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Appraisal of state-of-the-art

Application of miniaturized immunoassays to discovery pharmacokinetic bioanalysis

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ABSTRACT

Introduction: Pharmacokinetic properties of biotherapeutics are an important aspect of preclinical drug development. The lead identification and optimization space is characterized by aggressive timelines, large sample numbers, a variety of species and matrices, and limited reagent and sample volumes all of which represent challenges for traditional microtiter plate assays. Since the Gyrolab immunoassay platform can accommodate small sample volumes and automated assay processing, we evaluated the workstation as an alternative to the plate-based assays. **Methods:** Three representative example assays — a generic anti-human IgG, a target specific and an anti-drug capture assay — were investigated in detail for accuracy and precision performance and their application to bioanalytical support for preclinical pharmacokinetic studies. Different animal matrices were tested in the assays and during study support. **Results:** Gyrolab procedures could be closely modeled after regular microtiter plate assays. The small reagent volumes necessary for Gyrolab allowed studying serial bleeds of transgenic mice with only 10 μL of blood sample. During development and during study support, the Gyrolab performance was similar to what can be expected from plate-based systems with accuracy and precision within 100 ± 20% or less. **Discussion:** Overall, the technology was well suited to support quantitation of biotherapeutics using small volume samples from different preclinical species. Limited operator involvement for assay processing allowed for reduced staffing and training. However, high instrument costs and a single source of reagent supplies represent risks when moving assays further into long-term applications such as clinical studies. Despite interest in the bioanalytical field, this is the first detailed investigation of bioanalytical applications of Gyrolab in pharmacokinetic studies.

1. Introduction

The advent of biopharmaceuticals brought a new focus on immunoassays as a core technology for bioanalytical support of pharmacokinetic (PK) and immunogenicity studies. While immunoassays have a long history, most formats are still microtiter plate-based without technological advances comparable to small molecule analysis by liquid-chromatography–mass spectrometry (LC–MS). Plate-based immunoassays, mostly enzyme-linked immunosorbent assays (ELISA), typically require long development times of up to several weeks, large reagent and sample volumes of 100–200 μL/well, and well-trained operators to achieve high accuracy and precision (David, 2005). Complete ELISA automation using robotic liquid handlers has addressed through-put in some laboratories. However, this can be cumbersome to set up, in particular if small sample volumes need to be transferred with high accuracy, and contract research organizations often have no access to robotic equipment. As bioanalytical support is increasingly out-sourced, immunoassays should be robust and well-developed in order to minimize involvement of the sponsor in technical trouble-shooting (Ray et al., 2010).

The biopharmaceutical discovery space on the other hand is characterized by aggressive timelines, large sample numbers, a variety of animal species and sample matrices, and limited available critical reagent and sample volumes. The implementation of a flexible assay design, such as "generic" anti-human antibody assays (Stubenrauch, Wessels and Lenz, 2009; Yang et al., 2008), could address a few of these challenges. Some of the advantages of LC–MS were also attempted to be transferred into biotherapeutics development but have not quite matured yet (Ezan, Dubois, & Becher, 2009; Ezan & Bitsch, 2009). In particular, limited sample volumes still represent an obstacle for immunoassays leading to increased animal numbers per study for smaller species to accommodate volume requirements. This can become an issue when studying transgenic models with limited colony sizes. Assay miniaturization as used for biomarker discovery (Ellington, Kullo, Bailey, & Klee, 2010; Jokerst et al., 2009; Templin, Stoll, Bachmann, & Joos, 2004) has not found wide-spread application for pharmacokinetic immunoassays.

The Gyrolab immunoassay platform (Gyros, Uppsala, Sweden) was developed to address several of the challenges outlined above. It requires

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minimal sample and reagent volumes, almost no hands-on time and 112 data points can be generated within 1 h. Details of the technology can be found in the manufacturer's web page (http://www.gyros.com). Briefly, immunoassays are carried out on a special compact disk (CD). Reagents and samples flow through nano-scale channels etched into the CD over a streptavidin-coated bead column where the immunosandwich is assembled. The detection antibody is fluorescently labeled to allow visualization by laser. The Gyrolab is completely integrated allowing fully automated immunoassays without operator oversight.

Although several applications of the platform have been published (Kange et al., 2005; Lund et al., 2010; Rivera, Ekholm, Inganas, Paulie and Gronvik, 2005; Eriksson et al., 2006) including immunogenicity and pharmacokinetic assays, (Singh et al., 2010; Yeung, Stevenson, Osterlund, & Amaravadi, 2008; Hamuro, Qiu, Verch, Song, Liao and Fernandez-Metzler, 2009; Mora, Obenauer-Kutner and Vimal, 2010; van der Woude et al., 2010), detailed information regarding platform performance still is limited. We evaluated Gyrolab-based PK immunoassays of biotherapeutics in the discovery space with the objective of addressing limitations in sample volumes, staffing and turn-around times. In this manuscript we describe our experiences with Gyrolab performance using three representative assays.

2. Materials and methods

2.1. Common reagents

Animal matrices were obtained from Bioreclamation, Inc. (Liverpool, NY). StartingBlock (PBS) Blocking Buffer, Thermo-Fast 96 skirted PCR plates and Matrix Screenmates 0.75 and 1.4 mL round-bottom storage tubes were purchased from Thermo Fisher Scientific Inc (Norristown, PA). Rexxip F Detection Buffer, Bioaffy 200 CDs and microplate foil sealers were purchased from Gyros US Inc. (Monmouth Junction, NJ). Immunoassays were run on the Gyrolab automated system (Gyros AB, Uppsala, Sweden).

