26

Target Validation in Drug Discovery

Robert A. Blake

Summary

The process of target validation identifies and assesses whether a molecular target merits the development of pharmaceuticals for therapeutic application. The most valuable application of high content screening to target validation is at the early stages of the process when genetic methods (including RNA interference—RNAi) are being applied to many potential targets. At this stage both throughput and indepth analysis are required. This process is illustrated using various examples from the area of oncology target validation. The Akt signal transduction pathway is used to illustrate an efficient way of identifying HCS compatible reagents for use in assay development. RNAi transfection methods are discussed. A description is given of an HCS assay that simultaneously measures two nodes of the Akt pathway: Akt substrate phosphorylation and RPS6 phosphorylation. Another example of an assay measuring proliferation (DNA synthesis) and apoptosis (Histone H2B phosphorylation) within the same cell population is used to illustrate the combination of typical phenotypic assays.

Key Words: Akt; apoptosis; BrdU; DNA synthesis; high content screening; histone H2B, oncology; phospho-histone H2B; phospho-RPS6; PRAS40; phospho-PRAS40; 4E-BP1; phospho-4E-BP1; RNAi, RPS6; siRNA, S-phase; target validation.

1. Introduction

The term "target validation" describes the process of demonstrating that a molecular target is a therapeutically relevant pharmacological target. In reality a target is not truly "validated" until late stage clinical trials are complete and the mechanism of action understood. "Target validation" is also used to describe the very early stages of this process that precede the development of pharmacological reagents—a stage that is very reliant on genetic methods of modulation of the target. These genetic methods include RNAi or small interfering RNA (siRNA; a gene silencing technology that is finding a major application in target validation; *see* Chapter 18 on RNAi and **Note 1**).

It is this earliest stage of target validation, when multiple targets are being processed in parallel, that high content screening is most valuable. HCS is able to merge the detailed biological measurements often required for target validation with the throughput required for processing multiple targets. The focus of this chapter will be the application of HCS to the early phase of the target validation process using RNAi techniques for decreasing gene expression. This process will be illustrated by a description of methods used to identify and validate oncology targets that are associated with the Akt signal transduction pathway and affect cellular proliferation or apoptosis. The general principles behind these examples can be extended to other areas of biology and target validation projects.

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

2. Materials

1. HCS cell imaging equipment: Cellomics, Inc. Arrayscan with Compartmental Analysis Bioapplication.
2. 96-well plate washer: for example, Titertek MAP C2 Quadrant.
3. Microtiter plate liquid handling equipment: for example, Titertek Multidrop.
4. Microtiter plate 96/384-well-to-96/384-well liquid handling equipment: for example, Matrix PlateMate® Plus, Caliper LifeSciences RapidPlate®.
5. Transfection reagents: siRNA, positive and negative control siRNA, Oligofectamine.
6. Tissue culture reagents: DMEM growth medium. OptiMEM1 serum reduced medium. Fetal bovine serum (FBS).
7. Human lung carcinoma cell line A549, Human prostate cancer cell line PC3.
8. Insulin-Arg (Upstate Biotechnology 01-207), IGF-I (Upstate Biotechnology 01-189), LY294002 (Calbiochem, cat. no. 440202), Rapamycin (Calbiochem [EMD Biosciences, San Diego, CA] cat. no. 553210).
9. Antibodies to components of signal transduction pathway or biological process under study:
   a. Rabbit antiphospho-Ser235/236-RPS6 antibody (Cell Signaling, cat. no. 2211).
   b. Biotinylated antiphospho-PRAS40-Thr246 (biotinylated form of Biosource, cat. no. 44-1100G).
   c. Antibromodeoxyuridine, mouse IgG1, monoclonal (anti-BrdU) (Molecular Probes/Invitrogen, cat. no. A21300).
   d. Antiphospho-Histone H2B rabbit polyclonal (UBI, cat. no. 07-191).
10. Fluorophore-conjugated secondary antibodies:
    a. Alexa Fluor® 546 goat antirabbit IgG (H + L) highly cross-adsorbed (Molecular Probes [Invitrogen Corp., Carlsbad, CA] A-11035).
    b. Alexa Fluor® 488 goat antirabbit IgG (H + L) highly cross-adsorbed (Molecular Probes, cat. no. A-11034).
    c. Alexa Fluor® 546 goat antimouse IgG1 (Molecular Probes, cat. no. A-21123).
    d. Alexa Fluor® 488 goat antimouse IgG1 (Molecular Probes, cat. no. A-21121).
11. BisBenzimide (Hoechst, cat. no. 33258; Sigma, cat. no. B-1782 [St. Louis, MO]).
12. 96-well clear bottom black wall tissue culture plates (e.g., ViewPlate-96 Black, PerkinElmer, cat. no. 6005182).
13. Plate seals (e.g., ThinSeals from Excelscientific [Wrightwood, CA] cat. no. 100-THIN-PLT).
14. 4% w/v formaldehyde in PBS.
15. 0.5% v/v Triton X-100 in PBS.
16. 5-bromo-2′-deoxyuridine (BrdU; Sigma B9285).
17. 2.4 *M* HCl.
18. Phosphate buffered saline (PBS), pH 7.6.

