

**Instruction Manual**  
**for**  
**Tyrosine Kinase Assay Kit**  
**Colorimetric Detection**  
**Catalog # 17-315**

Direct ELISA system for the colorimetric detection of protein tyrosine phosphotransferase activity using a biotinylated peptide substrate and a monoclonal anti-phosphotyrosine-HRP conjugate detection antibody.

Sufficient reagents for 96 assays per kit.

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**DIAGNOSIS OF DISEASE IN HUMANS.**  
**DO NOT USE IN HUMANS.**

## I. STORAGE AND STABILITY

**Storage:** Upon receipt, store individual components at recommended temperatures. Store components 12-440A, 12-443, 20-278 and 20-279 at -20°C. Store all other components at 4°C.

**Stability:** Components stable for 6 months from date of shipment if stored and handled correctly.

## II. ASSAY OVERVIEW

The Tyrosine Kinase Assay Kit, (Colorimetric detection, Catalog # 17-315, or Chemiluminescent detection, Catalog # 17-331) is designed to measure the phosphotransferase activity of tyrosine kinases from a variety of sources including crude preparations such as cell lysates or column fractions, immune complexes containing the tyrosine kinase of interest, or pure recombinant enzyme. By virtue of the strong biotin-streptavidin interaction ( $K_D \cong 10^{-15}M$ ), the peptide substrate is efficiently captured, and the stringency of washes can be adjusted as needed to help reduce non-specific background in the ELISA.

The non-radioactive kinase assay is a two-step peptide phosphorylation/detection assay kit. In the first stage, a biotinylated substrate peptide containing tandem repeats of Poly (Glu<sub>4</sub>-Tyr) is incubated with a tyrosine kinase enzyme sample in the presence of non-radioactive ATP and a Mn<sup>2+</sup>/Mg<sup>2+</sup> co-factor cocktail. The kinase assay can be performed with substrate peptide either in solution, or bound to the well of a streptavidin-coated microplate. The second step involves the detection of phosphorylated substrate by Direct Enzyme Linked Immunosorbent Assay (ELISA) using a monoclonal anti-phosphotyrosine-HRP (Horseradish Peroxidase) antibody conjugate. Tetramethylbenzidine (TMB) is provided as the HRP substrate for the colorimetric detection version of the kit (Catalog # 17-315), and LumiGLO<sup>®</sup> chemiluminescent substrate (Catalog # 20-212) is provided for the chemiluminescence detection version of the kit (Catalog # 17-331).

The colorimetric detection version of the Tyrosine Kinase Assay Kit (Catalog # 17-315) is provided with a 96-well plate containing removable strip-wells so that the ELISA can be performed on more than one occasion. The chemiluminescence detection version of the Tyrosine Kinase Assay Kit (Catalog # 17-331) is provided with 2, white 96-well solid plates. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination. The non-radioactive assays have several advantages over currently used radioactive methods. The methods are highly sensitive, fast and simple to perform while eliminating the hazards that accompany the use of radioactive materials. The chemiluminescent version of the kit provides an alternative detection method that is preferred by many investigators. Either method can be adapted for automation and high-throughput drug targeting and validation studies.

Also provided with this kit is a phosphorylated Poly (Glu<sub>4</sub>-Tyr) biotinylated peptide [Poly (Glu<sub>4</sub>-phospho-Tyr)]. This peptide can be added to a reference well, thereby serving as a positive control for the Direct-ELISA portion of this assay.

To assay the tyrosine kinase activity of enzymes immobilized on a solid support (e.g. kinase samples in immune complexes bound to agarose beads) the *in vitro* kinase assay must be performed with the peptide in solution, followed by peptide binding to the plate. An alternative procedure is included in this manual. Protein A-agarose (Catalog # 16-125) or Protein-G-agarose (Catalog # 16-266), required for capturing immune complexes are available separately, as are the Tyrosine Kinase Reaction Buffer, 5X (Catalog # 20-278) and the Poly (Glu<sub>4</sub>-Tyr) Peptide, biotin conjugate (Catalog # 12-440, a 100µg pack size) since the immune-complex protocol requires use of larger amounts of these components.

The end user should determine the linear range of the assay empirically. This assay can be performed at room temperature (25°C), 30°C, or 37°C, with incubation times of up to 30 minutes. Further incubation or higher levels of incorporation may not be linear and may therefore not give a true representation of kinase activity.

