Cardiac troponin I: a case study in rational antibody design for human diagnostics

P.J.Conroy¹, R.J.O’Kennedy and S.Hearty¹†

Biomedical Diagnostics Institute, National Centre for Sensor Research and School of Biotechnology, Dublin City University, Dublin 9, Ireland

¹To whom correspondence should be addressed.
E-mail: Stephen.hearty@dcu.ie

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In vitro diagnostic (IVD) platforms provide rapid and accurate determination of disease status. The clinical performance of antibody-based diagnostic platforms is paramount as the information provided often informs the medical intervention taken and, ultimately, the patient’s outcome. Breaking down such an immuno-IVD device into its component elements, the biorecognition entity is key to the analytical specificity of the test. Furthermore, tailored optimisation of the antibody is often necessary to impart the desired biophysical properties for the specific application. This tailoring is now widely facilitated by advances in combinatorial approaches to antibody generation, molecular evolution strategies and the availability of truly high-throughput (HT), refined surface plasmon resonance-based screening tools. In this paper, we demonstrate a rational, knowledge-driven approach to the generation of epitope-specific antibodies for the early detection of cardiovascular disease, discuss the merits of the approaches taken and offer a perspective on HT strategies to mining large antibody libraries. These results highlight the expedience of such methodologies for the development of truly superior cardiovascular disease biorecognition elements.

Keywords: cardiac troponin I/high-throughput screening/phage display/recombinant antibody/surface plasmon resonance

Introduction

Cardiovascular disease is the greatest cause of mortality in the western world and costs the EU economy €192 million per year (Allender et al., 2008). In the continuum of acute coronary syndrome (ACS), cardiac biomarkers now play a key role in the diagnosis, prognosis and risk stratification of patients (Vittorini and Clerico, 2008; McDonnell et al., 2009). The cardiac troponins (cTn) are part of the regulatory complex involved in cardiac striated muscle contraction and consist of Troponin I (cTnI), C (TnC) and T (cTnT) subunits. cTnI is the current gold standard biochemical marker for definitive diagnosis of acute myocardial infarction (AMI), triage ranking, due to its high negative predictive value (NPV), and patient prognosis (Eriksson et al., 2006; Melanson et al., 2007; McDonnell et al., 2009).

Current new-generation cTnI assays have limits of detections in the 10–40 pg/ml range and these are typically laboratory-based automated platforms (Tate, 2008; McDonnell et al., 2009). Point-of-care (POC) provides a near-patient approach to clinical diagnosis and in modern management of ACS the time from sample to decision is crucial (Amadio et al., 2010). These devices provide simple, high-throughput (HT) analysis in small bench top or hand-held instruments. The latter integrates the key analytical steps through ‘state-of-the-art’ micro-fabrication approaches. A number of POC systems exist, typically for creatine kinase, myoglobin and troponin measurement based on the lateral flow immunoassay concept, and have demonstrated potential benefits in emergency departments (McDonnell et al., 2009). The use of point-of-care testing (POCT) potentially reduces the patient turnaround time, hospital stay times and, most importantly, impacts on the patient’s outcome. POCT has met with mixed opinions where proponents argue for reduced testing time and operator input/training, whereas opponents point to questions of accuracy (McDonnell et al., 2009). The use of such devices requires improved analytical sensitivity to detect the lower clinically relevant cTn concentrations (Tate, 2008). Research into increasingly sophisticated POC platforms potentially permits the development of more advanced systems using novel signal transduction platforms (Conroy et al., 2009), modified surfaces, microfluidics (Dimov et al., 2011) and detection systems (Kurzbuch et al., 2008; Hill et al., 2011). The trend towards miniaturisation (nano- and micro-) complicates the process in terms of the ancillary components required but also introduces challenges for the type and quality of antibody developed for such applications.

The current emphasis on epitope specific targeting of cTnI relies on multiple capture and reporter combinations to accurately detect the analyte. Owing to the complexity of the cTnI protein, commercial assays employ sandwich-type formats and are dominated by the use of monoclonal and polyclonal antibody reagents (Panteghini et al., 2001). In most cases this permits the detection of many of the various forms of cTnI and the cTn complex but not necessarily with the same sensitivity leading to overestimation or underestimation of cTn concentration. The massive sensitivity improvements over the last number of years, moving towards high-sensitivity assays, have come about by refinement of assay steps; increasing incubation times, enrichment steps (e.g. using magnetic beads) and signal amplification advances (Tate, 2008). However, improving antibody sensitivity, affinity and modularity have received little attention. At present, no commercial recombinant antibody-based cTnI assays are available.

The amino acid composition of cTnI (Fig. 1) illustrates the well-established areas of importance for the generation of
epitope-specific antibodies. The first generation of cTnI assays were compromised due to several non-specific issues resulting from the presence of secondary conditions for the patient, pre-analytical factors and non-specific interference (Panteghini, 2001; Eriksson et al., 2005; Eriksson et al., 2006; Melanson et al., 2007; McDonnell et al., 2009). During AMI, cTnI is represented in a number of diverse forms, of which the binary complex of TnC–cTnI is the predominant circulating form with minor forms of the tertiary complex (TnC–cTnI–cTnT) and free cTnI. The heterogeneity of the circulating population of cTnI is further complicated by the complex interplay present challenges to analytical detection. In this study, we investigated the generation of highly specific, high-affinity antibodies, for improved cTnI diagnostics in the critical N-terminal, cardiac-specific region (amino acid sequences 1 and 2). The challenges surrounding cTnI in a clinical diagnostic setting have existed for many years and due to the complexity of the protein, current emphasis focuses on specific epitope targeting. We successfully employed synthetic peptide immunogens to guide avian and murine responses to the target regions, isolating cTnI-specific antibodies by downstream protein screening regimes. Traditional hybridoma and recombinant antibodies were isolated in conjunction with support from HT surface plasmon resonance (SPR)-based screening.

