

## 1.0 INTRODUCTION

The phosphorylation of proteins by protein tyrosine kinases (PTKs) is critical to the normal regulation of many biological mechanisms, including cell growth, proliferation, differentiation, and metabolism (1, 2). Defects in these pathways have been implicated in a number of human diseases, including cancer (1, 2). Conventional tyrosine kinase assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera's PTK Red Assay is simple, sensitive, non-radioactive, homogeneous, and is formatted for high-throughput screening. Additionally, the use of the red-shifted fluorescently labeled phosphopeptide tracer has the added benefit of minimizing background fluorescence interference occasionally found in compound libraries.

This assay kit contains all materials necessary to analyze protein tyrosine phosphorylation using fluorescence polarization (FP) as the detection method. Product numbers P2882 and P2883 contain enough reagents sufficient for one hundred and one thousand 100  $\mu$ L assays, respectively. These kits are formatted for use with either a multi-well plate instrument capable of measuring FP in 96-well plates or a single-well FP instrument, such as the Beacon® 2000 System (PanVera Corporation). These kits will also work in 384- and 1536-well plates by using reduced reagent volumes.

## 2.0 THEORY

### 2.1 What is Fluorescence Polarization?

Fluorescence polarization is a technique for monitoring molecular interaction in a homogenous environment at equilibrium. The binding of a small, fluorescent molecule to another, larger molecule can be quantified by a change in the rate of rotation of the fluorescent molecule. Plane-polarized light is used to excite fluorescent molecules in solution; if the excited molecule remains stationary during the period of excitation (4 nanoseconds for fluorescein) the emitted light will remain highly polarized. However, if the excited molecule can tumble or rotate during this period, the emitted light will be depolarized (3, 4).

Since FP is a measure of this tumbling rate, it is directly related to the molecular volume of the fluorescent molecule. An increase in molecular volume, due to biological processes such as receptor-ligand binding, antibody-antigen binding, DNA hybridization or DNA-protein binding, or a decrease in molecular volume due to enzymatic degradation or dissociation, can be measured directly by FP.

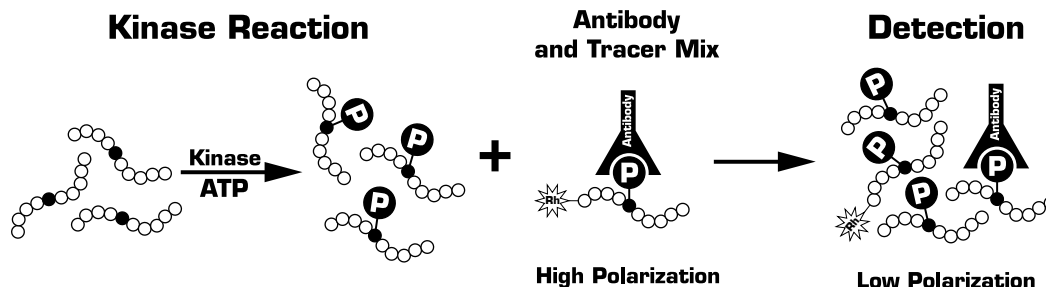
The measured FP value is the weighted average of the FP values of the individual bound and free fluorescent molecules and is therefore a direct measure of the fraction bound. These data can be handled exactly the same way as traditional radioligand-binding assay data. Fluorescence polarization values are plotted against the logarithm of the receptor concentration, resulting in the familiar saturation-binding isotherm.

