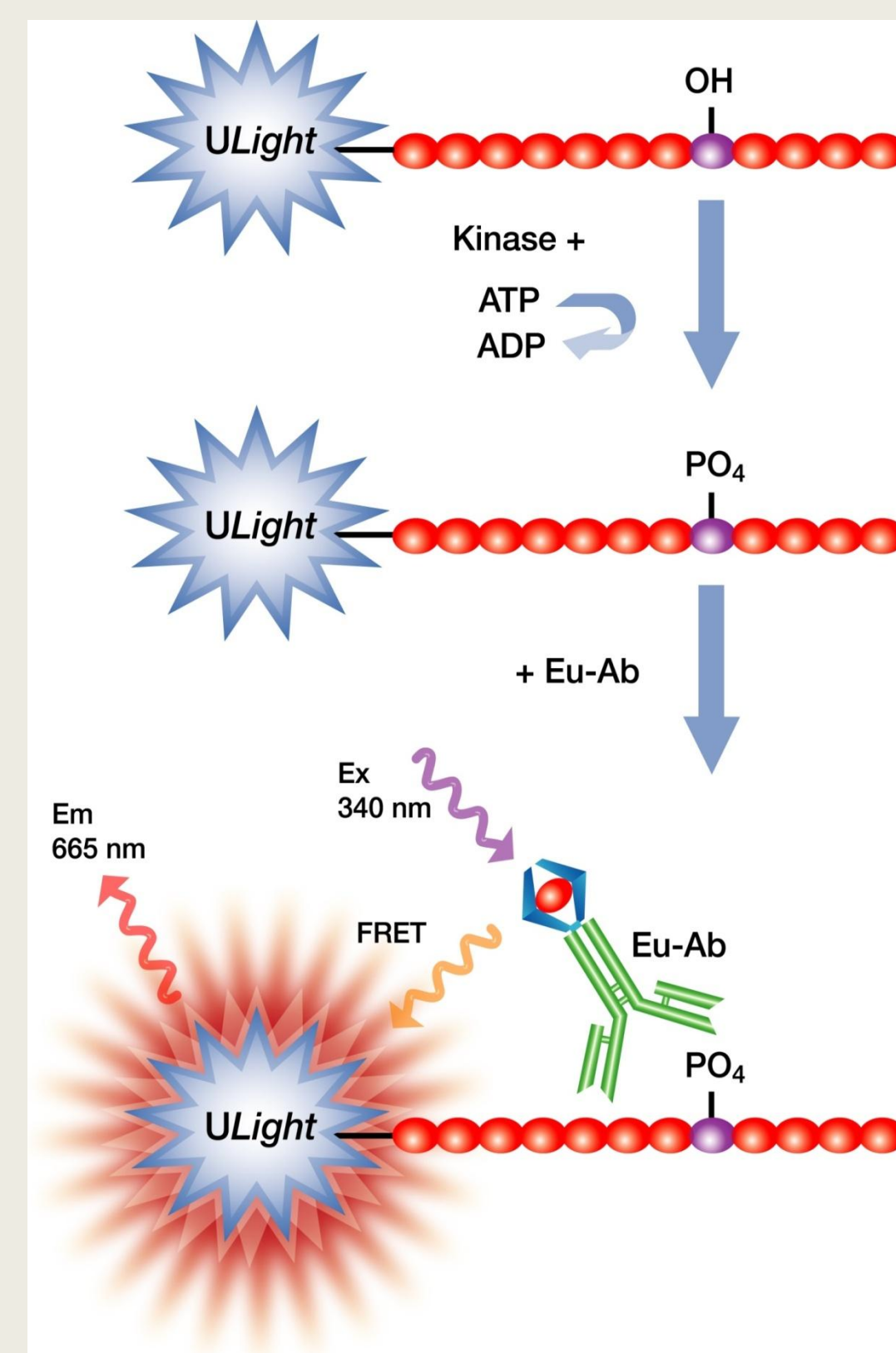


## 1 Abstract

Tyrosine (Tyr) kinases have emerged as one of the most important classes of drug targets due to their crucial role in cellular signalling. LANCE® Ultra time-resolved fluorescence resonance energy transfer (TR-FRET) kinase assays use europium-labeled anti-phospho-substrate antibodies with ULight™-labeled substrates. LANCE Ultra reagents include a series of six ULight-labeled substrates for tyrosine kinases that have been characterized with a panel of 83 receptor and non-receptor tyrosine kinases. A first group of substrates includes ULight-peptides whose sequences are derived from known tyrosine substrate proteins (JAK 1, CDK1 and IRS-1). These short peptidic substrates display excellent performance with specific subsets of Tyr kinases, but not with others. A second group of Tyr kinase substrates includes the large ULight-poly GAT and ULight-poly GT substrates, which are generic for the vast majority of Tyr kinases. These random co-polymers of glutamine, tyrosine and alanine are heterogeneous in size and tend to aggregate over time. Accordingly, we have developed a new ULight-labeled substrate that combines the advantages of both short peptides and large co-polymers. The ULight-TK peptide is a generic tyrosine kinase substrate that is homogeneous in size (28 amino acids). Its proprietary sequence includes eight Tyr residues placed in different amino acid contexts to favour its interaction with a majority of Tyr kinases. This new LANCE Ultra product can be used in combination with any of the three LANCE europium-anti-phosphotyrosine antibodies currently available (i.e., PT66, PY20 and P-Tyr-100). In this poster, we present the profiling of the ULight-TK peptide against a panel of 83 tyrosine kinases and the development of an EphA4 tyrosine kinase assay in a 384-well format using the ULight-TK peptide and PT66. Overall, data demonstrate that the ULight-TK peptide is suitable for over 85% of assayed kinases and enables the development of robust ( $Z'$ =0.79) Tyr kinase assays suitable for HTS. The ULight-TK peptide is therefore a valuable addition to the LANCE Ultra panel of tyrosine kinase substrates.

## 2 LANCE Ultra Kinase Assay Principle



LANCE Ultra is a TR-FRET technology for kinase assays. In a typical Tyr kinase assay, the binding of a Eu-labeled anti-phospho-Tyr antibody to the phosphorylated ULight-labeled substrate brings donor and acceptor molecules into close proximity. Following irradiation of the kinase reaction at 320 or 340 nm, the energy from the Eu donor is transferred to the ULight acceptor dye which, in turn, generates light at 665 nm. The intensity of the light emission is proportional to the level of ULight-substrate phosphorylation.

