PolarScreen™ PDK1 Threonine Kinase Assay Kit, Far Red Protocol

Part # PV3346

Lit. # 839-0408650 040804

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1.0 INTRODUCTION

PDK1 (3-Phosphoinositide-Dependent Protein Kinase-1) activates the conventional PKCs and PKCζ (zeta) though phosphorylation at Threonine 500 in the 'activation loop.' PDK1 also phosphorylates Protein Kinase B (PKB/Akt) at Threonine 308 in the presence of phosphatidylinositol-3,4,5-triphosphate. Active PKB/Akt inactivates Glycogen Synthase Kinase-3 (GSK-3), eventually leading to the dephosphorylation and activation of glycogen synthase and the stimulation of glycogen synthesis. Because of the role PDK1 plays in insulin-induced glycogen synthesis and PKC activation, it is potentially an important target for metabolic drug research. Conventional threonine kinase assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera®'s PolarScreen™ PDK1 Threonine Kinase Assay Kit, Far Red assay kit is a major advance because it is simple, sensitive. Non-radioactive, homogeneous, and formatted for high-throughput screening. This assay kit contains all the material necessary to analyze PDK1-like kinase activity via PDK1 phosphorylation using fluorescence polarization (FP). This kit contains reagents to perform eight-hundred 20 µL assays (Part No. PV3346), and is formatted for use with a multi-well plate instrument capable of measuring FP in 384- or 1536-well plates.

2.0 ASSAY THEORY

Fluorescence polarization (FP) measures the rotational freedom of a fluorophore during its excited-state lifetime. When a small fluorescent molecule is excited with polarized light, it is free to tumble in solution before re-emitting a photon. Because of this rotation, the light emitted from the small fluorescent molecule is depolarized. In contrast, a large fluorescent molecule (or a complex of a small fluorescent molecule bound to a larger molecule) does not undergo significant rotation before emitting a photon. Thus, when a large fluorescent molecule or a complex of a small fluorescent molecule bound to a larger molecule is excited with polarized light, it will emit light that is polarized.

The principle of an FP kinase assay is illustrated in Figure 1. In the first stage of the assay, the kinase reaction is allowed to proceed under normal conditions, typically in the presence of an inhibitor or a library compound that is a potential inhibitor. After the reaction is complete, tracer and antibody are added to the reaction mix. The antibody can associate with either the labeled tracer (resulting in a high FP value) or the kinase-produced phosphorylated substrate (resulting in a lower FP value). The amount of antibody that is bound to the tracer is inversely proportional to the amount of phosphorylated substrate present, and in this manner kinase activity can be detected and measured by a decrease in the FP value

