Published in final edited form as:

Cancer Genet. 2012 October ; 205(10): 488–500. doi:10.1016/j.cancergen.2012.06.006.

An optimized technology platform for the rapid multiplex molecular analysis of genetic alterations associated with leukemia

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Abstract

Molecular methods play a critical role in the accurate diagnosis of leukemia by complementing morphologic, cytochemical, immunophenotypic, and cytogenetic analyses. We developed a multiplex RT-PCR method combined with liquid bead array cytometry for the rapid detection of genetic alterations associated with leukemia. Fusion transcripts corresponding to the most common recurrent chromosomal translocations were reproducibly detected in as low as 0.1 to 10 ng of total RNA with an analytical sensitivity of 0.01 to 1%. Multi-day, -lot, -operator, and -instrument precision studies for a total of 678 independent measures in 46 runs showed a very high reproducibility with 100% agreement between replicates. Using multiplex panels for four to twenty independent targets, we demonstrate the flexibility of the method to co-detect rare splicing isoforms, discriminate between multiple variants generated by unique cytogenetic abnormalities, identify distinct chromosomal partners involved with 11q23 or 17q21 rearrangements, and assess cryptic abnormalities not detectable by standard cytogenetics such as t(12;21), del(1p32), or *NPM1* mutations. Overall, three different internal control transcripts and 34 variants resulting from eighteen abnormal chromosomal sites were evaluated. These results underscore the value of the multiplex assay system as a sensitive and reliable technology platform for the characterization of relevant genetic alterations in leukemia.

Keywords

leukemia; fusion transcript; multiplex; RT-PCR; molecular diagnosis

Introduction

The classification of myeloid neoplasms and acute leukemias in clinically independent risk groups entails the analysis of multiple genetic alterations that provide entity-defining, therapy-determining and/or prognosis-relevant information (1). In many cases, recurrent chromosomal translocations or gene mutations can be detected by molecular methods based on the polymerase chain reaction (PCR) method. Molecular identification of the corresponding RNA markers by conventional reverse transcription (RT) PCR approaches is

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Conflict of interest: WLW and EL are employees of Asuragen Inc. FY declares no conflict of interest.

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however often impractical for routine clinical testing. For example, individual amplification reactions for multiple targets followed by sequencing using the current gold standard dideoxynucleotide chain terminator method would be too time-consuming and costly. Real-time RT-PCR is very sensitive and quantitative but lacks sufficient multiplexing capabilities. It can be labor-intensive, costly, and time-consuming when separate reactions have to be performed to screen for individual transcripts. For these reasons, real-time RT-PCR is better suited for applications such as monitoring of minimal residual disease after the identity of the specific genetic alterations and corresponding transcripts has been identified by other methods (2).

Several applications of multiplex RT-PCR in combination with regular gel-based detection have been reported (3,4). These methods showed appropriate performance but may not be attuned to the clinical setting due to the low throughput of the downstream procedure for signal detection and analysis. Gel-based or capillary electrophoresis methods are capable of differentially detecting multiple RT-PCR products (3–5). However, as the number of targets in the reaction increases, it becomes increasingly difficult to identify the amplicons based on size alone. Furthermore, the presence of potential non-specific PCR products can interfere with fragment size analysis and data interpretation. An additional confirmation of the PCR product sequences, using for example a probe hybridization approach, can further increase the confidence in results. The combination of RT-PCR and microarray detection has been shown to offer the desired specificity and sensitivity for expression profiling and screening of fusion genes in leukemia (5,6). Microarray-based platforms are powerful tools for discovering new biomarkers but owing to the high cost of consumables and relatively long turnaround time and low throughput, they may not be optimal for analyzing a large number of samples in the busy clinical laboratory setting.

Liquid bead array hybridization followed by flow cytometry detection may be better suited to the requirements of the diagnostic laboratory. This format has been generally applied to clinical analytes, including serologic, genetic, bacterial and viral targets using the CE-marked and FDA-cleared Luminex[®] 100 or 200 Systems (7–10). We took advantage of the 96-well plate format and automation capabilities of this platform to develop a rapid multiplex assay for the qualitative detection of twelve fusion transcripts resulting from seven chromosomal abnormalities commonly found in chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), or acute myeloid leukemia (AML). The assay was successfully evaluated with over 600 representative leukemia specimens in multiple laboratories highlighting its potential value as a complement to cytogenetic analyses (11,12). The goals of the present study were to expand the panel beyond the initial twelve targets, including for submicroscopic abnormalities that cannot be detected by classic cytogenetics, and to fully characterize the analytical sensitivity, specificity and precision of the assay system.

Materials and Methods

RNA specimens

Total RNA from the cultured cell lines BV173 (BCR-ABL1 e13a2), K562 (BCR-ABL1 e14a2), SupB15 (BCR-ABL1 e1a2), ME1 (CBFB-MYH11 type A), REH (ETV6-RUNX1 e5e2), MV4 (MLL-AFF1 e9e5), NB4 (PML-RARA L form), Kasumi1 (RUNX1-RUNX1T1 e5e2), 697 (TCF3-PBX1 e13e2), OCI-AML3 (NPM1 mutation A), and HL60 as a negative control was isolated with the RNeasy Mini Kit according to manufacturer's instructions for cultured cells (Qiagen, Hilden, Germany). All human specimens used in this study were residual total RNA samples isolated from peripheral blood or bone marrow specimens using laboratory-validated RNA extraction methods and acquired by Asuragen from various sources under approved investigational protocols. Specimens were de-identified and no

protected health information or other information identifying patients were released to Asuragen. Molecular testing results were compared to matching metaphase cytogenetic or interphase FISH data for research purpose only and were not reported to treating physicians or patients or used for treatment decision. Synthetic RNAs corresponding to specific fusion transcripts, splicing variants, or mutations were prepared using standard *in vitro* transcription methods and diluted in HL60 total RNA. Bi-directional sequencing using the BigDye[®] terminator method (ACGT Inc., Wheeling, IL, USA) was performed on every plasmids and cell line RNA and on specific residual clinical specimens to confirm the identity of the fusion transcript or mutation and the presence of the binding sites for the primers and probes used in the multiplex molecular assays. RNA concentration was determined in every sample using a NanoDrop ND1000 (NanoDrop Technologies, Waltham, MA, USA).

