Development of an AlphaLISA MEK1 kinase assay using full-length ERK2 substrate.

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Introduction

The mitogen-activated protein kinases (MAPK) pathway, also referred to as the RAS/RAF/MEK/ERK signaling cascade, is involved in multiple cellular processes by relaying various upstream signals to cytosolic and nuclear targets. The ubiquitously expressed Ser/Thr kinases ERK1 and ERK2 (collectively referred to as ERK1/2) are specific effectors of the MAPK pathway and the dual-specificity kinases MEK1/2. Upon activation, RAF phosphorylates MEK1/2, which in turn sequentially phosphorylate tyrosine and threonine residues within the activation loop of their substrates, ERK1/2 (modeled in Figure 1A). The MAPK pathway is one of the most frequently mutated signaling pathways in human cancers which can lead to hyperactivation, cellular proliferation and tumor growth. Therefore, substantial efforts have been made to develop inhibitors that target different points in this pathway. Multiple inhibitors of MEK1/2 and other kinases in the cascade have been developed and tested for efficacy in treatment of a variety of cancer types over the past few decades.[1-3]

One challenge in developing *in vitro* enzymatic assays is the limitation of some assay technologies, such as TR-FRET, to measure phosphorylation of full length protein substrates due to strict distance limitations of common TR-FRET Donor and Acceptor molecules (~10 nM). One benefit of AlphaLISA™ technology is that it allows for the detection of large molecules and protein-protein interactions in a homogeneous, no-wash format by allowing for a broader distance between the Donor and Acceptor pairs. AlphaLISA assays require two bead types:

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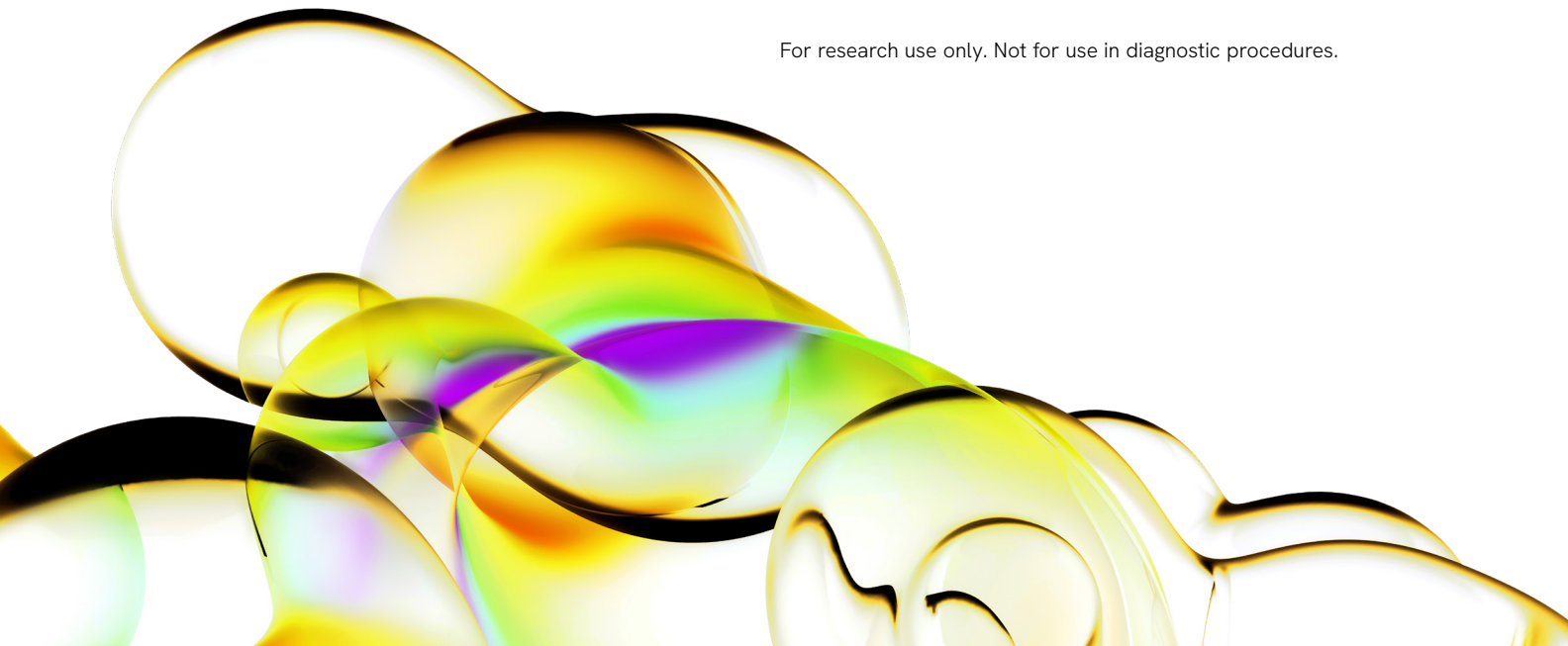

