Flow cytometric immunobead assay for fast and easy detection of PML-RARA fusion proteins for the diagnosis of acute promyelocytic leukemia

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Abstract (199 words; needs to be ≤ 200 words)

The PML-RARA fusion protein is found in approximately 97% of patients with acute promyelocytic leukemia (APL). APL can be associated with life-threatening bleeding complications when undiagnosed and not treated expeditiously. The PML-RARA fusion protein arrests maturation of myeloid cells at the promyelocytic stage, leading to accumulation of neoplastic promyelocytes. Complete remission can be obtained by treatment with all-trans-retinoic acid (ATRA) in combination with chemotherapy. Diagnosis of APL is based on the detection of t(15;17) by karyotyping, FISH or PCR. These techniques are laborious and demand specialized laboratories. We developed a fast (performed within 4 to 5 hours) and sensitive (detection of at least 10% malignant cells in normal background) flow cytometric immunobead assay for the detection of PML-RARA fusion proteins in cell lysates using a bead-bound anti-PML capture antibody and a PE-conjugated anti-RARA detection antibody. Testing of 163 newly diagnosed patients (including 46 APL cases) with the PML-RARA immunobead assay showed full concordance with the *PML-RARA* PCR results. Since the applied antibodies recognize outer domains of the fusion protein, the assay appeared to work independently of the *PML* gene breakpoint region. Importantly, the assay can be used in parallel with routine immunophenotyping for fast and easy diagnosis of APL.

Keywords: PML-RARA protein, t(15;17), APL, immunobead, flow cytometry

Introduction

The presence of t(15;17) with the *PML-RARA* fusion gene is considered to be the hallmark of acute promyelocytic leukemia (APL), also known as acute myeloid leukemia type M3 (AML-M3).[1] Initially t(15;17) was assumed to be present in all APL patients. However, over the last 10 years a sizeable minority of APL cases (~5%) has been identified as lacking this classical translocation, but containing the *PML-RARA* fusion gene. Such non-classical *PML-RARA* fusion genes can be caused by chromosomal aberrations, such as insertions or complex chromosomal aberrations, which may be missed by cytogenetics and FISH, but are detected by PCR.[2-4]

The breakpoints in the *RARA* gene are all located in intron 2 (~15 kb), whereas the vast majority of breakpoints in the *PML* gene cluster in the breakpoint cluster region (bcr) of intron 6 (bcr1; ~ 55% of cases), exon 6 (bcr2; ~5% of cases) or intron 3 (bcr3; ~40% of cases).[3] Two APL cases with t(15;17) have been reported with breaks in *PML* exon 7 (Figure 1).[5] A rare subgroup of APL cases (2 to 3%) has variant *RARA* gene translocations with a non-PML fusion partner. So far five alternative *RARA* gene partners have been identified in APL cases. The *PLZF* gene appears to be the most frequent alternative partner and is present in ~1% of all APL cases (Table 1).[6-11]

Finally, in ~1% of APL molecular techniques have not identified a *RARA* gene rearrangement.[2] Further studies are needed to asses what genetic event causes APL characteristics in these patients. In some of these cases the PCR technique may have given false-negative results due to unusual positioning of the *PML* or *RARA* gene breakpoints, not covered by the applied primers.[5]

APL patients with the *PML-RARA* fusion gene aberration have been shown to be highly sensitive to retinoid differentiating agents, such as all-trans retinoic acid (ATRA). Combination therapy (ATRA and chemotherapy) has significantly improved treatment outcome in APL patients. The long-term outcome is now favorable because of the low risk of relapse and prevention of life-threatening coagulopathy at diagnosis. Also in recent years, As_2O_3 was introduced as a new and efficient treatment alternative for APL patients. Consequently, stem cell transplantation (SCT) in first remission is no longer recommended.[12,13]

It should be noted that APL patients with the most frequent variant fusion gene, the *PLZF-RARA* fusion gene (Table 1), are not sensitive to ATRA treatment.[11,13,14] However, other rare variant APL cases with *NPM-RARA* and *NUMA-RARA* fusion genes are sensitive to ATRA treatment, comparable to PML-RARA positive cases.[11]

Since the first description of APL, life-threatening bleeding problems have been identified as the most notorious manifestation of the disease. This coagulopathy in many APL patients leads to pulmonary and cerebral hemorrhages, if the appropriate treatment regimen is not initiated instantly.[12,13,15] Even upon treatment, APL-related coagulopathy takes 5 to 8 days to improve. Many hematologists consider the correct diagnosis in a patient with APL a medical emergency, because of the coagulopathy manifestations, which continue to be a major cause of death in APL patients. Consequently, a positive diagnosis of APL is of utmost importance for patient care in leukemia treatment. Such diagnosis preferably should be provided within hours and not within days.

Although the clinical and cytomorphological picture of APL seems relatively clear, the leukemic cells in a subset of patients (5 to 10%) do not exhibit the typical APL morphology.[16] Consequently, it is strongly recommended to make a fast and accurate diagnosis of the *PML-RARA* aberration. Because of the risk of early death due to bleeding, best clinical practice recommends that APL should be excluded in each patient with newly diagnosed AML.[11,12]

At present, the diagnosis of PML-RARA positive APL cases is based on the result of cytomorphology and immunophenotyping combined with karyotyping, fluorescence *in situ* hybridization (FISH), and/ or PCR. Immunophenotyping results are typically completed on the same day, but the other techniques are time consuming (usually 1 to 2 days for FISH and PCR techniques and 1 to 2 weeks for karyotyping) and are restricted to specialized laboratories with well-trained personnel. Therefore, novel methods are needed for fast and easy diagnosis of PML-RARA positive APL. We have developed a new antibody-based flow cytometric immunobead assay for specific detection of the PML-RARA fusion protein, comparable to our BCR-ABL immunobead assay.[17] To allow detection of all known PML-RARA fusion proteins independent of the breakpoint (bcr1, bcr2, bcr3, and the rare bcr in exon 7), antibodies were generated that recognize immunogenic epitopes located upstream of the breakpoint cluster regions (upstream of bcr3) for PML and downstream of the break point region in intron 2 known for RARA. The antibodies were combined into a prototype immunobead assay in which the anti-RARA antibody was used as a capture antibody and the anti-PML antibody as a

phycoerythrin (PE)-conjugated detection antibody. After initial testing on cell lines and a small series of patient samples, the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708) evaluated 163 acute leukemias at diagnosis, which showed full concordance between PCR and immunobead results.

