

# Detection of internal tandem duplications in the *FLT3* gene by different electrophoretic methods

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## Abstract

**Background:** In acute myeloid leukemia (AML), the internal tandem duplication (ITD) in the juxtamembrane domain of the *FLT3* (Fms-like tyrosine kinase 3) gene is one of the most frequent genetic alterations associated with poor prognosis.

**Methods:** A complex evaluation of the analytical properties of the three most frequently used detection methods – PCR followed by agarose (AGE), polyacrylamide (PAGE) or capillary electrophoresis (CE) – was performed on 95 DNA samples obtained from 73 AML patients.

**Results:** All the three methods verified the presence of a mutant allele in 20 samples from 18 patients. AGE and PAGE could detect the presence of 1%–2% mutant allele, while the detection limit of CE was 0.28%. However, acceptable reproducibility (inter-assay CV <25%) of the mutant allele rate determination was only achievable above 1.5% mutant/total allele rate. The reproducibility of the ITD size determination by CE was much better, but the ITD size calculated by PeakScanner or GeneScan analysis was 7% lower as compared to values obtained by DNA sequencing. The presence of multiple ITD was over-estimated by PAGE and AGE due to the formation of heteroduplexes.

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**Conclusions:** This study suggests the use of PCR+CE in the diagnostics and the follow-up of AML patients. The data further supports the importance of proper analytical evaluation of home-made molecular biological diagnostic tests.

**Keywords:** acute myeloid leukemia; agarose gel electrophoresis; capillary electrophoresis; fms-like tyrosine kinase 3 (*FLT3*); internal tandem duplication (ITD); polyacrylamide gel electrophoresis.

## Introduction

The Fms-like tyrosine kinase 3 (*FLT3*) plays an important role in the activation of hematopoietic stem cells. Binding to its ligand results in the induction of different signaling pathways known to regulate cell proliferation, differentiation and apoptosis of these cells. As a result, alteration in functionality of *FLT3* can be important in the development of hematological malignancies (1–4). Three different types of activating mutations have been described in the *FLT3* gene: a point mutation at nucleotide position 869 of the tyrosine kinase domain (TKD), point mutations in the exons coding the juxtamembrane domain (JM) of the protein and the internal tandem duplication (ITD) in the JM domain (1–4). These mutations are one of the most frequent genetic alterations in acute myeloid leukemia (AML) and show poor prognosis, especially in patients without cytogenetic alterations, those classified as the intermediate prognostic group (3–6).

From the three types of the *FLT3* mutations, the *FLT3*-ITD shows the highest frequency and clinically seems to be the most important. Here, a 3–400-base-long DNA fragment is duplicated (rarely triplicated) in the region of exon 14, intron 14 and exon 15 and sometimes short nucleotide additions are also combined with the duplication. In spite of the large nucleotide insertions the reading always remains in frame, resulting in an *FLT3* protein with a longer JM domain showing a reduced inhibitory effect on the TKD domain (1, 3–7).

The detection of the *FLT3*-ITDs is based on a PCR using primers designed for the 5' end of exon 14 and the 3' end of exon 15. The presence of a longer PCR product compared to that amplified from the wild-type allele shows the presence of the ITD (8). Different electrophoretic methods are available for the analysis of these PCR products. Single stranded conformational polymorphism (9), conformational sensitive gel electrophoresis (10), temperature gradient capillary electrophoresis (11), high-resolution melting analysis (11) and denaturing HPLC (dHPLC) (12, 13) are sensitive methods for the detection of small genetic alterations, like ITD, but are rather

time-consuming and labor-intensive. A simple agarose gel electrophoresis (AGE) is quite economical and easy to handle (7, 8, 14–16). Polyacrylamide gel electrophoresis (PAGE) has higher resolution and sensitivity but is more demanding and, perhaps, is less suitable for routine diagnostic purposes (17–19). Data are somewhat contradictory, but several lines of evidence suggest that the size of the ITD and the rate of mutant allele might have important prognostic information in AML (2, 13, 15, 16, 20–29). Semi-quantitative AGE or PAGE (14, 20), dHPLC (12, 13), the Agilent Bioanalyser (30, 31), capillary electrophoresis (CE; e.g., GeneScan analysis) (13, 17, 19, 22–29, 32, 33) or real-time quantitative PCR (34, 35) can provide information about these parameters.

The application of home-made molecular genetic tests in the field of hemato-oncology is rather wide-spread in routine diagnostic laboratories. The proper evaluation of the analytical properties of these methods prior to their routine application – especially in the case of the quantitative tests – is essential. As such, we compared the analytical properties of PCR combined with AGE, PAGE and CE – the three most frequently used analytical methods – in the detection of FLT3-ITD in samples of AML patients.

## Materials and methods

### AML patients

Altogether 73 adult patients (age: 51.7±18.5 years, 36 males, 37 females) with AML were enrolled in this study. Patients were followed-up by the 2nd Department of Internal Medicine of the Medical and Health Science Center, University of Debrecen, Debrecen and by the Department of Hematology of the András Józsa County Hospital, Nyíregyháza. Detailed clinical data was available in the case of 51 patients (Supplementary Table 1). The work was assigned by the Local Ethical Committee of the University of Debrecen and informed consent was obtained from each donor. We have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

### Amplification of exon 14, intron 14 and exon 15 of the *FLT3* gene

Genomic DNA was isolated from peripheral blood of AML patients using the QIAmp DNA Blood Minikit (Qiagen, Valencia, CA, USA). The region of exon 14, intron 14 and exon 15 of the *FLT3* gene was amplified by a PCR using forward (5'-ATT TAG GTA TGA AAG CCA GC-3') and reverse (5'-CTT TCA GCA TTT TGA CGG CAA CC-3') primers (Applied Biosystems, Foster City, CA, USA) (8). For CE analysis the forward primer was labeled at the 5'-end by 6-carboxy-fluorescein (FAM). Each 25 µL reaction included 200–500 ng of genomic DNA, 5 pmol of each primer, 400 µM dNTP, 2 mM MgCl<sub>2</sub> and 1 U AmpliTaq Gold polymerase (Applied Biosystems). When samples were prepared for the CE analysis, 0.5 pmol of each primers (FAM-labeled forward and non-labeled reverse) and 10 mM MgCl<sub>2</sub> was used in the PCR amplification. The PCR was performed using the GeneAmp 2700 PCR system (Applied Biosystems) under the following conditions: denaturation at 95°C for 12 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 10 min terminated the amplification.

