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## INTRODUCTION

*BCR-ABL1* measurement by RT-qPCR is integral to both the diagnosis and monitoring of Chronic Myeloid Leukaemia (CML), and has been central to the remarkable improvement in patient outcomes seen in this disease. As a provider of External Quality Assessment (EQA)/Proficiency Testing (PT) in this area, UK NEQAS for Leucocyte Immunophenotyping (UKNEQAS LI) has a unique perspective on the changing face of *BCR-ABL1* testing in CML with access to participants' technical data.

In order to assess the impact of technical standardisation and the development of the International Scale (IS) on the accuracy of *BCR-ABL1* testing, we have reviewed our EQA trial data from 2007-2015.

## METHOD(S)

Three sets of EQA samples are issued to participants per annum with each comprising two lyophilised cell line samples containing a mix of *BCR-ABL1* (e14a2) positive K562 cells in a background of *BCR-ABL1* negative HL60 cells.

K562 and HL60 cell lines were obtained from DSMZ (Braunschweig, Germany). HL60 and K562 cultures tested negative for HIV, hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human T-lymphotropic virus type I and II (HTLV-I/II), human herpes virus 8 (HHV-8), and mycoplasma by PCR. Cell lines were grown in RPMI 1640 (Gibco ThermoFisher) medium supplemented with 10% fetal bovine serum (Gibco ThermoFisher). Pre-defined dilutions of K562 cells in HL60 are then prepared and supplemented with Human Albumin Serum and Adonitol (Sigma-Aldrich). 1mL aliquots containing 10-20x10<sup>6</sup> cells are transferred into 3mL glass ampoules for freeze drying. Samples are pre cooled at -50°C for 24h before being placed in the freeze drier at a temperature of -50°C and a vacuum of 10mbar is applied. Samples are dried for 24 hours. Samples are then distributed to participants at ambient temperature; there are currently 190 active participants in UKNEQAS LI's *BCR-ABL1* Quantification programme.

Participants in the programme are asked to measure the *BCR-ABL1*/Reference Gene levels in each sample using their normal laboratory protocols and to report the results (either converted to the IS, unconverted or both) as well as certain methodological details.

The data used in this study is taken from trial data submitted to UKNEQAS LI since the programme's inception in 2007 (21 trial issues, 42 samples). The transient inclusion of HEL and Jurkat cell-lines alongside K562 and HL60 cells in a number of trial samples means that the variance of these samples cannot be meaningfully compared to the 'standard' cell line mix of K562 and HL60, thus only 32 samples (from 16 trials), manufactured with K562 and HL60s only, are included in this study.

As the nature of error in RT-qPCR is multiplicative, rather than additive, data distributions from RT-qPCR based EQA/PT testing programmes produce a lognormal distribution; an asymmetric distribution of results with a strong positive skew.

As such, in order to normalise the distribution to allow the calculation of dispersion using parametric methods, log transformation was required. Log<sub>10</sub>, log<sub>2</sub> and natural log transformation were all applied to our data; producing data sets of indistinguishable distribution thus log<sub>10</sub> was applied to all. As log transformed results of below one produce a negative result, and those above one produce a positive result, we applied a constant of one to all participants *BCR-ABL1* results prior to transformation.

Co-efficient of variation ( $cv = \sigma/\mu$ ) was used to compare dispersion of participant results for different samples, as it is scale insensitive and allows the comparison of dispersion between samples with vastly different means. SDs were used to compare dispersion of unconverted and IS data from the same sample as these did not have vastly different means. Whilst dispersion was calculated using log transformed data, where a meaningful data midpoint was required medians were used as log transformed means (geometric means) are difficult to interpret, and back transforming log transformed means is not recommended as it leads to underestimation.

