

LabChip® GXII Touch™  
protein characterization  
system, ProteinEXact™ assay

## Authors:

Séverine Fagète<sup>a</sup>, Antsa Rabenifara Estoppey<sup>a</sup>,  
Alexandra Martiné<sup>a</sup>, Rémi Bussat<sup>a</sup>, Francesca  
Fanizzi<sup>a</sup> and Pierre-Alain Girod<sup>a</sup>, Carole  
Poutriquet<sup>b</sup>

<sup>a</sup>Selexis SA, 14 chemin des Aulx, CH-1228  
Plan les Ouates, Switzerland

<sup>b</sup>PerkinElmer, Inc.  
Waltham, MA

## Microcapillary electrophoresis analysis of asymmetrical bispecific antibody products by the ProteinEXact™ assay using the LabChip® GXII Touch™ protein characterization system

### Introduction

In the area of next-generation antibody therapeutics, bispecific antibodies (bsAb) with their exclusive dual binding functions, predominate in the market.<sup>1</sup> The wide diversity of their formats is

only limited by our imagination.<sup>2,3</sup> Therefore, ensuring quality assessment and characteristic analysis has become a real challenge, especially in cell line development in view of isolating clones expressing the right molecule. Among the different subclasses of bsAbs, asymmetrical molecules are of particular interest as they offer the possibility to discriminate the unwanted forms – homodimers – from the active form – heterodimer – by the size.

Here we evaluated a microfluidic electrophoretic separation technology, the LabChip® GXII Touch™ system, which offers fast-automated analysis and quantitation of protein samples.<sup>4,5</sup> The system provides information on concentration and molecular weight, as well as percent purity, charge variants or glycan profiling. Practically no development is required as different application assays are already available as chip kits.

We focused on the  $\mu$ CE-SDS sizing method and on the comprehensive analysis of the performance of the ProteinEXact™ assay, defined as a highly sensitive quantitation and sizing assay<sup>6</sup>. Results highlight that the LabChip® GXII Touch™ system can serve as an integrated analytical workflow solution in the cell line development process for asymmetrical bsAbs, providing sensitive, high resolved and reproducible separation and quantification of bsAb forms in cell culture supernatant.