2.2. Conjugation

All conjugations were performed according to the manufacturer's recommendations. The mouse anti-human TNF RII IgG_{2A} monoclonal antibody (mAb) (R&D Systems, Minneapolis, MN, USA, cat no. MAB2261) and recombinant mouse TNF-α (Millipore, Temecula, CA, USA, cat no. GF027) were conjugated to biotin using the EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce, Rockford, IL, USA). A 50-fold molar excess of biotin was added to the protein solution and incubated for an hour at ambient temperature. Reactions were performed in phosphate-buffered saline (PBS).

Mouse anti-human IgG Fc-specific mAb (Southern Biotech, Birmingham, AL, USA, cat no. 9040-01), was conjugated using the Alexa Fluor-647 MAb Labeling Kit (Molecular Probes, Inc., Eugene, OR, catalog no. A-20186). Antibody was concentrated to 1 mg/mL prior to conjugation step using Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore, Billerica, MA, catalog no. UFC505024), as per manufacturer's recommendations. Reaction was incubated for an hour at an ambient temperature.

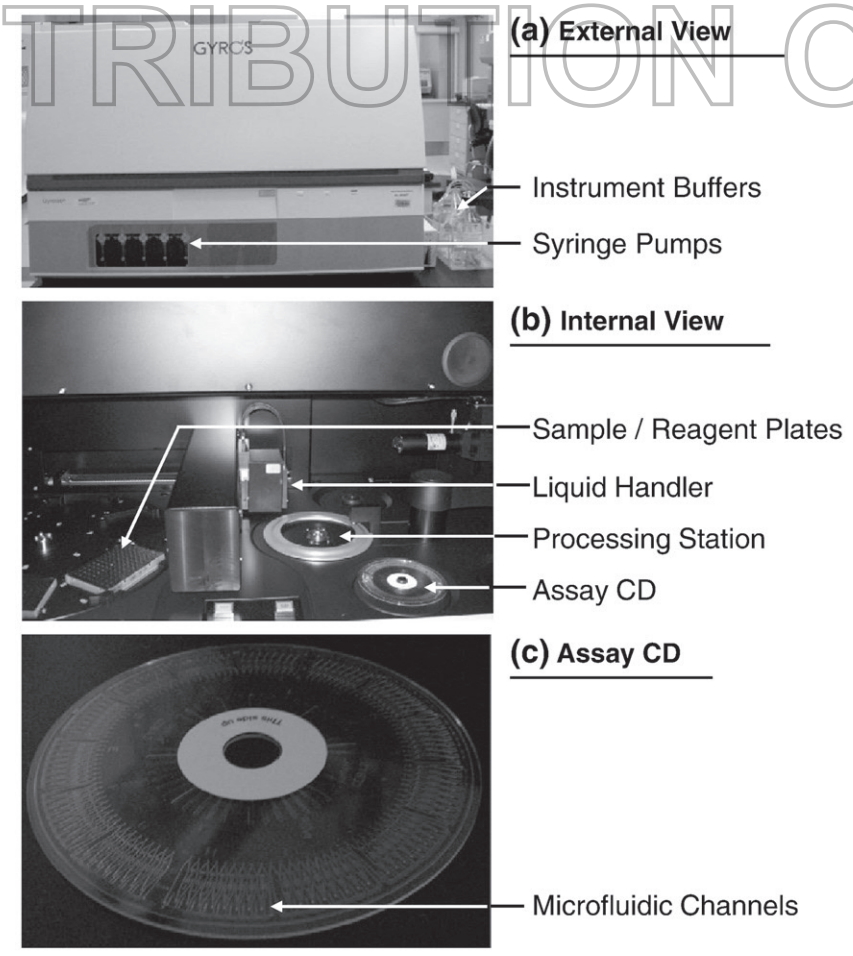

Fig. 1. Photographic representations of the Gyrolab instrument. (a) Closed view from outside of the instrument; (b) open view onto the instrument deck where samples, reagents and assay CDs are loaded and process; and (c) assay CD with view of the microfluidic channels.

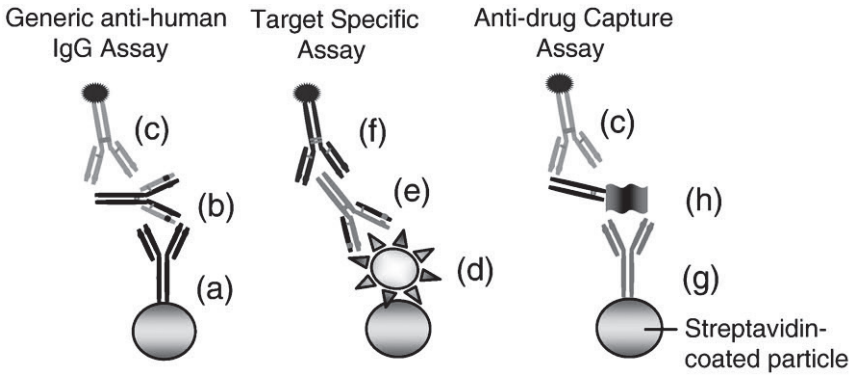

Fig. 2. Schematic diagrams of three different assay formats as indicated. (a) Biotin Ms anti-Hu IgG1κ mAb; (b) Hu IgG mAb analyte; (c) Alexa647 Ms anti-Hu IgGγ Fc mAb, (d) Biotin Ms TNFa; (e) Ms anti-Hu TNFa mAb analyte; (f) Dylight649 labeled Dk anti-Ms IgG (H + L) mAb; (g) Biotin labeled Ms anti-TNFaRII IgG2A mAb; (h) Hu Fc-TNFaRII analyte; (c) Alexa647 Ms anti-Hu IgGγ Fc mAb.

