CiToxLAB
Safety and Health Research Laboratories

TOXICOLOGY SERVICES

- General toxicology:
  - Rodents
  - Non-rodents: dogs, NHPs and minipigs
- Infusion
- Inhalation
- Dermal
- Ocular
- Immunotoxicology
- Reproductive toxicology including minipigs and NHPs
- Carcinogenicity studies also in rasH2 and p53+/- mice
- Genetic toxicology: ICH compliant package
- *In vitro* toxicology: BCOP, MUSST, DPRA, Photo 3T3, Episkin™
- Agrochemical / Chemical / REACH
- QSAR
- Physical chemistry
- Ecotoxicology: wide range of test species

SAFETY PHARMACOLOGY

- Integrated Safety Pharmacology in Toxicology Studies
  - CV (JET), BP
  - Respiratory (JET), plethysmography
  - CNS (FOB) and JET-EEG
- Safety pharmacology core battery
- Early safety pharmacology screening
  - hERG
  - Rodent and non-rodent LVP telemetry
  - Anesthetized models: ECG, ABP, LVP and QA

DMPK AND BIOMARKERS

- Radiolabelled DMPK: in all species
- Bioanalysis LC-MS/MS, GC-MS/MS, LC-ICP/MS, ELISA, RIA
- Toxicogenomics, miRNA: Affymetrix™ Accredited service provider, Next Generation Sequencing (Illumina®)
- Immunology: 10-color flow cytometer, Luminex, Mesoscale

SPECIALIZED EXPERTISE

- Juvenile studies including minipigs
- Fertility studies in rodents and NHPs
- Radiation safety and efficacy studies
- Tissue Cross Reactivity: human and animal tissue banks
- Gene therapy vector biodistribution via qPCR
- ES cell testing: devTOX™ and cardioTOX™ (with Stemina)
- Lead optimization and predictive toxicology services: Leadscreen™

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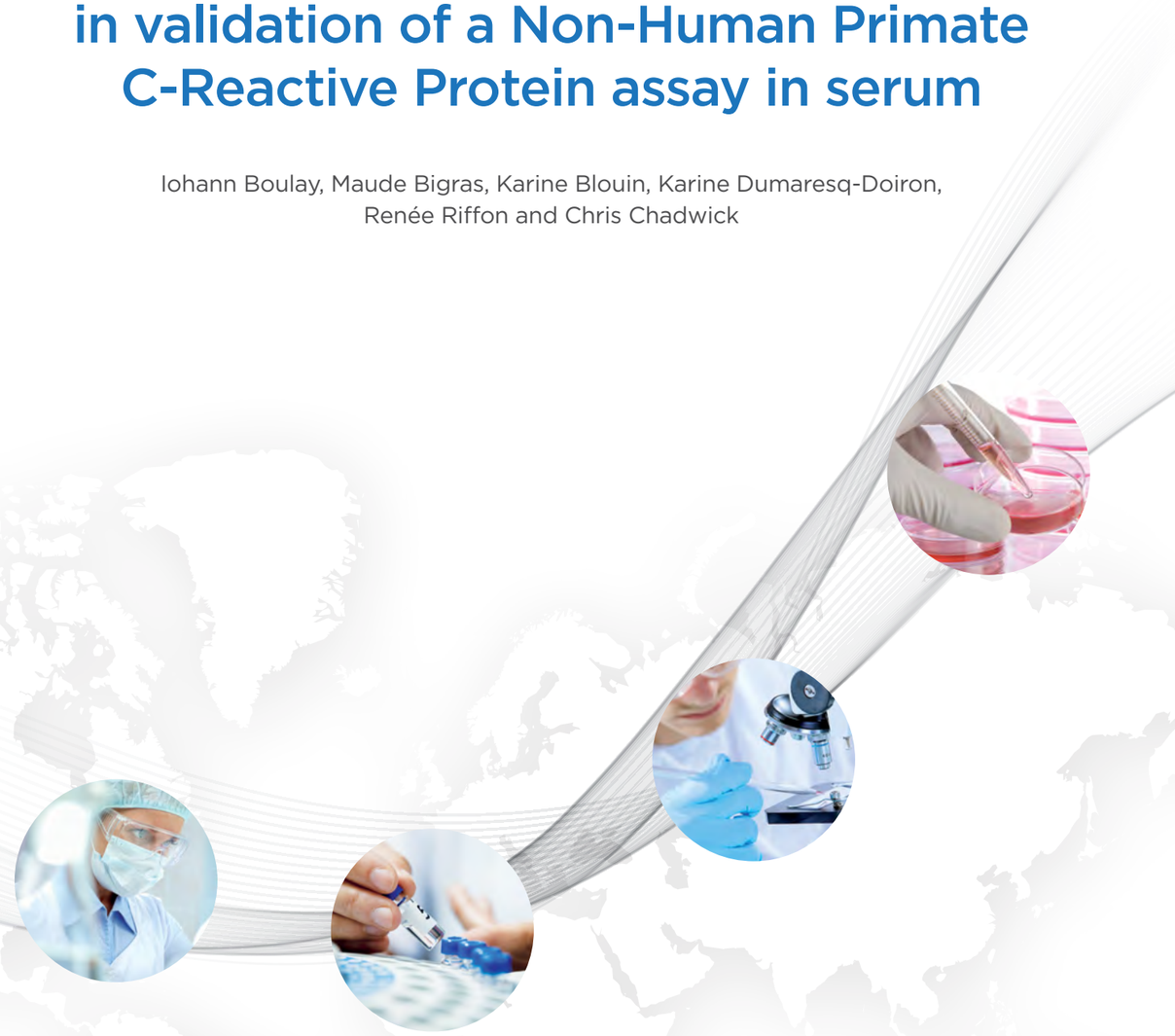
SPARCL™: Use of a novel technology in validation of a Non-Human Primate C-Reactive Protein assay in serum

