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Short communication

Qualification of a microfluidics-based electrophoretic method for impurity testing of monoclonal antibodies

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ABSTRACT

In this work, we present a comprehensive evaluation of the Agilent Bioanalyzer, a microfluidics-based electrophoretic device that was used for impurity testing of a monoclonal antibody (mAb). We compared the system to SDS-PAGE, both operated under non-reducing conditions and found a significant improvement of accuracy for the Bioanalyzer. In addition, the latter exhibited a larger assay range and lower limit of quantitation (LOQ) based on a predefined total error limit of ±30%. However, during method qualification applying a three-factor nested design with two operators performing duplicate measurements per day, each on 4 different days, we observed unpredictable recurring quantitative outliers using the chip-based system. In-depth analysis on multiple runs with various chip lots confirmed the above finding and indicated that most likely on-chip dye labeling and/or post-column background fluorescence elimination are not compatible with the large size of the intact antibody as similar findings were observed for myosin used as upper marker for time correction. Interestingly, after reducing the intact antibody into light and heavy chain, we resolved the outlier issue. Eventually, requalification of the micro-fabricated analytical device under reducing conditions revealed only 1 out of 32 quality control samples (QCs) exceeding the ±30% total error limits.

1. Introduction

IGN311 is a humanized monoclonal antibody (mAb). It binds to the carbohydrate antigen Lewis Y and is used as a passive immunotherapeutic agent. IGN311 can directly destroy tumor cells by complement activation and by the activation of cytotoxic effector cells.

For the quality control of mAbs (e.g. stability and release testing), a comprehensive portfolio of analytical methods is required. Among these, SDS-PAGE, a method that has been introduced for the analysis of polypeptides and proteins several decades ago [1] is suggested for purity testing and molecular characterization by regulatory guidelines and pharmacopoeia monographs [2,3].

Chip-based electrophoresis systems have been developed recently for the analysis of DNA, RNA, and protein [4–11]. For the latter, a commercially available micro-fabricated and miniaturized analytical device on a glass chip was developed where the sample is detected by laser-induced fluorescence using non-covalently bound dyes that bind to SDS–protein complexes [12,13].

The advantages of this microfluidics-based technology over traditional gel-based systems are reported to be a significant reduction in analysis time and an improved accuracy with respect to sizing and quantitation [13,14]. In addition, an electropherogram and a virtual gel are generated by the software that enables easy documentation and evaluation of the results.

This system has been applied to the analysis of half-antibodies in IgG4 samples [15,16]. Both studies compared some quantitative performance characteristics of the chip-based system to the conventional SDS-PAGE method and found the microfluidics-based technology being superior regarding linearity and accuracy. Another study analyzing mAbs from cell culture supernatants reported good quantitative and qualitative correlation between the chip- and the gel-based system, both operated under reducing conditions [17]; however, the latter study did not report important assay performance characteristics for any of the methods and a detailed quantitative comparison using typical qualification/validation criteria was not attempted. Recently, Chen and co-workers applied a microchip-based assay to the screening of mAb product quality and compared it to conventional capillary electrophoresis [18]. Their work mainly focused on the ability of the

Abbreviations: LOQ, limit of quantitation; QCs, quality control sample; mAb, monoclonal antibody; ANOVA, analysis of variances; TCA, time-corrected area.

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system to discriminate between glycosylated and non-glycosylated mAb heavy chains and presented a simple glycan typing assay. They also included preliminary assay qualification data.

This work aims at a comprehensive comparison of a chip-based electrophoretic device under non-reducing and reducing conditions with conventional non-reducing SDS-PAGE, which was developed for stability testing detecting impurities in drug substance/product. Both systems were qualified using IGN311 (purity) as surrogate reference for unknown impurities in a three-factor nested design with two operators performing duplicate measurements per day, each on 4 different days. With this design, we emphasized on long-term assay robustness, which is of utmost importance for quality control of pharmaceutical products.

2. Materials and methods

2.1. Materials

Dilution buffer (1× PBS without Ca and Mg) was purchased from PAA, Austria. Acetic acid 100% and ethanol absolute was obtained from Merck, Austria. Cell culture grade water was purchased from PAA, Austria and β-mercaptoethanol was received from Merck, Austria.

