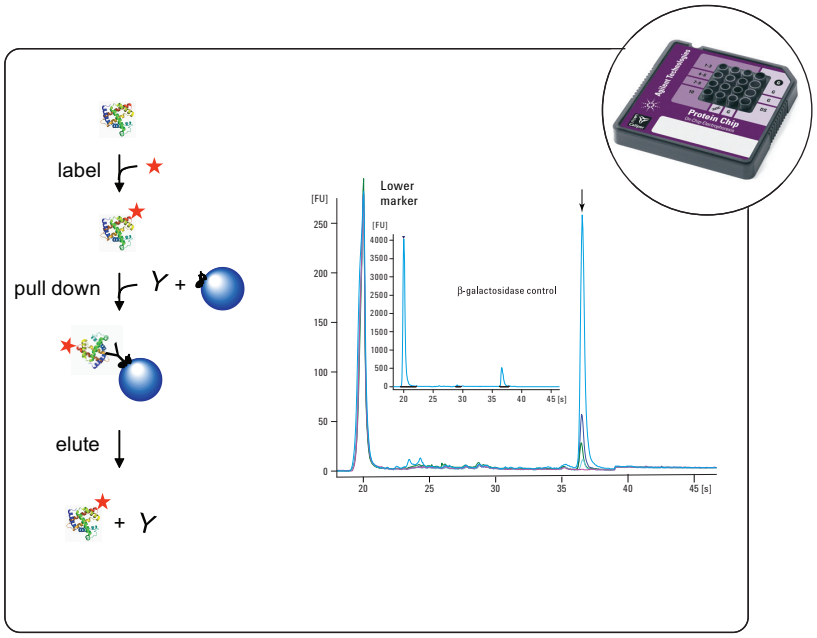
Immunoprecipitation combined with microchip capillary gel electrophoresis for detection and quantification of β-galactosidase from crude *E. coli* cell lysate in bioprocess technology

Application Note

Quantitative Proteomics and Protein Identification



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Abstract

This Application Note describes the development of an immunoprecipitation method in combination with microfluidic on-chip analysis on the Agilent 2100 Bioanalyzer for sensitive, selective and quantitative detection of endogenous β-galactosidase in crude *E. coli* lysates. The effects of antibody selection and the importance of preclearing steps for sensitive and specific detection of the target protein are discussed. Quantification of β-galactosidase was achieved via an external calibration curve based on various spiking levels of pure β-galactosidase into *E. coli* lysate. Results proved the described approach to be quantitative over orders of magnitudes and highly reproducible.

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Introduction

Immunoprecipitation (IP) of proteins from whole cell or tissue extracts is a powerful technique for highly sensitive and specific detection of weakly expressed proteins and physical interactions between proteins. IP can enrich proteins of interest to improve sensitivity when compared to immunoblotting of whole cell extracts. In a typical experiment, cells (for example, derived from a bioreactor) or tissue (for example from a human biopsy) extracts are lysed under nondenaturing conditions. The protein under investigation is immunoprecipitated from the extract using a solid-phase matrix, linked to an antibody directed against the protein of interest. Usually the analysis of the precipitated material is performed by planar gel electrophoresis[1,2].

Determination of β-galactosidase in bacterial strains is important in biotechnology, clinical and environmental analysis as well as, food and molecular microbiology. The determination of β-galactosidase concentrations plays a crucial role for *lac* operon control especially in bioprocess technology and genetic engineering. This is an example of large scale gene control, because the *lacZ* gene structure encodes this enzyme, which is necessary for lactose catabolism[3-5]. Most of the methods for determining the concentration of this protein are based on spectroscopic methods (photometry), using the enzymatic activity towards O-nitrophenyl-β-D-galactopyranoside (ONPG)[6,7].

This Application Note describes a strategy for protein identification and quantification that combines IP with automated SDS-Microchip Capillary Gel Electrophoresis (IP-MCGE). Several antibody dilutions were tested to find the optimum antibody-antigen ratio. Spiking experiments helped to determine the overall recovery rate and quantification of β-galactosidase in cell extracts from different *E. coli* strains.

The workflow for IP-MCGE is similar to that previously described[8]. Protein G coated magnetic beads were used instead of Protein A beads.

