Chapter 20

Quantification of Recombinant Products in Yeast

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Abstract

Quantification of various proteins expressed in yeast can be performed by different methods. In this respect, classical as well as advanced techniques can be applied, where the analysis of crude supernatants is of special interest in screening but also manufacturing.

The following chapter addresses the analytical background of the introduced methods followed by specific recommendations for the quantification of different products of industrial interest. The method portfolio includes electrophoresis, chromatography, and ELISA as classical techniques, but also biosensor-based, microfluidic and automated, miniaturized methods are introduced. Furthermore, individual strengths and perceived limitations are summarized.

Although prominent examples are described, it should be noted that individual modifications are required according to host and cultivation mode.

Key words Protein quantification, Electrophoresis, HPLC, ELISA, Bio-layer interferometry, Microfluidics CD technology, Surface plasmon resonance, Electrochemiluminescence assays, Microchip-based assays

1 Introduction

Biotechnology has traditionally employed pro- and eukaryotic organisms as production systems. In microbial production, predominantly yeasts and bacteria share beneficial features although some limitations for the production of human proteins are evident. The increasing attractiveness of yeasts for both basic research and industrial manufacture requires efficient production strategies and full characterization of the various products based on established, but even more in future, innovative new analytical perspectives [1–4].

Yeast platforms for recombinant protein production have several explicit advantages:

1. Rapid growth rate, coupled with ease of high cell density fermentation.
2. High levels of productivity in protein-free medium.

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3. Absence of endotoxin and bacteriophage contamination.
4. Ease of genetic manipulation of well-characterized yeast expression vectors.
5. Absence of known human pathogenicity in the spectrum of lytic viruses.
6. Diverse posttranslational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments.
7. The ability to produce secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves [5].

In screening, cultivation and manufacturing various analytical methods for qualitative as well as quantitative analysis are needed. In respect to different scenarios such as (1) small and variable amounts of secreted protein of interest, (2) small volumes in early stages, (3) variable media composition, and (4) variable matrices caused by impurities, sufficient analytical methods need to be implemented for clone screening, media optimization, and process control purposes. Thereby, optimized strain selection and process-related product quantification but also verification of product quality are enabled. To fulfill these high standards, analytical methodologies need:

1. To be carefully assessed and developed according to the intended purpose.
2. To be reliable under different conditions.
3. To be selective and/or sensitive.
4. To be optimized for a broad dynamic working range.
5. To be reproducible and robust.
6. To be of high throughput and automatization.
7. To have data formats transferable into databases.
8. To have an efficient time and effort ratio to obtain information.

In the following chapter, classical, but also advanced, analytical methods, the theoretical background of each technique, applications, and selected examples is introduced. As it can be seen, even for one particular protein, different methods are possible, and the setup often depends on the equipment and established technologies already available in a certain laboratory or institution. Therefore, no overall recommendations can be given.

2 Classical Quantification Methods

2.1 Electrophoresis

2.1.1 Background

Protein gel electrophoresis is a well-established technique based on the movement of charged molecules in response to an electric field. The migration rate (units/cm^2/Vs) defines the rate of movement of the individual molecules. Meanwhile, numerous techniques are applicable for crude culture supernatants of different origins. The separation is based on the complex relationship between the physical characteristics of both, the electrophoresis system and the protein. The individual performance depends on the strength of the electric field, the temperature of the system, the pH, ion type, and concentration of the buffer as well as size, shape, and charge of the proteins [6, 7]. A typical workflow includes method selection, sample preparation, gel and buffer preparation, separation, and protein detection combined with data evaluation.

In research, but also manufacture, polyacrylamide gel electrophoresis (PAGE) is commonly applied and optimized for the intended application. Polyacrylamide gels are stable, chemically inert, electrically neutral, hydrophilic, and transparent for optical detection at wavelengths above 250 nm [6]. No particular gel type and/or buffer is suitable for all proteins. In principle, native and denatured PAGE can be distinguished, of which the latter one is favored [8]. In this case denaturing buffer systems [9] come into place. The anionic detergent sodium dodecyl sulfate (SDS) is non-covalently bound to proteins in a stoichiometric ratio of 1.4 g SDS per 1 g protein, leading to an overall negative charge of the protein. The caused rod shape of the protein enforces the migration primarily by the size enabling the molecular weight estimation. In case of the analysis of subunits, which are bridged by disulfide bonds, reductive conditions can be applied. Thiol-reducing agents which disrupt intra- and intermolecular disulfide bonds, such as 2-mercaptoethanol (ß-ME) or dithiothreitol (DTT), are predominately used. Disruption of hydrophobic interactions between and within proteins can be performed either with nonionic (NP-40, Triton X-100) or zwitterionic (CHAPS, sulfobetaines) detergents [10, 11]. In addition, chaotropic agents that disrupt hydrogen bonds as well as hydrophobic interactions, both between and within proteins, such as urea or thiourea are in use (*see* **Note 1**). The optimization of qualitative and quantitative protein electrophoresis that offers the highest resolution in the size range of interest may require some experimentation prior to use in routine analysis. Therefore, selected protein standards in the intended size range are suggested as well as protein content analysis and dilution series of unknown samples. In completion, Western blot analysis is recommended to test the protein integrity in a qualitative manner.

Depending on the aim and the available imaging equipment, different staining techniques are offered [12, 13]. Samples can be

Table 1
List of the most relevant protein staining dyes used in electrophoresis

Staining dye	Attributes/characteristics
Coomassie (Brilliant or R-250) blue stain	Most popular, anionic dye; for almost all proteins, good quantitative linearity, medium sensitive, favorable for mass spectroscopy for additional identification, adequate price
Fluorescent stain	High sensitivity, wide linear range (four orders of magnitude), simple and robust, but more expensive
Silver stain	Highest sensitivity, but low dynamic range, time-consuming, less reproducible, not sufficient reproducibility for quantification
Negative stain	Very rapid; proteins appear as clear areas; zinc and copper stains do not require fixation; less applicable for quantification
Specific protein stains	Specific for different protein classes, such as phosphoproteins
	Qualitative more than quantitative, preferable for identification

stained prior and/or after the separation, for qualitative and/or quantitative analysis (*see* **Note 2**). This enables more or less sensitive, specific, and unspecific labeling (Table 1). Data analysis is performed with different imaging devices producing electropherograms and may employ sophisticated software for data processing. State-of-the-art imaging systems are, among others, flatbed densitometers, charge-coupled device (CCD) cameras, or laser-based scanner systems.

Electrophoresis is commonly applied in basic research, but due to further developments and automatization combined with advanced methods, it is still of great importance in the manufacture of recombinant proteins. Due to the advantages but also the known limitations (Table 2) regarding high throughput, standardization, analysis, and data management, improved technical innovations have been established to fulfill the needs especially in R&D and manufacturing. Meanwhile, automated devices of several suppliers are commercially available. Most of them use microfluidic devices to overcome the electrophoretic limitation [14]. All of them are intended to analyze small volumes of samples, and analysis of several samples in parallel significantly reduces processing time. Furthermore, they apply more sensitive staining dyes for quantification and provide software solutions for analysis and data management. Detection is preferentially performed with fluorescence dyes enabling sensitive quantification of proteins with an appropriate dynamic working range. This approach implies efficient separation of the target protein and ideally requires the availability of a defined standard for the quantitative calibration.

