SUPPLEMENTARY APPENDIX

# Minimal residual disease analysis by eight-color flow cytometry in relapsed childhood acute lymphoblastic leukemia

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**Supplementary Information**

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## Supplemental Materials and Methods

*Patients: clinical characteristics, strategic groups and treatment*

Clinical characteristics of the study cohort are depicted in the Table S1. Stratification of the patients into standard- (S1), intermediate- (S2) and high-risk (S3, S4) treatment strategic groups was performed using immunophenotype, time to relapse and site of relapse parameters as shown in the Table S2.[1] For all strategic groups, induction treatment consisted of the two multi-agent chemotherapy blocks F1/F2, followed by the consolidation phase, which was randomised to the therapy arm A with alternating R courses (R2/R1/R2) and arm B with a more continuous treatment including idarubicin (protocol II-IDA).[2] For all high-risk patients, allogeneic HSCT directly followed consolidation treatment. For intermediate-risk patients the allocation to HSCT was dependent on the MRD level measured after induction treatment: patients with MRD$<10^{-3}$ received five alternating R1/R2 blocks followed by maintenance therapy and those with MRD$\geq 10^{-3}$ two R blocks and allogeneic HSCT.[3]

*Parallel MRD sample analyses by PCR and FCM*

A total of 263 follow-up BM samples from 122 patients was measured by FCM in parallel to PCR-MRD at multiple time points during treatment. The majority of patients (75.4%) were allocated to the intermediate risk group. Given that induction and consolidation therapy blocks are similar for intermediate and high risk groups within the ALL-REZ BFM 2002 protocol, the BCP-ALL samples from the high-risk groups (24.6%) were also included into comparison of FCM and PCR methodologies. The time points of sample acquisition were: after induction (35%), during consolidation (35%), before HSCT (18%) and during maintenance therapy (12%).

Measurements were performed centrally in the PCR-MRD reference laboratory (Charité, Berlin) and in the FCM-MRD research laboratory (Charité, Berlin). Since PCR-MRD analysis

had therapeutic implications in the ALL-REZ BFM 2002 protocol, priority was given to this technique if the cell number was insufficient for both methods. The monitoring of MRD was performed from April 2009 to December 2012.

*Sample collection and preparation*

BM aspirates were collected at relapse diagnosis and at defined time points during relapse treatment as described above. Initial sample prepartion was performed in the central PCR-MRD reference laboratory. BM samples were enriched with mononuclear cells using Ficoll–Isopaque (Pharmacia Amersham, Uppsala, Sweden) gradient centrifugation, washed twice, resuspended in PBS, and separated into two fractions. The fraction for molecular analysis was directly processed to DNA using the Nucleo-Spin Tissue kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany). The fraction for FCM analysis was supplemented with 10% FCS, stored at $4^0$C and measured within one (80% of cases) or two to three days (20% of cases).

*MRD assessment by PCR*

PCR-MRD measurements were performed as described previously.[2,4,5] Briefly, clonal antigen-receptor gene rearrangements were identified in leukemic cell DNA at relapse diagnosis by PCR, heteroduplex analysis and Sanger sequencing. Specific and sensitive real-time quantitative PCR assays were established and MRD quantification was performed using at least two MRD PCR targets with a quantitative range of at least $\geq 10^{-3}$ and a sensitivity of $5x/1x10^{-4}$. PCR analysis with only one sensitive marker was allowed in cases with positive MRD results $\geq 10^{-3}$. Analysis of quantitative MRD results was performed according to the guidelines of the EuroMRD group.[6]

*MRD assessment by FCM*

FCM was performed using a BD CANTO II flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using BD FACSDiva (Becton Dickinson) and Kaluza (Beckman-Coulter, Miami, FL, USA) software. It should be noted that the Kaluza software uses fluorescence intensity scala in the range $10^{-1}$ to $10^3$, while the FACSDiva tool applies the more common $10^0$ to $10^4$ scala range. For comparability, we multiplied the mean fluorescence intensity (MFI) values from the Kaluza software by ten. The compensation matrix was set up using BD CompBeads (Becton Dickinson) for fluorochrome-conjugated antibodies and using normal blood cells for the Syto 41 dye. Quality control was performed using BD Cytometer Setup & Tracking Beads (Becton Dickinson). The antibody tubes tested are shown in Table

S3. The list of antibodies, antibody clones, fluorochromes, and manufacturers is provided in Table S4.

