Molecular Profiling of Diffuse Large B-Cell Lymphoma Subtypes on the ICEPlex® System

Jörk Nölling[1], Angela M.B. Collie[2], Jeffrey J. Lin[2], Brian T. Hill[2], Tomas Radivoyevitch[3], Eric D. Hsi[2], and Lilly Kong[1]
[1]PrimeraDx, Mansfield, MA 02048, [2]Cleveland Clinic, Cleveland 44195; [3]Case Western Reserve University, Cleveland 44106

PrimeraDx The Multiplex PCR Company

Cleveland Clinic

Abstract

Introduction: Molecular profiling of diffuse large B-cell lymphoma (DLBCL) has emerged as an important prognostic tool for affected patients. Current molecular profiling technologies are not capable of providing quantitative, specific, and sensitive assays that are also able to examine large numbers of analytes simultaneously, in particular when using material extracted from formalin-fixed paraffin-embedded (FFPE) specimens. To overcome these limitations, we developed a novel high-multiplex gene expression profiling 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.

Materials and Methods: Primer design followed a proprietary approach, extension tag amplification (ETA), that allows amplification and detection of low quality RNA targets with template size less than 100 nucleotides from FFPE specimens. Primers were designed across exon junctions and were screened *in silico* for potential sequence interaction using our software. Multiplexed reactions were optimized for target identification and amplification performance on the ICEPlex instrument using RNA from ABC and GCB DLBCL cell lines. Relative quantification of target gene expression was achieved by normalization to reference genes. The assay was further tested for analytical performance, amplification efficiency, dynamic range and precision, using FFPE-extracted cell line RNA.

Results: A real-time 19-plex expression profiling assay for DLBCL tumor subtyping was developed on the ICEPlex System. The single-reaction assay comprised of a 17-gene panel, established as biomarkers for DLBCL subtyping (Wright classifier and Lossos outcome predictor), and two reference genes. Multiplex assay performance showed similar PCR efficiencies for all targets. Less than 1ng of FFPE-extracted material was sufficient to enable classification of DLBCL cell lines into GCB or ABC subtypes.

Conclusion: We have developed a novel 19-plex quantitative expression profiling assay designed for DLBCL tumor classification into GCB or ABC subtypes from FFPE specimens. This enabling work will be applied to a clinical validation set of DLBCL patients treated with immunochemotherapy. This assay has the potential to provide rapid and accurate subclassification and prognostic information in DLBCL.

1. Wright G et al. PNAS. 100(17): 9991-9996, 2003.
2. Lossos IS et al. NEJM. 350(18):1828-37, 2004.

Technology Overview

Unlike any other detection system, 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).

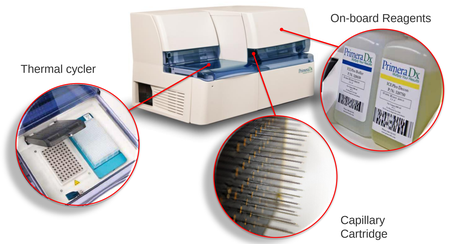


Figure 1. Components of ICEPlex fully automated real-time PCR and CE system

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).

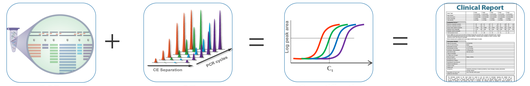


Figure 2. Principles of the multiplex real-time PCR detection on the ICEPlex System

Materials and Methods

Primer design: Primers for 17 genes, established as biomarkers for DLBCL subtyping, and two reference genes were selected 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 83-102nt (Table 1). Primer candidates were screened for multiplex compatibility using CrossHyb program (PrimeraDx, Mansfield, MA) and, if necessary, redesigned until a set of matching primers were selected. Primer specificity was confirmed by Primer BLAST from the National Center for Biotechnology Information (Bethesda, MD). 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 (IDT,) 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 114-181nt (Table 1).

RNA Isolation: Total RNA from fresh frozen cell lines, OCI-LY3 and SUDHL6, was isolated with the mirVana mRNA isolation kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. For isolation of RNA from formalin-fixed paraffin-embedded cell lines, the AllPrep DNA/RNA FFPE kit (Qiagen, Germantown, MD) was used, following the recommended protocols for RNA isolation.

RT-PCR setup and amplification conditions: RT reactions were set up in 20ul reactions using 20U of SuperScript III (Invitrogen, Grand Island, NY) according to the manufacturer's recommendation. After addition of RNA template, the reactions were incubated for 30min at 50° C followed by 5min at 90° C. For subsequent PCR, 4ul of the RT reaction was used. PCR reactions were prepared in 25ul volumes and contained 5U of AptaTaq Δexo DNA polymerase (Roche Diagnostics, Indianapolis, IN), 1x multiplex buffer (PrimeraDx, Inc., Mansfield, MA), 3.5mM $MgCl_2$, 1x multiplex primer mix and 0.25x of the Universal Calibrators (PrimeraDx, Inc., Mansfield, MA). PCR reactions were subjected to thermocycling in a standard 96-well PCR plate on the ICEPlex System. PCR amplification conditions were as follows:

- 96° C for 10 minutes
- 17 cycles at 64° C for 40sec., 72° C for 40sec. and 96° C for 10sec.
- 28 cycles at 64° C for 110sec., 72° C for 150sec., 96° C for 10sec.