Although tyrosine kinases do not exhibit tight specificity for particular substrates, the Poly (Glu<sub>4</sub>-Tyr) substrate peptide included in the kit is phosphorylated by many tyrosine kinases, some may not recognize the peptide as a substrate. Each kit contains sufficient reagents for 96 (Catalog # 17-315) or 192 (Catalog # 17-331) individual assays.

A recombinant kinase (Src, active, Catalog # 14-326) has been used for internal testing of this assay kit, and is available separately.

### III. SYSTEM COMPONENTS

#### A. Provided Kit Components

##### Streptavidin Coated Strip Plate

Catalog #20-183

One streptavidin coated plate containing 12 strips of 8 wells each.

##### Poly (Glu<sub>4</sub>-Tyr) Peptide, biotin conjugate

Catalog # 12-440A

One vial containing **12.5µg** peptide in 250µl sterile distilled water.

##### Poly (Glu<sub>4</sub>-Phospho-Tyr) Peptide, biotin conjugate

Catalog # 12-443

One vial containing **5µg** in 50µl sterile 0.1M sodium bicarbonate.

##### Tyrosine Kinase Reaction Buffer, 5X

Catalog # 20-278

One vial containing **5ml** of 5X Tyrosine Kinase Reaction Buffer (5XTKRB): 100mM Tris-HCl, pH 7.4, 50mM MgCl<sub>2</sub>, 5mM MnCl<sub>2</sub>, 5mM dithiothreitol, 1mM ATP.

##### Sodium Orthovanadate, 50mM

Catalog # 20-279

One vial containing **500µl** of Sodium Orthovanadate, pH 10.0.

##### 20X TBS, 2% Tween<sup>®</sup>-20

Catalog # 20-202

One vial containing **50ml** of 1M Tris, 3M NaCl, 2% Tween-20, pH 7.4.

##### 10% BSA in TBS

Catalog # 20-191

One vial containing **10ml** of 10% BSA in TBS, pH 7.4 containing 0.05% Kathon<sup>®</sup>.

##### Anti-phosphotyrosine, recombinant 4G10<sup>™</sup>, HRP conjugate:

Catalog # 16-184B

One vial containing **25µl** of IgG<sub>2bκ</sub>-HRP conjugate in PBS containing 5mg/ml BSA and 0.05% Kathon<sup>®</sup>.

##### TMB (tetramethylbenzidine) Substrate Reagent A

Catalog # 20-182a

One vial containing **7.5ml** of 0.04% (w/v) TMB solution.

##### TMB (tetramethylbenzidine) Substrate Reagent B

Catalog # 20-182b

One vial containing **7.5ml** of 0.02% hydrogen peroxide in citric acid buffer.

#### B. Required Materials Not Provided

- Enzyme preparation or cell extract containing active enzyme(s).
- Reagent troughs for multichannel pipettes
- 2M sulfuric acid
- 5M HCl
- Microtiter plate washer (optional), shaker or a platform vortex
- Shaking incubator
- Wash bottle or multichannel dispenser for washing
- Timer
- 96-well plate spectrophotometer capable of measuring absorbance of at 450nm.
- Variable volume (5-200µl) pipet + tips
- TBS
- Plastic plate sealer film

#### C. Stock Solutions

Prepare the following solutions in advance of performing the Tyrosine Kinase Assay:

**1X TBS/T:** Dilute 50ml 20X TBS, 2% Tween<sup>®</sup>-20 (Catalog # 20-202) with 950ml of sterile water to create a working solution of 1X TBS/T. Store at room temperature.

**ELISA Blocking Buffer:** Add 90ml of 1X TBS/T to 10ml 10% BSA in TBS (Catalog # 20-191) to make a working solution of 1% BSA in TBS/T.

**2M Sulfuric Acid:** Dilute stock solution with sterile water to a final 2M concentration.

**Substrate Solution:** For each 8-well strip to be used, prepare 1ml of 1µg/ml Substrate Solution by diluting 20µl Poly (Glu<sub>4</sub>-Tyr) Peptide, biotin conjugate (Catalog # 12-440A) with 980µl 1X ELISA Blocking Buffer. Vortex the vial prior to diluting.

**Reference Solution:** Prepare 250µl of 1µg/ml Reference Solution by diluting 2.5µl Poly (Glu<sub>4</sub>-phospho-Tyr) Peptide, biotin conjugate (Catalog # 12-443) with 247.5µl 1X ELISA Blocking Buffer. Vortex the vial prior to diluting.

