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# Evaluation of an immunoassay for human-specific quantitation of therapeutic antibodies in serum samples from non-human primates

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ABSTRACT

Pharmacokinetic characterization of therapeutic antibodies plays an important role during preclinical and clinical development. However, accurate pharmacokinetic evaluation of therapeutic antibodies in serum samples from non-human primates is often complicated by insufficient specificity of the assays to measure drug levels. The present paper describes the use of a murine monoclonal antibody in an immunoassay format to specifically and quantitatively measure human therapeutic antibodies in serum from non-human primates. This murine antibody is directed against a unique epitope on the constant region CH2 domain of all isotypes of human immunoglobulin G (IgG). The antibody, designated anti-human Fcγ-pan: R10Z8E9, does not cross-react with serum from mouse, rat, and the non-human primates marmoset, rhesus macaque, cynomolgus monkey and baboon when using an enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance technology (Biacore) format for measurement of the therapeutic antibody. Use of the antibody anti-human Fcγ-pan: R10Z8E9 as capturing and detection reagent allowed human-specific quantitation of total therapeutic antibody anti-IGF-1R in spiked cynomolgus monkey serum via a Sandwich ELISA format. In contrast, a commercially available polyclonal antibody (PAB) directed to the Fcγ fragment of human IgG only specifically measured the therapeutic antibody in buffer samples, but not in serum from cynomolgus monkeys. This generic human IgG assay was already applied in several pharmacokinetic studies in cynomolgus monkeys to determine serum levels of different therapeutic antibodies, including the anti-IGF-1R. Validation of the assay for a humanized IgG1 therapeutic antibody against a membrane protein revealed a lower limit of quantitation of 8 ng/mL in undiluted serum. Intra-assay and inter-assay precision was characterized by a coefficient of variation of less than 10% and accuracy was within 15%. Dilutional linearity was evidenced by a recovery of 98.7–114% of expected concentrations. In conclusion, the monoclonal antibody anti-human Fcγ-pan: R10Z8E9 provides a standard means for human-specific quantitation of therapeutic antibodies with high sensitivity in serum samples from non-human primates in a generic human IgG assay.



## 1. Introduction

Monoclonal antibodies are now established as a key therapeutic modality for a range of diseases [1]. Pharmacokinetic properties play an important role in the development of these macromolecules prompting the need for accurate pharmacokinetic evaluation of therapeutic antibodies which is often complicated by a number of factors [2]. Bioanalytical methods are essential for any pharmacokinetic study, but for many therapeutic proteins and antibodies the immunoassay and assay methodologies are often insufficient due to lack of specific assay reagents. Sometimes the estimation of pharmacokinetic parameters becomes even assay dependent.

Phage display and transgenic mice technology platforms have increasingly been used to create fully human antibodies for development and the first fully human therapeutic monoclonal antibodies meanwhile have been approved [3]. Preclinical pharmacokinetic characterization of these therapeutic monoclonal antibodies often has to be performed in non-human primates because of insufficient target cross-reactivity of the therapeutic antibody in rodent and non-rodent laboratory animals, such as mouse, rat, rabbit and dog. The cynomolgus monkey is a preferred non-human primate for preclinical studies with therapeutic antibodies because it often provides sufficient cross-reactivity of the target with the therapeutic antibody. However, non-human primates such as the cynomolgus monkey show a high sequence homology of their immunoglobulins with human immunoglobulins. Immunoglobulin variable region genes from cynomolgus monkeys were shown to have 85–98% homology with human immunoglobulin sequences [4]. The sequence of cynomolgus mon-

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key immunoglobulin G (IgG) constant region was 95, 93, 95 and 95% similar to that of the human IgG1, IgG2, IgG3 and IgG4 constant regions [5]. This high protein sequence homology for IgG constitutes a disadvantage for the bioanalytical measurement of the concentration of human therapeutic antibodies in non-human primate serum samples because it leads to an assay interference by unspecific binding of the matrix IgG. The background concentration of about 10–30 mg/mL of immunoglobulin in the serum of cynomolgus monkeys [6] is in an excess of about 100-fold to up to 10 million-fold compared with typical therapeutic antibody serum concentrations of between 1 ng/mL and 100 μg/mL [7,8] and, thus, can influence the specificity of the bioanalytical assay.

In case that the bioanalytical assay for measurement of serum concentrations of therapeutic antibodies employs the soluble antigen for capture or detection of the analyte, i.e. the therapeutic antibody, the impact of the high sequence homology and high background concentration may consist in reducing the sensitivity of the bioanalytical assay. However, in many cases a soluble antigen is not available for assay purposes because it may be too expensive or because it forms part of a membrane receptor, e.g. the chemokine receptor CCR5, and cannot be expressed as a binding-competent antigen with proper folding. In such situations, bioanalytical assays for measurement of antibody serum levels make use of anti-idiotypic antibodies for capture or detection of the analyte. However, such anti-idiotype antibodies specifically have to be generated for each new therapeutic antibody in a time- and cost-consuming manner. Thus, there is a need for improved methods using assay reagents which do not cross-react with the host's background and can be used in a generic way, i.e. independent of the target-specificity of the novel therapeutic antibody.

