NGS-assisted DNA-based digital PCR for a personalised approach to the detection and quantification of residual disease in CML patients

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Funding was received from the LEUKA charity to complete this work.

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Abstract (200)

Recent studies indicate that 40% of chronic myeloid leukaemia (CML) patients who achieve sustained undetectable BCR-ABL1 transcripts on tyrosine kinase inhibitor (TKI) therapy remain disease-free after drug discontinuation. In contrast, 60% experience return of detectable disease and have to re-start treatment, thus highlighting the need for an improved method of identifying patients with the lowest likelihood of relapse. Here we describe the validation of a personalised DNA-based digital PCR approach for quantifying very low levels of residual disease, which involves the rapid identification of t(9;22) fusion junctions using targeted next generation sequencing coupled with the use of a digital PCR (dPCR) platform. t(9;22) genomic breakpoints were successfully mapped in samples from 32 of 32 patients with early disease. Disease quantification by DNA-dPCR was performed using the Fluidigm BioMark platform on 46 follow-up samples from 6 of the 32 patients, including 36 samples that were in deep molecular remission (MR). Digital PCR detected persistent disease in 81% of MR samples, out-performing both RT-dPCR (25%) and DNA-based qPCR (19%). We conclude that dPCR for *BCR-ABL1* DNA is the most sensitive available method of residual disease detection in CML and may prove useful in the management of TKI withdrawal.

Introduction

The fusion oncogene *BCR-ABL1* is the hallmark for chronic myeloid leukaemia (CML) (1). The BCR-ABL1 tyrosine kinase protein is responsible for the cellular phenotype of CML and is therefore a rational target for therapy via tyrosine kinase inhibition, a treatment approach that has revolutionised patient outcome (2). The measurement of BCR-ABL1 transcripts via reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is the most widely used method for monitoring residual disease in patients with CML, an essential aspect of modern disease management(3). While tyrosine kinase inhibitors (TKI) are routinely administered indefinitely, recent studies indicate that 40% of patients who achieve undetectable BCR-ABL1 by RT-qPCR (molecular remission with 5 log reduction on the international scale, MR^5) on imatinib that is sustained for at least two years, will remain disease-free after drug discontinuation (4), raising the possibility of an "operational cure" in this subgroup of patients. However, the safe introduction of a TKI withdrawal strategy would require a reliable and cost-effective method for the identification of those patients with the lowest likelihood of relapse. The probability of relapse post-withdrawal is likely to be related to persistence of residual disease, which may include transcriptionally quiescent TKI-resistant leukaemic stem cells (4, 5), at a level that is below the threshold of detection by the 'gold-standard' RT-qPCR (10^{-5})(6, 7). A means of detecting these cells that does not depend on oncogene transcription might be clinically valuable.

Here we describe a DNA-based method of detecting and quantifying low levels of *BCR-ABL1* positive disease that improves on previous methodologies in two key areas. Firstly, the identification of *BCR-ABL1* fusion junctions is undertaken by targeted next generation sequencing allowing the rapid generation of high-performing DNA based hydrolysis probe assays that are specific to the unique molecular footprint of each patient's CML clone. Secondly we sought to further enhance the sensitivity of a DNA-based approach by optimising the technique for use on a digital PCR (dPCR) platform, which provides absolute molecular quantification without the need for a standard curve. When applied to samples with undetectable disease by RT-qPCR, DNA-based digital PCR (dPCR) provided a marked improvement in sensitivity, not only over RT-qPCR, but also compared to real-time qPCR of DNA and to digital RT-PCR (RT-dPCR).

Materials and Methods

Patient Cohort

To validate the NGS part of this technique, we studied 32 CML patients treated at the Hammersmith Hospital. Twenty two patients were on treatment with TKI and 10 had received allogenic stem cell transplants (alloSCT). RNA extracted from diagnostic samples had been previously used to establish the fusion type using multiplex PCR as previously described (8). Ethical approval was provided and informed consent was obtained from all patients in accordance with the declaration of Helsinki. Forty-six follow up samples from six of these patients (A1-A6; median: 5 samples per patient) collected at least 28 months from diagnosis (follow up range: 28-178 months) were used to compare the sensitivities of different residual disease measuring technologies, as described below. Thirty-six of these samples were in deep molecular remission (MR) as defined in Cross et al, 2015 (9), while the remaining 10 were at the level of MR^3 (previously termed major molecular response) or above. Of the six patients, A1 and A6 received TKI therapy and the remaining four were post-transplant (A2 to A5).

Next Generation Sequencing identification of fusion junctions

1) DNA extraction

Peripheral blood cell lysates (PBCL) prepared using RLT buffer (Qiagen, Manchester, UK; cat number 79216) and stored at minus 80°C were collected from all 32 patients either from the time of diagnosis, or when the level of residual disease was ≥ 10% by BCR-ABL1 RT-qPCR. DNA was extracted from 200µl of the PBCLs using the QIAamp DNA blood mini kit (Qiagen, cat number 51106) following the manufacturer's protocol on the QIAcube robotic workstation (Qiagen). DNA was quantified using the Qubit fluorometric method

(Invitrogen, Qubit® dsDNA BR Assay kit, cat number Q32850). DNA was further assessed for impurities using the 260/280 and the 260/230 ratios measured using a spectrophotometer. The integrity of the DNA was assessed on a 2% agarose gel.

2) Targeted NGS fusion mapping

Targeted Next Generation Sequencing (NGS)

Library preparation was performed using Illumina's TruSeq DNA sample prep and PCR kits, according to manufacturer's instructions (Illumina, Cambridge, UK; ref numbers 15025064 and 15027084). Briefly, 1µg of DNA from each sample was fragmented using the Covaris DNA shearing system (Covaris S2, Massachusetts, USA). The fragments were end-repaired and ligated with Illumina adaptors and barcodes for multiplexing. Individual library quality and size were assessed by running an aliquot of each library on a bioanalyzer using a high sensitivity DNA chip (2100 Bioanalyzer, Agilent Technologies) and/or capillary gel electrophoresis (Qiagen© Qiaxcel system). The average insert size was 400bp (ranging from 100 to 900bps). Individual libraries were purified using AgenCourt Apure XP beads (A63881, Beckman Coulter), quantified using Qubit and an equimolar amount of each library was pooled together into a final multiplexed library. The 32 patients were grouped into four batches, comprising multiplexes of 6, 12, 8 and 6 patients, respectively.

For the targeted sequencing, each pooled library was hybridised twice overnight with a pool of synthetic oligonucleotide probes custom-designed using Illumina's DesignStudio online portal to specifically capture the entire coding and non-coding regions of the *BCR* and *ABL1* genes (100kb upstream and downstream from each gene, respectively). Probe details and coordinates are provided as a BED file (Supplementary Table 1). Sequence capture was followed by extensive washing and purification steps according to the manufacture's protocol (Illumina, TruSeq Custom enrichment kit-24 reactions-boxes one and two, ref numbers 15022030 and 15022031). Bridge amplification, cluster generation and 150bp paired-end sequencing were performed on an Illumina MiSeq following captured library quantification by qPCR using Kapa library quantification kit (kit code KK4835, KBiosciences). The median read coverage across *BCR* and *ABL1* targets was 100x (range 50x to 150x, with minimum base call quality of Q20.

Bioinformatics-based prediction of fusion junctions

Bioinformatics analysis of sequencing reads was performed according to the following steps. First, TruSeq adaptor sequences were removed using the Cutadapt(10) software. Trimmed reads were then aligned to the human genome reference assembly GRCh37 using the BWA-MEM(11) aligner software (v 0.7.2). Duplicate reads were marked in the BAM files and the coverage metrics for targeted regions were collected using the Picard (v1.85; http://picard.sourceforge.net/) software. CREST(12) and BreakDancer(13) software were used to extract split reads (single reads composed of material from two non-contiguous genomic regions) and discordant pairs of reads (in which individual reads in a pair do not map at the expected distance and/or orientation), respectively, that were likely to mark the site of structural rearrangement (Figure 1).

3) Fusion junction confirmation

All predicted fusion junction sequences were validated via conventional Sanger sequencing. Primer sequences are listed in Tables 1. For the validation of the *ABL1-BCR* fusion junctions predicted by split reads, primers were designed at least 200bp upstream and downstream of the putative fusion junctions, while in cases where the fusion was alluded to via discordant read pairs, primers were designed 200bp upstream and downstream of the end of the read closest to the predicted junction on either side of the junction. The Primer3(14) online software V.0.40 was used for primer design.

Qiagen Fast Cycling PCR kit (QIAGEN, Cat. No. 203743) was used for amplification of junction sequences, which contains a high fidelity HotStart Taq *Plus* DNA polymerase capable of functioning over long distances. For all PCRs, two negative controls, one sample of pooled DNA extracted from eight CML patients (pool1) and DNA extracted from a normal individual (CSC) were included in addition to a no template control (NTC). PCR was performed using the following temperature cycling parameters: 94°C for 5 min, 30 cycles of 94°C for 30 sec, (55-65)°C for 30 sec, 72°C for 37 sec, followed by a final extension step at 72°C for 10 min.

For sanger sequencing of PCR products, two independent cycling PCR reactions, one forward and one reverse, was performed per patient sample using ABI's BigDye Terminator V3.1™ Cycle Sequencing kit (Applied Biosystems, Foster City, USA) under the following conditions: 96°C for 1 min, 25 cycles of 96°C for 10 sec, 55°C for 15 sec, 60°C for 4 min, prior to storage at at 4°C. PCR products were purified with BigDye XTerminator™ Purification kit following manufacturers' protocol. Sequencing was performed on the ABI 3500xl Genetic Analyzer (ABI, Life technologies Ltd, Foster City, USA). Sequence quality and traces were analysed using the ABI's Sequence Analysis software V5.2.

Sequences obtained by Sanger sequencing were aligned to the reference genome for *BCR* and *ABL1* using BLAST(15) (Basic Local Alignment Search Tool; NCBI) or BLAT(16) (Blast Like Alignment Tool; UCSC) genome alignment tools.

Residual Disease quantification

The sensitivity of dPCR in identifying and quantifying *BCR-ABL1*-positive residual disease in 46 post-treatment CML samples from six patients was compared to that of three other quantitative PCR methods, namely RT-qPCR, qPCR and RT-dPCR. Each was performed as follows:

1) DNA-based dPCR (dPCR)

Design and optimisation of TaqMan FAM-MGB Hydrolysis Probe assays

For six patients (A1-A6) qPCR assays were designed specific to each patient's fusion junction using Primer Express 3 software v3.0.1 (ABI, Life technologies Ltd, Foster City, USA) and the AutoDimer tool (17). Assays were designed specific to the *BCR-ABL1* fusion junction where available (four patients) or to *ABL1-BCR*, if the *BCR-ABL1* junction sequence was unavailable (two patients) (Table 2). Primer specificity was assessed using a SYBR Green melt curve assay (Sigma Ltd, Missouri, USA) following manufacturer protocol, in addition to capillary electrophoresis.

