



BMA Biomedicals



Peninsula Laboratories

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Peptide ELISA Protocol Nr. III

EIAH - high sensitivity absorbance assays

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Technical Help and Ordering Information:

Please e-mail your questions or requests to info@bma.ch

Note: Device Limited to Investigational Use by Law.
For Research Use Only, not for Use in Diagnostic Procedures

STORAGE

After you receive the kit, store it in the refrigerator (4 - 8°C) for up to 1 year. Long term storage, improper storage conditions and large temperature fluctuation cycles may cause precipitates in the TMB solution and in the ELISA buffer concentrate. These precipitates should not affect the assay noticeably. Nevertheless, if you observe such precipitates, we recommend removing them by filtration prior to usage.

KIT COMPONENTS

Component Description	EIAH	EIAS (human)	EIAS (rat)	EIAS (mouse)	Available Separately
ELISA Buffer Concentrate (50 ml 20x concentrate)	✓	✓	✓	✓	Y-1055
96-well Immunoplate with Plate Sealer	✓	✓	✓	✓	*
Antiserum (lyophilized powder)	✓	✓	✓	✓	*
Standard (lyophilized powder with 1 µg target peptide)	✓	✓	✓	✓	*
Biotinylated Tracer (lyophilized powder)	✓	✓	✓	✓	*
Streptavidin-HRP (100 µl 200x concentrate)	✓	✓	✓	✓	*
TMB-H ₂ O ₂ Stock Solution (1.5 ml)	✓	✓	✓	✓	*
Substrate Buffer (15 ml)	✓	✓	✓	✓	*
Stop Solution 2 N HCl (15 ml)	✓	✓	✓	✓	*
Standard Diluent - Peptide-free human Serum (9 ml)		✓			Y-1060
Standard Diluent - Peptide-free rat Serum (8 ml)			✓		Y-1065
Standard Diluent - Peptide-free mouse Serum (8 ml)				✓	*
Datasheet	✓	✓	✓	✓	*
Protocol	✓	✓	✓	✓	*
* Please inquire					
The following materials are not included but are recommended for EIAH kits. They are not required for EIAS kits.					
Extraction kit (with 50 SEP-Columns and Buffers A & B)					S-5000
Buffer A					Y-1040
Buffer B					Y-1045
Sep-Column (200 mg)					Y-1000
Sep Column adapter					*

Please consult "PREPARE KIT COMPONENTS" on page 7.

Materials Not Provided

- 96-well microtiter plate reader set up to measure 450 nm and 650 nm
- 96-well plate washer and shaker (optional)
- Distilled or deionized water, or comparable quality
- Curve fitting software (optional, use free online services)
- Test tubes, pipettes and various other standard laboratory items
- Sample Extraction Kit S-5000 (recommended for EIAH Kits)

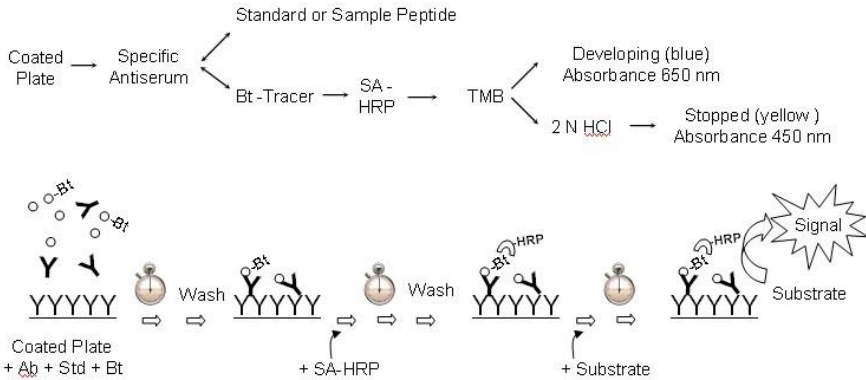
SUMMARIZED PROTOCOLS

The enclosed datasheet indicates which protocol should be used and contains all necessary information for the kit.

Please read the booklet for more detailed protocols and for background information.

