

## Reagents Provided

**Phycoerythrin (PE)-conjugated goat polyclonal anti-human LAG-3:** Supplied as 75 µg of antibody in 1 mL saline containing up to 0.5% BSA and 0.1% sodium azide.

**Isotype:** goat IgG

## Reagents Not Provided

- PBS (Dulbecco's PBS)
- BSA

## Storage

Reagents are stable for **twelve months** from date of receipt when stored in the dark at 2° - 8° C.

## Intended Use

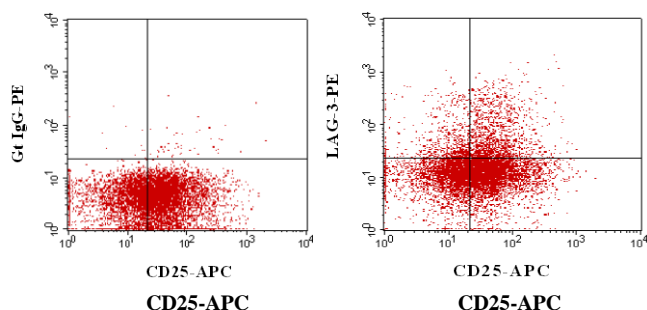
Designed to quantitatively determine the percentage of cells bearing LAG-3 within a population and qualitatively determine the density of LAG-3 on cell surfaces by flow cytometry.

## Principle of the Test

Washed cells are incubated with the phycoerythrin-labeled polyclonal antibody, which binds to cells expressing LAG-3. Unbound phycoerythrin-conjugated antibody is then washed from the cells. Cells expressing LAG-3 are fluorescently stained, with the intensity of staining directly proportional to the density of expression of LAG-3. Cell surface expression of LAG-3 is determined by flow cytometric analysis using 488 nm wavelength laser excitation and monitoring emitted fluorescence with a detector optimized to collect peak emissions at 565 - 605 nm.

## Reagent Preparation

**Phycoerythrin-conjugated goat anti-human LAG-3:** Use as is; no preparation necessary.



CD25+ T-cells were stained with PE-conjugated anti-human LAG-3 (Catalog # FAB2319P, right dot plot) or PE-conjugated isotype control (Catalog # IC108P, left dot plot).

## Sample Preparation

**Peripheral blood cells:** Whole blood should be collected in evacuated tubes containing EDTA or heparin as the anticoagulant. Contaminating serum components should be removed by washing the cells three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) by centrifugation at 500 x g for 5 minutes. Transfer 50 µL of packed cells to a 5 mL tube for staining with the monoclonal antibody. Whole blood will require lysis of RBC following the staining procedure.

**Cell Cultures:** Continuous cell lines or activated cell cultures should be centrifuged at 500 x g for 5 minutes and washed three times in an isotonic PBS buffer (supplemented with 0.5% BSA), as described above, to remove any residual growth factors that may be present in the culture medium. Cells should then be resuspended in the same buffer to a final concentration of 4 x 10<sup>6</sup> cells/mL and 25 µL of cells (1 x 10<sup>5</sup>) transferred to a 5 mL tube for staining.

Note: Adherent cell lines may require pretreatment with 0.5 mM EDTA to facilitate removal from substrate. Cells that require trypsinization to enable removal from substrate should be further incubated in medium for 6 - 10 hours on a rocker platform to enable regeneration of the receptors. The use of the rocker platform will prevent reattachment to the substrate.

## Sample Staining

- 1) Cells should be Fc-blocked by treatment with 1 µg of human IgG/10<sup>5</sup> cells for 15 minutes at room temperature prior to staining. Do not wash excess blocking IgG from this reaction.
- 2) Transfer 25 µL of the Fc-blocked cells (up to 1 x 10<sup>6</sup> cells) or 50 µL of packed whole blood to a 5 mL tube.
- 3) Add 10 µL of PE-conjugated LAG-3 reagent.
- 4) Incubate for 30 - 45 minutes at 2° - 8° C.
- 5) Following this incubation, remove unreacted LAG-3 reagent by washing the cells twice in 4 mL of the same PBS buffer (*note: whole blood will require an RBC lysis step at this point using any commercially available lysing reagent, such as R&D Systems Whole Blood Lysing Kit, Catalog # WL1000*).
- 6) Finally, resuspend the cells in 200 - 400 µL of PBS buffer for final flow cytometric analysis.
- 7) As a control for analysis, cells in a separate tube should be treated with PE-labeled goat IgG antibody.

This procedure may need modification, depending upon final utilization.

## Background Information

LAG-3 (Lymphocyte activation gene-3), also known as CD223, is a member of the immunoglobulin superfamily (IgSF). The mature LAG-3 protein is a 496 amino acid (aa) membrane protein with a 421 aa extracellular region which contains four IgSF domains, a 21 aa transmembrane region and a 54 aa cytoplasmic region. LAG-3 and CD4 molecules share < 20% amino acid sequence homology but have similar structure.<sup>1,2</sup> Both molecules bind to MHC class II. LAG-3 binds to MHC class II with higher affinity compared to CD4. Both LAG-3 and CD4 genes are located on the distal part of the short arm of chromosome 12.

LAG-3 is expressed on activated T cells and NK cells, but not on resting T cells. Studies using LAG-3<sup>-/-</sup> mice have shown significant delay of T cell apoptosis following antigen stimulation and increased size of memory T cell pools following infection.<sup>3,4</sup> It also has been reported that anti-LAG-3 antibodies up-regulate T cell activation by blocking the interaction of LAG-3 with MHC class II. The recent study has demonstrated that LAG-3 is selectively expressed on activated CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Reg</sub> cells and plays a role in their suppressive activity.<sup>5</sup> This evidence indicated LAG-3 binds to MHC class II and negatively regulates T cell activation through LAG-3 signaling, unlike the interaction of CD4 with MHC class II which plays a positive role in T cell activation. On the other hand, studies have shown that binding of LAG-3 to MHC class II molecules on antigen presenting cells induces maturation of dendritic cells and cytokine secretion by monocytes through MHC class II signal transduction.<sup>6</sup> Taken together, LAG-3 may have two major functions, to negatively regulate T cell activation through LAG-3 signaling and to stimulate antigen presenting cells which express MHC class II.

## References

1. Triebel, F. *et al.*, 1990, J. Exp. Med. **171**:1393.
2. Baixeras, E. *et al.*, 1992, J. Exp. Med **176**:327.
3. Workman, C.J. and D.A. Vignali, 2003, Eur. J. Immunol. **33**:970.
4. Workman, C.J. *et al.*, 2004, J. Immunol. **172**:5450.
5. Huang, C.T. *et al.*, 2004, Immunity **21**:503.
6. Andreae, S., Buisson, S. and F. Triebel, 2003, Blood **102**:2130.

**Warning:** Contains sodium azide as a preservative - sodium azide may react with lead and copper plumbing to form explosive metal azides. Flush with large volumes of water during disposal.