EGFR and human EphA4 receptor tyrosine kinase assays for compound screening: Performance evaluation of LANCE® *Ultra*™ TR-FRET assay platform

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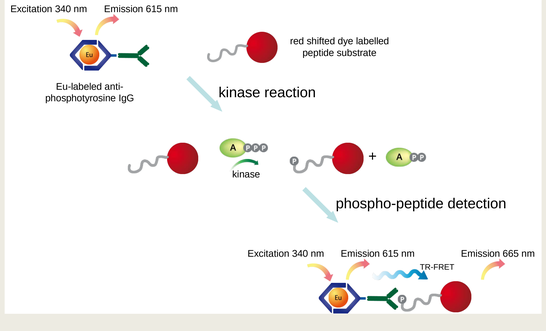
1 Abstract

Tyrosine kinases are implicated in the etiology of several disease steps including neoplastic development and progression. Tyrosine kinase signaling pathways normally prevent deregulated proliferation and contribute to sensitivity to apoptotic stimuli. These signaling pathways are often altered in cancer cells. Thus in the area of drug development it becomes important to have quick and reliable methods for kinase analysis. In the present study, assays were developed for the EGF and EphA4 receptor tyrosine kinases to evaluate the performance of two TR-FRET assay platforms with regard to key attributes for compound screening. TR-FRET tyrosine kinase assays were performed using both the LANCE® *Ultra* kinase assay technology and an alternative europium-based TR-FRET platform from a different supplier (Supplier I). The signal:background (S:B) ratios, kinase concentration requirements, and responses to inhibitors were evaluated for both assay platforms. Using the same source of EphA4 kinase, both assays were capable of achieving a S:B ratio greater than 3:1, a general criteria for an acceptable assay. The LANCE Ultra assay also required 4-fold lower amounts of EphA4 kinase and was capable of generating S:B ratios as high as 20. For EGFR kinase, the S:B value of 3:1 was surpassed by the LANCE assay and marginally achieved by the alternative TR-FRET assay. Kinase inhibition studies for EphA4 kinase were performed using three kinase inhibitors: Staurosporine, AG1478 (4-(3-Chloroanilino)-6,7-demthoxyquinzoline) and PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo [3,4-d]pyrimidine. Our data indicated that both assays gave IC50 values close to previously reported values. However, a lower amount of kinase was needed for the inhibitor studies using the LANCE *Ultra* kinase assay platform.

2 Introduction

Kinase assay setup



LANCE *Ultra* and Supplier I assay configurations.

Both assay technologies relied on anti-phosphotyrosine antibodies labeled with Europium chelates: pTyr-100, PT66 and PY20 IgGs were tested with LANCE *Ultra* while only PY20 was available from Supplier I. LANCE *Ultra* and Supplier I assays were performed using U*Light*™- and Alexa-647 labeled peptide substrates respectively.

3 Materials & Methods

Microplates

384-well white ProxiPlate™ (PerkinElmer #6008280)

Kinase Reaction Buffer

EphA4: 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT

EGFR: 50mM HEPES, 10mM $MgCl_2$, 1mM EDTA, 2mM DTT, 0.01% Tween 20

EGFR Assay Optimization

Antibody testing: Kinase reactions contained 2.5 µM ATP (control: no ATP) and were performed for 1 hr at 23 °C (10 µL reaction volume in 384-well white ProxiPlate) and stopped by addition of 6 mM EDTA. Then 2 nM Eu-antibody (20 µL final reaction volume) was added and assays were incubated 4 hrs, then read on the EnVision® Multilabel Plate Reader.

EGFR kinase (Invitrogen #PV3872) was used at 500 pM in the 10 µL reaction for assay optimizations, and at the concentrations indicated for the kinase titration experiments. The poly-GT substrates from both suppliers were used at the optimal concentration recommended by each supplier (100 nM in the 10 µL reaction).

LANCE *Ultra* vs Supplier I comparison: Kinase reactions were performed as described above, except that kinase concentration was varied from 31.3 pM to 500 pM. The pairs of poly-GT substrates and antibodies were from the respective suppliers (PerkinElmer: P-Tyr-100 antibody, Supplier I: PY20 antibody).

EphA4 Assay Optimization

Antibody testing: Kinase reactions contained 36 µM ATP (control: no ATP) and were performed for 1 hr at 23 °C (10 µL reaction volume in 384-well white ProxiPlate) and stopped by addition of 6 mM EDTA. Then 2 nM Eu-antibody (20 µL final reaction volume) was added and assays were incubated 2 hrs, then read on the EnVision Multilabel Plate Reader.

EphA4 kinase (Invitrogen #PV3651) was used at 119 pM in the 10 µL reaction for assay optimizations, and at the concentrations indicated for the kinase titration experiments. The poly-GT substrates from both suppliers were used at the optimal concentration recommended by each supplier (100 nM in the 10 µL reaction).

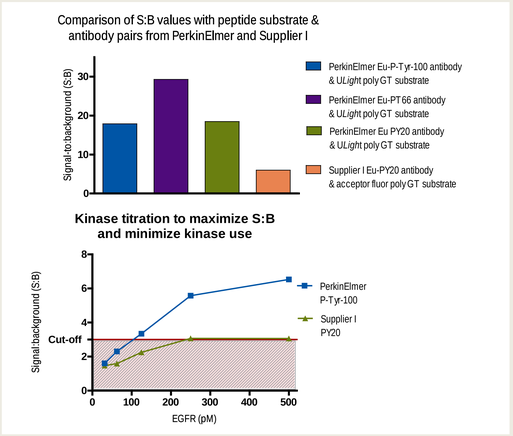
LANCE *Ultra* vs Supplier I comparison: Kinase reactions were performed as described above, except that kinase concentration was varied from 3.7 pM to 120 pM. The pairs of poly-GT substrates and antibodies were from the respective suppliers (PerkinElmer: PT66 antibody, Supplier I: PY20 antibody).

