

## Affimers as anti-idiotypic affinity reagents for pharmacokinetic analysis of biotherapeutics

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### ABSTRACT

Therapeutic antibodies are the fastest growing class of drugs in the treatment of cancer, and autoimmune and inflammatory diseases that require the concomitant development of assays to monitor therapeutic antibody levels. Here, we demonstrate that the use of Affimer nonantibody binding proteins provides an advantage over current antibody-based detection systems. For four therapeutic antibodies, we used phage display to isolate highly specific anti-idiotypic Affimer reagents, which selectively bind to the therapeutic antibody idiope. For each antibody target the calibration curves met US Food and Drug Administration criteria and the dynamic range compared favorably with commercially available reagents. Affimer proteins therefore represent promising anti-idiotypic reagents that are simple to select and manufacture, and that offer the sensitivity, specificity and consistency required for pharmacokinetic assays.

### METHOD SUMMARY

Anti-idiotypic Affimer proteins that bind therapeutic antibodies are introduced as alternative affinity reagents to traditional antibodies. A nonbridging ELISA assay for pharmacokinetic analysis of these biotherapeutics in serum is developed.

### KEYWORDS

Affimer reagents • anti-idiotypic • biotherapeutics • diagnostics • pharmacokinetics

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### LAY ABSTRACT

The fastest growing class of drugs are derived from human proteins that are modified to target the processes that cause the disease. Owing to the presence of similarly structured proteins in the blood, it is currently extremely difficult to specifically detect the therapy over the levels of normal human proteins in patients. However, it is vitally important to be able to monitor the levels of these therapies in the blood in order to ensure patients receive appropriate dosages. This paper describes the use of artificially derived proteins, called Affimers, to detect four commonly used therapies. Overall, we demonstrate that Affimer reagents provide a valid approach for monitoring dosing of these types of drugs.

Monoclonal antibody (mAb) drugs have been a resounding clinical and commercial success, applied across a range of therapeutic areas with a particularly significant impact in the treatment of cancer, autoimmune and inflammatory diseases [1–4]. High-interaction surface area binding gives mAbs greater specificity and so lower off-target, toxic effects compared with small-molecule drugs [3]. By the end of 2017, 68 mAbs or biosimilars were in clinical use and the market exceeded US\$98 billion in sales, having grown every year since 2013 [5]. This trend looks set to continue; therapeutic antibodies are the fastest growing class of drugs and in 2017 a record number of ten were granted their first marketing approvals in the USA or EU [1,6]. Further acceleration in mAb approvals is expected, with a considerable pipeline of over 550 antibodies in clinical development and over 50 in late-stage clinical trials at the end of 2017 [1,6].

The advance in antibody therapeutics requires a parallel development of pharmacokinetic (PK) assays that

can monitor mAb concentration and distribution in patients to guide dosage during clinical trials and practice [7,8]. In PK assays, the natural antigen may not be the optimal mAb capture reagent if it is expensive, hazardous or not reliably available, or when measurements of antigen-bound drug are required [7,9,10]. As therapeutic antibodies are highly humanized and present among up to a million-fold excess of human IgG in serum, it is particularly challenging to generate capture reagents that avoid cross-reactivity [10–12]. Anti-idiotypic reagents that bind to the unique idiotopes of the antibody variable regions are required to specifically target the therapeutic antibody in PK assays [10,13]. Specific anti-idiotypic reagents are also required for immunogenicity assays [7,14–16], affinity purification [17], analytical studies [18] and vaccine development [19–21].

Antibodies are important as successful anti-idiotypic reagents, but their selection and production can be complex and certain limitations exist [7,10–12,18,22,23]. With traditional immunization methods it is hard to select for specific binding to the antibody idiope, as binding to other regions of the humanized mAb target molecules results in serum cross-reactivity [7,23,24]. Extensive screening may be required and long development times may not match the pace of drug development [7,12,25]. mAb technology can, however, be used to more easily generate anti-idiotypic reagents. Antibody production is also dependent on animals or mammalian cell culture to ensure correct folding, glycosylation and cysteine oxidation [23,26]. This complex manufacture is expensive and some antibodies have lot-to-lot reproducibility issues, meaning extensive validation is required to ensure consis-

**Table 1. Number of clones in the primary screen.**

Target	Clones screened by iQue	Hits sequenced	Unique sequences	Clones selected for ELISA tests
Trastuzumab	360	Top 96	21	21
Rituximab CDR mAb	192	192	102	17
Adalimumab CDR mAb	192	192	15	15
Ipilimumab CDR mAb	192	192	113	16

CDR: Complementary-determining region; mAb: Monoclonal antibody.

tency throughout the drug evaluation process [27–29]. mAbs will not have significant lot-to-lot variation but there is still a desire for complementary or alternative reagents to overcome some limitations. Alternatives include anti-idiotypic antibody fragments (antigen-binding fragments [Fabs]) [7], single-chain variable fragments [11,30], camelid nanobodies [31], shark variable new antigen receptors [10] and llama single-domain antibodies [32], and when directed selection and recombinant production is implemented reagents can often have improved consistency and specificity.

Currently, there is a particular interest in developing antibody mimetics based on nonimmunoglobulin scaffold proteins with randomized, selectable binding regions, as they can be engineered to have desirable properties [23,33–35]. Many scaffolds exist [23]; anti-idiotypic DARPins [22] and monobodies [18] have been generated, but not validated as reagents in PK assays. A promising alternative binding protein is the Affimer reagent, which is based on a cystatin scaffold with two variable, nine amino acid loops that allow high-affinity binding to a range of target molecules [36,37]. Affimer reagents have been used in numerous assays from studying protein function to diagnostics [37–47] and exhibit key characteristics that make them potentially suitable as anti-idiotypic reagents [45]. They can be rapidly identified, incorporating the use of negative selection [48] to direct binding towards the idio type and reduce cross-reactivity. Additionally, the small, stable scaffold can be reproducibly produced at high yield in *E. coli*, to give a reliable, consistent supply [36,37,48]. Here we confirm the suitability of Affimer binders as anti-idiotypic reagents; Affimer reagents against four therapeutic antibodies have been generated, characterized and

validated for use in PK assays. Lot-to-lot reproducibility is evaluated and comparisons made with anti-idiotypic Fabs to assess the advantages of Affimer reagents in terms of specificity, detection range and flexibility of assay format.

