

THE COMBINED EFFECTS OF FORMALIN FIXATION AND INDIVIDUAL STEPS IN TISSUE PROCESSING ON IMMUNO-RECOGNITION

DENNIS OTALI, ROB STOCKARD, DENISE OELSCHLAGER, UPENDER MANNE, AND WILLIAM E. GRIZZLE
SUPPORTED IN PART BY THE EARLY DETECTION RESEARCH NETWORK AND THE COOPERATIVE HUMAN TISSUE NETWORK

- VARIABLES AFFECTING IMMUNORECOGNITION IN IHC
 - Antigen-Antibody
 - Secondary Detection System
 - Tissue
 - Fixation
 - Tissue processing
 - Shrinkage of Cells

Purpose of Study

- Antigen masking in immunohistochemistry (IHC) has mainly been attributed to fixation
- Very little literature on effects of tissue processing on IHC staining
- The purpose of this study was to determine whether the effects of fixation and tissue processing on IHC staining could be additive

Tissue Processing Model

- Removal of free water with graded ETOHs 70%-1hr, 80%-1hr, 90%-1hr, absolute-3hr.
- Development of hydrophobic environment- with xylene, 2hr.
- Infiltration with paraffin within hydrophobic environment

Hypothesis

- Fixation and tissue processing modify conformation of Ag, preventing Immunodetection of some Ags
- Ki67 detection on frozen tissues fixed in NBF, but not FFPE.
- PCNA can be detected in FFPE

Can epitope masking be reversed?

Antigen retrieval

Heating temp pressure cooker

pH-EDTA pH=8

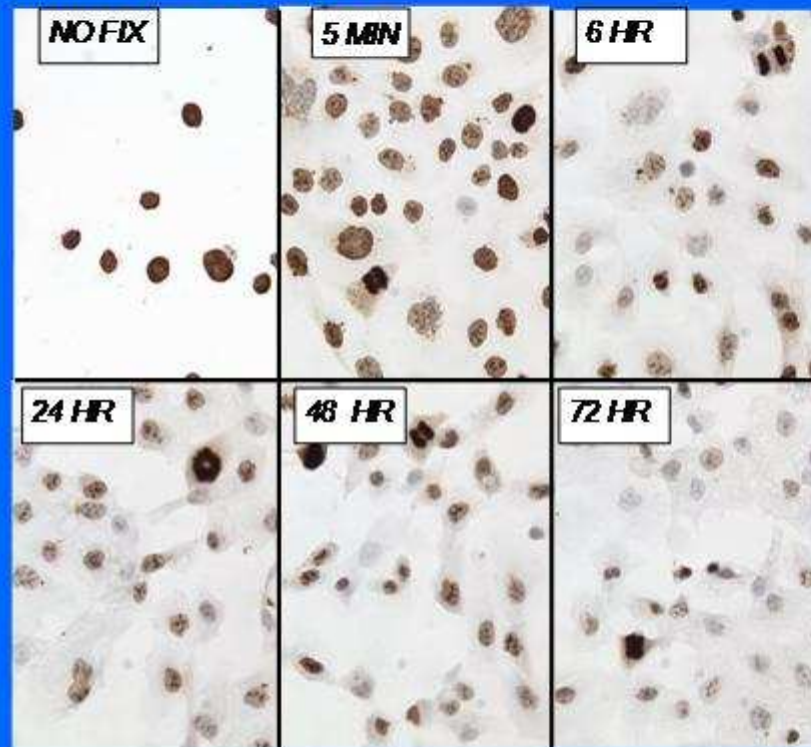
Methodology

- Slide preparation
 - slides labeled/identified
 - Coated with polylysine
 - Autoclaved
- Cell model
 - Cell culture – DU145 & SKOV3
 - Wash off media – fresh tissue
- Fixation
 - No fixation, 5m, 6h, 24h, 48h 72h
- Tissue Processing
 - No fixation with each of other steps in TP e.g. 70% ETOH
- . IHC with and without AR for Ki-67 and PCNA; staining at the same time.
- . Photography
- . Evaluation

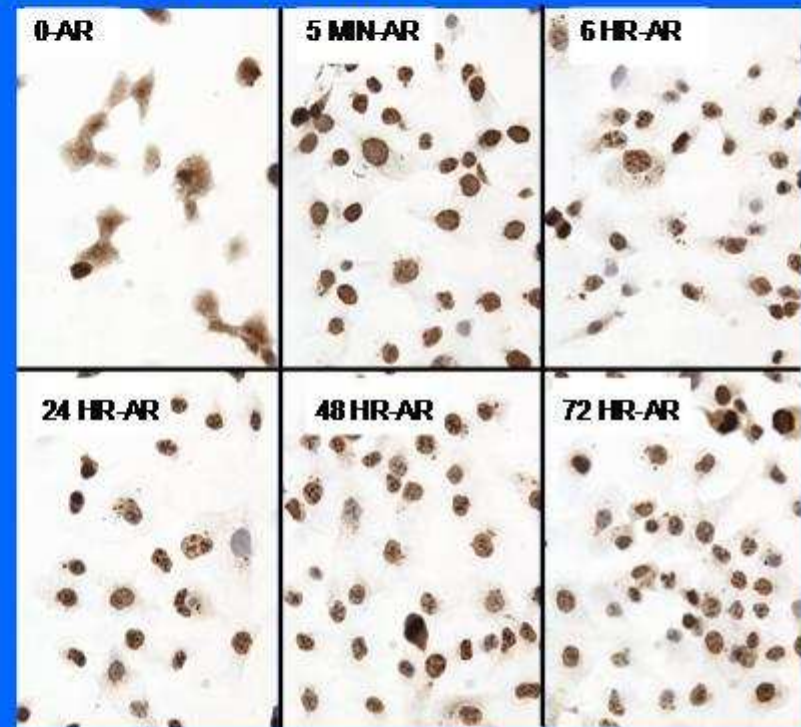
Effect of 10% NBF Fixation on DU145 Cells

STEP 1	Formalin at various timepoints
STEP 2	70% ETOH for 1 hr
STEP 3	80% ETOH for 1 hr
STEP 4	90% ETOH for 1 hr
STEP 5	100% ETOH for 3 hr
STEP 6	Xylene for 2 hr
STEP 7	Paraffin for 2 hr

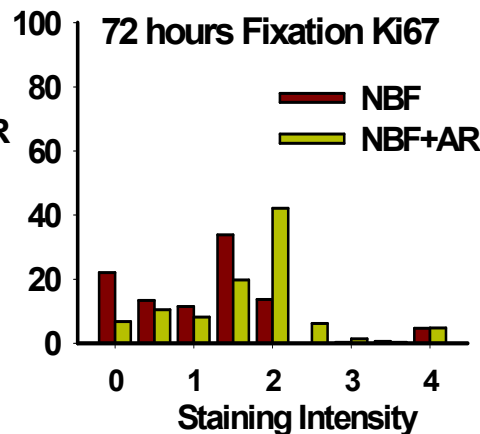
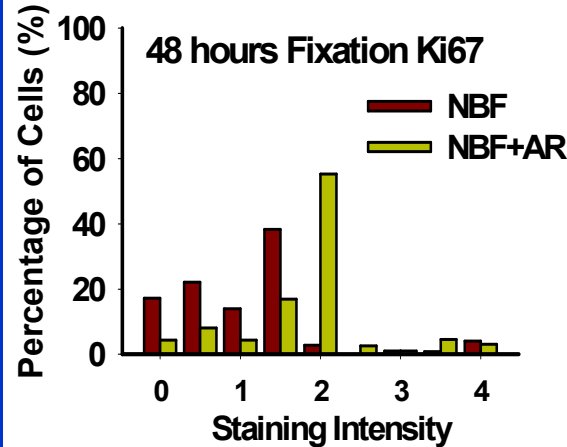
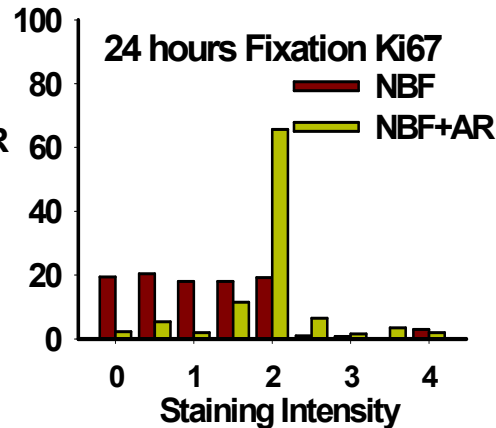
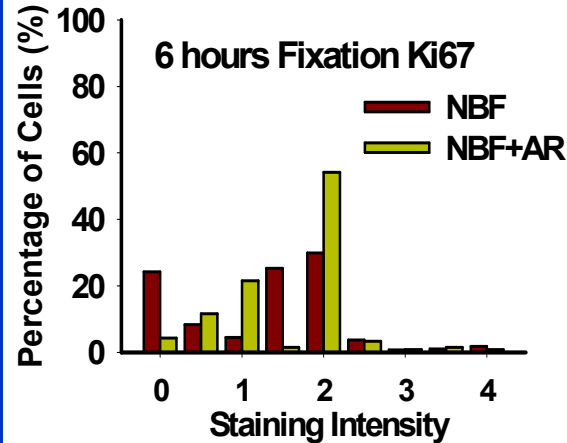
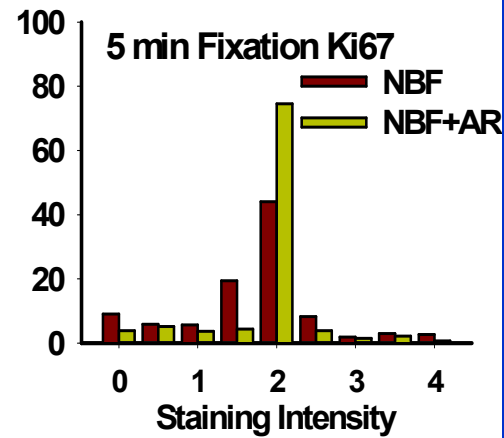
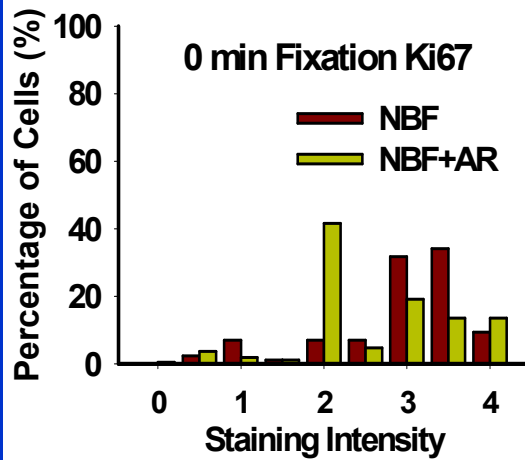
STAINING OF KI-67-STEP 1



