Unlocking formalin-fixed paraffin-embedded (FFPE) samples with CytoScan® Cytogenetics Solution

Introduction
CytoScan® High-Density (HD) Cytogenetics Solution is an analysis research tool used for genome-wide, high-resolution DNA copy number and single nucleotide polymorphism (SNP) detection. With 2.68 million markers that have been designed to detect copy number gains and losses, loss of heterozygosity (LOH), and regions identical-by-descent and uniparental disomy (UPD), CytoScan® HD Array provides genome-wide coverage of OMIM® and RefSeq constitutional and cancer genes on a single microarray. It has been adopted by researchers for studying both constitutional cytogenetics and hematological malignancies with DNA extracted from blood, bone marrow, buccal cells, saliva, fresh and frozen tissues, and direct and cultured cells. Formalin-fixed paraffin-embedded (FFPE) samples represent the largest source of biological material for human cancer studies, but handling these samples in the laboratory can be challenging.

This application note demonstrates the performance of FFPE-derived DNA with the CytoScan® assay and details a modified workflow, which has been utilized to develop an external reference set, which is now available for improving the quality of results with this difficult sample type. This workflow includes a commercially available genomic DNA extraction method, quality control guidelines, and data analysis parameters that entail the suggested use of an FFPE-derived external reference set. These workflow modifications are suggested for research with FFPE samples less than five years old. To assess research applications for cancer copy number with samples greater than five years old, we recommend Affymetrix® OncoScan™ FFPE Express 2.0 Service.

Modified workflow
DNA isolation
We have evaluated a number of commercially available methods for DNA extraction from FFPE samples for research use with the CytoScan assay. For a detailed overview of the recommended procedure, see the CytoScan® DNA Purification from FFPE Tissue, PIN 703117* available at http://www.affymetrix.com/estore/browse/products.jsp?productId=prod520004#1_1. In short, each sample is heat-deparaffinized, and tissues are disrupted with lysis buffer and Proteinase K overnight. DNA is then isolated using the QIAGEN® DNeasy® Blood & Tissue Kit, after which DNA quantification is performed with spectrophotometry.

Quality control
The current user manual for the CytoScan assay has been optimized for use with blood-derived and fresh and frozen tissue samples. In this application for FFPE samples, we suggest a set of QC metrics to increase overall array success rate, which are described in this section.

DNA quantity and yield should be determined with a method that detects double-stranded material, and post-PCR quality and yield assessments are made with an agarose gel.

DNA quantity and quality may be measured with a spectrophotometer such as the NanoDrop. The A260/280 ratio should be above 1.8 to ensure the quality of the DNA. (Note: the spectrophotometer may represent an overestimate of double-stranded DNA. There are other methods, such as Picogreen® dsDNA Quantitation Reagent or Qubit®, which provide a more accurate assessment of true double-stranded DNA yield.) The percentage of double-stranded material compared to other nucleic acid moieties in the sample will vary depending on tissue type, fixation method, degradation profile, and calibration of the spectrophotometer. Therefore, if your research laboratory has the capability to perform Picogreen® dsDNA Quantitation Reagent or Qubit®, we encourage these methods as alternatives to gain accuracy in DNA yield. The yield requirement for the assay (250 ng at 50 ng/ul) does not vary from the user manual.

If, when starting the CytoScan assay with 250 ng of gDNA quantified by spectrophotometry, you routinely get a lower than expected amount of amplified product, we suggest either increasing the starting material to 1 μg or utilizing one of the alternative quantitation methods mentioned above.

The yield of PCR product is directly correlated to the quality of array data with respect to functional resolution, sensitivity, and specificity. The SNPQC and Median Absolute Pairwise Difference (MAPD) represent measurements of signal-to-noise, which have a direct association to functional resolution, sensitivity, and specificity.
Results and data analysis

Normalization with reference sets

CytoScan® Cytogenetics Solution includes Affymetrix® Chromosome Analysis Suite (ChAS) Software, which is used for processing and interpreting the data across a wide range of cytogenetic research applications. The analysis methodology utilizes an external reference set to serve as a universal baseline for making copy number estimates across the genome. The use of the external reference set adds flexibility to interpretation since different baseline samples can be incorporated over time.