For more information, see our on-line Fluorescence Polarization Applications Guide at:

<http://www.panvera.com/tech/appguide/index.html>

### 2.2 Assay Theory

FP can be exploited in a PTK assay because Rhodamine derivative-labeled phosphopeptides (Rh-phosphopeptides, also referred to as the "tracer") and phosphopeptides or phosphoproteins generated during a kinase reaction will compete for binding to anti-phosphotyrosine antibodies. During detection and when there are no kinase reaction products present, a significant portion of the Rh-phosphopeptide tracer will be bound by the anti-phosphotyrosine antibody, resulting in a high polarization value. However, after a tyrosine kinase reaction has occurred, phosphorylation can be detected because the anti-phosphotyrosine antibodies will bind to both the Rh-phosphopeptide tracer and the reaction products, decreasing the amount of bound tracer and thus decreasing the fluorescence polarization value of the sample. This is illustrated below. If enough competitor phosphopeptide is generated during the reaction, the fluorescent tracer can be completely displaced from the anti-phosphotyrosine antibodies and the emitted light will be totally depolarized. Thus, the change in fluorescence polarization value is directly related to tyrosine kinase activity.



In this kit, the binding to phosphotyrosines is sequence-independent. To demonstrate this, at concentrations relevant to an *in vitro* kinase reaction, the following tyrosine-phosphorylated peptides have been shown to completely displace the Rh-phosphopeptide tracer from the anti-phosphotyrosine antibody included in this kit:

Peptide Substrate Sequence	Enzymes That Utilize These Substrates
poly(Glu, Tyr) 4:1	Zap70, EGF Receptor, pp60 <sup>c-Src</sup> , Src N1, Src N2, Lck
ADEYLIPQQ	EGF Receptor
DEDYIQD	EGF Receptor
KVEKIGEGTYGVV	Fyn, Lck, Lyn, pp60 <sup>c-Src</sup>
RRDIYETD	Insulin Receptor
EYLIPQ	EGF Receptor
EILYNMDEG	EGF Receptor
KQVVDSDATYNVIK	EGF Receptor
QEEYSAM	H-ras 1/p21
TAENAEYLRVAPQ	EGF Receptor
SLDNPDYQQDFFP	EGF Receptor
SVQNPVYHNQPLN	EGF Receptor
FLPVPEYINQSVP	EGF Receptor
HTDDEMTGYVATR	MEK6

### 3.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed. All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. The chemical, physical, and toxicological properties of these products may not have been thoroughly investigated. We recommend the use of gloves, lab coats, eye protection and a fume hood when working with any chemical reagents.

### 4.0 KIT COMPONENTS

#### 4.1 Materials Provided

Description	Composition	P2882		P2883	
		Size	Part #	Size	Part #
Anti-Phosphotyrosine Antibody, 10X	Anti-phosphotyrosine antibody in 20 mM Tris (pH 7.5), 0.02% NaN <sub>3</sub>	1 mL	P2840	10 mL	P2841
PTK Red Tracer, 10X	Rhodamine derivative-labeled phosphopeptide in 20 mM Tris (pH 7.5), 0.02% NaN <sub>3</sub>	1 mL	P2891	10 mL	P2892
PTK Competitor	100 μM phosphopeptide in nanopure water	200 μL	P2655	200 μL	P2655
PTK Quench Buffer	100 mM EDTA (pH 7.5)	1 mL	P2846	10 mL	P2847
FP Dilution Buffer	20 mM Tris (pH 7.5), 0.02% NaN <sub>3</sub>	5 mL	P2838	50 mL	P2839

#### 4.2 Materials Required but not Supplied

- Any PTK Substrate, such as poly(Glu, Tyr) 4:1 or a peptide containing tyrosine
- Kinase
- ATP
- Kinase Reaction Buffer
- Multi-well plates suitable for fluorescence polarization or 6mm borosilicate tubes
- Multi-well fluorescence polarization instrument, such as a TECAN Ultra with 535 nm excitation and 590 nm emission interference filters or a Beacon® 2000 Fluorescence Polarization instrument (Panvera Corporation) with 525 nm excitation and 590 nm emission interference filters
- Pipeting devices
- Reagent reservoir(s)
- Laboratory timer
- Red (FP) Standardization Kit (Panvera Part No. P2888). This kit is recommended as a polarization standard for red-shifted assays.
- Beacon® FP One-Step Standardization Kit (Panvera Part No. P2581). This kit is recommended to validate instrument performance and may be required as a polarization standard to calibrate certain instruments.