1. Henze G, v Stackelberg A, Eckert C. ALL-REZ BFM - the consecutive trials for children with relapsed acute lymphoblastic leukemia. Klin Padiatr. 2013;225 Suppl 1:S73-78.
2. Eckert C, von Stackelberg A, Seeger K, et al. Minimal residual disease after induction is the strongest predictor of prognosis in intermediate risk relapsed acute lymphoblastic leukaemia - long-term results of trial ALL-REZ BFM P95/96. Eur J Cancer. 2013;49(6):1346-1355.
3. Eckert C, Henze G, Seeger K, et al. Use of allogeneic hematopoietic stem-cell transplantation based on minimal residual disease response improves outcomes for children with relapsed acute lymphoblastic leukemia in the intermediate-risk group. J Clin Oncol. 2013;31(21):2736-2742.
4. Eckert C, Biondi A, Seeger K, et al. Prognostic value of minimal residual disease in relapsed childhood acute lymphoblastic leukaemia. Lancet. 2001;358(9289):1239-1241.
5. Eckert C, Landt O, Taube T, et al. Potential of LightCycler technology for quantification of minimal residual disease in childhood acute lymphoblastic leukemia. Leukemia. 2000;14(2):316-323.
6. 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):604-611.

Table S1. Clinical characteristics of the presented study cohort

| **Variable** | | | |
|---|---|---|---|
| | | **Total cohort** | |
| | | n | % |
| | Total | 122 | 100 |
| **Gender** | Male | 74 | 60,7 |
| | Female | 48 | 39,3 |
| **Age at relapse** | <5 years | 20 | 16,4 |
| | ≥5 - <10 years | 41 | 33,6 |
| | ≥10 years | 61 | 50,0 |
| **Time to relapse*** | Very early | 14 | 11,5 |
| | Early | 31 | 25,4 |
| | Late | 77 | 63,1 |
| **Site** | Bone marrow isolated | 84 | 68,9 |
| | Bone marrow combined | 35 | 28,7 |
| | Extramedullary isolated | 3 | 2,5 |
| **Immunophenotype** | Pro-B ALL | 2 | 1,6 |
| | Common ALL | 89 | 73,0 |
| | Pre-B AL | 11 | 9,0 |
| | Biphenotypic | 2 | 1,6 |
| | No data | 18 | 14,8 |
| **Strategic group**** | S2 | 92 | 75,4 |
| | S3 | 16 | 13,1 |
| | S4 | 14 | 11,5 |
| **Randomisation** | Arm A | 88 | 72,1 |
| | Arm B | 29 | 23,8 |
| | None | 5 | 4,1 |
| **HSCT** | Yes | 56 | 45,9 |
| | No | 51 | 41,8 |
| | No data | 15 | 12,3 |
| **Outcome/events** | Continuous complete remission | 85 | 69,7 |
| | Cytological non-response | 6 | 4,9 |
| | Therapy related death | 8 | 6,6 |
| | 2$^{nd}$ relapse | 18 | 14,8 |
| | Secondary malignancy | 2 | 1,6 |
| | Loss to follow-up | 3 | 2,5 |

*** Time to relapse definitions:**
Very early (<18 months after diagnosis)
Early (≥18 months after diagnosis and ≤6 months after regular completion of initial treatment)
Late (>6 months after regular completion of initial treatment)

**** Strategic group definition:** s. Table S2

Table S2. Definition of strategic groups for treatment stratification

| **Immunophenotype** | BCP-ALL | | | T-cell ALL | | |
|---|---|---|---|---|---|---|
| **Site of relapse** | isolated extramedullary | combined bone marrow/ extramedullary | isolated bone marrow | isolated extramedullary | combined bone marrow/ extramedullary | isolated bone marrow |
| **Time to relapse** | | | | | | |
| very early | S2 | S4 | S4 | S2 | S4 | S4 |
| early | S2 | S2 | S3 | S2 | S4 | S4 |
| late | S1 | S2 | S2 | S1 | S4 | S4 |

Table S3. Combinations of fluorochrome-conjugated antibodies and nucleic acid dye Syto 41 tested in the study

| **Laser** | stain | "CD58"-tube | "CD38"-tube | experimental | experimental | experimental |
|---|---|---|---|---|---|---|
| **blue** | FITC | CD58 | CD38 | CD72 | CD58 | cyBcl2 |
| | PE | CD10 | CD10 | CD10 | CD10 | CD10 |
| | PerCP-Cy5.5 | CD19 | CD19 | CD19 | CD19 | CD19 |
| | PC7 | CD34 | CD34 | CD34 | CD24 | CD34 |
| **red** | APC | CD22 | CD22 | CD86 | CD22 | cyTdT |
| | APC-Cy7 | CD20 | CD20 | CD20 | CD44 | CD20 |
| **violet** | Syto 41 | Syto 41 | Syto 41 | Syto 41 | Syto 41 | Syto 41 |
| | HV500 | CD45 | CD45 | CD45 | CD45 | CD45 |

