SUPPLEMENTARY APPENDIX

BCR-ABL1 genomic DNA PCR response kinetics during first-line imatinib treatment of chronic myeloid leukemia

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Supplements

Methods

Patient characteristics and samples

All patients in TIDEL-II commenced first-line imatinib treatment at a dose of 600 mg daily, with selective dose escalation (800 mg daily) or switching to nilotinib (400 mg twice daily) for patients who failed to meet pre-defined time-dependent molecular response targets.

516 paired samples from total white cells were analysed by both DNA techniques (QPCR and dPCR) and mRNA RQ-PCR. 330 paired samples were analysed by DNA Q-PCR and 186 paired samples by DNA dPCR. Details of the patients' transcript types and methods of analysis are summarized in Supplemental Table 1. The slightly higher proportion of patients with e13a2 transcripts (54%) possibly reflects the more efficient amplification (in long range PCR) of *BCR* breakpoints involving the shorter intron following exon 13, so that these breakpoints were more easily identified. However, the overall clinical characteristics and treatment responses of the selected cohort were not significantly different from those of the entire TIDEL-II study population of 210 patients (Supplemental Table 2). Seven patients withdrew from the study after a median of 9.5 months of therapy (range 3.7-24) because of blast crisis (n=1), development of a kinase domain mutation with (n=2) or without progression (n=1), TKI intolerance (n=1), non-compliance (n=1), or pregnancy (n=1).

RQ-PCR

The achievement of a major molecular response (MMR; ≤0.1%) or $MR^{4.5}$ (≤0.0032%) required confirmation at 2 consecutive measurements. We defined the sensitivity of *BCR-ABL1* detection for each individual sample based on the Europe Against Cancer formula.[1] In

our laboratory a sample with UMRD requires a minimum of 400 000 *BCR* transcripts to demonstrate that $MR^{4.5}$ is achieved.[2]

Breakpoint detection and Feasibility and Reproducibility of patient-specific assays for *BCR-ABL1* DNA

In the current paper we used a multiplex long-range PCR screening approach for the breakpoint detection with a success rate of 78%. Additional breakpoints can be detected by simplex long-range PCR screening, or by using additional breakpoint detection methods, but it was not necessary for us to do so in this study.

It was possible to design specific and efficient Q-PCR primers and probes for every patient in who we were able to detect the breakpoint. This included patients in whom one or both breakpoints were in a genomic repeat element. Repetitive elements are not a major problem because 1) specificity can be achieved as long as one of the primers is not located in a repeat element; and 2) repetitive elements are conserved, but not always identical, so that it usually possible to find a stretch of unique sequence within the repeat. Two examples of assay design involving repetitive elements are shown in Supplemental Figure 1.

23 samples new batch extracted from 3 patients have been re-analysed by DNA Q-PCR and the results have been compared with the original data showing agreement and confirming the reproducibility of the data (Supplemental Figure 2).

Design and validation of patient-specific assays for *BCR-ABL1* DNA

Patient-specific genomic DNA assays were designed using the Primer Express® Software Version 3.0 with a TaqMan FAM-MGB or FAM-TAMRA probe (Life Technologies, Carlsbad, CA, United States) and with a forward primer located in *BCR* as near as possible to the *BCR-ABL1* junction, and a reverse primer located in *ABL1*. Assay performance was

assessed following minimum information for publication of quantitative real-time PCR experiments guidelines.[3] To ensure accurate disease quantification, we also followed the European Minimal Residual Disease Consortium recommendations for qPCR in Ph-negative acute lymphoblastic leukemia, in which DNA extracted from each patient's diagnostic sample was used for preparing standard curves (assuming the diagnostic sample is 100% leukaemic),[4] and we follow the recommendations of van der Velden and colleagues for the detection of MRD by real-time quantitative PCR.[5] The efficiency of every assay was calculated by interpolation of a standard curve obtained with patient-specific diagnostic DNA 10-fold serial diluted on non-human buffered DNA (Thermofisher, Waltham, Massachusetts, United States) starting from 50 ng. The efficiency of the reaction was calculated as $\eta=(10^{-1/slope}-1)*100$. A regression curve with a slope of -3.32 and $R^2=1$ give to an optimal efficiency of 100%. Acceptable values are between 90-110% with a slope in a range between -3.0 and -3.9 with $R^2>95\%$. Assays with an unacceptable efficiency were discarded and re-designed (Supplemental Figure 3). Assays were additionally tested for non-specificity using pooled normal human DNA. The *GUSB* gene was used as control. The GUSB gene has been chosen since is rarely involved in the Ph translocation and is present in 2 copies per cell (although any autosomal gene or intergenic sequence could be used for DNA quantification, as, so long as its sequence is specific, and not repeated throughout the genome and highly conserved).

