# SPA G-Protein Coupled Receptor Assay

For use with [$^{35}$SGTPγS] 500 tubes

## Product Booklet

Code: RPNQ0210

## Handling

### Safety warnings and precautions

**Warning:** ***For research use only.***

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

**Caution: For use with radioactive material.**

All chemicals should be considered as being potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with water.

Note that the assay protocol requires the use of dithiothreitol and sodium azide.

**Warning:** Dithiothreitol is harmful.

**Warning:** Sodium azide is toxic.

Please follow the manufacturers' safety data sheets relating to the safe handling and use of these materials.

### Storage

Store at -15°C to -30°C.

### Expiration

The expiration date is stated on the package and will be at least 4 weeks from the date of dispatch.

## Components of the assay system

**This pack contains the following assay components, sufficient material for 500 tubes. Components for this kit should be stored as directed below.**

**SPA bead**

Wheat-germ agglutinin (WGA)-coated SPA bead (750 mg) based on polyvinyltoluene (PVT) and containing scintillant, lyophilized. Store at -15°C to -30°C. Reconstitute using
8.5 ml of 50 mM Tris buffer, pH 7.4, containing 0.1% (w/v) sodium azide to give a bead stock concentration of ~90 mg/ml.

**Note: Once reconstituted, the beads must be stored at 2–8°C. DO NOT FREEZE.**

**5 x Assay buffer**

25 ml of assay buffer is supplied as a 5 x concentrate. Store at -15°C to -30°C. This buffer contains: 100 mM HEPES, 500 mM NaCl, 50 mM $MgCl_2$ and 5 mM EDTA, pH 7.4. Prior to use, dilute to 125 ml by the addition of 100 ml of water. Dithiothreitol (DTT) (not supplied) should then be added to this diluted (1 x) buffer prior to use.

# Description

The SPA G-protein coupled receptor assay is a single well homogeneous technique using the scintillation proximity assay (SPA) principle. The SPA system is based on a polyvinyltoluene bead containing scintillant. The outer surface of the bead has been modified by a coating of wheat-germ agglutinin (WGA) which enables receptors to bind. The beads are allowed to interact with the receptor to enable binding to occur. This assay can be used for the direct quantification of agonist-induced or inverse agonist-induced activity for receptors which are coupled to the GTP-binding proteins (G-proteins). The G-proteins are heterotrimeric moieties, composed of α, β and γ subunits (see section on background and references).

Assays are carried out in the presence of added [$^{35}$S]GTPγS and guanosine diphosphate (GDP) as well as mono- and divalent cations. GDP, $Mg^{2+}$ and $Na^{+}$ (chlorides) seem to be absolute requirements for G-protein coupled receptor activity. In the resting state (receptor unoccupied), the G*a* subunit is bound to GDP but is not or only partially associated with the receptor. In this resting state, there is a low (basal) rate of GDP [$^{35}$S]GTPγS exchange. The radiolabel associated with the G-protein-receptor complex activates the SPA beads and results in photon production and resulting SPA counts, but at a relatively low basal level.

However, upon the binding of a suitable ligand to the receptor, the GDP dissociates from the G*a* unit. This allows [$^{35}$S]GTPγS to bind to a greater extent than the basal-level binding, resulting in higher SPA counts. It is this level of agonist-induced enhancement of SPA counts that indicates the potency of a particular agonist or inverse agonist.

**If no GDP was added to the system, basal rates of GDP [$^{35}$S]GTPγS exchange would be too high and potential agonist effects would be masked. Agonist effects are therefore only observed in the presence of added GDP.**

There is no need in this SPA format, unlike that in conventional filter-based systems, to separate the receptor-G-protein-[$^{35}$S]GTPγS complex from the free [$^{35}$S]GTPγS. The SPA system will detect only that radioactivity which is associated with the bead.

After the assay has been set up and the components incubated for a desired period, the beads are centrifuged or allowed to settle prior to counting. This step is required to eliminate the high non-specific signal which would result if the free [$^{35}$S]GTPγS in solution was counted with the beads in co-suspension.

The assay is designed for use in both single reaction tubes or 96-well microplate format. The SPA protocol is very simple to perform, and compared to traditional methodology has many advantages:

- No harvesting or filtration step to separate bound from free [$^{35}$S]GTPγS is required
- Only pipetting steps are necessary
- Use of liquid scintillant is not required
- The system is amenable to automation.