## 3 Materials, Protocol and Optimization

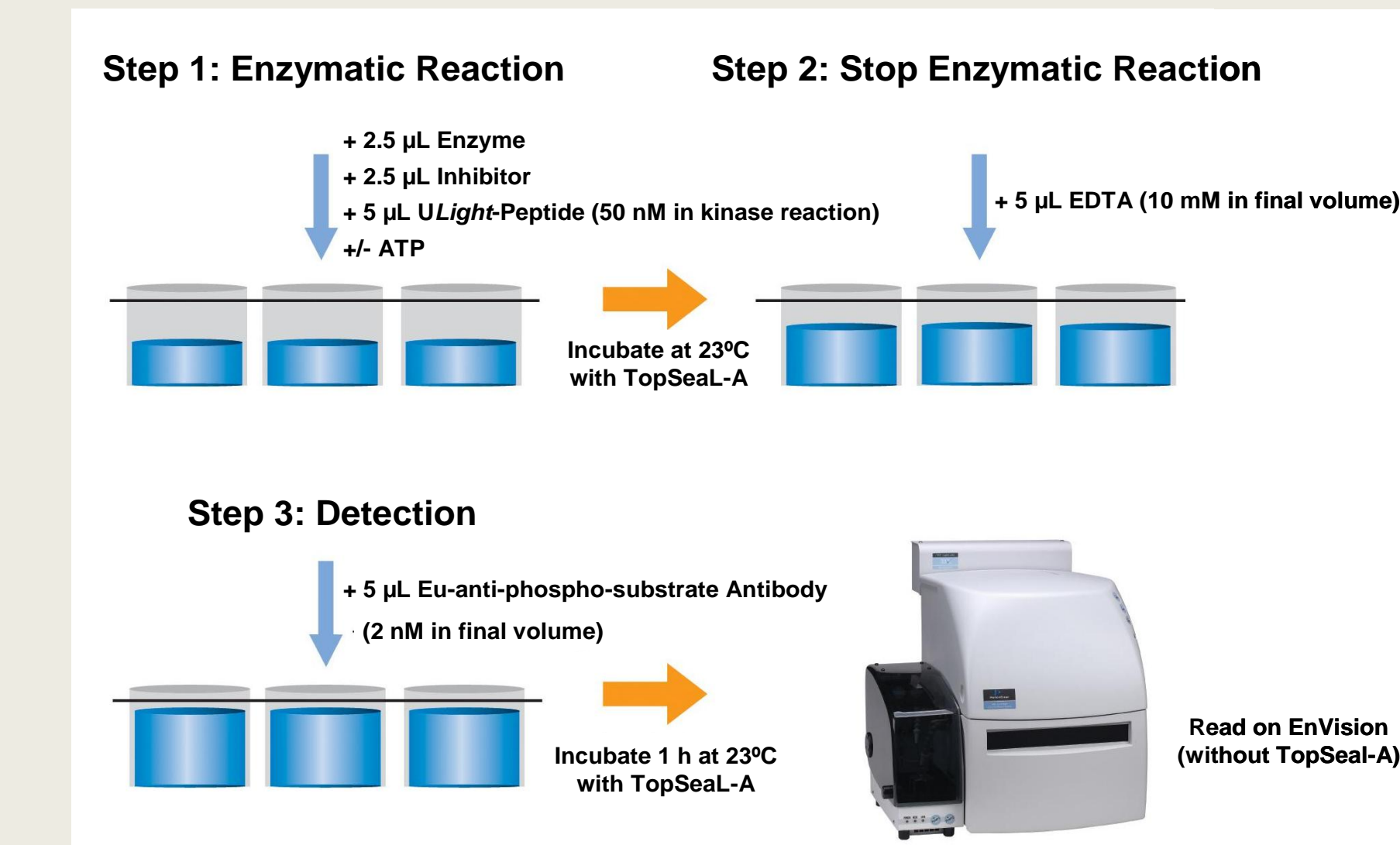
### Materials

- ULight™ -TK peptide PerkinElmer # TRF0127-M
- Eu-anti-phosphotyrosine (PT66) PerkinElmer # AD0068
- LANCE® Detection Buffer, 10X PerkinElmer # CR97-100
- OptiPlate™-384, white PerkinElmer # 6007299
