ZEUS *ELISA*™ NTx Urine Test System

REF NTx9006 IVD **Rx Only**

INTENDED USE

The ZEUS ELISA NTx Urine Test System is an immunoassay that provides a quantitative measurement of the excretion of cross-linked N-telopeptides of type I collagen (NTx) in human urine samples and can be used as an indicator of human bone resorption. Elevated levels of urinary NTx indicate elevated human bone resorption. Measurement of NTx is intended for use in predicting skeletal response (bone mineral density) to hormonal antiresorptive therapy in postmenopausal women. Measurement of NTx is also intended for the use in therapeutic monitoring of antiresorptive therapy in postmenopausal women, antiresorptive therapy in individuals diagnosed with osteoporosis, antiresorptive therapy in individuals diagnosed with Paget's disease of bone, estrogen-suppressing therapies and identifying the probability for a decrease in bone mineral density after one year in postmenopausal women receiving calcium supplement relative to those treated with hormonal antiresorptive therapy. This test is for *in-vitro* diagnostic use only.

The measurement range of the ZEUS ELISA NTx Urine Test System is 20 to 3000 nM Bone Collagen Equivalents (BCE).

SIGNIFICANCE AND BACKGROUND

Bone is a dynamic and evolving tissue. Bone tissue undergoes constant bone turnover which involves the destruction of bone (bone resorption) followed by the construction of new bone (bone formation)(1). In mammals, this continuous remodeling of bone is accomplished through a coupled process of osteoclast mediated bone resorption, followed by osteoblast mediated bone formation (2). This process is necessary for normal development and maintenance of the skeleton. When balanced, resorption and formation result in healthy bone tissue. If there should be an abnormal imbalance in these two processes, this may result in changes in skeletal mass and shape.

There are many documented markers of bone resorption. Most fall into one of four categories: collagen degradation products, non-collagenous proteins, osteoclastic enzymes, and osteocyte activity markers (1). NTx falls into the collagen degradation product category. Approximately 90% of the organic matrix of bone tissue is type I collagen. NTx are generated from the amino terminus of the type 1 collagen by cleavage of the N-terminal region by cathepsin K during the resorption phase of bone turnover (1).

Bone turnover markers such as NTx have clinical utility in the comprehensive evaluation of osteoporosis. Elevated concentrations of NTx mean elevated levels of bone resorption. When interpreted with caution and with a good understanding of its natural variability, measurement of NTx may provide information that supplements osteoporosis management and provides useful clinical information about the conditions that alter bone turnover (3).

PRINCIPLE OF THE ASSAY

The ZEUS NTx Urine assay is a competitive-inhibition enzyme-linked immunosorbent assay (ELISA) that utilizes microwells as the solid phase onto which NTx has been adsorbed. NTx in the specimen competes with the solid phase NTx for binding sites of a monoclonal antibody labeled with horseradish peroxidase. The amount of antibody bound to the solid phase is therefore inversely proportional to the amount of NTx in the specimen. Quantitation of the NTx concentration in the specimen is determined spectrophotometrically and calculated from a standard calibration curve. Assay values are corrected for urinary dilution by urinary creatinine analysis and expressed in nanomoles bone collagen equivalents per liter (nM BCE) per millimole creatinine per liter (mM creatinine).

KIT COMPONENTS

A	1 Plate	Antigen coated 96-microwell plate. 12 x 8-well strips coated with purified human NTx antigen.
B	0.4 mL vial	Antibody Conjugate Concentrate. Solution containing purified murine monoclonal antibody directed against NTx conjugated to horse radish peroxidase and Proclin™ 300 (0.1%). Supplied as a 100X concentrated conjugate.
C	30 mL bottle	Antibody Conjugate Diluent. Buffered reagent with protein stabilizers, into which Antibody Conjugate Concentrate is diluted. ProClin™ 300 (0.05%) included as a preservative.
D	125 mL bottle	30X Wash Buffer Solution. One bottle containing a 30X ionic detergent solution.
E	0.9 mL bottle	Chromogen Reagent. One vial containing a solution of 3,3',5,5' – tetramethylbenzidine in dimethyl-sulfoxide. Supplied as a 100x concentrate.

