Establishment of GPCR Expression Cell Lines Using SNAP-tag Technology: a Case Example of Urotensin II Receptor

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Abstract: ***Objective:*** G-protein coupled receptors (GPCRs) represent crucial cell surface receptors in the drug discovery process. Here we present a post-translationally labeling strategy that uses SNAP-tag® technology to identify stably transfected GPCRs clones exemplified by the human urotensin II (UII) receptor (UT). ***Materials and methods:*** In this study, we transfected SNAP-UT into HEK293A cells to generate stable cell clones. ***Results:*** In total, 77.5%, 8.6%, 4.7%, and 2.2% of clones had fluorescence intensities that were 2-, 4-, 8-, and 16-fold higher than the control, respectively. In the recombinant clones, the fluorescence intensities were parallel well with the RNA levels of UT. For example, the fluorescence intensity of clone A8 was four times higher than clone A50, and qRT-PCR results showed that the UT mRNA levels in A8 were 3.7 times higher than A50. The binding assay result also suggested that the structure of the recombinant receptor was consistent with wild-type UT. Furthermore, the downstream signals conducted by the recombinant UT were matching with the wild-type. In recombinant clones, both Inositol-1-phosphate ($IP_{1)}$ accumulations and calcium flux levels rose dose-dependently under UII stimulation, and significantly prominent in clone A8 than A50. ***Conclusion:*** Our results indicate that SNAP-tag approach on GPCR cell line establishment is fast and simple, furthermore, can quantitatively provide recombinant gene expression levels.
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1. Introduction

G-protein-coupled receptors (GPCRs), which bear hallmark 7-transmembrane domain, constitute the largest and most diverse family of transmembrane receptors. GPCRs respond to various extracellular molecules and activate intracellular signal transduction and, ultimately, cell response. GPCRs have been considered as one of the most important targets with regard to drug discovery. Thus, a mammalian cell line with recombined GPCR expression would be valuable in understanding target receptor-related signal transduction and developing drug screening models for discovering the agonists and antagonists. However, establishing such a cell line is frequently time-consuming. In addition, the expression of GPCRs is often unstable and requires being monitored for 12 passages or more (Schucht et al.,2011).

Typically, the development of a stable GPCR-expressing cell line entails transfection with a plasmid that encodes GPCR. The transgene integrates randomly into the cell genome, and positive clones are selected by antibiotic selection and identified by various methods. However, despite successful plasmid integration into genomic DNA, the resulting heterologous expression is usually low or absent. Early and efficient identification of positive clones can help discard the clones whose final expression levels are not sufficiently high and reduce the workload tremendously.

There are many methods of evaluating the heterologous expression of GPCRs, the most common of which is to measure specific binding to an appropriate radioligand. Although radioligand binding can quantitate GPCR expression, it requires relatively large amounts of cells. Functional assays can also be used, such as measuring intracellular Ca^{2+}, IP_3, and cAMP levels on stimulation. Although these methods can qualitatively identify positive clones, they do not provide quantitative information that can differentiate high- and low-expressing clones (Hermans,2004). Immunoassays can also be performed, but a highly specific antibody is usually difficult to be obtained for most GPCRs. Thus, methods that accurately quantify protein expression in simple and specific way and require few cells are valuable for the efficient selection of clones.

Fluorescent labeling is a key technique in detecting a protein of interest, but conventional genetic fusion with fluorescent proteins has several

shortcomings. For example, the autofluorescence proteins (e.g. GFP) might interrupt some important GPCR functional assays such as calcium flux assay and Tag-lite™ technology based assays. Post-translationally labeling methods that use a genetically encodable tag and synthetic probes that target the tag can overcome these limitations. SNAP-tag technology was developed by Covalys Biosciences AG (Witterswil, Switzerland) and is based on a post-translationally tag-probe labeling approach. The SNAP-tag can be fused easily to the N-terminus of a protein by cloning the gene of interest into an appropriate generic SNAP plasmid. This tag, two-thirds the size of GFP, is a mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase that reacts specifically and attaches covalently to benzylguanine (BG) derivatives, leading to irreversible labeling of the SNAP-tag with a synthetic probe. Thus, proteins of interest — eg GPCRs — can be detected easily by fluorescence microscope or on an automatic microplate reader. The SNAP-tag allows specific fluorescent labeling of the protein without autofluorescence, rendering the recombinant GPCR expression system valuable for certain fluorescence-based assays(Stein et al.,2013).

