Clinical Laboratory Experience of Gene Expression Profiling of 2,384 solid tumors

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Abstract

Background: Optimizing therapeutic selections for cancer patients using the molecular profile of their tumor is an unmet need. Currently, many providers use results of tumor profiling by platforms including IHC, FISH and mutational analyses to identify potential cancer treatments. Gene expression profiling with microarrays also has the potential to help classify solid tumor subtypes and assist with therapy selections based on the under or over-expression of particular transcripts.

Methods: Solid tumor samples are assessed by board-certified pathologists to ensure the presence of at least 20% tumor nuclei. Total RNA is extracted from tumor tissues (FFPE, frozen and RNAlater-preserved tissues). In addition, extracted RNA is verified to be of sufficient quality (using spectrophotometric metrics and qPCR results on a housekeeping gene RPL13a). The RNA sample is then subjected to a whole genome (29,285 transcripts) microarray analysis using Illumina cDNA-mediated annealing, selection, extension and ligation (DASL) process with the HumanHT-12 v4 beadChip (Illumina Inc., San Diego, CA). After direct hybridization and scanning of the bead array, the expression of a subset of 88 transcripts are then compared to tissue-specific normal control pools and the statistical significance of the difference between the patient tumor sample and control is determined.

Results: The median Ct value from the RPL13a qPCR results for both frozen samples (N=70) and RNAlater preserved samples (N=851) was 24. The median Ct value for FFPE samples (N=1463) was 27. The ability of this assay to detect expression changes is directly proportional to the amount of tumor nuclei present in the patient sample. Dilution experiments showed that samples with 20% tumor nuclei are ~65% concordant for gene expression information compared to samples with >95% tumor nuclei with an R-squared value >0.99. Our intra-assay variability (same chip; same operator) was extremely low and the R-squared value was 0.99. The inter-assay variability (different chip; different operators) was somewhat lower with R-squared value of 0.93. The expected variability with internal proficiency (different reagent lots; different months; different operators) was >0.8.

Conclusions: This microarray assay has been a reliable and robust method to perform gene expression profiling on routine solid tumor samples, including FFPE, submitted for clinical molecular profiling.

Background

DNA microarrays for gene expression analysis rely on the use of nuclei acid polymer probes complementary to known gene transcripts that are immobilized on a glass surface. Direct hybridization of this probe array on glass is then used with patient tumor samples to ascertain the relative amounts of a given transcript.

In 2009 we reviewed commercially available microarray platforms and decided that the Illumina whole genome DASL gene expression platform was most aligned with our intent to validate a gene expression array platform that was designed specifically for additional sample types such as FFPE, and a platform that was amenable to high-throughput production testing in a clinical diagnostic laboratory.

Methods

Total RNA isolated from either FFPE, frozen or RNAlater preserved tissues is converted to cDNA using biotinylated oligo(dT) and random nonamer primers (Figure 1). The use of both oligo(dT) and random nonamer primers optimize cDNA synthesis of degraded RNA fragments, such as those obtained from FFPE tissue. The biotinylated cDNA is then annealed to the DASL Assay Pool (DAP) probe groups. Probe groups contain oligonucleotides specifically designed to interrogate each target sequence in the transcripts. High locus specificity is achieved in two ways, first by the requirement that both members of an oligonucleotide pair must hybridize in close proximity for an assay signal to be generated, and second by the removal of excess mishybridized oligonucleotides by washing after the annealing step.

Figure 1

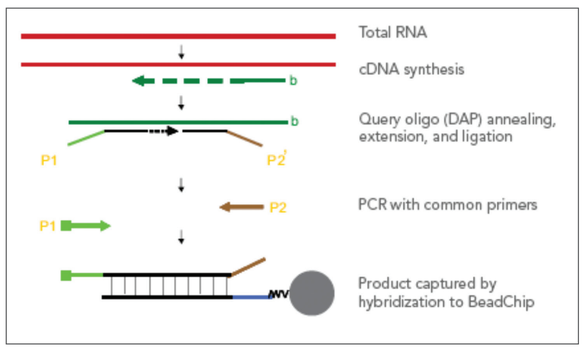

Figure 1 – Gene Expression Profiling with the Whole Genome DASL Assay (Source: Figure 2 from Illumina Data Information Sheet 1)

The resulting PCR products are then hybridized to the Human HT-12 expression BeadChip to determine the presence or absence of specific gene transcripts (see Table 1 below).