**Materials and methods**

Human cTnI antigen (catalogue #1210) was purchased from Life Diagnostics (USA). Biacore™ chips (CM5—Research Grade) and other Biacore™-related reagents used acquired from GE Healthcare (Sweden). Cell culture reagents were obtained from Fisher Scientific (Ireland) and general reagents, of the highest available grade, were purchased from Sigma–Aldrich (Ireland). Consumables were DNase and RNase ‘free’ for all molecular components of this work and research (Fiorentini et al., 2008; El-Awady et al., 2010; Mahajan et al., 2010). However, the main challenge that remains is the isolation of native epitope-binding antibodies (i.e. on the native protein) from a heterogeneous population of synthetic peptide-binding clones (Niman et al., 1983; Hearty and O’Kennedy, 2011) derived from a peptide-stimulated immune antibody library and especially if selection is carried out entirely on a synthetic form as opposed to the native protein. Binding to the native epitope will be dictated by cumulative, defined contextuality.

In this study, we investigated the generation of highly specific, high-affinity antibodies, for improved cTnI diagnostics in the critical N-terminal, cardiac-specific region (amino acid sequences 1 and 2). The challenges surrounding cTnI in a clinical diagnostic setting have existed for many years and due to the complexity of the protein, current emphasis focuses on specific epitope targeting. We successfully employed synthetic peptide immunogens to guide avian and murine responses to the target regions, isolating cTnI-specific antibodies by downstream protein screening regimes. Traditional hybridoma and recombinant antibodies were isolated in conjunction with support from HT surface plasmon resonance (SPR)-based screening.

**Fig. 1.** Amino acid composition of cTnI illustrating the various sources of interference from its numerous interactions with other Tn subunits, non-specific interference and pre-analytical factors. Contributory pre-analytical factors include the use of anti-coagulants in the collected blood sample, where additives including; heparin (charge association), ethylenediaminetetraacetic acid (disrupts TnC association) and the presence of fibrin strands (incomplete clotting) lead to erroneous cTnI measurements. Non-specific interferences relate to auto-antibodies, human anti-mouse antibodies (HAMA), masked epitopes, rheumatoid factor (RF), heterophilic antibodies, activated complement components and the structural heterogeneity of the circulating cTnI population. The protein can also undergo modifications such as oxidation, reduction and phosphorylation and the C- and N-terminal regions are susceptible to truncation. Combined, these modifications result in considerable cTnI heterogeneity and the complex interplay present challenges to analytical detection. In this study, the development of anti-peptide 1 and 2 antibodies, as key reagents for cardio-specificity, is addressed. This figure was adapted from Eriksson et al. (2006).
were purchased from Fisher Scientific (Ireland) and Cruinn Diagnostics (Ireland), unless otherwise stated. Commercial control cTnI-specific antibodies (mAb 19C7 and 228) were sourced from Hystex Ltd. (Finland). All absorbance measurements were carried out using a Tecan Sapphire™ or Sunrise™ absorbance reader.

Immunisation and fusion
Six-week-old female Balb/c mice (Harlan UK Ltd., UK) were immunised by intraperitoneal injection of 200 μl 50 μg peptide-2-keyhole limpet haemocyanin (KLH) conjugate in phosphate-buffered saline (PBS) emulsified 1:1 with Freund’s complete adjuvant (FCA) and boosted with 25 μg peptide-2-KLH in PBS emulsified in Freund’s incomplete adjuvant (FICA) until a suitable titre was observed. The mice were sacrificed by cervical dislocation and the spleen removed. The fusion of the B-lymphocytes with mouse spleen cells was harvested, resuspended in Dulbecco’s modified Eagle’s medium (DMEM) lacking fetal calf serum (FCS), counted and fused with the myeloma cells at a ratio of 6:5:1 in the presence of 50% (w/v) PEG. Hybridomas were selectively cultured in DMEM containing 5% (v/v) FCS, 5% (v/v) Briclone (Archport, Dublin, Ireland) and HAT supplement.

Hybridoma screening
Direct-binding enzyme-linked immunosorbent assay. cTnI (1 μg/ml in PBS) was coated onto a Nunc™ enzyme-linked immunosorbent assay (ELISA) plate and blocked with 5% (w/v) semi-skimmed milk in PBS (PBSM). Crude supernatants were diluted 1:1 in 1% (w/v) PBSM with 0.02% (v/v) semi-skimmed milk in PBS (PBSM). Crude supernatants were diluted 1:1 with Freund’s complete adjuvant (FCA) and boosted with 25 μg peptide-2-KLH in PBS emulsified in Freund’s incomplete adjuvant (FICA) until a suitable titre was observed. The mice were sacrificed by cervical dislocation and the spleen removed. The fusion of the B-lymphocytes with the immortalised mouse were harvested, resuspended in Dulbecco’s modified Eagle’s medium (DMEM) lacking fetal calf serum (FCS), counted and fused with the myeloma cells at a ratio of 6:5:1 in the presence of 50% (w/v) PEG. Hybridomas were selectively cultured in DMEM containing 5% (v/v) FCS, 5% (v/v) Briclone (Archport, Dublin, Ireland) and HAT supplement.