Iohann Boulay, Maude Bigras, Karine Blouin, Karine Dumaresq-Doiron, Renée Riffon and Chris Chadwick



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SPARCL™: Use of a novel technology in validation of a Non-Human Primate C-Reactive Protein assay in serum

Iohann Boulay [1], Maude Bigras [1], Karine Blouin [1], Karine Dumaresq-Doiron [1], Renée Riffon [1] and Chris Chadwick [2]

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INTRODUCTION

Biomarker assays have become more widely used over the years due to their role in drug discovery and development. C-Reactive Protein (CRP) is an acute-phase protein synthesized by the liver and released in response to tissue injury, infection or inflammation (1). CRP is an annular (ring-shaped), pentameric protein which level increases following interleukin-6 secretion by macrophages and T cells. Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via the C1Q complex (2).

Up to now CRP has been quantified using ELISA (Enzyme-Linked Immunosorbent Assay), but alternative methodology is being explored to facilitate the integration of CRP analysis in toxicology studies.

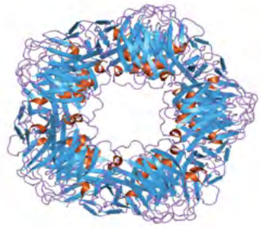

Figure 1. Pentameric C-reactive protein

METHODOLOGY

SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) novel technology is a proximity-dependent, homogenous, chemiluminescent detection method that allows rapid and cost effective immunoassay development, validation and sample analysis. In a SPARCL assay, a chemiluminescent substrate (acridan) is brought into the proximity of an oxidative enzyme (horseradish peroxidase: HRP) through a specific antigen/antibody interaction. A flash of light proportional to the quantity of analyte present in the sample is generated upon addition of a trigger solution containing H2O2 and para-hydroxycinnamic acid (pHCA). There is no need to remove excess reactants, as acridan-conjugated antibodies distant from HRP produce no signal. Furthermore, to enhance the signal to noise ratio, a background reducing agent can be added to minimize the background signal from unbound reactants (3).

