A Homogeneous TR-FRET Assay for Inhibitors of FRAP1 (mTOR) Kinase Activity

Connie S. Lebakken, Laurie Reichling, Steve Riddle, Kevin L. Vedvik, Leisha Kopp, Rica Bruinsma, Robert Horton and Kurt Vogel
Invitrogen Corporation • 501 Charmany Drive • Madison, Wisconsin 53719 • USA

Introduction

The mammalian Target of Rapamycin (mTOR, FRAP1) is a serine/threonine protein kinase that has been shown to regulate multiple cellular responses including cell growth, proliferation, motility, survival and protein synthesis. mTOR kinase activity is regulated by several upstream signaling pathways and its dysregulation has been implicated in several forms of cancer. We have produced a recombinant active form of human mTOR and have developed an HTS-compatible Time-Resolved FRET (TR-FRET) based assay to screen for inhibitors of mTOR *in vitro*. A physiological substrate of mTOR, 4E-BP1, has been expressed and purified as a fusion with green fluorescent protein (GFP). A terbium-labeled anti-phosphospecific 4E-BP1 antibody is utilized to detect phosphorylation of the substrate by this recombinant mTOR. This approach enables robust and sensitive detection of compounds that target both the catalytic and non-catalytic sites of mTOR including wortmannin and FKBP12-rapamycin. Additionally, we present data utilizing a FRET-based kinase assay to detect inhibitors of mTOR.

References

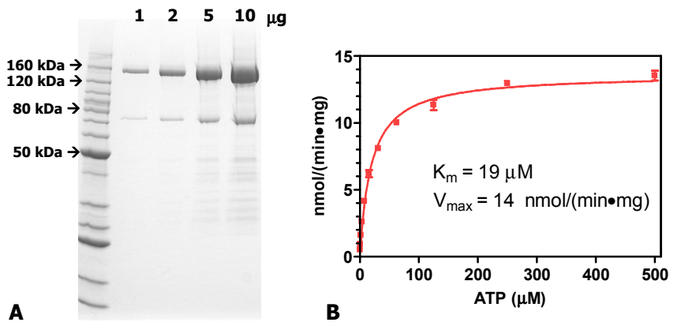
(1) Riddle, S.M., Vedvik, K.L., Hanson, G.T., and K.W. Vogel. (2006) Time-resolved fluorescence resonance energy transfer kinase assays using physiological protein substrates: application of terbium-fluorescein and terbium-green fluorescent protein fluorescence resonance energy transfer pairs. Anal. Biochem. 356 (1): 108-116.

More information on this kinase and assay at:

www.invitrogen.com/kinase

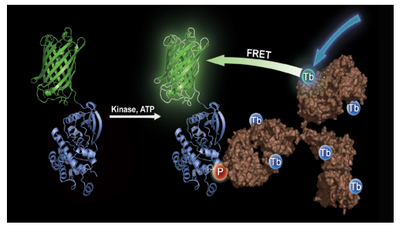
www.invitrogen.com/lanthascreen

Figure 1 – Characterization of recombinant mTOR by SDS-PAGE and radiometric assay



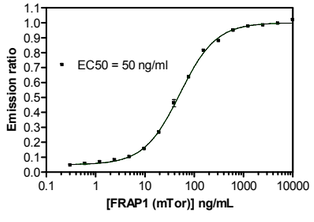
Truncated, GST-tagged mTOR was purified from baculovirus-infected insect cells. The expression construct contains the FRB, FAT, kinase, and FATC domains, but lacks the HEAT repeats. (A) Purity was assessed by Coomassie-stained SDS-PAGE. (B) ATP K_m was determined by radiometric assay using 40 µM 4E-BP1 as the substrate in 50 mM HEPES pH 7.5, 0.01% polysorbate 20, 1 mM EGTA, and 10 mM $MnCl_2$.

Figure 2 – Principle of GFP-Based TR-FRET Kinase Assay



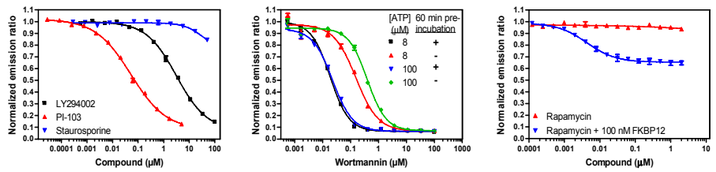
TR-FRET kinase assays are often performed with fluorophore-labeled peptide substrates. Although many kinases will phosphorylate such substrates, others show a strong preference for native protein substrates. By expressing native protein substrates as GFP fusions, robust assays have been developed for such problematic kinases. (1) After phosphorylation of a GFP-tagged substrate, a terbium-labeled phospho-specific antibody is added, resulting in FRET from terbium to GFP. Typically, the ratio of the acceptor (GFP) emission to donor (Tb) emission is calculated (Emission ratio). For mTOR, the reagents used are a GFP fusion of 4E-BP1 and a terbium labeled anti-pT46 4E-BP1 antibody.