	Fast protocol, add ST, AB, BT at once	Used to increase sensitivity and signal	Most frequently used protocol	Used to increase sensitivity	Used mostly with EIAS type kits	Used to increase signal	Used for a few special kits
	I	II	III	IV	V	VI	VII
Competition Phase: ST and BT for AB	50 µl ST	50 µl ST	50 µl ST	50 µl ST	25 µl AB RT 1 hr	50 µl ST	100 µl AB RT 2 hr Wash 3x
	25 µl AB	25 µl AB RT 1 hr	25 µl AB RT 1 hr	25 µl AB 4°C o/n RT 1 hr	50 µl ST RT 2 hr	25 µl AB	100 µl ST RT 2 hr
	25 µl BT RT 2 hr	25 µl BT 4°C o/n RT 1 hr	25 µl BT RT 2 hr	25 µl BT RT 2 hr	25 µl BT 4°C o/n RT 1 hr	25 µl BT 4°C o/n RT 1 hr	25 µl BT 4°C o/n RT 1 hr
Wash	Wash 5 times with ELISA buffer						
HRP	100 µl Streptavidin-HRP (dilute 1:200 in ELISA buffer before using)						
Wait	Incubate at RT for 1 hour						
Wash	Wash 5 times with ELISA buffer						
TMB	100 µl TMB-Chromogenic-Solution (dilute TMB-H ₂ O ₂ 1:21 in Substrate Buffer)						
Dev.	Incubate at RT for 10 minutes (adjust according to color development)						
Stop	100 µl Stop Solution						
Read	Read absorbance at 450 nm (yellow)						

BASIC NOTIONS AND FACTS



This ELISA kit is a competitive immunoassay. The antiserum is captured by antibodies coated on a 96-well plate. A constant concentration of Bt-tracer (biotinylated tracer) and varying concentrations of unlabeled standard or sample peptide compete for binding specifically to the antiserum. Captured Bt-tracer is subsequently bound by streptavidin-conjugated horseradish peroxidase (SA-HRP), which produces a soluble colored product after a substrate is added.

The sequence of the standard peptide is shown on the datasheet (note that large protein sequences are usually not shown).

The standard is used to make a standard curve in the range specified in the kit's datasheet. Standard curves are S-shaped (on a semi-log plot) but for a few kits they appear to be almost linear over the kit's range. The measuring range is the range of standard concentrations near the middle or near the IC_{50} of the standard curve. Unknown sample concentrations are measured by comparing their absorbance with the standard curve.

We include sufficient reagents for 96 determinations.

Variation • Accuracy • Extraction • Cross-reactivity

The kit's IC_{50} , or the shape of the standard curve, may exhibit some variation but this will not affect the kit's accuracy in the measuring range. The kit accurately measures sample peptides if the following conditions are met:

- **Both samples and standards must be measured in the same diluent and under the same conditions** (same microtiter plate).
- **The kit's antiserum must not cross-react appreciably with other factors present in the sample.** Cross-reactivity tables are included with each kit. The user may wish to test the cross-reactivity with other peptides.
- **The sample peptides must be identical to the kit's standard.** Ideally the kit's synthetic standard mimics the natural peptide perfectly. Sometimes, however, natural peptides exist as families of species related by a common or similar sequence. Also, natural peptides may be modified enzymatically or spontaneously, may exist in complexes, and may assume alternative structural forms. In these cases the kit might not measure the exact concentration of a particular natural peptide species, but it may still be used for relative average measurements.
- **Sample extraction.** Factors present in serum can bind to EIAH kit components. The effects can vary from negligible to complete obliteration of signal. Therefore, sample extraction may be required prior to using EIAH kits. In many cases, we have specially formulated cognate **extraction-free** (EIAS) kits that can be used without extraction for human, rat, or mouse serum.

DETAILED PROTOCOL

There are seven different protocols, I - VII. **The enclosed datasheet states which protocol should be used**, the correct protocol is always included with the kit in printed form. All protocols can be downloaded from www.bma.ch.

ELISA buffer and Diluent. Antiserum and Bt-tracer are always reconstituted and used in ELISA buffer. The standards and samples are prepared in "standard diluent" (or diluent).

- For EIAH kits the diluent is also ELISA buffer.
- For EIAS (extraction-free) kits it is the species-specific treated serum provided with the kit.

If there is no interference with the kit's components, you should use your own diluent for your samples and standards. However, the standard curve should show similar characteristics as the one from the datasheet.

Room Temperature. Reagents, samples, and the plate should be brought to room temperature before use.

Shakers. Shakers (optional) may help lower the experimental variation of duplicates (recommended at 60 rpm).

Blank Wells. Blanks will give you the background to be subtracted from all readings. These should not be confused with the "S0 Standards" which contain no standard peptide and which will yield the highest readings. Blank readings will not influence concentration calculations - thus, they are optional.