## 5.0 STORAGE AND STABILITY

The Anti-Phosphotyrosine Antibody, 10X should be stored at -20°C. Once thawed, it should be stored at +4°C. The PTK Red Tracer, 10X and the PTK Competitor should be stored at -20°C, thawed when needed, and then refrozen. Avoid subjecting these reagents to repeated freeze/thaw cycles. The FP Dilution Buffer and Kinase Quench Buffer can be thawed upon receipt and stored at 20–30°C. All reagents are stable for 6 months from the date of receipt.

## 6.0 DETERMINING THE IC<sub>50</sub> FOR THE PTK COMPETITOR

The following instructions for testing and validating the kit are optional, but highly recommended for a first-time user. Generating a standard curve demonstrates the type of data that will be generated during an enzymatic reaction. It is also recommended that you generate this type of standard curve using the tyrosine-phosphorylated form of the peptide substrate you intend to use for your PTK reaction. Once you have the IC<sub>50</sub> for the tyrosine-phosphorylated form of the substrate of your choice, these data will give you some idea (based on the turnover rate of your enzyme) of how much enzyme you will need, how much substrate your PTK reaction will require, and how long the reaction should be run.

### 6.1 96-well Plate Format

1. In a 96-well plate suitable for fluorescence polarization, pipette 90 µL of FP Dilution Buffer into well A1. Then add 10 µL of the 100 µM PTK Competitor. The concentration of the PTK Competitor in well A1 is now 10 µM.
2. Dispense 50 µL FP Dilution Buffer into wells A2 to A12 and wells B1 to B4 (15 wells total).
3. Perform a 2-fold serial dilution of the PTK Competitor by transferring and mixing 50 µL from well A1 into well A2.
4. Repeat this process (from well A2 to A3, then from well A3 to A4 etc.) until well B4 is reached.
5. Discard 50 µL from well B4. There should be 50 µL in every well from A1 to B4.
6. **VORTEX** PTK Red Tracer, 10X.
7. Add 10 µL of the Anti-Phosphotyrosine Antibody, 10X and 10 µL of the PTK Red Tracer, 10X to every well from A1 to B4 (16 wells total). Add 30 µL of FP Dilution Buffer to each well. The tracer, antibody, and buffer can also be pre-mixed, and then added all at once (please see Section 6.4). This step will reduce the concentration of all of the detection components to 1X and reduce the concentration of PTK Competitor in each well by 2-fold (*i.e.* well A1 now has a competitor concentration of 5 µM, while B4 has a competitor concentration of approximately 0.153 nM).
8. Mix gently, cover the microwell plate to reduce evaporation and protect the reagents from light, and incubate for at least 30 minutes at room temperature.
9. Measure the polarization values of your samples, following the procedures required by your FP instrument. We recommend that you use 100 µL FP Dilution Buffer as your blank and a 1:10 dilution of the Red Polarization Standard (Panvera Part No. P2889, which is found in the Red (FP) Standardization Kit, Panvera Part No. P2888) as your low polarization standard, if necessary. You may also want to have controls for the antibody/tracer mixture without competitor and for the tracer alone to determine the high and low polarization values for the assay itself. See Section 6.3.
10. Perform non-linear regression on a semi-log plot of the data (mP vs. log [Competitor]). The IC<sub>50</sub> of the competitor control should be between 5 and 20 nM. Sample data generated on the TECAN Polarion microplate reader (n = 4) appear at the end of Section 6.4.

