CAT CERULOPLASMIN SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: CER-SP-8

INTRODUCTION

Ceruloplasmin (CP) is a copper-transport protein of ~150 kDa that is synthesized in the liver and circulates in blood. It is a positive acute phase protein that is elevated in serum following injury, infection and disease. In studies at Life Diagnostics, Inc., we found serum CP levels ranging from 100 to 800 μg/ml.

PRINCIPLE OF THE ASSAY

The cat CP SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 2) assay uses two affinity purified CP-specific antibodies. One is conjugated to horseradish peroxidase (HRP) and the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to CP they are brought into molecular proximity. With the addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence. Acridan conjugated antibodies distant from HRP produce no signal. This principle allows the development of a homogeneous assay that allows rapid measurement of CP concentrations.

The HRP and acridan conjugated antibodies provided with the kit are mixed with standards and diluted samples in wells of the 96-well SPARCL™ plate provided with the kit[2]. After incubation for 30 minutes on a shaker at 25°C and 150 rpm, the plate is placed into a luminometer. Trigger solution containing hydrogen peroxide is injected into each well and luminescence is immediately measured. The concentration of CP is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-cat CP HRP conjugate **Store ≤ -70°C**
- Anti-cat CP acridan conjugate **Store ≤ -70°C**
- Cat CP stock **Store ≤ -70°C**
- Denaturing buffer; SDB10-1, 10 ml
- Diluent; CSD50-1, 2 x 50 ml
- Trigger solution; TS7-1, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

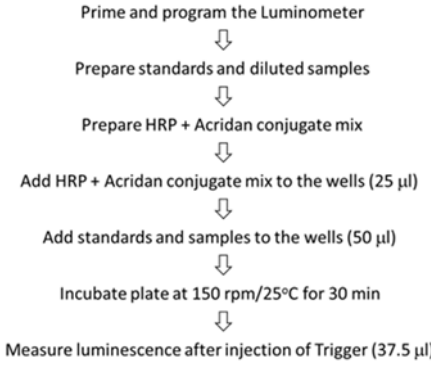
- Precision pipettes and tips
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection/measurement
- Graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and CP stock at or below -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 2-8°C. The SPARCL™ plate should be kept in a sealed bag with desiccant and antioxidant. The kit will remain stable for at least six months from the date of purchase, provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. Please take the time to completely read all instructions before starting your assay. Contact us if you need clarification.
2. All reagents used in the assay should be allowed to reach room temperature (25°C) before use.
3. If crystals form in the denaturing buffer, agitate the sealed bottle in warm water until dissolved.
4. It is important that standards and samples be added to the SPARCL™ plate quickly. If testing large numbers of samples, rather than pipetting standards and samples directly into the white SPARCL™ plate using a single channel pipettor, we recommend the following. First, pipette an excess volume of standards and samples into appropriate wells of the clear 96-well plate. Then use an 8- or 12-channel multipipettor to quickly and efficiently transfer 50 μl aliquots to the appropriate wells of the white SPARCL™ plate. The wells of the clear plate hold a maximum volume of 300 μl.
5. Follow the sequence of events below when running the assay.



STANDARD PREPARATION

The cat CP stock is comprised of pure cat CP in denaturing buffer at a concentration of 200 μg/ml.

1. Prepare the 200 ng/ml standard as follows:
   a. Mix 5.0 μl of the 200 μg/ml stock with 95 μl of CSD50-1 diluent to give a 10 μg/ml stock.
   b. Mix 10.0 μl of the 10 μg/ml stock with 490 μl of CSD50-1 to give the 200 ng/ml standard.
2. Label 7 polypropylene tubes as 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 ng/ml and dispense 150 μl of CSD50-1 into each.
3. Pipette 150 μl of the 200 ng/ml CP standard into the tube labeled 100 ng/ml and mix. This provides the 100 ng/ml CP standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

Please Note: Use the standards within one hour of preparation.

