Antibody Beacon™ Tyrosine Kinase Assay Kit (A-35725)

Quick Facts

Storage upon receipt:

- ≤6°C
- If frozen, avoid freeze-thaw cycles
- Protect from light

Ex/Em: 492/517 nm

Number of Assays: 400

Assay Volume: 50 µL

Introduction

Tyrosine kinases play an important role in a variety of cellular pathways and processes, as well as pathologies such as cancer.[1] The Antibody Beacon™ Tyrosine Kinase Assay Kit (A-35725) provides a simple and robust assay for measuring the activity of tyrosine kinases and their inhibitors and modulators. The assay is based on a detection complex comprised of small-molecule tracer ligand that exhibits quenched fluorescence when bound to anti-phosphotyrosine antibodies. In the presence of phosphotyrosine-containing peptides, the ligand is rapidly displaced and there is an increase in fluorescence (Figure 1).

Each component of the detection complex has been optimized for minimal background fluorescence, maximal displacement of the ligand in the presence of phosphotyrosine-containing peptides and a large increase in fluorescence upon displacement. The approximately 4-fold fluorescence enhancement of the ligand upon displacement by the phosphopeptide provides excellent signal-to-background discrimination (Figure 2). The excitation and emission spectra of the Oregon Green 488 dye closely match those of fluorescein thus making this assay readily compatible with any fluorescence multiwell plate reader. The antibody component provides detection specificity for phosphotyrosine peptides and minimal interference from coexisting assay components such as ATP (up to 1 mM) or reducing agents, such as dithiothreitol (up to 2 mM).

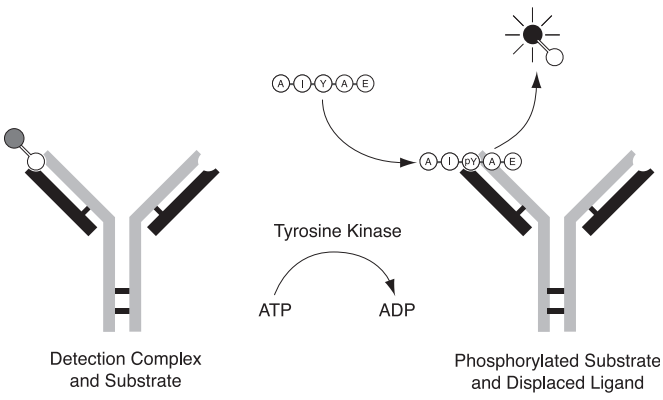

Figure 1. Antibody Beacon tyrosine kinase assay reaction scheme.

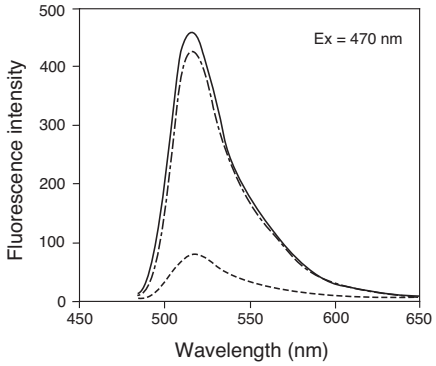

Figure 2. Fluorescence response of the Antibody Beacon detection complex in the presence of a phosphotyrosine-containing peptide. The fluorescence of Oregon Green 488 ligand (—— solid line), Antibody Beacon detection complex (– – – – dashed line) or the Antibody Beacon detection complex plus phosphorylated Abl substrate peptide (EAIpYAAPFAKKK; – – – – – dashed line) was measured in kinase assay buffer. In the presence of the phosphopeptide, the Oregon Green 488 ligand was displaced from the Antibody Beacon complex and exhibited a 4-fold enhancement over the fluorescence of the Antibody Beacon complex in buffer alone.

The Antibody Beacon tyrosine kinase assay, unlike most other commercially available tyrosine kinase activity assays, allows for the real-time monitoring of kinase activity. Real-time assays are possible due to the rapid dissociation of the detection complex and the capacity for the assay components to be simultaneously combined. The assay is also easily adapted for measuring the effec tiveness of tyrosine kinase inhibitors (see Figure 3). Additional features of the assay include its use of unlabeled peptide substrates, its low limit of detection (≤50 nM of phosphotyrosine-containing peptide) and its broad signal window, indicated by a Z′ factor[2] of 0.9.

