



# Z'-LYTE™ KINASE ASSAY KIT – TYR 3 PEPTIDE PROTOCOL

Part # PV3192

Lit. # 762-038341 020304

## TABLE OF CONTENTS

|      |                                                                    |    |
|------|--------------------------------------------------------------------|----|
| 1.0  | INTRODUCTION .....                                                 | 2  |
| 2.0  | ASSAY THEORY .....                                                 | 2  |
| 3.0  | Z'-LYTE™ KINASE ASSAY KIT – TYR 3 PEPTIDE MATERIALS PROVIDED ..... | 3  |
| 4.0  | MATERIAL REQUIRED, BUT NOT SUPPLIED .....                          | 3  |
| 5.0  | STORAGE AND STABILITY .....                                        | 3  |
| 6.0  | Z'-LYTE™ ASSAY CONSIDERATIONS .....                                | 4  |
| 6.1  | Assay Controls .....                                               | 4  |
| 6.2  | Signal Stability .....                                             | 4  |
| 6.3  | Solvent Tolerances .....                                           | 4  |
| 6.4  | Assay Plates .....                                                 | 4  |
| 6.5  | Assay Volumes .....                                                | 4  |
| 6.6  | Incubation Temperature .....                                       | 4  |
| 6.7  | Kinase Reaction .....                                              | 4  |
| 6.8  | Development Reaction .....                                         | 4  |
| 7.0  | ASSAY OPTIMIZATION PROCEDURE .....                                 | 5  |
| 7.1  | Reagent Preparation.....                                           | 5  |
| 7.2  | Kinase Dilution .....                                              | 5  |
| 7.3  | Kinase Reaction .....                                              | 6  |
| 7.4  | Development Reaction .....                                         | 6  |
| 7.5  | Stop Step and Fluorescence Detection .....                         | 6  |
| 7.6  | Data Analysis.....                                                 | 6  |
| 8.0  | SCREENING PROCEDURE .....                                          | 7  |
| 8.1  | Reagent Preparation.....                                           | 7  |
| 8.2  | Assay Protocol.....                                                | 8  |
| 8.3  | Sample Data .....                                                  | 9  |
| 9.0  | DATA ANALYSIS .....                                                | 10 |
| 9.1  | Calculate Emission Ratio .....                                     | 10 |
| 9.2  | Calculate Percent Phosphorylation .....                            | 10 |
| 9.3  | Calculate Z'-factor Values .....                                   | 11 |
| 10.0 | REFERENCES .....                                                   | 11 |
| 11.0 | PURCHASER NOTIFICATION .....                                       | 12 |

## 1.0 INTRODUCTION

Protein kinases and phosphatases regulate many critical biological mechanisms, including metabolism and cell growth, proliferation, and differentiation. Aberrations in the activity of the kinases and phosphatases involved in signal transduction have been linked to many human diseases. The discovery of more than 600 kinases and phosphatases encoded by the human genome has spurred development of rapid screening techniques for potential drugs against these enzymes.

We have developed the proprietary Z'-LYTE™ technology to address the need for quickly available assays to identify potential kinase and phosphatase inhibitors. This robust, room-temperature, homogeneous assay method uses fluorescence resonance energy transfer (FRET) between coumarin and fluorescein for detection. Reaction progress is quantitated with a ratiometric approach that reduces the effects of well-to-well variations. Therefore the results produce both low coefficients of variation (CVs) and high Z'-factor values, even when only a small percentage of the substrate is phosphorylated. Z'-LYTE™ technology is highly compatible with automated high-throughput screening (HTS) systems and can readily meet the growing demand for new assays to screen for inhibitors of a broad array of tyrosine and serine/threonine protein kinases and phosphatases.

The Z'-LYTE™ Kinase Assay Kit Tyr 3 Peptide is designed to accurately and reliably screen potential kinase inhibitors in a 20-μL, two-hour, room-temperature reaction. The reagent volumes listed in the Components Section are sufficient for 800 (20-μL) assays in 384-well assay plates; however, the assay is readily modified for ultra-miniaturized HTS applications (to as small as a 1.6-μL assay volume) with no loss of quality. This Z'-LYTE™ Kinase Assay Kit provides a flexible, addition-only, screening assay that yields Z'-factor values >0.7. For a list of kinases identified that phosphorylate the Z'-LYTE™ Tyr 3 Peptide, refer to [www.invitrogen.com/zlyte](http://www.invitrogen.com/zlyte).

