Equine Luteinizing Hormone (LH)

ELISA Quantitation Kit

Catalog number: 40-289-23551

Enzyme Immunoassay for the Quantitative Determination of Luteinizing Hormone in Horse Serum

FOR RESEARCH USE ONLY

Store at 2 to 8°C.

INTENDED USE

For the quantitative determination of the Luteinizing Hormone concentration in horse serum.

INTRODUCTION

The estrous cycle of the mare is characterized by an extended and highly variable pre-ovulatory period, and breeding attempts during early estrus are not associated with high pregnancy rates. Monitoring the mare’s uterus and ovaries with trans-rectal ultrasoundography and palpation provides useful information on characteristics that are associated impending ovulation, but ultra-sonography alone is not a reliable predictor of ovulation (1). The pre-ovulatory portion of the equine estrous cycle is characterized by a prolonged LH surge that extends through late estrous and ovulation, and peaks one day after detectable ovulation (2). Because the pre-ovulatory LH surge spans the optimum time periods for fertile insemination, monitoring of LH concentrations during estrus might be a useful adjunct to ultra-sonography and rectal palpation for prediction of ovulation in natural estrous cycles (3).

LH is a glycoprotein hormone composed of an alpha and beta subunit. The alpha subunit is common to all glycoprotein hormones (TSH, FSH, and eCG) and the beta subunit confers specificity. This property lends itself to the development of highly specific matched pair antibodies for equine LH.

PRINCIPLE OF THE TEST

The Luteinizing Hormone (LH) Elisa kit is a monoclonal – monoclonal sandwich ELISA. A mouse monoclonal antibody to Equine LH Beta Subunit is pre-coated to plastic micro well strips. During incubation, LH from calibrators and samples bind to the precoated antibody. The wells are washed to remove unbound components and a biotin conjugated mouse monoclonal antibody to LH Alpha Subunit is added to complete the antibody sandwich. The wells are washed to remove any unbound detection antibody and incubated with streptavidin - HRP. The wells are washed again to remove excess streptavidin -HRP and incubated with tetramethylbenzidine (TMB) substrate resulting in a blue colored solution. A sulfuric acid solution is then added to the wells to stop reaction and turning the solution yellow. The intensity of the yellow is directly proportional to the concentration of LH in sample. LH levels are quantified by measuring the absorbances at 450 nm and comparing the absorbance vs. concentration generated from the standard curve.

SPECIMEN COLLECTION AND PREPARATION

Serum should be prepared from a whole blood specimen obtained by acceptable medical techniques. This kit is for use with serum samples without additives only.
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Reagents

Materials provided with the kit:
- ANTI-EQUINE LUTEINIZING HORMONE ELISA MICRO WELL STRIPS (EP23551)
  96 wells, 12 strips
- LUTEINIZING HORMONE LH CALIBRATOR (CR23551)
  (1μg/mL), 25 μL
- WASH SOLUTION CONCENTRATE (WS0001)
  (20X), 25mL
- DILUENT BUFFER CONCENTRATE (DB0001)
  (5X), 25mL
- DETECTION ANTIBODY – BIOTIN (DA23551)
  1mL
- STREPTAVIDIN–HRP (SA0001)
  1mL
- TMB SUBSTRATE SOLUTION (TMBAB and TMBB)
  6mL & 6mL
- STOP SOLUTION (ST0001)
  12mL

WARNING: Avoid contact with skin

Materials required but not provided:
- PRECISION PIPETTES AND TIPS: 20 ML, 100 ML, 200 ML., AND 1 ML.
- DISTILLED WATER.
- DISPOSABLE PIPETTE TIPS.
- VORTEX MIXER.
- ABSORBENT PAPER OR PAPER TOWEL.
- A MICROTITER PLATE READER AT 450 NM WAVELENGTH, WITH A BANDWIDTH OF 10 NM OR LESS AND AN OPTICAL DENSITY RANGE OF 0-2 OD OR GREATER.
- GRAPH PAPER.