Quantification of *BCR-ABL1* DNA by quantitative real-time PCR

Genomic DNA was extracted by phenol/chloroform method from the same peripheral blood leukocytes used for the molecular monitoring of the mRNA. The Qubit Fluorometer (Life Technologies, Carlsbad, CA, United States) was used to determine the concentration and both

NanoDrop 8000 Spectrophotometer (Life Technologies, Carlsbad, CA, United States) and agarose gel were used to check the gDNA quality and integrity. The amount of amplifiable DNA in each sample was measured using quantitative real-time PCR (Q-PCR) as previously described.[6] Briefly, on the same plate were ran standard curves for both *BCR-ABL1* and the *GUSB* control gene. Standard curves for *GUSB* control gene were prepared using 10-fold serial dilutions of *GUSB* plasmid, and standard curves for *BCR-ABL1* used serial dilutions of the patient's diagnostic DNA (assuming that the diagnostic sample was 100% leukaemic) in non-human buffered DNA (Thermofisher, Waltham, Massachusetts, United States) starting from 100 ng. Results were reported as *BCR-ABL1/GUSB%* by interpolation from a standard curve. The log of each known concentration based on the dilution series of GUSB (x-axis) was plotted against the Ct values of the *BCR-ABL1* from their relative standard curve (y-axis). The concentration of *BCR-ABL1* in each target ($BCR\text{-}ABL1_{target}$) was then derived by the formula $BCR\text{-}ABL1_{target}$=10^(intercept-CT $mean_{target}$)/slope. The number of leukemic cells (%) was then calculated by dividing the concentration of $GUSB_{target}$ by the concentration of $BCR\text{-}ABL1_{target}$. The sensitivity was determined by the amount of DNA according to the GUSB copy number.

Quantification of BCR-ABL1 DNA by digital PCR

The Q-PCR method was adapted to use in dPCR using the BioMark HD System with the dPCR 37k integrated fluidic circuits (Fluidigm Corporation, South San Francisco, California, United States). Each integrated fluidic circuit consists of 48 panels (sample inlets) each containing 770 reaction chambers of 0.85 nL reaction volume. Reaction mixes were prepared with 3 µL of 2x TaqMan® Gene Expression Master mix (Life Technologies), 0.6 µL of 20x GE Sample Loading Reagent (Fluidigm), 0.24 µL of patient-specific primers and probe at a final concentration of respectively 400 nM and 200 nM, 1.5 µL of gDNA and nuclease free

water in a final volume of 6 µL. Four microliters of the prepared reaction mixes were loaded into the corresponding inlets of each array chip, and the BioMark IFC controller MX (Fluidigm) was used to uniformly partition each reaction mix from the loading inlets through channels into the reaction chambers. The dPCR was then performed using the BioMark HD System, and the data were collected using the Data Collection software version 4 (Fluidigm). The Digital PCR Analysis software version 4.0.1 (Fluidigm) was used to set the Cq threshold (Ct) and range, and to count the number of positive chambers (*H*) out of the total number chambers (*C*) per panel from which the Poisson distribution was used to estimate the average number of template copies per chamber (λ), were: $\lambda = -\ln(1 - H/C)$.[7,8] Thus target copies per reaction volume were estimated as (Est target) = λ * *C*.[8] Based on the manufacturer's instructions only about 16% of the initial volume per well ends up in the reaction chambers, therefore the limit of detection was determined based on the amount (ng) of gDNA used for the *BCR-ABL1* quantification normalized on the number of copies of *GUSB* in dPCR. Since *GUSB* is diploid, the average estimated target number calculated by the Fluidigm software was divided by 2 then divided by the total amount of gDNA used for *GUSB* in dPCR to calculate the *GUSB* number of cells per ng (*GUSB* cells/ng). The limit of detection (LOD) was calculated by multiplying the total ng of gDNA used for *BCR-ABL1* quantification by the number of cells/ng. For samples at or near $MR^{4.5}$ the number of panels was increased to achieve a LOD of at least 5-logs (i.e. one in 100 000 cells). The sensitivity calculated for undetectable results by qRT-PCR ranged from 4.6 to 6.5 logs. A median of 7 panels (range, 1-18 panels) was used per follow-up sample, with a median of 3.7 µg of total gDNA (range, 5 ng-12 µg). To avoid saturation of the panels 5 ng of DNA were used for samples at diagnosis and for *GUSB*. The minimal residual disease was then determined by dividing the *BCR-ABL1* Estimated targets per *GUSB* cells/ng values, and multiplying by 100 to obtain the *BCR-ABL1/GUSB* ratio %.

Analysis of *BCR-ABL1* DNA at diagnosis uses only the dPCR values. Analysis of *BCR-ABL1* DNA during treatment uses only relative quantification. For Q-PCR this is based on the standard curve. For dPCR this is based on a mathematical correction that expresses the follow-up level relative to the patient's individual baseline value.

Supplemental References

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4. van der Velden VH, Cazzaniga G, Schrauder A, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia. 2007;21(4):604-11.

5. van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia. 2003;17(6):1013-34.

6. Ross DM, Branford S, Seymour JF, et al. Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR. Leukemia. 2010;24(10):1719-24.

7. Dube S, Qin J, Ramakrishnan R. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PloS one. 2008;3(8):e2876.

8. Huggett JF, Foy CA, Benes V, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clinical Chemistry. 2013;59(6):892-902.

