



CytoCHECK SPAchip™ Calcium *single*-Detection Kit

Instructions for Use of Product: CCS-003CT

Research Use Only
MADE IN SPAIN

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CytoCHECK SPChip™ Calcium single-Detection Kit

All technical literature is available on the Internet at www.a4cell.com

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Product Reference: 77G!\$\$' 7H'

For use with: Cell imaging platforms (confocal microscope, HCS/HCA readers are recommended) and flow cytometers.

1. General Introduction

CytoCHECK SPChip™ assay kits are novel fluorescence assays developed by A4Cell that brings together the fields of nanotechnology and cell biology. CytoCHECK SPChip™ are composed of fluorescently labeled silicon microparticles -SPChips®- that can be internalized in the cytosol of cultured cells and monitor changes for long periods of time.

CytoCHECK SPChip™ Calcium Single-Detection Kit allows measurement of intracellular free Calcium levels by changes in fluorescence intensity, which allows a more comprehensive study of the living single-cell physiology and maximizes the performance of most of imaging analyzers.

CytoCHECK SPChip™ Single-Detection kits are optimized for its use with microscopes and HCS/HCA analyzers with confocal capability and 20X or over magnification objectives, yet epi-fluorescence microscopes and imaging system with fixed wavelength filters can be also used¹. Flow cytometers have additionally been validated to analyze intra and extracellular SPChips.

SPChips are used as common fluorophores and chemical probes for cell biology. After resuspending them in the assay buffer, a SPChip® to cell ratio of 2:1 is recommended; or simply add 1 µl per well to the cells in a standard 96-well plate and incubate overnight to allow SPChip® to be incorporated in the cytosol². SPChip® will remain in the cytosol for longer than one month to monitor the evolution of the culture.

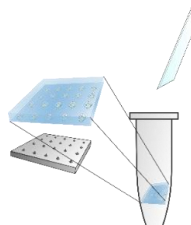
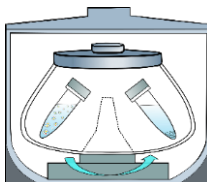
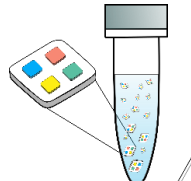
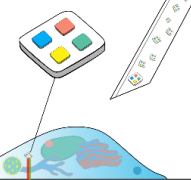
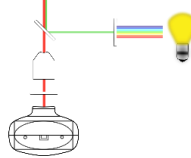
! Storage Information

Upon receipt, each kit can be stored protected from light at 2-6°C. If preferred, for saving room in the fridge or cold-room, components can be stored separately. In this case, the multi-well plate can be stored at room temperature and reagent tubes (ASSAY buffer and SPChips®) at 2-6°C protected from light.

¹ For using CytoCHECK SPChip™ kits in non-confocal imaging systems,

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

2. CytoCHECK SPChip™ Calcium Single Detection Kit Workflow:

	SPChip® Assay Workflow	Notes	
ASSAY SPChip® Preparation	Dissolve ASSAY SPChip® tube	Thoroughly dissolve ASSAY SPChip® 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 SPChip®-containing pellet.	
	Resuspend	Resuspend the SPChips® in 1mL ASSAY Buffer	
	Store	Repeat x2. Resuspend the ASSAY SPChips® in 100 µL ASSAY Buffer to obtain a stock solution of 2.5x10 ⁴ SPChips®/µL ASSAY SPChips® stock solution can be stored protected from light at 4°C for at least 6 months.	
Cell Assay	Cell Culture	Seed cells in the multi-well plate provided with the kit. Incubate the cells up to 70-80% confluence ¹ .	
	SPChip Dilution	Dilute CONTROL and ASSAY SPChips® in cell culture medium to obtain a final SPChip to cell ratio of 2:1.	
	Addition to Cell Culture	Aspire the cell medium and add SPChip®-loaded fresh culture medium.	
	Incubation	Incubate overnight in a cell incubator (37°C, 5% CO ₂) to allow SPChip® internalization by the cells.	
Fluorescence Quantification	Data Acquisition	Use green fluorescence channel to image SPChip® detection (Excitation 488 nm, Emission 520 nm)	
	Data Analysis		

¹ This cell culture protocol only provides a guideline, and it should be modified depending on specific needs. Please, read the whole protocol before starting.

3. Contents and Storage (CytoCHECK SPACHIP™ Calcium Single Detection)

Each CytoCHECK SPACHIP™ Calcium Single-Detection Kit contains:

Materials		Units
Sterile ASSAY buffer tube	3 mL	1
CONTROL SPACHIPS® tube (non-fluorescent, ready to use)	~10 ⁴ CONTROL SPACHIPS®/4 uL	1
ASSAY SPACHIPS® tube (embedded in a solid fluorescence-protective soluble film)	~2.5x10 ⁶ ASSAY SPACHIPS®	1

