

# High throughput screening using G-protein coupled receptors as the target

## *plate-based and biochromatographic technologies*

The G-protein coupled receptor (GPCR) family constitutes the largest class of cell surface receptors and GPCRs are the targets for more than 50% of the marketed drugs. The therapeutic importance of these receptors has produced a number of high throughput screens aimed at the discovery of new drug candidates. These screens, which range from cell-based functional screens to a newly developed liquid chromatographic approach using immobilised cellular membranes, are the subject of this review.

The G-protein coupled receptor (GPCR) family constitutes the largest class of cell surface receptors. Characterised GPCRs are a key target for the development of small molecule drugs and more than 50% of marketed drugs are active at these receptors<sup>1</sup>. In addition to characterised GPCRs, it is estimated the mammalian genome contains about 1,000 genes that encode for approximately 10,000 GPCRs<sup>1,2</sup>. Only a few hundred of the possible GPCRs have been identified and described, the rest are classified as 'orphan receptors' whose activities have not been established. Thus, the 'de-orphanisation' of these receptors presents the pharmaceutical industry with the opportunity to develop new therapeutic targets and new classes of drugs.

The de-orphanisation of receptors, or target validation, and the identification of lead drug candidates have been approached predominately through the use of high throughput screens (HTS).

Since GPCRs constitute an important family of targets, a wide variety of HTS have been developed. The majority of these screens are based upon microtiter plate technology, the dominant technological format for HTS, although a new column-based technology is also available. These screens are discussed below.

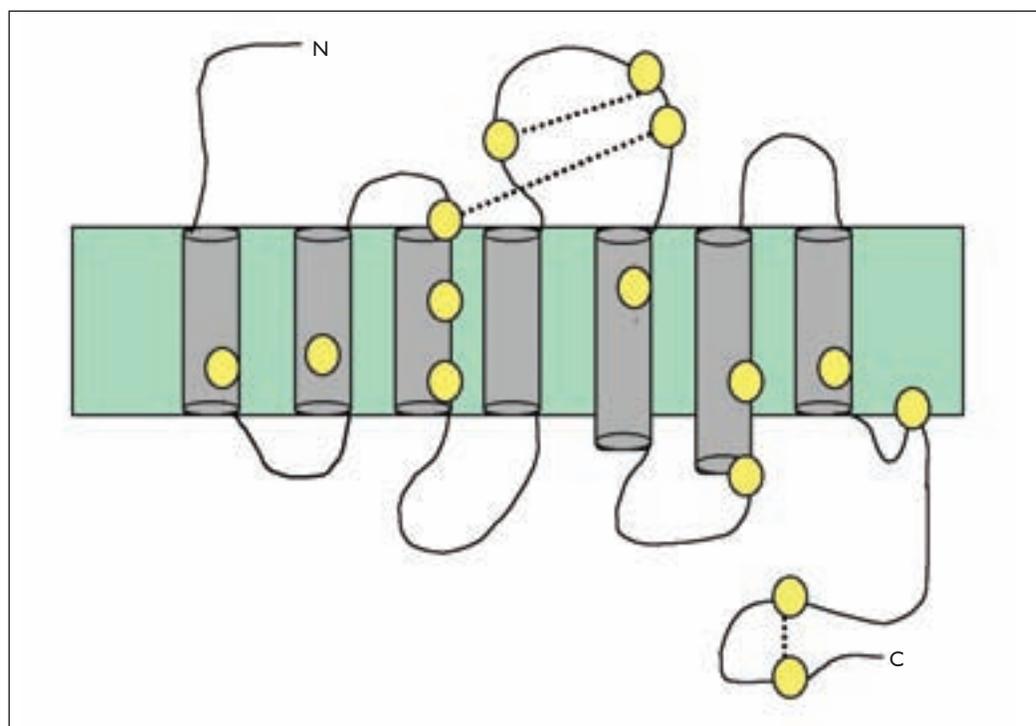
### **Background**

A vast majority of mammalian cell-surface receptors are coupled to a trimeric signal-transducing G-protein. GPCRs are a superfamily of proteins that mediate an enormous range of physiological processes, including responses to hormones, neurotransmitters and extracellular stimuli such as light, taste, odorants and pain in virtually every cell<sup>1,3-5</sup>. Despite the remarkable diversity of the activating ligands, all GPCRs show a common three-dimensional structural motif of seven transmembrane (TM)  $\alpha$ -helices linked by alternating intracellular

