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A quantitative LC–MS/MS method for comparative analysis of capture-antibody affinity toward protein antigens☆

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ABSTRACT

A mass spectrometry-based antibody selection procedure was developed to evaluate optimal 'capture' monoclonal antibodies that can be used in a variety of analytical measurement applications. The isotope-dilution liquid chromatography-tandem mass spectrometry (ID LC–MS/MS) methodology is based on the use of multiple-reaction monitoring of tryptic peptide fragments derived from protein antigens. A panel of monoclonal antibodies (mAb) was evaluated based on a quantitative determination of relative binding affinity to human cardiac troponin I following immunoprecipitation. Dissociation constants (K_d) were determined for 'bound mAb–antigen' vs. 'unbound antigen' using non-linear regression analysis. Relative quantification of both antigen and antibody was based on the use of stable isotope-labeled synthetic peptides as internal standards. Optimal 'capture' mAbs were determined through evaluation of relative K_d constants of all monitored peptide transitions. A panel of six pre-screened candidate capture mAbs was concluded to consist of two subsets of mAbs, each with statistically equivalent K_d constants as determined using NIST Standard Reference Material (SRM) 2921 – Human Cardiac Troponin Complex. This ID LC–MS/MS method is shown to be capable of quantitatively differentiating mAbs based on relative binding affinities. Selection of optimal capture mAbs can be applied toward a number of analytical applications which require metrological traceability and unbiased quantification.

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1. Introduction

Many quantitative and qualitative measurement procedures utilize an affinity reagent, commonly an antibody, for capture and detection of protein or small molecule targets. Capture antibodies are routinely used in a variety of analytical applications, notably for enzyme-linked immunosorbent assays (ELISAs), protein arrays, surface plasmon resonance (Biacore), immunohistochemistry, immunostaining, microfluidic sensors, and affinity purification, among others. There is often a diversity of commercially available antibodies specific to any given target antigen. For antigens that are large molecules, such as proteins, monoclonal antibodies specific to many distinct regions of the molecule, or polyclonal antibodies with multiple specificities, are commercially available. Subsequently, it can be difficult to choose among antibodies when developing an antibody-based measurement. Monoclonal antibodies (mAbs) are often the preferable capture reagents when high measurement specificity is needed. A key factor in choosing a suitable mAb is its relative affinity to an antigen – a property that depends on non-colavent interactions, specificity and cross-reactivity, antigen stability, and environmental conditions, among many factors. Unfortunately, information about the antigen-antibody binding affinity is generally not provided with commercial antibody preparations. Accordingly, it is necessary to have a tool for objective selection of mAbs during immunoassay development.

Selection of mAbs for an immunoassay is routinely performed through empirical optimization of assay signal using a panel of available antibodies. Yet, empirical approaches provide little information regarding the binding chemistry of the immunoassay. Immunoassay is inherently prone to chemical interferences and other types of measurement bias. Measurement bias in an immunoassay can be difficult to identify as a result of the indirect relationship between what is being measured (a fluorescence or chemiluminescent signal, often from an enzyme reaction) and the measurand of interest. It is difficult to determine if observed measurement bias is due to an impairment of the antigen-antibody binding or impairment to signal generation. An empirical approach to antibody selection does not address bias issues at the antibody selection stage. Despite limitations, antibody-based measurement

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procedures can yield high measurement sensitivity and specificity that cannot often be achieved with other approaches. With their potential for high measurement sensitivity and specificity, antibody-based assays are attractive approaches as reference measurement procedures for measuring low-level analytes in complex sample matrices of standard reference materials at NIST. However, without examination of the chemistry involved, antibody-based measurements lack the metrological rigor that is a necessary component of a reference measurement procedure. The use of LC–MS as a detection technique can effectively limit bias during quantitative measurements of K_d based on three criteria for target specificity – precursor and product ion measurement, and retention time matching.

Detailed affinity characterization is often performed using surface plasmon resonance (SPR) based on Biacore technology [1]. Although SPR offers the advantages of real-time observation of the binding event for the determination of equilibrium constants and on/off rates, this technology suffers from similar problems to other ligand-binding assays, namely the potential for selectivity problems leading to bias and inaccurate quantification. Additionally, it should be noted that SPR has difficulty quantifying molecules with small cross-sections or low solubility, and suffers from poorer detection sensitivity than LC–MS methods [2–4]. A related methodology is the use of high performance affinity chromatography for affinity and rate constant measurement, however, this method also uses indirect measurement of analyte spectral properties [5]. Mass spectrometry-based methods are most suited to our goal of affinity characterization using a *higher order* methodology – something that cannot be provided solely by SPR or related technologies.

Here, we demonstrate an approach to select capture mAbs for a reference measurement procedure based on analytical scrutiny of the chemical interaction steps in a measurement cascade. This approach will be applied to the development of a measurement process that utilizes a mAb to capture a protein analyte along with mass spectrometry for protein quantification. Through the direct measurement of both the antibody and antigen with mass spectrometry in the antibody selection process, the measurement selectivity of an LC–MS/MS approach should help reveal bias that may have remained unseen through a routine selection approach.

