



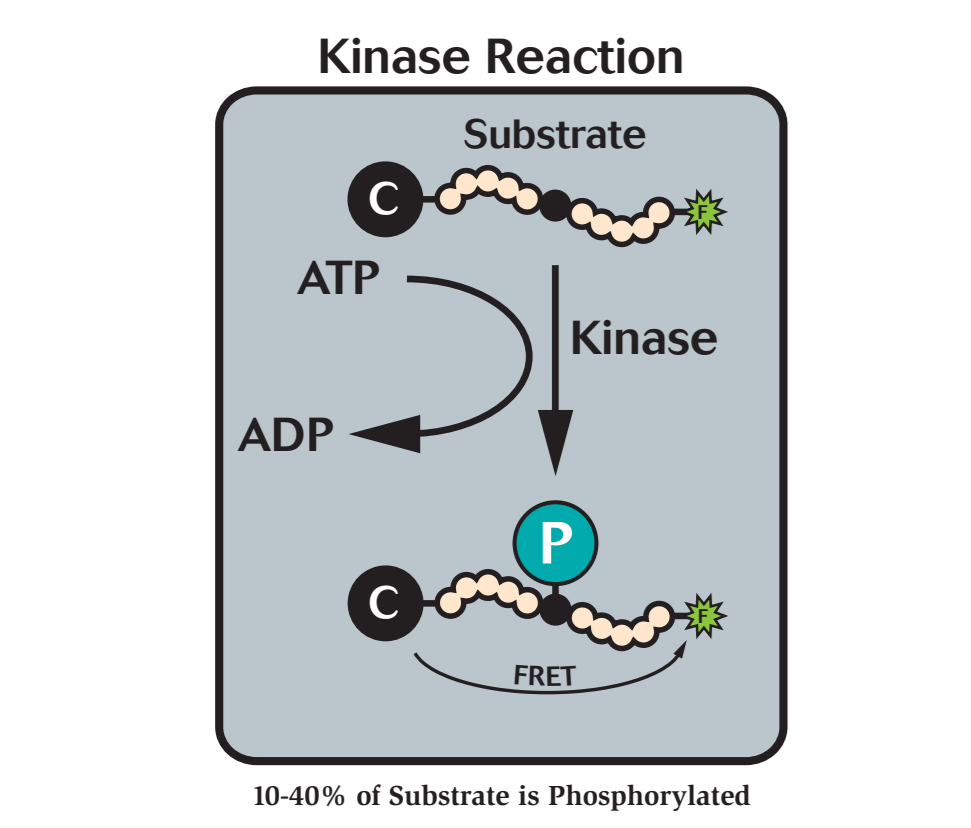
# BROAD KINASE COVERAGE WITH A PANEL OF Z'-LYTE™ SUBSTRATES

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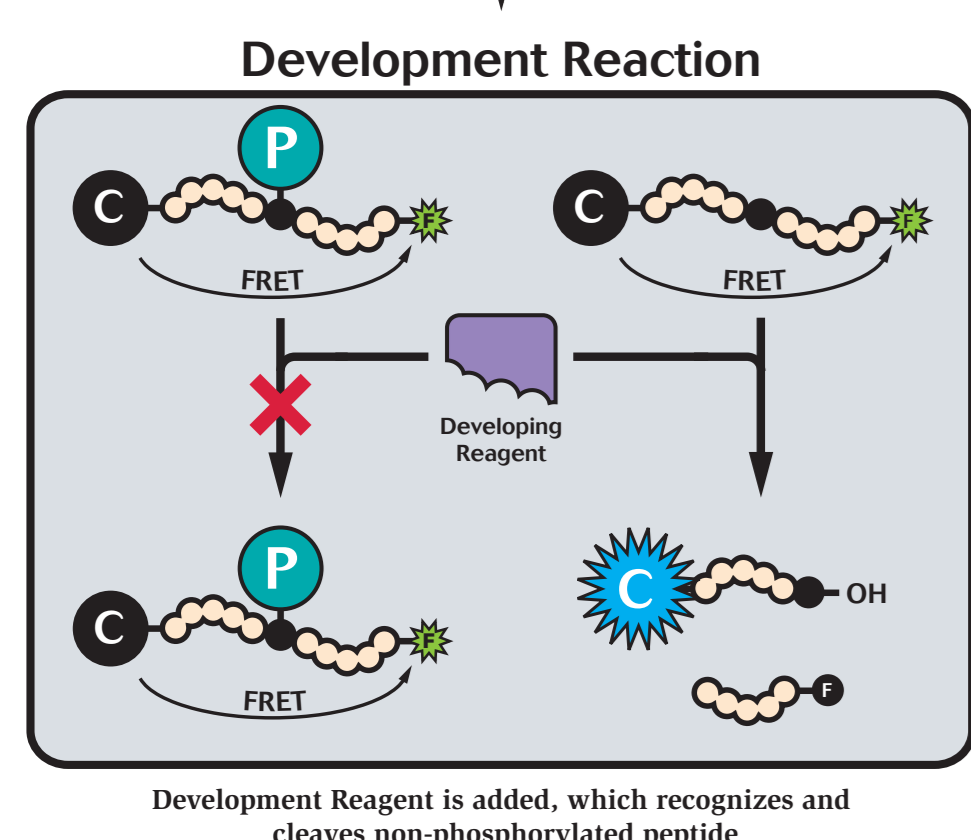
## 1 Abstract

Data from the human genome project suggest that 518 protein kinases exist, of which 90 are tyrosine kinases. Because of the high association between kinases and many human diseases, kinases represent the second largest drug screening target. A single technology to assay these targets would prove a valuable and enabling tool for these drug discovery efforts. The Z'-LYTE™ technology offers a FRET-based assay that requires no radioactive substrates or expensive antibodies and is highly compatible with HTS or uHTS applications. To demonstrate the broad coverage of the Z'-LYTE™ technology, we screened more than 115 different conventional kinases against a panel of 8 serine/threonine and 4 tyrosine substrates. Our results demonstrate that 38 tyrosine kinases and 47 serine/threonine kinases, at reasonable concentrations, phosphorylated at least one substrate on the panel. This represents a > 70% success rate.