Materials and Methods

Cell lines

Several leukemic cell lines were used to investigate the specificity of the PML-RARA immunobead assay. The NB4 cell line with t(15;17) and *PML-RARA* transcripts was used as positive control; the *PML* breakpoint in this cell line is located in bcr1 (intron 6).[18] Several other leukemic cell lines were used as negative controls: ME-1 with inv(16) and *CBFB-MYH11* transcripts,[19] MV4-11 with t(4;11) and *MLL-AF4* transcripts,[20,21] K562 with t(9;22) and *BCR-ABL* transcripts,[22, 23] 697 with t(1;19) and *E2A-PBX1* transcripts,[24] RCH-ACV with t(1;19) and *E2A-PBX1* transcripts,[25] KASUMI-1 with t(8;21) and *AML1-ETO* transcripts,[26] and REH with t(12;21) and *TEL-AML1* transcripts.[27] All leukemic cell lines used in this study are available from the German Collection of Microorganisms and Cell cultures (DSMZ GmbH, Braunschweig, Germany).

Healthy controls

Peripheral blood (PB) samples were obtained from healthy donors after informed consent according to the local Medical Ethics Committee guidelines. Total white blood cells (WBC) were obtained by NH_4Cl lysis of the erythrocytes. PB mononuclear cells (PBMC) were obtained by ficoll separation. To mimic leukemic samples with varying tumor loads, cells of the PML-RARA expressing cell line NB4 were mixed at different concentrations in either PML-RARA negative cell lines or in normal PBMC or WBC.

Patient samples

Freshly collected or frozen bone marrow (BM) or PB samples were obtained from patients in accordance with the Declaration of Helsinki. All participants in the EuroFlow Consortium (EU-FP6 grant LSHB-CT-2006-018708) obtained approval from the local Medical Ethics Committee for secondary use of remaining diagnostic material of patients suspected to have a hematological malignancy. Cell samples were processed by either NH_4Cl lysis or ficoll separation.

Antibodies for specific detection of PML and RARA domains

Antibodies were raised against recombinantly expressed epitopes of PML and RARA domains that are present in all known fusion protein variants, which result from various breakpoint regions. The PML domain located before the first Zinc finger domain was used for immunization (Figure 2A). For the generation of anti-RARA antibodies, the C-terminal domain of RARA was recombinantly expressed and used for immunization (Figure 2B).

The recombinant proteins were expressed in HEK 293FS cells and after purification were used for immunization of SJL (anti-PML) or Balb/c (anti-RARA) mice using subcutaneous immunization techniques optimized at BD Diagnostics (Durham, NC, USA), followed by fusion of spleen cells with the P3X,653 cell line for hybridoma formation. This resulted in the generation of the anti-PML 5D8 and the anti-RARA 9G4.16 antibodies.

Prototype immunobead assay

The here described and tested prototype immunobead assay for detection of the PML-RARA fusion protein was essentially developed as described for the BCR-ABL immunobead assay.[17] Small adaptations were made in the pretreatment and lysis reagents to improve the detection of the PML-RARA fusion protein, which has a nuclear localization. This is in contrast to the cytoplasmically expressed BCR-ABL fusion protein. In short, to inhibit protease activity before cells lysis, intact cells were pretreated for 10 minutes on ice with the protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, (AEBSF, Sigma; 15 mM AEBSF, 15 µl of 1M stock in PBS) and phenylmethanesulfonyl fluoride (PMSF, Sigma; 1mM, 5 µl of 200 mM stock in ethanol) in PBS at a concentration of 10^7 cells/mL. Next, cells were gently spun down (5 minutes at 500*g* at 4°C) and washed with 1 mL of 5% FBS in PBS. After removal of the supernatant the cells were resuspended in BD Pharmingen cell lysis buffer supplemented with 0.1% SDS, 2 mM MgCl, 50 U/ mL of Benzonase, and the BDBaculogold protease inhibitor cocktail solution diluted as described by the manufacturer (BD Biosciences, San José, CA, USA). After 15 minutes' incubation on ice, the lysate was spun down at 14,000 rpm (20,000g) for 10 min at 4ºC to remove cell debris, and the supernatant was transferred to a fresh Eppendorf tube then used in the cytometric bead assay.

For the cytometric bead assay both antibody conjugates were diluted in PBS with 5% FBS, and 50 µL of cell lysate was incubated together with 50 µL of anti-RARA

9G4.16 antibody conjugated to beads (6,000 beads) and 50 µL anti-PML 5D8 PE conjugated antibody, for 2 hours at room temperature while being shaken. Incubation was performed in either a 96 well filter plate (Multiscreen HTS filter plates, MSBVN1250, Millipore, Tullagreen, Ireland) or in BD Falcon 12x75mm tubes. After incubation, the filter plate was washed twice with 200 µL of BD CBA-wash buffer and after the final drain, the beads were resuspended in the plate with 200 µL of BD CBA wash buffer. The beads incubated in BD Falcon 12x75mm tubes were washed one time by the addition of 1 mL of BD CBA wash buffer, and after pelleting the beads for 7 minutes at 500*g* at 4 ºC, the beads were resuspended in 300 µL of BD CBA wash buffer. Analysis was performed on the results obtained from at least 1000 beads that were acquired on a BD FACS Canto II or BD LSR II flow cytometer using BD FACSDiva software, version 6.1 (BD Biosciences). Instruments were set-up according to the BD Cytometer Setup and Tracking (CS&T) module and subsequently photo multipliers were adjusted using capture beads according to the manufacturer's recommendations, resulting in median MFI values of 20,000, 40,000 and 100 for the APC, APC-Cy7 and PE channels, respectively.