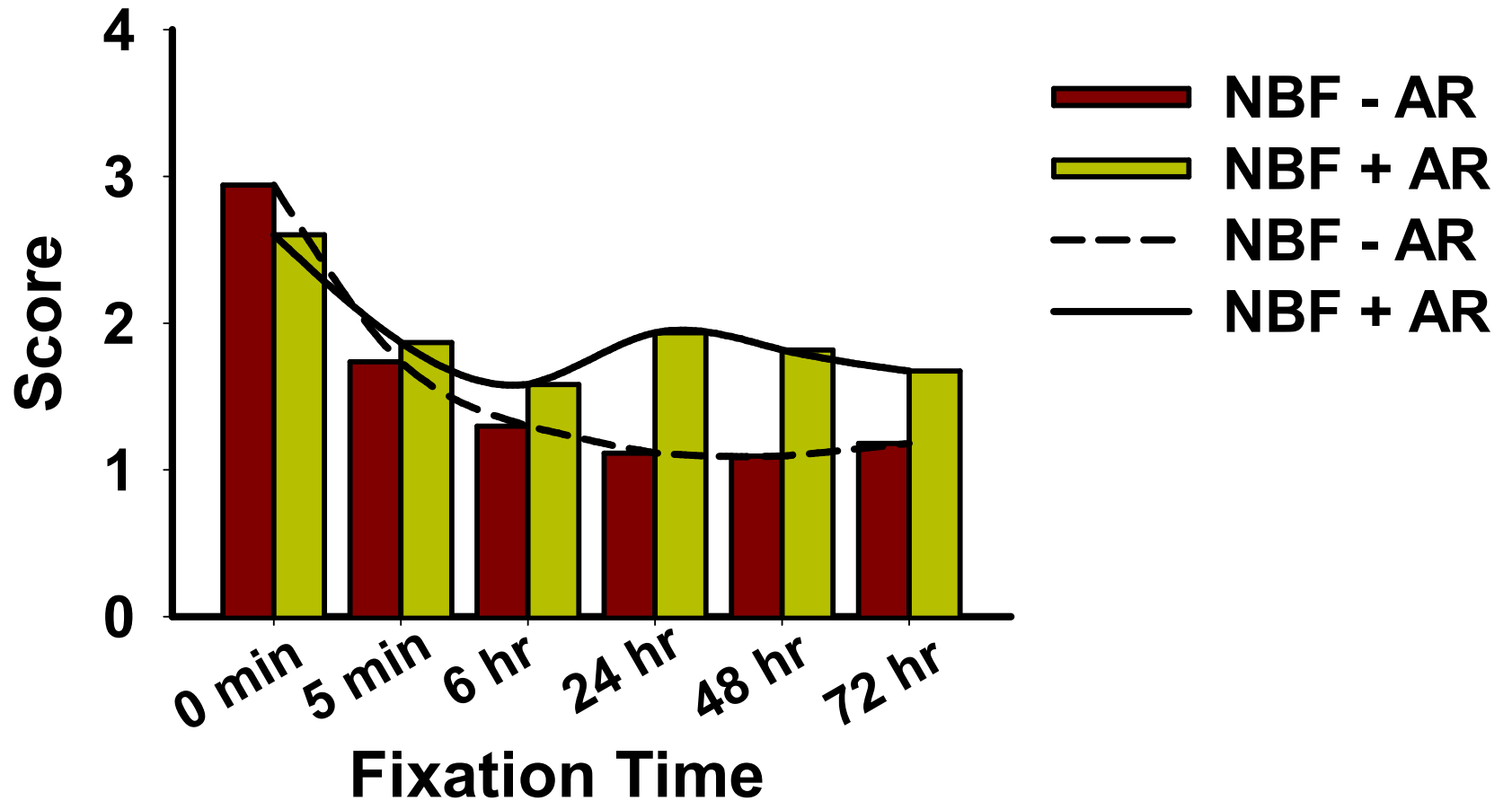
STAINING OF KI-67 with AR-STEP 1



THE EFFECT OF 10% NBF FIXATION ON DU145 CELLS WITH AND WITHOUT AR Ki67

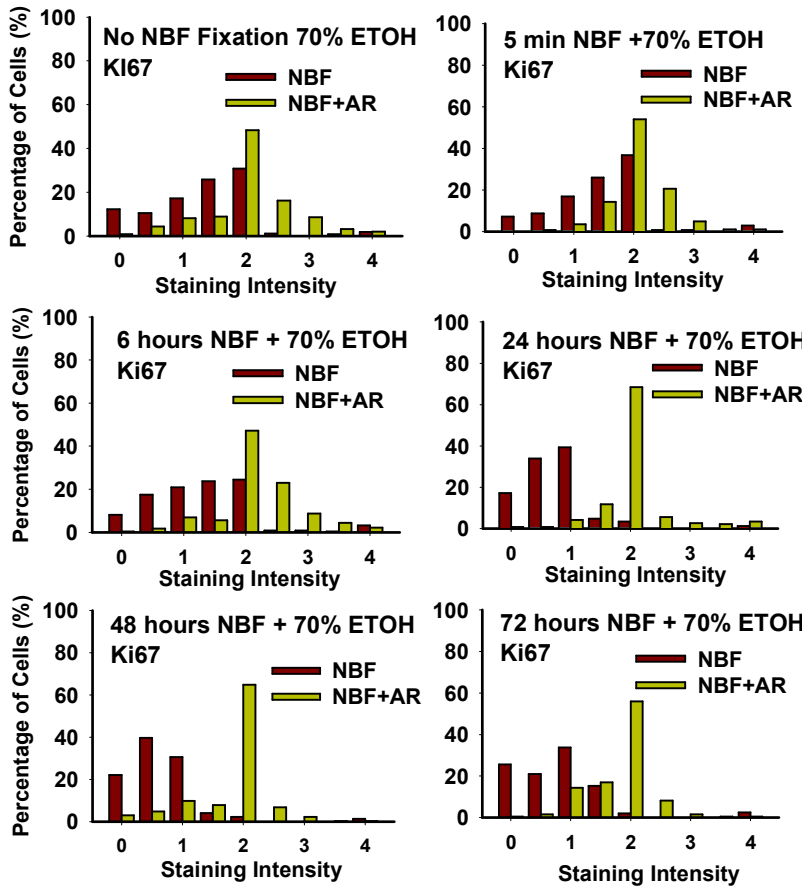


Staining score vs time in 10% NBF, Ki67 DU145

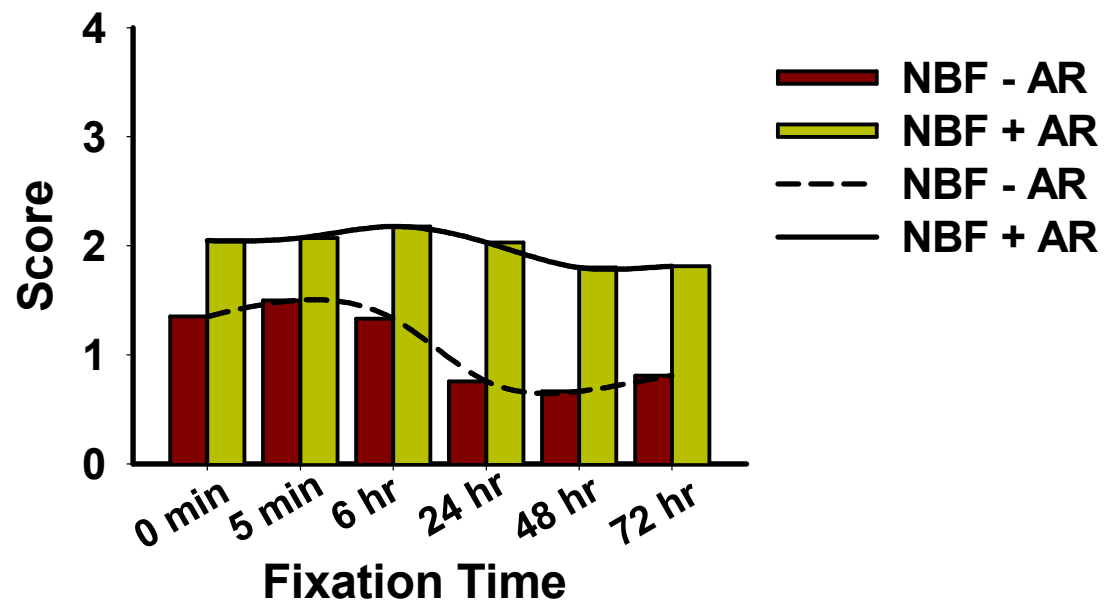


Effect of 10% NBF +70% ETOH on DU145 Cells

STEP 1	Formalin for various timepoints
STEP 2	70% ETOH for 1 hr
STEP 3	80% ETOH for 1 hr
STEP 4	90% ETOH for 1 hr
STEP 5	100% ETOH for 3 hr
STEP 6	Xylene for 2 hr
STEP 7	Paraffin for 2 hr