### 6.2 Single-tube Format

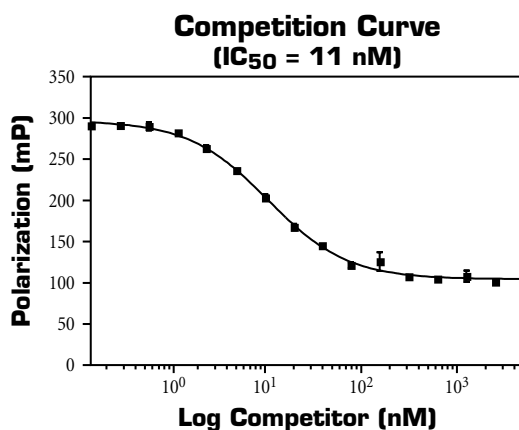
1. Label sixteen borosilicate tubes #1 through #16. Pipette 90 µL of FP Dilution Buffer into tube #1. Then add 10 µL of the 100 µM PTK Competitor. The concentration of the PTK Competitor in tube #1 is now 10 µM.
2. Dispense 50 µL FP Dilution Buffer into tube #2 through #16 (15 tubes total).
3. Perform a 2-fold serial dilution of the PTK Competitor by transferring and mixing 50 µL from tube #1 into tube #2.
4. Repeat this process (from tube #2 to #3, then from tube #3 to #4 etc.) until tube #16 is reached.
5. Discard 50 µL from tube #16. There should now be 50 µL in all 16 tubes.
6. **VORTEX** PTK Red Tracer, 10X.
7. Add 10 µL of the Anti-Phosphotyrosine Antibody, 10X and 10 µL of the PTK Red Tracer, 10X to every tube. Add 30 µL of FP Dilution Buffer to each tube. The tracer, antibody, and buffer can also be pre-mixed, and then added all at once (please see Section 6.4). This step will reduce the concentration of all of the detection components to 1X and reduce the concentration of PTK Competitor in each tube by 2-fold (*i.e.* tube #1 now has a competitor concentration of 5 µM, while tube #16 will have a competitor concentration of approximately 0.153 nM).
8. Mix gently, cover the tubes to reduce evaporation and protect the reagents from light, and incubate for at least 30 minutes at room temperature.
9. Measure the polarization values of your samples, following the procedures required by your FP instrument. We recommend that you use 100 µL FP Dilution Buffer as your blank and a 1:10 dilution of the Red Polarization Standard (Panvera Part No. P2889, which is found in the Red (FP) Standardization Kit, Panvera Part No. P2888) as your low polarization standard, if necessary. You may also want to have controls for the antibody/tracer mixture without competitor and for the tracer alone to determine the high and low polarization values for the assay itself. See Section 6.3 for recommended controls.
10. Perform non-linear regression on a semi-log plot of the data (mP vs. log [Competitor]). The IC<sub>50</sub> of the competitor control should be between 5 and 20 nM. Sample data (n = 4) appear at the end of Section 6.4.

### 6.3 Standard Curve Controls

To demonstrate the minimum polarization value of this detection system under the standard curve conditions, add 10  $\mu$ L of the PTK Red Tracer, 10X to 90  $\mu$ L of the FP Dilution Buffer in a single microplate well or borosilicate tube. The minimum polarization value represents conditions in which the tracer has been completely displaced from the antibody (*i.e.* total phosphorylation of the substrate by a kinase). To demonstrate the maximum polarization value of this detection system under the standard curve conditions, add 10  $\mu$ L of the PTK Red Tracer, 10X and 10  $\mu$ L of the Anti-Phosphotyrosine Antibody, 10X in 80  $\mu$ L of the FP Dilution Buffer. The high polarization value represents conditions in which the tracer is completely bound by the antibody (*i.e.* no phosphorylated substrate has been generated by a kinase that can compete for binding sites and displace the tracer from the antibody).

