BioLegend®

Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit

# Mouse IL-35 Heterodimer

Cat. No. 440507

ELISA Kit for Accurate Quantitation of Mouse IL-35 Heterodimer from Cell Culture Supernatant, Serum, Plasma, and Other Biological Fluids

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***It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.***

## Table of Contents

## Introduction:

IL-35 is a member of the IL-12 cytokine family. It is a heterodimeric protein composed of EBI-3 (Epstein-Barr virus-induced gene 3) and p35 (IL-12A) subunits. EBI-3 (IL-27B) is a 34 kD glycoprotein that is related to the p40 (IL-12B) subunit of IL-12. p35 is a 35 kD glycoprotein that is related to the p19 (IL-23A) and p28 (IL-27A) subunits of IL-23 and IL-27, respectively.

IL-35 is a new anti-inflammatory cytokine with many reported biological functions. One of the proposed functions of IL-35 is regulating T regulatory (Treg) cell. IL-35 is expressed by T regulatory cells. It expands $CD4^+CD25^+$ Treg cells, suppresses the proliferation of $CD4^+CD25^-$ effector cells, and inhibits Th17 cell polarization.

IL-35 also suppresses collagen-induced arthritis or Helicobacter-induced colitis in animal models. High levels of IL-35 were found in the plasma samples of acute myeloid leukemia patients, and synovial fluid samples of patients with rheumatoid arthritis. Relative levels of IL-35 in biological samples and its significance are not fully understood at the time of this kit release.

LEGEND MAX™ Mouse IL-35 Heterodimer ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a rat monoclonal anti-mouse IL-35 antibody. The Detection Antibody is a biotinylated goat polyclonal anti-mouse IL-12 antibody. This kit is specifically designed for the accurate quantitation of mouse IL-35 heterodimer from cell culture supernatant, serum, plasma, and other biological fluids. This kit is analytically validated with ready-to-use reagents.

## Materials Provided:

| Description | Quantity | Volume (per bottle) | Part # |
|---|---|---|---|
| Anti-Mouse IL-35 Pre-coated 96 well Strip Microplate | 1 plate | | 77039 |
| Mouse IL-35 Detection Antibody | 1 bottle | 12 mL | 77040 |
| Mouse IL-35 Standard | 1 vial | lyophilized | 77042 |
| Avidin-HRP D | 1 bottle | 12 mL | 78237 |
| Assay Buffer F | 1 bottle | 25 mL | 79859 |
| Wash Buffer (20X) | 1 bottle | 50 mL | 78233 |
| Substrate Solution F | 1 bottle | 12 mL | 79132 |
| Stop Solution | 1 bottle | 12 mL | 79133 |
| Plate Sealers | 4 sheets | | 78101 |



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## Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker

### Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="6">Store opened reagents between 2°C and 8°C, and use within one month.</td></tr>
<tr><td>Avidin-HRP D</td></tr>
<tr><td>Assay Buffer F</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution F</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

### Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

**Specimen Collection and Handling:**

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at -70°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles.

**Reagent and Sample Preparation:**

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water.
2. Reconstitute the lyophilized Mouse IL-35 Standard by adding the volume of Assay Buffer F to make the 150 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.
3. In general, serum, plasma and tissue culture supernatant samples are analyzed without dilutions. However, if dilutions are required, use the control culture medium or Assay Buffer F as the sample diluent.