EphA4 Inhibition Studies

LANCE *Ultra*: 5 µl EphA4 were added to each well followed by 2.5 µL inhibitors diluted in 4% DMSO and 2.5 µL U*Light*™ polyGT (100 nM final). Kinase reactions contained 22.5 µM ATP and were performed for 1 hr at 23 °C (10 µL reaction volume in 384-well white ProxiPlate) and stopped by addition of 6 mM EDTA. Then 2 nM Eu-PT66 (20 µL final reaction volume) was added and assays were incubated 4 hrs and read on the EnVision Multilabel Plate Reader.

Supplier I assay: 5 µL EphA4 were added to each well followed by 2.5 µL inhibitors diluted in 4% DMSO and 2.5 µl Alexa-647 polyGT (100nM final). Kinase reactions contained 22.5 µM ATP and were performed for 1 hr at 23 °C (10 µL reaction volume in 384-well white ProxiPlate) and stopped by addition of 6 mM EDTA. Then 2 nM Eu-PTY20(20 µL final reaction volume) was added and assays were incubated 4 hrs and read on the EnVision Multilabel Plate Reader.

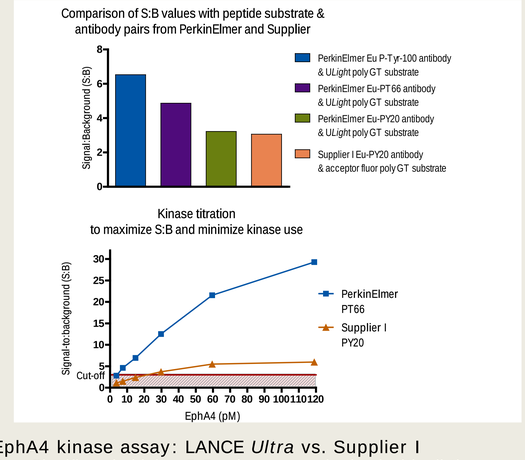
4 EGFR Assay Optimization



EGF receptor kinase assay: LANCE *Ultra* vs. Supplier I

Top: Two of the three antibodies available from PerkinElmer gave significantly higher S:B values than the one antibody available from Supplier I. Bottom: Using the PerkinElmer P-Tyr-100 antibody and substrate, S:B values over 6.5 were achievable. The only EGFR kinase assay from Supplier I (based on PY20 antibody) was barely able to achieve the S:B value of 3:1 needed for an acceptable assay.

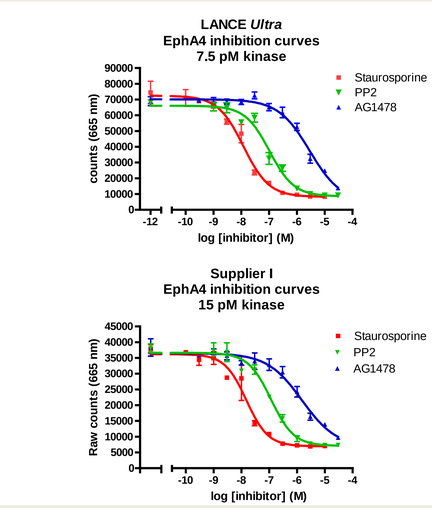
5 EphA4 Assay Optimization



EphA4 kinase assay: LANCE *Ultra* vs. Supplier I

Top: Significantly higher S:B values were obtained with all three antibodies available from PerkinElmer than with the one antibody available from Supplier I. Bottom: The best available antibody and substrate was used from each supplier. The PerkinElmer assay achieved S:B values over 20, and needed 4-fold less kinase to achieve a S:B value above the S:B cut-off of 3:1 for an acceptable assay. Lower kinase consumption means a lower cost per assay point.

6 EphA4 Inhibition Studies



Small compound inhibition of EphA4 activity.

Kinase inhibition studies were performed using three kinase inhibitors: Staurosporine, AG1478 and PP2. Both assay technologies generated IC_{50} values in agreement to those previously reported values. A two-fold lower amount of kinase was needed for the inhibitor studies using the LANCE *Ultra* kinase assay platform.

7 Summary

LANCE *Ultra* and Supplier I TR-FRET reagents were compared for their capacity to deliver robust performance for EGFR and EphA4 kinase screening. During the course of this study, we found that:

- Careful antibody selection is key to produce sensitive assay conditions: P-Tyr-100 and PT66 antibodies were shown to generate the best assay conditions for EGFR and EphA4, respectively, when assays were developed with LANCE *Ultra* reagents.
- PY20, the only antibody available from Supplier I, was not the antibody of choice for the EGFR or EphA4 assay, based on data from LANCE *Ultra* assays.
- For EGFR kinase, the S:B value of 3:1 was surpassed by the LANCE assay and marginally achieved by the alternative TR-FRET assay. The LANCE *Ultra* assay also required 4-fold lower amounts of EphA4 kinase and was capable of generating S:B ratios as high as 20.
- EphA4 kinase inhibition studies performed using three kinase inhibitors indicated that both assays gave appropriate pharmacology based on IC_{50} values.
- It is worth noting that the lower amount of kinase required by the LANCE *Ultra* kinase assay platform allows for cost savings, as well.

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