LAYOUT

Seven-Point Standard Curve Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S0	S0	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

Blk = blank S = standards U = unknown samples

PREPARE SAMPLES

- 1. Sample extraction.** Sample extraction is recommended for serum or plasma samples used in **EIAH - high sensitivity absorbance assays**. It may be less important for tissue culture samples. See "Suggested Protocol for Sample Extraction" below for details. The kit may still be used without extraction but this may cause unexpected results due to the possible binding between serum proteins and kit components. **EIAS - extraction-free absorbance assays** can be used with human, rat or mouse serum or plasma (according to its designation) without performing an extraction.
- 2. Sample concentration.** The concentration of the target molecule must be within the measuring range of the kit (most precise results will be achieved in the linear part of the standard curve around the IC_{50}). If the concentration range of your sample is difficult to estimate, prepare it at different concentrations such that one of the samples should lie within the measuring range.

PREPARE KIT COMPONENTS

Lyophilized kit components should not be re-hydrated until they are needed. **Please check the included datasheet for the appropriate protocol.**

- 1. Equilibrate unopened kit components to room temperature.** Avoid accumulation of moisture, do not open reagents and immunoplate while they are cold.
- 2. ELISA buffer.** Dilute the ELISA buffer concentrate 1 in 20 with water and mix well. Example: mix the 50 ml contained in the kit with 950 ml of water.
- 3. Standard diluent.** Depending on the type of kit (check datasheet) the standards and samples are diluted in the following diluent:
 - EIAH type ELISA kit: working dilution of **ELISA buffer**
 - EIAS type ELISA kit: species-specific treated **serum**
- 4. Standard.** Add 1 ml of standard diluent to the vial of lyophilized standard peptide (1 µg) and mix gently. If samples are to be extracted and re-suspended in ELISA buffer as described below, use ELISA buffer as a diluent. Otherwise, we encourage customers to use their own diluent such that standards and samples will be treated equally. For extraction-free (**EIAS**) kits we provide peptide-free human, rat, or mouse serum as a diluent, but again, customers should use their own sample diluent provided it does not bind appreciably to the antiserum.
- 5. Standard curve.** Make serial dilutions of the standard to cover the range of this kit. Please check the included datasheet for the appropriate range and dilution suggestions.
- 6. Antiserum.** Add 5 ml of ELISA buffer and mix gently.
- 7. Biotinylated tracer.** Add 5 ml of ELISA buffer to the vial of lyophilized biotinylated peptide and mix gently. Please check the datasheet for exceptions.

Protocol III

1. **Into each well of the immunoplate add**
50 µl standard or sample (in ELISA buffer)
25 µl antiserum (in ELISA buffer)
Add 75 µl ELISA buffer to blank wells.
2. **Incubate at room temperature for 1 hour.**
3. **Add 25 µl Bt-tracer** (in ELISA buffer) per well including the blanks.
4. **Incubate at room temperature for 2 hours.**
5. **Wash immunoplate 5 times with 300 µl per well of ELISA buffer.** Be careful not to cross-contaminate between wells in the first wash/dispensing cycle. In each wash cycle empty plate contents with a rapid flicking motion of the wrist, then gently blot dry the top of plate on paper towels. Dispense 300 µl of ELISA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
6. **Add 100 µl per well of streptavidin-HRP.** Tap or centrifuge the SA-HRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in ELISA buffer (60 µl in 12 ml) and mix gently. Add 100 µl to all wells, including the blanks.
7. **Incubate at room temperature for 1 hour.**
8. **Prepare TMB chromogenic solution** immediately before use by mixing 20 parts of the Substrate buffer (citrate, brought to room temperature) with 1 part TMB - H₂O₂ Stock Solution. This dilution should be used within 15 minutes after preparation.
9. **Wash immunoplate 5 times (see step 3).**
10. **Add 100 µl per well of freshly prepared TMB chromogenic solution.** Add to all wells, including the blanks.
11. **Incubate at room temperature (usually 10 minutes).** This can be adapted according to how fast the color reaction takes place. You may read the developing blue color at 650 nm to decide when to stop the color reaction.
12. **Terminate reactions by adding 100 µl 2 N HCl per well.**
13. **Read absorbance at 450 nm within 15 minutes** (and optionally at 650 nm for background correction).

DATA ANALYSIS

Plot data and calculate results. We recommend that you use curve fitting software for your data analysis. Plate readers often include such software packages, otherwise use free online tools that support 4 parameter logistic fitting (4PL) also called dose-response curve, i.e. <https://mycurvefit.com/>. Statistical software such as Origin™, Prism™ or R are also capable of such calculations. This is, however, not essential and you may opt to plot manually on semi-log paper or use a spreadsheet program.

