spectragenetics

Application note KIT-01 series
FAP-based GPCR Internalization Assay Kits

Assay Principle

This cell-based assay detects and quantifies a prevalent measure of GPCR response: agonist-stimulated receptor internalization. It likewise detects and quantifies constitutive internalization, and can be used to detect and quantify antagonist activity on the basis of inhibition of agonist-stimulated internalization.

The assay employs a fluorogen-activating protein tag (FAP-tag) that is fused to the receptor's extracellular N-terminus. The FAP binds malachite green, a non-fluorescent fluorogen dye that acquires strong far-red fluorescence upon binding (Perkins and Bruchez, 2020).

The assay uses a special fluorogen molecule (MSSC) comprised of a malachite green moiety joined to a Cy3 moiety by a short linker that contains a reduction-sensitive disulfide bond.

HEK 293 cells are seeded in microwells into which transfection-ready vector DNA has been printed. As the cells attach and spread, they take up the DNA and express FAP-tagged receptors.

To begin the assay, MSSC is added to each test well. The MSSC binds to FAP-tagged receptors at the cell surface, yielding:

- Far-red 'direct fluorescence' when the malachite green moiety is excited by red light.
- Far-red 'FRET fluorescence' when the Cy3 moiety is excited by green light.

Receptors that are not on the surface do not become fluorescent because MSSC does not cross the plasma membrane.

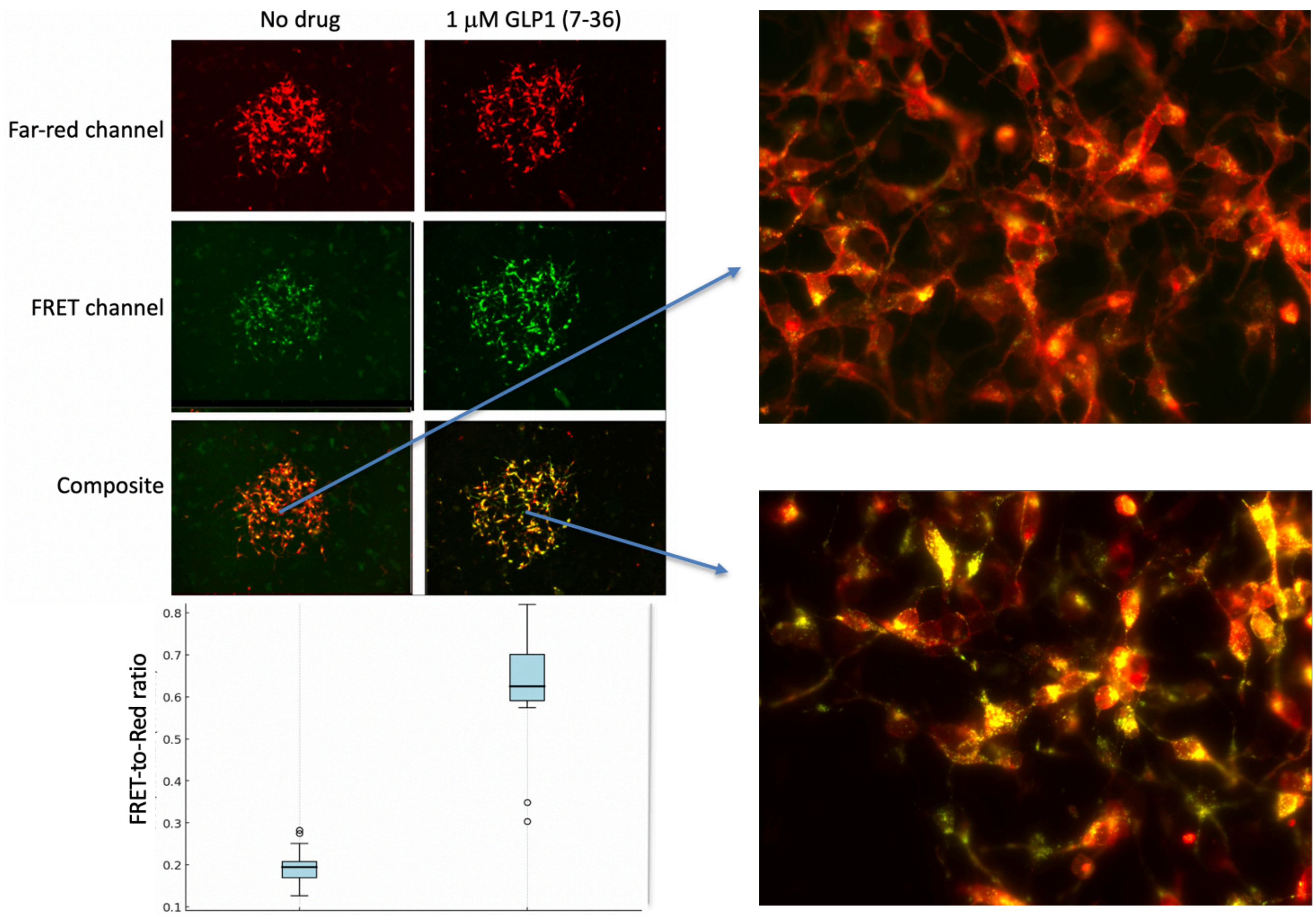
In a typical experiment, candidate agonists are added to some of the wells along with fluorogen, while control wells receive just the fluorogen. After a number of minutes (usually 20 - 40) the membrane-impermeant reducing agent TCEP is added. This eliminates FRET signal from surface-exposed receptors, but does not affect FRET from internalized receptors.

