

## A Functional Assay for GPR55: Envision Protocol

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### Abstract

AlphaScreen® SureFire® assay is a novel technology that combines luminescent oxygen channeling technology, nano-beads, and monoclonal antibodies to detect the level of a selected protein in a volume lower than 5  $\mu$ l. This method is more sensitive compared with the traditional enzyme-linked immunosorbent assays (ELISA), and can detect an increasing number of new targets. Here, we described a method for AlphaScreen® SureFire® assay that targets ERK1/2 phosphorylation, a primary downstream signaling pathway that conveys activation of GPR55 by L- $\alpha$ -lysophosphatidylinositol (LPI) and certain cannabinoids.

**Key words** AlphaScreen, Enspire, AlphaLISA, GPR55, Phytocannabinoids, High-throughput screening, Cannabinoid receptor

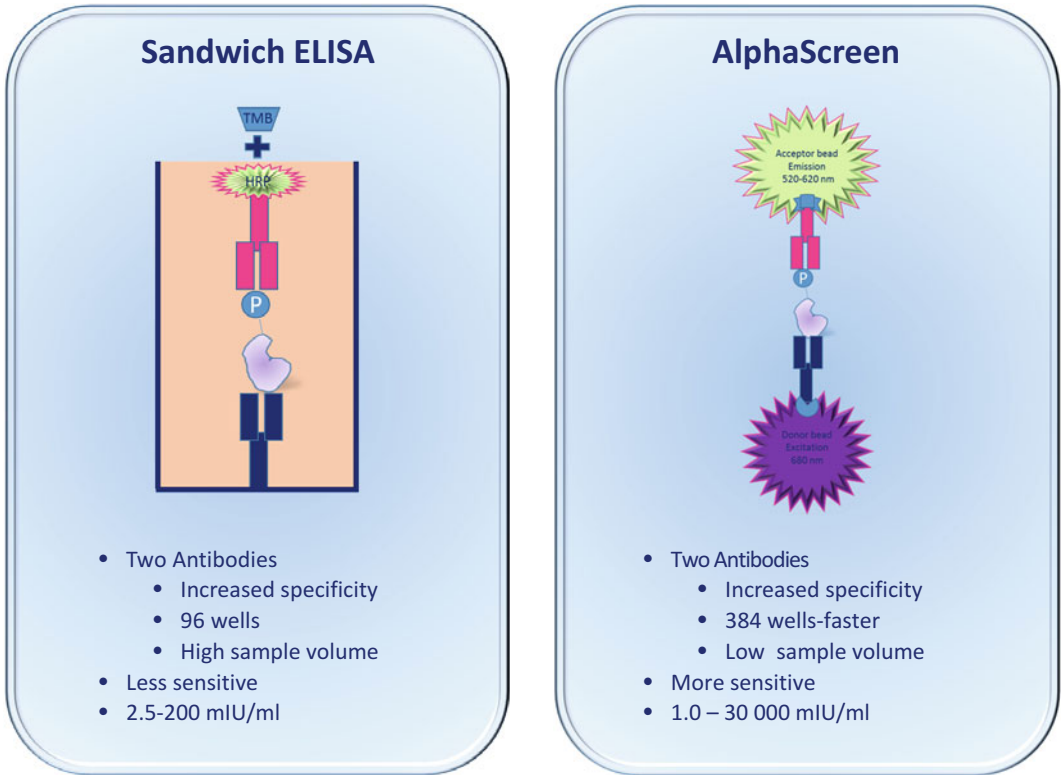
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### 1 Introduction