Epitope region specificity mapping on Biacore™ 4000. A CM5 chip (research grade) surface was modified with neutrAvidin® (Pierce, USA) by EDC–NHS coupling chemistry. Individual spots were modified within each of the four flow cells with an excess (10 μM) of biotinylated cTnI peptides (spot 1: peptide 1, spot 2: peptide 2, spot 4: peptide 3 and spot 5: peptide 4) using the hydrodynamic addressing functionality of the Biacore™ 4000 instrument. The crude hybridoma supernatants were diluted 1:5 in Hepes-buffered saline containing 0.05% (v/v) P20 surfactant (1X HBS-EP+) and injected across the immobilised peptides at 30 μl/min.

Construction of the anti-peptide-1 phage displayed library
The anti-cTnI peptide-1 single-chain fragment variable library (scFv) was constructed as described by Andris-Widhopf et al. (2000). In brief, an adult leghorn chicken was immunised with 100 μg of peptide-1-KLH in PBS emulsified 1:1 with FCA and boosted with 50 μg of peptide-1-KLH in PBS emulsified 1:1 with FICA. The chicken was sacrificed once a strong antiserum titre was observed. DNA was extracted from spleen and bone marrow of the animal and complementary DNA was synthesised using a SuperScript III kit (Invitrogen, USA). Antibody variable genes were amplified from the cDNA using primers published by Andris-Widhopf et al. (2000). The variable genes were assembled via a glycine–serine linker and cloned into the pComb3XSS phagemid vector. The cloned antibody library was subsequently transformed into electrocompetent Escherichia coli XL-1 Blue cells (Stratagene, USA) and displayed on filamentous phage.

Selection of anti-cTnI peptide-1-specific scFVs
The transformed library was subjected to four sequential rounds of bio-panning using mAb 20B3 to present cTnI to the library. The library was first depleted of non-specific 20B3-binding scFv by incubation for 1 h at room temperature (RT) while rolling in an immunotube coated with 50 μg/ml 20B3 mAb in PBS. For bio-panning, a fixed concentration of 20B3 mAb (50 μg/ml) was applied to the wells of a microtitre plate. Varying amounts of cTnI antigen (75 μg/ml over eight wells followed by 75, 50 and 25 μg/ml over four wells) were captured for presentation to the phage-displayed library with fixed washing per round. The rescued phage library was resuspended in 1% (w/v) KLH in PBS with 0.02% (w/v) NaN₃ and 100 μl/well of the preparation applied to the captured antigen. After incubation for 1 h at RT, shaking at ~150 rpm, the wells were washed with 200 μl × 5 PBSTM and 200 μl × 5 PBS. After bio-panning, the cTnI-bound phage population was recovered by trypsin elution (10 mg/ml PBS) and incubation for 30 min at 37°C. The eluted population was infected into E. coli XL-1 Blue cells (Stratagene, USA), and the expression of phage–scFv induced by co-infection with VCSM13 helper-phage (Stratagene, USA).

Soluble expression
The output phage round corresponding to enrichment of specific scFv–harbouring phage was identified by polyclonal phage ELISA. This was prepared for soluble expression by infecting a 10 μl sample into the non-suppressor strain E. coli TOP10F (Invitrogen, USA) which were then plated on Luria–Bertani agar plates at 37°C overnight. A total of 300 clones were randomly selected and grown in 96-well plates containing super broth supplemented with 100 μg/ml carbenicillin and 1X 505 supplement (50% glyceral (w/v), 5% glucose (w/v)). The cultures were induced overnight with 0.5 mM isopropyl-β-d-galactopyranoside, and whole cell suspensions were lysed by three successive freeze–thaw cycles. The cell debris was removed by centrifugation of the plates at 4000 rpm for 30 min at 4°C separating the ‘scFv-rich’ supernatant.

Screening of the selected WT clones
Sandwich ELISA screening. The mAb 20B3 (1 μg/ml) was coated onto an ELISA plate in triplicate, stored at 4°C overnight followed by blocking with 5% (w/v) PBSM. Three different concentrations of cTnI (36, 6 and 0 nM) in 1% (w/v) PBSTM were applied to separate plates. The soluble expressed scFvs were diluted 1:3 in 1% (w/v) PBSTM and 100 μl/well applied over the three plates. After incubation for 1 h at 37°C and washing (PBST × 3 and PBS × 3) a
1:2000 dilution of an anti-HA HRP-labelled secondary antibody (Roche, USA) was applied to the plate. The plate was incubated and washed as above followed by addition of 100 µl/well substrate (Insight Biotech), quenching with 10% (v/v) HCl and absorbance measurement at 450 nm.

**Biacore™ 4000-based screening.** A CM5 (research grade, GE Healthcare) chip was functionalised with ~6000 RU of anti-HA epitope pAb (Thermo Fisher, USA) by EDC-NHS coupling chemistry over spots 1, 2, 4 and 5. ScFv-enriched lysates were diluted 1:5 in 1X HBS-EP⁺ (GE Healthcare), housed in temperature-controlled racks (10°C) and analysed in a capture approach over the anti-HA surface at 25°C. The diluted lysates were injected over the spots 1 and 5 of each flow cell at a flow rate of 10 µl/min for 2 min. cTnI (25 nM in 1X HBS-EP⁺) was then passed over the entire flow cell at 30 µl/min, data collected at 1 Hz/s and reference subtracted online by spots 2 and 4. The surface was regenerated with a 30 s pulse of 20 mM NaOH in between cycles. The data were evaluated using the dedicated BiaEvaluation™ software.

