Rat *BioActive* Intact PTH ELISA Kit

Immutopics

Immutopics, Inc.

Enzyme-Linked ImmunoSorbent Assay (ELISA) for the Quantitative Determination of Rat Bioactive Intact Parathyroid Hormone Levels in Plasma or Cell Culture Media

96 Test Kit

For RESEARCH Use Only

Cat. #60-2700

Not for use in diagnostic procedures

Store at 2 - 8ºC Upon Receipt

INTENDED USE

This kit is intended for research use only in the determination of rat bioactive intact PTH levels in plasma or cell culture media.

INTRODUCTION

Rat intact parathyroid hormone (PTH) is an 84 amino acid polypeptide produced by the parathyroid gland with its biological activity residing in the N-terminal region of the peptide. PTH plays an important role in maintaining the concentration of ionized calcium within the limits needed to achieve normal metabolic functions. When serum calcium levels are decreased the parathyroid gland increases secretion of the hormone which results in increased mobilization of calcium from skeletal reserves into the circulation. When levels of serum calcium are increased the secretion of PTH is reduced.

The similarities between rat and human physiology relative to calcium metabolism make rat an excellent live-animal model for studying human skeletal disease and in the pre-clinical evaluation of pharmacologic agents that may alter bone remodeling. Quantitation of only full-length biologically active rat intact PTH with this kit can provide a precise and sensitive assessment of changes in bone and mineral metabolism.

TEST PRINCIPLE

The Rat BioActive Intact PTH ELISA Kit is a two-site enzyme-linked immunosorbent assay (ELISA) for the measurement of PTH in rat plasma or cell culture media. Two affinity purified goat polyclonal antibodies have been selected to detect only the full-length biologically active intact form of rat PTH. The antibody which recognizes epitopes within the C-terminal portion of the peptide is biotinylated for capture. The other antibody which recognizes the initial N-terminal epitope is conjugated with the enzyme horseradish peroxidase (HRP) for detection.

A sample containing rat intact PTH is incubated simultaneously with the biotinylated capture antibody and the HRP conjugated detection antibody in a streptavidin coated microtiter well. Intact PTH (1-84) contained in the sample is immunologically bound by the capture antibody and the detection antibody to form a “sandwich” complex:

Well/Avidin-Biotin Anti-Rat PTH — Rat Intact PTH — HRP Anti-Rat PTH

At the end of this incubation period, the well is washed to remove any unbound antibody and other components. The enzyme bound to the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microtiter plate reader. The enzymatic activity of the antibody complex bound to the well is directly proportional to the amount of bioactive intact PTH in the sample. A standard curve is generated by plotting the absorbance versus the respective intact PTH concentration for each standard on linear or logarithmic scales. The concentration of rat bioactive intact PTH in the samples is determined directly from this curve.

REAGENTS: Preparation and Storage

Store the kit at 2-8ºC upon receipt. **Store the standards and controls at -20ºC or below after reconstitution.** For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature and mix by gentle swirling and inversion. Reagents from different kit lot numbers should not be combined or interchanged.

1. **STREPTAVIDIN COATED MICROTITER PLATE (40-0010)**
   One plate with 12 eight well strips and frame (96 wells total). This reagent should be stored in the foil pouch with desiccant at 2 - 8°C and is stable until the expiration date on the kit.
2. **BIOTINYLATED RAT BIOACTIVE INTACT PTH ANTIBODY (40-2710)**
   One vial containing 2.7 mL of biotin labeled anti-rat PTH in TRIS buffered saline with protein stabilizers and a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C and is stable until the expiration date on the kit.
3. **HRP CONJUGATED RAT BIOACTIVE INTACT PTH ANTIBODY (40-2720)**
   One vial containing 2.7 mL of horseradish peroxidase conjugated anti-rat PTH in a stabilized protein solution with a non-azide, non-mercury preservative. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.

