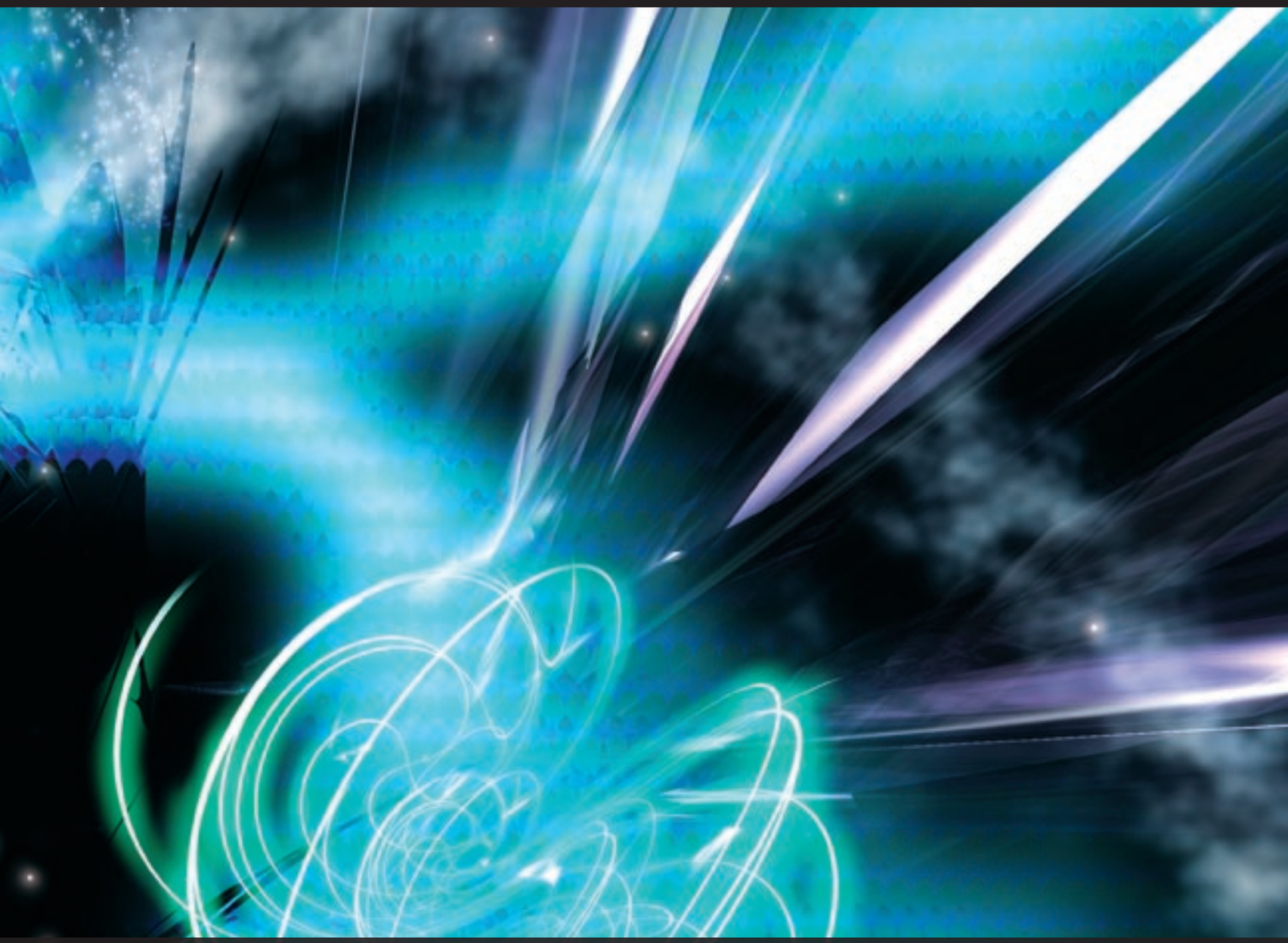


Z′-LYTE™ Kinase Assay Platform

Z'-LYTE™ Kinase Assay Platform

- Non-radioactive assay format for screening a diverse collection of over 185 kinase targets
- Assay flexibility allows inhibitors with varying modes of action to be identified
- Rigorous validation ensures robust and reproducible assay results
- Compatibility with automated high-throughput screening systems

