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 and Rat cardiac Troponin-I assay in serum

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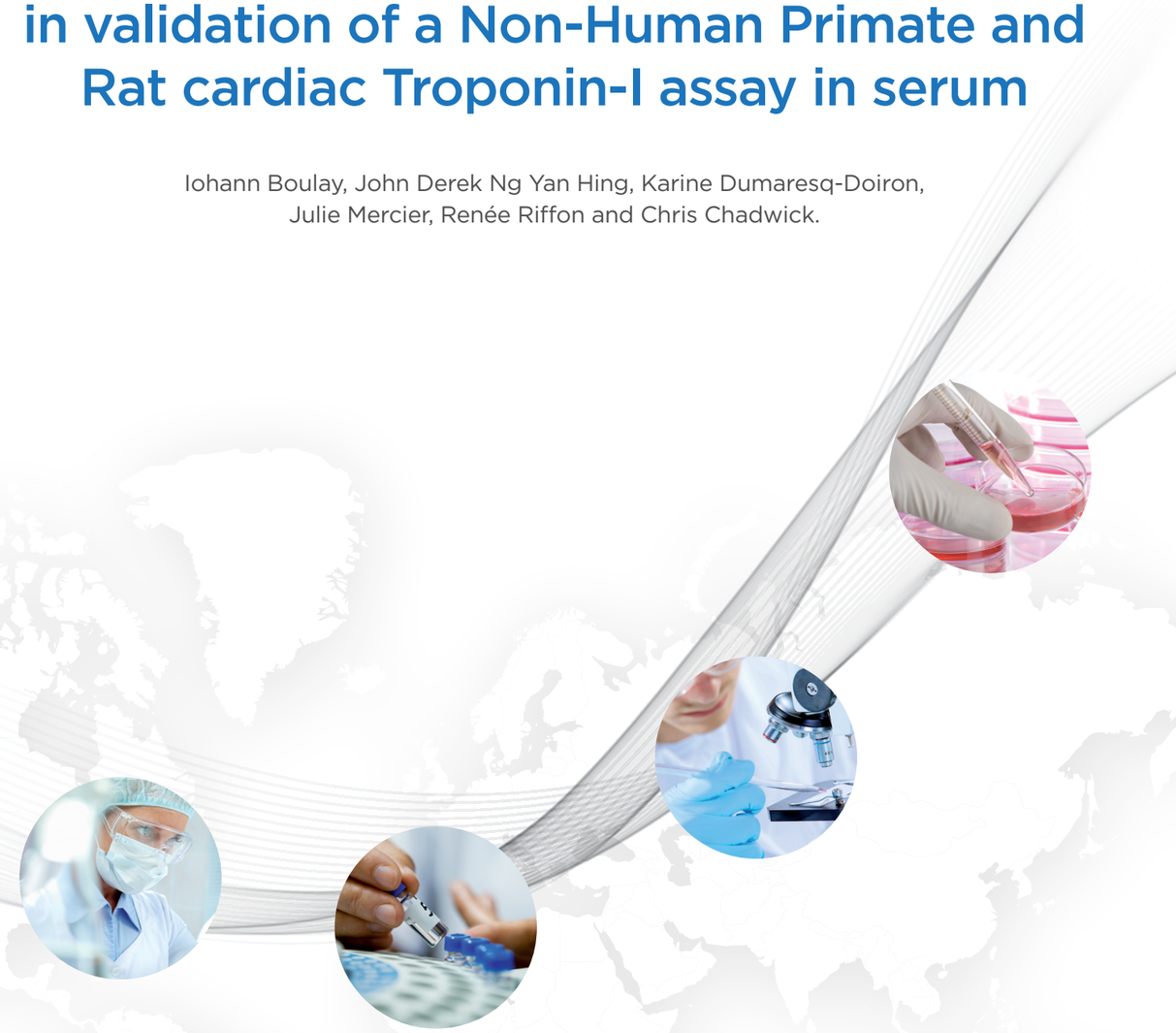

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SPARCL™: Use of a novel technology in validation of a Non-Human Primate and Rat cardiac Troponin-I assay in serum

Iohann Boulay [1], John Derek Ng Yan Hing [1], Karine Dumaresq-Doiron [1], Julie Mercier [1], Renée Riffon [1] and Chris Chadwick [2].

1. CiToxLAB North America - 2. Life Diagnostics, Inc.

ABSTRACT

Purpose : Biomarker assays have gained precedence over the years due to their role in drug discovery and development. Cardiac Troponin-I (cTnI), one of the three Troponin-I isoforms, is released into the bloodstream from damaged cardiac muscle cells. The evaluation of cTnI serum levels therefore allows assessment of the extent of cardiac injury. Historically quantified by traditional ELISA, an alternative assay methodology was explored to facilitate integration of cTnI analysis in toxicology studies.

