ION BIOSCIENCES

Instructions

# Multidrug Resistance Activity Kit

## Kit Contents (Cat # 3330-10)

| Label | Name | Size | Quantity | Storage |
|---|---|---|---|---|
| Reagent A | 1000X Cyclosporine A in Ethanol | 50 µL Vial | 1 | -20°C |
| Reagent B | Calcein AM, 4 mM in DMSO[1] | 50 µL Vial | 2 | -20°C |
| Reagent C | 1X HEPES-Buffered Hanks Balanced Salt Solution (1X HHBSS)* | 100 mL Bottle | 1 | 4°C* |
| Reagent D | 50X TRS[2]* | 4 mL Bottle | 1 | 4°C* |

**1X HHBSS and 50X TRS[2] reagents are shipped frozen but may be stored at refrigerated temperatures upon receipt. 1000X Cyclosporine A and Calcein AM solutions should always be stored frozen.*

## Description

ION's Multidrug Resistance (MDR) Activity kit is an effective solution for detecting MDR1 and MRP1 activity and compounds susceptible to MDR-mediated efflux. ION's MDR Activity kit is compatible with fluorescence microscopy, flow cytometry, and fluorescence plate readers using FITC/fluorescein settings.

Calcein AM is a membrane-permeant, non-fluorescent dye that enters cells passively. Once inside the cytosol of cells, intracellular esterases convert it to fluorescent Calcein (Ex/Em: 495 nm/515 nm), resulting in uniform cytosolic fluorescence. Drug efflux transporters, such as P-glycoprotein (Pgp, MDR1) and multidrug-resistance-associated protein (MRP1), actively extrude Calcein AM from inside the cell before esterases can convert it to Calcein. The presence of additional MDR substrates or inhibitors of MDR expression results in decreased Calcein AM efflux, causing a measurable increase in intracellular fluorescence. In addition to identifying MDR substrates and inhibitors, this kit can also be used to evaluate the activity of MDR transporters in cells.

When following our protocol, ION's MDR Activity kit provides enough reagents to make 100 mL of working solution, enough for ten 96- or 384-well plates or 80 flow cytometry samples. The actual number of assays will vary according to optimal dye concentrations for your application.

## Laboratory Procedures

### Getting Started

Before you begin, make sure that you have all the additional reagents and materials you will need for the successful completion of your experiment. Although ION's MDR Activity kit contains the key reagents you will need to prepare your cells for analysis, your experiments will likely require other reagents which are not included in your MDR Activity kit. Notably, compounds to be tested, buffers and solvents for the dissolution of these compounds, and reagents necessary for cell culture are not included.

In addition to reagents - a microscope, fluorescence plate reader, or flow cytometer that is capable of providing an excitation source at ~ 495 nm and measuring emission at ~515 nm is required. Compatible filters are FITC or GFP.

## General Considerations

1. Optimal Calcein AM concentrations will vary depending on cell type and application. Recommended Calcein AM concentrations range between 1 µM and 2 µM.
2. Minimize freeze-thaw cycles of Calcein AM and Cyclosporine A solutions.
3. Aqueous solutions of Calcein AM are susceptible to hydrolysis; therefore, all working solutions should be used as quickly as possible and no later than 4 hours after preparation.
4. Calcein cannot withstand fixation after staining.
5. Serum-containing solutions may increase extracellular fluorescence. If conducting an assay with serum present, we recommend including TRS$^2$ to minimize extracellular fluorescence.
6. Cyclosporine A can be used as a positive control. Use at a concentration of 5 µM (1:1000 dilution) for maximum efficacy.
7. Molecules that affect esterase activity, intracellular ATP production, or that bind to alternative MDR binding sites may result in an inaccurate classification of MDR substrates.

## Plate Reader Assay

1. For adherent cells, plate cells overnight, seed ~30,000 cells/well/100 µL in a 96-well plate or ~10,000 cells/well/25 µL in a 384-well plate. When using this assay kit, make sure to use cells that possess high levels of MDR activity such as CHO K1, HCT-8, U2OS, or cells that have been genetically engineered to overexpress MDR proteins.
2. Remove the 1000X Cyclosporine A (Reagent A) and one vial of Calcein AM (Reagent B) from the freezer and allow to warm to room temperature. Protect reagents from light.
3. Prepare the test compound solutions at the desired concentrations in 1X HHBSS (Reagent C) or a similar buffer of your choice.
4. For a positive control, make the appropriate amount of Cyclosporine A solution needed, enough for 100 µL/well to be evaluated. For example, add 1 µL of 1000X Cyclosporine A (Reagent A) to 0.999 mL of the buffer used in step 3.
5. Remove medium from the wells of the plate and add 100 µL/well of test compound solutions, including your positive control solution from step 4. Incubate for 15 - 30 minutes at 37 °C or room temperature.
6. Prepare dye loading solution that contains 2X Calcein AM, 4 µM (1:1000 dilution) in 1X HHBSS (Reagent C). For example, add 10 µL of Calcein AM (Reagent B) to 10 mL of 1X HHBSS (Reagent C) for a full plate. Vortex briefly to mix.
7. Optional: Add 400 µL of 50X TRS$^2$ (Reagent D) to the dye loading solution. Adjust 1X HHBSS (Reagent C) volume in step 6 to 9.6 mL. TRS minimizes extracellular fluorescence and is recommended when long incubation times with dye loading solution or serum containing media are used.
8. After the 15 - 30 minute incubation period from step 5 is complete, add dye loading solution prepared in step 6 or 7 directly to wells. Add 100 µL/well to a 96-well plate.
9. Incubate cells for an additional 30 minutes at 37 °C or room temperature. Protect from light.
10. Measure fluorescence using a fluorescence plate reader. For Calcein, use Ex/Em ~495 nm/515 nm or FITC settings.

