

# A Bridging Immunogenicity Assay Using SPARCL™ Technology

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## INTRODUCTION

Evaluating the immune response in patients is an important aspect associated with biological drug safety assessment. The presence of anti-drug antibodies (ADA) can not only have various clinical consequences, including an altered pharmacokinetic (PK) profile, but also affect ligand-binding assays used for PK assessment. Sensitive immunogenicity assays for ADA detection are critical during biological drug development.

SPARCL™ technology is a homogeneous assay technology where a chemiluminescent compound (acridan) and HRP are brought into close proximity as a result of the specific binding event. Quantification of an analyte is achieved by measuring the light signal that is generated upon addition of a trigger solution without the need to remove excess unbound reagents. A generic bridging immunogenicity assay is described here based on SPARCL™ technology. The assay uses mouse IgG (drug) and goat anti-mouse IgG (ADA). Biotin-labeled and fluorescein (FITC)-labeled mouse IgG are used in conjunction with acridan-labeled streptavidin and an HRP-conjugated anti-FITC antibody to form the SPARCL™ immune-complex.

## MATERIALS AND INSTRUMENTATION

**Lumigen SPARCL™ Detection Kit:** Lumigen, Inc. (Catalog No. SDK-10K); included in the kit are SPARCL Labeling Reagent, BuHTM Borate Buffer Pack, SPARCL Background Reducing Agent (BGR) and SPARCL Trigger Solution.

**Mouse IgG:** Purchased from Jackson ImmunoResearch (Catalog No. 015-000-003).

**Biotin-Conjugated Mouse IgG:** Purchased from Jackson ImmunoResearch (Catalog No. 015-060-003).

**FITC-Conjugated Mouse IgG:** Purchased from Jackson ImmunoResearch (Catalog No. 015-090-003).

**Streptavidin:** Purchased from SouthernBiotech (Catalog No. 7100-01).

**HRP-Conjugated Rabbit Polyclonal Anti-FITC Antibody:** Purchased from Abcam (Catalog No. ab196968).

**Rat Serum:** Purchased from Bioreclamation (Catalog No. RATSRM).

**Assay Buffer:** 1xPBS containing 0.05% Tween-20 and 1% BSA.

**Acid Solution Used for Acid-Treatment:** 300 mM acetic acid.

**Base Solution Used for Neutralization:** 1.5 M TRIS base.

**Luminescence Plate Reader:** SpectraMax L (Molecular Devices).

**PP-Microplate White 96-Well Plate Flat-Bottom:** Greiner Bio-One (Ref# 655207).

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## METHOD

### SpectraMax L Settings

Mode:	Fast Kinetic, Luminescence
Integration Point/Count:	Integrate 0.02 seconds, repeat 50 times (total read time is 1 second)
Sensitivity:	Photon counting, correction is set to none
Automix:	Not used
Assay Plate Type:	96-well Standard
Wells to Read:	Enter in the well information based on plate layout
Injection and Delay:	P-injection is set to “off” M-Injection volume of 75 uL, baseline read delay is set to “zero”, Injection speed set to 230 uL/second, Shake after injection is set to “zero”, number of baseline reads is set to “zero”
Injection Wells:	Enter the well information based on your needs
Dark Adaptor:	Off
AutoRead	Off

### Assay Sensitivity—Standard Curve

- 1) Prepare acridan-labeled streptavidin following standard procedure indicated in Lumigen SPARCL™ Detection Kit.
- 2) Prepare working solutions of the following reagents with the assay buffer: biotin-conjugated mouse IgG (1 µg/mL, 1:1,000 dilution from the commercial stock), FITC-conjugated mouse IgG (1 µg/mL, 1:1,000 dilution from the commercial stock), acridan-labeled streptavidin (2 µg/mL, 1:125 dilution from the labeling reaction mixture) and HRP-conjugated rabbit polyclonal anti-FITC antibody (1 µg/mL, 1: 250 dilution from the commercial stock).
- 3) Prepare 1:1 mixture solution of biotin-conjugated mouse IgG and FITC-conjugated mouse IgG by combining equal volumes of the working solutions.
- 4) Prepare goat anti-mouse IgG (ADA) standards by serial dilution in rat serum (neat) at 10,000, 4,000, 1,000, 500, 250, 62.5, 31.3, 15.6, 0 ng/mL.
- 5) To each well of a 96-well plate, add 50 µL of antibody mixture solution from Step-3 and 25 µL of mouse IgG standard in rat serum.
- 6) Incubate with moderate shaking at room temperature for 30 minutes.
- 7) Prepare 1:1 mixture solution of acridan-labeled streptavidin and HRP-conjugated rabbit polyclonal anti-FITC antibody by combining equal volumes of the working solutions.
- 8) Add 50 µL of the solution from Step-7.
- 9) Incubate with moderate shaking at room temperature for 30 minutes.
- 10) Add 8 uL of SPARCL BGR to each well.
- 11) Read the plate on SpectraMax-L by injecting 75 µL of SPARCL Trigger Solution to each well.

