Introduction

Monoclonal antibodies (mAbs) represent the fastest growing class of biotherapeutics.1 As of June 2012, 36 therapeutic mAbs had been approved or undergone review by the US Food and Drug Administration and European Medicines Agency,2 and new mAb candidates are entering clinical trials at a rate of over 40 molecules per year.3 Successful development of a therapeutic mAb requires a deep understanding of pharmacokinetic (PK) and pharmacodynamic (PD) parameters, as well as safety and efficacy. Information from a range of bioanalytical methods, including PK assays and tissue cross-reactivity (TCR) studies are used as metrics during mAb development. PK assays often measure therapeutic mAb concentrations in circulation following administration of therapeutics and provide a surrogate for assessing drug exposure at the site of action. Data from PK assays are critical for establishing appropriate dosing regimens. Therapeutic mAb PK data are usually generated by ligand binding assays (LBAs) which typically must be able to quantitate ng/mL levels of a recombinant humanized mAb or human mAb...
(collectively referred to here as rhumAb) in a complex biological matrix that may contain 10–15 mg/mL of human immunoglobulin G (huIgG). LBAs generally use one or more carefully selected monoclonal or polyclonal antibody reagents to attain the desired level of sensitivity and selectivity for the therapeutic mAb candidate. The availability of LBA reagents with satisfactory selectivity for a target mAb can be on the critical path for generation of decision-enabling PK data; however, current reagent generation practices for LBAs are time-consuming. It can take 3–6 months to generate reagents for one mAb program depending on the types of reagents needed (e.g., soluble recombinant antigen, anti-ID mAb). In addition, lot-to-lot consistency could be an issue for reagents such as polyclonal antibodies (pAbs).

Data from TCR studies, which are used to characterize the binding of a therapeutic mAb to antigen on tissues, are critical for safety assessment. These studies can be used to support selection of animal species for safety studies and to identify potential off-target toxicities. TCR studies are most frequently conducted using immunohistochemistry (IHC) staining of a panel of human tissues. Due to the high degree of similarity between therapeutic rhumAb candidates and endogenous huIgGs, an IHC assay reagent must be able to distinguish the specific binding of a rhumAb to antigen or its cross-reactive epitope from non-specific background, which is often from endogenous huIgGs present in human tissues. For this reason, most IHC assays use a format where the test mAbs are directly biotinylated and then applied to tissue samples, followed by avidin-HRP, which then generates an assay signal from an HRP substrate. This format eliminates the need for a secondary antibody reagent that can distinguish rhumAb candidates from endogenous huIgGs. Therefore, the use of a biotinylated therapeutic reagent has a technical advantage over alternative IHC staining methods for human tissue. Labeling of the test mAb, however, can sometimes lead to altered binding to target and cause undesirable excessive or insufficient staining in IHC assays.

An assay reagent that could be used to support PK and IHC assays for multiple therapeutic rhumAb candidates and that has minimal cross-reactivity with endogenous huIgGs would be highly desirable. Several years ago, we developed a novel PK assay that quantified humanized mAbs in cynomolgus monkey serum. The assay used a pan-mAb specific assay reagent. Several labs have subsequently adopted similar approaches to accelerate their development of PK assays for animal studies; however, this type of pan-mAb specific assay reagent approach has not yet been successfully applied to samples from human subjects. Most therapeutic mAbs in use or under development are humanized or fully human IgGs. These rhumAbs typically share 95% or more framework sequence identity with human germline sequences. Therefore, it has been a tremendous challenge to generate an assay reagent that is capable of binding selectively to low levels of rhumAbs in the presence of the significant molar excess levels of endogenous huIgGs that occur in human blood and tissues.

Therapeutic rhumAbs may be generated by identifying variable regions that bind to the target of interest and coupling these to human constant regions. For humanized antibodies, the variable domains contain donor antibody-derived complementarity-determining region (CDRs) and human-derived framework sequences. Alternatively, variable domains may be selected from combinatorial libraries comprising natural or synthetic CDR segments contained in a human framework sequence. Several strategies have been used to select candidate human frameworks, including an approach based on a human consensus sequence framework. Consensus sequences are based upon the most common amino acid found at a particular position from a collection of endogenous huIgG sequences; they may contain sequence motifs that rarely occur in natural huIgGs. We hypothesized that, for a group of rhumAbs that were constructed using the same consensus sequence framework, a sequence motif that is shared among them might potentially be utilized to facilitate generation of a panel-specific assay reagent that could then be used to develop assays for all of these rhumAbs. To verify this, we immunized mice with a cocktail of rhumAbs derived from a common human consensus sequence framework. We identified a unique mAb (clone 10C4) that showed specific reactivity with multiple rhumAbs during hybridoma screening, but which did not react with purified polyclonal human IgGs. We conducted a series of studies characterizing 10C4 using various techniques, including X-ray crystallography and mutagenesis. In addition, we evaluated the performance of 10C4 in different rhumAb PK and IHC assays.

