Unlocking formalin-fixed, paraffin-embedded (FFPE) samples with the CytoScan HD Cytogenetics Solution

Introduction

The Applied Biosystems™ CytoScan™ High-Density (HD) Cytogenetics Solution is an analysis research tool used for genome-wide, high-resolution detection of DNA copy number and single-nucleotide polymorphisms (SNPs). With 2.68 million markers that have been designed to detect copy number gains and losses, loss of heterozygosity (LOH), regions identical by descent, and uniparental disomy (UPD), the CytoScan HD Array provides genome-wide coverage of OMIM™ and RefSeq constitutional and cancer research 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 the CytoScan assay with FFPE-derived DNA and details a modified workflow that has been developed to help ensure high-quality research data. This workflow includes a DNA isolation method, quality control steps, and data analysis parameters. These workflow modifications have been verified for research with FFPE samples less than 5 years old. For copy number applications with cancer research samples more than 5 years old, we recommend Applied Biosystems™ OncoScan™ FFPE Services.

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 this procedure, see the user manual for CytoScan DNA Purification From FFPE Tissue (Pub. No. 703117), available at **thermofisher.com/manuals**. 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 is quantified by spectrophotometry.

Quality control

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

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

DNA quantity and quality may be measured with an instrument such as the Thermo Scientific™ NanoDrop™ spectrophotometer. The A_{260}/A_{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 with the Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Reagent or Invitrogen™ Qubit™ 4 Fluorometer, that provide more accurate assessment of true double-stranded DNA yield.) The percentage of double-stranded DNA compared to other nucleic acid species in the sample will vary depending on tissue type, fixation method, degradation profile, and calibration of the spectrophotometer. Therefore, if your laboratory has the capability to perform Quant-iT or Qubit assays, we encourage these methods as alternatives for more accurate assessment of DNA yield. The yield requirement for the assay (250 ng at 50 ng/µL) does not vary from that stated in the user manual.

If, when starting the CytoScan assay with 250 ng of gDNA quantified by spectrophotometry, the amount of amplified product is routinely lower than you expect, we suggest either increasing the starting material to 1 µg or quantitating by one of the alternative methods mentioned previously.

The yield of PCR product is directly correlated to the quality of subsequent array data with respect to functional resolution, analytical sensitivity, and specificity. The SNPQC and Median Absolute Pairwise Difference (MAPD) represent measurements of signal-to-noise ratios, which are directly associated with functional resolution, analytical sensitivity, and specificity.

Figure 1 shows an example of SNPQC and MAPD as functions of PCR yield. For the SNPQC metric, higher numbers represent higher signal-to-noise ratios; conversely, for the MAPD metric, higher numbers represent lower signal-to-noise ratios. The QC metrics and significance of each are further explained in the data analysis section.

Once the PCR step of the CytoScan assay has been completed, a 2% TBE agarose QC gel should be used to determine the quantity and quality of the PCR product. You should also verify that the PCR product distribution is between 500 bp and 2,000 bp, as shown in Figure 2. If the sample does not pass this step, do not proceed with array hybridization.

The gel profile may vary depending on the percentage of double-stranded gDNA relative to the rest of the sample, which varies depending on tissue type, fixation method, degradation profile, and calibration of the spectrophotometer. Therefore, in an effort to recover samples that have not passed this QC gel stage, we suggest either increasing the assay starting material to 1 µg or quantitating the DNA by one of the suggested methods such as the Quant-iT or Qubit assay.

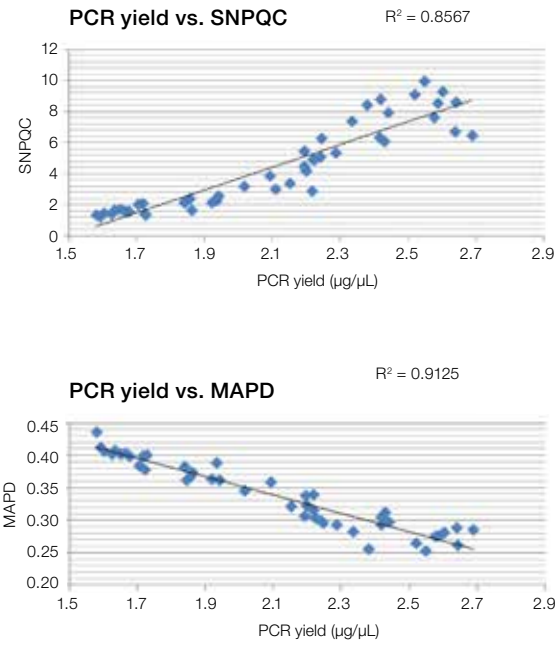

Figure 1. Assay QC parameters as a function of PCR yield.

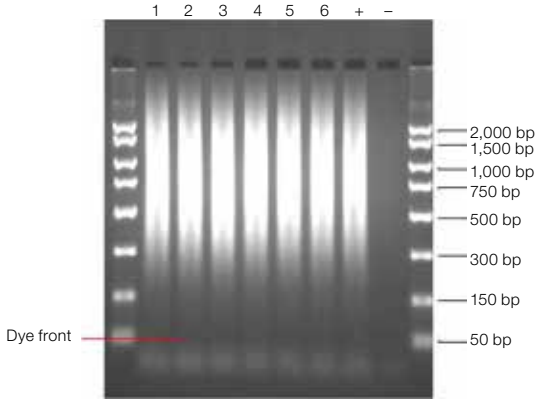

Figure 2. Example of a gel showing PCR products that pass QC.

