Review of Qualification Data for Cardiac Troponins

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Biomarker Qualification Review

1. EXECUTIVE SUMMARY

This is a review by the Biomarker Qualification Review Team (BQRT) of a submission by PJ O'Brien, WJ Reagan, MJ York, and MC Jacobsen for the nonclinical qualification of cardiac troponins T and I measured in serum/plasma as biomarkers of cardiac morphologic damage.

a. Background

The Biomarker Qualification Process at the FDA evaluates proposals for biomarker qualification submitted by scientists from multiple organizations. This document reviews the data contained in the 2008-2009 O'Brien et al. submission supporting the request for qualification of the use of cardiac troponins T and I in nonclinical drug development studies in rats, dogs, and monkeys. This is the first submission received by the BQRT seeking qualification of a serum/plasma biomarker as an indicator of cardiac morphologic injury.

This submission was based upon work already published and is the first literature-based submission reviewed by the BQRT. This is also a situation of reverse translation, as the cardiac troponins are already accepted and used in both human and veterinary medicine as the preferred standard for detecting the presence and extent of myocardial necrosis and injury.

The majority of work supporting the qualification of the cardiac troponins was generated prior to recently established recommendations for biomarker qualification and a portion of the data does not meet the new guidelines. In the opinion of the BQRT the strength of the data outweigh the limitations and warrants the qualification of cardiac troponins for use in non-clinical drug development.

b. Sources of Data and Major Findings

In the original application, O'Brien et al cited 240 papers from the peer reviewed scientific literature. At the request of the BQRT, a subset of 90 references was identified by the sponsor as the most critical references for the review. The sponsors noted that any one study considered in isolation was insufficient to support qualification. The studies required collective review.

Rats: In rats, circulating cardiac troponins T (cTnT) and I (cTnI) were at least as effective as histopathology in identifying cardiac damage. Increases in circulating troponins preceded the changes found with routine light microscopy and hematoxylin & eosin (H&E) staining. When special histochemical stains and/or immunostaining for the troponins were examined, it was found that the increase in troponins could be correlated with morphologic changes detectable by these alternate methods. Cardiac troponins were also found to outperform creatine kinase (CK) isoforms and lactate dehydrogenase (LDH) isoforms in the detection of cardiac injury.

Dogs: In dogs, circulating cTnT and cTnI were at least as effective as histopathology using H&E staining in identifying cardiac damage. The cardiac troponins were found to be more sensitive biomarkers of myocardial injury than CK, CK-MB, aspartate aminotransferase (AST) and LD. Tissue data were supportive of the validity of early increases in circulating troponins as markers of injury by showing loss of immunostaining in cardiac myocytes within 0.5 hour after coronary

occlusion. These findings were supported by electron microscopic evidence of necrosis while H&E stained light microscopy sections showed edema only.

Non-human primates (NHP): The database for non-human primates is less extensive than that for rats or dogs. Histology was used as a reference standard in two of the five studies submitted. The primate studies are based on the premise that 1) cTns are as effective in detecting cardiac injury in non-human primates as in human primates 2) cTns are expected to behave in a manner consistent with the other species in which troponins have been examined. The data, though limited, show that circulating troponins in non-human primates can be measured reliably by commercially available assays approved for human in vitro diagnostic use and suggest that troponins can detect cardiac damage in non-human primates consistent with findings from other species.

Quantitative relationships between cTns and damaged myocardium: Studies in rats, dogs, pigs and humans demonstrated a quantitative correlation between the amount of TnT and TnI lost from ischemic, infarcted and remodeled areas of the myocardium and the levels of the respective biomarker in circulation.

c. Limitations of the Data

The limitations of the data are those that might be expected for a biomarker evolving slowly over time, through the work of independent investigators.

1. There were peer-reviewed publications for over 40 different compounds tested using the cardiac troponins as indicators of cardiac toxicity. By species, the following number of chemicals were tested and reported in the literature: rats (25), dogs (>7), rabbits (5), mice (3), and monkeys (2). Where reports did not specify an exact number of proprietary chemicals, the symbol ">"was used to indicate the minimum number of chemicals identified. Known cardiotoxins such as isoproterenol and doxorubicin were tested multiple times in several species and represented a large proportion of the database.

Table1. Summary of the Number of Cardiotoxic Chemicals Tested per Species

Species	Total # of chemicals tested in the species	# of cardiotoxic chemicals	# of Non-cardiotoxic chemicals tested	Unknown or proprietary
Rat	25	12	4	9
Mouse	>7	7		Remainder
Dog	>7	6	1	Remainder
Monkey	2	2		
Rabbit	5	4	1	

Note: The designation ">" in the table indicates that some references did not specify the exact number of proprietary compounds tested in that set of studies.

2. Histopathological evaluation was not standardized across studies. Certain aspects of the histological evaluations did not meet recently described criteria for optimum characterization: These include:

    i. Lack of consistent severity of lesion grading scales. There is no universal grading system for cardiac morphologic damage and the scales used were of necessity established by individual investigators or consortia,.

    ii. Lack of standardized/illustrated scales of histologic lesions (lexicons). Numerous investigators used pre-specified criteria and lexicons that were in local rather than general use.

    iii. Lack of standardized system of evaluation. The details of slide processing and evaluation were not always clear. Frequently the publications did not specify whether the pathologist was blinded to treatment groups during assessment of slides

3. Comparisons to other biomarkers (e.g., clinical chemistry, imaging, electrocardiogram (ECG)) were not always conducted in the same manner from study to study.

4. The published database for primates was not as extensive as that for rodents and dogs. Due to the cost of primates, the majority of studies available did not include euthanasia and histology.

d. Strengths of the Data

Strengths of the data include

1. Consistency of results across laboratories, time and experimental protocols used, showing a robustness to the biomarker.
2. Inclusion of retrospective and prospectve safety assessment as well as veterinary clinical investigations of naturally occurring diseases.
3. Availability of information for both sexes of each species for which qualification was sought.
4. Generation of baseline values and normal ranges from control animals in the majority of studies. Several studies investigated reference ranges for clinically healthy animals.
5. Description of factors creating variability and high background that need to be minimized, avoided, or controlled..
6. Description of factors creating true false positives and elevations in troponin secondary to other conditions.
7. Description of factors creating false negatives.
8, Early examination of the applicability of immunologically-based assays for multiple species occurred early in the evolution of this research.
9. Use of different materials for calibration and quality control could be considered both a strength and a limitation. A human standard is now available from the National Institute of Standards and Technology.Purified dog, rat and non-human primate cTnI standards are available commercially.
10. Use of various statistical approaches to analyze the data depending upon the goals of the particular study.

Additional Issues: Histology

At least eight publications evaluating cardiac tropoins as biomarkers specifically state that the histopathologist was blinded to sample identification. Two additional publications indicate blinding, but it is not clear who was blinded to what information. Although more than 20 other cited publications utilized histopathology, no specific statements were found that indicated whether the biomarker technician or the histopathologist were blinded to sample identification.

Several publications raised the issue of examining a limited number of tissue sections to evaluate the sensitivity of troponin measurements relative to macroscopic or microscopic pathology. The ability of one or even a few histologic sections to adequately represent the whole organ is an issue, particularly when the lesions are focal or of minimal severity. While the use of a single histologic section is an accepted practice in safety assessment studies, it is not clear if this practice is appropriate for hypothesis-driven scientific investigation of biomarkers.

The lack of blinding and use of inadequate numbers of sections in histologic evaluation means that these studies fall short of the specific recommendations in the draft guidance *Use of Histology in Biomarker Qualification Studies* designed to improve the efficiency of biomarker characterization. In the case of the troponins, while the majority of studies in the data base do not indicate blinding, the consistency of results found under different experimental conditions and different laboratories provides some reassurance about data integrity.

Increases in circulating troponins without apparent histologic correlate was investigated by several different investigators from the perspectives of 1)understanding the kinetics of release and correlation with the nature and severity of histologic changes; 2)determining whether there was a threshold of damage necessary for detectable increases in circulating levels of troponins; 3) correlating established histological and biochemical measurements of myocardial damage with key molecular events in early stages of myocardial damage, including cTn tissue content and immunoreactivity. These assessments included histology with special histochemical stains in addition to H&E, immunochemical techniques and electron microscopy. Multiple tissue sections were taken for the different histochemical techniques but without discussion about how many sections were necessary for definitive investigation. Despite this shortcoming, the independent investigations established collectively that early increases in circulating troponins were not predictive of damage, but corresponded to active morphologic damage that was not reliably detected with H&E staining.

e. BQRT Conclusions

Based upon consideration of the strengths and limitations of the database, the BQRT concludes that the data contained in the submission support the qualification of the circulating (serum/plasma) cardiac troponins for the following contexts of use as sought by the sponsors. It must be noted, the sponsors state the necessity for appropriate analytical methodology for quantification of the troponins and good study design. The conclusions of the BQRT must begin with the caveat that the absence of an increase in circulating troponins in one safety assessment study cannot be taken as conclusive proof of a lack of cardiac structural damage. To have confidence in the validity of negative troponin data,the non-clinical assessment must include well validated troponin assays, appropriate timing of sample collection, understanding of the metabolite profiles between species, and some understanding of the drug's mechanism of action. At this time it is not possible to make general recommendations for how to determine the optimum time of sample collection for all therapeutics. Evaluating circulating troponins in studies of varying duration with different sampling protocols may be necessary depending on the mechanism of damage.

All of the BQRT conclusions are based on the assumptions that the above conditions have been met.

1. The BQRT agrees that measurement of cardiac troponins can identify the presence and extent of cardiac structural damage in an appropriately designed and conducted study.An absence of an increase in circulating cardiac troponins, however, does not necessarily demonstrate absence of cardiac structural damage.

2. The BQRT agrees with the sponsor's recommendation for application of the context of use:

a) cardiac structural damage has occurred with previous testing of the compound. The role of testing for this context of use is to get a better estimate of the lowest toxic dose or a good estimate of the highest non-toxic dose to help choose doses for human testing by using the cardiac troponins as a clinical chemistry correlate to the histology. In this case, lower doses without increases in cardiac troponins may be used to support a no observed effect level (NOEL) identified by histology. Troponin measurement is not used in this context to gain primary evidence of cardiac toxicity.

b) the compound being tested is of a chemical or pharmacologic class with known cardiac structural toxicity. Measurement of circulating troponins in this context is to augment standard chemical and histopathological tests that have revealed no structural toxicity at the maximum doses used. The purpose is to provide additional evidence using a more sensitive assessment of morphologic cardiotoxic potential of the drug.

3. The BQRT agrees with the proposal to use troponins for reflex testing. Based on the sponsor's definition, reflex testing is initiated in response to unexpected post-mortem morphological findings. Reflex testing refers to the specific and retrospective testing for a clinical pathology correlate to the morphological findings using the reserve or stored serum/plasma samples that were collected

in the same study in which these morphological effects occurred. Additionally, reflex testing introduces the clinical pathology correlative test into subsequent safety assessment studies. In this application, troponin is proposed as a reflex test, included in the studies conducted subsequent to that in which cardiac morphological effects occurred.

f. BQRT Recommendations

Overall, the BQRT agreed with the recommendations made by O'Brien et al.That is,

1.The cardiac troponins are qualified for use in rats and dogs as described above in BQRT conclusions.

2 Because of the paucity of published data and inconsistent results, it is not possible at this time to define a qualified context of use in non-human primate (NHP). Since some individual investigators have successfully used cTn to monitor cardiotoxicity in NHP studies, we highly encourage the collection of troponin data in NHP to increase the existing database.

Additionally the BQRT has the following recommendation:

3. When an official format for submission of biomarker data becomes available, we recommend that sponsors use this format to submit additional cardiac troponin data. The submission of troponin results in a structured format should facilitate the collection, storing, linking and analysis of troponin data with other data submitted using such standards. The analysis of standardized troponin data should stimulate a dialogue between the Agency and sponsors with respect to the enhanced use of this biomarker in drug development.

2. BACKGROUND

a. Overview of the Problem

Recently, cardiovascular toxicity has accounted for the withdrawal of several drug products including tegaserod (withdrawn 2007), sibutramine (withdrawn 2010), and rosiglitazone (2010, in Europe). Analysis of 238 pharmaceuticals in clinical trials prior to 1999 indicated 17% produced cardiovascular toxicity in man (Olson et al., 2000). While the number of compounds abandoned in early development because of cardiotoxicity is not known, significant attrition of candidate drugs in the preclinical setting is likely. Thus, the sum effect of market withdrawals, and clinical, and preclinical attrition make cardiovascular toxicity a major safety issue in drug development. Furthermore. cardiotoxicity is a limiting factor in the use of a wide variety of drugs, including anticancer (Yeh, 2006) and anti-retroviral agents (Lewis, 2004). Heart failure has been associated primarily with the anthracyclines, and also with mitoxantrone, mitomycin, trastuzumab and possibly other chemotherapeutics (Albini 2010). Different aspects of highly active antiretroviral therapy (HARRT) have pathophysiological sequelae, contributing both to cardiovascular disease and, in the case of nucleoside reverse trascriptase inhibitors (NRTI), cardiovascular toxicity (Lewis, 2004; Farrugia, 2009). For such drugs, it is especially important to have sensitive and reliable safety biomarkers to monitor and direct therapeutic strategy.

Drug-induced cardiotoxicity can be broadly categorized as structural or functional. Structural damage may be a direct effect of the drug or secondary e.g. the result of vascular injury leading to ischemia. Functional damage may occur as a result of drug interaction with cardiac ion channels, leading to prolonged QTc interval and subsequent arrhythmia. This functional toxicity may or may not cause an increase in circulating levels of a cellular component, i.e. biomarker. A biomarker that detects a structural change may not necessarily detect a functional alteration and vice versa.

b. Currently Available Tools

Histopathology,electrocardiography (ECG), echocardiography, and blood biomarkers (e.g. creatine phosphokinase (CK), lactate dehydrogenase (LDH), and serum aspartate aminotransferase (AST)) are currently available tools to evaluate possible cardiac toxicity in the non-clinical setting.

Histopathology

In safety assessment, cardiac histopathology typically consists of examinationof 1-2 tissue sections, approximately 5 microns in thickness stained with H&E. This standardized technique may miss lesions, especially subtle and/or focal injury. While H&E is an excellent histochemical stain for general examination, the preferred stains for early detection of myocardial injury include phosphotungstic acid hematoxylin, trichrome, and periodic acid-Schiff (Vargas et al, 1999).

Biomarkers

CK is frequently used for non-clinical safety assessment of cardiac damage. AST and LDH are also used but less frequently. Biomarkers are not used in isolation for the diagnosis of myocardial injury; in non-clinical safety assessments, cardiac biomarkers are used in conjunction with histopathology. In clinical practice, cardiac biomarkers are interpreted in the context of other data such as evidence

of myocardial ischemia or pathological findings. Table 2 summarizes the biomarkers that have been used or proposed for clinical use to diagnose myocardial morphologic injury in the setting of ST-elevation myocardial infarction.

Table 2. Biomarkers for the Evaluation of Patients with ST-Elevation Myocardial Infarction

Biomarker	Molecular Weight (D)	Range of Times to Initial Elevation (hr)	Mean Time to Peak Elevations (nonreperfused)	Time to Return to Normal Range
Frequently used in clinical practice				
MB-CK	86,000	3-12	24 hr	48-72 hr
cTnI	23,500	3-12	24 hr	5-10 d
cTnT	33,000	3-12	12 hr-2d	5-14 d
Infrequently used in clinical practice				
Myoglobin	17,800	1-4	6-7 hr	24 hr
MB-CK tissue isoform	86,000	2-6	18 hr	Unknown
MM-CK tissue isoform	86,000	1-6	12 hr	38 hr

From Antman and Braunwald, 2008.

All of the above biomarkers are proteins found in cardiac myocytes and their appearance in the blood is indicative of cardiac myocyte necrosis or injury. Of those listed, CK-MB, cTnI, cTnT, and myoglobin have achieved clinical acceptance not only for acute myocardial infarction (AMI) but for other forms of myocardial injury as well, including myocarditis, pericarditis, sepsis, heart contusion, cardiac transplant rejection and drug-induced myocardial damage. However, CK isoenzymes, and myoglobin lack specificity for cardiac damage. Detailed discussion of the limitations of CK-MB and myoglobin may be found in Appendix 1.

In this application, the proposed biomarkers were assessed against a number of different comparators. Not all comparators were used in all studies. The sensitivity of the troponins has been compared to the standard blood-derived biomarkers, ECG, echocardiography, and histology, both clinically and non-clinically.

c. Background Information on the Proposed Biomarker

Biology of the troponins

Troponins C, T and I make up the cardiac troponin complex which is located on the thin filament of the cardiac myocyte contractile apparatus and regulates the actin-myosin interaction needed for muscle contraction. Troponins T and I each have two different adult isoforms one of which is specific for cardiac muscle and the other for skeletal muscle. Troponin C isoforms are identical in skeletal and cardiac muscle. A more detailed discussion of the biology of cardiac troponins can be found in Appendix 2.

Current use in clinical practice

In clinical practice, cardiac troponins are widely used to detect ischemic cardiac injury in patients with acute coronary syndrome (ACS). In 2000, the American College of Cardiology and the European Society of Cardiology declared cTns the preferred biomarker for myocardial infarct, based upon the high sensitivity and essentially absolute specificity of cTns (Joint European Society of Cardiology/American College of Cardiology Committee, 2000).

Although the knowledge of cardiac troponins is based largely on studies in the ACS population, the tissue specificity of cTns has led to a new understanding of numerous pathological conditions in human and veterinary medicine by raising awareness of cardiac morphologic damage secondary to other disease processes, including left ventricular hypertrophy, congestive heart failure, myocarditits, pulmonary embolism, blunt trauma, sepsis, stroke, seizure activity, renal disease, and diabetes mellitus (Kelley et al 2009).

The utility of cardiac troponins as clinical markers of drug-induced cardiac toxicity is unclear and requires further study (e.g. anticancer drugs). (Cardinale D et al., 2000, Cardinale et al., 2010). Although the increasing sensitivity of cardiac troponin assays may advance the ability to detect drug-induced subclinical myocardial injury, the optimal use of cardiac troponins in non-ACS clinical drug development trials, including when to use them and how to interpret and manage changes in troponin levels, remains an area of active discussion.

Database supporting the use of troponins in preclinical safety assessments

As biomarkers, cTns (I and T) are unusual in that they have already achieved acceptance in human and veterinary medicine and are now being reverse translated for use in preclinical safety assessments.

At least 68 published non-clinical toxicology studies have reported using cTns to detect drug-induced cardiac morphologic damage. The reports include evaluations of various classes of drugs, including alpha and beta adrenergic agents, peroxisome proliferator activator receptor (PPAR) agonists, phosphodiesterase inhibitors, anthracyclines and other anti-neoplastic drugs. These studies have been conducted in several species, including mice, rats, dogs, pigs, and rabbits. More recently there have been published studies on macaques (Yin et al, 2007), marmosets (Hanton et al, 2008), rhesus monkeys (Apple et al, 2008), and cynomolgus monkeys (Apple et al, 2008; Schultze et al, 2008, Minomo et al 2009,Zabka et al 2009).

Since the submission of this qualification package, additional work has been added to the published literature some of which has been considered in this review. Troponins are already being used for preclinical safety assessments in some drug development programs (as indicated by verbal communications during meetings of the ILSI Cardiac Troponins Working Group and the records of several investigational new drug applications (INDs) and new drug applications (NDAs). Much of these data are not available in the published literature and therefore were not available to the sponsors for inclusion in the qualification material.

Tables 2 and 3 summarize the mechanisms used to induce cardiac morphologic damage for the study of troponins in the general published literature and in the core 90 studies of the application. Details of pharmacology and mechanisms of damage weren't available for proprietary compounds. Studies were also cited for mouse, cat, ferret, rabbit, cow and pig. While the information provided is part of the overall dataset, qualification was not requested for these species. Therefore, those studies are not included in the subsequent tables.

Table 3: Published Reports of Pharmacologic Agents Used for Characterization of Tns in Non-Human Species

species	Pharmacologic agent	Assay	reference
mouse	doxorubicin	T, LDI	O'Brien97a-11; Feleszko 00; Hou 05, 06, 07; Cole 06; Kogan 07; Shuai 07
	epirubicin	T	Santucci 07
	Cannabinoid quinone	T	Kogan 07
	Cobra venom	T	Cher 05
	A-PPAR agonist	Bay I	Pruimboom-Brees 06
	NNRTI		deJonghe 08
	unidentified compounds	BAY I	O'Brien 06-30
rat	isoprenaline	T, BCA	Bleuel 95; Bertinchant 00 Bertsch 97, 99
	Orciprenaline	T	Acikel 03; Hassan 05; O'Brien06-60; Herman 06; York 07; Hasic 07;
	isoproterenol	T, BAY, LDI, BV, TRI, DPC, Sing, AA, BCA, DBO	Senthil 07; Kurata 07; Rajadurai 07; Hasic 07; Schultz 08; Mikaelian 08; Zhang 08; Apple 08
	daunorubicin	T	Ali 02, deNigris 08
	doxorubicin	T, BCA, DRG	Herman 98, 99, 01; Della Torre 01; Dowd 01; Childs 02 (shown non-toxic at low dose); Ahmed 03; de Nigris 08 Bertinchant 03, 04; Koh 04
rat	Mitoxantrone	T	Herman 01
	idarubicin	T	Della Torre 01
	Cyclopentenyl cytosine	T	Schimmel 05 (shown nontoxic)
	cyclophosphamide	DBO, LDI	Mythili 06, Yavuz 08
	N-acetylcolchinol-O-phosphate	T	Gould 07
	alcohol	T	Patel 01
	Viper venom	DBO	Luksic 08
	methidathion	Bay	Yavuz 04
	Bis(2)chloroethoxymethane	T	Dunnick 04
	allylamine	Bay	Brady 06
	PDE3 inhibitor	T	Zhang 06
	Delta-PPAR agonist	LDI	Yue 08
	cobalt	T	O'Brien 06-15
	WF10 chlorite matrix immunosuppressant		Hansen 01 (shown nontoxic)
	unidentified compounds	Bay BV; Bay	O'Brien 06-20; Serra 08

*Immunoassays, analyzers and ELISA kits: ABT = cTnI ELISA kit, AboaTech Ltd, Turku, Finland; AA = cTnI, Axsym, Abbot Laboratories, Abbott Park, USA; Bay = cTnI, Advia Centaur or Automated Chemiluminescence System, (formerly Chiron Diagnostic Corporation, Halstead, Essex, UK) Bayer Healthcare Diagnostics, Newbury, UK; BCA = cTnI Access, Beckman-Coulter, High Wycombe, UK; BCU = Unicel DXI, Beckman-Coulter, Fullerton, Calif.; BV = cTnT, Bioveris Europe, Whitney, Oxford, UK; DBD = cTnI, Dimension, Dade Behring Liederbach, Germany; DBO = cTnI, Opus, Dade Behring Liederbach, Germany; DBS = cTnI, Stratus, DPC = cTnI, Immulite, Diagnostic Products Corporation, Llanberis, UK; DRG = cTnI ELISA kit, DRG International, Mountainside, NJ, USA; LDI = Rat cTnI I ELISA Kit, Life Diagnostics,Inc West Chester, PA, NNRTI = non-nucleoside reverse transcriptase inhibitor; PPAR = peroxisome proliferator activated receptor.