Results

Generating mAb 10C4. A hybridoma process using mice immunized with a cocktail of antigens containing 7 structurally-related rhumAbs (rhumAb15–21 in Fig. 1) was used to generate mAb 10C4. During the hybridoma supernatant screening, one of a total of 864 clones, 10C4, was found to have good reactivity with tested rhumAbs, but little binding with commercially available huIgGs and rituximab (Rituxan®), a chimeric antibody sharing greater than 99% sequence identity in constant domains with most tested rhumAbs, but having different variable domains (Tables S1 and S2). Evaluation of purified 10C4 against a broad panel of rhumAbs revealed that 10C4 binds to 22 of the 25 tested rhumAbs, but is nearly non-reactive with commercially available huIgGs purified from human sera (Fig. 1). The 25 unique rhumAbs are directed against a range of therapeutic targets, including those in circulation and expressed on cells. The rhumAbs are composed of consensus sequence framework derived from human variable domain of heavy chain (VH) subgroup III and variable domain of light chain (VL) subgroup I and constant regions with human IgG1 heavy and kappa light chains. These results suggested that 10C4 recognizes a unique motif common among the binding rhumAbs that is distinct from endogenous huIgGs. mAb 10C4 did not recognize the chimeric antibody, which suggests that the unique binding epitope for 10C4 is likely located in the Fab region instead of the Fc region. This was confirmed by additional data showing 10C4 binds to seven representative rhumAb Fabs that were either enzymatically cleaved or expressed directly, but not to huIgG Fc region based upon these rhumAbs (data not shown).
Analyzing molecular details of the 10C4-therapeutic rhumAb interaction by X-Ray crystallography. RhumAb6 was chosen as a model molecule to study the interaction between 10C4 and rhumAbs. Structure determination employed 10C4 Fab generated from cleavage of a mouse-human chimeric 10C4 IgG because poorly diffracting crystals were obtained using 10C4 Fab derived from the hybridoma 10C4 (data not shown).

The 10C4/rhumAb6 interface is large, burying about 1320 Å² from solvent on each protein. As shown in Figure 2, the epitope on rhumAb6 Fab is extensive and conformational, with 10C4 contacting residues distributed over \( \text{V}_{\text{H}}, \text{V}_{\text{L}}, \text{C}_{\text{H1}}, \text{C}_{\text{L}} \) domains of rhumAb6. These contact residues are heavily concentrated in the heavy chain, especially within the variable domain of the heavy chain. For example, 79% of all contact residues within 4.0 Å are from the heavy chain, with the remaining 21% coming from the light chain. In addition, 73% of all the heavy chain contact residues are within the variable domain. Thus, 10C4 contacts a broad area on the “side” of rhumAb6-Fab, with a main epitope in the variable domain but extending into the constant region. The paratope on 10C4 is mainly in the \( \text{V}_{\text{H}} \) domain. As shown in Table 1, the amino acids at positions 61 and 62 vary among all tested rhumAbs, which suggests that these 2 residues are not critical in determining whether a rhumAb binds to 10C4 or not. The mutagenesis data were consistent with this hypothesis: mutations of residues 61 and 62 individually or together in rhumAb25 did not improve the binding of this antibody to 10C4.

Analyzing the 10C4-therapeutic rhumAb interaction by mutagenesis. To confirm our hypothesis that the key contact residues on rhumAb6 were conserved among 10C4-binding rhumAbs, we aligned the amino acid sequences of the 25 tested rhumAbs (Table 1). These results revealed that 17 of the 19 (89%) key contact residues within 3.5 Å on rhumAb6 were well-conserved across all rhumAbs. The only two non-conserved residues are located at positions 61 and 62, near the C-terminal of \( \text{V}_{\text{H}} \) CDR2. We examined residues in “near-by” positions 61 and 62 because these amino acids might influence the conformation of the epitope. Position 63 was interesting because the three non-10C4-binding rhumAbs all coded L, whereas all the 10C4-binding rhumAbs had either F or V (Table 1). Position 65 was also of note because all three non-binding rhumAbs coded S. The 10C4-binding rhumAbs varied among G65, D65, R65 or N65. These observations led us hypothesize that the 10C4 non-binding behavior of all these three rhumAbs could be related to the region of 61–65 that contains P61, S62, L63 and S65. To test this, we chose rhumAb25, a 10C4 non-binding mAb and designed six mutants involving replacement with the amino acids found at these positions in rhumAb6: P61Q, S62K, L63F, S65G, P61Q/S62K and P61Q/S62K/L63F/S65G (QKFG).

Binding of 10C4 to wild type (WT) rhumAb25 as well as its mutants was evaluated by ELISA and Biacore. Results from both assays demonstrated that only two of the six mutants, QKFG and L63F, had significantly improved binding to 10C4 compared with the WT antibody (Fig. 3). The other four mutants had no detectable changes in binding properties. A key difference between these two sets of 10C4-binding mutants is the amino acid residue at position 63. A change from leucine to phenylalanine at this position drastically changed the binding ability of rhumAb25 to 10C4 (Fig. 3). Interestingly, mutant L63F clearly showed higher binding to 10C4 than mutant QKFG, which also contains the L63F mutation. This observed difference in binding is likely due to additional mutations at positions 61, 62 and 65 in mutant QKFG. Q61 and K62 are the 10C4 contact residues in rhumAb6 based on X-ray crystallography. As shown in Table 1, the amino acids at positions 61 and 62 vary among all tested rhumAbs, which suggests that these 2 residues are not critical in determining whether a rhumAb binds to 10C4 or not. The mutagenesis data were consistent with this hypothesis: mutations of residues 61 and 62 individually or together in rhumAb25 did not improve the binding of this antibody to 10C4.

