



Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/175858/>

Version: Published Version

Article:

Scott, S., Cartwright, A., Francis, S. et al. (2021) Assessment of droplet digital polymerase chain reaction for measuring BCR-ABL1 in chronic myeloid leukaemia in an international interlaboratory study. *British Journal of Haematology*, 194 (1). pp. 53-60. ISSN: 0007-1048

<https://doi.org/10.1111/bjh.17521>




Reuse

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Assessment of droplet digital polymerase chain reaction for measuring *BCR-ABL1* in chronic myeloid leukaemia in an international interlaboratory study

Stuart Scott,^{1,2}  Ashley Cartwright,¹ Sebastian Francis,³ Liam Whitby,¹ A. Pia Sanzone,⁴ André Mulder,⁵ Sara Galimberti,⁶ Stephanie Dulucq,^{7,8}  Carole Mauté,⁹ Calogero Lauricella,¹⁰ Matthew Salmon,^{11,12} Susan Rose,¹³ Josh Willoughby,¹⁴ Nancy Boeckx,^{15,16} Niels Pallisgaard,¹⁷ Jacqueline Maier,¹⁸ Elisabeth O. Leibundgut,¹⁹ Hana Zizkova,²⁰ Lih Ling Goh,²¹ Chinh Duong,^{22,23} Wing F. Tang,²⁴ Edmond Ma,^{25,26}  Yogesh Shivakumar,²⁷ Lan Beppu,²⁸ Prasanthi Bhagavatula²⁹ and Andrew Chantry^{2,3}

¹Sheffield Teaching Hospital NHS Foundation Trust, UK National External Quality Assessment Services (NEQAS) for Leucocyte Immunophenotyping, ²Faculty of Medicine Dentistry and Health, Department of Oncology and Metabolism, University of Sheffield, ³Department of Haematology, Sheffield Teaching Hospitals NHS Foundation Trust, ⁴Advanced Therapies, National Institute for Biological Standards and Control, Potters Bar, UK, ⁵Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, Netherlands, ⁶Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, ⁷Laboratory of Hematology, University Hospital Centre Bordeaux, Pessac, ⁸University of Bordeaux, INSERM, U1218, Bordeaux, ⁹Haematology Laboratory, Hôpital Saint-Louis, Paris, France, ¹⁰Department of Laboratory Medicine, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy, ¹¹Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury, ¹²University of Southampton, School of

Summary

Measurement of BCR activator of RhoGEF and GTPase -ABL proto-oncogene 1, non-receptor tyrosine kinase (*BCR-ABL1*) mRNA levels by reverse transcription quantitative polymerase chain reaction (RTqPCR) has been critical to treatment protocols and clinical trials in chronic myeloid leukaemia; however, interlaboratory variation remains a significant issue. Reverse transcriptase droplet digital PCR (RTddPCR) has shown potential to improve testing but a large-scale interlaboratory study is required to definitively establish this. In the present study, 10 *BCR-ABL1*-positive samples with levels ranging from molecular response (MR)^{1.0}–MR^{5.0} were tested by 23 laboratories using RTddPCR with the QXDX BCR-ABL %IS kit. A subset of participants tested the samples using RTqPCR. All 23 participants using RTddPCR detected *BCR-ABL1* in all samples to MR^{4.0}. Detection rates for deep-response samples were 95.7% at MR^{4.5}, 78.3% at MR^{4.7} and 87.0% at MR^{5.0}. Interlaboratory coefficient of variation was indirectly proportional to *BCR-ABL1* level ranging from 29.3% to 69.0%. Linearity ranged from 0.9330 to 1.000 (average 0.9936). When results were compared for the 11 participants who performed both RTddPCR and RTqPCR, RTddPCR showed a similar limit of detection to RTqPCR with reduced interlaboratory variation and better assay linearity. The ability to detect deep responses with RTddPCR, matched with an improved linearity and reduced interlaboratory variation will allow improved patient management, and is of particular importance for future clinical trials focussed on achieving and maintaining treatment-free remission.

Keywords: *BCR-ABL1*, external quality assessment (EQA), Quality, CML, RTddPCR.

Medicine, Southampton, ¹³West Midlands Regional Genetics Laboratory, Birmingham Women's and Children's Hospitals NHS Foundation Trust, Birmingham, ¹⁴Sheffield Diagnostic Genetics Service, Sheffield Children's Hospital NHS Foundation Trust, Sheffield, UK, ¹⁵Department of Laboratory Medicine, University Hospitals Leuven, Leuven, ¹⁶Department of Oncology, KU Leuven, Leuven, Belgium, ¹⁷Department of Surgical Pathology, Zealand University Hospital, Roskilde, Denmark, ¹⁸Department of Transfusion Medicine, University Hospital Leipzig, Leipzig, Germany, ¹⁹Department of Haematology, Inselspital, Bern University Hospital, Bern, Switzerland, ²⁰Department of Molecular Genetics, Institute of Hematology and Blood Transfusion, Praha, Czech Republic, ²¹Personalized Medicine Service, Tan Tock Seng Hospital, Singapore, Singapore, ²²Genetics and Molecular Biology, National Institute of Hematology and Blood Transfusion, Hanoi, ²³Department of Hematology and Blood Transfusion, University of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam, ²⁴Department of Pathology, Division of Haematology, Queen Mary Hospital, ²⁵Department of Pathology, The University of Hong Kong, ²⁶Pathology, Hong Kong Sanatorium and Hospital, Hong Kong, Hong Kong, ²⁷Department of Genomics, Strand Life Sciences, Bangalore, India, ²⁸Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, and ²⁹Digital Biology Group, Bio-Rad, Pleasanton, CA, USA