3. Methods

The initial step in any target identification and validation process is defining the criteria that the prospective targets should meet. In this example the criteria are defined as follows: (1) the target must modulate components of the Akt signal transduction pathway (*see* **Fig. 1**); (2) the target must regulate either proliferation or apoptosis of cancer cells. The next step is the development of assays to survey the biology of interest. The following sections describe: (1) a process to identify suitable HCS reagents to probe the Akt signal transduction pathway; (2) a specific assay that surveys two individual nodes of the Akt signal transduction pathway; (3) an assay that simultaneously monitors effects on proliferation and apoptosis. The cell-lines selected for this study include: A549 a lung adenocarcinoma cell-line that has an upregulated Akt pathway probably resulting from a deletion of the tumor suppressor kinase LKB1 ***(1,2)*** and PC3 cells (a prostate cancer cell-line that has an up-regulated Akt pathway because of deletion of the tumor suppressor PTEN ***(3)*** (*see* also **Note 1**).

3.1. Reagent Identification

The initial limiting factor in developing HCS assays is usually the identification of suitable reagents to track the biology under study; that is, identify suitable antibodies that work sufficiently well for immunofluorescence staining to be applied to HCS. Unfortunately the manufacturers of commercial antibodies do not always accurately describe the performance of their products and

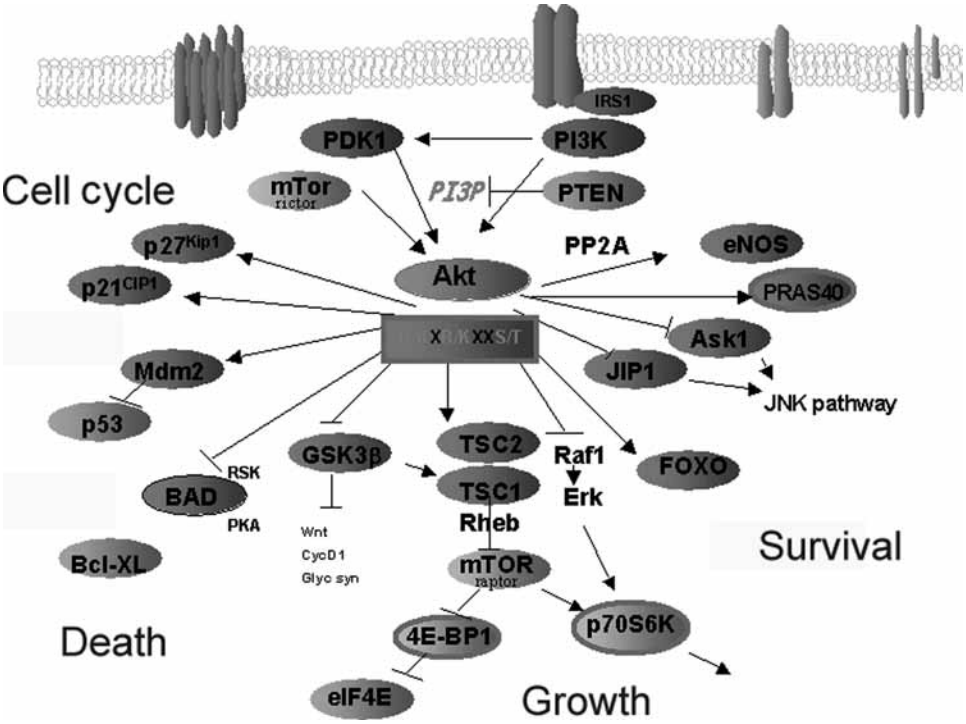

Fig. 1. Schematic representation of the Akt signal transduction pathway: The PI3Kinase/Akt/mTor pathway is generally involved in the regulation of cell survival and growth. It is has been widely implicated in many types of cancer. Akt kinases phosphorylate substrates within the generic amino-acid sequence R/K-X-R/K-X-X-S/T and often regulate binding to 14-3-3 proteins.

the choice of cell-line often affects the usefulness of an antibody owing to differences in epitope expression. Often the most efficient approach is to screen all available reagents together for direct comparison of their performance. The example used to illustrate this process is the survey of multiple reagents of components of the Akt signal transduction pathway. The method is based on testing the immunofluorescence-staining pattern for each antibody on cells, in which the Akt pathway is stimulated or inhibited (*see* **Fig. 2**). The objective is to identify antibodies whose immunofluorescence-staining pattern is modulated in a way that makes sense according to the biology of the pathway and individual protein (e.g., a decrease in phospho-staining of Akt substrates following PI3 Kinase inhibition).

1. Collect aliquots of antibodies to components of the Akt signal transduction pathway (proteins, phosphoproteins, and so on) and dispense each into a polypropylene 96-well plate, grouping antibodies according to species of origin (enables easier dispensing of the appropriate secondary antibody later).
2. Seed four 384-well black wall clear bottom tissue culture plates with A549 (4000 cells per well; 2.5% FCS); incubate overnight (*see* **Note 2** and **3**).
3. Dispense inhibitors of the Akt/mTor pathway as follows: add the PI3Kinase inhibitor LY294002 (final concentration 50 μ*M*) to plate nos. 1 and 2 to the alternating columns 2, 4, 6, and so on. Add the mTOR inhibitor rapamycin (final concentration 100 n*M*) to plate nos. 3 and 4 to the alternating columns 2, 4, 6, and so on (**Note 4**).
4. Dispense activators of the Akt/mTor pathway as follows: to plate nos. 1 and 3 add insulin (concentration 500 ng/mL) to the alternating rows B, D, F, and so on. To plate nos. 2 and 3 add IGF1 (concentration 50 ng/mL) to the alternating rows B, D, F, and so on.
5. Fix the cells as follows: 40 min after addition of the insulin and IGF1 add an equal volume of 4% formaldehyde directly to the tissue culture medium to fix the cells; incubate at room temperature for 1 h (**Note 5**).

