DATA SHEET

Bovine CCL2 ELISA Kit

Cat. No. E11-800

Components Supplied

- **Bovine CCL2 Pre-Coated 96-well Strip Plate,** 1 each
- **Bovine CCL2 Standard**, 20 ng/vial, 2 each
- **Bovine CCL2 Detection Antibody,** 12 ml
- **10X Dilution Buffer B**, 25 ml
- **HRP Solution,** 12 ml
- **TMB Substrate,** 12 ml
- **Stop Solution,** 12 ml
- **20X Wash Buffer,** 50 ml
- **Sealing Tape,** 6 sheets

Introduction

Enzyme linked immunosorbent assay (ELISA) for the detection of Bovine CCL2 in cell culture supernatants. Kit contains sufficient components to quantitate Bovine CCL2 protein concentration in up to 40 samples, tested in duplicate.

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium

Background

Chemokine (C-C motif) ligand 2 (CCL2) is a small cytokine belonging to the CC chemokine family that is also known as monocyte chemotactic protein-1 (MCP-1). CCL2 recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection.[1-2] This chemokine is produced as a protein precursor containing signal peptide of 23 amino acids and a mature peptide of 76 amino acids.[3-4] It is a monomeric polypeptide, with a molecular weight of approximately 13kDa. The cell surface receptors that bind CCL2 are CCR2 and CCR4.[5]

CCL2 is found at the site of tooth eruption and bone degradation. In the bone, CCL2 is expressed by mature osteoclasts and osteoblasts and is under the control of nuclear factor κB (NFκB). CCL2 causes the degranulation of basophils and mast cells, an effect potentiated by pre-treatment with IL-3 and other cytokines.[6-7]

Principle of the Assay

This kit is based on a sandwich ELISA. Bovine CCL2 present in the test sample is captured by anti-bovine CCL2 antibody that has been pre-adsorbed on the surface of microtiter wells. After sample binding, unbound proteins and molecules are washed off, and a biotinylated detection antibody is added to the wells to bind to the captured CCL2. A strepavidin-conjugated horseradish peroxidase (SA-HRP) is then added to catalyze a colorimetric reaction with the chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine). The colorimetric reaction produces a blue product, which turns yellow when the reaction is terminated by addition of dilute sulfuric acid. The absorbance of the yellow product at 450 nm is proportional to the amount of CCL2 analyte present in the sample and a four-parameter standard curve can be generated. The CCL2 concentrations in the test samples can then be quantified by interpolating their absorbance from the standard curve generated in parallel with the samples. After factoring sample dilutions, the CCL2 concentrations in the original sample can finally be calculated.

Procedure Overview

1. Add 100 µl of standard or sample to well.
   Note: Run each standard or sample in duplicate.
2. Cover plate and incubate at room temperature (20-25°C) for 1 hour.
3. Wash plate FOUR times.
4. Add 100 µl of Bovine CCL2 Detection Antibody to each well.
5. Cover plate and incubate at room temperature for 1 hour.
6. Wash plate FOUR times.
7. Add 100 µl of HRP Solution to each well.
8. Cover plate and incubate at room temperature for 30 minutes.
9. Wash plate FOUR times.
10. Add 100 µl of TMB Substrate Solution to each well.
11. Develop the plate in the dark at room temperature for 30 minutes.
12. Stop reaction by adding 100 µl of Stop Solution to each well.
13. Measure absorbance on a plate reader at 450 nm.

Additional Materials Required

- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene or polyethylene tubes to prepare standard and samples – do not use polystyrene, polycarbonate or glass tubes
- A container to prepare 1X Dilution Buffer B
- A container to prepare 1X Wash Buffer
- A wash bottle or an automated 96-well plate washer
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm

Precautions

- Store all reagents at 2-8°C. Do not freeze reagents.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Solution. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain preservatives. Wear gloves while performing the assay. Please follow proper disposal procedures.

Standard and Sample Handling and Preparation

1X Dilution Buffer B Preparation

- Prepare 1X Dilution Buffer B by diluting 25 ml of 10X Dilution Buffer B into 225 ml of ultra pure water. Mix well. Store reconstituted 1X Dilution Buffer B at 2-8ºC for up to six (6) months. Do not use 1X Dilution Buffer B if it becomes visibly contaminated during storage.

Standard Preparation

1. Reconstitute 20 ng standard vial with 1 ml of 1X Dilution Buffer B to achieve a concentration of 20,000 pg/ml. Mix well. Dilute 500 µl of reconstituted standard in 500 µl 1X Dilution Buffer B. Mix well. This is the top standard with a final concentration of 10,000 pg/ml.
2. Label seven (7) tubes, one for each additional standard curve point: 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312.5 pg/ml, 156.3 pg/ml, and 0 pg/ml.
3. Pipette 500 µl of 1X Dilution Buffer B into tubes.
4. Serial dilute the 10,000 pg/ml standard 1:1 with 1X Dilution Buffer B. Perform dilution by mixing 500 µl of the previous standard with 500 µl of 1X Dilution Buffer B. Continue until reach standard value of 156.3 pg/ml.
5. Use 1X Dilution Buffer B only as the zero standard value.

Sample Handling

- Cell culture supernatants may be tested in this ELISA.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of sample or standard is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Store samples to be assayed within 24 hours at 2-8ºC. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.

- If the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

Sample Preparation

- If it is suspected that the concentration of the sample exceeds the highest point of the standard curve, prepare one or more dilutions of the sample in 1X Dilution Buffer B until the desired concentration is obtained. For example, a 1:100 dilution could be prepared by adding 10 µl of sample to 990 µl of 1X Dilution Buffer B in a clean, fresh tube and mixing well. If a final dilution of 1:10,000 is desired, the dilution could be prepared by adding 10 µl of the 1:100 sample dilution to 990 µl 1X Dilution Buffer B in a separate tube and mixing well.

