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

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Introduction
Molecular characterization of diffuse large B-cell lymphomas (DLBCL) provides important prognostic and potentially therapeutic information. Although the original classification of DLBCL has been centered in the cell line (GBM) and subtypes defined based on the available knowledge of their biology, this classification has not been validated in a clinical setting, primarily because it cannot be performed on formalin-fixed, paraffin-embedded (FFPE) specimens.

Materials and Methods
Microarray Gene Expression Profiling and Immunohistochemical Algorithms: Twenty-three de novo DLBCL clinical samples, with paired frozen tissue and FFPE tissue from patients at our institution were examined. DLBCL subtype classification was performed using three published gene sets (Hans, Choi, Takahashi)10; Gene expression profiling was performed on frozen tissue, and molecular subtype was assigned based on Wright classification.

ICEPlex® DLBCL Assay Design: Primers were designed for 17 genes utilized previously in DLBCL subclassification (weakly positive, negative, and low positive expression). Primers were designed to amplification of short template regions, ranging in size from 123-1102 bp. To establish discrimination of target-specific amplicons on the ICEPlex platform, it was decided to aim for amplicon slightly larger than the size, reverse primers were designed to FAM or TYLE fluorescent dyes and both forward and reverse primers were designed to allow separation by melting temperature (Tm) of the TATA binding protein (TBP), membrane metallo-endopeptidase (MME), and transferrin receptor (TFRC targeted sequences.

Results
The ICEPlex assay was performed on isolated RNA from two cell lines, one GBM cell line (U266) and one diffuse large B-cell lymphoma (SUDHL-6). Following quantification, the multiplex assay was performed on 3 frozen DLBCL clinical samples, 2 FFPE-embedded cell lines, and 7 FFPE DLBCL clinical samples using 12.5 ng RNA per reaction. Crossing threshold time for each of the 17 genes was normalized to two housekeeping genes, a PCR protocol was then optimized utilizing the lyophilized bead technology (Eton). 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 GBM cell line (U266) and one diffuse large B-cell lymphoma (SUDHL-6). Following quantification, the multiplex assay was performed on 3 frozen DLBCL clinical samples, 2 FFPE-embedded cell lines, and 7 FFPE DLBCL clinical samples using 12.5 ng RNA per reaction. Crossing threshold time for each of the 17 genes was normalized to two housekeeping genes, a PCR protocol was then optimized utilizing the lyophilized bead technology (Eton). Generating amplicons ranging in size from 114-181 nucleotides (Table 1).

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 and validated on the ICEPlex system. The ICEPlex DLBCL assay allowed discrimination of ABC and GCB cell lines using < 1 mg of RNA from FFPE-isolated material, with comparable results between frozen and FFPE-derived cell line and clinical samples. The clinical FFPE DLBCL samples were examined and molecular subtype classification based on the ICEPlex® DLBCL assay showed high correlation with frozen-derived samplings in microarray expression profiling.

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