A Novel NO Wash Probeniceid-Free Calcium Assay for Functional Analysis of GPCR and Calcium Channel Targets

Jinfang Liao*, Qin Zhao, Xing Han and Zhenjun Diwu

AAT Bioquest, Inc., 520 Mercury Drive, Sunnyvale, CA 94085

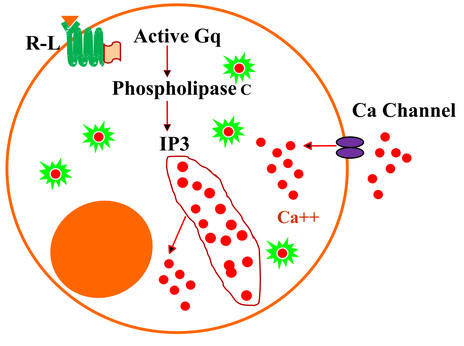
Introduction

The Calcium influx assay is a preferred method for monitoring the activities of GPCR and calcium channels. We have developed a novel no wash probeniceid-free Cal 520™ Calcium Assay for cell-based calcium mobilization high-throughput screening assays. Cal 520™ AM is a new fluorescent calcium-sensitive dye developed for monitoring GPCR and calcium channel targets with a significantly improved signal to noise ratio and intracellular retention.

Cal 520™ AM ester is non-fluorescent. Once it enters the cells, the lipophilic AM blocking groups are cleaved by intracellular esterases, resulting in a negatively charged fluorescent dye that stays inside cells. When cells are stimulated with bioactive compounds, the receptor signals release intracellular calcium. As the dye binds to Ca^{2+} inside the cells, fluorescence intensity is greatly enhanced.

In this study, the signal intensity and S/N (signal to background) ratio of probeniceid-free Cal 520™ Calcium Assay Kit, BD™ PBX Calcium Assay Kit, Fluo-4 Direct, and Calcium-4, along with Fluo-3 AM and Fluo-4 AM were evaluated with different receptor signaling pathways using several cell lines including CHO-M1, HEK-293 and Jurkat cells. Fluo-4 Direct, Calcium-4, Fluo-3 AM and Fluo-4 AM which are easily pumped out by organic-anion transporters. Probeniceid-free Cal 520™ Calcium Assay kit and Cal 520™ AM has much better cellular retention than other existing calcium indicators in addition to its significantly higher S/N ratio. It requires no organic-anion transporter inhibitors (such as probenecid) present in the assay system. Cal 520™ AM is an improved fluorescent indicator for the measurement of intracellular calcium. The high S/N ratio and better intracellular retention make the Cal 520™ calcium assay a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

Cal 520™ Calcium Assay Principle



[Ca^{2+}] Increase via Gq or calcium channel is measured by Cal 520™ fluorescence enhancement.

Material and Methods

1. CHO-M1 or HEK cells were plated at 96-well black wall/clear bottom costar plate at 37°C incubator for overnight.
2. For Wash Assay: Take growth medium off, incubate the cells with Fluo-3 AM, Fluo-4 AM or Cal 520™ AM at 37°C for 90 min, then at room temperature for 30 min.
3. For No Wash Assay: The cells were incubated with Fluo-4 Direct, Calcium 4, BD™ PBX Calcium Assay Kit, Cal 520 ™ NW kit or Cal 520™ PBC-free kit at 37°C for 90 min, then at room temperature for 30 min..
4. Run calcium efflux experiments on Flexstation.

Plating cells for overnight

↓

Aspirate growth medium (for wash assay)

↓

Dye loading for 1-2 hours at 37 °C

↓

Run calcium assay at Ex/Em = 485-490/520-530nm

Results

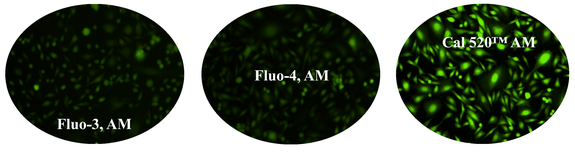


Figure1. **Response of endogenous P2Y receptor to ATP in CHO-M1 cells in the absence of probenecid.** CHO-M1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µl of 4 µM Fluo-3 AM, Fluo-4 AM or Cal 520™ AM in HHBS were added into the wells, and the cells were incubated at 37 °C for 2 hour. The dye loading medium were replaced with 100 µl HHBS, 50 µl of 300 µM ATP were added, and then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

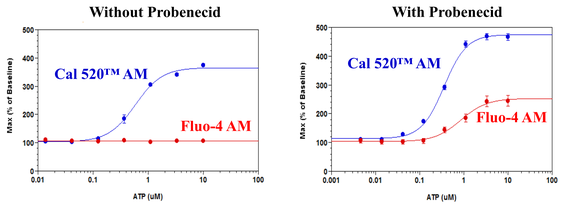


Figure 2. **ATP dose response on endogenous P2Y receptor in CHO-M1 cells with or without probenecid.** CHO-M1 cells were seeded overnight in 50,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The growth medium was removed and every one third of the plate were incubated with 100 µl of 5 µM or Cal 520™ AM or Fluo-4 AM in HHBS with or without 2.5 mM probenecid, and the cells were incubated at 37 °C for 2 hour. ATP (50 µL/well) was added using Flexstation to achieve the final indicated concentrations.

