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Monitoring of troponin release from cardiomyocytes during exposure of toxic substances using surface plasmon resonance biosensing

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Abstract

Troponin T (TnT) is a useful biomarker for studying drug-induced toxicity effects on cardiac cells. In this paper, we describe how a surface plasmon resonance (SPR) biosensor was applied to monitor the release of TnT from active HL-1 cardiomyocytes *in vitro* when exposed to cardiotoxic substances. Two monoclonal human TnT antibodies were compared in the SPR immunosensor for analysing of the TnT release. The detection limit of TnT was determined to 30 ng/ml in a direct assay set-up and to 10 ng/ml in a sandwich assay format. Exposure of doxorubicin, troglitazone, quinidine and cobalt chloride to the cardiomyocytes for periods of 6 to 24 hours gave significant SPR responses, while substances with low-toxicity showed insignificant effects (ascorbic acid, methotrexate). The SPR results were verified with a validated immuno-chemiluminescence method which showed a correlation of $r^2 = 0.790$.

Keywords: SPR, Troponin T, Cardiotoxicity, *In vitro* toxicity, HL-1 Cardiomyocytes, Cytotoxicity screening

Abbreviations: SPR, surface plasmon resonance; RU, resonance units; cTnT, cardiac troponin T; mAb, monoclonal antibody; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; SD, standard deviation; SEM, standard error of mean

Introduction

Three troponin forms are released from heart muscle cells during cardiac injury: troponin T (TnT), troponin I (TnI), and troponin C (TnC) [1]. The release is caused by a combination of a disruption of the cardiomyocyte membrane and a dissociation of the thin-filament troponin complex in the cell [2-3].

This mechanism makes cardiac troponins sensitive clinical biomarkers for all heart muscle related diseases [4-7]. It also makes the cardiac troponins useful as biomarkers when investigating cardiotoxicity effects induced by drugs [8-12]. In addition to that, troponins also are potential markers for testing of toxicants of other origin, such as cosmetic ingredients and other additives used in consumer products and which require more thorough testing according to new safety regulations [13].

Biosensors have attracted significant interest for developing sensitive troponin assays. In particular surface-based immunosensors, such as surface plasmon resonance (SPR) [14], total internal reflection fluorescence and surface acoustic wave sensors [15] provide a powerful assay format due to their ability to capture protein analytes by specific antibodies raised towards distinct epitopes [16] in rapid and parallel analytical setups.

For troponins SPR has been applied for analysis of the cardiac troponin I form (cTnI) in clinical blood samples [17] using conventional SPR instrumentation as well as fibre-optical SPR devices [18]. SPR has for this purpose also been applied to cTnT [20-21] and TnC analyses [22]. These assays were developed to facilitate rapid and more specific methods compared to existing ELISA based methods (e.g. Elecsys [16]).

For the analysis of drug-induced cardiotoxicity effects troponins are not exploited, except a few recent reports [9, 23]. For *in vitro* toxicity assaying this requires testing system, with relevant cardiac cells exhibiting a typical pacemaker phenotype [e.g. 24], i.e. showing beating rates characteristics of ventricular cardiomyocytes and which can be monitored in close proximity to a sensitive analytical device. In particular biosensors have appropriate analytical attributes for this purpose – being adaptable to miniaturization, allowing rapid sampling and specific detection, having the potential of simultaneously analyzing several analytes (biomarkers) and compensating for changing media background during culture testing.

The design of the SPR sensor chips is here particularly useful, due to their robust liquid conduit flow systems. Also, the SPR chips have the advantage of allowing parallel on-line analysis, and can be further expanded into multi-protein microarray devices as has been shown recently [25].

To our knowledge, SPR has not yet been applied to drug-induced cardiotoxicity testing. In this paper, we demonstrate the use of the SPR technique for testing a few well-known cardiotoxic compounds on a beating cardiomyocyte cell line, HL-1, and compare the performance and sensitivity with an existing *in vitro* method.

Experimental

Materials

All SPR reagents, including HBS-EP, amine-coupling kit, and regeneration solutions, were obtained from GE Healthcare (Uppsala, Sweden). The antibodies used were 1F11 (Abcam, UK), 1C11, and 7G7 (HyTest, Finland). Human cardiac troponin T was purchased from HyTest (Finland). The Elecsys 2010 system and reagents were obtained from Roche. For cell cultivation was used a Claycomb medium, norepinephrine, and fibronectin (all from Sigma), fetal bovine serum (Lot 6L0730) (JRH Biosciences, UK), L-glutamine (Invitrogen), penicillin and streptomycin (Gibco, USA), gelatine (BDH Prolabo, Germany), T-25 flasks (Sarstedt, Germany), and 48-well plates (VWR, Sweden). The test substances, doxorubicin, quinidine, cobalt chloride and ascorbic acid, were obtained from chemical companies.

Cardiomyocyte culture

Cardiomyocyte cells of the cell line HL-1 was provided by Professor William C Claycomb, Louisiana State University, New Orleans, LA, USA, and were propagated as previously described [24, 26]. The cells were maintained in Complete Claycomb Medium supplemented with 10% fetal bovine serum, 10 µM norepinephrine (dissolved in 30 mM L-ascorbic acid), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in T-25 flasks. Flasks and multi-well plates were coated with 0.2% gelatine and 5 µg/ml fibronectin. The HL-1 cells showed characteristic cardiac beating activity. All cultures were incubated at 37 ˚C in 5% CO_2.

Preparation of SPR surfaces

The cTnT antibodies were covalently immobilized on CM5 sensor chips in a Biacore 2000 system (GE Healthcare, Uppsala, Sweden) using amine coupling. HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) was used as running buffer at a continuous flow of 5 µl/min. The carboxylated dextran matrix was activated with a 7 min injection of a 1:1 ratio of 0.1 M NHS/0.4 M EDC. The antibodies were

coupled to the surface with a 10 min injection (150 µg/ml in 10 mM sodium acetate buffer, pH 4.5), followed by a 7 min injection of 1 M ethanolamine (pH 8.5) to block the remaining activated groups. The surface was washed 5 x 3 min with 10 mM glycine-HCl, pH 2.5. The immobilization levels of antibodies were 9,000-13,000 RU.

Toxicity testing protocols

Two testing protocols were used in the study. The first protocol used a direct immunoassay procedure were the TnT analyte was directly detected on the sensor chip after binding to the antibody ligand. In the second protocol a sandwich assay procedure was applied where a secondary antibody amplified the detection (cf. Fig. 1).

In both protocols HL-1 cells were seeded 20 h prior to drug exposure in 48-well plates at a density of 1.5×10^5 cells per well. Stock solutions were made of doxorubicin, quinidine, cobalt chloride and ascorbic acid in H_2O, methotrexate in 0.1 M sodium carbonate buffer and troglitazone in DMSO. Further dilutions to test concentrations were made in complete Claycomb medium supplemented as described above. At the start of the experiment, the overnight medium was replaced by media supplemented with drug compounds, or by media containing solvent as controls. After 24 h, the media samples were withdrawn from the cells, centrifuged at 13,000*g* for 10 min, and immediately analyzed undiluted on SPR sensor chips prepared as described above.

The assayed biochips were continuously purged with a flow of HBS-EP at 5 µl/min into which samples was injected for 3 min.