Further characterization of the two binding mutants demonstrated that, compared with the WT antibody, binding affinities...
Thus, in assays where target interference is anticipated or known to be an issue, an anti-ID mAb might be less appealing than pAbs as an assay detection reagent, but this would need to be evaluated on a case-by-case basis. CDR-specific pAbs are typically affinity purified using the CDR of a rhumAb. In general, CDR-specific pAbs can tolerate interference from soluble target better than anti-ID mAbs because they comprise a mixture of antibodies with different specificities, subclasses and affinities; however, CDR-specific pAbs may not have good lot-to-lot consistency in reagent performance. In addition, generating an anti-ID mAb or a CDR-specific pAb for each rhumAb therapeutic candidate is costly and time-consuming. In theory, anti-huIgG polyclonal antibodies from commercial sources can be applied to multiple rhumAbs, but, in practice, these reagents often produce assays with high background signal (and consequently poor assay sensitivity) because they are unable to discriminate between rhumAb therapeutics and endogenous huIgGs.

10C4, with its unique features, could potentially overcome drawbacks associated with the four categories of assay reagents. We evaluated the utility of 10C4 in different rhumAb clinical PK assays and compared its performance with that of anti-ID mAbs and pAbs. Representative case studies based upon rhumAb1, rhumAb2 and rhumAb17 are summarized in following sections.

Case study 1: Significantly reduced assay background compared with anti-huIgG pAb. 10C4 is panel-specific to rhumAbs, with...
very minimal binding to endogenous huIgGs. This feature can be very useful to help overcome the high and variable background issue often encountered in assay development. We used 10C4 in two different rhumAb clinical PK assays and evaluated its performance against alternative reagents. The assay for rhumAb2 used the recombinant therapeutic target as the capture reagent and was developed for use with colorectal cancer (CRC) patient sera. The assay for rhumAb17 used an affinity-purified goat anti-rhumAb17 CDR pAb as the capture reagent and was developed for use with sera from rheumatoid arthritis (RA) patients. Assay formats with anti-huIgGs as detection reagents were originally selected as the best performing PK assay formats for these two assays (data not shown). Using anti-huIgG pAb as detection reagents, we discovered that serum samples from therapeutic mAb naïve patients exhibited unacceptably high and variable background. As shown in Figure 4A, the O.D. signals in 41 CRC and 32 RA therapeutic mAb naïve patient sera were 0.066 ± 0.068 (mean ± standard deviation) and 0.057 ± 0.071, respectively and some of the individual samples had O.D. signals greater than 0.3 or 0.4. This caused a reduction of assay sensitivities and precluded accurate quantification of rhumAb at low concentrations in patient samples. Using 10C4 as the detection reagent, after normalizing the O.D. signals against positive controls in the assays, the aforementioned high and variable assay backgrounds were greatly reduced to 0.028 ± 0.003 and 0.033 ± 0.003 in the new rhumAb2 and rhumAb17 PK assays, respectively.

**Case study 2: Improved assay sensitivity compared with anti-huIgG pAbs and anti-ID mAb.** The 10C4-based assays overall provide higher signal-to-background ratios and therefore better sensitivities compared with anti-huIgG pAb based assays due to the lower and less variable assay background mentioned above. As shown in Figure 4B, in a rhumAb1 PK assay where the therapeutic target was used as capture reagent, biotin-10C4/streptavidin-HRP was compared with two HRP-anti-huIgG pAbs as detection reagent. The assay using 10C4 had higher signal-to-background ratios than the two assays using anti-huIgG as detection reagents. At the highest rhumAb1 concentration tested (0.781 ng/mL), the assays using 10C4, anti-huIgG (heavy and light chain-specific), or anti-huIgG (Fc-specific) had signal-to-background ratios of 6.1, 1.5 and 1.8, respectively.

Anti-ID mAbs are usually more specific than pAbs, but more specificity does not always mean greater sensitivity. In some cases, anti-ID mAbs compete with soluble therapeutic targets for binding to rhumAbs, which could lead to suboptimum assay

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**Figure 3.** 10C4 binding to the wild type (WT) and mutants of rhumAb25 on (A) Biacore assay and (B) ELISA. rhumAb6 was used as a positive control. O.D: optical density.
### Table 1. Sequence alignment of amino acid sequences of 25 tested rhumAbs

| rhumAb | LC residues | HC residues |
|--------|-------------|-------------|
|        | FR1 | FR4 | C\textsubscript{1} | C\textsubscript{1} | FR1 | FR4 | C\textsubscript{1} | C\textsubscript{1} | FR1 | FR4 | C\textsubscript{1} | C\textsubscript{1} | FR1 | FR4 | C\textsubscript{1} | C\textsubscript{1} | FR1 | FR4 | C\textsubscript{1} | C\textsubscript{1} |
| rhum-Ab1 | M Q S P A K G E | E N F K N E L S S E L S Y |
| rhum-Ab2 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab3 | M Q S P A K G E | Q R F K G E L S S E L S Y |
| rhum-Ab4 | M Q S P A K G E | P S V K G E L S S E L S Y |
| rhum-Ab5 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab6 | M Q S P A K G E | Q K F K G E L S S E L S Y |
| rhum-Ab7 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab8 | M Q S P A K G E | G K F K G E L S S E L S Y |
| rhum-Ab9 | M Q S P A K G E | Q K F K G E L S S E L S Y |
| rhum-Ab10 | M Q S P A K G E | P N F K D E L S S E L S Y |
| rhum-Ab11 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab12 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab13 | M Q S P A K G E | E I F K G E L S S E L S Y |
| rhum-Ab14 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab15 | M Q S P A K G E | D S V K G E L S S E L S Y |
| rhum-Ab16 | M Q S P A K G E | A D F K R E L S S E L S Y |
| rhum-Ab17 | M Q S P A K G E | Q K F K G E L S S E L S Y |
| rhum-Ab18 | M Q S P A K G E | P S V K G E L S S E L S Y |
| rhum-Ab19 | M Q S P A K G E | Q K F K D E L S S E L S Y |
| rhum-Ab20 | M Q S P A K G E | Q R F K G E L S S E L S Y |
| rhum-Ab21 | M Q S P A K G E | P S V K G E L S S E L S Y |
| rhum-Ab22 | M Q S P A K G E | D S V E G E L S S E L S Y |
| rhum-Ab23‡ | M Q S P A K G E | P S L K S E L S S E L S Y |
| rhum-Ab24‡ | M Q S P A K G E | P S L K S E L S S E L S Y |
| rhum-Ab25‡ | M Q S P A K G E | P S L K S E L S S E L S Y |