Table 2
Summary of performance considerations in electrophoresis

Advantages	Perceived limitations
Crude supernatant	Non-specific
Various gel formats available	Nonselective
Different staining opportunities	Protein disintegration possible
Small sample volume	Interference with other proteins—purity
Qualitative/quantitative	Interference with salts, detergents, organic solvents, sulfones, etc.
	High viscosity (DNA, carbohydrates)
	Limited sample throughput Time-consuming
	Standardization Operator-dependent analysis Data management and documentation

2.1.2 Example

In this example a standardized workflow for SDS-PAGE and Western blot analysis, performed with the Amersham WB analyzer, is described [15]. The applied analyzer consists of two instrument units, one for electrophoresis and scanning to separate SDS-coupled proteins in a polyacrylamide gel and one for the full Western blot workflow including transfer, probing, and drying. The system is based on fluorescence detection and can be used to scan gels after electrophoresis as well as polyvinylidene (PVDF) cards after probing and drying in a Western blot experiment. For pre-labeling the fluorescent dye, Cy5 is used, which binds via its NHS ester to the lysine residues and the N-terminus of the proteins. There is no need of post-staining, and the obtained signal represents the total protein content. Depending on the protein, a limit of detection in the pg range is attainable, and calibration can be usually done in the ng range. Additionally, the target protein can be identified via a secondary antibody labeled with Cy3. Standardized protocols help to deliver consistent and quantifiable data. The use of Western blot analysis to detect a specific protein is highly recommended and confers an advantage over SDS-PAGE alone because the identity of the target protein can be determined with a higher degree of confidence. In this context it is advisable to run samples under reduced (DTT in the loading buffer) and non-reduced conditions. For example, by adding DTT, heavy and light chain of an antibody can be identified, after cleaving of the intramolecular disulfide bonds. However, prior to starting the analysis, it is recommended to perform a Bradford protein assay to estimate the total protein concentration in the supernatant. Several suppliers offer this simple colorimetric assay as a ready-to-use test kit, which has the advantage of getting fast results, which enables the selection of an adequate sample pre-dilution within the calibration range [15–17].

Product: Monoclonal antibody (mAb) [16, 17].

Host: Not specified.

Aim: Standardized quantification of antibody.

Method: SDS-Page, Western blot.

Sample matrix: Culture supernatant.

Equipment: Amersham Western blotting (WB) system.

Sample preparation:

1. Dilute each sample from culture media accordingly with 0.15 M NaCl.
2. Pre-labeling with Cy5 is performed according to an Amersham standard protocol: mix 17 μL labeling buffer (Tris-based buffer containing SDS, pH 8.7), 2 μL sample, and 1 μL Amersham WB Cy5 dye, and let the labeling reaction take place for 30 min at room temperature.
3. For reduced samples, add 20 μL Amersham WB loading buffer (Tris-Cl, SDS loading buffer) with 40 mM DTT to the sample, and heat 3–5 min at 95 °C.
4. For non-reduced samples, use Amersham WB loading buffer directly without DTT.
5. Dilute mAb standard (7.8–500 ng), and pre-label as described above.

Method Description:

Electrophoresis:

1. Load an Amersham WB molecular weight marker (10–225 kDa, labeled with both Cy3 and Cy5), samples, and a dilution series of the mAb standard (20 μL each) onto an Amersham WB gel card 8–18% (gel card for resolution of proteins ranging between 3.5 and 225 kDa).
2. Default electrophoresis settings of 600 V, and a maximum current of 50 mA is recommended.
3. Scan the gel after completed electrophoresis at a sensitivity setting of 5, and evaluate the data with Amersham WB software.

Western Blot Analysis:

1. Transfer samples (after electrophoresis) to a PVDF membrane using the Amersham WB tank transfer protocol: perform the transfer in a buffer with 192 mM glycine, 25 mM Tris, and 20% ethanol with default settings of 100 V for 30 min.
2. Use the Amersham WB default protocols to perform the blocking and probing procedures: first block the membranes in 3% BSA for 10 min.

3. Target the antibody by rabbit antihuman κ antibody (diluted 1:20,000 in PBS-T).
4. Use Amersham WB Cy3-labeled anti-rabbit IgG as secondary antibody for detection (diluted 1:2500 in PBS-T).
5. The probing volumes are 10 μL, and probing times are 60 and 30 min for primary and secondary antibodies, respectively.
6. Dry membranes after probing, for 10 min in an Amersham WB dryer.
7. Scan membranes in an Amersham WB scanner in Cy3 (Ex 550 nm/Em 570 nm) and Cy5 (Ex 650 nm/Em 670 nm) channels at a sensitivity setting of 2, and evaluate data with the Amersham WB software.

2.2 ELISA: Enzyme-Linked Immunosorbent Assay

2.2.1 Background

In 1971, the enzyme-linked immunosorbent assay (ELISA) was first described by Engvall and Perlmann [18, 19]. This technique is applicable to detect a specific target protein or antigen in crude culture supernatants or even cell extracts and has meanwhile replaced the formerly used radioimmunoassay, which has potential health risks. The development of antigen-specific monoclonal antibodies [20] accelerated the acceptance and applicability of this technology. Furthermore, the possibility to chemically link enzymes to antibodies, which enables a measurable signal in solutions containing appropriate substrates, strengthens the ELISA platform. With the development of fluorescence technology, signal generation using fluorophore-labeled antibodies has also become prevalent, especially in multiplex arrays.

2.2.2 Procedure

Although many variants of ELISA have been developed and are used in different configurations, they all include the following elements:

1. Coating/capture: direct or indirect immobilization of antigens to the surface of polystyrene microplate wells.
2. Plate blocking by addition of irrelevant protein (such as bovine serum albumin or casein) or other molecules (the most common nonionic detergent blocker is Tween 20; normal concentrations are about 0.01–0.1%) to cover all unsaturated surface-binding sites of the microplate wells.
3. Probing/detection by incubation with antigen-specific antibodies that selectively bind to the antigens.
4. Detection of the signal that is generated by a direct or secondary tag on the specific antibody.

Washing steps ensure that only specific (high-affinity) binding events are maintained to cause a signal at the final step (*see* **Note 3**) [21].

Coating of an antigen which represents the target protein in product quantification can be performed either traditionally (direct coating) or by capture (indirect coating) in recommended buffer (*see* **Note 4**). If the antigen is present at very low concentrations or does not adhere well to the plastic, alternative sandwich or capture ELISA is advantageous (*see* **Note 5**). Sandwich ELISAs are very popular for complex protein samples (*see* **Note 6**). For this approach, two different antibodies are required; the first antibody (bound to the plate) is called the capture antibody or coating antibody, whereas the second antibody detects the immobilized antigen (detection antibody). Combinations of monoclonal (mainly as the coating antibody) and polyclonal antibodies (detection antibody) can be used (*see* **Note 7**). Blocking buffers usually consist of formulations to prevent non-specific binding of proteins to the plate [22]. An optimal blocking buffer maximizes the signal-to-noise ratio but does not react with the antibodies or target protein (*see* **Note 8**). Some assays may benefit from the addition of a surfactant such as Tween 20 to the blocking solution. Surfactants might minimize hydrophobic interactions between the blocking protein(s) and the antigen or the antibodies. Typically, a final concentration of 0.05% (v/v) Tween 20 is used (*see* **Note 9**) [23, 24]. Both direct and indirect detection can be performed, with indirect detection often being preferred because in most of the assays, this technique is more sensitive. Nevertheless, direct detection is generally faster than indirect detection, and potential background signal from secondary antibody cross-reactivity with the coating antibody is also reduced (*see* **Note 10**).

The increasing number of stable fluorophores in the visible and infrared ranges has made fluorescent signal detection very popular. For this approach, either the detection antibody or the secondary antibody is labeled. When a multiplexing technique is applied, detection antibodies from different species are used in order to distinguish the separate signals [25]. The detection limit is typically approx. 100 pg/well.

