Description

The CD40:CD40L TR-FRET Assay Kit is designed to measure binding activity of CD40 (cluster of differentiation 40) to CD40L (CD40 ligand) for screening and profiling applications using TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer). It utilizes Terbium-labeled donor and a labeled acceptor to complete the TR-FRET pairing. The CD40:CD40L TR-FRET Assay Kit comes in a convenient 384-well format, with enough recombinant biotinylated CD40 (amino acids 21-193), recombinant CD40L (amino acids 116-261), Tb-Labeled Donor and Dye-Labeled Acceptor and assay buffer for 384 reactions.

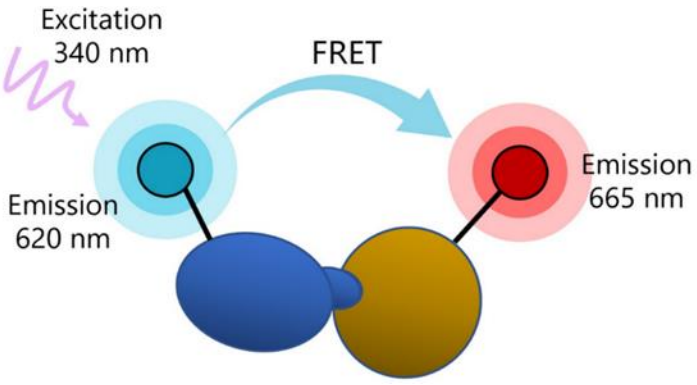

Figure 1: CD40:CD40L TR-FRET Assay Kit schematic.

A sample containing terbium-labeled donor, dye-labeled acceptor, CD40, CD40L, and an inhibitor is incubated for 90 minutes. The fluorescence intensity is then measured using a fluorescence reader. In the presence of binding of CD40 to CD40L, energy transfer occurs due to the proximity of the donor and acceptor. Disruption of the binding results in decrease of energy transfer. Fluorescence intensity at λ=665 nm corresponds directly to the binding of CD40 to CD40L.

Background

CD40 (cluster of differentiation 40), also known as TNFRSF5 ((tumor necrosis factor)-receptor superfamily 5) is a type I transmembrane protein involved in the activation of antigen presenting cells (APCs), such as dendritic cells, B cells and macrophages. It can also be found in epithelial and endothelial cells, and tumor cells. CD40L (CD40 ligand), also known as TNFSF5 and CD154, is a type II membrane glycoprotein that exists in cells in membrane bound (mCD40L) and soluble (sCD40L) forms. It is found at high levels in activated $CD4^+$ T cells, and at lower levels in Th1, Th2, Th17 and Tregs. Expression can also be induced in NK cells, $CD8^+$ T cells, basophils, and others. CD40 and CD40L are stimulatory immune checkpoints, and their signaling is mediated by different TRAF (TNF receptor associated factor), in a cell and stimuli dependent mode. For example, it mediates the activation of the NF-κB (nuclear factor kappa-B) pathway. CD40 is implicated in Hyper Ig-M immunodeficiency, where IgM is found at high levels in the serum, while other IgG are present at lower-than-normal levels, and patients have higher risk of developing autoimmune diseases and cancer. The role of CD40 and CD40L as immune checkpoints makes them highly attractive targets in cancer therapy, and several clinical trials using anti-CD40 or anti-CD40L agonist antibodies or increasing their expression are underway targeting both hematological and solid tumors. The inhibition of CD40:CD40L interaction is also clinically relevant, and clinical trials have been focusing on treatment options for lupus, rheumatoid arthritis and ALS (amyotrophic lateral sclerosis). Further studies and development of refined therapies will continue to benefit the cancer therapy field and patients suffering from autoimmune disorders.

Applications

- Study enzyme kinetics.
- Screen small molecule inhibitors in high throughput screening (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
79102	CD40, Fc Fusion (IgG1) Avi-Tag, Biotin Labeled*	15 µg	-80°C
71191	CD40L (CD154), His-Tag (Human)	2 µg	-80°C
30017	Anti-His Tb-Labeled Donor	2 x 10 µl	-20°C
	Dye-Labeled Acceptor	2 x 10 µl	-20°C
79311	3x Immuno Buffer 1	4 ml	-20°C
79969	White, non-binding Corning low volume microtiter plate	1	Room Temp

** The concentration of protein is lot-specific and will be indicated on the tube containing the protein.*

Tb-labeled donor and dye-labeled acceptor are products of PerkinElmer.