Symbol	Protein	Wright Panel	Lossos Panel	Template Size (nt)	Amplicon Size (nt)	Label
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1			100	166	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. Genes 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

Simultaneous Profiling of 19 mRNA Targets in One Reaction

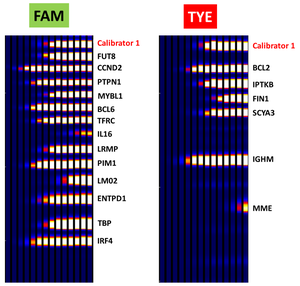
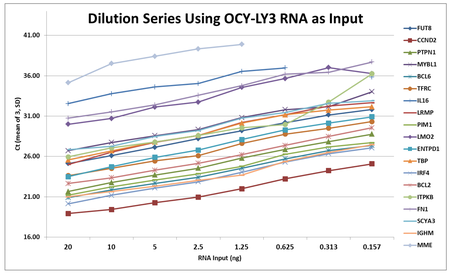


Figure 3. Simultaneous detection of 19 mRNA targets with the ICEPlex DLBCL assay. Using cDNA generated from 10ng of SUDHL6, ten targets are detected in the FAM channel and six targets in the TYE channel (Table 1). To allow relative quantification of target expression, two reference targets, TFRC and TBP, are monitored in the FAM channel. Each channel also detects calibrators that are used as size standards and absolute quantification.

Input Dose Response and PCR Efficiency



Target	Series SD
FUT8	0.25
CCND2	0.21
PTPN1	0.17
MYBL1	0.45
BCL6	0.16
TFRC	0.29
IL16	0.49
LRMP	0.28
PIM1	0.22
LMO2	0.33
ENTPD1	0.21
TBP	0.29
IRF4	0.19
BCL2	0.28
ITPKB	0.65
FN1	0.69
SCYA3	0.33
IGHM	0.21

Figure 4. Dose response on amplification of individual targets in the ICEPlex DLBCL Assay. cDNA generated from OCI-LY3 RNA was diluted 2-fold, from 20ng to 0.156ng, and run in triplicate PCR reactions on the ICEPlex platform. Mean C_t values were plotted against cDNA input. To highlight data consistency, standard deviations derived from replicate PCR reactions for each dilution data point were averaged for the entire dilution series per target (C_t cut-off > 35) and shown in the table.

	FF		FFPE	
Target	SUDHL6	OCI-LY3	SUDHL62	OCI-LY33
FUT8	85%	103%	99%	104%
CCND2		113%		110%
PTPN1	95%	97%	92%	94%
MYBL1	97%	99%	98%	107%
BCL6	101%	107%	93%	144%
TFRC	98%	101%	93%	95%
IL16	99%	94%	88%	126%
LRMP	84%	86%	81%	101%
PIM1	96%	106%	93%	102%
LMO2	99%	86%	98%	92%
ENTPD1	81%	90%	57%	91%
TBP	91%	102%	95%	86%
IRF4	90%	98%	84%	95%
BCL2	93%	100%	101%	108%
ITPKB	83%	100%	104%	116%
FN1		99%		76%
SCYA3	102%	108%	106%	147%
IGHM	105%	108%	119%	127%
MME	70%	84%	86%	

Table 2. PCR Efficiencies for fresh frozen and FFPE cell line material. cDNA generated from fresh frozen (FF) and FFPE material from cell lines SUDHL6 and OCI-LY3 was diluted 2-fold, from 20ng to 0.156ng, and run in triplicate PCR reactions on the ICEPlex platform. PCR efficiencies were calculated using the slope of C_t values against the log input values using the formula $E=10^{(-1/Cs)}-1$.
Results show efficiency values ranging from 90-110% for the majority of the targets for both FF- and FFPE-derived material. Empty cells indicate insufficient data to calculate PCR efficiencies due to low or no expression detection.

DLBCL Cell Line Subtyping

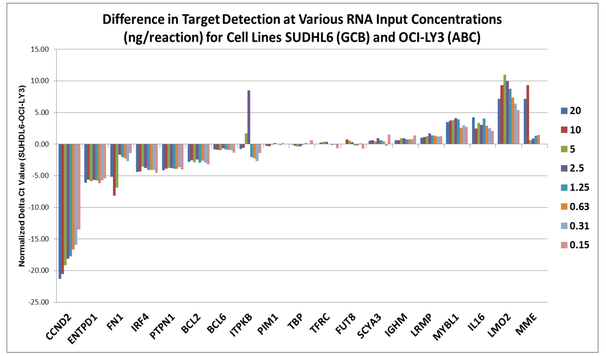


Figure 5. Differential target detection pattern for ABC- and GCB cell line RNAs at various input levels. RNA was extracted from FFPE cell line materials (SUDHL6 and OCI-LY3), diluted in 2-fold dilution steps, starting with 100ng, to cover a range of > 1000-fold input, and converted to cDNA. 20% of the RT reaction was used for PCR, performed in triplicate. For relative quantification target abundance C_t values for each target were normalized ($Norm_{ct} = Target_{ct} - ((Ref1_{ct} + Ref2_{ct})/2)$) and used to calculate the target detection difference for each cell line. Targets CCND2, ENTPD1, FN1, IRF4, PTPN1 and BCL2 were detected at higher levels in OCI-LY3 while MME, LMO2, IL16 and MYBL1 showed higher levels in SUDHL6. In contrast, targets PIM1, FUT8, SCYA3 and IGHM showed little potential in discriminating between SUDHL6 and OCI-LY3. These differential detection patterns were generally consistent over the entire RNA input range.

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.
- Testing of the ICEPlex DLBCL assay on DLBCL cell lines of different subtype, SUDHL6 (GCB) and OCI-LY3 (ABC), showed good dose response over a 1000-fold RNA input range in 2-fold dilution series.
- PCR efficiencies, calculated for both fresh frozen and FFPE-derived cell line material, showed similar efficiencies for most targets in the range of 90-110%.
- The ICEPlex DLBCL assay allowed discrimination of ABC and GCB cell lines based on specific target expression patterns generated from < 1ng of RNA from FFPE-isolated material.

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