   NOTE: Make a Working Antibody Solution by pipetting equal volumes of Rat Bioactive Intact PTH Biotinylated Antibody and Rat Bioactive Intact PTH HRP Conjugated Antibody prior to use. Mix only the volume required for immediate use. Mix well to ensure homogeneity.
4. **RAT INTACT PTH STANDARDS (40-2531 to 40-2536)**
   Six vials each containing rat intact PTH (1-84) lyophilized in a protein matrix with a non-azide, non-mercury preservative. **Refer to vial label for exact concentration.** Before use reconstitute the vial with the rat intact PTH concentration of 0 pg/mL with 2.0 mL of deionized water. Before use reconstitute each of the other five vials of standards with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

   Use the standards immediately after reconstitution; freeze the unused portion for later use. After reconstitution the standards are stable until the expiration date on the kit box when stored at -20ºC or below with up to 3 freeze/thaw cycles.
5. **RAT INTACT PTH CONTROLS I & II (40-2541 & 40-2542)**
   Two vials each containing rat intact PTH (1-84) lyophilized in a protein matrix with a non-azide, non-mercury preservative. **Refer to vial label for control ranges.** Before use reconstitute each control with 1.0 mL of deionized water. Allow the vials to sit for approximately 20 minutes with occasional gentle swirling and inversion. Assure complete reconstitution before use.

   Use the controls immediately after reconstitution; freeze the unused portion for later use. After reconstitution the controls are stable until the expiration date on the kit box when stored at -20ºC or below with up to 3 freeze/thaw cycles.
6. **ELISA WASH CONCENTRATE (40-0041)**
   One bottle containing 20 mL of a 20 fold concentrate. Before use dilute the contents to 400 mL with deionized water and mix well.
   Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution should be stored at room temperature and is stable until the expiration date on the kit.

7. **ELISA HRP SUBSTRATE (40-0026)**
   One bottle containing 11 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 - 8°C protected from light and is stable until the expiration date on the kit.
8. **ELISA STOP SOLUTION (40-0030)**
   One bottle containing 11 mL of 1 M sulfuric acid. This reagent may be stored at room temperature or at 2 - 8°C and is stable until the expiration date on the kit box.
9. **PLATE SEALER (10-2016)**
   Two included in kit; use to prevent evaporation and cross-contamination of wells.

SAFETY PRECAUTIONS

Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid (i.e. ELISA HRP Substrate and ELISA Stop Solution). In case of contact with any of these reagents, wash thoroughly with water. TMB is a suspected carcinogen. Use Good Laboratory Practices. Wash hands before eating. Do not eat, drink or smoke in the work area.

MATERIALS REQUIRED BUT NOT PROVIDED

1. 1.0 mL and 2.0 mL volumetric pipettes for reconstituting standards and controls.
2. Precision pipets capable of delivering 25 µL, 50 µL and 100 µL.
3. Aluminum foil.
4. Automated microtiter plate washer OR
5. Repeating dispenser for delivering 350 µL and suitable aspiration device.
6. Container for storage of wash solution.
7. Spectrophotometric microtiter plate reader capable of reading absorbance at 450 nm and 595 - 650 nm.
8. Deionized water.
9. Horizontal rotator capable of maintaining 180 - 220 RPM.
10. Timer.

SPECIMEN COLLECTION

The intact PTH molecule is unstable, resulting in decreased immunoreactivity over time. Sample collection and storage procedures should be carried out in an expeditious manner. **Due to the variable lability of the molecule, measurement of the bioactive intact PTH concentration should be made using EDTA plasma or cell culture media. (Serum is no longer recommended as an appropriate sample.)** Fifty microliters of plasma or culture media are required to assay the sample in duplicate. Centrifuge the sample and separate the plasma or media from the cells. Samples should be assayed immediately or stored frozen at -20ºC or below. Avoid repeated freezing and thawing of specimens.

The use of various anesthetics can cause significant elevations in blood PTH concentrations. It is therefore imperative to use consistent sample collection procedures within studies. (See ref. #4)

