

Protein A ELISA Kit

*For the detection of
MabSelect SuRe™ ligand*

**User Guide
P/N 9333-1**

Disclaimer

This ELISA kit is intended FOR RESEARCH USE ONLY. It is not intended for use as a diagnostic in humans or animals.

Questions

See the "Troubleshooting" section at the end of this protocol. Customers can obtain technical support by calling (800) 622-2259.

Trademarks

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INTRODUCTION

Repligen's Protein A ELISA kit (P/N 9333-1) has been designed for the specific detection of MabSelect SuRe™ ligand in the presence or absence of IgG antibodies and provides accurate, precise, and linear quantitation of the residual ligand.

Our Protein A ELISA kit is a reliable, easy-to-use, highly sensitive assay that measures small amounts of residual ligand in therapeutic protein products. Testing occurs in several different phases of development and commercial manufacturing that may include:

- Process development for leaching characteristics of the resin under specific conditions
- Manufacturing, typically from eluted samples taken throughout several points in the purification process
- Finish product release to document process containment levels and lot-to-lot consistency

The kit is supplied with GE Healthcare's MabSelect SuRe™ ligand for use as an internal protein standard. To quantitate other Protein A variants, please order Repligen's Protein A ELISA Kit, part number 9000-1.

OVERVIEW OF ASSAY FORMAT

The polystyrene microtiter wells provided in this kit are coated with anti-Protein A antibodies. MabSelect SuRe™ Standard (Reagent B) and Test Samples are diluted with sample diluent (Reagent A) and incubated with the immobilized antibodies. Captured MabSelect SuRe™ ligand is then detected by the addition of Biotinylated anti-Protein A

probe (Reagent C). The high substitution of the probe allows maximum binding of Streptavidin Peroxidase conjugate (Reagent D). The final detection step involves adding Tetramethylbenzidine; TMB (Reagent E) to give a highly sensitive colorimetric reaction. The color intensity is proportional to the amount of MabSelect SuRe™ ligand present in the sample.

ASSAY SENSITIVITY

The numerical results of this assay are expressed as nanograms per milliliter (ng/mL) of SuRe ligand. The sensitivity of the assay is typically 0.1 ng/mL which corresponds to a sensitivity or limit of quantitation of 0.8 ppm Protein A per antibody using the Buffer Exchange or Dilute & Go methods or a sensitivity of 0.032 ppm using the Boil & Boost method.

Assay characterization using each of the three sample preparation methods is available as Technical Notes, please contact Customer Service for these documents.

MATERIALS

Reagents/Components Provided:

- Reagent A: Sample Diluent (5X). Contains acetic acid, sodium chloride, Tween-20. (CAUTION: Corrosive). 20 mL
- Reagent B: MabSelect SuRe™ Standard solution, concentration of 1.0 mg/mL in sterile water. 200 µL
- Reagent C: Rabbit anti-Protein A Biotin probe. Contains probe with 0.02% sodium azide. 200 µL

- Reagent D: Streptavidin Horseradish Peroxidase conjugate. 200 µL
- Reagent E: TMB substrate contains 3,3',5,5'-tetramethylbenzidine in buffer. 20mL
- Anti-Protein A coated microtiter wells. 96 wells with plate holder and dessicant packs.
- PBS Packs: 2 packs. Final volume of each pack when reconstituted is 1L.

Note: Reagents are specific to the kit lot and should be discarded once all plate strips have been consumed.

Reagents/Components/ Equipment Required But Not Supplied

- Distilled water or HPLC Grade Water (preferred)
- PBS for antibody sample preparation
- 1L graduated cylinder
- 1.5 mL Eppendorf tubes
- 15 and 50 mL plastic centrifuge tubes
- Tween-20
- Reagent reservoir dishes
- Serological pipettes (5 mL, 10 mL)
- Parafilm or Plate sealers
- 1L bottle top Filter, 0.22 µm
- 1N Phosphoric acid
- Micropipettors P20, P200, P1000, and 12-channel pipettor
- ELISA plate reader, with wavelength capability at 450 nm
- Timer

- Vortex mixer
- Microcentrifuge
- Waterbath (for Boil & Boost sample preparation)

STORAGE

Reagent B, MabSelect SuRe™ Standard solution should be stored at –20 °C. All other reagents should be stored at +2-8 °C when not in use.

