SZABO SCANDIC

Part of Europa Biosite

Produktinformation

- Forschungsprodukte & Biochemikalien
- Zellkultur & Verbrauchsmaterial
- Diagnostik & molekulare Diagnostik
- Laborgeräte & Service

Weitere Information auf den folgenden Seiten!
See the following pages for more information!

Lieferung & Zahlungsart

siehe unsere Liefer- und Versandbedingungen

Zuschläge

- Mindermengenzuschlag
- Trockeneiszuschlag
- Gefahrgutzuschlag
- Expressversand

SZABO-SCANDIC HandelsgmbH

Quellenstraße 110, A-1100 Wien

T. +43(0)1 489 3961-0

F. +43(0)1 489 3961-7

mail@szabo-scandic.com

www.szabo-scandic.com

linkedin.com/company/szaboscandic

SLP-76 (Total) TR-FRET Assay Kit

Powered by Bioauxilium's THUNDER™ TR-FRET Technology

Item No. 500223

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	480 wells Quantity/Size
400269	Europium-Labeled SLP-76 (Total) Antibody	1 vial/5 µl	1 vial/25 µl
400270	Acceptor-Labeled SLP-76 (Total) Antibody	1 vial/20 µl	1 vial/100 µl
400211	Lysis Buffer 2 (5X)	1 vial/1 ml	4 vials/1.25 ml
400225	Detection Buffer (10X)	1 vial/50 µl	1 vial/250 µl
400268	SLP-76 Positive Control Lysate	1 vial/100 µl	1 vial/500 µl
400257	Phosphatase Inhibitor Cocktail (100X)	1 vial/50 µl	1 vial/250 µl

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

Do not mix or substitute reagents or materials from other kit lots or kits. Kits are quality control tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

We cannot guarantee the performance of the product outside the conditions detailed in this kit booklet.

The kits are designed for the detection of endogenous cellular proteins across a wide variety of cell lines. However, until each cell line in particular is tested, the possibility of the presence of undetectable levels of the target protein cannot be excluded.

Users should ensure that their cell line has measurable levels of the target protein. Expression levels of signaling proteins in different cell types vary widely. The cell line used for the assay validation of this kit is shown in the figures starting on page 30.

Before You Start

Please note the following:

ONLY white plates should be used for TR-FRET.

DO NOT modify the assay protocol or volumes.

DO optimize the cell density, serum starvation (optional), and stimulation or inhibition parameters.

ALWAYS use the included positive control lysate for every assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -80°C and used before the expiration date indicated on the outside of the box.

THUNDER™ General Information

THUNDER™ TR-FRET Cell Signaling Assay Kits are designed for the semi-quantitative measurement of phosphorylated and/or total (both phosphorylated and unphosphorylated) proteins in cell lysates using homogeneous (no wash) TR-FRET technology. The kits are compatible with both adherent and suspended cells.

THUNDER™ TR-FRET Cell Signaling Assay Kits are based on Bioauxilium's enhanced proprietary time-resolved Förster resonance energy transfer (TR-FRET) technology. THUNDER™ assays can be read on most commercially available TR-FRET-compatible plate readers (a list of suitable TR-FRET readers can be found at www.Bioauxilium.com). TR-FRET-based assays are homogeneous because they do not require any washing or separation steps. In addition, the THUNDER™ assays use a standardized, simple, and rapid "add-incubate-measure" protocol with a single step reagent addition. This streamlined assay protocol dramatically decreases hands-on time and provides a powerful alternative to cumbersome, error-prone and time-consuming techniques such as Western blot and ELISA.

THUNDER™ TR-FRET Cell Signaling Assay Kits contain the essential reagents necessary to carry out the measurement of signaling proteins in cells, with the exception of the plate(s).