SNAP-tag technology can be used to construct an artificial cell membrane anchor as a fitting for artificial extracellular functionalities of eukaryotic cells (von et al.,2012).Post-translational labeling methods that use a genetically encodable tag and synthetic probes that target the tag can control the labeling ratio precisely for multicolor labeling of living cells (Yano & Matsuzaki,2009).The SNAP-tag fusion at the N-terminus of GPCRs does not alter their expression, and the SNAP-tag can be labeled with 100% efficacy; further, the 23-kDa SNAP-tag and its specific fluorescent substrate label do not affect the binding of GPCR to its ligand or its function (Maurel et al.,2008; Zwier et al.,2010; Leyris et al.,2011).

Urotensin II (UII), a cyclic 11-13 residue peptide that is expressed in motoneurons of the spinal cord, is a potent vasoconstrictor (Coulouarn et al.,1998; Ames et al.,1999), the effects of UII are mediated by binding to urotensin II receptor (UT), which is expressed in endothelium, smooth muscle, heart and pancreas (Ames et al.,1999). UT is an attractive drug target for many cardiovascular diseases.

In this study, we established stable cell lines that expressed different levels of recombined UT using SNAP-tag technology. The binding affinity with UII and biological activity such as inositol trisphosphate (IP_3) and calcium influx upon stimulation were measured. Our results showed that SNAP-tag technology is a valuable method on establishing reliable GPCR-expressing cell lines in different levels.

2. Material and Methods

2.1Cell culture

All cell culture media and supplements were from Gibco (Grand Island, USA). The HEK293A kidney cell line was obtained from Invitrogen (USA), and were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose), supplemented with 10% fatal bovine serum (FBS), and cultured at 37℃ and in a humidified 5% CO2 atmosphere; the media was changed every 3-4 days. Stable SNAP-UT-HEK293A cell clones were maintained in the corresponding culture media, supplemented with 0.6 mg/ml G418.

2.2 Construction of Tag-lite™ SNAP-UT plasmid

The human urotensin II receptor (hUT) mRNA sequence (GenBank number: NM_018949; UTS2R) was used. The genome sequence of hUT lacks introns, we amplified the hUT gene from human genomic DNA by PCR using the following primers: for the full-length clone, sense 5'-GAAGGTGTTGCCTGATTTGCT-3' and anti-sense 5'-GGGCCTGTGATTTGGGAGTT-3', 1377bp. PCR-amplified UT and Taglite™ SNAP-plasmid (cat no. PT8SNAPNEO, Cisbio Bioassays, France) were digested with EcoRV and XhoI and separated by agarose gel electrophoresis, the 6038-bp fragment from the plasmid and the 1183-bp fragment of UT were recovered and ligated to generate Tag-lite™SNAP-UT plasmid.

2.3 Transfection assay and generation of stable clones

The transfection protocol was performed as report (John,2008). HEK293A cells were seeded in a 6-well plate, and after an overnight incubation, the cells were transfected with Tag-lite™SNAP-UT by FuGENE® HD Transfection Reagent (Roche, Switzerland). Forty-eight hours after transfection, the cells were trypsinized to obtain a single cell suspension, divided into 5 fractions, and subcultured in 150mm dishes with fresh media, containing 1500mg/L G418 (cat no.110131-035, Gibco, Japan). Ten days later, tiny cellular colonies appeared at the bottom of dishes. Colonies that were average in size and well isolated from other colonies were trypsinized using PYREX® 6x8mm Cloning Cylinders (Corning, U.S.A.), transferred to a 12-well plate, and cultured in fresh media, containing 1500mg/L G418.

