INTRODUCTION

A. CLINICAL PHYSIOLOGY

The ImmuChem™ Total Estrogens (TE) kit detects the combined concentrations of unconjugated estradiol-17ß (E2) and estrone (E1). It does not detect estrogen sulfates or glucuronides. It will not distinguish between free E1 or E2 or E2 bound to circulating binding proteins. TE measurements are an approximation of plasma estrogenicity since E2 is biologically more active than E1 and the E2/E1 ratio may fluctuate widely under varying physiological and pathological conditions.

In prepubertal children and in the male, circulating TE (E1 + E2) concentrations are low and noncyclic. In the ovulating female, TE (E1 + E2) concentrations are highly variable depending on the phase of the menstrual cycle. In the postmenopausal or oophorectomized woman, TE (E2 + E1) concentrations are low and noncyclic.

B. CLINICAL APPLICATIONS

1. ASSESSMENT OF ESTROGEN STATUS: The measurements may be utilized to evaluate the estrogen status in children and adults where the clinician is not concerned about the E2/E1 ratio.

2. HUMAN MENOPAUSAL GONADOTROPIN (HMG) OVULATION INDUCTION CONTROL: The most recent advance in the monitoring of HMG dosage is the measurement of serum estrogens by radioimmunoassay.

II. PRINCIPLE OF THE TEST

In the ImmuChem™ Total Estrogens assay, the reaction follows the Mass Action Law and the labeled and non-labeled analyte binds to the antibody in proportion to their relative concentrations. The amount of radioactive analyte which binds is inversely proportional to the amount of unlabelled analyte in the sample. When compared to a series of calibrators, a dose response curve can be constructed which plots the counts bound versus the dose of the analyte. Because of the inverse relationship between the dose of the analyte and the counts bound, counts bound will decrease with increasing concentration of nonlabeled analyte as shown in the sample standard curve in Section XI.

III. REAGENTS PROVIDED AND LABEL COLOR CODE (100 Tube Kit)

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>LABEL COLOR</th>
<th>VOLUME OR QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Buffer</td>
<td>Tan</td>
<td>2 x 52 mL</td>
</tr>
<tr>
<td>Anti-Total Estrogens</td>
<td>Yellow</td>
<td>11 mL</td>
</tr>
<tr>
<td>Standards (6)</td>
<td>Green</td>
<td>5 mL ea.</td>
</tr>
<tr>
<td>Second Antibody</td>
<td>Red</td>
<td>11 mL</td>
</tr>
<tr>
<td>Estradiol-125I</td>
<td>Blue</td>
<td>11 mL</td>
</tr>
</tbody>
</table>

IV. REAGENTS DESCRIPTION AND PREPARATION

A. DILUENT BUFFER

0.005% rabbit gamma globulins in 0.1M phosphate buffered saline, pH 7.0, containing gelatin.

STORAGE: 2 to 8°C.
STABILITY: Refer to expiration date on kit vial.

B. ANTI-TOTAL ESTROGENS

Estradiol-17ß 3-Carboxymethyl ether-BSA was used as the antigen to generate antiserum in rabbits. The antiserum binds 50-60% of the Estradiol-17ß 125I derivative in the absence of nonradioactive Total Estrogens. The antiserum has been diluted with diluent buffer.

STORAGE: 2 to 8°C.
STABILITY: Refer to expiration date on kit vial.

C. TOTAL ESTROGENS STANDARDS

Six standards are provided at the following concentrations: 2.5 pg/0.5 mL, 5 pg/0.5 mL, 10 pg/0.5 mL, 25 pg/0.5 mL, 50 pg/0.5 mL, and 100 pg/0.5 mL. The standards have been diluted with diluent buffer.

STORAGE: 2 to 8°C.
STABILITY: Refer to expiration date on kit vial.

D. PRECIPITATING ANTISERUM (SECOND ANTIBODY)

Goat anti-rabbit gamma globulins in 0.01M phosphate buffered saline, pH 7.5. 0.1 mL of this precipitating antiserum will precipitate all the antibody bound antigen per predetermined incubation time.

