CoreHTS® PROTEIN KINASE C ASSAY KITS PROTOCOL*

Part # P2747, P2748, P2940, P2941

Lit. # L0827 Rev. 05/02

Note: This protocol replaces L0098 (PanVera® Part No. P2747), L0099 (PanVera® Part No. P2748) and L0609 (PanVera® Part No. P2940 and P2941). The kit components (with the exception of the Anti-phosphoserine Antibody included with the PKC Red Tracer) and basic procedures have not been altered.

KIT COMPONENTS

Description	Composition	P2747		P2748		P2940		P2941	
		Amount	Part #	Amount	Part #	Amount	Part #	Amount	Part #
Anti-Phosphoserine Antibody, 4X	Anti-phosphoserine-containing peptide antibody in BGG/Phosphate buffer (pH 7.4) with 0.02% NaN_3	2.5 mL	P2749	25 mL	P2752	2.5 mL	P2749	25 mL	P2752
PKC Green Tracer, 50X	Fluorescein-labeled phosphopeptide tracer in BGG/Phosphate buffer (pH 7.4) with 0.02% NaN_3	200 µL	P2756	2 mL	P2757	--	--	--	--
PKC Red Tracer, 50X	Rhodamine derivative-labeled phosphopeptide tracer in BGG/Phosphate buffer (pH 7.4) with 0.02% NaN_3	--	--	--	--	200 µL	P3002	2 mL	P3003
PKC Competitor, 10 µM	10 µM phosphopeptide in water	100 µL	P2750	100 µL	P2750	100 µL	P2750	100 µL	P2750
PKC Standard Curve Dilution Buffer	BGG/Phosphate buffer (pH 7.4) with 0.02% NaN_3	8 mL	P2751	50 mL	P2946	8 mL	P2751	50 mL	P2946
Kinase Quench Buffer	500 mM EDTA (pH 8.0)	1.0 mL	P2825	10 mL	P2832	1.0 mL	P2825	10 mL	P2832

STORAGE AND STABILITY

Description	Storage Temperature	Notes	Part #
Anti-Phosphoserine Antibody, 4X	–20˚C	Once thawed, should be stored at +4˚C	P2749, P2752
PKC Green Tracer, 50X	–20˚C	Thaw when needed and then refreeze. **Vortex before use.**	P2756, P2757
PKC Red Tracer, 50X	–20˚C	Thaw when needed and then refreeze. **Vortex before use.**	P3002, P3003
PKC Competitor, 10 µM	–20˚C	Thaw when needed and then refreeze.	P2750
PKC Standard Curve Dilution Buffer	20–30˚C	Thaw upon receipt.	P2751
Kinase Quench Buffer	20–30˚C	Thaw upon receipt.	P2825, P2832

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

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, as yet, have been thoroughly investigated. We recommend that you use gloves, lab coats, eye protection and a fume hood when working with any chemical reagents.

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1.0 INTRODUCTION

The phosphorylation of serine and threonine residues in proteins by Protein Kinase C (PKC) family members is critical to the normal regulation of many biological mechanisms, including the modulation of membrane structure and cytoskeletal reorganization, receptor desensitization, transcriptional control, cell growth and differentiation, and mediation of immune response. PKCs also play a role in memory, learning, and long-term potentiation. The PKCs influence cellular events via their activation by second messenger pathways that involve the production of diacylglycerol (1, 2). The *in vivo* regulation of PKC family members involves a combination of the subcellular location of the enzyme(s) and their substrate(s). Identification of specific functions of the different isoforms is dependent on the development of isoform-specific inhibitors (3, 4).

The substrate used in this kit contains the R-X-X-S/T consensus motif, which is recognized by a number of serine/threonine kinases. The activity of PKA, GSK3β, and PKB has been detected using this assay. Other serine/threonine kinases whose substrates contain a similar serine or threonine consensus motif or the motif R-X-S/T may also have measurable activity in this assay.