## MATERIALS & METHODS

### Target QC

The target antibodies were trastuzumab (anti-HER2; Roche, Switzerland), rituximab CDR (complementary-determining regions) mAb (anti-CD20-hIgG4-mab14; Invivogen, CA, USA), adalimumab CDR mAb (anti-TNF $\alpha$ -hIgG1-mab1; Invivogen) and ipilimumab CDR mAb (Anti-CTLA4-hIgG1-mab1; Invivogen). Each target antibody concentration was checked by measuring  $A_{280}$  and dividing by the extinction coefficient. A 1- $\mu$ g aliquot of each was analyzed by SDS-PAGE (Bolt 4–12% Bis-Tris; Life Technologies, CA, USA) to determine quality and purity. A 100- $\mu$ g aliquot of each target mAb was then biotinylated by incubating in a 10 $\times$  excess of EZ-Link Sulfo-NHS-LC-Biotin (21327, 1 mg no-weigh format; Thermo Fisher Scientific, MA, USA) for 2 h on ice. Free biotin was removed by buffer exchange, the concentration measured and a 1- $\mu$ g aliquot analyzed by SDS-PAGE (as above) to check purity. Biotinylation was then confirmed by western blot analysis; 200 ng of biotinylated target mAb was separated by SDS-PAGE (as above) and transferred onto nitrocellulose using an iBlot system (Thermo Fisher Scientific). The membrane was then blocked using 1 $\times$  TBS (50 mM Tris, 150 mM NaCl; both Sigma, MO, USA) + 3% bovine serum albumin (BSA; Sigma) pH 7.4 for 1 h at room temperature with gentle agitation on a roller mixer. It was then washed for 3  $\times$  5 min using TBS-T (1 $\times$  TBS + 0.05% Tween 20; Sigma) and detected using a 1:10,000 dilution of streptavidin horseradish peroxidase (HRP; Abcam, UK) in 1 $\times$

TBS + 3% BSA pH 7.4 for 1 h at room temperature. The membrane was then washed as described previously and protein detected using Amersham ECL detection reagent (GE Healthcare Life Sciences, IL, USA), analyzed by chemiluminescence using a G:Box gel doc system (Syngene, India).

### Phage display

Phage display was performed as described previously [48]. Briefly, targets were submitted to three rounds of phage display, with deselection against antibodies of similar isotype used to remove cross-specific binders from the phage output. For trastuzumab, a therapeutic antibody cocktail (rituximab, Humira, Avastin and human IgG1) was used for deselection. For rituximab, adalimumab and ipilimumab CDR mAbs, deselection was performed with anti-CTLA4 hIgG4, anti-CTLA4 hIgG1 and anti-TNF $\alpha$  hIgG1 antibodies, respectively.

### Primary screen

Following phage display, the Affimer coding regions resulting from panning rounds 2 and 3 were subcloned into pEtLECTRA vectors: cHA-His6-Cys for trastuzumab and cHA-His6 for the other mAbs. Colonies were picked (Table 1) and Affimer proteins were expressed in 1-ml cultures and purified using Ni-NTA resin (Qiagen, Germany). A primary screen was performed using the iQue Screener (Intellicyete, NM, USA). Biotinylated targets and deselection targets were immobilized onto QSH DevScreen iQue beads following the manufacturer's instructions (Intellicyete). Affimer protein concentrations were normalized to 2.5  $\mu$ g/ml and 10  $\mu$ l added to 10- $\mu$ l prepared beads in a 384-well plate. Alexa488-conjugated anti-HA antibody (BioLegend, CA, USA) was used to quantify Affimer reagent binding. Clones were sequenced to identify unique

sequences (Table 1).

### Affimer protein expression

Medium-scale recombinant Affimer protein production (50–100 ml) was performed to generate 1–2 mg of Affimer material, which was purified by Ni-NTA magnetic beads (Qiagen). 5 ml LB (made using LB [Lennox] EZ mix powder; Sigma) containing 50 µg/ml kanamycin sulphate (Sigma) and 1% (w/v) glucose (Sigma) was inoculated with a sequence-checked glycerol stock for the Affimer reagent of interest and grown for 16 h at 37°C, 220 rpm. 1 ml of starter culture was used to inoculate 100 ml Terrific Broth (Melford, UK) containing 50 µg/ml kanamycin sulphate and cultures were grown at 37°C, 220 rpm until an OD<sub>600</sub> of 0.6–0.8 was reached. The temperature was then reduced to 25°C, IPTG (Sigma) added to a final concentration of 1 mM and cultures incubated at 25°C, 220 rpm overnight. Cells were harvested by centrifugation (10,000×g, 10 min, 4°C) and resuspended in Affimer purification lysis buffer (pH 8.0) comprising 50 mM sodium phosphate (Sigma), 500 mM sodium chloride (VWR, PA, USA), 30 mM imidazole (Sigma), 10% (v/v) SoluLyse (Genlantis, SA, USA), 25 U/ml Benzonase Nuclease HC (Millipore, MA, USA) and for the trastuzumab Affimer reagent 5 mM TCEP (Generon, UK) was also added. The resuspended cells were sonicated for 5 min (10 s on, 10 s off), then the insoluble fraction was removed by centrifugation (10,000×g, 30 min, 4°C) and the supernatant filtered (0.45 µm) to remove any remaining insoluble material. Cleared lysates were purified with Ni-NTA magnetic beads (Qiagen) using an automated process (washed in 50 mM imidazole, eluted in 400 mM imidazole). Purified trastuzumab Affimer reagents were buffer exchanged into pH 6.5 CBS (100 mM sodium citrate tribasic dehydrate + 150 mM sodium chloride; both Sigma) + 5 mM TCEP + 0.02% sodium azide (Severn Biotech, UK). Other Affimer reagents were buffer exchanged into pH 7.4 PBS (100 mM sodium phosphate + 150 mM sodium chloride; both Sigma) + 0.02% sodium azide. The concentration (A280/extinction coefficient) and purity (1 µg aliquot analyzed by SDS-PAGE) of all purified Affimers was measured as described previously.