2.3. Immunoassays

All calibrators and quality controls (QCs) were prepared in a matrix comparable to the samples and were diluted according to the established minimum required dilution in assay diluent prior to analysis. Plate wash buffer consisted of PBS + 0.01% (v/v) Tween 20. The plates were sealed with foil sealers and centrifuged for 5 min at 2000 *g* to remove air bubbles, then transferred to the Gyrolab instrument and analyzed using Bioaffy 200 CDs. The vendor installed *Bioaffy 200 3-step C-A-D v1* method was used with changes as outlined in the assay sections below.

2.4. Generic anti-human IgG assay in cynomolgus monkey serum

Samples were diluted 1:20 in assay diluent (StartingBlock Blocking Buffer) prior to analysis. Capture and detection reagents were applied at 100 μg/mL of Biotin labeled anti-human $IgG1_\kappa$-specific mAb (BD Pharmingen, San Jose, CA, USA, cat no. 555790), in assay diluent and 12.5 nmol/L of Alexa-647 labeled anti-human IgG Fc-specific mAb in Rexxip F. The instrument was run as previously described, using the manufacturer's method with a 1% PMT setting.

2.5. Generic anti-human IgG assay in mouse plasma

Two different assays were developed: (A) a Gyrolab-based format similar to the assay described above for monkey serum. The basic assay set-up was the same with monkey serum being replaced by mouse plasma.

(B) A microtiter-plate ELISA based format was developed as well. Plates (Costar, cat #9018) were coated for 16–20 h at 4°C with anti-human $IgG1_\kappa$-specific mAb (BD Pharmingen, San Jose, CA, USA, cat no. 555750) in Bicarbonate buffer (Pierce, cat #28382). After a blocking step in assay diluent (phosphate-buffer saline supplemented with 3% BSA), calibrators, QCs and samples were applied at a 20× dilution in assay diluent for 1 h at room temperature while shaking. Subsequently, bound antibody was detected using anti-human IgG Fc-specific mAb conjugated to horse-radish peroxidase (Southern Biotech, Birmingham, AL, USA, cat #9040-05). The assay was developed with 3,3',5,5'-Tetramethylbenzidine (TMB, KPL, cat #50-76-00), and optical density was measured at 450 nm. All steps were separated by three washes with phosphate buffer saline supplemented with 0.05% Tween-20.

2.6. Target specific assay in mouse plasma

Samples were minimally diluted 1:10 in assay diluent (PBS) prior to analysis. Capture and detection reagents were applied at 25 μg/mL of biotin labeled mouse TNF-α in StartingBlock and 100 nmol/L of donkey anti-mouse IgG (H+L)-specific mAb conjugated to Dylight649 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, cat no. 715-495-150) in Rexxip F. The instrument

Table 1
Summary of assay accuracy and precision. Assay QCs were measured in triplicates over the course of several days. Assay concentrations are displayed post application of the minimal required dilution.

a: Inter-assay accuracy and precision in matrix

	Nominal sample conc (ng/mL)	Target specific assay				Generic anti-human IgG assay			
		Average conc	n	Mean % CV	Mean % bias	Average conc	n	Mean % CV	Mean % bias
ULOQ	2000.00	1994.90	13	3.7	−0.3	1999.80	12	4.1	0.0
QC 1	1500.00	1516.67	29	8.0	1.1	1569.52	26	9.3	4.4
QC 2	150.00	150.18	28	7.0	0.1	152.08	26	6.9	1.4
QC 3	15.00	15.65	29	3.8	4.2	15.45	26	8.6	2.9
LLOQ	5.00	5.05	13	7.1	1.0	5.15	12	10.7	2.8

b: Intra-assay accuracy and precision in matrix

	Nominal sample conc (ng/mL)	Target specific assay				Generic anti-human IgG Assay			
		% CV range		% Bias range		% CV range		% Bias range	
ULOQ	2000.00	1.6	24.5[a]	−5.0	3.0	2.3	5.7	−4.8	4.6
QC 1	1500.00	2.6	11.9	−11.3	8.8	2.3	14.9	−6.9	12.3
QC 2	150.00	3.2	8.9	−9.3	6.9	−6.9	7.7	−5.6	8.2
QC 3	15.00	2.2	8.9	−2.6	8.7	4.0	19.1	−5.2	10.2
LLOQ	5.00	0.5	10.4	−13.6	7.4	0.5	5.6	−0.4	12.3
	Assay range	5–2000 ng/mL				5–2000 ng/mL			
	Matrix	5% Mouse plasma				5% Cynomolgus monkey serum			

[a] 12 out of 13: highest %CV = 9.8%.

was run as previously described, using the manufacturer's method with a 0.2% PMT setting.

2.7. Anti-drug capture assay in mouse serum

There is no minimum required dilution for this assay; therefore, samples requiring dilution were diluted in 100% serum prior to analysis. Capture and detection reagents were applied at 12.5 μg/mL biotin labeled mouse anti-human TNF RII IgG_{2A} mAb in StartingBlock and 12.5 nM of Alexa647 labeled mouse anti-human IgG mAb in Rexxip F. The instrument was run as described above, using the manufacturer's method with a 1% PMT setting.

