Phosphotyrosine HTRF® Assay

Catalog No. 17-10015

96-wells

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 645-5476
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Introduction

The phosphotyrosine HTRF® Assay (Cat. # 17-10015) 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.

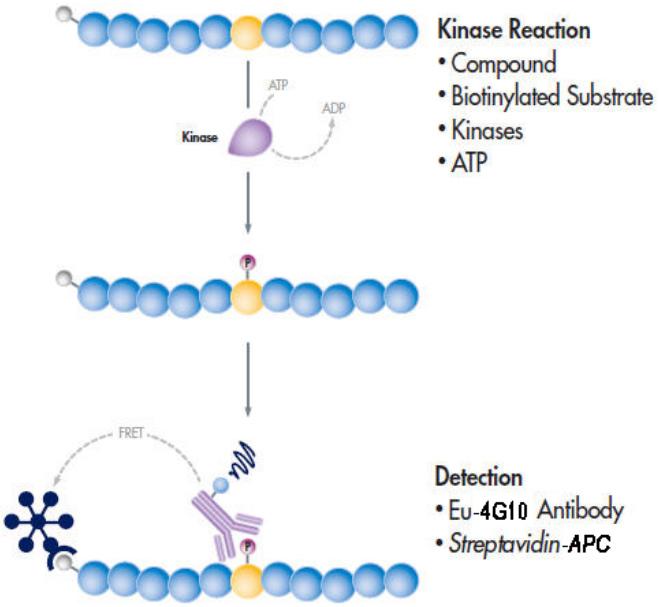
The assay has a wide dynamic range and high precision, making this assay a valuable new tool for the study of tyrosine phosphorylation.

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 2-8º C

1. Black 96 well plate: (Part No. R5013) One 96-well black plate.
2. Detection buffer: (Part No. CS206478) One bottle containing 5 mL of detection buffer.

Store at -20º C

3. 10X KinEASE buffer: (Part No. CS206508). 1 vial containing 1 mL of kinase reaction buffer.
4. Biotinylated TK1 Peptide: (Part No. CS206504). 1 vial containing 60 µL of Biotinylated TK1 Peptide Stock at 50µM.
5. Biotinylated TK2 Peptide: (Part No. CS206505). 1 vial containing 60 µL of Biotinylated TK2 Peptide Stock at 50µM.
6. Biotinylated TK3 Peptide: (Part No. CS206506). 1 vial containing 60 µL of Biotinylated TK3 Peptide Stock at 50µM.
7. Biotinylated Phosphotyrosine Peptide Standard: (Part No. 90192). One vial containing 10 µg lyophilized biotinylated phosphotyrosine peptide standard.
8. Streptavidin-APC: (Part No. CS206501). 1 vial containing 360 µL of streptavidin-APC stock at 1 µM.
9. Eu-cryptate-conjugated 4G10® anti-phosphotyrosine: (Part No. CS206507). 1 vial containing 30 µL Eu-cryptate conjugated 4G10 anti-phosphotyrosine antibody stock at 35 µg/mL.
10. ATP: (Part No. CS206509). 1 vial containing 50 µL of ATP stock at 10mM.
11. DTT: (Part No, 90499). 1 vial containing 100 µL of DTT stock at 1M.
12. $MnCl_2$: (Part No. CS206510). 1 vial containing 100 µL of $MnCl_2$ stock at 0.1M.
13. $MgCl_2$: (Part No. CS206511). 1 vial containing 100 µL of $MgCl_2$ stock at 1M.
14. Sodium Orthovanadate: (Part No. CS206465). 1 vial containing 50 µL of sodium orthovanadate stock at 50 mM.
15. 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. 96-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 -20ºC 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.

2. **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 due to it.
3. **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.
4. **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 Km. 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 Km. 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 Km 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.

