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Native Calcium GPCR Assays

For Fluorescent Detection of Activated GPCRs

User Manual

Please refer to the updated
"Cell Culture and Handling Procedure"
attached at the end of this user manual

Simple Solutions for Complex Biology

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ATTENTION:

Read the entire product insert prior to beginning the assay. Refer to the data sheets for additional information on cell-line specific media requirements.

NOTES:

NOTES:

LEGAL SECTION

This product and/or its use is covered by one or more of the following U.S. patents #6,342,345 B1, #7,135,325 B2, #8,101,373 B2 and/or foreign patents, patent applications, and trade secrets that are either owned by or licensed to DiscoveRx® Corporation. This product is for *in vitro* use only and in no event can this product be used in whole animals. The right to use or practice the inventions in the foregoing patents (including method of use claims) by using or propagating this product is granted solely in connection with the use of appropriate Detection Reagents (protected under trade secret) purchased from DiscoveRx Corporation or its authorized distributors.

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The cells and detection reagents (collectively Materials) purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Protein:Protein Interaction Assay (Assay) that employs genetically modified cells and vectors (collectively, the "Cells"), and related detection reagents (the "Reagents") (collectively referred to as "Materials"). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

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For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

INTENDED USE & TECHNOLOGY PRINCIPLE

Native Calcium GPCR Assays provide a fast, reliable method for detection of changes in intracellular calcium. Cells expressing a receptor of interest that signals through calcium are pre-loaded with a calcium-sensitive dye and then treated with compound. Upon stimulation, the receptor signals release of intracellular calcium, which results in an increase of dye fluorescence. The assay provides a homogenous assay format for detection of calcium mobilization and measurement of intracellular calcium provides valuable information on the activation status of GPCRs and ion channels. Signal is measured on a fluorescent plate reader equipped with fluidic handling capable of detecting rapid changes in fluorescence upon compound stimulation. These assays are designed to be compatible with both 96-well and 384-well plate formats.

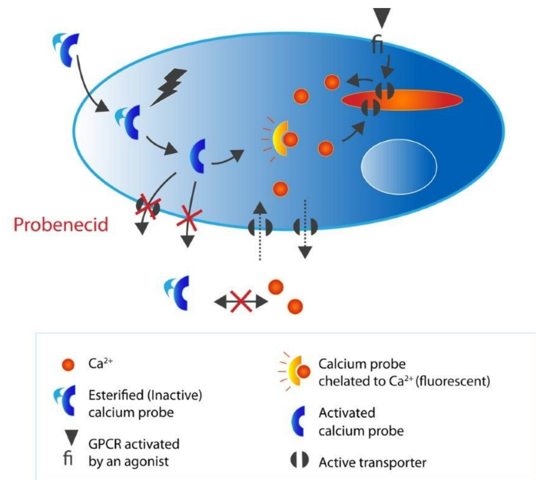


Figure 1. Native Calcium GPCR Assay Principle. Native Calcium Cell Lines overexpress naturally Gq coupled, wildtype GPCRs and are designed to detect calcium mobilization in response to agonist stimulation of the receptor.

NOTES:

TROUBLESHOOTING GUIDE (CONTINUED)

PROBLEM	CAUSE	SOLUTION
Cells growing slowly	U2OS grows slower than CHO-K1 or HEK 293	Average doubling time is 3 days, so please observe cells under microscope and monitor cell health
	Slow growing clones	Using DiscoveRx functionally validated and optimized media and reagents improves assay performance
EC_{50} is right-shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in buffer containing 0.1% BSA
		Non-binding surface plates may be necessary for hydrophobic compounds
High well-to-well variability in Z' study	Problems with plate type and compound solubility	Z' studies should be performed with automation
		It may be necessary to test plate types and compound stability

For additional information or technical support, please call **1.866.448.4864** (US) **+44.121.260.6142** (Europe).