**Light-chain shuffling of selected WT clone**

The heavy-chain region of the selected scFv (180) was amplified by polymerase chain reaction using the same primers that were used to construct the initial V₄₇ fragments from the cDNA. The light chain was amplified from the cDNA as for the WT library generation. The mutant (MT) library was constructed and subjected to bio-panning ‘in-solution’.

**Selection of improved anti-cTnI peptide-1-specific MT scFvs**

The rescued MT library was first depleted of non-specific binders by incubation with 20 µl streptavidin-coated paramagnetic beads (NEB, USA) for 1 h at RT with shaking (150 rpm). The depleted library (150 µl) was incubated with biotinylated peptide-1 concentration for each round of panning (50 µl of 4X required concentration) shaking (150 rpm) for 45 min at RT. Streptavidin-coated beads (30 µl washed × 5 with PBST) were added to the biotinylated peptide phage mixture and incubated for a further 15 min at RT while shaking (150 rpm). The bead-peptide-antibody complexes were recovered by application of a magnet and washed (500 µl/wash of PBST × 5 and 500 µl/wash of PBS × 5) followed by elution with 500 µl of 10 mg/ml trypsin in PBS. The eluted phage population was infected into E. coli XL-1 Blue cells, and the expression of phage-scFv induced by co-infection with VCSM13 helper-phage (Stratagene, USA). The number of washes per round remained constant with gradual reduction in the available free peptide antigen in the range 10, 1, 0.5 and 0.1 nM.

**Screening of the selected mutated library**

The selected MTs were screened using SPR (Biacore™ 4000; GE Healthcare) and the chip surface (CM5 research grade) was prepared as described in ‘screening of the selected WT clones’. In this case, the inclusion of a zero concentration of cTnI allowed for the acquisition of double-reference subtracted kinetic data. Initially, the clones were differentiated by percentage left (% left) analysis in comparison with the WT in the capture screening approach and in ‘2 over 2’ kinetics. For the ‘2 over 2’ analysis, a differential density of anti-HA pAb was created during immobilisation taking advantage of the lag time between activation and contact of the protein on the inner spots. The ‘scFv-rich’ lysates were diluted (1:10 in 1X HBS-EP⁺) and scFvs were captured (10 µl/min flow rate and contact time of 2 min) followed by application of two cTnI concentrations (25 and 75 nM) in addition to a zero antigen concentration (30 µl/min flow rate, 2 min contact time followed by 10 min dissociation). The data were evaluated using the dedicated BiaEvaluation™ software.

**Comparison of WT, MT and Hytest equivalent antibodies**

The optimised scFv and mAb dilutions were obtained by titration and selecting the dilution corresponding to the midpoint of the curve. Double-strength dilutions (i.e. 2-fold midpoint dilution from the titration) were prepared and further diluted 1:1 with a free cTnI concentration range (200 to 0.39 nM, including zero cTnI in 1% (w/v) PBSTM) and incubated at 37°C for 1 h in 1.5 ml tubes. The inhibition mix (100 µl) was then applied to a plate coated with cTnI (0.5 µg/ml in PBS). After 1 h of incubation at 37°C the plate was washed, the secondary antibody applied and the plate absorbance read at 450 nm. Each clone A₀ (absorbance at zero cTnI) was divided into the response at each free cTnI concentration resulting in an inhibition curve.

**Results**

The antibody generation campaigns for epitope regions 1 and 2 were undertaken based on the differential serum responses to the peptide antigens. In the case of peptide-1-KLH, immunisations in a chicken yielded a cTnI-specific response, but this was not found in mouse and the opposite trend was seen with peptide-2-KLH (by ELISA, Supplementary Figure S1). Strong native protein (cTnI)-binding responses were observed in both synthetic peptide immunisation regimes and facilitated subsequent fusion (region 2) and library building (region 1) experiments.

