Rapid and Quantitative Cell-Based Immunoaffinity Kinase Assay in a 96-well Format

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A wide range of protein kinases have been implicated in human diseases and, as a result, kinase inhibitors are of great interest as therapeutic drugs. Robust quantitative assays that measure kinase activity in a cellular context are crucial to the mechanistic study of kinase inhibitors. We present an *in vitro* kinase assay that measures selected kinase activities in cell lysate preparations. In this method immunoaffinity purification of a selected kinase and a homogeneous kinase assay (LANCE®) are carried out in the same well of a 96-well plate. This method offers several advantages over traditional immunoprecipitation/kinase assays including a 96-well plate format that enables the handling of multiple samples with consistency and increased throughput, and a homogeneous detection method that avoids the use of multiple wash steps and radioisotopes. We demonstrate the robustness of this technique by evaluating the activity of FMS-related tyrosine kinase 3 (Flt3) in SEM cell lysates. This assay has been extended to other kinases by using target selective antibodies and substrates.

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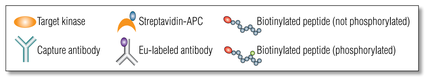
Assay Principal

Design of cell-based immunoaffinity kinase assay in a 96-well format. Key assay steps are illustrated above, including LANCE® detection.

Materials:

1. 10X Cell Lysis Buffer (CST #9803)
2. 4X Kinase Assay Buffer (CST #9805)
3. 10 mM ATP (CST #9804)
4. Gastric Precursor (Tyr87) Biotinylated Peptide (CST #1310)
5. 1.25 M DTT
6. 50 mM EDTA
7. Blocking Buffer
8. 96-well plate
9. SEM cell lysate
10. Streptavidin: Surelight®-APC (Perkin Elmer #CR130-100)
11. Eu-W1024-labeled anti-phospho-tyrosine Ab (Perkin Elmer #AD0203)



Add cell lysate to capture antibody-coated well and incubate

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Wash to remove unbound proteins

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Add substrate and ATP and incubate

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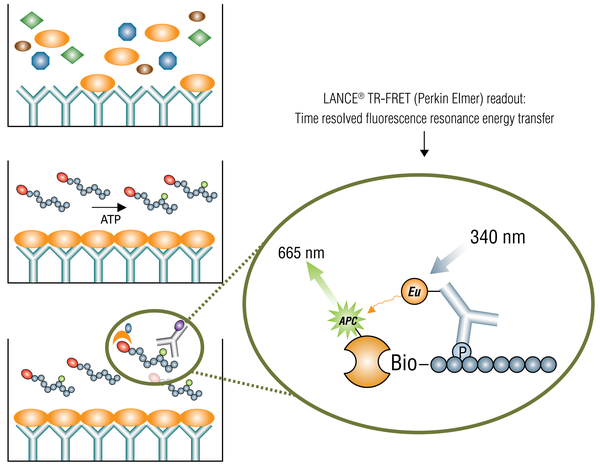
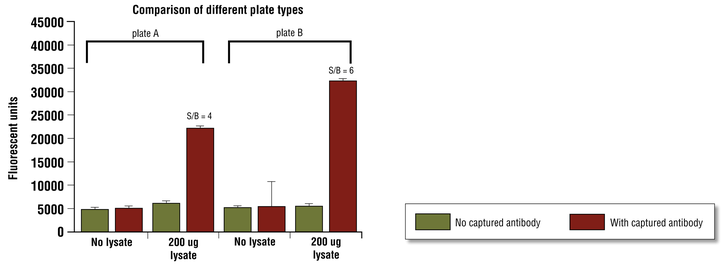
LANCE® detection

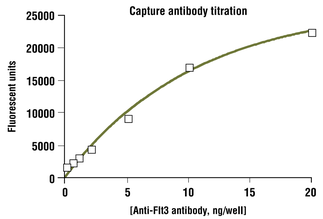
Diagram of Assay Principle



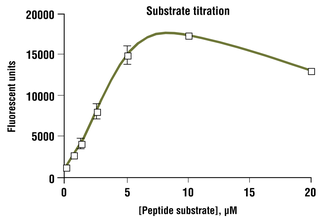
Assay Optimization

Comparison of immunoaffinity kinase assay in two different plate types. Plate B was selected for use in the remainder of the study due to better signal to background ratio (S/B) as compared to plate A.

