PROTOCOL

Peptide Enzyme Immunoassay (EIA)

EIAH - high sensitivity absorbance assays
EIAS - extraction-free absorbance assays
EIAF - highest sensitivity fluorescence assays

CAUTION: INVESTIGATIONAL DEVICE
LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE
FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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STORAGE

After you receive the kit, store the lyophilized components and standard diluent at a constant -20 °C for up to one year from the kit's assembly date. The remaining components should be stored in the refrigerator (2-4 °C) also up to 1 year. Long term storage, improper storage conditions and large temperature fluctuation cycles may result in the accumulation of precipitates in the TMB solution and in the EIA buffer concentrate. These precipitates should not affect the assay noticeably. Nevertheless, if you observe such precipitates, we recommend that you avoid them by allowing them to sink to the bottom.

KIT COMPONENTS

Component Description	EIAH	EIAS (Human)	EIAS (Rat)	EIAF	Available Separately
EIA buffer concentrate (50 ml 20 X concentrate)	√	√	√	√	Y-1055
96-well immunoplate with acetate plate sealer	√	√	√	√	*
Antiserum (lyophilized powder)	√	√	√	√	*
Standard (1 µg lyophilized powder).	√	√	√	√	*
Biotinylated tracer (lyophilized powder)	√	√	√	√	*
Streptavidin-HRP (100 µl 200 X concentrate)	√	√	√	√	*
TMB substrate solution (11 ml TMB and H_2O_2)	√	√	√		*
Stop solution 2 N HCl (15 ml)	√	√	√		*
Fluorescent substrate solution FS1 (10.8ml)				√	*
Fluorescent substrate solution FS2 (1.2 ml)				√	*
Fluorescence kit stop solution (15 ml)				√	*
Standard diluent 8 ml (peptide-free human serum)		√			Y-1060
Standard diluent 8 ml (peptide-free rat serum)			√		Y-1065
Datasheet	√	√	√	√	*
Protocols	√	√	√	√	*
* - Please inquire					
The following materials are not included but are recommended for for EIAH and EIAF kits. They are not required for EIAS kits.					
Extraction kit (with 50 Sep-columns and buffers A and B)					S-5000
Buffer A					Y-1040
Buffer B					Y-1045
Sep-Column (200 mg)					Y-1000
Sep-Column adapter					Y-1010

Additional Materials Not Provided

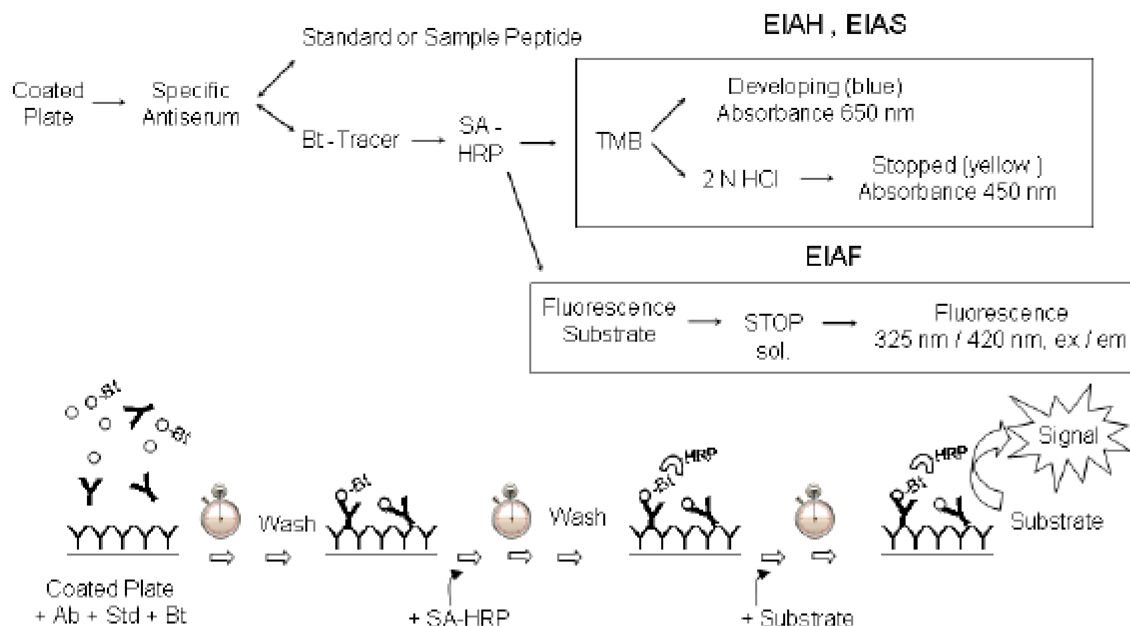
- For EIAH and EIAS absorbance assays: 96-well microtiter plate reader set up to measure 450 nm and 650 nm
- For EIAF Fluorescence assays : a fluorescence plate reader is required (325nm / 420nm, ex/em)
- 96-well plate washer and shaker **(optional)**
- Sterile deionized or USP water
- Curve fitting software (**optional**)
- Test tubes, pipettes and various other standard laboratory items