IGN311 drug substance was produced in SP2/0 cells according to GMP guidelines by BioInvent International AB (Lund, Sweden). It is a cytolytic, fully humanized monoclonal IgG1 antibody directed against the Lewis Y carbohydrate antigen. IGN311 is based on the murine Lewis Y specific mAb ABL364. Upon humanization, the molecular structures mediating binding to Lewis Y remained unchanged whereas all other parts were exchanged with human immunoglobulin structures [19].

2.2. Microfluidics-based device

Microfluidics-based electrophoresis was performed using the Agilent 2100 Bioanalyzer with the Protein 200 Lab Chip Kit, supplied by Agilent Technologies, Waldbronn, Germany and applied according to the manufacturer's instructions.

The chip priming station was adjusted by changing the base plate position to position "A" and adjusting the syringe clip to the middle position. Afterwards, the Bioanalyzer 2100 Expert Software was started and the corresponding assay selected. Then, 25 μl of the dye concentrate were combined with 650 μl gel matrix and centrifuged for 15 min at 5200 rpm in a micro-centrifuge. The destaining solution consisted of 650 μl gel matrix without dye concentrate and was also centrifuged as described. Then, 4 μl of protein sample were combined with 2 μl of sample buffer (with or without β-mercaptoethanol), mixed, and heated for 5 min at 95 °C. Afterwards, the samples were left to cool down to room temperature, spun down, and filled up with 84 μl deionized water. After loading the gel-dye mix into the chip using the chip priming station, 6 μl of each sample, 6 μl ladder and 12 μl destaining solution were pipetted in the corresponding wells on the chip. Finally, the chip was inserted in the Bioanalyzer and the run was started.

2.3. Non-reducing SDS-PAGE

Non-reducing SDS-PAGE was performed on a Multiphor II Electrophoresis System using the power supply EPS 3501 XL (Amersham, Austria) with homogeneous 7.5 ExcelGels and ExcelGel SDS buffer strips, supplied by Amersham, Austria.

The fixing solution consisted of 400 ml ethanol, 100 ml acetic acid, and 500 ml cell culture grade water. The staining solution contained 1 tablet PhastGel Blue R (Amersham, Austria) dissolved in 400 ml destaining solution and filtered using a sterile filter (Stericup from Millipore, Germany). The destaining solution was 250 ml ethanol, 80 ml acetic acid, and 670 ml cell culture grade water.

Thirty microliters of samples were blended with 15 μl dilution buffer and 15 μl LDS-PAGE sample buffer (Invitrogen, Germany), mixed using a vortex and shaken for 2 min at 85 °C in a thermomixer.

The electrophoresis system was cooled to 15 °C, BayolF (Serva, Germany) was pipetted onto the cooling plate, and the gel was positioned with the sample wells at the cathodic side. Then, cathodic and anodic SDS buffer strips were applied accordingly. Ten microliters of sample and marker (Mark12 from Amersham, Austria) were loaded onto the gel using the preformed sample wells. Electrical settings were 600 V, 50 mA, 30 W. Running time was 80 min.

Immediately after electrophoresis, the buffer strips were removed and the gel immersed in fixing solution for 30 min. Then, the staining solution was heated to 60 °C and the gel stained for 15 min. Afterwards, the gel was rinsed twice in distilled water and destained by changing the destaining solution several times until the background was clear. After completed destaining, the gel was scanned using a flatbed scanner with 600 dpi (color and black/white) and analyzed using GelScan Pro gel evaluation software (BioSciTec, Germany).

2.4. Qualification design

Two operators performed duplicate measurements per day, each on four different days resulting in eight qualification experiments.

2.4.1. Microfluidics-based device

Each qualification experiment comprised a four-level calibration using IGN311 from 25 to 200 μg/ml and four QCs (duplicates of 50 and 100 μg/ml) run on one chip. For the determination of the response function, three dilution series including 25, 50, 100, and 200 μg/ml were performed on three chips. To analyze the repeatability within a single chip, five chips with a sample concentration of 500 μg/ml and three chips with 200 μg/ml were performed under non-reducing conditions and seven runs with a sample concentration of 500 μg/ml were performed under reducing conditions. Four different chip lots were used for the qualification (four lots per operator) and five chip lots for the repeatability experiments. To evaluate memorizing effects within the separation channel of the chip, a run with alternately loaded blank and IGN311 (200 μg/ml) was performed.