See page 546 for legend to Table 1.
sensitivities. In the rhumAb2 clinical PK assay, the recombinant therapeutic target was used as capture reagent. Two anti-ID mAbs were originally identified as the best two among a panel of anti-ID antibodies against rhumAb2 in a screening assay where these anti-ID mAbs were captured on soluble target coated plate via binding to rhumAb2 (Fig. 4C inset). When the two anti-ID mAbs were tested together with 10C4 as detection reagents, the 10C4 based assay had an apparently higher sensitivity than assays using either of the two anti-ID mAbs (Fig. 4C).

**Case study 3: Improved assay tolerance to target interference compared with anti-huIgG pAbs and anti-ID mAb.** Because 10C4 recognizes a motif common to multiple rhumAbs, it potentially can tolerate more target interference than anti-ID mAbs, yet may be more specific than a typical anti-species pAb. We assessed assay tolerance to target interference in three rhumAb2 clinical PK assays. In all three assays, rhumAb2 was captured by the recombinant soluble target coated on a plate and was detected by anti-huIgG pAbs, an anti-ID mAb, or 10C4. After spiking different concentrations of recombinant soluble target into control samples containing 1000 ng/mL of rhumAb2, sample recovery in all three assays decreased upon increasing soluble target levels. The 10C4-based assay, however, exhibited a greater tolerance to spiked target than the anti-ID based assay (Fig. 4D), as expected. As revealed in the crystal structure of 10C4 Fab and rhumAb6 Fab complex, 10C4 recognizes the “side” of the rhumAb, thus the epitope is unlikely to overlap the unique CDR of rhumAbs. As a result, 10C4 has the advantage of being less susceptible to target competition compared with anti-ID mAbs. While the anti-huIgG based assay had the best tolerance to target interference among the three assays, it had unacceptably high and variable assay background as mentioned earlier (see Fig. 4A).

Additionally, in the rhumAb1 clinical PK assay where the therapeutic target was used as the capture reagent, we found the 10C4-based assay showed comparable tolerance to target interference to two other pAb-based assays (Fig. 4E).

**Case study 4: 10C4-based assay in support of clinical sample analysis.** The unique performance characteristics of 10C4 have enabled it to be used in assays to quantitate rhumAbs in human serum samples from clinical studies. As an example, MNRP1685A is a phage-derived human mAb that specifically binds to neuropilin-1 (NRP1). A PK ELISA that used recombinant human NRP1 as the coat reagent and biotinylated 10C4 as the detection antibody was successfully developed and validated. The assay has high sensitivity and good precision, and it was successfully used to analyze MNRP1685A PK samples from a Phase 1a study in cancer patients; this work was described and discussed by Xin et al.20

In **IHC assay development.** As mentioned in the introduction, most IHC assays currently rely on directly biotinylated therapeutic mAb candidates to recognize antigen presented in tissues and detection by avidin-HRP. The caveat of using this direct format is that labeling of the therapeutic mAb candidate could potentially alter its molecular characteristics and therefore complicate further data interpretation. With the unique panel-specific feature of 10C4, we hypothesized that the native forms of mAb therapeutics binding to antigens can be detected.

**rhumAb14 IHC assay development.** In the rhumAb14 IHC assay, using a format with direct labeling of rhumAb14 with biotin, weak positive staining was observed on positive control human tissues (Figs. 5A) as opposed to negative staining of negative control human tissues (Fig. 5B). There are several potential reasons for the observed weak positive staining, including altered binding of rhumAb14 to the target after biotinylation. As positive controls are necessary in IHC assays to assess method validity, an alternative assay protocol is needed to obtain a good positive staining. Therefore, a 10C4-based assay was developed where unlabeled rhumAb14 was first applied to tissue samples, and Biotin-10C4 was used as secondary detection reagent. Using this assay, much stronger positive staining was observed in the positive control human tissues (Fig. 5C, compare with 5A), while no staining was observed on negative control human tissues (Fig. 5D). On the other hand, when Biotin-anti-huIgG was used to detect unlabeled rhumAb14 in the assay, excessive background staining that precluded interpretation of data was observed for both positive and negative control human tissues (Fig. 5E and 5F). We also tested binding of Biotin-10C4 to both positive and negative control tissues in the absence of rhumAb14 to rule out the possibility of non-specific binding of Biotin-10C4 to the target antigen. Negative staining was observed in both cases, as expected (Fig. 5G and 5H).

