Serum CrossLaps® ELISA

English

For the quantification of degradation products of C-terminal telopeptides of Type-I collagen in human serum and plasma

The Serum CrossLaps® ELISA kit is for *in vitro* use only.
Nordic Bioscience Diagnostics is not responsible for any other use of the kit or consequence hereof than the one specified above. Neither for misuse, e.g. use deviating from the procedure described in this manual.

Furthermore, Nordic Bioscience Diagnostics A/S is not to be made responsible for any diagnoses or conclusions made by the user or third party based on the results obtained with the Serum CrossLaps® ELISA kit nor for any consequences such interpretations may cause.

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INTRODUCTION

Intended use
The Serum CrossLaps® ELISA is an enzyme immunological test for the quantification of degradation products of C-terminal telopeptides of Type-I collagen in human serum and plasma. **The Serum CrossLaps® ELISA assay is intended for in vitro diagnostic use as an indication of human bone resorption and may be used as an aid in**

A. Monitoring bone resorption changes of
1) Anti-resorptive therapies in postmenopausal women:
a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

2) Anti-resorptive therapies in individuals diagnosed with osteopenia;
a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

B. Predicting skeletal Response (Bone Mineral Density) in postmenopausal women undergoing anti-resorptive therapies
a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

Limitations
The use of the test has not been established to predict the development of osteoporosis or future fracture risk.
The use of the test has not been established in hyperparathyroidism or hyperthyroidism.
When using the test to monitor therapy, results may be confounded in patients afflicted with clinical conditions known to affect bone resorption e.g. bone metastases, hyperparathyoidism or hyperthyroidism.
Serum CrossLaps® ELISA results should be interpreted in conjunction with clinical findings and other diagnostic results and should not be used as a sole determinant in initiating or changing therapy
Do not interchange Serum CrossLaps® ELISA values with Urine CrossLaps® ELISA values.

Summary and explanation of the test
Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primarily in bone (1). During renewal of the skeleton, Type I collagen is degraded, and small peptide fragments are excreted into the bloodstream. These fragments can be measured by Serum CrossLaps® ELISA The measurements of the specific degradation products of Type I collagen in both urine (2) and serum (3) by a competitive CrossLaps® ELISA have been reported.
The sandwich assay has been reported as useful for follow-up of anti-resorptive treatment of patients with metabolic bone diseases (3-17).

Principle of the procedure
The Serum CrossLaps® ELISA is based on two highly specific monoclonal antibodies against the amino acid sequence of EKAHD-β-GGR, where the aspartic acid residue (D) is β-isomerized. In order to obtain a specific signal in the Serum CrossLaps® ELISA, two chains of EKAHD-β-GGR must be cross-linked.

Standards, control, or unknown serum samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of a mixture of a biotinylated antibody and a peroxidase-conjugated antibody. Then, a complex between CrossLaps® antigens, biotinylated antibody and peroxidase-conjugated antibody is generated, and this complex binds to the streptavidin surface via the biotinylated antibody. Following the one-step incubation at room temperature, the wells are emptied and washed. A chromogenic substrate is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance is measured.

PRECAUTIONS

The following precautions should be observed in the laboratory:

- Do not eat, drink, smoke or apply cosmetics where immunodiagnostic materials are being handled
- Do not pipette by mouth.
- Wear gloves when handling immunodiagnostic materials and wash hands thoroughly afterwards
- Cover working area with disposable absorbent paper

Warnings

For *in vitro* use only.

- All reagents and laboratory equipment should be handled and disposed of as if they were infectious.
- Do not use kit components beyond the expiry date and do not mix reagents from different lots.

HAMA interference

Some individuals have antibodies to mouse immunoglobulins (HAMA), which can cause interference in immunoassays that employ murine monoclonal antibodies, such as Serum CrossLaps®. In rare cases, the content of HAMA exceeds the capacity of the blocking agent incorporated into Serum CrossLaps® leading to a false-positive test result. Therefore, Serum CrossLaps® values should be used only in conjunction with information available from the clinical evaluation of the patient.

Storage

Store the Serum CrossLaps® ELISA kit upon receipt at 2-8°C. Under these conditions the kit is stable up to the expiry date stated on the box.