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**Figure 1**

Schematic illustration of a seven-helical transmembrane G-protein coupled receptor inserted in its membrane. The yellow circles are the cysteine residues on the helices



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Continued on page 72

(I-III) and extracellular (II-IV) loops (Figure 1). The extracellular loops and the N-termini are known to be involved in ligand binding while the intracellular loops and the C-termini are linked to G-protein recognition and activation.

GPCRs are a critical element in the signalling pathway which converts extra-cellular stimuli into intra-cellular functions. Generally, ligand or drug binding to a GPCR produces conformational changes in the receptor protein that promote the association of the receptor with a distinct heterotrimeric ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits) G-protein. Interaction of the activated G-protein triggers the exchange of GTP for GDP on the  $\alpha$ -subunit leading to the dissociation of the complex from  $\beta\gamma$ -subunits. The bound  $\alpha$ -GTP subunit and the free  $\beta\gamma$ -subunits can then interact with specific effectors. For example, activated  $\alpha_s$  and  $\alpha_i$ -subunits regulate intracellular level of cAMP by mediating activation or inhibition of adenylyl cyclase respectively. The up-regulation or down-regulation of cAMP leads to the observed physiological responses<sup>1</sup>.

Classification of GPCRs can be based on parameters such as amino acid similarity, ligand structure and G-protein coupling preferences. Based on conserved amino acid sequences, three major subfamilies have been identified: Rhodopsin related (type A), calcitonin related (type B) and those that are related to the metabotropic glutamate/calcium sensor receptors (type C)<sup>1</sup>. The GPCRs can also be

classified based on the receptor's preference for  $G_s$ ,  $G_{i/o}$ ,  $G_{q/11}$  or  $G_{12/13}$  subtype of the  $\alpha$ -G protein<sup>1</sup>.

## Plate-based HTS screening

The reported HTS for GPCR ligands are based upon the measurement of intra-cellular calcium or cAMP. The methods utilising the measurement of intra-cellular calcium require the introduction of reporter genes into the target cells and spectrophotometric measurements via colorimetric, chemiluminescence or fluorescence<sup>6</sup>. The cAMP-based methods require pretreatment, incubations, lysis and immunoassays<sup>7</sup>. The cAMP levels are measured by either scintillation counting of incorporated radiolabels or with an ELISA-like assay. These methods and the companies offering these HTS are summarised below and in Table 1.

### The measurement of intra-cellular calcium

The first HTS developed for GPCRs employed a fluorescent probe that underwent a rapid intensity change when it interacted with calcium (*cf* Synapticorp, Table 1). This approach is demonstrated by the measurement of calcium changes in serotonin receptors<sup>8</sup>. In this procedure, the cells are pre-incubated with a membrane permeable  $Ca^{+2}$  sensitive fluorescence label, such as Fluo-3-AM. The fluorescence readings are then taken for 10 seconds prior to the addition of the drug.

The fluorescent probes were detected using a

Fluorescent Imaging Plate Reader (FLIP-R). This reader was developed for the sole purpose of allowing HTS of GPCRs that have calcium as another messenger. HTS using the FLIP-R technology has been developed for a variety of proteins including: 5-HT-7<sup>9</sup> and  $\alpha$ -2A Adrenoreceptors<sup>10</sup>.

In most cases, these assays use Fluo-3-AM as the calcium sensitive dye.