Materials Needed But Not Supplied

1. A plate reader equipped with a TR-FRET option
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Culture plate: 96-well clear, flat-bottom polystyrene tissue culture-treated plate(s) for culturing cells when using the 2-plate (transfer) assay protocol. *NOTE: Do not use this type of plate for the 1-plate (all in one well) assay protocol.*
5. Detection plate (96-well plate option): Half-area, 96-well white plate(s) for TR-FRET detection when using the 2-plate (transfer) assay protocol
6. Detection plate (384-well plate option): Low-volume, 384-well white plate(s) for TR-FRET detection when using the 1- or 2-plate assay protocols
7. Adhesive sealing film for plates
8. Orbital microplate shaker

INTRODUCTION

Background

SLP-76 is an adapter protein and a member of the SLP-76 family of adapters that has a role in regulating the immune response downstream of T cell receptors.[1] It is composed of an N-terminal region containing three tyrosine phosphorylation motifs, a proline-rich region, and a C-terminal SH2 domain.[1,2] SLP-76 is expressed in hematopoietic cells and localized to the cytoplasm but can be translocated to the plasma membrane at the T cell-to-antigen presenting cell (APC) junction following T cell receptor activation and SLP-76 interaction with certain molecules.[1] SLP-76 is phosphorylated by the Syk family kinase ZAP-70 in the N-terminal region, which induces interactions with a variety of proteins, including the Tec family kinase Itk. It also associates with the adapter protein GADS and PLCγ1 *via* the proline-rich region and the adapter protein ADAP and hematopoietic progenitor kinase 1 (HPK1) *via* the SH2 domain. Phosphorylation of SLP-76 at serine 376 (Ser^{376}) in the SH2 domain by HPK1 induces an interaction with 14-3-3 adapter proteins, followed by ubiquitination and proteolytic degradation of SLP-76, which provides negative feedback to T cell receptor signaling.[1,2] Mutations in *LCP2*, the gene encoding SLP-76, resulting in a loss of SLP-76 protein expression are associated with T and B cell immunodeficiency, impaired platelet aggregation, and severe neutrophil defects.[3] SLP-76 is expressed ectopically in human chronic lymphocytic leukemia (CLL) cells, where it mediates B cell signaling, and protein levels of SLP-76 are positively associated with the rate of disease progression.[4]

About This Assay

This SLP-76 (Total) TR-FRET Assay Kit uses a homogeneous TR-FRET assay method amenable to rapid measurement of total protein levels in cells. This SLP-76 (Total) TR-FRET Assay Kit is suitable for screening a large number of samples. The signal is stable at room temperature for at least 24 hours, affording flexibility in read times. The amount of reagents provided is sufficient for testing either 96 or 480 total protein wells, depending on the size of the kit.

Principle Of This Assay

This assay is based on the traditional sandwich immunoassay principle (Figure 1, below). Following cell treatment, cells are lysed with the specific lysis buffer provided in the kit. Then, the target protein in the cell lysates is detected with a pair of fluorophore-labeled antibodies reactive to human samples.

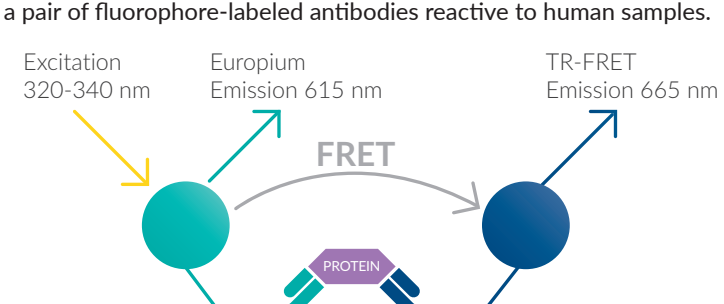


Figure 1. Schematic of the TR-FRET cell signaling assay principle

The first antibody is labeled with a long-lifetime donor fluorophore (a europium chelate; Eu-Total Ab) and the second with a far-red acceptor fluorophore (FR-Total Ab). The binding of the two labeled antibodies to distinct epitopes on the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Eu chelate molecules with a flash lamp (320 or 340 nm) or laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which, in turn, emit a TR-FRET signal at 665 nm. The signal at 665 nm is proportional to the concentration of target protein in the cell lysate. Residual energy from the Eu chelate generates light at 615 nm, which can be used as an internal standard to normalize light emitted at 665 nm.

TR-FRET assays exhibit very low background fluorescence levels and high signal-to-background (S/B) ratios. The data can be expressed and analyzed as either the signal at 665 nm or the 665 nm/615 nm ratio. The ratiometric measurement further increases assay reproducibility and robustness.

