Novel Substrates for Fluorescence-based Protein Tyrosine Kinase Assays

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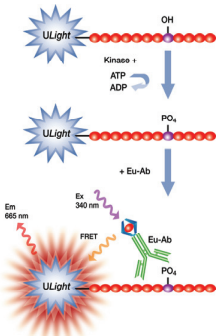
1 Introduction



Protein tyrosine kinases (PTK) are involved in the regulation of many aspects of the cell cycle, including proliferation, differentiation, secretion and apoptosis. Aberrant protein kinase expression or function is a cause or consequence of many human diseases like cancer and atherosclerosis. As a result, kinases became very important targets in drug discovery. This has prompted the development of many kinase assay technologies such as time-resolved fluorescence energy transfer (TR-FRET).

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

Six U*Light*-labeled PTK substrates were developed. The substrates are poly GT (4:1), poly GAT(1:1:1), three peptides of 14-18 amino acids in length derived from known phosphorylated targets: CDK1 (Tyr15), IRS-1 (Tyr983), and JAK-1 (Tyr1023), and a fourth peptide containing a novel sequence phosphorylated by most tyrosine kinases (TK peptide; patent pending). These peptide targets were initially validated using PTKs cited in the literature. For example, the JAK-1 peptide was assayed with the enzyme JAK3 (Wang et al 2003). Our objective was to further evaluate these substrates with a large number of human PTKs in order to determine the applicability of the substrates to assay other PTKs.

The results demonstrated that poly GT, poly GAT, and the TK peptide are universal substrates, whereas the other peptide substrates work only with certain kinases. Due to its small size, the TK peptide may offer unique advantages in assay development compared to the large poly GT and poly GAT substrates in regard to assay reproducibility and signal stability. To our knowledge, this is the first time such extensive analysis of human PTK activity versus different substrates has been undertaken.

2 Materials and Methods

Materials - U*Light*-poly GT (4:1), U*Light*-poly GAT (1:1:1), U*Light*-IRS-1 (Tyr983), U*Light*-JAK-1 (Tyr1023), U*Light*-CDK1 (Tyr15), U*Light*-TK, U*Light*-Streptavidin, and Europium-anti-phospho-Tyr (PT66) Antibody were from PerkinElmer LAS, Inc (PKI). All kinases were recombinant (human origin) and purchased from Carna Biosciences.

General assay procedure - The assays were performed in White OptiPlate™-384 microplates from PKI. All assay incubations were performed at room temperature and the microplates were sealed with TopSeal-A™ (PKI) during that time. The fluorescence signals were read using an excitation filter of 320 nm and an emission filter of 665 nm on an EnVision® Multilabel Reader (PKI). The final assay volume was 20 µL.

Determination of kinase concentration – 0 to 30 nM of each kinase was incubated with 50 nM of biotinylated phospho-Tyr-peptide in the presence of 200 µM ATP for 2 h. The reaction was stopped by adding 10 mM EDTA and 50 nM U*Light*-Streptavidin for 5 min. 2 nM Europium-anti-phospho-Tyr (PT66) Antibody was added and incubated for another 1 h before reading the fluorescence.

Kinase profiling – The kinase was incubated with 50 (CDK1, JAK-1 and TK) or 100 nM (IRS-1, poly GT and poly GAT) substrate in the presence or absence of 200 µM ATP. The enzyme reaction proceeded for 2 h before stopping with 10 mM EDTA for 5 min. 2 nM Europium-anti-phospho-Tyr (PT66) Antibody was added and incubated for another 1 h before reading the fluorescence. The signal to background (S/B) ratio was calculated by dividing the fluorescence signal of the reaction with ATP by the signal from the reaction without ATP.

3 The Substrates

U*Light*-poly GT (4:1) & U*Light*-poly GAT (1:1:1)
Polypeptide sequences : polymers of amino acids (20-50 kDa) in which the ratios of amino acids are as indicated (G = glutamic acid, T = tyrosine, A = alanine)
Origin: these synthetic polypeptides were originally described by Braun et al 1984

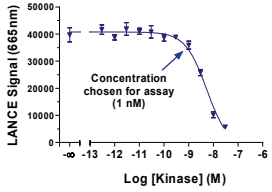
U*Light*-CDK1 (Tyr15)
Peptide sequence: CAGAGKIGEGT**Y**GVVYK
Origin: peptide derived from amino acids 9-20 of human cyclin-dependent kinase 1 (CDK1). The peptide is a substrate for several Src family kinases (Cheng et al 1992)

