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Calcium NoWash^{PLUS} (Ca NW^{PLUS}) Assay Kit

Fluorescent Detection

User Manual: 90-0091 Series

Simple Solutions for Complex Biology

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For more detailed information on products, bibliographies and applications please visit our website www.discoverx.com. For any technical inquiries please contact technical support department at support@discoverx.com or call 510-979-1415 Option 5.

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LEGAL SECTION

Limited Use License Agreement

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INTENDED USE & TECHNOLOGY PRINCIPLE

This kit provides a homogenous assay format for detection of calcium mobilization. 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.

The DiscoveRx Ca NW^{PLUS} Assay Kit provides 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.

MATERIALS PROVIDED

Number of Assays	**10 x 96- or 384-well plates**	**20 x 96- or 384-well plates**	**100 x 96- or 384-well plates**
Material	**Amount**		
	90-0091	**90-0091L**	**90-0091XL**
Dye Loading Buffer (DLB)	1 x 100 mL	2 x 100 mL	Not included*
Ca NW^{Plus} Dye	1 tube (500 µg ea)	2 tubes (500 µg ea)	10 tubes (500 µg ea)
Additive A	10 mL	20 mL	5 x 20 mL

*20 mM HEPES in Hank's Balanced Salt solution (HBSS), pH 7.4.
Upon receipt, store all reagents at -20°C. If required DLB can be stored at 4°C. Additive A can be frozen and thawed up to 3 times without loss in performance. Aliquot Additive A if necessary and store at -20°C until needed.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

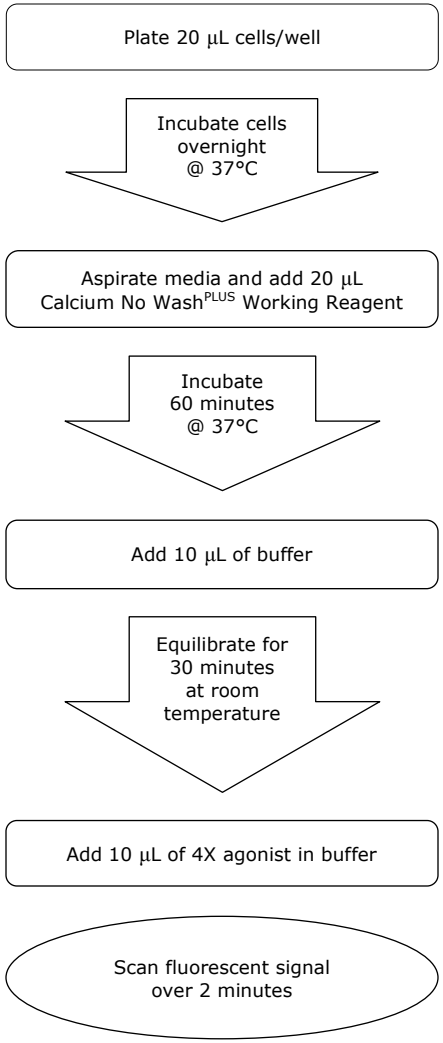
Material	**Suggested Vendor/Cat. #**
AssayComplete™ Revive Reagent	DiscoveRx, Cat. #92-0016RM Series
AssayComplete Cell Culture Kit	DiscoveRx, Cat. #92-0018/19/20/21/22G Series
AssayComplete Preserve Freezing Reagent	DiscoveRx, Cat. #92-0017FR Series
AssayComplete Cell Detachment Reagent	DiscoveRx, Cat. #92-0009
AssayComplete Cell Plating Reagent	DiscoveRx, Cat. #93-0563R Series
Black 96-well or 384-well Clear bottom TC Treated, Sterile Microplates	DiscoveRx, Cat. #92-0024 (384-well)
Probenecid	Sigma, Cat. #P8761
DLB: 90-0091XL Only	Preferred Vendor

NOTES:

GENERAL RECOMMENDATIONS

1. Prepare the dye concentrate with anhydrous DMSO. Anhydrous DMSO can be prepared by storing DMSO stocks over activated molecular sieves (4A). This will reduce the hydrolysis of the dye when stored as a concentrate.
2. Optimal dye loading time may vary for each cell type. If insufficient signal is observed, increasing dye loading incubation time or titrating cell density may improve assay signal.
3. Cell density should be optimized for each cell type.
4. Room temperature equilibration is a recommended approach if temperature effects are observed upon compound addition.
5. If dye loading is performed at 37°C and a signal dip is observed when compound is added, it may be necessary to allow plates to equilibrate to room temperature for 30 minutes before starting the assay.
6. Dispensing tip height and speed should also be optimized to minimize dispense effects on the assay signal.
7. Use of poly-D-lysine coated plates is suggested for best performance, especially when using HEK 293 cells.
8. Use freshly prepared probenecid. Add 0.72 g of probenecid to 3.5 mL 1N NaOH, followed by 6.5 mL DLB to make a 250 mM Probenecid working solution. Mix vigorously until dissolved. Exercise appropriate caution while handling this chemical.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



PREPARATION OF REAGENTS

Ca NW^{PLUS} Working Reagent:
If you are using the 90-0091 or 90-0091L kits the dye loading buffer is supplied with the kit, proceed to step 1.

The 90-0091XL kit does not include the dye loading buffer. For 100 microplates please prepare a total of 1L of 20 mM HEPES in Hank's Balanced Salt Solution (HBSS), pH 7.4 and then proceed to step 1.

1. Thaw reagents to room temperature and mix.
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 and mix well the following components and mix well:
   a) 9 mL of Dye Loading Buffer.
   b) 1 mL of Additive A.
      NOTE:
      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. (Refer to page 7 for more details).
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.

PREPARATION OF CELLS

Harvest and resuspend cells in AssayComplete™ Cell Culture media to desired concentration and seed onto microplates. We recommend plating 10000 cells/well in a 384-well plate and 50000 cells/well in a 96-well plate. Optimal cell densities must be established for each cell line and plate type. Allow cells to adhere overnight under standard cell culture conditions.

ASSAY PROTOCOL

1. Remove microplates containing cells from the incubator.
2. Aspirate media from cells and replace with 20 µL Ca NW^{PLUS} Working Reagent.
3. Incubate cells for 1 hour at 37°C, 5% CO_2.

4. Add 10 µL of buffer HBSS/20 mM HEPES and equilibrate at room temperature for 30 mins.
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.

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)

REPRESENTATIVE DATA

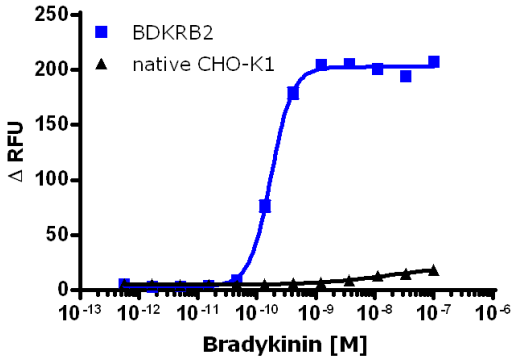


Figure 1. PathHunter® CHO-K1 BDKRB2 β-Arrestin Cells (DiscoveRx, Cat. #93-0189C2). Cells were plated in a 384-well plate at 10,000 cells/well. Target cells and native parental cells were then stimulated with the known agonist Bradykinin (DiscoveRx, Cat. #92-1053). Calcium mobilization was detected using the Calcium No Wash^{PLUS} Detection Kit according to the recommended protocol. The EC_{50} for agonist was estimated at 0.17 nM.