Example Experiment

Detecting p53 Activation in COS-7 Cells Following Hydroxyurea Treatment

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I. Background

Accurate quantification of protein expression and/or post-translational modifications is important for advancing both basic and translational research. Given the flexibility, reproducibility, and higher throughput of the In-Cell Western™ Assay, it offers a convenient alternative to Western blotting and is a powerful platform for meaningful *in situ* analyses. The In-Cell Western microplate format can be used to analyze:

- Protein phosphorylation and signaling (1 - 3)
- Off-target effects of drugs on signaling pathways (4)
- Timing and kinetics of signaling events (5 , 6)
- Quantification of viral load (7 - 11)
- Genotoxicity assays (12, 13)
- Cell proliferation and apoptosis assays (14)
- Bacterial-induced epithelial signaling (15)
- Glycoprotein analysis (16, 17)
- Library screening (18 - 20)
- Screening of monoclonal antibody clones (21)

II. Introduction

P53 is a transcription factor that regulates the expression of numerous genes in response to stress stimuli (22). Many of these genes play important roles in apoptosis, senescence, cellular metabolism, the DNA damage response, and the oxidative stress response (23). In response to DNA damage, p53 governs the activation of specific gene expression programs to achieve the desired cellular outcomes through multiple layers of regulation (24). For example, p53 is phosphorylated at several sites by different protein kinases, each triggering a specific response (25). DNA damage stimulates p53 phosphorylation at Ser15 and Ser20, disrupting the interaction between p53 and the oncoprotein, MDM2, its negative regulator (26).

Understanding how new therapies impact this complex pathway is of great importance to the drug discovery field. For example, 50% of cancers lose p53 function as a result of mutations that produce single amino-acid substitutions in the core DNA-binding domain (27). In other cancers, wild-type p53 is targeted for degradation by cellular (MDM2) or viral (E6) oncoproteins (28, 29). As a result, many aggressive forms of cancers are resistant to

traditional chemotherapy regimens owing to the lack of p53-dependent apoptosis (30). Therefore, developing cell-based assays that can identify compounds capable of exploiting the p53 pathway by either displaying synthetic lethality in cancers with p53 mutations or by looking for non-genotoxic activators of the p53 response, is of great importance. In the following example, we illustrate how the In-Cell Western™ Assay can be utilized to quantify p53 activation after drug treatment in the p53-wildtype cell line, COS-7.

III. Experimental Design

p53 activation is monitored using an antibody that detects endogenous levels of p53 that have been phosphorylated on serine residue, 15. This antibody does not recognize p53 that has been phosphorylated on other sites. Phosphorylated p-53 (Ser15) levels are then normalized against total ERK1 protein levels. Hydroxyurea, a potent teratogen and anticancer agent, induces oxidative stress and activates a DNA damage response pathway (31, 32). Therefore, we investigated the effects of hydroxyurea on p53 activation in COS-7 cells by In-Cell Western.

IV. Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Rabbit Secondary Antibody (LI-COR P/N 925-32211 or 926-32211)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (LI-COR P/N 925-68070 or 926-68070)
- Intercept® (PBS) Blocking Buffer

> **Note:** Odyssey® Blocking Buffer was used in the original experiment and has been discontinued. Intercept Blocking Buffer is now available instead of Odyssey Blocking Buffer.

Additional Reagents

- 1X PBS wash buffer
- COS-7 cells (ATCC; CRL-1651)
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Black-sided 96-well or 384-well microplates with clear well bottoms

- Hydroxyurea (Sigma®, P/N H8627)
- Anti-phospho-p53 (Cell Signaling Technology, P/N 9286)
- Normalization antibody: Anti-total ERK1 (Santa Cruz Biotechnology, P/N SC-94)

> **Note:** This has been discontinued. Suitable alternatives may be obtained from Cell Signaling Technology.

- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100

V. Prepare Cells

1. Allow COS-7 (ATCC; CRL-1651) cell growth in a T75 flask using standard tissue culture procedures until ~80% confluency is achieved (~1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).
2. Remove growth media and wash cells with sterile 1X PBS (room temperature (RT)).
3. Add trypsin and incubate 3-5 minutes at 37 °C to displace cells.
4. Neutralize displaced cells with culture media and pellet by centrifugation (500 x g).
5. Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube.