**Hybridoma screening**

To ensure isolation of an optimal anti-cTnI epitope region 2 monoclonal antibody, a ‘data-rich’ ranking approach was adopted using ‘steady-state’ and ‘flow-based’ analyses. Individual wells (1170) were screened from the fusion progeny by direct binding ELISA to ensure specificity for the peptide, albeit in the context of the adsorbed native protein, which can itself be non-ideal (Spangler, 1991). By applying an arbitrary ‘cut-off’ limit of 0.4AU, 112 individual positive wells were identified for scale-up (Fig. 2A). Epitope-region specificity was confirmed by mapping experiments using SPR, discounting issues with reactivity against the other cTnI synthetic peptides and linker chemistries (Fig. 2B) and the positive monoclonal antibodies were found to exclusively react with the synthetic peptide 2. In such steady-state analysis, the signals obtained are due to unknown combinations of intrinsic affinity and the relative abundance of the secreted antibody in the supernatant (Harriman et al., 2009) which provides little in the way of information regarding kinetic parameters (‘on-’ or ‘off-rate’). Accordingly, ‘off-rate’ ranking was carried out against the peptide 2 surface by SPR. The stability of the interactions...
for the top 24 performing antibodies (Fig. 2C) were categorised by % left and four (Supplementary Table S2) selected, with values >90% left, for subsequent expansion. After scale-up and cloning by limiting dilution, the analysis was modified to include capture ranking by SPR (Biacore™ 4000) using a panel identified from the ‘off-rate’ ranking experiments. An anti-mouse Fc-specific surface captured the antibodies from crude supernatants followed by monitoring of the association and dissociation of cTnI. Figure 2D illustrates the cTnI-binding profile for 19 clones which demonstrates the binding of the protein in a solution-phase context more representative of the scenario in which the mAb will be deployed in a clinical IVD setting. In addition, the profiles compared favourably with the equivalent mAb 228 (Hytest, Finland) where % left values were calculated from the dissociation phase by comparing a time-point early in the dissociation to a late time-point (Supplementary Table S3). Of note, the difference in ‘% left’ values in this approach, using the native protein, was substantial compared with the Biacore™ 4000-based ranking against the synthetic peptide. This highlights the importance of integrating a number of screening approaches to ensure success during hybridoma selection experiments. The clone 20B3 was ultimately selected (IgG1) and kinetic analysis (Biacore™ 3000) using the 1:1 Langmuir binding with drifting baseline model gave an affinity (equilibrium dissociation constant, $K_D$) of 1.84 nM (Supplementary Figure S2).

Recombinant antibody development

The wild-type (WT) scFv library was constructed, as described by Andris-Widhopf et al. (2000), from cDNA reverse-transcribed from avian mRNA (isolated from chicken immunised with peptide-1-KLH) and transformed into E. coli creating a library of $9 \times 10^8$ members. The phage-displayed library was bio-panned in a selection methodology designed to ensure the isolation of a scFv panel for compatible use in a sandwich immunoassay with the 20B3 mAb. This was achieved within four rounds of selection using cTnI which was first captured by surface-adsorbed 20B3 mAb to present the antigen to the scFv library in a native assay representative context. Employing this selection methodology, with due consideration for the isolated antibodies intended application, obviated the requirement to mine for suitable assay pairings post-bio-panning and removed the need to reselect or rescreen. After four rounds of bio-panning, the phage population was assessed initially for specific enrichment by polyclonal phage ELISA (Supplementary Figure S3) and diversity by single colony (18 clones) restriction digest (Supplementary Figure S4). Three hundred individual colonies were subsequently screened in a combination of sandwich ELISA and HT capture approach (% left ranking) using SPR. The sandwich ELISA-based analysis challenged the scFv against 6-fold differing cTnI concentrations, expressed as a ratio, as adapted from the time-resolved fluorescence-
based ranking of hybridomas by Daigo et al. (2006). This type of analysis was valuable as quantitative kinetic analysis of antibodies is arduous due to the variant antibody concentrations in each lysate preparation. In this case, higher ratios were attributed to increased affinity (Supplementary Figure S5). This analysis was subsequently paired with an evaluation of the ‘off-rate’ of cTnI from the captured scFvs by HT-SPR. The capture approach (Fig. 3A) typically allowed for 300 clones to be analysed in 12 h. Three distinct scFv populations were identified from a plot of ‘stability early’ versus ‘stability late’ (Fig. 3B). Post-panning, the library retained some genetic diversity but after specific screening a panel of clones (four) revealed a narrow consensus sequence (data not shown), which was not surprising given the extremely focused screening criteria applied, and the WT clone 180 was selected for further analysis.

To ensure clinical sensitivity, the affinity of the antibody was a crucial factor and a chain shuffle mode of mutagenesis was undertaken to effect in vitro affinity maturation. The WT V_{H,180} was light chain shuffled against the catalogue of V_{L} from the original cDNA amplification. In the case of the constructed MT library (3.1 × 10^8 members), to improve the statistical probability of isolating high affinity V_{L}–V_{H} combinations, solution-phase bio-panning was chosen using biotinylated synthetic peptide 1. After bio-panning (four rounds) the output (Supplementary Figure S6) was screened for improved MTs using a capture-based and ‘2 over 2’ kinetic configuration. In the capture approach, the MTs were assessed by ‘% left’ ranking in comparison to the WT_180. A dramatic improvement in stability of the binding event was observed (Fig. 4A), towards 70–94% compared with the WT_180 (63%). From the same data, preliminary kinetic constants were assigned to the clones in a HT manner. Each clone was analysed using a single antigen concentration plus a zero control (Fig. 4B). The ‘on/off rate’ map of the 192 MT clones and the WT_180 clone with the affinity graduations graphically represented provided an insight into the apparent k_{d}/k_{a} (Fig. 4C). With little variance in k_{a} a dramatic ‘slowing’ of the k_{d} was apparent. Given that affinity (K_{D}) = k_{d}/k_{a}, this dramatic improvement in k_{d} was advantageous, giving rise to a sharp improvement in the affinity values. To further increase confidence in the results but retaining a significant number of clones to analyse (15 plus the WT_180) a refined ‘2 over 2’ kinetic analysis facilitated the determination of K_{D} using two scFv capture densities with two antigen concentrations plus a zero. Using the concept of ‘2 over 2’ (Fig. 5A) representative curves for the WT_180 and a selected MT_2B12 (Fig. 5B) illustrates the MTs dramatic improvement in k_{d} and affinities in excess of the WT_180. However, the magnitude of improvement (K_{D} values moving towards 10^{-12}–10^{-15} M) approached instrument limitations.