Multiplex assays

Multiplex reactions for twelve fusion transcripts and an endogenous control transcript (GAPDH) were performed in 96-well plates using the Signature[®] LTx v2.0 Kit (for research use only, not for use in diagnostic procedures, Asuragen Inc., Austin, TX, USA) as described in Gocke et al. (11). The protocol for the prototype expanded panels detecting six additional ALL splicing variants or nineteen different fusion transcripts was the same except that modified primer mixes and bead mixes were used at the PCR and hybridization steps, respectively. For the ten other prototype assays, the RT reactions were performed using up to 5 μ L of test sample and the Signature[®] RT Reagents (General Purpose Reagents, Asuragen Inc.) in a final volume of 20 μ L. Mixtures were directly incubated for 45 minutes at 42°C without heat-denaturation step. The resulting cDNA samples (5 μ L) were amplified by multiplex PCR using the Signature[®] DNA AMP Reagents (General Purpose Reagents, Asuragen Inc.), optimized panel-specific PCR primer mixes, and 2.5 units of AmpliTaq[®] Gold (Applied Biosystems, Carlsbad, CA, USA). Amplification reactions were performed in a final volume of 25 μ L with 40 to 45 cycles consisting of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec after an initial denaturation step at 94°C for 10 minutes. The PCR products (5 μ L) were then hybridized to optimized panel-specific bead mixes using the Signature[®] Hyb Reagents (General Purpose Reagents, Asuragen Inc.). Hybridization reactions were performed in a final volume of 50 μ L for 30 min at 52°C followed by addition of 25 μ L of Reporter Solution (24.5 μ L of Signature[®] Hyb Buffer plus 0.5 μ L of Signature[®] Conjugate). The PCR products bound to bead-conjugated probes were detected by flow cytometry using a Luminex[®] 100 or 200 System (Luminex Corp., Austin, TX, USA). The Median Fluorescent Intensity (MFI) detected by the Luminex System on at least fifty beads for each target-specific bead population was subsequently analyzed in Excel (Microsoft Corp., Redmond, WA, USA). A sample was called positive for a given target when the corresponding MFI signal was greater than a qualitative cut off set at 350 MFI. A no RNA control (Signature[®] Diluent) and a negative control (400 ng of HL60 total RNA) were included in every run. During the course of the study three different GeneAmp[®] PCR System 9700 (Applied Biosystems) were used for the RT, PCR and hybridization steps and three different Luminex[®] Systems were used for the detection step.

Primers and probes

To detect chromosomal targets not represented in commercially available panels, PCR primer and bead mixes were prepared using oligonucleotides (Biosearch Technologies, Novato, CA, USA) purified by reverse phase HPLC, diluted at 100 μ M in water and stored below -15°C. Probes carried a 5' amino-C12 modification, reverse PCR primers were 5'-biotinylated and forward PCR primers were unmodified. Primer mixes were formulated in water to obtain a final concentration of 10 to 35 nM of each primer per PCR. For carbodiimide coupling, 10E6 carboxylated beads (Luminex Corp) resuspended in 50 μ L of

0.1 M MES pH 4.5 were mixed with 200 pmol of 5' amine-modified oligonucleotides in the presence of 1 mg/mL of EDC for 15 minutes at room temperature in the dark. After two consecutive washes with 0.02% Tween-20 and 0.1% SDS, conjugated beads were resuspended in 200 μ L of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored in the dark at 2 to 8°C. Bead mixes were formulated in 2.25 M TMAC, 37.5 mM Tris, 3 mM EDTA, 0.075% Sarkosyl, pH 8.0 to obtain at least 2,000 of each spectrally distinct bead population per hybridization reaction. The target-specific sequences of the probes (-p), forward (-f) and reverse (-r) primers were: MLL-AF6-p1 CCAAAAGAAAAGGATTTGGAGTTC, MLL-AF9-p1 CCAAAAGAAAAGTCTGAACAACC, MLL-AF9-p2 GCATGTAGAGTCTGAACAACC, MLL-ELL-p1 TGGACTTTAAGGATTCTGTTTC, DEK-CAN-p1 GATTTGCAAAAAGGAAATTCGG, OTT-MAL-p1 ATTGTCCGTGCTTTGAAAAG, SIL-TAL-p1 TCAGCTCCGCGGAAGTTG, NUMA-RARA-p2 CCCCTCCAGCCATTGAGA, PLZF-RARA-p1 TGGCTCATTGAGCCATTGAG, PLZF-RARA-p2 AGACCCATACTGCCATTGAG, STAT5-RARA-p2 GGAAGTTTGATTCCCATTGAG, GUS-p1 TGAACAGTCACCGACGAGAG, MLL-f1 GTCCAGAGCAGAGCAAACAG, MLL-f2 GGAGGCTTAGGAATCTTGACTTC, DEK-f1 AAGTTGAAGAAACCCCTACAGA, OTT-f1 AGCAGTTCCTGGATTCCCCT, SIL-f1 CGCTCCTACCCTGCAAACA, NUMA-f1 GGTTCTCCCGATTATGGCAAC, PLZF-f1 GCTTCCTGATAACGAGGCTGTG, PLZF-f3 AAAGCCTTTGTCTGTGATCAGT, STAT5-f1 CAGGCCCATGACCTACTCATTA, GUS-f2 CACCAGGATCCACCTCTGAT, AF6-r1 GTTGCAAAGTTTCCAGCAGCT, AF9-r1 CCACTTCATCTGATTCTCTCTC, AF9-r2 CCACTTCATCTGATTCTCTCTC, ELL-r1 GAGATGTGCCCTTGGCTTC, CAN-r1 CATCATTCACATCTTGGACAGCA, MAL-r1 TGTGCATCCTGACCAGCTC, TAL-r1 CCGAGGAAGAGGATGCACA, RARA-r2 GCTTGTAGATGCGGGGTAGA, GUS-r2 CCTGGTTTCATTGGCAATCTTC.

Experimental design

For input range studies, total RNA samples were prepared by ten-fold serial dilution in Signature® Diluent. For analytical sensitivity studies, total RNA samples were prepared by ten-fold serial dilution in a background of HL60 total RNA, aliquoted and stored below -15°C until testing. When cell lines were not available, synthetic transcripts prepared by in vitro transcription were diluted at 5.E3 copies per ng of HL60 total RNA, aliquoted and stored below -15°C. This concentration corresponds to a very conservative estimate for a transcript expressed at low level in white blood cells (five copies per cell) with a RNA extraction yield of about 1 pg of total RNA per cell (1,000 white blood cells per ng of purified RNA). Synthetic transcripts were then further diluted and tested like the cultured cell line samples. For example, 400 ng of undiluted material contained two million copies of target transcript and a 1% dilution sample (4 ng in 400 ng of HL60 total RNA) contained 2.E4 copies. For the sub-panels precision studies total RNA purified from eight translocation-positive cell lines was used either undiluted (100%) or diluted at 0.1% in HL60 total RNA (400 ng input). For the five other targets (CBFB-MYH11 types D and E, PML-RARA L, S and V forms) the samples consisted of 2.E6 or 2.E3 copies of the corresponding synthetic transcripts spiked in 400 ng of HL60 total RNA. BCR-ABL1 (three targets) or ETV6-RUNX1, MLL-AFF1 and TCF3-PBX1 samples were tested in triplicate in four runs and in singleton in eight runs (twelve runs total, twenty replicates per sample) by four operators using three thermal cyclers, three flow cytometers and the CML or ALL prototype sub-panels. CBFB-MYH11 (three targets) and RUNX1-RUNX1T1 or PML-RARA (three targets) samples were tested in triplicate in two runs and in singleton in six runs (eight runs total, twelve replicates per sample) by three operators using three thermal cyclers, two flow cytometers and the AML or APL prototype sub-panels.

