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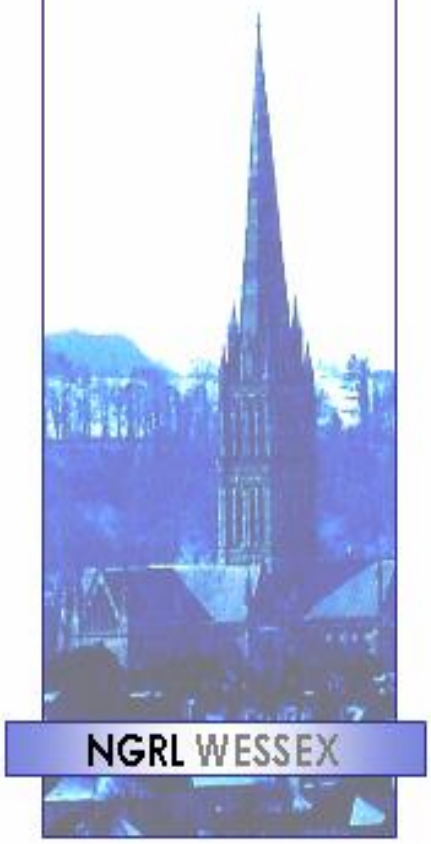
NHS

Reference Reagents



Freeze Dried Cell Lines as reference materials for standardisation of *BCR-ABL* RQ-PCR methods: report of field trial evaluation

May 2008

Title	Freeze Dried Cell Lines as reference materials for standardisation of *BCR-ABL* RQ-PCR methods: report of field trial evaluation
NGRL Ref	NGRLW_NIBSC_*BCR_ABL*_1.0
Publication Date	May 2008
Document Purpose	Dissemination of information about production and field trial evaluation of reference materials for *BCR-ABL* RQ PCR methods
Target Audience	Laboratories performing *BCR-ABL* RQ-PCR and participants of NGRL (Wessex) / NIBSC field trial.
NGRL Funded by	

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 freeze dried cell lines as candidate reference materials for the standardisation of *BCR-ABL* RQ-PCR protocols.

- Eight reference standards were prepared by diluting K562 cells (b3a2 expressing cell line) into two *BCR-ABL* negative cell lines: HL60 and KG1. Cell mixtures with four levels of *BCR-ABL* in each negative cell line were produced and freeze dried at $3x10^6$ cells/vial.

- The performance of the freeze dried cells was assessed by an international field trial (June - October 2007) that involved 14 laboratories (7 EU, 4 USA, 3 Asia/Australasia) using 7 different protocols and 9 different RQ-PCR platforms.

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

- The median number of copies obtained for *ABL*, *BCR* and *GUSB* for the HL60 material were 1.16E+05, 2.60E+05 and1.70E+05 respectively and for the KG1 material were 1.02E+05, 5.85E+05 and 1.13E+05 respectively.

- The coefficient of variation for % *BCR-ABL* / control gene values obtained from all labs were comparable to those obtained in the IS conversion factor rounds administered by the Mannheim laboratory using primary patient samples. There was no statistical difference between the CVs obtained from either cell line for any of the control genes.

- 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 / convert the data to the reference material values.

- Both freeze dried cell line mixes appear to be suitable for development of *BCR-ABL* reference reagents and accelerated degradation studies of the material are ongoing.

- A medium scale pilot freeze dry study using the HL60 / K562 cell mixture is planned for mid-2008 in collaboration with NIBSC and the European Collection of Cell Cultures (ECACC).

- If this pilot is successful, a large scale culture of the two cell lines will be performed to enable the production of 3000 vials for each level of *BCR-ABL* (12,000 vials total).

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. It has been shown previously that good quality RNA can be extracted from freeze dried K562 cells[6] and therefore one possible solution is the use of freeze dried cell line mixtures.

The aim of this collaborative study was to produce and perform a preliminary assessment of the use of freeze dried cell line mixtures as universal reference materials. As an initial step we identified cell lines in which the relative ratio of the three recommended control genes – *BCR, ABL* and *GUSB* – was similar to that seen in normal leucocytes. We surveyed 26 haemopoietic cell lines (ACC42, Jurkat, JVM2, Loucy, MOLT3, NALM76, PEER, REH, RS4-11, T-ALL1, THP1, HeLa, MV4-11, Karpas, Caco2, ML-1, ML-2, 1E8, ALLPo, NB4, KG1, KG1a, HL60, Kasumi1, NOMO1, SKM1) in up to 4 different centres and identified KG1 and HL60 as the best candidates to take forward for detailed evaluation. This report details the preparation of pilot reference materials and their evaluation in an international field trial that involved 14 laboratories (7 EU, 4 USA, 3 Asia/Australasia) using 7 different protocols and 9 different RQ-PCR platforms

2. MATERIALS AND METHODS

2.1 Cell lines and preparation of reference materials

HL60 and KG1 cell lines were obtained from DSMZ and the K562 cell line was obtained from the Hammersmith Hospital, London, UK. All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma Aldrich). For evaluation purposes we aimed to produce four dilutions of K562 in both HL60 and KG1 that corresponded roughly to 10% (Level 1), 1% (Level 2), 0.1% (Level 3) and 0.01% (Level 4) *BCR-ABL-ABL*.

9 x 10^8 HL60 and KG1 cells harvested from cultures in exponential phase were centrifuged at 1300rpm (Centra CL3, 243 Rotor, Thermo IEC) for 5 minutes and resuspended in 150ml ice cold 2X PBS (6 x 10^6 cells / ml). The Level 1 HL60 / K562 cell mix was prepared by resuspending 1.2 x 10^7 K562 cells in 2.4 x 10^8 HL60 cells (40ml of HL60 cell solution in 2X PBS). The Level 1 KG1 / K562 cell mix was prepared by resuspending 2.4 x 10^7 K562 cells in 2.4 x 10^8 KG1 cells (40ml of KG1 cell solution in 2X PBS). Level 2, 3 and 4 cell mixes for each of the two *BCR-ABL* negative cell lines HL60 and KG1 were prepared by performing 10 fold serial dilutions of the Level 1 samples in the 2X PBS cell suspensions. Aliquots from each dilution were lysed directly into Trizol (Invitrogen) for subsequent comparison with the freeze dried cells.

2.1 x 10^8 cells (35ml at 6 x 10^6 cells / ml in 2X ice cold PBS) for each of the four *BCR-ABL* level dilutions for each cell line were transferred to the National Institute for Biological Standards and Control (NIBSC) on ice within 6 hours after processing. On the same day, 5ml glass vials were filled with 3 x 10^6 cells (0.5ml) for freeze drying. The drying process took 65-67 hours and a total 475 vials were produced.

K-562 Cells in HL-60 cells	Level 1	PM-07-011-A	(60 vials)
K-562 Cells in HL-60 cells	Level 2	PM-07-011-B	(58 vials)
K-562 Cells in HL-60 cells	Level 3	PM-07-011-C	(60 vials)
K-562 Cells in HL-60 cells	Level 4	PM-07-011-D	(60 vials)
K-562 Cells in KG-1 cells	Level 1	PM-07-012-A	(57 vials)
K-562 Cells in KG-1 cells	Level 2	PM-07-012-B	(60 vials)
K-562 Cells in KG-1 cells	Level 3	PM-07-012-C	(60 vials)
K-562 Cells in KG-1 cells	Level 4	PM-07-012-D	(60 vials)

2.2 Pilot field trial (27th April – 1st June 2007)

A pilot analysis of freeze dried reagents was carried out by the Mannheim, Marseilles and NGRL (Wessex) laboratories. The aims of the pilot study were to:

(i) test two different resuspension protocols.

(ii) perform an initial assessment to determine if the freeze dried cells were suitable for more detailed analysis, ie. that RNA could be extracted by different centres, that *BCR-ABL* and the three control genes could be amplified at levels comparable to normal leukocytes, that the level of *BCR-ABL* corresponded roughly to the dilutions.

(iii) test if the protocol was comprehensible and unambiguous

2.2.1 Freeze dried cell mixtures

Sixteen 5ml glass vials containing the freeze dried cell mixtures were supplied to each lab. These were labelled as follows:

K562 Cells in HL60 cells	Level 1	PM-07-011-A	(2 vials)
K562 Cells in HL60 cells	Level 2	PM-07-011-B	(2 vials)
K562 Cells in HL60 cells	Level 3	PM-07-011-C	(2 vials)
K562 Cells in HL60 cells	Level 4	PM-07-011-D	(2 vials)
K562 Cells in KG1 cells	Level 1	PM-07-012-A	(2 vials)
K562 Cells in KG1 cells	Level 2	PM-07-012-B	(2 vials)
K562 Cells in KG1 cells	Level 3	PM-07-012-C	(2 vials)
K562 Cells in KG1 cells	Level 4	PM-07-012-D	(2 vials)

Eight samples (1 vial each of PM-07-011-A to D & PM-07-012-A to D) were resuspended using protocol A and the remaining 8 samples were resuspended using protocol B.

2.2.1.1 Resuspension protocol A

- Carefully open the glass vial trying to minimise the dispersion of any freeze dried material that has adhered to the rubber cap of the vial during transit.
- Gently resuspend freeze dried cells in 300µl nuclease free water
- Incubate for 20 min at room temperature
- Transfer the suspension to a 1.5ml tube
- Centrifuge at 15000*g* for 30min
- Discard the supernatant
- Resuspend the pellet in 1ml Trizol

2.2.1.2 Resuspension protocol B

- Carefully open the glass vial trying to minimise the dispersion of any freeze dried material that has adhered to the rubber cap of the vial during transit.
- Resuspend freeze dried cells directly in 1ml Trizol. Ensure that the material is fully lysed by repetitive pipetting.
- Transfer Trizol lysate to 1.5ml tube

2.2.2 Pre-freeze dried material

In addition to the freeze dried cells, a further eight samples were also analysed. These were prepared directly from the cell dilutions supplied to NIBSC but were lysed directly into Trizol prior to freeze drying. The samples were labelled as follows:

K562/ HL60 Level 1 Pre freeze dried
K562/ HL60 Level 2 Pre freeze dried
K562/ HL60 Level 3 Pre freeze dried
K562/ HL60 Level 4 Pre freeze dried

K562/ KG1 Level 1 Pre freeze dried
K562/ KG1 Level 2 Pre freeze dried
K562/ KG1 Level 3 Pre freeze dried
K562/ KG1 Level 4 Pre freeze dried

2.2.3 Analysis of samples

RNA was extracted from the pre-freeze dried samples and the samples resuspended using protocols A and B (24 samples total). The entire 1ml Trizol lysate was used for each RNA extraction. cDNA synthesis and RQ-PCR for *BCR-ABL* and the control gene(s) were performed for all samples using the laboratory's established method and standards to give absolute copy number and %*BCR-ABL* / control gene values.

The following data were recorded: date of RNA extraction, total µg of RNA, A_{260} / A_{280}, A_{260} / A_{230}, date of cDNA synthesis, final volume of cDNA reaction, volume of cDNA added to RQ-PCR, date of RQ-PCR, *BCR-ABL* transcript value (Ct value and copy number) control gene(s) transcript value (Ct value and copy number), *BCR-ABL* / control gene(s) (%) before conversion to IS, *BCR-ABL* / control gene(s) (%) converted to IS.

2.3 Large field trial evaluation (29th June – 7th September 2007)

Following the pilot analysis, laboratories participating in the International *BCR-ABL* Standardization Group were emailed to ask if they would like to participate in the full evaluation. Fourteen laboratories responded (see Appendix 1) from Europe (n=7), USA (n=4), Australia (n=1), Hong Kong (n=1) and Korea (n=1). Vials were distributed to all laboratories by courier at ambient temperature.

Twenty four 5ml glass vials were supplied labelled as follows:

K-562 Cells in HL-60 cells Level 1 PM-07-011-A (3 vials)
K-562 Cells in HL-60 cells Level 2 PM-07-011-B (3 vials)
K-562 Cells in HL-60 cells Level 3 PM-07-011-C (3 vials)
K-562 Cells in HL-60 cells Level 4 PM-07-011-D (3 vials)

K-562 Cells in KG-1 cells Level 1 PM-07-012-A (3 vials)
K-562 Cells in KG-1 cells Level 2 PM-07-012-B (3 vials)
K-562 Cells in KG-1 cells Level 3 PM-07-012-C (3 vials)
K-562 Cells in KG-1 cells Level 4 PM-07-012-D (3 vials)

The cells were resuspended using the following protocol:

- Carefully open the glass vial trying to minimise the dispersion of any freeze dried material that has adhered to the rubber cap of the vial during transit.
- Resuspend freeze dried cells in either 1ml Trizol or 600µl RLT Buffer (QIAGEN) depending on the RNA extraction method used in your laboratory.
- Ensure that the material is fully lysed by repetitive pipetting.
- Transfer lysate to 1.5ml tube

RNA was extracted in 3 batches on different days. For each batch, one vial of each *BCR-ABL* level for both KG1 and HL60 were used (figure 1). The entire 1ml Trizol or 600µl RLT lysate was used for each RNA extraction. Two cDNA reactions were performed for each of the 3 extracted RNA samples at each *BCR-ABL* level. These were performed on different days, giving a total of 48 cDNA samples. RQ-PCR for *BCR-ABL* and the control gene(s) was performed on each of the 6 cDNA samples for each *BCR-ABL* level in separate quantitative runs. Samples and data were analysed using the laboratory's established methods.

The following data were recorded: date of RNA extraction, total µg of RNA, A_{260} / A_{280}, A_{260} / A_{230}, date of cDNA synthesis, final volume of cDNA reaction, volume of cDNA added to RQ-PCR, date of RQ-PCR, *BCR-ABL* transcript value (Ct value and copy number) control gene(s) transcript value (Ct value and copy number), *BCR-ABL* / control gene(s) (%) before conversion to IS, *BCR-ABL* / control gene(s) (%) converted to IS.

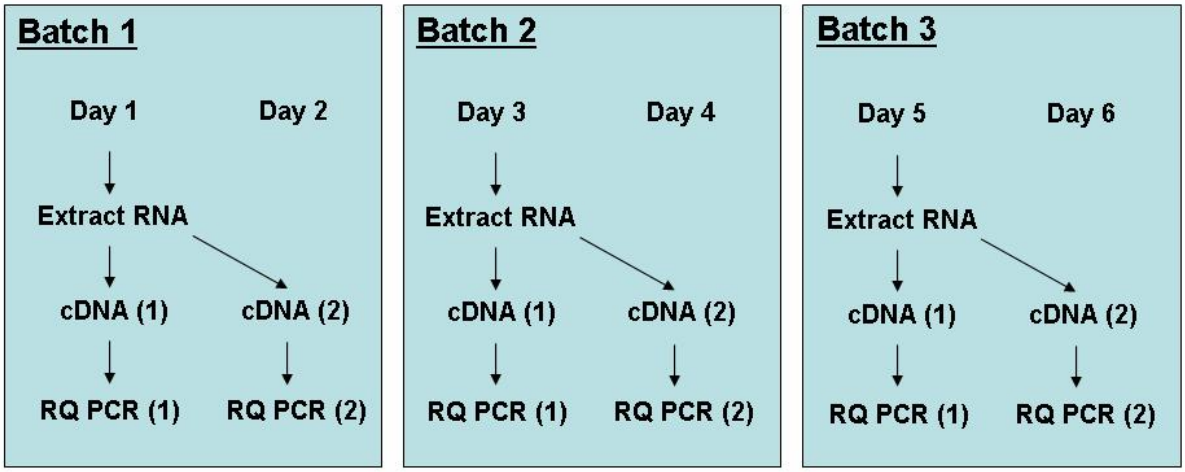


Figure 1: Schematic flow diagram showing suggested order of analysis for the minimal number of reverse transcription reactions and RQ-PCR runs.

2.4 Accelerated degradation studies

For accelerated degradation studies, 2 vials of each reference material were stored at 56°C, 45°C and 37°C and 4 vials of each material were stored at -20ºC. These samples will be analysed at 6 months post freeze drying.

3. RESULTS OF PILOT FIELD TRIAL

3.1 Amount of RNA Extracted

The total amount of RNA extracted from all samples was assessed by optical density and the mean and median for each resuspension protocol are shown in table 1. Extraction of RNA from samples stored for 2 months at room temperature was repeated by lab C to make a preliminary assessment of the stability of the samples.

Sample type	HL60 µg RNA (Mean)	HL60 µg RNA (Median)	KG1 µg RNA (Mean)	KG1 µg RNA (Median)
PBS reconstitution	8.4	8.0	4.6	2.9
Trizol resuspension	31.6	31.3	33.5	35.0
Pre freeze dried	33.6	32.6	40.5	41.1
2 months at room temp PBS reconstitution	8.3	8.7	3.4	1.8
2 months at room temp Trizol resuspension	36.0	36.1	39.1	39.5
2 months at room temp Pre freeze dried	35.5	35.3	44.5	43.5

Table 1: Mean amount of total RNA extracted from each cell line prior to freeze drying, when resuspended in water and lysed directly into Trizol.

The amount of RNA extracted using resuspension protocol A was significantly lower than either the amount of RNA extracted when the cells were lysed directly in Trizol (protocol B) or from the amount of RNA extracted from the pre-freeze dried material. There was no significant difference between the amount of RNA extracted using protocol B (direct Trizol lysis) and the amount of RNA extracted from the pre-freeze dried material (p=0.551, 2 sample T-test).

3.2 RQ-PCR for BCR-ABL and control genes

3.2.1 Absolute copy numbers (b3a2 and control gene transcripts)

The absolute copy numbers obtained from the RQ-PCR analysis of each control gene and b3a2 in the three testing labs are shown in table 2 (HL60) and table 3 (KG1). Results are shown for the freeze dried cells resuspended using protocol A (PBS), protocol B (Trizol), prior to freeze drying and for freeze dried cells tested at 2 months after storage at ambient temperature (*ABL* only).

3.2.1.1 ABL

There was no significant difference in the absolute *ABL* copies obtained using protocol A and B for HL60 or KG1 (p=0.284, p=0.777, respectively). The number of *ABL* copies in the pre-freeze dried material were not significantly different to sample prepared using protocols A and B (HL60: p= 0.174 and p= 0.072 respectively; KG1: p= 0.464 and p= 0.377, respectively)

3.2.1.2 BCR

There was no significant difference in the absolute *BCR* copies obtained using protocol A and B for HL60 or KG1 (p=0.127, p=0. 0.108, respectively). The number of *BCR* copies in the pre-freeze dried material were not significantly different to sample prepared using protocols A and B (HL60: p= 0.186 and p= 0.401 respectively; KG1: p= 0.190 and p= 0.250, respectively)

3.2.1.3 GUSB

There was no significant difference in the absolute *GUSB* copies obtained using protocol A and B for HL60 or KG1 (p= 0.259, p= 0.827 respectively). The number of *GUSB* copies in the pre-freeze dried material were not significantly different to sample prepared using protocols A and B for KG1 and protocol B for HL60 (KG1: p= 0.443 and p= 0.537, respectively; HL60 p=0.216). There was a statistically different number of copies of *GUSB* determined when comparing the pre freeze dried HL60 material and HL60 samples prepared using protocol A (p= 0.026).

a)

Lab ID	*ABL* (PBS)	*ABL* (Trizol)	*ABL* (Pre freeze dry)
Lab A	8.58E+04	1.86E+05	1.12E+05
Lab B	6.51E+04	2.06E+04	1.26E+04
Lab C	9.71E+04	1.18E+05	4.88E+04
Lab C 2 months	4.01E+04	5.86E+04	1.01E+05

b)

Lab ID	*BCR* (PBS)	*BCR* (Trizol)	*BCR* (Pre freeze dry)
Lab B	1.97E+05	8.36E+04	6.44E+04
Lab C	2.94E+04	2.21E+04	NT

c)

Lab ID	*GUSB* (PBS)	*GUSB* (Trizol)	*GUSB* (Pre freeze dry)
Lab A	3.66E+05	3.78E+05	2.77E+05
Lab B	3.94E+05	2.19E+05	1.95E+05
Lab C	1.31E+05	1.16E+05	7.20E+04

d)

Lab ID	Lab A	Lab B	Lab C	Lab C 2 months
Level 1 PBS	2.05E+04	4.01E+04	2.70E+04	1.14E+04
Level 1 Trizol	4.71E+04	1.58E+04	4.34E+04	1.69E+04
Level 1 (Pre freeze dry)	3.06E+04	1.16E+04	7.23E+03	3.09E+04
Level 2 PBS	2.23E+03	2.58E+03	2.89E+03	1.03E+03
Level 2 Trizol	5.36E+03	1.28E+03	3.32E+03	1.57E+03
Level 2 (Pre freeze dry)	3.14E+03	7.59E+02	6.67E+02	3.26E+03
Level 3 PBS	2.15E+02	1.66E+02	4.23E+02	1.40E+02
Level 3 Trizol	4.75E+02	7.10E+01	3.95E+02	1.25E+02
Level 3 (Pre freeze dry)	3.00E+02	5.06E+01	1.06E+02	2.90E+02
Level 4 PBS	1.10E+01	2.15E+01	2.95E+01	1.36E+01
Level 4 Trizol	3.60E+01	7.43E+00	5.96E+01	1.04E+01
Level 4 (Pre freeze dry)	3.70E+01	2.33E+00	5.60E+01	3.70E+01

Table 2: Absolute copy number of control gene transcripts obtained for the HL60 / K562 cell line mixes a) *ABL*, b) *BCR*, c) *GUSB* and d) b3a2. NT= not tested.

a)

Lab ID	*ABL* (PBS)	*ABL* (Trizol)	*ABL* (Pre freeze dry)
Lab A	1.64E+05	2.18E+05	1.48E+05
Lab B	5.53E+04	1.18E+04	1.17E+04
Lab C	5.78E+04	7.48E+04	5.68E+04
Lab C 2 months	2.74E+04	9.01E+04	1.11E+05

b)

Lab ID	*BCR* (PBS)	*BCR* (Trizol)	*BCR* (Pre freeze dry)
Lab B	5.24E+05	1.79E+05	1.51E+05
Lab C	4.80E+04	4.84E+04	NT

c)

Lab ID	*GUSB* (PBS)	*GUSB* (Trizol)	*GUSB* (Pre freeze dry)
Lab A	1.96E+05	2.47E+05	1.98E+05
Lab B	2.62E+05	1.63E+05	1.58E+05
Lab C	3.50E+04	5.83E+04	5.23E+04

d)

Lab ID	Lab A	Lab B	Lab C	Lab C 2 months
Level 1 PBS	6.34E+04	5.31E+04	8.11E+03	3.32E+04
Level 1 Trizol	6.98E+04	7.60E+03	3.04E+04	2.78E+04
Level 1 (Pre freeze dry)	6.87E+04	9.58E+03	3.44E+04	7.84E+04
Level 2 PBS	8.22E+03	4.72E+03	1.21E+03	2.56E+02
Level 2 Trizol	1.11E+04	1.12E+03	3.43E+03	3.65E+03
Level 2 (Pre freeze dry)	7.85E+03	8.23E+02	1.28E+03	9.49E+03
Level 3 PBS	6.00E+02	4.61E+02	6.64E+02	2.67E+01
Level 3 Trizol	9.10E+02	1.23E+02	2.95E+02	3.39E+02
Level 3 (Pre freeze dry)	3.50E+02	9.17E+01	3.01E+02	7.14E+02
Level 4 PBS	7.00E+01	5.32E+01	3.25E+01	2.89E+00
Level 4 Trizol	1.10E+02	1.34E+01	3.73E+01	3.28E+01
Level 4 (Pre freeze dry)	7.50E+01	9.61E+00	2.98E+01	3.82E+01

Table 3: Absolute copy number of control gene transcripts obtained for the KG1 / K562 cell line mixes a) *ABL*, b) *BCR*, c) *GUSB* and d) b3a2. NT= not tested.

3.2.2 % BCR-ABL / control gene(s)

Table 4 (HL60) and table 5 (KG1) show the % *BCR-ABL* / control gene levels for each reference material after testing in the three laboratories. Mean values are shown for the freeze dried cells resuspended using protocol A (PBS), protocol B (Trizol) and prior to freeze drying and for freeze dried cells tested at 2 months after storage at ambient temperature.

	ABL				***BCR***		***GUSB***		
	Lab A	**Lab B**	**Lab C**	**Lab C 2 mths**	**Lab B**	**Lab C**	**Lab A**	**Lab B**	**Lab C**
L1 PBS	18.430	35.907	34.929	24.204	15.771	45.421	5.778	10.92	13.869
L2 PBS	2.520	3.689	3.537	3.344	1.320	5.704	0.547	0.517	1.237
L3 PBS	0.251	0.374	0.430	0.283	0.092	0.377	0.056	0.039	0.116
L4 PBS	0.019	0.063	0.023	0.041	0.014	0.041	0.003	0.008	0.010
L1 Trizol	21.258	47.884	31.679	22.624	17.086	92.308	13.183	7.962	11.240
L2 Trizol	2.596	5.223	3.132	2.565	1.203	8.168	1.289	0.459	1.011
L3 Trizol	0.273	0.473	0.411	0.220	0.097	0.665	0.128	0.033	0.065
L4 Trizol	0.025	0.076	0.044	0.025	0.012	0.066	0.010	0.004	0.008
L1 Pre FD	27.586	60.933	42.035	32.605	16.044	NT	12.288	6.058	10.688
L2 Pre FD	2.892	6.467	4.904	2.991	1.144	NT	1.076	0.289	0.903
L3 Pre FD	0.286	0.518	0.373	0.241	0.088	NT	0.107	0.026	0.027
L4 Pre FD	0.030	0.024	0.041	0.029	0.004	NT	0.013	0.002	0.006

Table 4: % *BCR-ABL* / control gene mean values obtained for the HL60 / K562 cell line mixes pre freeze drying and following the two different resuspension protocols for labs A, B and C (NT= not tested; Pre FD = pre freeze dry; L1 – L4 = levels 1 -4 *BCR-ABL*)

	ABL				*BCR*		*GUSB*		
	Lab A	**Lab B**	**Lab C**	**Lab C 2 mths**	**Lab B**	**Lab C**	**Lab A**	**Lab B**	**Lab C**
L1 PBS	28.655	110.23	44.807	34.837	12.069	33.216	31.418	21.14	33.587
L2 PBS	4.717	8.661	6.173	5.300	0.926	3.956	3.914	1.913	5.068
L3 PBS	0.459	0.820	0.458	0.485	0.078	0.402	0.325	0.187	0.765
L4 PBS	0.054	0.085	0.067	0.075	0.010	0.039	0.037	0.018	0.077
L1 Trizol	29.910	71.174	33.115	24.174	4.739	78.592	31.193	4.701	32.415
L2 Trizol	4.939	10.402	4.592	4.320	0.666	4.543	3.871	0.736	4.487
L3 Trizol	0.422	1.013	0.500	0.428	0.061	0.839	0.389	0.070	0.564
L4 Trizol	0.055	0.100	0.051	0.040	0.007	0.083	0.045	0.008	0.070
L1 Pre FD	34.813	52.772	35.796	36.129	5.395	NT	31.727	6.589	39.431
L2 Pre FD	5.292	11.114	5.818	5.717	0.752	NT	3.676	0.552	4.487
L3 Pre FD	0.460	0.961	0.565	0.428	0.065	NT	0.336	0.054	0.612
L4 Pre FD	0.044	0.083	0.053	0.061	0.005	NT	0.029	0.006	0.045

Table 5: % *BCR-ABL* / control gene mean values obtained for the KG1 / K562 cell line mixes pre freeze drying and following the two different resuspension protocols for labs A, B and C (NT= not tested; Pre FD = pre freeze dry; L1 – L4 = levels 1 -4 *BCR-ABL*)

4. RESULTS OF LARGE FIELD TRIAL

14 laboratories participated in the field trial and all labs returned data; 7 participants from Europe, 4 from the United States of America, 2 from Asia and 1 from Australasia (Appendix A).