2.2.3 Detection

For enzymatic detection two enzymes are preferably used. Alkaline phosphatase (AP) is a large enzyme used in a minority of assays. Its size (140 kDa) limits the amount of signal that can be generated. It is rather difficult to conjugate more than one or two molecules of the enzyme to each molecule of an antibody. AP is less sensitive and is also prone to stability issues unless stored and handled correctly. Alternatively, horseradish peroxidase (HRP) is a more commonly used enzyme. The small size of HRP (40 kDa) allows more molecules to be coupled to antibodies, and this can boost signal generation. Enzymatic signal generation requires the catalysis of a substrate to produce a colored or fluorescent compound or chemiluminescence (visible light) [26]. Colorimetric substrates form a

soluble, colored product that accumulates over time relative to the amount of enzyme present in each well (*see* **Note 11**). When the desired color intensity is reached, the product absorbance is either measured directly or in some cases a stop solution is added. Colorimetric substrates for horseradish peroxidase are TMB (3,3′,5,5′-tetramethylbenzidine), OPD (o-phenylenediamine dihydrochloride), and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), while for alkaline phosphatase pNPP (para-nitrophenylphosphate) is commonly used.

For small concentrations or sometimes for higher diluted samples, signal amplification is required to improve the signal-to-noise ratio. A commonly used technique is based on the biotin-streptavidin binding [27]. Biotinylation (biotin labeling) typically results in multiple biotin tags per detection antibody molecule, thus allowing more than one streptavidin molecule to bind to each moiety. Additionally, enzyme-conjugated streptavidin, appropriately diluted in wash buffer, is added. Finally, appropriate substrate solution is added to each well. After incubation at room temperature, color change is attained. The combined effect of this multiple labeling results in higher number of enzymes in the final, very stable immune complex. This increases the catalysis of the substrate and gives stronger signals. The used microplates are typically made of polystyrene, but also other materials, such as polypropylene, polycarbonate, and nylon, are available (*see* **Note 12**). Many plates are now gamma-irradiated to generate a positive charge, which facilitates enhanced coating.

Meanwhile, several pre-activated plates exist for specific, oriented binding of the coating antibody [28]. These plates are pre-coated with protein A or protein G. However, they are not recommended for sandwich ELISAs because of potential cross-reactivity with detection antibodies and/or secondary antibodies. For biotinylated samples or biotinylated coating antibodies, plates that are pre-coated with streptavidin are ideal. Some assays require direct immobilization of a histidine-tagged protein, in which nickel- or copper-coated plates are suitable (*see* **Note 13**). For group-specific attachment of molecules via amines or thiols to form a covalent bond, maleic anhydride- or maleimide-activated plates are provided, which enables direct attachment of peptide antigens that do not coat well by passive adsorption due to their small size.

2.2.4 Data Evaluation

If a quantitative result is needed, the simplest way to proceed is to average the triplicate, in minor applications the duplicate, of the standards readings and deduct the reading of the blank control sample. Next, plot the standard curve to find the line of best fit. A variation is to plot the data using semilog, log/log, log/logit, and its derivatives—the 4 or 5 parameter logistic models. Any dilutions made need to be adjusted for at this stage. Typically, the working

Table 3
Factors that impact ELISA signal generation

Factor	Variable characteristic
Assay plate	Material, well shape, pre-activation
Coupling buffer	Composition, pH
Capture antibody	Specificity, concentration, affinity, incubation time, and temperature
Blocking buffer	Composition, concentration, cross-reactivity
Target antigen	Conformation, stability, available epitope(s), matrix effects
Detection antibody	Specificity, concentration, affinity, incubation time and temperature, cross-reactivity
Enzyme conjugate	Type of enzyme, type of conjugate, activity, concentration, cross-reactivity
Wash step	Buffer composition, volume, duration, frequency
Substrate	Sensitivity, lot-lot consistency, age
Signal detection	Filters, imaging instrument, exposure time

range should be 0.1–1000 ng/mL. ELISAs belong to the most sensitive immunoassays available. The typical detection range is 0.1–1 fmole or 0.01–0.1 ng. However, the test sensitivity depends upon the particular characteristics of the antibody-antigen interaction.

By these examples the high variability and great number of applications become evident. Factors which significantly affect the assembly of the immune complexes are listed in Table 3, while the advantages and known limitations of the ELISA technique are summarized in Table 4.

2.2.5 *Examples*

In the first described sandwich ELISA, detection antibody is conjugated with horseradish peroxidase (HRP). The added OPD is a chromogenic substrate, which reacts with horseradish peroxidase conjugates, resulting in an orange soluble end product, and can be read spectrophotometrically. Development of the color is dependent on the amount of present HRP. Further dilution of the sample may be required, if color develops too quickly. This reaction is terminated by addition of sulfuric acid altering the pH of the system. Upon stopping, OPD changes in color from yellow to orange, and OD readings of OPD substrates increase by more than twice compared to the corresponding nonstop values. Alternatively, peroxidase substrates such as ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) or TMB (3,3′,5,5′-tetramethylbenzidine) are more frequently used. TMB is meanwhile recommended because it is not carcinogenic and no hazardous stopping solution is required. Furthermore, it is

Table 4
Summary of applicability and limitations of ELISA techniques

Advantages	Perceived limitations
Crude supernatant	Capture failure and unpredictable signal intensities may occur because large immune complexes are formed
Various plate formats available Various antibodies available	Selected antibodies may recognize many or all different isoforms but also only some variants
Different detection opportunities	High background signal and low or absent signal require additional optimization work Matrix effects/interferences
Small sample volume	Standard curves need to be appropriate for different samples Poor standard curve linearity Poor dynamic range
Qualitative/quantitative Automatization Sensitive applications	Nonautomated assays are time-consuming Reproducibility needs to be verified Cost-effective when assaying many samples
Fairly robust	

two times more sensitive than OPD. It produces a blue color measurable at a wavelength of 650 nm. For low-concentration assays, the reaction can be stopped with sulfuric acid [29, 30].

Product: 3D6 single-chain Fv-Fc anti-HIV-1 antibody [29]

Host: Pichia pastoris.

Aim: Quantification of secreted antibody.

Method: Sandwich ELISA.

Sample matrix: Culture supernatant.

Equipment: 96-well immunosorbent plates, microplate reader (492 nm/620 nm).

Method description:

1. Coat 96-well immunosorbent plates with 0.33 μg/mL goat antihuman IgG (γ-chain specific) antibody, diluted in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 9.6) at 4 °C overnight.
2. After incubation step, wash the plates three times using washing buffer (phosphate-buffered saline, containing 0.1% Tween 20, pH 7.4).
3. Affinity purified 3D6scFv-Fc can be used as a standard at a starting concentration of 100 ng/mL.
4. Dilute standards and samples serially in washing buffer containing 1% bovine serum albumin, and apply onto the pre-coated plates.
5. Allow incubation for 1 h.

6. Incubate captured 3D6scFv-Fc with 0.5 μg/mL horseradish peroxidase-conjugated goat antihuman IgG (γ-chain specific) antibody for 1 h.
7. Use 100 mg/mL o-phenylenediamine diluted in 0.15 M citric acid buffer, pH 5.0 containing 0.02% H_2O_2 for staining.
8. Stop the reaction with 25% H_2SO_4.
9. Measure absorption at 492 nm (620 nm reference wavelength) using a microplate reader.

Product: Human albumin (HSA) [29]

Host: Pichia pastoris.