Selection of optimal capture mAbs using a mass spectrometry-based approach is demonstrated here for human cardiac troponin I (cTnI). Troponin, a well-studied serum protein complex, is both a sensitive and specific diagnostic marker for heart muscle damage [6–9]. This protein complex, consisting of three regulatory subunits (designated C, T, and I), is integral to muscle contraction and is released from tissue into the bloodstream as a result of damage to cardiac muscle. Routinely, cTn concentrations are measured in serum using commercially available immunoassay kits for patients presenting with chest pains or acute coronary syndrome in order to differentiate stable angina from a suspected myocardial infarction [10]. Additionally, elevated serum cTn concentrations are prognostically important to many of the conditions in which they are used for diagnosis [11]. Due to patent regulations, a single manufacturer produces the antibodies used in cTnT immunoassay kits. No patent exists for cTnI measurement, resulting in many different clinical immunoassay platforms available targeting cTnI. Unfortunately, measurements using cTnI commercial assays suffer from poor standardization, high clinical variability, and poor diagnostic specificity [12,13]. Efforts by the International Federation of Clinical Chemistry's Working Group for the Standardization of Troponin I (IFCC WG-TNI) to standardize clinical cTn measurements using certified reference materials (in the form of cTnI-positive human serum pools) are dependent on the establishment of a reproducible and repeatable reference measurement procedure based on well-characterized measurements of cTnI [14]. While the work presented here evaluates optimal capture mAbs against cardiac troponin I, the comparative approach presented here is significant as a generally applicable mass spectrometry-based methodology for antibody selection. This approach was developed with considerations of being universally applicable for mAb–antigen interactions.

2. Experimental

2.1. Materials

Human cardiac troponin complex was obtained as Standard Reference Material (SRM) 2921 from the National Institute of Standards and Technology (NIST) and is described in detail in a Certificate of Analysis [15]. All other reagents used in this analysis were obtained from commercial sources. Isotopically labeled ^{15}N and/or ^{13}C synthetic peptides of each troponin subunit and all IgG isotypes and their unlabeled analogs were purchased through AnaSpec, Inc. (San Jose, CA) and are detailed in Supplementary Table 1. Human cardiac troponin I monoclonal antibodies were purchased through HyTest Ltd (Turko, Finland). α-GAPDH mAb (used for negative control) was purchased through Calbiochem (San Diego, CA). Dynabead MyOne Tosyl-activated magnetic beads were purchased through Invitrogen Corp. (Oslo, Norway). Rapigest SF surfactant was purchased through Waters Corp. (Milford, MA). Sequencing grade modified porcine trypsin was purchased through Promega (Madison, WI). Bovine serum albumin (BSA), sodium borate, trifluoroacetic acid, Tween-20, Tris, sodium chloride, dithiolthreitol (DTT), iodoacetamide (IAM), sodium azide, and hydrochloric acid (HCl) were purchased through Sigma-Aldrich. High purity LC–MS grade water/formic acid and acetonitrile/formic acid were purchased from Honeywell – Burdick and Jackson.

2.2. Preparation of mAb – magnetic bead conjugates

Each of six α-cTnI monoclonal antibodies and one α-GAPDH mAb (negative control) was separately immobilized to a solid magnetic bead support under optimized conditions according to a modification of the manufacturer's (Dynal) instructions for use. Briefly, mAb–bead conjugates were formed by covalent interaction of activated hydroxyl groups on the bead surface to amino groups located [ideally] within the IgG constant region. Antibodies (approximately 125 μg) were activated for 15 min at room temperature by addition of 0.0025% TFA and neutralized in 0.1 mol/L Na_3BO_3 buffer (pH 9.5). A ratio of 40 μg mAb/mg of Dynabeads was used for optimal coating. 3.12 mg of prewashed (3× in borate buffer) magnetic beads were added to the activated mAbs. Bead concentration during coating was ≈40 mg/mL. mAbs were crosslinked to the magnetic beads with addition of 3 mol/L ammonium sulfate (1 mol/L final concentration, in 0.1 mol/L Na_3BO_3 buffer, pH 9.5). The mixture was rotated end-over-end for 96 h at 25 °C. Next, the supernatant was discarded and the mAb–bead complex was incubated in PBS containing 0.5% (w/v) BSA/0.05% (v/v) Tween-20 for 16 h at 37 °C to block uncoated bead surfaces and limit non-specific binding. Finally, the mAb–bead conjugates were washed 3× in PBS containing 0.1% (w/v) BSA/0.05% Tween 20/0.02% (w/v) sodium azide (preservative) and stored at 4 °C. Each mAb–bead complex was prepared to a final [mAb] of ≈2 μmol/L based on stoichiometric crosslinking.