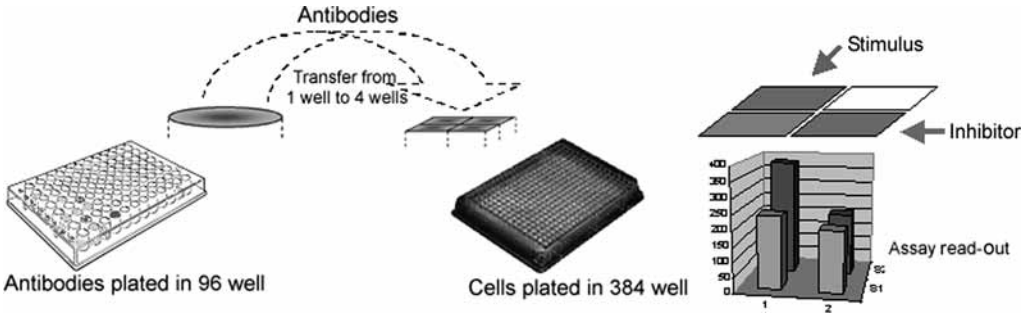

Fig. 2. Schematic depicting the process described in **Subheading 3.1** by which antibodies are screened for changes in immunofluorescence staining patterns in cells treated with activators or inhibitors of the Akt pathway.

6. Wash (four times) with 100 µL of PBS and add 0.5% Triton X-100 (in PBS) to permeabilize the cells. Incubate for 5 min and then wash (**Notes 6** and **7**).
7. Stain the cells with the various primary antibodies as follows:
   a. Transfer 1 µL of each antibody from the stock 96-well plate to a second polypropylene plate. Dilute with 200 µL of PBS.
   b. Remove the PBS from each of the 384-well tissue culture plates (by inverting and flicking over a sink). Transfer 40 µL of each of the diluted antibody from the 96-well plate to the cluster of four wells that correspond to the same position in the 384-well plate. Incubate overnight at 4°C. Wash (four times) with 100 µL of PBS.
8. Stain the cells with appropriate secondary antibodies as follows (**Note 2**).
   a. Prepare the appropriate fluorophore labeled secondary antibodies Alexa-fluor 488 conjugated anti-mouse and/or antirabbit IgG (Molecular Probes) (depending on the species of origin of the primary antibodies at a 1/1000 dilution in PBS containing 1 µg/mL Hoechst dye.
   b. Remove the PBS from each of the 384-well tissue culture plates and transfer 50 µL of each of the diluted secondary antibodies to the appropriate wells according to species of origin of the primary antibody. Incubate for 2 h at room temperature. Wash (four times) with 100 µL of PBS.
9. Seal the plates with plastic plate seals.
10. Read the plates on a Cellomics, Inc. Arrayscan plate reader using the "Compartmental Analysis" Bioapplication using XF100-Hoechst in channel 1 (focus channel) and the AlexaFluor488 in channel 2 with the XF100/FITC (1 s fixed exposure; *see* **Note 8**).
11. Data analysis: Export the following well features:
    a. MEAN_CircTotalIntCh2.
    b. MEAN_CircAvgIntenCh2.
    c. MEAN_RingTotalIntenCh2.
    d. MEAN_RingAvgIntenCh2.
    e. MEAN_CircRingAvgIntenDiff.
    f. MEAN_CircRingAvgIntenRatio.

These features will capture variations in the staining intensity in the nuclear and cytoplasmic regions (*see* **Note 9**). Compare the data from your untreated, stimulated, and inhibited samples and identify antibodies whose immunofluorescence pattern is consistently modulated by activation or inhibition of the signaling pathway. Determine whether the changes in the immunofluorescence make sense according to the biology of the individual proteins. **Figure 3** shows examples of antibodies identified using this procedure.

Fig. 3. *(Opposite page)* Thr 246 site (Biosource no. 44-1100G) shows a similar pattern. The antibody to the phospho-Ser235/236-RPS6 antibody (Cell Signaling no. 2211) strongly stains the cytoplasm in control A549 cells. Transfection with siRNA to Akt2 dramatically diminishes the cytoplasmic staining pattern. An antibody to the mTor substrate 4E-BP1's phospho-Thr37/46 site (Cell Signaling no. 9459) gives a diffuse cytoplasmic and nuclear stain in control A549 cells. Transfection with siRNA to Akt2 reduces the cytoplasmic but not the nuclear staining.