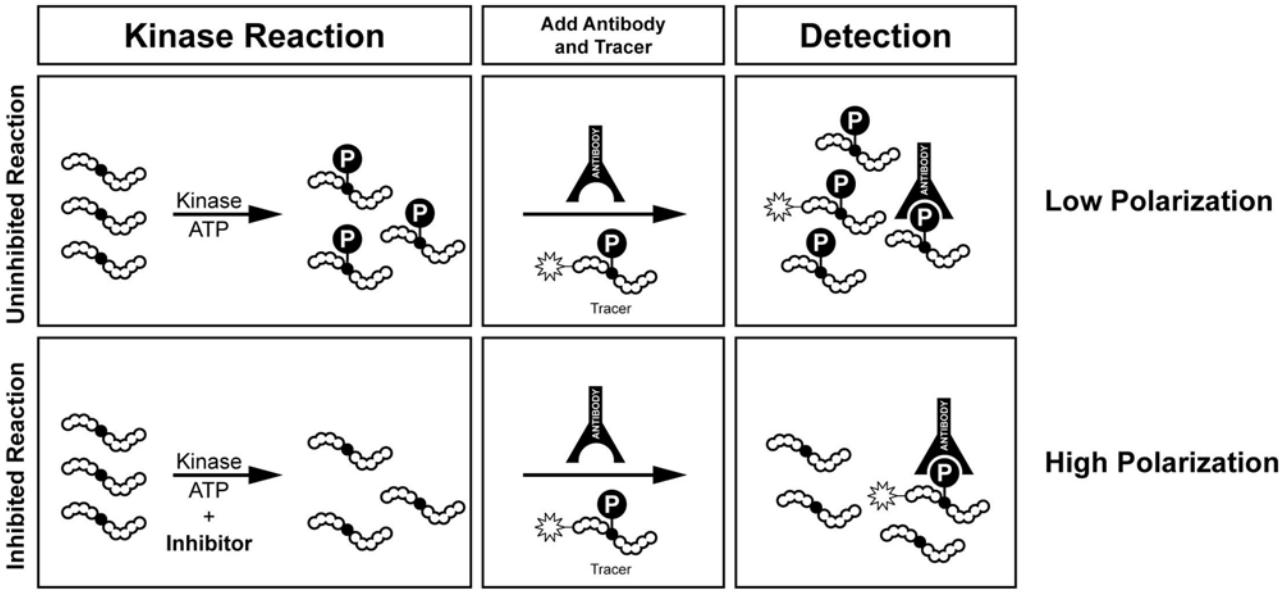


Figure 1. Schematic Illustration of an FP-based kinase assay.

3.0 KIT COMPONENTS

3.1 Materials Provided

Description	Composition	Size	Part #
anti-pThr (PDK1) Antibody, 10X	Anti-phosphothreonine peptide-specific antibody in FP Dilution Buffer (pH 7.5)	1.6 mL	PV3347
PDK1 Far Red Tracer, 10X	Fluorophore-labeled phosphopeptide in FP Dilution Buffer (pH 7.5)	1.6 mL	PV3348
PDK1 Competitor	5 µM phosphopeptide in FP Dilution Buffer (pH 7.5)	500 µL	P2918
Kinase Quench Buffer	500 mM EDTA (pH 8.0)	1 mL	P2825
FP Dilution Buffer	Proprietary Buffer (pH 7.5)	14 mL	PV3330

3.2 Materials Available Separately

- PDK1 Peptide Substrate, 100 µM - Non-phosphorylated (GVTTKTFAGTP) Invitrogen Part No. P2925, 1mL

3.3 Materials Required but not Supplied

- Kinase
- ATP
- Kinase Reaction Buffer
- Multiwell plates suitable for FP
- Multiwell fluorescence instrument with FP capability, with suitable excitation and emission filters (Excitation: 610/20 nm, Emission: 670/40 nm). Filters meeting these specifications are available as part number XF45 from Omega Optical (www.omegafilters.com).
- Pipetting devices
- Reagent reservoir(s)
- Laboratory timer

4.0 STORAGE AND STABILITY

Description	Storage Temp.	Notes
anti-pThr (PDK1) Antibody, 10X	-20°C	Minimize repeated freeze/thaw cycles
PDK1 Far Red Tracer, 10X	-20°C	Minimize repeated freeze/thaw cycles. Vortex prior to use
PDK1 Competitor	-20°C	Minimize repeated freeze/thaw cycles. Vortex prior to use.
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 if stored and handled properly.

5.0 DETERMINING THE IC_{50} FOR THE PDK1 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 phosphorylated form of the peptide or protein substrate that 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 GVTTKTFAGTP.

5.1 Multiwell Plate Format

1. In a multiwell plate suitable for FP measurements (black), pipette 8 µL of PDK1 Competitor (VORTEXED) and 24 µL FP dilution buffer into well A1.
2. Dispense 16 µL FP Dilution Buffer into wells A2 to A18.
3. Perform a 2-fold serial dilution of the PDK1 Competitor by transferring and mixing 16 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 16 µL from well A18. There should be 16 µL in every well from A1 to A18.
6. VORTEX PDK1 Far Red Tracer, 10X.
7. Add 2 µL of the anti-pThr (PDK1) Antibody,10X, followed by 2 µL of the PDK1 Far Red Tracer, 10X, to every well from A1 to A18. This step will reduce the concentration of all of the detection components to 1X and reduce the concentration of the competitor in each well to range from 1 µM (well A1) to 8 pM (well A18) by a series of 2-fold dilutions.

Note: Premixing the tracer and antibody is not recommended due to slow equilibration between the phosphorylated-peptide product and the tracer competing to bind to the antibody.

8. Mix the solutions and cover the plate to reduce evaporation. Protect the plate from light. Incubate for 2 hours at room temperature to reach equilibrium. Note that the plate may be read at earlier timepoints, but that the readings will not reflect equilibrium binding conditions.
9. Following the procedures required by your instrument, read the plate.
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 35 nM. Sample data (n = 4) generated on a TECAN Ultra (Tecan, Research Triangle Park, NC) appear in **Section 5.3**.