### Agarose gel electrophoresis

The PCR products (10 µL) were loaded with 0.5 µL loading dye (bromophenolblue: 4.2 mg/mL; xylen cyanide: 4.2 mg/mL; glycerol: 50 v/v%; Sigma, St. Louis, MO, USA) into slots of a 10×15 cm 3% agarose (Cambrex, East Rutherford, NJ, USA) gel in TBE buffer using the wide-cell horizontal electrophoresis system (Biorad, Hercules, CA, USA). The electrophoresis was performed using a power of 10–11 V/cm for 1–2 h. The TBE buffer was changed one to two times during runs in order to prevent excessive warming. The bands were visualized by 0.4 µg/mL ethidium bromide (Sigma) that was mixed into the gel and running buffer.

### Polyacrylamide gel electrophoresis

The PCR products (10 µL) were loaded with 0.5 µL loading dye into slots of a 15×20 cm 6% polyacrylamide/bisacrylamide (29:1) gel (Biorad) in TAE buffer using the Amersham SE-600 system (Amersham Pharmacia, Piscataway, NJ, USA). The electrophoresis was performed using a power of 10–11 V/cm for 1–2 h. The gel was stained after the run by 1 µg/mL ethidium bromide for 20 min at room temperature.

### Isolation of PCR products from the polyacrylamide gel

The FLT3-ITD PCR products amplified from the wild and mutant alleles were isolated from the polyacrylamide gel by the “crush and soak” procedure. In short, a disposable surgical knife was used to cut out the bands from the gel that were crushed against the wall of a microfuge tube (1.5 mL) with a disposable pipette tip. To the gel slice we added one to two times of the gel slice volume of elution buffer (NH<sub>4</sub>-acetate 0.5 M; MgSO<sub>4</sub> 15 mM; EDTA 1 mM, 0.1% SDS; Sigma). The tube was incubated for 12–16 h at 37°C on a rotary platform and was centrifuged (13,800 g, 1 min, +4°C). The supernatant was transferred to a new tube while the polyacrylamide pellets were washed again with 0.5 mL elution buffer. After a repeated centrifugation the second supernatant was combined with the first eluate. The PCR product extracted from the gel was further purified using absolute ethanol and 3 M Na-acetate (Sigma).

### Capillary electrophoresis

The PCR products containing the 6-FAM-labeled forward primer were diluted 8-times in molecular biology grade water (Promega, Madison, WI, USA). The diluted sample (1 µL) was mixed with 10 µL formamide (Hi-Di™ formamide, Applied Biosystems) and 0.5 µL GeneScan 500-ROX internal size standard (Applied Biosystems) and denaturated for 3 min at 96°C. The capillary electrophoresis of the samples was performed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The technical parameters were the following: capillary length and diameter: 47 cm and 50 µm; capillary temperature: 60°C; polymer: POP6; run power and time: 15.0 kV and 60 min; injection power and time: 2 kV and 5–15 s. The data collection was performed using the ABI Prism™ Collection software (Applied Biosystems), while the runs were analyzed by the PeakScanner 1.0 and in certain cases by the GeneScan® 3.1 software (Applied Biosystems). For size determination the local Southern method was applied, unless the size of a certain peak exceeded the upper range of the size standard, when linear regression was used. In samples of de novo cases we accepted signals with relative fluorescence unit (RFU) >50 as peaks and in follow-up samples with known peak-localizations even lower RFU values (above 15) were evaluated. The area under the wild-type and mutant peaks was also determined and the

rate of mutant allele was calculated in two different ways: (i) mutant/total allele rate =  $AUC_{\text{mutant peak}} / (AUC_{\text{mutant peaks}} + AUC_{\text{wild-type peak}}) \times 100$ ; and (ii) mutant/wild-type allele rate =  $AUC_{\text{mutant peak}} / AUC_{\text{wild-type peak}}$ .

### Bidirectional sequencing of the ITDs

The PCR products were cleaned from the unbound dNTP, primers and other assay components by a spin column centrifugation (Amicon® Ultra – 0.5 mL, 30K; Millipore, Carrigtowhill, Ireland). A cyclic termination end sequencing reaction was performed using the BigDye Terminator kit (v. 3.1, Applied Biosystems) according to the manufacturers' instruction. The unbound labeled dNTP was removed by centrifugation using the DyeEx® 2.0 Spin kit (Qiagen). The purified product (1 µL) was mixed with 10 µL formamide (Hi-Di™ formamide) and denaturated for 3 min at 96°C. The capillary electrophoresis of the samples was performed using ABI PRISM 310 Genetic Analyzer. The technical parameters were the following: capillary length and diameter: 47 cm and 50 µm; capillary temperature: 50°C; polymer: POP6; run power and time: 15.0 kV and 36 min; injection power and time: 2 kV and 5–15 s. The data collection and analysis was performed using the ABI Prism™ Collection and Sequencing Analysis 3.4.1 softwares.

### Identification of FLT3-TKD mutations

The determination of the presence of the FLT3-TKD mutations – as part of the general diagnostic procedure for AML patients – was performed by a separate PCR+RFLP technique based on the article of Yamamoto et al. (36). The frequency of FLT3-TKD mutation was 2/75 AML patients; combined FLT3-ITD and -TKD mutations were not present. In order to make the study population more homogenous we have enrolled in the analysis only the 73 individuals without FLT3-TKD mutations.

### Statistical analysis

The descriptive statistics were calculated with the help of Microsoft Excel software. The differences in the laboratory parameters between the ITD-positive and -negative AML patients were evaluated by the Mann-Whitney U-test, the gender distribution by the  $\chi^2$ -test, while the survival data between these groups was compared by the Kaplan-Meier analysis. The correlation between the different ways of ITD size determination was evaluated by Pearson's regression analysis. Significant difference was considered in the case of  $p < 0.05$ . Statistical analysis was performed with the help of the "Statistica" software.