The present paper reports such an improved method developed for human-specific quantitation of therapeutic antibodies in serum from non-human primates. This immunoassay makes use of a capture and detection antibody highly specific for an epitope found on all sub-classes of human immunoglobulin G which does not cross-react with IgG from non-human primates. The experiments reported in the present work demonstrate by two different methods the specificity of the human-specific antibody in comparison with other commercially available anti-human IgG antibodies which cross-react with primate IgG. This human-specific antibody is also shown to lack cross-reactivity with rodent and other non-rodent animal IgG and may extend its future application to other animal species. In this work, the generic human IgG enzyme-linked immunosorbent assay (ELISA) was developed and validated for the determination of a membrane-targeting therapeutic monoclonal antibody (MAb 1) in cynomolgus monkey serum and its application to measure the serum concentration of a second therapeutic antibody (anti-IGF-1R) after administration to cynomolgus monkeys is presented, thus demonstrating the generic applicability of the ELISA.

Although recently a Generic Immunoglobulin Pharmacokinetic assay has been used to quantify a therapeutic antibody in 10% cynomolgus monkey serum by use of sheep polyclonal antibodies (PABs) against human IgGs which were stripped of cynomolgus monkey cross-reacting antibodies [9], the generic human IgG assay described here confers the advantage of using a monoclonal antibody against human IgG and the associated long-term supply security, and consistent batch quality. Furthermore, it reacts with a defined epitope on the CH2 domain of IgG and no separation steps are required to achieve limited cross-reactivity. In summary, the monoclonal antibody anti-human Fcγ-pan: R10Z8E9 provides a standard means for human-specific quantitation of therapeutic antibodies with high sensitivity in serum samples from non-human primates.

## 2. Experimental

### 2.1. Chemicals and reagents

The therapeutic monoclonal antibody used in the experiments was a recombinant fully human monoclonal IgG1 antibody directed against the insulin-like growth factor-1 receptor (anti-IGF-1R). The therapeutic antibody was produced at Roche Diagnostics GmbH, Penzberg, Germany. The human-specific murine IgG1 monoclonal antibody was directed against a conformation epitope on the CH2 domain of all four sub-classes of human Fcγ [10] as evidenced by KD values in the range from $3.55 \times 10^{-9}$ to $3.88 \times 10^{-10}$ M (IgG1: $1.77 \times 10^{-10}$ M; IgG2: $3.88 \times 10^{-10}$ M; IgG3: $3.55 \times 10^{-9}$ M; IgG4: $3.18 \times 10^{-10}$ M; measured by Biacore). This antibody was designated anti-human Fcγ-pan: R10Z8E9. The antibody-producing cell line was provided by Roy Jefferis, University of Birmingham, and was deposited at Deutsche Sammlung von Mikroorganismen and Zellkulturen, DSMZ, Braunschweig, Germany, as DSM ACC2708. The antibody is available upon request by contacting Roy Jeffries and reimbursement of the associated costs. Serum samples from one chimpanzee, seven rhesus macaques, five marmoset monkeys and four baboons were obtained by standard methods from individual zoo animals. Serum samples from 20 different individual cynomolgus monkeys were provided by Covance, Muenster, Germany. Batches of pooled cynomolgus monkey sera for use as matrix in the ELISA for calibration standards (CS) and quality control (QC) samples as well as for dilution of analyte samples were from Covance, Muenster, Germany, and from Bioreclamation Inc., Hicksville, NY, USA. Serum from dog, rat and CD1 and NMRI mice was obtained from commercial sources (Charles River, Wilmington, MA, USA). Human serum samples were provided by the serum bank of Roche Diagnostics GmbH, Penzberg, Germany. All serum samples were stored at −80 °C. Dako GmbH, Hamburg, Germany, was the supplier of the polyclonal anti-human IgG-Bi antibody (cat. no. E0428) and of the polyclonal anti-human IgG:F(ab)'2-HRP (cat. no. P0406). Chemicon (now Millipore), Billerica, MA, USA was the supplier of the monoclonal anti-human IgG MK1A6 (cat. no. CBL101) and of the monoclonal antibody anti-human IgG-HRP (cat. no. AP113P). Dianova, Hamburg, Germany, delivered the monoclonal anti-human IgG:F(ab)'s-HRP (cat. no. 109-066-098). All other labelled and unlabelled antibodies were from Roche Diagnostics GmbH, Mannheim, Germany. The following reagents were also obtained from Roche Diagnostics GmbH, Mannheim, Germany: phosphate-buffered saline (PBS)-polysorbate 20 (Tween 20) (cat. no. 11332465-001), bovine serum albumin (BSA) (cat. no. 11726536-001) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (cat. no. 11684302-001). All other chemicals were of analytical grade.