Received 6 January 2021; accepted for publication 21 March 2021

Correspondence: Stuart Scott, UK NEQAS for Leucocyte Immunophenotyping, Pegasus House, 4th Floor Suite, 463a Glossop Road, Sheffield S10 2QD, UK.
E-mail: stuart.scott@ukneqasli.co.uk

Introduction

Sequential measurement of BCR activator of RhoGEF and GTPase-ABL proto-oncogene 1, non-receptor tyrosine kinase

(*BCR-ABL1*) mRNA levels by reverse transcription quantitative polymerase chain reaction (RTqPCR) is now embedded in standard patient management protocols for chronic myeloid leukaemia (CML)¹ and is a validated surrogate outcome

for traditional clinical outcomes in trials of new therapies for patients with CML.² It has played a seminal role in the remarkable improvement in patient survival seen in this disease, where the majority of patients with chronic phase disease now have a normal life expectancy.³

This improvement in life expectancy has led to a change in emphasis for the clinicians and researchers treating CML, from extending patient survival to curing the disease. Current aims for treatment in CML are to achieve a faster molecular response (MR), preventing progression to blastic phase disease allowing a stable deep MR and tyrosine kinase inhibitor (TKI) treatment discontinuation for treatment-free remission (TFR).^{4,5} With RTqPCR being used to monitor the speed of response, and define eligibility for TFR ever more accurate and precise measurement of *BCR-ABL1* is desirable, to ensure that these important treatment decisions are made on the best data available with optimal comparability between laboratories. Efforts to standardise the current 'gold standard' approach for *BCR-ABL1* measurement, RTqPCR,^{6,7} including the development of the International Scale (IS),^{8–10} has reduced inter- and intralaboratory variation in *BCR-ABL1* measurement;¹¹ however, the inherent complexity of RTqPCR means that inter- and intralaboratory variation remains a significant issue for laboratories,^{8,11} that still needs to be addressed.

It has been speculated that reverse transcriptase droplet digital PCR (RTddPCR) may overcome some of the variability inherent to RTqPCR, particularly when measuring very low *BCR-ABL1* transcript levels. RTddPCR separates a bulk PCR reaction into thousands of droplet-based single molecule reactions, allowing a more accurate quantification of the number of target molecules. In contrast to RTqPCR, RTddPCR does not rely on the indirect relationship between the amplification of the *BCR-ABL1* transcript in patient samples and a calibration curve to quantify *BCR-ABL1* levels, a complex approach that is vulnerable to potential error. Instead, it is a direct measurement of the number of copies of the target molecule. Encouragingly, initial studies have displayed a lower limit of detection (LOD) and limit of quantification compared to RTqPCR.^{12–15} The Life After Stopping TKIs (LAST) study showed that the superior LOD of RTddPCR over RTqPCR may allow for less frequent monitoring, resulting in less hospital visits for patients.¹⁶ Large-scale, multicentre studies are required to assess if the benefits of RTddPCR shown in single-centre studies can be replicated in a multicentre study.

Methods

A total of 10 cell-line based *BCR-ABL1* positive samples (labelled A–J in a random order) with *BCR-ABL1* levels ranging from MR^{1.0} (10% *BCR-ABL1*^{IS})–MR^{5.0} (0.001% *BCR-ABL1*^{IS}) were manufactured from a mixture of *BCR-ABL1* (e14a2)-positive K562 cells in a background of *BCR-ABL1*-negative HL60 cells. K562 and HL60 cell lines were obtained from the

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The HL60 and K562 cultures tested negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), human T-lymphotrophic 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 (Thermo Fisher, Waltham, USA) medium supplemented with 10% fetal bovine serum (Thermo Fisher). Pre-defined dilutions of K562 cells in HL60 cells were then prepared and freeze dried in 3-ml glass ampoules to contain 9×10^6 cells. Samples were dried for 24 h. Prior to distribution, to establish that the manufactured samples accurately represented the target values, and to ensure sample quality and homogeneity, a minimum of three selected samples were subjected to (phenol chloroform) RNA extraction, complementary DNA (cDNA) synthesis and *BCR-ABL1* quantification. This was performed using the Qiagen Ipsogen *BCR-ABL1* Mbc kit (Qiagen, Hilden, Germany) and the Applied Biosystems 7500 RQ-PCR machine (Thermo Fisher) and the QXDX *BCR-ABL1* %IS kit (Bio-Rad, Hercules, CA, USA) and QX200 Auto DG system (Bio-Rad). Data analysis was performed in line with UK recommendations.¹⁷ Sample quality was defined as RNA OD260/280 ratio of between 1.8 and 2.2 and *ABL1* levels >100 000/replicate. The percentage *BCR-ABL1*^{IS} results from pre issue testing were required to be within 1.2-fold of the target value, as this is approaching the degree of reproducibility seen within laboratories^{8,18} (Table SI). Stability of trial samples was ensured by measuring *ABL1* levels on a further three vials at trial closure. Samples A–G were formulated to contain *BCR-ABL1* levels between MR^{1.0} (10% *BCR-ABL1*^{IS})- and MR^{4.0} (0.01% *BCR-ABL1*^{IS})- at half log intervals, important to detect within standard treatment protocols. Samples H–J represented 'deep-response' levels [MR^{4.5} (0.0033% *BCR-ABL1*^{IS})-, MR^{4.7} (0.002% *BCR-ABL1*^{IS})- and MR^{5.0} (0.001% *BCR-ABL1*^{IS})-], important to detect in treatment discontinuation protocols.

