Multi-Site Evaluation of a Multiplex Assay for the Rapid Detection of Leukemia-Associated Fusion Transcripts

Christopher D. Gocke[1], Hayley Newell[2], Wailairat Laosinchai-Wolf[3], Cecilia Higgs[1], Judith Karp[1], Mike Griffiths[2], Joanne Mason[2], and Emmanuel Labourier[3]

[1]Johns Hopkins University School of Medicine, Baltimore, Maryland, USA | [2]West Midlands Regional Genetics Laboratory (WMRGL), Birmingham, UK | [3]Asuragen Inc., Austin, Texas, USA

SUMMARY

- The Signature® LTx v2.0 Kit (RUO)* is a qualitative assay for the detection of 12 fusion transcripts resulting from 7 chromosomal abnormalities associated with AML, ALL and CML
- The multiplexed and flexible assay format is compatible with the clinical laboratory setting and the development of expanded panels for other relevant transcripts and mutations
- Preliminary research data indicate that the Signature® technology platform has the analytical and clinical performance required to complement current standard cytogenetic methods**

INTRODUCTION

Modern therapy for leukemia is based on the principle of risk stratification. Recurring genetic abnormalities commonly found in leukemia, including balanced chromosomal translocations, are often associated with either an unfavorable or favorable prognosis enabling the use of more or less toxic interventions. Knowledge of the specific genetic abnormality can also facilitate the use of targeted therapies. At the molecular level, the chromosomal breakpoints can vary over a wide region within the genes involved, and it is often necessary to identify the specific fusion transcript variant expressed by leukemic cells for subsequent molecular measurement of patient response during treatment and for assessment of residual disease.

Accurate, rapid and sensitive molecular methods are therefore required to complement standard cytogenetic techniques and speed up the routine workup of leukemia. The main objective of this study was to evaluate in a clinical laboratory setting the potential utility of a multiplex molecular assay for the simultaneous detection of 12 leukemia fusion transcripts and to establish its preliminary performance relative to standard cytogenetic methods.

MATERIALS AND METHODS

Total RNA was isolated from peripheral blood or bone marrow specimens using laboratory-validated methods at site 2 (JHU) or 3 (WMRGL). Residual RNA samples were tested with the Signature ® LTx v2.0 Kit (RUO)* at each site. Fifty independent specimens from site 2 were also tested at site 1 (Asuragen). Total RNA was reverse transcribed into cDNA and amplified by multiplex PCR using target-specific, biotin-modified primers. GAPDH transcripts were co-amplified in each sample and concurrently analyzed to serve as endogenous internal controls. The PCR products were then sorted on a liquid bead array containing oligonucleotide probes specific for each marker and detected using the Luminex® 200 System. Qualitative calls (positive or negative for each target) were determined relative to a fixed cut off signal set at 350 MFI, above the distribution of negative signals (Figure 1). All archived specimens in this study were de-identified and no protected health information was released.

	Rep.	Signal	Min. MFI	Max. MFI	Med. MFI	Avg. MFI	St. Dev.
No RNA (blank)	36	396	0	218	86	90	40
HL60 cell line RNA	36	396	0	212	91	95	40
Control RNA	16	176	0	182	87	84	41
Overall	88	968	0	218	89	91	40

Figure 1. Distribution of negative signals. Limit of blank and normal range studies were performed by testing in triplicate a no RNA control and HL60 total RNA in 12 runs with 3 operators, 3 thermal cyclers and 3 Luminex systems over multiple days at site 1 (36 replicates). Total RNA purified with 2 different extraction methods from 8 healthy control whole blood specimens were also tested at 2 input volumes representing a mass input range from 75 to 1,400 ng per RT reaction. A total of 968 individual probes results were generated.

