ANIARA

Manufactured By: Medicago AB

Protein L Ligand Leakage ELISA kit

Product no A10-0027

Instructions for Use

Read before use:

- *The sample preparation method recommended in this instruction (i.e. heat treatment) has been successfully used for some target molecules, including mAb and Fab. Some Fabs may, however, require additional optimization. Preliminary experiments with heat treatment of sdAb molecules have not been successful.*

- *The protein L reference is sent frozen and should be kept frozen.*

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Background and intended use

The Protein L Ligand Leakage ELISA Kit is a quantitative enzyme-linked immunosorbent assay. This ELISA-kit has been designed to detect and quantify ligand leakage in eluates from Protein L affinity chromatography medium containing immunoglobulin (Ig) or Ig-fragments.

Protein L was originally isolated from the bacterium *Peptostreptococcus magnus*, and is used as ligand in chromatography media for capture of Ig and Ig fragments. Protein L binds to the variable region of the kappa light chain of the antibody, without interfering with the antigen-binding site. This allows Protein L to bind a wider range of Ig classes and subclasses than other antibody-binding proteins. In addition, Protein L also binds antibody fragments such as Fabs, single-chain variable fragments and domain antibodies which contain the kappa light chain.

The Protein L Ligand Leakage ELISA is a sandwich ELISA with 96 microtiter wells in modules of 12 strips, coated with an affinity purified anti-Protein L antibody. The amount of Protein L in a sample is determined using a standard curve, prepared by spiking a reference sample with Protein L.

Sample preparation is required in order to separate the Ig or Ig-fragments from Protein L. For mAbs and Fabs, the samples are prepared by boiling and centrifugation before incubation in the strip wells.
Note that for small Ig-fragments like sdAbs, this sample preparation procedure does not work. Additional optimization of the sample preparation is required for sdAbs.

There is a need to dilute the reference standard curve (from Protein L reference provided in the kit) in a ***Reference sample***. The Reference sample should be isolated by a different purification method, see more information in the sample preparation section below.

If samples do not contain any Ig or Ig-fragments, there is no need to boil the samples. They can be used directly in the assay.

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Warnings and precautions

1. Before starting the assay, read the instructions carefully. Use the valid version of the instructions provided with the kit.
2. Do not mix components from different kit batches.
3. Follow good laboratory practice and local safety guidelines.
4. The Substrate solution (EC-blue Enhanced) is irritating to eyes, respiratory tract and skin. The Stop solution is an acid which can cause burns. Handle with care.
5. Do not pipette by mouth.
6. Do not use kit components beyond date of expiration.

Storage and stability

- Store kit components at +4ºC
- Store the Protein L reference at -20 ºC or -80ºC.
- Stability 12 months

Kit contents

Component	Quantity	Handling
Anti-Protein L coated microtiter plate	1 plate 96 wells 12 × 8-well strips	Ready to use. Let the plate reach room temperature before opening the bag. If only part of the plate is used, store unused strips in a sealed bag at +4ºC.
HRP-conjugated anti-Protein L pAb	1 × 130 µl	Dilute 100x in PBS-Tween. Store protected from light.
Protein L reference (600 ng/ml)	1 × 200 µl	Dilute in your reference dilution buffer (see below). **Store working aliquots at -20/-80ºC.** **Avoid repeated freezing/thawing**
EC-Blue Enhanced™	1 × 13 ml	TMB substrate, ready to use. Store protected from light. Let the substrate reach room temperature before use.
Stop solution	1 × 7 ml	Ready to use.
PBS-Tween tablet	1 × 6 tablets	Dissolve 1 tablet in 500 ml ultra-pure water
Plate sealing tape	1	Remove tape cover, seal the plate during incubations.

Materials required but not supplied

1. Micropipettes and pipette tips, range 10-1000 µl
2. Vials, tubes, and racks
3. Ultra-pure water
4. Reference sample (see sample preparation)
5. Water bath
6. Vortex mixer
7. Centrifuge
8. Wash bottle or automatic microtiter plate washer
9. Microtiter plate reader reading absorbance at 450 nm