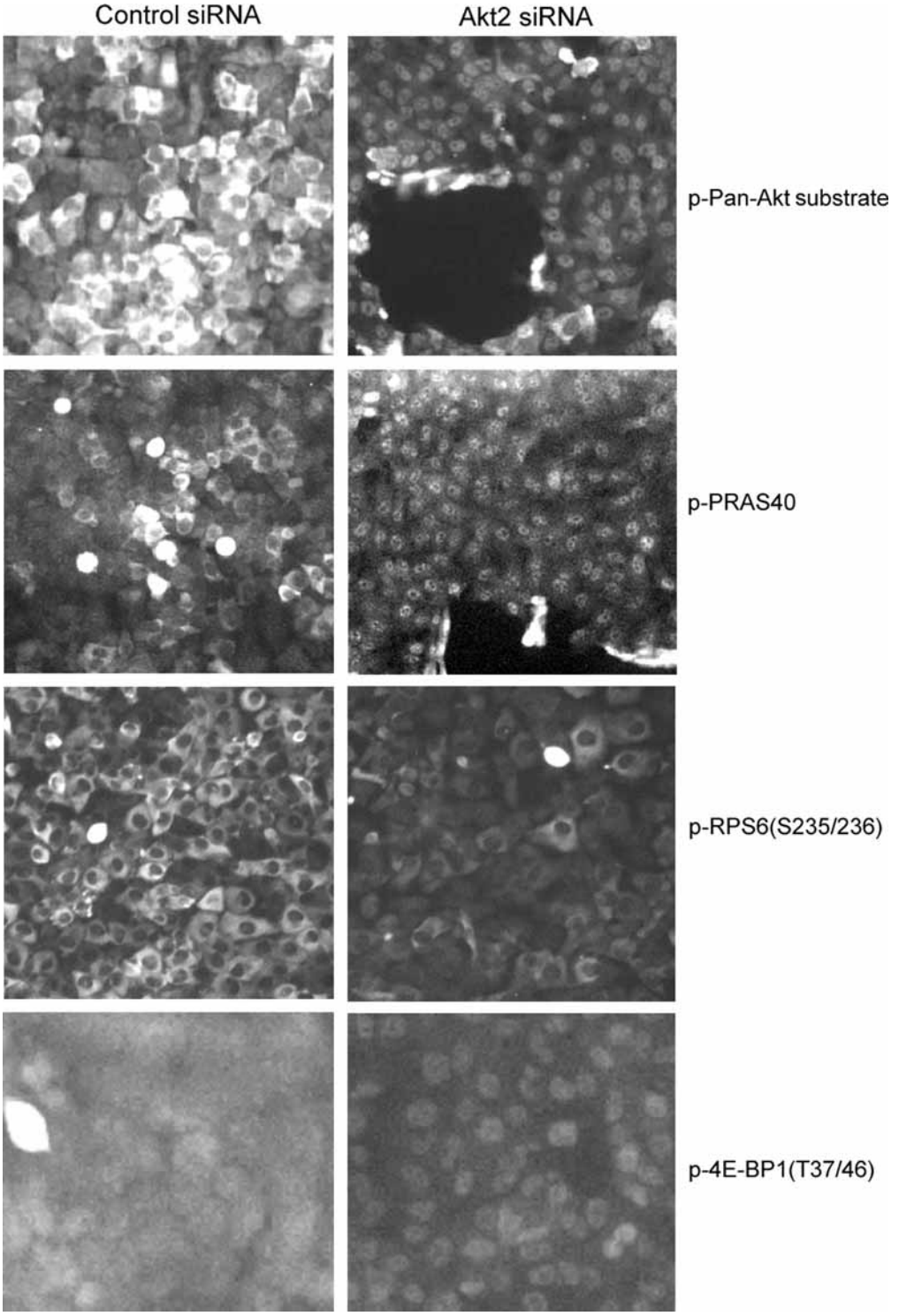

Fig. 3. Examples of antibodies identified using the process described in **Subheading 3.1.** The phospho-Serine/Threonine Akt substrate antibody (Cell Signaling no. 9611) recognizes both cytoplasmic and nuclear proteins in control A549 cells. Transfection with siRNA to Akt2 or inhibition of PI3Kinase (not shown) reduces cytoplasmic staining but not nuclear staining. The phospho-antibody to the Akt substrate PRAS40

3.2. Multiplexed Phospho-RPS6, Phospho-PRAS40 HCS Assay

Two antibodies that were identified using the method described in **Subheading 3.1.** were: antiphospho-PRAS40, which provides a measurement of Akt activity *(4)*; and antiphospho-RPS6_235/236, which provides a measurement of the p70 S6Kinase/mTor arm of the pathway *(5,6)*. This section describes an HCS assay in which both of these read-outs are combined to provide simultaneous measurements on two sections of the Akt pathway. This is an example of a multiplexed HCS assay that can be used to test siRNA for the purposes of early stage target validation (*see* **Note 10**). Other assays developed in **Subheading 3.1.** could also be applied.