- TopSeal™-A PerkinElmer # 6005185
- Envision® PerkinElmer # 2104-0010

All kinases were recombinant (human origin) and purchased from Carna Biosciences.

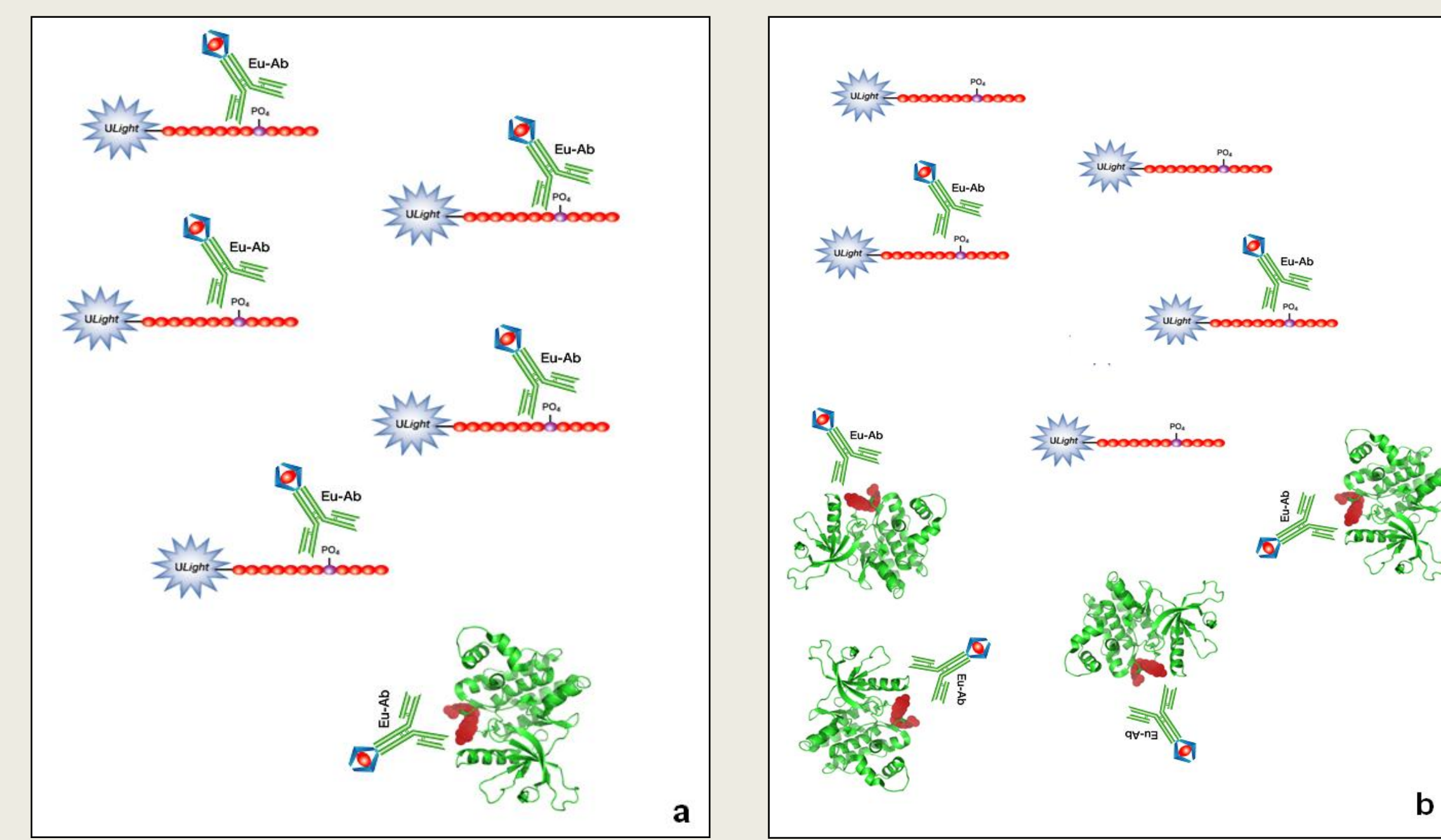
Kinase Buffer: 50 mM HEPES pH 7.5, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, 2 mM DTT and 0.01% Tween-20.

