1.0 INTRODUCTION

The retinoblastoma protein (Rb) is known as a tumor suppressor protein that acts via inhibition of cell cycle progression from the G_1 to the S phase. Evidence also supports a more general function of Rb at the transcriptional and cellular levels (1). In addition to its involvement in not only regulating an elusive cell cycle checkpoint, Rb may also have roles in oncogenic viral infection, cellular differentiation and apoptosis. Rb's control over cell cycle progression is determined by its phosphorylation by Cyclin Dependant Kinases (CDKs). One site on Rb that is phosphorylated and has an important impact on the regulation of cellular events is Threonine 252 (2, 3). Because of CDK and Rb's involvement in many important cell-regulatory processes, they are potentially important targets for therapeutic drug discovery research. Conventional kinase assays are tedious, utilize radioactive reagents, and are not easily automated or converted to a high-throughput format for drug screening. PanVera®'s CDK Assay Kit, Rb^{ING} is a major advance because it is simple, sensitive, non-radioactive, homogeneous and formatted for high-throughput screening. This assay kit contains all the materials, except the kinase, necessary to analyze CDK-like kinase activity via phosphorylation of Rb at Thr252 (Rb^{ING}) using fluorescence polarization (FP) as the detection method. The CDK Assay Kits, Rb^{ING} contain reagent volumes sufficient for one hundred (PanVera® Part No. P2928) and one thousand (PanVera® Part No. P2929) 100 µL 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 also work in 384- and 1536-well plates 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 (4, 5).

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.

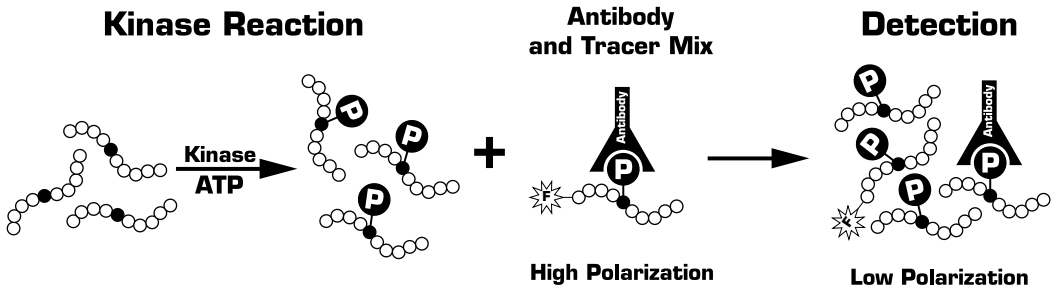
The measured FP value is the weighted average of the FP values of the individual bound and free fluorescent molecules and is therefore a direct measure of the fraction bound. These data can be handled exactly the same way as traditional radioligand-binding assay data. Fluorescence polarization values 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 CDK Assay because fluorescein-labeled phosphopeptides (F-phosphopeptides, also referred to as the "tracer") and phosphopeptides or phosphoproteins generated during a kinase reaction will compete for binding to anti-phosphothreonine peptide-specific antibodies. When there are no kinase reaction products present, a significant portion of the F-phosphopeptide tracer will be bound by the anti-phosphothreonine peptide-specific antibody, resulting in a high polarization value. However, after a CDK reaction has occurred, phosphorylation can be detected because the anti-phosphothreonine peptide-specific antibodies will bind to both the F-phosphopeptide tracer and the non-fluorescent 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-phosphothreonine peptide-specific antibodies and the emitted light will be totally depolarized. Thus, the change in FP is directly related to CDK kinase activity.