## 2.0 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 (**Figure 1**). The peptide substrate is labeled with two fluorophores—one at each end—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 residue in the synthetic peptide substrate. In the secondary reaction (the Development Reaction), a site-specific protease (the Development Reagent) recognizes and cleaves non-phosphorylated peptides. Phosphorylated peptides exhibit suppressed cleavage by the Development Reagent. Cleavage disrupts FRET between the donor (*i.e.*, coumarin) and acceptor (*i.e.*, fluorescein) fluorophores on the peptide, whereas uncleaved, phosphorylated peptides maintain FRET. A ratiometric method, which calculates the ratio (the Emission Ratio) of donor emission to acceptor emission after excitation of the donor fluorophore at 400 nm, quantitates reaction progress, as shown in the equation below.

$$\text{Emission Ratio} = \frac{\text{Coumarin Emission (445 nm)}}{\text{Fluorescein Emission (520 nm)}}$$

This ratiometric method for quantitating reaction progress offers the significant benefit of reducing data fluctuations arising from well-to-well variations in peptide concentration and signal intensities. As a result, the assay generates data with very high Z'-factor values (>0.7), even when a low percentage of the peptide substrate is phosphorylated.

Both cleaved and uncleaved FRET-peptides contribute to the fluorescence signals, and therefore to the Emission Ratio. The extent of phosphorylation of the FRET-peptide can be calculated from the Emission Ratio. The Emission Ratio will remain low if the FRET-peptide is phosphorylated (*i.e.*, no kinase inhibition) and will be high if the FRET-peptide is non-phosphorylated (*i.e.*, kinase inhibition).

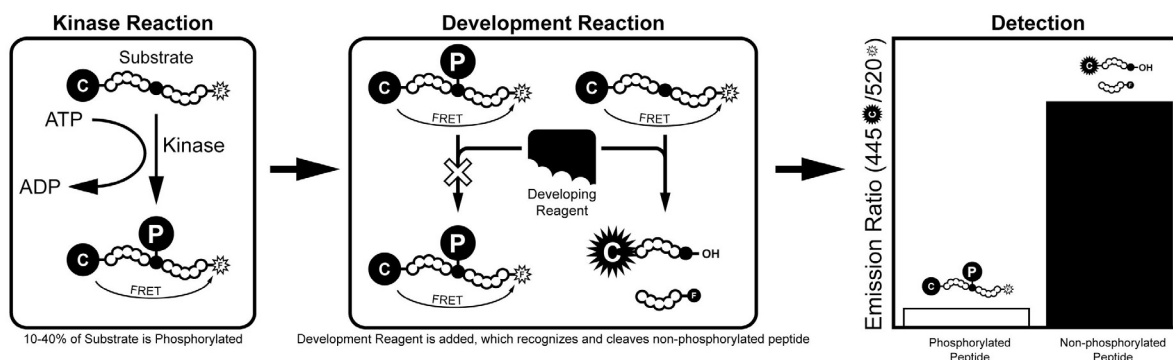


Figure 1. Schematic diagram of the Z'-LYTE™ biochemical assay.

**3.0 Z'-LYTE™ KINASE ASSAY KIT–TYR 3 PEPTIDE MATERIALS SUPPLIED**

| Reagent                        | Description                                                               | Quantity | Part No. |
|--------------------------------|---------------------------------------------------------------------------|----------|----------|
| Z'-LYTE™ Tyr 3 Peptide         | 1 mM in DMSO                                                              | 20 µL    | PV3275   |
| Z'-LYTE™ Tyr 3 Phospho-peptide | 1 mM in DMSO                                                              | 10 µL    | PV3276   |
| 5X Kinase Buffer               | 250 mM HEPES (pH 7.5), 50 mM MgCl <sub>2</sub> , 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 B           | Proprietary Buffer                                                        | 20 mL    | P3127    |
| Stop Reagent                   | Proprietary Reagent                                                       | 5 mL     | P3094    |