## Results

### Comparison of the performance of AGE, PAGE and CE on FLT3-ITD detection in samples of patients with AML

Altogether 95 DNA samples of 73 adult patients with AML were tested for the presence of FLT3-ITD using the three different electrophoretic methods. Two independent samples were tested in the case of 18 and three samples in the case of two patients. We could detect the presence of at least one mutant allele in 20 samples of 18 patients, irrespective of the detection method used. Detailed clinical data was available only in

the case of 51 patients and the ITD-positivity rate was 12/51 in this cohort. Similarly to previously published data (1–6) the FLT3-ITD positive patients had higher white blood cell count and LDH values and the overall and disease-free survival period was shorter in this group (Supplementary Table 1).

Based on the CE method the size of the ITD detected in the positive samples varied between 9 and 178 bp (median 40 bp), while the rate of the mutant to wild type or the mutant to total alleles was 0.006–3.714 (median: 0.144) and 0.48%–77.8% (median: 12.5%), respectively. In nine of the 18 ITD-positive patients more than one type of the mutant allele could be detected (multiple ITDs). The number of the cases with two and three mutant peaks on CE was seven and two, respectively.

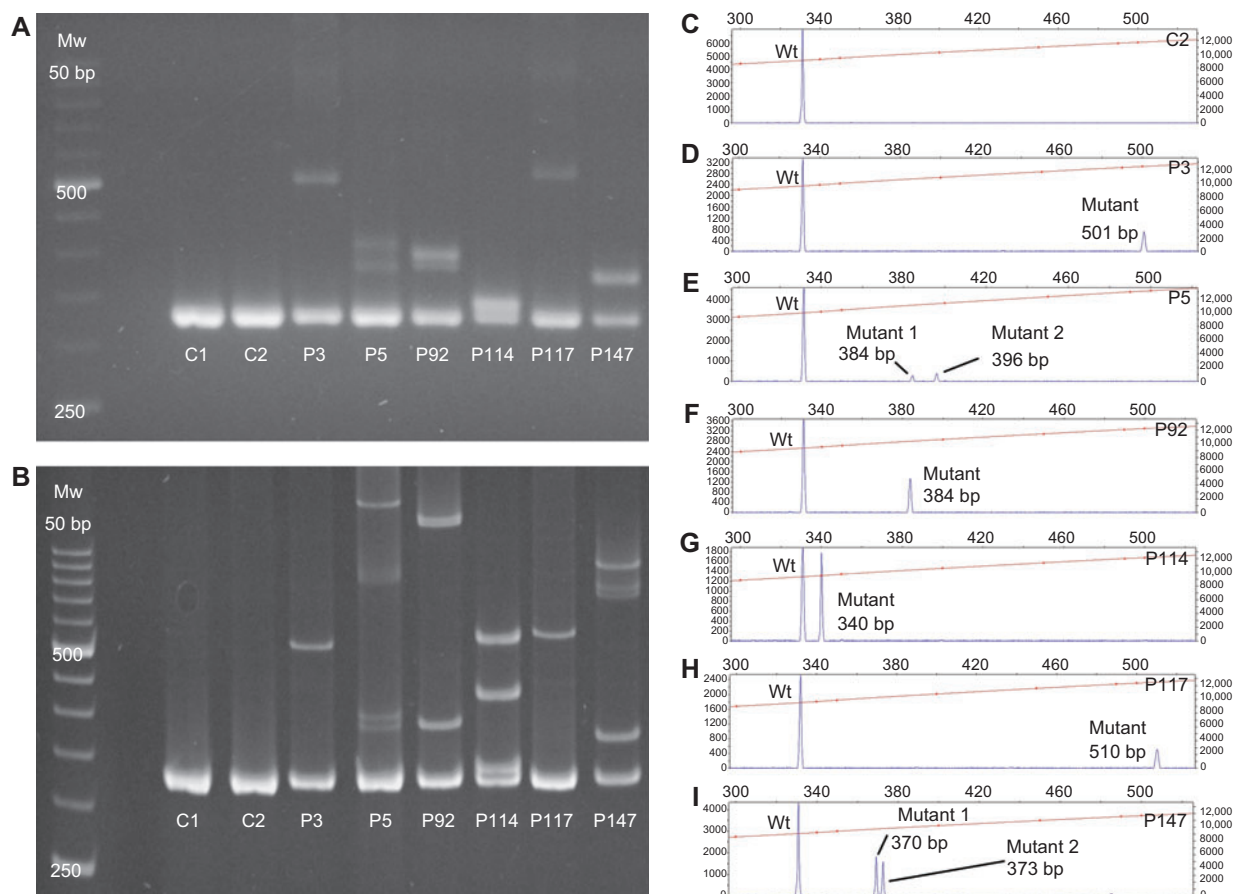
Although all three detection methods could identify the FLT3-ITD positive samples, there was a discrepancy concerning the number, size and intensity of the mutant alleles present (Figure 1). Consequently, the analytical properties of the three electrophoretic methods used for FLT3-ITD detection were evaluated.

### Evaluation of the analytical properties of AGE, PAGE and CE on FLT3-ITD detection: reproducibility

In order to evaluate the intra- and inter-assay reproducibility of the tested electrophoretic methods two FLT3-ITD positive samples (P35 and P80) were amplified 10-times in the same PCR assay on the same day and once daily on the following 9 days. With CE, sample P35 contained one major mutant peak and a hardly visible minor peak at 387 bp and 339 bp, respectively. In sample P80, we detected a major mutant peak at 355 bp and a very faint peak at 398 bp (Supplementary Figure 1).

Table 1 contains the reproducibility data of the size quantitation by CE. Here, the intra-assay variability was calculated in two ways. First, the two positive samples were amplified 10-times in the same PCR on the same day and then each PCR product was injected independently into the capillary of the ABI310 analyzer (Table 1, "10 cups"). Second, one of the 10 PCR products from both samples was injected 10-times subsequently into the analyzer (Table 1, "1 cup"). Regarding the size of the individual wild-type and mutant peaks, we observed very good intra- and inter-assay reproducibility (the CV% was typically below 0.2%). The size of the ITD was calculated as the size of the mutant allele minus the size of the wild-type allele. The intra- and inter-assay CV% of ITD was below 1% and 2%, respectively, if the ITD size was larger (above 24 bp; sample P35–ITD2; sample P80–ITD1 and –ITD2). In the case of ITD1 of sample P35 a smaller, 9 bp insertion could be observed with a relatively higher intra- and inter-assay CV% (7% and 11%).