ASSAY OVERVIEW

Please read the entire protocol completely before running the assay. The **Native Calcium Assay Procedure** and **Quick Start Procedure** sections in this booklet contain detailed information about how to run the assay. Refer to the cell-line specific datasheet for additional information on the optimized cell plating reagent and reference ligand recommended for the assay.

Assays should be run using a fresh split of low-passage cells that have not been allowed to reach confluency for more than 24 hours. Following treatment of the cells with compound, GPCR activity is detected by adding a working solution of fluorescent detection reagents using a simple, mix-and-read protocol.

The following steps are required to monitor GPCR activity using a Native Calcium GPCR Cell Line (Figure 2).

1. Plate cells.
2. Dilute and add compounds or antibodies.
3. Perform functional assay in agonist, antagonist or allosteric modulator mode.

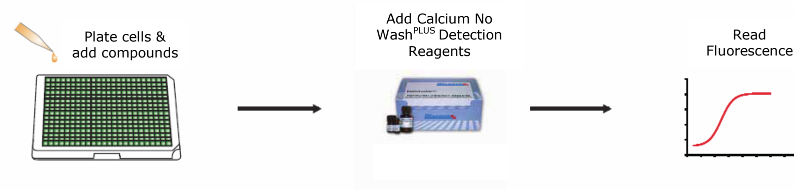


Figure 2. Simple fluorescent assay protocol for monitoring GPCR activity in response to compound challenge.

MATERIALS PROVIDED

Description	Contents	Storage
Native Calcium GPCR Cell Line*	2 vials	Liquid N_2 (vapor phase)

*Please refer to the cell line specific datasheet for detailed information on the Native Calcium GPCR Cell Line you are testing.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

Equipment	Materials
• Green V-Bottom PP Ligand Dilution Plates, 10 plates/pack (DiscoveRx, Cat. #92-0011) • 384-well Black Clear Bottom TC treated, Sterile w/lid, 10 plates/pack (DiscoveRx, Cat. #92-0024) • Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar) • Hemocytometer • Cryogenic Freezing Container (Nalgene, Cat. #1.5100-0001 or similar) • Cryogenic Freezer Vials (Fisher Scientific, Cat. #375418 or similar) • Multimode or fluorescence plate reader • Single and multi-channel pipettes and tips • Tissue culture disposables and plastic ware (T25 and T75 flasks, etc.)	• Calcium NoWash^{PLUS} Assay Kit (DiscoveRx, Cat. #90-0091, #90-0091L or #90-0091XL) • AssayComplete™ Revive Reagent (DiscoveRx, Cat. #92-0016RM Series) • AssayComplete Cell Culture Kits (DiscoveRx, Cat. #92-0018/19/20/21/22G Series) • AssayComplete Preserve Freezing Reagent (DiscoveRx, Cat. #92-0017FR Series) • AssayComplete Cell Detachment Reagent (DiscoveRx, Cat. #92-0009) • AssayComplete Cell Plating (CP) Reagent (DiscoveRx, Cat. #93-0563R Series) • Probenecid (Sigma, Cat. #P8761) • GPCR control agonist • GPCR test compound(s) and/or antagonists

±Please refer to the cell line specific datasheet to determine catalog numbers for the media and reagent requirements for the Native Calcium Cell Line you are testing.

FROZEN CELL HANDLING PROCEDURE

To ensure maximum cell viability, thaw the vial and initiate the culture as soon as possible upon receipt. If continued storage of the frozen vials is necessary, store vials in the vapor phase of liquid nitrogen (N_2). **DO NOT** store at –80°C for extended periods as this could result in significant loss in cell viability.

