.535	15.235	1.225	0.146	11.958	0.127	0.035	27.458	0.011	0.007	58.224
16	Trizol	11.137	1.956	17.561	1.793	0.325	18.136	0.211	0.179	85.215	0.033	0.034	102.141
17	QIAGEN	5.814	0.347	5.961	0.784	0.091	11.638	0.135	0.060	44.376	0.022	0.021	96.069
18	Trizol	9.137	1.044	11.422	1.226	0.178	14.547	0.147	0.029	19.904	0.011	0.005	43.746
19	QIAGEN	4.183	2.137	51.085	0.641	0.395	61.625	0.064	0.024	37.264	0.005	0.001	24.457
20	RNAzol	4.118	0.932	22.628	0.436	0.119	27.337	0.049	0.019	38.144	0.004	0.002	37.369
21	Trizol	234.484	82.750	35.290	1996.727	3409.123	170.736	-	-	-	-	-	-
22	Trizol	21.728	5.956	27.412	0.898	1.194	132.917	0.001	0.002	173.205	-	-	-
24	QIAGEN	4.725	2.856	60.452	0.768	0.190	24.690	0.081	0.035	42.800	0.022	0.024	108.816
25	Trizol	5.182	0.568	10.971	0.293	0.268	91.563	0.052	0.021	41.174	0.010	0.004	39.389
26	Trireagent	13.251	5.503	41.531	1.524	0.331	21.745	0.208	0.141	67.698	-	-	-
28	Trizol	16.036	1.818	11.337	1.761	0.217	12.334	0.211	0.036	17.234	0.025	0.023	92.729
29	Trizol	9.803	0.844	8.615	1.240	0.571	46.094	0.146	0.019	12.787	0.015	0.009	60.361

Table 14: Mean, SD and CV values for %b3a2 / *ABL* for each laboratory for the b3a2 aRNA reference materials following RNA extraction

ABL Unxtracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	Trizol	16.995	0.691	4.066	1.932	0.449	23.239	0.192	0.052	27.047	0.017	0.007	39.709
3	Trizol	10.161	2.370	23.324	1.327	0.190	14.292	0.139	0.049	35.160	0.015	0.005	32.999
4	QIAGEN	2.339	0.812	34.741	0.239	0.159	66.580	0.006	0.007	121.724	0.002	0.003	143.111
5	Trizol	4.089	0.570	13.950	0.597	0.056	9.408	0.070	0.030	43.375	0.013	0.012	96.754
6	QIAGEN	4.377	0.762	17.404	0.210	0.205	97.706	0.050	0.017	34.641	0.004	0.001	25.000
7	QIAGEN	7.002	1.094	15.628	1.141	0.084	7.399	0.140	0.020	14.180	0.010	0.001	9.726
8	Trizol	9.267	3.217	34.715	1.106	0.443	40.084	0.148	0.083	55.986	0.015	0.004	26.991
9	Trizol	7.153	1.524	21.308	0.977	0.176	17.993	0.160	0.092	57.669	0.007	0.002	25.893
10	QIAGEN	2.081	1.339	64.365	0.263	0.128	48.646	0.038	0.040	105.363	0.005	0.007	140.885
11	Trizol	7.358	1.045	14.202	0.859	0.103	12.015	0.098	0.002	1.991	0.011	0.003	28.372
12	Trizol	2.523	0.458	18.138	0.379	0.105	27.846	0.055	0.025	46.303	0.008	0.004	47.161
13	Trizol	7.000	0.608	8.679	1.021	0.101	9.886	0.073	0.064	87.041	0.011	0.002	19.283
14	Roche	14.179	6.327	44.618	2.432	1.658	68.195	0.163	0.073	44.925	0.028	0.026	91.194
15	QIAGEN	9.903	0.592	5.981	0.948	0.195	20.607	0.094	0.016	17.535	0.010	0.001	8.632
16	Trizol	13.049	1.247	9.556	1.916	0.309	16.133	0.182	0.024	13.312	0.019	0.007	35.236
17	QIAGEN	5.916	0.667	11.272	0.797	0.058	7.260	0.083	0.010	11.866	0.010	0.004	36.330
18	Trizol	9.214	1.010	10.964	1.472	0.063	4.310	0.153	0.019	12.238	0.016	0.003	18.073
19	QIAGEN	6.264	1.144	18.263	1.000	0.288	28.849	0.098	0.042	43.007	0.008	0.004	49.490
20	RNAzol	5.327	1.244	23.346	0.657	0.572	87.063	0.052	0.036	69.501	0.005	0.004	88.199
21	Trizol	241.934	40.272	16.646	222.218	63.116	28.403	145.779	88.288	60.563	48.042	49.510	103.057
22	Trizol	101.997	87.544	85.830	9.428	10.885	115.448	1.811	2.913	160.876	-	-	-
24	QIAGEN	4.811	2.384	49.563	0.735	0.183	24.853	0.073	0.006	8.715	0.007	0.001	16.498
25	Trizol	3.645	1.207	33.115	0.485	0.249	51.326	0.043	0.024	56.014	0.005	0.002	44.298
26	Trireagent	9.819	1.214	12.359	1.238	0.133	10.745	0.100	0.024	23.624	0.007	0.002	24.243
28	Trizol	12.407	1.596	12.864	1.813	0.647	35.710	0.197	0.045	22.954	0.024	0.008	33.894
29	Trizol	12.231	2.588	21.161	1.717	0.173	10.065	0.189	0.029	15.083	0.018	0.001	5.273

Table 15: Mean, SD and CV values for %b3a2 / *ABL* for each laboratory for the b3a2 aRNA reference materials following heat lysis (unextracted)

a)

BCR Extracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	Trizol	184.100	135.119	73.394	17.074	4.819	28.222	4.791	7.154	149.319	0.523	0.905	173.205
8	Trizol	97.684	33.689	34.488	14.917	0.817	5.475	1.770	0.756	42.723	0.579	0.486	84.075
13	Trizol	31.305	16.342	52.204	4.241	1.954	46.062	0.795	1.144	143.881	0.163	0.159	97.865
15	QIAGEN	11.234	0.826	7.353	1.273	0.058	4.530	0.145	0.032	22.132	0.011	0.006	59.240
21	Trizol	295.241	73.712	24.967	58.605	73.647	125.668	-	-	-	-	-	-
25	Trizol	25.198	5.867	23.283	0.837	0.235	28.105	0.287	0.080	28.019	0.036	0.010	28.082
28	Trizol	82.560	9.678	11.723	8.641	2.137	24.725	0.929	0.190	20.395	0.118	0.099	83.933

b)

BCR Unextracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	Trizol	141.061	24.306	17.231	20.688	5.332	25.775	2.598	0.664	25.564	0.297	0.200	67.435
8	Trizol	154.877	22.114	14.278	19.093	4.285	22.440	2.681	0.812	30.289	0.274	0.039	14.066
13	Trizol	55.042	6.234	11.326	7.416	1.444	19.478	0.476	0.416	87.223	0.075	0.014	18.182
15	QIAGEN	13.040	1.303	9.992	1.370	0.087	6.331	0.129	0.020	15.557	0.014	0.001	4.536
21	Trizol	443.922	8.905	2.006	57.520	10.212	17.753	6.275	3.372	53.741	0.912	0.843	92.426
25	Trizol	20.076	4.162	20.730	1.135	0.316	27.863	0.184	0.070	38.010	0.023	0.010	44.011
28	Trizol	86.096	23.507	27.303	9.155	2.912	31.805	1.127	0.277	24.598	0.123	0.043	34.858

Table 16: Mean, SD and CV values for %b3a2 / *BCR* for each laboratory for the b3a2 aRNA reference materials following a) RNA extraction and b) heat lysis (unextracted)

a)

GUS Extracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	Trizol	128.653	5.493	4.270	17.413	2.477	14.225	1.701	0.195	11.463	0.190	0.073	38.265
5	Trizol	17.925	2.792	15.577	1.967	0.778	39.551	-	-	-	-	-	-
6	QIAGEN	202.370	106.748	52.749	29.253	14.654	50.092	4.193	1.991	47.477	0.330	0.066	19.871
7	QIAGEN	191.288	113.226	59.191	19.879	5.949	29.925	2.014	0.754	37.450	0.213	0.088	41.267
8	Trizol	153.316	68.610	44.751	18.184	5.544	30.491	2.261	0.749	33.116	0.714	0.521	72.988
9	Trizol	202.467	105.602	52.158	20.699	6.927	33.464	2.153	1.395	64.828	-	-	-
12	Trizol	32.995	21.791	66.042	1.823	0.972	53.287	2.963	-	-	-	-	-
13	Trizol	86.143	38.349	44.518	11.350	5.292	46.625	2.306	3.501	151.784	0.711	0.090	12.671
15	QIAGEN	58.579	10.443	17.828	6.320	0.880	13.926	0.741	0.133	17.947	0.060	0.024	39.122
16	Trizol	281.595	49.464	17.566	37.491	2.852	7.607	3.775	2.552	67.594	0.614	0.565	91.944
19	QIAGEN	260.480	52.044	19.980	23.313	9.323	39.991	2.440	0.020	0.802	0.217	0.075	34.419
20	RNAzol	231.013	78.853	34.134	18.298	4.792	26.191	2.107	0.576	27.338	0.216	0.117	54.228
21	Trizol	366.411	146.192	39.898	28.769	33.905	117.856	-	-	-	-	-	-
22	Trizol	404.684	227.230	56.150	17.934	26.898	149.984	0.035	0.049	141.421	-	-	-
23	T & Q	142.667	45.092	31.607	11.467	3.979	34.704	1.433	1.274	88.869	0.063	0.066	105.505
25	Trizol	12.608	1.166	9.245	1.417	0.522	36.848	1.086	0.353	32.476	0.150	0.036	24.382
26	Trireagent	214.515	83.479	38.915	28.814	7.755	26.916	3.672	2.208	60.113	-	-	-
28	Trizol	253.489	118.609	46.791	31.101	18.818	60.506	2.923	1.067	36.497	0.342	0.168	49.204
29	Trizol	189.601	40.743	21.489	21.963	11.942	54.376	2.287	0.532	23.240	0.273	0.199	72.950

b)

GUS Unextracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	Trizol	123.687	29.387	23.759	15.619	3.376	21.615	1.463	0.138	9.423	0.209	0.052	24.701
5	Trizol	15.031	3.416	22.724	2.190	0.087	3.954	0.255	0.115	45.135	0.049	0.053	106.624
6	QIAGEN	113.547	25.701	22.635	8.485	5.791	68.252	1.160	0.353	30.467	0.083	0.040	48.497
7	QIAGEN	86.793	38.638	44.518	13.764	4.718	34.278	1.637	0.616	37.631	0.128	0.058	45.869
8	Trizol	211.399	24.918	11.787	27.815	6.111	21.970	2.667	0.910	34.127	0.334	0.022	6.437
9	Trizol	184.529	59.161	32.061	27.353	13.988	51.139	4.292	2.273	52.961	0.149	0.044	29.731
12	Trizol	17.571	9.401	53.502	2.288	0.828	36.170	0.313	0.137	43.703	0.037	0.019	50.248
13	Trizol	142.954	9.190	6.429	19.574	2.333	11.917	1.297	1.151	88.764	0.206	0.060	29.317
15	QIAGEN	33.880	5.886	17.375	3.375	0.452	13.395	0.316	0.095	30.200	0.036	0.006	15.477
16	Trizol	305.542	11.447	3.747	39.440	6.318	16.020	3.806	0.076	2.002	0.366	0.106	28.798
19	QIAGEN	206.248	41.089	19.922	30.083	1.047	3.479	2.736	0.200	7.320	0.281	0.048	17.083
20	RNAzol	207.785	70.868	34.107	40.908	46.993	114.876	1.460	0.995	68.156	0.171	0.062	36.168
21	Trizol	285.758	24.884	8.708	33.536	3.854	11.494	3.543	1.879	53.041	0.518	0.507	97.869
22	Trizol	638.016	270.313	42.368	55.518	26.368	47.494	6.604	7.337	111.086	-	-	-
23	T & Q	64.833	15.332	23.649	7.247	0.525	7.251	0.720	0.044	6.054	0.090	0.023	25.019
25	Trizol	73.666	17.365	23.573	5.114	2.838	55.489	0.959	0.269	28.063	0.121	0.052	42.496
26	Trireagent	231.839	106.629	45.993	25.087	9.519	37.946	1.502	0.696	46.347	0.117	0.041	34.775
28	Trizol	319.540	59.678	18.676	29.468	3.080	10.451	2.962	0.194	6.538	0.389	0.334	85.913
29	Trizol	251.108	53.347	21.245	29.115	6.945	23.855	3.258	0.714	21.923	0.280	0.082	29.093