Table 1

Probes	Description	Number
NM	Coding transcripts, well established annotations	27,253
XM	Coding transcripts, provisional annotations	426
NR	Non-coding transcripts, well established annotations	1,580
XR	Non-coding transcripts, provisional annotations	26
Total		**29,285**

Table 1 – RefSeq* Content of the HumanHT-12 v4 BeadChip. *Release 38 Source: http://www.ncbi.nlm.nih.gov/RefSeq/

After hybridization, the HumanHT-12 Expression BeadChips are scanned using the iScan system from Illumina. This scanner incorporates high-performance lasers, optics, and detection systems for rapid, quantitative scanning of the bead chip array. Gene expression data from previously scanned normal tissues are used to assess relative gene expression changes in a given tumor sample. The normal reference samples are composed of a pool of three individually quality controlled (RNA quality, normal diagnosis from H&E slide by a board certified pathologist) normal reference tissue samples.

Results

Figure 2

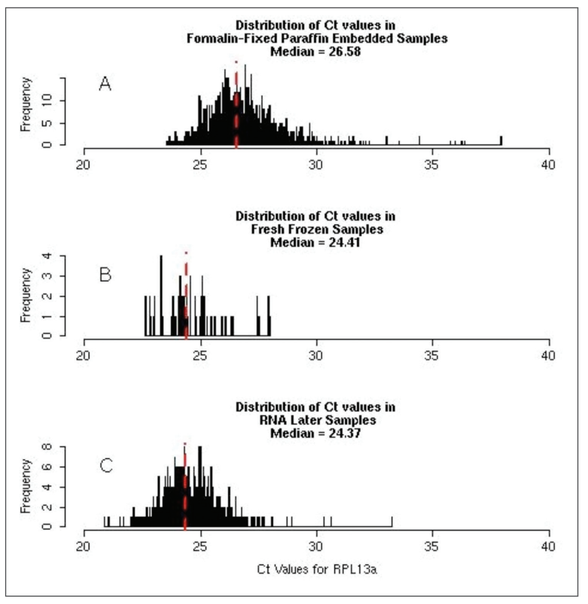

Figure 2 – Distribution of Ct values for the various sample types A, FFPE samples. B, Frozen samples. C, RNA later preserved samples. The red vertical dashed line indicates the median value observed for each distribution.

Figure 3

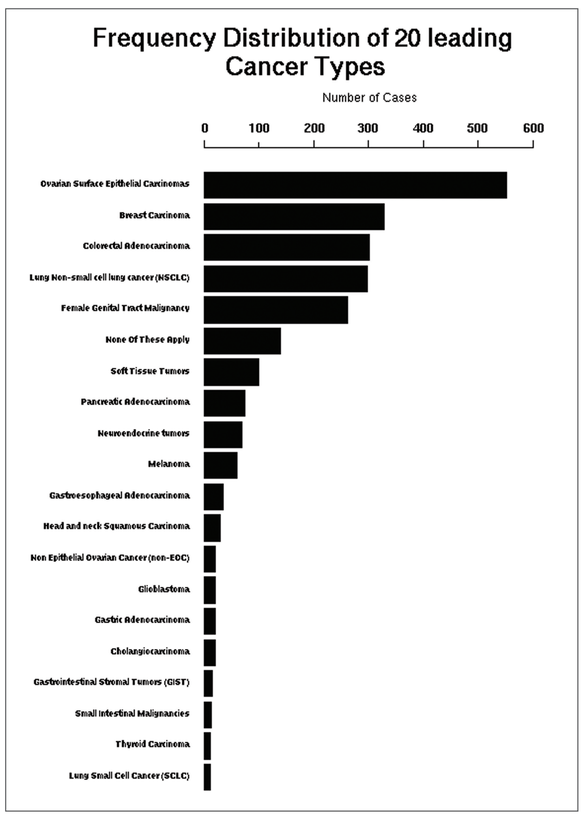

Figure 3 – Frequency distribution of cancer types where microarray analysis was completed as part of routine molecular profiling of solid tumors.

