

Application Note

P-Tyr-100 Insulin Receptor Tyrosine Kinase Assay



Introduction

Protein tyrosine kinases play a key role in signal transduction and normal cell growth. They are also involved in numerous proliferative diseases like cancer and atherosclerosis, in addition to a number of autoimmune diseases. For that reason the tyrosine kinases are often targeted in research and HTS laboratories for new drug discovery. We have designed a very robust, low cost, generic tyrosine kinase assay using a new P-Tyr-100 AlphaScreen™ kit. The AlphaScreen P-Tyr-100 assay is designed to measure the activity of tyrosine kinases with very high sensitivity and specificity. Until recently, two antibodies were commonly used to detect tyrosine kinase activity: PY20 and PT66. The new antibody clone P-Tyr-100 binds to a broader range of peptides with a higher affinity, since its binding activity is independent of the adjacent amino acid sequence. Using the AlphaScreen detection platform, P-Tyr-100 has shown superior phosphotyrosine detection characteristics and results in an assay with signal-to-background (S/B) ratios of over 250 and signals over 150,000 cps. Due to the robustness of this homogeneous format assay, we also endeavored to reduce screening costs for HTS by stretching the Alphascreen P-Tyr-100 reagents.

Principles of AlphaScreen Technology

AlphaScreen is a bead based non-radioactive Amplified Luminescent Proximity Homogeneous Assay. When a biological interaction brings the beads together, a cascade of chemical reactions act to produce a greatly amplified signal. On laser excitation, a photosensitizer in the “Donor” bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead, generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm.

In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result no signal is produced.

AlphaScreen provides a highly versatile, sensitive, homogeneous and miniaturizable means to efficiently perform assay development and HTS resulting in higher throughput at lower costs.

To maximize AlphaScreen signal detection, the AlphaQuest® HTS Microplate Analyzer and Fusion-Alpha™ Multilabel Reader were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes.

For further details on AlphaScreen technology, refer to *A Practical Guide to Working with AlphaScreen* (reference no. S4077).

AlphaScreen P-Tyr-100 Assay for Insulin

The AlphaScreen P-Tyr-100 assay kit was used to test the activity of the soluble β -insulin receptor kinase domain (IRKD), derived from the cytoplasmic portion of the human insulin receptor. IRKD is constitutively active and retains its substrate specificities, kinetic constants and autophosphorylation sites without the requirement for hormone-mediated activation. The assay uses the broad spectrum substrate poly[Glu:Tyr] (4:1), and is based on the binding between the phosphorylated polypeptide and the anti-phosphotyrosine antibody P-Tyr-100 conjugated to Acceptor beads (Figure 1). This assay is also applicable to other tyrosine kinases using either poly[Glu:Tyr] or poly[Glu:Ala:Tyr] as substrates.

Materials and Methods

The AlphaScreen P-Tyr-100 Assay Kit is composed of Donor-streptavidin and Acceptor-P-Tyr-100 beads.

IRKD enzyme was purchased from Calbiochem, c-Src was from Upstate Biotechnology Inc. and EGF Receptor from Sigma. Biotinylated poly[Glu:Tyr] (4:1) and poly[Glu:Ala:Tyr] (1:1:1) are available through PerkinElmer's custom services (for more information please contact assayservices@perkinelmer.com).

The AlphaScreen P-Tyr-100 assay involves the following three steps:

1. Mix IRKD enzyme, biotinylated poly[Glu:Tyr] and ATP in a well of a 384-well plate (ex.: PerkinElmer OptiPlate™ 384 well plates cat. No. 6007290 and 6007299); incubate for 30 minutes at room temperature (RT).
2. Quench by adding the detection buffer containing EDTA, Donor-Streptavidin and Acceptor-P-Tyr-100 beads; incubate for 1 hour at RT.
3. Detect AlphaScreen signal using either an AlphaQuest HTS Microplate Analyzer or a Fusion-Alpha Multilabel Reader.

All assays were performed in white, opaque 384-well plates (ex.: PerkinElmer OptiPlate 384 well plates cat. no. 6007290 and 6007299) in a final volume of 25 μ L. In all experiments except for enzyme titration, IRKD was used at 50 pg/well, poly[Glu:Tyr] and ATP at 0.5 nM and 100 μ M respectively. Donor-Streptavidin and Acceptor-P-Tyr-100 beads were used at 20 μ M each.