PRE-ASSAY PREPARATION

Assay Optimization

A critical step in performing any cell-based assay is the optimization of cell culture and treatment conditions. The following protocol assumes that both the cell number and treatment conditions have been previously optimized, as these key parameters often vary for each cell line. It is, therefore, strongly recommended to optimize these parameters in order to maximize the assay signal and ensure optimum performance with a high S/B ratio.

Cell number, serum starvation (optional), and stimulation or inhibition time (at either room temperature or 37°C) should be optimized for each cell line and target protein. Cell numbers that are too high or too low can negatively influence the activation of intracellular signaling pathways. Cell seeding densities of 40,000-80,000 cells/well for adherent cells or 100,000-200,000 cells/well for suspended cells are generally acceptable for most cell lines. Of note, the optimal length of time for stimulation can vary widely among cell lines from a few minutes to more than one hour. As such, a time-course study is strongly recommended to determine the optimal stimulation time, ideally at both room temperature and 37°C, since incubation temperature has an effect on the kinetics of target protein stimulation. Additional assay development guidelines are available on Bioauxilium's website (www.Bioauxilium.com).

Reagent Preparation

The instructions described below are for testing the entire number of wells in each kit. Adjust volumes accordingly when testing fewer wells.

Bring all reagents to room temperature prior to use.

Centrifuge all tubes before use to improve recovery of content (2,000 x g, 10-15 seconds).

Mix the lysis and detection buffers and the positive control lysate by vortexing gently before use. Do NOT vortex the antibodies.

Use ultrapure water (18 MΩ·cm) to dilute the lysis and detection buffers.

NOTE: It is recommended to test all samples and controls at least in duplicate.

NOTE: ALWAYS include a positive control using the positive control lysate provided.

1. **Supplemented Lysis Buffer**

   Supplemented Lysis Buffer 2 (1X) for the 2-Plate (Transfer) Assay Protocol with Adherent Cells: The supplemented Lysis Buffer 2 (1X) is designed for use in the 2-plate (transfer) assay protocol using adherent cells (see page 17). Each well requires 50 µl of supplemented Lysis Buffer 2 (1X). Dilute the Lysis Buffer 2 (5X) (Item No. 400211) with ultrapure water and add the Phosphatase Inhibitor Cocktail (100X) (Item No. 400257), which contains sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), and glycerophosphate at 100, 200, and 200 mM, respectively, to final NaF, Na_3VO_4, and glycerophosphate concentrations of 1, 2, and 2 mM, respectively. *NOTE: It is mandatory to supplement Lysis Buffer 2 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 2 (1X) at 4°C; it will be stable for approximately two days.

OR

Supplemented Lysis Buffer 2 (5X) for the 2-Plate (Transfer) Assay Protocol with Suspension Cells or the 1-Plate Assay Protocol: The supplemented Lysis Buffer 2 (5X) is designed for use in the 2-plate (transfer) assay protocol using suspension cells (see page 19) or the 1-plate (all in one well) assay protocol for adherent or suspension cells (see page 24). Each well requires 10 µl (transfer) or 3 µl (all in one well) of supplemented Lysis Buffer 2 (5X). Directly add the Phosphatase Inhibitor Cocktail (100X) (Item No. 400257), which contains sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), and glycerophosphate at 100, 200, and 200 mM, respectively, to final NaF, Na_3VO_4, and glycerophosphate concentrations of 5, 10, and 10 mM, respectively. *NOTE: It is mandatory to supplement Lysis Buffer 2 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 2 (5X) at 4°C; it will be stable for approximately two days.

2. **Detection Buffer (1X)**

   Dilute 50 µl (96 wells) or 250 µl (480 wells) Detection Buffer (10X) (Item No. 400225) with 0.45 ml (96 wells) or 2.25 ml (480 wells), respectively, of ultrapure water. Store unused Detection Buffer (1X) at 4°C; it will be stable for approximately two days.

3. **Antibody Detection Mix (4X)**

   Prepare and mix just before use. *NOTE: Due to the low reagent volumes, the antibodies are diluted with Detection Buffer (1X) directly in the vial when assaying only 96 wells.*

   NOTE: Each well requires 5 µl of Antibody Detection Mix (4X).