**Discussion**

Therapeutic mAb development programs depend upon reliable and specific assays for PK and safety assessments. The data generated from these assays are important for elucidating PK/PD relationships, measuring therapeutic mAb exposure, assessing safety margins and establishing appropriate dose regimens. It is therefore essential to develop and deploy robust and sensitive assays for each therapeutic mAb development program. The importance of generating and characterizing high-quality critical reagents for such assays has been articulated in two white papers focusing on LBA21 and tissue cross-reactivity studies, respectively. Antibody-based reagents are often used in these assays to confer selectivity for the cognate ligand. These reagents, however, can take months to develop and can therefore be on the critical path for drug development.
When developing a set of related therapeutic mAb candidates, a panel-specific assay reagent that is capable of specifically recognizing these mAbs over endogenous huIgGs and that has consistent batch-to-batch performance, would be highly desirable. Sometimes a high tolerance to target interference is also needed. Despite an increasing awareness of the critical role that assay reagents play in enabling drug development, it has
not been possible to develop reagents for clinical assay development that simultaneously possess all these features.

We wanted to see if it was feasible to accelerate the process of assay and reagent development. A unique hybridoma process was used to generate the mAb 10C4. mAb 10C4 is directed against an epitope that is broadly conserved across the panel of therapeutic mAbs included in the cocktail of immunogens. This conserved epitope confers “panel-specific” immunoreactivity on 10C4.

The panel-specific property of 10C4 is different from all other commercially available anti-huIgG antibodies because 10C4 has minimal cross-reactivity with endogenous huIgGs. The cross-reactivity of assay reagents to endogenous huIgGs contributes to high and variable assay background. Depending on the assay format and analyte of interest, the reported prevalence of interfering antibodies in patient sera varies from < 0.05% to 40%. These matrix and analyte of interest, the reported prevalence of interfering antibodies that simultaneously possess all these features.

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The panel-specific property of 10C4 is different from all other commercially available anti-huIgG antibodies because 10C4 has minimal cross-reactivity with endogenous huIgGs. The cross-reactivity of assay reagents to endogenous huIgGs contributes to high and variable assay background. Depending on the assay format and analyte of interest, the reported prevalence of interfering antibodies in patient sera varies from < 0.05% to 40%. These include pre-existing autoantibodies against therapeutic targets, heterophilic antibodies with broad species specificity to antibodies, human anti-animal antibodies, and rheumatoid factors (autoantibodies in RA patient sera) against the Fc portion of antibodies. Sometimes, the elevated and variable background, as seen in the PK assay and IHC study examples described earlier, may preclude the development of acceptable assays for timely PK and safety assessment of a therapeutic mAb. The development of 10C4 addresses these issues primarily due to its unique “panel-specific” property, which arises from its distinct epitope. This epitope is likely shared among a group of rhumAbs, but not among all human immunoglobulins, as suggested by the crystal structure and also by prevalence analysis of key contact residues.

The Fab residues forming the 10C4 epitope on rhumAb6 are contributed by a total of 6 different germline genes: V\textsubscript{H}\textsuperscript{10}, J\textsubscript{H}\textsuperscript{1}, C\textsubscript{G}1, V\textsubscript{L}J3, and C\textsubscript{L}. Except for the constant regions, these germline segments combine more or less randomly in immunoglobulins. An evaluation of the residues forming the 10C4 epitope in human immunoglobulins in the Kabat database showed that the combination of them all in one mAb has a relatively low probability (3.6%; Table S5). Subtle structural differences between antibodies that contain this exact motif in the context of a different framework or isotype may also contribute to the specificity of 10C4. The specificity of 10C4 for rhumAbs with the V\textsubscript{H}3 and V\textsubscript{L} frameworks also suggests that it may not bind rhumAbs with other frameworks.

Besides the “panel-specific” feature that is beneficial to PK and IHC assay development, 10C4 also has a much greater tolerance compared with conventional anti-ID mAb reagents for soluble targets in PK samples. Unlike most anti-ID antibodies that recognize idiotopes located within CDRs of a therapeutic mAb, 10C4 recognizes an epitope that is mostly composed of non-CDR residues and thus is slightly away from the antigen-binding site. There has been much recent industry dialog about the utility of data from both “free” vs. “total” PK assays. PK assays based on 10C4 will allow the measurement of both target-bound and unbound drug concentrations.

While the majority of 10C4-contacting residues are non-CDR, it is worth noting that other CDR residues might also contribute to the rhumAb-10C4 binding interactions. For example, rhumAb9 and rhumAb6 have very different binding profiles, as shown in Figure 1; however, they both contain the same 19 contact residues that were revealed by X-ray crystallography of rhumAb6. In fact, these two rhumAbs have the same amino acid sequences in all other framework regions and constant domains. The only difference is in their CDR residues.

Evaluation of interactions between 10C4 and rhumAb25 by mutagenesis showed that residue 63 in the C-terminal region of V\textsubscript{H} CDR2 on rhumAbs plays a critical role in determining whether a rhumAb can bind to 10C4. Interestingly, both phenylalanine and valine are found at position 63 in many 10C4-binding rhumAbs (Table 1). Although residue 63 is not among the key contact residues on rhumAb6, the C-terminal residue is 64, and rhumAb25 had no effect on rhumAb25 binding to its target. As the C-terminal region of V\textsubscript{H} CDR2 is often less important to target binding than many other CDRs, this potentially provides an opportunity to improve binding of a therapeutic mAb to 10C4 without compromising target-binding.