4.1 Methodologies used

4.1.1 Control genes analysed

Four control genes were analysed in this study: *ABL* (12 labs), *BCR* (6 labs), *GUSB* (8 labs) and *G6PD* (1 lab). Eight labs tested the samples using more than one control gene.

4.1.2 RQ-PCR machines used

Nine different type of RQ-PCR machines were used in this study: ABI 7000 (3 labs), ABI 7500 (2 labs), ABI 7700 (1 lab), ABI 5700 (1 lab), Corbett RotorGene 6000 (1 lab), Roche LightCycler 1.5 (1 lab), Roche LightCycler 2.0 (3 labs), Roche LightCycler 480 (1 lab), Stratagene MX3000P (1 lab).

4.1.3 RQ-PCR methods

4.1.3.1 RNA Extraction methods

Two RNA extraction methods were used; 9 labs used Trizol (Invitrogen) and 5 used RNeasy kits (QIAGEN).

4.1.3.2 RT-PCR method cited

Eight of the participants cited Gabert et al. (2003)[1] as one of the references used as their RQ-PCR protocol. Other references cited are listed in the reference section [7 – 13].

4.1.3.3 RT-PCR method and primers used

Twelve labs reported the use of random hexamers for reverse transcription. The final concentrations ranged from 6µM - 125µM. One lab used a proprietary method and another lab did not provide information. The amount of RNA added to the cDNA reactions varied from 0.3 – 10 µg.

4.1.3.4 Standard type and source

12 labs used plasmid DNA as standards and these were either made in house or obtained from another lab (10) or purchased from Ipsogen (3). One lab used cDNA standards derived from the K562 cell line and another did not run standards but used a ΔΔCt method for calculating % *BCR-ABL*.

4.2 Field trial data analysis

4.2.1 RNA Extraction

The mean and median amount of RNA extracted from each reference material is shown in table 6.

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**

Table 6: Mean and median amounts of RNA extracted for each reference material (n=14)

4.2.2 Mean copy numbers obtained for control genes

The mean copy number values obtained for each control gene in each lab are given in table 7. The values have not been corrected for variation in the amount of RNA extracted and used in the cDNA reaction or amount of cDNA added to the RQ-PCR and therefore the data are not comparable between labs. For the labs that tested more than one control gene the relative amount of each control gene in relation to *ABL* are shown in figure 2.

Lab ID	Cell line mix	*ABL*	*BCR*	*GUSB*
1	**HL60**	NT	5.78E+05	NT
1	**KG1**	NT	1.25E+06	NT
2	**HL60**	1.16E+05	NT	NT
2	**KG1**	9.87E+04	NT	NT
3	**HL60**	1.77E+04	NT	3.92E+04
3	**KG1**	2.00E+04	NT	2.40E+04
4	**HL60**	3.49E+04	NT	1.06E+05
4	**KG1**	4.09E+04	NT	6.41E+04
5	**HL60**	2.84E+05	NT	NT
5	**KG1**	4.78E+05	NT	NT
6	**HL60**	5.63E+05	4.53E+05	8.20E+05
6	**KG1**	6.74E+05	1.03E+06	5.65E+05
7	**HL60**	3.30E+05	NT	NT
7	**KG1**	4.26E+05	NT	NT
8	**HL60**	2.70E+05	NT	5.21E+05
8	**KG1**	2.94E+05	NT	3.45E+05
9	**HL60**	3.38E+04	6.71E+04	2.34E+05
9	**KG1**	3.61E+04	1.36E+05	1.62E+05
10	**HL60**	1.56E-01	8.30E-01	8.81E-01
10	**KG1**	1.67E-01	1.73E+00	5.39E-01
11	**HL60**	1.00E+05	NT	NT
11	**KG1**	1.02E+05	NT	NT
12	**HL60**	4.13E+05	NT	NT
12	**KG1**	5.20E+05	NT	NT
13	**HL60**	6.10E+04	2.16E+04	7.15E+04
13	**KG1**	6.15E+04	5.14E+04	4.28E+04

Table 7: Mean copy number values obtained for each control gene in each lab (NT = not tested). Copy numbers for *G6PD* were not given.

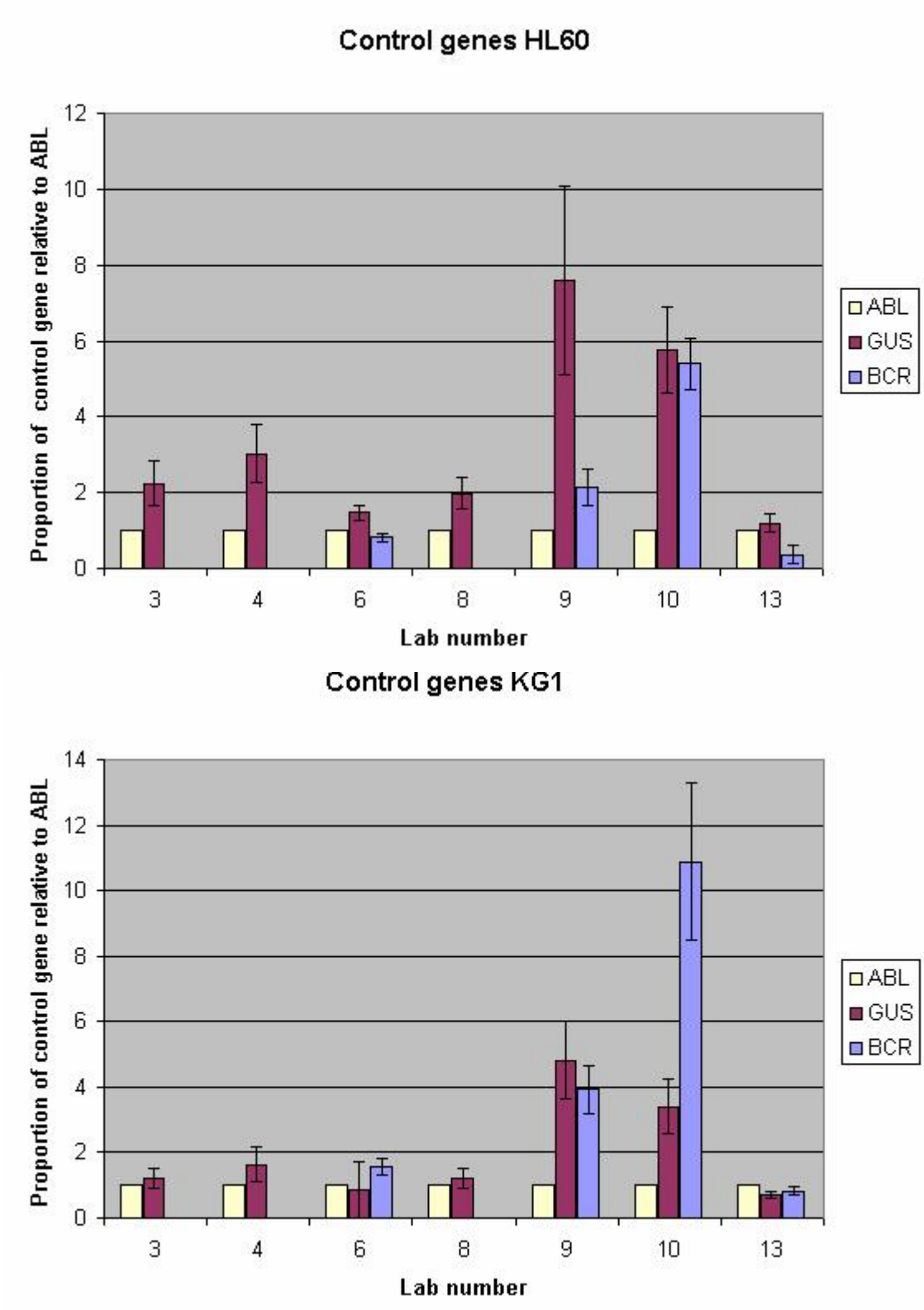


Figure 2: The relative amounts of each control gene in relation to *ABL* for laboratories who tested more than one control gene

4.2.3 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 8 (*ABL* and *G6PD*) and 9 (*BCR* and *GUSB*). There was no statistical difference between the CVs obtained for HL60 and KG1 at any level of *BCR-ABL* (2-sample t-test).

HL60 ABL	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	17.738	1.247	7.03	2.014	0.153	7.58	0.181	0.040	22.12	0.015	0.004	27.36
3	27.252	8.432	30.94	1.958	0.339	17.31	0.227	0.049	21.79	0.019	0.006	29.12
4	18.789	4.409	23.47	3.110	1.056	33.95	0.245	0.046	18.93	0.039	0.012	31.14
5	16.276	4.219	25.92	1.956	0.380	19.41	0.189	0.045	23.86	0.019	0.006	29.70
6	51.783	6.279	12.13	6.224	0.665	10.69	0.622	0.028	4.52	0.069	0.022	31.50
7	22.533	4.875	21.64	2.433	0.594	24.42	0.275	0.046	16.86	0.022	0.012	53.96
8	14.038	2.430	17.31	1.566	0.128	8.20	0.142	0.044	30.85	0.012	0.005	42.85
9	35.950	9.591	26.68	4.227	0.600	14.19	0.464	0.056	12.10	0.058	0.024	41.62
10	15.898	1.250	7.86	1.926	0.403	20.92	0.170	0.036	21.43	0.018	0.003	17.82
11	19.149	2.065	10.78	2.253	0.421	18.67	0.232	0.053	22.98	0.025	0.007	27.12
12	22.177	5.954	26.85	1.923	0.359	18.65	0.230	0.074	32.19	0.024	0.004	15.12
13	36.558	7.425	20.31	5.175	1.790	34.60	0.467	0.128	27.41	0.039	0.007	18.31

KG1 ABL	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
2	20.055	1.546	7.709	2.178	0.202	9.290	0.256	0.070	27.485	0.034	0.011	32.414
3	31.103	5.868	18.866	2.719	0.442	16.270	0.382	0.057	14.856	0.028	0.004	13.180
4	37.853	8.548	22.582	6.426	1.671	26.011	0.676	0.114	16.931	0.083	0.015	18.616
5	23.466	6.220	26.507	3.496	0.762	21.807	0.333	0.051	15.364	0.025	0.003	13.776
6	71.146	6.720	9.445	10.741	2.311	21.517	1.139	0.140	12.260	0.113	0.018	16.175
7	29.737	7.127	23.968	4.225	0.725	17.163	0.435	0.080	18.319	0.037	0.015	41.060
8	21.744	4.268	19.628	2.745	0.486	17.715	0.256	0.054	20.933	0.024	0.007	30.556
9	56.205	6.954	12.373	7.707	1.436	18.637	0.803	0.128	15.941	0.062	0.020	32.279
10	25.791	4.248	16.472	2.397	0.579	24.143	0.531	0.182	34.163	0.048	0.006	13.639
11	22.849	3.853	16.863	3.487	0.575	16.492	0.363	0.076	21.016	0.039	0.008	19.868
12	32.158	11.999	37.312	3.739	1.086	29.038	0.339	0.124	36.512	0.032	0.012	37.685
13	-	-	-	6.036	0.631	10.457	0.662	0.045	6.811	0.072	0.011	15.831

HL60 G6PD	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
14	25.6667	3.0111	11.7315	2.5500	0.4037	15.8327	0.2083	0.0611	29.3416	0.0186	0.0188	100.9536

KG1 G6PD	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
14	31.1667	6.8240	21.8951	3.0333	0.2160	7.1217	0.2417	0.0902	37.3256	0.0372	0.0202	54.3316

Table 8: Mean, SD and CV values for %*BCR-ABL* / A*BL* and %*BCR-ABL* / *G6PD* for each laboratory for the HL60 / K562 and KG1 / K562 reference materials. There was no statistical difference between CVs for the HL60 and KG1 cell line mixes. (2 sample t-test: HL60 CV vs KG1 CV for *ABL* Level 1, 2, 3 and 4: p=0.999, 0.999, 0.724, 0.142).

HL60 BCR	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	53.167	19.498	36.673	5.983	1.440	24.067	0.557	0.127	22.827	0.047	0.023	50.102
6	75.149	11.206	14.912	7.020	1.048	14.922	0.750	0.100	13.386	0.084	0.029	33.944
9	21.215	3.693	17.405	2.141	0.494	23.066	0.226	0.027	12.058	0.021	0.009	43.685
10	3.474	0.214	6.167	0.310	0.060	19.199	0.032	0.007	22.359	0.003	0.000	14.718
13	106.505	23.691	22.244	10.881	1.043	9.585	1.113	0.194	17.436	0.100	0.022	21.747

KG1 BCR	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	43.167	12.797	29.646	4.867	1.449	29.767	0.558	0.199	35.649	0.083	0.037	44.036
6	61.661	9.592	15.556	6.585	1.598	24.273	0.656	0.098	15.013	0.066	0.012	18.390
9	17.308	2.229	12.880	1.819	0.243	13.340	0.200	0.049	24.667	0.016	0.004	27.488
10	3.302	0.249	7.529	0.224	0.041	18.426	0.041	0.009	22.603	0.004	0.001	15.235
13	-	-	-	8.057	1.170	14.523	0.735	0.091	12.351	0.077	0.017	22.535

HL60 GUS	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	11.851	2.296	19.376	0.773	0.198	25.598	0.113	0.035	31.222	0.010	0.002	22.989
4	7.508	2.061	27.448	0.938	0.199	21.226	0.081	0.014	17.433	0.014	0.006	46.639
6	41.828	4.353	10.406	3.832	0.546	14.235	0.411	0.024	5.853	0.046	0.016	33.698
8	9.506	3.266	34.356	0.734	0.085	11.556	0.064	0.014	21.951	0.007	0.004	57.179
9	6.967	2.031	29.150	0.609	0.120	19.636	0.063	0.009	14.285	0.006	0.003	54.843
10	3.043	0.605	19.874	0.304	0.057	18.788	0.031	0.011	33.835	0.003	0.000	14.940
13	33.303	3.915	11.757	2.880	0.789	27.408	0.336	0.031	9.242	0.025	0.005	20.769

KG1 GUS	Level 1			Level 2			Level 3			Level 4		
Lab ID	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
3	38.931	10.718	27.530	1.963	0.334	17.006	0.321	0.063	19.743	0.019	0.004	18.407
4	29.701	7.599	25.586	4.845	2.299	47.450	0.432	0.136	31.471	0.043	0.008	18.713
6	105.357	11.210	10.640	12.177	1.400	11.494	1.238	0.124	10.025	0.121	0.021	17.377
8	25.474	6.899	27.083	2.245	0.398	17.739	0.194	0.041	21.073	0.018	0.006	32.046
9	12.817	2.716	21.186	1.705	0.295	17.298	0.183	0.081	44.436	0.013	0.004	34.931
10	9.559	0.772	8.079	0.757	0.156	20.575	0.134	0.042	31.741	0.013	0.003	20.998
13	-	-	-	7.624	0.711	9.325	0.883	0.171	19.367	0.088	0.024	27.788

Table 9: Mean, SD and CV values for %*BCR-ABL / BCR* and %*BCR-ABL / GUSB* for each laboratory for the HL60 / K562 and KG1 / K562 reference materials There was no statistical difference between CVs for the HL60 and KG1 cell line mixes. (2 sample t-test: HL60 CV vs KG1 CV for *BCR* Level 1, 2, 3 and 4: p=0.671, 0.657, 0.377, 0.409; HL60 CV vs KG1 CV for *GUSB* Level 1, 2, 3 and 4: p=0.728, 0.949, 0.305, 0.139).

4.2.4 Linear regression

The linear regression of the log transformed data obtained from all labs are summarised in figures 3 (*ABL*), 4 (*BCR*) and 5 (*GUSB*). Data were plotted against reference material values which were assigned as 10% (level 1), 1% (level 2), 0.1% (level 3) and 0.01% (level 4) *BCR-ABL* / Control Gene (this is an arbitrary designation to illustrate the principal that these reagents can be used to normalize variables between laboratories. It does not mean that these reagents were actually 10%, 1% etc. on the IS). The regression equations for the log transformed data for each cell line and each control gene are given in table 10 and the individual regression plots for each lab are shown in appendix B.

Lab ID	HL60 *ABL*	KG1 *ABL*
2	Log(y)= 0.2596 + 1.025 log(x)	Log(y)= 0.3545 + 0.9295 log(x) ♠
3	Log(y)= 0.3564 + 1.041 log(x)	Log(y)= 0.4863 + 0.9988 log(x)
4	Log(y)= 0.3915 + 0.9168 log(x) ♠	Log(y)= 0.7261 + 0.8953 log(x) ♠
5	Log(y)= 0.2494 + 0.9786 log(x)	Log(y)= 0.4440 + 0.9881 log(x)
6	Log(y)= 0.7644 + 0.9677 log(x)	Log(y)= 0.9645 + 0.9373 log(x) ♠
7	Log(y)= 0.3748 + 0.9993 log(x)	Log(y)= 0.5590 + 0.9726 log(x)
8	Log(y)= 0.1507 + 1.031 log(x)	Log(y)= 0.3821 + 0.9939 log(x)
9	Log(y)= 0.6129 + 0.9359 log(x) ♠	Log(y)= 0.8228 + 0.9884 log(x)
10	Log(y)= 0.2353 + 0.9884 log(x)	Log(y)= 0.4835 + 0.8856 log(x) ♠
11	Log(y)= 0.3294 + 0.9650 log(x)	Log(y)= 0.4757 + 0.9296 log(x) ♠
12	Log(y)= 0.3290 + 0.9766 log(x)	Log(y)= 0.5100 + 1.005 log(x)
13*	Log(y)= 0.6084 + 0.9891 log(x)	Log(y)= 0.7813 + 0.9615 log(x) ♠

Lab ID	HL60 *BCR*	KG1 *BCR*
1^	Log(y)= 0.7267 + 1.031 log(x)	Log(y)= 0.6970 + 0.9103 log(x) ♠
6	Log(y)= 0.8674 + 0.9881 log(x)	Log(y)= 0.8030 + 0.9912 log(x)
9	Log(y)= 0.3288 + 1.003 log(x)	Log(y)= 0.2495 + 1.012 log(x)
10	Log(y)= -0.4852 + 1.002 log(x)	Log(y)= -0.5089 + 0.9528 log(x)
13*	Log(y)= 1.028 + 1.007 log(x)	Log(y)= 0.8962 + 1.012 log(x)

Lab ID	HL60 *GUSB*	KG1 *GUSB*
3	Log(y)= 0.00401 + 1.005 log(x)	Log(y)= 0.4511 + 1.069 log(x)
4	Log(y)= -0.07129 + 0.9300 log(x) ♠	Log(y)= 0.5730 + 0.9570 log(x)
6	Log(y)= 0.6092 + 0.9902 log(x)	Log(y)= 1.060 + 0.9813 log(x)
8	Log(y)= -0.1189 + 1.062 log(x)	Log(y)= 0.3483 + 1.055 log(x) ♠
9	Log(y)= -0.1999 + 1.025 log(x)	Log(y)= 0.1706 + 1.001 log(x)
10	Log(y)= -0.5144 + 0.9880 log(x)	Log(y)= -0.01101 + 0.9329 log(x) ♠
13*	Log(y)= 0.4990 + 1.035 log(x)	Log(y)= 0.8904 + 0.9693 log(x)

Lab ID	HL60 *G6PD*	KG1 *G6PD*
14	Log(y)= 0.3725 + 1.052 log(x)	Log(y)= 0.4687 + 0.9926 log(x)

Table 10: Linear regression equations of log transformed %*BCR-ABL* / Control gene data for each lab and cell line. * Level 1 KG1 not tested. ^ Analysis of repeat samples. ♠ Gradient statistically different to reference (CI 95%)

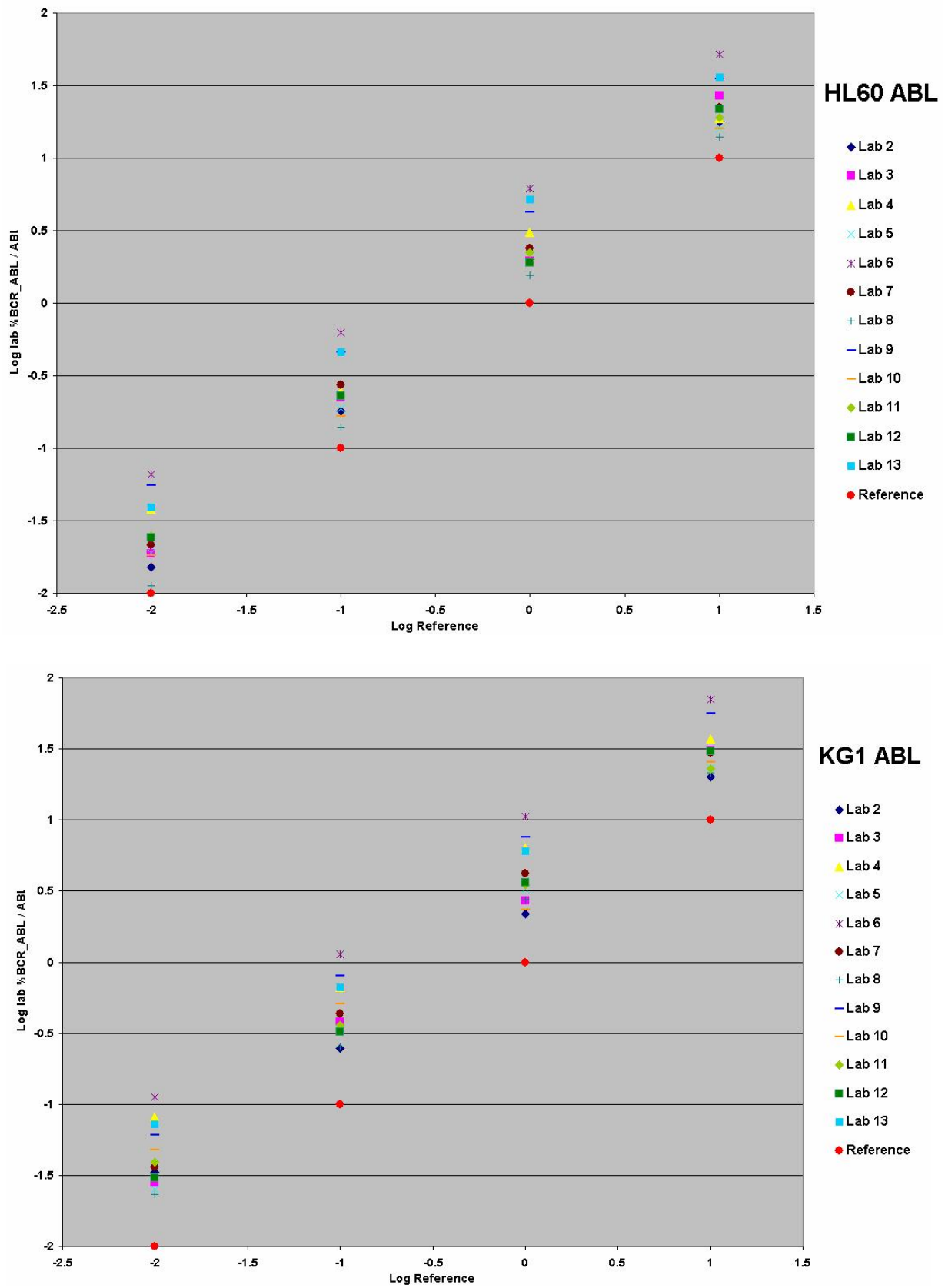
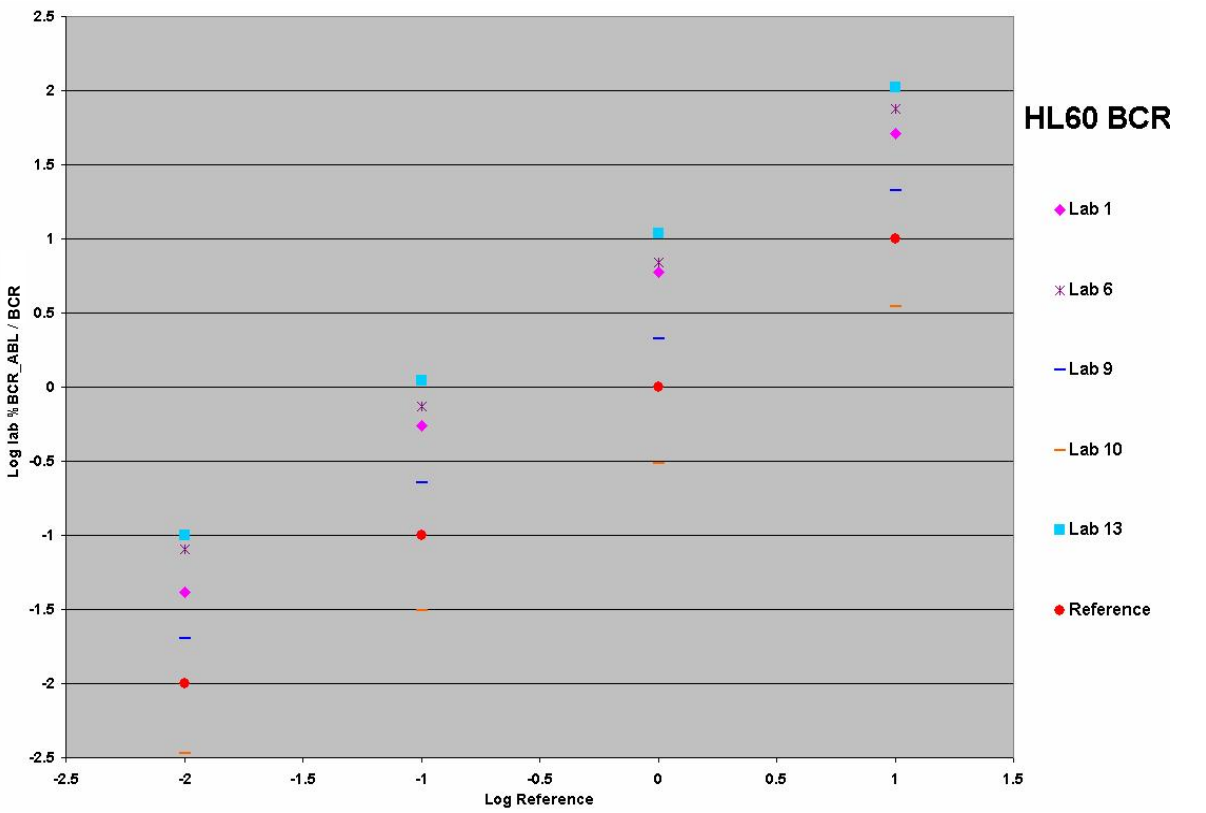


Figure 3: Summary of the linear regressions of the log transformed data obtained from all labs testing *BCR-ABL / ABL* in HL60 and KG1 reference material. The reference materials have been assigned values of 10%, 1%, 0.1% and 0.01% *BCR-ABL / ABL*.