2.8. In vivo studies

Sprague–Dawley rats, CD1 mice or cynomolgus monkeys, respectively, were dosed intravenously with study compounds on day 1. Serial serum samples were taken at different time-points post dose to quantitate the dosed therapeutic by immunoassay. Immunogenicity was not assessed. All animal procedures were in accordance with

Table 2
Comparison of different minimal sample dilutions in the anti-drug capture assay. QCs were diluted and quantitated in multiple replicates as indicated. A, B: Assay performance using a mouse plasma sample matrix. C: Assay performance using a rat plasma sample matrix. Assay concentrations are displayed post application of the minimal required dilution.

a: Inter-assay accuracy and precision in mouse serum

	Nominal sample conc (ng/mL)	100% Serum				50% Serum			
		Average conc	n	Mean % CV	Mean % bias	Average conc	n	Mean % CV	Mean % bias
ULOQ	1000.00	927.15	4	5.1	−7.9	976.38	2	4.2	−2.4
QC 1	750.00	773.01	6	1.0	3.0	782.09	4	4.3	4.1
QC 2	75.00	74.83	6	1.2	−0.2	74.48	4	7.5	−0.7
QC 3	7.50	7.42	6	5.6	−1.1	7.46	4	8.6	−0.6
LLOQ	2.50	2.62	4	4.1	4.7	2.59	2	10.1	3.3
	Nominal sample conc (ng/mL)	20% Serum				5% Serum			
		Average conc	n	Mean % CV	Mean % bias	Average conc	n	Mean % CV	Mean % bias
ULOQ	1000.00	999.51	2	2.4	0.0	996.65	2	0.8	−0.3
QC 1	750.00	778.48	4	0.5	3.7	757.43	4	7.0	1.0
QC 2	75.00	71.38	4	2.6	−5.1	68.94	4	9.9	−8.8
QC 3	7.50	8.56	4	5.5	12.3	7.78	4	22.	3.6
LLOQ	2.50	3.13	2	5.0	20.1	5.41	2	38.	53.8

b: Intra-assay accuracy and precision in mouse serum

	Nominal sample conc (ng/mL)	100% Serum				50% Serum			
		% CV range		% Bias range		% CV range		% Bias range	
ULOQ	1000.00	0.5	0.5	−14.1	−3.1	*	*	−5.6	0.5
QC 1	750.00	2.9	3.1	1.9	3.9	5.3	5.3	1.1	6.9
QC 2	75.00	5.4	7.7	−1.3	1.1	1.9	1.9	−6.3	4.4
QC 3	7.50	0.7	3.7	−7.4	4.0	2.4	2.4	−7.0	5.2
LLOQ	2.50	3.7	3.7	0.0	7.4	*	*	−4.2	9.7
	Assay range	2.5–1000 ng/mL				2.5–1000 ng/mL			
	Matrix	100% Mouse serum				50% Mouse serum			
	Nominal sample conc (ng/mL)	20% Serum				5% Serum			
		% CV range		% Bias range		% CV range		% Bias range	
ULOQ	1000.00	*	*	−1.8	1.6	*	*	−0.9	0.2
QC 1	750.00	3.3	3.3	3.3	4.0	1.3	1.3	−4.2	5.6
QC 2	75.00	2.8	2.8	−7.1	−3.2	3.4	3.4	−16.9	−1.7
QC 3	7.50	3.2	3.2	8.8	15.6	6.8	6.8	−14.3	16.7
LLOQ	2.50	*	*	17.2	22.8	*	*	36.5	63.7
	Assay range	5–1000 ng/mL				7.5–1000 ng/mL			
	Matrix	20% Mouse serum				5% Mouse serum			

c: Inter-assay accuracy and precision in rat serum

	Nominal sample conc (ng/mL)	100% Rat serum			
		Average Conc	n	Mean % CV	Mean % Bias
ULOQ	800.00	819.21	4	9.4%	2.3%
QC 1	150.00	151.24	4	2.7%	0.8%
QC 2	15.00	14.85	4	3.1%	−1.0%
QC 3	1.50	1.59	4	6.1%	5.9%
LLOQ	0.80	0.88	4	18.2%	9.0%
	Assay range	0.8–800 ng/mL			
	Matrix	100% Rat serum			

*Data not available. **Upper end not determined beyond 1000 ng/mL.

established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

3. Results

3.1. General observations

The Gyrolab immunoassay workstation combines specialized robotic liquid handling, assay processing and fluorescent reader functions into an integrated platform (Fig. 1a,b). Assays are carried out on CDs containing microfluidic channels controlled through hydrophobic valves (Fig. 1c). Centrifugational force is applied to overcome the hydrophobic barriers and thus to open the valves. In our assays, the assembly of an immunosandwich in microtiter plates could be closely mimicked in the Gyrolab with each step separated by washes and multiple layers of capture, sample and detection added consecutively. The instrument software allowed controlling a number of factors including flow rate and signal amplification, i.e., the photomultiplier tube (PMT) setting. The sample/assay volume is fixed and dependent on the CD used which are available with 20, 200 and 1000 nL sample volumes. They differ mainly in the size of the Streptavidin column allowing for greater analyte capacity. Even the largest CD consumes much smaller amounts of sample than traditional plate-based technologies (1 μL versus 25–200 μL). However, larger volumes need to be loaded on the instrument deck for the robotic needles to function properly. We found 10–30 μL loaded in the sample plate to be most reliable avoiding the risk of air bubbles being trapped. Unused sample remains in the plate allowing for potential repeat testing if necessary. A brief centrifugation step of the sample and reagent plates prior to the run also helped reducing the risk from air bubbles. For small signal responses such as in the anti-drug capture assay presented below, an increased PMT setting can be used to amplify the assay response above instrument noise. Due to increased background as well, this approach had limitations with respect to assay sensitivity but successfully was implemented to improve assay range and performance. While the instrument allowed for extensive customization, we found the manufacturer's recommended program settings to be suitable for most applications that we tested

We used the Gyrolab to support 14 different analytes including therapeutic antibodies, proteins and peptides. Study subjects included regular and transgenic mice strains and different rat strains (serum and plasma); rhesus monkeys (serum, plasma and cerebral spinal fluid); and cynomolgus monkeys (serum). We chose three different assay formats (Fig. 2) as representative examples to demonstrate technology performance. All three assays used nanoliter volumes of samples and reagents, were fully automated and required minimal sample dilution due to the large dynamic range. The assays were successfully applied to analyze preclinical PK study samples (see below). Calibrator and QC concentrations below were calculated after application of the minimal required dilutions, i.e., stated concentrations are applicable to the actual assay and need to be multiplied for samples.

