SER/THR KINASE ASSAY KIT*, CROSSTIDE, RED OR GREEN PROTOCOL

Part # P3101 or P3103

762-038343 121803

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1.0 INTRODUCTION

Phosphorylation of proteins is critical to the regulation of many biological mechanisms, including cell growth, apoptosis, and differentiation (1). Kinases are often grouped by their ability to recognize common sequence motifs within protein substrates, therefore, assays using substrates which are based on a common motif can sometimes be used to measure the activity of multiple kinases. Crosstide is a synthetic peptide corresponding to the sequence surrounding serine 9 on Glycogen Synthase Kinase-3β (GSK-3β) and serine 21 on Glycogen Synthase Kinase-3α (GSK-3α). Because the Crosstide motif is phosphorylated by many serine/threonine kinases, PanVera®'s Crosstide assay allows the researcher to study many kinases, including, but not limited to Akt1, Akt2, Akt3, MSK1 and PKA, using a single assay format. The assay is simple, sensitive, non-radioactive, scaleable, homogeneous and formatted for high-throughput screening. PanVera®'s Crosstide assay kit contains all the reagents (except for the kinase, substrate, ATP, and kinase reaction buffer) necessary to analyze kinase activity via Crosstide phosphorylation using fluorescence polarization (FP) as the detection method. This kit contains reagent volumes sufficient for eight-hundred 20 µL assays (Invitrogen Part No. P3101 Red or P3103 Green), and is formatted for use with a multi-well plate instrument capable of measuring FP in 96-, 384-, or 1536-well plates.

2.0 ASSAY THEORY

Fluorescence polarization (FP) measures how quickly a fluorophore rotates during its fluorescence lifetime (the period after it has been excited by plane-polarized light, but before it emits light). Small molecules tumble quickly in solution, so emit relatively depolarized light, but larger molecules and complexes tumble more slowly, and emit more highly polarized light. Therefore, small molecules yield low polarization values and large molecules yield high polarization values (**Figure 1**).

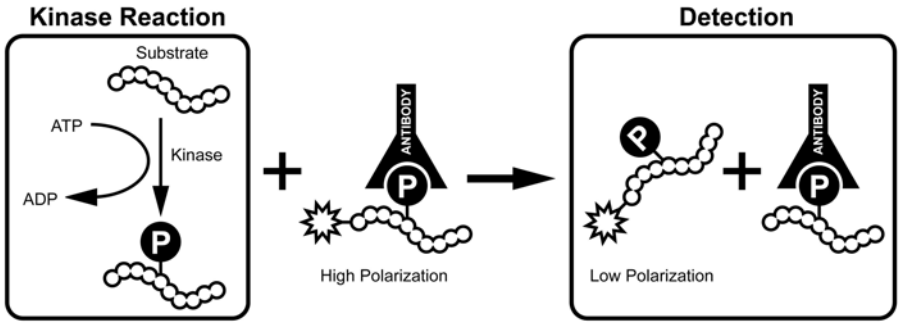


Figure 1. Schematic Illustration of an FP-based kinase assay.

In this assay, a small fluorescently labeled phosphopeptide tracer and a phospho-specific antibody (a relatively large molecule) are added to a kinase reaction. Initially, the phosphopeptide tracer binds to the antibody, forming a large complex and yielding a high polarization value. The kinase reaction phosphorylates the substrate, however, to produce an unlabeled phosphopeptide that displaces the phosphopeptide tracer and itself binds to the antibody. The released phosphopeptide tracer yields a low polarization value; the more active the kinase, the more phosphopeptide tracer is displaced and the lower the polarization value. Kinase activity can be detected and measured by monitoring the decrease in polarization value during the assay.

3.0 KIT COMPONENTS

3.1 Materials Provided

Description	Composition	Red - P3101		Green - P3103	
		Size	Part #	Size	Part #
Crosstide Antibody, 10X	Anti-phosphoserine peptide-specific antibody in Proprietary Buffer (pH 7.5)	2 mL	P3105	2 mL	P3105
Crosstide Red Tracer, 10X	Rhodamine-labeled phosphopeptide in Proprietary Buffer (pH 7.5)	2 mL	P3102	--	--
Crosstide Green Tracer, 10X	Fluorescein-labeled phosphopeptide in Proprietary Buffer (pH 7.5)	--	--	2 mL	P3104
Crosstide Competitor	5 µM phosphopeptide in Proprietary Buffer (pH 7.5)	100 µL	P3108	100 µL	P3108
Kinase Quench Buffer	500 mM EDTA (pH 8.0)	1 mL	P2825	1 mL	P2825
FP Dilution Buffer	Proprietary Buffer (pH 7.5)	15 mL	P3106	15 mL	P3106