Concerning the quantitation of the mutant/wild-type and mutant/total allele rate we obtained variable results (Table 2). In the case of sample P35–ITD2 and sample P80–ITD1 the mutant/total allele rate was 65% and 12%, and the intra- and inter-assay CV% were below 5%. The reproducibility was worse when the mutant/total allele rate was ~1.2%–1.5% (sample P80–ITD2), showing an intra-assay CV% of 10%–14% and an inter-assay CV% of 25%. When the mutant/



**Figure 1** Analysis of FLT3-ITD-positive and -negative DNA samples by three different electrophoretic methods.

Genomic DNA was amplified as described in Materials and methods. The PCR products (10  $\mu$ L) were loaded on 3% agarose (A) and 6% polyacrylamide gel (B). For capillary electrophoretic analysis a different PCR protocol was used with a fluorescently labeled forward primer. The samples were measured by an ABI310 analyzer, the size of the PCR products were determined by the PeakScanner software based on a ROX-500 size-standard (C, D, E, F, G, H, I). The size of the PCR product amplified from the FLT3 wide-type allele is 331 bp, and any fragments larger than this indicate the presence of ITD. (C, controls; P, patients.)

total rate was below 1% (sample P35–ITD1) the reproducibility was poor: CV% were above 50%.

Although quantitative data was not gathered from the agarose and polyacrylamide gel electrophoretograms in our study, we performed the reproducibility analysis in the case of these methods as well. Evaluating with agarose and polyacrylamide gel electrophoresis, the major mutant bands were clearly visible with similar size and intensity for each day and each run while the minor mutant bands were absent (data not shown).

#### Evaluation of the analytical properties of AGE, PAGE and CE on FLT3-ITD detection: analytical sensitivity

As a next step the detection limit of the three different electrophoretic methods was compared. The DNA samples of ITD-positive patients (P35 or P80) were serially diluted in an ITD-negative DNA. The DNA concentration in the FLT3-positive and negative samples was equal. The rate of the mutant to total alleles in the original samples was determined by CE and PeakScanner analysis and this rate in the diluted

DNA samples was calculated based on the initial value and the dilution factor (Figure 2A and B). These DNA mixes were amplified using unlabeled primers in the FLT3-PCR and 10  $\mu$ L of the PCR products were loaded into an agarose or a polyacrylamide gel (Figure 2A and B). The presence of the dominant mutant allele (mutant 2 in the case of P35) could be detected typically at 1%–2% both on agarose and polyacrylamide gel (Figure 2A and B). The minor mutant alleles were not detectable by these methods.

The serially diluted ITD-positive DNA was also tested using a fluorescently labeled forward and unlabeled reverse primer during PCR, followed by denaturation of the PCR products and analysis by CE. The measured rate of the mutant to wild-type (Figure 2D) or mutant to total (Figure 2C) alleles was determined by PeakScanner analysis. These rates were expressed as a function of the calculated mutant rates in the diluted samples. As two different samples in different dilutions were tested, the data of three independent experiments was pooled (Figure 2C and D). In order to find the lowest point of the linear part of the dilution curves a running cut-

**Table 1** Analytical performance of FLT3-ITD size quantitation done by capillary electrophoresis.

	Wild-type allele <sup>a,b</sup>	Mutant allele 1 <sup>a,b</sup>	Mutant allele 2 <sup>a,b</sup>	ITD 1 <sup>b</sup>	ITD 2 <sup>b</sup>
<b>Sample P35<sup>a</sup></b>					
Intra-assay variation (1 cup) <sup>c</sup>	330.9±0.2 (0.1%) <sup>d</sup>	339.2±0.6 (0.2%)	387.2±0.1 (0.1%)	8.3±0.6 (7.2%)	56.1±0.3 (0.5%)
Intra-assay variation (10 cups) <sup>c</sup>	331.2±0.1 (0.1%)	338.9±0.4 (0.1%)	386.9±0.1 (0.1%)	7.7±0.5 (6.5%)	55.7±0.1 (0.2%)
Inter-assay variation <sup>c</sup>	330.6±0.5 (0.2%)	339.7±0.6 (0.2%)	387.1±0.1 (0.1%)	9.1±1.0 (11.0%)	56.5±0.6 (1.1%)
<b>Sample P80<sup>a</sup></b>					
Intra-assay variation (1 cup) <sup>c</sup>	330.8±0.2 (0.1%) <sup>d</sup>	355.2±0.1 (0.1%)	398.4±0.1 (0.1%)	24.4±0.2 (0.8%)	67.6±0.3 (0.4%)
Intra-assay variation (10 cups) <sup>c</sup>	331.0±0.2 (0.1%)	355.2±0.1 (0.1%)	398.1±0.2 (0.1%)	24.1±0.2 (0.8%)	67.1±0.4 (0.6%)
Inter-assay variation <sup>d</sup>	330.3±0.5 (0.2%)	355.2±0.1 (0.1%)	398.5±0.3 (0.1%)	24.8±0.4 (1.6%)	68.1±0.7 (1.0%)

<sup>a</sup>Two FLT3-ITD positive samples (P35 and P80) containing two mutant alleles were analyzed. <sup>b</sup>The size of the wild-type and mutant alleles was determined by CE using the ABI310 analyzer and the PeakScanner software. The size of the ITDs was calculated as the size of the mutant alleles minus the size of the wild-type allele. <sup>c</sup>The intra-assay variability was calculated in two ways. First, the two positive samples were amplified 10-times in the same PCR on the same day and then each PCR product was injected independently into the capillary of the ABI310 analyzer ("10 cups"). Second, one of the 10 PCR products from both samples was injected 10-times subsequently into the analyzer ("1 cup"). <sup>d</sup>Data presented show the mean±SD values calculated from the 10 determinations. CV% (presented in brackets) was calculated as "SD/mean×100". <sup>e</sup>The two FLT3-ITD positive samples were amplified and analyzed once on 10 subsequent days.

off from 0.1% to 0.5% for the calculated mutant/total allele rate (Figure 2C) and 0.001–0.005 for the calculated mutant/wild-type allele rate (Figure 2D) was used and a linear regression line was inserted upon the data points above the actual cut-off. The best regression coefficient was observed in the case of 0.2% and 0.002 cut-offs for the calculated mutant/total and mutant/wild-type allele rates (R=0.989 and R=0.990, respectively). The analytical sensitivity of the assay was calculated using the 10 data points with the lowest calculated mutant allele rates. The mean+3 SD of these values were 0.28% and 0.0028, defining the detection limit of the measured mutant/total and mutant/wild-type allele rates (Figure 2C and D).