Table 3 continued: Published Reports of Pharmacologic Agents Used for Characterization of Tns in Non-Human Species

species	Pharmacologic agent	assay	reference
Dog	phenylpropanolamine		Crandell 05
	norepinephrine	T	Masuda 02
	isoproterenol	T, Sing	Feng 05, Schultze 08, Apple 08
	dobutamine	Bay	O'Brien 06-8
	doxorubicin	T,BCA	Christiansen 02, 03, DeFrancesco 02,Selting 04
	Viper venom	T, Bay	Segev 08
	PDE4	Bay	O'Brien06-8
	unidentified compounds	Bay	O'Brien 06-24
monkey	isoproterenol	Sing	Schultze 08, Apple 08
	PDE3 (marmoset)	Bay	Hanton 08
rabbit	isoproterenol	DBO	Pinelli 04a,b; Felten 04; Saeed 06a,b.
	daunorubicin	T, ABT	Adamcova 99, 02a,b, 03, 07; Machácková 00, 01; Simunek 03, 04, 05; Sterba 06a,b, 07a,b; Potácová 07a,b; Popalova 08
	dimethoxybenflurone	T	Machácková 00, 01; Adamcová 01
	oracin	T	Machácková 00; Adamcová 01
	Pyridoxal isonicotinoyl hydrazone	T	Adamcová 02

*Immunoassays, analyzers and ELISA kits: ABT = cTnI ELISA kit, AboaTech Ltd, Turku, Finland; AA = cTnI, Axsym, Abbot Laboratories, Abbott Park, USA; Bay = cTnI, Advia Centaur or Automated Chemiluminescence System, (formerly Chiron Diagnostic Corporation, Halstead, Essex, UK) Bayer Healthcare Diagnostics, Newbury, UK; BCA = cTnI Access, Beckman-Coulter, High Wycombe, UK; BCU = Unicel DXI, Beckman-Coulter, Fullerton, Calif.; BV = cTnT, Bioveris Europe, Whitney, Oxford, UK; DBD = cTnI, Dimension, Dade Behring Liederbach, Germany; DBO = cTnI, Opus, Dade Behring Liederbach, Germany; DBS = cTnI, Stratus, DPC = cTnI, Immulite, Diagnostic Products Corporation, Llanberis, UK; DRG = cTnI ELISA kit, DRG International, Mountainside, NJ, USA; LDI = Rat cTnI I ELISA Kit, Life Diagnostics, Inc West Chester, PA, NNRTI = non-nucleoside reverse transcriptase inhibitor; PPAR = peroxisome proliferator activated receptor.

Table 4: Summary of agents and mechanisms of damage included in the sponsor's selected core studies

species	Agent or mechanism of damage	
dog	Unspecified "Compound X"	
	Isoproterenol	IV (1 study) and sc (1 study)
	Doxorubicin	IV (2 studies) and IC (3 studies)
	Atrial pacing	2 studies
	Left coronary occlusion	1 study
	Natural disease	1 study
	Aging	1 study
	Endogenous hypercholesterolemia	1 study
rat	Isoproterenol	IV (1study) and sc (5 studies) ip
	Doxorubicin	IV (3 studies) and ip (1 study)
	Mitoxantrone	1 study
	PDE3 inhibitor	1 study
	Fenthion	1 study
	Methidathion	1 study
	Aging	1 study
	Left coronary occlusion	2 studies
	Microcystin	1 study
monkey	Norepinephrine (rhesus)	11 different assays compared
	Left coronary occlusion (cynomologous)	11 different assays compared
	Hydrochlorthiazide (cynomologous)	
	Minoxidil (marmoset)	

d. intended application of the biomarker

The sponsor proposes the qualification of cardiac troponins I and T for the following uses:

1. as circulating biomarkers of acute or ongoing cardiac structural damage in preclinical studies in rats, dogs and non-human primates if

a) cardiac structural damage has occurred with previous testing of the compound. The role of testing here is to get a better estimate of the lowest toxic dose or a good estimate of the higest non-toxic dose to help choose doses for human testing. Circulating troponins are not used in this situation to gain primary evidence of cardiac toxicity.

b) the compound being tested is of a chemical or pharmacologic class with known cardiac structural toxicity. Measurement of circulating troponins is to augment when standard chemical and histopathological tests have revealed no structural toxicity at the maximum doses used. The purpose here is to gain a sensitive assessment of toxic potential.

2. to collect troponin data in a voluntary, exploratory manner to expand the database for nonhuman primate, to evaluate compounds with marked electrophysiological effects on the heart and to address emerging technologies with improved analytic sensitivity.

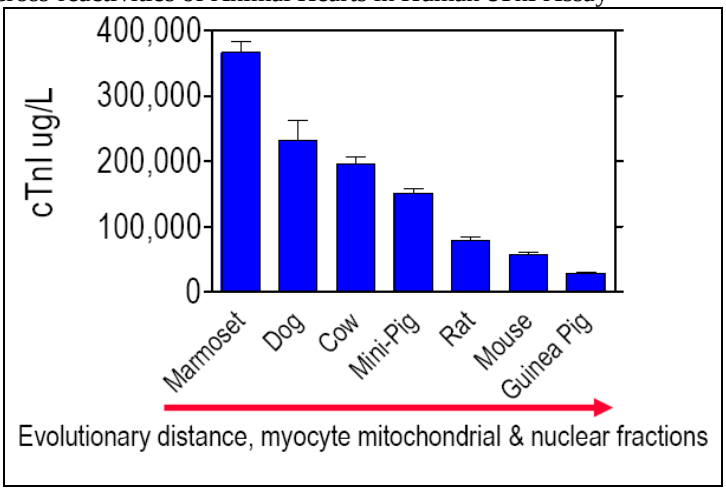
3. for reflex testing. According to the sponsor, reflex testing is specific and retrospective testing for a clinical pathology correlate to unexpected post-mortem morphological findings using the reserve or stored serum/plasma samples collected in the same study in which these morphological effects occurred. Additionally, reflex testing introduces the clinical pathology correlative test into subsequent safety assessment studies. In this application, troponin is proposed as a reflex test, included in the studies conducted subsequent to that in which cardiac morphological effects occurred.

e. Technical aspects of cardiac troponin measurement

Cardiac Tn is measured by immunoassay, typically using commercially-available reagents developed for automated clinical immunoanalyzers. Some point-of-care immunoassays are available. More than a dozen cTn assays and analyzers have been approved by CDRH for use in human samples. There are no automated immunoassays developed specifically for use in animals, although there are species-specific ELISA assays.

Troponin structure is highly conserved across mammalian species. The region commonly targeted for immunoassay antibody production differs between dogs and humans by only 1 amino acid out of 83. The rodent sequence differs by 6 amino acids(Malouf et al, 1992; Rishniw et al, 2004, 2005; Hastings 1997)Many assays designed for human samples may be used for laboratory animals (O'Brien 1997a,b, 2006) if the antibody or antibodies used recognize the conserved epitopes. Animal size impacts reactivity with different assays since smaller mammals have higher metabolic rates. The additional cell volume fraction that needs to be allocated to nuclei and mitochondria is done at the expense of myofibrillar volume.

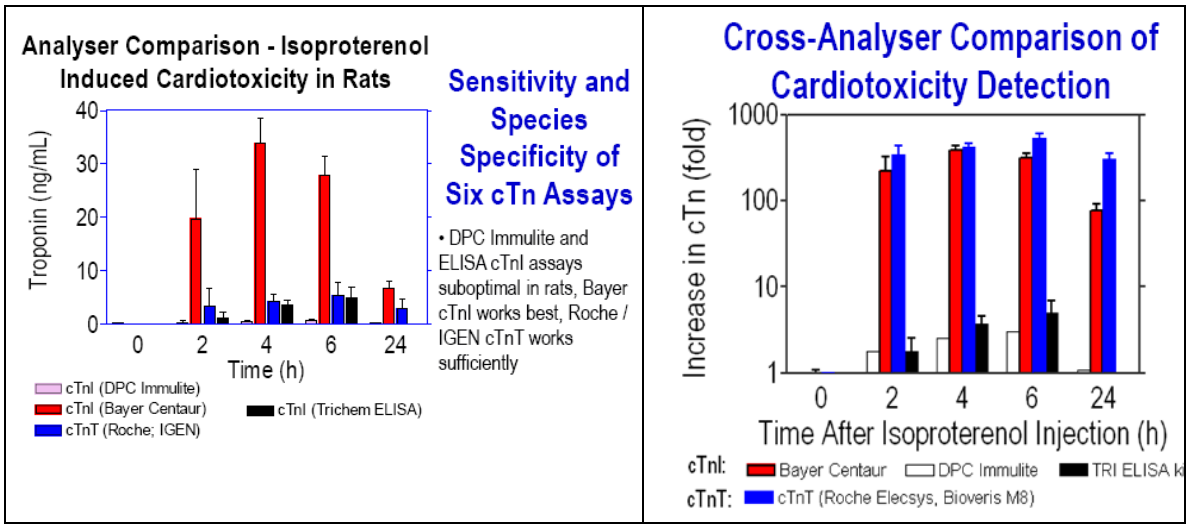
Figure 1. Cross-reactivities of Animal Hearts in Human cTnI Assay



From: O'Brien PJ, Landt Y, Ladeson JH. 1997. Clin Chem.

Some submitted reports performed direct comparisons of assays in rats, dogs and primates. The results demonstrated that not all assays were equally effective for each species. Figure 2 displays the variability in sensitivity and species specificity of six cTn assays.

Figure 2a,b. Cross-species sensitivity and specificity of various cTn assays in rats



From: O'Brien PJ et al. , 2006.

The National Academy of Clinical Biochemistry (NACB) and International Federation of Clinical Chemistry (IFCC) have published detailed guidelines for the clinical implementation of cardiac biomarker assays. Some of the key recommendations include determining the distribution of values in a healthy reference population, the statistical determination of the 99th percentile cutoff for the reference population and of the concentration corresponding to the 10% coefficient of variation (CV, total imprecision). The NACB and IFCC also recommend that for new assays, the laboratory should explore preanalytical factors such as effects of storage time and temperature, effect of glass vs plastic tubes and gel separator tubes and the influence of anticoagulants (Apple et al., 2007).

Consensus *clinical* recommendations (Babuin and Jaffe, 2005) have been developed to identify an "abnormal" value. The abnormal value may vary according to sex, age, and genetic background.

Consensus *safety assessment* recommendations for use of troponins do not appear in the published literature. The sponsors of this qualification proposal provide recommendations for the implementation of assays in preclinical safety assessment based upon the IFCC guidelines. The sponsors' recommendations are provided the next section.

f. Kinetics of Release

The troponins are released from cardiac tissue during the active phase of cell lysis and return to baseline following termination of active pathogenesis (Wallace et al, 2004; O'Brien et al, 2006). Progressive or continuing cell injury shows a sustained increase in serum troponins (Selting et al, 2004; Popelova et al, 2008).

The kinetics of TnI and TnT are similar even though they differ somewhat in intracellular compartmentation. The majority of troponins are myofibril-bound; the minority is found as a soluble cytoplasmic pool, which may serve as a precursor pool for the synthesis of the troponin complex. The cytosolic fraction of cTnT in human myocardium is estimated to be 6 % of total TnT and 2.8 % for cTnI. This distribution may vary from one species to another. The biological half-life of cTnT is 120 minutes, and 90 minutes for cTnI, presumably for humans (Adamcova et al., 2002). The troponins are cleared from the circulation with a half-life of approximately 6 hours in rats (O'Brien et al 2006).

In human and animal studies, cTn has been demonstrated to be released within minutes of myocardial injury and show peak circulating levels as early as 2 to 6 hours, depending on the duration and severity of the cardiac injury. With naturally-occurring myocardial infarction in humans, cTn typically rises within 4-6 hours and peaks at 18 to 24 hours. High sensitivity assays can demonstrate a rise in cTn within 2 hours. At 72 hours, cTn results correlate with scintigraphic measurement of infarct size. With coronary occlusion restricted to 90 minutes, experimental models of myocardial infarct in dogs, showed peak cTn levels in 3 to 4 hours (O'Brien et al, 1997a). In rats treated with a single, subcutaneous toxic dose of isoproterenol, cTnI and cTnT serum concentrations peaked within 2 to 6 hours (O'Brien et al, 2006). In these rat and dog models, cTnI increases were correlated with histopathological score and infarct size at 3 hours.

As for other organ biomarkers, the amount of cTnI release is affected by tissue content. Partial depletion of tissue cTnI with prior injury, inanition and weight loss, and heart failure may be associated with decreased amounts of release (Feng, 2004; O'Brien et al., 1995; O'Brien 2006). Weight loss of 25% in rats produces a 50% decrease in cTnT content of myocardium. Heart failure in rats and humans produces approximately 30% decrease in tissue cTnI. For regulatory applications of the biomarker, the kinetics of release, clearance, and depletion of tissue levels have implications for the timing of sample collection and for the possibility of false negative values.

While the sponsors cited 4 studies for the kinetic summary immediately above, the core studies of the application include 5 rat studies that examined kinetics of release and clearance, 11 dog studies in which samples for multiple time points were collected, and 3 primate studies in which multiple time points were examined. The sponsor's summary table of these studies is provided as Appendix 3.

g. Correlation of cardiac troponins with histological change in the myocardium

Animal models of human myocardial infarct provided the first evidence of the relationship between ischemic injury and cTn increase in blood, showing the myocardium to be the source of the circulating cTn (Ricchiuti et al., 1998). The models demonstrated that peak increases of blood concentration of troponins were correlated with the degree of histopathological change in dog, rat, pig, and mouse models of acute myocardial infarct (see Table 5). The only references examined were those pertaining to the species listed in the context of use. Points that are still under debate are

1. Can elevations of circulating troponins be caused by reversible damage? The mechanism of troponin release in these circumstances is unclear.
2. Is a significant amount of troponins released into the blood following apoptosis of myocardial cells?

Table 5. Studies correlating circulating troponins and cardiac histopathology

Studies Demonstrating Correlation Between Serum cTn and Cardiac Histopathology		
Drug or treatment	Species	Author
Ischemia	dog	Voss et al, 1995
Ischemia	dog	Ricchiuti et al, 1998
Ischemia	dog	Rempis et al, 2000
Ischemia	dog	O'Brien et al, 1997a
Ischemia	pig	Feng et al, 1998.
Ischemia	rat	Bertinchant et al, 2002, 2003
Ischemia	mouse	Metzler et al, 2002
Ischemia	mouse	Manikandan et al, 2004
Doxorubicin	rat	Herman et al, 1999
Doxorubicin	rat	Bertinchant et al, 2004
Doxorubicin	rabbit	Adamcova et al, 2007
Isoproterenol	rabbit	Pinelli et al, 2004a, b
Isoproterenol	rat	O'Brien et al, 2006
Isoproterenol	rat	Herman et al, 2006
Isoproterenol	rat	York et al, 2007
Isoproterenol	rat	Kurata et al, 2007
Isoproterenol	rat	Hasic et al, 2007
Isoproterenol	rat	Zhang et al, 2008
Isoproterenol	rat	Schultze et al, 2008
Isoproterenol	rat	Mikaelian et al, 2008
PDE3 inhibitor	rat	Zhang et al, 2006
PDE3 inhibitor - Minoxidil	marmoset	Hanton et al, 2008
Isoprenaline	rat	Bertinchant et al, 2000
Cyclophosphamide	rat	Mythili et al, 2006
X	dog	O'Brien et al, 2006
Spontaneous cardiomyopathy	rat	O'Brien et al, 2006
Allylamine	rat	Brady et al, 2006
PPAR α agonist	mice	Pruimboom-Brees et al, 2006
N-acetylcolchinol-*O*-phosphate	rat	Gould et al, 2007
Non-nucleoside reverse transcriptase inhibitor	mice	De Jonghe et al, 2008
Organophosphate	rat	Yavuz et al, 2008

A number of studies examined quantitative relationships between cTns and damaged myocardium. Voss et al(1995) examined human hearts obtained at necropsy from patients who had died of myocardial infarction. The investigator also examined dogs after 3 weeks of coronary occlusion. Infarct size originally did not correlate well with serum cTnT or CK-MB concentrations. When the data were separated by infarct location, correlations improved, possibly related to differences in tissue concentrations of cTnT and CK-MB between different locations in the heart. Ricchiuti et al (1997) studied distribution of cTnI and cTnT in postinfarction left ventricular remodeled myocardium at 2 months in a porcine heart failure model. At 2 months postinfarct in a porcine heart failure model (LVR), both Western blot and biochemical assay analyses were performed on left ventricular myocardium remote from the infarct zone in ligated animals (n=8). Results were compared with data from the left ventricular myocardium from similar sized control (unligated) pigs (n=7). Autoradiograms from Western blot analysis showed that the protein mass for cTnI and cTnT in LVR hearts decreased 80% ($P < 0.001$) and 40% ($P < 0.02$), respectively, when compared with

nondiseased tissue. Similarly, the concentrations for cTnI and cTnT in LVR hearts decreased 42% ($P < 0.05$) and 70% ($P < 0.001$), respectively, compared with nondiseased normal tissue. The investigators concluded that cTnI and cTnT in the blood is proportional to chronic loss of cTnI and cTnT from injured myocardium associated with left ventricular remodeling. Ricchiuti (1998) also examined troponin distribution in dogs, 3 weeks following coronary artery occlusion (left anterior descending artery, LAD). The serum profiles for time vs mean cTnI and cTnT concentrations in 6 dogs after occlusion showed peak concentrations at 1 day and 5 days, respectively. The concentrations of troponins were similar in all nonischemic zones. However, cTnI and cTnT decreased signifcantly in the LV ischemic tissues. Loss of cTnT, but not cTnI in ischemic LV tissues correlated signifcantly with infarct size 3 weeks after the infarction. Biochemical alterations suggest that the increases in serum troponins after the infarction parallel the decreases in tissue concentrations of troponins.

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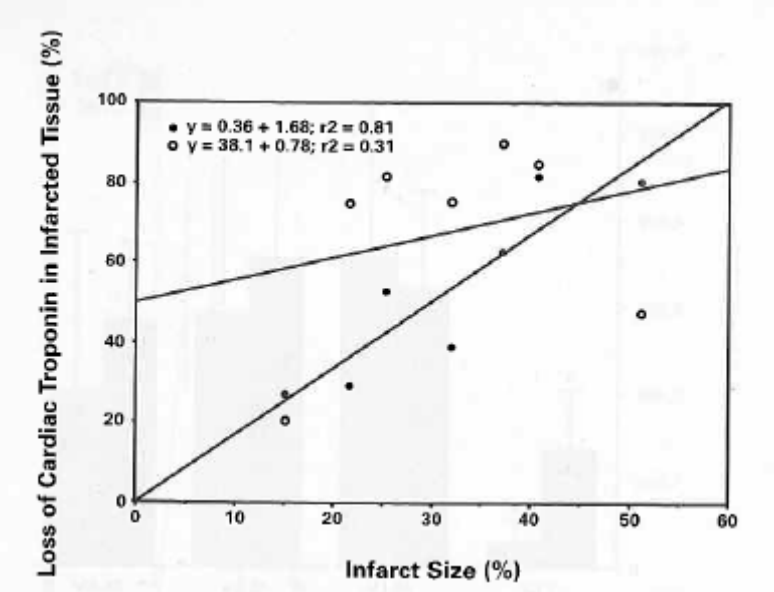


Figure 3.
From Ricchiuti et al 1998. Linear regression between the myocardial infarct size(reported as percentage of volume infarct of tissue, in abscissa) and the loss of cardiac troponins in infarcted tissue (expressed in percentages on the x-axis). Two regression lines are drawn,corresponding with regression of myocardial infarction size with loss in infracted tissue of cardiac troponins T(black circle) and I(open circle).