Method : A SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) assay was used herein to validate a Non-Human Primate and Rat cTnI quantification method. SPARCL is a novel technology that allows rapid and cost effective immunoassay development, validation and sample analysis. The principle lies on proximity assay in homogenous phase. Two affinity purified cTnI-specific antibodies were used, one conjugated to HRP and one to acridan, a chemiluminescent substrate. When these two antibodies are brought into close proximity due to specific binding and when a solution containing hydrogen peroxide is added, HRP catalyzes oxidation of proximal acridan molecules causing a chemiluminescent reaction, which is captured by a plate-based luminometer.

Results : Following adaptation of a commercially available cTnI Non-Human Primate and Rat assay, an advanced method validation was performed, according to industry guidelines. In order to better characterize the performance of the assay, serum samples were obtained from animals which underwent heart ligature, which significantly increased the cTnI levels over 8 hours. These samples were used to define precision, ruggedness, parallelism, stability and minimum required dilution. An acceptable standard curve fit was obtained between 0.1 and 5.0 ng/mL. Quantification range was confirmed using precision samples at different endogenous cTnI levels and the ones at low, medium and high concentrations were then used as quality controls (QCs) for plate acceptance, once their reference concentrations were determined. Parallelism was proven using serum samples from different Non-Human Primates and Rats in order to span the widest range of dilution folds. Moreover, samples at low and high concentrations were used to demonstrate stability after 3 freeze-thaws, overnight at room temperature and after long-term storage at -70˚C.

Conclusion : A novel method for the quantification of cTnI in Non-human primate and Rat serum samples was successfully validated. The SPARCL assay was found to present short assay run time since no wash are required. It allowed a high throughput for method validation and sample analysis with an analytical range that covered relevant concentrations upon cardiac injury in the Non-human primate and Rat.

METHODOLOGY

SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) technology is a proximity dependent, non-separation, chemiluminescent detection method. In a SPARCL assay, a chemiluminescent substrate (acridan) is brought into the proximity of an oxidative enzyme (horseradish peroxidase, HRP) through the 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 H_2O_2 and para-hydroxycinnamic acid (pHCA). There is no need to remove excess reactants, acridan conjugated antibodies distant from HRP produce no signal. Furthermore, to enhance signal to noise ratio, a background reducing agent can be added to minimize the background signal from unbound reactants (1).

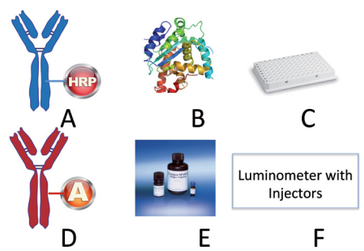


Figure 1. SPARCL key components. A. HRP labeled Antibody. B. Immunoassay target (analyte). C. 96 well low binding white plate. D. Acridan labeled Antibody. E. SPARCL Kit. F. Luminometer.

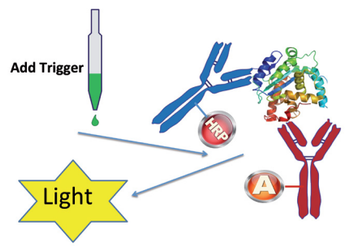


Figure 2. A Representative SPARCL assay. Specific antibody and antigen interaction brings acridan and HRP into close proximity, the addition trigger solution then causes a flash of light.

INTRODUCTION

Cardiac Troponin-I :

Troponin I is a subunit of the troponin complex (Tn), which is a heteromeric protein bound to the thin filament. Troponin complex plays an important role in the regulation of skeletal and cardiac muscle contraction. Troponin complex consists of three subunits: troponin T (TnT), troponin I (TnI) and troponin C (TnC). The subunits are held together by non-covalent interactions. The TnI subunit is responsible for inhibiting actomyosin formation at low intracellular Ca2+ concentrations. Being expressed only in cardiac tissue, Troponin I has been a preferred biomarker for myocardial infarction for a long time (2).

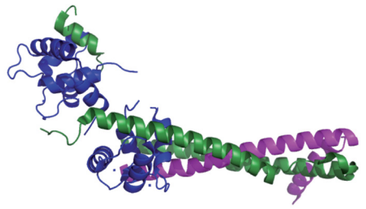


Figure 3. Ribbon representation of the human cardiac troponin core complex. Blue = troponin C; green = troponin I; magenta = troponin T (3).

Assay workflow :

Rat and Monkey cTnI SPARCL kits from Life Diagnostics, Inc. were selected and slightly adapted for use.

- affinity-purified cTnI-specific antibodies are pre-mixed with background reducing agent
- antibodies are mixed with standards, positive controls or samples in a 96-well white plate.
- the whole mixture is incubated at room temperature for 30 minutes.
- once placed in the luminometer, the trigger solution is injected into each well and luminescence is immediately measured.