The samples were shipped at ambient temperature to 26 study participants in 15 countries in Europe, Asia and North America. All participants in the study were asked to extract RNA and perform reverse transcription using their standard in-house protocol and test the samples with the QXDX *BCR-ABL1* %IS kit, using either the QX200 auto or manual DG system. Results from the QXDX *BCR-ABL1* %IS kit are reported on the IS by using an assay specific conversion factor determined by comparing the assay to an IS reference value. Samples A–G used two reaction wells. Samples H–J used four reaction wells. Participants were asked to independently analyse RTddPCR data using the QuantaSoft (version 1.7.4) and QXDx Reporter Tool software. Where participants were currently reporting *BCR-ABL1* quantification results using RTqPCR, all study samples were run using local methodology for processing and analysis for comparison to the RTddPCR results ($n = 11$).

Table I. Summary of all participants reverse transcriptase droplet digital polymerase chain reaction (RTddPCR) results for samples A–J.

	Sample F (MR ^{1.0})	Sample E (MR ^{1.5})	Sample C (MR ^{2.0})	Sample D (MR ^{2.5})	Sample G (MR ^{3.0})	Sample B (MR ^{3.5})	Sample A (MR ^{4.0})	Sample J (MR ^{4.5})	Sample I (MR ^{4.7})	Sample H (MR ^{5.0})
<i>n</i>	23	23	23	23	22†	23	23	23	23	23
Average*	11.348	4.011	1.265	0.401	0.134	0.039	0.015	0.005	0.003	0.003
SD	3.792	1.211	0.377	0.117	0.041	0.016	0.008	0.003	0.002	0.002
CV, %	33.4	30.2	29.8	29.3	30.5	40.4	52.9	59.7	61.6	69.0

CV, coefficient of variation; MR, molecular response; *n*, number of participant results submitted; SD, standard deviation.

*Average of participant *BCR-ABL*^{IS} results.

†One participant did not return results for sample G as it did not pass local quality control measures.

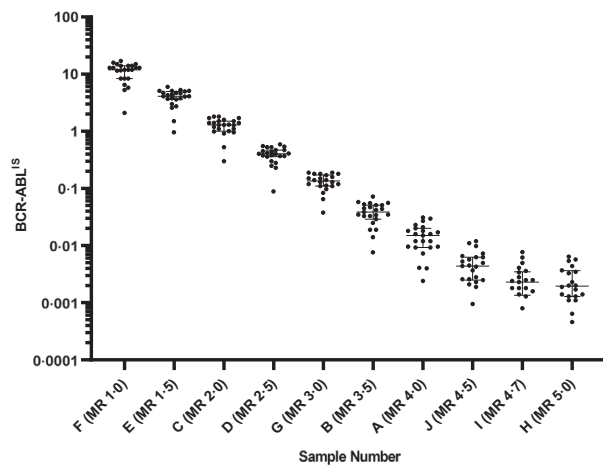


Fig 1. *BCR-ABL*^{IS} results reported for samples A–J for all participants who performed reverse transcriptase droplet digital polymerase chain reaction (RTddPCR). Black dots represent individual participant results. Long horizontal line represents average. Short horizontal line represents standard deviation.

Final *BCR-ABL*^{IS} levels for each sample were then calculated and reported independently by each participant (along with methodological information) using a dedicated online data entry page. Method averages were calculated using an arithmetic mean and significance calculated using a two-tailed *t*-test. A *P* < 0.05 was defined as statistically significant.

Methodological variance was calculated using coefficient of variation (CV) with an *F*-test for two-sample variance used to calculate significance in Microsoft Excel. A *P* < 0.05 was defined as statistically significant. Assay linearity was calculated using a simple linear regression (*R*²) in GraphPad Prism (version 8.3.1). *R*² > 0.99 was deemed satisfactory. A Fisher *r*-to-*z* transformation was used to create a *z* value that was used to assess the significance of difference between the two correlation coefficients. A *P* < 0.05 was defined as statistically significant.

Results

Results were returned by 23/26 (88.0%) participants. All returned RTddPCR data (Table I; Fig 1) with 11 participants

returning both RTqPCR and RTddPCR data (Table II; Fig 2).