3.2. Generic Anti-human IgG Assay

An anti-human IgG assay was used to quantitate human, humanized and human–mouse chimeric antibodies in animal matrices. The following example demonstrates the performance of an assay measuring a human-chimeric antibody against TNF-α in cynomolgus monkey serum. The assay was executed on five separate days, over the course of several months (Table 1). Each run consisted of a standard curve ranging from 5 ng/mL to 2000 ng/mL and multiple sets of QCs (n = 26 for daily run QCs). The lower limit of quantitation (LLOQ) was 5 ng/mL, the upper limit of quantitation (ULOQ) was 2000 ng/mL with an anchor point at 2 ng/mL. The observed accuracy ranges were 0.0 to 4.4% (inter-assay, Table 1a) and −6.9 to 12.3% (intra-assay, Table 1b). The precision ranges were 4.1 to 10.7% (inter-assay) and −6.9 to 19.1% (intra-assay). Samples were linear upon dilution with a mean accuracy bias of −9.4% (15% CV). Comparable assay performance was observed during study analysis (Table 3) and when using other analytes and animal matrices (data not shown). When plotting analyte concentrations in serial serum samples against time, expected profiles typical for antibody therapeutics were obtained (Fig. 3a).

3.3. Comparison of Gyrolab and traditional ELISA

The generic anti-human IgG assay was also developed in a microtiter plate-based ELISA format and compared with the Gyrolab assay. ELISA calibrators ranged from 4 to 250 ng/mL whereas Gyrolab allowed for a greater range from 5 to 2000 ng/mL. Analytical recovery and precision of QCs during study support were comparable between

Table 3
In-study accuracy and precision of daily run QCs during preclinical study support. Duplicates of each QC level were applied in each assay. Assay concentrations are displayed post application of the minimal required dilution.

a: In-study inter-assay accuracy and precision

	Generic anti-human IgG assay					Target specific assay					Anti-drug capture assay				
	Nominal sample conc (ng/mL)	Average sample conc (ng/mL)	n	Mean % CV	Mean % bias	Nominal sample conc (ng/mL)	Average sample conc (ng/mL)	n	Mean % CV	Mean % bias	Nominal sample conc (ng/mL)	Average sample conc (ng/mL)	n	Mean % CV	Mean % bias
QC 1	1500.00	1646.51	4	1.8	8.9	1500.00	1470.83	12	5.3	−2.0	750	759.43	13	3.8	1.2
QC 2	150.00	155.60	4	0.9	3.6	150.00	156.58	12	5.4	4.2	75	74.68	13	1.5	−0.4
QC 3	15.00	16.01	4	1.0	6.3	15.00	14.98	12	2.3	−0.1	7.5	7.65	14	2.4	2.0

b: In-study intra-assay accuracy and precision

	Generic anti-human IgG assay					Target specific assay					Anti-drug capture assay				
	Nominal sample conc (ng/mL)	% CV range		% Bias range		Nominal sample conc (ng/mL)	% CV range		% Bias range		Nominal sample conc (ng/mL)	% CV range		% Bias range	
QC 1	1500.00	2.9	4.1	7.8	10.0	1500.00	4.1	10.4	−8.3	2.4	750	0.9	8.4	−5.3	4.5
QC 2	150.00	2.1	4.8	3.0	4.2	150.00	1.5	11.2	−2.2	7.3	75	1.5	4.7	−2.7	1.5
QC 3	15.00	3.5	5.8	5.6	6.9	15.00	7.2	9.3	−2.7	1.8	7.5	0.3	10.7	−0.9	5.7
Assay range	5–2000 ng/mL					5–2000 ng/mL					2.5–1000 ng/mL				
Matrix	5% Cynomolgus monkey serum					5% Mouse plasma					100% Mouse serum				