### **Assay Sensitivity—Standard Curve under Acid-Dissociation Condition**

- 1) Prepare working solutions of all antibody reagents following the same procedure as described above.
- 2) Prepare goat anti-mouse IgG (ADA) standards by serial dilution in rat serum (neat) at 10,000, 4,000, 1,000, 500, 250, 62.5, 31.3, 15.6, 0 ng/mL.
- 3) To each well of a 96-well plate, add 60  $\mu$ L of 300 mM acetic acid and 15  $\mu$ L of mouse IgG standard in rat serum.
- 4) Incubate with moderate shaking at room temperature for 30 minutes.
- 5) Immediately before use prepare a mixture solution by combining working solutions of biotin-conjugated mouse IgG (1  $\mu$ g/mL), FITC-conjugated mouse IgG (1  $\mu$ g/mL) and 1.5 M TRIS base (1:1:0.52, v/v/v).
- 6) Add 63  $\mu$ L of the solution from Step-5.
- 7) Incubate with moderate shaking at room temperature for 30 minutes.
- 8) Prepare a mixture solution by combining working solutions of acridan-labeled streptavidin (2  $\mu$ g/mL) and HRP-conjugated rabbit polyclonal anti-FITC antibody (1  $\mu$ g/mL) (1:1, v/v).
- 9) Add 50  $\mu$ L of the solution from Step-8.
- 10) Incubate with moderate shaking at room temperature for 30 minutes.
- 11) Add 8  $\mu$ L of SPARCL BGR to each well.
- 12) Read the plate on SpectraMax-L by injecting 100  $\mu$ L of SPARCL Trigger Solution.

### **Drug Tolerance Experiment without Acid Pre-Treatment**

- 1) Prepare working solutions of all antibody reagents following the same procedure as described above.
- 2) Prepare a goat anti-mouse IgG (ADA) standard in rat serum (neat) at 500 ng/mL.
- 3) Prepare rat serum samples containing 2x mouse IgG (free drug) at 0, 0.50, 1.0, 2.0, 10, 20, 50, 100  $\mu$ g/mL.
- 4) Prepare rat serum samples containing 250 ng/mL of goat anti-mouse IgG (ADA) and 1x mouse IgG (free drug) at 0, 0.25, 0.50, 1.0, 5.0, 10, 25, 50  $\mu$ g/mL by combining equal volume of the solution from Step-2 and that from Step-3. Incubate with moderate shaking at room temperature for 60 minutes.
- 5) Prepare a mixture solution by combining biotin-conjugated mouse IgG (1  $\mu$ g/mL) and FITC-conjugated mouse IgG (1  $\mu$ g/mL) (1:1, v/v).
- 6) To each well of a 96-well plate, add 50  $\mu$ L of the solution from Step-5 and 15  $\mu$ L of rat serum sample from Step-4.
- 7) Incubate with moderate shaking at room temperature for 10 minutes, then 4°C for overnight without shaking.
- 8) Prepare a mixture solution by combining working solutions of acridan-labeled streptavidin (2  $\mu$ g/mL) and HRP-conjugated rabbit polyclonal anti-FITC antibody (1  $\mu$ g/mL) (1:1, v/v).
- 9) Add 50  $\mu$ L of the solution from Step-8.
- 10) Incubate with moderate shaking at room temperature for 60 minutes.
- 11) Add 8  $\mu$ L of SPARCL BGR to each well.
- 12) Read the plate on SpectraMax-L by injecting 75  $\mu$ L of SPARCL Trigger Solution.