3.2 Materials Available Separately

- Crosstide Substrate, 1 mM - Non-phosphorylated (GRPRTSSFAEG) Invitrogen Part No. P3107, 200 µL

3.3 Materials Required but not Supplied

- Kinase
- ATP
- Kinase Reaction Buffer
- Multiwell plates suitable for fluorescence polarization
- Multiwell fluorescence polarization instrument with suitable interference filters (**Green:** 485 nm excitation and 530 nm emission; **Red:** 535 nm excitation, 590 nm emission)
- Pipeting devices
- Reagent reservoir(s)
- Laboratory timer
- FP One-Step Reference Kit (Invitrogen Part No. P3088). This kit is recommended to validate instrument performance and may be required as a polarization reference to calibrate certain instruments.

4.0 STORAGE AND STABILITY

Description	Storage Temperature	Notes
Crosstide Antibody, 10X	-20°C	Minimize repeated freeze/thaw cycles
Crosstide Red Tracer, 10X	-20°C	Minimize repeated freeze/thaw cycles; Vortex the Tracer before use
Crosstide Green Tracer, 10X	-20°C	Minimize repeated freeze/thaw cycles; Vortex the Tracer before use
Crosstide Competitor	-20°C	Minimize repeated freeze/thaw cycles
Kinase Quench Buffer	20–30°C	Thaw upon receipt
FP Dilution Buffer	20–30°C	Thaw upon receipt

All reagents are stable for 6 months from the date of receipt.

5.0 DETERMINING THE IC_{50} FOR THE CROSSTIDE 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 one can expect during an enzymatic reaction.

We recommend that you also generate a standard curve for the serine-phosphorylated form of the peptide substrate you intend to use for your enzyme reaction. You may be able to use a longer sequence substrate than that recommended for use in the kit; however, the substrate must contain the core sequence GRPRTSSFAEG.

5.1 Multiwell Plate Format

1. In a multiwell plate suitable for fluorescence polarization, pipette 20 µL of Crosstide Competitor (P3108 - **VORTEXED**) into well A1.
2. Dispense 10 µL FP Dilution Buffer (P3106) into wells A2 to A18.
3. Perform a 2-fold serial dilution of the Crosstide Competitor by transferring and mixing 10 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 A18 is reached.
5. Discard 10 µL from well A18. There should be 10 µL in every well from A1 to A18.
6. **VORTEX** Crosstide Tracer, 10X (P3102 or P3104).
7. Add 2 µL of the Crosstide Tracer, 10X (P3102 or P3104) and 2 µL of the Crosstide Antibody, 10X (P3105) to every well from A1 to A18. Add 6 µL of FP Dilution Buffer (P3106) to each well. This step will reduce the concentration of all of the detection components to 1X and reduce the concentration of the Competitor in each well by 2-fold (*i.e.,* well A1 now has a competitor concentration of 2.5 µM, while A18 has a competitor concentration of approximately 0.02 nM).

   Note: **Premixing the tracer and antibody is not recommended** due to slow equilibration (> 4 hours Red Assay, overnight Green Assay) between the phosphorylated-peptide product and the premixed Tracer:Antibody complex.

8. Mix, cover the multi-well plate to reduce evaporation and protect the reagents from light, and incubate **Red Assays for 1 hour** at room temperature and **Green Assays for 4 hours** at room temperature to reach equilibrium.
9. Measure the polarization values of your samples, following the procedures required by your FP instrument. We recommend that you use 20 µL FP Dilution Buffer (P3106) as your blank and the One-Step (FP) Reference Kit (Invitrogen Part No. P3088) for your low and high polarization references, if necessary. We recommend that you also run controls for the antibody/tracer mixture (without competitor) and for the tracer alone to determine the respective high and low polarization values for the assay itself. See **Section 6.2**.
10. Perform non-linear regression on a semi-log plot of the data (mP vs. [Competitor]). The representative IC_{50} value of the competitor control is generally less than 40 nM. Sample data (n = 4) generated on a TECAN Ultra (Research Triangle Park, NC) appear in **Section 6.3**.

5.2 Standard Curve Controls

To demonstrate the **maximum polarization value** of this detection system under the standard curve conditions, add 2 µL of the Crosstide Tracer, 10X and 2 µL of the Crosstide Antibody, 10X in 16 µL 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). To demonstrate the **minimum polarization value** of this detection system under the standard curve conditions, add 2 µL of the Crosstide Tracer, 10X to 18 µL of the FP Dilution Buffer in a single well of a multi-well plate. The minimum polarization value represents conditions in which the tracer has been completely displaced from the antibody.

5.3 Results and Discussion

The results of a competition curve for both the Crosstide Green and Red Assays are shown in **Figure 2**. The IC_{50} for the Crosstide competitor was 4 nM (n = 4) in the Red Kit (incubation time = 1 hr) and 2 nM (n = 4) in the Green kit (incubation time = 4 hrs).