Table 17: Mean, SD and CV values for %b3a2 / *BCR* for each laboratory for the b3a2 aRNA reference materials following a) RNA extraction and b) heat lysis (unextracted)

a)

ABL Extracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	Trizol	23.029	3.324	14.435	2.594	0.502	19.367	0.395	0.065	16.374	0.030	0.011	37.722
3	Trizol	28.658	1.949	6.802	4.076	0.925	22.700	0.358	0.037	10.424	0.042	0.009	21.377
4	QIAGEN	27.054	4.368	16.144	4.258	1.819	42.733	0.458	0.125	27.229	0.068	0.029	42.782
5	Trizol	6.138	1.311	21.359	0.764	0.436	57.142	0.157	0.012	7.600	0.099	0.098	99.211
6	QIAGEN	21.777	8.862	40.694	2.637	0.409	15.501	0.273	0.142	52.040	0.040	0.010	25.000
8	Trizol	17.718	7.607	42.935	3.832	2.850	74.382	0.452	0.627	138.938	0.083	0.052	62.616
9	Trizol	6.395	1.942	30.362	0.617	0.416	67.428	0.103	0.067	64.660	-	-	-
10	QIAGEN	2.917	1.286	44.097	0.375	0.059	15.788	0.099	0.036	36.673	0.000	0.000	173.205
11	Trizol	13.064	2.045	15.651	1.724	0.248	14.376	0.145	0.021	14.242	0.019	0.007	39.630
12	Trizol	4.279	2.058	48.091	0.714	0.379	53.150	0.201	0.313	155.779	-	-	-
13	Trizol	14.696	2.881	19.602	4.128	1.576	38.176	0.406	0.041	10.082	0.087	0.011	12.457
14	Roche	19.466	6.450	33.134	3.400	1.700	50.010	0.314	0.257	81.601	0.038	0.016	41.775
16	Trizol	24.163	3.262	13.500	3.166	0.968	30.577	0.390	0.136	34.817	-	-	-
18	Trizol	23.372	5.029	21.515	2.930	0.606	20.668	0.351	0.120	34.118	0.074	0.068	90.985
20	RNAzol	12.142	2.749	22.640	1.439	0.433	30.124	0.153	0.153	100.107	0.015	0.002	14.911
21	Trizol	395.889	81.988	20.710	373.540	183.734	49.187	-	-	-	-	-	-
22	Trizol	65.138	29.670	45.550	6.985	4.439	63.549	0.639	0.459	71.906	0.000	0.000	87.693
25	Trizol	15.716	3.668	23.340	3.024	0.543	17.958	0.246	0.113	45.994	0.024	0.014	57.338
28	Trizol	34.054	2.905	8.530	4.431	1.312	29.604	0.478	0.283	59.344	0.049	0.008	16.226
29	Trizol	29.970	1.780	5.940	3.686	0.654	17.749	0.324	0.077	23.718	0.043	0.014	31.937

b)

ABL Unextracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	Trizol	26.686	4.621	17.317	3.211	0.327	10.187	0.351	0.090	25.500	0.035	0.009	26.508
3	Trizol	23.433	3.061	13.063	2.765	0.155	5.603	0.269	0.061	22.878	0.034	0.009	26.173
4	QIAGEN	17.745	4.355	24.542	3.378	2.387	70.683	0.292	0.184	63.096	0.039	0.007	17.008
5	Trizol	5.537	0.224	4.037	0.821	0.137	16.718	0.060	0.034	57.687	0.005	0.000	9.350
6	QIAGEN	9.520	3.067	32.217	1.487	0.430	28.926	0.150	0.026	17.638	0.014	0.005	37.796
8	Trizol	21.700	14.559	67.090	2.156	0.944	43.766	0.989	1.351	136.519	0.042	0.029	68.688
9	Trizol	6.648	1.439	21.648	0.878	0.237	26.943	0.088	0.024	27.711	0.008	0.001	15.125
10	QIAGEN	3.703	0.847	22.878	1.124	1.040	92.579	0.033	0.030	91.824	0.009	0.014	151.292
11	Trizol	16.825	3.771	22.416	2.060	0.327	15.860	0.223	0.046	20.635	0.025	0.007	28.543
12	Trizol	2.606	0.182	6.974	0.351	0.187	53.375	0.041	0.047	112.792	0.019	0.025	130.000
13	Trizol	20.463	6.951	33.969	2.609	0.638	24.467	0.283	0.078	27.699	0.031	0.011	35.627
14	Roche	11.704	6.012	51.365	1.448	0.957	66.061	0.374	0.319	85.256	0.022	0.009	41.660
16	Trizol	19.370	2.278	11.760	2.381	0.279	11.709	0.278	0.043	15.355	0.035	0.010	29.676
18	Trizol	17.820	0.873	4.901	2.442	0.179	7.329	0.272	0.021	7.672	0.032	0.008	25.420
20	RNAzol	9.941	6.377	64.146	2.456	1.191	48.498	0.258	0.120	46.354	0.033	0.025	76.016
21	Trizol	365.593	36.640	10.022	379.411	28.363	7.475	288.502	29.039	10.065	84.070	43.403	51.627
22	Trizol	98.447	57.131	58.032	16.524	3.128	18.932	1.918	0.848	44.217	0.114	0.102	89.350
25	Trizol	12.615	2.926	23.193	1.812	0.597	32.957	0.187	0.064	34.281	0.016	0.006	39.169
28	Trizol	30.261	4.047	13.374	4.670	0.500	10.717	0.349	0.023	6.644	0.042	0.013	30.227
29	Trizol	30.494	4.178	13.701	4.306	0.487	11.313	0.386	0.045	11.724	0.039	0.015	37.344

Table 18: Mean, SD and CV values for %b2a2 / *ABL* for each laboratory for the b2a2 aRNA reference materials following a) RNA extraction and b) heat lysis (unextracted)

a)

BCR Extracted												
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	Trizol	172.899	35.783	20.696	28.983	21.136	72.927	2.062	2.747	133.236	2.174	-	-
8	Trizol	293.905	56.232	19.133	41.034	20.024	48.799	5.221	5.578	106.829	1.425	0.934	65.527
13	Trizol	106.347	25.076	23.580	30.420	22.710	74.657	2.355	0.320	13.594	0.489	0.029	5.850
21	Trizol	653.637	231.274	35.383	124.287	100.375	80.761	190.395	-	-	-	-	-
25	Trizol	56.635	9.913	17.502	9.250	2.316	25.042	1.309	0.219	16.729	0.146	0.067	45.664
28	Trizol	206.765	18.690	9.039	23.936	11.110	46.413	2.887	2.353	81.517	0.270	0.039	14.434

b)

BCR Unextracted												
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	Trizol	84.348	12.285	14.565	9.949	3.476	34.939	1.514	0.097	6.390	0.238	0.117	49.405
8	Trizol	399.430	155.117	38.835	37.183	5.315	14.293	15.099	18.950	125.512	0.622	0.327	52.620
13	Trizol	143.473	6.117	4.263	17.270	1.871	10.832	2.025	0.397	19.606	0.233	0.068	29.079
21	Trizol	1040.518	171.669	16.498	108.322	13.130	12.121	13.609	1.510	11.098	1.809	0.952	52.595
25	Trizol	61.998	20.962	33.810	10.876	3.184	29.275	1.052	0.069	6.543	0.105	0.020	19.179
28	Trizol	208.217	11.198	5.378	24.344	1.322	5.429	2.201	0.493	22.406	0.233	0.024	10.397

Table 19: Mean, SD and CV values for %b2a2 / *BCR* for each laboratory for the b2a2 aRNA reference materials following a) RNA extraction and b) heat lysis (unextracted)

a)

GUS Extracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	Trizol	388.517	31.066	7.996	41.363	6.082	14.705	3.817	1.105	28.949	0.501	0.080	15.955
5	Trizol	16.701	11.437	68.484	2.724	1.871	68.689	0.430	0.171	39.738	0.256	0.248	96.941
6	QIAGEN	572.580	161.092	28.134	68.043	10.998	16.163	6.550	5.295	80.838	0.800	0.408	51.051
8	Trizol	458.747	222.169	48.429	106.483	95.673	89.848	14.093	21.041	149.303	1.777	0.869	48.896
9	Trizol	219.186	103.690	47.307	16.725	18.555	110.940	3.473	2.890	83.218	-	-	-
12	Trizol	23.868	9.714	40.699	4.179	1.638	39.205	1.156	1.822	157.639	-	-	-
13	Trizol	362.691	102.847	28.357	55.485	20.308	36.602	7.238	1.800	24.868	1.510	0.464	30.724
16	Trizol	450.789	87.861	19.490	61.143	9.323	15.248	8.395	3.056	36.406	-	-	-
20	RNAzol	603.664	181.549	30.075	53.678	12.772	23.795	4.797	4.323	90.122	0.545	0.086	15.720
21	Trizol	869.997	226.070	25.985	172.777	103.112	59.679	92.837	160.799	173.205	-	-	-
22	Trizol	986.949	549.867	55.714	111.905	87.985	78.625	11.134	10.055	90.303	0.004	0.001	26.771
23	T & Q	437.333	285.115	65.194	57.267	20.723	36.187	21.403	18.213	85.095	1.926	2.335	121.226
25	Trizol	242.144	169.973	70.195	54.952	10.125	18.426	6.685	2.516	37.641	0.440	0.217	49.387
28	Trizol	634.371	68.813	10.848	77.024	23.764	30.852	8.153	4.345	53.287	0.881	0.151	17.172
29	Trizol	577.690	87.629	15.169	60.989	16.404	26.896	5.107	0.918	17.967	0.696	0.196	28.135

b)