### Standard Protocol



### Determination of Optimal Kinase Concentration for Profiling

- Several kinases contain phospho-Tyr that can bind to the Eu-anti-P-Tyr Antibodies, thus interfering with signal from the phosphorylated substrate.
- Enzyme titrations were therefore performed to determine up to which concentration each kinase could be used without interfering with the assay signal.
- Kinase concentration is then selected so that it does not interfere markedly with signal.

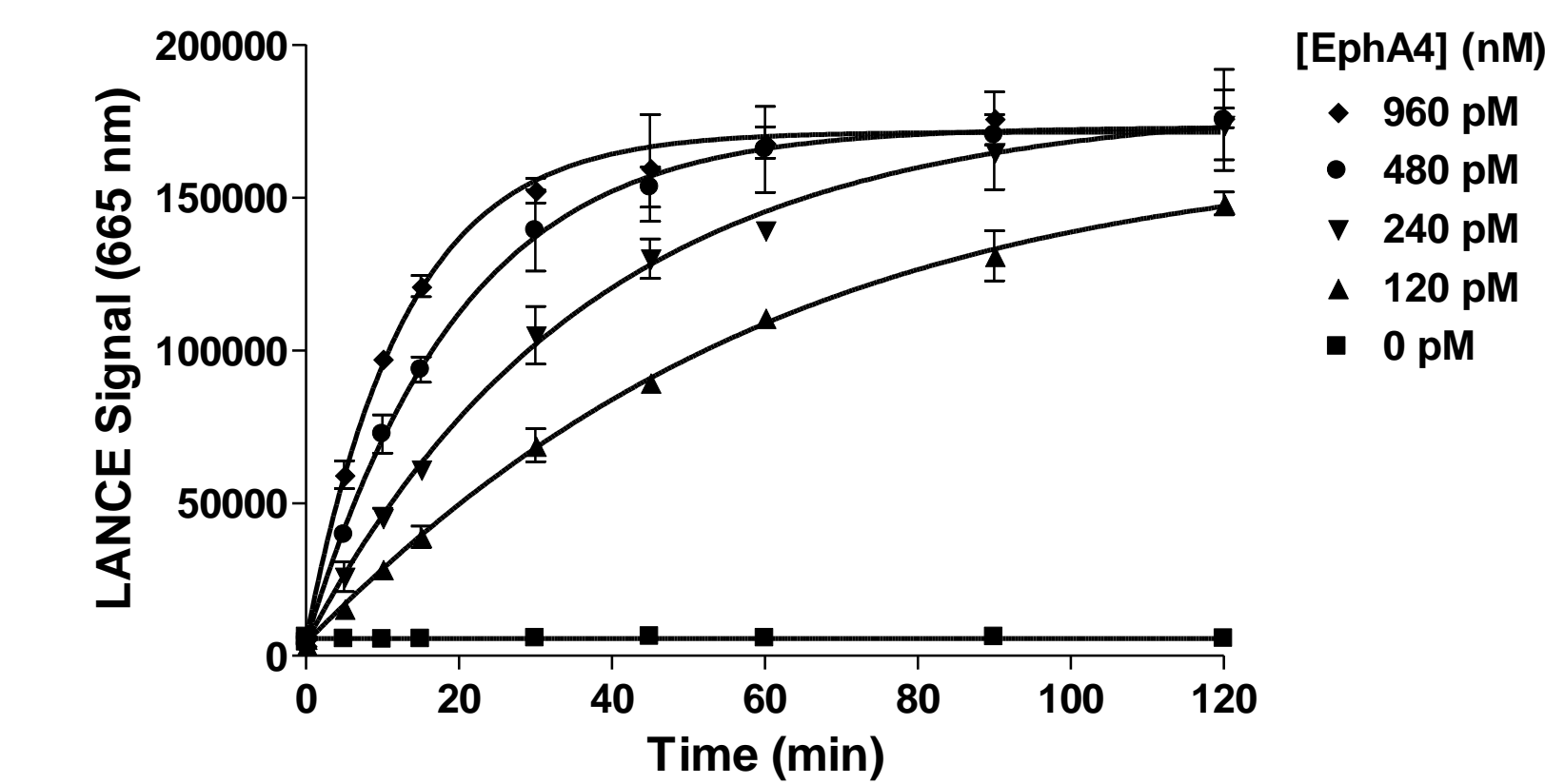


Low concentrations of phosphorylated kinase

At higher concentrations of phosphorylated kinase, the Eu-antibody is depleted (less Eu-Ab is available to bind ULight peptide = lower signal at 665 nm)

