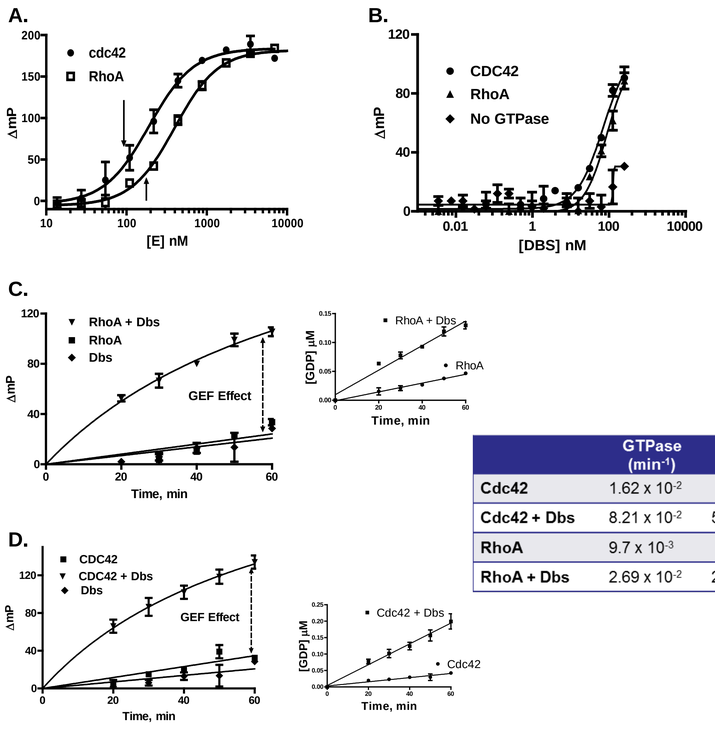
A High Throughput Assay for Rho Guanine Nucleotide Exchange Factors Based on the Transcreener® GDP Assay

ROBERT G. LOWERY[1], KENT L. ROSSMAN[2], MEERA KUMAR[1], TOM ZIELINSKI[1]
[1]BellBrook Labs, Madison, WI; [2]Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Overview

Rho family GTPases act as molecular switches controlling cell growth, movement and gene expression by cycling between inactive GDP- and active GTP-bound conformations. Guanine nucleotide exchange factors (GEFs) positively regulate Rho GTPases by accelerating GDP dissociation to allow formation of the active, GTP-bound complex. Rho proteins are directly involved in cancer pathways, especially cell migration and invasion, and inhibiting GEFs holds potential as a therapeutic strategy to diminish Rho-dependent oncogenesis. Methods for measuring GEF activity suitable for high throughput screening (HTS) are limited. We developed a simple, generic biochemical assay method for measuring GEF catalytic activity based on the fact that GDP dissociation is generally the rate-limiting step in the Rho GTPase catalytic cycle, and thus addition of a GEF causes an increase in steady state GDP formation. We used the Transcreener® GDP Assay, which relies on selective immunodetection of GDP, to measure the GEF- dependent stimulation of steady state GTP hydrolysis by small GTPases using Dbs as a GEF for Cdc42 and RhoA and P-Rex1 for Rac1. The assay is well suited for HTS, with a homogenous format and far red fluorescence polarization readout, and it should be broadly applicable to diverse Rho GEF/GTPase pairs.

Development of GEF assays with Transcreener GDP Assay



	GTPase (min^{-1})	GEF Effect
Cdc42	1.62 x 10^{-2}	
Cdc42 + Dbs	8.21 x 10^{-2}	5.1x
RhoA	9.7 x 10^{-3}	
RhoA + Dbs	2.69 x 10^{-2}	2.8x

Figure 3. Development of GEF assays for Dbs/RhoA and Dbs/Cdc42. A. GTPases are first titrated to identify the concentration that produced 20% of the maximal signal: 39 nM Cdc42 and 78 nM RhoA. **B.** Titration of Dbs into limiting GTPase to determine optimimal Dbs concentration (125 nM). **C, D.** Continuous assays are used to determine rates of GTPase activity in the presence and absence of Dbs and polarization data is converted to GDP formation using a standard curve as in Fig. 1C. **Table:** The ratio of rates in the presence and absence of DBS is used to determine the GEF effect.