**4.0 MATERIALS REQUIRED, BUT NOT SUPPLIED**

- Kinase
- A fluorescence plate reader with the appropriate filter sets installed for detecting the fluorescence emission signals of coumarin and fluorescein. The recommended excitation wavelength is 400 nm and the recommended emission wavelengths are 445 nm and 520 nm, respectively. Select an excitation filter with a bandwidth appropriate for coumarin but not fluorescein. Select emission filter sets with appropriate bandwidths so that the emission signals of coumarin and fluorescein do not overlap. We chose 400 nm (12 nm bandwidth) for the excitation wavelengths and 445 nm (12 nm bandwidth) and 520 nm (12 nm bandwidth) for the emission wavelengths. Other similar filter sets may be suitable.
- 384-well assay plates. We recommend Corning® 384-well low-volume assay plates for serial dilutions (Corning® Part No. 3676). Other low-volume plates, while not tested, may be suitable.
- 12-channel, multi-channel pipette and any pipetting devices capable of accurately delivering repeated volumes of 2.5 µL and 5 µL.
- 96-well assay plate that can accommodate 300 µL.

**5.0 STORAGE AND STABILITY**

The Z'-LYTE™ Kinase Assay Kit is shipped on dry ice. Store entire kit at -80°C. Following initial use, store as detailed in the Table below. All reagents are stable for 6 months from the date of purchase, if stored and handled properly.

| Reagent                        | Part No. | Storage Temperature | Notes                                |
|--------------------------------|----------|---------------------|--------------------------------------|
| Z'-LYTE™ Tyr 3 Peptide         | PV3275   | -20°C               |                                      |
| Z'-LYTE™ Tyr 3 Phospho-peptide | PV3276   | -20°C               |                                      |
| 5X Kinase Buffer               | PV3189   | 20–30°C             |                                      |
| ATP                            | PV3227   | -20°C               |                                      |
| Development Reagent A          | PV3295   | -80°C               | Avoid more than 5 freeze/thaw cycles |
| Development Buffer B           | P3127    | 20–30°C             |                                      |
| Stop Reagent                   | P3094    | -80°C               | Avoid more than 5 freeze/thaw cycles |

## 6.0 Z'-LYTE™ ASSAY CONSIDERATIONS

This kit contains sufficient reagents to perform 800 (20-μL) assays in 384-well plates. This flexible assay can accommodate alternative reaction conditions, such as changes in assay volumes, kinase and Development Reagent A concentrations, reaction times, and incubation temperatures.

### 6.1 Assay Controls

The 0% Phosphorylation and 100% Phosphorylation Controls allow you to calculate the percent phosphorylation achieved in a specific reaction well. The 0% Inhibition and 0% Phosphorylation (100% Inhibition) Controls define the dynamic range in a screen. Control wells do not include any kinase inhibitors.

#### **0% Phosphorylation Control (100% Inhibition Control)**

The maximum Emission Ratio is established by the 0% Phosphorylation Control (100% Inhibition Control), which contains no ATP and therefore exhibits no kinase activity. This control will yield 100% cleaved peptide in the Development Reaction.

#### **100% Phosphorylation Control**

The 100% Phosphorylation Control, which consists of synthetically phosphorylated peptide, is designed to allow for the calculation of percent phosphorylation. This control will yield a very low percentage of cleaved peptide in the Development Reaction.

#### **0% Inhibition Control**

The minimum Emission Ratio in a screen is established by the 0% Inhibition Control, which contains active kinase. This control is designed to produce a recommended 20–40% phosphorylated peptide in the Kinase Reaction and to yield 60–80% cleaved peptide in the Development Reaction.

### 6.2 Stability of Reagent Dilutions and Assay Signal

All diluted reagents are stable at room temperature for 6 hours, and with no loss of assay performance. Once developed, the Emission Ratio of the 20-μL assay does not change appreciably for up to 18 hours, if the assay plate is covered and protected from light.

### 6.3 Solvent Tolerances

The 10-μL Kinase Reaction can tolerate up to 2% DMSO without affecting assay results.

### 6.4 Assay Plates

We recommend Corning® 384-well low volume non-binding surface assay plates with a working volume range of 2–35 μL. Use comparable black, low binding assay plates when using larger or smaller assay volumes.

### 6.5 Assay Volumes

The final assay volume is 20 μL. You can successfully adapt the protocol for different reaction volumes (1.6–100 μL) if you use the component concentrations specified in this protocol.