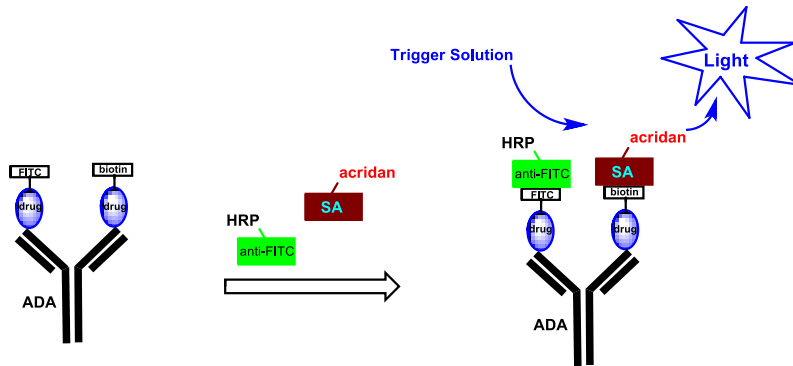
### **Drug Tolerance Experiment with Acid Pre-Treatment**

- 1) Prepare working solutions of all antibody reagents following the same procedure as described above.
- 2) Prepare a goat anti-mouse IgG (ADA) standard in rat serum (neat) at 500 ng/mL.
- 3) Prepare rat serum samples containing 2x mouse IgG (free drug) at 0, 0.50, 1.0, 2.0, 10, 20, 50, 100 µg/mL.
- 4) Prepare rat serum samples containing 250 ng/mL of goat anti-mouse IgG (ADA) and 1x mouse IgG (free drug) at 0, 0.25, 0.50, 1.0, 5.0, 10, 25, 50 µg/mL by combining equal volume of the solution from Step-2 and the solution from Step-3. Incubate with moderate shaking at room temperature for 60 minutes.
- 5) To each well of a 96-well plate, add 60 µL of 300 mM acetic acid and 15 µL of rat serum samples from Step-4.
- 6) Incubate with moderate shaking at room temperature for 30 minutes.
- 7) Immediately before use prepare a mixture solution by combining working solutions of biotin-conjugated mouse IgG (2 µg/mL), FITC-conjugated mouse IgG (2 µg/mL) and 1.5 M TRIS base (1:1:1.04, v/v/v).
- 8) Add 38 µL of the solution from Step-7.
- 9) Incubate with moderate shaking at room temperature for 60 minutes.
- 10) Prepare a mixture solution by combining working solutions of acridan-labeled streptavidin (4 µg/mL) and HRP-conjugated rabbit polyclonal anti-FITC antibody (2 µg/mL) (1:1, v/v).
- 11) Add 25 µL of the solution from Step-10.
- 12) Incubate with moderate shaking at room temperature for 60 minutes.
- 13) Add 8 µL of SPARCL BGR to each well.
- 14) Read the plate on SpectraMax-L by injecting 100 µL of SPARCL Trigger Solution.

### **RESULTS**

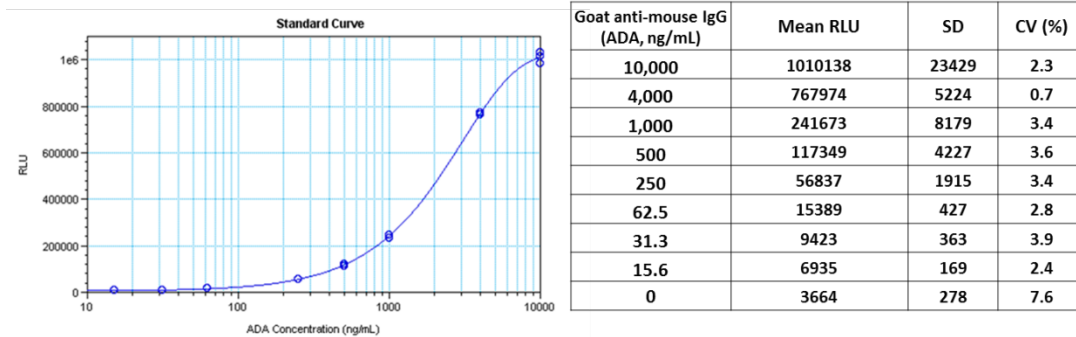
A generic SPARCL™ bridging assay is configured using mouse IgG (drug) and goat anti-mouse IgG (ADA) to demonstrate the utility of SPARCL™ technology for immunogenicity applications. The assay uses biotin-labeled and fluorescein (FITC)-labeled mouse IgG in conjunction with acridan-labeled streptavidin and an HRP-conjugated anti-FITC antibody (Figure 1). This generic configuration can be easily adapted to any biological drug in general without using specialty reagents except SPARCL labeling compound which is included in Lumigen SPARCL Detection Kit.

**Figure 1: Assay Configuration**



The assay protocol includes two incubation steps and requires no wash. Figure 2 shows a typical standard curve in 100% rat serum. The bridging assay displays a wide dynamic range with tight CVs and a calculated sensitivity (3SD) of 2.3 ng/mL.