DNA extracted from each patient's diagnostic or high-level disease sample was used to prepare standard curves to evaluate assay efficiency, quantitative range and limit of detection. Primer and hydrolysis probe concentrations were optimised by real-time PCR using a matrix of 8 different concentrations run on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Life technologies Ltd, Foster City, USA). The combination with the highest fluorescence and lowest quantification cycle (Cq) values was subsequently used. The standard curves produced acceptable slopes (-3.2 to -3.6) and correlation co-efficiencies (0.98-0.99%), acceptable amplification efficiency figures (80-110%) and acceptable dynamic ranges, in line with the expected theoretical sensitivity range. Quantitative ranges (LoQ) varied from 10^{-3} to 10^{-4} and sensitivity ranges (LoD) varied from 10^{-4} to $5x10^{-5}$ (Supplementary Table 2). SYBR Green assays showed a unique melting curve per assay and capillary gel electrophoresis produced a unique band of the expected size.

Preamplification

Measurement of residual disease was carried out by dPCR in the follow-up samples either directly using unamplified DNA or including a preamplification step according to a previously published protocol (18) using TaqMan Preamp Master Mix (Invitrogen, part no.

4391128). In brief, a total reaction mixture of 20µl was prepared using 10µl of 2X preamp mix, 5µl of 0.2X patient-specific assay, 2µl of distilled water and 150ng DNA in 3µl. Preamplification was performed using the following thermocycling conditions: 95^{o}C for 10 min, then 14 cycles of 96^{o}C for 15 sec and 60^{o}C for 4 min. Amplification products were diluted 1:5 in 1X TE, pH 8 and subsequently used for dPCR quantification. We evaluated the efficiency of preamplification of DNA extracted from 46 follow-up samples from six CML patients using a standard curve of 4 points per assay prepared from each of the corresponding six patients' early disease material. The standard curves produced acceptable slopes (-3.1 to -3.8) and correlation co-efficiencies (0.994-0.998%) and acceptable amplification efficiency metrics (81-114%) (Supplementary Table 3). Of the 46 follow-up samples, ten had previously demonstrated RT-qPCR positivity at MR^3 or above and thus served as positive controls; all ten amplified within expected ranges.

dPCR quantification

Microfluidic digital PCR was performed using the Fluidigm Biomark HD (Fluidigm, California, USA) and the 48.770 dPCR array containing 770 x ~ 0.84nl reaction partitions in each of the 48 panels (Fluidigm, dPCR 37k IFCs, 100-6151).

Reaction mixes of 6µl were prepared containing 3µl of 2X ABI Fast Advanced Master Mix (Invitrogen, Foster City, USA; cat no. 4444965), 0.6µl of 20X GE sample loading reagent (Fluidigm, cat no. 85000820), 0.3µl of 20X patient specific TaqMan assay, 0.3µl distilled water and 1.8µl of (50ng/ul) template DNA. A partition size of nine panels per sample was used to allow the quantification of an equal amount of DNA as in qPCR (150ng). Five microliters of the prepared reaction mixes were loaded into the corresponding inlets on each array chip and the BioMark IFC controller MX (Fluidigm) was used to uniformly partition the reactions across the panels. Thermocycling was performed using the fast mode (5.5°C/min) as follows: 95°C for 60 sec, 40 cycles of 96°C for 5 sec followed by 60°C for 20 sec. Data was collected using The Data Collection software v4. Data analysis was performed using the Digital PCR Analysis Software v4. The positive partitions per 9 panels for each assay were grouped (k), and Poisson distribution was used to estimate the average number of template copies (λ) per partition in all of the 9 panels (n) where λ= -ln(1-k/n)(19). Estimated target copy number per 1µl and per reaction volume were calculated in addition to the 95% confidence intervals to account for the counting uncertainty. The formulas used for these calculations are as follows and are formatted into Supplementary Table 4 and 5 which could be used as a template: The probability of having more than one target molecule per partition P=k/n where k is the number of positive partitions and n is the total number of partitions; Standard deviation from the mean of P is estimated as S= SQRT((P*(1-P))/n); the upper and lower 95% confidence interval (CI) of the P are estimated as P_L and P_H = (P +/- (1.96*S)); the upper and lower 95% CI of the λ is estimated as λ_L and λ_H = -ln(1-P_L) and -ln(1- P_H); Est target copies per reaction volume (Est targets) = λ*n; The upper and lower 95% CI of the Est targets (Est targets-$_L$ and Est targets-$_H$) = λ_L*n and λ_H*n; Est target per 1µl= (Est Targets ÷ (DNA volume per panel*number of panels)); the upper and lower 95% CI of Est targets per 1µl = (Est targets-$_L$ ÷ (DNA volume per panel*number of panels)) and (Est targets-$_H$ ÷ (DNA volume per panel*number of panels)).

Positive and negative controls were run using the same partition size used per sample for each of the six DNA assays to assess platform function, amplification protocol and level of background noise. Early disease samples from each of the six patients were used as positive controls. DNA from a normal donor and from a pool of eight CML samples were used as negative controls. Three NTC panels were included in each array to rule out contamination. The threshold and the Cq ranges were manually set specific to each assay and its background noise, but consistent across all panels of the same assay (Table 3 and Supplementary Figures 1-7).

Linearity and the dynamic range of the Fluidigm platform was previously assessed(18). However, we also performed a limited experiment to assess these two parameters in our hands. We generated a series of dilutions of two synthetic oligonucleotides each containing the fusion sequence of one CML patient (A-3 and A-6; GeneArt, Life technologies Ltd, Foster City, USA). Two-fold dilutions of 4 to 512 molecules per panel were run in duplicate panels on the 48.770 chip array. Linearity was maintained at 4 molecules per panel (lambda of 0.005; approximately equal to 2 copies/1μl); therefore we considered this a conservative limit for reliable quantification Supplementary Figure 8). All the normal controls we tested showed complete negativity, additionally, none of the normal controls showed any amplification. We therefore chose a range of 0.5 and 3 molecules per panel to be considered positive outside the quantitative range. These thresholds have been previously applied to other tests in the measurement of residual disease in acute lymphoblastic leukaemia using antigen receptor targets (20).

2) RT-qPCR

After the patients' specific fusion type was identified in the diagnostic sample by multiplex PCR using previously described protocols (7, 8), RT-qPCR was used to identify residual disease in each of the 46 follow samples as previously described using best practice methodology(6, 7, 21, 22). RNA was extracted using Qiagen RNeasy Mini kit (Qiagen cat no 74106) and Qiagen Qiacube robot (Qiagen). Reverse transcription and cDNA synthesis were performed using M-MLV reverse transcriptase (Invitrogen, cat no 28025-013) and random hexamers (Invitrogen, cat no 48190-018) as described in an earlier publication (6).

RT-qPCR was performed using a duplex MGB TaqMan assay (22) (a modified version of the Europe Against Cancer (EAC) assay described in Gabert et al 2003 (23)) with 2x TaqMan Fast Advance Master Mix (ABI, cat no 4444965) in a final 20ul reaction. The level of expression of target BCR-ABL1 molecules was expressed as a percentage ratio between BCR-ABL1 and ABL1 to obtain a normalised value for the gene independent of the integrity of the RNA and the efficiency of the RT reaction.

3) RT-dPCR

Positive and negative controls were used to assess the amplification protocol and to establish the Cq range and the quantification threshold. cDNA from each patient's presentation sample in addition to the in-house standardised "Wessex" plasmid, which contains the e14a2 BCR-ABL1 cDNA junction, were used as positive controls. cDNA from a healthy individual was used as negative control. A partition size of 15 panels per sample was used to allow the quantification of an equal volume of cDNA as in RT-qPCR (3μl). All cDNA used for RT-dPCR quantification were the same used on the RT-qPCR platform to exclude variability introduced by the RT step. Three NTC panels were included in each array to rule out contamination. Using the Digital PCR Analysis Software v4 (Fluidigm) the quantification threshold was determined to be 0.03. The Cq range was manually defined as 20-32 (Table 3 and supplementary Figure 9).

Running a series of two-fold dilutions prepared using the ERM®-AD623 plasmid (produced by the European Commission for Reference Materials), a reference vector containing the *BCR-ABL1* exonic fusion, we established linearity and found that accurate quantification was possible down to 4 molecules per panel (2 copies/μl) as described for DNA-dPCR (Supplementary Figure 10).

Total reaction mixes, volume per inlet, thermocycling conditions and data analysis software were as described for dPCR of DNA. Threshold and amplification range across the panels of the same assay were set according to the parameters defined during the validation stage.

4) qPCR of *BCR-ABL1* DNA

Hydrolysis probe assays were as previously detailed for dPCR. Assay performance was assessed following MIQE guidelines(24). In order to ensure accurate disease quantification we also followed the EuroMRD recommendations for qPCR in Ph-negative acute lymphoblastic leukaemia (20) in which DNA extracted from each patient's diagnostic sample is used to prepare standard curves (Supplementary Table 2). Patient specific standard curves were prepared by performing five log dilutions of each patient's diagnostic sample including two half log dilutions at the end of the scale. The samples were diluted in tRNA buffer (Sigma-Aldrich). We used Albumin as a control gene to normalize the total amount of quantifiable DNA included per assay to 150ng/3ul/20ul reaction. The standards were run in triplicate, and in sextuplicate for the values below 10^{-3}, in order to assess assay performance.

The limit of quantification (LoQ) for each assay was determined based on the following criteria: 1) The presence of specific amplification in sexduplicates; 2) Acceptable Cq variance between the sexduplicates of the same dilution point is ≤ 0.5 (for Cq < 30), ≤ 1 (for Cq between 31-33), ≤ 1.5 (for Cq >34) Cq; 3) Mean Cq variance between log dilution points to be 2.6-4 and between half log dilutions to be 0.5-1.5 Cq; 4) The lowest Cq with a positive signal to be at least 1 Cq lower than the Cq of the background.

The limit of detection (LoD) was defined as the lowest dilution point at which the following could be demonstrated: 1) the presence of specific amplification in at least one of the Sexduplicates in the same dilution point. 2) Mean Cq variance between the highest and the lowest dilution points to be <20 Cq. 3) The lowest Cq with a positive signal to be at least 1 Cq lower than the Cq of the background.

DNA from healthy donors and a pool of 8 CML patients were included as negative controls to establish background noise in addition to an NTC. Positive signal within the negative controls was considered the result of unspecific amplification and was taken into account while defining the quantitative and sensitivity ranges where the mean Cq values of the replicates are required to be ≤ 3 Cq above background value. The assays were run on the StepOnePlus^{TM} Real-Time PCR System and data were analysed using the StepOnePlus^{TM}Software v2.2.2. Input DNA quantities were normalised to Albumin and 150ng of total DNA was used per reaction. QPCR assays were performed in 3 independent runs; each plate containing sexduplicates per FU sample, triplicates for the Negative controls and NTC in addition to the optimised standard curve as described earlier. Repeatability and precision for qPCR runs were tested using ANOVA variance analysis using Microsoft Excel 2013 and SPSS on Cq values within LoQ and no significant difference was present among replicates (data not shown). However, standard deviation increased greatly between replicates falling within the LoD as expected.