Specificity and sensitivity testing of the PML-RARA prototype immunobead assay

The specificity of the PML-RARA prototype kit was confirmed by the analysis of cell lysates from leukemic cell lines with different types of fusion proteins (see above).

The sensitivity of the PML-RARA prototype assay was determined by analyzing serial dilutions of the NB4 cell line into both PBMC and WBC. All analyses were performed in duplicate. MFI values detected in 100% PBMC and 100% WBC were used as negative control values.

Testing of the PML-RARA prototype immunobead assay by the EuroFlow Consortium.

The PML-RARA prototype immunobead assay was tested in nine EuroFlow laboratories on BM and/or PB samples of 163 leukemia patients at initial diagnosis, including 46 APL patients (according to morphology and/or immunophenotyping). Because of the rarity of APL samples, a large fraction of the patient samples was used from local cell banks. Only six of the 46 APL samples were fresh cell samples. To facilitate the standardized collection of patient data with accompanying results from PML-RARA testing, a Case Report Form (CRF) was developed for each sample with

drop-down menus for all relevant information. This CRF was electronically linked to a corresponding Microsoft Excel file to guarantee transfer of data without typing errors.

The PML-RARA prototype immunobead assay was performed according to recommendations developed at BD Biosciences (see above). However, in 3 laboratories without a refrigerated microcentrifuge, centrifugation of lysates was performed at 20,000*g* for 2 minutes at room temperature (RT) instead of 10 minutes at 4°C. Storage of the recovered lysates at -80°C was allowed in order to analyze multiple samples collectively for better comparison with several positive and negative controls. Controls were provided by Dynomics (lysates from 10% or 20% NB4 in PBMC as positive controls and negative control lysates derived from PBMC).

PCR analysis of PML-RARA transcripts

PCR analysis of the different types of *PML-RARA* fusion transcripts was performed with the standardized Europe Against Cancer (EAC) protocols using TaqMan based real-time quantitative PCR (RQ-PCR; see Gabert *et al.* 2003 for details).[27] The *ABL* or *MGUS* internal control genes for the RQ-PCR studies were selected from the EAC study by Beillard *et al.*[28] According to the EAC guidelines, if too few control gene copies were detected in patients without *PML-RARA* transcripts, those patient samples were excluded from the study because of the low quality of the RNA.[28]

Statistical analysis

Statistical analyses using the Mann-Whitney *U* test were performed to compare mean MFI values between different PML-RARA positive samples, between different *PML* breakpoint types (bcr1, bcr2, bcr3) and between different experimental conditions, e.g. microcentrifugation at room temperature for 2 min versus at 4°C for 10 min and sample analysis on the same day versus overnight. The relation between MFI values and age of the samples was evaluated by Spearman's rank correlation coefficient. A value of $p < 0.05$ was regarded to be statistically significant.

Results

Validation of the PML-RARA prototype immunobead assay on cell lines and a small number of patient samples

Antibodies were developed against regions within PML and RARA proteins, that are present in all described PML-RARA fusion proteins (Figure 2). The anti-PML antibody 5D8 was raised against exposed, non-homologous amino acid sequences with sufficient antigenicity. The involved region is located upstream of the breakpoint cluster regions (Figure 2A). Using the same criteria, the anti-RARA antibody was raised against sequences downstream of the RARA breakpoint region (Figure 2B). In the prototype PML-RARA immunobead assay, the anti-RARA antibody is used as the capture antibody and the anti-PML antibody as the PE-conjugated detection antibody (Figure 3).

The specificity of the assay was determined by testing leukemic cell lines with t(15;17) or other translocations. The PML-RARA prototype immunobead was shown to be specific, since only the t(15;17) positive NB4 cell line gave high PE-fluorescent signals, whereas the other cell lines tested exhibited low background signals (Figure 4A). The sensitivity of the PML-RARA immunobead assay was determined by dilution of the t(15;17) positive NB4 cell line in both WBC and PBMC. The PML-RARA specific fluorescent signal was still detectable at concentrations as low as 10% of the NB4 cell line (Figure 4B). This sensitivity is sufficient, since PB and BM samples of most APL patients at diagnosis, contain 70% to 90% leukemic cells. Testing of a small series of APL patients with BCR1 or BCR3 PML-RARA variants showed that the immunobead assay was positive in all APL patients and the results were independent of the *PML* breakpoint position (Figure 4C).

Testing of the PML-RARA prototype immunobead assay by the EuroFlow Consortium.

The PML-RARA prototype immunobead assay was distributed to nine EuroFlow laboratories. A series of 163 leukemia patients was evaluated, consisting of 112 AML patients (including 46 APL patients), one CML patient in accelerated phase, 34 B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients, and 16 T-ALL patients. For each analysis of patient samples, both a positive and negative control lysate were used to verify the proper performance of the immunobead assay.

MFI values of the non-APL patient samples had a median value of 114 (range 100 to 173) and a mean of 119 (SD 15.4). The MFI values of the AML (non-APL) samples were slightly higher than the MFI values of the BCP-ALL samples and T-ALL samples: mean of 121 (SD 18.3) versus 118 (SD 10.4) and 113 (SD 8.7), respectively. The cut-off between negativity and positivity was based on the MFI values of negative AML (non-APL) samples, using 3-SD values, resulting in an MFI value of 176. Consequently, a safe MFI cut-off value of 180 was defined. All tested PCR-negative samples and negative controls had an MFI below this cut-off value. Based on the MFI cut-off value and the values of the positive samples, several ranges of positive MFI values were arbitrarily defined to cluster the leukemias according to the expression level of PML-RARA protein: high level positivity: MFI ≥1,000; medium level positivity: MFI ≥500, but <1,000; low level positivity: MFI ≥180, but <500; negativity: MFI <180.

Full concordance (163/163; 100%) was observed between the *PML-RARA* PCR results for fusion transcript detection and the prototype PML-RARA immunobead results for fusion protein detection (Tables 2 and 3 and Figure 5). A total of 46 APL patients (22 children and 24 adults) were positive for PML-RARA with both methods. All other 117 leukemia patients including 66 AML patients (18 children and 48 adults) 1 CML patient (accelerated phase), 34 BCP-ALL patients and 16 T-ALL patients, were PML-RARA negative in both assays (Table 2).

