Validation of a ligand binding assay

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Conclusions

Gyrolab™ xP Workstation has been used to validate a ligand binding assay for a recombinant therapeutic antibody in human serum. Validation was performed essentially in accordance with accepeted regulatory guidelines. The performence characteristics of the validated assay are summarized below:

- Analytical Range: 2–500 µg/L
- Minimum Required Dilution: 1/20 corresponding to 5% serum
- Sample-to-sample Carry Over: <1:166,600
- Selectivity: 6/9 samples within acceptance limits at 2 µg/L
- Dilutional Linearity: Approximately two orders of magnitude
- Inter-Run QC Sample Precision: 10.9–20.7%
- Inter-Run QC Sample Accuracy: 1.4–13.3%

This example shows that assay validation is readily performed on a Gyrolab xP Workstation demonstrating excellent performance. The system is well suited to address analytical challenges for macromolecules in ligand binding assays in environments where regulatory requirements apply.

Introduction

Ligand binding assays are commonly used for quantification of macromolecular drug candidates assessing pharmacokinetic (PK) and pharmacodynamic (PD) properties of a drug. In most instances various forms of immunoassays specific for the drug candidates have to be developed in order to quantitatively assess drug concentrations in samples generated during pre-clinical and clinical studies. These assays must be subject to validation procedures determining the conditions under which assays are performing properly for the intended use. A number of different aspects have to be considered during validation of assays. These have been described in detail by regulatory authorities and by experts in the field (1,2,3,4).

Over the last several years new technologies have been developed that addresses many of the weaknesses of currently used technologies potentially improving the overall assay performance. Gyrolab Workstation is one of these platforms that can generate high quality analytical data at high throughput in a fully automated manner. The technology is based on microfluidic principles taking advantage of rapid reaction kinetics to shorten TAT and increase throughput. The miniaturized format means reduced time for assay development, reduced consumption of samples and reagents, and increased tolerance against matrix effects.

The purpose of this work has been to validate an assay developed for an approved therapeutic monoclonal antibody in human serum on Gyrolab Workstation, essentially adhering to the guidelines in effect. The validation procedure described serves to illustrate how validation of an assay can be performed and what performance can be expected from similar efforts.

Materials and methods

Validation procedure

The validation procedure employed essentially follow recommendations (3).The procedure is described in Figure 1.

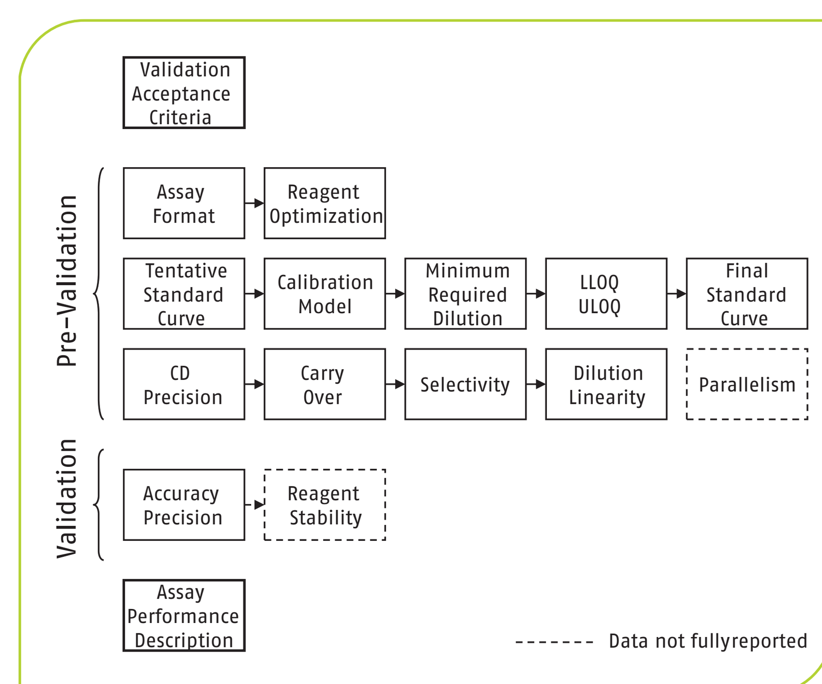

Fig 1. Illustration of the validation procedure.

Assay description

An indirect antibody assay was developed for an approved therapeutic monoclonal antibody drug molecule (DM) of the IgG1 subclass based on immobilization of biotinylated recombinant target molecule (TM) to the streptavidin coupled capture column within the Bioaffy CD followed by addition of samples containing DM. Bound DM was detected using a mouse monoclonal antibody directed against human IgG that was labeled with Alexa Fluor 647 (Invitrogen AB, Täby, Sweden) and diluted in Rexxip F (Gyros AB, Uppsala, Sweden). On column fluorescence detection was carried out automatically within the Workstation.