**Figure 2: Typical Standard Curve**



- 1) 25  $\mu$ L (1.0  $\mu$ g/mL) FITC-mouse IgG, 25  $\mu$ L (1.0  $\mu$ g/mL) biotin-mouse IgG and 25  $\mu$ L standard in rat serum, 30 min incubation.
- 2) Premixed solution: 25  $\mu$ L (1.0  $\mu$ g/mL) HRP-rabbit anti-FITC, 25  $\mu$ L (2.0  $\mu$ g/mL) acridan-streptavidin, 30 min incubation.
- 3) 8  $\mu$ L BGR, 75  $\mu$ L SPARCL trigger.

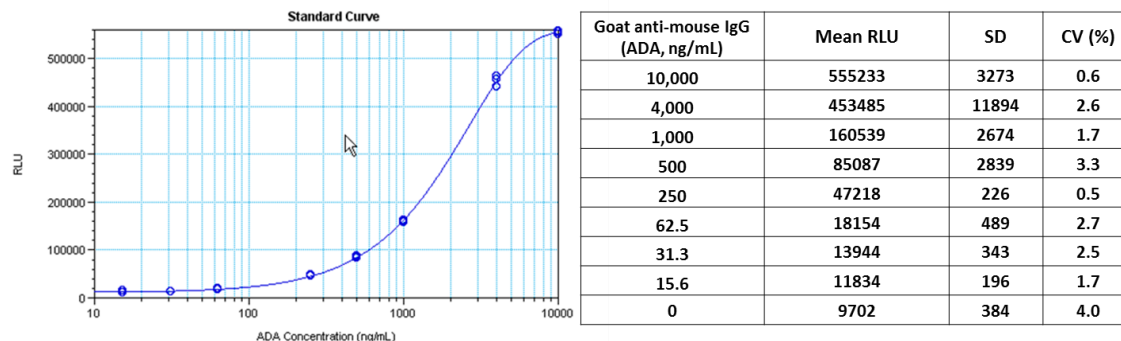
In immunogenicity testing presence of the biological drug in the sample can interfere with the ADA detection. Samples containing circulating biotherapeutic drug could exhibit assay interference due to competition between the circulating drug and the “reagent drug” used in the bridging assay. To overcome this challenge, acid-treatment is often used as a counter measure to dissociate the drug-ADA complex. It is crucial that a bridging assay maintains its performance under such conditions.

To examine this particular aspect, the performance of the SPARCL<sup>TM</sup> bridging assay has been evaluated with an added acid-treatment step. A slightly modified protocol is used where a base is included for neutralization purpose, and a reduced sample volume utilized to accommodate the total volume in each well, which also allows the test to take place in the same well without

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sample transfer after acid-dissociation. As shown in [Figure 3](#), the acid-treatment has very little effect on assay performance. The assay displays similar dynamic range and calculated sensitivity (3SD, 1.9 ng/mL).

**Figure 3: Standard Curve under Acid-Dissociation Conditions**



- 1) 60  $\mu$ L of 300 mM acetic acid and 15  $\mu$ L of mouse IgG standard in rat serum, 30 min incubation.
- 2) Premixed solution: 25  $\mu$ L (1.0  $\mu$ g/mL) biotin-mouse IgG; 25  $\mu$ L (1.0  $\mu$ g/mL) FITC-mouse IgG; 13  $\mu$ L of 1.5 TRIS base, 30 min incubation.
- 3) Premixed solution: 25  $\mu$ L (1.0  $\mu$ g/mL) HRP-rabbit anti-FITC; 25  $\mu$ L (2.0  $\mu$ g/mL) acridan-streptavidin, 30 min incubation.
- 4) 8 $\mu$ L BGR, 100 $\mu$ L SPARCL trigger