SUMMARIZED PROTOCOLS

<u>The enclosed datasheet indicates which protocol should be used.</u>
Please read this entire booklet for more detailed protocols and for background information.

	Fast protocol, add Std▶Ab▶Bt at once	Used to increase sensitivity and signal	Most frequently used protocol	Used to increase sensitivity	Used mostly with extraction-free kits	Used to increase signal	Sequential protocol used for a few special kits	IIIF Protocol for fluorescence EIA
	I	II	III	IV	V	VI	VII	EIAF
Competition Phase Ab+Std+Bt	50ulStd	50ulStd	50µlStd	50µlStd	25µlAb	50µlStd	100µlAb	50µlStd
					RT 1 hr		RT 2 hr	
							Wash 3x	
	25µlAb	25µlAb	25µlAb	25µlAb	50µlStd	25µlAb	100µlStd	25µlAb
		RT 1 hr	RT 1 hr	4 C o/n	RT 2 hr		RT 2 hr	RT 1 hr
				RT 1 hr				
	25µlBt	25µlBt	25µlBt	25µlBt	25µlBt	25µlBt	25µlBt	25µlBt
		4 C o/n			4 C o/n	4 C o/n	4 C o/n	
	RT 2 hr	RT 1 hr	RT 2 hr	RT 2 hr	RT 1 hr	RT 1 hr	RT 1 hr	RT 2 hr
Wash	Wash five times with EIA buffer							
SA-HRP	100 µl Streptavidin-HRP. Dilute 1/200 in EIA buffer before using							
Wait	RT 1 hr							
Wash	Wash five times with EIA buffer							
Substr.	**100 µl TMB**							**100 µl Substrate**
Devel.	RT ≤ 1 hr Read 650 nm (blue) before adding stop sol. if unsure of timing							RT 1 hr
STOP	**100 µl 2 N HCl**							**100 µl STOP**
READ	Read absorbance at 450 nm (yellow)							Read fluorescence

BASIC NOTIONS AND FACTS



Our **EIA** kits are competitive immunoassays. 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 **SA-HRP** (streptavidin-conjugated horseradish peroxidase), which produces a soluble colored or fluorescent product after a **substrate** is added.

The **sequence of the standard** peptide is shown on the datasheet. It is also in our catalog and on our web site (www.BACHEM.com) as the "**antigen sequence"** (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 **IC50** 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 · Crossreactivity

The kit's IC50, 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.

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A) Both samples and standards must be measured in the same diluent and under the same conditions (same microtiter plate).

B) 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, some of which may be available from our catalog.

C) 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. In addition, natural peptides may be enzymatically or spontaneously modified, 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.

D) Sample extraction. Factors present in serum can bind to EIAH or EIAF kit components. The effects can vary from negligible to complete obliteration of signal. Sample extraction may, therefore, be required prior to using EIAH or EIAF 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 PROTOCOLS

• **Different protocols and Datasheets**. There are seven different absorbance protocols: (I - VII) and one fluorescence protocol. The enclosed datasheet states which protocol should be used.
• **EIA buffer and Diluent.** The antiserum and the Bt-tracer are always reconstituted and used in EIA buffer. The standards and samples are prepared in "standard diluent" (or diluent). For EIAH and EIAF kits the diluent is also EIA buffer but for EIAS (extraction-free) kits it is either the human, rat or mouse treated serum provided with the kit. If there is no adverse interference with the kit's components you should use you own diluent for your samples and standards – you may do this as long as you obtain a standard curve that is similar to the one provided with 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

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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 especially for serum samples. It may not be as important for some 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.