Fig 2. Illustration of the assay design used for validation.

Samples and assay conditions

DM was spiked in pools or individual, undiluted human sera (Sera Laboratory International, Haywords Heath, UK (distributor for Bioreclamation in Europe)), and further diluted in Rexxip A (Gyros AB) to the desired matrix concentration.

Assays were performed in less than 60 minutes per CD using a standard method designed for the Bioaffy 200 CD (Gyros AB).

Results

Optimization of reagents

After titrating the detection reagent, it was used at 12.5 nM in Rexxip F in all reported experiments.

To optimize assay performance the amount of capture reagent was titrated using different proportions of biotinylated capture reagent and biotinylated BSA at different proportions. A molar ratio 50:50 generated the best results in terms of noise in the low end of the curve and broadest possible measuring range in the upper part of the curve (data not shown).

Selection of calibration model

The dose-response relationship algorithm fit was evaluated using 4- and 5-parameter logistic functions weighted against response and concentration, respectively, on back-calculated standards in Rexxip A covering a range of 0.18–3000 µg/L. In total 4 alternatives were evaluated. Using the maximum Relative Error (%) on the back-calculated standards as evaluation criteria (Fig 3) it was concluded that a 5-parameter logistic function weighted against "Response" was the best option.

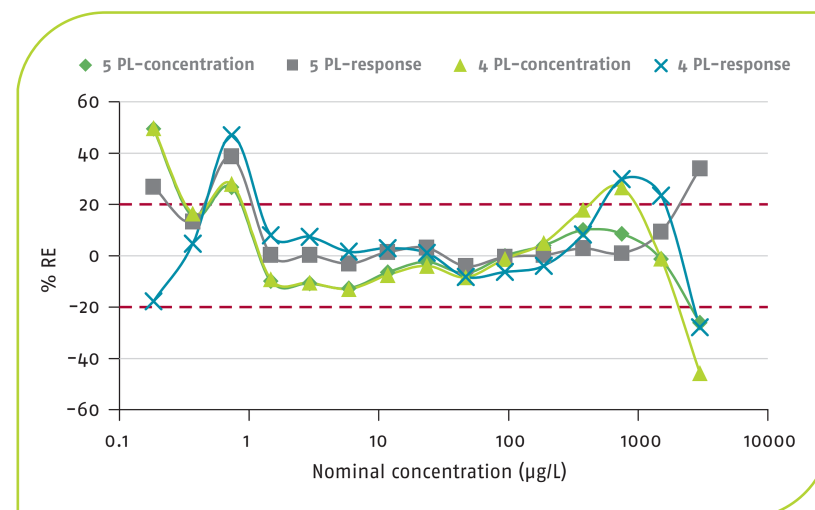

Fig 3. Back-calculated Relative Error (%) using different algorithm settings based on multiple serially diluted standard points (n=15) in Rexxip A.

Minimum required dilution (MRD)

In order to determine the optimal sample dilution factor different standard curves were prepared in different concentrations (40 to 1%) of pooled serum diluted in Rexxip A. Analysis of the data set suggested the most optimal sample concentration is 5% (data not shown). The results are shown in Figure 4.

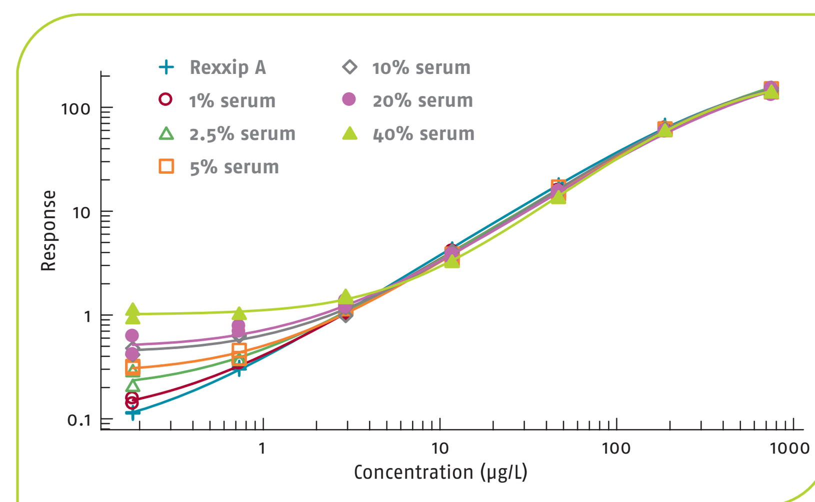

Fig 4. Determination of minimal required dilution (MRD) using a wide concentration range of analyte in 1 to 40% serum concentration.