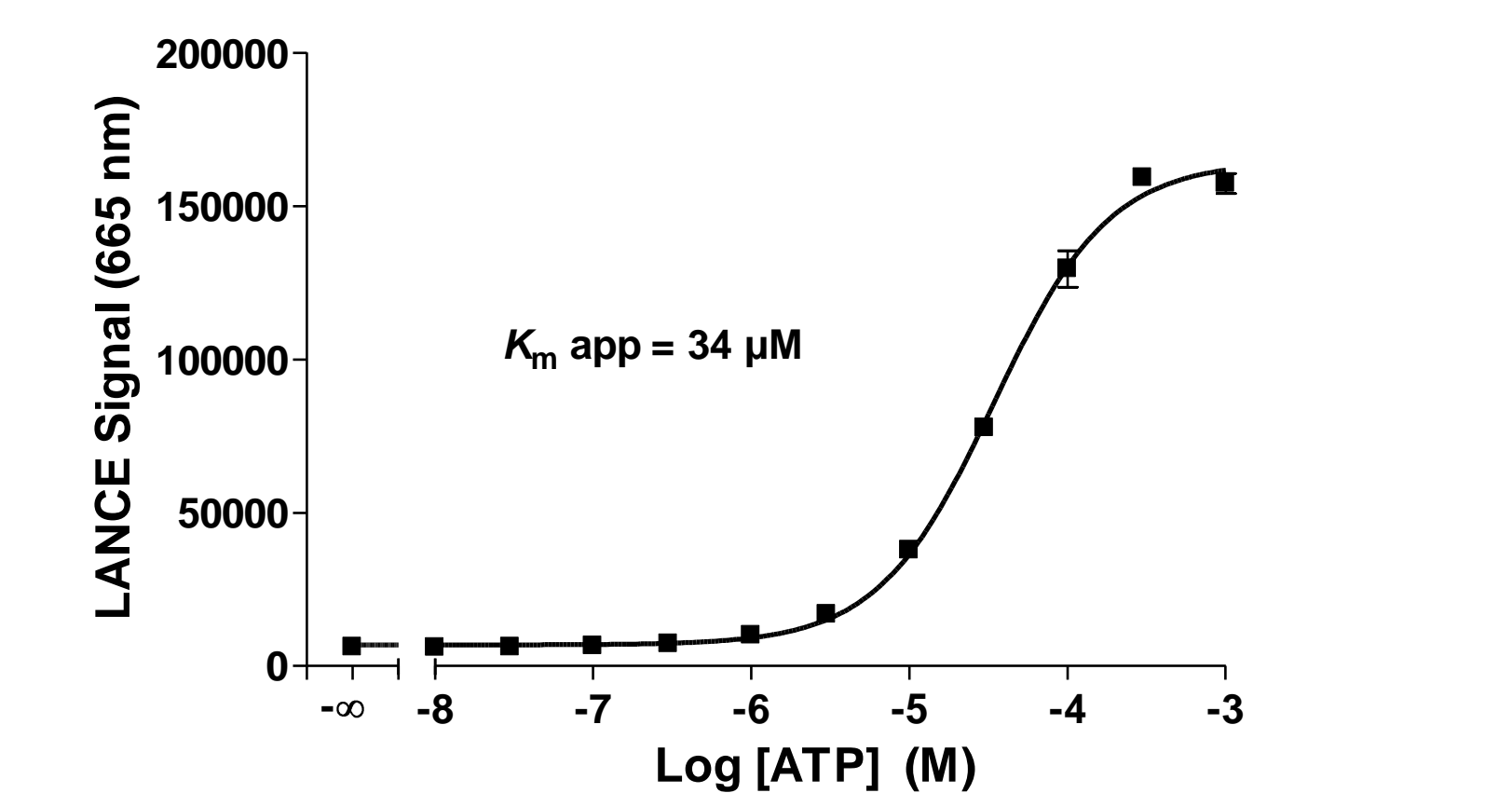
## 4 EphA4 Assay Development

### Enzyme Titration and Time Course



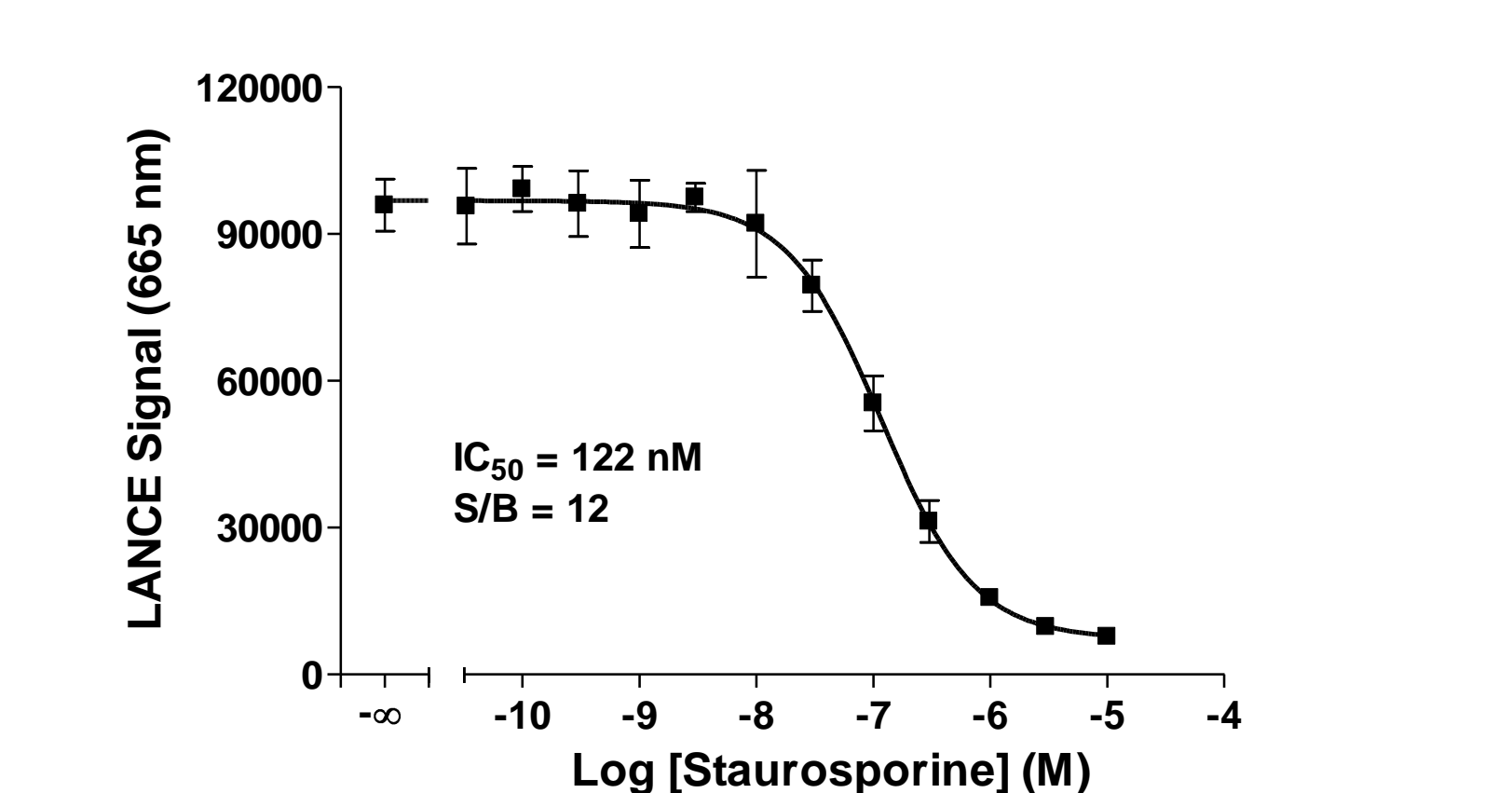
The EphA4 enzyme was incubated at concentrations ranging from 120 to 960 pM with 50 nM ULight-TK peptide and 200 µM ATP. Kinase reactions were terminated after 0 to 120 min by the addition of EDTA.