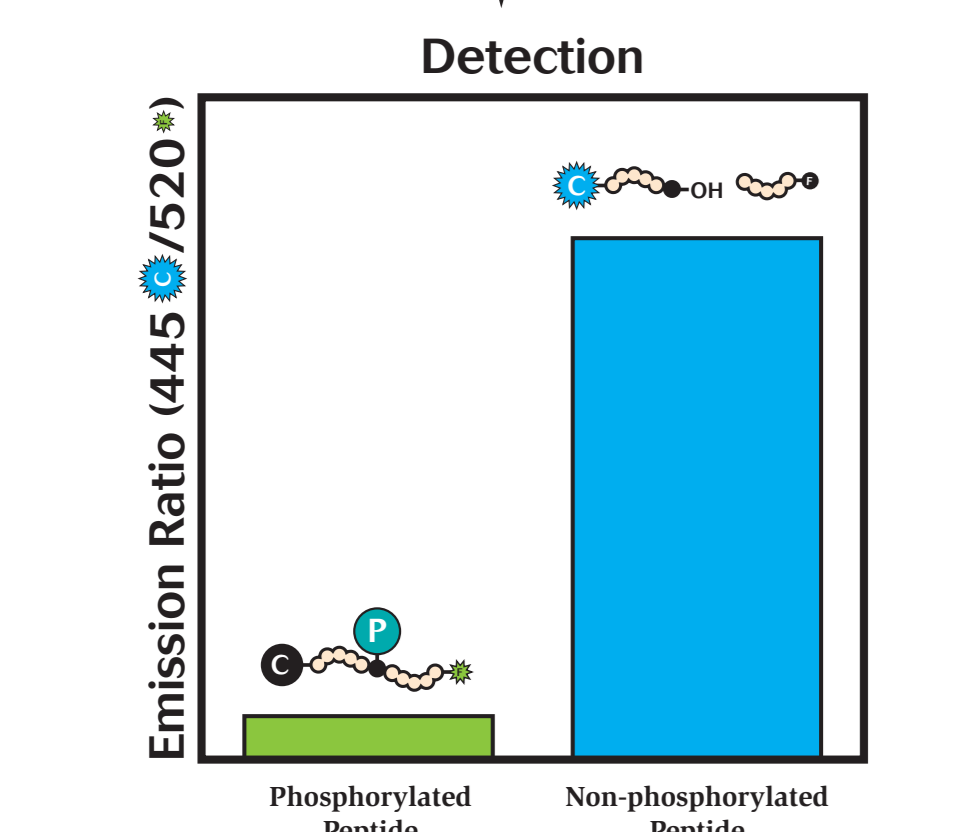
## 2 Assay Principle



In a 10 µL Kinase Reaction, the kinase transfers the γ-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 that 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 activity.



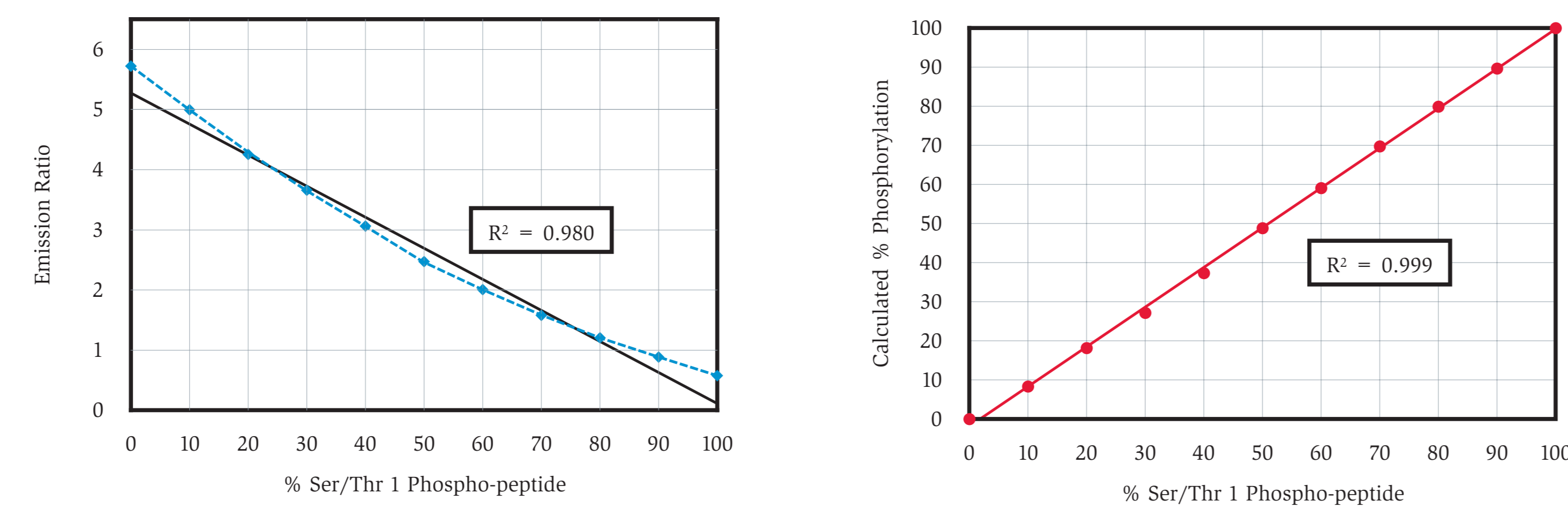
A ratiometric read-out of the donor emission over the acceptor emission quantitates reaction progress. The ratio is low if the peptide is non-phosphorylated, and high if the peptide is phosphorylated. Compounds that inhibit kinase activity will therefore produce a high ratio, and are easily distinguished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

$$\text{Emission Ratio} = \frac{\text{Coumarin Emission (445 nm)}}{\text{Fluorescein Emission (520 nm)}}$$