## RESULT(S)

As shown in Fig 1, UKNEQAS LI have dispatched 32 samples that can be meaningfully compared at dilution levels of 0.002%-5% K562 cells in a HL60 background, giving a median % *BCR-ABL1* IS calculated from all participants' results ranging from 0.01-16.7 (R<sub>2</sub> = 0.94). Distortions away from linearity due to the underestimation by participants of *BCR-ABL1* IS ~10% were evident in this data set (Fig 1). When variation was assessed in UK NEQAS LI's data set at differing levels of *BCR-ABL1* transcript (Fig 2) it can be seen that it increases with lower transcript levels, particularly below 1% *BCR-ABL1* IS.

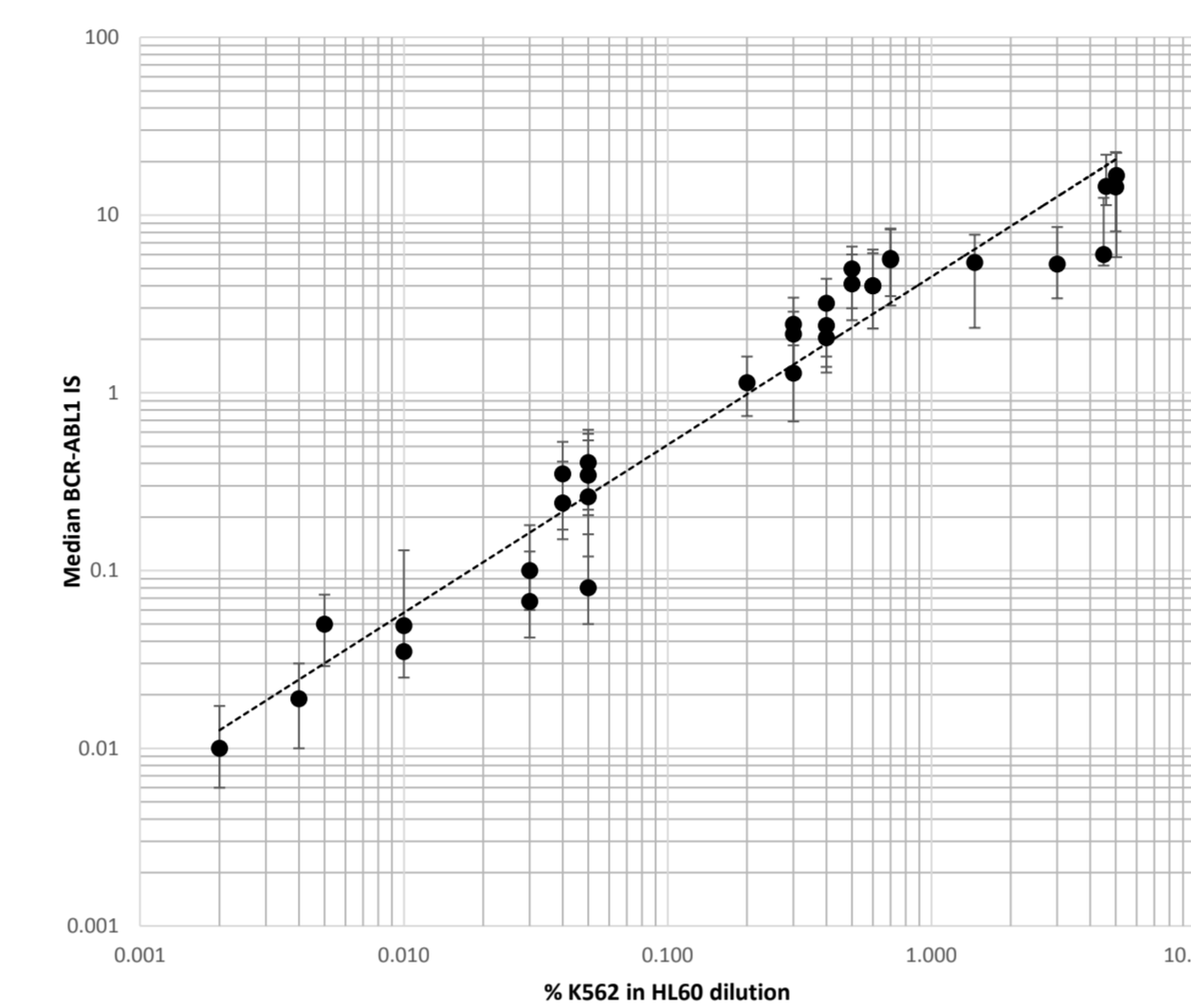


Figure 1: XY plot demonstrating the correlation between K562 dilution and consensus median *BCR-ABL1* IS values.

