INSTRUCTIONS

Smad2 Redistribution® Assay

For High-Content Analysis

044-01.04

Number	Description
R04-044-01	Recombinant MDA-MB-468 cells stably expressing human Smad2 (GenBank Acc. NM_005901) fused to the C-terminus of enhanced green fluorescent protein (EGFP). MDA-MB-468 cells are adherent epithelial cells derived from human breast adenocarcinoma. Expression of EGFP-Smad2 is controlled by a standard CMV promoter and continuous expression is maintained by addition of G418 to the culture medium.

Quantity: 2 cryo-vials each containing 1.0 x 10^6 cells in a volume of 1.0 ml Cell Freezing Medium.

Storage: Immediately upon receipt store cells in liquid nitrogen (vapor phase).

Warning: Please completely read these instructions and the material safety data sheet for DMSO before using this product. This product is for research use only. Not intended for human or animal diagnostic or therapeutic uses. Handle as potentially biohazardous material under at least Biosafety Level 1 containment. Safety procedures and waste handling are in accordance with the local laboratory regulations.

CAUTION: This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Please review Material Safety Data Sheet before using this product.

Introduction

The Redistribution® Technology

The Redistribution® technology monitors the cellular translocation of GFP-tagged proteins in response to drug compounds or other stimuli and allows easy acquisition of multiple readouts from the same cell in a single assay run. In addition to the primary readout, high content assays provide supplementary information about cell morphology, compound fluorescence, and cellular toxicity.

The Smad2 Redistribution® Assay

The transforming growth factor β (TGFβ) family of cytokines plays important roles in cell growth, cell differentiation, apoptosis and other cellular functions. TGFβs signal through cell surface receptors (e.g. ALKs and BMPRs) having serine-/threonine kinase activity and downstream Smad proteins. There are five receptor activated Smads (Smads 1,2,3,5, and 8). Smad2 and Smad3 are activated in response to TGF-β or Activin signals, while Smad1, Smad5, and Smad8 are activated in response to bone morphogenetic protein signals.

Binding of TGFβ to the cell surface receptors results in activation of receptor kinases, which then phosphorylate and activate downstream Smad2 and Smad3 proteins. Phosphorylated Smad2 and Smad3 then form heteromeric complexes with Smad4 and translocate to the nucleus where they activate transcription of multiple TGFβ response genes. Once bound to a TGFβ responsive element, Smad2, Smad3, and Smad4 interact with co-activators such as p300/CBP with intrinsic histone acetyl-transferase activity, or co-repressors such as Ski, Sno, and TGIF, which interact directly with histone deacetylase containing complexes and repress the ability of the Smads to activate TGFβ target genes [1,2].

The Smad2 Redistribution® Assay is designed to assay for inhibitors of TGF-β1-induced Smad2 translocation by monitoring the translocation of a GFP-Smad2 fusion protein from the cytoplasm to the nucleus. SB431542 is used as a reference antagonist, and compounds are assayed for their ability to inhibit TGF-β1-stimulated nuclear translocation of Smad2.

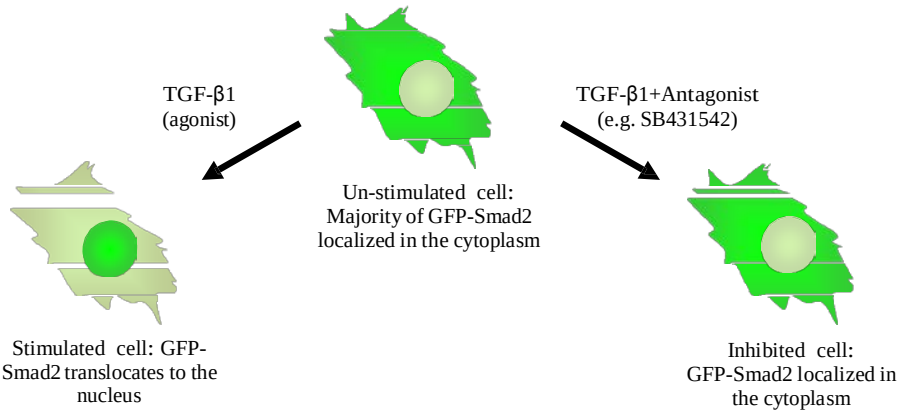


Figure 1: Illustration of the Smad2 translocation event.