Supplemental Figures

Supplemental Figure 1: Feasibility of the DNA Q-PCR assay. Example of assay design for 2 patients (NEP138 and PAH133) with repetitive elements at the breakpoint. The Q-PCR with Sybr Green showed no non-specific amplification and a unique melting curve.

NEP138

- Fusion Sequence (*BCR-ABL1*)

aacctattattcatggaccccaaacttgttcctcttatgtcctgtccctttgaggggcaccaccatccacccgcatggccaagccagaaaccgtggtctgct
ctccctccgttaaatgccattctccatcagtgaggcttcttagtcatctctggctgcctggccaggccctggctgtggcctcctccctggtctttgtagctctg
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ctttttgtgtgaaaacctcatatcttttaat

- Repetitive elements

```
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CTCTCCCTCCGTTAAATGCCATTCTCCATCAGTGAGGCTTCTTAGTCATC
TCTGGCTGCCTGGCCAGGCCCTGGCTGTGGCCTCCTCCCTGGTCTTTGTA
GCTCTGGATATCCCTGCAGAAAGGGTCCCCACTACCAGGCCTCTCCATCC
CCAGTCTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNAAAACCTCATATCTTTTAAT
```

Line1 164bp

NEP138

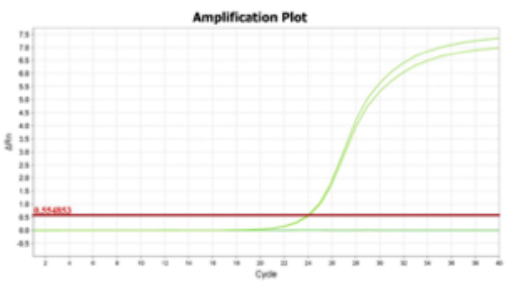
- Assay design: FAM-MGB probe

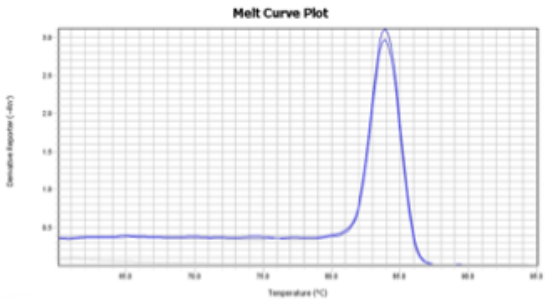
aacctattattcatggaccccaaacttgttcctcttatgtcctgtccctttgaggggcaccaccatccacccgcatggccaagccagaaaccgt
ggtctgctctcccctccgttaaatgccattctccatcagtgaggcttcttagtcatctctggctgcctggccaggccctggctgtggcctcctccc
tggtctttgtagctctggatatccctgcagaaagggtccccactaccaggcctctccatccccagtc* tca* tttcacgttcctaccagcaccc
ttgaacgccagaattcctggtatcctcggctgaactggctattgtcggtatttttaattttagccattctagtgagtatctcatcatggttttgctttc
cctgacagctaatgaggtggattgagcatctttttgtgtgaaaacctcatatctttttaat

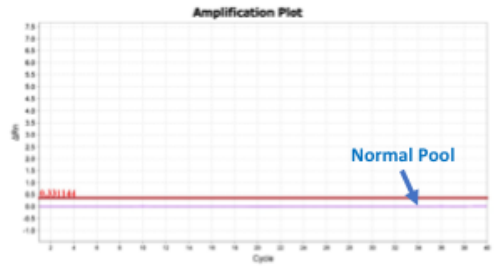
Amplicon length: 155 bp

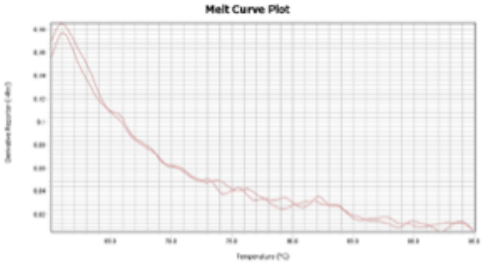
- Primers test

NEP138 **BCR/ABL1**









PAH133

- Fusion Sequence (*BCR*-*ABL1*)

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ccccactcccgtcctcccagccctc*ct*tcttttactcttaaaagcatttattccgccgggcgtggtggcacacgcctgtaatcccagcaat
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- Repetitive elements

```
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNCCCCCCCCAAAAAAAAGCATTTATTAGGAAA
CAGGAAAGACTGAACAAGAACAAGAGAACTAAGCATGTAACTCAAGAAGC
```