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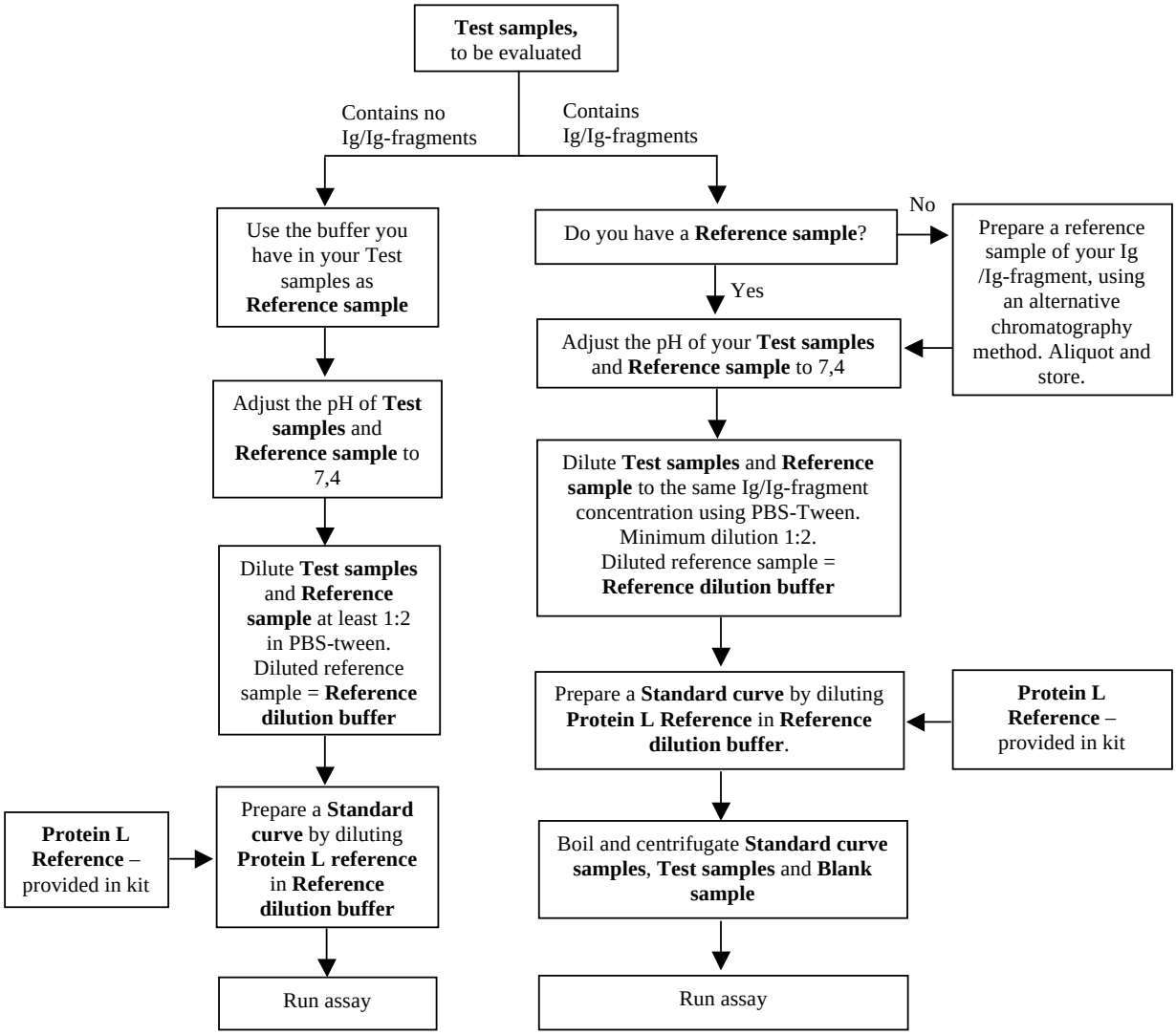
Sample preparation

Schematic overview



Reference sample (Ig/Ig-fragment sample without Protein L)

As different target molecules, at different concentrations, may have different effect on the assay performance, there is a need to dilute the Protein L reference (provided in the kit) in a ***Reference sample***. The reference sample should contain the same Ig or Ig-fragment in an equivalent buffer and at the same concentration as the ***Test samples*** to be assayed (i.e. elution samples from the Protein L affinity chromatography medium). Importantly, the Ig or Ig-fragments of the reference sample should be prepared by use of an alternative purification strategy (i.e. not involving Protein L affinity chromatography medium, for example a suitable ion exchange column). Aliquot the Reference sample and store at suitable conditions, e.g. -20ºC or -80ºC, to be used for several different Protein L ELISA assays. If your Test samples do not contain any Ig or Ig-fragments, use your test sample buffer as Reference sample.

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- If needed, adjust the pH of your reference sample to 7.4.
- Dilute the reference sample in PBS-Tween at least 1:2, to match the concentration in your samples.
- Use this solution (***Reference dilution buffer***) for dilution of the Protein L reference supplied in the kit, in order to get a standard curve (see below).

Test samples - pH and dilution

- Adjust the pH of all samples to approximately 7.4.
- Dilute all samples at least 1:2 in PBS-Tween. Make sure all samples contain equal amounts of target molecule (Ig/Ig-fragment), the same concentration as in your Reference dilution buffer (see above).
- For some samples containing Ig-fragments, the assay results may be improved by adjusting the final concentration of Tween 20 to 0.05 %.
- Samples containing Ig or Ig-fragments need to be further prepared by boiling and centrifugation (see below). Samples without Ig or Ig-fragments may be used directly in the assay.

Standard curve samples

- Protein L reference should be diluted 1:10 to reach the highest point in the standard curve (60 ng/ml). Dilute the Protein L reference in your Reference dilution buffer. Then make a serial dilution in steps of 1:2. See example in the table below. Vortex each sample and change pipette tip before making the next dilution.