Conventional PKC assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera®'s PKC Assay Kit is a major advance, because it is simple, sensitive, non-radioactive, and homogenous, and formatted for high throughput screening using fluorescence polarization (FP) as the detection method. These kits contain reagents sufficient for 100 (PanVera® Part No. P2747 or P2940) or 1,000 (PanVera® Part No. P2748 or P2941) high-throughput PKC assays. The PKC isoforms, substrate and buffers required to run PKC assays are not supplied in the kit (see **Section 7.0** and **8.0**). These kits are formatted for use with instruments capable of measuring FP in 96-, 384- and 1536-well plates. The following PKC isoforms, available from PanVera®, have been successfully tested with this kit: PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCζ, PKCη and PKCθ (alpha, betaI, betaII, gamma, delta, epsilon, zeta, eta and theta, respectively).

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 (5, 6).

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/fpguide/index.html

2.2 Assay Theory

FP technology can be exploited in a PKC assay because fluorescein-labeled or rhodamine derivative-labeled phosphopeptides (the Tracer) and any unlabeled phosphopeptides or phosphoproteins generated during a kinase reaction will compete with each other for binding to Anti-phosphoserine Antibody (**Figure 1**). When there are no kinase reaction products present, a significant portion of the tracer will be bound by the Antibody, resulting in a high polarization value. However, after an enzyme reaction has occurred, the Antibody will bind to both the reaction products and the Tracer, decreasing the amount of bound Tracer and thus decreasing the fluorescence polarization of the sample. If enough phosphopeptide is generated during the reaction, the fluorescent Tracer can be completely displaced from the Antibody and the emitted light will be completely depolarized. Thus, the change in FP is directly related to kinase activity.

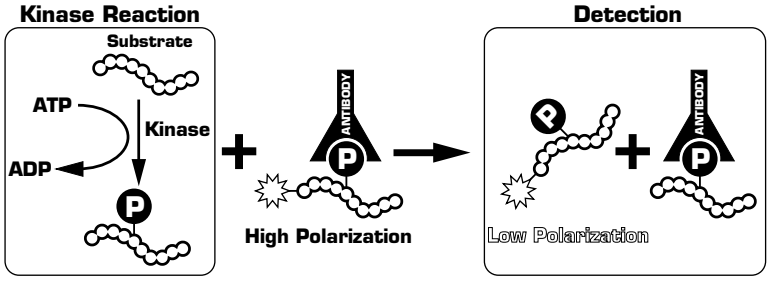


Figure 1. Principle of the Fluorescence polarization-based Protein Kinase C assay.

4.0 MATERIALS

4.1 Materials Available Separately from PanVera

- Peptide Substrate, 100 µM (RFARKGSLRQKNV), PanVera® Part No. P2760
- Protein Kinase C isoform(s). PanVera® currently supplies the following PKC isoforms:

Isoform	Quantity	
	5 µg	20 µg
PKCα	P2232	P2227
PKCβI	P2291	P2281
PKCβII	P2254	P2251
PKCγ	P2233	P2228
PKCδ	P2293	P2287
PKCε	P2292	P2282
PKCζ	P2273	P2268
PKCη	P2633	P2634
PKCθ	P2996	--
Panel of all 9 isoforms	1 µg each – P2352	

4.2 Materials Required but Not Supplied

- Kinase (see **Section 7.1.3** and **8.1.3**)
- ATP (5X solution for **Section 7.1.4**, 10X solution for **Section 8.1.4**)
- 1 M HEPES (pH 7.4)
- 10% TRITON® X-100 (v/v)
- 10% NP-40 (v/v)
- 1 M $MgCl_2$
- 1 M $CaCl_2$
- Phosphatidylserine
- Diacylglycerol
- 125 mM Sodium Vanadate
- Dithiothreitol (DTT)
- Pipetting devices
- Hamilton syringe
- Laboratory timer
- Multiwell plates suitable for fluorescence polarization
- Multiwell fluorescence polarization instrument with suitable excitation and emission interference filters and appropriate dichroic mirrors (Green Ex: 485 nm, Em: 530 nM; Red Ex: 535 nm, Em: 590 nm)
- FP One-Step Standardization Kit (PanVera® Part No. P2581) or Red (FP) Standardization Kit (PanVera® Part No. P2888). These kits are recommended for validating instrument performance and may be required as a polarization standard to calibrate certain instruments.