2.3. Immunoprecipitation (IP) of cTn

Human cTn complex from NIST SRM 2921 was thawed at room temperature for 15 min, diluted in 120–130 μL of 0.2% (w/v) BSA, and added at eight different levels (≈2.5, 7.8, 13.3, 18.9, 24.5, 30.0, 35.5, 41.4 pmol) to a constant molar addition of mAb–bead complex (≈40 pmol mAb) corresponding to cTn concentrations during IP of ≈0.016, 0.049, 0.082, 0.12, 0.15, 0.18, 0.22, to 0.25 μmol/L. These

eight samples were replicated for each of six cTnI mAbs (labeled as MF4, M18, 3C7, 19C7, 560, and 267) and one α-GAPDH mAb used as a negative control. The design was intended to cover the entire optimal IP range 0.2–0.8 for $\text{bound}_{[\text{mAb–cTnI}]}/\text{total}_{[\text{mAb}]}$ at eight discrete points along the predicted K_d curve. Samples were rotated end-over-end for 3 h at 25 °C to ensure equilibrium. mAb–bead–antigen complexes were isolated using a magnet (Dynal Magnetic Particle Concentrator) and the supernatant was removed and discarded prior to being washed twice with ≈400 μL TBST (0.1% (v/v) Tween-20, 20 mmol/L Tris, 137 mmol/L NaCl) containing 0.1% (w/v) BSA, followed by a single wash of ≈400 μL 0.1% (w/v) BSA in water. Supernatant was removed and discarded prior to digestion.

The washed mAb–bead–antigen complexes were reconstituted in 40 μL 0.1% Rapigest surfactant in 100 mmol/L NH_4HCO_3 and boiled for 2 min. Disulfide linkages in the denatured proteins were reduced by the addition of ≈5 μL of 50 mmol/L DTT and shaken at 60 °C for 30 min. Cysteine residues were alkylated in the dark for 30 min using iodoacetamide at a final concentration of 15 mmol/L. Samples were tryptically digested without bead removal using a 40:1 total protein: trypsin ratio for 16 h at 37 °C with shaking. Rapigest was cleaved by addition of 0.1 mol/L HCl and removed by centrifugation at 4 °C. Finally, eight isotopically labeled peptides corresponding to each subunit of the cTn complex and to each IgG (mAb) isotype were spiked into the sample at a constant concentration (≈0.18 μmol/L and 0.36 μmol/L, respectively) to serve as an internal standard for isotope dilution quantification. Final unlabeled: labeled molar mass ratios ranged from ≈0.15 to 2.5 among the eight points along the saturation curve (see Supplementary Table 2). Matrix-matched calibration solutions were prepared from NIST SRM 2921 at six discrete concentrations ranging from ≈0.023 to 0.41 μmol/L corresponding to unlabeled: labeled mass ratios of ≈0.12–2.65 following the addition of internal standard. Calibration solutions were carried through the identical experimental procedures as the samples using an equimolar mixture of all mAbs and by omitting the wash steps to ensure complete recovery. Similarly, parallel experiments designed to test reproducibility associated with the preparation of the mAb–bead complex were performed, and will be discussed in the Results section.

2.4. ID MS analysis

Liquid chromatographic separation was achieved using a Zorbax (Agilent) SB-C_{18} reversed-phase analytical column (2.1 mm × 150 mm, 3.5 μm particles) at a flow rate of 200 μL/min. Peptide elution was accomplished using an increasing linear gradient of organic/aqueous solvent (ACN/H_2O) up to 50% B over 33 min, followed by a column wash and re-equilibration. Mobile phases A and B consisted of 0.1% (v/v) formic acid in H_2O and ACN, respectively. Column temperature was maintained at 35 °C; autosampler plate temperature control was set at 5 °C. Blank injections were monitored for sample carry-over. An Agilent 1200 LC system (Santa Clara, CA) was coupled in-line with an Applied Biosystems API 5000 triple quadrupole mass spectrometer (Foster City, CA) equipped with a standard micro-flow source. Ions were detected using a scheduled multiple-reaction monitoring (MRM) method in positive ion mode with a target scan time of 1 s and a MRM detection window of 30 s. Two fragmentation transitions were monitored per peptide from a total of five cTn peptides (three from cTnI, one from cTnT, and one from cTnC) and three IgG peptide isotypes (one each for IgG_1 IgG_{2a}, and IgG_{2b}) for both the isotopically labeled, and unlabeled peptides. The optimum peptides were selected using a previously described procedure [16] and the OrgMassSpecR computer program [17]. Optimized source and fragmentation parameters (declustering potential, entrance potential, collision energy, and collision cell exit potential) were selected independently for all peptide transitions by monitoring MS-response over a broad range and noting maximum signal intensity (see Supplementary Table 3). Isotopically labeled peptide analogs were additionally infused to validate the optimization results and to verify negligible isotope effect. During data acquisition, all source and fragmentation parameters were set identically for unlabeled/labeled pairs: collision gas = 3.4×10^4 Pa (5 psi), unit resolution in Q1 & Q3, curtain gas (CUR) = 4.1×10^5 Pa (60 psi), intensity threshold = 0, ion source gas 1 (GS1) = 2.8×10^5 Pa (40 psi), settling time = 5 ms, ion source gas 2 (GS2) = 2.8×10^5 Pa (40 psi), pause between mass ranges = 5 ms, ion spray voltage (IS) = 5000 V, *x*-axis spray position (vert.) = 0 mm, capillary temperature (TEM) = 500 °C, *y*-axis spray position (horiz.) = 7 mm, target scan time = 1.3 s, interface heater = ON, and MRM detection window = 60 s. Data acquisition and peak integration was performed using Analyst v1.5 software (Applied Biosystems). Peaks were identified manually and were automatically selected by the Analyst Quantitation Wizard; peak areas were integrated by Analyst using a bunching factor = 1, number of smooths = 0, and all other parameters set to default values. All peak integrations were visually inspected and manually integrated when necessary.