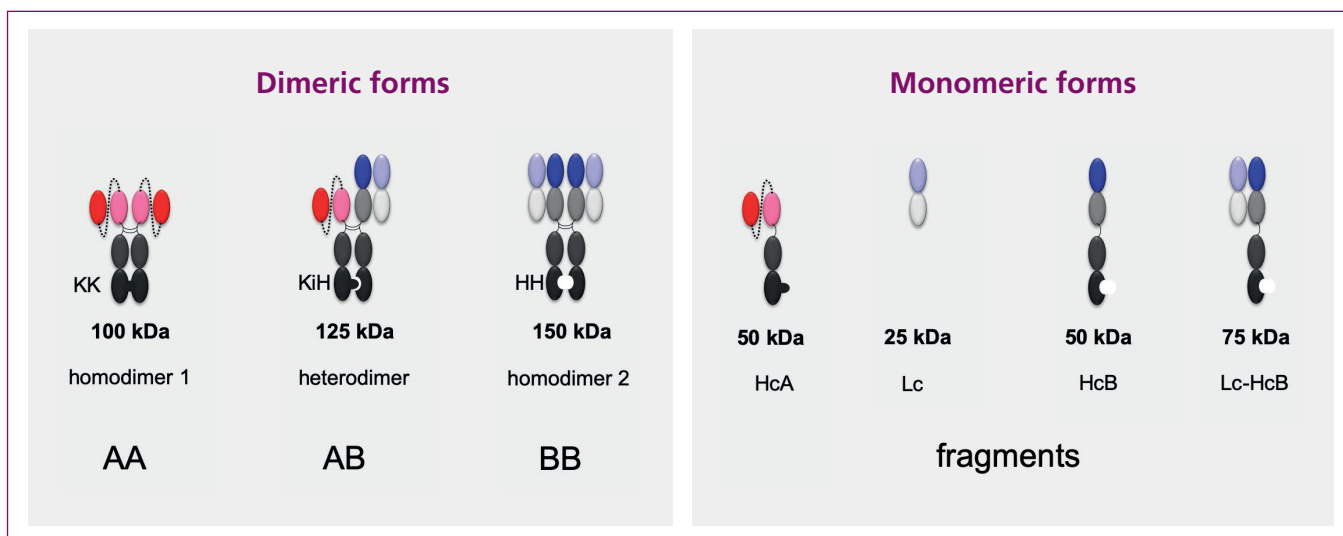


Figure 1: Case study bsAb format and derivative forms. Schematic representation of the heterodimer KiH-Fc-Fab/scFv bispecific antibody format used in this study (left panel, AB), the derivatives homodimers (left panel, AA and BB) and the non-associated half antibodies and single polypeptide chains (right panel, fragments).

## 2. Material and methods

### 2.1. BsAb format design and quality attributes

A Fab-scFv bsAb format was chosen as a model molecule (Fig. 1, left panel, heterodimer AB). It results from the assembly of three polypeptide chains, a scFc-Fc of around 50 kDa, a classical Hc of 50 kDa and its associated light chain (Lc) of 25 kDa (Fig. 1, right panel, HcA, HcB and Lc, respectively). Knob-into-hole (KiH) mutations were introduced in the Fc fragment 7 in order to promote heterodimerization (Fig1. Left panel). Despite this forced pairing, alternative mispaired dimeric bsAb forms, the homodimers (Fig. 1, left panel, AA and BB), are still co-expressed as impurities. They differ in size, respectively 100 kDa and 150 kDa, as compared to the 125 kDa heterodimeric form (Fig.1 left panel). Therefore, quality analysis of an asymmetrical bsAb product will be focused on the heterogeneous distribution of co-existing forms (or purity) based on their size, as a distinctive attribute.

### 2.2. bsAb samples

Two clonal cell lines, stably expressing the bsAb forms at different proportion, were generated using Selexis SUREtechnology™ platform<sup>®</sup>. A 10-day fedbatch supernatant of these CHO-M expressing cells was clarified by centrifugation and used as sample for analysis.

### 2.3. $\mu$ CE-SDS separation by LabChip<sup>®</sup>

All experiments were performed on the LabChip<sup>®</sup> GXII Touch™ HT protein characterization system (PerkinElmer) using microchip capillary electrophoresis-based separation (Figure 2). Molecular weight sizing and percent purity analysis of bsAb samples was evaluated using the ProteinEXact™ assay (PerkinElmer) offering sizing range of 6.5 kDa-250 kDa. All the reagents were part of the ProteinEXact™ reagent kit except the iodoacetamide and the 2-mercaptoethanol, both purchased from Sigma Aldrich. The samples were prepared according to the manufacturer's instructions for the ProteinEXact™ assay (Figure 2) and run using the default injection and separation conditions of the instrument.