STORAGE: Frozen.
STABILITY: Refer to expiration date on kit vial.

E. 17ß-ESTRADIOL 125I DERIVATIVE

This radioactive material contains less than 4 µCi per vial for 100 tube kit on the date of shipment. 0.1 mL of this radioactive derivative will provide approximately 50,000 cpm at 75% counter efficiency on the date of shipment.

STORAGE: 2 to 8°C.
STABILITY: Refer to expiration date on kit vial.
V. SPECIMEN COLLECTION AND HANDLING

VI. EQUIPMENT AND REAGENTS REQUIRED

A. Pipettors and/or pipettes.
B. Water bath, 37-45°C.
C. Test tube rack.
D. Laboratory vortex mixer.
E. Disposable glass tubes for extraction.
F. Absorbent paper for blotting.
G. Compressed air or nitrogen.
I. Pipette and/or pipette tips.
J. Water bath, 37-45°C.
K. Laboratory vortex mixer.
L. Pipette and/or pipette tips.
M. Test tube rack.
N. Centrifuge-refrigerated (preferred) or room temperature.

VII. LIMITATIONS, PRECAUTIONS AND GENERAL COMMENTS

A. Strict adherence to the protocol is advised for reliable performance. Any changes or modifications may affect the precision and accuracy of the kit and, therefore, are the responsibility of the user.
B. Care must be taken that clinical samples do not contain any exogenous radioactivity since it may interfere with the results.
C. A standard curve must be established with every assay.
D. The reagents in this kit are designed specifically for the quantitation of Total Estrogens in humans. Anyone doing animal research work must establish their own conditions as the plasma.
E. The reagents supplied in this kit are for IN-VITRO DIAGNOSTIC USE.
F. The use of grossly hemolized or lipemic samples should be avoided.
G. The reagents supplied in this kit are for IN-VITRO DIAGNOSTIC USE.
H. The kit reagents and materials are intended for use as an integral unit. Do not mix various lots of any component reagent within an individual run.

H. RADIOACTIVE MATERIALS

Please observe the following precautions when handling this radioactive material:

1. This radioactive material may be received, acquired, possessed and used only by those licensed to do so. Its use is solely for in-vitro clinical or laboratory tests not involving the internal or external administration of the materials, i.e. radioactivity, to humans or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of, and with a general license from, the U.S. NRC or the state with which the U.S. NRC has entered into agreement for the exercise of regulatory authority.
2. Immediately upon receipt of the kit, check for breakage and verify the contents as per the packing list. Should there be any breakage or questions regarding this kit’s contents, immediately notify your representative.
3. Kit reagents should be stored and used only at clean, designated work stations of the laboratory. Although the exposure to radiation from the small amount of isotope supplied is negligible, it is a good practice to designate a storage area at least 10 feet from any work station. Furthermore, persons under the age of 18 should not be permitted to handle radioactive material or enter into an area where it is either stored or used.
4. Should there be spillage of any of the radioactive material, the following cleanup procedure is recommended: while wearing rubber gloves, blot the spillage onto an absorbent material. Dispose of this material as radioactive waste.
5. The pipetting of radioactive material by mouth should be avoided. Smoking, eating or drinking while performing tests involving radioactive material should not be permitted. Lastly, persons handling radioactive material should wash their hands immediately after handling and prior to leaving the laboratory area.

VI. SPECIMEN COLLECTION AND HANDLING

Plasma: Draw blood into a green capped (Heparin) Vacutainer™ tube. After separation, store the plasma in a refrigerator (can be stored for up to one week) or store frozen.

Serum: Draw blood into a red capped Vacutainer™. Allow the blood to clot for at least thirty minutes at room temperature. Separate the serum and store under the same conditions as the plasma.