Reagent Preparation

1. All reagents should be allowed to reach room temperature (18- 25°C) before use.
2. To prepare Wash Buffer (1X): Add 25 ml of Wash Buffer (20X) to 475 ml of DI water. The diluted Wash Buffer is stable at 2- 8°C for 30 days. Mix well before use.
3. To prepare Diluent Buffer (1X): Add 25 ml of Diluent Buffer (5X) to 100 ml of DI water. The diluted Wash Buffer is stable at 2- 8°C for 7 days. Mix well before use.
4. To prepare Detection Antibody – Biotin remove 1mL of Detection Antibody – Biotin and add 10 ml of Diluent Buffer 1X.
5. To prepare Streptavidin-HRP remove 1mL of Detection Antibody – Biotin and add 10 ml of Diluent Buffer 1X.
6. To prepare LH calibrator follow table below. The LH calibrator is supplied at a concentration of 1 μg/ml. Standards need to be prepared immediately prior to use. Mix well and avoid bubbles. The calibrator should be aliquoted and stored frozen avoiding multiple freeze-thaw cycles.

<table>
<thead>
<tr>
<th>Standard</th>
<th>ng/mL</th>
<th>Amount</th>
<th>Volume of 1x Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10 μL of Calibrator</td>
<td>990 μL</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>500 μL standard 1</td>
<td>500 μL</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>500 μL standard 2</td>
<td>500 μL</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>500 μL standard 3</td>
<td>500 μL</td>
</tr>
<tr>
<td>5</td>
<td>0.625</td>
<td>500 μL standard 4</td>
<td>500 μL</td>
</tr>
<tr>
<td>6</td>
<td>0.313</td>
<td>500 μL standard 5</td>
<td>500 μL</td>
</tr>
<tr>
<td>7</td>
<td>0.156</td>
<td>500 μL standard 6</td>
<td>500 μL</td>
</tr>
</tbody>
</table>
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**ASSAY PROCEDURE**

**Step-by-Step Method (Perform all steps at room temperature)**

1. Standards, Controls, and Samples  
   A. Dilute the standards (CR23551) in 1X Diluent buffer (DB0001) according to the table on page 2. Adding 975 ul of 1X diluent buffer into the provided aliquot of standard and performing serial dilutions at a 1:2 (one part sample, one part diluent) ratio to achieve the proper concentrations:  
   B. Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards. 1:15 – 1:30 dilution is recommended  
   C. Transfer 100 μl of standard, control or sample to assigned wells (EP23551). The standards and each sample should be measured in duplicate or triplicate as space permits.  
   D. Incubate plate for 1 hour at room temperature.  
   E. After incubation, decant samples and standards. Fill each well with appropriately diluted Wash Solution (WS0001) and aspirate. Repeat 4-5 times.

2. Detection Antibody  
   A. Transfer 100 μl of diluted Detection Antibody (DA23551) to each well.  
   B. Incubate 1 hour at room temperature.  
   C. After incubation, remove detection antibody solution and wash each well 5 times as in Step 1.E.

3. Streptavidin-HRP  
   A. Transfer 100 μl of diluted Streptavidin-HRP (SA0001) substrate solution to each well.  
   B. Incubate for 1 hour at room temperature  
   C. After incubation, remove detection antibody solution and wash each well 5 times as in Step 1.E.

4. Enzyme Substrate Reaction  
   A. Prepare the Substrate solution by mixing equal parts, Solution A and Solution B. (TMBA and TMBB)  
   B. Transfer 100 μl of mixed TMB substrate solution to each well.  
   C. Incubate for 8 to10 minutes  
   D. Stop the reaction, by adding 100ul of the Stop Solution (ST0001) to each well.

5. Plate Reading  
   A. Using a microplate reader, read the plate at the wavelength that is appropriate for the substrate used (450 nm for TMB).