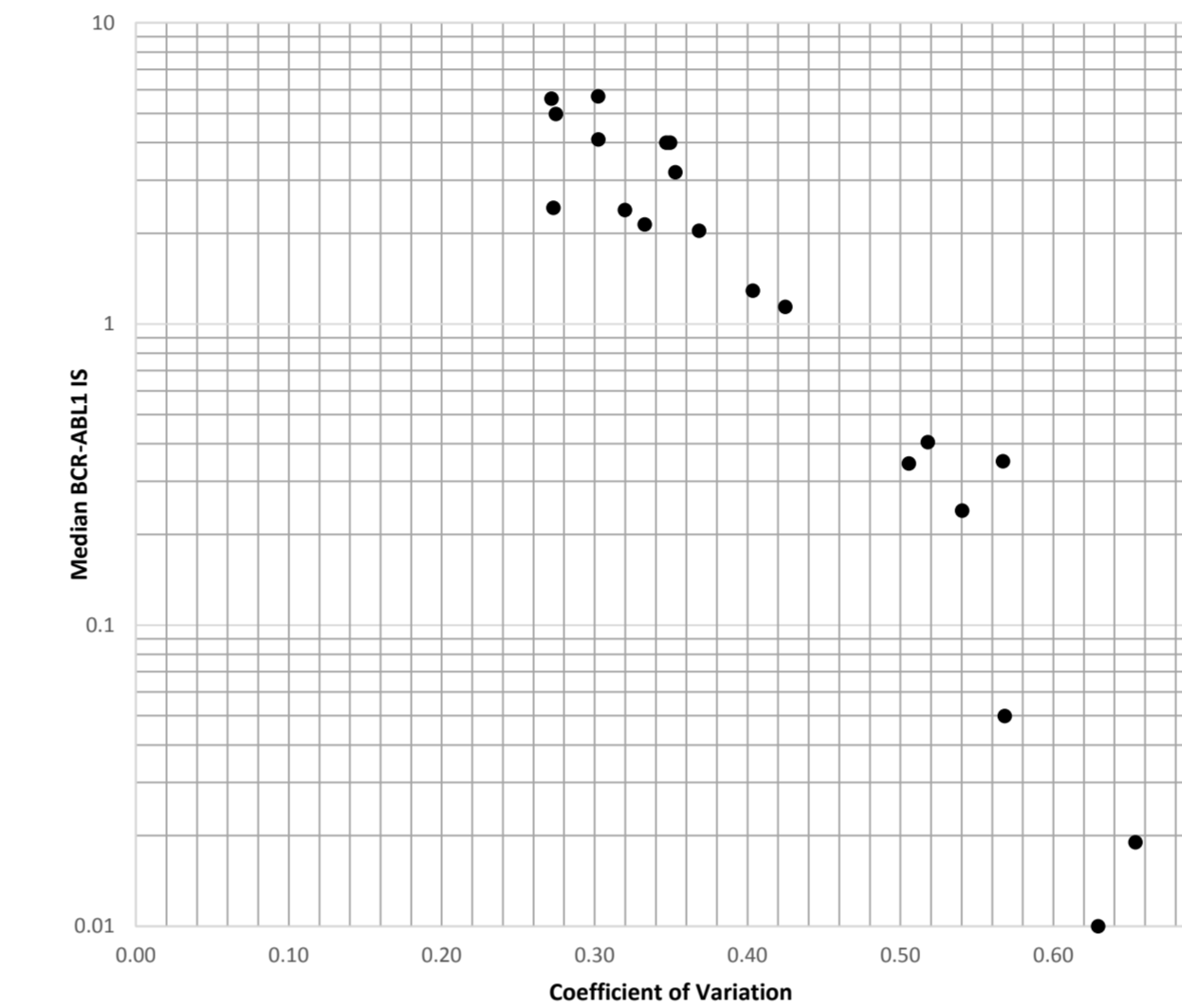


Figure 2: XY plot demonstrating the correlation between consensus median *BCR-ABL1* IS values and coefficient of variation