MATERIALS

Specimen collection

Collect blood by venipuncture taking care to avoid haemolysis. Separate the serum from the cells within 3 hours after collection of blood. It is recommended to freeze (< -18°C) samples immediately.

For optimal results it is recommended to draw blood as fasting morning samples (18).

Also for monitoring the individual patient, follow-up samples should be collected under same conditions as the baseline sample.

When analysing plasma, both heparia and EDTA plasma may be used.

Materials supplied
Before opening the kit, read the section on **Precautions.** The kit contains reagents sufficient for 96 determinations.

Streptavidin coated microtitre plate (MTP)
Microwell strips (12x8 wells) pre-coated with streptavidin. Supplied in a plastic frame.
CrossLaps® Standard (Vial A)
One vial (min. 1.5 mL/vial) of ready-for-use PBS-buffered solution with protein stabiliser and preservative.
CrossLaps® Standards (Vial B-F)
Five vials (min. 0.4 mL/vial) of ready-for-use, CrossLaps® standard in a PBS-buffered solution with protein stabiliser and preservative. The exact concentration is stated on each vial.
Control (Vial CO 1-2)
Two vials (0.5 mL/vial) of ready-for-use, desalted urinary antigens of human origin in a PBS-buffered solution with protein stabiliser and preservative. Please refer to enclosed techical datasheet for control range.
Biotinylated Antibody (Vial no. 1)
One vial (0.25 mL) of a concentrated solution of a biotinylated monoclonal murine antibody specific for degradation products of C-terminal telopeptides of Type I collagen, raised. Prepared in a buffered solution with protein stabiliser and preservative.
Peroxidase Conjugated Antibody (Vial no. 2)
One vial (0.25 mL) of a concentrated solution of a peroxidase conjugated murine monoclonal antibody specific for degradation products of C-terminal telopeptides of Type I collagen. Prepared in a buffered solution with protein stabiliser and preservative.
Incubation Buffer (Vial no. 3)
One vial (min. 19 mL) of a ready-for-use buffered solution with protein stabiliser, detergent and preservative.
Substrate Solution (Vial TMB)
One vial (min. 12 mL) of a ready-for-use tetramethylbenzidine (TMB) substrate in an acidic buffer.
Please note that the chromogenic substrate might appear sligthy blueish.
Stopping Solution (Vial ST)
One vial (min. 12 mL) of ready-for-use 0.18 mol/L sulfuric acid.
Washing Buffer (Vial W)
One vial (min. 20 mL) of a concentrated washing buffer with detergent and preservative.
Sealing tape
Adhesive film for covering wells during incubation.

Materials required - not supplied
- Containers for preparing the Antibody Solution and the Washing Solution
- Precision micropipettes to deliver 50-200 µL
- Distilled water
- Precision 8- or 12-channel multipipette to deliver 100 µL, and 150 µL
- Microwell mixing apparatus
- Microtiter plate reader

ASSAY PROCEDURE

Assay Procedure
Mix all reagents and samples before use (avoid foam)

Prior to use, prepare and equilibrate all solutions to room temperature. **Perform the assay at room temperature (18-22°C).**

1 Preparation of the Antibody Solution:
ATTENTION: Prepare the following **Antibody Solution** maximum 30 minutes before starting the assay. Mix the solutions in vial no. 1 (Biotinylated Antibody), vial no. 2 (Peroxidase Conjugated Antibody) and vial no. 3 (Incubation Buffer) in the volumetric ratio 1+1+100 in an empty container. Mix carefully and avoid formation of foam. **Prepare a fresh solution before each run of the assay.**

2 One Step incubation
Pipette 50 µL of either **Standards** (vial A-F), Control (vial CO), or unknown samples into appropriate wells followed by 150 µL, of the **Antibody Solution.** Cover the immunostrips with sealing tape and incubate for 120±5 minutes at room temperature (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

3 Washing
Wash the immunostrips 5 times manually with **Washing Buffer** (vial W) diluted 1+50 in distilled water. Using an automated plate washer, follow the instructions of the manufacturer or the guidelines of the laboratory. Usually 5 washing cycles are adequate. Make sure that the wells are **completely emptied** after each manual or automatic washing cycle.