## 9 References

Rodems, S. *et al.* (2002) *ASSAY Drug Devel. Technol.* **1**:9-19  
Kleman-Leyer, K. *et al.* (2003) *Drug Disc. Devel.* **6**:81-2.

## 3 Linear Conversion



The degree of linearity of Emission Ratio to the actual % Phospho-peptide varies between peptides (left panel) and in some cases may be less than ideal. The use of the % Phosphorylation equation (shown below) transforms nonlinear Emission Ratio data to a nearly perfect linear fit with actual % Phospho-peptide (right panel).

$$\% \text{ Phosphorylation} = 1 - \frac{(\text{Emission Ratio} \times F_{100\%}) - C_{100\%}}{(C_{0\%} - C_{100\%}) + [\text{Emission Ratio} \times (F_{100\%} - F_{0\%})]}$$

Emission Ratio = Coumarin/Fluorescein ratio of sample wells

C<sub>100%</sub> = Coumarin signal of the 100% Phosphorylation Control

C<sub>0%</sub> = Coumarin signal of the 0% Phosphorylation Control

F<sub>100%</sub> = Fluorescein signal of the 100% Phosphorylation Control

F<sub>0%</sub> = Fluorescein signal of the 0% Phosphorylation Control

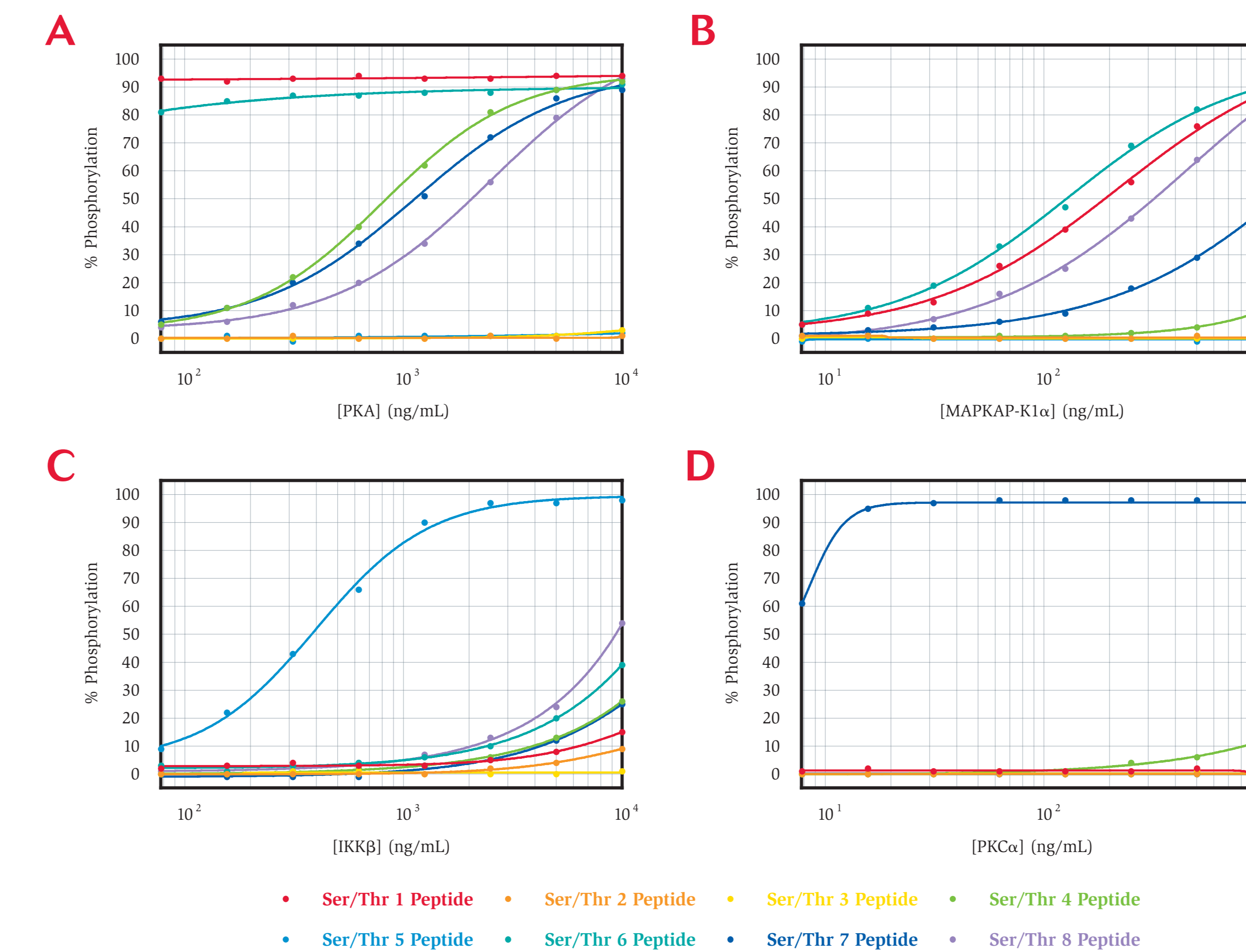
## 8 EC<sub>50</sub> Values in the Z'-LYTE™ Assay