When applying sandwich assay protocol, the sample injection was followed by a 3 min injection of indicated antibody (2 µg/ml in HBS-EP). The surface was regenerated by injection of 10 mM glycine-HCl pH 2.5, for 1 min.

Human cTnT reference samples were diluted to various concentrations in HBS-EP or complete supplemented Claycomb media.

Immunochemiluminescence method

The cTnT concentration in the withdrawn culture samples was also determined by an automated immunochemiluminescence method (Elecsys 2010, Roche). The assay was carried out according to the manufacturer's recommendations. A biotinylated mouse monoclonal antibody (M11.7) linked to streptavidin coated paramagnetic beads was used for antigen capture. A mouse monoclonal antibody (M7) labelled with ruthenium trisbypyridyl complex was used for detection.

Statistical analysis

For cell-free experiments, the response from fresh solution (HBS-EP or complete supplemented Claycomb medium) was subtracted as background. For cell experiments, medium from untreated cells was used as background. Data from sandwich assays and drug testing are presented as means ± SEM. The interassay comparison was analyzed using linear regression.

Results and Discussion

Evaluation of antibodies against cTnT for SPR analysis

Three monoclonal antibodies against cTnT were evaluated for their usefulness as ligands in an SPR-based assay for testing drug-induced cardiotoxicity (see Fig. 2). The 1F11 and 1C11 antibodies are both reported to bind to a conserved region of cTnT, and therefore to recognize cTnT from several different species, including human and mouse. The 7G7 antibody recognizes a more variable region of cTnT, and is human-specific. Each of the three antibodies was immobilized in separate flow channels on an SPR sensor chip. The amount of immobilized antibody per channel was between 8,000 and 13,000 RU. A fourth flow channel on the same chip was first amine-activated and then deactivated with ethanolamine and used as reference surface.

Injection of 3 µg/ml of cTnT in complete Claycomb medium for 3 min resulted in typical SPR response curves for all three antibodies (Fig. 2a). The reference surface showed a change in refractive index only, with no binding during the injection. Three-minute injections of increasing concentrations of cTnT in HBS-EP (10-3000 ng/ml) are shown in Fig. 2b. Antibody 7G7 gave the highest response, followed by 1F11. As well as giving the lowest response, 1C11 also showed a decreasing response after a repeated number of injections ($n > 30$) (data not shown). Antibodies 7G7 and 1F11 showed no significant reduction in response ($n > 100$). The lower detection limits for cTnT with 7G7 and 1F11 antibodies were estimated at 30 ng/ml, defined as background response $\pm$ 2 standard deviations.

To compare the background effects on the SPR response signal of a typical growth medium designed for cardiomyocyte culturing, we performed spike-in experiments with cTnT dissolved in both the complete Claycomb medium and the SPR running buffer (HBS-EP) (Fig. 3). As seen in the figure, the complete Claycomb medium gave an additional background response of approximately 100 RU regardless of cTnT concentration. The Claycomb medium had no effect on the detection limit.

Thus, the cTnT antibodies considered best suited for SPR assaying were antibody 7G7 and 1F11. As mentioned above, while the 7G7 antibody is human-specific, the 1F11 binds to a more conserved region of cTnT and recognizes cTnT from several different

species. An assay using 1F11 will therefore be compatible with cell lines and primary cells from different origin. A benefit of this is that the same assay setup can be applied when analyzing and comparing preclinical toxicity data from animal with human trials.

The nonspecific adsorption of biomolecules from the cell culture medium gave a background signal of approximately 100 RU. This did not interfere with the antibody/antigen interaction and did not affect the detection limit. This background signal may vary between batches of media components. Therefore these factors need be kept constant within an experiment.

Sandwich assay

In an attempt to improve the performance of the assay, we investigated whether a sandwich design could lower the detection limit. Since cTnT is a 36 kDa protein, adding a secondary antibody of 170 kDa should greatly increase the response (cf. Fig 1). The 7G7 mAb was immobilized on the surface followed by injection of cTnT for 3 min (Fig. 4a). The 1F11 mAb was then injected for 3 min. The response measured after the mAb injection was approximately 2.5 times higher for all concentrations compared to cTnT only. Injection of 1F11 without a prior injection of cTnT gave a background response of 8 RU, which was subtracted from the sandwich assay values above. Using 1F11 as immobilized capture antibody and 7G7 as secondary antibody gave similar results (Fig. 4b). Using this sandwich assay, the detection limit was improved to10 ng/ml.

Thus, the sandwich assay with 7G7 and 1F11 increased the response and improved the detection limit three-fold; however, this approach could only be used for human cardiomyocytes since 7G7 is human-specific.

cTnT stability and measurability over time

We investigated the influence of the sample storage time before analysis of the cTnT samples. Aliquots of cTnT were added to complete supplemented Claycomb medium. These were stored at + 20 °C, + 4 °C, and - 70 °C for 24 hours. Fresh samples were then prepared, and all samples were analyzed by SPR (Fig. 5). Regardless of temperature, the

samples stored overnight gave significantly lower responses than freshly-prepared samples. Similar results were observed using the Elecsys 2010 for cTnT analysis. All further cTnT measurements were therefore performed on fresh samples.

The cTnT measurability was strongly reduced when samples were stored, regardless of temperature and analytical method. This may have been caused by degradation as previously reported [27], or the fact that cTnT can form tightly bound tetramers *in vitro* [28] which may block the antibody binding sites. Hessel *et al*. [2] showed a degradation of cTnT in cell culture medium after cell death and the formation of degradation products. The magnitude of signal loss we observed was similar to the loss of intact cTnT in their study after 24 hours.

Compound testing

Compounds with or without known cardiotoxic effect and the murine cardiomyocyte cell line HL-1 were used to investigate whether SPR analysis of cTnT released into the cell culture medium can be used as a measure of cardiotoxicity. The HL-1 cells were exposed to doxorubicin in a concentration range of 1.25-320 µM for 6 and 24 hours respectively, and analyzed on the SPR using the 1F11 mAb in a direct assay (Fig. 6). The response of medium from cells incubated without drug was subtracted as background. Cells incubated for 6 hours showed a response of 44 RU at the highest doxorubicin concentration only. After 24 hours a dose-response curve was observed with a maximum response of 20 RU at a doxorubicin concentration of 20 µM. The response decreased at higher doxorubicin concentrations. This is most likely due to cTnT being released at a earlier time point and the loss of measurability described above.

The SPR responses from HL-1 cells exposed to troglitazone in the concentration range 1.25-160 µM for 24 h are shown in Fig. 7a, and quinidine in the concentration range 100-850 µM in Fig. 7b. The dose-response curve showed a maximum response of 20 RU at a troglitazone concentration of 80 µM, and 21 RU at a quinidine concentration of 550 µM.

Again the response decreased at higher compound concentrations. The effects of cobalt chloride in the concentration range 5-320 µM is shown in Fig. 7c. The maximum effect was 11 RU at 80 µM.

As a negative control we exposed HL-1 cells to non-toxic levels of ascorbic acid in the range 0.14-35 mM (Fig. 7d) and methotrexate in the range 5 nM to 295 µM (Fig. 7e) for 24 h. No significant response to ascorbic acid was observed. In the methotrexate experiment the SPR response was approximately 8 RU regardless of compound concentration, indicating that methotrexate gave a slightly increased background. No dose response was observed.