   Antibody Detection Mix (4X) (96 wells): Add 255 µl of Detection Buffer (1X) into the vial containing 5 µl of Europium-Labeled SLP-76 (Total) Antibody (Eu-Total Ab; Item No. 400269). Add 240 µl of Detection Buffer (1X) into the vial containing 20 µl of Acceptor-Labeled SLP-76 (Total) Antibody (FR-Total Ab; Item No. 400270). Gently mix the diluted Eu-Total Ab and FR-Total Ab solutions together.

   OR

   Antibody Detection Mix (4X) (480 wells): Gently mix 25 µl of Eu-Total Ab (Item No. 400269) with 1,275 µl of Detection Buffer (1X). Gently mix 100 µl of FR-Total Ab (Item No. 400270) with 1,200 µl of Detection Buffer (1X). Gently mix the diluted Eu-Total Ab and FR-Total Ab solutions together.

   Store unused Antibody Detection Mix (4X) working solution at 4°C; it will be stable for approximately two days.

4. **Positive Control Lysate**

   The SLP-76 Positive Control Lysate (Item No. 400268) is supplied ready to use. The thawed positive control lysate can be aliquoted, refrozen at -80°C, and thawed at least three more times.

TR-FRET Plate Reader Settings

We recommend reading the TR-FRET assays at two wavelengths, detecting both the emission from the Eu chelate donor fluorophore at 615 nm and the acceptor fluorophore at 665 nm. Table 1, below, provides instrument settings to be used as guidelines.

	TR-FRET-compatible Plate Reader	
Parameter	**Flash lamp excitation**	**Laser excitation**
Excitation filter	320 nm (or 340 nm)	N/A
Emission filter	615 nm (or 620 nm)	615 nm (or 620 nm)
Delay time	90 µs	50 µs
Flash energy level	100% or High	100%
Number of flashes	100	20
Window (integration time)	300 µs	100 µs

Table 1. Recommended TR-FRET plate reader settings

ASSAY PROTOCOL

Workflow

The THUNDER™ TR-FRET Cell Signaling Assay workflow consists of 3 simple steps.

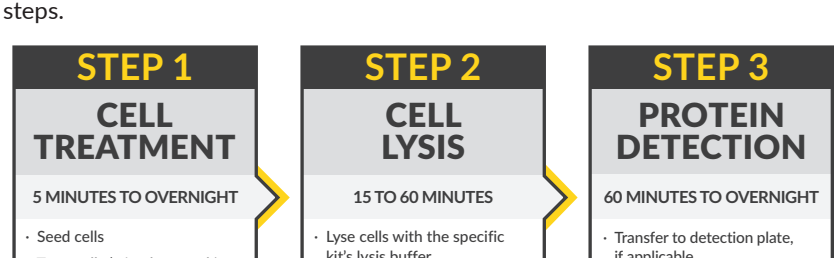


Figure 2. Assay workflow

Assay Summary

The THUNDER™ TR-FRET Cell Signaling Assays can be run using one of two possible protocols.

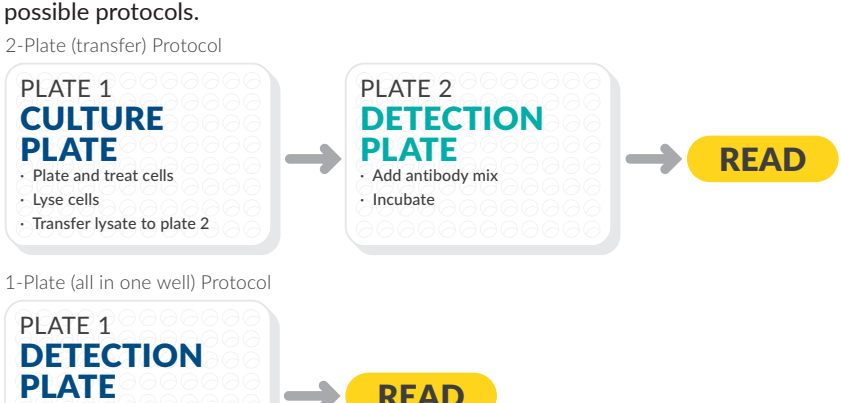


Figure 3. Assay summary Each assay uses the same total volume of 20 µl for TR-FRET detection. The 2-plate protocol is conducted in either a half-area 96-well or low-volume 384-well plate(s), while the 1-plate protocol is conducted in a low-volume 384-well plate(s).