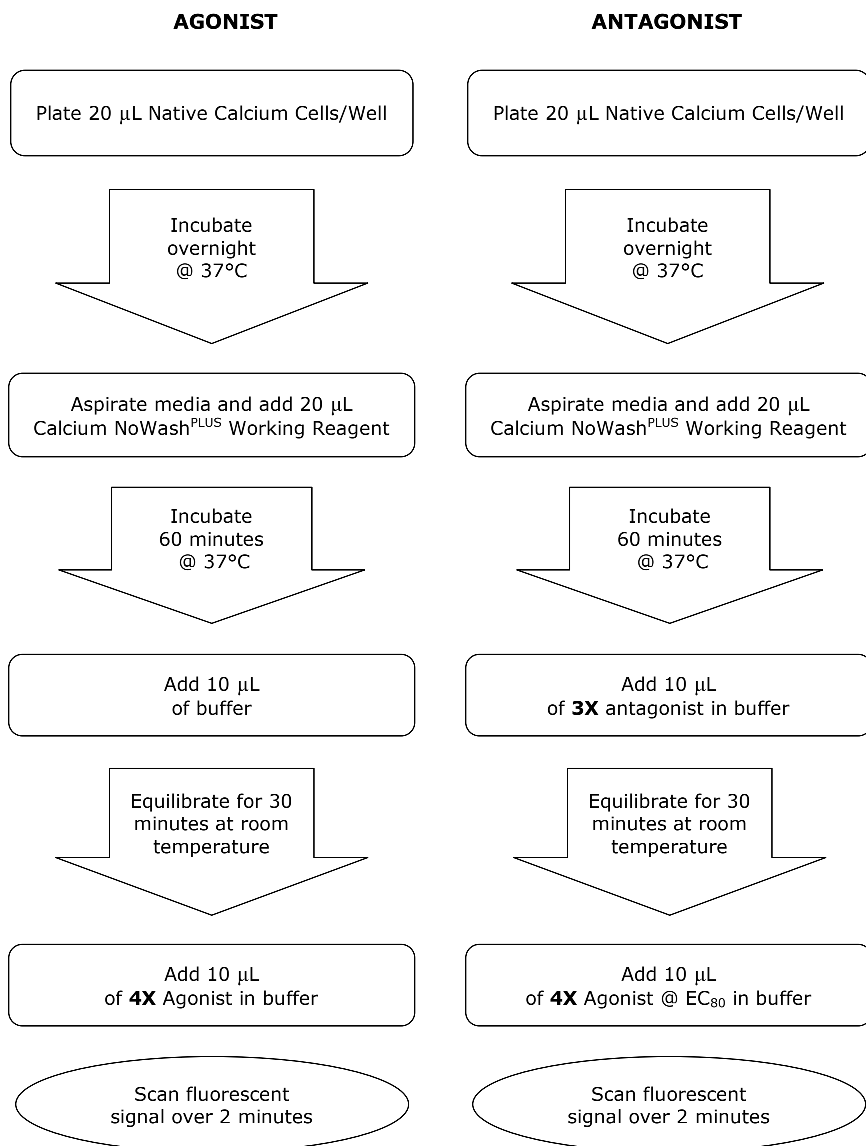
CELL PLATING REAGENT REQUIREMENT

Each Native Calcium GPCR Cell Line has been validated for optimal assay performance using the recommended AssayComplete Cell Plating (CP) Reagent and control ligand as indicated in the cell line specific datasheet. **For optimal performance, always use the CP Reagent recommended for the cell line and DO NOT substitute at any time.**

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No Response	Improper cell growth conditions	See datasheet for cell culture conditions
	High DMSO/solvent concentration	Maintain DMSO/solvent at <1% in serial dilutions of compounds.
	Improper ligand used or improper ligand incubation time	See datasheet for recommended ligand and assay conditions
	Improper preparation of ligand (agonist or antagonist)	Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased Response	Higher passages give reduced performance	Native Calcium cells are stable up to 10 passages. Use low passage cells whenever possible
	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscopy
Low or No Signal	Improper preparation of detection reagents	Detection reagents should be prepared just prior to use and are sensitive to light.
	Problem with cell growth, cell viability, cell adherence or cell density	See datasheet for cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 sec/well.
Experimental S:B does not match datasheet value	For cell pools, S:B may vary greatly from passage to passage or day to day	Prepare a clonal cell line or use lower passage number cells.
		Repeat the assay
		Confirm assay conditions
	Improper preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands

QUICK-START PROCEDURE



SOLVENTS AND PREPARATION OF COMPOUND DILUTIONS

Native Calcium GPCR Assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). As solvents can affect assay performance, optimize the assay conditions accordingly if other solvents or solvent concentrations are required.