F	30 mL bottle	Buffered Substrate. One bottle containing a buffered hydrogen peroxide solution.	
G	25 mL bottle	Stopping Reagent. One bottle containing a solution of 1N sulfuric acid.	
1	0.4 mL vial	1 nM BCE Calibrator	
2	0.4 mL vial	30 nM BCE Calibrator	
3	0.4 mL vial	100 nM BCE Calibrator	Various amounts of purified NTx antigen in a buffered diluent.
4	0.4 mL vial	300 nM BCE Calibrator	Contains ProClin™ 300 (0.05%) as a preservative.
5	0.4 mL vial	1000 nM BCE Calibrator	
6	0.4 mL vial	3000 nM BCE Calibrator	
I	0.4 mL vial	Level I Urine Control	Human urine with known NTx concentration. ProClin™ 300 (0.10%)
II	0.4 mL vial	Level II Urine Control	included as a preservative.
	Plate Sealers	1 pad	

Note: Bovine Serum or Bovine Serum Albumin is present in some components

PRECAUTIONS

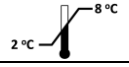
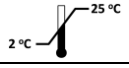
1. **For *in vitro* diagnostic use only.**
2. The Antigen Coated 96-well Plate, Calibrators, and Urine Controls contain human urine and/or antigen processed from human bone tissue. Although each lot of urine and bone has been documented to be non-reactive for HIV 1, HIV 2, HBsAg, HCV and RPR by FDA approved methods, these materials should be handled as potentially infectious and should be disposed of properly.
3. The Stopping Reagent contains 1N sulfuric acid. Danger: Causes severe skin burns and eye damage.
4. The Chromogen Reagent contains 3,3',5,5'- tetramethylbenzidine and dimethylsulfoxide. Warning - Dimethylsulfoxide is readily absorbed through the skin. May cause skin irritation or serious eye irritation.
5. ProClin is included as a preservative in most reagents, at concentrations listed in the reagent section. May cause skin irritation or serious eye irritation.
6. Assay plate, Assay Calibrators, Urine Controls: Contain materials of human origin.
7. Urine specimens may contain infectious agents and should be disposed of properly. Decontamination is most effectively accomplished with a 0.5% solution of sodium hypochlorite (1:10 dilution of household bleach) or by autoclaving one hour at 121 ºC. Do not autoclave solutions containing sodium hypochlorite. Do not combine sodium hypochlorite solution with acid.
8. Never pipette reagents or clinical specimens by mouth.
9. Wear protective gloves and clothing when handling specimens and reagents. Wash hands thoroughly after use.
10. Do not use reagents beyond their expiration dates.
11. Do not mix components from different lots of the ZEUS NTx Urine assay kits.
12. Microwell strips must be kept desiccated. Reseal unused microwell strips in the pouch containing desiccant.
13. Do not reuse microwells. Dispose of properly after use.
14. Perform the assay procedure in a controlled laboratory environment that adheres to the stated incubation requirements. Avoid extreme environmental conditions during the procedure.

Note: Safety data sheets are available for professional users upon request.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Single and multichannel pipettes capable of delivering 25 µL, 100 µL and 200 µL volumes.
2. Disposable pipette tips.
3. Disposable plastic containers for reagent mixing and pipetting reservoirs.
4. Automated microwell washer.
5. Microwell or microstrip spectrophotometric reader. The reader must read at 450 nm with a 630 nm reference filter and detect absorbances from 0 to 3.000 (or greater) optical density units.
6. Software capable of calculating a 4-parameter curve fit.
7. Deionized water.

STORAGE CONDITIONS

	When not in use, the kit should be stored at 2 – 8 °C. Reagents must be allowed to reach room temperature before use. Do not expose the reagents to temperatures greater than 30 °C or less than 2 °C.
	Diluted (1X) wash solution may be stored at room temperature for up to one month.

SPECIMEN COLLECTION AND STORAGE

1. Collect a second void of the morning (spot) urine specimen or a 24-hour urine specimen in an appropriate collection device with a tight fitting lid.
2. DO NOT ADD PRESERVATIVE TO URINE SPECIMEN.

3. Specimens with visible whole blood contamination or visible hemolysis may interfere with the assay and should be discarded. Collection of a new specimen is recommended.
4. Store refrigerated (2 – 8 ºC) for up to 72 hours or at room temperature for up to 24 hours. Store frozen (–20 ºC or below) for longer term storage. Specimens may undergo three freeze/thaw cycles.
5. When monitoring therapy, baseline samples should be collected prior to initiation of therapy. Subsequent specimens for comparison should be collected at the same time of day as the baseline specimen.