For participants who submitted both unconverted and IS results, the standard deviation in both data sets was compared. In all samples the IS standard deviation was lower than the unconverted, suggestive of less variability; however, when an F test was performed no significant difference could be identified ( $P > 0.05$ ).

UKNEQAS LI have observed and reported in multiple trial reports that different methods of converting to the IS produce consistently different median *BCR-ABL1* IS results trial-on-trial. With over 20 different methods of converting to the IS reported to UKNEQAS LI we have graphically displayed only those methods used by greater than 5 participants in all trials analysed (Fig 3). A one-way ANOVA confirmed that the differences observed are statistically significant ( $P < 0.05$ ). The median results provided by participants using the Qiagen Ipsogen IS MMR kit are the highest followed by the Napoli conversion factor, with the Mannheim conversion factor and Cepheid GeneXpert assay giving the lowest results. Across all trials analysed, the Qiagen Ipsogen IS MMR kit gives results between 2.0 and 7.3x higher than the Cepheid GeneXpert (median 2.8x).

Despite the large number of instruments and kits commercially available all assays have basically performed well, aside from participants using Roche Lightcycler in conjunction with the *G6PDH* Roche kit who failed to detect *BCR-ABL1* in a number of low level samples.

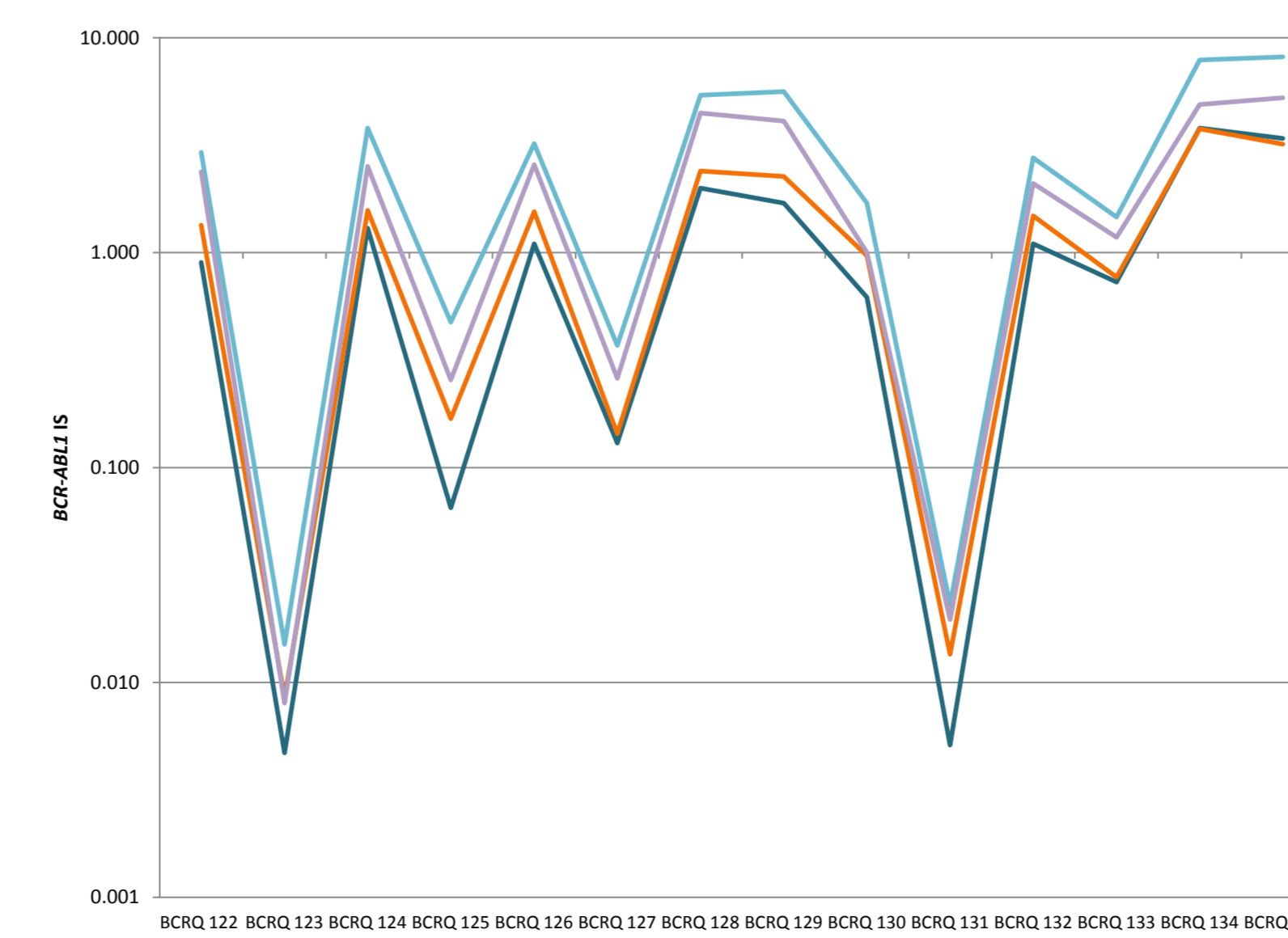


Figure 3: Line plot to show the median *BCR-ABL1* IS results submitted to UKNEQAS, by method of converting to the IS

## CONCLUSION(S)

As can be seen in Fig 1 and 2 measurement inaccuracies exist at both high and low levels of *BCR-ABL1* quantification. It was previously predicted that *BCR-ABL1* was being underestimated at levels >10% (1) and we have confirmed this finding in our dataset (Fig 1). This phenomenon, caused by the primers designed to amplify the *ABL1* transcript, also amplifying the *BCR-ABL1* transcript, has