Donor beads and Acceptor beads. Donor beads contain a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O_2, singlet oxygen, upon illumination at 680 nM. With a four µsec half-life, singlet oxygen can diffuse approximately 200 nM in solution. If an Acceptor bead is within that distance, energy is transferred from the singlet oxygen to provoke a chemical cascade in the Acceptor bead, resulting in emission of a sharp peak of light at 615 nM. If the Donor bead is not in proximity (within approximately 200 nM) of an Acceptor bead, the singlet oxygen falls to ground state and no signal is produced. Here, we utilize a full-length ERK2 substrate to measure the kinase activity of MEK1 using an easy to develop AlphaLISA assay.

In the AlphaLISA assay developed for the detection of phosphorylated ERK2 (illustrated in Figure 1B) a full-length ERK2 substrate labeled with a GST tag is detected by a rabbit anti-phospho-ERK2 IgG that is bound to an anti-rabbit IgG-conjugated Donor Bead and a Glutathione (GSH)-conjugated Acceptor Bead. Therefore, light emission is proportional to the phosphorylation state of the full-length ERK2 substrate.

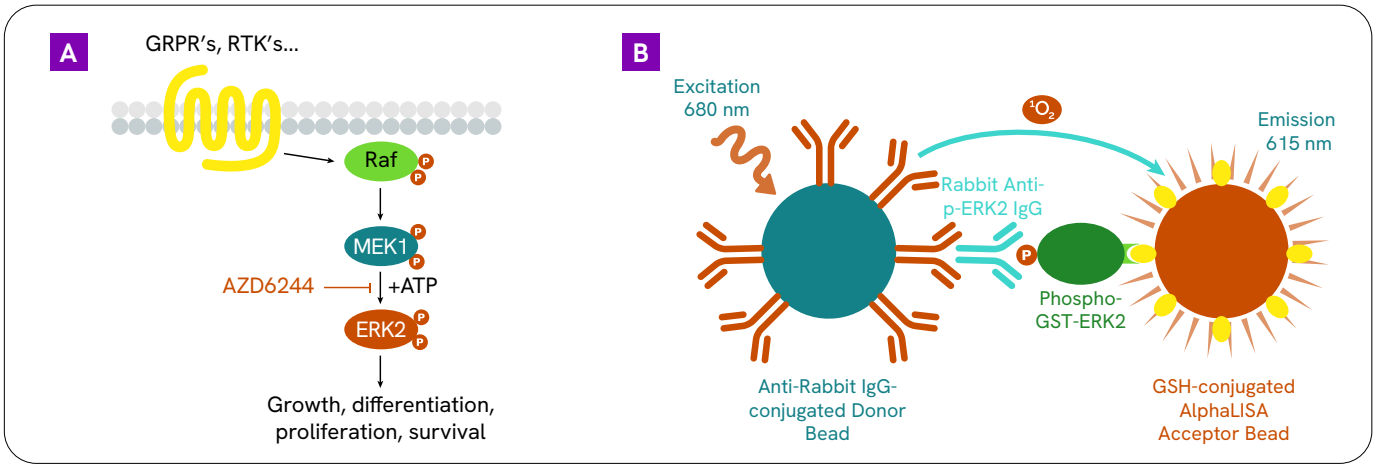

Figure 1: Schematic representations of the A) MEK1/ERK2 kinase cascade and B) the AlphaLISA ERK2 phosphorylation assay.

This technology has several distinct advantages including high signal to background, wide dynamic range, and an extremely simple, straight-forward, no-wash protocol. In addition, Revvity offers a large range of Alpha toolbox beads specific for binding to a variety of peptide tags or antibody species, providing a myriad of options for rapidly developing and validating assays for full-length proteins and protein-protein interactions. This technical note illustrates one example of how these toolbox beads may be used to efficiently develop an assay for assessing MEK1 phosphorylation of a native ERK2 substrate.