As with dPCR we also investigated whether increasing the concentration of input DNA improves qPCR sensitivity. Standard curves were prepared using presentation DNA from patients A-2 and A-4 at concentrations of 150ng/3ul, 300g/3ul and 500ng/3ul to evaluate the effect of DNA concentration on Cq values. Higher DNA concentration produced no appreciable effect on the dynamic range of the qPCR assays. In fact, the LoQ was reduced when 500ng/3ul was used as a starting material (data not shown).

Results

Breakpoint Identification

Using bioinformatics tools, we identified clusters of reads that predicted at least one t(9;22)-associated genomic fusion junction in all of the 32 patients tested in this study. The junctions were predicted either to a base pair resolution via split reads which appeared to directly cover the fusion junction, or via discordant pairs of aligned reads in which each read in the pair mapped to different genomic loci, thus inferring indirectly the likely location of the junction (illustrated in Figure 1a). All predicted breakpoint locations were consistent with the exonic *BCR-ABL1* fusions identified in each patient by multiplex PCR.

In keeping with a balanced translocation in which two reciprocal fusion genes are formed, we found evidence of two fusion junctions in 11 of the 32 cases (34%), corresponding to both *BCR-ABL1* (located on the derivative chromosome 22), and its reciprocate, *ABL1-BCR* (situated on the derivative chromosome 9). In a further 16 cases (50%), an *ABL1-BCR* fusion junction was not detected, while in the remaining 5 cases (16%) we were unable to detect the *BCR-ABL1* junction. Nevertheless, evidence of the location of at least one of the two disease-specific breakpoint junctions was successfully obtained in all 32 patients (Supplementary Table 8).

In the majority of split reads or discordant read pairs both *BCR* and *ABL1* sequences ran in the same genomic direction (eg 5' to 3' *BCR* joined to 5' to 3' *ABL1*). However, in a minority of patients (3/32) junction spanning reads were obtained in which *BCR* and *ABL1* sequences ran in opposite genomic directions (eg 5' to 3' *ABL1* joined to 3' to 5' *BCR*). This is consistent with previous breakpoint mapping studies in CML that have reported the presence, in a minority of patients, of small inversions of intronic sequence at the junction sites of one of the derivative chromosomes (Figure 1b) (25, 26). For each breakpoint, primers were designed to amplify across the fusion junctions. All *BCR-ABL1* and *ABL1-BCR* fusion junctions predicted by NGS for all 32 patients were successfully amplified and validated by Sanger sequencing of the PCR amplicons. (Table 2).

Comparison of quantification methods

The aim of this study was to compare the specificity and sensitivity of different nucleic acid substrates (DNA or RNA) and different platforms, namely RT-qPCR, qPCR, dPCR and RT-dPCR in the detection of low levels of BCR-ABL1-positive disease. To carry out this comparison we tested 46 samples collected during routine clinical management from six patients. The patients were selected as they had been classified as good responders and had sustained deep molecular remission for a median of 7 years (range 2-14 years). Ten of the 46 samples were used as positive controls. All had a residual disease level of MR^3 or above by RT-qPCR (Table 4 and Figure 2: samples A1_46; A2_1; A3_28; A4_8, 9, 10; A5_15, 16; A6_21, 22. Also see supplementary Tale 6 and 7 for additional details). The remaining 36 samples were considered negative by RT-qPCR since either zero or <10 molecules of BCR-ABL1 transcript had been detected (21 and 15 patients, respectively), which, when assessed along with control gene transcript number produced residual disease levels of MR^4 (8 patients), $MR^{4.5}$ (17 patients) or MR^5 (11 patients; Table 4 and supplementary table 6 and 7).

1) DIGITAL PCR (dPCR) Quantification

Sixteen (44%) of the 36 negative MR samples were positive by dPCR when performed without a preamplification step (Table 4 and supplementary Table 6 and 7). Eight of these samples were positive above the threshold for accurate quantification while eight were positive below this threshold. Introducing a preamplification step of 14 cycles further increased the number of positive samples to 29 (80.5%) (Table 4 and supplementary Table 6 and 7). Twenty seven out of the 29 samples were positive above the threshold for accurate quantification with a mean number of molecules per microliter of 67 (range 2 to 416), while the remaining two were positive below this threshold. In the context of individual patients, *BCR-ABL1* DNA was detected in at least one MR sample from all six patients (Figure 2). The 10 control samples that showed detectable disease by RT-qPCR (MR^3 or above) were all positive by dPCR well within the quantification limit (Table 4 and supplementary Table 6 and 7).

2) RT-dPCR Comparison

Nine (25%) of the 36 MR samples showed quantifiable transcripts by RT-dPCR (mean 29 molecules/µl, range 5 to 67) after preamplification (Table 4 and supplementary Table 6 and 7). The positive samples were detected in three of the six patients (Figure 2). Only four of the samples were positive without preamplification. Twenty three of the 27 samples that remained negative by RT-dPCR were found to be positive by dPCR, thus RT-dPCR failed to detect disease in 85% of the samples in which a positive result was obtained by DNA dPCR. The 10 control samples that were MR^3 or above by RT-qPCR were all positive by RT-dPCR (Table 4 and supplementary Table 6 and 7).

Among MR samples, we observed no consistent proportional relationship between the level of transcript detected by RT-dPCR and the level of *BCR-ABL1* DNA assessed by dPCR (Figure 3 and Table 4). For example, BCR-ABL1 transcript was frequently detectable in MR samples from patient A1 despite relatively low levels of DNA (mean 8 molecules/µl), while in patient A3, transcript was undetectable in any MR sample by RT-dPCR even though *BCR-ABL1* DNA consistently measured substantially higher (mean 57 molecules/µl). Finally, oncogene transcript was more frequently detected in samples estimated to be in MR from patients on TKI therapy (7/14; 50%) than from patients who had received a bone marrow transplant (1/22; 5%), implying a deeper level of remission post-transplant than while on TKI.

3) qPCR Comparison

Nineteen percent of the 36 MR samples (7/36; from three of the six patients) were positive by qPCR for the *BCR-ABL1* genomic breakpoint. All seven samples had also tested positive by dPCR, Thus qPCR failed to detect positivity in 76% (22/29) of the dPCR positive samples. The 10 MR^3 or above control samples were all positive using this method (Table 4 and supplementary Table 6 and 7). Four samples (A4-14 and A5-17, 18, 10) remained negative by all four quantification methods. These results might represent “true negative” readings particularly as they belong to patients who had received alloSCT.

Discussion

In our study we report a novel method of detecting and quantifying low levels of residual disease in peripheral blood samples from patients with CML in whom BCR-ABL1 transcriptions are undetectable using RT-qPCR. Our DNA-based method avoids laborious breakpoint mapping by the use of targeted high throughput NGS, and provides improved sensitivity by employing a digital PCR platform for residual disease quantification.

The potential benefits of using genomic DNA (gDNA) for residual disease detection in CML are numerous (27-32). In addition to being more stable and easier to extract than RNA, use of gDNA eliminates the variability introduced by the RT step, and allows target cell detection regardless of transcriptional activity. The latter feature ensures the detection of quiescent cells as well as allowing the quantification of true cellular disease burden rather than a mean level of oncogene expression. Furthermore, the possibility of amplifying benign BCR-ABL1-positive clones, reported to be found in healthy individuals (33) is eliminated. A resulting improvement in sensitivity of at least 1 log compared to an RNA-based assay has been estimated for a DNA-based qPCR approach (29).

However, a major obstacle to the routine adoption of DNA monitoring is the inherent difficulty of mapping the genomic breakpoints of a translocation that occurs across a wide area of intronic sequence, and often involves additional events such as deletions, insertions, duplications and inversions. The principle of using target enrichment NGS, which simplifies breakpoint characterisation significantly, has been previously described (34), but earlier enrichment technology was unsuited to clinical use, being costly with laborious bait generation and sequencing workflows. In contrast, the technique we describe, with which we successfully mapped a disease-specific junction in all 32 patients tested, is relatively simple, cost effective and suited to a high throughput laboratory.

Previous studies using qPCR have reported the detection of *BCR-ABL1*-positive disease in a substantial proportion of patients with undetectable transcripts by RT-qPCR (27-30). However, one limitation of the application of DNA-based hydrolysis probe assays on a real-time qPCR platform is the need for positive control material with which to generate a standard curve. The use of patient's presentation material for this purpose compromises accuracy and sensitivity because patients rarely present with 100% BCR-ABL1 positive cells in the peripheral blood (35-38). The method we present circumvents this constraint by use of a digital PCR platform. By assigning an absolute value to the target molecules, it allows precise quantification without the need for a patient-specific standard curve.

In this proof of principle analysis, we explored the impact of changing 1) the PCR substrate, 2) the PCR platform or 3) both these variables on the sensitivity of residual disease detection compared to the current gold-standard method for clinical management, RT-qPCR. The application of a standard BCR-ABL1 hydrolysis probe assay to cDNA using a digital PCR platform (RT-dPCR) showed a modest improvement in sensitivity, detecting disease in over 11% and 25% of post-treatment samples, without and with preamplification, respectively in which BCR-ABL1 transcripts were undetectable by RT-qPCR (Table 4 and supplementary Table 6 and 7). This is in keeping with previous findings (18, 39). The application of hydrolysis probe assays specific to patients' *BCR-ABL1* DNA fusion using a real-time platform (qPCR) also provided an improved detection rate compared to RT-qPCR, identifying disease cells in 19% of the same samples. The highest sensitivity, however, was obtained by the use of the DNA assays on a digital PCR platform (dPCR), which detected disease in 44% of the MR samples. Introducing a preamplification step further increased the sensitivity allowing residual disease detection in 80% of the samples (Table 4 and supplementary Table 6 and 7). Digital PCR preceded with 14 cycles of preamplification step for the *BCR-ABL1* DNA fusion point was therefore by far the most sensitive available method for the detection and quantification of residual disease. Preamplification is increasingly becoming a required step in the assessment of nucleic acid quantities in samples with limited amount of target molecules. However, preamplification could be associated with bias; therefore when used, it needs to be carefully assessed and preamplification efficiency cautiously monitored per assay to ensure accurate quantification (18, 40, 41).

DNA based qPCR has previously been used to assess the correlation between detection of *BCR-ABL1* at the time of stopping TKI therapy and disease recurrence (27). Retrospective analysis of serial samples from 18 patients who had been in MR^5 for at least two years has shown no significant correlation between the presences of *BCR-ABL1* before stopping imatinib suggesting that perhaps factors other than the presence of residual disease cells must contribute to the probability of stable MR after withdrawal of imatinib (27). One possible factor could be related to the level of residual disease present which could become a risk factor for predicting relapse once a certain threshold is crossed. A method of absolute quantification would be of great value to establish such a threshold if present. We are currently investigating the impact of residual disease level as assessed by dPCR at the time of treatment withdrawal on outcome within the UK based DESTINY clinical trial. If validated in clinical trials of stopping TKI, the technique will permit a more personalised approach to recommendations for dose reduction or drug cessation in individual patients, ensuring that therapy is withdrawn only from patients with the highest chance of long-term remission. In practical terms this would require that genomic breakpoints be identified in a patient as soon as they are diagnosed allowing the design and optimisation of a patient-specific assay. Patients' response to therapy would then be monitored via standard RT-qPCR until they have reached MR; hereafter routine monitoring would be augmented with DNA quantification by dPCR and would benefit from the publication of standardised guidelines as with RT-qPCR.

