Description

Recombinant HEK293 cell line expressing the full length human CD40 (also known as Tumor necrosis factor receptor superfamily member 5; TNFRSF5). Expression is confirmed by flow cytometry and western blot. An NF-κB luciferase reporter construct is stably integrated into the genome. Thus, the firefly luciferase gene is controlled by 4 copies of an NF-kB response element located upstream of a TATA promoter. Following activation by a CD40 agonist, NF-κB transcription factors bind to the DNA response elements to induce transcription of the luciferase gene. Activation of CD40 is quantified by measuring luciferase activity.

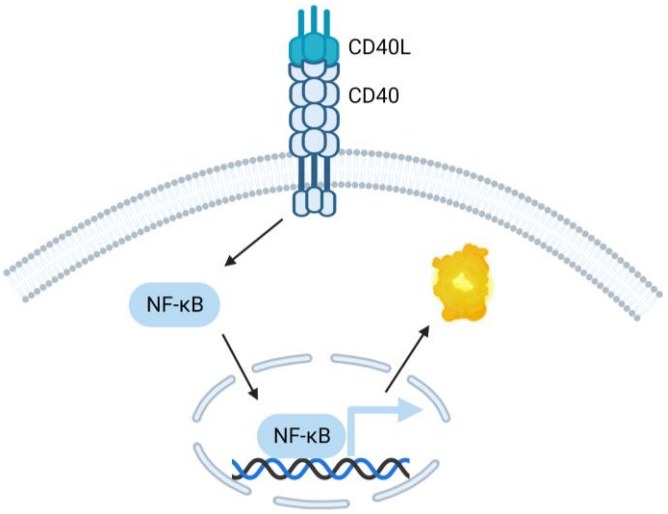

Illustration of CD40/NF-κB Luciferase Reporter HEK293 Cell Line

Background

CD40, a member of the TNF receptor superfamily, is expressed in B lymphocytes, antigen presenting cells (APCs) such as monocytes, basophils, and dendritic cells, and in non-immune cells such as endothelial and epithelial cells. The protein is overexpressed in several types of carcinomas. Interaction of CD40 with CD40 ligands (CD40L, CD154) on $CD4^+$ T helper lymphocytes triggers the expression of intercellular adhesion molecule (ICAM) and other pro-inflammatory cytokines. CD40:CD40L signaling increases the activation of antigen-specific T cells. CD40 also activates NF-κB-dependent signaling in response to lipopolysaccharide (LPS) found on Gram negative bacterial pathogens. Furthermore, agonistic CD40 monoclonal antibodies activate antigen presenting cells (APC) and promote anti-tumor T-cell responses in addition to fostering cytotoxic myeloid cells with the potential to control cancer in the absence of T cell immunity. CD40 is considered a potential target for cancer immunotherapy.

Application(s)

- Screen and characterize potential agonists or inhibitors of CD40
- Study the effect of CD40 agonists and antagonists on NK-κB signaling in a cellular system

Materials Provided

Components	Format
2 vials of frozen cells	Each vial contains 2 x 10^6 cells in 1 ml of cell freezing medium (BPS Bioscience #79796)

Parental Cell Line

HEK293, Human Embryonic Kidney, epithelial-like cells, adherent

Mycoplasma Testing
The cell line has been screened to confirm the absence of Mycoplasma species.

Materials Required but Not Supplied

These materials are not supplied with the cell line but are necessary for cell culture and cellular assays. BPS Bioscience's reagents are validated and optimized for use with this cell line and are highly recommended for best results. Media components are provided in the Media Formulations section below.

Media Required for Cell Culture

Name	Ordering Information
Thaw Medium 1	BPS Bioscience #60187
Growth Medium 1A	BPS Bioscience #79528

Materials Used in the Cellular Assay

Name	Ordering Information
human TNFα	R&D Systems #210-TA
CD40L	BPS Bioscience #71191
CD40L antibody	BioLegend #310827
ONE-Step™ Luciferase Assay System	BPS Bioscience #60690
White clear-bottom 96-well cell culture plate	
Luminometer	

Storage Conditions

Cells are shipped in dry ice and should immediately be thawed or stored in liquid nitrogen upon receipt. Do not use a -80°C freezer for long term storage. Contact technical support at support@bpsbioscience.com if the cells are not frozen in dry ice upon arrival.

Media Formulations
For best results, it is *highly recommended* to use these validated and optimized media from BPS Bioscience. Other preparations or formulations of media may result in suboptimal performance.

