



KAMIYA BIOMEDICAL COMPANY

Dog Estradiol (E2) ELISA

**For the quantitative determination of dog E2 in
serum, plasma and tissue homogenates**

Cat. No. KU-092

For Research Use Only.

Product Information
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INTENDED USE

The Dog Estradiol (E2) ELISA is for the quantitative determination of dog E2 in serum, plasma and tissue homogenates. For research use only.

PRINCIPLE

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. Calibrators or samples are added to the appropriate microtiter plate wells with an antibody specific for E2 and Horseradish Peroxidase (HRP) conjugated E2. The competitive inhibition reaction is launched between with HRP labeled E2 and unlabeled E2 with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of E2 in the sample. The color development is stopped and the intensity of the color is measured.

PERFORMANCE**Detection Range**

40 pg/mL - 1,400 pg/mL.

Sensitivity

The minimum detectable dose of dog E2 is typically less than 25 pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest dog E2 concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero calibrator added by their three standard deviations.

Specificity

This assay has high sensitivity and excellent specificity for detection of dog E2.

No significant cross-reactivity or interference between dog E2 and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between dog E2 and all the analogues, therefore, cross reaction may still exist.

Precision**Intra-assay Precision (Precision within an assay): CV% <15%**

Three samples of known concentration were tested twenty times on one plate to assess.

Inter-assay Precision (Precision between assays): CV% <15%

Three samples of known concentration were tested in twenty assays to assess.

Limitations of the Procedure

- **For research use only.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate higher values than the highest calibrator, dilute the samples and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the immunoassay, the possibility of interference cannot be excluded.

COMPONENTS

Reagents	Quantity
Assay plate	1 (96 wells)
Calibrator	6 x 0.5 mL
HRP-conjugate	1 x 6 mL
Antibody	1 x 6 mL
Substrate A	1 x 7 mL
Substrate B	1 x 7 mL
Wash Buffer (20x concentrate)	1 x 15 mL
Stop Solution	1 x 7 mL
Plate sealer for 96 wells	4

Calibrator Concentration

Calibrator	0	1	2	3	4	5
Concentration (pg/mL)	0	40	120	350	700	1,400

STORAGE

Unopened kit: Store at 4 °C. Do not use the kit beyond the expiration date.

Opened kit: May be stored up to 1 month at 4 °C.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 600 nm - 630 nm.
2. An incubator which can provide stable incubation conditions up to 37 °C ± 0.5 °C.
3. Squirrt bottle, manifold dispenser, or automated microplate washer.
4. Absorbent paper for blotting the microtiter plate.
5. 100 mL and 500 mL graduated cylinders.
6. De-ionized or distilled water.
7. Pipettes and pipette tips.
8. Test tubes for dilution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

Serum

Use a serum separator tube (SST) and allow samples to clot for two hours at room temperature or overnight at 4 °C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or store samples in aliquot at -20 °C or -80 °C for later use. Avoid repeated freeze/thaw cycles.

Plasma

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1,000 x g at 4 °C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20 °C or -80 °C. Avoid repeated freeze/thaw cycles.

Tissue Homogenates

100 mg tissue was rinsed with 1X PBS, homogenized in 1 mL of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5,000 x g, 4°C. The supernate was removed and assayed immediately. Alternatively, aliquot and store samples at -20°C or -80°C. Centrifuge the sample again after thawing before the assay. Avoid repeated freeze-thaw cycles.

Note:

1. Kamiya Biomedical Company is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤ 1 month) or -80°C (≤ 2 months) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
5. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
6. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
7. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.
8. Please predict the concentration before assaying. If values for these are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.
9. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

REAGENT PREPARATION

Note:

1. **Kindly use graduated containers to prepare reagent.**
2. Making serial dilution in the wells directly is not permitted.
3. Bring all kit components to room temperature (18-25°C) before use for 30 minutes.
4. Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

Wash Buffer (1x)

If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 15 mL of Wash Buffer Concentrate (20x) into deionized or distilled water to prepare 300 mL of Wash Buffer (1x).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and calibrators be assayed in duplicate.

1. Prepare all reagents and samples as directed in the previous sections.
2. Determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Set a **Blank** well without any solution
4. Add 50 μ L of **Calibrator** and **Sample** per well. Calibrator needs to be tested in duplicate.
5. Add 50 μ L of **HRP-conjugate** to each well (not to Blank well), then 50 μ L **Antibody** to each well. Mix well and then incubate for one hour at 37°C.
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with **Wash Buffer** (200 μ L) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 10 seconds, complete removal of liquid at each step is

essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

7. Add 50 μ L of **Substrate A** and 50 μ L of **Substrate B** to each well, mix well. Incubate for 15 minutes at 37°C. Keep the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50 μ L of **Stop Solution** to each well, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm.

Note:

1. The final experimental results will be closely related to operation skills of the end users and the experimental environments.
2. Sample or reagents addition: Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
5. Controlling of reaction time: Observe the change of color after adding Substrates (e.g. observation once every 10 minutes). Substrates should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. Substrates are easily contaminated. Substrates should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the Substrates. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrates.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples as instructed;
2. Set a Blank well without any solution;
3. Add 50 μ L calibrator or sample to each well;
4. Add 50 μ L HRP-conjugate to each well (not to Blank well);
5. Add 50 μ L Antibody to each well;
6. Incubate 60 minutes at 37°C;
7. Aspirate and wash 3 times;
8. Add 50 μ L Substrate A and 50 μ L Substrate B to each well.
9. Incubate 15 minutes at 37°C. **Protect from light;**
10. Add 50 μ L Stop Solution to each well. Read at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Using professional graphing software to make a calibration curve is recommended.

Average the duplicate readings for each calibrator and sample and subtract the average optical density of Blank.

Create a calibration curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a calibration curve by plotting the mean absorbance for each calibrator on the x-axis against the concentration on the y-axis and draw a best fit

curve through the points on the graph. The data may be linearized by plotting the log of the E2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

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