Key Determinants for Successful Formalin Fixed Paraffin Embedded Tissue Sample Prep for Downstream Applications

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Introduction

Formalin-fixed paraffin embedded (FFPE) tissue samples allow the analysis of archival tissue samples that would otherwise be too costly to maintain. However, obstacles need to be addressed when using, selecting, and processing FFPE tissues for down-stream applications. Formalin fixation results in fragmentation and crosslinking between intra-cellular macromolecules, rendering molecular assays challenging. Due to the challenges that researchers face when working with FFPE samples, we sought to determine if there are factors that predict success for genomic screening. We reviewed the outcome of over 2,000 molecular assays performed at BioIVT using nucleic acids derived from FFPE samples to determine correlation of screening results with the following factors: 1) percentage of necrosis, 2) time of fixation, 3) recovery type, and 4) age of block. We were unable to identify a single dominant factor that determines successful outcomes in downstream molecular applications, including surprisingly the age of the block. Due to these findings, we believe that the best way to obtain results from FFPE samples is by working with an experienced team able to provide proper sample selection (including sample diagnosis, samples size, and tissue composition) and tailored nucleic acid isolation.

Methods

This study used a quantitative descriptive design to review the outcomes of molecular annotation assays conducted over a three year time period. The data were reviewed against single factors that have been speculated to affect downstream results. In all studies nucleic acid extractions were performed from formalin-fixed paraffin embedded (FFPE) specimens for qPCR or Next Generation Sequencing assays. The samples used in this study are representative of archival FFPE tissue samples collected using various methods and time points of fixation that can be applied to all archival collections.

For the qPCR dataset, 2,893 samples were included. For the NGS dataset 681 samples were included.

Key determinates for evaluation are defined below:

Sample Necrosis is the estimated percentage of the entire specimen that is necrotic. The value is determined by a board certified pathologist.

Fixation Time is the time in hours that a sample was exposed to formalin and/or formaldehyde

Recovery Type represents surgical samples preserved according to a known protocol versus surgical archive samples that may not have been preserved according to a known protocol

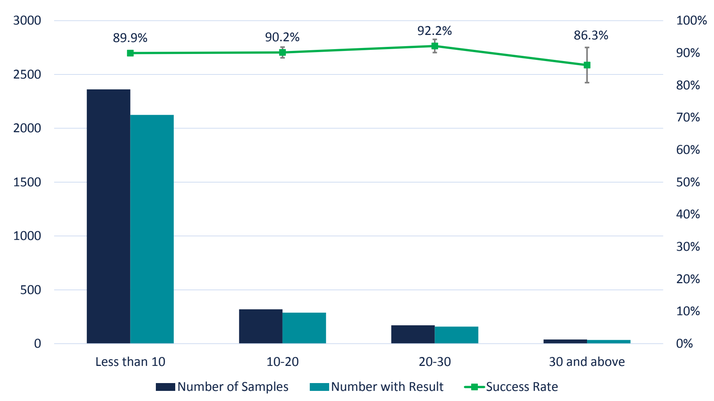
Age of the block measures the time from fixation to nucleic acid isolation

In-house isolations were performed using one 40µm section, one 20µm section, or two 10µm sections. DNA was isolated using either QIAGEN QIAamp DNA FFPE Tissue kit or QIAGEN Allprep DNA/RNA FFPE Kit, according to manufacturer's instructions. External isolation of DNA from FFPE samples used two to four 5µm sections on slides and were macro-dissected for input with a Siemens Tissue Preparations System.

The data resulting from each assay were compiled and reviewed for successful analysis. Samples that yielded a result were reviewed as successful and samples that were not able to generate a result (inconclusive) were deemed unsuccessful. The success probability and interval was estimated using the Wilson score.