2.4.2. Non-reducing SDS-PAGE

Each qualification experiment comprised a four-level calibration using IGN311 from 0.125 to 1 μg load (corresponds to 25–200 μg/ml) and four QCs (duplicates of 0.25, and 0.5 μg load which corresponds to 50 and 100 μg/ml) run on one gel. For the determination of the response function, a gel was run with four concentrations of IGN311 (0.125, 0.25, 0.5, and 1 μg load) in quintuplicate that were randomly arranged on the gel.

2.5. Data evaluation

Areas from calibrators were back-calculated on their corresponding regression. The back-calculated values of all runs were used to estimate bias and intermediate precision for all concentrations eventually providing accuracy profiles (total error) for both methods.

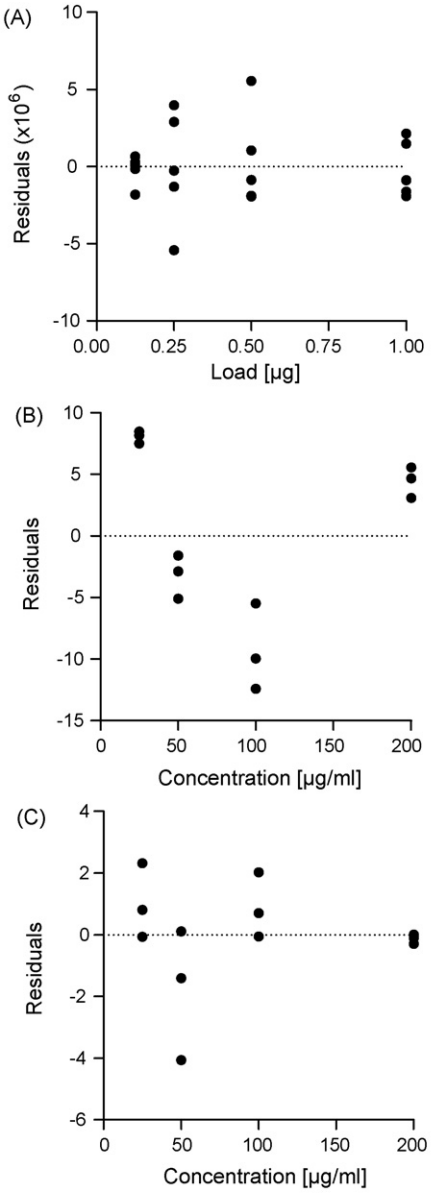

Fig. 1. Residual plot from linear regression of IGN311 load/concentration vs. area for SDS-PAGE (A) and the microfluidics-based device (B), and from quadratic regression for the microfluidics-based device (C), all operated under non-reducing conditions.

The total error (derived from bias and precision) of the method was calculated as following (in % relative error):

$$\%\text{R.E.} = \left(\frac{100}{\mu}\right) \times (Z - \mu) \pm 2s_{\text{IP}}$$

where z is the overall mean (by averaging all back-calculated values for each concentration level), μ the nominal concentration and s_{IP} the standard deviation (estimated by calculating the standard deviation from all back-calculated values for each concentration level). This provides for a concentration-dependent, simplified β-expectation interval within a future single value will be located with 95% probability.

In addition, the recovery of the QCs was calculated using the corresponding regression curve for both, SDS-PAGE and the microfluidics-based device.

3. Results

3.1. Assay performance under non-reducing conditions

3.1.1. Response function—linearity

For data evaluation, absolute integrated densities for the SDS-PAGE method and time-corrected areas (TCA) for the microfluidics-based device were used. To describe the concentration–response (area) relationship of IGN311, linear regression was used.

For SDS-PAGE under non-reducing conditions, no significant trend in the residuals was observed visually for the four concentrations tested (Fig. 1A). Based on d'Agostino/Pearson and Bartletts test at $p = 0.05$, normality of residuals and homogeneity of variances over the entire concentration range was confirmed and the linear regression model was suggested as response function of choice. Eventually, analysis of variances (ANOVA) with lack-of-fit test revealed no significant deviation from linearity at $p = 0.05$ ($n = 20$).

For the microfluidics-based device under non-reducing conditions, visual evaluation of residuals from linear regression revealed a non-random pattern (Fig. 1B) which was confirmed by statistical analysis. ANOVA with lack-of-fit test revealed a significant deviation from linearity at $p = 0.05$. Thus, linear regression may not be the optimum model and data were fit to a quadratic equation.

For the non-linear regression, visual inspection (Fig. 1C) and statistical analysis (ANOVA with lack-of-fit test) showed no trend in residuals making the quadratic equation the model of choice for the microfluidics-based device.