METHOD

Pre-Assay Preparation

1. **All Kit Components** – Allow all kit components to equilibrate to room temperature (including the frozen Mabselect SuRe™ Standard, Reagent B).
2. **1X Sample Diluent** - Dilute 4.0 mL of Reagent A (5X Sample Diluent) in 16 mL of distilled water in a 50 mL plastic centrifuge tube. Vortex for 5-10 seconds, or invert 10-15 times for thorough mixing. The 1X Sample Diluent is stable for 2 weeks at room temperature.
3. **PBS Solution** - Dissolve the contents of one PBS pack in 800 mL of dH₂O. Mix in a beaker on a stir plate until dissolved. Add dH₂O to a final volume of 1L. Mix well. Filter PBS solution through a 0.22 µm filter.
4. **PBS-Tween-20 Wash Solution** - Pour 700 mL of the PBS solution (prepared & filtered above) into 1L graduated cylinder. To this add 700 µL Tween-20. Mix well. Save the remaining 300 mL PBS solution for the final ELISA wash. Filter PBS-Tween solution through a 0.22 µm filter.
5. **TMB Substrate Solution** – For full plate assay use the whole bottle of TMB. For half plate assays aliquot 8 mL of

TMB into a 15 mL conical centrifuge tube, cover the tube with aluminum foil, and return bottle to the refrigerator.

6. **Test Sample(s)** – Allow all test samples to equilibrate to room temperature.

7. **10% Tween 20** – Prepare a 10% Tween 20 solution if using Boil & Boost sample preparation method.

Note: *An ideal room temperature range of 60-72 °F (16-22 °C) is important for optimum assay performance.*

Microtiter Plate Preparation

1. Before removing the plate from its protective pouch, design the experiment. Consider the appropriate number of strip wells, samples to test, dilutions, duplicates vs. triplicates, etc.

Note: *When using triplicate data points and four dilution steps the plate can accommodate either one assay for the analysis of six samples or two assays for the analysis of two samples. However, assay setup can be tailored to the investigator's needs. See Figure 1 (page 13) for suggested plate setup for single sample analysis using triplicate data points. Experimental design should take into consideration that strips must be full length to function in strip holder.*

2. With gloved hands, remove the plate sealer from the plate and remove the strips that are not needed. Carefully wrap them in Parafilm and place them back into the foil pouch with desiccant pack and refrigerate.

3. Make sure the strips are properly snapped in the strip well holder. Do not leave the plate exposed to the environment, cover with a plate sealer or Parafilm.

Note: Before starting the assay, make sure the plate is fully equilibrated to room temperature. This is critical for proper assay performance.

Test Sample Preparation

Sample preparation methods for the Protein A ELISA assay have been optimized to allow end users to select the method which is most appropriate for their assay needs. The following table can be used to assist in method selection:

Preparation Method Overview

Desired LOQ	Input Sample Conc. Constraint	Method	Description
~ 0.8 ng/mg	N/A	A – Buffer Exchange	Samples are buffer exchanged into PBS by dialysis or spin column, then diluted to 0.5 mg/mL in PBS prior to performing <u>Preparation of Test Sample Dilutions</u>
~ 0.8 ng/mg	≥5.0 mg/mL antibody	B – Dilute & Go	Samples are diluted in PBS 0.1% Tween 20 at least ten-fold, to 0.5 mg/mL, before performing <u>Preparation of Test Sample Dilutions</u>
~ 0.03 ng/mg	≤15.0 mg/mL of antibody	C – Boil & Boost	Samples are first diluted to ≤15 mg/mL if necessary in neutral buffer. Sample composition is then adjusted to 0.1% Tween 20. Samples are boiled for 5 minutes and centrifuged prior to performing <u>Preparation of Test Sample Dilutions</u>

Method Attribute Table

	A – Buffer Exchange	B – Dilute & Go	C – Boil & Boost
High Performance	X	X	X
Assay completion < 2 hrs	X	X	X
Reduced Sample Preparation Steps		X	
Enhanced Limit of Quantitation			X
High starting sample concentration			X

Method A – Buffer Exchange

Samples to be run using this method must be buffer exchanged into PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, 0.003 M potassium chloride, pH 7.2-7.4), and diluted to a protein concentration of ≤ 0.5 mg/mL prior to running the assay. This may be accomplished by dialysis or use of a desalting column.