### 6.4 Pre-mixing Antibody and Tracer

In Sections 6.1 and 6.2, antibody and tracer were added individually to the FP Dilution Buffer to generate the standard curve. To reduce the number of pipetting/dispensing steps, the antibody and tracer can also be pre-mixed and added in a single step. To pre-mix the antibody and tracer, add 10  $\mu$ L of the Anti-Phosphotyrosine Antibody, 10X, 10  $\mu$ L of PTK Red Tracer, 10X, and 30  $\mu$ L of FP Dilution Buffer per well or tube requiring antibody and tracer in your experiment. The total volume of this 2X complex should be equivalent to 50  $\mu$ L multiplied by the total number of wells requiring antibody and tracer. The addition of 50  $\mu$ L of this 2X complex to the 50  $\mu$ L of FP Dilution Buffer or kinase assay mix already in the tubes or wells generates the final, 1X working complex.



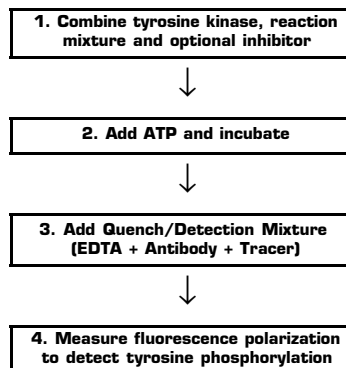
## 7.0 Protein Tyrosine Kinase Assay

### 7.1 Tyrosine Kinase Assay Outline

Each complete reaction must contain an appropriate buffer system, enzyme, ATP, substrate, PTK Red Tracer, and Anti-Phosphotyrosine Antibody. EDTA is then used to quench kinase activity.

There are two modes of analysis for this assay: kinetic and end-point. The kinetic mode is useful for rapidly optimizing enzyme concentration, reaction time, substrate concentration, and buffer conditions. The end-point assay mode is useful when screening kinase inhibitors or activators.

In the end-point mode (Section 7.4), four general steps are required. First, the reaction mixture, including the substrate and optional inhibitor, is combined. ATP is then added to start the reaction. Following an incubation, a quench/detection mixture containing EDTA, Anti-Phosphotyrosine Antibody and tracer is added to stop the kinase reaction and initiate the competition for antibody binding. Finally, polarization values are measured to determine the extent of tyrosine phosphorylation. If a kinetic experiment is performed (Section 7.3), the components of Steps 1 and 3 (without EDTA) are combined first and then Steps 2 and 4 are executed simultaneously.



**Note:** The following protocol is configured for use with 96-well plates and a multiwell FP instrument. However, the protocol can also be performed in 384- and 1536-well plates. If you are using a single-tube FP instrument such as the Beacon® 2000 Instrument, use borosilicate tubes. The antibody contained in this kit is highly specific for phosphotyrosine and therefore its binding affinity is salt-dependent. Salt from the enzyme preparation and substrates (*i.e.* ATP and poly(Glu, Tyr) 4:1) should be minimized as it may reduce the dynamic range of the assay. We recommend titrating ATP and substrate, such as poly(Glu, Tyr) 4:1, to determine the optimal concentration for use with the particular kinase of interest. Concentrations of ATP and poly(Glu, Tyr) 4:1 exceeding 4 mM and 50  $\mu$ g/L, respectively will reduce the dynamic range of the assay to less than 85% of the maximum. If the enzyme of interest has been eluted from a salt column, use a control with the same salt concentration to test any effects on the detection mixture.

## 7.2 Experimental Controls

To demonstrate the minimum polarization value of this detection system under the conditions of your reaction, **DO NOT** add the Anti-Phosphotyrosine Antibody to one reaction as described in Step 2 of Section 7.3 or Step 1 of Section 7.4.2. Substitute this volume with FP Dilution Buffer. This is referred to as the “No Antibody” control throughout the remainder of this protocol. The “No Antibody” control will indicate the minimum polarization value of the reaction system, which simulates a kinase reaction that has completely displaced the phosphopeptide tracer from the Anti-Phosphotyrosine Antibody. If a time-course experiment was performed, the polarization value should decrease with time. If tyrosine kinase inhibitors were titrated into the reaction, the polarization value should remain high as the kinase inhibitor concentration is increased, while low or zero concentrations of PTK inhibitor will have low polarization values, indicating that the PTK reaction was able to proceed largely (or totally) uninhibited.