Sensitivity of the assay is comparable to existing filter methods and assay precision is high. Substantial time saving can be achieved over existing methodology. The assay can be used to identify potential ligands that are active at G-protein coupled receptors and could be adapted to a high throughput format.

# The use of [$^{35}$S] with SPA

This kit requires the use of [$^{35}$S]GTPγS, which is a departure from the isotopes, such as [$^{3}$H], which are commonly used in SPA assays. [$^{35}$S] has a relatively high maximum energy (0.167 MeV) with a concomitantly greater path length (0.32 mm in water) of the β-particle. What this means for this SPA-based assay is that, were the free [$^{35}$S]GTPγS to be counted with the SPA beads in co-suspension, the non-specific proximity effects (excitation of bead fluor by unbound isotope) would be significant. This can be overcome either by allowing the beads to settle out under gravity (which can take up to 6 hours) or by pelleting the beads using a centrifuge.

With some receptor preparations, assay drift over time has been noted. In these cases, settling procedures may not be applicable.

## Critical parameters

**The following points are critical.**

- The 5 x concentrate assay buffer supplied (100 mM HEPES, 500 mM NaCl, 50 mM $MgCl_2$, 5 mM EDTA, pH 7.4) must be diluted 5 x into AnalaR-grade water and have dithiothreitol (DTT) added prior to use. Add 5 mg DTT for every 30 ml diluted (1 x) assay buffer employed. Store the 1 x assay buffer containing DTT at 2–8°C for no longer than 1 week.
- This assay is designed to be used in conjunction with guanosine 5'[-γ-$^{35}$S] thiotriphosphate, triethylammonium salt ([$^{35}$S]GTPγS), available from commercial suppliers.
- Evaluation studies have been carried out with [$^{35}$S]GTPγS using cloned rat adenosine A1 receptor expressed in CHO cells or cloned human muscarinic $M_1$ receptor expressed in CHO cells. These and other putative G-protein coupled receptors, can be obtained by in-house purification (1), cloning (2) or purchased from commercial suppliers.
- Prior to use, the beads (750 mg) should be reconstituted into a TRIS-based buffer at pH 7.4 containing sodium azide. This produces a storage stock of bead at a concentration of ~90 mg/ml (see section on assay protocol).
- Variation on the recommended amount of bead added per assay well or tube may affect assay characteristics.
- Antimicrobial agents are not included in the assay components. Users should be aware that contamination may occur when reagents are stored for long periods. If such agents are added, it remains the responsibility of the users to assess the effects on the assay.
- Ensure suitable blanks and controls are included in all assays.
- Users may notice a difference in the signal-to-noise ratios obtained depending on whether the beads are pelleted or allowed to settle. Long settling times can lead to count drifts.
- When researchers are using highly coloured samples, colour quench correction may be necessary.

## Additional equipment and reagents required

The following materials and equipment are required but not supplied:

- Bead reconstitution buffer, 50 mM Tris buffer, pH 7.4, containing 0.1% (w/v) sodium azide (8.5 ml).
- Dithiothreitol (DTT). Add 5 mg DTT for every 30 ml diluted 1 x assay buffer employed. Store the assay buffer containing DTT at 2–8°C for no longer than 1 week.
- Suitable source of cloned receptor (see overleaf).
- Guanosine 5'-[γ-$^{35}$S]-thiotriphosphate, triethylammonium salt ([$^{35}$S]GTPγS).
- Guanosine 5'-γ-thiophosphate (GTPγS), for non-specific binding determination. 1 mM stock made by dissolving 5.63 mg GTPγS into 10 ml 1 x assay buffer containing DTT. Dispense in 1 ml aliquots into labelled Eppendorf tubes and store at -80°C.
- Guanosine 5'-diphosphate (GDP). 1 mM stock made by dissolving 4.43 mg GDP into 10 ml 1 x assay buffer containing DTT. Dispense in 1 ml aliquots into Eppendorf tubes and store at -80°C.
- Scintillation counter for microplates or microcentrifuge tubes
- 96 well microplates compatible with microplate scintillation counter or microcentrifuge tubes, such as Eppendorfs.
- Self adhesive microplate seals
- AnalaR or similar analytical grade water
- Pipetting equipment, either manual or automated systems (10 µl, 50 µl, 100 µl, 200 µl, 1 ml)
- Centrifuge, with rotor suitable for microplates or microcentrifuge tubes (optional)
- Ice bath for the temporary storage of all reagents
- Adenosine deaminase enzyme, if working with adenosine receptor homogenates.

