

## **University of New Mexico Assay Overview:**

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### **HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases**

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### **Assay Background and Significance:**

Ras and related small molecular weight GTPases function in the regulation of signaling and cell growth, and collectively serve to control cell proliferation, differentiation and apoptosis [Tekai et al. 2001; Wennerberg et al. 2005]. Ras family members (Ras, Rap, Ral, Rheb among others) are recognized their control of growth factor receptor signaling cascades. When mutant or hyperactivated, Ras family members contribute contribute to oncogenesis. The Ras-related GTPases are divided into four subfamilies with the Rab proteins regulating membrane transport, Rho proteins (including Rac and Cdc 42) regulating cytoskeletal rearrangements and responses to signaling, Arf/Sar proteins regulating membrane and microtubule dynamics as well as protein transport, and Ran protein controlling nucleocytoplasmic transport. Ran is the subject of an independent screening initiative (Weiss, K. UC Berkeley) and Arf family members are membrane anchored via their N-termini in contrast to other family members, which are anchored via their C-termini [Kahn et al. 2006]. This project focuses on representative Ras, Rho, and Rab family members to validate the approach for the identification of new chemical compounds with novel therapeutic potential in cell signaling and growth control.

Ras and Ras-related GTPase functions are tightly regulated, and dysregulation is causal in a wide variety of human diseases. Proper functioning of Ras and Ras-related GTPases is regulated at the level of localization and nucleotide binding and hydrolysis. Ras mutations resulting in impaired GTP hydrolysis and plasma membrane hyperactivation are linked to many human cancers [Farnsworth et al. 1991; Sukumar et al. 1983; Taparowski et al. 1982; Boylan et al. 1990; Hruban et al. 2004; Abrams et al. 1996]. Point mutations in the Rab and Rho GTPases are also causal in diverse human diseases affecting pigmentation, immune, and neurologic functions [Houlden et al. 2004; Verhoeven et al 2003; Williams et al. 2000; Bahaderan et al. 2003; and preliminary findings]. Rab and Rho mutants identified in human disease act as dominant negatives either due to a failure to bind GTP or due to inappropriate coupling of the active proteins with downstream effectors. To date, inhibition of Ras and Ras-related proteins has relied largely on altering membrane recruitment with various drugs affecting prenylation [Morgillo-Flee HY, 2006; Russell RG, 2006; Park, et al. 2002]. Generally, Ras proteins must be farnesylated for proper membrane localization, while Rab and Rho proteins are geranylated. Such strategies lack specificity and are problematic because each of these prenylation machineries is required for the proper function of many Ras superfamily members. Rational drug design has only recently been applied to identify the first two small molecule inhibitors of Rho GTPase family members [Gao, et al. 2004; Nassar et al. 2006]. Therefore, broadly testing the Ras-related GTPases as targets for small molecule inhibitors and activators is expected to identify new classes of compounds that may be useful in the treatment of human disease, as well as in unraveling the molecular details of how Ras-related GTPases function.

The assay described here is a no-wash fluorescent GTP-binding assay adapted to multiplexed, high-throughput measurements whereby multiple GTPases are simultaneously

screened against the MLSCN library. The specificity is based on the observation that individual GTPases including wt and activated forms exhibit measurably distinct affinities for Bodipy-FI-GTP vs GTP. The multiplex assay involves the binding of fluorescent GTP to G protein-GST fusion proteins on GSH beads. A set of six G proteins (Rac 1 wt, Rab7 wt, Rac 1 activated, Ras wt, Rab 2 wt., CDC wt) are arrayed under conditions of divalent molecule depletion in the assay reported here.



## Protocol:

Bead sets are coated with individual GST-small G proteins, blocked with 0.1% BSA in Buffer (0.01% NP-40; 30mM HEPES pH 7.5; 100mM KCl; 20mM NaCl; 1mM EDTA) and incubated overnight at 4 degrees C. Beads are washed in wash buffer (0.1% BSA and 1mM DTT). The different bead sets, acquired from Duke Scientific, have similar size (~ 4 micron diameter) however they are distinguished by varied magnitude of emission at 665 +/-10 nm with excitation at 635 nm.

The assay is conducted in 384-well microplates in a total well volume of 10.1 microliters (5 microliters of bead mixture, 0.1 microliters of test compound, and 5 microliters of 200nM Bodipy-FL-GTP in buffer containing BSA and DTT for a final concentration of GTP of 100nM). Positive Controls, which contains bead mixture and fluorescent GTP but no test compound, are located in columns 1 and 2 on plate. Negative Controls, bead mixture with fluorescent GTP and 0.5 mM unlabeled GTP, are collected from a separate test tube. Plates are placed on rotators and incubated for 40-45 minutes at 4 degrees C.

