CytoTRACK SPAchip®
pH DUAL-DETECTION KIT

Instructions for Use
Product Reference: D-001-PHGR

For use with flow cytometers and cell imaging platforms
For research use only

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General Introduction

SPAchip® based assay kits are novel cell-based assays for living single-cell developed by A4Cell that bring together the fields of nanotechnology and cell biology. CytoCKECK 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.

CytoTRACK SPAchip® pH Dual-Detection Kit is the combination of the two technologies addressed to measure intracellular pH: CytoCHECK SPAchip® pH red single-detection and CytoCHECK SPAchip® pH green single-detection. The product combines these two pH detection technologies so that each of them is internalized in one cell line for co-culturing applications. While tracking the two different cell subtypes and their interaction, measurements of intracellular pH levels by changes in fluorescence intensity are feasible at real time. The advantages of this product are the combination of our two CytoCHECK pH detection kits, where the selection of the SPAchip® to the corresponding cell line will depend on the features of each. Red emission SPAchip® is recommended for applications which need to overcome high green autofluorescence signals and increase SNR. Green emission SPAchip® advantages are its easiness due to the possibility of using common GFP fluorescence filters and its easy linear response.

CytoTRACK SPAchip® pH Dual-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.

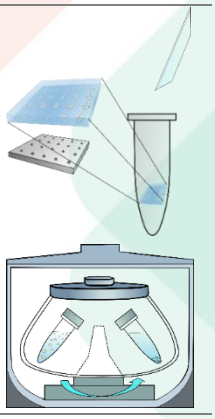
SPAchip® kits can be handled as common fluorophores and chemical probes for cell biology. After resuspending them in the assay buffer (a SPAchip®-to-cell ratio of 2:1 is recommended), an overnight incubation is required to allow SPAchip® to be incorporated in the cytosol[2]. SPAchip® will remain in the 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 2-8°C.

CytoTRACK SPAchip® Dual-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).
	↓	**Centrifugate** tubes at 4300xg for 10 min.
	Wash with Assay Buffer (x2)	Discard supernatant and keep SPAchip® containing pellet. **Resuspend** the SPAchips in 1mL ASSAY Buffer. **Repeat x2**.
	↓	**Resuspend** the ASSAY SPAchips in 100 µL ASSAY Buffer to obtain a stock solution of approximately 2.5x10⁴ SPAchips/µL.
	Resuspend	ASSAY SPAchip® stock solution can be **stored** protected from light at 4°C for up to 6 months.
	↓	
	Store	



[1] For using CytoTRACK SPAchip® kits in non-confocal imaging systems.
[2] CytoTRACK 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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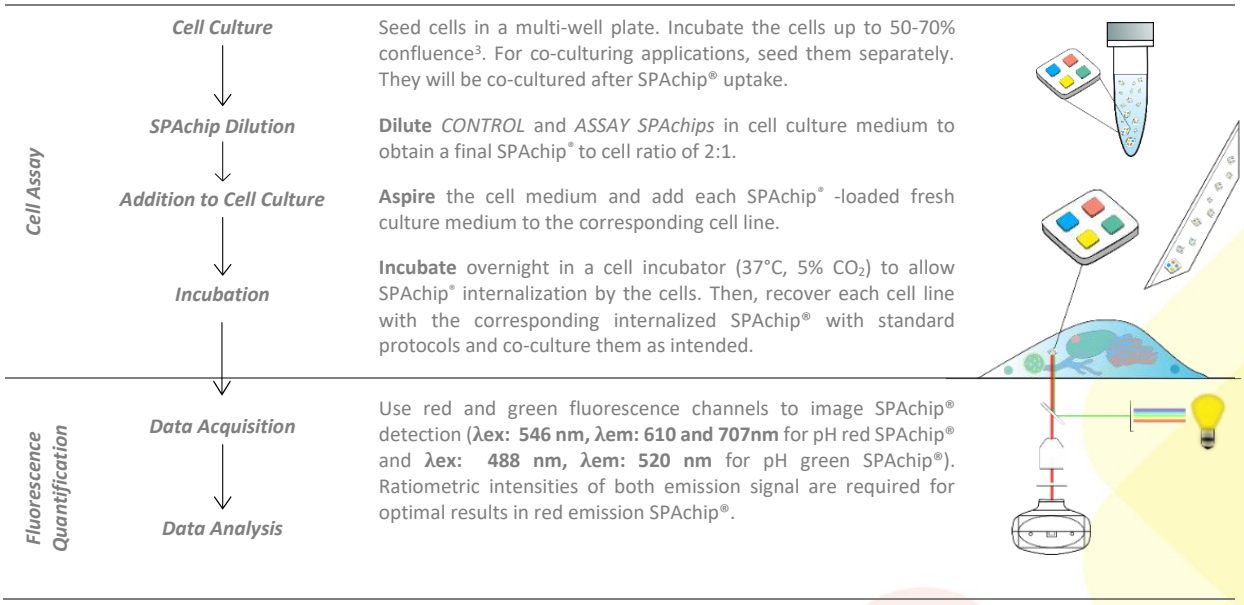