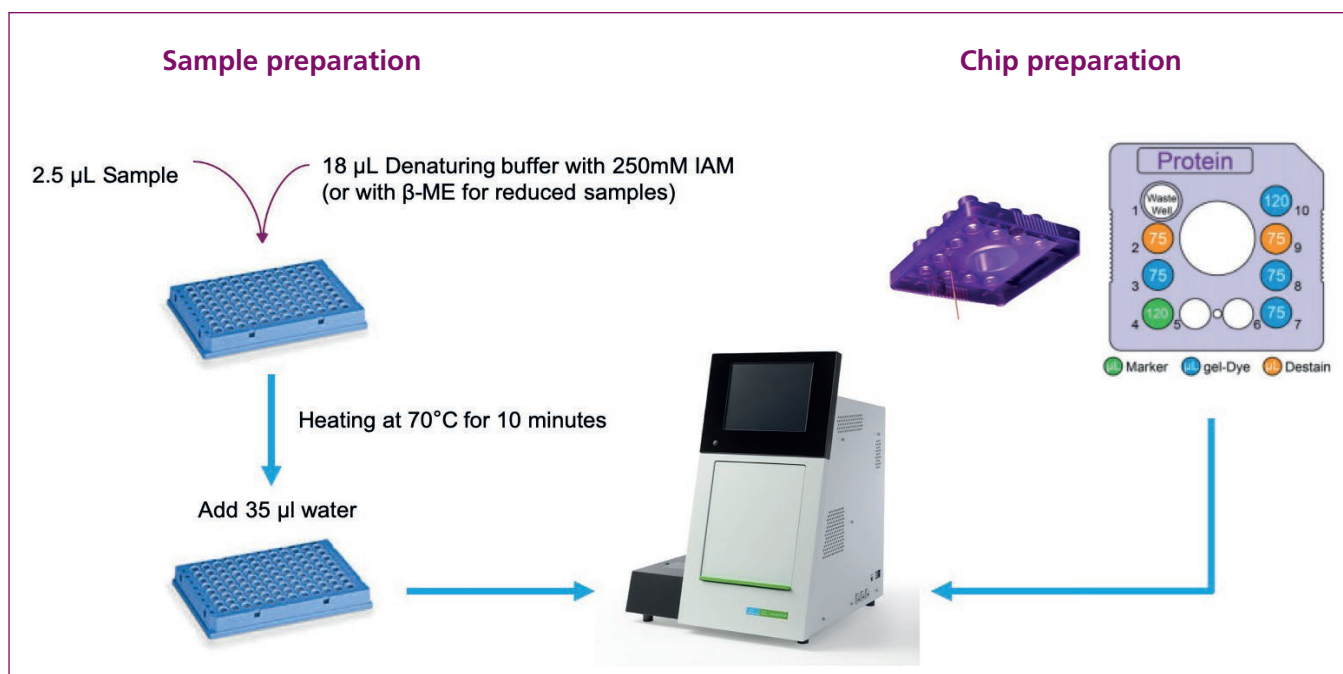


Figure 2: Workflow of the analytical platform constituted of the ProteinExact™ assay and the LabChip® GXII Touch™ instrument. Characterization of a protein sample is accomplished by the preparation of the sample in desired conditions in 96-well plate in parallel to preparation of the LabChip® chip. Both are then inserted into the LabChip® GXII Touch™ instrument for automated analysis.

## 2.4. SDS-PAGE

Conventional 4-12% Bis-Tris SDS-PAGE gel was run under reducing and non-reducing conditions using Invitrogen reagents. The Novex Sharp pre-stained protein standard from Invitrogen was used as a ladder. For non-reduced samples, 1 µL of sample was mixed with 2.5 µL of LDS buffer and 6.5 µL of distilled water. For reduced samples, 1 µL of sample was mixed with 2.5 µL of LDS buffer, 1 µL of NuPage Sample reducing agent and 5.5 µL of water. Before gel loading, reduced samples were heated to 70°C for 10 minutes.

## 2.5. Statistics

Purity of a form has been determined using the corresponding peak areas given by the µCE-SDS analysis. Concentrations given by the LabChip® GX Reviewer software have been used to assess the detection and quantitation limits of each form. Using a curve comparing the theoretical and given concentrations, the intercept of the curve has been assigned to the “background” titer ( $y_0$ ). The standard deviation of  $y_0$  ( $\sigma$ ) and the slope of the curve have been used to determine the detection and quantitation limits (ref ICH)

The limit of detection (LOD) is described as the concentration from which the signal is significant enough to claim the presence of the analyte and was calculated as follow:

$$\text{LOD} = \frac{3.3 \sigma}{S}$$

The limit of quantitation (LOQ) is described as the lowest concentration giving a precise and accurate measurement and was calculated as follow:

$$\text{LOQ} = \frac{10 \sigma}{S}$$

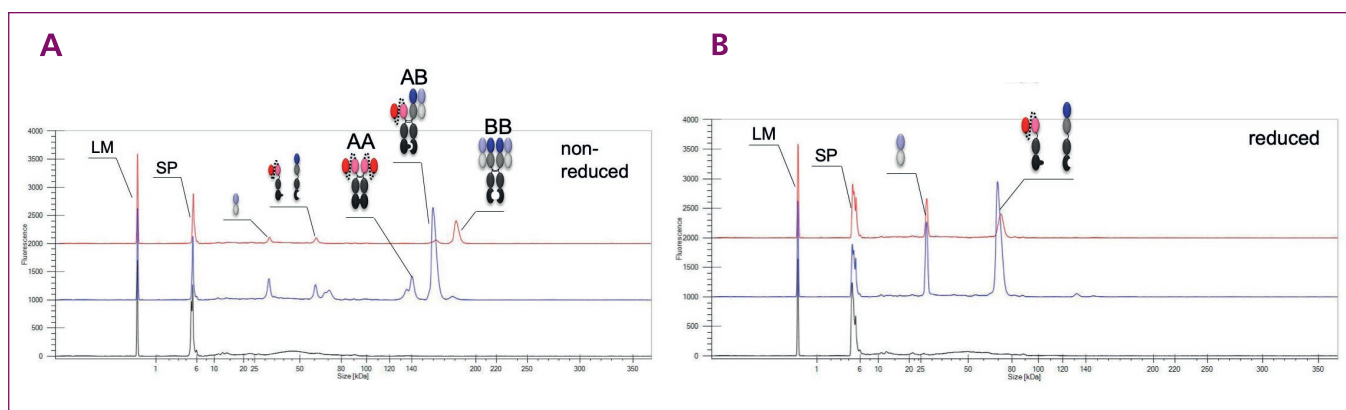


Figure 3: Electropherograms of bsAb clones using ProteinEXact™ assay on the LabChip® GXII Touch™ system. Shown are the electropherograms of unpurified supernatants from two bsAb clones (bsAb1, red; bsAb2, blue) under non-reduced (A) versus reduced (B) conditions. Specific peaks are identified, and associated form is assigned. Lower marker (LM) is used to normalize and align sample data. (System Peak (SP) is the signal resulting from residual fluorescence and inherent to the chip. Mock supernatant (black) consists in supernatant collected from a culture of CHO-M seed train.

### 3. Results and discussion

#### 3.1. Specific identification of bsAb forms

We first examined the possibility to identify the different forms of two different bsAb supernatant samples on LabChip® electropherograms using a protein sizing ladder containing proteins of different molecular weight. Under denaturing non-reduced conditions, efficient separation based on the size of the three different main forms AA, AB and BB, was achieved with high resolution and specificity as no interference was recorded with the mock supernatant (Figure 3A). Under reduced conditions (Figure 3B), all the derivative bsAb monomeric forms were assigned, further demonstrating the high specificity and easy monitoring of sample purity in crude supernatant by the combination of ProteinEXact™ assay with LabChip® workstation analysis.

When comparing data on LabChip® assays with traditional SDS-PAGE gels, we consistently observed a size shift of 20-30 kDa toward high molecular weight in all the identified forms in this study AA, AB and BB. Virtual SDS gels (Figure 4A) were compared to traditional SDS PAGE (Figure 4B). All the dimeric forms were clearly identified using both techniques with similar sensitivity. An additional band was nonetheless detected on non-reduced SDS PAGE around 70 kDa but it was ascribed to a CHO-M host cell protein (HCP). We also observed similar size shift in other similar protein subjects, when we use LabChip® assays (Selexis unpublished data).

This shift may be due to the complexity of molecular interactions between protein molecules, and electro kinetics details within micro channels. For examples, the stabilized SDS-protein micelle complex may carry less negative charges to cause the slow migration, i.e., higher MW in LabChip® assay observation. Large micellar complex might also experience more hydrodynamic drag force from boundaries when they flow through micro channels. Less SDS molecules can bind onto proteins in the confined microchannels condition, than the bulk SDS-PAGE gel. Both number of SDS molecules, and time of binding process are limited in LabChip® assays. SDS-PAGE gel experiment may inherent some unknown factors to shift molecular weight data lower, which caused this discrepancy. We may choose to use other more precise methods, such as liquid chromatography and mass spectrometers, in order to find out details.