5.2 Standard Curve Controls

To demonstrate the **maximum FP value** of this detection system under the standard curve conditions, mix:

- 16 µL FP Dilution Buffer.
- 2 µL PDK1 Far Red Tracer, 10X
- 2 µL anti-pThr (PDK1) Antibody, 10X

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 FP value** of this detection system under the standard curve conditions, mix:

- 6 µL FP Dilution Buffer
- 10 µL PDK1 competitor
- 2 µL anti-pThr (PDK1) Antibody, 10X
- 2 µL PDK1 Far Red Tracer, 10X

The minimum FP value represents conditions in which the tracer has been completely displaced from the antibody (*i.e.,* total phosphorylation of the substrate by a kinase).

5.3 Results and Discussion

The results of a competition curve for the assay are shown in **Figure 2**. The IC_{50} for the PDK1 Competitor was 6 nM (n = 4).

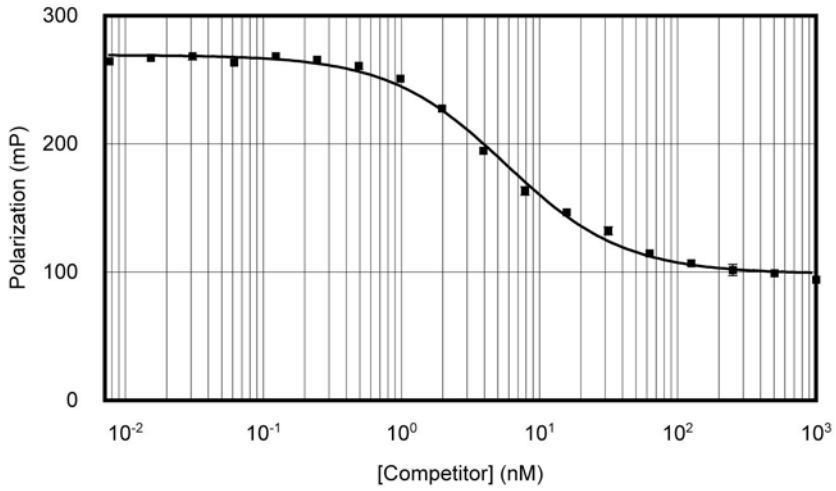


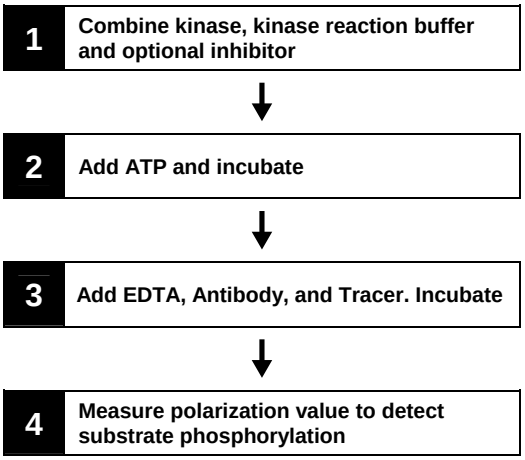
Figure 2. Typical competition curve results. The IC_{50} for the competitor was 6 nM (n = 4).

6.0 FAR-RED FP SERINE/THREONINE KINASE ASSAY, CROSSTIDE

6.1 Kinase Assay Outline

Each complete kinase reaction must contain a kinase reaction buffer, enzyme, ATP and substrate (Invitrogen offers PDK1 Peptide Substrate, 100 µM, Part No. P2925). EDTA is then used to quench kinase activity at the desired time point, and antibody and tracer are then added.

For an end-point mode kinase reaction, four general steps are required. First, the kinase reaction buffer, substrate and optional inhibitor(s), are combined. ATP is then added to start the reaction. After the desired reaction time, the kinase reaction can be stopped with EDTA (Kinase Quench Buffer). Antibody and tracer should then be added **separately** to initiate the competition for antibody binding. Finally, the plate is read and the polarization values are measured to determine the extent of substrate phosphorylation, which is directly related to kinase activity.



Note: The following protocol is configured for use with 384-well plates.

6.2 Experimental Controls

- To demonstrate the maximum polarization value of this detection system under the conditions of your reaction, set up at least one well with all of your kinase reaction and detection components, except the ATP. Substitute this volume with FP Dilution Buffer and any necessary additional matrix components such as DMSO. Perform the rest of the experiment as described. This sample is referred to as the "No ATP" control. The "No ATP" control should have a high polarization value since no competitor phosphopeptide is produced during the enzyme reaction to displace the phosphopeptide tracer from the antibody.
- To demonstrate the minimum polarization value of this detection system under the conditions of your reaction, prepare at least one well as described in **Section 5.2** that contains any additional matrix components such as DMSO. This is referred to as the "minimum polarization" control throughout the remainder of this protocol. The "minimum polarization" 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 DMSO stocks of compounds are to be tested or screened, we recommend a final DMSO concentration of 1% or less. In general, assay plates should include a "zero" inhibitor sample ("vehicle" or buffer control) containing the appropriate amount of DMSO (or other solvent) to measure its effect on the kinase reaction.