VII. EQUIPMENT AND REAGENTS REQUIRED

In addition to the materials provided with the kit, the following materials are required:

A. Pipettors and/or pipettes.
B. Gamma counter.
C. Water bath, 37-45°C.
D. Laboratory vortex mixer.
E. Test tube rack.
F. Centrifuge-refrigerated (preferred) or room temperature.
H. Disposable glass tubes for extraction.
I. Compressed air or nitrogen.
J. 10 x 75 mm glass tube for RIA.
K. Absorbent paper for blotting.

VIII. ASSAY PROCEDURE

A. ASSAY PREPARATIONS

1. Set up assay in consecutively numbered 10x75 mm disposable glass test tubes. (DO NOT USE PLASTIC TUBES). Refer to the protocol (Section IX) as a guide only. Pipet all reagents directly from shipping vials.

B. EXTRACTION OF SERUM OR PLASMA SAMPLE

1. Add 0.1 mL* or 0.6 mL of serum or plasma to an appropriate glass disposable tube.

*NOTE: This volume to be used only for HMG Ovulation Induction Control samples.
2. Add 6 mL of ethyl acetate: hexane, mixed in a ratio of 3:2, to the serum or plasma.
3. Shake or vortex mix vigorously for 60 seconds and allow the phases to separate.
4. Withdraw 5 mL of the organic phase (top phase) and evaporate under air or nitrogen.
5. Reconstitute the sample residue (4) with 2.5 mL of diluent buffer and incubate at room temperature for 30 minutes or longer. Swirl or gently mix during this incubation period.
6. Withdraw 0.5 mL aliquots for assay.

NOTE: Each 0.5 mL is equivalent to 0.1 mL serum or plasma if 0.6 mL of sample was extracted, or 0.0166 mL if 0.1 mL of sample was extracted. The dilution factors are 10 and 60 respectively.

C. ASSAY STEPS

1. Add 0.6 mL of diluent buffer to tubes numbers 1 and 2, and 0.5 mL to tubes 3 and 4 (see Protocol, Section IX).
2. Add 0.5 mL of TOTAL ESTROGENS STANDARD (2.5-100pg) to tube numbers 5-16.
3. Add 0.5 mL of reconstituted sample (see Section VIII, B,(6) to tube numbers 17 to end of assay.
4. With the exception of tube numbers 1 and 2, add 0.1 mL of ANTI-TOTAL ESTROGENS to all the assay tubes.
5. Add 0.1 mL of 17β-ESTRADIOL 125I to all the assay tubes.
6. Mix all the assay tubes, then incubate for 90 minutes at room temperature.
7. After incubation (6), add 0.1 mL of SECOND ANTIBODY to all the assay tubes and incubate at room temperature for 60 minutes.
8. After 60 minutes incubation (7), centrifuge all the assay tubes at 2300-2500 rpm (1000 x g) for 15 minutes. Aspirate or decant the supernatant (if decanting, blot the rim of the test tubes on absorbent paper before turning right side up).
9. Count the precipitate in a gamma counter.
10. Calculate the results using the supplied formula or use any type of RIA data reduction system.

D. QUALITY CONTROL

Serum pools or commercially available controls containing a low, normal, and high concentration of total estrogens should be assayed routinely as unknowns. The concentrations of these controls should be plotted on a run to run basis using a Levy-Jennings type system in order to assess the performance and reliability of the assay. For further information, see:


<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>Diluent Buffer (mL)</th>
<th>Standard or Sample (mL)</th>
<th>Anti-Total Estrogens (mL)</th>
<th>E₂ 125I (mL)</th>
<th>2nd Antibody (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>Blank (NSB)</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>3,4</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6</td>
<td>2.5 pg</td>
<td>0</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,8</td>
<td>5 pg</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,10</td>
<td>10 pg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11,12</td>
<td>25 pg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13,14</td>
<td>50 pg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15,16</td>
<td>100 pg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17,18</td>
<td>Control 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19,20</td>
<td>Patient Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
X. CALCULATIONS

A. Take the average of all duplicate tubes (samples and standards). Subtract the Blank (NSB) value from the average of the samples and standards. Divide this value by zero standard value. This yields the percent bound.

