Description

The NFAT Luciferase Reporter RAW 264.7 Cell Line is a murine RAW 264.7 cell line that expresses the stably integrated firefly luciferase reporter under the transcriptional control of NFAT (Nuclear Factor of Activated T-cells)-responsive elements.

This cell line has been validated to respond to mouse RANKL, Calcimycin (A23187) and PMA. It was also tested with cyclosporin A after activation with mouse RANKL or Calcimycin (A23187) and PMA.

Background

Nuclear Factor of Activated T cells (NFAT) is a family of 5 transcription factors of near ubiquitous expression, known to have a central function in the immune system, for example by inducing the expression of various cytokines (such as IL-2, IL-3, IL-4, and TNFα (tumor necrosis factor alpha)) in T cells. NFATs cooperate with multiple other proteins to regulate distinct gene expression programs that determine the fate and function of T cell populations. The NFAT family also plays important roles in the nervous system, in the heart, and in skeletal muscles. In resting T cells, the NFAT protein is phosphorylated and confined to the cytoplasm in an inactive state. In response to a stimulus, an influx of calcium activates the Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin, which rapidly dephosphorylates the serine-rich region (SRR) and SP-repeats in the amino termini of NFAT proteins. This results in a conformational change that exposes a nuclear localization signal, promoting NFAT translocation to the nucleus. In the nucleus, NFAT proteins cooperate with other transcriptional regulators to induce gene expression. Through their role in the immune system, NFATs are involved in inflammation and these transcription factors are considered promising therapeutic targets in a variety of immune-related diseases.

RAW 264.7 is a murine macrophage cell line that was transformed by the Abelson murine leukemia virus (A-MuLV) and is useful for studies involving immunoreactivity. The use of a luciferase reporter allows for easy readouts in cellular assays.

Applications

- Monitor the activity of NFAT signaling in response to stimulants such as mouse RANKL and Calcimycin (A23187).
- Screen activators or inhibitors that specifically target the NFAT pathway.

Materials Provided

Components	Format
2 vials of frozen cells	Each vial contains ≥1x 10^6 cells in 1 ml of Cell Freezing Medium (BPS Bioscience #79796)

Parental Cell Line

RAW 264.7, mouse monocyte/macrophage cell line, 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 6	BPS Bioscience #60183
Growth Medium 6K	BPS Bioscience #84148

Materials Required for Cellular Assay

Name	Ordering Information
Mouse RANKL	R&D Systems #462-TEC-010/CF
Calcimycin (A23187)	BPS Bioscience #84149
Cyclosporin A	BPS Bioscience #84150
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich #P-8139
ONE-Step™ Luciferase Assay System	BPS Bioscience #60690
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, the use of validated and optimized media from BPS Bioscience is *highly recommended*. 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 to maintain selective pressure on the cell population expressing the gene of interest.

Cells should be grown at 37˚C with 5% CO_2. BPS Bioscience's cell lines are stable for at least 10 passages when grown under proper conditions.

Media Required for Cell Culture

Thaw Medium 6 (BPS Bioscience #60183):
DMEM medium supplemented with 10% FBS, 1% Penicillin/Streptomycin.

Growth Medium 6K (BPS Bioscience #84148):
DMEM medium supplemented with 10% FBS, 1% Penicillin/Streptomycin plus 4 µg/ml of Puromycin.

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 6.

   Note: 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 6.

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 6 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 6K.

Cell Passage

1. Aspirate the medium, wash the cells with phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+}, and detach the cells from the culture vessel with 0.05% Trypsin/EDTA.

2. Once the cells have detached, add Growth Medium 6K and transfer to a tube.

3. Spin down cells at 300 *x g* for 5 minutes, remove the medium and resuspend the cells in Growth Medium 6K. Seed into new culture vessels at the recommended sub-cultivation ratio of 1:6 to 1:8 twice a week.

Cell Freezing

1. Aspirate the medium, wash the cells with PBS without Ca^{2+}/Mg^{2+}, and detach the cells from the culture vessel with 0.05% Trypsin/EDTA.

2. Once the cells have detached, add Thaw Medium 6 and count the cells.

3. Spin down the cells at 300 *x g* for 5 minutes.

4. Remove the medium and resuspend the cells in 4°C Cell Freezing Medium (BPS Bioscience #79796) at ~2 x 10^6 cells/ml.

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

6. Transfer the vials to liquid nitrogen the next day for long term 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

- 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.
- All conditions should be performed in triplicate.
- Assay A should include “Stimulated Cells”, “Background Control” and “Unstimulated Control” conditions.

- Assay B should include "Stimulated Cells, No Compound", "Stimulated Cells, Test Compound", "Background Control" and "Unstimulated Control" conditions.
- It is recommended to use a final concentration of 0.1% DMSO or less for optimal results.