U*Light*-IRS-1 (Tyr983)
Peptide sequence : CKKSRGDY**M**TMQIG
Origin: peptide derived from residues 979-989 of mouse Insulin receptor substrate 1 (IRS-1). The peptide contains the YMxM motif shown to be a consensus site for PTK phosphorylation (Shoelson et al 1992, Garcia et al 1993)

U*Light*-JAK-1 (Tyr1023)
Peptide sequence : CAGAGAIETDKEY**Y**TVKD
Origin: peptide derived from amino acids 1015-1027 of human Janus kinase 1 (JAK-1). The peptide is a substrate for JAK3 (Wang et al 2003)

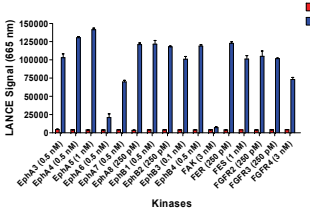
U*Light*-TK
Peptide sequence and origin : patent pending

4 Determination of Kinase Concentration



Several kinases contain phospho-Tyr residues that can bind to the antibody in the assay and compete with the phosphorylated substrate; therefore, the kinase concentration has to be chosen so that it does not interfere in the signal generation. Preliminary tests with the kinase and a phospho-peptide were performed to determine the concentration of kinase to use in the assay. Enough ATP was added to allow for potential protein autophosphorylation to occur. The fluorescence signal was plotted versus the concentration of enzyme and the highest concentration of enzyme providing minimal or no interference of signal was chosen for determination of activity versus the various U*Light*-substrates. The competition curve for JAK2 kinase is illustrated.

5 Kinase Profiling



Kinase	S/B ratio
EphA3	22.4
EphA4	31.5
EphA5	35.5
EphA6	5.4
EphA7	17.2
EphA8	32.3
EphB1	29.9
EphB2	28.8
EphB3	24.7
EphB4	29.8
FAK	1.7
FER	31.0
FES	24.9
FGFR2	27.1
FGFR3	24.4
FGFR4	17.7

A panel of 16 kinases were tested with the substrate U*Light*-TK. The results clearly demonstrate that this substrate can be used to assay numerous kinases.