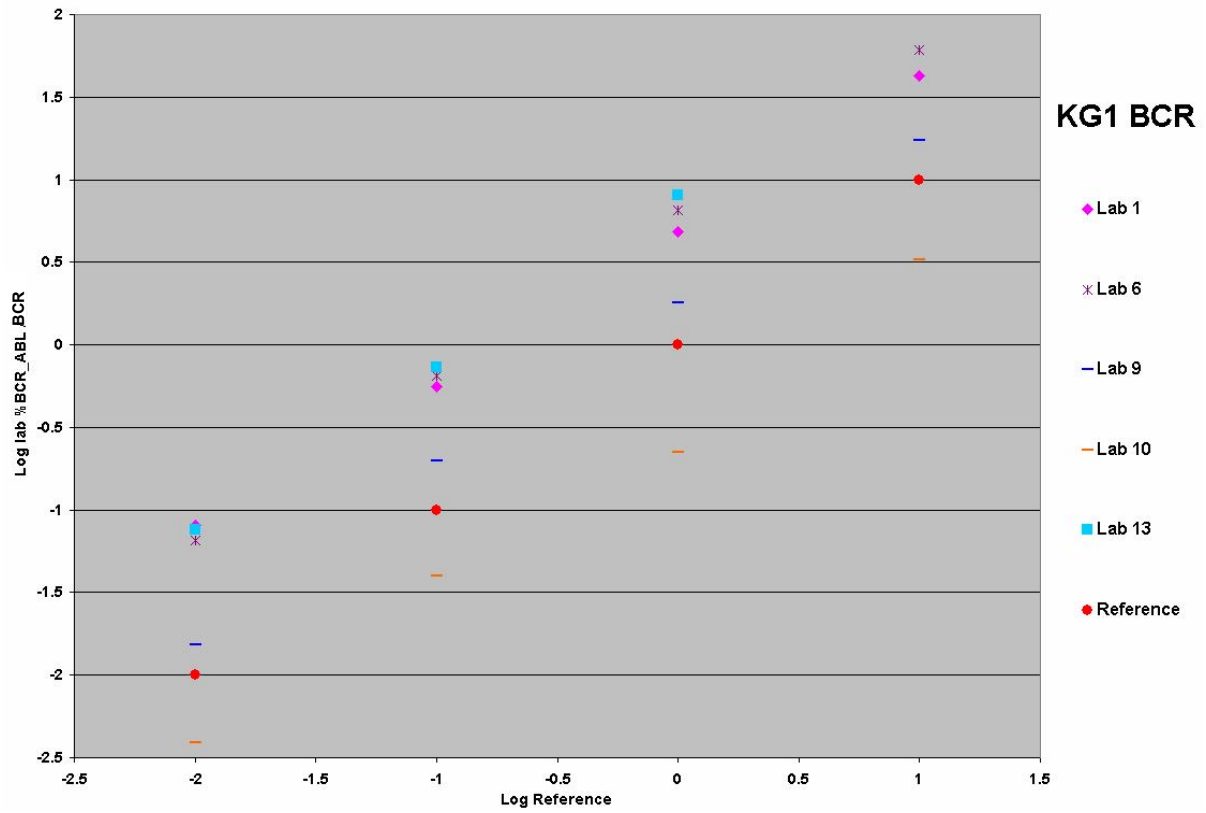
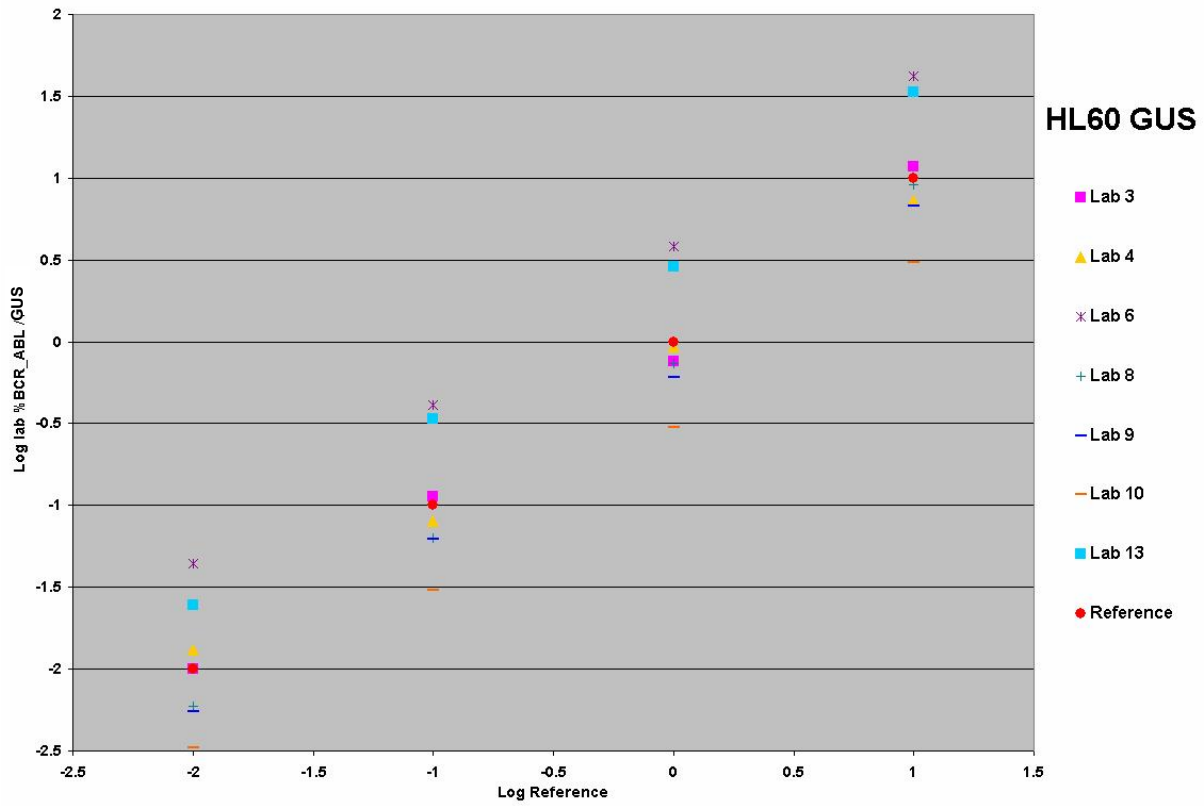


Figure 4: Summary of the linear regressions of the log transformed data obtained from all labs testing *BCR-ABL / BCR* in HL60 and KG1 reference material. The reference materials have been assigned values of 10%, 1%, 0.1% and 0.01% *BCR-ABL / BCR*.



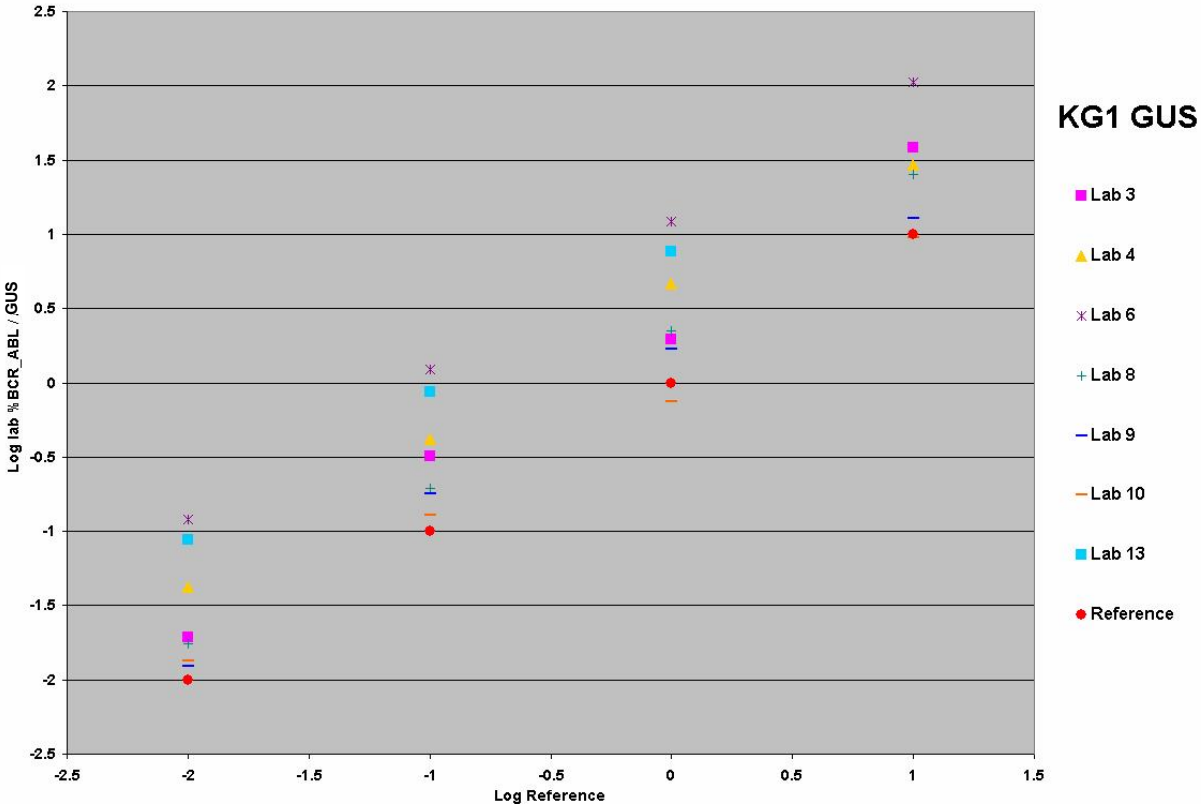


Figure 5: Summary of the linear regressions of the log transformed data obtained from all labs testing *BCR-ABL / GUSB* in HL60 and KG1 reference material. The reference materials have been assigned values of 10%, 1%, 0.1% and 0.01% *BCR-ABL / GUSB*.

4.3 Use of prototype reference materials for standardisation

Using the linear regression equations shown in table 10 it is possible to use the reference material to generate a conversion / calibration factor which would allow all laboratories to report %*BCR-ABL* / control gene results on a uniform scale.

Using the HL60 *ABL* data as an example the data have been standardised using two methods:

4.3.1 Correct Y to scale of X by inverting the regression

Log transformed data from twelve laboratories were calibrated to the reference standards using the slope and intercepts from the regression equations for HL60 *ABL*. The equation used to correct the lab data to the standards or 'reference data set' was:

$$Y_{corrected} = (Y - a) / b$$

Where a = intercept
b = slope

The graph of all the corrected lab results for log transformed data of %*BCR-ABL / ABL* (HL60) are shown in figure 6 (uncorrected data can be seen in figure 3, top panel)

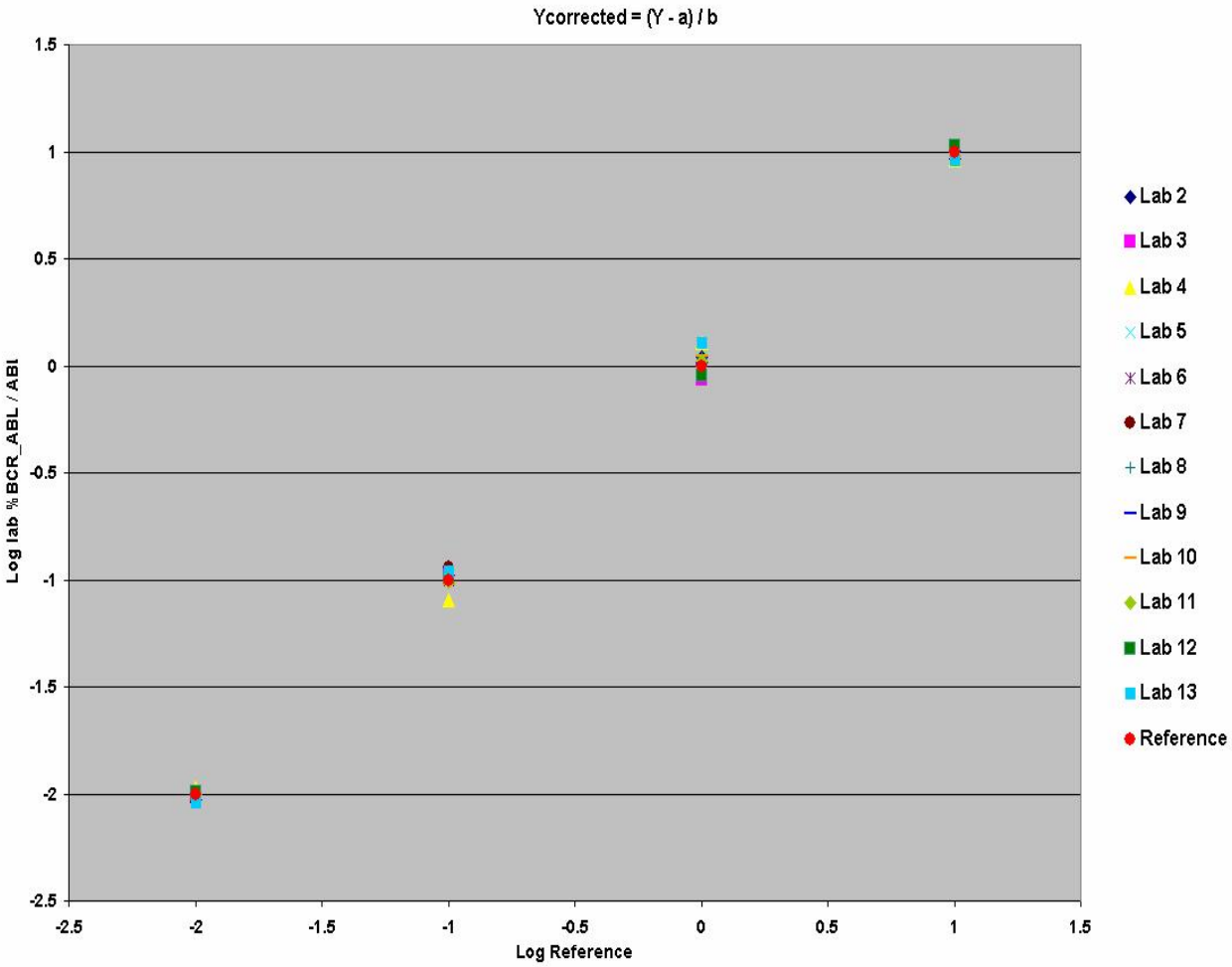


Figure 6: Log transformed data for HL60 %*BCR-ABL / ABL* calibrated to reference standards using the slope and intercepts from the regression equations.

4.3.2 Using IS conversion factor method from Adelaide

Log transformed data from twelve laboratories were calibrated to the reference standards using the slope and intercepts from the regression equations for HL60 *ABL*. The process used to correct the lab data to the standards or 'reference data set' was:

$$\log y = (b \times \log MMR_{IS}) + a$$

$$\text{Conversion factor} = MMR_{IS} / \text{antilog } y$$

Where MMR_{IS} = 0.1
a = intercept
b = slope

The graph of all the corrected lab results for log transformed data of %*BCR-ABL / ABL* (HL60) is shown in figure 7 (uncorrected data can be seen in figure 3, top panel).

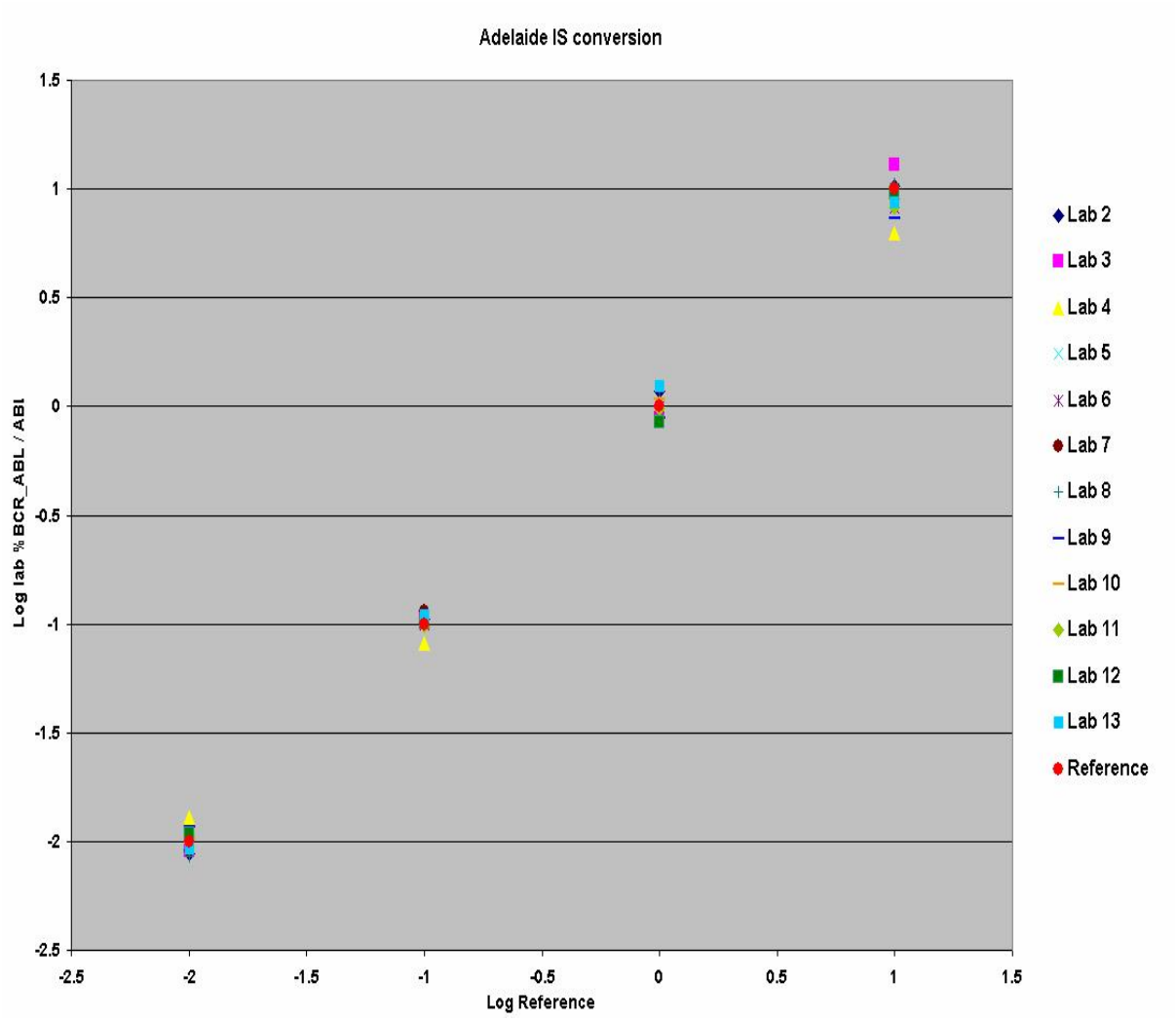


Figure 7: Log transformed data for HL60 %*BCR-ABL / ABL* calibrated to reference standards using the IS conversion factor as calculated by the Adelaide laboratory

4.3.3 Converted % BCR-ABL / ABL data (HL60)

The laboratory data and resulting log transformed and unconverted data for each standardisation method are shown below:

4.3.3.1 Un- transformed data

Original Untransformed Data (%BCR/ABL / ABL)

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
10	17.7240	26.8587	18.6682	15.9611	51.7505	22.3774	13.9216	35.0439	15.8705	19.1380	21.6182	36.3119
1	2.0095	1.9535	3.0944	1.9361	6.2128	2.4020	1.5635	4.2235	1.9137	2.2457	1.9136	5.1747
0.1	0.1800	0.2247	0.2445	0.1865	0.6218	0.2733	0.1404	0.4615	0.1671	0.2281	0.2287	0.4584
0.01	0.0151	0.0187	0.0383	0.0190	0.0663	0.0215	0.0113	0.0556	0.0182	0.0249	0.0244	0.0390

Ycorrected data

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
10	9.2223	10.7260	9.1081	9.4301	9.5765	9.4557	9.1861	9.8975	9.4759	9.7061	10.7132	9.1651
1	1.1026	0.8649	1.2825	1.0923	1.0712	1.0134	1.1018	1.0319	1.1146	1.0538	0.8948	1.2784
0.1	0.1047	0.1083	0.0805	0.1000	0.0993	0.1151	0.1064	0.0969	0.0946	0.0985	0.1016	0.1103
0.01	0.0093	0.0100	0.0106	0.0097	0.0098	0.0091	0.0092	0.0101	0.0100	0.0099	0.0103	0.0091

IS corrected data

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
10	10.3267	12.9923	6.2575	8.5559	8.2645	9.4256	10.5679	7.3725	8.9886	8.2698	9.6033	8.7247
1	1.1708	0.9449	1.0372	1.0378	0.9922	1.0118	1.1869	0.8885	1.0839	0.9704	0.8501	1.2433
0.1	0.1049	0.1087	0.0819	0.1000	0.0993	0.1151	0.1066	0.0971	0.0947	0.0986	0.1016	0.1101
0.01	0.0088	0.0091	0.0128	0.0102	0.0106	0.0091	0.0086	0.0117	0.0103	0.0108	0.0108	0.0094

4.3.3.2 Log transformed data

Original log transformed data

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
1	1.2486	1.4291	1.2711	1.2031	1.7139	1.3498	1.1437	1.5446	1.2006	1.2819	1.3348	1.5600
0	0.3031	0.2908	0.4906	0.2869	0.7933	0.3806	0.1941	0.6257	0.2819	0.3514	0.2819	0.7139
-1	-0.7448	-0.6484	-0.6118	-0.7293	-0.2064	-0.5634	-0.8525	-0.3358	-0.7769	-0.6419	-0.6407	-0.3387
-2	-1.8213	-1.7277	-1.4171	-1.7203	-1.1787	-1.6667	-1.9467	-1.2546	-1.7410	-1.6034	-1.6134	-1.4093

Ycorrected data

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
1	0.9648	1.0304	0.9594	0.9745	0.9812	0.9757	0.9631	0.9955	0.9766	0.9870	1.0299	0.9621
0	0.0424	-0.0630	0.1081	0.0384	0.0299	0.0058	0.0421	0.0137	0.0471	0.0228	-0.0483	0.1067
-1	-0.9799	-0.9652	-1.0943	-1.0001	-1.0032	-0.9388	-0.9731	-1.0137	-1.0241	-1.0065	-0.9929	-0.9576
-2	-2.0301	-2.0020	-1.9727	-2.0128	-2.0079	-2.0429	-2.0343	-1.9955	-1.9995	-2.0029	-1.9890	-2.0400

IS corrected data

Reference	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13
1	1.0140	1.1137	0.7964	0.9323	0.9172	0.9743	1.0240	0.8676	0.9537	0.9175	0.9824	0.9407
0	0.0685	-0.0246	0.0159	0.0161	-0.0034	0.0051	0.0744	-0.0513	0.0350	-0.0130	-0.0705	0.0946
-1	-0.9794	-0.9638	-1.0865	-1.0001	-1.0031	-0.9389	-0.9722	-1.0128	-1.0238	-1.0063	-0.9931	-0.9580
-2	-2.0559	-2.0431	-1.8918	-1.9911	-1.9754	-2.0422	-2.0664	-1.9316	-1.9879	-1.9678	-1.9658	-2.0286

5. CONCLUSIONS

5.1 Pilot field trial

The aims of the initial pilot trial were to (i) test two different resuspension protocols, (ii) perform an initial assessment to determine if the freeze dried cells were suitable for more detailed analysis, ie. that RNA could be extracted by different centres, that *BCR-ABL* and the three control genes could be amplified at levels comparable to normal leukocytes, that the level of *BCR-ABL* corresponded roughly to the dilutions and (iii) test if the protocol was comprehensible and unambiguous.

Given that the amount of RNA extracted was significantly lower using protocol A, compared to both protocol B (direct Trizol lysis) and the pre-freeze dried material, protocol B was selected for the large field trial.

Although, as expected, there was variation in absolute numbers between the three centres all three control genes (*ABL*, *BCR* and *GUSB*) were detected at high level. The b3a2 *BCR-ABL* transcript was detected at four levels in both the HL60 and KG1 cell lines and the % *BCR-ABL* / control gene values showed 10 fold reductions between levels. The level 4 standard (assigned as 0.01% *BCR-ABL* / control gene) was detected by all three laboratories.

Minor modifications to the protocol were made in response to the feedback that was received.

5.2 Large Field trial

The aims of the large field trial were to (i) assess the performance of KG1/K562 and HL60/K562 across a wide variety of platforms and assay protocols and, if successful, (ii) provide data for the selection of one combination to take forward for the development of accredited reference reagents.

Fourteen laboratories took part in the field trial. Two RNA extraction protocols were used and over half of the laboratories cited Gabert et al. (2003) as their RT-PCR method [1]. Random hexamers were used for reverse transcription by all labs who returned the methodology forms.

The mean and median amount of RNA extracted per vial from the HL60 reference material were 30.5 and 29.6µg respectively. The mean and median amount of RNA extracted per vial from the KG1 reference material were 30.9 and 31.4µg respectively. This is much higher than that obtained from typical clinical samples suggesting that the number of cells per vial could be reduced and / or the contents of each vial could be used for several independent tests once resuspended in Trizol.

It was not possible to directly compare the absolute copy numbers obtained from the different labs as many different protocols were used and sufficient information to standardize these results was not available. However, the median copies obtained for *ABL*, *BCR* and *GUSB* (lab 10 excluded) for the HL60 material were 1.16E+05, 2.60E+05 and1.70E+05, respectively, and for the KG1 material 1.02E+05, 5.85E+05 and 1.13E+05, respectively. For labs who tested more than one control gene the relative amounts of *ABL* : *BCR* and *ABL* : *GUSB* were calculated. As can be seen from figure 2, the ratios varied quite substantially between labs. In general, *GUSB* appears to be the control gene with the highest relative level of expression in HL60 cells and in most labs the amount of *ABL* and *GUSB* expressed in KG1 cells were comparable.

The coefficient of variation for % *BCR-ABL* / control gene values obtained from all labs were comparable to those obtained in the IS conversion factor rounds administered by the Mannheim laboratory (M.Muller, Mannheim, personal communication) and other studies[2]. There was no statistical difference between the CVs obtained from either cell line for any of the control genes.

For the purpose of this field trial and to demonstrate how this type of reference material could be used to standardize reporting, values of 10%, 1%, 0.1% and 0.01% *BCR-ABL* / control gene were arbitrarily assigned to the level 1, level 2, level 3, and level 4 material respectively. Linear regression plots were produced for log transformed lab data plotted against the log transformed reference standard values. The resulting regression equations obtained by least squares analysis were used to standardize / convert the data to the reference material values by solving the equation for the MMR value (0.1%). As can be seen in figures 6 and 7 the reference material can be used in this way to greatly improve the comparability of the data between labs. It is important to emphasise that this analysis was designed to illustrate the way that reference reagents could be used. Other methods of transforming

the raw laboratory data are possible that may be superior. This is an area that needs further work in future trials and / or when accredited reagents are available for use.

Some of the linear regression plots showed statistically significant variation in the slope of the line when compared to the reference standard suggesting that the assays may not be linear for this reference material (Method Validator software, CI 95%). These were most prevalent in the KG1 *ABL* analyses (6 labs, 46%).

Overall, it appears that both freeze dried cell line mixes could be suitable for development of *BCR-ABL* reference reagents. RNA (median 30µg/vial) was successfully extracted from freeze dried cell mixtures shipped worldwide at ambient temperature. The HL60/K562 and KG1/562 cell mixes performed equally well producing CVs that were comparable to those expected for primary patient samples.

6. FUTURE WORK

Accelerated degradation studies with this batch of freeze dried material are ongoing and results will be reported in due course. A further medium scale pilot freeze dry study using the HL60 / K562 cell mixture is planned for mid-2008 in collaboration with NIBSC and the European Collection of Cell Cultures (ECACC). We will assess the use of 1.5×10^6 cells freeze dried into glass ampoules, plus the logistics of growing up and processing large scale cell cultures. ECACC will be responsible for the large scale grow up of the two cell lines, staff from NGRL (Wessex) will prepare the cell line mixes and dilutions at ECACC and transfer them to NIBSC for freeze drying within 24 hours.