> **Note:** To maintain cell integrity, do not pipet or vortex during pellet disruption.

6. Resuspend cells in 20 mL of complete media and count cells using a hemocytometer.
7. Reconstitute and dilute cells with complete media to a concentration of 100,000 cells/mL.
8. Manually mix the cell suspension thoroughly.
9. Under sterile conditions, dispense 200 µL of the cell suspension per well in a 96-well plate (20,000 cells plated per well).
10. Incubate cells at 37 °C with 5% CO_2 and monitor cell density until ~80% confluency is achieved.

VI. Treat Cells

1. Warm serum-free media (DMEM, Gibco) to 37 °C.
2. Remove complete media from plate wells by aspiration or manual displacement.
3. Add either serum-free media for resting cells (mock) or serum-free media with serial concentrations of Hydroxyurea ranging 0.04 - 20 mM for activated cells. Add 100 µL of resting or activation media per well.
4. Incubate at 37 °C with 5% CO_2 overnight (16 - 24 hours).

VII. Fix and Permeabilize Cells

Fix Cells

1. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

2. When incubation period is complete, carefully remove activation media manually or by aspiration to avoid detaching the cells.
3. Using a multi-channel pipettor, add 150 µL of fresh Fixing Solution (RT). Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow incubation on bench top for 20 minutes at RT with no shaking.

Permeabilize Cells

1. Prepare Triton® Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

2. Remove Fixing Solution to an appropriate waste container (contains formaldehyde).
3. Using a multi-channel pipettor, add 200 µL Triton Washing Solution (RT). Add the solution down the sides of the wells carefully to avoid detaching the cells.
4. Allow plate to shake on a rotator for 5 minutes at RT.
5. Repeat washing steps 4 more times, removing wash manually each time.

Important: Do not allow cells to become dry during washing. Immediately add the next wash after manual disposal.

VIII. Block Cells

1. Using a multi-channel pipettor, add 150 µL of Intercept® Blocking Buffer to each well. Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells.
2. Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

IX. Primary Antibodies

Dilute Primary Antibodies

1. Dilute the two primary antibodies in Intercept Blocking Buffer. Combine the following antibodies for phospho-p53 target analysis, using total ERK1 for normalization:

   Phospho-p53; mouse (1:400 dilution in the combined solution; Cell Signaling Technology, P/N 9286)

   Total ERK1; rabbit (1:100 dilution in the combined solution; Santa Cruz Biotechnology, P/N SC-94)
2. Mix the primary antibody solution thoroughly before adding to wells.

Incubate with Primary Antibodies

1. Remove blocking buffer from the blocking step and add 50 µL of the desired primary antibody or antibodies in Intercept Blocking Buffer to cover the bottom of each well.
2. Make sure to include control wells without primary antibody to serve as a source for background well intensity. Only add 50 µL of Intercept Blocking Buffer to control wells.
3. Incubate with primary antibody overnight with gentle shaking at 4 °C.

Wash

1. Prepare Tween® Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS + 0.1% Tween 20	1000 mL

2. Remove primary antibody solution.
3. Using a multi-channel pipettor, add 200 µL Tween Washing Solution (RT). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
4. Allow wash to shake on plate shaker for 5 minutes at RT.
5. Repeat washing steps 4 more times.

X. Secondary Antibodies

Dilute Secondary Antibodies

1. Dilute the fluorescently-labeled secondary antibodies in Intercept® Blocking Buffer as specified below. To lower background, add Tween 20 to the diluted antibody to a final concentration of 0.2%. Recommended dilution range is 1:200 to 1:1,200.
   Goat anti-Rabbit IRDye® 800CW (1:800 dilution in the combined solution)
   Goat anti-Mouse IRDye 680RD (1:800 dilution in the combined solution)

   > **Note:** Minimize exposure of the antibody vials to light.

2. Mix the antibody solutions and add 50 µL of the secondary antibody solution to each well.

Incubate with Secondary Antibodies

1. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

Wash

1. Remove secondary antibody solution.
2. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution at RT (see "Wash" on the previous page). Add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.
3. Allow wash to incubate on a plate shaker for 5 minutes at RT.
4. Repeat washing steps 4 more times. Protect plate from light during washing.

