Abstract # 5279

Establishing Robust Pharmacodynamic Immunofluorescence Assays of Clinical Biopsies at the National Cancer Institute

Optimized Quality Control Procedures for the Evaluation of DNA Damage Response and Epithelial Mesenchymal Transition (EMT) Biomarkers

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Introduction

Robust PD assay results are valuable for informing decisions about the development of new agents and combinations. The National Cancer Institute's Division of Cancer Treatment and Diagnosis (DCTD) develops and validates PD assays to obtain information about drug effect on intended molecular targets in first-in-human trials, which will enable clinical decisions. Our group utilizes quantitative immunofluorescence assays (qIFA) of PD biomarkers in FFPE slides prepared from pre- and post-dose tumor biopsies collected from patients on early-phase clinical trials. Stringent methods are employed during fixation, blocking, and microtomy to maximize the success rate of generating sufficient slides with optimal areas for analysis and to ensure proper preservation of labile epitopes such as phosphorylated proteins. Flanking H&E slides are utilized to assess the quality of the tumor biopsies. Pathology-guided analysis, as appropriate for the intended molecular measurements, allows the assay operator to focus on tumor regions of interest and avoid normal tissue or other confounding regions compromised by sample-handling artifacts and helps to ensure a non-subjective analysis.

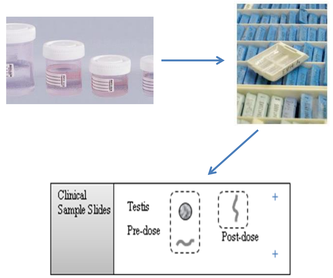
1 Robust procedures have been established for the collection and preservation of tumor needle biopsy specimens for pharmacodynamic biomarker analyses: DCTD SOP340507

Images (left, middle) provided by Dr. Melinda Hollingshead, Ph.D., DVM, BTB, NCI, showing collection method used for preclinical tumors. This process is mirrored as closely as possible during the collection of human tumor biopsies.

- Pairs of biopsies are collected from patients on early-phase trials, at two time points.
- Collection method originally validated using mouse xenograft models, with evaluation of ischemia time and impact on biomarker stability.
- Biopsies are collected, placed in pre-chilled cryogenic vials, and frozen within 2 minutes of collection.
- Samples are stored at or below -80°C prior to analysis.
- Biopsy pairs are analyzed in parallel in all pharmacodynamic assays.

Figures and Discussion

2 Optimized procedures for FFPE blocking and microtomy in preparation for quantitative pharmacodynamic IFA analyses of DNA damage–induced biomarkers: DCTD SOP340522



- Pre- and post-dose biopsies are fixed and paraffin blocked in parallel with a positive biomarker control tissue.
- Positive control acts as a fixation and staining control for evaluation of positive biomarker staining on each clinical slide.
- Methods applied to help maintain optimal and full-face longitudinal presentation of the biopsy during sectioning.
- Sections from two blocks containing the pre- and post-dose are sectioned onto a series of slides in parallel.

3 Sectioning schema and IFA use designations based on H&E evaluations: DCTD SOP340522

Slide Designations:
Slide numbers refer to **order** in the optimal 35-slide range based on visual inspection

H&E	IFA	Backup-1	Backup-2	Backup-3
1	2	3	4	5
	6	7	8	9
	10	11	12	13
	14	15	16	17
18	19	20	21	22
	23	24	25	26
	27	28	29	30
	31	32	33	34
35				

- 50 slides are prepared to allow for evaluation of a significant portion of the tumor biopsy.
- 35 slides are designated as optimal by the histology laboratory based on a visual inspection of the slides.
- The designated range should have sufficiently large pre- and post-dose sections at or near full-face longitudinally and contain sufficient control tissue for the IFA analysis.
- **Steps are taken to ensure the proper storage of the paraffin slides, including paraffin dipping after microtomy, which is particularly critical for the preservation of labile epitopes, such as phosphorylated MET.**

Slide Designations:

H&E	IFA	Backup-1
1	2	3
	4	5
	6	7
	8	9
	10	11
	12	13
	14	15
	16	17
18		

This designation scenario is used:
- If there are fewer than 35 slides prepared that meet the visual inspection criteria.
- When slide 35 fails to meet sufficiency based on H&E review process, but slides 1 and 18 are sufficient.

Slide Designations:

H&E	IFA	Backup-1
18	19	20
	21	22
	23	24
	25	26
	27	28
	29	30
	31	32
	33	34
35		

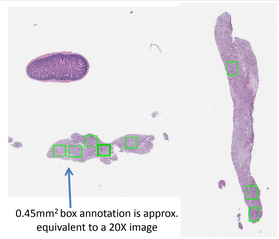
This designation scenario is used:
- When slide 1 fails to meet sufficiency based on H&E review process, but slides 18 and 35 are sufficient.

4 H&E slide quality control review by a pathologist

Representative slide for a pre-dose and post-dose biopsy pair for a patient on a clinical trial protocol. The positive control tissue in this study was mouse jejunum.

- The following H&E review criteria are customized to the intended biomarker measurements:
  - ✓ Presence of neoplastic tissue.
  - ✓ Morphology of each section should indicate sufficient nuclear and cellular definition
  - ✓ Sufficient cellularity should be present in each section so that at least one 20x field (~0.45mm²) can be analyzed per slide.
  - ✓ Minimum number of regions of interest for analysis are preset by the quantitative biomarker analysis protocol (typically 8).