1. Contents and Storage

Each CytoTRACK SPAchip® pH Dual-Detection Kit contains:

Materials		Units
ASSAY green SPAchip® tube (embedded in a solid fluorescence-protective soluble film)	~2.5x10^6 ASSAY SPAchips	1
ASSAY red SPAchip® tube (embedded in a solid fluorescence-protective soluble film)	~2.5x10^6 ASSAY SPAchips	1
ASSAY buffer tube (Sterile, cell culture suitable)	8 mL	1
CONTROL SPAchip® tube (non-fluorescent, ready-to-use)	~5x10^5 CONTROL SPAchips/200 µL	1

*2.*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)

[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.

- 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:
- Intracellular pH calibrators

3. 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 each ASSAY SPAchip® tube and mix until complete solubilization of the membrane (vortexing required).

2-Centrifugate the tubes at approximately 4300xg (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.

4-Repeat steps 1 and 2.

5-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 **$2.5x10^7$ SPAchips/ml**. Once prepared, Assay SPAchip® tube can be stored at 2-8°C 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 for chips internalization in a multi-well plate following standard protocols. See an example of plate template in Figure 1: *ctrl SPAchips* are non-fluorescent chips; rows C, D, E, F are intracellular pH calibration wells where measurements should be interpolated; *cell line 1* and *2* are the cells for the desired tracking which will be seeded after SPAchips internalization in wells *sample 1* and *2* creating the co-culture.

2-Incubate the cells for SPAchips internalization 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 the different ASSAY SPAchip® stock solutions in each 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.

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

5- In the multi-well plate, aspire the cell medium and add 100 µL of CONTROL SPAchip® dilution to the control wells (Figure 1). Vortex the tube right before use. Add 100 µL of ASSAY SPAchip®-containing fresh culture medium per well to the corresponding cell line (*cell line 1* and *2* in the example). Homogenize the solution by pipetting up and down often.

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

7-Recover the cells with standard protocols and co-culture them in wells *sample 1* and *2* (Figure 1). These wells contain the co-cultured cells with internalized chips for correct tracking of each cell subtype and pH measurements of interest.

8- If quantification of the measurements is needed, use some wells of the plate for calibrating the system (see example of plate template below in Figure 1: controls and intracellular pH calibrators in columns 1- 4). In such case, follow calibrator manufacturer's instructions.

9- 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.

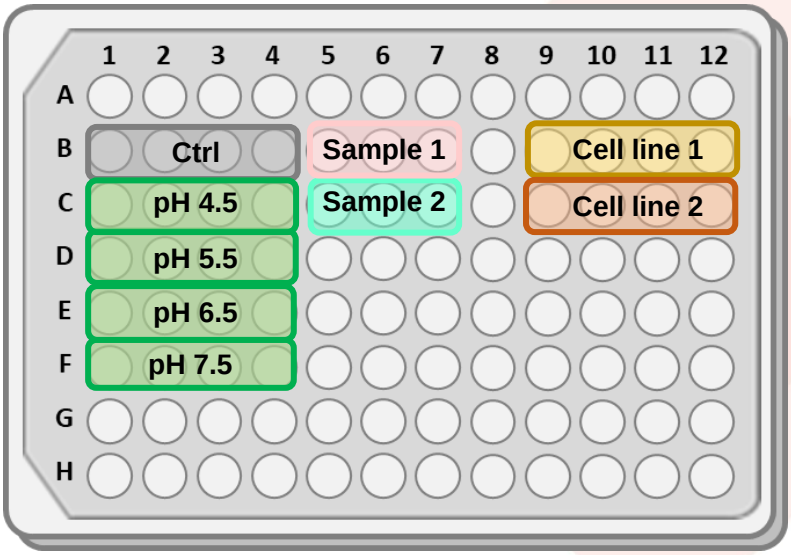

Figure 1: Example of 96-well plate configuration with controls and calibrators.

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c. SPAchip® Data Collection

- *Image Acquisition*:

1- Use transmitted light to localize at least three fields of interest per well.

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: Excitation with 546 nm and emission with 610 and 707 nm for red SPAchip® and excitation with 488 nm and emission with 520 for green SPAchip®. Check acquisition parameters are adjusted to all emission wavelengths.