ASSAY PROCEDURE

Preparatory Steps

1. **Allow all specimens and reagents to equilibrate to room temperature (18 – 28 ºC) for at least one hour before performing the assay**. To facilitate warming, remove reagents from the kit box. Frozen urine specimens may be thawed at 37 ºC, in either a water bath or an incubator, then brought to room temperature prior to use in the assay. The Chromogen Reagent contains dimethylsulfoxide, which may solidify when refrigerated but is liquid at room temperature.
2. Prepare the working strength wash solution. Dilute 30X Wash Concentrate 1:30 with deionized water (1 part 30X Wash Concentrate to 29 parts deionized water) and mix for a minimum of five (5) minutes. This solution is stable for one (1) month at room temperature.
3. Create a plate map indicating location of calibrators, controls and urine specimens. It is recommended that calibrators, controls and urine specimens be run in duplicate microwells. An example of a plate map is provided below for a ZEUS NTx Urine assay with 4 specimens:

	1	2	3
A	1 nM BCE Calibrator	1000 nM BCE Calibrator	Specimen #1
B	1 nM BCE Calibrator	1000 nM BCE Calibrator	Specimen #1
C	30 nM BCE Calibrator	3000 nM BCE Calibrator	Specimen #2
D	30 nM BCE Calibrator	3000 nM BCE Calibrator	Specimen #2
E	100 nM BCE Calibrator	Level I Urine Control	Specimen #3
F	100 nM BCE Calibrator	Level I Urine Control	Specimen #3
G	300 nM BCE Calibrator	Level II Urine Control	Specimen #4
H	300 nM BCE Calibrator	Level II Urine Control	Specimen #4

4. Using a clean disposable plastic container, dilute the Antibody Conjugate Concentrate 1:101 using the Antibody Conjugate Diluent. As a guideline, for each Antigen Coated microwell strip used, dilute 20 µL Antibody Conjugate Concentrate into 2 mL of Antibody Conjugate Diluent. Mix gently by inversion only. Do not vortex or use a magnetic stir bar. Avoid foaming. Use the working strength conjugate solution within one hour of preparation. Do not reuse the container.
5. Prior to pipetting, gently mix the Calibrators, Controls and urine specimens. Avoid foaming. Allow cloudy or turbid specimens to settle 5 to 10 minutes prior to pipetting. Urine specimens containing particulates may be centrifuged before use.
6. Remove the appropriate number of microwell strips from the sealed foil pouch. Place any unused strips back in the pouch, resealing the pouch along the zipper. Do not remove the desiccant pillow from the foil pouch.

Specimen and Antibody Incubation

Once the assay has been started, complete it without interruption.

7. Following the plate map generated in Step 3, pipette 25 µL of each Calibrator, Control, or urine specimen into the bottom of the designated microwells. Use a calibrated pipettor and new pipette tips for each Calibrator, Control, or urine specimen.
8. Using a multichannel pipettor, deliver 200 µL of the working strength conjugate solution into each microwell. Apply a plate sealer and swirl the plate gently on a flat surface for 5-10 seconds to ensure mixing.
9. Incubate the plate at room temperature (18 - 28 ºC) for 90 ± 5 minutes.
10. Prepare the Chromogen/Buffered Substrate solution during the last 10 minutes of incubation by making a 1:101 dilution of the Chromogen Reagent into the Buffered Substrate. As a guideline, for each Antigen Coated microwell strip used, dilute 20 µL of the Chromogen Reagent into 2 mL of the Buffered Substrate. Pipette the Buffered Substrate into a clean plastic disposable container. Thoroughly mix the Chromogen Reagent prior to pipetting. Add the Chromogen Reagent to the Buffered Substrate Reagent and invert gently to mix. Do not vortex, shake vigorously or use a magnetic stir bar to mix. Use the Chromogen/Buffered Substrate solution within 30 minutes of preparation. The Chromogen/Buffered Substrate solution should be colorless when mixed. A blue color indicates that the reagent has been contaminated and must be discarded. Do not reuse the Chromogen/Buffered Substrate solution container.
11. At the end of the incubation period, carefully remove and discard the plate sealer. Wash the plate five (5) times with the working strength wash solution using an automated plate washer. The automated washer must dispense at least 350 µL of the working strength wash solution per well. Between wash cycles, wells should be filled with working strength wash solution. When wash procedure is complete, grasp the plate frame at the center of each side and invert, blotting on an absorbent paper towel. Immediately add the prepared Chromogen/Buffered Substrate solution as described below.