Due to the loss of measurability of cTnT in solution over time, mainly cTnT released at the end of the experiment is detected. Also, untreated cells and cells exposed to low levels of the toxic compound continue to grow during the 24 hour incubation. This is not the case for cells exposed to highly toxic levels where lesser cells per well remain after the exposure period. This makes timing of the release of cTnT an important factor. If the incubation period is too short only very high concentrations of the toxic compound will have time to act on the cells. If the incubation time is too long the results will be strongly influenced by cTnT degradation and uneven cell numbers. The 24 hour incubation time was considered to be an appropriate trade-off. When testing a new potentially toxic compound, a broad concentration range and several time points need to be tested to rule out toxicity.

Interassay comparison of Biacore 2000 and Elecsys 2010

To validate the SPR assay, we made a comparison between SPR (Biacore 2000) and Elecsys 2010. The Elecsys 2010 system is routinely used to analyze cTnT levels in human plasma to diagnose acute myocardial infarction. HL-1 cells were exposed to 2.5-20 µM doxorubicin or 80 µM troglitazone for 24 h. Cell culture medium was analyzed for cTnT with both methods, and cTnT levels in medium from untreated cells were subtracted as background. Figure 8 shows a correlation plot between the two methods. A linear correlation with an r^2 value of 0.790 was observed. Thus, the correlation between SPR and the clinically approved Elecsys method was verified. The value of the

correlation factor reflects the methodological differences of the methods, including the different antibody specificities. Published comparative data between different cTnT assays are scanty, partly explained by the shortage of commercial assay providers. However, very recently a comparison between the fourth generation TnT and a high-sensitive cTnT method was published that showed great variation in bias across the entire measurement range [29]. In the low concentration range (10-30 ng/L) the bias varied from ca 20-290 ng/L, and at higher concentrations the bias was less pronounced. This is probably due to different molecular forms of TnT are excreted into plasma when the cardiac infarction varies in magnitude and that these molecular forms are measured with different specificity. Also in acute myocardial infarction, the intact troponin subunits rapidly disappear from the circulation during the early hours after infarction, while immunoreactive troponin fragments remain [11]. Furthermore it could be noted that for cTnI, for which there are a number of instruments and methods available, including antibodies and calibrators from several manufacturers, the results vary 20-fold to 40-fold in the reported clinical investigations [1, 29]. We think that development of new SPR assays may in future studies contribute to the further understanding of these fundamental questions.

Conclusion

A novel SPR based assay for measuring cardiotoxicity using an actively beating cardiomyocyte cell line and cTnT as a biomarker was developed. As emphasized in this article, the SPR-based cTnT assay is particularly suitable for study of drug-induced cardiotoxicity. The concentration of cTnT in cell culture medium from HL-1 cells exposed to toxic compounds is well within the detection range of this method.

The efficiency and sensitivity of the method was demonstrated using four compounds of different toxicity and two non-toxic compounds. The benefits of SPR based assay methods are their short analysis time, no need for sample pre-treatment or labelling and that very small sample volumes are required. Also, SPR methods can easily be integrated into cell culture systems for automatic and on-line measurement.

When evaluating the cardiotoxicity of drugs, however, it is necessary to consider several myopathic effects; for example, effects related to angina, ventricular hypotrophy, and congestive heart failure conditions [6]. Also, the predictive value can be improved by monitoring the release of troponins and natriuretic peptides (ANP, BNP) in combination with other factors such as endomyocardial biopsy and heart rate variability [7]. Moreover, the release of troponins is associated with myocyte cell death, and has also been shown to be a predictive factor for cardiac dysfunction, particularly in children when measured serially.

Recent SPR methods allow parallel analysis using several antibody ligands, thus enhancing throughput and allowing fine-tuning for compensation for subtile epitope and media deviations [30].

In this study we has demonstrated how SPR can be used as a platform for a cardiotoxicity assay based on one biomarker, cTnT. The applied system with four flow cells allows parallel assaying of up to three biomarkers (and one reference surface) to be analyzed in parallel. With high-throughput array-based SPR systems, the number of biomarkers that can be studied simultaneously increases drastically.

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Figure legends

Fig. 1. Immuno-assays for monitoring drug-induced cardiotoxicity used in the study. The thin filament-bound troponins are released from the cardiomyocytes exposed to a toxic compound, as an effect of membrane disintegration and by dissociation of the thin filament-troponin complex. Extracellular cTnT is sampled and assayed by a direct or a sandwich assay on an SPR dextran-gold chip with immobilized antibody.

Fig. 2. SPR sensorgrams and response curves for the three monoclonal antibodies for cTnT injections. Antibody 7G7 (black), antibody 1C11 (red), antibody 1F11 (green), and control (blue). (a) Sensorgrams for 10 µg/mL of cTnT during 3 min intervals. (b) Spike-in experiment with human cTnT in concentrations between 10 and 3000 ng/mL.

Fig. 3. Comparison of SPR responses in Claycomb medium and SPR buffer with the 1F11 antibody. Human cTnT was added to complete Claycomb culture medium or HBS-EP (blue) at concentrations between 10 and 3000 ng/mL of cTnT.

Fig. 4. Responses of cTnT in the sandwich assay format. (a) 7G7 antibody was immobilized on the surface. Human cTnT was injected for 3 min at concentrations 10-100 ng/mL, followed by injection of 1F11 antibody for 3 min. The relative response was measured directly after cTnT injection (blue) and again after the 1F11 injection (red). (b) 1F11 antibody was immobilized on the surface. Human cTnT was injected for 3 min at concentrations 10-100 ng/mL, followed by injection of 7G7 antibody for 3 min. The relative response was measured directly after cTnT injection (blue) and again after the 7G7 injection (red). The error bars represent mean values of n=3.

Fig. 5. Influence of storage time before analysis of cTnT samples. 300ng/mL cTnT were added to complete supplemented Claycomb medium. Samples were stored at + 22

°C, + 4 °C, and - 70 °C for 24 hours. Fresh samples were then prepared, and all samples were analyzed by SPR.

Fig. 6. The effect of doxorubicin on HL-1 cells. HL-1 cells were exposed to doxorubicin for 6 hours (a) or 24 hours (b) in the concentration range 1.25-320 µM. The amount of cTnT released into the cell culture medium was analyzed by SPR. The error bars represent mean values of n=3.

Fig. 7. The effect of toxicants on HL-1 cells. The HL-1 cells exposed to toxicants for 24 h at varying concnetrations. The amount of cTnT released into the cell culture medium was analyzed by SPR. (a) exposure of troglitazone in the range of 1.25 to 160 µM. (b) exposed to quinidine in the range of 100 to 850 µM, (c) exposure to cobalt chloride in the range of 5 to 320 µM. (d) exposure of ascorbic acid in the range of 140 µM to 35 mM, and (e) exposure of methotrexate in the range of 5 nM to 295 µM. The error bars represent mean values of n=3.

Fig. 8. Correlation between Biacore 2000 and Elecsys 2010. HL-1 cells were exposed to doxorubicin or troglitazone at different concentrations. Cell culture medium was analyzed for cTnT content by Biacore 2000 and Elecsys 2010. A correlation between the two methods was observed with r^2=0.790.

Fig. 1.

