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1. Introduction

Protein kinases regulate many critical biological mechanisms, including metabolism and cell growth, proliferation, and differentiation. Aberrations in the activity of the kinases involved in signal transduction have been linked to many human diseases. The discovery of more than 518 kinases encoded by the human genome has spurred development of rapid screening techniques for potential drugs against these enzymes.

Z´-LYTE™ Kinase Assays are non-radioactive assays for screening inhibitors of many members of the tyrosine and serine/threonine protein kinase families. These robust, room-temperature, homogeneous assays use fluorescence resonance energy transfer (FRET) between coumarin and fluorescein for detection. Reaction progress is quantitated with a ratiometric approach (coumarin emission/fluorescein emission) that reduces the effects of well-to-well and day-to-day variations. This results in low standard deviations and high Z´-factors (> 0.7), even when only a small percentage of the Z´-LYTE™ Peptide Substrate is phosphorylated.

Z´-LYTE™ Kinase Assay Kits are designed to accurately and reliably screen potential kinase inhibitors in a two-hour, room-temperature reaction. The reagent volumes in each kit are sufficient for 800 20-µl assays in 384-well assay plates. However, the assay is scalable for ultra-miniaturized high-throughput screening (HTS) applications (assay volumes as low as 1.6 µl) with no loss of data quality.

For a list of identified kinases that phosphorylate Z´-LYTE™ Peptide Substrates, go to www.invitrogen.com/zlyte.

2. Assay Theory

The Z´-LYTE™ biochemical assay employs a FRET-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. The assay uses a synthetic peptide substrate (the Z´-LYTE™ Peptide Substrate) that is labeled with a donor fluorophore (coumarin) and an acceptor fluorophore (fluorescein) that make up a FRET pair. In the primary reaction (the Kinase Reaction), the kinase transfers the γ-phosphate of ATP to a single tyrosine, serine, or threonine residue on the substrate, while the presence of a kinase inhibitor in the primary reaction suppresses phosphorylation.

In the secondary reaction (the Development Reaction), a site-specific protease (the Development Reagent) is added at an optimized concentration. The development buffer quenches the Kinase Reaction, while the protease recognizes and cleaves non-phosphorylated Z´-LYTE™ Peptide Substrate at a substantially higher rate than phosphorylated substrate. Cleavage disrupts FRET between the donor and acceptor fluorophores on the non-phosphorylated substrate, while uncleaved, phosphorylated substrate maintains FRET (**Figure 1**). Concentrations of all reagents are carefully optimized to maximize the assay window for each Z´-LYTE™ Peptide Substrate. Reaction progress is quantitated by calculating the emission ratio. The method for this is described in Section 7.1.

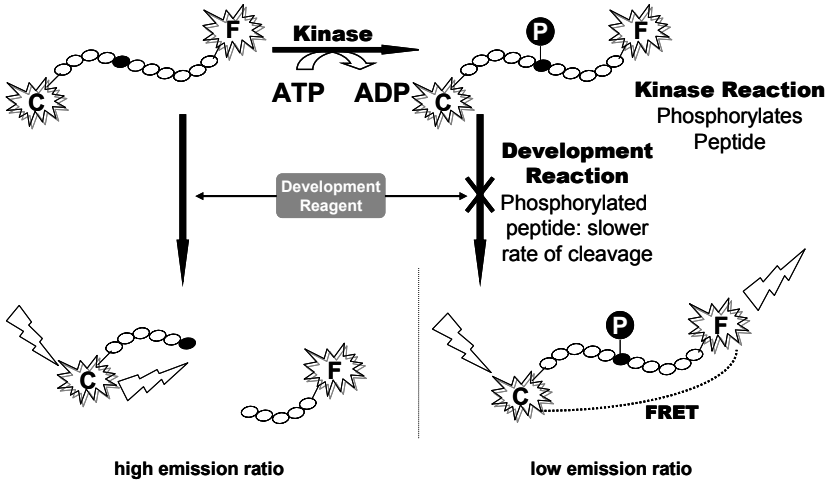


Figure 1. Diagram of the antibody-free Z´-LYTE™ biochemical assay technology.

3. Advantages of the Kit

Z´-LYTE™ Kinase Assay Kits offer the following advantages:

- Numerous validated Z´-LYTE™ Peptide Substrates are available, for assaying more than 180 protein kinases.
- Antibody-free assays enable rapid development of new substrates for emerging targets.
- The ATP concentration can vary from sub-K_M to saturating, depending on the experimental goals, without affecting the validity of the results.
- Assays are compatible with automated HTS systems.

4. Kit Contents

This kit provides sufficient reagents to run two 384-well assay plates at a 20-µl final assay volume. You will need additional kits if you plan to perform all the assays described in this manual.

Component	Description	Quantity	Component Catalog No.
Z´-LYTE™ Ser/Thr 13 Peptide Substrate	1 mM in DMSO	20 µl	PV3794
Z´-LYTE™ Ser/Thr 13 Phospho-peptide	1 mM in DMSO	10 µl	PV3795
5X Kinase Buffer	250 mM HEPES (pH 7.5), 50 mM $MgCl_2$, 5 mM EGTA, 0.05% Brij-35	4 ml	PV3189
ATP	10 mM in water	500 µl	PV3227
Development Reagent A	Proprietary reagent	50 µl	PV3295
Development Buffer	Proprietary buffer	5 ml	P3127
Stop Reagent	Proprietary reagent	5 ml	P3094

5. Storage and Stability

This kit is shipped on dry ice. Upon receipt, store the entire kit at –80°C. After initial use, store the components as detailed in the table below. All reagents are stable for 6 months from the date of purchase, if stored and handled properly.

Reagent	Storage Temperature (after initial use)	Notes
Z´-LYTE™ Ser/Thr 13 Peptide Substrate	–20 °C	
Z´-LYTE™ Ser/Thr 13 Phospho-peptide	–20 °C	
5X Kinase Buffer	20–30 °C	
ATP	–20 °C	
Development Reagent A	–80 °C	Avoid more than five freeze/thaw cycles
Development Buffer	20–30 °C	
Stop Reagent	–80 °C	Avoid more than five freeze/thaw cycles

6. Materials Required But Not Supplied

The following materials are required in the following protocols, but not supplied in the kit:

- Protein kinase of interest
- Any buffer supplements required for optimal protein kinase activity. Some common buffer supplements include $CaCl_2$, calmodulin, $MnCl_2$, DTT, and lipids. For further details regarding the addition of supplements to Invitrogen kinases, see the SelectScreen™ Kinase Profiling Service Screening Protocol and Assay Conditions at www.invitrogen.com/kinaseprofiling.

- A fluorescence plate reader with the appropriate filter sets installed for detecting the fluorescent emission signals of coumarin and fluorescein (see the **Note About Filter Sets**, below), or a plate reader equipped with a monochromator, such as the TECAN Safire™.
- 384-well assay plates. We recommend black Corning® 384-well, low-volume, round-bottom (non-binding surface) assay plates. Other black-walled, low-binding assay plates, while not tested, may be suitable.
- Multi-channel pipettor (12 channels) or any pipetting device that can accurately deliver repeated volumes of 2.5 µl and 5 µl.
- 96-well assay plates that can accommodate 300 µl per well.

Note about Filter Sets

The recommended excitation wavelength is 400 nm and the recommended emission wavelengths are 445 nm and 520 nm (for coumarin and fluorescein, respectively). Select an excitation filter with a bandpass appropriate for coumarin that will not excite fluorescein. Select emission filter sets with appropriate bandpasses so that the emission signals of coumarin and fluorescein do not overlap. We recommend 400 nm (12 nm bandpass) for the excitation wavelength and 445 nm (12 nm bandpass) and 520 nm (12 nm bandpass) for the emission wavelengths. Similar filter sets may be suitable. For additional information on filter sets for a specific instrument, contact Invitrogen's Drug Discovery Technical Support at 1-800-955-6288, press 3, then extension 40266.

7. Data Analysis

7.1 Emission Ratio

Use **Equation 1** to calculate the ratio of the signal intensity of the donor fluorophore to the signal intensity of the acceptor fluorophore upon excitation of the donor fluorophore at 400 nm.

Equation 1:

$$\text{emission ratio} = \frac{\text{coumarin emission signal intensity (at 445 nm)}}{\text{fluorescein emission signal intensity (at 520 nm)}}$$

Both cleaved and uncleaved Z´-LYTE™ Peptide Substrates contribute to the fluorescent signals, and therefore to the emission ratio. The emission ratio will remain low if the substrate is phosphorylated (*i.e.*, little or no kinase inhibition has occurred) and will be high if the substrate is non-phosphorylated (*i.e.*, kinase inhibition has occurred). This ratiometric method for quantitating reaction progress greatly reduces data fluctuations arising from pipetting errors or "edge effects." As a result, Z´-LYTE™ assays generate data with very high Z´-factors (> 0.7), even when only a small proportion of the Z´-LYTE™ Peptide Substrate has been phosphorylated (*i.e.*, even when only a small change in emission ratio is detected).

