CytoCHECK SPAchip®

CALCIUM GREEN SINGLE-DETECTION KIT

Instructions for Use

Product Reference: S-002-CAG

For use with flow cytometers and cell imaging platforms

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For research use only

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1. General Introduction

CytoCHECK SPAchip® assay kits are novel cell-based assays for living single-cell developed by A4Cell that bring together the fields of nanotechnology and cell biology. CytoCHECK SPAchip® kits are composed of fluorescently labeled silicon microparticles -SPAchips®- that can be internalized in the cytosol of cultured cells to monitor changes in specific intracellular analyte concentrations for long periods of time.

CytoCHECK SPAchip® Calcium Green Single-Detection Kit allows measurement of intracellular calcium levels by changes in fluorescence intensity, which allows a more comprehensive study of the living single-cell physiology and maximizes the performance of imaging platforms and flow cytometers.

CytoCHECK SPAchip® Single-Detection kits are optimized for its use with confocal microscopes and HCS/HCA analyzers with 20X or over magnification objectives, yet epi-fluorescence microscopes and imaging system with fixed wavelength filters can be also used[1]. Flow cytometers have additionally been validated to analyze intra and extracellular SPAchips®.

SPAchip® kits can be handled as common fluorophores and chemical probes for cell biology. After resuspending them in the assay buffer (a SPAchip®-to-cell ratio of 2:1 is recommended), an overnight incubation is required to allow SPAchip® to be incorporated in the cytosol[2]. SPAchip® will remain in the cytosol for longer than one month to monitor the evolution of the culture.

! *Storage Information*

Upon receipt, each kit can be stored protected from light at 2-8ºC.

[1] For using CytoCHECK SPAchip® kits in non-confocal imaging systems,

[2] CytoCHECK SPAchip® kits are optimized for its use with adherent cells. Some cells may need a lipofection step to maximize SPAchip internalization. Please contact with A4Cell for further information or questions about your cell model.

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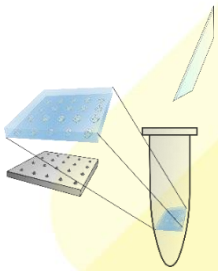
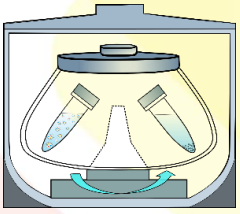
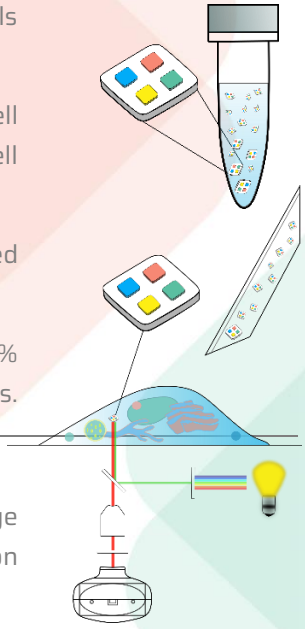
CytoCHECK SPAchip® Calcium Single-Detection Kit Workflow

	SPAchip® Assay Workflow	Notes	
ASSAY SPAchip® Preparation	***Dissolve ASSAY SPAchip® tube***	Thoroughly **dissolve** ASSAY SPAchip® tube solid film in assay buffer (vortexing might be required)	
	Wash with Assay Buffer (x2)	**Centrifugate** tubes at. 4300xg for 10 min. Discard supernatant and keep SPAchip® containing pellet. **Resuspend** the SPAchips in 1mL ASSAY Buffer **Repeat x2**.	
	Resuspend	**Resuspend** the ASSAY SPAchips in 100 µL ASSAY Buffer to obtain a stock solution of $2.5x10^4$ SPAchips/µL	
	Store	*ASSAY* SPAchip® stock solution can be **stored** protected from light at 4ºC for up to 6 months.	
Cell Assay	***Cell Culture***	Seed cells in a multi-well plate. Incubate the cells up to 50-70% confluence[3].	
	SPAchip Dilution	**Dilute** *CONTROL* and *ASSAY SPAchips* in cell culture medium to obtain a final SPAchip® to cell ratio of 2:1.	
	Addition to Cell Culture	**Aspire** the cell medium and add SPAchip®-loaded fresh culture medium.	
	Incubation	**Incubate** overnight in a cell incubator (37ºC, 5% CO_2) to allow SPAchip® internalization by the cells.	
Fluorescence Quantification	***Data Acquisition***	Use green fluorescence channel to image SPAchip® detection (excitation 488 nm, emission 520 nm)	
	Data Analysis		