If you purchased an Extraction-Free Kit (EIAS) you may use it for the measurement of 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 (in a region around the IC50). If you cannot estimate the concentration range of your sample you can prepare it at different concentrations such that one of the samples may be 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. Do not open reagents and immunoplate while they are cold. Equilibrate at room temperature before opening to avoid accumulation of moisture.
2 - EIA buffer. Dilute the EIA buffer concentrate to 1,000 ml with sterile deionized or USP pure water (18 MOhm) and mix well.
3 - Standard. Add 1 ml of standard diluent buffer to the vial of lyophilized standard peptide (1 µg) and vortex. If samples are to be extracted and re-suspended in EIA buffer as described below, use EIA 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 kits we provide peptide-free human, rat or mouse serum as a diluent, but again, customers should use their own sample diluent provided it

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does not bind appreciably to the antiserum. range of this kit. Please check the included datasheet for the appropriate range**.**
5 - Antiserum. Add 5 ml (10 ml for protocol VII) of EIA buffer and vortex.
6 - Bt-tracer. Add 5 ml of EIA buffer to the vial of lyophilized biotinylated peptide and vortex.
7 - EIAF substrate solution. Mix 90% Substrate Solution FS1 and 10% Substrate Solution FS2 (9:1, v:v).

PROTOCOL I (Std.Ab.Bt)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)
25 µl Bt-tracer (in EIA buffer)
Add 50 µl diluent, 25 µl EIA buffer and 25 µl Bt-tracer to blank wells.
2 - Incubate at room temperature for 2 hours.
3 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
4 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
5 - Incubate at room temperature for 1 hour.
6 - Wash immunoplate 5 times (see step 3).
7 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
8 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
9 - Terminate reactions by adding 100 µl 2 N HCl per well.
10 - Read absorbance at 450 nm within ten minutes.

PROTOCOL II (Std.Ab1hr.BtON)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)
Add 50 µl diluent and 25 µl EIA buffer to blank wells.
2 - Incubate at room temperature for 1 hour. Shorter preincubations may result in lower sensitivity.
3 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
4 - Incubate at 4°C overnight. Shorter incubation times may result in low signal. For best results re-equilibrate to RT before proceeding
5 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.

6 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
7 - Incubate at room temperature for 1 hour.
8 - Wash immunoplate 5 times (see step 5).
9 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
10 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
11 - Terminate reactions by adding 100 µl 2 N HCl per well.
12 - Read absorbance at 450 nm within ten minutes.

PROTOCOL III (Std.Ab1hr.Bt)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)
Add 50 µl diluent and 25 µl EIA buffer to blank wells.
2 - Incubate at room temperature for 1 hour. Shorter preincubations may result in lower sensitivity.
3 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
4 - Incubate at room temperature for 2 hours.
5 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
6 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
7 - Incubate at room temperature for 1 hour.
8 - Wash immunoplate 5 times (see step 5).
9 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
10 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
11 - Terminate reactions by adding 100 µl 2 N HCl per well.
12 - Read absorbance at 450 nm within ten minutes.

PROTOCOL IV (Std.AbON.Bt)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)
Add 50 µl diluent and 25 µl EIA buffer to blank wells.
2 - Incubate at 4°C overnight. Shorter pre-incubations may result in lower sensitivity.
3 - Incubate at RT for 1 hour. This is to allow the plate to reach room temperature.
4 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
5 - Incubate at room temperature for 2 hours.

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6 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
7 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
8 - Incubate at room temperature for 1 hour.
9 - Wash immunoplate 5 times (see step 6).
10 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
11 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
12 - Terminate reactions by adding 100 µl 2 N HCl per well.
13 - Read absorbance at 450 nm within ten minutes.

