TECHNICAL DATA SHEET

THUNDER™ Phospho-BTK (Y223) + Total BTK
TR-FRET Cell Signaling Assay Kit

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CATALOG NUMBERS KIT-BTKPT-500
400 points for phospho-BTK and 100 points for total BTK

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

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PRODUCT DESCRIPTION

This assay kit measures intracellular levels of **phospho-BTK (Y223)** and **total BTK** proteins 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 pairs, one pair that recognizes **BTK** phosphorylated at **Tyr223** and another pair that recognizes **total** (both phosphorylated and unphosphorylated) **BTK**.

SPECIES REACTIVITY

Human (Swiss-Prot Acc.: Q06187; Entrez-Gene Id: 695).

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

TR-FRET ASSAY PRINCIPLE

The **Phospho-BTK (Y223) + Total BTK** 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-BTK (Y223)** and **Total BTK** in the cell lysates are detected in separate wells with two pairs of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (single-step reagent addition; no wash steps). For detection of the phosphorylated protein, one antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The same approach is used for the second antibody pair detecting the total protein (Eu-Ab3 and FR-Ab4). The binding of the two matched labeled antibodies to distinct epitopes on the target protein (either **phospho-BTK** or **total BTK**) 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-BTK (Y223)** and **Total BTK** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

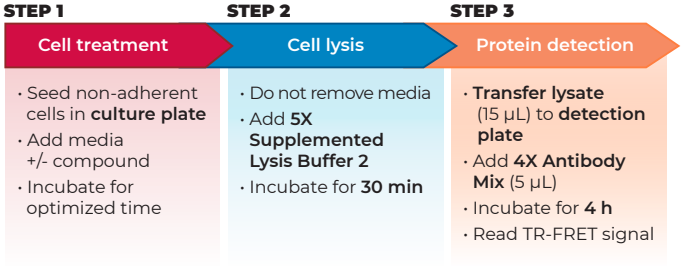

Figure 2 Assay workflow using the 2-plate (transfer) protocol.

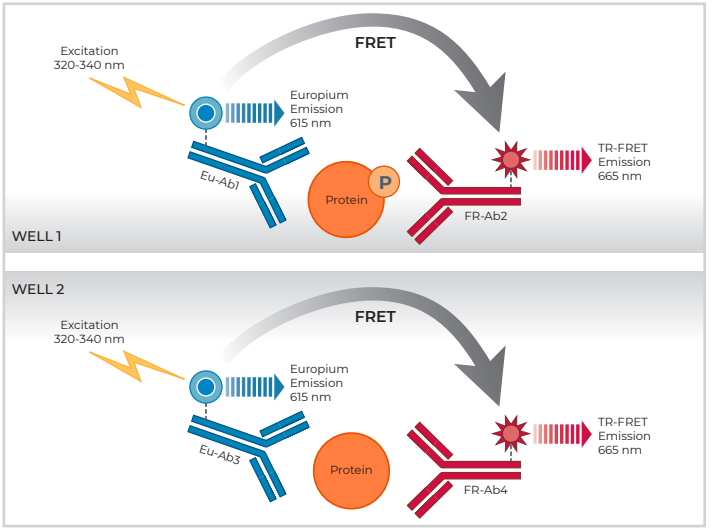

Fig. 1 Schematic representation of the TR-FRET cell signaling assay principle.

KIT COMPONENTS

KIT COMPONENTS	500 points*
Eu-labeled phospho-BTK (Y223) antibody (Eu-Ab1)	20 µL
Acceptor-labeled phospho-BTK (Y223) antibody (FR-Ab2)	80 µL
Eu-labeled total-BTK antibody (Eu-Ab3)	5 µL
Acceptor-labeled total-BTK antibody (FR-Ab4)	20 µL
Lysis Buffer 2 (5X)	5 ml
Detection Buffer (10X)	250 µL
Positive control cell lysate	200 µL
Phosphatase Inhibitor Cocktail (100X)	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).

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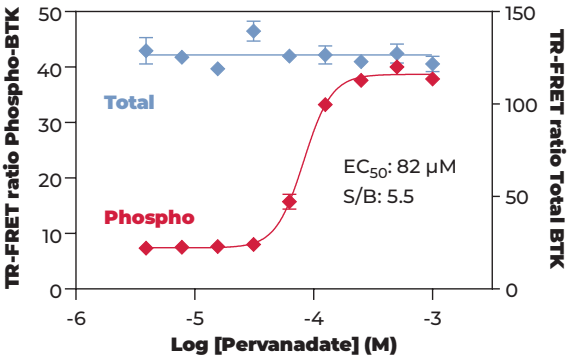
Phospho-BTK (Y223) + Total BTK

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-BTK (Y223) and total-BTK in Raji cell lysates using the 2 plate assay protocol.

