ESTRAMET™ Urinary 2MeoE Assay Kit

ENZYME IMMUNOASSAY KIT FOR URINARY ESTROGEN METABOLITES (2-METHOXYESTROGEN)

PLEASE NOTE: Upon arrival of kit, please remove standards, sample diluent, positive controls, and alkaline phosphate conjugate from kit and store at -20°C or lower until use. Store all other vials and plates at 4-8°C until use. DO NOT FREEZE PLATES

REF IA52021

For Research Use Only. Not for Use in Diagnostic Procedures.
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# ENZYME IMMUNOASSAY KIT FOR URINARY ESTROGEN METABOLITES (2-METHOXYESTROGEN)

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## I. Intended Use

The ESTRAMET™ Urinary 2-Methoxyestrogen Metabolite kit provides materials for the quantitative measurement of total urinary 2-methoxyestrogens (2MeoE) in the urine of males and females.

## II. Summary and Explanation of the Test

The ESTRAMET™ urinary 2MeoE estrogen metabolite kit determines the combined concentrations of urinary 2-methoxyestrogens (2MeoE), that is, the sum of 2-methoxyestrone + 2-methoxyestradiol + 2-methoxyestradiol, respectively. The 2-methoxyestrogens arise in vivo from conversion of 2-hydroxyestrogen metabolites to 2-methyl ether derivatives by catechol-O-methyl transferases (COMTs). Amounts of the 2MeoE, therefore, are dependent upon both the activity of COMT and levels of 2-hydroxyestrogens. The relative amounts of 2-methoxyestrogen metabolites may be significant in that these naturally occurring estrogen metabolites have been found to display anti-tumorigenic, anti-angiogenic, and cytostatic activity (1,2) as well as hypocholesterolemic effects (3). Very recent studies further suggest that 2-methoxyestrogens attenuate renal and cardiovascular injury and associated mortality by reducing vascular and interstitial cell inflammation and proliferation (4). Whereas 2-methoxyestradiol is recognized to have the greatest cytostatic activity under most circumstances (1,2), combined levels of other 2-methoxyestrogens are much greater, and their contribution to cytostatic and other activities are, therefore, significant. For example, while 2-methoxyestrone was found to have 57 per cent of the activity of 2-methoxyestradiol in inhibition of tubulin polymerization (1), levels of 2-methoxyestrone in urine are 3 to 10 times higher than the former (see “Comparison with Other Methods”, below). While many studies over the past 10 years have increased our understanding of how 2-methoxyestrogens exert their pleiotropic effects, the exact molecular mechanisms of action are not yet clear (2). It is increasingly evident, however, that the key structural element that confers the multiple beneficial properties of 2-methoxyestrogens is the 2-monomethyl ether/3-hydroxy group on the A-ring of an estrogen. Other substituents affect the bioavailability, half-life, and distribution of individual 2-methoxyestrogen metabolites. The present ESTRAMET™ 2MeoE urinary estrogen metabolite assay utilizes an extremely specific high affinity monoclonal antibody specific to this molecular structure.

## III. Principle of the Test
The present test may be described as a competitive, solid-phase enzyme immunoassays (EIA). In this assay format, the antibody is captured on the solid phase and the antigen (estrogen metabolite) is labeled with the enzyme. In the test, binding of the antigen-enzyme conjugate by the antibody is inhibited by free antigen (in the standards, or test sample). Since a restricted number of antibody binding sites are available, the enzyme activity bound to the solid phase in the presence of free antigen is lowered. When enzyme substrate is added to the washed solid phase, the enzyme product (e.g., colored dye) concentration is inversely proportional to the concentration of the free antigen. In the current assay kits, monoclonal antibody to estrogen metabolites are captured directly to the solid phase (wells of 8 x 12 polystyrene microtiter plates). The estrogen metabolite 2MeoE has been conjugated to alkaline phosphatase enzyme (AP).

The urinary forms of 2MeoE are found mainly as the 3-glucuronide and the 3- or 3,16α-glucuronides, respectively. Glucuronides require removal of the sugars before recognition by the monoclonal antibody in the EIA kit. The estrogens are deconjugated of both glucuronic acid and sulphate by use of a mixture of β-glucuronidase and arylsulphatase enzyme isolated from the snail *Helix pomatia* (mixture is called here "Deconjugating Enzymes"). In practice, an aliquot of urine is diluted 1:20 with a buffer containing the enzymes and incubated until deconjugation is complete. The enzyme digest is then neutralized and used directly in the assay.