2.5. Non-linear curve fitting and error determination

Troponin concentrations as determined by ID MS analyses were fitted to non-linear curves on a two-dimensional plot consisting of parameters of 'bound antibody-antigen' [Ab–Ag] versus 'free antigen' [Ag]. The model [18,19] that relates the free troponin and the bound troponin measurements is:

$$[\text{AbAg}] = \frac{(B_{\max})[\text{Ag}]}{K_d + [\text{Ag}]}$$

In this work, we treat the thermodynamics of Ab–Ag binding as a reversible bimolecular reaction with a K_a representing the affinity of the Ag for the Ab–bead conjugate, and $K_d = K_a^{-1}$. For the equilibrium reaction Ab + Ag ⇔ AbAg,

$$K_d = \frac{[\text{Ab}][\text{Ag}]}{[\text{AbAg}]} = \frac{1}{K_a} \quad (1)$$

The mass action law [1] can be rewritten as:

$$[\text{AbAg}] = K_a[\text{Ab}][\text{Ag}] = \frac{([\text{Ab}_{\text{tot}}] - [\text{AbAg}])[\text{Ag}]}{K_d} \quad (2)$$

or

$$[\text{AbAg}] = \frac{[\text{Ab}_{\text{tot}}][\text{Ag}]}{K_d + [\text{Ag}]} \quad (3)$$

where $\text{Ab}_{\text{tot}} = [\text{Ab}] + [\text{AbAg}]$, and when Ab_{tot} is written as equal to $B_{\max}$ the equilibrium data can be plotted as:

$$y = \frac{(B_{\max})(x)}{K_d + x} \quad (4)$$

so that the concentration of [AbAg] complex increases linearly with increasing [antigen] at low [antigen], and the slope tapers off at higher [antigen] to reach an asymptotic plateau at $B_{\max}$ as all Ab is saturated. The dissociation constant K_d has units of mol/L. For a high-affinity Ab–Ag equilibrium, a $K_d \approx 10^{-9}$ mol/L is typical [20], being, as expected, approximately an order of magnitude lower than the dissociation constants determined for the best binding mAbs in our cTnI panel. It is suggested [21–23] that immobilization of an antibody on a solid surface – in this case on magnetic beads – can affect the binding constants for an Ab–Ag equilibrium. Therefore, what is being measured here is the immobilized non-intrinsic binding affinity – which also mimics the immobilization approach that is used in developing immunoassays where mAbs are bound to solid supports via reactive amine groups.

A description of the non-linear response curve-fitting parameters is provided in Supporting Data A. The data analysis was

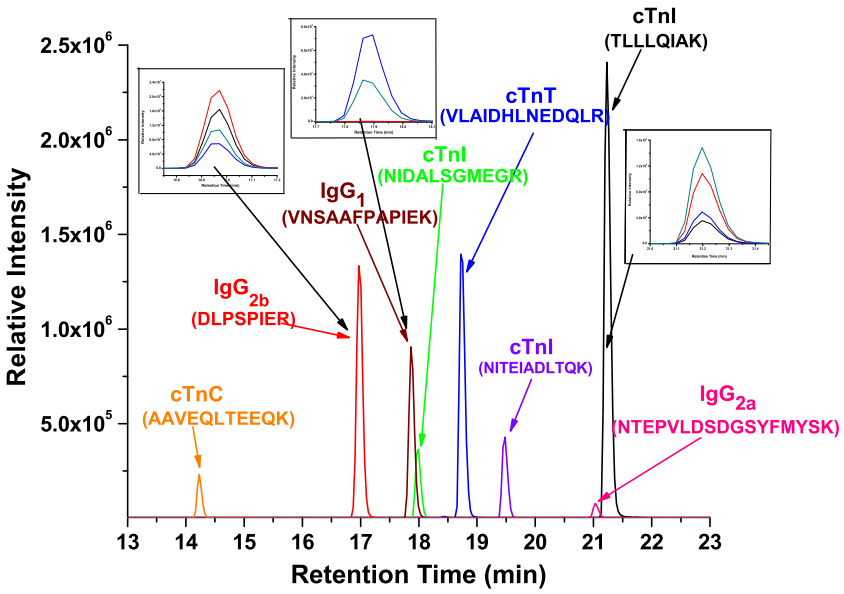

Fig. 1. A representative ID MS (MRM) total ion chromatogram of a cTn digest following an immunoprecipitation using an IgG2b mAb. Three cTnI peptides were monitored along with one cTnC, one cTnT, IgG1, IgG2a, and IgG2b peptides. Inserts represent the individual components of the TIC for each peak. Two labeled and two unlabeled transitions were monitored for all peptides. Note that for IgG1, only the two labeled peaks are quantifiable due to the fact that the assay was performed using an IgG2b mAb.

performed using the R programming language nls function; the script is also included as Supporting Data B.

