INSTRUCTIONS

PKA Redistribution® Assay

For High-Content Analysis

045-01.05

Number	Description
R04-045-01	Recombinant CHO-K1 cells stably expressing the catalytic domain of human Protein Kinase A (PKAcat) (GenBank Acc. NM_002730) fused to the N-terminus of enhanced green fluorescent protein (EGFP). CHO-K1 cells are adherent epithelial cells derived from Chinese hamster ovary. Expression of PKAcat-EGFP 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 PKA Redistribution® Assay

The cAMP-dependent protein kinase A (PKA) is a ubiquitous serine/threonine protein kinase. PKA is recognised as a major mediator of intracellular cAMP signals in eukaryotes. The PKA holoenzyme is an R2C2 tetramer consisting of a regulatory (R) dimer and two catalytic (C) subunits. Four isoforms of the regulatory subunit (RIα, RIβ, RIIα, RIIβ) and three isoforms of the catalytic subunit (Cα, Cβ, and Cγ) have been described. The catalytic subunits in the assembled tetramer are generally believed to be catalytically inactive. Gs-protein activation leads to activation of adenylate cyclases, which generate cAMP. Binding of two molecules of cAMP to each R subunit causes the release and activation of the C subunits. Dissociated catalytic subunits phosphorylate cytoplasmic substrates. To date, a large number of cytoplasmic and a few nuclear PKA substrates have been reported. The amino-termini of the catalytic subunits are myristoylated, a post-translational modification usually associated with membrane attachment. However, catalytic subunits are freely mobile within cells when detached from the regulatory subunits [1].

The PKA Redistribution® Assay is designed to assay for the concentration of cAMP in the cytoplasm by monitoring translocation of the catalytic domain of PKA fused to GFP (PKAcat-EGFP) from cytoplasmic aggregates to a uniform cytoplasmic localization [2,3]. In unstimulated cells, the PKAcat-GFP is found in highly fluorescent aggregates in the cytoplasm. Upon stimulation with forskolin, an activator of adenylate cyclase, cAMP is generated, leading to cAMP binding and activation of PKAcat-EGFP which results in dispersion of PKAcat-EGFP from the bright aggregates. Forskolin is used as reference compound in the assay [4]. Test compounds causing foci dispersion of PKA are considered activators of adenylate cyclase leading to generation of cAMP.

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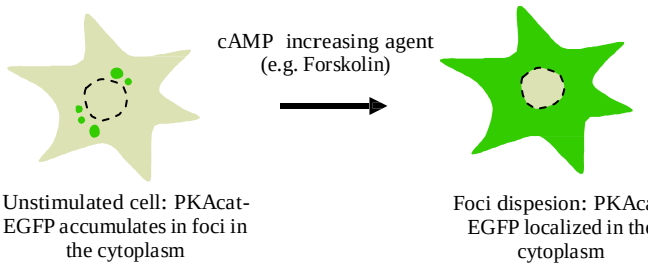


Figure 1: Illustration of the PKAcat translocation.

Additional materials required

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

- Ham's F12 with L-Glutamine (Thermo Scientific, Fisher Scientific cat.# SH30026)
- 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)
- Forskolin (Sigma, cat.# F6886)
- 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: Ham's F12 supplemented with 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: Ham's F12 supplemented with 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% FBS.
- Assay Buffer: Ham's F12 supplemented with 1% Penicillin-Streptomycin and 10% FBS and 10 mM Hepes Buffer.
- Control Compound Stock: 100 mM Forskolin stock solution in DMSO. Prepare by dissolving 25 mg Forskolin (MW = 410.5) in 609 µl DMSO. Store at -20°C.
- 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. As early as possible, create and store at least one aliquot of cells for back-up.

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 1:12 to 1:24 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 250g (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 25, 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 100,000 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 10,000 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 in a 37ºC, 5% CO_2, 95% humidity incubator prior to starting the assay.

Assay protocol

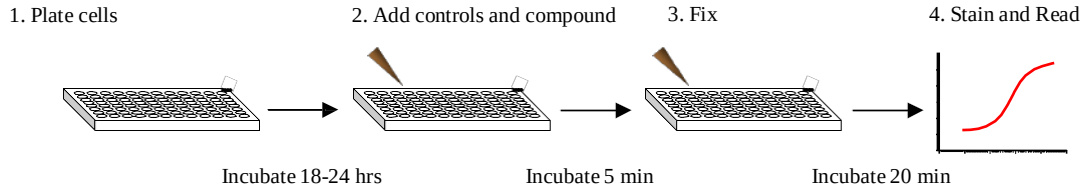


Figure 2: Quick assay workflow overview

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.
2. Prepare controls and test compounds:
   - Dilute controls and test compounds in Assay Buffer to a 2X 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	2X concentration	Final assay concentration	Final DMSO concentration
Negative control	12 ml	----	60 µl	0.5% DMSO	----	0.25%
Positive control	12 ml	12 µl Forskolin	48 µl	100 µM Forskolin	50 µM Forskolin	0.25%

3. Add 100 µl 2X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
4. Incubate cell plate for 5 minutes in a 37ºC, 5% CO_2, 95% humidity incubator.
5. Fix cells by gently decanting the buffer and add 150 µl Fixing Solution per well.
6. Incubate cell plate at room temperature for 20 minutes.
7. Wash the cells 4 times with 200 µl PBS per well per wash.
8. Decant PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution.
9. 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

The dispersion of PKA aggregates 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 10x objective or higher magnification.

The primary output is the formation/dispersion of spots in the cytoplasm. The data analysis should therefore report an output that corresponds to area or intensity of spots in the cytoplasm.