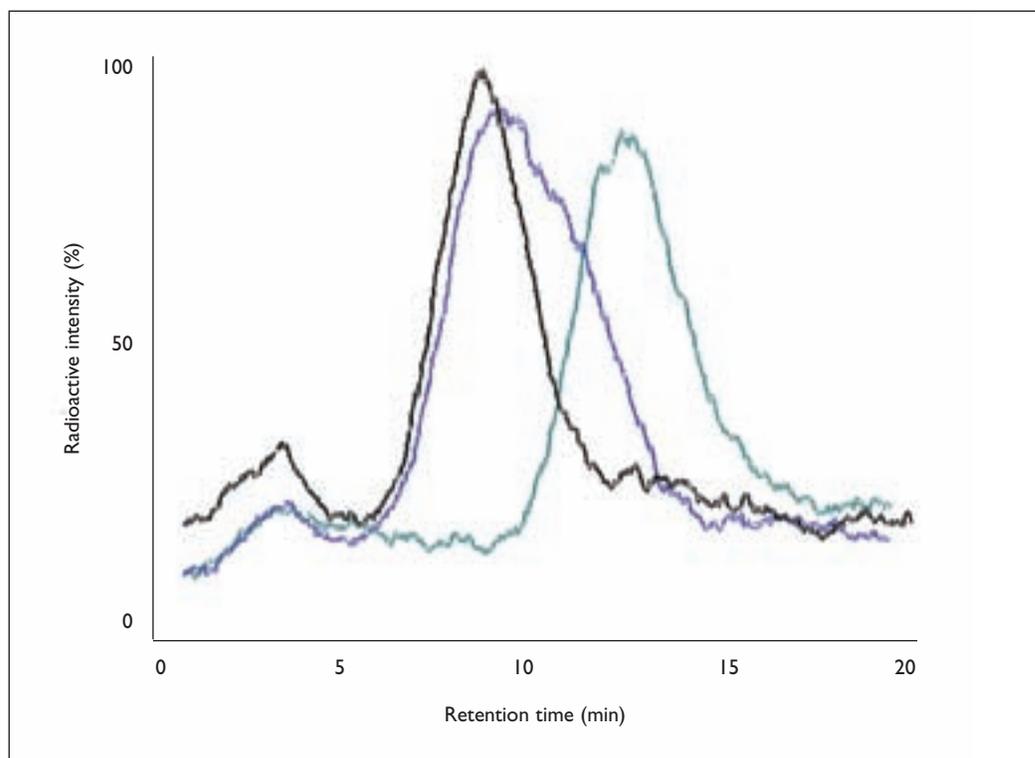
However, while assays based on FLIP-R technology represent an advance in HTS, they are time consuming, in particular when the experimental parameters must be varied. Thus, they have limited

**Table I:** Partial list of companies advertising commercially available GPCR plate-based and biochromatographic assay technologies

COMPANY NAME	APPLIED TECHNOLOGY ON GPCRS	MEASURING FACTOR/DETECTION METHOD	WEBSITE ADDRESS
Euroscreen sa	Cells expressing aequorin protein. Ca <sup>2+</sup> level is increased and binds to Aequorin, 20sec light emitted	Ca <sup>2+</sup> level/luminometers	www.euroscreen.be
Synaptic Pharmaceutical Corporation	Mammalian-cell based functional assay carried out with in-house cell transfection	Ca <sup>2+</sup> level/FLIPR	www.synapticcorp.com
Aptus Pharmaceuticals Inc	Ligand binding and functional assays in assay plate readers	Ca <sup>2+</sup> level and cAMP level/microplate readers and FLIPR	www.apтусpharma.com
Bio-Quant Image Analysis Corporation	Two platforms applied: Chemotaxis and cell activation (Ca <sup>2+</sup> ). Use of developed resistant fluorescent and or BioLuminescence dyes	Ca <sup>2+</sup> level and Chemotaxis (cell motility)/fluorescent and FLIB-R dye (fluorescence Infrared Bio-Luminescence)	www.bio-quant.com
DiscoverX Corporation	HitHunter™, endogenous cAMP competes with conjugated ED-cAMP introduced in cells to complement EA in the assay. Signal detected	cAMP assay/luminometer or fluorometer	www.discoverx.com
Meso-Scale Diagnostics, LLC	Immobilised GPCR membranes in assay plates. Introduced chemiluminescence labelled ligand	Ca <sup>2+</sup> level and cAMP level/microplate readers and luminescence	www.meso-scale.com
Norak Biosciences, Inc	Fused b2-arrestin protein with fluorescent protein (GFP) in cells. b-arrestin translocated through receptor conformational changes	Genetic modification, implement fluorescent protein (GFP) into cells/fluorescent readers	www.Norakbio.com
Arena Pharmaceuticals, Inc	CART (Constitutively Activated receptor) technology. Modified active GPCRs in cells are monitored	Genetically modified, Ca <sup>2+</sup> level and cAMP/fluorescence and luminescence	www.arenapharm.com
RETT Corporation	Immobilised receptors, drug transporters and enzymes used in a biochromatography format	Binding studies/mass spectrometry, fluorescence or luminescence can be adapted	www.rettcorp.com