Putative G-protein coupled receptors can be obtained by in-house purification or cloning or purchased from commercial suppliers.

Guanosine 5'-[γ-$^{35}$S] thiotriphosphate, triethylammonium salt ([$^{35}$S] GTPγS), is available from PerkinElmer.

Dithiothreitol (DTT), guanosine 5'-γ-thiophosphate (GTPγS), guanosine 5'-diphosphate (GDP) and adenosine deaminase can be purchased from commercial suppliers such as Sigma Chemical Company Ltd.

# Assay procedure

## Storage

Store the reagents at -15°C to -30°C before use.

## Reagent preparation

**Assay buffer containing dithiothreitol (1 mM)**

Add 100 ml of water to the 5 x concentrate buffer provided. Store the diluted 1 x buffer at -15°C to -30°C. Add 5 mg DTT for every 30 ml 1 x assay buffer employed. Store the 1 x assay buffer containing DTT at 2–8°C for no longer than 1 week.

**Bead in storage and assay buffer**

**1.** Prior to use, the lyophilised beads (750 mg) should be reconstituted into storage buffer (8.5 ml 50 mM Tris buffer at pH 7.4 containing 0.1% (w/v) sodium azide). Mix thoroughly to ensure dispersion. This produces a storage stock of bead at a concentration of ~90 mg/ml. Keep the beads in this buffer at 2–8°C. **DO NOT FREEZE.**

**2.** For one 96-well plate, remove 150 mg (1.66 ml) of SPA bead in storage buffer into a clean glass container. Add 1 x assay buffer (3.33 ml) to the bead suspension and vortex mix gently. This produces a bead working stock at 30 mg/ml. Store on ice and use within 5 hours. Do not store this working stock of bead in assay buffer for later use once this 5 hour period has expired. Please note that a small excess volume of bead in assay buffer will be generated to allow for pipette variation.

**[$^{35}$S]GTPγS dilutions (4–8 nM)**

The objective is to add the [$^{35}$S]GTPγS in 10 µl 1 x buffer into a total assay volume of 200 µl. The desired final concentration of $^{35}$[S]GTPγS is 200–400 pM. A working stock of 4–8 nM is therefore required
(200–400 pM multiplied by the (20 x) dilution factor). The example below is sufficient for 1 x 96-well plate or 96 tubes. Scale up for larger numbers if required. For **one** 96-well microplate, and for a total assay volume of 200 µl, the following calculations need to be carried out:

$$\frac{\text{Radioactive concentration (mCi/ml)}}{\text{Specific activity (Ci/mMol)}} \times 1000 = \text{[stock ligand] (µM)}$$

For example, for [$^{35}$S]GTPγS at a specific activity of 1250 Ci/mMol and at a concentration of 1mCi/ml, the molarity would be 1/1250 x 1000 = 800 nM. For a working stock of 4 nM, we therefore need, in this example, to dilute the main stock 800/4 = ~200-fold. Therefore, using this same example, take 6 µl stock (6 µCi) and dilute 200-fold to
1200 µl in 1 x assay buffer. Store on ice.

**NOTE: The example quoted above assumes a radioactive concentration of 1 mCi/ml. The [$^{35}$S] isotope decays with a half-life of 87.4 days. The [$^{35}$S]GTPγS tracer is normally supplied at a radioactive concentration (RAC) of 1 mCi/ml (1 µCi/µl) at the reference date. Refer to the decay table (see appendix 1) and calculate the actual RAC on the particular date of use.**

**Example calculation:** [$^{35}$S]GTPγS at 5 days past the reference date. At this date, the actual RAC is 96.1% of the reference RAC. Thus:

Reference RAC = 1 µCi/µl
Reference (+ 5 days) RAC = 0.961 µCi/µl

Therefore, the molarity of the stock in the example quoted at reference (+ 5 days) will be 0.961/1250 x 1000 = 769 nM. Recall using the same example, that the molarity of the stock at the reference date (1 mCi/ml at 1250 Ci/mMol) was 800 nM.

