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Data Sheet

LAG3 / NFAT Reporter - Jurkat Recombinant Cell Line

Catalog #: 71278

Product Description
Recombinant Jurkat T cell expressing firefly luciferase gene under the control of NFAT response elements with constitutive expression of human LAG3 (lymphocyte-activation gene 3, CD223, GenBank Accession # NM_002286).

Background
Lymphocyte-activation gene 3 (LAG3, CD223) is a cell surface protein that belongs to the immunoglobulin (Ig) superfamily. LAG3 is expressed on activated T cells, natural killer cells, B cells, and plasmacytoid dendritic cells. Its main ligand is MHC class II, to which it binds with higher affinity than CD4. It negatively regulates cellular proliferation, activation, and homeostasis of T cells, in a similar fashion to CTLA-4 and PD-1, and has been reported to play a role in Treg suppressive function. A number of LAG3 antibodies are in preclinical development for treatments for cancer and autoimmune disorders. LAG3 may be a better immune checkpoint inhibitor target than CTLA-4 or PD-1 since antibodies to these two checkpoints are only activating effector T cells, and not inhibiting Treg activity where an antagonist LAG3 antibody can both activate effector T cells (by downregulating the LAG3 inhibiting signal) and inhibit induced (i.e. antigen-specific) Treg suppressive activity.

Application
- Screen for activators or inhibitors of LAG3 signaling in a cellular context
- Characterize the biological activity of LAG3 and its interactions with ligands

Format
Each vial contains $2x10^6$ cells in 1 ml of 10% DMSO

Storage
Immediately upon receipt, store in liquid nitrogen.

Mycoplasma Testing
The cell line has been screened using the PCR-based Venor®GeM Mycoplasma Detection kit (Sigma-Aldrich) to confirm the absence of *Mycoplasma* species.

General Culture Conditions

Thaw Medium 2 (BPS Bioscience Cat. #60184): RPMI1640 medium (Life Technologies #A10491-01) supplemented with 10% FBS (Life Technologies #26140-079), 1% Penicillin/Streptomycin (Hyclone #SV30010.01)

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Growth Medium 2A (BPS Bioscience Cat. # 60190): Thaw Medium 2 (BPS Bioscience Cat. #60184) plus 1 mg/ml of Geneticin (Life Technologies #11811031) and 200 µg/ml of Hygromycin B (Life Technologies #10687-010).

Cells should be grown at 37°C with 5% CO_2 using Growth Medium 2A (BPS Bioscience Cat. #60190).

To thaw the cells, rapidly thaw the frozen cells from liquid nitrogen in a 37°C water-bath, then transfer the entire contents of the vial to a tube containing 10 ml of Thaw Medium 2 (**no Geneticin and Hygromycin B)**. Spin down the cells, remove supernatant and resuspend cells in 5 mls of pre-warmed Thaw Medium 2 **(no Geneticin and Hygromycin B)**. Transfer the resuspended cells to a T25 flask and incubate at 37°C in a 5% CO_2 incubator until the cells are ready to be split. Cells should be split before they reach ~$2x10^6$ cells/ml. At first passage, switch to Growth Medium 2A **(contains Geneticin and Hygromycin B).**

To passage the cells, dilute cell suspension into new culture vessels at no less than $0.2x10^6$ cells/ml. Subcultivation ratio: 1:5 to 1:10 twice a week. Cells should be split before they reach $2x10^6$ cells/ml.

*Note: Just after thawing, the cells may grow at a slower rate. It is recommended to split the cells at no less than 0.4*x10^6 cells/ml *at the beginning of culturing. After ~two passages, the cell growth rate increases and the cells can be split to 0.2x10^6 cells/ml.*

To freeze down the cells, spin down cells, and resuspend cell pellet in 4°C Freezing Medium (10% DMSO + 90% FBS) to ~$2x10^6$ cells/ml. Dispense 1 ml of cell aliquots into cryogenic vials. Place vials in an insulated container for slow cooling and store at -80°C overnight. Transfer to liquid nitrogen the next day for storage. It is recommended to expand the cells and at an early passage freeze down more than 10 vials of cells for future use.

