# STAT1 (Phospho-Tyr$^{701}$) TR-FRET Assay Kit

*Powered by Bioauxilium's THUNDER™ TR-FRET Technology*

Item No. 500285

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# GENERAL INFORMATION

## Materials Supplied

| Item Number | Item Name | 96 wells Quantity/Size | 480 wells Quantity/Size |
|---|---|---|---|
| 400417 | Europium-Labeled STAT1 (Phospho-Tyr$^{701}$) Antibody | 1 vial/5 µl | 1 vial/25 µl |
| 400418 | Acceptor-Labeled STAT1 (Phospho-Tyr$^{701}$) Antibody | 1 vial/20 µl | 1 vial/100 µl |
| 400209 | Lysis Buffer 1 (5X) | 1 vial/1 ml | 4 vials/1.25 ml |
| 400225 | Detection Buffer (10X) | 1 vial/50 µl | 1 vial/250 µl |
| 400419 | STAT1 Positive Control Lysate | 1 vial/100 µl | 1 vial/200 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 31.

## 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

Signal transducer and activator of transcription 1 (STAT1), also known as STAT1α, is a transcription factor and member of the STAT protein family with roles in innate and adaptive immunity.[1] It is composed of an N-terminal domain that is essential to protein-protein interactions and dimerization, a DNA-binding domain that facilitates nuclear import and export, as well as DNA binding, a linker domain, a tail segment, and a transactivation domain that facilitates transcription of target genes.[2] STAT1β is an isoform of STAT1 that is formed by alternative splicing, lacks the 38-amino acid transactivation domain, and is considered a dominant-negative regulator of STAT1 transcriptional activation.[3,4] Upon phosphorylation by JAKs at tyrosine 701 ($Tyr^{701}$), STAT1 dimerizes and is translocated to the nucleus to activate transcription of IFN-γ-inducible genes.[4] STAT1 is also phosphorylated in a JAK-independent manner by various kinases, including EGFR, PDGFR, and Abl.[5]

## About This Assay

This STAT1 (Phospho-$Tyr^{701}$) TR-FRET Assay Kit uses a homogeneous TR-FRET assay method amenable to rapid measurement of phosphorylated protein levels in cells. This STAT1 (Phospho-$Tyr^{701}$) TR-FRET Assay Kit is robust, with a Z' value of 0.85, and 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 phosphorylated 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.

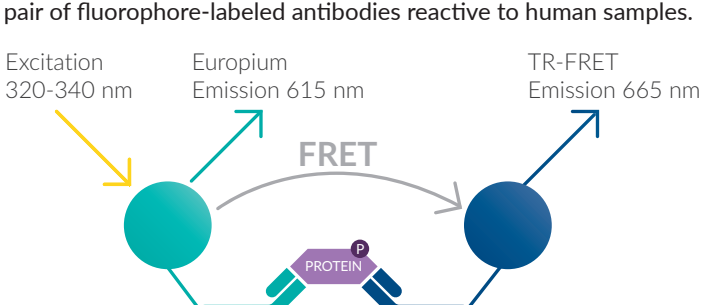


**Figure 1. Schematic of the TR-FRET cell signaling assay principle**

The first antibody is labeled with a long-lifetime donor fluorophore (a europium chelate; Eu-Phospho Ab) and the second with a far-red acceptor fluorophore (FR-Phospho 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 1 (1X) for the 2-Plate (Transfer) Assay Protocol with Adherent Cells:** The supplemented Lysis Buffer 1 (1X) is designed for use in the 2-plate (transfer) assay protocol using adherent cells (see page 18). Each well requires 50 µl of supplemented Lysis Buffer 1 (1X). Dilute the Lysis Buffer 1 (5X) (Item No. 400209) 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 1 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 1 (1X) at 4°C; it will be stable for approximately two days.