B. Formula:

\[ \text{%B/Bo} = \frac{\text{CPM (sample)} - \text{CPM (blank, NSB)}}{\text{CPM (0 standard)} - \text{CPM (blank, NSB)}} \times 100 \]

\[ \text{CPM} = \text{Average counts of duplicates.} \]

\[ \text{Sample} = \text{Particular serum or standard being calculated.} \]

\[ \text{Blank NSB} = \text{Blank tube (also known as non-specific binding tube).} \]

\[ \text{0 Standard} = \text{0 tube (also known as 100% binding tube)} \]

Example: Control II (from Section XI):

\[ \frac{6656 - 770}{10927 - 770} \times 100 = 58\% \]

C. Plot percent bound (y-axis) against the Total Estrogen Standards (x-axis) ranging from 2.5 - 100 pg. This yields the standard curve. Convert sample values as follows: pg read off the standard curve x 10 or x 60 = pg/mL of Total Estrogens (see Note in Section VIII, B, 6).

D. ALTHOUGH THE STANDARD CURVE IS LINEAR THROUGHOUT ITS ENTIRE RANGE, IT IS ERRONEOUS TO EXTRAPOLATE A VALUE FOR A PATIENT SAMPLE THAT BINDS EITHER HIGHER OR LOWER THAN THE STANDARD CURVE.

XI. SAMPLE ASSAY

These calculations are for example only. The user must construct a standard curve each time the assay is run.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CPM</th>
<th>Avg. CPM</th>
<th>Avg. CPM - Blank</th>
<th>Bound</th>
<th>Total Estrogens Conc. (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (NSB)</td>
<td>718</td>
<td>822</td>
<td>770</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 pg/tube</td>
<td>10952</td>
<td>10902</td>
<td>10927</td>
<td>10157</td>
<td></td>
</tr>
<tr>
<td>2.5 pg/tube</td>
<td>10141</td>
<td>9372</td>
<td>9756</td>
<td>8986</td>
<td>89</td>
</tr>
<tr>
<td>5.0 pg/tube</td>
<td>8528</td>
<td>8395</td>
<td>8461</td>
<td>7691</td>
<td>76</td>
</tr>
<tr>
<td>10 pg/tube</td>
<td>6764</td>
<td>7030</td>
<td>6897</td>
<td>6127</td>
<td>60</td>
</tr>
<tr>
<td>25 pg/tube</td>
<td>5029</td>
<td>5043</td>
<td>5036</td>
<td>4266</td>
<td>42</td>
</tr>
<tr>
<td>50 pg/tube</td>
<td>3899</td>
<td>3899</td>
<td>3899</td>
<td>3129</td>
<td>31</td>
</tr>
<tr>
<td>100 pg/tube</td>
<td>2970</td>
<td>2757</td>
<td>2864</td>
<td>2094</td>
<td>21</td>
</tr>
<tr>
<td>Control I</td>
<td>10624</td>
<td>10744</td>
<td>10684</td>
<td>9914</td>
<td>98</td>
</tr>
<tr>
<td>Control II</td>
<td>6654</td>
<td>6658</td>
<td>6656</td>
<td>5886</td>
<td>58</td>
</tr>
<tr>
<td>Control III</td>
<td>5162</td>
<td>5570</td>
<td>5366</td>
<td>4596</td>
<td>45</td>
</tr>
</tbody>
</table>

SAMPLE STANDARD CURVE

NOTE: This curve serves only as an example. Sample values should not be derived from it.