Determination of LLOQ and ULOQ

LLOQ and ULOQ were determined by spiking 6 individual sera at 100% serum concentration with different concentrations of analyte. Upon further dilution to the predetermined working concentration of 5% serum in Rexxip A, analyte concentrations of 1, 2, 4, and 8 µg/L for LLOQ studies and 300, 400, 500, 600 and 800 µg/L for ULOQ samples were generated, respectively. The results from analysis are illustrated in Table 1 and 2 for LLOQ and ULOQ, respectively.

Table 1. Determination of LLOQ. The mean concentration of 6 replicates of each sample was determined and the CV (%), Relative Error (%) and Total Error (%) were calculated. Figures in red represent values outside acceptance limits (3).

	Sample ID (n=6)	Expected Concentration (µg/L)	Mean Concentration (µg/L)	CV (%)	Relative Error (%)	Total Error (%)
Individual 1	QC1	8	7,99	4,16	-0,12	4,3
	QC2	4	4,51	4,76	12,81	17,6
	QC3	2	2,25	13,61	12,40	26,0
	QC4	1	1,11	15,72	10,81	26,5
Individual 2	QC1	8	8,84	3,35	10,47	13,82
	QC2	4	4,63	2,96	15,78	18,75
	QC3	2	2,30	6,05	14,80	20,85
	QC4	1	1,15	8,30	14,80	23,11
Individual 3	QC1	8	8,47	3,75	5,82	9,6
	QC2	4	4,21	4,76	5,29	10,1
	QC3	2	1,79	6,51	-10,41	16,9
	QC4	1	0,62	14,64	-38,27	52,9
Individual 4	QC1	8	8,11	2,74	1,43	4,2
	QC2	4	4,45	3,73	11,23	15,0
	QC3	2	2,16	3,91	8,13	12,0
	QC4	1	1,14	45,36	13,94	59,3
Individual 5	QC1	8	8,95	3,46	11,84	15,3
	QC2	4	4,81	4,45	20,32	24,8
	QC3	2	2,09	5,32	4,66	10,0
	QC4	1	1,14	16,85	13,78	30,6
Individual 6	QC1	8	8,03	2,80	0,36	3,2
	QC2	4	4,20	4,30	4,88	9,2
	QC3	2	1,76	12,49	-12,24	24,7
	QC4	1	0,55	15,66	-45,49	61,2

Table 2. Determination of ULOQ. The mean concentration of 6 replicates of each sample was determined and the CV (%), Relative Error (%) and Total Error (%) were calculated. Figures in red represent value outside acceptance limits (3).

	Sample ID (n=6)	Expected Concentration (µg/L)	Mean Concentration (µg/L)	CV (%)	Relative Error (%)	Total Error (%)
Individual 1	QC1	800	825,87	23,13	3,23	26,36
	GC2	600	593,00	8,40	-1,17	9,57
	QC3	500	538,82	11,96	7,76	19,73
	QC4	400	436,88	6,53	9,22	15,75
	QC5	300	307,64	3,98	2,55	6,53
Individual 2	QC1	800	964,93	33,98	20,62	54,60
	GC2	600	875,65	30,51	45,94	76,45
	QC3	500	544,24	11,26	8,85	20,11
	QC4	400	411,38	6,95	2,85	9,79
	QC5	300	305,97	4,62	1,99	6,61
Individual 3	QC1	800	801,08	19,78	0,14	19,91
	GC2	600	685,37	14,25	14,23	28,48
	QC3	500	547,14	5,91	9,43	15,34
	QC4	400	433,71	5,76	8,43	14,19
	QC5	300	319,52	5,43	6,51	11,94
Individual 4	QC1	800	1080,60	15,94	35,08	51,01
	GC2	600	698,42	13,68	16,40	30,08
	QC3	500	532,16	11,30	6,43	17,74
	QC4	400	441,19	7,14	10,30	17,43
	QC5	300	321,97	4,22	7,32	11,55
Individual 5	QC1	800	1124,38	32,21	40,55	72,76
	GC2	600	597,42	13,19	-0,43	13,63
	QC3	500	473,55	8,88	-5,29	14,17
	QC4	400	423,81	2,75	5,95	8,71
	QC5	300	317,90	3,70	5,97	9,67
Individual 6	QC1	800	1044,68	19,68	30,58	50,27
	GC2	600	671,56	16,61	11,93	28,53
	QC3	500	591,43	6,48	18,29	24,77
	QC4	400	458,00	6,12	14,50	20,62
	QC5	300	332,60	4,39	10,87	15,26

In summary, the LLOQ and ULOQ were determined to be 2 µg/L and 500 µg/L, respectively, covering an analyte concentrations range of 250.