ASSAY PROCEDURE

1. Place a sufficient number of Streptavidin Coated Strips in a holder to run PTH standards, controls and unknown samples.
2. Pipet 25 µL of standard, control, or sample into the designated or mapped well. Freeze the remaining standards and controls as soon as possible after use.
3. Pipet 50 µL of the Working Antibody Solution consisting of equal volumes of Rat Bioactive Intact PTH Biotinylated Antibody and Rat Bioactive Intact PTH HRP Conjugated Antibody into each well.
4. Cover the plate with one plate sealer, then cover with aluminum foil to avoid exposure to light.
5. Incubate plate at room temperature for three hours on a horizontal rotator set at 180 - 220 RPM.
6. Remove the aluminum foil and plate sealer. **Using an automated microtiter plate washer aspirate the contents of each well. Wash each well five times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents.** A suitable aspiration device may also be used.
7. Pipet 100 µL of ELISA HRP Substrate into each of the wells.
8. Re-cover the plate with the Plate Sealer and aluminum foil. Incubate at room temperature for 30 minutes on a horizontal rotator set at 180 - 220 RPM.
9. Remove the aluminum foil and plate sealer. Read the absorbance at 595 nm (see Note) within 5 minutes in a microtiter plate reader against the 0 pg/mL Standard wells as a blank.
10. Immediately pipet 100 µL of ELISA Stop Solution into each of the wells. Mix on horizontal rotator for 1 minute.
11. Read the absorbance at 450 nm within 10 minutes in the microtiter plate reader against a reagent blank of 100 µL of Substrate and 100 µL of Stop Solution.

    If dual wavelength correction is available set the Measurement wavelength to 450 nm and Reference wavelength to absorbance used in step #9.

    NOTE: Absorbance may be read at wavelengths from 595 nm to 650 nm depending upon available filters.

PROCEDURAL NOTES

1. It is recommended that all standards, controls and samples be assayed in duplicate. The average absorbance reading of each duplicate should then be used for data reduction and the calculation of results.
2. Store light sensitive reagents (i.e. HRP Conjugated Antibody, the Working Antibody Solution consisting of combined Biotinylated Antibody and HRP Conjugated Antibody, and ELISA HRP Substrate) in the original amber bottles or other suitable container which is well protected from light.
3. Store any unused Streptavidin Coated Strips in the resealable aluminum pouch with desiccant to protect from moisture.
4. The sample and all reagents should be pipetted carefully to minimize air bubbles in the wells.
5. The sequence and timing of each reagent addition is important as both the immunological and enzymatic reactions are in kinetic modes. The washing step is also an important part of the total assay procedure. **The use of an automated microtiter plate washer is strongly recommended.** All pipeting and washing steps should be performed such that the timing is as consistent as possible.
6. Samples with values greater than the highest standard should be diluted 1:10 with the 0 pg/mL Standard and reassayed. Multiply the result by 10. (See Limitations, # 1 and # 2)
7. Plasma or cell culture media samples may contain fibrin clots or cellular debris. Freeze/thaw of plasma samples may accelerate clot formation. These samples must be centrifuged and decanted prior to assay to remove all particulate material which can cause random high non-specific binding on well surface.
8. Rarely, upon opening the streptavidin plate, small white crystals may be observed in some of the wells. This is entirely cosmetic and will not affect the assay. This condition is reported by other kit manufacturers and results from the final stabilizing buffer used in the coating process.

CALCULATION OF RESULTS

The two absorbance readings taken before and after the addition of the ELISA Stop Solution allow for the construction of two standard curves using the rat intact PTH standards contained in the kit**. Refer to the individual vial label for exact concentration.** The primary curve used for calculation of results is the second reading taken after the addition of the ELISA Stop Solution and read at 450 nm. This data utilizes the absorbance values obtained with the first five standards. The first reading taken before the addition of the ELISA Stop Solution and read at 595 nm – 650 nm is intended to extend the analytical range to the value of the sixth (highest) standard provided in the kit**. It should be used only for sample results that fall between the value of the fifth and sixth standard.** Results obtained with this reading should not replace the on-scale reading at 450 nm. Each curve should be generated as follows:

Primary Procedure — Read at 450 nm

1. Calculate the average absorbance for each pair of duplicate assay wells.
2. Subtract the average absorbance of the 0 pg/mL Standard from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by plotting the corrected absorbance of the first five standard levels on the ordinate against the standard concentration on the abscissa using linear-linear or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of the rat bioactive intact PTH results.

The intact PTH concentration of the controls and samples are read directly from the standard curve using their respective corrected absorbance. If log-log graph paper or computer assisted data reduction programs utilizing logarithmic transformation are used, samples having corrected absorbance between the 0 pg/mL Standard and the next highest standard should be calculated by the formula:

$$\text{Value of unknown} = \frac{\text{Corrected Absorbance (unknown)}}{\text{Corrected Absorbance (2nd Std.)}} \times \text{Value of the 2nd Std.}$$

Secondary Procedure — Read at 595 nm - 650 nm

1. Calculate the average absorbance for each pair of duplicate assay wells.
2. The standard curve is generated by plotting the absorbance of the three highest standards on the ordinate against the standard concentration on the abscissa using linear-linear or log-log graph paper.
3. The intact PTH concentration of samples reading only between the fifth and sixth standard are read directly from this standard curve.