PROTOCOL V (Ab1hr.Std2hr.BtON)

1 - Into each well of the immunoplate add 25 µl antiserum (in EIA buffer)**.** Add 25 µl EIA buffer to blank wells.
2 - Incubate at room temperature for 1 hour.
3 - Add 50 µl standard or sample (in diluent)**.** Do not wash plate before adding. Add 50 µl diluent to blank wells.
4 - Incubate at room temperature for 2 hours. Shorter preincubations may result in lower sensitivity.
5 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
6 - Incubate at 4°C overnight. For best results re-equilibrate to RT before proceeding
7 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
8 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
9 - Incubate at room temperature for 1 hour.
10 - Wash immunoplate 5 times (see step 7).
11 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
12 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
13 - Terminate reactions by adding 100 µl 2 N HCl per well.
14 - Read absorbance at 450 nm within ten minutes.

PROTOCOL VI (Std.Ab.BtON)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)

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25 µl Bt-tracer (in EIA buffer)
Add 50 µl diluent, 25 µl EIA buffer and 25 µl Bt-tracer to blank wells.
2 - Incubate at 4°C overnight. For best results re-equilibrate to RT before proceeding
3 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
4 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
5 - Incubate at room temperature for 1 hour.
6 - Wash immunoplate 5 times (see step 3).
7 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
8 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.
9 - Terminate reactions by adding 100 µl 2 N HCl per well.
10 - Read absorbance at 450 nm within ten minutes.

PROTOCOL VII (Ab2hr.wash.Std2hr.BtON)

Note: for this protocol only, the antiserum needs to be resuspended in 10 ml EIA buffer.

1 - Into each well of the immunoplate add 100 µl antiserum (in EIA buffer)**.** Add 100 µl EIA buffer to the blank wells.
2 - Incubate at room temperature for 2 hours.
3 - Wash immunoplate 3 times with 300 µl/well of EIA buffer. 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 EIA buffer into each well and gently shake for at least a few seconds.
4 - Add 100 µl standard or sample (in diluent). (Add 100 µl diluents to blank wells)
5 - Incubate at room temperature for 2 hours. Shorter preincubations may result in lower sensitivity.
6 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
7 - Incubate at 4°C overnight. For best results re-equilibrate to RT before proceeding
8 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very careful not to cross-contaminate between wells in the first wash/dispensing cycle. Thorough washing is essential. See step 3.
9 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
10 - Incubate at room temperature for 1 hour.
11 - Wash immunoplate 5 times (see steps 3 and 8).
12 - Add 100 µl/well of TMB solution. Add to all wells, including the blanks.
13 - Incubate at room temperature (usually 30 - 60 minutes). You may read the developing blue color at 650 nm and use the data for your calculations.

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14 - Terminate reactions by adding 100 µl 2 N HCl per well.
15 - Read absorbance at 450 nm within ten minutes.

PROTOCOL EIAF (IIIF Std.Ab1hr.Bt)

1 - Into each well of the immunoplate add
50 µl standard or sample (in diluent)
25 µl antiserum (in EIA buffer)
Add 50 µl diluent and 25 µl EIA buffer to blank wells.
2 - Incubate at room temperature for 1 hour. Shorter preincubations may result in lower sensitivity.
3 - Rehydrate the Bt-tracer (in EIA buffer) **and add 25 µl / well.**
4 - Incubate at room temperature for 2 hours.
5 - Wash immunoplate 5 times with 300 µl/well of EIA buffer. Be very 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 EIA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
6 - Add 100 µl /well of streptavidin-HRP. Tap or centrifuge the SAHRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in EIA buffer (60 µl / 12 ml) and vortex. Add 100µl to all wells, including the blanks.
7 - Incubate at room temperature for 1 hour.
8 - Wash immunoplate 5 times (see step 5)
9 - Add 100 µl/well of the combined substrate solution. Make sure to prepare it by mixing 90% Substrate Solution FS1 and 10% Substrate Solution FS2 (9:1, v:v). Add to all wells, including the blanks.
10 - Incubate at room temperature 60 minutes. You may read the plate after setting the fluorimeter to excitation: 325 nm / emission 420 nm before 60 minutes and use the data for your calculations, but terminating the reaction (next step) will result in a stronger signal
11 - Terminate reactions by adding 100 µl stop solution per well.
12 - Read fluorescence (ex 325 nm / em 420 nm)

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. This is, however, not essential and you may opt to plot manually on semi-log paper. You can also use a spreadsheet program. Should you need help with the latter method we recommend the following procedure.