If the pilot is successful, a large scale grow up of the two cell lines will be performed to enable us to produce 3000 vials for each level of *BCR-ABL* (12,000 vials total). ECACC will grow 40 litres HL60 (c. 1.8×10^{10} cells) and 3 litres K562 (1.5×10^9). NGRL staff will prepare cell mixes and dilutions and freeze drying of the cells will take place at NIBSC. At the time of writing this large scale production is scheduled for Autumn 2008, with performance evaluation and the accreditation process to commence shortly afterwards.

How exactly these reference reagents will be used remains to be established, but this scale of production is small compared to the requirements of testing labs worldwide. An informal survey of opinions from the International *BCR-ABL* Standardization Group suggests that there are probably at least 300 laboratories worldwide performing RQ-PCR for *BCR-ABL*, with many more keen to establish this methodology. The planned large scale grow up would only enable 10 vials at each dilution to be distributed to each laboratory, or 2 vials per annum over a 5 year period. Currently it is uncertain how often laboratory calibration needs to be performed, but clearly it is not feasible to scale up production of freeze dried cell lines to enable calibration to be performed by all laboratories on a frequent basis. A more likely scenario is that the freeze dried cell lines will be accredited as primary reference reagents that will define the IS. These reagents will be made available on a limited basis to companies, reference laboratories etc. who are engaged in the production of secondary reference reagents. Such secondary reagents will be calibrated against the primary reagents and generally available to testing laboratories.

7. ACKNOWLEDGMENTS

We would like to thank

- all field trial participants for their assistance with this project
- Dr Andy Chase, Wessex Regional Genetics Laboratory for assistance with cell culture
- Elaine Gray, Ross Hawkins, Paul Metcalfe and Paul Matejtschuk (NIBSC) for freeze drying the reagents and their assistance with the field trial design

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Appendix A: Field Trial Participants

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Appendix B: Linear regression plots

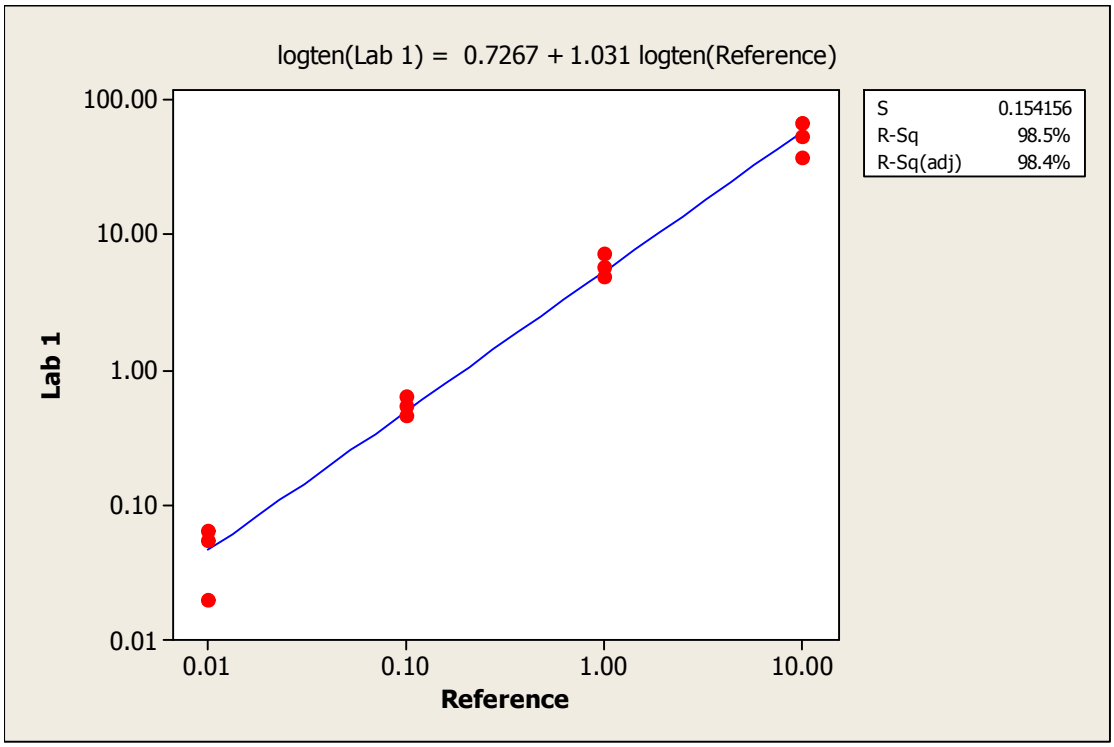
Summary of data

Lab	ABL			BCR			GUS		
	Gradient	S	R2 (Adj)	Gradient	S	R2 (Adj)	Gradient	S	R2 (Adj)
HL60									
1				1.031	0.154	98.4%			
2	1.025	0.055	99.8%						
3	1.041	0.077	99.6%				1.005	0.106	99.2%
4	0.917	0.100	99.1%				0.930	0.120	98.8%
5	0.979	0.088	99.4%						
6	0.968	0.077	99.5%	0.988	0.090	99.4%	0.990	0.092	99.4%
7	0.993	0.074	99.6%						
8	1.031	0.093	99.4%				1.062	0.133	98.9%
9	0.936	0.093	99.3%	1.003	0.086	99.5%	1.025	0.122	99.0%
10	0.988	0.067	99.7%	1.002	0.052	99.8%	0.988	0.069	99.6%
11	0.965	0.068	99.6%						
12	0.977	0.076	99.6%						
13	0.989	0.087	99.5%	1.007	0.053	99.8%	1.035	0.051	99.8%
KG1									
1				0.910	0.084	99.4%			
2	0.930	0.090	99.4%						
3	0.999	0.076	99.6%				1.069	0.129	98.9%
4	0.895	0.090	99.3%				0.957	0.127	98.7%
5	0.988	0.101	99.2%						
6	0.937	0.073	99.6%	0.991	0.063	99.7%	0.981	0.045	99.8%
7	0.973	0.083	99.5%						
8	0.994	0.083	99.5%				1.055	0.065	99.7%
9	0.988	0.103	99.2%	1.012	0.083	99.5%	1.001	0.106	99.2%
10	0.886	0.128	98.5%	0.953	0.112	99.0%	0.933	0.110	99.0%
11	0.930	0.071	99.6%						
12	1.005	0.156	98.3%						
13	0.962	0.026	99.9%	1.012	0.056	99.6%	0.969	0.054	99.6%

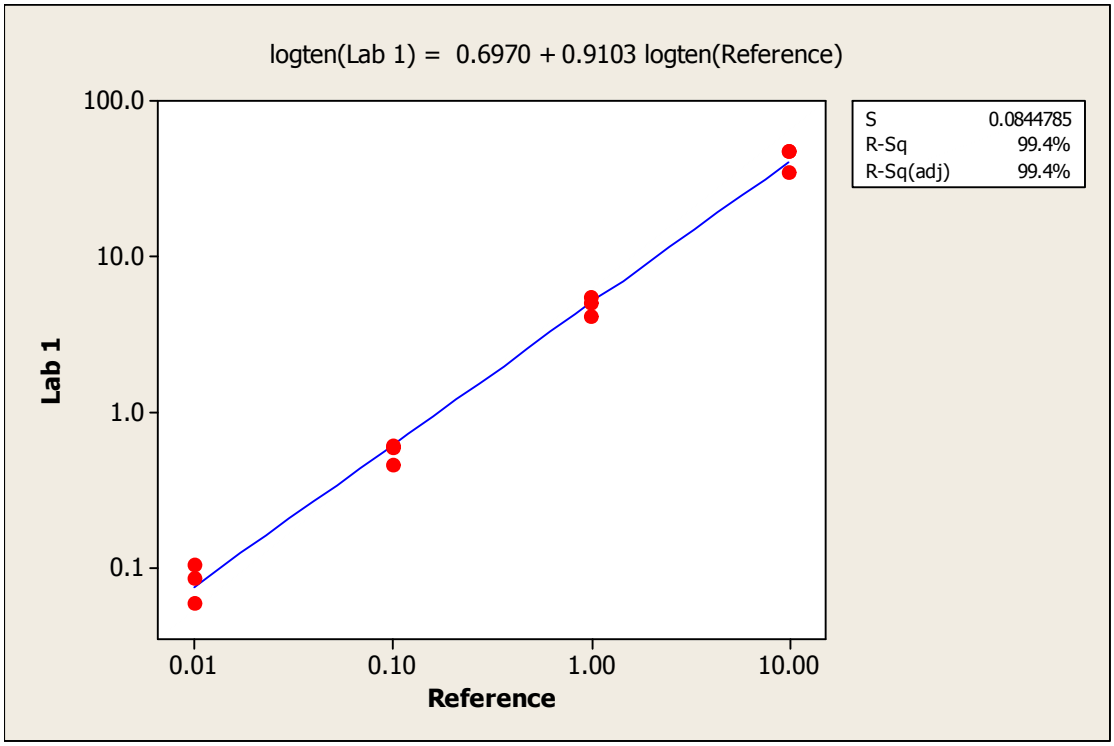
	G6PD		
	Gradient	S	R2 (Adj)
14 HL60	1.052	0.075	99.6%
14 KG1	0.993	0.119	98.9%