In Corning® low-volume NBS 384-well plates, 30 kinases from Invitrogen Corporation were titrated with their optimal Z'-LYTE™ peptide substrate to generate EC<sub>50</sub> values. Titrations were performed in the presence of 10, 50, and 100 µM ATP in the Kinase Reactions. EC<sub>50</sub> values ranged from 7 pg/well to 50 ng/well and varied with the amount of ATP used in the Kinase Reaction.

Kinase	EC <sub>50</sub> Values (per well)		
	10 µM ATP	50 µM ATP	100 µM ATP
Abl1 (ng)	2.4	1.6	1.4
Arg (ng)	2.6	1.1	0.5
Akt1 (ng)	4.6	1.4	1.2
Akt2 (ng)	52	11	6.5
Akt3 (ng)	5.6	2.0	1.8
CHK1 (ng)	44	7.6	3.4
Csk (ng)	ND	10	6.2
Fgr (ng)	9.3	5.3	4.6
FLT3 (ng)	ND	40	19
Fyn (ng)	50	22	23
GSK3α (pg)	34	16	11
Hck (ng)	2.2	1.2	1.0
Lck (ng)	47	16	11
Lyn A (ng)	8.7	4.1	3.7
Lyn B (ng)	9.7	4.4	3.1

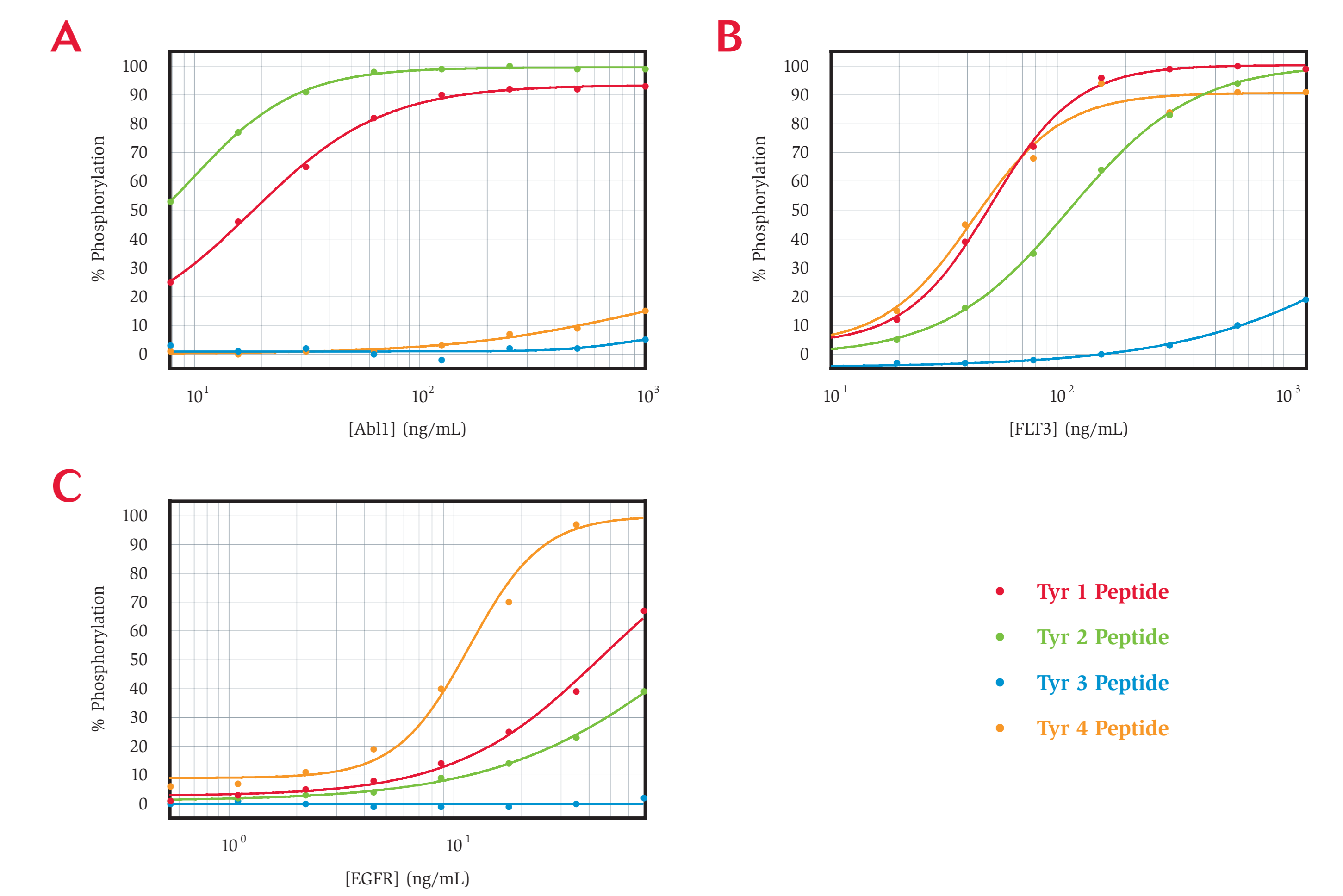
Kinase	EC <sub>50</sub> Values (per well)		
	10 µM ATP	50 µM ATP	100 µM ATP
PKA (pg)	41	29	24
PKCα (pg)	120	49	42
PKCβ1 (pg)	51	30	16
PKCβII (pg)	450	320	250
PKCγ (pg)	37	13	7
PKCδ (pg)	150	90	10
PKCε (pg)	59	59	69
PKCζ (pg)	44	35	25
PKCη (pg)	81	57	33
PKCθ (ng)	1.5	1.2	1.1
PKCι (ng)	1.5	0.6	0.6
Src (ng)	38	11	7.9
Src N1 (ng)	10.9	3.8	3.7
Src N2 (ng)	27	12	8.0
YES (ng)	30	13	9.6