To validate each Native Calcium GPCR Assay, reference ligand was diluted using the AssayComplete Cell Plating (CP) Reagent recommended for the cell line (containing the appropriate solvent). For antibodies or other compounds that may be sensitive to serum and/or other assay components, dilutions can be prepared in either Hanks Buffered Salt Solution (HBSS) + 20 mM HEPES + 0.1% Bovine Serum Albumin (BSA) or OptiMEM® + 0.1% BSA without affecting assay performance.

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Cells are shipped in 2 vials on dry ice and contain approximately 1 x 10^6 cells per vial in 1 mL of AssayComplete Preserve Freezing Reagent. The following procedures are for safely storing and removing cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice. If cells arrive thawed, do not proceed, contact technical support.
2. Frozen cells must be immediately transferred to liquid N_2 storage or thawed and put into culture upon arrival.
3. When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N_2 inside the vial to evaporate.
4. Proceed with the thawing protocol in the following section.

SAFETY WARNING: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Some cryovials can leak when submerged in liquid N_2. Upon thawing, the liquid N_2 present in the cryovial converts back to its gas phase which can result in the vessel exploding.

CELL THAWING AND PROPAGATION

The following procedures are for thawing, seeding and expanding the cells, and for maintaining the cultures once the cells have been expanded. Cells are free of contamination prior to shipment and care should be taken in their handling to avoid contamination.

NOTE:
Face shield, gloves and a lab coat should be worn during the thawing procedure.

1. Pre-warm 15 mL AssayComplete Revive Media in a 37°C water bath.
2. Place the frozen cell vials ***briefly*** (10 seconds to 1 min) in a 37°C water bath under sterile conditions until only small ice crystals remain and the cell pellet is almost completely thawed. Caution: Longer incubation may result in cell death.
3. To remove DMSO from the media, carefully transfer the thawed cells to a sterile 15 mL tube and then fill tube with 10 mL pre-warmed AssayComplete Revive Media. Centrifuge at 300 x g for 4 minutes to pellet cells.
4. Remove media without disturbing cell pellet and resuspend in 5 mL of prewarmed AssayComplete Revive Media. Transfer cells to a T25 flask and incubate for 24 hours at 37°C, 5% CO_2.
5. After 24 hours, gently remove AssayComplete Revive Media (being careful not to disturb the cell monolayer) and replace with 5 mL of pre-warmed complete AssayComplete Cell Culture Media.
6. Once the cells become >70% confluent in the T25 flask, aspirate media and wash cells with 5 mL PBS. Aspirate PBS and dissociate cells with 0.5 mL AssayComplete Cell Detachment Reagent and resuspend in 5 mL of AssayComplete Cell Culture Media. Transfer the entire cell suspension to a T75 flask containing 15 mL of AssayComplete Cell Culture Media for continued growth.
7. Passage the cells every 2-3 days, based on the doubling time of the cell line, using AssayComplete Cell Detachment Reagent. For routine passaging, prepare a 1:3 dilution of cells in a total volume of 15 mL AssayComplete Cell Culture Media. Transfer 5 mL of the diluted cells to each new T75 flask.

   NOTE:
   To maintain logarithmic growth of the cells, cultures should be maintained in a subconfluent monolayer.
8. Each Native Calcium GPCR Cell Line has been found to be stable for at least 10 passages with no significant drop in assay window or shift in EC_{50}.
9. Assay performance and cellular response can be assessed by treating the cells with reference agonist. **Refer to the cell line specific datasheet for the recommended control agonist for your Native Calcium GPCR Cell Line.** For antagonist assays, cells can be pretreated with varying doses of antagonist/inhibitor compounds followed by agonist challenge, typically at an EC_{80} concentration.