Lab 1

a) HL60 *BCR*

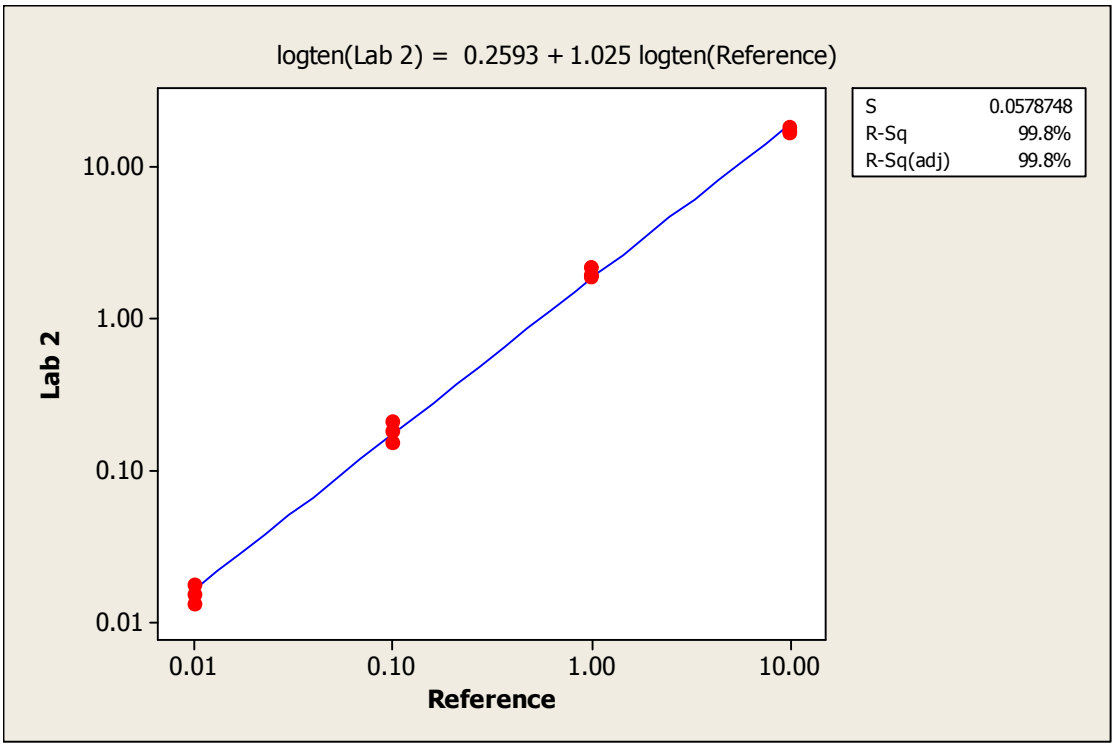


b) KG1 *BCR*

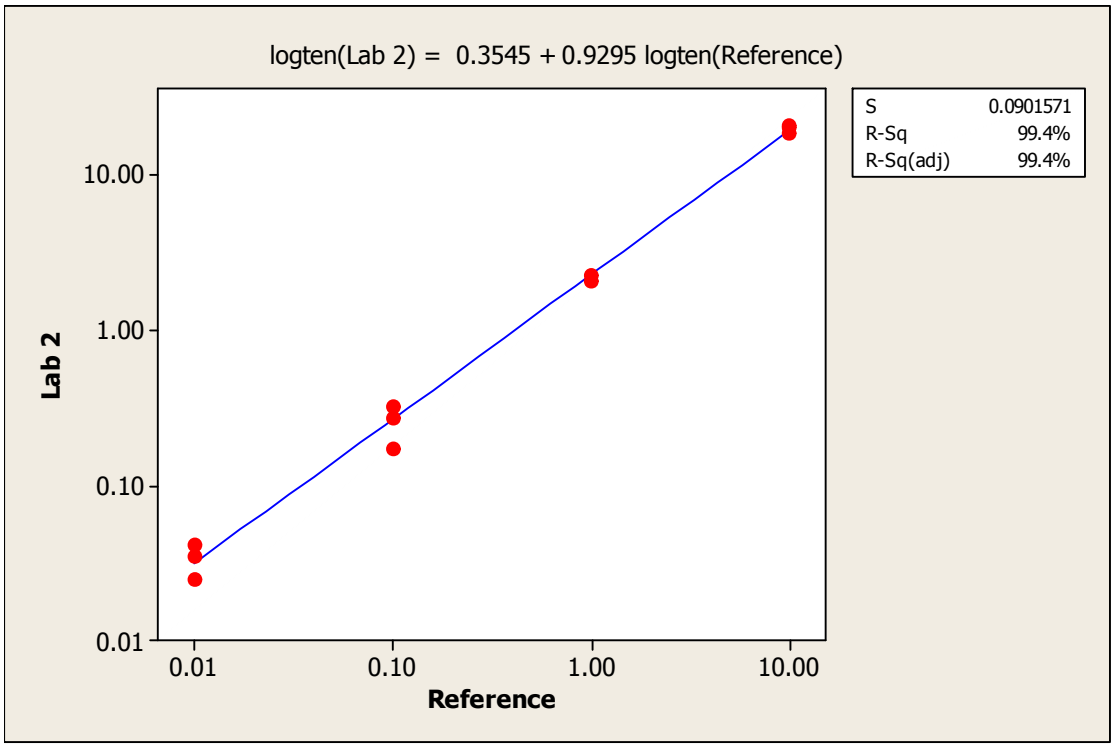


Lab 2

a) HL60 *ABL*

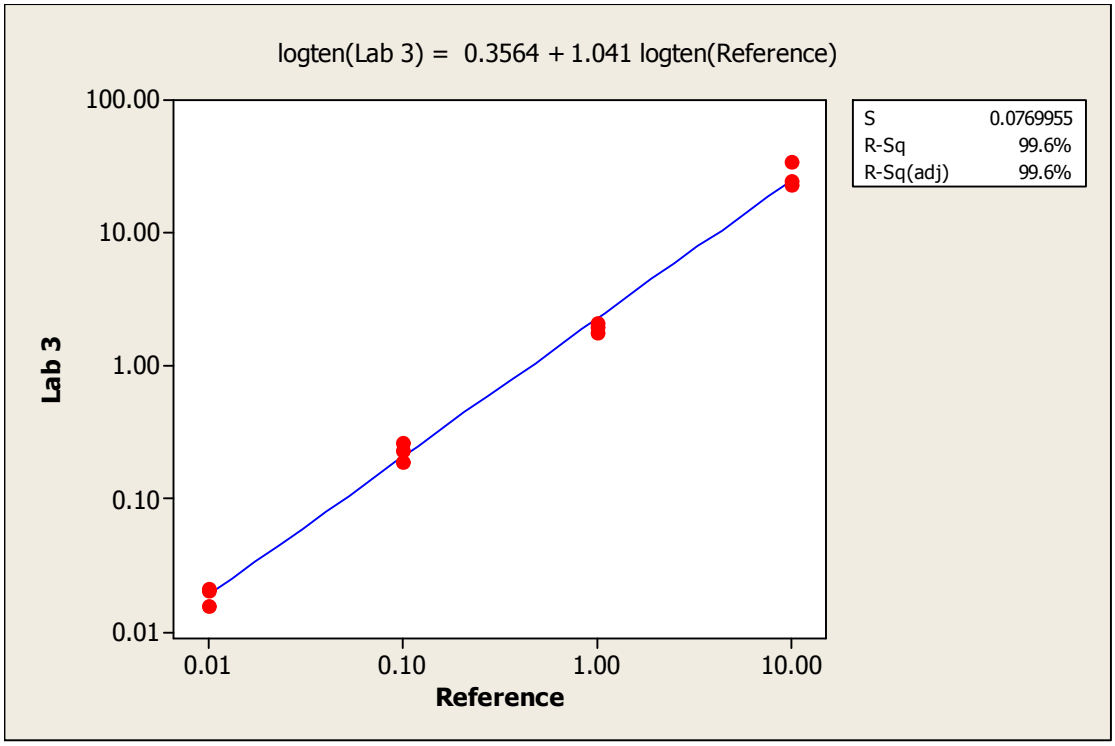


b) KG1 *ABL*

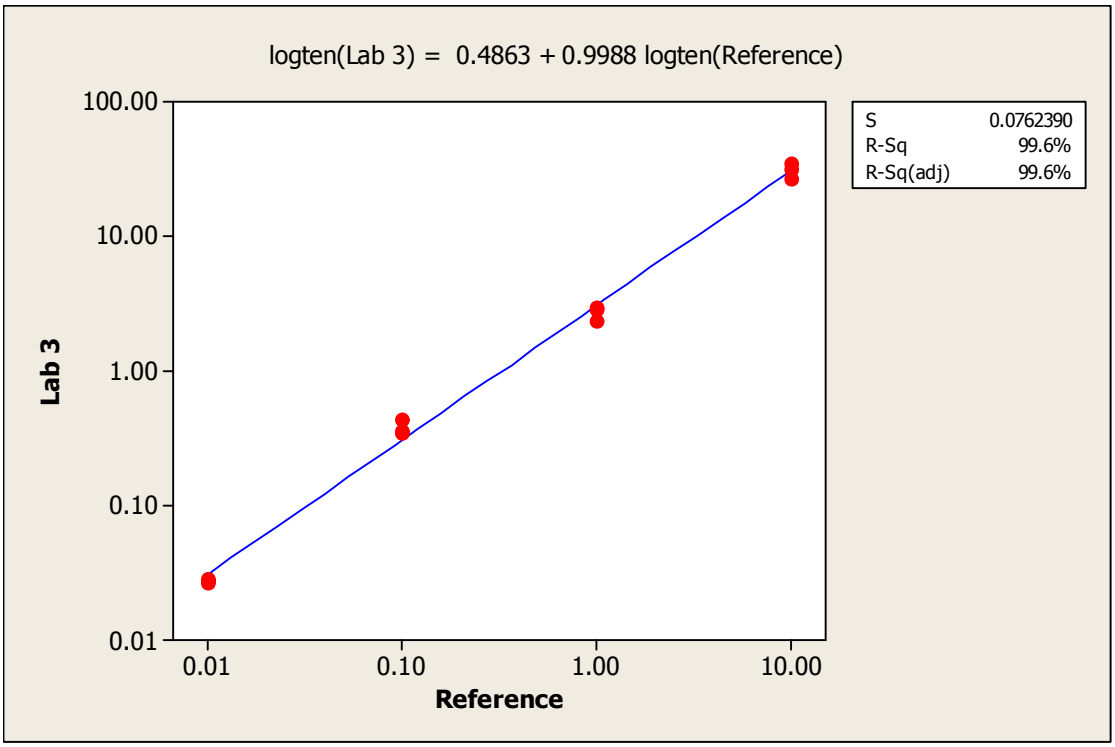


Lab 3

a) HL60 *ABL*

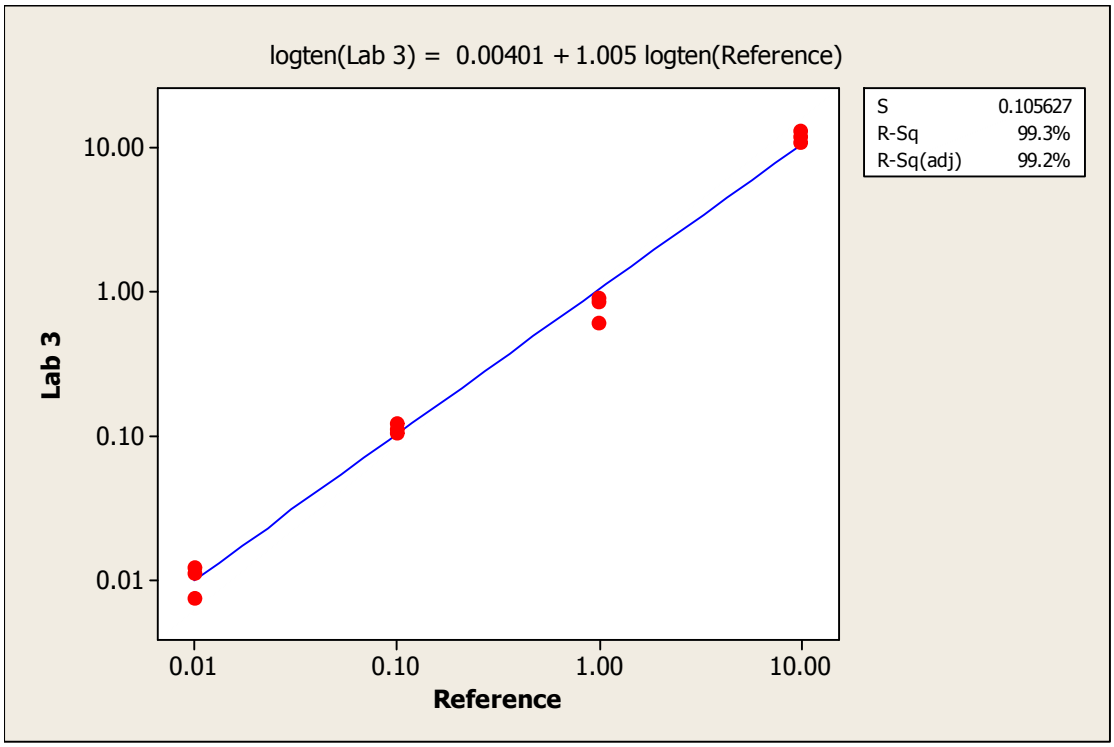


b) KG1 *ABL*

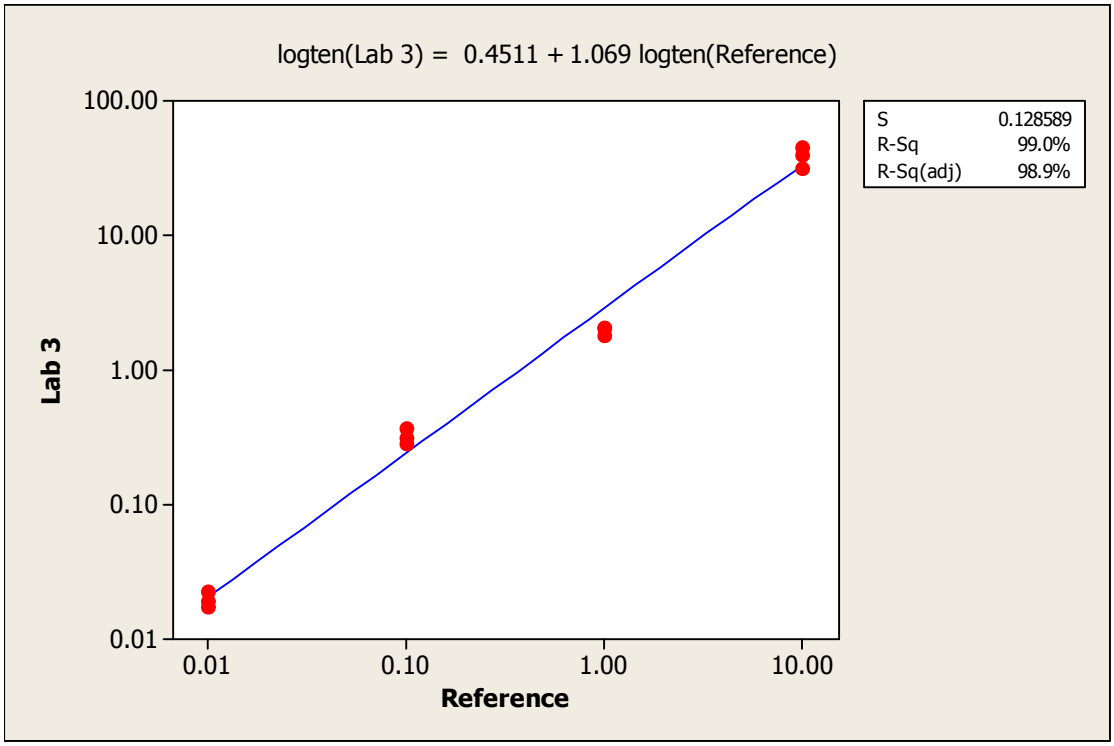


Lab 3

a) HL60 *GUSB*

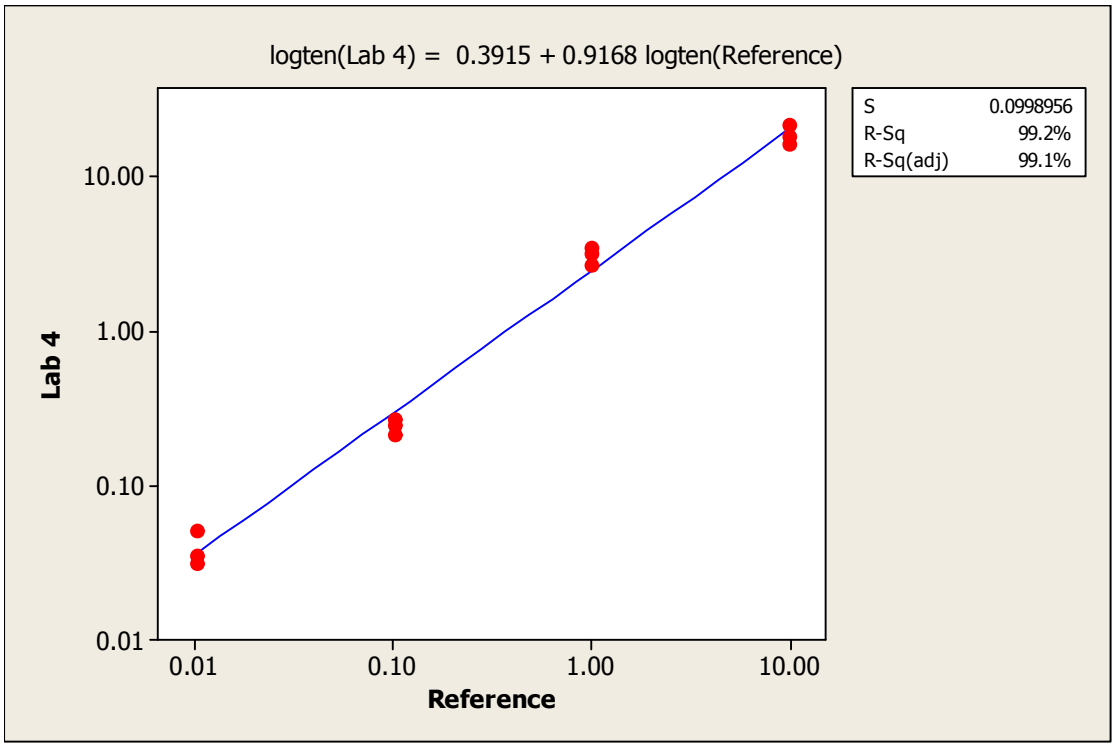


b) KG1 *GUSB*

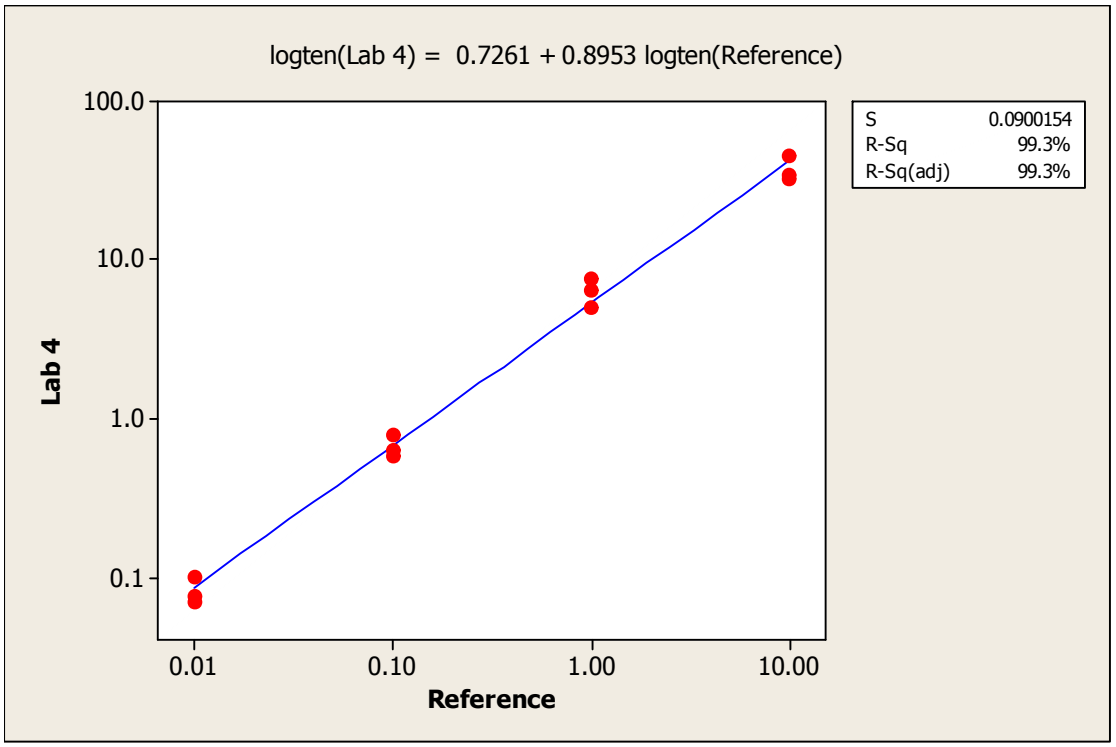


Lab 4

a) HL60 *ABL*

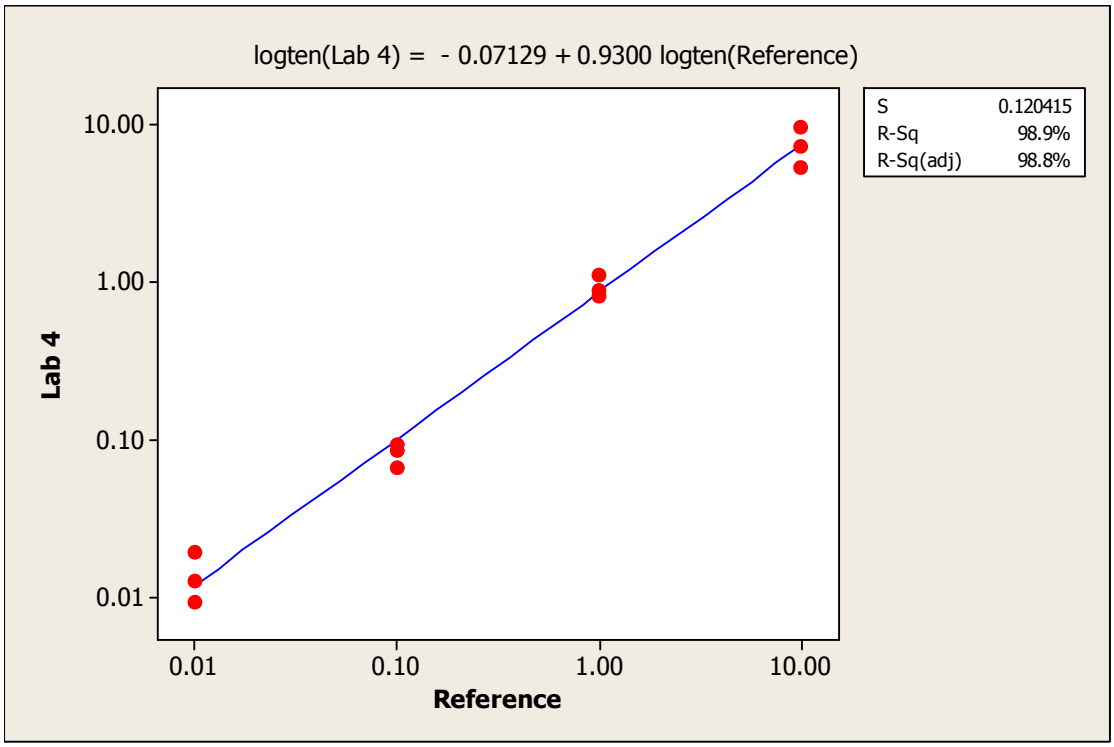


b) KG1 *ABL*

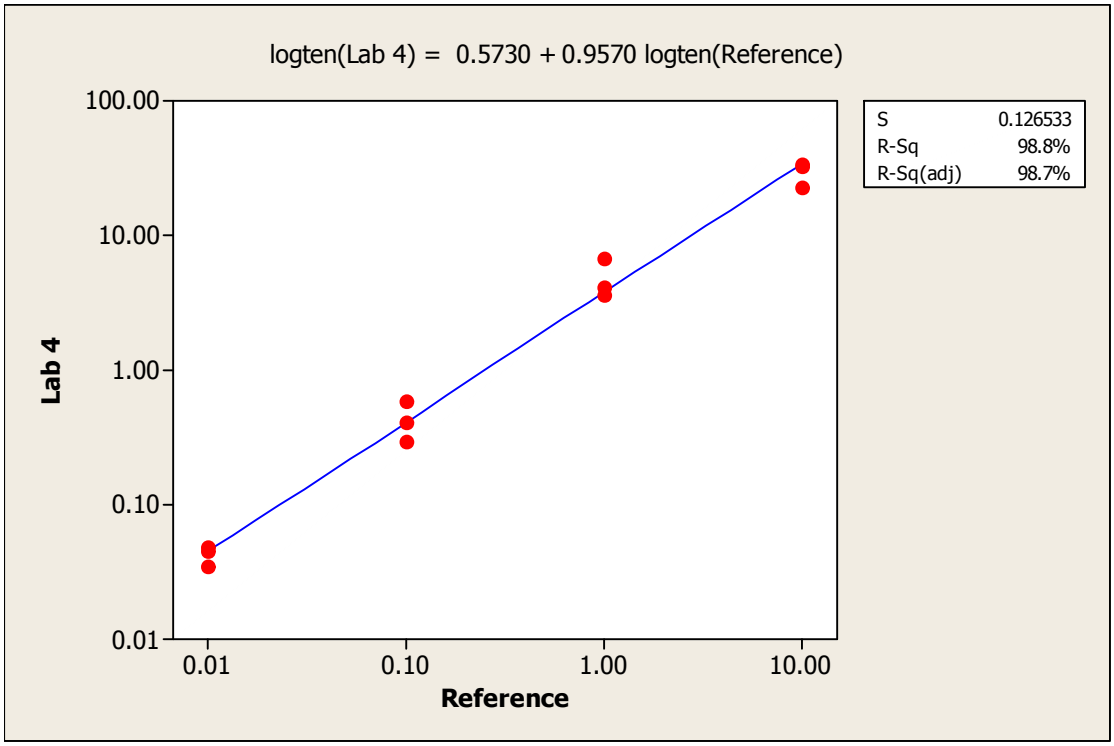


Lab 4

a) HL60 *GUSB*

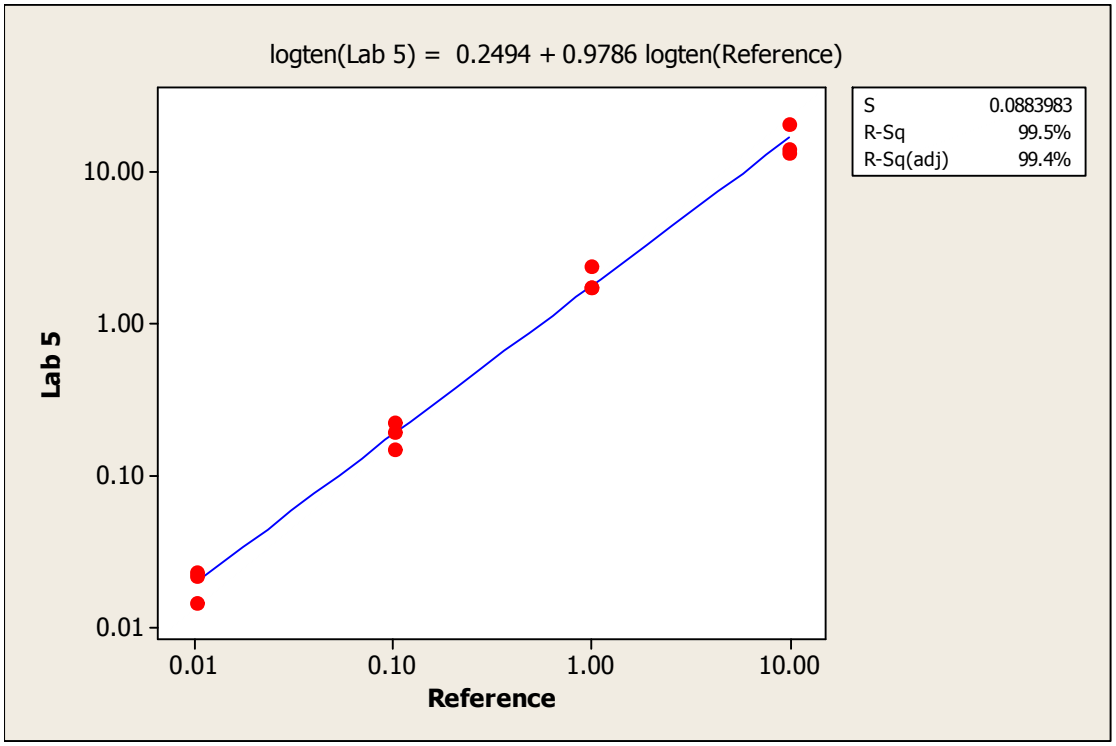


b) KG1 *GUSB*

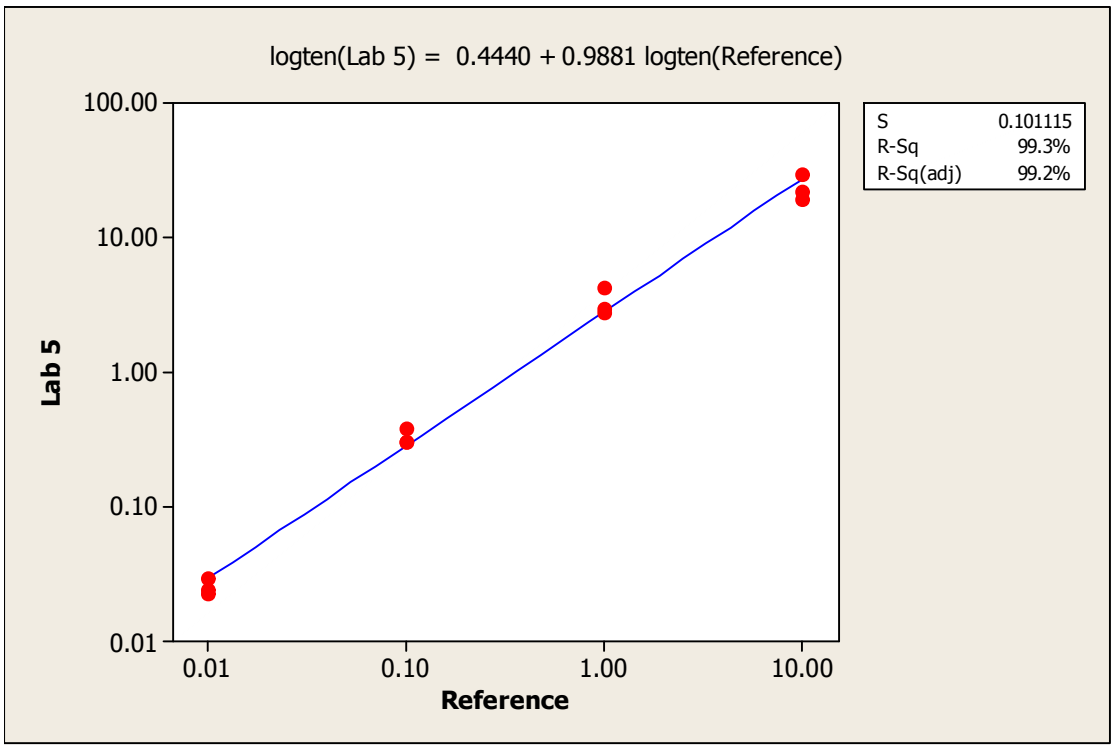


Lab 5

a) HL60 *ABL*