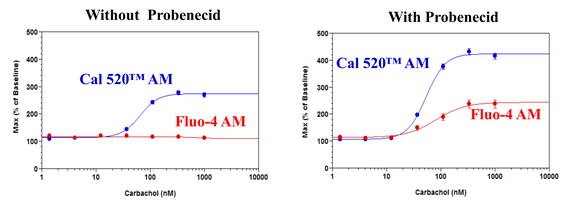


Figure 3. **Carbachol dose response on enxogenous M1 receptor in CHO-M1 cells with or without probenecid.** CHO-M1 cells were seeded overnight in 50,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The growth medium was removed and every one third of the plate were incubated with 100 µl of 5 µM or Cal 520™ AM or Fluo-4 AM in HHBS with or without 1 or 2.5 mM probenecid, and the cells were incubated at 37 °C for 2 hour. ATP (50 µL/well) was added using Flexstation to achieve the final indicated concentrations.

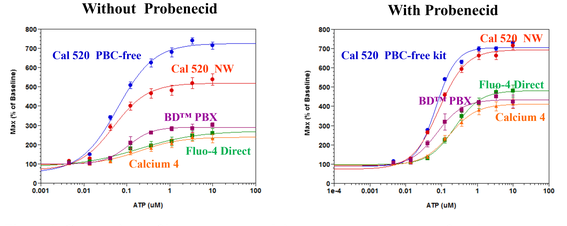


Figure 4. **Comparison of homogeneous calcium assays on response of endogenous P2Y receptor to ATP in CHO-M1 cells in the absence of probenecid.** CHO-M1 cells were seeded overnight in 50,000 cells per 100 µl per well in a 96-well black wall/clear bottom costar plate. The cells were incubated with 100 µl of Cal 520™ PBC-free Ca Kit, Cal 520™ NW kit, BD™ PBX Ca kit, Fluo-4 direct or Calcium 4 in HHBS with or without 1 or 2.5 mM probenecid for 2 hours at 37 °C, 5% CO_2 incubator. ATP (50 µL/well) was added using Flexstation to achieve the final indicated concentrations.

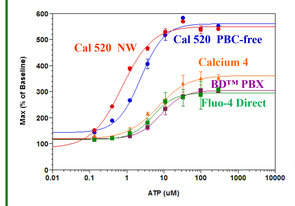


Figure 5. **Comparison of homogeneous calcium assays on response of endogenous P2Y receptor to ATP in HEK-293 cells.** HEK-293 cells were seeded overnight in 30,000 cells per 100 µl per well in a 96 well black wall/clear bottom costar plate. The cells were incubated with 100 µl of 5 different calcium assay kits in HHBS for 2 hours at 37 °C, 5% CO_2 incubator. ATP (50 µL/well) was added using Flexstation to achieve the final indicated concentrations. The EC_{50} of ATP for Cal 520 ™ PBC-free Ca Kit is 2 µM, Cal 520 ™ NW kit is 1 µM, BD™ PBX Ca kit is 8 µM, Fluo-4 direct is 5 µM, and Calcium 4 is 7 µM respectively.

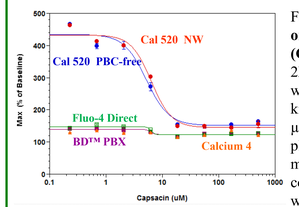


Figure 6. **Comparisons of homogeneous calcium assays on concanavalin A induced capacitative clacium entry (CCE) in JurKat cells.** Jurkat cells were suspended at $2X10^6$ cells per ml in calcium-free HHBS buffer, cells were incubated with 100 µl of 5 different calcium assay kits in calcium-free HHBS buffer at $2X10^5$ cells/well/100 µL in a 96-well black wall/clear bottom poly-d lysine plate for 1.5 hr at 37 °C incubator. At the end of the 10 min incubation, 10 µL /well of channel opener concanavalin A at 3 µg/ml (final in well concentration) with or without channel inhibitor capsacin were added into the cells to achieve the final indicated concentrations. 50µL/well of HBSS with additional 24 mM (5X) calcium was added using FlexStation.

Summary

Cal 520™ AM is a novel calcium indicator for measuring intracellular calcium. The high S/N ratio and good intracellular retention make the probenecid-free Cal 520™ calcium assay a robust tool for evaluating GPCR and calcium channel targets (especially for those interfered with probenecid), as well as for screening their agonists and antagonists. Cal 520™ Calcium Assay has the following benefits and features:

- ❑ **Enable probenecid-free Ca^{2+} assays.**
- ❑ **Significantly higher S/N ratio than any other commercially available fluorescent Ca^{2+} assays.**
- ❑ **Essentially identical spectra to Fluo-3, Fluo-4, and Fluo-8™.**

AAT Bioquest®
Advancing Assay Technologies