**Figure 2**

Elution profile of zonal affinity chromatography on immobilised  $\mu$ -opioid receptor obtained from cell membrane. Displacement of the 1.5nM Naloxone, antagonist, is shown by other agonists. From right to left, 1.5nM Naloxone, 0.15mM Levorphanol, 0.15mM Methadone. Bed volume 0.4ml, flow rate 0.2ml/min



Continued from page 70

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Continued on page 75

potential as an HTS assay. Furthermore, this technique detects changes in calcium concentration, which makes it incapable of detecting GPCRs that may act via different messengers.

Ng et al<sup>11</sup> have developed an alternative approach based upon the transcription of calcium regulated reporter genes, such as luciferase. In this method, a change in intra-cellular calcium concentration translates into a sustained luminescent readout due to luciferase production. These assays have several advantages over the FLIP-R assays including their robustness, intense readouts and linearity over a large concentration range. However, this technique also has several disadvantages including long incubation times, inability to determine kinetic parameters, existence of false positive detection when screening for antagonists and finally the reporter genes have low sensitivity for small changes in calcium concentrations.

In an attempt to optimise the luminescent assays, Euroscreen (Table 1) developed a luminescent assay using aequorin as an alternative to the existing reporter genes<sup>6</sup>. Aequorin is also a calcium sensitive photo-protein that has previously been used for calcium detection. In order to activate the protein molecular oxygen from apoaequorin and its co-factor and coelenterazine are needed. A conformational change is induced upon interaction with

calcium resulting in the oxidation of coelenterazine and a subsequent emission of a blue flash is seen<sup>6</sup>. Aequorin has a low affinity for calcium ( $K_d=10\mu M$ ), which is around biological levels for calcium concentrations, and the protein is essentially specific for calcium since it does not significantly bind to other divalent cations. This modified luminescent assay offers many advantages over fluorescent dyes, which display non-selective localisation and are more sensitive to changes in their environment. However, this screening method requires a pre-incubation of four hours at room temperature to 18 hours at 4°C, and the length of the incubation is dependent on the cell line and the type of receptor<sup>6</sup>. In addition, these assays can only be done in selective cell types and are unable to identify GPCRs with other second messengers.

### The measurement of intra-cellular cAMP

It is estimated that 30% of GPCRs have cAMP as their second messengers. Agonist binding to these receptors results in an activation of the G<sub>s</sub> protein, followed by upregulation of adenylate cyclase activity and conversion of ATP to cAMP and inorganic pyrophosphate (P<sub>i</sub>). The level of cAMP increases rapidly as the binding of one ligand produces several hundred cAMPs. If an antagonist is added, the G<sub>i</sub> protein is activated producing an inhibition of cAMP production. Any cAMP

## High Throughput Screening

present prior to activation of the  $G_i$  protein will be degraded by cAMP phosphodiesterases.

HTS has been developed for these receptors (*cf* Aptus Pharmaceuticals, Inc, Table 1), which rely on the ability to detect small changes in cAMP levels in cellular lysates. Radioimmunoassay is the most common method of detection and quantification of changes in cAMP concentrations, but this technology is not optimal for HTS due to the long reading time and the washing steps. To avoid this, the radioimmunoassays have been replaced by homogeneous scintillation proximity and Flashplate assays<sup>7</sup>. Both methods eliminate the wash steps making it easier for automation. However, you are still left with long incubations, usually overnight, making the screen a two-day process, not including cell preparation time. An alternative approach utilises a novel chemiluminescent assay which provides a higher throughput<sup>7</sup>. A broad dynamic range allows cAMP samples from 60fmol to 6nmol to be measured without the acetylation or dilution protocols required by other methods. Data collection occurs within two hours of cell lysis, providing a format with higher

throughput. However, this technology can only detect cAMP and no other second messenger.