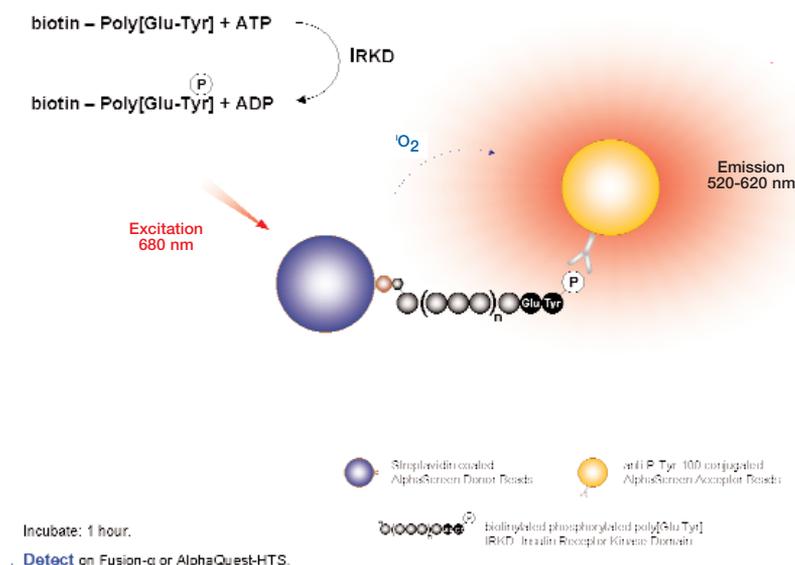


Figure 1. Phosphorylated polypeptide bound by streptavidin-coated Donor beads and by specific anti-phospho-MBP antibodies bound to Protein A-conjugated Acceptor beads.

Materials and Methods (continued)

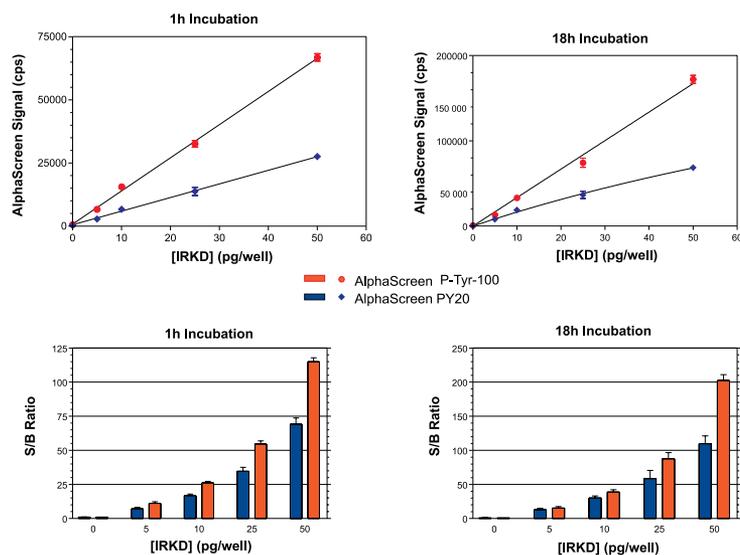


Figure 2. Enzyme Titration at 1 and 18 hour incubation.

Results

Higher Performance of AlphaScreen P-Tyr-100

Using a generic substrate, poly[Glu:Tyr], AlphaScreen P-Tyr-100 significantly outperformed AlphaScreen PY20. At 50 pg enzyme per well, total counts were 2.4-fold higher with AlphaScreen P-Tyr-100, with a corresponding S/B ratio increase of >1.5 fold (Figure 2), showing that P-Tyr-100 has a higher affinity and sensitivity for the substrate than PY20. A signal of approximately 2800 cps and an S/B ratio of 11, could be obtained in one hour using as low as 5 pg of enzyme per well and 0.5 nM substrate. Extending the incubation time to 18 hours further increases these values.

High Sensitivity of AlphaScreen P-Tyr-100 for Phosphotyrosyl Peptides

High sensitivity of detection was determined for two sequence-specific substrates. Peptide substrates for Lck and Src were tested in the presence of an excess of non-phosphorylated peptides. A total of 1 μ M phosphorylated and non-phosphorylated biotinylated peptides was used to simulate substrate conversion by the kinases. At all ratios of phosphorylated/total peptides tested, the specificity of detection was significantly higher with Alpha-Screen P-Tyr-100 than with PY20, as shown by the high S/B results (Figure 3). At very low concentrations of phosphorylated peptides (<3%), only AlphaScreen P-Tyr-100 reached a significant level of detection.

AlphaScreen P-Tyr-100 Assay is Applicable to a Wide Range of Tyrosine Kinases

Three tyrosine kinases were tested against two synthetic substrates. Insulin receptor, c-Src and Epidermal Growth Factor Receptor (EGFR) were assayed using either poly[Glu:Tyr] (4:1) or poly[Glu:Ala:Tyr] (1:1:1). Very high signal and S/B ratios were generated with the three kinases but with clear substrate preferences (Figure 4). Both c-Src and EGFR phosphorylated the two substrates but with some selectivity. The Insulin receptor, however, was 100-fold selective for poly[Glu:Tyr].