#### Evaluation of the analytical properties of AGE, PAGE and CE on FLT3-ITD detection: resolution

A 9 bp difference between the PCR products amplified from the wild-type and mutant alleles in sample P114 could be

detected by all of the applied electrophoretic methods (Figure 1A, B and G). On the contrary, in the case of sample P147, a 370 and a 373 bp mutant allele could be detected by CE, but this 3 bp difference could not be resolved by agarose and polyacrylamide gel electrophoresis (Figure 1A, B and I).

#### Analysis of multiple bands detected in FLT3-ITD positive samples by PAGE and AGE

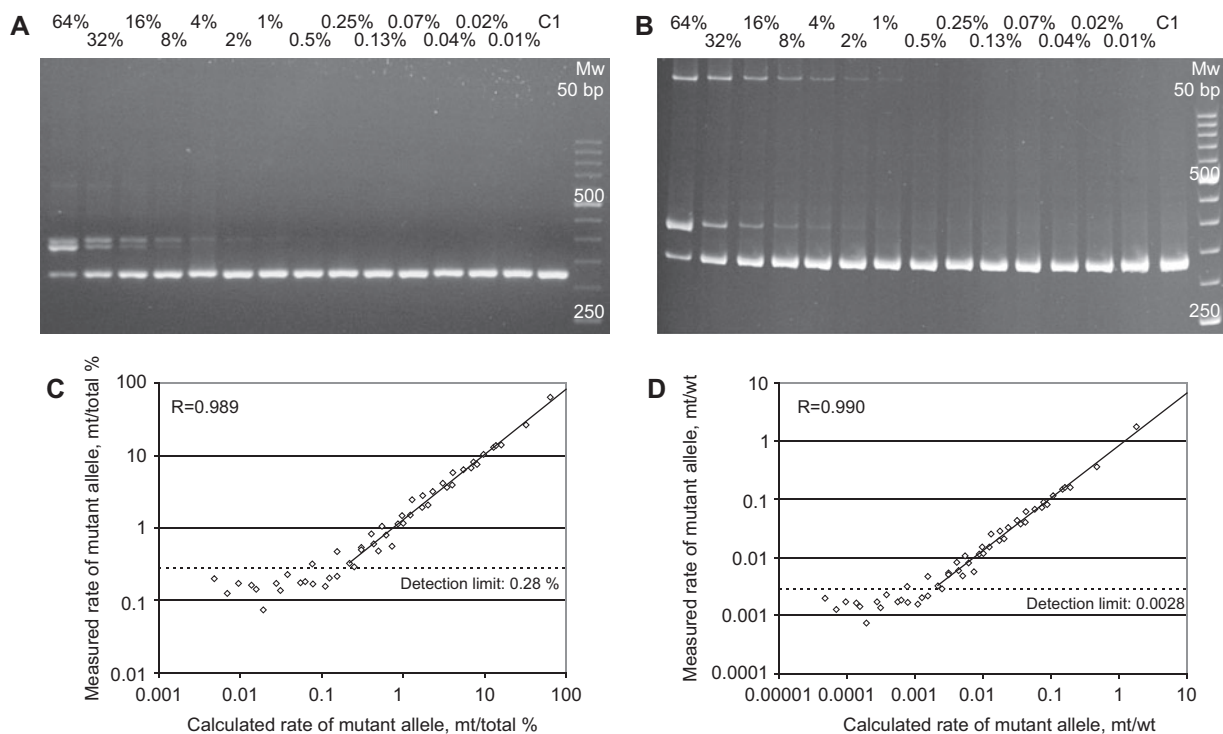
In the 20 FLT3-ITD positive DNA samples, we could detect two, three and four mutant bands with higher molecular weight in 12, three and two cases and two, three and zero cases using PAGE and AGE, respectively. The majority of these larger "extra" bands were not present on the CE runs (Figure 1).

In order to reveal their nature – in the case of Patient N°55 – the wild-type (band 1=331 bp), mutant 1 (band 2=approx. 350 bp) and mutant 2 (band 3=approx. 550 bp) bands were

**Table 2** Analytical performance of FLT3-ITD rate quantitation done by capillary electrophoresis.

	Mutant 1/total, % <sup>a,b</sup>	Mutant 2/total, % <sup>a,b</sup>	Mutant 1/wild type <sup>a,b</sup>	Mutant 2/wild type <sup>a,b</sup>
<b>Sample P35<sup>a</sup></b>				
Intra-assay variation (1 cup) <sup>c</sup>	0.39±0.20 (51.3%) <sup>d</sup>	63.9±0.5 (0.8%)	0.011±0.006 (54.5%)	1.790±0.035 (2.0%)
Intra-assay variation (10 cups) <sup>c</sup>	0.43±0.34 (79.1%)	65.3±1.6 (2.5%)	0.012±0.009 (75.0%)	1.911±0.114 (6.0%)
Inter-assay variation <sup>c</sup>	0.63±0.36 (57.1%)	65.7±2.4 (3.7%)	0.020±0.012 (60.0%)	2.052±0.203 (9.9%)
<b>Sample P80<sup>a</sup></b>				
Intra-assay variation (1 cup) <sup>c</sup>	11.9±0.1 (0.8%) <sup>d</sup>	1.5±0.1 (6.7%)	0.138±0.001 (0.7%)	0.018±0.002 (11.1%)
Intra-assay variation (10 cups) <sup>c</sup>	12.6±0.2 (1.6%)	1.2±0.1 (8.3%)	0.146±0.003 (2.1%)	0.014±0.002 (14.3%)
Inter-assay variation <sup>c</sup>	12.5±0.5 (4.0%)	1.3±0.3 (23.1%)	0.144±0.007 (4.9%)	0.016±0.004 (25.0%)