Functional Validation and Assay Performance
Expression of human LAG3 in LAG3/NFAT Reporter-Jurkat cell line was confirmed by FACS.

The functionality of the cell line was validated using a LAG3 cell-based assay. In this assay, LAG3/NFAT Reporter-Jurkat cells are used as effector cells and Raji cells are used as target cells. When these two cells are co-cultivated, addition of superantigen cross-links the TCR complexes on effector cells and the MHC class II on target cells, resulting in TCR activation and the expression of the NFAT luciferase reporter in effector cells. However, LAG3 and MHC class II interaction prevents this cross-linking event and, therefore, inhibits the TCR activation and the expression of NFAT-responsive luciferase in effector cells. This inhibition can be specifically reversed by LAG3 neutralizing antibody. LAG3 neutralizing antibody blocks LAG3: MHC class II interaction and promotes T cell activation by superantigen, resulting in reactivation of the NFAT responsive luciferase reporter.

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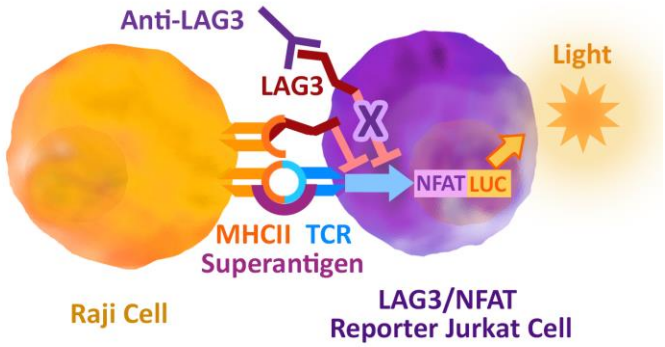

Materials Required but Not Supplied

- Raji cell (ATCC # CCL-86)
- Assay medium: RPMI1640 + 10% FBS + 1% Penicillin/Streptomycin (Thaw Medium 2 BPS Bioscience #60184)
- Growth Medium 2A (BPS Bioscience #60190)
- Small glass vials
- Tissue culture grade water
- Superantigen (SEE, Toxin Technology # ET404), (stock = 1µg/ul, prepared in tissue culture grade water, in a glass vial and stored at-20°C, minimize freeze/thaw cycles). Consult the Appendix section for guidance on using SEE superantigen in this assay.
- Anti-LAG-3 neutralizing antibody (BPS Bioscience #71219)
- 96-well tissue culture-treated white clear-bottom assay plate
- One-Step luciferase assay system (BPS Bioscience # 60690) or other luciferase reagents for measuring firefly luciferase activity
- Luminometer

Protocol

1. Dilute superantigen stock 1:100 in tissue culture grade water in a small glass tube to 10ng/µl. Additional intermediate dilutions should be prepared in tissue culture grade water in small glass tubes as needed. The final dilution of superantigen should be prepared in assay medium in a plastic conical tube. The final concentration of superantigen should be determined by the user according to the guidelines given in the Appendix section.

2. Prepare a serial dilution of anti-LAG-3 antibody in assay medium (the concentration of antibody here is 5x of the final treatment concentration of antibody).

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3. Harvest LAG3/NFAT-reporter Jurkat cells by centrifugation and resuspend in assay medium at 1x10^{6}cells/ml.

4. Harvest Raji cells by centrifugation and resuspend in assay medium containing superantigen (prepared in step 1) at 0.75x10^6 cells/ml. Preincubate the cells at 37° in a CO_2 incubator for 30 minutes.

5. Combine 40 µl of LAG3/NFAT Reporter Jurkat cells (1x10^6 cells/ml) and 20 µl of diluted anti-LAG3 antibody per well in 96-well white clear-bottom assay plate. Preincubate the cells at 37° in a CO_2 incubator for 30 min.

6. Add 40 µl per well of Raji cells in superantigen from step 4 to the LAG3/NFAT Reporter Jurkat cells with anti-LAG3 antibody from step 5. Mix the plate gently.

