A Multiplex Bead Array-based Assay for Rapid Identification of Common Leukemia Associated Translocations

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ABSTRACT

Background: Chromosomal translocations that produce chimeric transcripts are useful as molecular markers of chronic myelogenous leukemia (CML), acute promyelocytic leukemia (AML-M3) and subtypes of acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). Reverse transcription PCR is the most common method of fusion transcript detection in a diagnostic specimen. However, this typically requires individual assays for each transcript. Hence, a single tube assay that is rapid, specific, and allows detection of a range of leukemia-associated transcripts is highly desirable. **Methods:** We evaluated a single tube Luminex bead-array assay (Ambion Diagnostics, Austin, TX), to simultaneously detect 10 leukemia-associated fusion transcripts: b2a2, b3a2 and e1a2 forms of bcr-abl [t(9;22), CML], AML1-ETO [t(8;21), AML-M2], E2A-PBX1 [t(1;19), ALL], TEL-AML1 [t(12;21), AML], PML-RARα long and short forms [t(15;17), AML-M3], CBFβ-MYH11 [inv16, AML-M4], and MLL-AF4 [t(4;11), ALL], and GAPDH as an internal control. A total of 82 peripheral blood or bone marrow aspirate specimens, previously tested by individual gel-based PCR or quantitative real time PCR (qRT-PCR), were analyzed by the bead-array in a 96-well format. Two-step reverse transcription PCR was performed prior to analysis by the bead-array assay. Four fold increase in fluorescent signal above background was considered positive for fusion transcript presence. Tumor burden in each specimen was calculated by FISH for CML and by manual blast counts for ALL and AML. For establishing lower sensitivity limits, post-treatment specimens of CML with low disease burden were analyzed. **Results:** In specimens with >10% tumor cells, the concordance between the bead-array assay and our PCR assays was 99% (69/70), with detection of the correct fusion transcripts in 7/7 cases with b2a2, 15/16 b3a2, 9/9 e1a2, 9/9 AML1-ETO, 2/2 E2A-PBX1, 8/8 PML-RARα (long), 2/2 PML-RARα (short) and 11/11 CBFβ-MYH11. The normalized bcr-abl fusion transcript level (qRT-PCR) in one discordant specimen was 4%. Fusion transcripts were not detected in 12 post-treatment CML specimens with <10% of tumor as determined by FISH. The levels of the normalized bcr-abl transcripts determined by qRT-PCR in these specimens ranged from 0.01-40%. **Conclusion:** This multiplex bead-array assay correctly identified the transcript fusion type present in diagnostic specimen with high specificity, thus useful for rapid diagnostic screening and classification.

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is most commonly used to detect the fusion transcripts resulting from leukemia-associated translocations. Since setting up individual assays for each transcript type is laborious, we explored the possibility of using a microsphere (bead) based flow cytometric assay (Luminex Corp., Austin, TX) that has both liquid hybridization kinetics and the capability to analyze multiple analytes in a single reaction as a platform for detection of the most common leukemia translocations. In this study, we evaluated the ability of a multiplex bead array-based assay, the LTx complete panel developed by Ambion Diagnostics (Austin, TX) for rapid and specific identification of the fusion transcript present in diagnostic leukemia specimens.

The technology is based on the binding of a labeled, specific target sequence to a complementary probe bound to a capture bead of a specific color. Prior to detection, each target transcript is reverse transcribed to cDNA and then amplified by PCR using a biotin-labeled primer. The resulting mix of PCR products are denatured and hybridized to the capture beads, stained with streptavidin-phycoerythrin, and analyzed by flow cytometry (Figure 1).

Within the Luminex cytometer, one laser (red) detects the color of the bead indicative of the specific translocation, while a second laser (green) detects if PCR product is associated with that bead. This detection sequence takes ~10 seconds/specimen, with completion of entire 96-wells in 15 minutes.

The bead based technology with the ability to test for multiple chimeric transcripts in a single reaction with a small amount of input RNA has distinct advantage in screening limited diagnostic specimens. In contrast to singleplex qRT-PCR assays, it is a more efficient and cost-effective screening tool with rapid turn around time.

METHODS

- A total of 82 specimens (55 BM and 27 PB), previously tested by individual gel-based PCR, qRT-PCR, or FISH/karyotyping were analyzed in a 96-well format.
- Conventional PCR assays were performed to detect fusion transcripts AML1/ETO, CBFβ/MYH11 and PML-RARα. PCR products were analyzed using a 2% agarose gel with subsequent blotting and detection with biotin-labeled probes using the NEBlot® Phototope® and Phototope Star Detection Kits (New England Biolabs, Inc., Ipswich, MA).
- TaqMan qRT-PCR was performed to detect and quantitate BCR-ABL transcripts[1].
- To determine tumor burden, two hundred cells were evaluated by FISH on CML bone marrow aspirate specimens while manual blast counts were used for ALL and AML specimens.
- For the Luminex bead assay 500 ng of RNA from each specimen was converted to cDNA using translocation specific primers. 2 µl cDNA was then used in PCR using translocation-specific primers, one of which is biotin-labled. 2 µl of PCR product was hybridized with a mixture of beads bearing probes for each translocation for 30 minutes at 52 C. The specimens were then allowed to bind the streptavidin-phycoerythrin for 10 minutes prior to analysis (Figure 1). Each specimen was run through the LS100 Cytometer (Luminex Corp.) for detection of bound product.
- Assay sensitivity was determined by dilution series of undiluted, 1:2, 1:4, 1:8 and 1:16 of two positive cell lines and two positive specimens into HL-60 RNA.
- Interassay variability was assessed by repeating 4 positive specimens 2 additional times at 15 and 45 days after the initial run.

