TECHNICAL DATA SHEET

THUNDER™ Phospho-RIPK1 (S166) TR-FRET Cell Signaling Assay Kit

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CATALOG NUMBERS

KIT-RIPK1P-100 (100 tests)
KIT-RIPK1P-500 (500 tests)
KIT-RIPK1P-2500 (2500 tests)
KIT-RIPK1P-5000 (5000 tests)
KIT-RIPK1P-10000 (10000 tests)

Store at -80°C
For research use only.
Not for use in diagnostic procedures.

Visit Product Page

PRODUCT DESCRIPTION

This assay kit measures intracellular levels of **phospho-RIPK1 (S166)** protein in cell lysates using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit is compatible with both adherent and suspension cells.

SPECIFICITY

This assay kit contains two specific and selective antibody, one that recognizes **RIPK1** phosphorylated at **Ser166**, and another that recognizes total (both phosphorylated and unphosphorylated) RIPK1.

SPECIES REACTIVITY

Human (Swiss-Prot Acc.: Q13546; Entrez-Gene Id: 8737).

Other species should be tested on a case-by-case basis.

TR-FRET ASSAY PRINCIPLE

The **Phospho-RIPK1 (S166)** assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 1). The THUNDER™ Cell Signaling assay workflow consists of 3 steps (Figure 2). Following cell treatment, cells are first lysed with the specific Lysis Buffer provided in the kit. Then **Phospho-RIPK1 (S166)** in the cell lysates is detected with a pair of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (single-step reagent addition; no wash steps). One antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). 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 Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of **Phospho-RIPK1 (S166)** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

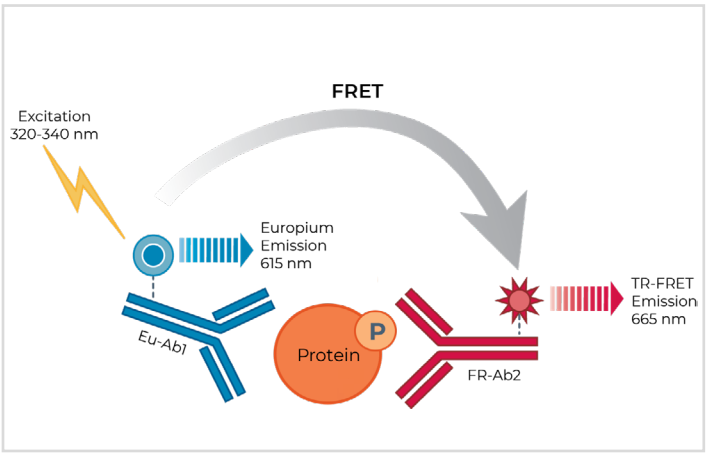


Figure 1 Schematic representation of the TR-FRET cell signaling assay principle.

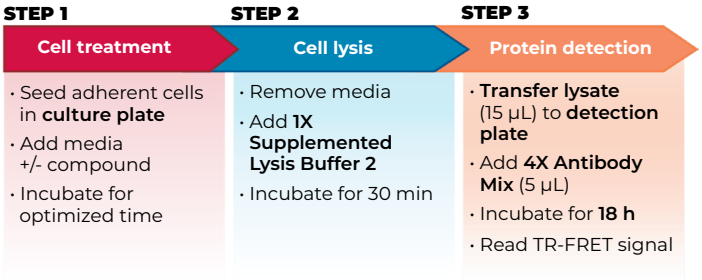


Figure 2 Assay workflow using the 2-plate (transfer) protocol.

KIT COMPONENTS

	100 points*	500 points*
Eu-labeled Phospho-RIPK1 (S166) antibody (Eu-Ab1)	5 µL	25 µL
Acceptor-labeled Phospho-RIPK1 (S166) antibody (FR-Ab2)	20 µL	100 µL
Lysis Buffer 2 (5X)	1 mL	5 mL
Detection Buffer (10X)	50 µL	250 µL
Positive control cell lysate	100 µL	500 µL
Phosphatase Inhibitor Cocktail (100X)	50 µL	250 µL

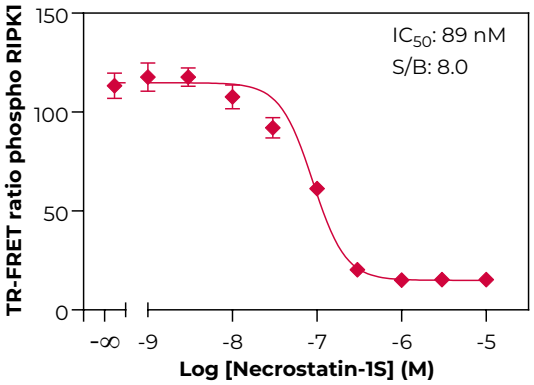
* The number of assay points is based on an assay volume of 20 µL in half-area 96-well or low-volume 384-well assay plates using the kit components at the recommended concentrations (refer to the User Manual).