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	P2928		P2929	
		Size	Part #	Size	Part #
Ab, 4X, CDK Assay, Rb[ING]	Anti-phosphothreonine peptide-specific antibody in 20 mM Tris (pH 7.5), 0.02% NaN_3	2.5 mL	P2934	25 mL	P2935
Rb[ING] Tracer, 10X	Fluorescein-labeled phosphopeptide in 20 mM Tris (pH 7.5), 1 mM DTT, 0.02% NaN_3	1 mL	P2936	10 mL	P2937
Rb[ING] Competitor, 5 µM	5 µM phosphopeptide in 20 mM Tris (pH 7.5), 0.02% NaN_3	500 µL	P2950	500 µL	P2950
Kinase Quench Buffer	500 mM EDTA (pH 8.0)	1 mL	P2825	10 mL	P2832
FP Dilution Buffer	20 mM Tris (pH 7.5), 0.02% NaN_3	5 mL	P2838	50 mL	P2839

4.2 Materials Available Separately

- Rb[ING] Peptide Substrate, Non-phosphorylated, 100 µM (INGSPRTPRRGQNR) PanVera® Part No. P2939, 1 mL

4.3 Materials Required but not Supplied

- Kinase
- ATP
- Kinase Reaction Buffer
- Multiwell plates or 6 mm borosilicate tubes suitable for fluorescence polarization
- Multiwell fluorescence polarization instrument or a Beacon® 2000 Fluorescence Polarization instrument with suitable 485 nm excitation and 530 nm emission interference filters
- Pipeting devices
- Reagent reservoir(s)
- Laboratory timer
- 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 4X Antibody, CDK Assay (PanVera® Part No. P2934 or P2935) should be stored at –20˚C. Once thawed, it should be stored at +4˚C. The 10X Rb[ING] Tracer (PanVera® Part No. P2936) and the Rb[ING] Competitor (PanVera® Part No. P2938) should be stored at –20˚C, thawed when needed, and then refrozen. Vortex the 10X Rb[ING] Tracer before use. Avoid subjecting these reagents to repeated freeze/thaw cycles. The FP Dilution Buffer (PanVera® Part No. P2838) and Kinase Quench Buffer (PanVera® Part No. P2825) 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_{50} FOR THE Rb[ING] 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 INGSPRTPRRGQNR. 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 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.

6.1 Multiwell Plate Format

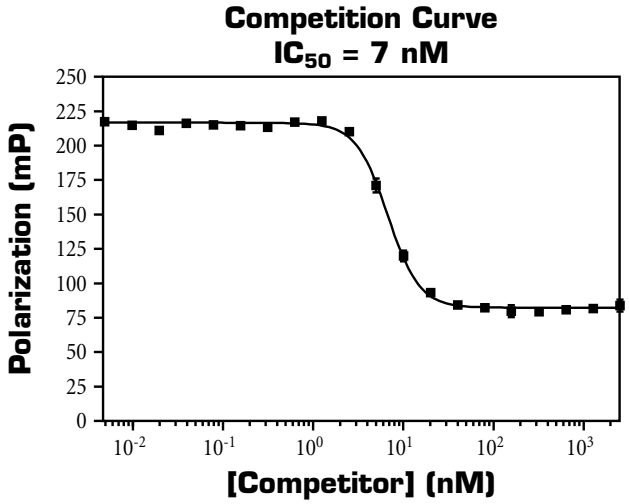
1. In a multiwell plate suitable for FP, pipette 100 µL of 5 µM Competitor (PanVera® Part No. P2950 - **VORTEXED**) into well A1.
2. Dispense 50 µL FP Dilution Buffer (PanVera® Part No. P2838 or P2839) into wells A2 to A12 and wells B1 to B6 (17 wells total).
3. Perform a 2-fold serial dilution of the 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 B6 is reached.
5. Discard 50 µL from well B6. There should be 50 µL in every well from A1 to B6 (18 wells total).
6. **VORTEX** 10X Tracer (PanVera® Part No. P2936 or P2937).
7. Add 25 µL of the 4X Antibody (PanVera® Part No. P2934 or P2935) and 10 µL of the 10X Tracer to every well from A1 to B6 (18 wells total). Add 15 µL of FP Dilution Buffer to each well. (**Do not pre-mix the tracer and antibody** or displacement by the phosphopeptide competitor will not occur.) 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 B6 has a competitor concentration of approximately 0.02 nM).
8. Mix, cover the multi-well plate to reduce evaporation and protect the reagents from light, and incubate for 1 hour at room temperature.
9. Measure the polarization values of your samples, following the procedures required by your FP instrument. We recommend that you use 25 µL of 4X Ab and 75 µL FP Dilution Buffer as your blank and the Beacon® FP One-Step Standardization Kit (PanVera® Part No. P2581) for your low and high polarization standards, if necessary. We recommend that you may also run controls for the antibody/tracer mixture (without competitor) and for the tracer alone to determine the low and high polarization values for the assay itself. See Section 6.3.
10. Perform non-linear regression on a semi-log plot of the data (Polarization vs. [Competitor]). Representative IC_{50} values of the competitor control are generally between 5 and 40 nM. Sample data (n = 3) generated on a TECAN Ultra (Research Triangle Park, NC) appear in Section 6.3.