In technical terms, a direct comparison between the sensitivities of the templates and platforms used in this study would be of great value. A more systematic approach would be required to address such an endeavour. Estimating the limit of detection (LoD) and the limit of quantification (LoQ) of each platform using templates extracted from a normalized cell count or performing serial dilutions of reference materials after converting their values to the international scale would provide a good strategy for establishing the average improvement of sensitivity. Such attempts have been made in the past albeit being sporadic with limited number of samples demonstrating an improved sensitivity of 1 to 3 log for the RT-dPCR platform compared to the conventional real time quantitative platform RT-qPCR (39, 42), in addition to 1 log improved sensitivity using DNA as a template compared to cDNA on a quantitative real time platform (27, 29).

The technologies described allow the assignment of absolute quantities to both *BCR-ABL1* DNA and RNA targets, facilitating for the first time direct comparison of mean expression versus cellular disease burden. In future it will therefore be important to explore not only whether the risk of relapse following withdrawal is a feature of the number of residual CML cells, but also whether it relates to the degree of transcriptional activity in those cells. We observed that 8% (3/36) of the samples were positive by RNA but negative by DNA based methods (Table 4 and supplementary Table 6 and 7). Conversely, in MR samples that showed detectable *BCR-ABL1* DNA there was heterogeneity in the detectability of transcript by RT-dPCR that appeared to be unrelated to the amount of *BCR-ABL1* DNA detected. It should be borne in mind that RT and cDNA synthesis steps remain a potential source of variation affecting cDNA concentration, and therefore these results should be interpreted with caution. Nevertheless, the relationship between residual oncogene DNA and RNA and the impact of this ratio on outcome after withdrawal requires further investigation.

Conflict of Interest

The Authors declare no conflict of interest.

Author contribution

Mary Alikian, Planned and conducted the experiments in addition to data analysis and manuscript preparation; Peter Ellery, conducted qPCR experiments and data analysis; Martin Forbes, conducted qPCR experiments and data analysis; Gareth Gerrard, contributed in experimental design and provided continuous discussion and troubleshooting; Dalia Kasperaviciute, Bioinformatics data analysis; Alona Sosinsky, Bioinformatics data analysis; Michael Mueller, Bioinformatics data analysis; Alexandra Whale, helped with dPCR experimental design and data analysis; Dragana Milojkovic, helped with sample selection and commented on the manuscript; Jane Apperley, helped with sample selection and commented on the manuscript; Jim Huggett, helped with dPCR experimental design and data analysis and facilitated access to the Fluidigm BioMark platform; Letizia Foroni, helped with experimental design and data analysis and helped in manuscript preparation; Alistair G Reid, contributed in editing the manuscript.

Acknowledgements

We thank Michaela Noseda, and Prof Michael Schneider at the NHLI and Prof Phillip Froguel and Petros Takousis at the Centre for Computational Genomics and Genomic Medicine, Imperial College London for providing access to their facilities and the Fluidigm platform. We are grateful for support from the NIHR Biomedical Research Centre Funding Scheme and also thank the Imperial College High Performance Computing Service for providing computing resources and support. This work is dedicated to the memory of Professor John M Goldman who instigated and guided the preliminary part of this study.

Supplementary information is available at the Journal of Molecular Diagnostics website (http://www. http://jmd.amjpathol.org//).

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Figures

Figure 1

Pictorial representation of breakpoint mapping read types obtained by NGS of *ABL1* and *BCR* sequences in a patient with CML. a) *Right hand panel*: an example of split reads. These single reads are composed of material from two non-contiguous regions on the reference genome that map directly across the fusion junction and are thus capable of identifying breakpoints to base pair resolution. Based on the sequence information obtained from these reads, sequencing primers (grey arrows) are designed for confirmation by Sanger sequencing. **b)** *Left hand panel*: an example of discordant pairs of reads in which individual reads in a pair map to different genes. Clusters of discordant read pairs allow indirect estimation of the position of the fusion site to within up to 1kb. **c)** The effect of an inversion at the fusion site on read orientation. *BCR* and *ABL1* components of the split read run in opposite genomic directions.

Figure 2

Comparison of the sensitivity of 4 methods of disease quantification in 46 samples from 6 CML patients. Quantification results are shown for the follow-up samples of six patients with CML according to subestrate type and platform used. The four techniques are listed on the left hand side. Samples are represented by circles on a linear timeline (not to scale), with the earliest sample on the far left. The vertical dotted lines represent the point of acheivement of MR^4 or lower (based on RT-qPCR anaysis of the proceding sample). Black-filled circles represent samples in which disease was detectable and quantifiable by that technique. Grey-filled circles represent samples in which disease was detecable below the limit of reliable quantification (LoD). White-filled circles represent samples in which disease was undetectable. dPCR and RT-dPCR values are after preamplification.

Figure 3

Comparison of the DNA-based dPCR and RT-dPCR readings in 46 samples from 6 CML patients. DNA dPCR results are shown on the left (blue bars) and RT-dPCR results are shown on the right (green bars). The vertical red dotted lines represent the point of acheivement of MR^4 or lower (based on RT-qPCR anaysis of the proceding sample). Precise readings are provided in Table 5 and supplementary Table 5. Plotted dPCR and RT-dPCR values are after preamplification. The barcharts depict five senarios: 1)DNA is detected while RNA is not. 2)RNA is detected while DNA is not. 3) Both DNA and RNA are detected, but DNA quantity is higher than RNA. 4) Both DNA and RNA are detected with higher RNA quantity compared to DNA. 5)Neither DNA nor RNA are detected.

Tables

Table 1

Details of the primers used to characterise BCR-ABL1 fusion junctions in 32 CML patients. Primers were designed based on the results of targeted next generation sequencing.

PATIENT	FUSION GENE	PRIMER	SEQUENCE	Strand	AMPLICON SIZE (in bp)	Tm (ºC)
A1	ABL1-BCR	F	5' GTTCCTACCAGCACCCTTGA 3'	+	580	61
		R	5' CTCTCTCCCAACCCCATTC 3'	-		
A2	BCR-ABL1	F	5' TTCACGCCAGACCACAATTA 3'	+	233	61
		R	5' ACTGAAACCAGCCAATGGAC 3'	-		
	ABL1-BCR	F	5' GCTCTAGGCTTTCCTCAGCA 3'	+	299	61
		R	5' TGTGATGACAGGGATGGGTA 3'	-		
A3	BCR-ABL1	F	5' CCCATGACACTGGCTTACCT 3'	+	778	61
		R	5' GCTCTTTGCCCACTCCACTA 3'	-		
	ABL1-BCR	F	5' CTTAGGCACCAGCTCGTAGG 3'	+	938	61
		R	5' GGGAAACACCAGCGTTTATG 3'	-		
A4	ABL1-BCR	F	5' AACCCAAAAAGGAGGACTTGA 3'	+	336	61
		R	5' ACGGCGACACACAATACAAA 3'	-		
A5	BCR-ABL1	F	5' GATGCTGACCAACTCGTGTG 3'	+	340	61
		R	5' CCCAGGGATGGTAAAAACCT 3'	-		
	ABL1-BCR	F	5' AAAATGTACGGGGGAGAAGC 3'	+	235	61
		R	5' AGATCCAAGGCACAGAGCAT 3'	-		
A6	BCR-ABL1	F	5' TCTTGCGCAGATGATGAGTC 3'	+	932	61
		R	5' TGAACAACCTCCCTGTTTCC 3'	-		

B1	BCR-ABL1	F	5' GCTGTTTGCGCTCACATTTAC 3'	+	201	61
		R	5' CAGCCTCCCAAGTAGCTGAG 3'	-		
B2	BCR-ABL1	F	5' TGTCATCGTCCACTCAGCCAC 3'	+	246	65
		R	5' GGCAGACAGAGTGAGACTCCATC 3'	-		
B3	BCR-ABL	F	5' GGAATTGTTTTTCCCGGAGT 3'	+	286	61
		R	5' CCAGGAACAGGCTTTGTTTAA 3'	-		
	ABL1-BCR	F	5' CCATTGGTGAACTGCTCCTT 3'	+	230	61
		R	5' CGGCCTCGAGAAACTTACAC 3'	-		
B4	BCR-ABL	F	5' GATGAGTCTCCGGGGCTCTA 3'	+	230	61
		R	5' CTCGTTCTGTCGCTAGGGTG 3'	-		
B5	BCR-ABL	F	5' GCTCTTACAGACCATGTGGGT 3'	+	284	61
		R	5' AGGACTGAGGCTGGAAGTCA 3'	-		
B6	BCR-ABL	F	5' TTGTGCTGGTTGATGCCTTC 3'	+	200	61
		R	5' TGCAATCTCTCTCTCCAAGGA 3'	-		
B7	BCR-ABL	F	5' TGGACAAGGTGGGTTAGGAG 3'	+	280	61
		R	5' AGTAAGAGCTGACGTGTATTGTGC 3'	-		
	ABL1-BCR	F	5' CCAGGGTCTCATAACCAAGG 3'	+	310	61
		R	5' AAGGTTCCAAGGACAGCAGA 3'	-		
B8	BCR-ABL	F	5' CATGTCCACTTCTCCCCACA 3'	+	211	60
		R	5' TTCACGTGATCCCTCTGCCT 3'	-		
B9	BCR-ABL	F	5' ACTTCTCCAGCACTGAGCTG 3'	+	258	61
		R	5' GTCCCAATTAACGGTGGAAA 3'	-		
B10	BCR-ABL	F	5' ATCGTCCACTCAGCCACTG 3'	+	664	61
		R	5' TTATCAACATTCACATCTCACAGG 3'	-		
	ABL1-BCR	F	5' CAGCTGAAGAAGGTCTGGATTAGTA 3'	+	332	61
		R	5' CTGGTAAGCTTTCTGTCTCCACA 3'	-		
B11	BCR-ABL1	F	5' GTGAAGGCTGGTAACACATGAG 3'	+	384	61
		R	5' CCCAACCAGCTCACTTTACB 3'	-		

