



Transcreener™ KINASE Assay Case Study: PKA Kinase Inhibitor Screen

The Transcreener™ KINASE Assay is compatible with serine/threonine and tyrosine protein kinases using protein or peptide acceptor substrates, lipid kinases using lipid acceptor substrates, and metabolic kinases using carbohydrate substrates, as well as ATPases. The Transcreener™ KINASE Assay was designed as a simple two-part, endpoint assay that consists of the kinase reaction (10 μ L) followed by the addition of ADP Detection Mixture (10 μ L) for a final volume of 20 μ L.

Transcreener™ KINASE Assay Template:

1

Perform Kinase Reaction (10 μ L volume)

Add to each well of a 384-well assay plate:

- 2.5 μ l of 4% DMSO or 4X Test Compounds (40 μ M compound in 4% DMSO).
- 5 μ l of 2X Kinase Reaction Mix (includes buffer, MgCl₂, kinase, and substrate).
- 2.5 μ l of 4X ATP Solution to begin the kinase reaction.
- Mix on a plate shaker and incubate at room temperature (20-25°C) for 1 hour.

2

Add ADP Detection Mixture (10 μ L volume)

- Dispense 10 μ l of the ADP Detection Mixture into each well. Detection mixture consists of Stop and Detect Buffer, ADP Tracer and ADP Antibody.
- Mix on a plate shaker and incubate at room temperature (20-25°C) for 1 hour.

3

Measure the fluorescence polarization

- Configure the fluorescence polarization (FP) plate reader to measure the fluorescence polarization of far-red fluorophores. Recommended excitation and emission wavelengths are 612 nm and 670 nm, respectively. Set the temperature of the FP plate reader to 30°C.
- The wells that do not contain the Ab ('No Ab Control') should be set to 20 mP. The wells that do not contain tracer ('No Tracer Control') should be used as a reference blank and a sample blank (for buffer subtraction).
- Measure the fluorescence polarization (FP) of the assays.

Case Study Template:

1. PKA Kinase Reaction

The case study that follows is designed for use as a template to aid in assay development and primary inhibitor screening. The 10 μ L PKA kinase reaction was performed in 50 mM HEPES (pH 7.5), 4 mM MgCl₂, 2 mM EGTA,

1% DMSO (test compound solvent), 0.01% Brij-35, 50 μ M kemptide, 12 ng/mL PKA and 10 μ M ATP. However, buffer components (including [ATP]) ideal for any enzyme target can be used.

1.0 Prepare 4X test compounds in 4% DMSO (2.5 μ L/ well).

In this case study test compounds from the Gen-Plus compound library were prepared at 40 μ M in 4% DMSO. Four percent DMSO was added to the “No PKA” control wells, the “PKA “ control wells (~20% ATP conversion; 0% inhibition) and to the ADP/ATP standard curve wells.

2.0 Prepare 2X “PKA” and “No PKA” Reaction Mixes (5 μ L/well). The assay window will be determined from these reactions after the addition of ATP.

2.1. Prepare 2X “No PKA” Reaction Mix:

The 2X “No PKA” Reaction Mix consists of 100 mM HEPES (pH 7.5), 8 mM MgCl₂, 4 mM EGTA, 0.02% Brij-35 and 100 μ M kemptide (Sigma). This control reaction will define the upper mP limit of the assay window (0% ATP Conversion; 100% inhibition) and will be used in the preparation of the standard curve.

2.2. Prepare 2X “PKA” Reaction Mix:

PKA kinase (Invitrogen; 24 ng/mL) was prepared in 100 mM HEPES (pH 7.5), 8 mM MgCl₂, 4 mM EGTA, 0.02% Brij-35 and 100 μ M kemptide. This reaction will define the lower mP limit of the assay window (~20% ATP conversion; 0% inhibition).

3.0 Prepare a 4X ATP Solution for initiating the PKA reaction (2.5 μ L/well). Prepare a 4X ADP Solution for preparing the ADP/ATP standard curve:

3.1. 4X ATP Solution:

The 4X ATP Solution (40 μ M ATP) is used to start the kinase reaction and to prepare the Standard Curve. The 4X ATP solution was added to all wells containing the “PKA” and “No PKA” reaction mixes.

3.2. 4X ADP Solution:

Dilute the 500 μ M ADP (provided) 1:12.5 to 40 μ M using deionized water. The 4X ADP Solution is used to prepare the Standard Curve.

3.3. 4X ADP/ATP Mixtures for Standard Curve:

To generate a twelve-point 10 μ M ADP/ATP Standard Curve, prepare 4X ADP/ATP Mixes as described below. These 12-point ADP/ATP standard curve samples are added to wells containing 2X ‘No PKA’ Reaction Mix.

Table 1. ADP/ATP Standard Curve

% ATP Conversion	Concentration of ADP (μ M)/ ATP (μ M)	Volume of 40 μ M ADP/40 μ M ATP (final volume = 100 μ L)
0	0 μ M / 40 μ M	0 μ L / 100 μ L
2	0.8 μ M / 39.2 μ M	2 μ L / 98 μ L
4	1.6 μ M / 38.4 μ M	4 μ L / 96 μ L
6	2.4 μ M / 37.6 μ M	6 μ L / 94 μ L
8	3.2 μ M / 36.8 μ M	8 μ L / 92 μ L
10	4 μ M / 36 μ M	10 μ L / 90 μ L
15	6 μ M / 34 μ M	15 μ L / 85 μ L
20	8 μ M / 32 μ M	20 μ L / 80 μ L
30	12 μ M / 28 μ M	30 μ L / 70 μ L
40	16 μ M / 24 μ M	40 μ L / 60 μ L
60	24 μ M / 16 μ M	60 μ L / 40 μ L
100	40 μ M / 0 μ M	100 μ L / 0 μ L

4.0 Mix the plate and incubate for 1 hour at room temperature.

Although the Transcreener™ KINASE Assay was developed for a one-hour, room temperature (20-25°C) reaction, the enzyme reaction can be performed at elevated temperatures or extended times (to reduce the enzyme requirement).