### ELISA validation

Selected Affimer binders (Table 1) were tested by ELISA. Affimer proteins were passively adsorbed onto Maxisorp plates at 200 nM overnight at 4°C. Plates were washed with 1× PBS-T (PBS diluted from 10× PBS; Gibco + 0.05% Tween 20; Sigma), using 3 × 300 µl per well on a BioTek (VT, USA) 405-plate washer. Wells were then blocked with 1× casein-blocking buffer (diluted in PBS from 10× casein-blocking buffer; Sigma) for 2 h at room temperature with gentle agitation. Plates were washed as previously and incubated with biotinylated target (at target dependent dilution) for 1 h at room temperature with gentle agitation. Plates were washed again and bound target was detected using a 1:10,000 dilution of streptavidin poly-HRP (Pierce, WI, USA) for 1 h at room temperature with gentle agitation and visualized using tetramethylbenzidine (TMB; Surmodics, MN, USA). TMB incubation time was target dependent, usually 5–10 min, and ODs were read at 450 nm and 630 nm (reference wavelength).

Affimer binders selected from the validation ELISA were then tested in a sandwich ELISA. Affimer reagents were passively adsorbed onto Maxisorp plates at 1 µg/ml overnight at 4°C. Plates were washed and blocked as described previously before incubation with titrated target (twofold dilution from 2000 ng/ml) for 1 h at room temperature with gentle agitation. Plates were washed again and bound target was detected with a 1:100,000 dilution of HRP-conjugated anti-hlgG antibody (Bethyl, TX, USA) for 1 h at room temperature with gentle agitation and visualized using TMB (Surmodics). TMB incubation time was target dependent, usually 5–10 min, and ODs were read at 450 nm and 630 nm (reference wavelength).

### Assay optimization

The sandwich ELISA protocol was used to test selected Affimer proteins for specificity, lot-to-lot variation and full curve metrics.

A modified sandwich ELISA protocol was used for Fabs (anti-Trastuzumab HCA168, anti-Rituximab HCA186 and anti-Adalimumab HCA202; BioRad, CA, USA). They were passively adsorbed onto Maxisorp plates at 5 µg/ml overnight at 4°C, washed as described previously and blocked with

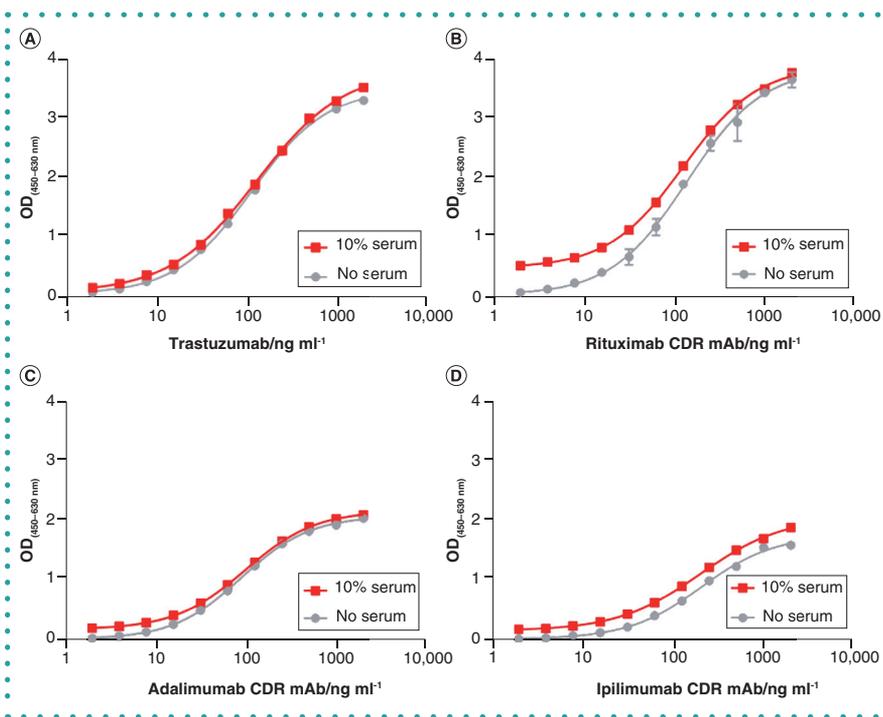
5% BSA in PBS-T (the manufacturer recommended blocking). Plates were washed again then the titrated target series was incubated for 1 h at room temperature with gentle agitation before a final wash. Bound target was detected with a 1:10,000 dilution of mouse anti-human IgG (Fc) CH2 domain antibody (anti-hFc; BioRad) for 1 h at room temperature with gentle agitation and visualized using QuantaBlu (following manufacturer's protocol; Thermo Fisher Scientific).

Flexibility of detection methods was tested using the sandwich ELISA protocol with the following minor adaptations: the two generic antibodies (anti-hlgG; Bethyl at 1:100,000 and anti-hFc; BioRad at 1:10,000) used the protocol as described except that visualization was with QuantaBlu. The anti-hlgG Affimer reagent (0.5 µg/ml) was pre-incubated with a 1:15,000 dilution of streptavidin-HRP (Abcam) in blocking buffer before addition to test plate for detection (1 h at room temperature with gentle agitation) and visualization with TMB.