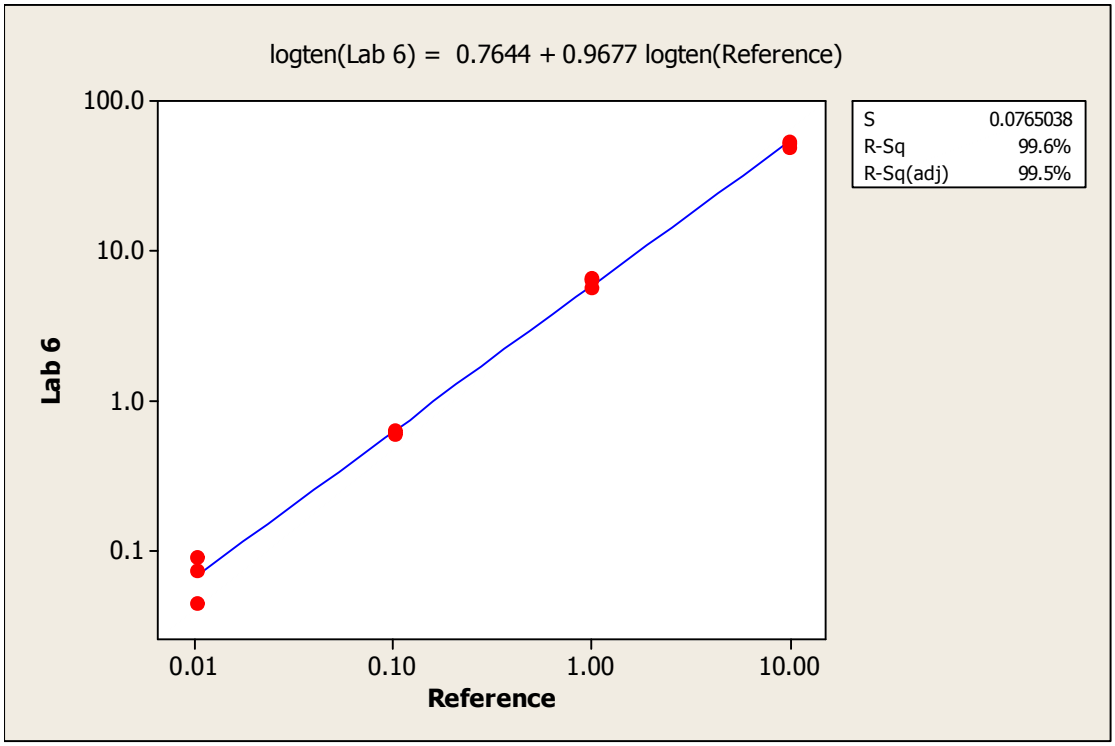


b) KG1 *ABL*

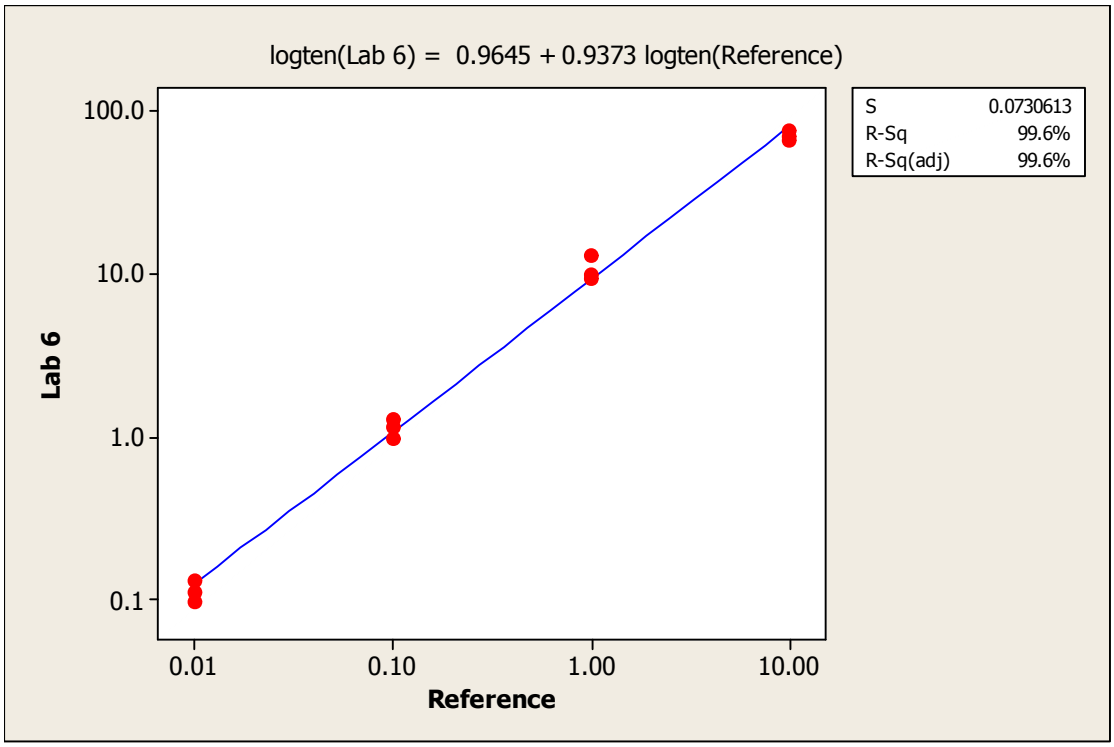


Lab 6

a) HL60 *ABL*

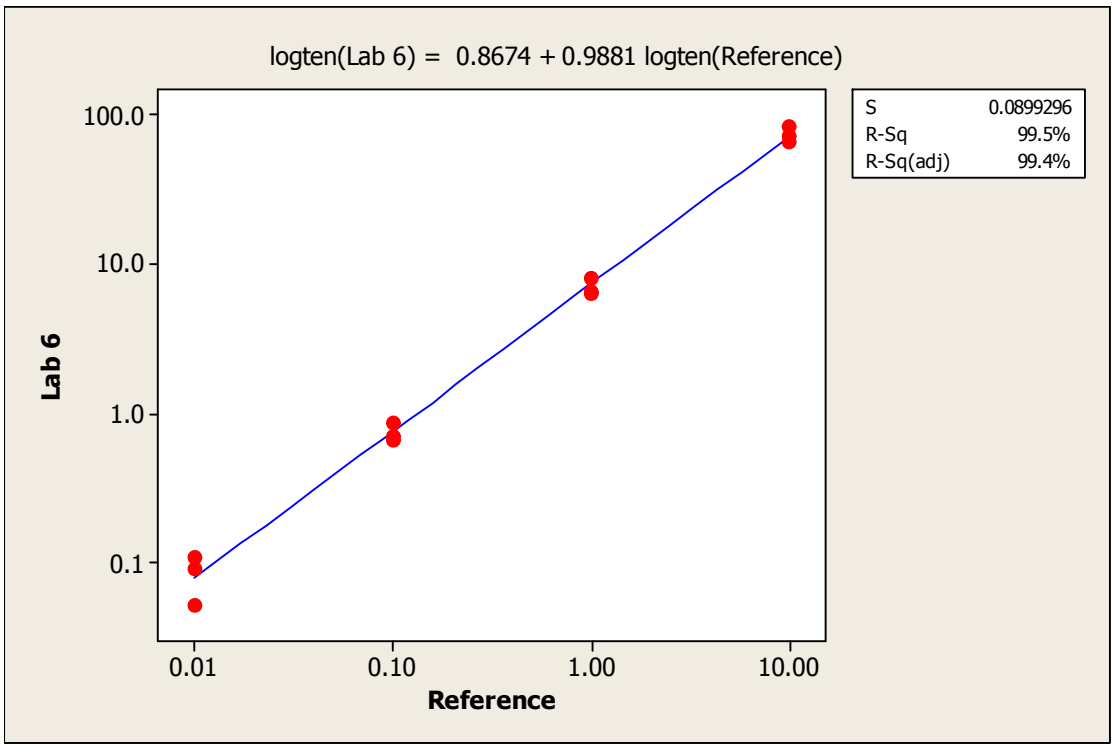


b) KG1 *ABL*

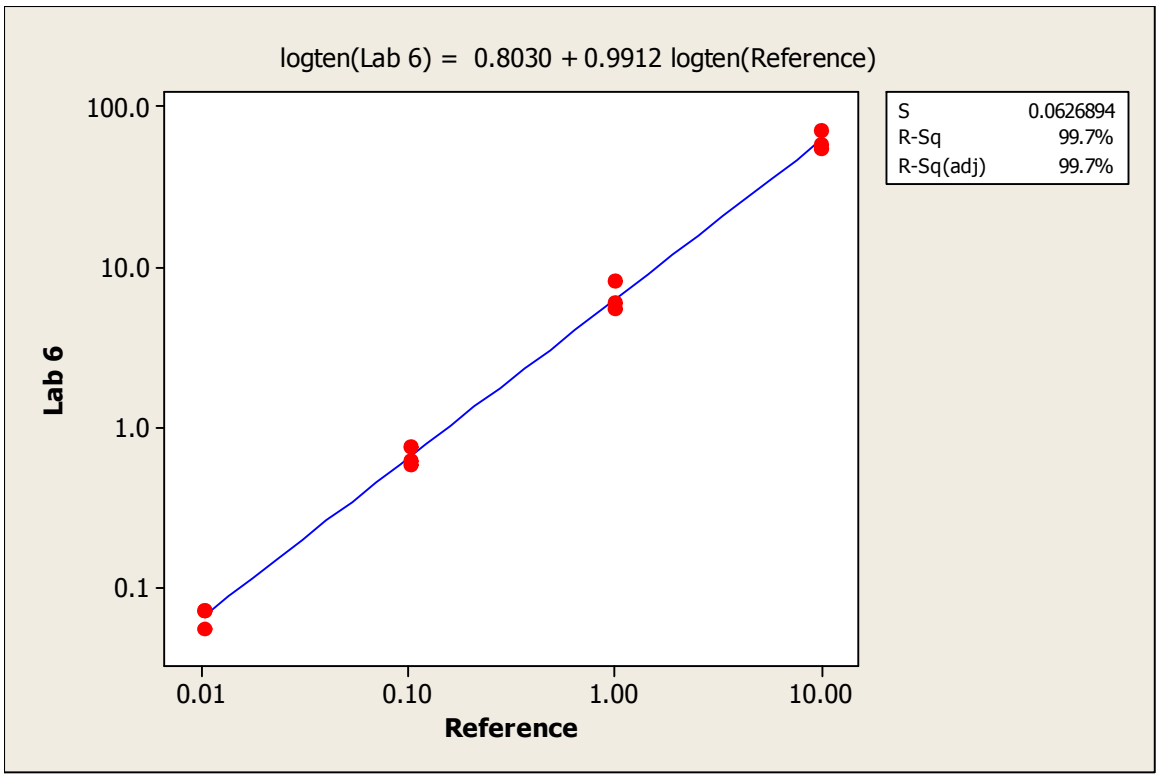


Lab 6

a) HL60 *BCR*

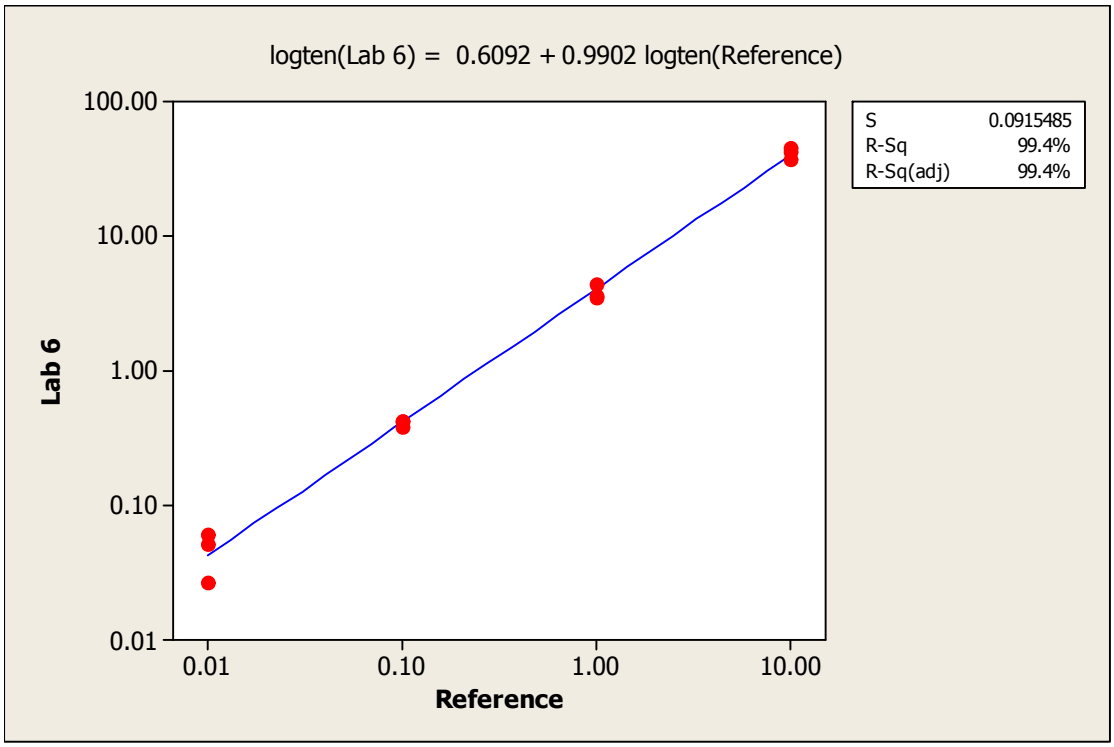


b) KG1 *BCR*

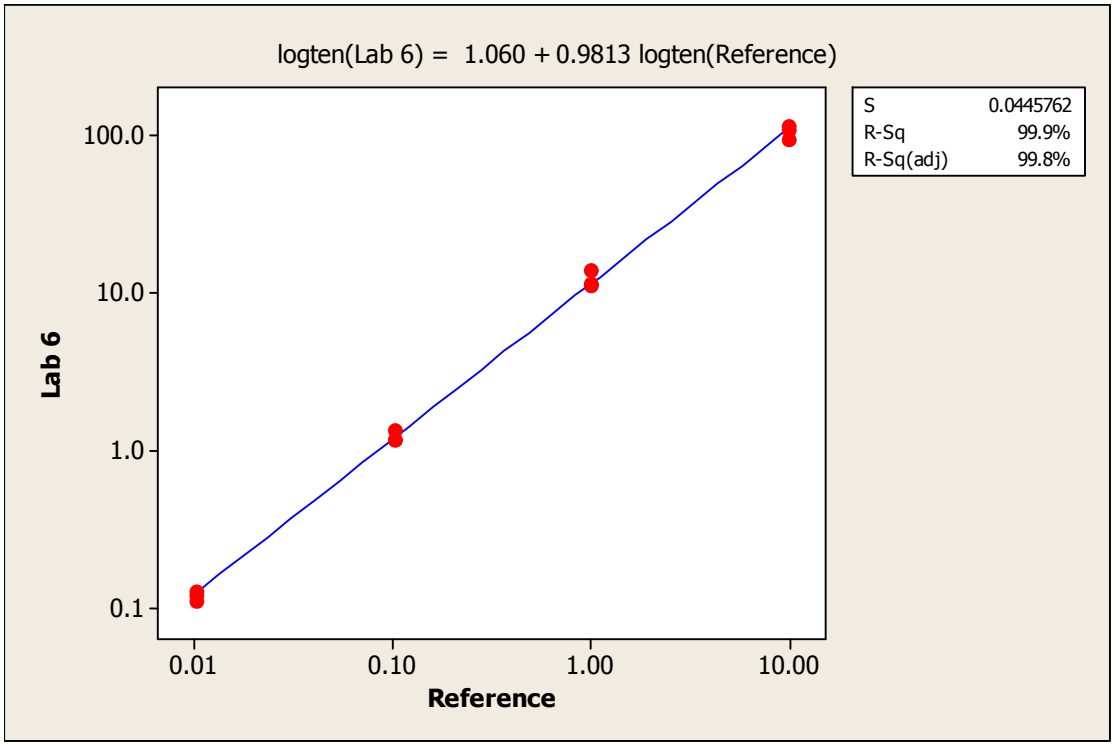


Lab 6

a) HL60 *GUSB*

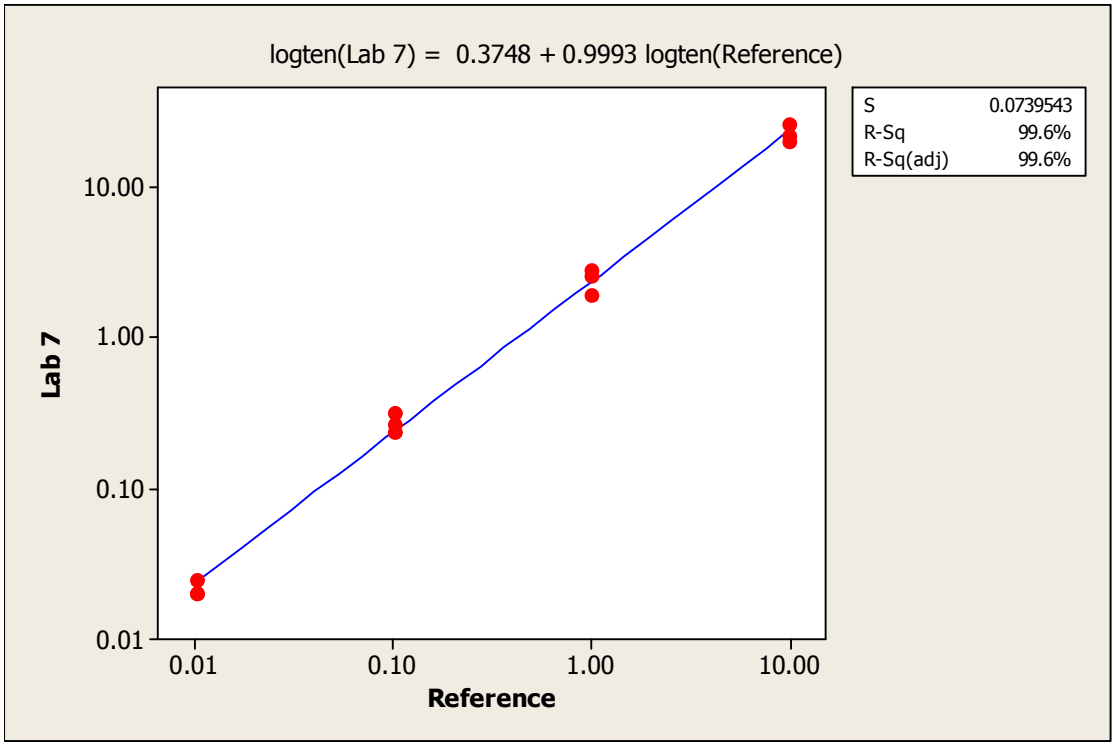


b) KG1 *GUSB*

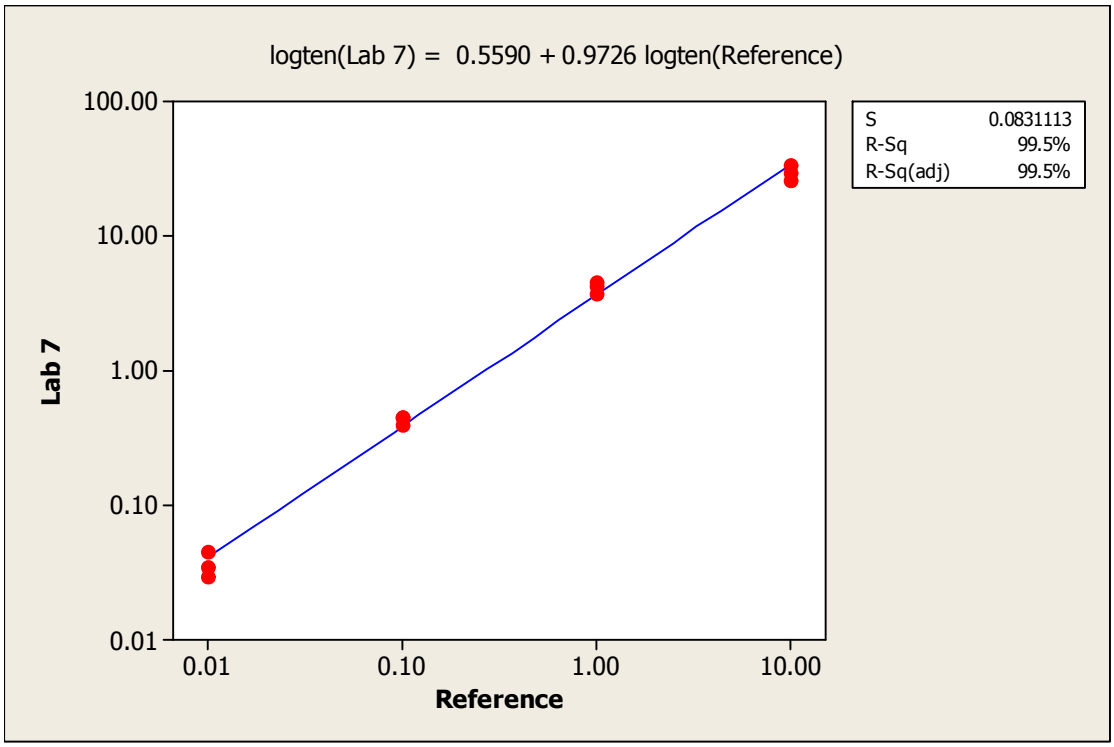


Lab 7

a) HL60 *ABL*

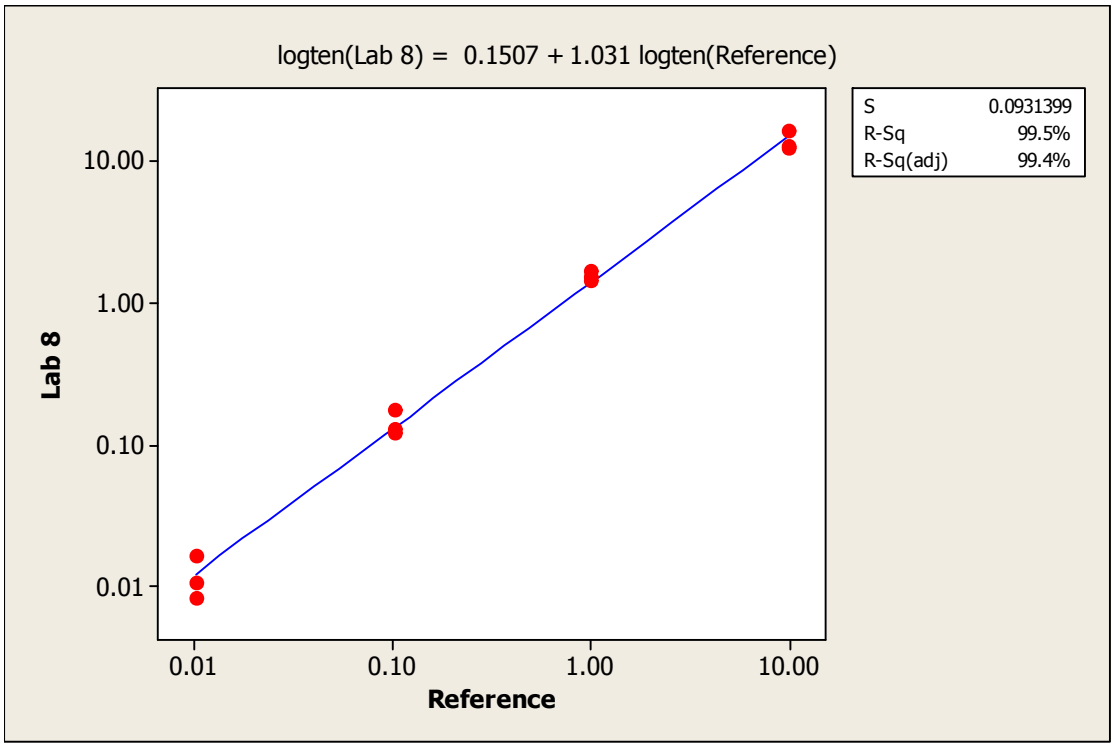


b) KG1 *ABL*

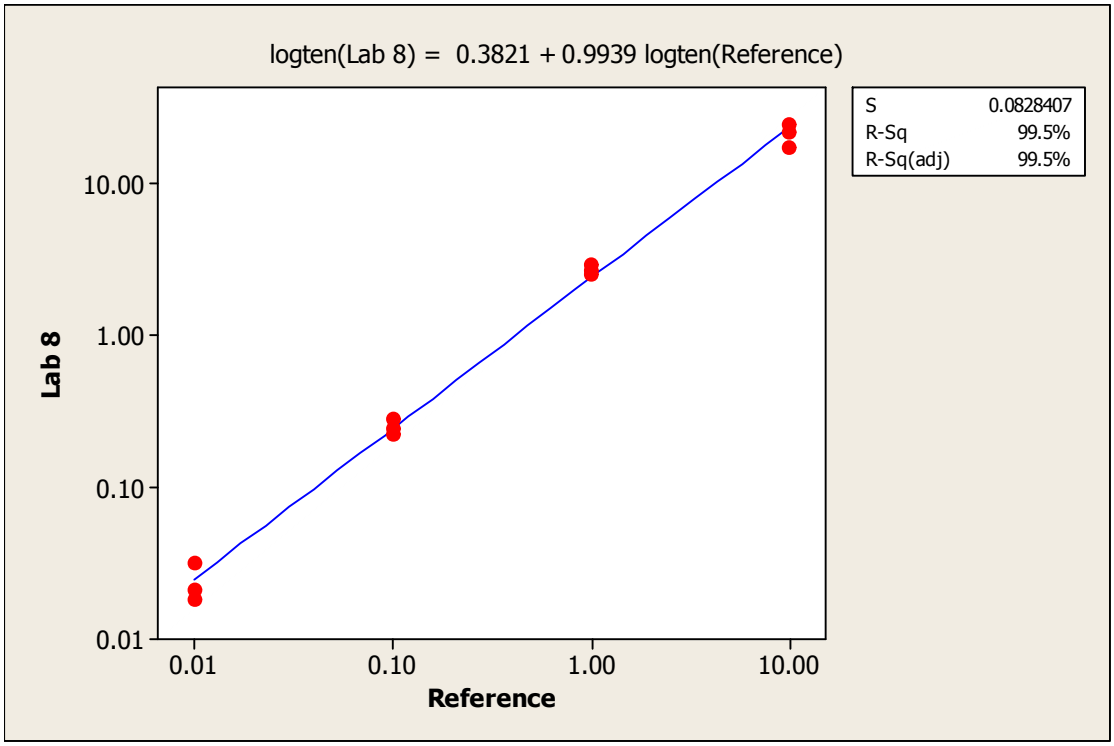


Lab 8

a) HL60 *ABL*

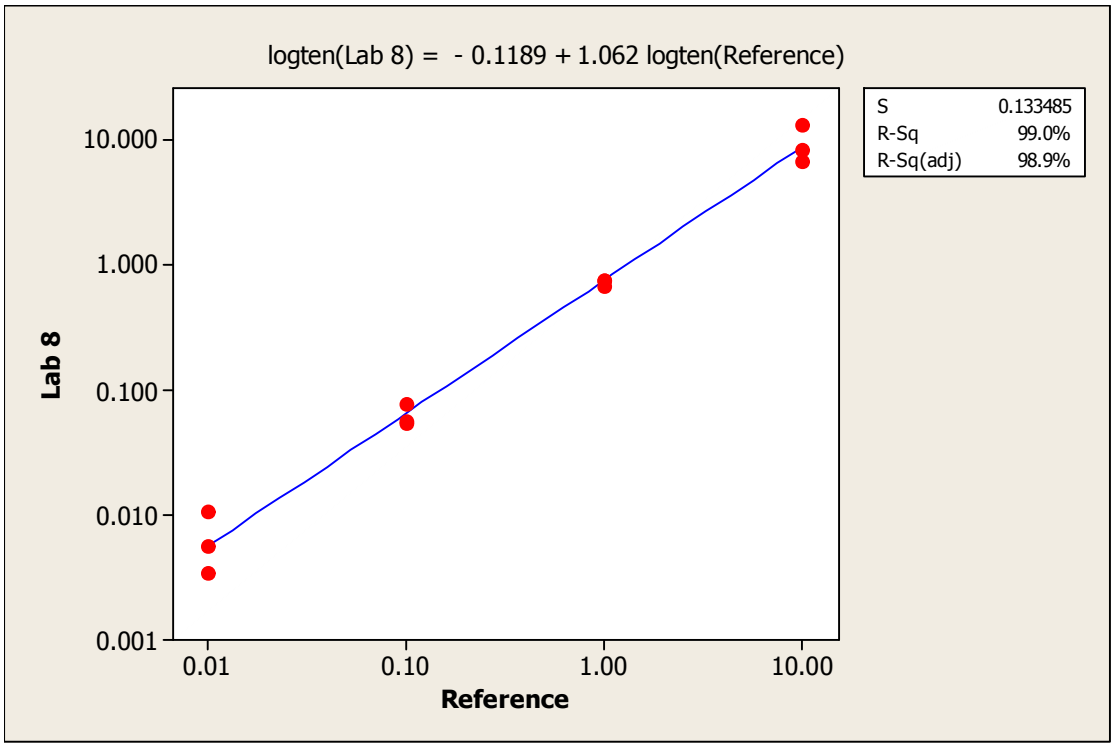


b) KG1 *ABL*

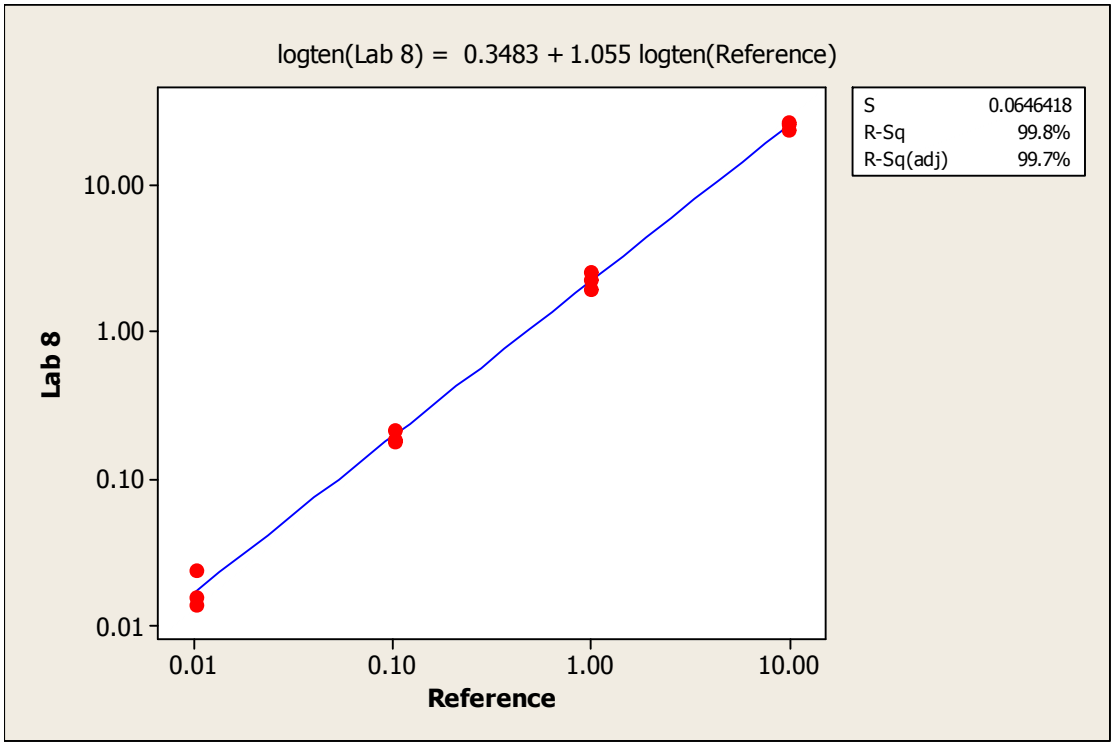


Lab 8

a) HL60 *GUSB*

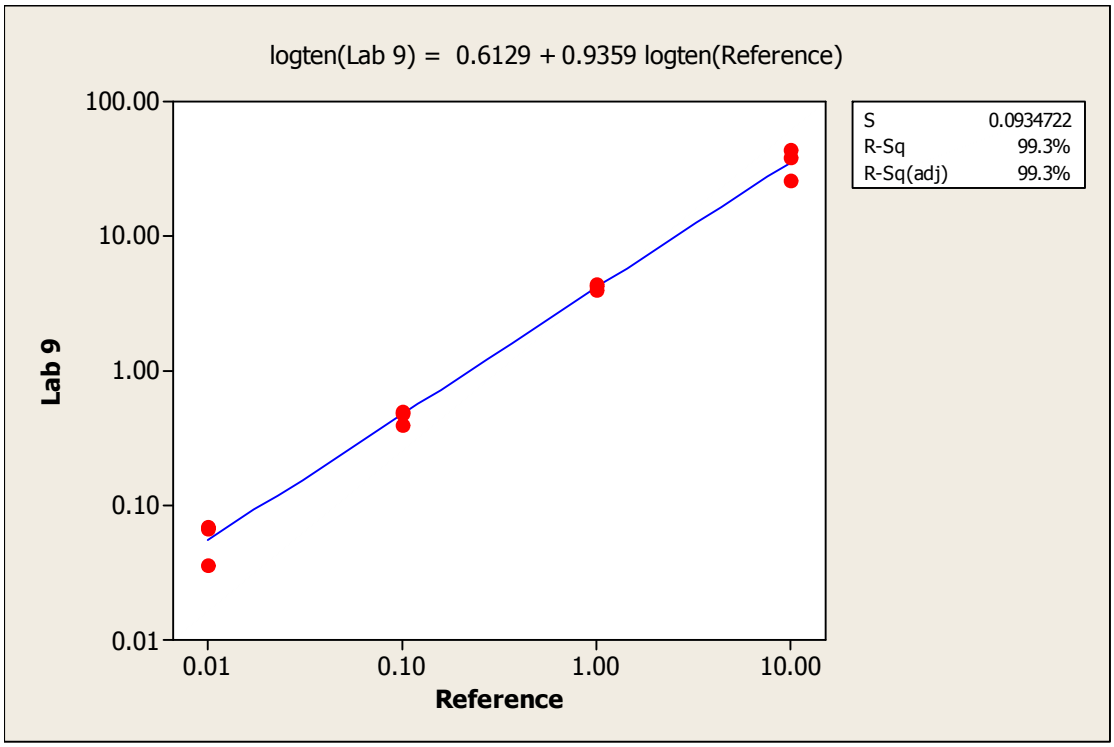


b) KG1 *GUSB*