Experimental

Materials

Dynabeads Protein G and Dynamag-2 were purchased from Invitrogen (Carlsbad, CA, USA), Protein LoBind tubes from Eppendorf (Hamburg, Germany), anti-β-galactosidase antibody, β-galactosidase and Tween 20 from Sigma (Taufkirchen, Germany), the second anti-β-galactosidase antibody from Acris (Herford, Germany), Coomassie Plus Assay Reagent and the Protease Inhibitor Cocktail Kit from Pierce Biotechnology (Rockford, IL, USA). The Agilent 2100 Bioanalyzer and High Sensitivity Protein 250 (HSP-250) Kit were from Agilent Technologies (Waldbronn, Germany). *E. coli* BL21 (DE3) and *E. coli* JM109 (β-galactosidase deficient strain) were generous gifts from Ch. Herwig (Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria). Bacto peptone was purchased from Difco (Franklin Lakes, NJ, USA). NaCl, lactose and NH_4Cl were obtained from Fluka (Taufkirchen, Germany). Na_2HPO_4 was purchased from Riedl-de-Haen (Hannover, Germany). Yeast extract, KH_2PO_4 and NaH_2PO_4 were from Merck (Darmstadt, Germany), and $MgSO_4$ and $CaCl_2$ from Roth (Karlsruhe, Germany). The applied centrifuge was from Sigma (Osterode am Harz, Germany), incubator from Infors (Bottmingen, Switzerland), spectrophotometer from Hitachi (Tokyo, Japan) and ultrasonificator from Branson (Danbury, CT, USA).

Sample Preparation

Ten milliliters of Luria-Bertani (LB) broth was inoculated from a frozen stock of *E. coli* BL21 (DE3) and incubated overnight at 37 °C. One milliliter of this culture was transferred to 10 mL of minimal media for *E. coli* (5 g glucose or lactose, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.12 g $MgSO_4$, 0.01g $CaCl_2$ in 1L distilled water) that was prepared with lactose as a sole carbon source instead of glucose. The mixture was incubated overnight at 37 °C. Five milliliters of the culture were transferred to 500 mL of minimal media with lactose and incubated for two days at 37 °C (OD_{600} ≈3). The culture was centrifuged at 3,750 × g for 15 min at 4 °C. Pellets were washed once with distilled water and centrifuged again under the same conditions. The pellets were suspended in 50 mM sodium phosphate buffer pH 8.3 (2.6 mM NaH_2PO_4, 47.4 mM Na_2HPO_4, 300 mM NaCl) and lysed by ultrasonification on ice to prevent heating of the sample. Cell debris was removed by centrifugation at 4 °C (15 min, 3,750 × g). Total protein content of the cell lysate was determined with the Coomassie Plus Assay Reagent to be approximately 3 mg/mL. Protease inhibitor cocktail was added to the lysate according to manufacturer's instructions then the lysate was stored at –20 °C. Cell lysates from the β-galactosidase deficient control strain JM109 were prepared similarly, except that JM109 was grown on LB media only.

Protein labeling

All proteins were labeled as described in the Agilent High Sensitivity Protein 250 Kit guide[9]. Ten microliters of *E. coli* lysate (approximately 30 µg total protein) was mixed with 1 µL of dye and incubated on ice for 30 min. The labeling reaction was stopped by the addition of 1 µL of ethanolamine and continued incubation on ice for 10 min. After labeling, Tween 20 was added to a final concentration of 0.1%. IP was performed in Protein LoBind tubes.

Immunoprecipitation

The IP workflow includes four steps: preclearing, antibody incubation, immunocomplex capturing, and immunocomplex elution.

Sample preclearing to reduce background: 1 µL of fresh Protein G beads (approximately 30 mg/mL) were washed twice with 100 µL of wash buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl, 0.1% Tween 20). The sample was incubated with 10 µL labeled sample for 10 min on ice. Tubes were vortexed every 2-3 min to keep the beads in suspension. After 10 min, the supernatant was collected and beads were discarded.

Antigen and antibody incubation: Several dilutions (1:10, 1:100, 1:200, 1:500) of two different anti-β-galactosidase antibodies were prepared in wash buffer. One microliter of the antibody dilution was added to the precleared sample and incubated for 1 h on ice.

Immunocomplex capturing: After incubation, 1 µL of freshly washed Protein G beads (2 × 100 µL wash buffer) was added to the sample. After 10 min incubation on ice with vortexing every 2–3 min, the supernatant was discarded and the beads were washed three times with 200 µL wash buffer.