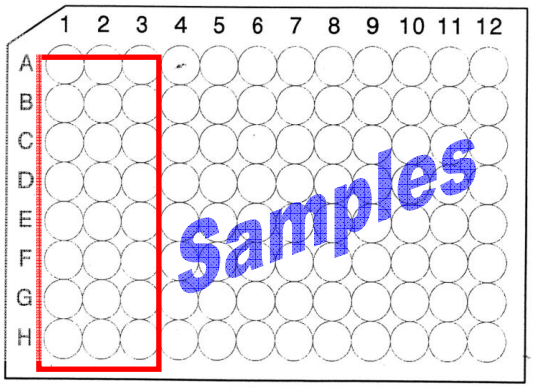
Biotinylated Phosphotyrosine Peptide Standard serial dilutions should be setup along with various concentration of streptavidin-APC to keep Biotin/Streptavidin ratio fix at 8/1.

Use 3 columns to setup the standard. Cover the unused remaining wells with plate sealer.

Wells Layout:

STD (uM)

1.1
2.0.2
3.0.04
4.0.008
5.0.0016
6.0.00032
7.0.000064
8.0



1. Label 7 test tubes #1-7 and "0 dose". Add 100 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.
   4.8 µL of 50 µM stock
   30 µL of streptavidin-APC (part No. CS206501)
   85.2 µL of Detection Buffer (part No. CS206478)
   Total 120 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 25 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 (25 µL)	Final assay conc. (50 µL)	Conc. in standard solution (25 µL)	Final assay conc. (50 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 25 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	-	-	5 µL	-	-	-
Biotin TK Substrate (5µM)	5 µL	5 µL	5 µL	-	-	-
Kinase (2 ng/µL)	-	5 µL	5 µL	-	-	-
ATP (500µM)	5 µL	5 µL	5 µL	-	-	-
Biotin-p-Tyr Std (as table 2)	-	-	-	25µL	-	-
1X Kinase Buffer	15µL	10 µL	5 µL	-	25µL	25µL

6. A total 25 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 25 µL/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 µM 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 25 µL 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 µsec
Integration time	400 µsec
Z Height	0.2 µm

HTRF Ratios are calculated as follows:

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

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 24 replicates for each of no biotin-p-Tyr detection mixture as background signal and biotin-p-Tyr detection mixture as positive signal.

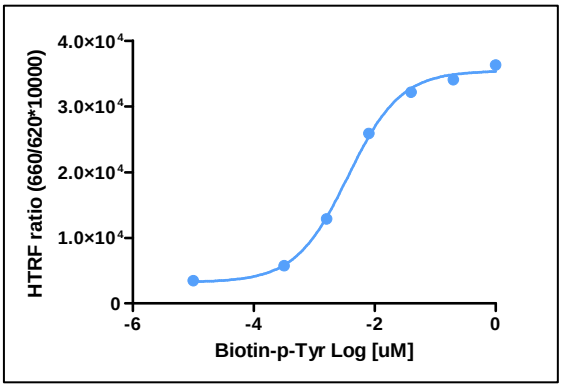


Figure 1. Dose response curve of Biotinylated-phosphotyrosine peptide.
Phosphotyrosine-containing peptide from 0~1 µM was incubated with Europium 4G10 and streptavidin APC. TR-FRET signal was determined, and a non linear curve fit with Sigmoidal Dose Response was applied.

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.

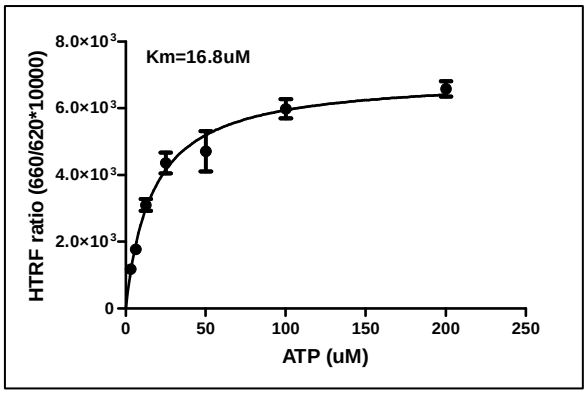


Figure 2. Determination of ATP dependence of active Src kinase by TR-FRET.
10ng active Src kinase was applied with Biotin-TK3 substrate. The Km value may be used for establishing ATP concentration when screening compounds.

