Version 26-12/24

User's Manual

Human Parathormone 1-34 ELISA Kit

REF **DEIA-BJ695**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

General Description

Parathyroid Hormone (pTH) (1-34), is a peptide fragment of the naturally occurring human parathyroid hormone. pTH is secreted from cells of the parathyroid glands and finds its major target cells in bone and kidney. It is an important regulator of calcium and phosphorus metabolism, playing a role in calcium homeostasis of bone, kidney, breast, and placenta. pTH (1-34) administration suppresses cardiovascular calcification and down-regulates aortic osteogenic programs driven by diabetes and dyslipidemia. This ELISA was developed with serum from rabbits immunized with pTH (1-34) coupled to a carrier protein.

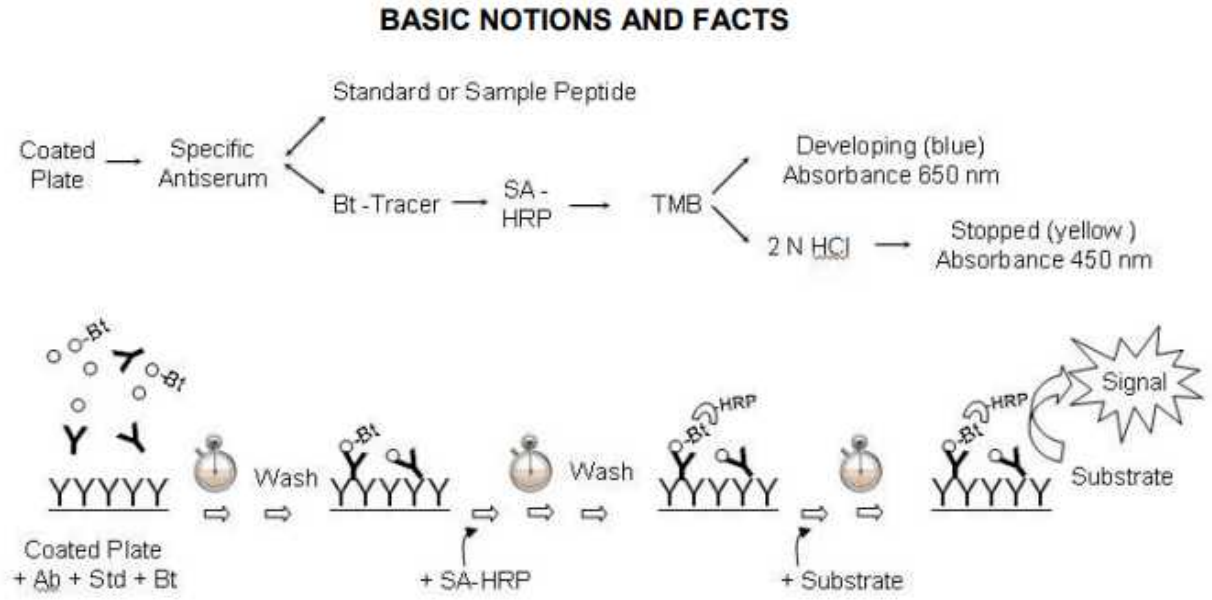
Principles of Testing

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.

Immunogen: Synthetic peptide H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-GlyLys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-LeuGln-Asp-Val-His-Asn-Phe-OH) coupled to carrier protein.

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.



Reagents And Materials Provided

1. ELISA buffer concentrate (50ml 20× concentrate)
2. 96-well immunoplate with plate sealer
3. Antiserum (lyophilized powder)
4. Standard (1mg lyophilized powder)
5. Biotinylated tracer (lyophilized powder)
6. Streptavidin-HRP (100µl 200× concentrate)
7. TMB substrate stock solution (1.5ml)
8. TMB substrate buffer (15ml citrate buffer)
9. Stop solution 2 N HCl (15ml)
10. Protocols

The following materials are not included but are recommended.