Reagents

- Kinase: MEK1 wt (ProQinase, #0550-0000-3)
- Substrate: ERK2 (MAPK1), GST N-terminal (Carna, #04-143-11)
- Phospho-Substrate: p-ERK2, GST N-terminal (EMD Millipore, #14-550)
- Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), Rabbit mAb (CST, #4370S)
- ATP (Sigma, #A7699)
- DTT (Sigma, #43816)
- MEK1 inhibitor: Selumetinib (AZD6244) (Selleckchem, #S1008)
- EDTA (Sigma, #E7889)
- DMSO (Sigma, #D2650)
- AlphaLISA PPI Detection Buffer (Revvity, #AL015C)
- Glutathione AlphaLISA Acceptor Beads (Revvity, #AL109C)
- Anti-Rabbit IgG (Fc specific) AlphaLISA Acceptor Beads (Revvity, #AL104C)
- Glutathione Alpha Donor Beads (Revvity, #6765300)
- Anti-Rabbit IgG Alpha Donor Beads (Revvity, #AS105D)
- AlphaPlate™-384 microplates (Revvity, #6005350)

AlphaLISA assay protocol

The AlphaLISA full-length MEK1 kinase (ERK2 phosphorylation) assay involves the following steps:

1. Mix MEK1 kinase, GST-labeled full-length ERK2 substrate, inhibitor compound to be tested, and ATP in the wells of a 384-well AlphaPlate and incubate for an appropriate time (e.g., one hour) in kinase assay buffer (50 mM HEPES pH 7.5, 100 mM $MgCl_2$, 0.01% final, 2 mM DTT).
2. Quench kinase activity with AlphaLISA PPI detection buffer containing EDTA (10 mM in final volume) and rabbit IgG specific to phosphorylated ERK2.
3. Add AlphaLISA Acceptor Beads and Donor Beads (both at 20 µg/mL final) conjugated with Glutathione (GSH) and anti-rabbit IgG and incubate at room temperature.
4. Read plate on an Alpha-enabled microplate reader.

Data collection and analysis

The AlphaLISA assays were measured using a Revvity EnVision™ multimode plate reader using default values for standard Alpha detection. Enzyme and inhibitor titration curves were plotted and fitted with non-linear regression in GraphPad Prism®. All data points were a minimum of triplicate wells.

Cross-titration of ERK2 substrate and detection antibody concentrations

The first step in developing this assay is to determine the starting concentrations for the GST-labeled full-length ERK2 substrate and anti-phospho-antibody to use in subsequent experiments. For this, we started with an already activated (phosphorylated) GST-tagged ERK2 substrate to avoid the added variable of MEK1 kinase concentration and a rabbit monoclonal antibody that detects endogenous levels of ERK2 when dually phosphorylated at Thr185 and Tyr187. Additionally, this allowed us to test two AlphaLISA bead configurations to determine the optimal orientation of Donor and Acceptor beads. The results of this cross-titration of can be found in Figure 2.

Since the signal to background was significantly higher in wells containing GSH Acceptor beads and anti-rabbit Donor beads (Figure 2B), we chose to use this bead configuration in subsequent experiments for testing enzymatic activity with a full-length, inactive ERK2 substrate. We then repeated the experiment with inactive substrate, a set concentration (8 nM) of MEK1 kinase and excess ATP. The data from this test, shown in Figure 3, demonstrate that the AlphaLISA assay can detect phosphorylation of full-length ERK2 by MEK1.