GUS Unextracted													
Lab ID	RNA Method	Level 1			Level 2			Level 3			Level 4		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	Trizol	299.575	77.439	25.850	34.464	11.464	33.263	3.569	0.402	11.274	0.461	0.086	18.762
5	Trizol	19.467	1.079	5.542	3.024	0.983	32.514	0.204	0.132	64.574	0.019	0.007	38.671
6	QIAGEN	289.970	89.586	30.895	40.613	6.672	16.429	3.587	1.475	41.113	0.317	0.159	50.362
8	Trizol	501.649	209.172	41.697	56.404	11.219	19.891	17.048	22.457	131.725	0.803	0.470	58.480
9	Trizol	198.774	79.642	40.067	25.218	10.981	43.546	2.543	1.411	55.473	0.228	0.100	43.852
12	Trizol	12.823	1.109	8.652	1.875	1.141	60.824	0.230	0.256	111.174	0.081	0.095	117.805
13	Trizol	431.422	56.471	13.090	44.114	3.167	7.178	5.950	1.446	24.309	0.599	0.130	21.680
16	Trizol	443.526	71.314	16.079	49.950	6.147	12.307	6.013	0.706	11.743	0.673	0.150	22.353
20	RNAzol	288.628	257.326	89.155	58.787	10.188	17.330	5.408	0.600	11.101	0.608	0.160	26.368
21	Trizol	561.052	125.055	22.289	60.243	5.989	9.941	7.053	1.616	22.907	0.971	0.375	38.619
22	Trizol	1385.611	1027.654	74.166	185.190	132.737	71.676	21.341	9.748	45.678	1.499	1.715	114.406
23	T & Q	227.667	71.696	31.492	24.167	2.043	8.453	2.590	0.251	9.676	0.243	0.035	14.432
25	Trizol	308.312	93.434	30.305	40.133	12.652	31.525	3.831	1.673	43.661	0.384	0.131	34.074
28	Trizol	551.241	170.195	30.875	69.458	23.344	33.609	5.793	1.655	28.579	0.647	0.257	39.720
29	Trizol	491.049	26.913	5.481	67.197	4.934	7.343	6.340	1.589	25.061	0.545	0.226	41.482

Table 20: Mean, SD and CV values for %b2a2 / *GUSB* for each laboratory for the b2a2 aRNA reference materials following a) RNA extraction and b) heat lysis (unextracted)

3.3.2.1 Overall CV for aRNA samples analysis

The overall median CVs for the b3a2 and b2a2 aRNA samples tested after RNA extraction and direct heat lysis (unextracted) are shown in table 21 and table 22 respectively. The p values are given for a 2-sample t-test analysis for the difference in CVs for the extracted and unextracted samples (calculated using full data set).

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	

Table 21: Overall median CVs for the b3a2 aRNA samples tested after RNA extraction and direct heat lysis (unextracted). * statistically different at a confidence of 90% (i.e p<0.1)

Protocol	Control gene	Level 1	p	Level 2	p	Level 3	p	Level 4	p
Extracted	**ABL**	21.437	0.840	30.351	0.375	36.673	0.507	40.702	0.708
Unextracted	**ABL**	22.032		21.700		27.705		36.486	
Extracted	**BCR**	19.914	0.778	60.863	0.005*	81.517	0.244	30.049	0.873
Unextracted	**BCR**	15.532		13.207		15.352		39.242	
Extracted	**GUSB**	30.075	0.438	36.187	0.075*	80.838	0.043*	30.724	0.986
Unextracted	**GUSB**	30.305		19.891		28.579		38.671	

Table 22: Overall median CVs for the b2a2 aRNA samples tested after RNA extraction and direct heat lysis (unextracted). * statistically different at a confidence of 90% (i.e p<0.1)

3.3.3 Linear regression for b3a2 aRNA samples

The linear regression of the log transformed data obtained from all labs are summarised in figures 12 and 13 (*ABL*), figure 14 (*BCR*) and figure 15 (*GUSB*). Data have been plotted against reference material values which have been calculated as 100% (level 1), 10% (level 2), 1% (level 3) and 0.1% (level 4) *BCR-ABL* / Control Gene based on the input copy number of each aRNA construct. The regression equations for the log transformed data for each cell line and each control gene are given in table 23 (*ABL*), table 24 (*BCR*) and table 25 (*GUSB*). The individual regression plots and associated data for each lab are shown in appendix B.

Lab ID	b3a2 / ABL Heat Lysed	R^2 (Adj)	b3a2 / ABL Extracted	R^2 (Adj)
2	Log(y)= -0.7574 + 1.006 log(x)	99.0%	Log(y)= -0.7548 + 0.8662 log(x)	97.2%
3	Log(y)= -0.8752 + 0.9519 log(x)	99.1%	Log(y)= -0.7854 + 0.9279 log(x)	97.0%
4	Log(y)= -1.975 + 1.119 log(x)	84.4%	Log(y)= -1.333 + 0.9791 log(x)	82.3%
5	Log(y)= -1.157 + 0.8912 log(x)	95.9%	Insufficient data points	-
6	Log(y)= -1.390 + 0.9970 log(x)	98.8%	Log(y)= -0.8710 + 0.8677 log(x)	95.6%
7	Log(y)= -0.9660 + 0.9471 log(x)	99.0%	Log(y)= -0.8919 + 0.9426 log(x)	96.9%
8	Log(y)= -0.8978 + 0.9234 log(x)	97.4%	Log(y)= -0.8787 + 0.8035 log(x)	95.8%
9	Log(y)= -1.046 + 0.9937 log(x)	97.2%	Log(y)= -1.175 + 0.9747 log(x)	96.5%
10	Log(y)= -1.687 + 1.009 log(x)	85.6%	Log(y)= -1.780 + 1.099 log(x)	83.4%
11	Log(y)= -1.013 + 0.9400 log(x)	99.6%	Log(y)= -1.238 + 0.9381 log(x)	94.8%
12	Log(y)= -1.285 + 0.8430 log(x)	97.7%	Insufficient data points	-
13	Log(y)= -0.9860 + 0.9369 log(x)	99.6%	Log(y)= -0.9978 + 0.8143 log(x)	77.8%
14	Log(y)= -0.6409 + 0.8763 log(x)	94.4%	Log(y)= -0.4082 + 0.8465 log(x)	82.1%
15	Log(y)= -1.022 + 1.003 log(x)	99.7%	Log(y)= -0.9504 + 0.9955 log(x)	98.7%
16	Log(y)= -0.7540 + 0.9603 log(x)	99.3%	Log(y)= -0.6277 + 0.8267 log(x)	94.7%
17	Log(y)= -1.082 + 0.9402 log(x)	99.3%	Log(y)= -0.9274 + 0.8428 log(x)	95.9%
18	Log(y)= -0.8331 + 0.9255 log(x)	99.5%	Log(y)= -0.9299 + 0.9734 log(x)	98.9%
19	Log(y)= -1.084 + 0.9760 log(x)	97.4%	Log(y)= -1.291 + 0.9750 log(x)	97.3%
20	Log(y)= -1.240 + 1.005 log(x)	95.1%	Log(y)= -1.363 + 0.9897 log(x)	98.7%
21	Log(y)= 1.699 + 0.4629 log(x)	27.3%	Insufficient data points	-
22	Log(y)= -0.7154 + 1.348 log(x)	59.4%	Insufficient data points	-
24	Log(y)= -1.159 + 0.9335 log(x)	98.6%	Log(y)= -1.031 + 0.8344 log(x)	93.0%
25	Log(y)= -1.383 + 0.9743 log(x)	96.8%	Log(y)= -1.259 + 0.9096 log(x)	93.3%
26	Log(y)= -1.041 + 1.049 log(x)	99.4%	Log(y)= -0.7399 + 0.9180 log(x)	94.9%
28	Log(y)= -0.7157+ 0.916log(x)	98.7%	Log(y)= -0.7369 + 0.9800 log(x)	96.4%
29	Log(y)= -0.7569 + 0.9402 log(x)	99.6%	Log(y)= -0.8889 + 0.9496 log(x)	98.0%

Table 23: Linear regression equations of log transformed %b3a2 / *ABL* data for the heat lysed and extracted aRNA samples for each lab.

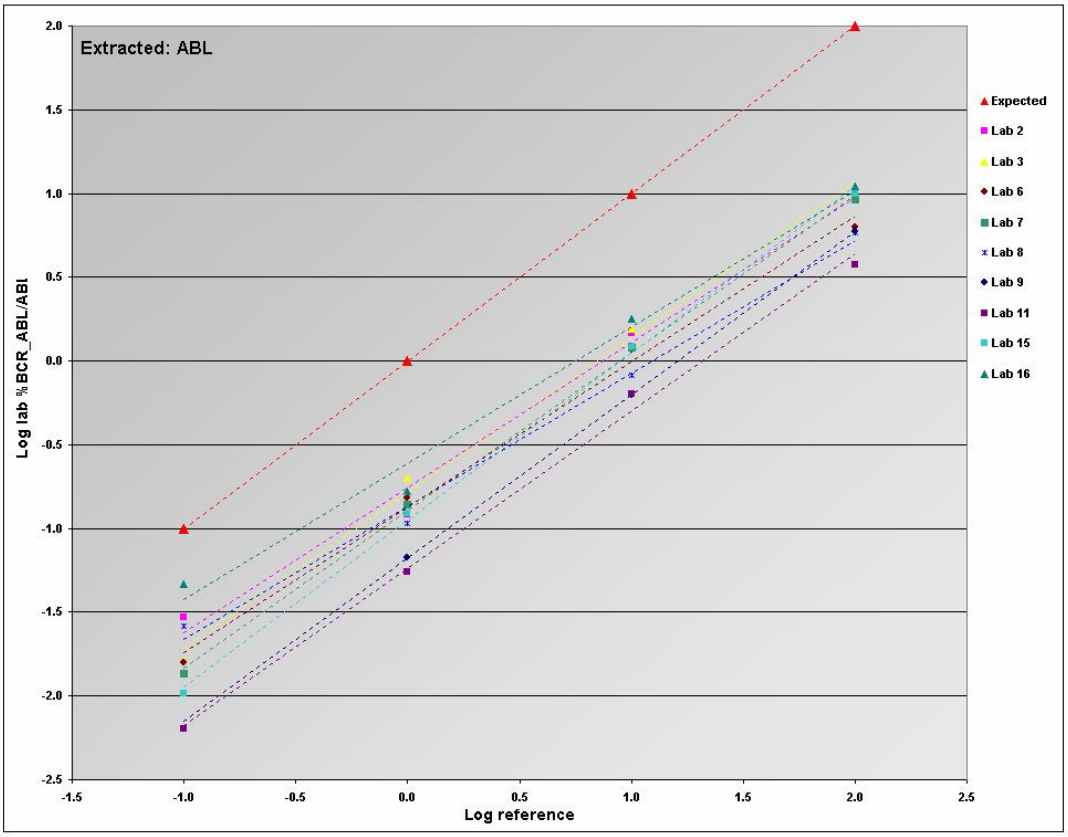
Lab ID	b3a2 / BCR Heat Lysed	R^2 (Adj)	b3a2 / BCR Extracted	R^2 (Adj)
1	Log(y)= 0.3594 + 0.9124 log(x)	97.8%	Log(y)= 0.6739 + 0.7023 log(x)	81.3%
8	Log(y)= 0.3718 + 0.9113 log(x)	99.3%	Log(y)= 0.3500 + 0.7976 log(x)	95.0%
13	Log(y)= -0.1506 + 0.9626 log(x)	99.6%	Log(y)= -0.2056 + 0.7788 log(x)	73.5%
15	Log(y)= -0.8739 + 0.9954 log(x)	99.9%	Log(y)= -0.9343 + 1.015 log(x)	98.7%
21	Log(y)= 0.5444 + 1.116 log(x)	77.2%	Insufficient data points	-
25	Log(y)= -0.7585 + 0.9707 log(x)	97.3%	Log(y)= -0.6082 + 0.9214 log(x)	94.9%
28	Log(y)= 0.0217 + 0.9470 log(x)	98.7%	Log(y)= -0.0564 + 0.9880 log(x)	96.9%

Table 24: Linear regression equations of log transformed %b3a2 / *BCR* data for the heat lysed and extracted aRNA samples for each lab.