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   Final cell density of LAG-3/NFAT Reporter- Jurkat cells and Raji cells is 4 x10^4 cells/well and 3 x10^4 cells/well, respectively. Final concentration of antibody is 1x. Final volume is 100 µls in each well. Set up each treatment in at least triplicate.

   Add 100 µl of assay medium to cell-free control wells (for determining background luminescence).

   Incubate the plates at 37°C in a CO_2 incubator for 5 to 6 hours.

7. After ~5 to 6 hour incubation, 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.
   If using luciferase reagents from other vendors, follow the manufacturer's assay protocol.

8. Data Analysis: Subtract the average background luminescence (cell-free control wells) from the luminescence reading of all wells.
   The fold induction of NFAT luciferase reporter expression = background-subtracted luminescence of antibody treated well / average background-subtracted luminescence of untreated control wells.

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Figure 1. Characterization of biological activity of anti-LAG3 neutralizing antibody in LAG3 cell-based assay using the LAG3/NFAT Reporter-Jurkat cells co-cultured with Raji cells.

LAG3/NFAT Reporter-Jurkat cells (BPS Bioscience #71278) were incubated with anti-LAG3 neutralizing antibody (BPS Bioscience # 71219), Raji cells (ATCC # CCL-86), and superantigen. After incubation, ONE-Step™ Luciferase reagent (BPS Bioscience #60690) was added to the cells to measure NFAT activity.

The fold induction is equal to background-subtracted luminescence of antibody-treated well / background-subtracted luminescence of untreated-control wells of each respective cell line.

Dose response of anti-LAG3 neutralizing antibody in LAG3/NFAT Reporter-Jurkat cells.