3.1.2. Assay accuracy, range, and limit of quantitation (LOQ)

For the determination of assay bias, precision, assay range, and LOQ, a nested design (2 operators; each 4 runs on different days) was performed, resulting in eight individual regressions for IGN311. The same response values were back-calculated on their corresponding regression providing data for the calculation of bias and precision.

For SDS-PAGE under non-reducing conditions, Table 1 shows bias and intermediate precision for IGN311 for all loads included in assay qualification and Fig. 2A shows the accuracy profile (total error) based on data from Table 1. For predefined acceptance limits (±30% total error), the LOQ was identified at 0.25 µg as the 0.125 µg load exceeded the acceptance limits. Thus, the total assay range is 0.25–1.0 µg. Depending on the amount of sample (purity) eventually loaded onto the gel, impurity may be quantified over a wide range in future assays using IGN311 as surrogate reference (external standard calibration) for unknown impurities.

For the microfluidics-based device under non-reducing conditions, Table 2 shows bias and intermediate precision for IGN311 for all concentrations tested using the microfluidics-based device. Again, data from Table 2 were used to compute the accuracy profile (total error) for all concentrations under non-reducing conditions (Fig. 2B) and acceptance limits were set to ±30% total error. Compared to the results obtained for SDS-PAGE, the microfluidics-based device revealed significantly lower system variability (bias and intermediate precision) eventually resulting in an appreciably narrower accuracy profile being within acceptance limits over the

Table 1
Bias and intermediate precision for IGN311 using SDS-PAGE operated under non-reducing conditions.

Load [µg]	Bias [%]	Intermediate precision [%]
0.125	9.2	37
0.25	0.65	12
0.5	3.5	10
1	−0.78	2.4

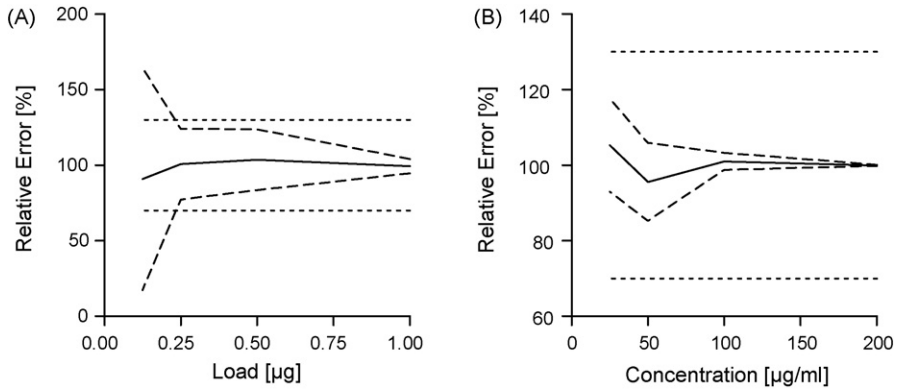

Fig. 2. Accuracy profile (total error) for IGN311 showing bias (solid lines) and simplified 95% β-expectation limits (dashed lines) for SDS-page (A) and the microfluidics-based device (B), both operated under non-reducing conditions. Dotted lines are ±30% acceptance limits.

Table 2
Bias and intermediate precision for IGN311 using the microfluidics-based device operated under non-reducing conditions.

Concentration [µg/ml]	Bias [%]	Intermediate precision [%]
25	5.3	6.2
50	−4.4	5.1
100	0.99	1.1
200	−0.059	0.068

whole range. Thus, the LOQ is 25 µg/ml (corresponds to 0.125 µg load for the SDS-PAGE) and the assay ranges from 25 to 200 µg/ml, which is significantly larger compared to the SDS-PAGE.

3.1.3. Recovery of QCs

For both methods, QCs (0.25 µg and 0.5 µg load for non-reducing SDS-PAGE and 50 µg/ml and 100 µg/ml for the microfluidics-based device), which were applied to qualification gels and chips, respectively, were analyzed on the corresponding calibration curve and the recovery in % was calculated (Fig. 3).

For SDS-PAGE, only 2 out of 32 QCs were found to exceed the ±30% acceptance limits (Fig. 3A); however, the microfluidics-based device under non-reducing conditions revealed ∼25% of the QCs being considerably outside the predefined acceptance criterion (Fig. 3B) which disables the current procedure for routine analysis.