Extraction kit (with 50 Sep-columns and buffers A and B)

1. Buffer A
2. Buffer B
3. Sep-Column (200 mg)
4. Sep-Column adapter

Materials Required But Not Supplied

1. 96-well microtiter plate reader set up to measure 450nm and 650nm
2. 96-well plate washer and shaker **(optional)**
3. Distilled or deionized **water**, or comparable quality
4. Curve fitting software (optional)
5. Test tubes, pipettes and various other standard laboratory items

Storage

After you receive the kit, store the lyophilized components and standard diluent at -20°C for up to one year from the kit's assembly date. The remaining components should be stored in the refrigerator (4 - 8°C) also 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 to avoid them by allowing them to sink to the bottom.

Specimen Collection And Preparation

Sample extraction. Sample extraction is recommended for serum or plasma samples. 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. **Extraction-Free Kit (EIAS)** can be used to assay human, rat, or mouse serum or plasma (according to its designation) without performing an extraction.

Sample concentration. The concentration of the target molecule must be within the measuring range of the kit (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.

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.

C_{18} 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

SEP-COLUMN containing 200mg of C_{18}

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 column)

You may also consider purchasing Extraction kits, which include SEP-columns and buffers

Withdrawal and Preparation of Plasma

Collect blood samples (2 - 6ml) into a chilled syringe and transfer into a polypropylene tube containing EDTA (1mg/ml of blood) as an anticoagulant and Aprotinin (500KIU/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×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×g to 17,000×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 1ml Buffer B followed by 3× 3ml 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 IC_{50} (within the measuring range).

Reagent Preparation

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 50ml contained in the kit with 950ml of water.
3. **Standard.** Add 1ml of standard diluent buffer to the vial of lyophilized standard peptide (1µg) and vortex gently. If samples are to be extracted and re-suspended in ELISA buffer as described below, use ELISA buffer as a diluent.
4. **Standard curve.** Make serial dilutions of the standard to cover the range of this kit. **Please check the included datasheet for the appropriate range.**
5. **Antiserum.** Add 5ml of ELISA buffer and vortex.
6. **Biotinylated tracer.** Add 5ml of ELISA buffer to the vial of lyophilized biotinylated peptide and vortex. **Please check the datasheet for exceptions.**

Assay Procedure

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). 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.

1. **Into each well of the immunoplate add**

   50µl standard or sample (in diluent)

   25µl antiserum (in ELISA buffer)

   Add 50µl diluent and 25µl ELISA buffer to blank wells.
2. **Incubate at room temperature for 1 hour. Shorter incubation may result in lower sensitivity.**
3. **Rehydrate the Bt-tracer** (in ELISA buffer) **and add 25µl per well.**
4. **Incubate at room temperature for 2 hours.**

5. **Wash immunoplate 5 times with 300µl 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 300µl of ELISA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.
6. **Add 100µl 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 (60µl in 12ml) and vortex gently. Add 100µl to all the wells, including blanks.
7. **Incubate at room temperature for 1 hour.**
8. **Prepare TMB chromogenic solution** immediately before use by mixing 20 parts of the TMB substrate buffer (citrate, brought to room temperature) with 1 part TMB – H_2O_2 solution (TMB substrate stock). This dilution should be used within 15 minutes after preparation.
9. **Wash immunoplate 5 times (see step 5).**
10. **Add 100µl per well of TMB solution.** Add to all the wells, including blanks.
11. **Incubate at room temperature (usually 10 to 30 minutes).** You may read the developing blue color at 650nm and use the data for your calculations.
12. **Terminate reactions by adding 100µl 2N HCl per well.**
13. **Read absorbance at 450nm within fifteen minutes.**

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

Quality Control

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.

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.

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. 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.