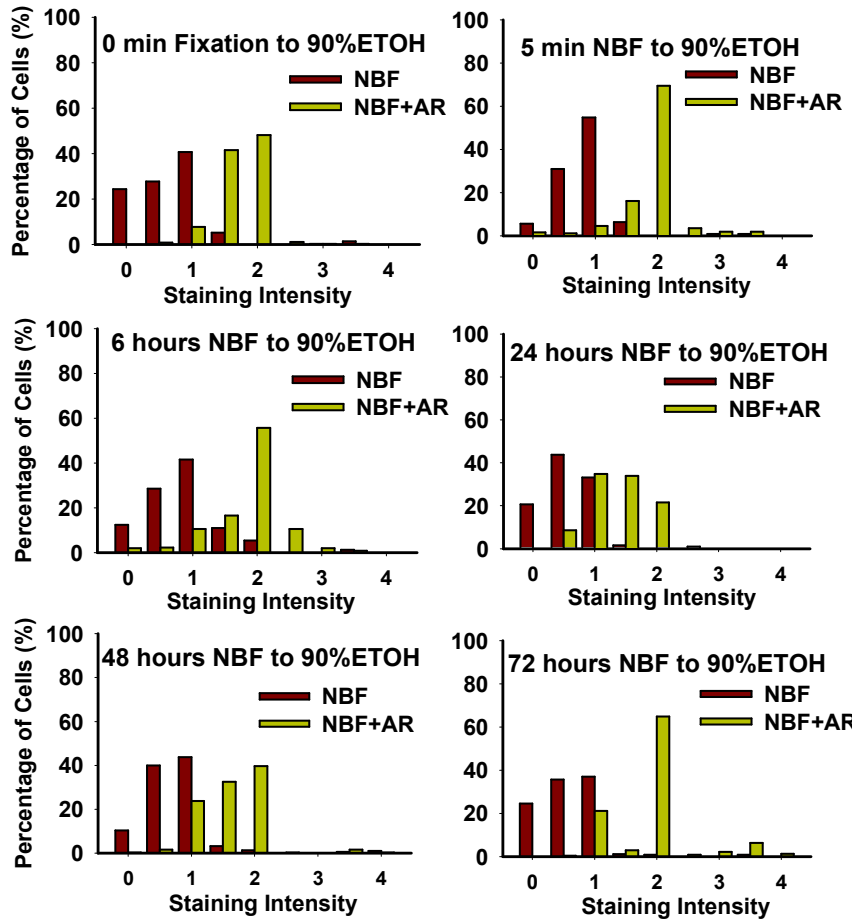


Staining score vs time in 10% NBF, 70% ETOH, Ki67 DU145

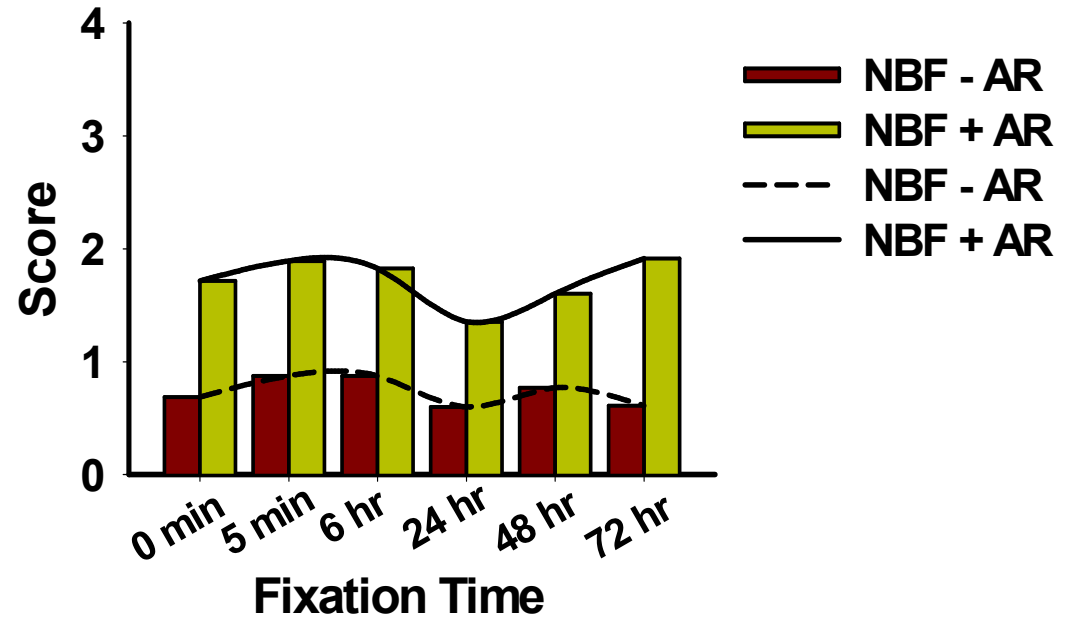


Effect of 10% NBF +90%ETOH on DU145 Cells

STEP 1	Formalin for various timepoints
STEP 2	70% ETOH for 1 hr
STEP 3	80% ETOH for 1 hr
STEP 4	90% ETOH for 1 hr
STEP 5	100% ETOH for 3 hr
STEP 6	Xylene for 2 hr
STEP 7	Paraffin for 2 hr

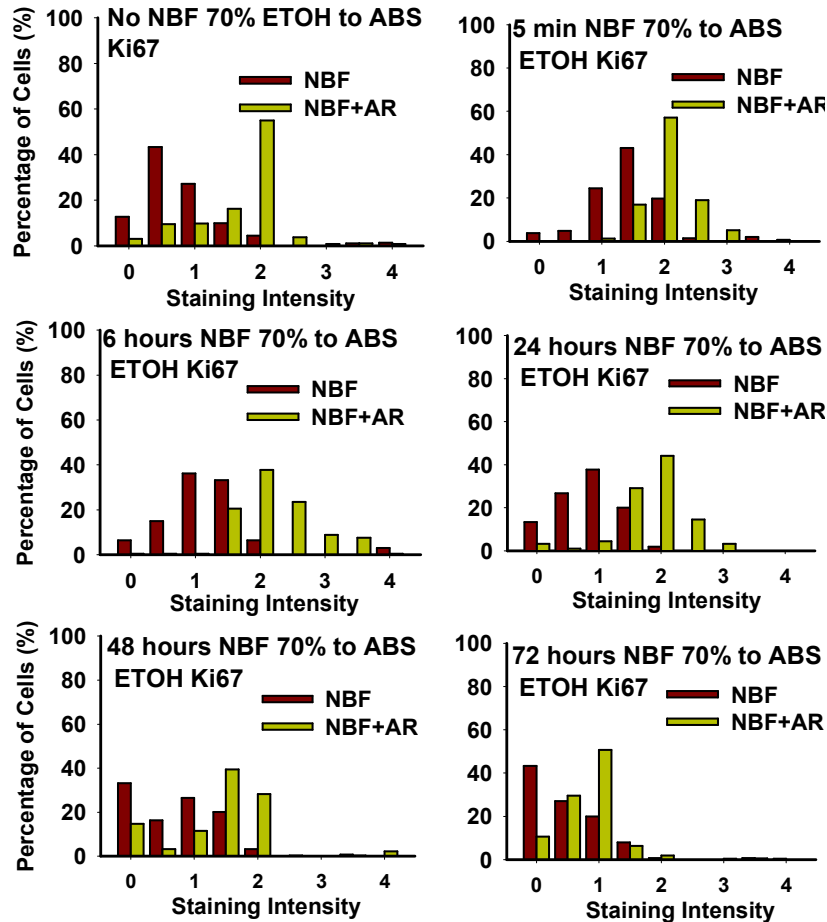


Staining score vs time in 10% NBF, 70- 90% ETOH, Ki67 DU145

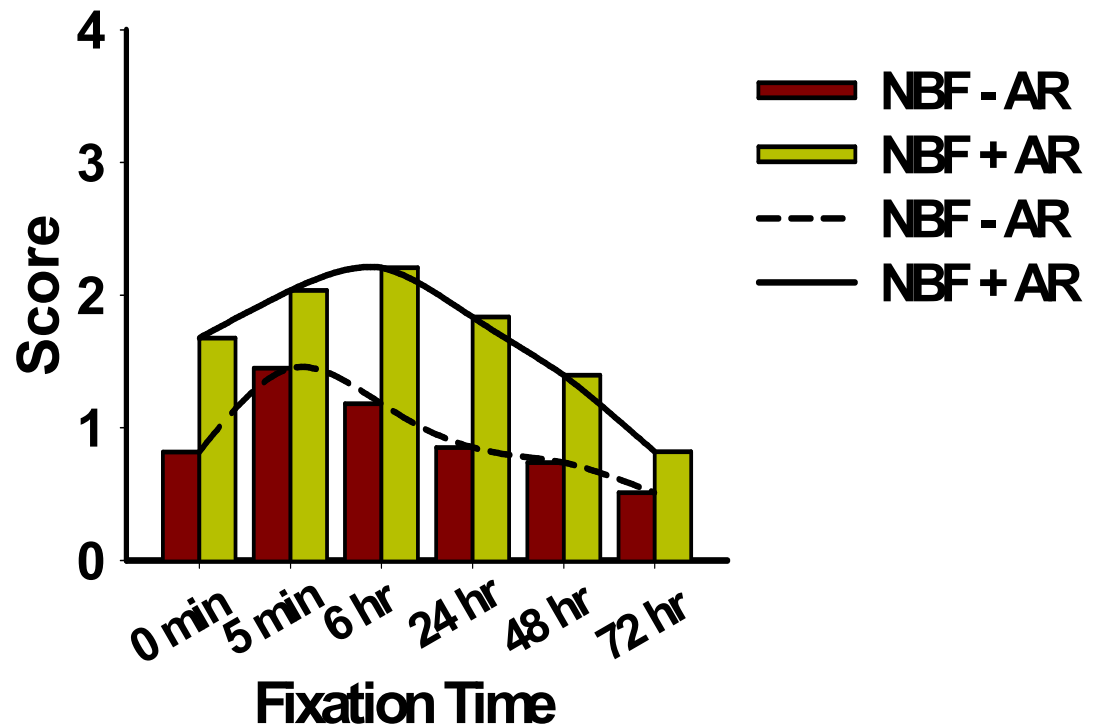


Effect of 10% NBF Fixation - 100% ETOH on DU145 cells

STEP 1	Formalin for various timepoints
STEP 2	70% ETOH for 1 hr
STEP 3	80% ETOH for 1 hr
STEP 4	90% ETOH for 1 hr
STEP 5	100% ETOH for 3 hr
STEP 6	Xylene for 2 hr
STEP 7	Paraffin for 2 hr