If you e-mail us (contact information on front cover) we will be happy to send you a working Excel spreadsheet for fitting your curve within Excel.

SUGGESTED PROTOCOL FOR SAMPLE EXTRACTION

We have provided an excess amount of standard that you may use to determine if extraction is required. For example, if you are working with serum, you may spike it with known amounts of standard and check if they are accurately determined by the assay with and without extraction. Extraction eliminates potentially interfering substances, such as albumin. Extraction may also be necessary to concentrate the sample to within the measuring range. As with any purification technique, recovery of the desired substance is likely to be incomplete. Therefore, both optimization and quantification of the extraction procedure are recommended for more accurate determinations. While we cannot provide you with extraction optimization and quantification protocols, we have included enough standard in the kit should you wish to use it for this purpose.

C₁₈ Sep-Column Extraction Method

The following generic protocol is meant to help users with extracting their samples. It should be applicable to different biological fluids but should not be thought of as an optimized protocol for any particular antigen.

Required Materials (must be purchased separately)

- SEP-Column containing 200 mg of C₁₈ (Cat. No. Y-1000)
- Buffer A: 1% trifluoroacetic acid (TFA, HPLC Grade). (Acidifies plasma sample to remove interfering proteins such as albumin) (Cat. No. Y-1040)
- Buffer B: 60% acetonitrile (HPLC Grade), 1% TFA, and 39% distilled water. (Elutes peptide from column) (Cat. No. Y-1045)

You may also consider purchasing Extraction kits (Cat. No. S-5000), which include SEP-columns and buffers A & B for 50 samples

Withdrawal and Preparation of Plasma

Collect blood samples (2 - 6 ml) into a chilled syringe and transfer into a polypropylene tube containing EDTA (1 mg/ml of blood) as an anticoagulant and Aprotinin (500 kIU/ml of blood) as a protease inhibitor at 4°C. Do not use heparinized tubes as they may interfere with the assay. Vacutainers with EDTA are acceptable.

Centrifuge blood at 1,600 x g for 15 minutes at 4°C.

Collect the top (plasma) layer.

Proceed to extraction immediately or freeze at -70°C for later use.

Extraction Procedure

1. Add an equal amount of Buffer A to the plasma.
2. Centrifuge at 6,000 x g to 17,000 x g for 20 minutes at 4°C.
3. Transfer supernatant to a new tube discarding any pellet that may be present.
4. Equilibrate a SEP-Column by washing with 1 ml Buffer B followed by 3 x 3 ml Buffer A.
5. Load the plasma solution onto the equilibrated SEP-Column.
6. Slowly wash the column with Buffer A (3 ml, twice) and discard the wash. A light vacuum (10 sec/drop) may be applied to the column.
7. Elute the peptide slowly with Buffer B (3 ml, once) and collect eluant in a polypropylene tube. A light vacuum may be applied as in previous step.
8. Freeze-dry eluant to dryness using a dry ice/methanol bath to freeze the sample and a centrifugal concentrator to evaporate it.
9. Dissolve the residue in a suitable volume of ELISA buffer such that the concentration of the substance of interest will fall close to the IC₅₀ (within the measuring range).

TROUBLESHOOTING

Often, problems may arise from alterations to the protocol. Please check that the expiration date has not passed and store the kit properly (see page 2).

Can the kit be used more than once?

Although we do not guarantee the performance of our kits on a subsequent use, the end user should be able to use the kit multiple times if the reconstituted specific antiserum, standard, and Bt-tracer and standard diluent are stored at or below a constant -20°C and the remaining of the components are kept dry and refrigerated (2-4°C). Freezing aliquots of the reconstituted components may further extend multiple use lifetime.

What are the sources of inaccurate readings?

- Exceeding the OD range of plate reader
- Dirt or grease on the bottom of the plate - wipe with 70% ethanol
- Air bubbles or foaming in wells

The standard curve does not look right.

If you wait too long to read, the curve will be flattened at the top. If you are not familiar with the kit we recommend you read the plate several times while the signal is still developing. For EIAH and EIAS absorbance assays the developing blue color (at 650 nm) will be less intense compared to that of the terminated reactions (yellow absorbance at 450 nm) but the data are still good and this way you won't risk losing the lower end of the range.

Some curves are almost linear, check the datasheet for a typical plot of the product you are using - it may be normal for that particular product.

The IC₅₀ is not as expected.