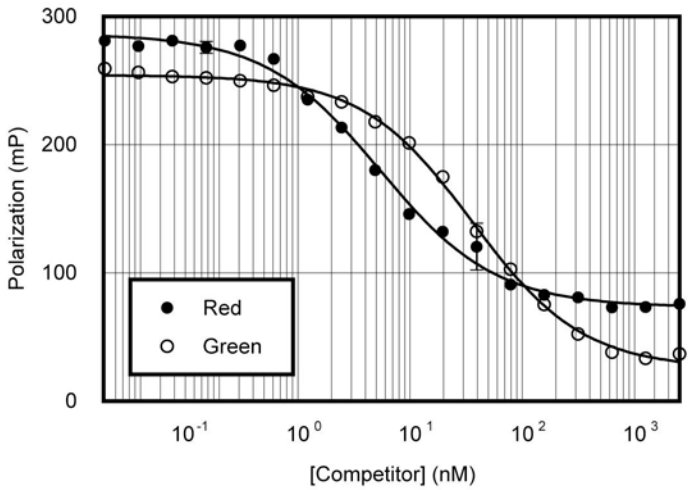


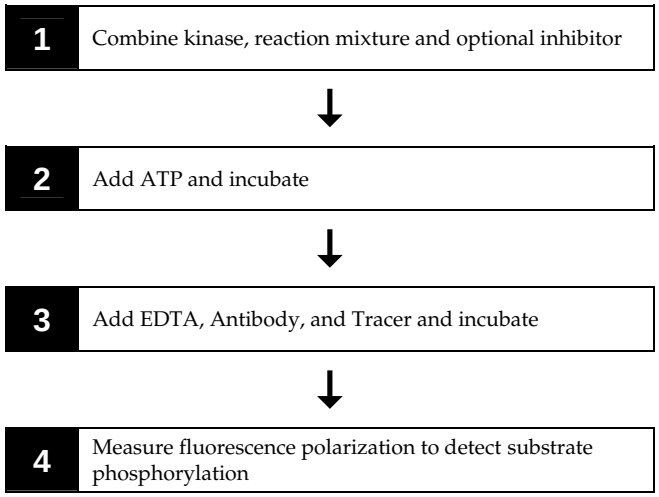
Figure 2. Crosstide Red and Green Assays Competition Curves (n = 4).

6.0 SERINE/THREONINE KINASE ASSAY, CROSSTIDE

6.1 Kinase Assay Outline

Each complete Serine/Threonine Kinase Assay reaction must contain an appropriate buffer system, enzyme, ATP, substrate, Tracer and Antibody. EDTA is then used to quench kinase activity.

For an end-point mode kinase reaction, four general steps are required. First, the reaction mixture, substrate (Invitrogen offers Crosstide Substrate, 1 mM Part No. P3107) and optional inhibitor(s), are combined. ATP is then added to start the reaction. After the desired incubation time, the kinase reaction can be stopped with EDTA (Kinase Quench Buffer). Crosstide Antibody and the Crosstide Tracer should then be added **separately** to initiate the competition for antibody binding. Finally, polarization values are measured to determine the extent of Crosstide phosphorylation.



Note: The following protocol is configured for use with 384-well plates and a multi-well FP instrument. Assay volumes may be reduced however this would require further optimization by the customer.

6.2 Experimental Controls

To demonstrate the **minimum polarization value** of this detection system under the conditions of your reaction, do not add the Antibody to one reaction as described in Step 2 of **Section 6.3.2**. 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 fluorescent phosphopeptide tracer from the anti-phosphoserine peptide-specific antibody. If a time-course experiment is performed, the polarization value should decrease with time.

To demonstrate the **maximum polarization value** of this detection system under the conditions of your reaction, set up one tube or well with all of your kinase reaction and detection components, except the ATP, which is added in Step 2 of **Section 6.3.1**. Substitute this volume with FP Dilution Buffer. 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 since no competitor phosphopeptide was produced during the enzyme reaction to displace the phosphopeptide tracer from the antibody.

If kinase inhibitors are to be tested or screened, we recommend that you initially serially dilute the inhibitors in 100% DMSO or another 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, followed by another 10-fold dilution into the reaction(s), producing 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 kinase reaction. If kinase inhibitors were titrated into the reaction, the polarization value should remain high as the kinase inhibitor concentration is increased. Low or zero concentrations of kinase inhibitor will have low polarization values, indicating that the reaction was able to proceed largely (or totally) uninhibited.