Aim: Quantification of HSA.

Method: Sandwich ELISA.

Sample matrix: Culture supernatant.

Equipment: Human Albumin ELISA Quantitation Set containing affinity purified human albumin coating antibody (1 mg mL^{-1}), human reference serum (22 mg mL^{-1}), HRP-conjugated human albumin detection antibody (1 mg mL^{-1}), 96-well plates, TMB One Component HRP Microwell Substrate, microplate reader.

Method description:

1. Dilute coating antibody 1:1000 in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) to a concentration of 1 μg/mL immediately prior to its addition to the wells (per plate: 10 μL antibody per 10 mL buffer).
2. Transfer 100 μL to each well, and incubate plate overnight at 4 °C or 1 h at room temperature on a rotary shaker (*see* **Note 14**).
3. Wash plate with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) using a plate washer.
4. Add 200 μL blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) to each well.
5. Incubate for 30 min at room temperature on a rotary shaker.
6. Wash each well five times with wash buffer.
7. Thaw 400 μL "ready-to-use" HSA standard aliquot (400 ng/mL) (*see* **Note 15**).
8. Dilute samples in dilution buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) within the concentration range of the standards (*see* **Note 16**).
9. Pipet 100 μL dilution buffer from row B to G of the 96-well plate.
10. Transfer 200 μL blank (dilution buffer) standard or sample to assigned wells in row A (line 1 for blank, lines 2 and 3 for standards, lines 4–12 for samples).
11. For the dilution series, take 100 μL/well from row A, and dilute it 1:2 until the last row is reached.
12. Incubate for 1 h at room temperature on a rotary shaker.

13. Wash each well four times.
14. Dilute HRP conjugate 1:30,000 in dilution buffer.
15. Transfer 100 µL to each well.
16. Incubate 60 min at room temperature on a rotary shaker.
17. Wash each well five times.
18. Add 100 µL of TMB One Component HRP Microwell Substrate to each well.
19. Develop the enzymatic color reaction for 10–15 min in the dark at room temperature (the substrate reaction yields a blue solution).
20. Stop TMB reaction by applying 100 µL stop solution (2 M H_2SO_4) to each well, and tap plate gently to mix (color changes from blue to yellow) (*see* **Note 17**).
21. Read plate at 450 nm for TMB substrate using a microplate reader.
22. For data analysis, average the duplicate absorbance values for each standard, control, and sample and subtract the average blank from each value.
23. Create a standard curve using software that generates a four-parameter logistic (4-PL) curve fit, and calculate HSA concentration of samples (*see* **Note 18**).
24. Multiply obtained values by the dilution factor to determine the concentration of undiluted samples.

Product: Hepatitis B surface antigen (HBsAg) [30]

Host: Pichia pastoris.

Aim: Determination of soluble HBsAg.

Method: Sandwich ELISA.

Sample matrix: Cell extract.

Sample preparation:

1. Centrifuge aliquots of induced cells at 10,000 × *g* at 4 °C.
2. Wash with sterile water.
3. Resuspend cells in breaking buffer (25 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L DTT) using 6.0 mL breaking buffer/g wet cells for 18 h at 4 °C.
4. Disrupt cells in a homogenizer (4 passes at chamber pressures of 13,000 psi).

Method description:

1. Clarify cell extracts.
2. Dilute extracts into a series of fivefold with 20 mmol/L PBS (pH 7.2).

3. Add 100 µL of the diluted extracts to each well of a 96-well plate coated with anti-HBsAg polyclonal antibody.
4. Incubate the 96-well plate for 1 h at 37 °C.
5. Empty and tape dry the plate.
6. Wash with PBS containing 0.02% Tween 20 5 times.
7. Add 100 µL horseradish peroxidase (HRP)-conjugated mouse anti-HBsAg to each well.
8. Incubate for 1 h at 37 °C.
9. Empty and tape dry the plate.
10. Wash five times as described above.
11. Add 100 µL of substrate (OPD), and allow the plate to develop for 30 min at 37 °C.
12. Stop the reaction by adding 50 µL of 2 mol/L H_2SO_4.
13. Read each well of the plate at a double wavelength of 450 nm/620 nm.
14. Make a reference curve with a series of standard dilution of HBsAg (0~4 ng).

2.3 HPLC: High-Performance Liquid Chromatography

2.3.1 Background

High-performance liquid chromatography (HPLC) is well established to separate, identify, and quantify individual components. It is feasible for purified analytes but also crude culture supernatants. HPLC operates under the basic principle that the separation of compounds in a sample into its constituent parts depends on the relative affinities of different molecules for the mobile phase and the stationary phase used for the separation. The column is packed with a stationary phase composed of irregularly or spherically shaped particles, a porous monolithic layer, or a porous membrane. The injected sample is forced through the column by an appropriate liquid with adjusted composition and flow rate. Based on the overall performance, conventional HPLC, u-HPLC (ultra-HPLC), and micro HPLC systems (µHPLC), which needs special equipment, can be distinguished [31, 32]. Depending on the available equipment and in particular the stationary phase and the related flow rate, two performances are applied: conventional systems create pressures between 6000 and 9000 psi, while u-HPLC systems reach pressures above 20,000 psi and are characterized by higher speed, resolution, and sensitivity making them ideally suited for use with MS (mass spectroscopy). U-HPLC systems were designed with (1) low dwell volume that is a term that refers to gradient separations, (2) extra column volume, (3) smaller particle size (sub-2 micron), and (4) dispersion that is minimized. More theoretical plates can reach better separation. For HPLC columns up to 2000 and more plates are ideal [33]. Small injection volumes for improved resolution and symmetric, sharp peaks are

recommended (*see* **Note 19**). The tube is packed with tiny particles up to 5 micron followed by pumping the liquid phase through the column. Columns might be either made of glass, synthetic material, or polished stainless steel and are between 50 and 300 mm long.

The interaction of different molecules involves several chemical and/or physical interactions. For the analysis of supernatants, guard columns are recommended to remove small interfering particles and/or contaminants so that protection of the analytical column is ensured (*see* **Note 20**). Once separated, the molecules will be eluted and are detected by a flow-through device, called detector. Various detection principles are available such as UV spectroscopy, fluorescence, refractive index, mass spectrometry, light scattering, and electrochemical detectors [34]. In certain circumstances more than one detector can be operated in serial (*see* **Note 21**).

Instrumentation of HPLC

The HPLC instrumentation might be very diverse, consisting of different components:

1. Up to four mobile phase reservoirs as stock of mobile phase (s) for complex analysis.
2. A degasser, which degasses the mobile phase(s).
3. A pump in different designs, suitable as solvent delivery system, enabling the flow of the mobile phase.
4. The sample valve injector as sample delivery system, which introduces the sample to stationary phase.
5. The column for separation.
6. The column compartment, used to control the temperature of the column.
7. A detector, to detect each eluted component.
8. Data acquisition, to convert data from the detector to meaningful results.
9. A waste collector of liquid waste [35].

Mode of Separation

1. Normal Phase

   Polar stationary phases, typically silica, combined with relative nonpolar mobile phases (hexane, methylene chloride, chloroform, and diethyl ether). Suitable for somewhat polar to highly polar samples. Molecular weight range 10^0–10^4.

2. Reverse Phase

   The stationary phase is nonpolar to somewhat polar in nature, while the mobile phase is relatively polar and uses gradients of water and methanol or acetonitrile. It works on the principle of

hydrophobic interactions. Samples are nonpolar to somewhat polar. Molecular weight range: 10^0–10^4.