2.4 Identification positive clone with Tag-lite™ SNAP-Lumi4-Tb

The clones were seeded in 96-well plates for identification. After 24-h incubation at 37 °C in 5% CO_2, the media was removed and 100 μL of 100 nM

fluorescent SNAP-tag substrate — Tag-lite^{TM} SNAP-Lumi4-Tb (cat no.SSNPTBC, Cisbio Bioassays, France) was added. After incubation for 1 h at 37 ^{o}C, the cells were washed 4 times with 100μL Tag-lite^{TM} labeling medium. The Tb fluorescence intensity was read on an HTRF compatible reader (M5, Molecular Devices Company, U.S.A.) at excitation 343 nm and emission 620 nm. The signal, which corresponds to the specific labeling of the GPCR, is calculted as follows: Specific signal = Δ620 = Positive 620 nm signal - Negative 620 nm signal. Positive 620 nm signal = Fluorescence intensity at 620 nm, obtained with cells that express the SNAP-tag receptor. Negative 620 nm signal = Fluorescence intensity, obtained with cells without the SNAP-tag receptor.

2.5 Realtime qRT-PCR

The quantification of human UII mRNA in the cell clones was performed with Realtime quantitative reverse transcription polymerase chain reaction (qRT-PCR), The primers of human UII (sense 5'-GGCAACGCCTACACGCTGGT-3' and antisense 5'-GGCAGGCACAGGCTCTTGGG-3', 401bp) and human GAPDH (sense 5'-AATGGGCAGCCGTTAGGAAA -3' and antisense 5'- GCCCAATACGACCAAATCAGAG -3', 166bp) were used in the experiment. The reactions were performed with SsoFast EvaGreen Supermix (cat no. 172-5200, Bio-Rad, USA) as described in the manufacturer's protocol, and in the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). PCR conditions were as follows: 2 min at 98°C; 40 cycles of 5 s at 95°C, 10 s at 55 °C and 30 s at 72°C; followed by a melting curve analysis step (temperature gradient from 60°C to 95°C with +0.3°C per cycle). We applied the comparative CT (ΔΔCT) method for the relative quantification of transcription levels. All samples were normalized to endogenous GAPDH levels. The samples were measured in duplicate and the experiments were performed twice.

2.6 Homogeneous time-resolved fluorescence-based UT–urotensin II binding assay

Clonal SNAP-UT HEK293A cells were trypsinized. suspended in 150 nM SNAP-Lumi4-Tb labeling solution (1×10^6 cell/ml), incubated at 37 ^{o}C for 1 h, and centrifuged; the pellets were washed with Tag-lite^{TM} labeling medium for 4 times. Labeled cells were distributed into 384-well plate at 5×10^4 cell/25μl/well. FITC-UII and UII were added at 5μl/well. The final concentrations of FITC-UII and UII were 10^{-7}M and 10^{-5} M respectively. Saline was used as the control. Fluorescence intensities (ex 343 nm, em 620 nm/520 nm) were read on a HTRF-compatible reader (M5, Molecular Devices Company, U.S.A.).

2.7 IP1 detection

IP-One Tb assay (cat no.62IPAPEB, Cisbio Bioassays, France) was used. IP_1, a downstream metabolite of IP_3, accumulates in cells following Gq receptor activation and is stable in the presence of LiCl, making it ideal for GPCR (Gq) functional assays. IP_1 was detected per the manufacturer's instructions. In brief, cells were seeded in a 96-well plate and incubated at 37 ^{o}C overnight. The supernatant was removed, and tested compounds were added and incubated for 0.5-1 h at 37 ^{o}C. HTRF$^{©}$ reagents were added and the cells were incubated for 60 min at room temperature. The fluorescence intensity was readed on an HTRF compatible reader (M5, Molecular Devices Company, U.S.A.) at excitation 343 nm, emission 620 nm and emission 665 nm. The results were expressed as the ratio of 665 nm/620 nm. Standard curves were constructed by plotting the ratio versus IP_1 concentration.