Powerful fluorescent kinase assay technology

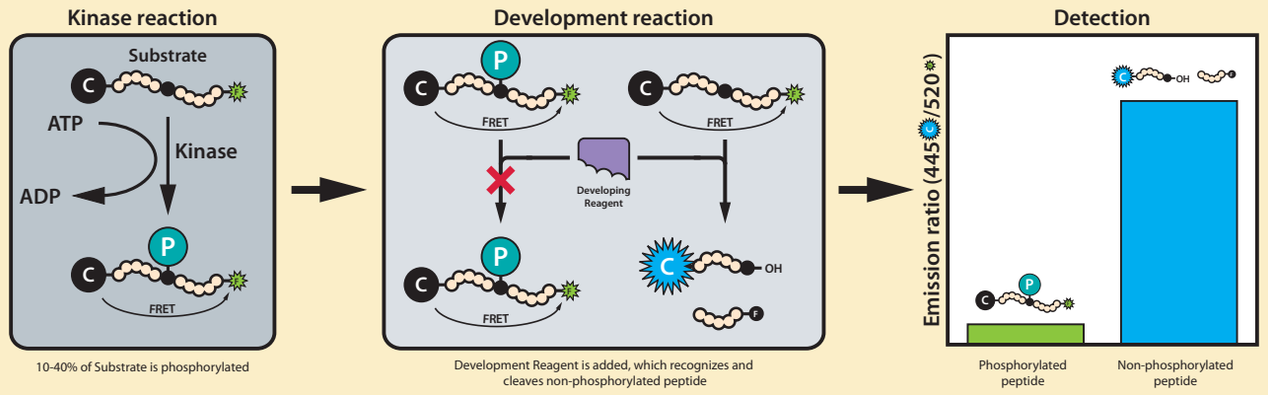
Kinases are a major drug screening target, and as such the growing discovery and characterization of this target class necessitates the need for quick assay development. Invitrogen's proprietary Z´-LYTE™ Kinase Assay Platform provides a universal Fluorescence Resonance Energy Transfer (FRET)-based assay platform that requires no expensive radioactive substrates or antibodies and is highly compatible with high-throughput screening (HTS) applications. With the Z'-LYTE™ platform, assays can be performed in as little as 2 hours saving you time and effort by eliminating the need to optimize your own assays.

The Z´-LYTE™ kinase assay kits are designed to accurately and reliably screen potential kinase inhibitors and to further assess their selectivity profiles. Kits use the broadest fluorescent kinase assay technology currently available and are suitable for both Serine/Threonine and Tyrosine kinases. Greater than 185 kinase assays have been validated using this format, and more continue to be developed. To see whether your kinase of interest can be assayed using the Z'-LYTE™ Kinase Assay Platform, refer to the Reactivity Table on our website at **www.invitrogen.com/zlyte**. If no Z'-LYTE™ Peptide Substrate works with your kinase of interest, but the sequence of the substrate is known, a custom Z'-LYTE™ Peptide can by synthesized and validated.

Robust, homogeneous assay

The Z′-LYTE™ assay method (Figure 1) is based on FRET between coumarin and fluorescein (1). The assay employs a fluorescence-based, coupled-enzyme format and utilizes the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. Ratiometric measurements result in a low percent coefficient of variation allowing for high Z′-values (2) at a low percent phosphorylation. Complete flexibility in ATP concentrations allows for the detection of both competitive and allosteric inhibitors.

Figure 1 — Schematic of the Z´-LYTE™ assay



In a 10 µl **Kinase Reaction**, the kinase transfers the gamma-phosphate of ATP to a single Serine, Threonine or Tyrosine residue in the synthetic peptide substrate (2 µM). The peptide is labeled with two fluorophores (coumarin and fluorescein) — one at each end, to make up a FRET pair.

In the **Development Reaction**, 5 µl of a site-specific protease recognizes and cleaves non-phosphorylated peptides. Phosphorylation of peptides suppresses cleavage by the protease. Cleavage disrupts FRET between the coumarin and the fluorescein on the peptide. Uncleaved, phosphorylated peptides maintain the FRET signal.