### ATP Titration



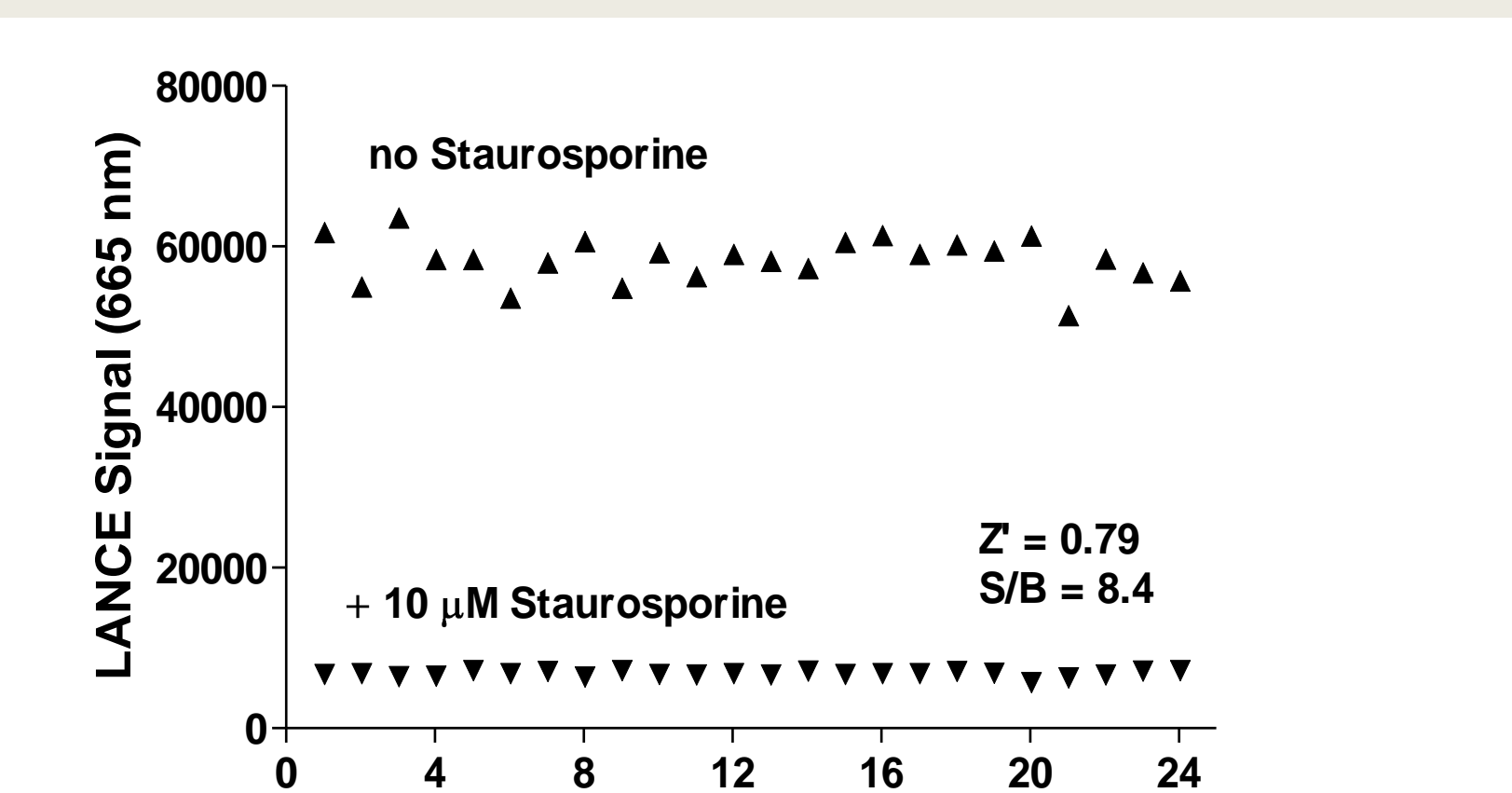
Serial dilutions of ATP ranging from 10 nM to 1 mM were incubated with 120 pM EphA4 enzyme and 50 nM of ULight-TK peptide. Kinase reactions were terminated after 60 min by the addition of EDTA.

