APPENDICES
APPENDIX-I

ESTIMATION OF TRACP-5b IN HUMAN SERUM SAMPLES

Method: ELISA method

Kit supplier: IDS, Boldon, UK.

Principle:
1. The plate is coated with anti-TRACP antibodies (monoclonal).
2. Calibrators, control and patient samples are added.
3. Releasing reagent is added.
4. Dissociation of active TRACP-5b from the binding proteins.
5. TRACP-5b is bound by the anti TRACP antibodies.
6. Incubation with pNPP substrate.

The reaction is stopped by adding sodium hydroxide. The absorption is read photometrically.

Reagents provided with the kit:
Microplate coated with monoclonal anti-TRACP antibody (mouse) and BSA, control, calibrators, wash buffer, sample diluent, releasing reagent, substrate buffer, substrate tablets (p-nitrophenyl phosphate), stop solution.

Materials required:
Distilled water, micropipettes (adjustable), incubator, microplate shaker and microplate reader with filter for 405 nm.

Test procedure:
1. Cut the aluminium bag above the zip fastener and take out the required number of microplate wells.
2. Add 100 µl Sample Diluent as blank to the first two wells of the plate.
3. To the next wells, add 100 µl each of Calibrators, Control and samples in duplicates.
4. Add 50 µl Releasing Reagent to each well.
5. Seal the microplate with incubation cover foil and incubate for 60 min (± 5 min) at room temperature with constant shaking at 850-950 rpm.
6. After incubation wash the microplate wells four times with 300 μl wash buffer per well. Pay attention that all wells are filled.

7. After washing tap microplate wells on filter paper.

8. Do not allow the wells to dry out, proceed immediately.

9. Add 100 μl Substrate Solution to each well.

10. Seal the microplate with incubation cover foil and incubate for 60 min (± 5 min). At 37 °C (± 1°C).

11. Stop the reaction by adding 25 μl of Stop Solution to each well. Ensure for a good mixing by shaking gently.

12. Clean microplate wells from underneath before the photometric reading and take care that there are no air bubbles in the wells.

13. The reading should be done within 15 min after adding the Stop Solution.

**TABLE 20**

**TEST PROCEDURE:**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Calibrators</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Diluent</td>
<td>100 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calibrators</td>
<td>-</td>
<td>100 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>100 μl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100 μl</td>
</tr>
<tr>
<td>Releasing Reagent</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

Incubate for 60 min (± 5 min) at room temperature with constant shaking at 850-950 rpm, wash 4x with 300 μl wash buffer.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Calibrators</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Solution</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

Incubate for 60 min (± 5 min) at 37 °C (± 1°C).

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Calibrators</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop Solution</td>
<td>25 μl</td>
<td>25 μl</td>
<td>25 μl</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

Photometric reading at 405 nm

Control value: 2.61 U/L  
Intra-assay variation: 5.4%  
Inter-assay variation: 8.9%
ESTIMATI0N OF URINE CROSSLAPS OF TYPE-I COLLAGEN

Method: ELISA

Kit suppliers: Nordic Bioscience Diagnostics, Herlev, Denmark.

Principle of the procedure:

The urine crossLaps ELISA assay is based on the competitive binding of the anti-CrossLaps antibodies to either soluble CrossLaps antigen or to CrossLaps antigen-coated micro-titre wells. Standards, controls, or unknown samples are pipetted into the appropriate micro-titre wells. Then the anti-CrossLaps antibodies are added and incubation takes place for 1 hour at 18-22 °C. The wells are washed and peroxidase conjugated anti-rabbit immunoglobulin is added. The wells are then incubated again for 1 hour at 18-22 °C. After a second washing step the wells are incubated for 15 min with a chromogenic substrate. The reaction is stopped and the absorbance is measured.

Reagents provided with the kit:

immunostrips pre-coated with CrossLaps antigen, CrossLaps standards, urine control, peroxidase conjugated antibody, substrate solution, stopping solution, washing solution, sealing tape.

Materials required:

Micropipettes (50 μl, 100 μl), microwell mixing apparatus (300 rpm), microtiter plate reader.

Assay Procedure:

For optimal performance of the assay it is important to comply with the instructions given below. Prior to use, prepare and equilibrate all solutions to room temperature. Perform the assay at 18-22 °C.

Determine the number of strips needed for the assay. It is recommended to test all samples in duplicate. In addition, for each run a total of 14 wells are needed for standards and control. Place the appropriate number of strips in the plastic frame. Store unused immunostrips in the tightly closed foil bag with desiccant capsules.
1. Pipette Standards or Samples:
   Pipette 15 µl of either Standards (vial A-F), Control (vial CO) or unknown samples into appropriate wells.

2. Incubation in Immunostrips:
   Add 100 µl Primary Antibody Solution (vial no.1) to each well. Cover the immunostrips with sealing tape and incubate for 60 min ± 5 minutes at 18-22 °C on a micro-titre plate mixing apparatus (300 rpm).