Note: *The PBS packs provided in the kit are not intended for this buffer exchange. They are to be reconstituted and used as directed in the ELISA protocol.*

Method B – Dilute & Go

The Dilute & Go method is designed to dilute out any interfering substances. It has been shown to perform with common process buffers such as 100 mM Citrate, Glycine, and Acetate buffers neutralized with Tris-base. The assay should be characterized with process specific buffers and proteins for performance.

Protein A-purified antibody samples with starting concentrations greater than 5.0 mg/mL may be diluted directly into phosphate buffered saline (PBS) with 0.1% Tween 20 to reach a final concentration of 0.5 mg/mL prior to running the assay.

Note: *No buffer exchange is required when the dilution step is performed. If sample concentration is less than 5.0 mg/mL Dilute & Go is not recommended and the user should proceed with Method A.*

Method C – Boil & Boost

The Boil & Boost method is designed for high input antibody concentrations. It has been shown to perform with common process buffers such as 100 mM Citrate and Acetate neutralized with Tris-base at antibody concentrations up to 15.0 mg/mL. The assay should be characterized with process specific buffers and proteins for performance.

Note: *Protein A recovery in Glycine buffers or with > 0.2% Polysorbates was observed to be significantly lower than other buffers when the Boil & Boost method was used. It is recommended that samples containing Glycine or high concentrations of surfactants be buffer exchanged into PBS prior to running the Boil & Boost method.*

Add at least 0.5 mL of each sample to 1.5 mL centrifuge tubes (the assay procedure will require 0.25 mL). Tween 20 should be added to each sample to a final concentration of 0.1%. Create a pin hole in the cap of each centrifuge tube and boil for 5 minutes in a water bath. After cooling the

samples centrifuge the tubes at 13,000 x g or 11,000 rpm for 5 minutes. Boiling causes dissociation from Protein A and precipitation of IgG. Transfer the supernatant to a new tube (optional). The supernatant will be used when preparing sample dilutions in the assay procedure.

MabSelect SuRe™ Standard Dilutions

1. When Reagent B is completely thawed, vortex to mix. If reagent remains on the sides or cap of the tube, briefly spin in a micro-centrifuge.

2. Label three 1.5 mL Eppendorf tubes "Tube 1", "Tube 2", and "Tube 3". Prepare the most concentrated standard solution (1.6 ng/mL "Tube 3") by diluting Reagent B with 1X Sample Diluent, as follows (vortex each tube thoroughly between dilutions):

Tube	MabSelect SuRe™ Std.	1X sample Diluent
1	10 µL of Reagent B	990 µL
2	10 µL of Tube 1	990 µL
3	16 µL of Tube 2	984 µL

3. Place "Tube 3" (1.6 ng/mL MabSelect SuRe™ Standard) off to the side.

Preparation of Test Sample Dilutions

1. After test samples have been prepared and are at the appropriate starting concentration (pg 8-11) label an Eppendorf tube for each Test Sample. Add 200 µL of **5X Sample Diluent (Reagent A)** to each. Next add 550 µL of

distilled water to each of these tubes. Vortex for 5 to 10 seconds to ensure thorough mixing.

2. Test Samples should be fully equilibrated to room temperature before diluting. Add 250 μ L of each Test Sample to the labeled tubes. Vortex for 5 to 10 seconds to ensure thorough mixing. These are the first 1:4 starting sample dilutions. Place these tubes off to the side with the 1.6 ng/mL standard dilution ("Tube 3").

3. Let each Test Sample and the 1.6 ng/mL Standard dilution sit for 10 minutes before pipetting into the assay plate.

4. During the 10 minute incubation, wash the plate three times. Fill the wells with distilled water by using a wash bottle or automated plate-washing system. Aspirate or dump the liquid and repeat. After the third wash, the plate must be dried. To effectively dry the plate, pound the plate firmly, four times on clean paper towels.

Setup of Standard Curve and Test Sample Dilutions on Assay Plate.

Note: The following pipetting and suggested dilution instructions are specific to a single sample assay, as shown in Figure 1. Analogous steps should be taken when performing the assay according to your personal design. Alternatively users may choose to prepare standards and samples in a dilution plate and transfer to assay plate.

