Time-Resolved Fluorescence Based GTP Binding Assay for $G_s$-Protein Coupled Receptors

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Abstract

G-protein coupled receptors (GPCRs) participate in a wide range of cell signaling pathways and are believed to play an important role in a variety of disease states. Receptor ligand binding assays in the past have often been used in high throughput screening (HTS) to develop therapeutics for GPCR targets. However, functional assays such as cAMP, Ca²⁺ flux and GTP binding are becoming significant technologies in the HTS laboratory to measure efficacy of compounds. Functional assays can detect the ligand binding event as well as the functionality of the binding event. When doing GTP binding assays, the response from a receptor ligand-binding event activates the GPCR, which in turn exchanges bound GDP for GTP. Recently a new technology has been introduced which allows GTP binding detection in a non-radioactive assay that is suitable for HTS. The DELFIA GTP Binding Assay Kit from PerkinElmer Life and Analytical Sciences has successfully been used to demonstrate GPCR functional assays for Gi and Gq-protein coupled receptors. Gs-protein coupled receptors are more problematic due to the slower hydrolysis rate of GTP compared to Gi-coupled receptors. Therefore, the turnover of inactive Gs to active Gs is much slower causing a decrease in the amount of GTP binding that occurs. PerkinElmer Life and Analytical Sciences has optimized conditions to successfully format a Time Resolved Fluorescence Gs-protein coupled receptor functional assay yielding acceptable results.
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Introduction

Add to Acro Well filter plate:
buffer components
GPCR membranes compounds

→ Add GTP-Eu
→ Filter & wash
→ Measure at 615 nm

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Materials

- DELFIA GTP Binding Assay Kit PerkinElmer, Cat. #AD0167
  - 10X GTP Wash Solution
  - 5 M NaCl
  - 250 mM HEPES (pH 7.4)
  - 1 M MgCl₂
  - GDP (lyophilized)
  - GTPgS (lyophilized)
  - Eu-GTP (lyophilized)
  - 50 mg/ml saponin
  - AcroWell filter plate

- Human Beta 2 Adrenergic Receptor PerkinElmer, Cat. #RBHBE2M
- Epinephrine Sigma, Cat. #E4250

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Methods

> Add in the following order to AcroWell filter plate:

- 40 µl of β2 adrenergic receptor membranes
- 20 µl of 5X conc. agonist or buffer blank
- 40 µl of 10 nM Eu-GTP

- Incubate 60 min or indicated time
- Filter and Wash twice with 300 µl of 1X GTP wash buffer
- Read 615 nm emission on Victor²V, EnVision, ViewLux or other TRF enabled reader

Results

GDP was titrated to final concentrations of 0.1, 1, 3, and 10 µM. For each concentration of GDP, MgCl₂ was run at 1, 3, 10, and 20 mM final concentration. The basic buffer was 50 mM HEPES. Eu-GTP was used at a final concentration of 4 nM. The membrane amount used was 5 µg membrane protein/well. Incubation was performed for 1 h at room temperature while mixing. Wells were filtered prior to washing 2X with Wash Buffer and subsequent reading of the 615 nm emission on a Victor²V multilabel plate reader.
Human β2 adrenergic receptor (5 µg membrane protein) was incubated in buffer containing: 50 mM HEPES, 0.1 µM GDP, 3 mM MgCl₂, 4 nM Eu-GTP, and various amounts of NaCl ranging from 0-200 mM in the presence or absence of 100 µM epinephrine for 60 min. Wells were filtered prior to washing 2X with Wash Buffer and subsequent reading of the 615 nm emission on a Victor2V multilabel plate reader.

Saponin was added to final concentrations of 0, 1.2, 4, 12, 40, 120, and 400 µg/mL. In addition, the buffer contained 50 mM HEPES, 0.1 µM GDP, 3 mM MgCl₂, and 4 nM Eu-GTP. The membrane amount used was 5 µg/well. Wells were filtered prior to washing 2X with Wash Buffer and subsequent reading of the 615 nm emission on a Victor2V multilabel plate reader.
Various amounts of human β2 adrenergic receptor (0-20 µg membrane protein) was incubated in buffer containing: 50 mM HEPES, 0.1 µM GDP, 3 mM MgCl₂, 15 mM NaCl, 40 µg/mL saponin, and 4 nM Eu-GTP in the presence or absence of 100 µM epinephrine for 60 min. Wells were filtered prior to washing 2X with Wash Buffer and reading the 615 nm emission on a Victor²V multilabel plate reader.

Human β2 adrenergic receptor (5 µg membrane protein/well) was incubated in buffer containing: 50 mM HEPES, 0.1 µM GDP, 3 mM MgCl₂, 15 mM NaCl, 40 µg/mL saponin, and 4 nM Eu-GTP in the presence or absence of 100 µM epinephrine for times ranging from 15 to 120 min. Wells were filtered prior to washing 2X with Wash Buffer and reading the 615 nm emission on a Victor²V multilabel plate reader.
Conclusions

- Easy to optimize, non-radioactive GTP binding assay
- Previously shown to work with G_i-coupled receptors, now shown here to work with β2 adrenergic receptor, a G_s-coupled GPCR
- Optimized conditions for Eu-GTP binding described
- S/B = 2 obtained with as short as 60 min incubation
- Assay can automated in combination with liquid handling to yield daily throughput of 200 plates