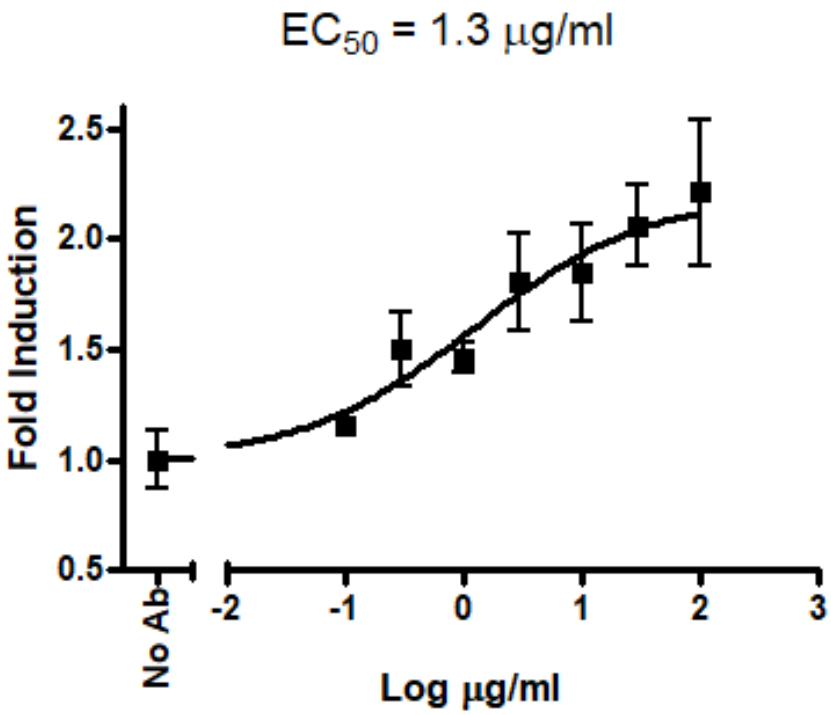

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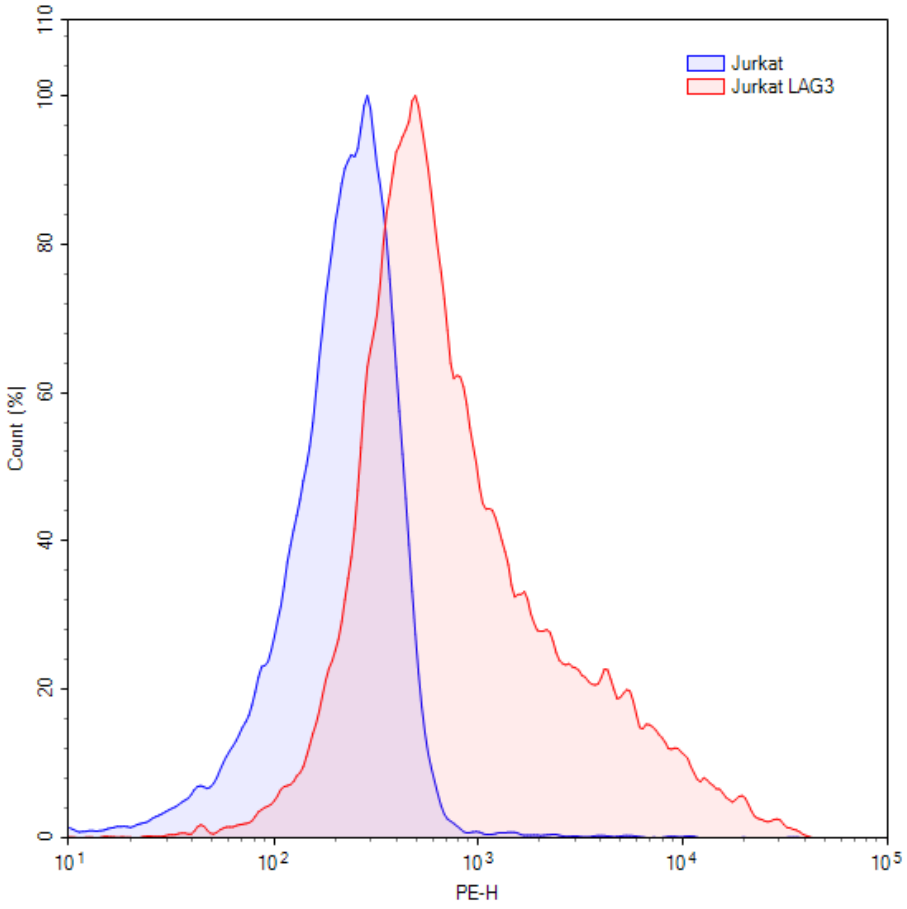
Figure 2 FACS analysis of cell surface expression of LAG3 in LAG3/NFAT Reporter-Jurkat cells.

LAG3/NFAT Reporter-Jurkat or control Jurkat cells were stained with PE-labeled anti-LAG3 antibody (BPS Bioscience# 71226) and analyzed by FACS. Y-axis is the cell count. X-axis is the intensity of PE.



	Samples	Subset	Cell Count
	LAG3/NFAT Reporter – Jurkat	Live singlet	13,800
	Control Jurkat	Live singlet	16,019

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Sequence

Human LAG3 sequence (accession number NM_002286)

MWEAQFLGLLFLQPLWVAPVKPLQPGAEVPVVWAQEGAPAQLPCSPTIPLQDLSLLRRAGVTWQHQPDSG
PPAAAPGHPLAPGPHPAAPSSWGPRPRRYTVLSVGPGGLRSGRLPLQPRVQLDERGRQRGDFSLWLRPAR
RADAGEYRAAVHLRDRALSCRLRLRLGQASMTASPPGSLRASDWVILNCSFSRPDRPASVHWFRNRGQGR
VPVRESPHHHLAESFLFLPQVSPMDSGPWGCILTYRDGFNVSIMYNLTVLGLEPPTPLTVYAGAGSRVGL
PCRLPAGVGTRSFLTAKWTPPGGGPDLLVTGDNGDFTLRLEDVSQAQAGTYTCHIHLQEQQLNATVTLAI
ITVTPKSFGSPGSLGKLLCEVTPVSGQERFVWSSLDTPSQRSFSGPWLEAQEAQLLSQPWQCQLYQGERL
LGAAVYFTELSSPGAQRSGRAPGALPAGHLLLFLILGVLSLLLLVTGAFGFHLWRRQWRPRRFSALEQGI
HPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQL

Appendix

The following technical notes are intended to supplement the information provided in the data sheet. These notes are intended to aid the investigator in achieving optimal results when using the LAG3/NFAT reporter-Jurkat cell line. It is important to maintain proper cell health for both the LAG3/NFAT Jurkat and the Raji cell lines throughout the culture and experimental processes. The LAG3/NFAT Jurkat cells remain stable and functional for at least 15 passages. See the General Culture Conditions section above for more information.

SEE Superantigen Optimization

We have identified potency differences from different lots of SEE superantigen (Toxin Technology, #ET404) (Figure 1: Dose responses in the LAG3/NFAT assay for two example lots of SEE). It is important to test a dose range of each lot of SEE in order to identify the optimal concentration for the LAG3 assay. The SEE superantigen dose response can be performed by following the experimental protocol provided above and using assay media in place of the antibody.

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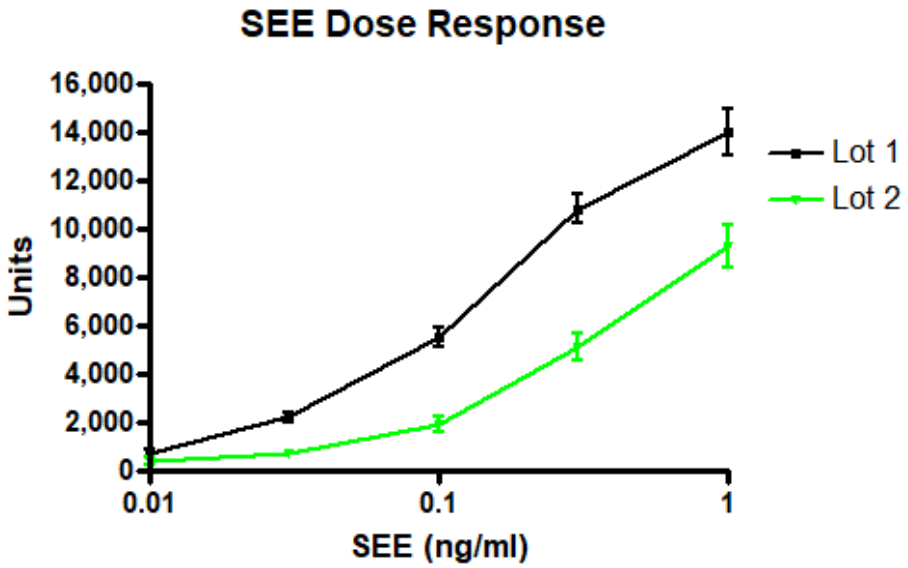
Figure 1: Example dose responses from 2 lots of SEE



Optimizing the Dose

We have observed that the raw signal as well as the fold stimulation from a given concentration of SEE can vary significantly from assay to assay, even when keeping the luminometer settings and the lot of SEE superantigen the same. Typically, very low signal (less than 100-200 units on our luminometer, for example) results in high standard deviations and data that is not reliable. Alternately, a very high signal for wells without added LAG3 antibody (greater than 10,000 units on our luminometer, for example), may reduce or eliminate the stimulation window when antibody is added. An example of this can be seen in figure 2 which shows the assay performance and fold stimulation by LAG3 antibody from the same lots of SEE superantigen depicted in figure 1.