Smad2 nuclear translocation can be inhibited by TGF-βR inhibitors, thereby blocking Smad2 phosphorylation and subsequent nuclear import. In this assay, the TGF-β1 receptor kinase inhibitor SB431542 [3,4] is used as reference antagonist. Compounds inhibiting TGF-β1-induced cytoplasm to nucleus translocation of Smad2 could interfere directly with Smad2 translocation or act upstream of Smad2.

Additional materials required

The following reagents and materials need to be supplied by the user.

- RPMI 1640 without L-Glutamine (Thermo Scientific, Fisher Scientific cat.# SH3009602)
- L-Glutamine supplement, 200 mM (Thermo Scientific, Fisher Scientific cat.# SH30034)
- Fetal Bovine Serum (FBS) (Thermo Scientific, Fisher Scientific cat.# SH30071)
- Penicillin/Streptomycin, 100X solution (Thermo Scientific, Fisher Scientific cat.# SV30010),
- Trypsin-EDTA, 0.05% (Thermo Scientific, Fisher Scientific cat.# SH30236)
- G418, 50mg/ml (Thermo Scientific, Fisher Scientific cat.# SC30069)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, cat.# BP231)
- Dulbecco´s Phosphate-Buffered Saline (PBS), w/o calcium, magnesium, or Phenol Red (Thermo Scientific, Fisher Scientific cat.# SH30028)
- Hepes Buffer, 1 M, Free Acid (liquid) (Thermo Scientific, Fisher Scientific cat.# SH30237)
- Bovine Serum Albumin (BSA) Cohn Fraction V (MP Biomedicals, cat.# ICN841032)
- SB 431542 hydrate (Sigma-Aldrich, cat.# S4317)
- Transforming Growth Factor-β1 (TGF-β1), human recombinant (EMD Chemicals, cat.# 616455)
- 4 mM HCl
- Hoechst 33258 (Fisher Scientific, cat.# AC22989)
- Triton X-100 (Fisher Scientific, cat.# AC21568)
- 10% formalin, neutral-buffered solution (approximately 4% formaldehyde) (Fisher Scientific, cat.# 23-305-510)
  Note: is not recommended to prepare this solution by diluting from a 37% formaldehyde solution.
- 96-well microplate with lid (cell plate) (e.g. Nunc 96-Well Optical Bottom Microplates, Thermo Scientific cat.# 165306)
- Black plate sealer
- Nunc EasYFlasks with Nunclon Delta Surface, T-25, T-75, T-175 (Thermo Scientific, cat.# 156367, 156499, 159910

Reagent preparation

The following reagents are required to be prepared by the user.

- Cell Culture Medium: RPMI supplemented with 1mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% FBS.
- Cell Freezing Medium: 90% Cell Culture Medium without G418 + 10% DMSO.
- Plate Seeding Medium: RPMI supplemented with 1mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 1% FBS.
- Assay Buffer: RPMI supplemented with 1mM L-Glutamine, 1% Penicillin-Streptomycin, 1% FBS and 10 mM Hepes.
- Control Stock: 10 mM SB 431542 stock solution prepared in DMSO. Prepare by dissolving 5 mg SB 431542 (MW=384.4) in 1.3 ml DMSO. Store at -20°C.
- 10% BSA: 1 g BSA dissolved in purified water to a final volume of 10 ml.
- TGF-β1 Agonist Stock: 2 µg/ml TGF-β1 stock solution prepared in 4 mM sterile HCl containing 0.1% BSA. Prepare by dissolving 2 µg TGF-β1 in 1 ml 4 mM sterile HCl containing 0.1% BSA.
- Fixing Solution: 10% formalin, neutral-buffered solution (approximately 4% formaldehyde).
  Note: It is not recommended to prepare this solution by diluting from a 37% formaldehyde solution.
- Hoechst Stock: 10 mM stock solution is prepared in DMSO.
- Hoechst Staining Solution: 1 µM Hoechst in PBS containing 0.5% Triton X-100. Prepare by dissolving 2.5 ml Triton X-100 with 500 ml PBS. Mix thoroughly on a magnetic stirrer. When Triton X-100 is dissolved add 50 µl 10 mM Hoechst 33258. Store at 4°C for up to 1 month.