### 2.2. Enzyme-linked immunosorbent assay for determination of specificity of binding

An enzyme-linked immunosorbent assay was used to evaluate the *specificity of binding* to human immunoglobulin (IgG) of various anti-human IgG antibodies in comparison with cynomolgus monkey serum and to evaluate the cross-reactivity of the human IgG-specific monoclonal antibody anti-human Fcγ-pan: R10Z8E9 versus various animal species. The ELISA was performed on microtiter plates (MTP, Maxisorp, from Nunc, Germany) at room temperature which were first coated with cynomolgus monkey serum or human serum diluted to 20% in carbonate buffer (pH 9.6) for 1 h. After washing three times with phosphate-buffered saline-polysorbate 20 (Tween 20), all wells of the MTP were blocked with PBS/3% bovine serum albumin for 1 h. Then the wells of the MTP were incubated for 1 h with different anti-human IgG antibodies (horseradish peroxidase- (HRP-) unconjugated or -conjugated

according to the instructions of the corresponding manufacturers. After washing three times, wells incubated with HRP-conjugates were directly processed for enzymatic reaction/detection of bound anti-human immunoglobulin. The other wells were incubated for 1 h as appropriate with anti-digoxigenin- (Dig)-, anti-mouse IgG- or streptavidin-peroxidase (POD)-conjugates (all reagents from Roche Diagnostics, Germany). The POD comprised in the POD-conjugates catalyzed the color reaction of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate. The signal was measured by an ELISA reader at a wavelength of 405 nm (reference wavelength: 490 nm).

The *cross-reactivity* of the human IgG-specific monoclonal antibody anti-human Fcγ-pan: R10Z8E9 versus different animal species was evaluated in the same MTP-ELISA format as described above. The wells of an MTP were coated with serum from the respective animal and the assay was performed as described above using the Dig-labelled monoclonal antibody anti-human Fcγ-pan: R10Z8E9 to detect human IgG. An anti-Dig POD-labelled antibody was used to catalyze the colorigenic reaction with substrate ABTS.

### 2.3. Biacore assay for determination of specificity of binding

The specificity of the murine anti-human IgG monoclonal antibody anti-human Fcγ-pan: R10Z8E9 was evaluated in a *second assay system*. These experiments were performed with the Biacore® 2000 instrument (Biacore, Uppsala, Sweden) using a CM5 sensor chip (BR-1000-14; Biacore, Uppsala, Sweden). Coating of an antibody to this chip was achieved by standard amine coupling. Unless otherwise stated, all incubations were performed in HBS buffer (HEPES, NaCl, pH 7.4) at 25 °C. A saturating amount of anti-human Fcγ-pan: R10Z8E9 and polyclonal anti-human Fc antibody (Dianova, Hamburg, Germany), respectively, was immobilized by amine coupling on different flow cells of the same CM5-chip. All animal sera were diluted in HBS buffer containing 1 mg/mL CM-dextran at a final concentration of 1%. Binding was analyzed by injection of the 1 in 100 diluted sera and incubation for 60 s. Dissociation was measured by washing the chip surface with HBS buffer for 180 s. Using BIAevaluation software V4.1 from Biacore, Sweden, the dissociation constant values (=$K_{Diss}$) were calculated with a 1:1 Langmuir fitting model. For all animal sera this calculation was based on the assumption that the IgG level is 15 mg/mL. The signal values 80 s after start of the injection of the test antibody have been chosen for the comparison of the amount of IgG bound (resonance signal units (RU) in Tables 1 and 2).

### 2.4. Preparation of calibration standards and quality control samples

Stock solutions for CS and QC were prepared separately. The CS and QC stock samples used for the evaluation were made in 100% cynomolgus monkey pooled serum and stored as single use aliquots at −20 °C. The CS stock sample was spiked with MAb 1 to achieve a concentration 100-fold higher than that of the highest calibrator standard. At the time of assay, a single use aliquot of the stock solution was thawed and stepwise 1:2 diluted to yield calibrator concentrations of 500, 250, 128, 64, 32, 16 and 8 ng/mL. Four separate QC samples were prepared at stock concentrations representing high (375 ng/mL), medium (200 ng/mL) and low (24 ng/mL) undiluted serum concentrations and the LLOQ (8 ng/mL).