Imaging on Thermo Scientific Arrayscan HCS Reader
This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 10x objective (0.63X coupler), XF100 filter sets for Hoechst and FITC, and the SpotDetectorV3 BioApplication. The output parameter used was SpotTotalIntenPerObject. The minimally acceptable number of cells used for image analysis in each well was set to 250 cells.

Other BioApplications that can be used for this assay include CompartmentalAnalysisV2 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:

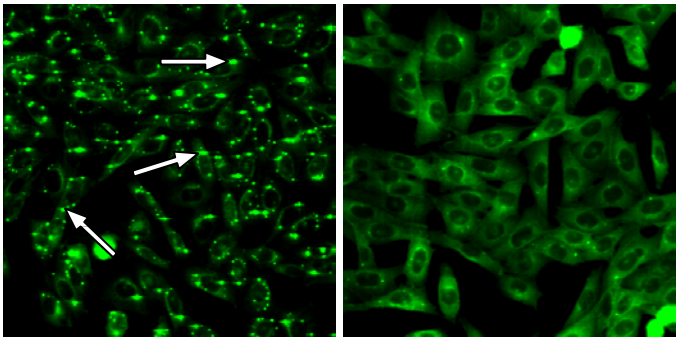
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 PKA Redistribution® assay is designed to assay for the concentration of cAMP in the cytoplasm by monitoring translocation of the catalytic domain of PKA fused to GFP (PKAcat-GFP) from cytoplasmic aggregates to a uniform cytoplasmic localization. Forskolin is used as a reference compound, and test compounds are assayed for their ability to disperse PKAcat-GFP from the bright aggregates.

Representative images of PKA Redistribution® assay cells treated with forskolin are shown in Figure 3.



DMSO-treated cells Forskolin-treated cells

Figure 3. Translocation of PKAcat-GFP. Cells were treated with or without 50 µM forskolin for 5 min. Arrows indicate the aggregates of PKAcat-EGFP in the cytoplasm of untreated cells detected by the image analysis algorithm.

Figure 4 shows a representative concentration response curve of the reference compound forskolin in the PKA assay. The EC_{50} of forskolin is ~3 µM.

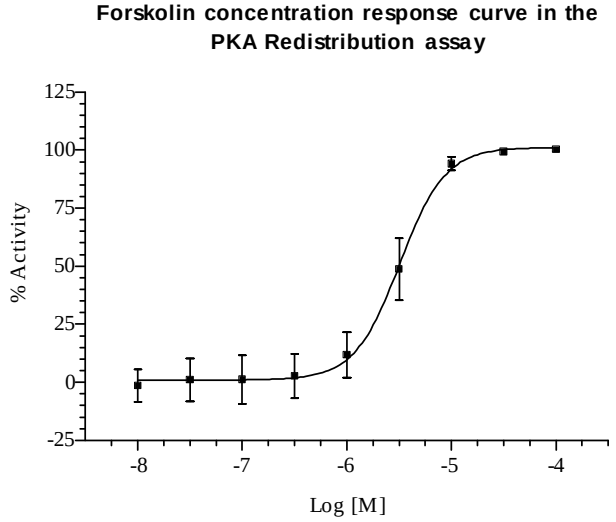


Figure 4. Concentration response curve of forskolin in the PKA assay (n=16). The EC_{50} is approximately 3 µM. Concentration response was measured in 9 point half log dilution series. Cells were treated with compound for 5 min. Cells were then fixed and spot dispersion was measured using the imaging was performed using the Cellomics ArrayScan V^{TI} Reader and the SpotDetectorV3 BioApplication. % activity was calculated relative to the positive (50 µM forskolin) and negative control (0.25% DMSO)

Product qualification

Assay performance has been validated with an average Z'=0.67±0.08. The cells have been tested for viability. The cells have been tested negative for mycoplasma.

Related Products

Product #	Type	Product description	Cell line
R04-017-02	Profiling & Screening	Gq-coupled GPCRs – NFATc1 Redistribution® Assay	U2OS
R04-045-02	Profiling & Screening	Gs/Gi-coupled GPCRs – PKA Redistribution® Assay	CHO-K1
R04-046-01	Profiling & Screening	β2-AR:PKA Redistribution® Assay	CHO-K1
R04-047-01	Profiling & Screening	GlucagonR:PKA Redistribution® Assay	CHO-K1
R04-048-01	Profiling & Screening	NK1:NFATc1 Redistribution® Assay	U2OS
R04-067-01	Profiling & Screening	S1P1:PKA Redistribution® Assay	CHO-K1
R04-088-01	Profiling & Screening	M1:NFATc1 Redistribution® Assay	U2OS
R04-072-01	Profiling & Screening	M2:PKA Redistribution® Assay	CHO-K1
R04-073-01	Profiling & Screening	M3:NFATc1 Redistribution® Assay	U2OS
R04-078-01	Profiling & Screening	AT1:NFATc1 Redistribution® Assay	U2OS
R04-079-01	Profiling & Screening	MCH1:NFATc1 Redistribution® Assay	U2OS
R04-081-01	Profiling & Screening	MOR1:PKA Redistribution® Assay	CHO-K1

References

1. Feliciello A et al., J Mol Biol 308, 99-114, 2001.
2. Almholt K et al., Cell Signal. 16, 907-20, 2004.
3. Zaccolo M et al., Nat Cell Biol. 2, 25-29, 2000.
4. Seamon KB & Daly JW., Adv Cyclic Nucleotide Protein Phosphorylation Res. 20, 1-150, 1986.

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For European customers:
The PKA Redistribution cell line is genetically modified with a vector expressing the catalytic domain of Protein Kinase A 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.

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