The following procedures have been optimized for this cell line. It is strongly recommended that an adequately sized cell bank is created containing cells at a low passage number.

Cell thawing procedure

1. Rapidly thaw frozen cells by holding the cryovial in a 37°C water bath for 1-2 minutes. Do not thaw cells by hand, at room temperature, or for longer than 3 minutes, as this decreases viability.
2. Wipe the cryovial with 70% ethanol.
3. Transfer the vial content into a T75 tissue culture flask containing 25 ml Cell Culture Medium and place flask in a 37ºC, 5% CO_2, 95% humidity incubator.
4. Change the Cell Culture Medium the next day.

Cell harvest and culturing procedure

For normal cell line maintenance, split 1:8 every 3-4 days. Maintain cells between 5% and 95% confluence. Passage cells when they reach 80-95% confluence. All reagents should be pre-warmed to 37ºC.

1. Remove medium and wash cells once with PBS (10 ml per T75 flask and 12 ml per T175 flask).
2. Add trypsin-EDTA (2 ml per T75 flask and 4 ml per T175 flask) and swirl to ensure all cells are covered.
3. Incubate at 37ºC for 3-5 minutes or until cells round up and begin to detach.
4. Tap the flask gently 1-2 times to dislodge the cells. Add Cell Culture Medium (6 ml per T75 flask and 8 ml per T175 flask) to inactivate trypsin and resuspend cells by gently pipetting to achieve a homogenous suspension.
5. Count cells using a cell counter or hemocytometer.
6. Transfer the desired number of cells into a new flask containing sufficient fresh Cell Culture Medium (total of 20 ml per T75 flask and 40 ml per T175 flask).
7. Incubate the culture flask in a 37ºC, 5% CO_2, 95% humidity incubator.

Cell freezing procedure

1. Harvest the cells as described in the “Cell harvest and culturing procedure”, step 1 – 5.
2. Prepare a cell suspension containing 1 x 10^6 cells per ml (5 cryogenic vials = 5 x 10^6 cells).
3. Centrifuge the cells at 250x g (approximately 1100 rpm) for 5 minutes. Aspirate the medium from the cells.
4. Resuspend the cells in Cell Freezing Medium at 1 x 10^6 cells per ml until no cell aggregates remain in the suspension.
5. Dispense 1 ml of the cell suspension into cryogenic vials.
6. Place the vials in an insulated container or a cryo-freezing device (e.g. Nalgene "Mr. Frosty" Freezing Container, Thermo Scientific, Fisher Scientific cat.# 15-350-50) and store at -80ºC for 16-24 hours.
7. Transfer the vials for long term storage in liquid nitrogen.

Cell plating procedure

The cells should be seeded into 96-well plates 18-24 hours prior to running the assay. Do not allow the cells to reach over 95% confluence prior to seeding for an assay run. The assay has been validated with cells up to passage 24 split as described in the “Cell harvest and culturing procedure”.

1. Harvest the cells as described in the “Cell harvest and culturing procedure”, step 1-5 using Plate Seeding Medium instead of Cell Culture Medium.
2. Dilute the cell suspension to 150,000 cells/ml in Plate Seeding Medium.
3. Transfer 100 µl of the cell suspension to each well in a 96-well tissue culture plate (cell plate). This gives a cell density of 15,000 cells/well.
   Note: At this step, be careful to keep the cells in a uniform suspension.
4. Incubate the cell plate on a level, vibration-free table for 1 hour at room temperature (20-25°C). This ensures that the cells attach evenly within each well.
5. Incubate the cell plate for 18-24 hours at 37ºC, 5% CO_2, 95% humidity incubator prior to starting the assay.