Our data show that 10C4 binds to most “panel” rhumAbs with equilibrium dissociation constants ranging from double-digit nanomolar to submicromolar (Biacore assay, data not shown). This might be because 10C4 mainly uses its heavy chain in contact with rhumAbs, as suggested by the 10C4-rhumAb6 crystal structure. Perhaps increasing the involvement of the 10C4 light chain could improve the binding affinity. The use of FR3...
of V<sub>H</sub> in recognizing antigen is relatively rare, but otherwise the 10C4 residues involved in binding rhumAb6 are typical of those used by an antibody binding to a large protein antigen. Even with only moderate affinities, 10C4 demonstrated superior performance in both PK and IHC assays compared with alternative anti-ID mAbs and anti-huIgG pAbs.

The unique features and excellent performance characteristics of 10C4 mentioned above have made it superior to many conventional assay reagents. As summarized in Table 2, 10C4 is...
more specific than pAbs, while less prone to target interference than anti-ID mAbs. As a mAb, 10C4 provides good stability and lot-to-lot consistency. Moreover, in IHC assays, 10C4 eliminates the need to biotinylate the test rhumAb, which could alter its binding to target and so affect staining results. In addition, 10C4 can be used for PK assays that support non-clinical studies of rhumAbs in non-human primates, mice and rats, further streamlining PK assay development. To date, 10C4 has been used for 15 non-clinical and clinical PK and IHC assays. Our use of 10C4 has greatly saved time in development and maintenance of each assay that it is used in and thereby reduced the duration of the critical path to decision-enabling PK and IHC data for multiple therapeutic mAbs. It also has eliminated the cost of developing analyte specific mAb and pAb reagents for each assay.

In summary, we describe here for the first time a strategy to develop a panel specific reagent that can expedite the development of multiple clinical assays for structurally-related therapeutic mAbs. This provides a novel approach to bioanalytical assay reagent generation. The concept described here should be applicable to other groups of rhumAbs sharing common features.

Materials and Methods

Generation of hybridoma clones and purification of mAb 10C4.

A mixture of 2 μg of each of the following rhumAbs was suspended in monophosphoryl-lipid A and trehalose dicorynomycolate (MPL+TDM) adjuvant (Corixa, Hamilton): rhumAb15, rhumAb16, rhumAb17, rhumAb18, rhumAb19, rhumAb20 and rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injected in each hind footpad of a BALB/c mouse at 3 to 4 d intervals. Initial sera were titered against rhumAb21. The mixture was injecte

Table 2: Performance characteristics of 10C4 in PK and IHC assays compared with other commonly used reagents

| Reagent performance characteristics | 10C4 anti-ID mAb | Anti-CDR purified pAb | Commercial anti-huIgG pAb | Therapeutic target |
|-------------------------------------|----------------|-----------------------|----------------------------|-------------------|
| High specificity                    | +++            | +                     | -                          | ++                |
| Good tolerance to target interference| ++             | -                     | +++                        | -                 |
| Good stability                      | +++            | ++                    | +                          | +++               |
| Lot-to-lot consistency              | +++            | +++                   | -                          | -                 |
| Can be used across projects         | +++            | -                     | +++                        | -                 |
| Applicable as a detection Ab in IHC studies when native drug is used | +++ | + | + | - |

Note: Any performance characteristics with two plus signs above means acceptable.
μL) were added to a microtiter plate followed by 50 μL of supernatant from individual hybridoma clones. The plate was incubated at room temperature in the dark for overnight before the addition of 10 μg of streptavidin coated magnetic beads in a volume of 150 μL. After incubation, the plates were read on an IGEN M384 (IGEN International, Inc.) analyzer. Data were collected and reported in electrochemiluminescence units (ECLU). Medium A was used a background control.

10C4 binding to rhumAb therapeutics and endogenous huIgGs on ELISA. A 384-well microtiter plate was coated with 25 μL of donkey F(ab’), anti-huIgG (Fc fragment specific) (Jackson ImmunoResearch) at a concentration of 0.25 μg/mL in PBS. The plate was incubated at 2–8°C overnight and then washed three times with wash buffer. The plate was blocked for approximately 2 h with 50 μL per well assay diluent at room temperature with agitation. After washing, 25 μL of 0.1–75 ng/mL recombinant huIgG therapeutics and huIgG purified from human sera from two vendors (Jackson ImmunoResearch; Vital Products) diluted in assay diluent were added to each well and incubated at room temperature for approximately 1 h with agitation. Unbound huIgGs were removed by washing the plate three times, then 25 μL per well of 10C4-biotin conjugate diluted to 1 μg/mL was added and incubated at room temperature for approximately two hours with agitation. The plate was then washed 3 times and 25 μL of avidin D conjugated to HRP (Vector Laboratories) diluted to 50 ng/mL in assay diluent was added to each well and incubated for approximately 30 min at room temperature with agitation. Excess avidin D-HRP conjugate was removed after washing the plate three times, then 25 μL per well of TMB substrate (Kirkegaard and Perry Laboratories) and the absorbance was read at 450 nm after subtraction at 630 nm.

