Molecular Subtype Characterization of Formalin-Fixed, Paraffin-Embedded Diffuse Large B-Cell Lymphoma Samples on the ICEPlex® System

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Introduction

Molecular characterization of diffuse large B-cell lymphoma (DLBCL) provides important prognostic, and potentially therapeutic, information. Although the original classification of DLBCL into germinal center B cell-like (GCB) and activated B cell-like (ABC) subtypes was based on microarray gene expression profiling, this technique is not currently feasible in a clinical setting, primarily because it cannot be performed on formalin-fixed, paraffin-embedded (FFPE) specimens. Surrogate studies to assign classification, such as immunohistochemistry, have shown variability between laboratories and may not yield reliable results. Therefore, we have developed a novel multiplex real-time PCR assay on the automated ICEPlex System which allows differentiation between germinal center B cell-like (GCB) and activated B cell-like (ABC) subtypes of DLBCL from FFPE specimens.

Technology Overview

ICEPlex integrates a PCR thermal cycler that accommodates a standard 96-well PCR plate, a capillary electrophoresis (CE) system with a replaceable CE cartridge, and fluidic pumps with on-board reagents. Since the ICEPlex System has two solid state lasers (488nm and 639nm excitation) and a spectrophotometer with CCD camera, two different dyes can be detected simultaneously in single well (Figure 1).

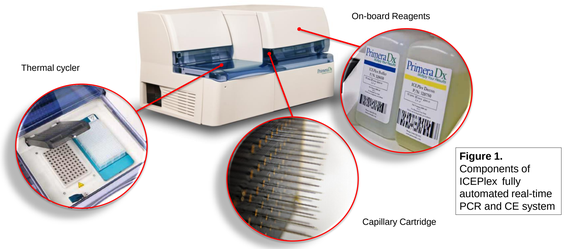

Figure 1. Components of ICEPlex fully automated real-time PCR and CE system

Unlike other real time PCR technologies, STAR (Scalable Target Analysis Routine) technology detects and quantifies fluorescently labeled PCR products with unique sizes on the ICEPlex System by sequential sampling and separation using capillary gel electrophoresis (CE) in real time. Multiple quality controls and calibrators can be embedded in the PCR reaction to ensure quality of the reported results. Built-in software automatically generates amplification curves by converting the fluorescent signals corresponding to the detected PCR amplicons once the run on ICEPlex is complete. Cycle thresholds (C_t) for all PCR targets are reported in the Clinical Report (Figure 2).

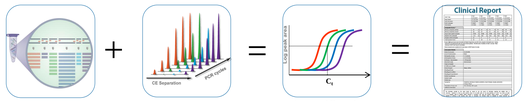

Figure 2. Principles of the multiplex real-time PCR detection on the ICEPlex System

ICEPlex system and ICEPlex DLBCL Assay have not been approved for IVD by the FDA. The presented information is for demonstration purpose only.

Materials and Methods

Microarray Gene Expression Profiling and Immunohistochemical Algorithms: Twenty-seven *de novo* DLBCL clinical samples, with paired frozen tissue and FFPE tissue, from patients at our institution were selected. DLBCL subtype classification into GCB and non-GCB (NGC) subtypes by immunohistochemistry (IHC) was performed using three published algorithms (Hans, Choi, Tally).[1,2,3] Gene expression profiling using microarrays was performed on frozen tissue, and molecular subtype was assigned based on Wright classification.[4]

ICEPlex DLBCL Assay Design: Primers were designed, for 17 genes utilized previously in DLBCL subtype classification (Wright classifier[4] and Lossos[5] outcome predictor) and two reference genes, in unique target regions spanning exon-exon junctions (Table 1). To accommodate detection of highly degraded template RNA, primers were designed to amplify short template regions, ranging in size from 83-102 nucleotides. To allow discrimination of target-specific amplicons on the ICEPlex platform by fluorescent label and by size, reverse primers were labeled with FAM or TYE fluorescent dyes and both forward and reverse primers were equipped with 5'-nucleotide tags to allow extension of the original template size (extension tag amplification, ETA), generating amplicons ranging in size from 114-181 nucleotides (Table 1).

ICEPlex Assay: The ICEPlex assay was performed on isolated RNA from two cell lines, one GCB subtype (SUDHL6) and one ABC subtype (OCI-LY3). Following optimization, the multiplex assay was performed on 3 frozen DLBCL clinical samples, 2 FFPE-embedded cell lines, and 27 FFPE DLBCL clinical samples using 12.5 ng RNA per reaction. Crossing threshold data for each of the 17 genes was normalized using two housekeeping genes. A PCR score was then calculated using the normalized data and published gene weights.[4] Cases within 5% of the mean were considered unclassified (UNCL) while higher scores were classified as ABC and lower scores were classified as GCB. The ICEPlex subtype classification was compared against the microarray subtype classification. Sensitivity and specificity were calculated.