Color Development and Measurement

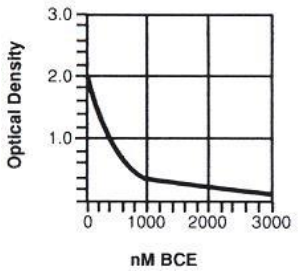
12. Pipet 200 µL of the Chromogen/Buffered Substrate solution prepared in step 10 into each well using a multichannel pipettor. Cover the plate with a new plate sealer.
13. Incubate at room temperature for 15 ± 1 minutes. A blue color will develop in wells containing bound antibody-horseradish peroxidase conjugate.
14. Following incubation, carefully remove and discard the plate sealer. Using a multichannel pipettor, add 100 µL of Stopping Reagent to each well in the same order as addition of the Chromogen/Buffered Substrate Reagent. Wells which have developed a blue color will now turn yellow.
15. Swirl the plate gently on a flat surface for 5 -10 seconds to ensure mixing. Allow the plate to sit at room temperature for 5 minutes before reading absorbance values.
16. Within 30 minutes of adding the Stopping Reagent, read the absorbance of the Calibrators, Controls, and urine specimens. Use a microwell plate reader at 450 nm with a reference filter of 630 nm. The reader must have a maximum optical density reading of ≥3.

ANALYSIS OF RESULTS

1. Determine concentration values (nM BCE) of Controls and urine specimens using a 4-parameter curve fitting equation.

EXAMPLE:

CALIBRATION CURVE



2. Assay results are valid if the following criteria are met:
    a. The mean absorbance value of the 1 nM BCE Calibrator must be ≥1.500.
    b. The span of the calibrator curve (difference between absorbance values of the 1 nM BCE and 3000 nM BCE Calibrators) must be ≥1.300.
3. The recommended coefficient of variation (% CV) between urine specimen concentration value (nM BCE) duplicates is ≤20%. Specimens with >20% CV should be rerun.
4. The lower limit of detection is 20 nM BCE (assay value, does not include creatinine correction).
5. Urine specimens that exceed 3000 nM BCE may be diluted 1:5 in a urine specimen or pool of urine known to be within the range of 200-500 nM BCE, and retested. When using urine as a diluent, the nM BCE of the urine diluent should be confirmed by testing it as a specimen in the same plate as the diluted unknown specimen. The dilution factor and background (the diluent nM BCE) should be incorporated into the final calculation.

    Example: 1040 nM BCE assay value derived from a 1:5 dilution of a 4000 nM BCE specimen using a urine diluent of known ZEUS NTx Urine assay value (300 nM BCE)

    1040 nM BCE - (0.8 x 300 nM BCE) = 800 nM BCE
    800 nM BCE x 5 (dilution factor) = 4000 nM BCE

    Note: 1:5 dilutions represent 80% diluent (0.8), 20% specimen contribution.

6. Report the concentration values for urine specimens as nM BCE/mM creatinine, as shown in the following example:

Assay value	=	360 nM BCE
Urinary creatinine	=	60 mg/dL creatinine
		11.3*
	=	5.3 mM creatinine
360 nM BCE	=	68 nM BCE/mM creatinine
5.3 mM creatinine		

    *Note: Conversion factor used to convert mg creatinine per dL to millimole creatinine per liter.

7. These Urine Control ranges have been established by the manufacturer. It is recommended that each laboratory establish its own control ranges.

LIMITATIONS OF THE PROCEDURE

While the ZEUS NTx Urine assay is used as an indicator of bone resorption, the use of this test has not been established to predict development of osteoporosis or future fracture risk. Use of this test has not been established in primary hyperparathyroidism or hyperthyroidism. When using the ZEUS NTx Urine assay to monitor therapy, results may be confounded in patients afflicted with clinical conditions known to affect bone resorption, e.g., metastases to bone. While a ZEUS NTx Urine assay value provides a measure of the level of bone resorption, a single ZEUS NTx Urine assay value cannot provide the rate of bone resorption as reported results do not contain a measure of time. The ZEUS NTx Urine assay results should be interpreted in conjunction with clinical findings and other diagnostic results.

PERFORMANCE CHARACTERISTICS

Expected Values

Urine NTx concentrations are dependent upon multiple factors. Using the prospectively collected specimens used for the comparative study outlined below, we can report the NTx values for these groups.