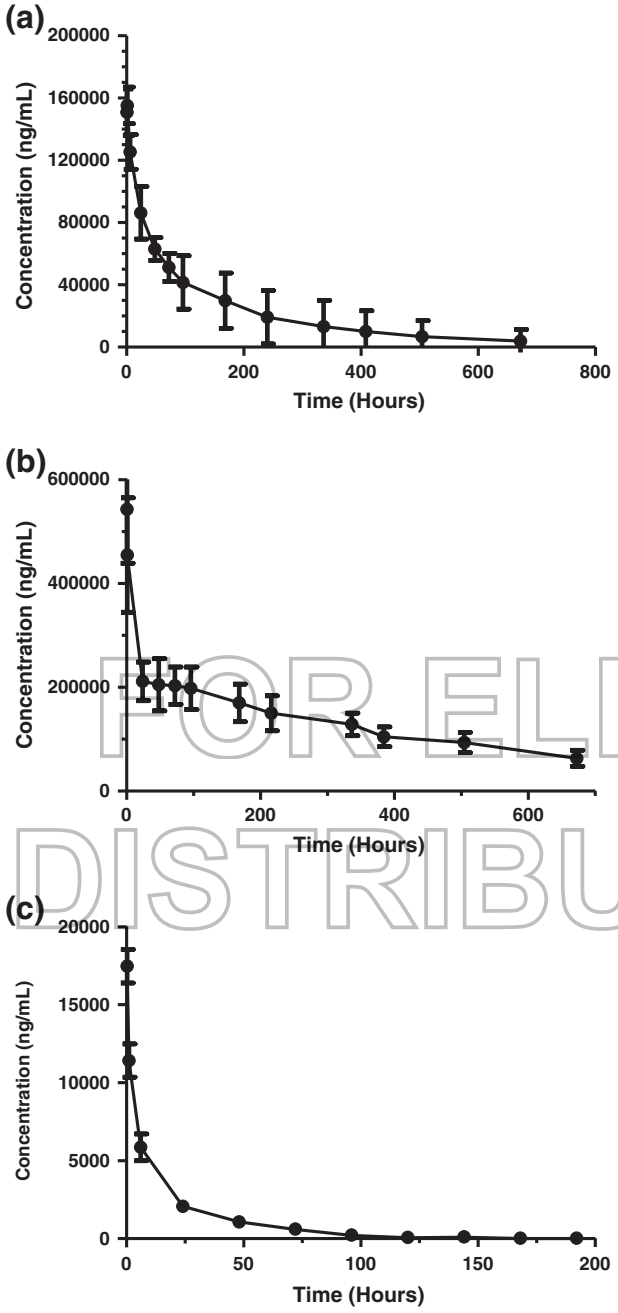

Fig. 3. Concentration versus time curves of PK study data obtained using Gyros technology in three different assay formats: (a) Generic anti-human IgG Assay: Cynomolgus monkeys were dosed with 5 mg/kg body weight of anti-human TNF-α mAb. (b) Target specific assay: CD1 mice were dosed with 10 mg/kg body weight of anti-mouse TNF-α mAb. (c) Anti-drug capture assay: Sprague Dawley rats were dosed with 1 mg/kg body weight of TNF-α RII-Fc fusion protein. Three subjects in each study were averaged together and the calculated mean concentrations were plotted against the sampling time with error bars representing the 95%CI.

both methods (Table 4). When plotting analyte concentrations in serial serum samples against time, expected profiles were achieved with both methods (Fig. 4). However, side-by-side comparisons with the same study samples were not carried out. Thus, conclusions of PK comparability are limited.

3.4. Target specific assay

The quantitation of a mouse surrogate antibody against mouse TNF-α in mouse plasma required the development of a target specific capture assay. Use of an anti-mouse IgG antibody in a mouse sample matrix caused increased assay background. Therefore, the standard PMT settings were adjusted resulting in reduced background and signal (Fig. 5a). This also improved back-calculated standard concentrations for both the 0.2% and the 0.5% PMT setting resulting in more accurate analytical recovery of QCs (Table 5). Interestingly, the 1% PMT setting exhibited analytical recovery issues in the upper end of the standard curve rather than the lower end (Fig. 5b). The calibrators in the final assay at 0.2% PMT ranged from 5 ng/mL to 2000 ng/mL with an anchor point at 2 ng/mL. The LLOQ was 5 ng/mL and the ULOQ was 2000 ng/mL. To characterize assay performance, six individual runs were performed over the course of several months using different reagent lots and multiple instruments (Table 1). Assay accuracy ranged from −0.3 to 4.2% (inter-assay, Table 1a) and −13.6 to 8.8% (intra-assay, Table 1b). Assay precision ranged from 3.7 to 8.0% (inter-assay) and 0.5 to 24.5% (intra-assay). The latter value (24.5%) can be contributed to a single data point (n = 13), which when removed decreases the range to 0.5 to 9.8%. Samples were linear upon dilution with a mean accuracy bias of 2.9% (6.3% CV). Similar assay performance was observed during study analysis (Table 3). Similar to the human–mouse chimeric antibody measured with the generic assay, analyte versus time concentration profiles obtained with the target-specific capture assay were typical for antibody therapeutics (Fig. 3b).

3.5. Anti-drug capture assay

Quantitation of TNF-α receptor II fused to a human Fc domain was accomplished by capture with an anti-TNF-α receptor II mAb combined with detection by anti-human IgG_{Fc}-specific mAb. This strategy employed stable antibody reagents and allowed for whole molecule quantitation.

Calibrators and QCs were prepared in matrix containing 5, 20, 50 or 100% mouse serum to assess potential matrix effects in the Gyrolab technology. Assay performance was characterized during several runs. Calibrators ranged from 2.5 ng/mL to 1000 ng/mL with an anchor point at 1 ng/mL. The LLOQ was 7.5 ng/mL in 5% plasma and 2.5 ng/mL in 100% plasma. The ULOQ was not affected negatively and was kept at 1000 ng/mL. Higher ULOQs with increasing serum concentrations were not investigated. While increasing sample dilution resulted in decreased sensitivity, all four serum concentrations demonstrated similar accuracy and precision in the mid to upper range of the assay (Table 2a and b). Assay performance of the 100% serum assay during study analysis was comparable to the qualification runs (Table 3). To demonstrate assay application to PK time course studies, we chose a rat study since the assay was also qualified in rat (Table 2c) and cynomolgus monkey serum (data not shown). The mouse and rat matrices performed similarly with respect to analytical recoveries and precision of QCs (Tables 2c and 3). When plotting analyte concentration in rat serum against time (Fig. 3c) expected curve shapes were obtained.

4. Discussion

We evaluated and applied the Gyrolab nano-scale immunoassay technology as a platform to support preclinical PK studies. Three representative assays were selected to demonstrate the Gyrolab performance in more detail.

A generic anti-human assay was developed as a platform approach to quantitate multiple human antibody drug candidates in animal sera while reducing assay development time. While this assay was successfully applied to support multiple PK discovery studies in mice, rats, rhesus and cynomolgus monkeys, we showed representative data for the latter. The target specific and anti-drug capture assays were developed for the analysis and quantitation of mouse antibodies in mouse plasma and a receptor fused to an IgG Fc-domain.