6 Profiling Data

	Family	Kinase	Kinase concentration (nM)	S/B ratios					
				ULight-poly GT	ULight-poly GAT	ULight-CDK-1	ULight-IRS-1	ULight-JAK-1	ULight-TK
Cytoplasmic Tyrosine Kinases	ABL	ABL	0.25	13.1	11.1	1.3	1.1	1.2	4.1
	ABL	ABL [T315I] (ABL1)	0.5	6.8	6.4	1.3	1.2	1.3	4.8
	ACK	ACK	20	27.3	27.2	4.8	1.5	2.0	17.3
	ABL	ARG	0.5	13.2	12.7	1.4	1.2	1.4	10.6
	SRC	BLK	0.5	16.5	7.8	9.6	1.1	2.6	31.0
	TEC	BMX	0.5	13.2	9.0	2.2	1.1	1.2	7.4
	SRC	BRK	3	17.4	3.0	1.4	1.3	1.1	2.4
	TEC	BTK	0.25	11.8	19.6	3.1	1.0	1.5	6.8
	CSK	CSK	20	10.4	20.3	1.7	1.3	3.9	27.2
	CSK	CTK	20	12.6	18.8	1.0	1.0	1.0	2.5
	FAK	FAK	3	20.0	13.0	1.0	1.0	1.0	1.7
	FER	FER	0.25	12.9	17.1	16.1	9.1	5.8	31.0
	FER	FES	1	17.1	19.0	10.3	7.2	4.0	24.9
	SRC	FGR	0.1	25.1	17.7	4.1	1.1	3.2	29.3
	SRC	FRK	0.5	20.8	22.9	1.3	1.1	2.2	9.7
	SRC	FYN	0.25	12.8	10.4	19.7	1.0	2.3	25.9
	SRC	HCK	0.25	21.3	13.2	12.8	1.2	5.1	29.7
	TEC	ITK	0.5	9.8	8.5	2.5	1.6	1.6	8.4
	JAKA	JAK1	7	17.6	16.4	1.3	3.7	19.5	13.7
	JAKA	JAK2	1	8.9	7.9	7.2	21.4	41.8	35.5
	JAKA	JAK3	0.5	6.7	6.9	1.3	6.0	37.3	16.1
	SRC	LCK	0.5	10.4	16.5	6.1	1.0	1.7	12.3
	SRC	LYNa	0.5	15.6	8.9	14.3	1.1	7.4	32.5
	SRC	LYNb	1	18.6	13.2	24.0	1.2	18.3	33.1
	FAK	PYK2	0.25	15.5	7.7	2.3	1.1	1.3	2.9
	SRC	SRC	0.5	15.1	18.4	16.8	1.1	5.9	31.7
	SRC	SRM	0.5	18.3	20.1	8.0	1.3	2.0	32.5
	SYK	SYK	0.5	2.3	4.1	1.2	1.8	20.0	24.6
	TEC	TEC	0.5	25.4	15.4	2.2	1.0	1.8	18.6
	ACK	TNK1	1	9.3	15.2	2.4	1.6	1.2	6.8
	TEC	TXK	3	11.0	18.9	3.2	1.2	1.3	5.9
	JAKA	TYK2	4	6.2	5.3	1.3	7.3	15.2	7.3
	SRC	YES	0.25	15.3	14.0	12.8	1.0	3.0	28.0
	SYK	ZAP70	4	11.1	15.3	1.1	1.1	6.7	7.6
Receptor Tyrosine Kinases	ALK	ALK	4	9.8	7.2	2.6	16.8	3.9	8.8
	ALK	AXL	4	9.8	5.6	1.5	1.6	1.2	1.8
	DDR	DDR1	0.5	2.0	3.0	1.0	1.1	1.0	1.0
	DDR	DDR2	0.5	4.1	5.1	1.0	4.9	1.0	1.0
	EGFR	EGFR	0.5	1.7	5.0	1.8	2.5	19.0	10.0
	EGFR	EGFR [T790M]	0.5	18.1	10.8	1.1	1.2	3.9	3.3
	EPH	EphA1	0.5	24.1	11.4	3.1	1.2	1.2	10.8
	EPH	EphA2	0.25	12.3	7.1	2.1	1.8	3.6	26.9
	EPH	EphA3	0.5	17.4	8.0	4.4	3.5	3.8	22.4
	EPH	EphA4	0.5	12.6	11.3	6.7	2.4	6.4	31.5
	EPH	EphA5	1	18.0	21.9	19.2	7.6	21.2	35.5
	EPH	EphA6	0.5	14.2	15.5	1.2	1.1	1.3	5.4
	EPH	EphA7	0.5	22.7	11.1	1.4	1.3	1.5	17.2
	EPH	EphA8	0.25	16.4	7.8	3.1	1.6	5.6	32.3
	EPH	EphB1	0.5	25.1	7.9	14.1	8.4	10.6	29.9
	EPH	EphB2	0.25	27.9	13.2	13.1	3.3	5.7	28.8
	EPH	EphB3	0.1	29.0	11.2	8.3	2.3	4.0	24.7
	EPH	EphB4	0.5	13.7	4.0	12.6	4.3	12.0	29.8
	FGFR	FGFR1	0.5	2.9	5.0	1.6	13.5	30.8	26.4
	FGFR	FGFR2	0.25	7.8	11.6	1.6	15.5	33.2	27.1
	FGFR	FGFR3	0.25	16.0	10.7	1.4	8.1	27.6	24.4
	FGFR	FGFR4	3	9.2	5.6	1.2	3.4	20.8	17.7
	VEGFR	FLT1	0.25	22.2	15.4	1.1	1.9	10.0	4.1
	PDGFR	FLT3	0.1	20.8	13.2	3.6	4.5	25.0	25.9
	VEGFR	FLT4	0.25	6.9	6.5	1.8	11.4	36.9	20.0
	FGFR	FMS (CSFR)	0.25	14.6	6.2	1.2	1.4	6.3	9.1
	EGFR	HER2	10	9.3	8.4	1.1	1.1	1.4	1.4
	EGFR	HER4 (ERBB4)	0.1	25.2	14.4	1.1	1.1	1.5	3.1
	INSR	IGF1R	0.25	21.8	14.6	3.1	15.2	12.8	25.1
	INSR	INSR	20	18.3	25.4	1.1	3.2	1.6	4.0
	INSR	IRR	0.5	21.6	9.1	2.4	15.6	12.7	19.2
	VEGFR	KDR	0.25	8.6	4.3	1.3	5.1	30.8	16.9
	PDGFR	KIT	2	13.0	21.7	1.0	1.0	3.3	1.5
	ALK	LTK	1	21.3	25.1	2.4	3.6	2.7	13.7
	AXL	MER	0.25	29.2	15.9	9.7	5.6	3.8	12.9
	MET	MET	0.5	17.0	13.2	4.1	4.5	19.7	24.2
	MUSK	MUSK	1	2.5	10.6	1.8	1.1	3.9	3.1
	PDGFR	PDGFRa	0.5	17.8	10.6	1.3	2.4	9.0	8.0
	PDGFR	PDGFRb	0.25	11.2	12.5	1.6	2.0	3.0	4.7
	RET	RET	0.5	1.4	3.2	1.5	6.9	23.6	20.3
	MET	RON	0.25	27.7	25.7	1.3	4.4	4.9	3.5
	ROR	ROR1	20	3.4	1.5	0.9	1.0	1.0	1.1
	ROR	ROR2	20	4.2	1.4	1.0	0.9	1.0	1.0
	SEV	ROS	0.25	23.8	9.3	4.4	20.5	7.7	24.5
	TIE	TIE2	0.5	19.3	9.4	1.6	2.1	5.4	17.7
	TRK	TRKA (NTRK1)	0.5	16.3	17.5	5.5	11.8	15.1	23.2
	TRK	TRKB (NTRK2)	0.5	22.9	26.3	7.0	11.1	17.0	28.0
	TRK	TRKC (NTRK3)	0.5	34.1	22.3	1.1	1.7	1.4	6.4
	AXL	TYRO3	0.25	21.5	9.1	4.7	4.8	2.1	6.1