Lab ID	b3a2 / GUSB Heat Lysed	R^2 (Adj)	b3a2 / GUSB Extracted	R^2 (Adj)
3	Log(y)= 0.2194 + 0.9340 log(x)	99.3%	Log(y)= 0.2302 + 0.9560log(x)	99.4%
5	Log(y)= -0.6018 + 0.8960 log(x)	94.6%	Insufficient data points	-
6	Log(y)= -0.0424 + 1.033 log(x)	97.9%	Log(y)= 0.4927+ 0.9090 log(x)	96.6%
7	Log(y)= 0.1010 + 0.9430 log(x)	97.0%	Log(y)= 0.2829 + 0.9770 log(x)	97.3%
8	Log(y)= 0.4520 + 0.9430 log(x)	99.3%	Log(y)= 0.4618 + 0.8150 log(x)	95.0%
9	Log(y)= 0.3444 + 1.010 log(x)	95.8%	Log(y)= 0.2820+ 0.9970 log(x)	94.3%
12	Log(y)= -0.5597+ 0.8910 log(x)	97.0%	Insufficient data points	-
13	Log(y)= 0.2783 + 0.9570 log(x)	99.5%	Log(y)= 0.2270+ 0.7750 log(x)	70.1%
15	Log(y)= -0.4753 + 0.9960 log(x)	99.5%	Log(y)= -0.2001 + 0.9940 log(x)	99.3%
16	Log(y)= 0.5617 + 0.9810 log(x)	99.6%	Log(y)= 0.6767 + 0.8670 log(x)	96.5%
19	Log(y)= 0.4354 + 0.9630 log(x)	99.5%	Log(y)= 0.3535 + 1.0240 log(x)	99.1%
20	Log(y)= 0.2421 + 1.0840 log(x)	92.3%	Log(y)= 0.2940 + 1.010 log(x)	98.2%
21	Log(y)= 0.3088 + 1.131 log(x)	78.4 %	Insufficient data points	-
22	Log(y)= 0.1990 + 1.333 log(x)	66.0%	Insufficient data points	-
23	Log(y)= -0.1123 + 0.958 log(x)	99.6%	Log(y)= -0.0988 + 1.142 log(x)	94.6%
25	Log(y)= -0.6578 + 0.909 log(x)	96.9%	Log(y)= -0.1594 + 0.598 log(x)	90.8%
26	Log(y)= 0.17473 + 1.109 log(x)	98.4%	Insufficient data points	-
28	Log(y)= 0.4665 + 1.013 log(x)	97.1%	Log(y)= 0.4512 + 0.9610 log(x)	96.3%
29	Log(y)= 0.4562 + 0.982 log(x)	99.2%	Log(y)= 0.3342 + 0.970 log(x)	97.3%

Table 25: Linear regression equations of log transformed %b3a2 / *GUSB* data for the heat lysed and extracted aRNA samples for each lab.

a)



b)

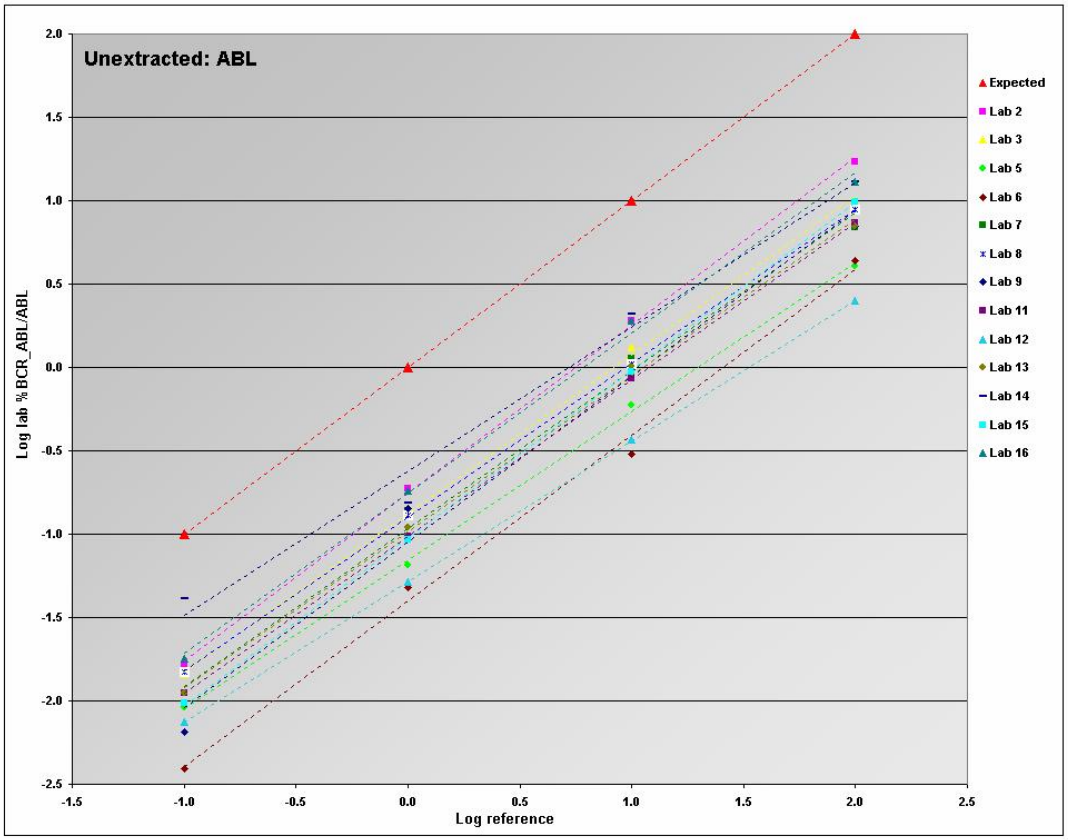
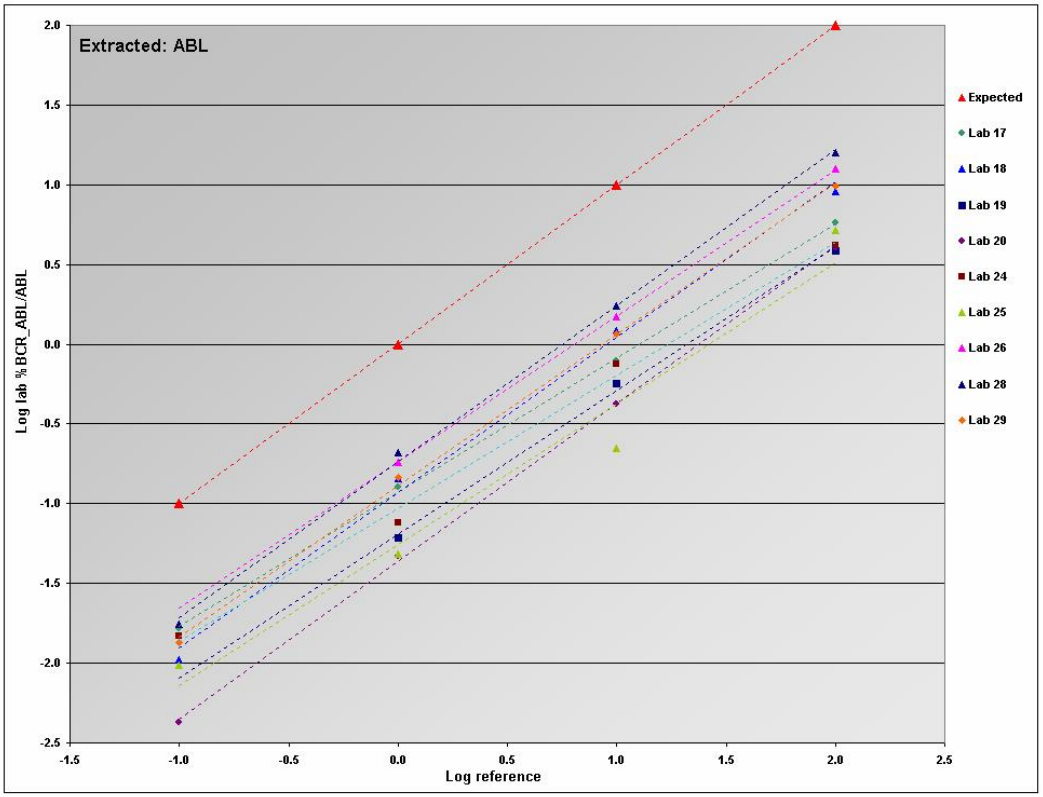


Figure 12: Summary of the linear regressions of the log transformed %b3a2 / *ABL* data obtained from labs 2 - 16 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)

a)



b)

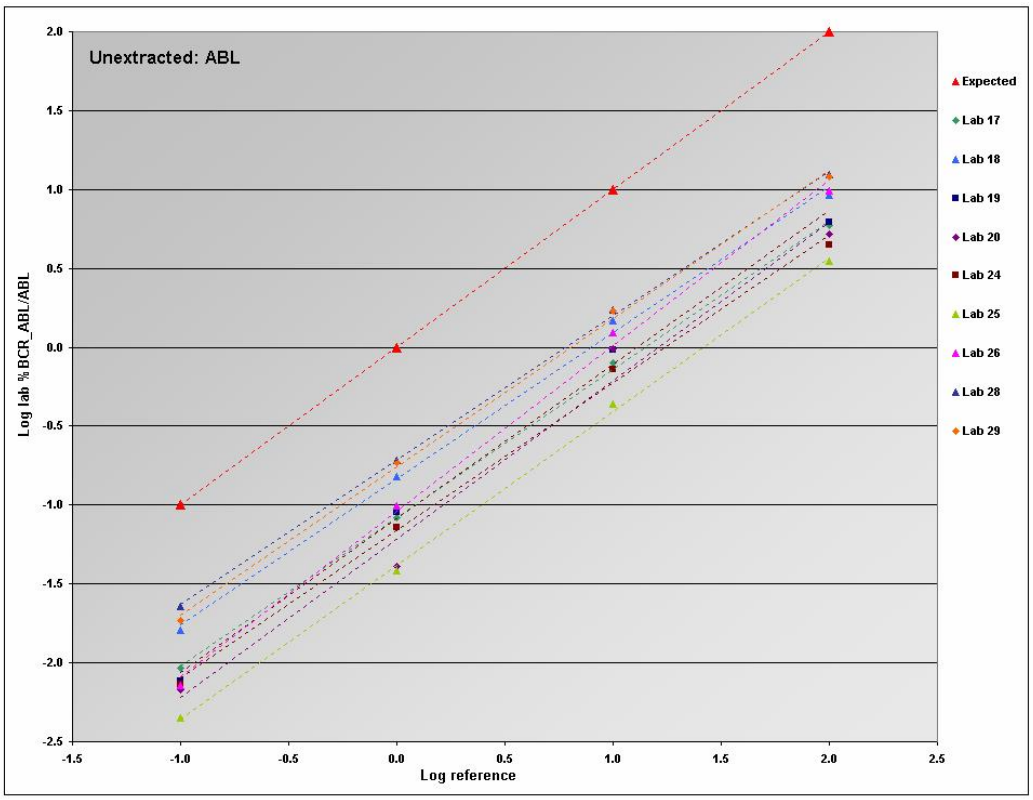
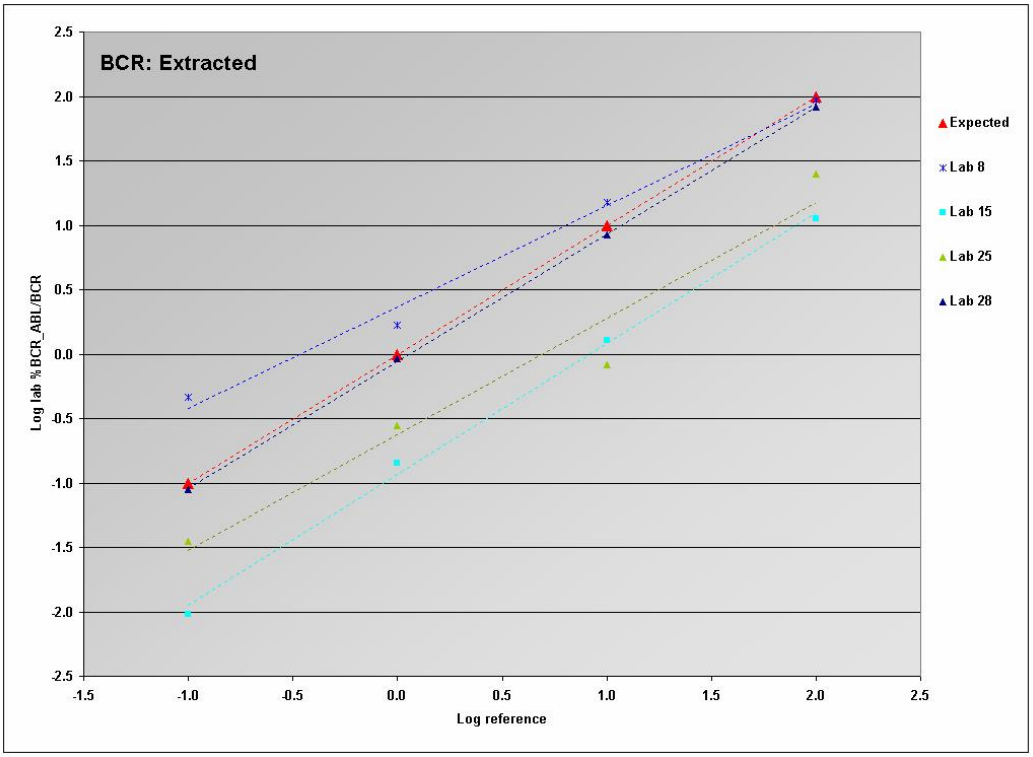