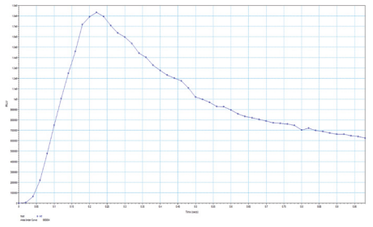


Figure 4. Example of a SPARCL™ flash luminescence signal for the highest rat cTnI standard. Luminescence was measured every 0.02 seconds for 1 second.

RESULTS

Precision and Ruggedness :

Precision samples at ULOQ and high concentrations were prepared by spiking the monkey cTnI stock (Reference standard) in Cynomolgus monkey serum, while for precision medium, low and LLOQ levels, unspiked Cynomolgus monkey serum from animals that underwent coronary (LAD) ligation surgery were used. Similarly, precision samples at ULOQ concentration were prepared by spiking the rat cTnI stock (Reference standard) in Sprague-Dawley rat serum, while for precision high, medium, low and LLOQ levels, unspiked Sprague-Dawley rat serum from animals that underwent surgery were used.

Table 1. Monkey cTnI assay: Intra- and Inter-Assay Precision Results and Ruggedness (inter-analyst precision).

Sample ID	Intra-assay precision n = 3 per run		Inter-assay precision Overall n = 18		Ruggedness	
	Mean (range; ng/mL)	%CV (range)	Overall Mean (ng/mL)	Overall %CV	Mean analyst A (ng/mL)	Mean analyst B (ng/mL)
ULOQ	4.71 - 5.44	**1.1 - 5.4**	5.04	**6.5**	4.85 **%difference**	5.23 **-7.5**
High	3.49 - 3.82	**1.2 - 4.7**	3.68	**4.1**	3.63 **%difference**	3.73 **-2.6**
Medium	1.27 - 1.47	**0.9 - 4.7**	1.34	**6.4**	1.39 **%difference**	1.29 **7.8**
Low	0.26 - 0.35	**0.5 - 7.0**	0.31	**11.5**	0.33 **%difference**	0.29 **12.2**
LLOQ	0.18 - 0.24	**0.9 - 11.2**	0.21	**11.7**	0.22 **%difference**	0.20 **7.8**

Table 2. Rat cTnI assay: Intra- and Inter-Assay Precision Results and Ruggedness (inter-analyst precision).

Sample ID	Intra-assay precision n = 3 per run		Inter-assay precision Overall n = 18		Ruggedness	
	Mean (range; ng/mL)	%CV (range)	Overall Mean (ng/mL)	Overall %CV	Mean analyst A (ng/mL)	Mean analyst B (ng/mL)
ULOQ	4.81 - 5.22	**2.1 - 6.1**	5.01	**4.6**	5.02 **%difference**	5.00 **0.5**
High	3.07 - 3.35	**1.1 - 4.2**	3.19	**4.3**	3.24 **%difference**	3.14 **3.0**
Medium	0.68 - 0.80	**0.6 - 4.7**	0.74	**6.8**	0.78 **%difference**	0.70 **10.3**
Low	0.16 - 0.19	**1.1 - 4.3**	0.17	**6.3**	0.18 **%difference**	0.17 **8.3**
LLOQ	0.075 - 0.099	**0.6 - 9.5**	0.09	**10.4**	0.09 **%difference**	0.08 **13.7**

Parallelism :

Positive samples generated through coronary ligation were tested for parallelism. Depending on the cTnI levels present in the samples, various dilution series were generated as to fall within the quantification range. Results from the dilution at MRD were used as reference for evaluation of the % difference upon dilution. Acceptable parallelism was also demonstrated in Wistar rats (between 8- to 198-fold; data not shown).

Table 3. Parallelism in various monkey individuals.

Sample ID	Dilution factor (range)	Adjusted Result (ng/mL)	% difference (range)	Overall %CV
Female 1 (Cyno)	4.0 - 10.0	3.07 - 3.68	**4.2 - 18.1**	**6.4**
Male 1 (Cyno)	4.0 - 30.4	7.72 - 9.70	**-21.7 - 1.0**	**7.9**
Male 2 (Cyno)	4.0 - 30.4	4.78 - 5.40	**-8.5 - 3.8**	**4.6**
Male 3 (Cyno)	4.0 - 10.0	1.31 - 1.55	**5.6 - 18.1**	**8.6**
Female 2 (Cyno)	4.0 - 14.9	2.59 - 2.81	**-0.9 - 7.2**	**3.3**
Female 1 (Rhesus)	4.0 - 10.0	1.78 - 1.93	**-7.0 - 0.8**	**3.6**
Female 2 (Rhesus)	4.0 - 30.4	4.59 - 5.24	**-11.1 - 2.3**	**5.3**

Table 4. Parallelism in various rat individuals (Sprague-Dawley)