## IV. TYROSINE KINASE ASSAY PROCEDURE

**Safety Warnings and Precautions:** The Tyrosine Kinase assay 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.

### A. General Notes

1. If the enzyme to be assayed is in solution, follow sections B, C and D of this protocol. If the enzyme is immobilized (i.e. in an immune complex), refer to Appendix B.
2. Prepare the **Substrate** and **Reference** solutions (see Section III-C, Stock Solutions) the same day of the assay. Discard unused solutions at the completion of the assay.
3. The Reference peptide provided (**Poly (Glu<sub>4</sub>-phospho-Tyr) Peptide, biotin conjugate, Catalog # 12-443**) serves as a positive control for the direct-ELISA portion of this assay. It is recommended to include at least one reference well (10ng of Reference Peptide + 90ng Substrate Peptide) each time the assay is performed. Alternatively, a Reference series containing increasing amounts of Reference peptide. See Section V, Appendix A.
4. The amount of purified enzyme or lysate needed for the assay has to be determined empirically to ensure that the assay is within the linear range. If enzyme sample is limiting, establishing linearity of the assay may also be achieved by varying the reaction incubation time in Section IV-C (*In vitro* Kinase Reaction for Enzymes in Solution) of this procedure. For time courses, delay the addition of the tyrosine kinase assay mixture such that all end points will coincide.
5. When performing washes manually, avoid introducing bubbles when dispensing liquids into the well strips, and ensure each well is filled with buffer, but not overflowing to avoid cross-contamination between wells. Empty wells with a wrist-flick motion over an appropriate receptacle, and while still inverted, blot any remaining moisture on clean paper towels. If an automated plate washer is used, follow manufacturer's recommendation for operation.
6. Agitation of the wells during the ELISA wash steps (Section IV-C, D) is recommended to reduce non-specific background. If microtiter plate agitator is not available, a platform vortex at a low setting can be used (e.g. level 1 of Fisher's Genie II platform vortex). Generally, less washing is required for purified kinase reactions, than for crude cell lysate reaction mixtures. If background problems occur, simply increase the number and/or duration of washes.
7. A brief water rinse is recommended prior to the addition of the TMB substrate mixture (Section IV-D, Phosphotyrosine detection) to remove traces of Tween<sup>®</sup>-20 which can interfere with HRP activity. TBS can be used in place of water. All traces of moisture must be removed before the addition of the TMB substrate since volume fluctuations between wells affects OD measurement accuracy.
8. The amount of time the HRP reaction with the provided TMB substrate is allowed to proceed will vary based on the experimental conditions. Stop the reaction at first trace of color in negative control wells (no-enzyme or 0ng Reference Peptide wells). This is a very important step to help keep background levels low.
9. Well to well variations of the plastic (background) can be determined by reading the plate at 570nm. The 570nm values should then be subtracted from the 450nm values.

## B. Binding Peptides to Well-Strips

Both the Substrate and Reference peptides are biotinylated and bind to the streptavidin present in the well-strips. This phase of the procedure can be performed a day in advance of the remainder of the assay. The white frame that holds the individual well-strips should be retained for future use.

1. Remove the Streptavidin Coated Strip Plate (Catalog # 20-183) from the foil pouch. Remove any strips of microtiter wells not required for the assay. Return unused strips to the foil and store at 4°C.
2. Add 100µl **ELISA Blocking Buffer** per well. Let stand for 15 minutes at room temperature to rehydrate the wells.
3. Remove **ELISA Blocking Buffer** from wells with a wrist-flick or aspirate wells using a microtiter plate washer. See *General Note 5*.
4. Add 100µl/well **Substrate Solution** (1µg/ml **Poly (Glu<sub>4</sub>-Tyr) Peptide, biotin conjugate**) to each experimental well. Substrate Solution preparation is described in Section III-C, Stock Solutions. See *General Note 2*.
5. Add up to 100µl/well **Reference Solution** (1µg/ml **Poly (Glu<sub>4</sub>-phospho-Tyr) Peptide, biotin conjugate**) to each reference well or prepare a Reference Series (see Appendix II and General Notes 2 and 3) using 1, 5, 10, and 20 µl/well of **Reference Solution** and sufficient quantities of **Substrate Solution** to bring the final volume/well to 100µl in each of the reference wells.
6. Cover wells with a plastic plate sealer film. Incubate strip wells for 1 hour at 37°C.
7. Remove peptide solution and wash all wells three times with **1X TBS/T** to remove any unbound peptide.
8. Add 300µl/well **ELISA Blocking Buffer** and incubate 1-2 hours at 37°C or overnight at 4°C.