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

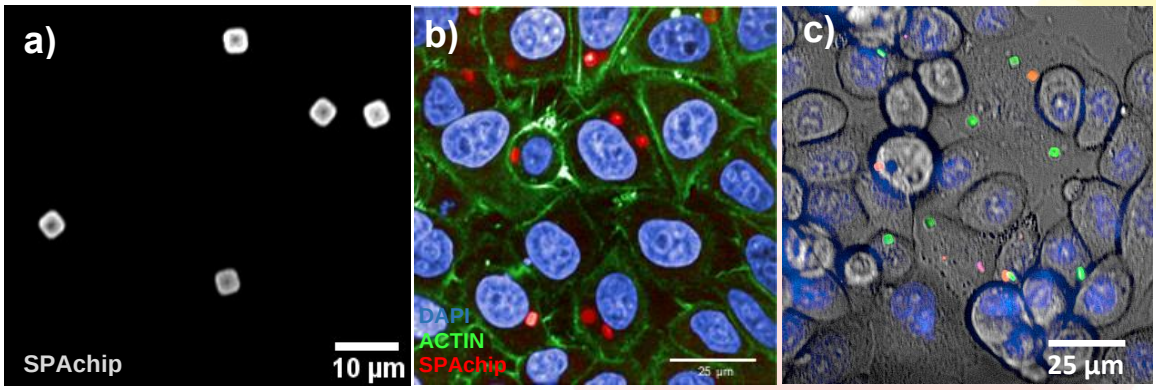

Figure 2: **a**) Fluorescence signal of SPAchips in an imaging plate with cell culture medium. **b**) Representative image of HeLa cells with internalized SPAchips (red) stained with DAPI (blue) and phalloidin (green). **c**) Representative brightfield image of a co-cultured with HeLa cells (red SPAchip®) and Cal-51 (green SPAchip®) and HOECHST tinction.

- *Flow Cytometry*:

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

2- Create fluorescence histograms for red and green channels. 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 the two SPAchip® fluorescent channel signals (emission at 610 and 707 nm for red SPAchip® and emission at 520 nm for green SPAchip®) and measure the intensities of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software. Afterwards, only ratio of red fluorophore measurements can be taken to increase signal to noise ratio.

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

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

3- Export your data to a spreadsheet software.

4-To quantify intracellular pH, use the fluorescence of the calibration wells to plot ratiometric or mean fluorescence intensity (MFI) vs calibration pH as a control curve. Interpolate the values of the sample wells which fits to a sigmoidal curve in case of red SPAchips (Figure 3A) or a line in the case of green SPAchips (Figure 3B). It is important to interpolate sample values in the intracellular calibration curves instead of extracellular media.

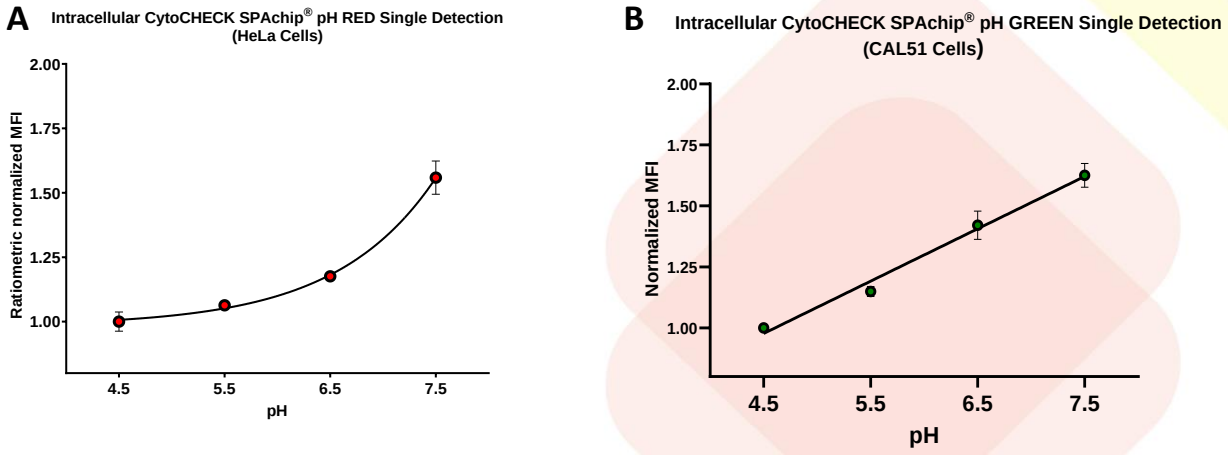

Figure 3: Red and green CytoTRACK® pH Detection KIT at different pH conditions using commercial calibrators inside cells. A) Graph showing ratiometric normalized fluorescence intensity values of intracellular CytoCHECK SPAchip® pH RED Single Detection at different pH conditions using intracellular calibrators in HeLa cells. Ratiometric values were obtained by dividing λem2=707/ λem1=610 nm emission signals in HCS-Operetta equipment with the excitation in the range λex=546/15 nm. B) Graph showing normalized relative fluorescence intensity values of intracellular CytoCHECK SPAchip® pH GREEN Single Detection at different pH conditions using intracellular calibrators in CAL51 cells.