### 6.6 Incubation Temperature

For optimal results, perform the standard assay at 20–25°C. If temperatures fall below this range, increase the incubation times for the Kinase and Development Reactions.

### 6.7 Kinase Reaction

Determine the optimal kinase concentration, ATP concentration, and reaction time empirically for each kinase (Section 7.0). We recommend using near- $K_M$  ATP concentrations and a kinase concentration that phosphorylates 20–40% of the Z'-LYTE™ Tyr 3 Peptide in a one-hour, room-temperature incubation.

### 6.8 Development Reaction

At the concentration specified in this protocol, Development Reagent A will completely cleave all non-phosphorylated Z'-LYTE™ Tyr 3 peptides in the one-hour, room-temperature Development Reaction.

## 7.0 ASSAY OPTIMIZATION PROCEDURE

This section provides a recommended method for optimizing the Z'-LYTE™ Kinase Assay Kit–Tyr 3 Peptide for use with a particular kinase. The only variable is the kinase concentration. You can easily modify the protocol to examine the effects of ATP concentration or incubation time on the assay. Use the resulting experimental data as a guide for choosing an appropriate kinase concentration within the linear range to obtain the desired percent phosphorylation with an acceptable Z'-factor value. Experimental factors, such as incubation time and reaction temperature, affect the actual percent phosphorylation obtained. The volumes provided below are sufficient for 400 (20- $\mu$ L) assays.

### 7.1 Reagent Preparation

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

#### 1X Kinase Buffer

Dilute 2 mL 5X Kinase Buffer to 1X with water and any supplements required by the kinase.

#### 2X Kinase Solution

Determine the desired maximum final kinase concentration for the assay. Prepare 300  $\mu$ L of kinase in 1X Kinase Buffer to 2X the desired maximum concentration. Mix gently by pipetting; **do not vortex**.

#### Peptide/ATP Mixture

Determine the desired ATP concentration for the assay. Prepare 2250  $\mu$ L of Peptide/ATP Mixture by diluting 9  $\mu$ L Z'-LYTE™ Tyr 3 Peptide and ATP in 1X Kinase Buffer. Use enough ATP so that the final concentration of ATP in this mixture is 2X the desired final concentration used in the assay. Mix thoroughly.

#### Phospho-peptide Solution

Add 2  $\mu$ L Z'-LYTE™ Tyr 3 Phospho-peptide to 498  $\mu$ L 1X Kinase Buffer.

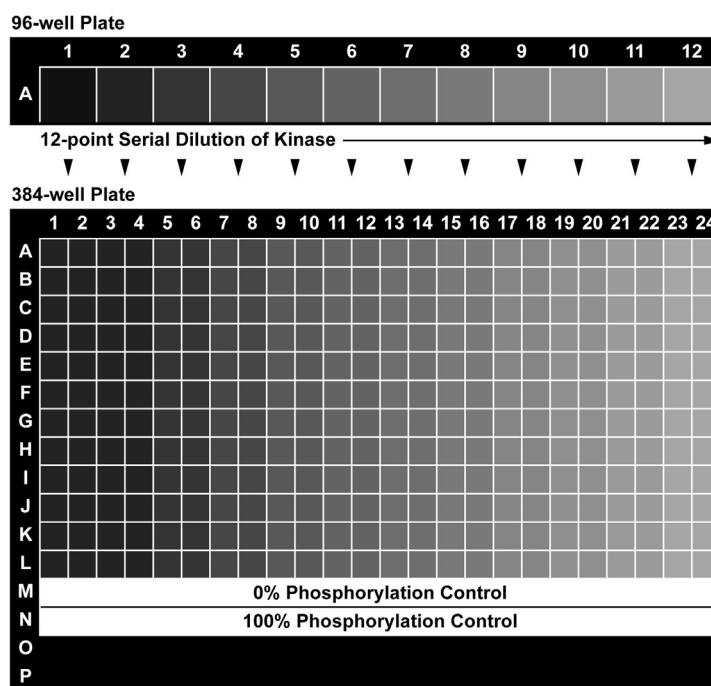
#### Development Solution

Combine 3.3  $\mu$ L Development Reagent A with 2197  $\mu$ L Development Buffer B to make the Development Solution. Mix gently by pipetting; **do not vortex**.