All 23 participants using RTddPCR detected *BCR-ABL* in all samples down to MR^{4.0}. Detection rates for the deep-response samples were 95.7% at MR^{4.5} (0.0033% *BCR-ABL*^{IS}-), 78.3% at MR^{4.7} (0.002% *BCR-ABL*^{IS}-) and 87.0% at MR^{5.0} (0.001% *BCR-ABL*^{IS}-) (Table SII). Interlaboratory CV ranged from 29.3% at MR^{2.5} (0.33% *BCR-ABL*^{IS}-) to 52.9% at MR^{4.0} (0.01% *BCR-ABL*^{IS}-) (Table I). For deep-response samples, the CV was 59.7% at MR^{4.5} (0.0033% *BCR-ABL*^{IS}-), 61.6% at MR^{4.7} (0.002% *BCR-ABL*^{IS}-) and 69.0% at MR^{5.0} (0.001% *BCR-ABL*^{IS}-) (Table I). Linearity (*R*²) was assessed for all participants using RTddPCR results from MR^{1.0} (10% *BCR-ABL*^{IS}-) to MR^{4.0} (0.01% *BCR-ABL*^{IS}-) and ranged between 0.9330 and 1.000 (average 0.9936) (Fig 3; Table SIII). Overall, 20/23 laboratories had a satisfactory assay linearity with *R*² > 0.99.

In total, 11 participants performed both RTddPCR and RTqPCR. When the average percentage *BCR-ABL*^{IS} results reported by participants using RTqPCR was compared to the average percentage *BCR-ABL*^{IS} result for participants using RTddPCR, the results were seen to be comparable, with no statistically significant difference demonstrated (Table II).

There was no statistically significant difference seen in the median *ABL1* control gene levels calculated from participant's RTddPCR data compared to RTqPCR (Table SIV; Table SV).

When detection rates were compared for participants who performed both RTddPCR and RTqPCR, all participants detected *BCR-ABL* in all samples down to MR^{4.0} (0.01% *BCR-ABL*^{IS}-), using both techniques. For deep-response samples, RTddPCR showed a detection rate of 90.9% at MR^{4.5} (0.01% *BCR-ABL*^{IS}-), 81.8% at MR^{4.7} (0.033% *BCR-ABL*^{IS}-) and 81.8% at MR^{5.0} (0.001% *BCR-ABL*^{IS}-) compared to 90.9% at MR^{4.5} (0.033% *BCR-ABL*^{IS}-), 90.9% at MR^{4.7} (0.02% *BCR-ABL*^{IS}-) and 72.7% at MR^{5.0} (0.001% *BCR-ABL*^{IS}-) for RTqPCR (Table SIV).

Interlaboratory CV was lower across all 10 samples for RTddPCR when compared to RTqPCR (Table II, Fig 2) with RTddPCR CV ranging from 18.5% for sample D [MR^{2.5} (0.33% *BCR-ABL*^{IS}-)] to 44.1% for sample A [MR^{4.0} (0.01%

Table II. Summary of data from participant's who submitted RTqPCR and RTddPCR results for samples A–J.

		Sample F (MR ^{1.0})	Sample E (MR ^{1.5})	Sample C (MR ^{2.0})	Sample D (MR ^{2.5})	Sample G (MR ^{3.0})	Sample B (MR ^{3.5})	Sample A (MR ^{4.0})	Sample J (MR ^{4.5})	Sample I (MR ^{4.7})	Sample H (MR ^{5.0})
RTddPCR	<i>n</i>	11	11	11	11	11	11	11	11	11	11
	Average*	12.356	4.209	1.363	0.436	0.143	0.042	0.019	0.004	0.003	0.003
	SD	2.433	0.827	0.254	0.081	0.028	0.010	0.008	0.003	0.001	0.001
	CV, %	19.7	19.6	18.7	18.5	19.4	25.1	44.1	70.6	46.8	49.3
RTqPCR	<i>n</i>	11	11	11	11	11	11	11	11	11	10†
	Average*	11.733	3.955	1.331	0.393	0.123	0.035	0.013	0.006	0.003	0.002
	SD	4.659	1.390	0.501	0.155	0.051	0.021	0.008	0.007	0.002	0.001
	CV, %	39.7	35.2	37.7	39.5	41.3	58.4	59.4	116.6	75.4	71.4

CV, coefficient of variation; MR, molecular response; *n*, number of participant results submitted; RTddPCR, reverse transcriptase droplet digital polymerase chain reaction; RTqPCR, reverse transcription quantitative polymerase chain reaction; SD, standard deviation.

*Average of participant *BCR-ABL1^{IS}* results.

†One participant did not return results for sample H as it did not pass local quality control measures.