RESULTS

	BCR-ABL1 e13a2	BCR-ABL1 e14a2	BCR-ABL1 e1a2	CBFB-MYH11 A	CBFB-MYH11 D	ETV6-RUNX1	MLL-AFF1	PML-RARA bcr 1	PML-RARA bcr 3	RUNX1-RUNX1T1	TCF3-PBX1	GAPDH
LTx Negative Control	127	110	107	93	125	145	96	31	57	136	48	5560
LTX NTC (no RNA Control)	81	114	120	138	121	99	98	110	144	107	127	106
LTX Positive Control	3472	4577	3907	3756	3044	2490	4890	5126	4027	4542	3375	5390
HL60 (400 ng)	83	48	24	36	59	50	54	20	33	19	0	5682
HL60 + BCR-ABL1 e13a2	4049	72	148	80	123	102	79	142	113	139	46	6193
HL60 + BCR-ABL1 e14a2	56	4535	73	119	97	121	100	80	97	54	62	5787
HL60 + BCR-ABL1 e1a2	75	117	1791	101	80	93	114	219	88	129	83	6217
HL60 + CBFB-MYH11 A	106	97	142	3992	112	109	138	88	126	120	23	5909
HL60 + CBFB-MYH11 D	76	161	85	70	3600	129	100	57	147	94	117	6068
HL60 + ETV6-RUNX1 e5e2	152	79	106	124	99	2624	116	64	13	61	124	6507
HL60 + MLL-AFF1 e9e5	103	73	148	142	77	143	5572	100	44	157	156	5852
HL60 + MLL-AFF1 e10e4	95	71	91	127	180	50	2080	132	81	117	40	6087
HL60 + PML-RARA bcr1	96	110	162	96	117	73	83	5876	70	97	101	6024
HL60 + PML-RARA bcr3	159	41	86	106	161	116	41	125	4266	95	86	6205
HL60 + RUNX1-RUNX1T1 e5e12	131	29	69	86	77	87	118	109	109	5236	127	5843
HL60 + TCF3-PBX1 e13e2	131	110	126	88	53	52	105	66	189	108	3315	6289

Figure 2. Representative examples with control materials. Median fluorescence intensity (MFI) signals are shown for the 3 controls included in the Signature® LTx v2.0 Kit, a total RNA sample isolated from a translocation-negative cell line (HL60), and 12 different synthetic fusion transcripts prepared by in vitro transcription and spiked in a background of HL60 RNA. Positive signals above the qualitative cut off are highlighted in orange. Preliminary analytical specificity was also confirmed with total RNA isolated from cell lines expressing fusion transcripts specific for 8 out of the 11 probes included in the assay (data not shown). All samples were confirmed by Sanger sequencing.

	Site 1	Site 2	Site 3	Total
At presentation	50	57	58	165
At follow up	0	12	2	14
Cytogenetics Pos.	14	30	60	104
Cytogenetics Neg..	36	39	0	75
Healthy donor	8	0	12	20
Total	58	69	72	199

Category	Count
AML	94
ALL	38
CML	27
MDS	8
AL mix lineage	5
MPN	3
Other	4
Healthy donor	20
Total	199

Chrom. Abn.	Count
t(9;22)	41
t(15;17)	19
inv(16)	14
t(8;21)	12
t(4;11)	8
t(1;19)	5
t(12;21)	5
n/a	95
Total	199

Figure 3. Study design and sample set. A total of 179 residual total RNA samples from study subjects previously evaluated by karyotyping and/or FISH (and independent molecular tests for site 3) were evaluated with the Signature® LTx v2.0 Kit at 3 independent sites. Among this set, 104 specimens were positive by cytogenetics for one of the 7 different chromosomal abnormalities included in the Signature assay design. Fourteen specimens were from follow up analyses, including 5 relapse cases positive for t(9;22), t(8;21) or t(4;11). The clinical set was supplemented with 20 RNA samples from control healthy donors. The "other" category included 1 poorly differentiated hematologic malignancy, 1 blastic plasmacytoid dendritic cell neoplasm, 1 Felty syndrome, and 1 treated T-PLL with no residual disease, all negative by cytogenetics at site 2.

	Site 1	Site 2	Site 3	Total	% agreement
BCR-ABL1 e13a2	3	7	6	16	100
BCR-ABL1 e14a2	2	3	6	11	90.9†
BCR-ABL1 e1a2	2	7	6	15	93.3†
ETV6-RUNX1	0	0	5	5	100
MLL-AFF1	0	0	8	8	100
CBFB-MYH11 A	2	4	6	12	100
CBFB-MYH11 D	1	0	1	2	100
PML-RARA bcr 1	0	2	5	7	100
PML-RARA bcr 3	0	7	5	12	100
RUNX1-RUNX1T1	4	2	6	12	100
TCF3-PBX1	0	0	5	5	100
Positive	14	32	59	105	100
Negative	44	37	12	93	97.9†
Fail	0	0	1	1	n/a
Total	58	69	72	199	n/a
% agreement	100	97.1†	100	n/a	99.0†