Immunocomplex elution: Washed beads were mixed with 20 µL elution buffer (sample buffer from the HSP-250 kit containing 35 mM dithiothreitol mixed with water in a volume ratio of 1:2) and heated to 95 °C for 5 min. The samples were ready for on-chip deposition and analysis after spinning down.

Negative control to monitor unspecific binding: 10 µL of labeled *E. coli* BL21 (DE3) lysate was treated like all other IP samples without the addition of antibody.

On-chip analysis

On-chip sample analysis was performed according to the HSP-250 Kit Guide[9].

Quantification

An external calibration with a β-galactosidase deficient *E. coli* strain JM109 was performed for β-galactosidase quantification in *E. coli* BL21 (DE3). β-galactosidase was spiked into *E. coli* JM109 lysate (approximately 12 mg/mL) at specific levels (625 to 12500 pg/µL corresponding to 0.0006% to 0.0116% of the total protein) and immunoprecipitated with 1:1000 diluted anti-β-galactosidase antibody (Sigma).

Time-corrected peak area of the β-galactosidase signal for each concentration obtained by means of the Agilent 2100 Expert Software was plotted against the corresponding amount of β-galactosidase. The concentration of unknown endogenous β-galactosidase in *E. coli* BL21 (DE3) was calculated based on this calibration curve.

Results and discussion

High levels of β-galactosidase can be detected in whole *E. coli* lysates without sample pretreatment by means of the Agilent High Sensitivity Protein 250 kit and the Agilent 2100 Bioanalyzer (Figure 1). However, the specificity of an antigen and antibody interaction can help reduce background signals, increasing the signal of the protein of interest and the dynamic range for reliable quantification.

E. coli lysate was investigated by combining β-galactosidase IP with the HSP-250 protein kit. Crude cell lysate was labeled with the fluorescent dye provided by the kit and immunoprecipitated with an anti-β-galactosidase antibody and Protein G magnetic beads. Introduction of a sample preclearing step eliminated nonspecific protein binding to the beads and significantly reduced unwanted background signals (Figure 2).