   **OR**

   **Supplemented Lysis Buffer 1 (5X) for the 2-Plate (Transfer) Assay Protocol with Suspension Cells or the 1-Plate Assay Protocol:** The supplemented Lysis Buffer 1 (5X) is designed for use in the 2-plate (transfer) assay protocol using suspension cells (see page 20) or the 1-plate (all in one well) assay protocol for adherent or suspension cells (see page 25). Each well requires 10 µl (transfer) or 3 µl (all in one well) of supplemented Lysis Buffer 1 (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 1 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 1 (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 STAT1 (Phospho-Tyr$^{701}$) Antibody (Eu-Phospho Ab) (Item No. 400417). Add 240 µl of Detection Buffer (1X) into the vial containing 20 µl of Acceptor-Labeled STAT1 (Phospho-Tyr$^{701}$) Antibody (FR-Phospho Ab) (Item No. 400418). Gently mix the diluted Eu-Phospho Ab and FR-Phospho Ab solutions together.

   **OR**

   **Antibody Detection Mix (4X) (480 wells)**: Gently mix 25 µl of Eu-Phospho Ab (Item No. 400417) with 1,275 µl of Detection Buffer (1X). Gently mix 100 µl of FR-Phospho Ab (Item No. 400418) with 1,200 µl of Detection Buffer (1X). Gently mix the diluted Eu-Phospho Ab and FR-Phospho 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 STAT1 Positive Control Lysate (Item No. 400419) 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.

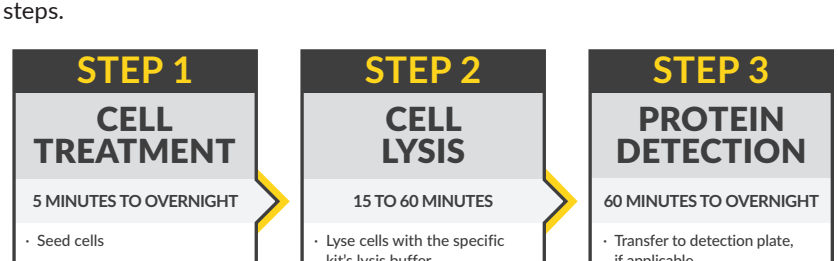


**Figure 2. Assay workflow**

## Assay Summary

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

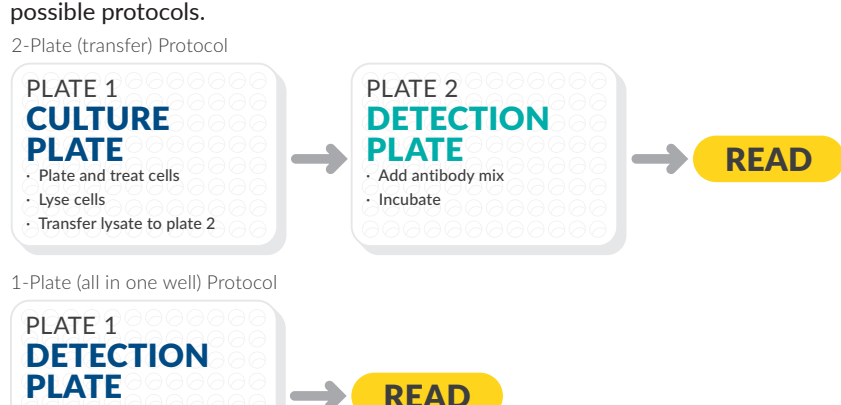


**Figure 3. Assay summary** Each assay uses the same total volume of 20 µl for TR-FRET detection. The 2-plate protocol is conducted in either a half-area 96-well or low-volume 384-well plate(s), while the 1-plate protocol is conducted in a low-volume 384-well plate(s).

## 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 22).

#### 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 1 (1X). The volume of supplemented Lysis Buffer 1 (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 1 (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 STAT1 Positive Control Lysate (undiluted) and 15 µl of Lysis Buffer 1 (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 4 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 24).*

## 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 23).

### 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 1 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 1 (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 19).

## 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 1 (1X)* | | | |
| | Incubate 30 minutes on an orbital shaker | | | |
| Protein Detection | 15 µl lysate | | | |
| | 5 µl Antibody Detection Mix (4X) | | | |
| | Cover and incubate 4 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 1 (5X)* | | | |
| | Incubate 30 minutes on an orbital shaker | | | |
| Protein Detection | 15 µl lysate | | | |
| | 5 µl Antibody Detection Mix (4X) | | | |
| | Cover and incubate 4 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 1 (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 27-29).