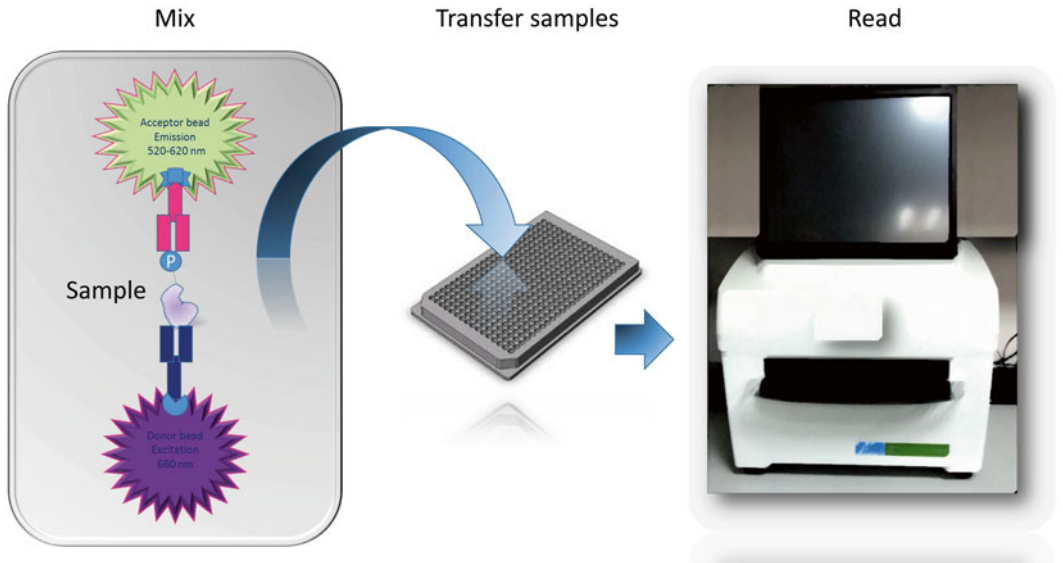
AlphaScreen® SureFire® assay is an alternative technology to the traditional ELISA (Fig. 1).

It is faster and more sensitive than ELISA, requires a micro-quantity of the tested sample, and can be performed in 384 well plates (Fig. 2). This upscale enables the translation to high-throughput screening platforms that employ robotic devices at drug discovery laboratories.

AlphaScreen® SureFire® assay has been applied to study the level of phosphorylated proteins in various fields of research, including GPCR signaling, epigenetics, kinase research, and protein-protein interactions. Using this method, we have recently studied the pharmacological actions of synthetic cannabinoids and phytocannabinoids at the human GPR55 receptor [1]. The latter is activated by the endogenous lipid LPI [1–4], and appears to highly regulate cancer cell function. In selected cancer cells, GPR55 is highly expressed and its activation by LPI increases cell migration, invasion, and proliferation [5–7]. Based on these studies, many resources have been allocated to identify GPR55 inhibitors [1, 8]. Other important



**Fig. 1** Time is money. A comparison between traditional sandwich ELISA and AlphaScreen® SureFire® assays. Note the number of samples, time, and sensitivity that AlphaScreen® SureFire® assay offers



**Fig. 2** Illustration of AlphaScreen® SureFire® technology. Steps of the assay are highlighted

clinical targets for identifying GPR55 inhibitors emerged from studies which showed that activation of GPR55 contributes to inflammation and enhances neuropathic pain [9]. We have identified GPR55 enhancers, inhibitors, and modulators of LPI-induced activation of human GPR55 [1]. Our results also suggest that GPR55 has an allosteric binding pocket and point to a cross-talk between the cellular messengers of NFAT-ERK1/2 signaling cascades [1]. Another example is related to the lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) [10]. The latter has been shown to directly interact with C-reactive protein (CRP) in addition to binding to oxLDL [10]. These findings suggest that CRP, a risk factor for cardiovascular events, promotes endothelial dysfunction and amplifies vascular inflammation by engaging LOX-1.

