Rapid, ELISA-based measurement of protein tyrosine kinase activity using the K-LISA™ Kit

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The K-LISA™ PTK Screening Kit is a colorimetric kit in a 96-well plate format, which provides a rapid and sensitive method for detection of protein tyrosine kinase (PTK) activity in purified or partially purified kinase preparations, cell lysates and tissue extracts. The kit features a multiplex array of three synthetic, random polypeptide substrates of glutamic acid, alanine and tyrosine, which allow comparison of specificity differences among PTKs.

PTKs phosphorylate many different intracellular proteins and play key roles in signal transduction pathways that control a variety of critical cellular functions, and have been implicated in many diseases, including immunological disorders and cancer. As a result, PTKs are important targets in signal transduction and drug discovery research.

PTKs readily accept synthetic substrates composed of random polymers devoid of serine and threonine, but containing different molar ratios of glutamic acid (E) and tyrosine (Y)^{1–4}; glutamic acid, alanine (A) and tyrosine^{4,5}; or glutamic acid, alanine, lysine and tyrosine⁴. Indeed, some synthetic PTK substrates have much lower Michaelis-Menten constants than corresponding physiological substrates⁴. Different PTKs exhibit preferences for specific molar ratios of the amino acids forming the polypeptide substrates. For example, the β -insulin receptor (β -InsR)^{4,5} preferentially phosphorylates E₄Y compared to E₆A₃Y, whereas the epidermal growth factor receptor (EGFR) and the Abelson tyrosine kinase (c-Abl) preferentially phosphorylate E₆A₃Y compared to E₄Y⁵. Nonetheless, the E₄Y substrate is a broadly accepted PTK substrate⁵ and has been successfully used in an ELISA-based format to evaluate purified PTK activity^{1,2} and to assess PTK activity in cytosolic breast cancer samples⁶.

Here we demonstrate the ability of the K-LISA™ PTK Screening Kit to detect PTK activity using microunit to milliunit amounts of purified PTKs, less than 20 ng total protein from tissue extracts and less than 5 μ g total protein from cultured cell lysates. In addition, experiments to distinguish between the activities of two EGFR variants at less than 100 pM are described to address the kit's utility in mutational analyses.

The K-LISA™ PTK Screening Kit

The K-LISA™ PTK Screening Kit is a nonradioactive, rapid assay that can be completed in 1.5–2 h using simple colorimetric detection. The kit includes two precoated and preblocked 96-well plates

with sealers, which can be separated into 8-well strips. All required assay components are supplied with the exception of BSA, 2-mercaptoethanol and 0.5 N H₂SO₄. A lyophilized rat spleen extract is also supplied as a positive control. Each 8-well strip includes two each of blank, E₄Y, EAY and EY polypeptide coated wells. The assay can detect PTK activity in less than 5 μ g total protein from human cell lysates and in less than 20 ng total protein from tissue extracts such as rat spleen. Moreover, the assay's high sensitivity allows measurement of purified PTK activity in the microunit (for example, FLT3, c-Abl, β -InsR) to milliunit (for example, Src, EGFR) range. The assay kit procedure entails diluting the sample into reaction buffer, a 30 minute

Figure 1 | Activities and substrate preferences of purified PTKs. The K-LISA™ PTK Screening Kit detected β -InsR, FLT3 and c-Abl activities with microunit (μ U) sensitivity (1 U = 1 nmol phosphate transferred to E₄Y per min at 30 °C in the presence of 100 μ M ATP), whereas Src activity was detected at milliunit (mU) sensitivity (1 U = 1 pmol phosphate transferred to E₄Y per min at 30 °C in the presence of 100 μ M ATP). Data were obtained using a microplate reader (plate background subtracted at 595 nm). The four-parameter fits are from baseline corrected data ($r^2 = 0.96$ to 0.99). Backgrounds were typically 0.05–0.07 absorbance units. Error bars are s.e.m.

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incubation at 30 °C followed by phosphotyrosine recognition using a peroxidase-conjugated anti-phosphotyrosine mAb, and detection with the tetramethylbenzidine substrate.

