RayBio® Human LAG3 / LSECtin Ligand Binding Assay

Inhibitor Screening ELISA Kit

User Manual Version 1.0
July 16, 2024

Catalog numbers: BAH-LAG3-CLEC4G-1 (1 plate kit)
BAH-LAG3-CLEC4G-2 (2 plate kit)
BAH-LAG3-CLEC4G-5 (5 plate kit)

Caution:
Please read manual carefully
before starting your experiment.

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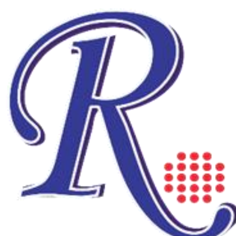

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RayBio® Ligand Binding Assay
LAG3 / LSECtin

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Please read the entire manual carefully before starting your experiment.

I. STORAGE

The kit may be stored at 4°C up to 1 month from the date of shipment. Shelf life can be extended up to 6 months by storing protein (Item F) and antibodies (Items C and D, if included), at -20°C immediately upon delivery. All other kit components should be stored at 4°C.

II. MATERIALS PROVIDED

ITEM	COMPONENT	Quantity
A*	LAG3 coated 96 well microplate	1 plate
B	20X Wash Buffer Concentrate	1 vial (25 ml)
E2	5X Assay Diluent	1 vial (15 ml)
F	LSECTin Protein	2 vials+
C	Detection Antibody	2 vials+
D	HRP Conjugated Anti-IgG	1 vial+
H	TMB One-Step Substrate Reagent	1 vial (12 ml)
I**	Stop Solution	1 vial (8 ml)

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*
***Contains 0.2 M Sulfuric Acid*
+See vial for reagent volume.

III. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Shaker.
3. Pipettes capable of accurately delivering 2 µl to 1 ml volumes.
4. Pipettes capable of delivering 1 – 25 ml volumes for reagent preparation.
5. Graduated cylinders: 100 ml and 1 liter
6. Distilled or deionized water.
7. Tubes to prepare sample dilutions.

IV. REAGENT PREPARATION

NOTE: *Thaw all reagents, buffers, and "Test Reagents"* to room temperature immediately before use. If wash buffers contain visible crystals, mix gently (at room temperature) until dissolved.*

NOTE: *Briefly centrifuge (~1,000g) Ligand Protein (Item F) and antibodies (Items C and D, if present) before opening to ensure maximum recovery.*

<table>
<tr><th>ITEM</th><th>COMPONENT</th><th>PREPARATION</th><th>STORAGE AFTER PREPARATION</th></tr>
<tr><td>A</td><td>LAG3 coated 96-Well Microplate</td><td>No Preparation</td><td>6 months at 4°C</td></tr>
<tr><td>B</td><td>20X Wash Buffer Concentrate</td><td>Dilute 20-fold** with distilled or deionized water</td><td rowspan="2">1 month at 4°C</td></tr>
<tr><td>E2</td><td>5X Assay Diluent</td><td>Dilute 5-fold with distilled or deionized water to prepare "1X Assay Diluent"</td></tr>
<tr><td>F</td><td>LSECTin Protein[+]</td><td>Bring total volume (per tube) to 60 uL with 1X Assay Diluent to prepare "100x Ligand Protein Concentrate."

Dilute 100-fold with 1X Assay Diluent to prepare "1X Protein Solution." Leave enough undiluted 100x Ligand Protein Concentrate to prepare test reagents.</td><td rowspan="3">5 days at 4°C</td></tr>
<tr><td>C</td><td>Detection Antibody[+]</td><td>Bring total volume (per tube) to 55 uL with 1X Assay Diluent".