The default reference set in ChAS Software was derived from phenotypically healthy whole-blood and cell line samples. This enables processing across a wide variety of different samples, including blood, amniocytes, chorionic villus sampling, buccal, saliva, bone marrow, and fresh tissue.

Since FFPE samples introduce different sources of variability relative to the default blood/cell reference set included with the software, we have developed an FFPE-specific reference set from 160 normal tissue renal, breast, lymph, and colon samples from multiple laboratories. The FFPE universal reference set can be downloaded as CEL files from www.affymetrix.com.

The individual CEL files can be used to create separate tissue-specific reference sets depending on your application; these files are also available for download at www.affymetrix.com. Furthermore, reference sets may also be developed by your own laboratory to capture the fixation method and additional sources of variability that the universal reference set may not completely reflect. New reference sets can be built within ChAS Software as outlined in the software user manual Affymetrix® Chromosome Analysis Suite 1.2.2 User Manual, PIN 702943, which can be downloaded from http://media.affymetrix.com/support/downloads/manuals/chas_software_user_manual.pdf. However, when getting started with FFPE samples, we highly recommend first using the universal FFPE reference set outlined here to determine whether or not such improvements are necessary.

In order to start analyzing data from FFPE samples in ChAS Software the following steps should be followed:

The SNPQC metric is based on a constitutional cytogenetics assumption of having homozygote clusters across the majority of the genome, which is not the case for cancer samples due to heterogeneity and biological complexity. Therefore, samples may not reach the SNPQC threshold of 15. For this reason we suggest that you initially disable the SNPQC metric in the software. Each lab will have its own baseline of SNPQC as a function of the FFPE sample source, so we recommend monitoring the values over time. These data points can be used to establish a new baseline for SNPQC. Once this baseline value has been defined, SNPQC should be re-enabled in ChAS Software.

The Waviness SD metric is likely to exceed the default threshold 2. Within ChAS Software, load the CEL files in the reference set creation utility as shown in Figure 3. Note: Once the CEL files are processed and converted into a reference set, steps 1 and 2 no longer need to be followed for subsequent runs of the software.

3. Begin analyzing your FFPE tumor samples by selecting the reference set you just created during the CEL file processing step. Tip: When creating your FFPE reference set, make sure to assign it an intuitive name (such as “FFPE Universal Reference”) so you can identify your built reference within the software for future runs of FFPE tumor samples.

Figure 4 (see page 4) shows example data with the default reference set as well as the FFPE reference set.

Recommendations for array QC and analysis parameter adjustments
ChAS Software reports three QC metrics, which provide insight into performance for each CytoScan® Array. The metrics are Median Absolute Pairwise Difference (MAPD), SNPQC, and Waviness SD. The MAPD metric provides an assessment of signal-to-noise for log2 ratios relative to the selected reference set. This is the key metric for copy number functional resolution, and we recommended that you use it primarily for assessing CytoScan Array performance with FFPE samples, particularly because MAPD is robust against high biological variability in log2 ratios induced by conditions such as cancer. The default QC threshold within the ChAS Software for MAPD has been established from blood-derived samples, so for use of FFPE samples, we recommend tracking your laboratory MAPD values over time to establish a baseline of acceptable performance, which can then be monitored for deviations. This application note is intended to provide guidelines on the use of FFPE on CytoScan® Cytogenetics Solution.

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The Waviness SD metric is likely to exceed the default threshold 2.
settings and not reflect technical variation but rather complex biological effects such as heteroploidy and tumor heterogeneity when using FFPE samples. For this reason, we recommend disabling the Waviness SD metric.

**Conclusion**

This application note demonstrates an approach when using FFPE-derived DNA with the CytoScan® assay in research applications. With FFPE samples, CytoScan® Cytogenetics Solution shows improved data quality when carefully quantitating DNA, implementing QC steps, and applying an FFPE-specific reference set and data analysis parameters. The assay protocol modifications described in this application note are easy to implement in your lab with no need for additional equipment or software. Visit [www.affymetrix.com/cytoscan](http://www.affymetrix.com/cytoscan) to learn more about CytoScan Cytogenetics Solution.

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