<sup>a</sup>Two FLT3-ITD positive samples (P35 and P80) containing two mutant alleles were analyzed. <sup>b</sup>The area under the peaks (AUC) of the wild-type and mutant alleles was determined by CE using the ABI310 analyzer and the PeakScanner software. The rate of the mutant alleles was calculated as  $AUC_{\text{mutant allele}}/AUC_{\text{mutant+wild-type alleles}}$  or as  $AUC_{\text{mutant allele}}/AUC_{\text{wild-type allele}}$ . <sup>c</sup>The intra-assay variability was calculated in two ways. First, the two positive samples were amplified 10-times in the same PCR on the same day and then each PCR product was injected independently into the capillary of the ABI310 analyzer ("10 cups"). Second, one of the 10 PCR products from both samples was injected 10-times subsequently into the analyzer ("1 cup"). <sup>d</sup>Data presented show the mean±SD values calculated from the 10 determinations. CV% (presented in brackets) was calculated as "SD/mean×100". <sup>e</sup>The two FLT3-ITD positive samples were amplified and analyzed once on 10 subsequent days.



**Figure 2** Sensitivity of agarose-, polyacrylamide- and capillary electrophoresis used for identification of FLT3-ITD.

A serial dilution of an ITD-positive DNA sample (P35) was prepared using an ITD-negative DNA with the same concentration. These mixes were amplified as described in Materials and methods and 10  $\mu$ L of the PCR product was loaded into an agarose (3%, A) and a polyacrylamide (6%, B) gel. The numbers above the lanes show the mutant/total allele rates calculated in the DNA mixes. The size of the PCR products amplified from the FLT3 wild-type and mutant allele are 331 and 387 bp, respectively. For capillary electrophoretic analysis a different PCR was applied with a fluorescently labeled forward primer using the DNA dilution series described above. The area under the wild-type and mutant peaks was determined by the PeakScanner software and the measured AUC rates (C: mutant/total, D: mutant/wild type) were expressed against the calculated rates. The regression lines on part C and D show the best fit on the points above 0.2% mutant/total and 0.002 mutant/wild-type rate, while the detection cut-offs were defined as the mean+3 SD of the lowest 10 points, respectively. Parts A and B show one representative experiment out of three, while parts C and D present the pooled data of three independent determinations.

cut from the polyacrylamide gel (Figure 3A, lane 'O'). Their DNA content was extracted and was further tested. First the DNA sequence was determined by bidirectional sequencing. Comparison of the wild-type (band 1) and mutant 1 (band 2) sequences revealed a 21-bp-long duplication in intron14/exon15 (c.1773\_1793dup21) in mutant 1 (Figure 3C and D). In mutant 2 (band 3) the mixture of the two sequences could be observed even after repeated excision and amplification of this band (Figure 3E).

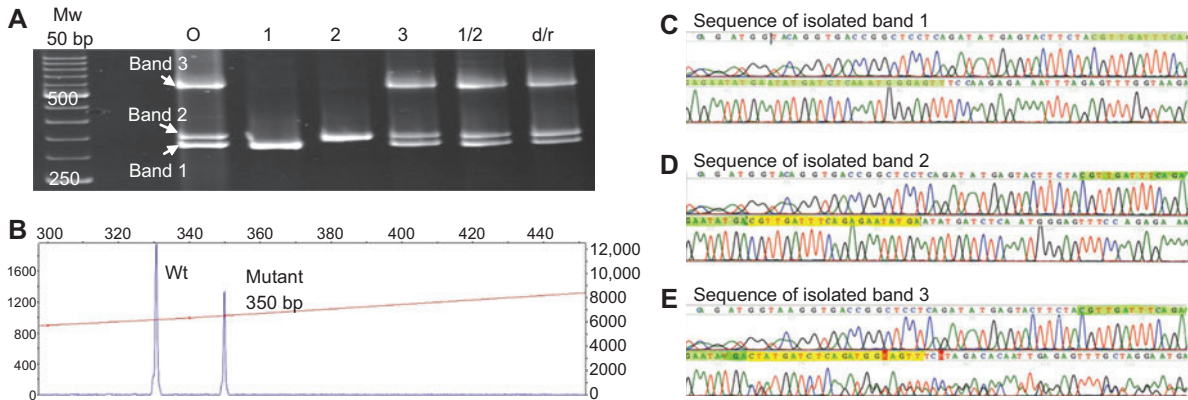
This can suggest that the mutant band 2 is a heteroduplex of the wild-type and the mutant 1 bands. In order to prove this hypothesis two experiments were performed. First, the DNA of the wild-type and the mutant 1 bands isolated from the gel was mixed and amplified together in the FLT3-ITD PCR (Figure 3A, lane "1/2"). Second, the two isolated DNA were amplified independently by the FLT3-PCR, and then mixed, denaturated at 95°C for 10 min and cooled down slowly (1°C/min) to 4°C (Figure 3A, lane "d/r"). In both cases the wild-type, mutant 1 and mutant 2 bands were visible by PAGE.

Finally, the presence of the two mutant bands was also analyzed by a separate PCR using fluorescence primers and CE by ABI 310 analyzer. Here, the heteroduplexes were first

eliminated by denaturing the PCR products by formamide and heating before loading into the capillary. CE revealed the presence of the wild-type allele (331 bp) and only one peak was amplified from a mutant allele (350 bp; Figure 3B).