†Two specimens from Ph+ ALL or CML cases under treatment and in cytogenetic remission were found low positive with Signature at site 2

		Cytogenetics		
		Pos	Neg	Total
Signature	Pos	103	2†	105
	Neg	0	93	93
	Total	103	95	198
Positive % agreement		100 (96.4-100)		
Negative % agreement		97.9 (92.6-99.4)		
Overall % agreement		99.0 (96.4-99.7)		

Figure 4. Summary of results. The correct fusion transcript was detected with the Signature® v2.0 assay in 103 specimens positive by cytogenetics, including 5 relapse cases at sites 2 and 3. Among the 95 specimens negative by cytogenetics, 2 were found low positive by Signature at site 2 (768 and 946 MFI for e14a2 or e1a2, respectively). This discrepancy can be explained by the difference in analytical sensitivity between the 2 methods (the 2 cases were Ph+ at presentation and negative by FISH at follow up). One Ph+ CML specimen failed amplification at site 3 (no GAPDH endogenous control signal) corresponding to an overall failure rate of 0.5% (1.4% at site 3).

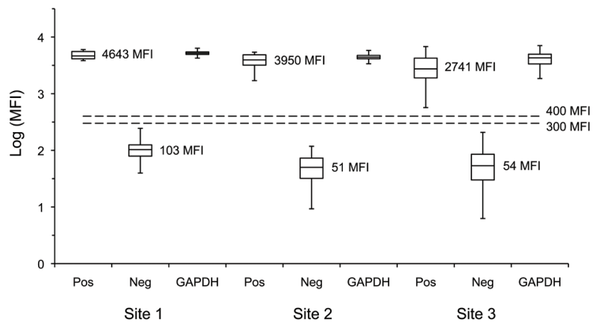

Figure 5. Quantitative analysis. The graph shows the distribution of positive, negative and GAPDH endogenous control signals (MFI) in the log space for each of the 3 sites. The boxes represent the 25th, 50th (median) and 75th percentiles of the signal distributions for each category. The tails of the distributions are indicated by whiskers corresponding to 1.5 IQR (interquartile range, that is the 75th percentile value minus the 25th percentile value) or the maximum/minimum value of the distributions if those values were within ±1.5 IQR. The median MFI of the positive and negative signal distributions at each site are shown. These results indicate that a qualitative cut off between 300 and 400 MFI is appropriate for all 3 sites.

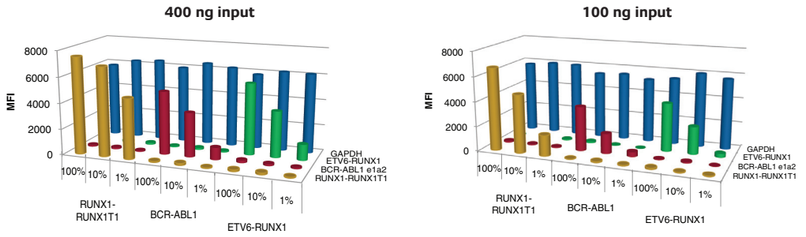

Figure 6. Analytical sensitivity. Preliminary sensitivity was assessed using total RNA isolated from cell lines expressing RUNX1-RUNX1T1, BCR-ABL1 (e1a2) or ETV6-RUNX1. Total RNA was tested either undiluted (100%) or diluted at 10 or 1% in a background of total RNA isolated from the translocation-negative cell line HL60. The graphs show representative examples of MFI signals generated by the 3 probes of interest and by the GAPDH endogenous control probe. All samples generated signals above the 350 MFI cut off at 400 ng input (left) but not at 100 ng input (right). Although fusion transcripts are reproducibly detected in 10 to 1,000 ng of undiluted RNA from positive cell lines (data not shown), the input for optimal analytical sensitivity is 400 to 1,000 ng per RT reaction.