**Fig. 3.** Screening for wild-type scFv. (A) Capture screening: the surface was composed of a polyclonal anti-HA antibody on spots 1, 2, 4 and 5 of each flow cell (FC 1–4). The outer spots (1 and 5) were the active spots where recombinant antibodies were captured from crude bacterial lysates (two scFv per FC with four FC on the system = 8 scFv per cycle). A fixed antigen concentration was then passed over the captured scFvs with the inner spots (2 and 4) acting as online references. (B) HT ‘stability early’ versus ‘stability late’ profile: plotting ‘stability early’ versus ‘stability late’ values for each of the 300 clones illustrates the overall stability of the binding event. From the graph, three distinct populations were identified—red circle: lower expressing clones with % left values ≥ 60, green circle: higher expressing clones with % left values ≥ 60 and non-circled: those clones with % left values < 60. The blue diagonal line represents ideal, irreversible binding where no dissociation occurs and therefore the % left value is 100%.
or underlying antigen quality issues contributed to the unexpectedly stable binding profile. Indeed in full kinetic analysis the ‘flatness’ of the dissociation phase preventing reliable fitting of the kinetic constants (data not shown).

Thus, to further validate the affinity improvement, the top performing MT (MT_2B12) and the WT_180 were compared by half maximal inhibitory concentration (IC₅₀) value which is the effective antigen concentration required to decrease antibody binding by 50% at equilibrium. IC₅₀ values were obtained from three independent analyses (in duplicate) of an optimised inhibition ELISA, including the equivalent monoclonal antibody (19C7). 19C7 had a mean IC₅₀ of 1883 ± 340 ng/ml, the WT_180 an IC₅₀ of 583 ± 104 ng/ml and the MT_2B12 an IC₅₀ of 210 ± 17 ng/ml. The analysis shows the clear superiority of the recombinant antibodies generated in the course of this work compared with the monoclonal (3.2- and 9.0-fold, respectively) and the MT_2B12 clone showed additional improvement over the WT_180 (2.8-fold).

Discussion
The ubiquitous deployment of antibodies on IVD platforms attests their significant utility for a plethora of analytes relating to a panoply of indicators, not least in the clinical diagnostics arena (Hearty and O’Kennedy, 2011). A wide range of antibodies are available for diagnostically relevant human targets which consistently fail to perform optimally on a diagnostic platform, due to inconsistencies in their validation and poor performance in their intended application which we contend is obstinately due to the fact that selection is not suitably representative of the endpoint application. In fact, the issue of antibody validation is a cause of considerable consternation among researchers (Bordeaux et al., 2010; Colwill and Graslund, 2011). The antibody development approaches described here sought to meet the current need for highly specific, high-affinity and modular antibodies for application to human diagnostic platforms.

Epitope specificity was paramount and was the driving force behind the focused immunisation strategies undertaken. The differential responses found in both chicken and mouse to the synthetic peptides dictated the antibody generation approaches undertaken (combinatorial approach and hybridoma, respectively). The difference in animal response aside, the validity of the peptide-based immunisation regimes is clear as in both cases anti-peptide antibodies capable of specific recognition of the native protein were generated successfully.

Hybridoma technology was exploited to successfully isolate an anti-epitope-2 region-specific antibody from a murine repertoire. HT-binding interaction analysis proved to
be an efficient means of assessing the binding characteristics of the secreted hybridoma-derived antibodies and are acknowledged to provide valuable information to support the antibody selection processes, e.g. on SPR-based instruments (Safsten et al., 2006) or microarrays (Moller et al., 2008; Rieger et al., 2009). In parallel, the screening process must be designed to closely mimic the eventual analytical format that will be used to ensure the antibody’s effectiveness in that format. From the initial fusion, 9.57% of wells were confirmed to exhibit native protein epitope binding. To systematically evaluate each clone in a judicious manner, a multi-platform analysis regime was employed with the initial focus on epitope region (on Biacore™ 4000) and protein specificity (by ELISA and Biacore™ 3000). The ability to rapidly acquire information dramatically increased the chances of success, minimising sources of error not related to chromosome loss or competition by non-secreting cells. This ‘data-rich’ approach directly facilitated the successful isolation of a panel of antibodies for the cardiac-specific N-terminal epitope-2 region of cTnI. Although in theory the immune system has the ability to continually evolve its antibodies far below single digit nanomolar affinity, does not frequently manifest in vivo (Hearty and O’Kennedy, 2011) and gives credence to the concept of an affinity ceiling (Foote and Eisen, 1995; Batista and Neuberger, 2000). Therefore, accessing the true affinity potential of the natural immune repertoire in vitro using the well-established B-cell immortalisation techniques is relatively inefficient and the generation truly high-affinity reagents is inherently difficult (Hearty and O’Kennedy, 2011). Combinatorial molecular biology permits access to this true affinity potential in practically any species unlike traditional hybridoma technology which is typically, though not exclusively, limited to rodents and rabbits. This was borne-out in the course of this research where the monoclonal antibody generated exhibited a classical single digit nanomolar affinity with the combinatorial approach generating sub-nanomolar affinity antibodies. Combinatorial libraries can generate diversities which far exceed the natural repertoire as the combinations of heavy and light chains brought together are truly stocastic (Hearty and O’Kennedy, 2011). In such a case, an artificial representation of the repertoire is created, expanded by randomising the combinations and subsequently recovering the functional entity. It becomes much less of a question of isolation of an antibody that binds a particular antigen, but rather a superlative antibody which performs efficiently in its desired application. It is now possible to readily access diversities far exceeding natural immunity (Lerner, 2006) and to rationally focus the library specificity using knowledge-driven selection regimes and refined HT-screening methodologies.