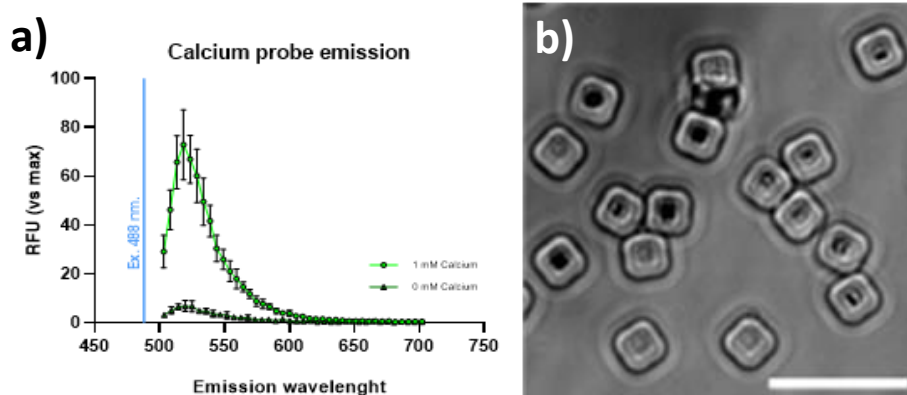


Figure 1: a) Emission spectra of Calcium--sensing probes when excited at the indicated wavelength. b) Brightfield image of SPACHIPS® once in solution (Image from Torras et al. 2015 DOI: 10.1002/adma.201504164) Scale bar = 10 μ m.

4. Materials to Be Supplied by the User

- Pipettes and pipette tips (1-10 μ L, 2-20 μ L, 20-200 μ L, 200-1000 μ L)
- Multi-channel pipettes and pipette tips (50-300 μ L) (not mandatory but desirable)
- Vortex mixer
- Mini-centrifuge
- Reagent reservoirs
- 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 ...)

5. Assay procedure (CytoCHECK SPACHIP™ Calcium Single Detection Kit: 77 G!\$\$' 7 H'

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

A. ASSAY SPACHIPS® preparation

- 1- Add 1 mL of assay buffer to ASSAY SPACHIPS® tubes and mix until complete solubilization of the membrane (vortexing is desirable).
- 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.
- 4- Carefully, aspire and discard supernatant (**Look out not to aspire the pellet!!!**). Resuspend the pellet in **100 μ L of assay buffer** to obtain a SPACHIP® stock solution. This results in approximately **2.5×10^4 SPACHIPS®/ μ L**. Once prepared, ASSAY SPACHIPS® can be stored at 2-6°C protected from light for up to 6 months.

B. Assay protocol (standard protocol for 96-well plate)³:

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

1- Seed cells of interest in the multiwell plate provided with the kit following standard protocols. (See an example of plate template in figure 3)

2- Incubate the cells until 70-80% confluence is reached, approximately 10⁴ 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 SPACHIPS[®]* stock solution in cell culture medium to obtain a final *SPACHIP[®]* to cell ratio of 2:1. Alternatively, simply calculate 1 uL of stock per assay well and dilute 1:100 in cell culture medium. Vortex the *ASSAY SPACHIPS[®]* tube right before using it. Pipette up-and-down twice to homogenize the *ASSAY SPACHIPS[®]* 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 SPACHIPS[®]* dilution to the control wells C1 and C2. 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 *SPACHIPS[®]*. Internalization rate may vary depending on the cell subtype but should be over 25%.

7- If quantification is needed, use the first column of the plate for calibrating the system (controls and Calcium flux calibrators, see example of plate template below (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.

³ For cells resistant to SPACHIPS internalization, lipofection may be necessary. Please, contact A4Cell for further information.

	CONTROL SPAchips 1	Sample 1.1	Etc...								
	CONTROL SPAchips 2	Sample 1.2									
	+A23187.1	Sample 2.1									
	+A23187.1	Sample 2.2									
	+BAPTA-AM 1	Sample 3.1									
	+BAPTA-AM 2	Sample 3.2									

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

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.

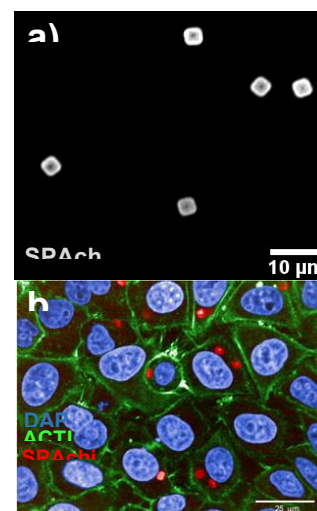


Figure 4: a) Fluorescence signal of SPAchips® in an imaging plate with cell culture medium. b) Representative image of HeLa cells with internalized SPAchips®.

- Flow Cytometry Analysis:

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.

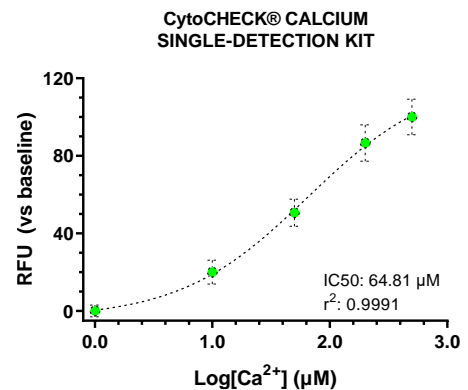


Figure 5: Graph showing normalized relative fluorescence intensity values of intracellular *CytoCHECK SPAchip*™ Calcium Single Detection at different Calcium concentrations conditions.