Alu 274bp

PAH133

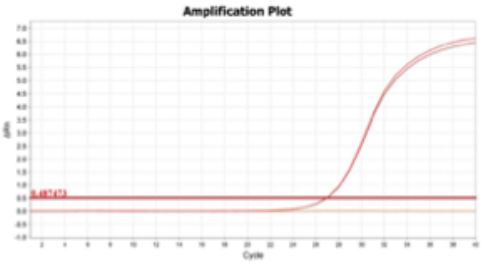
- Assay design: FAM-MGB probe

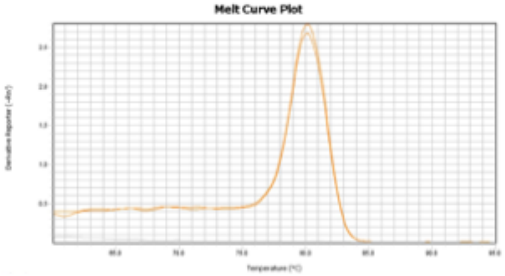
gctgctgggtggttgaggagatgcacggcttctgttcctagtcacaaggctgcagcagacgctcctcagatgctctgtgccttggatctggcc
ccactcccgtcctcccagccctc*ct*tcttttactcttaaaagcatttattccgccgggcgtggtggcacacgcctgtaatcccagcaatttgg
gaggctgaggtgggtggatcacaagttcaggcgttcaagaccagcctggccaagatggtgaaaccctgtctcttactaaaaatacaaaaatt
agctgggcttggtggcagctgcctatagctactcgggaggctgaggcaggataatcccttgaacccggggtacagaggttgcagtgagcc
gagatcgcgccattgcactccagcctgggtgacagagtgagacaccgcccccccccccaaaaaaaagcatttattaggaaacaggaaa
gactgaacaagaacaagagaactaagcatgtaactcaagaagccaga