1. Resuspend the siRNA according to manufacturers instructions in RNAase free H_2O at a final concentration of 20 μ*M*.
2. Seed A549 or PC3 cells at a density of 2000 cells per well (100 μL DMEM, 2.5% FBS, no antibiotics) in a 96-well. Incubate overnight at 37°C, 5% CO_2 (**Notes 8, 12,** and **13**).
3. Prepare the siRNA transfection mixes as follows in separate wells of a 96-well polypropylene plate (The volumes listed are sufficient for a single well of a 96-well plate and must be scaled up according to the number of samples being transfected. The final siRNA concentration is 25 n*M*.):
   a. Prepare Mix-A by adding 0.15 μL of 20 μ*M* siRNA to 16.85 μL of OptiMEM in a polypropylene 96-well plate (this is enough for transfecting a single well of a 96-well plate. Scale accordingly).
   b. Prepare Mix-B in bulk to add to Mix-A. For a single transfection well blend 0.6 μL of Oligofectamine with 2.4 μL of OptiMEM. Scale these volumes up to be sufficient for the total number of wells required including excess to account for dispensing dead-volume.
   c. Mix 3 μL of Mix-B with 17 μL Mix-A (Single transfection volume. Scale accordingly.) Mix gently by pipeting up and down a couple of times. Let the total mixture stand for 20 min (room temperature).
   d. Transfer 20 μL of the MixA/B blend to the each well of the tissue culture plates. Return the tissue culture plates to the incubator (**Notes 13–15**).
4. Change the cells into a no serum "starvation medium" as follows: 24 h after transfection wash the cells in DMEM by gently aspirating off the existing medium; wash with 200 μL DMEM per well; repeat the aspiration and dispense 100 μL of DMEM.
5. Fix the cells as follows: 72 h after transfection fix the cells by adding 100 μL of 4% formaldehyde to each well and incubating for 1 h at room temperature (**Note 4**).
6. Permeabilize the cells as follows: aspirate the formaldehyde from each well and add 100 μL of 0.5% v/v Triton X-100 in PBS. Incubate for 5 min at room temperature. Wash three times with 200 μL PBS (**Note 6**).
7. Stain for phospho-RPS6_S235/236 as follows:
   a. Add 50 μL of PBS containing a 1/100 dilution of rabbit antiphospho-Ser235/236-RPS6 antibody and incubate overnight at 4°C. Wash four times with 200 μL PBS (pH 7.4).
   b. Add 50 μL of PBS containing a 1/1000 dilution of Goat-antiRabbit-Alexa546 and 1 μg/mL Hoechst 33258. Incubate at room temperature for 2 h. Wash four times with 200 μL PBS (pH 7.4) (**Note 16**).
8. Stain for phospho-PRAS40 as follows:
   a. Incubate with 1/500 dilution of biotinylated antiphospho-PRAS40 antibody overnight at 4°C. Wash.
   b. Incubate with 500 μL of a 1/1000 dilution of AlexaFluor 488 conjugated Streptavidin in PBS; Incubate 2 h at room temperature. Wash leaving 200 μL PBS in the wells. Seal the plates (**Note 16**).
9. Read on the Cellomics Arrayscan using BioApplication "Compartmental Analysis" with reference and autoexposure wells activated and defined as the negative control (uninhibited) wells. Channel 1—nuclei, XF53-Hoechst, Channel 2—pRPS6, XF53—TexasRed, Channel 3—pPRAS40, XF53—FITC. Autoexposure peak target (percentile): Ch1—40%, Ch2—24%, Ch3—24%. RingAvgIntenLevelHighCh2_CC set to 2. CircRingAvgIntenDiffLevelLowCh3_CC set to 0.1 (these are suggested starting values *see* **Note 17**).
10. Retrieve the following data:
    a. Percentage of cells positive for pRPS6 staining: HighRingAvgIntenCh2 (%).
    b. Percentage of cells positive for cytoplasmic vs nuclear pPRAS40 staining: LowCircRingAvgIntenDiffCh3 (%).
    c. Cell density: ObjectPerFieldCount (**Notes 8, 9, 17–19**).

3.3. Proliferation and Apoptosis Multiplexed Assay

This section describes an HCS assay that addresses the second target validation criteria defined in **Subheading 3.** that the target must regulate either proliferation or apoptosis of cancer cells. In this example DNA synthesis is used as a measurement of proliferation and is monitored using the incorporation of the nucleotide derivative bromo-deoxyuridine into newly synthesized DNA. The apoptotic marker used here is the phosphorylation of Histone H2B in the nucleus. Histone H2B is phosphorylated by the kinase MST1, following its cleavage and activation by the Caspase 3 ***(7)***.