Table S4. Fluorochromes, clones and manufacturers of the antibodies tested in the study

| Fluorochrome | Antibody | Clone | Manufacturer |
|---|---|---|---|
| FITC | CD58 | 1C3 | BD Biosciences |
| | CD38 | HIT2 | BD Biosciences |
| | CD72 | J4-117 | BD Biosciences |
| | Bcl2 | 124 | Dako |
| PE | CD10 | HI10a | BD Biosciences |
| PerCP-Cy5.5 | CD19 | SJ25C1 | BD Biosciences |
| PC7 | CD34 | 8G12 | BD Biosciences |
| | CD24 | ML5 | BD Biosciences |
| APC | CD22 | S-HCL-1 | BD Biosciences |
| | CD86 | 2331 (FUN-1) | BD Biosciences |
| | TdT | E17-1519 | BD Biosciences |
| APC-Cy7 | CD20 | L27 | BD Biosciences |
| APC-H7 | CD44 | G44-26 | BD Biosciences |
| Syto 41 | Syto 41 | | Life Technologies (Invitrogen) |
| HV500 | CD45 | HI30 | BD Biosciences |

## Results and Discussion

*Regeneration burst in the MRD samples from patients with relapsed BCP-ALL*

Density plot presentation demonstrates similar positions of the CD19+CD20-/+CD10+/++ B cell progenitors in the MRD (A) and control (D) samples. However, the MRD sample reveals a considerably higher percentage of the early CD19+CD20-CD10++CD34+ cells as compared to the "classical" regeneration pattern (encircled dots, 48% in Fig1A vs 8% in Fig. 1D)