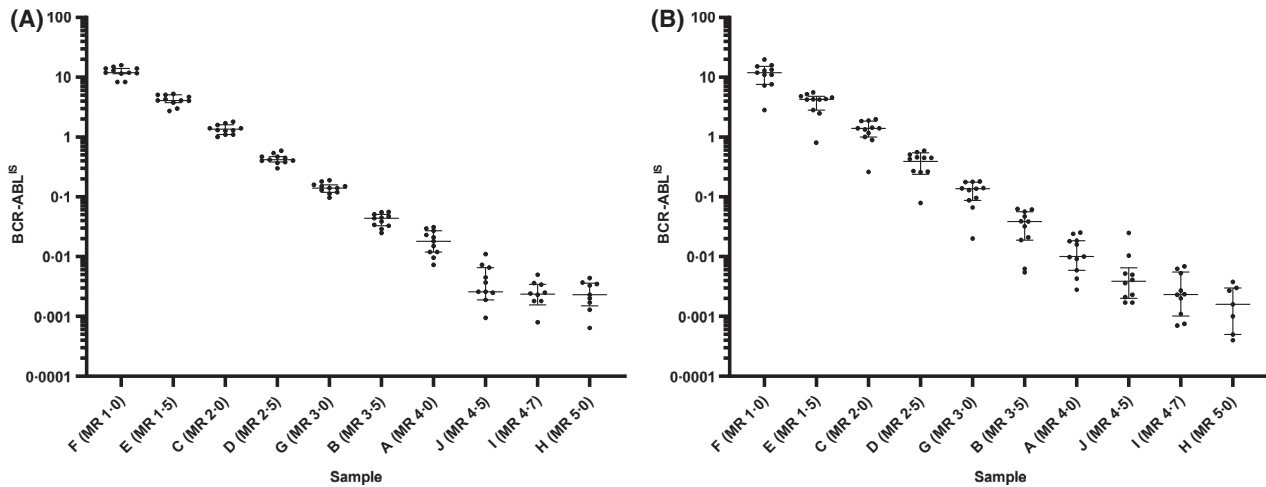


Fig 2. *BCR-ABL1^{IS}* results reported for samples A–J for participants who performed reverse transcriptase droplet digital polymerase chain reaction (RTddPCR) (A) and reverse transcription quantitative polymerase chain reaction (RTqPCR) (B). Black dots represent individual participant results. Long horizontal line represents average. Short horizontal line represents standard deviation.

BCR-ABL1^{IS} compared to a range of 35.2% for sample E [MR^{1.5} (3.3% *BCR-ABL1^{IS}*)] to 59.4% for sample A [MR^{4.0} (0.01% *BCR-ABL1^{IS}*)] for RTqPCR for samples between MR^{1.0} (10% *BCR-ABL1^{IS}*) and MR^{4.0} (0.01% *BCR-ABL1^{IS}*). For deep-response samples, the CV ranged from 46.8% for sample I [MR^{4.7} (0.0033% *BCR-ABL1^{IS}*)] to 70.6% for sample J [MR^{4.5} (0.033% *BCR-ABL1^{IS}*)] for participants using RTddPCR compared to a RTqPCR CV range of 71.4% for sample H [MR^{5.0} (0.001% *BCR-ABL1^{IS}*)] to 116.6% for sample J [MR^{4.5} (0.033% *BCR-ABL1^{IS}*)]. A statistically significant lower CV for RTddPCR ($P < 0.05$) could be demonstrated in seven of the 10 samples (Table SVII). A statistically significant lower CV for RTddPCR could not be shown in sample E [MR^{1.5} (3.3% *BCR-ABL1^{IS}*)], sample A [MR^{4.0} (0.01% *BCR-ABL1^{IS}*)] and sample H [MR^{5.0} (0.01% *BCR-ABL1^{IS}*)].

Nine out of 11 participants RTddPCR results showed better linearity from MR^{1.0} (10% *BCR-ABL1^{IS}*) to MR^{4.0} (0.01%

BCR-ABL1^{IS}) (average $R^2 = 0.9996$) compared to RTqPCR (average $R^2 = 0.9940$) (Fig 4; Table SVIII) with six of these nine being shown to be a statistically significant difference (Table SVIII). One participant showed the same linearity with both RTddPCR and RTqPCR, and one participant showed better linearity with RTqPCR, although this was not a statistically significant difference. All 11 participants using RTddPCR had a satisfactory $R^2 > 0.99$; eight of 11 participants using RTqPCR had a satisfactory $R^2 > 0.99$.

Discussion

Measurement of *BCR-ABL1* has long since been central to the management of patients with CML. The European LeukemiaNET (ELN) 2020 recommendations for CML mandate patients *BCR-ABL1* levels are monitored molecularly every 3 months by qPCR, regardless of whether they have achieved

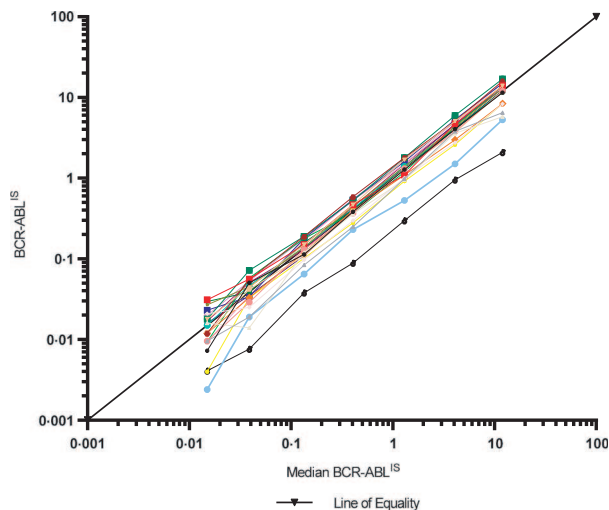


Fig 3. Linear regression for all participants who performed reverse transcriptase droplet digital polymerase chain reaction (RTddPCR) for samples A–G. Coloured lines represent individual participant’s results. [Colour figure can be viewed at wileyonlinelibrary.com]

a MR^{3.0} while on TKI therapy. The *BCR-ABL*^{IS} levels at 3, 6, 12 and 18 months are important assessment points and determine the response to TKI therapy, with key decisions being made on continuation or switching of treatment. Measurement *BCR-ABL1* at deep-response levels is also used in the management of pregnancy in younger patients,^{1,19} as well as for bone marrow transplantation.²⁰ TFR is now an achievable goal for a proportion of patients with CML.