2.8 Calcium flux assay

Calcium flux assay was performed using the wash-free Fluo-8 calcium assay (cat no. HD03-0010, HD Biosciences, China) per the manufacturer's instructions. Cells were used to seed 96-well plates. After overnight incubation at 37 ^{o}C, the media were replaced with 100μl 1×Fluo-8 Calcium assay loading solution. The cells were incubated at 37 ^{o}C for 30 minutes and at room temperature for another 30 minutes until use. The fluorescence intensities (ex 490 nm, em 525 nm) were read immediately on a plate reader (FlexStation 3, Molecular Devices Company, U.S.A.) once the tested compound was added.

2.9 Statistical analysis

All data were analyzed using SPSS 10.0 and are presented as means±standard deviation. The experiments were repeated at least 3 times independently. Statistical difference was evaluated by paired Student's test between treated and untreated control group, a p value <0.05 was considered statistically significant.

3. Results and Discussions

GPCRs comprise about 900 members, are targeted by about 25% of the 100 top-selling drugs (Hopkins & Groom,2002; Overington et al.,2006). Thus, establishment of stable cell lines that express specific GPCR is critical for GPCR-targeted drug discovery and studies on receptor function. In this study, we used human urotensin II receptor (UT) as an example to demonstrate the efficiency of this technique. We established UT-expressing cell lines using SNAP-tag technology. This simple, reliable, quantitative, and rapid method can accurately identify clones that express the gene of interest with minimal amount of cells and with different expression levels.

The resulted plasmid, SNAP-UT, was transfected into HEK 293A cells, and hundreds clones were picked for further identification. To confirm that UT was expressed in the clones, we labeled the clones with Tag-lite^{TM} SNAP-Lumi4-Tb, and the fluorescence intensities of Tb were measured on an HTRF-compatible reader. The fluorescence intensities of recombinant clones were monitored for more than 12 passages. The clones which fluorescence intensities remained higher and stable for 12 passages were chosen for further study. In addition, we verified the integration of SNAP-UT into HEK293A genomic DNA in these clones by PCR, and confirmed the heterologous expression of human UII in the cell clones by Realtime qRT- PCR. Our results showed that the human UII mRNA levels were parallel with the fluorescence intensities. Further, we confirmed the structure of recombinant UT by UII binding assay, and the related intracellular signal changes under UII stimulation by calcium influx and IP_1 accumulation assays

3.1 Development and identification of SNAP-UT-HEK293A cell clones

Many cell lines are commonly used to establish stable GPCR clones, such as human embryonic kidney293 (HEK293), Chinese hamster ovary (CHO), AtT20, BV2, N18, and U-2 OS osteosarcoma cells (Atwood et al.,2011)-(Ames et al.,2004). In this study, we chose HEK293A as the host cell line, which was derived from HEK293 by Invitrogen. The adherent property of HEK293A facilitates receptor binding and signal transduction studies. The host cells should have no or very low endogenous expression of the target GPCR. To this end, HEK293 cells are suitable for stable expression of heterologous UT (Ames et al.,1999).

The Tag-lite^{TM} SNAP plasmid contains the geneticin (G418) resistance gene; thus, G418 was used to select for positive clones. HEK293A cells have relatively higher G418 resistance — 1500μg/ml G418 was needed to kill all the HEK293A cells within 7 days. Thus after transfected with SNAP-UT plasmid, G418-resistant clones were screened under 1500μg/ml G418. Candidate clones were seeded in 96-well plates for further identification with Tag-lite^{TM} SNAP-Lumi4-Tb. In this assay, when recombinant hUT protein was expressed on the cell surface, the cells could be labeled with with Tb dye.