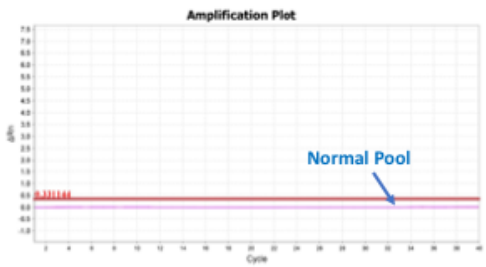
Amplicon length: 79 bp

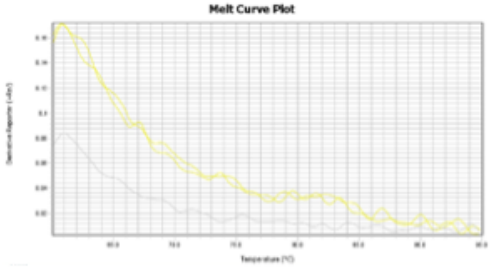
- Primers test

PAH133 **BCR/ABL1**

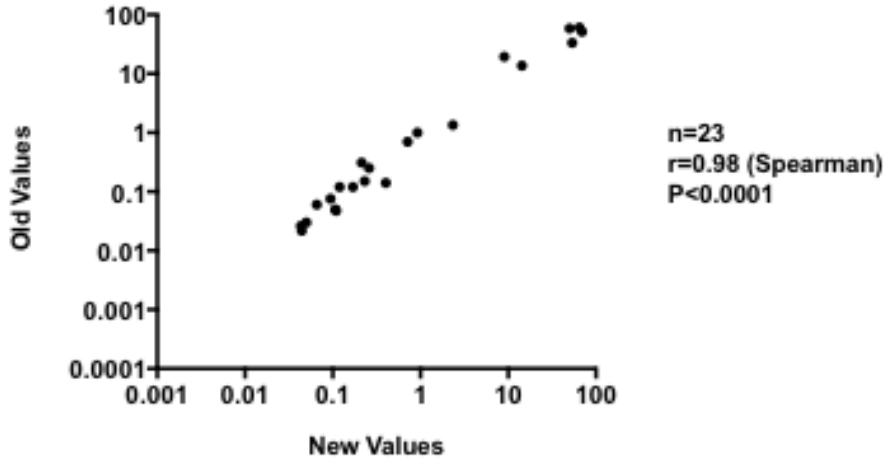


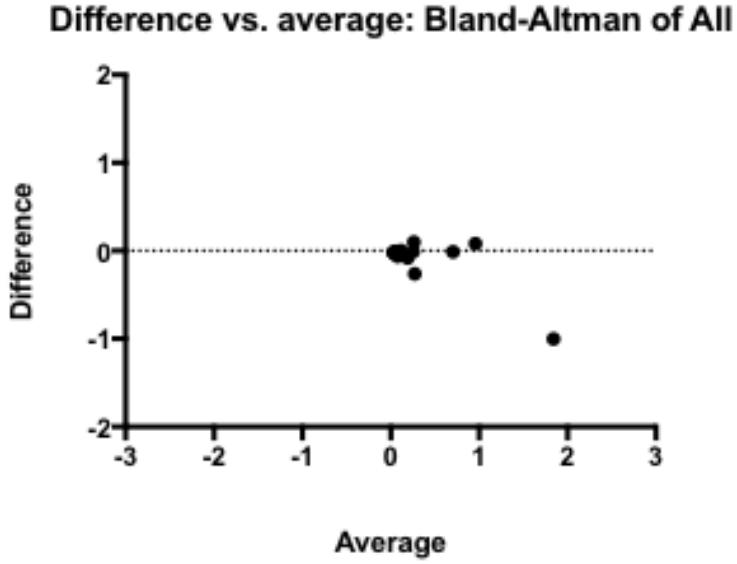




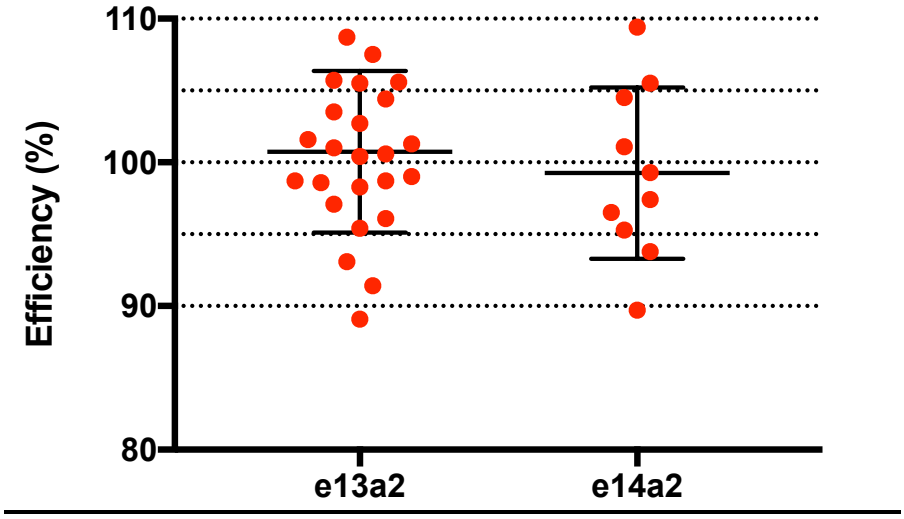


Supplemental Figure 2: reproducibility of the DNA Q-PCR method. A second DNA extraction and Q-PCR was performed for 23 samples and the results from the first and second batch were compared using Spearman Correlation and Bland-Altman.



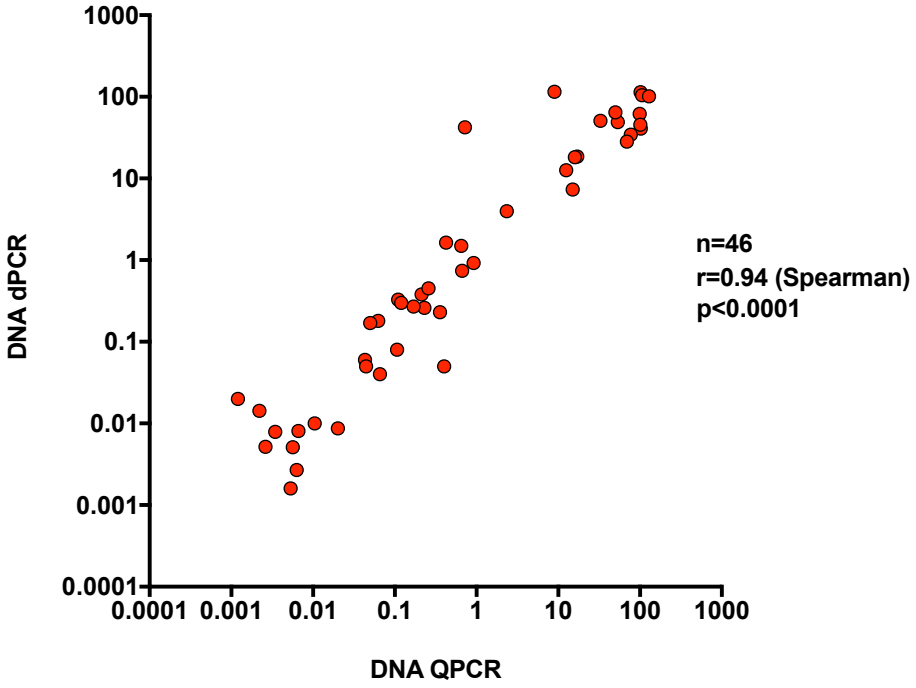


Supplemental Figure 3 - Efficiency of DNA Q-PCR assays for the e14a2 and e13a2 comparison.



Supplemental Figure 4 – Agreement between Q-PCR and dPCR for *BCR-ABL1* DNA. **(A)** Correlation between *BCR-ABL1* DNA measured by QPCR and dPCR. **(B)** Bland-Altman plot showing the agreement between the two DNA methods. Values from the time of diagnosis (pre-treatment) are excluded.