1. Day 1: seed the cells (in 2.5% FBS) into black wall clear bottom tissue culture plates (4000 cells per well) (**Notes 2** and **11**).
2. Day 2: carry out siRNA transfections as described in **Subheading 3.2.**
3. Day 5: at the start of the experimental period during which DNA synthesis is being measured, add BrdU (20 μ*M*) and incubate for 4 h.
4. (1) At the end of 4 h fix the cells by adding 100 μL of 4% formaldehyde directly to the tissue culture medium in the well and leave for 1 h at room temperature. (2) Aspirate off the formaldehyde and wash four times with PBS (200 μL/well).
5. Permeablize the cells with 0.5% v/v Triton X-100 in PBS (50 μL/well). Incubate 5 min. Wash with PBS (**Note 6**).
6. Treat the cells with 2.4 *M* HCl (50 μL/well) at 37°C (inside incubator) for 20 min. Wash thrice with PBS (200 μL/well) (**Note 20**).
7. Stain the cells with 50 μL/well of PBS containing: 1/500 dilution of mouse antibromodeoxyuridine; 1/1000 dilution of antiphospho-Histone H2B, Hoechst dye 1 μg/mL. Incubate overnight. Wash four times with PBS (200 μL/well) (**Note 16**).
8. Stain the cells with 50 μL of 1/1000 dilution of AlexaFluor546 antirabbit IgG in PBS for 1 h at room temperature and wash.
9. Stain the cells with 50 μL of 1/1000 dilution of AlexaFluor488 antimouse IgG in PBS for 1 h at room temperature and wash.
10. Seal the plates.
11. Read on the ArrayScan using Compartmental Analysis Channel 1 Hoechst/XF53; channel 2 FITC/XF53; channel 3 TRITC/XF53, ×10 objective. Assay parameters: reference wells off. Autoexposure peak target percentile 24% for channels 2 and 3. CircRingAvgIntenDiffLevelHighCh2 = 30. CircRingAvgIntenDiff-LevelHighCh3 = 50. (These are suggested starting values, *see* **Note 17**.)
12. Retrieve the following data:
    a. Percentage of BrdU positive cells: HighCircRingAvgIntDiffCh2 (%)—(Percentage of cells with BrdU staining in nucleus at a threshold intensity greater than background cytoplasmic staining).
    b. Percentage of Phospho-Histone H2B positive cells: HighCircRingAvgIntDiffCh3 (%)—(Percentage of cells with Nuclear phospho-Histone H2B staining (**Fig. 4**) in nucleus at a threshold intensity greater than cytoplasmic staining).
    c. Cell density: ObjectPerFieldCount (**Notes 8, 9, 17,** and **18**).

4. Notes

1. Cell-line selection is an important aspect to target validation. Not only will it affect the quality of the assay, it might affect how targets behave and how well specific antibodies work. There is no easy answer to this apart from using all available genomic information and testing different cell-lines for siRNA effects and protein knockdown.
2. HCS assays require frequent dispensing of reagents and cells into plates. Automated or semi-automated dispensing systems are invaluable. The TiterTek Multidrop is a useful device for this. If using it to seed cells, make sure you flush it through with 70% ethanol followed by sterile PBS first. If you have issues seeding cells at low density try treating the tubing with 50% serum before seeding cells.
3. You should seed cells in, and stain, all wells of a multiwell plate. Empty wells can cause HCS readers to temporarily lose the correct focal plane. This will slow down the read-time as the HCS reader tries to re-establish the correct focal height and search for objects.
4. When adding compounds dissolved in DMSO or other organic solvents always prepare an intermediate dilution (e.g., 10X final concentration) in tissue culture medium and add this to the cells.

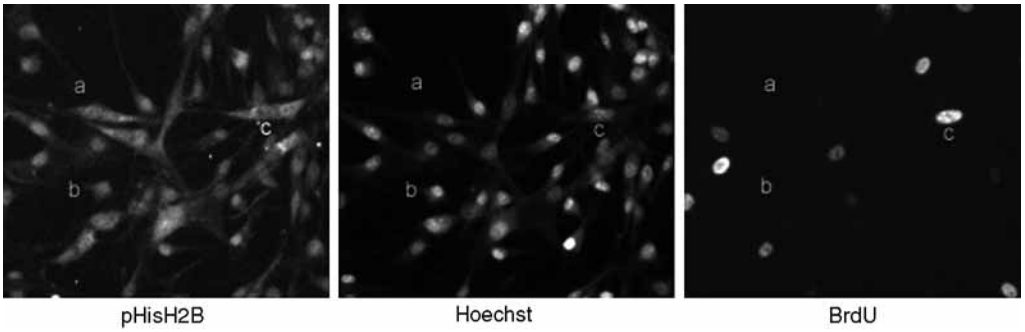

Fig. 4. The three images show the same field of A549 cells stained for phospho-Histone H2B (apoptosis marker), DNA (Hoechst) and newly synthesized DNA (BrdU). Cell **(A)** is an example of a cell that is negative for both nuclear phospho-Histone H2B staining and BrdU. It is has not started DNA synthesis nor has it gone into apoptosis. Cell **(B)** is an example of a cell that has nuclear phospho-Histone H2B staining but not BrdU staining. It represents part of the apoptotic cell-population. Cell **(C)** is an example of a cell that does not have nuclear phospho-histone H2B and stains positive for BrdU. It is part of the cell population that has begun DNA synthesis.

100% DMSO added to cells will descend undiluted to the bottom of the well and kill the cells where it lands. Make sure that the final DMSO concentration does not adversely affect the cells. one percent v/v is a typical concentration to use.