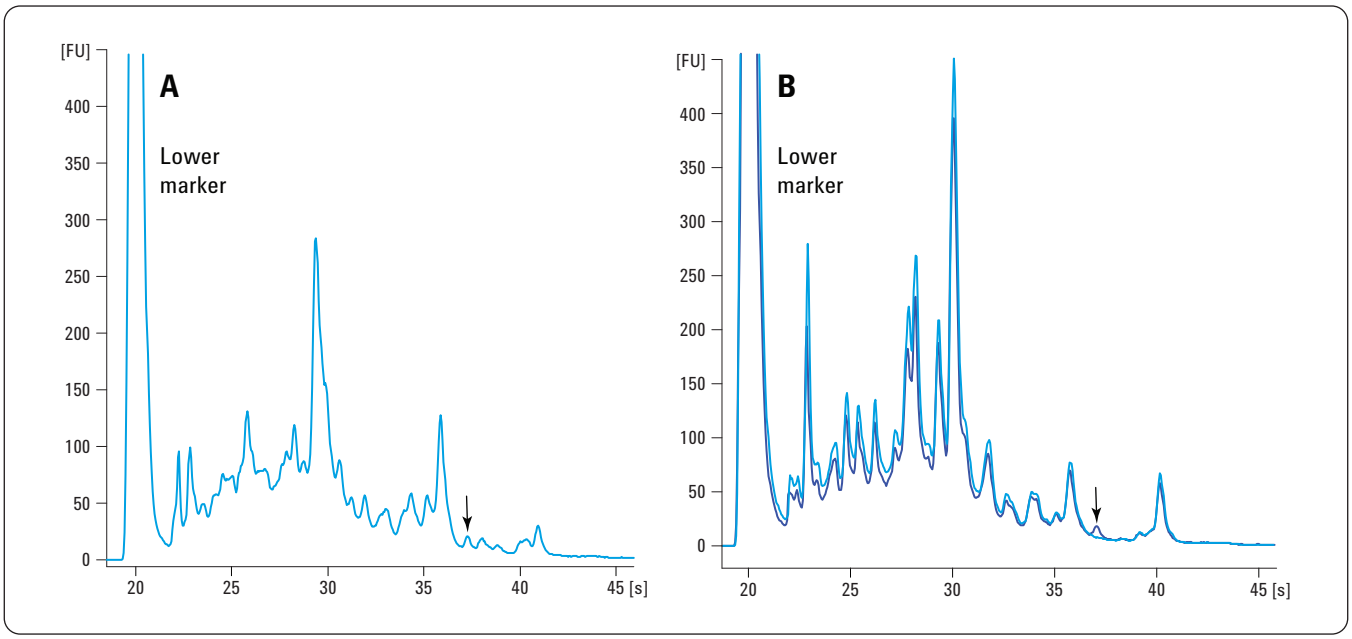

Figure 1
Total *E. coli* lysate before immunoprecipitation. A: β-galactosidase (arrow) containing *E. coli* strain BL21 (D3) B: β-galactosidase deficient *E. coli* strain JM109 (light blue) and the same strain spiked with 0.5 µg/µL (4.6%) β-galactosidase (arrow, blue).

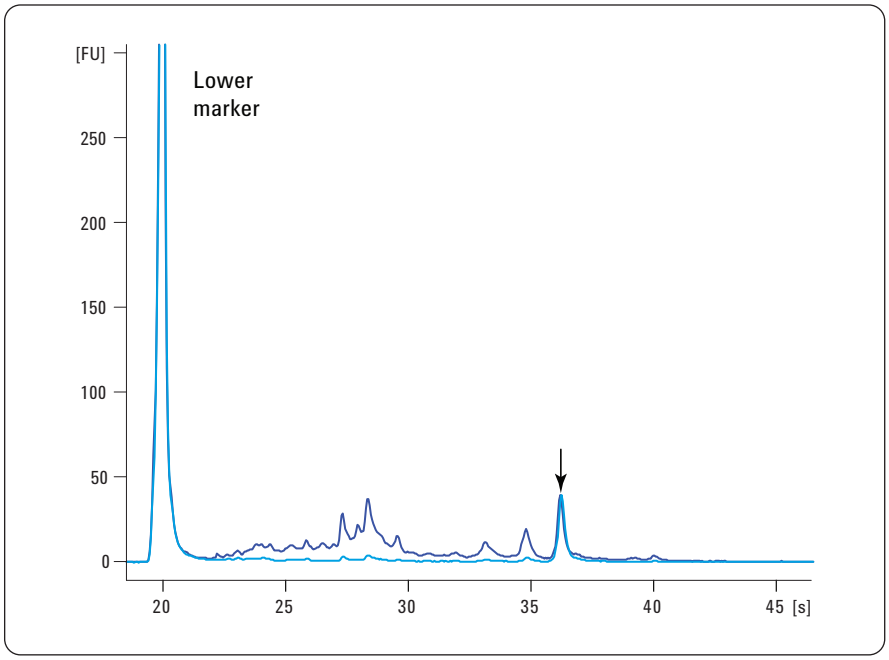

Figure 2
Comparison of IP with (light blue) and without (blue) preclearing of *E. coli* lysate with Protein G coated magnetic beads. IP with 1:1000 diluted Sigma antibody.

Antibody selection plays a very important role in IP. Recovery rate of the target protein depends on the specific affinity of the antibody used. For the given study, two different anti-β-galactosidase antibodies were tested (Sigma G8021, concentration 29 mg/mL, and Acris R1064, concentration 80 mg/mL). Both antibodies were diluted 1:100 and IP was performed with crude *E. coli* BL21 cell lysate. The difference in β-galactosidase recovery after IP is shown in Figure 3. Higher specificity of the antibody allows the application of lower antibody concentrations, enhancing the signal-to-noise and the overall sensitivity of the IP-MCGE method (Figure 4).

A critical point for a reliable IP experiment in terms of reproducibility and low limits of detection is the antigen-to-antibody ratio. The optimal antibody concentration was determined through dilution experiments. IP was performed with different antibody dilutions (Figure 4) and the results were evaluated for protein recovery.

Electropherograms of immunoprecipitated samples show very few additional signals in the molecular mass range between 10 and 95 kDa compared to β-galactosidase control. This suggests a very low degree of nonspecific binding of cell components to anti-β-galactosidase antibody or Protein G beads. This data implies that no protein degradation or aggregation occurred during sample treatment (Figure 4).

