CytoCHECK SPAchip®

pH RED SINGLE-DETECTION KIT

Instructions for Use

Product Reference: S-001-PHR

For use with flow cytometers and cell imaging platforms
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 RED SPAchip® pH Single-Detection Kit allows measurement of intracellular pH levels by changes in fluorescence intensity, which allows a more comprehensive study of the living single-cell physiology and maximizes the performance of most imaging analyzers. Its signal in red makes it feasible for applications with high green autofluorescence such as organoids cultures. Moreover, due to its ratiometric behaviour, signal to noise ratio is increased leading to more precise curves for interpolation.

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® pH RED 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 approximately $2.5x10^4$ 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 red fluorescence channels to image SPAchip® detection (λex: 546 nm, λem: 610 and 707 nm). Ratiometric intensities of both emission signals are required for optimal results.
	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® pH 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

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:

- Intracellular pH calibrators

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.

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 1: *ctrl* wells are non-fluorescent SPAchips; *pH 4.5*, *5.5*, *6.5* and *7.5* are intracellular calibration wells where measurements should be interpolated; *sample 1* and *2* are the measurements of interest whose values should be interpreted according to the calibrators.

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

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

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 a 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 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%.

7- If quantification is needed, use some wells of the plate for calibrating the system (controls and intracellular pH calibrators in the columns 1-4, see example of plate template below in Figure 1). 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.

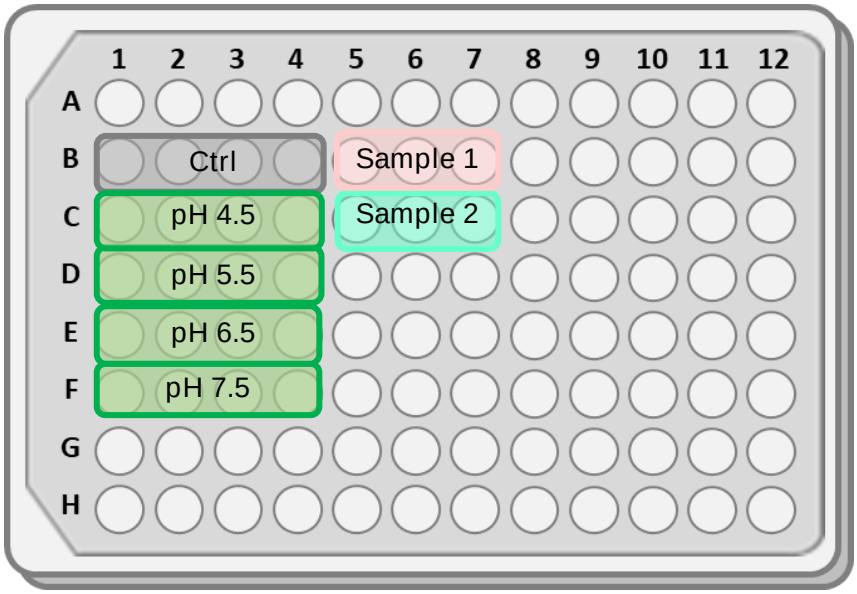

Figure 1: Example of 96-well plate configuration with positive controls and calibrators.

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c. SPAchip® Data Collection

- *Image Acquisition*:

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

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 to avoid pixel saturation. As a ratiometric fluorophore, check the acquisition parameters are adjusted in both emission wavelengths.

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

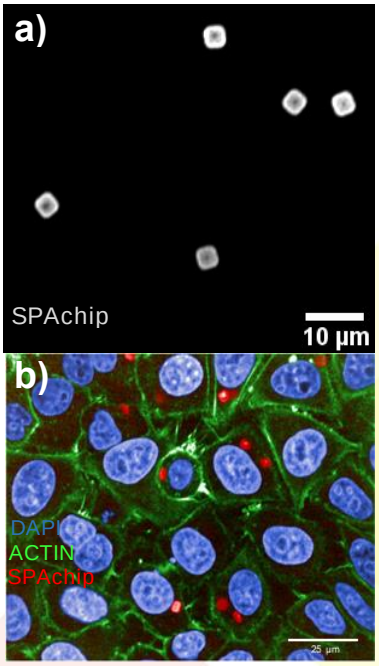

Figure 2: a) Fluorescence signal of SPAchips in an imaging plate with cell culture medium. b) Representative image of HeLa cells with internalized SPAchips (red) stained with DAPI (blue) and phalloidin (green).

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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 red 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 580 and 665nm) and measure the intensities of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software. Afterwards, ratio of these 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 fluorescence units vs calibration pH as a control curve. Interpolate the values of the sample wells which fits to a sigmoidal curve (Figure 3B) to obtain the intracellular pH value of the sample wells. It is important to interpolate sample values in the intracellular calibration curves instead of extracellular media.

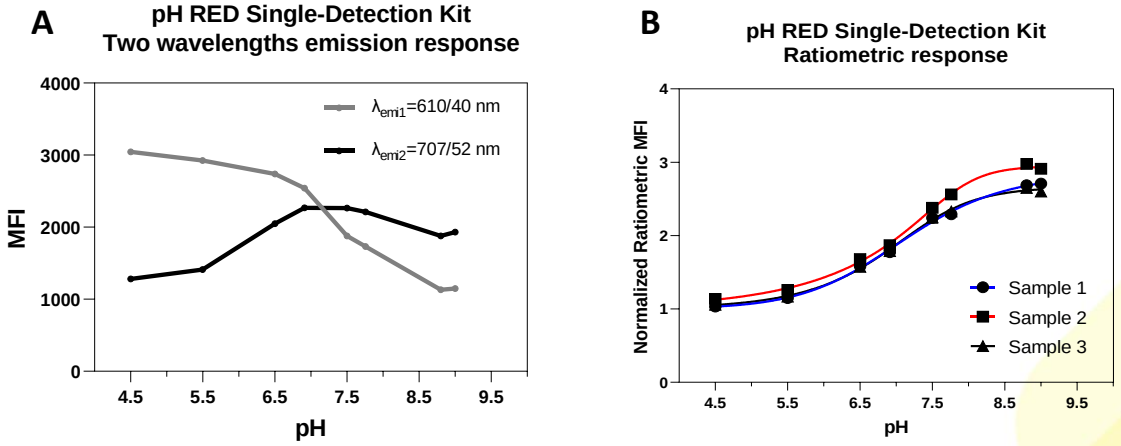

Figure 3: CytoCHECK SPAchip® pH RED Single Detection at different pH conditions using commercial calibrators. A) Graph showing emission signal at two different wavelengths for pH 4.5 – 9.0. B) Graph showing ratiometric normalized fluorescence intensity values at different pH. Ratiometric values were obtained by dividing λ_{emi2}/ λ_{emi1} emission signals in HCS-Operetta equipment with the excitation in the range λ_{exc}=545/15 nm.

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