**Therefore, for the desired working stock (4–8 nM), adjust the dilution accordingly as the radioisotope decays and the RAC decreases.**

**Guanosine 5'-γ-thiophosphate (GTPγS) (1 mM stock)**

(for non-specific binding determination)
1 mM stock made by dissolving 5.63 mg GTPγS into 10 ml
1 x assay buffer containing DTT. Dispense in 1 ml aliquots into labelled Eppendorf tubes and store at -80°C. Prior to use, dilute 5 x into
1 x assay buffer to give a working stock of 200 µM. Final concentration in assay = 10 µM.

**Guanosine 5'-diphosphate (GDP) (1 mM stock)**

1 mM stock made by dissolving 4.43 mg GDP into 10 ml 1 x assay buffer containing DTT. Dispense in 1 ml aliquots into Eppendorf tubes and store at -80°C. Prior to use, dilute 10 x into 1 x assay buffer to give a working stock of 100 µM. Final concentration in assay = 5 µM.

**Receptor in assay buffer**

For **one** 96-well microplate, thaw out the desired quantity of receptor and gently vortex mix to disperse. Remove the required volume of receptor stock and dilute to a total of 10 ml into cold 1 x assay buffer. Store on ice.

**Guanosine 5'-γ-thiophosphate (GTPγS) (200 µM)**

Dilute a 1 ml aliquot of 1 mM stock 5 x into assay buffer.

**Guanosine 5'-diphosphate (GDP) (100 µM)**

Dilute a 1 ml aliquot of 1 mM stock 10 x into assay buffer.

## Assay protocol (see table 1)

**(final assay volume 200 µl)**

The following protocol illustrates the general assay procedure, whether it is to be performed in tubes or microplates.

1. Prepare reagents as described in the previous section.
2. Label appropriate plates/wells.
3. To each well, add 10 µl assay buffer.
4. To each well, add 10 µl agonist (in appropriate solvents) at a suitable concentration (see additional information section on solvent compatibility) **or** add 10 µl 1 x assay buffer (**zero agonist control).**
5. Add 10 µl GDP (100 µM) per well.
6. Add 10 µl non-radioactive GTPγS (200 µM) to each well for non-

   specific binding (NSB) determination OR add 10 µl 1 x assay buffer for total count determination ($B_0$).
7. Add 10 µl [$^{35}$S]GTPγS (4–8 nM) to each well.
8. Add 100 µl receptor per well (pretreated with adenosine deaminase enzyme if studying adenosine receptors. 1 unit of enzyme for every 500 µl diluted receptor. The receptor/enzyme mixture is incubated for
   0.5–1.0 hour at 25°C and can then be used directly in the assay without further purification). Allow to stand for 5 minutes.
9. Add 50 µl SPA bead (30 mg/ml).
10. Seal plate with appropriate stickers.
11. Allow plates to incubate at room temperature for 0.5–1.0 hour. The optimal time should be determined by the researcher.
12. For assays in plates: The beads may be pelleted using appropriate centrifuge equipment. GE Healthcare has found 10 minutes at 1000 x g in a Beckman J-6M centrifuge with Beckman Matched-Weight Microplate Carriers in a JS-4.2 rotor to be sufficient.

    For assays in tubes: Pellet beads in a microcentrifuge at 1000–2000 rpm for 10 minutes.
13. Count each well for 1 minute in a β-scintillation counter (see subsection on counting, below). Ideally, counting should be performed within 0.5–1.0 hour. This is to minimise assay count drift over time that has been observed with some receptors.

## Counting

1. Scintillant should not be added to the assay.
2. If the scintillation counter has removable racks compatible with assay tubes, the tubes can be loaded directly into the racks. Alternatively, assay tubes can be placed into standard liquid scintillation vials and loaded into the counter.
3. For counters fitted with spectrum analysis packages, the suitable window opening should be determined and the machine set accordingly. Windows for other counters should be set wide open.
4. When researchers are using highly coloured samples, colour quench correction may be necessary. Please contact your local GE Healthcare representative for further information.
5. The following machines are compatible with SPA technology: Wallac 1450 MicroBeta™ 96-well microplate
   Packard TopCount™ 96-well microplate Conventional scintillation counters

**Please note:** The SPA counts obtained will depend on the type of counter used and the absolute efficiency of the instrument.