### 2.5. Enzyme-linked immunosorbent assay set-up for validation

The suitability of the anti-human Fcγ-pan: R10Z8E9 antibody for quantitation of the total serum concentration of active and inactive, i.e. *total therapeutic antibodies*, was evaluated in an MTP-ELISA format (Fig. 1). In this generic human IgG assay, all antibody

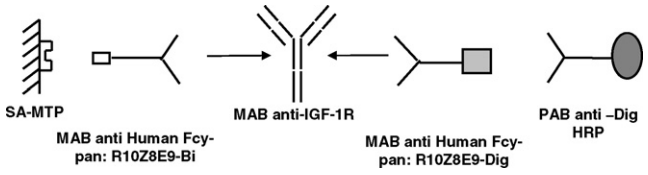



**Fig. 1.** Measurement of total therapeutic antibody anti-IGF-1R in an MTP-ELISA format.

solutions were prepared in dilution buffer containing 50 mM potassium phosphate buffer, with 150 mM NaCl, pH 7.5 and 0.5% bovine serum albumin. All incubation steps were performed at room temperature. To prepare digoxigenylated anti-human Fcγ-pan: R10Z8E9, a solution of the monoclonal antibody (MAB) anti-human Fcγ-pan: R10Z8E9 in phosphate buffer was adjusted to pH 8.5 and to a concentration of about 2 mg/mL. Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester was dissolved in DMSO and added to the antibody solution in a molar ratio of 1:5. The reaction was stopped after 60 min by adding L-lysine, and the surplus of the labelling reagent was removed by dialysis against 50 mM potassium phosphate buffer, with 150 mM NaCl, pH 7.5. Biotinylated MAB anti-human Fcγ-pan: R10Z8E9 or polyclonal antibody directed against human Fc was bound to streptavidin-coated microtiter plates (SA-MTP), obtained from MicroCoat Biotechnologie GmbH, Bernried, at a concentration of 0.5 μg/mL, in the first step. This solution was incubated for 1 h. The excess of unbound antibody was removed by washing. Samples and standards obtained by spiking cynomolgus serum (5%, v/v) with the therapeutic humanized IgG1 monoclonal antibody directed against a membrane protein (designated MAb 1) were simultaneously pre-incubated with 0.05 μg/mL of digoxigenylated MAB anti-human Fcγ-pan: R10Z8E9 (anti-human Fcγ-pan: R10Z8E9-Dig) for 1 h. Pre-incubation was preferred over a sequential procedure for its increased sensitivity. Thereafter, the mixture was added to wells of a SA-MTP coated with the biotinylated anti-human IgG antibodies and incubated for 1 h. After washing, a polyclonal anti-digoxigenin-horseradish peroxidase conjugate (Roche, cat. no. 11633716001) was used to detect the bound digoxigenylated MAB anti-human Fcγ-pan: R10Z8E9. A dilution of 50 mU was incubated for 1 h. The HRP of the antibody–enzyme conjugates catalyzed the color reaction of ABTS substrate. The signal was measured by ELISA reader at 405 nm wavelength (reference wavelength: 490 nm). Absorbance values of each serum sample were determined in triplicates.

### 2.6. Validation of the analytical method

The generic human IgG assay was validated for the bioanalytical measurement of the concentration of MAb 1 in 5% cynomolgus serum samples. Previously, pilot experiments had been performed with anti-IGF-1R to evaluate the influence of the percentage of serum in the sample (2.5, 5, 10 and 20% versus buffer) on the assay read out. Four independent calibration curve preparations with seven calibrator samples (stock concentrations diluted 1:20 in the assay to yield final concentration range of 25–0.39 ng/mL) and a blank sample were measured in duplicates on one plate to define a suitable range for accurate measurement. Four independent assays were performed to take into account a potential variability by dilution of the calibrators. The performance of four assay runs and duplicate measurements of each sample results in a total of eight measurements thought to be suitable for statistical evaluation. The limit of detection (LOD) was defined as the concentration of analyte corresponding to twice the mean OD value of the blank. According to the FDA guideline for bioanalytical method validation [11], the LLOQ of the ELISA was defined as the lowest concentration of a validation sample which can be determined with an accuracy and

precision of 75–125% and less than 25% CV. One vial of each QC sample was freshly thawed and diluted with assay buffer to 5% serum content (1:20) to yield final concentrations of 18.75, 10, 1.2 and 0.4 ng/mL. To determine intra-assay precision and accuracy, one calibration curve and six replicates (from six separate vials) of each QC were measured on one plate in duplicates. Results were calculated as percent recovery from target concentration (accuracy) and percent coefficient of variation (precision). Inter-assay precision and accuracy was calculated by separate analysis of QC duplicates in 11 independent assays. Dilutional linearity was assessed by preparing spiked serum samples with a concentration that was 1000-fold higher than the highest calibrator (25 ng/mL) and stepwise diluted 1:2 in assay diluent containing 10% pooled serum as standard matrix. Matrix effects in individual serum samples were evaluated by spiking 10 individual neat serum samples (five female, five male) with the reference standard at high and low QC concentrations and compared to the respective unspiked (blank) and QC samples.

### 2.7. Application of the generic ELISA to analysis of serum samples from cynomolgus monkeys dosed with anti-IGF-1R

The method was applied to the determination of primate serum concentrations from one male and one female cynomolgus monkey following a single intravenous administration of 1 mg/kg anti-IGF-1R. Blood samples were taken from an iv line into a test tube before and repeatedly after administration of the therapeutic antibody and stored at room temperature for at least 30 min to allow clotting. Serum was prepared by centrifugation at 1000 × *g* for 15 min and aliquots were taken, immediately frozen and stored at −80 °C until assayed. Before analyzing the serum samples, the generic ELISA was validated for anti-IGF-1R as described previously for MAb 1.