Performing the Assay: 2-plate (Transfer) Protocol

Adherent Cells

This is a transfer protocol that is conducted in two different types of plates: cell culture and lysis are conducted in a 96-well culture plate(s), whereas detection is conducted in either a white, half-area 96-well assay plate(s) or a white, low-volume 384-well assay plate(s), with a volume of 20 µl per well for TR-FRET detection. A summary of this protocol is provided in Table 2 (see page 21).

Cell Treatment

1. Dispense 50 µl of cells at the pre-optimized density into a 96-well tissue culture-treated plate(s) in appropriate culture medium.
2. Incubate overnight at 37°C and 5% CO_2.
3. Cell Stimulation or Inhibition

   Stimulation: Add 50 µl of stimulator (2X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

   No treatment: Add 50 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

   OR

   Inhibition: Add 25 µl of inhibitor (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 25 µl of stimulator (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

   No treatment: Add 25 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 25 µl of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Carefully remove the cell culture medium by aspirating the supernatant.
2. Immediately add 50 µl of supplemented Lysis Buffer 2 (1X). The volume of supplemented Lysis Buffer 2 (1X) may be optimized from 25-100 µl.
3. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (1X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

1. Carefully pipette 15 µl of cell lysate from the 96-well culture plate to a well of either a white, half-area 96-well or a white, low-volume 384-well plate.
2. It is recommended to add 15 µl of SLP-76 Positive Control Lysate (undiluted) and 15 µl of Lysis Buffer 2 (1X) (negative control) to separate assay wells.
3. Add 5 µl of Antibody Detection Mix (4X) to each of the assay wells.
4. Cover the plate(s) with a plate sealer and incubate for 18 hours at room temperature.
5. Gently remove the adhesive plate sealer. Read on a TR-FRET-compatible plate reader. *NOTE: The same plate can be read several times without a negative effect on the assay performance, and the signal is stable for at least 24 hours at room temperature.*

NOTE: A summary of this pipetting protocol is provided in Table 4 (see page 23).

Suspension Cells

This is a transfer protocol that is conducted in two different types of plates: cell culture and lysis are conducted in a 96-well culture plate(s), whereas detection is conducted in either a white, half-area 96-well assay plate(s) or a white, low-volume 384-well assay plate(s), with a volume of 20 µl per well for TR-FRET detection. A summary of this protocol is provided in Table 3 (see page 22).

Cell Treatment

1. Dispense 20 µl of cells at the pre-optimized density into a 96-well tissue culture-treated plate(s) in appropriate culture medium.
2. Directly proceed to cell stimulation or inhibition or incubate 2-4 hours at 37°C and 5% CO_2 prior to stimulation or inhibition. This step may be optimized.
3. Cell Stimulation or Inhibition

   Stimulation: Add 20 µl of stimulator (2X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

   No treatment: Add 20 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

   OR

   Inhibition: Add 10 µl of inhibitor (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 10 µl of stimulator (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

   No treatment: Add 10 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 10 µl of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Add 10 µl supplemented Lysis Buffer 2 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (5X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

Following cell lysis, proceed to the TR-FRET detection step as described for the standard 2-plate (transfer) protocol for adherent cells (see page 18).

2-Plate (Transfer) Assay Summary

Step	Adherent Cells			
Cell Treatment	Stimulation	No Treatment	Inhibition	No Treatment
	50 µl cells	50 µl cells	50 µl cells	50 µl cells
	Incubate cells overnight			
	50 µl stimulator (2X)	50 µl serum-free medium	25 µl inhibitor (4X)	25 µl serum-free medium
	Incubate for pre-optimized time			
			25 µl stimulator (4X)	25 µl serum-free medium
			Incubate for pre-optimized time	
Cell Lysis	Remove media			
	50 µl supplemented Lysis Buffer 2 (1X)*			
	Incubate 30 minutes on an orbital shaker			
Protein Detection	15 µl lysate			
	5 µl Antibody Detection Mix (4X)			
	Cover and incubate 18 hours			
	Read TR-FRET signal			