7.2 Percent Phosphorylation and Calibration Curve

The Z´-LYTE™ Peptide Substrate functions as a 0% phosphorylation control for the assay, and each kit also contains a Z´-LYTE™ Phospho-peptide, which functions as a synthetic 100% phosphorylation control. These controls establish minimum and maximum emission ratio values on your assay plate, and are also used to convert experimental emission ratios to calculated percent phosphorylation values.

As **Figure 2** illustrates, in a control reaction containing both Z´-LYTE™ Phospho-peptide and Z´-LYTE™ Peptide Substrate, as the percentage of phospho-peptide increases, the emission ratio value decreases. The graph of the emission ratio to the phospho-peptide percentage may be less linear for some substrates than for others (*e.g.*, some substrates demonstrate a larger change in the magnitude of the fluorescein signal between the 0% and 100% phosphorylation controls). **Equation 2** is necessary to linearize this relationship (as shown in **Figure 3**), and eliminates the need for a traditional standard curve. **Equation 2** also calculates the extent of phosphorylation of each sample well (containing kinase), based on the 0% and 100% phosphorylation control wells.

Equation 2:*

$$\text{percent phosphorylation} = 1 - \left(\frac{(\text{emission ratio} \times F_{100\%}) - C_{100\%}}{(C_{0\%} - C_{100\%}) + [\text{emission ratio} \times (F_{100\%} - F_{0\%})]} \right) \times 100$$

where:

emission ratio = Ratio of coumarin/fluorescein emission signal intensities from sample wells

$C_{100\%}$ = Average coumarin emission signal intensity of the 100% phosphorylation control

$C_{0\%}$ = Average coumarin emission signal intensity of the 0% phosphorylation control

$F_{100\%}$ = Average fluorescein emission signal intensity of the 100% phosphorylation control

$F_{0\%}$ = Average fluorescein emission signal intensity of the 0% phosphorylation control

*To enter the equation into Microsoft® Excel, use the format below:

$$= 100^* (1–(((\text{emission ratio} * F_{100\%}) – C_{100\%})/((C_{0\%} – C_{100\%}) + (\text{emission ratio} * (F_{100\%} – F_{0\%}))))$$

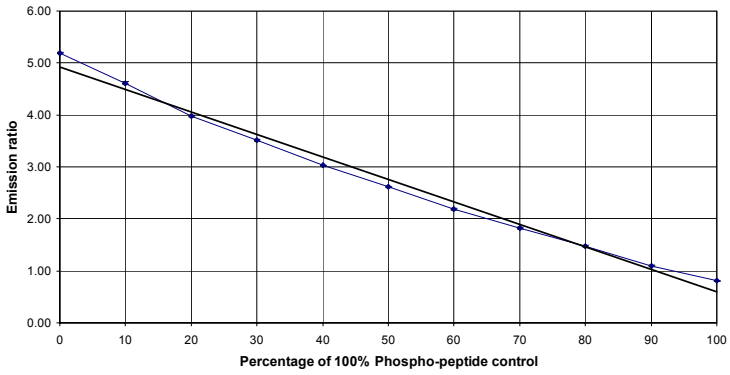


Figure 2. Decrease in emission ratio as a function of the increase in the percentage of the 100% phosphorylation control. A set of 10 solutions containing increasing percentages (from 0% to 100%, in 10% increments) of the Z´-LYTE™ Ser/Thr 13 Phospho-Peptide were mixed with the Z´-LYTE™ Ser/Thr 13 Peptide Substrate in 1X Kinase Buffer (2 µM final total peptide concentration in a 10 µl reaction) and dispensed into the wells of a 384-well assay plate. After the addition of development solution and a one-hour, room-temperature incubation to cleave the Z´-LYTE™ Ser/Thr 13 Peptide Substrate, the coumarin and fluorescein emission signals were measured on a Tecan Safire™. The experimental emission ratio (curved line) was calculated and plotted for each sample. A straight line is shown for comparison.

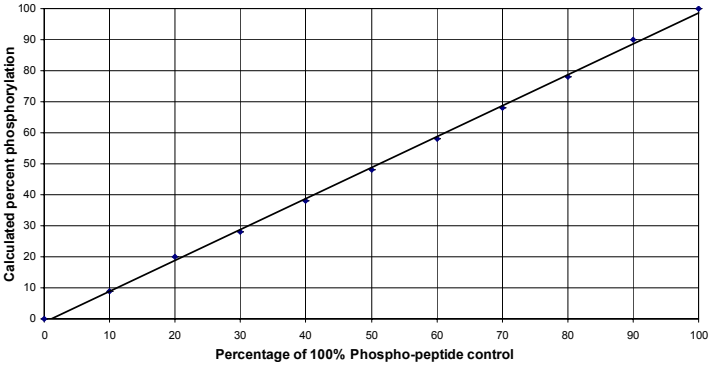


Figure 3. Linearization of emission ratio data using Equation 2. The figure shows the calculated percent phosphorylation, linearized using **Equation 2**, plotted against the percentage of 100% phosphorylation control. **Equation 2** eliminates the need to prepare a standard curve using intermediate proportions of the Z´-LYTE™ Ser/Thr 13 Peptide Substrate and the Z´-LYTE™ Ser/Thr 13 Phospho-peptide. The R^2 value = 0.9991.

8. Assay Considerations

The Z´-LYTE™ assay can accommodate alternative reaction conditions, such as changes in assay volumes or kinase concentrations. We have carefully optimized Development Reagent concentrations, reaction times, and incubation temperatures to provide the best assay window. If you alter these parameters, you will need to re-optimize the assay protocol for the new parameters.

8.1 Assay Controls

The 0% phosphorylation and 100% phosphorylation controls establish the minimal and maximal emission ratio values on an assay plate and also enable you to calculate the percent phosphorylation achieved in a specific reaction well. These controls also enable you to calculate the percent phosphorylation achieved in the 0% inhibition control wells (active kinase, no compound). The 0% inhibition and 0% phosphorylation (100% inhibition) controls define the dynamic range in a given assay. Control wells contain no kinase inhibitor.

Z´-LYTE™ Kinase Assays require the following controls:

0% phosphorylation control (100% inhibition control)
This control contains Z´-LYTE™ Peptide Substrate with either no kinase or kinase with no ATP present, thereby yielding 100% cleaved substrate in the development reaction. It establishes the maximal emission ratio value for an assay plate.

100% phosphorylation control
This control uses Z´-LYTE™ Phospho-peptide in the development reaction, which yields a very low percentage of cleaved peptide. It establishes the minimal emission ratio value on an assay plate. The 100% phosphorylation control, in conjunction with the 0% phosphorylation control, enables you to calculate the percent phosphorylation of all sample wells and the 0% inhibition control wells.

0% Inhibition Control
This control includes Z´-LYTE™ Peptide Substrate, active kinase, and inhibitor compound solvent, but lacks inhibitor compound. This control is designed to produce the recommended 20–50% levels of phosphorylated substrate in the kinase reaction. As shown in **Figures 2 and 3** (the calibration curve), 20–50% of the phosphorylated substrate will produce an emission ratio that falls between the emission ratios of the 0% phosphorylation and 100% phosphorylation controls. The percentage of phosphorylated Z´-LYTE™ Peptide Substrate in the 0% inhibition control wells may vary day-to-day within this 20–50% range, depending on assay conditions; such variations are expected.

8.2 Stability of Diluted Reagents

All diluted buffers and peptides supplied in the kit are stable at room temperature for up to 6 hours, with no loss in assay performance. We recommend using the diluted Development Reagent within two hours of preparation. Although each unique protein kinase has a different level of stability after dilution, we recommend using the diluted kinase as soon as possible after dilution. If this is not possible, store the diluted kinase on ice.

8.3 Addition of Stop Reagent

Add Stop Reagent after the one-hour development reaction, unless you will read the assay plate immediately after this incubation step.

8.4 Stability of Assay Signal

After the Stop Reagent has been added (following the one-hour development reaction), the emission ratio of the assay will not change appreciably for up to 18 hours if the assay plate is covered and sealed (*e.g.*, with foil or stacked) to prevent evaporation and to protect from light. If the assay plate is covered and sealed for an extended period of time, we recommend that you centrifuge the assay plate to reduce the effect of evaporation.

8.5 Solvent and Reagent Tolerances

Although adding the solvents and reagents in the following table up to the specified tolerance limits will not affect the development reaction, they may affect the kinase reaction. Perform the appropriate controls to determine the tolerance limits of the kinase of interest before adding solvents/reagents to the kinase reaction.

Solvent/Reagent	Tolerance Limits
DMSO	2% (v/v)
DTT	1 mM
Ethanol	4% (v/v)
Glycerol	4% (v/v)
Methanol	4% (v/v)

8.6 Assay Plates and Volumes

We recommend using black Corning® 384-well, low-volume, round-bottom (non-binding surface) assay plates with a working volume range of 2–35 µl. For larger or smaller assay volumes, use comparable black, low-binding assay plates.

The final assay volume in the following protocols is 20 µl. You can adapt each protocol for different final reaction volumes (1.6–100 µl) if you use the same component concentrations.