## Protocol summary table

**Table 1. SPA protocol**

| Component | Total binding ($B_0$) | Non-specific binding (NSB) |
|---|---|---|
| 1 x buffer | 20 µl | 10 µl |
| agonist **or** 1 x buffer (zero agonist control) | 10 µl | 10 µl |
| GDP (100 µM) | 10 µl | 10 µl |
| GTPγS (200 µM) | – | 10 µl |
| [$^{35}$S]GTPγS (4-8 nM) | 10 µl | 10 µl |
| receptor | 100 µl | 100 µl |
| SPA bead (1.5 mg) | 50 µl | 50 µl |

# Assay performance

**Figure 1a.** Filter assay showing PIA-induced [$^{35}$S]GTPγS binding to rat A1 receptor membranes.

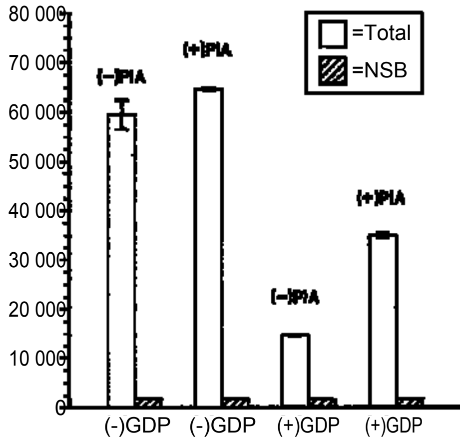


**Figure 1a.** Binding of [$^{35}$S]GTPγS (377 pM) to Chinese hamster ovary (CHO)-expressed rat adenosine A1 receptor membranes (Biosignal) (1.25 U, 19.25 µg per well) was performed as described in the protocol. The receptor was preincubated with adenosine deaminase (1 U enzyme per 500 µl diluted receptor) for 0.5 hour at 25°C, and used directly in the assay. Filter assays were carried out in microplates in the presence of 5 µM GDP and 10 µM (-)-$N^6$-(2-phenylisopropyl-adenosine) [PIA] in 200 µl total assay volume, at 25°C for 1.5 hours, followed by harvesting (Skatron cell harvester) on to GF filter mats and counting, after drying, in a BetaPlate™ counter using Meltilex™

solid scintillant. Plots are shown (figure 1a) as duplicates (range bars) for total and NSB (non-specific binding). NSB was determined in the presence of 10 µM GTPγS.

In the absence of GDP, there is no significant difference in specific counts whether agonist is present (62853 cpm) or absent (57586 cpm) (agonist enhancement = 1.09 x). This is because there is a high basal rate of GDP [$^{35}$S]GTPγS exchange, where any agonist effects, if present, have been masked. However, upon addition of GDP, there is an agonist enhancement effect (33278 cpm/12975 cpm, enhancement 2.56 x) (see table 2). Note that with GDP added, the overall counts are lowered, due to the lower basal exchange rate.

**Figure 1b.** SPA showing PIA-induced [$^{35}$S]GTPγS binding to rat A1 receptor membranes

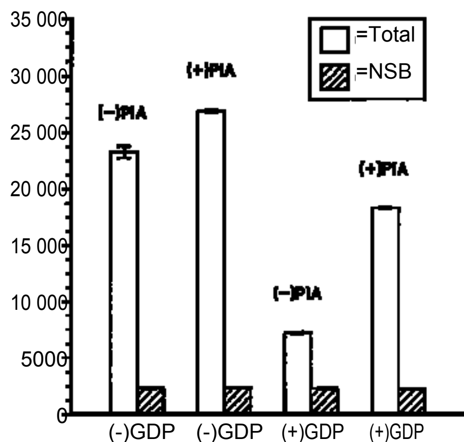

**Figure 1b**. SPA was performed essentially as above, except that 1.5 mg WGA-coated SPA beads was added to the assay mix prior to incubation (1.5 hours), followed by counting in a MicroBeta scintillation counter after centrifugation. The plots (figure 1b) are shown as duplicates (range bars) for total and NSB (non-specific binding). NSB was determined in the presence of 10 µM GTPγS. From figure 1b, in the absence of GDP, as in the filter assay (figure 1a), there is no significant count difference whether agonist is present or absent (agonist enhancement =1.17 x). In the presence of GDP, one can identify an agonist response to PIA (3.27 x).

It can be thus be seen that the two assay formats are comparable and that in the presence of GDP, agonist responses are observed (see table 2 below; data shown as mean specific cpm).