[3] This cell culture protocol only provides a guideline, and it should be modified depending on specific needs. Please, read the whole protocol before starting.

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2. Contents and Storage

Each CytoCHECK SPAchip® Calcium Single-Detection Kit contains:

Materials		Units
ASSAY SPAchip® tube (embedded in a solid fluorescence-protective soluble film)	~2.5x10^6 ASSAY SPAchips	1
ASSAY buffer tube (Sterile, cell culture suitable)	5 mL	1
CONTROL SPAchip® tube (non-fluorescent, ready-to-use)	~2.5x10^5 CONTROL SPAchips/100 uL	1

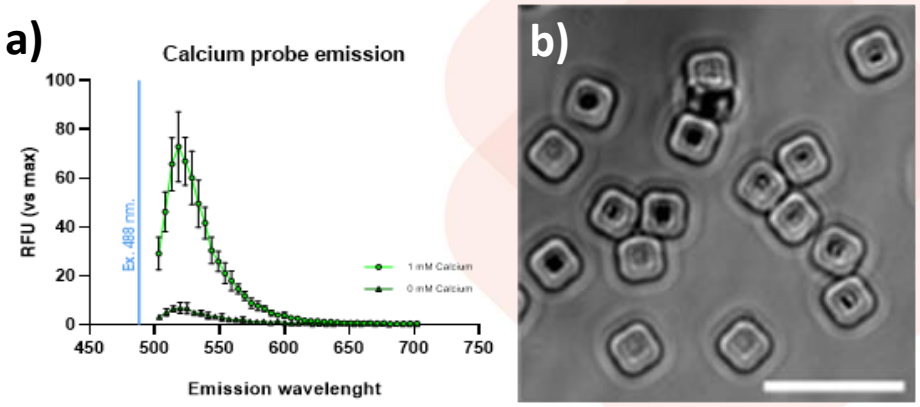

***Figure 1**: **a)** Emission spectra of Calcium--sensing probes when excited at the indicated wavelength. **b)** Brightfield image of SPAchips once in solution (Image from Torras et al. 2015 DOI: 10.1002/adma.201504164) Scale bar = 10 μm.*

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3. Materials to be Supplied by the User

- Pipettes and pipette tips (1-10 µL, 2-20 µL, 20-200 µL, 200-1000 µL)
- Multi-channel pipettes and pipette tips (50-300 µL) (not mandatory but desirable)
- Vortex mixer
- Mini-centrifuge
- Reagent reservoirs
- Cell culture plate (multi-well)
- Cell culture facilities
- Cell culture media (phenol red free is recommended)
- Cell imaging system (HCS/HCS, Confocal microscope, imaging plate reader, etc...). At least 20x magnification objectives are required for quantitative analysis.
- Image analysis software (Contact A4Cell Staff for support)

If quantitative analysis is required, it will be necessary:

- Calcium flux positive and negative controls (for example, A23187 and BAPTA-AM ...)

4. Assay Procedure

NOTE: Work under sterile conditions. Protect the tubes from light, specially from UV light.

a. ASSAY SPAchip® Preparation

1- Add 1 mL of assay buffer to ASSAY SPAchip® tubes and mix until complete solubilization of the membrane (vortexing required)

2- Centrifugate the tubes at approx. 4300 xg (8000 RPM in a 6-cm minispin rotor) for 10 minutes.