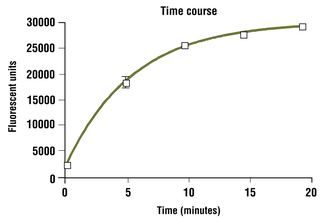




Capture antibody titration for Flt3 immunoaffinity kinase assay. Increasing amounts (0 to 20 ng/well) of anti-Flt3 antibody (CST #3462) were coated in a well. Kinase activity was measured using a LANCE® detection method. 10 ng/well capture Ab (CST #3462) was selected for use in the remainder of the study.

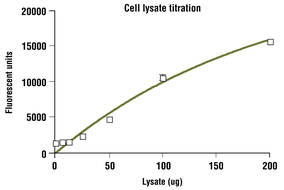


Substrate titration for Flt3 immunoaffinity kinase assay. Increasing concentration (up to 20 uM) of Flt3 substrate peptide (CST #1310) was used to measure Flt3 kinase activity. 5 uM was selected for use in the remainder of the study.



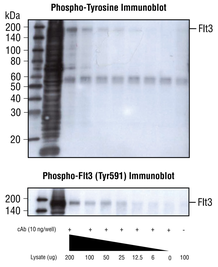
Time course of Flt3 immunoaffinity kinase assay. Each well was coated with 10 ng capture Ab (CST #3462) and 5 mM Flt3 substrate peptide (CST #1310) was used for each reaction. A reaction time of 60 minutes was selected for use in the remainder of the study.

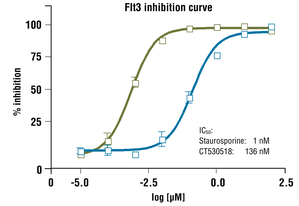
Assay Specific



◀ *Cell lysate titration for FLT3 immunoaffinity kinase assay. Increasing amounts of SEM cell lysate were added to wells and the immunoaffinity kinase assay was carried out as outlined in previous figures. The activity of captured Flt3 was determined using a LANCE® detection method.*

Upon completion of the assay, plates were washed and SDS sample buffer was added to each well to remove the contents. Contents of the wells were analyzed by SDS-Page followed by Western blotting using Phospho-Tyrosine Ab (CST #9411) (top panel) and Phospho-FLT3 (Tyr591) Ab (CST #3466) (bottom panel). MW marker in lane 1, cell lysate in lane 2 and control well lacking Flt3 capture antibody in lane 10 (last lane). ▶





◀ *Inhibition of Flt3 kinase activity by treatment with staurosporine (general kinase inhibitor) or CT530518 (Flt3 specific inhibitor). Captured Flt3 kinase was exposed to increasing concentrations of inhibitor for 5 min. followed by addition of ATP and substrate. The IC50 values determined for staurosporine and CT530518 in this assay are consistent to those reported in the literature.*

Summary

A cell-based immunoaffinity kinase assay has been developed in a 96-well plate format. Using Flt3 containing SEM cells as an example, we demonstrate that this assay format can selectively measure kinase activity in cell lysate using a homogeneous LANCE® detection method. IC50 values determined using this assay are consistent to those reported in literature. Compared to traditional immunoprecipitation/kinase assays, this method has the follow advantages:

1. A 96-well plate format that eases handling of multiple samples
2. An in-plate immunoseparation process that replaces a traditional immunoprecipitation method
3. An antibody-based detection method that eliminates radioactive waste generation
4. A homogeneous detection method (LANCE®) that eliminates multiple wash steps