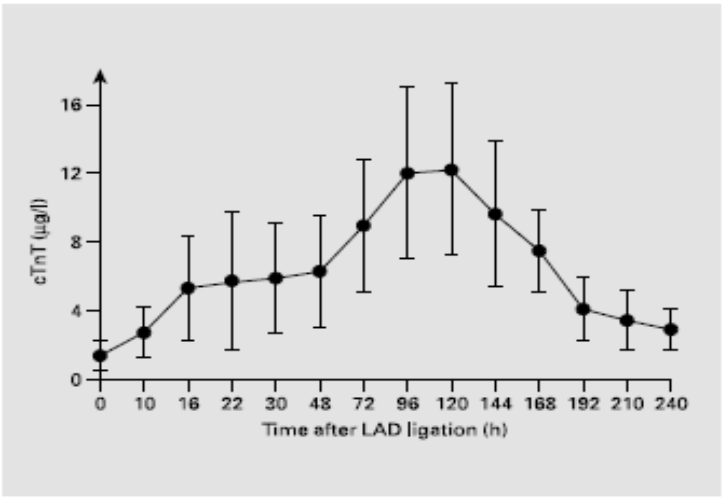


Figure 4.
Remppis et al (2000) used coronary artery ligation to generate infarcts in dogs. Infarct sizes were determined using the triphenyltetrazolium chloride method and compared with the serum concentrations of cTnT at each time point between 4 and 168 hours after left anterior descending artery (LAD) ligation. A significant correlation was found between cTnT levels at 96 hours ($p = 0.0001$, $r = 0.83$) or cumulative cTnT

levels and relative infarct size (p=0.0010, r=0.74)

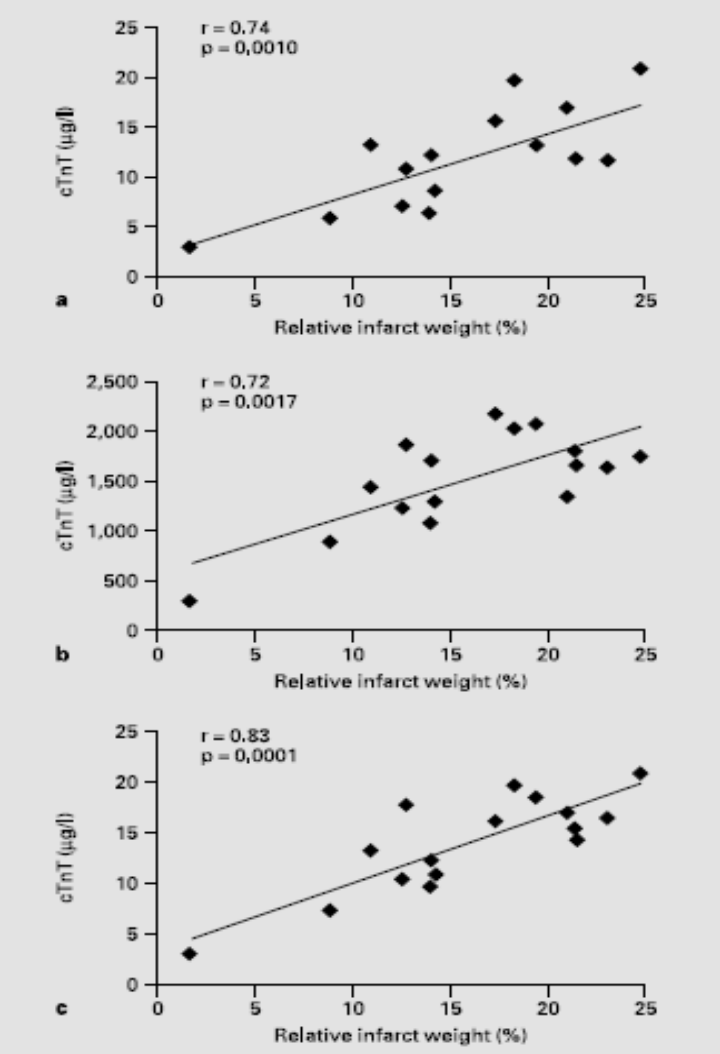
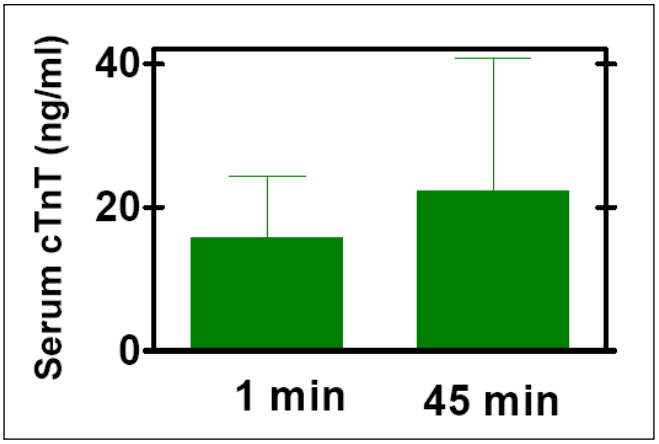


Figure 5. Correlation of cTnT values with relative infarct weights.
From Remppis et al 2000. cTnT time release curve. Values are mean±SD. **a**.96-hour cTnT levels. **b** Cumulative cTnT levels **c**. Peak cTnT levels

h. Factors that may increase background cTn in blood unrelated to treatment

Cardiac puncture under anesthesia is occasionally used for blood collection in small species. Collection of blood samples by this method causes marked elevation of cTn. Occurrence of marked elevation at 1 minute indicates the source is from penetration of the myocardium and not from increased circulating levels. If blood is collected by cardiac puncture, the physical damage to the heart will cause an increase in troponin levels that may confound interpretation of subtle signals.

Figure 6. Increases in serum cTn due to blood collection by cardiac puncture



O'Brien PJ, et al. 1997. Lab Anim Sci

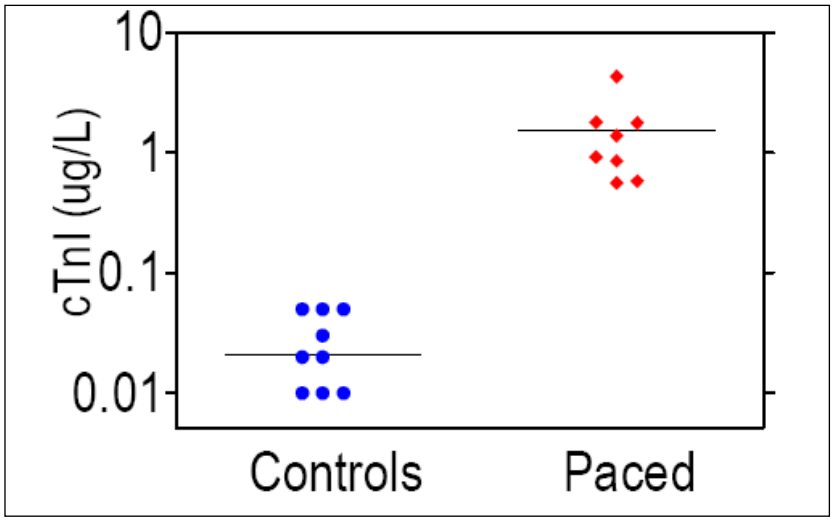
Atrial pacing in safety assessment studies was also listed as a factor contributing to background increases in cardiac troponins. There is some inconsistency in the literature over the degree or duration of atrial pacing needed to cause increases in troponins. Lee et al (2009), performed transcutaneous cardiac pacing (TCP) for 1 hour (5 dogs) or 3 hours (5 dogs) using an automated external cardiac pulse generator and a transdermal electrode. Serum concentrations of CK-MB fraction and cardiac troponin I and activities of AST, CK, and LDH were evaluated the day before (baseline) and at intervals until 7 days after TCP. The serum cTnI concentration was markedly increased only in the long-duration TCP group at days 0, 1, and 2. Although serum cTnI concentration was increased at days 0 and 1 after the short-duration TCP, these values were not significantly different from the baseline value and were within reference limits. The authors suggest that a reason for the failure of other researchers (Madsen et al 1988; Syverud et al 1983) to detect increases in circulating concentrations of cardiac biomarkers (eg, CK-MB) and enzyme activities (eg, CK and AST) in dogs and humans in previous studies might be the short (30 minutes)duration of TCP . They also note that both skeletal muscle and cardiac muscle were damaged by TCP and that the magnitude and duration of increases in serum AST, CK and LDH activities were directly correlated to the duration of TCP.

Another investigator examined atrial pacing in safety pharmacology studies (O'Brien et al, 2006). Eight of these dogs had been paced under isoflurane anaesthesia. They were subjected to four periods of 30 minutes duration each, in which atrial tachycardia was induced using pacemakers. In each period, at 5 minute intervals, hearts were progressively paced in increments of 10 from 150 to 200 beats per minute. Blood samples were collected 2 hours after commencement of pacing. The increase shown (figure 4)is indicative of a cumulative pacing phenomenon of greater than half an hour and correlates with the results of Lee et al 2009.

Syverud et al (1983) reported micro-infarcts in 5 of the 10 dogs studied and subsequently reported (Kicklighter et al 1985) microscopic lesions that involved less than 5% of the right ventricular free

wall and less than 1% of the left ventricular posterior wall in 10 of 10 dogs studied under similar stimulation conditions. Since cTns were not measured in these early studies, it is unclear how much cardiac damage is required in dogs before significant increases in cTn can be detected. Detection of troponin release following micro-infarcts may require a high sensitivity assay. However, in a dog model of coronary occlusion (O'Brien et al 1997), serum TnT concentrations correlated with time of reperfusion and with infarct size (ranging in size from 2.00 -18.72 g as determined by special staining of the ischemic areas).

Figure 7. Effect of Atrial Pacing on Blood cTn



From:
O'Brien PJ, et al. 2006.

Atrial pacing itself should be considered in evaluating these results. The mechanism of myocyte damage is different in the different methodologies. Transcutaneous pacing requires relatively high levels of electrical charge to produce electrical capture of the heart and depends on the amplitude, duration of the pacing current, and the size and placement site of the electrodes. These parameters are not necessarily standardized between investigators (Lee, Nam and Hyun, 2010.). The highest density of electrical current is found at the skin and underlying skeletal muscle where the electrodes are placed. Increased CK-MB is expected due to the skeletal muscle damage. The primary mechanism of injury for both skeletal muscle and cardiac muscle is direct electrical damage. The high ventricular rate methods of pacing cause myocardial damage via the rate of myocardial contraction. (Nath and Haines 1995; Nath et al. 1994.)

Other iatrogenic factors that have been demonstrated to increase cTns include excessive restraint stress, excessive exertion (Chen et al, 2000), anoxia during anesthesia resulting in cardiac ischemic injury.

Age- and sex-dependent spontaneous cardiomyopathy occurs in clinically healthy Sprague-Dawley rats (O'Brien et al, 2006). Males 8 months had 10-fold greater serum cTnI levels than 3-month old males and age-matched females correlating to mild degenerative change in myofibers. Analysis of cTns has allowed recognition of this condition as wide-spread. Historically, only rare, incidental, treatment-independent findings were noted by histopathological examination (Kemi et al. 2000).

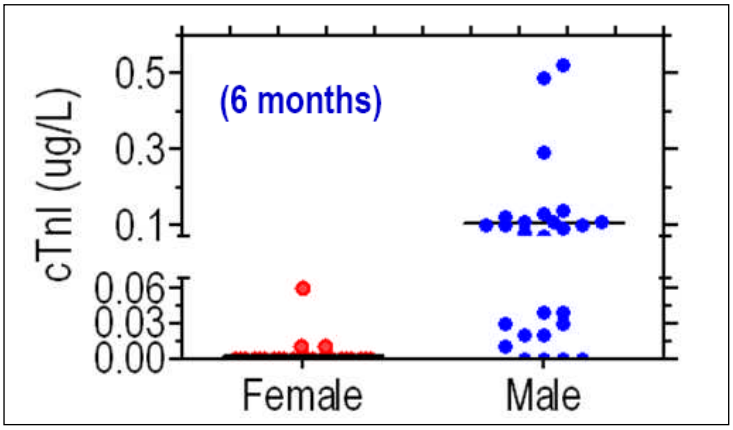


Figure 8 O'Brien et al, 2006.

i. Interpretation of troponin data

As with any other clinical chemistry parameter, the significance of change or lack of change must be interpreted in the context of all clinical information or research data. There is sometimes an expectation that new biomarkers allow for clear-cut decision making without the need for other data.

Consideration of the entire clinical scenario is necessary for the diagnosis of myocardial infarction. The universal definition of myocardial infarction (Thygesen et al., 2007) requires detection of a rise and/or fall of cardiac biomarkers (preferably troponin) with at least one value above the 99th percentile of the upper reference limit together with evidence of myocardial ischemia from at least one of the following: clinical symptoms, ECG changes, or imaging data.

Recently, cardiac troponin assays with increasing sensitivity have become available for clinical use. Some literature suggests that even minor troponin elevation may be prognostic with respect to death, myocardial infarction, or the development of congestive heart failure. (Bonaca et al., 2010, Omland et al., 2009, Miller et al., 2009, de Lemos et al., 2010, deFilippi et al., 2010, Kociol et al., 2010).

Increases in circulating troponins due to cardiac damage secondary to conditions (e.g. renal damage, electrolyte imbalances) other than a primary cardiotoxicity are sometimes referred to as "non-specific increases" or "false positives." In an editorial for the New England Journal of Medicine, Morrow (2009) states:

> It is essential to differentiate between the tissue specificity of troponin for cardiomyocyte injury and the clinical specificity for myocardial infarction, which is defined by ischemia as the mechanism of injury. The adoption of troponin has revealed the occurrence of myocardial injury in many conditions in which it was not previously detected with the use of CK-MB. Such detection has given the impression of an increased number of false positive results. However, this occurrence does not impugn the tissue specificity of troponin but rather underscores that myocardial injury may result from a variety of mechanisms.

Morrow's point about multiple mechanisms of cardic damage is relevant in the context of safety assessment. A drug may not induce primary cardiac toxicity, but when tested at toxicologic doses typical of safety assessment studies, may cause damage secondary to other toxicities. The interpretation of the results should distinguish between primary and secondary toxicity.

The limitations of the comparator method used to determine cardiac disease or damage should be considered. For example, in studies where it is reported that there is no ECG evidence of cardiac disease, it should be remembered that ECGs have limited ability to identify myocardial damage. Therefore, these studies should not necessarily be perceived as evidence of a false positive troponin result.

An active controversy in the troponin literature is whether troponin is released only after irreversible cardiac damage or if it is possible to see elevations after reversible damage. There is no consensus definition of what constitutes reversible damage. The hypothesis behind the reversible damage elevations theory (RDET) is that an unbound cytoplasmic pool of troponin may leak out of the myocytes in the absence of damage to the contractile apparatus (Lippi et al, 2009).

j. Presentations of data

Most studies present the data in tabular form (example below) or graphically.

Table 6. Comparison of the AUCs of Fabp3, MLC1, cTnI, cTnT, AST, LDH, CK following carbofuran (CAF) or isoproterenol (ISO) treatment.

	Fabp3		
	Control	CAF	ISO
AUC (ng h/mL)	48.36 ± 5.82	110.42 ± 12.20*	103.77 ± 18.04*
	MLC1		
	Control	CAF	ISO
	4.53 ± 0.50	38.47 ± 4.58*	40.61 ± 12.60*
	cTnI		
	Control	CAF	ISO
	0.20 ± 0.09	1.68 ± 1.09	29.31 ± 9.84*
	cTnT		
	Control	CAF	ISO
	0.24 ± 0.14	2.48 ± 0.33	27.64 ± 10.57*
	AST		
	Control	CAF	ISO
	1836 ± 101	3293 ± 163**	2845 ± 449
	LDH		
	Control	CAF	ISO
	3516 ± 944	3096 ± 139	4846 ± 996
	CK		
	Control	CAF	ISO
	5653 ± 361	6090 ± 1399	6409 ± 747

Data are expressed as means±S.E. The statistical significance of differences between treated and control groups (n = 4, respectively) was determined using Dunnett's multiple comparison test. * $p < 0.05$. **$p<0.01$.
From: Tonomura et al. 2009.

Another common presentation of troponin data was a graph of the serum concentration of the biomarker compared to time. Figure 6 compares troponin T release to CK-MB and myosin light chain in a dog model of subarachnoid hemorrhage, a situation of secondary cardiac damage.

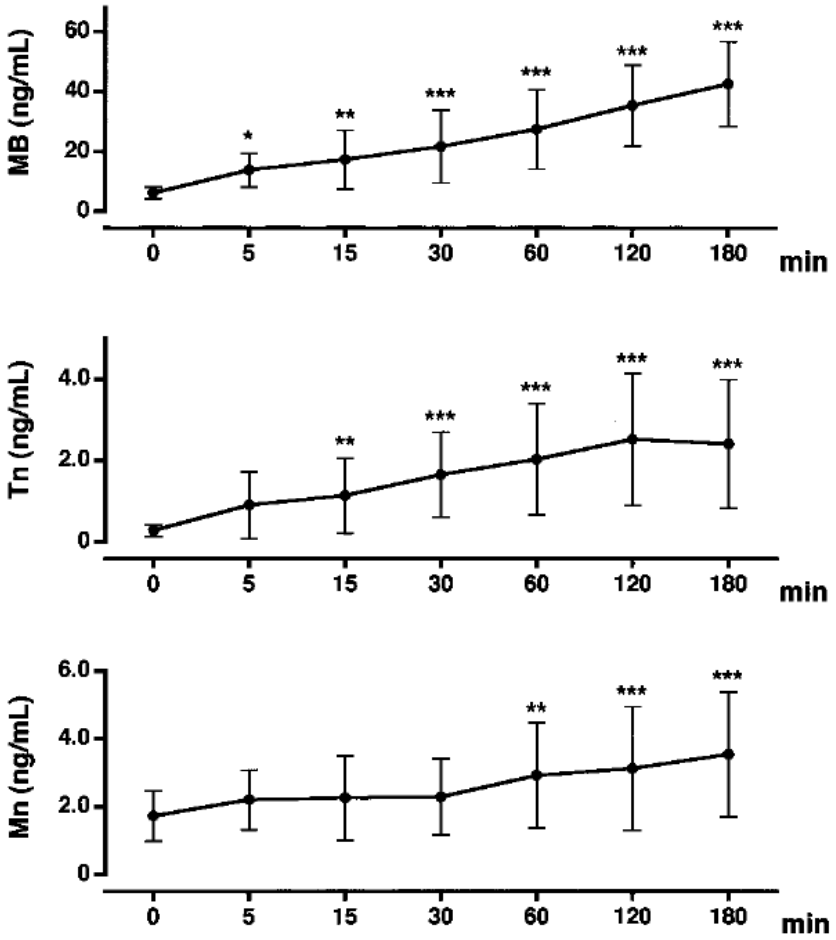


Figure 9. Time course of serum concentrations of CK-MB (MB), troponin T (Tn), and myosin light chain (Mn) after sub-arachnoid hemorrhage. Values are mean±SD of 18 dogs. *p<0.05, **p<0.01, ***p<0.001 vs baseline values. From: Masuda et al. 2002

The study of Tonomura et al (2009) in rats compares the sensitivity and specificity of several biomarkers for either skeletal muscle or cardiac muscle damage and represents one of the few non-clinical ROC analyses for the cTns. The ROC curve shown below indicates cTnT, followed closely by cTnI, was superior to the other biomarkers for detecting cardiac toxicity. The ROC curve is shown below along with the AUC values for the different biomarkers.

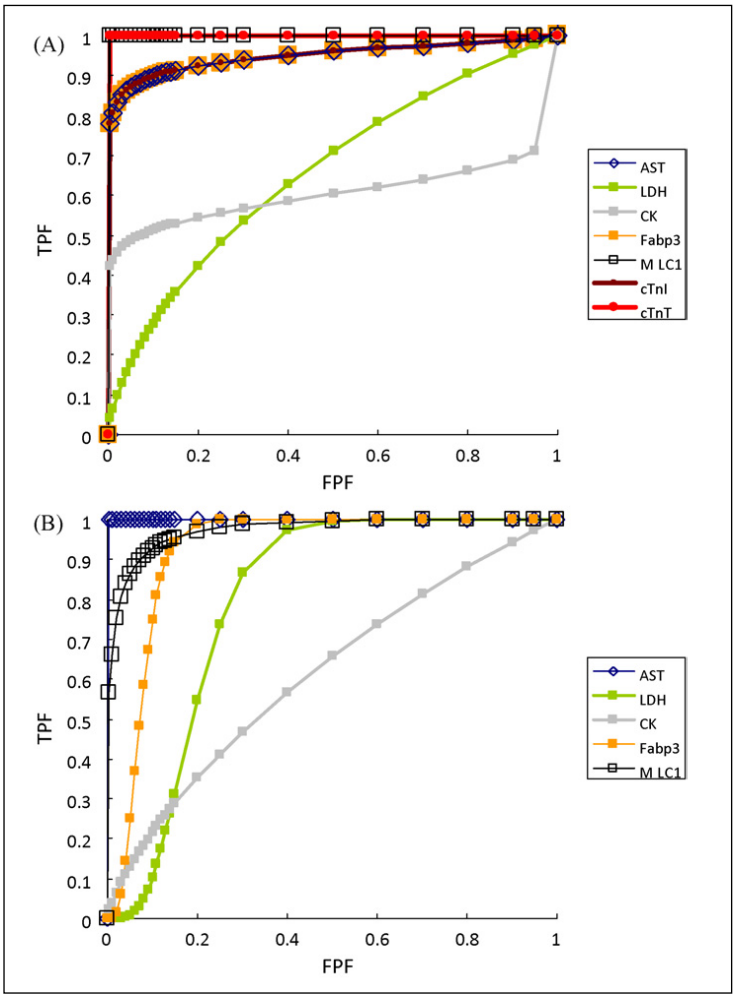


Figure 10. ROC Analysis of pathology versus circulating biomarkers
ROC analysis of heart and skeletal muscle pathology versus the AUC of AST, LDH, CK, Fabp3, MLC1, cTnI and cTnT obtained by blood chemistry. The indicated parameters were analyzed by ROC analysis. TPF=true positive; FPF= false positive.
(A) ROC curve for cardiotoxicity biomarkers (n=12 in respective curves)
(B) ROC curve for skeletal muscle toxicity biomarkers (n=12 in respective curves).
From : Tonomura et al. 2009.

3. DATA FOR SPECIES INCLUDED IN THE QUALIFICATION

a. Data provided for the rat

O'Brien et al cited 20 publications as critical to the qualification of troponins for use in the rat. The work was primarily conducted in the Sprague-Dawley but also included work in 5 other strains. One study specifically stated that both sexes of rats were examined, one study examined female rats only and the remainder of the work assessed male rats only. Eight of the studies used isoproterenol as the agent of cardiac damage, 4 studies used doxorubicin, 5 studies used other named pharmacologic agents and 3 studies used unnamed proprietary compounds. Two studies used physical damage (coronary occlusion) and 1 study addressed the influence of aging. Thirteen studies examined multiple time points and 5 studies examined the kinetics of clearance and release. Three studies also used a comparison to traditional biomarkers. A summary of this information is provided in the table below.

Table 7: Summary of core rat studies

Parameter	
Strains studied (# studies)	CRL(1), CR(1), Fischer 344(1), SD(7), SHR(3), SHR CRL(1), Wistar(6)
Studies using males only	18
Studies using females only	1(Wistar)
Studies using both sexes	1(SD)
Drugs used (# studies)	Isoproterenol (8), doxorubicin(4),mitoxantrone(1),PDE3 inhibitor(1), fenthion(1), methidathion(1), microcystin(1), unnamed proprietary (3)
Other mechanisms of damage used	Natural aging (1), coronary artery occlusion/ligation(2)
Multiple time points sampled	13 studies
Correlation to histology	Reference standard for all studies
Was damage quantitated	References Qiu 2009, 135. 150,
Severity scale used	Refs 239,28,93,30,240,152
Kinetics of clearance and release	5 studies
Comparison of assays	2 studies
≥sensitivity than histology for early lesions	6 studies
Comparison to other biomarkers	3 (LD, LD2, CK and CKMB, ALT, AST, GLD, aldolase

b. Specific studies for the rat

The majority of studies showed troponins in rats to be at least as effective as light microscopy with H&E staining for detecting minimal changes. Depending upon the timing of the first tissue sample and the techniques used, troponins were also demonstrated to be more sensitive than light microscopy with routine H&E staining. Because of the significance of these findings, some of the individual publications will be discussed.

Several studies compared troponins to the traditional biomarkers of LD isoforms, CK and CKMB and explored the sensitivity of detection of the troponins. While the investigators worked independently, the studies considered together provided a consistent and more complete picture of the relationship of troponin release to histological detection of cardiac damage.