**Table 2.**

| | (+) PIA | (-)PIA | Enhancement |
|---|---|---|---|
| (-) GDP filter | 62853 | 57586 | 1.09 x |
| (-) GDP SPA | 24602 | 20958 | 1.17 x |
| (+) GDP filter | 33278 | 12975 | 2.56 x |
| (+) GDP SPA | 16077 | 4910 | 3.27 x |

**Figure 2.** SPA showing the effect of sodium chloride on PIA-induced [$^{35}$S]GTPγS binding to rat A1 receptor membranes in the presence of GDP.

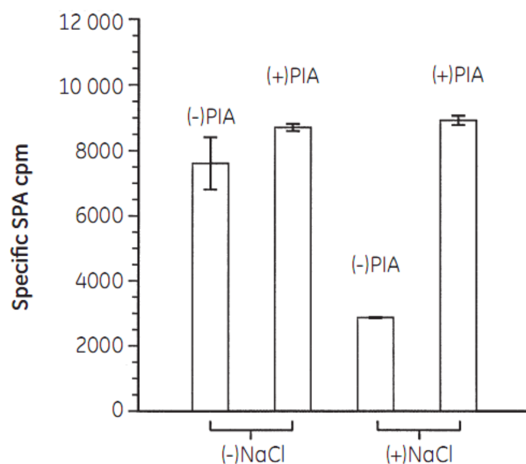


**Figure 2.** SPA was performed essentially as above, except that experiments were carried out in the presence or absence of 100 mM NaCl. The plots (figure 2) are shown as duplicates (range bars) for specific binding. Non-specific binding (NSB) was determined in the presence of 10 µM GTPγS. Data shown is in the presence of 5 µM GDP.

The agonist-induced (+GDP) enhancement (3.11 x) of [$^{35}$S]GTPγS binding in the presence of 100 mM NaCl is almost completely negated (1.13 x) in the absence of NaCl. The respective control values (-GDP) are 1.16 x (+ NaCl) and 1.05 x (-NaCl) (data not shown).

**Figure 3.** SPA showing the effect of increasing GDP on the agonist-induced binding of [$^{35}$S]GTPγS binding to rat A1 receptor membranes.

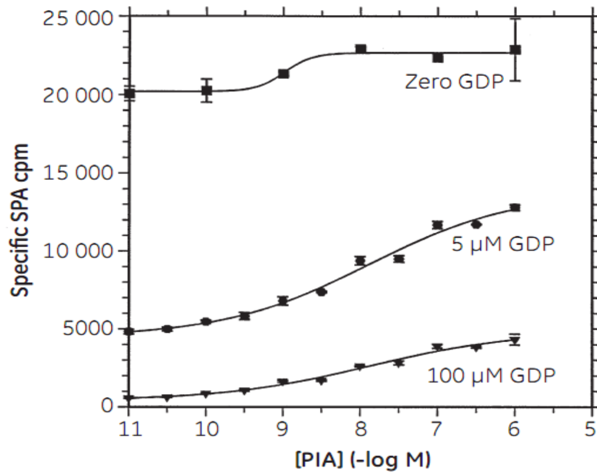


**Figure 3.** SPA was performed essentially as above, except that experiments were carried out in the presence of varying agonist, at three different concentrations of GDP (zero, 5 µM and 100 µM).
The plots (figure 3) are shown as duplicates (range bars) for specific binding. Non-specific binding was determined in the presence of 10 µM GTPγS. Data shown is curve fitted using the FigP™ software programme (logistic sigmoid-dose vs effect). The $EC_{50}$ for PIA at either 5 µM or 100 µM GDP is 7.9 nM.

From above, one can see the general trend of increasing counts with increasing [ligand]. However, although as expected, the count levels decrease as the level of GDP increases (basal exchange levels decrease), the maximum agonist enhancement values are 1.13 x, 2.65 x and 6.66 x at zero, 5 µM and 100 µM GDP respectively.