### 7.2 Kinase Dilution

**Note:** Use a 96-well plate that can accommodate 300  $\mu$ L.

1. Dispense 140  $\mu$ L 1X Kinase Buffer to wells A2–A12 of a 96-well plate. Do not add buffer to well A1, because it will contain the highest kinase concentration.
2. Dispense 280  $\mu$ L Kinase Solution to well A1 of the 96-well plate.
3. Perform a two-fold serial dilution of the Kinase Solution by titering 140  $\mu$ L from well A1 through well A12. Discard the final 140  $\mu$ L so that 140  $\mu$ L is left in wells A1–A12 of the 96-well plate.
4. Using a 12-channel pipette, transfer 5  $\mu$ L diluted kinase from each well in row A (wells A1–A12) of the 96-well 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.



### 7.3 Kinase Reaction (384-well plate)

1. Dispense 5  $\mu$ L 1X Kinase Buffer to each well in rows M and N (wells M1–N24).
2. Dispense 5  $\mu$ L Phospho-peptide Solution to each well in row N.
3. Add 5  $\mu$ L Peptide/ATP Mixture to each well in rows A–M.
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).

### 7.4 Development Reaction

1. Add 5  $\mu$ L Development Solution to each well in rows A–N.
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).

### 7.5 Stop Step and Fluorescence Detection

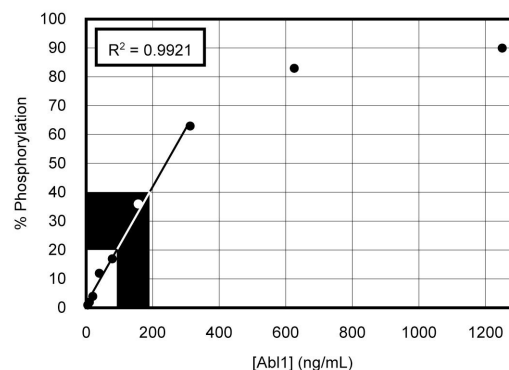
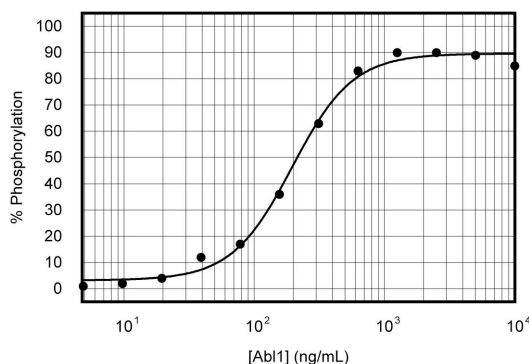
1. Add 5  $\mu$ L Stop Reagent to each well in rows A–N.
2. Shake the assay plate on a plate shaker for 30 seconds to insure homogenous reaction mixtures.
3. Measure the Coumarin (Ex. 400 nm, Em. 445 nm) and Fluorescein (Ex. 400 nm, Em. 520 nm) emission signals on a fluorescence plate reader.

### 7.6 Data Analysis

Refer to **Section 9.0** for instructions on calculating the Emission Ratio, Percent Phosphorylation, and Z'-factor value for each kinase concentration tested following the Assay Optimization Procedure. Choose the appropriate kinase concentration for the Screening Procedure (**Section 8.0**) based upon the Z'-factor values within the linear range. We recommend using near- $K_M$  ATP concentrations and a Kinase concentration that phosphorylates 20–40% of the Z'-LYTE™ Tyr 3 Peptide in a one-hour, room-temperature incubation.

**Table 1. Percent Phosphorylation and Z'-factor values corresponding to Kinase concentration. Representative sample data generated for Abl1 (Invitrogen Part No. P3049) with the Z'-LYTE™ Kinase Assay Kit–Tyr 2 Peptide**

| [Abl1] (ng/mL) in Kinase Reaction | Percent Phosphorylation | Z'-factor Value |
|-----------------------------------|-------------------------|-----------------|
| 4.9                               | 1%                      | < 0.50          |
| 9.8                               | 2%                      | 0.54            |
| 19.5                              | 4%                      | 0.75            |
| 39.1                              | 12%                     | 0.90            |
| 78.1                              | 17%                     | 0.91            |
| 156.2                             | 36%                     | 0.96            |
| 312.5                             | 63%                     | 0.97            |
| 625                               | 83%                     | 0.98            |
| 1,250                             | 90%                     | 0.99            |
| 2,500                             | 90%                     | 0.99            |
| 5,000                             | 90%                     | 0.99            |
| 10,000                            | 90%                     | 0.98            |



## 8.0 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- $\mu$ L) assays.

### 8.1 Reagent Preparation

**Note:** Thaw and store the kinase and Development Reagent A 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 supplements required by the kinase. In the screen, because the test compounds are in 4% DMSO, the 10- $\mu$ L Kinase Reaction will contain all the kinase components in 1X Kinase Buffer and 1% DMSO.