*Note: If conducting an assay using a fluorescence microscope, use GFP or FITC filters to acquire images. If using non-adherent cells, follow flow cytometry protocol until step 10. Then add cell suspensions to wells of a microplate and centrifuge your plate before acquiring fluorescence data.

## Flow Cytometry Assay

1. Remove the 1000X Cyclosporine A (Reagent A) and one vial of Calcein AM (Reagent B) from the freezer and allow to warm to room temperature. Protect reagents from light.
2. Prepare your test compounds at the desired concentrations in 1 mL of in 1X HHBSS (Reagent C) or a similar buffer of your choice. To prepare the positive control, add 1 µL of the 1000X Cyclosporine A (Reagent A) solution to 0.999 mL of the same buffer.
3. If using adherent cells, detach cells from the culture dish and suspend cells in cell culture medium at a concentration of ~1-2 x $10^6$ cells/mL. For non-adherent cells, suspend in medium at your desired cell concentration.
4. Prepare tubes for each assay condition in triplicate by adding 250 µL of the cell suspension from step 3 to a minimum of 3 tubes. Add an extra tube for an unstained cell control, if desired.
5. Centrifuge cells and remove the medium.
6. Resuspend a single tube of cells in 125 µL of test compound solution or positive control solution. Repeat in triplicate (3 tubes total) for each test compound. Also include a vehicle control condition of 125 µL of your buffer containing the same DMSO concentration as your test compound solutions.
7. Incubate tubes for 15 - 30 minutes at room temperature.
8. During incubation period, prepare a dye loading solution that contains 2X Calcein AM, 4 µM (1:1000 dilution) in 1X HHBSS. For example, add 10 µL of Calcein AM (Reagent B) to 10 mL of 1X HHBSS (Reagent C). Vortex briefly to mix.
9. After the 15 - 30 minute incubation period from step 7 is complete, add 125 µL of dye loading solution prepared in step 8 to each tube.
10. Incubate cells for an additional 30 minutes at room temperature. Protect from light.
11. After the completion of step 10, centrifuge cells and aspirate your dye loading solution and test compound buffer. Resuspend cells in your preferred flow cytometry (FACS) buffer.
12. Analyze cells using a flow cytometer. To detect Calcein AM (+) cells, use FITC settings.

## Example Results

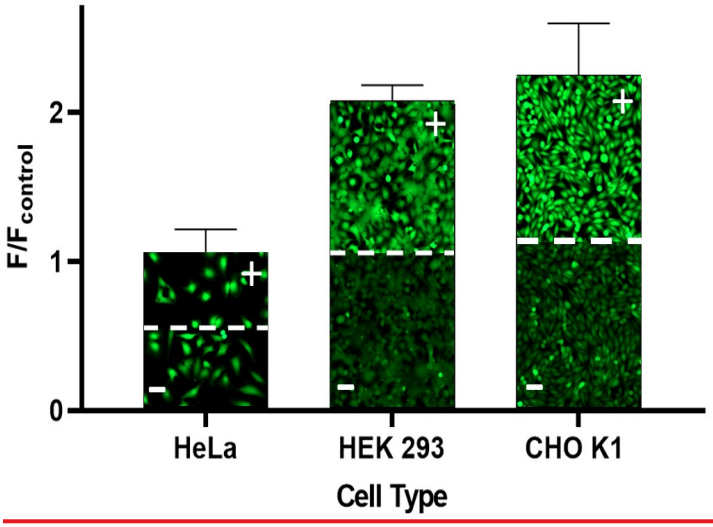


Figure 1. Calcein fluorescence in HeLa, HEK 293, and CHO K1 cells in the presence (+, top half of bar) or absence (-, bottom half of bar) of ~5.0 µM CsA. Cells that do not express the transporter, such as HeLa cells, do not show a change in fluorescence when CsA is added. Fluorescence (Ex: 495 nm, Em: 515 nm) was recorded on a BioTek® Cytation 5 plate reader. Images were acquired using GFP filters, Ex: 469/35 nm, Em: 525/39 nm, and 4X objective on a BioTek® Cytation 5. Reported ratios are calculated using fluorescence "bottom-read" data. Error bars represent standard deviation.

## Laboratory Procedures - Footnotes

[1] Ethidium homodimer I is a 2 mM solution in a 1:3 ratio of DMSO:$H_2O$.

[2] Calcein AM, 4 mM in DMSO is not a regular catalog item available for purchase, however, ION Biosciences does stock a 2 mM in DMSO solution for sale (see Table 1 below), which can be used in place of Reagent B by simply doubling the volume used in any of the procedures described in this protocol.

## Additional Information

Additional dye indicator and buffer reagents can be purchased either directly from our website or by contacting our Sales Department. Custom and bulk sizes are also available. Contact Sales for more information.

| Table 1 | Additional Reagents | Available Sizes | |
|---|---|---|---|
| Kit Label | Name | Size | Catalog # |
| Reagent A | 1000X Cyclosporine A in Ethanol | Not a Regular Catalog Item Contact Sales to Purchase | |
| Alternative to Reagent B | Calcein AM, 2 mM in DMSO | 0.5 mL x 1 Vial | 5030 |
| Reagent C | 1X HEPES-Buffered Hanks Balanced Salt Solution (1X HHBSS) | 100 mL Bottle | 7001 |
| Reagent D | 50X TRS | 20 mL Bottle | 7060A |