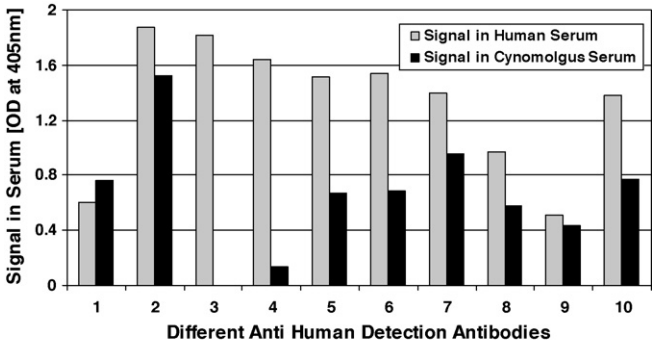
## 3. Results and discussion

### 3.1. Specificity of binding of anti-human Fcγ-pan: R10Z8E9 in the ELISA

The specificity of various anti-human IgG antibodies was evaluated in an MTP-ELISA system. The results showed a 58- and 8.7-fold elevated signal of human serum in comparison with cynomolgus monkey serum for the monoclonal antibody anti-human Fcγ-pan: R10Z8E9 which indicated a high human specificity of this antibody directed against a unique epitope on all isotypes of human IgG (Fig. 2). All other tested antibodies directed against human IgG showed a high cross-reactivity for cynomolgus monkey IgG as evidenced by a signal ratio ranging from 0.84 to 2.20 (Fig. 2). Except for the chimpanzee, the MAB anti-human Fcγ-pan: R10Z8E9 showed a very low cross-reactivity with serum from mice, rat, dog, marmoset, rhesus macaque, cynomolgus monkey and baboon as evidenced by a more than 14-fold elevated signal in human serum versus animal serum in the MTP-ELISA using an anti-Dig POD conjugate for quantitation (Fig. 3).

### 3.2. Specificity of binding of anti-human Fcγ-pan: R10Z8E9 in the Biacore assay

The surface plasmon resonance (SPR) analysis of the different animal sera confirmed the results seen in the MTP-ELISA. The MAB anti-human Fcγ-pan: R10Z8E9 did not cross-react with mice, rat and any monkey species as evidenced by a lack of binding (Tables 1a and 1b). Only the IgG comprised in human and chimpanzee serum was detected. In contrast to the MTP-ELISA, low affinity binding of dog serum to anti-human Fcγ-pan: R10Z8E9 was measured (Table 1a). Cross-reactivity of the anti-human Fcγ-pan: R10Z8E9 with dog serum was about 27% and, thus, the dog should be excluded from application of the assay. The relatively high $K_{Diss}$ for dog IgG (correlating to inferior binding) as compared to human IgG with a $K_{Diss}$ difference of more than 440-fold indicated that this low interaction did not interfere significantly in an immunoassay. In contrast to the anti-human IgG MAB anti-human Fcγ-pan: R10Z8E9, the polyclonal anti-human Fc antibody showed a high cross-reactivity with sera of dog and all tested monkey species.




| | |
|---|---|
| 1 | MAB anti Human IgG; MK1A6 (Chemicon,CBL101) |
| 2 | PAB anti Human IgG-Bi (Dako,E0428) |
| 3 | MAB anti Human Fcγ-pan: R10Z8E9-Dig (Roche) |
| 4 | MAB anti Human Fcγ-pan: R10Z8E9 (Roche) |
| 5 | MAB anti Human Agg-IgG; IgM-Dig (Roche) |
| 6 | MAB anti Human Kappa; R12Z6H9-Dig (Roche) |
| 7 | MAB anti Human IgG: F(ab′)2-HRP (Dianova, no.109-066-098) |
| 8 | MAB anti Human IgG-HRP (Chemicon, no. AP113P) |
| 9 | PAB anti Human IgG: F(ab′)2-HRP (Dako, no. P0406) |
| 10 | MAB anti Human IgG-HRP (Boehringer Mannheim) |

**Fig. 2.** Reactivity of different anti-human antibodies with human and cynomolgus monkey serum in an MTP-ELISA system.

### 3.3. Validation of the generic ELISA

As can be seen in Fig. 4a, in buffer the human-specific MAB anti-human Fcγ-pan: R10Z8E9 and a commercial polyclonal anti-human IgG antibody are equally suitable for determination of the total concentration of the therapeutic anti-IGF-1R antibody, as evidenced by overlapping steep standard curves. However, in the presence of cynomolgus monkey serum, the polyclonal anti-human IgG antibody in contrast to MAB anti-human Fcγ-pan: R10Z8E9 was not