Activities and substrate preferences of purified PTKs

The sensitivity and range response of the K-LISA™ PTK Screening Kit is demonstrated in the activity profiles of purified FLT3, c-Abl, β -InsR and Src PTKs. Figure 2 illustrates sensitivity in the picomolar range. Moreover, the kit's multiplex-based design shows different substrate preferences for these PTKs. For example, FLT3 preferentially phosphorylated EAY over E₄Y and EY (FLT3 phosphorylated these latter two substrates almost equivalently), whereas Src preferred the EY substrate over EAY and greatly preferred EAY over E₄Y.

PTK activity in tissue extracts and human cell lysates

Protein kinases typically represent 1–2% of the total protein from cell or tissue extracts, only about 1% of which are protein tyrosine kinases. Notably, the K-LISA™ PTK Screening Kit can detect tyrosine kinase activity in less than 20 ng total protein from rat spleen extract, which contains about 2 pg of PTK (data not shown).

To examine the robustness and sensitivity of the K-LISA™ PTK Screening Kit, PTK activity was measured in cell lysates (5 μ g/well total protein) from cultures of A431, HeLa and HEK 293 cells (Fig. 3). Because A431 cells are rich in EGFR and Src, whereas HEK 293 cells are rich in FLT3 and c-Abl, it was of interest to ascertain whether the PTKs in these cell lysates had differential phosphorylation consistent with the substrate preferences denoted in Figure 1 and for EGFR (Fig. 3). Indeed, the strong phosphorylation of the E₄Y and EY substrates by the A431 lysate and of the EAY substrate by the HEK 293 lysate is consistent with the substrate preferences of the purified PTKs.

Comparison of the PTK activity of EGFR-WT and EGFR-ct1022

PTK activities of immunopurified wild-type EGFR (EGFR-WT) and a truncated form of EGFR (EGFR-ct1022) were compared for mouse B82L cells that had been transfected to contain either human EGFR-WT or EGFR-ct1022. The carboxy-terminal region of EGFR is a common site for various naturally occurring mutations^{7,8}, contains the calpain hinge region near amino acid 1,037 (ref. 7), and may control both substrate specificity and have an inhibitory function^{7–9}. Figure 3 illustrates the PTK activity responses from EGFR-WT and EGFR-ct1022. Both forms of EGFR preferentially phosphorylated the E₄Y substrate, but EGFR-ct1022 had 1.5 to 2 times greater activity than

Figure 3 | Comparison of PTK activity of EGFR-WT and EGFR-ct1022. EGFR proteins were immunopurified from mouse B82L cells transfected to contain either human EGFR-WT or human EGFR-ct1022. Detection was performed using EGFR-WT and EGFR-ct1022 at 76 pM and 89 pM concentrations, respectively (that is, 1 ng/100 μ l).

EGFR-WT. These results are consistent with a previously reported fluorescence polarization study of these two forms of EGFR⁷. Notably, the sensitive K-LISA™ PTK Assay Kit distinguished the activity differences between EGFR-WT and EGFR-ct1022 at concentrations of only 76 pM and 89 pM, respectively (1 ng/100 μ l).

Summary

This ELISA-based assay kit offers a rapid, sensitive method to monitor PTK activity and detect substrate specificity in cell and tissue extracts as well as purified enzyme preparations. The assay had picomolar sensitivity for purified PTKs and detected PTK activity using microgram and nanogram amounts of total protein extracted from human cells and rat spleen tissue extract, respectively. The inclusion of three substrates adds a ratiometric component to the comparison of these samples. Moreover, this kit is sensitive enough to detect differences between two forms of EGFR receptor at concentrations <100 pM. The K-LISA™ PTK Screening Kit is a useful tool for studying kinase inhibitors and mutational effects or for rapid assessment of PTK activity in a variety of sample types.

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Figure 2 | PTK activity of human cell lysates. Values shown have been corrected for background and are the average of triplicate measurements. Error bars are s.e.m.