Data analysis

For all experiments, qualitative positive or negative calls were determined for each of the probes relative to a single cut off value set at 350 MFI. Percent agreement between methods was calculated by comparing the qualitative positive or negative calls obtained with the multiplex assays at the probe or sample level against the known presence or absence of each target in each sample. Quantitative analyses of MFI signals at the probe level were performed in Excel (Microsoft Corp.). In the LTx input study the GAPDH probe signal was the average MFI generated by the eight cell line samples in three runs at each input level. The average negative signal (Neg.) was calculated using all negative probe signals at all inputs combined (seven samples x four input levels x three runs) for the fusion transcript-specific probes or using the “no RNA control” reactions for the GAPDH probe. In the LTx sensitivity study the average negative signal (Neg.) was calculated for all negative probe signals at all dilution levels combined (eleven samples x three dilution levels x three runs).

Results

Validation of a 12-target multiplex assay

The leukemia translocation panel (LTx) was developed and optimized to detect ten different fusion transcripts on ten distinct beads, two variants resulting from t(4;11) on a single bead and GAPDH transcripts as an internal endogenous control on an independent bead (Table 1, LTx column). The twelve targets (plus one control) were assayed in a single reaction. The multiplex assay was first validated by testing various inputs of total RNA purified from eight well-characterized translocation-positive leukemic cell lines in three independent runs (Figure 1A). A dose-response was observed for the output Median Fluorescence Intensity (MFI) signals between 1 and 1,000 ng of total RNA with signal saturation above 100 ng input for all targets including GAPDH. The expected fusion transcripts were specifically detected above the qualitative positive/negative cut off value (350 MFI) in every sample except for BCR-ABL1 e1a2 and ETV6-RUNX1 at 1 ng input. The background signals were low at all inputs (see Figure 1B for the ME1 cell line and Supplemental Table 1 for all samples) with negative probe signals ranging from 74 to 105 MFI on average (Figure 1A).

To further validate the assay analytical sensitivity we next tested in three independent runs the eight cell line RNA serially diluted in a background of total RNA from the translocation-negative cell line HL60 and keeping the input constant at 400 ng per RT reaction (Figure 1C). For the two PML-RARA and the CBFB-MYH11 type D targets we used *in vitro* transcribed synthetic transcripts spiked in HL60 total RNA to mimic a sample purified from translocation-positive leukemic cells (see Materials and Methods). The expected fusion transcripts were reproducibly detected at 1% dilution or 2.E4 copies of target per RT reaction with a signal on average at least two-fold above the qualitative cut off (Figure 1C). The signal to noise ratio was high with negative probe signals ranging from 56 to 87 MFI on average. Raw MFI data for all probes with the MV4 cell line (MLL-AFF1 e9e5) and the PML-RARA bcr3 (S form) synthetic transcript are shown in Figure 1B and representative examples for all samples are shown in Supplemental Table 1.

Establishment of multiplex sub-panels with additional transcript variants

Many of the balanced chromosomal translocations included in the WHO classification of acute leukemia are characterized by multiple genomic breakpoints and/or multiple alternative splicing isoforms. In ALL, t(4;11), t(12;21) and t(1;19) all generate variants at the transcript level (2,13–15). We developed an optimized eleven-target multiplex assay containing the five ALL fusion transcripts from the LTx panel and six additional variants relevant in infant, pediatric or adult ALL (Table 1, column ALL). BCR-ABL1 e1a2 and two ETV6-RUNX1, six MLL-AFF1, or two TCF3-PBX1 variants were specifically detected on

four individual beads using cell line RNA and synthetic transcripts (Figure 2A). Serial dilutions of total RNA from translocation-positive cell lines demonstrated an analytical sensitivity of 0.1% at 400 ng input for each bead (Figure 2B). Repeat testing in three independent runs of synthetic transcripts serially diluted in HL60 total RNA further showed that each target was reproducibly detected at 2.E3 copies per RT reaction (Supplemental Figure 1A). This assay design for increased coverage of ALL variants was also successfully implemented in two other multiplex prototype assays: a six-bead, thirteen-target assay including detection of BCR-ABL1 e13a2 and e14a2 on two additional beads and in an eleven-bead, eighteen-target assay for the twelve LTx panel targets with co-detection of the six additional ALL variants on the ETV6-RUNX1-, MLL-AFF1- or TCF3-PBX1-specific beads (Supplemental Figures 1B and 1C).

Following the same rationale, a seven-bead assay focused on targets relevant in AML was developed. In addition to the five targets present in the LTx panel, the assay specifically detected the rarer CBFB-MYH11 type E and PML-RARA bcr2 variants on two distinct beads (Figure 3A). Analytical experiments with translocation-positive cell lines and synthetic transcripts confirmed that the multiplex assay also reached 0.1% sensitivity for each bead (Figure 3B) and reproducibly detected at least 2.E3 copies of each transcript in 3 independent runs (Supplemental Figure 2). Additional experiments (Supplemental Figure 3) demonstrated that the panels could be further split in sub-panels each containing only three to four beads and focusing exclusively on fusion transcripts relevant in CML (Table 1, column CML), ALL, AML or acute promyelocytic leukemia (APL). Overall, each fusion transcript was specifically detected in as low as 0.1 to 0.4 ng of total RNA with a corresponding analytical sensitivity of 0.01% for RUNX1-RUNX1T1 and 0.1% for the other targets (Table 3 and Supplemental Figures 1, 2 and 3).

Analytical precision studies

In the analytical characterization experiments described above singleton testing in independent runs generated the same qualitative positive/negative assay results, suggesting good method precision. To validate both the within-run (repeatability) and between-run (reproducibility) precision of the twelve-target LTx panel a study was performed using three independent lots of reagents each tested in two independent runs by two operators. In each run the three controls provided with the reagents and twelve different positive samples consisting of 1.E5 copies of synthetic transcript spiked in a background of 400 ng of HL60 total RNA were tested in triplicate resulting in 270 independent measures overall (Table 2). There was 100% qualitative agreement between replicates and between runs. At the probe level, a total of 2,970 individual results were generated during the study (fifteen samples x three replicates x six runs x eleven multiplexed assays). Each probe generated the expected positive or negative signal relative to the 350 MFI cut off with a high signal to noise ratio (see representative examples in Supplemental Table 1). The average signal for the 414 positive probe results was 3,764 MFI and was 102 MFI for the 2,556 negative probe results with 95% of the negative signals below 177 MFI (data not shown).