8.7 Incubation Conditions

For optimal results, perform the standard assay at room temperature (20–25°C). If the temperature falls below this range, you may need to increase the incubation times for the kinase and development reactions. Changing the incubation times may also require that you re-optimize the assay protocol.

We recommend that you protect the assay plates from light (*e.g.*, by loosely covering them with foil, stacking them and/or incubating them in the dark) during the one-hour incubation steps.

8.8 Recommendations for the Kinase Reaction

Determine the optimal kinase concentration, ATP concentration, and reaction time empirically for each kinase (see **Assay Optimization Procedure,** page 7). We recommend using ATP at near $K_{M\ [apparent]}$ concentrations (see **Determining ATP Km [apparent],** page 11; for the pre-determined ATP $K_{M\ [apparent]}$ concentrations of kinases that Invitrogen supplies, see www.invitrogen.com/kinaseprofiling). We also recommend using a kinase concentration that phosphorylates 20–50% of the Z´-LYTE™ Peptide Substrate in a one-hour, room-temperature (20–25°C) incubation.

Some protein kinases require buffer supplements for optimal activity. Common buffer supplements include $CaCl_2$, calmodulin, $MnCl_2$, DTT, and lipids. If you add supplements to the kinase reaction buffer, use the same reaction buffer composition for the assay controls (0% and 100% phosphorylation controls, 0% inhibition controls). For further details, see the SelectScreen™ Kinase Profiling Service Screening Protocol and Assay Conditions at www.invitrogen.com/kinaseprofiling.

8.9 Recommendations for the Development Reaction

When you dilute Development Reagent A as specified in the Development Reagent Certificate of Analysis, it will completely cleave all non-phosphorylated Z´-LYTE™ Peptide Substrate in the one-hour, room-temperature (20–25°C) development reaction. We have chosen the concentration of the Development Reagent to maximize the assay window.

9. Assay Optimization Procedure

This section provides a recommended method for optimizing the Z´-LYTE™ Ser/Thr 13 Peptide Substrate for use with a particular kinase. The only variable is the kinase concentration. The assay is optimized for a one-hour, room-temperature incubation; however, you can modify the protocol to examine the effects of ATP concentration or the incubation time on the kinase reaction. Use your experimental data to guide your choice of an appropriate kinase concentration within the linear range to obtain the desired percent phosphorylation with an acceptable Z´-factor. Experimental factors such as incubation time and reaction temperature affect the actual percent phosphorylation obtained, which can exhibit day-to-day variations. The volumes provided below are sufficient for the 384-well assay plate layout described in this section.

9.1 Prepare Reagents

Note: Thaw and store the kinase and Development Reagent on ice before diluting any reagents. Equilibrate all other assay components to room temperature.

1. Add 2 ml of 5X Kinase Buffer to 8 ml water to prepare 10 ml of **Kinase Buffer A**.

   Note: The final 1X concentration of **Kinase Buffer A** is 50 mM HEPES (pH 7.5), 1 mM EGTA, 0.01% Brij-35, and 10 mM $MgCl_2$.

2. Take a 5-ml aliquot of **Kinase Buffer A** and add any supplements required for optimal kinase activity to 2X the final desired 1X concentration per 10 µl of kinase reaction **(Kinase Buffer X)**. For example, if you want **Kinase Buffer X** to have a final $MnCl_2$ concentration of 2 mM, then supplement this 5 ml aliquot with 4 mM $MnCl_2$.

   Points to consider:

   - Because the final 1X concentration of **Kinase Buffer A** contains 1 mM EGTA, if your kinase is calcium-dependent, the final 1X $CaCl_2$ concentration per 10 µl kinase reaction should be ≥ 2 mM. For these kinases, you may want to prepare your own **Kinase Buffer A** to eliminate or reduce the concentration of EGTA.
   - Some tyrosine kinases require concentrations of manganese as the co-factor for ATP rather than the 10 mM concentration of magnesium as supplied in **Kinase Buffer A**. For some of these tyrosine kinases, adding additional $MnCl_2$ as a supplement to Kinase Buffer A may be optimal. For others, making a

Kinase Buffer A composition by eliminating or reducing the concentration of $MgCl_2$ and adding additional $MnCl_2$ may be optimal.

- Some protein kinases require a reducing agent, such as DTT, for optimal activity. If you add DTT as a supplement to the kinase reaction, the final 1X DTT concentration should be less than the threshold described in **Solvent and Reagent Tolerances**, page 6.
- If you supplement the buffer with additional divalent cations, the total 1X amount per 10 µl kinase reaction should be less than 15 mM. Otherwise, the Development Buffer will not be able to completely quench the kinase reaction.
- If you add any additional buffer supplements to the kinase reaction, use the identical buffer composition as the kinase reaction for the assay controls.

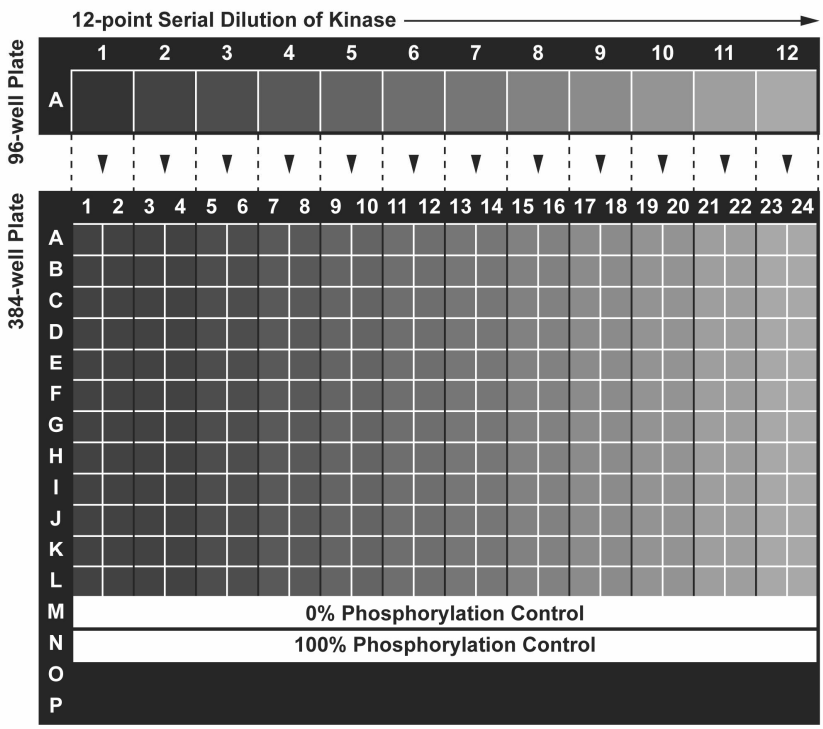
3. Choose the maximal final kinase concentration to be tested in the assay, and prepare a 2X Kinase Solution by diluting the kinase to 2X the maximal concentration in **Kinase Buffer X** to a final volume of 300 µl. Mix gently by pipetting; do not vortex. Use the diluted kinase immediately following dilution and store on ice.
4. Prepare 2X Z´-LYTE™ Peptide Substrate/2X ATP Mixture. Determine the desired ATP concentration for the assay (to determine the ATP $K_{M\,[apparent]}$ for a particular kinase, see page 11). Prepare the 2X Z´-LYTE™ Peptide Substrate/2X ATP Mixture by adding 8 µl Z´-LYTE™ Peptide Substrate, the appropriate amount of ATP, and the remaining amount as **Kinase Buffer A** to equal a total volume of 2,000 µl. The final concentration of ATP in this mixture should be 2X the desired final concentration used in the assay. Mix thoroughly.

   Note: Generally, if you use a lower concentration of ATP in your assay, you will need a greater amount of protein kinase per well to phosphorylate a given percentage of Z´-LYTE™ Peptide Substrate.
5. Prepare 2X Z´-LYTE™ Phospho-peptide Solution by adding 2 µl Z´-LYTE™ Phospho-peptide to 498 µl of **Kinase Buffer A**.
6. Prepare the Development Solution by referring to the Development Reagent Certificate of Analysis enclosed as a separate document with this kit. The Certificate of Analysis indicates the correct dilution for each lot of Development Reagent into Development Buffer. Dilution factors for the Development Reagent can vary from lot to lot.

9.2 Kinase Dilution

Use a 96-well assay plate that can accommodate up to 300 µl per well to create a dilution series of 12 kinase concentrations. The 384-well assay plate will contain 24 replicates (n = 24) of each kinase concentration from the 96-well assay plate. These 24 replicates of each kinase concentration, along with the 24 replicates of the 0% phosphorylation control (see the template on the next page), will be used to determine a Z´-factor calculation.

1. Dispense 140 µl of **Kinase Buffer X** to wells A2–A12 of a 96-well assay plate. Do not add buffer to well A1, because it will contain the highest kinase concentration.
2. Dispense 280 µl of kinase solution (in **Kinase Buffer X**) to well A1 of the 96-well assay plate.
3. Perform a two-fold serial dilution of the kinase solution by titering 140 µl from well A1 through well A12. Discard the final 140 µl so that wells A1–A12 of the 96-well assay plate contain 140 µl.
4. Using a multi-channel pipette, transfer 5 µl diluted kinase from each well in row A (wells A1–A12) of the 96-well assay plate to duplicate columns of the first 12 rows (rows A–L) of a 384-well assay plate, as shown in the template to the right. This means that cells A–L in columns 1 and 2 of the 384-well assay plate will receive 5-µl aliquots from cell A1 of the 96-well assay plate, cells A–L in columns 3 and 4 of the 384-well assay plate will receive 5-µl aliquots from cell A2 of the 96-well assay plate, and so on.