3.1.4. Within-chip repeatability experiments

We investigated the nature of this phenomenon in more detail and ran 8 chips loaded with 200 and 500 µg/µl IGN311, respectively and again found 1–2 significant outliers (>1.5 and 3 interquartile ranges, respectively) on 6 of 8 chips (Fig. 4A).

Furthermore, there was a random distribution of outliers on the chips which does not allow omitting defined well positions on the chip enabling reproducible operation in future experiments.

We also evaluated if the upper marker myosin – normally applied for time correction – may be used as internal standard compensating for e.g. sample introduction variation. This approach was suggested previously for the analysis of half-antibody species [16]; however, run-specific correction measures did not resolve the above issue. Detailed evaluation of electropherograms revealed that myosin also underwent strong area variations within a chip; however, they did not correlate to those of the mAb. Interestingly, this phenomenon has not been reported previously for IgG which used myosin as reference for calculating the concentration of light and heavy chain of the antibody [17].

We can also exclude potential adsorption of the mAb onto the separation channel (each of the 10 samples migrates through the same channel) which would then elute with time over the following runs. We did not observe any 'memorizing' effects of consecutive runs using a chip with alternately loaded blank and 200 µg/ml IGN311 (Fig. 4B).

We assumed that the described phenomenon was analyte rather than chip/system-related and hypothesized if the size or nature of the intact antibody (non-reducing conditions) may be responsible for the recurring outliers. Thus, we ran 7 chips at a concentration of 500 µg/ml IGN311 under reducing conditions. Fig. 5 shows scatter plots of TCA for light chain (LC, panel A) and heavy chain (HC,

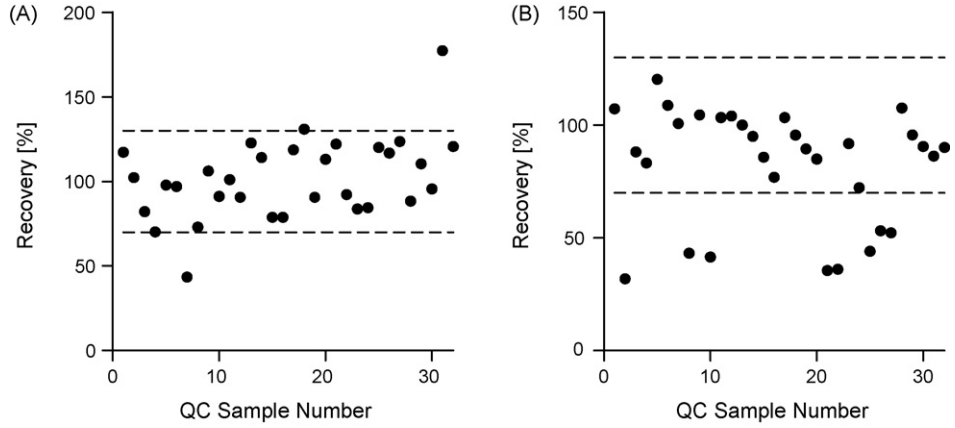

Fig. 3. Recovery in % of QCs analyzed on corresponding regression curve during method qualification of SDS-PAGE (A) and the microfluidics-based device (B), both operated under non-reducing conditions. Dashed lines are ±30% acceptance limits.

