Application Note

Z'-LYTE® Kinase Assay from Invitrogen™ on Synergy™ 4

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Introduction

Here we describe the use of the Synergy 4 Hybrid Microplate Reader to measure the output from Invitrogen's Z'-LYTE® assay technology.

Z'-LYTE® technology is a homogeneous assay format based on fluorescence resonance energy transfer (FRET), and is a universal platform for screening and profiling protein kinases. The assay uses a peptide substrate labeled with two different fluorophores (coumarin donor and fluorescein acceptor) on each end. These two fluorophores make up a FRET pair, which allows the transfer of energy when both are present on the same molecule. The initial kinase reaction transfers the ϒ-phosphate of ATP to a tyrosine or serine/threonine residue on the substrate (Figure 1). In a secondary reaction, referred to as the development reaction, a site-specific protease recognizes and cleaves only non-phosphorylated peptide, while phosphorylated substrate remains uncleaved (Figure 2). Uncleaved phosphorylated product will exhibit FRET emission, while cleaved peptides will not. Upon excitation of coumarin at 400 nm, the FRET signal is measured as a ratio between the coumarin donor emission at 445-460 nm and the fluorescein acceptor emission at 520-535nm.

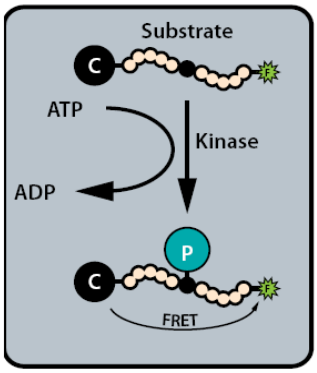

Figure 1. Z'-LYTE® Kinase Reaction. The kinase transfers the gamma-phosphate of ATP to a single tyrosine, serine or threonine residue in a synthetic FRET-peptide.

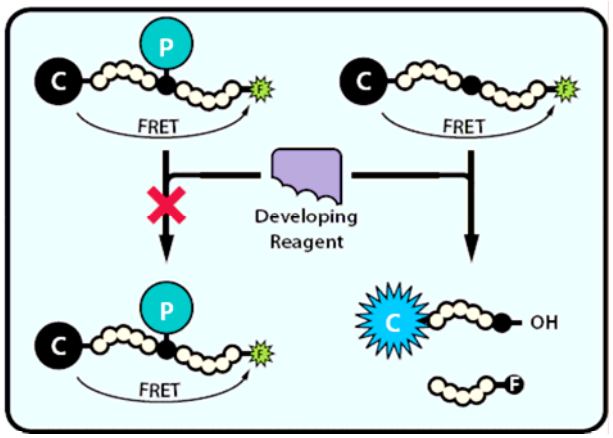

Figure 2. Z'-LYTE® Development Reaction. Phosphorylation of FRET-peptides suppresses cleavage by the Development Reagent. Cleavage disrupts FRET between the coumarin donor and fluorescein acceptor fluorophores on the FRET-peptide, whereas uncleaved, phosphorylated FRET-peptides maintain FRET.

Materials and Methods

A Z'-LYTE® Kinase Assay Kit (catalog number PV3193) was obtained from Invitrogen (Carlsbad, CA). The assay was carried out according to the kit instructions. Briefly, 5X kinase buffer (250 mM HEPES (pH 7.5), 50 mM MgCl2, 5 mM EGTA, 0.05% BRIJ-35) was diluted to 1X using distilled water. The phosphopeptide positive control (Z'-LYTE® Tyr 4 phospho-peptide) and the non-phosphorylated negative control (Z'-LYTE® Tyr 4 peptide) were then diluted 1:500 with 1X kinase buffer to 2 µM. A series of mixtures, each with 2 µM peptide ranging from 100% phosphopeptide to 0%, were then made by combining various amounts of phosphorylated and non-phosphorylated peptides to mimic the results of a kinase reaction. Working development solution was then made by diluting 12 µL of the provided stock solution with 388 µL of the provided development buffer. Aliquots (20 µL) of each peptide mixture were added to the wells of a Corning 3914 solid white microplate in replicates of 8. The development reaction was initiated by the addition of 10 µL of working development-solution to all the wells. The plates were allowed to incubate at RT for 60 minutes. Reactions were then stopped by the addition of 10 µL of stop solution.

Reactions were quantitated using a BioTek Synergy 4 Hybrid Microplate Reader. Reader control, blank subtraction, ratiometric calculations and graph plotting were carried out using Gen5™ Data Analysis Software.

Results

Figure 3 illustrates the relationship between the 460 nm / 535 nm ratio and the extent of phosphorylation present, which can be described using a 4-parameter logistic fit. 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). This data demonstrates the ability of the Synergy 4 Hybrid Microplate Reader to measure the FRET signal of the Z'-LYTE® Kinase assay kit from Invitrogen.

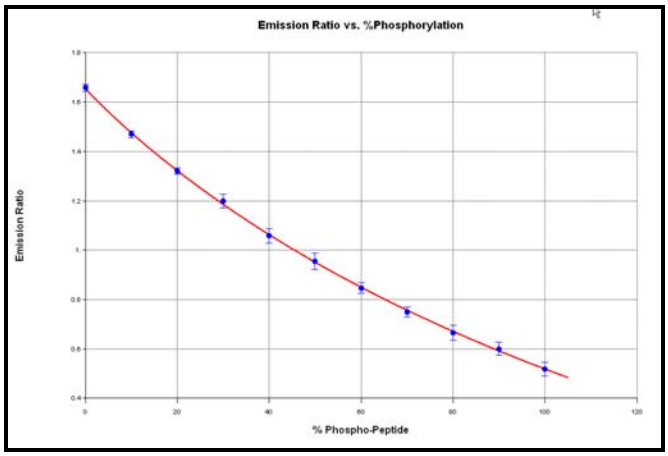

Figure 3. Emission Ratio for various degrees of phosphorylation

Z'-LYTE® is a registered trademark of Invitrogen.