Cells are then fixed and the wells are imaged by fluorescence microscopy, with direct fluorescence displayed in red and FRET fluorescence displayed in green. Because FRET signal comes only from internalized receptors, the ratio of FRET to red signal reflects the extent of receptor internalization.

Example assay results for human glucagon like peptide receptor (GLP1R).

Direct fluorescence is shown in red, FRET in green. Stimulation time was 20 minutes.

In the composite images, green and red combine to give orange or yellow. The more yellow the color, the greater the extent of internalization of the receptor.

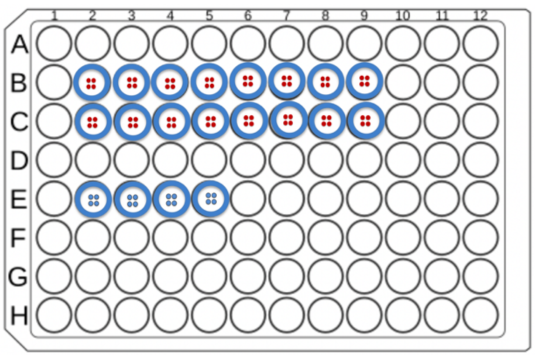


Contents of Kit

Each kit provides all materials and reagents needed for the assay. 16 wells support expression of your GPCR, and 4 wells support expression a positive control receptor (ADRB2).

- **Black 96-well test-plate** with lipid nanoparticles/vector DNA printed in 20 wells. Store test plate at minus 20° C or at room temperature.
- **White 96 well source-plate** with 40 µL medium in 20 wells. Store source plate at minus 20° C.
- **Cryovial** with frozen HEK 293 cells. Store at minus 80° C.
- Growth medium: Optimem / 5% FBS. 2 mL. **Green vial**. Store at minus 20° C.
- Reducing agent: 250 mM TCEP in water. 1 mL. **Yellow vial**. Store at minus 20° C.
- Fixing agent: 12 % paraformaldehyde (PFA) in water. 1 mL. **Red vial**. Store at minus 20°C
- PBS: 2 mL **White vial**. Store at minus 20° C or 4° C.
- Foil sealing sheet for storage of test-plate
- Box of large-bore 200 µL pipet tips

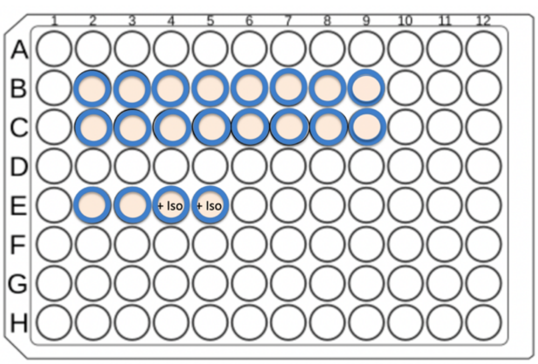
Schematic of test-plate (black plate)



Lipid nanoparticle spots are printed in 20 wells, four spots to a well, as shown.