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

4. Incubation with Peroxidase Conjugated Antibody:
   Add 100 µl of peroxidase conjugated antibody (vial no.2) to each well, cover with sealing tape, and incubate for 60 min ± 5 minutes at 18-22 °C on the mixing apparatus (300 rpm).

5. Washing:
   Proceed as described in step 3.

6. Incubation with Chromogenic Substrate Solution:
   Pipette 100 µl of the Substrate Solution (vial TMB) into each well, cover with sealing tape, and incubate for 15 min ± 2 minutes at 18-22 °C in the dark on the mixing apparatus (300 rpm).
   
   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 no.3.

7. Stopping of color reaction:
   Pipette 100 µl of Stopping Solution (vial ST) into each well.

8. Measurement of absorbance:
   Measure the absorbance at 450 nm with 650 nm as reference within two hour.
Calculation of corrected CrossLaps value:

For each sample the CrossLaps concentration (µg/L) and the creatinine concentration (mM = mmol/L) should be determined. For determination of creatinine the method by Jaffe or Equivalent is recommended.

The following equation corrects the CrossLaps concentration for variation in urine concentration.

\[
\text{Corrected CrossLaps value (µg/mmol) = CrossLaps (µg/L)}/ \text{Creatinine (mM)}.
\]

Control value: 186.53 µg/mmol creatinine
Intra-assay variation: 7.6%
Inter-assay variation: 6.3%
ESTIMATION OF ESTRADIOL IN HUMAN SERUM SAMPLES

Method: Radioimmunoassay method

Kit supplier: Diasorin, Italy.

Principle:
The Radioimmunoassay of estradiol is a competition assay. Samples and Calibrators are incubated 3 hours (1 hour for samples of women under ovulation induction) with $^{125}\text{I}$-labeled estradiol, as tracer, in antibody-coated tubes. After incubation, the content of tubes is aspirated and bound radioactivity is measured. A calibration curve is established and unknown values are determined by interpolation from the curve.

Reagents provided with the kit:
Anti-estradiol antibody-coated tubes, $^{125}\text{I}$-labeled estradiol, tracer buffer, calibrators, control serum.

Materials required:
- pipetting devices (100 μl, 500 μl)
- vortex mixer
- orbital shaker
- aspiration system and gamma counter.

Assay Procedure:
1. Dilution of the concentrated tracer (55X)
   - Pour carefully the contents of the tracer vial into the tracer buffer vial.
   - Mix gently to avoid foaming before dispensing.
   - Store the reconstituted tracer at 2-8 °C until expiration date of the kit.
### TABLE 21

**TEST PROCEDURE:**

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Additions</strong>&lt;sup&gt;*&lt;/sup&gt;</td>
<td><strong>Incubation</strong></td>
<td><strong>Counting</strong></td>
</tr>
<tr>
<td>To antibody coated tubes, add successively: 100 µl of calibrator, control or sample and 500µl of tracer Mix</td>
<td>Incubate for 3 hours at 18-25 °C with shaking (350 rpm) (1 hour for samples of women under ovulation induction)</td>
<td>Aspirate carefully the contents of tubes (except the 2 tubes &lt;&lt; total cpm&gt;&gt;) Count bound cpm (B) and total cpm (T) for 1 min.</td>
</tr>
</tbody>
</table>

* Add 500 µl of tracer to 2 additional tubes to obtain total cpm.

Control value: 104.9 pg/ml
Intra-assay variation: 6.3%
Inter-assay variation: 7.2%
ESTIMATION OF HUMAN ERYTHROCYTE SEDIMENTATION RATE

Method: Westergren method

Principle:
When anticoagulated whole blood is allowed to stand, red blood cells settle out. The rate at which they fall is known as the erythrocyte sedimentation rate and is a rough measure of abnormal concentrations of acute phase proteins and immunoglobulins. This property makes ESR a sensitive, but nonspecific, indicator of tissue damage and inflammation.

Reagents and equipment:
1. Westergren pipettes, sodium citrate, EDTA, sodium chloride.
2. Vertical rack: This special rack is equipped with a leveling bubble device to ensure that the tubes are held in a vertical position. This fitting on the rack should be clean and uncracked to prevent leakage of the diluted blood.

Procedure:
1. Mix the blood citrate or blood EDTA-saline mixture thoroughly.
2. Aspirate a bubble free specimen into a clean and dry Westergren pipette. Fill to the '0' mark.
3. Do not pipette by mouth. Place the pipette into vertical rack at 20° to 25° in an area free from vibrations and direct sunlight.
4. After 6 minutes, read the distance in millimeters from the bottom of the plasma meniscus to the top of the sedimented erythrocyte.
5. Record the value as millimeters in 1 hour.

Reference values:
Female- 13 mm/hr (younger than 50 years)
20 mm/hr (older than 50 years)