Achievement of a deep MR has been a major criterion for eligibility for treatment discontinuation studies.⁵

Despite initial studies showing the potential to deliver more accurate and precise data on which these important treatment decisions could be made, RTddPCR has yet to gain widespread adoption for the measurement of *BCR-ABL1*; however, it is showing an increased uptake for the detection of other haematological variants. There has been a steady increase in participants using digital PCR in the UK National External Quality Assessment Services (NEQAS) for Leucocyte Immunophenotyping (LI) JAK2 p.Val617Phe Mutation Status external quality assessment (EQA) programme (Figure S1) (from 0.7% of participants in 2015 to 8.2% in 2020) and the UK NEQAS LI KIT p.Asp816Val in Mast Cell Disease EQA programme (from 8.3% of participants in 2015 to 24.0% in 2020); however, growth has been minimal in the *BCR-ABL1* (Major) Quantification programme (from 0.2% of participants in 2015 to 1.0% in 2020). A lack of interlaboratory validation studies, the extensive validation required for a quantitative minimal residual disease test, concerns about the comparability of results to legacy data, the lack of well-established best practise guidelines and the need to establish an IS conversion factor for laboratory developed RTddPCR tests are likely some of the limiting factors for laboratories considering adopting RTddPCR for *BCR-ABL1* measurement that have stymied its growth.

The average *BCR-ABL*^{IS} results submitted by participants using RTqPCR in the present study was compared to that from participants using RTddPCR and were seen to be comparable, with no statistically significant difference

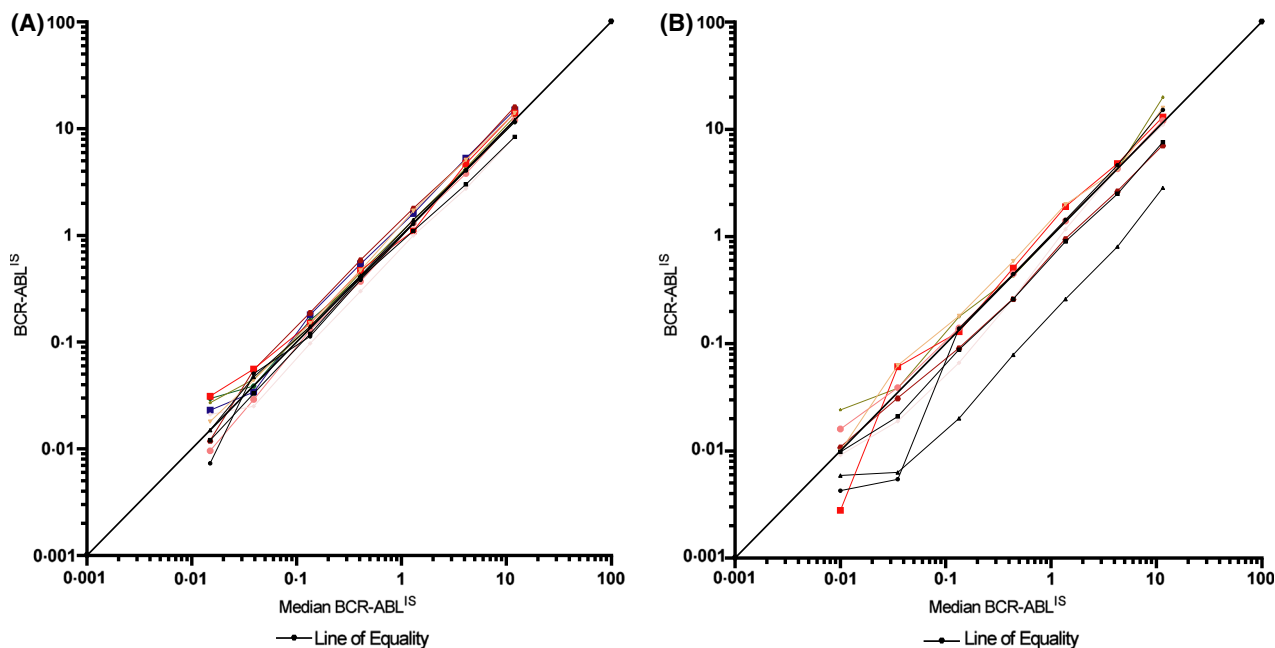


Fig 4. Linear regression for participants who performed both reverse transcriptase droplet digital polymerase chain reaction (RTddPCR) (A) and reverse transcription quantitative polymerase chain reaction (RTqPCR) (B) for samples A–G. Coloured lines represent individual participant’s results. [Colour figure can be viewed at wileyonlinelibrary.com]

demonstrated. This provides confidence to laboratories looking to adopt RTddPCR that the RTddPCR percentage *BCR-ABL*¹⁵ results can meaningfully compared to legacy percentage *BCR-ABL*¹⁵ RTqPCR data in longitudinal analysis. Nonetheless, comparability of results must still be validated on a centre-by-centre basis. Both methods achieved sufficient *ABL1* control gene levels (median for participants using RTqPCR was >80 000 for all samples; median for participants using RTddPCR was >100 000 for all samples) (Table SIV; Table SV) to allow laboratories to adhere to current best practise requirements for measuring deep MR using both methods.²¹

Both RTqPCR and RTddPCR showed good linearity and sensitivity; however, less interlaboratory variation was demonstrated in RTddPCR results in all samples down to MR³⁻⁰, with four of the five samples tested by laboratories showing a statistically significant difference. Furthermore, nine out of 11 participants using RTddPCR showed improved assay linearity compared to RTqPCR down to and beyond MR³⁻⁰ (0.1% *BCR-ABL*¹⁵). This is indicative of the precision and accuracy inherent to digital PCR due to it being an end-point, binary enumeration method²² that reduces the potential for under- or overestimating *BCR-ABL1* levels. RTddPCR had a reduced interlaboratory variation in all deep-response samples when compared to RTqPCR, with a statistically significant difference being demonstrated in two-thirds of the deep-response samples.