5.0 DETERMINING THE IC_{50} FOR THE COMPETITOR

The following instructions for testing and validating the kit are optional, but are highly recommended for a first-time user. Generating a standard curve demonstrates the type of data that you can expect during an enzymatic reaction.

Note: 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 RFARKGSLRQKNV. It will be necessary to determine the IC_{50} of the phosphorylated form of your substrate using your buffer conditions. Determining the IC_{50} for your substrate of choice will give you some idea of how much enzyme you will need, how much substrate your reaction will require, and how long the reaction should be run.

Quick Procedure	Expanded Procedure
1. Dispense 50 µL of PKC Standard Curve Dilution Buffer into 11 wells.	To determine the IC_{50} for the PKC Competitor using a 96-well plate, dispense 50 µL of PKC Standard Curve Dilution Buffer (PanVera® Part No. P2751) into wells A2 to A12 (11 wells total) of a multiwell plate suitable for fluorescence polarization.
2. Add 20 µL of the 10 µM PKC Competitor and 80 µL of the PKC Standard Curve Dilution Buffer to an empty well.	In well A1, add 20 µL of the 10 µM PKC Competitor (PanVera® Part No. P2750 – **vortexed**) and 80 µL of the PKC Standard Curve Dilution Buffer. The concentration of PKC Competitor in well A1 is now 2 µM.
3. Perform a two-fold serial dilution.	Perform a two-fold serial dilution of the 2 µM PKC Competitor (in well A1) through well A12.
4. Discard 50 µL from the last well.	Discard 50 µL from well A12. There should now be 50 µL in all twelve wells.
5. Prepare 650 µL of a 2X Tracer/Antibody Mix.	Prepare 650 µL of a 2X Tracer/Antibody Mix by adding 26 µL of **vortexed** PKC Green Tracer (PanVera® Part No. P2756 or P2757) or PKC Red Tracer (PanVera® Part No. P3002 or P3003) Tracer, 50X to 325 µL 4X Antibody (PanVera® Part No. P2749 or P2752) and 299 µL of PKC Standard Curve Dilution Buffer.
6. Add 50 µL of the 2X Tracer/Antibody Mix to each well.	Add 50 µL of the 2X Tracer/Antibody Mix to each well. The concentration of PKC competitor in well A1 is now 1 µM.
7. Mix gently and cover the plate. Incubate for 2 hours at room temperature.	Mix gently, cover the plate to prevent evaporation and protect the reagents from light, and incubate for 2 hours at room temperature.
8. Measure the polarization values of your samples.	Measure the polarization values of your samples, following the procedures required by your FP instrument. We recommend that you use 25 µL of the antibody mixed with 75 µL of PKC Standard Curve Dilution Buffer as your blank. Use the FP One-Step Standardization Kit (PanVera® Part No. P2581) for the Green Kit, or the Red Polarization Standard [From the Red (FP) Standardization Kit; PanVera® Part No. P2888] for the Red Kit, diluted 1:10 in PKC Standard Curve Dilution Buffer, as your low polarization standard. We recommend that you also run controls for the Tracer/Antibody mixture (without competitor) and for the Tracer alone to determine the respective high and low polarization values for the assay itself. For more information on standard curve controls, please see **Section 5.1**.
9. Analyze the data.	Perform nonlinear regression on a semi-logarithmic plot of the data (Polarization vs. [Competitor]). Representative IC_{50} values of the competitor control are generally between 10-50 nM. Sample data (n = 3) appear in **Figure 2.**

5.1 Standard Curve Controls

To demonstrate the minimum polarization value of this detection system under the standard curve conditions, add 2 µL of the 50X PKC Tracer to 98 µL of the PKC Standard Curve Dilution Buffer in a single well. 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 2 µL of the 50X PKC Tracer and 25 µL of the 4X Antibody to 73 µL of the PKC Standard Curve 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).