1. Using a 12-channel pipettor add 100 μ L of 1X Sample Diluent into columns 1-3 rows B-G and columns 4-6 rows A-C.
2. Transfer 200 μ L of the 1.6 ng/mL MabSelect SuRe™ Standard solution (Tube 3) into wells 1H-3H.
3. Transfer 200 μ L of 1:4 Test Sample dilution into wells 4D-6D.
4. Make 2-fold serial dilutions of the MabSelect SuRe™ Standard and Test Samples by transferring 100 μ L from each set of triplicate wells into the well directly above it. Mix well by pipetting 5 times. It is not necessary to change tips for each row (in a single sample assay format).
5. After making the last MabSelect SuRe™ Standard serial dilution in wells 1C-3C remove 100 μ L from wells 1C-3C and discard. Also discard 100 μ L from the final Antibody Sample dilution in wells 4A-6A.

Figure 1. Representative Plate Setup for One Antibody Sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	Plate Blank			1:32								
B	0			1:16								
C	0.05			1:8								
D	0.1			Sample #1, 1:4								
E	0.2											
F	0.4											
G	0.8											
H	1.6											

ELISA Testing

1. After the MabSelect SuRe™ Standards and Test Sample dilutions have been prepared on the plate, cover the plate with Parafilm or plate sealer and incubate at room temperature for 30 minutes.

2. At the end of the 30-minute incubation, dump or aspirate the solution from the wells. Using a wash bottle or automated plate-washing system, wash the plate with PBS-Tween-20 solution. Dump or aspirate the fluid from the wells. Repeat the wash three more times, for a total of four washes. After the fourth wash, remove excess liquid or bubbles by pounding the plate firmly, four times on clean paper towels.

3. Briefly vortex the Reagent C vial. If reagent remains on the sides or cap of the tube, briefly spin in a micro-centrifuge. Prepare the Rabbit anti-Protein A Biotin probe solution. For a full plate assay prepare 12 mL by adding 70 μ L of Reagent C to 12 mL of prepared PBS-Tween-20 in a 15 mL conical centrifuge tube. For a half plate assay prepare 6 mL by adding 35 μ L Reagent C to 6 mL PBS-Tween-20. Mix solution thoroughly.

4. Using a 12-channel pipettor, add 100 μ L of the diluted Reagent C probe solution to each well containing Test Sample or Standard. Leave wells 1A-3A (Plate Blanks) empty.

5. Cover the wells with a plate sealer or Parafilm and incubate at room temperature for 30 minutes. After thirty minutes, wash the wells four times with PBS-Tween-20. Dry

the plate by pounding the plate firmly four times on clean paper towels.

6. Briefly vortex the Reagent D vial. If reagent remains on the sides or cap of the tube, briefly spin in a micro-centrifuge. For a full assay plate prepare 12 mL of Streptavidin Horseradish Peroxidase conjugate solution by adding 12 μ L of Reagent D to 12 mL of PBS-Tween-20 in a 15 mL conical tube. For a half plate assay prepare 6 mL by adding 6 μ L of Reagent D to 6 mL PBS-Tween-20. Mix solution thoroughly.

7. Add 100 μ L of the diluted Reagent D conjugate solution to each well containing Test Sample or Standard. Leave wells 1A-3A (Plate Blanks) empty.

8. Cover the wells with a plate sealer or Parafilm and incubate at room temperature for 30 minutes.

9. After the 30-minute incubation, dump or aspirate the conjugate solution from the wells and pound dry. Wash the plate twice with PBS-Tween 20. Repeat the washing procedure two more times using **PBS only**, for a total of four washes. Dry the plate by pounding the plate firmly, four times on clean paper towel.

Note: Before proceeding with the next step the TMB substrate solution should be at room temperature. This should be no more than 72 °F. If the lab is too warm, move the assay to a cooler location for the development step.

10. Using a multi-channel pipettor, add 100 μ L of the TMB substrate to each of the wells, INCLUDING 1A-3A (Plate Blanks).

11. Incubate the plate for **exactly 4 minutes**. Stop the reaction by addition of 100 μ L of 1N phosphoric acid to each of the wells, including 1A-3A, in the same order of pipetting for the TMB substrate solution.

Note: *Other strong acids typically used as stop solutions in ELISA assays may be substituted for 1N phosphoric acid. If bubbles are present in the wells, agitate slightly before reading.*

12. Read the plate at 450 nm.

Suggested Calculation of Data

1. Calculate the mean absorbance value for the plate blank wells (A1-A3) and subtract from all remaining wells on the plate (including the 0 ng/mL standard curve). Determine the average absorbance values of each Standard concentration and all Test Samples.