3. Size Exclusion (Gel Permeation)

   The column is filled with small, porous, silica, or polymeric particles. Analytes are separated roughly to their molecular size, more precisely according to the hydrodynamic radius, resulting in broader peak widths compared to other techniques. The solvent is polar or nonpolar. Molecular weight range 10^0–10^6.

4. Ion Exchange

   Ion exchange resins are made of insoluble, high-molecular-weight functionalized material. This technique is applicable to highly polar to ionic molecules. The mobile phase is an aqueous buffer, where both pH and ionic strength can be adjusted. Sometimes organic solvents are added to moderate the solvent strength. Molecular weight range 10^0–10^4.

5. Affinity

   It is the most selective type, utilizing the specific interaction between one kind of solute molecule and a second molecule immobilized on the stationary phase.

Elution Techniques

1. Isocratic Elution

   In this case one composition of the mobile phase throughout the whole procedure is used. This elution is a good choice for simple separations but is limited to an increasing peak width by time that is linear with the number of theoretical plates.

2. Gradient Elution

   The mobile phase composition is changed during the separation process. A gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower peaks. Stepwise and linear gradients are most popularly applied for complex samples where a better separation is required.

Effective Method Development

Analytical method development is a critical process when using HPLC for pharmaceutical analysis. Although this technique is commonly applied, both advantages and limitations are recognized (Table 5). A method needs to (1) satisfactorily separate the desired components, (2) generate accurate and constant results over time, and (3) be reproducible and robust. The most critical steps are (a) sample preparation, (b) the analysis, and (c) standardization. All individual parameters should be investigated before the final method optimization is performed (*see* **Note 22**). Method validation can finally be performed according to the ICH guidelines. Due to the high flexibility of this technique, individual working ranges and limits are adjustable. Nevertheless, it can be considered that for

Table 5
Summary of applicability and limitations of HPLC analysis

Advantages	Perceived limitations
Applicable for diverse analyte/sample types—crude supernatant	Lack of an ideal universal detector General costs
Excellent accuracy and precision	Relatively difficult for novices
Qualitative and quantitative	Time-consuming standardization
High flexibility	Operator-dependent analysis
Automated operation	Interference with other proteins—purity
High separation power for proteins and related substances	Interference with salts, detergents, organic solvents, sulfones, etc.
Sensitive detection	High viscosity (DNA, carbohydrates)
Coupling with mass spectroscopy	Limited sample throughput
Highly reproducible	Large quantities of expensive organics
Compared to other chromatographic methods—quick and efficient	Data management and documentation Complex troubleshooting
Short analysis time for single compounds	Long running time for multi-compound analysis

many applications this technique is less sensitive than other quantification methods and, however, more selective.

2.3.2 Examples

The following examples give an overview of the applicability of HPLC for the analysis of different products from yeasts. Although in the examples no guard columns are used, they are highly recommended to protect the analytical column against particulates. Additionally, gradients need to be adapted because the complex sample matrices can have an enormous impact on the separation. For the calibration of a quantitative HPLC method, either a purified material of the analyte or when available a USP reference standard should be used. Equal injection volumes and concentrations for standards and samples are recommended. For detection DAD detection system is favorable, for recording wavelengths in parallel, e.g., 280 and 214 nm (*see* **Note 23**) [36–41].

Product: Insulin precursor (IP) [36]

Host: Pichia pastoris.

Aim: Quantification of secreted IP in clone screening.

Method: Reversed-phase HPLC (RP-HPLC).

Sample matrix: Clarified culture broth.

Equipment: HPLC system equipped with a binary pump system, an auto-injector, a column oven, and an UV-VIS detector

(280 nm). As analytical column, a SUPELCOSIL™ LC-304, 5 μm, 300 Å, 4.6 × 250 mm, is applied.

Sample preparation:

1. Filtrate an aliquot of a *P. pastoris* culture broth through a syringe filter (0.2 μm).
2. Mix a filtered aliquot with an equal volume of solvent A.
3. Human insulin CRS from European Pharmacopoeia (EP) as standard is used.

Method description:

1. Solvent A (0.15% (v/v) TFA in Milli-Q water).
2. Solvent B (0.15% (v/v) TFA in acetonitrile).
3. Elution is performed with a flow rate of 1 mL/min and a gradient shown in Table 6 and illustrated in Fig. 1.

Product: Hepatitis B surface antigen (HBsAg) [38]

Host: Pichia pastoris (intracellular production of HBsAg).

Aim: Determination of total HBsAg.

Method: Reversed-phase HPLC (RP-HPLC).

Sample matrix: Cell lysate.

Equipment: HPLC system consisting of a binary pump, an auto-injector, column oven, and an UV-VIS detector (214 nm). A SUPELCOSIL™ LC-DB-18, 5 μm, 4.6 × 150 mm column, is used for separation.

Sample preparation:

Preparation of Cell Lysate

1. Determine the cell concentration via optical density (600 nm) with a spectrophotometer.
2. Centrifuge samples corresponding to 100 OD units (1 mL) at 15,000 × *g* for 15 min to get a cell pellet.

Table 6
Gradient for IP quantification by RP-HPLC

Time (min)	Solvent (%B)	Function
0–6	10	Equilibration
6–41	10–43	Separation
41–43	43–100	Column cleaning
43–53	100–10	Readjustment
53–60	10	Equilibration

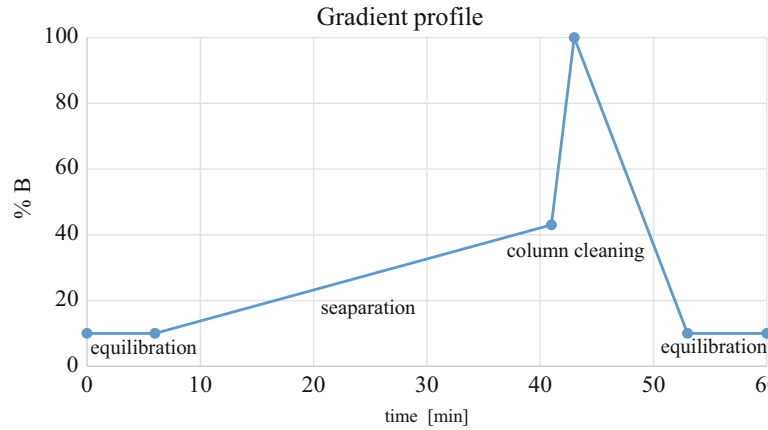

Fig. 1 Applied gradient for IP quantification by RP-HPLC illustrating the equilibration, separation, and cleaning of the column

3. Wash the cell pellet with 1 mL 10 mmol/L phosphate buffer, pH 7.2.
4. Mix it with 0.6 g glass beads of 0.45 mm diameter suspended in 500 µL of lysis buffer (10 mmol/L phosphate buffer (pH 8), 5 mmol/L EDTA, 500 mmol/L NaCl, 8% (v/v) glycerol).
5. The cells are lysed by 20 cycles of 1 min vortexing at maximum speed followed by 4–5 min resting on ice.
6. After the final cycle, transfer the lysate into a fresh tube.
7. Rinse the glass beads twice with lysis buffer, and pool with the initial lysate to obtain a final volume of 1 mL.
8. Suspend a 100 µL aliquot of the cell lysate in an equal volume of solubilization reagent (8% (w/v) SDS, 50% (v/v) β-mercaptoethanol, 1 mol/L DTT).
9. Boil for 15 min.
10. Clarify sample by centrifugation and filtrate through a syringe filter (0.2 µm).