9.3 Kinase Reaction (384-well assay plate)

1. Dispense 5 µl of **Kinase Buffer X** to each well in rows M and N (wells M1–N24).
2. Dispense 5 µl of 2X Z´-LYTE™ Phospho-peptide Solution to each well in row N (wells N1–N24).
3. Add 5 µl of 2X Z´-LYTE™ Peptide Substrate/2X ATP Mixture to each well in rows A–M (wells A1–M24).
4. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
5. Incubate the assay plate for one hour at room temperature (20–25°C).

9.4 Development Reaction

1. Add 5 µl of Development Solution to each well in rows A–N (wells A1–N24).
2. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
3. Incubate the assay plate for one hour at room temperature (20–25°C).

9.5 Stop Step and Fluorescence Detection

1. Add 5 µl of Stop Reagent to each well in rows A–N (wells A1–N24).
2. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
3. Measure the coumarin and fluorescein emission signals on a fluorescence plate reader (excitation: 400 nm; emission 445 and 520 nm, respectively).

9.6 Analyze Data

1. Calculate the emission ratio for each sample and control well (see **Equation 1** page 4).
2. Calculate the percent phosphorylation for each sample and control well (see **Equation 2**, page 4).
3. Calculate the Z´-factor for each set of kinase concentrations (n = 24). The Z´-factor indicates the quality of an assay; Z´-factors ≥ 0.5 classify an assay as excellent. To calculate the Z´-factor, use the wells in row M, which

contain no kinase, as the 0% phosphorylation (100% inhibition) control wells, and the wells in a particular set of duplicate columns containing the identical kinase concentration as the 0% inhibition control wells. Repeat this calculation for each of the 12 kinase concentrations used.

- From the emission ratio values calculated in Step 1, calculate the average and standard deviation for each set of kinase concentrations and the control wells (n = 24).
- Calculate the Z´-factor for each set of kinase concentrations (n = 24) using the following equation:

Equation 3:

$$\text{Z´-factor} = 1 - \left(\frac{[(3 * \text{standard deviation}_{100\% \text{ Inhibition}}) + (3 * \text{standard deviation}_{0\% \text{ Inhibition}})]}{(\text{mean}_{100\% \text{ Inhibition}} - \text{mean}_{0\% \text{ Inhibition}})} \right)$$

4. Based upon the Z´-factors calculated above, choose an appropriate kinase concentration for subsequent experiments that yielded a Z´-factor > 0.5. We recommend using ATP concentrations near $K_{M\,[apparent]}$ (see page 11) and a kinase concentration that phosphorylates 20–50% of the Z´-LYTE™ Peptide Substrate in the one-hour, room-temperature incubation. By selecting a kinase concentration that phosphorylates 20–50% of substrate, assay results will fall within the lower end of the linear range of the titration curve. Therefore, the assay can sensitively detect "hits" in a screening assay. Further, if you do or do not want to detect competitive inhibitors for ATP in a screen, you can perform the assay near, below, or above the ATP $K_{M\,[apparent]}$ concentration. **Figure 4** and **Table 1** show representative data.

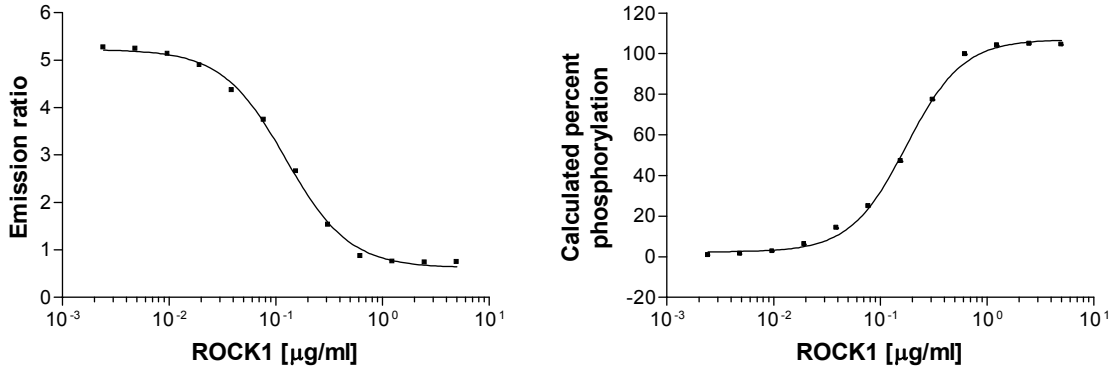


Figure 4. Representative sample data generated for ROCK1 (Invitrogen catalog no. PV3691) with the Z´-LYTE™ Ser/Thr 13 Peptide Substrate. The assay was performed in a 384-well assay plate following the assay optimization procedure (described starting on page 7) in 1X Kinase Buffer supplemented with 100 µM ATP (final 1X concentration per 10 µl kinase reaction). The graph on the left plots the emission ratio values as a function of kinase concentration and the graph on the right plots the calculated percent phosphorylation as a function of kinase concentration. Curve fitting (sigmoidal dose response, variable slope) and data presentation were generated using Prism® 4.0 software from GraphPad Software, Inc.

Table 1. Percent phosphorylation and Z'-factor corresponding to kinase concentrations (calculated from the representative data generated in **Figure 4**).

Kinase concentration (ng/ml)	Mean emission ratio (n = 24)	Emission ratio stdev (n = 24)	Emission ratio 3*stdev	Emission ratio %CV	Emission ratio Z'-factor	Calculated percent phosphorylation
5,000	0.738	0.020	0.060	2.70	0.97	104
2,500	0.726	0.013	0.038	1.75	0.98	105
1,250	0.747	0.017	0.052	2.32	0.97	104
625	0.861	0.019	0.057	2.22	0.97	100
312.5	1.525	0.055	0.164	3.59	0.94	77
156.25	2.652	0.059	0.176	2.22	0.91	47
78.13	3.737	0.056	0.167	1.49	0.85	25
39.06	4.360	0.109	0.328	2.51	0.59	14
19.53	4.886	0.049	0.146	0.99	0.51	6
9.77	5.131	0.041	0.122	0.79	-0.01	3
4.88	5.231	0.047	0.141	0.90	-1.38	1
2.44	5.262	0.043	0.129	0.82	-2.48	1
0	5.318	0.022	0.067	0.42	N/A	0

10. Determining ATP $K_{m\ [apparent]}$

This section will help you determine an estimated ATP $K_{M\ [apparent]}$ value for a protein kinase of interest. Because all Z´-LYTE™ kinase assays are performed at 2 µM Z´-LYTE™ Peptide Substrate (final 1X concentration per 10-µl kinase reaction), the ATP $K_{M\ [apparent]}$ value determined may or may not be a true reflection of the real ATP K_M value. This discrepancy arises because the concentration of Z´-LYTE™ Peptide Substrate used (2 µM) may be below, equal to, or above the substrate K_M value. Therefore, when the Z´-LYTE™ kinase assays are performed under the recommended reaction conditions of 2 µM Z´-LYTE™ Peptide Substrate, the ATP concentration found to give ½ maximal velocity is called ATP $K_{M\ [apparent]}$ (*i.e.*, the ATP K_M under the Z´-LYTE™ kinase assay conditions).

This procedure uses a dual matrix involving a kinase titration (n = 2) down the 384-well assay plate against a number of variable ATP concentrations across the 384-well assay plate. Therefore, this protocol will also enable the user to determine the concentration of kinase needed per reaction to obtain a certain percent phosphorylation at a given ATP concentration. The volumes provided below are sufficient for the assay plate layout described on page 14.

Note: We use the following protocol to determine ATP $K_{M\ [apparent]}$ values. All kinase and ATP dilutions are prepared individually (rather than by serial dilutions) to reduce error propagation.

10.1 Prepare 0% and 100% phosphorylation controls

Note: Thaw and store the kinase and Development Reagent on ice before diluting any reagents. Equilibrate all other assay components to room temperature.

1. Add 2 ml of 5X Kinase Buffer to 8 ml water to prepare 10 ml of **Kinase Buffer A**.

   Note: The final 1X concentration of **Kinase Buffer A** is 50 mM HEPES (pH 7.5), 1 mM EGTA, 0.01% Brij-35, and 10 mM $MgCl_2$.

2. Prepare 1,500 µl of 8 µM Z´-LYTE™ Peptide Substrate by adding 12 µl of 1 mM Z´-LYTE™ Peptide Substrate to 1,488 µl of Kinase Buffer A (4X Z´-LYTE™ Peptide Substrate).

3. Prepare 200 µl of 4 µM Z´-LYTE™ Peptide Substrate by adding 100 µl of 4X Z´-LYTE™ Peptide Substrate (prepared in Step 2) to 100 µl of Kinase Buffer A (2X Z´-LYTE™ Peptide Substrate).