No difference in performance was seen in the ability to detect *BCR-ABL1* in 'deep-response' samples required by treatment discontinuation studies; however, stochastic sampling error (Poisson noise) limited the possibility of reliable detection of *BCR-ABL1* when potentially detecting a single molecule (Cross *et al.*, 2015²¹), given the standard sample input into the assay. To demonstrate a statistically significant difference would require an impractical number of replicates in a study of this scale. The effect of increasing blood draw volumes and RNA sample input, although potentially challenging clinically and costly, should be the focus of future studies.

The present study has demonstrated that RTddPCR using the QXDX *BCR-ABL* %IS kit is a viable alternative to the current 'gold standard' RTqPCR. Quantifying samples directly with no reliance on standard curves, it offers decreased interlaboratory variation and better assay linearity when directly compared to RTqPCR. It suggests that RTddPCR using the QXDX *BCR-ABL* %IS kit will allow more comparable management of patients with CML in a diverse range of clinical scenarios. It also establishes a baseline performance for RTddPCR using the QXDX *BCR-ABL* %IS kit for the measurement of *BCR-ABL1* for a diverse group of laboratories in a range of different countries. This reproduces the improved accuracy of RTddPCR shown in single-centre studies,¹²⁻¹⁵ while additionally demonstrating the robustness of the approach when applied to a diverse range of laboratories (research, clinical, translational) in a

wide variety of countries. However, with only 11 laboratories testing both RTqPCR and RTddPCR, this data will need to be replicated in a larger dataset using a more diverse range of digital PCR approaches. The impact RNA extraction and cDNA synthesis diversity on RTddPCR variability was not assessed in the present study and should be the focus of future standardisation projects.

The cost of Bio-Rad's QXDX *BCR-ABL* %IS kit is similar to the automated Cepheid Xpert[®] cartridge-based systems, but is more expensive than conventional laboratory developed RTqPCR tests, especially when the extra wells for deep-response detection are considered.²³ This is off set by the lack of requirement for a standard curve and the reduced validation required when using a Conformité Européenne (CE) marked kit given the imminent changes to Europe's *in vitro* diagnostic (IVD) regulations and their impact on laboratory developed tests.

The ability to detect deep responses with RTddPCR using the Bio-Rad QXDX *BCR-ABL* %IS kit, matched with an improved linearity and reduced interlaboratory CVs, compared to RTqPCR, offers the potential for better classification of patients according to the ELN 2020 criteria at both major MR and deep-response level.

Acknowledgments

This work was supported by an unrestricted educational grant from Bio-Rad who additionally provided RTddPCR reagents. The supporters did not have any role in the study design or analysis of data or writing in the present work. We thank Adam Corner and the team at Bio-Rad for technical advice provided over the course of the study. Stuart Scott designed the research study, analysed the data and wrote the paper. Ashley Cartwright analysed the data and reviewed the manuscript. Sebastian Francis wrote the clinical aspects of the discussion and reviewed the manuscript. Andrew Chantry and Liam Whitby reviewed the manuscript. Prasanthi Bhagavatula tested and analysed the study samples. All other participants tested and analysed the study samples and reviewed the manuscript. Stuart Scott has served on the advisory boards of Novartis and Amgen and has provided educational lectures for Novartis.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Percentage of participants using droplet digital PCR in UK NEQAS LI programmes by participation year.

Table SI. Study sample constitution information.

Table SII. *BCR-ABL1* detection rate in samples A–J for all participants using RTddPCR.

Table SIII. Linearity (R^2) for all participants who performed RTddPCR for samples A–G.

Table SIV. *ABL1* copy number levels reported by participants using RTqPCR.

Table SV. *ABL1* copy number levels reported by participants using RTddPCR.

Table SVI. *BCR-ABL1* detection rate in samples A-J for participants using RTddPCR and RTqPCR.

Table SVII. *F*-test for two-sample variances to show statistical significance between RTddPCR and RTqPCR results.

Table SVIII. Linearity (R^2) for participants who performed both RTddPCR (A) and RTqPCR (B) for samples A–G.