Note: *Method of calculation for Standard Curve should be based on internal standards. Other curve fits may be used as deemed appropriate.*

2. Calculate the standard curve:

Linear fit

Plot each Standard Curve concentration (ng/mL MabSelect SuRe™ ligand) on the x-axis versus the corresponding mean

absorbance value on the y-axis. Using linear regression, calculate the best fitting straight line through the points of the standard curve (See Figure 2.)

4 parameter fit

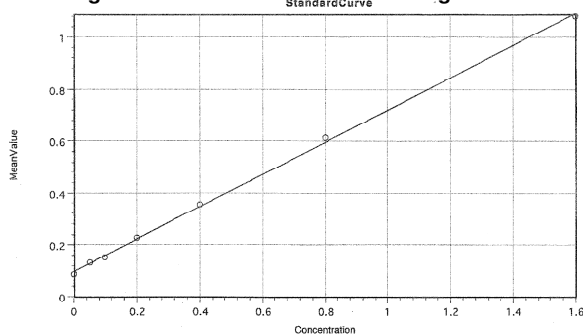
The standard curve may be constructed using a 4 parameter logistic curve fitting program. Such a fit is the acknowledged reference model for sigmoidal immunoassay data.^{6,7} The regression line can be used to determine the MabSelect SuRe™ ligand concentration [PA] for the samples.

$$[\text{PA}] \times \text{Sample Dilution} = C \text{ (ng/mL)}$$

To determine the ng/mg of MabSelect SuRe™ ligand in each sample well use the following formula:

$$\text{ng/mg} = \frac{\text{Mean Conc. [ng/mL]}}{\text{mg/mL of Antibody per well (e.g. 0.125mg/mL)}}$$

Figure 2. Standard Curve Linear Regression



Linear Fit: $y = A + Bx$

	A	B	R ²
STD (Standard: Concentration vs MeanValue)	0.0986	0.622	0.999

SPECIFICITY

This Protein A ELISA Kit is supplied with GE Healthcare's MabSelect SuRe™ ligand for use as an internal standard. For detection of other variants of Protein A please order part number 9000-1.

TROUBLESHOOTING

Problem	Cause	Solution
Problem pipetting enough of required reagent.	Splashing of reagent on sides or cap of reagent tube during vortexing, shipping or handling	Quickly centrifuge reagent tube in a micro-centrifuge.
Inconsistent ppm results between sample dilutions.	Antibody was not fully equilibrated in PBS, pH 7.2-7.4, before assay.	Re-dialyze sample at 1:100 in PBS, pH 7.2-7.4 and re-run assay.
	The protein concentration in the undiluted sample was > 0.5 mg per mL.	Check protein concentration via A280 or Lowry assay.
"Outliers": One replicate has an abnormally high or low absorbance value.	Small amount of peroxidase conjugate left on plate before color development (i.e., wells were not thoroughly washed).	Discard outliers and average duplicates. Ensure thorough washing in any subsequent ELISA testing.
	Precipitated TMB was not adequately redistributed and settled to the bottom.	Tap corner of plate against palm before reading.
Color: development time to reach 1.0 AU is > 4-5 minutes.	TMB Substrate solution, Reagent E, was not at RT before adding to wells.	Solution can be warmed with lukewarm tap water before adding to wells.
	Cool room temperature.	Use room temperature incubator set at 20-25 °C for all incubations or allow wells to develop longer than 4 minutes.

Background signal is >0.150	Color development for TMB-substrate was >4 minutes	Start timer immediately after adding TMB substrate to 1.6 ng/mL Standard wells.
	Temperature of TMB-substrate >25°C	
	Insufficient plate washing	Store TMB in a location that is between 20-25°C until use.
		Wash the plate 4 x's at each wash step and pound plate firmly to dry.
O.D. values are consistently high for all samples.	Buffer component interference	Buffer exchange sample into a neutral buffer or perform a greater fold dilution into a neutral buffer (Page 9)
Low recovery of Protein A in samples		

ADDITIONAL REFERENCES

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(7) Smith, W. C. and G. S. Sittampalam (1998) "Conceptual and statistical issues in the validation of analytic dilution assays for pharmaceutical applications." J Biopharm Stat 8(4): 509-32.



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