3. Results and discussion

Standardization of immunoassay measurement procedures is of vital importance for the clinical community. Here, a method was developed to quantitatively select among monoclonal antibodies from a panel of high-performing, pre-screened cTnI mAbs based on their relative binding affinities using ID MS.

The amino acid sequence of cTnI is shown in Supplementary Fig. 1. Those regions against which each mAb was raised are highlighted, as are the amino acid sequences for which isotopically labeled peptides were synthesized for use as internal standards. cTnI is known to have multiple phosphorylation sites [24], most notable are those at Ser-23 and Ser-24 which have widely accepted functional significance in the control of cardiac contractility [25,26], and which are located on a unique N-terminal extension of the cTnI variant not found in skeletal TnI [27,28]. A representative MRM trace is provided in Fig. 1. Each experiment monitored two unique precursor-to-product ion transitions for each of five cTn peptides and three IgG peptides, for both the labeled and unlabeled peptide analogs. Three peptides originating from the cTnI subunit were quantified along with one peptide each from the cTnT and cTnC subunits. Although the cTnI subunit contains the binding epitope for all mAbs used in this study and for any corresponding immunoassay, we also quantified peptides from the T and C subunits to validate the stability of the intact cTn complex during the experimental procedure. In all cases, signal was detected from IgG peptide transitions specific for the antibody isotype used in that assay; the isotopically labeled IgG peptide transitions were detected in all assays. IgG peptides were previously selected from the antibody constant region by digesting a representative IgG isotype, and measuring a set of theoretical MRM transitions, as described for the cTn peptide selection (see Supplementary Fig. 2 and Supplementary Table 4).

Calibration curves were developed for each peptide transition and exhibited linear regression R^2 values >0.99 (see Supplementary Fig. 3). K_d constants were determined as [cTn_{free}] concentration at one-half of the maximum [$bound_{cTn}$] concentration ($\frac{1}{2}B_{max}$) using an iterative fit to all nonlinear curve data. All data points are corrected for by subtracting the binding to a non-specific antibody (α-GAPDH) measured at each of the eight antigen concentrations. Non-specific binding of cTn to the bead surface and/or to the F_C region of the antibody estimated from α-GAPDH assays was typically negligible suggesting that our washing protocols were sufficient, but not excessive, as the assays were optimized incrementally to reduce non-specificity.

A best-fit non-linear saturation curve for each mAb assay is provided in Fig. 2 for [$bound_{cTn}$] versus [$free_{cTn}$] (for a representative peptide transition). The highest affinity binder (lowest K_d) among the mAbs was determined to be antibody 19C7, with a mean $K_d = 7.4 \times 10^{-8} \pm 3.5 \times 10^{-8}$ mol/L. Table 1a provides the dissociation constants of all other mAb–bead conjugates quantified in this manner. Mean K_d's range over two orders of magnitude from 7.4×10^{-8} mol/L (19C7) to 2.6×10^{-6} mol/L (M18) with a median $K_d = 1.4 \times 10^{-7}$ mol/L and a mean K_d among all mAbs = 5.9×10^{-7} mol/L. The antibody panel is separated statistically into three discrete groupings with mAbs 19C7, 560, MF4, and 267 being statistically indistinguishable as optimal capture antibodies, and mAbs 3C7 and M18 being poorer choices, in that order. Table 1b details K_d constants determined for individual peptide transitions. Two optimal 'capture' mAbs as determined from this work (19C7 and 560) do support the results determined by the IFCC WG-TNI, and moreover, are known to bind the cTnI 'stable region', making them suitable choices for immunoassay development. However, because the WG-TNI's evaluation of the optimal capture mAb was determined using a pair-wise assessment using both a capture and detection antibody, we cannot absolutely compare the analytical approaches. Here, application of the ID MS approach is meant only to demonstrate its general use as a complementary quantitative tool for the selection of antibodies for subsequent immunoassay development.

The use of isotopically labeled peptides (as opposed to labeled proteins) as internal standards in our ID MS experiments means that the cTn digestion efficiency is not accounted for by the peptide calibration curves. It is therefore evident that different tryptic peptides within the cTn complex yield different saturation curves that cluster for precursor-to-product ion transitions originating from the same tryptic peptide. We can use corresponding transitions from a single tryptic peptide as validation of the ID MS