York et al (2007) examined a dose-response effect of cardiac damage at different time points and compared troponins to LD and CK isoforms. Female Hanover Wistar rats were given intraperitoneal isoproterenol (iso) or the vehicle of phosphate buffered saline (PBS). Samples were taken at multiple timepoints for histology sampling, immunochemical analysis, and comparison of cTnI and cTnT, LD and isozymes, CK and isozymes, ALT, AST, glutamate dehydrogenase (GLD), and aldolase.

Using light microscopy and H&E staining, histologic lesions were first identified at 4 hours after dosing. Cardiac troponins were elevated at 2 hours after drug administration in each of the iso-

treated groups but not in the control animals. This finding was in the absence of identified histologic lesions. A threshold dose of iso for elevated Tns was not identified.

The histologic assessment of cardiac lesions at 2 hours post-dosing is displayed in Table below, with the changes quantified on a scale of 0 to 4 (0= no lesion, 4= most severe), and the mean (SD) of the grades expressed as the myodegeneration score. At 2 hours postdosing, a total of 3 animals, including 1 control and 1 each treated with 8.0 and 10.0 mg/kg ISO, showed minimal (grade 1) lesions characterized as chronic myodegeneration. Given the results from the time course study, these observable lesions were considered to predate the start of the study and therefore represent background pathology.

By 24 hours after dosing, lesions were detectable at all doses of iso. The circulating levels of cTnI were back to baseline at doses ≤ 6 mg/kg. Possibly, the damage was complete (no longer ongoing) by 24 hours, accounting for the return to baseline.

Table8. Comparison of serum biomarkers from female rats treated with isoproterenol and euthanized 2 hours post-dosing.

ISO dose (mg/kg)	cTnI (μg/L)	LD (U/L)	LD1 (%)	LD2 (%)	CK (U/L)	CKMB (%)	Myodegeneration score
0 (Control)	<0.030 (0.000)	2324.2 (656.1)	1.90 (0.22)	2.08 (0.43)	849.2 (255.2)	14.20 (2.59)	0 (0)
0.25	0.796 (0.581)*	1803.6 (385.6)	3.86 (1.05)**	4.02 (1.55)*	671.2 (145.9)	9.98 (0.90)*	0 (0)
	[25.5]	—	[1.0]	[0.9]	—	—	—
0.5	1.192 (1.602)	1702.0 (311.3)	3.16 (1.14)	3.18 (1.60)	619.8 (94.5)	11.42 (4.71)	0 (0)
	[38.7]	—	[0.7]	[0.5]	—	—	—
1.0	0.790 (1.124)	1993.2 (371.0)	2.84 (0.59)*	2.76 (0.95)	669.0 (120.0)	9.06 (2.87)*	0 (0)
	[25.3]	—	[0.5]	[0.3]	—	—	—
0 (Control)	0.060 (0.042)	2303.2 (387.4)	1.82 (0.43)	1.82 (0.33)	871.8 (161.2)	9.86 (1.15)	0 (0)
2.0	8.498 (4.629)**	1670.0 (439.4)*	5.08 (1.86)**	6.76 (3.33)*	568.8 (107.6)**	15.40 (2.82)**	0 (0)
	[140.6]	—	[1.8]	[2.7]	—	[0.6]	—
4.0	15.722 (10.080)**	1551.2 (380.6)*	7.82 (4.04)*	10.26 (6.41)*	523.6 (151.4)**	15.80 (1.37)***	0 (0)
	[261.0]	—	[3.3]	[4.6]	—	[0.6]	—
6.0	15.626 (17.366)	1650.8 (660.0)	5.66 (2.86)*	7.78 (4.67)*	479.0 (159.4)**	13.16 (4.00)	0 (0)
	[259.4]	—	[2.1]	[3.3]	—	[0.3]	—
0 (Control)	<0.030 (0.000)	2528.0 (754.1)	1.92 (0.53)	1.44 (0.05)	969.6 (255.6)	11.38 (2.43)	0.2 (0.4)
8.0	14.804 (8.980)**	3486.2 (1067.8)	3.86 (0.74)***	5.74 (1.68)***	1165.6 (255.8)	10.70 (1.83)	0.2 (0.4)
	[492.5]	[0.4]	[1.0]	[3.0]	[0.2]	—	—
10.0	7.726 (7.421)*	2612.8 (568.7)	4.72 (1.42)**	6.54 (2.20)***	978.2 (141.5)	13.04 (3.06)	0.2 (0.4)
	[256.5]	—	[1.5]	[3.5]	—	[0.1]	
20.0	3.200 (3.264)	2642.2 (402.1)	3.84 (1.53)*	3.90 (2.36)*	819.4 (118.4)	12.30 (3.70)	0 (0)
	[105.7]	—	[1.0]	[1.7]	—	[0.1]	—

Values are mean(SD). The mean severity grade of the cardiac lesions is presented as the myodegeneration score.
[a]The experiment was conducted in 3 parts, according to the dose level of ISO administered; part 1, at 0.25, 0.5, 1.0 mg/kg; part 2, at 2.0, 4.0, 6.0 mg/kg; part 3, at 8.0, 10.0, 20.0 mg/kg; each part of the experiment had a separate control group. There were 5 animals in each dose level group.
*Significantly different from the control animals, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
[bc]TnI was measured on the ACS: 180SE. LD1 and LD2 are expressed as % of total LD1 to LD5. CKMB is expressed as % of total CKMB, CKMM and CKBB.
[] indicates the "fold increase" of the mean value over the control mean value.
[c]Cardiac lesions were graded on a scale: 0=absent; 1=minimal; 2=slight; 3=moderate; 4=moderately severe; the mean of the severity grade score is presented as the myodegeneration score.

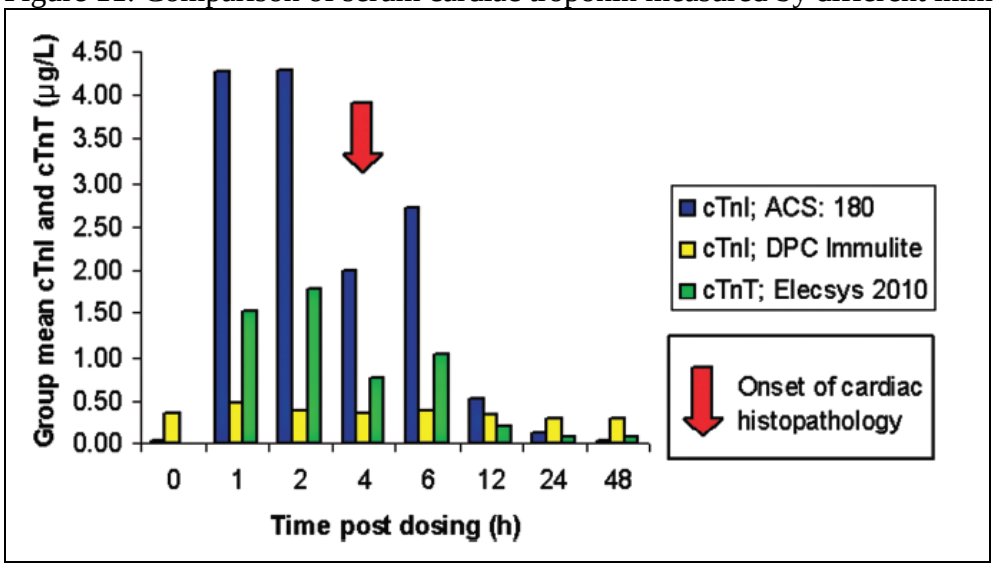
Table 9. Comparison of serum biomarkers from female rats treated with isoproterenol and euthanized 24 hours post-dosing.

ISO dose (mg/kg)	cTnI (μg/L)	LD (U/L)	LD1 (%)	LD2 (%)	CK (U/L)	CKMB (%)	Myodegeneration score
0 (Control)	<0.030 (0.000)	2174.3 (1030.8)	2.08 (1.15)	1.20 (0.45)	897.5 (417.4)	13.73 (4.32)	0 (0)
0.25	<0.030 (0.000)	2243.5 (421.4)	3.40 (0.71)	2.08 (0.51)*	945.0 (278.2)	12.60 (4.52)	1.5 (0.6)**
	-	—	[0.6]	[0.7]	[0.1]	—	—
0.5	<0.030 (0.000)	2103.5 (706.1)	2.35 (0.96)	1.48 (0.46)	647.8 (262.6)	9.48 (4.67)	1.5 (0.6)**
	-	—	[0.1]	[0.2]	—	—	—
1.0	0.320 (0.600)	2253.5 (362.2)	4.13 (2.45)	2.03 (0.85)	733.0 (127.3)	6.13 (0.22)*	2.0 (0.8)**
	[9.7]	—	[1.0]	[0.7]	—	—	—
0 (Control)	<0.030 (0.000)	2007.8 (108.5)	2.65 (0.90)	1.48 (0.36)	690.5 (80.9)	9.10 (1.53)	0 (0)
2.0	<0.030 (0.000)	3012.8 (1830.7)	2.18 (0.68)	1.45 (0.19)	874.8 (348.4)	16.03 (10.53)	1.0 (0.8)*
	—	[0.5]	—	—	[0.3]	[0.8]	—
4.0	<0.030 (0.000)	1649.5 (748.1)	3.40 (1.79)	1.73 (0.75)	570.8 (215.4)	11.28 (7.01)	2.3 (1.0)**
	—	—	[0.3]	[0.2]	—	[0.2]	—
6.0	0.653 (1.205)	2497.8 (1016.4)	4.45 (2.56)	2.40 (1.00)	687.8 (170.7)	11.30 (3.21)	3.0 (1.2)**
	[20.8]	[0.2]	[0.7]	[0.6]	—	[0.2]	—
0 (Control)	<0.030 (0.000)	2017.0 (379.3)	2.80 (0.37)	1.50 (0.08)	759.0 (109.6)	15.35 (2.23)	0 (0)
8.0	0.188 (0.198)	1967.5 (253.5)	4.15 (1.39)	1.85 (0.19)*	804.3 (41.4)	10.65 (1.82)*	2.8 (0.5)***
	[5.3]	—	[0.5]	[0.2]	[0.1]	—	—
10.0	0.573 (1.085)	1718.0 (420.9)	4.38 (1.69)	2.08 (0.67)	598.0 (158.1)	12.03 (3.04)	3.3 (0.5)***
	[18.1]	—	[0.6]	[0.4]	—	—	—
20.0	6.200 (6.657)	1825.8 (403.8)	9.33 (4.35)*	4.98 (4.75)	592.5 (80.7)*	10.23 (1.72)*	3.3 (1.0)***
	[205.7]	—	[2.3]	[2.3]	—	—	—

All other information as Table 3, except there were 4 animals in each ISO dose level group.

Another facet of this study was the comparison of the immunoassays. As shown in Figure 11, the ACS assay was most sensitive to the rat samples while the DPC Immulite showed little sensitivity for this species. These results are consistent with work from other investigators.

Figure 11. Comparison of serum cardiac troponin measured by different immunoassays



Group mean levels of serum cardiac troponin I (cTnI) measured with the ACS: 180SE (Bayer) and the DPC Immulite (Diagnostic Products), and cardiac troponin T (cTnT) measured with the Elecsys 2010 (Roche), in the female HanoverWistar rat at a series of time points after the administration of a single dose of isoproterenol at 50.0 mg/kg (intraperitoneal injection; 4 animals at each time point). The time of onset of histopathological findings in the heart is also shown. York et al. 2007.

The work of Mikaelian et al. (2008) was notable for the use of different histochemical stains in the examination of the early histologic changes. Male Wistar rats were given a single subcutaneous dose of sterile water (control) or isoproterenol (0.5 mg/kg). Necropsies were performed at 0.5 hours, 1 hours, 3 hours, 6 hours, 12 hours, 1 day, 3 days, 7 days and 14 days.

Phosphotungstic acid hematoxylin (PTAH) histochemical stain identified myocardial damage consisting of prominent myofiber contraction bands at 0.5 hours. In the same study, the earliest detection of damage using H&E staining occurred at 3 hours. A critical finding was that elevations in circulating troponin correlated with damage identified with the more sensitive PTAH staining at the earlier time points. This is consistent with troponins as integral elements of the myofilament being susceptible to proteolysis and with decreased immunolabelling for cTnI in the areas of contraction bands.

Other immunohistochemical studies have also been performed in dogs, pigs and rats following coronary occlusion with similar results to the above findings. The light microscopy sections (H&E staining) showed only edema while electron microscopy data supported necrosis concordant with loss of troponin immunostaining (Fishbein, 2003).

Early increases in troponins were investigated by concurrent use of immunostaining for cTnT, cTnI and TUNEL staining. One example of such investigation was the work of Zhang et al (2008). using isoproterenol to generate lesions. Alterations of interstitial edema, the basement membrane, and myocyte integrity were considered reversible a designation that may be debatable given Fishbein's EM data. Serum levels of cTnT showed significant changes that paralleled the severity of the lesion scores (see table below).

Immunohistochemical staining for cTnT demonstrated alteration of the characteristic striated pattern due to decreased tissue immunoreactivity and associated with increases in serum cTnT. In conjunction with more severe lesions, the serum levels of cTnT were ten-fold above the levels detected in association with milder lesions (8–64 µg/kg Iso) and were elevated ~100 times above those levels found in saline-control animals.

By 48 hours, cardiac tissue morphology was characterized by the predominance of dense connective tissue interspersed with the remnants of dying or dead cardiac myocytes. At this time, the levels of serum cTnT were reduced to less than 1 ng/mL, consistent with relatively rapid clearance of serum cTnT after cessation of active damage. The results of this published report were summarized in the table 10 below.

Table 10. Histologic Findings and Serum Levels of cTnT in Rats Following Different Doses of Isoproterenol

—Potential signs of irreversible or reversible myocardial injury and serum levels of cTnT in rats following treatment with various doses of isoproterenol.

		Reversible myocardial findings					Irreversible myocardial injury				Cardiac troponin T	
Iso dose (μg/kg)	Time (hr)	Interstitial edema	Leukocytes	Macrophages	BM damage	Myocardial degeneration	Apoptosis	Necrosis	CM damage	Interstitial fibrosis	Immunoreactivty of cardiac tissues	Serum levels (ng/mL)
0	all	—	—	—	—	—	—	—	—	—	Normal	0.015[a]
8–16	3	—	—	—	—	—	Rarely +/-	—	—	—	Minimal ↓	0.07–0.27
32–64	3	+	+	—	+	+	Rarely +/-	—	—	—	Minimal ↓	0.19–0.26
32–64	6	+	+	—	+	+	Rarely +/-	—	—	—	Minimal ↓	0.13–0.21
125	3	+	+	—	++	++	+	++	++	—	↓↓	2.68
250–500	3	+++	+++	—	+++	+++	++	+++	+++	—	↓↓↓↓	6.8–13.63
125–250	6	+++	+++	+	+++	+++	++	+++	+++	—	↓↓↓↓	7.74–9.1
500	6	++	++	++	+++	+++	++	+++	+++	—	↓↓	3.7
125–500	12	++	++	+++	+++	+++	+	+++	+++	+	↓↓↓	1.3–3.5
125–500	24	+	+	+++	+++	+++	+	++	++	++	↓↓	0.46–5.5
125–500	48	+/–	+/–	+	++	++	—	+/–	+/–	+++	↓	0.06–0.18

[a] Serum levels of cTnT range in saline-treated rats: 0—0.18 ng/mL.

Abbreviations: BM = Myocardial basement membrane; CM = Cell membrane of cardiac myocytes.

Keys to myocardial lesions:—(no changes); +/– (no remarkable changes); + (mild changes); ++ (moderate changes); and +++ (severe changes).

Keys to cTnT immunoreactivity: normal (no changes of immunoperoxidase staining intensity); diffuse (diffuse reduction of staining intensity by image analysis); ↓ (reduction of staining intensity in a few cardiac myocytes); ↓↓ (reduction of staining intensity in groups); and ↓↓↓ (extensive and diffuse reduction of staining intensity).

Zhang et al, 2008.

Schultze et al. (2008) indirectly explored early troponin increases in a comparison of the high sensitivity cTnI Erenna (Singulex) assay to a standard sensitivity (Beckman Access) cTnI assay. Rats received either 0.5 mg/kg or 8 mg/kg isoproterenol.

Figure 9. Rat cTnI measured by Erenna vs Beckman assays
From Schultze et al 2008.

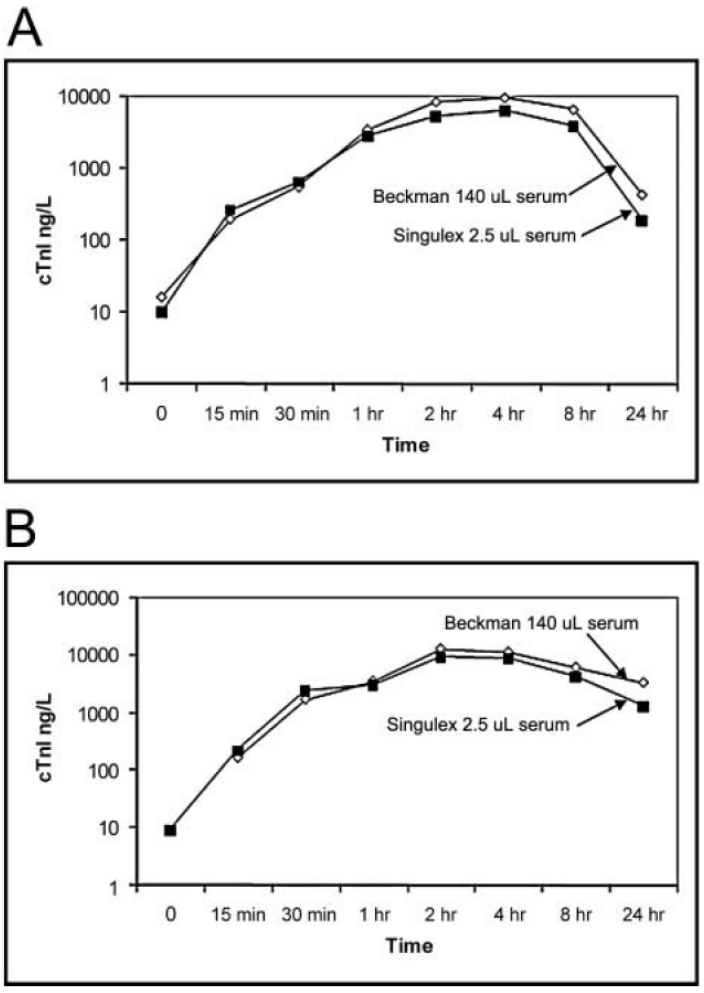


FIGURE 2.—Rat cTnI concentrations measured by Singulex Erenna and Beckman Access assays after administration of isoproterenol (panel A, 0.5 mg/kg and panel B, 8 mg/kg). Each data point represents average cTnI values from five rats. In the case of predose zero with the Access assay, three of five rats were measurable in the 0.5 mg/kg dose group and zero of five were measurable in the 8 mg/kg dose group.

Measured with the Erenna assay, cTnI concentration was quantifiable at all time intervals tested (pre-dose, fifteen minutes to seventy-two hours) in all animals following isoproterenol. As early as fifteen minutes post-administration, marked increases from baseline cTnI values were observed. Representative data, using the rat response as an example, are shown on the left. Cardiac TnI values obtained for identical specimens using the Beckman Access assay are presented, as well. In the predose rats, cTnI was measurable in all ten animals with the Erenna assay and measurable in only three animals (all in the 0.5 mg/kg iso dose, Figure A) with the Access assay. Both assays showed parallel increases and decreases in cTnI values.

The Erenna assay was tested in a preliminary experiment with dilutions of rat serum covering a range of 5-50µl. In subsequent work, cTnI values were obtained from rats using 2.5 µl and 50 µl of serum from the same animal. Using 2.5 µL of serum, all samples provided measurable cTnI (range 3–441 ng/L) with a linear regression correlation of R2 = 0.97 (2.5 µL vs 50 µL serum). The results suggest that 2.5 µL of rat serum can be used to accurately measure cTnI with the Erenna assay and demonstrate that new assay technology requires validation before adoption into safety assessment protocols.

The reference from Qui (2009) may be seen as equivocal, or not supportive of the qualification. Intravenous microcystin was given at 0, 14 and 87 mcg/kg to 5, 5 and 10 male Wistar rats. An

equivalent volume of saline solution was administered to five controls.Twenty-four hours after the administration, the rats were euthanized and blood was collected. Troponin I was measured with the Beckman Coulter AccuTnI kit of the ACCESS 2.

This study is somewhat paradoxical in that the low dose of microcystin dose produced myocardial damage including enlarged cells with enlarged and often bizarrely-shaped nuclei, cytoplasmic vacuolization and degenerative muscle fibres but no increase in serum TnI. At the high dose of microcystin, disarray of myocardial fibres and the degenerative muscle fibres with myocytolysis were the most prominent features with pycnotic changes in the nuclei, occasional malformation of blood vessels and slight infiltration of lymphocytes. The authors stated that the "significant elevations of serum CK and cTnI…were in accordance with histopathological observations." It is not clear what amount of necrotic tissue was produced in these rats and why it did not produce a detectable increase in troponins. Was the sample taken too late, after resolution of the damage or was there a problem with implementation of the assay?

Table 11. Changes in physiologic and biochemical parameters in rats treated with microcystin

Parameter	0 μg kg^{-1}	14 μg kg^{-1}	87 μg kg^{-1}	Dead rats
Mean arterial pressure, mmHg	90.9 ± 3.3	83.6 ± 0.4*	33.1 ± 5.2**	–
Heart rate, bmp	419.5 ± 80.6	350.0 ± 50.5	159.3 ± 59.8**	–
Plasma ALT activity, IU/L	40.5 ± 1.5	40.0 ± 2.2	335.3 ± 145.8**	255.7[a]
Plasma ALP activity, IU/L	270.0 ± 10.0	305 ± 23.0*	271.3 ± 49.4	338.0[a]
Plasma AST activity, IU/L	144.5 ± 28.5	190.0 ± 24.9*	395.3 ± 117.7**	433.9[a]
Plasma LDH activity, IU/L	1316.0 ± 138.0	1337.7 ± 204.5	1667.7 ± 446.2**	2178[a]
Plasma CK activity, IU/L	2151.0 ± 116.0	3041.0 ± 198.3**	3475.7 ± 228.9**	6126[a]
Plasma cTn I content, ng/mL	0.02 ± 0.01	0.02 ± 0.01	0.1 ± 0.04**	0.43–0.53[b]

The values are expressed as mean S.E. ($N = 5$). The significance levels observed are $p < 0.05$ (*) and $p < 0.01$ (**) in comparison to control group values.

[a] The plasma biochemical indicators of the first dead rat.