5. There are several methods for fixing cells including cold alcohols and formaldehydes. Acetone should never be used as it degrades plate plastics. Formaldehyde (or paraformaldehyde) is most commonly used. Avoid using formaldehyde solutions containing methanol, as this will destroy fine cytoskeletal structures within cells. If you want to retain loosely attached cells (e.g., mitotic cells) consider adding formaldehyde directly into the medium instead of removing the medium first (this will require a slightly longer fixing period approx 1 h). Cold methanol or ethanol require the medium to be aspirated first and are more difficult to work with in a scaled-up and automated process. They should be used only if the antibodies require it.
6. There are a couple of golden rules on washing multiwell plates: do not wash the cells off and do not allow the cells to dry out (this causes artifacts in immunofluorescent staining). Automated plate washers greatly aid throughput and provide consistency. Set the dispense speeds to be gentle. Make sure aspirating tips do not come too close to the cell monolayer and leave a protective layer of liquid. Wash four times with just less than the maximum well volume; for example, 200 μL for a 96-well plate, 100 μL for a 384-well plate. Be sure to always flush the plate washer with distilled water after use otherwise salt buildup will compromise performance. One recommended plate washer is the Titertek MAP C2 Quadrant, which can handle 96- and 384-well plates.
7. The concentration of detergent required to permeabilize different cell-types might differ. Also permeabilizing the nuclear membrane requires higher detergent concentrations than the cytoplasmic membrane. Typically 0.5% v/v Triton X-100 in PBS is sufficient for most cell-types.
8. Exposure times should be set to ensure that typical images are not over or under exposed and remain within the linear range. It is best to use autoexposure options to minimize the effects of experiment-to-experiment variation in staining intensity. When surveying many different antibodies on the same plate you should set a fixed exposure time that works for the majority of staining intensities.
9. Different HCS instruments apply different names to the output features but the general principle remains the same. Use spatial and temporal changes in staining intensity to determine whether a protein is moving within the cell, has increased or decreased covalent modification and so on. You can use changes in absolute fluorescence intensity or changes in the ratio or difference between compartments of the cell depending on what makes sense regarding the biology and the images.
10. The practical limitations to multiplexing assays derive from the following: Primary antibodies must be of different species so that cross-reactivity by secondary antibodies does not occur. Alternatively primary antibodies can by directly conjugated to fluorophores (however, such staining is not as stable and

tends to fade probably resulting from diffusion of antibodies off the epitope). The use of biotinylated antibodies enables two primary antibodies of the same species to be used (as is the case for the pRPS6 pPRAS40 assay described in **Subheading 3.2.**). Other limitations derive from the ability of HCS readers to distinguish different fluorophores.

11. Serum concentration can greatly affect the performance of an assay. The serum concentration needs to be sufficient to maintain healthy cells but too high a serum concentration can reduce the sensitivity of cells to antiproliferative effects of siRNA or their effects on signal transduction pathways. Typically 2.5% v/v fetal calf serum was used in the assays described here but the optimal concentration should be determined experimentally.
12. Cell density can have significant effects on assay performance. For example the phospho-RPS6 staining described in **Subheading 3.2.** is greatly reduced as cells approach confluence. The phospho-Histone H2B signal increases in confluent cell-layers as the percentage of apoptotic cells increase. Also, depending on the cell-type, confluent cell layers are more likely to peal off during washing procedures.
13. It is important to establish a set of positive and negative control siRNAs for any assay. For example siRNA to Akt family members for Akt substrate assays. Not only are these essential for assay development but they also provide transfection controls while screening and enable quality control of the process.
14. Sterility of tissue culture might be a concern to newcomers to HCS based target validation. Transfection of multiple siRNA into multiple plates usually requires robotic systems that are not enclosed in tissue culture hoods. siRNA transfection requires that antibiotics be omitted. The experiments typically last 48–72 h, which might be long enough for an infection to develop. Take comfort from the fact that it is not as bad as it sounds. As long as you ensure that all solutions, dispensing devices, plates and tips are sterile, briefly exposing tissue culture plates to unfiltered air rarely results in a contamination within the timeframe of typical siRNA experiments.
15. Optimal siRNA transfection protocols can differ between cell-lines. Test different lipid transfection reagents and concentrations. Monitor the decrease in a control protein (e.g., lamin, β-catenin) by immunofluorescence staining (best) or by Western blot and the monitor the health of the cells. Oligofectamine from Invitrogen usually works fairly well.
16. There are some common staining artifacts that can occur. The presence of a single bright punctuate spot in the nucleus is likely to be resulting from insufficient time for unbound primary antibody to diffuse out of the nucleus between washing and addition of the secondary antibody (diffusion out of the nucleus is slower than the cytoplasm because of the nuclear membrane). Trapped unbound primary antibody forms an immune-complex with the secondary antibody resulting in the bright punctuate spot. This can be remedied by allowing extra time between the primary antibody washing step and the addition of the secondary antibody.
17. Many features of the image analysis will have to be optimized by the user and cannot be defined here. Ensure that the object identification method is correctly identifying the nuclei by varying background correction and the object identification parameters. Ensure that the mask defining the nucleus is retained within the nuclear area. Ensure that the ring defining the cytoplasm extends outside of the nucleus and does not (for the most part) extend beyond the cytoplasm. Define intensity thresholds etc to define responding and nonresponding cells.
18. Population analysis measurements permit you to register cells as responders or nonresponders defined by response thresholds. Population analysis is useful for improving an assay's dynamic range and in many cases provides more biologically meaningful data than averaged responses. The thresholds should be determined experimentally. Some Bioapplications (e.g., Cellomics, Inc., Compartmental Analysis) allow you to define these thresholds on a plate-by-plate basis using reference wells to establish a nonresponsive baseline.
19. Cells can also be scored for cytoplasmic staining intensity above a threshold by setting the RingSpot intensity to pick up a minimum staining intensity and thresholding on the number of spots. Several other approaches exist that the user should experiment with.
20. Inhibition of the Akt pathway by siRNA or PI3Kinase inhibitors reduces Akt substrate phosphorylation in the cytoplasm but less so in the nucleus. One explanation for this is that Akt signaling is more rapid and transient in the cytoplasm than in the nucleus ***(10)***. Also the different members of the 14-3-3 family of proteins that bind to phosphorylated Akt substrates show differential localization between cytoplasm and nucleus ***(11)***.