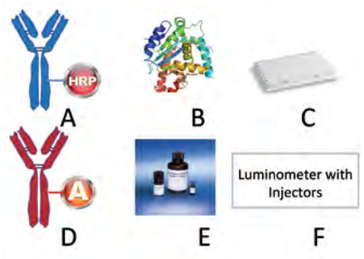

Figure 2. SPARCL key components. A. HRP labeled Antibody. B. Immunoassay target (analyte). C. 96 well low binding white plate. D. Acridan labeled Antibody. E. SPARCL Kit. F. Luminometer

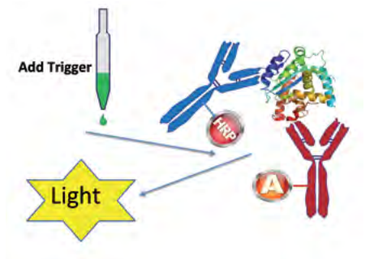

Figure 3. A Representative SPARCL assay. Specific antibody and antigen interaction brings acridan and HRP into close proximity, the addition trigger solution then causes a flash of light

Assay workflow

The Monkey CRP SPARCL kit produced by Life Diagnostics, Inc. was selected and slightly adapted for use.

- affinity-purified CRP-specific antibodies are mixed with standards, positive controls or serum samples in a 96-well white plate
- background-reducing reagent is added after sample incubation
- the plate is placed in a luminometer, then the trigger solution is injected into each well and luminescence is immediately measured.

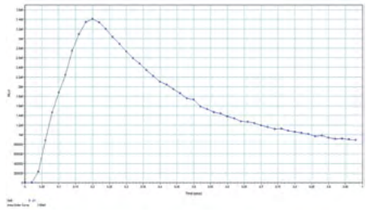

Figure 4. Example of a SPARCL™ flash luminescence signal for the highest CRP standard. Luminescence was measured every 0.02 seconds for 1 second.

RESULTS

Precision and Ruggedness

Precision samples consisted of Cynomolgus monkey serum containing endogenous CRP. For ULOQ (Upper Limit of Quantification) and high levels, serum from animals induced with LPS (lipopolysaccharide) was used. An intra-assay precision assessment was also performed in Rhesus monkey serum.

Table 1. Intra- and Inter-Assay Precision Results and Ruggedness (inter-analyst precision) in Cynomolgus monkey sera

Sample ID	Intra-assay precision		Inter-assay precision		Ruggedness	
	Mean (range; ng/mL)	%CV (range)	Overall Mean (ng/mL)	Overall %CV	Mean analyst A (ng/mL)	Mean analyst B (ng/mL)
ULOQ	222.5 - 367.5	1.3 - 21.8	249.3	15.1	246.1	254.6
					%difference	-3.4
High	144.8 - 187.1	3.1 - 13.6	171.7	13.3	160.6	184.0
					%difference	-13.7
Medium	46.22 - 54.47	1.1 - 5.7	50.34	7.1	50.08	50.63
					%difference	-1.1
Low	12.05 - 15.31	2.0 - 7.2	13.03	9.5	12.55	13.56
					%difference	-7.7
LLOQ	3.520 - 5.127	1.3 - 7.0	4.357	12.6	4.053	4.694
					%difference	-14.7

Table 2. Intra-Assay Precision Results in Rhesus monkey sera

Sample ID	Intra-assay precision	
	Mean (ng/mL)	%CV
ULOQ	297.8	8.4
High	211.6	6.0
Medium	53.08	4.4
Low	14.98	2.0
LLOQ	3.934	1.8

Selectivity

Monkey serum from individuals with low and high levels of endogenous CRP was spiked with an amount of CRP equivalent to the endogenous level.