To further evaluate the precision of the technology platform, repeat tests were also systematically performed with the prototype sub-panels at both high and low levels of target transcripts (see Materials and Methods). In eight to twelve independent runs using a minimum of three operators, three thermal cyclers and two flow cytometers for each sub-panel there was 100% agreement between replicates and between runs for a total of 408 independent measures (Table 3). Quantitative analysis at the probe level for the thirteen different positive samples tested at high or low levels showed that the target-specific MFI distributions were consistent with the initial sensitivity experiments (Figure 4). The median values ranged from 5,726 to 8,218 MFI at high input with 95% of the signals within 5,280 and 8,710 MFI. Importantly, 100% of the positive signals were above the cut off at low

target level with 95% of the signals above 590 MFI and the lowest individual probe signals obtained for BCR-ABL1 e1a2, ETV6-RUNX1 or PML-RARA bcr3 at 410, 494 and 492 MFI, respectively (data not shown).

Establishment of multiplex panels with additional chromosomal targets

In addition to balanced chromosomal translocations, submicroscopic genetic abnormalities not detectable by standard or molecular cytogenetics, such as *NPM1* mutations, can also provide information paramount for the classification of leukemia (1). The multiplex assay system was further optimized to detect *NPM1* mutant transcripts and relevant AML fusion transcripts in a single reaction. RUNX1-RUNX1T1, three CBFB-MYH11 variants and the three most common *NPM1* mutations (A, B and D) were specifically detected on five individual beads using cell line RNA and synthetic transcripts (Figure 5A). With this assay the *NPM1* mutant transcripts were detected on a single bead and either GAPDH or *NPM1* wild-type transcripts were used as internal endogenous controls (Supplemental Figure 4A). Serial dilutions and repeat testing in three independent runs demonstrated an analytical sensitivity of 0.1% at 400 ng input for both mutant and fusion transcripts (Supplemental Figure 4B). Synthetic transcripts were reproducibly detected at 2.E3 copies per RT reaction including with an eight-bead assay that detected three additional PML-RARA variants in the same reaction (Table 1, column AML and Supplemental Figure 4C).

RARA at 17q21 can be rearranged with several chromosomal partners other than *PML* at 15q24 (16). A seven-bead multiplex assay was also developed to distinguish between the fusion transcripts generated by translocations involving *RARA* and *PML*, *NUMA1*, *STAT5B*, or *ZBTB16* (Table 1, column APL). With this assay target transcripts were specifically detected on six beads, the *RARA* exon 3 bead further confirmed the presence of any *RARA*-containing transcripts and GUS transcripts were amplified and detected on an independent bead to serve as an internal endogenous control (Figure 5B). Similarly, *MLL* at 11q23 has many translocation gene partners (17) and several of these molecular variants were included in a large nineteen-target multiplex assay format (Table 1, column Exp). Each of the twelve targets from the LTx panel was specifically detected with the addition of four beads to identify MLL-MLLT4, MLL-ELL and two MLL-MLLT3 variants (Figure 5C). The expanded panel also included three additional beads to detect RBM15-MKL1 and DEK-NUP214, two fusion transcripts relevant in AML, and SIL-TAL1, a fusion transcript resulting from a cytogenetically cryptic deletion in T-ALL (Table 1, column Exp and Figure 5C). Overall, the technology platform was shown to be compatible with 34 distinct transcript variants in a flexible multiplex assay format for three to nineteen targets plus an endogenous control (Table 1).

Evaluation of representative clinical specimens

To assess the compatibility of the multiplex technology with specimens commonly encountered in the clinical setting, four disease-focused panels were each evaluated with 20 independent residual total RNA samples from CML, ALL, AML or APL cases at presentation (Figure 6). The qualitative molecular results (positive or negative for a given target) were compared to cytogenetic data for the fusion transcripts or to bi-directional sequencing for the presence or absence of *NPM1* mutations in AML specimens with normal karyotype. For each sub-panel a perfect positive agreement was observed between the molecular assays and the reference methods (Figure 6E). One specimen negative for t(8;21) by cytogenetic was found positive for RUNX1/RUNX1T1 with the AML panel (AML-18 with a relatively low positive signal at 2,434 MFI, Figure 6D). This specimen was likely below the limit the detection of cytogenetic or carried a cryptic translocation as it was confirmed positive by bi-directional sequencing (data not shown). The corresponding overall agreement between the multiplex assays and the reference methods was 98.8% (79/80, 95%

confidence intervals: 93.3 to 99.8%). At the probe level, the 56 positive MFI signals were all robust and at least 3.6-fold above the cut off with a mean of 6,234 MFI. The mean and standard deviation for the 229 negative probe signals were 77 and 41 MFI respectively, confirming that the high signal to noise of the technology is appropriate for the accurate qualitative analysis of relevant genetic alterations in leukemia specimens.

Discussion

The qualitative assay system described in the present study involves multiplex RT-PCR amplification, capture of the fluorescent PCR products onto bead-probe complexes in solution and rapid bead sorting and fluorescence analysis on a flow cytometer. By optimizing every step in the procedure we have developed specific and sensitive assays compatible with the workflow of the molecular laboratory. The procedure is streamlined with a five to six hour turnaround time and a 96-well plate format amenable to high-throughput configuration and automation. Data analysis is also simple and rapid, relative to a single qualitative cut off value, further reducing operator and total assay time. The system integrates external plate/run controls, such as a negative control and a pool control positive for every target (see examples in Supplemental Table 1), to assess the validity of the RT, PCR, hybridization and detection steps in every run. Co-amplification and co-detection of an internal endogenous transcript, such as GAPDH, NPM1 or GUS, further controls for the entire process in every tested sample, including RNA quality and quantity, presence of residual inhibitors, or protocol errors, and ultimately protects from potential false negative results.