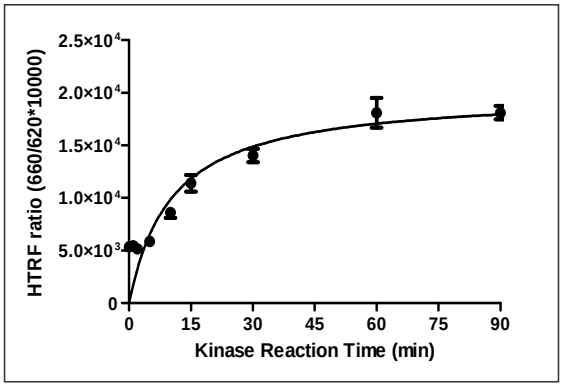


Figure 3. Kinetics of substrate phosphorylation by active FGFR1 kinase domain.
The kinase reaction was complete by 60 minutes at 30°C.

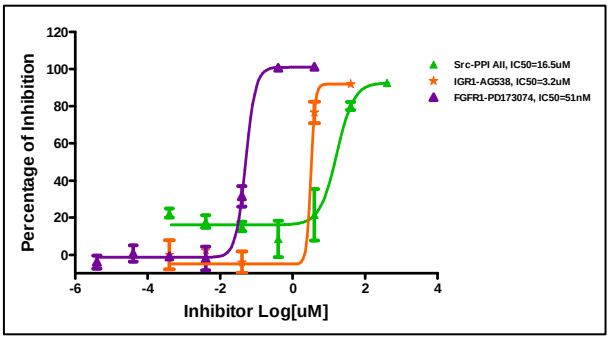


Figure 4. Determination of IC50 values for kinase-selective inhibitors.
The assay contained 10ng kinase, 100 µM ATP, and 0.5 µM biotinylated substrate, was incubated for 60 minutes at 30°C. Data obtained by TR-FRET was analyzed by non-linear curve fit with sigmoidal dose response with variable slope.

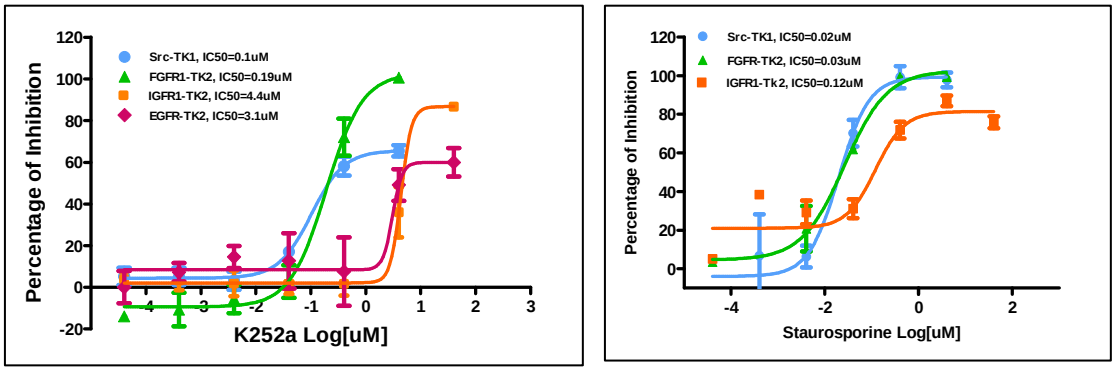


Figure 5. Determination of IC50 values of universal ATP-competitive inhibitors with a panel of kinases.

The assay contained 10ng kinase, 20 µM ATP, and 1 µM 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

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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 $MgCl_2$
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 $MnCl_2$

Kinase	Millipore Part	Recommended Substrate	Recommended Kinase Reaction Condition
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 $MgCl_2$
PDGFRα (h)	14-467	Biotin-TK2	5mM $MgCl_2$, 1mM $MnCl_2$
PDGFRβ (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 $MnCl_2$
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
No signal or weak signal in all wells	Missing components or key steps	Check to make sure all components were added in the appropriate steps and amounts.
	Biotinylated-phosphotyrosine Peptide is no longer active or has reduced activity	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.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures Incorrect assay temperature	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.
No detectable signal in samples	Low phosphotyrosine level or missing key components.	Check the kinase reaction setup procedure, make sure ATP, biotin-TK substrate and $MgCl_2$, $MnCl_2$ are added to the reaction buffer.

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