Materials Required but Not Supplied

- Fluorescent microplate reader capable of measuring Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET)
- Adjustable micropipettor and sterile tips

Storage Conditions

This assay kit will perform optimally for up to **6 months** from date of receipt when the materials are stored as directed.

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Contraindications

The CD40:CD40L TR-FRET Assay Kit is compatible with up to 1% final DMSO concentration.

Assay Protocol

- All samples and controls should be performed in duplicate.
- The assay should include “Positive Control”, “Negative Control” and “Test Inhibitor” conditions.
- We recommend maintaining the diluted protein on ice during use.
- For detailed information on protein handling please refer to Protein FAQs (bpsbioscience.com).
- We recommend using a purified anti-human CD40L antibody as internal control. If not running a dose response curve for the control inhibitor, we recommend running the control inhibitor at 0.1X, 1X and 10X the IC_{50} value shown in the validation data below.

1. Prepare **1x Immuno Buffer 1** by diluting 3-fold the **3x Immuno Buffer 1** with distilled water.

   Note: Make only the amount needed for the assay. The remaining 3x Immuno Buffer 1 can be stored as single use aliquots at -20°C.

2. Dilute Anti-His Tb-Labeled Donor 100-fold with 1x Immuno Buffer 1 (5 µl/well).

3. Dilute Dye-Labeled Acceptor 100-fold with 1x Immuno Buffer 1 (5 µl/well).

   Note: Make only the amount needed of diluted Tb-Labeled Donor and Dye-Labeled Acceptor for the assay. The remaining solution can be stored as single use aliquots (minimum volume of 5 µl) at -20°C.

4. Prepare the Test Inhibitor (2 µl/well): for a titration prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 20 µl.

   4.1 If the Test Inhibitor is water-soluble, prepare 10-fold more concentrated serial dilutions of the inhibitor than the desired final concentrations in 1x Immuno Buffer 1.

   For the positive and negative controls, use 1x Immuno Buffer 1 (Diluent Solution).

   OR

   4.2 If the Test inhibitor is soluble in DMSO, prepare the test inhibitor at a concentration 100-fold higher than the highest desired concentration in 100% DMSO, then dilute the inhibitor 10-fold in 1x Immuno Buffer 1 to prepare the highest concentration of the 10-fold intermediate dilutions. The concentration of DMSO is now 10%.

   Using 1x Immuno Buffer 1 containing 10% DMSO to keep the concentration of DMSO constant, prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations.

   For positive and negative controls, prepare 10% DMSO in 1x Immuno Buffer 1 (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

   Note: The final concentration of DMSO should not exceed 1%.

5. Thaw **CD40**, on ice. Briefly spin the tube to recover the full content.

6. Dilute CD40 to 8 ng/µl with 1x Immuno Buffer 1 (3 µl/well).

7. Prepare a Master Mix (13 µl/well): N wells x (3 µl of diluted CD40 + 5 µl of diluted Anti-His Tb Donor + 5 µl of diluted Dye-Labeled Acceptor).

8. Add 13 µl of Master Mix to each well.

9. Add 2 µl of diluted test inhibitor solution to the “Test Inhibitor” wells.

10. Add 2 µl of Diluent Solution to the "Negative Control" and "Positive Control" wells.

11. Add 5 µl of 1x Immuno Buffer 1 to the Negative Control" wells.

12. Thaw **CD40L**, on ice. Briefly spin the tube to recover the full content.

13. Dilute CD40L to 0.7 ng/µl with 1x Immuno Buffer 1 (5 µl/well).

14. Add 5 µl of diluted CD40L to the "Positive Control" and "Test Inhibitor" wells.

Component	**Negative Control**	**Positive Control**	**Test Inhibitor**
Master Mix	13 µl	13 µl	13 µl
Test Inhibitor	-	-	2 µl
Diluent Solution	2 µl	2 µl	-
1x Immuno Buffer 1	5 µl	-	-
Diluted CD40L (0.7 ng/µl)	-	5 µl	5 µl
Total	**20 µl**	**20 µl**	**20 µl**

15. Incubate at Room Temperature for 90 minutes.

16. Read fluorescence intensity of the samples in a microplate reader capable of measuring TR-FRET.

Instrument Settings

Two sequential measurements should be conducted. Tb-donor emission should be measured at 620 nm followed by dye-acceptor emission at 665 nm. Data analysis is performed using the TR-FRET ratio (665 nm emission/620 nm emission).