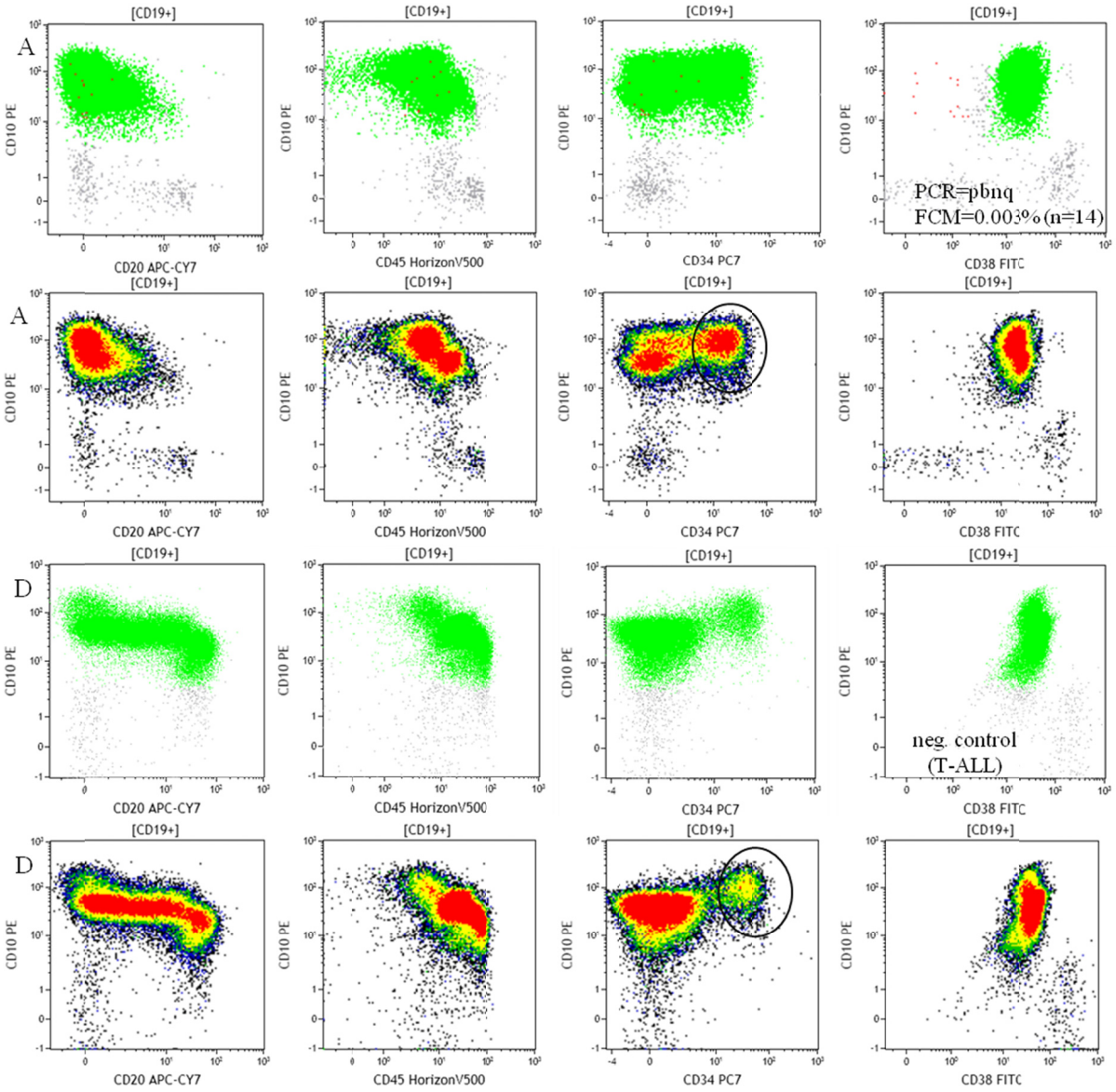


Figure S1. Dot plot and density plot presentations of the samples with a high regeneration background (depicted also as dot plots in the Fig. 1A and Fig. 1D).

Table S5. Antigen expression levels in normal (hematogones) and leukemic (BCP-ALL) cells

| Marker | mean / cases | B cell precursor | | absolute fold difference* | p-value** |
|---|---|---|---|---|---|
| | | normal | leukemic | | |
| CD24 | MFI | 444 | 457 | 1.03 | n.s.*** |
| | n | 17 | 12 | | |
| CD38 | MFI | 375 | 63 | 5.95 | .000 |
| | n | 50 | 50 | | |
| CD22 | MFI | 68.5 | 117 | 1.71 | .000 |
| | n | 50 | 50 | | |
| CD58 | MFI | 60.0 | 92.1 | 1.54 | .000 |
| | n | 50 | 50 | | |
| CD72 | MFI | 33.5 | 19.0 | 1.76 | .03 |
| | n | 18 | 10 | | |
| CD44 | MFI | 26.1 | 25.1 | 1.04 | n.s. |
| | n | 17 | 12 | | |
| bcl-2 | MFI | 24.7 | 63.3 | 2.56 | .001 |
| | n | 33 | 22 | | |
| TdT | MFI | 23.0 | 21.7 | 1.06 | n.s. |
| | n | 33 | 22 | | |
| CD86 | MFI | 2.5 | 7.2 | 2.88 | n.s. |
| | n | 18 | 10 | | |

* ratio MFI normal / MFI leukemic, if MFI normal > MFI leukemic;
ratio MFI leukemic / MFI normal, if MFI leukemic > MFI normal