Table 2. Assay summary for the 2-plate (transfer) protocol with adherent cells
*The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

2-Plate (Transfer) Assay Summary				
Step	**Suspension Cells**			
Cell Treatment	Stimulation	No Treatment	Inhibition	No Treatment
	20 µl cells	20 µl cells	20 µl cells	20 µl cells
	20 µl stimulator (2X) OR incubate for 2-4 hours, then add 20 µl stimulator (2X)	20 µl serum-free medium OR incubate for 2-4 hours, then add 20 µl serum-free medium	10 µl inhibitor (4X) OR incubate for 2-4 hours, then add 10 µl inhibitor (4X)	10 µl serum-free medium OR incubate for 2-4 hours, then add 10 µl serum-free medium
	Incubate for pre-optimized time			
			10 µl inhibitor (4X)	10 µl serum-free medium
			Incubate for pre-optimized time	
Cell Lysis	10 µl supplemented Lysis Buffer 2 (5X)*			
	Incubate 30 minutes on an orbital shaker			
Protein Detection	15 µl lysate			
	5 µl Antibody Detection Mix (4X)			
	Cover and incubate 18 hours			
	Read TR-FRET signal			

Table 3. Assay summary for the 2-plate (transfer) protocol with suspension cells
*The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

	Untreated Cells	Treated Cells	Positive Control Lysate	Negative Control Lysate
Cell lysate (untreated cells)	15 µl			
Cell lysate (treated cells)		15 µl		
Positive Control Lysate			15 µl	
Lysis Buffer 2 (1X)				15 µl
Antibody Detection Mix (4X)	5 µl	5 µl	5 µl	5 µl
Total assay volume	20 µl	20 µl	20 µl	20 µl

Table 4. Summary of pipetting protocol 2-Plate (transfer) protocol after lysis and prior to TR-FRET

Performing the Assay: 1-plate (All in One Well) Protocol

Adherent and Suspension Cells

This is an all-in-one-well protocol. No transfer step is needed. Conduct the assay in a white, low-volume 384-well assay plate(s) with a total assay volume of 20 µl per well. A summary of this protocol is provided in Tables 5-7 (see pages 26-28).

Cell Treatment

1. Dispense 8 µl of cells at the pre-optimized density in serum-free medium into a white, low-volume 384-well assay plate(s).
2. Cell Stimulation or Inhibition

   Stimulation: Add 4 µl of stimulator (3X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

   No treatment: Add 4 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

   OR

   Inhibition: Add 2 µl of inhibitor (6X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 2 µl of stimulator (6X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

   No treatment: Add 2 µl of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 2 µl of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Add 3 µl of supplemented Lysis Buffer 2 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (5X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

1. Add 15 µl of SLP-76 Positive Control Lysate (undiluted) and 15 µl of Lysis Buffer 2 (1X) (negative control) to separate assay wells.
2. Add 5 µl of Antibody Detection Mix (4X) prepared in Detection Buffer (1X) to each of the assay wells.
3. Cover the plate(s) with a plate sealer and incubate for 18 hours at room temperature.
4. Gently remove the adhesive plate sealer. Read on a TR-FRET-compatible plate reader. *NOTE: The same plate can be read several times without a negative effect on the assay performance, and the signal is stable for at least 24 hours at room temperature.*

1-Plate Assay Summary				
Step	**Adherent or suspension cells**			
White, low-volume 384-well plate(s)				
Cell Treatment	Stimulation	No treatment	Inhibition	No treatment
	8 µl cells	8 µl cells	8 µl cells	8 µl cells
	4 µl stimulator (3X)	4 µl serum-free medium	2 µl inhibitor (6X)	2 µl serum-free medium
	Incubate for pre-optimized time			
			2 µl stimulator (6X)	2 µl serum-free medium
			Incubate for pre-optimized time	
Cell Lysis	3 µl supplemented Lysis Buffer 2 (5X)*			
	Incubate 30 minutes on an orbital shaker			
Protein Detection	15 µl of SLP-76 Positive Control Lysate and 15 µl of Lysis Buffer 2 (1X) to separate wells			
	5 µl Antibody Detection Mix (4X)			
	Cover and incubate 18 hours			
	Read TR-FRET signal			