#### 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 1 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 1 (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 STAT1 Positive Control Lysate (undiluted) and 15 µl of Lysis Buffer 1 (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 4 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 1 (5X)* | | | |
| | Incubate 30 minutes on an orbital shaker | | | |
| Protein Detection | 15 µl of STAT1 Positive Control Lysate and 15 µl of Lysis Buffer 1 (1X) to separate wells | | | |
| | Antibody Detection Mix (4X) | | | |
| | Cover and incubate 4 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 1 (5X) | 3 µl | 3 µl | | |
| Lysis Buffer 1 (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 1 (5X) | 3 µl | 3 µl | | |
| Lysis Buffer 1 (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 STAT1 Positive Control Lysate must generate an S/B ratio of at least 2 when compared to the negative control (Lysis Buffer 1 (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 STAT1 (Phospho-Tyr$^{701}$) 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.

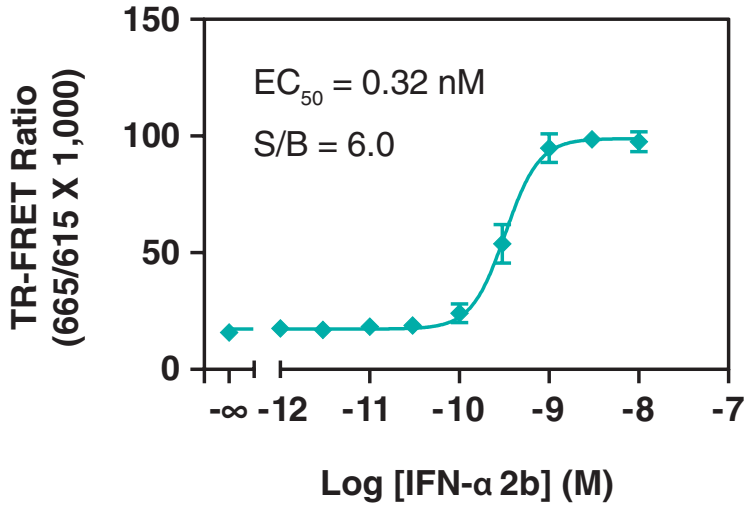


**Figure 4. Stimulation of STAT1 phosphorylation at Tyr$^{701}$ in B lymphocytes** B lymphocytes were seeded at 200,000 cells/well in triplicate and incubated with serial dilutions of IFN-α 2b for 15 minutes at room temperature.

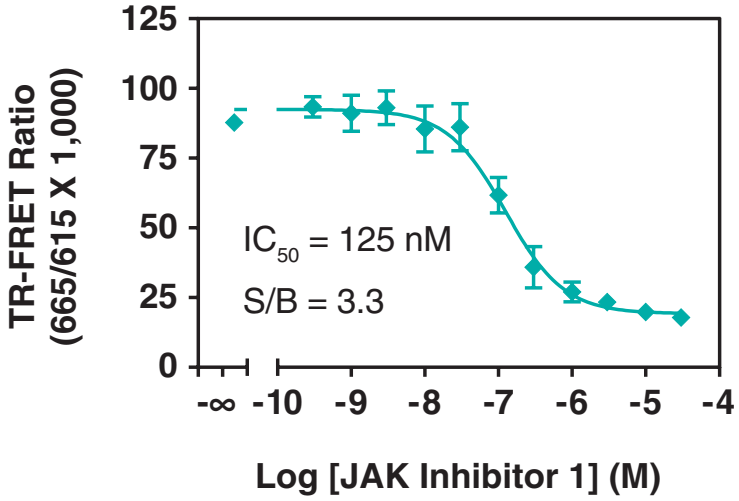


**Figure 5. Inhibition of STAT1 phosphorylation at Tyr$^{701}$ in B lymphocytes** B lymphocytes were seeded at 200,000 cells/well in triplicate and incubated with serial dilutions of the JAK inhibitor 1 for 30 minutes at room temperature then stimulated with 1 nM of IFN-α 2b for 15 minutes at room temperature.