## RESULTS & DISCUSSION

### Affimer reagent selection

An Affimer phage display library [36] was screened against four therapeutic antibodies: trastuzumab (Herceptin) and mAbs containing the CDR corresponding to the parental therapeutics rituximab (Rituxan), adalimumab (Humira) and ipilimumab (Yervoy). Screening was performed in three successive rounds against biotinylated target mAbs. Each target underwent SDS-PAGE to assess the molecular weight and purity and biotinylation was confirmed by western blot analysis. A negative selection procedure [48] was used to direct Affimer binding towards the antibody idiotype and deselect against the antibody isotype. Small amounts of isotype-specific antibody were premixed with the phage library in the second and third panning rounds, to block cross-reactive Affimer binders and selectively isolate specific anti-idiotypic Affimer reagents. Outputs from the phage panning were subcloned, recombinantly produced and purified in a small-scale automated process. A high-throughput primary screen was performed using the bead-based iQue screener (Intellicyt), with target and deselection targets immobilized onto different beads, such that selective Affimer ►



**Figure 1.** Sandwich ELISA dose response curves for target therapeutic antibodies with (A) anti-trastuzumab (B) anti-rituximab CDR mAb, (C) anti-Adalimumab CDR mAb and (D) anti-ipilimumab CDR mAb Affimer reagent capture surfaces. Detection of each target therapeutic antibody was compared in buffer (gray) and 10% human serum (red). Anti-hlgG-HGF was used as detection reagent, with TMB substrate visualization read at 450 nm (minus 630 nm reference). Data points are the mean of triplicate measurements and error bars indicate standard deviation from the mean. NB: the majority of the error bars are so small as to be occluded by the data point symbol. CDR: Complementary-determining region; mAb: Monoclonal antibody.

binders with no cross-reactivity were identified. Clones were sequenced and a subset found to be unique. Of these, the highest affinity binders identified from iQue screening were selected for medium-scale (50–100 ml) protein production and purification, prior to further ELISA validation. The number of clones analyzed from the primary screen is summarized for each target in Table 1.

An ELISA was used to validate the Affimer proteins as capture reagents for the biotinylated antibody targets. The best-performing Affimer reagents were then tested in a sandwich format, using an Affimer capture surface for the nonbiotinylated mAb target and HRP-conjugated anti-human IgG antibody (anti-hlgG-HRP) as a detection reagent. Based upon the results of this screen, a lead candidate Affimer reagent for each target was chosen for further characterization. The lead Affimer candidate was selected to have the greatest target specificity and a binding strength optimal for the required assay range.

### Performance characterization of Affimer reagents in PK assay

A sandwich ELISA was developed to assess the performance of the selected anti-idiotypic Affimer reagents in quantifying therapeutic antibody concentrations. The calibration range of the assay is 1.95–2000 ng/ml, corresponding to a serum concentration of 0.0195–20 µg/ml with samples diluted 1:10. This exemplifies the potential of Affimer binders as critical PK assay reagents, with the scope for clinically relevant serum concentrations to be measured if samples are diluted further and curves run in more diluted matrix [49–53]. The anti-idiotypic Affimer reagent was used as capture reagent for the target mAb, detection was with anti-hlgG-HRP and visualization with 3,3', 5,5"-TMB as the HRP substrate. In this assay format each Affimer reagent detected its target mAb across a broad dynamic range (Figure 1). PK assays aim to measure the target therapeutic in human serum samples, so require minimal

sample matrix effects; that is, minimal variation in assay performance due to the serum matrix in which the assay is performed. A sample matrix effect was observed in the detection of the rituximab CDR mAb in 10% human serum (Figure 1B), noted as an increase in background absorbance relative to measurements in buffer. For all other targets, minimal sample matrix effects were observed and each Affimer reagent displayed comparable detection of the target mAb in 10% human serum as shown in buffer (Figure 1). This confirms the success of the selection protocols in generating anti-idiotypic Affimer reagents that specifically detect clinically relevant mAb concentrations within a human sample matrix. The robustness of Affimer reagent performance in the presence of serum overcomes any need for advance sample preparation.

Target specificity of the anti-idiotypic reagents within patient sample matrices is crucial to ensure the efficacy of the assay, as the predominance of natural antibodies in the matrix may give rise to inaccurate measurements if any cross-reactivity exists. In the sandwich assay format, each Affimer reagent was highly specific in binding its therapeutic antibody target, showing no cross-reactivity in the presence of high concentrations (1 mg/ml) of alternative mAbs, which contain highly homologous constant domain regions to the other therapeutic antibodies and human IgGs (Figure 2). This highlights the value of the deselection protocols in driving binding towards the idio type and demonstrates the high specificity of the isolated Affimer reagents.

### Affimer PK assay calibration curves assessed against regulatory criteria

The performance of the calibration curves for each anti-idiotypic Affimer sandwich assay was assessed using section III, B, 2 of the US FDA criteria for bioanalytical method validation [54]. Calibration curves were assessed in terms of the accuracy and precision of mAb quantification within a 10% human serum matrix. Triplicate measurements at each nominal concentration (1.95–2000 ng/ml as twofold dilutions) of target were performed and a four-parameter logistic regression was fitted as a calibration curve. For each measurement, an interpolated concentration was then back-calculated.