**Figure 4.** SPA showing McN-A343-induced [$^{35}$S]GTPγS binding to human muscarinic M1 receptor membranes.

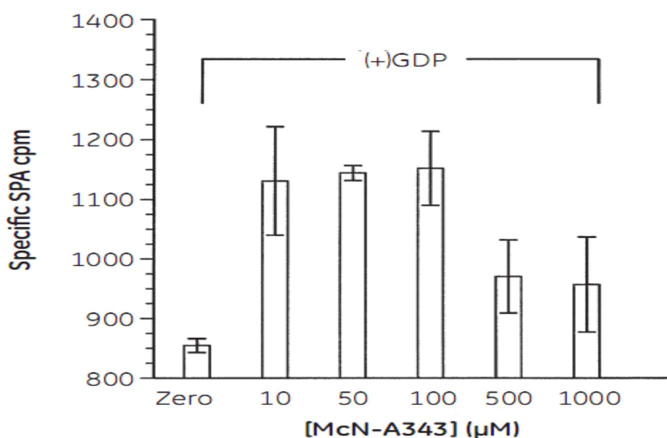


**Figure 4.** Binding of [$^{35}$S]GTPγS (377 pM) to Chinese hamster ovary (CHO)-expressed human muscarinic M1 receptor membranes (Biosignal) (1 U, 3 µg per well) was performed as described in the protocol. SPA was performed essentially as above, except that experiments were carried out in the presence of zero, 10 µM, 50 µM, 100 µM, 500 µM and 1000 µM agonist (McN-A343). The plots (figure 4) are shown as duplicates (range bars) for specific binding. Non-specific binding (NSB) was determined in the presence of 10 µM GTPγS.
Experiments were carried out in the absence (data not shown) or presence of 5 µM GDP.

In the presence of GDP and 10 µM, 50 µM or 100 µM agonist (McN-A343), there is a modest but obvious agonist enhancement (1.35 x) of [$^{35}$S]GTPγS binding compared to the basal effect in the absence of GDP (1.04 x). The agonist effect is attenuated at higher concentrations (500 µM and 1000 µM) of agonist. Although these enhancement values are lower than the previous cases with the adenosine receptor, this muscarinic receptor system still demonstrates G-protein receptor coupling using McN-A343 as the agonist.

# Additional information

## Solvent compatibility

The assay is compatible with DMSO and acetic acid which can be used for dissolving putative drug compounds, but in limited quantities. Lower SPA counts have been observed if the volume per well of these solvents exceeds 10 µl (5% of total volume). Solvents such as acetone, triethylamine and various halocarbons such as dichloromethane

are not suitable. If suitable organic solvents are employed, ensure appropriate control wells are set up.

## Receptor variability

There are many factors which can contribute to observed signals in the presence of agonists (see section on background and references). The majority of G-protein-coupled receptors (GCRs) should demonstrate enhanced [$^{35}$S]GTPγS binding in the presence of agonists. However in some cases, reported GCRs may not give the expected results in [$^{35}$S]GTPγS based assays. A publication (3) by

Biosignal Inc. (Montreal) highlights the reasons why this may occur. A summary of Biosignal's findings based on cloned receptors is shown below:

- **Low level expression of some receptor subtypes.** Where obvious differences (10 fold in some cases) in receptor expression were obtained, the higher-expressing systems (CCKA/NK2 receptors) showed greater agonism sensitivity. Low level (0.2 pmol/mg) expression for the AT1 receptor expressed in Rat1 cells showed little or no response. However, higher levels of expression do not always guarantee high efficacy. Biosignal found that CHO-cell expressed
  V1A receptors (at 0.9 pmol/mg) protein did not demonstrate significant agonist-induced count enhancement. This was even though there were previous literature reports that receptor preparations (at 0.8 pmol/mg protein) exhibited agonist-induced



  inositol phosphate production and intracellular $Ca^{2+}$ mobilisation, both of which are physiological events characteristic of agonism linked via G-proteins at this receptor.
- **Expressing receptor subtypes within a family, but in different expression systems**, leads to variation in agonist responses. This was demonstrated for the dopamine D1, D5 and D2s subtypes, where only the D5 receptor expressed in GH4 cell lines, demonstrated agonist responses. The other receptors, expressed to the same levels, but in different cell lines (L), showed only weak responses.
- **Preference of some cell lines for the expression of particular**

  **receptor subtypes.** Weak agonist effects were observed for the CHO-expressed $5HT_7$ receptor (predominantly found in the brain), whereas strong agonism was demonstrated for the D5 receptor (also found predominantly in the brain), but expressed in the pituitary-derived GH4 cell line.
- **The method of preparation and freezing of the receptor preparation** may be an important factor.
- **Optimisation of assay conditions is still very important.** Biosignal states that concentrations of $Mg^{2+}$, $Mn^{2+}$, $Na^{+}$ and GDP all need to be optimised as well as the assay incubation times.

## Background and references

A large majority of extracellular signalling factors such as neurotransmitters and hormones bind to and activate guanine nucleotide-binding (G-protein) coupled receptors. The G-proteins are heterotrimeric moieties composed of α, β and γ subunits (4,5).