B12	BCR-ABL1	F	5' GGAAACAGGGAGGTTGTTCA 3'	+	671	60
		R	5' GCTACAAGGAACACGCAACA 3'	-		
C1	BCR-ABL1	F	5' GCCCATGACACTGGCTTACC 3'	+	228	61
		R	5' CACACCAAGCCTCCCAAGTT 3'	-		
C2	ABL1-BCR	F	5' GAGATGGAGTTTCACCGTGC 3'	+	170	61
		R	5' GCAAACCAGTGACTCGAAGT 3'	-		
C3	BCR-ABL1	F	5' CCCATGACACTGGCTTACCT 3'	+	224	60
		R	5' TGGCCTGCTTGGTTAACTTT 3'	-		
C4	ABL1-BCR	F	5' TTTGGTGGTTGGGTTGCAAA 3'	+	183	61
		R	5' TATGAGGCAGCCAGAGACAG 3'	-		
C5	BCR-ABL1 (+/-)	F	5' CTAGGCAGTGGGCACCTGTA 3'	+	2722	61
		R	5' GTAAGAAATCTTTGTGTCTACCCTAAGG 3'	+		
	ABL1-BCR (+/-)	F	5' GTGGTCTGTGTAAGAAATCTTTGTG 3'	+	220	61
		R	5' GGCAGTGGGCACCTGTAAT 3'	+		
C6	BCR-ABL1	F	5' CTGGAGTCCGGGTGTCCTC 3'	+	206	61
		R	5' TCACGTTTGAGGCTGTGGAA 3'	-		
	BCR-ABL1 (+/-)	F	5' ATGATGACAGTGAGTGTGGCC 3'	-	282	61
		R	5' TGTCGCATTGAAAGATGACACTTA 3'	-		
	ABL1-BCR (+/-)	F	5' GCTGCACGATTAGTGTTGTACATT 3'	-	285	
		R	5' AACTCCCTGGCATGGTGG 3'	-		61
C7	BCR-ABL1	F	5' CCCATGACACTGGCTTACCT 3'	+	300	61
		R	5' CATACTCCGTTCCAGCGG 3'	-		
	ABL1-BCR	F	5' ATGATCTCATCCGCTGGAAC 3'	+	350	61
		R	5' CCAGCTCCCAGGATCTGAG 3'	-		

C8	BCR-ABL1	F	5' CCCCGTTTCCGTGTACAGG 3'	+	239	60
		R	5' CTAGGACCCTGGAGCACTGT 3'	-		
D1	BCR-ABL1	F	5' TGCACCTCTTTTCCAACCTC 3'	+	248	61
		R	5' TGCAGCCAGTCCCTTAGTCT 3'	-		
D2	BCR-ABL1	F	5' TCTGCTGTCCTTGGAACCTT 3'	+	185	61
		R	5' ATGAGGGAAGAGGGAGGAGA 3'	-		
D3	BCR-ABL1	F	5' TGTGACCTTCTCCATGTCCA 3'	+	206	61
		R	5' TCTGCCACACAAAGAACCTG 3'	-		
	ABL1-BCR	F	5' CAAGGAACTGCCCTATTCCA 3'	+	300	61
		R	5' CAACATTCGTTCACTCAGTCG 3'	-		
D4	ABL1-BCR	F	5' TTGGGAAGAGAAGGGAACCT 3'	+	200	61
		R	5' AGGCAGTGTCACAGCACAAC 3'	-		
D5	BCR-ABL1	F	5' TCACGCCAGACCACAATTAG 3'	+	244	60
		R	5' GTCCCAATTAACGGTGGAAA 3'	-		
	ABL1-BCR (+/-)	F	5' TGTAATCCCAGCAATTTGGGA 3'	+	189	65
		R	5' CACAGCAGGCTGCCTGG 3'	+		
D6	BCR-ABL1	F	5' GAGTTGGAGACCAGCCTGAC 3'	+	183	61
		R	5' CCTCCCTCACCTCCACAAA 3'	-		
	ABL1-BCR	F	5' CCAACCCCTGCCCTTTTAAA 3'	+	191	61
		R	5' CGATTCTCTTCCCTCAGCCT 3'	-		

Table 2

Sequences of primer and probe components of 6 patient-specific hydrolysis probe assays for the BCR-ABL1 or ABL-BCR DNA junction used for dPCR and qPCR

Patient	Fusion	Assay*	Sequence	Tm	Length (bp)
A1	ABL1-BCR	A1_ABL1-BCR_F	5' GTTTAGTTGATGACACACCTGACTCTAA 3'	58	116
		A1_ABL1-BCR_R	5' CCCAGGCTGGAATGCAGT 3'	58.2	
		A1_ABL1-BCR_P	6FAM- 5' CCTGGCGGAGGTTG 3'	70	
A2	BCR-ABL1	A2_BCR-ABL1_F	5' GGTGATGTGGAAAAGACCTGTGA 3'	59	127
		A2_BCR-ABL1_R	5' CATCCACATATATAGGACTCCCAACAC 3'	59	
		A2_BCR-ABL1_P	6FAM- 5' CTTCTCCATGTCCACTTC 3'	70	
A3	BCR-ABL1	A3_BCR-ABL1_F	5' CAGATCCTGGGAGCTGGTGA 3'	59.8°C	145
		A3_BCR-ABL1_R	5' GATGGTGTTTCACCACATTAGCC 3'	59.2°C	
		A3_BCR-ABL1_P	6FAM- 5' CGGATCACAAGGTCA 3'	69°C	
A4	ABL1-BCR	A4_ABL1-BCR_F	5' ATCACATAACCTAAAACTTAACATTGACACC 3'	60	109
		A4_ABL1-BCR_R	5' CGCTAACAAAGGCAGACAAAAAG 3'	59	
		A4_ABL1-BCR_P	6FAM-5' TGGAAAGAGACTTAAAAAG 3'	69	
A5	BCR-ABL1	A5_BCR-ABL1_F	5' TGATGGGACTAGTGGACTTTGGTT 3'	59.1°C	97
		A5_BCR-ABL1_R	5' TCTACACCCATGTGGGAGCAG 3'	59°C	
		A5_BCR-ABL1_P	6FAM-5' AGAAGGAAGAGCTATGCTT 3'	70°C	
A6	BCR-ABL1	A6_BCR-ABL1_F	5' GGATACTACTTTTTTTTTCCTTTCCCTC 3'	58.4° C	162
		A6_BCR-ABL1_R	5' GTAACATTAACTGTTGGAAAACATGTCTTAG 3'	58.9 $^{\circ}$C	
		A6_BCR-ABL1_P	6FAM- 5' CTTAAATAGCTCTAGTTCCCT 3'	70°C	

Table 3

dPCR and RT-dPCR threshold and Cq parameta defined for each of the 6 assays performed on DNA (A1-A6) and one assay performed on cDNA (WESSEX plasmid).

Analytical method	Assay	Cq range	threshold
dPCR	A1	20-32	0.05
dPCR	A2	20-32	0.05
dPCR	A3	20-32	0.05
dPCR	A4	18-40	0.03
dPCR	A5	16-40	0.02
dPCR	A6	20-40	0.025
RT-dPCR	WESSEX plasmid	20-32	0.03

Table 4

Table showing the Sanger-validated junction sequences of BCR-ABL1 and/or ABL1-BCR fusion genes in 32 CML patients.

Table showing the Sanger-validated junction sequences of BCR-ABL1 and/or ABL1-BCR fusion genes in 32 CML patients.

Patient	**Fusion***	**Sequence****
A1	*ABL1-BCR*	5'TAACCTGCATAAGTATGAGGAAGAAAAGTGKGAACWGAATCATAACTAAAGGAAAGAAGAACATTGCTGTCT TAAGCAGATCACGCCTTTGTCGTTATACAGWAWGGTACAATAATCCATTACACATCTCTTTGATTCATCTTGTCTG TTAAATTTCTTTATAAAATTTGTTTAGTTGATGACACACCTGACTCTAATATAAGAGTTGAATGTTGTATCACTTTTT TTTTTCCT**GGCGGAGGTTGCAGTGAGCCGAGCTTGTGCCACTGCATTCCAGCCTGGGCGACAGAGCAAGACTCC GCCTCAAAAAAAAAAAAAAAAAGTTCCTAGAAACAGCAAAATGTGGAGACAGAAAGCTTACCAGGGATTGTT GGGGAATGGGGTTGGGAGAGAGGACTAACTGCAGATGAACCCAAGGGGGACTTTTTAGGTGA** 3'

A2	*ABL1-BCR*	5'GCTCTAGGCTTTCCTCAGCAGTCTGGTAATCCTGGTTGTCCATTCATGCTTA**GGGTATTTTGTGAATAAAGCAA** **AGACGCGCGTCTACAGGGACACAGCTGAGCCAAACTGGAACGAGGTGAGGAACTGATTCCACAAGGGCCCAG** **CCTGCCAGGTGGGGCACAGGATATTTTCCACTGGAGTAATCTAAACCTTCAGGGGCTCCCTGAGGGCTTCCCCC** **GAAGGCAGTGCTGCTGAATTCCAGGAATCTCCTGGGGGGCCAGTAGGTGACGTGTCCAAGAGTTTTATCTCCTT** **GTTCCTGAAAACCTTACCCATCCCTGTCATCACA** 3'
A2	*BCR-ABL1*	5'**GTTTAATTTTTAAAAAGAAAATTACAACCTTTTTTTTTTATTTTTATTTTTTCTGATTCTGCAAATAACACCTGCT** **CTTACAGACCATGTGGGTGATGTGGAAAAGACCTGTGACCTTCTCCATGTCCACTTCTCCCCACAGATCTGTACT** **GCACCCTGGAGGTGGATTCCT**ACATGGGAATGGAAGGGGTGTGGAGTCCC 3'
A3	*BCR-ABL1*	5'**AAGGACTCATCGGGCAGGGTGTGGGGAAACAGGGAGGTTGTTCAGATGACCACGGGACACCTTTGACCCTG** **GCCGCTGTGGAGTGTTTGTGCTGGTTGATGCCTTCTGGGTGTGGAATTGTTTTTCCCGGAGTGGCCTCTGCCCTC** **TCCCCTAGCCTGTCTCAGATCCTGGGAGCTGGTGAGCTGCCCCCTGCAGGTGGATCGAGTAATTGCAGGGGTTT** **GGCAAGGACTTT**GCGGATCACAAGGTCAGGAGATCGAGACCATCCTGGCTAATGTGGTGAAACACCATCTCTAC TAAAAATACAAAAAATTAGCCGGGCGTGGTGGCGGGCGCCTGTAGTCCCAGCTACTCAGGAGGCTGAGGCAGG AGAATGGCGTGAACCCTGAAGGCGGAGCTTGCAGTGAGCCGAGATGGCGCCACTGCACTCCAGCCTGGGCAAC AGAGCAAGACTCCGCCTCAGAAAAAAAAAAAGAAAAGAAATGCCCCGTAGAGGCTCT 3'
A3	*ABL1-BCR*	5'TAAACATTAACTCCCCTTTAGTATTTCATTTCTAGGGTTTCCAGCCTTGGTAACAATTACCAAGAACTTTGCTGTC ATGGGGTACAGCTCTAGGCTGCTGACAGGATCTTCATACTGGAGGAGACTATACTTTTATAGCCAATTCCAACTG CCCCAGAGATAACTTGGATAAACACTGGCTCCTCCACTACCTCCTAATCCCTGCTGGGTTTTACACTGTGGGGAGG TTT*GGGAGGCC***GAGTTGGAGACCAGCCTGACCAACATGGTGAAACCCTGTGTCTACTAAAAATACAAAGATTA** **GCCAGGCTAGGCAGTGGGCACCTGTAATCACAACTGCTTGGGAGGCTGAGGGAAGAGAATCGCTTGAACCCA** **GGAGGCGGAGGTTGCAGTGAGCCGAGCTTGTGCCACTGCATTCCAGCCTGGCGACAGAGCAG** 3'