MFI values in patients with positivity for the PML-RARA immunobead assay

The vast majority of PML-RARA positive patients had high MFI values (65%) or medium MFI values (24%) clearly above the cut-off value determined at MFI 180. Low MFI values were found in only 11% of the positive patients (5 cases): 192, 195, 273, 339, and 444 (Figure 5). The 5 cases consisted of four adult patients and one child (Table 3). The diagnosis sample with the lowest MFI value of 192 contained only 37% leukemic cells, which was substantially less than the typical leukemic cell percentages in most of the other APL samples (Figure 6). The diagnosis samples with the MFI values of 195 and 273 had a time lapse between sampling and processing of 3 days (65 and 60 hours, respectively), which most likely has resulted in protein degradation (see below).

Detection of PML-RARA proteins, derived from all three PML breakpoints

The immunobead assay was designed to detect all PML-RARA fusion proteins that originate from the bcr1, bcr2, and bcr3 breakpoints in the *PML* genes. As

summarized in Table 3, all three types of breakpoint regions in the PML gene were included in the 46 APL/AML cases with a positive PCR result for PML-RARA transcripts: 21 cases with bcr1 (46%), 1 case with bcr2 (2%), and 24 cases with bcr3 (52%). This implies that indeed the anti-PML and anti-RARA antibodies recognize epitopes that are sufficiently upstream and downstream of the PML and RARA breakpoint cluster, respectively (Figure 5). The seemingly lower MFI values in APL patients with bcr1 breaks as compared to APL patients with bcr3 breaks did not reach statistical significance (p=0.052 by Mann-Whitney *U* test; Figure 5).

Relation between PML-RARA protein level and percentage of leukemic cells

To evaluate whether differences in MFI levels between leukemias might be related to the percentage of leukemic cells present in the tested samples, MFI values were plotted against APL cell percentages (Figure 6). MFI values (^{10}log transformed) from APL patients with bcr1 breaks seemed to have some correlation with the percentage of leukemic cells (Spearman's rho 0.048; correlation coefficient 0.45), whereas no significant relation was observed for patients with bcr3 breaks (Spearman's rho 0.3).

Microcentrifugation of cell lysates and temperature

The 46 PML-RARA positive patients were evaluated for effects resulting from temperature differences during microcentrifugation. A total of 27 cases were in the 10 min 4°C group and had a median MFI value of 1,460 and a mean of 3,042 (SD 6,098). The high mean value and the high SD value appeared to be caused by the fact that this group contained 4 of the 5 low MFI values and the single very high MFI value of 32,160. The other 19 cases were in the 2 min RT group with a median MFI value of 1,697 and a mean value of 2,108 (SD 1,769). The Mann-Whitney *U* test did not show a significant difference (p=0.7). Consequently, the temperature of the microcentrifugation step does not seem to influence the results of the PML-RARA immunobead assay.

No effect of time lapse between sampling and processing of the patient material within the first 36 hours

To understand whether longer transportation and/or processing times might have a negative impact on the quality and reliability of the PML-RARA immunobead assay, the EuroFlow laboratories were asked to make a fair estimate of the time lapse between

sampling and processing of the patient material. These assessed and estimated time lapses were reported in the CRFs. The time lapse was reported in 30 of the 46 PML-RARA positive cases: 10 cases were processed on the same day (within 8 hours), 17 cases on the next day (18 to 36 hours), and 3 cases were processed on the third day or later (>48 hours). Comparison of the MFI values did not show a clear decrease between cases processed within 8 hours (same day) and 18 to 36 hours (next day) ($p=0.9$ by Mann-Whitney *U* test; Figure 7). We cannot draw conclusions from the three samples processed on the third day or later (>48 hours), other than the fact that 2 of the 3 samples had a very low MFI value (MFI: 195 and 273).

These results indicate that processing within 36 hours does not harm the performance of the PML-RARA prototype immunobead assay. The results of patient samples processed after 36 hours (after two days) should be analyzed with caution.

Paired blood (PB) and bone marrow (BM) samples

In the study, nine paired fresh BM and PB samples were analyzed, six of which were derived from APL patients at time of diagnosis and three from non-APL patients. Fully comparable results were obtained in the six paired PB-BM APL samples (Figure 8), indicating that the intra-individual PML-RARA protein expression in leukemic cells from BM and PB is fully comparable.

Discussion

The PML-RARA fusion protein is the hallmark of APL and is present in 92% of APL patients with the classical t(15;17) translocation and in 5% of APL patients with non-classical genetic aberrations such as insertions or complex chromosomal aberrations. PML-RARA positive APL patients often present with coagulopathy and without the right treatment, 40% of the patients develop pulmonary and cerebral hemorrhages, which can be lethal. Therefore, APL is a medical emergency, and the diagnosis of PML-RARA positive APL should be triaged fast enough to enable patients to enter a correct treatment protocol as soon as possible.

In this study, we developed a simple and fast flow cytometric immunobead assay for detection of PML-RARA fusion proteins based on the same principle as described for the BCR-ABL protein immunobead assay.[17] For the detection of PML-RARA fusion proteins in cell lysates, antibodies were raised against the N-terminal domain of PML, upstream of the breakpoint region BCR3 and present in all known PML-RARA fusion proteins, and against the C-terminal domain of RARA (downstream of the RARA breakpoints in intron 2). The antibodies were either bead-coupled or labeled with a fluorochrome. PML-RARA proteins were detected in the immunobead assay using a bead-bound capture antibody against one side of the fusion protein (anti-RARA) and a fluorochrome-conjugated detection antibody against the other side of the fusion protein (anti-PML).

The prototype immunobead assay appeared to be specific for the detection of the PML-RARA fusion proteins since a positive signal was obtained only when the epitopes of both the anti-PML and anti-RARA antibodies were present in a protein lysate. No background signals were obtained in cell lines with wild type proteins or other fusion proteins. The sensitivity of the PML-RARA prototype immunobead assay was at least 10% for the NB4 cell line. The suitability of the novel immunobead assay was further shown by detection of PML-RARA fusion proteins originating from both the bcr1 (downstream) and bcr3 (upstream) breakpoints in the *PML* gene.