Assay protocol

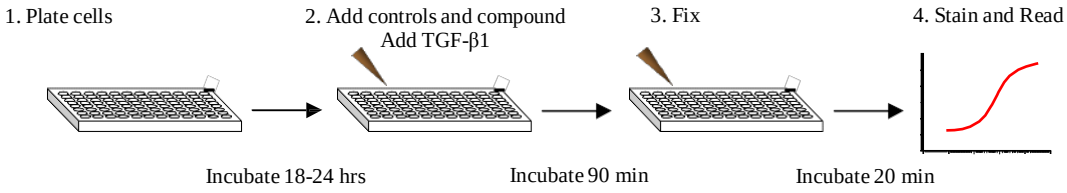


Figure 2. Quick assay workflow overview

The following protocol is based on 1x 96-well plate.

1. Before initiating the assay:
   - Prepare Assay Buffer. Ensure Assay Buffer is pre-warmed to 20-37ºC.
   - Dilute controls and test compounds in Assay Buffer to a 4X final concentration. (Volumes and concentrations are indicated below). A final DMSO concentration of 0.25% is recommended.
   - Mix controls for 1x 96–well plate as indicated below:

	Assay Buffer	Control Stock	DMSO	4X concentration	Final assay concentration	Final DMSO concentration
Negative control	6 ml	----	60 µl DMSO	1% DMSO	----	0.25%
Positive control	6 ml	7.2 µl SB 431542	52.8 µl DMSO	12 µM SB 431542	3 µM SB 431542	0.25%

2. Prepare 4X TGF-β1 Agonist Solution (12 ng/ml):
   - Prepare fresh by mixing 36 µl 2 µg/ml TGF-β1 Agonist Stock with 6 ml Assay Buffer. Use the TGF-β1 Agonist Solution within 20 minutes after preparation.
3. Add 50 µl 4X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
4. Add 50 µl 4X TGF-β1 Agonist Solution to appropriate wells of the cell plate.
5. Incubate cell plate with lid for 90 minutes in a 37ºC, 5%, CO_2, 95% humidity incubator.
6. Fix cells by gently removing the buffer and add 150 µl Fixing Solution per well.
7. Incubate cell plate with lid at room temperature for 20 minutes.
8. Wash the cells 4 times with 200 µl PBS per well per wash.
9. Remove PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution.
10. Seal plate with a black plate sealer. Incubate at room temperature for at least 30 minutes before imaging. The plate can be stored at 4ºC for up to 3 days in the dark.

Imaging

Imaging in general

The translocation of Smad2 can be imaged on most HCS platforms and fluorescence microscopes. The filters should be set for Hoechst (350/461 nm) and GFP/FITC (488/509 nm) (wavelength for excitation and emission maxima). Consult the instrument manual for the correct filter settings.

The translocation can typically be analyzed on images taken with a 10-20x objective or higher magnification.

The primary output in the Smad2 Redistribution® assay is the translocation of Smad2 from cytoplasm to nucleus. The data analysis should therefore report an output relating to the GFP fluorescence intensities in the nucleus and the cytoplasm.

Imaging on Thermo Scientific Arrayscan HCS Reader

This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 20x objective (0.63X coupler), XF100 filter sets for Hoechst and FITC and the Redistribution V3 BioApplication. The output used was MEAN_CircRingAvgIntenRatioLog (Log of the ratio of average fluorescence intensities of nucleus and cytoplasm (well average)). The minimally acceptable number of cells used for image analysis in each well was set to 200 cells.

Other BioApplications that can be used for this assay include Molecular TranslocationV2, CompartmentalAnalysisV2, NucTransV2 and ColocalizationV3.

High Content Outputs

In addition to the primary readout, it is possible to extract secondary high content readouts from the Redistribution® assays. Such secondary readouts may be used to identify unwanted toxic effects of test compounds or false positives. In order to acquire this type of information, the cells should be stained with a whole cell dye which allows for a second analysis of the images for determination of secondary cell characteristics.

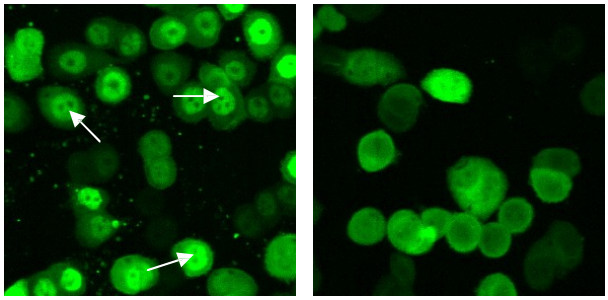
Examples of useful secondary high content outputs:

Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

The thresholds for determining compound cytotoxicity or fluorescence must be determined empirically. Note that the primary translocation readout in some cases may affect the secondary outputs mentioned above.