A4	*ABL1-BCR*	5'AACCCAAAAAGGAGGACTTGAGATTTCTGATTTATAAACAGTTCGTCAAAAAAGACAGATCACATAACCTAAAA CTTAAGATTGACACCTA**AAGAAGCCACCTATTTTGCTGGAGAAGTGTTTGGAAAGAGACTTAAAAAGGCTTTTT** **GTCTGCCTTTGTTAGCGCTTATGTTTACTTGGTAGGTTTCCATAGTGTTTTTCCGCAGAAGTTGGAATGAGCTCCC** **GTGACCCTCTTGGCTTCCGGAAGCCTGGGGCTCTGCTGGTGGGGCTGGGACAGGGACTGTAGGGTCCTGCTTG** **CTTGTCTCGGGGTGTTGGGTTTGTATTGTGTGTCGCCG** 3'
A5	*BCR-ABL1*	**5'GATGCTGACCAACTCGTGTGTGAAACTCCAGACTGTCCACAGCATTCCGCTGACCATCAATAAGGAAGGTGG** **GCCCCCCCGTTTCCGTGTACAGGGCACCTGCAGGGAGGGCAGGCAGCTAGCCTGAAGGCTGATCCCCCCTTCCT** **GTTAGCACTTTTGATGGGACTAGTGGACTTTGGTTCAGAAGGAAGAGCTATGCTTGTTAGGGCCTCTTGTCTCC** **TCCCAGGAG**AGCTGCTCCCACATGGGTGTAGAAACTGGTTTTATCCTATCTGTGTGACCTCCAATAGCTGCAAGTT GGAAATCTTGATTTAGAGTGGACCTAGGTTTTTACCATCCCTGGG 3'
A5	*ABL1-BCR*	5'AAAATGTACGGGGGAGAAGCACATTACACAGTATCAATCAGAAAGGAATATTTCTGTCTGAGCTTTCACTTTGA GTGGAGGGCAAAGAAAAAAAATCACATGAGAACTTGTGGCTGCTGACCAGC**AGTTTCTCCCTGAGTGGCTGCTG** **CTGGGTGGTTGAGGAGATGCACGGCTTCTGTTCCTAGTCACAAGGCTGCAGCAGACGCTCCTCAGATGCTCTGT** **GCCTTGGATCT** 3'
A6	*BCR-ABL1*	5'**CTGGGAGCTGGTGAGCTGCCCCCTGCAGGTGGATCGAGTAATTGCAGGGGTTTGGCAAGGACTTTGACAGA** **CATCCCCAGGGGTGCCCGGGAGTGTGGGGTCCAAGCCAGGAGGGCTGTCAGCAGTGCACCTTCACCCCACAGC** **AGAGCAGATTTGGCTGCTCTGTCGAGCTGGATGGATACTACTTTTTTTTTCCTTTCCCTCT**AAAAAAAAAAAAAAT CTTAAATAGCTCTAGTTCCCTGAATTATTTTCTCCTAAGACATGTTTTCCCAACAGTTAATGTTACTTATTTCATCTTC TGATCTTACTCATATTATCCACCTTTCACTTAAATTGGAAATTTTCTGCCATTTTACAAAGTTCAAGACTTTC 3'

B1	*BCR-ABL1*	5'**GAGAGCAGTGTCGTGAAAAGACTGTGGTGCTGTTTGCGCTCACATTTACATTTCCTAAAATTCTTTAAACCCT** **ACACTTGG**CTGAGGCGGGTGTATCACCTGGGTCAGGAGTTCGAGACCAGTCTGGCCAACATGGTGAAACCCCGT CTCTACTAAAATATAAAAAAAAT 3'
B2	*BCR-ABL1*	5'**TGTCATCGTCCACTCAGCCACTGGATTTAAGCAGAGTTCAAGTAAGTACTGGTTTGGGGAGCAGG**TACAGGG GTGAGCCTCCACGCCGGGCCTCTCTTTTTAAGATAATAAAAATTACTAAACATCATAATGACATGATTTGGGATTA CTCTTTCAGAGAAACAGTTCCTAAAGGTTTACTTCAGTTAGTGA 3'
B3	*BCR-ABL1*	5'**CCTGGGAGCTGGTGAGCTGCCCCCTGCAGGTGGATCGAGTAATTGCAGGGGTTTGGCAAGGACTTTGACAG** **ACATCCCCAGGGGTGCCCGGGAGTGTGGGGTCCAAGCCAGGAGGGCTGTCAGCAGTGCACCTT**AGTGGGTGG TTTTAGAATTCCACTTCAGATGGCACTGACCTTTCCTTAATACAGCTGGTTTCACTTAATACATTTTGTTTAAACAAA GCCTGTTCCTGG 3'
B3	*ABL1-BCR*	5'TCCATTGGTGAACTGCTCCTTGCCTTGAGGTCCTGTAGCTAGTTTGCCACTGAGTATTTCCTTCAAAAAACAGGA AGGATGCCCTGATATTATG**CAGCAGAGCAGATTTGGCTGCTCTGTCGAGCTGGATGGATACTACTTTTTTTTTCC** **TTTCCCTCTAAGTGGGGGTCTCCCCCAGCTACTGGAGCTGTCAGAACA** 3'
B4	*BCR-ABL1*	5'**GATGAGTCTCCGGGGCTCTATGGGTTTCTGAATGTCATCGT**TCATCCCGGCCAACATGGTGAAACCCCGTCTCT ACTAACCATACAAAAATTAGCTGGGCGTGGTGGCGCTTGCCTGTGTAATCCCAGCTACTCAGGAGGCTGAGGCA GGAGAATCGCTTTAACCAGGGAGTCGGAGGTTGCAGTGAACCGAGATCGCGCCATTGCACTCCACCCTAGCGAC AGAACGAG 3'
B5	*BCR-ABL1*	5'**TCTTACAGACCATGTGGGTGATGTGGAAAAGACCTGTGACCTTCTCCATGTCCACTTCTCCCCACAGATCTGTA** **CTGCACCCTGGAGGTGGATTC**CCTGGTCAGGTCTGCATTTGAGAAAGATGGCCCTGATAGTAGGGAGTGGAGGT GGAATGGCCAAAGGGGGACCCAGCCAAGGGCGTGGTAGAGCATGGAAAGGACAGAAGTGATGAGAGATTGAG GAGGAAAAGTGC 3'

B5	*ABL1-BCR*	5'AGATTCTTCAGAGGAGGCCCCTCCAGCCACTCAGAACTTCATCATTCCAAAAAAGGAGATCCA*T***CCTGCTCTTAC** **AGACCATGTGGGTGATGTGGAAAAGACCTGTGACCTTCTCCATGTCCACTTCTCCCCACAGATCTGTACTGCACC** **CTGGAGGTGGATTCCTTTGGGTATTTTGTGAATAAAGCAAAGACGCGCATCTA** 3'
B6	*BCR-ABL1*	5'**GGCCTCTGCCCTCTCCCCTAGCCTGTCTCAGATCCTGGGAGCTGGTGAGCTGCC**CAAAAATAATAAATAAATAA ATAAATAAATAAATAAATAAATAAATAAATAAAGCTATGCATTTCCCACAAATACTGCTTAAAAAAAAAAGAACTA TTCTTTGCTATCCTTGGAGAGAGAGATTG 3'
B7	*BCR-ABL1*	5'**TGGACAAGGTGGGTTAGGAGCAGTTTCTCCCTGAGTGGCTGCTGCTGGGTGGTTGAGGAGATGCACGGCTTC** **TGTTCCTAGTCACAAGGCTGCAGCAGACGCTCCTCAGATGCTCTGTGCCT**TGTCTGACTTCTCTGAGCATCTGTG GGGTTTGTTTGTTTTTAAACCTGAAGAATTGGGATAATCTGTTTCCTCACAGAATCATTACGCAGATGCAATACAG TTAATGAA 3'
B7	*ABL1-BCR*	5'CCAATGCTTTAATCCACTTGGAATTTATTCTTGATGTGAAGGGTCAGCATCATCCACTGGTCGGGAGCCTGATCC TTGGAGCCAGGCAGACCTGGGTTGAGTCCATCTCCGCCTGTTTCCAGCTGTGGGCAAGGT**CTGGCCCCACTCCCG** **TCCTCCCAGCCCTCCTCTCCTCCAGCTACCTGCCAGCCGGCACTTTTGGTCAAGCTGTTTTGCATTCACTGTTGCAC** **ATATGCTCAGTCACACACACAGCATACGCTATGCACATGTGTCCACACACACCCCACCCACATCCCACATCACCC** **CGACCCCC** 3'
B8	*BCR-ABL1*	5'**CATGTCCACTTCTCCCCACAGATCTGTACTGCACCCTGGAGGTGGATTCCTTTGGGTATTTT**CGGATCACTTGA GGTCAGGAGTTCGAGACCACCCTGGCCAACATGGTGAAACCTCGTCTCTACTAAAAATACAAAAATTAGTCGGCA TGGTGGCACACACCTATAGTCCCACCTACATGGGAGGCTGAGGCAGAGGGATCACGTGAA 3'
B9	*BCR-ABL1*	5'**CTCCAGCACTGAGCTGCTTCCTGTGCCCCACAGTGGCCTGGAGTCCCCTTTGCCTTAACTCTTTGCCCCATAGTA** **C**AGCGTGGCCCCCAGCCCCGGCACCAGCCCCGGTAGAGCCACGCCGGATGGTGACGGCGGCGTCCGGGACCCC ACAGTGCGGGCTCCCCCGAAAAAGTTTGAGAAAGCCAA 3'