Note: Thaw Media do *not* contain selective antibiotics. However, Growth Media *do* contain selective antibiotics, which are used for maintaining the presence of the transfected gene(s) over passages.
Cells should be grown at 37°C with 5% CO_2. BPS Bioscience's cell lines are stable for at least 15 passages when grown under proper conditions.

Media Required for Cell Culture

Thaw Medium 1 (BPS Bioscience #60187):
MEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1 mM Na pyruvate, 1% Penicillin/Streptomycin.

Growth Medium 1A (BPS Bioscience #79528):
MEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1 mM Na pyruvate, 1% Penicillin/Streptomycin plus 100 µg/ml Hygromycin B and 400 µg/ml Geneticin.

Media Required for Functional Cellular Assay

Thaw Medium 1 (BPS Bioscience #60187):

MEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1 mM Na pyruvate, 1% Penicillin/Streptomycin.

Cell Culture Protocol

Cell Thawing

1. Swirl the vial of frozen cells for approximately 60 seconds in a 37°C water bath. As soon as the cells are thawed (it may be slightly faster or slower than 60 seconds), quickly transfer the entire contents of the vial to a tube containing 10 ml of pre-warmed Thaw Medium 1 **(no Geneticin or Hygromycin)**.
   Leaving the cells in the water bath at 37°C for too long will result in rapid loss of viability.

2. Immediately spin down the cells at 300 x g for 5 minutes, remove the medium and resuspend the cells in 5 ml of pre-warmed Thaw Medium 1 **(no Geneticin or Hygromycin)**.

3. Transfer the resuspended cells to a T25 flask or T75 flask and incubate at 37°C in a 5% CO_2 incubator.

4. After 24 hours of culture, check for cell attachment and viability. Change medium to fresh Thaw Medium 1 **(no Geneticin or Hygromycin)** and continue growing in a 5% CO_2 incubator at 37°C until the cells are ready to passage.

5. Cells should be passaged before they are fully confluent. At first passage and subsequent passages, use Growth Medium 1A **(contains Geneticin and Hygromycin)**.

Cell Passage

1. Aspirate the medium, wash the cells with phosphate buffered saline (PBS), and detach the cells from the culture vessel with 0.05% Trypsin/EDTA.

2. Once the cells have detached, add Growth Medium 1A and transfer to a tube. Spin down cells at 300 x g for 5 minutes, remove the medium and resuspend the cells in Growth Medium 1A **(contains Geneticin and Hygromycin)**. Seed into new culture vessels at the desired sub-cultivation ratio of approximately 1:5 once or twice weekly.

Cell Freezing

1. Aspirate the medium, wash the cells with phosphate buffered saline (PBS), and detach the cells from the culture vessel with 0.05% Trypsin/EDTA.

2. Once the cells have detached, add Growth Medium 1A and count the cells.

3. Spin down the cells at 300 x g for 5 minutes, remove the medium and resuspend the cells in 4°C Freezing Medium (BPS Bioscience #79796, or 10% DMSO + 90% FBS) at ~2 x 10^6 cells/ml.

4. Dispense 1 ml of cell aliquots into cryogenic vials. Place the vials in an insulated container for slow cooling and store at -80°C overnight.

5. Transfer the vials to liquid nitrogen the next day for storage.

Note: It is recommended to expand the cells and freeze at least 10 vials at an early passage for future use.

A. Validation Data

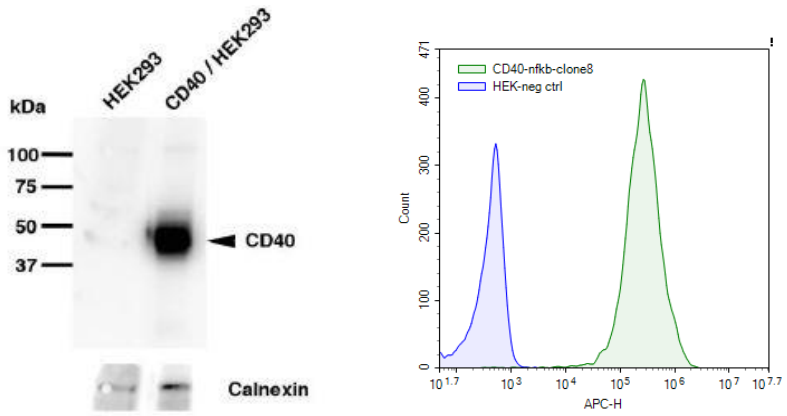

Figure 1: Expression of human CD40 in CD40/NFkB Luciferase Reporter HEK293 Cell Line. Expression of CD40 protein was assessed by western blot (left) using a specific CD40 antibody (Santa Cruz Biotechnology #sc-975). Calnexin (Cell Signaling Technology #2433) was used as a loading control. CD40/NFkB Luciferase Reporter HEK293 were stained with APC-labeled anti-human CD40 antibody (Clone 5C3, Biolegend #334323) and analyzed by flow cytometry (right). Y-axis is the % cell number. X-axis is the intensity of PE.