A. Treatment of NFAT Luciferase Reporter RAW 264.7 Cell Line with mouse RANKL or with Calcimycin (A23187) and phorbol 12-myristate 13-acetate (PMA)

1. Seed NFAT Luciferase Reporter RAW 264.7 cells at a density of 30,000 cells (for mRANKL) or 100,000 cells (for A23187 + PMA) in 90 µl/well of Thaw Medium 6 into a white clear-bottom 96-well cell culture plate. Leave a few empty wells as cell-free control wells ("Background Control").

2. Incubate the cells at 37°C with 5% CO_2 overnight.

3. Prepare a serial dilution of mouse RANKL, or a serial dilution of A23187 combined 30 nM PMA, at 10-fold the final concentration of mouse RANLK or A23187 in Thaw Medium 6 (10 µl/well).

4. Add 10 µl of the diluted mRANKL or A23187 with PMA to the "Stimulated Cells" wells.

5. Add 10 µl of Thaw Medium 6 to the "Unstimulated Control" wells.

6. Add 100 µl of Thaw Medium 6 to the "Background Control" wells (cell-free wells).

7. Incubate the cells at 37°C with 5% CO_2 overnight.

8. Add 100 µl of ONE-Step™ Luciferase reagent per well.

9. Incubate the plates at Room Temperature (RT) for ~10 minutes.

10. Measure luminescence using a luminometer.

11. The "Background Control" luminescence value should be subtracted from all readings.

12. Data Analysis: Subtract the average background luminescence from the luminescence reading of all other wells. The fold induction of luciferase reporter expression is the average background-subtracted luminescence of stimulated wells divided by the average background-subtracted luminescence of unstimulated control wells.

$$Fold\ induction = \frac{Luminescence\ of\ \ Stimulated\ Wells - avg.background}{Avg.Luminescence\ of\ Unstimulated\ Wells - avg.background}$$

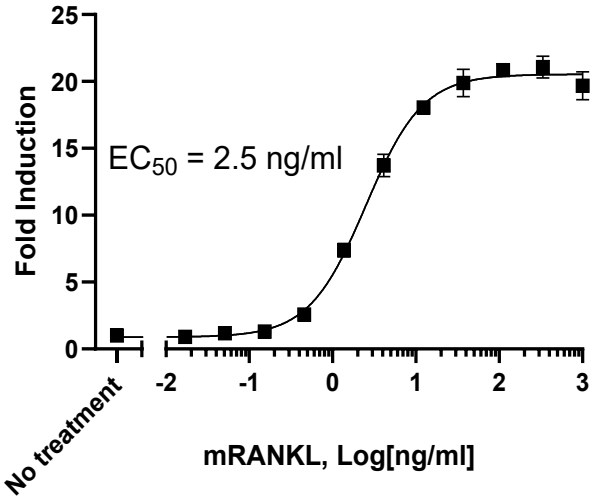

Figure 1. NFAT reporter activity in response to mouse RANKL in the NFAT Luciferase Reporter RAW 264.7 Cell Line.

NFAT Luciferase Reporter RAW 264.7 cells were treated with increasing concentrations of mouse RANKL. The results are shown as relative fold induction of luciferase reporter expression.

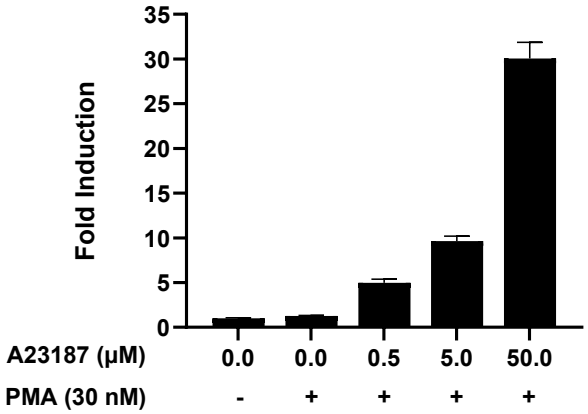

Figure 2. NFAT reporter activity in response to A23187 with 30 nM PMA in the NFAT Luciferase Reporter RAW264.7 Cell Line.

NFAT Luciferase Reporter RAW264.7 cells were treated with increasing concentrations of A23187 combined with 30 nM PMA. The results are shown as relative fold induction of luciferase reporter expression.

B. Inhibition of mRANKL- or A23187-induced NFAT reporter activity by Cyclosporin A in the NFAT Luciferase Reporter RAW 264.7 Cell Line

1. Seed NFAT Luciferase Reporter RAW 264.7 cells at a density of 30,000 cells (for mRANKL) or 100,000 cells (for A23187 + PMA) in 80 µl/well of Thaw Medium 6 into a white clear-bottom 96-well cell culture plate. Leave a few empty wells as cell-free control wells ("Background Control").