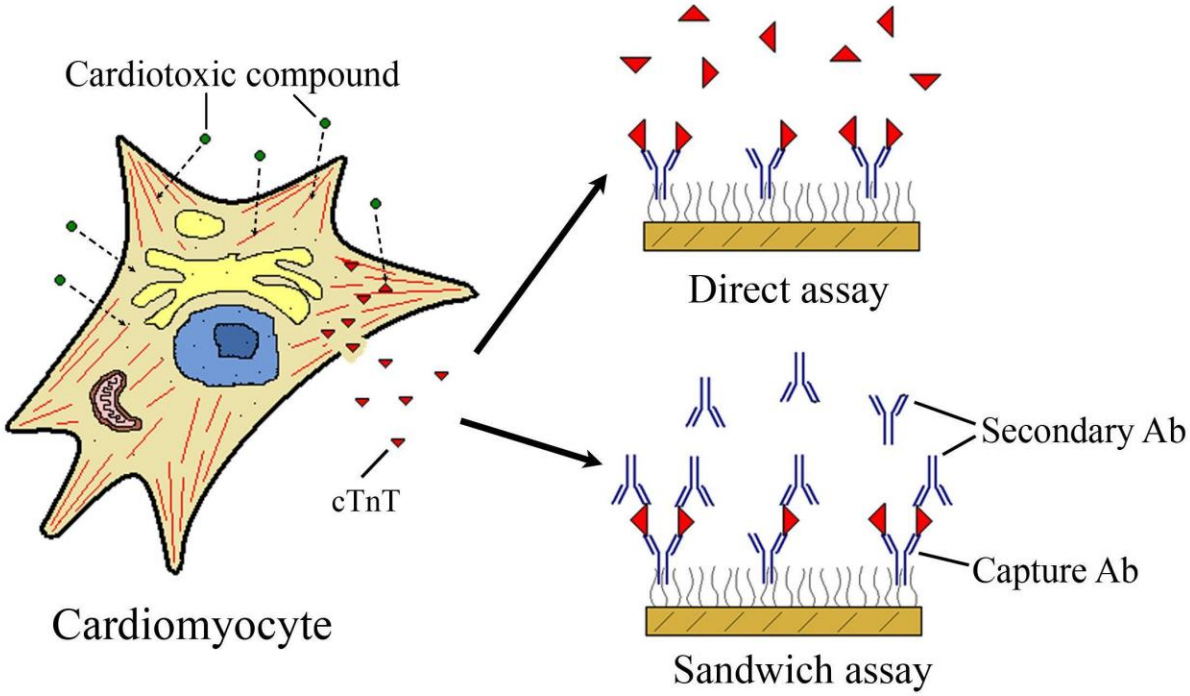
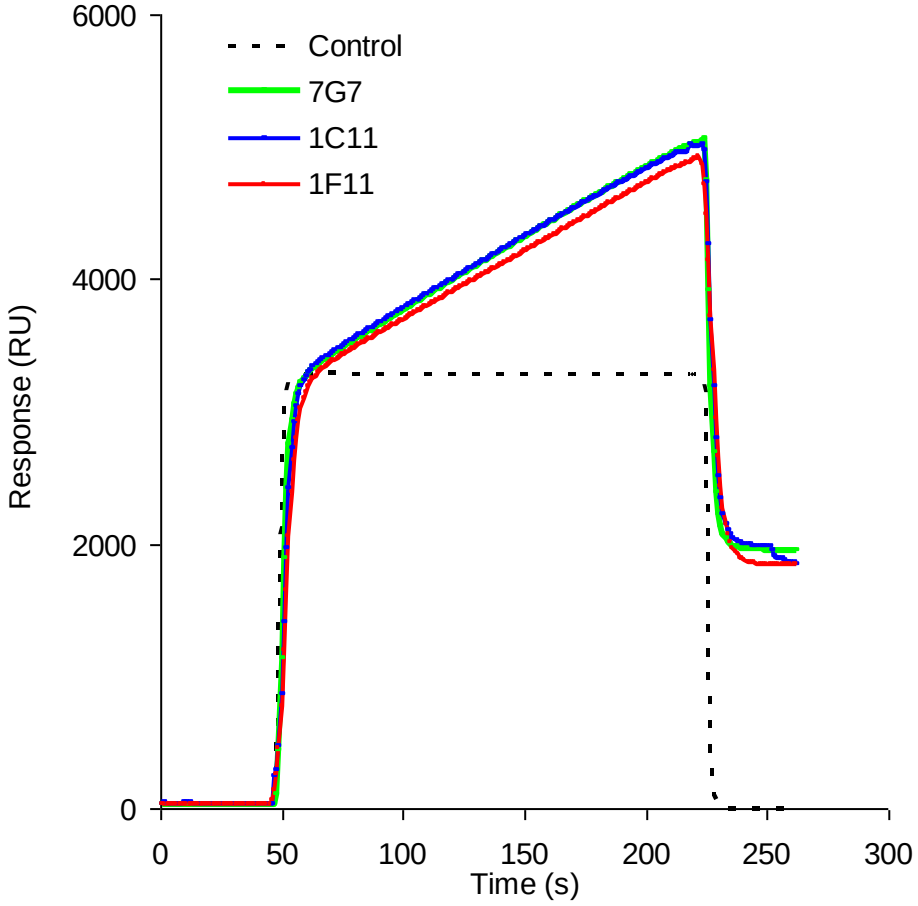

Cardiotoxic compound
cTnT
Cardiomyocyte
Direct assay
Secondary Ab
Capture Ab
Sandwich assay

Fig. 2a.


6000
4000
2000
0
Response (RU)
Control
7G7
1C11
1F11
0
50
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150
200
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300
Time (s)

Fig. 2b.