[b] The range of cTn I content of all the dead rats.

Qui et al 2009.

c. Data Provided for the Dog

The submission included thirteen published studies and a pre-print of a manuscript. Four studies specified the inclusion of both sexes. The remainder of reports either did not specify sex or were restricted to males. The dog studies were more varied than the rat studies and included the effects of natural disease and clinical treatment, including troponin monitoring of treatment related effects. Two experimental physical mechanisms of damage were studied in addition to drug-induced cardiac damage. One study of naturally occurring disease considered the effects of aging. The clinical cases by their nature could not include histologic analysis of the hearts. Several of the non-clinical research studies were not terminal and did not include histology data. Other comparators and monitors of cardiac function were used, including ECG and echocardiography but not in a fashion to allow for efficacy comparisons to troponins.

Table 12. Summary of Core Dog Studies

Parameter	
breeds studied in laboratory research	Beagle (6), Foxhound (2) mixed breed (4) not specified (1) Note: numerous breeds were studied in the clinical work
Studies using males only	Not specified
Studies using females only	Not specified
Studies using both sexes	4 (3 Beagle studies, 1 mixed breed study)
Drugs used	Compound X, isoproterenol (2),doxorubicin (5;1 study used dogs with naturally occurring lymphoma or osteosarcoma)
Other mechanisms of damage used	Atrial pacing, left coronary occlusion, natural cardiac disease, endogenous hypercatecholemia
Multiple time points sampled	11 studies
Correlation to histology	4 studies, 5 studies did not collect histology data
Other comparators	Clinical signs (3) echo (1), ECG(1),traditional blood biomarkers(4) circulating catecholamines (1), function (1)
Was damage quantitated	1 study (Ref 150)
Severity scale used	N/A
Kinetics of clearance and release	N/A
Comparison of assays	3 studies
Finding ≥sensitivity than histology for early lesions	N/A

d. Specific studies for the dog

The work of Serra et al, addressed possible confounding factors for troponin interpretation. This report also reiterated the necessity of assay validation within a lab.

Cardiac damage secondary to severe conditions or diseases, is a concern for interpretation of troponin results in toxicology studies. In this report, cTnI was increased in conditions such as pancreatitis, marked anemia, uncontrolled Addison's and Cushing's disease, renal disease, severe weight loss and vomiting. Approximately 25% of clinically healthy aged dogs had troponin levels within the control reference range. The remainder of the dogs were determined to be without cardiac or serious disease yet had somewhat elevated cTn levels.

In the setting of a veterinary teaching hospital Serra et al, assessed 6 new assays in 500 animals of 7 species. Animals from drug-safety studies (250 dogs, 76 horses, 48 bovine, 62 cats, 50 dogs, 100 rats,12 rabbits, 20 mice) were assessed also, primarily for assay evaluation. Healthy animals and clinic cases without cardiac or serious disease served as controls.

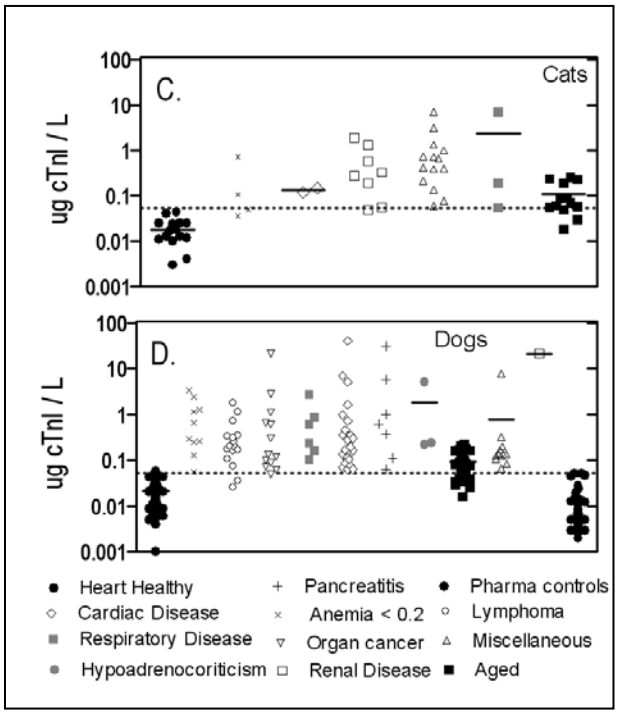


Figure 13 Comparison of cTnI values for healthy and diseased animals From Serra M, Papaconstantinou, M Adamcova, PJ O'Brien. Veterinary and toxicologic applications of cardiac troponin for detection of active and ongoing cardiac injury in animals. Values in figure from the Centaur cTnI assay

Sample volume was discussed as well as the evaluation of several new assays. Mesoscale Discovery provides a commercially available cTn assay for sample volumes as low as 25 µl. MSD®96-well Multi-Spot® rat cardiac injury panel measures cTnI, cTnT and FABP3 using a 4-spot MULTI-SPOT plate which is pre-coated with the three capture antibodies on separate spots within each well allowing simultaneous quantification of multiple markers in a single sample volume. The cTnT assay was effective in rats but not dogs nor any of the other species in which it was tested. The MSD cTnI assay was only poorly effective in rats and dogs.

Another assay evaluated was the ADVIA Centaur TnI-Ultra™ a three-site sandwich immunoassay that uses direct chemiluminometric technology. Samples with insufficient volume for analysis were diluted to bring the volume to the required amount. This is important for studies in small mammals or when there is multiple use of samples with consequent volume restriction. A diluent compatible with the sample matrix must be used.

There are other examples of elevation of cTn in the absence of clinical cardiac disease. A retrospective observational study (Porciello et al 2008) found that 70% of dogs and 70% of cats with azotaemic renal failure and 70% of dogs with a variety of systemic non-cardiac diseases have elevated cTnI levels using a decision limit of 0.18 ng/mL for cTnI. Cardiac troponin I concentrations did not correlate with the degree of azotaemia, presence of murmurs, hypertension or type of non-cardiac illness. As the study was retrospective, pre-existing cardiac disease could not be

ruled out nor could additional testing be conducted. Also, the primary disease conditions studied all have a high probability of cardiac involvement. The results are supported by a subsequent study by Sharkey et al (2009) in which approximately 80% of dogs with renal insufficiency, but no overt clinical signs of cardiac disease, had significantly higher serum cTnI concentrations (median, 0.35 ng/mL range 0.2-26 ng/ml) than did healthy dogs (0.20 ng/mL; range 0.2-0.4 ng/ml). The authors propose that these results can be explained by subclinical cardiac damage similar to that proposed by Freda et al (2002). Similar results can be seen in human medicine and have been interpreted to mean that there are conditions that can result in clinically silent myocardial injury or altered elimination of cTns (Sharma et al 2006; deFilippi et al 2007).

The sponsors maintain that dogs with cardiac arrhythmias, tachycardia, and cardiac effusion with dyspnea may have elevated levels of cTn (O'Brien et al 1997). Several reports on this topic illustrate that interpretation of clinical data requires careful consideration.

Prosek et al (2007) reported that in a group of 48 dogs with respiratory distress, congestive heart failure (CHF) was diagnosed in 22 dogs based on echocardiography and radiographic examinations. Plasma cTnI concentrations were not significantly different between the dogs without heart disease (0.29 ng/mL, 95% CI 0.12-0.72 ng/mL) and the dogs with CHF (0.42 ng/mL, 95% CI 0.18-0.97, P = .53). In contrast, NT-proANP, BNP, and ET-1 had AUC_{ROC} values of 0.946, 0.886, and 0.849, respectively, and appeared useful for distinguishing between dogs with cardiac and noncardiac causes of dyspnea, whereas cTn did not. While this is biologically plausible, the only detail about TnI measurement was the use of an unspecified ELISA and no mention of standardized material or positive serum for quality assurance. In contrast, there was a half page of detail about the natriuretic assays in the same report.

In a study to evaluate methods of detecting occult dilated cardiomyopathy (DCM), Oyama et al (2007) diagnosed DCM in 21 of 118 dogs based on ECG and echocardiographic examination. Although plasma levels of cTnI increased in the dogs with DCM (0.21 ± 0.10 ng/mL) compared to clinically normal dogs (0.06 ± 0.01 ng/mL), ROC analysis indicated brain natriuretic peptide had a higher AUC_{ROC} value (0.844) with higher sensitivity (95%) and specificity (62%) compared with cTnI with a AUC_{ROC} value (0.74) with higher sensitivity (89%) and specificity (42%).

A drawback to this report is the minimal information provided about the troponin assay used and if any validation was performed for the laboratory. However, it is reasonable to see superiority of BNP for detection of this disease state. The natriuretic peptides are released in response to increased stress of the myocardial wall, presumably early in the course of disease. At this early point in the pathogenesis there may be considerably less myocyte necrosis to release troponins. Similarly, in humans, assay of ANP and BNP concentrations can help clinicians distinguish cardiac from noncardiac causes of dyspnea.

Table 13 Comparison of BNP, ANP and cTnI for detection of Occult DCM in 21 of 118 dogs

Assay	AUC	*P* value*	Cutoff value	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
BNP	0.844	< 0.001	6.210 pg/mL	95.2	61.9	98.4	35.1
cTnI†	0.740	< 0.001	0.030 ng/mL	88.9	41.8	95.0	23.2
ANP	0.658	0.005	0.244 nmol/L	85.7	47.4	93.9	26.1

*Values were considered significant at $P < 0.05$.
AUC = Area under the receiver-operating curve. NPV = Negative predictive value. PPV = Positive predictive value.

From Oyama MA, Sisson DD, Solter PF.2007.

e. Data Provided for the Non-Human Primate

The published database for the non-human primate is less extensive than that for either rat or dog. The five studies cited as central to the qualification studied Cynomolgus monkeys, Rhesus monkeys and Marmosets. The studies provided minimal correlation with histopathology. Three of the non-human primate studies focused on assessment of the assays. Overall, the number of animals examined was low and there were little data to show a temporal or quantitative correlation between morphologic damage and circulating levels of troponins. The studies assumed that troponins would behave in a manner consistent with what had been reported for other species.

Since the submission of this qualification package, a number of studies have been added to the published literature for primates and are supportive of the use of cTns for safety assessment. These studies will be discussed in more detail below but are not included in the summary table of core studies.

Table 14. Summary of Core Primate Studies

Parameter	
breeds studied	Cynomolgus (3 studies) rhesus(1study) marmoset (1study)
Studies using both sexes	4 studies
Studies with unspecified sex(es)	1 study
Drugs used	Isoproterenol(1study), norepinephrine(1 study),hydrochlorthiazide (1 study), minoxidil(1study)
Other mechanisms of damage used	Left coronary occlusion
Multiple time points sampled	3 studies
Correlation to histology	2 studies; 3 studies did not collect histology
Other comparators	Traditional biomarkers, ECG
Was damage quantitated	N/A
Severity scale used	N/A
Kinetics of clearance and release	N/A
Comparison of assays	3 studies
≥sensitivity than histology for early lesions	NA

f. Specific Studies for the Non-Human Primate

Hanton et al.(2008) attempted to assess the cardiotoxicity of the vasodilator minoxidil in marmosets. A single oral dose of 150mg/kg was given to one male and three females and 200mg/kg was given to two males. One male and one female were kept as controls and received the vehicle alone. The animals were examined by ECG and echocardiography 1 hour after dosing. At the same time, blood was obtained for clinical chemistry. All treated animals showed changes in the ECG consisting of increased T wave amplitude, depression of ST segment, and/or deep and enlarged S waves with no detectable ST segment. The echocardiograms showed treatment-related effects on cardiac function. The animals were euthanized 24 hours after dosing and histology was generated. All animals, including controls, showed increased troponin levels. Treatment-related findings were reported in all treated animals and included myocardial necrosis, coronary arteriopathy and degeneration of renal tubules. There is a temporal disconnect between the blood samples and the echocardiographic and electrocardiographic data. The presentation also makes it difficult to correlate the different modes of clinical analysis.

Table 15. Plasma troponin in animals treated with minoxidil before dosing (day -1) and approximately 1 hour after treatment (day1)

Dose (mg/kg)	Animal number	Troponin concentrations (μg/L)	
		Day −1	Day 1
Control	M3.1	<0.03	0.39
	F3.1	0.1	0.34
150	M3.2	0.08	1.69
	F3.2	<0.03	0.58
	F3.3	<0.03	0.46
	F3.4	<0.03	NA
200	M3.3	0.04	3.45
	M3.4	0.18	0.54

From Hanton et al.. 2008.

Table 16. Incidence of myocardial necrosis after treatment with minoxidil

Localization	Number of affected animals in the group		
	150 mg/kg		200 mg/kg
	Males ($n = 1$)	Females ($n = 3$)	Males ($n = 2$)
Left ventricle	1	3	2
Right ventricle	1	2	1
Interventricular septum	0	1	0
Left atrium	0	0	1
Right atrium	0	2	0

From Hanton et al. 2008.

Takeuchi et al(2008)) examined the association between hydrochlorothiazide-induced hypokalemia and subsequent myocardial necrosis in four female Cynomolgus monkeys. All animals were euthanized the day after the final dose of hydrochlorothiazide and samples from all organs and tissues were collected.

Plasma potassium changed profoundly on day 3. One of the treated animals showed an increase in cTnI on day 3 while two others showed a significant increase on day 5. Three of the four animals showed some degree of myocardial necrosis that correlated roughly with the degree of hypokalemia. However, the four treated animals and 1 control monkey all showed fluctuations and increases in measured troponin levels. The treated animals showed greater degrees of troponin increase than the control animal, with no explanation offered for the increase reported for the control animal. AST showed no discernible changes over the course of the study. CK showed an increase in one animal on Day 3 followed by steady declines. The ECG tracings showed flattened T-waves in two animals, both of which showed marked hypokalemia and myocardial necrosis. The timing of sample collection and deteriorating general condition of the animals in this study may have contributed to the variability of the troponin measurements.

Several of the manuscripts used primates to make comparisons of commercially available assay systems. Apple et al (2008) used samples from 2 species of non-human primates, to compare seven assays used frequently in safety assessment. Rhesus monkeys were infused with norepinephrine for 2 hours and anesthetized adult male and female Cynomolgus monkeys were subject to ligation of the left anterior descending artery.

No histology was performed for primates in this study. A unique feature of this work was the examination of the imprecision profiles of the different assays at the low end of the troponin concentration range. The variability or imprecision of each assay was greatest at low concentrations, similar to the clinical situation.Table 17 compares assay characteristics of different cTn assays.

Table 17. Statistical comparison of different cTn assays

Linear regression analysis statistics for dilution studies by animal species/strain for each cTn assay compared to the relative dilution factor of each pool tested.[a]

	SD rat		Wistar rat		Beagle dog		Rhesus monkey		Cynomolgus monkey	
Analyzer	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept
Architect	3.60	−0.91	4.30	−5.7	0.96	0.11	6.38	−0.43	1.28	0.10
Centaur first generation	5.10	0.63	5.91	0.08	4.52	−0.69	20.0	−0.18	6.07	0.50
Access	5.02	−0.74	6.39	−0.92	1.81	−0.17	9.12	−1.35	2.03	0.23
Dimension	5.89	−1.00	6.20	0.30	1.13	−0.09	7.10	0.61	2.36	0.03
Vitros	0.10	−0.07	0.10	−0.09	1.77	−0.21	7.21	0.20	2.12	0.57
Immulite	0.18	0.13	0.17	0.29	5.64	−3.63	23.5	−3.20	3.01	0.04
Elecsys	1.23	0.12	1.08	0.11	0.06	−0.04	0.89	0.05	0.73	0.28
Enzyme immunoassay	1.22	0.59	1.05	0.67	1.40	−0.34	6.23	−2.01	2.65	−2.27
Tosoh[b]					1.03	−0.65	7.08	0.66	1.84	0.19

[a] Negative, low, medium, and high pools were assigned values of 0, 1, 2, and 4, respectively, for this linearity study. All r values >0.99.
[b] No data reported for Tosoh assay for rats owing to lack of response (all values <0.6).

From: Takeuchi et al. 2008.

Another study cited as pivotal examined the high sensitivity Erenna Assay(Schulze et al, 2008), described above in Section 3a. The point of the study was to examine the ability of the new technology assay to measure troponins in animal models of chemically-induced cardiotoxicity. Commercially available monkey purified cTnI was used as a standard for comparison as well as the human NIST material. Because the monkeys were not euthanized at study conclusion, no histologic examination of heart tissue was conducted.

A preliminary evaluation of analytical assay performance was performed. Linear responses (R^2 = 0.99) were observed for the standard curves for all species. The data suggests that the Erenna immunoassay can be used for determination of monkey cTnI concentrations.

Table 18. Baseline values using the Erenna assay

Species of healthy animal	cTnI results	Number of animals
Male monkey	5.3 ng/l	3
Female monkey	4.4 ng/l	3
dog	1.0 – 4.6 ng/l	4
rat	9.5 ng/l	20
Values from control, vehicle treated animals		
rats	9-20 ng/l	10
dogs	2-3 ng/l	3

From: Schultze et al 2008..

g. Studies for Primates Published after Submission of the Troponin Qualification Material

Minomo et al (2009) first investigated the tissue distribution of cardiac troponins in three clinically healthy, drug naïve male Cynomolgus monkeys. Tissue distribution in nine organs (including the heart, liver, and kidneys) showed that TnT and TnI were distributed specifically in the heart, and were not detected in other tissues. Changes in blood biomarker levels and histopathological changes in cardiac tissue were measured after myocardial injury induced by concomitant administration of isoproterenol (ISO) and vasopressin (VASO). While cTnT was measured by the Roche assay which has been demonstrated as sensitive for Cynomologous samples, cTnI was measured with a species specific ELISA. This assay was described as validated but no references or data were provided.

Compared with pre-dosing, TnT and TnI were markedly increased in the ISO + VASO groups, in which severe histopathological changes were observed. The relationship of biomarker levels with the severity of myocardial injury was determined using Spearman's correlation coefficient calculated between C(max)/ AUC of the biomarkers and necrosis/vacuolation scores in the heart. The high correlation between necrosis/vacuolation in the heart and TnT/TnI levels suggest that TnT and TnI possess high sensitivity and specificity for myocardial injury and are useful biomarkers for detection of drug-induced myocardial injury in Cynomolgus monkeys.The information generated in this work was consistent with similar work in the rat and dog.

Zabka et al 2009 examined a previously undescribed spontaneous cardiomyopathy in Cynomologous monkeys. The pathology was identified during histologic examination of four clinically healthy animals. The ECGs were not remarkable. There were no clinical signs of heart failure and no gross findings at necropsy. Routine light microscopy showed chronic cardiomyopathy ranging from mild (1 animal), moderate(1 animal) and severe (2 animals).

The authors used phosphotungstic acid hematoxylin (PTAH) for detection of early cardiac myocyte injury and the retention of troponin immunostaining to describe the level of activity of the cardiomyopathy. The disrupted cardiomyocytes were immunoreactive to desmin and troponin-I

antibodies and had a normal cross-striation pattern by PTAH, indicating the chronic cardiomyopathy was not associated with active cardiomyocyte damage. The consistent distribution and morphology of the cardiomyopathy suggested a common etiology and pathogenesis. The features were reminiscent of chronic catecholamine-induced experimental cardiomyopathy and stress cardiomyopathy in monkeys and humans, respectively. The focus of the report was the documentation of a spontaneous heart lesion identified in clinically healthy monkeys that should be considered during interpretation of toxicology studies.

4. STRENGTHS AND LIMITATIONS OF THE DATA

There are several strengths of the troponin literature that are indicative of the scientific maturity of the research field.

1. Consistency of results across labs

   The studies selected to support the qualification of troponins have been conducted by numerous independent investigators from academia, industry, human clinical medicine and veterinary clinical medicine. The troponin assays have become more sensitive in the 10-15 years that the primary database was generated. Investigators encompassed a full spectrum of sophistication in the implementation of new clinical chemistry assays. Pharmacologic agents as well as physical methods have been used to induce cardiac damage. Other phenomena studied include natural disease in animals and aging in both rats and dogs. Regardless of the means of causing damage, the majority of studies show consistency of the specificity, sensitivity and utility of the troponins. The need to collect samples within a specific window of time following a discrete injury has been a consistent finding.

   Over the years, there has been no standardization of study design. While histology was used as a reference standard for many, but not all, studies, the grading scales were not standardized between the labs. The general consistency of results under the variety of conditions of use and testing supports the robustness of the biomarker.

2. Nature of the studies

   The studies included retrospective, and prospective safety assessment studies as well as veterinary clinical investigations of a variety of naturally occurring diseases. The naturally occurring conditions encompassed a variety of cancers, adrenal and renal pathologies, trauma, dilated cardiomyopathy, mitral valve degeneration, regurgitation and dysplasia, Wolf-Parkinson-White syndrome, ventricular tachycardia, respiratory conditions, heartworm disease, arrythmogenic right ventricular cardiomyopathy (ARVC), gastric dilatation and volvulus, patent ductus arteriosus, blunt chest trauma, dyspnea, ehrlichiosis, babesiosis, leptospirosis, snake evenomations and bacterial/viral infections.

3. Information available for both sexes

   In each of the species cited for qualification, studies have been conducted to some extent

in both sexes.

4. Baseline values, normal ranges

While almost all studies included control animals, several published studies were for the purpose of establishing reference ranges for clinically healthy animals. In some cases the clinically healthy animals were compared to animals with defined conditions such as congenital or acquired heart disease.

5. Factors that may increase background cTn in blood unrelated to treatment

Factors that need to be minimized, avoided or controlled have also been described. These include methods of blood collection, excessive restraint stress, excessive exertion, anoxia during anesthesia causing cardiac ischemia, atrial pacing in safety pharmacology studies, age, and sex-dependent spontaneous cardiomyopathy in Sprague-Dawley rats.

6. Causes of false positive values

False positive values fall into two general categories:1) true false positives and 2) elevations secondary to other conditions. True false positives are rare, usually in response to: heterophile antibodies, rheumatoid factor, macroenzymes (high molecular mass complexes formed by polymerization of enzymes, serum and multiple other components, the formation of which is associated with several autoimmune and liver diseases), circulating antibodies from immunotherapies, vaccinations or blood transfusions, fibrin clots, immunocomplexes and malfunction of analyzers.

Cardiac damage or disease may occur secondarily to other conditions causing increased troponin values. These are not false positive results per se, but are not due to primary cardiotoxicity and should be appropriately distinguished.

7. Causes of false negative values

Causes of false negative values include use of an inappropriate assay for the species under study, inappropriate timing of sample collection, incorrect sample storage, and plasma samples instead of serum (McNeil, 2007; Lippi et al. 2009).