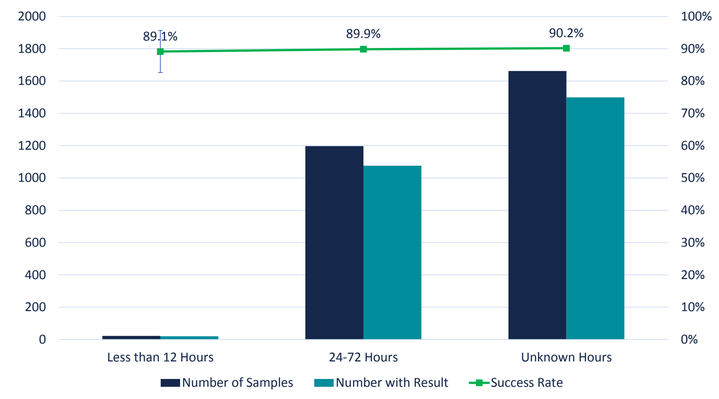
Results & Discussion

Percentage of Necrosis and qPCR Results



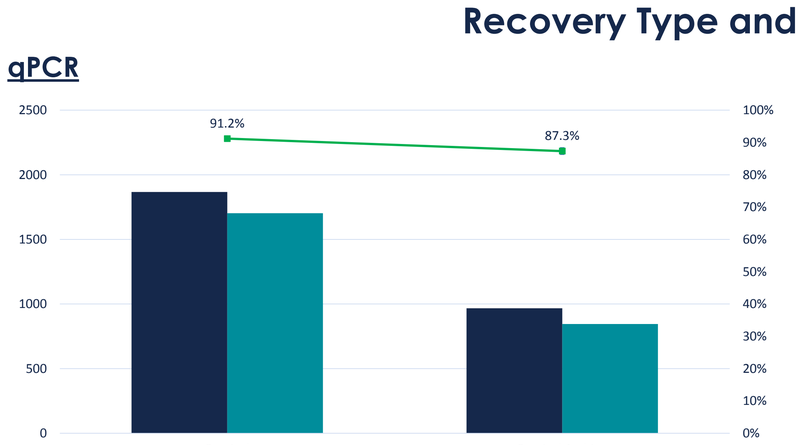
Necrosis levels below 30% do not have a negative impact on successful molecular assay results. The data demonstrate that the success rate trends lower as the level of necrosis increases. (BioIVT standard QC policies quarantine samples with a necrosis rate above 30% upon initial pathology review).

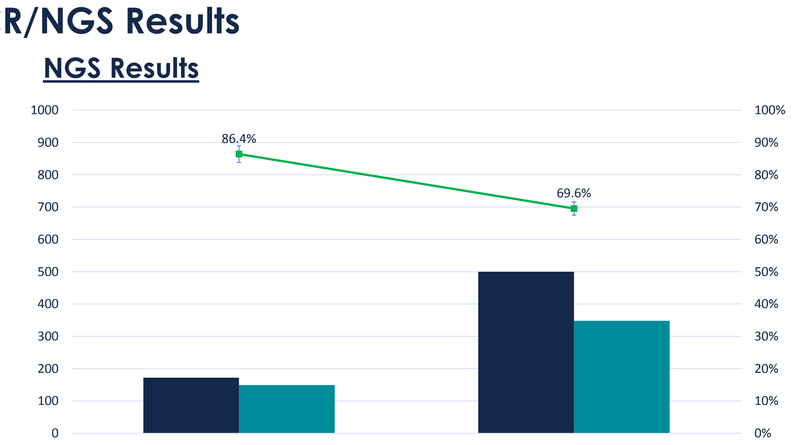
Fixation Time and qPCR Results



Fixation time was assessed as the amount of time samples were placed in formalin/formaldehyde. Standard fixation protocol for ASTERAND™ Human Tissue Samples is 12 and 72 hours. For archived samples, the time of fixation may be unknown. As no significant difference exists between cohorts, fixation time is not a good indicator of success rate. Further slicing of the 12-72 hour cohort (data not shown), was unable to determine a significant difference.

