Biochemical assays for selectivity profiling across the entire PI3 kinase family

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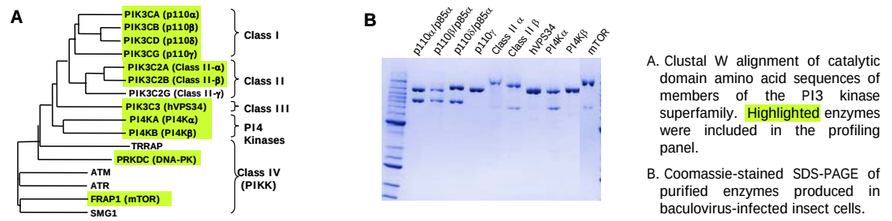
The Human Kinome

Introduction

The selectivity of small molecule kinase inhibitors impacts both efficacy and off-target toxicity and is therefore crucial to effective development of therapeutics. Although PI3 kinase and mTOR inhibitors are a major focus of drug discovery efforts, tools and methods for assessing selectivity of these inhibitors have been lacking. We describe the development of fluorescence-based, HTS-compatible assays for all types of PI3 kinase family members, including Class I, II and III PI3 kinases, Type III PI4 kinases, and PIKKs (mTOR, DNA-PK). Detection of lipid kinase activity was achieved using the new Adapta™ assay, which detects ADP formation using a Europium→Alexa Fluor® 647 TR-FRET pair. Assays for lipid kinases were formulated with stable, optimized lipid substrate formulations and highly purified enzymes produced in insect cells. Inhibition of mTOR and DNA-PK was measured with LanthaScreen™ TR-FRET kinase assays. The selectivity of eight small molecule inhibitors was examined across the family-wide panel.

Figure 1 – PI3 Kinase Superfamily



A. Clustal W alignment of catalytic domain amino acid sequences of members of the PI3 kinase superfamily. Highlighted enzymes were included in the profiling panel.

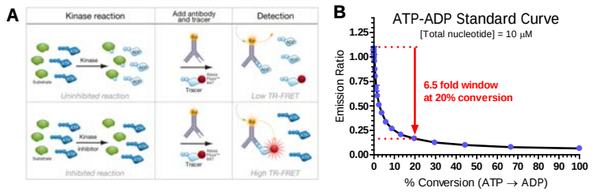
B. Coomassie-stained SDS-PAGE of purified enzymes produced in baculovirus-infected insect cells.

Figure 2 - ATP $K_{m,\ app}$ values for lipid kinases

Enzyme	[Enzyme] (µg/mL)	ATP $K_{m,app}$ (µM)
p110α/p85α	2	59 +/- 9 µM
p110β/p85α	15	680 +/- 140 µM
p110δ/p85α	5	211 +/- 37 µM
p110γ	20	16 +/- 4 µM
Class II-α	0.5	104 +/- 19 µM
Class II-β	5	214 +/- 11 µM
PIK3C3 (hVPS34)	1.5	24 +/- 1 µM
PI4Kα	15	152 +/- 11 µM
PI4Kβ	0.6	128 +/- 8 µM

To facilitate inhibitor studies, ATP $K_{m,\ app}$ values were determined for all lipid kinases in radiometric assays under the same assay conditions as those used for the high throughput Adapta™ assays. Assay conditions are summarized in Figure 5B and the enzyme concentrations used in the table above. 50 µL kinase reactions were performed at room temperature for between 15 and 40 minutes with trace amounts of γ-^{32}P ATP. Reactions were stopped by adding 200 µl 1M HCl. Reaction products were extracted by addition of 400 µL 1:1 mixture of chloroform:MeOH and 125 µL of the organic phase was counted in a liquid scintillation counter.

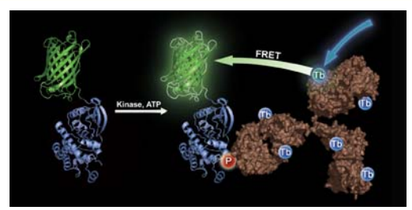
Figure 3 – Adapta™ Assay Schematic (for lipid kinases)



A. The Adapta™ assay is performed in two steps. First, a 10 µL standard kinase reaction is performed in the presence or absence of inhibitor. In the second step, formation of ADP is detected by adding a europium-labeled anti-ADP antibody, Alexa Fluor® 647 labeled ADP, and EDTA. ADP formed by uninhibited kinase disrupts the antibody-tracer interaction, resulting in a low TR-FRET signal. Inhibited kinase forms less ADP, resulting in an intact antibody-tracer interaction and a high TR-FRET signal.

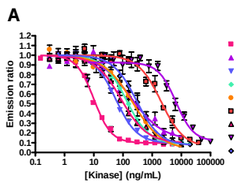
B. A standard curve was generated by using various ratios of ATP and ADP such that the total nucleotide concentration was 10 µM.

Figure 4 – LanthaScreen™ Assay Schematic (for mTOR & DNA-PK)



LanthaScreen™ TR-FRET kinase assays are performed with fluorescein-labeled peptide substrates or GFP fusions to a physiological protein substrates. After phosphorylation of the substrate, a terbium-labeled phospho-specific antibody is added, resulting in FRET from terbium to GFP or fluorescein. Typically, the ratio of the acceptor (GFP or Fl) emission to donor (Tb) emission is calculated. For mTOR, the reagents used are a GFP fusion of 4E-BP1 and a terbium labeled anti-pT46 4E-BP1 antibody. For, DNA-PK, the substrate used is a fluorescein-labeled peptide derived from p53.