Figure 13: Summary of the linear regressions of the log transformed %b3a2 / *ABL* data obtained from labs 17 - 29 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)

a)



b)

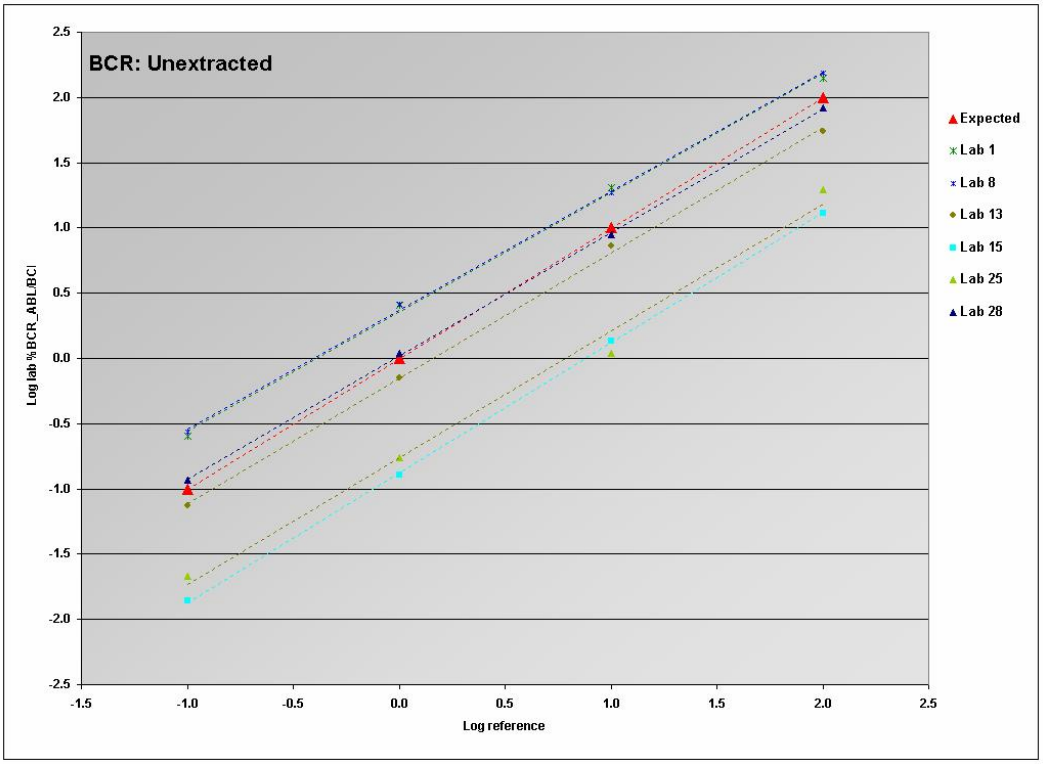
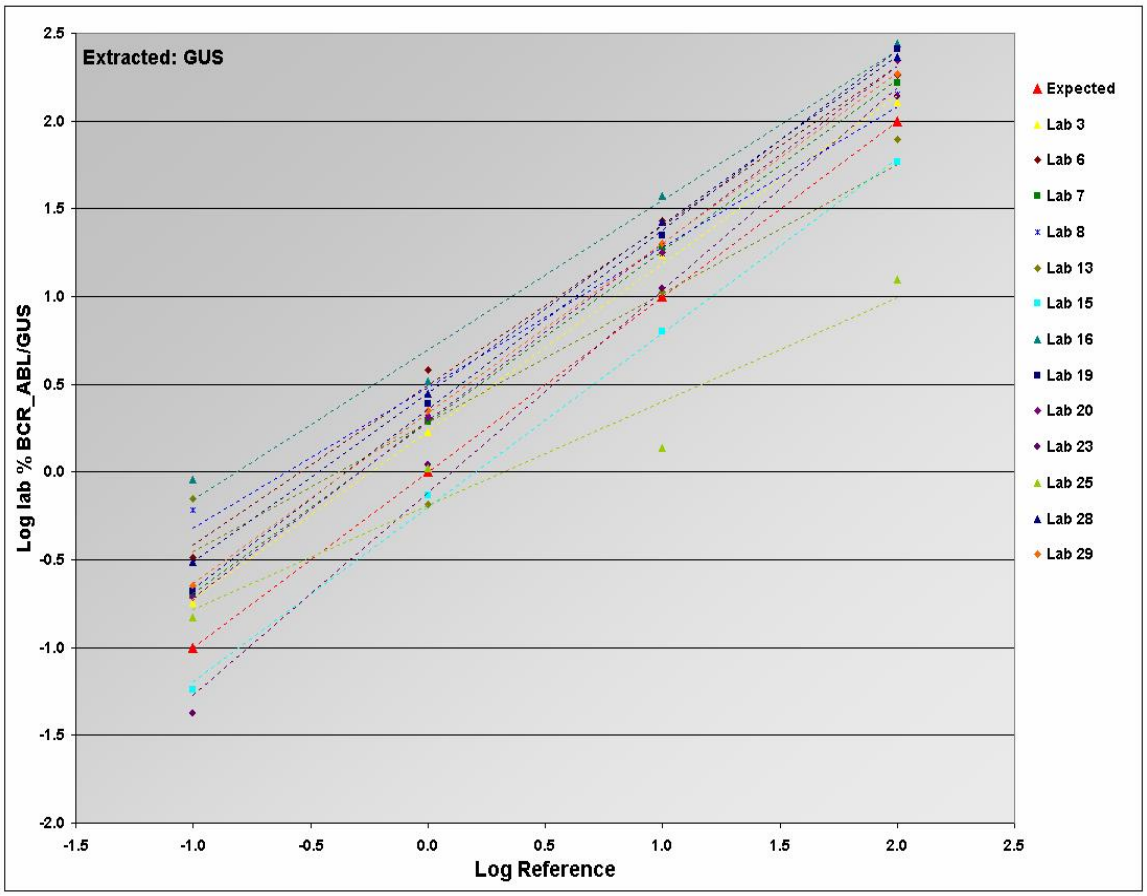
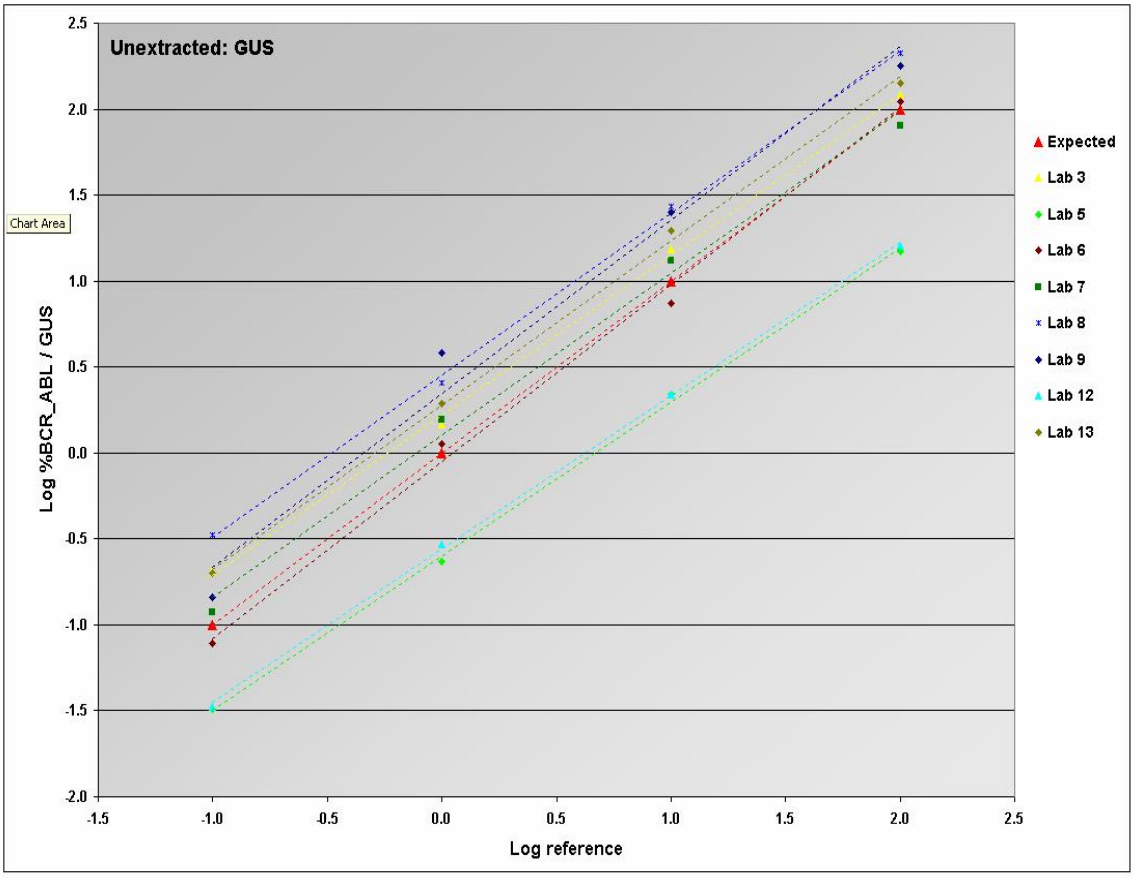


Figure 14: Summary of the linear regressions of the log transformed %b3a2 / *BCR* data obtained from labs 1 - 29 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)

a)