Fig. 5. Analysis of wild-type and mutant scFv by ‘2 over 2’ kinetic evaluation. (A) ‘2 over 2’ kinetics: the surface was again composed of a polyclonal anti-HA antibody on spots 1, 2, 4 and 5 of each flow cell (FC 1–4). In this case, two scFvs were captured (one on spots 1 and 2 with a second on spots 4 and 5) at two different densities (high density on spots 1 and 5 with low density on spots 2 and 4). Two cTnI concentrations (25 and 75 nM in 1X HBS-EP+) and a zero concentration were then passed over the two capture densities and referenced against the unmodified surface on spot 3. The two densities of each clone interacting with three cTnI concentrations formed the basis of kinetic constant determination using the 1:1 Langmuir binding model. Representative curves from ‘2 over 2’ experiment: scFv_180 (B) and scFv_2B12 (C) with the high (solid boxes) and low (dashed boxes) density scFv capture levels for two cTnI concentrations (the data for the 25nM sample was deemed of insufficient quality and was omitted from global fitting). The curves (blue) for each scFv are fitted with a kinetic model (1:1 Langmuir binding model) using a global fit (black).
Phage display (Rader and Barbas, 1997; Kehoe and Kay, 2005) has become the ‘work-horse’ of antibody isolation due to its robust nature and growing accessibility. Antibody isolation can be tailored as demonstrated by the numerous successful applications of phage display to selection campaigns (Conroy et al., 2009). However, there are multiple display technologies available including ribosome display (Hoogenboom, 2005; Dufner et al., 2006) and yeast display (Gai and Wittrup, 2007), which have particular merit in the isolation of very-high-affinity binders. Tailoring selections towards ‘on-rate’ is difficult due to a number of factors such as antigen/antibody size, charge and diffusion which is particularly true for equilibrium interactions. Selections therefore typically assess ‘off-rate’ as a convenient metric to differentiate the pool of recombinant antibodies within the library (Zahnd et al., 2010; Hearty and O’Kennedy, 2011). In the case of the anti-peptide-1 scFv, a library of $9 \times 10^8$ members was created and bio-panned by a stringent solid-phase capture approach using the isolated monoclonal antibody, 20B3, to extract compatible antibody pairings. To mitigate against the limitations of diffusion rates and for optimal performance at the temperatures envisaged for the assay, the selections were carried out at RT and with orbital shaking of the plate. Differentiation of the most promising lead candidates from a background of multiple affinities is crucial. To do so guided by measurements from direct-binding ELISA alone provides no means to evaluate the clones by quantitative kinetics. In this case, we opted for a differential antigen concentration sandwich assay and apparent ‘off-rate’ ranking on the Biacore™ 4000. In the case of the sandwich ELISA, it served two functions: to identify optimal pairings with the monoclonal antibody and to discriminate higher-affinity antibodies based on the hypothesised ranking format demonstrated by Daigo et al. (2006). When combined with apparent ‘off-rate’ ranking data the results obtained from the ELISA appeared to be unaffected by relative antibody concentration in the lysate as both low and high expressers, identified from their respective positions on the ‘off-rate’ map, had similar ratios. The value of HT-SPR-based instrumentation as a favoured technology for monitoring molecular interactions is due to the fact that the interactions can be monitored in ‘real-time’ without ancillary labels. Such information was acquired in analyses of $\sim 12$ h for 300 clones with relatively conservative use of antigen. This campaign attests the significant power of the combinatorial library approach combined with focused selection which was designed to take large library diversities to a very narrow population or to a consensus (Hearty and O’Kennedy, 2011), as in this case.

The primary determinant of immunoassay specificity and sensitivity is the antibody. The issue of specificity was well defined early in the antibody generation process and to ensure optimal sensitivity, the affinity of the antibody was of crucial importance. Many modes of mutagenesis are available, broadly divided into random and site-directed strategies (Dufner et al., 2006). It is known from crystal structure determination and for genetic reasons that the $V_H$ dominates antibody-binding interactions and thus a light chain shuffle was a valid method to improve the antibody-binding energies (Lerner, 2006). A secondary library based on the WT_180 $V_H$ contained many combinations of light chains from the original chicken immune $V_L$ repertoire, in essence the libraries overlap greatly, and the solution-phase selection strategy was undertaken to isolate higher-affinity $V_L$–$V_H$ combinations. The true power of SPR-based HT-screening tools was exemplified in the screening of the mutated library. Inclusion of zero antigen concentrations for each clone allowed estimation of ‘one shot’ apparent $k_d$ and $k_d$ values providing insights into affinity. Use of the WT_180 as a benchmark throughout the analysis allowed clear differentiations to be drawn in a parallel comparison with the MT lead candidates in qualitative analyses for preliminary ranking. The dramatic improvement in apparent ‘off-rate’ was indicative of improved affinity and was further verified for a subset of clones using ‘2 over 2’ kinetics, where two antigen concentrations over two densities of capture provide four curves to assign more accurate assessment of affinity. Critical to the success of the screening campaigns was the rational, well-designed bio-panning experiments tailored to select for the most optimal antibody pairings for cTnl-diagnostic applications. The selected antibodies represent the most desirable outcome from the selection campaigns (sub-nanomolar affinity and epitope region-specificity). The ability to access these high-value and high-affinity reagents was supported by the