Table 3. Selectivity in various Cynomolgus monkeys

Sample ID	CRP Concentration (ng/mL)				%RE
	Unspiked Measured	Spiked	Expected	Spiked Measured	
Female 1- High	78.55	75.00	153.55	183.89	19.8
Female 2 - High	41.61	35.00	76.61	83.22	8.6
Female 3 - High	115.86	95.00	210.86	268.33*	NA
Male 1 - High	71.64	75.00	146.64	169.28	15.4
Male 2 - High	96.58	75.00	171.58	211.68	23.4
Male 3 - High	106.28	95.00	201.28	387.10*	NA

Sample ID	CRP Concentration (ng/mL)				%RE
	Unspiked Measured	Spiked	Expected	Spiked Measured	
Female 1- Low	18.41	12.00	30.41	29.91	-1.6
Female 2 - Low	13.94	12.00	25.94	28.85	11.2
Female 3 - Low	14.20	12.00	26.20	28.42	8.5
Male 1 - Low	19.59	12.00	31.59	33.74	6.8
Male 2 - Low	15.49	12.00	27.49	28.94	5.3
Male 3 - Low	13.27	12.00	25.27	25.90	2.5

*Result above quantification range, % relative error cannot be determined

Parallelism

Samples from non-treated and LPS-induced individuals were tested for parallelism. Depending on the CRP levels present in the samples, serial dilutions were prepared to bring the samples into the quantification range. Results at the MRD (Minimum Required Dilution) or beyond (depending on the dilution range) were used for reference in the evaluation of the % difference upon dilution.

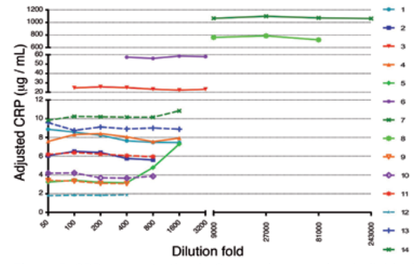

Figure 5. CRP concentration in Cynomolgus monkey individual sera upon dilution (between 50- to 243 000-fold)

Table 4. Parallelism in various Cynomolgus monkeys

Sample ID	Dilution factor (range)	Adjusted Result (µg/mL)	% difference (range)	Overall %CV
Female 1	100 - 3.200	22.30 - 25.84	-10.5 to 4.2	5.6
Female 2	50 - 1.600	7.557 - 8.417	-0.3 to 10.5	4.5
Female 3	400 - 3.200	56.13 - 58.59	-2.2 to 2.1	1.8
Female 4	50 - 800	5.949 - 6.384	-3.8 to 3.2	2.7
Female 5	50 - 400	1.782 - 1.862	2.0 to 4.4	1.9
Female 6	50 - 1.600	9.829 - 10.835	3.3 to 9.7	3.2
Female 7 (LPS induced)	9.000 - 243.000	1.061 - 1.097	-0.3 to 3.1	1.5

Sample ID	Dilution factor (range)	Adjusted Result (µg/mL)	% difference (range)	Overall %CV
Male 1	50 - 1.600	7.455 - 8.867	-17.3 to -3.4	7.5
Male 2	50 - 800	5.605 - 6.516	-7.6 to 7.4	6.5
Male 3	50 - 1.600	3.162 - 7.300	-2.8 to 76.7	39.3
Male 4	50 - 400	3.075 - 3.417	-10.5 to -2.3	5.2
Male 5	50 - 800	3.658 - 4.211	-13.3 to 0.8	6.7
Male 6	50 - 1.600	8.746 - 9.579	-9.1 to -5.0	3.2
Male 7 (LPS induced)	9.000 - 81.000	722.0 - 785.7	-5.4 to 3.1	4.3

Table 5. Parallelism in various Rhesus monkeys

Sample ID	Dilution factor (range)	Adjusted Result (µg/mL)	% difference (range)	Overall %CV
Female 1	50 - 1.600	7.845 - 8.278	0.7 to 5.4	2.3
Female 2	50 - 800	3.353 - 3.928	-15.8 to -1.6	6.3
Female 3 (LPS induced)	4.800 - 19.200	795.2 - 833.5	1.0 to 4.7	2.5
Female 4 (LPS induced)	4.800 - 19.200	772.5 - 808.9	0.5 to 4.6	2.6
Male 1	50 - 800	3.580 - 3.944	-1.1 to 8.6	4.2
Male 2	50 - 800	3.861 - 4.231	-3.0 to 6.2	3.4
Male 3	50 - 800	3.956 - 4.130	-1.4 to 2.9	1.8