Extracted: GUS
Log lab % BCR_ABL/GUS
Log Reference
Expected
Lab 3
Lab 6
Lab 7
Lab 8
Lab 13
Lab 15
Lab 16
Lab 19
Lab 20
Lab 23
Lab 25
Lab 28
Lab 29

b)


Unextracted: GUS
Chart Area
Log %BCR_ABL / GUS
Log reference
Expected
Lab 3
Lab 5
Lab 6
Lab 7
Lab 8
Lab 9
Lab 12
Lab 13

c)

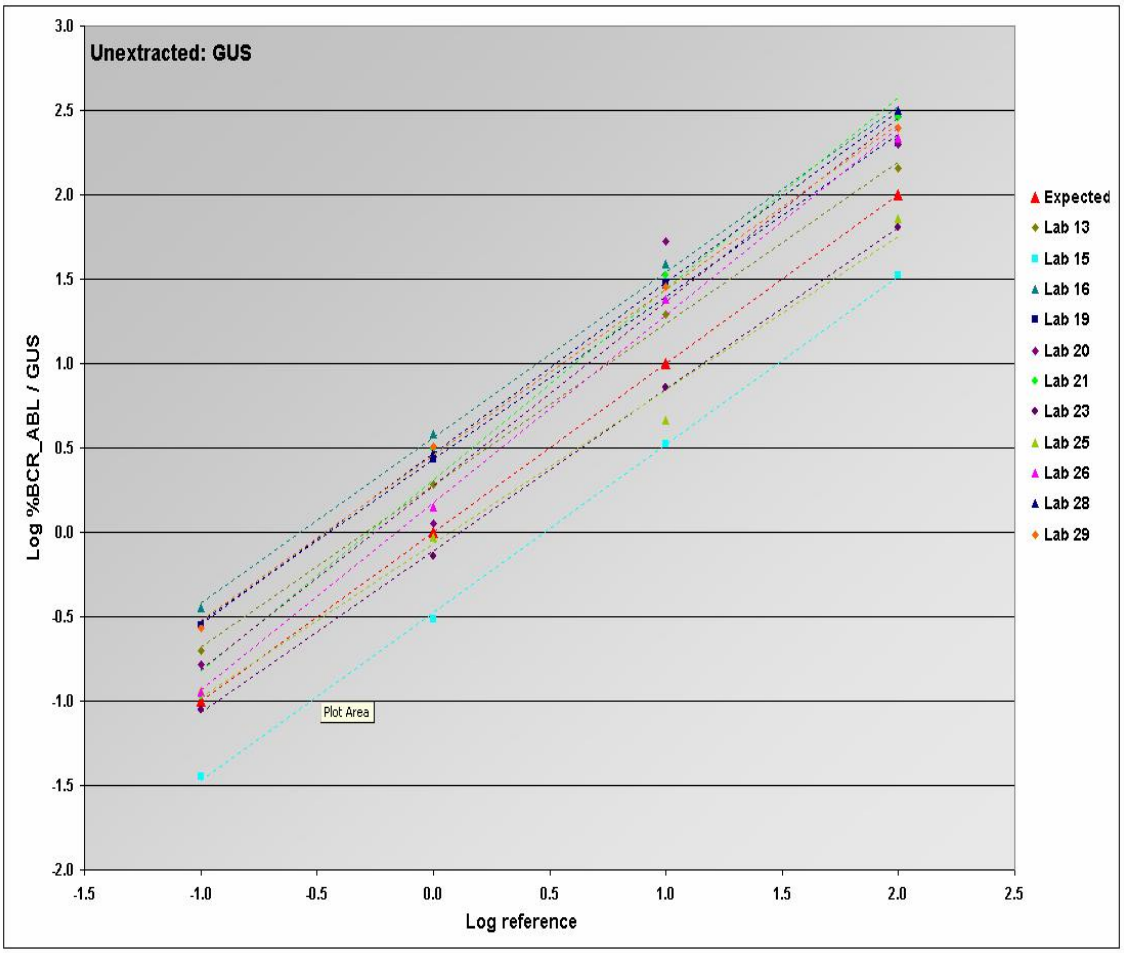


Figure 15: Summary of the linear regressions of the log transformed %b3a2 / *GUSB* data obtained from labs 1 - 29 using the aRNA material a) taken through an RNA extraction procedure, b) heat lysed (unextracted) before adding to cDNA reaction (labs 3-13) and c) heat lysed (unextracted) before adding to cDNA reaction (labs 13-29).

3.3.4 Linear regression for b2a2 aRNA samples

The linear regression of the log transformed data obtained from all labs are summarised in figure 16 (*ABL*), figure 17 (*BCR*) and figure 18 (*GUSB*). Data have been plotted against reference material values which have been calculated as 100% (level 1), 10% (level 2), 1% (level 3) and 0.1% (level 4) *BCR-ABL* / Control Gene based on the input copy number of each aRNA construct. The regression equations for the log transformed data for each cell line and each control gene are given in table 26 (*ABL*), table 27 (*BCR*) and table 28 (*GUSB*). The individual regression plots and associated data for each lab are shown in appendix C.

3.3.5 r^2 values for linear regressions

The number of labs where the r^2 value for the linear regression was >98% are shown in table 29.

Lab ID	b2a2 / ABL Heat Lysed	R^2 (Adj)	b2a2 / ABL Extracted	R^2 (Adj)
2	Log(y)= -0.4805 + 0.9620 log(x)	99.4%	Log(y)= -0.5239 + 0.9540 log(x)	98.9%
3	Log(y)= -0.5385 + 0.9550 log(x)	99.5%	Log(y)= -0.4212 + 0.9570 log(x)	99.5%
4	Log(y)= -0.5231 + 0.8990 log(x)	96.8%	Log(y)= -0.3241 + 0.8840 log(x)	97.7%
5	Log(y)= -1.2442 + 1.0270 log(x)	97.4%	Log(y)= -0.6491 + 0.6610 log(x)	88.9%
6	Log(y)= -0.8695 + 0.9500 log(x)	98.6%	Log(y)= -0.5343 + 0.9190 log(x)	97.3%
8	Log(y)= -0.4866 + 0.8790 log(x)	88.6%	Log(y)= -0.4462 + 0.8250 log(x)	83.9%
9	Log(y)= -1.0962 + 0.9780 log(x)	99.2%	Log(y)= -1.1216 + 0.9290 log(x)	87.9%
10	Log(y)= -1.3513 + 1.0400 log(x)	88.6%	Log(y)= -1.4017 + 0.9800 log(x)	87.6%
11	Log(y)= -0.6603 + 0.9470 log(x)	99.3%	Log(y)= -0.7957 + 0.9670 log(x)	99.0%
12	Log(y)= -1.4356 + 0.9130 log(x)	78.6%	Insufficient data points	-
13	Log(y)= -0.5672 + 0.9410 log(x)	98.5%	Log(y)= -0.3096 + 0.7660 log(x)	97.7%
14	Log(y)= -0.7177 + 0.8790 log(x)	93.4%	Log(y)= -0.5310 + 0.9260 log(x)	95.9%
16	Log(y)= -0.5531 + 0.9200 log(x)	99.6%	Log(y)= -0.4215 + 0.9030 log(x)	98.2%
18	Log(y)= -0.5669 + 0.9200 log(x)	99.7%	Log(y)= -0.3196 + 0.8060 log(x)	96.9%
20	Log(y)= -0.6482 + 0.8350 log(x)	91.9%	Log(y)= -0.8078 + 0.9570 log(x)	98.7%
21	Log(y)= 2.2570 + 0.2200 log(x)	56.5%	Insufficient data points	-
22	Log(y)= 0.0524 + 1.0250 log(x)	91.5%	Log(y)= -1.0796 + 1.6270 log(x)	86.8%
25	Log(y)= -0.7942 + 0.9718 log(x)	98.1%	Log(y)= -0.6514 + 0.9719 log(x)	97.0%
28	Log(y)= -0.4131 + 0.9738 log(x)	99.3%	Log(y)= -0.3560 + 0.9539 log(x)	98.7%
29	Log(y)= -0.4221 + 0.9783 log(x)	99.1%	Log(y)= -0.4424 + 0.9652 log(x)	99.2%

Table 26: Linear regression equations of log transformed %b2a2 / *ABL* data for the heat lysed and extracted aRNA samples for each lab.

Lab ID	b2a2 / BCR Heat Lysed	R^2 (Adj)	b2a2 / BCR Extracted	R^2 (Adj)
1	Log(y)= 0.1797 + 0.8540 log(x)	98.5%	Log(y)= 0.4126 + 0.8660 log(x)	70.7%
8	Log(y)= 0.7542 + 0.9110 log(x)	92.5%	Log(y)= 0.7315 + 0.8130 log(x)	90.6%
13	Log(y)= 0.2946 + 0.9340 log(x)	99.5%	Log(y)= 0.4696 + 0.8030 log(x)	96.6%
21	Log(y)= 1.1344 + 0.9290 log(x)	98.9%	Insufficient data points	-
25	Log(y)= -0.005 + 0.9277 log(x)	98.7%	Log(y)= 0.0501 + 0.8717 log(x)	98.2%
28	Log(y)= 0.3562 + 0.9906 log(x)	99.8%	Log(y)= 0.3837 + 0.9640 log(x)	97.6%

Table 27: Linear regression equations of log transformed %b2a2 / *BCR* data for the heat lysed and extracted aRNA samples for each lab.

