RAT ANTI-KLH IgG SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: KLHG-SP-2

INTRODUCTION

Drug candidates are routinely screened for evidence of immune system regulation. It is important that the immune response is neither enhanced nor diminished since such effects may lead to hypersensitivity or increased susceptibility to infection. Determination of the effect of a drug candidate on anti-KLH antibody levels allows assessment of immune system regulation (ref 1). Animals are immunized with KLH while undergoing drug treatment and serum is collected at appropriate times after immunization. Typically, serum collected 5-7 days after immunization is used for measurement of anti-KLH IgM levels, and serum collected 14+ days post immunization is used to measure anti-KLH IgG levels. Comparison of anti-KLH IgG or IgM levels in drug-treated, versus control groups reveals effects on the immune response.

PRINCIPLE OF THE ASSAY

The rat anti-KLH IgG SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 2) assay uses two different antibody conjugates: HRP conjugated affinity purified rabbit anti-KLH IgG and acridan conjugated anti-rat IgG.

Rat serum or plasma samples are diluted and incubated alongside rat anti-KLH IgG standards in the wells of a KLH-coated white microtiter plate for 30 minutes. The plate is then washed to remove non-KLH reactive rat IgG and other serum components. A mixture of rabbit anti-KLH IgG HRP and anti-rat IgG acridan is then added to the wells and incubated for 30 minutes. During this step, rabbit anti-KLH IgG HRP binds to the many unoccupied epitopes on KLH and acridan conjugated anti-rat IgG binds to rat anti-KLH IgG that is also bound to KLH. HRP-conjugated rabbit anti-KLH IgG and acridan conjugated anti-rat IgG antibodies are thereby brought into close proximity. Following a wash step, the addition of hydrogen peroxide (trigger solution) facilitates HRP catalyzed oxidation of proximal acridan molecules causing a flash of chemiluminescence. The level of rat anti-KLH IgG in the sample is proportional to the luminescence. Actual values are derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-KLH HRP stock **Store ≤ -70°C**
- Anti-rat IgG acridan stock **Store ≤ -70°C**
- Rat Anti-KLH IgG stock (2 vials)
- KLH coated white plate (12 x 8-well)
- Sample diluent; YD50-1, 50 ml
- Conjugate diluent; CSD10-1, 10 ml
- 20x Wash solution; TBS50-20, 50 ml
- Trigger solution; TS7-1, 7 ml

Materials required but not provided:

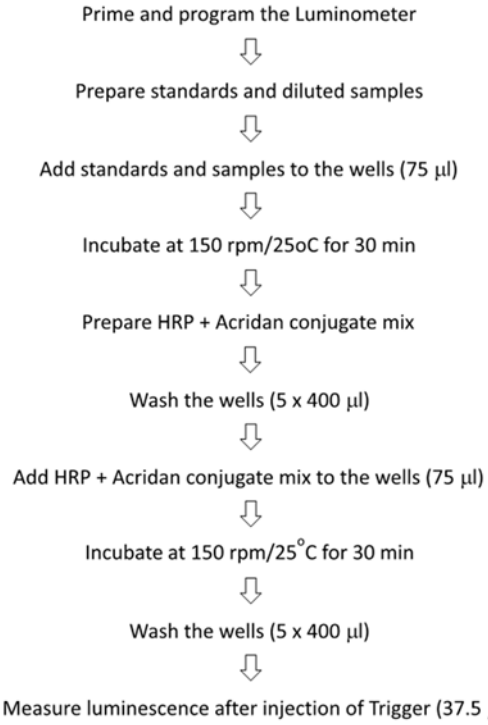
- Precision pipettes and tips
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection/measurement
- Curve fitting software

STORAGE

Store the HRP and acridan conjugates at or below -70°C. The remainder of the kit should be stored at 2-8°C. The KLH coated white plate should be kept in a sealed bag with desiccant. 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

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. Follow the sequence of events below when running the assay.



WASH SOLUTION PREPARATION

The wash solution is provided as a 20x stock. Prior to use dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water.