The average S/B ratio was calculated, for each enzyme:substrate pair, from two independent experiments.

S/B ratio > 10
S/B ratio > 5 and < 10
S/B ratio > 3 and < 5

7 Data Analysis

Kinase representation:

	Receptor	Cytoplasmic	Total
Number of families	16	9	25
Number of kinases	49	34	83

Assay performance:

Substrate	Highest S/B ratio obtained	Kinase(s)
U*Light*-polyGT	34	TRKC
U*Light*-polyGAT	27	ACK
U*Light*-CDK1	24	LYNb
U*Light*-IRS-1	21	JAK2, ROS
U*Light*-JAK-1	42	JAK2
U*Light*-TK	36	JAK2, EphA5

Selectivity of the substrates

U*Light*-poly GT (4:1) & U*Light*-poly GAT (1:1:1) & U*Light*-TK
77 kinases had an S/B ratio > 3 for U*Light*-poly GT.
81 kinases had an S/B ratio > 3 for U*Light*-poly GAT.
72 kinases had an S/B ratio > 3 for U*Light*-TK.

U*Light*-CDK1 (Tyr15)
33 kinases, from 12 families, had an S/B ratio > 3.
10/33 kinases are from the SRC family (cytoplasmic). Only 2/12 kinases (BRK and FRK) from the SRC family did not phosphorylate the U*Light*-CDK1 substrate.
9/33 kinases are from the EPH family (receptor).

U*Light*-IRS-1 (Tyr983)
32 kinases, from 14 families, had an S/B ratio > 3. Only 2 of these families (6 kinases) are of the cytoplasmic type: JAKA and FER.

U*Light*-JAK-1 (Tyr1023)
50 kinases, from 19 families, had an S/B ratio > 3.

8 Conclusions

83 human PTKs were assayed with six substrates using the TR-FRET technology. The general conclusions are:

- It was possible to find at least one substrate for every kinase tested.
- Poly GT, poly GAT, and TK are general substrates for PTKs. They worked with over 87% of PTK enzymes.
- The CDK1 peptide was a selective substrate, as mostly kinases of the SRC and EPH families phosphorylated this peptide.
- The IRS-1 peptide was also a selective peptide, as mostly kinases of the receptor type were active with this peptide.
- On the other hand, the JAK-1 peptide was quite generic in that 50 kinases from 19 different families phosphorylated this substrate.
- Kinase concentration should be optimized carefully as many PTKs are phosphorylated on tyrosines and could interfere in the assays.

9 References

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