

Chapter 2

Determination of Serum Estradiol Levels by Radiometric and Chemiluminescent Techniques

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Abstract

The ability to precisely measure circulating levels of hormones is a foundation of modern endocrinology. For assisted reproductive technologies such as in vitro fertilization (IVF), accurate determination of circulating levels of estradiol are crucial for patient management, retrieval of fertilizable oocytes, and successful pregnancy outcome. For many years, circulating levels of estradiol were determined by radioimmunoassay. More recently, nonradioactive techniques such as ELISAs or chemiluminescent approaches have replaced traditional radioimmunoassays. In the current chapter, we outline the procedures for analysis of circulating levels of estradiol by both radioimmunoassay and chemiluminescent techniques.

Key words: Radioimmunoassay, chemiluminescence, estradiol, Immulite, hormone detection.

1. Introduction

A major breakthrough in the field of endocrinology was the development of sensitive, specific, quantitative assays to determine the concentrations of hormones. In 1977, Dr. Rosalyn Yalow received the Nobel Prize in Medicine for the development of a radioimmunoassay (RIA) for insulin. The RIA developed by Drs. Yalow and Berson replaced bioassays and was the first technique that allowed for the precise measurement of previously undetectable levels of hormones (1). RIA is based upon a competitive binding reaction where a known amount of radiolabeled hormone “competes” with an unknown quantity of the hormone found in serum (or other biological fluid or tissue) for an antibody against the hormone. As the concentration of the hormone in the sample increases, more of the hormone binds to

the antibody and displaces the radiolabeled hormone. Thus as the hormone concentration increases, the ratio of the antibody-bound radiolabeled hormone to free radiolabeled hormone decreases. The antibody bound hormones are separated from the unbound hormones and the radioactivity measured. The amount of hormone present in the serum can be determined by plotting the outcomes on a binding curve.

Since the early discovery of RIA, kits have become commercially available to measure a vast array of hormones. In our laboratory, we use the Siemens Coat-A-Count kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA) to measure estradiol. The estradiol Coat-A-Count kits are a solid phase RIA based on a specific antibody to estradiol immobilized to the wall of a polypropylene tube. ^{125}I -labeled estradiol competes for a fixed time with estradiol in the patient sample for antibody binding sites on the wall of the tube. After incubation, separation of bound estradiol from free hormone is achieved by decanting. The tube is then counted in a gamma counter, the counts per minute (CPM) being inversely related to the amount of estradiol present in the patient sample. In other words, the higher the concentration of estradiol in the serum, the more radiolabeled estradiol is displaced from the antibody binding sites resulting in lower counts per minute. The quantity of estradiol in the sample is determined either through a computer program or by comparing the counts to a calibration curve.

Although the RIA technique is extremely sensitive and specific, nonradioactive techniques have gained increasing popularity in recent years primarily due to the problems associated with storage and disposal of radioisotopes. Our laboratory has replaced the radiometric estradiol assay with a chemiluminescent procedure. The Siemens IMMULITE 1000 Estradiol procedure is based upon the same principles as the RIA but instead uses a solid-phase, competitive binding chemiluminescent enzyme immunoassay. Instead of radiolabeled hormone, alkaline phosphate conjugated estradiol is employed. The detection of alkaline phosphate conjugated estradiol is accomplished by the ability of alkaline phosphate to hydrolyze a phosphate ester of a chemiluminescent substrate (adamantyl dioxetane). The cleavage of the chemiluminescent substrate is detected spectrophotometrically by a luminometer (2). The photon counts can be used in the same manner as the radiometric counts to determine the concentration of estradiol in the sample. In this chapter, we detail the methodology to determine the serum concentrations of estradiol by both RIA and chemiluminescence. This procedure is the cornerstone to assess a woman's responsiveness to exogenous gonadotropin stimulation during assisted reproductive technology procedures.

2. Materials

2.1. Specimen (Serum) Collection and Storage

1. Evacuated Serum Collection Tubes (BD Vacutainer Systems, Preanalytical Solutions, Franklin Lakes, NJ).
2. Holder/Adapter—use with the evacuated collection system. (BD Vacutainer Systems).
3. Tourniquet.
4. Alcohol Wipes—70% isopropyl alcohol.
5. Gauze sponges—for application on the site from which the needle is withdrawn.
6. Adhesive bandages/tape—protects the venipuncture site after collection.
7. Sharps disposal unit—BD Vacutainer Systems (*see Note 1*).
8. Gloves.