Reading Mode	Time Resolved
Excitation Wavelength	340±20 nm
Emission Wavelength	620±10 nm
Lag Time	60 µs
Integration Time	500 µs
Excitation Wavelength	340±20 nm
Emission Wavelength	665±10 nm
Lag Time	60 µs
Integration Time	500 µs

CALCULATING RESULTS

Calculate the FRET value by using the following formula:

$$FRET = \frac{S_{665} - \left(\frac{Tb_{665}}{Tb_{620}} \times S_{620}\right)}{S_{620}} \times 1000$$

S_{665} = Sample value measured at 665 nm, S_{620} = Sample value measured at 620 nm, Tb_{665} = Tb only or Blank value measured at 665 nm, Tb_{520} = Tb only or Blank value measured at 520 nm.

The FRET value calculated for the negative control should be subtracted from all other measurements and can be set as 0%. The FRET value from the "Positive Control" can be set as 100% activity.

$$\%\ Activity = \frac{FRET_S - FRET_{neg}}{FRET_P - FRET_{neg}} \times 100\%$$

$FRET_S$ =FRET value for samples of Test Inhibitor, $FRET_{Sub}$ = FRET value for the Substrate Control, and $FRET_p$ = FRET value for the Positive Control (no inhibitor).

Example Results

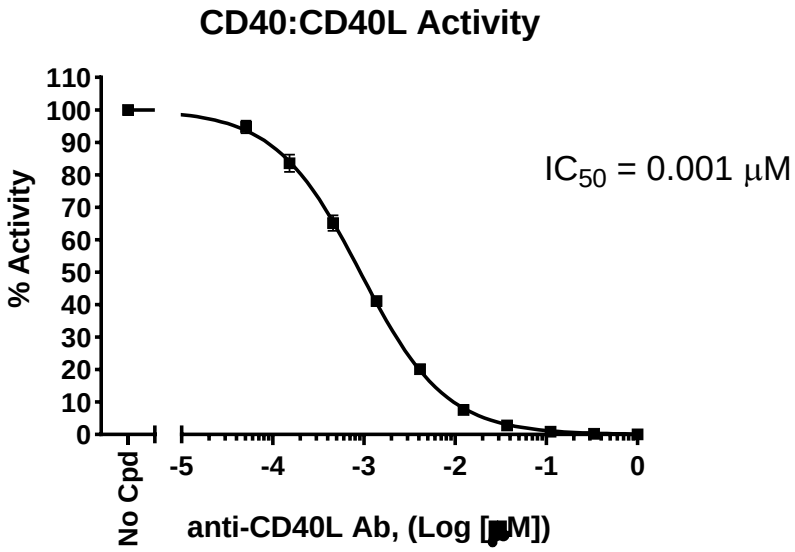

Figure 2: Binding activity of CD40 to CD40L in the presence of an anti-CD40L antibody.
CD40 binding to CD40L was measured in the presence of increasing concentrations of the inhibitor Ultra-LEAF™ Purified anti-human CD40 (BioLegend #310827).

Data shown is representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For further questions, email support@bpsbioscience.com

References

Annis A., *et al.,* 2004 *J. Amer. Chem. Soc.* 126(4): 15495-15503.
Yan, T.*, et al.,* 2001 *J. Cellular Biochem*. 83(2): 320-325.
Tang T., *et al.*, 2021 *Pharmacol The* 219:107709.

Related Products

Products	*Catalog #*	*Size*
CD40, Fc fusion (Human) Recombinant	71174	25 µg/100 µg
CD40 - HEK293 Cell Line	71257	2 vials
CD40/NF-κB Reporter Luciferase Reporter HEK293 Cell Line	60626	2 vials
CD40:CD40L[Biotinylated] Inhibitor Screening Kit	79257	96 reactions

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