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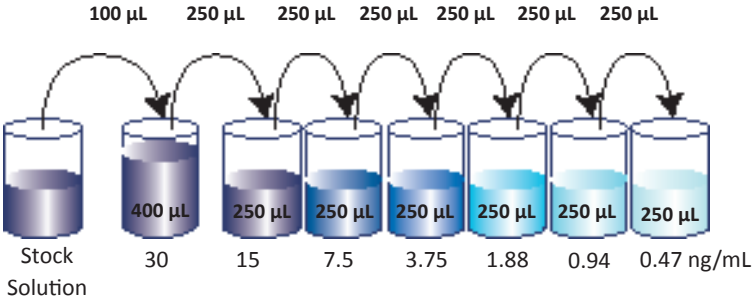
**Assay Procedure:**

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 µL of the 30 ng/mL top standard by diluting 100 µL of the 150 ng/mL standard stock solution into 400 µL of Assay Buffer F in a polypropylene tube. Starting with the 30 ng/mL top standard, perform six two-fold serial dilutions by mixing and then transferring 250 µL to the next tube. The final mouse IL-35 standard concentrations in the tubes are 30 ng/mL, 15 ng/mL, 7.5 ng/mL, 3.75 ng/mL, 1.88 ng/mL, 0.94 ng/mL, and 0.47 ng/mL, respectively. Assay Buffer F serves as the zero standard (0 pg/mL) .




4. Wash plate 4 times with at least 300 µl 1X Wash Buffer per well and blot residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 µL of Assay Buffer F to each well that will contain either standard dilutions or samples.

6. Add 50 µL of standard dilutions or samples to the appropriate wells.

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking.

8. Discard the plate contents into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 µL of Mouse IL-35 Detection Antibody solution to each well, seal

the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

11. Add 100 µL of Avidin-HRP D solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

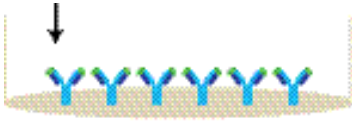
13. Add 100 µL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing mouse IL-35 should turn blue in color with intensity proportional to concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 µL of Stop Solution to each well. The well color should change from blue to yellow.

15. Read absorbance at 450 nm within 20 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

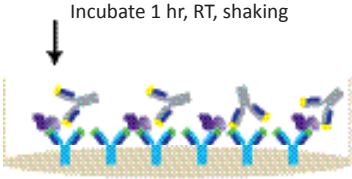
## Assay Procedure Summary

1. Wash 4 times
Add 50 µL Assay Buffer F

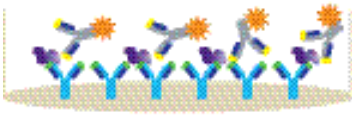


2. Add 50 µL standards or samples
Incubate 2 hr, RT, shaking

3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 1 hr, RT, shaking



4. Wash 4 times
Add 100 µL Avidin-HRP D solution
Incubate 30 min, RT, shaking



5. Wash 5 times
Add 100 µL Substrate Solution F
Incubate 15 min, RT, in the dark

6. Add 100 µL Stop Solution

7. Read absorbance at 450 nm and 570 nm

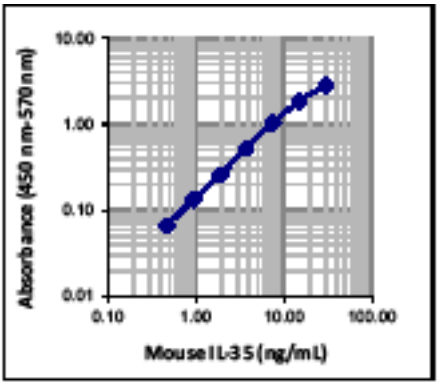
## Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

## Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



**Performance Characteristics:**

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines, each at 50 ng/mL.

| | |
|---|---|
| Mouse | IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-22, IL-23, IL-27, IL-34, GM-CSF, TACI, CCL1, CXCL1, CXCL9, IFNγ and TNFα |
| Human | IL-12p40, IL-12p70, IL-23, IL-27, IL-35, IL-2, IL-4, IL-5, IL-6, IL-8, IL-11, IL-17A, IL-17F, FGF-b, IFNγ and TNFα |

*Sensitivity:* The average minimum detectable concentration of mouse IL-35 is 0.08 ± 0.04 ng/mL.