Set up a spreadsheet as shown below (note that the values on the spreadsheet are merely illustrative and are not necessarily typical for this particular kit). If you e-mail us we will be happy to send you the actual working Excel spreadsheet shown below.

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Data Analysis

blanks
Stds
samples

II - Replace the cell contents below with your own data, according to the given layout.

Copy and paste the plate reader data into here: (Make sure the layout is correct)

	Duplicates		Duplicates		Duplicates		Duplicates		Duplicates		Duplicates	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.000	1.001	1.001	1.009	1.009	1.017	1.017	1.025	1.025	1.033	1.033
B	0.277	0.175	1.002	1.002	1.010	1.010	1.018	1.018	1.026	1.026	1.034	1.034
C	0.346	0.290	1.003	1.003	1.011	1.011	1.019	1.019	1.027	1.027	1.035	1.035
D	0.527	0.476	1.004	1.004	1.012	1.012	1.020	1.020	1.028	1.028	1.036	1.036
E	0.816	0.936	1.005	1.005	1.013	1.013	1.021	1.021	1.029	1.029	1.037	1.037
F	1.398	1.361	1.006	1.006	1.014	1.014	1.022	1.022	1.030	1.030	1.038	1.038
G	1.609	1.608	1.007	1.007	1.015	1.015	1.023	1.023	1.031	1.031	1.039	1.039
H	1.605	1.547	1.008	1.008	1.016	1.016	1.024	1.024	1.032	1.032	1.040	1.040

0.000 ◄ Blank average

III - Enter standards concentration below in the ng/ml column

Note: To include So in the plot enter an arbitrary small number (e.g. 0.01) but not ZERO in C69

Note: To extrapolate the "fit" red curve add more concentrations immediately above (C61 and C60) and below Std data points (C68).

	ng/ml	signal	FIT	stdev
			1.752	
			1.752	
Enter S1	10.000	0.226	0.242	0.0718
Enter S2	2.500	0.318	0.303	0.0393
Enter S3	0.625	0.501	0.492	0.0355
Enter S4	0.156	0.878	0.899	0.0848
Enter S5	0.039	1.379	1.361	0.0263
Enter S6	0.010	1.609	1.621	0.0007
	0.001		1.735	
S0 (zero)		1.576	1.752	

4 - Adjust parameters a b c d (green cells) to optimize the fit.

0.625	0.5014	IC50	
0.876	3	0.156	0.8781	0.159

Calculations of initial curve parameters

1.609	1.000	0.159	0.226	◄parameters first bet (please adjust green cells)
1.752	0.936	0.123	0.217	After you dump your data in the B46:M53 area
(max) a	slope) b	(IC50) c	(min) d	the first plot you'll see above will not be perfectly

fitted to your data. You must adjust the parameters in the green cells above to optimize the fit of the red curve to your data.

0.0325 ◄This number gets smaller as the fit gets better

5 - Read unknown concentrations below. NOTE: trust results only if ODs are within the measuring range.

SPIKED (if you have "spiked" samples with known concentrations enter them in the F column)

▼ ▼ If the signal readings are as expected the result will be 100% X-reactivity

Smpls	duplic. 1	duplic 2	Average	ng/ml	ng/ml	%Xreact
U1	1.001	1.001	1.001	0.117	10000	0%
U2	1.002	1.002	1.002	0.117	10000	0%
U3	1.003	1.003	1.003	0.117	10000	0%

Set up an 8 X 12 area to match the layout of the plate and dump the plate reader data in it.

Calculate the average of the blanks on another cell as indicated by the arrows starting from cells A1 and A2.

Enter the concentration of the standards (see under ng/ml in figure).

Calculate the average of the ODs of the standards and subtract the background (blank) as indicated by the arrows for the last standard (cells H1 and H2).

Plot a standard curve on a semi-log scale. Use the y axis for the average of the OD readings (minus the blank average) and the x axis for the standard concentrations in ng/ml.

Use the equation shown below to calculate the values on the "FIT" column and plot a smooth line of FIT values versus standard concentrations. Then change the parameters a (max), b (slope), c (IC50), and d (min), until you are satisfied that fit is good.