X-Ray crystallographic study. Generation of chimeric 10C4. 10C4 Fab used in the X-ray crystallographic study was generated from a mouse-human chimeric 10C4 (referred as ch10C4). RT-PCR was employed to clone the mouse 10C4 heavy and light chain variable domains as chimeric constructs with human constant domains. Total RNA was isolated from 10C4 hybridoma cells using the RNeasy kit (Qiagen, Inc.). The variable domains were amplified from RNA by RT-PCR using the Superscript III One Step kit (Invitrogen) and oligonucleotide primers specific to the mouse variable domains. Forward primers were designed on the basis of determination of the N-terminal sequences of the heavy and light chains of the antibody purified from hybridoma cells. The sequence of the heavy chain forward primer was:

5’-GGT GCA GCC CTG AGC CAG TGT G GG CAG TCT GGA CCT GAC TGT GGT TCT GCA CTT GCT CTA GAA

and for the light chain:

5’-GTT GCA GCC AGC TGT GAG TGG TCA GGT GGT TTC TCA CC-3’

The PCR products were purified by agarose gel electrophoresis, digested with restriction enzymes EcoRV and RsrII for the light chain, or PvuII and ApaI for the heavy chain and ligated into similarly digested human kappa light chain and human IgG1 heavy chain expression plasmids, respectively. Dideoxynucleotide sequencing was used to confirm the identity of the chimeric plasmids.

The chimeric antibody was expressed in Chinese hamster ovary (CHO) cells and purified from the cell culture medium by a column containing MabSelect Sure resin (GE Healthcare) according to manufacturer’s instruction. Eluted antibody was concentrated and then subjected to size exclusion chromatography using Superdex 200 resin in 50/100 HiLoad column (GE Healthcare). Fractions containing monomeric antibody were pooled, then concentrated and buffer-exchanged to PBS.

Preparation of Fabs. Fabs of ch10C4 and rhumAb6 were generated through limited lys C digestion similar to the published procedure.34

In brief, ch10C4 and rhumAb6 were subjected to proteolysis with lysyl endopeptidase (Wako Chemicals) according to the manufacturer’s instructions. After the reactions were stopped, the digest was diluted with sodium Acetate (pH 5.0) and loaded onto a 5 mL HiTrap SPFF column (GE Healthcare). Eluted Fabs were confirmed by mass spectrometry on an Agilent 6210 Time-of-Flight TOF LC/MS (Agilent, Inc.).

Crystallization. Solutions of ch10C4 Fab and rhumAb6 Fab were mixed in a molar ratio of 1:1.5, incubated at 4°C for 1 h and then concentrated to 5 mL. This solution was passed over a 320 mL Highload Superdex 200 SEC column (GE Healthcare) at 1 mL/min, collecting 1.5 mL fractions. Pooled fractions from the earliest eluting absorbance maximum were concentrated to 40 mg/mL and subjected to sparse-matrix crystallization screens in sitting drops. Crystals grew from a drop made with reservoir containing 0.2 M NaCl, 0.1 M Na/K phosphate (pH 6.5) and 25% (v/v) PEG 1000. A rod-like crystal was harvested, plunged into liquid nitrogen and used for X-ray data collection.

Data collection, structural determination and refinement. Data collected at ALS beamline 5.0.2 using 0.9730 Å X-rays extended to 2.95 Å resolution and were determined to belong to space group P2_12_1. Data reduction was accomplished using HKL2000 and elements of the CCP4 suite.36 These data suffered from dose-dependent intensity decay. The structure was solved by molecular replacement (PHASER) using variable region and constant region fragments from an anti-HER2 Fab structure (pdb 1FVD). The assignment of Fabs as either the rhumAb6 (human sequence) or the ch10C4 (with murine variable domains) was made according to the trace of framework region #1 in the heavy chains. One constant region was not placed during molecular replacement but was placed manually using the program Coot after phases were improved by refinement using REFMACS.38 The final model shows 99.3% of residues within allowed and generously allowed Ramachandran space. Data collection and refinement statistics are shown in Table S6.
Design and characterization of mutants. Expression and purification of rhumAb25 variants. Amino acid substitutions in the V<sub>H</sub> domain of rhumAb25 were constructed using oligonucleotide-directed mutagenesis following the protocol of Kunkel. <sup>39</sup> Desired substitutions were confirmed by dideoxynucleotide DNA sequencing. Antibodies were expressed in human 293 T cells by co-transfection of heavy and light chain DNA, supernatants were harvested at 7 d post transfection, and antibody was purified on Protein A-Sepharose.

Screening rhumAb25 mutants binding to 10C4 on Biacore. Binding of 10C4 to rhumAb25 mutants was assessed on a Biacore T100 instrument (GE Healthcare) using an indirect capture format. Briefly, goat anti-human IgG (Fc fragment specific) (Jackson ImmunoResearch Laboratories) was coupled onto two flow cells (FCs) of a Series S CM5 sensor chip (GE Healthcare). The capture levels were ~10000 response units (RUs), using a standard amine coupling and blocking procedure recommended by the manufacturer. Selected rhumAb25 mutants were injected and captured only on FC2 (~190RU). FC1 that contains the same amount of goat anti-human IgG was served as a reference FC.

mAb 10C4 diluted to 15 μg/mL in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.05% surfactant P20) was injected over FC1 and FC2 at a flow rate of 5 μL/min for 5 min. The binding activity of mutants to immobilized 10C4 was reference FC subtracted, and was reported as the response five seconds before the end of each injection. The sensor chip was regenerated by 10 mM glycine (pH 1.5). To ensure that any potential differences observed between the mutants are beyond assay variation, binding of 10C4 to each mutant was repeated twice in randomized orders. The experiments were performed at 25°C.

Kinetics affinity measurement of rhumAb25 variants binding to 10C4 or antigen. Experiments measuring the kinetics of rhumAb25 variants (WT and selected mutants) binding to 10C4 or antigen of rhumAb25 were performed similarly as described above except for the changes described below. Goat anti-human IgG was coupled onto both FC1 and FC2 of a sensor chip, and rhumAb25 variants were indirectly captured only on FC2 at approximate levels of 100RU.

mAb 10C4 at various concentrations diluted into HBS-EP buffer was injected into the 2 FCs at a flow rate of 40 μL/min for 5 min, and the dissociation of 10C4 was allowed to proceed for 10 min. The experiments were performed at 25°C.