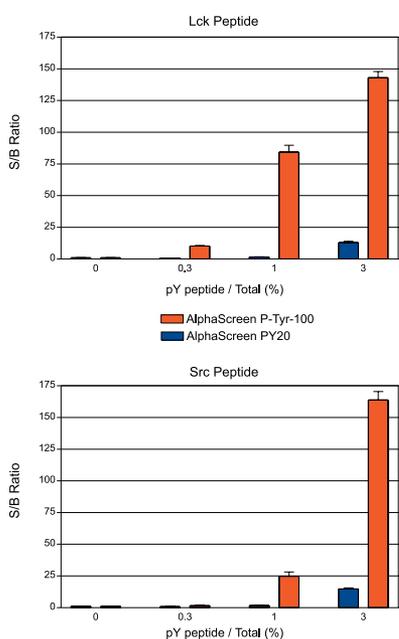


Figure 3. Sensitivity of P-Tyr-100 Assay is Applicable to a Wide Range of Tyrosine Kinases.

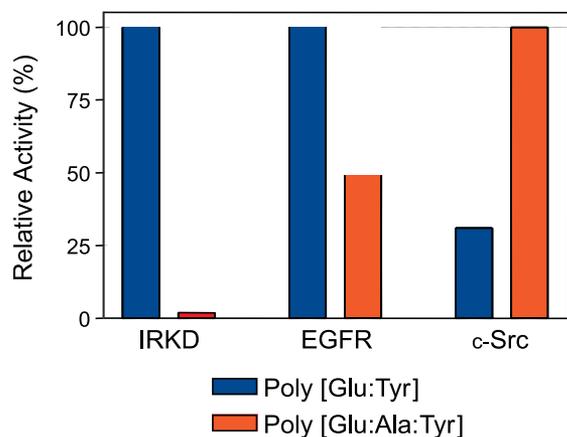


Figure 4. Substrate Preferences for Three Tyrosine Kinases.

Minimizing Reagent Use in the AlphaScreen P-Tyr-100 Assay.

Due to the very high signal and S/B ratio observed with the AlphaScreen P-Tyr-100 assay kit (typically >150,000 cps and >250 S/B after 18-hours incubation), we tested the lower detection limit of IRKD activity by decreasing the concentration of detection reagents (Donor and Acceptor beads) in order to reduce screening costs. As shown in Figure 5, reagents could be stretched to 1/20 of their standard recommended concentration (20 µg/mL down to 1 µg/mL), while maintaining a good signal (1612 cps) and S/B ratio of 13 after 18 hours of incubation. These results show that AlphaScreen P-Tyr-100 assay reagents can be stretched down to 1.0 µg/mL.

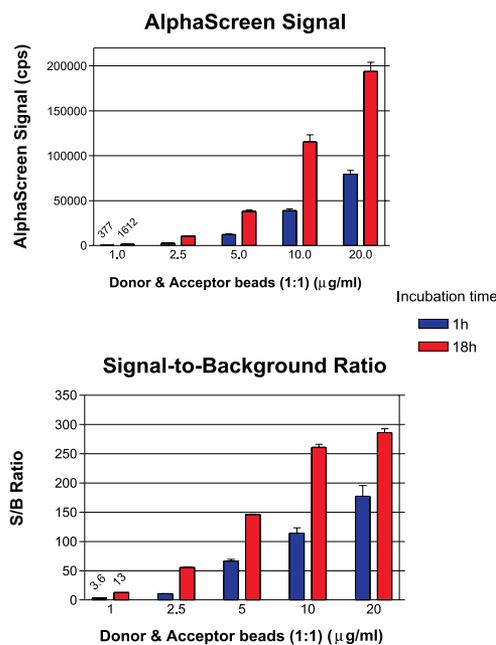


Figure 5. Stretching of P-Tyr-100 Reagents.

Conclusion

Using the AlphaScreen P-Tyr-100 assay kit, very high signal and S/B ratios can be obtained, even at very low enzyme and substrate concentrations. The Alpha-Screen P-Tyr-100 Tyrosine Kinase assay was found to be extremely specific and sensitive to phosphotyrosine detection. The assay can be used with sequence-specific as well as generic poly[Glu:Tyr] and poly[Glu:Ala:Tyr] substrates. Due to the superior sensitivity and broader range of substrate sequence detection by the antibody, the AlphaScreen P-Tyr-100 kit is the assay of choice for all tyrosine kinase research and drug discovery.

After testing the effect of stretching the reagents for use in high throughput screening, we conclude that the reagent cost for AlphaScreen can effectively be reduced for these types of assays.



PerkinElmer Life and Analytical Sciences, 710 Bridgeport Avenue, Shelton, CT 06484 USA (800) 762-4000 or (+1) 203-925-4602

PerkinElmer Life and Analytical Sciences, Imperiastraat 8, BE-1930 Zaventem Belgium

Technical Support: in Europe: techsupport.europe@perkinelmer.com in US and Rest of World: techsupport@perkinelmer.com

Belgium: Tel: 0800 94 540 • France: Tel: 0800 90 77 62 • Netherlands: Tel: 0800 02 23 042 • Germany: Tel: 0800 1 81 00 32 • United Kingdom: Tel: 0800 89 60 46

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