The model SPARCL<sup>TM</sup> bridging assay has been evaluated for drug tolerance under common acid-treatment conditions, and also under conditions using long incubation times without an acid. The latter is often used when acidic conditions cannot be used due to incompatibility of either the drug or ADA, to allow maximum exchange between the bound-drug and the “reagent drug”. Samples are prepared by combining a rat serum containing mouse IgG (drug) with a rat serum spiked with or without goat anti-mouse antibody (ADA), and incubating to allow drug-ADA complex formation. Data in [Table 1](#) are from an experiment with overnight incubation at 4°C but no acid-treatment. As shown below, ADA at a concentration of 250 ng/mL can be detected in the presence of a drug concentration up to at least 10  $\mu$ g/mL. [Table 2](#) indicates the results from a typical acid dissociation experiment using 300 mM acetic acid. In this case, a sample (15  $\mu$ L) is first treated with acid (60  $\mu$ L) by incubating for 30 minutes. A neutralization solution (1.5 M TRIS base) containing the biotin- and FITC-labeled drug is then added followed by incubation. Subsequent addition of acridan-streptavidin and an HRP-conjugated anti-FITC antibody leads to immune complex formation for SPARCL signal detection. Under such conditions, a sample containing ADA at 250 ng/mL and 50  $\mu$ g/mL of drug gives distinguishable SPARCL signal from a sample containing no ADA. Acid-dissociation provides significant improvement in drug tolerance for the SPARCL bridging assay.

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**Table 1: Drug Tolerance without Acid-Treatment**

Goat anti-mouse (Drug) (µg/mL)	250 ng/mL Mouse IgG (ADA)					0 ng/mL Mouse IgG (ADA)				
	Rep1	Rep2	Rep3	Mean	CV (%)	Rep1	Rep2	Rep3	Mean	CV (%)
0	54367	51656	52654	52892	2.6	3557	3260	3424	3413	4.4
0.25	5911	6302	5894	6035	3.8	3525	3310	3483	3439	3.3
0.50	5766	6079	5928	5924	2.6	3519	3477	3470	3489	0.8
1.0	5821	5877	5729	5809	1.3	3581	3955	3742	3759	5.0
5.0	4529	4474	4405	4469	1.4	3371	3418	3358	3382	0.9
10	3863	3740	3721	3775	2.0	3474	3378	3294	3382	2.7
25	3595	3574	3516	3561	1.1	3272	3596	3270	3379	5.6
50	3283	3339	3387	3336	1.6	3381	3432	3312	3375	1.8

- 1) 25 µL (1.0 µg/mL) FITC-mouse IgG, 25 µL (1.0 µg/mL) biotin-mouse IgG and 15 µL sample in rat serum, overnight incubation at 4°C.
- 2) Premixed solution: 25 µL (1.0 µg/mL) HRP-rabbit anti-FITC; 25 µL (2.0 µg/mL) acridan-streptavidin, 60 min incubation.
- 3) 8µL BGR, 75µL SPARCL trigger.

**Table 2: Drug Tolerance with Acid-Dissociation**

Goat anti-mouse (Drug) (µg/mL)	250 ng/mL Mouse IgG (ADA)					0 ng/mL Mouse IgG (ADA)				
	Rep1	Rep2	Rep3	Mean	CV (%)	Rep1	Rep2	Rep3	Mean	CV (%)
0	87943	91374	91956	90424	2.4	14247	14101	15612	14653	5.7
0.25	64123	64549	63690	64120	0.7	14025	14649	14400	14358	2.2
0.50	56344	60808	57659	58270	3.9	14333	15130	13944	14469	4.2
1.0	50533	52114	51670	51439	1.6	13648	13942	13787	13792	1.1
5.0	32259	32323	31209	31930	2.0	13673	14152	13992	13939	1.7
10	23341	23267	22273	22960	2.6	13681	13288	14090	13686	2.9
25	17656	18663	18398	18239	2.9	13355	12965	14449	13590	5.7
50	15649	16466	16238	16118	2.6	14199	13193	13970	13787	3.8

- 1) 60 µL of 300 mM acetic acid and 15 µL of sample in rat serum, 30 min incubation.
- 2) Premixed solution: 12.5µL (2.0 µg/mL) biotin-mouse IgG; 12.5 µL (2.0 µg/mL) FITC-mouse IgG; 13 µL of 1.5 M TRIS base, 60 min incubation.
- 3) Premixed solution: 12.5 µL (2.0 µg/mL) HRP-rabbit anti-FITC; 12.5 µL (4.0 µg/mL) acridan-streptavidin, 60 min incubation.
- 4) 8µL BGR, 100µL SPARCL trigger.

## CONCLUSION

SPARCL™ technology provides a versatile tool for development of sensitive bridging assays for immunogenicity applications. As indicated in this study, a SPARCL bridging assay offers distinct advantages including:

- 1) Simple protocol with no wash step
- 2) Use of readily available generic reagents
- 3) High sensitivity, reproducibility and drug tolerance
- 4) Inexpensive instrumentation

Such assays represent great opportunities for cost saving while maintaining uncompromised assay performance.

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