2.2. Radioimmunoassay

1. Reagents for radiometric measurement of estradiol are obtained in kit form from Siemens Medical Solutions Diagnostics, Los Angeles, CA. All reagents should be stored at 2–8°C and are stable for at least 30 days after opening. A single 100 tube kit (TKE21) (*see Note 2*) contains the following:
 - a. Estradiol Antibody Coated Tubes: 100 polypropylene tubes coated with rabbit antibodies to estradiol and packaged in zip-lock bags.
 - b. (125I) Estradiol: Iodinated synthetic estradiol in liquid form, ready to use. Each vial contains 105 ml.
 - c. Zero standard (A Calibrator), 1 vial, 5 ml.
 - d. Estradiol Calibrators B through G: 6 vials, 2 mls each. The calibrators contain respectively 20, 50, 150, 500, 1800, and 3600 picograms of estradiol per milliliter (pg/ml); equivalently: 0.07, 0.18, 0.55, 1.84, 6.61, and 13.2 nanomoles per liter (nmol/L).
2. Quality control specimens: CON6 Multivalent Control Module (Siemens, Catalog Number CON6) is an assayed, human serum based, tri-level control. Each set of CON6 controls consists of lyophilized material which is reconstituted by adding exactly 6.0 ml of distilled or deionized water. Let it stand for 30 minutes, then mix by inversion until completely dissolved. The controls are stable for 7 days at 2–8°C or for 2 months frozen at –20°C. Aliquot if necessary in tightly capped plastic containers to avoid repeated freeze-thaw cycles and evaporation.
3. Disposable polypropylene tubes, 12 × 75 mm.

4. Pipettor for 100–1000 μ l.
5. Disposable 100 and 1000 μ l tips.
6. Vortex mixer.
7. Centrifuge.
8. Distilled or deionized water.

2.3.
Radioimmunoassay
Analysis and
Quantification of
Estradiol

1. Gamma counter (Beckman, Inc).

2.4.
Chemiluminescence

1. Reagents are obtained in kit form (Catalog Number LKE21) from Siemens Medical Solutions Diagnostics.
2. All reagents, test units, and wedges should be stored at 2–8°C and are stable until the expiration dates stated on the packaging.
3. There are three components in each kit (the test units, reagent wedge, and adjustors) which represent a matched set. Bar-codes encode information about the test, including expiration dates, component lot numbers, adjustment and master curve parameters.
4. Estradiol Test Units: 25 test units per re-sealable pack. Each bar-coded-labeled test unit contains one bead coated with polyclonal anti-estradiol antibody. The test unit packs are packaged in re-sealable bags with desiccant and are stable until the expiration date on the label when stored refrigerated at 2–8°C.
5. Estradiol Reagent Wedge: One barcode reagent wedge, containing 7.5 ml bovine calf intestine alkaline phosphatase conjugated to estradiol in human protein based matrix. Store refrigerated at 2–8°C to maintain stability until the expiration date on the wedge label (*see Note 3*).
6. Estradiol Adjustors: one set of two vials. 2 ml each, designated low and high concentrations of estradiol in processed human serum. Store refrigerated at 2–8°C for 30 days after opening. Stable at –20°C for 6 months after reconstitution.
7. Quality control specimens: CON6 Multivalent Control Module (Siemens, Catalog Number CON6) is an assayed, human serum based, tri-level control. Each set of CON6 controls consists of lyophilized material which is reconstituted by adding exactly 6.0 ml of distilled or deionized water. Let it stand for 30 minutes, then mix by inversion until completely dissolved. The controls are stable for 7 days at 2–8°C or for

2 months frozen at -20°C . Aliquot if necessary in tightly capped plastic containers to avoid repeated freeze-thaw cycles and evaporation.