XII. EXPECTED PHYSIOLOGICAL RANGES

<table>
<thead>
<tr>
<th>Males</th>
<th>Prepubertal:</th>
<th>&lt;40 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult:</td>
<td>40-115 pg/mL</td>
<td></td>
</tr>
<tr>
<td>Females: Prepubertal:</td>
<td>&lt;40 pg/mL</td>
<td></td>
</tr>
<tr>
<td>Postmenopausal or castrate:</td>
<td>&lt;40 pg/mL</td>
<td></td>
</tr>
<tr>
<td>HMG Treatment (therapeutic range):</td>
<td>400-800 pg/mL</td>
<td></td>
</tr>
<tr>
<td>Female Cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – 10 days:</td>
<td>61.394 pg/mL</td>
<td></td>
</tr>
<tr>
<td>11 – 20 days:</td>
<td>122-437 pg/mL</td>
<td></td>
</tr>
<tr>
<td>21 – 30 days:</td>
<td>156-350 pg/mL</td>
<td></td>
</tr>
</tbody>
</table>

XIII. PERFORMANCE CHARACTERISTICS

A. PARALLELISM (linearity of dilutions)

Two female patient samples were extracted, reconstituted with dilution buffer, further diluted and assayed. Results shown represent actual values multiplied back by the indicated dilution factor.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Neat</th>
<th>1:2</th>
<th>1:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>640</td>
<td>580</td>
<td>624</td>
</tr>
<tr>
<td>34</td>
<td>700</td>
<td>660</td>
<td>688</td>
</tr>
</tbody>
</table>

All values are in pg/mL.

B. RECOVERY OF EXOGENOUS TOTAL ESTROGENS

To demonstrate the accuracy of the method, known amounts of Estrogens were added to aliquots of Estradiol free serum. The results are shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of Estrogens Added (pg/mL)</th>
<th>Amount of Estrogens Expected (pg/mL)</th>
<th>Amount of Estrogens Recovered (pg/mL)</th>
<th>% Estrogens Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>50</td>
<td>47</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>111</td>
<td>111</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>250</td>
<td>238</td>
<td>95</td>
</tr>
</tbody>
</table>

AVERAGE = 100%
C. PATIENT SAMPLE CORRELATION

Fifteen laboratory samples covering representative male and female values were assayed with a tritiated (\(^3\)H) method requiring chromatography and the method described herein. The following data were obtained:

D. INTRA-ASSAY VARIATION (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>120</td>
<td>430</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>120</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>130</td>
<td>430</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>135</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>135</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>135</td>
<td>390</td>
<td></td>
</tr>
</tbody>
</table>

Mean 53 129 422
S.D. 2.9 7.4 43
% C.V. 5.5% 5.7% 10.2%

E. INTER-ASSAY VARIATION (n = 7)

<table>
<thead>
<tr>
<th>II (pg/mL)</th>
<th>HMG (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>1150</td>
</tr>
<tr>
<td>120</td>
<td>1250</td>
</tr>
<tr>
<td>140</td>
<td>900</td>
</tr>
<tr>
<td>110</td>
<td>1085</td>
</tr>
<tr>
<td>140</td>
<td>800</td>
</tr>
<tr>
<td>125</td>
<td>1000</td>
</tr>
</tbody>
</table>

Mean 126 998
S.D. 13.7 174
% C.V. 10.9% 17.4%

XIV. SPECIFICITY OF THE ANTISERUM

The following ligands have been checked for cross reactivity with this antiserum. The percentages indicate the cross reactivity at 50% displacement as compared to the total estrogens curve.

COMPOUND | % CROSS REACTION
----------|------------------
Estradiol-17\(\beta\) | 100.00
Estrone | 100.00
Estradiol-17\(\alpha\) | 7.0
Equilin | 2.5
Testosterone | <0.1
Dihydrotestosterone | <0.1
Cholesterol | <0.1
Pregnenolone | <0.1
Pregnenolone sulfate | <0.1
17\(\alpha\)-Hydroxyprogrenolone | <0.1
Progesterone | <0.1
17\(\alpha\)-Hydroxyprogesterone | <0.1
20\(\alpha\)-Dihydroprogesterone | <0.1
DOC | <0.1
11-Desoxycortisol | <0.1
Cortisol | <0.1
Coricosterone | <0.1
Aldosterone | <0.1
Androstenedione | <0.1
DHEA | <0.1
Androsterone | <0.1
Etocholanolone | <0.1

XV. REFERENCES