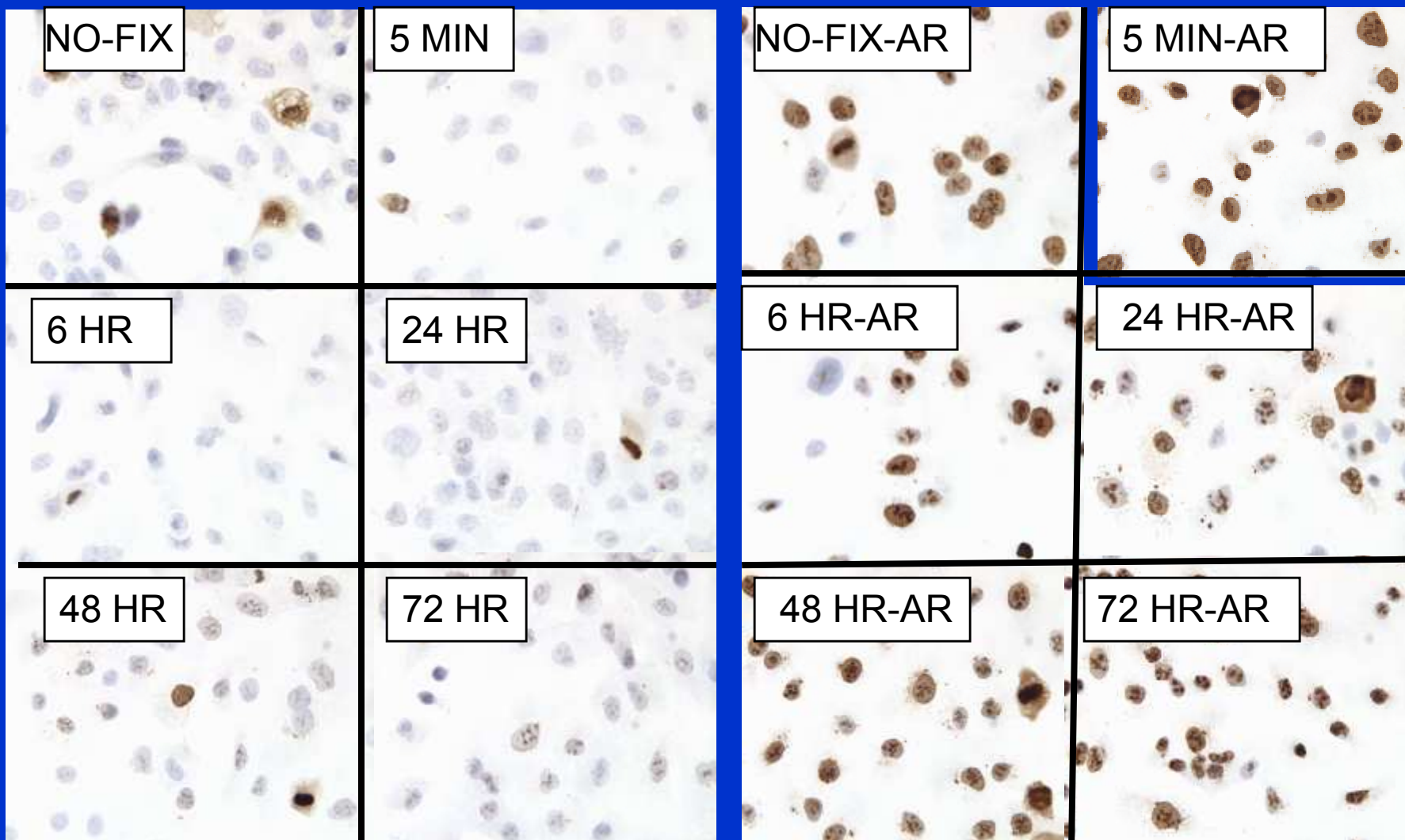


Staining score vs time in 10% NBF, 70% to absolute ETOH, Ki67 DU145



STAINING KI-67 STEP 70% ETH-XYLENE

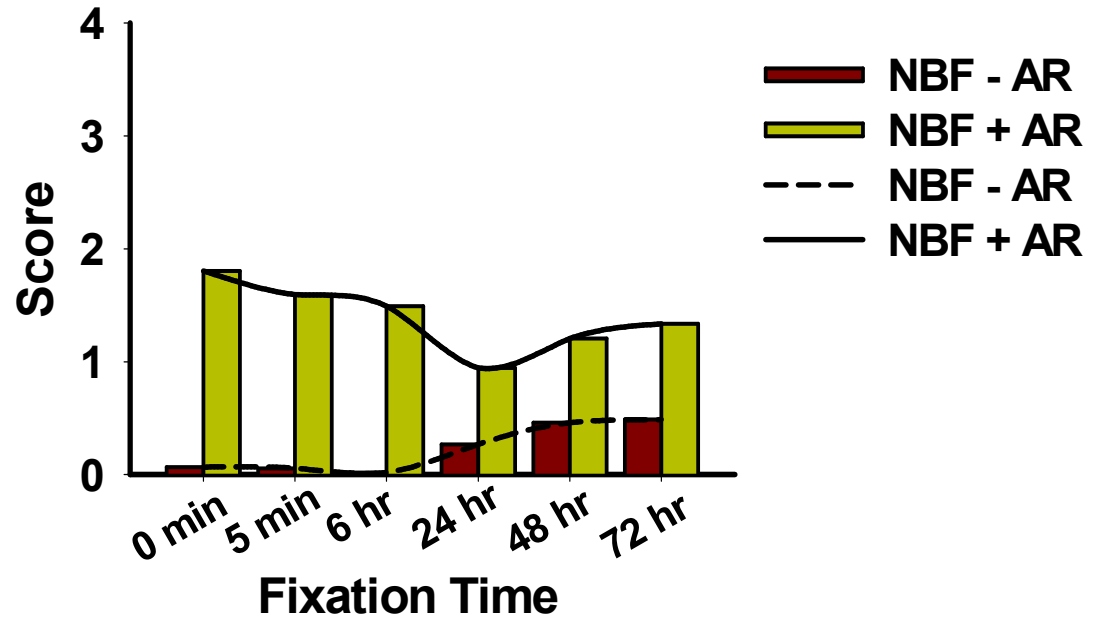
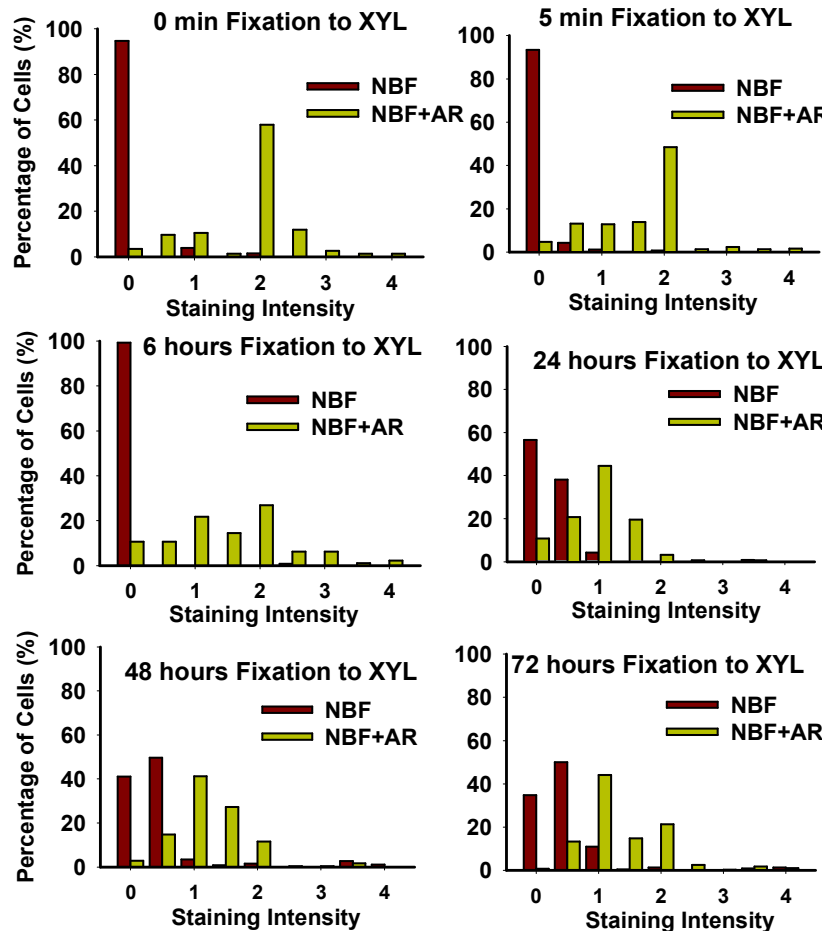
STAINING KI-67-AR STEP 70% ETH-XYLENE