** unpaired t-test

*** not significant

*FCM analysis of the CD19-CD34+CD22+ fraction in regenerating BM samples*

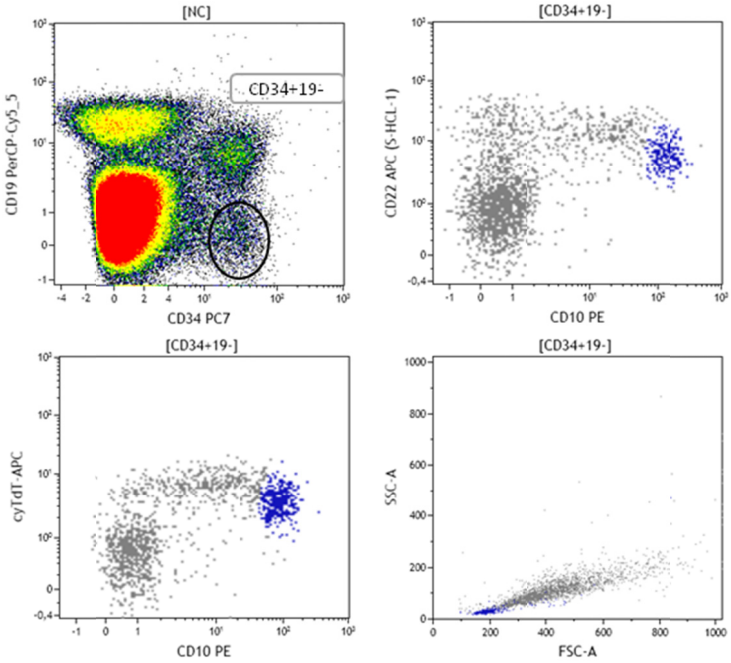


Figure S2. Presence of CD22+CD10+ cells (blue points) in the CD19-CD34+ fraction in the samples with regeneration background. These cells express TdT in the cytoplasm (cyTdT) and reveal low forward and side scatter (FSC and SSC).

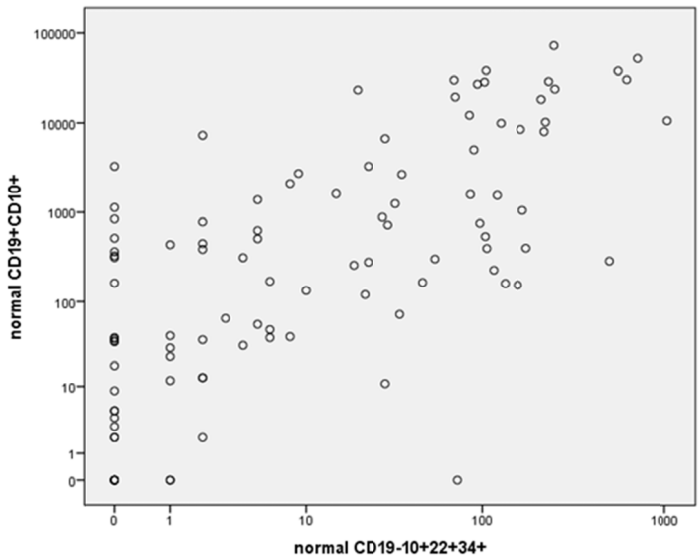


Figure S3. Quantitative relation between cell counts of CD19-CD10+CD22+CD34+ cells and CD19+CD10+ B cell progenitors in MRD samples. Spearman correlation coefficient rs=0.68, $p<0.001$, n=101.

*Detection limit analyis of FCM-MRD*

Table S6. FCM-MRD performance above and below the FCM detection limit of 10 cells (CD38-tube series, n=104)

| FCM+ cell count range | FCM+ samples | PCR+FCM+ | PCR-FCM+ | %PCR+FCM+ |
|---|---|---|---|---|
| pbnq (1-9)* | 14 | 7 | 7 | 50 |
| 10-19 | 4 | 4 | 0 | 100 |
| 20-29 | 5 | 5 | 0 | 100 |
| 30-39 | 3 | 2 | 1 | 67 |
| 40-49 | 3 | 3 | 0 | 100 |

* in order to investigate count levels below 10 cells, we did not set the lowest limit for positive cell counts. Absolute FCM+ cell counts in the PCR+ cohort were 1, 3, 3, 6, 6, 7, 8. Absolute FCM+ cell counts in the PCR- cohort were 1, 3, 4, 4, 6, 9, 9.

*Supplemental discussion point: investigating samples near to detection limit*

The increase of discordant cases in the samples approaching detection limit can be illustrated by the example in which, similarly to our study, a testing sample is divided in two parts to be measured by two different methods. If the initial sample contains a low number, e.g. only two cells, there is a 50% combinatorial probability that one part of the sample will contain two cells and another one will be negative (2/0 and 0/2, truly discordant) and a 50% probability that both parts will contain one cell (1/1and 1/1, truly concordant). Therefore, investigating samples near to detection limit would objectively provide discordant cases, being characterized in that the frequencies of -/+ and +/- discordant combinations are similar.

Table S7. PCR performance in intrapatient MRD series with highly discordant and concordant FCM- and PCR-MRD levels