A-



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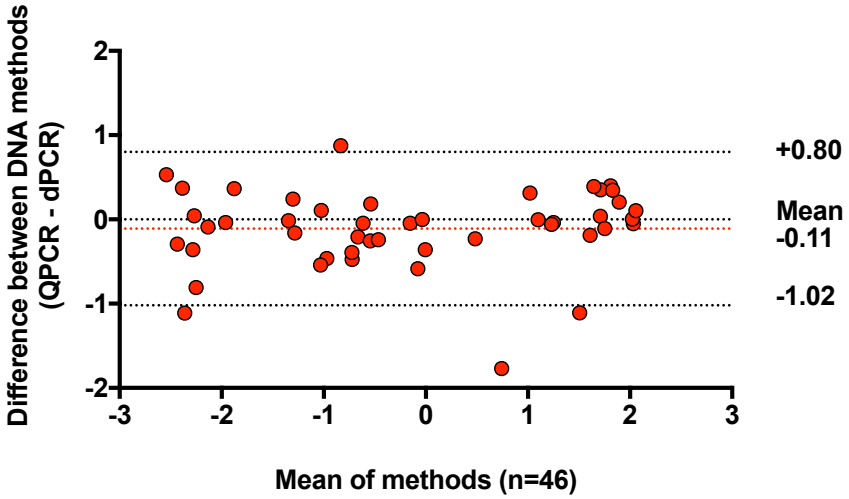
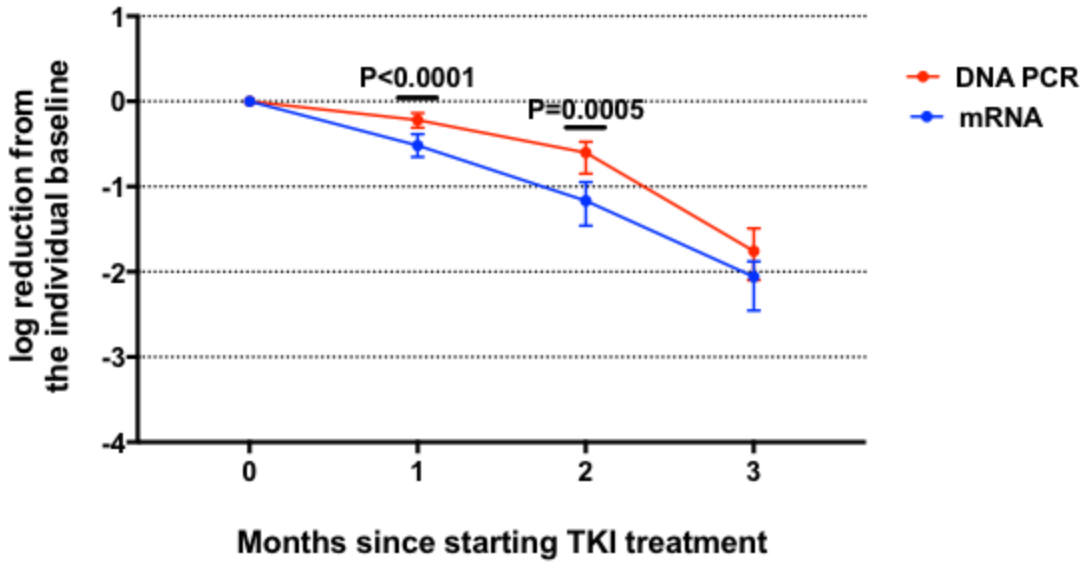
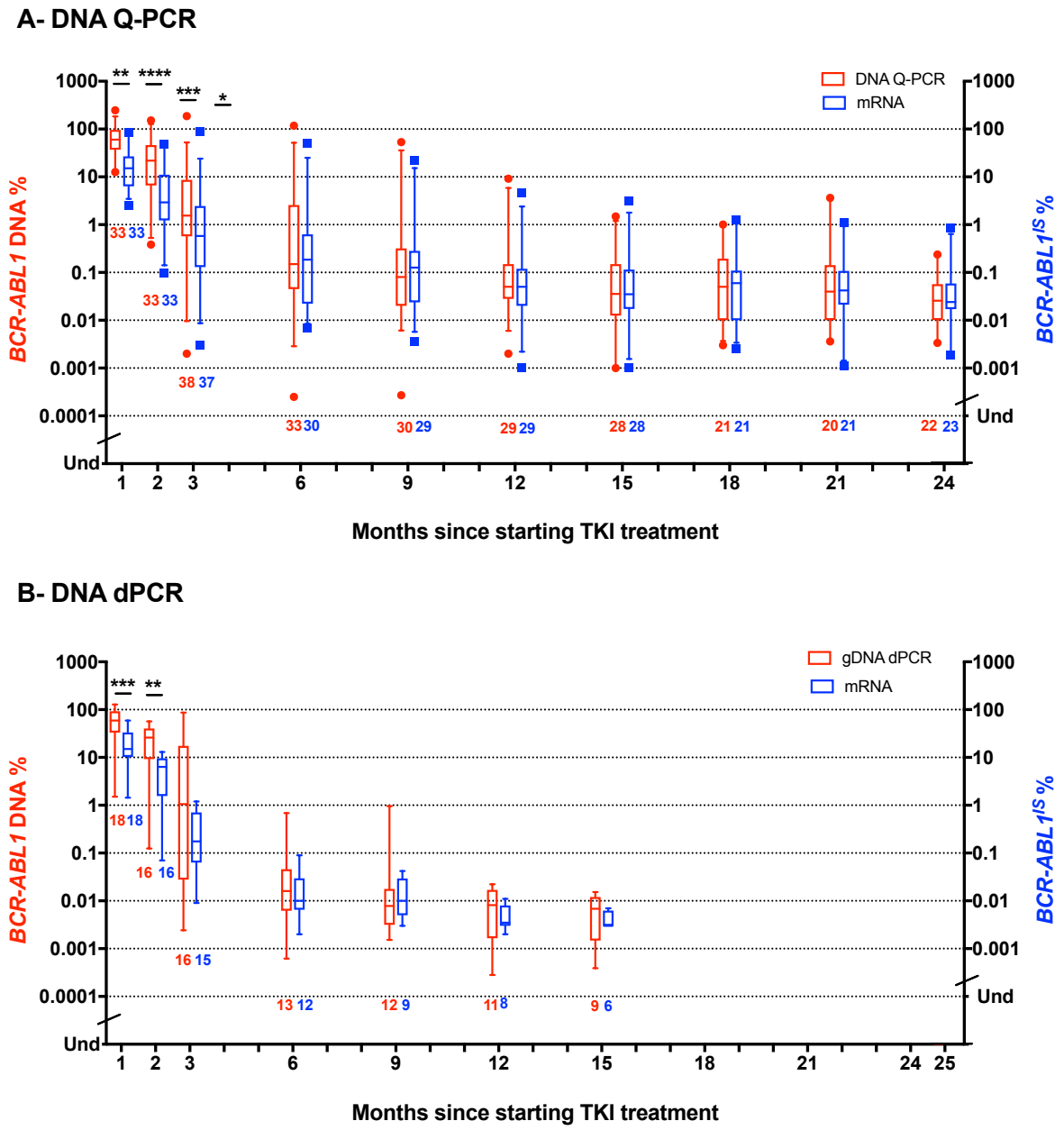