4. Dispense 5 µl of the 2X Z´-LYTE™ Peptide Substrate to wells A21–P22 of the 384-well assay plate (see **Assay Plate Layout,** page 14).

5. Prepare 500 µl of 4 µM Z´-LYTE™ Phospho-peptide by adding 2 µl of 1 mM Z´-LYTE™ Phospho-peptide to 498 µl of Kinase Buffer A (2X Z´-LYTE™ Phospho-peptide).

6. Dispense 5 µl of 2X Z´-LYTE™ Phospho-peptide to wells A23–P24 of the 384-well assay plate (see **Assay Plate Layout,** page 14).

10.2 Prepare 2X Z´-LYTE™ Peptide Substrate/2X ATP

1. Prepare 500 µl of 1 mM ATP by adding 50 µl of 10 mM ATP to 450 µl of Kinase Buffer A.
2. Prepare the following dilutions from either 10 mM or 1 mM ATP (prepared in Step 1) in wells A1–A10 of a 96-well assay plate (non-binding) that can accommodate up to 300 µl per well:

Well	Vol. (µl) of 10 mM ATP	Vol. (µl) of 1 mM ATP	Vol. (µl) of Kinase Buffer A	Intermediate 4X ATP Conc. (µM)	Final 1X ATP Conc. (µM)
A1	50	—	75	4000	1000
A2	25	—	100	2000	500
A3	12.5	—	112.5	1000	250
A4	7.5	—	117.5	600	150
A5	5	—	120	400	100
A6	3.75	—	121.2	300	75
A7	—	25	100	200	50
A8	—	12.5	112.5	100	25
A9	—	5	120	40	10
A10	—	2.5	122.5	20	5

Note: You can easily modify the protocol to perform assays using ATP concentrations other than those described in the table above. Adjust the volumes of ATP and Kinase Buffer A appropriately to maintain the desired 4X ATP concentration in a 125 µl volume.

3. Dispense 125 µl of 4X Z´-LYTE™ Peptide Substrate (prepared in Section 10.1, Step 2) to wells A1–A10 of the 96-well assay plate (each well A1–A10 contains 125 µl of the appropriate ATP concentration prepared above).
4. Using a multi-channel pipette, mix the contents of wells A1–A10 of the 96-well assay plate by pipetting up and down.
5. Using a multi-channel pipette, transfer 5 µl of the 2X Z´-LYTE™ Peptide Substrate/2X ATP dilution mixtures from wells A1–A10 of the 96-well assay plate to duplicate columns of all 16 rows (rows A–P) of a 384-well assay plate, as shown in the template in Section 10.6. This means that cells A–P in columns 1 and 2 of the 384-well assay plate will receive 5-µl aliquots from cell A1 of the 96-well assay plate, cells A–P in columns 3 and 4 of the 384-well assay plate will receive 5-µl aliquots from cell A2 of the 96-well assay plate, and so on.

10.3 Prepare Kinase Titration

1. Take a 3.5-ml aliquot of Kinase Buffer A and add any supplements required for optimal kinase activity to 2X the final desired 1X concentration per 10-µl kinase reaction (Kinase Buffer X). For example, if you want Kinase Buffer X to have a final $MnCl_2$ concentration of 2 mM, then supplement this 3.5-ml aliquot with 4 mM $MnCl_2$ (see **Points to Consider** under Step 2 on page 7).
2. Choose the maximal final kinase concentration to be tested in the assay, and prepare a 2X Kinase Solution by diluting the kinase to 2X the maximal concentration in **Kinase Buffer X** to a final volume of 330 µl. Mix gently by pipetting; do not vortex. Place the diluted kinase on ice and use immediately following dilution.

   Note: This protocol starts the kinase titration at 10 µg/ml (2X = 20 µg/ml). You can modify the protocol to start the titration at either a higher or lower concentration depending on the kinase. Do not change any volumes or other dilution factors (adjust the 2X starting concentration as needed).
3. Prepare 400 µl of a 1/200 dilution of the desired kinase concentration by adding 2 µl of 2X Kinase Solution (prepared in Step 2) to 398 µl of Kinase Buffer X.

   Note: The concentration of the 1/200 dilution prepared here will be at 0.1 µg/ml (2X starting concentration = (20 µg/ml)/200 = 0.1 µg/ml). You can easily modify the protocol to start the titration at either a higher or lower concentration depending on the kinase. Do not change any volumes or other dilution factors (adjust the 2X starting concentration as needed).
4. Prepare the dilutions in the following table from the 2X Kinase Solution (20 µg/ml kinase dilution prepared in Step 2) or the 0.1 µg/ml kinase dilution (prepared in Step 3) in the designated wells in column 1 of a 96-well assay plate (non-binding) that can accommodate up to 300 µl per well.

Well	Vol. of Kinase Buffer X added (µl)	Vol. of 2X Kinase Solution added (µl)
A1	—	225.0
B1	169	56.3
C1	211	14.1
D1	221	3.5

Well	Vol. of Kinase Buffer X added (µl)	Vol. of 1/200 2X Kinase Solution added (µl)
E1	49	175.8
F1	181	43.9
G1	214	11.0
H1	222	2.7

5. Using a multi-channel pipette, mix the contents of wells A1–H1 of the 96-well assay plate by pipetting up and down.
6. Add 75 µl of **Kinase Buffer X** to the wells in column 2 (wells A2–H2) of the same 96-well assay plate.
7. Using a multi-channel pipette, transfer 75 µl of the diluted kinase from wells A1–H1 to the corresponding wells in column 2 (wells A2–H2). This means that 75 µl each is transferred from well A1 to well A2, from well B1 to well B2, and so on). Mix the contents of the wells thoroughly by pipetting up and down. The 2X concentrations of kinase in column 2 will now be ½ the 2X concentration in column 1.
8. Using a multi-channel pipette, transfer 5 µl of the 2X kinase dilutions from each well in column 1 (wells A1–H1) of the 96-well assay plate to each well in alternating rows (rows A, C, E, G, I, K, M, and O) in all columns (columns 1–24) of the 384-well assay plate (see **Assay Plate Layout**, page 14).
9. Using a multi-channel pipette, transfer 5 µl of the 2X kinase dilutions from each well in column 2 (wells A2–H2) of the 96-well assay plate to each well in alternating rows (rows B, D, F, H, J, L, N, and P) in all columns (columns 1–24) of the 384-well assay plate.
10. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
11. Incubate the assay plate for one hour at room temperature (20–25°C).

