Ratiometric Calcium Assay Kit

Item No. 35761

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Label	Item Name	Quantity/Size	Storage
Reagent A	Fura-2 AM	10 vials/50 µg	-20°C
Reagent B	TRS (50X)	1 bottle/2 ml	4°C
Reagent C	Pluronic® F-127 (100X)	1 vial/1 ml	4°C
Reagent D	HEPES-buffered Hanks' Balanced Salt Solution (1X)	1 bottle/100 ml	4°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with the Ratiometric Calcium Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorescence plate reader capable of rapid kinetic acquisition, with a read time of ~2-5 seconds per well or faster, and dual-excitation fluorescence detection (ex. 340/380 nm, em. 505 nm); the capability for automated injection on the plate reader is highly recommended (see **Instrument Settings** on page 6 for more information)
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. Black-walled, clear-bottom 96-well cell culture plates
4. DMSO
5. Cell culture supplies (*e.g.*, culture medium, PBS, culture plates)
6. Agonists or compounds to stimulate calcium flux

INTRODUCTION

Background

Fura-2 AM is a widely used ratiometric Ca^{2+} indicator.[1] Once inside cells, intracellular esterases cleave the AM ester group, trapping the fluorescent dye within the cytoplasm.[2] Binding of Ca^{2+} alters the excitation spectrum of the dye: Ca^{2+}-bound Fura-2 is excited at 340 nm, while Ca^{2+}-free Fura-2 is excited at 380 nm.[1] Both forms emit fluorescence at 505 nm. The ratio of fluorescence intensities at these excitation wavelengths (340/380) provides a quantitative measurement of intracellular Ca^{2+} levels. Ratiometric measurements are advantageous because they reduce artifacts from photobleaching, heterogenous dye loading, and variable cell morphology.[1,2]

About This Kit

This Ratiometric Calcium Assay Kit provides the reagents necessary to perform no-wash, ratiometric Ca^{2+} flux assays compatible with fluorescence plate readers. Individual components are provided to allow flexibility in optimizing assay conditions for different cell types and experimental systems.

When prepared according to the recommended protocol, this kit provides sufficient reagents to generate approximately 100 ml of dye loading solution, which is enough to process ten 96-well plates when using 100 µl per well. The total number of assays may vary depending on the concentration and experimental conditions required for a given application.

PREPARATION

Instrument Settings

Since Ca^{2+} flux responses often occur within seconds of agonist addition, a plate reader capable of rapid kinetic acquisition (2-4 seconds per well or faster) is recommended. The instrument should be capable of measuring fluorescence using excitation wavelengths of 340 and 380 nm with emission detection at 505 nm.

Instruments equipped with automated injectors are strongly recommended because they allow compounds to be added during fluorescence acquisition and improve the ability to capture rapid Ca^{2+} responses. If an automated injection system is not available, compounds may be added manually during fluorescence acquisition. In this case, it is recommended that compounds be added to a small number of wells at a time to ensure that early Ca^{2+} responses are captured.

Reagent Preparation

NOTE: Allow all reagents to equilibrate to room temperature prior to use

1. **Fura-2 AM - (Reagent A)**

   Each vial contains 50 µg of lyophilized Fura-2 AM. Reconstitute the contents of each vial with 25 µl of DMSO to prepare a 2 mM stock solution. Vortex until fully dissolved and centrifuge briefly to collect all contents at the bottom of the tube.

   When used at 5 µM or less, one vial provides a sufficient amount to prepare working solutions for one 96-well plate. If additional wells will be assayed, reconstitute additional vials as needed. If the entire vial will not be used all at once, prepare single-use aliquots and store at -20°C, protected from light, where it will be stable for one month. Avoid repeated freeze-thaw cycles.

2. **TRS (50X) - (Reagent B)**

   This vial contains 2 ml of TRS (50X). TRS is a membrane-impermeant dye that masks extracellular fluorescence. *NOTE: In some cases, TRS may interfere with the response of certain Ca^{2+}-modulating compounds. If this occurs, the assay can be performed without TRS.*

   If the entire bottle will not be used all at once, prepare single-use aliquots and store at -20°C, protected from light. Avoid repeated freeze-thaw cycles.

3. **Pluronic® F-127 (100X) - (Reagent C)**

   This vial contains 1 ml of Pluronic® F-127 (100X). Pluronic® F-127 improves the solubility of hydrophobic AM-ester dyes and promotes uniform dye loading into cells.

