



**Highest Standards for  
Research Kits™**

***Manual  
LAG-3, Soluble (human)  
Detection Set (For ELISA  
Application)***

**[Lymphocyte Activation Gene-3, Soluble (human) Detection Set; CD223, Soluble (human) Detection Set]**

***For Research Use Only***

**APO-54N-017**

**Version 6 (31-AUG-07)**



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## 1. INTENDED USE

The LAG-3, Soluble (human) Detection Set is to be used for the *in vitro* quantitative determination of soluble human LAG-3, in human serum or cell culture supernatant. This Detection Set is for research use only.

## 2. INTRODUCTION

The lymphocyte activation gene-3 (LAG-3, also called CD223), a member of the immunoglobulin superfamily (IgSF) related to CD4, binds to the major histocompatibility complex (MHC) class II molecules but with higher affinity than CD4 (1). Several alternative mRNA splice-variants of human LAG-3 have been described, two of them encoding potential secreted forms: LAG-3V1 (*i.e.* the D1-D2 domains of the protein, 36 kDa) and LAG-3V3 (D1-D3, 52 kDa)(1,2). The longer form was detected by ELISA in the serum of healthy individuals as well as of tuberculosis patients with a favorable outcome (3). LAG-3 expression by T cell clones correlated with IFN- $\gamma$  production, and hence soluble LAG-3 has been suggested as a serological marker of Th1 responses (4).

### References:

1. Triebel, F. (2003). LAG-3: a regulator of T-cell and DC responses and its use in therapeutic vaccination. *Trends Immunol.* 24, 619-622.
2. Triebel, F., Hacene, K., and Pichon, M.F. (2005). A soluble lymphocyte activation gene-3 (sLAG-3) protein as a prognostic factor in human breast cancer expressing estrogen or progesterone receptors. *Cancer Lett.*
3. Lienhardt, C., *et al.* (2002). Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo. *Eur. J. Immunol.* 32, 1605-1613.
4. Annunziato, F. *et al.* (1996). Expression and release of LAG-3-encoded protein by human CD4+ T cells are associated with IFN-gamma production. *Faseb J* 10, 769-776.

## 3. PRINCIPLE OF PROCEDURE

This assay is a sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) developed for the direct measurement of human soluble LAG-3 (sLAG-3) in serum and cell culture supernatants. A monoclonal antibody specific for human LAG-3 (hLAG-3)(11E3) is coated onto the wells of the supplied microtiter strips. Samples and concentration standards of sLAG-3 are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, sLAG-3 is recognized by the addition of a biotinylated monoclonal antibody specific for hLAG-3 (17B4)(biotin). After removal of excess biotinylated antibody, streptavidine-peroxidase is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of sLAG-3 in the samples.

#### 4. MATERIALS PROVIDED

- 1 vial (50µg) LAG-3:Fc (human) (recombinant) (Prod. No. APO-50N-059)
- 1 vial (100µg) MAb to LAG-3 (human) (17B4) (Biotin) (Detection Antibody) (Prod. No. APO-20N-052B)
- 1 vial (100µg) MAb to LAG-3 (human) (11E3) (Capture Antibody) (Prod. No. APO-20N-051).

#### 5. MATERIALS REQUIRED

- PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, 0.2 µM filtered
- Wash Buffer: 0.1% Tween<sup>®</sup> 20 in PBS.
- Blocking Buffer: 2% bovine serum albumin (BSA) in PBS, 0.2 µM filtered
- ELISA Buffer: 2% BSA and 0.1% Tween<sup>®</sup> 20 in PBS
- 2M sulphuric acid (H<sub>2</sub>SO<sub>4</sub>)
- Tetramethylbenzidine (TMB) substrate kit (BD PharMingen)
- HRP labelled streptavidin (DAKO)
- HPLC grade water

#### 6. PRODUCT SPECIFICATION

**Number of Assays:** This detection Set contains sufficient materials to run ELISAs on 2 x 96-well plates.

**Specificity:** The monoclonal antibodies used in this detection Set are specific for measurement of natural and recombinant human LAG-3. They do not cross-react with mouse or rat LAG-3.

**Sensitivity:** 0.05 ng /ml (range 0 to 4 ng /ml)

**Stability:** Stable at least 12 months after receipt when stored at +4°C.

## 7. GENERAL ELISA PROTOCOL

### PREPARATION OF REAGENTS

1. Dilute the desired amount of capture antibody (11E3) (0.5mg/ml) to a concentration of 5µg/ml in PBS without carrier protein.
2. Dilute the desired amount of detection antibody (17B4) (Biotin) (0.5mg/ml) to a concentration of 0.5µg/ml in ELISA buffer.
3. Dilute the standard protein (rhLAG-3:Fc) (0.5mg/ml) in ELISA Buffer. A seven point standard curve using 2-fold serial dilutions in ELISA Buffer is recommended. Suggested standard points are 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml and 62.5pg/ml.
4. If measuring serum or plasma, dilute samples in ELISA buffer.
5. To reduce interference from rheumatoid factor (RF) in the serum, Ig can be cleared by treating serum dilutions with 0.05 volume of protein G-Sepharose (Amersham Biosciences) twice for one hour at 4°C before pelleting the beads and collecting supernatants (precleared sera)<sup>1</sup>.