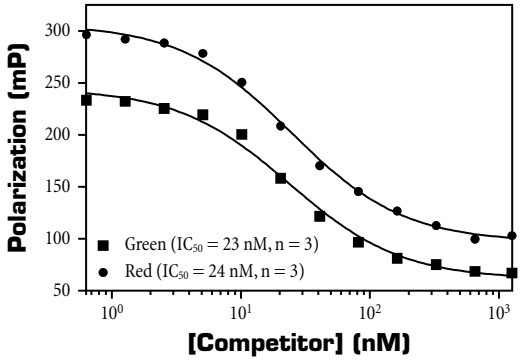


Figure 2. Typical Standard Curve Results. The IC_{50} for the PKC Green Kit was 23 nM (n = 3) and the IC_{50} for the PKC Red Kit was 24 nM (n = 3). Note that the error bars were smaller than the data points

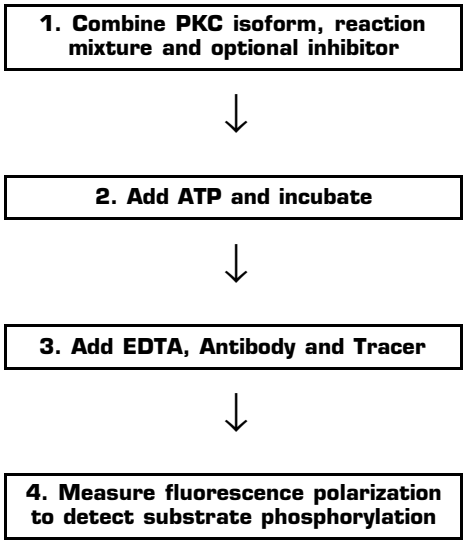
6.0 PROTEIN KINASE C ASSAY INFORMATION

6.1 PKC Kinase Assay Outline

Each complete PKC Assay Outline reaction must contain an appropriate buffer system, enzyme, ATP, substrate, PKC Green or Red Tracer and Anti-phosphoserine Antibody and EDTA to quench the 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 7.0**), four general steps are required. First, the reaction mixture, including the substrate (PanVera® offers PKC Peptide Substrate, Part No. P2760) and optional inhibitor, are combined. ATP is then added to start the reaction. Following an incubation, the kinase reaction is stopped with a mixture of EDTA (Kinase Quench Buffer), Antibody and Tracer, which also initiates the competition for antibody binding. Finally, polarization values are measured to determine the extent of substrate phosphorylation. If a kinetic experiment is performed (**Section 8.0**), the components of Steps 1 and 3 (without EDTA) are combined first and then Steps 2 and 4 are executed simultaneously.



Note: This protocol can also be performed in 384- or 1536-well plates using reduced reagent volumes.

6.2 Use of Inhibitors

When kinase inhibitors are screened (either as a single data point or through an IC_{50} determination), we recommend that you initially serially dilute the inhibitors in 100% DMSO or another solvent. Perform a 20-fold dilution of the inhibitors once from 100X (in 100% DMSO) to 5X (in 5% DMSO) using buffer as the diluent. By adding 10 µL of a 5X inhibitor to an end-point kinase reaction in a final volume of 50 µL (a 5-fold dilution), the final concentration of inhibitor will be 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 enzyme reaction. If 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 enzyme inhibitor will have low polarization values, indicating that the enzyme reaction was able to proceed largely (or totally) uninhibited.

6.3 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 7.3** or **Section 8.2**. Substitute this volume with PKC Standard Curve 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 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 well with all of your kinase reaction and detection components, ***except the ATP***, which is added in Step 2 of **Section 7.4.1** or Step 3 of **Section 8.3**. Substitute this volume with PKC Standard Curve 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 Tracer from the Antibody.

To blank you instrument, we recommend that you create a "No Tracer" control that contains all of the reaction and detection components (except the Tracer) at their final concentration. These controls are summarized in **Appendix I**.

6.4 Other Considerations

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 PKC isoforms.

Through optimization, you may find that higher or lower concentrations of EDTA (or another quenching reagent entirely) may be required for your specific PKC isoform. Be aware that high concentrations of EDTA may interfere with Antibody binding.

7.0 PROTEIN KINASE C ASSAY: END-POINT

7.1 Preparation of Reaction Components

In addition to several supplied components, the following reagents (not supplied) will be required to perform an end-point PKC reaction.

7.1.1 10X Kinase Buffer (5 µL required per assay)

Prepare a solution containing 200 mM HEPES (pH 7.4), 50 mM $MgCl_2$ 1 mM $CaCl_2$ and 0.2% NP-40.