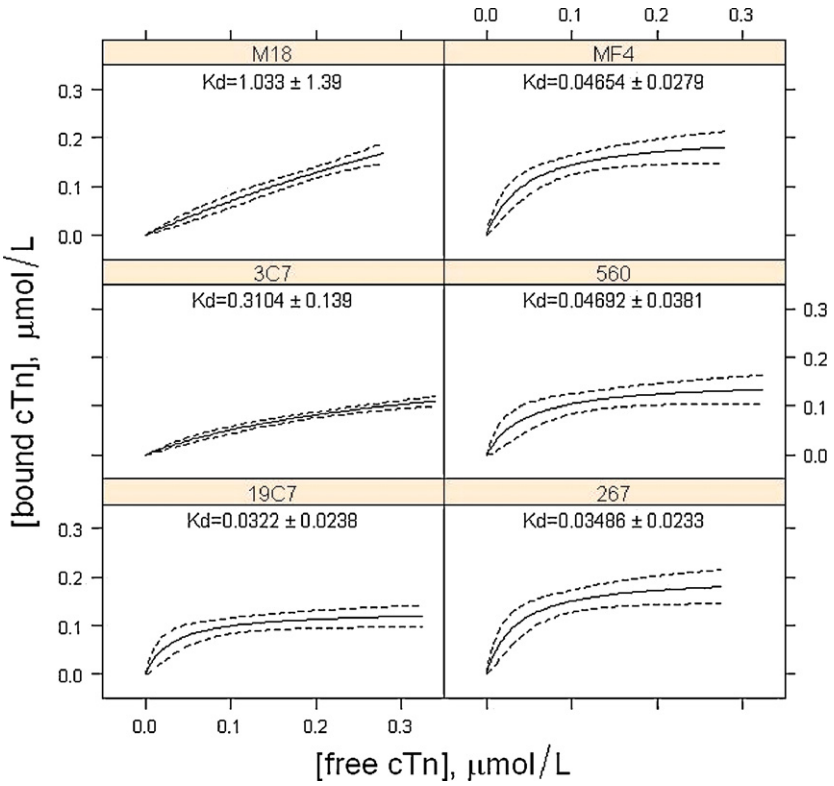

Fig. 2. Saturation curves are provided with 95% confidence intervals for all mAbs of a representative MRM transition (450.3 / 685.5). A value of the nonintrinsic K_d with uncertainty is given in units of μmol/L.

measurements. Further, we can directly compare data from each peptide transition for all mAbs. Peptide transitions will produce dissimilar saturation curves due to differences in digestion efficiencies, while transitions originating from the same tryptic peptides cluster together. Table 2a provides a representative data set of K_d and B_{max} values determined for each transition of antibody 19C7, demonstrating the effect of digestion efficiencies. Dissimilar data observed between tryptic peptides suggests that we are making relative measurements and that saturation curves from these data sets represent relative values of K_d (non-intrinsic K_d) specific not only to the efficiency of the enzymatic digestion for a particular peptide, but also to the use of a solid support during the IP. The goal of the experiment is to quantitatively differentiate binding affinities of each mAb to cTnI under these given conditions. Therefore, the optimal 'capture' mAb can be determined by either comparing K_d constants from a single peptide transition across all mAb assays, or by comparing K_d constants using the mean of all peptide transition data for each antibody. In both cases, within error limits, the relative K_d among all mAbs is unchanged. Fig. 3 shows a representative plot of the K_d constants determined for (a) the individual transition 450.3/572.4, and (b) for the mean of all transitions of all mAb assays (95% confidence interval). A similar pattern is observed for other individual peptide transitions as detailed in Table 1b. This data suggests that the top four performing mAbs in our panel are in fact, indistinguishable within error limits. This is not surprising noting that our mAb panel was pre-screened for the best

Table 1
Dissociation constants of cTnI antibodies.

	Mean K_d (μmol/L)				Standard deviation (μmol/L)					
(a) Mean dissociation constant (K_d) of all monitored peptide transitions for each cTnI antibody										
Antibody										
19C7	0.0740				0.035					
560	0.119				0.082					
MF4	0.133				0.076					
267	0.145				0.10					
3C7	0.513				0.19					
M18	2.60				1.2					
Peptide transition	TLLQIAK		AAVEQLTEEQK		NIDALSGMEGR		NITEIADLTQK		VLAIDHLNEDQLR	
	450.3/572.4	450.3/685.5	623.3/1018.5	623.3/675.4	581.8/749.4	581.8/935.4	623.3/1004.5	623.3/747.4	512.6/662.3	512.6/718.9
(b) Dissociation constant (K_d) of individual peptide transitions for all cTnI antibodies										
Antibody										
19C7	0.0226	0.0322	0.0589	0.0363	0.0975	0.0875	0.116	0.125	0.0867	0.0770
560	0.0292	0.0469	0.0852	0.0493	0.116	0.106	0.231	0.289	0.125	0.116
267	0.0198	0.0349	0.0759	0.0393	0.192	0.181	0.330	0.273	0.150	0.153
MF4	0.0282	0.0465	0.0951	0.0512	0.191	0.183	0.221	0.241	0.144	0.131
3C7	0.354	0.310	0.394	0.359	0.827	0.669			0.593	0.600
M18	1.76	1.03	2.859		3.59				4.14	2.22

Table 2
mAb constants for clone 19C7.

Peptide	Transition	K_d	B_{max}
(a) K_d and B_{max} values – the effect of digestion efficiency on transitions from different precursor ions			
AAVEQLTEEQK	623.3 → 1004.5	0.116	0.0216
AAVEQLTEEQK	623.3 → 747.4	0.125	0.0182
NIDALSGMEGR	581.8 → 749.4	0.0975	0.0363
NIDALSGMEGR	581.8 → 935.4	0.0875	0.0400
VLAIDHLNEDQLR	512.6 → 662.3	0.0867	0.0703
VLAIDHLNEDQLR	512.6 → 718.9	0.0770	0.0794
NITEIADLTQK	623.3 → 1018.5	0.0589	0.107
NITEIADLTQK	623.3 → 675.4	0.0363	0.135
TLLQIAK	450.3 → 572.4	0.0226	0.149
TLLQIAK	450.3 → 685.5	0.0322	0.131