8. Awareness of assay issues

The existence of commercially available assays for clinical use was beneficial to the non-clinical research. The applicability of immunologically- based assays for multiple species was examined early in the evolution of the research in numerous studies comparing different assays and published conclusions about the relative efficacy of certain assays for different species. Issues, such as dilution of small volume samples and storage conditions, have been raised in a number of studies and have been subsequently addressed.

9. Standardized material

Depending upon the nature of the study and when it was conducted, investigators have used different materials for calibration and quality assurance. This lack of consistent use of reference material can be considered a limitation as well as a strength. A number of the referenced publications included a human reference material from the National Institute of Standards and Technology (NIST), NIST-2921, a ternary complex of ITC purified from human cardiac tissue. Other species-specific reference materials included cTnI purified from rat and /or dog myocardium. A few publications used a dilution of cardiac homogenate from the species of interest (rat or dog). Purified dog, rat and non-human primate cTnIs are commercially available from Hytest (Turku, Finland) and were included in several published studies.

10. Statistical analysis

There was no standard approach to statistical analysis of data. The Receiver Operator Characteristic (ROC) curve that has been used extensively in other biomarker qualification submissions has received little utilization in the animal studies of troponins. A variety of methods have been used depending upon the goals of the particular study and the overall assessment.

The limitations of the data are those that would be expected for a biomarker evolving slowly, over time, through the work of independent investigators.

1. The number, scope and variety of published reports of chemicals tested differed by species. There were peer-reviewed publications for over 40 different compounds tested using the cardiac troponins as indicators of cardiac toxicity. By species, the following number of chemicals were tested and reported in the literature: rats (25), dogs (>7), rabbits (5), mice (3), and monkeys (2) The designation ">" indicates that some references did not specify the exact number of proprietary compounds tested in that set of studies. Many of the proprietary compounds were not identified. The preponderance of isoproterenol and doxorubicin in the database is a limitation in that a restricted number of mechanisms of toxicity have been examined.

2. Comparisons to other biomarkers (e.g., clinical chemistry, imaging, electrocardiogram (ECG)) were not uniformly conducted. In the absence of procedural information, it is probably reasonable to assume that assessments made with other technologies were made with knowledge of the troponin values.

3. The published database for primates was not as extensive as that for rodents and dogs. Due to the cost of primates, the majority of studies available did not include euthanasia and the use of histological evaluation.

4. Histopathological evaluation was not standardized across studies. Certain aspects of the histological evaluations did not meet recently described criteria for optimum characterization:

a. Lack of consistent severity of lesion grading scales. There is no universal grading system for cardiac morphologic damage and the scales used were of necessity established by individual investigators or consortia,.

b. Lack of standardized/illustrated scales of histologic lesions (lexicons). Numerous investigators appeared to use pre-specified criteria and lexicons that were in local rather than general use.

c. Lack of standardized system of evaluation. The details of slide processing and evaluation were not always clear. In particular, the subject of blinded evaluation was frequently not specified. Therefore, it is probably reasonable to assume that a majority of the evaluations were performed in an open, unblinded fashion.

The recently enumerated paradigm of focused, efficient biomarker characterization recommends blinded histology evaluation. At least eight publications evaluating cardiac tropoins as biomarkers specifically state that the histopathologist was blinded to treatment.

Two publications from Herman and colleagues (Herman et al 2001; Thompson et al 2010) specifically state that the pathologist was blinded to treatment. In a personal communication, Dr. Herman indicated that the laboratory's standard operating policy has been that the troponin analyses are completely blinded to all study information and that the histopathologist is blinded to treatment. This communication implies that all studies from this laboratory used blinded evaluation of histopathology. However, several publications (Herman et al 1999; Zhang et al 2006; Zhang et al 2008) from this laboratory do not specifically state that the pathologist was blinded to treatment.

In addition to the publications from Herman and colleagues, at least six publications from other laboratories (Bertinchant et al 2000; Dowd, et al 2001; Bertinchant et al 2004; Yavuz et al 2008; Engle et al 2009; Varga et al 2009) have specifically stated blinded evaluation of histopathology was used in studies evaluating troponin measurements. Two additional publications (Yavuz, et al 2004; York et al 2007) indicate blinding, but it is not clear who was blind to what.

Although more than 20 other cited publications utilized histopathology, no specific statements were found that indicated blinding of either the biomarker technician or the histopathologist.

Several publications (Létienneet al 2006; Mikaelian et al 2008; Varga et al 2009) in discussing the sensitivity of troponin measurements relative to macroscopic or microscopic pathology raised the problem of examination of a limited number of tissue sections. The ability of one or even a few histologic sections to adequately represent the whole organ is an issue particularly when the lesions are few in number and small in size.

It is interesting to note that when circulating troponin increases were detected without correlating histopathology lesions in H&E stained sections, the research community investigated these observations from the perspectives of 1)understanding the kinetics of release and correlation with the nature and severity of histologic changes; 2)the determination of whether there was a threshold of damage necessary for detectable increases in circulating levels of troponins; 3) correlation of established histological and biochemical measurements of myocardial damage to key molecular

events in early stages of myocardial damage, including cTn tissue content and immunoreactivity. These assessments included histology with special histochemical stains in addition to H&E, immunochemical techniques and electron microscopy. Multiple tissue sections were taken for the different histochemical techniques but there was a lack of discussion as to how many sections are necessary for definitive investigation. Despite this shortcoming, the independent investigations established collectively that early increases in circulating troponins were not predictive, but corresponded to morphologic damage that was not reliably detectable with H&E staining. The sum of these independent studies was also a detailed examination of troponin behavior in response to tissue injury and determination of sensitivity relative to H&E tissue sections. The investigators did not see the sensitivity of the troponins as predictive of damage, but as early reporting of the fact of damage that had already occurred. A detailed presentation of this issue may be found in Section 3.b. Specific Studies for the Rat.

5. BQRT CONCLUSION

The literature database for the cardiac troponins is substantive, with the strengths outweighing the limitations. These proteins were first identified over 4 decades ago. Since the recognition of the protein fractions comprising the functional complex, troponins T and I have evolved into the preferred biomarker for use in diagnosis of myocardial necrosis and injury in human and veterinary medicine. There has been a slow evolution and maturation of the database into the form that gives confidence as to the reliability for application in safety assessment. This is not unexpected as most of the work was performed by independent investigators outside of the recently enumerated paradigm for focused, efficient characterization of new biomarkers.

In an appropriately designed and conducted study, the circulating cTns can be used to show that myocardial damage has occurred and to estimate the extent of thisdamage. There are several possible scenarios:

1. A drug in development produces a signal for cardiac damage that requires further exploration. In this situation, circulating troponins may be used in several ways, including characterization of the timecourse of damage and identification of a no observed adverse effect level (NOAEL). Histology is typically used as the indicator of the dose(s) where damage occurs. However, an increase in circulating cTns with no histology finding may indicate that a lesions or lesions were not captured on sectioning and that the NOAEL requires further exploration.
2. A drug in development is a member of a class of drugs known to be associated with cardiac morphologic damage. Circulating troponins may be included in the safety assessment to determine the safety margins early in development.
3. A dose-related incidence of cardiac damage or necrosis is identified in the histology for a safety assessment study. If a dose-related incidence of cardiac damage is found in the histology, retained plasma or serum from that study can be analyzed for cardiac troponins (reflex testing) . This practice may generate data about the nature and timing of damage as well as the NOAEL.

The isoforms of T and I found in the mature heart are organ specific. These molecules are subject to significant modification when released into the blood. The profiles of fragments and complexes

released, as well as post-translational modifications, are incompletely described and the subject of ongoing research. It is unclear if these profiles are indicative of mechanisms of damage or have any additional prognostic value. At the present time, the fragments, modifications, and complexes may contribute to assay variability and differences in assay sensitivity.

There was additional information, unavailable to the sponsors or inaccessible for proprietary reasons, that would have added additional strength to the qualification proposal. Various investigational new drugs (INDs) and new drug applications (NDAs) indicate that cardiac troponins are already in use for regulatory decision making and have been requested by some FDA Divisions on a case by case basis. Personal communications indicate that cTns are also in relatively regular use at certain pharmaceutical companies. However, much of these data were not available in the published scientific literature and therefore were not available for the sponsors to evaluate or to include in this qualification package.

A striking note in the database is inconsistency followed by consistency. That is, many investigators over many years, primarily working independently of each other, have contributed to the overall understanding of cardiac troponins. As noted earlier in this review, there is a lack of standardized study design, histological lexicons and evaluation, general histological methods, and statistical and analytical methods. The methods of cardiac damage explored have ranged from clinically healthy aging to a variety of natural diseases, including clinical cases of canine chemotherapy, experimental drug-induced damage, some standard toxicology studies, and physical methods of damage such as pacing and coronary artery ligation. Despite the timespan, number of investigators, variety of approaches, methods of analysis, and multiple species, the results have been consistent for the specificity, sensitivity and utility of the cardiac troponins for detecting cardiac morphologic damage. These results suggest a robustness to the biomarker that is encouraging.

6. RECOMMENDATIONS FOR IMPLEMENTATION

The BQRT concurs with the sponsors' recommendations for the implementation of cardiac troponins in a safety assessment laboratory. The procedures as enumerated by the sponsors are listed below.

1. The Study

Controls - Values for cTn in a preclinical toxicology study should be compared with the concurrent and historical controls and pretreatment values where available.

- Statistical comparison of biomarker group values in different treatment groups is needed.
- Abnormal individual values should be identified,

Rapid kinetics require early sampling. Because of cTn's rapid rise following injury and relatively short half-life, concentration should be assessed early (e.g. within 24 hours) after study initiation (in at least one study in each species used) as well as at study termination, where acute myocardial injury is anticipated.

2. The Assay

New assays should be subject to appropriate analytical validation and biological qualification in laboratory animal species prior to use. Newer generation assays that may involve changes in antibodies or increased sensitivity of the assay from the previous generation should undergo repeat analytical validation to ensure consistency of cardiac troponin detection.

3. The Conduct of Analytical Validation

Guidelines for consideration in the evaluation of cTn analytical performance characteristics using a commercial platform are listed below and are those parameters evaluated using standard techniques for validation of automated immunoassays. Since minor increases in troponin may be significant, detailed assessment of the sensitivity of an assay as well as imprecision at the limits of detection of the assay are especially important. The user may conduct a verification strategy (reduce the scope of the analytical validation) to confirm assay performance data supplied by the manufacturer.

A. Determine the limit of detection (LOD) for the assay in species /strain by measurement of the negative serum/plasma pools prior to imprecision testing. If using commercial sera, identify that blood collection procedure was appropriate to minimize background cTn levels (eg, not collected via cardiac puncture). Alternatively the user may wish to calculate the limit of quantitation of the assay based on a pre-determined acceptable coefficient of variation (CV), typically 20%.

B. Confirm the linearity and dynamic range of the assay using the manufacturer's recommended calibration materials. Comparing the response of the same calibration material diluted in negative serum/plasma human and species-specific serum/plasma pools will facilitate assessment of the potential for species specific matrix effects on the dynamic range/response of the cTn assay.

C. Be aware of the antibodies employed in the assay and cTn epitope recognition sites (Apple 2007).

D. Evaluate the assay imprecision, assessing repeatability or within run imprecision and the total or within-laboratory precision following the general principles of the committee and laboratory standards institute protocol EP05-A2 (2004).

E. Assess species specific imprecision profiles at low level concentrations of cTn using a modified CLSI protocol (Apple et al. 2008, Panteghini et al. 2004), and compare these results to clinical and manufacturer's performance.

F. Users should assess the cTn assay response with either

i. Species-specific cTn standards in species-appropriate cTn-negative matrix
ii. Animal model of experimentally-induced myocardial injury with histopathology comparison
iii. With stored and previously identified cTn positive samples.

G. Peform quality control of the assay using the manufacturer's recommended materials. Including species specific cTn positive and negative sera may help monitor any impact of variation in reagent lots and repeat calibrations.

H. Assess appropriate sample type and stability (initial room temperature v 2-8°C) and long term storage (-20°C to -80°C) for the intended conditions of use. Additionally, determine stability of sample testing following repeated freeze-thaw cycles (5 times).

I. Establish cTn reference ranges for relevant animal species and assay platforms and identify the 99th percentile in each preclinical laboratory. Consistent application of these assays will allow inter-study comparisons and aid interpretation. Individual laboratories should establish relevant population reference ranges considering species, strain, age and gender of models used in that laboratory and the procedure used in blood sampling, if varied.

7. RECOMMENDATIONS FOR CDER REVIEWERS

During the review, the BQRT identified several issues pertinent for reviewers who will have to assess cTn data. It is the opinion of the BQRT that a study report which includes cTn data should include the following:

1. The sponsor should show or reference data to demonstrate that the assay used is appropriate for the non-clinical species used in the study. These data should be based on the recommendations found in Section 6.3 of this review ("Conduct of Analytical Validation").
2. The sponsor should provide some rationale for the timepoints at which samples were collected.
3. Other information should be generated and collected in the study so that the sponsor will have a reasonable dataset to be able to distinguish primary drug-induced cardiotoxicity from damage secondary to other effects of the drug. That is, hematology, clinical chemistry, urinalysis and histologic analysis of organs and tissues other than the heart should be considered.
4. The testing facility should have reference ranges for the species, sex, and age of animals used.
5. At this time, there is insufficient data to recommend the use of one cardiac troponin over another.

8. APPENDICES

APPENDIX 1: Limitations of traditional biomarkers of cardiac morphologic damage.

Creatine Kinase: Creatine kinase (CK) is an enzyme that catalyses the transfer of phosphate from creatine phosphate to ADP to form ATP. There are 3 major CK isoenzymes identified (MM, MB, and BB) that exhibit some degree of tissue specificity. CK-MM is the principal form in skeletal muscle, both CK-MM and CK-MB are present in myocardium, and CK-BB is the predominant form in brain and kidney. The MB isozymes of CK can also be present in small intestine, tongue, diaphragm, uterus, and prostate. CK-MB can be found in the blood of clinically healthy people. The cutoff value for an abnormal elevation is usually set a few units above the upper reference limit for a given laboratory. Elevation of serum CK-MB is considered a reasonably specific marker of acute myocardial infarction (AMI), but elevations of CK-MB occur in some disease states in which there is skeletal muscle injury, following intramuscular injections or vigorous exercise, and in association with administration of certain drugs, such as benzodiazepines and tricyclic antidepressants.

Creatine Kinase Isoforms: Isoforms of the MM and MB isoenzymes have been identified. Certain isoforms can appear in the blood quite rapidly, perhaps as soon as 1 hour, after the onset of infarction.

Myoglobin: Myoglobin is a low molecular weight heme protein monomer that serves as an oxygen reservoir to temporarily provide oxygen to muscle tissues under hypoxic conditions. Myoglobin is found in all muscle types and therefore lacks specificity for cardiac myocyte injury. In addition, there are significant interspecies and even intraspecies variations in muscle myoglobin content. Serum concentrations of myoglobin increase rapidly following muscle injury, with peak values observed 1–4 hr post-AMI. Isolated measurements of serum myoglobin are generally considered nondiagnostic. Elevated serum myoglobin at presentation is considered a negative prognostic indicator in AMI and may reflect of the extent of myocardial damage.

Lactate Dehydrogenase (LDH): The L-lactate dehydrogenases are enzymes that catalyze the reversible oxidation of L-lactate to pyruvate. LDH is found in numerous tissues and tissue content is highly variable. There are 5 major isoenzymes based on electrophoretic mobility. Of these, LDH1 is found in highest concentration in the heart, thereby conferring some diagnostic utility to LDH isoenzyme profiling. Aside from the lack of cardiac-specificity, serum levels of LDH do not peak until 0.5–2 days and may remain elevated for up to 2 weeks following AMI. The lack of tissue-specificity and broad diagnostic window limit the diagnostic utility of serum LDH analyses.

References for Appendix 1

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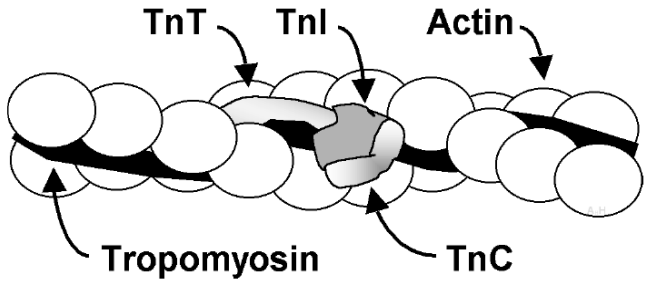
Apple FS. 1999. Tissue specificity of cardiac troponin I, cardiac troponin T and creatine kinase-MB. Clin Chim Acta. 1999 Jun 30;284(2):151-9.

APPENDIX 2: Biology of the troponins

The sarcomere is the basic structural and functional unit of the myocardial cell. Each cardiac sarcomere is comprised of interdigitating thick and thin filaments that present a striated appearance via light microscopy, similar to the sarcomere of skeletal muscle. The thick filaments are composed primarily of myosin. Actin is the main constituent of the thin filaments. The tropomyosin-troponin complex is located periodically along the actin thin filament strands within the sarcomeres of all types of striated muscles (fast twitch, slow twitch, and cardiac) but not smooth muscle, where calmodulin regulates contraction. The interaction between the actin and myosin filaments is the basis for striated muscle contraction.

The contractile process in striated muscle is controlled by tropomyosin and the troponin complex. Tropomyosin lies in the long pitched grooves on either side of the actin filament. It is thought that at low calcium concentrations, tropomyosin sterically prevents the interaction of actin with myosin. The tropomyosin molecules shift their positions slightly when calcium concentrations are increased, which allows for the interaction of myosin and actin to produce the contractile response.



From: Wallace et al 2004

The subunit proteins of the troponin complex are troponin T (TnT; 37 kDa), troponin I (TnI; 22 kDa), and troponin C (TnC; 17 kDa). Different forms of TnT and TnI are found in cardiac, fast twitch and slow twitch skeletal muscle. TnC is common to all muscle types.

Each muscle specific troponin type is encoded by separate genes. During fetal muscle development, all three troponin T proteins (cardiac, fast and slow twitch) are expressed simultaneously. Mid-gestation, the cTnT gene is upregulated in cardiac myocytes and suppressed in skeletal myocytes, initiating terminal differentiation for these two cell types. Cardiac troponin I is not detectable in fetal tissue, only in adult cardiac tissue.

In healthy human myocardium, two adult isoforms of cTnT have been described. The isoform cTnT1 is predominant, with cTnT2 reportedly increased in end-stage heart failure. The regions of difference between these two isoforms are outside of the epitope binding sites of the commercially available cTnT immunoassay and so currently indistinguishable by that methodology.

cTnI is well conserved across phyla with up to 91% sequence homology among most mammals. The canine and feline cTnI genes were recently cloned and sequenced and found to be 95 and 96% homologous, respectively, to the human cTnI gene. (192, Selting).

The troponin molecules are subject to a number of post-translational modifications including proteolysis, glycosylation, phosphorylation, oxidation and formation of complexes with other troponin molecules or fragments. Any of these alterations occurring *in situ* may alter the function of the complex or the interaction with the troponin-tropomyosin complex. The release of altered forms of troponins raises the following questions:

1. Is the profile of post-translational modification indicative of the mechanism of damage?
2. Does the profile of post-translational modification have prognostic value?
3. Does the profile of post-translational modification alter the measured levels (are the measured epitope sites affected?)?

As an example of multiple circulating forms, the dominant species of cTnI detectable in serum following ACS are non-covalent ternary complexed cTnT-I-C (TIC complex) and binary complexed cTnI-C (IC complex) although complexed cTnI-T (IT complex) and free forms are also present. Circulating free cTn can be detected soon after the initial ischaemic insult and prior to the detection of complexed forms. It has been proposed that cytosolic troponins are released from cardiac tissue into the extracellular compartment during ischaemia while proteolytic degradation as a result of cellular necrosis is required to liberate troponins bound to the contractile apparatus. However, these processes are a matter of debate.

Degradation products of both cTnI and cTnT are also present in the serum. Eleven modified cTnI products have been reported and the number and extent of modified proteins change over the time course following the acute event. Some cTnT fragments have also been detected, but these modified forms occur less frequently than the cTnI modifications. Troponin degradation products and covalent complexes similar to those isolated from ischemic cardiac tissue have been identified in serum following myocardial infarction and include both phosphorylated and non-phosphorylated

species. The modification of cardiac troponin prior to release into the circulation appears to be extensive and progressive.

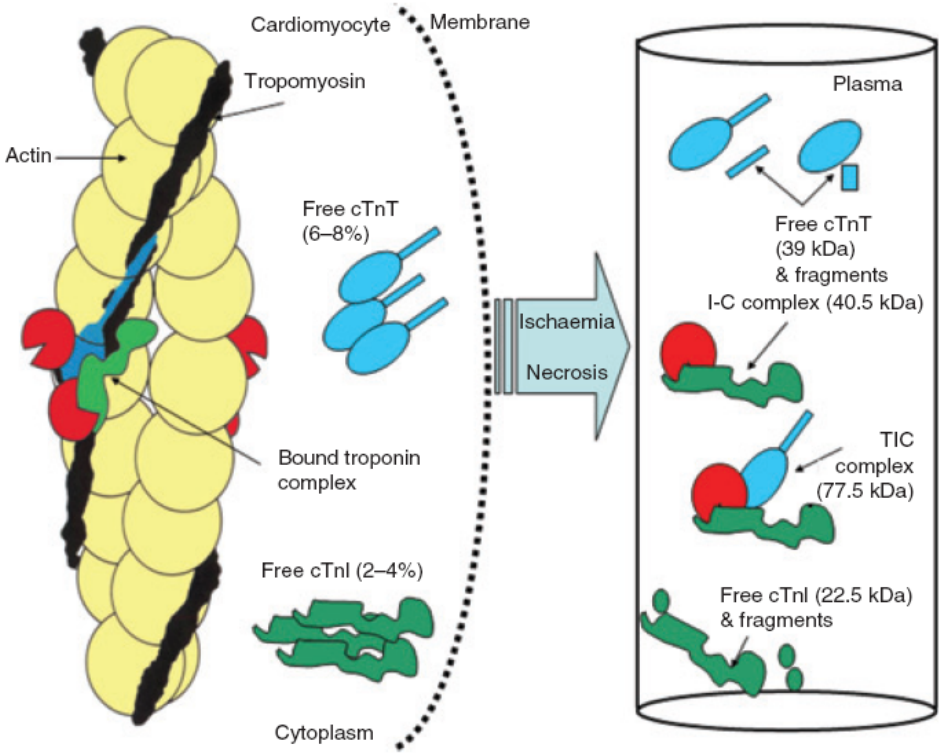


Figure 1 Structure of the cardiac troponin complex and troponin forms released following ischaemia or necrosis. The troponin complex is composed of three proteins: troponin T, troponin I, troponin C – TnT, TnI and TnC – and is located on the thin filament of the cardiomyocyte. Most intracellular troponin is bound to the myofibril with only 6–8% of cTnT and 2–4% of cTnI existing in the cytoplasm. Following ischaemia and necrosis, troponin from the cytoplasm and enzymatically digested bound complexes are released into the circulation. Circulating TnI exists mainly as cTnI-TnC and some cTnT-cTnI-TnC complex with little free or fragmented cTnI. The phosphorylated forms are not depicted here as it is currently not known if they exist in complex or free form

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References for Appendix 2

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Appendix 3. Sponsor's Tables of Key References

Summary of Selected Studies on Preclinical Use of cTn

In support of Request for Qualification by FDA of Cardiac Troponin as a Blood Biomarker for Non-Clinical Toxicology Studies

O'Brien[1] PJ, Reagan[2] W, York[3] M, Jacobsen[4] M.