Method description:

1. Solvent A (0.15% (v/v) TFA in Milli-Q water).
2. Solvent B (2-propanol: acetonitrile in 80:20 (v/v)).
3. Column temperature: 70 °C.
4. Gradient elution is performed at a flow rate of 1 mL/min as shown in Table 7.

Product: Human epidermal growth factor (hEGF) [39]

Host: Hansenula polymorpha.

Aim: Quantification of hEGF.

Method: Reversed-phase HPLC (RP-HPLC).

Sample matrix: Culture supernatants.

Table 7
Gradient for HBsAg quantification by RP-HPLC

Time (min)	Solvent (%B)	Function
0–4	45	Equilibration
4–10	45–95	Separation
10–13	95	Column cleaning
13–14	95–45	Readjustment
14–18	45	Equilibration

Table 8
Gradient for hEGF quantification by RP-HPLC

Time (min)	Solvent (%B)	Function
0–5	0–15	Separation
5–40	15–50	Separation

Equipment: HPLC system consisting of a binary pump, an autoinjector, and an UV-VIS detector (215 nm). A Hypersil C18 column, 5 μm, 4.6 × 150 mm column, is used for separation.

Sample preparation:

1. Centrifuge culture supernatants and filtrate through a syringe filter (0.2 μm).

Method description:

1. Solvent A (0.1% (v/v) TFA in Milli-Q water).
2. Solvent B (0.1% (v/v) TFA in acetonitrile).
3. After equilibration, gradient elution is performed at a flow rate of 1 mL/min shown in Table 8.

Product: mAb [41]

Host: Pichia pastoris.

*Aim: Q*uantification of mAb.

Method: Protein A HPLC.

Sample matrix: Culture supernatant.

Equipment: HPLC system consisting of a binary pump, an autoinjector, and an UV-VIS detector (280 nm). For mAb quantitation, a POROS A 20 column, 2.1 × 30 mm, is used.

Table 9
Alternatives for buffer B for mAb quantification by protein A HPLC

Buffer B
100 mM citrate, pH 2.5
100 mM acetate, pH 2.5
100 mM phosphate, pH 2.5
100 mM glycine, pH 2.5
12 mM HCl, pH 1.9

Table 10
Gradient for mAb quantification by protein A HPLC

Time (min)	Solvent (%B)	Function
0–0.5	0	Equilibration/wash; 14 column volumes
0.51–1.5	100	Elution; 29 column volumes
1.51–3.0	0	Re-equilibration; 43 column volumes

Sample preparation:

1. Centrifuge and filtrate (syringe filter 0.2 μm) culture supernatant samples before loading onto the column.
2. Dilute samples, if required, in equilibration buffer.
3. For quantitation of the specific mAb, standard curves should be generated from purified material.

Method description:

1. Equilibration/wash buffer (buffer A), e.g., 50 mM phosphate, 150 mM NaCl, pH 7.0.

   In principle, the molar concentration of phosphate in buffer A can range from 25 to 100 mM with a pH ranging from 6.6 to 7.5 with or without sodium chloride up to 150 mM and has to be adapted on the specific requirements.
2. Elution buffer (buffer B): as buffer B either the same buffer as buffer A with a pH ranging from 2.0 to 3.5 is used or alternatives shown in Table 9,
3. Flow rate is 3 mL/min with gradient shown in Table 10 and illustrated in Fig. 2 (*see* **Note 24**).

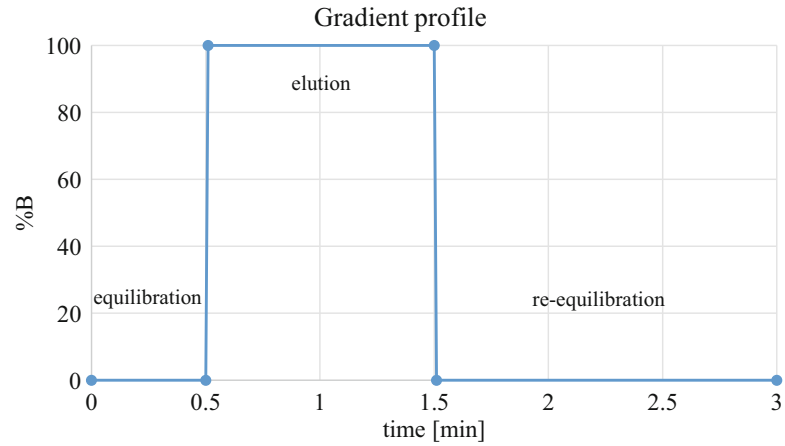

Fig. 2 Elution profile including equilibration, elution, and re-equilibration for mAb quantification by protein A HPLC

3 Advanced Methods for Quantification

3.1 Bio-layer Interferometry

3.1.1 Background

Over the last years, the implementation of biosensor technologies for qualitative and quantitative assays has markedly advanced. One promising biosensor platform, using bio-layer interferometry (BLI), is investigated by FortéBio; meanwhile PALL provides a real-time measuring performance based on a label-free technology. In particular, quantification and measurement of biomolecular interactions are intended [42]. This optical analytical technique utilizes the interference pattern of white light reflected from two surfaces: (1) a layer of immobilized protein on the biosensor tip and (2) an internal reference layer. The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip surface, which results in a wavelength shift ($\Delta\lambda$) as shown in Fig. 3. This change in thickness of the biological layer is a direct indicator for the specific interaction of the molecules, and by a certain calibration, the increase of signal intensities is used for quantification. Interactions are measured in real time, providing the ability to monitor binding specificity, rates of association and dissociation, or concentration profiles, with high precision and accuracy.

This non-fluidic technique is either performed in 96- or 384-well plate formats. Biosensors with different ligands (e.g., streptavidin; proteins A, G, and L; nickel-charged Tris-nitriloacetic acid (Tris-NTA) for his-tagged proteins; antihuman IgG Fc capture (AHC)) coated on a biocompatible matrix are commercially available. The dynamic range for a standard assay is assessed between 1.3 and 150 μg/mL. Signal enhancement, such as metal chelator (DAB), combined with a horseradish peroxidase-tagged antibody, is described.

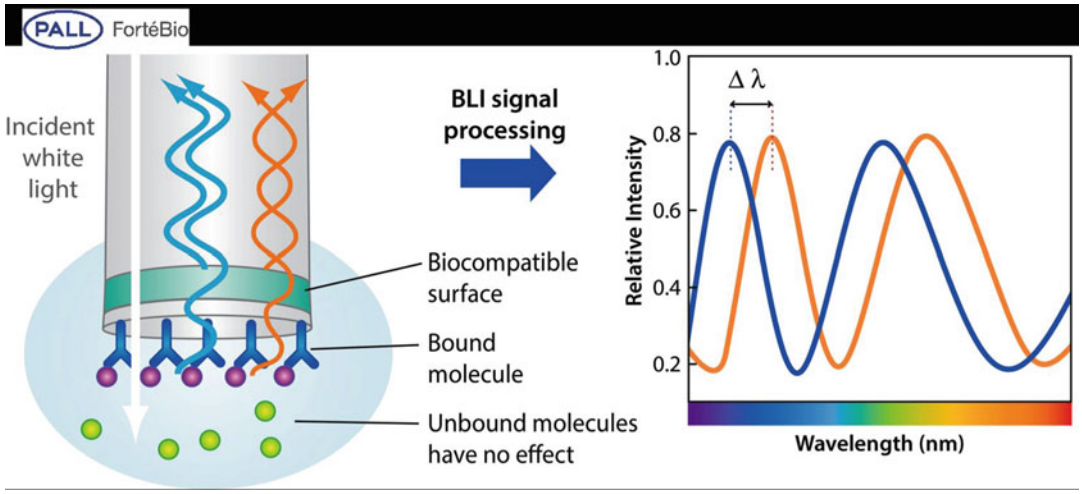

Fig. 3 Schematic drawing of the bio-layer interferometry principle of an individual tip surface. A layer of immobilized protein on the biosensor tip and an internal reference layer are shown on the left side. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real time. The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift, $\Delta\lambda$ as shown on the right side of the figure. Modified with permission from Pall FortéBio, USA