Peptide	Transition	K_d	Expanded uncertainty
(b) K_d with expanded uncertainty for triplicate bead preparations			
AAVEQLTEEQK	623.3 → 1004.5	0.0732	0.0303
AAVEQLTEEQK	623.3 → 747.4	0.124	0.0404
NIDALSGMEGR	581.8 → 749.4	0.102	0.0437
NIDALSGMEGR	581.8 → 935.4	0.105	0.0338
NITEIADLTQK	623.3 → 1018.5	0.0744	0.0174
NITEIADLTQK	623.3 → 675.4	0.0787	0.0274
TLLQIAK	450.3 → 572.4	0.0275	0.0168
TLLQIAK	450.3 → 685.5	0.0343	0.0129

available choices prior to this work. Nonetheless, the ID MS approach is successful in eliminating mAbs 3C7 and M18 from further consideration. Future work aims to express isotopically labeled cTn complex in a native three-dimensional state to use as an internal standard to be spiked into the assay prior to enzymatic digestion, and which should account for variability in peptide digestion efficiency.

It is apparent that reproducibility of an immunoassay is highly dependant on the preparation of the mAb–magnetic bead conjugate. We tested reproducibility of our experimental technique for preparation of the mAb–bead conjugate by preparing three unique batches of mAb–bead conjugates for the antibody 19C7 and implementing equivalent immunoassays, tryptic digestions, and ID MS quantification. Independent curves were fit for each series of replicates. Significant differences between the replicates were tested at 95% confidence limits. In all cases, the K_d, and B_{max} values were found to be indistinguishable for any given transition, and could therefore be treated as a single data set. Fig. 4 illustrates 95% confidence intervals for data sets of each peptide transition. In Table 2b we provide values of the K_d parameter with error limits when estimated by a series of replicates for each transition. It is further demonstrated in this example that data sets cluster among tryptic peptide precursor ions.

The incorporation of isotopically labeled IgG peptides in our internal standard is useful for estimating the amount of mAb bound to the magnetic bead surface – regardless of orientation. All mAbs were assumed to be proportionally crosslinked to the bead surface in all orientations so that the amount of 'active' Ab remained consistent between assays. Tryptic digestions of cTn (bound to mAb) were performed directly on the bead surfaces; therefore we are capable of measuring mAb tryptic peptides simultaneously with cTn peptides during LC–MS/MS analysis. Isotopically labeled IgG peptides for each mAb isotype were incorporated into the internal standards as discussed above. The concentration of IgG at all points on the saturation curve was quantified and used as a normalization factor for $cTnI_{free}$ to account for differences in the amount of mAb–bead added at any given cTn concentration. In most cases, this normalization factor approximates a value of one (see Supplementary Table 5), as any minor quantitative differences are attributed to pipetting inconsistencies or microheterogeneity of the slurry. The ability of

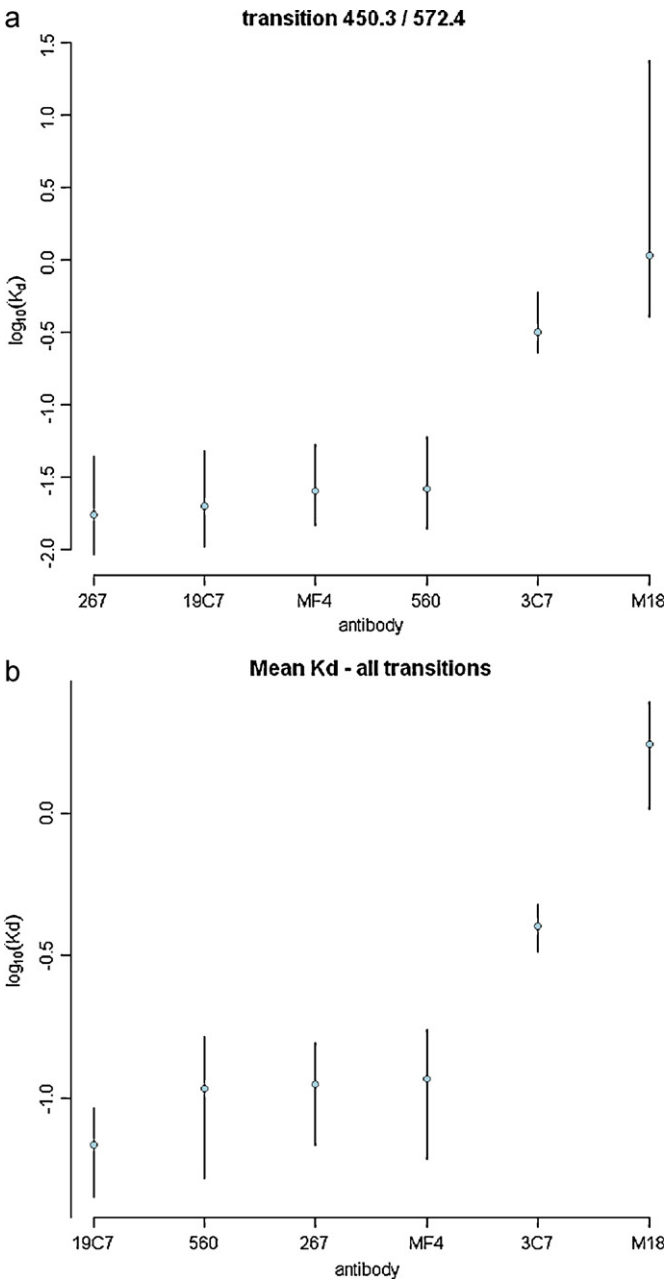

Fig. 3. (a) K_d constants are plotted for each mAb considering a representative peptide transition (450.3 / 572.4), and (b) estimates for the mean of the K_d constants considering all monitored peptide transitions for each mAb, with all error bars indicating a 95% confidence interval of the mean.