4 Incubation with chromogenic substrate solution
Pipette 100 µL of the **Substrate Solution** *(*vial TMB) into each well and incubate for 15±2 minutes at room temperature (18-22°C) in the dark on the mixing apparatus (300 rpm). Use sealing tape.
Do not pipette directly from the vial containing TMB substrate but transfer the needed volume to a clean reservoir. Remaining substrate in the reservoir should be discarded and not returned to vial TMB.

5 Stopping of colour reaction
Pipette 100 µL of the **Stopping Solution** *(*vial ST) into each well.

6 Measurement of absorbance
Measure the absorbance at 450 nm with 650 nm as reference within two hours.

Limitations of the procedure
- If the absorbance of a sample exceeds that of **Standard F,** the sample should be diluted in **StandardA** and re-analysed.

QUALITY CONTROL

Good Laboratory Practice (GLP) requires the use of quality control specimens in each series of assays in order to check the performance of the assay. Controls should be treated as unknown samples, and the results analysed with appropriate statistical methods.

RESULTS

Calculation of results
A quadratic curve fit can be used.

Alternatively, calculate the mean of the duplicate absorbance determinations. Construct a standard curve on graph paper by plotting the mean absorbances of the six standards A-F (ordinate) against the corresponding CrossLaps® concentrations (abscissa). Determine the CrossLaps® concentration of the controls and each patient sample by interpolation.

Example of results obtained:

Standards/ Controls/ Samples	CrossLaps® conc. (ng/mL)	$A_{450-650}$ (nm) Obs 1/ Obs 2	Mean $A_{450-650}$ (nm)	Interpolated CrossLaps® conc. (ng/mL)
Standard A	0.000	0.041 / 0.040	0.041	
Standard B	0.120	0.128 / 0.125	0.127	
Standard C	0.339	0.324 / 0.313	0.319	
Standard D	0.700	0.644 / 0.631	0.638	
Standard E	1.442	1.278 / 1.265	1.272	
Standard F	2.172	1.904 / 1.969	1.937	
Control CO 1		0.286 / 0.296	0.291	0.305
Sample I		0.216 / 0.215	0.216	0.216
Sample II		0.373 / 0.398	0.386	0.416
Sample III		0.859/ 0.886	0.873	0.982

Please note: The data above are for illustration only and should not be used to calculate the results of any run.

Performance characteristics
Detection limit: 0.020 ng/mL CrossLaps®
This is the concentration corresponding to three standard de viations above the mean of 21 determinations of the blank ("CrossLaps® Standard A").

Imprecision
The imprecision of the Serum CrossLaps® ELISA was evaluated for three serum samples. The results are summarised in the table below:
InterAssay Variation (n=12)

Mean (ng/mL)	SD (ng/mL)	CV (%)
0.273	0.022	8.1
0.393	0.021	5.4
0.488	0.032	6.5

IntraAssay Variation (n=21)

Mean (ng/mL)	SD (ng/mL)	CV (%)
0.242	0.013	5.4
0.375	0.019	5.0
0.476	0.024	5.1

Dilution/Linearity
The Serum CrossLaps® ELISA is linear in the range 0.020 ng/mL to 3.380 ng/mL of CrossLaps®.

Serum samples with the concentration of 0.460-0.668 ng/mL CrossLaps® were diluted with standard A and the concentration of CrossLaps® were determined with Serum CrossLaps® ELISA. The serum neat sample is set to 100%.

The data below is calculated from 3 different runs:

Dilution Procedure		
Serum [%]	Standard A [%]	Recovery [% of expected value]
100	0	100
90	10	103
80	20	103
70	30	101
60	40	102
50	50	105
40	60	109
30	70	107
20	80	97
10	90	100
Mean		103

Interference:
The interference of Ditaurobilirubin, Hemoglobin and IntraLipid on the measurement of CrossLaps® in serum by Serum CrossLaps® Step ELISA was investigated.
In the concentration listed below no interference was detected:

Ditaurobilirubin	600 mg/L
Hemoglobin	10 g/L
IntraLipid	10 g/L

Expected values
It is advisable for a laboratory to establish its own range of normal and pathological values. As an example, the mean values and standard deviations for various populations are given below. For further reading, please refer to the reference list. All samples were morning fasting samples from healthy individuals.