### On-line screens using immobilised GPCR

Alternative approaches to plate-based HTS technologies involve the application of immobilised GPCR membranes on matrices and surfaces. One method reported by Meso Scale Discovery<sup>TM</sup> (Table 1) uses array technology and electro-chemiluminescence for receptor-ligand binding study or functional assay detection. In this system, target membranes are immobilised in assay plates and labelled marker ligands are introduced as a 'TAG'. Binding studies or *in situ* generation of cAMP studies are then conducted within 90 seconds. Specific features of this technique are the HTS platform, low incubation times, no-washing steps and fast detection. However, the detection should be performed within a few hours as the immobilisation of these membranes shows stability for a maximum of seven hours.

A non-plate-based HTS is biochromatography, which is based upon target biopolymers



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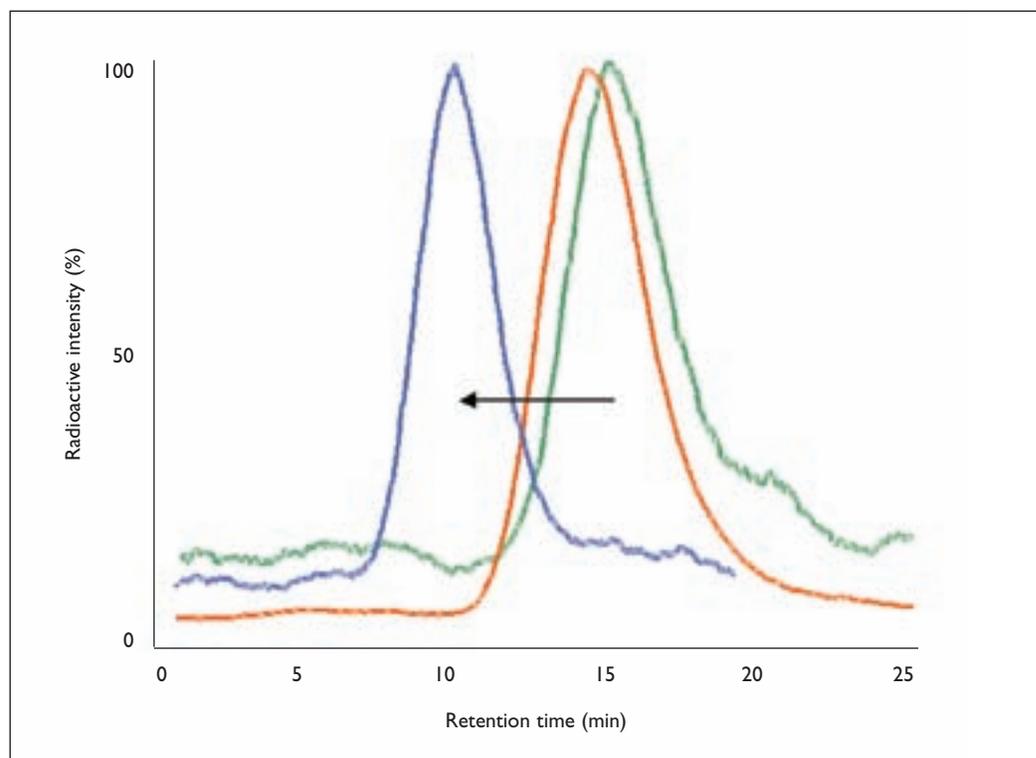
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If you are looking for a high-quality source of GPCR targets, don't look further. At Euroscreen, we have the widest choice of GPCRs available as off-the-shelf recombinant cell lines or membrane preparations on the market. We can rapidly produce any amount of ready-to-use membranes for binding and GTP-gamma-S tests. If you are into functional testing, we can provide cell lines with GPCRs coupled to calcium for any application including use with our AequoScreen<sup>TM</sup> technology or we could simply test your compounds for you. Recognized as leaders in the field of GPCR research and receptor de-orphanization, we take pride in the quality of our products and strive to offer you the best support and the widest choice of biological tools for your GPCR drug discovery requirements.