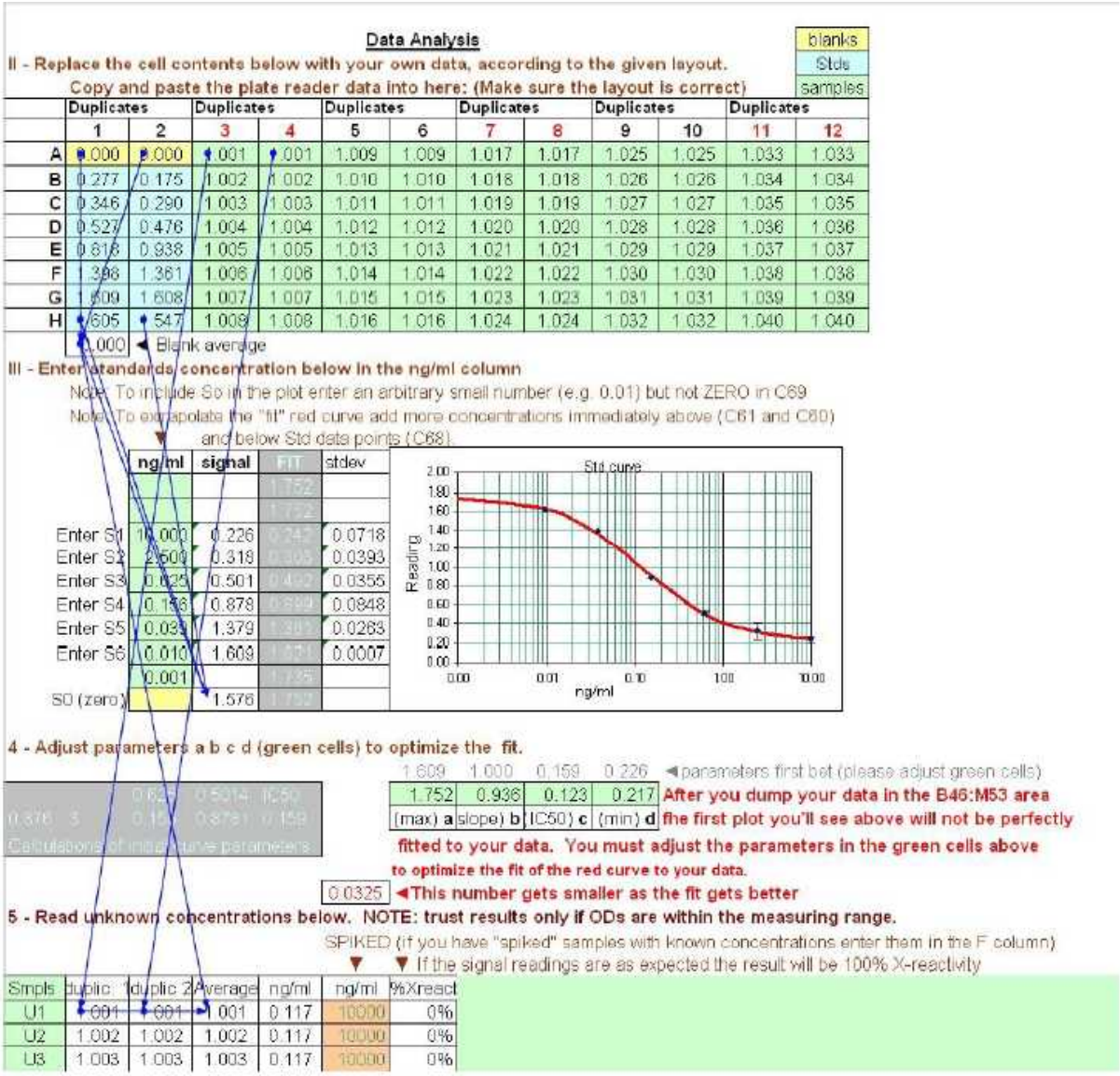
D) Sample extraction. Factors present in serum can bind to EIAH kit components. The effects can vary from negligible to complete obliteration of signal. Therefore, sample extraction may be required prior to using EIAH kits. In many cases, we have specially formulated cognate **extraction-free** (EIAS) kits that can be used without extraction for human, rat, or mouse serum.