**Table 2. Calibration curve accuracy and precision metrics.**

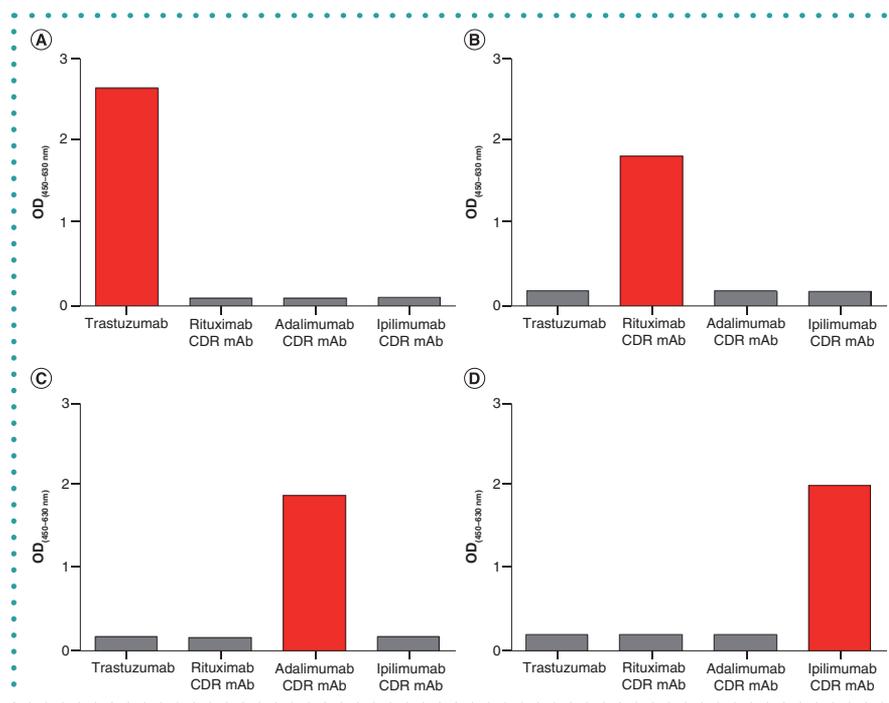
		Trastuzumab	Rituximab CDR mAb	Adalimumab CDR mAb	Ipilimumab CDR mAb
Quantifiable range (ng/ml)	ULOQ	1000	1000	1000	1000
	LLOQ	ca. 8	ca. 2	ca. 4	ca. 15
Inter-assay	% CV	2.3–6.2	2.0–19.4 <sup>†</sup>	1.4–7.1	2.7–11.4
	% recovery	98.1–104.5	95.8–104.6	98.4–106.1	94.4–110.9
Intra-assay	% CV	2.8–24.9 <sup>†</sup>	0.7–19.5	1.2–17.3 <sup>†</sup>	0.3–12.0
	% recovery	96.2–109.6	84.0–122.3 <sup>†</sup>	95.4–112.7	91.7–122.6 <sup>†</sup>

<sup>†</sup>Values approaching FDA limits obtained at a limit of quantification.

CDR: Complementary-determining region; LLOQ: Lower limit of quantification; mAb: Monoclonal antibody; ULOQ: Upper limit of quantification.

lated from this curve. Intra-assay accuracy was determined by percentage recovery; the mean interpolated concentration as a percentage of the nominal concentration. Intra-assay precision was determined by the coefficient of variation (% CV); the standard deviation of the interpolated concentration as a percentage of the mean. This was repeated in a total of three separate experiments and the local means used to calculate inter-assay accuracy (% recovery) and precision (% CV). Each of the calibration curves for the four anti-idiotypic Affimer reagents meet the following FDA performance requirements; at least 75% and a minimum of six non-zero standards (inside the anchor points) have intra-assay and inter-assay calibration metrics of  $\leq 20\%$  CV and 80–120% recovery, with  $\leq 25\%$  CV and 75–125% recovery at the lower and upper limits of quantification (LLOQ/ULOQ) (Table 2) [54]. The LLOQ and ULOQ are the lowest and highest amount of analyte that can be quantitatively determined with acceptable precision and accuracy, giving the quantifiable range for each therapeutic mAb.

The quantifiable range of ipilimumab CDR mAb is comparable to that of a commercially available ELISA kit (10–1000 ng/ml) [55]. The other Affimer ELISAs offer a wider quantifiable range than commercially available ELISA kits for trastuzumab (11–300 ng/ml), rituximab (3–300 ng/ml) and adalimumab (30–1000 ng/ml) [56–58]. As the anti-idiotypic Affimer-based assay calibration curves meet accuracy and precision standards over wide detection ranges, this reduces the need for dilutions and repetitions, allowing for a wider range



**Figure 2.** Specificity of therapeutic antibody detection in a sandwich ELISA using (A) anti-trastuzumab (B) anti-rituximab CDR mAb (C) anti-adalimumab CDR mAb and (D) anti-ipilimumab CDR mAb Affimer reagent capture surfaces. For each capture surface, detection of 1 mg/ml trastuzumab, rituximab CDR mAb, adalimumab CDR mAb and ipilimumab CDR mAb was compared in a 10% human serum matrix. Anti-hlgG-HRP was used as detection reagent, with TMB substrate visualization read at 450 nm (minus 630 nm reference).

CDR: Complementary-determining region; mAb: Monoclonal antibody.

of samples to be analyzed within a single assay. Furthermore, this is achieved with just a single specific capture reagent and universal detection format, rather than requiring two separate specific anti-idiotypic reagents, as in the case of many PK bridging assays [24]. Antibodies are not limited to the bridging format but sufficiently high-quality reagents are required.

### Consistent lot-to-lot reproducibility in the performance of Affimer reagents

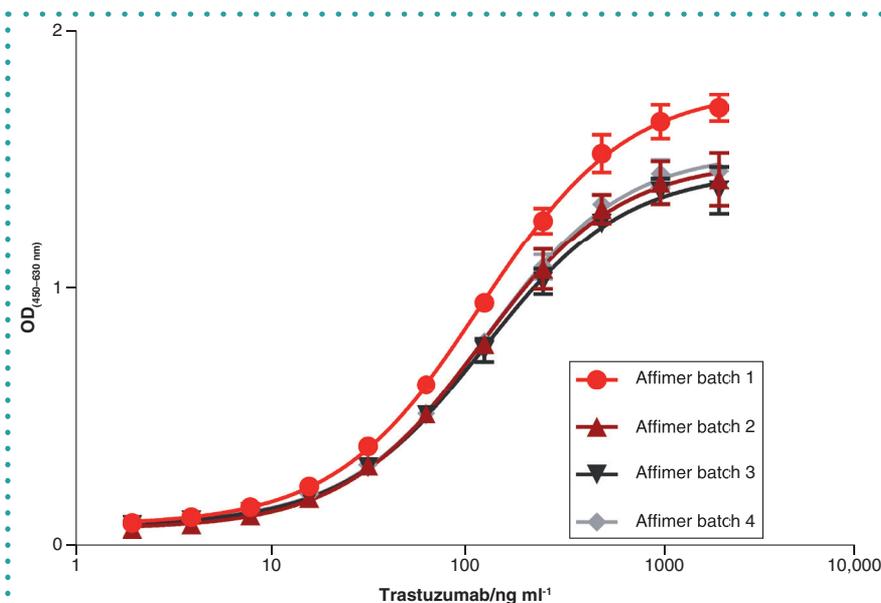
Once validated for use in PK assays, it is important that a reliable and consistent supply of the anti-idiotypic reagent is available over the course of clinical development and usage. Anti-idiotypic antibodies can suffer from poor lot-to-lot reproducibility; therefore, extensive standardization may be required between lots to ensure ▶