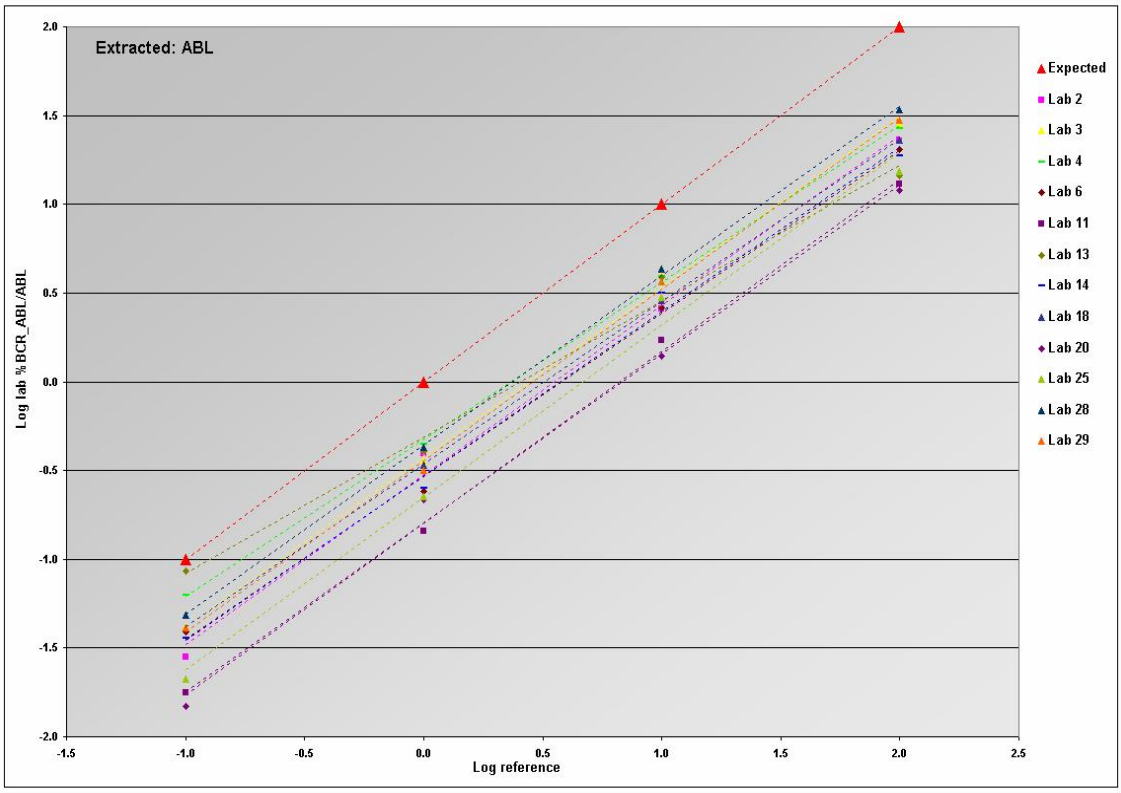
Lab ID	b2a2 / GUSB Heat Lysed	R^2 (Adj)	b2a2 / GUSB Extracted	R^2 (Adj)
3	Log(y)= 0.5788 + 0.9390 log(x)	99.1%	Log(y)= 0.6304 + 0.9720 log(x)	99.4%
5	Log(y)= -0.7068 + 1.0320 log(x)	98.5%	Log(y)= -0.2202 + 0.6310 log(x)	81.1%
6	Log(y)= 0.5116 + 1.002 log(x)	98.3%	Log(y)= 0.7931 + 0.9810 log(x)	96.1%
8	Log(y)= 0.8445 + 0.9250 log(x)	92.4%	Log(y)= 0.9347+ 0.8420 log(x)	81.9%
9	Log(y)= 0.3456 + 0.9840 log(x)	97.8%	Log(y)= 0.3227 + 0.9380 log(x)	81.2%
12	Log(y)= -0.7218 + 0.9100 log(x)	79.9%	Insufficient data points	-
13	Log(y)= 0.7299 + 0.9460 log(x)	99.5%	Log(y)= 0.9213 + 0.8010 log(x)	98.1%
16	Log(y)= 0.7651 + 0.9380 log(x)	99.7%	Log(y)= 0.9081+ 0.8710 log(x)	98.3%
20	Log(y)= 0.7122 + 0.8650 log(x)	93.7%	Log(y)= 0.7603 + 1.000 log(x)	99.2%
21	Log(y)= 0.8675 + 0.9270 log(x)	99.1%	Insufficient data points	-
22	Log(y)= 1.1161 + 1.0210 log(x)	91.6%	Log(y)= 0.0389 + 1.6170 log(x)	83.8%
23	Log(y)= 0.3877 + 0.9850 log(x)	99.6%	Log(y)= 0.8364 + 0.8870 log(x)	73.3%
25	Log(y)= 0.5596 + 0.9756 log(x)	98.5%	Log(y)= 0.6634 + 0.9030 log(x)	94.6%
28	Log(y)= 0.7755 + 0.9900 log(x)	98.4%	Log(y)= 0.8934 + 0.9579 log(x)	98.9%
29	Log(y)= 0.7571 + 0.9968 log(x)	99.1%	Log(y)= 0.7740 + 0.9838 log(x)	99.2%

Table 28: Linear regression equations of log transformed %b2a2 / *GUSB* data for the heat lysed and extracted aRNA samples for each lab.

	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%)

Table 29: The number of labs where the r^2 value for the linear regression was >98%

a)



b)

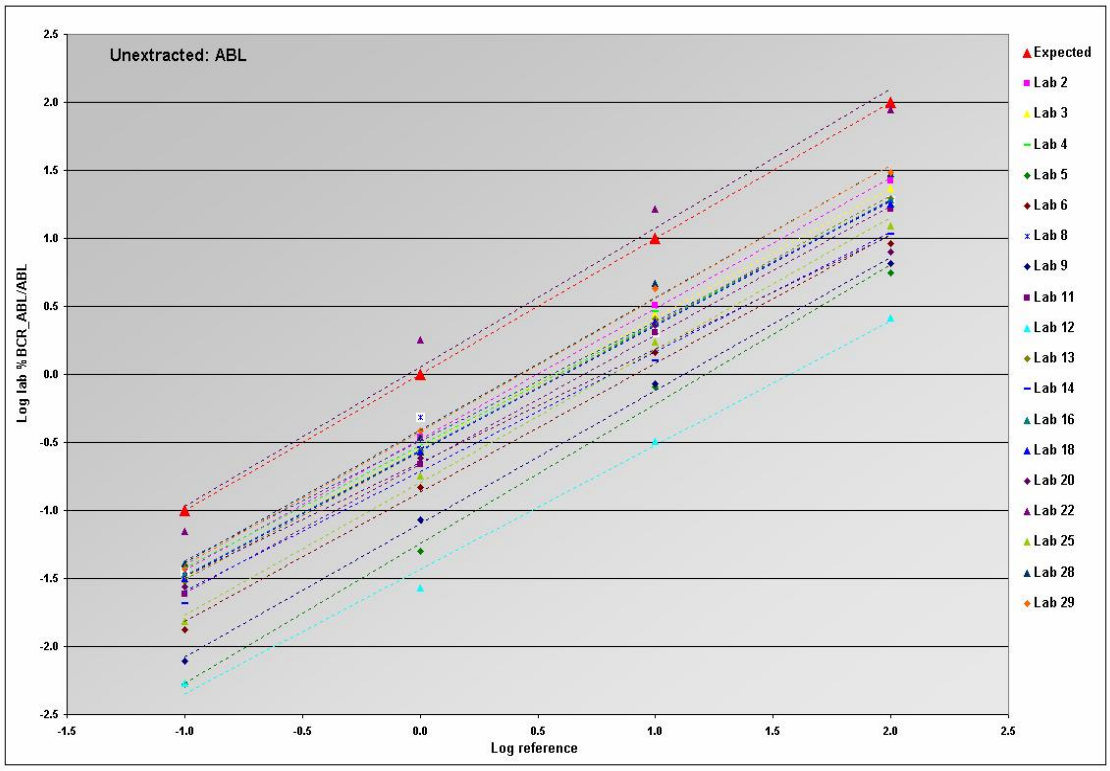
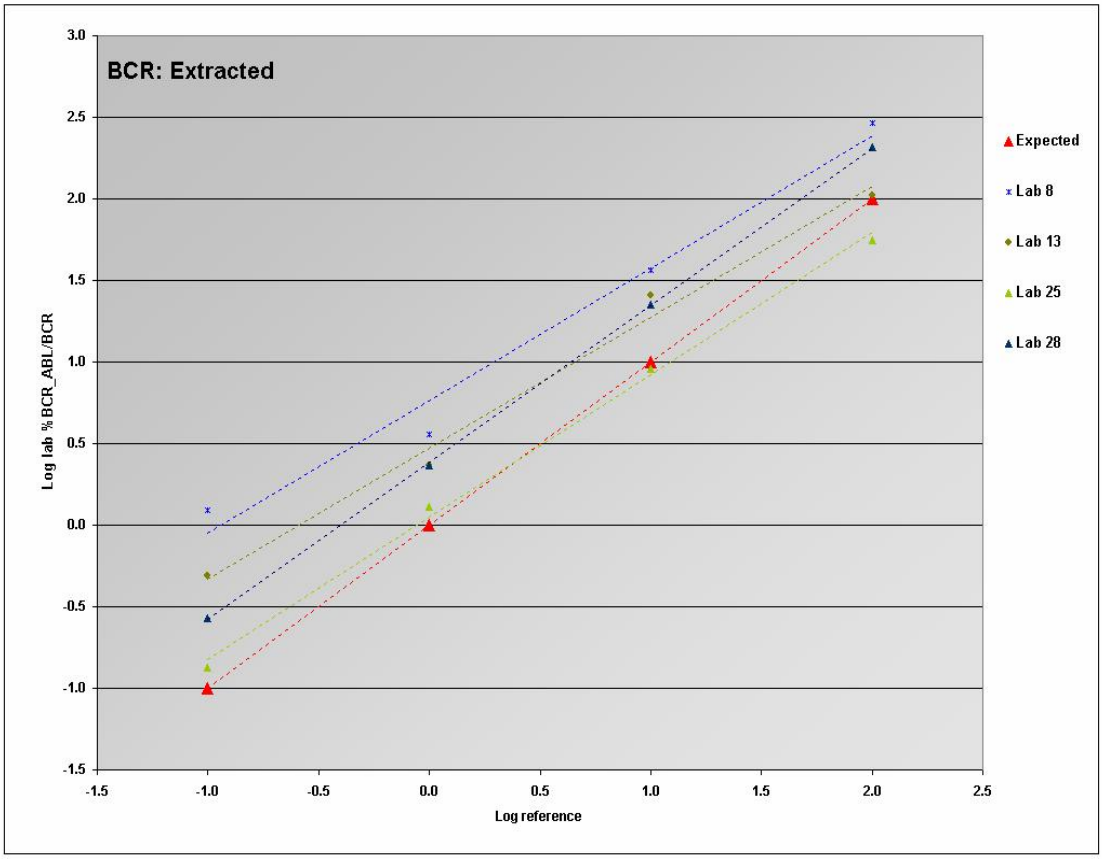


Figure 16: Summary of the linear regressions of the log transformed %b2a2 / *ABL* data obtained from labs 2 - 29 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)

a)



b)