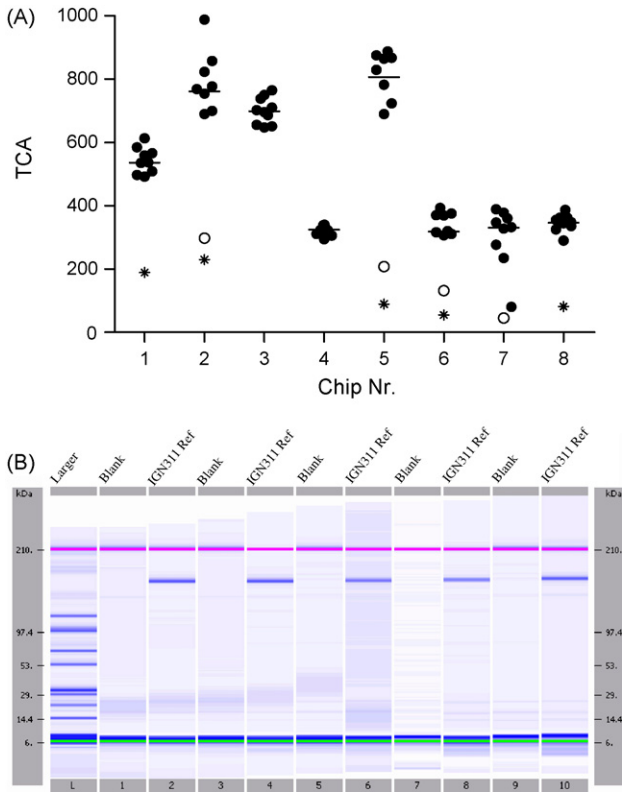

Fig. 4. Scatter plot of TCA for repeated measurements (10 runs per chip) of 500 μg/ml IGN311 (chip 1–5) and 200 μg/ml IGN311 (chip 6–8) using the microfluidics-based device operated under non-reducing conditions. Solid lines are medians, open circles are outliers with >1.5 interquartile ranges, and stars are outliers with >3 interquartile ranges (A). Electropherogram of alternately loaded blank and IGN311 (200 μg/ml) starting from lane 1 (B).

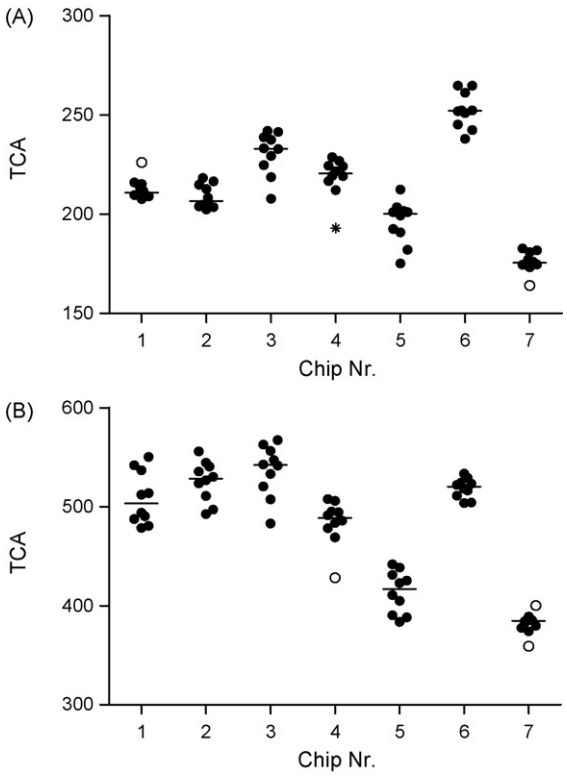

Fig. 5. Scatter plot of TCA of LC (A) and HC (B) for repeated measurements (10 runs per chip, 7 chips in total) of 500 μg/ml IGN311 using the microfluidics-based device under reducing conditions. Solid lines are medians, open circles are outliers with >1.5 interquartile ranges, and stars are outliers with >3 interquartile ranges.

panel B). Indeed, only few outliers were identified and a significantly improved overall performance was achieved compared to operation under non-reducing conditions. Actually, the outliers are mostly due to the very low variability of the remaining samples.

Based on these findings, we assume that on-chip dye labeling and/or post-column background fluorescence elimination is somewhat error prone for large proteins such as intact antibodies (~150 kd) or myosin (~210 kd) using the microfluidics-based device. Both proteins showed significant area variations which did not correlate and thus, may not be attributed to sample introduction issues. As already mentioned above, these findings were not reported previously and other authors reported reproducible performance for the analysis of mAbs even under non-reducing conditions which may also be due to the different microfluidics-based system used in their study [18].

3.2. Performance of the microfluidics device under reducing conditions

Based on the preliminary results obtained under reducing conditions, we decided to re-qualify the microfluidics-based system using LC and/or HC of IGN311 as surrogate reference for unknown impurities.

3.2.1. Response function—linearity

As for the microfluidics-based device used under non-reducing conditions, operation with reduced mAbs revealed a non-random residual pattern using linear regression (data not shown). The visual finding was confirmed by ANOVA with lack-of-fit test revealing significant deviation from linearity at $p = 0.05$. Again, quadratic equation proved to be the fit of choice with visual inspection of residuals showing no deviation from linearity (data not shown). In addition statistical analysis using ANOVA with lack-of-fit test showed no significant trend at $p = 0.05$ for the LC and only a slight deviation from a random residual distribution for the HC ($p = 0.044$); however, the quadratic fit was still found to be appropriate to describe the concentration–response relationship.