STANDARD PREPARATION

1. Reconstitute the lyophilized rat anti-KLH IgG stock as described on the vial label. Mix gently until dissolved.
2. Label 8 polypropylene tubes as 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 ng/ml.
3. Into the tube labeled 1000 ng/ml, pipette the volume of diluent (YD50-1) detailed on the stock vial label. Then add the indicated volume of stock and mix gently. This provides the 1000 ng/ml standard.
4. Dispense 200 µl of diluent into the tubes labeled 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 ng/ml.

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

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5. Pipette 200 µl of the 1000 ng/ml anti-KLH IgG standard into the tube labeled 500 ng/ml and mix. This provides the 500 ng/ml anti-KLH IgG standard.
6. Similarly prepare the remaining standards by two-fold serial dilution.

If future use of the reconstituted IgG stock is intended, it should be stored frozen at or below minus 20°C.

SAMPLE PREPARATION

Serum or heparinized plasma should be prepared as quickly as possible after blood collection. Do not use EDTA or citrate plasma unless samples are diluted at least 100-fold (EDTA and citrate inhibit HRP causing false low values). All samples should be similarly processed (i.e., storage times and temperatures should be the same). If samples cannot be assayed immediately they should be frozen at or below –20°C. Avoid repeated freeze-thaws.

Anti-KLH IgG levels are not detectable in serum from rats not exposed to KLH. We found that when rats were injected with KLH (0.24 mg/kg, i.v), levels increased to 1.636±0.886 mg/ml (mean±SD, n=9). The levels of anti-KLH IgG depend on the route of injection, adjuvant, amount of KLH injected, and the timing of serum collection. Researchers must therefore determine the optimum dilution factor(s) for their samples. In the study mentioned above, we found that all samples were within range of the standard curve when tested at a dilution of 10,000-fold. All dilutions must be performed with the YD50-1 diluent provided with the kit.

CONJUGATE PREPARATION

Prepare the conjugate mix 5-10 min prior to use according to the instructions detailed on the box that contains the HRP and acridan conjugates. The clear diluent (CSD10-1) must be used for dilution of the conjugates.

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 at least 1 ml of trigger solution.
3. Place the injection needle into the injection port, (necessary 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 KLH coated plate is provided as a 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 KLH-coated 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant. Store unused strips at 2-8°C.
3. Dispense 75 µl of standards and diluted samples into each well (we recommend that samples and standards be tested in duplicate).
4. Cover the plate and incubate at 25°C for 30 minutes on an orbital shaker (150 rpm).
5. Aspirate the well contents and wash the wells (5 x 400 µl) using a plate washer.
6. Remove remaining droplets of wash solution by striking the inverted plate firmly on a pad of paper towels.
7. Dispense 75 µl of conjugate mix into the wells.
8. Cover the plate and incubate on an orbital micro-plate incubator shaker at 25°C and 150 rpm for 30 minutes.
9. After the 30-minute incubation wash the wells as detailed in steps 5 & 6 of this section.
10. Place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 µl).
11. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

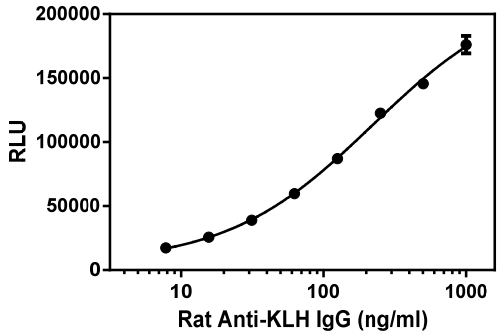
CALCULATION OF RESULTS

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 curve fitting software, construct a standard curve by plotting the sum of the RLU values for the standards versus the $\log_{10}$ of the anti-KLH IgG concentration and fit to a sigmoidal, 4PL model.
4. Derive the corresponding concentration of anti-KLH IgG 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 anti-KLH IgG in the original sample.
6. If the sum of the RLU values of the 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 values plotted on the Y-axis versus $\log_{10}$ anti-KLH IgG concentrations on the X-axis is shown below. This curve is for illustration only and should not be used to calculate unknowns.

Anti-KLH IgG (ng/ml)	RLU
1000	176111
500	145630
250	122504
125	87119
62.5	59819
31.25	38855
15.63	25709
7.81	17334



REFERENCES

1. JR Picotti et.al. T-cell-dependent antibody response: Assay development in cynomolgus monkeys. Journal of Immunotoxicology, 2:191-196 (2005)
2. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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