Antigen binding to rhumAb25 was assessed using the antigen at various concentrations being injected over the captured mAb25 antibodies at a flow rate of 50 μL/minute for five minutes. During preliminary runs, dissociation of the antigen from rhumAb25 was observed to be very slow, possibly approached or reached the detection limits of the instrument. <sup>40</sup> To derive more reliable dissociation rate constants, the experiments were performed at 37°C to increase the dissociation rate constants, <sup>31</sup> and the dissociation time was set to 1 h.

Binding kinetic parameters (association and dissociation rate constants and dissociation equilibrium constant) were calculated with the Biacore T100 Evaluation Software (version 2.0.1; GE Healthcare). Three independent experimental runs were performed using three separate sensor chips and the results were averaged and summarized (see Results section).

rhumAb1 human PK assay. 96-well microtiter plates were coated overnight with 100 μL of recombinant human rhumAb1 target extracellular domain (ECD) at a concentration of 0.5 μg/mL in coating buffer. After washing the plates with wash buffer, plates were blocked with 200 μL of assay diluent. Samples were diluted 1:20 with assay diluent. After blocking, the plates were washed with wash buffer and then incubated with samples at room temperature for approximately one hour. The plates were again washed with wash buffer. HRP conjugated donkey F(ab’)<sub>2</sub>, anti-human IgG (H+L) (Jackson ImmunoResearch Code 709-036-149), HRP conjugated goat F(ab’)<sub>2</sub>, anti-human IgG (Fc fragment specific) (Jackson ImmunoResearch Code 109-036-098), or biotinylated 10C4 with avidin-D HRP conjugate (Vector Catalog A2004) was added as the detection reagent. After washing, TMB was used as the peroxidase substrate and the oxidized TMB was measured by absorbance at 450 nm.

In addition, target interference has been evaluated. A general description of the method is shown below. The method was used in other PK assays when target interference was evaluated. Briefly, a set of normal human serum samples was spiked with a constant concentration of the test rhumAb antibodies and increasing concentrations of soluble target. These samples were then stored at or below −60°C until they were analyzed in PK assays.

rhumAb2 human PK assay. A 96-well microtiter plate was coated by an overnight incubation with 100 μL of recombinant human rhumAb2 target ECD at a concentration of 2 μg/mL in coating buffer. After washing the plates with wash buffer, plates were blocked with 200 μL of assay diluent. Samples were diluted 1:20 with sample diluent (PBS with 0.5% BSA, 0.05% polysorbate 20, 0.35 M NaCl, 0.25% CHAPS, 5 mM EDTA, 0.05% ProClin 300, pH 7.4). After blocking the plates with 200 μL of assay diluent, the plate was washed with wash buffer and then incubated with samples at room temperature for approximately two hour. The plates were again washed with wash buffer. HRP conjugated goat F(ab’)<sub>2</sub>, anti-human IgG (Fc fragment specific), biotinylated 10C4 with avidin-D HRP conjugate, biotinylated anti-ID mAbs (clone 16A8 or 16H3) against rhumAb2 with avidin-D HRP conjugate was added as the detection reagent. After washing, TMB was used as the peroxidase substrate and the oxidized TMB was measured by absorbance at 450 nm.

rhumAb17 human PK assay development. 96-well microtiter plates were coated either by two hour incubation at room temperature or overnight incubation at 4°C with 1 μg/mL of affinity purified goat anti-rhumAb17 in coating buffer. Plates were washed with wash buffer and then blocked with assay diluent. Samples were diluted 1:100 with sample diluent (PBS with 0.5% BSA, 1% normal goat serum, 0.05% Polysorbate20, 0.05% ProClin 300, 0.25% CHAPS, 5 mM EDTA, 0.35 M NaCl, pH 9.0). After blocking, the plates were washed with wash buffer and then incubated with samples at room temperature for one hour. The plates were again washed with wash buffer and then incubated with one of the following detection reagents: peroxidase-conjugated, F(ab’)<sub>2</sub>, goat anti-human IgG (Fcγ fragment specific) or biotinylated mAb10C4 and Avidin-D HRP conjugate. TMB was
used as the peroxidase substrate and oxidized TMB was measured by absorbance at 450 nm (with subtraction at 650 nm). *rhumAb14* immunohistochemistry assay development. Immunohistochemistry (IHC) assay was performed on 5 μm thick frozen tissue sections embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA). Sections were mounted on glass slides, air-dried and fixed in acetone for 5 min at room temperature. Sections were then blocked for endogenous peroxidase activity with glucose oxidase solution for 60 min at room temperature and for endogenous biotin with avidin/biotin block (Vector Labs), respectively. Non-specific IgG binding sites were then blocked with 3% BSA (Roche AG). Sections were incubated with either biotinylated rhumAb14, or unconjugated rhumAb14 with biotinylated 10C4 antibodies and subsequently incubated with Vectastain ABC Elite Peroxidase reagent (Vector Labs) following manufacturer’s instructions. Slides were then incubated with a metal enhanced DAB colorimetric reagent (Thermo Scientific), counterstained with Mayer’s hematoxylin, dehydrated and coverslipped.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials can be found here: www.landesbioscience.com/journals/mabs/article/24822/
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