Hundreds of candidate clones were picked and identified with Tag-lite^{TM} SNAP-Lumi4-Tb, time-resolved emission fluorescence at 620 nm was observed at an excitation wavelength of 343 nm. Compared with non-transfected control cells, 93.1% candidate clones had higher fluorescence intensity, 77.5%, 8.6%, 4.7%, and 2.2% of which had intensities that were 2-, 4-, 8-, and 16-fold higher than the control, respectively. Of these clones, we chose 14 cell clones with stronger fluorescence intensities and good morphology for subculture. Although the fluorescent intensity of some clones decreased significantly after subculture, clone A8 maintained higher UT expression up to passage 12.

As shown in figure.1, the fluorescence intensities of clone A8 equaled or exceeded that of transiently transfected HEK293T cells. HEK293T is a partially adherent cell line, which is suitable for most transient transfection experiments with GPCRs. At passage 12, the fluorescence intensity of clone A8 maintained 4.5-fold higher than that of transiently transfected HEK293A cells (Fig.1). Suggesting that clone A8 experiences higher transcription and translation, and the integration locus in the host cells was stable. We chose clone A8, and clone A50 that had one quarter of fluorescence intensity compared with A8 for further validation.

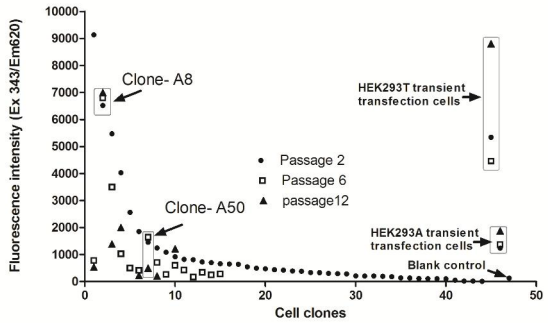

Figure 1. Identification of positive SNAP-UT-HEK293A clones. The clones were seeded in a 96-well plate, tagged by Tag-liteTM SNAP-Lumi4-Tb and detected the fluorescences intensities. The fluorescence of recombinant clones were monitored for 12 passages, and the HEK293A and HEK293T cell lines were transcient transfected SNAP-UT and repeated for three times as control.

3.2 hUT RNA transcript in SNAP-UT-HEK293A cell clones

We amplified cDNA from clone A8 at passage 13, clone A50 at passage 6 and HEK293A cells for the heterologous expression validation. The quantification of human UT mRNA was performed by Realtime PCR; samples were normalized to endogenous GAPDH levels. In our study, the fluorescence intensity of clone A8 was around 3.8 times higher than clone A50; and the UT mRNA level of clone A8 was 3.7 times higher than clone A50 (Figure. 2). Our results showed that the fluorescence levels of recombined clones were well parallel with the recombined mRNA levels, which suggests the fluorescence levels of recombined clones could be used to represent recombinant gene

expression levels. Many studies on GPCR have used HEK293 cells as the expression systems, because they are often regarded as 'blank slates' (Atwood et al.,2011). Our results showed that HEK293A had the hardly detectable endogenous expression of UT. We conclude that the UT transcription in clone A8 was attributed to recombinant UT.