#### Comparison of FLT3-ITD size quantitation determined by CE and DNA sequencing

It is known from previous publications that the FLT3-ITDs always result in an in-frame genetic alteration, i.e., the ITD size is a multiple of 3 (1, 3, 4). Based on our data the ITD size determination performed by CE revealed values that did not correspond strictly to this rule. Therefore, in 10 cases we directly sequenced the DNA of ITD-positive samples and compared the number of duplicated/inserted nucleotides in the dominant mutant allele to the ITD size determined by CE (Table 3). The correlation between the two methods was very strong ( $R=0.999$ ), but the size of the duplication/insertion was 7% lower if it was determined by CE ( $\text{Size}_{\text{sequencing}} = 1.07 \times \text{Size}_{\text{CE}} + 0.5$ ) (Supplementary Figure 2A). This alteration was not peculiar to the PeakScanner software, which is a freeware program for PCR fragment analysis, as after analyzing the



**Figure 3** Larger extra bands observed in the presence of ITD on polyacrylamide gel electrophoretograms are heteroduplexes of PCR products amplified from the wild-type and the mutant alleles.

DNA sample of P55 was amplified using the PCR described in Materials and methods. The PCR product of the wild-type allele (331 bp, band 1) and two extra bands with higher molecular weight (band 2 and 3) could be observed using polyacrylamide gel electrophoresis (lane “O”, part A). The three bands were excised from the gel and were reamplified separately using the same PCR (Lane “1”, “2” and “3”, part A). Then the isolated bands 1 and 2 were mixed in equal concentrations and were amplified again in the same PCR reaction (lane “1/2”, part A) or the isolated bands 1 and 2 were amplified independently, were mixed in equal rate and were denaturated at +95°C followed by slow cooling to +4°C (lane “d/r”, part A). The isolated and reamplified bands were also sequenced. Band 1 showed the wild-type sequence (C), band 2 had a 21 bp ITD (c.1773\_1793dup21; D), while the sequence of band 3 was the mixture of the two former ones (E). Capillary electrophoresis of fluorescently labeled and denaturated PCR products revealed only the wild-type and one mutant peak of 350 bp (B).

raw data of 27 ITD peaks of 17 positive samples with the GeneScan software perfectly matching results were achieved ( $\text{Size}_{\text{GeneScan}} = 1.0 \times \text{Size}_{\text{PeakScanner}} + 0.1$ ;  $R = 0.999$ ; Supplementary Figure 2B).

## Discussion

FLT3-ITD is one of the most frequent genetic alterations in adult patients suffering from AML. In our study the rate of ITD-positivity in the whole study cohort was 24.7% (18/73), showing good agreement with the majority of published data (3, 4, 6, 7). Moreover, the rate of ITD-positive samples was similar for all the three detection methods – AGE, PAGE,

CE – used, indicating a comparable analytical sensitivity for the three techniques. On the other hand, some minor mutant bands detectable by CE were not visible on the agarose or polyacrylamide gels. Here, the detection limit of the assays – determined by serial dilution of an ITD-positive DNA sample – was 1%–2% for AGE and PAGE and 0.28% for CE. In the majority of the former FLT3-ITD publications similar detection sensitivities were found in the case of AGE and PAGE (17, 19). Concerning CE a detection threshold of 0.8%–2% was described for FLT3-ITD quantitation in five publications (18, 37–40), while 3%–10% in another four papers (25, 41–43). The somewhat lower detection limit of CE observed in our study might be explained by the low RFU threshold used for peak identification by us. In new samples we accepted signals

**Table 3** Comparison of FLT3-ITD size determined by PaekScanner and GeneScan analysis and DNA sequencing.

Sample <sup>a</sup>	DNA sequencing <sup>a</sup>	PeakScanner <sup>b</sup>	GeneScan <sup>b</sup>
P13	c.1773_1793dup21 <sup>a</sup>	19.2±0.3 bp <sup>c</sup>	19.7±0.4 bp
P16	c.1742_1777dup36	32.7±0.2 bp	33.4±0.9 bp
P35	c.1776_1833dup58+c.1833_1834insCC	56.5±0.6 bp	56.9±0.5 bp
P55	c.1773_1793dup21	19.1±0.2 bp	18.9±0.2 bp <sup>c</sup>
P58	c.1772_1833dup62+c.1833_1834insGAGA	61.5±0.2 bp	60.9±1.4 bp
P80	c.1734G>A+c.1734_1735insCAACCAACCAACCATGCGAGGGCCTCC	24.8±0.4 bp	25.0±0.7 bp
P92	c.1790_1835dup46+c.1835_1836insCCTCCCCACGG	52.5±0.3 bp	52.5±0.3 bp
P113	c.1762_1827dup66	61.3±1.1 bp	61.5±1.0 bp
P114	c.1788_1789insGGGGTCCCT	9.0±0.1 bp	9.1±0.1 bp
P125	c.1786_1837+3dup55+c.1837+3_1837+4insCC	52.3±0.6 bp	52.0±1.3 bp

<sup>a</sup>The DNA of 10 ITD-positive samples was directly sequenced and the location and number of duplicated/inserted nucleotides in the dominant mutant allele was determined. <sup>b</sup>The size of the wild-type and mutant alleles was determined by CE using the ABI310 analyzer and the PeakScanner or GeneScan programs. The size of the ITDs was calculated as the size of the mutant alleles minus the size of the wild-type allele. <sup>c</sup>Data presented are mean±SD of 3–10 independent determinations.

with RFU >50 as peaks and in follow-up samples with known peak-localizations even RFU values above 15 were accepted. In this way – based on a maximal RFU signal of 6000 – a detection limit below 1% could be obtained. Only some information about the RFU threshold values is available in former FLT3-ITD studies. Murphy et al. (42) used RFU=150 as a threshold, while Lin et al. (18) mentioned RFU=110 as a very low peak height reaching a maximal FLT3-ITD detection sensitivity of 2%–3%. To ensure the presence of ITD-mutants with low signal intensity, analysis was repeated and declared positive only if the result was reproducible.

Although mutant alleles with very low rates were not detectable by AGE and PAGE, several extra bands with larger size were visible that were absent in the electrophoretograms of CE. We performed a detailed analysis of the isolated PCR products amplified from the wild-type and mutant alleles using DNA sequencing and heteroduplex testing, and could prove that the large extra bands corresponded to the heteroduplexes formed by the wild-type and mutant alleles. Previous publications also suggest this phenomenon (30, 42, 43). Based on the absence of mutant alleles with very low rates and the presence of heteroduplexes on AGE and PAGE, the method that is suitable for the reliable detection of multiplex ITDs is CE.