SAMPLE PREPARATION

Samples must be denatured and diluted prior to testing:

[1] The SPARCL technology was developed by Lumigen Corp.

[2] The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

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Denaturation.

1. For each sample to be tested dispense 90 µl of denaturation buffer into a microcentrifuge tube.
2. Mix 10 µl of each serum or plasma sample with 90 µl of denaturation buffer (this represents a 10-fold dilution of the sample).
3. Incubate at room temp for at least 10 minutes (but no longer than 1 hour) then proceed to the further-dilution step.

Further-dilution.

1. For each sample to be tested dispense 95 µl and 490 µl of CSD50-1 diluent into separate microcentrifuge tubes.
2. Mix 5.0 µl of each denatured sample with 95 µl of diluent (this represents a 200-fold dilution of the original sample).
3. Mix 10.0 µl of the 200-fold diluted sample with 490 µl of diluent to give a 10,000-fold dilution of the sample.

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. If necessary, after thawing, briefly centrifuge to ensure that the contents are at the bottom of the tubes. Prepare the mix shortly before use using the CSD50-1 diluent provided with the kit.

LUMINOMETER SETUP

1. The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
2. Prime the luminometer injection port with 1 ml of trigger solution.
3. Place the injection needle into the injection port as needed for BMG luminometers.
4. Program the luminometer to inject 37.5 µl of trigger solution per well and to measure from time zero for 1 second (50 x 0.02 second intervals).
5. Define the format of the assay using the luminometer software.
6. Because the white SPARCL™ plate is provided as 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the luminometer is programmed to inject trigger solution only into the wells being used.
7. We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
8. There are a number of manufacturers of luminometers that are equipped to run a SPARCL™ assay. Please contact Life Diagnostics or Lumigen (www.lumigen.com) to discuss your luminometer.

PROCEDURE

1. Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
2. Secure the desired number of SPARCL™ 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant and antioxidant. Store unused strips at 2-8°C.
3. Aliquot 25.0 µl of conjugate mix into each well.
4. Dispense 50.0 µl of standards and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
5. Incubate on an orbital micro-plate shaker at 150 rpm 25°C for 30 minutes.
6. After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 µl).
7. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

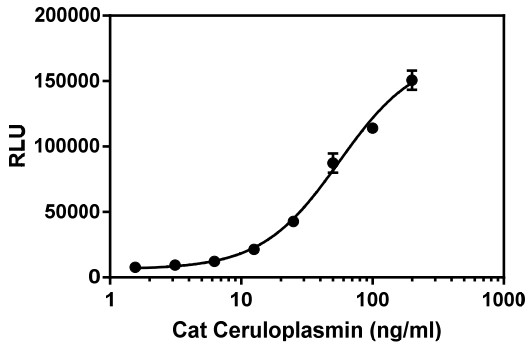
CALCULATION OF RESULTS

1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells. We routinely use the sum of RLU values from a 100-980 ms data collection window.
2. Determine the sum of RLU values within the data collection window for the standards and samples.
3. Using graphing software, construct a standard curve by plotting the sum of the RLU values for the standards versus the $\log_{10}$ of the CP concentration and fit to a sigmoidal, 4PL model.
4. Derive the corresponding concentration of CP in the samples from the standard curve (remember to derive the concentration from the antilog).
5. Multiply the derived concentration by the dilution factor to determine the concentration of CP in the original sample.
6. If the sum of the RLU values of diluted samples fall outside the standard curve, samples should be appropriately diluted and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with sum of RLU plotted on the Y-axis versus $\log_{10}$ CP concentrations on the X-axis is shown below. This curve is for illustration only and should not be used to calculate unknowns. A standard curve must be run for each experiment.

CP (ng/ml)	RLU
200	150756
100	114012
50	87340
25	42723
12.5	21447
6.25	12248
3.13	9358
1.56	7778



REFERENCES

1. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

Rev 021618. For technical assistance please email us at techsupport@lifediagnostics.com