## 4 Z'-LYTE™ Ser/Thr Kinase Substrate Panel 1



The Z'-LYTE™ Ser/Thr Kinase Substrate Panel 1 provides a quick and reliable assay to test the reactivity of a serine/threonine kinase against 8 Z'-LYTE™ peptide substrates. The eight Z'-LYTE™ Ser/Thr peptides were specifically chosen to provide substrates for different protein kinase families within the human kinome. **Panel A-C** PKA, MAPKAP-K1α, and IKKβ were serially diluted in Kinase Buffer (50 mM HEPES pH 7.5, 0.01% Brij-35, 10 µM MgCl<sub>2</sub>, 1 mM EGTA) in a 96-well plate according to the assay protocol and profiled against the Panel 2 µM substrate and 800 µM ATP in the kinase reactions. PKA and MAPKAP-K1α phosphorylated several peptide substrates to varying degrees. Z'-LYTE™ Ser/Thr 1 Peptide is the best substrate for PKA, Z'-LYTE™ Ser/Thr 6 Peptide for MAPKAP-K1α, and Z'-LYTE™ Ser/Thr 5 Peptide for IKKβ because they yield maximal phosphorylation, even at low kinase concentrations. **Panel D** PKCα was serially diluted in Kinase Buffer supplemented with required lipid (diacylglycerol and phosphatidyl serine) under similar conditions as the other serine/threonine kinases. PKCα phosphorylated only Z'-LYTE™ Ser/Thr 7 Peptide substrate.

## 6 Z'-LYTE™ Tyr Kinase Substrate Panel 1



The Z'-LYTE™ Tyr Kinase Substrate Panel 1 provides a quick and reliable assay to test the reactivity of a tyrosine kinase against 4 Z'-LYTE™ peptide substrates. **Panel A-B** Abl1 and FLT3 were serially diluted in Kinase Buffer and profiled against the Panel 2 µM substrate and 800 µM ATP in the kinase reactions. Z'-LYTE™ Tyr 2 Peptide is the best substrate for Abl1 and Z'-LYTE™ Tyr 1 Peptide for FLT3 because they yield maximal phosphorylation, even at low kinase concentrations. **Panel C** EGFR was serially diluted in Kinase Buffer supplemented with 2 mM MnCl<sub>2</sub> and 1 mM DTT under similar conditions as the other tyrosine kinases and Z'-LYTE™ Tyr 4 Peptide is the optimal substrate.

## 5 Z'-LYTE™ Ser/Thr Reactivity Table

In Corning® low-volume NBS 384-well plates (Corning # 3676), 68 commercially available serine/threonine kinases were tested against the Z'-LYTE™ Ser/Thr Kinase Substrate Panel 1 in the presence of 800 µM ATP in the Kinase Reactions. Z'-LYTE™ peptides that were phosphorylated at least 30% with ≤100 ng/well kinase were considered a viable substrate for that particular kinase. If more than one substrate was found, the top 3 peptide substrates were ranked. 47 Ser/Thr Kinases were identified to work with one or more of the 8 Z'-LYTE™ peptides, which are amenable to being used in high-throughput screening.

Ser/Thr Kinases	Kinase Family	Ser/Thr Peptide		
		1st	2nd	3rd
Akt1	AGC	6	8	7
Akt2	AGC	6	7	1
Akt3	AGC	6	7	1
AMPK	CAMK	4	-	-
Aurora A	Other	1	-	-
CamKIIα	CAMK	7	-	-
CaMKII	CAMK	4	5	1
CDK2/CycA	CMGC	3	8	-
CDK3/CycE	CMGC	3	1	-
CDK6	CMGC	8	-	-
CDK7/CycH	CMGC	4	5	1
CHK1	CAMK	7	-	-
CHK2	CAMK	7	4	-
CK1δ	CKI	5	-	-
ERK2	CMGC	3	-	-
GSK3α	CMGC	9	-	-
IKKα	Other	5	4	3
IKKβ	Other	5	8	6
MAPKAP-K1α/RSK1	AGC	6	1	8
MAPKAP-K1β/RSK2	AGC	6	1	8
MAPKAP-K1γ/RSK3	AGC	6	1	8
MAPKAP-K2	CAMK	4	-	-
MAPKAP-K3	CAMK	4	-	-
MAPKAP-K5	CAMK	4	-	-