[1]University College Dublin, Ireland; [2]Pfizer Inc, Groton Connecticut, [3]GlaxoSmithKline, Ware, Herts, UK; [4]AstraZeneca, Macclesfield, UK.

	Species Strain / Breed Weight Gender	A) Treatment compound & route B) Dose or injury C) Time-points D) Number per group	Tn Assay Used	Reference range mean ±SD (ug/L)	Tn change compared to reference	Histology correlation – statistic value	Major finding / conclusion	Supports qualification
1.	Dog Beagle M & F	A) **Compound X**, per os B) 0, 2.5, 12.5, 25 mg/kg SID for 28 d C) Monitor at 0, 1, 2, 29 d D) 24 (6 per group)	Centaur cTnI (1st generation)	0.013 ± 0.016	10 – 20 x	Odds of increase in cTn being due to chance – 0.0001	cTnI was as effective as histology in identifying cardiotoxicity.	Yes
Note: 31 healthy control beagles were used to set the reference range. All 3 dogs having increased cTn levels had myocardial necrosis on histopathological exam at day 29.								
2.	Dog Beagle M & F	A) **Isoproterenol, iv** B) 4 µg /kg/min for 20 min C) Monitor:0, 1, 2, 4, 8, 24, 48 h D) 3 treated	a) Singulex Erenna cTnI b) Beckman Access cTnI	a) 0.001 - 0.0046 b) <0.02	a) 100 - 1000 x	Not determined by this study as dogs were recovered	Peak within 4 hours	Yes
Note: 7 healthy control beagles were used to set the reference range. The Beckman assay was performed only on the control dogs.								
3.	Dog Beagle M & F	A) **Isoproterenol**, subcutaneous B) 1 mg /kg twice, 24 h apart C) Monitor:0, 4, 8, 10, 24 h D) 2 treated	a) Beckman Access cTnI b) non-commercial cTnT	< 0.7 or not detectable	a) 3 and 55 ug/L b) 19 and 1 ug / L	Severe coagulative necrosis was found in both dogs	a) cTnI effective at detection of isoproterenol cardiotoxicity in dogs b) Peak at ~8 hours c) Tn release after 2nd dose was ~1/3 lower than after 1st dose	Yes
Note: Reference ranges were not established but noted to be very low, <0.7 or not detectable. Values reported for cTn change are after the first dose.								
4.	Dog Foxhound 30 – 35 kg	A) **Doxorubicin**, intracoronary B) 0.3 mg/kg weekly for 5 wk C) Monitor 0, 6, 12 wk D) 6 (1 dog died after 4th administration)	Centaur cTnI (1st generation)	0.02 ± 0.025	Mean values increased 85 fold	All dogs had marked increases in cTn and characteristic histological changes of doxorubicin cardiotoxicity including cytoplasmic vacuolation, interstitial fibrosis, hemorrhages and dystrophic calcifications.	cTn useful for detection and assessment of chronic doxorubicin cardiotoxicity associated with cumulative dose in dogs. It indicates lack of recovery from cardiac insult..	Yes
Note: 6 healthy control fox hounds were used to set the reference range. cTnI values remained increased for 6 wk following the last dose of doxorubicin (1.3 ± 0.2 ug/L). The doxorubicin treatment produced dilated card								
5.	Dog Foxhound 30 – 35 kg	A) **Doxorubicin**, intracoronary B) 0.3 mg/kg weekly for 5 wk followed by partial ventriculectomy C) Monitor 0, 6, 12 wk D) 6 (2 dogs died)	Centaur cTnI (1st generation)	0.028 ± 0.026	Mean values increased 64 fold	All dogs had marked increases in cTn and characteristic histological changes of doxorubicin cardiotoxicity	cTn useful for detection and assessment of chronic cardio toxicity associated with cumulative dose in dogs	Yes

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Note: This study is similar to 4) above except that 1 week after the doxorubicin treatments, a partial ventriculectomy was performed. 6 healthy control fox hounds were used to set the reference range. This report included the 6 dogs reported in 4) above (ie reference 50). cTnI values remained increased for 6 wk following the last dose of doxorubicin (1.8 ± 0.4 ug/L).									
6.	Dog mixed breed - 13 with lymphoma, 31 with osteo-sarcoma 12-73 kg	A) **Doxorubicin iv** B) ~1 mg/kg (30 mg/m²) ea 2 or 3 wk for 5 treatments C) Monitor: pretreatment, prior to 3rd & 5th treatment, & every 12 wks until progressive neoplastic disease, death or 1 yr after diagnosis D) 44 dogs	Beckman Access cTnI	<0.03 (this is the lower limit of detection of the assay)	0 – 15 fold	cTn increased before clinical signs occurred in dogs that developed doxorubicin cardiomyopathy (10 / 44 dogs); dogs with clinical signs that underwent postmortem had histopathology changes	1) cTn useful for early detection of chronic cardiotoxicity due to cumulative dose in dogs 2) Peak cTn after final treatment or 3 or 6 mo later 3) 1 / 3 had mild increase in cTn but not clinical signs 3) ¼ had mild increase in cTn, clinical signs, and cardiac morphological changes	Yes	192
20 healthy dogs, as indicated by clinical examination and history, were used for setting the reference range.. All treated animas received 150 mg of doxorubicin / m² body surface in total. 44 dogs were studied in total (plus 20 do healthy controls) divided in 3 groups (1) dogs without cardiac deterioration (n=20), (2) dogs with cardiac deterioration but without clinical signs (n=14) , (3) dogs with cardiac deterioration plus clinical signs of cardiomyopathy (n= Clinical signs of cardiac disease were arrhythmias on electrocardiograms or decreased fractional shortening on echocardiogram. Histopathological findings included increased amount of adipose tissue, focal interstitial replacem fibrosis with calcification, degenerate myocytes, and myofiber vacuolation. Mean serum cTnI concentration increased significantly compared to baseline during the course of monitoring among groups. Peak cTnI values occurred most commonly at the last treatment (fifth doxorubicin) or at the 3- or 6-month post-treatment sample.									
7.	Dog (breed and age not identified)	A) **Doxorubicin**, intracoronary B) ~1 mg/kg twice ea 2 wk C) Monitor:: 0, 1, 2, 14, 15, 16 d; 8 wk D) 7 dogs treated	Beckman Access cTnI	<0.03 (this is the lower limit of detection of the assay)	8 - 880 fold	All dogs developed cardiomyopathy and had increased cTn. 4 of the7 dogs that died prior to the completion of the study had higher cTnI concentrations than dogs that completed the study with no overlap of values. No histopathologic data was reported in this study.	1) peak cTn within 48 h 2) dogs with cTn > 0.8 died (4) 3) cTn useful for detection and assessment of chronic toxicity associated with cumulative dose in dogs	Yes	192
Reference range data is the same as for the study above, both studies are reported in the same publication. For all of these dogs, there was a peak in serum cTnI concentration within 48 h of one or both doxorubicin administrat (peak values among dogs were 0.20 - 9.30 ug/L and gradually climbed over 8 weeks (highest value 26.38 ug / L).									
8.	Dog Mixed breed	A) **Doxorubicin iv** B) ~1 mg/kg (30 mg/m²) ea 2 wk for 3 to 6 treatments C) Monitor: 0. 1, 5, 7, 14 d after ea treatment D) 2	Elecsys cTnT	<0.05	2 - 6 x	Not determined	1) cTn increased after 5 doses (2 dogs) and peaked after the last dose 2) cTn useful for detection and assessment of chronic toxicity associated with cumulative dose in dogs	Yes	60
15 normal dogs aged from 1-10 yrs to set the reference range. 2 dogs with neoplasia received doxorubicin as chemotherapy. Peak cTnT concentration in the 1st dog occurred 3 wk after the 6th dose (cumulative dose of 180 mg/m The 2nd dog had increased cTnT after a cumulative doxorubicin dose of 150 mg/m², and cTnT increased further after the last. One dog died of suspected doxorubicin cardiotoxicity (ventricular arrhythmias) 6 weeks after the last doxorubicin administration while still in complete remission.									
9.	Dog Beagle	A) **Atrial pacing –** 4 bouts of 30 min each at 150 – 200 beats per minute C) Monitor 2 h after start pacing D) 8 dogs	Centaur cTnI (1st generation)	0.013 ± 0.016	40 – 330 fold	Not determined	2 h tachycardia (100 -150% increased heart rate) increases cTn in all dogs	Yes	152
31 healthy control beagles were used to set the reference range. Paced dogs were subjected to four periods of 30 min duration each, in which atrial tachycardia was induced under isoflurane anesthesia using pacemakers. In e period, hearts were paced at a rate progressing each 5 min in increments of 10 from 150 to 200 beats per minute. Blood samples were collected 2 hours after commencement of pacing.									
10.	Dog Beagles	A) **Atrial pacing** induced by transdermal electrode. B) To induce bradyarrhythmia, diltiazem (20 to 50 mg) was administered.	TOSOH AIA-pack second-generation cardiac troponin I	<0.05	1 - 7 fold	Histologic examination was not conducted in this study. Cardiac tissue injury was confirmed by	cTn was found to be the more sensitive biomarker of myocardial injury than any other	Yes	Lee 09

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		C) Blood was sampled on days –1, 0 (immediately after short- or long-duration pacing), 1, 2, 3, 5, and 7. D) 5 dogs had transcutaneous pacing for 1 h, and 5 for 3 h.	assay.			measurement of classical enzyme biomarkers.	cardiac biomarker enzymes examined (CK, CK-MB, AST, LDH)		
Baseline concentration of dogs before pacing was used to set the reference range. There was 7.3-fold increase immediately after pacing in the long-duration group and persistently elevation until day 2, although only 100% increase in cTnI for the short duration group and only for 1 day.									
11.	Dog Mixed breed 18 kg	A) **Left coronary occlusion** B) 90 min C) Monitor 9 after 60, 90, 150, 210, 270, 360 min. 5 more dogs after recovered for 24 h D) 14	Boerhinger cTnT (1st and 2nd generation assays)	0.01 ± 0.00	40 – 2500 fold	Pearson coefficient r = 1 infarcted tissue quantitated by a) weight after visualizing with trypan blue exclusion; b) loss of tissue CK activity; and c) increase in cardiac LD1 and LD2 isozymes	1) Myocardial injury is detected and quantifiable with cTnI in dogs - Infarct size precisely correlated with cTnT 3 – 4 h after infarct 2) cTnT more effective than LD and CK isozymes 3) release kinetics defined	Yes	150
14 healthy dogs were used to establish the reference range. 9 dogs were studied for the relationship between infarct size and cTnT; 5 dogs were studied for the relationship between LD1 and LD2 with cTnT.									
12.	Dog Mixed breed M & F	A) **Natural cardiac disease** B) not relevant C) not relevant D) 6 healthy, 9 with symptomatic heart disease, 6 symptomless but with cardiac anomalies	Centaur cTnI (1st generation)	0.013 ± 0.016	~100 x	An increase in cTnI correlated with cardiac symptoms	cTn increased in all dogs suffering from severe cardiac disease as well as in those suffering from dysrhythmias, dyspnea, ventricular hypertrophy, or murmur	Yes	150
Note: 31 healthy control beagles were used to set the reference range. Cardiac diseases included aortic or pulmonic stenosis, pericardial effusion without dyspnea, patent ductus arteriosus, or tricuspid dysplasia									
13.	Dogs Beagle 15 kg	**A) Endogenous** hypercatecholemia Perforation of basilar artery causing subarachnoid hemorrhage C) Monitor: 0, 5, 15, 30, 60, 120, 180 min D) 18	Elecsys cTnT	0.28 ± 0.15 At 0 min	~10 fold	cTnT correlated with CK-MB and myosin light chain and with occurrence of deterioration of cardiac function	1) cTnT highly correlates with increased catecholamines r = 0.83 2) cTnT increases markedly with injury within 15 min and peaks within 2-3 h	Yes	134
14.	Rat SD 7 wks M	A) **Isoproterenol, subcutaneous** B) 100 mg/kg C) Monitor: 0, 2, 4, 6, 24 h D) 30 (6 per time point)	a) Centaur cTnI (1st generation) b) M8 Bioveris cTnT c) Elecsys cTnT d) Immulite cTnI e) Trichem cTnI	a) 0.008 ± 0.0123 b) <0.08 c) <0.01 d) <0.2 e) <1	a) 6700x b) 121 x c) 800 x d) 3 x e) 7 x	cTnI increased in all cases with histopathological change. The severity of histopathological change highly correlated (Pearson coefficient of 1) with serum cTn concentration during the first 6 hours when cTn was increasing. However, cTnI rose earlier than histopathological change, which persisted longer than cTnI increases.	1) cTnI was more sensitive than histology in identifying early cardiotoxicity in rats 2) kinetics of release and clearance ~6 h half-life after injury; return to normal 60 h 3) Several commercial cTnI assay have inadequate sensitivity: Immulite, Trichem	Yes	152
Reference ranges were established from the 30 study rats prior to their treatment. Maximum changes seen in cTn are indicated for each assay. Histopathological scores for severity of degenerative change were defined as 1 for no degenerative change, 2 for minimal and multifocal necrosis, 3 as for 1 but with mineralization, 4 as for 3 but with mild degenerative change, inflammation and edema, and 5 as for 4 but with moderate degenerative change. Minimal multifocal necrosis of cells was detected at 2 hours. This progressed with mineralization detected at 4 and 6 hours and inflammation with edema detected at 24 hours.									
15.	Rat SD 7 wks M	A) **Isoproterenol, iv** B) 0, 1, 4 mg/kg C) Monitor at 3 and 24 h D) 30 (5 per group)	a) Centaur cTnI (1st generation) b) Life Diagnostics cTnI rat specific	a) 0.008 ± 0.0123 b) <1	a) 7400 x b) 12 x	Not determined	Compared to the Centaur assay, the Life Diagnostics assay has too low a dynamic range (1/60th) of values to be much effective in assessing myocardial injury	Yes	152

References for the above table

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16.	Rat CR 7 wks M	A) **Isoproterenol sq** B) 0, 0.04, 0.4, 4 mg/kg C) Monitor at 4 h D) 3-4 per group	Centaur cTnI (1st generation)	0.01 ± 0.00	350 x	All rats with histopathological change had serum cTnI increased.	cTnI as effective as histology in identifying isoproterenol cardiotoxicity in rats, and more effective than CK & LD isozymes	Yes	121
The normal range was defined by the cTn values obtained from healthy animals treated with saline (controls). Minimal focal myofibrillar degeneration, was found with treatment, proving early myocardial damage. There was no difference in the degree of histological changes among groups, unlike the biomarkers which had dose-dependent increases, suggesting they are more sensitive than histopathology.									
17.	Rat SD 8 wk	A) **Isoproterenol sq** B) 0, 0.008, 0.016, 0.032, 0.064, 0.125, 0.250 mg/kg C) Monitor: 3, 6, 12, 24, 48 h	Elecsys cTnT	0—0.18	< 1000 x	cTnI was increased whenever there was histopathological change. At low doses was almost a 10 fold increase, while histopathologic damage was found in only a small number of treated animals.	cTnT increase more sensitive than histological change in assessing isoproterenol toxicity in rats. Indicates increased severity of myocardial injury over time.	Yes	240
The reference range was set by values obtained from healthy animals treated with saline and used as controls. The classification scheme followed for histopathologic evaluation, is as follows: 0 = normal myocardial cells;1 = scant number of myocardial cells (< 5%) showing necrosis, partial absence of myocardial BM, a few apoptotic cells (one or two cells/high magnification field in an affected area); 2 = 5–15% of myocardial cells showing necrosis, partial absence of myocardial BM, no more than four apoptotic cells per field; 3 = multiple foci of necrotic myocardial cells (16–25%), partial absence of myocardial BM, up to five apoptotic cells/field; 4 = myocardial cells (26–35%) showing necrosis in confluent areas, marked loss of myocardial BM, up to six apoptotic cells/field; 5 = more than 35% of myocardial cells showing necrosis in multiple massive or coalescent areas (which also show hyper contraction bands and myofibrillar loss), complete absence of myocardial BM, seven or more apoptotic cells/field).								
18.	Rat CRL 7 wks M	A) **Isoproterenol sq** B) 0, 0.5 mg/kg C) Monitor at 0.5, 1, 3, 6, 12 h, 1, 3, 7, 14 d D) 5 per group	Beckman Access cTnI	<0.03 Rats treated with saline were used for setting the reference range.	< 1000 x	When there was histopathological change there was cTn increase, although cTn were detected above normal range at 0.5 h for the first time and peaked at 3 hr, while histology showed first changes at 6 hr and reached maximum severity at 3 days.	1) Serum cTnI is more sensitive than histopathology at detecting isoproterenol cardiotoxicity. 2) Release & clearance kinetics: increase in 0.5 h; peak in 3 h; half-life ~6 h, return to normal in 72 h	Yes	144
19.	Rat Fischer 344 5-6 wk M	A) **Isoproterenol sq** B) 0, 0.5, 8 mg/kg (3 groups) C) Monitor: 0.5, 1, 2, 4, 8, 24 h D) 5 / group	a) Beckman Access cTnI B) Singulex Erenna cTnI	a) <0.02 b) 0.009 – 0.020 Rats treated with saline were used for the reference range.	< 1000 x	When there was histopathological change, there was increased cTnI. At early time points cTnI was increased without histopathological change.	1) cTnI increases in isoproterenol cardiotoxicity in rats earlier than myocardial histopathologic change 2) Release & clearance kinetics: increase in 0.5 h, peak in 4 h, half-life 4-6 h	Yes	189
Histopathologic findings in the hearts of rats administered either 0.5 or 8 mg/kg of isoproterenol were similar in character and distribution but were slightly more severe in the animals given 8 mg/kg. Myocardial lesions were most easily recognized in samples collected twenty-four hours after dosing and consisted of discrete and variably sized areas of acute myocardial necrosis scattered throughout the ventricles, with a predilection for the subendocardium and papillary muscles.									
20.	Rat Wistar 7-9 wk, females	A) **Isoproterenol ip** B) 8, 16, 24, 32, 40, 48 mg/kg C) Monitor 1, 2, 4, 6, 12, 24, 48 h D) 3-6 rats / group	Immulite cTnI Bayer ACS 180 cTnI Elecsys cTnT	0.368 ± 0.038 <0.03 considered normal, all controls were in this range <0.01 considered normal, same as above	< 1.5x < ~1500 x <170 x	1) cTn was increased prior to histopathological change was detected (1 versus 4 h) 2) cTn correlated best with histological indicators of acute change	kinetics of release and clearance: increase in 1 h, peak at 2 h; half-life ~ 4-5 h, mostly cleared by 48 h peak effect seen at 48 mg/kg	Yes	235
4 experiments were done in this paper : *Experiment 1a*: Immunoreactivity and Linearity of the cTnI response, *Experiment 1b*: Study on the Stability of cTnI in Serum (serum was tested at 4°C, -20°C, -80°C) *Experiment 2*: Time-Course Study (1 to 48 hours post dosing), *Experiment 3*: Dose-Response Study (8.0 to 48.0 mg/kg Isoproterenol), *Experiment 4:* Dose-Response Study (0.25 to 20.0 mg/kg Isoproterenol) Histological findings included increased myofibre swelling and eosinophilia, acute myodegeneration with swollen eosinophilic myofibers, neutrophilic infiltration and chronic myodegeneration at 24 hours after the administration of ISO with loss of myofibers and a mononuclear cell infiltration.									
21.	`Rat	**A) Isoproterenol sq**	a) Beckman	a) 0.086 ± 0.21	a) < 30 x	cTnI of 0.35 corresponded to minimal	1) cTnI and cTnT are effective at		