To increase confidence in positive results the assay system was designed to incorporate specificity at both the PCR amplification and probe hybridization steps. Most fusion transcripts can be detected using a pair of exonic primers flanking the genomic breakpoints and a probe specific for the exon junctions (Supplemental Figure 5). This design facilitates the careful selection of primer sequences with similar melting temperatures. This requirement, essential for efficient multiplex PCR, cannot always be fulfilled with primers targeting exon junctions. In contrast, the probes sequence content is less critical and can be restricted to the few nucleotides around the exon junction as amplicon detection is performed under conditions optimized for rapid solution hybridization kinetics (less than 30 minutes) independently of the melting temperature (18). To further distinguish between fusion transcript variants, a single forward or reverse primer can be combined with multiple complementary primers and probes specific for different exon junctions (Strategy #1). For example, the three BCR-ABL1 variants are amplified with a single *ABL1* exon a2 reverse primer and CBFB-MYH11 A and D with a single *CBFB* exon 5 forward primer. In certain cases, when distinguishing between multiple variants does not provide additional information or when one of the variants is extremely rare (e.g., MLL-AFF1), a common exonic probe can be used in combination with multiple primers targeting each exon (Strategy #2). If one of the variants contains a small exon that does not significantly impact amplicon length and PCR efficiency, which is the case for ETV6-RUNX1 e5e2 or TCF3-PBX1 e13e2i27, a single primer pair can be used. Finally, for complex chromosomal rearrangements resulting in unknown junction sequences, both strategies can be combined (Strategy #3). For example, PML-RARA bcr2, also called V form, is characterized by the inclusion of a variable number of *RARA* intron 2-derived nucleotides (2,19). The presence of this variant can be revealed by a unique signal on a probe specific for *RARA* exon 3 with the absence of signal on two probes targeting the bcr1 or bcr3 exon junctions (Figure 3A).

Analytical specificity was verified for every target with well-characterized cell line or synthetic samples and analytical sensitivity was validated through repeat testing of dilution series. By optimizing primers concentration and probes/amplicons length, every target in the

original leukemia translocation panel was detected at about 1% cell line dilution or 2.E4 copies per RT reaction (Figure 1). This level of sensitivity, equivalent to the detection of one leukemia cell in a background of 100 normal cells, is superior to current clinical reference methods such as fluorescence in situ hybridization, Sanger sequencing or karyotyping (5 to 20%) and was shown to be adequate for the characterization of leukemia samples at diagnosis (11,12). Using an optimized RT system and PCR buffer (see Materials and Method) we demonstrated that the multiplex assay system can be further enhanced to reach 0.1 to 0.01% dilution or 2.E3 copies per RT reaction. Using an insertion-specific assay design (Supplemental Figure 5, Strategy #4), NPM1 mutant transcripts were also detected at low levels even in the presence of a large excess of competing wild-type transcripts expressed in both normal and leukemia cells (20–22). Following appropriate clinical validation, this improved analytical sensitivity could extend the utility of the technology platform to other applications such as semi-quantitative monitoring of treatment response beyond cytogenetic remission or earlier qualitative detection of molecular relapse (11,21).

Repeat testing with cell line or synthetic samples also validated the repeatability and reproducibility of the method. Six runs for a total of 270 replicates were performed with the LTx panel and forty runs for a total of 408 replicates with the focused sub-panels (Tables 2 and 3). Thus, 678 independent measures in 46 runs were obtained during the course of the precision studies with 100% qualitative agreement between replicates. Although the assays output is a qualitative positive or negative call, quantitative analyses further demonstrated the high signal to noise ratio of the technology and the validity of the unique cut off set at 350 MFI (Figure 4). The average positive signal was 6,551 MFI for high positive samples (undiluted cell line RNA or 2.E6 copies) and 1,789 MFI for low positive samples (0.1% dilution or 2.E3 copies), that is 18.7- or 5.1-fold above the cut off, respectively. With the LTx panel, the average positive signal in mid-level samples (1.E5 copies) was 10.8-fold above the cut off (3,764 MFI). The average negative probe signal in these experiments was 102 MFI with a maximum at 273 MFI and 95% of the signals between 0 and 177 MFI (data not shown). These results are in agreement with an independent limit of blank study showing that 95% of the signals generated in known negative samples are at least two-fold below 350 MFI for each probe (11).

The leukemia translocation panel was initially designed to target seven genetic abnormalities defining clinically relevant leukemia entities of myeloid or lymphoblastic lineages (Table 1, column LTx). t(9;22), t(12;21), t(1;19), t(4;11), t(8;21), inv(16) and t(15;17) all play a critical role in the accurate risk-based stratification of CML, ALL or AML (1). In this study we demonstrated that the multiplex assay system can be further optimized to co-detect rare transcript variants and identify additional chromosomal targets. One of the expanded panels included fusion transcripts resulting from thirteen different well-characterized and clinically important cytogenetic abnormalities (Table 1, column Exp). t(4;11) and the three additional translocations involving *MLL* at 11q23, t(6;11), t(11;19) and t(9;11), are all relevant in acute leukemia where they are found at different frequencies in adult and pediatric AML or ALL (17). t(9;11) *MLL*-*MLLT3* also defines an independent AML entity in the 2008 WHO classification (1). Similarly, t(1;22) *RBM15*-*MKL1*, found in one third of the *de novo* megakaryocytic AML, and t(6;9) *DEK*-*NUP214*, found in up to 5% of adult AML, both characterize independent entities with distinct morphologic and clinical features (1,23,24).

Although in theory all 34 target transcripts evaluated here could be further combined in a single-well reaction assay, such a large panel would be extremely difficult to validate for every target and every disease entity. Sub-panels focused on specific leukemia classes and with an optimal molecular coverage for increased clinical sensitivity may be more practical. In ALL for example, the variants *ETV6*-*RUNX1* e5e3 and *TCF3*-*PBX1* e13e2i27 are generated by alternative splicing in 5 to 10% of the t(12;21)- or t(1;19)-positive cases

(14,15). In addition, specific variants can have a different prevalence in distinct populations. For example, the t(4;11) breakpoint occurs most frequently in *MLL* introns 9 or 10 in pediatric and adult ALL and in intron 11 in infant ALL (13). As a consequence, *MLL*-*AFF1* fusion transcripts involving exons 9 or 10 are present in about 85% of adult and pediatric patients while exon 11-containing variants are predominantly found in infants (2). Thus, a multiplex molecular assay including ten variants from these three translocations (Table 1, column ALL), plus *BCR*-*ABL1* found in 65% of adults and 80% of children with t(9;22)-positive ALL, would cover a range of genetic alterations associated with an unfavorable to very good prognosis and relevant in the infant, pediatric and adult ALL populations (1,2,13–15,25).