1X Wash Buffer Preparation

- Prepare 1X Wash Buffer by diluting 20X Wash Buffer in ultra pure water. For example, if preparing 1 L of 1X Wash Buffer, dilute 50 ml of 20X Wash Buffer into 950 ml of ultrapure water. Mix well. Store reconstituted 1X Wash Buffer at 2-8ºC for up to six (6) months. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

Assay Procedure

Sample Incubation

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in the foil pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

1. Add 100 µl of appropriately diluted standards or samples to each well. Run each standard, sample, or blank in duplicate.
2. Carefully cover wells with a new adhesive plate cover. Incubate for one (1) hour at room temperature, 20-25°C.
3. Carefully remove adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

   Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Detection Antibody Incubation

- Only remove the required amount of Detection Antibody reagent for the number of strips being used.

1. Add 100 µl of Detection Antibody to each well containing standard, sample or blank. Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover. Incubate plate for one (1) hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

HRP Solution Incubation

- Only remove the required amount of HRP Solution for the number of strips being used.

1. Add 100 µl of HRP Solution to each well containing sample or blank.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

TMB Substrate Incubation and Reaction Stop

- Only remove the required amount of TMB Substrate Solution and Stop Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µl of TMB Substrate Solution into each well.
2. Allow the enzymatic color reaction to develop at room temperature (20-25°C) in the dark for 30 minutes. Do NOT cover plate with a plate sealer. The substrate reaction yields a blue solution.
3. After 30 minutes, stop the reaction by adding 100 µl of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

Calculation of Results

- Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.

1. Prepare a standard curve to determine the amount of Bovine CCL2 in an unknown sample. Plot the average absorbance obtained for each standard concentration on the vertical (Y) axis versus the corresponding Bovine CCL2 concentration on the horizontal (X) axis using graph paper or curve-fitting software.
2. Calculate the Bovine CCL2 concentration in unknown samples using the prepared standard curve. Determine the amount of Bovine CCL2 in each unknown sample by noting the Bovine CCL2 concentration (X axis) that

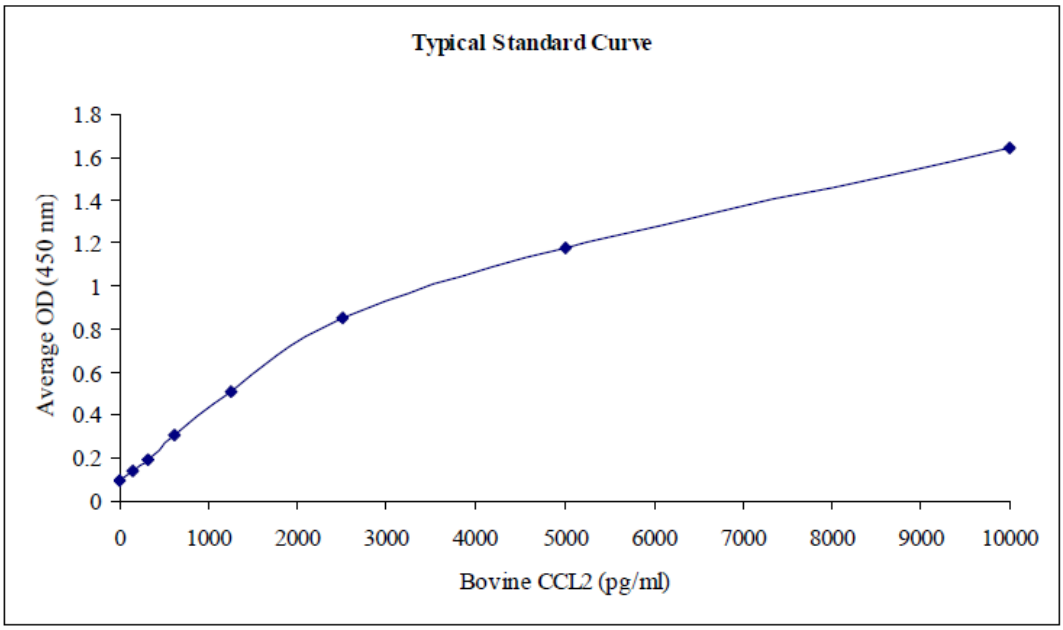
correlates with the absorbance value (Y axis) obtained for the unknown sample.

3. If the sample was diluted, multiply the Bovine CCL2 concentration obtained by the dilution factor to determine the amount of Bovine CCL2 in the undiluted sample.

Performance Characteristics

Typical Standard Curve

- This typical standard curve was generated using the Bovine CCL2 ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.



Assay Range: 10,000-156.3 pg/ml

- Suggested standard curve points are 10,000 pg/ml, 5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312.5 pg/ml, 156.3 pg/ml, and 0 pg/ml.

Representative Data

- PBMCs were harvested by ficoll density gradient from day old whole blood collected from an apparently healthy cow. The PBMCs were suspended at 0.6 x 10^6 cells/ ml in RPMI medium containing 10% serum. PBMCs were

stimulated with phytohemagglutinin (PHA; 10 µg/ml), staphylococcal enterotoxin B (SEB; 5 µg/ml) or phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) and inomycin (500 ng/ml). Cell-free supernatants were harvested following three days stimulation and run in the Bovine CCL2 ELISA. The levels of Bovine CCL2 detected are as follows:

Stimulant	Bovine CCL2 (pg/ml)
Unstimulated	49,541
PHA	20,598
SEB	9,588
PMA/Ionomycin	267