RAT CYSTATIN-C SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: CYSC-SP-2

INTRODUCTION

Cystatin-C, a protein of ~13 kDa, is used as a biomarker of kidney function in rats (refs 1-2).

PRINCIPLE OF THE ASSAY

The rat cystatin-C SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two rat cystatin-C specific antibodies. One is conjugated to horseradish peroxidase (HRP), the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to cystatin-C they are brought into 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 cystatin-C concentrations.

Diluted serum or plasma samples and standards are mixed with the HRP and acridan-conjugated antibodies in the wells of the 96-well SPARCL™ plate[2] provided with the kit. 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 cystatin-C is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-cystatin-C HRP conjugate. **Store ≤** -70°C
- Anti-cystatin-C acridan conjugate. **Store ≤** -70°C
- Cystatin-C stock. **Store ≤** -70°C
- Diluent (CSD50-1), 2 x 50 ml
- Trigger solution, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

- Precision pipettes and tips
- Polypropylene tubes
- Vortex mixer
- Micro-Plate incubator/shaker
- Luminometer capable of simultaneous injection & measurement
- PC graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and cystatin-C stock at -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.

GENERAL INSTRUCTIONS

The dilution buffer and 8-well strips used in the assay should be allowed to reach room temperature (25°C) before use.

STANDARD PREPARATION

The cystatin-C stock is comprised of Sprague Dawley rat serum of known cystatin-C concentration[3] diluted in a stabilizing buffer. Thaw the stock shortly before use.

1. Label six polypropylene tubes[4] as 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/ml.
2. Into the tube labeled 50 ng/ml, pipette the volume of diluent detailed on the cystatin-C stock vial label. Then add the indicated volume of cystatin-C stock and mix gently. This provides the 50 ng/ml standard.
3. Dispense 150 µl of diluent into the tubes labeled 25, 12.5, 6.25, 3.13 and 1.56 ng/ml.
4. Pipette 150 µl of the 50 ng/ml cystatin-C standard into the tube labeled 25 ng/ml and mix. This provides the 25 ng/ml cystatin-C standard.
5. Similarly prepare the remaining standards by serial dilution.

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

SAMPLE PREPARATION

Serum or plasma samples should be diluted at least 50-fold with diluent to avoid matrix effects. In studies at Life Diagnostics, we found levels of 1562±254 ng/ml (mean±SD, n=9) in serum from Sprague Dawley rats. Optimal dilutions must be determined by the end user; however, we suggest an initial dilution of 100-fold (2.5 µl of serum mixed with 247.5 µl of diluent). Use only the diluent provided with the kit.

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 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 a 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the

[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.

[3] Cystatin-C concentration of the serunm was determined using recombinant rat cystatin-C as reference standard.

[4] Dilutions of standards can be performed in wells A1-A6 of the clear untreated 96-well plate provided with the kit. This allows rapid transfer of standards to the white SPARCL™ plate using a multipipettor. Diluted samples can also be first aliquoted into appropriate wells of the clear polystyrene plate and subsequently transferred to the SPARCL™ plate with a multipipettor. If using this method, ensure that an excess volume is aliquoted into the clear plate in order to ensure complete transfer of 50 µl aliquots to the SPARCL™ plate.

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.

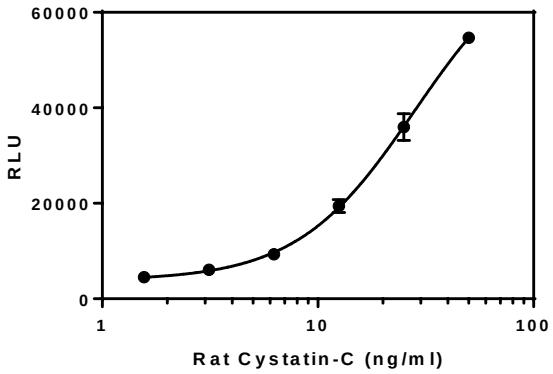
CALCULATION OF RESULTS

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 usually eliminate data generated in the first 100 msec.
2. Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus $\log_{10}$ of the cystatin-C concentration in ng/ml.
3. Fit data to a sigmoidal 4-parameter logistic equation.
4. Derive the concentration of cystatin-C in the diluted samples from the standard curve (remember to derive the antilog).
5. Multiply the derived concentration by the dilution factor to determine the actual concentration of cystatin-C in the sample.
6. If the RLU values of diluted samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve is for illustration only and should not be used to calculate unknowns.

Cystatin-C (ng/ml)	RLU
50	54692
25	35962
12.5	19413
6.25	9324
3.13	6057
1.56	4507



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2. Dokumaccioglu E et. Al. The effect of sulfforaphane on the levels of serum cystatin-c in acetoaminophen induced nephrotoxicity in rats. Dicle Medical Journal. 43(3):383-389 (2016)
3. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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