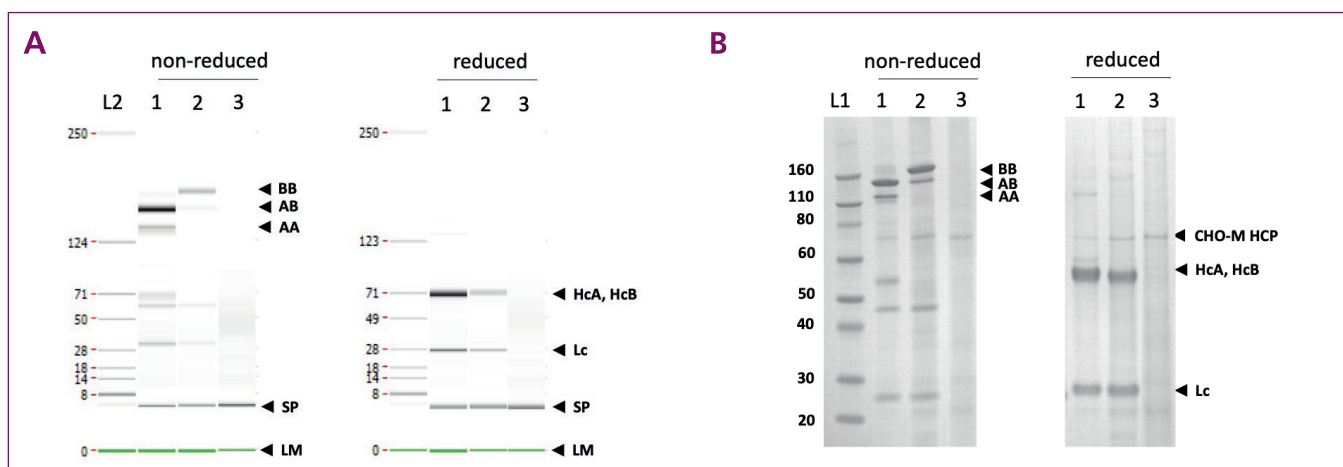


Figure 4: Comparative bsAb separation analysis using different molecular weight sizing methods. (A) Virtual LabChip® GXII Touch™ gel from  $\mu$ CE-SDS. (B) Traditional SDS-PAGE. Both reduced and non-reduced conditions are presented. LM, low molecular weight internal control of the ProteinEXact™ assay; SP, system peaks.

Table 1: Precision of the ProteinEXact™ assay using LabChip® GXII Touch™ system

Samples	Forms	Precision <sup>a</sup>			
		Precision repeatability <sup>b</sup>		Intermediate precision <sup>c</sup>	
		Mean	RDS%	Mean	RSD%
bsAb1	AA1	17.3	2.4	16.6	5.2
	AB1	79.5	0.6	80.3	1.2
	BB1	3.2	3.1	3.1	5.1
bsAb2	AA2	0.0	-	0.0	-
	AB2	11.6	0.4	11.8	5.3
	BB2	88.4	0.1	88.2	0.7

a. precision herein refers to the percentage of corrected peak area of the bsAb form of interest, calculated as the mean of the repeats. RSD values are also calculated on peak area.

b. precision repeatability was evaluated by preparing triplicates of each bsAb supernatant samples, tested in exactly the same conditions (n = 6).

c. intermediate precision was evaluated by testing the bsAb supernatant samples in triplicate by two analysts, each using a different ProteinEXact™ chip and a different LabChip® GXII Touch™ system. In addition, the analysis was repeated on a different day by one analyst on the same equipment for a total of 12 conditions (n = 12).

### 3.2. Reproducible assessment of bsAb purity

Precision of the ProteinEXact™ assay was assessed in terms of repeatability (intra-experiment sample repeat) and intermediate precision (different analyst, different equipment and different day) under non reduced conditions<sup>9</sup>. Two bsAb supernatant samples with different total form concentrations determined by ELISA, bsAb1 at 965 ug/mL and bsAb2 at 272 ug/mL and different distribution of the forms (Figure 1) were used for the purpose of addressing

the concentration effects. Relative standard deviation (RSD) was calculated on the average values. As shown in table 1, precision repeatability was good at 0.6% RSD and 0.4% RSD for the heterodimer AB form and 3.1% RSD or better for all the other homodimeric forms of the two bsAb tested. These values are in accordance with the acceptance criteria  $\leq 1\%$  for drug substance (heterodimer AB) and  $\leq 5\%$  for minor components (homodimers AA and BB). Intermediate precision was also evaluated at 1.2 % and 5.3 %RSD for the heterodimer AB form and 5.2% RSD or better for AA and BB forms. While a bit higher than precision repeatability these values are still very close or under the acceptance criteria of  $\leq 2\%$  statistical RSD. Last, no notable effect of neither the total concentration of the sample or the distribution of the different forms was shown to have an impact on measurements, further demonstrating the good precision performance of the system in bsAb purity assessment.