historically been dismissed (1), as it was thought that when patients express very high levels of disease precise measurement was of limited clinical importance. However, with a glut of publications in recent years advocating *BCR-ABL1* IS levels at 3 months (2), or *BCR-ABL1* halving times from diagnosis (3), as the strongest predictors of prognosis this would no longer seem to be the case. If 'Early Molecular Response (EMR)' is to become a key predictor of response, it would seem the underestimation of *BCR-ABL1* ~10%, shown in our data, takes on greater importance.

Prior to the conception of 'EMR', the main goal of the treatment of CML with TKIs has been the achievement of the 'major molecular response ( $MMR \leq 0.1$  *BCR-ABL1* IS)'. In line with previous publications (1), variation in our EQA data sets is inversely proportional to transcript levels, and this is particularly marked in samples with <1% *BCR-ABL1* IS, inferring an increased risk of laboratories misclassifying the success/failure of treatment at the key clinical decision point of 0.1% *BCR-ABL1* IS.

The lower SDs in our IS data sets, compared to our unconverted data sets, are encouraging; however, the inability to prove these differences in variability are statistically significant show that the impact of the IS has been limited. Yet more intriguing is the phenomenon whereby different modes of converting to the IS (whether it be conversion factor or commercial kit based) are producing consistently different median results (Fig 3). Whilst there is no obvious mechanism behind this; this information points to imperfections in the process of converting to the IS.

Our data suggests that in order to fully realise the benefits of improved treatment protocols further refinement of RT-qPCR protocols, or perhaps a switch to new technologies such as droplet digital PCR, to allow better quantification of 'Early' and 'Deep' Molecular Response will be required.

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