6.2 Single-tube Format

1. Label eighteen borosilicate tubes #1 through #18. Pipette 100 µL of 5 µM Competitor (PanVera® Part No. P2950 - **VORTEX**) into tube #1.
2. Dispense 50 µL FP Dilution Buffer (PanVera® Part No. P2838 or P2839) into tube #2 through #18 (17 tubes total).
3. Perform a 2-fold serial dilution of the 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 #18 is reached.
5. Discard 50 µL from tube #18. There should now be 50 µL in all 18 tubes.
6. **VORTEX** 10X Tracer (PanVera® Part No. P2936 or P2937).
7. Add 25 µL of the 4X Antibody (PanVera® Part No. P2934 or P2935) and 10 µL of the 10X Tracer to every tube. Add 15 µL of FP Dilution Buffer to each tube. (**Do not pre-mix the tracer and antibody** or displacement by the phosphopeptide will not occur.) This step will reduce the concentration of all of the detection components to 1X and reduce the concentration of the Competitor in each tube by 2-fold (*i.e.,* tube #1 now has a competitor concentration of 2.5 µM, while tube #18 will have a competitor concentration of approximately 0.02 nM).
8. Mix, cover the tubes to reduce evaporation and protect the reagents from light, and incubate for 1 hour at room temperature.
9. Measure the polarization values of your samples, following the procedures required by your FP instrument. It is recommended that you use 25 µL of 4X Ab and 75 µL FP Dilution Buffer as your blank and the Beacon® FP One-Step Standardization Kit (PanVera® Part No. P2581) for your low and high polarization standards, if necessary. We recommend that you also run controls for the antibody/tracer mixture (without competitor) and for the tracer alone to determine the low and high polarization values for the assay itself. See Section 6.3.
10. Perform non-linear regression on a semi-log plot of the data (Polarization vs. [Competitor]). Representative IC_{50} values of the competitor control are generally between 5 and 40 nM. Sample data (n = 3) generated on a TECAN Ultra (Research Triangle Park, NC) appear in Section 6.3.

6.3 Standard Curve Controls

To demonstrate the minimum polarization value of this detection system under the standard curve conditions, add 10 µL of the 10X Tracer to 90 µL of the FP Dilution Buffer in a single well of a multi-well plate 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 µL of the 10X Tracer and 25 µL of the 4X Antibody in 65 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).



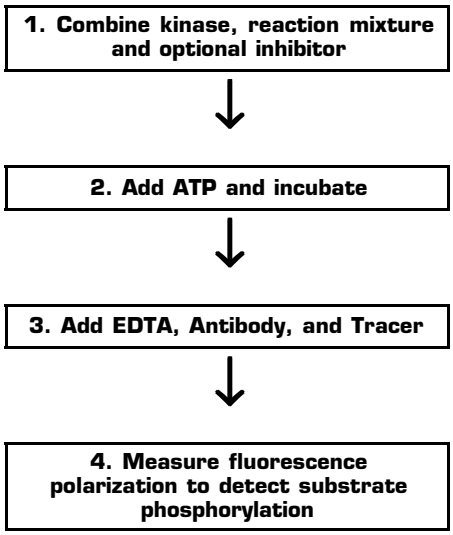
7.0 CYCLIN-DEPENDENT KINASE ASSAY, Rb[ING]