Category	n =	Average NTx (nM BCE)	Min NTx (nM BCE)	Max NTx (nM BCE)	Median NTx (nM BCE)
Males, < 25 YOA	25	1821	33	3150	1740
Males, 26-50 YOA	25	743	128	1862	704
Males, > 50 YOA	25	780	79	2602	744
Females, 18-35 YOA	25	723	64	2685	525
Females, > 50 YOA	50	601	49	1852	481

Intra and Inter-Assay Precision

Intra and Inter-Assay precision were evaluated on one lot of the ZEUS ELISA NTx Urine Test System. Briefly, four patient samples were identified that spanned the reportable range of the assay. These four specimens were tested in quadruplicate on each day. The assay was repeated on five days resulting in 20 replicates for each specimen. The results of the Intra-Assay and Inter-Assay precision study are depicted in the table below:

ZEUS ELISA NTx Urine		Intra-Assay Precision					Inter-Assay Precision
		Day1	Day2	Day3	Day4	Day5	All days
Sample 1	Mean	2226	2678	2275	2449	2504	2426
	Standard Deviation	15.66	42.49	82.36	62.19	16.60	173.15
	Percent CV	0.7%	1.6%	3.6%	2.5%	0.7%	7.1%
Sample 2	Mean	1069	1493	1253	1203	1375	1279
	Standard Deviation	70.75	29.74	30.31	44.01	46.89	154.65
	Percent CV	6.6%	2.0%	2.4%	3.7%	3.4%	12.1%
Sample 3	Mean	441	664	507	496	554	532
	Standard Deviation	29.54	34.50	32.50	13.91	22.11	80.66
	Percent CV	6.7%	5.2%	6.4%	2.8%	4.0%	15.2%
Sample 4	Mean	45	89	70	77	71	70
	Standard Deviation	7.63	4.65	7.09	13.15	9.54	16.56
	Percent CV	16.9%	5.3%	10.1%	17.0%	13.5%	23.5%

Lot-to-Lot Precision

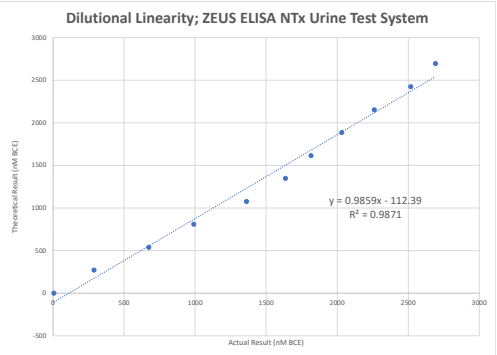
Four samples were chosen that spanned the reportable range of the assay. These samples were tested in 10 replicate wells for each run. Each run was repeated twice for a total of 20 replicates per sample. This was repeated on a second lot of the ZEUS ELISA NTx Urine Test System. The results of this lot-to-lot precision study are depicted in the table below:

	ZEUS ELISA NTx Urine Test System Lot 1			ZEUS ELISA NTx Urine Test System Lot 2			Both Lots Combined		
	Average (nM BCE)	Standard Deviation	Percent CV	Average (nM BCE)	Standard Deviation	Percent CV	Average (nM BCE)	Standard Deviation	Percent CV
Sample 1	2562	75.19	2.9%	2423	98.72	4.1%	2492	111.51	4.5%
Sample 2	1361	83.45	6.1%	1280	129.24	10.1%	1321	114.90	8.7%
Sample 3	596	55.27	9.3%	538	60.48	11.2%	567	64.13	11.3%
Sample 4	71	18.73	26.3%	69	10.10	14.7%	70	14.91	21.4%

Dilutional Linearity Study:

A sample that was near the upper end of the reportable range of the assay was selected for this study. This sample was diluted to various concentrations using the assay calibrator diluent as a 0 nM BCE diluent. The results of this dilutional linearity study are depicted below:

Amount of Urine Sample	Amount of Calibrator Diluent	Result (nM BCE)	Expected or Theoretical Result
100%	0%	2692	2692
90%	10%	2517	2423
80%	20%	2260	2154
70%	30%	2034	1884
60%	40%	1815	1615
50%	50%	1637	1346
40%	60%	1361	1077
30%	70%	990	808
20%	80%	673	538
10%	90%	287	269
0%	100%	6.5	0