### 3.3. Sensitive bsAb quantification with low level impurity detection

The performance of the ProteinEXact™ assay using the LabChip® protein characterization system was further investigated through the determination of the sensitivity thresholds of bsAb quantitation in crude supernatant. Accuracy could not be directly assessed for bsAb samples as we did not have another method to discriminate and quantify the different bsAb forms. Nevertheless, in order to be able to run the linearity assay, we used a classical IgG as a standard reference for which the protein concentration was determined by using a Nanodrop™ fluorometer (Thermo Fisher). We then run this standard reference in parallel to our bsAb samples on the ProteinEXact™ assay and determined the assay accuracy. We found a value of 98.8 %, which validates the experimental approach and allows for the determination of the first data point, the concentration of the bsAb forms contained in non-diluted supernatant. In order to cross-validate this first point, LabChip® system values of the area under the curve of the three different forms were cumulated to artificially get the experimentally defined total concentration and compared it to total IgG quantification results by ELISA. As a result, we found an accuracy average value of 92.7 %, further confirming the relevance of our approach (data not shown). Figure 5 illustrates the linearity of the quantification assay using the titers obtained from serial dilutions versus the mean fluorescence of the peak area. Excellent linearity with a coefficient of determination ( $R^2$ ) of 0.9904, 0.9905 and 0.9977 for AA, AB and BB respectively (Figure 5 and Table 2). The limits of detection (LOD) and quantification (LOQ), above 5.8  $\mu\text{g/mL}$  and 17.7  $\mu\text{g/mL}$ , respectively, were similar for all the bsAb forms and therefore were not impacted by the dimeric format nature.

Table 2: BsAb detection and quantitation limits of the ProteinEXact™ assay using LabChip® GXII Touch™ system

Dimeric forms (non reduced)	Concentration of undiluted sample <sup>a</sup> ( $\mu\text{g/ml}$ )	LOD ( $\mu\text{g/ml}$ )	LOQ ( $\mu\text{g/ml}$ )	$R^2$
AA	207.3	5.8	17.7	0.9904
AB	833.4	5.5	16.6	0.9905
BB	212.6	4.4	13.4	0.9977

Note: This experiment is representative of three different bsAb supernatant samples, each tested in triplicate ( $n = 3$ ).

a. concentration of undiluted sample was determined using ProteinEXact™ assay with the LabChip® system.