### Enzyme Inhibitor Curve



Serial dilutions of Staurosporine ranging from 30 pM to 10 µM (final concentrations in 2% DMSO) were incubated with 120 pM EphA4 enzyme, 50 nM ULight-TK peptide and 35 µM ATP. Kinase reactions were terminated after 60 min by the addition of EDTA.

### Z'-factor Determination



EphA4 enzyme at 120 pM was incubated with 50 nM ULight-TK peptide and 35 µM ATP, with or without 10 µM staurosporine (final concentrations in 2% DMSO). Kinase reactions were terminated after 60 min by the addition of EDTA.

## 5 Profiling Data

### Receptor Tyrosine Kinases

Kinase	Kinase concentration (nM)	S/B ratio
ALK	4	8.8
AXL	4	1.8
DDR1	0.5	1.0
DDR2	0.5	1.0
EGFR	0.5	10.0
EGFR [T790M]	0.5	3.3
EphA1	0.5	10.8
EphA2	0.25	26.9
EphA3	0.5	22.4
EphA4	0.5	31.5
EphA5	1	35.5
EphA6	0.5	5.4
EphA7	0.5	17.2
EphA8	0.25	32.3
EphB1	0.5	29.9
EphB2	0.25	28.8
EphB3	0.1	24.7
EphB4	0.5	29.8
FGFR1	0.5	26.4
FGFR2	0.25	27.1
FGFR3	0.25	24.4
FGFR4	3	17.7
FLT1	0.25	4.1
FLT3	0.1	25.9
FLT4	0.25	20.0
FMS (CSFR)	0.25	3
HER2	10	1.4
HER4 (ERBB4)	0.1	3.1
IGF1R	0.25	25.1
INSR	20	4
IRR	0.5	19.2
KDR	0.25	16.9
KIT	2	1.5
LTK	1	13.7
MER	0.25	12.9
MET	0.5	24.2
MUSK	1	3.1
PDGFRa	0.5	8
PDGFRb	0.25	4.7
RET	0.5	20.3
RON	0.25	3.5
ROR1	20	1.1
ROR2	20	1.0
ROS	0.25	24.5
TIE2	0.5	17.7
TRKA (NTRK1)	0.5	23.2
TRKB (NTRK2)	0.5	28.0
TRKC (NTRK3)	0.5	6.4
TYRO3	0.25	6.1

### Cytoplasmic Tyrosine Kinases

Kinase	Kinase concentration (nM)	S/B ratio
ABL	0.25	4.1
ABL [T315I] (ABL1)	0.5	4.8
ACK	20	17.3
ARG	0.5	10.6
BLK	0.5	31.0
BMX	0.5	7.1
BRK	3	2.4
BTK	0.25	6.8
CSK	20	27.2
CTK	20	2.5
FAK	3	1.7
FER	0.25	31.0
FES	1	24.9
FGR	0.1	29.3
FRK	0.5	9.7
FYN	0.25	25.9
HCK	0.25	29.7
ITK	0.5	8.4
JAK1	7	13.7
JAK2	1	35.5
JAK3	0.5	16.1
LCK	0.5	12.3
LYNa	0.5	32.5
LYNb	1	33.1
PYK2	0.25	2.9
SRC	0.5	31.7
SRM	0.5	32.5
SYK	0.5	24.6
TEC	0.5	18.6
TNK1	1	6.8
TXK	3	5.9
TYK2	4	7.3
YES	0.25	28.0
ZAP70	4	7.6

S/B ratio ≥ 10 S/B ratio ≥ 5 and ≤ 10 S/B ratio ≥ 3 and ≤ 5

The average S/B ratio (as +/- ATP) was calculated, for each enzyme:substrate pair, from two independent experiments.

Assay conditions:  
Kinase reaction, 2h in 10µL  
- ULight-TK peptide: 50nM  
- ATP: 200µM  
Detection, 1h in 20µL final volume  
- EDTA: 10mM  
- Eu-PT66: 2nM

## 6 Summary

- ULight-TK is a synthetic 28-amino acid peptide containing eight Tyr residues placed in different amino acid contexts. Its proprietary design makes it an ideal substrate for the vast majority of Tyr kinases.
- ULight-TK allows the straightforward development of robust Tyr kinase assays suitable for HTS (EphA4,  $Z'$ =0.79).
- ULight-TK was used for kinase profiling and was a suitable substrate for 72 out of the 83 Tyr kinases tested.
- LANCE Ultra ULight-TK assays can be easily automated and miniaturized to both the 384-low volume and the 1536-well formats.
- ULight-TK is now available as part of the new LANCE Ultra KinaSelect™ TK kit (TRF0301-D), together with the europium-labeled anti-phosphotyrosine antibody PT66 and LANCE detection buffer.