By examining our patient samples we could compare the resolution of the applied electrophoretic methods, too. While any of the three methods could differentiate between bands/peaks which were 9 bp apart, a 3-bp-difference could be resolved only by CE. A similar superior resolution of CE-based FLT3-ITD analysis was observed in two previous publications (18, 27), further supporting the importance of this technique in multiple ITD detection. The presence of multiple ITDs – determined by CE – in our study was 50% (9/18), showing somewhat higher values compared to the previously published 11%–32% (7, 13, 19–22, 27, 33, 42, 44). This might be explained by the lower threshold used for the definition of “peaks” on the CE electrophoretograms.

Concerning the size of ITD, basically three different techniques have been used for its quantitation so far. DNA sequencing (7, 14, 21, 23, 26, 27, 29), CE (22, 24, 33, 40, 43–45) and application of the Agilent Bioanalyzer (30, 31, 46) showed ITD sizes varying from 3 to 400 bp, with a median changing between 24 and 70 bp. Our results were within these limits, as the observed ITD size range was 9–178 bp, with a median of 40 bp. Our data showed a good reproducibility of the ITD size determination by CE, as an inter-assay CV% of 11% was achievable even in the case of the shortest insertion (9 bp). The number of duplicated/inserted nucleotides was not divisible by three in every case, this has been reported in two former publications (30, 45), but not in other studies using Agilent Analyzer or CE. This phenomenon can be explained by the observed variation of about  $\pm 0.5$  bp in the size of the wild-type and mutant peaks on the CE electrophoretograms that can result in a  $\pm 1.0$  bp change in the ITD-size. As such, the method that can provide the exact ITD size is direct sequencing of patient DNA samples or sequencing the isolated – and cloned – ITD fragments. Following the former approach we sequenced 10 samples with ITD and always found the duplication/insertion inframe, but these ITD sizes

were slightly larger as compared to data obtained by CE and PeakScanner or GeneScan analysis. Based on literature data and our own experience, we suggest the usage of DNA sequencing for exact ITD size determination and especially because recent data emphasized that even the location of the duplication/insertion might have prognostic importance (13).

Five different methods were used so far to determine the rate of the mutant allele in samples of AML patients: semi-quantitative AGE or PAGE (14, 20), dHPLC (12, 13), Agilent Bioanalyzer (30, 31, 46), real-time quantitative PCR (34, 35) and CE (13, 17, 19, 22–29, 32, 33). Concerning the expression of the results two major approaches were described in literature; the mutant/wild-type (13, 23–28, 32) or the mutant/total allele rate (17, 19, 20, 22, 30, 46) was used with similar frequency. As such we calculated both rates in our analysis. The median of the mutant/wild-type allele rate in our patient group was 0.144 (range: 0.006–3.714), while median of the mutant/total allele rate showed 12.5% (range: 0.48%–77.8%), this seems to be somewhat lower than previously published data (range for all data and for median of the mutant/wild-type rate: 0.008–32.56 and 0.530–0.836; range for all data and for median of the mutant/total rate: 1%–100% and 30%–47%). Most probably this can be explained again by the lower RFU limit used for peak definition in our study. Concerning the reproducibility of these determinations our results indicated that the mutant/wild-type and the mutant/total allele rate can be determined with an inter-assay CV% <25% above 0.020 and 1.5%, respectively, giving the quantitation limit of these determinations.

The clinical importance of the presence of multiple ITDs and the ITD-size determination seems to be conflicting, and cannot be suggested for routine diagnostic application at the moment (13, 20–24). With regards to the clinical importance of the mutant allele rate quantitation, literature data is more solid and several articles showed that higher ITD-allele rate – and especially the loss of the wild-type allele (15, 16) – is associated with worse overall- and disease-free survival and a higher relapse rate in AML (3, 13, 20, 22, 24–29). Here, the major problem is how to define “high” rate. Mostly the median of the allele rate values measured in a patient group was used as cut-off, but this varied from 0.530 to 0.836 for the mutant/wild-type allele rate and 30%–47% in the case of the mutant/total rate. Based on literature data and our own experience, we suggest the usage of the mutant/total allele rate that gives more clear information to the clinician as it can be scaled from 0% to 100%. The development of a general diagnostic cut-off needs further effort, either by pooling the data presently available in literature or by initiating large prospective trials. Until this is achieved, the 50% limit can be used for samples obtained from de novo AML cases, as at this time-point the majority of cells are malignant, where a mutant/total rate above 50% means the presence of cells with homozygous ITD (47).

The application of home-made molecular genetic tests in the detection and quantitation of FLT3-ITD is rather widespread in scientific literature. On the other hand – to the best of our knowledge – no complex evaluation of analytical properties of the applied methods is published before. In our work



we defined the precision, inaccuracy, analytical and functional sensitivity, resolution, and ability to detect multiplex ITDs of a PCR+CE-based quantitative method and compared these parameters – where applicable – to that of PCR+AGE/PAGE-based assays. The novel findings concerning the analytical performance of these tests were the following; (i) though the CE-based assay has a very high analytical sensitivity for detection of FLT3-ITD mutant alleles (0.28%) the quantitation of the rate of the mutant allele can be done reliably only if it is higher than 1.5%; (ii) the quantitation of the ITD size by CE can be done with high reproducibility but the accuracy is less perfect (7% lower ITD-size values compared to DNA sequencing); (iii) the PCR+AGE/PAGE-based methods are not reliable for identification of the presence of multiplex ITDs because of heteroduplex formation. Our data further supports the importance of the proper analytical evaluation of home-made molecular biological diagnostic tests.

In conclusion, our experimental results and data of former publications clearly indicate that a CE-based test seems to be the most suitable method for the detection and quantitation of the mutant/total allele rate of FLT3-ITD, and can be used even for routine diagnostic purposes. This assay can supplement the diagnostics and follow-up of patients with AML. The detection of multiple ITDs and quantitation of the size of the duplication/insertion cannot be suggested for clinical usage at the moment. For research purposes, proper estimation of multiple ITD can be done by CE if the rate of the mutant allele is above 0.28%, and DNA sequencing can provide reliable information about the size and location of ITD.

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