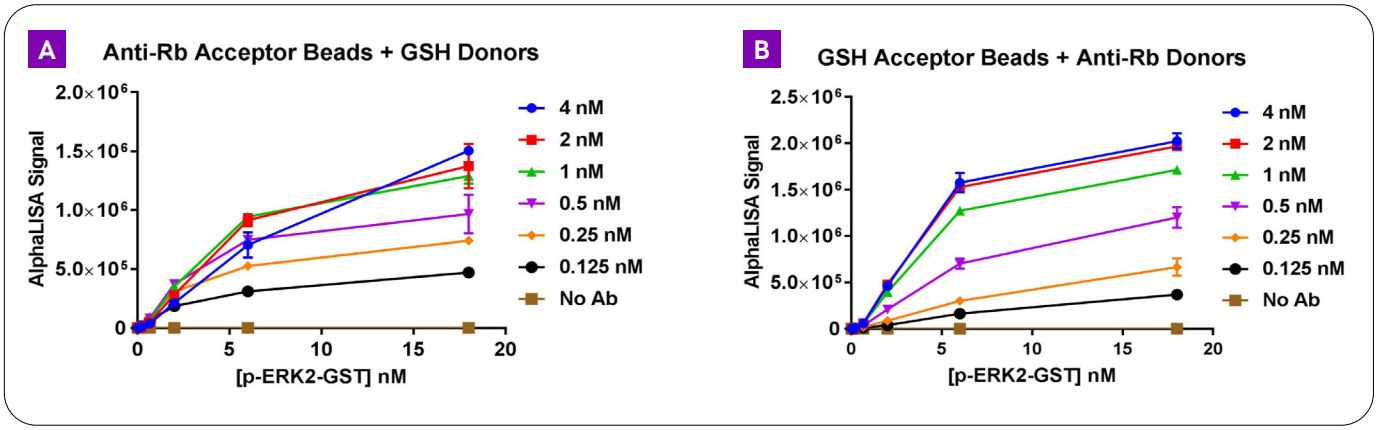

Figure 2: Cross-titration of activated (phosphorylated) ERK2 substrate and anti-phospho ERK2 antibody with two bead configurations. The substrate and antibody were pre-incubated for 1.5 hours before addition of either (A) anti-rabbit Acceptor beads and GSH Donor beads or (B) GSH Acceptor beads and anti-rabbit Donor beads and plates read after an additional one hour incubation.

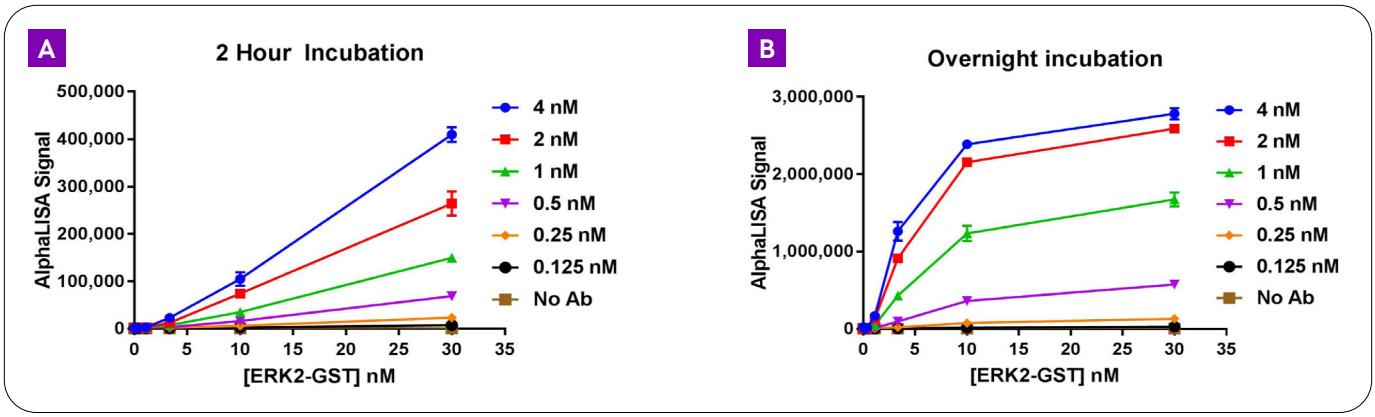

Figure 3: Cross-titration of anti-phospho-ERK antibody and full-length GST-tagged ERK2-substrate activated with 8 nM MEK1 and excess (400 µM) ATP. The kinase assay ran for (A) two hours and the plate was read again after (B) overnight incubation (18 hours total). Note: Kinase activity for this assay was allowed to continue overnight.

Note here that 8 nM MEK1 is not enough to fully phosphorylate the higher concentrations of substrate after only a two-hour incubation (Figure 3A) as can be observed after overnight incubation (Figure 3B). As a result of this experiment, we chose to use 5 nM ERK2-GST substrate (concentration in enzyme reaction) and 2 nM of anti-p-ERK2 antibody (concentration in final assay volume) in subsequent experiments.

Enzyme titration

The next step was to perform a full titration of MEK1 enzyme for a one-hour incubation to determine working concentrations going forward. In addition, after stopping the enzyme reaction with 10 mM EDTA, the effect of increasing incubation time for the antibody and Alpha beads was also examined. Results from this experiment (shown in Figure 4) indicate that optimal phosphorylation of the substrate was achieved after one hour by using 3.3 - 10 nM MEK1. Also, allowing the assay to incubate overnight significantly improved the assay window suggesting that additional time is needed for the assay to reach equilibrium. This allows for complete binding of the anti-rabbit antibody and beads to their respective sites. If a shorter assay time is required, other anti-phospho-ERK2 antibodies or toolbox beads (such as Protein A coated beads) may be tested.