REPRESENTATIVE DATA

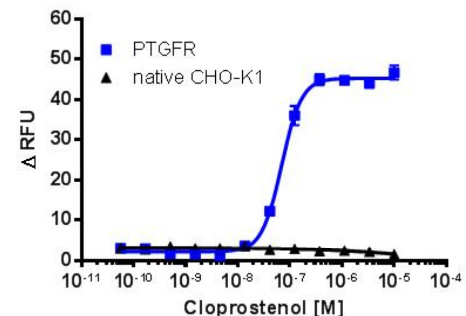


Figure 10. CHO-K1 PTGFR Cell Line (95-1009C2). Cells were plated in a 384-well plate at 10,000 cells/well. Target cells and native parental cells were then stimulated with the known agonist Cloprostenol (DiscoveRx, Cat. #92-1180). Calcium mobilization was detected using the Calcium NoWash^{PLUS} Detection Kit (DiscoveRx, Cat. #90-0091) according to the recommended protocol. The EC_{50} for agonist was estimated at 73 nM.

DAY 2: AGONIST COMPOUND ADDITION AND SIGNAL DETECTION

1. Aspirate media from cells and replace with 20 µL Ca NW^{PLUS} Working Reagent.
2. Incubate cells for 1 hour at 37°C, 5% CO_2.
3. Add 10 µL of HBSS/20 mM HEPES and equilibrate assay plate for 30 minutes at room temperature.
4. Prepare serial dilutions of the compound for a 12-point dose response curve. We recommend that the highest final concentration be **50X** the expected EC_{50} of the compound.
5. Add 10 µL of **4X** agonist in HBSS/20 mM HEPES.
6. Measure compound activity using fluorescence reader with appropriate settings (excitation at 494 nM and emission at 516 nM). Signal is monitored over 2 minutes at 2 second intervals. We recommend that you pre-read the assay plates for 5 seconds prior to agonist addition.

ANTAGONIST COMPOUND ADDITION AND SIGNAL DETECTION

1. Aspirate media from cells and replace with 20 µL Ca NW^{PLUS} Working Reagent.
2. Incubate cells for 1 hour at 37°C, 5% CO_2.
3. Prepare serial dilutions of the compound for a 12-point dose response curve. We recommend that the highest final concentration be **50X** the expected IC_{50} for the compound.
4. Add 10 µL of **3X** antagonist in HBSS/20 mM HEPES, and equilibrate assay plate for 30 minutes at room temperature.
5. Add 10 µL **4X** EC_{80} agonist in HBSS/20 mM HEPES.
6. Measure compound activity using fluorescence reader with appropriate settings (excitation at 494 nM and emission at 516 nM). Signal is monitored over 2 minutes at 2 second intervals. We recommend that you pre-read the assay plates for 5 seconds prior to agonist addition.

DATA ANALYSIS

1. Dose curves in the presence and absence of compound are plotted using GraphPad Prism or Activity Base.
2. For agonist mode assays, percentage activity is calculated using the following formula:

   % Activity = 100% x (Mean RLU of test sample - Mean RLU of vehicle control)/ (Mean MAX RLU control ligand - Mean RLU of vehicle control)
3. For antagonist mode assays, percentage inhibition is calculated using the following formula:

   % Inhibition = 100% x (1 - (Mean RLU of test sample - Mean RLU of vehicle control) / (Mean RLU of EC_{80} control - Mean RLU of vehicle control)

CELL FREEZING PROTOCOL

The following procedures are for freezing cells from confluent T225 flasks. If smaller flasks are used, adjust the volumes accordingly. Care should be taken in handling to avoid contamination.