During **Detection**, a ratiometric read-out of the donor emission over the acceptor emission quantitates reaction progress. The ratio is low if the peptide is phosphorylated, and high if the peptide is non-phosphorylated. Compounds that inhibit kinase activity will therefore produce a high ratio, and are easily distinguished from potential protease inhibitors that produce a low ratio.

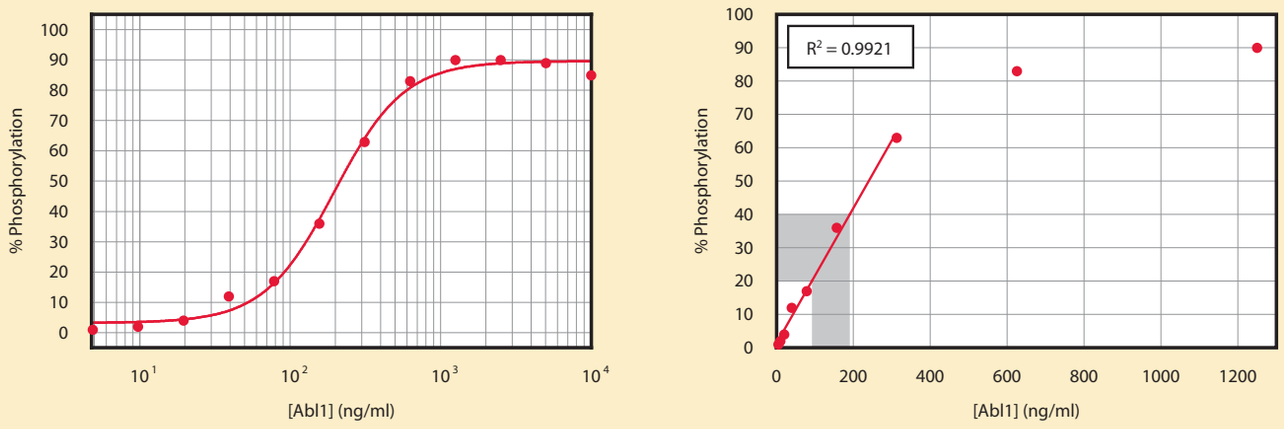
Excellent Z′-values at low percent phosphorylation

In the Z′-LYTE™ assay, reaction progress is quantitated with a ratiometric approach (coumarin emission/fluorescein emission) that reduces the effects of well-to-well and day-to-day variations. The results produce both low standard deviations and high Z′-values, even when only a small percentage of the Z′-LYTE™ peptide substrate is phosphorylated (Table 1). Staying within the linear range of the kinase activity, as determined by the precise calculation of percent phosphorylation in each assay well, ensures that the assay is not biased against weak or strong kinase inhibitors (Figure 2).

Table 1 — Percent phosphorylation and Z′-values corresponding to kinase concentration

[Abl1] in the kinase reaction (ng/ml)	Percent phosphorylation	Z′-value
4.9	1%	<0.50
9.8	2%	0.50
19.5	4%	0.75
39.1	12%	0.90
78.1	17%	0.91
156.2	36%	0.96
312.5	63%	0.97
625	83%	0.98
1,250	90%	0.99

Figure 2 — Percent phosphorylation as a function of Abl1 concentration

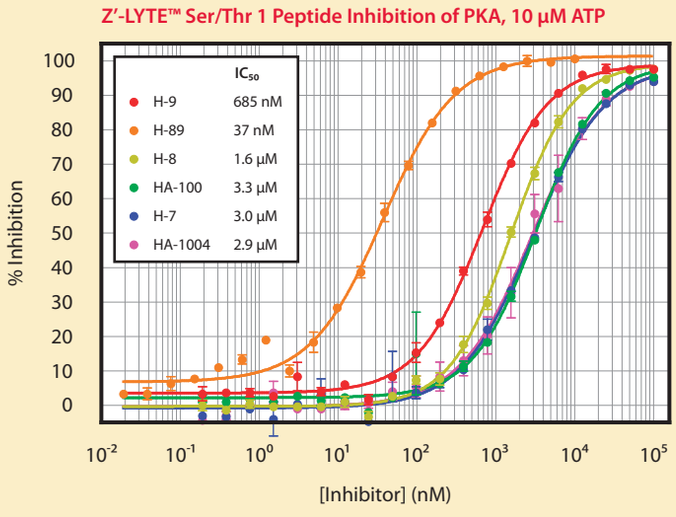


Representative sample data generated for Abl1 for the Z′-LYTE™ Kinase Assay Kit – Tyr 2 Peptide. The kinase titration data shown for Abl1 demonstrates that a very good Z′-value is achieved even when a very low percentage of substrate is phosphorylated.