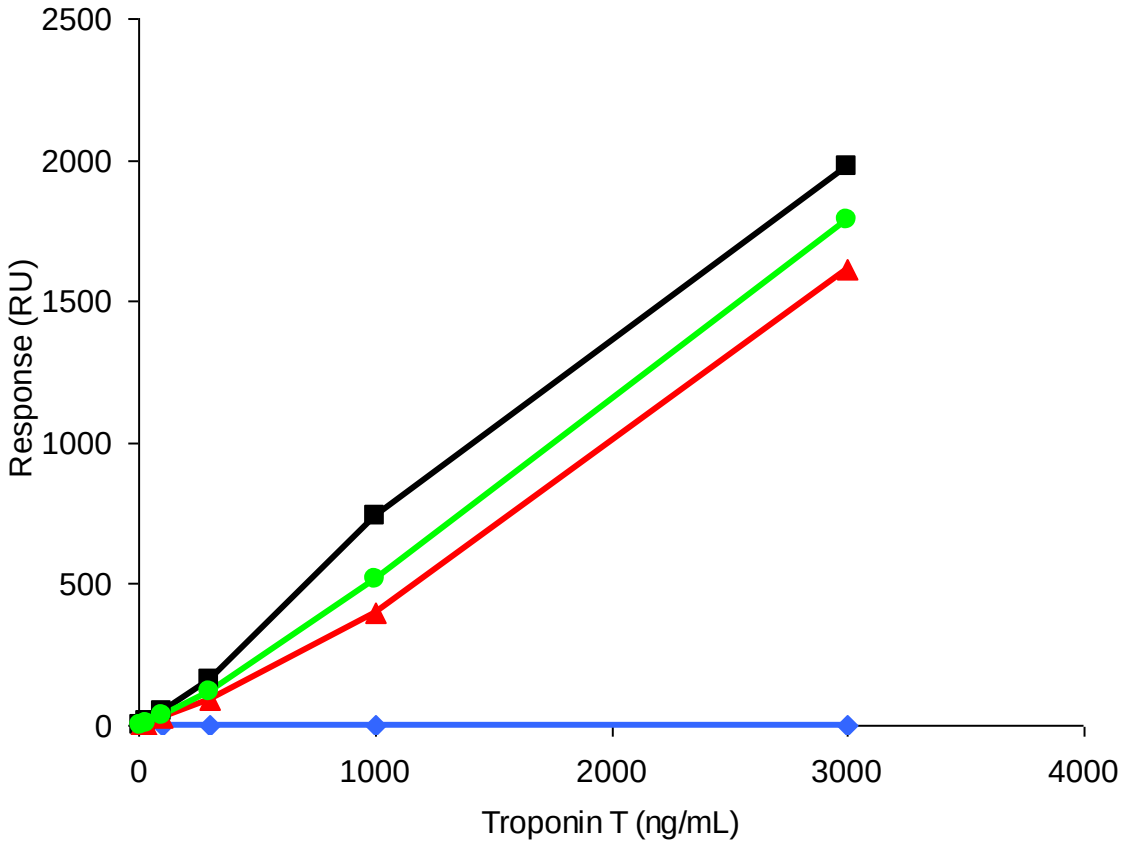

2500
2000
1500
1000
500
0
Response (RU)
0
1000
2000
3000
4000
Troponin T (ng/mL)

Fig. 3.

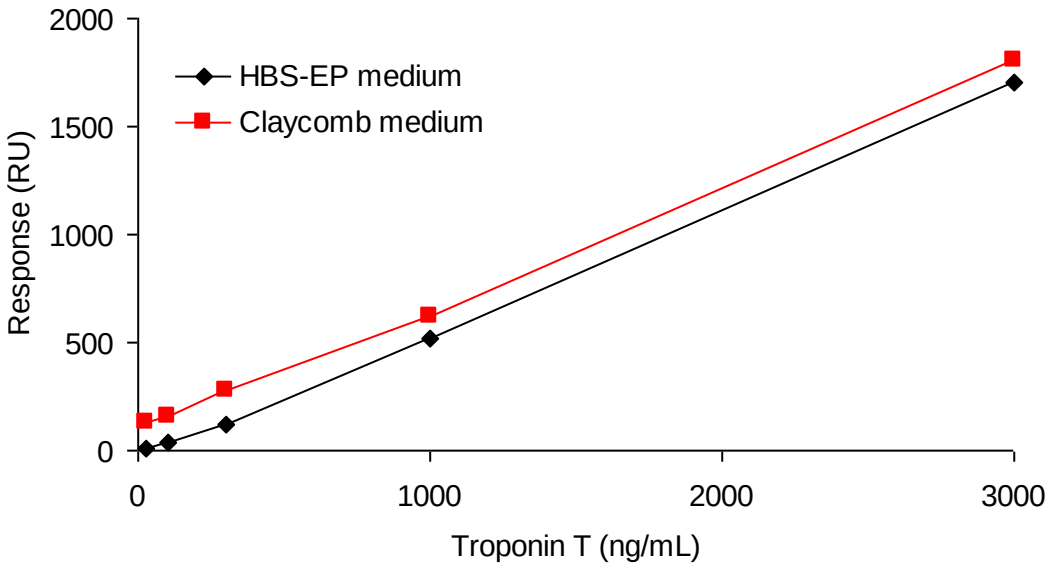

2000
1500
1000
500
0
Response (RU)
HBS-EP medium
Claycomb medium
0
1000
2000
3000
Troponin T (ng/mL)

Fig. 4a.

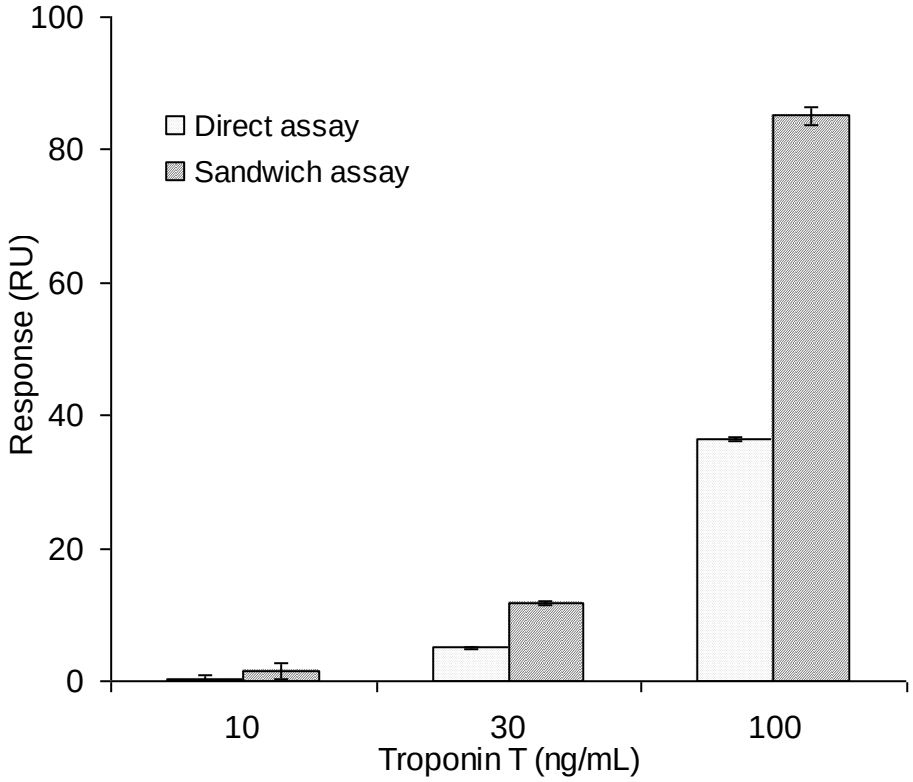

100
80
60
40
20
0
Response (RU)
Direct assay
Sandwich assay
10
30
100
Troponin T (ng/mL)

Fig. 4b.