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	Wistar 310 g	B) 4 mg/kg C) Monitor: 2, 4, 6, 24 h D) 36	Access cTnI b) Elecsys cTnT	b) 0.04 ± 0,07	(1.1±2.3; n=14) b) < 150x (3.6 ±3.1, n=12)	histopathological change, 0.7 to marked histopathological change	detection and assessment of isoproterenol cardiotoxicity in rats 2) kinetics of release and clearance: increase in 2 h, peak at 4 h; half-life ~ 5 h.	Yes	30
Reference range data was obtained from 18 control study rats. Histopathological changes were graded as : no changes; 1+ mild (focal myocyte damage or small multifocal degeneration with slight degree of inflammatory process); 2+ moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process); 3+ marked necrosis with diffuse inflammatory process)									
22.	Rat SHR CRL 12 wk M	**A) Doxorubicin iv**; B) 1 mg/kg C) 1 week after 2, 4, 6, 8, 10, or 12 weekly doses D) 37 (6 groups of 5 with a control group of 7 for saline injections)	Elecsys cTnT	0.02 ± 0.01	< 20x	All rats with increased cTn had histopathological changes (Spearman coefficient r = 0.9); no saline controls had either. Severity of histopathological change correlated with cTnT. There also was a correlation of tissue cTnT decrease with serum cTnT increase.	1) cumulative dose- dependent increase in cTnT 2) blood cTnT as effective as histology in identifying doxorubicin cardiotoxicity in rats and correlates with its severity	Yes	93
The reference range was determined from all 37 rats of the study prior to their treatment: The serum cTnT levels and the total cumulative doses of doxorubicin showed a high degree of correlation. Even at the lower doxorubicin dosage, alterations in serum cTn were evident. The magnitude of the increase in serum levels of cTnT shows a positive correlation with the cardiomyopathy scores derived from microscopic examination of cardiac tissues.									
23.	Rat Wistar 17 wk M	A) **Doxorubicin iv** B) 1.5 mg/kg weekly C) 1, 4, 5, 6, 7, 8, 9 wk D) **44**: 7 groups of 5 treated rats, 9 controls	a) Beckman Access cTnI b) Elecsys cTnT	a) 0.03 – 0.043 b) < 0.01	a) < 150 x b) < 10 x	All treated rats developed increased cTn and histopathological change. Also maximal cTn T and I correlated with functional impairment (r = 0.81) as determined by echocardiography.	cTn useful for detection and assessment of chronic doxorubicin cardiotoxicity associated with cumulative dose (7.6 mg/kg) in rats whereas CK-MB was not	Yes	28
Many of the DOX rats died prematurely of toxicity during the 9-week period: 2 by 5 wks, 8 by 6 wks, 17 by 7 weeks, 22 by 8 weeks, and 24 by 9 weeks. Histopathological findings were classified as perivascular fibrosis, interstitial fibrosis, myocyte vacuolisation and degeneration, and then graded using the following scale in each heart: no changes, + very slight, ++ slight, +++ moderate, ++++ marked. Histological exam was made of 17 of the DOX-treated rats at 6 and 9 weeks after the last DOX dose and in all the control rats.									
24.	Rat SD 6-8 wk M	A) **Doxorubicin ip**; B) 15 mg/kg ± doxorubicin exacerbating agents (COX inhibitors) C) Monitor at 4 h D) 16 controls, 12 doxorubicin treated, 27 doxorubicin + exacerbant treated	Boerhinger ES 300 cTnT	< 0.01	Up to 7 fold	Increases in serum cTnT occurred in treatment groups that also had increased cardiac myocyte apoptosis or increased serum lactate dehydrogenase.	cTn effective at detection of minimal doxorubicin cardiotoxicity in rats and for discrimination of exacerbating agents	Yes	66
Reference range was lowest limit of detection for the assay - all 16 control animals were below that limit in this study. Control groups had either DMSO or the CoX-2 inhibitors. There were no increases in serum cTnT in control groups. Animals in the groups with increased serum cTnT also had a) apoptosis in cardiac sections as measured by TUNEL assay, and b) increase in serum LD									
25.	Rat SHR 12 wk M	A) **Doxorubicin iv** B)1.0 mg/kg/wk ± partial antidote (dexrazoxane) for 12 wk C)Monitor : 0, 6, 9, 12 wk D)5 / group	Elecsys cTnT	0.03 ± 0.02	< ~30	Tn increase from 9th wk treatment; all treated animals had histopathological changes and Tn increase which were both partial reversed by the antidote	1) cTn useful for detection and assessment of chronic doxorubicin cardiotoxicity in rats associated with cumulative dose 2) cTn useful for assessment of protection from cardiotoxicity	Yes	94
26.	Rat SHR 12 wk M	**A) Mitoxantrone iv** B) 0.5 mg/kg/wk ± partial antidote (dexrazoxane) for 12 wk C) Monitor: 0, 6, 9, 12 wk D) 5 / group	Elecsys cTnT	0.03 ± 0.02	< ~10	Tn increase from 9th wk treatment; all treated animals had histopathological changes and Tn increase which were both partial reversed by the antidote	1) cTn useful for detection and assessment of chronic toxicity associated with cumulative dose 2) cTn useful for assessment of protection from cardiotoxicity	Yes	94
27.	Rat SHR	A) **PDE3 inhibitor**, subcutaneous B) 0, 100, 200 mg/kg	Elecsys	0.02 ± 0.01	Up to 50	Correlation of increase serum Tn with a) loss of tissue Tn, and b) severity	cTn is effective biomarker of drug-induced myocardial injury.	Yes	239

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	10 wk M	C) Monitor: 24 h D) 21 controls, 16 treated	cTnT		fold	of histopathology change.			
Animals from control group treated with saline or DMSO alone were used for setting the reference range. Histopathologic exam was performed in all treated animals and the lesions were divided in groups (0-5) according to their severity. Apoptotic cells were measured using the TUNEL method. Histological grades of injury were: 0 = no alteration; 1 = minimal inflammation; 2=mild inflammation and necrosis; 3 = multiple foci with inflammation and necrosis as well as mild-to-moderate hemorrhage and edema; 4 = confluent areas of inflammation and necrosis as well as severe hemorrhage and edema; 5 = diffuse, severe inflammation, necrosis, hemorrhage and edema									
28.	Rat SD 160 – 250 g	**A) Organophosphate fenthion** sq B) 0, 0.8 g/kg, 0.8 g/Kg + partial antidote (diphenhydramine) C) Monitor at 24 h D) 8 / group	Life Diagnostics cTnI ELISA rat specific	<1	2 – 4 fold	Pearson coefficient = 1 for group Tn and histopathologic change	cTn effective biomarker of antidotal effects on cardiotoxicity of organophosphate	Yes	232
Myocardial histopathological changes of edema, inflammation, vacuolization, and necrosis were significantly higher in organophosphate-treated animals, which had highest cTnI. This was significantly decreased by the partial antidote treatment. The antidote-treatment group had intermediate cTnT values to those of controls and organophosphate-treated groups. It's noteworthy that samples for this study were obtained by cardiac puncture, which is known to increase background cTn levels and to reduce sensitivity for detection of treatment-induced cTn.									
29.	Rat Wistar 230 – 250 g M	**A) Organophosphate** (methidathion), oral gavage B) 0, 5 mg/kg, 5 mg/kg + partial antidote (vit E & C) for 5 d / wk for 4 wk C) Monitor: at 4 wk D) 6-8 / group	Chiron ELISA cTnI	<1	<3x	Treatment produced increased cTn and histopathological evidence for necrosis and both were partially decreased by antidote	1) Chronic cumulative toxicity is measureable by cTnI 2) cTn can be used to assess myocardial protective effects of agents on cardiotoxicity	Yes	231
It's noteworthy that samples for this study were obtained by cardiac puncture, which is known to increase background cTn levels and reduce sensitivity for detection of treatment-induced cTn.									
30.	Rats SD M & F	A) **Aging** compared to 3 mo B) not relevant C) not relevant D) 12 M, 12 F at 3 mo; 83 M, 82 F at 6 mo; 26 M, 27 F at 8 mo	Centaur cTnI (1st generation)	0.008 ± 0.0123 (3 mo)	10 x (M)	Pearson coefficient r = 0.6	1) Tn may be more effective than histology at detecting minimal change: 2) SD have age & gender dependent spontaneous cardiomyopathy detected with cTn – M & some F at > 6 mo	Yes	152
31.	Rat SD	A) **Left coronary occlusion** B) 90 min C) Monitor 10, 70, 130, 190, 270 min later D) 5 rats	Boerhinger ES300 cTnT	0.01 ± 0.00	10,000 x	Pearson coefficient r = 1 infarcted tissue quantitated by a) weight after visualizing with trypan blue exclusion and b) loss of tissue CK activity	1) Infarct size precisely correlated with cTnT 3 – 4 h after infarct 2) release kinetics defined	Yes	150
32.	Rat Wistar 200-250 g M	B) **Left coronary occlusion** C) Monitor 1, 2, 4, 8, 16, 32 d D) 6 rats per group	Elecsys cTnT	0.02 ± 0.01	< ~350 x	infarcted tissue quantitated by visualizing by loss of tetrazolium reductase staining; infarction associated with cTn increase	cTn an effective biomarker of myocardial infarct in rat	Yes	135
It's noteworthy that samples for this study were obtained by cardiac puncture, which is known to increase background cTn levels and reduce sensitivity for detection of treatment-induced cTn.									
33.	Rat Wistar 180-200 g M	A) **Microcystin** iv; B) 14 or 87 ug/kg (ie 0.16 and 1.0 x LD50) C) 24 h D) 5 at low dose, 10 at high dose; and 5 controls treated with saline.	ACCESS 2 Beckman Coulter	0.02 ± 0.01	4-25 fold	Severity of histopathological change was mild in the low dose group, more severe in the high dose group of rats hat survived and marked in the group that died at the high dose.	Severity of cTn increased is related to the severity of the histopathological change.	Yes	Qiu 2009
Compared to controls, there was a 5-fold increase of cTnI in the high-dose group amongst rats that survived, but 20 – 25-fold in rats that died at high dose. There was no significant cTnI increase in the low-dose group. Microcystin caused disorganization of cell structure and loss of adherence between cardiomyocytes. At the 0.16LD50 dose, myocardial damage included enlarged cells with enlarged and often bizarre-shaped nuclei, occasional cytoplasmic vacuolization and partial degenerative muscle fibres. There were pyknotic changes in nuclei, and slight infiltration of lymphocytes. At the 1LD50 dose, disarray of myocardial fibres and the degenerative muscle fibres with myocytolysis were the most prominent features. Vacuolization and degenerative cardiac muscle fibres were obvious in dead rats. These rats also had marked depletion of mitochondrial staining in cardiac tissue.									
34.	Mouse BALB/c &	A) **Isoproterenol** sq; B) 0.1, 1, 10, or 50 mg/kg single	Life Diagnostics	< 0.14	Up to	Increased cTnI was progressive with dose and occurred concurrently with	1) More than mild increase in cTnI corresponds to cardiac	Yes	Engel

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	CD-1 F	dose C) Monitor 1, 4, 8, 24, 48, or 72 h D) 3 – 10 / group	cTnI ELISA Mouse specific		~100 fold	cardiac necrosis. Strain differences in cTnI increase coincided with differences in severity of cardiac myocyte injury.	necrosis 2) cTnI is more sensitive than histopathology, CK, FAB-3, and AST.		2009
Lesions scores were: 0 = normal, 1 = minimal (very few, small foci affected, ~2-5 myofibers per histological section), 2 = slight (identifiable at low magnification but estimated to involve < 8% of tissue), 3 = moderate (easily identifiable, 8 - 20% of tissue affected), 4 = marked (20 - 50% of tissue affected), and 5 = severe (>50% affected). Isoproterenol-induced increases in cTnI were both greater and more sustained in BALB/c than in CD-1 mice, consistent with increased incidence and severity of morphological changes (greater number of muscle fibers involved or greater total volume of injury).									
35.	Mouse BALB/c F	A) **Kinase inhibitors** po; B) 10 mL / kg SID for 4 d C) Monitor 4 h after last dose D) 135 mice: 3-4 / dose	Life Diagnostics cTnI ELISA Mouse specific	< 0.14	Up to ~30 fold	Compounds could be divided into 3 groups based on cTnI that corresponded to risk of histological necrosis in 4 day studies.	cTnI can be used effectively for derisking drug discovery programmes involving kinase inhibitors for cardiotoxicity	Yes	Engel 2009
Reference range is based on the lower limit of detection of the assay and ~95% mice having values lower than this. A clear difference was observed across compounds, suggesting a significant off-target component to the acute cardiotoxicity caused by these kinase inhibitors and justifying further exploration of structure-activity relationships to retain pharmacological activity while minimizing compound-related cardiotoxicity. Compounds used in 2-day screening studies from which all measured cTnI concentrations fell into the low-risk category were not observed to cause cardiac necrosis in 4-day studies. Compounds that produced cTnI concentrations in the medium-risk category demonstrated a dose margin in which compound administration did not cause cardiac necrosis. All compounds that caused cTnI concentrations in the high-risk category caused cardiac necrosis in 4-day studies.									
36.	Mice	A) **Compound Y** per os B) 0, 1, 100 mg/kg/day for 12 d C) Monitor: 12 d D) 30; 10 / dose	a) Centaur cTnI (1st generation) b) M8 Bioveris cTnT	0.0 ± 0.0	3.5 ± 2.3	Complete concordance between occurrence of cTnT increase and histopathology change. Both only occurred at the high dose.	1) cTnI as effective as histology in identifying cardiotoxicity. 2) cTnI effective in chronic toxicity studies	Yes	152
Control mice had undetectable levels of cTnI									
37.	Mice CRL 7 wks M & F	A) **Doxorubicin** ip B) 10 mg/kg SID for 5d C) Monitor: 5d D) 11: 3 controls, 4 M & 4 F treated	Boerhinger ES 300 cTnT	0.19 ± 0.03	~50 x	Marked cardiac degeneration associated with increased cTnT	cTnT effective in mice for detection of doxorubicin cardiotoxicity	Yes	150
38.	Mice C57BL/6 20-25 g 6-8 wk M	A) **Doxorubicin** ip B) 20 mg/kg SID for 4d ± partial antidote (tetrathiomolybdate Cu-chelator) C) Monitor: 4d D) 24: 10 (doxorubicin), 10 (doxorubicin + TM), 4 (saline)	Life Diagnostics cTnI ELISA Rat specific	<0.1 Animals treated with saline were used for setting the reference range.	< 80 x	Treatment produced increased cTn and histopathological evidence for necrosis and both were partially decreased by antidote	cTn can be used to assess myocardial protective effects of agents on doxorubicin cardiotoxicity in mice	Yes	101
39.	Mice C57BL/6 7-8 wk	A) **Doxorubicin** ip B) 20 mg/kg SID for 4d ± partial antidote (tetrathiomolybdate Cu-chelator) C) Monitor: 4d D) 20: 5 (TM + doxorubicin), 5 (TM + Cu + Do), 5 (saline), 5 (Dox)	Life Diagnostics cTnI ELISA Rat specific	<1 Animals treated with saline were used for setting the reference range	< 10 x	Not determined, but increases were shown to correlate with the classical biomarkers of cardiotoxicity (CK-MB and LD)	cTn can be used to differentiate myocardial protective effects of TM on cardiotoxicity	Yes	102
40.	Mice Metallothio neine KO 6-8 wk M	**A) Doxorubicin** ip B) 15 mg/kg ± partial antidote (Zn) for 4 days C) Monitor: 24 h D) 4 groups of 7 / group	Elecsys cTnT	< 0.03	~ 14 x	Treatment produced increased cTn and histopathological evidence for necrosis and both were partially decreased by antidote	cTn can be used to detect myocardial protective effects of zinc and metallothionein gene knockout on doxorubicin cardiotoxicity in mice	Yes	197
41.	Mice	A) **left coronary occlusion**			4-fold	Pearson r = 0.841 infarcted tissue	cTnT is an accurate measure of		

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	C57BL/6J 22-25 g M	B) C) Monitor 24 h D) 32 mice used	Roche TnT Cardiac Reader	Not determined	variation in infarct size & cTn	quantitated by visualizing by loss of tetrazolium reductase staining	infarct size	Yes	142
42.	Mice CRL (PPARα KO & wild type) 10 wk	A) **PPARα agonist per os** B) 0, 1.25, 5, 10, 20 mg/kg SID for 12 d C) Monitor: 12 d D) 5-6 per group	Bayer ACS: 180 cTnI	<0.02	Up to 18 ug/L	Correlation with histopathology change and with serum CK-MB	cTnI as effective as histopathology and CK-MB in detection of PPARα cardiotoxicity in mice	Yes	170
Control mice had undetectable levels of cTnI									
43.	Monkey Cyno-molgus M & F 2.5 – 5 kg	A) **Isoproterenol, iv** B) 4 µg /kg/min for 20 min C) Monitor: 0, 2, 4, 8, 24, 48, 72, 92 h D) 2 treated	a) Singulex Erenna cTnI b) Beckman Access cTnI	a) 0.001 - 0.020 b) <0.02	a) 0.033 - >5 b) 10	Not determined by this study as monkeys were recovered	Peak within 4 hours	Yes	189
Reference range for Beckman assay is set at the limit of detection of the assay, as cTnI could not be detected in control monkey serum. In two monkeys, values of 0.033 and > 5 ug/L were noted two hours post-dosing, which gradually decreased to 0.0011 and 0.187 ug/L by forty-eight hours, respectively. The monkey with the highest Singulex cTnI developed a Beckman Access cTnI of 10.									
44.	Monkey Rhesus 4.8 – 8 kg	A) **Norepinephrine** B) 20 ug/kg/min for 2 h C) Monitor: 2, 3, 4, and 5 d D) at least 4	11 analysers studied	Not determined	>0.8	Not determined	Abbott, Bayer Ultra, Beckman, and Dade assays gave good responses.	Yes	15
This study focused on assessing cross-species reactivity, assay imprecision, and assay sensitivity. The number of animals is not indicated, although it is stated that animals were killed on 4 different days and their samples were pooled in order to generate specimens of 0.8, 0.4, 0.2, 0.1, and 0.05 ug/L.									
45.	Monkey Cyno-molgus M & F	**A) Left coronary occlusion** B) 4 h C) Monitor at 4 h D) at least 2	11 analysers studied	Not determined	>0.8	Not determined	Abbott, Bayer Ultra, Beckman, and Dade assays gave good responses. Weak responses were observed with species-specific cTnI and Roche cTnT.	Yes	15
This study focused on assessing cross-species reactivity, assay imprecision, and assay sensitivity. The number of animals is not indicated, although it is stated that animals were killed on 4 different days and their samples were pooled.									
46.	Monkey Cyno-molgus 3 - 4 kg; 3-4 yr F	A) **Hydrochlorothiazide per os** B) 50 mg/kg/day for 1 or 2 wk C) Bled at days 1,3,5,7,11, one h pre- and 4 h post-treatment D) 1 control and 4 treated - 2 for 1 wk, 2 for 2 wk.	Life Diagnostics ELISA Monkey-specific	Control animal bled 18 times: 0.094 – 0.184	0 - 4 fold transient increases during period of hypokalemia	Mild to moderate focal myocardial hemorrhages and necrosis was found in 3 of 4 treated animals but not the control. Mild elevation in cTnI was obvious in 2 of 3 with histopathologic change. The animal with greatest histopath change had highest cTnI.	There was good correspondence between the occurrence of increase in cTn and histopathological change.	Yes	Takeuchi 2008
The myocardial degeneration was shown to be related to hypokalemia produced by the treatment with diuretic. There were no changes in other cardiac biomarker enzymes (CK, LD, AST, ALD). The lack of clear increase in cTn in one of the 3 animals with myocardial change was considered to be possibly related to the timing of the collection of blood for measurement.									
47.	Marmoset 24 – 31 mo M & F	A) **Minoxidil** by gastric lavage B) 0, 150, 200 mg/kg C) 1 – 3 h after each dose D) 7: 2 controls 5 treated	Centaur cTnI (1st generation)	0.03 – 0.18	3 – 100 fold	All marmosets with histopathological evidence of necrosis had increased cTnI	cTn is effective biomarker of minoxidil-induced cardiovascular injury in marmosets	Yes	87
48.	Rabbit NZ White 3 kg M	A) **Isoproterenol ip** B) 3 mg/kg C) Monitor: 1 h D) 6 animals / group	Dade-Behring cTnI	<0.3 Animals treated with saline were used for setting the reference range	< 20 x	Treatment produced both histopathological evidence of necrosis & increased cTn	cTn is effective for detection and assessment of cardiotoxicity in rabbits	Yes	162

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49.	Rabbit Chinchilla 3.4 kg M	A) **Daunorubicin iv** B) 3 mg/kg weekly for 10 wk C) Monitor: 1, 5, 8, 11 wk D) 42	Elecsys cTnT	<0.01 Animals of the control group were used to set the upper limit.	< 20 x	Mild increases occurred at 5 weeks with marked increases from 8 weeks corresponded to marked histopathology change at 10 wk	cTn can be used effectively in chronic studies	Yes	164
50.	Ferret M	A) **Cardiac puncture** B) hypodermic needle C) 1 and 45 min D) 7	Boerhinger ES 300 cTnT	0.01 ± 0.00	2000 x	Not determined	Blood collection by cardiac puncture cannot be used in evaluating cardiotoxicity	Yes	150
51.	Cattle Jersey / Holstein 494±85 kg F	A) **Monensin** po B) 1 dose 30, 40 or 50 mg/kg C) Monitor at baseline 12, 24, 36, 48, 72, 96, 120, 144 h D) 10	Centaur cTnI (2nd generation)	0.01–0.02	Up to 3900-fold	High correlation (r = 0.7, Pearson) between cTnI and quantitative measure of histopathological lesion.	1) cTn I can be effectively used as a quantitative biomarker of myocardial injury and myocardial function 2) cTn is more effective than CK 3) kinetics of release and clearance defined	Yes	Varga 2009
Semiquantitative histopathologic examinations of the heart were performed in each cow. A scoring system with regard to the magnitude of myocardial injury was established and a total heart score was compared with maximum cTnI concentration measured after monensin administration. Five hearts from healthy cows served as controls. cTnI was significantly associated with left ventricular shortening fraction (r^2 = 0.51; p <0 .02) and myocardial histopathologic lesion score (r^2 = 0.49; p < 0.021). All 7 cows with evidence of myocardial necrosis had a cTnI concentration > 1.									
52.	Pig Pietrain 20-30 kg M & F	A) $FeCl_3$ intracoronary, topical B) 45 min exposure to strip with 20 or 50% w/v solution C) bled at 3 and 6 h after exposure D) 10	Boerhinger ES 300 cTnT	0.071 ± 0.031	14-fold (0.997 ± 0.106)	$FeCl_3$ in all 10 cases produced marked infarction, loss of tissue ATP, and myocardial necrosis. In all cases cTnT was substantially increased.	cTnT can be effectively used as a quantitative biomarker of myocardial injury	Yes	Dogne 2005
Reference range was determined from the 10 pigs prior to the experiment. Amount of infarction was identified and quantitated by use of Evans blue and tetrazolium dye and planimetry. A significant elevation of TnT concentration was observed after topical application of ferric chloride to the coronary artery and throughout the 6 h of ischemia.									
53.	Pig Landrace 18-25 kg M	A) **Left coronary occlusion ± cariporide** B) 30 then 48 h reperfusion C) 5 min before occlusion, at mid-point of occlusion, at 5, 15, 30 min, 1, 6, 24 and 48 h reperfusion D) 21 pigs: 9 with occlusion & perfusion; 6 with perfusion; 6 with occlusion, perfusion and cariporide	AxSYM System (Abbot Laboratories)	0.5 ± 0.3 for sham	30 – 1600 fold	least-squares linear regression analysis showed a linear relationship between myocardial infarct size and maximal plasma troponin with high correlation of r = 0.86.	1) Myocardial injury is detected and quantifiable with cTnI in dogs - Infarct size precisely correlated with cTnI 6 h after infarct 2) Infarct size reduction by cardioprotective drug was precisely quantitated by measurement of cTnI 3) kinetics of release and clearance defined	Yes	Letienne 2006
Myocardial infarct size was measured using Evans blue and vital tetrazolium stain and image analysis. In non-occluded, cTn rose to 14.3 ± 3.5 at 6 h reperfusion and returned to normal by 48 h. Occluded pigs without cardioprotective drug had 0.2 ± 0.2 at baseline that rose to 767 ± 152 at 6 h. Occluded pigs with cardioprotective drug had 0.6 ± 0.2 at baseline that rose to 288 ± 59 at 6 h.									

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