Note that the IC₅₀ reported with each of our products is based on the concentration of the prepared standards before they are added to the assay solution.

A difference by a factor of two or three may be normal for some kits and may be caused by the time it takes to equilibrate the binding of the tracer and the standard. This will be especially true for pre-incubation protocols. If possible you should always include your own reliable standard at a concentration close to the expected IC₅₀ to check the accuracy of the kit.

In cases where the standard curves are almost rectilinear, accurate IC₅₀ values cannot be calculated. Using excessive amounts of antiserum or tracer, or using a degraded standard may elevate the IC₅₀.

There is too much variation in duplicated readings.

There are only trivial explanations for this such as: (a) poor mixing, (b) poor pipetting technique or faulty pipettes, (c) kit reagents not allowed to equilibrate to room temperature before use, (d) cross-contamination of samples, e.g. droplets or spray from one well to the next, (e) bubbles or foaming in the wells, or finger prints or dirt on the bottom of the plate, etc.

The readings are lower than expected.

The color intensity has little to do with the accuracy of the kit, as long as the slope in the measuring range is normal, but, if the intensity is extremely low, and assuming that you have waited long enough, this may mean that one of the components (antiserum, Bt-tracer, SA-HRP, TMB substrate) was added in low amounts or was degraded due to incorrect storage or excessive freeze-thawing.

The curve looks OK but the results seem implausible.

- Possibly you used different solvents or conditions for standards and samples
- The antiserum may bind to another antigenically similar peptide
- Antigen was lost during extraction or extraction did not eliminate interfering factors

Make sure that the kit's antigen is the same as the target that you are trying to measure. Sometimes the kit's antigen is a peptide that is part of but not the complete natural protein. If so the kit can still be used for determining relative concentrations but not necessarily for determining the absolute concentration of the complete protein antigen.

REFERENCES

- 1) **T. Porstmann and S.T. Kiessig** Enzyme immunoassay techniques. An Overview. J. Immunol. Methods **150**, 5-21 (1992)
- 2) **S. Avrameas** Amplification systems in immunoenzymatic techniques. J. Immunol. Methods **150**, 23-32 (1992)
- 3) **E. Bucht et al.** A rapid extraction method for serum calcitonin. Clin. Chim. Acta, **195**, 115-124 (1991)

SAFETY PRECAUTIONS

The physical and chemical properties of the reagents contained in this kit have been tested individually. Reagents do not contain ingredients which have been determined to be health hazards and which comprise greater than 1% of the mixture or which could be released from the mixture in concentrations that would exceed OSHA permissible exposure limits.

Hazardous Ingredients:

The lyophilized standard, antiserum and biotinylated tracer contain thimerosal or ProClin™ 150 as preservative. For a complete list see MSDS of individual kit.

Physical and Chemical Data:

Components are stable in closed containers under normal temperatures and pressures. No hazardous polymerization is known.

Fire and Explosion Data:

Components are non-combustible with negligible fire hazard when exposed to heat or flame. Fire fighting media should be appropriate to burning material.

Health Hazards:

Components may be harmful by inhalation, ingestion, or skin absorption and may cause skin irritation or eye irritation. In case of eye contact, flush eye with water and contact a physician. In case of skin contact, wash skin with soap and water.

Reactivity Data:

Components are stable in closed containers under normal temperatures and pressures.

Spill and Disposal Procedures:

For spills, ventilate area and wash spill site. For disposal, please dispose in accordance with local regulations.

Handling and Storage Information:

Safety glasses, gloves, and a full-length lab coat should be worn to prevent unnecessary contact.

The above information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. It is the user's responsibility to determine the suitability of this information for the adoption of safety precautions as may be necessary. Chemoforma AG or its Division BMA Biomedicals shall not be held liable for any damage resulting from the handling or use of the above product.

GUARANTEE AND LIMITATION OF REMEDY

Chemoforma AG or its Division BMA Biomedicals make no guarantee of any kind, expressed or implied, which extends beyond the description of materials in this kit, except that these materials and kit will meet our specifications at the time of delivery. The customer's remedy and Chemoforma AG or its Division BMA Biomedicals' sole liability hereunder are limited at Chemoforma AG or its Division BMA Biomedicals' option to refund the purchase price or replace material that does not meet our specifications. By the acceptance of our products, the customer indemnifies and holds Chemoforma AG or its Division BMA Biomedicals harmless against and assumes all liability for the consequences of its use or misuse by the customer, its employees or others.

Date: ___/___/_____ Product Number: S-_____ Lot number: _____

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