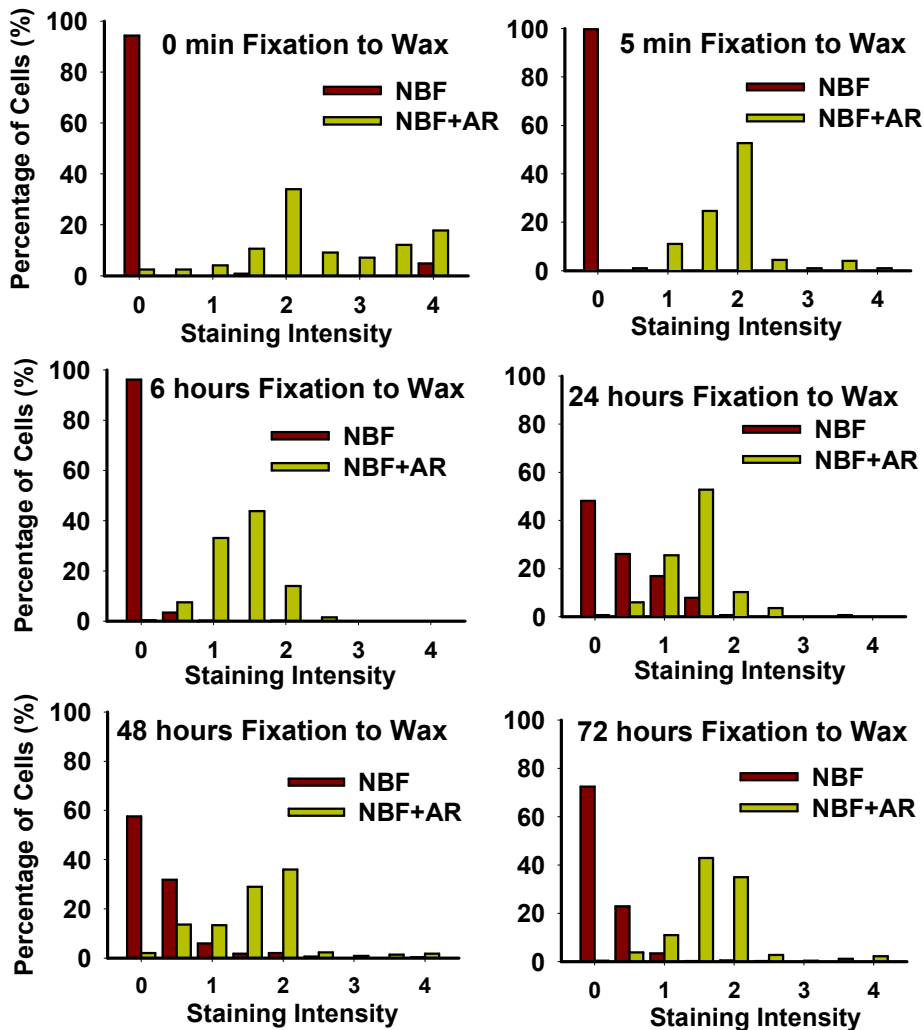


Effect of 10% NBF Fixation - XYLENE on DU145 Cells

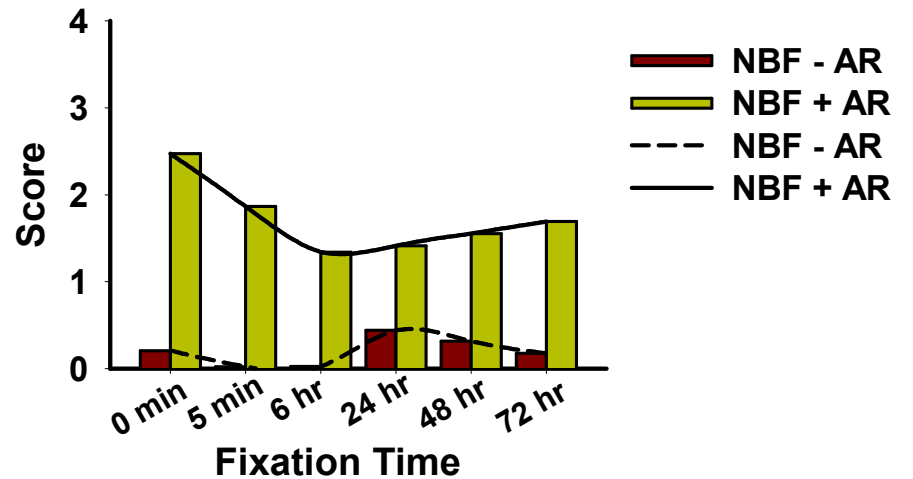
STEP 1	Formalin for various timepoints
STEP 2	70% ETOH for 1 hr
STEP 3	80% ETOH for 1 hr
STEP 4	90% ETOH for 1 hr
STEP 5	100% ETOH for 3 hr
STEP 6	Xylene for 2 hr
STEP 7	Paraffin for 2 hr

Staining score vs time in 10% NBF, 70% ETOH to xylene, Ki67 DU145

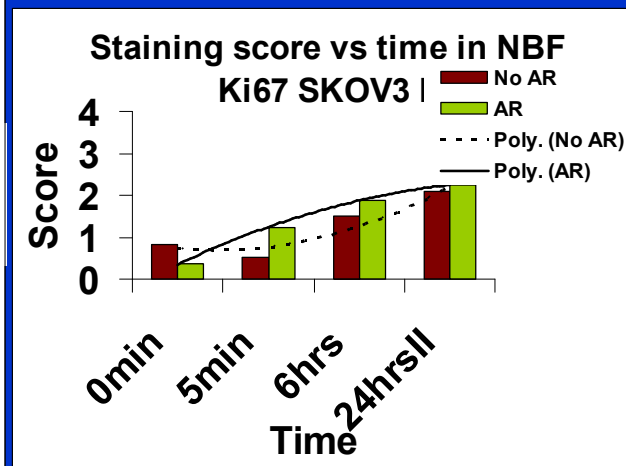
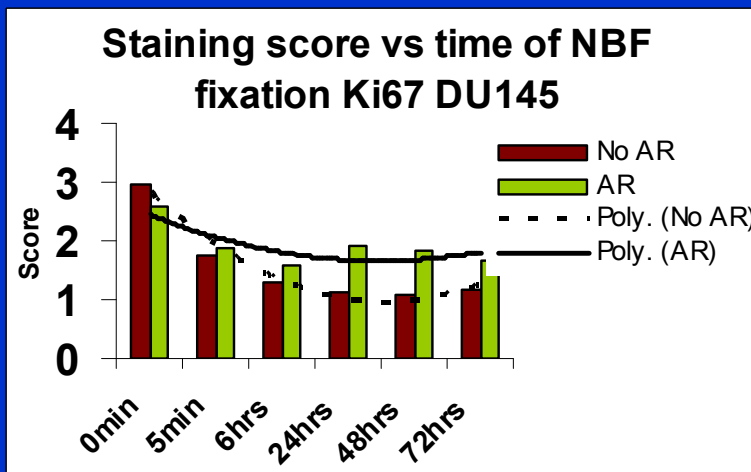




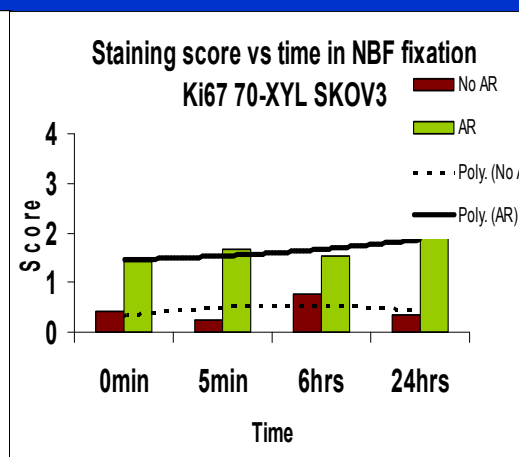
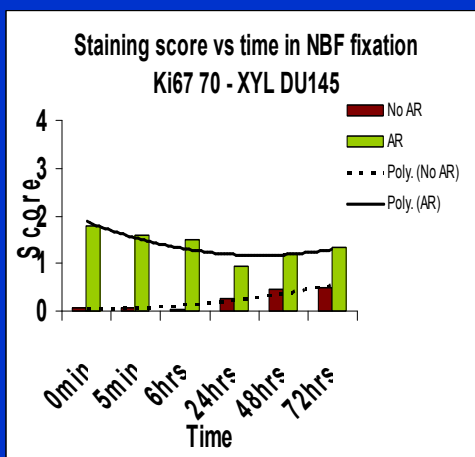
Staining score vs time in 10% NBF, 70% -absolute ETOH, xylene to paraffin wax, Ki67 DU145



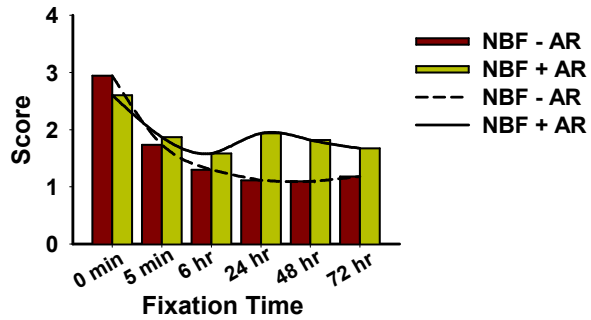
DU145/SKOV3 comparison 10% NBF fixation Ki67



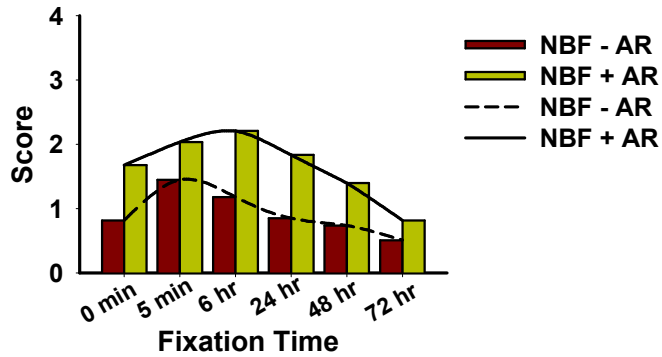
DU145/SKOV3 comparison 10% NBF, 70-Xylene Ki67