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 kinase required should be determined experimentally.

For the end-point assay, a kinase reaction mix is prepared such 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. After quenching with EDTA, 2 µL of anti-pThr (PDK1) Antibody, 10X, 2 µL of PDK1 Far Red Tracer, 10X (**VORTEXED**), and FP Dilution Buffer (to bring the final total volume to 20 µL) are added to each well. This addition reduces the concentration of each component of the kit to its final 1X concentration. The plate is then read and the FP values are measured 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 kinase reaction buffer and peptide substrate.
2. 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.

6.3.2 Quench/Detection Phase

1. Quench the kinase reaction(s) at the end of the incubation. We recommend that you add EDTA at the minimal concentration necessary to stop the kinase reaction. 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.

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

2. After quenching the reaction, add 2 µL of anti-pThr (PDK1) Antibody, 10X and 2 µL of PDK1 Far Red Tracer, 10X (**VORTEXED**) to each well. Add FP Dilution Buffer to a final volume of 20 µL.

Note: Premixing the tracer and antibody is not recommended due to slow equilibration between the phosphorylated-peptide product and the Tracer competing to bind to the antibody.

3. Cover the plate to reduce evaporation and to protect from light, Incubate for 2 hours at room temperature to reach equilibrium.
4. Measure the polarization value of each reaction following the procedures required by your instrument.

7.0 QUICK START PROTOCOLS

Competition Curve Quick Start Chart:

		Assay Reaction(s)	Maximum Polarization Value	Minimum Polarization Value
	Reagents	**Competitor + Antibody + Tracer**	**Antibody + Tracer**	**Antibody + Tracer + Competitor**
Binding Equilibrium	PDK1 Competitor (Diluted to desired test concentration)	16 µL	- -	10 µL (Undiluted)
	anti-pThr (PDK1) Antibody, 10X	2 µL	2 µL	2 µL
	PDK1 Far Red Tracer, 10X	2 µL	2 µL	2 µL
	FP Dilution Buffer	- -	16 µL	6 µL

Mix assay plate and incubate for 2 hours or more at room temperature to reach equilibrium. Plate may be read at earlier time points but values will not represent equilibrium values.

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Read Plate

Kinase Reaction Quick Start Chart:

		Assay Reaction(s)	Maximum FP	Minimum FP
	Reagents	**Kinase + Test Compound + ATP**	**No ATP**	**High Concentration Competitor**
Kinase Reaction	2X Kinase Reaction (Includes Kinase, Substrate, Test Compounds, and Kinase Reaction Buffer)	5 µL	5 µL*	4 µL*
	2X ATP	5 µL	- -	- -
	PDK1 Competitor	- -	- -	10 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	2 µL	2 µL	2 µL
	anti-pThr (PDK1) Antibody, 10X	2 µL	2 µL	2 µL
	PDK1 Far Red Tracer, 10X	2 µL	2 µL	2 µL
	FP Dilution Buffer	4 µL	4 µL	- -

Mix assay plate and incubate for 2 hours or more at room temperature to reach equilibrium. Plate may be read at earlier time points but values will not represent equilibrium values.

↓

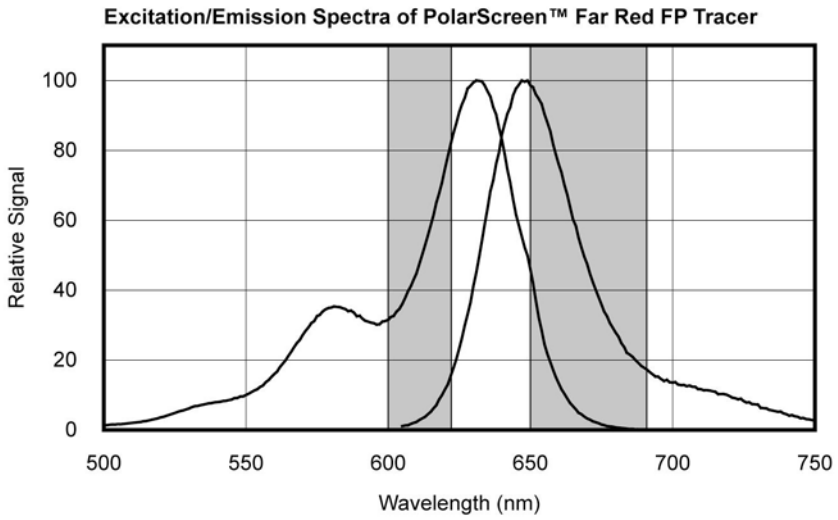
Read Plate

*Should contain appropriate concentrations of matrix components such as DMSO.