After further optimization, the prototype immunobead assay was distributed for large-scale testing to nine diagnostic laboratories enrolled in the EuroFlow consortium. The results obtained with the PML-RARA immunobead assay were fully concordant with

the PCR results in a series of 163 patient samples including 46 APL patients, 66 non-APL AML patients, one CML patient in accelerated phase of the disease, 34 BCP-ALL patients and 16 T-ALL patients.

In the series of APL patients included in this study, the adult and childhood cases were equally represented and they showed comparable levels of PML-RARA fusion protein. The detection of PML-RARA fusion proteins in the 46 APL cases studied at diagnosis was independent of the type of PML breakpoint (bcr1, bcr2, or bcr3). This implies that the location of the epitopes used for generation of the anti-PML and anti-RARA antibodies were chosen sufficiently upstream and downstream of the known breakpoint clusters. Analysis of the MFI levels detected with the prototype immunobead assay did not show a clear correlation with the percentages of leukemic cells present in the samples. This could imply that different APL cases may express different levels of PML-RARA fusion proteins. This explanation is supported by the evaluation of PML-RARA expression levels in paired BM and PB samples, which showed clear patient-to-patient differences between the six APL patients analyzed. In contrast, comparable PML-RARA expression levels were detected within in the paired BM and PB samples of the same patient.

Processing of leukemia samples in the same day, or the first day after sampling gave comparable results. However, it is not clear from this study whether a longer time lapse between sampling and processing would lead to suboptimal results (too few samples tested), but it is advised to keep the time lapse between sampling and processing within 36 hours. Since it is not known whether protein integrity is affected during transportation and the time lapse between sampling and processing, it would be advantageous to supplement the immunobead assay with an internal control for the assessment of protein quality in the cell lysates, as also proposed for the previously reported study performed with the BCR-ABL protein kit.[17]

We conclude that the flow cytometric immunobead assay is a very powerful diagnostic tool for detection of PML-RARA fusion proteins in leukemic cells. Because of the severe course of APL, a clinical assay with high specificity and sensitivity is critically needed to positively identify APL patients within a total group of AML patients.[11,12] Comparable to the BCR-ABL immunobead assay described previously, the PML-RARA immunobead assay also exhibits several obvious advantages over classical PCR and FISH techniques. The immunobead assay is independent of the breakpoint position in the fusion gene, there is no need for special laboratory facilities, the assay requires no

specialized equipment other than a standard flow cytometer, the results can be obtained in approximately 4 hours, and the assay can be run in parallel to routine immunophenotyping without the need for extra technician time. It should be noted that the sensitivity of the current prototype PML-RARA immunobead assay is at least 10%, which is less sensitive than PCR and also slightly less sensitive than FISH, but sufficiently sensitive for the formal diagnosis of acute leukemia. Consequently, the novel PML-RARA immunobead assay will contribute to an earlier diagnosis of APL and thereby will enable clinicians to start specific treatment as soon as possible. Use of the novel PML-RARA immunobead assay hopefully will prevent the patients from developing any life threatening bleeding problems because of early stage treatment.

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Conflict of interest

EHA Dekking, AL Noordijk, PM Vermeulen and MT de Man are employees of Dynomics, a spin-off company of Erasmus MC, Rotterdam, The Netherlands; JJM van Dongen and VHJ van der Velden are inventors of the immunobead assay (patent PCT/NL01/00.945), which has been licensed by Erasmus MC to Dynomics; R Varro and H Wai are employees of BD Biosciences; EP Dixon is employee of BD Diagnostics; JJM van Dongen is stockholder of Dynomics and advised Dynomics in the development and

optimization of the immunobead assay. It is thanks to this crucial combination of disciplines (the University setting of Erasmus MC, the spin-off company Dynomics, the Industry represented by BD Biosciences and the EuroFlow Consortium, where the clinic matches the diagnostic) that we have been provided with a complete chain of events, which has led us from invention through development and production to end up in a final clinical testing, which we present here, the description of a new diagnostic product for the diagnosis of APL.

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Legends

Figure 1. Structure of the *PML* and *RARA* genes with the breakpoint regions and the corresponding fusion gene transcripts. A. The *PML* gene contains three well-defined small breakpoint cluster regions (bcr's): bcr1 in intron 6, bcr2 in the downstream part of exon 6, and bcr3 in intron 3. In addition two rare breakpoints in intron 7 have been reported. In the *RARA* gene the breakpoints cluster in intron 2 (~15 kb). **B.** The three well-defined BCR's and the rare intron 7 breaks in the *PML* gene result in four different *PML-RARA* fusion transcripts. Percentages refer to the relative frequency of the various PML-RARA variants.

Figure 2. Selection of the PML and RARA constructs for raising anti-PML and anti-RARA antibodies. A. To increase the chance of making an anti-PML antibody that worked in the immunobead assay, the PML immunogen had to meet specific criteria. The PML protein domains encoded by exon 1 to 3 (upstream of all known breakpoints) were analyzed with various web-based programs to identify exposed regions (accessibility for the antibody), non-homologous regions (no cross-reactivity with other human proteins) and antigenic determinants (difference between human and mouse). Accordingly, part of the PML domain before the first Zinc finger domain was selected for cloning, protein expression, protein purification, and subsequent immunization. The anti-PML antibody 5D8 was used in the immunobead assay. **B.** Using the same selection criteria, the C-terminal domain of RARA were used for cloning, protein production, and subsequent immunization. The anti-RARA antibody 9G4.16 was used in the immunobead assay.

Figure 3. Configuration of the prototype PML-RARA immunobead assay. The bead-bound anti-RARA antibody serves as the capture antibody, whereas the phycoerynthrin (PE)-conjugated anti-PML antibody serves as the detection antibody. The leukemia cells are lysed and incubated with beads and the detection antibody, then washed and evaluated by flow cytometry for PE positivity of the immunobeads.