Populations	Number of subjects	Mean Values* (ng/mL)	95% range (ng/mL)
Post-menopausal women	193	0.439	0.142 – 1.351
Pre-menopausal women	226	0.287	0.112 – 0.738
Males	125	0.294	0.115 – 0.748

*Geometric mean value and corresponding 95% confidence interval

Day to Day Individual Variation
The Day to Day Intra-individual Variation was assessed by analyzing serum samples (morning fasting) from 11 healthy post menopausal women at five time points over 2 weeks.

Subject No	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Mean (ng/mL)	SD (ng/mL)	CV (%)
1	0,423	0,428	0,396	0,460	0,445	0,430	0,024	6
2	0,461	0,523	0,500	0,535	0,539	0,512	0,032	6
3	0,850	0,731	0,761	0,782	0,764	0,778	0,045	6
4	0,377	0,468	0,455	0,499	0,440	0,448	0,045	10
5	0,918	0,834	0,791	0,781	0,714	0,808	0,075	9
6	0,268	0,249	0,246	0,257	0,258	0,255	0,009	3
7	0,431	0,457	0,468	0,494	0,506	0,471	0,030	6
8	0,666	0,587	0,670	0,595	0,728	0,655	0,063	10
9	0,323	0,357	0,341	0,409	0,345	0,355	0,033	9
10	0,419	0,520	0,541	0,491	0,470	0,488	0,047	10
11	0,353	0,472	0,429	0,464	0,400	0,424	0,049	12

CLINICAL DATA

The Serum CrossLaps® ELISA has been used to monitor treatment in several clinical studies and the CrossLaps® values have been compared to Bone Mineral Density (BMD_{spine}) measurements.

All the clinical studies presented below were performed according to the European Standard for good clinical practice (GCP and GLP).
Most of the clinical studies presented here were conducted on white Danish women. However, several studies have been published showing that other demographic groups display similar CrossLaps® decrease in response to anti-resorptive therapies (9-13).

For all the data presented below fasting morning samples have been used.

The Bone mineral density was measured at the Lumbar spine (L 1 - L4).
The change in the bone mineral density is presented below α-BMD. α -BMD is defined as the slope of the linear regression line for BMD_{spine} versus time (years) for the period of treatment. In most cases the calculation of α-BMD involves a minimum of 5 BMD_{spine} measurements. The α-BMD thus represents the % change in BMD_{spine} per year.
Because there to this date is no universal agreement as to what constitutes positive BMD response we have calculated the sensitivity and specificity using two different cut-off values for α-BMD; α-BMD>0 and α-BMD> 1.
The sensitivity is defined as the percent of the study population with a positive BMD response and who have a % change from baseline of Serum CrossLaps® ELISA which is 40% or greater.
The specificity is defined as the percent of the study population without a positive BMD response and who have a % change from baseline of Serum CrossLaps® ELISA that is less than 40%.

Bisphosphonate studies
Below is shown the Serum CrossLaps® ELISA data from two different bisphosphonate studies.

Alendronate

- Women between age 40 and 59 years, 6 months to 3 years since menopause
- 12 participants on placebo (500 mg calcium)
- 42 participants on active treatment (5 mg (n=16), 10 mg (n=14), 20 mg (n=12)) Alendronate and 500 mg calcium)
- Treatment period: 2 - 3 years

Serum CrossLaps® ELISA

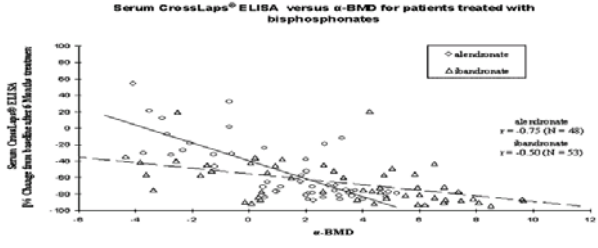
	Placebo group **Mean (ng/mL)** **SD** **SEM** *(95% Confidence Int.)*			Alendronate group **Mean (ng/mL)** **SD** **SEM** *(95% Confidence Int.)*		
Baseline	0.676 (0.543-0.809)	0.235	0.068	0.603 (0.540-0.666)	0.208	0.032
After 6 months treatment	0.623 (0.498-0.748)	0.221	0.064	0.191 (0.144-0.238)	0.153	0.024