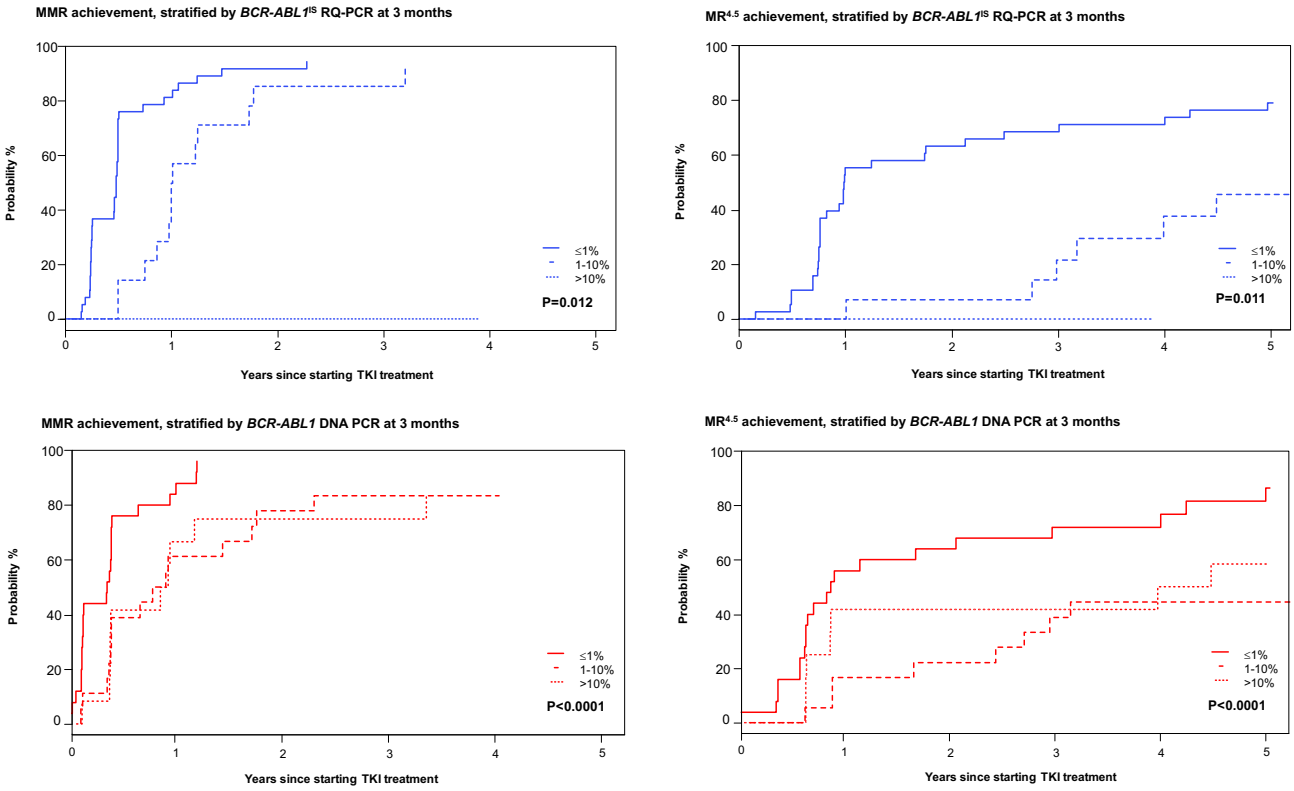
Supplemental Figure 5 - Log reduction from the individual baseline.