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## 2 Materials

1. *Drugs.* All compounds were dissolved in DMSO, and 10 mM stocks were kept at  $-20^{\circ}\text{C}$ . LPI was stored at  $-80^{\circ}\text{C}$  for up to 3 months.
2. *Cells.* Untransfected HEK293 cells are grown in Dulbecco's modified Eagle's medium (Gibco) containing 2 mM L-glutamine medium (Gibco), and 10 % fetal bovine serum. Cells are split at a ratio of 1:2 or 1:4, according to the required cell density. For HEK293 cells that stably express the tagged-human GPR55 receptor (hGPR55-HEK293), the GPR55 receptor is tagged with a triple hemagglutinin epitope (HA) at the N terminus (3xHA-GPR55), preceded by the signal sequence from the human growth hormone (HGH; residues 1–33), and is subcloned into pcDNA 3.1 vector. Transfected hGPR55-HEK293 cells are grown in a medium prepared with 500 ml of Dulbecco's modified Eagle's medium/F12 (Gibco), 50 ml of 10 % new born calf serum (Gibco), 5 ml of G-418 (50 mg/ml), 5 ml of L-glutamine (Sigma), and 3 ml of penicillin/streptomycin solution (containing 10,000 units of penicillin (base) and 10,000  $\mu\text{g}/\text{ml}$  of streptomycin from Gibco).
3. *Serum-free Medium.* For transfected cells, add to DMEM/F12 medium 5 ml of G418 and 5 ml of L-glutamine. For untransfected cells, add to DMEM/F12 medium 5 ml of L-glutamine without G418.
4. *Assay Medium.* Prepare serum-free/phenol-free "assay medium" with Dulbecco's modified Eagle's medium/F-12 containing 2 nM L-Glutamine (Gibco).
5. *AlphaScreen® Kit.* AlphaScreen® SureFire™ Phospho-ERK $\frac{1}{2}$  is from PerkinElmer, and its components are listed in Table 1.

**Table 1**  
**Materials supplied with the kit**

Lysis buffer ×5	5 × 2 ml	Store at 4 °C
Activation buffer	1 × 2 ml	Store at room temperature or 4 °C Warm up to 37 °C on assay day
Reaction buffer	2 × 1.7 ml	Store at 4 °C
Control un-stimulated cell lysate	50 µl	Aliquot and store at -20 °C
Control stimulated cell lysate	50 µl	Aliquot and store at -20 °C
Protein A IgG kit		Store at 4 °C in the dark

6. *Positive/Negative Control Lysates*. Lysates are provided with the AlphaScreen® Kit. Reconstitute them with double-distilled water (DDW) according to the manufacturer's instructions, and freeze aliquots at -20 °C.
7. *Plates*. Perform the assay in 384 well white ProxiPlates according to the manufacturer's instructions.

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### 3 Methods

1. *Cell Maintenance*. Add cells to 10 ml of medium, centrifuge for 5 min at 200 × g, remove supernatant, add 10 ml of fresh growth medium, seed in a T75 flask, place in the incubator in a humidified atmosphere at 37 °C and 5 % CO<sub>2</sub>; refresh medium after 24 h.
2. *Plating hGPR55-HEK Cells for Assay*. The following protocol yields about five 96 well plates. Harvest cells from two T75 flasks by washing once with dissociation buffer (from Sigma), add 10 ml of dissociation buffer, collect cells into a 50 ml tube, add 10 ml of growth medium, centrifuge at 200 × g for 3 min, resuspend in 20 ml of growth medium. Count cells, seed 40,000 cells/well in a 96 well plate. Cells should be 100 % confluent on the next day.
3. *Prepare hGPR55-HEK for Assay*. Remove the growth medium from the cells, and replace with 100 µl of serum-free medium. Serum-starve the cells for 48 h. Both media should be warmed up to 37 °C (*see Note 1*).
4. *ERK1/2 MAP-kinase Phosphorylation Assay*

Follow these steps:

1. Design your assay plate (method described below is for a 96 well plate).
2. Warm up the assay medium to 25 °C.

3. Prepare 1× lysis buffer by diluting 1:5 with DDW the lysis buffer ×5, supplied with the kit. For example, mix 1000 ml of lysis buffer with 4000 ml of DDW. Keep on ice (*see* **Note 2**).
4. Prepare drugs at the required test dilutions. Keep the concentrations of the solvent at a constant level throughout the experiment. Typically, final concentrations of 0.1 or 0.2 % DMSO are used.
5. Label plate and remove medium.
6. Wash once with assay medium.
7. Remove medium from cells.
8. Add the tested drugs at the desired volume.
9. Incubate at 37 °C. Typically, to measure LPI-induced ERK1/2 phosphorylation an incubation of 20 min is recommended. However, the time should be selected based on preliminary experiments.
10. Remove medium from cells.
11. Keep the plate on ice.
12. Add 50 µl/well of 1× lysis buffer.
13. Store at –80 °C for at least 1 h (*see* **Note 3**).