In AML, unique entity-defining chromosomal translocations associated with a favorable prognosis can also generate multiple variants at the transcript level. For example, *CBFB*-*MYH11* type E and *PML*-*RARA* bcr2 are two relatively rare variants found in about 5% of inv(16)- or t(15;17)-positive AML (19,26). Mutations in the *NPM1* gene also define a provisional entity associated with favorable prognosis in the 2008 WHO classification of myeloid neoplasms and are observed in 50 to 60% of cytogenetically normal *de novo* AML (1,20,22). Thus, a single multiplex molecular assay for ten fusion or *NPM1* mutant transcripts (Table 1, column AML) would cover the most common genetic alterations that define a favorable risk group in AML. Among these targets, t(15;17) *PML*-*RARA* is associated with specific morphologic and clinical features and is in general sufficient for the diagnosis of greater than 95% of APL regardless of the blast count (1). *RARA* at 17q21 can however be rearranged with several other chromosomal sites that correlate with different levels of responsiveness to *all-trans* retinoic acid treatment (1,16). A sub-panel focusing on *RARA* molecular partners (Table 1, column APL) would further facilitate the identification of these specific APL sub-classes.

Many of the genetic alterations used to assess risk and classify leukemia can be challenging to detect by cytogenetic analyses. In some cases, such as t(6;9) *DEK*-*NUP214*, the abnormality is so subtle that it can be missed by conventional karyotyping (24). In other cases, the chromosomal rearrangement involves regions with similar banding patterns and is virtually undetectable. For example, t(12;21) is rarely identified by routine cytogenetics, yet the corresponding fusion transcript *ETV6*-*RUNX1* is found in 20 to 30% of pediatric ALL, and *SIL*-*TAL1*, a fusion transcript resulting from a cytogenetically cryptic interstitial deletion at 1p32, is commonly found in childhood T-cell ALL (27,28). Likewise, gene mutations, such as small four-nucleotide long insertions in *NPM1* exon 12 at 5q35, cannot be detected by standard or molecular cytogenetics due to their submicroscopic nature. The fact that these frequent genetic alterations are reliably identified by the multiplex assay system further stresses the complementary role of cytogenetic and molecular analyses in the clinical assessment of leukemia.

For routine use in the clinical setting, novel molecular methods must be compatible with the workflow of the molecular laboratory and their clinical accuracy must be validated against other well-characterized cytogenetic and molecular methods. By evaluating eighty representative clinical specimens we observed 98.8% agreement between such methods and disease-focused sub-panels (Figure 6). In a separate blinded multicenter retrospective study designed to fully assess the many sources of pre-analytical, analytical, and clinical variability associated with different specimen collection and testing sites, an overall agreement of 98.6 % was reported for the LTx panel using 280 adult and pediatric acute or chronic leukemia specimens (11). An independent prospective comparator study on 330 adult acute leukemia at presentation also demonstrated 100% clinical sensitivity for this panel and highlighted the potential high frequency of false negative results with standard cytogenetics (12). The six-bead, thirteen-target ALL prototype panel shown in Supplemental

Figure 1B was also evaluated in the clinical setting with sixty representative archived total RNA samples resulting in 100% agreement relative to cytogenetic methods or independent singleplex, laboratory-validated molecular tests (data not show). The results from these studies, combined with the analytical validation experiments presented here for thirteen different panels, demonstrate the sensitivity, precision and accuracy of the method. We conclude that multiplex RT-PCR followed by liquid bead array detection is a general technology platform suitable for the flexible analysis of clinically actionable genetic alterations in leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Bernard Andruss for critical review of the manuscript and Kerri Poe, Zhe Yang and Mari Anguiano for their contributions during the early development of the multiplex platform technology. This work was supported in part by grants R43CA115129, R44CA115129, R43CA130501 and R44CA130501 from the National Cancer Institute to FY and EL.

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Figure 1.

Analytical characterization of the twelve-target multiplex assay. (A) Input range study with eight different translocation-positive cell lines tested in three independent runs at 1, 10, 100 or 1,000 ng input. The graph shows the average MFI signals generated by each positive probe at each input level relative to the 350 MFI cut off value (dash line). Neg.=average negative probe signal. (B) Representative examples of assay output (MFI) at various inputs of total RNA from the ME1 cell line and at various dilution levels of total RNA from the MV4 cell line or synthetic PML-RARA S transcripts. Target-specific positive signals above the qualitative cut off (350 MFI) are highlighted. (C) Analytical sensitivity study with eight different translocation-positive cell lines and three synthetic transcripts (CBFB-MYH11 type D, PML-RARA L and S forms) tested in three independent runs either undiluted (100%) or diluted at 10 or 1% in a background of total RNA isolated from the translocation-negative cell line HL60 at a final input of 400 ng per RT reaction. The graph shows the average MFI signals generated by each positive probe at each dilution level relative to the 350 MFI cut off value (dash line). Neg.=average negative probe signal.

A

	BCR- ABL1	ETV6- RUNX1	MLL- AFF1	TCF3- PBX1	GAPDH
HL60 400 ng	52	0	29	24	5032
No RNA Control	40	20	81	0	92
SupB15 400 ng	5726	22	6	87	4556
REH 400 ng	56	5822	42	45	4749
HL60 + 2.E6 ETV6-RUNX1 e5e3	50	6630	7	21	4582
MV4 400 ng	40	52	6556	35	4594
HL60 + 2.E6 MLL-AFF1 e9e4	96	4	5252	71	4631
HL60 + 2.E6 MLL-AFF1 e10e5	73	27	7449	64	4805
HL60 + 2.E6 MLL-AFF1 e10e4	52	61	5142	78	4510
HL60 + 2.E6 MLL-AFF1 e11e5	58	37	5889	41	4762
HL60 + 2.E6 MLL-AFF1 e11e4	50	34	4898	91	4983
697 400 ng	74	49	25	6323	4687
HL60 + 2.E6 TCF3-PBX1 e13e2i27	17	40	123	5065	4981

Figure 2. Sub-panel with additional ALL variants. (A) Representative example of results (MFI) with the indicated cell line RNA and synthetic transcripts using a prototype multiplex assay co-detecting eleven different fusion transcripts on four beads. Target-specific positive signals above the qualitative cut off (350 MFI) are highlighted. (B) Evaluation of analytical sensitivity. Total RNA purified from translocation-positive cell lines was tested either undiluted (100%) or diluted at 10, 1% or 0.1% in a background of total RNA isolated from the translocation-negative cell line HL60 at a final input of 400 ng per RT reaction. The graph shows only the MFI signals generated by the target-specific and GAPDH endogenous control probes relative to the 350 MFI cut off value (dash line).

