

# Development of Calibrators and Controls as Quality Control Tools for Clinical Implementation of Quantitative Immunofluorescence Assays for Pharmacodynamic Biomarkers of Molecular Targeted Agents in Tumor Biopsies

Ferry-Galow, K.V.<sup>1,\*</sup>, Navas, T.<sup>1,\*</sup>, Lawrence, S.M.<sup>1,\*</sup>, Mutreja, K.<sup>1</sup>, Butcher, D.<sup>2</sup>, Hollingshead, M.<sup>3</sup>, Parchment, R.E.<sup>1</sup>, Tomaszewski, J.E.<sup>4</sup>, Doroshov, J.H.<sup>4</sup>, and Kinders, R. J.<sup>1</sup>

<sup>1</sup>Laboratory of Human Toxicology and Pharmacology and <sup>2</sup>Pathology/Histotechnology Laboratory, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research,

Frederick, Maryland 21702; <sup>3</sup>Biological Testing Branch, Developmental Therapeutics Program, National Cancer Institute, Frederick, Maryland 21702;

<sup>4</sup>Division of Cancer Treatment and Diagnosis and Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892, \*Authors contributed equally



**SAIC** Frederick

## Introduction

The application of robust pharmacodynamic (PD) assays as part of clinical trial design is valuable for confirming putative drug mechanisms in patients. Our group supports the development, validation, and clinical implementation of PD assays, including quantitative immunofluorescence (qIFA) measurements in formalin-fixed, paraffin-embedded (FFPE) human tumor biopsies. One such assay is a multiplex qIFA method, referred to as Rx1-IFA4, for evaluating biomarker modulation in response to DNA-damaging agents.

RX1-IFA 4 Panel ID	Antigen Description	PD Marker
$\gamma$ H2AX	Histone H2AX phosphorylated at Ser139	DNA Damage (ds breaks)
cCasp3	Cleaved Caspase 3	Apoptosis
Ki67	Nuclear protein associated with cellular proliferation	Proliferative Index
DAPI	Fluorescent stain for DNA	Nuclear mask

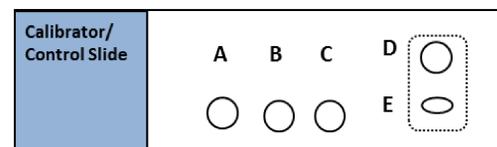
- Integral quality control tools developed and validated to support clinical implementation of a multiplex PD IFA include:
  - Xenograft tissue calibrators, which serve as reference standards representing a range of biomarker expression levels in a known responsive model with an active drug
  - Positive and negative controls for each biomarker included on the calibrator slide and on each clinical slide which serve as QC checks for analytical performance
- Methods to reproducibly produce, qualify, and store the calibrator/control slides have been developed.

## Materials and Methods

- Human breast cancer cell line MDA-MB-231 was used to produce xenograft tumors in athymic nude mice. Animals were treated with vehicle or a single dose of a CIAP inhibitor at 3 or 20 mg/kg and tumors were collected, quartered, and flash frozen 7 hr post-dose.<sup>1</sup>
- Tumor quadrants and control tissues were FFPE treated, and slides were prepared as described.<sup>2,3</sup>
- Slides were stained using a Bond-Max Autostainer (Leica Microsystems) with biotinylated-  $\gamma$ H2AX mAb (Millipore, #16-193) plus Alexa-660-streptavidin (Invitrogen, #S21377), anti-Ki67-FITC (Abcam, clone SP6), anti-cCasp3 (R&D Systems, #MAB835) plus Alexa-546 anti-rabbit (Invitrogen, #A-11035), and DAPI.
- Images were captured using a Nikon Eclipse 80i (Nikon) microscope and Q-Imaging Retiga-2000R fast 1394 mono-cooled camera (Qimaging), and analyzed using a custom macro developed with Image-Pro (Media Cybernetics) to quantitate % nuclear area positive (%NAP) for  $\gamma$ H2AX and Ki67, and % cytoplasmic area positive (%CAP) for cCasp3.