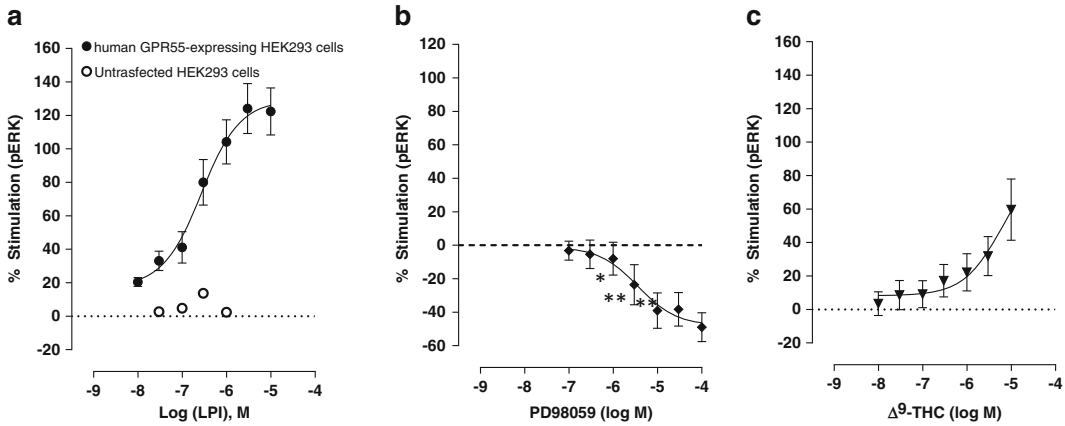
### 3.1 ERK1/2 Phosphorylation Assay

To test the level of ERK1/2 phosphorylation, follow these steps:

1. Warm up Activation buffer from the kit to room temperature.
2. Thaw the assay plate (from the above **step 13**) at room temperature.
3. Transfer 4 µl of the lysed cells from each well into a well of the 384 ProxiPlate.
4. Transfer 4 µl of control lysate (supplied with the kit) into a well of the 384 ProxiPlate.
5. Prepare AlphaScreen® beads-containing ERK1/2 assay mix, as detailed in Table 2.
6. Add 7 µl per well of ERK1/2 assay mix.
7. Seal the ProxiPlate and protect from light.
8. Incubate the ProxiPlate for 3.5 h at 23–25 °C in the dark.

**Table 2**  
**ERK1/2 assay mix**

Assay mix for 100 wells
600 µl of Reaction Buffer
100 µl of Activation Buffer
10 µl of Donor Beads
10 µl of Acceptor Beads



**Fig. 3** (a) Mean log concentration-response curves of LPI effect on ERK1/2 phosphorylation in hGPR55-HEK293 cells after 20 min ( $n=4$  each in triplicate). No significant differences are observed in basal levels of phosphorylated ERK1/2 in untransfected HEK293 cells and hGPR55-HEK293 cells incubated for 20 min in 0.1 % DMSO ( $n=2$  each in duplicate). (b) LPI-induced stimulation of ERK1/2 phosphorylation is attenuated by 10  $\mu$ M PD98059, a MEK1 inhibitor, that significantly reduces basal pERK levels. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by means of one-sample  $t$ -test. (c) Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min stimulation at 37 °C with  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) ( $n=3$ ). Each symbol represents the mean percentage change in bound phosphorylated ERK1/2  $\pm$  SEM over the basal level

9. Incubate plate at room temperature and read with the Envision system (PerkinElmer), by using AlphaScreen® settings.

### 3.2 Data Analysis

Raw data can be presented as “Envision units,” defining basal level (in the presence of vehicle) as zero. Results are presented as means and variability as SEM or 95 % confidence limits (CL) of the percent stimulation of phosphorylated ERK1/2 above the basal level. Data can be analyzed by using nonlinear analysis of log agonist *versus*-response curves with Prism 5.0 program (GraphPad, San Diego, CA) (Fig. 3).

When curves cannot be fitted by nonlinear analysis of log agonist *versus*-response curves, statistical significance of the stimulation can be determined with an unpaired Student’s  $t$ -test at each specific concentration. Results are considered significant only when F-test comparing the variance is not significantly different.

## 4 Notes

1. All media should be warmed up to 37 °C, unless specified otherwise.
2. Portions of the diluted lysis buffer can be stored frozen (–20 °C) for subsequent experiments.
3. Lysates can be frozen (–80 °C) at this stage, to be assayed later.

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