Carry over

Carry over was determined for individual sample needles by aspirating and dispensing, first a blank sample containing 5% serum in Rexxip A, followed by a sample at an analyte concentration of 50 mg/L in 5% serum, and then a fresh second blank. Between sample processing, needles were wash according to the standard method in the wash station. The results indicate that blanks processed before and after analyte containing samples did not differ significantly in response, nor did they reach the detection limit of the assay. Thus the experimentally determined carry over was <0.3/50000 = <1:166,600. (Fig 5)

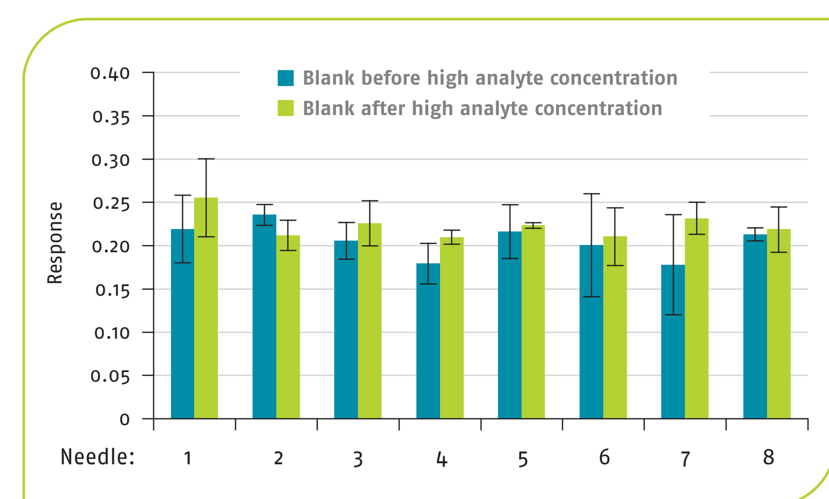

Fig 5. Carry over of selected assay by separate transfer of samples (blanks, 50 mg/L, blanks) in triplicates by 8 different needles. All responses were below the detection limit for the assay and did not differ significantly.

Selectivity

Sera from 9 different individuals were spiked with analyte at a concentration of 40 µg/L in undiluted serum. Samples were further diluted to 5% serum concentration in Rexxip A and analyzed for analyte contents. 6/9 samples passed the acceptance criteria in correspondence with 4-6-30 rule (4). Data are shown in Table 3.

Table 3. Evaluation of analyte selectivity. Results outside acceptance criteria are indicated in red (n=3).

Sample	Expected Concentration (µg/L)	Average concentration (µg/L)	CV (%)	Relative Error (%)
1	2,00	2,33	5,35	16,57
2	2,00	2,02	8,83	1,08
3	2,00	2,59	3,92	29,27
4	2,00	3,70	6,92	84,91
5	2,00	2,65	6,27	32,59
6	2,00	2,59	4,99	29,55
7	2,00	2,24	1,43	12,17
8	2,00	2,21	8,9	10,71
9	2,00	2,61	4,63	30,26

Dilution Linearity

The linearity-of-dilution was determined by spiking analyte into 100% serum from 5 different individuals at approximately 1000 times the ULOQ. Spiked samples were first diluted to 5% serum concentration and then serially diluted covering concentrations exceeding 3 orders of magnitude and analyzed for analyte concentration. The concentrations determined analytically were then back-calculated and compared to expected concentrations. The results are illustrated in Figure 6 and indicate that the dilution linearity of the assay is approximately two orders of magnitude which is likely to be sufficient for most analytical situations.