3.2.2. Assay accuracy, range, and LOQ

The same nested design qualification strategy as described in Section 3.1.2 was used for evaluating the performance of the microfluidics-based device under reducing conditions.

Table 3 shows bias and intermediate precision for IGN311 LC and HC for all concentrations applied. Errors were found to be in the same range compared to the system operated under non-reducing conditions (Table 2) and again appreciably lower than the accuracy obtained for SDS-PAGE (Table 1).

Based on the data from Table 3, we computed total errors for LC and HC (Fig. 6A and B, respectively) revealing similar accuracy profiles as obtained for the non-reducing chip-based system. Again, compared to SDS-PAGE (non-reducing), assay variability was found to be significantly reduced meeting acceptance limits of ±30% total error over the entire range with an LOQ of 25 μg/ml.

Table 3
Bias and intermediate precision (s_{IP}) for IGN-311 LC and HC using the microfluidics-based device operated under reducing conditions.

Concentration [μg/ml]	Bias, LC [%]	s_{IP}, LC [%]	Bias, HC [%]	s_{IP}, HC [%]
25	5.3	6.2	−5.7	9.4
50	−4.4	5.1	4.0	6.8
100	0.99	1.1	−0.79	1.4
200	−0.059	0.068	0.04	0.07

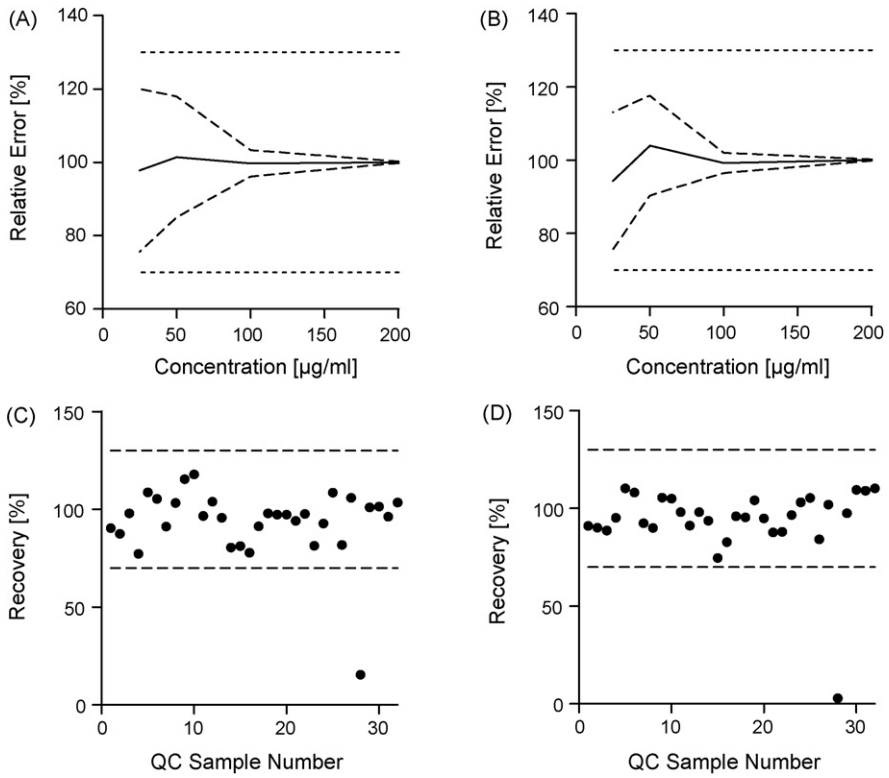

Fig. 6. Accuracy profile (total error) for IGN311 showing bias (solid lines) and simplified 95% β-expectation limits (dashed lines) for LC (A) and HC (B) using the microfluidics-based device operated under reducing conditions. Dotted lines are ±30% acceptance limits. Recovery in % of LC (C) and HC (D) of QCs analyzed on corresponding regression curve during method qualification of the microfluidics-based device operated under reducing conditions. Dashed lines in panels C and D are ±30% acceptance limits.