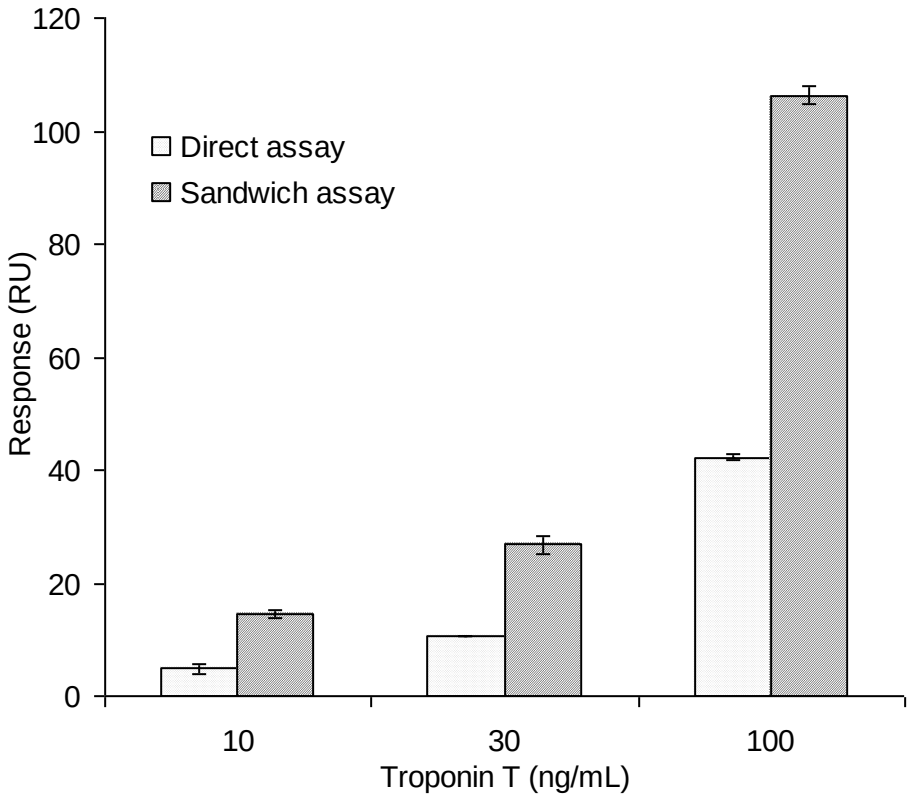

120
100
80
60
40
20
0
Response (RU)
Direct assay
Sandwich assay
10
30
100
Troponin T (ng/mL)

Fig. 5.

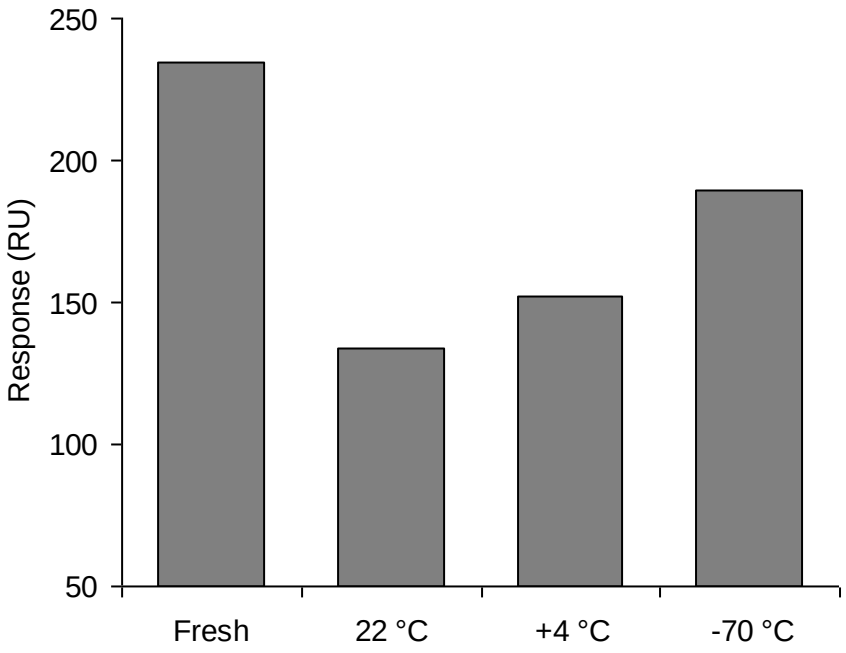


Fig. 6a.

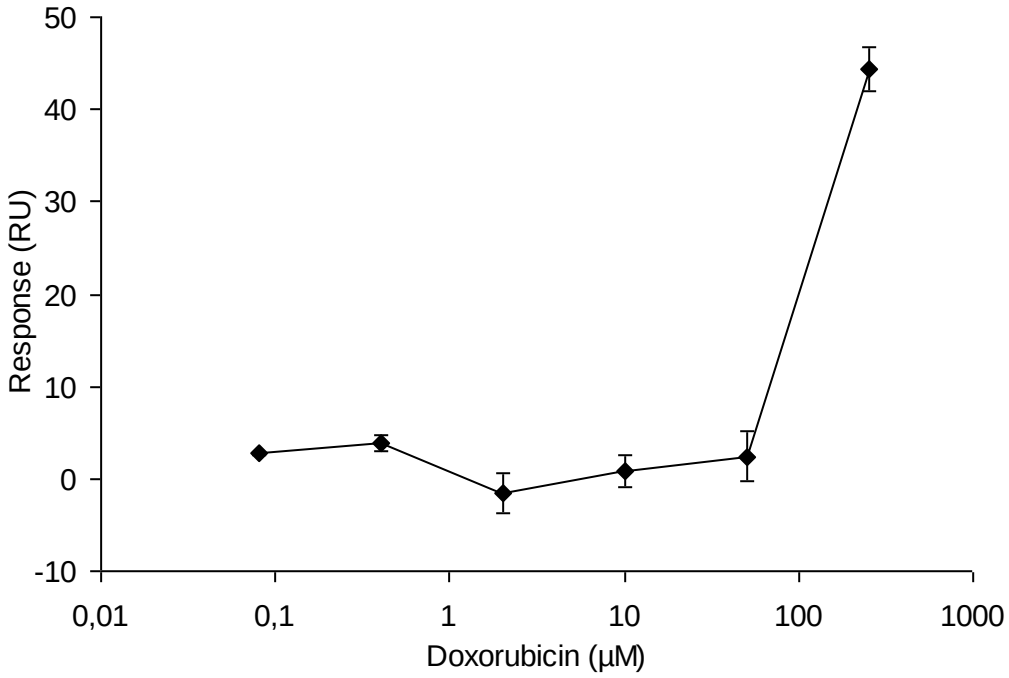

50
40
30
20
10
0
-10
Response (RU)
0,01
0,1
1
10
100
1000
Doxorubicin (µM)

Fig. 6b.

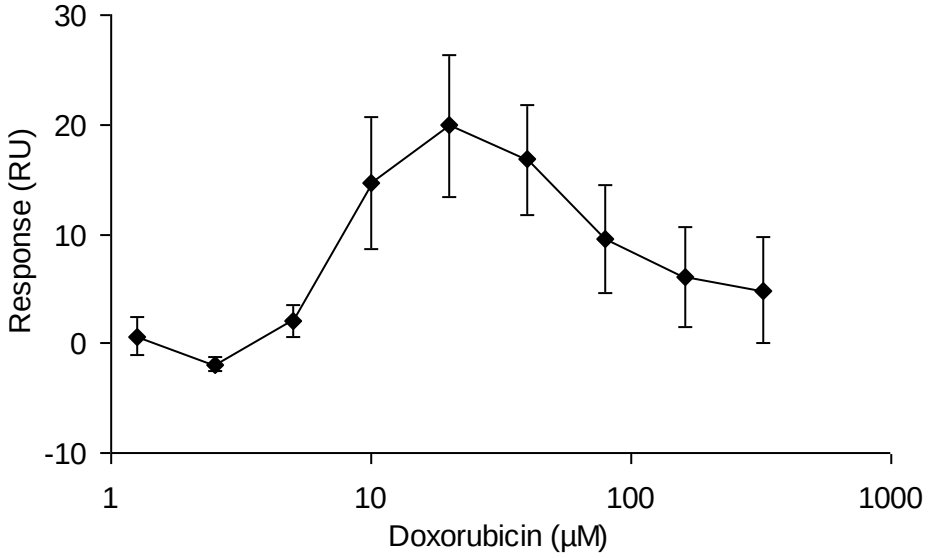

30
20
10
0
-10
Response (RU)
1
10
100
1000
Doxorubicin (µM)

Fig. 7a.