7.1.2 10X Lipid Solution (5 µL required per assay)

Note: The following steps will provide sufficient 10X Lipid Solution (1 mg/mL phosphatidylserine, 0.20 mg/mL diacylglycerol) to perform one PKC assay. If you wish to run multiple reactions, prepare sufficient 10X Lipid Solution for all reactions (scale up the amount of each component proportionally).

1. Using a Hamilton syringe cleaned with methanol and a glass tube, combine 5 µg of phosphatidylserine (PS) and 1 µg of diacylglycerol (DAG).
2. Evaporate the solvent(s) with a nitrogen stream.
3. Resuspend the dried lipids in 5 µL of 10 mM HEPES (pH 7.4) and 0.3% TRITON® X-100.
4. In 3-minute intervals, alternate between vortexing and incubating in a hot-water bath until the lipids have dissolved. This process generally takes about 30 minutes. The resulting solution should be stable for one day on ice or it can be stored for extended periods at –20˚C.

7.1.3 50X Protein Kinase C (1 µL required per assay)

We recommend that you dilute the PKC isoforms to your desired 50X concentration in 10 mM HEPES (pH 7.4), 5 mM DTT and 0.01% TRITON® X-100. This 50X stock will be diluted to a 1X working concentration in the PKC reaction.

7.1.4 5X ATP Solution (10 µL required per assay)

The final ATP concentration required by your assay system should be determined experimentally.

7.2 Prepare the PKC Reaction Mixture

The end-point assay protocol uses a 50 µL PKC reaction, followed by the addition of 50 µL of a Quench/Detection Mixture (100 µL final volume). To prepare the PKC Reaction Mix (for a 100 µL final assay volume), combine the reagents as described below. **Vortex the Tracer** prior to adding it to the reaction mixture. Once all components are added, mix gently (do not vortex).

Note: The final assay volume can be scaled to any volume you desire. However, the kinase reaction will always be diluted by the addition of the PKC Quench/Detection Mixture. The final assay volume must be greater than the minimum volume required by your instrument, but less than the maximum capacity of the microwell that you are using.

Composition of the End-point PKC Reaction Mixture

Reagent	# of Reactions			Final Concentration in 50 µL PKC reaction
	1	100	1000	
10X Kinase Buffer	5.0 µL	500 µL	5.0 mL	1X (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$ 0.1 mM $CaCl_2$ and 0.02% NP-40)
10 µM Peptide Substrate	1.0 µL	100 µL	1.0 mL	200 nM
10X Lipid Solution	5.0 µL	500 µL	5.0 mL	1X (0.1 mg/mL PS, 0.02 mg/mL DAG)
50X Protein Kinase C	1.0 µL	100 µL	1.0 mL	1X
1.25 mM Sodium Vanadate	2.0 µL	200 µL	2.0 mL	50 µM
Nanopure Water	16.0 µL	1600 µL	16.0 mL	--
Total Volume	**30 µL**	**3.0 mL**	**30.0 mL**	**- -**

7.3 Prepare the 2X Quench/Detection Mixture (for a 100 µL final assay volume)

1. Mix 2 µL of the 50X Tracer, 25 µL of the 4X Antibody, 2 µL Kinase Quench Buffer (PanVera® Part No. P2825 or P2832) and 21 µL PKC Standard Curve Dilution Buffer.
2. You may use more or less Kinase Quench Buffer (or another quenching reagent) for your specific kinase. Please note that high EDTA concentrations are known to interfere with the antibody/phosphopeptide binding.

Note: 50 µL of 2X Quench/Detection Mixture is required per 50 µL reaction. If you wish to run multiple reactions, prepare sufficient 2X Quench/Detection Mixture for all reactions (scale up the volume of each component proportionally).