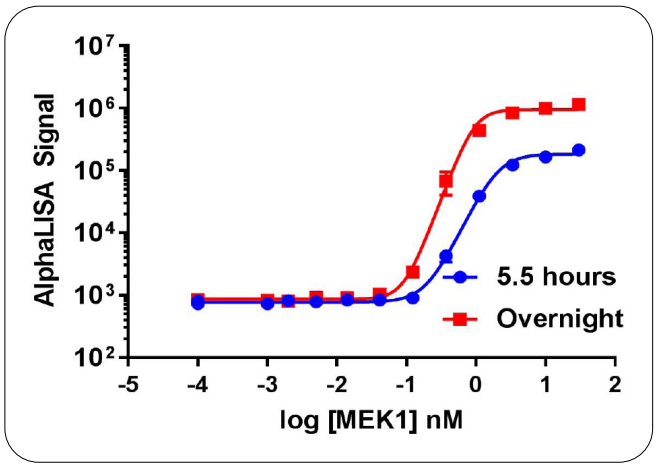

Figure 4: MEK1 kinase concentration effects on phosphorylation of ERK2 and the effect of incubation time on assay performance. Effects of a full range titration of MEK1 enzyme (from 30 nM) on phosphorylation of 5 nM GST-ERK2 substrate with one hour incubation and excess ATP (400 µM) and testing of increased incubation time. The EC_{50} for MEK1 is 2 nM for 5.5 hours and 0.93 nM for overnight incubation.

MEK1 inhibitor titration

To test for specificity of the assay for measuring ERK2 phosphorylation by MEK1 kinase, we examined the effects of a known inhibitor, Selumetinib. Selumetinib is a noncompetitive, highly selective, allosteric inhibitor of MEK1 that has been tested in clinical trials for treatment of metastatic colorectal cancer and KRAS positive non-small cell lung carcinoma.[1,3] Figure 5 illustrates both the effectiveness of Selumetinib to inhibit ERK2 phosphorylation by MEK1 (with an EC_{50} of 0.59 µM) and the protocol followed for the inhibitor assay.

Assay variability and DMSO tolerance

The tolerance of an assay for negligible concentrations of DMSO is important because many inhibitor compounds are stored in DMSO so we tested assay performance in response to a wide range of DMSO concentrations. We find the assay is tolerant to DMSO to approximately 0.625% in the enzymatic reaction demonstrating about a 16.7% reduction in signal (Figure 6A). Assay variability was assessed with three concentrations of inhibitor and 14 replicate wells using 5 nM MEK1, 400 µM ATP, and 2 µM of inhibitor, a concentration above the measured IC_{50}. The assay performs extremely well for use in screening workflows with Z′ values well over 0.6 (Figure 6B). Further optimization steps can be done to reduce assay volume and the last two steps could be combined (antibody and bead addition) to simplify the protocol further for higher throughput screening paradigms.