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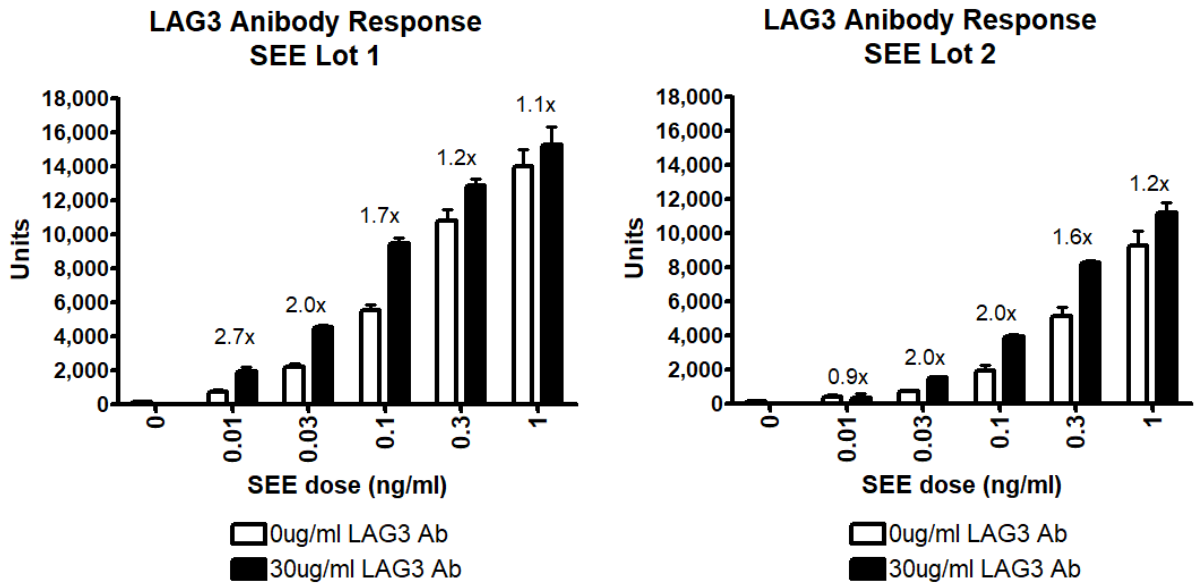
Figure 2: Identifying the best dose of SEE based on the LAG3 antibody response



An optimal LAG3 antibody window can usually be achieved at doses of SEE superantigen that are in the proper range (several hundred to the low thousands of counts on our luminometer, for example). In the data shown in figure 2 for SEE lot 1, we would recommend 0.01-0.03ng/ml, and for lot 2 we would recommend 0.03-0.1ng/ml. Each researcher needs to determine the optimal dose using their lot of SEE superantigen, the cells cultured in their lab and their luminometer. For best results, we suggest identifying the concentration range of SEE superantigen which gives a sufficient antibody signaling window. Future experiments should then be performed at two doses of SEE superantigen, one at the higher end and one at the lower end of this range.

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Summary of Recommendations

1) Include 3 wells of "no SEE superantigen" as a control in each assay. The SEE superantigen-treated wells should give a signal greater than this control.
2) Optimize the assay system by performing a dose curve of each lot of SEE superantigen.
3) Optimize the assay system by testing the LAG3 antibody at several doses of SEE superantigen to identify the SEE superantigen dose range which gives a sufficient antibody signal window.
4) Run each assay at 2 doses of SEE superantigen to account for assay to assay signal variability.

The LAG3 / NFAT Reporter cell line is considered to have performed successfully if:

1) Wells with SEE superantigen have a higher signal than wells without SEE superantigen.
2) 30 ng/ml of the LAG3 antibody results in an approximately two-fold increased signal versus wells without antibody.

Related Products

Product	Cat. #	Size
NFAT Reporter – Jurkat cell line	60621	2 vials
Anti-LAG3 neutralizing antibody	71219	100 µg
ONE-Step™ Luciferase Assay System	60690-1	10 ml
ONE-Step™ Luciferase Assay System	60690-2	100 ml
PE labeled anti-LAG3 antibody	71226-1	50 µg
PE labeled anti-LAG3 antibody	71226-2	100 µg
PD-1/NFAT Reporter-Jurkat cell line	60535	2 vials
TCR Activator/PD-L1-CHO cell line	60536	2 vials
LAG3 (CD223), Fc fusion (Human)	71146	100 µg
LAG3 (CD223), Biotin-labeled (Human) HiP™	71147	50 µg

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