**Table 3. Anti-Trastuzumab Affimer batch accuracy and precision metrics.**

Trastuzumab batch comparison					
Calibration range (ng/ml)		Inter-batch		Intra batch	
LLOQ	ULOQ	%CV	%Recovery	%CV	%Recovery
Ca. 8	1000	0.9–13.7	99.0–117.9 <sup>†</sup>	2.2–20.0 <sup>†</sup>	92.8–120.1 <sup>†</sup>

<sup>†</sup>Values approaching FDA limits obtained at a limit of quantification.

LLOQ: Lower limit of quantification; ULOQ: Upper limit of quantification.



**Figure 3.** Lot-to-lot reproducibility of the anti-trastuzumab Affimer reagent as a capture surface in a sandwich ELISA. For four separate lots of anti-trastuzumab Affimer capture reagent the dose–response curves of trastuzumab in a 10% human serum matrix are compared. Anti-hIgG-HRP was used as detection reagent, with TMB substrate visualization read at 450 nm (minus 630 nm reference). Data points are the mean of triplicate measurements and error bars indicate standard deviation from the mean.

assay performance is maintained [12,25]. This can lead to delays in the drug development process. Affimer reagents should offer low lot-to-lot variability due to their simple and robust bacterial manufacturing process. The reproducibility of the anti-trastuzumab Affimer binder was assessed as an example of lot-to-lot consistency in these reagents. Four separate lots were manufactured and compared by analyzing the consistency of the sandwich ELISA calibration curves (Figure 3) in terms of accuracy and precision.

With each Affimer lot, triplicate measurements of 1.95–2000 ng/ml trastuzumab (twofold dilution series) were made in 10% human serum (Figure 3) and concentrations then back-calculated from the calibration curve. Although the curve for lot 1 was slightly higher than the other

three assessed, the interpolated values of each curve (intra-curve), as well as the mean of all four curves (inter-curve), met the criteria for accuracy and precision, within the previously validated quantifiable range (7.8–1000 ng/ml) (Table 3). This confirms lot-to-lot consistency in assay performance and demonstrates the high reproducibility of the Affimer reagent. Anti-idiotypic Affimer proteins therefore offer the assurance of supply required for critical reagents in the bioanalysis of potential therapeutics, preventing delays due to extensive lot-to-lot normalization.

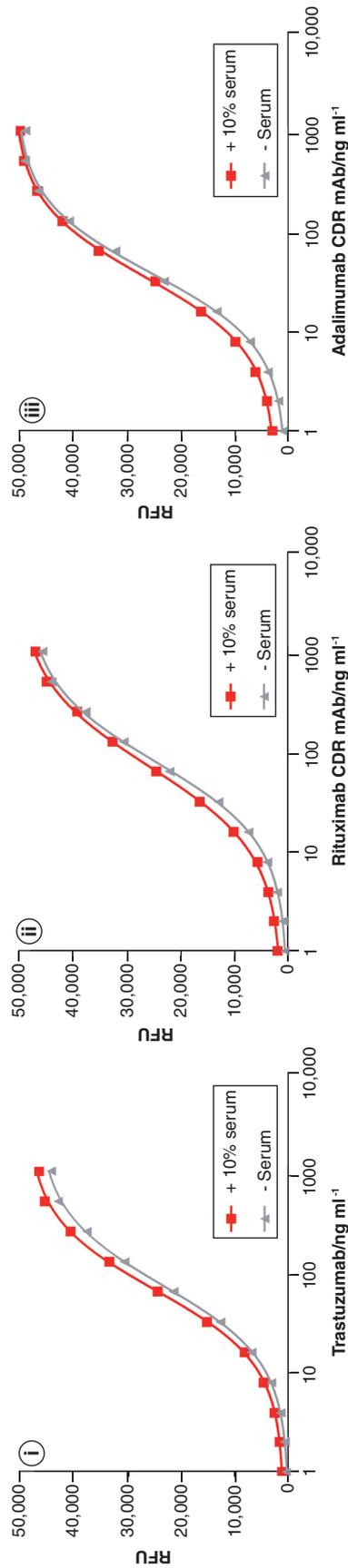
### Comparison of Affimer- & Fab-based PK assay performance

Anti-idiotypic Fabs are alternative reagents that can be selected *in vitro* and produced recombinantly [11,59]. The selected anti-