Table 4
Comparison of the Generic anti-human IgG assay in a Gyrolab-based and a microtiter plate ELISA-based format. (A) Data from a monoclonal antibody without a known target, (B) data from a human therapeutic antibody. Assays were applied to study sample analysis, and QC performance was evaluated over the course of two assays on different days. Analytical recovery and precision of individual replicates from all assays are summarized. QCs were minimally diluted 20× as required by the assay protocol. Assay concentrations are displayed post application of the minimal required dilution.

In-Study Inter-assay Accuracy and Precision										
	Gyrolab anti-human IgG assay					Plate-based ELISA anti-human IgG assay				
	Nominal sample conc (ng/mL)	Average sample conc (ng/mL)	n	Mean % CV	Mean % bias	Nominal sample conc (ng/mL)	Average sample conc (ng/mL)	n	Mean % CV	Mean % bias
A										
QC 1	1500.00	1503.46	4	4.2	0.2	160.00	157.56	6	5.5	−1.5
QC 2	150.00	147.27	4	1.3	−1.9	40.00	40.10	6	1.0	0.3
QC 3	15.00	15.33	4	3.5	2.2	4.00	4.03	6	5.0	0.9
B										
QC 1	1500.00	1557.50	4	2.6	3.7	160.00	159.53	4	4.1	−0.3
QC 2	150.00	144.64	4	1.7	−3.7	40.00	38.49	4	3.9	−3.9
QC 3	15.00	14.92	4	6.3	−0.6	4.00	4.08	4	6.5	2.0

Similar accuracy and precision were observed for all three assay formats demonstrating the versatility of the instrument for various formats, analytes and animal matrices. The observed range differences between the target specific and generic assay formats versus the anti-drug capture format may be attributed to the serum concentration rather than the assay format. Since matrix effects appear to be limited, greater assay sensitivities could easily be achieved by the use of 100% matrix instead of diluted serum. However, more detailed studies need to be undertaken to determine whether the Gyrolab technology is less prone to matrix effects by design or whether this is assay dependent similar as for ELISA.

In general, Gyrolab-based assays exhibited similar performance with respect to accuracy and precision to what can be expected from plate-based assays. We compared Gyrolab- and microtiter plate-based assays for the two different analytes. Both technologies exhibited similar analytical recoveries and precision. However, the Gyrolab

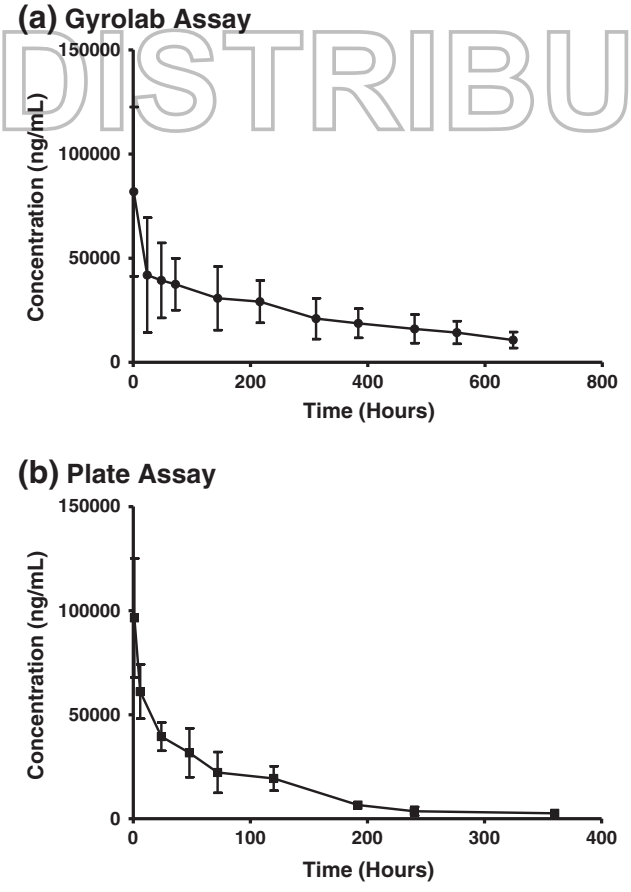

Fig. 4. Concentration versus time curves of PK study data obtained using the generic anti-human IgG assay in (a) the Gyrolab-based and (b) the microtiter plate ELISA-based format. PK studies were carried out separately for each assay by dosing mice with 10 mg/kg body weight of antibody and taking serial blood plasma samples over time. Three subjects in each study were averaged together and the calculated mean concentrations were plotted against the sampling time with error bars representing the 95%CI.

