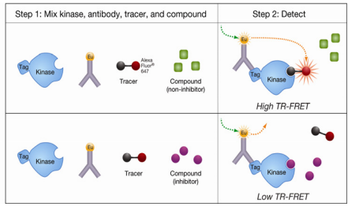
A flexible kinase inhibitor assay platform for active, non-activated, and impure kinase preparations

Caroline Wessely, Connie Lebakken, Laurie Reichling, Jason Ellefson, Kun Bi and Steve Riddle, Discovery and ADMET Systems, Madison, WI, USA, 53719

ABSTRACT

Kinase activity-based assays are cost-effective and widely used to drive early drug discovery and lead identification, however, they typically require purified, active kinase preparations be produced that are shown to phosphorylate a known substrate. While it is possible to satisfy these requirements for the well-studied kinases, there are many kinases which lack one or more of these requirements, such as CDK8 and STK33, making drug discovery efforts difficult. To solve for these assay development problems, we have developed a TR-FRET-based binding assay platform which allows for characterization of compounds with kinases that are not sufficiently purified or do not demonstrate measurable activity against putative or potential substrates. This assay addresses kinase assay requirements early in the drug discovery process, and should enable more targets to be screened sooner, resulting in compounds being made available for lead optimization faster. The ability to detect and characterize binding of compounds to non-activated kinases is also a benefit to the lead optimization process, as compounds that bind preferentially to kinases in their non-activated state, such as Imatinib (Gleevec®, Novartis), are sometimes preferred due to their selectivity. In this poster, we present solutions for kinases that are difficult to address with activity assays and provide a comparison of compound affinities for active and non-activated forms of a diverse set of kinases to enable faster lead optimization.

Figure 1. LanthaScreen® Eu Kinase Binding Assay



- ➤Easy-to-optimize, mix-and-read assay format
- ➤Broaden target coverage to include challenging kinases
  - •No substrate required
  - •Analyze low activity or non-active kinases
  - •Specificity driven by epitope tag
- ➤Complement existing work flow for downstream applications
  - •Easily detect Type I, II or III (allosteric) inhibitors
  - •Track slow binding inhibitors in real-time
  - •Compare on/off rates

Table 1. Assay Conditions for Inhibitor Screening

LanthaScreen® Eu Kinase Binding Assays to evaluate inhibitors are typically performed by addition of 3 components each at 3X the final desired concentration as follows:

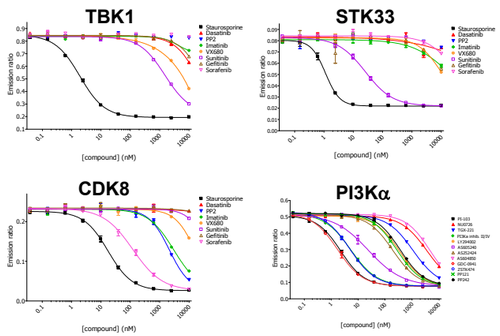
STEP 1. Add 5 µL of test compound
STEP 2. Add 5 µL of kinase/antibody mixture
STEP 3. Add 5 µL of tracer
STEP 4. Incubate for 1 hour at room temperature and read plate

Typical final assay conditions are 5 nM kinase, 2 nM antibody, and the tracer equal to or near the Kd value.

Protocols and assay conditions for specific kinases can be found at http://www.invitrogen.com/bindingassaytable