To demonstrate the maximum polarization value of these reagents under the conditions of your reaction, add to at least one tube or well, all of your kinase reaction and detection components, *except the ATP*, which is added in Step 3 of Section 7.3 or Step 2 of Section 7.4.1. Substitute this volume with FP Dilution Buffer and perform the rest of the experiment as described. This sample is referred to as the “No ATP” control throughout the remainder of this protocol. The “No ATP” control should have a high polarization value because no phosphopeptide was generated to compete with the phosphopeptide tracer for binding to the antibody.

If kinase inhibitors are to be tested or screened, we recommend that you initially serially dilute the inhibitors in 100% DMSO or your preferred solvent. Perform a 10-fold dilution of the inhibitors once from 100X (in 100% DMSO) to 10X (in 10% DMSO) using kinase reaction buffer as the diluent. A 10-fold dilution of the 10X inhibitor into the PTK reaction(s) will result in a final concentration of 1X (in 1% DMSO). Include a “zero” inhibitor sample (“vehicle” or buffer control) containing the appropriate amount of DMSO or another solvent to measure its effect on the PTK reaction.

## 7.3 Kinetic Assay

In the kinetic assay, all of the reaction and detection reagents (FP Dilution Buffer, ATP, substrate, Anti-Phosphotyrosine Antibody, and the PTK Red phosphopeptide tracer) are present during the reaction phase of the assay. Phosphorylation can be monitored in real-time as the reaction proceeds; the polarization value of the sample will be reduced as phosphotyrosine-containing peptides are generated and compete with the tracer for binding to the antibody.

1. Set up a PTK reaction without ATP in a volume of less than 50  $\mu$ L. Remember that the final volume of 100  $\mu$ L will include 50  $\mu$ L of detection reagents (Section 7.3, Step 2) and ATP (Section 7.3, Step 3). A typical reaction will require a protein tyrosine kinase, an appropriate buffer system, peptide substrate, and ATP (which is added last to initiate the reaction). Sample conditions for the EGF receptor are 20 mM HEPES (pH 7.4), 5 mM  $MgCl_2$ , 2 mM  $MnCl_2$ , 50  $\mu$ M  $Na_3VO_4$ , 1 nM EGF Receptor, 10  $\mu$ M ATP, and 2 ng/ $\mu$ L poly(Glu, Tyr) 4:1.
2. Once you have dispensed the reactants into a multi-well plate or borosilicate tube, add 10  $\mu$ L of the Anti-Phosphotyrosine Antibody, 10X, and 10  $\mu$ L of the PTK Red Tracer, 10X (**VORTEXED**), to each sample. Add 30  $\mu$ L FP Dilution Buffer to each well (the tracer, antibody, and buffer can also be pre-mixed, then added all at once. Please see Section 6.4). Preincubate each sample at your desired reaction temperature.
3. Add an ATP solution of the appropriate concentration to each sample to start the reaction. The final volume should be brought to 100  $\mu$ L with water. A “No ATP” control (substitute water or buffer for ATP) should be included in every experiment.
4. Quickly transfer your microwell plate or borosilicate tube to your FP instrument (operating at your preferred reaction temperature) and begin reading the fluorescence polarization value(s) of your sample(s); continue to measure the polarization value(s) of your sample(s) as long as necessary.

## 7.4 End-point Assay

The end-point PTK assay is divided into two phases: the reaction phase and the detection phase. The reaction phase is performed almost exactly as a traditional radioactive kinase assay, except that no radioactive label is required.