Sample analysis is conducted with the HyperCyt(R) high throughput flow cytometry platform [Kuckuck, et al., 2001; Ramirez, et al., 2003]. Flow cytometric data of light scatter and fluorescence emission at 530 +/- 20 nm (FL1) and 665 +/- 10 nm (FL8) are collected via Cyan (Dako). The resulting time-resolved single data file per plate is analyzed by IDLQuery software to determine the compound activity in each well. Gating based on FL8 emission distinguishes the beads coated with different proteins, and the median fluorescence per bead population is calculated.

### Calculations:

In order to get a significant measurement of the effect by a compound on a particular protein, 25 beads with that particular protein bound is the minimum number of beads to be collected from a well. When less than 25 beads are counted, the result for that protein is considered missing. Compounds from missing wells is given PUBCHEM\_ACTIVITY\_OUTCOME = 4, and are automatically assigned a PUBCHEM\_ACTIVITY\_SCORE of 0. In this set of 138,773 compounds, for Rac activated mutant there are only 51 missing compounds.

When the measured emission is potentially attributed to the innate fluorescence of the compound, these compounds are flagged as "Possible Fluorescent Compound" (see column titled PUBCHEM\_ASSAYDATA\_COMMENT) and the results from a fluorescent compound is considered to be 'inconclusive' (PUBCHEM\_ACTIVITY\_OUTCOME = 3, PUBCHEM\_ACTIVITY\_SCORE = 0). Assessment of fluorescent compound is made by comparing the influence of compound fluorescence on all the proteins in one well. Difference between Sample fluorescence and Positive Control fluorescence are calculated for all the different proteins in a well. The following equation describes this difference (CompoundFLinRacWT) for RacWT;

$\text{CompoundFLinRacWT} = \text{RacWTSampleFL} - \text{PCntrlRacWTFL}$

with RacWTSampleFL being the Sample fluorescence and PCntrlRacWTFL the Positive Control fluorescence of RacWT coated beads. Next, the coefficient of variation (CV) of all these CompoundFL from the different protein beads (RacWT, Rab7, RacACT, RasWT, Rab2, Cdc) in the same well are calculated. If the CV was less than 30%, meaning the compound attributed fluorescence was very similar between all the different proteins, then the compound was flagged as a potential fluorescent compound. In this set of 138,773 compounds, for Rac activated mutant there are only 2810 fluorescent compounds.

Due to potential systematic trends in data over the entire plate (whole plate trends), normalization is utilized to calculate the percent activity of the test compound. Whole plate trends of the positive controls are evaluated by linear regression. Then due to the plate location of a sample, a calculated Positive Control value (notated with WPcalc) is utilized for calculating % activity normalized by whole plate:

$\% \text{ Activity} = 100 \times (\text{SampleFL} - \text{NCntrl}) / (\text{PCntrl\_WPcalc} - \text{NCntrl})$

where all variables are Median Fluorescence Intensity associated with the bead set bound with a specific protein. SampleFL is for beads in wells containing test compound, PCntrl\_WPcalc is the well-specific, calculated value based on whole plate linear regression of wells without test compounds, and NCntrl is for measurement in presence 0.5 mM unlabeled GTP. Baseline of % Activity is 100%.

Maximum PUBCHEM\_ACTIVITY\_SCORE of 100 was given for the primary screening. The absolute difference between baseline and %Activity was used to calculate PUBCHEM\_ACTIVITY\_SCORE:

$\text{PUBCHEM\_ACTIVITY\_SCORE} = |100\% - \% \text{Activity}|$

A compound was considered "Active" if the change in %Activity was greater than 20% from baseline (i.e.,  $\text{PUBCHEM\_ACTIVITY\_SCORE} \geq 20\%$ ). PUBCHEM\_ACTIVITY\_OUTCOME is indicated as 2 for "Active" and 1 for "Non-active".

For "Active" compounds, the type of activity is indicated in column entitled "ActivityType". "Inhibitor" is a compound with  $< 80\%$  Activity and "Activator" is a compound with  $> 120\%$  Activity.

Average Zprime for this screen was 0.90 +/- 0.03.

Keywords: NIH Roadmap, NMMLSC, high throughput flow cytometry, GTPase, multiplex bead-based screening