	BCR-ABL1 e13a2	BCR-ABL1 e14a2	BCR-ABL1 e1a2	ETV6-RUNX1	MLL-AFF1	TCF3-PBX1	GAPDH
No RNA control	61	33	5	34	61	0	42
HL60 (400ng)	57	64	28	45	25	76	4829
HL60 + BCR-ABL1 e13a2	1516	0	48	5	32	2	4475
HL60 + BCR-ABL1 e14a2	7	3782	46	0	114	29	4252
HL60 + BCR-ABL1 e1a2	44	40	4011	0	0	24	4800
HL60 + ETV6-RUNX1 e5e2	9	33	29	4478	151	0	4769
HL60 + ETV6-RUNX1 e5e3	47	31	75	3633	4	59	4433
HL60 + MLL-AFF1 e9e5	92	116	1	35	2991	96	5026
HL60 + MLL-AFF1 e10e4	45	0	82	37	2206	42	5091
HL60 + MLL-AFF1 e9e4	134	51	36	29	1652	32	4769
HL60 + MLL-AFF1 e10e5	9	31	61	34	2983	25	4968
HL60 + MLL-AFF1 e11e4	41	34	67	81	2742	42	4549
HL60 + MLL-AFF1 e11e5	27	19	32	13	3212	36	4874
HL60 + TCF3-PBX1 e13e2	78	1	39	205	30	2620	4611
HL60 + TCF3-PBX1 e13e2i27	29	25	49	0	121	1672	5056

	CBFB-MYH11 A	CBFB-MYH11 D	CBFB-MYH11E	PML-RARA bcr 1	PML-RARA bcr 3	PML-RARA RARA e3	RUNX1-RUNX1T1	NPM1 Mut	NPM1 WT
No RNA control	72	46	56	62	68	50	81	36	40
HL60 (400 ng)	21	93	37	51	68	57	57	65	5966
HL60 + NPM1 mut A	43	81	95	0	36	63	3	4127	5849
HL60 + NPM1 mut B	115	66	64	99	83	42	47	4247	5397
HL60 + NPM1 mut D	84	74	29	52	0	2	69	4880	6319
HL60 + CBFB-MYH11 A	3580	52	50	33	49	52	37	48	6125
HL60 + CBFB-MYH11 D	0	3612	27	80	34	53	0	78	6299
HL60 + CBFB-MYH11 E	28	42	4228	67	52	19	89	97	6072
HL60 + PML-RARA bcr1	30	101	72	3836	78	4921	62	97	6023
HL60 + PML-RARA bcr3	48	66	82	121	1493	1345	57	62	6486
HL60 + PML-RARA bcr2	49	72	69	33	53	2538	58	102	6694
HL60 + RUNX1-RUNX1T1 e5e12	66	41	71	26	0	66	4293	124	5463

Figure 7. Signature® panel expansion. Representative example of results with 2 prototype assays detecting a total of 23 different targets prepared by in vitro transcription and spiked in a background of translocation- and mutation-negative HL60 RNA. One assay is focused on targets commonly found in ALL or CML (top panel) and co-detects 6 additional rare fusion transcripts: ETV6-RUNX1 e5e3, MLL-AFF1 e9e4, e10e5, e11e4, or e11e5, and TCF3-PBX1 e13e2i27. GAPDH is used as an endogenous control. The other assay contains various markers associated with favorable prognosis in AML (bottom panel). The assay detects CBFB-MYH11 type E, PML-RARA bcr2 (or V form) and the 3 most common NPM1 mutations (A, B and D). A positive signal only on the probe specific for RARA exon 3 indicates detection of PML-RARA bcr2. For this assay, GAPDH (data not shown) or the NPM1 wild type sequence (NPM1 WT) can be used as an endogenous control.

CONCLUSION

The Signature® LTx v2.0 assay (RUO)* is compatible with representative RNA samples extracted from various lymphoid and myeloid malignancies, including AML, CML, ALL, MDS and MPN. The assay helped resolve complex cytogenetic cases and positively identified the expected fusion transcript in RNA samples from cases with low blast count or at relapse. The multiplex assay format and rapid time to results (about 5 hours from purified RNA) are compatible with the clinical laboratory workflow. Additional analytical experiments confirmed assay specificity, established a preliminary sensitivity of 1%, and showed that the panel content can be increased to include additional rare variants and other relevant biomarkers. Overall, the assay is a sensitive, specific and flexible molecular tool attuned to the clinical laboratory workflow that can complement current standard cytogenetic methods and help further improve and streamline the risk-based classification of leukemia**.

*For research use only. Not for use in diagnostic procedures.
**Preliminary research data. The performance characteristics of these assays have not yet been established.