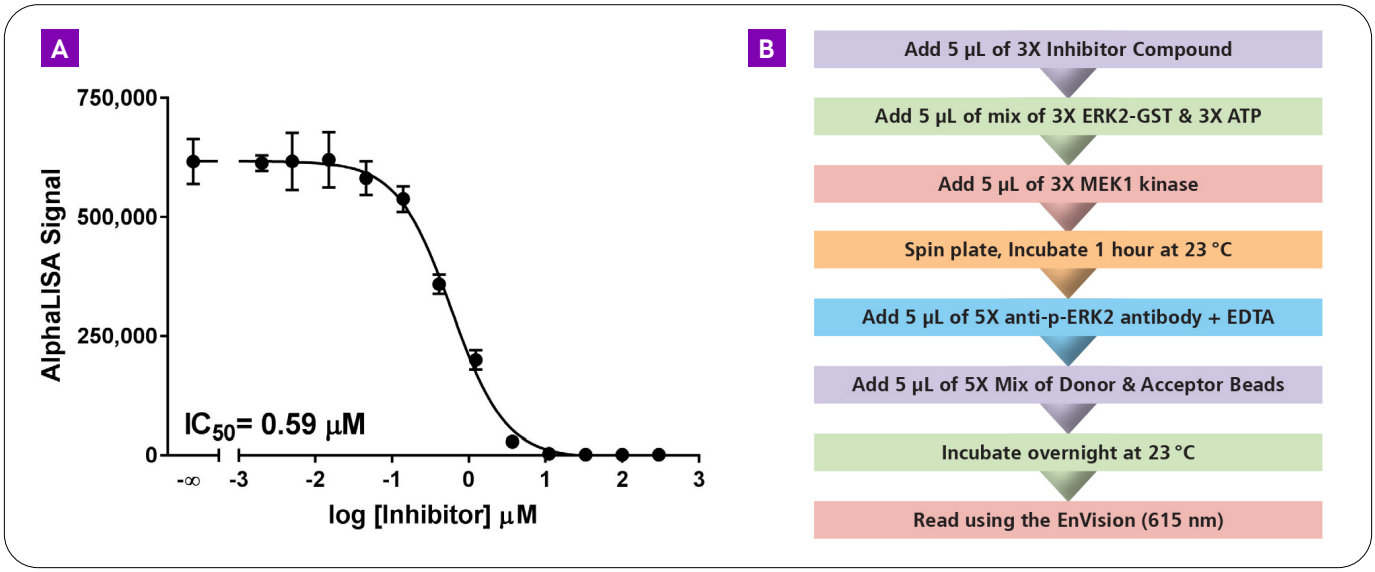

Figure 5: Assay inhibition and full protocol. (A) Titration of the MEK1 inhibitor Selumetinib and a (B) schematic illustrating protocol used for the inhibitor assay.

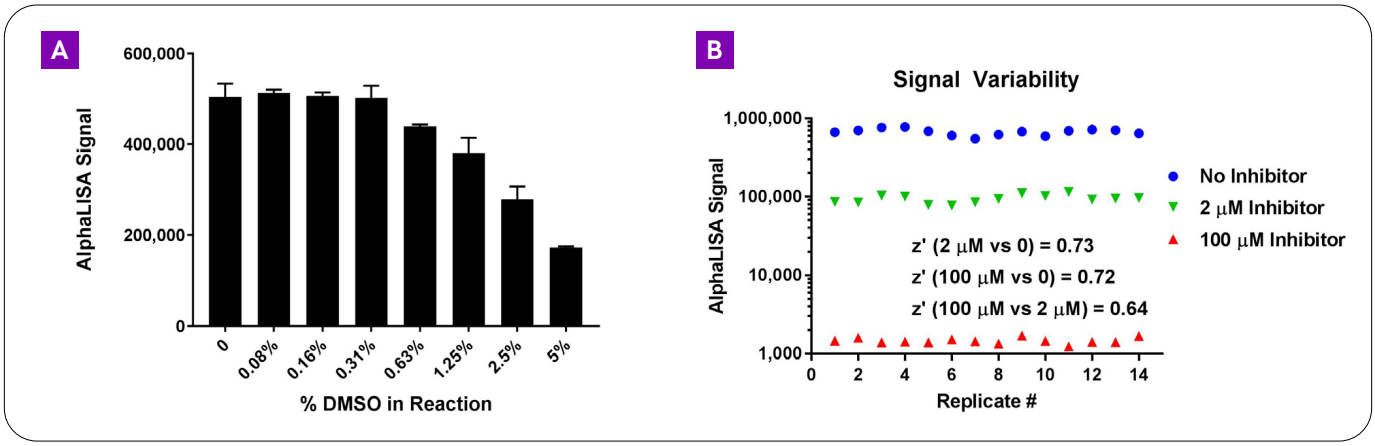

Figure 6: AlphaLISA MEK1 assay performance. (A) DMSO tolerance and (B) assay signal variability was assessed using three concentrations of the inhibitor, Selumetinib, and assay conditions described in Figure 5.

Summary

AlphaLISA technology provides a straight-forward, no-wash format for rapid development of assays for both detection of full-length proteins and measurement of protein-protein interactions. This technical note describes an example of how Alpha toolbox beads may be combined with any substrate of interest and specific detection antibody to quickly and easily develop an assay for assessment of kinase phosphorylation of a full-length substrate, such as ERK2.

References

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3. Wu, P-K & Park, J-I. (2015). MEK1/2 Inhibitors: Molecular Activity and Resistance Mechanisms. *Seminars in Oncology*, Vol 42(6): 849-862.

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