We recommend that you initially test several time points to determine the optimal incubation time for your enzyme and assay system. However, we have found that an incubation of at least 90 minutes at room temperature is sufficient to achieve a complete reaction when using nanogram or picogram amounts of most enzymes.

Through optimization, you may find that higher or lower concentrations of EDTA (or another quenching reagent entirely) may be required for your specific enzyme.

6.3 End-point Assay

The end-point assay is divided into two phases: the reaction phase and the detection phase. The reaction phase is performed similar to a traditional radioactive kinase assay, except that no radioactive label is required. The concentrations of substrate, ATP and enzyme required should be determined experimentally.

For the end-point assay, a kinase reaction mix is prepared in a volume of less than 10 µL, so that the final reaction volume (including ATP and inhibitors) will equal 10 µL. The reaction is started by the addition of ATP and incubated for the desired length of time. The reaction is then quenched with EDTA. Add to each well 2 µL of Crosstide Tracer, 10X (**VORTEXED**), then 2 µL of Crosstide Antibody, 10X, and FP Dilution Buffer (to bring the final total volume to 20 µL). 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.

6.3.1 Reaction Phase

1. In a volume of less than 10 µL, set-up a reaction without ATP. A typical reaction will require a protein kinase, an appropriate buffer system and peptide substrate. Optionally, inhibitor or test compound will be included in this step.
2. Once you have dispensed the reaction mixture into a multi-well plate, add an appropriate amount of ATP to each well to start the reaction. The total volume should now be 10 µL. Please note that a "No ATP" control (substitute water or buffer for ATP) should be included in every experiment.
3. Cover and 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 have found that an incubation of at least 90 minutes at room temperature is sufficient to achieve a complete reaction when using nanogram or picogram amounts of most enzymes.

6.3.2 Quench/Detection Phase

1. Quench the kinase reaction(s) at the end of the incubation. We recommend that you add EDTA to a final concentration of 15 mM. Add 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.

   Note: EDTA at a concentration greater than 20 mM is known to interfere with the antibody/phosphopeptide binding.

2. Add 2 µL of Crosstide Tracer, 10X (**VORTEXED**) to each well.
3. Add 2 µL of Crosstide Antibody, 10X to each well.

   Note: **Premixing the tracer and antibody is not recommended** due to slow equilibration (> 4 hours Red Assay, overnight Green Assay) between the phosphorylated-peptide product and the premixed Tracer:Antibody complex.

4. Add FP Dilution Buffer to a final volume of 20 µL.
5. Cover the wells or tubes to reduce evaporation and protect them from light, then incubate **Red Assays for 1 hour** at room temperature and **Green Assays for 4 hours** at room temperature to reach equilibrium.
6. Measure the fluorescence polarization value of each reaction following the procedures required by your FP instrument.

7.0 QUICK START PROTOCOLS

7.1 Competition Curve Quick Start Chart

		Assay Reaction(s)	Maximum Polarization	Minimum Polarization
	Reagents	**Competitor + Antibody + Tracer**	**Antibody + Tracer**	**Free Tracer**
Binding Equilibrium	Competitor [Diluted to desired test concentration(s)]	10 µL		
	Crosstide Antibody, 10X	2 µL	2 µL	
	Crosstide Tracer, 10X	2 µL	2 µL	2 µL
	FP Dilution Buffer	6 µL	16 µL	18 µL

Mix assay plate and incubate the 20-µL Red Assays for 1 hour at room temperature and the 20-µL Green Assays for 4 hours at room temperature to reach equilibrium.

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Read Plate

7.2 Kinase Reaction Quick Start Chart

		Assay Reaction(s)	Maximum Polarization	Minimum Polarization
	Reagents	**Kinase + Test Compound + ATP**	**No ATP**	**No Antibody**
Kinase Reaction	2X Kinase Reaction (Includes Kinase, Substrate, Test Compounds, and Kinase Reaction Buffer)	5 µL	5 µL	5 µL
	2X ATP	5 µL		5 µL
	Water (or buffer used to dilute ATP)		5 µL	

Mix assay plate and incubate the 10-µL Kinase Reaction for 90 minutes at room temperature.

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Quench/Detection Reaction	Kinase Quench Buffer (Dilute your specific Quench Buffer to 10X)	2 µL	2 µL	2 µL
	Crosstide Antibody, 10X	2 µL	2 µL	
	Crosstide Tracer, 10X	2 µL	2 µL	2 µL
	FP Dilution Buffer	4 µL	4 µL	6 µL

Mix assay plate and incubate the 20-µL Red Assays for 1 hour at room temperature and the 20-µL Green Assays for 4 hours at room temperature to reach equilibrium.

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8.0 REFERENCES

1. Hunter, T. (1995) *Cell* **80:**225-36.
2. Cross D.A. *et al.* (1995) *Nature* **378:**785-9.
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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