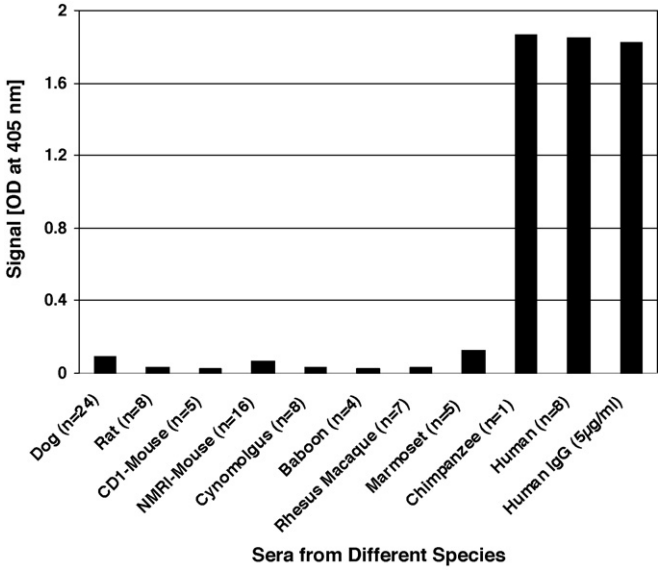



**Fig. 3.** Species cross-reactivity of human IgG-specific MAB anti-human Fcγ-pan: R10Z8E9 using an anti-Dig POD conjugate for quantitation in an MTP-ELISA format (arithmetic mean; sample size indicated in parenthesis).

**Table 1a**
Binding signals (RU) of sera from mouse, rat, dog, cynomolgus monkey and human to anti-human Fcγ-pan: R10Z8E9 MAB and a polyclonal anti-human Fcγ antiserum in the Biacore system and the corresponding dissociation constants ($K_{Diss}$).

| Sample (serum) | MAB anti-human Fcγ-pan: R10Z8E9 | | Anti-human Fcγ PAB (Dianova) | |
|---|---|---|---|---|
| | Bound (RU) | $K_{Diss}$ (M) | Bound (RU) | $K_{Diss}$ (M) |
| Human | 2377 | $1.83 \times 10^{-10}$ | 2399 | $5.64 \times 10^{-11}$ |
| Cynomolgus | 8 | No binding | 1929 | $6.24 \times 10^{-11}$ |
| CD1-mouse | 2 | No binding | 0 | No binding |
| NMRI mouse | 5 | No binding | 3 | No binding |
| Rat | 25 | No binding | 92 | $5.28 \times 10^{-8}$ |
| Dog | 634 | $8.12 \times 10^{-8}$ | 925 | $6.21 \times 10^{-10}$ |

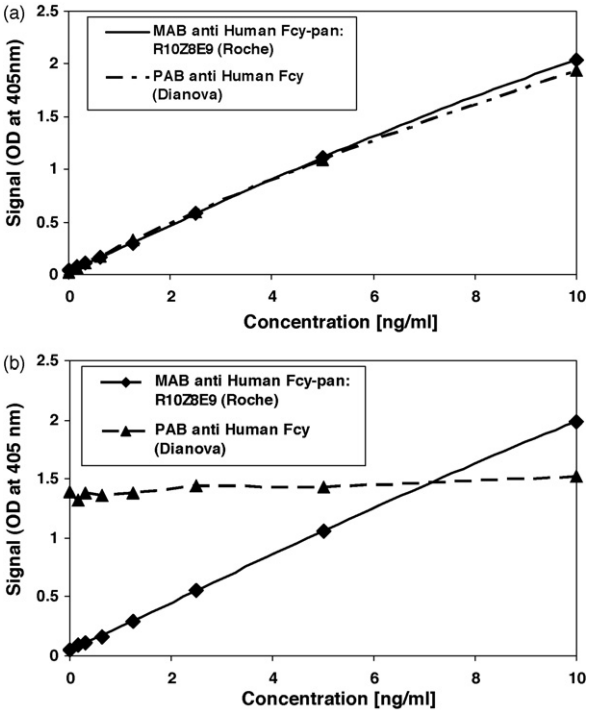



**Fig. 4.** (a) Comparison of standard curves for total therapeutic antibody anti-IGF-1R with MAB anti-human Fcγ-pan: R10Z8E9 or with a polyclonal anti-human Fcγ antibody in buffer (PBS-T, 0.5% BSA) in the generic human IgG assay. (b) Comparison of standard curves for total therapeutic antibody anti-IGF-1R with MAB anti-human Fcγ-pan: R10Z8E9 or with a polyclonal anti-human Fcγ antibody in 5% (v/v) cynomolgus monkey serum in the generic human IgG assay.