Figure 4

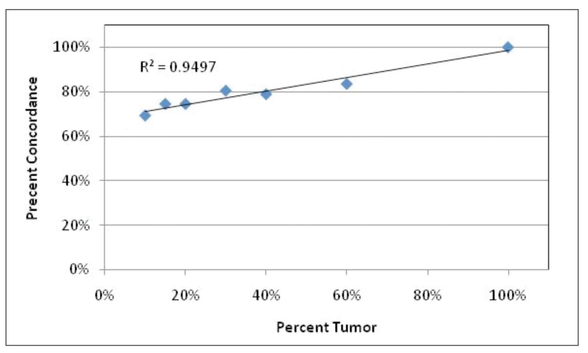

Figure 4 – Concordance of gene expression data across 80 target genes versus percent tumor nuclei in dilution experiments with 7 independent tumor samples using the Human Ref-8 bead chip.

Figure 5

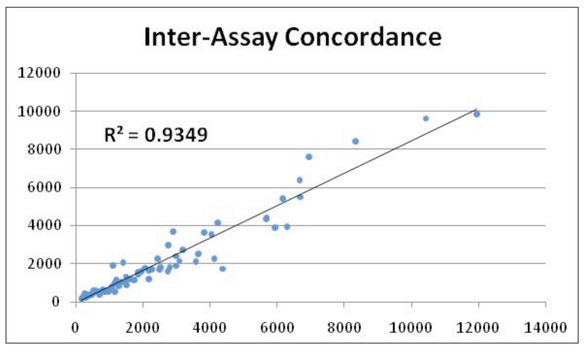

Figure 5 – Concordance of gene expression results Inter-assay experiments (different chips, different operators, same lot of reagents. Each data point represents the relative fluorescence units (RFU) for a given marker (N=80) using the Human Ref-8 bead chip.

Table 2

Source of variability	Scenario	R-squared value
Intra-assay	Same chip, same reagent lots, same operator	0.99
Inter-assay	Different chips, same reagent lots, different operator	0.93
Proficiency	Different chips, different reagent lots, different months, different operators	>0.8

Table 2 – Main sources of variability seen in Illumina microarray performance

Study Highlights

We validated the Illumina microarray platform with FFPE samples, Frozen samples and RNA later preserved samples.

Both frozen and RNA later preserved tissue samples exhibited similar values of RNA quality as shown by the nearly identical Ct values. Since RNA later-preserved samples performed equivalent to frozen tissue samples we are able to use normal frozen tissue samples as the "reference" samples for RNA later preserved tumors.

Conclusions

Specimen requirements for routine profiling of solid tumor samples include: RNA concentration of 40 ng/ul (200 ng total input), A260/A280 ratio > 1.5, Ct for RPL13a gene must be <38.

Despite showing larger Ct value than both frozen and RNA-later preserved samples, RNA extracted from FFPE specimens that meet our stringent QC metrics routinely provide quality expression profiling results.

This microarray assay has been a reliable and robust method to perform gene expression profiling on routine solid tumor samples, including FFPE, submitted for clinical molecular profiling.

References

1. Whole-Genome DASL® HT Assay for Expression Profiling in FFPE Samples, Data Sheet RNA Analysis, 5pgs, Pub. No. 470-2010-005 as of 14 October 2010, Illumina Inc., San Diego, CA.
2. Whole-genome gene expression profiling of formalin-fixed, paraffin-embedded tissue samples, April C, Klotzle B, Royce T, et al., 2009, PLos ONE, Vol 4, issue 12, e8162,10pp.
3. Whole genome gene expression analysis using DASL chemistry-Validation Notebook, Dec 2009 [Initial Illumina microarray platform validation study for FFPE and Frozen sample types], Caris Life Sciences, Phoenix, AZ.