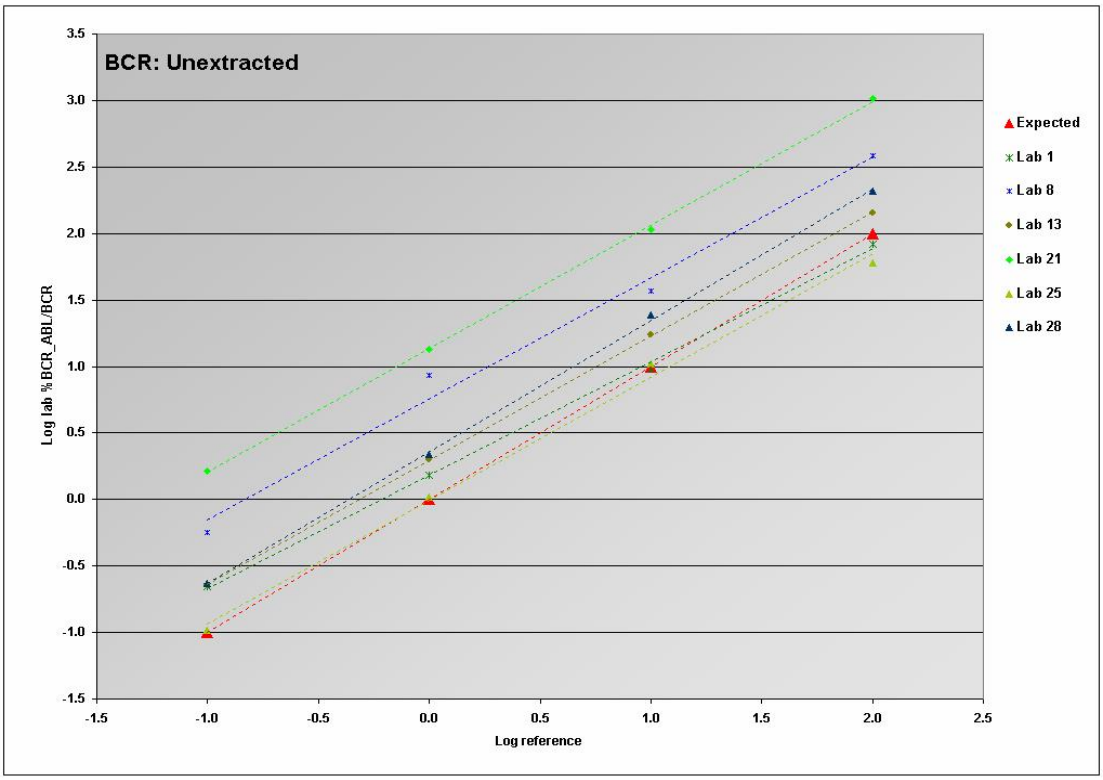
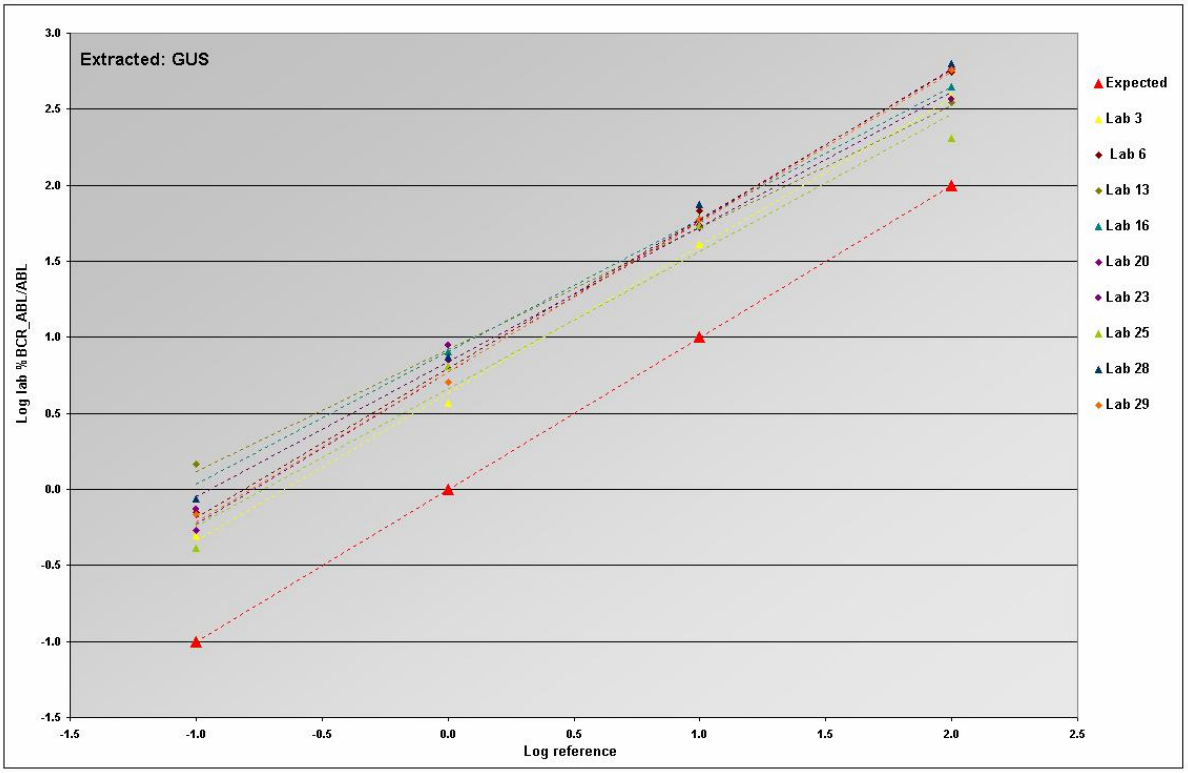


Figure 17: Summary of the linear regressions of the log transformed %b2a2 / *BCR* data obtained from labs 1- 28 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)



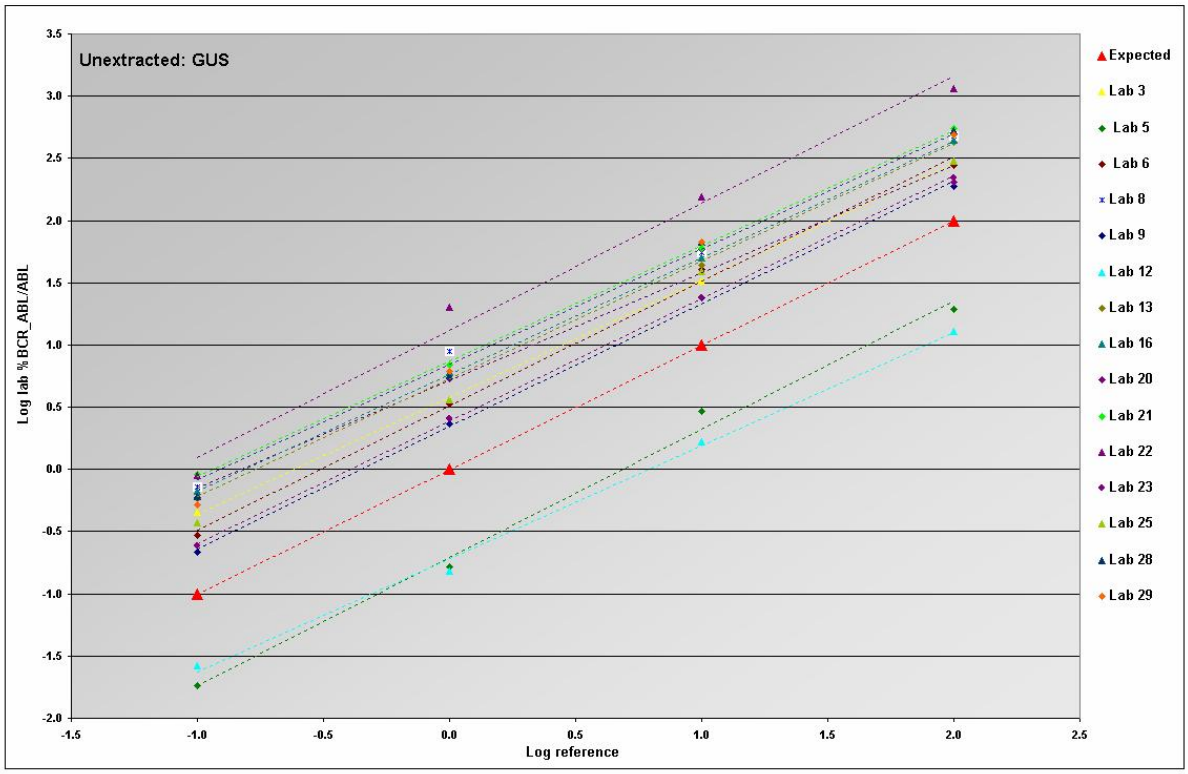


Figure 18: Summary of the linear regressions of the log transformed %b2a2 / *GUSB* data obtained from labs 1- 28 using the aRNA material a) taken through an RNA extraction procedure b) heat lysed before adding to cDNA reaction (Unextracted)

4. CONCLUSIONS

The aim of this collaborative study was to produce and assess the use of aRNA as a candidate reference material for the standardisation of *BCR-ABL* RQ-PCR protocols. Nine prototype aRNA reference materials were produced all of which were estimated to contain $3x10^4$ copies/µl of the control genes *ABL*, *BCR* and *GUSB*. Four levels of b3a2 and b2a2 aRNA were added to the background of control gene aRNA to produce samples with predicted %*BCR-ABL* / Control gene values of 100% (level 1), 10% (level 2), 1% (level 3) and 0.1% (level 4). A control gene only sample was also included for analysis.

The performance of the aRNA prototype reference material was assessed by an international field trial (October - December 2007). aRNA samples were shipped at ambient temperature by express courier to 29 laboratories (22 EU, 3 USA, 4 Asia/Australasia). The aRNA samples were tested following RNA extraction (7 different protocols) or after direct heat lysis using 3 different control genes on 14 different RQ-PCR platforms. Seventeen labs and 8 labs made modifications to the field trial protocol for the extracted and direct heat lysed samples respectively. For the purposes of this report the copy number values have been corrected for variation in the volume of aRNA extracted and used in the cDNA reaction and the amount of cDNA added to the RQ-PCR so that the data between labs are comparable.

The expected copy number for each control gene in each aRNA sample was $3x10^4$ copies/µl (based on the copy number supplied by Asuragen). The median number of copies/ul obtained for *ABL*, *BCR* and *GUSB* for the b3a2 aRNA samples following RNA extraction were $8.13x10^3$, $3.46x10^2$ and $4.09x10^2$ respectively and following direct heat lysis were $1.25x10^5$, $1.25x10^4$ and $7.72x10^3$ respectively.

The median number of copies/ul obtained for *ABL*, *BCR* and *GUSB* for the b2a2 aRNA samples following RNA extraction were $6.32x10^3$, $4.86x10^2$ and $3.80x10^2$ respectively and following direct heat lysis were $1.16x10^5$, $1.57x10^4$ and $7.9x10^3$ respectively. The overall 12-fold loss in control gene copy number observed when samples were analysed after RNA extraction appeared to be related to the RNA extraction protocol used. Labs using a Trizol protocol showed a median 28-fold loss and those using QIAGEN protocols showed a median 2-fold loss. For the direct heat lysed samples the median copy numbers of *BCR* and *GUSB* were approximately 2 and 4 fold less than the expected copy number. The median copy number of *ABL* for the direct heat lysed samples was approximately 4-fold higher than the expected copy number. The *ABL* copy number values were based on an estimated OD_{260} conversion factor and not the NIST-traceable phosphate assay which probably accounts for the unexpectedly high values for this gene.

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 failed to detect level 3 (n=2) and level 4 (n=6) b3a2 transcripts respectively and 6/22 labs failed to detect level 3 (n=1) and level 4 (n=6) b2a2 transcripts respectively. For the heat lysed aRNA samples level 4 b3a2 and b2a2 could not be detected by one lab. This was most likely due to the loss of recovery of the aRNA following RNA extraction using the Trizol protocol.

The coefficient of variance for the %*BCR-ABL* / control gene values for the extracted and heat lysed samples were statistically different (90% confidence; i.e. $p<0.1$) for 7 analyses (b3a2: *ABL* Level 4, *BCR* Levels 1 & 4, *GUSB* Level 1; b2a2: *BCR* Level 2, *GUSB* Levels 2 & 3). The CVs for the extracted samples were generally higher than those for the heat lysed samples.

Linear regression plots were produced for log transformed lab data plotted against the log transformation of the reference standard values for the b3a2 and b2a2 a RNA samples. The r^2 values for the linear regression of the b3a2 samples were >98% in 53% and 18% of labs for the heat lysed and extracted samples respectively. The r^2 values for the linear regression of the b2a2 samples were >98% in 63% and 34% of labs for the heat lysed and extracted samples respectively. The r^2 values for the freeze dried cell line field trial (report available at http://www.ngrl.org.uk/ Wessex/downloads.htm) were >98% for all laboratories.

Overall, the aRNA samples worked well when directly heat lysed prior to cDNA synthesis but further 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. The 9 reference samples used in this study contained a total of 0.9 to $1.2x10^5$ copies/µL of aRNA. At this very low RNA input, RNA extraction protocols have to be optimized to obtain efficient and

reproducible recovery of RNA. The aRNA samples will undergo a further round of field trial evaluation with the aim of establishing them as secondary reference reagents. For this field trial, 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.

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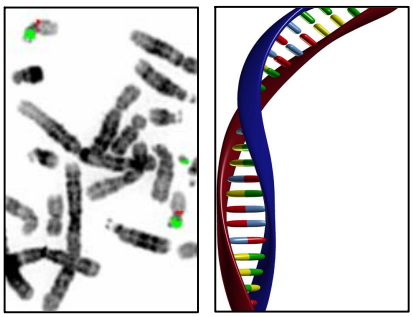

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