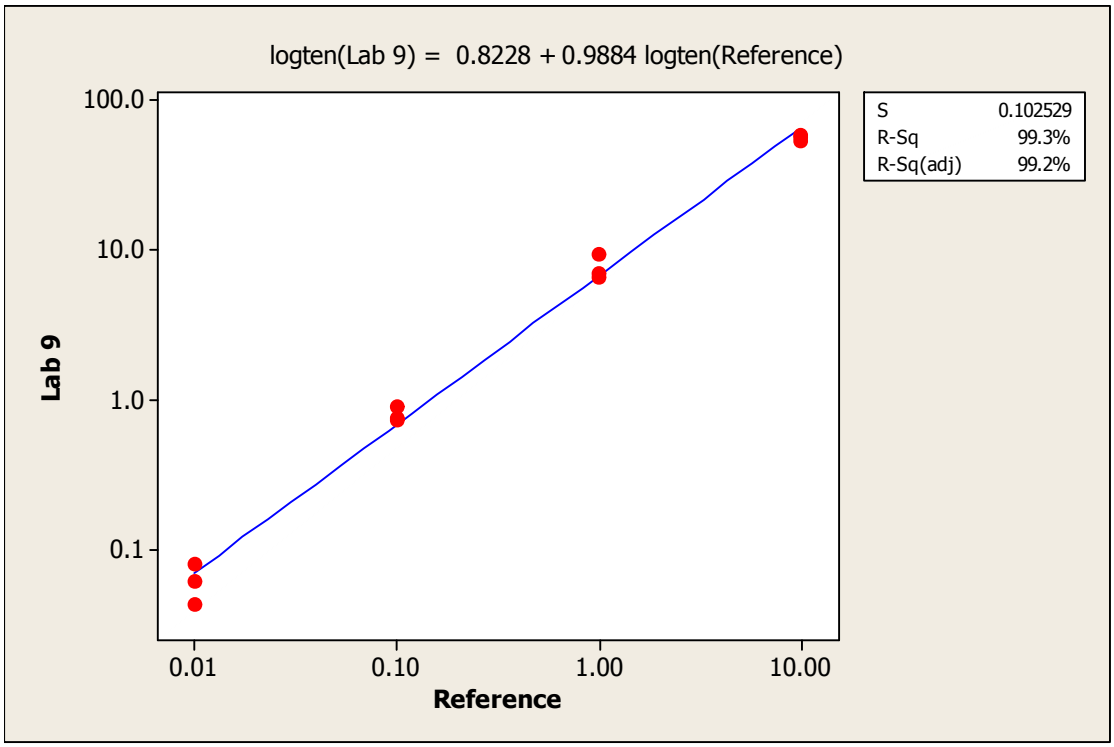


Lab 9

a) HL60 *ABL*

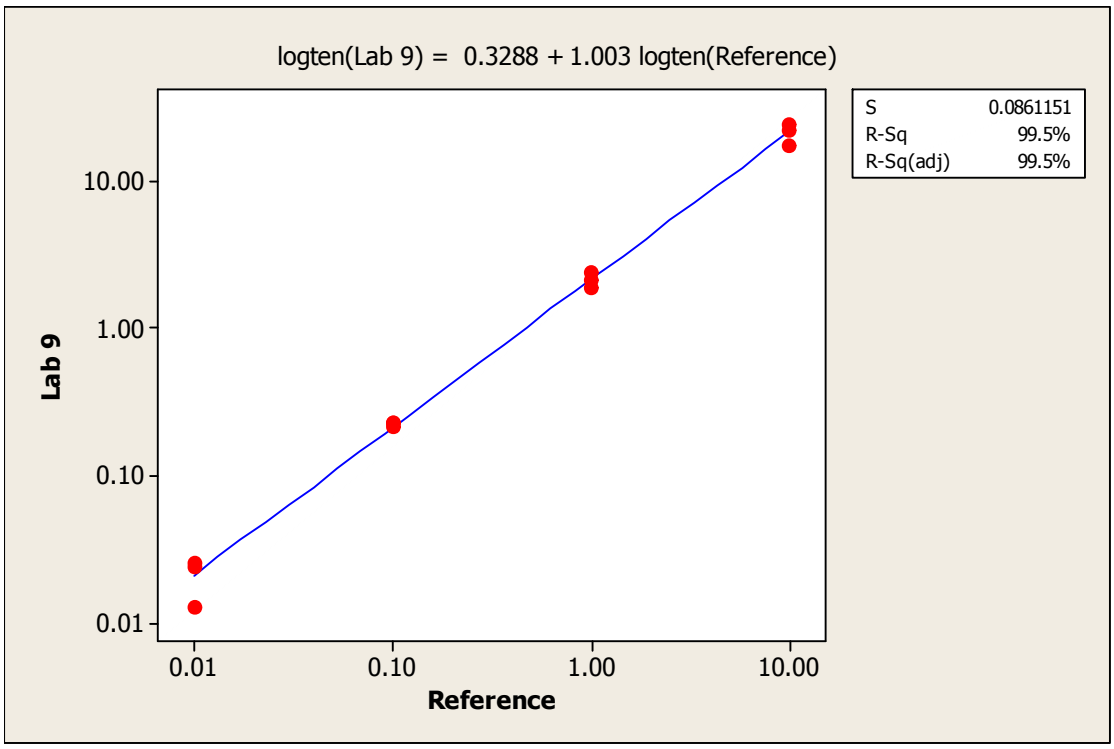


b) KG1 *ABL*

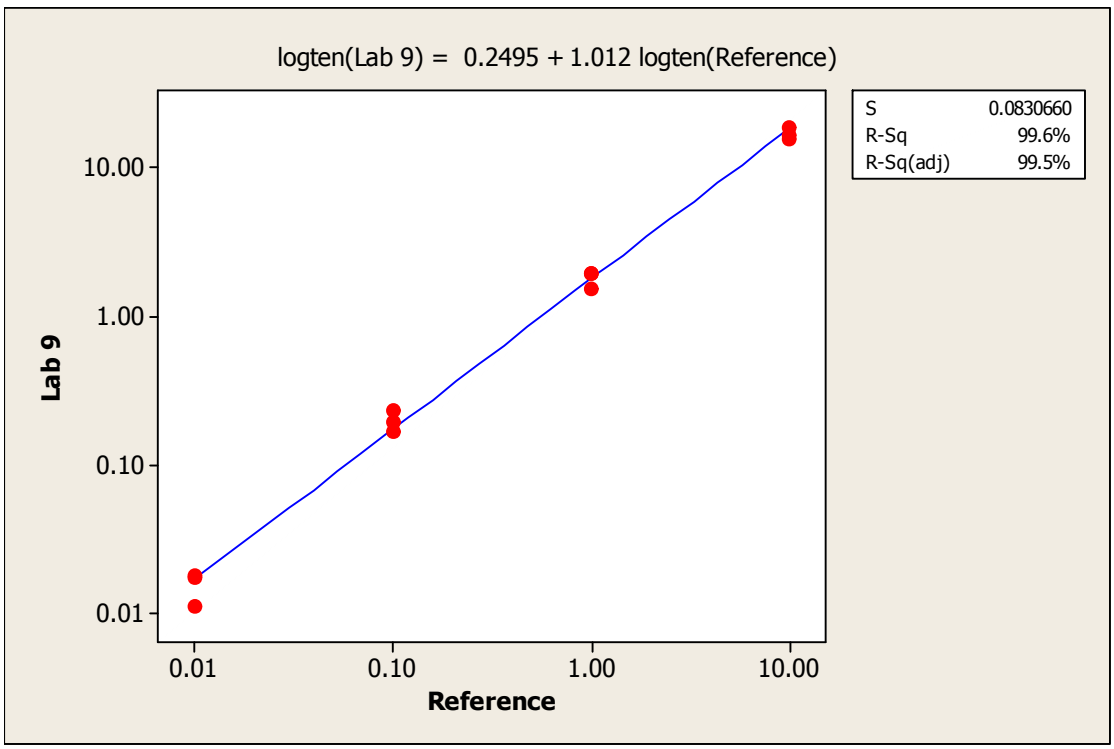


Lab 9

a) HL60 *BCR*

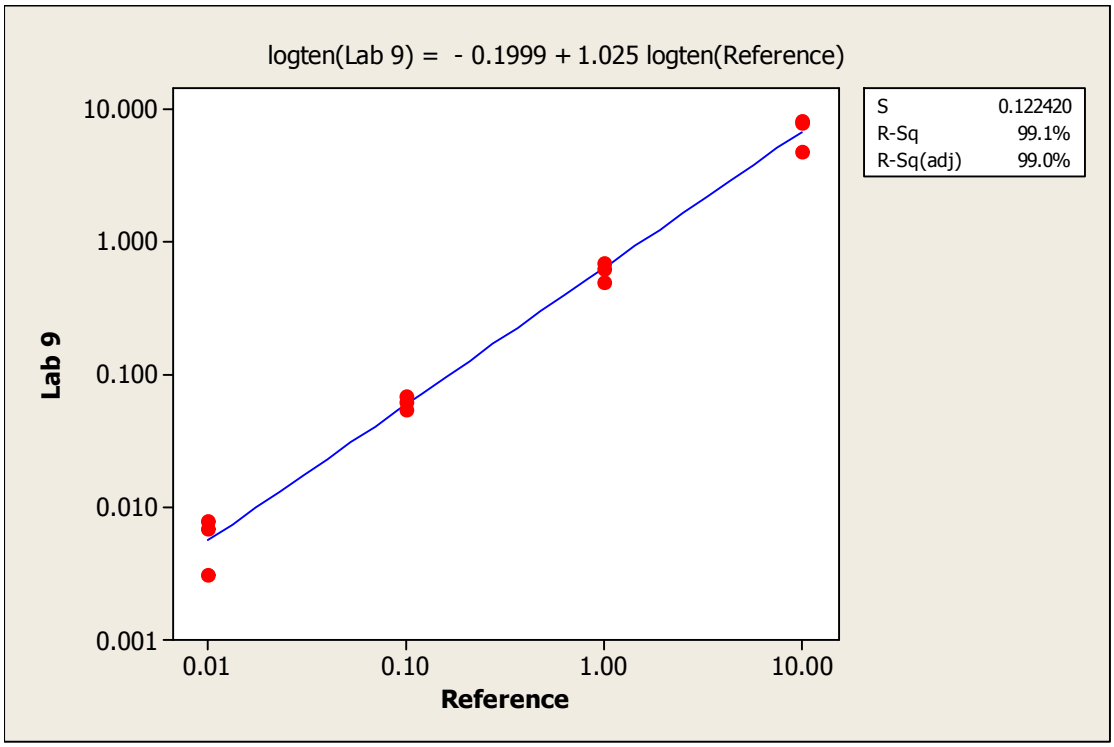


b) KG1 *BCR*

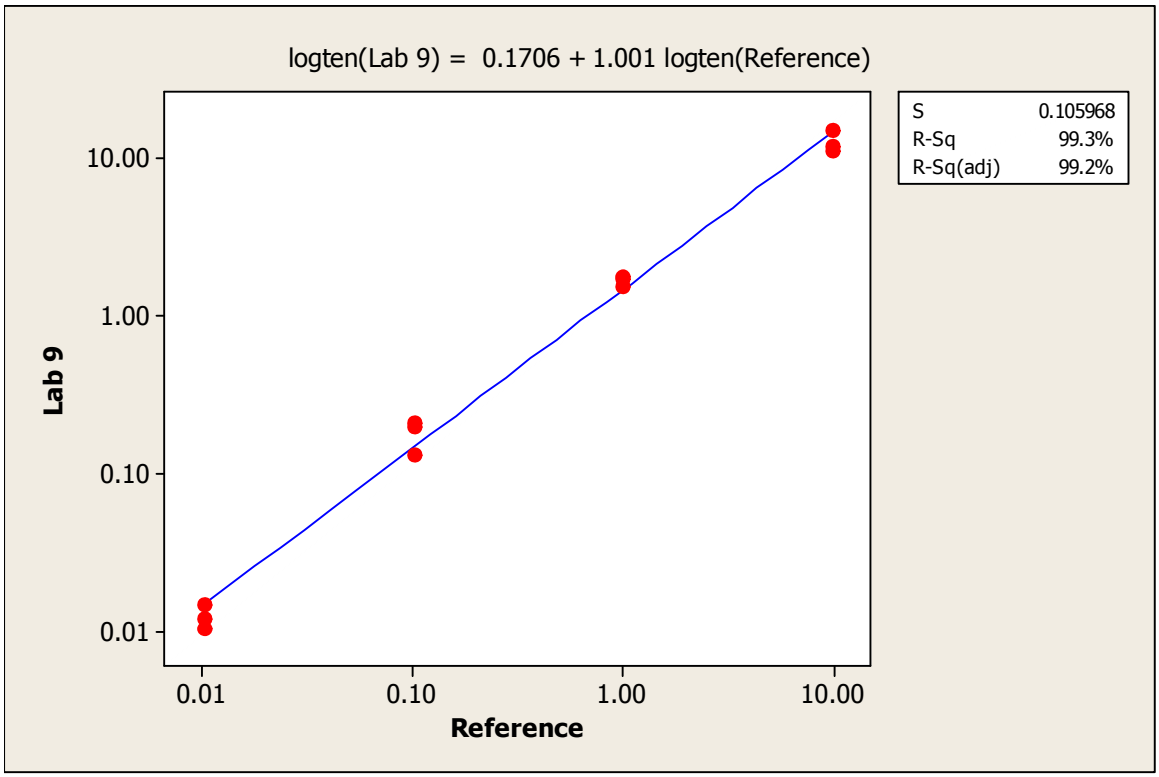


Lab 9

a) HL60 *GUSB*

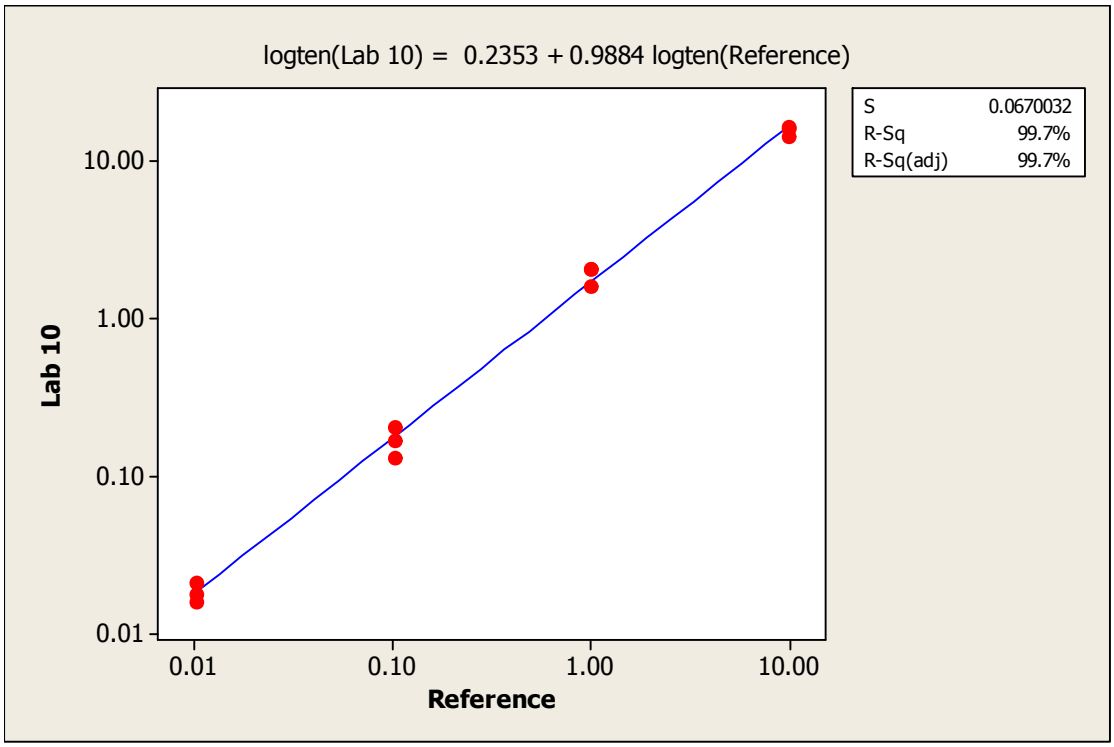


b) KG1 *GUSB*

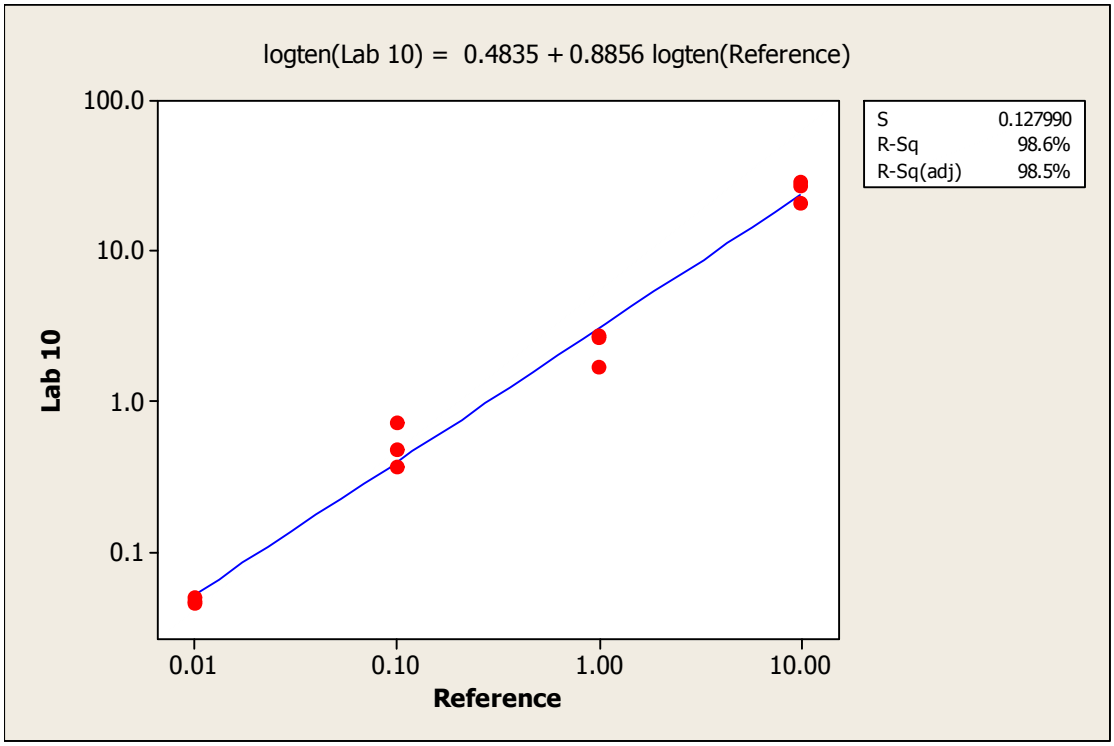


Lab 10

a) HL60 *ABL*

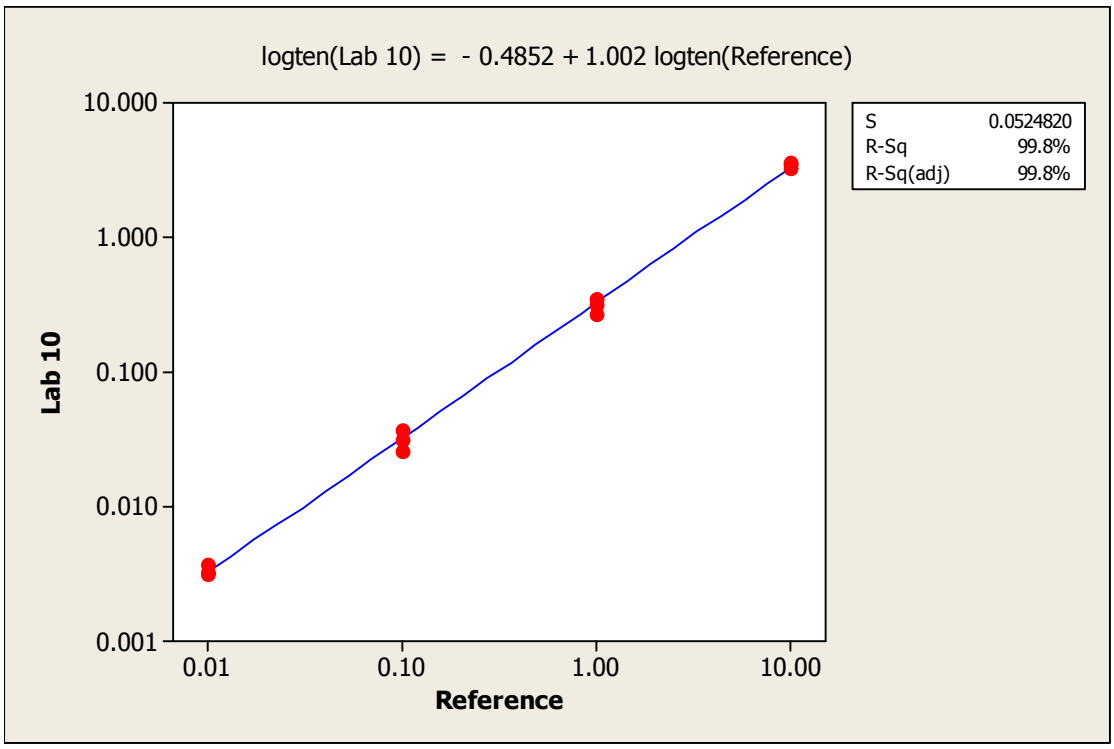


b) KG1 *ABL*

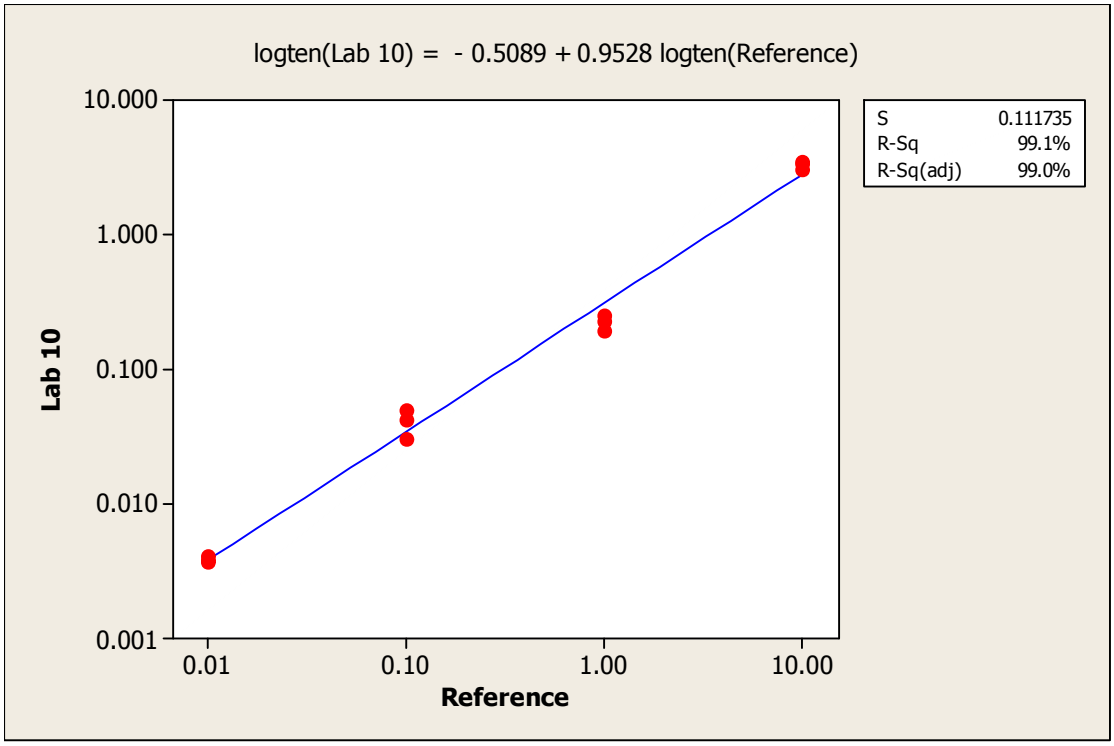


Lab 10

a) HL60 *BCR*

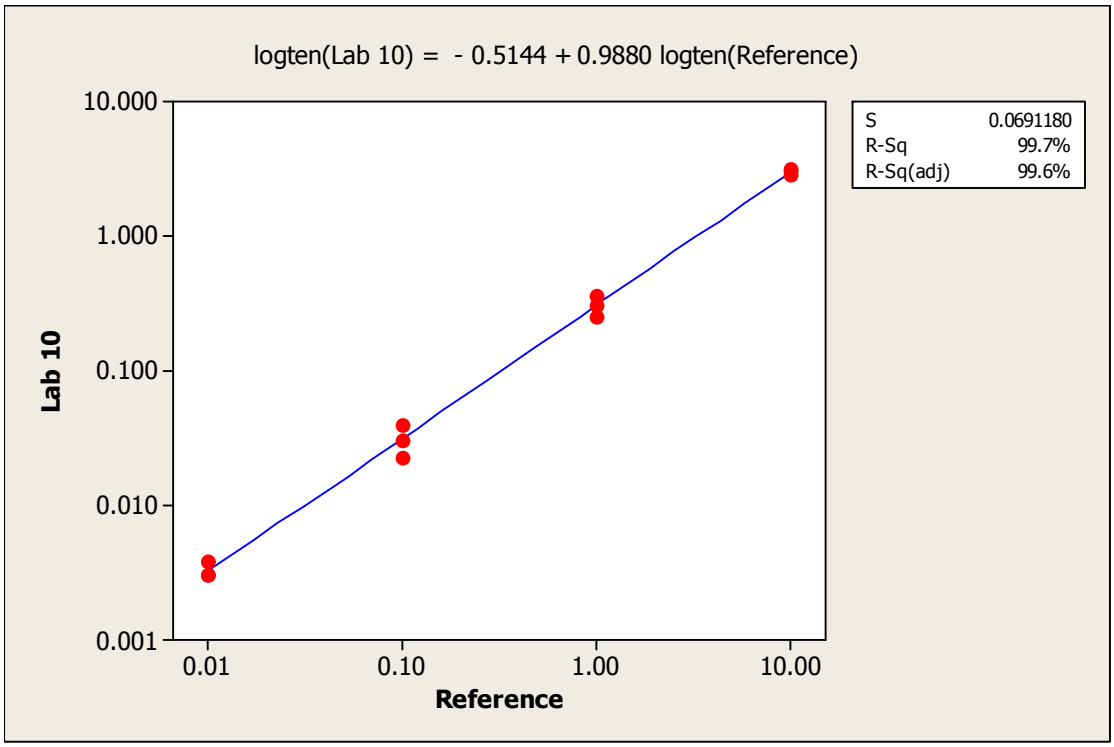


b) KG1 *BCR*

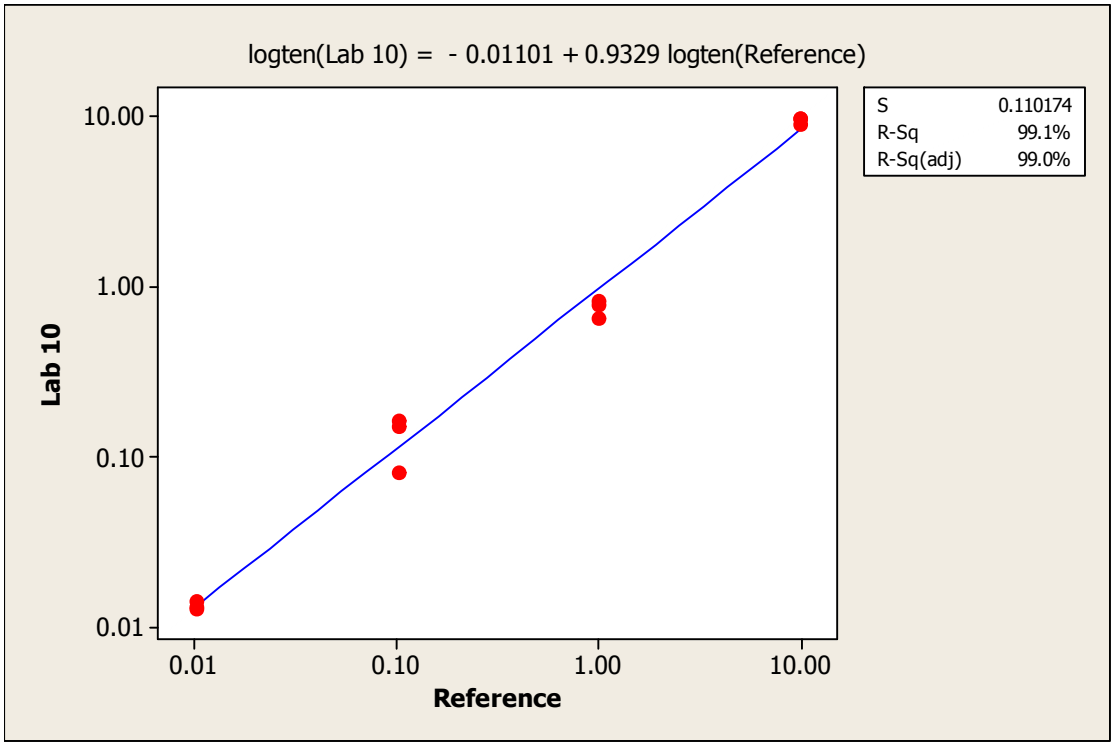


Lab 10

a) HL60 *GUSB*

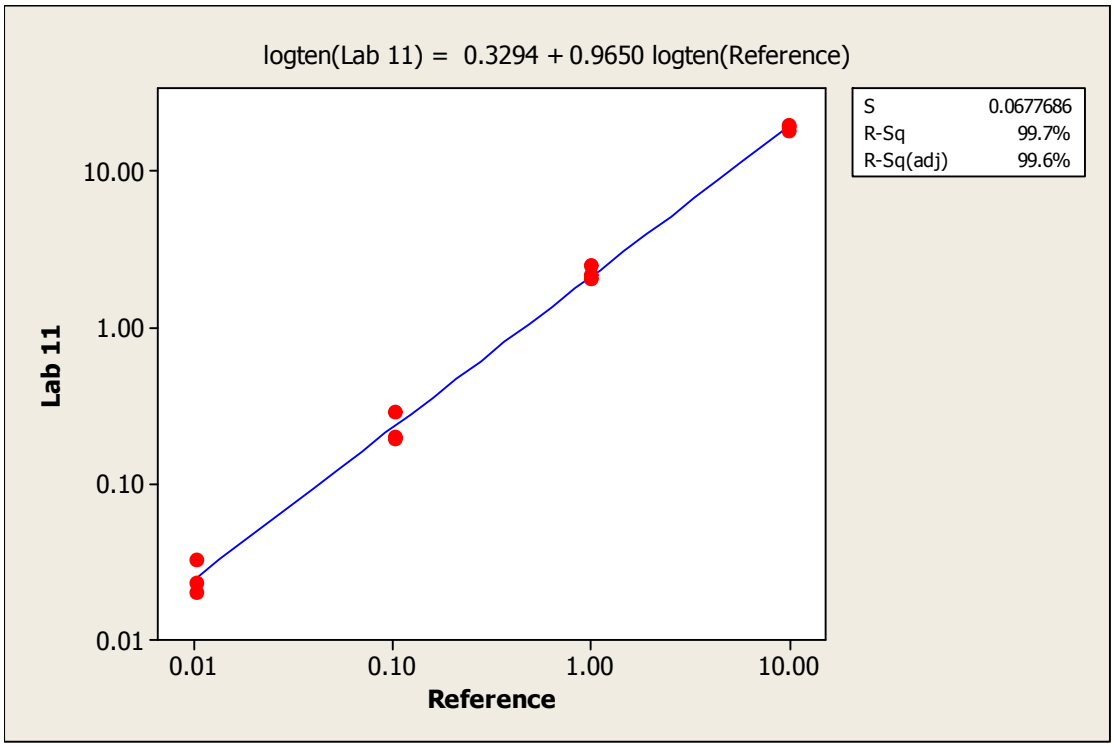


b) KG1 *GUSB*

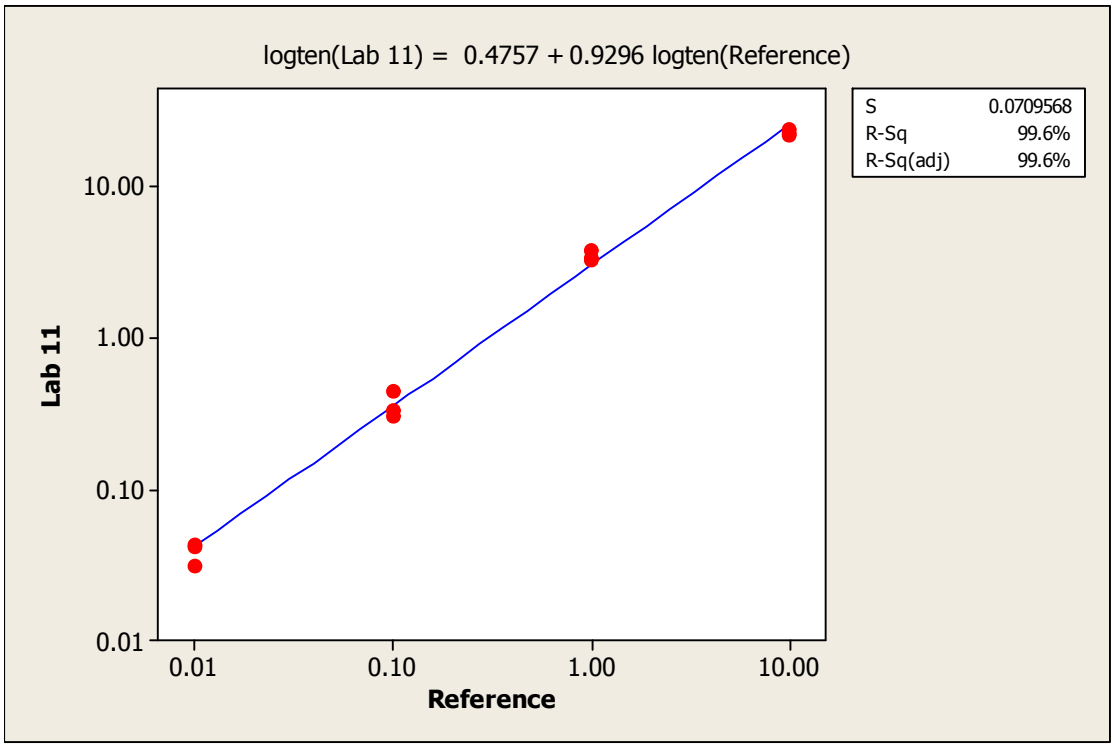