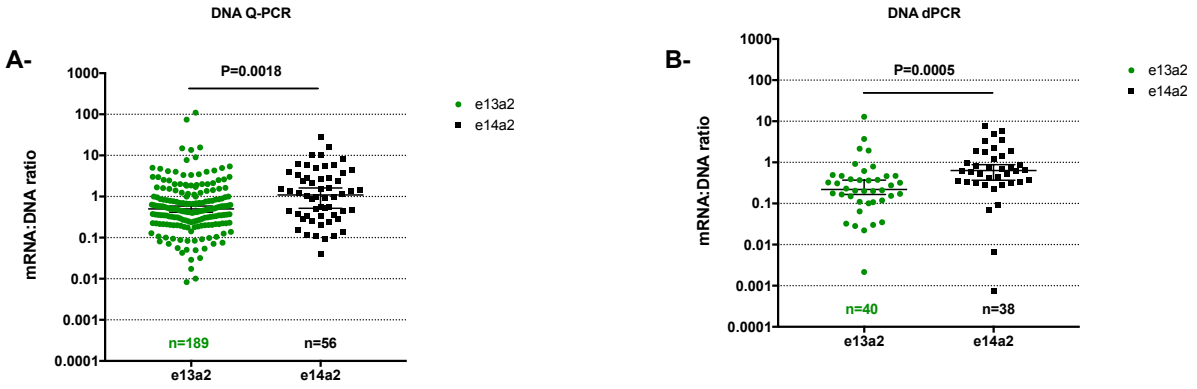
Supplemental Figure 6 - Comparison between mRNA and either (A) DNA Q-PCR or (B) DNA dPCR methods for the quantification of *BCR-ABL1*. The mRNA (blue) and DNA (red) values (number, median and interquartile range) are shown for individual time points up to 24 months. Undetectable values are excluded. *P<0.05; **P<0.01; ***P<0.0001; Und, undetectable.



Supplemental Figure 7 - *BCR-ABL1* mRNA and DNA levels at 3 months are predictive of later molecular responses. The cumulative incidence of MMR and $MR^{4.5}$ according to *BCR-ABL1* mRNA values **(A, B)** and DNA values **(C, D)** at the 3 month landmark is shown in a competing risk analysis.



Supplemental Figure 8 - *BCR-ABL1* transcript type and molecular response. Comparison of the *BCR-ABL1* expression ratio (**(A)** mRNA:DNA Q-PCR or **(B)** mRNA:DNA dPCR for e13a2 and e14a2 transcripts (horizontal lines represent median and 95% CI).



Supplemental tables

Supplemental table 1: Number of patients per different transcripts type analysed by either DNA QPCR or dPCR. 25 e13a2 and 10 e14a2 were monitored by DNA Q-PCR and 7 e13a2 and 7 e14a2 were monitored by dPCR. 4 patients (2 e13a2, 11 samples and 2 e14a2, 12 samples) originally monitored by DNA Q-PCR were additional monitored by DNA dPCR for a total of 40 e13a2 samples and 38 e14a2 samples, and are indicated as +2.

	In the cohort (N, 59)	DNA Q-PCR	DNA-dPCR
e13a2, N	32	25	7 (+2)
e14a2, N	17	10	7 (+2)
both, N	9	5	4
e13a3, N	1	0	1
Total Patients, N	59	40	19 (+4)

N= number of patients

Supplemental Table 2: Patient Characteristics.

	TIDEL-II population	Study Cohort
Number	210	59
Age years, median	50 (range, 17-81)	48 (range, 18-76)
Female	44% (n=92)	49% (n=29)
Sokal score, median	0.82 (range, 0.46-8.08)	0.78 (range, 0.56-8.08)
High	18% (n=38)	24% (n=14)
Intermediate	30% (n=62)	22% (n=13)
Low	45% (n=95)	51% (n=30)
Missing	7% (n=15)	3% (n=2)
Transcript type		
e13a2	40% (n=85)	54% (n=32)
e14a2	38% (n=80)	29% (n=17)
both	20% (n=42)	15% (n=9)
e13a3	1% (n=1)	2% (n=1)
e1a2	1% (n=2)	0

n= number of patients

Supplemental Table 3: Characteristics of three patients with unusually low *BCR-ABL1* expression at diagnosis.

Patient ID	Age at Diagnosis	Sokal Score	Sex	Transcript Type	Therapy Snapshot	Progression/ Event	WCC	Spleen Size	Platelets	Blasts	CCA/Ph+	iFISH (*BCR-ABL1*%)
#1	31	intermediate	Male	b2a2	600mg IM > Early Withdrawal	Blast Crisis - Mutation: H396P	15.4	14.00	366.00	1.00		99.4
#2	40	low	Female	b2a2, b3a2	600mg IM > 800mg IM > 800mg NIL > Early withdrawal	Mutations: G250E and Y253H	9.4	1.00	730.00	0.00		N/A
#3	42	high	Female	b2a2	600mg IM > 800mg IM > 800mg NIL		1.7	25.00	617.00	11.00	t(9;22;16)[20]	87.8

IM: imatinib; NIL: nilotinib; WCC: white cell count; CCA: conventional cytogenetic analysis; Ph+: Philadelphia positive; iFISH: interphase fluorescence in situ hybridisation; N/A: not available.