8. Chemiluminescent Substrate (Catalog Number LSUBX): Each box of Chemiluminescent Substrate includes two bottles of Chemiluminescent Substrate. Prior to use, the Chemiluminescent Substrate is stored refrigerated at $2-8^{\circ}\text{C}$. The substrate can remain on the instrument for up to 30 days.
9. Probe Wash: Each box of Probe Wash (Siemens Catalog Number LPWS2) contains two bottles of Probe Wash Concentrate that should be stored at room temperature. Before using, each 100 ml bottle must be diluted with 900 ml of distilled water (1:10 dilution).
10. Immulite 1000 sample cups (Catalog Number LSCP).
11. Immulite cups holder set (Catalog Number LCH).
12. Transfer pipets (Sarstedt, Newton, NC).
13. Centrifuge.
14. Distilled or deionized water.

3. Methods

3.1. Specimen (Serum) Collection and Storage

1. Label vacutainer collection tube with patient identification information.
2. Don gloves and prepare the collection site (*see Note 4*) by swabbing the area with alcohol wipes.
3. Place a tourniquet above the collection area tight enough to make the vein bulge. Insert the needle into the vein at a 15 degree angle.
4. Push the vacutainer (blood specimen collection tube) into the holder, keeping the needle steady. The vacutainer will automatically begin filling. Draw the correct volume of blood allowing the vacuum in the tube to be exhausted (*see Note 5*).
5. When the collection is finished, remove the needle from the collection site by pulling it out at the same angle at which it was inserted.
6. Remove the tourniquet.
7. Apply a dry gauze to the site and apply pressure. Immediately dispose of the collection needle by placing it in a sharps container. Do NOT recap the needle.
8. Gently invert the tube 5 times.

9. After 2 or 3 minutes, remove the gauze and cover the collection site with an adhesive bandage.
10. Allow the specimen to coagulate by sitting at room temperature (22–26°C) 20–30 minutes.
11. Centrifuge at 1300–1800 *g* for 10 minutes to separate the serum from the cells.
12. The samples may be stored at 2–8°C for 7 days, or up to 2 months frozen at –20°C (*see* **Note 6**).

3.2.

Radioimmunoassay

1. Allow all reagents, specimens and CON6 controls to come to room temperature (22–26°C) before using (*see* **Note 7**).
2. Label two plain (uncoated) 12 × 75 mm polypropylene tubes as T (total counts) and two plain (uncoated) 12 × 75 mm polypropylene tubes as NSB (nonspecific binding) (*see* **Note 8**).
3. Label Estradiol Ab-Coated Tubes as A (0 pg/ml, maximum binding) and B through G (20, 50, 150, 500, 1800, and 3600 pg/ml) in duplicate (total of 14 tubes). Label additional antibody-coated tubes, also in duplicate for controls and patient samples controls.
4. Pipet 100 µl of the zero calibrator A into the NSB and A tubes (0 pg/ml) and 100 µl of each of the calibrators B through G into correspondingly labeled tubes. Pipet 100 µl of each control and patient sample into the tubes prepared (*see* **Notes 9–11**).
5. Add 1.0 ml of (125I) estradiol to every tube and vortex all tubes.
6. Cap the total count tubes to avoid accidental decantation at the end of incubation.
7. Cover and incubate at room temperature for 3 hours.
8. Decant thoroughly all tubes except the total count tubes (*see* **Note 12**).

3.3.

Radioimmunoassay Analysis and Quantification of Estradiol

1. Count each tube for 1 minute in a gamma counter. Results will be expressed as counts per minute (CPM).
2. For each pair of tubes calculate the average NSB corrected counts per minute. This is accomplished by subtracting the NSB from each tube as follows: Average NSB corrected CPM (or Net Counts) = CPM – Average NSB CPM. Determine the binding of each pair of tubes as a percentage of maximum binding (MB), with the NSB-corrected counts of the A tubes (0 pg/ml) taken as 100%: Percent Bound = (Net Counts/Net MB Counts) × 100. Note: The calculation can be simplified by omitting the correction for nonspecific binding; samples within range of the calibrators yield virtually the same results when Percent Bound is calculated from the Average CPM.

3. Most gamma counters will have a program to determine binding kinetics. However if necessary use logit-log graph paper, plot Percent Bound on the vertical (probability or ordinate) axis against Concentration on the horizontal (logarithmic or abscissa) axis for each of the nonzero calibrators, and draw a line approximating the path of these points (**Fig. 2.1**). Results for the unknowns may then be calculated from the line by interpolation (3) (*see Note 13*).