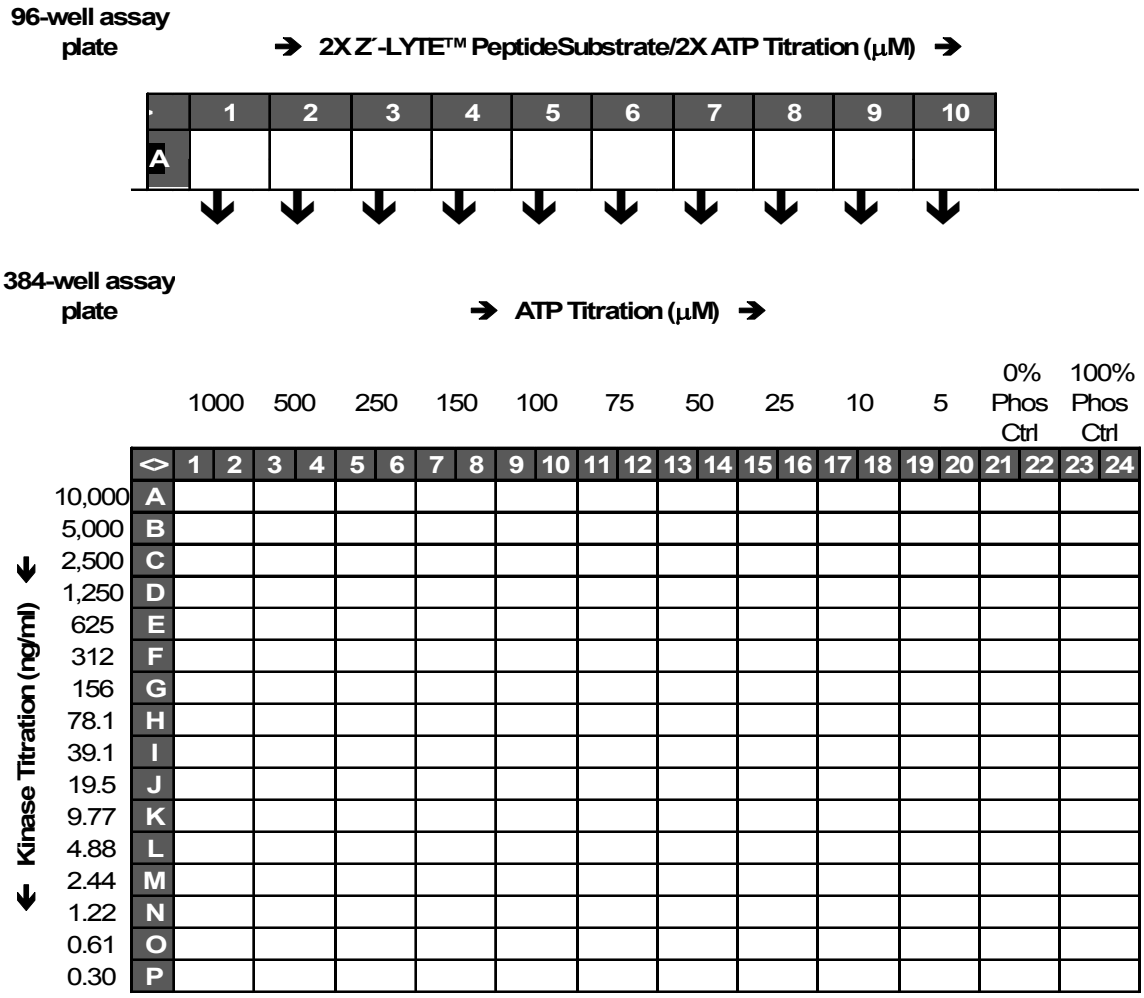
10.4 Prepare Development Solution

1. Prepare Development Solution as specified in the Development Reagent Certificate of Analysis included with this kit. The Certificate of Analysis indicates the correct dilution for each lot of Development Reagent into Development Buffer. Dilution factors for the Development Reagent can vary from lot to lot.
2. Add 5 µl of Development Solution to each well in the 384-well assay plate.
3. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
4. Incubate the assay plate for one hour at room temperature (20–25°C).

10.5 Stop Step and Fluorescence Detection

1. Add 5 µl of Stop Reagent to each well in the 384-well assay plate.
2. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
3. Measure the coumarin and fluorescein emission signals on a fluorescence plate reader (excitation: 400 nm; emission 445 and 520 nm, respectively).

10.6 Assay Plate Layout



10.7 Analyze Data

1. Calculate the emission ratio for each sample well and control well (see **Equation 1**, page 4).
2. Calculate the percent phosphorylation for each sample well and control well (see **Equation 2**, page 4).
3. Graph the percent phosphorylation (y-axis) versus the log ATP concentration (x-axis) for each kinase concentration. For representative data, see Figure 5a.
4. Graph the percent phosphorylation (y-axis) versus the log kinase concentration (x-axis) for each ATP concentration. For representative data, see Figure 5b.

From the graph generated in Step 3 above, select kinase concentrations that plateau between 20–50% phosphorylation. Using an appropriate graphing software program, determine the ½ maximal values (EC_{50}) of ATP for each kinase concentration and average the values to calculate the ATP $K_{M\,[apparent]}$ value. Also, by using an appropriate graphing software program, you can determine the amount of kinase needed to phosphorylate the Z´-LYTE™ Peptide Substrate to the recommended 20–50% at a given ATP concentration from the graph generated in Step 4.

Note: The ATP $K_{M\,[apparent]}$ value determination described in this section was chosen for simplicity and ease of use. At Invitrogen, we also verify these values by graphing the reaction velocity (nmol/min/mg) versus [ATP] utilizing the Michaelis-Menton curve fitting technique. For further details about this methodology, contact Invitrogen's Drug Discovery Technical Support at 1-800-955-6288, press 3, then extension 40266.

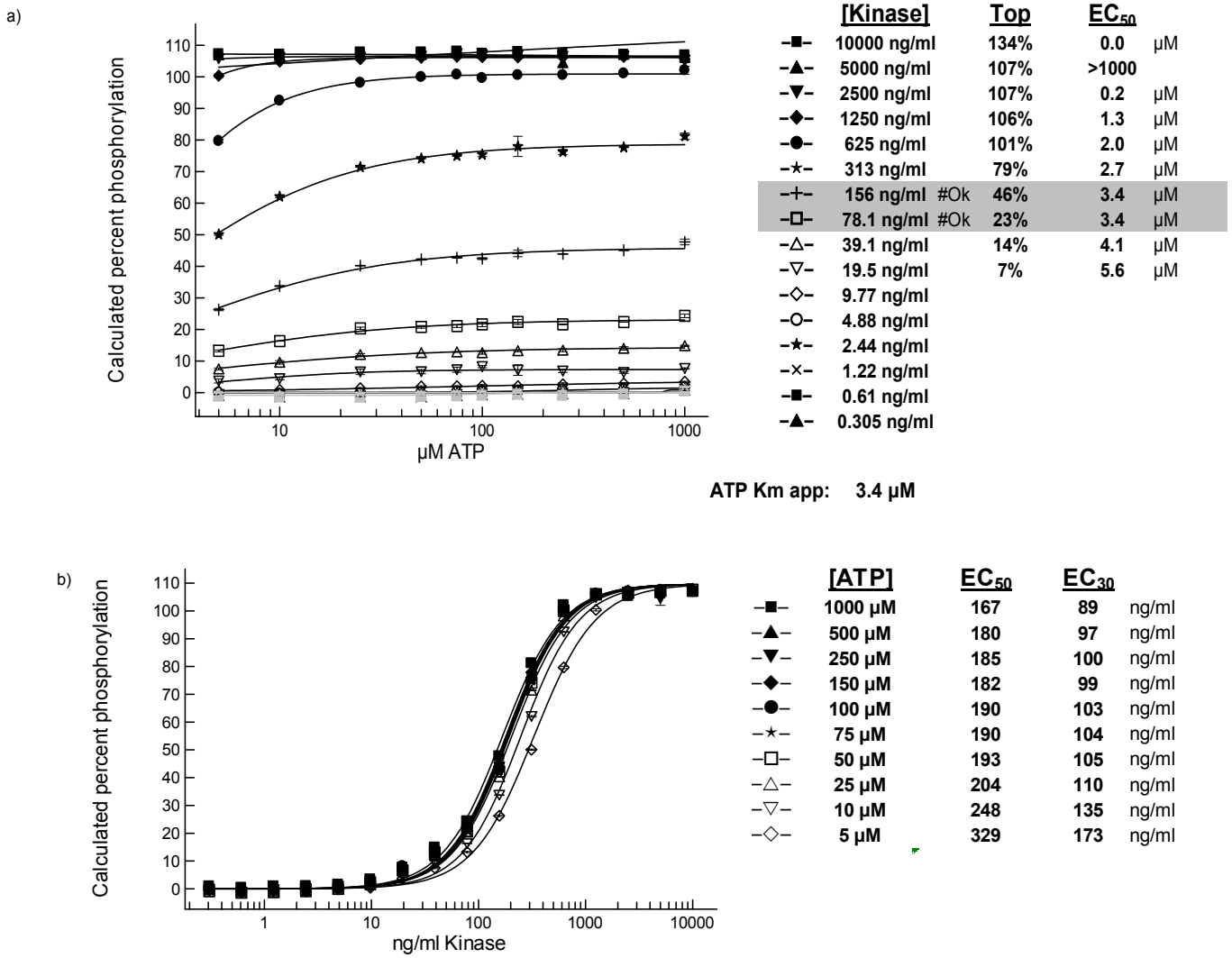


Figure 5. Representative sample data generated for ROCK1 (Invitrogen catalog no. PV3691) with the Z´-LYTE™ Ser/Thr 13 Peptide Substrate. The assay was run in a 384-well assay plate following the procedure for the determination of ATP $K_{M\,[apparent]}$ in 1X Kinase Buffer. Graph (a) shows a plot of ATP concentration versus percent phosphorylation at multiple kinase concentrations. For plots that plateau between 20–50% phosphorylation, the EC_{50} for ATP was averaged to determine the ATP $K_{M\,[apparent]}$. Graph (b) shows a plot of kinase concentration versus percent phosphorylation at multiple ATP concentrations. The concentrations of kinase that gave 50% phosphorylation (EC_{50}) and 30% phosphorylation (EC_{30}) were calculated at a given ATP concentration. Curve fitting and data presentation were performed using XL*fit*® 4.1 software (ID Business Solutions Inc. (IDBS), Emeryville CA).

11. Determining IC_{50} Values for Test Compounds

This section describes how to determine IC_{50} values for test compounds for a particular protein kinase of interest. The IC_{50} values for test compounds can vary depending on a number of reaction conditions. Using higher concentrations of kinase in a reaction will generally yield a greater percentage of phosphorylated substrate (at a given ATP concentration) and will require more test compound to inhibit 50% of kinase activity (*i.e.*, a higher IC_{50} value). In addition, because most test compounds are competitive inhibitors against ATP, the observed IC_{50} value can vary depending on the ATP concentration used in an assay (a higher ATP concentration in the reaction will require more test compound to inhibit 50% of kinase activity). Therefore, we recommend using a kinase concentration that phosphorylates 20–50% of the Z´-LYTE™ Peptide Substrate at the ATP $K_{M\,[apparent]}$ value. Although you can modify the kinase and ATP concentrations for the particular reaction conditions of choice, such modifications can affect your IC_{50} values.

Note: The volumes listed below are sufficient to test two compounds or two reaction conditions. When assaying more compounds and/or testing additional reaction conditions, scale up the volumes

appropriately. As shown in the assay plate template (see **Assay Plate Layout**, page 18), you can test up to eight compounds/reaction conditions per 384-well assay plate.