For the end-point assay, a kinase reaction mix is prepared in a volume of less than 50  $\mu$ L, so that the final reaction volume (including ATP and Kinase Quench Buffer) will equal 50  $\mu$ L. The reaction is started by the addition of ATP, incubated for the desired length of time, then quenched with EDTA, bringing the final volume of the mixture to 50  $\mu$ L. Then, 10  $\mu$ L each of the Anti-Phosphotyrosine Antibody, 10X, and PTK Red Tracer, 10X (**VORTEXED**), and 30  $\mu$ L of FP Dilution Buffer are added to each well (the tracer, antibody, and buffer can also be pre-mixed, then added all at once. Please see Section 6.4). This addition reduces the concentration of each component of the kit to its final 1X concentration. The polarization values are then read and the data analyzed.

It should be noted that the reaction can be run in any volume, keeping in mind that the quenched kinase reaction will be diluted with the addition of antibody and tracer. The final volume must be less than the maximum volume of the microplate well you are using.



## 7.4.1 Reaction Phase

1. In a volume of less than 50  $\mu$ L, set-up a PTK reaction without ATP. A typical reaction will require a protein tyrosine kinase, an appropriate buffer system, peptide substrate, and ATP. Sample conditions for the EGF receptor are 20 mM HEPES (pH 7.4), 5 mM  $MgCl_2$ , 2 mM  $MnCl_2$ , 50  $\mu$ M  $Na_3VO_4$ , 1 nM EGF Receptor, 10  $\mu$ M ATP, and 2 ng/ $\mu$ L poly(Glu, Tyr) 4:1.
2. Once you have dispensed the reaction(s) into a multi-well plate or borosilicate tube, add ATP (at the appropriate concentration) to each well to start the reaction. The total volume should now be 50  $\mu$ L. A "No ATP" control (substitute water or buffer for ATP) should be included in every experiment.
3. Incubate at your preferred reaction temperature. Incubation time will vary depending on the amount of kinase used, as well as the turnover rate of your kinase. We recommend running an initial time-point experiment to determine the optimal incubation length.
4. Quench the kinase reaction(s) at the end of the incubation. We recommend that you add EDTA to a final concentration of 6 mM and bring the final volume of each quenched PTK reaction to 50  $\mu$ L. After the addition of EDTA, mix and incubate for 5 minutes at room temperature. You may use more or less EDTA (or another quenching reagent) for your specific kinase. Under the sample reaction conditions described in this protocol, EDTA at a final concentration greater than 12 mM is known to interfere with the Anti-Phosphotyrosine Antibody/phosphopeptide binding.
5. Proceed to the Detection Phase of the experiment in Section 7.4.2.

## 7.4.2 Detection Phase

1. After quenching the PTK reaction, add 10  $\mu$ L of the Anti-Phosphotyrosine Antibody, 10X and 10  $\mu$ L of the PTK Red Tracer, 10X (**VORTEXED**), and 30  $\mu$ L of FP Dilution Buffer to each well. (The tracer, antibody, and buffer can also be pre-mixed, then added all at once. Please see Section 6.4).
2. Cover the wells or tubes to reduce evaporation and protect the reagents from light, then incubate for 30 minutes at room temperature. Fluorescence polarization values can be measured for up to 48 hours.
3. Measure the fluorescence polarization value of each reaction following the procedures required by your FP instrument. We recommend that you use 100  $\mu$ L FP Dilution Buffer as your blank and a 1:10 dilution of the Red (FP) Standardization Kit (PanVera Part No. P2888) for your low polarization standard, if necessary.

## 8.0 REFERENCES

1. Hunter, T. (1995) *Cell* **80**:225-36.
2. Pawson, T. (1995) *Nature* **373**:573-580.
3. Checovich, W.C., Bolger R.E. and Burke, T. (1995) *Nature* **375**:254-6.
4. Jameson, D.M. and Sawyer, W.H. (1995) *Meth. Enzymol.* **246**:283-300.

\*United States and International Patents Pending

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