Results and data analysis

Normalization with reference sets

The CytoScan HD Cytogenetics Solution includes Applied Biosystems™ 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. The default reference enables processing across a wide variety of different samples, including blood, amniocytes, chorionic villi, buccal cells, saliva, bone marrow, and fresh tissue.

Since FFPE samples introduce different sources of variability relative to the default blood and 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; please contact your field applications scientist (FAS) for access.

The individual CEL files can be used to create separate tissue-specific reference sets depending on your application. 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 user manual. 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.

To start analyzing data from FFPE samples in ChAS Software, simply follow the reference-building instructions outlined in the ChAS Software user manual. When building the reference, be sure to use the desired CEL files whether for universal, tissue-specific, or lab-specific reference sets. Once the CEL files are processed and converted into a reference set, the reference no longer needs to be built for subsequent runs of the software.

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 3 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 MAPD, SNPQC, and Waviness SD. The MAPD metric provides an assessment of signal-to-noise for $\log_2$ 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 $\log_2$ ratios induced by conditions such as cancer. The default QC threshold within 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 with the CytoScan HD Cytogenetics Solution.

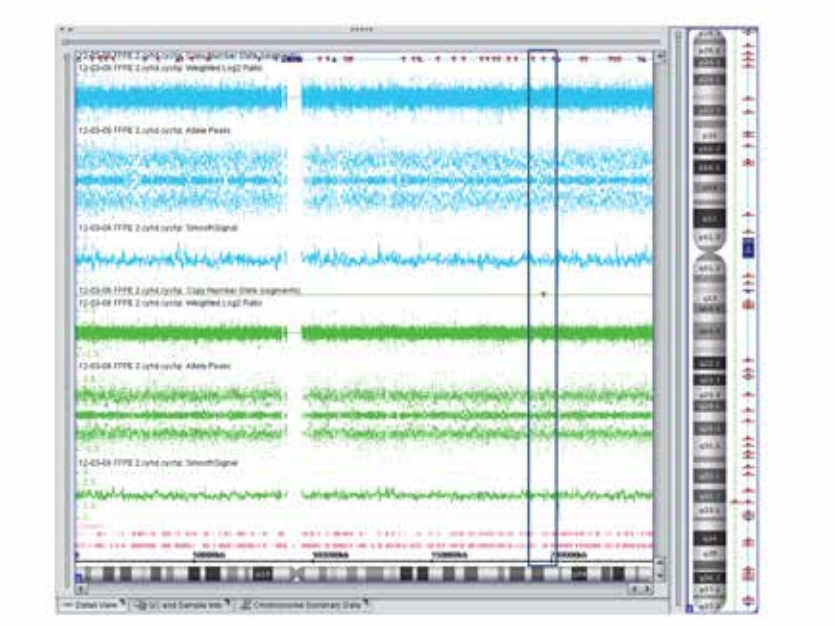

Figure 3. Example data without the tissue-specific reference set and with the reference set implemented. In this figure, the $\log_2$ ratios and allelic difference plots are provided for the case where an FFPE tumor sample is analyzed relative to the standard software-supplied blood reference set (top panel, blue), and this same FFPE tumor sample is analyzed relative to the recommended FFPE reference set outlined in this application note (bottom panel, green). This figure illustrates the improvement in signal-to-noise ratio when using an FFPE reference set that substantially improves data quality. In this example, the use of the FFPE reference set resulted in the one correct copy number call being summarized while the blood-derived reference set resulted in additional calls. This increase in CNV call specificity for the FFPE reference set is highlighted in the vertical rectangular selection.

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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 research 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 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. Table 1 provides a summary of reported QC metrics.

Table 1. Summary of QC metrics.

QC metric	Utility	Recommendation
MAPD	Assesses signal-to-noise for $\log_2$ ratios between adjacent genomic markers relative to selected reference set.	Use this as the primary metric for copy number functional resolution.
SNPQC	Estimates the distributions of homozygous and heterozygous alleles and calculates the distance between them.	Disable initially; collect SNPQC values and monitor over time. Use these data points to establish a new SNPQC threshold.
Waviness SD	Measures genome-wide variation of probes.	Disable when analyzing FFPE-derived samples.

Conclusion

This application note demonstrates an approach for using FFPE-derived DNA with the CytoScan assay in research applications. With FFPE samples, the CytoScan HD Cytogenetics Solution shows improved data quality when DNA is carefully quantitated, QC steps are implemented, and an FFPE-specific reference set and analysis parameters are used. The assay protocol modifications described in this application note are easy to implement in your lab without additional equipment or software. Visit **thermofisher.com/cytoscan** to learn more about the CytoScan HD Cytogenetics Solution.

Acknowledgment

Dr. Federico Monzon, Director of Molecular Pathology of the Cancer Genetics Laboratory at Baylor College of Medicine, was instrumental in offering his guidance toward optimizing this workflow.

Find out more at **thermofisher.com/rh**

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