Sample ID	Dilution factor (range)	Adjusted Result (ng/mL)	% difference (range)	Overall %CV
Female 1	8.0 - 19.9	2.07 - 2.61	**7.9 - 23.2**	**8.1**
Male 1	8.0 - 60.8	8.62 - 9.86	**2.5 - 13.5**	**4.7**
Male 2	8.0 - 43.0	4.15 - 5.74	**10.7 - 32.2**	**11.3**
Male 3	8.0 - 60.8	6.80 - 9.39	**10.2 - 32.0**	**10.7**
Female 2	8.0 - 151.2	12.13 - 15.61	**2.6 - 25.1**	**9.9**
Female 3	8.0 - 151.2	15.83 - 19.27	**2.0 - 19.6**	**7.2**
Female 4	8.0 - 113.6	11.21 - 13.25	**2.5 - 16.7**	**6.1**
Female 5	8.0 - 113.6	10.84 - 13.60	**-2.3 - 20.2**	**8.6**

Figure 5: Troponin I (cTnI) concentration in NHP individual sera upon dilution (between 4- to 30-fold).

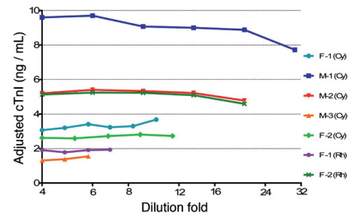
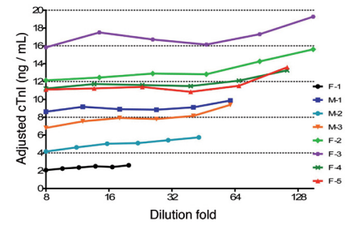


Figure 6: Troponin I (cTnI) concentration in Rat individual sera upon dilution (between 8- to 151-fold).



Stability : The same positive sera samples used as detailed above were also used to assess the various stability parameters. Long-term stability is currently ongoing but was demonstrated to be at least 29-days when stored at -70°C in non-human primate serum during assay development.

Table 5. NHP cTnI assay: Stability results.

Conditions	Bench-top		3 Freeze-thaw cycles	
Sample ID	Low	High	Low	High
Reference concentration (ng/mL)	1.55	5.35	1.55	5.35
Mean	1.83	6.31	1.78	6.17
%CV	**3.0**	**1.3**	**2.1**	**1.0**
%RE	**18.0**	**18.0**	**14.9**	**15.3**
n	3	3	3	3

Table 6. Rat cTnI assay: Stability results.

Conditions	Bench-top		3 Freeze-thaw cycles	
Sample ID	Low	High	Low	High
Reference concentration (ng/mL)	1.39	25.51	1.39	25.51
Mean	1.36	22.17	1.47	23.40
%CV	**1.1**	**1.5**	**1.3**	**3.6**
%RE	**-2.1**	**-13.1**	**5.6**	**-8.3**
n	3	3	3	3

Cardiac Troponin release in NHP and Rat : The assay could be successfully used to study cTnI release kinetic upon coronary (LAD) ligation. Significant increases were demonstrated between 4-8 hours post-surgery.

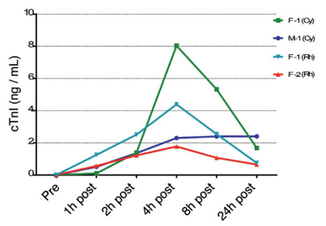


Figure 7: Troponin I (cTnI) concentration in NHP sera before and after coronary ligation.

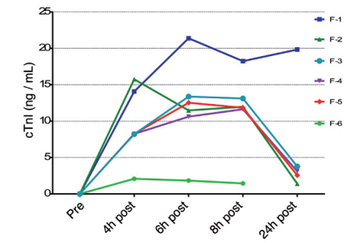


Figure 8: Troponin I (cTnI) concentration in Sprague-Dawley rat sera before and after coronary ligation.

CONCLUSION

A novel method for the quantification of cTnI in Non-human primate and Rat serum samples was successfully validated. The SPARCL assay was found to present short assay run time since no wash are required. It allowed a high throughput for method validation and sample analysis with an analytical range that covered relevant concentrations upon cardiac injury in the Non-human primate and Rat.

REFERENCES

1. Text and Figures 1 and 2 courtesy of Beckman Coulter Inc. - 2. Cardiac Troponin I booklet (Feb 2015), HyTest - 3. Takeda S, Yamashita A, Maeda K, and Maeda Y (2003). Nature 424(6944): 35–41. - 4. Guidance for Industry: Bioanalytical Method Validation (2001), CDER and CVM. - 5. Jean W. Lee et al. (2005). Pharm Res 23: 312-328 - 6. Valentin M.-A. et al., J. Pharmaceutical and Biomedical Analysis, 2011, 55: 869-77.