Symbol	Protein	Wright Panel	Lossos Panel	Template Size (nt)	Amplicon Size (nt)	Label
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1			100	156	FAM
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)			87	115	FAM
IGHM	immunoglobulin heavy constant mu			102	146	TYE
IL16	interleukin 16			98	142	FAM
IRF4	interferon regulatory factor 4			96	181	FAM
ITPKB	inositol-trisphosphate 3-kinase B			97	125	TYE
LRMP	lymphoid-restricted membrane protein			98	148	FAM
MME	membrane metallo-endopeptidase			99	160	TYE
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1			99	129	FAM
PIM1	pim-1 oncogene			98	153	FAM
PTPN1	protein tyrosine phosphatase, non-receptor type 1			100	125	FAM
CCND2	cyclin D2			84	119	FAM
LMO2	LIM domain only 2 (rhombotin-like 1)			102	159	FAM
BCL6	B-cell CLL/lymphoma 6			100	133	FAM
BCL2	B-cell CLL/lymphoma 2			87	119	TYE
FN1	fibronectin 1			92	129	TYE
SCYA3	chemokine ligand 3			95	136	TYE
TBP	TATA binding protein	Reference Gene		100	175	FAM
TFRC	transferrin receptor	Reference Gene		88	137	FAM

Table 1. Gene targets for the ICEPlex DLBCL assay were selected based on the Wright et al. (2003) and Lossos et al. (2004) classification. Template size, amplicon size and fluorescent label for each target are indicated. Reference genes are boxed.

Results

DLBCL Case #	Microarray Gene Expression Profiling Molecular Subtype	ICEPlex Assay Molecular Subtype	ICEPlex Assay Molecular PCR Score	Hans IHC Molecular Subtype	Choi IHC Molecular Subtype	Tally IHC Molecular Subtype
30	ABC	ABC	-1.64	NGC	NGC	NGC
6	ABC	ABC	-1.39	NGC	NGC	NGC
19	ABC	ABC	-1.12	NGC	NGC	NGC
16	ABC	ABC	-1.09	NGC	NGC	NGC
33	ABC	ABC	-0.96	NGC	NGC	NGC
10	ABC	ABC	-0.89	NGC	NGC	NGC
18	ABC	ABC	-0.66	NGC	NGC	NGC
11	ABC	ABC	-0.56	NGC	NGC	NGC
21	ABC	ABC	-0.39	NGC	NGC	NGC
12	UNCL	ABC	-0.31	GCB	GCB	NGC
34	ABC	ABC	-0.27	NGC	NGC	NGC
17	UNCL	ABC	-0.22	NGC	NGC	NGC
2	GCB	UNCL	-0.12	GCB	GCB	NGC
31	UNCL	UNCL	-0.05	GCB	GCB	GCB
13	UNCL	UNCL	0.00	GCB	GCB	NGC
4	ABC	UNCL	0.00	GCB	GCB	GCB
25	GCB	GCB	0.38	GCB	GCB	GCB
36	UNCL	GCB	0.47	GCB	GCB	GCB
5	GCB	GCB	0.57	GCB	GCB	GCB
7	GCB	GCB	0.61	GCB	GCB	NGC
15	GCB	GCB	0.67	GCB	GCB	GCB
32	GCB	GCB	0.90	GCB	GCB	GCB
1	GCB	GCB	0.99	GCB	GCB	GCB
3	GCB	GCB	1.06	GCB	GCB	GCB
23	GCB	GCB	1.28	GCB	GCB	GCB
14	GCB	GCB	1.53	GCB	GCB	GCB
20	GCB	GCB	1.65	GCB	GCB	GCB

Table 2. ICEPlex Assay Molecular Subtyping Classification Results for 27 FFPE DLBCL Specimens

Comparison between the ICEPlex molecular subtyping results and microarray gene expression profiling shows high correlation. In classified cases, the ICEPlex assay showed 91% accuracy in assigning ABC cases and 91% accuracy in assigning GCB cases, compared to microarray expression profiling. When unclassified cases (UNCL) were considered to be non-GCB cases, the ICEPlex assay showed 94% accuracy in assigning non-GCB cases and 91% accuracy in assigning GCB cases The assay demonstrated 100% specificity and 100% sensitivity, as compared to ABC classification with microarray expression profiling for the cases able to be classified by the ICEPlex assay. When IHC was used to determine subtype in unclassified microarray cases, the specificity was 92% and the sensitivity was 100% for ABC classification.

PCR efficiency values for each of the 19 genes ranged from 90-110% for the majority of the targets for both FF-derived and FFPE-derived cell line material. Gene detections patterns were consistent over a greater than 1000 fold RNA input range, and allowed less than 1 ng of RNA from FFPE samples to be used per reaction.

The assay showed similar results between frozen and FFPE-derived cell line and clinical samples (Table 3).

DLBCL Sample	ICEPlex Assay Molecular Subtype FROZEN	ICEPlex Assay Molecular Subtype FFPE
OCI-LY3 Cell Line	ABC	ABC
SUDHL6 Cell Line	GCB	GCB
DLBCL Case # 13	UNCL	UNCL
DLBCL Case # 18	ABC	ABC

Table 3. Comparison of the ICEPlex DLBCL Assay on Frozen-derived and FFPE-derived samples shows high correlation between and accurate assessment of molecular subtype.

Conclusions

- A novel quantitative 19-plex mRNA expression profiling assay designed to allow DLBCL tumor classification on FFPE specimens in a single tube PCR reaction was developed on the ICEPlex system.
- The ICEPlex DLBCL assay allowed discrimination of ABC and GCB cell lines using < 1ng of RNA from FFPE-isolated material, with comparable results between frozen and FFPE–derived cell line and clinical samples.
- 27 clinical FFPE DLBCL samples were examined and molecular subtype classification based on the ICEPlex DLBCL assay showed high correlation with frozen-derived samples in microarray expression profiling.
- This potentially robust multiplex assay provides rapid molecular classification that is feasible for routine laboratory use in a clinical setting and may allow rapid determination of molecular subtype for clinical trials, and potentially treatment selection.

References

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