Figure 4. Detection of PML-RARA fusion protein in cell lines and patient samples with the prototype PML-RARA immunobead assay. (**A**) Cell lysates from cell lines with different translocations expressing various fusion proteins: ME1 with (inv16)

expressing CBFβ-MYH11, MV4;11 with t(4;11) expressing MLL-AF4, 697 with t(1;19) expressing E2A-PBX1, RCH-ACV with t(1;19) expressing E2A-PBX1, Kasumi with t(8;21) expressing AML1-ETO, REH with t(12;21) expressing TEL-AML1 did not give a PE fluorescence signal in the PML-RARA immunobead assay, whereas the cell line NB4 with t(15;17) expressing PML-RARA (bcr1) gave a strong PE signal. (**B**).The PML-RARA positive NB4 cell line was diluted into PBMC of a healthy individual and cell lysates were prepared. The immunobead assay was carried out in triplicate for every diluted sample. At a concentration of 10 % NB4 cells a signal-to-noise (s/n) ratio compared to the PBMC background of 2.7 was obtained. (**C**) The novel PML-RARA immunobead assay was carried out on a series of cell lysates of AML patients without the t(15;17) translocation(samples 1-5), APL patients with translocations in the *PML* gene involving both the BCR1 and BCR3 region and of two APL patients in which the breakpoint region involved in the t(15;17) translocation was not determined (samples 6-13), and of healthy individuals (samples 14-19). Strong positive PE signals were only obtained in the cell lysates of APL patients independent of the bcr involved.

Figure 5. Results of the EuroFlow testing of the prototype PML-RARA immunobead assay. MFI values were plotted per patient group. The results of the RQ-PCR analyses are indicated with symbols (bcr1, bcr2, bcr3, and negative). The seemingly lower MFI values in APL patients with bcr1 breaks as compared to APL patients with bcr3 breaks did not reach statistical significance (Mann Whitney *U* test: p= 0.052). Further details about the PML-RARA positive samples are given in Figures 6-8.

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Figure 7. Comparison of PML-RARA fusion protein expression levels between paired BM and PB samples of 9 acute leukemias at diagnosis.

In all paired BM and PB samples of the same patient, fully comparable MFI values were observed. Remarkably, the MFI values of the BM and PB levels in the 6 APL cases

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Figure 8. Level of PML-RARA fusion protein expression versus time lapse in sample processing.

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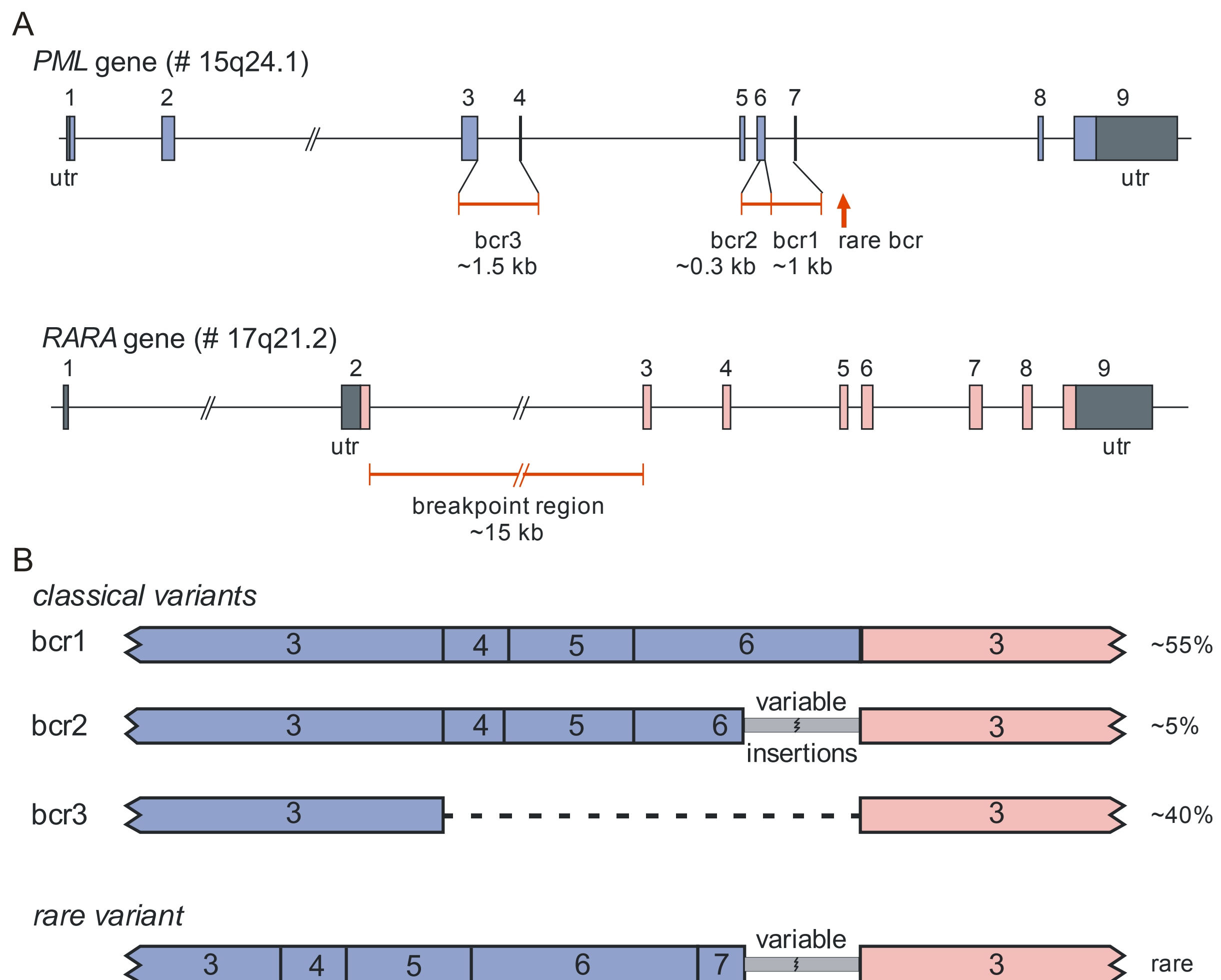
Figures