IV. Comparison with other Methods

The ESTRAMET™ enzyme immunoassay (EIA) for 2-methoxyestrogens has been validated by comparison to urinary levels of 2-methoxyestrone (2MeoE1) as determined by Gas Chromatography–Mass Spectroscopy (GC-MS) (5). Levels of 2-methoxyestradiol and 2-methoxyestriol have been difficult to measure routinely by GC-MS, as levels are much lower than that of 2MeoE1. One recent report using HPLC-MS, however, reported levels of 2-methoxyestradiol to be only 10% to 30% of that of 2MeoE1, whereas 2MeoE3 was not determined (6). The correlation (multiple R) between levels of 2-methoxyestrogens (2MeoE1 + 2MeoE2 + 2MeoE3..) as determined by the ESTRAMET™ 2MeoE EIA versus 2MeoE1 by the cited GCMS method for 18 female urine samples was 0.90 with values of 0.4 to 42 nanomoles/liter. Furthermore, using the EIA kits, we have demonstrated 100% recovery of steroid spiked into urine samples, and recovery of steroid with sample dilution. The mean within assay variability is about 6%, the mean between assay variability is about 10%, for the experienced operator. Urinary estrogen is ordinarily normalized to urinary creatinine concentration in units of nanograms estrogen per mg creatinine.

Levels of urinary 2MeoE in the urine samples vary greatly, with greatest values in premenopausal women (about 2 to 12 ng/ml), and much lower levels in postmenopausal women and men (about 0.3 to 3 ng/ml) for 24-hour urine collections.

V. Kit Contents

CHEMICAL HAZARD: Some of the reagents in this kit contain sodium azide as a preservative. For all such solutions, the concentration of sodium azide is 0.05% or less. Sodium azide may react with metals including lead and copper plumbing to form explosive azide salts. Dispose of reagents by flushing down the drain with large volumes of water.

1. **Deconjugating Enzymes** (21 mL/vial): Enzyme from *Helix pomatia* with glucuronidase and sulphatase activities. Store at 4-8°C. Contains 0.05% azide as preservative.

2. **Neutralization Buffer** (22 mL/vial): A buffered solution to neutralize the acidic deconjugation reaction. Store at 4-8°C. Contains 0.05% azide as preservative.

3. **2MeoE1:AP Conjugate Diluent** (10 mL/vial): A buffered solution to dilute 2MeoE1:alkaline phosphatase conjugates. The Conjugate Diluent solution contains monoclonal antibody to 2MeoE (blue-colored). Store at 4-8°C. Contains 0.05% azide as preservative.

4. **AP Substrate** (22 mL/vial): A basic solution of stabilized paranitrophenyl phosphate (pNPP). Store at 4-8°C.

5. **Microtiter Plate** (96-well plate with adhesive covers): One plate coated with antibody to mouse immunoglobulin to capture specific antibody-estrogen: alkaline phosphatase enzyme complex. Store at 4-8°C. Contains 0.05% azide as preservative.
6. **2-MeoE1:Alkaline Phosphatase Conjugate** (5-10 µL in 500 µL/vial). Store at -20°C.

7. **Positive Control Urine** (0.3 mL). Store at or below -20°C.

8. **Standards** (0.3 mL): 10, 5, 2.5, 1.25, 0.625, and 0.3125 ng/ml **2-methoxyestrone** in 1mL vial. Store at or below -20°C.

9. **Sample Diluent** (1 mL): Used to dilute premenopausal urine 1:4 with before assay, or if assayed values are found to be greater than 10 ng/mL. Store at or below -20°C.

10. **Microtubes** (one box): 1.1 mL racked, 96 tubes/rack (do not substitute).

11. **Plate Sealers** (3 each): Adhesive.

**VI. Storage and Stability**

**PLEASE NOTE:** Upon arrival of kit, please remove standards, sample diluent, positive controls, and alkaline phosphatase conjugate from the kit and store at -20°C until use. Store all other components, including plates, at 4-8°C until use. (DO NOT FREEZE PLATES). Do not reuse standards after thawing. The date of kit expiration is typically 90 days after the kit lot number (month/date/yr) if components are stored properly. Do not mix and match components from different lots of kits.

**VII. Specimen Collection and Handling**

Either 24-hour or first-morning void collections are recommended. Freeze/thaw samples at least once before use in the EIA. Urine for long-term storage is best collected with addition of ascorbic acid to urine to prevent oxidation of labile metabolites. 50 mg of ascorbic acid may be added to 50 mL of urine (1 mg/mL). Addition of greater than 5 mg/mL ascorbate may interfere with determination of creatinine. However, untreated urine stored at 4°C for up to 48 hours after collection may be used for assay after freezing and thawing once. The urine container should be labeled, immediately placed at 4°C, and frozen at or below -20°C within 48 hours. Samples without ascorbate may be stored frozen at -20°C for several months without loss of metabolites. Avoid long-term storage of frozen urine samples in frost-free refrigerators and multiple freeze-thawing before assays. Remove all precipitate by centrifugation before use in the assay.