7.4 End-point Assay Procedure

Each PKC reaction, when complete, must contain an appropriate buffer system, PKC isoform, phosphatidylserine, diacylglycerol, ATP, peptide substrate, PKC Tracer, Antibody, an optional inhibitor, and PKC Quench Buffer (EDTA), which are added at the end of the reaction to quench the end-point kinase 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, 30 µL of the PKC Reaction Mixture (**Section 7.4.1**) and 10 µL an optional inhibitor or PKC Standard Curve Dilution Buffer is prepared in a volume of 40 µL. After the addition of 10 µL of a 5X ATP to start the reaction, the volume of each assay should equal 50 µL. Following an incubation, the reaction(s) are quenched by the addition of 50 µL the 2X Quench/Detection Mixture (containing EDTA, Antibody and Tracer; **Section 7.4.2**), bringing the final volume of the mixture to 100 µL. 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 kinase reaction will be diluted two-fold with the addition of the 2X Quench/Detection Mixture. The final volume must be less than the maximum volume of the multiwell plate you are using (and greater than the minimum volume required by your instrumentation).

7.4.1 Reaction Phase

Quick Procedure	Expanded Procedure
1. Set-up a reaction without ATP	In a volume of 30 µL, set-up a reaction without ATP using the PKC Reaction Mixture described in **Section 6.4.2**. The final reaction volume, including 10 µL of a 10X ATP solution and 10 µL optional inhibitor, vehicle or buffer, will be 50 µL prior to the addition of the detection reagents.
2. Add ATP to start each reaction.	Add 10 µL of a 5X ATP solution to each sample to start the reaction. After the addition of ATP, the final volume should be 100 µL. A "No ATP" control (substitute water or buffer for ATP) should be included in every experiment. The volume should now be 50 µL.
3. Proceed to the Detection Phase.	Proceed to the Detection Phase of the experiment in **Section 7.4.2**.

7.4.2 Detection Phase

Quick Procedure	Expanded Procedure
1. Quench the kinase reaction(s) with the 2X Quench/Detection Mixture.	At the end of the incubation, quench the kinase reaction(s) by adding 50 µL of the 2X Quench/Detection Mixture (containing EDTA, the Tracer and the Antibody; **Section 7.3**).
2. Incubate for 1 hour at RT.	Mix, cover the wells or tubes to reduce evaporation and protect them from light, and incubate for 1 hour at room temperature.
3. Measure the FP value of each reaction.	Measure the fluorescence polarization value of each reaction following the procedures required by your FP instrument.

8.0 PROTEIN KINASE C ASSAY: KINETIC

8.1 Preparation of Reaction Components

In addition to several supplied components, the following reagents (not supplied) will be required to perform a kinetic PKC reaction.

8.1.1 10X Kinase Buffer (10 µL required per assay)

Prepare a solution containing 200 mM HEPES (pH 7.4), 50 mM $MgCl_2$ 1 mM $CaCl_2$ and 0.2% NP-40.

8.1.2 10X Lipid Solution (10 µL required per assay)

Note: The following steps will provide sufficient 10X Lipid Solution (1 mg/mL phosphatidylserine, 0.20 mg/mL diacylglycerol) to perform one PKC assay. If you wish to run multiple reactions, prepare sufficient 10X Lipid Solution for all reactions (scale up the amount of each component proportionally).

1. Using a Hamilton syringe cleaned with methanol and a glass tube, combine 10 µg of phosphatidylserine (PS) and 2 µg of diacylglycerol (DAG).
2. Evaporate the solvent(s) with a nitrogen stream.
3. Resuspend the dried lipids in 10 µL of 10 mM HEPES (pH 7.4) and 0.3% TRITON® X-100.
4. In 3-minute intervals, alternate between vortexing and incubating in a hot-water bath until the lipids have dissolved. This process generally takes about 30 minutes. The resulting solution should be stable for one day on ice or it can be stored for extended periods at –20˚C.

8.1.3 50X Protein Kinase C (2 µL required per assay)

We recommend that you dilute the PKC isoforms to your desired 50X concentration in 10 mM HEPES (pH 7.4), 5 mM DTT and 0.01% TRITON® X-100. This 50X stock will be diluted to a 1X working concentration in the PKC reaction.

8.1.4 10X ATP Solution (10 µL required per assay)

The final ATP concentration required by your assay system should be determined experimentally.

