Application Note

BellBrook Labs

Direct detection of RGS GAP Activity using the RGScreen™ Platform to Discover Small Molecule Inhibitors for RGS Proteins

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Introduction

Transcreener® is a universal, high throughput biochemical assay platform based on the detection of nucleotides, which are formed by thousands of cellular enzymes—many of which catalyze the covalent regulatory reactions that are central to cell signaling and are of great value as targets in drug discovery.

The RGScreen Platform uses proprietary Gα protein variants with altered GTP hydrolysis and GDP dissociation rates that enable detection of RGS protein GAP activity by the Transcreener GDP Assay. The RGScreen Platform is available in two different detection outputs-Fluorescent Polarization (FP), and Fluorescent Intensity (FI). The Transcreener GDP Assay is a far-red competitive immunoassay for GDP detection.

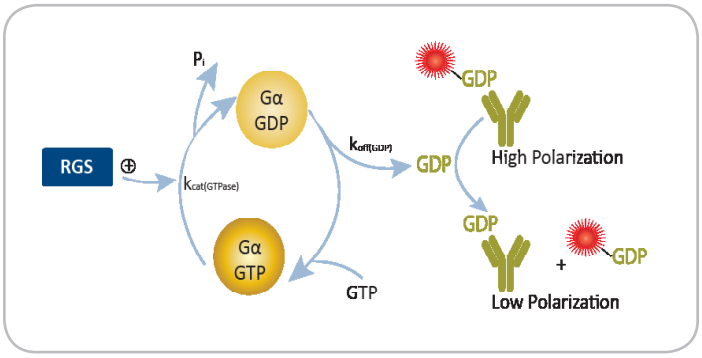

Figure 1. Detection of GDP using the Transcreener GDP Assay, a competitive fluorescence polarization immunoassay, offers an HTS compatible approach for directly measuring RGS GAP effects, however GDP dissociates from isolated Gα proteins very slowly, so RGS GAP effects are not detectable using steady state GTPase assays. To overcome this kinetic limitation, we combined Gα mutations that increased GDP dissociation and decreased GTPase activity curves used to determine GDP production.

This Application note will serve as a guide to set up and run a RGS Screen/Assay.

Step 1) Titrate RGS protein in the presence of Gα protein to determine optimal concentration of RGS protein for the assay.

Step 2) Screen for Inhibitors.

Step 3) Analyze Data.

Step 4) Run Dose Response Curves.

Materials and Methods

Instrument: Microplate Reader capable of measuring Fluorescence Polarization.

Microplates: Corning® 384 Well Low Volume Black Round Bottom PS NBS™ Microplate (Product #3676).

Reagent	Catalog #
Transcreener® GDP FP Assay	3009-1K
GDP Alexa 633 Tracer, 400 nM	2043
$G\alpha_{i1}$(R178M/A326S)	8002
RGS4 (29-198)	8005
GDP Antibody	2044
5 mM GTP	2046
5 mM GDP	2045
Buffer Components:	Not Provided
500 mM EDTA	
1000 mM Tris	
500 mM $MgCl_2$	
100% DMSO	

Step 1) Titrate RGS protein in the presence of Gα protein to determine the optimal concentration of RGS protein for the assay.

1. Serially titrate the RGS protein in a volume of 10 µL of 20 mM Tris buffer (pH 7.5).
2. Add 10 µL of detection mix comprising of GDP Alexa 633 tracer, GDP antibody, GTP and Gα protein in a buffer with 20 mM Tris, 2 mM EDTA and 20 mM $MgCl_2$. The final concentrations of the components in 20 µL reactions were 4 nM tracer, 10 µg GDP antibody, 10 µM GTP and 0.6 ng/µL of Gα protein.
3. The optimal concentration of the antibody was determined in a separate experiment by running an antibody binding curve in the presence of 10 µM GTP. Mix the plate on a plate shaker, cover with a plate seal, and incubate at 30° C.
4. The plates were read at 15 minute intervals to measure fluorescence polarization.