7.1 Cyclin-Dependent Kinase Assay, Rb[ING] Outline

Each complete CDK Assay, reaction must contain an appropriate buffer system, enzyme, ATP, substrate, Tracer and 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 (PanVera® offers Peptide Substrate, Part No. P2939) and optional inhibitor, are combined. ATP is then added to start the reaction. Following an incubation, stop the kinase reaction with EDTA (Kinase Quench Buffer), then add anti-phosphothreonine peptide-specific antibodies and tracer **separately** to initiate the competition for antibody binding. Finally, polarization values are measured to determine the extent of substrate 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 multi-well FP instrument. However, the protocol can also be performed in 384- or 1536-well plates using reduced volumes. You may also use borosilicate tubes if you are using a single-tube FP instrument such as the Beacon® 2000 Instrument.

7.2 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 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 fluorescent phosphopeptide tracer from the anti-phosphothreonine 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 3 of Section 7.3 or Step 2 of Section 7.4.1. 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 CDK 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 CDK 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 CDK 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 CDK inhibitor will have low polarization values, indicating that the CDK 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. Be aware that high concentrations of EDTA may interfere with anti-phosphoserine peptide-specific antibody/phosphopeptide binding.

7.3 Kinetic Assay

In the kinetic assay, all of the reaction and detection reagents (FP Dilution Buffer, kinase, ATP, substrate, Antibody, and 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 phosphothreonine-containing peptides are generated and compete with the phosphopeptide tracer for binding to the antibody.

1. In a volume of less than 50 µL, set-up a CDK reaction without ATP. The final reaction volume (including ATP) will be 50 µL prior to the addition of the detection reagents. A typical reaction will require a protein threonine kinase, an appropriate buffer system, peptide or protein substrate, and ATP (which is added last to initiate the reaction).
2. Once you have dispensed the reactants into a multi-well plate or borosilicate tube, add 25µL of the 4X Antibody and 10 µL of the 10X Tracer (**VORTEXED**), to each sample. **DO NOT** pre-mix the tracer and antibody or displacement by the phosphopeptides generated during your kinase reaction *will not* occur. Add 15 µL of FP Dilution Buffer to each well. 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 µL with water. A "No ATP" control (substitute water or buffer for ATP) should be included in every experiment.
4. Quickly transfer your multi-well plate or borosilicate tube 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.

7.4 End-point Assay

The end-point CDK-Rb[ING] 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 50 µL, so that the final reaction volume (including ATP and Kinase Quench Buffer) will equal 50 µL. 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 µL. Then, 25 µL of the 4X Antibody, 10 µL of the 10X Tracer (**VORTEXED**), and 15 µL of FP Dilution Buffer are added to each well. 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 multi-well plate you are using.

7.4.1 Reaction Phase

1. In a volume of less than 50 µL, set-up a CDK reaction without ATP. A typical reaction will require a protein kinase, an appropriate buffer system and peptide substrate.
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 µL. Please note that 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 10 mM and bring the final volume of each quenched CDK reaction to 50 µL. 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. EDTA at a concentration greater than 20 mM is known to interfere with the anti-phosphothreonine 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 CDK reaction, add 25 µL of the 4X Antibody, 10 µL of the 10X Tracer (**VORTEXED**), and 15 µL of FP Dilution Buffer to each well. **DO NOT** pre-mix the tracer and antibody or displacement by the phosphopeptides generated during your kinase reaction *will not* occur.
2. Cover the wells or tubes to reduce evaporation and protect them from light, then incubate for 1 hour at room temperature.
4. Measure the fluorescence polarization value of each reaction following the procedures required by your FP instrument.

8.0 REFERENCES

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