XI. Image

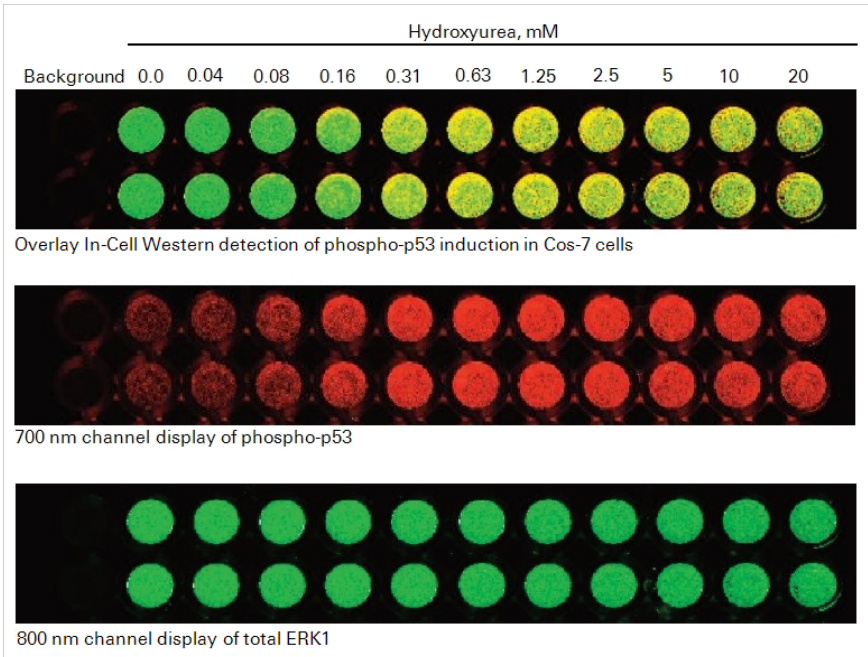
1. After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for several weeks (sealed and protected from light).
2. Before plate scanning, clean the bottom plate surface and the Odyssey® Imager scanning bed (if applicable) with moist, lint-free tissue to avoid any obstructions during scanning.
3. Scan plate with detection in both 700 and 800 nm channels.

Suggested Scan Settings

All settings may require adjustment for optimal data quality. Higher resolutions or scan qualities can be used, but the scan time will increase.

Instrument	Resolution	Scan Quality	Intensity Setting (700 nm)	Intensity Setting (800 nm)
Odyssey Classic	169 µM	lowest	5	5
Odyssey CLx	169 µM	lowest	Auto Mode	Auto Mode
Odyssey Sa	200 µM	lowest	7	7
Aerius™ Imager	200 µM	lowest	7	7

XII. Experimental Results



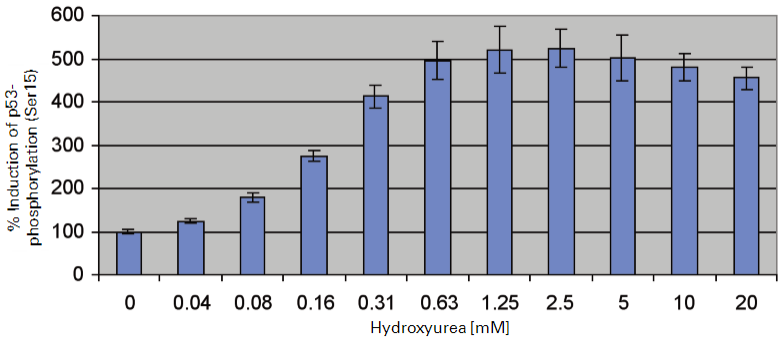


Figure 1. Dose response of Cos-7 cells to Hydroxyurea as measured by specific antibody detecting phosphorylated-p53 (Ser15) using total ERK1 for normalization. The image represents a 96-well two-color In-Cell Western™ Assay with the 700 and 800 nm channels detecting phosphorylated-p53 (Ser15) and total ERK1, respectively. Background wells were incubated with secondary antibody but no primary antibody. The graph represents the average of four sets of technical replicates, demonstrating the percent induction of phosphorylated-p53 (Ser15).

XIII. References

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