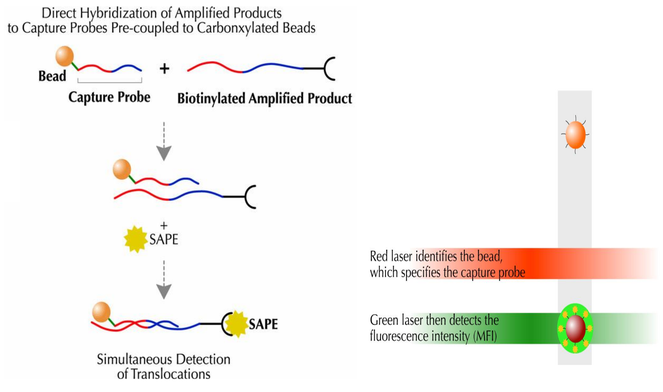


Figure 1. Schematic of LTx Assay

RESULTS

- In involved specimens with >10% tumor cells, the concordance between the LTx assay and the conventional PCR or qRT-PCR and/or FISH assays was 99% (69/70) with one discordant case occurring in a patient with presumed t(4;11)(q21;q23) identified by cytogentics and not detected by the LTx assay (Table 1).
- Interassay variability as shown in Figure 2 is greater in specimens with high fluorescence signal.
- The detection sensitivity was 6% using two patient specimens (100% tumor cells, positive for BCR-ABL; 46% tumor cells, positive for CBFβ/MYH11). For cell lines Kasumi (AML1/ETO), and B-15 (e1a2), the sensitivity was 12% and 25% respectively.
- 12 post-treatment qRT-PCR positive CML specimens with <10% tumor results were negative by LTx assay.
  - –4 of 12 showed no tumor cells by FISH
  - –8 of 12 showed 1-10% tumor cells by FISH

Table 1. Concordance of results between detection methods.

TESTS	RT-PCR	LTx BDA
	+VE	+VE
AML/ETO	10	10
inv16A	11	11
e1a2	10	10
b2a2	16	11*
b3a2	22	15*
RARA-LS	11	11
	FISH/Cytogenetics	**LTx BDA**
	+VE	+VE
E2A/PBx	2	2
AF4	4	3**

* Discordant cases consisted of 12 post-treatment CML specimens.
** In this one discordant case of presumed AF4-MLL, no standard PCR was done for confirmation.

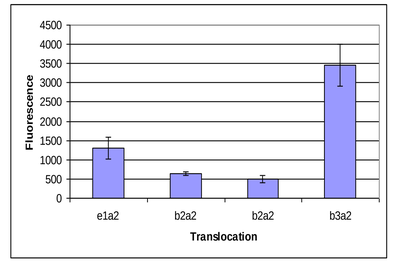
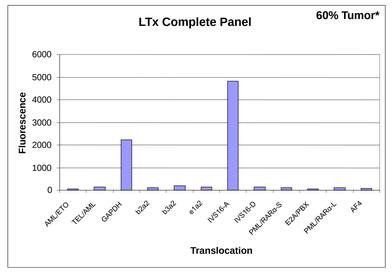
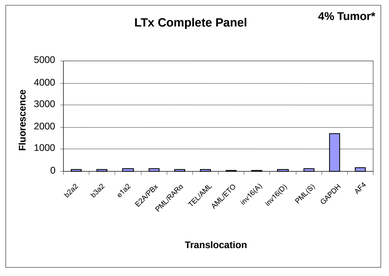


Figure 2. Run to run variation for four specimens.





*** Determined by FISH or manual blast count.**

Figure 3. Detection sensitivity for specimens with high and low tumor load.

CONCLUSIONS

- The Luminex LTx complete panel multiplex bead-array assay correctly identified fusion transcripts present in diagnostic specimens (>10% tumor) with high specificity making it useful for rapid screening and classification of newly diagnosed leukemias.
- The assay has distinct advantage of screening limited diagnostic specimens for multiple chimeric transcripts as it requires only 500 ng of total RNA.
- Inter-assay results do vary but would only affect detection and classification of those specimens whose fluorescence signal is significantly low (<500 units).

REFERENCES

1. Luthra R, Sanchez-Vega B, Jeffrey Medeiros L. TaqMan RT-PCR assay coupled with capillary electrophoresis for quantification and identification of bcr-abl transcript type. Mod Pathol. 2004 Jan;17(1):96-103.