Determine rank order and potency

To validate the Z′-LYTE™ technology for kinase screening, the Z′-LYTE™ Kinase Assay Kit was used to screen the LOPAC™ library in 384-well format with protein kinase A (PKA) and Ser/Thr Peptide 1. The screen resulted in the identification of PKA inhibitors. These inhibitors were flagged as initial hits of >50% inhibition, and follow-up IC_{50} determinations gave appropriate rank order and potency values (Figure 3).

Figure 3 — Z′-LYTE™ Ser/Thr 1 Peptide inhibition of PKA

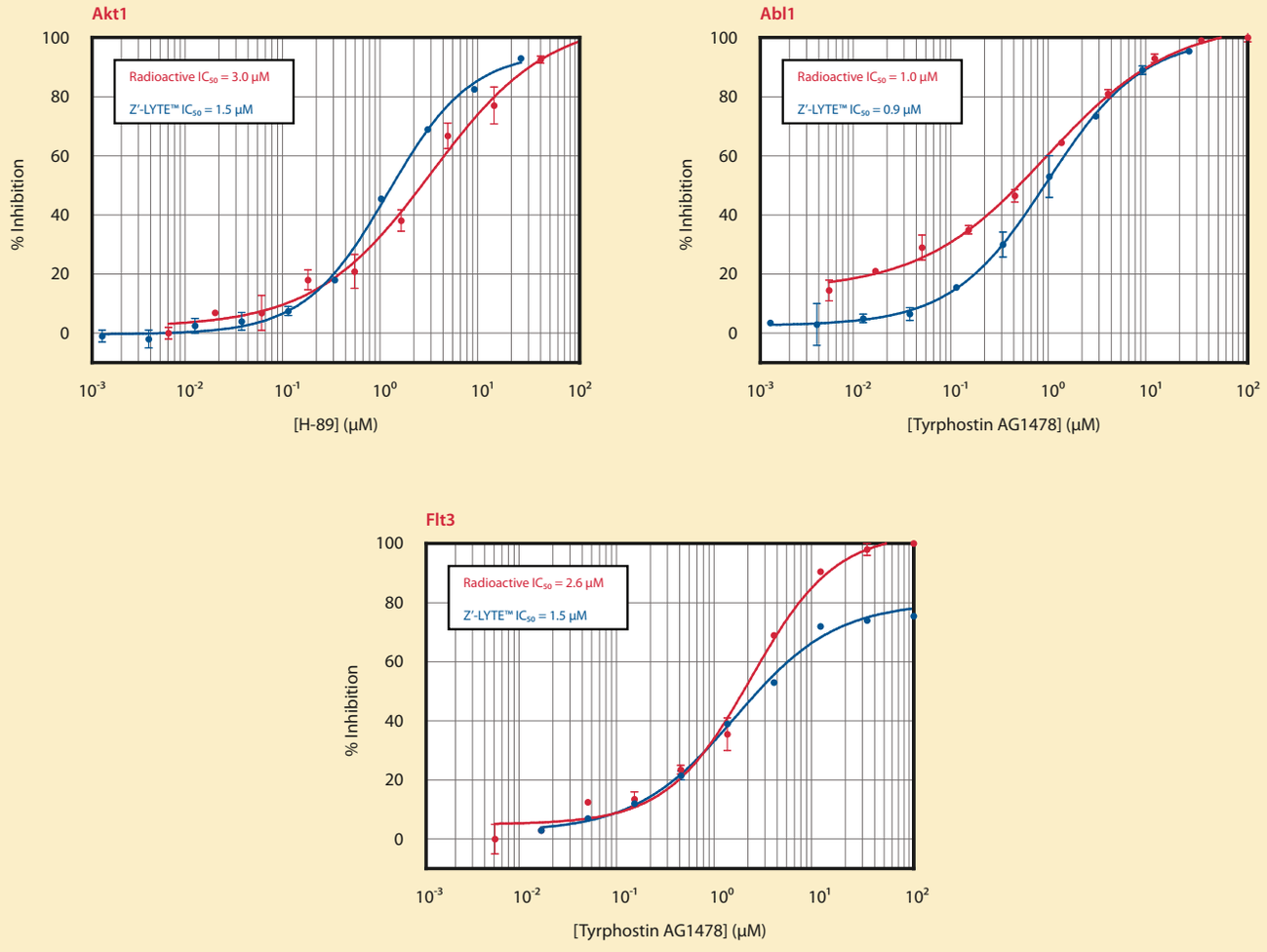


Six inhibitors were assayed against PKA using the Z′-LYTE™ Kinase Assay Kit – Ser/Thr 1 Peptide at 10 µM ATP. The inhibitor titration data demonstrates that the assay is able to distinguish very potent inhibitors with IC_{50} values that correlate closely with published literature values. Z′-values were calculated based on controls for each assay, and ranged between 0.82–0.91.

Rigorous validation and consistent results make Z′-LYTE™ an ideal platform for selectivity profiling

By using Invitrogen's portfolio of Z′-LYTE™ kits and kinase enzymes, assay panels may be assembled to rapidly assess the selectivity of focused libraries, chemical arrays, and lead compounds. Selectivity data derived from Z′-LYTE™ assays shows strong correlation with traditional radioactive filter-capture methods (Figure 4), but without the expense and effort. The breadth of