University of Michigan Comprehensive Cancer Center	SOP-BCR-5.17	**Double Immunofluorescence Staining for RAD-51 and H2Ax Foci in Cells**	Author: S. Clouthier Approved: M. Wicha	Rev: 0	Issued: 6/19/2013 Revised:

1.0 Purpose

The purpose of SOP 5.17 is to state the procedure double Immunofluorescence Staining for RAD-51 and H2Ax Foci in Cells.

2.0 Scope

SOP is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Procedure

3.1 Plate 200k-300k cells into each well of two-well glass chamber slides (Collagen-coated for SUM159)
3.2 Irradiate cells with γ–irradiation at 4 Gray.
3.3 Change media immediately after irradiation and return cells to incubator.
3.4 After 8-24 hours (HCC1954: 15 hours, SUM149: 8 hours, SUM159: 12 hours), wash cells with room-temperature 1x PBS. Repeat this step three times.
3.5 Fix cells in room-temperature (RT) 2% ParaFormaldehyde (diluted in PBS). Incubate at room temperature for 15 minutes.
3.6 Wash cells with room temperature 1x PBS. Repeat this step three times.
- If necessary, cells can be stored in PBS at 4ºC overnight.

3.7 Permealize in 0.1% Triton X-100 (in PBS) for five minutes at room temperature. (Can use small glass slide holder).
3.8 Wash cells with room temperature 1x PBS. Repeat this step three times.
3.9 Use the blue blocking solution from AEC Staining Kit (Invitrogen 85-8943) or 10% Goat Serum from Invitrogen. This step takes 10 minutes.
3.10 **H2AX Primary Antibody** (-20ºC Ab box in freezer 1; Cat#16-193, Millipore JBW301)
- 1:1,000 dilution in 1x PBS
- Incubate for 1 hour at room temperature

3.11 Wash cells with room temperature 1x PBS. Repeat this step three times.
3.12 **Turn off the lights.** Proceed with the following steps in the **DARK.**
3.13 **Alexa-fluor 546 Goat Anti-Mouse (Red)** (Invitrogen A11030 located in Freezer #2)
- 1:2,000 dilution in 1x PBS.
- Incubate for 30 minutes.

3.14 Wash cells with room temperature 1x PBS. Repeat this step three times.
3.15 Use the **blue blocking solution** from AEC Staining Kit (Invitrogen 85-8943) or 10% Goat Serum from Invitrogen. This step takes 20 minutes.
3.16 **RAD-51 Primary Antibody** (4ºC; SantaCruz sc-8349; located in Unconjugated Ab Box in Fridge #1)
- 1:200 dilution in 1x PBS.
- Incubate for 1 hour at room temperature.

3.17 Wash cells with room temperature 1x PBS. Repeat this step three times.
3.18 **Alexa-fluor 488 Goat Anti-Rabbit (Green)** (Invitrogen A11008; located in 2º Ab Box in Fridge #1)
- 1:1,000 dilution in 1x PBS (1:200 too much backround).
- Incubate for 30 minutes.

3.19 Wash cells with room temperature 1x PBS. Repeat this step three times.

3.20 **Mount slides with Invitrogen ProLong Gold Antifade Reagent with DAPI** (cat # P-36931; located in Freezer #4)
3.21 Store slides overnight in the **DARK** at room temperature.
3.22 Analyze slides and capture photos after overnight storage

4.0 Applicable References

5.0 Change Description

Revision	Date	Reference	Description of Change