EXAMPLE DATA AND STANDARD CURVE

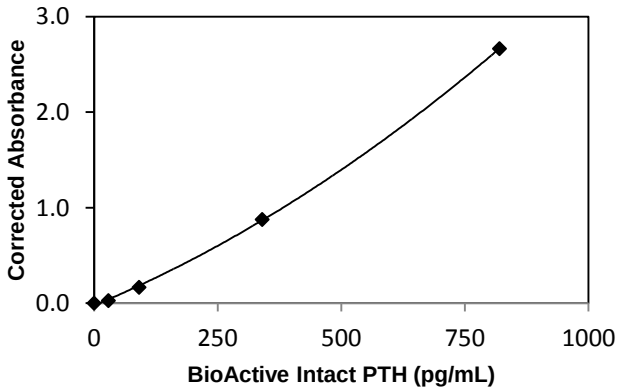
The following are representative examples of data and the resulting standard curves from the primary and secondary procedures. **These curves should not be used in lieu of a standard curve run with each assay.**

PRIMARY ASSAY - 450 nm

WELL I.D.	ABS	AVERAGE ABS	CORRECTED ABS	RESULTS pg/mL
Reagent Blank	0.000			
	0.000	0.000		
0 pg/mL	0.003			
	0.001	0.002	0.000	
29 pg/mL	0.032			
	0.025	0.029	0.027	
91 pg/mL	0.168			
	0.166	0.167	0.166	
340 pg/mL	0.881			
	0.876	0.878	0.876	
820 pg/mL	2.721			
	2.611	2.666	2.664	
Control I	0.085			
	0.079	0.082	0.080	53
Control II	0.362			
	0.376	0.369	0.367	162
Sample 1	0.271			
	0.270	0.271	0.269	127
Sample 2	0.706			
	0.720	0.713	0.711	282
Sample 3	2.972			
	2.920	2.946	2.944	*

* > 820 pg/mL; calculate using secondary assay.

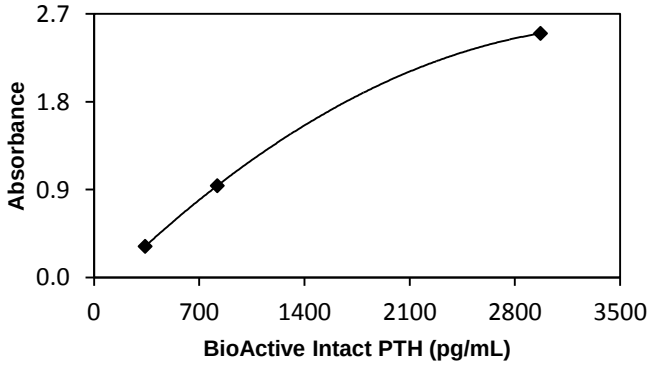
Rat *BioActive* Intact PTH ELISA
Primary Assay



SECONDARY ASSAY - 595 nm

WELL I.D.	ABS	AVERAGE ABS	RESULTS pg/mL
0 pg/mL	0.013		
	0.020	0.017	
340 pg/mL	0.319		
	0.320	0.319	
820 pg/mL	0.958		
	0.921	0.940	
2970 pg/mL	2.529		
	2.471	2.500	
Sample 3	1.023		
	1.067	1.045	965

Rat *BioActive* Intact PTH ELISA
Secondary Assay



LIMITATIONS OF THE PROCEDURE

1. The lowest concentration of rat bioactive intact PTH measurable is 3 pg/mL (assay sensitivity) and the highest concentration of rat bioactive intact PTH measurable without dilution is the value of the highest standard.
2. The reagents in this Rat BioActive Intact PTH ELISA kit have been optimized so that the high dose "hook effect" is not a problem for samples with elevated intact PTH values. Samples with rat intact PTH levels between the highest standard and 200,000 pg/mL will read greater than the highest standard and should be diluted 1:10 with the 0 pg/mL Standard and reassayed for correct values.
3. Grossly lipemic serum or plasma samples may affect the immunological response and it is recommended that results obtained with such samples be scrutinized accordingly.