3-Carefully, aspire and discard supernatant and wash pellets with 1 mL of solubilization medium. Repeat steps 1 and 2.

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4-Carefully, aspire and discard supernatant (**Look out not to aspire the pellet!!!**). Resuspend the pellet in **100 µL of assay buffer** to obtain a SPAchip® stock solution. This results in approximately **2.5x10⁴ SPAchips/µl**. Once prepared, Assay SPAchip® *tube* can be stored at 2-8ºC protected from light for up to 6 months.

b. ASSAY PROTOCOL (standard protocol for 96-well plate with adherent cells)[4]:

NOTE: ASSAY and CONTROL SPAchip® dilutions suggested in this section are suggested for 96-well plates. Optimization might be required for different cell types or multiwell plates.

1- Seed cells of interest in a multi-well plate following standard protocols. (See an example of plate template in Figure 2)

2- Incubate the cells until 50-70% confluence is reached, approximately 10^4 cells/well (times may vary depending on the cell type).

3- Prepare a 1:100 dilution of the non-fluorescent CONTROL SPAchips in cell culture medium. Mix thoroughly (vortex). Do not spin the tubes.

4-Dilute ASSAY SPAchip® stock solution in cell culture medium to obtain a final SPAchip® to cell ratio of 2:1. Alternatively, simply calculate 1 µL of stock per assay well and dilute 1:100 in cell culture medium. Vortex the ASSAY SPAchip® tube right before using it. Pipette up-and-down twice to homogenize the ASSAY SPAchip® solution and do not spin the tube.

! Optimization may be required depending on the cell type and assay conditions.

5- In the multi-well plate, aspire the cell medium and add 100 µL of CONTROL SPAchip® dilution to the control wells (see Figure 2). Vortex the tube right before use. Add 100 µL ASSAY SPAchip®-containing fresh culture medium per well. Homogenize the solution by pipetting up and down often.

6- Incubate overnight in a cell incubator to let the cells to internalize the SPAchip®. Internalization rate may vary depending on the cell subtype but should be over 25%.

[4] For cells resistant to SPAchips internalization, lipofection may be necessary. Please, contact A4Cell for further information.

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7- To include reference values, use some wells of the plate for calibrating the system (controls, calcium ionophores and/or calcium sequestrant such as Br-A23187 or BAPTA-AM in Figure 2). In such case, follow calibrator manufacturer's instructions.

8- Perform the experiment with your read-out platform. For long-time multiple-measurements assay (for example, monitorization during a week or month), keep the plate in proper conditions between each measurement and change the medium every 24-48 hours, depending on the cell subtype.

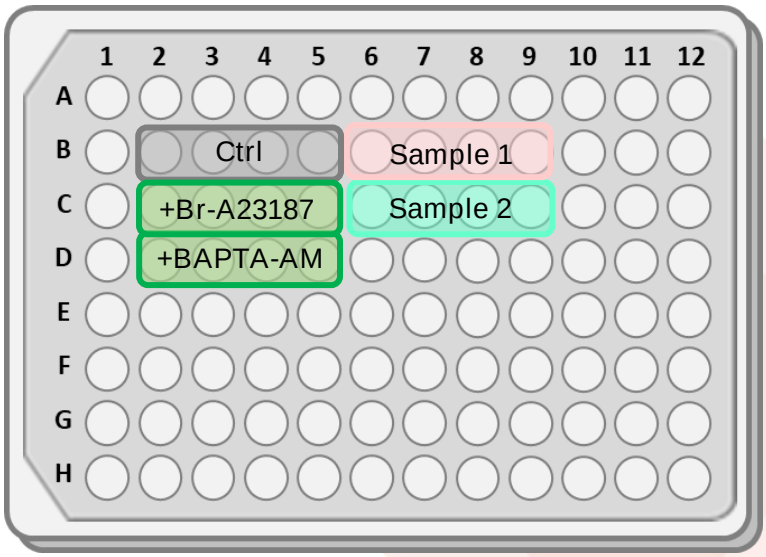

Figure 2: Example of 96-well plate configuration with positive controls and calibrators