*Recovery:* Four levels of known concentrations of IL-35 were spiked into two pooled mouse sera, and then analyzed with the LEGEND MAX™ Mouse IL-35 Heterodimer ELISA Kit. On average, 97% of IL-35 was recovered from serum.

*Linearity:* Two pooled mouse serum samples were spiked with high concentrations of IL-35 were diluted with Assay Buffer F to produce sample concentrations within the dynamic range of the assay. On average, 99% of the expected cytokine was detected from diluted serum samples

*Intra-Assay Precision:* Two samples with different mouse IL-35 concentrations were tested with 16 replicates in one assay.

| Concentration | Sample 1 | Sample 2 |
|---|---|---|
| Number of Replicates | 16 | 16 |
| Mean Concentration (ng/mL) | 14.1 | 3.7 |
| Standard Deviation | 0.6 | 0.2 |
| % CV | 4.3 | 5.4 |

Inter-Assay Precision: Two samples with different concentrations of mouse IL-35 were assayed in three independent assays.

| Concentration | Sample 1 | Sample 2 |
|---|---|---|
| Number of Assays | 3 | 3 |
| Mean Concentration (ng/mL) | 14.6 | 3.7 |
| Standard Deviation | 0.6 | 0.3 |
| % CV | 4.1 | 8.1 |

*Biological Samples:* Serum: Fresh individual C57BL/6 mouse serum (n=20) and CD-1 mouse serum (n=20) samples (from a commercial source) were assayed for basal levels of mouse IL-35. Mouse IL-35 levels in all samples tested are below the lowest standard curve point, 0.47 ng/mL.

## Troubleshooting Guide:

| Problem | Probable Cause | Solution |
|---|---|---|
| High Background | Background wells were contaminated | Avoid cross-well contamination by using the provided plate sealers.<br>Use multichannel pipettes and change tips between pipetting samples and reagents. |
| | Insufficient washes | Increase number of washes.<br>Increase soaking time between washes prior to addition of substrate solution. |
| | TMB Substrate Solution was contaminated | TMB Substrate Solution should be clear and colorless prior to addition to wells.<br>Use a clean container prior to pipetting substrate solution into wells. |
| No or poor signal | Detection Antibody, Avidin-HRP or Substrate solution were NOT added | Rerun the assay and follow the protocol. |
| | Wrong reagent or reagents were added in wrong sequential order | |
| | Insufficient plate agitation | The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing. |
| | The wash buffer contains Sodium Azide (NaN3) | Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity. |
| | Incubations were done at an inappropriate temperature, timing or without agitation | Rerun the assay and follow the protocol. |
| Low or poor standard curve signal | The standard was incorrectly reconstituted or diluted | Adjust the calculations and follow the protocol. |
| | Standard was inappropriately stored | Store the reconstituted standard stock solution in polypropylene vials at -70°C.<br>Avoid repeated freeze-thaw cycles. |
| | Reagents added to wells with incorrect concentrations | Check for pipetting errors and the correct reagent volume. |



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| Problem | Probable Cause | Solution |
|---|---|---|
| Signal is high, standard curves have saturated signal | Standard reconstituted with less volume than required | Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol. |
| | Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long | Rerun the assay and follow the protocol. |
| Sample readings are out of range | Samples contain no or below detectable levels of the analyte | If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications. |
| | Samples contain analyte concentrations greater than highest standard point | Samples may require dilution and analysis. |
| High variation in samples and/or standards | Multichannel pipette errors | Confirm that pipette calibrations are accurate. |
| | Plate washing was not adequate or uniform | Ensure pipette tips are tightly secured.<br>Ensure uniformity in all wash steps. |
| | Non-homogenous samples | Thoroughly mix samples before assaying. |
| | Samples may have high particulate matter | Remove particulate matter by centrifugation. |
| | Cross-well contamination | Do not reuse plate sealers.<br>Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate. |

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**ELISA Plate Template**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |


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