Materials

Contents

- **Oregon Green 488 ligand, Antibody Beacon reagent for phosphotyrosine detection** (Component A), 200 µL of a 2.5 µM solution in 1X reaction buffer

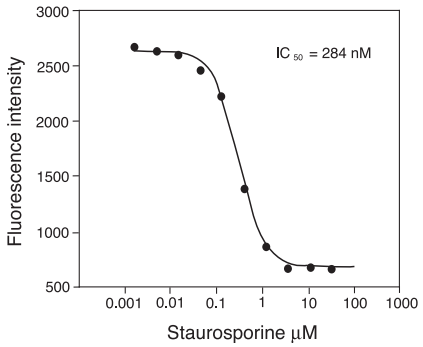

Figure 3. Inhibition of src kinase by staurosporine. Varying concentrations of staurosporine were incubated with src kinase (25 U/mL in reaction buffer) for 20 minutes at 37°C. The Antibody Beacon tyrosine kinase detection complex, kinase substrate (poly(Glu:Tyr), 4:1) and ATP were then added to each well, and the reactions were incubated at 37°C. After 1 hour, fluorescence was measured in a fluorescence microplate reader using excitation at 485 nm and emission at 535 nm.

- **Anti-phosphotyrosine antibody, P-Tyr-100** (Component B), 200 µL of a 5 µM (0.75 mg/mL) solution in pH 7.4 buffer containing 5 mM sodium azide
- **2X Tyrosine kinase reaction buffer** (Component C), 25 mL of 100 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM EGTA, 0.02% Brij 35, pH 7.5 (at 22°C)
- **Tyrosine kinase substrate # 1** (Component D), 400 µL of a 10 mg/mL poly(Glu:Tyr) solution, 4:1 ratio, in dH_2O
- **Tyrosine kinase substrate # 2** (Component E), 400 µL of a 10 mg/mL poly(Glu:Ala:Tyr) solution, 6:3:1 ratio, in dH_2O
- **Dithiothreitol (DTT)** (Component F), 31 mg
- **Adenosine triphosphate (ATP)** (Component G), 200 µL of a 100 mM solution in buffer
- **Reference phosphotyrosine-containing peptide** (Component H), 50 µL of a 500 µM solution of phospho-pp60 c-src (521–533) in dH_2O; sequence = TSTEPQpYQPGENL; MW = 1543.7

The kit includes sufficient material to perform ~400 assays using a 50 µL volume.

Storage and Handling

Upon receipt, components should be stored at 2–6°C until required for use. For long-term storage, the kit can be stored at ≤–20°C. If frozen, Component B should be divided into single-use aliquots to avoid multiple freeze-thaw cycles. When stored properly, the kit components should be stable for at least 6 months.

Experimental Protocols

Overview

Three sample protocols are provided below in the sections *Tyrosine Kinase Activity Assay, Tyrosine Kinase–Inhibition Assay* and *Phosphopeptide-Detection Assay*. Each protocol describes the preparation for and the implementation of 100 separate assays, each with a reaction volume of 50 µL. The Antibody Beacon Tyrosine Kinase Assay Kit provides sufficient material for 400 such assays. Typically, the assays are performed in a 384-well microplate, in a "half-well" 96-well microplate or in a standard 96-well microplate. The sample protocols can be modified for a different number of assays or for a different assay volume.

Positive and Negative Controls

It is important to incorporate positive and negative controls into the experimental design. Various controls for consideration are listed below.