8.2 Prepare the PKC Reaction Mixture

The assay protocol described in **Section 8.3** involves a 100 µL kinetic PKC reaction. Each kinetic PKC reaction, when complete, must contain an appropriate buffer system, PKC isoform, phosphatidylserine, diacylglycerol, ATP, peptide substrate, Tracer, Antibody and an optional inhibitor. Please note that the ATP should be added last to initiate the reaction. To prepare this PKC Reaction Mix, combine the reagents as described below. **Vortex the Tracer** prior to adding it to the reaction mixture. Once all components are added, mix gently (do not vortex).

Note: The final assay volume can be scaled to any volume you desire. The final assay volume must be greater than the minimum volume required by your instrument, but less than the maximum capacity of the microwell that you are using.

Composition of the Kinetic PKC Reaction Mixture

Reagent	# of Reactions			Final Concentration in 100 µL PKC reaction
	1	100	1000	
10X Kinase Buffer	10 µL	1 mL	10 mL	1X (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$ 0.1 mM $CaCl_2$ and 0.02% NP-40)
10 µM Peptide Substrate	2.0 µL	200 µL	2 mL	200 nM
10X Lipid Solution	10 µL	1 mL	10 mL	1X (0.1 mg/mL PS, 0.02 mg/mL DAG)
50X Protein Kinase C	2.0 µL	200 µL	2 mL	1X
1.25 mM Sodium Vanadate	4.0 µL	400 µL	4 mL	50 µM
50X Tracer	2.0 µL	200 µL	2 mL	1X
4X Antibody	25 µL	2.5 mL	25 mL	1X
Nanopure Water	15 µL	1.5 mL	15 mL	--
Total Volume	**70 µL**	**7.0 mL**	**70 mL**	**- -**

8.3 Kinetic Assay Procedure

In the kinetic assay, all of the reaction and detection reagents (kinase, ATP, substrate, buffer, Antibody and Tracer) are present during the kinase assay. Phosphorylation can be monitored in real-time as the reaction proceeds; the polarization value of the sample will be reduced as phosphoserine-containing peptides are generated and compete with the phosphopeptide tracer for binding to the antibody.

Quick Procedure	Expanded Procedure
1. Set-up a reaction without ATP	In a volume of 70 µL, set-up a reaction without ATP or inhibitor using the PKC Reaction Mixture described in **Section 8.2**. The final reaction volume, including 20 µL optional inhibitor, 10 µL of a 10X ATP solution or buffer, will be 100 µL.
2. Add optional inhibitor or vehicle.	Add 20 µL optional inhibitor, vehicle or buffer. The volume of each reaction should now be 90 µL.
3. Add ATP to start each reaction.	Add 10 µL of a 10X ATP solution to each sample to start the reaction. After the addition of ATP, the final volume of each reaction should be 100 µL. A "No ATP" control (substitute water or buffer for ATP) should be included in every experiment.
4. Quickly transfer the plate to your FP instrument and begin measurements.	Quickly transfer the plate to your FP instrument (operating at your preferred reaction temperature) and begin reading the fluorescence polarization values of your sample(s); continue to measure the polarization values of your samples as long as necessary.

9.0 REFERENCES

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2. Nishizuka, Y. (1986) *Science* **233:**305-12.
3. Wilkinson, S.E. and Hallam, T.J. (1994) *Trends Pharmacol. Sci.* **15:**53-7.
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5. Checovich, W.C., Bolger, R.E. and Burke, T. (1995) *Nature* **375:**254-6.
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APPENDIX I. PKC REACTION AND EXPERIMENTAL CONTROLS