Dilute 100-fold with 1X Assay Diluent to prepare "1X Detection Antibody"</td></tr>
<tr><td>D</td><td>HRP Conjugated Anti-IgG[+]</td><td>Dilute 1000-fold with 1X Assay Diluent to prepare "1X HRP Conjugated Anti-IgG"</td></tr>
<tr><td>H</td><td>TMB Substrate</td><td rowspan="2">No Preparation</td><td rowspan="2">6 months at 4°C</td></tr>
<tr><td>I</td><td>Stop Solution</td></tr>
</table>

* *"Test Reagent" refers to the samples, or potential inhibitors, to be tested. The binding assay is compatible with various types of Test Reagents, including serum, plasma, peptides, antibodies, small molecules, and proteins.*

** *A 20-fold dilution is the same as 1-part 20X Wash Buffer Concentrate and 19-parts water.*

[+] *100 µl of each reagent is needed per well. Scale up according to number of wells run per assay. We recommend that the calculated volume be multiplied by 1.2 to compensate for pipetting error and to ensure that there is sufficient volume to fill all the wells.*

V. "TEST REAGENT" SAMPLE PREPARATION

Dose Response. When evaluating a potential inhibitor, it is highly recommended to perform a titration curve. This approach will help the researcher empirically determine the dose-responsive range of Test Reagent as well as the lowest level that yields detectable inhibition. It will also help confirm whether the inhibition is real; that is, whether the inhibition increases as more Test Reagent is applied. ***Note***: The researcher should determine an appropriate serial dilution based on the known properties of their Test Reagent. From the result of this dilution series, the best dilution of the Test Reagent can be empirically determined.

Preparation of Reactions. Since the Test Reagent putatively competes with the ligand for binding to receptor, it is critical that the ligand be present at the same concentration in every well. That is, the concentration of ligand protein is held constant while the Test Reagent (or vehicle blank) varies from well to well.

Replicates. It is recommended that at least 2 replicates are run for all Test Reagents and controls. Therefore, replicates should be taken into account when calculating the volumes to be prepared. Each well will contain a final volume of 100 µl. It is recommended that 125 µl be prepared for each replicate to account for volume loss cause by normal pipetting error.

Test Reagent Serial Dilution Series.

1. Label a series of tubes, which will be used for the Test Reagent's serial dilutions.

2. Prepare the starting dilution ("Dilution 1") of the Test Reagent dilution series as follows:

   a. Mix Test Reagent + 1.25 µl 100x Ligand Protein Concentrate.

   b. Bring total volume to 125 µl with 1x Assay Diluent.

   Note*: Volume of Test Reagent should be determined by researcher. Above instructions are for one replicate. Volumes should be scaled up depending on the number of replicates run.*

3. Create remaining serial dilutions. Below instructions are based on 10-fold serial dilutions. Adjust volumes depending on your desired dilution.

   a. Pipette 112.5 µl 1X Protein Solution to each of the remaining tubes of your Test Reagent dilution series.

   b. Pipette 12.5 µl from the most concentrated dilution ("Dilution 1") into the second most concentrated dilution ("Dilution 2"). Mix well with pipette or vortex.

   c. Repeat Step 3b for each serial dilution until the final concentration is reached.

Vehicle Blank Serial Dilution Series.

1. It is **strongly recommended** that each unique vehicle blank is evaluated for unintended vehicle effects. If a vehicle effect is observed, the researcher should run a vehicle blank dilution series in place of the "reagent blank" negative control on all subsequent assays.

   Note: *The vehicle blank is the solution or buffer that the Test Reagent is in. For example, if the Test Reagent is in dimethyl sulfoxide (DMSO), a parallel set of tubes should be prepared with DMSO vehicle blank in an identical manner as the test reagent.*

2. Create the vehicle blank dilution series in same way as the Test Reagent serial dilution series, using the vehicle in place of the Test Reagent.

Assay Controls

1. Prepared 1X Protein Solution serves as the "0 mM" positive control.

   Note: This positive control does not contain the Test Reagent and is necessary to use as a reference to determine whether the Test Reagent is an inhibitor. This control is required every time the assay is performed.

2. Prepared 1X Assay Diluent serves as the "reagent blank" negative control.

   Note*: This negative control does not contain the Test Reagent or the 1X Protein Solution. If you do not include the vehicle blank, you must include this Reagent Blank negative control every time the assay is performed.*

   Note*: Vehicle blank controls are preferred over the Reagent Blank control.*

VI. ASSAY PROCEDURE

Note*: All incubations and wash steps must be performed at room temperature under gentle rocking or rotation (~1-2 cycles/sec).*

1. Bring all reagents to room temperature (18 - 25°C) before use.

2. Design your experiment and label removable 8-well strips as appropriate for your experiment. See Figure 2 below for an example.