3.2.3. *Recovery of QCs*

Finally, we analyzed QCs (50 and 100 µg/ml, respectively) on the corresponding regression and calculated the recovery in % (Fig. 6C and D). An example electropherogram of the 200 µg/ml reference is presented in Fig. 7. Indeed, preliminary results from the chip-based system running under reducing conditions, which basically showed absence of frequent unpredictable outliers (Fig. 5) was confirmed for LC and HC, respectively. For both, only 1 outlier violating the predefined ±30% total error criterion was found which translates into a probability of ~3%. Thus, significant improvement over the non-reducing system was achieved (compare Fig. 3B) meeting the high reproducibility and system

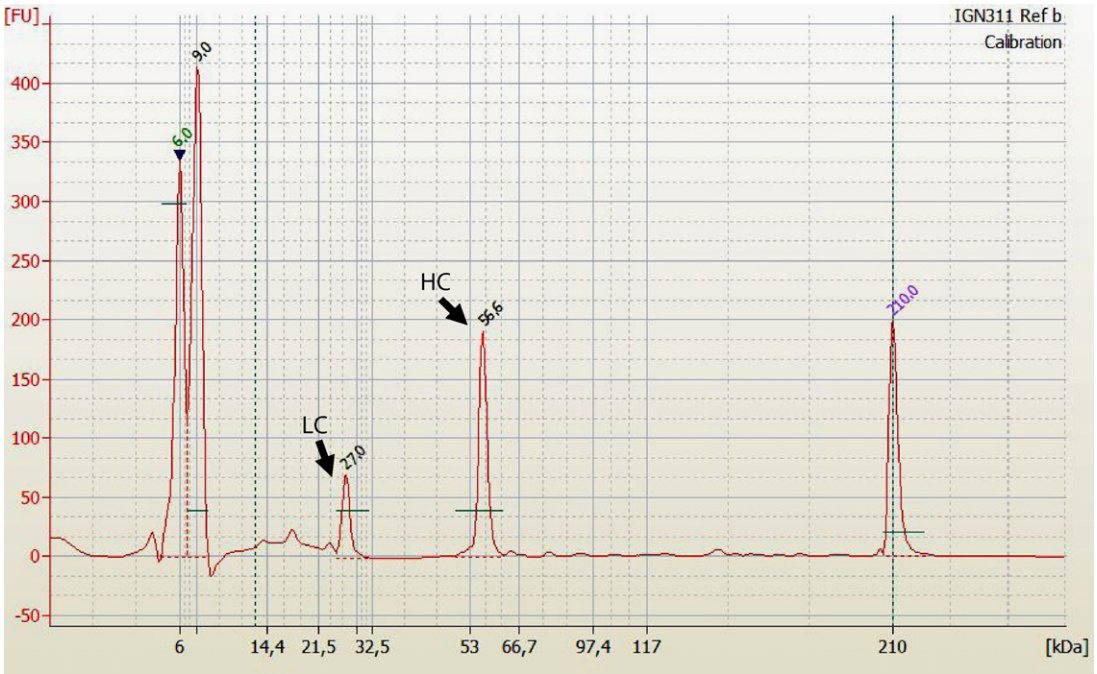

Fig. 7. Microfluidics-based system under reducing conditions: example electropherogram of the 200 µg/ml reference; LC and HC are indicated.

stability requirements for analytical applications in quality control.

4. Conclusion

The Agilent Bioanalyzer, a microfluidics-based electrophoretic device proved to be applicable for reliable impurity analysis for mAbs using the purity itself as surrogate reference for not yet determined degradation and by-products. The device was found to be notably more accurate compared to the widely used SDS-PAGE system which confirms previous findings [15,16]. In our setup, the micro-fabricated system exhibited a broader assay range accompanied by a lower LOQ based on a predefined total error criterion of ±30%. A quadratic equation was used as the concentration–response relationship was significantly non-linear.

Under non-reducing conditions however, the chip-based device revealed randomly recurring outliers observed in several experiments with diverse chip lots, a phenomenon which has not been addressed previously by other authors using the system under non-reducing conditions [16,18]. Due to the given, fixed manufacturer's instructions, manipulations on reagents or even the chip itself are not feasible. However, we hypothesized that the large size of the intact antibody may be the cause of the above mentioned outliers as detailed analysis of multiple runs/chip also revealed a similar finding for the upper marker (myosin) used for time correction of area responses. Indeed, operating under reducing conditions with separate LC and HC resolved this issue and provided with a fast and reliable assay ready to be implemented in the quality control of mAbs.

The major advantage of the microfluidics-based device is the fast processing of samples. The total run time of one chip (10 samples) including sample preparation and evaluation is approximately 1 h, whereas the run time for a typical gel-based application including evaluation is roughly 5 h. Furthermore, the handling of the microfluidics-based technology is much more convenient and does not require the extensive and time-consuming staining procedure of conventional gel electrophoresis.

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