		Full Reaction		Controls (for End-point Assay)			
	Reagents	End-point	Kinetic	"No Antibody"	"No Tracer"‡	"No ATP"	"Vehicle"
PKC Reaction Mixture	10X Kinase Buffer (**Section 7.1.1** and **8.1.1**)	5 µL	10 µL	5 µL	5 µL	5 µL	5 µL
	10 µM Peptide Substrate	1 µL	2 µL	1 µL	1 µL	1 µL	1 µL
	10X Lipid Solution (**Section 7.1.2** and **8.1.2**)	5 µL	10 µL	5 µL	5 µL	5 µL	5 µL
	50X Protein Kinase C (**Section 7.1.3** and **8.1.3**)	1 µL	2 µL	1 µL	1 µL	1 µL	1 µL
	1.25 mM Sodium Vanadate	2 µL	4 µL	2 µL	2 µL	2 µL	2 µL
	Nanopure Water	16 µL	15 µL	16 µL	16 µL	26 µL	16 µL
	5X Inhibitor/Buffer/Solvent (**Section 7.4.1** and **8.3**)	10 µL	20 µL	10 µL	10 µL	10 µL	10 µL§
	5X or 10X ATP Solution (**Section 7.1.4** and **8.1.4**)	10 µL	10 µL	10 µL	10 µL	0 µL	10 µL
Quench/Detection	50X PKC Green or Red Tracer (**Section 7.3** and **8.2**)	2 µL	2 µL	2 µL	0 µL	2 µL	2 µL
	4X Antibody (**Section 7.3** and **8.2**)	25 µL	25 µL	0 µL	25 µL	25 µL	25 µL
	Kinase Quench Buffer (**Section 7.3**)	2 µL	0 µL	2 µL	2 µL	2 µL	2 µL
	PKC Standard Curve Dilution Buffer	21 µL	0 µL	46 µL	23 µL	21 µL	21 µL
	Total Volume at Detection	**100 µL**	**100 µL**	**100 µL**	**100 µL**	**100 µL**	**100 µL**

‡The "No Tracer" control should be used as the blank when calibrating your instrument.
§If screening kinase inhibitors, this volume should add the same type and concentration of solvent used in your compound library.

APPENDIX II. USE OF PANVERA® PKC ISOFORMS WITH THIS ASSAY KIT

PanVera® PKC isoforms are human recombinant proteins expressed in insect cells infected with recombinant baculovirus. The isoforms are greater than 95% pure (90% for PKCη and PKCθ), which is determined by visual inspection of a Coomassie® Blue-stained SDS-PAGE gel. Each isoform is supplied at lot-specific concentrations in 20 mM HEPES (pH 7.4), 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.05% TRITON® X-100 and 50% Glycerol [PKCθ is in 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% NP-40, 5 mM DTT and 20% Glycerol]. For long-term storage, they should be kept –80˚C; if they are to be used within one month, they can be stored at –20˚C. The performance of PanVera®'s PKC isoforms is guaranteed for six months from the date of purchase if stored and handled properly.

When using PanVera®'s PKC isoforms in this assay (**Figure 3**), we recommend that you dilute the enzymes to your desired 50X concentration in 10 mM HEPES (pH 7.4), 5 mM DTT, and 0.01% TRITON® X-100. Diluted isoforms are stable for at least 2 hours at 4˚C. Once the PKC isoforms have been added to your reaction mixture (prior to the addition of ATP), their activity will be moderately enhanced (by approximately 10% per hour) the longer they are preincubated with the buffers, substrate, and phospholipids contained in the reaction mixture. We have observed this effect for up to 4 hours.

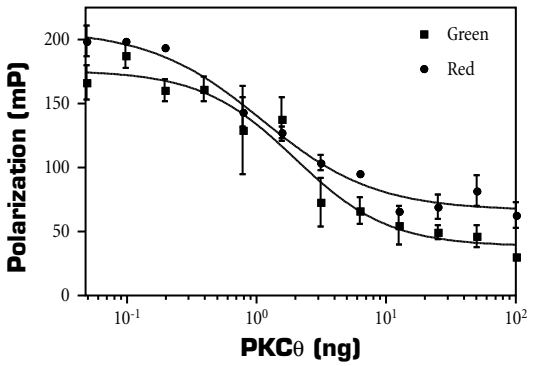

Figure 3. PKCθ Titration Using PanVera®'s PKC Assay Kit, Green or Red. A 2-fold serial dilution of enzyme from 100 ng to 0.1 ng was performed. Final reaction conditions were 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 10 µM Na_3VO_4, 10 µM ATP, and 1 µM PKC substrate. After a 90 minute incubation at room temperature, 25 µL of 4X PKC Antibody, 1X final PKC Green or Red Tracer and PKC Dilution Buffer were added for a final volume of 100 µL in each well. Please note that the buffer conditions used in these experiments were optimized for PKCθ.

*United States and International Patents pending.

The performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.

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