**CALCULATION OF RESULTS**

1. Average the duplicate or triplicate readings from each standard, control, and sample.  
2. Subtract the zero reading from each averaged value above.  
3. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. Other curve fits may also be used.  
4. A standard curve should be generated for each set of samples (see examples below)  
5. Determine the concentration of unknowns from the standard curve using absorbance readings for the samples.
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**EXAMPLE OF STANDARD CURVE**

Results of a typical standard run with optical density readings at 450nm are below. Each user should obtain his or her own data and standard curve.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ng/mL)</th>
<th>Absorbance (450nm)</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>St01</td>
<td>10</td>
<td>3.492</td>
<td>0.2</td>
</tr>
<tr>
<td>St02</td>
<td>5</td>
<td>3.208</td>
<td>1.6</td>
</tr>
<tr>
<td>St03</td>
<td>2.5</td>
<td>2.569</td>
<td>0.2</td>
</tr>
<tr>
<td>St04</td>
<td>1.25</td>
<td>1.693</td>
<td>1.4</td>
</tr>
<tr>
<td>St05</td>
<td>0.625</td>
<td>0.963</td>
<td>0.5</td>
</tr>
<tr>
<td>St06</td>
<td>0.313</td>
<td>0.532</td>
<td>0.3</td>
</tr>
<tr>
<td>St07</td>
<td>0.156</td>
<td>0.307</td>
<td>3</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.085</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**EXPECTED VALUES AND SENSITIVITY**

Luteinizing Hormone circulates in mares during the estrous cycle from 1 to 20 ng/ml of whole blood.

**TECHNICAL HINTS**

1. Change pipette tips between each addition of standard, sample and reagents to avoid cross-contamination.

2. Standards and samples should be pipetted to the bottom of the wells; all other reagents should be added to the side of the wells to avoid contamination. When pipetting, avoid bubbles and foam.

3. Make sure that all buffers are not contaminated or expired. When troubleshooting ELISA results, it is recommended to prepare new buffers.
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4. Do not add sodium azide to the buffers.

5. Sample and Conjugate dilutions should be prepared shortly before use.

6. When preparing serial dilutions, wipe excess antibody/analyte from pipette tips to ensure accurate dilutions.

7. Incubation time of the HRP Substrate will depend on the formulation used and the intensity of the color change. The high standard should have an O.D. reading of about 2.0 and the low standard should have an O.D. reading above background.

8. The Stopping solution should be added to the wells in the same order as the HRP Substrate to ensure uniform chromogen reaction.

References

1. Pierson RA, Ginther OJ. Ultrasonic evaluation of the preovulatory follicle in the mare. Theriogenology 1985; 24; 359-368


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TROUBLESHOOTING

1. Problem: Low absorbance
   □ Incorrect dilutions or pipetting errors.
   □ Improper incubation times.
   □ Improper preparation of the TMB substrate.
   □ Wrong filter on microtiter reader. The wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
   □ Kit materials or reagents are contaminated or expired.
   □ Incorrect reagents used.

2. Problem: High Absorbance
   □ Cross contamination from other samples or positive control.
   □ Incorrect dilutions or pipetting errors.
   □ Improper washing.
   □ Wrong filter on microtiter reader.
   □ Contaminated buffers or enzyme substrate.
   □ Improper incubation times.
   □ Kit materials or reagents are contaminated or expired.

3. Problem: Poor Duplicates
   □ Inadequate preparation (e.g., mixing) of specimens.
   □ Incorrect dilutions or pipetting errors.
   □ Technical errors.
   □ Inconsistency in following ELISA protocol.
   □ Inefficient washing.

4. Problem: All wells are positive
   □ Contaminated buffers or enzyme substrate.
   □ Incorrect dilutions or pipetting errors.
   □ Kit materials or reagents are contaminated or expired.
   □ Inefficient washing.

5. Problem: All wells are negative
   □ Procedure not followed correctly.
   □ Contaminated buffers or enzyme substrate.
   □ Contaminated conjugate.
   □ Kit materials or reagents are contaminated or expired.