kinase coverage and the robustness of the technology make the Z′-LYTE™ assay the format of choice for Invitrogen's SelectScreen™ Kinase Profiling Service, where you can cost-effectively determine the inhibitory profile and mechanism of action of lead compounds.

Figure 4 — Assay comparison data between Z′-LYTE™ assay and radioactive methods

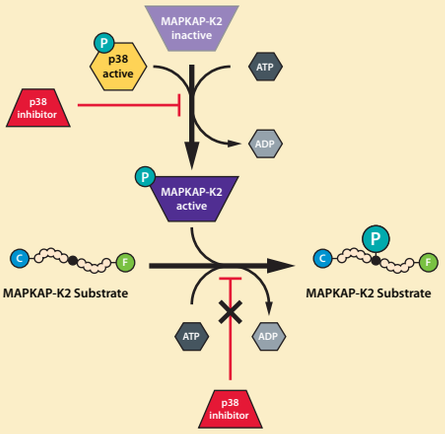


Data from 3 kinases assayed with both the Z′-LYTE™ assay and with radioactive filter-capture method in the presence of representative inhibitors. All assays were performed at the $K_{m(app)}$ for ATP as determined for each assay. The Z′-LYTE™ assays required less enzyme as compared to the radioactive assay. There is excellent IC_{50} value correlation between the two assay formats.

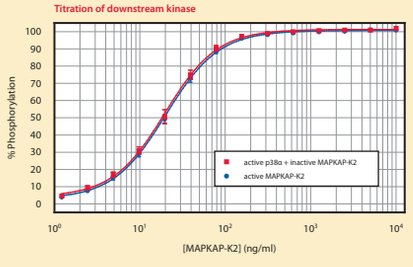
Assaying pathway cascades using Z′-LYTE™ Kinase Assay Platform

Z′-LYTE™ technology can be used to conduct pathway assays, an approach for screening inhibitors of upstream kinase targets where peptide substrates are difficult to generate. When peptide substrates are not applicable for a particular kinase, a pathway-based approach provides a viable way of evaluating that kinase's activity without the need for substrate-based or whole protein-based assays. This is demonstrated in Figure 5 which shows the development and validation of a MAPK14 (p38α)/MAPKAPK2 pathway assay using the Z′-LYTE™ platform.