4. Differences in protein concentration and protein type between samples and standards in an immunoassay contribute to "protein effects" and dose biases. When measuring low protein concentration culture media samples against high protein concentration standards, it is recommended that like samples be assayed together in the same assay to minimize this bias.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls with known levels of rat intact PTH. Immutopics recommends that all assays include the laboratory's own rat intact PTH controls in addition to those provided with this kit.

PERFORMANCE CHARACTERISTICS:

SENSITIVITY

The sensitivity of the rat bioactive intact PTH assay as determined by the 95% confidence limit on 20 duplicate determinations of the 0 pg/mL Standard is 3 pg/mL.

PRECISION

To assess intra-assay precision the mean and coefficient of variation were calculated from 20 duplicate determinations of two samples each performed in a single assay.

Mean Value (pg/mL)	Coefficient of Variation
54	3.9 %
157	2.5 %

To assess inter-assay precision the mean and coefficient of variation were calculated from duplicate determinations of two samples performed in 20 assays.

Mean Value (pg/mL)	Coefficient of Variation
55	8.9 %
163	7.8 %

PARALLELISM

Serum and plasma samples were diluted with the 0 pg/mL Standard and assayed. Results in pg/mL are as follows:

SAMPLE	DILUTION	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	undiluted	87		
	1:2	41	43	95
	1:4	20	22	91
2	undiluted	138		
	1:2	66	69	96
	1:4	32	35	91
3	undiluted	222		
	1:2	106	111	95
	1:4	47	56	84

RECOVERY

Various amounts of rat intact PTH were added to three different rat plasma samples and assayed. Results in pg/mL are as follows:

SAMPLE	ORIG. VALUE	AMOUNT ADDED	OBSERVED VALUE	EXPECTED VALUE	% O/E
1	17	29	45	46	98
		57	76	74	103
		86	100	103	97
2	19	46	64	65	98
		91	112	110	102
		137	155	156	99
3	14	17	30	31	97
		34	47	48	98
		50	65	64	102

CROSS-REACTIVITY

The Rat BioActive Intact PTH ELISA kit is specific for the full-length intact 1-84 form of the molecule. N-terminal 1-34, mid-region and C-terminal 39-84 and other non 1-84 fragments will not be measured. Cross-reactivity to other mammalian species is not known.

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Immutopics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Immutopics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights which vary from state to state.

REFERENCES

1. Schultz VL, Garner SC, Lavigne JR, Toverud SU, "Determination of bioactive rat parathyroid hormone (PTH) concentrations in vivo and in vitro by a 2-site homologous immuno-radiometric assay", *Bone and Mineral* 27 (1994) 121-132.
2. Jara A, Bover J, Lavigne J, Felsenfeld A, "Comparison of Two Parathyroid Hormone Assays for the Rat: The New Immunoradiometric and the Older Competitive Binding Assay", *J Bone Miner Res*, Vol. 9, No. 10, 1994, p. 1629.
3. Rucinski B, Mann GN, Epstein S "A new rapid and reproducible homologous immunoradiometric assay for amino-terminal parathyroid hormone in the rat," *Calcif Tissue Int*, 1995, 56:83-87.
4. Schultz VL, Boass A, Garner SC, Toverud SU, "Several Anesthetics, but Not Diethyl Ether, Cause Marked Elevation of Serum Parathyroid Hormone Concentration in Rats", *J Bone Miner Res*, Vol. 10, No. 9, 1995, p. 1298.
5. Urena P, Kubrusly M, Mannstadt M, Hruby M, et al., "The renal PTH/PTHrP receptor is down-regulated in rats with chronic renal failure", *Kidney International*, Vol. 45 (1994), pp. 605-611.
6. Mallya SM, Buchberger G, Arnold A, "Effects of anesthetic agents on serum parathyroid hormone and calcium concentrations in mice." *Veterinary Anesthesia and Analgesia,* 2007, 34: 403-407.

CLIENT SERVICES

To place an order or for technical assistance, contact Immutopics International at (800) 681-6665 or (949) 369-9207 or FAX to (949) 369-9405 or e-mail: clientservices@immutopicsintl.com.

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