Positive controls:

- **Oregon Green 488 ligand in the absence of antibody.** The Oregon Green 488 ligand (Component A) at 25 nM represents the maximum possible fluorescence signal
- **Tyrosine kinase control.** A high-activity level of a known-to-be-active tyrosine kinase, along with substrate and ATP
- **Phosphopeptide control.** The product of the tyrosine kinase reaction, at a high concentration (e.g., 50 µM)
- **No-inhibitor control.** For the tyrosine kinase–inhibition assay format

Negative controls:

- **No-kinase control.** The detection complex alone (i.e., the Oregon Green 488 ligand bound and quenched by the antibody) represents the minimum possible fluorescence signal
- **No-ATP control.**
- **No-substrate control.**

Substrates

Two generalized tyrosine kinase substrates are provided with the Antibody Beacon Tyrosine Kinase Assay Kit. Tyrosine kinase substrate #1 (Component D), which is poly(Glu:Tyr) in a 4:1 ratio, is suitable for use with many tyrosine kinases; however, it is not recommended for use with the tyrosine kinase Abl. Tyrosine kinase substrate #2 (Component E), which is poly(Glu:Ala:Tyr) in a 6:3:1 ratio, is suitable for use with Abl. For optimal assay performance, users are encouraged to provide peptide substrates with selectivities known to match the kinases under investigation. In such cases, the best concentration to use will have to be determined empirically.

Stock and Working Solutions

1.1 Prepare a 1 M stock solution of dithiothreitol (DTT). Add 200 µL of dH_2O to the vial containing DTT (Component F), and mix thoroughly to dissolve the contents. When not in use, store at ≤–20°C.

1.2 Prepare a working solution of 1X kinase buffer plus DTT (1X KB + DTT). Prepare 1X KB + DTT freshly, on the day of the experiment. To make 10.0 mL of 1X KB + DTT, a volume easily sufficient for one hundred 50 µL–reactions, mix the following:

- 5.0 mL 2X KB (Component C)
- 5.0 mL deionized H_2O (dH_2O)
- 20 µL 1 M DTT (prepared in step 1.1)

Tyrosine Kinase Activity Assay

2.1 Prepare the tyrosine kinase sample. Prepare the kinase (user-supplied) in 1X KB + DTT (prepared in step 1.2). For concentrated kinase preparations, simply dilute the kinase into 1X KB + DTT (note **A**). For unknown activity levels, several concentrations should be prepared for each sample. Note that the kinase

sample will be further diluted 2-fold in the assay. For each assay, a 25 µL volume will be used, and assays in triplicate are recommended.

2.2 Prepare the Antibody Beacon detection complex plus substrate. Mix the following:
- 1.80 mL 1X kinase buffer + DTT
- 50 µL Oregon Green 488 ligand (Component A)
- 50 µL anti-phosphotyrosine antibody, P-Tyr-100 (Component B)
- 100 µL tyrosine kinase substrate #1 or #2 (Component D or E)

Note that the detection complex plus substrate (62.5 nM ligand, 125 nM antibody and 500 µg/mL substrate), as prepared, will be further diluted 2.5-fold (to 25 nM, 50 nM and 200 µg/mL, respectively) in the assay. For each assay, a 20 µL volume will be used, and assays in triplicate are recommended.

2.3 Prepare 5 mM ATP. Mix the following:
- 570 µL 1X kinase buffer + DTT
- 30 µL 100 mM ATP (Component G)

For each assay, a 5 µL volume will be used, and the final concentration will be 0.5 mM in the reaction (note **B**).

2.4 Prepare positive and negative control samples. Minimally, include a no-kinase control; i.e., use 1X KB + DTT in place of the kinase sample in step 2.5, below. In addition, to see the maximal fluorescence signal, set up a well with 25 nM of the Oregon Green 488 ligand alone, in the absence of antibody (and lacking also kinase, substrate and ATP). For example, mix 0.5 µL of Oregon Green 488 ligand (Component A) and 49.5 µL of 1X KB + DTT in an empty microplate well; or, better, mix these in the same ratio in a larger volume and then use 50 µL per well.

2.5 Set up and start the reactions. Add the components (prepared above), in the following order, to the wells of the 384-well microplate (note **C**), and mix thoroughly after each addition:
- 25 µL kinase sample
- 20 µL Antibody Beacon detection complex plus substrate
- 5 µL 5 mM ATP

After mixing the first two components, pre-incubate the microplate at the desired reaction temperature. Start the reactions by adding the ATP (note **D**), and continue incubating at the reaction temperature.

2.6 Read the fluorescence. The Oregon Green 488 ligand has excitation/emission maxima at ~492/517 nm. Read the fluorescence in a fluorescence microplate reader using the same settings as for fluorescein (e.g., 485/530 nm). The Antibody Beacon tyrosine kinase assay is continuous (not terminated); therefore, fluorescence can be measured at multiple time points to follow the kinetics of the reaction.

Tyrosine Kinase–Inhibition Assay

3.1 Prepare the tyrosine kinase. Prepare the kinase sample at a predetermined, appropriate concentration in 1X KB + DTT (prepared in step 1.2; see also note **A**). Note that the kinase sample will be further diluted 4-fold in this assay format. For each assay, a 12.5 µL volume will be used, and assays in triplicate are recommended.

3.2 Prepare the inhibitor samples. Prepare an appropriate dilution series of the inhibitor in 1X KB + DTT. Include buffer alone as a no-inhibitor control. Note that these inhibitor samples will be further diluted 4-fold in the assay. For each concentration, a 12.5 µL volume will be used in each assay.

3.3 Prepare the Antibody Beacon detection complex plus substrate. Mix the following:
- 1.80 mL 1X kinase buffer + DTT
- 50 µL Oregon Green 488 ligand (Component A)
- 50 µL anti-phosphotyrosine antibody, P-Tyr-100 (Component B)
- 100 µL tyrosine kinase substrate #1 or #2 (Component D or E)

Note that the detection complex plus substrate (62.5 nM ligand, 125 nM antibody and 500 µg/mL substrate), as prepared, will be further diluted 2.5-fold (to 25 nM, 50 nM and 200 µg/mL, respectively) in the assay. For each assay, a 20 µL volume will be used, and assays in triplicate are recommended.

3.4 Prepare 5 mM ATP. Mix the following:
- 570 µL 1X kinase buffer + DTT
- 30 µL 100 mM ATP (Component G)

For each assay, a 5 µL volume will be used, and the final concentration will be 0.5 mM in the reaction (note **B**).

3.5 Prepare control samples. Include a no-kinase control; i.e., use 1X KB + DTT in place of the kinase sample in step 3.6, below. In addition, to see the maximal fluorescence signal, set up a well with 25 nM of the Oregon Green 488 ligand alone, in the absence of antibody (and lacking also kinase, substrate and ATP). For example, mix 0.5 µL of Oregon Green 488 ligand (Component A) and 49.5 µL of 1X KB + DTT in an empty microplate well; or, better, mix these in the same ratio in a larger volume and then use 50 µL per well.

3.6 Set up and start the reactions. Add the components (prepared above), in the following order, to the wells of the 384-well microplate (note **C**), and mix thoroughly after each addition:
- 12.5 µL kinase sample
- 12.5 µL inhibitor sample
- 20 µL Antibody Beacon detection complex plus substrate
- 5 µL 5 mM ATP

After adding the first two components, pre-incubate the microplate at the desired reaction temperature and, again, after adding the third component. Start the reactions by adding the ATP (note **D**), and continue incubating at the reaction temperature.

3.7 Read the fluorescence. The Oregon Green 488 ligand has excitation/emission maxima at ~492/517 nm. Read the fluorescence in a fluorescence microplate reader using the same settings as for fluorescein (e.g., 485/530 nm). The Antibody Beacon tyrosine kinase assay is continuous (not terminated); therefore, fluorescence can be measured at multiple time points to follow the kinetics of the reaction.

Phosphopeptide-Detection Assay

The Antibody Beacon tyrosine kinase assay can be used to detect phosphotyrosine-containing peptides in solution. The amount of the phosphopeptide present in a given sample is determined by use of a standard curve, derived from samples of the same phosphopeptide at known concentrations. In the sample protocol below, the provided reference phosphotyrosine-containing peptide, phospho-pp60 c-src (Component H), is serially diluted for preparing a standard curve.

4.1 Prepare a phosphopeptide working solution. Dilute the provided 500 µM solution of the reference phosphotyrosine-containing peptide, phospho-pp60 c-src (Component H), to 100 µM by mixing 12 µL plus 48 µL of 1X KB + DTT (prepared in step 1.2) to have a sufficient working solution for a standard curve based upon single determinations, or by mixing 36 µL plus 144 µL of 1X KB + DTT for a standard curve based upon triplicate determinations.

4.2 Set up a 2-fold dilution series. Pipet 50 µL of the 100 µM phosphopeptide solution (prepared in step 4.1) to the first well of the microplate row or column. Pipet 25 µL aliquots of 1X KB + DTT into adjacent wells. Sequentially remove 25 µL from the first well, mix with the buffer in the second well; remove 25 µL and mix with the buffer in the third well; etc. Remove and discard 25 µL from the final well in the series. In the end, there should be a series of wells, each with a 25 µL volume and with concentrations beginning with 100 µM and diminishing by factors of two. Note that the final concentrations will be further reduced by 2-fold in the assay.

4.3 Prepare a no-phosphopeptide control sample. Add 25 µL of 1X KB + DTT to one or more empty wells of the microplate.

4.4 Prepare the Antibody Beacon detection complex. Mix the following:

- 2.40 mL 1X KB + DTT
- 50 µL Oregon Green 488 ligand (Component A)
- 50 µL anti-phosphotyrosine antibody, P-Tyr-100 (Component B)

Adjust the volumes proportionally to have sufficient solution to have 25 µL per reaction (note **E**). For each assay, a 25 µL volume will be used, and assays in triplicate are recommended.

4.5 Add the Antibody Beacon detection complex. Add 25 µL of the detection complex (prepared in step 4.4) to each sample, including the no-phosphopeptide control sample. Mix well.

4.6 Prepare a no-antibody control sample. To see the maximal fluorescence signal, set up a well with 25 nM of the Oregon Green 488 ligand alone, in the absence of antibody. For example, mix 0.5 µL of Oregon Green 488 ligand (Component A) and 49.5 µL of 1X KB + DTT in an empty microplate well; or, better, mix these in the same ratio in a larger volume and then use 50 µL per well.

4.7 Read the fluorescence. After a brief incubation period (e.g., 10 minutes) at the desired temperature, read the fluorescence in a fluorescence microplate reader using the same settings as for fluorescein.

4.8 Prepare the standard curve. Plot the fluorescence readings versus the concentration of the phosphotyrosine-containing peptide in the samples.

Notes

[A] The tyrosine kinase reaction buffer is provided at a 2X concentration. In each of the sample protocols, the buffer is diluted to 1X prior to use. However, there are circumstances where having the 2X concentration is beneficial, for example, for mixing 1:1 with very dilute kinase samples or for preparing specialized kinase buffers that have additional required ingredients. In some cases, users may wish to replace the provided kinase buffer with a buffer better suited for the particular kinase under study. The final phosphate concentration should not exceed 5 mM.

[B] The recommended 5 mM ATP working solution results in a final ATP concentration of 0.5 mM in the assay. This ATP concentration may be too high for some applications and can be reduced as appropriate.

[C] For the Antibody Beacon tyrosine kinase assays or tyrosine kinase–inhibition assays, pre-blocking the microplate may be essential for consistent results. One method for pre-blocking a 384-well microplate is the following: prepare a 0.25% solution of Mowiol® 4-88 (Calbiochem cat# 475904) in dH_2O; pipet 80 µL of the 0.25% Mowiol solution into each well of the microplate; incubate 1 hour at room temperature; then empty the microplate and blot away the excess solution with paper towels or tissues. Rinse the microplate three times with dH_2O, emptying the wells as completely as possible and blotting the plate after each rinse. The plate is ready to use once it is completely dry.

[D] The ATP solution can be pre-mixed in a 1:4 ratio with the Antibody Beacon detection complex-plus-substrate mixture. However, pre-mixing the ATP in this way makes it more difficult to set up a no-ATP control reaction.

[E] In the phosphopeptide-detection protocol, as described, there is no substrate and no ATP present. In some circumstances, it may be desirable to include one or both of these components for a better-controlled determination.

References

1. Drug Discovery Today 5, 344 (2000); **2.** J Biomol Screen 4, 67 (1999).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
A-35725	Antibody Beacon™ Tyrosine Kinase Assay Kit *400 assays*	1 kit

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