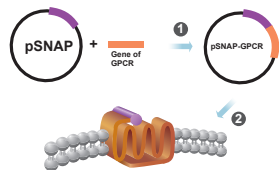


Tag-lite™, the new HTRF solutions for cell surface receptor studies and screening. Application to GPCR dimerization and highly selective ligand binding assays

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Introduction



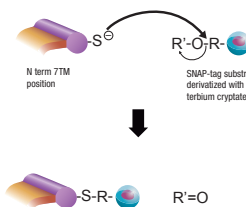
Cisbio introduces Tag-lite, a new technological concept for the investigation of cell surface receptors. Tag-lite combines HTRF[®] and SNAP-tag technology, a unique method to accurately label a protein of interest with a fluorescent dye. Tag-lite can be used in a comprehensive range of applications such as

receptor mechanistic and dimerization, ligand binding assays, and second messenger assessment.

SNAP-tag is a suicide enzyme which can be fused to the N-terminal position of a GPCR's 7TM fragment. A plasmid construction can be engineered ¹ and once transfected into cells, leads to the expression of the GPCR of interest tagged with the SNAP ² at the cell membrane surface.

For this platform, Cisbio has developed highly selective derivatized SNAP substrates. These substrates are labeled with HTRF fluorophores such as terbium cryptate (Lumi4-Tb) and green or red HTRF acceptors. As the Tag-lite SNAP substrates are non-permeating, only the SNAP tagged GPCR expressed at the cell surface can be labeled with the appropriate HTRF-compatible fluorophores.

No fluorescence resonance energy transfer (FRET) can occur within intracellular compartments where proteins accumulate during GPCR internalization or maturation processes.



Tag-lite cellular platform

Tag-lite plasmids and reagents form a comprehensive cellular platform, enabling the use of the same biological material (reverse transfection or stable cell line) to address ligand binding, GPCR dimerization and functional assays.

Application	HTRF reagents
Ligand binding assay	Tag-lite SNAP-Lumi4Tb + Red- Green-Ligand
	Lumi4Tb-Ligand + Tag-lite Green or Red
GPCR dimers	Tag-lite SNAP-Lumi4Tb + Tag-lite SNAP-Green or Red
Functional assay	Gi/s: cAMP kit
	Gq: IP-One kit

Material and methods

SNAP-tag-GPCR plasmid construction: Plasmid constructions were engineered with the SNAP-tag generic plasmid and the gene coding for the GPCR of interest. SNAP-tag is expressed on the N-terminal position of the receptor.

Reverse transfection: The assays were run in 96 well plates with cell culture treatment (GREINER). Reverse transfections were carried out with 200ng/well of the SNAP-tag-GPCR plasmid, 0.8µL/well of Lipofectamine2000 (Invitrogen) and 100 000 cells/well (COS-7). Plates were incubated 24h at 37°C+5%CO2 for expression of the SNAP-tag-GPCR and adherence of the cells.

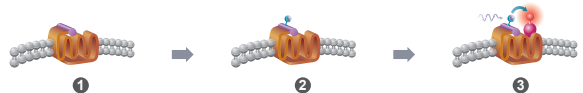
Covalent labeling of SNAP-tag-GPCR: After aspiration of the cell culture medium, Tag-lite SNAP-Lumi4Tb diluted in Tag-lite labeling medium at 100nM was added under 100µL/well and incubated 1h at 37°C for SNAP-tag-GPCR labeling. Then the excess of Tag-lite SNAP-Lumi4Tb was washed 4 times with 100µL/well of Tag-lite labeling medium, to obtain SNAP-tag-GPCR-Lumi4Tb receptors.

IP-One assay: The functional activation of V1a receptors by increasing the concentration of vasopressin was assessed with the IP-One kit. The assay measures inositol 1 phosphate (IP1) accumulation in the presence of lithium chloride. Vasopressin EC50 were measured and compared on both SNAP-tag and wild V1a receptors.

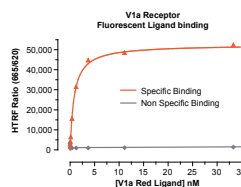
Ligand binding assay: All small molecule and peptide ligands were labeled with the HTRF red dye, using proprietary chemistry and methods. The total binding (TB) was the HTRF signal between the SNAP-tag-GPCR-Lumi4Tb and the red ligand. The non specific binding (NSB) was measured with a 100 fold excess of non-labeled ligand. $SB = (TB - NSB) / NSB$

Detection of V1a receptor homodimers at the surface of COS-7 cells: HTRF signal was measured with increasing concentrations of Tag-lite SNAP red substrate in the presence of Tag-lite SNAP-Lumi4Tb substrate 100 nM.