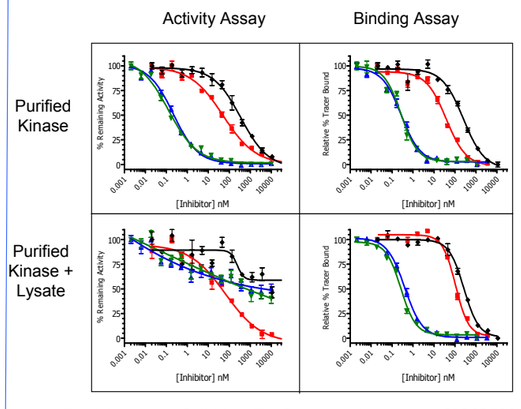
RESULTS

Figure 2. Assays for challenging targets



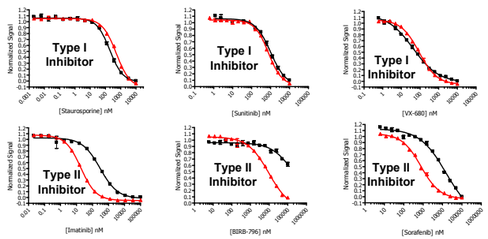
Robust assays have been developed for four potential oncology targets, including p110α/p85α (PIK3CA/PIK3R1), CDK8, STK33, and TBK1 with IC_{50} values that comparable to literature values. One advantage of a binding assay format over activity assays is the ability to rapidly develop assays for kinases with low intrinsic activity (e.g. CDK8) or without knowledge of the substrate (e.g. STK33).

Figure 3. Binding assays are resistant to contaminating kinases



Assay of EGFR inhibitors in absence (Purified Kinase) or presence (Purified Kinase + Lysate) of 300 mg/ml HeLa cellular lysate, either assayed in a traditional TR-FRET activity assay (Left panels) or in the kinase binding assay (Right panels). Lack of potent inhibition by EGFR specific inhibitors (erlotinib, gefitinib) in the activity assay in the presence of cellular lysate indicates that some of the activity being measured is from contaminating kinases in the lysate.

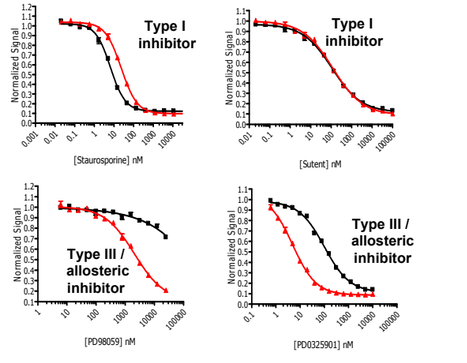
Figure 4. Higher affinity interactions of Type II inhibitors to non-activated Abl



IC_{50} (nM)	active	non-active
Staurosporine	192	507
Sunitinib	1000	770
VX-680	43	89
Imatinib	292	18
BIRB-796	>50,000	10,000
Sorafenib	15,000	770

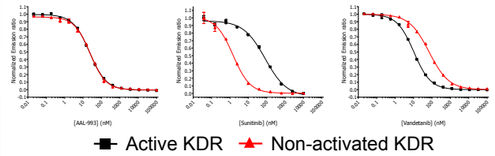
Competitive binding experiments were performed under optimized conditions for active Abl and Abl which had been treated with phosphatase (Non-activated Abl). The assay detects the higher affinity interactions of non-activated Abl and the Type II inhibitors imatinib, BIRB-796, and sorafenib, whereas the IC_{50} values detected for the Type I inhibitors staurosporine, sunitinib and VX-680 are similar between the two activation states.

Figure 5. Preferential binding of Type III (allosteric) inhibitors to non-activated MEK1



Competitive binding experiments were performed under optimized conditions for active and non-activated preparations of MEK1. The non-activated MEK1 used was wild-type protein that was not treated with any upstream activators. The active MEK1 used was a constitutively active mutant (S218D, S222D). The Type I inhibitors sunitinib and staurosporine displayed less than a 4-fold difference in affinity between the activation states whereas the allosteric and non-ATP competitive inhibitors PD98059 and PD0325901 display greater than 20-fold higher affinity for the non-activated state of MEK1. The ability to sensitively detect PD98059 provides an example of a compound that is detected in a binding assay, but not in a direct kinase activity assay.