Table 5. Assay summary for the 1-plate (all in one well) protocol with adherent or suspension cells
*The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

	Untreated Cells	Treated Cells	Positive Control	Negative Control
Suspension cells	8 µl	8 µl		
Stimulator (3X)		4 µl		
Serum-free Medium	4 µl			
Positive Control Lysate			15 µl	
Lysis Buffer 2 (5X)	3 µl	3 µl		
Lysis Buffer 2 (1X)				15 µl
Antibody Detection Mix (4X)	5 µl	5 µl	5 µl	5 µl
Total assay volume	20 µl	20 µl	20 µl	20 µl

Table 6. Summary of pipetting protocol 1-Plate (all in one well) protocol using a stimulator

	Untreated Cells	Treated Cells	Positive Control	Negative Control
Suspension cells	8 µl	8 µl		
Inhibitor (6X)		2 µl		
Stimulator (6X)		2 µl		
Serum-free Medium	4 µl			
Positive Control Lysate			15 µl	
Lysis Buffer 2 (5X)	3 µl	3 µl		
Lysis Buffer 2 (1X)				15 µl
Antibody Detection Mix (4X)	5 µl	5 µl	5 µl	5 µl
Total assay volume	20 µl	20 µl	20 µl	20 µl

Table 7. Summary of pipetting protocol 1-Plate (all in one well) protocol using an inhibitor

ANALYSIS

Calculations

1. TR-FRET data are typically calculated and presented ratiometrically using the following formula:

   [(665 nm/615 nm) x 1,000]

2. Calculate the TR-FRET ratio for each well.
3. TR-FRET assays are homogeneous; do not subtract average negative control data (no lysate) from any other well readings.
4. For concentration-response curves, analyze data according to a nonlinear regression using the four-parameter logistic equation (sigmodal dose-response curve with variable slope) and a $1/Y^2$ data weighting.
5. Assay quality control: The undiluted SLP-76 Positive Control Lysate must generate an S/B ratio of at least 2 when compared to the negative control (Lysis Buffer 2 (1X) only). If this is not the case, your reader is not compatible with THUNDER™ TR-FRET Cell Signaling Assay Kits.

NOTE: The positive control lysate is provided as a control reagent, not for conducting a standard curve.

Performance Characteristics

Representative Data

Data shown here are examples of data typically generated with the SLP-76 (Total) TR-FRET Assay Kit. The TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation) using the recommended plate reader settings. Note that both the TR-FRET ratios and S/B ratios will vary from one TR-FRET-compatible reader to another. In addition, note that excitation with a laser (337 nm) generates higher counts and, usually, higher S/B ratios.

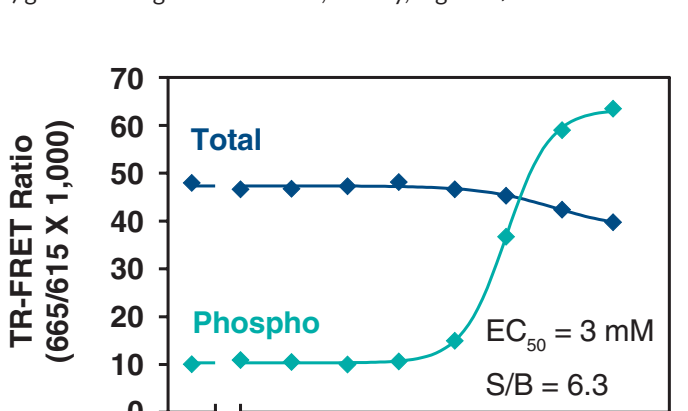
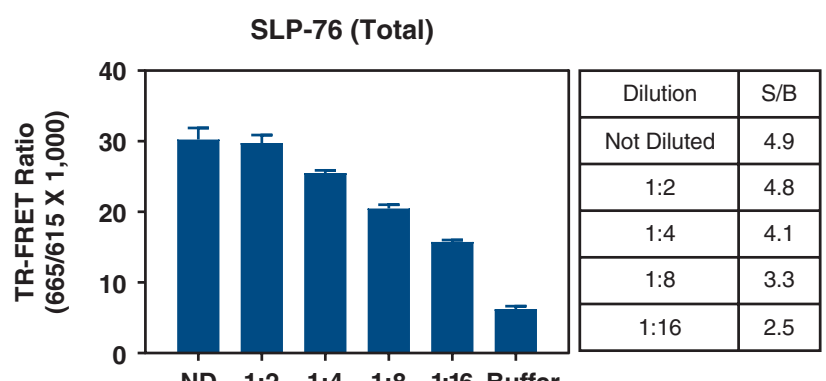


Figure 4. Stimulation of SLP-76 phosphorylation at Ser^{376} in Jurkat cells Jurkat cells were seeded at 400,000 cells/well in triplicate and incubated with serial dilutions of H_2O_2 for 15 minutes at RT.