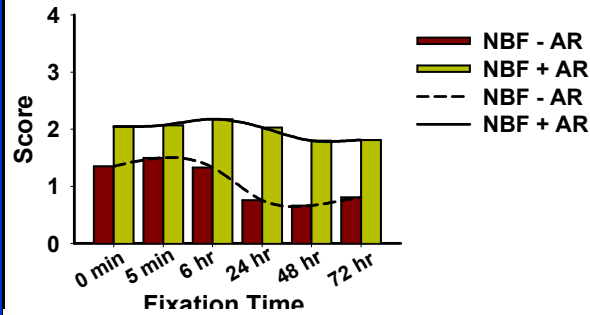
Staining score vs time in 10% NBF, Ki67 DU145



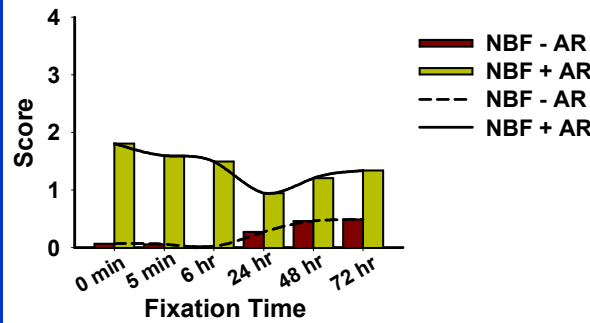
Staining score vs time in 10% NBF, 70% to absolute ETOH, Ki67 DU145



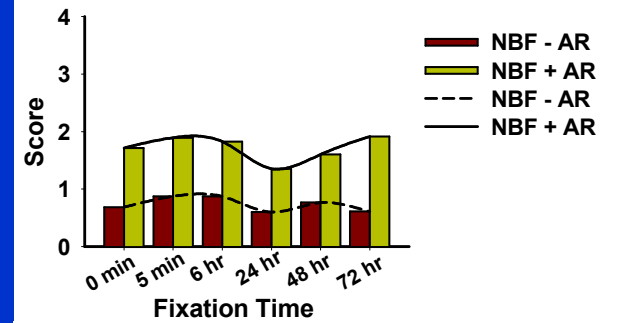
Staining score vs time in 10% NBF, 70% ETOH, Ki67 DU145



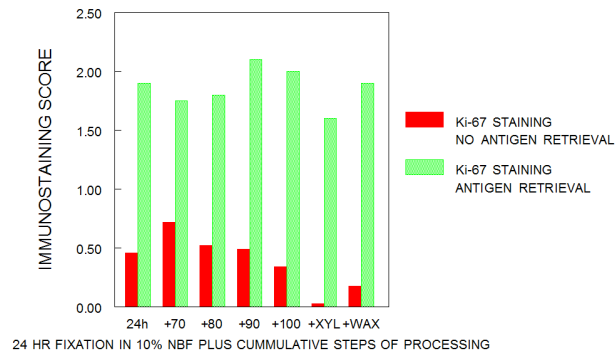
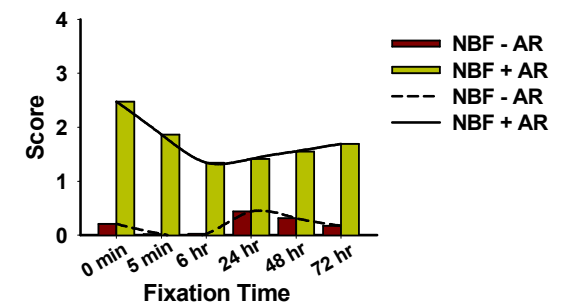
Staining score vs time in 10% NBF, 70% ETOH to xylene, Ki67 DU145



Staining score vs time in 10% NBF, 70- 90% ETOH, Ki67 DU145



Staining score vs time in 10% NBF, 70% -absolute ETOH, xylene to paraffin wax, Ki67 DU145



24 HR FIXATION IN 10% NBF PLUS CUMMULATIVE STEPS OF PROCESSING

Ki67 Summary

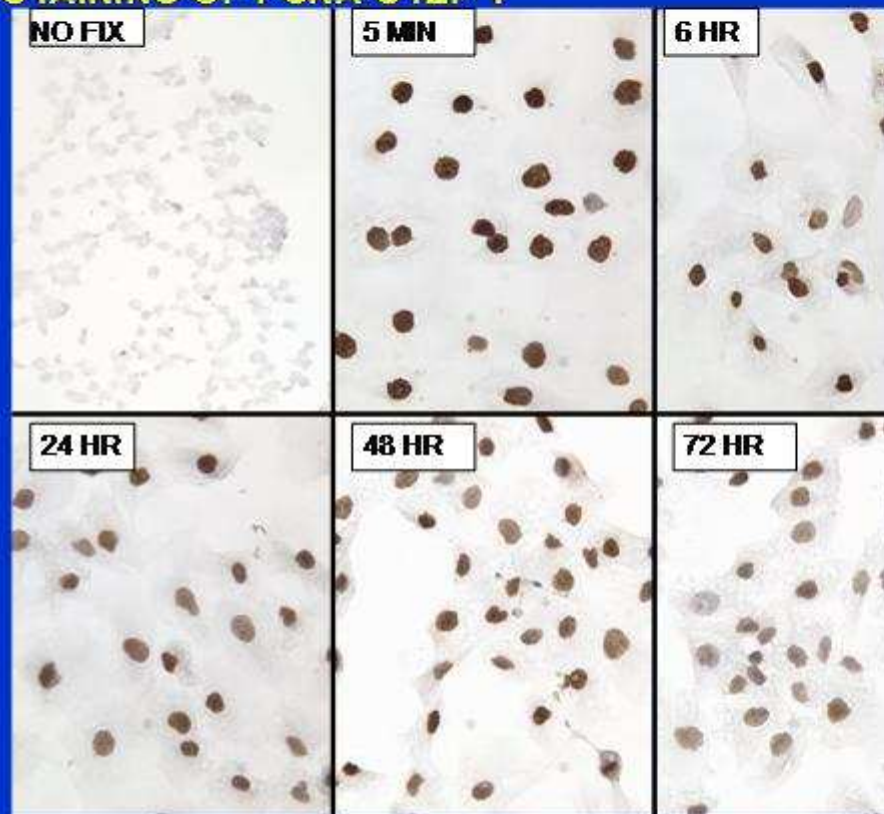
Immunorecognition of Ki67 somewhat decreases with increase in duration in 10% NBF – most of loss is recovered with AR

- Fixation + TP (Hydrophobic environment) in combination masks most of the Ki67 antigen; recovery is partial

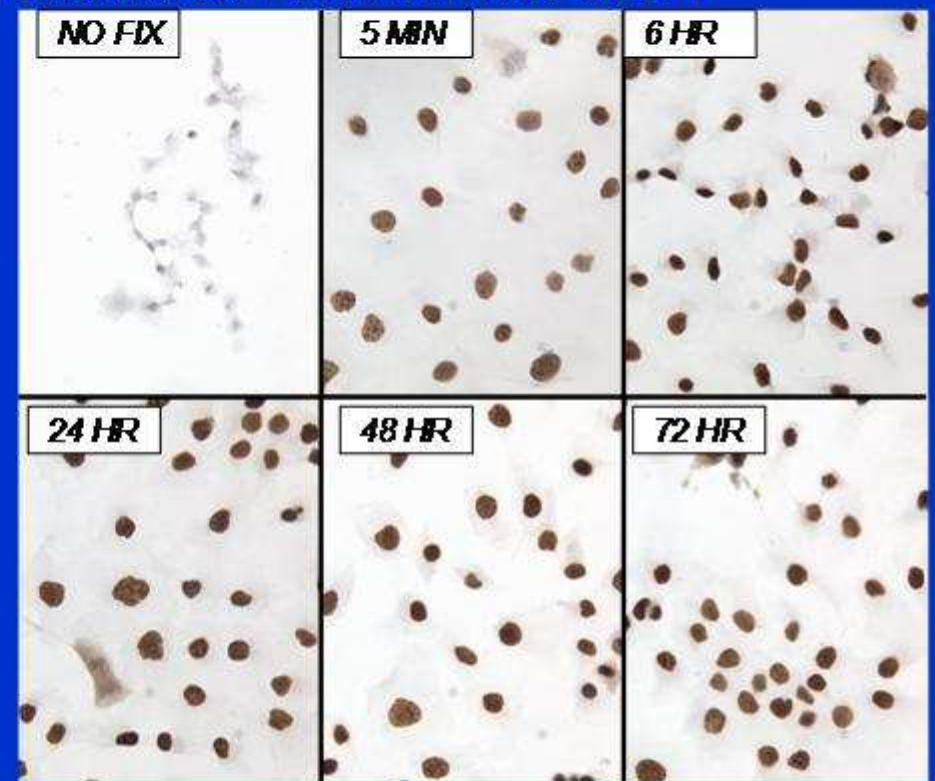
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STAINING OF PCNA-STEP 1