Lab 11

a) HL60 *ABL*

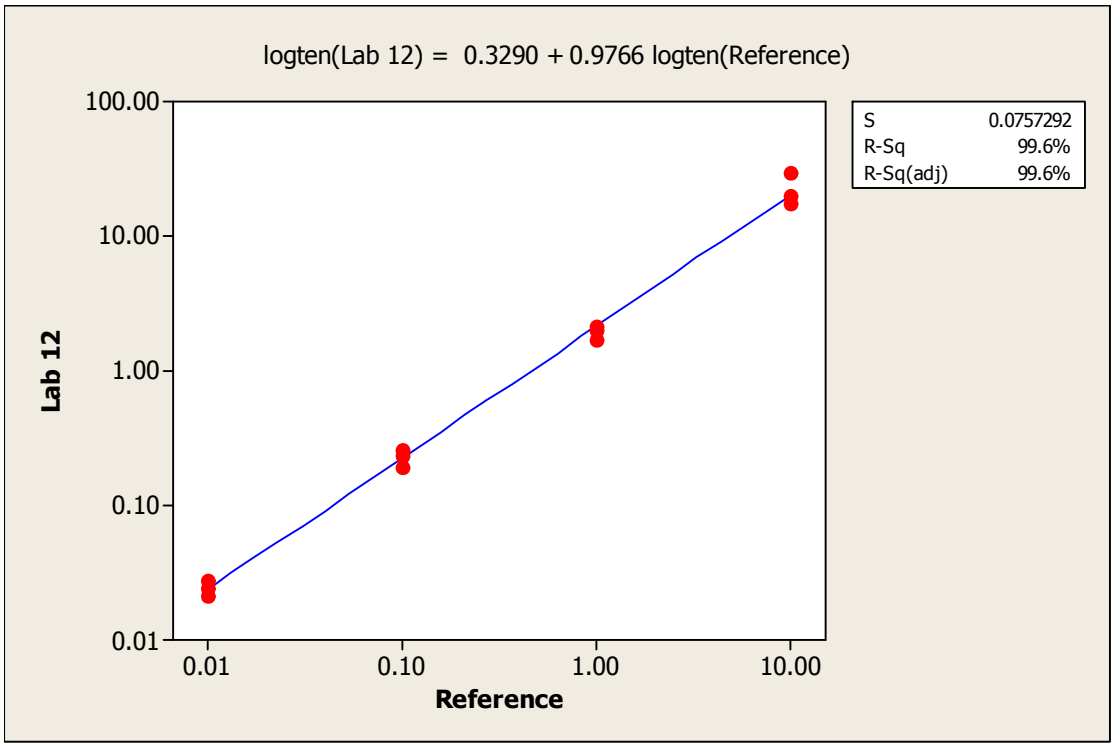


b) KG1 *ABL*

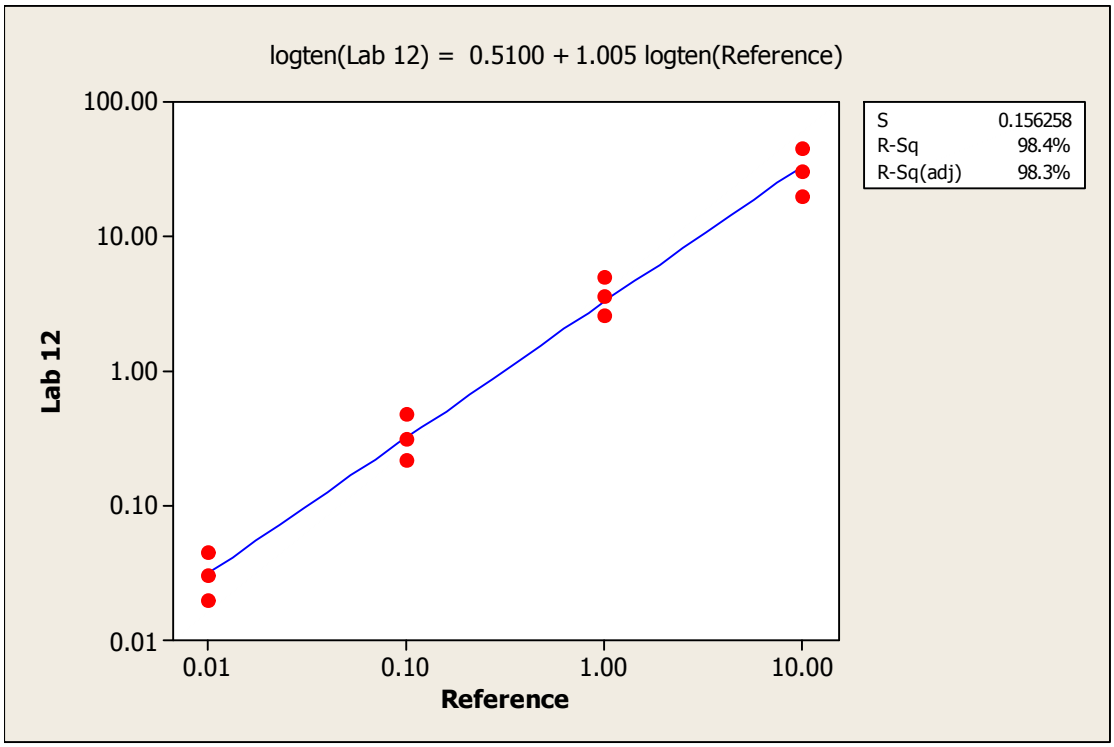


Lab 12

a) HL60 *ABL*

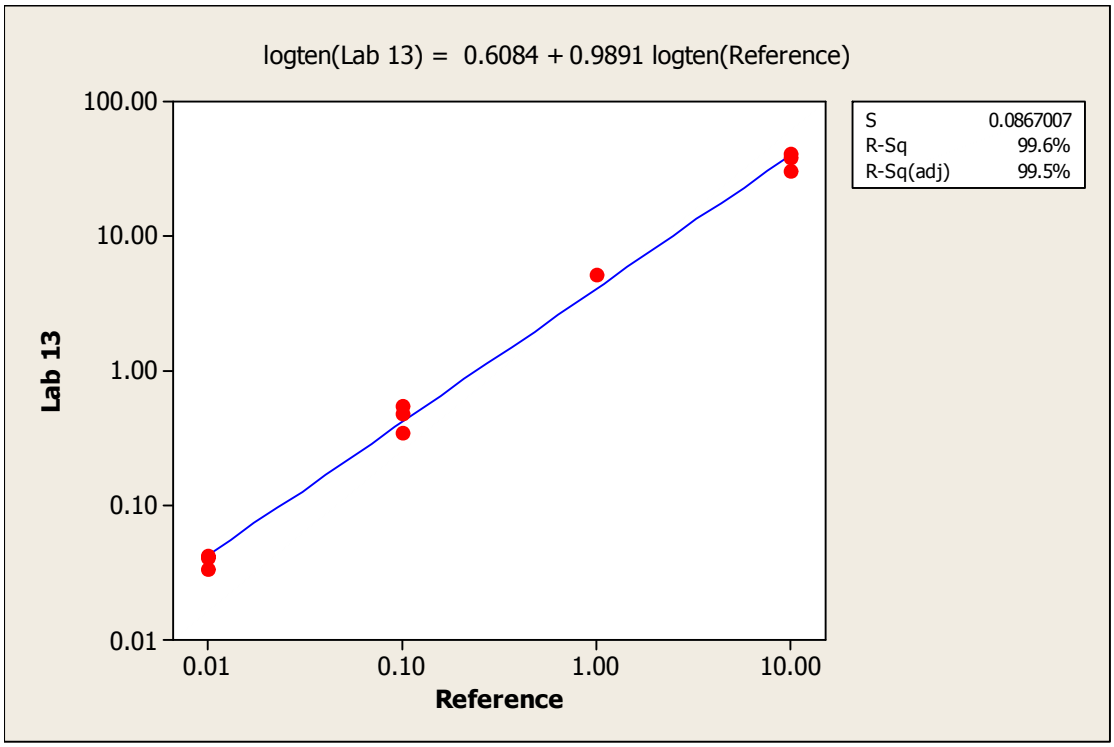


b) KG1 *ABL*

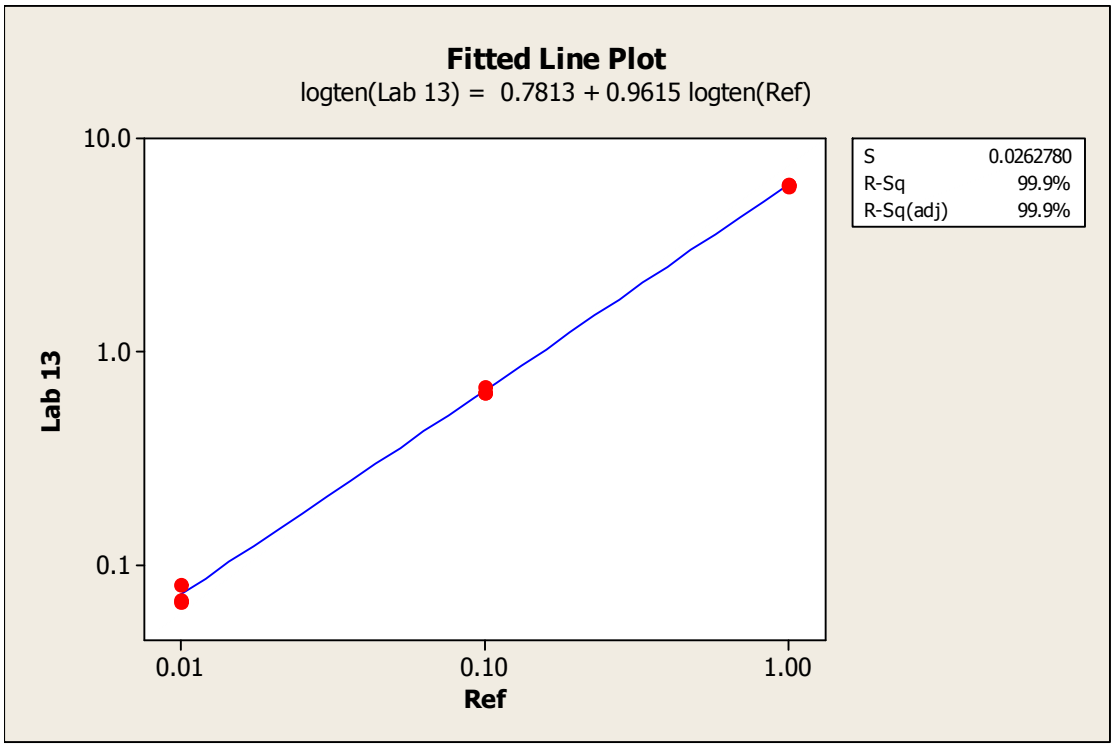


Lab 13

a) HL60 *ABL*

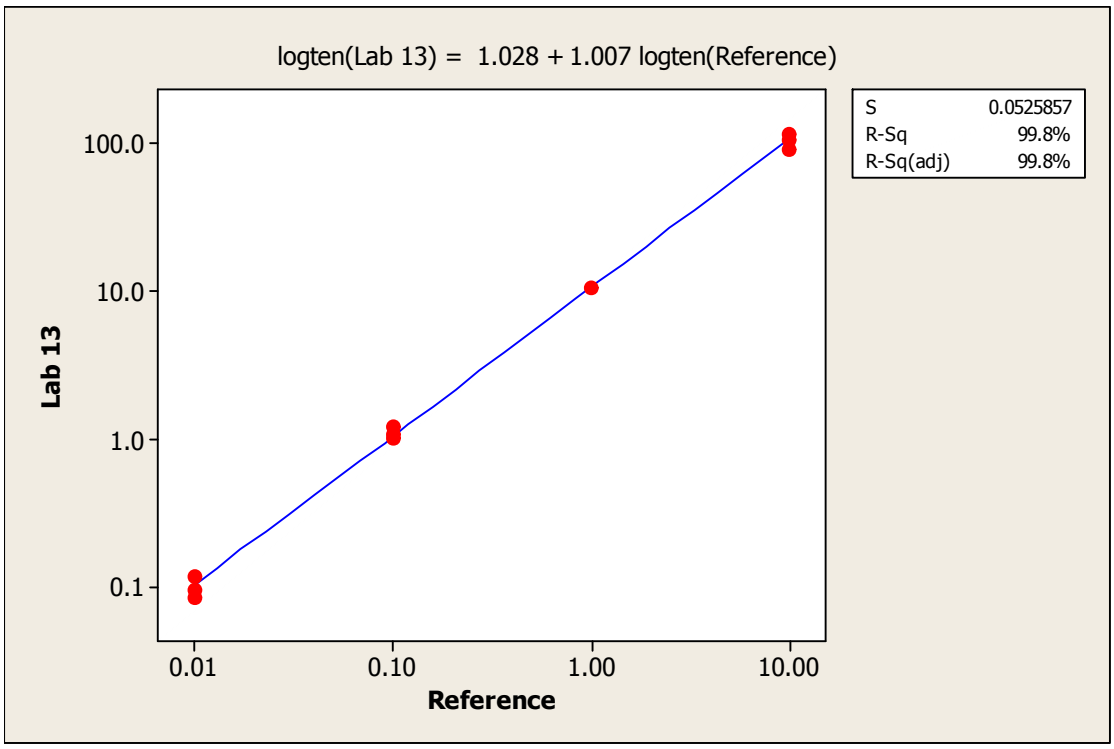


b) KG1 *ABL*

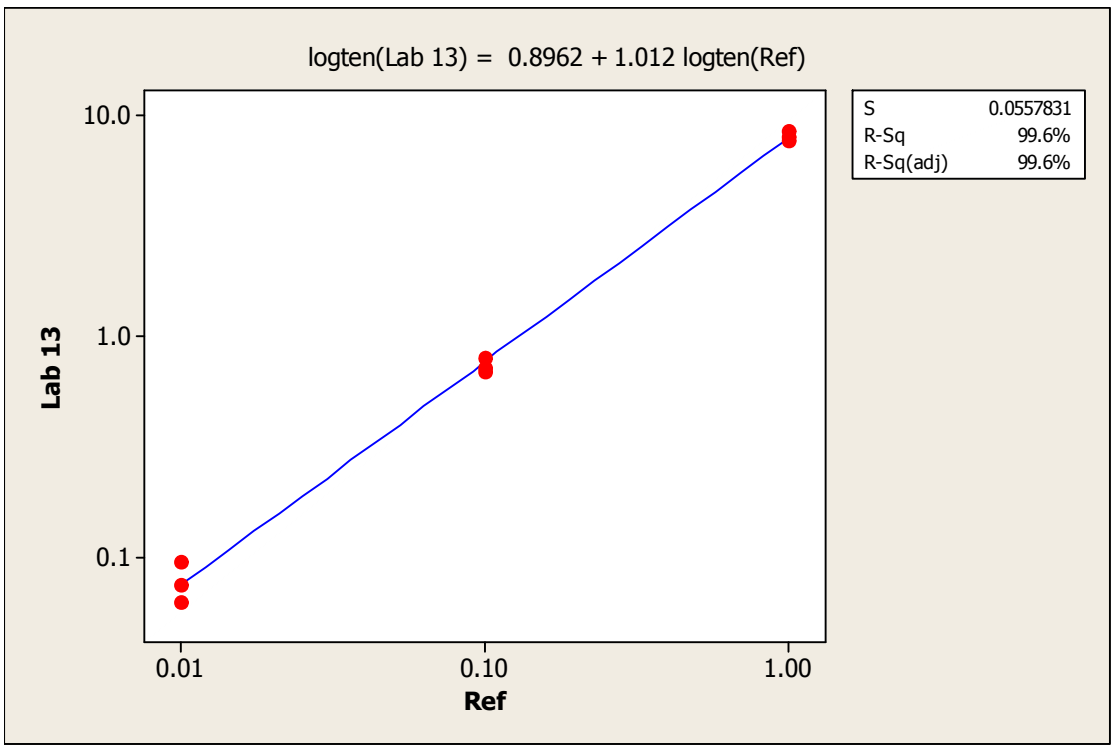


Lab 13

a) HL60 *BCR*

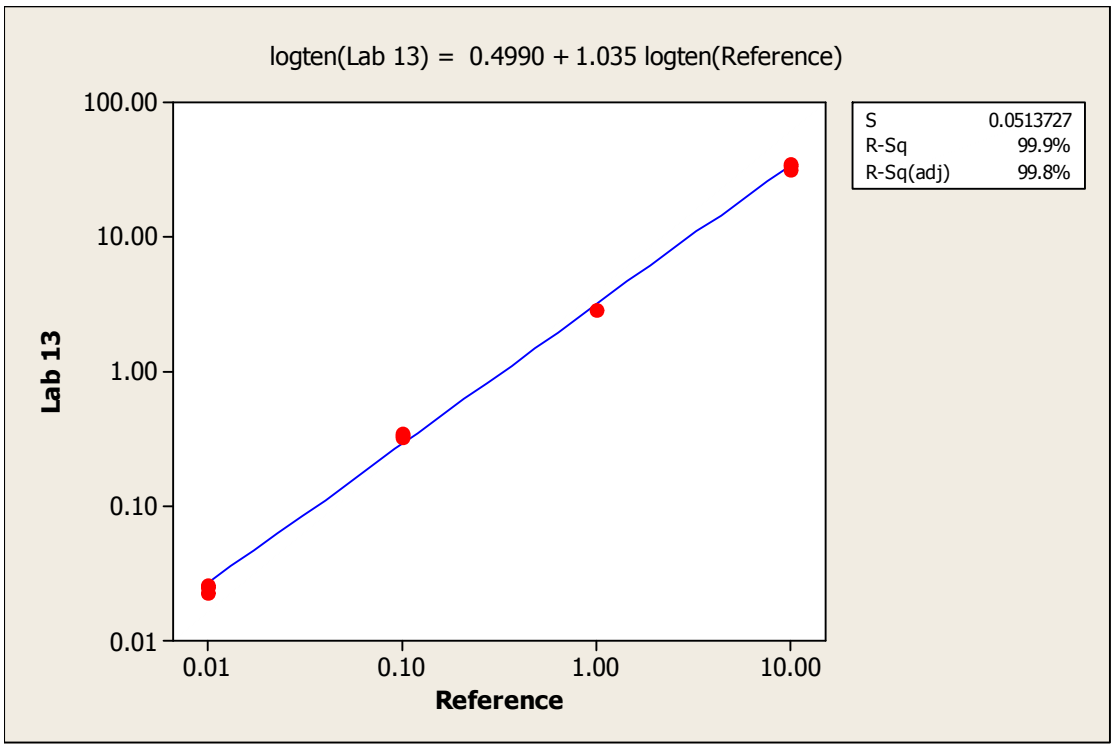


b) KG1 *BCR*

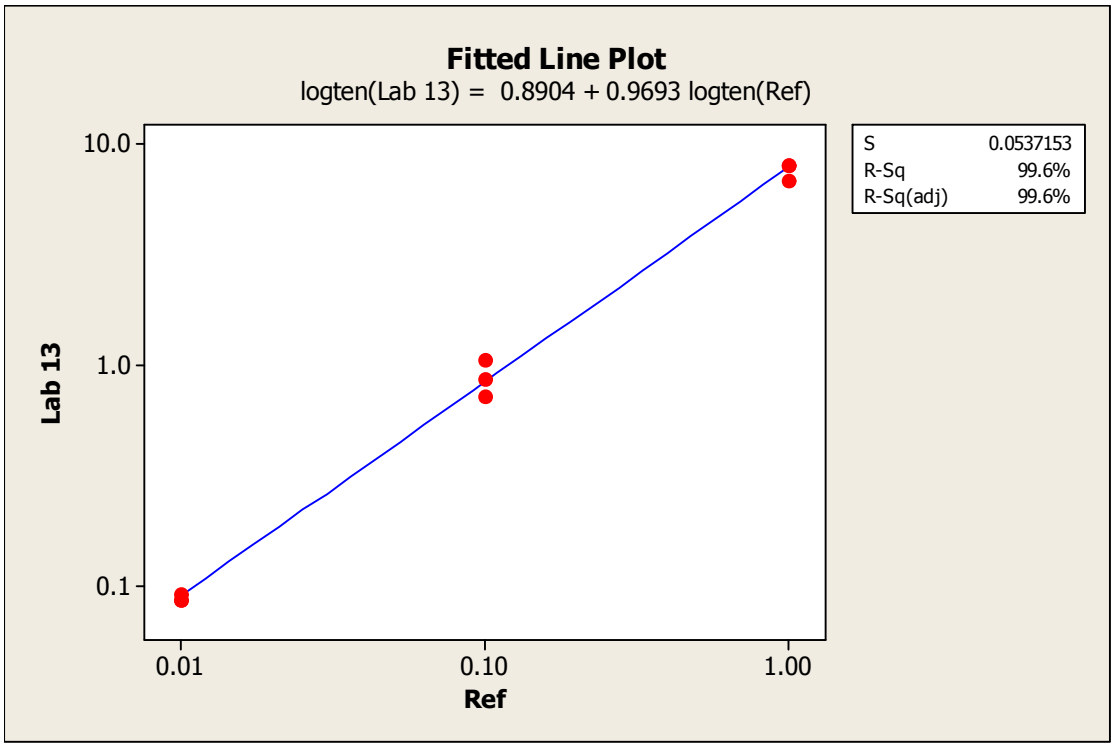


Lab 13

a) HL60 *GUSB*

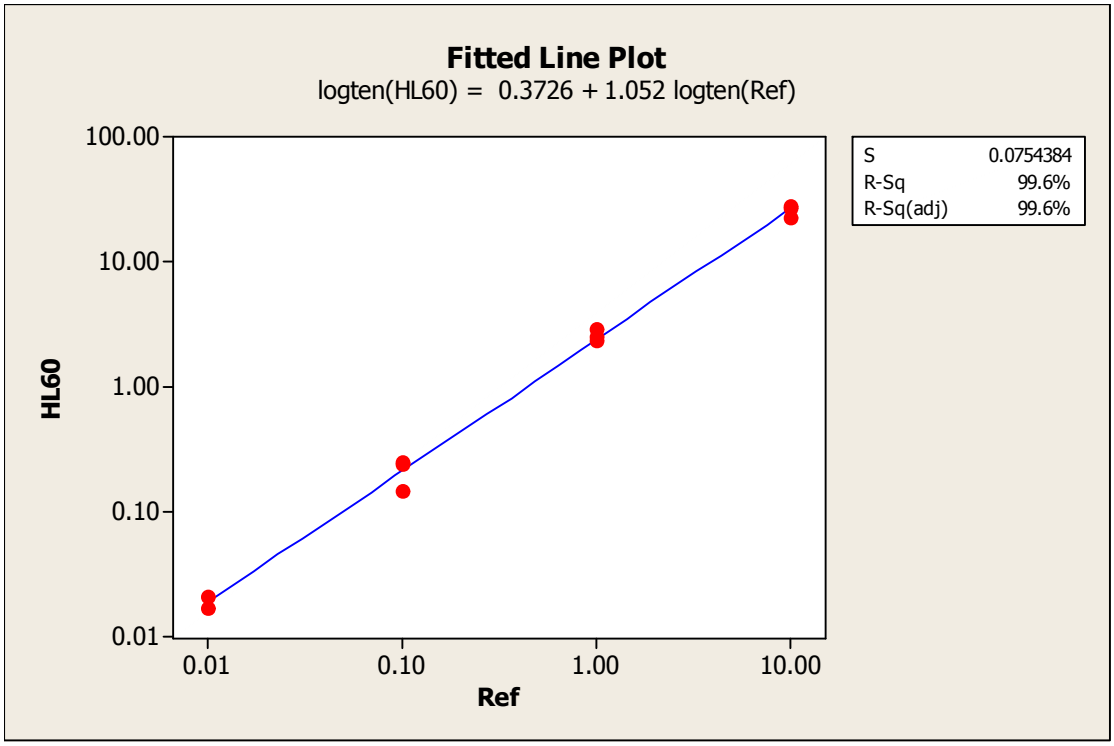


b) KG1 *GUSB*

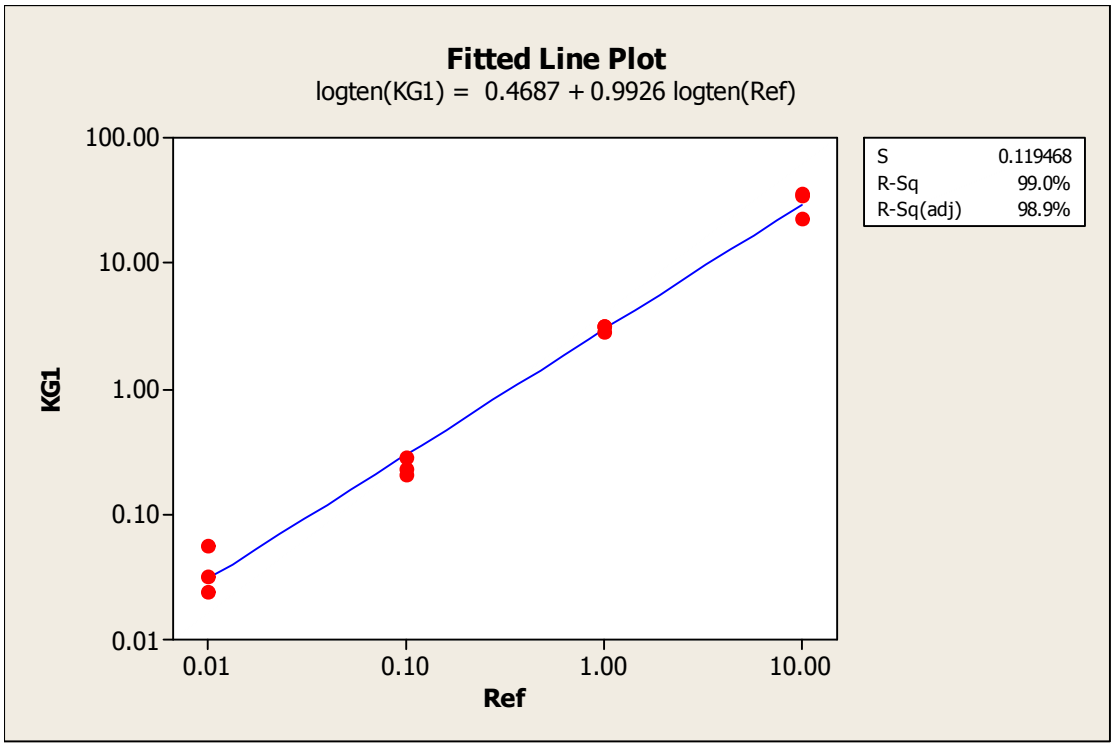


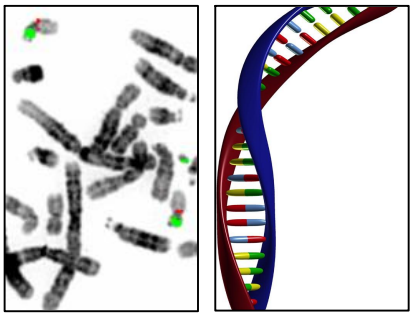
Lab 14

a) HL60 *G6PD*



b) KG1 *G6PD*





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