Biomolecules in crude lysates, culture supernatants, as well as purified samples can be analyzed [43]. Dilution of samples and standards needs to be performed externally by hand or automated, prior to transfer into the microwell plates. Due to microplate format, automatization for high-throughput purposes can be achieved by additional equipment. Test samples after cell removal, e.g., by centrifugation, but also with cell-containing suspensions can be applied if they are not interacting with the surface. Molecules and particles in the solution do not contribute to the signal. Only attached molecules are measured. During assay development unspecific binding needs to be carefully evaluated. Additionally, various buffers for individual washing steps and other required solutions, such as blocking solvents, are also placed in defined wells. The assay itself is subsequently performed automatically. Different sensors can be regenerated, according to established regeneration procedures, to minimize sensor costs. A summary of the applicability of bio-layer interferometry is given in Table 11.

3.1.2 Example

In principle, mAb concentration is calculated via the binding rate of antibodies to protein A sensor tips. A typical calibration range is between 0.25 and 300 µg/mL depending on the antibody as shown in Fig. 4. IgG standards for calibration should be as similar to the molecule to be analyzed, and also the matrix should match as closely as possible to the sample matrix, e.g., by diluting standards in freshly prepared media. Generally, the assay setting consists of an

Table 11
Summary of applicability and limitations of BLI

Advantages	Perceived limitations
Suitable for crude supernatants	Medium dynamic range
Various sensor formats available	Equipment costs
Label-free detection	Medium sensitivity without signal enhancement
Small sample volume	Interference with other proteins—unspecific binding
Qualitative/quantitative Accurate and precise	Sensor costs—regeneration Noise and drift optimization—additional time
Real time	Detectability for small molecules ($\leq$10 kDa)—optimization needed
Non-fluidic	
Significant throughput and flexibility	

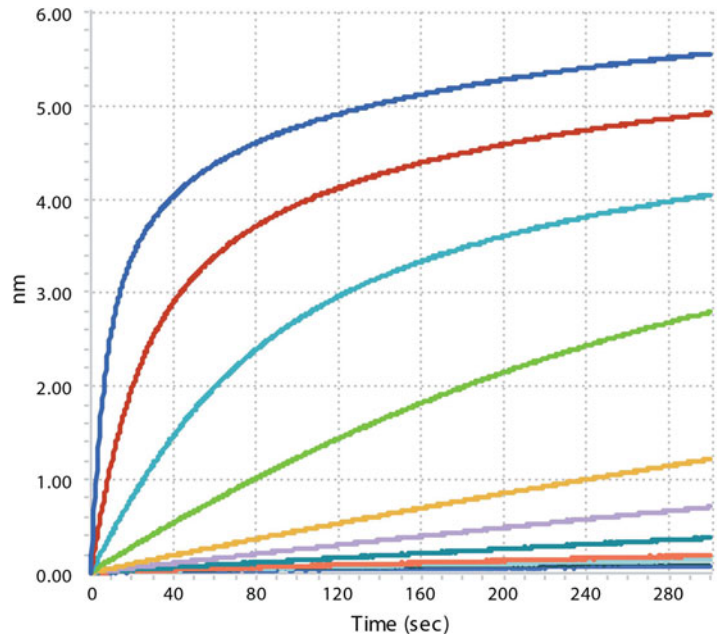

Fig. 4 Concentration-dependent binding curves from IgG standards pre-diluted in media to protein A biosensors. Standard concentration ranges between 0.25 (orange), 0.5 (dark green), 1 (lilac), 3 (yellow), 10 (light green), 30 (cyan), 100 (red), and 300 (blue) μg/mL. Reproduced with permission from Pall FortéBio, USA

equilibration, mAb loading, and regeneration step for the protein A sensors. Usually, biosensor tips can be reused up to ten times [44, 45].

Product: mAb [45].

Host: Not specified.

Aim: Quantification of mAb.

Method: Bio-layer interferometry (BLI) with protein A biosensors.

Sample matrix: Serum-free cell culture supernatants.

Equipment: Octet QK system.

Sample preparation: Centrifugation of culture supernatants.

Method description:

1. Use black 96-well plates for the measurements and sensor equilibration, respectively.
2. Equilibrate sensor tips by dipping in 200 µL equilibration buffer for 10 min (PBS, 0.1 Vol% Tween 20).
3. Prepare a second 96-well plate by pipetting in one row 200 µL regeneration buffer (10 mM glycine-HCL, pH 2.5) and in a second row 200 µL equilibration buffer (e.g., rows 11 and 12).
4. Transfer 50 or 25 µL culture supernatant starting from row 1.
5. Add 150 or 175 µL equilibration buffer, respectively, to each sample well in order to obtain a total volume of 200 µL.
6. Proceed with calibration standard in the same way, e.g., add to 50 µL standard (pre-diluted in media) 150 µL equilibration buffer.
7. Transfer the well plate in the well plate holder of the instrument.
8. Shake plates at 1000 rpm for 5 s to mix samples before each measurement.
9. Determine binding rates over a time interval of 300 s.
10. Calculate binding rates with the Octet software.
11. After each measurement, biosensor tips may be regenerated by washing in regeneration buffer (row 11) subsequently in equilibration buffer (row 12) for 5 s.

3.2 Microfluidics CD Technology

3.2.1 Background

Alternatively to the non-fluidic sensor-based technique, nanoliter microfluidics CD technology has been recently developed [46]. This technology is established as an open platform using miniaturized chambers located on a rotating CD (96 chambers per CD). Each chamber is filled with 15 nL affinity capture materials coated on modified beads (Fig. 5). Different equipment formats for one or more CDs are available for automated immunoassays at nanoliter-scale with parallel processing.

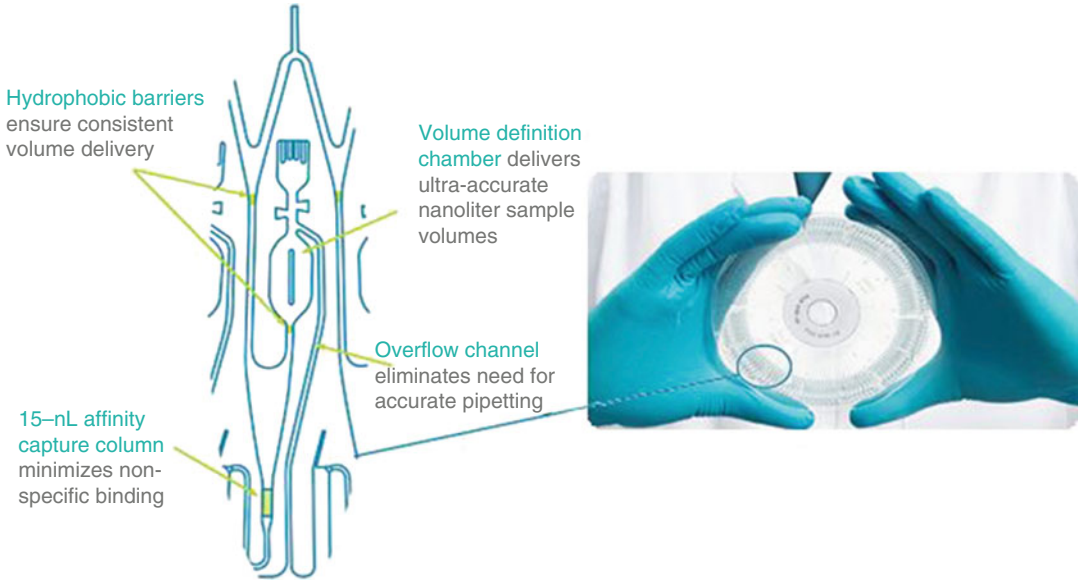

Fig. 5 Sketch of the nanoliter-scale device from Gyrolab Systems. Reproduced with permission from Gyros Protein Technologies, Sweden

