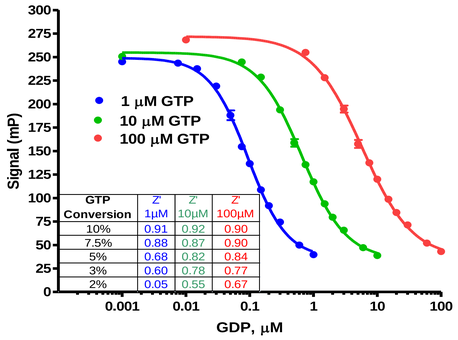
Investigation of Different GTPase Classes with the Transcreener® GDP Assay

Karen Kleman*, Thomas Zielinski, Andrew Kopp, Robert G. Lowery
BellBrook Labs LLC, Madison, WI

Introduction and Summary

The Transcreener® GDP Assays are single step, homogenous, fluorescent assays for direct detection and screening of GTPases. Available with fluorescence polarization (FP) and fluorescence intensity (FI) readouts, they provide a safe, HTS-compatible alternative to cumbersome radioassay methods and are more sensitive and less subject to interference than colorimetric phosphate detection methods. Based on BellBrook's proprietary nucleotide immmunodetection technology, the Transcreener® GDP Assays are compatible with any enzyme class that produces GDP. Interrogate inhibitors and accelerators of GTPase activity, studying both intrinsic GTPase activity and modulator proteins, which regulate GTPase activity. Here we show enzyme data for various classes of GTPases. RGScreen uses kinetically altered Gα subunits of heterotrimeric G proteins in combination with the Transcreener® GDP Assay to provide a robust HTS method for identifying inhibitors of RGS protein GAP activity: from initial screen to profiling hits. The GDP assays can also measure the GEF regulation of the GTPase activity of Gα subunits by modulators such as RIC8A, during non-receptor mediated pathways. The GTPase activity of monomeric small G proteins, including both GAP and GEF regulation can be investigated. The Transcreener® GDP assay can also interrogate other classes of GTPase domain containing proteins, like LRRK2, whose kinase activity is regulated by the GTPase domain.



GTP Conversion	Z' 1µM	Z' 10µM	Z' 100µM
10%	0.91	0.92	0.90
7.5%	0.88	0.87	0.90
5%	0.68	0.82	0.84
3%	0.60	0.78	0.77
2%	0.05	0.55	0.67

Figure 1. The Transcreener® GDP Assay Allows Direct Detection of GDP Formation in a Homogenous, HTS Compatible Format. Standard curves mimicking enzyme reactions were constructed for conversion of GTP to GDP using initial GTP concentrations of 1 µM, 10 µM, and 100 µM GTP; as GDP was added, GTP was decreased proportionately. Fluorescence polarization assays were performed in black Corning® 384 Well Black Flat Bottom Microplates (Part #3654). Assays were read in a Tecan Safire²™ multiwell reader. All points were done in 16 replicates so that Z' values could be determined (inset table).

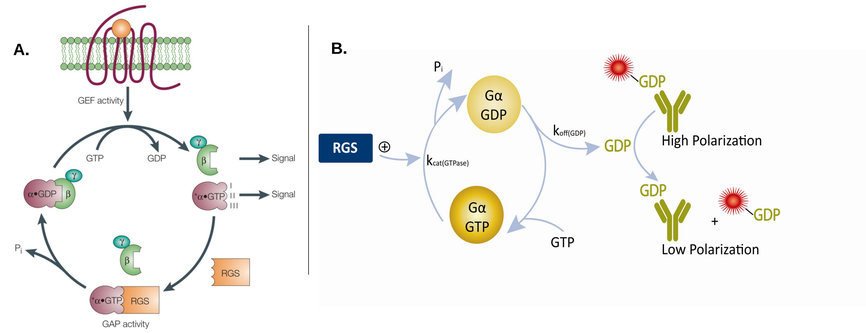
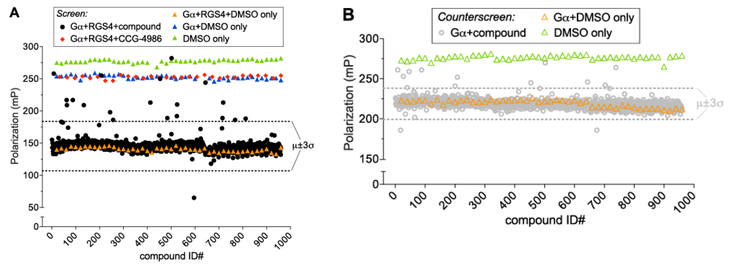
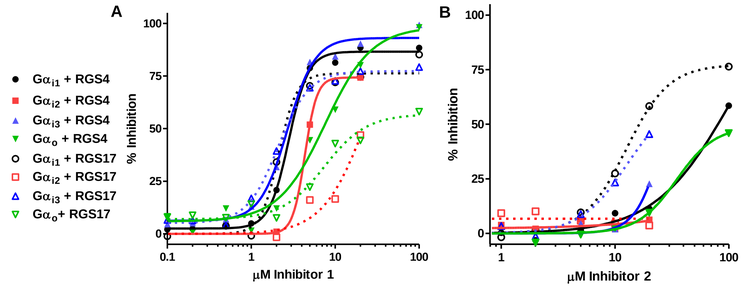
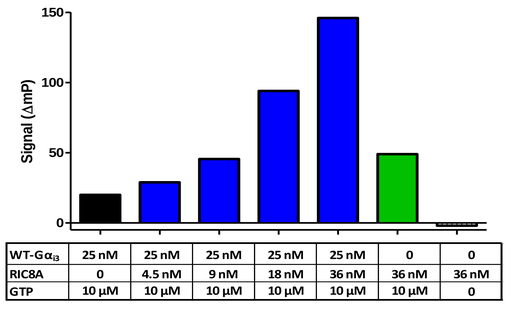


Figure 2. RGScreen Couples Kinetically Altered Gα Proteins With the Transcreener® GDP Assay Enabling Direct Detection of RGS Protein GAP Activity. **A)** RGS proteins act catalytically on Gα-GTP complexes to stimulate GTP hydrolysis rates as much as 100-fold, thereby attenuating GPCR agonist signals. **B)** 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 to measure RGS GAP activity.