1. Remove T225 flasks from incubator and place in the tissue culture hood. Aspirate the media from the flasks.
2. Add 10 mL PBS into each T225 flask and swirl to rinse the cells. Aspirate PBS from flask.
3. Add 5 mL of AssayComplete Cell Detachment Reagent to the flask. Rock the flask back and forth gently to ensure the surface of the flask is covered. Incubate at 37°C, 5% CO_2 for 2–5 minutes or until the cells have detached.
4. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
5. Add 8–10 mL of AssayComplete Revive Media to each T225 flask. Rinse the cells from the surface of the flask using the added media. Remove the cells from the flask and transfer to a 50 mL conical tube. (If necessary, add an additional 5 mL of media to the flask and rinse to collect the remaining cells and transfer the additional volume to the 50 mL conical tube).
6. Count the cells using a hemocytometer.
7. Centrifuge the collected cells at 300 x g for 4 minutes.
8. After centrifugation, discard the supernatant. Resuspend the cell pellet in AssayComplete Preserve Freezing Reagent. Based on the cell number obtained from Step 5, dilute the resuspended cells to a concentration of 1.2×10^6 cells/mL using AssayComplete Preserve Freezing Reagent.
9. Transfer 1 mL cells to each 2 mL cryogenic tube. (Keep cells on ice during this process and transfer to a cryogenic container pre-chilled at 4°C).
10. Transfer tubes to –80°C and store overnight. Transfer tubes into the vapor phase of a liquid N_2 tank for long-term storage.

NATIVE CALCIUM ASSAY PROCEDURE

The steps outlined below provide the assay volumes and procedures for performing Ca^{2+} mobilization assays using Native Calcium GPCR Cell Lines and Calcium No Wash ($CaNW^{PLUS}$) Detection Kit. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least *duplicate* wells for each dilution. Signal is measured on a fluorescent plate reader equipped with fluidic handling, capable of detecting rapid changes in fluorescence upon compound stimulation.

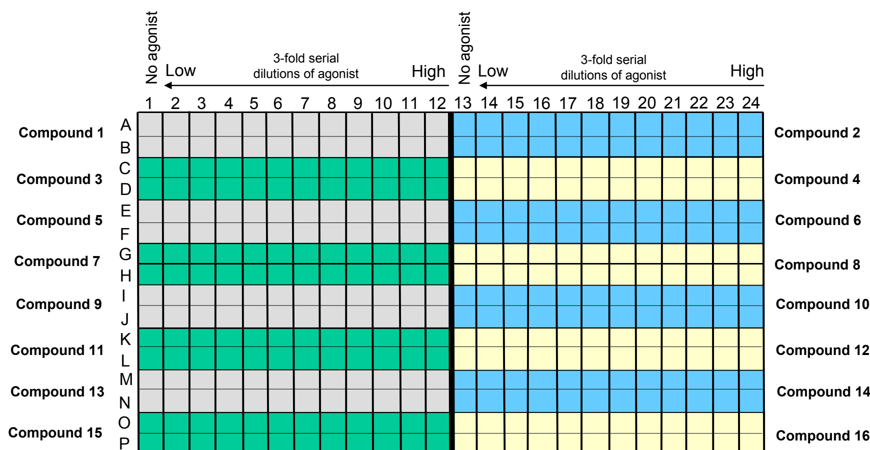


Figure 9. This plate map shows 12-point dose curves with 2 data points at each concentration. Plate map allows 16 compounds to be tested in duplicate per 384-well plate.

PREPARATION OF REAGENTS

Preparation of Calcium NW^{PLUS} Working Reagent:

1. Thaw reagents to room temperature and mix gently.
2. Add 110 µL **anhydrous** DMSO to 500 µg Ca NW^{PLUS} Dye tube and vortex to reconstitute dye. Allowing the pellet to soak in DMSO will facilitate reconstitution. It is very important to ensure that the dye goes completely into solution (solution should be dark yellow).
   a) Store reconstituted dye at -20°C.
   b) This reconstituted dye in DMSO reagent can be frozen and thawed up to 3 times without loss in performance.
   c) Aliquot if necessary.
3. To prepare Working Reagent for one microplate, combine the following components and mix well:
   a) 9 mL of Dye Loading Buffer.
   b) 1 mL of Additive A.
      NOTE:
      It is important to add Additive A before the dye.
   c) 10 µL of reconstituted Ca NW^{PLUS} Dye.
   d) 100 µL of freshly prepared 250 mM probenecid solution.
4. Working Reagent is stable for 24 hours at room temperature. Total signal may drop over longer time periods. Prepare Ca NW^{PLUS} Working Reagent as needed.