Dilution	S/B
Not Diluted	4.9
1:2	4.8
1:4	4.1
1:8	3.3
1:16	2.5

Figure 5. Jurkat control cell lysate titration (QC Test) for phospho-Ser^{376} SLP-76 and total SLP-76 The SLP-76 (Total) and SLP-76 (Phospho-Ser^{376}) TR-FRET Assay Kit is routinely quality control tested using Jurkat cell lysates treated with H_2O_2. Jurkat cells were cultured in a T175 flask, centrifuged and resuspended at 20 million cells/ml, and stimulated with 30 mM of H_2O_2 for 15 min at RT. Following cell lysis using Lysis Buffer 2 (5X) at a final dilution of 1X, lysates were serially diluted with Lysis Buffer 2 (1X) and tested in triplicate in separate wells for total SLP-76 and phospho-Ser^{376}-SLP-76. *NOTE: Due to the high sensitivity of the Total kit components, lysates from the T175 flask required at least a 1:2 predilution in order to be within the dynamic assay range.*

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Assay S/B ratio is <2 for the positive control lysate *versus* the negative control (*i.e.* Lysis Buffer (1X) alone)	Plate reader and/or settings not suitable for TR-FRET assays Use of low-quality water for reagent preparation Use of black plates Plate read with the adhesive plate sealer	Use a filter-based instrument to read the plate(s). Ensure the correct excitation and emission filters and mirror module have been used. Use recommended instrument settings. Optimize the delay time, measurement window, and number of flashes. Only use ultrapure water for preparation of the Lysis and Detection Buffers. Only use white plates. The plate sealer MUST be removed before reading the plate(s).
Low S/B ratio in the cellular experiment	Suboptimal cell culture and/or treatment conditions Use of a different lysis buffer than the one included in the kit Lack of phosphatase inhibitors in the lysis buffer Use of low-quality water for reagent preparation Use of black plates	Use the positive control lysate to determine whether the poor signal comes from the kit reagents or from the cellular experimental conditions used in the assay. Optimize cell culture conditions. Too high OR low cell numbers can affect basal and maximal activation. Ensure the cell passage number is not too high OR low and that cells are behaving as expected (*i.e.* doubling time, viability). The lysis buffer MUST be supplemented with the Phosphatase Inhibitor Cocktail (100X) (final concentrations depend on the use of 1X or 5X lysis buffer). Additional phosphatase inhibitors and/or protease inhibitors are typically NOT required. The assay S/B ratio might be increased by decreasing the volume of lysis buffer used to lyse the cells to 25 µl to increase the target protein concentration in the lysate. Only use ultrapure water. Only use white, opaque plates.

References

1. Wu, J.N. and Koretzky, G.A. The SLP-76 family of adapter proteins. *Semin. Immunol.* **16(6)**, 379-393 (2004).
2. Soini, L., Leysen, S., Davis, J., *et al.* The 14-3-3/SLP76 protein-protein interaction in T-cell receptor signalling: A structural and biophysical characterization. *FEBS Lett.* **595(3)**, 404-414 (2021).
3. Lev, A., Lee, Y.N., Sun, G., *et al.* Inherited SLP76 deficiency in human causes severe combined immunodeficiency, neutrophil and platelet defects. *J. Exp. Biol.* **218(3)**, e20201062 (2020).
4. Dezorella, N., Katz, B.-Z., Shapiro, M. S., *et al.* SLP76 integrates into the B-cell receptor signaling cascade in chronic lymphocytic leukemia cells and is associated with an aggressive disease course. *Haematologica* **101(12)**, 1553-1562 (2016).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.