**VIII. Required Equipment and Materials**

1. The following equipment is **absolutely** required for accurate results:
   1.1 An individual pipette able to accurately deliver 10 µL
   1.2 An 8- or 12- channel pipette able to accurately deliver 75–200 µL/channel
   1.3 An automatic microtiter plate washer
   1.4 A pH meter capable of measuring to the tenth’s place
   1.5 An automated plate reader with 405 nm filter capable of accurate endpoint and/or kinetic analyses of data with automatic curve fitting by four-parameter fit or equivalent.

2. Additional materials are required to create a plate washing buffer. The **recipe for 1 Liter of the Tris Buffer Saline (TBS), pH 7.4/0.05% Tween-20 buffer** (use of pre-titrated Tris is highly recommended) used for all plate washing steps is below. Add components to a 1 L container in the order indicated.
   2.1 1 L of glass distilled or deionized water
   2.2 1.51 grams of Tris (10 mM) - use Trizma, pH 7.4, Sigma P/N T-7693 or equivalent
   2.3 8.76 grams of NaCl (150 mM )
   2.4 Check pH and adjust solution to pH 7.4 with 1 N HCl, if necessary
   2.5 0.5 grams Tween- 20 (0.05% weight:volume)

**IX. Assay Parameters**
1. The sensitivity of the 2MeoE assay is about 0.10 ng/ml (after urine dilution of 1:40 in assay).

2. The within and between assay CV’s for the metabolite assays are generally less than 6 and 10%, respectively, for the experienced operator.

3. The EIA has been shown to demonstrate 100% recovery of metabolites with "spiking" pure metabolites to urine samples, and parallelism with dilution to at least 1:4 with the provided Sample Diluent

4. Assay incubation time is 3 hours at room temperature, with 60 minutes for development of alkaline phosphatase activity (yellow color). Kinetic reading and analysis (of slopes) of the assay is more rapid and may also be done. The entire assay requires about 6 hours to perform (5,7).

5. Urine samples for premenopausal women are to be prediluted 1:4 (1 part urine to 3 parts diluent) with sample diluent before assay to avoid the need to reassay the sample. Multiply the resultant value by 4 to obtain the undiluted value.

6. Each laboratory should establish its own range of expected values. Typical values are between 0.5 and 8 ng/ml for a spot urine collection.

X. Basic Protocol for the ESTRAMET™ Kit

1. Remove the white Styrofoam box containing the kit reagents and the plates from the refrigerator. Remove all bottles and vials from the freezer to bring all solutions to room temperature (at least one hour at room temperature).

2. Remove urine samples from –20°C storage and bring to room temperature. Mix and centrifuge samples to obtain clear sample. It is important to remove all precipitate from the samples before use, as these may interfere in the assay.

3. Arrange microtubes in the microtube rack, such that the 2MeoE standards, in triplicate (columns 1, 2, 3) for each standard in rows (B, C, D, E, F and G). Keep A1, A2 and A3 wells for plate blanking (only AP Substrate is added to these wells). Arrange microtubes for positive control in wells H1-H3. Arrange triplicate tubes for each urine sample in Columns 4-12, rows A-H. This provides for 6 standards, 1 control, and 25 unknowns on each plate.

4. Aliquot 10 µL of each standard (0.3125, 0.625, 1.25, 2.5, 5, and 10 ng/mL of 2MeoE1) and urine samples into each prearranged microtube. Change pipette tips between samples. We recommend 1:4 dilution of premenopausal urine with sample diluent (1 part urine to 3 parts sample diluent).

5. Aliquot 190 µL of the Deconjugating Enzymes solution from a reservoir into each microtube, and mix by vortexing. This gives a 1:20 dilution of the standards and sample. Seal the tubes tightly with the adhesive plate sealer provided.

6. Incubate (deconjugate) the samples at room temperature (20-23°C) for 2 hours. Exact temperature control is necessary for reproducibility.

7. While the samples are incubating, prepare Tris-buffered saline (TBS), pH 7.4 with 0.05% Tween-20 for upcoming plate washing steps (refer to section VIII. #2 for recipe). This solution may be prepared up to 24 hours prior to use.

8. Neutralize the deconjugated samples after 2 hours by adding 200 µL of the Neutralization Buffer to each microtube. Vortex to mix, and reseal if not used immediately. Samples and standards are now diluted 1:40. Deconjugated samples are best used within one-half hour.

9. Wash the 2MeoE EIA plate six (6) times with Tris-buffered saline (TBS), pH 7.4/0.05% Tween-20 buffer. Wash plate allowing each wash to remain in wells at least 10-15 seconds before decanting. Allow the initial wash to remain in the wells of the microtiter plate for 5 minutes before completely decanting liquid. Cover
the washed plate immediately with the adhesive plate cover until just before use. Proceed to next step as soon as possible after washing. Good results cannot be guaranteed if plates are washed by hand.