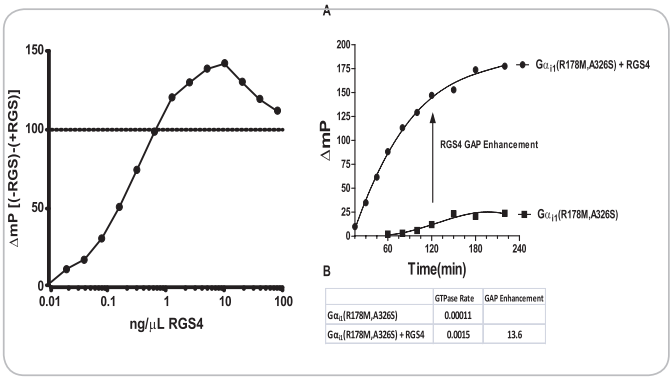

Figure 2. (A) RGS4 titration in the presence of 0.6 ng/µL of Gα protein. An optimal concentration of 2.0 ng/µL was determined based on this titration. B) Example of $G\alpha_{i1}$ (R178M,A326S) RGScreen™ Assay activity with RGS4 (Part# 8005). Dashed line is in the absence and solid line is in the presence of 2.0 ng/µL RGS4. Transcreener GDP assay reagents were added to enzyme reactions at time zero at 30°C and plates were read at intervals starting at 15 min. Reactions contained 1 ng/µL $G\alpha_{i1}$ (R178M,A326S), 20 mM Tris 7.5 pH, 1 mM EDTA, 10 mM $MgCl_2$, 10 µL GTP, 10 µL/mL GDP Antibody, and 4 nM GDP AlexaFluor® 633 Tracer in a final 20 µL volume. Control reactions lacked $G\alpha_{i1}$ protein. (B) GTPase Rates (µmol GDP x mg^{-1}x min^{-1}) were determined from GTP/GDP standard curves used to determine GDP production.

Step 2) Screen for Inhibitors.

1. Prepare compounds so they will be at desired concentration in assay. In this example, compounds are diluted to 100 µL in 20% DMSO (final concentration in 20 µL reaction of 10 µM compound/2% DMSO). Add 2 µL of compound to wells of a 384 well plate, rows A-P/columns 3-22. Add 2 µL 2% DMSO control to remainder of wells.
2. Prepare remainder of reagents on ice. Prepare Tracer Control by making assay buffer without GDP antibody. Prepare background Buffer Control by making assay buffer without GDP tracer. Add 18 µL Tracer Control to rows A-C/column 1. Add 18 µL Buffer Control to rows D-F/column 1. *Note: the remainder of column 1 and all of column 24 are available for other controls or standard curves to examine reaction progress.*
3. Prepare $G\alpha_{i1}$ alone control by adding 0.6 ng/µL $G\alpha_{i1}$ (R178M/A326S) to chilled assay buffer. Always add $G\alpha_{i1}$ (R178M/A326S) immediately before plating. Add 18 µL of $G\alpha_{i1}$ alone control to rows (A-P)/column 23.
4. Prepare ($G\alpha_{i1}$ + RGS4) mix by adding 2 ng/µL RGS4 and 0.6 ng/µL $G\alpha_{i1}$ (R178M/A326S) to chilled assay buffer. Always add $G\alpha_{i1}$ (R178M/A326S) immediately before plating. Add 18 µL of ($G\alpha_{i1}$ + RGS4) mix to rows A-P/columns 2-22. Column 2 contains the ($G\alpha_{i1}$ + RGS4) control.
5. Immediately mix reaction and then incubate at 30° C for ~75 minutes.
6. Measure fluorescence polarization using 635 nm excitation and 670 nm emission.

Step 3) Analyze Data.

1. The user must determine the threshold for what is considered a hit. In general, any compound that falls outside the average mP of ($G\alpha_{i1}$ + RGS4) ± 3 Standard deviations (µ ± 3) could be considered a hit. Compounds which were less than µ - 3, are potential inhibitors of the reaction, while compounds greater than µ + 3 are potential enhancers of the reaction.
2. Assay and compound screen robustness can be assessed by determining the Z′ and Z-factor respectively. Determine the Z′ using the average mP and standard deviation values of the $G\alpha_{i1}$ alone control (Column 23) and the ($G\alpha_{i1}$ + RGS4) control (Column 2) Figure 3. Z′ = 1-(((3(σ$G\alpha_{i1}$ alone + σ$G\alpha_{i1}$ + RGS4 control))/[µmP$G\alpha_{i1}$ alone - µmP$G\alpha_{i1}$ + RGS4 control]) Determine the Z-factor by using the average mP and standard deviation values of the $G\alpha_{i1}$ alone control (Column 3) and values of the compound wells (rows A-P/columns 3-22). Z-factor = 1-(((3($G\alpha_{i1}$ alone + $G\alpha_{i1}$ + RGS4 compounds))/[µmP$G\alpha_{i1}$ alone - µmP$G\alpha_{i1}$ + RGS4 compounds]).