References

- Hochhaus A, Baccarani M, Silver RT, Schiffer C, Apperley JF, Cervantes F, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia*. 2020;**34**:966–84.
- European Medicines Agency. Appendix 4 to the guideline on the evaluation of anticancer medicinal products in man Condition Specific Guidance [Internet]. 2015 [cited 2018 May 4]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/02/WC500201945.pdf.
- Bower H, Björkholm M, Dickman PW, Höglund M, Lambert PC, Andersson TM. Life expectancy of patients with chronic myeloid leukemia approaches the life expectancy of the general population. *J Clin Oncol*. 2016;**34**:2851–7.
- Hochhaus A, Masszi T, Giles FJ, Radich JP, Ross DM, Gómez Casares MT, et al. Treatment-free remission following frontline nilotinib in patients with chronic myeloid leukemia in chronic phase: results from the ENESTfreedom study. *Leukemia*. 2017;**31**:1525–31.
- Saussele S, Richter J, Guilhot J, Gruber FX, Hjorth-Hansen H, Almeida A, et al. Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *Lancet Oncol*. 2018;**19**:747–57.
- Branford S, Cross NCP, Hochhaus A, Radich J, Saglio G, Kaeda J, et al. Rationale for the recommendations for harmonizing current methodology for detecting BCR-ABL transcripts in patients with chronic myeloid leukaemia. *Leukemia*. 2006;**20**:1925–30.
- Müller MC, Cross NCP, Erben P, Schenk T, Hanfstein B, Ernst T, et al. Harmonization of molecular monitoring of CML therapy in Europe. *Leukemia*. 2009;**23**:1957–63.
- Branford S, Fletcher L, Cross NCP, Müller MC, Hochhaus A, Kim DW, et al. Desirable performance characteristics for BCR-ABL measurement on an international reporting scale to allow consistent interpretation of individual patient response and comparison of response rates between clinical trials. *Blood*. 2008;**112**:3330–8.
- White HE, Matejtschuk P, Rigsby P, Gabert J, Lin F, Lynn Wang Y, et al. Establishment of the first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA. *Blood*. 2010;**116**:e111–e117.
- Cross NC, White HE, Ernst T, Welden L, Dietz C, Saglio G, et al. Development and evaluation of a secondary reference panel for BCR-ABL1 quantification on the International Scale. *Leukemia*. 2016;**30**:1844–52.
- Scott S, Travis D, Whitby L, Bainbridge J, Cross NC, Barnett D. Measurement of BCR-ABL1 by RT-qPCR in chronic myeloid leukaemia: findings from an International EQA Programme. *Br J Haematol*. 2017;**177**:414–22.
- Jennings LJ, George D, Czech J, Yu M, Joseph L. Detection and quantification of BCR-ABL1 fusion transcripts by droplet digital PCR. *J Mol Diagn*. 2014;**16**:174–9.
- Andersen RF, Pallisgaard N. Exceeding MR5.0 sensitivity in routine BCR-ABL1 analysis using multiplex digital PCR. *Blood*. 2014;**124**:4540.
- Mori S, Vagge E, le Coutre P, Abruzzese E, Martino B, Pungolino E, et al. Age and dPCR can predict relapse in CML patients who discontinued imatinib: the ISAV study. *Am J Hematol*. 2015;**90**:910–4.
- Franke GN, Maier J, Wildenberger K, Cross M, Giles FJ, Müller MC, et al. Comparison of real-time quantitative PCR and digital droplet PCR for BCR-ABL1 monitoring in patients with chronic myeloid leukemia. *J Mol Diagn*. 2020;**22**:81–9.
- Atallah E, Schiffer CA, Radich JP, Weinfurt KP, Zhang M-J, Pinilla-Ibarz J, et al. Assessment of outcomes after stopping tyrosine kinase inhibitors among patients with chronic myeloid leukemia: a nonrandomized clinical trial. *JAMA Oncol*. 2021;**7**:42–50.
- Faroni L, Wilson G, Gerrard G, Mason J, Grimwade D, White HE, et al. Guidelines for the measurement of BCR-ABL1 transcripts in chronic myeloid leukaemia. *Br J Haematol*. 2011;**153**:179–90.
- Cross NC. Standardisation of molecular monitoring for chronic myeloid leukaemia. *Best Pract Res Clin Haematol*. 2009;**22**:355–65.
- Carlier P, Bernard N, Lagarce L, Dautriche A, Béné J, Fouilhé Sam-Lai N, et al. Pregnancy outcome among partners of male patients receiving imatinib, dasatinib or nilotinib in chronic myeloid leukemia: reports collected by the French network pharmacovigilance centers. *Arch Gynecol Obstet*. 2017;**295**:269–71.
- Chalandon Y, Passweg JR, Schmid C, Olavarria E, Dazzi F, Simula MP, et al. Outcome of patients developing GVHD after DLI given to treat CML relapse: a study by the chronic leukemia working party of the EBMT. *Bone Marrow Transplant*. 2010;**45**:558–64.
- Cross NC, White HE, Colomer D, Ehrencrona H, Faroni L, Gottardi E, et al. Laboratory recommendations for scoring deep molecular responses following treatment for chronic myeloid leukemia. *Leukemia*. 2015;**29**:999–1003.
- Yoo HB, Park SR, Dong L, Wang J, Sui Z, Pavšič J, et al. International comparison of enumeration-based quantification of DNA copy-concentration using flow cytometric counting and digital polymerase chain reaction. *Anal Chem*. 2016;**88**:12169–76.
- Kockerols CCB, Valk PJ, Levin MD, Pallisgaard N, Cornelissen JJ, Westerweel PE. Digital PCR for BCR-ABL1 quantification in CML: current applications in clinical practice. *HemaSphere*. 2020;**4**:e496.