11.1 Prepare Kinase Reaction Buffer

Note: Thaw and store the kinase and Development Reagent on ice before preparing dilutions. Equilibrate all other assay components to room temperature.

1. Add 1 ml 5X Kinase Buffer to 4 ml water to prepare 5 ml of Kinase Buffer A.

   Note: The final concentration of Kinase Buffer A is 50 mM HEPES (pH 7.5), 1 mM EGTA, 0.01% Brij-35, and 10 mM $MgCl_2$.

2. Take a 2 ml aliquot of **Kinase Buffer A** and add any supplements required for optimal kinase activity to 2X the final desired 1X concentration per 10 µl kinase reaction (**Kinase Buffer X**). For example, if you wish **Kinase Buffer X** to have a final $MnCl_2$ concentration of 2 mM, then supplement this 2 ml aliquot with 4 mM $MnCl_2$ (see **Points to Consider** under Step 2 on page 7).

3. Prepare 4 ml of **complete kinase reaction buffer** by combining a 2 ml aliquot of **Kinase Buffer X** with a 2 ml aliquot of **Kinase Buffer A**.

11.2 Prepare Test Compounds

1. Thaw the concentrated stock of test compound (usually stored in 100% DMSO) at room temperature.

2. Add 10 µl of 100% DMSO to row A, columns 2–12 (wells A2–A12) in a 96-well assay plate (non-binding surface). To well A1, add 15 µl of the concentrated stock of test compound. This will be the highest concentration of test compound in a 10-point titration curve. Use a concentration that is 100X the final desired 1X concentration per 10 µl kinase reaction. For example, if the final 1X concentration of 100 µM is desired for the highest concentration of test compound in the 10 point titration curve, add 15 µl of 10 mM test compound to well A1.

3. Titrate the 100X test compound three-fold across the assay plate from well A1 to well A10. To perform this three-fold titration, transfer 5 µl of the 100X test compound from well A1 to the 10 µl of 100% DMSO in well A2. Repeat for wells A2–A10. Discard the final 5 µl from well A10 so that all wells contain 10 µl. Do not titrate the compound into wells A11 and A12, because these will be your vehicle-only (DMSO) control for the 0% inhibition, 0% phosphorylation, and 100% phosphorylation controls. This completes the 10-point three-fold titration of test compound at 100X concentration.

4. Transfer a 2 µl aliquot of the 100X test compound titration series from each well in row A of the 96-well assay plate (wells A1–A12) to row B (wells B1–B12). Add 48 µl of complete kinase reaction buffer to each well in row B to dilute the 100X three-fold titration series of test compound to 4X (the DMSO will also be diluted, to 4%).

5. Transfer 2.5 µl of the 4X concentrated three-fold titration series of test compound from row B of the 96-well assay plates to quadruplicate wells of a 384-well assay plate (see **Assay Plate Layout**, page 18). Each well in columns 21–24 should contain 2.5 µl of 4% DMSO (no compound) in complete kinase reaction buffer. This 2.5 µl addition of 4X test compound in 4% DMSO produces a 1X concentration of test compound in 1% DMSO for a 10 µl kinase reaction.

6. Dispense 2.5 µl of complete kinase reaction buffer to each well in columns 23–24 of the 384-well assay plate.

11.3 Prepare Reagents

1. Prepare 500 µl of 4 µM Z´-LYTE™ Phospho-peptide by adding 2 µl of 1 mM Z´-LYTE™ Phospho-peptide to 498 µl of complete kinase reaction buffer.

2. Dispense 5 µl of the 4 µM Z´-LYTE™ Phospho-peptide (2X) to each well in column 24 of the 384-well assay plate (see **Assay Plate Layout**, page 18).

3. Prepare 1000 µl of 4 µM Z´-LYTE™ Peptide Substrate (2X)/2X kinase solution in complete kinase reaction buffer. If necessary, prepare an intermediate dilution of the kinase in complete kinase reaction buffer before preparing the Z´-LYTE™ Peptide Substrate (2X)/2X kinase solution. Use the appropriate 2X kinase concentration in the assay to phosphorylate 20–50% of the 0% inhibition controls at the ATP concentration desired (see **Sections 9 and 10**).

4 µl of 1 mM Z´-LYTE™ Peptide Substrate µl kinase = (1000 µl) × ([kinase]$_{2x\ Assay}$ µg/ml)/([kinase]$_{stock}$ µg/ml) µl **complete kinase reaction buffer** = 996 µl – (____µl kinase)
∴ 4 µl of 1 mM Z´-LYTE™ Peptide Substrate + (__µl kinase) + (__µl complete kinase reaction buffer)

4. Dispense 5 µl of the Z´-LYTE™ Peptide Substrate (2X)/2X kinase solution to each well in columns 1–23.
5. Prepare 1000 µl of 4X ATP in complete kinase reaction buffer. Use the appropriate 4X ATP concentration at the desired kinase concentration to achieve 20–50% phosphorylation of the 0% inhibition controls in the assay.

___µl ATP = (1000 µl) × ([ATP]$_{4x\ Assay}$ µM)/([10 mM ATP stock]) ___µl **complete kinase reaction buffer** = 1000 µl – (___µl ATP)
∴ (____µl ATP) + (____ µl complete kinase reaction buffer) = 1000 µl

6. Dispense 2.5 µl of the 4X ATP in complete kinase reaction buffer to each well in columns 1–22.
7. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
8. Incubate the assay plate for one hour at room temperature (20–25°C).

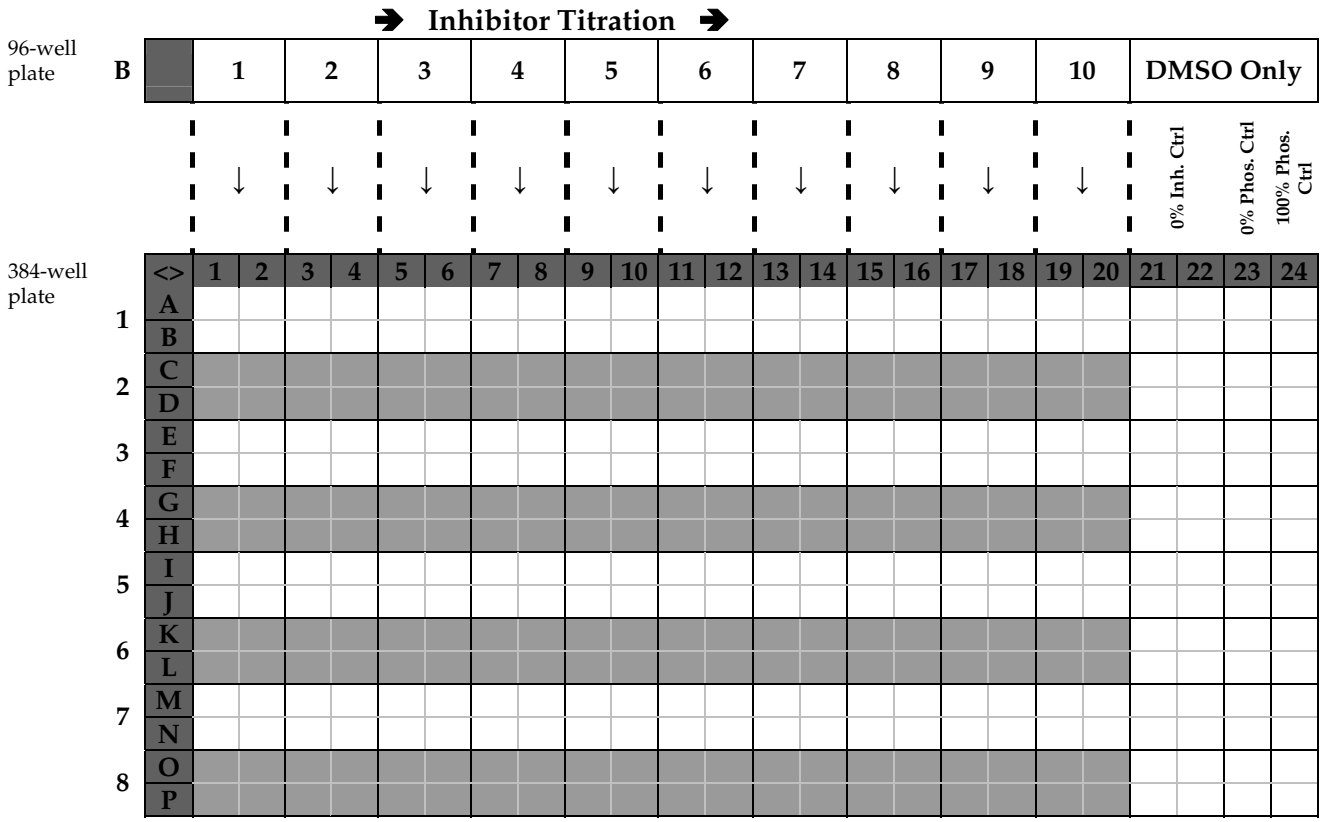
11.4 Prepare Development Solution

1. Prepare Development Solution as specified in the Development Reagent Certificate of Analysis included with this kit. The Certificate of Analysis indicates the correct dilution for each lot of Development Reagent into Development Buffer. Dilution factors for the Development Reagent can vary from lot to lot.
2. Add 5 µl of Development Solution to each well in the 384-well assay plate.
3. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
4. Incubate the assay plate for one hour at room temperature (20–25°C).