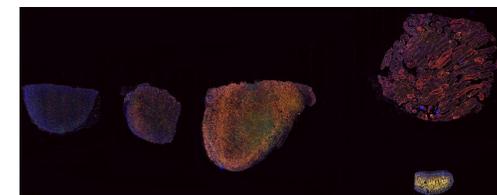
## Results

### 1 Calibrator/Control Slide Layout



Section	Tissue Description
A	MDA-MB-231 Xenograft, Vehicle
B	MDA-MB-231, 3 mg/kg CIAP Inhibitor
C	MDA-MB-231, 20 mg/kg CIAP Inhibitor
D	Mouse Testes
E	Mouse Jejunum

### 2 Rx1-IFA4 Calibrator/Control Aperio® Image



Panel ID	Color	Reporter
$\gamma$ H2AX	Red	Alexa-660 conjugated streptavidin
cCasp3	Yellow	Goat anti-rabbit Alexa-546
Ki67	Green	FITC direct conjugate
DAPI	Blue	DAPI

### 3 Pilot Lot Calibrator/Control Slide Performance Data

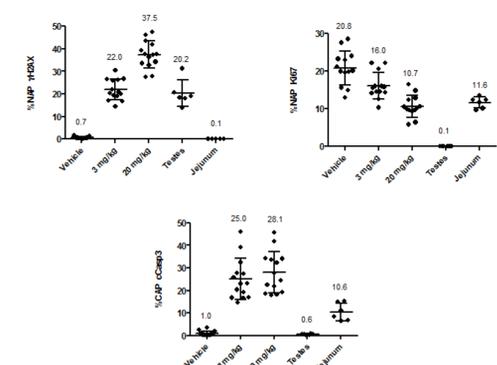


Figure 3: Rx1-IFA4 data for  $\gamma$ H2AX (%NAP – nuclear area positive), Ki67 (%NAP), and cCasp3 (%CAP – cytoplasmic area positive) as enumerated by image capture followed by analysis with a custom Image-Pro macro. Analyses of 15 sets of xenograft tumor calibrators and 6 mouse tissue controls are summarized.

### 4 Comparison of Performance of Two Lots of Calibrator/Control Slide Blocks

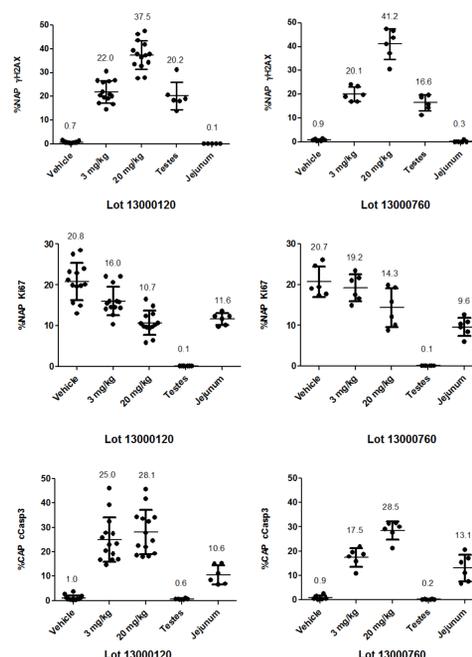


Figure 4: Rx1-IFA4 data showing comparable performance for  $\gamma$ H2AX, Ki67 and cCasp3 across two lots of slide blocks. Individual calibrator blocks were prepared from different tumor quadrants derived from the same drug-treated animal. Lot 13000120 (15 Analyses) and Lot 13000760 (6 Analyses) results, and the mean value for each group are shown.

### 5 Pilot Lot Calibrator/Control Slide Stability Data

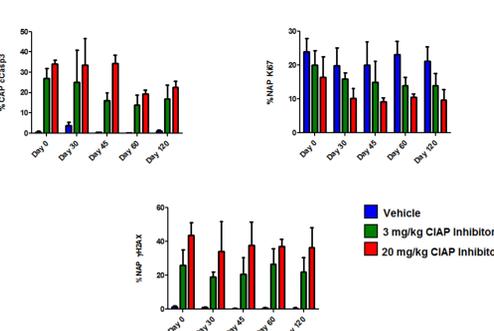


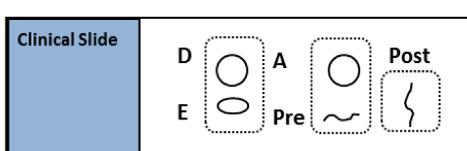
Figure 5: Rx1-IFA4 data showing comparable performance for cCasp3, Ki67, and  $\gamma$ H2AX analyzed over a period of 120 days. Based on a drop in %CAP signal for cCasp3 at Day 60, the stability of cut calibrator/control slides was set at 6 weeks.