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**Figure 3**

Zonal elution profile of immobilised  $\beta_2$ -Adrenergic receptor from cell membrane on IAM chromatographic support. Elution peak of 5, 50nM CGP 12177 and displacement of the 5nM with 0.1mM propranolol. Flow rate 0.2ml/min, bed volume 0.4ml



Continued from page 72

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Continued on page 76

immobilised in a flow system. In this format, chromatographic retention and/or peak shape are the probes of ligand-target interactions. While biochromatography cannot match the absolute quantity of data obtained from 1536-, 2080- and 2456-well plates, the information content of the data is greater and direct online structural information can be obtained.

The higher information content contained in biochromatographic data reflects the fact that the fundamental processes of drug action, absorption, distribution, excretion and receptor activation are dynamic in nature and, in this manner, similar to the basic mechanisms involved in chromatographic retention. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic, hydrogen bonding) determine the behaviour of compounds in both biological and chromatographic environments<sup>12</sup>.

In the past few years, this approach has been reported for the online screening of complex mixtures using biochromatography techniques and stationary phases containing immobilised trans-membrane receptors and transporters and cytosolic and membrane-bound enzymes<sup>13</sup> (see RETT Corporation, Table 1). The phases have included immobilised nicotinic acetylcholine receptors alone<sup>14,15</sup> or co-immobilised with the  $\gamma$ -aminobutyric acid receptor and N-methyl-D-aspartate

receptor<sup>16</sup>. The ABC transporter P-glycoprotein has also been immobilised<sup>17,18</sup>, as have the enzymes glutamine synthetase<sup>19</sup>, phenyl N-methyl transferase<sup>20</sup> and dopamine  $\beta$ -hydroxylase<sup>21</sup>.

GPCRs have also been adapted to the biochromatography format in our laboratory. This is illustrated by the development of immobilised  $\sigma$ ,  $\kappa$  and  $\mu$ -opioid and  $\beta_2$ -adrenergic receptor columns. Representative results from displacement experiments performed using a column containing an immobilised  $\mu$ -opioid receptor and a column containing an immobilised  $\beta_2$ -adrenergic receptor are presented in Figures 2 and 3, respectively<sup>22</sup>.

Since the receptor is immobilised together with the lipid bilayer from the tissue or cell line from which the GPCR was isolated, the result is that this biochromatographic approach can be adapted to any member of the GPCR family. The technology permits the immobilisation of all known or orphan GPCRs with a rapid screening of a large number of compounds against each receptor. The method can be used to identify agonists, reverse agonist, antagonists and non-competitive antagonists that bind to these receptors, classifying the receptors in a specific family, and subsequently obtaining first generation drugs for therapeutic purposes.

Since the method is focused on binding affinities and not on functional assays, it is not dependent on the messenger employed by the GPCRs. Most

importantly, the cells do not have to be genetically altered to introduce a reporter gene, nor do they have to be incubated, lysed and the lysate run through an ELISA-like assay or scintillation counted. Also, tissue samples can be studied. The immobilised GPCR columns are stable and can be adapted to standard chromatographic equipment and mass-spectrometers.

The biochromatographic approach can be used with single compounds, combinatorial libraries, complex biological mixtures and phage libraries. Furthermore, differential screening techniques allow cell lines transfected with a gene expressing a particular GPCR to be compared with the non-transfected cell line to identify specific binding to the GPCR of interest.

Even though the method does not directly produce functional data, it identifies new proteins and ligands, thus de-orphanising the receptor. With current HTS methods an agonist would have to be initially identified in order to be able to identify inhibitors of the receptors function. Thus, the technology is competitive with most current HTS methods. **DDW**

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*chromatographic phases and glass surfaces and the application of the resulting liquid chromatographic columns to high throughput screening. Dr Beigi has published seven papers and 15 abstracts.*

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Continued from page 75

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