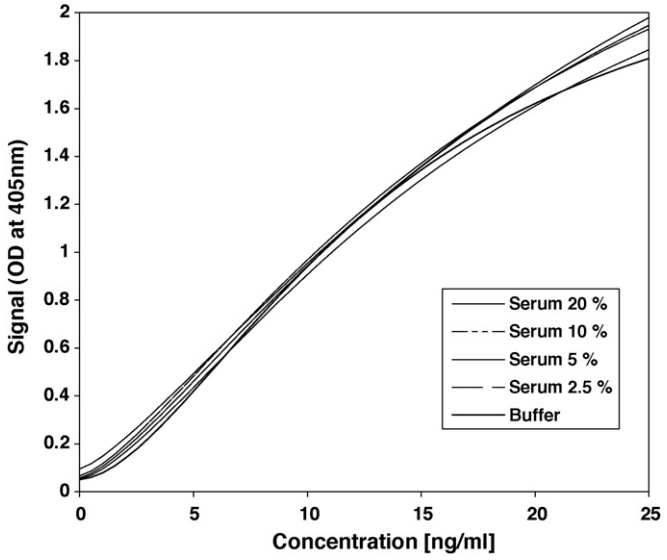



**Fig. 5.** Influence of the concentration of cynomolgus serum on the assay read out (optical density) in the generic human IgG assay for anti-IGF-1R.

**Table 2**
Determination of the limit of detection (LOD) in the generic human IgG assay for MAb 1 in cynomolgus serum (four different calibration runs with duplicate measurements each).

| | Calibrators (ng/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | 0 |
| Mean OD | 1.456 | 0.979 | 0.579 | 0.318 | 0.178 | 0.102 | 0.065 | 0.021 |
| SD | 0.017 | 0.037 | 0.024 | 0.024 | 0.009 | 0.005 | 0.004 | 0.001 |
| CV (%) | 1.1 | 3.8 | 4.1 | 7.5 | 5.0 | 5.3 | 5.7 | 4.4 |

suitable for measurement of the total therapeutic anti-IGF-1R antibody (Fig. 4b). The signals of the standard concentrations for the polyclonal anti-human IgG antibody did not differentiate resulting in a flat, horizontal standard curve.

The pilot experiments on the influence of the concentration of cynomolgus serum in the measurement sample did not reveal relevant differences in the optical density (Fig. 5), and, thus, the 5% cynomolgus serum concentration was selected and kept constant in the measurement samples after dilution. Experiments for determination of the limit of detection in the human generic IgG assay for MAb 1 in cynomolgus serum revealed a LOD of 0.18 ng/mL (Table 2) in the measurement range, corresponding to a LOD of 3.6 ng/mL in undiluted serum samples. The LLOQ was determined at 0.4 ng/mL after dilution in 5% serum corresponding to 8 ng/mL in undiluted

**Table 1b**
Binding signals (RU) of different monkey sera and human serum to anti-human Fcγ-pan: R10Z8E9 MAB and a polyclonal anti-human Fcγ antiserum in the Biacore system and the corresponding dissociation constants ($K_{Diss}$).

| Sample (serum) | MAB anti-human Fcγ-pan: R10Z8E9 | | Anti-human Fcγ PAB (Dianova) | |
|---|---|---|---|---|
| | Bound (RU) | $K_{Diss}$ (M) | Bound (RU) | $K_{Diss}$ (M) |
| Human | 1274.0 | $1.77 \times 10^{-10}$ | 1854.2 | $2.81 \times 10^{-11}$ |
| Cynomolgus 1 | 2.9 | No binding | 1591.9 | $6.64 \times 10^{-11}$ |
| Cynomolgus 2 | 2.8 | No binding | 1413.1 | $5.21 \times 10^{-11}$ |
| Cynomolgus 3 | 6.3 | No binding | 1899.0 | $1.15 \times 10^{-10}$ |
| Baboon | 0 | No binding | 1209.8 | $7.33 \times 10^{-11}$ |
| Marmoset | 5.1 | No binding | 433.9 | $1.02 \times 10^{-9}$ |
| Chimpanzee | 1077.5 | $2.21 \times 10^{-10}$ | 1967.5 | $<1.00 \times 10^{-12}$ |
| Rhesus macaque | −2.9 | No binding | 1409.9 | $4.86 \times 10^{-11}$ |

**Table 3**
Determination of intra-assay and inter-assay precision and accuracy in the generic human IgG assay for MAb 1 in cynomolgus serum.

| | MAb 1 concentration (ng/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Intra-assay ($n = 6$) | | | | Inter-assay ($n = 11$) | | | |
| | High QC | Mid QC | Low QC | LLOQ | High QC | Mid QC | Low QC | LLOQ |
| Spiked (expected concentration) | 18.75 | 10 | 1.2 | 0.4 | 18.75 | 10 | 1.2 | 0.4 |
| Mean of measured concentration | 18.64 | 10.19 | 1.17 | 0.34 | 18.07 | 10.07 | 1.15 | 0.36 |
| SD of measured concentration | 0.22 | 0.46 | 0.05 | 0.02 | 0.57 | 0.53 | 0.08 | 0.03 |
| Precision (%CV) | 1.2 | 4.5 | 4.3 | 5.6 | 3.2 | 5.2 | 6.9 | 9.7 |
| Accuracy (%recovery) | 99.4 | 101.9 | 97.5 | 85.9 | 96.4 | 100.7 | 95.5 | 88.9 |
| Accuracy range (%recovery) | 97.8–101.3 | 96.8–109.3 | 93.0–102.3 | 81.5–92.3 | 90.6–100.3 | 93.4–111.3 | 83.5–107.2 | 78.6–107.6 |