MRM assays to simultaneously quantify many analytes allows us to use this mass spectrometry-based approach rather than relying on accurate calibration of a pipette, or on gravimetry which assumes increasing error at very low volumes.

The results discussed here for the selection of an optimal 'capture' mAb were found to be in agreement with those determined by the IFCC WG-TNI using orthogonal approaches [14,29]. Quantitative accuracy and precision of ID MS techniques, and the selectivity and specificity of MRM measurements can provide necessary confidence to antibody selections [30]. Although it is not a high-throughput approach, ID MS possesses superior robustness and reproducibility as compared to empirical pair-wise immunoassay performance optimization; this platform can monitor hundreds of measurands simultaneously within an assay, and can validate quantitative results concurrently using multiple signature peptides

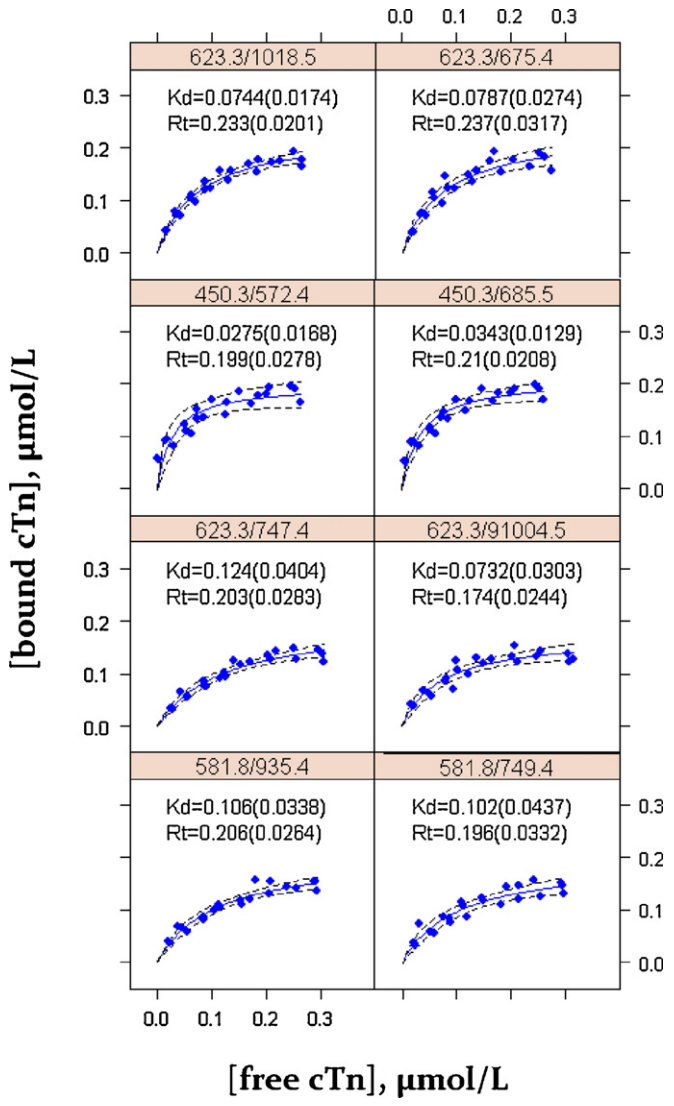

Fig. 4. Saturation curves are generated from triplicate preparations of mAb-bead constructs carried through immunoprecipitation and ID MS analysis. Curves are provided with 95% confidence intervals for each peptide transition for the representative mAb, 19C7. K_d constants and RT values are provided for each peptide transition along with associated uncertainties (in parentheses).

and multiple transitions per peptide. Here we demonstrate that MS-based selection of antibodies is an ideal complementary tool to immunoassay development.

4. Conclusions

We have shown that an LC–MS/MS immunoassay platform can quantitatively select optimal mAbs to be used as capture antibodies for development of an antibody-based measurement procedure for the clinically relevant protein cardiac troponin I. The metrological rigor associated with ID MS provides a tool for objective selection of mAbs in analytical applications. Based on data of relative K_d constants, we have selected clone 19C7 as a candidate capture mAb for further development. A well-characterized reference measurement procedure will provide the clinical community the necessary tools for inter- and intra-laboratory standardization of troponin I quantification with essential metrological traceability. Mass spectrometry is shown to be an invaluable complementary tool for immunoassay development and antibody characterization based on improved target specificity, true metrological traceability, and unbiased target quantification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.07.037.

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