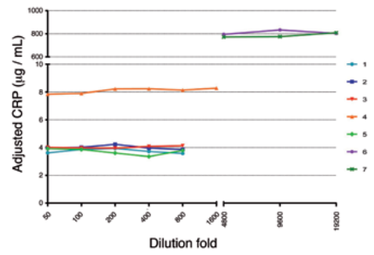

Figure 6. CRP concentration in Rhesus monkey individual sera upon dilution (between 50- to 19 200-fold)

Stability

Samples containing high and low levels of endogenous CRP were used to assess the various stability conditions. Long-term stability is currently ongoing, but was demonstrated to be at least 70-days when stored at -70°C.

Table 6. Monkey CRP assay: Stability results

Conditions	Bench-top for 18h		3 Freeze-thaw cycles		Long-term stability (70 days) at -70°C	
Sample ID	Low	High	Low	High	Low	High
Reference concentration (ng/mL)	13.03	171.68	13.03	171.68	13.03	171.68
Mean	13.38	168.89	12.77	150.26	12.95	150.23
%CV	1.5	8.1	2.3	7.6	2.2	3.3
%RE	2.7	-1.6	-2.0	-12.5	-0.6	-12.5
n	3	3	4	4	4	4

Hemolysis

Hemolysis was shown to have no impact on CRP measurement.

Table 7. Impact of hemolysis in various monkey individuals

Sample ID	Adjusted Result (µg/mL)	Hemolyzed sample Adjusted Result (µg/mL)	% RE
Male 1	5.254	4.558	-13.2
Male 2	4.073	3.797	-6.8
Female 1	2.966	2.814	-5.1

C-reactive protein induction in NHP

The assay could be successfully used to study CRP induction kinetic as it has been demonstrated that CRP significantly increases from 8h after dosing animals with LPS. The assay could also be used in the study of inflammation response to coronary (LAD) ligation, in which CRP increase was observed shortly after cardiac Troponin-I (cTnI) was released (as measured using another validated SPARCL cTnI assay).

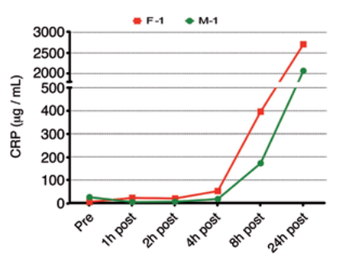

Figure 7. CRP concentration in Cynomolgus sera before and after LPS treatment

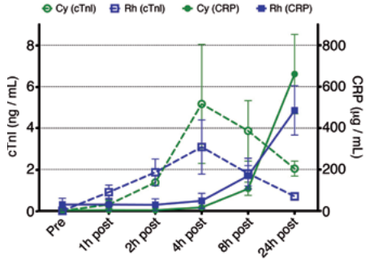

Figure 8. cTnI and CRP concentrations in Cynomolgus and Rhesus (n=2 each) monkey sera before and after coronary ligation

CONCLUSION

A novel method for the quantification of CRP in monkey serum samples, SPARCLTM, was successfully validated. The SPARCL assay presents an advantage of short assay run times since no washing is required. It allows high sample throughput, and the analytical range covers relevant concentrations in non-human primates.

REFERENCES

1. Du Clos T. W. Annals of Medicine, 2000, 32(4): 274-8 - 2. Thompson D. et al. Structure. 1999; 7(2): 169-177 - 3. Text and Figures 1 and 2 courtesy of Beckman Coulter Inc. - 4. Guidance for Industry: Bioanalytical Method Validation (2001), CDER and CVM. - 5. Jean W. Lee et al. Pharm Res. 2005; 23: 312-328 - 6. Valentin M.-A. et al., J. Pharm. Biomed. Anal. 2011; 55: 869-77. SPARCL is a trademark of Lumigen, Inc. and Beckman Coulter, Inc.