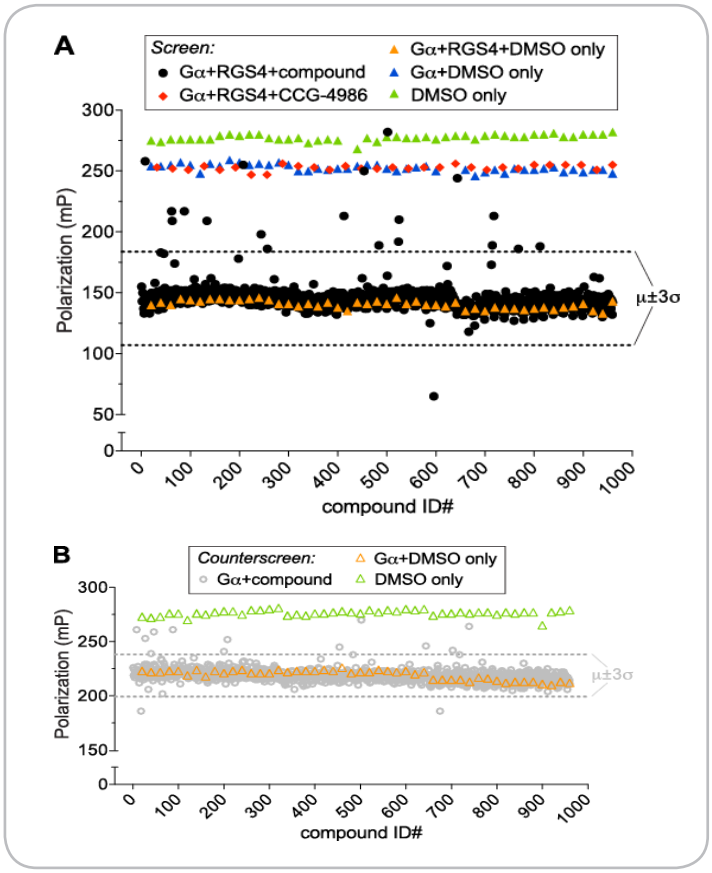

Figure 3. Pilot GenPlus Library Screen with Doubly-Mutated (DM) $G\alpha_{i1}$ in the Presence (A) and Absence (B) of RGS 4. GDP assay components were added to wells containing 50 nM $G\alpha_{i1}$ DM with (A) or without (B) 250 nM RGS4 protein and 10 µM compound (in 0.5% [v/v] final concentration of DMSO), 150 µM of reactive RGS4 inhibitor CCG-4986, or 0.5% DMSO only, as indicated in the legends. The range of signal observed (3 standard deviations [σ] about the mean [µL]) is denoted by the dashed lines for the 960-compound library screen using RGS4 and $G\alpha_{i1}$ DM. The Z′ in A at 120 minutes was 0.83, which reflects only the RGS4-dependent increase in GTPase activity of the controls. The Z factor for the GenPlus library screen was 0.73, which was obtained by excluding values from wells containing hit compounds. Of the 17 hits in A that were outside 3 StdDevs, 10 were excluded as GTPase inhibitors by B, thus the RGS4-specific hits rate was 0.7%.

Step 4) Run Dose Response Curves.

The compounds that were considered as hits specific to RGS activity were selected for profiling.

1. Serially titrate the compounds in a master plate. Transfer 2 µL of the compounds to two different 384 well plates. Add 2 µL of DMSO at 2% DMSO (serves as negative control) to row P.
2. Prepare remainder of reagents on ice. Prepare Tracer Control by making assay buffer without GDP antibody. Prepare background Buffer Control by making assay buffer without GDP tracer. Add 18 µL Tracer Control to wells P1-P4. Add 18 µL Buffer Control to wells P5-P8.
3. Prepare $G\alpha_{i1}$ alone by adding 0.6 ng/µL $G\alpha_{i1}$ (R178M/A326S) to chilled assay buffer. Always add $G\alpha_{i1}$ (R178M/A326S) immediately before plating. Add 18 µL of $G\alpha_{i1}$ alone control to set of compounds in plate 1.
4. Prepare ($G\alpha_{i1}$ + RGS4) mix by adding 2 ng/µL RGS4 and 0.6 ng/µL $G\alpha_{i1}$ (R178M/A326S) to chilled assay buffer. Always add $G\alpha_{i1}$ (R178M/A326S) immediately before plating. Add 18 µL of ($G\alpha_{i1}$ + RGS4) mix to compounds in another plate.
5. Immediately mix reaction and incubate at 30° C for ~75 minutes.
6. Measure fluorescence polarization using 635 nm excitation and 670 nm emission.