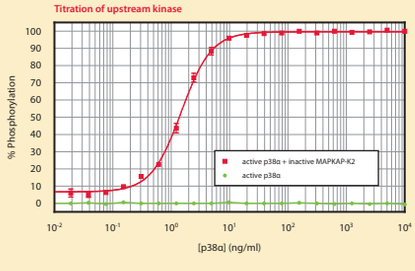
Figure 5 — The Z′-LYTE™ kinase assay pathway approach



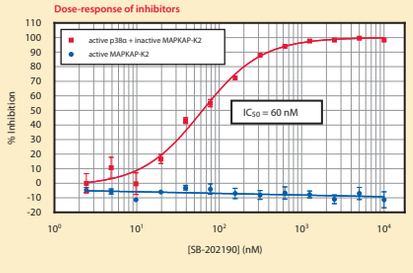
A pathway-based approach can be used to screen for inhibitors of upstream kinase targets, for which peptide substrates are difficult to generate. One example, shown here, is a Z′-LYTE™ p38α pathway assay where active p38α phosphorylates inactive MAPKAP-K2 which in-turn phosphorylates the MAPKAP-K2 Z′-LYTE™ substrate. With this method, it is vital that the active upstream kinase cannot directly phosphorylate the Z′–LYTE™ peptide substrate. Once lead compounds are identified, they will need to be screened against the activated downstream kinase to ensure that they only inhibit the target kinase. Similarly, MEK1 could be screened using another cascade with active MEK1, inactive ERK2, and the ERK2 Z′–LYTE™ substrate (data not shown).



Inactive MAPKAP-K2 was titrated in the presence of excess active p38α and compared to active MAPKAP-K2. These curves overlap, demonstrating the integrity of Invitrogen's purified, active MAPKAP-K2 protein kinase. From these data, the concentration of inactive MAPKAP-K2 which resulted in nearly 100% phosphorylation (500 ng/ml) was selected to be used in the cascade assay. This ensures that the phosphorylation of the MAPKAP-K2 Z′-LYTE™ substrate by activated MAPKAP-K2 is not rate-limiting.



Active p38α was titrated in the absence of inactive MAPKAP-K2 to demonstrate p38α's inability to directly phosphorylate the MAPKAP-K2 Z′-LYTE™ substrate. From the titration of active p38α, the concentration of active p38α which resulted in approximately 30% phosphorylation (2 ng/ml) was selected to be used in the cascade assay.