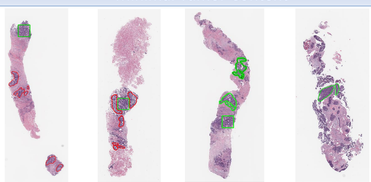
5 H&E Slide Annotation



0.45mm² box annotation is approx. equivalent to a 20X image

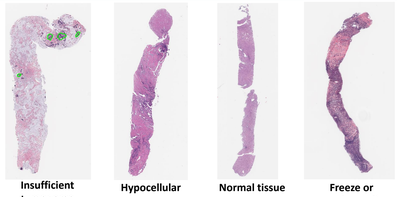
- The clinical pathologist determines whether the sections are sufficient for the intended analysis.
- The review allows the assay operator to select the optimal range of slides to stain and quantitatively analyze for the biomarker(s) of interest.
- Pathology-guided regions allow the assay operator to focus on tumor regions of interest and avoid normal tissue and other confounding regions.
- For nuclear biomarkers, such as γH2AX and related DNA damage repair biomarkers, the pathologist annotates areas of sufficient tumor content and viability.

6A Common Findings during H&E QC Review: Minimal Tumor Content



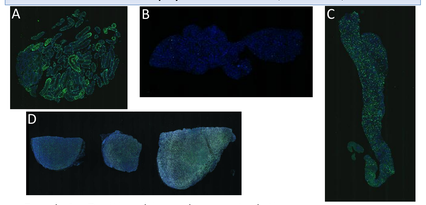
- Many samples are found with small areas of analyzable tumor area. In this case, the operator will use the annotated H&E image to locate these analyzable areas of the tissue.
- Quantitative biomarker reporting requires analysis of a minimum of 8 analyzable tumor areas from each tissue.
- Next-generation assays currently in clinical feasibility testing will also rely on software and biomarker-driven tumor area masking for quantitative biomarker analysis of the tumor areas of these highly heterogeneous specimens.

6B Common Findings during H&E QC Review: Insufficient for Analysis



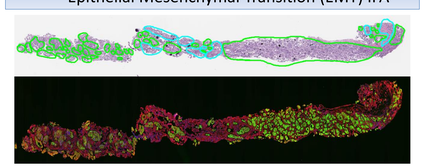
- In these cases, the specimens do not advance to the assay operator for analysis, which eliminates the analysis and possible reporting of inappropriate biomarker data.
- The clinical sample data report for these patient samples will provide an explanation to the clinical investigator regarding the insufficiency of the tumor materials for analysis.

7 Quantitative γH2AX Analysis of a Pre-dose and Post-dose Biopsy: SOP340523, 340533, 340534



- Panel A: Positive biomarker control tissue, mouse testes, is processed in parallel with each pair of clinical biopsies. This tissue shows consistently strong staining for γH2AX, serving as a fixation and staining control for each clinical slide.
- Panel B shows a pre-dose and panel C shows a post-dose biopsy from a patient on clinical trial. The drug treatment led to an increased level of γH2AX in this patient's tumor, which is analyzed and reported using clinically validated methods.
- Panel D: Drug response calibrator tissues showing low, mid, and high levels of γH2AX in mouse xenograft tumor quadrants derived from vehicle- and drug-treated animals. These calibrators serve as a reference standard for drug effect on target and are included in each run of clinical slides.

8 H&E Slide Review in Preparation for Epithelial Mesenchymal Transition (EMT) IFA



- Representative H&E slide annotated by a pathologist is shown in the top panel, and a slide from the same patient stained for a multiplex assay to assess EMT is shown in the bottom panel.
- In this case the pathologist annotates areas to exclude from the analysis, such as normal tissue; stroma; necrotic areas and processing artifacts (blue); and all tumor areas (green).
- The quantitative IFA to assess tissue architecture is performed from a whole slide scan and involves analysis of the entire tumor biopsy, excluding only the areas inappropriate for analysis as designated by the pathology review.

Summary and Conclusions

- Our group supports the development, validation, and implementation of robust PD assays to evaluate drug mechanism of action during clinical trial evaluations of new drug regimens. Quantitative IFA measurements in needle biopsies is one such assay type.
- Integral quality control procedures are applied during each phase of these quantitative qIFA methods to help ensure quality from biopsy collection, tissue preservation via FFPE, microtomy, and H&E review through qIFA imaging, analysis, and reporting.
- Using pathology guidance to guide the assessment of the biopsy tissues, both to eliminate the analysis of inappropriate specimens and to guide the analysis of sufficient samples via annotation of a whole slide image, is a critical tool in our qIFA workflow.
- This process helps to streamline the analysis process and helps to ensure a more accurate and non-subjective analysis of tumor areas for pharmacodynamic biomarker modulation, both as applied to the evaluation of DNA damage response biomarkers, such as γH2AX, and as applied to whole tissue assessments, such as EMT.

References

1. DCTD Standard Operating Procedures SOP340507, 340522, 340523, 340534, 340534: http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm
2. Development of validated immunofluorescence assay for γH2AX as a pharmacodynamic marker of topoisomerase I inhibitor activity; Kinders RJ et al., 2010, Clin. Can. Res., 16(22); 5447.
3. Quantitative immunofluorescence assessment of MET and epithelial mesenchymal transition (EMT) biomarker modulation by antiangiogenic inhibitors in xenograft tumor tissues; Navas T et al., 2014, Cancer Res., 74; Abstract 1049.
4. Impact of HGF Knock-in Microenvironment on Epithelial-Mesenchymal Transition and Cancer Stem Cells in a Non-Small Cell Lung Cancer Xenograft Model; Navas T et al., 2015 AACR Abstract 5082.

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