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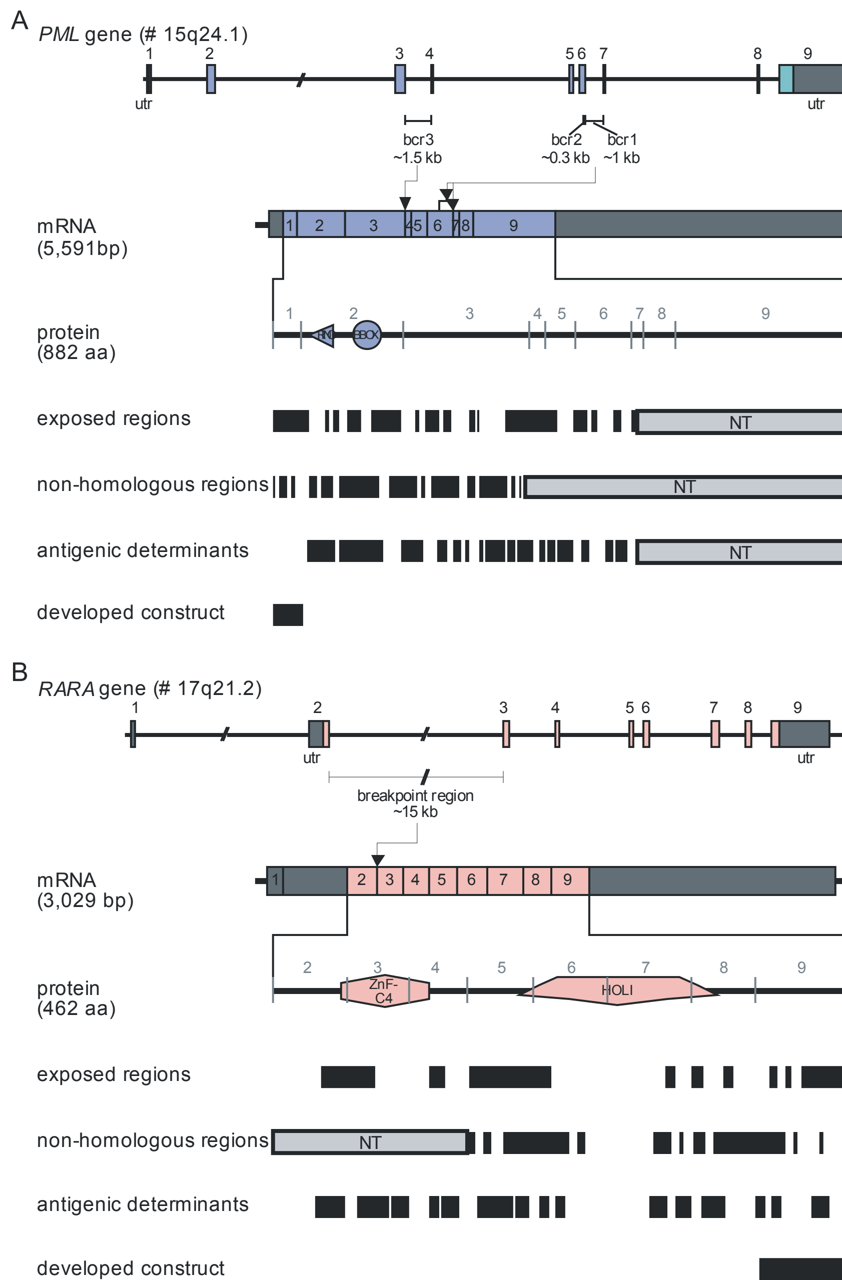

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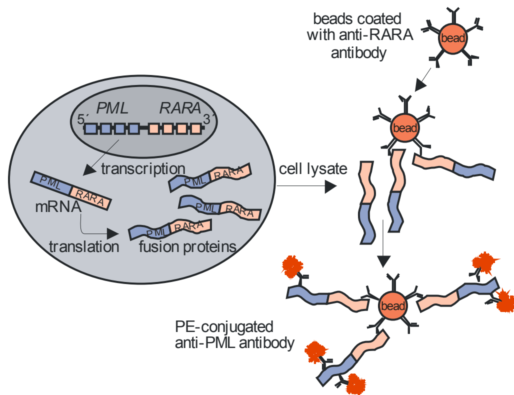

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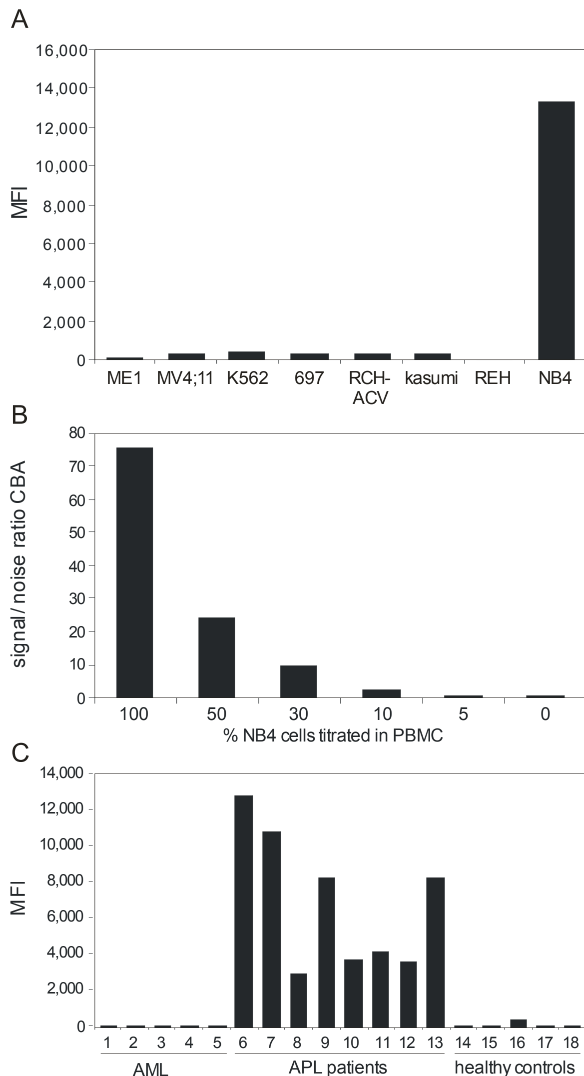