3. As prepared above in Section V, add **100 µl** of each Test Reagent, vehicle blank, "0 mM" positive control, and "reagent blank" negative control into appropriate wells as determined in step 2 below. Seal plate with included plate seals and incubate for **2.5 hours**.

   Optional*: This incubation step can be run overnight at 4°C with gentle shaking/rocking (~1-2 cycles/sec).*

4. Discard the solution over sink and wash **4 times** with <u>1x Wash Buffer</u>. Wash by filling each well with **300 µl** 1x Wash Buffer using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining 1x Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add **100 µl** of prepared <u>1x Detection Antibody</u> to each well. Incubate for **1 hour**.

6. Discard the solution. Repeat the wash as described in Step 4 above.

7. Add **100 µl** of the prepared <u>1x HRP Conjugated Anti-IgG</u> to each well. Incubate for **1 hour.**

8. Discard the solution. Repeat the wash as described in Step 4 above.

9. Add **100 µl** of <u>TMB One-Step Substrate Reagent</u> to each well. Incubate for **30 minutes**. Protect from light.

   ***Note**: Proceed immediately to step 10. Do **<u>NOT</u>** wash.*

10. Add **50 µl** of <u>Stop Solution</u> directly to each well. Read at 450 nm immediately.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th></tr>
<tr><td>A</td><td colspan="2" rowspan="7">Vehicle Blank Dilution Series
(In duplicate)</td><td colspan="2" rowspan="7">Test Reagent 1 Dilution Series
(In duplicate)</td><td colspan="2" rowspan="7">Test Reagent 2 Dilution Series
(In duplicate)</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td><td colspan="3">“0 mM”
Positive Controls</td><td></td><td></td><td></td></tr>
</table>

Figure 2. Example plate layout for RayBio Ligand Binding Assay. In this example, two Test Reagents (strips 3 – 6) and vehicle blank (strips 1 – 2) were serially diluted to prepare separate dilution series and tested in duplicate. “0 Mm” Positive controls were run in triplicate (wells H1 – H3). ***Note***: “Reagent blank” negative controls were not necessary in this example since the vehicle blank dilution series was tested. A “reagent blank” negative control <u>must</u> be included if not running the vehicle blank.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and Test Reagent samples as instructed.

2. Add 100 µl Test Reagent samples to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

3. Add 100 µl the prepared 1X Detection antibody solution to each well. Incubate 1 hour.

4. Add 100 µl the prepared 1X HRP-Conjugated Anti-IgG solution to each well. Incubate 1 hour.

5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes.

6. Add 50 µl Stop Solution directly to each well (do **NOT** wash after TMB step).

7. Read at 450 nm immediately.

VIII. DATA ANALYSIS

1. Calculate average optical density (OD) absorbance across all replicates for each Test Reagent, vehicle blank, and controls.

2. Subtract the “vehicle blank” or “reagent blank” OD values from your corresponding Test Reagent OD values.

3. Confirm that OD of highest Test Reagent concentration is lower than the “0 mM” positive control and increases as the Test Reagent concentration decreases.

4. Determine the percent binding inhibition (BI%):

$$BI\% = \frac{OD\ of\ \text{"0mM"}\ positive\ contol - OD\ of\ Test\ Reagent}{OD\ of\ \text{"0 mM"}\ positive\ control}\ X\ 100$$

IX. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
Low/no change in OD across Test Reagent dilution series	Test Reagent starting concentration too low	Increase Test Reagent concentration
	Test Reagent does not interfere (or minimally interferes) with ligand-receptor binding.	Consider alternative Test Reagents or more sensitive methods to examine Test Reagent.
High CV%	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low "0mM" positive control	Improper storage of kit	Upon receipt, the kit should be stored at 4°C
	Stop Solution	Stop Solution should be added to each well. The OD should be read immediately after adding the Stop Solution.
	Improper dilution of protein, primary or secondary antibody	Ensure correct dilution

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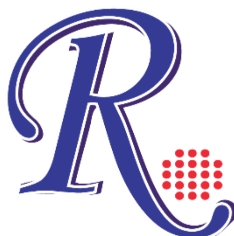

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