DAY 1: PREPARATION OF ASSAY PLATES

1. Harvest the cells as follows from a confluent T25 or T75 flask using Assay-Complete Cell Detachment Reagent. **Do not use Trypsin**.
   a) Remove AssayComplete Cell Culture Media.
   b) Gently wash cells with 5 mL PBS and aspirate.
   c) Add 0.5 mL AssayComplete Cell Detachment Reagent to each T25 flask or 1 mL to each T75 flask.
   d) Place the flask in the incubator for 5 minutes or until cells have detached.
   e) Add 3 mL of AssayComplete Cell Plating Reagent and transfer to a 15 mL conical tube.
2. Determine the cell density using a hemocytometer. Centrifuge the cells at 300 x g for 4 minutes to pellet cells. Remove supernatant.
3. Resuspend cells in CP Reagent at a concentration of 500,000 cells/mL (10,000 cells/20 µL). Transfer 20 µL of the cell suspension to each well of a black 384-well microplate (DiscoveRx, Cat. # 92-0024). If using 96-well plate seed 50,000 cells/100 µL in each well.
4. Incubate the plate overnight at 37°C, 5% CO_2.

DiscoverX

Technical Bulletin

Updated Cell Culture Protocol and Handling Procedure

Applies to: Cell Lines Only

Product Numbers: Several

Date: February 28, 2017

Related Documents: AssayComplete™ Product Lines Product Change Notification
discoverx.com/reagents

Dear Valued Customer,

DiscoverX constantly strives to deliver simple experimental workflows to ensure best performance of our assays. To ensure that these protocols work equally well in your hands, we have updated the “Cell Culture and Handling Procedure” for our cell lines.

The protocol is included in this bulletin and a summary of the updates is provided in Appendix 1. We are currently updating all our cell line user manuals to incorporate this updated protocol.

In the meantime, please use the attached protocol for thawing, propagation, and freezing of your cell lines until the revised cell line user manuals become available.

If you have any questions, please contact our technical support team at SupportUS@discoverx.com (in North America and Asia-Pacific) or SupportEurope@discoverx.com (in Europe).

Sincerely,

The DiscoverX team

Cell Culture and Handling Procedure

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells shipped in the vial). Upon receipt, the vials should be transferred to liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.
2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.
3. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid nitrogen inside the vial to evaporate.
4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line product datasheet for the specific AssayComplete products listed in the protocol below.

Contact technical support immediately if cells are thawed upon arrival.

A face shield, gloves, and a lab coat should be worn during these procedures.

Cryovials should be stored in the vapor phase. They are not rated for storage in the liquid phase of liquid nitrogen.

Cell Culture Protocol

The following procedures are for thawing cells in cryovials, seeding and expanding the cells, maintaining the cell cultures once the cells have been propagated, and preparing them for assay detection.

Care should be taken in cell handling to avoid contamination.

Cell Thawing

The following is a protocol for thawing cells in a T75 flask.

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.
2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask in a sterile tissue culture hood. Set aside for step 6 below. DO NOT add selection antibiotics to the thawing reagent.

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

3. Remove the cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them on dry ice prior to thawing.
4. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.
5. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.

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6. With a pipette, gently transfer the thawed cells to the pre-filled T75 flask and incubate at 37°C and 5% CO_2.
7. Maintain the cells in culture until they are >70% confluent. Then proceed to "Cell Propagation" instructions. Do not split if cells are below this confluency or growth issues may occur.