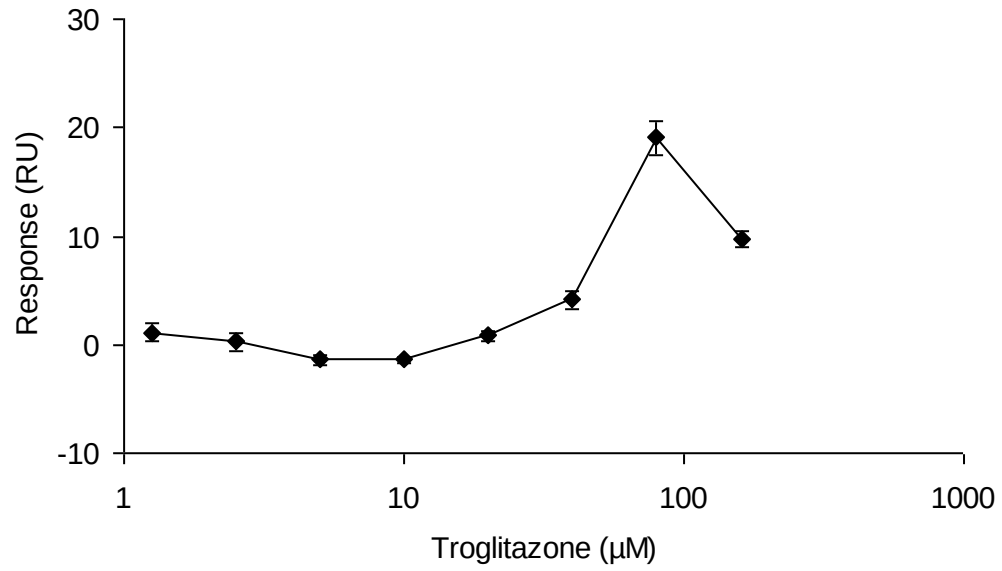

30
20
10
0
-10
Response (RU)
1
10
100
1000
Troglitazone (µM)

Fig. 7b.

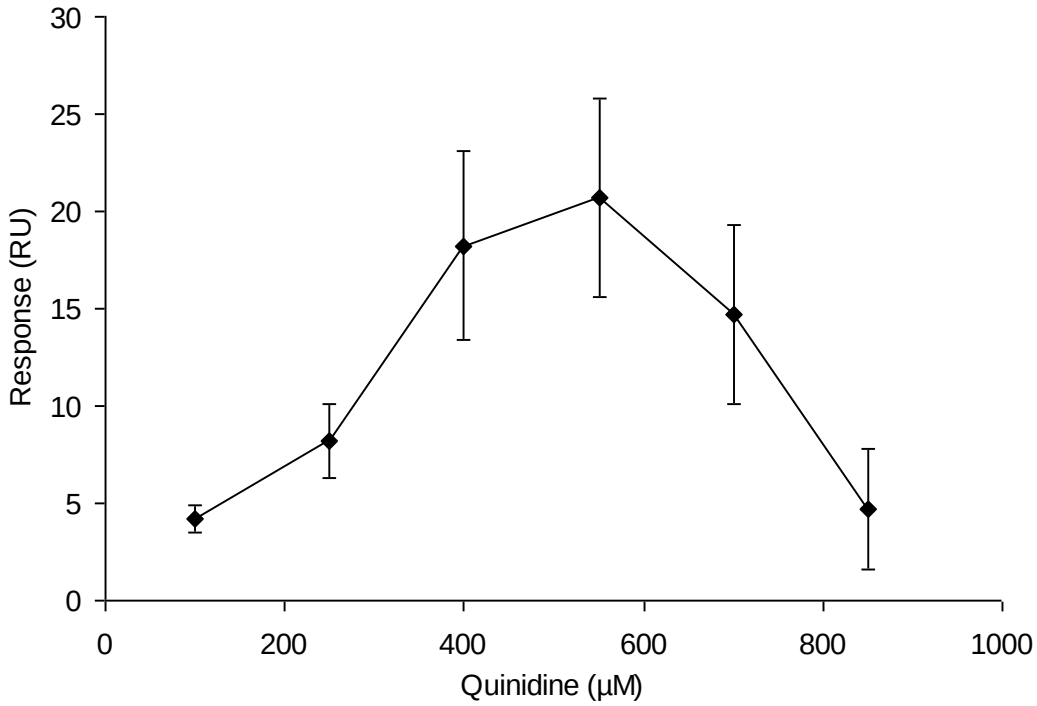

30
25
20
15
10
5
0
Response (RU)
0
200
400
600
800
1000
Quinidine (µM)

Fig. 7c

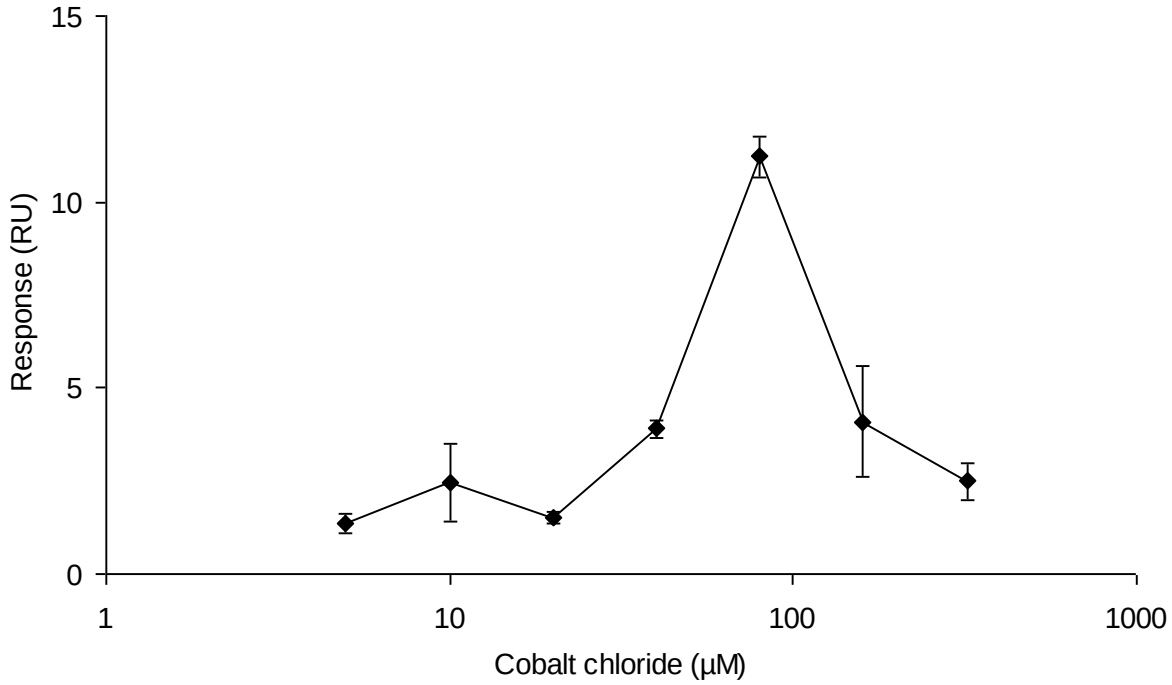

15
10
5
0
Response (RU)
1
10
100
1000
Cobalt chloride (µM)

Fig. 7d.