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

Next calculate the average of your sample readings and subtract the blank average (see arrows starting from A3 and A4, and the arrows leading to "Average").
Finally, you may use the "reverse" of the equation above to calculate the concentrations in ng/ml for all your samples.

$$x = c\left(\frac{y-a}{d-y}\right)^{1/b}$$

Caution: when you calculate sample concentrations using the "reverse" equation if y = d or y > a or y < d, the reading is out of range and the calculation will yield an error or a meaningless negative concentration.

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 little experience in 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
• SEP-COLUMN containing 200 mg of C18 Cat No. Y-1000
• Buffer A (BUFF-A): 1% trifluoroacetic acid (TFA, HPLC Grade). (Acidifies plasma sample to remove interfering proteins such as albumin)
• Buffer B (BUFF-B): 60% acetonitrile (HPLC Grade), 1% TFA, and 39% distilled water. (Elutes peptide from C18 column)
• You may also consider purchasing Extraction kits (Cat No. S-5000), which include SEP-columns and buffers

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 Apronitin (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 1,600xg for 15 minutes at 4°C.
• Collect the top (plasma) layer.
• Proceed to extraction immediately or freeze at -70°C for later use.

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Extraction Procedure
• Add an equal amount of Buffer A to the plasma.
• Centrifuge at 6,000xg to 17,000xg for 20 minutes at 4°C.
• Transfer supernatant to a new tube discarding any pellet that may be present.
• Equilibrate a SEP-COLUMN by washing with 1 ml Buffer B followed by 3 X 3 ml Buffer A.
• Load the plasma solution onto the equilibrated SEP-Column.
• 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.
• 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.
• Freeze-dry eluant to dryness using a dry ice/methanol bath to freeze the sample and a centrifugal concentrator to evaporate it
• Dissolve the residue in a suitable volume of EIA buffer such that the concentration of the substance of interest will fall close to the IC50 (within the measuring range).

TROUBLESHOOTING

Most problems are caused by poor technique or by alterations of the protocol. Please check that the expiration date has not passed and store the kit properly and according to the storage section on page 2.
• Can the kit be used more than once?
o 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? o exceeding the OD range of plate reader o dirt or grease on the bottom of the plate - wipe with 70% ethanol o air bubbles or foaming in wells.
• The standard curve does not look right. o 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 (absorb. 650 nm) will be less intense compared to that of the terminated reactions (yellow - absorb. 450 nm) but the data are still good and this way you won't risk losing the lower end of the range. For EIAF (fluorescence assays) you may use the same ex325nm/em 420nm settings before and after adding the stop solution.
o 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
o 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.
o 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.

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o In cases where the standard curves are almost rectilinear, accurate IC50 values cannot be calculated.
o Using excessive amounts of antiserum or tracer, or using a degraded standard may elevate the IC50.
• There is too much variation in duplicated readings.
o 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.
o The color or fluorescence 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, SAHRP, TMB, or fluorescence 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.
o Possibly you used different solvents or conditions for standards and samples.
o The antiserum may bind to another antigenically similar peptide
o Antigen was lost during extraction or extraction did not eliminate interfering factors
o 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.
• Will EIAF kits work with any fluorimeter?
o The light signal that is emitted from the sample well will have different characteristics depending on the instrument that is used. Our EIAF kits work well with SpectraMax microplate readers (Molecular Devices), which read one well at a time.

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

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The lyophilized standard, antiserum and biotinylated tracer contain thimerosal. The EIA buffer concentrate contains Tris and thimerosal. The buffer is in liquid form. The SA-HRP contains 0.01% methylisothiazolone, 0.01% bromonitrodioxane, and 10 ppm Proclin 300 as a preservative.

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. Peninsula Laboratories, LLC shall not be held liable for any damage resulting from the handling or use of the above product.

GUARANTEE AND LIMITATION OF REMEDY

Peninsula Laboratories, LLC makes 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 Peninsula Laboratories, LLC's sole liability hereunder are limited at Peninsula Laboratories, LLC's 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 Peninsula Laboratories, LLC harmless against, and assumes all liability for the consequences of its use or misuse by the customer, its employees or others.

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