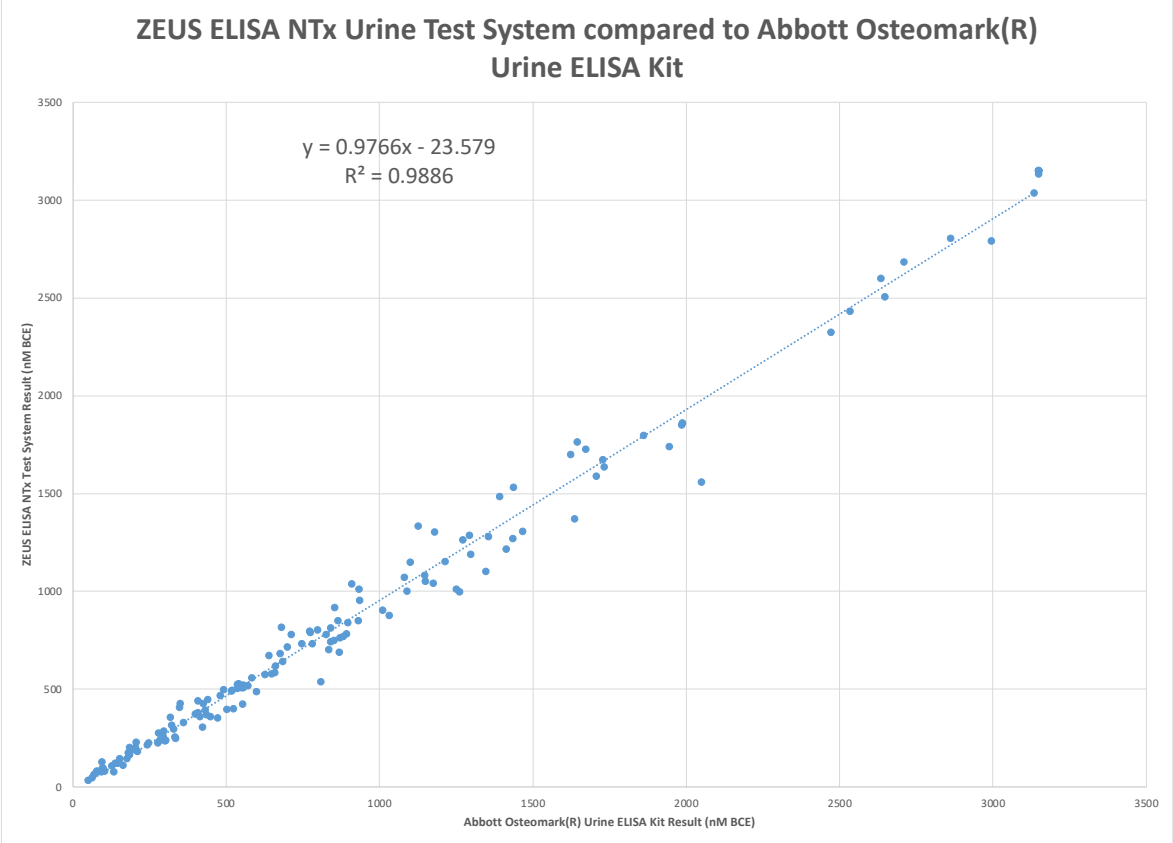


Comparative Study:

The ZEUS ELISA NTx Urine Test System was compared to the Abbott Osteomark® Test Kit in a comparative study. For this investigation, 150 urine specimens were prospectively collected from the Northeastern US. These specimens were collected as follows:

Category	n =	Average Age	Min Age	Max Age	Median Age
Males, < 25 YOA	25	19.04	15	24	20
Males, 26-50 YOA	25	37.4	26	50	38
Males, > 50 YOA	25	62.36	50	89	60
Females, 18-35 YOA	25	27.88	19	35	29
Females, > 50 YOA	50	59.48	50	81	58

The specimen cohort was tested on both the ZEUS and Abbott NTx ELISA products in duplicate. The mean of the duplicate values was calculated and plotted in a regression plot. The results of that comparative analysis are depicted below:



This study demonstrates that the ZEUS ELISA NTx Urine Test System is comparable to the Abbott Osteomark® NTx Urine kit across the entire reportable range of the assay.

REFERENCES

1. Shetty S, Kapoor N, Bondu JD, Thomas N, Paul TV. Bone turnover markers: Emerging tool in the management of osteoporosis. Indian J Endocrinol Metab. 2016 Nov-Dec;20(6):846-852. doi: 10.4103/2230-8210.192914. PMID: 27867890; PMCID: PMC5105571.
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