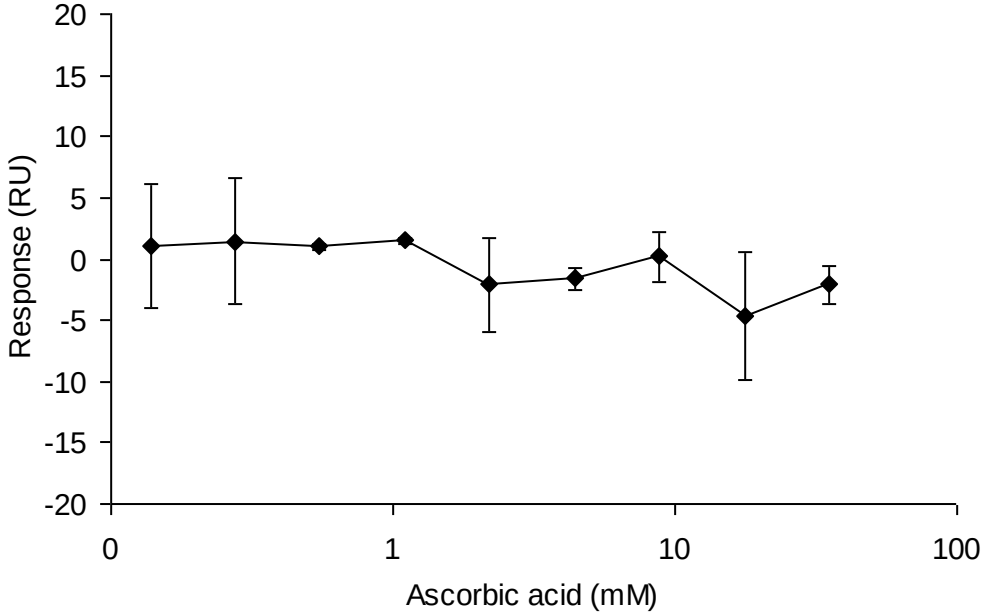

20
15
10
5
0
-5
-10
-15
-20
Response (RU)
0
1
10
100
Ascorbic acid (mM)

Fig. 7e.

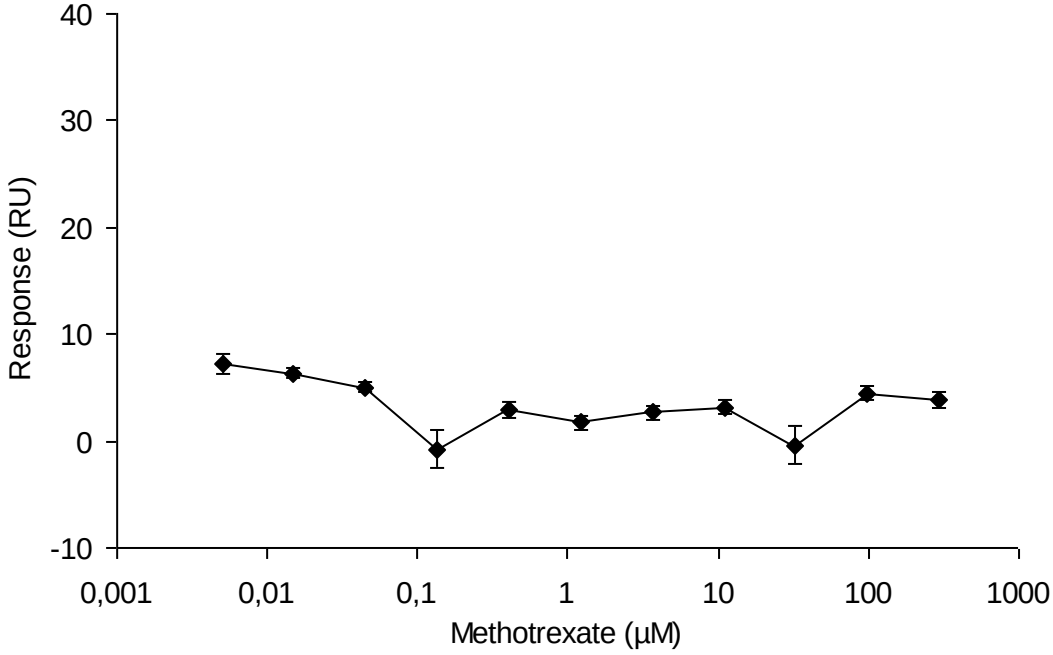
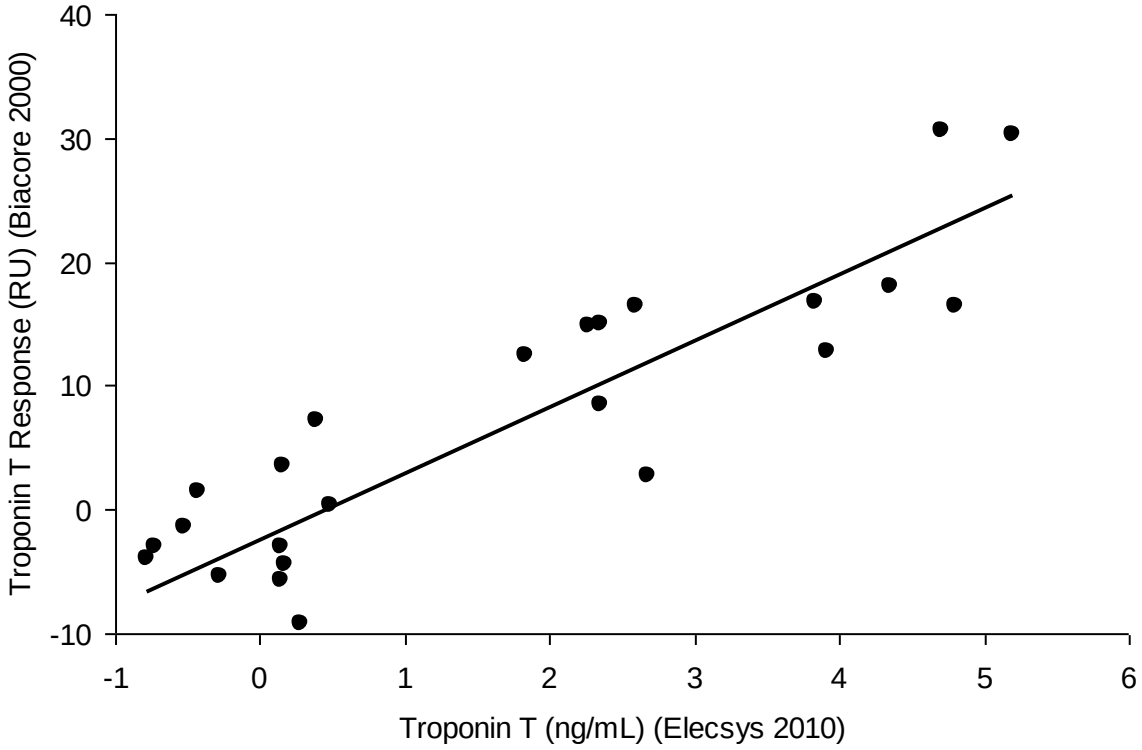

40
30
20
10
0
-10
Response (RU)
0,001
0,01
0,1
1
10
100
1000
Methotrexate (µM)

Fig. 8.


Troponin T Response (RU) (Biacore 2000)
40
30
20
10
0
-10
-1
0
1
2
3
4
5
6
Troponin T (ng/mL) (Elecsys 2010)