8.0 TECHNICAL CONSIDERATIONS

8.1 Filters

The excitation/emission spectra of the tracer used in PanVera®'s PolarScreen™ Ser/Thr Kinase Assay Kit, CREBtide, Far Red assay is shown below. Regions indicating recommended excitation and emission wavelengths and bandpasses (610/20 and 670/40, respectively) are indicated by the shaded bands. Although other filter combinations may perform satisfactorily we have found that this particular combination (available as filter set XF45 from Omega Optical, www.omegafilters.com) gives excellent results.



8.2 Dichroic Mirrors

A dichroic mirror (also known as a dichroic beam splitter) is used to reflect certain wavelengths of light to the sample for excitation, while allowing longer wavelengths of light to pass for detection. Because the dichroic mirror is in the path of both excitation and emission light, its characteristics are chosen such that the excitation light is reflected from the light source to the sample, and the emission light from the sample passes through the dichroic mirror before it is detected. To provide for instrument flexibility, "generic" or "50/50" dichroic mirrors are available which pass half of the excitation light as well as half of the emitted light (while reflecting the other half). The trade-off in using generic dichroic mirrors is a reduction in sensitivity. Although PanVera®'s PolarScreen™ Far-Red FP kinase assays have been tested using a 50/50 mirror instead of a dedicated dichroic, the errors associated with the resulting polarization measurements are increased. Therefore, we recommend the use of a dedicated dichroic mirror for use with the Far-Red fluorophore.

When using the recommended filter set, we recommend a dichroic mirror with a reflection cut-off centered between 625 and 635 nM. Such a dichroic is standard equipment on some plate readers (such as the Tecan Ultra). The instrument manufacturer for your specific instrument will be able to advise you on dichroic options that may be available as "stock" items. Alternatively, Omega Optical (www.omegafilters.com) sells a dichroic mirror meeting these specifications as part number XF2021. Contact your instrument manufacturer for information on installing such a mirror in your instrument.

8.3 Plates

We have optimized and validated this assay at 20 µL total detection volume in black Corning® Low-Volume, non-binding surface, round bottom 384-well plates (Corning® part #3676). Other plates may be used. Transparent plates should not be used. Performance at lower volumes or higher density may require additional optimization by the end-user.

8.4 Gain Settings

We recommend allowing the instrument to automatically determine the optimal gain settings, based on wells that contain free and fully bound tracer, such as would be present in a competition curve generated in section 5. Gain settings determined under such conditions may be fixed for subsequent assays performed in the same volume in the same type of plate.

8.5 Other Instrument Settings

When performing fluorescence polarization measurements, most instruments require the specification of a "G factor" (sometimes referred to as a "K factor"). This is a correction factor that is applied to the raw data such that the determined polarization value for a reference sample is set to a specific value. Polarization values for sample wells are then determined using this same correction factor. If a G-factor must be specified, you may set the G-factor using a well containing "1x" far-red tracer, with a reference polarization value arbitrarily set to 50. Alternatively, the G-factor can be set to "1". In this case the resulting polarization values will be different, but the relative differences between free and bound tracer will be identical.

Other instrument settings should be set according to your instrument manufacturer's recommendations. Aside from filter and dichroic settings, other instrument settings will likely remain the same as one would use with common fluorescein-based polarization measurements.

9.0 PURCHASER NOTIFICATION

PURCHASER SHALL USE THE PRODUCTS PURCHASED HEREUNDER (the "Products") SOLELY FOR THE PURPOSE OF CONDUCTING INTERNAL RESEARCH AT ITS ORGANIZATION ("RESEARCH"). Purchaser will not sell, transfer, disclose or otherwise provide access to the Products to any third party. Purchaser agrees that Purchaser shall not have the right to authorize any third party to use or sell any Products or derivatives thereof. Unless a license has been executed between Purchaser and Invitrogen Corporation explicitly providing otherwise, Purchaser will not: (i) reformulate or create derivatives or the Products; or (ii) use the Products for providing services to a third party (e.g. screening or profiling); (iii) use the Products in a diagnostic process; (iv) use the Products in a quality control or quality assurance process for the manufacture of a product for sale; or (v) use the Products as a component of a kit.