Cell Propagation

The following is a protocol for propagating cells once they become ≥70% confluent in a T75 flask.

1. Pre-warm AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.
2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate media from the T75 flask.
4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire surface of the flask to rinse the cells.
5. Gently aspirate PBS from flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
7. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with trypsin.
8. Incubate the flask at 37°C, 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

Prolonged treatment with Trypsin-EDTA may compromise cell viability

10. Add 4 mL of AssayComplete Thawing Reagent to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
12. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are included in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK 293	1:3
U2OS	1:2

For example, for CHO-K1 cells, add 4 mL of AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5th of the total reagent in the flask) into each new tissue culture flask.

13. Fill a new T75 or T225 flask with AssayComplete Thawing Reagent until the total volume equals 12 mL for T75 flasks (or 45 mL for T225 flasks), and add cell suspension (added volume determined at step 12) to the media in the flask. Transfer flask to a tissue culture incubator and incubate cells for 24 hours at 37°C and 5% CO_2.

To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

14. After 24 hours, examine the cells under a microscope. If the cells appear to be healthy (adhering to the surface of the flask with only a small number of cells remaining in suspension) exchange the AssayComplete™ Thawing Reagent with AssayComplete Cell Culture Reagent supplemented with the recommended concentration of selection antibiotic (refer to cell line datasheet to determine the correct Cell Culture Kit and recommended antibiotic and antibiotic concentration for your cell line); 12 mL for T75 flasks (or 45 mL for T225 flasks). Then return the flask to a tissue culture incubator. If the cells do not appear to be healthy or if confluency is <25%, incubate for an additional 24 to 48 hours to allow for additional cell recovery before executing this step.

15. Once the cells become ≥70% confluent in the flask, split the cells every 2 to 3 days, based on the doubling time of the cell line, using AssayComplete Cell Culture Reagent supplemented with the proper selection antibiotics. Typical split ratios for common cell backgrounds are included in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:10
HEK 293	1:5
U2OS	1:3

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 1 X 10^6 per vial).

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.

Care should be taken in handling to avoid contamination.

2. Gently aspirate the media from the flasks.
3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).

Keep cells on ice during this process and transfer to a cryogenic container.

6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.
7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
9. Add 5 mL AssayComplete Cell Culture Kit media to each T75 flask (or 15 mL to each T225 flask).
10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.
11. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional AssayComplete Cell Culture

Kit media to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.

12. For the purpose of determining the concentration of cells in the suspension:
    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.
    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.
13. Centrifuge the collected cells at 300 X g for 4 minutes.
14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.
15. Based on the total cell number calculated in step 12 above, re-suspend cells to the desired concentration (e.g. 1 X 10^6 to 2 X 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent.
16. Make 1 mL aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.
17. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

Keep cells on ice during this process to protect cell viability.

Appendix 1

Summary of updates in the “Cell Culture and Handling Procedure”

- When thawing cells, DO NOT centrifuge or vortex freshly thawed cells.
- Once cells are thawed, incubate cells in the AssayComplete Thawing Reagent without any selection antibiotics. Do not use selection antibiotics for the first passage to ensure robust recovery.
- For subsequent passages, supplement the cell line-specific cell culture media with appropriate selection antibiotic.
- For routine propagation and maintenance of adherent cell lines, use Trypsin-EDTA for detaching cells. Use of the AssayComplete Cell Detachment Reagent for routine cell passaging is not necessary.

Additionally, the recommendation for use of the Cell Detachment Reagent has been revised.

When preparing cells for the assay:

- Use AssayComplete Cell Detachment Reagent (Part number 92-0009) for detaching cells. Do not use trypsin for this step, especially in assays interrogating membrane-anchored receptors (e.g. GPCRs). Use of trypsin at this step can negatively affect assay results.
- Also, it is important to rinse the cells with 2 mL AssayComplete Cell Detachment Reagent, rather than with PBS, as rinsing with PBS may inhibit the detachment of cells from the flask surface.