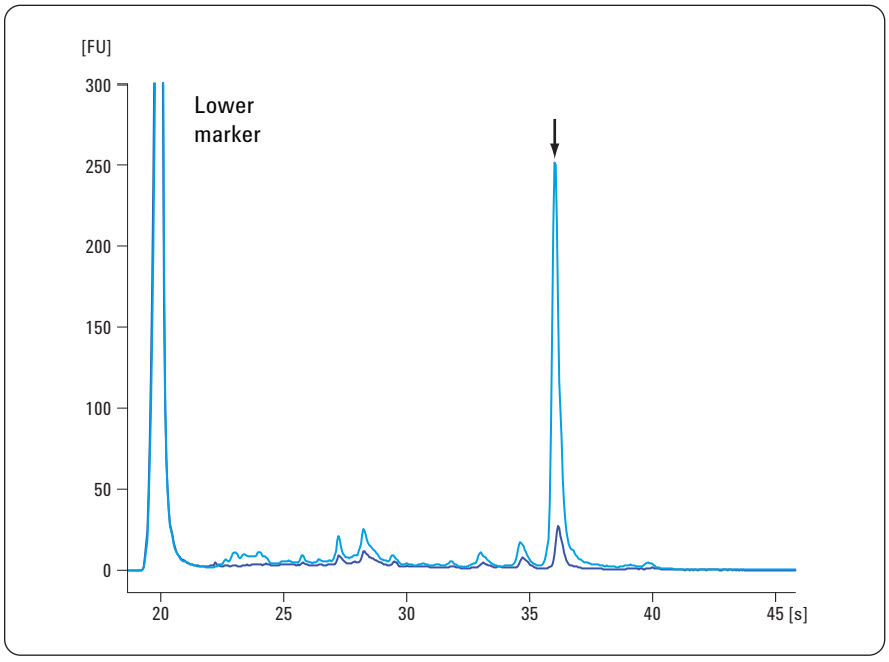

Figure 3
Comparison of two anti-β-galactosidase antibodies. Light blue: Sigma (mouse monoclonal, 29 mg/mL). Blue: Acris (rabbit polyclonal, 80 mg/mL). Both antibodies were diluted 1:100 and IP was performed with *E. coli* BL21 (D3).

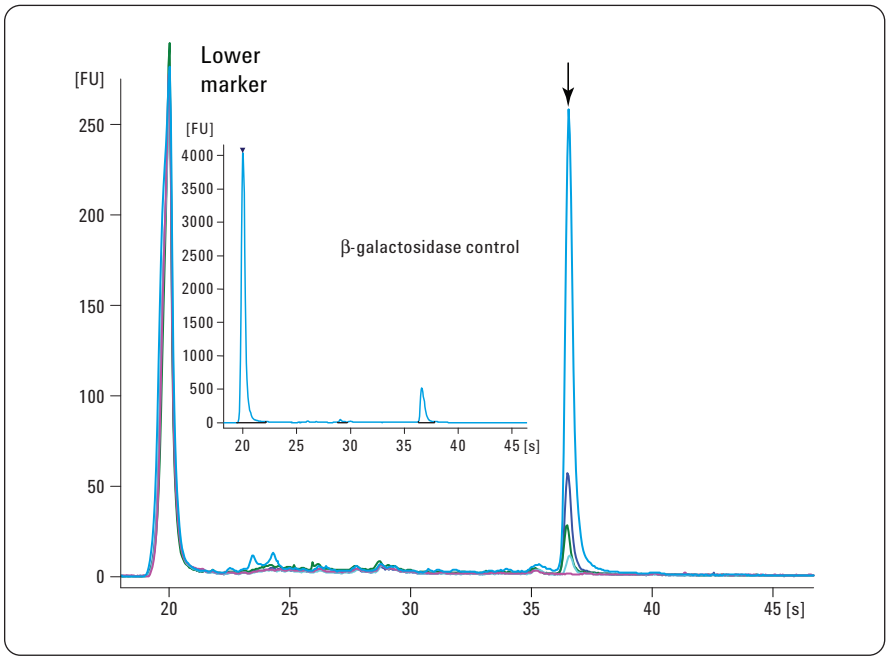

Figure 4
IP-MCGE of β-galactosidase containing *E. coli* BL21 (DE3) lysate with different concentrations of anti-β-galactosidase antibody (Sigma). Antibody dilution factors: 1:100 (light blue), 1:1000 (blue), 1:2000 (green), 1:5000 (turquoise), negative control (IP without AB) (pink). Inset: commercially available β-galactosidase protein (Sigma G3153, approximately 5 µg/µL) as control.