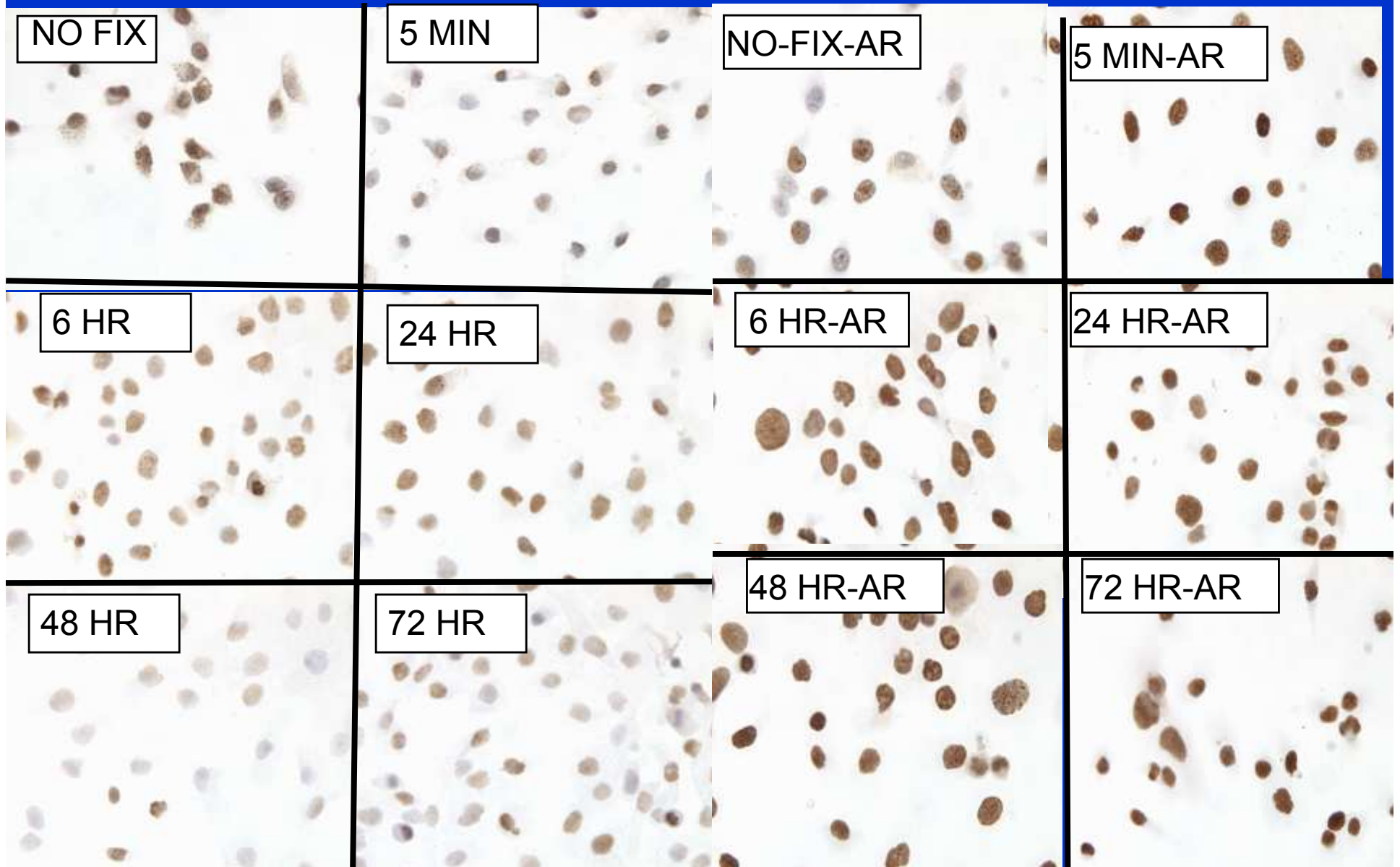


STAINING OF PCNA WITH AR-STEP 1

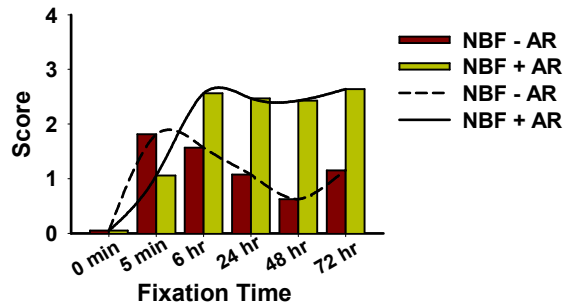


STAINING OF PCNA STEP 70% ETH-XYL

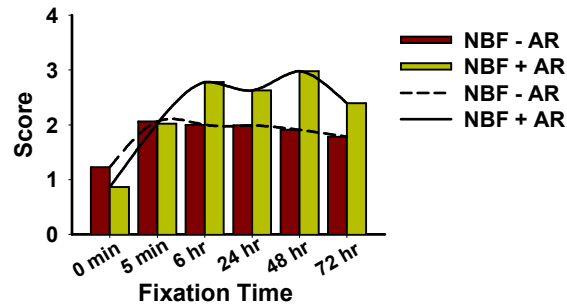
STAINING OF PCNA WITH AR STEP-70% ETH-ZYL



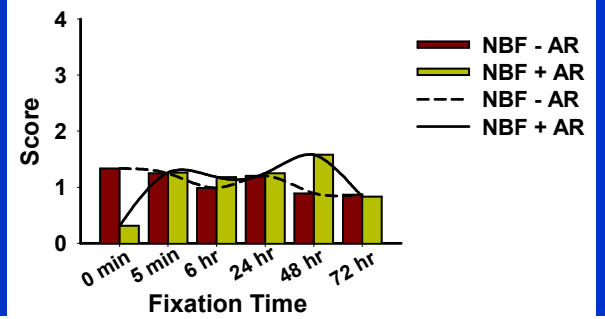
Staining score vs time in 10% NBF, PCNA DU145



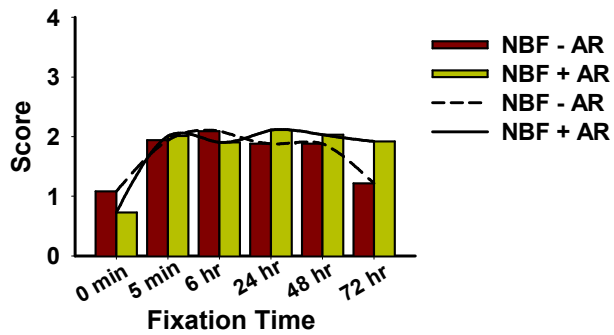
Staining score vs time in 10% NBF, 70% ETOH PCNA DU145



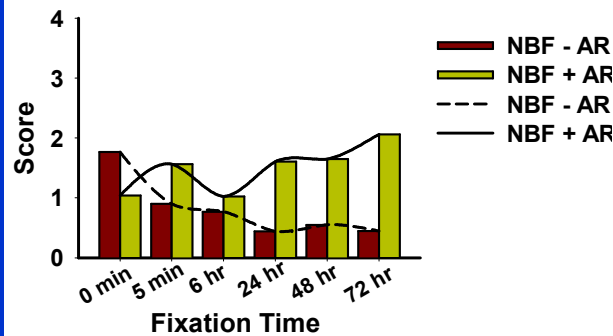
Staining score vs time in 10% NBF, 70-90% ETOH PCNA DU145



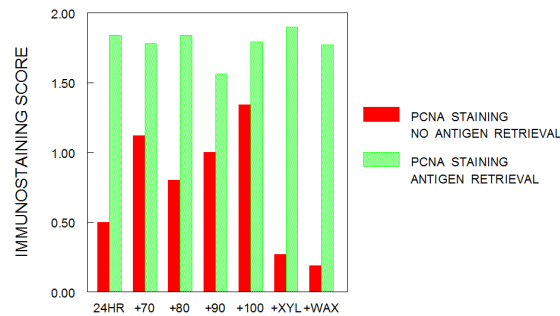
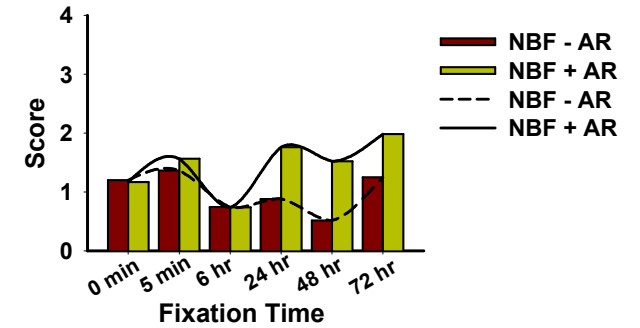
Staining score vs time in 10% NBF, 70% to absolute ETOH PCNA DU145



Staining score vs time in 10% NBF, 70% ETOH to xylene PCNA DU145



Staining score vs time in 10% NBF, 70% -absolute ETOH, xylene to paraffin wax, PCNA DU145

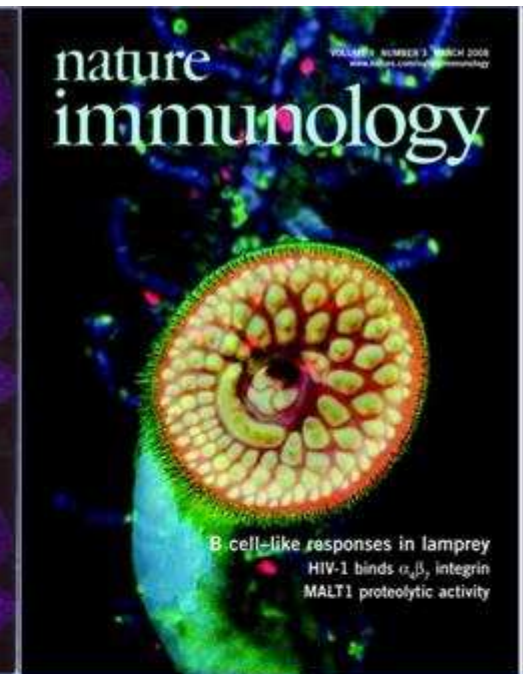
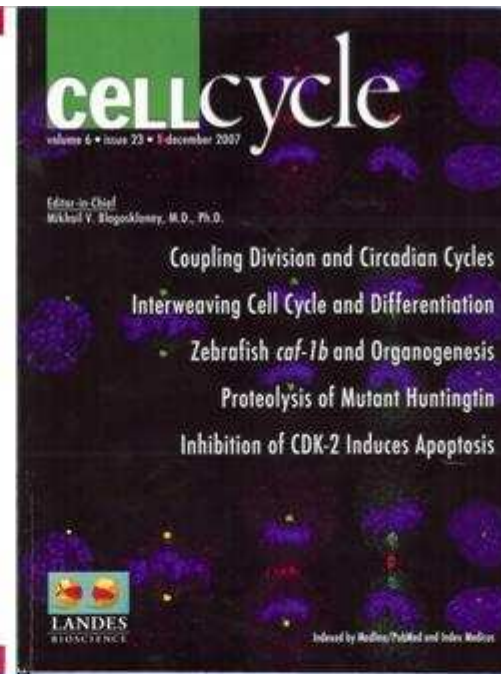


1) PCNA REQUIRES SOME FIXATION FOR IMMUNO-RECOGNITION

2) PCNA DOES NOT LOSE ALL IMMUNO-RECOGNITION WITH FIXATION AND TISSUE PROCESSING

3) MORE CONSISTENT IMMUNOSTAINING IS OBTAINED WITH ANTIGEN RETRIEVAL.