Standard (std)	
60 ng/ml	Add 60 µl Protein L reference to 540 µl Reference dilution buffer
30 ng/ml	Add 300 µl std 60 to 300 µl Ref. dilution buffer
15 ng/ml	Add 300 µl std 30 to 300 µl Ref. dilution buffer
7.5 ng/ml	Add 300 µl std 15 to 300 µl Ref. dilution buffer
3.75 ng/ml	Add 300 µl std 7.5 to 300 µl Ref. dilution buffer
1.88 ng/ml	Add 300 µl std 3.75 to 300 µl Ref. dilution buffer
0 ng/ml	300µl Reference dilution buffer

- If needed, the standard curve may be further diluted (below 1.88ng/ml), in the same manner.

Note: Make sure to always treat your Standard curve samples the same way as your Test samples. If your Test samples contain Ig or Ig-fragments and need boiling as sample preparation, your reference standard curve samples should be boiled too, at the same conditions.

Blank sample

PBS-Tween may be used as blank sample. An alternative is to use the same buffer as in your test sample. In this case, treat the blank sample the same way as your test samples (adjust pH, dilute in PBS-Tween, and boil).

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Boiling of samples

This step is necessary only if your samples contain Ig or Ig-fragments. Samples without Ig or Ig-fragments may be used directly in the assay.

- Boil test samples, blank sample and reference standard curve samples. Use tubes with screw cap. Do not use vials with skirt if using a heating block.
- Ig-containing samples should be boiled for 15min. Samples containing Ig-fragment should be boiled for 1 hour.
- Centrifuge samples for 2 minutes.
- Carefully mix the samples, without disrupting the pellet, before adding them to the plate.

Note: The protocol for sample preparation may be optimized for different Ig-fragments by altering the boiling time and dilution. Generally, smaller target molecules need longer boiling time.

Reagent preparation

- Microtiter strips are ready to use.

- 1 PBS-Tween tablet should be dissolved in 500 ml of ultra-pure water. The amount of PBS-Tween required is dependent on the washing procedure. Make sure to prepare enough PBS-Tween for the procedure used.

- HRP-conjugated anti-Protein L pAb should be diluted 1:100 in PBS-Tween. Dilute shortly before use.

- EC-blue Enhanced TMB substrate is ready to use.

- Stop solution is ready to use.

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Test procedure

Let all reagents reach room temperature before use.

1. Prepare reference standards and test samples (pH-adjustment, dilution, boiling and centrifugation). Carefully mix the samples without re-suspending the pellet before adding them to the wells. It is recommended to run all samples in duplicates (see suggested plate layout below).

2. Add 100 µl/well of reference standard, blank sample, and prepared test samples.

3. Seal the plate and incubate for 2 hours at room temperature.

4. Empty the plate and wash **3 times** with 350 µl PBS-Tween/well using an ELISA-plate washer, or by filling the wells to the top using a wash bottle. Tap the plate upside down against absorbent paper to empty it completely after washing.

5. Add 100 µl diluted conjugated antibody to the bottom of each well. Be careful not to contaminate the walls of the wells with the antibody solution.

6. Seal the plate and incubate for 2 hours at room temperature.

7. Empty the plate and wash **6 times** with 350 µl PBS-Tween/well using an ELISA-plate washer, or by filling the wells to the top using a wash bottle. Tap the plate upside down against absorbent paper to empty it completely after washing.

8. Add 100 µl EC-blue Enhanced (TMB substrate solution) to each well. Carefully tap the plate after adding the substrate.

9. Incubate the plate for 10 minutes at room temperature. Keep the plate away from direct sun light during the incubation.

10. Add 50 µl of the Stop solution to each well. Keep the same time interval between adding substrate and stop solution for all samples. Carefully tap the plate after adding the Stop solution to ensure mixing of substrate and stop solution.

11. Photometrical measurement of the optical density at 450 nm should be performed within 15 minutes.

Example plate layout, using sample duplicates:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Sample 1	Sample 1								
B	Std 0	Std 0	Sample 2	Sample 2								
C	Std 1.88	Std 1.88										
D	Std 3.75	Std 3.75										
E	Std 7.5	Std 7.5										
F	Std 15	Std 15										
G	Std 30	Std 30										
H	Std 60	Std 60										

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Analysis of results

Calculation of the Protein L concentration

- Calculate A_{450} mean values for your blank sample, test samples, and reference standard curve.
- Correct for background by subtracting the mean absorbance value of the blank sample.
- Plot the obtained values from your reference standard curve with Protein L concentration (ng/ml) on the x-axis (logarithmic scale) and A_{450} on the y-axis. If using a computerized curve fit, a four- or five-parameter logistic equation should preferably be used.
- Obtain the Protein L concentration (ng/ml) in your sample from the chart, by finding the corresponding Protein L concentration on your x-axis from your sample value of A_{450} on the y-axis.
- Remember to adjust for sample dilutions, in order to obtain the concentration in your original sample.

Performance

The measuring range of the kit is at least 0.5-60 ng/ml of Protein L for samples not containing Ig or Ig-fragments. The sensitivity of the assay may be reduced for samples containing Ig or Ig-fragments.

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