A 1:1000 dilution of the Sigma antibody was found to be appropriate for IP and was further used for a spiking experiment in which defined target protein concentrations were added to the β-galactosidase deficient *E. coli* lysate to generate a calibration curve. A linear calibration function between 625 – 12500 pg/µL (equaling 0.0006% to 0.0116% of the total protein) was obtained (Figure 5, Table 1) with an excellent correlation coefficient (R^2 = 0.9875). Within this concentration range, the overall recovery rate for β-galactosidase was calculated to be 46 ± 5% (average of the recoveries for all concentrations, data not shown). The amount of the endogenous β-galactosidase present in *E. coli* BL21 (DE3) was estimated based on the calibration curve, to 22.5 ng/µL (Table 2).

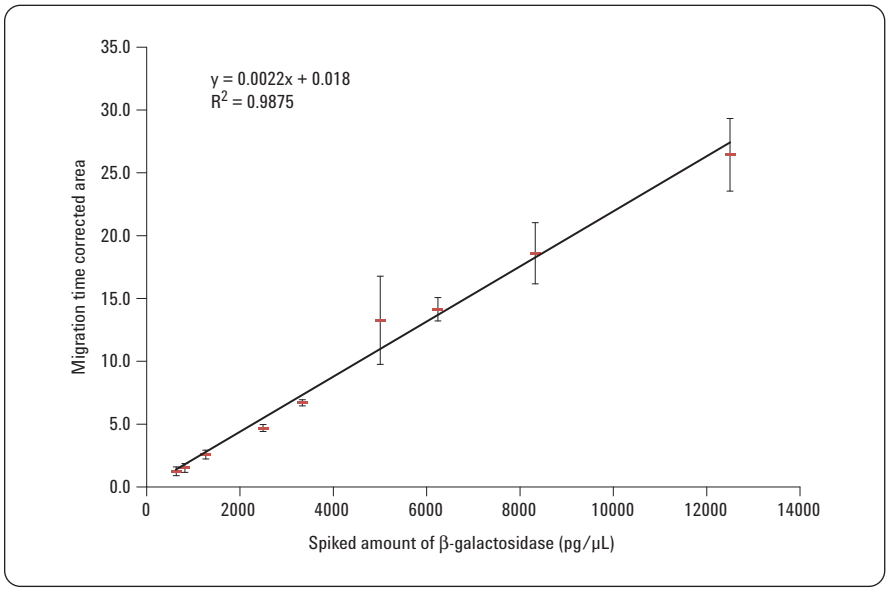

Figure 5
Calibration curve obtained from β-galactosidase spiked into *E. coli* JM109.

Conclusion

The combination of IP with the Agilent High Sensitivity Protein 250 kit on the Bioanalyzer delivers excellent results for the specific detection, identification, and quantification of endogenous proteins, such as β-galactosidase, in crude cell extracts derived from bioprocess optimization. It is sensitive and highly specific (dependent of the selected antibody). In addition, it has a shorter cycle time and consumes less reagents compared to classical Western blotting. Finally, it can quantify across a concentration range of 625 – 12500 pg/µL. The method combines the specificity of IP with the high sensitivity of microfluidic protein detection on the Bioanalyzer.

Spiked amount of β-galactosidase (pg/µL)	Migration time corrected peak area
12500	26.4 ± 2.9
8333	18.6 ± 2.4
6250	14.2 ± 0.9
5000	13.3 ± 3.5
3333	6.7 ± 0.2
2500	4.7 ± 0.3
1250	2.6 ± 0.3
833	1.5 ± 0.4
625	1.2 ± 0.4

Table 1
Calibration curve data.

Dilution factor of applied antibody	Migration time corrected peak area	Calculated amount of β-galactosidase (ng/µL)	Mean value (ng/µL) (n = 3)
1000	54.4	24.8	
2000	23.5	21.4	22.5 ± 1.9
5000	9.4	21.4	

Table 2
Quantification of endogenous β-galactosidase via calibration curve.

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www.agilent.com/genomics/bioanalyzer

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