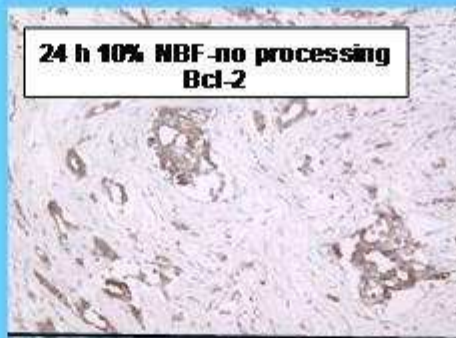
4) AS WITH Ki-67, LOSS OF IMMUNO-RECOGNITION OCCURS WITH BOTH FIXATION AND TISSUE PROCESSING WITH THE MAJOR CHANGE AT THE TRANSITION TO A HYDROPHOBIC ENVIRONMENT.



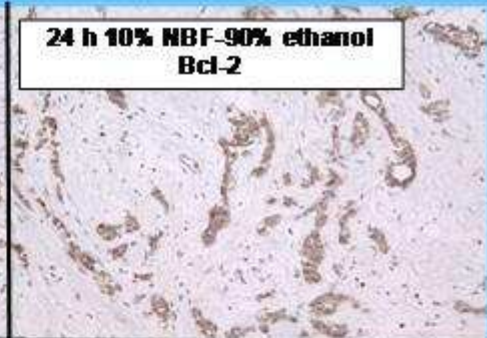
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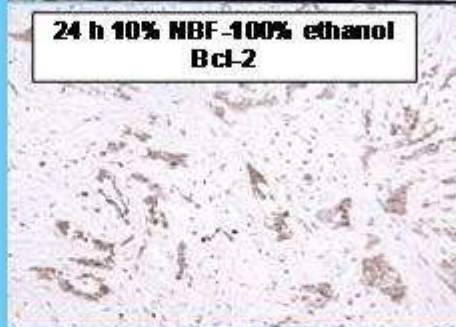
**24 h 10% NBF-no processing
Bcl-2**



**24 h 10% NBF-90% ethanol
Bcl-2**



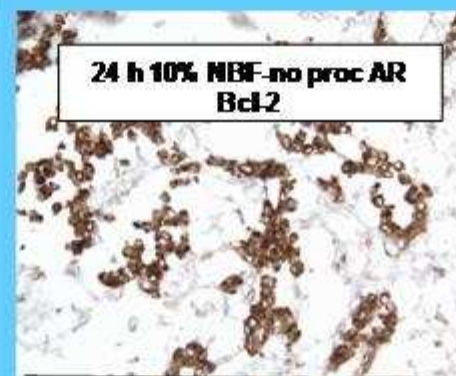
**24 h 10% NBF-100% ethanol
Bcl-2**



**24 h 10% NBF-xylene
Bcl-2**



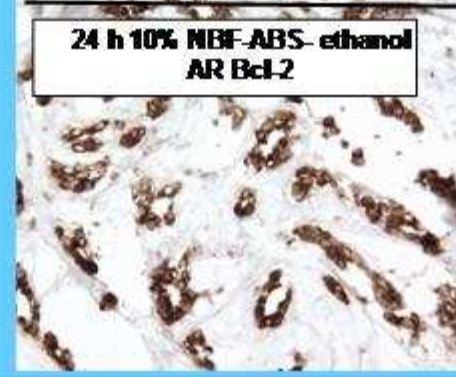
**24 h 10% NBF-no proc AR
Bcl-2**



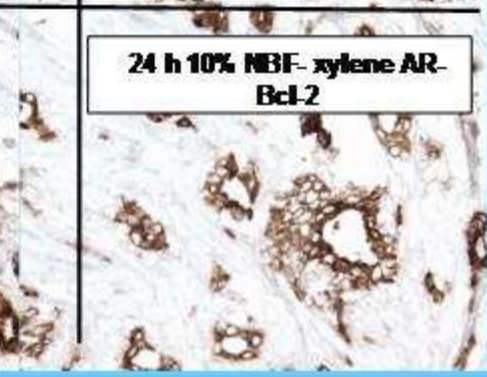
**24 h 10% NBF-90% ethanol
AR-Bcl-2**



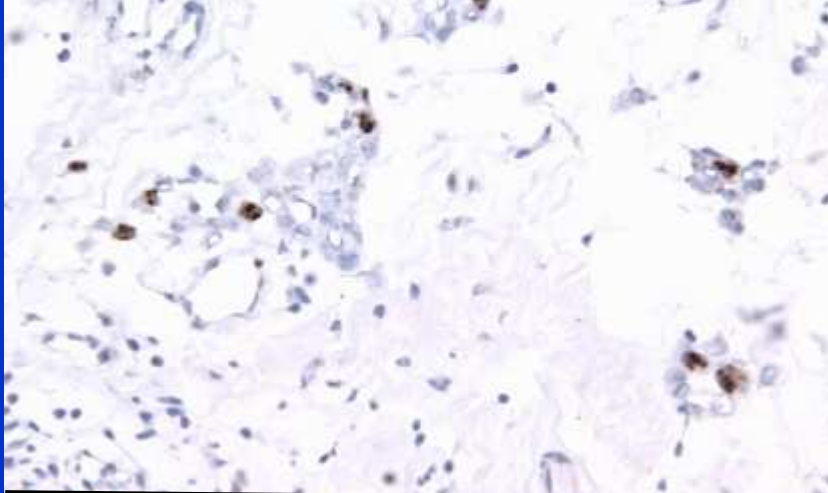
**24 h 10% NBF-ABS- ethanol
AR Bcl-2**



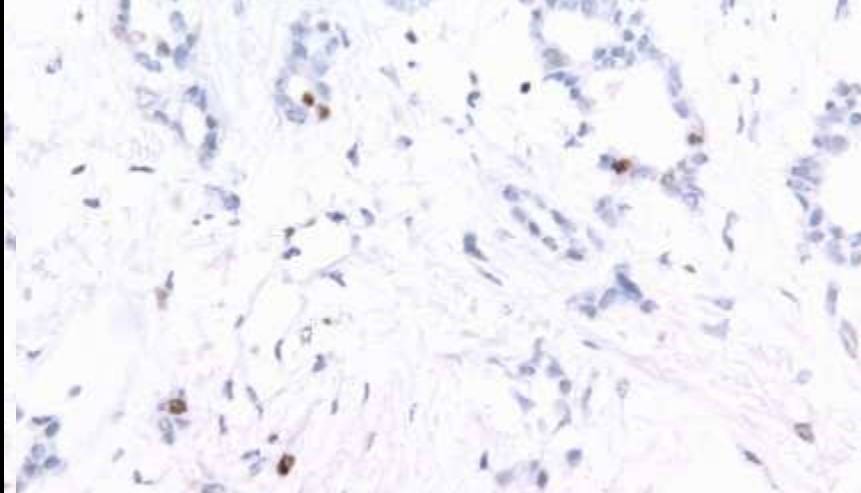
**24 h 10% NBF- xylene AR-
Bcl-2**



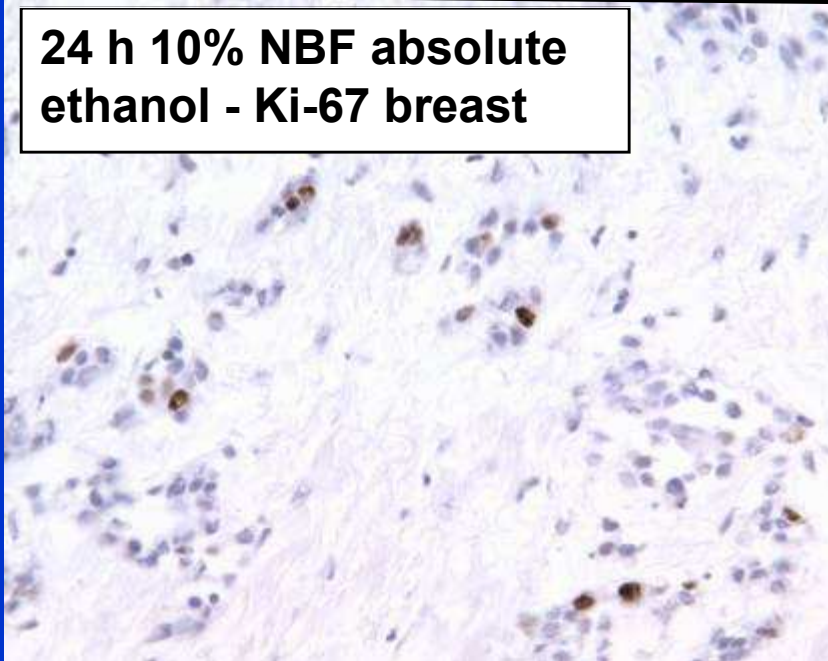
**24 h 10% NBF-no
process-Ki-67 breast**



**24 h 10% NBF-90%
ethanol-Ki-67 breast**



**24 h 10% NBF absolute
ethanol - Ki-67 breast**



**24 h 10% NBF-xylene-
Ki-67 breast**