Representative Data Examples

The Smad2 Redistribution® Assay monitors inhibition of TGF-β1-induced Smad2 translocation. Example images of the Smad2 Redistribution® Assay are illustrated in Figure 3. Figure 4 shows a concentration response curve of the reference compound SB431542 (a TGF-β receptor inhibitor) in the Smad2 assay. The EC_{50} value of SB431542 is approximately 150 nM in the assay.



DMSO-treated cells SB431542-treated cells

Figure 3. Translocation of EGFP-Smad2 stimulated with TGF-β1 in response to SB431542. Cells were treated with 3 ng/mL TGF-β1 in the absence or presence of 3 µM SB431542. Arrows indicate TGF-β1-mediated nuclear translocation detected by the image analysis algorithm.

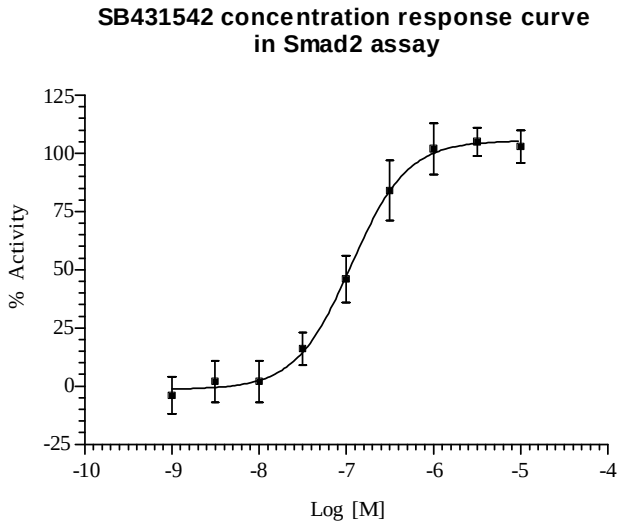


Figure 4: SB431542 concentration response curve in the Smad2 Redistribution® assay (n=8). Concentration response was measured in 9 point half log dilution series of SB431542. Cells were then fixed and the nucleus to cytoplasm translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the RedistributionV3 BioApplication. % activity was calculated relative to the positive (3 µM SB431542) and negative control (0.25% DMSO). The EC_{50} value of SB431542 is approximately 150 nM in the assay.

Product qualification

Assay performance has been validated with an average Z'=0.52±0.14. The cells have been tested for viability. The cells have been tested negative for mycoplasma and authenticated to be MDA-MB-468 cells by DNA fingerprint STR analysis.

Related Products

Product #	Type	Product description	Cell line
R04-044-01	CryoRedi	Smad2 Redistribution Assay	MDA-MB-468
8404201	HCS Reagent Kit	Smad4 activation kit	
8402101	HCS Reagent Kit	Smad3 activation kit	

References

1. Derynck R & Zhang YE, Nature 425, 577-584, 2003.
2. Shi Y and Massague J, Cell 113, 685-700, 2003.
3. Laping NJ et al., Mol Pharmacol. 62, 58-54, 2002.
4. Inman GJ et al., Mol Pharmacol. 62, 65-74, 2002.

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For European customers:
The Smad2 Redistribution cell line is genetically modified with a vector expressing Smad2 fused to EGFP. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

The Thermo Scientific Redistribution assays are part of the Thermo Scientific High Content Platform which also includes Thermo Scientific HCS Reagent Kits, Thermo Scientific Arrayscan HCS Reader, Thermo Scientific CellInsight Personal Cell Imager, Thermo Scientific ToxInsight IVT platform, BioApplication image analysis software and high-content informatics. For more information on Thermo Scientific products for high content and Cellomics, visit www.thermoscientific.com/cellomics, or call 800-432-4091 (toll free) or 412-770-2500. LC06623403

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