   If the entire bottle will not be used all at once, prepare single-use aliquots and store at -20°C, protected from light. Avoid repeated freeze-thaw cycles.

4. **HEPES-buffered Hanks' Balanced Salt Solution (1X) - (Reagent D)**

   This bottle contains 100 ml of HHBS (1X). It is used to prepare the working solutions and serves as the assay buffer during Ca^{2+} flux measurements.

ASSAY PROTOCOL

General Information

- The optimal working concentration of Fura-2 AM and the incubation times may vary depending on cell type, cell density, and experimental treatments. Preliminary experiments are recommended to determine optimal assay conditions for each application. See Table 1 below for recommendations.

Reagent	Function	Recommended Final Concentration
Fura-2 AM	Calcium probe	1-10 µM
TRS (Optional)	Masks extracellular fluorescence	1X
Pluronic® F-127	Improves solubility and cellular loading of Fura-2 AM	1X

Table 1. Recommended reagent concentrations

- Ca^{2+} flux responses are typically triggered by addition of an agonist or stimulus that activates Ca^{2+} signaling pathways. The concentration and timing of compound addition should be optimized for each experimental system.
- Fura-2 is susceptible to hydrolysis in aqueous solutions; therefore, all working solutions should be used as quickly as possible and no later than two hours after preparation for best results.
- For accurate interpretation of results, it is recommended to include the following controls:
  - Vehicle control wells
  - Positive control stimulus (*e.g.*, ionomycin or other Ca^{2+}-mobilizing agents)

Laboratory Procedures

NOTE: This protocol describes measurement of intracellular Ca^{2+} using Fura-2 AM in a 96-well plate format. Volumes may be adjusted proportionally for other plate formats.

Adherent Cells

1. Culture adherent cells according to standard procedures appropriate for the cell line used.
2. Seed cells into a black-walled, clear-bottom 96-well plate and allow them to adhere overnight. Cells should be seeded at a density that results in 80-90% confluence at the time of the assay.

   NOTE: Optimal seeding density may vary depending on cell type and assay conditions. Preliminary experiments are recommended to determine the optimal seeding density for each application.
3. Prepare the appropriate Fura-2 AM working solution following the table below. Scale as needed.

Reagent	+TBS	-TRS
HHBS (1X)	9,675 µl	9,875 µl
TRS (50X)	200 µl	--
Pluronic® F-127 (100X)	100 µl	100 µl
Reconstituted Fura-2 AM (2 mM)*	25 µl	25 µl

Table 2. Preparation of the Fura-2 AM working solution for adherent cells

**NOTE: Addition of 25 µl of 2 mM Fura-2 AM to the working solution results in a final concentration of 5 µM Fura-2 AM in the well. The amount of Fura-2 AM and HHBS (1X) may be adjusted to optimize the probe's concentration for different cell types and experimental conditions. Typical working concentrations of Fura-2 AM range from 1–10 µM.*

4. Remove the culture medium from the wells.
5. Add 100 µl of Fura-2 AM working solution to each well.
6. Incubate the plate at 37°C for 60 minutes in a 5% CO_2 incubator.
7. Transfer the plate to a fluorescence plate reader configured to measure fluorescence at 340 and 380 nm excitation with emission detection at 505 nm.
8. Begin kinetic fluorescence acquisition to establish a baseline signal.
9. After collecting baseline measurements, add test compounds, agonists, or vehicle control to the appropriate wells.

   NOTE: When adding compounds manually, it is recommended that compounds be added to a small number of wells at a time to ensure that rapid Ca^{2+} responses are captured.
10. Continue fluorescence acquisition to monitor changes in intracellular Ca^{2+} levels.

Suspension Cells

1. Culture suspension cells according to standard procedures appropriate for the cell line used.
2. Wash cells once and resuspend in HHBS (1X) at a concentration of 2-6 x 10^6 cells/ml.

   NOTE: Optimal cell density may vary depending on the cell type, growth characteristics, and assay conditions. Preliminary experiments are recommended to determine the optimal cell concentration for each application.
3. Transfer 50 µl of the cell suspension to the wells of a black-walled, clear-bottom 96-well plate.
4. Prepare the appropriate Fura-2 AM working solution following the table below. Scale as needed.