11.5 Stop Step and Fluorescence Detection

1. Add 5 µl of Stop Reagent to each well in the 384-well assay plate.
2. Shake the assay plate on a plate shaker for 30 seconds to mix the reactions thoroughly.
3. Measure the coumarin and fluorescein emission signals on a fluorescence plate reader (excitation: 400 nm; emission 445 and 520 nm, respectively).

11.6 Assay Plate Layout



Note: You can test up to eight compounds or reaction conditions on a 384-well assay plate.

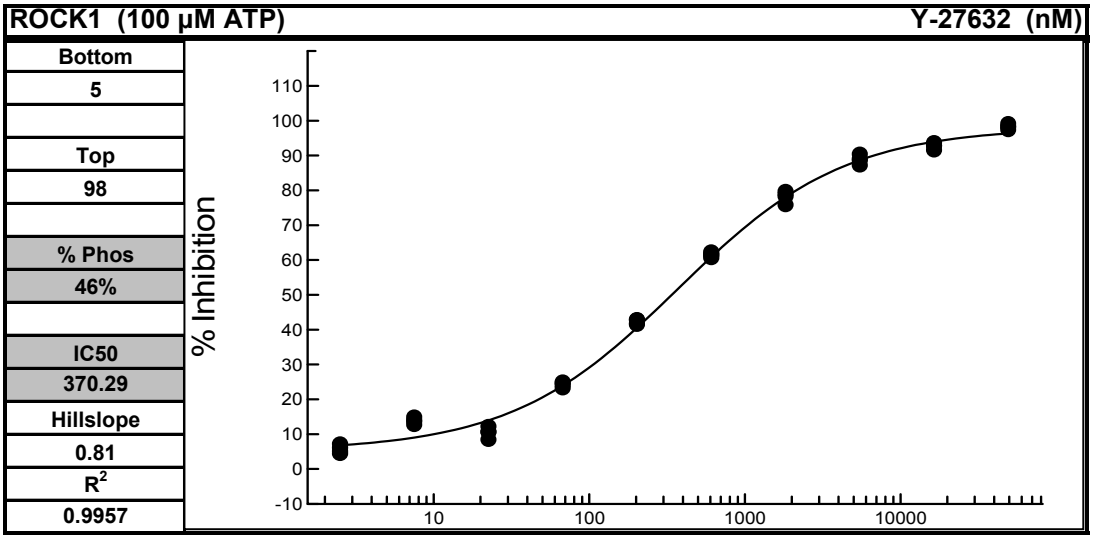
11.7 Analyze Data

1. Calculate the emission ratio for each sample and control well (see **Equation 1**, page 4).
2. Calculate the percent phosphorylation for each sample well and control well (see **Equation 2**, page 4).
3. Calculate the percent inhibition for each sample well in the inhibitor titration series using the following equation:

   Equation 4:

$$\text{percent inhibition} = 100 \times \left(1 - \left(\frac{\text{percent phosphorylation of test compound well}}{\text{percent phosphorylation of 0\% inhibition control}}\right)\right)$$

4. Graph the percent inhibition (y-axis) versus the log concentration of the text compound (x-axis) using an appropriate graphing software program. From this graph, calculate the test compound concentration that inhibits kinase activity by 50% (the IC_{50} value). For representative data, see Figure 6.



ROCK1 (100 µM ATP) **Y-27632 (nM)**

Y-27632 (nM)	ROCK1 (1)	ROCK1 (2)	ROCK1 (3)	ROCK1 (4)	Average	SEM
50000.000	98	99	97	98	98	1
16666.667	93	93	91	93	92	1
5555.556	88	90	87	89	89	1
1851.852	79	79	76	78	78	2
617.284	61	62	60	61	61	1
205.761	43	43	41	42	42	1
68.587	25	24	23	24	24	1
22.862	10	12	8	10	10	2
7.621	14	15	12	13	14	1
2.540	6	7	4	5	5	1

0% Inh. Control	2.849
0% Phos. Control	5.600
100% Phos. Control	0.883

Z'	0.90

Figure 6. Representative sample data generated for ROCK1 (Invitrogen catalog no. PV3691) with the Z´-LYTE™ Ser/Thr 13 Peptide Substrate. The assay was performed in a 384-well assay plate following the procedure for the determination of IC_{50} values of test compounds in 1X Kinase Buffer. The top graph shows a plot of percent inhibition versus log compound concentration. A concentration of kinase was used to achieve 20–50% phosphorylation at 10 µM ATP (the 0% inhibition control wells). The concentration of inhibitor that produces 1/2 inhibition of kinase activity (IC_{50}) was calculated using an appropriate graphing software program. The bottom table shows the percent inhibition values calculated for each test compound concentration (n = 4), which were used to generate the top graph. The table also shows the emission ratio values for the respective assay controls. Curve fitting and data presentation were performed using XL*fit*® 4.1 software (IDBS).

12. Quick Start Screening Procedure

Determine the necessary assay parameters, such as reaction times, incubation temperatures, and kinase and ATP concentrations, to produce the desired extent of phosphorylation in the kinase reaction before performing the screening procedure. Use this protocol as a guideline for performing a primary screen for kinase inhibitors and for characterizing hits to determine their potencies. The volumes provided below are sufficient for 400 20-µl assays.

12.1 Prepare Reagents

Note: Thaw and store the kinase and Development Reagent on ice prior to preparation of dilutions. Equilibrate all other assay components to room temperature.

1.33X Kinase Buffer

Dilute 2 ml of 5X Kinase Buffer to 1.33X with water and any required kinase supplements. In the screen, because the test compounds are in 4% DMSO, the 10 µl kinase reaction will contain all the kinase components in 1X Kinase Buffer and 1% DMSO.

4X Test Compounds

Prepare single concentrations of the test compounds (for primary screens) in 4% DMSO (in water) at four times the concentrations desired in the 10-µl kinase reactions.

Kinase/Z´-LYTE™ Peptide Substrate Mixture

Prepare 2250 µl of a kinase/Z´-LYTE™ Ser/Thr 13 Peptide Substrate Mixture by diluting the kinase to 2X the empirically determined optimal concentration (See **Sections 9 and 10**) and the Z´-LYTE™ Ser/Thr 13 Peptide Substrate to 4 µM (9 µl) in 1.33X kinase buffer. Mix gently by pipetting; **do not vortex**.

Phospho-peptide Solution

Add 2 µl of Z´-LYTE™ Ser/Thr 13 Phospho-peptide to 498 µl of 1.33X kinase buffer. Mix thoroughly.

ATP Solution

Prepare 1110 µl of an ATP solution by diluting the 10 mM ATP in 1.33X kinase buffer to 4X the desired ATP concentration.

Development Solution

Prepare Development Solution as specified in the Development Reagent Certificate of Analysis included with this kit. The Certificate of Analysis indicates the correct dilution for each lot of Development Reagent into Development Buffer. Dilution factors for the Development Reagent can vary from lot to lot.

12.2 Assay Protocol

Table 2. Protocol for the Z´-LYTE™ Kinase Assay Kit – Ser/Thr 13 Peptide Substrate. Add each component in the following order at the appropriate time points. In Step 1, initiate the kinase reaction by adding ATP.

		Assay Reaction(s)	Controls		
	Reagents	**Kinase + Test Compound**	**100% Inhibition (no ATP)**	**0% Inhibition (with ATP)**	**100% Phosphorylation**
Step 1	**Kinase Reaction (Primary Reaction)**				
	4X Test Compound (4% DMSO)	2.5 µl			
	4% DMSO		2.5 µl	2.5 µl	2.5 µl
	Kinase/Z´-LYTE™ Peptide Substrate Mixture	5 µl	5 µl	5 µl	
	Z´-LYTE™ Phospho-peptide Solution				5 µl
	1.33X Kinase Buffer		2.5 µl		2.5 µl
	4X ATP Solution	2.5 µl		2.5 µl	
	Mix contents of wells of assay plate and incubate the 10 µl Kinase Reaction for 1 hour at room temperature.	↓	The Kinase Reaction contains 1X inhibitor, 1X Kinase, 1X ATP, and 2 µM Z´-LYTE™ Ser/Thr 13 Peptide Substrate		
Step 2	**Development Reaction (Secondary Reaction)**				
	Development Solution	5 µl	5 µl	5 µl	5 µl
	Mix contents of wells of assay plate and incubate the 15 µl development reaction for 1 hour at room temperature.	↓			
Step 3	**Stop Step and Fluorescence Detection**				
	Stop Reagent	5 µl	5 µl	5 µl	5 µl

↓

Mix contents of wells of assay plate and measure fluorescence signals.
The 20-µl (final volume) assay contains 1 µM Z´-LYTE™ Ser/Thr 13 Peptide Substrate.

13. References

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14. Purchaser Notification

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