### a. *In vitro* Kinase Reaction for Enzymes in Solution

This assay has been performed at room temperature, 30°C, and 37°C using **Src, active** (Catalog # 14-326) and cell lysates frozen in the presence of 30% glycerol. Time points typically employed for the assay have been 0, 5, 10, 15, and 20 minutes (see Quality Control section in the Certificate of Analysis for an example assay results).

1. Thaw **Tyrosine Kinase Reaction Buffer, 5X** (Catalog # 20-278) and **Sodium Orthovanadate, 50mM** (Catalog # 20-279). **Note:** a precipitate may be observed in the thawed buffer. Vortex to bring into solution.
2. On ice, make a master mix of the Kinase Assay mixture for each enzyme concentration to be assayed, planning for 50µl/well. Use the proportions listed below, adding enzyme last. Mix gently.

For each 50µl reaction volume (1 well equivalent), add:

Tyrosine Kinase Reaction Buffer (5X stock)	10µl
Sodium Orthovanadate, 50mM	1µl
Purified enzyme or lysate	varies
Sterile, distilled water	Quantity sufficient to 50µl

3. Remove the ELISA blocking buffer from the wells. Place well-strips in micro-titer plate incubator set at the appropriate temperature.
4. To reference wells only; add 200µl **ELISA Blocking Buffer**.
5. To experimental wells, add 50µl/well of the **Kinase Assay mixture** (from step 2). Incubate wells for the desired time interval. If a time course is to be performed, see *General Note 4*.
6. Immediately remove the solution from all wells and rinse wells twice with 1X TBS/T to efficiently remove enzyme from experimental wells.
7. Proceed with four **TBS/T** washes, incubating the plate for 10 minutes at room temperature with agitation between buffer changes. Proceed to Section D of this procedure.
8. Perform a final wash in **ELISA Blocking Buffer** for 10 minutes prior to addition of antibody.

## D. Phosphotyrosine Detection

1. Remove TBS/T from the wells and add 100 $\mu$ l of **anti-phosphotyrosine, recombinant 4G10™, HRP conjugate** diluted 1:500,000 in **ELISA Blocking Buffer**. Incubate for 20-30 minutes at room temperature.
2. Rinse wells twice with 1X TBS/T, then proceed with five TBS/T washes, incubating the plate for a minimum of 10 minutes at room temperature with agitation between buffer changes.
3. During the last TBS/T wash prepare the TMB substrate. For each strip of wells prepare 1ml of a 1:1 (v/v) mixture of **TMB-Substrate A** (Catalog # 20-182a) and **TMB-Substrate B** (Catalog # 20-182b) and add 75 $\mu$ l per well.
4. Following the last TBS/T wash, rinse wells with sterile, distilled. Remove water with a wrist-flick and blot well-strips on paper towels. Ensure no traces of water remain. See *General Note 7*.
5. Add 75 $\mu$ l of the TMB mixture prepared in Step 3 to each well. Allow the reaction to proceed for up to 15 minutes. To keep background levels low, it is important to stop the reaction at the first trace of color in the control wells. See *General Note 8*.
6. Add 75 $\mu$ l of **2M Sulfuric Acid** to stop further color development.
7. Measure the absorbance in each well using a spectrophotometric plate reader at a wavelength of 450nm. See *General Note 9*.

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## V. APPENDICES

### A. Reference Series Preparation

Using the Substrate and Reference solutions prepared in Section III-C Stock Solutions, prepare a reference series using the volumes indicated in the table below.

Reference Peptide Concentration (ng/well)	Volume of Reference Solution to add ( $\mu$ l/well)	Volume of Substrate Solution to add ( $\mu$ l/well)
0	0	100
20	20	80
10	10	90
5	5	95
1	1	99

## B. Alternate Protocol for Assaying Immobilized Enzymes

The reagents in this kit can also be used to assay the activity of tyrosine kinases in immune complexes. Lysates should be prepared fresh using a suitable lysis buffer. Once the kinase of interest has been captured in an immune complex, the *in vitro* kinase reaction is performed with the substrate peptide in solution. The kinase reaction is then terminated, and the mixture is subsequently applied to the strip-wells, enabling binding of biotinylated substrate peptide. Phosphotyrosine detection proceeds as described above in the standard protocol.

Two kit components, which may become limiting if numerous assays are performed with this alternate protocol, are available separately: **Tyrosine Kinase Reaction Buffer, 5X** (Catalog # 20-278), required in the final wash of immune complexes at 1X-strength, and **Poly (Glu<sub>4</sub>-Tyr) Peptide, biotin conjugate** (Catalog # 12-440, 100 $\mu$ g pack size). Each assay point in this alternate procedure requires between 250-5000ng of substrate peptide.