A

	CBFB-MYH11			PML-RARA		RARA	RUNX1-	GAPDH
	Type A	Type D	Type E	L form	S form	Exon 3	RUNX1T1	
HL60 400 ng	45	69	43	42	29	48	28	5074
No RNA Control	53	71	88	65	50	44	67	44
ME1 400 ng	6041	75	58	84	45	44	51	4785
HL60 + 2.E6 CBFB-MYH11 B	39	6217	34	35	71	67	31	5014
HL60 + 2.E6 CBFB-MYH11 E	27	78	7287	45	51	55	52	4593
NB4 400 ng	49	59	57	5671	103	6254	52	4650
HL60 + 2.E6 PML-RARA S	28	30	43	36	5964	5813	28	5138
HL60 + 2.E6 PML-RARA V	60	52	64	42	42	6577	23	4964
Kasumi1 400 ng	78	8	45	30	44	56	7185	4656

Figure 3.

Sub-panel with additional AML variants. (A) Representative example of results (MFI) with the indicated cell line RNA and synthetic transcripts using a prototype multiplex assay discriminating between seven different fusion transcripts on seven beads. Target-specific positive signals above the qualitative cut off (350 MFI) are highlighted. The probe specific for *RARA* exon 3 detects all three forms of PML-RARA transcript. (B) Evaluation of analytical sensitivity. Total RNA purified from translocation-positive cell lines or synthetic fusion transcripts (PML-RARA L, S and V forms) were tested either undiluted (100%) or diluted at 10, 1% or 0.1% in a background of total RNA isolated from the translocation-negative cell line HL60 at a final input of 400 ng per RT reaction. The graph shows only the MFI signals generated by the target-specific and GAPDH endogenous control probes relative to the 350 MFI cut off value (dash line).

Figure 4.

Quantitative analysis of precision studies. The graph shows the distribution of the MFI signals generated by repetitively testing the high (100% of translocation-positive cell line RNA at 400 ng input or 2.E6 copies of synthetic transcript in 400 ng of HL60 total RNA) and low samples (0.1% dilution or 2.E3 copies of synthetic transcript in 400 ng of HL60 total RNA) for each of the thirteen probes in the sub-panels. The whiskers represent 100% of the signal distributions (minimum and maximum values), the boxes contain 50% of the signal distributions (25th to 75th percentile values), and the median values are represented by horizontal lines within the boxes. All signals were above the qualitative cut off set at 350 MFI (dash line).

A	CBFB-MYH11						NPM1		RUNX1		NPM1 WT	
	Type A	Type D	Type E	Mut	Mut	WT	Mut	Mut	Mut	Mut	WT	WT
HL60 400ng	23	43	43	37	43	8281						
No RNA Control	48	20	18	0	31	13						
ME1 400 ng	6579	100	78	52	45	7029						
OC43-AML3 400 ng	9	27	66	94	56	7226						
HL60 + 2.E6 NPM1 Mut B	57	36	56	7838	26	5852						
HL60 + 2.E6 NPM1 Mut D	0	47	40	8943	53	7317						
Kasumi1 400ng	72	104	62	9	8307	4956						

B	NUMA1-RARA		PML-RARA		RARA		STAT3B-RARA		ZBTB16-RARA		ZBTB16-RARA B		GUS	
	L form	S form	L form	S form	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3	Exon 3
HL60 400 ng	93	57	94	58	62	42	106	5430						
No RNA Control	83	45	99	99	67	51	143	56						
HL60 + 2.E6 NUMA1-RARA	4885	57	63	6097	11	113	4350							
NBM 400 ng	127	6178	105	7332	48	60	102	5091						
HL60 + 2.E6 PML-RARA S	116	80	7406	7110	64	125	101	5213						
HL60 + 2.E6 STAT3B-RARA	45	39	78	6677	6252	87	46	5028						
HL60 + 2.E6 ZBTB16-RARA A	117	4	72	7368	20	5175	91	4437						
HL60 + 2.E6 ZBTB16-RARA B	60	96	99	6759	64	76	5434	4862						

C	BCR-ABL1			CBFB-MYH11 A			ETV6-RUNX1		MLL-AFF1		PML-RARA		RUNX1-T1		TCF3-PBX1		GAPDH		DEK-NUP214		MLL-ELL		MLL-MLLT3 e11e9		MLL-MLLT4		RBM15-MKL1		SIL-TAL1	
	e13a2	e14a2	e1a2	Type A	Type D	Type E	Mut	Mut	Mut	Mut	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form	L form	S form
HL60	62	94	74	85	88	88	139	175	166	90	122	1444	85	124	137	218	70	137	167											
No RNA Control	94	136	122	120	77	76	92	91	118	41	70	67	109	31	120	166	217	151	193											
DPV173	3764	34	85	30	161	135	113	114	108	117	91	1989	34	82	122	194	178	95	121											
K562	142	5649	80	74	66	149	114	140	59	73	15	1783	133	120	75	125	150	159	79											
SupB15	45	88	1597	87	123	122	82	96	89	79	78	1830	87	94	32	102	162	141	130											
ME1	126	153	141	4678	89	111	113	153	144	108	135	2533	186	92	111	149	190	145	135											
HL60 + CBFB-MYH11 D	119	120	154	119	5963	72	133	119	157	92	105	2227	159	234	148	46	321	144	154											
REH	86	93	161	153	145	1642	224	151	143	95	116	2073	184	151	82	170	167	137	109											
MV4	115	107	122	161	109	81	3019	91	146	78	84	2047	101	194	183	243	189	117	121											
HL60 + MLL-AFF1 e10e4	107	128	148	195	147	80	2545	104	76	82	34	2915	105	154	179	83	216	120	167											
NB4	70	73	123	89	56	82	129	2696	91	93	119	2109	159	38	97	178	257	102	100											
HL60 + PML-RARA S	72	102	154	108	170	100	198	4599	100	154	2336	313	46	44	171	197	195	136												
Kasumi1	47	102	81	142	104	116	125	77	85	5108	124	1911	128	92	103	147	131	185	186											
697	91	114	103	91	65	80	87	159	76	35	3249	2610	110	130	149	178	205	55	97											
HL60 + DEK-NUP214	159	100	131	92	117	140	111	175	153	74	122	1853	2043	60	8	86	159	130	94											
HL60 + MLL-ELL	79	137	79	94	146	117	0	36	135	92	128	2534	99	2564	113	134	146	88	4											
HL60 + MLL-MLLT3 e11e9	159	99	83	64	75	91	174	140	109	110	52	2485	56	107	2098	113	80	58	86											
HL60 + MLL-MLLT3 e9e9	77	117	166	14	100	100	156	52	81	0	51	2155	152	143	134	3452	107	70	86											
HL60 + MLL-MLLT4	96	112	157	138	46	165	218	29	96	70	175	1492	96	39	190	92	2135	76	70											
HL60 + RBM15-MKL1	44	117	84	105	142	136	96	37	131	171	82	1621	121	158	186	218	107	2167	67											
HL60 + SIL-TAL1	168	107	159	90	90	85	110	154	107	75	94	1928	77	162	57	119	102	121	1934											

Figure 5.