Ibandronate

- Women less than 75 years, more than ten years after menopause and have a BMD forearm 1.5 SD or more below the standard for healthy pre-menopausal women
- 17 participants on placebo (1000 mg calcium)
- 36 participants on active treatment: (2.5 mg (n=20), 5 mg (n=16)) ibandronate and 1000 mg calcium
- Treatment period: 1 year

Serum CrossLaps® ELISA

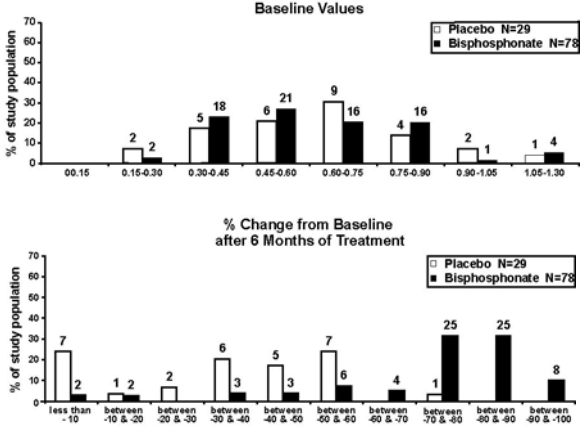
	Placebo group **Mean (ng/mL)** **SD** **SEM** *(95% Confidence Int.)*			Ibandronate group **Mean (ng/mL)** **SD** **SEM** *(95% Conf1dence Int.)*		
Baseline	0.590 (0.502-0.678)	0.185	0.045	0.614 (0.536-0.692)	0.240	0.040
After 6 months treatment	0.325 (0.241-0.409)	0.178	0.043	0.136 (0.093-0.179)	0.131	0.022



Using a cut-off for Serum CrossLaps® ELISA of 40% change from baseline the following sensitivities, specificities and 95% confidence intervals are obtained.
Below is indicated the actual fractions:

	Ibandronate		**Alendronate**	
	Sensitivity	Specificity	Sensitivity	Specificity
α-BMD>0	93% (81-99) 40/43	30% (7-65) 3/10	89% (73-97) 31/35	92% (64-100) 12/13
α-BMD>1	94% (81-99) 33/35	22% (6-48) 4/18	90% (73-98) 26/29	68% (43-87) 13/19

Below is shown distribution plots for the combined bisphosphonate studies. The number over each bar indicates the number of participants in each class.



HRT Studies
Below is shown the Serum CrossLaps® ELISA data from three different HRT studies.

Tibolone
- Women less than 75 years and more than ten years after meno pause
- 13 participants on placebo (400 mg calcium/day)
- 49 participants on active treatment (1.25 mg (n=25) or 2.5 mg (n=24) Tibolone and 400 mg calcium/day

- Treatment period 2 years

Serum CrossLaps® ELISA

	Placebo group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**	Tibolone group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**
Baseline	0.264 (0.217-0.311)	0.085	0.024	0.339 (0.302-0.376)	0.130	0.019
After 6 months treatment	0.287 (0.232-0.342)	0.099	0.028	0.192 (0.161-0.223)	0.113	0.016

HRT I

- Women more than 45 years, 1 to 6 years since menopause
- 42 participants on placebo (400 mg calcium/day)
- 120 participants on active treatment:

Days 1-16	Days 17-28
E 1 mg	E 1 mg + G 25 µg
E 2 mg	E 2 mg + G 25 µg
E 2 mg	E 2 mg + G 50 µg
E 1 mg + G 25 µg	E 1 mg + G 25 µg continuously

  E = estradiol-17β, G = gestodene, active treatment also receive 400 mg calcium/day
- Treatment period: 2 years.