2. Incubate the cells at 37°C with 5% CO_2 overnight.

3. Prepare a serial dilution of Cyclosporin A at 10-fold the final testing concentration in Thaw Medium 6 (10 µl/well).

   Note: For example, to test 100 nM Cyclosporin A, prepare 100 µM Cyclosporin A in DMSO and dilute it 100-fold in Thaw Medium 6. This results in 1 µM Cyclosporin A in Thaw Medium 6 containing 1% DMSO. This is the diluted Cyclosporin A at 10-fold the final testing concentration, resulting in a 0.1% final DMSO concentration when 10 µl of this solution is added to 90 µl of cells.

4. Add 10 µl of the diluted Cyclosporin A to the "Stimulated Cells, Test Compound" wells.

5. Add 10 µl of Thaw Medium 6 containing 1% DMSO to the "Stimulated Cells, No Compound" and "Unstimulated Control" (100% activity and 0% activity, respectively).

6. Incubate the cells at 37°C with 5% CO_2 for 1 hour.

7. Prepare mRANKL, or A23187 with 30 nM PMA, in Thaw Medium 6 at 10x the EC_{90} concentration, i.e. at 0.14 µg/ml for mRANKL and 400 µM for A23187 with 300 nM PMA (10 µl/well).

8. Add 10 µl of diluted mRANKL, or A23187 with PMA, to the "Stimulated Cells, Test Compound" and "Stimulated Cells, No Compound" wells.

9. Add 10 µl of Thaw Medium 6 to the "Unstimulated" wells.

10. Add 100 µl of Thaw Medium 6 to the "Background Control" wells.

11. Incubate the cells at 37°C with 5% CO_2 overnight.

12. Add 100 µl of ONE-Step™ Luciferase reagent per well.

13. Incubate the plates at RT for ~10 minutes.

14. Measure luminescence using a luminometer.

15. Data Analysis: Subtract the average background luminescence from the luminescence reading of all other wells. The percent activity is the average background control-subtracted luminescence of the inhibitor treated wells divided by the average background control-subtracted luminescence of the "Stimulated Cells, No Compound" wells (mRANKL or A23187 + PMA added, but no Cyclosporin A) multiplied by 100.

$$\%\ Activity = \left(\frac{Luminescence\ of\ \ inhibitor\ wells - avg.background}{Avg.Luminescence\ of\ positive\ control\ wells - avg.background}\right) X\ 100$$

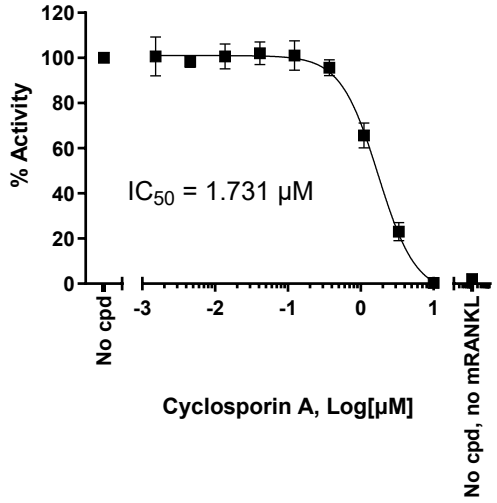

Figure 3. Inhibition of mRANKL-induced reporter activity by Cyclosporin A in the NFAT Luciferase Reporter RAW 264.7 Cell Line.
NFAT Luciferase Reporter RAW 264.7 cells were treated with increasing concentrations of cyclosporin A, in the presence or absence of mRANKL. The results are shown as relative fold induction of luciferase reporter expression.

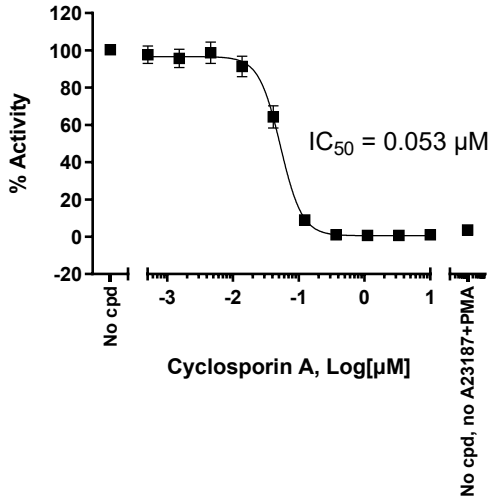

Figure 4. Inhibition of A23187+PMA- induced reporter activity by Cyclosporin A in the NFAT Reporter RAW 264.7 Cell Line.
NFAT Luciferase Reporter RAW 264.7 cells were treated with increasing concentrations of cyclosporin A, in the presence or absence of A23187 + PMA. The results are shown as relative fold induction of luciferase reporter expression.

Data shown is representative.

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Troubleshooting Guide
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