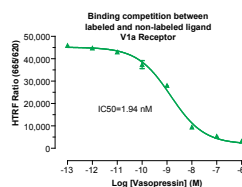
Tag-lite ligand binding assays



A transient transfection of SNAP-tag-V1a receptor was performed in COS-7 cells ¹. Covalent labeling of SNAP-tag-V1a receptor was carried out with Tag-lite SNAP-Lumi4Tb ². A homogeneous ligand binding assay was performed with a proprietary V1a ligand labeled with a red fluorescent dye ³. HTRF signal was measured after a 2 hour incubation.



Kd determination for V1a receptor: The NSB measured on V1a receptor with this Tag-lite assay was very low, as the ratio TB/NSB ≈ 40. The Kd (1.3 nM) was determined using the GraphPad Prism software.



IC50 and Ki determination for V1a receptor: Competition assay between V1a-red-ligand 1 nM and increasing concentrations of vasopressin. Based on the Cheng & Prusoff equation, vasopressin Ki is calculated.

GPCR	Compound	Radioactivity	Tag-lite
V1a	AVP	Ki=0.7nM (1), Ki=1.7 nM (2)	Ki=1.1 nM
V2	AVP	Ki=1.1nM (1), Ki=1.48 nM (2)	Ki=3.27 nM
CXCR4	SDF1alpha	Kd=24 nM (3)	Kd=40 nM, Ki=82 nM
	AMD3100	Ki=74 nM (4), Ki=106 nM (5)	Ki=63 nM

Characterization of V1a, V2 and CXCR4 receptors with Tag-lite platform: Ki and Kd were determined and compared radioactive ligand binding assays already published; results obtained with radioligand assay and Tag-lite platform were very similar.

Conclusion

With Tag-lite, the new HTRF-based cell-surface receptor platform, Cisbio provides reagents and technology to address the latest evolution in the GPCR drug discovery field. Our results with several GPCR and ligand types, either small molecules or peptidic by nature, demonstrate that the activity of both assay partners remains unaffected by the labeling procedure. Indeed, we show that both vasopressin and SDF1α labeled with a red acceptor bind the V1A and

V2 receptors, and CXCR4 receptor respectively. Ki obtained in each of the three cases (1.1 nM, 3.27 nM and 40 nM respectively) are in exact agreement with those published in the literature for the equivalent radioligand.

The test of these new fluorescence homogeneous methodologies is currently successfully expanding to a much broader range of cell surface receptors,

including those like chemokine which are known to be challenging. Based on these promising results, Tag-lite can be considered as a powerful alternative to conventional radioligand methods. In addition, we also show that the same cellular material expressing HTRF fluorophore labeled receptors can also be used for the study of receptor homo and heterodimerization.

(1) Durrroux, J. Med. Chem. 42, 1312-1319 (1999)
(2) Albizu, J. Med. Chem. 50, 4976-4985 (2007)

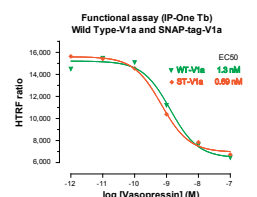
(3) Fricker, Biochem Pharm. 588-596 (2006)
(4) Gerlach, JBC, 14153-14160 (2001)

(5) Zhang, JBC, 24515-24521 (2002)
(6) Maurel, Nat. Methods 5, 561-567 (2008)

Receptor activity validation through functional assays

Reverse transfection of vasopressin 1a receptor was performed with the wild type (WT-V1a) and the SNAP-tag V1a (ST-V1a) of the receptor. Inositol 1-phosphate production induced by vasopressin stimulation was assessed by using the IP-One kit on the two different cellular models.

Studies confirmed that SNAP-tag fusion on the GPCR N-terminal do not affect either the GPCR binding of a specific ligand or the GPCR function. Vasopressin EC50 measured with IP1 accumulation assay were very similar on both WT-V1a and ST-V1a.



Tag-lite application to GPCR dimers studies

The combination HTRF and SNAP-tag is a powerful tool for investigating GPCR dimerization (6). We describe the detection of vasopressin 1a receptor homodimer in transfected COS-7 cells. Appropriate concentrations of both Tag-lite SNAP-Lumi4-Tb and Tag-lite SNAP-Red substrates were incubated with COS-7 expressing SNAP-tag-V1a.

Detection of V1a receptor homodimers at the surface of COS-7 cell: In the absence of red substrate all the SNAP-tags were labeled with the terbium substrate (1). The maximum HTRF signal was obtained at an appropriate ratio of both substrates (2); in an excess of red substrates, all the specific labeling sites were labeled with the red substrate (3).

The Tag-lite platform includes SNAP- and CLIP-tags. Both can be expressed as fusion proteins and labeled with their specific substrates. The main application of CLIP-tag is the labeling of a GPCR in conjunction with the SNAP-tag co-labeling of another GPCR. This approach was used to detect GPCR heterodimers (data not shown).