B10	*BCR-ABL1*	5'**CACTGGATTTAAGCAGAGTTCAAGTAAGTACTGGTTTGGGGAGGAGGGTTGCAGCGGCCGAGCCAGGGTCT** **CCACCCAGGAAGGACTAATCGGGCAGGGTGTGGGGAAACAGGGAGGTTGTTCAGATGACCACGGGACACCTT** **TGACCCTGGCCGCTGTGGAGTGTTTGTGCTGGTTGATGCCTTCTGGGTGTGGAATTGTTTTTCCCGGAGTGGCCT** **CTGCCCTCTCCCCTAGCCTGTCTCAGATCCTGGGAGCTGGTGAGCTGCCCCCTGCAGGTGGATCGAGTAATTGC** **AGGGGTTTGGCAAGGACTTT**CAGGC*G*TGCGCCACCATGCCTGGCCAATTTTTTTGTATTTTTAGTAGAGACGGGG TTTTGCTATGTTGGTCAGGCTGGTCTCAAACTCCTGACCTCAAGTGATCCGCCTGCCTTGGCCTCCCAAAGTGCTG GGATTACAGGCGTGAGCCACTGTACCTGGCCGATATTTTCTATTTTATTTCATTTTGAAAAAGATAGGCTGTGACT CATTAGTAGTGGATCTCATGACTAAGAATGGATCCTGACCCAAGTTTGAAGAGACTGGTAGGGGTAAATTTAGA GCCTGGGGCTCCAAAGTTTGTCTACCCAGTTTTAAATCCTGGCTTTCCCCTTAACTTGGGTGGGATCACAC 3'
B10	*ABL1-BCR*	5'TTTTCTTTTTCTTTTTTTTTGAGACAGTCTTACTCTGTTGCCCAGGCTGGAGTGCAGTGGTGTGATCTCGGCTTA CTGCAACCTCCACCTTCCAGGTTCAAGCAGTTCTCCTGTCTCAGCCTCCCAAGTAGCTGGGACTA**GGACTTTGACA** **GACATCCCCAGGGGTGCCCGGGAGTGTGGGGTCCAAGCCAGGAGGGCTGTCAGCAGTGCACCTTCACCCCACA** **GCAGAGCAGATTTGGCTGCTCTGTCGAGCTGGATGGATACTACTTTTTTTTTCCTTTCCCTCTAAGTGGGGGTCT** **CCCCCAGCTACTGGAGCTGTCAGAACAGTGAA** 3'
B11	*BCR-ABL1*	5'**GTGAAGGCTGGTAACACATGAGTTGCACTGTGTAAGTTTCTCGAGGCCGGGCGCAGTGGCTCATGCCTGTAA** **TCCCAGCACTTTGGGAGGCTGAGGCAGGTGGATCGCTTGAGCTCAGGAGTTGGAGACCAGCCTGACCAACATG** **GTGAAACCC**TACCAGTTACCATGCCGGAGTAACAGTTACC*T*CACTGGTATACCAGTTACCATGCCAGAGTGACTG ATTTTGACCAAGACCTTTGGGTTGTACAGAAGGGGTGAGACCATACCTGTCCTCAAGCATTTCCAACCTAGTGTA GAGAAAAACAGGAGCAAGATAAGAAC 3'

B12	*BCR-ABL1*	5'**TGTTTGTGCTGGTTGATGCCTTCTGGGTGTGGAATTGTTTTTCCCGGAGTGGCCTCTGCCCTCTCCCCTAGCCT** **GTCTCAGATCCTGGGAGCTGGTGAGCTGCCCCCTGCAGGTGGATCGAGTAATTGCAGGGGTTTGGCAAGGACT** **TTGACAGACATCCCCAGGGGTGCCCGGGAGTGTGGGGTCCAAGCCAGGAGGGCTGTCAGCAGTGCACCTTCAC** **CCCACAGCAGAGCAGATTTGGCTGCTCTGTCGAGCTGGATGGATACTACTTTTTTTTTCCTTTCCCTCTAAGTGG** **GGGTCTCCCCCAGCTACTGG***G*GGCATCCCTGACCTCCAAATGGTCTGTTTTGGCCTCCATTCCTATATCCTTTAAAT GACTGARAATGCAGCTGGTAAAGTTGGAARAATAAAGTTAACCAAGCAGGCCAGGCACGGTGGCTCACGCCTAT AATCCCAGCACTTAGGGAGGCCAAGGCGGGCAGATCACCTGAGGTCAATAGTTCGAGACCAGCCTGGCCAACAA GGTGAAACCCCATCTCTACTAAAAATACAAAAAATTAGCCAGGTGTGGTGGTGTGCACCTGTAGTCCCAGCTATT TGGRAGGCTGAGGTGGGAGAATCACTTGAACCTGGGAGGCGGAGGTTGCAGTGAGCTGAAATCGCGTACCACA CTC 3'
C1	*BCR-ABL1*	5'**GCCCATGACACTGGCTTACCTTGTGCCAGGCAGATGGCAGCCACACAGTGTCCACCGGATGGTTGATTTTGAA** **GCAGAGTTAGCTTGTCACCTGCCTCC**CAGCCTCTGCTTTTTCTGTTTCCTTCTTGATGGGAAGACTGGCAGCAGGA TTTACTTGGTTGCTACATTTGTGAATTTGTAAAATAATTCATGCTCTGCTAGGTCTGTAACTTGGGAGGCTTGGTGT G 3'
C2	*ABL1-BCR*	5'GAGATGGAGTTTCACCGTGCTGGCCAGGCTGGTGTCA**TATGCTCAGTCACACACACAGCATACGCTATGCACA** **TGTGTCCACACACACCCCACCCACATCCCAC** 3'
C3	*BCR-ABL1*	5'**CCCATGACACTGGCTTACCTTGTGCCAGGCAGATGGCAGCCACACAGTGTCCACCGGATGGTTGATTTTGAAG** **CAGAGTTAGCTTGTCACCTGCCTCCCTTTCCCGGGACAACAGAAGCTGACCTCTTTGATCTCTTGCGCAGATGAT** **GAGTCTCCGGGG***GGATGCC*GTTTTGGCCTCCATTCCTATATCCTTTA 3'
C4	*ABL1-BCR*	5'CTGCCATTTTTACTTGAAACTATGACAAACTATGGTTATTCA**CTTGGAATGGATGAATTACATGACATGCAGAT** **TGCACCTTCATAACATAATCTTTCTCCTGGGCCCCTGTCTCTGGCTGCCTCATAAACGCTGGTGTTTCCC** 3'

C5	*BCR-ABL1* *(+/-)*	5'**CTAGGCAGTGGGCACCTGTAATCACAACTGCTTGGGAGGCTGAGGGAAGAGAATCGCTTGAACCCAGGAGG** **CGGAGGTTGCAGTGAGCCGAGCTTGTGCCACTGCATTCCAGCCTGGGCGACAGAGCAAGACTCCGCCTCAAAA** **A**AAATATGAGGGAAAAAATGAACCTTCGCCCCTACACAGAAAAAACAATTCCAGGCCAACTATAAACATGAGAT GTAAGAGAGTGAAACTTCTTTGTGACCTTAGGGTAGACACAAAGATTTCTTACA 3'
C5	*ABL1-BCR* *(+/-)*	5'TGTAAGAAATCTTTGTGTCTACCCTAAGGTCACAAAGAAGTTTCACTCTCTTACATCTCATGTTTATAGTTGGCCT GGAATTGTTTTTTCTGTGTAGGGGCGAAGGTTCATTTTTTCCCTCATATT**TTTTTTGAGGCGGAGTCTTGCTCTGTC** **GCCCAGGCTGGAATGCAGTGGCACAAGCTCGGCTCACTGCAACCTCCGCCTCCTGGGTTCAAGCGAT** 3'
C6	*BCR-ABL1*	5'**TCTGGAGTCCGGGTGTCCTCGGGCAGGTCGCCAGAGAACCTGTCAAACCAAGAGTTGTATTGGCATCTGAGT** **AGGTTTCTGCTTGTCTCAGAGTCAGGTGTCTGAAATGTCCTGGGATCGATGGTAAGAGCAGCTAAGAGGTCT**G CTCATGGTTGGCATTTTATTTCTCCTGTCCAGAGACAATATTTCCACAGCCTCAAACGTGA 3'
C6	*BCR-ABL1* *(-/+)*	5'**CCCTGGCATGGTGGGAGCCCTGGCCA*A*CCTCAGCCACCCGTGCTTCTCCATGATGACAGTGAGTGTGGCCCGT** **CCCAGGCACAGACGCTTAGGTGTGTTCTGAGACATGCCAAGTATGTTCTTCACAAGACTCCCACAACCAGCATA** **AGGCACTCATCGCT**ATTTATAAATTCCAGTTCTAAATCTACGCACAATTTTTCATGTTTTCCATTCCGTACCTTCCAA GTAACTAATTCCTCTTGAAACCAGTATCAAAAATAAGTGTCATCTTTCAATGCGACA 3'
C6	*ABL1-BCR* *(-/+)*	5'ATTTCTGTCGCATTGAAAGATGACACTTATTTTTGATACTGGTTTCAAGAGGAATTAGTTACTTGGAAGG*TACG*G AATGGAAAACATAAAATTGTGCATAGATTTAGAACTGGAATTTATAAAT**AGCGATGAGTGCCTTATGCTGGTTGT** **GGGAGTCTTGTGAAGAACATACTTGGCATGTCTCAGAACACACCT*A*AGCGTCTGTGCCTGGGACGGGCCACAC** **TCACTGTCATCATGGAGAAGCACGGGTGGCTGAGG*T*TGGCCAGGGCTCCCACCATGCCAGGG** 3'
C7	*BCR-ABL1*	5'**CAGAGTTAGCTTGTCACCTGCCTCCCTTTCCCGGGACAACAGAAGCTGACCTCTTTGATCTCTTGCGCAGATGA** **TGAGTCTCCGGGGCTCTATGGGTTTCTGAATGTCATCGTCCACTCAGCCACTGG**ATAGATAAAATGACTTTCATC AAGCTCAAAATGTGGGAAATAATACTTTTCCTCTAAGCTCATATTTTGAAAAGTTGTTTGAGACTGTTATAAAATA ACTTGAGAATAGTCTGAGCATGATCTCATCCGCTGGAACGGAGTATG 3'

C7	*ABL1-BCR*	5'TCCGCTGGAACGGAGTATGAAATAAAACACGAAGCCTCTTTTTAGAAGGATCTGTTTTATAGATTTTTAAATTAC AGAGATTCCTTGAACTGGAGGATAATAGCGAAGGACCCACAAAGGAAATATGAATAAAGGCAGC**GGCAGAGCC** **AGGGTCTCCACCCAGGAAGGACTAATCGGGCAGGGTGTGGGGAAACAGGGAGGTTGTTCAGATGACCACGG** **GACACCTTTGACCCTGGCCGCTGTGGAGTGTTTGTGCTGGTTGATGCCTTCTGGGTGTGG** 3'
C8	*BCR-ABL1*	5'**CCCCGTTTCCGTGTACAGGGCACCTGCAGGGAGGGCAGGCAGCTAGCCTGAAGGCTG**ACAGTCTGAAGCCTT TGAATCTGGCTTCTTTCATTCAATATAATGGGTGAGGAGTCAACCACGTTGTTGCATGTTATCCACATTTCCTCCTT TTGTGTCTCTGAGTAGTATTCTCTTGCATAGAT 3'
D1	*BCR-ABL1*	5'**CTGCAGATGCTGWCCAACTCGTGTGTGAAATCCAGACTGTCCACAGCATTCCGCTGACCATCAATAAGGAAG** **GTGGGCCCCCCGTTTCCGTGTAC**AGGGCTAGAAGGGTCCTGGAGGTTTGCCACTTCTCCGTGTTGAGGTACAGA GTTCCTAGACTAAGGGACTGGCTGCATTGTTATGTGTGAGTGCTAGTCATGGTGCTGCCTGGCACCCACAAATGC CACAGCAGACCCATGGTCGAGGGAGA 3'
D2	*BCR-ABL1*	5'**CTGCTGTCCTGGAACCTTATTACACTTCGAGTCACTGGTTTGCCTGTAT***ACTGGA*GTCTGGATCTGACCAGTCTC CAGATGAAAACTCTTGCAACTTTCGTTTTTGGATAGTGCTCACCTCGTATCTGTACTCGTACCTGCTATTTCTAGGC GAATTGTCCCCTTTCTCCTCCCTCTTCCCTCAT 3'
D3	*BCR-ABL1*	5'**TGTGACCTTCTCCATGTCCACTTCTCCCCACAGATCTGTACTGCAC***T*CTGGAGGTCTCAGGTGATCTGCCCACCT CGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACTGTGCCCGGCCGGTTGAACGTTTTTAACATGTTTAGG GGAGATACATACTTATCTTTCTGTGAACCATCTCATCAGGTTCTTTGTGTGGCAGA 3'
D3	*ABL1-BCR*	5'CAAGGAACTGCCCTATTCCAAGCTCCAGAGGACCTGCTGAGTAGGTGCTTTTCAGGAGCTTGAAAAGACTGTTT CCAGGTCTACCCCCAGGGGTTAATTTAATTGGTCTGGGGAGAGGCCTAAGTATCAGTTTTTTTGTTTTGTT**GATGG** **CTGCCCTCTGCTGTGGCATCACTGTGTAACAATGGCGTGTACACCTCTCTGTCCCCACCAGTGCAGGGCCCTTCT** **CATCGTAGGGGCTTTAGCTGGGGTTTGTGGATCGACTGAGTGAACGAATGTTG** 3'