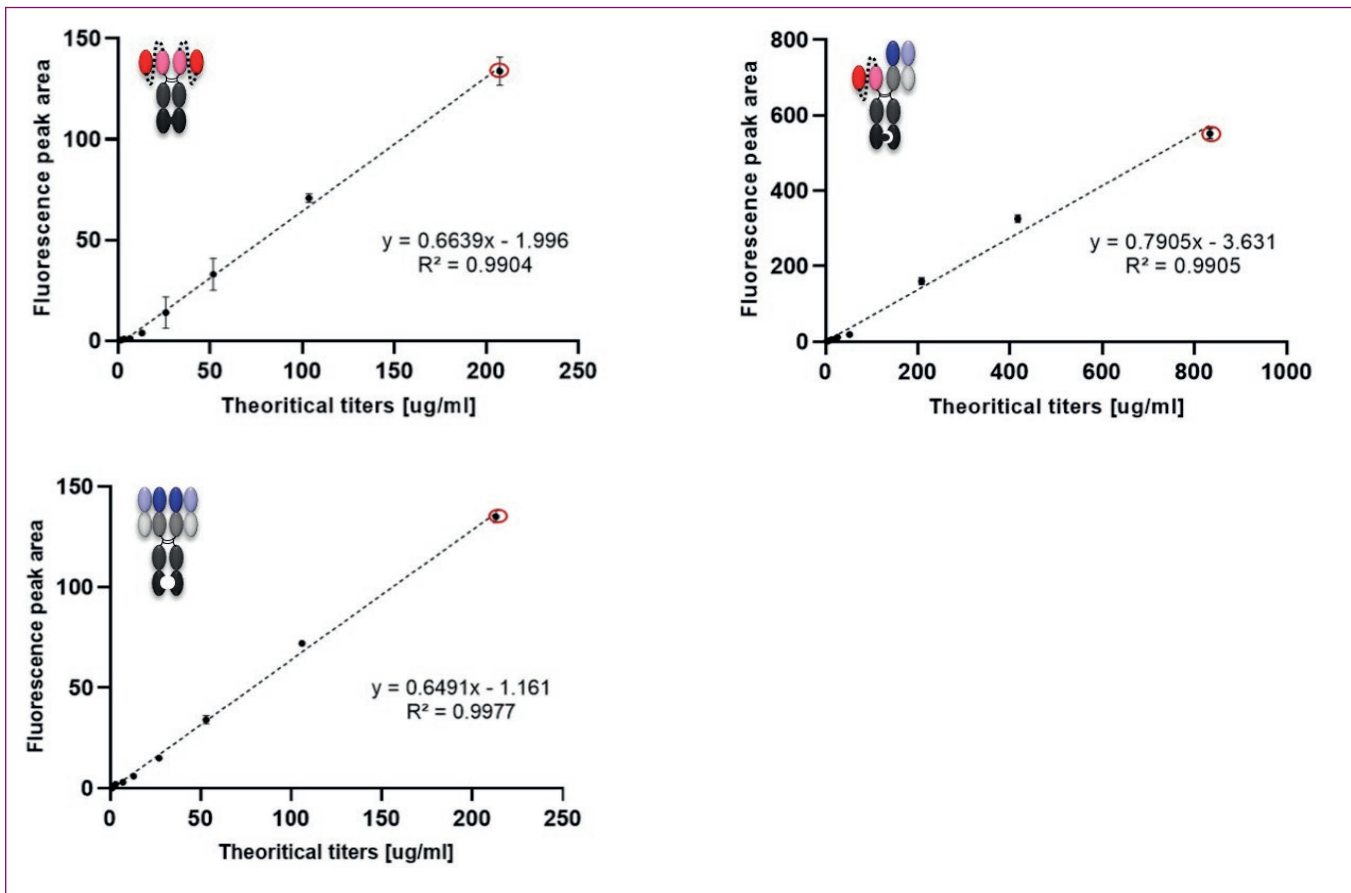


Figure 5: Linearity of the ProteinEXact™ assay for bsAb quantification. In red is shown the concentration of each form of the undiluted bsAb1 sample, experimentally determined by the assay in accordance to an internal IgG standard. Theoretical concentrations were then obtained by considering subsequent 1:2 serial dilutions of the sample.

## 4. Conclusions

In this study, we demonstrated that the ProteinEXact™ assay combined to the LabChip® GXII Touch™ instrument is an efficient system to rigorously analyze asymmetrical bsAbs based on molecular weight sizing. Its high resolution allows for accurate identification and sensitive quantification of bsAbs including low-expressing homodimeric forms, detected as impurities in crude supernatant.

In a word, this quality-controlled combinatorial platform, which provides highly reproducible results, turns to be an indispensable tool for asymmetrical bsAb purity and heterogeneity assessment in crude supernatant, at early stages of cell line development.

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### Abbreviations:

bsAb: bispecific antibody

CE-SDS: capillary electrophoresis Sodium Dodecyl Sulfate

CHO: Chinese Hamster Ovary

Hc: heavy chain

KIH: knob-into-hole

Lc: light chain

LOD: limit of detection

LOQ: limit of quantification

scFv: single chain fragment variable

### PerkinElmer, Inc.

940 Winter Street  
Waltham, MA 02451 USA

P: (800) 762-4000 or  
(+1) 203-925-4602

[www.perkinelmer.com](http://www.perkinelmer.com)



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