21. Seal plates with plasic plate seals with wells containing PBS approx 80% of well capacity. The presence of the liquid layer over the cells reduces photobleaching.
22. RNAi technology provides a quantum leap to high throughput target validation approaches. However it is an imperfectly understood phenomenon and carries with it several issues. For example decreased expression of a protein might not necessarily have the same impact as inhibiting an enzyme. Off-target effects can produce misleading results. Threshold effects are apparent. The current best solution is to use multiple siRNA for each gene and correlate the extent of protein decrease with the phenotypic effect but even this can be imperfect.
23. The availability of image analysis algorithms will limit the types of biology that can be studied. The most useful algorithms measure and compare fluorescence intensity within different compartments of the cell. Consider using these relative measurements as they take into account variation in immunofluorescence intensities between experiments and more closely match human pattern recognition. Consider using population analysis measurements. They often improve the dynamic range of an assay and are perhaps biologically more relevant than averaged values. The Compartmental Analysis bioapplication from Cellomics, Inc. is a versatile predefined algorithm and is extensively used in the assays described here.
24. Histone H2B is present in the cytoplasm as well as the nucleus in some carcinoma cell lines and other tissues *(8,9)*. The change observed in the phospho-Histone H2B pattern as cells become apoptotic is an increase in the nuclear phospho-Histone H2B staining.
25. The HCl treatment step in the BrdU protocol exposes the DNA and enables binding of the anti-BrdU antibody. This is the step that most frequently causes problems in this procedure. This step is sensitive to the HCl concentration, to the period of incubation and to temperature. Too low a concentration of HCl, too short an incubation or too cool a room temperature can cause the staining to be faint. Conversely too high an HCl concentration or too long or too warm an incubation will cause the Hoechst stain to be reduced. It is for these reasons that the HCl concentration, the period of incubation and the temperature of incubation must be consistent. In preparing the HCl solution always add the acid to the water and use appropriate safety measures. Often bottles of concentrated HCl do not provide the concentration in terms of molarity. Most stocks of approx 36–37% are approx 12 *M*.
26. If you do not get the expected experimental results make full use of the images available to you. They provide more information to help you trouble shoot the assays and determine the step that failed.

Acknowledgments

The author thanks the members of Exelixis Target Discovery team especially K. Ward who developed the pRPS6 assay and our collaborators at Bristol-Myers Squibb.

References

1. Mehenni, H., Lin-Marq, N., Buchet-Poyau, K., et al. (2005) LKB1 interacts with and phosphorylates PTEN: a functional link between two proteins involved in cancer predisposing syndromes. *Hum. Mol. Genet.* **14(15),** 2209–2219.
2. Jimenez, A. I., Fernandez, P., Dominguez, O., Dopazo, A., Sanchez-Cespedes, M. (2003) Growth and molecular profile of lung cancer cells expressing ectopic LKB1: down-regulation of the phosphatidylinositol 3′-phosphate kinase/PTEN pathway. Cancer Res. **63(6),** 1382–1388.
3. Hermans, K. G., van Alewijk, D. C., Veltman, J. A., van Weerden, W., van Kessel, A. G., and Trapman, J. (2004) Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines. *Genes Chromosomes Cancer* **39(3),** 171–184.
4. Kovacina, K. S., Park, G. Y., Bae, S. S., et al. (2003) Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. *J. Biol. Chem.* **278(12),** 10,189–10,194.
5. Choe, G., Horvath, S., Cloughesy, T. F., et al. (2003) Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. *Cancer Res.* **63(11),** 2742–2746.
6. Pende, M., Um, S. H., Mieulet, V., et al. (2004) S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol. Cell. Biol.* **24(8),** 3112–3124.
7. Cheung, W. L., Ajiro, K., Samejima, K., et al. (2003) Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. *Cell* **113(4),** 507–517.

8. Kato, M., Mochizuki, K., Kuroda, K., et al. (1991) Histone H2B as an antigen recognized by lung cancer-specific human monoclonal antibody HB4C5. *Hum. Antibodies Hybridomas* **2(2),** 94–101.
9. Kim, H. S., Cho, J. H., Park, H. W., et al. (2002) Endotoxin-neutralizing antimicrobial proteins of the human placenta. *J. Immunol.* **168(5),** 2356–2364.
10. Kunkel, M. T., Ni, Q., and Tsien, R. Y. (2005) Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. *J. Biol. Chem.* **280(7),** 5581–5587.
11. van Hemert, M. J., Niemantsverdriet, M., Schmidt, T., Backendorf, C., and Spaink, H. P. (2004) Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3 sigma and 14-3-3 zeta. *J. Cell Sci.* **117(Pt 8),** 1411–1420.