Reagent	+TBS	-TRS
HHBS (1X)	4,675 µl	4,875 µl
TRS (50X)	200 µl	--
Pluronic® F-127 (100X)	100 µl	100 µl
Reconstituted Fura-2 AM (2 mM)*	25 µl	25 µl

Table 3. Preparation of Fura-2 AM working solution for suspension cells

**NOTE: Addition of 25 µl of 2 mM Fura-2 AM to the working solution results in a final concentration of 5 µM Fura-2 AM in the well. The amount of Fura-2 AM and HHBS (1X) may be adjusted to optimize the probe's concentration for different cell types and experimental conditions. Typical working concentrations of Fura-2 AM range from 1–10 µM.*

5. To 50 µl of suspension cells in HHBS (1X), add an equal volume of Fura-2 AM working solution.
6. Incubate the plate at 37°C for 60 minutes in a 5% CO_2 incubator.
7. Transfer the plate to a fluorescence plate reader configured to measure fluorescence at 340 and 380 nm excitation with emission detection at 505 nm.
8. Begin kinetic fluorescence acquisition to establish a baseline signal.
9. After collecting baseline measurements, add test compounds, agonists, or vehicle control to the appropriate wells.

   NOTE: When adding compounds manually, it is recommended that compounds be added to a small number of wells at a time to ensure that rapid Ca^{2+} responses are captured.
10. Continue fluorescence acquisition to monitor changes in intracellular Ca^{2+} levels.

Calculations

1. Calculate the ratio of fluorescence intensities measured at 340 nm (Ca^{2+}-bound dye) and 380 nm (Ca^{2+}-free dye) for each time point.
2. Plot the 340/380 ratio as a function of time to visualize changes in intracellular Ca^{2+} concentration.

Example Data

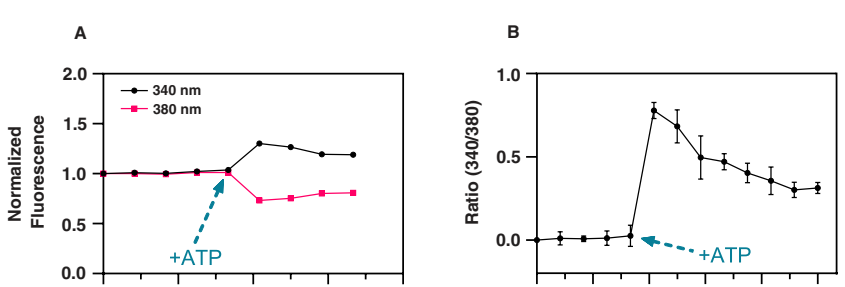

Figure 1. Measurement of intracellular Ca^{2+}. A549 cells were seeded in a black-walled, clear-bottom 96-well plate and loaded with 5 µM Fura-2 AM prepared according to the protocol described above. After incubation at 37°C for 60 minutes in a 5% CO_2 incubator, fluorescence was measured using a Cytation™ 5 multimode plate reader with excitation at 340 and 380 nm and emission detection at 505 nm. Baseline fluorescence was collected prior to addition of 1 mM ATP, which was added manually at the indicated timepoint. **A)** Normalized fluorescence traces at 340 and 380 nm excitation. **B)** The fluorescence ratio 340/380 for each time point. Addition of ATP produced a rapid increase in the fluorescence ratio consistent with increased intracellular Ca^{2+} levels. Error bars represent replicate wells.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Weak fluorescence signal	A. Suboptimal Fura-2 AM concentration was used B. Fura-2 AM precipitated	A. Increase concentration of Fura-2 AM or extend incubation time B. Ensure Fura-2 AM is first dissolved in DMSO; add Fura-2 AM after Pluronic® F-127 when preparing the working solution
No detectable Ca^{2+} response after agonist addition	A. Agonist concentration is too low B. Instrument read intervals are too slow to capture response	A. Verify compound is an agonist and optimize its concentration; use of a positive control, such as ATP or ionomycin, is highly recommended B. If an agonist is added manually, reduce the number of wells measured per run or use an instrument with automatic injectors
High background fluorescence	A. Serum esterases are hydrolyzing Fura-2 AM B. Extracellular dye contributing to signal	A. Remove culture medium prior to adding working solution B. Use TRS in the working solution

References

1. Kong, S.K. and Lee, C.Y. The use of fura 2 for measurement of free calcium concentration. *Biochem. Mol. Biol. Ed.* **23(2)**, 97-98 (2005).
2. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260(5)**, 3440-3450 (1985).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.