Ser/Thr Kinases	Kinase Family	Ser/Thr Peptide		
		1st	2nd	3rd
MSK1	AGC	1	6	7
p38β	CMGC	3	-	-
p38γ	CMGC	3	-	-
p38δ	CMGC	3	-	-
p70 S6	AGC	7	8	-
PAK2	STE	1	4	8
PIM1	CAMK	7	6	-
PKA	AGC	1	6	4
PKCα	AGC	7	-	-
PKCβI	AGC	7	-	-
PKCβII	AGC	7	4	-
PKCγ	AGC	7	-	-
PKCδ	AGC	7	-	-
PKCε	AGC	7	-	-
PKCζ	AGC	7	-	-
PKCη	AGC	7	-	-
PKCθ	AGC	7	-	-
PKCι	AGC	7	-	-
PKG	AGC	1	6	7
REDK	CMGC	6	2	4
ROCK	AGC	7	4	1
ROCK2	AGC	7	4	-
SGK1	AGC	6	7	4

## 7 Z'-LYTE™ Tyr Reactivity Table

In Corning® low-volume NBS 384-well plates, 45 commercially available tyrosine kinases were tested against the Z'-LYTE™ Tyr Kinase Substrate Panel 1 in the presence of 800 µM ATP in the Kinase Reactions. Z'-LYTE™ peptides that were phosphorylated at least 30% with ≤100 ng/well kinase were considered a viable substrate for that particular kinase. If more than one substrate was found, the top 3 peptide substrates were ranked. 38 Ser/Thr Kinases were identified to work with one or more of the 8 Z'-LYTE™ peptides, which are amenable to being used in high-throughput screening.

Tyr Kinases	Kinase Type	Tyr Peptide		
		1st	2nd	3rd
Abl1	Cytosolic	2	1	-
Abl2	Cytosolic	2	1	-
Axl	Receptor	2	-	-
Blk	Cytosolic	1	2	4
BMX	Cytosolic	1	2	4
Brk	Cytosolic	1	2	-
e-KIT	Receptor	2	1	3
c-MET	Receptor	2	1	-
CSF1R	Receptor	2	1	3
Csk	Cytosolic	2	1	-
EGFR	Receptor	4	-	-
EPHB4	Receptor	2	1	-
Fes/Fps	Cytosolic	2	1	-
FGFR1	Receptor	2	1	-
FGFR4	Receptor	2	4	-
Fgr	Cytosolic	2	1	-
FLT3	Receptor	1	2	3
Fyn	Cytosolic	2	1	-
Hck	Cytosolic	2	1	-

Tyr Kinases	Kinase Type	Tyr Peptide		
		1st	2nd	3rd
Hyl	Cytosolic	1	2	4
IGF1R	Receptor	1	2	-
IRKβ	Receptor	2	1	-
IRTK	Receptor	1	-	-
ITK	Cytosolic	2	1	-
Jak3	Cytosolic	2	1	-
KDR	Receptor	2	1	-
Lck	Cytosolic	2	1	-
Lyn A	Cytosolic	2	1	-
Lyn B	Cytosolic	2	1	-
PDGFR	Receptor	4	-	-
Ros	Receptor	1	4	2
Src	Cytosolic	2	1	-
Src N1	Cytosolic	2	1	-
Src N2	Cytosolic	2	1	-
SYK	Cytosolic	2	1	-
TIE2	Receptor	2	1	-
TrkA	Receptor	1	2	-
YES	Cytosolic	2	1	-