In the resting state (minus agonist), guanosine diphosphate is bound to the α subunit, and the heterotrimer is associated (usually weakly) with the low-affinity agonist receptor. High-affinity binding of an agonist to the receptor leads to a rapid dissociation of bound GDP
from the a subunit and equally rapid binding (via a guanylnucleotide exchange mechanism) of guanosine triphosphate. The binding of GTP to the α subunit effectively promotes the dissociation of the β/γ subunits from the trimeric complex. The free β/γ subunits then interact with a variety of effector proteins, leading to intracellular responses such as $K^{+}$ and $Ca^{2+}$ mobilisation, adenylate cyclase activation or inhibition and inositol phosphate production. These immediate intracellular responses signal the onset of direct
physiological responses such as electrical nerve-cell conductance, or indirect phenomena such as gene transcription and resultant protein synthesis. The G-proteins thus serve as a vital link between the extracellular activation of GCRs by agonists and intracellular events.

After the appropriate biological response has been signalled, the GTP-α subunit complex forces the GCR into a low affinity agonist state, leading to dissociation of the agonist from the receptor. Furthermore, in order to attenuate the biological responses when appropriate,
the GTP is hydrolysed to GDP by GTPase enzymes integral to the α subunit. The heterotrimeric G-protein complex reassociates and the resting state is restored.

When radioactive [$^{35}$S]GTPγS (an analogue of GTP) is added in the presence of agonist, to intact cells or membranes systems *in vitro*, the GDP GTP guanylnucleotide exchange can be monitored directly by trapping the [$^{35}$S]GTPγS/G-protein/GCR complex on to a filter
mat or an SPA bead. In the case of the filter assay, bound activity has to be separated from free [$^{35}$S]GTPγS prior to counting by liquid scintillation methods. In the case of the SPA method, there is no need to separate bound [$^{35}$S]GTPγS from free [$^{35}$S]GTPγS, and counting is performed directly in

the reaction tube or microplate well. [$^{35}$S]GTPγS is essentially non-hydrolysable (unlike the native GTP), leading to a relatively stable signal over a reasonable assay time period.

Added GDP is essential in order to observe agonist-induced [$^{35}$S]GTPγS binding. In the absence of GDP, no agonist-induced



effects are observed. The theory behind the use of added GDP is that in the absence of agonist, GDP lowers the basal count value (rate of GDP [$^{35}$S]GTPγS exchange) more than the agonist-stimulated values. Overall counts are thus lower in the presence of added GDP, but it is only under these conditions that the agonist effect becomes unmasked, and a count enhancement effect is seen.

Sodium ions (as well as magnesium or manganese ions) are also prerequisites in order to observe agonist-induced [$^{35}$S]GTPγS binding. In the presence of GDP, added $Na^+$ has a more pronounced inhibitory effect on basal rather than agonist-stimulated binding of [$^{35}$S]GTPγS binding. This, as is the case with added GDP, also leads to observed count enhancement in the presence of agonist. When sodium chloride is omitted from the assay, no agonist enhancement is observed in the presence of GDP. It is possible that in the presence of $Na^+$ and in the absence of agonist, there is an uncoupling of unoccupied receptors from G-proteins, leading to lower G-protein activity (and hence lower basal counts).

As highlighted above, current methods of assessing GCR activity include [$^{35}$S]GTPγS based filter binding assays. In addition, [$^{35}$S]GTPγS has been employed in autoradiographic studies. One can also monitor GTP hydrolysis (using [$^{33}$P]GTP) to GDP while bound to the α subunit. This technique requires the separation by charcoal of the released [$^{33}$P]phosphate.
However, the value of employing the SPA approach is that the technology offers a functional screening assay which can distinguish agonists at GCRs without lengthy separation methods.

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## Appendix 1: Radioactive decay table for [$^{35}$S]

| Days | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| -7 | 1.057 | 1.049 | 1.040 | 1.032 | 1.024 | 1.016 | 1.008 |
| 0 | 1.000 | 0.992 | 0.984 | 0.976 | 0.969 | 0.961 | 0.954 |
| 7 | 0.946 | 0.939 | 0.931 | 0.924 | 0.916 | 0.909 | 0.902 |
| 14 | 0.895 | 0.888 | 0.881 | 0.874 | 0.867 | 0.860 | 0.853 |

This table gives partial decay data for $^{35}$S radioisotope.

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