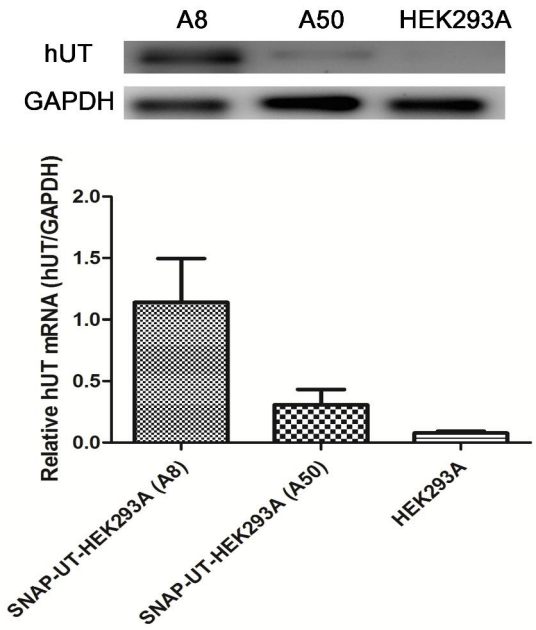

Figure 2. Recombinant UT RNA levels in SNAP-UT-HEK293A cell clones. Recombinant UT RNA from clone A8 at passage 13, clone A50 at passage 6 and HEK293A control were quantified by Realtime qRT-PCR. The PCR products of the agarose gel electrophoresis were the 25 cycle amplification products.

3.3 Recombinant receptor binds to UII

There are some studies showed that the fusion of the SNAP-tag to the N-terminus of GPCRs does not affect the binding or function of the A, B, or C GPCR families (Maurel et al.,2008; Zwier et al.,2010; Leyris et al.,2011). In this study, we examined whether the recombinant receptor in SNAP-UT-HEK293A cells could bind UII as wild type receptor by homogeneous time-resolved fluorescence (HTRF) assay. Lumi4-Tb pre-labeled cells from clone A8 were used to perform the binding assays. Compared with saline control, UII competed with FITC-UII binding to the receptor and thus decreased the fluorescence ratio 2.6-fold (Figure.3). Our result showed that UII could bind to the recombined UT, suggesting that the structure of the recombinant receptor is consistent with wild-type UT.

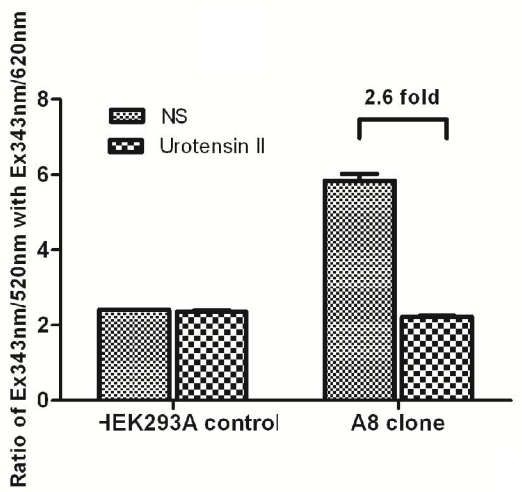

Figure 3. Result of UII competing with FITC-UII for the recombinant receptor by homogenous time-resolved fluorescence (HTRF) assay using SNAP-UT-HEK293A cell clone (A8) and HEK293A control. The data presented are means +/- SEM of triplicate wells (n=3).

3.4 Functional validations

We observed increase of the IP_1 and calcium influx signals in the SNAP-UT-HEK293A cell clones on UII stimulation, demonstrating that the recombinant cell lines are useful tools for UT signal transduction assays.

GPCRs activation is effected through 2 major G protein signal transduction pathways: The activation of Gs or Gi protein coupled receptor alters cyclic AMP (cAMP) levels, and the activation of Gq protein-coupled receptors inducing a transient increase of intracellular Ca^{2+}, triggered by inositol (1,4,5) tri-phosphate (IP_3). The lifetime of IP_3 is short (less than 30 s), and it is quickly transformed sequentially into IP_2 and IP_1. IP_1 is accumulated in the cell when LiCl is added to the medium and thus can be quantified precisely. Calcium influx and triggering of IP_3 mediate the UT signaling pathway; thus, we examined calcium influx and IP_1 levels under UII stimulation to evaluate the function of recombinant UT in recombinant clones

Our measurements on IP_1 showed that UII could dose-dependently increase the intracelluar IP_1 levels in the both recombinant clones A8 and A50. The increasing levels of clone A8 were much dramatic and higher than clone A50 when under the same UII concentration stimulations. The baseline level of IP_1 in clone A8 was higher than A50, and clone A50 had a similar baseline level compared with HEK293A control. The higher baseline of A8 might be attributed to nonspecific signal transduction by over expression of UT (Figure 4).