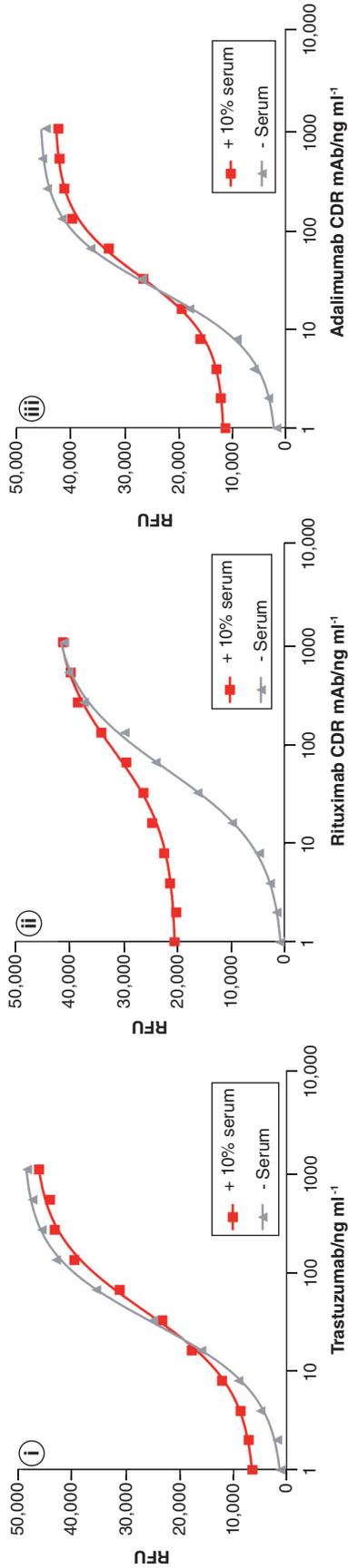
idiotypic Affimer reagents were compared with equivalent Fab reagents, as capture reagents for trastuzumab, rituximab CDR mAbs and adalimumab CDR mAbs, in a sandwich ELISA (Figure 4). Affimer proteins were coated at 1 µg/ml and Fabs at 5 µg/ml, to generate approximately equivalent molar concentrations of capture reagent (~70 nM and 96 nM, respectively). An Fc specific detection reagent, HRP-conjugated anti-human IgG (Fc) CH<sub>2</sub> domain antibody (anti-hFc-HRP), was used to prevent cross-reactivity with the Fab and the fluorogenic substrate QuantaBlu was used for visualization. Using this detection reagent, each Affimer capture reagent achieved a broad dynamic range for target detection with no clear sample matrix effects observed in 10% human serum (Figure 4A). The Fab capture reagents displayed reduced dynamic ranges for the detection of each target (Figure 4B). Greater sample matrix effects were observed in 10% human serum with the anti-adalimumab Fab and particularly the anti-rituximab Fab, compared with the equivalent Affimer binder (Figure 4B, ii & iii). This reduces the dynamic range of the Fab reagents still further in this assay format and may lead to inaccurate analysis in patient samples due to serum cross-reactivity issues.

The anti-idiotypic Affimer reagents therefore exhibit increased target specificity relative to the Fabs. A single capture Affimer reagent offers such specificity that a humanized therapeutic antibody can be detected in a background of human IgGs in serum, using a universal anti-human FC (anti-hFc) detection antibody. The Fab capture surface offers insufficient specificity for the use of a universal detection format; cross-reactivity leads to the capture of serum hIgGs and their detection by anti-hFc leads to matrix effects. This issue is not inherent to antibodies, but a sufficiently high-quality reagent would need to be

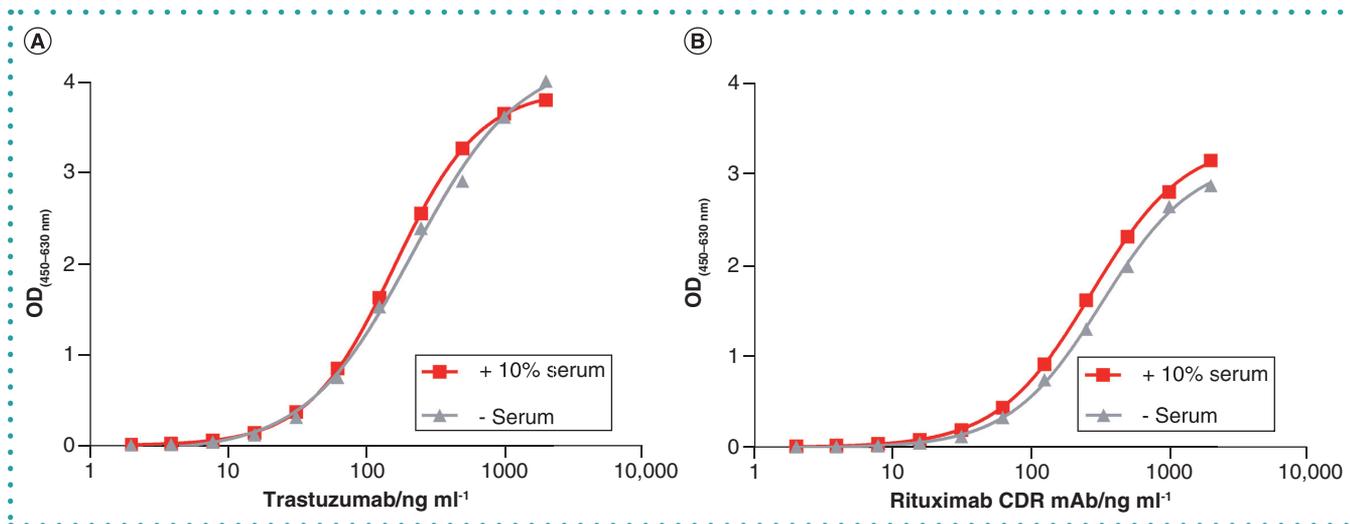
**(A) Anti-idiotypic Affimer capture reagent**



**(B) Anti-idiotypic Fab capture reagent**



**Figure 4.** Comparison of (A) anti-idiotypic Affimer reagents and (B) anti-idiotypic Fabs as capture reagents for (i) trastuzumab (ii) rituximab CDR mAbs and (iii) adalimumab CDR mAbs in a sandwich ELISA. Each anti-idiotypic Affimer capture reagent was coated at 1  $\mu$ g/ml (70 nM) and each anti-idiotypic Fab coated at 5  $\mu$ g/ml (96 nM). Dose-response curves were obtained for the target therapeutic antibody in buffer (gray) and a 10% human serum matrix (red). Anti-hFc-HRP was used as detection reagent, with fluorogenic QuantaBlu substrate visualization. CDR: Complementary-determining region; Fab: Antigen-binding fragment; mAb: Monoclonal antibody.