Figure 5 – Optimization of reaction conditions for lipid kinases in Adapta™ format



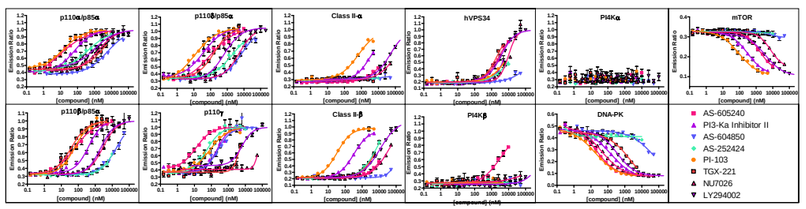
B

Enzyme	EC_{80} conc. of enzyme	Substrate	Assay Buffer
p110α/p85α	26 ng/mL (0.12 nM)	50 µM PIP2:PS	Kinase Buffer R+ 2 mM DTT
p110β/p85α	960 ng/mL (4.5 nM)	50 µM PIP2:PS	Kinase Buffer R + 2 mM DTT
p110δ/p85α	180 ng/mL (0.79 nM)	100 µM PIP2:PS	Kinase Buffer R + 2 mM DTT
P110γ	460 ng/mL (3.5 nM)	50 µM PIP2:PS	Kinase Buffer R + 2 mM DTT
Class II-α	1100 ng/mL (6.3 nM)	100 µM PI (no PS)	Kinase Buffer R + 2 mM DTT
Class II-β	7000 ng/mL (42 nM)	100 µM PI (no PS)	Kinase Buffer R + 2 mM DTT
PIK3C3 (hVPS34)	360 ng/mL (2.8 nM)	100 µM PI:PS	Kinase Buffer Q + 2 mM DTT + 5 mM MnCl2
PI4Kα	19000 ng/mL (160 nM)	100 µM PI:PS	PI4K Buffer + 2 mM DTT
PI4Kβ	1000 ng/mL (8.7 nM)	100 µM PI:PS	PI4K Buffer + 2 mM DTT

10 µL kinase reactions using a serial dilution of kinase were performed in the presence of 10 µM ATP and the substrate and buffer indicated in Table B. After 1 hr at room temperature, 5 µL of a detection mix was added, which was comprised of 12 nM Alexa Fluor 647® ADP Tracer, 6 nM Adapta™ Eu-anti-ADP Antibody, and 30 mM EDTA in TR-FRET dilution buffer. After 30 minutes, the plates were read on a BMG Pherastar or Tecan InfiniTE® F500 plate reader. The EC_{80} value for each kinase was then calclulated and used for inhibitor studies (Figure 6).

Kinase Buffer Q = 50 mM Hepes pH 7.3, 0.1% CHAPS, 1 mM EGTA
Kinase Buffer R = 50 mM Hepes pH 7.5, 3 mM MgCl2, 1 mM EGTA, 100 mM NaCl, 0.03% CHAPS
PI4K Buffer = 20 mM Tris pH 7.5, 5 mM MgCl2, 0.5 mM EGTA, 0.4% Triton X-100

Figure 6 – Inhibitor titrations across all lipid kinases, mTOR, and DNA-PK



For lipid kinases, 10 µL kinase reactions with variable amounts of inhibitor were performed as in Figure 5. The raw emission ratio values depicted were then converted to product formation (% conversion of ATP) which was calculated by converting emission ratio data to nucleotide concentrations based on a stardard curve, such as in Figure 3. The IC_{50} values calculated are listed in Figure 7.

For mTOR, 10 µL kinase reactions were performed with 35 ng/mL mTOR, 400 nM GFP-4EBP1, and 10 µM ATP in mTOR Buffer + 2 mM DTT and incubated for 60 minutes at room temperature. 10 µL of an antibody+EDTA mixture (4 nM Ab/20 mM EDTA) in TR-FRET dilution buffer was then added and the plates were read following a 30 minute incubation on a BMG Pherastar plate reader. DNA-PK reactions were performed as for mTOR, except in 1X Kinase Buffer A and with 1U/µL DNA-PK, 2.5µg/mL sonicated calf thymus DNA, 1 mM DTT, 1600 nM Fluorescein-p53 Peptide Substrate substrate, and 3 µM ATP. Detection was achieved with LanthaScreen™ Tb-anti-phospho-p53 [pSer15] antibody.

Figure 7 – Selectivity profile of commonly used PI3K/mTOR inhibitors

Compound	LY294002	NU7026	TGX-221	PI-103	PI3Kα Inhibitor II/IV	AS-252424	AS-604850	AS-605240
Enzyme								
p110α/p85α	280	600	1,300	5.9	19	90	1,400	2.8
p110β/p85α	500	580	13	12	48	3,600	3,500	5.0
p110δ/p85α	170	1,300	44	3.1	11	470	1,400	24
p110γ	3,500	>10,000	1,300	28	85	15	78	4.1
Class II-α	11,000	17,000	>10,000	100	670	>10,000	>50,000	4,200
Class II-β	910	2,900	940	4.7	48	640	49,000	3,100
PIK3C3 (hVPS34)	520	1,400	220	750	830	1300	16,000	300
PI4Kα	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
PI4Kβ	110,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	810
PRKDC (DNA-PK)	246	36	894	17	155	>10,000	>50,000	21
FRAP1 (mTOR)	3451	7,363	>10,000	91	299	>10,000	>10,000	1,717

LEGEND

IC_{50} (nM)
>10,000
2,000-10,000
501-2,000
100-500
10-100
<10

Conclusions

- The Adapta™ & LanthaScreen™ assays can be applied broadly to profile compounds against all lipid kinases in the PI3K family and the key PIKK enzymes mTOR and DNA-PK.
- For more information & details about the assay technologies & kinases profiled visit:
  - invitrogen.com/Adapta
  - invitrogen.com/LanthaScreen
  - invitrogen.com/Kinase
  - invitrogen.com/Kinasebiology

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