WT-Gαi3	25 nM	25 nM	25 nM	25 nM	25 nM	0	0
RIC8A	0	4.5 nM	9 nM	18 nM	36 nM	36 nM	36 nM
GTP	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM	0

Figure 3. Pilot Screen Utilizing RGScreen Platform. 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 [µ]) is denoted by the dashed lines for the 960-compound library screen using RGS4 and $G\alpha_{i1}$ DM. The Z' in figure 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 figure A that were outside 3 StdDevs, 10 were excluded as GTPase inhibitors by figure B, thus the RGS4-specific hits rate was 0.7%.

Figure 4. Profiling of Hits with RGScreen. An example of follow-up dose response curves with 2 inhibitors tested for their relative effects on RGS4 and RGS17 GAP activity with doubly-mutated $G\alpha_{i1}$ $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{o}$. Representative inhibition patterns are shown. **A)** Compound 1 exhibited greater potency with $G\alpha_{i1,2,\&3}$ than with $G\alpha_{o}$, with approximately equal potency for RGS4 and RGS17 with $G\alpha_{i1\&3}$, and higher potency/efficacy for RGS4 vs. RGS17 with $G\alpha_{i2\&O}$ **B)** Compound 2 had potency for RGS17 > RGS4 for $G\alpha_{i1\&3}$, and no inhibition with $G\alpha_{i2}$ or $G\alpha_{O}$.

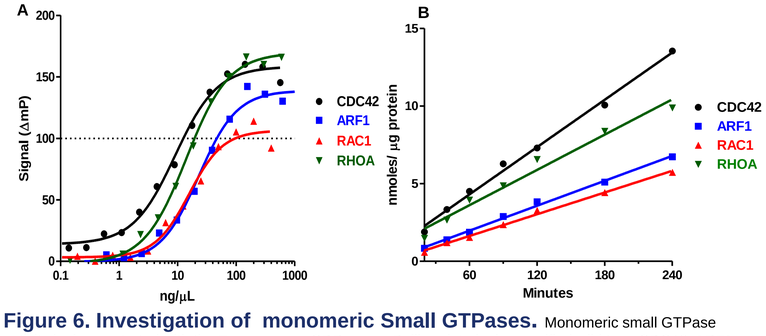


Figure 6. Investigation of monomeric Small GTPases. Monomeric small GTPase activity is demonstrated with the Transcreener GDP assay. Bellbrook Labs has purified wild type CDC42, ARF1, RAC1, and RHOA specifically to work with the GDP assay. **A)** CDC42, ARF1, RAC1 and RHOA were titrated in the presence and absence of 10 µM GTP(data not shown) along with Transcreener® GDP reagents. Data from a 2 hour time point is shown where polarization values are relative to controls that lacked enzymes. **B)** Reaction progression from figure A was plotted by converting signal to GDP product formation using a standard curve.

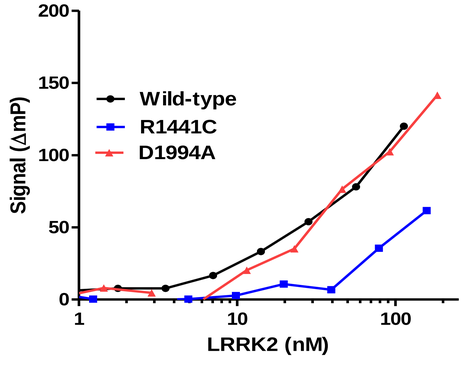


Figure 7. Detection of LRRK2 GTPase activity. GTPase activity of the wild type LRRK2, GTPase deficient (R1441C) and kinase-inactive (D1994A) mutants are demonstrated. These results show that the assay could potentially be used for detecting inhibitors of WT enzyme and activators of pathogenic, GTPase-deficient enzymes. This data also demonstrates that the GDP being produced is not the result of non-specific hydrolysis of GTP at the kinase domain (D1994A data). Varying concentrations of wild-type and LRRK2 mutants were incubated with GTP and Transcreener® GDP detection reagents for 2 hours in 384 well plates (20 µL), and fluorescence polarization was read in a BMG PHERAstar plate reader. Polarization values shown are relative to controls that lacked enzyme. Control reactions that lacked GTP showed no change in polarization (data not shown).

Conclusions and Future Directions

- **The Transcreener® GDP Assays are compatible with any enzyme class that produces GDP including: Gα subunits of heterotrimeric G proteins , monomeric small GTPases and other GTPase domain containing proteins. Modulators of GTPases, such as GAPs and GEFs are compatible .**
- **RGScreen uses kinetically altered Gα protein variants in combination with the Transcreener® GDP Assay to provide a robust HTS method for identifying inhibitors of RGS protein GAP activity.**
- **Bellbrook labs will soon be offering validated GTPases ,GEF and GAP proteins compatible with the Transcreener® GDP assay.**

Acknowledgements

Funding for this work was provided by NIH SBIR Grant #R44NS059082.

BellBrook Labs, 5500 Nobel Drive, Suite 250, Madison, WI 53711
866.313.7881 or 608.443.2400