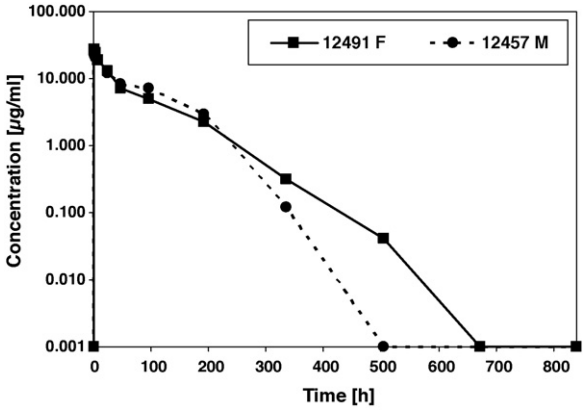



**Fig. 6.** Individual serum concentration–time curves for anti-IGF-1R administered to one female (12491F) and one male (12457M) cynomolgus monkey and measured with the generic human IgG assay.

serum with precision and accuracy data still in the acceptable range of less than 25% (Table 3). Intra-assay and inter-assay precision and accuracy values are summarized in Table 3. Intra-assay and inter-assay precision for high, medium and low QC concentrations showed mean CV values of less than 10%. Intra-assay and inter-assay accuracy for all three QC concentrations showed mean recovery values of 95.5–101.9%. Dilutional linearity was evidenced by recovery of 98.7–114% of expected concentrations in the measurement range of the assay after dilution by factor 1280–40960 (Table 4). Matrix effects on the measurement of high and low QC concentrations in cynomolgus serum were low. In neat sera of nine out of 10 individuals, recovery of the high QC concentration (18.75 ng/mL) was in the range of 86.6–107.0%; one outlier had a recovery of 65.0%. Recovery of the low QC concentration (1.2 ng/mL) was in the range of 81.2–116.3% in nine of 10 individuals (one outlier of 74.2%).

### 3.4. Application of the generic ELISA

In addition to the detailed assay validation for MAb 1, the assay was also successfully validated for five other therapeutic antibodies including anti-IGF-1R. The analytical procedure described was applied to serum samples obtained from two cynomolgus monkeys (one male, one female) which were intravenously administered a single dose of 1 mg/kg anti-IGF-1R. The serum concentration–time profiles for anti-IGF-1R administered to one female and one male cynomolgus monkey and measured with the present generic human IgG assay are depicted in Fig. 6. Evaluation of the mean pharmacokinetic parameters revealed a maximum concentration of 28.5 μg/mL achieved at 4.8 min. The antibody was eliminated from plasma with a mean half-life of 3.6 days (clearance of 0.577 mL/h/kg). Preliminary validation of the same generic human IgG assay for anti-IGF-1R revealed a LLOQ of 0.16 ng/mL in the measurement range of the assay, corresponding to 3.1 ng/mL in undiluted serum samples. Intra-assay ($n = 4$) and inter-assay ($n = 9$) precision and accuracy values were determined at 5.9 and 0.32 ng/mL of anti-IGF-1R in the measurement samples. Intra-assay and inter-assay precision for the high and low QC concentration showed mean CV values of less than 10.5%. Intra-assay and inter-assay accuracy for both QC concentrations showed mean recovery values of 93.3–100.4%.

**Table 4**
Dilutional linearity for MAb 1 in cynomolgus monkey pool serum measured in the generic ELISA.

| | Expected MAb 1 concentration (ng/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 19.4 | 9.7 | 4.8 | 2.4 | 1.2 | 0.6 |
| Recovery (%) | 98.7 | 113.4 | 109.0 | 100.0 | 105.8 | 114.0 |

## 4. Conclusions

A human generic IgG enzyme-linked immunosorbent assay was developed and validated for the quantitative determination of human therapeutic antibody MAb 1. The ELISA makes use of a murine antibody against a unique conformation epitope on the constant region CH2 domain of human IgG which is not shared with the cynomolgus monkey and other non-human primates. The generic human IgG1 assay was applied to quantify cynomolgus monkey serum concentrations of anti-IGF-1R after iv administration. In fact, this generic human IgG ELISA has already been used in the bioanalytical measurement of various novel human therapeutic antibodies and contributed to a more rapid development of the bioanalytical assays because of the generic applicability independent of the target of the antibody. Furthermore, the use of the murine antibody anti-human Fcγ-pan: R10Z8E9 as detection reagent in combination with the antigen as capturing reagent in Sandwich-type immunoassays can confer outstanding sensitivity (<1.5 ng/mL) and less interference with cynomolgus immunoglobulin unspecifically bound to the solid phase of the test system, e.g. immature IgG or IgM.

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