Serum CrossLaps® ELISA

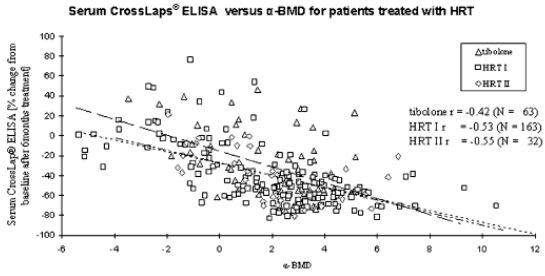
	Placebo group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**	HRT I group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**
Baseline	0.389(0.348-0.430)	0.136	0.021	0.411(0.386-0.437)	0.140	0.013
After 6 months treatment	0.396(0.349-0.443)	0.153	0.024	0.182(0.164-0.200)	0.098	0.009

HRT II

- Women between 65 and 70 years and BMC forearm below 1 SD of healthy pre-menopausal women
- 17 participants on placebo (1000 mg calcium/day)
- 15 participants on active treatment: 50 µg estradiol, 1 mg norethisterone and 1000 mg calcium/day

Serum CrossLaps® ELISA

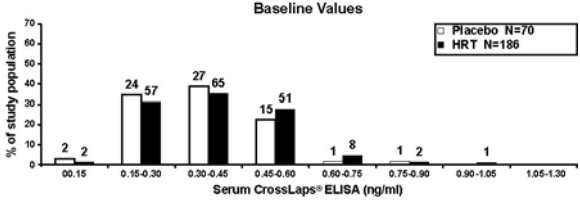
	Placebo group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**	HRT II group **Mean (ng/mL)** *(95% Confidence Int.)*	**SD**	**SEM**
Baseline	0.350(0.301-0.399)	0.099	0.025	0.371(0.306-0.436)	0.135	0.033
After 6 months treatment	0.293(0.240-0.346)	0.106	0.027	0.159(0.108-0.210)	0.106	0.026

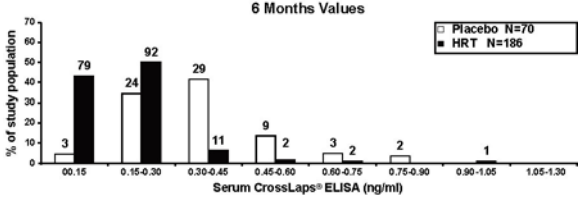


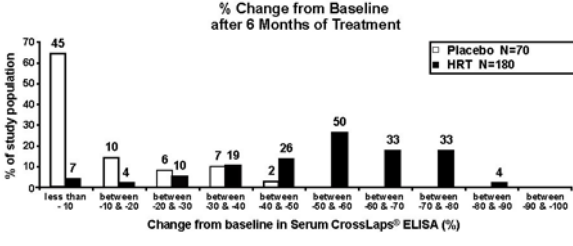
Using a cut-off for Serum CrossLaps® ELISA of 40% change from baseline the following sensitivities, specificities and 95% confidence intervals are obtained. Below is indicated the actual fractions:

	Tibolone		HRT I		HRT II	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
α-BMD>0	58% (44-72) 31/53	90% (56-100) 9/10	80% (72-87) 16/20	90% (77-97) 37/41	60% (39-79) 15/25	100% (59-100) 7/7
α-BMD>I	65% (49-79) 28/43	80% (56-94) 16/20	83% (74-94) 90/109	78% (64-88) 42/54	62% (38-82) 13/21	91% (59-100) 10/11

Below is shown distribution plots for the combined HRT studies. The number over each bar indicates the number of participants in each class.


Baseline Values
Placebo N=70
HRT N=186
% of study population
0
10
20
30
40
50
60
70
2
2
24
57
27
65
15
51
1
8
1
2
1
00.15
0.15-0.30
0.30-0.45
0.45-0.60
0.60-0.75
0.75-0.90
0.90-1.05
1.05-1.30
Serum CrossLaps® ELISA (ng/ml)


6 Months Values
Placebo N=70
HRT N=186
% of study population
0
10
20
30
40
50
60
70
3
79
24
92
29
11
9
2
3
2
2
1
00.15
0.15-0.30
0.30-0.45
0.45-0.60
0.60-0.75
0.75-0.90
0.90-1.05
1.05-1.30
Serum CrossLaps® ELISA (ng/ml)


% Change from Baseline
after 6 Months of Treatment
Placebo N=70
HRT N=180
% of study population
0
10
20
30
40
50
60
70
45
7
10
4
6
10
7
19
2
26
50
33
33
4
less than
- 10
between
-10 & -20
between
-20 & -30
between
-30 & -40
between
-40 & -50
between
-50 & -60
between
-60 & -70
between
-70 & -80
between
-80 & -90
between
-90 & -100
Change from baseline in Serum CrossLaps® ELISA (%)

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