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-RIPK1 (S166) in HT-29 cell lysates using the 2-plate assay protocol.

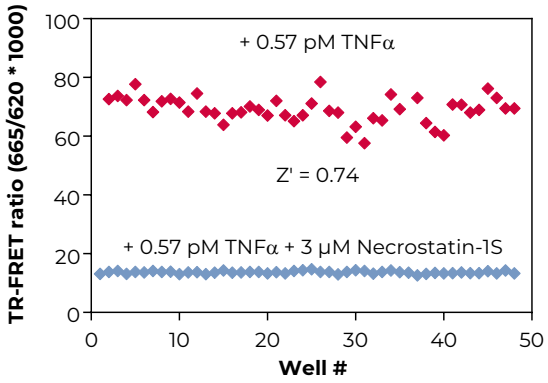
- Adherent cells were cultured overnight in a 96-well tissue culture plate (McCoy's 5A + 10% FBS).
- Following cell treatment, the media was removed and cells were lysed with the 1X **Lysis Buffer 2** (50 µL) supplemented with the 100X Phosphatase Inhibitor Cocktail diluted at 1X.
- Following a **30-min** incubation at room temperature (RT) on an orbital shaker (400 rpm), lysates (15 µL) were then transferred to a 384-well assay plate followed by addition of the labeled antibodies Eu-Ab1 and FR-Ab2 (5 µL) for detection of phospho-RIPK1 (S166).
- The plate was incubated at RT for **18 hours** and the TR-FRET signal was recorded at 665 and 615 nm (PHERASTAR® FSX; laser excitation).

INHIBITION OF PHOSPHO-RIPK1 (S166) IN HT-29 CELLS



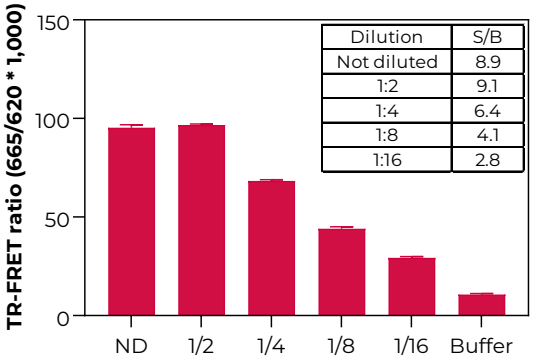
HT-29 cells (100,000 cells/well; in triplicate) were pre-incubated 20 minutes with 25 µM of Z-VAD(OMe)-FMK at 37°C. Serial dilutions of Necrostatin-1S were then added along with SM-164 at a final concentration of 100 nM; cells were incubated for an additional 10 minutes at 37°C. Finally, TNFα was added at a final concentration of 0.17 pM and cells were incubated for an additional 6 hours at 37°C. Data show that treatment of HT-29 cells with Necrostatin-1S inhibits phosphorylation of RIPK1 at S166 by TNFα.

Z'-FACTOR DETERMINATION IN HT-29 CELLS



HT-29 cells (100,000 cells/well; in triplicate) were pre-incubated 20 minutes with 25 µM of Z-VAD(OMe)-FMK at 37°C. A solution of SM-164 at a final concentration of 100 nM, without or with 3 µM of Necrostatin-1S, was added to the wells and cells were incubated for an additional 10 minutes at 37°C. Finally, TNFa was added at a final concentration of 0.57 pM and cells were incubated for an additional 6 hours at 37°C. The Z' factor value was determined after a 18h incubation time, using a total of 48 wells for each treatment group. The Z'-factor value of 0.74 indicates that the assay is robust and suitable for HTS.

HT-29 CONTROL LYSATE TITRATION (QC TEST) PHOSPHO-RIPK1 (S166)



Dilution	S/B
Not diluted	8.9
1:2	9.1
1:4	6.4
1:8	4.1
1:16	2.8

Quality Control: the Phospho-RIPK1 (S166) assay kit is routinely tested against TNFα treated HT-29 lysates. HT-29 cells were cultured in a T175 flask to 100% confluency and incubated in 25 µM of Z-VAD(OMe)-FMK for 20 min at 37°C before adding SM-164 at a final concentration of 100 nM. After an additional 10 min of incubation at 37°C, the cells were stimulated with 0.11 pM of TNFα for 6 hours at 37°C. Following cell lysis using 5 mL of 1X Lysis Buffer 2 supplemented with Phosphatase Inhibitor Cocktail, lysates were serially diluted with 1X Lysis Buffer 2 and tested in triplicate. Data show a linear relationship between lysate dilutions and TR-FRET ratio values.

FOR MORE INFORMATION ON DEVELOPING AND OPTIMIZING TR-FRET CELL SIGNALING ASSAYS, CONSULT THE USER MANUAL.