TRANSCREENER® GDP Assay

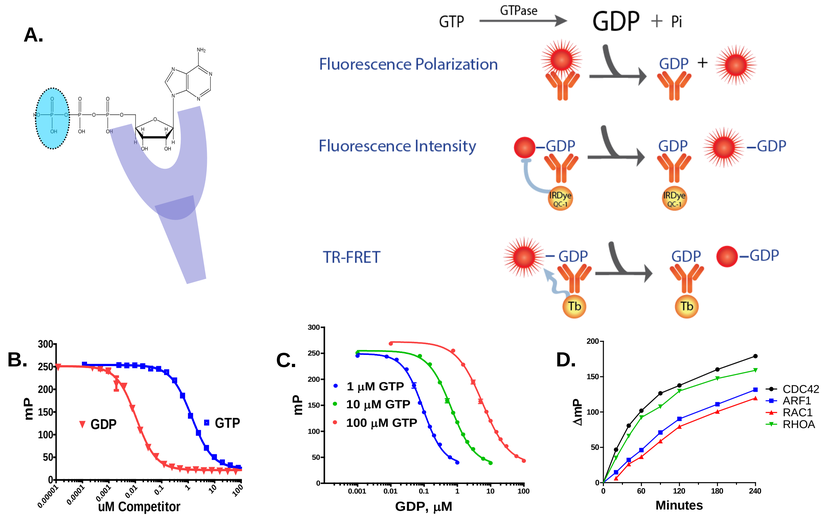
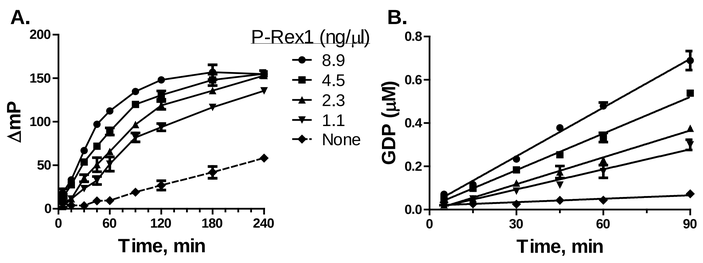


Figure 1. Selective immunodetection of GDP enables homogenous detection of GTPase activity. A. The Transcreener GDP Assay relies on highly selective antibodies and far red fluorescent tracers to allow homogenous detection of GDP with FP, FI or TR-FRET readouts. **B.** Greater than 100-fold selectivity enables detection of GDP in the presence of excess GTP. **C.** The dynamic range of the assay can be tuned for different initial GTP concentrations. **D.** GTP hydrolysis by small GTPases can be monitored by detecting GDP formation.

Detection of P-Rex1 GEF activity with Rac1



P-Rex1 (ng/μl)	Rac 1 GTPase (nM/min)	GEF Effect
None	0.53	
1.1	3.1	5.8x
2.3	4.1	7.8x
4.5	5.6	10.6x
8.9	7.5	14.1x

Figure 4: P-Rex1 simulates Rac1 GTPase activity. Reactions containing a limiting concentration of Rac1 (50 nM), 10 μM GTP, and Transcreener GDP detection reagents were titrated with P-Rex1 (DH-PH domain) and read at intervals. **A.** Time courses for P-Rex1 dependent stimulation of Rac1 GTPase activity. **B.** Polarization data from A. was converted to GDP formation using a standard curve as in Fig. 1C**. Table:** Rates of GDP formation by Rac1 and the effect of GEF stimulation.

GEF Assay Principle

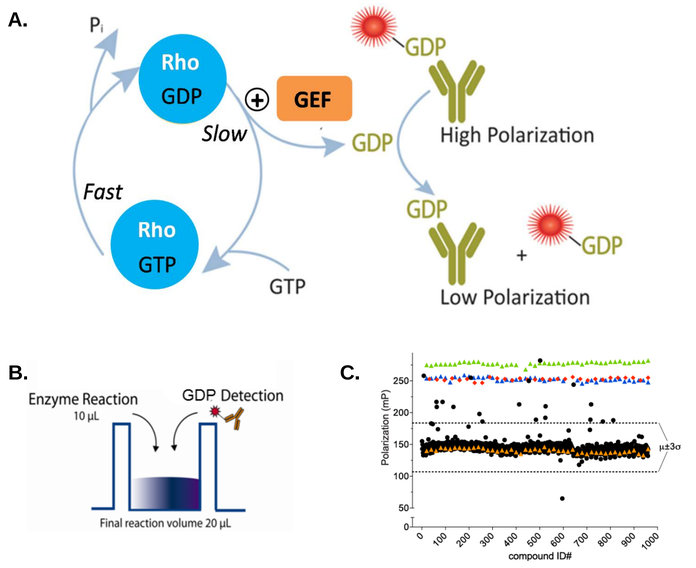


Figure 2. GEFs accelerate steady state GTP hydrolysis rates. A. By accelerating the rate limiting step of the GTPase catalytic cycle, GEFs enhance the steady state rates of GDP formation by GTPases, which can be detected using the Transcreener GDP Assay. The Transcreener GDP assay is designed for HTS with a mix-and-read format **(B)** and 24 hour reagent and signal stability for robust screening performance **(C)**.

Conclusions

- The Transcreener GDP Assay, an HTS-compatible method that relies on highly selective immunodetection of GDP with a far red fluorescent readout, can be used to detect GDP formation by small GTPases.
- By accelerating the release of GDP, the rate limiting step in the GTPase catalytic cycle, a GEF causes an increase in steady state GTPase activity, which can be readily detected with the Transcreener GDP Assay.
- The Transcreener GDP-based GEF assay was demonstrated for Dbs with RhoA and Cdc42 and for P-Rex1 with Rac1 and should be applicable to most if not all GEF/GTPase pairs, as GDP dissociation is generally rate limiting.
- The catalytic nature of the assay may provide advantages over fluor-GTP binding assays or protein-protein interaction assays in terms of sensitivity and the diversity of inhibitors identified.

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BellBrook Labs 866.313.7881 or 608.443.2400