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c. SPAchip® Data Collection

- *Image Acquisition*:

1- Use transmitted light to localize at least three fields of interest per well (if using fluorescent light sources use long-wavelength light -561nm and over- in order to avoid photobleaching of the calcium-sensitive probes).

2- Use z-stacks to capture and set the best focus and the z-position of each SPAchip®. In confocal systems, reflected light can be used to visualize the SPAchips and the cells. Brightfield images or cell masking stains are recommended for intracellular quantification.

3- Adjust the acquisition parameters of the test probes: Ex 488/Em 520 (FITC fluorescence filter or similar) to avoid pixel saturation. If using calibrators, use A23187-treated wells to adjust this value.

4- Launch the experiment and save the images. Do not change settings parameters while acquisition.

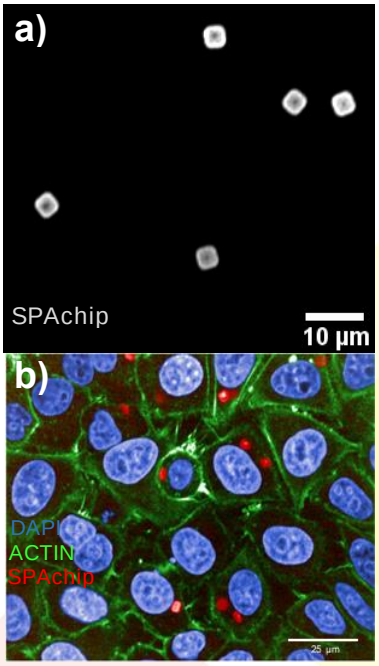

Figure 4: a) Fluorescence signal of SPAchips in an imaging plate with cell culture medium. b) Representative image of HeLa cells with internalized SPAchips (red) stained with DAPI (blue) and phalloidin (green).

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- *Flow Cytometry*:

1- Use FCS and SSC to locate SPAchips and cells independently by their size and complexity.

2- Create fluorescence histograms for green channel. Use cells treated with *CONTROL* SPAchips to set the fluorescence thresholds in both populations (free SPAchips and cells).

3- Analyze a tube of control cells treated with *ASSAY* SPAchips to verify the fluorescence threshold set in step 2 for gate "Cells". Positive cells will appear as a separate peak in the histogram.

d. Quantitative analysis

1- **A)** Image Analysis: Select SPAchip® fluorescent channel signal and measure the intensity of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software.

B) For flow cytometry assays: Analyze each sample with the adjusted gating parameters and quantify green channel detection (mean ± SD or median ± rSD).

2-Quantify the mean fluorescence of extra and intracellular SPAchip® populations.

3- Export your data to a spreadsheet software.

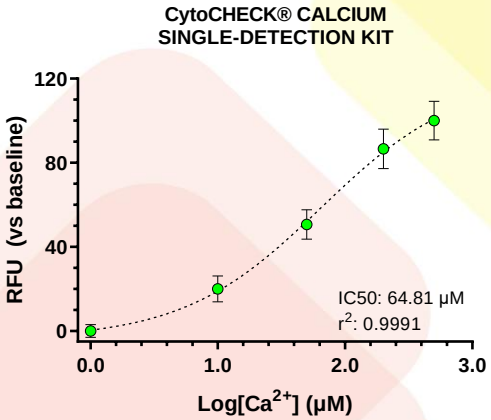

Figure 5: Graph showing normalized relative fluorescence intensity values of CytoCHECK SPAchip® Calcium Single Detection at different calcium concentration conditions.

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