### 6 Rx1-IFA4 Calibrator/Control Performance Specifications and Qualification Procedure

Section	$\gamma$ H2AX Criteria (%NAP)	cCasp3 Criteria (%CAP*)	Ki67 Criteria (%NAP)
A	$\leq 2\%$	Negative	$>10\%$
B	Set by Lot ( $>8\%$ NAP & $\geq 10\%$ Vehicle for 13000120)	Positive	--
C	Set by Lot ( $>20\%$ NAP & $\geq 25\%$ Vehicle for 13000120)	Positive	--
D	$>10\%$	Negative ( $\leq 4\%$ )	--
E	$\leq 1\%$	Positive ( $>4\%$ ) & $\geq 10\%$ Neg	$\leq 1\%$

Figure 6: Sub-lots of Rx1-IFA4 slides were produced from qualified calibrator/control blocks in increments of 20 or 40 slides at a time. To qualify a sub-lot, slide 1 and every 10<sup>th</sup> thereafter were stained and assayed. Specifications, derived during assay validation, were verified for **POSITIVE** and **NEGATIVE** control tissues for all biomarkers, as shown in color-coded values in the table above. Acceptable range of %NAP for  $\gamma$ H2AX for the xenograft calibrators were set for each sub-lot as a guidance for the operator for expected enumeration of this primary biomarker. Additionally, a digital Aperio® scan was generated for each sub-lot and used as a map to guide the operator to specific regions of optimal biomarker expression (also see Figure 2). \*cCasp3 assay readout is based on validation of a negative/positive cutoff of 4% CAP.

### 7 Rx1-IFA4 Clinical Slide Layout



Section	Tissue Description
Pre	Predose Biopsy
Post	Postdose Biopsy
A	MDA-MB-231 Xenograft, Vehicle
D	Mouse Testes
E	Mouse Jejunum

## Summary and Conclusions

- Our group supports the development, validation, and clinical implementation of robust PD assays to evaluate drug mechanism of action during clinical trial evaluations of new drug compounds and combination therapies. Multiplex qIFA measurements in needle biopsies of tumors collected pre- and post-dose is one such assay type.
- Integral quality control tools developed and validated to support clinical implementation of the multiplex qIFA Rx1-IFA4 include xenograft tumor calibrators derived from mice treated with compounds known to modulate the PD markers of interest and animal model-derived control tissues, which serve to verify the expected analytical performance for each biomarker on each calibrator/control and clinical slide.
- Methods developed to reproducibly generate, qualify, and store the xenograft calibrator/control slides for the multiplex qIFA Rx1-IFA4 were developed.
- Use of tumor quadrants allows for a large number of slides to be produced from a qualified calibrator/control block ( $\approx 150$  per block).
- Advantages over cell pellet slides derived from control or drug-treated cells include biomarker expression levels in cells rarely correlates with expression *in vivo* and accurate quantitative image analysis with Image Pro requires tissue context.

## References

- DCTD Standard Operating Procedure SOP340507; <http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>
- Development of a validated immunofluorescence assay for  $\gamma$ H2AX as a pharmacodynamic marker of topoisomerase I inhibitor activity; Kinders RJ, et al., 2010, *Clin. Can. Res.*, 16(22), 5447.
- DCTD Standard Operating Procedure SOP340522 (with modifications due to multiplex format); <http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>
- Prognostic and predictive impact of Ki-67 before and after neoadjuvant chemotherapy on PCR and survival: Results of the GeoparTrio trial; Von Minckwitz, G et al, 2012, *J.Clin. Onc.*, 30(15\_suppl), 1023.

Funded by NCI Contract No HHSN26120080001E

The Frederick National Laboratory for Cancer Research is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the USPHS Policy for the Care and Use of Laboratory Animals. All the studies were conducted according to an approved animal care and use committee protocol in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.).