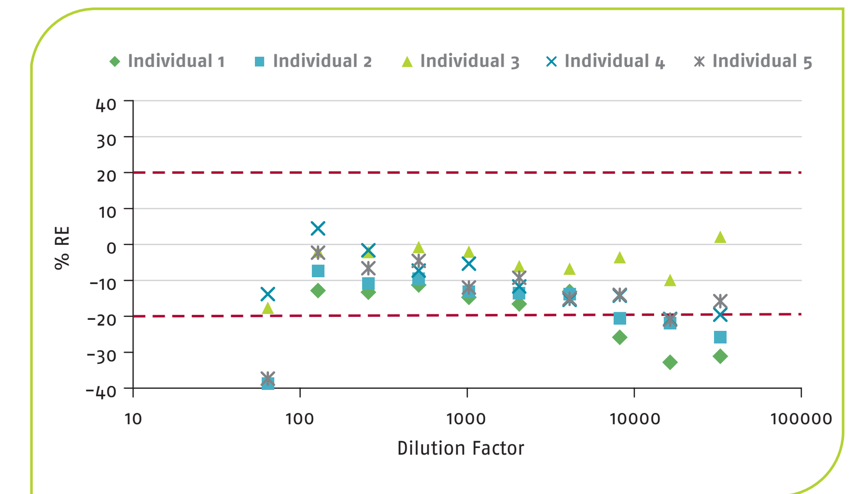

Fig 6. Linearity-of-dilution based on sequential dilutions of samples containing spiked analyte in 5% serum from 5 different individuals. Samples at low dilutions exceeding the ULOQ are not shown in the graph.

Intra-CD, inter-CD and inter-Run Precision

Five QC samples were prepared by spiking analyte into a human serum pool (100 %) at 20 x analyte concentrations, aliquoted and frozen at -20°C. Upon analysis, QC samples were thawed and further diluted to 5% in Rexxip A generating QC samples at 500, 375, 250, 5 and 2 µg/L, respectively.

Eight standards were prepared by serial dilution in 5% serum concentration in Rexxip A. Standards ranged from 1100 to 0.85 µg/L including anchor points (Table 4).

Table 4. Summary of inter CD standards in Run 3.

Standard Concentrations (µg/L)	Mean (µg/L)	Inter-CD Standard CV (%)	Relative Error (%)	Total Error (%)
0.85	0.85	20.5	0.4	20.9
2.55	2.64	10.1	3.4	13.5
7.64	7.56	2.8	-1.0	3.8
22.92	22.83	1.8	-0.4	2.2
91.67	92.76	2.8	1.2	4.0
275	269.96	1.5	-1.8	3.3
550	612.13	18.3	11.3	29.6
1100	1153.04	64.7	4.8	69.5

Six Runs were performed on separate days, each Run containing 5 CDs that were processed identically within each Run. Each CD was processed with duplicate standards, 4 blanks and the 5 QC samples in quadruplicate. 2 different operators performed the 6 runs (Tables 5–7).

Table 5. Summary of intra-CD performance in Run 3.

QC samples	Mean (µg/L)	Intra-CD CV (%)	Relative Error (%)	Total Error (%)
500	483.10	8.7	-3.4	12.1
375	343.88	7.6	-8.3	15.9
250	245.94	3.2	-1.6	4.9
5	4.84	3.5	-3.1	6.6
2	1.82	6.8	-8.9	15.7

Table 6. Summary of inter-CD performance in Run 3.

QC samples	Mean (µg/L)	Inter-CD CV (%)*	Relativ Error (%)	Total Error (%)
500	504.21	7.7	0.8	8.6
375	351.98	7.0	-6.1	13.1
250	242.79	3.7	-11.7	15.4
5	4.84	4.5	-3.1	7.6
2	1.94	6.9	-2.8	9.7

*Inter-CD CV (%) was calculated using ANOVA.

Table 7. Summary of inter-Run performance in Run 1–6.

QC samples	Mean (µg/L)	Inter-Run CV (%)*	Relative Error (%)	Total Error (%)
500	492.95	17.7	-1.4	19.1
375	336.54	14.6	-10.3	24.9
250	238.53	12.0	-13.3	25.2
5	4.76	10.9	-4.8	15.8
2	1.89	20.7	-5.7	26.4

*Inter-Run CV (%) was calculated using ANOVA.

Summary

An indirect antibody assay for a recombinant therapeutic antibody has been validated using Gyrolab Workstation. The results are summarized in Table 8.

Table 8. Validation Summary.

Parameter		Variable	Criteria	Results
Calibration Curve Fit				5-PL, Response weighting
LLOQ		Total Error (%)	30	2 µg/L
ULOQ		Total Error (%)	30	500 µg/L
QC Precision:	Intra-CD	CV (%)	10	3.2–8.7%
	Inter-CD	CV (%)	10	3.7–7.0%
	Inter-Run	CV (%)	20	10.9–20.7%
QC Accuracy:	Intra-CD	Relative Error (%)	20	1.6–8.9%
	Inter-CD	Relative Error (%)	20	0.8–11.7%
	Inter-Run	Relative Error (%)	20	1.4–13.3%
Carry over		Ratio	1:10000	1:166,600
Selectivity		Relative Error (%)	30	6/9 at 2 µg/L
Dilutional Linearity		Relative Error (%)	20	~2 orders of magnitude

Note. Data has not been back-calculated to 100 % serum concentration for any reported results.

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