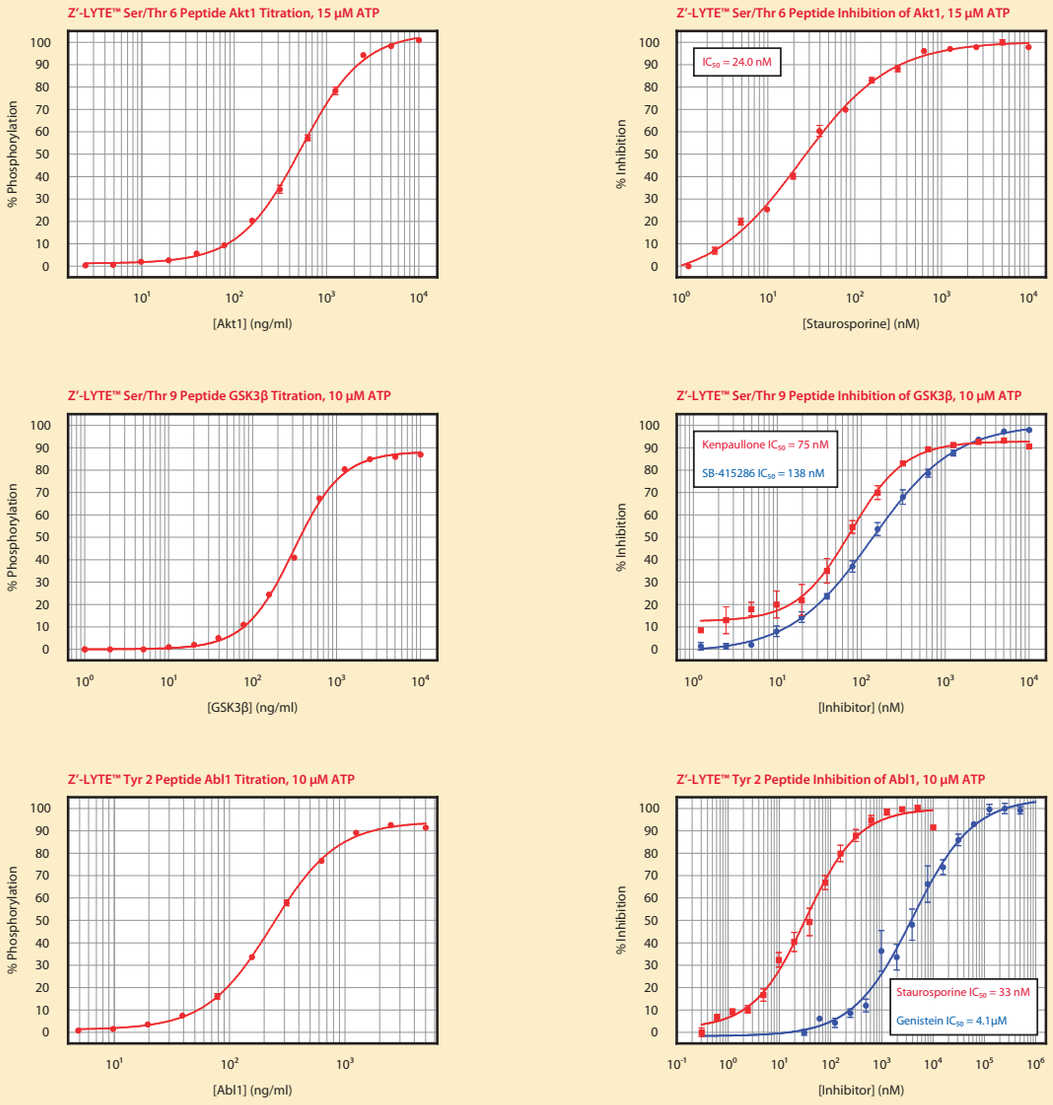


A known p38α inhibitor, SB-202190, was serially diluted in the presence of 2 ng/ml active p38α and 500 ng/ml inactive MAPKAP-K2. The dose-response curve with SB-202190 from the Z′–LYTE™ p38α cascade assay yielded an IC_{50} value of 60 nM (Biomol - IC_{50} for SB-202190 is 30 nM). The inhibitor did not inhibit the phosphorylation of the MAPKAP-K2 Z′-LYTE™ peptide substrate by active MAPKAP-K2.

Reliable kinase assay kits to screen and profile inhibitors of kinase activity

The Z´-LYTE™ Kinase Assay Kits are designed to accurately and reliably screen potential kinase inhibitors (Figure 6) in a 20 µl, two-hour, room-temperature assay under linear conditions and with near-K_m ATP concentrations for the kinase. The reagents contained in the kits are sufficient for 800 assays in 384-well plates and can be readily modified for ultra-miniaturized HTS applications with no loss of quality. The kits provide flexible, addition-only, screening assays that yield Z'-values >0.7. To verify that Invitrogen has a Z´-LYTE™ peptide for your kinase of interest, simply refer to the Reactivity Table on our website at **www.invitrogen.com/zlyte**.

Figure 6 — Representative sample kinase titrations and sample inhibitor IC_{50} data for the Z'-LYTE™ Detection Kinase Assay Kits



Representative validation data obtained by the Z'-LYTE™ Assays. Three different peptides (Z'-LYTE™ Ser/Thr 6, 9, and Tyr 2 Peptides) were tested through kinase titrations at 10 or 15 µM ATP concentrations. The same peptides were also tested in inhibition assays at 10 µM ATP concentration in IC_{50} format with sample inhibitors for each kinase.

Z′-LYTE™ instrumentation compatibilities

Invitrogen ensures that all platform technologies such as Z′-LYTE™ are validated on multiple instrument platforms where appropriate. The following tables provide an overview of the compatible instrument platforms for the Z′-LYTE™ technology (Tables 2 and 3).