### PLATE PREPARATION

1. Coat the wells by adding 100µl/well of diluted (5µg/ml) capture antibody (11E3) to a 96-well ELISA microplate (Nunc MaxiSorp™ flat-bottom 96 well plate is suggested). Cover the plate with plastic film and leave overnight at 4°C.
2. Aspirate the coated wells and add 300µl of Wash Buffer using a multichannel pipette or autowasher. Repeat the process for a total of three washes. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300µl of Blocking Buffer at room temperature for 2 hours.
4. Repeat the aspiration/wash as in step 2 for a total of three washes.

## ASSAY PROCEDURE

1. Add a total of 100µl/well diluted serum, plasma or rhLAG-3:Fc serial dilutions in ELISA Buffer to the plate.
2. Cover the plate with plastic film and incubate for 3 hours at room temperature.
3. Repeat the aspiration/wash as in step 2 of “Plate Preparation” for a total of four washes.
4. Add 100µl/well of the diluted (0.5µg/ml) detection antibody (17B4)(Biotin).
5. Cover the plate with plastic film and incubate for 1 hour at room temperature.
6. Repeat the aspiration/wash as in step 2 of “Plate Preparation” for a total of four washes.
7. Add 100µl/well of the diluted HRP labelled streptavidin in ELISA buffer (Amersham Biosciences; 1:5000). Cover the plate with plastic film and incubate for 30 min at room temperature in the dark.
8. Repeat the aspiration/wash as in step 2 of “Plate preparation” for a total of five washes.
9. Substrate development is conducted using Tetramethylbenzidine (TMB) substrate kit (follow manufacturer’s recommendations for use).
10. Stop the reaction by adding 50µl of 2M H<sub>2</sub>SO<sub>4</sub>. Tap the plate gently to ensure thorough mixing.
11. Measure the OD at 450nm in an ELISA reader.
12. Measure absorbance at 550nm and subtract these values from those obtained at 450nm to correct for optical imperfections in the microplate. If absorbance at 550nm is not possible, measure the absorbance at 450nm only.

**Note:** When the 550 nm measurement is omitted, absorbance values will be higher.

## 8. TECHNICAL HINTS AND LIMITATIONS

- Once reagents have been added to the plate, DO NOT let the plate dry at any time during the assay.
- Be sure no sodium azide is present in this assay, as this inhibits HRP enzyme activity.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can become contaminated thereby causing assay variability. Buffers containing a large quantity of protein should be made under sterile conditions and stored at 2-8°C or be prepared fresh daily.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedure.

## 9. CALCULATION OF RESULTS

- Average the duplicate readings for each standard, control and sample and subtract the average blank value.
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding LAG-3 concentration (pg/ml) on the horizontal axis (see **10.TYPICAL DATA**).
- Calculate results using graph paper or curve-fitting statistical software. The amount of LAG-3 in each sample is determined by interpolating for the absorbance value (Y axis) using the standard curve.
- If the test sample was diluted multiply the interpolated value by the dilution factor to calculate pg/ml of human soluble LAG-3 in the sample.

## 10. TYPICAL DATA

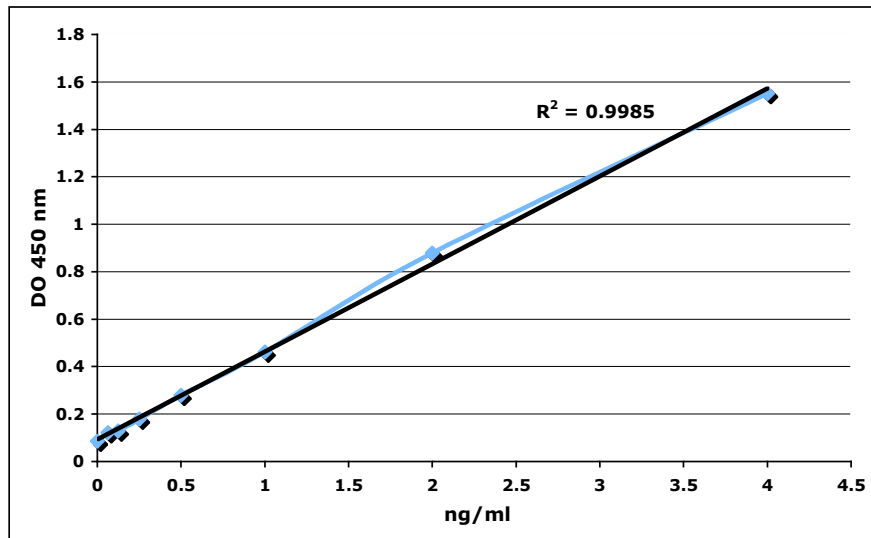


Figure: Standard curve

## 11. PRODUCT SPECIFIC LITERATURE REFERENCES:

*Expression and release of LAG-3-encoded protein by human CD4+ T cells are associated with IFN-gamma production:* F. Annunziato, et al.; FASEB J. **10**, 769 (1996).

*Preferential Th1 profile of T helper cell responses in X-linked (Bruton's) agammaglobulinemia:* A. Amedei, et al.; Eur. J. Immunol. **31**, 1927 (2001).

*Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo.* C. Lienhardt, et al.; Eur. J. Immunol. **32**, 1605 (2002).



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