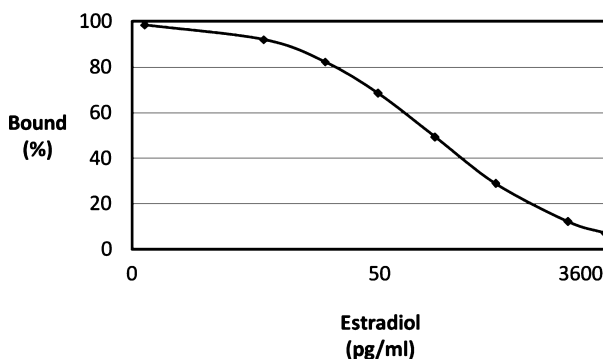


Fig. 2.1. Logit-Log standard curve for calculating estradiol concentration. The cpm for the estradiol standards are converted to percent bound which is plotted on the standard curve to generate the binding curve. The amount of estradiol in the patient sample is calculated from the binding curve.

3.4. Chemiluminescence

The Siemens IMMULITE 1000 Estradiol procedure is a solid-phase, competitive binding chemiluminescent enzyme immunoassay. The solid phase test unit contains a polystyrene bead coated with polyclonal rabbit anti-estradiol. The patient sample along with a known amount of estradiol conjugated to alkaline phosphatase are simultaneously introduced into the reaction tube containing the bead and incubated for 60 minutes at 37°C with intermittent agitation. During the incubation, estradiol in the sample competes with the alkaline phosphatase estradiol conjugate for a limited number of antibody binding sites on the bead. Unbound enzyme labeled conjugate and unbound sample are then removed by a centrifugal wash, after which substrate is added and the reaction tube is incubated for 5 minutes. The chemiluminescent substrate undergoes hydrolysis in the presence of alkaline phosphatase yielding the sustained emission of light. After the 5 minute incubation at 37°C, the light signal reaches a maximum and the photon counts are measured by the photomultiplier tube (PTM). Counts per second (cps) are converted to analyte concentrations (doses) using stored master curves. The IMMULITE 1000 calculates test results for controls and patient samples from the observed signal (counts per second), using a stored master curve (2) (*see Note 14*). The bound complex and thus also the photon counts, as measured by the luminometer, is inversely proportional to the concentration of estradiol in the sample (4).

1. Place the bags of estradiol test units UNOPENED at room temperature on the bench for 30 minutes. Do not open immediately after removing from the refrigerator as condensation will form on the beads.
2. Place the three CON6 controls on the bench and allow them to thaw while doing other set-up.
3. Place the Estradiol Reagent Wedge on the Reagent Carousel. Load the wedge into the Reagent Carousel by inserting the tab at the bottom of the wedge into the slot on the outer rim of the Reagent Tray. Press down firmly until it snaps into place.
4. Open the cap of the reagent wedge and remove bubbles in the reagent wedge. Do this by poking the liquid in the wedge with a pipette tip. Bubbles may cause false level sensing and a short sample or reagent draw.
5. Place the Reagent Carousel into the Reagent Chamber by positioning the Reagent Carousel so the line on the tray handle is aligned with the line on the carousel spindle. (The Reagent Carousel can only be seated on the carousel spindle if the two lines are properly aligned.) Close the lid covering the Reagent Carousel (**Fig. 2.2**).
6. Check the volume of water and probe wash on the LED level indicator on the left side instrument. Add water (labeled on the lid with a blue dot) if needed, (*see Note 15*). Add probe wash (yellow dot on lid) if needed.
7. Check the volume in the chemiluminescent substrate bottle (brown bottle on top of the Immulite). If it is empty, replace it with a new bottle. To replace, remove the old bottle by pulling it straight up off the spike. Remove the red cap that reads “flip off” from the new bottle and push the inverted bottle onto the spike. Push the gray prime button (just below the Chemiluminescent substrate bottle but above the fill level line) module 2 times (*see Note 16*).
8. Turn the Immulite monitor on and double click the Immulite 1000 icon. Press the “Run Immulite” button.
9. Enter your name or initials in the User ID box and press enter. The instrument will download programs. (This takes a few minutes.) The action takes you to the “Home” screen.
10. Clicking “Worklist” on the top tool bar will advance to the Worklist Entry Screen. At this screen, turn on “Auto Increment” by clicking it in the “Entry Options” panel on the left near the top of the screen. In the center of the screen, type the number “1” in the Sample Cup Box and press “Enter.”
11. Click “Control” on the left panel. Tab to “Control Name.” Click the down arrow to open the pull down box and select CON6. Press enter.

