The RNAi Consortium (TRC)
Broad Institute

TRC Laboratory Protocols
Protocol Title: **In-Cell Western (ICW) assay of V5 tagged ORF clones**
Current Revision Date: **10/20/2012**
RNAi Platform, Broad Institute, trc_info@broadinstitute.org

Brief Description:

This protocol is aimed to help users of our ORF-pLEX clones conduct In-Cell Western assay of ORF expression in high-throughput format (96-well plates).

Table of Contents:

Workflow Timeline:

The process involves the following steps:

Day 1	Fix cells. Stain with primary antibody overnight
Day 2	Stain with secondary antibody, DRAQ5/Sapphire700 and scan plates

These procedures should be carried out in accordance with biosafety requirements of the host institution.

Protocol:

I. Materials & Instrumentation

96-well black/clear bottom tissue culture plates (Corning #3904)
Paraformaldehyde (16%) (Electron Microscopy Sciences #15710)
Triton X-100 (Sigma #T9284)
anti-V5 antibodies: mouse (Invitrogen), rabbit (Sigma-Aldrich #V8137)
Infra-red (IR) secondary antibodies: anti-mouse 800, anti-rabbit 800 (Li-COR Biosciences)
DRAQ5/Sapphire700 (cellular staining for cell numbers) (Li-COR Biosciences)
Odyssey blocking buffer (Li-COR Biosciences #927-40000)
Odyssey scanner (Li-COR Biosciences)

40ml 4% formaldehyde / 0.1% Triton X-100 cell-fixing solution (Scale as needed):

10 ml 16% formaldehyde
200 μl 20% Triton X-100
30 ml PBS

Note: Formaldehyde is a hazardous chemical; all waste must be put in appropriate containers

Antibody mixes:

Notes:

- Spin antibody stocks 5 min @14,000 x g to remove aggregates which cannot be seen by eye. Pipette from the top.
- Prepare antibody mixes immediately prior to use.
- Choosing antibodies: In order to avoid secondary antibody cross-talking with the host cells, we recommend the primary antibodies be made from an organism differing from the host cells. For example, if you stain

human A549 cells, you can use anti-V5 antibody made from mouse, whereas if you stain mouse Hepa cells, you should avoid primary antibodies made from mouse (e.g. use rabbit anti-V5 antibody, instead).

Primary antibody mix (Scale as needed):
10 ml primary antibody mix in 0.1% Triton X-100
- 50 µl 20% Triton X-100
- 4 µl anti-V5 antibody (achieving 1:2,500 dilution)
- 10 ml PBS

Note: antibody dilution factor may vary

Secondary antibody/DNA staining mix (Scale as needed):
10 ml secondary antibody mix in 0.1% Triton
- 10 µl Anti-Mouse 800 IRDye (achieving 1:1,000 dilution)
- 10 µl Saphhire700 (achieving 1:1,000 dilution)
- 1 µl DRAQ5 (achieving 1:10,000 dilution)
- 50 µl 20% Triton
- 10 ml PBS

II. Instructions

Fixing cells:

1. The infected cells are in 96-well black/clear bottom tissue culture plates (Corning #3904) and are 3-4 days post-infection.
2. Remove the media.
3. Fix cells with 100µl 4% Formaldehyde/0.1% Triton X-100 per well.
4. Incubate plates for 30 min at room temperature (RT).
5. Remove fixative and wash with 200µl PBS 2 times.
6. Keep cells in 200µl of PBS.
7. Stain cells immediately or leave at 4^{o}C overnight for next day work.

Staining fixed cells with antibodies:

8. Remove 200µl PBS.
9. Block wells with 100µl Odyssey blocking buffer. Allow blocking for 1.5 hours at room temperature with moderate shaking on a plate shaker.
10. While waiting, prepare primary antibody mix.
11. Remove blocking buffer.

Note: In below step, we recommend that you carry some secondary antibody-only controls to assess its non-specific binding.

12. Excluding secondary antibody-only control wells, in all other wells, add 50uL freshly prepared **primary antibody mix.**
13. Incubate primary antibody for 2.5 hours at RT **or** overnight at 4 ^{o}C, with gentle shaking.
14. Remove antibodies.
15. Wash three times with 200µl 0.05% Tween 20/PBS
    - 40mL PBS
    - 100uL 20% Tween 20

In each wash, move plates to shaker and allow gentle shaking for 5 minutes.

16. Add 50µl per well of freshly prepared **Odyssey secondary antibody mix**, e.g. IRDye plus DRAQ5/Sapphire700.
17. Incubate at RT for 1 hour with shaking, with plates wrapped in aluminum foil.
18. Remove antibodies.
19. Wash three times with 200µl 0.05% Tween20/H_2O.
20. Keep cells in 100µl PBS.

21. Prior to scan, tap off the liquid.
22. Scan plates on Odyssey scanner, using plate settings and dual channels (700 and 800).

Revision Notes: