



Phosphotyrosine HTRF[®] Assay

Catalog No. 17-10000

384-well

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Italy: 848.845.645
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Introduction

The phosphotyrosine HTRF[®] Assay (Cat. # 17-10000) is a generic homogenous method for measuring tyrosine kinase activities using the industry standard monoclonal phosphotyrosine antibody, 4G10[®], tyrosine peptide substrate and a universal detection system. An increase in the HTRF[®] signal is observed as a result of the kinase activity. Inhibitor compounds can be screened during the kinase reaction for an IC 50 value determination.

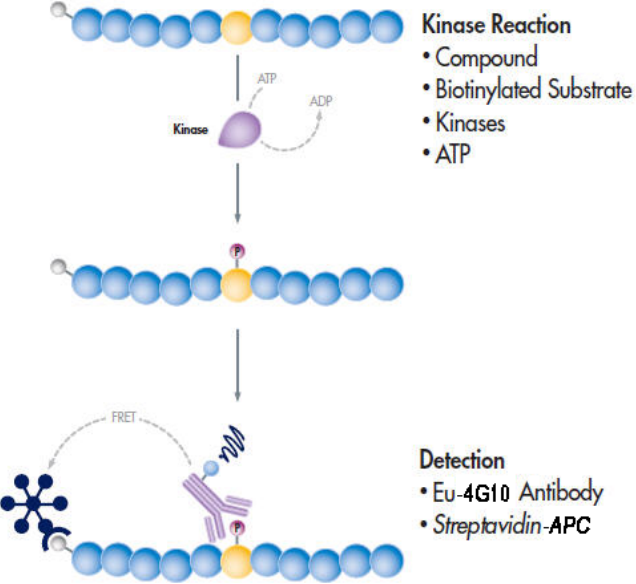
The assay has a wide dynamic range and high precision, making this assay a valuable new tool for the study of tyrosine phosphorylation.

Background

Cellular Signal Transduction through Tyrosine Phosphorylation Protein phosphorylation is often necessary for the initiation of biological processes such as cell growth, proliferation, and ubiquitin-mediated degradation. Tyrosine phosphorylation, in particular, often mediates receptor signaling events at the membrane by initiating protein-protein interactions in response to growth factors, hormones and cytokines. Research tools that detect tyrosine phosphorylation are central in understanding the significance of cellular signaling in many biological processes and disease origination. Millipore developed the Gold-Standard antibody (4G10) for Tyrosine Phosphorylation.

Tyrosine phosphorylation is one of the most common methods of research. There are many different assay formats available including fluorescence, ELISA, and IP. A number of homogeneous technologies (e.g. luminescence) are restricted by a reduction in signal intensity following assay measurement or prolonged incubations. Our patented HTRF[®] technology has many benefits including long signal stability. HTRF[®] fluorescence is not dampened by assay measurement, assay additives such as DMSO or extended incubations prior to reading. The advantages of the tremendous stability of HTRF[®] fluorescence include: time flexibility and assay security. In case of instrument failure, it is possible to read the microplates even after an extended period of time.

Assay Principle Flow Chart



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Kit Components

Store at 2-8° C

1. Black 384 well plate: (Part No. R5215) One 384-well black plate.
2. Detection buffer: (Part No. CS206478) Two bottles containing 5 mL each of detection buffer.
3. Plate Covers: Two plate covers.

Store at -20° C

4. 10X KinEASE buffer: (Part No. CS206508). Two vials containing 1 mL each of kinase reaction buffer.
5. Biotinylated TK1 Peptide: (Part No. CS206504). Two vials containing 60 µL each of Biotinylated TK1 Peptide Stock at 50µM.
6. Biotinylated TK2 Peptide: (Part No. CS206505). Two vials containing 60 µL each of Biotinylated TK2 Peptide Stock at 50µM.
7. Biotinylated TK3 Peptide: (Part No. CS206506). Two vials containing 60 µL each of Biotinylated TK3 Peptide Stock at 50µM.
8. Biotinylated Phosphotyrosine Peptide Standard: (Part No. 90192). One vial containing 10 µg lyophilized biotinylated phosphotyrosine peptide standard.
9. Streptavidin-APC: (Part No. CS206501). Two vials containing 360 µL each of streptavidin-APC stock at 1 µM.
10. Eu-cryptate-conjugated 4G10[®] anti-phosphotyrosine: (Part No. CS206507). Two vials containing 30 µL each Eu-cryptate conjugated 4G10 anti-phosphotyrosine antibody stock at 35 µg/mL.
11. ATP: (Part No. CS206509). Two vials containing 50 µL each of ATP stock at 10mM.
12. DTT: (Part No, 90499). Two vials containing 100 µL each of DTT stock at 1M.
13. MnCl₂: (Part No. CS206510). Two vials containing 100 µL each of MnCl₂ stock at 0.1M.
14. MgCl₂: (Part No. CS206511). Two vials containing 100 µL each of MgCl₂ stock at 1M.
15. Sodium Orthovanadate: (Part No. CS206465). Two vials containing 50 µL each of sodium orthovanadate stock at 50 mM.
16. Staurosporine: (Part No. CS201778). One vial containing 100 µL of staurosporine stock at 2.5 mM.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. Pipettors and tips capable of accurately measuring 1-1000 µL

4. Graduated serological pipettes
5. 384-well microtiter plate reader with luminescence readout.
6. Graphing software for plotting data or graph paper for manual plotting of data
7. Microcentrifuge tubes for standard and sample dilutions
8. Mechanical vortex
9. 1 liter container
10. Distilled or deionized water
11. DMSO to dissolve inhibitor compounds.

Storage

Maintain the unopened kit at -20°C until expiration date. Once opened, please follow component storage instructions. Avoid repeated freeze/thaw cycles, aliquot if necessary.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Technical Notes

- All kit reagents should be brought to room temperature (20°C to 25°C) just prior to use.
- Do not use reagents beyond the expiration date of the kit.
- Do not mix or interchange reagent from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minutes soak may reduce background values.

Reagents Preparation

1. **Prepare 1X working kinase buffer.** Provided kinase buffer is 10X stock (Part No. CS206508). Prepare the required amount per assay setup just before use. Working solutions cannot be stored and must be used immediately, discard any unused.

For example, follow table 1 below to prepare 1 mL of the 1X working kinase buffer.

Table 1.

Component	Part No.	Amount (μL)	Final concentration
10X KinEASE Buffer	CS206508	100	1X
1M MgCl_2	CS206511	5	5 mM
0.1M MnCl_2	CS206510	10	1 mM
1M DTT	90499	1	1 mM
50mM Sodium Orthovanadate	CS206465	2	0.1 mM
Distilled Water	N/A	882	N/A

***Note:** The appendix table A listed the recommended kinase reaction conditions. Please reference the recommended 1X reaction buffer column for the optimized buffer. MnCl_2 may not be needed for some kinases. Please adjust the preparation of 1X buffer accordingly.

- Prepare working inhibitor compounds solution.** Depends on the compound property, the end user will need to determine the suitable solvent to dissolve it to a concentrated stock. Typically, DMSO is a commonly used solvent. Then make further dilutions with 1X kinase buffer to give the final DMSO no more than 2% in the reaction to minimize any potential effects.
- Prepare working biotinylated tyrosine kinase peptide solution as substrate.** Total of 3 biotinylated tyrosine kinase (TK) peptides are included with the kit. All at 50 μM stock. Reference the appendix table attached in the end of this manual guide for a suitable TK substrate to the choice of kinase. Follow the optimization steps below to determine a proper final concentration for each kinase target. Then prepare a 5X concentrated working solution with 1X kinase buffer according to the determined proper final usage if inhibitor compounds screening will be applied to your target kinases.
- Prepare working solution of biotinylated phosphotyrosine standard.** Add 109.6 μL Distilled Water to the component 90192 to dissolve completely. This is a 50 μM solution.

Optimization of the Kinase Assay

Fix Biotin-substrate/Streptavidin-APC molar ratio to 8/1

- Kinase titration**

This allows the optimal kinase concentration for which the signal reaches around 80~90% of the maximum to be determined. A fixed concentration of the biotin-TK substrate (1 μM) and ATP (100 μM) should be tested with a range of kinase

concentrations (0.5-20ng/reaction in a two fold serial dilutions). Setup the enzymatic reaction to run for 60 minutes at 30-37°C.

- **Kinetic study**

Enzyme kinetic depends on the kinase and substrate concentrations. A time course is performed using the optimal kinase concentration determined at the kinase titration step, ATP (100 μM) and biotin-TK substrate (1 μM). The reaction is stopped at various end points by the addition of the detection reagents (1, 2, 5, 10, 15, 30, 60, 90 minutes). Determine the optimal reaction time when the signal reaches around 80-90% of the maximum. This optimal reaction time is used for the rest experiments.

- **Substrate titration**

This allows the determination of substrate K_m . Use the optimal kinase concentration determined from kinase titration step, fix concentration of ATP (100 μM) and test a range of the biotin-TK substrate (0.03-1 μM in a two fold serial dilutions). Adjust the streptavidin-APC concentration to have a fixed biotin/streptavidin molar ratio at 8/1. Reference to table 2 for making the adjustment. Let the kinase reaction run for 60 minutes at 30-37°C.

- **ATP titration**

This allows the determination of ATP K_m . Use the optimal kinase concentration, a fixed concentration of the biotin-TK substrate (1 μM) to test a range of ATP concentrations (3.125-200 μM in a two fold serial dilutions). The kinase reaction is stopped at 60 minutes at 30-37°C.

- **Inhibitor compounds IC 50 determination**

The kinase activity is tested over a broad range of inhibitor concentrations to generate a dose response curve. The test is generally run using the previously determined optimal assay conditions. A proper ATP concentration (reference the K_m value determined at the ATP titration step) should be considered for ATP-competitive compounds.

Suggested final concentration ranges of the assay components:

Component	Concentration range
Biotin-TK substrate	0.03-1 μM
Kinase	0.5-20 ng/reaction
ATP	3.125-200 μM
Streptavidin-APC	0.04-125 nM
Eu-4G10	Ready to use

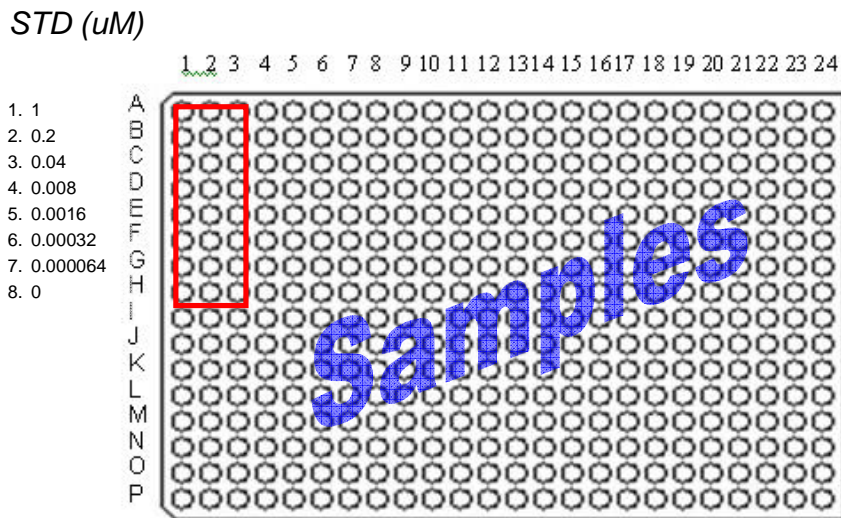
Assay Protocol

I. Setup a standard curve with provided biotinylated phosphotyrosine peptide.

Biotinylated Phosphotyrosine Peptide Standard serial dilutions should be setup along with various concentration of streptavidin-APC to keep Biotin/Streptavidin ratio fix at 8/1.

Use 3 columns to setup the standard in triplicates for each point. Cover the unused remaining wells with plate sealer.

Wells Layout:



1. Label 7 test tubes #1-7 and "0 dose". Add 48 μL of 1X kinase buffer to tubes #2-7 and "0 dose".
The biotinylated phosphotyrosine peptide standard solution prepared above is further used to make the #1 standard tube. It will be a 1 μM final in the assay.
 - 2.4 μL of 50 μM stock
 - 15 μL of streptavidin-APC (part No. CS206501)
 - 42.6 μL of Detection Buffer (part No. CS206478)
 - Total 60 μL .
2. Standards #2-7 are then prepared by performing a 5-fold serial dilution of the preceding standard. For example, to make Standard #2, remove 12 μL of Standard #1 and add it to tube #2 and vortex, and so on. Do not add any Standard to the "0 dose" tube.
3. Table 2 below showed the final biotinylated-phosphotyrosine (as biotin-p-Tyr) and Streptavidin-APC (as Strep-APC) concentrations in the standard solutions.

Table 2.

Biotin-p-Tyr Standard (μM)		Streptavidin- APC (nM)	
Conc. In standard solution (12.5 μL)	Final assay conc. (25 μL)	Conc. in standard solution (12.5 μL)	Final assay conc. (25 μL)
2	1	250	125
0.4	0.2	50	25
0.08	0.04	10	5
0.016	0.008	2	1
0.0032	0.0016	0.4	0.2
0.00064	0.00032	0.08	0.04
0.000128	0.000064	0.016	0.008
0	0	0	0

4. Dispense 12.5 μL /well of the standard solutions made above (reference table 3 below for the biotin-p-Tyr column).

II. Setup kinase activity samples, compounds can be added to the kinase reactions in a dose-response curve to determine IC 50 value.

5. Setup kinase reactions by applying the optimal condition determined during the assay optimization. Reference table 3 for reaction setup examples.

Note*. It is recommended to have a buffer only and Eu-4G10 only control for the first time the assay is setup (as highlighted columns in table 3).

Table 3.

Kinase Reaction	Negative	Positive	Inhibitor/ Compounds	Biotin-p-Tyr Standard	Eu-4G10 only	Buffer only
Inhibitor/Compounds	-	-	2.5 μL	-	-	-
Biotin TK Substrate (5 μM)	2.5 μL	2.5 μL	2.5 μL	-	-	-
Kinase (4 ng/ μL)	-	2.5 μL	2.5 μL	-	-	-
ATP (500 μM)	2.5 μL	2.5 μL	2.5 μL	-	-	-
Biotin-p-Tyr Std as table 2	-	-	-	12.5 μL	-	-
1X Kinase Buffer	7.5 μL	5 μL	2.5 μL	-	12.5 μL	12.5 μL

6. A total 12.5 μL reaction mix per well. Gently tap the plate to mix the solutions, cover with one plate sealer. Incubate for 60 minutes at 30-37°C on a plate shaker with gentle agitation.
7. Prepare the detection reagent:
- 1). For biotin-p-Tyr and Eu-4G10 only wells, dilute Eu-4G10 conjugate (part No. CS206507) 1:100 with detection buffer (part No. CS206478), vortex briefly to mix well. Make sure to prepare some extra for pipetting.

2). For buffer only wells, directly add 12.5 μ L/well detection buffer (part No. CS206501).

3). For negative, positive and inhibitor/compounds wells, prepare the detection reagents as below, Make sure to prepare some extra for pipetting.

Eu-4G10	1:100
Streptavidin-APC	1:8 if 1 μ M Biotin-TK substrate is applied in the kinase reaction.
DM C	Fill up the required volume.

* Adjust the dilution factor of streptavidin-APC to have a fix biotin/streptavidin molar ratio at 8/1 if a different concentration of biotin-TK substrate is applied.

8. Pipet 12.5 μ L detection reagent prepared in step 7 to each well accordingly. Gently tap to mix the solutions. Incubate for 60 minutes at room temperature on a plate shaker with gentle agitation. Protect from light.
9. Measure TR-FRET ratio on an appropriate reader according to the following parameters (these are guideline parameters based on Molecular Probes -Analyst, please also refer to parameters recommended in the instrument instruction manual):

Excitation	330 nm
Emission	660-50 nm and 620-35nm
Counting Delay	50 μ sec
Integration time	400 μ sec
Z Height	0.2 μ m

HTRF Ratios are calculated as follows:

$$HTRF \text{ Ratio} = \left(\frac{\text{Emission at } 660\text{nm}}{\text{Emission at } 620\text{nm}} \right) \times 10000$$

10. Data is interpreted by GraphPad PRISM nonlinear fit, Sigmoidal dose-response (variable slope).

Assay Results

Z' factor was calculated to be greater than 0.8 using 48 replicates for each of no biotin-p-Tyr detection mixture as background signal and biotin-p-Tyr detection mixture as positive signal.

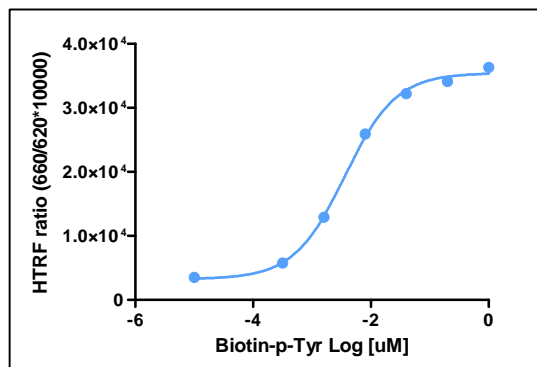


Figure 1. Dose response curve of Biotinylated-phosphotyrosine peptide.

Phosphotyrosine-containing peptide from 0~1 μM was incubated with Europium 4G10 and streptavidin APC. TR-FRET signal was determined, and a non linear curve fit with Sigmoidal Dose Response was applied.

NOTE: This data is presented for reference use only and should not be used to interpret actual assay results. A standard curve must be generated for each assay.

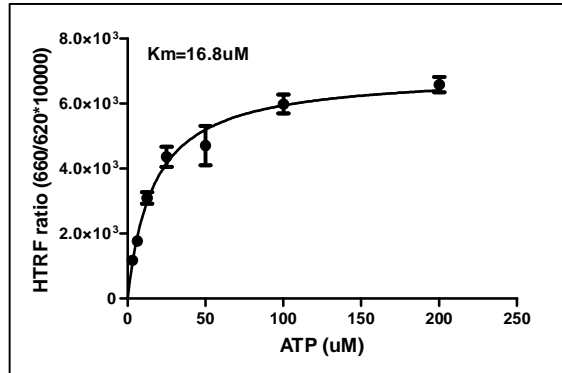


Figure 2. Determination of ATP dependence of active Src kinase by TR-FRET.

10ng active Src kinase was applied with Biotin-TK3 substrate. The Km value may be used for establishing ATP concentration when screening compounds.

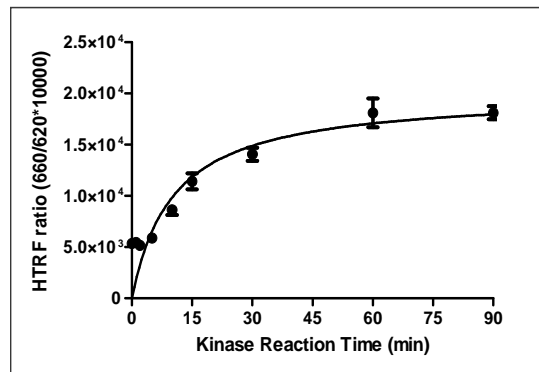


Figure 3. Kinetics of substrate phosphorylation by active FGFR1 kinase domain.

The kinase reaction was complete by 60 minutes at 30°C.

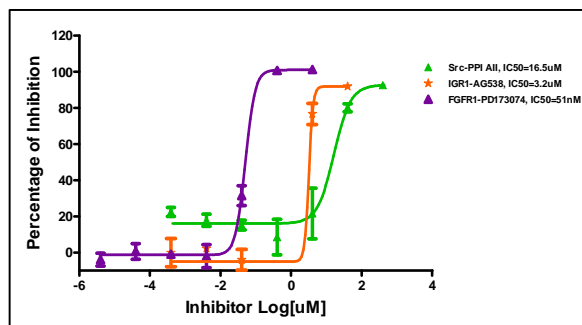


Figure 4. Determination of IC50 values for kinase-selective inhibitors.

The assay contained 10ng kinase, 100 μ M ATP, and 0.5 μ M biotinylated substrate, was incubated for 60 minutes at 30°C. Data obtained by TR-FRET was analyzed by non-linear curve fit with sigmoidal dose response with variable slope.

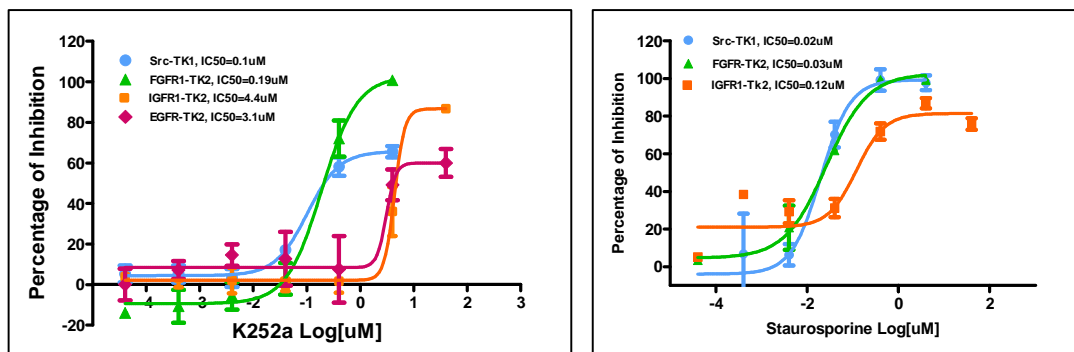


Figure 5. Determination of IC50 values of universal ATP-competitive inhibitors with a panel of kinases.

The assay contained 10ng kinase, 20 μ M ATP, and 1 μ M biotinylated substrate, and was incubated for 60 minutes at 30°C. Data obtained by TR-FRET was analyzed by non-linear curve fit with sigmoidal dose response with variable slope.

* K252a (alkaloid), a staurosporine analog, is a highly potent cell permeable inhibitor of CaM kinase and phosphorylase kinase. It acts by competition with the ATP binding site, non-selective protein kinase inhibitor.

References

1. Kanakura, Y., et al. (1991). *J. Biol. Chem.* 266:490.
2. Cohen, B., et al. (1990). *Proc. Natl. Acad. Sci.USA.* 87: 4458.
3. Druker, B. J., et al. (1989). *New Eng. J. Med.* 321: 1383.
4. O'Brian, C A and Ward, N E (1990). *J Natl Cancer Inst* 82: 1734-5.
5. Raychaudhuri, S.P., et al., *J. Invest. Dermatol.*, 122, 812-819 (2004).
6. 8. Winston, J.H., et al., *J. Pain.*, 4, 329-337 (2003).

Appendix A. Recommended Kinase Conditions.

Table of recommended reaction conditions for Upstate kinases (h = human; m = mouse):

Kinase	Millipore Part	Recommended Substrate	Recommended Kinase Reaction Condition
Abl (h)	14-529	Biotin-TK1	5mM MgCl ₂
Abl (T315I) (h)	14-522	Biotin-TK1	5mM MgCl ₂
Abl (m)	14-459	Biotin-TK1	5mM MgCl ₂
Arg (h)	14-521	Biotin-TK1	5mM MgCl ₂

Kinase	Millipore Part	Recommended Substrate	Recommended Kinase Reaction Condition
Arg (m)	14-460	Biotin-TK1	5mM MgCl ₂
Blk (m)	14-316	Biotin-TK1	5mM MgCl ₂
Bmx (h)	14-499	Biotin-TK1	5mM MgCl ₂
BTK (h)	14-552	Biotin-TK1	5mM MgCl ₂
CSK (h)	14-458	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Src (h)	14-326	Biotin-TK3	5mM MgCl ₂ , 1mM MnCl ₂
EGFR (h)	14-531	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
EphA2 (h)	14-560	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
EphB2 (h)	14-553	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
EphB4 (h)	14-554	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Fes/Fps (h)	14-473	Biotin-TK1	5mM MgCl ₂
FGFR1 (h)	14-582	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
FGFR4 (h)	14-583	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
FGFR3 (h)	14-464	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Flt1 (h)	14-562	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Flt3 (h)	14-500	Biotin-TK1	5mM MgCl ₂
Fms (h)	14-551	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Fyn (h)	14-441	Biotin-TK3	5mM MgCl ₂
IGF-1R (h)	14-465	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Insulin R (h)	14-466	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Lck (h)	14-442	Biotin-TK3	5mM MgCl ₂ , 1mM MnCl ₂
Lyn (h)	14-510	Biotin-TK1	5mM MgCl ₂ , 1mM MnCl ₂
Lyn (m)	14-315	Biotin-TK1	5mM MgCl ₂ , 1mM MnCl ₂
Met (h)	14-526	Biotin-TK1	5mM MgCl ₂
PDGFR α (h)	14-467	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
PDGFR β (h)	14-463	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Ret (h)	14-570	Biotin-TK1	5mM MgCl ₂
Ros (h)	14-527	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Rse (h)	14-535	Biotin-TK3	5mM MgCl ₂ , 1mM MnCl ₂
Syk (h)	14-314	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂

Kinase	Millipore Part	Recommended Substrate	Recommended Kinase Reaction Condition
Tie2 (h)	14-540	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂
Trk A (h)	14-571	Biotin-TK1	5mM MgCl ₂ , 1mM MnCl ₂
Trk B (h)	14-507	Biotin-TK2	5mM MgCl ₂
Yes (h)	14-478	Biotin-TK2	5mM MgCl ₂
ZAP-70 (h)	14-404	Biotin-TK2	5mM MgCl ₂ , 1mM MnCl ₂

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
<p>No signal or weak signal in all wells</p>	<p>Missing components or key steps</p> <p>Biotinylated-phosphotyrosine Peptide is no longer active or has reduced activity</p> <p>Plate reader or settings are not optimal</p> <p>Incorrect storage temperatures</p> <p>Incorrect assay temperature</p>	<p>Check to make sure all components were added in the appropriate steps and amounts.</p> <p>Make sure all components are stored at the recommended temperature and minimize the freeze/thaw cycle as manual recommends. Make aliquots of components when first thawed if planning more than one assay.</p> <p>Verify the measurement, read time, and filter on the plate reader.</p> <p>Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.</p>
<p>No detectable signal in samples</p>	<p>Low phosphotyrosine level or missing key components.</p>	<p>Check the kinase reaction setup procedure, make sure ATP, biotin-TK substrate and MgCl₂, MnCl₂ are added to the reaction buffer.</p>

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