<table>
<thead>
<tr>
<th>Avian antibodies</th>
<th>Benefit</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Simplistic Ab gene arrangement</td>
<td>Single primer sets. Efficient sampling of immune repertoire</td>
<td>Michael et al. (1998); Andris-Widhopf et al. (2000); Sayegh et al. (2000); Finlay et al. (2005); Finlay et al. (2006)</td>
</tr>
<tr>
<td>Phylogenetically distant</td>
<td>Respond strongly to mammalian protein antigens</td>
<td>Michael et al. (1998); Finlay et al. (2006); Hof et al. (2008)</td>
</tr>
<tr>
<td>Stable at higher core temperature (41°C)</td>
<td>Stable antibodies (half-life in order of months)</td>
<td>Narat (2003)</td>
</tr>
<tr>
<td>Increased CDRH3 length</td>
<td>Potential for greater diversity</td>
<td>Wu et al. (1993)</td>
</tr>
<tr>
<td>Lacking Fc effector function</td>
<td>Advantageous in diagnostic tests—reducing interference and false positives</td>
<td>Carlander and Larsson (2001); Narat (2003); Greunke et al. (2008)</td>
</tr>
<tr>
<td>Multiple antigen immunisations tolerated</td>
<td>Multiple $V$-gene specificities available from a single animal and reducing costs</td>
<td>Hof et al. (2008)</td>
</tr>
<tr>
<td>High antibody yield in egg</td>
<td>Non-invasive, plentiful source of polyclonal Ab</td>
<td>Narat (2003)</td>
</tr>
<tr>
<td>High ratio of B cells (100x compared with mouse)</td>
<td>Rich source of $V$-gene mRNA, facilitates efficient sampling of $V$-gene repertoire</td>
<td>Michael et al. (1998)</td>
</tr>
<tr>
<td>Not yet used in therapeutics</td>
<td>No HAMA responses—reduction in instances of false positives</td>
<td>Narat (2003)</td>
</tr>
<tr>
<td>Increased levels of antibody produced</td>
<td>Reduction in number of laboratory animals required leading to reduced costs</td>
<td>Narat (2003)</td>
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capability to exhaustively mine large numbers of clones in a HT, assay-relevant manner. During the screening campaigns, multi-parameter data were acquired which was most notably highlighted in the MT-screening campaign.

The use of chickens as diagnostic antibody generation hosts is potentially superior in terms of the antibody bio-physical properties and, accordingly, chicken antibodies have received considerable attention as superior entities for diagnostic applications (Carlander and Larsson, 2001; Narat, 2003; Kovacs-Nolan and Mine, 2004) (Table I). The area of POCT for cTnI is dominated by the use of monoclonal and polyclonal antibodies with no commercially available, approved recombinant examples available. Ylikotila et al. (2006) highlight the utility of recombinant Fab fragments, derived from the commercial (Hytest®) catalogue of mouse monoclonal antibodies, notably for the ability to site-specifically label the fragments. Key parameters for the successful exploitation of antibodies in POC-based applications include sensitivity, selectivity, stability, immobilisation, orientation, labelling and antibody size. The ability to develop more sensitive and robust systems is reliant on the modification of antibodies to improve these cumulative performance characteristics. The capacity to enhance recombinant antibodies with respect to their selection, modification and suitability for subsequent applications is of strategic importance to robust diagnostic biosensor development.

Herein, we have successfully demonstrated the rational development of anti-epitope region-specific antibodies for the cardiac biomarker, cTnI. The research described demonstrates the merit in applying a rational design-based approach to antibody generation and the comprehensive, multi-metric approaches taken to optimising each element of the antibody generation process is unique. The strategy can be applied to any antibody generation campaign and was particularly valid for this complex antigen. We have highlighted the significant advantages of ‘data-rich’ HT-based screening and the methodologies employed generated highly specific and high-affinity reagents for cTnI diagnostic platforms. The major advantage of the selection and screening approach is entirely underpinned by functional parameters. However, it is acknowledged that one area that could contribute further to the rationale of the strategy outlined is that of HT sequencing between the selection and screening phases, as this would eliminate the redundancy without compromising the analysis (Hearty and O’Kennedy, 2011). For the development of a low-cost, highly specific and sensitive POCT for cTnI diagnosis, the issues of affinity and specificity of antibodies have been comprehensively addressed. In an effort to fine tune specificity, targeted immunisation using synthetic epitope mimics is a valuable methodology to focus antibody library potential. Ultimately, the screening strategy employed must be representative of the endpoint application. Accordingly, identifying the optimal antibody and in particular, antibody pairs, benefits hugely from harnessing the selective power phage display and ensuring not just selection, but also screening, is a suitable representative of the endpoint application.

**Supplementary data**

Supplementary data are available at PEDS online.

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**Author Contributions**

S.H. and P.J.C. conceived and designed the experiments. P.J.C. performed the experiments and analysed the data. P.J.C., S.H. and R.O.K. interpreted the data and contributed to the writing of the manuscript.

**References**


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