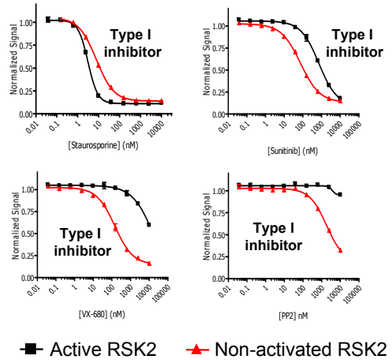
Figure 6. KDR (VEGFR2) inhibitor rank order potencies are phospho-state dependent



IC_{50} (nM)	active	non-active
Axitinib	1	0.35
Pazopanib	6.4	1.7
Sunitinib	49	1.5
Motesanib	6.2	14
AAL-993	22	24
Vatalanib	33	28
Vandetanib	10	70

Competitive binding experiments were performed under optimized conditions for activated (phosphorylated) KDR and non-activated KDR (non-phosphorylated). For a set of established, high affinity KDR inhibitors different rank order potencies were observed for the two phosphorylation states. Some compounds, such as AAL-993 had similar affinities for the two phosphorylation states. However, sunitinib had close to the highest affinity against non-activated KDR, whereas it had the lowest affinity versus active KDR. The opposite was observed for vandetanib, which had a seven-fold higher affinity for active KDR. These differences lead to the question of whether binding to active or non-activated KDR better predicted cellular potency, which is addressed in Figure 8.

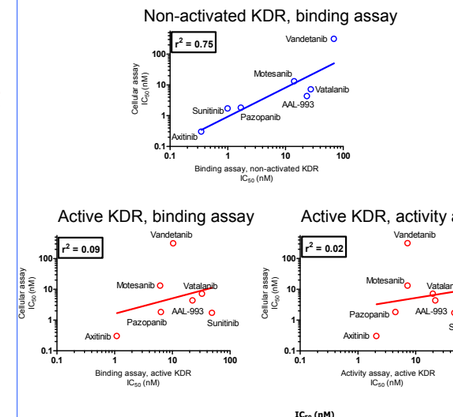
Figure 7. Preferential binding of some Type I inhibitors to non-active RSK2



IC_{50} (nM)	active	non-active
Staurosporine	3.1	7.6
Sunitinib	714	74
VX-680	>10,000	140
PP2	>10,000	1800

Competitive binding experiments were performed under optimized conditions for active and non-activated preparations of RSK2 (RPS6KA3). The non-activated RSK2 used was wild-type protein that was expressed in *E. coli* and not treated with any upstream activators. The active RSK2 used was wild-type protein that was activated by ERK2 and PDK1. Three Type I inhibitors tested bind with greater than 8-fold higher affinity to the non-activated state of RSK2.

Figure 8. Binding to non-activated KDR (VEGFR2) correlates with cellular potency



IC_{50} (nM)				
Activation state	non-active	active	active	
Assay format	binding	binding	activity	cellular
Axitinib	0.35	1.1	2.1	0.3
Sunitinib	1.0	49	47	1.7
Pazopanib	1.7	6.4	4.5	1.8
Motesanib	14	6.2	20	13
AAL-993	24	22	22	4.3
Vatalanib	28	33	20	7.1
Vandetanib	70	10	7.3	310

RESULTS AND CONCLUSIONS

- The LanthaScreen® Eu Kinase Binding Assay enables rapid development of assay for challenging oncology targets such as STK33, TBK1, CDK8, and PI3 kinases
- Unlike activity assays, the Kinase Binding Assay is insensitive to the presence of "contaminating" kinases
- Binding assays do not require the identification or use of substrates
- The Kinase Binding Assay enables affinity measurements for both non-active and active kinase states
- Type I, Type II, and Type III (allosteric) inhibitors can all display phosphorylation state specific binding
- Use of binding assays and non-activated kinases may, in some cases, be a better predictor of cellular potency

REFERENCES

Lebakken, C.A., Riddle, S.M., Singh, U., *et al. J Biomol Screen.* **14** 924-935 (2009)

http://www.invitrogen.com/bindingassay