2. ADP Detection

An antibody titration was performed prior to this study to determine the ideal antibody concentration for the kinase reaction conditions.

1.0 Prepare ADP Detection Mixtures (10 μ L/well):

The table below explains the preparation of the Transcreener™ ADP Detection Mixture for sample and control reactions. The volumes will fill a 384-well assay plate with overfill to accommodate pipetting requirements.

1.A. Prepare ADP Detection Controls:

These controls are used to calibrate the fluorescence polarization plate reader and should be added to kinase reaction mixture that includes substrate and ATP.

No Tracer Control: This control consists of 20 μ g/mL ADP Antibody in 1X Stop and Detect Buffer and is the reference blank for the 'No Ab' Control and the sample blank for all other wells (see table)

No Ab Control (free tracer): This control consists of 1X ADP Tracer in 1X Stop and Detect Buffer and is the reference sample for the tracer (set to 20 mP) (see table).

1.B. Prepare ADP Detection Mixture:

Prepare enough ADP Detection Mixture to accommodate the kinase reaction and controls. The ADP Detection Mixture consists of 1X Stop and Detect Buffer containing 1X ADP Tracer and 20 μ g/mL ADP Antibody (see table).

Table 2. ADP Detection Mixtures and Controls

Order of Addition	Component	Stock Conc.	Working Conc.	ADP Detection Mixture*	No Tracer Control	No Ab Control
1	deionized water	N/A	N/A	Sufficient for 4.5 mL final volume	Sufficient for 400 μ l final volume	356 μ l
2	Stop and Detect Buffer	10X	1X	450 μ l	40 μ l	40 μ l
3	ADP Far RedTracer	100X	1X	45 μ l	N/A	4 μ l
4	ADP Antibody	3200 μ g/mL varies w/lot see CoA	20 μ g/mL varies w/lot see CoA	28.1 μ L Calculated from C of A	2.5 μ L Calculated from C of A	N/A

* ADP Detection Mixture for Kinase Reactions, ADP/ATP Standard Curve, and "No Kinase Controls"

2.0 Add Detection Mixes to Plate: Add ADP Detection Mixture, "No Tracer" Control Mixture, and "No Ab" Control Mixture to appropriate wells and mix plate.

3.0 Incubate for 1 hour at room temperature (20-25°C).

NOTE: After adding the ADP Detection Components to the plate, the polarization signal is stable for at least 8 hours at room temperature (20-25°C).

4.0 Measure Fluorescence Polarization

Configure the fluorescence polarization (FP) plate reader to measure the fluorescence polarization of far-red fluorophores. A Tecan Ultra spectrofluorometer was used in this case study (Ex_{612nm} (10 nm bandwidth) and Em_{670nm} (25 nm bandwidth)).

NOTE: The Transcreener™ KINASE Assay has been successfully used on the following instruments, Tecan Ultra, Tecan Safire 2, Molecular Devices Analyst, Perkin Elmer Victor 2V, and Perkin Elmer Envision. Please see FAQs section of BellBrook Labs website for specific instrument filter settings.

5.0 Data Analysis

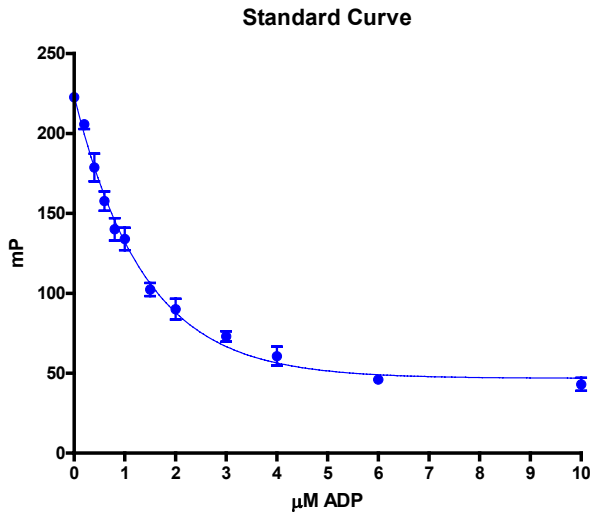
Using the Standard Curve, determine the μM ADP, % Conversion, and % Inhibition for each well.

5.1. Graphing the Standard Curve:

Plot mP versus μM ADP on a linear scale and use one-phase exponential decay curve fitting(Prizm software).

$$Y = \text{Span} * \exp(-K * X) + \text{Plateau}$$

Starts at $\text{Span} + \text{Plateau}$ and decays to Plateau with a rate constant K . The half life is $0.69/K$



5.2 Converting mP values to μM ADP:

Using the rearranged equation from the Standard Curve and the tabulated results, convert mP values to μM ADP.

$$\mu\text{M ADP} = \left(\frac{\ln((y - \text{Plateau}) / \text{span})}{-K} \right)$$

5.3 Determining % ATP Conversion:

$$\% \text{ ATP Conversion} = \left(\frac{\mu\text{M ADP}_{\text{SAMPLE}}}{\mu\text{M ADP}_{\text{starting}}} \right) * 100$$

5.4 Determining % Inhibition:

$$\% \text{ Inhibition} = \left(\frac{\mu\text{M ADP}_{\text{PKA}} - \mu\text{M ADP}_{\text{PKA} + \text{compd}}}{\mu\text{M ADP}_{\text{PKA}}} \right) * 100$$

6.0 Sample Data