12. At the “Control Lot#” box, select the lot number on the bottom of the list and press enter. At the “Control Level” box, select “4.” Then press “Accept.”
13. Because the Auto Increment was turned on in step 10, the sample cup number will now read “2.” Repeat steps 11–12 to enter the Controls 5 and 6 respectively.
14. Sample cup holders 1, 2, and 3 should remain in the sample cup holder rack. Place sample cups in the sample cup holders.
15. Vortex, then pipette CON6 controls 4, 5, and 6 into sample cup holders 1, 2, and 3 respectively using sample transfer pipets.
16. Sample Cup # now reads “4.” In the panel on the left, click “Patient” then tab to “Accession Number.” This is a required field on the Immulite 1000. You may either enter the patient accession number (i.e. hospital identifier number) or create your own identifier for your samples. Click the “Accept” at the bottom of the screen. This will advance the next screen.
17. Transfer a patient sample into a plastic sample cup and place the cup into Sample Cup Holder #4. The minimum volume required for estradiol is 125 μl . 100 μl is necessary as “dead volume” while 25 μl is the actual amount pipetted by the Immulite during operation (*see Note 17*). Ensure there are no bubbles on the surface of the sample or reagents. Bubbles may cause false level-sensing and a short sample or reagent draw.
18. Repeat steps 16–17 for each sample being assayed.
19. Press the green “GO” button on the Immulite screen. This message will appear: *DPC IMMULITE INITIALIZING. Please wait.* Next, the following prompt appears: *Remove all tubes from the load chain. Press GO when completed.* Do this.
20. Lift the cover on the Immulite. Remove any Test Units or sample cups between the first green arrow on the Load Platform and the Main Carousel.
21. After a brief initialization, the prompt will read: *Load water and substrate containers. Empty waste container and press GO.* Ignore this as you have already done this. Simply press GO.
22. The message will read: *IMMULITE is priming the syringes. Please wait.* The Immulite will prime the syringes once. As the Immulite primes the Hamilton Syringe Pump, watch for air bubbles in the lines and large air spaces in the syringes (**Fig. 2.2**). These should go away as the instrument primes the syringes. If there are large air spaces or bubbles when priming is finished, manually prime the pump again. Do this by pushing the “Prime” button on the pump. Repeat this until major bubbles and spaces are gone.

23. This message will now appear: *Prime the syringes, substrate and water. Press GO when done.* To do this, unscrew the long silver thumbscrew which is located below and to the left of the substrate bottle on the Substrate Heater. Gently lift the Substrate Heater and check for white precipitate at the end of the Substrate Heater Nozzle. If necessary, wipe the nozzle with a moist lint-free tissue with distilled water. Prime the Substrate Pump by pushing the black button on the substrate pump (**Fig. 2.2**). (Catch the waste substrate in a suitable container and discard it.) Push the prime button AT LEAST four times.
24. Lift the thin tubing next to the Substrate Heater pump. Push the black button on the Water Pump AT LEAST four times (**Fig. 2.2**). (Catch the waste water in a suitable container and discard it.)
25. Put the gray plastic connector for the thin tubing back into hole. Place the Substrate Heater pump back into place and tighten the thumbscrew. Press GO.
26. The Immulite will display the following prompt: *IMMULITE is priming the syringes. Please wait.* The large syringe will prime and the following message will appear: *Load reagents, samples and test units. Press GO to read reagents.*
27. The reagents in the reagent carousel, water, chemiluminescent substrate and probe wash were placed on board in earlier steps so it will not be necessary to load the reagents at this point. Only samples and test units must be loaded on the load platform (**Fig. 2.2**).
28. To print a copy of the worklist, click the “Worklist” box in the bottom left corner of the screen. To print, click the “Print Worklist” box at the bottom. To exit this screen, click “Close.”