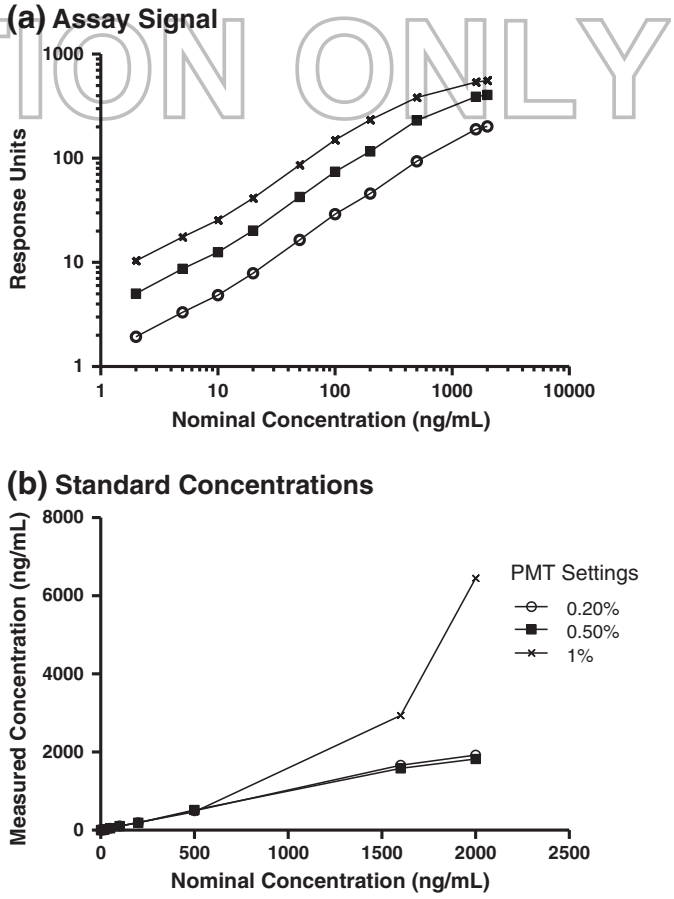

Fig. 5. Comparison of calibrator performance at different PMT settings in the target-specific assay. The signal response (a) and back-calculated mean calibrator concentrations (b) were evaluated against the nominal calibrator concentration for each PMT setting. Error bars representing the minimum and maximum values of two replicates are plotted.

Table 5
Comparison of different PMT settings in the target-specific assay. QCs were diluted and quantitated in multiple replicates as indicated. At PMT-setting 1%, one replicate at 2000 ng/mL measured above the calibrator range (indicated by *). Assay concentrations are displayed post application of the minimal required dilution.

Inter-assay accuracy and precision in mouse serum													
	Nominal sample conc (ng/mL)	0.2% PMT				0.5% PMT				1.0% PMT			
		Average conc	n	Mean % CV	Mean % bias	Average conc	n	Mean % CV	Mean % bias	Average conc	n*	Mean % CV	Mean % bias
QC 1	1500.00	1623.33	3	3.5	7.6	1493.33	3	1.0	−0.4	1890.00	2	3.7	20.6
QC 2	150.00	163.17	3	1.6	8.1	162.00	3	1.3	7.4	162.33	3	2.0	7.6
QC 3	15.00	15.60	3	5.3	3.8	15.48	3	6.0	3.1	15.85	3	7.4	5.4

assay had a larger assay range. This can be an advantage for PK studies as it reduces necessary sample dilutions that can be a potential cause for analytical bias or error. Although only exemplary data are presented here, this holds true for other analytes that we tested including a peptide that required anti-peptide mAb capture and detection. However, assay sensitivity appeared to be greater in some plate-based methods when using analyte-specific reagents with high affinity. Detailed studies are necessary to confirm these preliminary trends.

On the technical side, we found the Gyrolab instrument to be an efficient and versatile platform for PK discovery support. After some pioneering work at Merck, the manufacturer also started to offer an interface to link the instrument to the commonly used Watson PK laboratory information management system (LIMS). Since Gyrolab is an open platform, custom assays can be developed easily in contrast to automated, clinical immunoanalyzer systems that tend to be closed platforms.

The fully automated format significantly reduced hands-on operator time for assay development and study support even allowing overnight runs. In some cases, this reduced sample turn-around time to 24 h. As an integrated platform, operator training requirements are limited for Gyrolab and do not require cumbersome set-ups as needed for standard robotic liquid handlers. The nano-scale sample requirements can be of particular importance when supporting preclinical studies with limited sample volume such as serial bleeds from transgenic mouse models. Operator training could be minimal which can be advantageous when out-sourcing study support. In three years we had almost no downtime due to instrument malfunction. While standard maintenance procedures are simple, regular use of serum or plasma samples requires more frequent cleaning than recommended by the manufacturer. For multiple daily runs, the desorb procedure described in the manuals should be carried out on a weekly basis. One assay was found to require cleaning after each run. We speculate a combination of a polysaccharide target and the quality of the detection reagent being the underlying reason. Indications for a necessary desorb procedure are increasing assay background, random CV failures or decreasing accuracy.

On the other hand, reliance on a single supplier source for critical reagents, i.e. the CDs, could pose a risk for long-term study support. Bioveris is an example of clinical pharmacokinetic assays requiring redevelopment due to discontinued technology support after Roche's acquisition of the company.

While we used Gyrolab extensively in a discovery environment, we did not yet pursue regulated work, partially due to the associated 21CFR part 11 regulatory requirements. Some of the basic issues regarding regulatory compliance have been addressed by the manufacturer during the last two years, including the Watson LIMS interface mentioned above and a GxP compliance package for the instrument. Since Gyrolab not only is a robotic pipet station but also integrates reader functions to generate actual data, validation may represent a significant effort. Although Gyrolab validation has not been published, recent clinical applications suggest ongoing efforts in that direction (Singh et al., 2010).

Despite the potentially fast turn-around of a limited number of samples, Gyrolab is not a high-throughput platform. While multiple microtiter plates can be assayed in parallel, this is not possible in the Gyrolab. Thus, five CDs take five times the time of a single CD resulting in an approximate speed of 112 data points per hour. Format flexibility can also be more limited compared to plate-based assays. For example, dissociation and neutralization steps can only be carried out prior to the assay. This makes neutralization in the presence of immobilized capture reagent difficult. Although the technology lends itself to out-sourced bioanalytical support, only a few contract research organizations currently have the capability to support Gyrolab-based assays.

While the technology may not yet be used widely, it may have significant impact for immunoassay applications during drug development.

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