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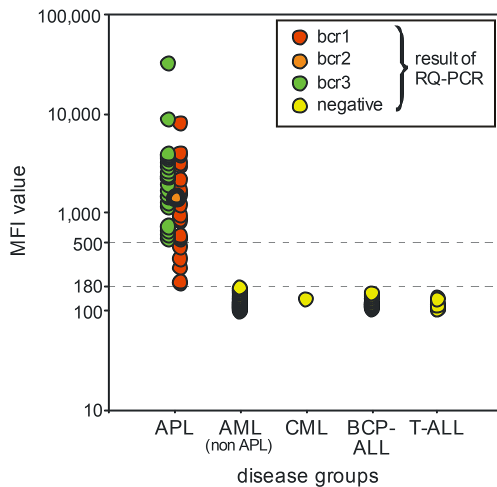

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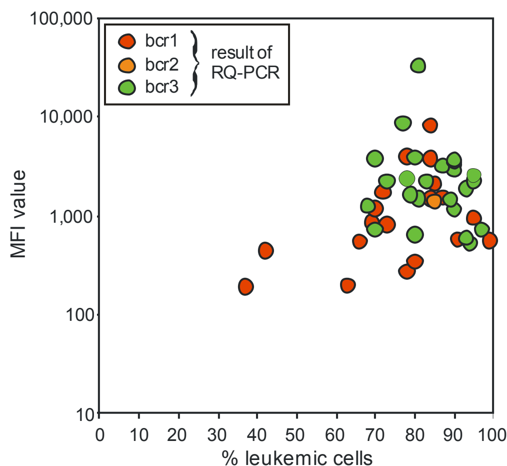

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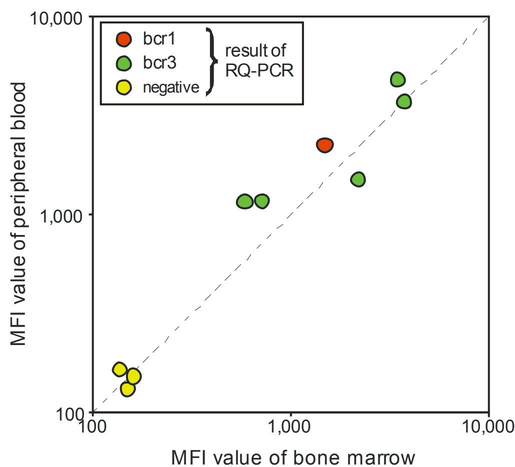

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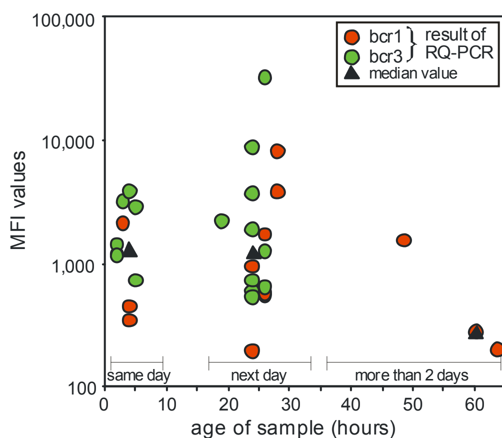

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Tables

Table 1. *RARA* fusion genes in APL

Chromosome aberration	Fusion gene	Relative frequency in APL
t(15;17) (q22;q21) (classical)	*PML-RARA*	~ 92%
Other 15q22 and 17q21 aberrations*	*PML-RARA**	~ 5%
t(11;17) (q23;q21)	*PLZF-RARA*	~ 1%
t(5;17) (q35;q21)	*NPM-RARA*	~ 0.5%
t(11;17) (q13;q21)	*NUMA-RARA*	rare
t(11;17) (q11;q21)	*STAT5B-RARA*	rare
complex der(17)	*PRKAR1A-RARA*	single case
no aberration detectable	no *RARA* gene rearrangement	~ 1%

- The non-classical 15q and 17q aberrations with PML-RARA fusion genes can be caused by several types of genetic aberrations, such as insertions and complex translocations.[2-4]

Table 2. Results of PML-RARA detection by PCR and the immunobead assay performed in 163 patient samples.

Newly diagnosed patients	No. of patients	PML-RARA mRNA positive	PML-RARA immunobead assay						
			positive	MFI positive		MFI negative			
				median	range	median	range	mean	3SD
APL (AML-M3)	46	46	46	1514	192-32.160	NA	NA	NA	NA
AML, non-APL	66	0	0	NA	NA	114	100-173	121	55
CML accelerated phase	1	0	0	NA	NA	131	NA	NA	NA
BCP-ALL	34	0	0	NA	NA	115	106-151	118	31
T-ALL	16	0	0	NA	NA	113	113-130	113	26
TOTAL	163	46	46	1514	192-32.160	114	100-173	119	46

NA= not applicable

Table 3. Results of PML-RARA detection by PCR and the novel immunobead assay in AML patients according to age, *PML* breakpoint and MFI value.

Leukemia type	**No. of samples**	**PML-RARA mRNA positivity**			**MFI of PML-RARA protein positivity**		
		bcr1	**bcr2**	**bcr3**	**low (180-500)**	**medium (500-1000)**	**high (>1000)**
APL							
- childhood	22	11	1	10	1	7	14
- adult	24	10	0	14	4	4	16
Other AML							
- childhood	18	0	0	0	0	0	0
- adult	48	0	0	0	0	0	0
TOTAL	112	21	1	24	5	11	30
		(46%)	(2%)	(52%)	(11%)	(24%)	(65%)