| Patient | % of leukemic blasts at relapse diagnosis | Number of PCR markers identified | Number of PCR markers used for MRD | Number of gene loci with >1 gene rearr. | Time point | **Quantitative results** (Ct-value $10^{-1}$ diution step) | | | | | MRD result | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | PCR-marker 1 | PCR-marker 2 | PCR-marker 3 | PCR-marker 4 | PCR-marker 5 | PCR (%) | FCM (%) |
| **#13** | 98 | 8 | 4 | 1 | d0 | (Ct=29.8) | (Ct=25.0) | (Ct=24.8) | (Ct=33.1) | | | |
| | | | | | 1 | 6,20E-03 | <5E-04 p.b.n.q. | <1E-04 negative | ≥1E-3 p.b.n.q. | | 0,6 | 0,06 |
| | | | | | 2 | 1,90E-02 | 8,40E-04 | 1,81E-03 | ≥1E-2 p.b.n.q. | | 1,9 | 0,17 |
| | | | | | 3 | 2,23E-02 | 7,40E-04 | 2,58E-03 | ≥1E-2 p.b.n.q. | | 2,2 | 0,25 |
| **#14** | 41 | 5 | 3 | 1 | d0 | (Ct=27.5) | (Ct=27.6) | (Ct=27.6) | | | | |
| | | | | | 1 | 6,29E-02 | 5,30E-02 | 2,20E-02 | | | 6,3 | 0,7 |
| | | | | | 2 | 3,09E-01 | 3,60E-01 | 1,90E-01 | | | 31 | 7 |
| | | | | | 3 | 1,70E-01 | 1,44E-01 | 6,00E-02 | | | 17 | 0,8 |
| **#15** | 38 | 5 | 5 | 1 | d0 | (Ct=26.5) | (Ct=32.3) | (Ct=27.5) | (Ct=25.3) | (Ct=25.2) | | |
| | | | | | 1 | ≥1E-02 p.b.n.q. | 8,81E-02 | 6,01E-01 | 3,32E-01 | 1,79E-01 | 60 | 7,2 |
| | | | | | 2 | 1,47E-01 | n.a. | 3,39E-01 | 2,15E-01 | 1,30E-01 | 34 | 2,5 |
| | | | | | 3 | n.a. | n.a. | 5,97E-03 | 1,44E-02 | 9,30E-03 | 1,4 | 0,2 |
| **#1** | 70 | 5 | 3 | 1 | d0 | (Ct=26.9) | (Ct=26.1) | (Ct=25.6) | | | | |
| | | | | | 1 | 1,06E-03 | 7,80E-04 | 6,30E-04 | | | 0,11 | 0,11 |
| | | | | | 2 | 4,60E-04 | 3,40E-04 | 1,80E-04 | | | 0,05 | 0,02 |
| | | | | | 3 | 8,00E-05 | 3,00E-05 | <1E-04 p.b.n.q. | | | 0,008 | 0,009 |
| **#2** | 94 | 2 | 1 | 0 | d0 | (Ct=26.8) | | | | | | |
| | | | | | 1 | 9,66E-03 | | | | | 1 | 3,9 |
| | | | | | 2 | 1,53E-02 | | | | | 1,5 | 1,9 |
| | | | | | 3 | 2,28E-02 | | | | | 2,3 | 1,2 |
| | | | | | 4 | 3,15E-02 | | | | | 3,1 | 2,9 |
| **#3** | 96 | 5 | 3 | 1 | d0 | (Ct=26.1) | (Ct=25.4) | (Ct=25.4) | (Ct=24.6) | | | |
| | | | | | 1 | 4,88E-03 | 2,11E-03 | 2,28E-03 | n.a. | | 0,49 | 0,53 |
| | | | | | 2 | 6,29E-03 | 1,38E-03 | <1E-04 negative | <1E-04 negative | | 0,63 | 0,32 |
| | | | | | 3 | 1,19E-03 | 2,40E-04 | 1,30E-04 | n.a. | | 0,12 | 0,11 |
| **#4** | 67 | 1 | 1 | 0 | d0 | (Ct=26.3) | | | | | | |
| | | | | | 1 | 1,98E-02 | | | | | 2 | 2 |
| | | | | | 2 | 3,54E-03 | | | | | 0,35 | 0,39 |
| | | | | | 3 | 1,85E-02 | | | | | 1,8 | 3 |
| | | | | | 4 | 5,69E-02 | | | | | 5,7 | 3,7 |

Interpretation

pt. 13: presence of different subclones (subclone 1: markers 1 and 4; subclone 2: markers 2 and 3), with different response kinetics. Application of the smaller subclone 1 (with the considerably higher Ct value) as the major one may result in over-interpretation of quantitative results

pt. 14: no subclones at relapse diagnosis

pt. 15: one smaller subclone

pt. 1: no sublcones, no different response kinetics

pt. 2: no subclones at relapse diagnosis

pt. 3: no obvious smaller subclone at diagnosis (similar Ct values), but different response kinetics during treatment

pt. 4: no subclones at relapse diagnosis