#### **4X Test Compounds**

Prepare single concentrations (for primary screens) or serial dilutions (for characterizing hits) of the test compounds in 4% DMSO (in water) at four times the concentrations desired in the 10- $\mu$ L Kinase Reactions.

#### **Kinase/Peptide Mixture**

Prepare 2250  $\mu$ L of a Kinase/Peptide Mixture by diluting the kinase to 2X the empirically determined optimal concentration (See **Section 7.0**) and the Z'-LYTE™ Tyr 3 peptide to 4  $\mu$ M (9  $\mu$ L) in 1.33X Kinase Buffer. Mix gently by pipetting; **do not vortex**.

#### **Phospho-peptide Solution**

Add 2  $\mu$ L of Z'-LYTE™ Tyr 3 Phospho-peptide to 498  $\mu$ L of 1.33X Kinase Buffer. Mix thoroughly.

#### **ATP Solution**

Prepare 1110  $\mu$ L of an ATP Solution by diluting the 10-mM ATP in 1.33X Kinase Buffer to 4X the desired ATP concentration.

#### **Development Solution**

Combine 3.3  $\mu$ L Development Reagent A with 2197  $\mu$ L Development Buffer B to make the Development Solution. Mix gently by pipetting; **do not vortex**.

## 8.2 Assay Protocol

**Table 2. Protocol for the Z'-LYTE™ Kinase Assay Kit.** 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/Peptide Mixture             | 5 µL   | 5 µL   | 5 µL   |        |
|        | Phospho-peptide Solution           |        |        |        | 5 µL   |
|        | 1.33 X Kinase Buffer               |        | 2.5 µL |        | 2.5 µL |
|        | 4X ATP Solution                    | 2.5 µL |        | 2.5 µL |        |

Mix 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™ Tyr 3 Peptide

| Step 2 | Development Reaction (Secondary Reaction) |      |      |      |      |
|--------|-------------------------------------------|------|------|------|------|
|        | Development Solution                      | 5 µL | 5 µL | 5 µL | 5 µL |

Mix assay plate and incubate the 15-µL Development Reaction for 1 hour at room temperature.



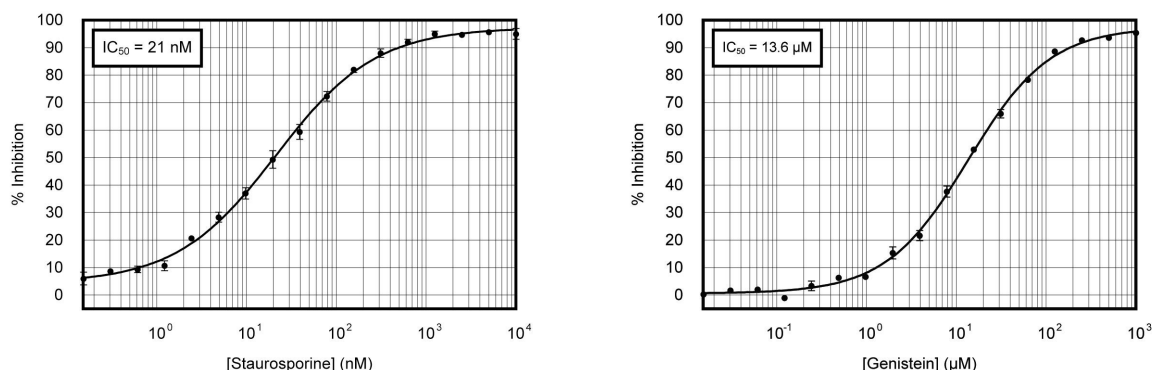
| Step 3 | Stop Step and Fluorescence Detection |      |      |      |      |
|--------|--------------------------------------|------|------|------|------|
|        | Stop Reagent                         | 5 µL | 5 µL | 5 µL | 5 µL |



Mix assay plate and measure fluorescence signals.  
The 20-µL (final volume) assay contains 1 µM Z'-LYTE™ Tyr 3 Peptide.