B: Functional characterization of the CD40/NF-κB Luciferase Reporter HEK293 Cell Line

Agonist dose-response

- The following assays are designed for 96-well format. To perform the assay in different tissue culture formats, the cell number and reagent volumes should be scaled appropriately.
- The experiments should be performed in triplicate.

1. The day before the experiment, plate CD40/NF-κB Luciferase Reporter HEK293 cells in a white clear-bottom 96-well cell culture plate at 30,000 cells/50 µl/well in Thaw Medium 1. Incubate the cells at 37°C in a 5% CO_2 incubator overnight. Keep three wells without cells to determine the background luminescence signal.
2. The next day, prepare serial dilutions of agonist TNFα, CD40L, or other desired agonist at concentrations 2-fold higher than the final desired concentrations in Thaw Medium 1.
3. Without removing the cell culture medium, add 50 µl/well of each agonist dilution to the cells. The total volume is now 100 µl/well.
4. Add 100 µl/well of Thaw Medium 1 to cell-free wells (background luminescence)
5. Incubate at 37°C, In a 5% CO_2 incubator for 6 hours.

6. Perform the luciferase assay using the ONE-Step™ luciferase assay system. Add 100 µl of One-Step luciferase reagent per well and rock gently at room temperature for ~30 minutes. Measure luminescence using a luminometer.
7. Data Analysis: Subtract the average background luminescence (cell-free control wells) from the luminescence reading of all wells. The fold induction is the average background-subtracted luminescence of agonist- treated well divided by the average background-subtracted luminescence of untreated control wells.

$$Fold\ induction = \frac{agonist\ treated\ cells - ave.background}{untreated\ cells - ave.background}$$

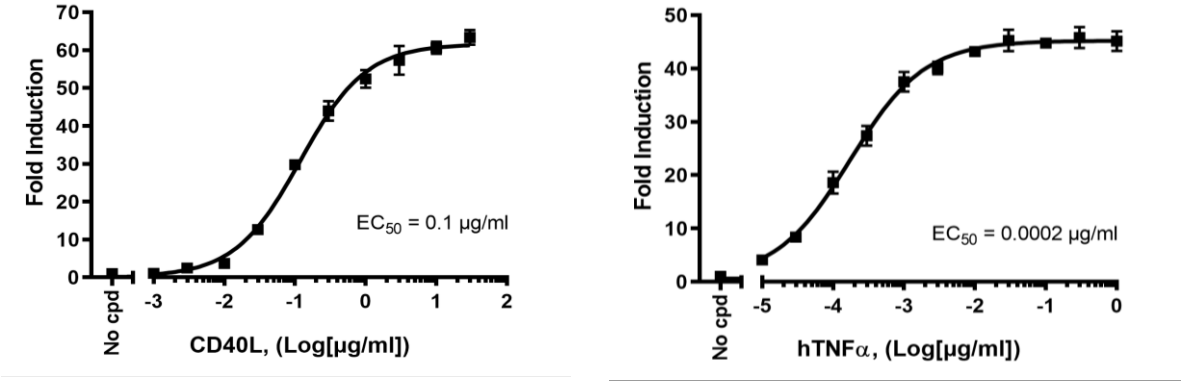

Figure 2: Dose response of agonist CD40L (left panel) and TNFα in CD40/NF-κB Luciferase Reporter HEK293 cells. The experiment was performed as described above.

Testing antibodies or inhibitors of CD40

- The following assays are designed for 96-well format. To perform the assay in different tissue culture formats, the cell number and reagent volumes should be scaled appropriately.
- The experiments should be performed in triplicate.

1. The day before the experiment, plate CD40/NF-κB Luciferase Reporter HEK293 cells in a white clear-bottom 96-well cell culture plate at 30,000 cells/50 µl/well in Thaw Medium 1. Incubate the cells at 37°C in a 5% CO_2 incubator overnight. Keep three wells without cells to determine the background luminescence signal.
2. The next day, prepare dilutions of neutralizing CD40L antibody or inhibitor(s) of interest at concentrations 4-fold higher than the final desired concentrations in Thaw Medium 1.
3. Prepare agonist CD40L (or another desired agonist) at a concentration 4-fold higher than the desired final concentration in Thaw Medium 1. In our assays we use a dose equal to the EC_{50} established for CD40L.