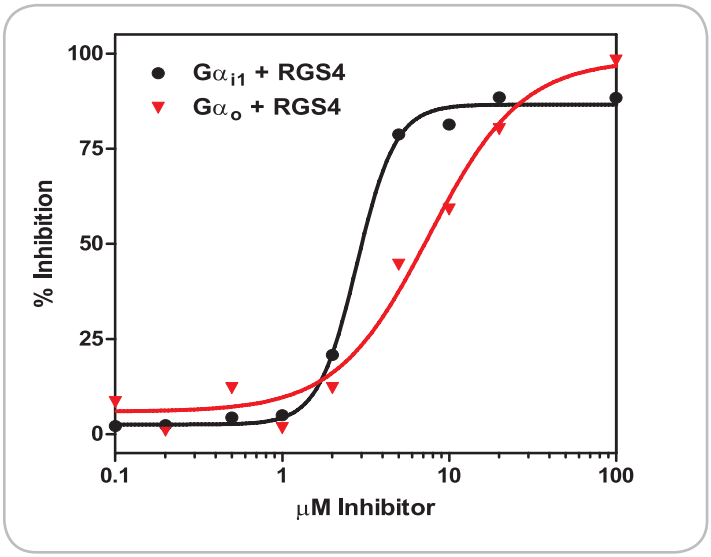

Figure 4. Follow-up dose response curves were performed with 160 compounds from the 50K compounds screen and tested for their relative effects on RGS4 and RGS17 GAP activity with doubly-mutated $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and Gαo. Shown here is an example of the effect of RGS4 on the doubly-mutated $G\alpha_{i1}$ and $G\alpha_{o}$.

Conclusions

The most direct way to detect RGS function is by measuring the increased GTPase activity of the associated Gα protein. However, the GTPase activity of isolated Gα proteins is limited by GDP dissociation, so RGS GAP activity cannot be measured using simple biochemical assays. The RGScreen™ platform overcomes this kinetic constraint by using proprietary Gα protein variants with altered GTP hydrolysis and GDP dissociation rates that enable detection of RGS GAP activity using the Transcreener® GDP Assay.

References & Notes

1. Thomas Zielinski, Adam J. Kimple, Stephanie Q. Hutsell, Mark D. Koeff, David P. Siderovski, and Dr. Robert G. Lowery: Two $G\alpha_{i1}$ Rate-Modifying Mutations Act in Concert to Allow Receptor-Independent, Steady-State Measurements of RGS Protein Activity. J Biomol Screen 2009 Dec;14(10):1195-206. Epub.
2. Sun W, Vanhooke JL, Sondek J, Zhang Q: High-throughput fluorescence polarization assay for the enzymatic activity of GTPase-activating protein of ADP-ribosylation factor (ARFGAP). J Biomol Screen. 2011 Aug;16(7):717-23. Epub 2011 May 18.
3. Bosch DE, Zielinski T, Lowery RG, Siderovski DP: Evaluating Modulators of Regulator of G-protein Signaling" (RGS) Proteins. Curr Protoc Pharmacol. 2012 Mar; Chapter 2: Unit 2.8.

Additional Information

Ordering Information

Please visit www.bellbrooklabs.com or contact BellBrook Labs for pricing for the Transcreener® Assays.
Custom quotes are available for bulk orders.

Phone Orders:
608.443.2400
866.313.7881

Fax Orders:
608.441.2967

Email Orders:
info@bellbrooklabs.com

Related Products

Transcreener® ADP² FP Assay	3010-1K
Transcreener® ADP² TR-FRET Red Assay	3011-1K
Transcreener® ADP² FI Assay	3013-1K
Transcreener® AMP/GMP Assay	3006-1K
Transcreener® UDP Assay	3007-1K
Transcreener® GDP FP Assay	3009-1K
Transcreener® GDP FI Assay	3014-1K
$G\alpha_{o}$ (R179M/A327S)	8001
$G\alpha_{i1}$ (R178M/A326S)	8002
$G\alpha_{i2}$ (R179M/A327S)	8003
$G\alpha_{i3}$ (R178M/A326S)	8004
RGS4 (29-198)	8005
RGS10 (26-151)	8006
RGS14 (56-207)	8007
RGS17 (72-206)	8008
RGS18 (75-223)	8009

Technical Information

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