Table 2 — Compatible filter-based instruments

	Tecan GENios Pro™	Tecan Ultra	Molecular Devices Analyst®	Perkin Elmer EnVision™
Excitation filter	405/20*	405/20*	405/35*	400/25*
Emission filter, donor	465/35*	465/35*	460/40*	460/25*
Emission filter, acceptor	535/20*	535/20*	530/25*	535/25*
Dichroic for excitation	330/420:440/850	50%	425	General dual
Dichroic for emission	330/420:440/850	320/500:520/800	425	General dual

* Wavelength in nm/bandpass

Table 3 — Compatible monochromator-based instruments

	Tecan Safire™ and Safire²™
Excitation filter	400/12*
Emission filter, donor	445/12*
Emission filter, acceptor	520/12*

* Wavelength in nm/bandpass

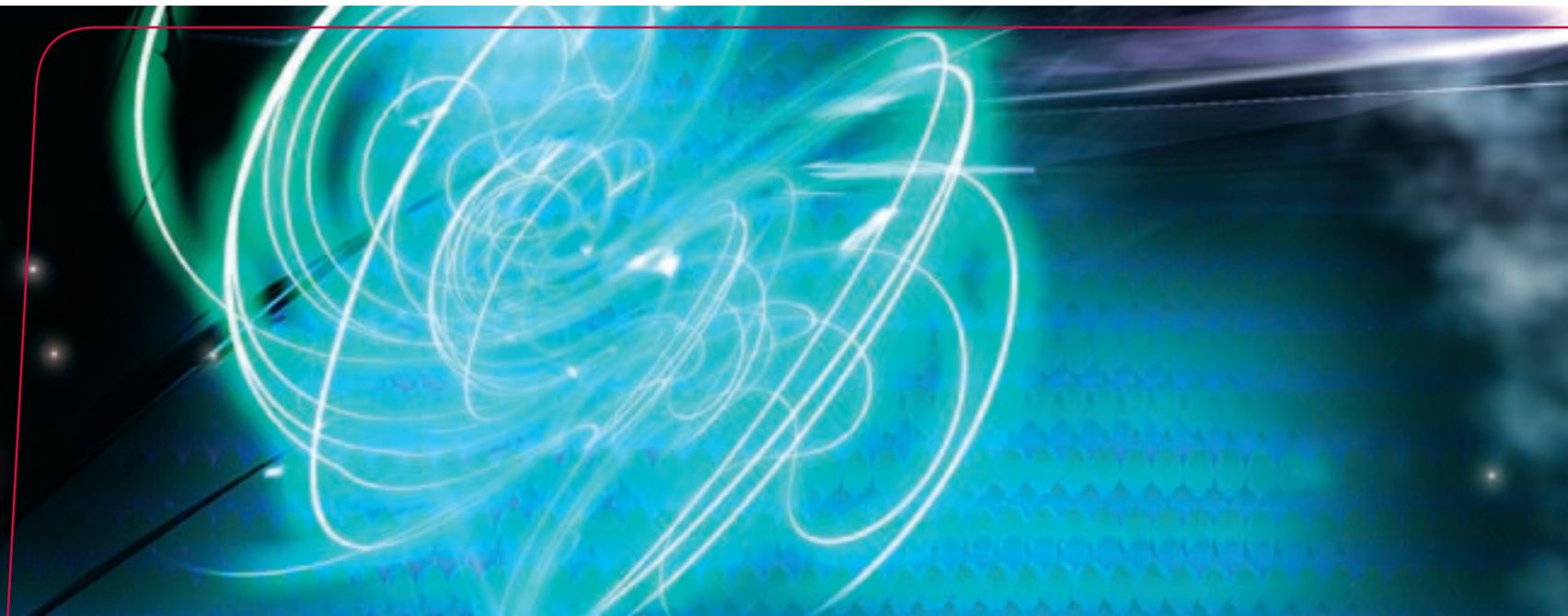
For detailed protocols and applications describing the use of the Z′-LYTE™ reagents on the above listed instruments, please see our website at **www.invitrogen.com/zlyte**. Other instruments may be compatible with Z′-LYTE™ reagents, but have not yet been validated by Invitrogen.

Ordering information

To rapidly develop assays for a broad array of kinase targets, order the Z′-LYTE™ Kinase Assay Kits today. To order or to learn more about Z′-LYTE™ technology, visit **www.invitrogen.com/zlyte**.

References

1. Rodems, S.M. *et al.* (2002) *ASSAY Drug Devel. Technol.* **1:**9–19.
2. Zhang, J.H. *et al.* (1999) *J. Biomol. Screen.* **4:**67–73.



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