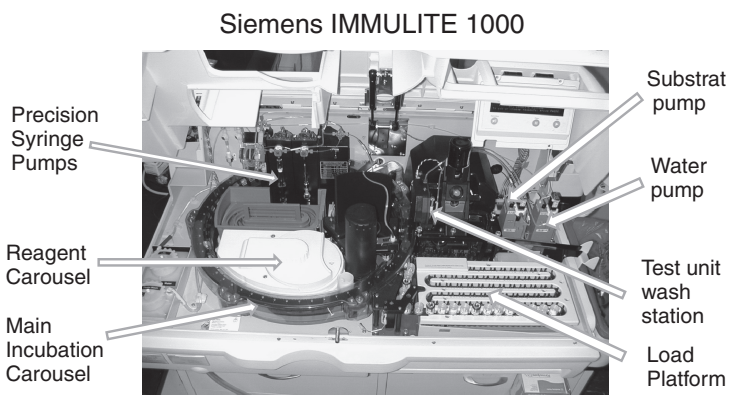


Fig. 2.2. Photograph illustrating the key components of the Siemens IMMULITE 1000 used for chemiluminescent detection of estradiol.

4. Notes

1. Needles should be placed in a proper disposal unit IMMEDIATELY after their use.
2. Estradiol kits are available in different sizes as follows: 100 tubes (catalog number TKE21), 200 tubes (TKE22), 500 tubes (TKE 25), and 1000 tubes (TKE2X).
3. When starting a new wedge, remove the foil seal and any gummy residue. Then check the surface of the liquid for bubbles. Break any bubbles with a disposable pipette tip.
4. The most common location for collecting blood is the median cubital vein which runs down the inner part of the forearm. This is an optimum collection site because the vein is close to the skin surface and there are not many nerves surrounding it.
5. If using a syringe and needle system manually pull back on the syringe plunger to fill the syringe with blood.
6. Aliquot, if necessary, to avoid repeated thawing and freezing of serum.
7. Before assay, allow the samples to come to room temperature and mix by gently swirling or inversion.
8. Because nonspecific binding in the Coat-A-Count procedure is characteristically low, the NSB tubes may be safely omitted without compromising accuracy or quality control.
9. Pipet directly into the bottom of the tubes.
10. Use a disposable tip micropipette and change the tip between samples to avoid carryover contamination.
11. Patient samples expected to contain concentrations of estradiol greater than the highest calibrator (3600 pg/ml) should be diluted in the zero calibrator before assay.
12. To greatly enhance precision it is important to remove all visible moisture from each tube.
13. Analysis may also be performed by implementation of the 4 parameter logistic.
14. Each new kit must be adjusted with the adjustors supplied in the kit before being used to process patient samples in order to ensure the applicability or the lot-specific stored master curve. Thereafter, while the same lot remains in use, the adjustment procedure for the IMMULITE 1000 estradiol assay should be repeated every 2 weeks. Criteria for calibration curve acceptance (slope and intercept) after adjustment have been established are lot specific. Each curve is electronically evaluated by the IMMULITE 1000 microprocessor.

Unacceptable adjustments are flagged. After adjustment, all controls must be within 2 SD range of the monthly calculated mean for acceptability of calibration curve adjustments. Siemens CON6 Multivalent Control Module is used as quality control material. Two of the three controls must be within the 2 SD range of the monthly calculated mean for acceptability of patient results. The upper limit of linearity for the estradiol assay is 2000 pg/dL. The linearity of the assay varies from 91 to 113% (see package insert for dilutions).

15. Distilled water of consistent quality is required. Water should meet NCCLS Type 1 reagent water standards at the time of preparation.
16. An alarm will sound when the bottle of chemiluminescent substrate is low. There will be enough chemiluminescent substrate in the Immulite to run 20–25 test units once the alarm sounds. The Immulite WILL run with the chemiluminescent substrate bottle removed.
17. While the minimum sample volume required for estradiol assay by the Immulite 1000 is 125 μ l, we recommended using at least 150 μ l. If the Immulite 1000 system senses insufficient volume, an error message appears and the Test Unit is shown as a white circle on the Home screen.

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