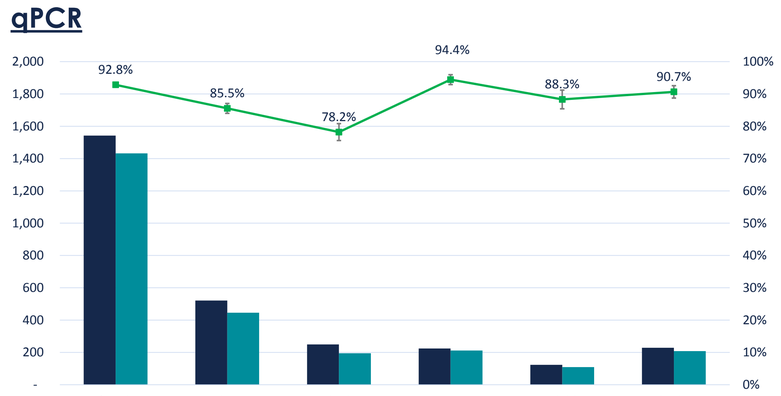
Recovery Type and qPCR/NGS Results

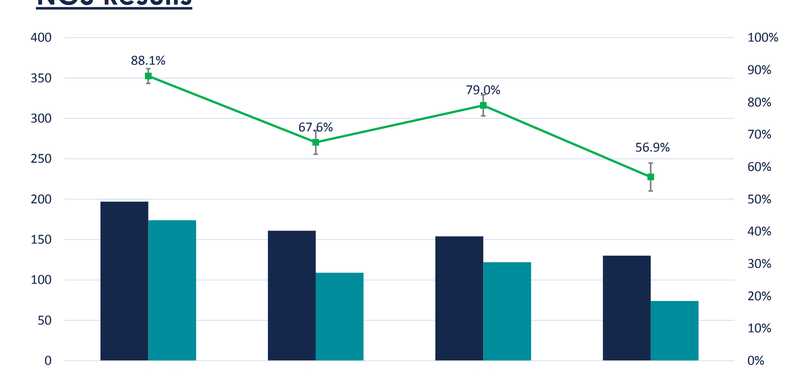




Recovery type was split into two separate cohorts: surgical and surgical archive. Surgical samples are excised and preserved according to a known protocol whereas surgical archive samples may not be. We sought to determine whether a difference in known processing would allow us to determine a cohort that is more likely to provide results. When assessing qPCR results, there was no difference between surgical and surgical archived samples to warrant choosing one cohort over the other. However, when assessing the NGS results, testing surgical samples (or at least samples with a known protocol) may allow for more samples with results. NGS assays may be more sensitive to fragmentation due to the complexity of the assay and often a requirement for longer amplicons. Further, NGS technology may be slightly behind qPCR when it comes to optimization for FFPE samples.

Age of the Block and qPCR/NGS Results





Age of Block at time of testing was assessed as the time between sample fixation and nucleic acid isolation in the laboratory for testing. The success rate does not follow a linear trend downward as the blocks get older, thus the age of the block is not a determining factor for success. This is true for qPCR as well as NGS assays.

Conclusions

An extensive bank of archived tissues containing valuable information can benefit research in clinical and molecular assays. But questions remain about the factors that lead to successful assays. The samples used in this study are representative of archival FFPE tissue samples collected using various methods and time points of fixation. We were unable to identify a single dominant factor that determines successful outcomes in downstream molecular applications; including, surprisingly, the age of the block. Our parameters included percent of necrosis, time of fixation, recovery type, and overall age of the block.

Despite not identifying a single variable that altered success, BioIVT has successfully obtained both qPCR and NGS results through our careful selection, isolation and screening process. We suggest that the best way to reach a goal for known screening results is to integrate the following steps:

1. Careful sample selection, including refined sample diagnosis, samples size, and tissue composition
2. Careful determination of proper isolation method for type of block and downstream analysis