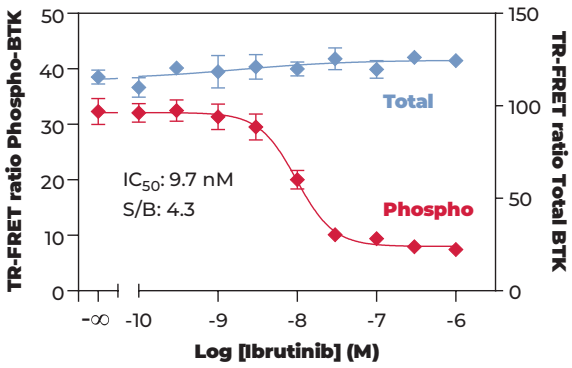
- Non-adherent cells were cultured in RPMI+10% FBS before being centrifuged and resuspended at the desired density in RPMI without serum.
- Following cell treatment, cells were lysed with the 5X **Lysis Buffer 2** (to a final concentration of 1X) supplemented with the 100X Phosphatase Inhibitor Cocktail diluted at 5X.
- 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 to separate wells of either the labeled antibodies Eu-Ab1 and FR-Ab2 (5 µL) for detection of phospho-BTK (Y223) or Eu-Ab3 and FR-Ab4 (5 µL) for detection of total BTK.
- The plate was incubated at RT for **4 hours** and the TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation).

STIMULATION OF PHOSPHO-BTK (Y223) IN RAJI CELLS



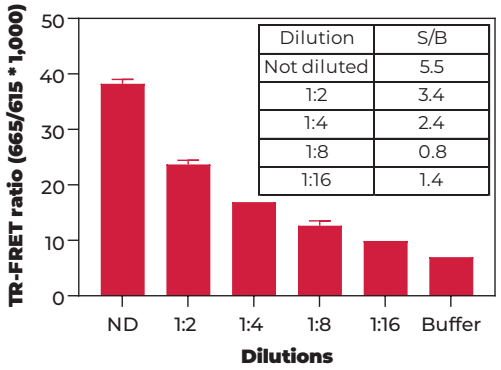
Raji cells (200,000 cells/well; in triplicate) were incubated with serial dilutions of Pervanadate for 30 min at 37°C. Data show that treatment of Raji cells with Pervanadate stimulates phosphorylation of BTK at Y223 but does not have a major effect on the levels of total BTK.

INHIBITION OF PHOSPHO-BTK (Y223) IN RAJI CELLS



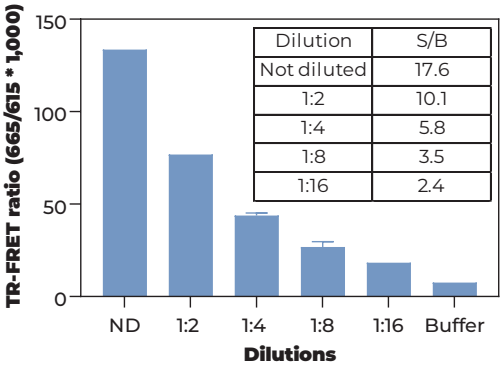
Raji cells (200,000 cells/well; in triplicate) were incubated with serial dilutions of the inhibitor Ibrutinib for 60 min at 37°C. Cells were then stimulated with 150 µM Pervanadate for 30 min at 37°C. Data show that treatment of Raji cells with Ibrutinib inhibits phosphorylation of BTK at Y223 by Pervanadate, but does not have a major effect on the levels of total BTK.

RAJI CONTROL LYSATE TITRATION (QC TEST) PHOSPHO-BTK (Y223)



Dilution	S/B
Not diluted	5.5
1:2	3.4
1:4	2.4
1:8	0.8
1:16	1.4

RAJI CONTROL LYSATE TITRATION (QC TEST) TOTAL-BTK



Dilution	S/B
Not diluted	17.6
1:2	10.1
1:4	5.8
1:8	3.5
1:16	2.4

Quality Control: the Phospho-BTK (Y223) + Total BTK assay kit is routinely tested against Pervanadate treated Raji lysates. Raji cells were cultured, centrifuged, resuspended at 10 million cells/mL and stimulated with 200 µM of Pervanadate for 30 min at 37°C. Following cell lysis with 5X Lysis Buffer 2 (final concentration: 1X), lysates were serially diluted with 1X Lysis Buffer 2 and tested in triplicate and in separate wells for phospho-BTK (Y223) and total BTK. 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.

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