Protein A-Agarose (Catalog #16-125) and Protein G-Agarose (Catalog #16-266) are also available from Upstate.

### Well Rehydration

1. Rehydrate required number of strip wells as described in Section IV-B, steps 1-3.
2. Remove the ELISA Blocking Buffer from the wells with a wrist-flick, and add 300 $\mu$ l of fresh **ELISA Blocking Buffer** to each well. Incubate for a minimum of 2 hours at 37°C or overnight at 4°C.

### Preparation of Antibody-protein A- or G-Agarose complexes

3. Determine the number of assay points needed. Include a negative control using a pre-immune or non-specific antibody.
4. Select protein A- (Catalog #16-125) or G-beads (Catalog #16-266) as appropriate for the primary antibody and wash with TBS.
5. Add 40 $\mu$ l of a 50% bead slurry to each microfuge tube (one tube representing a single assay point). Add 500 $\mu$ l TBS and a sufficient amount of immunoprecipitating antibody (known to quantitatively IP the kinase of interest). Mix end-over-end at 4°C for a minimum of 30min.
6. Pellet beads with a brief centrifugation. Discard supernatant to remove any unbound antibody. Store beads on ice.

### Immunoprecipitation//*In vitro* Kinase Assay

7. Dilute a freshly prepared lysate, containing protease and phosphatase inhibitors, to 1mg/ml in lysis buffer. Frozen lysates containing 30% glycerol can be used to assay some kinases, however activity will be reduced.
8. Pre-clear a sufficient amount of lysate with 100 $\mu$ l of protein A- or G-agarose beads per 1ml of lysate. Mix end-over end at 4°C for 30-60min. The same beads used to prepare the antibody-agarose bead complex should be used to pre-clear lysates.
9. Pellet beads with a brief microfuge spin.
10. Add pre-cleared lysates to microfuge tubes containing the antibody-bead complexes prepared above. To vary enzyme amounts to be assayed, vary the amount of lysate/microfuge tube (i.e. 0.25mg, 0.5mg, 1mg).

11. Add lysis buffer to 1ml/tube and incubate, mixing end-over-end for 1-2 hours at 4°C.
12. Collect immune complexes (Step 6 above) with a brief microfuge spin. Remove supernatant fraction and add 500µl TBS or lysis buffer to wash the beads. Resuspend beads with a brief vortex pulse (<1second at lowest setting).
13. Repeat the above wash step 3-5 times. During the wash steps, thaw **Tyrosine Kinase Reaction Buffer, 5X** (Catalog #20-278) and **Sodium Orthovanadate, 50mM** (Catalog #20-279). **Note:** a precipitate may be observed in the thawed buffer. Vortex to bring into solution.
14. On ice, make a master mix of the Kinase Assay mixture, planning for 50µl/tube. Use the proportions listed below. Mix gently.

For each 50µl reaction volume (1 tube equivalent), add:

Tyrosine Kinase Reaction Buffer (5X stock)	10µl
Sodium Orthovanadate, 50mM	1µl
Poly (Glu <sub>4</sub> -Tyr) Peptide, biotin conjugate (provided stock, 12.5µg in 250µl)	5µl
Sterile, distilled water	34µl

15. Perform a final wash of the immune complexes using 100-200µl of 1X Tyrosine Kinase Reaction Buffer. Remove as much buffer as possible, and proceed immediately to next step.
16. Add 50µl of Kinase Assay mixture prepared in step 14 to each tube and transfer tubes to an shaking incubator set at the desired temperature for the appropriate amount of time. (Refer to Section IV-C, *In vitro* Kinase Reaction for Enzymes in Solution).
17. Terminate the reaction; either by heat inactivation (5min at 95°C) or by reducing the pH to 2 with the addition of 2µl 5M HCl. Kinase-specific inhibitors can also be used.
18. Collect reaction product in microfuge tubes with a brief microfuge pulse.
19. Remove blocking buffer from strip wells (Step 2). Rinse once with TBS/T, then transfer supernatant from each reaction tube to individual wells. If reference wells are to be included, add Reference Solution to the appropriate wells at this point. Cover wells with a plastic plate sealer film and incubate for 1 hour at 37°C.
20. Rinse wells twice with 1X TBS/T, then proceed with five TBS/T washes, incubating the plate for a minimum of 10 minutes at room temperature with agitation between buffer changes. Proceed to Section IV-D of the standard procedure.

## C. References

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