Red spots: Receptor of interest. 16 wells
Blue spots: ADRB2 control. 4 wells.

Schematic of source-plate (white plate)



All wells contain 40 µL Optimem with 400 nM MSSC.

Positive control wells E4 and E5 additionally contain 4 µM isoproterenol, a potent ADRB2 agonist.

Assay Protocol

Day 1 — Cell Seeding

1. Bring the black test-plate and blue vial to room temperature.

2. Thaw the cryovial containing HEK 293 cells.

3. Add the contents of the green vial to the cryovial, mix, and use a large bore pipette tip (supplied) to add 90 µL to each of the 20 printed wells in the black test plate. Take care not to touch the tip to the bottom of the well where the printed spots are.

4. Incubate the plate overnight in a 37°C /CO_2 incubator. Transfection occurs as the cells attach and spread, so do not disturb the plate during this incubation.

Day 2 — Treatment, Reduction, Fixation

1. Before beginning the experiment, bring the yellow, red and white vials, and the white source plate, to room temperature..

2. Remove the foil seal from the source-plate, and load your test compound(s) into one or more of the 16 wells in rows B and C. Reserve at least one well as a negative control well (no test compound).

Add test compounds to the source plate wells. Compound concentration in the wells should be at four times the desired final concentration in the experiment. For example, if your compound stock solution is at 100 µM and you want the final concentration to be 1 µM, you would add 1.6 µL to the 40 µL of medium in the source well, yielding a 4 µM solution.

2. Remove the test plate from the incubator and carefully transfer 30 µL from each source-plate well to its corresponding test-plate well.

Use wide-bore pipette tips (supplied) to transfer the 30 µL. Hold the tip a millimeter or two above the meniscus and dispense the 30 µL so that a single drop falls into the center of the well. This results in effective mixing; no additional mixing manipulations are needed.

3. Return the test-plate to the incubator for the desired internalization time, usually **20 to 30 minutes**.

4. Remove the plate from the incubator and add 30 µL TCEP solution (yellow vial) to each well with a wide bore tip so that a single drop falls into the center of the well. Incubate at room temperature for **60-90 seconds.**

5. Add 30 µL PFA solution (red vial) to each well with a wide bore tip so that a single drop falls into the center of the well. Incubate at room temperature for **15 minutes**.

6. *Gently* remove most of the medium from each well and *gently* replace with 100 µL PBS (white vial).

Plates can be imaged immediately or stored at 4°C for later imaging. For storage times longer than a few days, seal the wells using the provided adhesive sealing foil to prevent evaporation.

Day 2 or Later — Imaging and quantification of receptor internalization

For direct imaging, use a standard **Cy5 filter cube**.
For FRET imaging, use a **Cy3 filter cube** with the **Cy3 emission filter replaced by a Cy5 emission filter** (~670 nm).

Obtain composite images with **direct fluorescence displayed in red** and **FRET fluorescence displayed in green**.

4x or 10x objectives, with their relatively large depth of field, give good output for quantification because surface and internal signal is captured in each image. When imaging, be sure to use identical exposure settings for experimental and control wells, and take care to use a short enough exposure times to avoid much pixel saturation.

Strong internalization responses are apparent from visual inspection of the composite images, as in the example given previously: control spots look red and experimental spots look orange or yellow. For receptors with high levels of constitutive internalization, response to agonist may be less apparent to the eye, yet be evident when green-to-red ratios are quantified. For image quantification, a drag-and-drop image analyzer designed for the assay is available on the SpectraGenetics website.

SpectraGenetics' Image Analyzer

An image analyzer for ratiometric quantification of two-color images is available free of charge to SpectraGenetics' customers.

An example of analyzer output for a control spot and an agonist-stimulated spot is shown below.

Image Analyzer for FAP-based Assays

Drop images here or click to browse
JPEG / PNG only

Selected Files

B2-B.png	51.4 KB	x
G2-B.png	69.0 KB	x

2 files selected

Analyze Images | Clear Results | Clear Files

Detection & Filtering

Min Brightness (Red): 50 Pixels below this threshold are not considered for object detection.

Min Object Size (pixels): 25

Object Mask Display: 3.0 Adjusts how strongly Object Mask colors respond to G:R ratios. For visualization only; does not affect calculated metrics.

Display & Metrics

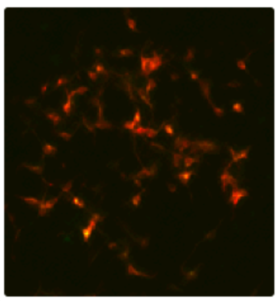
Primary ratio metric: Object-average

Choose between G:R calculated by pooling the red and green values across all objects, or G:R calculated by determining the G:R value for each object and averaging those values.

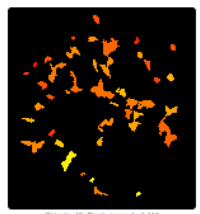
```
Ready.
[INFO] 1:30:42 PM: Added 1 file(s). Total: 1
[INFO] 1:30:49 PM: Added 1 file(s). Total: 2
[INFO] 1:31:06 PM: Starting analysis of 2 image(s)...
[INFO] 1:31:06 PM: Reading B2-B.png...
[INFO] 1:31:06 PM: Reading G2-B.png...
[INFO] 1:31:06 PM: Done.
```

B2-B.png

Original

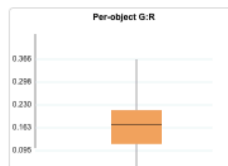


Object Mask (colored by G:R ratio)



Objects: 42; Pixels in mask: 6,416

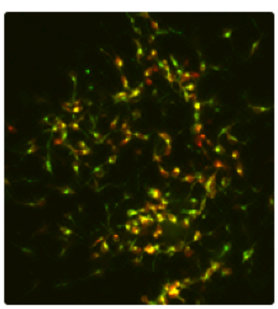
Per-Object Distribution



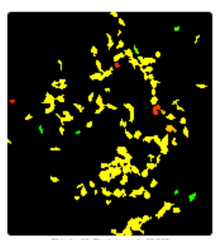
Object-avg G:R ☆ Primary	SD (Object G:R)	Mean Red	Mean Green
0.177	0.085	64.489	11.729

G2-B.png

Original

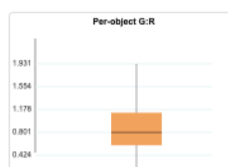


Object Mask (colored by G:R ratio)



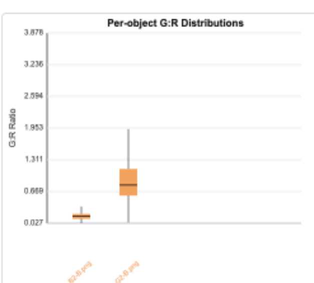
Objects: 66; Pixels in mask: 10,560

Per-Object Distribution



Object-avg G:R ☆ Primary	SD (Object G:R)	Mean Red	Mean Green
0.948	0.626	68.101	55.162

Combined Distributions



References

Perkins LA and MP Bruchez. 2020. Fluorogen activated protein toolset for protein trafficking measurements. Traffic 21: 333-348 PMID: 32080949

Moo EV, JR van Senten, H Brauner-Osborne, T Moller. 2021. Arrestin dependent and independent internalization of G-protein coupled receptors: methods mechanisms and implications on cell signaling. Molec. Pharm. 99:242-255.