Figure 3 – Kinase titration curve at ATP $_{Km(App)}$



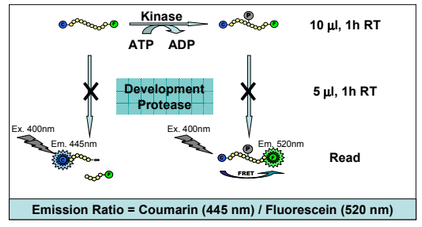
FRAP1 (mTOR) kinase titration using 400 nM GFP-4EBP1 and 8 µM ATP in 50 mM HEPES pH 7.5, 0.01% polysorbate 20, 1 mM EGTA, 10 mM MnCl2 and 1% DMSO. After a 1 hour room temperature incubation, the assay was stopped with 10 mM EDTA and phosphorylation was detected with 2 nM Tb-anti-pT46 4E-BP1 antibody. TR-FRET measurements were obtained on either a Tecan Ultra™ or BMG Pherastar™ plate readers. All assays were performed in Corning Low-volume 384-well plates (#3676).

Figure 4 – FRAP1 (mTOR) TR-FRET Assay Validation



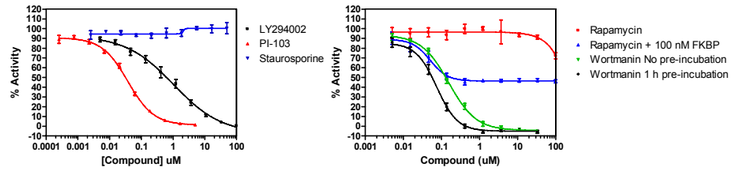
The TR-FRET FRAP1 (mTOR) assay was validated with several known mTOR inhibitors. As expected, LY294002 and PI-103 inhibited activity while staurosporine had little effect. Wortmannin, an ATP competitive, slow binding, irreversible inhibitor was tested at different ATP concentrations with or without a 60 minute pre-incubation. As expected, the inhibitor was more potent with a pre-incubation. Inhibition with rapamycin was completely dependent on FKBP12 (up to 1 uM rapamycin), but inhibition was only partial. The Z' value was 0.88 under these conditions.

Figure 5 – Principle of FRET-based Z'-LYTE™ assay



Z'-LYTE™ FRET based kinase assays are performed with peptide substrates labeled with a coumarin and fluorescein FRET pair, one at each end of the peptide. A specific protease site is designed into the peptide, but upon phosphorylation, the peptide becomes resistant to protease cleavage, thereby allowing detection of the phosphorylated peptide by excitation of the coumarin fluorophore at 400 nm and detection of the coumarin fluorophore at 445 nm and the fluorescein fluorophore at 520 nm. The acceptor emission to donor emission ratio is used with 0% and 100% phosphorylated peptide controls to calculate a % phosphorylation. Inhibitor titrations are generally performed at kinase concentrations which demonstrate 30% phosphorylation of the peptide.

Figure 6 –FRAP1 (mTOR) Z'-LYTE™ Assay Validation



The Z'-LYTE™ FRAP1 (mTOR) assay was also validated with the same set of inhibitors. One hour, 10 µL enzyme assays were performed with mTOR at EC_{30} (4 µg/ml), 2 µM Z'-LYTE™ Ser/Thr11 peptide substrate, and 10 µM ATP in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 1 mM EGTA, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT and 1% DMSO. Development reagent was added and after 1 hour of additional incubation fluorescence measurements were obtained on a Tecan Safire™ plate reader. The Z' value for this assay under these conditions was greater than 0.7.

Figure 6 – Comparison of results between fluorescent assay formats

Compound	(TR-FRET) IC_{50}	(Z'-LYTE™) IC_{50}
Rapamycin (with FKBP12)	4.3 nM	51 nM
Wortmannin (1 hr pre-incubation)	19 nM	75 nM
PI-103	52 nM	38 nM
Wortmannin (no pre-incubation)	155 nM	156 nM
LY294002	3.2 µM	1 µM
Staurosporine	>50 µM	>50 µM

Because the TR-FRET format uses 50—fold less kinase in the assay at the $K_{m(App)}$ for ATP (80 ng/ml compared to 4 µg/ml in the conditions presented here which correspond to 0.5 nM and 24 nM kinase in the assay respectively), the TR-FRET assay is able to discriminate between potencies of inhibitors with very low nM IC_{50} values.

Results and Conclusions

- Recombinant, active FRAP1 (mTOR) has been successfully expressed and purified.
- A robust, TR-FRET assay for FRAP1 (mTOR) kinase activity has been developed, utilizing a GFP-fusion of the native substrate 4E-BP1 and a terbium-labeled phospho-specific 4E-BP1 antibody.
- The FRAP1 (mTOR) TR-FRET kinase assay demonstrates the expected potencies for the known inhibitors rapamycin, wortmannin, and PI-103.
- A peptide-based FRET assay (Z'-LYTE™ format) has also been developed for FRAP1 (mTOR), with general agreement in rank-order potency between the two assay formats.
- Screening for mTOR inhibitors will be offered in Invitrogen's SelectScreen™ Profiling service beginning May 2007.