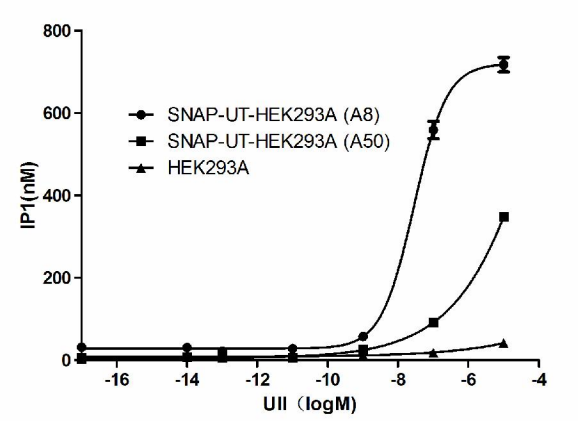

Figure 4. Intracellular IP1 levels under various concentrations of UII stimulation in SNAP-UT-HEK293A cell clones (A8 and A50) and HEK293A control. The data presented are means +/- SEM of triplicate wells (n=3).

The measurement of calcium flux is the most universal functional assay for GPCRs in drug screenings, due to the availability of cell-permeable Ca^{2+}-sensitive fluorescent dyes and automated real-time fluorescence plate readers. GPCRs that couple naturally to Gαq affect a ligand-dependent increase in intracellular Ca^{2+}. Moreover, Gαi/o, Gαs, or Gα12-coupled GPCRs can be switched to induce a rise in intracellular Ca^{2+} through the expression of a chimeric G-protein or promiscuous G-protein (Berridge,1993; Zhang & Xie,2012). In our study, UT couples to Gαq on UII stimulation, and intracellular Ca^{2+} levels increase dramatically for a short period, which can be detected by calcium indicators. The fluorescence intensity of calcium indicators correlates to intracellular Ca^{2+} levels, reflecting the activity of the stimulator. In clone A8, intracellular Ca^{2+} levels climbed on stimulation by UII dose-dependently (EC_{50} was 5.39×10^{-8} M) (Figure. 5). Previous studies reported that the EC_{50} of wild type UT under UII stimulation from calcium flux assay was around 1nM (Ames et al.,1999). Our results indicated that the function of recombined UT is consistent with the wild type UT.

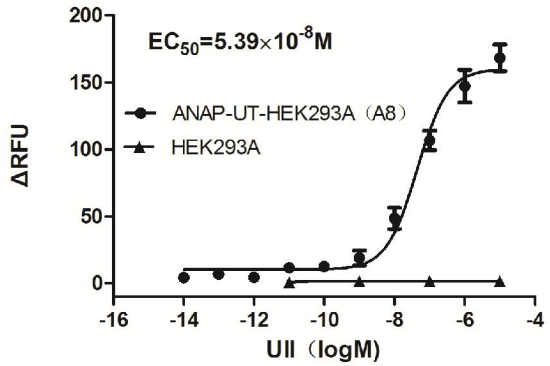

Figure 5. Calcium assay of SNAP-UT-HEK293A (A8) under UII stimulation. A8 and HEK293A control cells were loaded with Ca2+-sensitive fluorescent dye (Fluo-8) for 30min, and stimulated with various concentrations of UII, the fluorescent intensities were read. The data presented are means +/- SEM of triplicate wells (n=3).

In conclusion, we introduced a SNAP-tag at the N-terminal end of UT. Taking advantage of this tag, we successfully identified different levels UT expression cell clones. This approach is simple and fast, requires fewer cells, and provides quantitative information on recombinant GPCR expression level. In addition, this assay can be used to examine signal transduction pathways that are activated by GPCRs, accelerating the development of HTS models for the discovery of GPCR agonists/antagonists.

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