Calculation

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 (contact information on front cover) we will be happy to send you the actual working Excel spreadsheet shown below.



Set up an 8 × 12 area to match the layout of the plate and copy 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)

Make a standard curve by plotting the OD readings (minus the blank average) against 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 (IC_{50}), and d (min), until you are satisfied that fit is good.

$y = [(a-d) / (1 + (x/c)^b)] + d$

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 isolate x in the equation above to calculate the concentrations in ng/ml for all your samples:

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

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.

Interpretation Of Results

TROUBLESHOOTING

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

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 650nm) will be less intense compared to that of the terminated reactions (yellow absorbance at 450nm) 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 IC_{50} is not as expected

Note that the IC_{50} 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 IC_{50} to check the accuracy of the kit.

In cases where the standard curves are almost rectilinear, accurate IC_{50} values cannot be calculated.

Using excessive amounts of antiserum or tracer, or using a degraded standard may elevate the IC_{50}.

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.

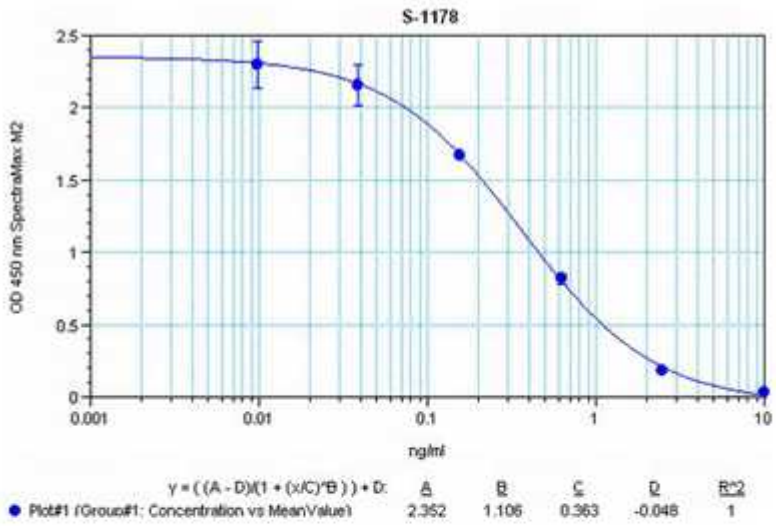
The antiserum may bind to another antigenically similar peptide

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.

Typical Standard Curve

Typical titration curve of pTH in a competitive ELISA with this antibody:



Suggested Preparation of Standards		
	ng/ml	Range: 0.01 to 10ng/ml
Stock	1000	
S1	10.00	Add 10µl Stock + 990µl diluent
S2	2.50	Add 200µl S1 + 600µl diluent
S3	0.63	Add 200µl S2 + 600µl diluent
S4	0.16	Add 200µl S3 + 600µl diluent
S5	0.04	Add 200µl S4 + 600µl diluent
S6	0.01	Add 200µl S5 + 600µl diluent
S0	0.00	500µl diluent

Detection Range

0 - 10 ng/ml

Specificity

PEPTIDE:	%:
Parathyroid Hormone (1-34) (human)	100
Parathyroid Hormone (1-44) (human)	0
Parathyroid Hormone (39-68) (human)	0
Parathyroid Hormone (1-84) (human)	0
[Asp^{76}] Parathyroid Hormone (39-84) (human)	0
Parathyroid Hormone (1-34) (rat)	0

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. The ELISA buffer concentrate contains Tris and thimerosal. The buffer is in liquid form. The SA-HRP contains 0.01% methylisothiazolone, 0.01% bromonitrodioxane, and 10ppm 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 International, Inc. shall not be held liable for any damage resulting from the handling or use of the above product.