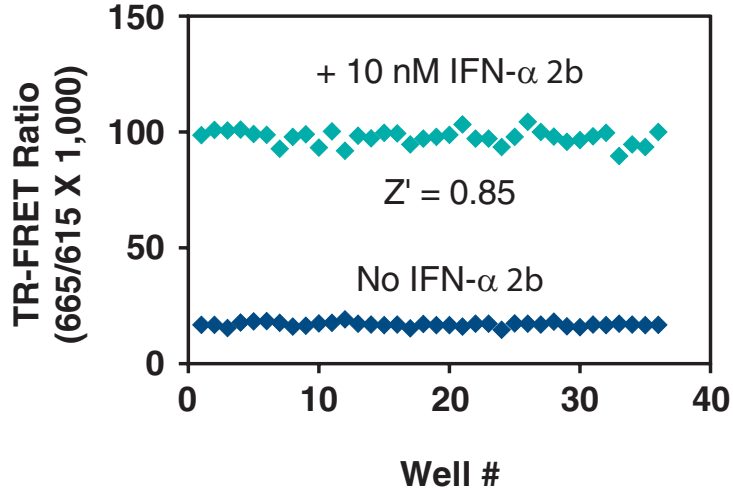


**Figure 6. Z'-Factor determination in B lymphocytes** B lymphocytes were seeded at 200,000 cells/well and incubated without or with 10 nM of IFN-α 2b for 15 minutes at room temperature. The Z'-factor value was determined using a total of 36 wells for each group. The Z'-factor value of 0.85 indicates that the assay is robust and suitable for high-throughput screening (HTS).

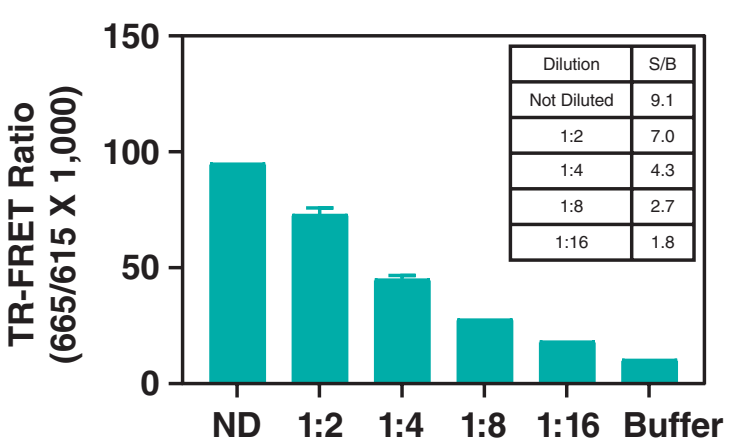


**Figure 7. B lymphocyte cell lysate titration (QC Test) for STAT1 (Phospho-Tyr$^{701}$)** The STAT1 (Phospho-Tyr$^{701}$) TR-FRET Assay Kit is routinely quality control tested using IFN-α 2b-treated B lymphocyte lystates. B lymphocytes were centrifuged and resuspended in serum-free RPMI at 60 million cells in 6 ml of media. Cells were then stimulated with 10 nM of IFN-α 2b for 15 minutes at room temperature. Following cell lysis using 3 ml of Lysis Buffer 1 (5X), lysates were serially diluted with Lysis Buffer 1 (1X) and tested in triplicate.

# RESOURCES

## 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<br><br>Use of low-quality water for reagent preparation<br><br>Use of black plates<br><br>Plate read with the adhesive plate sealer | Use a filter-based instrument to read the plate(s).<br><br>Ensure the correct excitation and emission filters and mirror module have been used.<br><br>Use recommended instrument settings. Optimize the delay time, measurement window, and number of flashes.<br><br>Only use ultrapure water for preparation of the Lysis and Detection Buffers.<br><br>Only use white plates.<br><br>The plate sealer MUST be removed before reading the plate(s). |

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Low S/B ratio in the cellular experiment | Suboptimal cell culture and/or treatment conditions<br><br>Use of a different lysis buffer than the one included in the kit<br><br>Lack of phosphatase inhibitors in the lysis buffer<br><br>Use of low-quality water for reagent preparation<br><br>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.<br><br>Optimize cell culture conditions. Too high OR low cell numbers can affect basal and maximal activation.<br><br>Ensure the cell passage number is not too high OR low and that cells are behaving as expected (*i.e.* doubling time, viability).<br><br>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.<br><br>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.<br><br>Only use ultrapure water.<br><br>Only use white, opaque plates. |



## References


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## NOTES



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