**Figure 5.** Sandwich ELISA dose response curves using anti-hIgG Affimer as detection reagent with (A) anti-trastuzumab and (B) anti-rituximab CDR mAb Affimer reagent capture surfaces. Therapeutic antibodies were detected in buffer (gray) and a 10% serum matrix (red). Visualization was with TMB substrate read at 450 nm (minus 630 nm reference).

generated. Otherwise, in this case a bridging assay format, using the anti-idiotypic Fab as capture and detection reagent, would be necessary to enhance specificity. However, bridging assays often require optimization of a low-capture reagent coating density, to prevent bivalent binding of the therapeutic antibody that precludes binding of the anti-idiotypic detection reagent [12]. The use of such low-capture reagent concentrations leads to low sensitivities and high susceptibility to inconsistencies [12]. To avoid this, two different anti-idiotypic reagents with non-overlapping binding sites would need to be found, which is very unlikely due to steric issues. Hence, the universal detection format afforded by the anti-idiotypic Affimer capture approach is extremely desirable and less optimization should be required from assay to assay. Furthermore, the enhanced dynamic range offered by the Affimer reagents reduces the need for multiple dilutions and minimizes repetition of measurements.

### Universal Affimer detection reagent

Both anti-hIgG-HRP and anti-hFc-HRP antibodies have been successfully used as detection reagents, alongside anti-idiotypic Affimer capture reagents. Further flexibility in detection format was demonstrated by the use of a universal anti-hIgG Affimer detection reagent (Figure 5), giving an Affimer-only assay format. The anti-hIgG Affimer was biotinylated and pre-incubated

with streptavidin-HRP prior to detection and visualization with TMB.

For detection of both trastuzumab and rituximab CDR mAbs, minimal matrix effects were observed in 10% serum (Figure 5) and the dynamic ranges were comparable to those obtained with anti-hIgG antibodies. The intra-assay curve metrics for trastuzumab in 10% serum gave a quantifiable range of 2–2000 ng/ml, with  $\leq 18.0\%$  CV and 93.9–115% recovery. In 10% serum rituximab CDR mAb had a quantifiable range of 7.8–2000 ng/ml, with  $\leq 17.7\%$  CV and 95.3–110.7% recovery. This again demonstrates the excellent specificity of the Affimer capture surfaces, allowing a number of different universal detection formats to be successfully employed, which offers assay developers flexibility to suit individual assay requirements. The Affimer sandwich assay format obviates the need for antibodies and each binding reagent is an easily manufactured recombinant protein. Furthermore, the detection reagent is universal, so should be applicable across assays, simplifying assay development and reagent manufacture.

The generation of anti-idiotypic affinity reagents against therapeutic mAbs presents a particular challenge. In order to specifically recognize a humanized therapeutic antibody within the high background of human IgGs present in serum, reagents must be highly specific for the mAb idiope. Here we demonstrate Affimer reagents to be easily selected and manufactured

non-immunoglobulin reagents that can address this challenge. Anti-idiotypic Affimer reagents against trastuzumab and CDR mAbs of rituximab, adalimumab and ipilimumab therapeutic antibodies have been identified and characterized. A simple negative selection protocol successfully drove binding towards the target idiope, generating highly specific Affimer binders that display minimal matrix effects in 10% human serum and no cross-reactivity with nonspecific mAbs, without any need for affinity maturation. Compared with equivalent Fabs, the anti-idiotypic Affimer capture surfaces offered such exquisite specificity that a flexible universal detection format (anti-hIgG antibody, anti-hFc antibody or anti-hIgG Affimer) can be used, avoiding bridging assays and simplifying assay development. A sandwich ELISA using anti-idiotypic Affimer capture and universal anti-hIgG-HRP antibody detection was developed within a 10% human serum matrix. Calibration curves met the FDA criteria for accuracy and precision over favorable dynamic ranges in comparison to Fabs and commercially available kits, confirming the applicability of anti-idiotypic Affimers to PK assays. The lot-to-lot consistency of the anti-trastuzumab Affimer was also confirmed, giving assurance of supply.

Overall, it is clear that the Affimer platform can be reliably used to develop anti-idiotypic reagents for immunoassays, to measure relevant concentrations of thera-

peutic mAbs with suitable accuracy and precision metrics for calibration curves. As well as being simple to identify and easy to produce, the anti-idiotypic Affimer reagents offer performance improvements in terms of high specificity, low matrix effects, broad dynamic ranges, flexible universal detection formats and low lot-to-lot variation. The fast reagent development time, ease of assay development and assurance of supply make the highly specific anti-idiotypic Affimers promising reagents to meet the demands of clinical development timelines and prevent delays, particularly in the growing biosimilars arena, where speed to market is essential. The development of antibody therapeutics continues to expand and anti-idiotypic Affimer reagents offer the potential for critical partner PK assays.

## FUTURE PERSPECTIVE

Increasing numbers of biologics entering drug development and achieving regulatory approval, combined with the rising rates of patients suffering with chronic diseases, ensures that critical reagents for pharmacokinetic studies will remain an essential building block within the drug development pipeline. Current pharmacokinetic assay reagents can be costly to produce and require long development times, which hinder the drug development process. As the technology driving non-immunoglobulin alternatives to antibodies expands, bioanalysts will continue to explore both antibody and non-antibody-based critical reagents when establishing PK and therapeutic drug monitoring assays. Reagent selection will depend upon superior performance, delivery speed and simple and cost-effective assay design and development. We foresee that these critical reagents may also have application in both drug monitoring and patient selection in the future.

## AUTHOR CONTRIBUTIONS

HA, CT and AAT performed phage display and isolation of Affimer reagents. AN, AD, HD, AW, RF and JN performed the ELISAs and validation experiments. HA, MJM, MJ and DCT conceived the idea, supervised the experiments and wrote the paper. All the authors read and edited the manuscript.

## FINANCIAL & COMPETING INTERESTS DISCLOSURE

MJM and DCT: Inventors of the Adhiron/Affimer technology and own personal shares in Avacta Life Sciences. The Adhiron patent (patent application number PCT/GB2014/050435) is owned by the University of Leeds and licensed to Avacta Ltd. AM, AD, HC, AW, RF, JN and MJ all work for Avacta Life Sciences. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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