D4	*ABL1-BCR*	5'CACAGACCTAGTCCTTTGTTGTGGTGCGAGGCTCAGT**GGGAGGGACATGGGAGCCCTCAGGGAAGTGGTAAGTGAATCCCAGAACCCTTAGACCAGTTCCCTTGAGGTAGTGTTGTGCTGTGACACTGC** 3'
D5	*BCR-ABL1*	5'**CAACCTTTTTTTTTTATTTTTATTTTTTCTGATTCTGCAAAT**CCCGGCACCAGCCCCGGTAGAGCCACGCCGGATGGTGACGGCGGCGTCCGGGGCCCCACAGTGCGGGCTCCCCCGAAAAAGTTTGAGAAAGCCAACTCGCCAGG 3'
D5	*ABL1-BCR (+/-)*	5'TGGGCCCTTCCTAGGCCTCGCCGCCCGCGCGCTCCCGCCTGCGCCCTCCCCGGGTCTTGTCTTTTTTTTCTTTCTTCC.....CTCTTCTCTTCTCTTCAGTTCTCTTATATTCTGTCTCTCTTTCTTTCTCTCTGTGTCTGTCTCTTTTCTCTTCTCT<u>T</u>**CTCAGCTACCCCCGCGCAATTGTGAGGGTGG** 3'
D6	*BCR-ABL1*	5'**GTTGGAGACCAGCCTGACCAACATGGTGAAACCCTGTGTCTACTAAAAATACAAAGATTAGCCGGGCTA**AAACAGTATCTTTTGGGTAAATTCTGGATTTTTTCATATATCCTGCC 3'
D6	*ABL1-BCR*	5'TTATATACTGCTACTTAGCTGTGCAGAGAAATTACAGCATTTAAAAAAATCACCAATCTGTCTTCCTCATAAGTCATCATCTAGAAATGGGGAGAAGCAAAGAGTGGTATTTTGGCCAGTTAATAAGGTCTTATATTC**GGCAGTGGGCACCTGTAATCACAACTGCTTGGGAGGCTGAGGGAAGAGAATCG** 3'
**Unless otherwise specified, fused strands run in the same genomic direction (+/+). Stands marked +/- or -/+ run in oposite genomic directions and are therefore evidence of micro-inversions present at the fusion junction.*		
***Key: bold text = BCR sequence; regular text = ABL1 sequence; underlined text = region of microhomology; italic = SNP, underlined and italic = insertion.*		

Table 5

Target quantities assessed using four molecular quantification methodologies in 46 follow-up samples from 6 patients with CML.

Patient demographics	**DNA and RNA copy numbers measured on Digital PCR platform**	**DNA and RNA copy numbers measured on Real Time Quantitative PCR platform**	

patient	sample	month from therapy start	dPCR (copy/ul)	dPCR (copy/ul)preamp	RT-dPCR (copy/ul)	RT-dPCR (copy/ul)preamp	qPCR		RT-qPCR (IS)		BCR-ABL1 (copy/3ul)	ABL1 (copy/3ul)	MR level
A1	**A1_46**	54	1622	¶	1475	¶	0.140000	*	3.114583	*	8847	68300	>MR^{3}
A1	**A1_37**	68	11	46	7	67	0.000814	†	0.004485	†	3	20000	MR^{4}
A1	**A1_38**	70	1	2	0	0	0.000641	†	0.000000	‡	0	16000	MR^{4}
A1	**A1_39**	71	0	0	0	38	0.000018	‡	0.000831	†	2	72000	MR^{5}
A1	**A1_40**	95	0	0	0	452	0.000284	‡	0.004530	†	4	26400	MR^{4}
A1	**A1_41**	134	1	1	0	5	0.000000	‡	0.000000	‡	0	71300	MR$^{4.5}$
A1	**A1_42**	163	1	1	0	0	0.000000	‡	0.000000	‡	0	68200	MR$^{4.5}$
A1	**A1_43**	169	1	9	0	0	0.000000	‡	0.000362	†	1	82700	MR^{5}
A1	**A1_44**	175	0	0	0	30	0.000000	‡	0.000000	‡	0	74900	MR$^{4.5}$
A1	**A1_45**	178	0	11	0	0	0.000000	‡	0.000000	‡	0	91500	MR$^{4.5}$
A2	**A2_1**	33	1833	¶	1167	¶	0.680000	*	24.916667	*	10000	12000	>MR^{3}
A2	**A2_2**	63	0	4	0	0	0.000000	‡	0.002833	†	9	95000	MR$^{4.5}$
A2	**A2_3**	81	0	5	0	0	0.000000	‡	0.003504	†	10	128000	MR^{4}
A2	**A2_4**	104	0	2	0	0	0.000000	‡	0.000000	‡	0	11400	MR^{4}

A2	**A2_5**	116	0	2	0	0	0.000000	‡	0.000000	‡	0	31200	MR[4]
A2	**A2_6**	122	0	3	0	0	0.000000	‡	0.000000	‡	0	93200	MR[4,5]
A2	**A2_7**	143	0	52	0	0	0.000000	‡	0.000000	‡	0	67100	MR[4,5]
A3	**A3_28**	48	75	-	68	-	0.001281	*	0.079929	*	409	153000	>MR[3]
A3	**A3_29**	92	3	53	3	1	0.000009	‡	0.000341	†	1	87800	MR[5]
A3	**A3_30**	93	6	55	0	0	0.000005	‡	0.000000	‡	0	89000	MR[4,5]
A3	**A3_31**	96	4	38	0	0	0.000019	‡	0.000000	‡	0	91600	MR[4,5]
A3	**A3_32**	102	4	64	0	0	0.000068	‡	0.000000	‡	0	117000	MR[5]
A3	**A3_33**	103	6	65	0	0	0.000027	‡	0.000549	†	2	109000	MR[5]
A3	**A3_34**	106	1	72	0	0	0.000022	‡	0.002072	†	7	101000	MR[4,5]
A3	**A3_35**	110	2	46	0	0	0.000029	‡	0.000516	†	3	174000	MR[5]
A3	**A3_36**	114	2	60	0	0	0.000016	‡	0.000000	‡	0	92800	MR[4,5]
A4	**A4_8**	33	808	¶	735	¶	0.163000	*	3.459297	*	4408	38100	>MR[3]
A4	**A4_9**	39	127	¶	116	¶	0.004000	*	0.287023	*	695	72400	>MR[3]
A4	**A4_10**	45	268	¶	243	¶	0.006000	*	0.475534	*	1460	91800	>MR[3]
A4	**A4_11**	55	0	416	1	62	0.000690	*	0.003866	†	6	46400	MR[4]

A4	**A4_12**	58	1	301	0	1	0.000250	†	0.000000	‡	0	114000	MR[5]
A4	**A4_13**	59	1	177	0	0	0.000101	†	0.000000	‡	0	68400	MR[4.5]
A4	**A4_14**	62	0	0	0	0	0.000000	‡	0.000000	‡	0	26500	MR[4]
A5	**A5_15**	28	235	¶	214	¶	0.045169	*	1.194137	*	1282	32100	>MR[3]
A5	**A5_16**	35	637	¶	579	¶	0.022476	*	0.586851	*	3474	177000	>MR[3]
A5	**A5_17**	66	0	0	0	0	0.000000	‡	0.000000	‡	0	89500	MR[4.5]
A5	**A5_18**	67	0	0	0	0	0.000000	‡	0.000000	‡	0	67100	MR[4.5]
A5	**A5_19**	69	1	4	0	0	0.000000	‡	0.000000	‡	0	81500	MR[4.5]
A5	**A5_20**	77	0	0	0	1	0.000000	‡	0.000823	†	6	218000	MR[5]
A6	**A6_21**	49	64	¶	58	¶	0.145103	*	0.163604	*	348	63600	>MR[3]
A6	**A6_22**	77	3	¶	2	¶	0.011997	*	0.013205	*	14	31700	>MR[3]
A6	**A6_23**	102	0	15	0	63	0.000443	*	0.000425	†	1	70300	MR[5]
A6	**A6_24**	104	0	212	0	0	0.000128	†	0.000906	†	2	66000	MR[5]
A6	**A6_25**	109	0	3	0	0	0.000000	‡	0.000000	‡	0	151000	MR[5]
A6	**A6_26**	110	0	90	0	0	0.000000	‡	0.000000	‡	0	24400	MR[4]
A6	**A6_27**	112	0	6	1	19	0.000000	‡	0.001349	†	3	66500	MR[4.5]

dPCR: DNA based digital PCR; RT-dPCR: RNA based digital PCR; qPCR: DNA based quantitative PCR; RT-qPCR: RNA based quantitative PCR													
*	Denotes a positive value within the LoQ of the standard curve.												
†	Denotes a positive value within the LoD of the standard curve.												
‡	Denotes a negative value												
¶	Denotes no preamplification												
MR^{3}	Major Molecular Response												
MR^{4}	Deep Molecular response specified as 4 log transcript level reduction on the IS (ref *Cross et al. Ann Hematol. 2015;94 Suppl 2:219-25*)												
MR$^{4.5}$	Deep Molecular response specified as 4.5 log transcript level reduction on the IS (ref Cross et al. Ann Hematol. 2015;94 Suppl 2:219-25)												
MR^{5}	Deep Molecular response specified as 5 log transcript level reduction on the IS (ref Cross et al. Ann Hematol. 2015;94 Suppl 2:219-25)												