10. Aliquot 75 µL of 1:40 diluted (neutralized) standards and samples to the corresponding wells of the washed 2MeoE plate with a multi-channel pipette, using the same set of pipette tips for the same corresponding samples on both plates. Change tips between sets of samples.

11. Prepare 2MeoE:Alkaline phosphatase enzyme conjugate solution by diluting the concentrated 2MeoE:Alkaline Phosphatase in 2MeoE:Conjugate Diluent Buffer (blue-colored) to the dilution shown on the small (0.5 mL) vial of 2MeoE:Alkaline Phosphatase. For example, if vial cites "Use at 1:2000," dilute 5 µL of 2MeoE:Alkaline Phosphatase within the 10 mL bottle of 2MeoE:Conjugate Diluent. Note: Do not blowout or eject excess sample from pipette tips - pipettes are designed to deliver microliter volumes, not contain that volume. Prepare at least 80 µL of diluted 2MeoE:Alkaline Phosphatase for each well/microtube in the final assay (e.g. about 8 mL for the whole plate). Pour into a plastic reagent reservoir.

12. Within one minute of preparing the 2MeoE:Alkaline Phosphatase conjugate solution, immediately aliquot 75 µL of the solution into each assay well of the 2MeoE assay plate already containing 75 µL of deconjugated, neutralized samples.

13. Use a plate rotator at 4-5 rps for 2 minutes to mix plate contents. Alternatively, tap the assay plate several times to gently mix the contents of each well. Cover the assay plate with the adhesive-coated plate cover supplied with the kit.

14. Incubate the assay for 3 hours at room temperature (20-23°C). Avoid setting the covered plate in areas susceptible to drafts or temperature changes.

15. Wash the plate six (6) times with TBS (pH 7.4)/0.05% Tween-20 after incubation, allowing a liquid dwell time of 10-15 seconds between washes. After the final wash, decant liquid completely by forcefully tapping the plate on paper towel several times.

16. Aliquot 100 µL of the AP Substrate (pNPP, paranitrophenyl phosphate) enzyme substrate solution to each well of the washed EIA plate with an 8-12 channel pipette as rapidly as possible. Use a plate rotator at 4-5 revolutions/sec. for 2 minutes to mix plate contents. Alternatively, tap the assay plate several times to gently mix the contents of each well.

17. Read the assay plate by kinetic or end point analysis at 405 nm. For endpoint analysis, read the plate 60 minutes after adding AP substrate. For kinetic analysis, start reading 5 minutes after adding AP Substrate and read every two minutes for at least 20 minutes. See step 16 above for comments on staggering assay plates.

XI. Standard Curve

Below is a typical standard curve for EIA of urinary 2MeoE. We highly recommend use of a four-parameter curve to fit the standards.
Figure: Standard curve for EIA of urinary 2-methoxyestrogen; nanograms/mL versus optical density at 405 nm (O.D. units) at 60 minutes after addition of enzyme substrate (para-nitrophenyl phosphate).

XII. Specificity

Table 1  Specificity and Affinity of Monoclonal Antibody 9D3 to 2-Methoxyestrogens.

<table>
<thead>
<tr>
<th>Estrogen Metabolite</th>
<th>Common Name</th>
<th>% Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,5[10]-Estratrien-3-ol-17-one 2-methyl ether</td>
<td>2-Methoxyestrone</td>
<td>100.0</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-3,17-ol 2-methyl ether</td>
<td>2-Methoxyestradiol</td>
<td>100.00</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-3,16α,17β-triol 2-methyl ether</td>
<td>2-methoxyestradiol</td>
<td>60.0</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-2,3-diol-17-one 2,3-dimethyl ether</td>
<td>2,3-Methoxyestrone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-2,3-diol-17-one</td>
<td>2-Hydroxyestrone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-3-ol-17-one</td>
<td>Estrone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-3-ol-17β-diol</td>
<td>Estradiol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>1,3,5[10]-Estratrien-3,16α-diol-17-one</td>
<td>16α-Hydroxyestrone</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Affinity (Ka, L/mol) = 1 x 10^{12}

XIII. References


LIABILITY: Complaints will be accepted in each mode – written or vocal. Preferred is that the complaint is accompanied with the test performance and results. Any modification of the test procedure or exchange or mixing of components of different lots could negatively affect the results. These cases invalidate any claim for replacement. Regardless, in the event of any claim, the manufacturer’s liability is not to exceed the value of the test kit. Any damage caused to the kit during transportation is not subject to the liability of the manufacturer.