Panels with additional relevant targets. (A) Representative example of results (MFI) with the indicated cell line RNA and synthetic transcripts using a prototype multiplex assay detecting seven AML fusion transcripts or MPM1 mutations (Mut) on five beads. The NPM1 wild-type transcript (WT) is used as endogenous control. (B) Representative example of results (MFI) with the indicated cell line RNA and synthetic transcripts using a prototype multiplex assay detecting seven APL fusion transcripts on seven beads. The GUS transcript is used as endogenous control and the probe specific for *RARA* exon 3 detects all *RARA*-containing transcripts. (C) Representative example of results (MFI) with the indicated cell line RNA and synthetic transcripts using a prototype multiplex assay detecting nineteen leukemia fusion transcripts on eighteen beads. For all figures, target-specific positive signals above the qualitative cut off (350 MFI) are highlighted.

Figure 6.

Evaluation of eighty representative clinical specimens. The indicated residual total RNA samples (400 to 1,500 ng input per RT reaction) were tested with independent sub-panels focused on (A) CML, (B) ALL, (C) APL, or (D) AML targets. Positive signals above the qualitative cut off (350 MFI) indicating the presence of a given target are highlighted. (E) Summary of results presented as two-by-two qualitative agreement tables relative to reference methods for each sub-panel and overall.

Table 1

Genetic alterations, target transcripts and examples of panel content

Category	Chromosomal target (location)	Corresponding RNA target	Panel				
			LTx	CML	ALL	AML	Exp
CML	t(9;22) (q34;q11)	BCR-ABL1 e13a2	✓	✓			✓
		BCR-ABL1 e14a2	✓	✓			✓
	t(9;22) (q34;q11)	BCR-ABL1 e1a2	✓	✓	✓		✓
ALL	t(12;21) (p13;q22)	ETV6-RUNX1 e5e2	✓		✓		✓
		ETV6-RUNX1 e5e3			✓		
	t(1;19) (q23;p13)	TCF3-PBX1 e13e2	✓		✓		✓
		TCF3-PBX1 e13e2i27			✓		
	t(4;11) (q21;q23)	MLL-AFF1 e9e4			✓		
		MLL-AFF1 e9e5	✓		✓		✓
		MLL-AFF1 e10e4	✓		✓		✓
		MLL-AFF1 e10e5			✓		
		MLL-AFF1 e11e4			✓		
		MLL-AFF1 e11e5			✓		
	del(1p32)	SIL-TAL1					✓
AML	NPM1 (5q35.1)	Mutant A (insTCTG)				✓	
		Mutant B (insCATG)				✓	
		Mutant D (insCCTG)				✓	
	t(8;21) (q22;q22)	RUNX1-RUNX1T1	✓		✓		✓
	inv(16) (p13q22)	CBFB-MYH11 A	✓		✓		✓
		CBFB-MYH11 D	✓		✓		✓
		CBFB-MYH11 E				✓	
	t(15;17) (q24;q21) ^a	PML-RARA ber1 (L)	✓		✓		✓
		PML-RARA ber3 (S)	✓		✓		✓

Category	Chromosomal target (location)	Corresponding RNA target	Panel				
			LTx	CML	ALL	AML	Exp
		PML-RARA bcr2 (V)		✓			✓
		ZBTB16-RARA A					✓
	t(11;17) (q23;q21)	ZBTB16-RARA B					✓
		NUMA1-RARA					✓
	t(17;17) (q21;q21)	STAT5B-RARA					✓
		RBM15-MKL1					✓
	t(1;22) (p13;q13)	DEK-NUP214					✓
	t(6;9) (p23;q34)	MLL-MLLT4					✓
		MLL-MLLT3 c9e9					✓
	t(9;11) (p22;q23)	MLL-MLLT3 e11e9					✓
		MLL-ELL					✓
	t(11;19) (q23;p13.1)	GAPDH	✓	✓	✓	✓	✓
Internal control	<i>NPM1</i> (5q35.1)	NPM1 wild-type				✓	
	<i>GUS</i> (7q11.21)	GUS					✓

LTx: 12-target leukemia translocation panel. Exp: expanded LTx panel.

^aUpdated name for t(15;17) (q22;q21) as *PML* is located at 15q24.

Table 2

Summary of precision study for the twelve-target multiplex assay

Sample type	Sample level analysis				Probe level analysis			
	Run	RNA	N	Precision (%) ^a	N	Pos	Neg	Precision (%) ^a
No RNA Control	6	1	18	100 (82.4–100)	198	0	198	100 (98.1–100)
Negative Control	6	1	18	100 (82.4–100)	198	0	198	100 (98.1–100)
Positive Control	6	1	18	100 (82.4–100)	198	198	0	100 (98.1–100)
BCR-ABL1	6	3	54	100 (93.4–100)	594	54	540	100 (99.4–100)
CBFB-MYH11	6	2	36	100 (90.4–100)	396	36	360	100 (99.0–100)
ETV6-RUNX1	6	1	18	100 (82.4–100)	198	18	180	100 (98.1–100)
MLL-AFF1	6	2	36	100 (90.4–100)	396	36	360	100 (99.0–100)
PML-RARA	6	2	36	100 (90.4–100)	396	36	360	100 (99.0–100)
RUNX1-RUNX1T1	6	1	18	100 (82.4–100)	198	18	180	100 (98.1–100)
TCF3-PBX1	6	1	18	100 (82.4–100)	198	18	180	100 (98.1–100)
Overall	6	15	270	100 (98.6–100)	2970	414	2556	100 (99.9–100)

N: number of results. Pos: number of positive results. Neg: number of negative results.

^a95% confidence intervals are indicated between parentheses.

Table 3

Summary of validation studies for multiplex sub-panels

Sample type	Input range (ng)	Sensitivity (%) ^a	Run	RNA	Precision		
					N	High (%)	Low (%) 95% CI
BCR-ABL1	0.4–1,000	0.1	12	6	120	100	100 96.9–100
CBFB-MYH11	0.1–1,000	0.1	8	6	72	100	100 94.9–100
ETV6-RUNX1	0.4–1,000	0.1	12	2	40	100	100 91.2–100
MLL-AFF1	0.4–1,000	0.1	12	2	40	100	100 91.2–100
PML-RARA	0.1–1,000	0.1	8	6	72	100	100 94.9–100
RUNX1-RUNX1T1	0.1–1,000	0.01	8	2	24	100	100 86.2–100
TCF3-PBX1	0.4–1,000	0.1	12	2	40	100	100 91.2–100

N: number of results. CI: confidence intervals.

^aAt 400 ng total RNA input per RT reaction.