   Note: to measure the effect of an inhibitor, cells must also be stimulated with an agonist. Without stimulation, the luciferase activity will be very low.

4. In a 96-well assay plate, add 25 µl/well of each dilution of neutralizing anti-CD40 L antibody prepared in step 2 and 25 µl/well of the agonist prepared in step 3. Make sure to keep un unstimulated control (negative control) and a stimulated control without inhibitor (positive control).
5. Mix and incubate at room temperature for 30 minutes.
6. Without removing the cell culture medium, add 50 µl of the CD40 agonist/inhibitor mix to the cells. The total volume is now 100 µl/well.
7. Add 100 µl of Thaw Medium 1 to cell-free control wells (background luminescence).
8. Incubate at 37°C in a 5% CO_2 incubator for 6 hours.
9. Perform the luciferase assay using the ONE-Step™ luciferase assay system. Add 100 µl of One-Step luciferase reagent per well and rock gently at room temperature for ~30 minutes. Measure luminescence using a luminometer.
10. Data analysis: Subtract the average background luminescence (cell-free control wells) from the luminescence reading of all wells. The percent luminescence is the average background-subtracted luminescence of the inhibitor-treated cells (agonist-treated well in the presence of inhibitor), divided by the average background-subtracted luminescence of positive control (agonist-treated well in absence of inhibitor) multiplied by 100%.

$$\%\ Luminescence = \left(\frac{Lum.\,of\ inhibitor\ treated\ cells - ave.\,background}{Lum.\,of\ positive\ control\ cells - ave.\,background}\right) x100$$

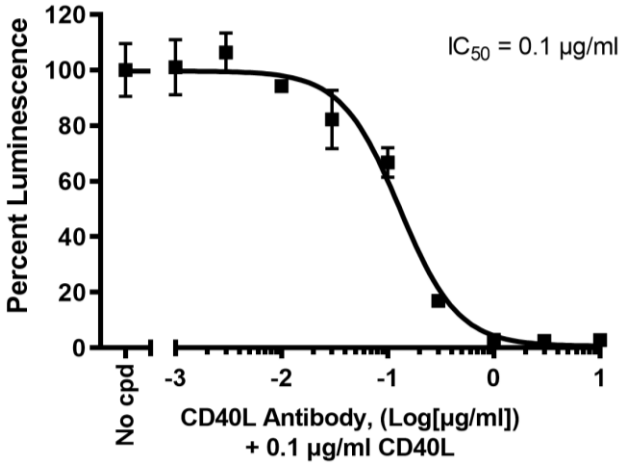

Figure 3. Neutralizing CD40L antibody inhibition of CD40L stimulation in CD40/NF-κB-luciferase reporter HEK293 cells. The experiment was performed as described before.

Sequence

NF-κB-Luciferase was cloned into the MCS of pCDNA3.1™ (+) vector (Invitrogen #V79020).

Human CD40 (NP_001241.1; Accession BC012419) was cloned into the MCS of pIREShyg3 vector (Clontech #631620).

MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECLPCGESEFLDTWNRETHCHQHKYCDP
NLGLRVQQKGTSETDTICTCEEGWHCTSEACESCVLHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCHPWTSCETKD
LVVQQAGTNKTDVVCGPQDRLRALVVIPIIFGILFAILLVLVFIKKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAAPVQETLHGC
QPVTQEDGKESRISVQERQ

References

1. Li G, *et al*. (2013) Human Genetics in Rheumatoid Arthritis Guides a High-Throughput Drug Screen of the CD40 Signaling Pathway. PLoS Genet. **9:** e1003487
2. Pontrelli P, *et al.* (2006) CD40L Proinflammatory and Profibrotic Effects on Proximal Tubular Epithelial Cells: Role of NF-kB and Lyn. *JASN*. **17**: 627.
3. Moschonas A, *et al.* (2012) CD40 Stimulates a "Feed-Forward" NF-κB- Driven Molecular Pathway That Regulates IFN-β Expression in Carcinoma Cells. *J Immunol.* **188**: 5521.

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Troubleshooting Guide

Visit bpsbioscience.com/cell-line-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com.

Related Products

Products	*Catalog #*	*Size*
CD40, Fc fusion Protein	71174	100 µg
CD40:CD40L[Biotinylated] Inhibitor Screening Assay Kit	79257	96 reactions
CD40 HEK293 Stable Cell Line	60625	2 vials
CD40:CD40L TR-FRET Assay	79258	384 reactions