### 8.3 Sample Data



**Figure 2. Dose-dependent inhibition of Abl1 Kinase by Staurosporine and Genistein in the Z'-LYTE™ Kinase Assay Kit.** Two inhibitors, Staurosporine and Genistein, were titrated in the Z'-LYTE™ Kinase Assay Kit – Tyr 2 Peptide (PV3191) to determine their potencies against Abl1 Kinase. Both inhibitors were serially diluted two-fold across 17 wells. The 10  $\mu$ L Kinase Reactions included 2  $\mu$ M Tyr 2 Peptide, 130 ng/mL Abl1 Kinase (P3049/26727C), and 10  $\mu$ M ATP. The experimentally derived  $IC_{50}$  values for Staurosporine and Genistein, defined as the inhibitor concentration that produces a half-maximal value, were 21 nM and 13.6  $\mu$ M, respectively. Error bars represent one standard deviation from the mean of three replicates. These assays were performed in a Corning® 384-well low volume assay plate and data were generated on a TECAN Safire™ monochromator-based fluorescence plate reader [Ex400; Em445; Em520. All filters had a 12-nm bandwidth.]. Curve fitting and data presentation were performed using Prism® software from GraphPad Software, Inc.

|                  | EGFR       | Src          | Abl1       | PKC $\alpha$  | PKA           |
|------------------|------------|--------------|------------|---------------|---------------|
| Literature Value | 22 $\mu$ M | > 50 $\mu$ M | 39 $\mu$ M | > 100 $\mu$ M | > 100 $\mu$ M |
| Z'-LYTE™         | 21 $\mu$ M | > 50 $\mu$ M | 27 $\mu$ M | > 100 $\mu$ M | > 100 $\mu$ M |

**Table 3. Selectivity Profiling.** The table above shows the results of Z'-LYTE™  $IC_{50}$  determinations for the EGFR inhibitor Genistein with several other kinases, and compares these data to the corresponding literature values. These data demonstrate that Z'-LYTE™ assays generate  $IC_{50}$  values that are very comparable to the literature values and provide a similar rank order of compound potency (EGFR used Z'-LYTE™ Tyr 4 Peptide; Src and Abl1 used Z'-LYTE™ Tyr 2 Peptide; PKC $\alpha$  used Z'-LYTE™ Ser/Thr 7 Peptide; PKA used Z'-LYTE™ Ser/Thr 1 Peptide; ATP concentration for all kinases was 10  $\mu$ M).

## 9.0 DATA ANALYSIS

### 9.1 Calculate Emission Ratio

The Emission Ratio for each well on the assay plate is calculated by dividing the coumarin emission signal (445 nm) by the fluorescein emission signal (520 nm).

$$\text{Emission Ratio} = \frac{\text{Coumarin Emission (445 nm)}}{\text{Fluorescein Emission (520 nm)}}$$

### 9.2 Calculate Percent Phosphorylation

The extent of phosphorylation of each sample well (containing kinase) is determined according to the 0% and 100% Phosphorylation Control wells. There is a non-linear relationship between Emission Ratio and Phosphorylation (see Figure 3), which the following equation accounts for:

$$\% \text{ Phosphorylation} = 1 - \frac{(\text{Emission Ratio} \times F_{100\%}) - C_{100\%}}{(C_{0\%} - C_{100\%}) + [\text{Emission Ratio} \times (F_{100\%} - F_{0\%})]}$$

where:

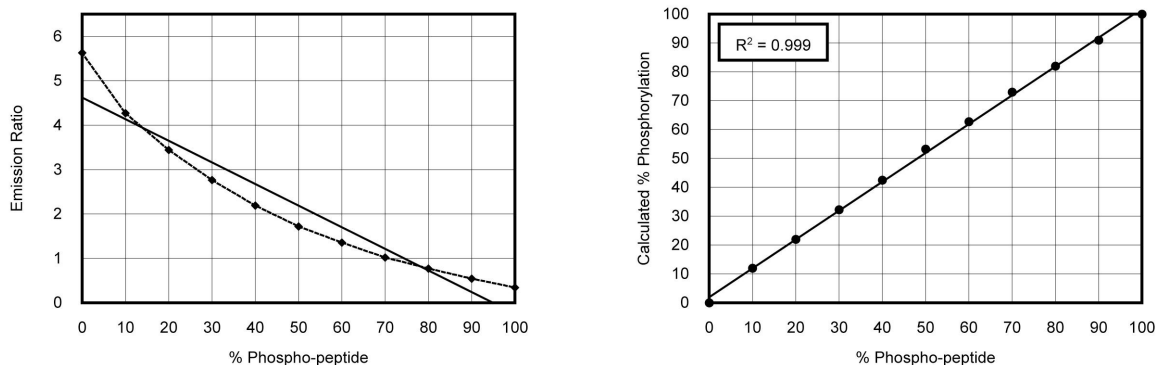
Emission Ratio = Coumarin/Fluorescein ratio of sample wells

$C_{100\%}$  = Average Coumarin emission signal of the 100% Phos. Control

$C_{0\%}$  = Average Coumarin emission signal of the 0% Phos. Control

$F_{100\%}$  = Average Fluorescein emission signal of the 100% Phos. Control

$F_{0\%}$  = Average Fluorescein emission signal of the 0% Phos. Control



**Figure 3.** In a 384-well plate, various concentrations of Z'-LYTE™ Tyr 3 Phospho-peptide were mixed with Z'-LYTE™ Tyr 3 Peptide, each to a final concentration of 2  $\mu$ M total Peptide in 10  $\mu$ L of Kinase Buffer. The plate was incubated for one-hour at room-temperature with the Development Solution to cleave all non-phosphorylated peptide. Coumarin and Fluorescein emission signals were measured on a TECAN Safire™. The Emission Ratio and the Percent Phosphorylation of each sample were calculated and plotted against the Percent of Phospho-peptide. The degree of linearity of Emission Ratio to Percent Phospho-peptide is peptide-dependent. Peptides that demonstrate a larger change in magnitude of the Fluorescein signals between the 0% and 100% Phosphorylation Controls will demonstrate a greater degree of non-linearity between Emission Ratio and Percent Phospho-peptide, necessitating linearizing the emission ratios using the equation.

### 9.3 Calculate Z'-factor Values

The Z'-factor indicates the quality of an assay; Z'-factor values of 0.5 or greater classify an assay as excellent. The Assay Optimization Procedure (see **Section 7.0**) uses the wells in row O, which contain no kinase, as the 100% Inhibition Control wells, and each well in the rows containing kinase as the 0% Inhibition Control wells.

1. Calculate the Emission Ratio for each well.
2. Calculate the average and standard deviations of the ratios in each row
3. Calculate the Z'-factor value, using the following equation:

$$Z'\text{-Factor} = 1 - \frac{(3 \times \sigma_{100\% \text{ Inhibition}}) + (3 \times \sigma_{0\% \text{ Inhibition}})}{\mu_{100\% \text{ Inhibition}} - \mu_{0\% \text{ Inhibition}}}$$

## 10.0 REFERENCES

---

1. Rodems, S. *et al.* (2002) *ASSAY Drug Devel. Technol.* **1**:9-19.
2. Kleman-Leyer, K. *et al.* (2003) *Drug Disc. Devel.* **6**:81-2.
3. Zhang, J.H. *et al.* (1999) *J. Biomol. Screen.* **4**:67-73.
4. Akiyama, T. *et al.* (1987) *J. Biol. Chem.* **262**:5592-5
5. Geissler, J. F. *et al.* (1990) *J. Biol. Chem.* **265**:22255-61

## 11.0 PURCHASER NOTIFICATION

### Limited Use Label License No. 155: Z'-LYTE™ Technology

This product is the subject of U.S. Patent No. 6,410,255 and foreign equivalents owned by Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Z'-LYTE™ Technology is covered by one or more of the following U.S. Patents, as well as corresponding pending or issued foreign patents:  
6,410,255; other U.S. patents pending.

Z'-LYTE™ is a trademark of Invitrogen Corporation.

Corning® is a registered trademark of Corning Incorporated.

Prism® is a registered trademark of GraphPad Software, Inc.

©2004 Invitrogen Corporation. All rights reserved. Reproduction without permission forbidden.