The assay workflow includes precise, automated control of centrifugal and capillary forces, which steer liquid flow-through. Samples are diluted externally and transferred into special microwell plates with a protection slide to prevent evaporation of the small volumes (10–max. 100 μL). Transfer of the samples into the reaction chambers is performed via a volume definition chamber that delivers accurate sample volumes, with hydrophobic breaks to ensure consistency in volume delivery. An overflow channel eliminates the need for manual pipetting accuracy, and a 15 nL affinity capture column mitigates non-specific binding. Once CDs are loaded into the workstation, up to five discs in series, the platform performs parallel sample processing via high-sensitivity, laser-induced fluorescence detection. The CDs are spun at controlled speeds to ensure optimal binding and uniform conditions for all assays.

Although this technique has been established rather for biological samples than for biotechnological samples, this technique became of great interest due the PAT (process analytical technology) initiative. A very broad quantification range (3–4 logs) with sensitivity down to 1 ng/mL is offered. Therefore, this technology is feasible both for clone screening and also product manufacture. Advantages and perceived limitations are shown in Table 12.

Table 12
Summary of applicability and limitations of microfluidics CD technology

Advantages	Perceived limitations
Crude supernatant—with little need for sample pretreatment	Interference with other proteins—assay dependent Data export is complex
Various gel formats available	High costs
Different staining opportunities	Semiautomated combined with external sample preparation
Small sample volume	Evaporation risk
Reduced reagent consumption	
High sensitivity and precision Reduced matrix interference Broad dynamic range—reduced dilution Robust and rugged Fast total assay time Adaptability to automation	

3.2.2 Example

The subsequent example describes a protocol to quantify intact human IgG (IgG1, IgG2, IgG4) in cell supernatants. For this semi-automated assay standards, pre-diluted samples, capture reagent, detection reagent, and wash solution have to be provided in a 96-well plate. Subsequent pipetting steps as well as incubation and detection are operated automatically. The advantage of miniaturization is that extremely low sample volumes are required. The provided kit (Gyrolab-hulgG-k) includes ready-made reagents and can be used together with the CDs, Bioaffy 20 HC (300–1000,000 ng/mL, 20 nL sample volume, 112 microstructures) or Bioaffy 1000 HC (1–20,000 ng/mL, 1000 nL sample volume, 96 microstructures). Furthermore, a Bioaffy 200 HC (10–1000 ng/mL, 200 nL sample volume, 112 microstructures) is available. Solutions are evenly distributed by rotation of the CD and pervade the microstructures. Combination of different CD formats enables quantification of human IgG over six orders of magnitude.

In principle, a biotinylated derivative of protein A (capture reagent) is introduced into a microstructure in the CD to saturate a capture column packed with porous beads coupled with streptavidin, followed by sample application, where intact IgG is captured in the capture column. Subsequently an Alexa Fluor 647 labeled F $(ab')_2$ fragment of antihuman IgG (detection reagent) is added. The corresponding signal in the capture column represents the total response from the sample [47].

Product: Human IgG (IgG1, IgG2, IgG4) [47].

Host: Not specified.

Aim: Quantification of human IgG.

Method: Microfluidics CD technology.

Sample matrix: Cell supernatants.

Equipment: Gyrolab workstation.

Sample preparation:

1. Centrifuge samples at 12,000 × *g* for 4 min to sediment any aggregates or particulates.
2. Remove supernatant carefully.

Method description:

1. Transfer 50 µL of the ready-to-use capture reagent, detection reagent, and wash buffer (PBS—0.01% Tween 20) directly to the microtiter plate according to the instructions.
2. Dilute samples in sample dilution buffer at least 1 + 1 by volume, but further dilution steps may be necessary depending on the working range of the particular version of IgG titer assay.
3. Select an appropriate IgG molecule at known concentration as standard, or use standard from Gyrolab which contains a human monoclonal IgG1 (4 mg/mL) in 50 µL.
4. Dilute standards also in sample dilution buffer.

Automated test sequence (approx. 1.5 h):

1. Wash step: microstructures are rinsed with wash buffer.
2. Application of the capture reagent including a short incubation.
3. Wash step.
4. Sample application including a short incubation.
5. Wash step.
6. Recording of the background signal for following fluorescent detection.
7. Application of the detection reagent including a short incubation.
8. Wash step.
9. Detection of the fluorescence signal.
10. Data evaluation is performed by the Gyrolab Evaluator.

3.3 Surface Plasmon Resonance

3.3.1 Background

Surface plasmon resonance (SPR), a biosensor-based technology, is currently of great interest for the analysis of various compounds, including proteins and peptides. Liedberg et al. initially published the optical phenomenon of surface plasmon resonance over 30 years ago [48]. It was first shown that the interaction of molecules in a label-free state can be measured in which the binding

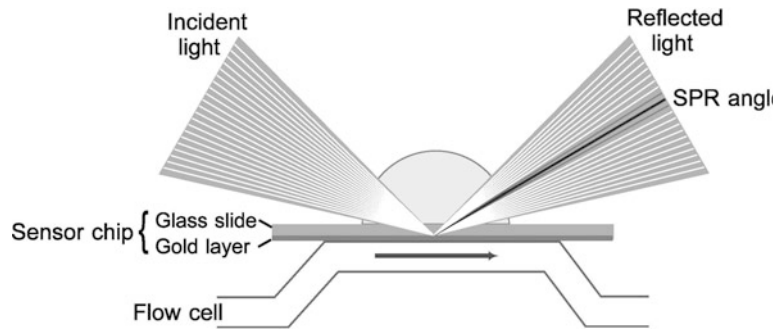

Fig. 6 Principle of surface plasmon resonance (SPR). Reproduced with permission from GE Healthcare Life Sciences

response is given in resonance units (RU) proportional to the molecular mass [49]. Biacore first commercialized this technique by offering a sensor chip platform in a microfluidic device, which controls the flow of buffer and samples across the sensor surface (Fig. 6).

In principle, SPR occurs in a thin contacting film placed at the interface between two media of different refractive indices. In Biacore systems (GE Healthcare Life Sciences), a 50 nm gold layer is sandwiched between a glass layer and the sample solution. Gold layers are preferred surfaces for SPR due to a well-defined reflectance minimum and due to the fact that it is amenable to covalently attach surface matrix layers. Furthermore, gold is mostly inert and suited for various buffer systems. For the efficient loading of various molecules, different surface layers are commercially available. The most popular surface is carboxymethylated dextran (CM). By certain modifications, capturing of the molecules from a solution can be optimized [50]. Covalent coupling can be performed either via free amino, thiol-disulfide, or aldehyde groups. Unbound and non-covalently bound material can be removed either with buffer or injection of suitable regeneration and cleaning solutions. The most critical step in assay development is the adjustment of proper immobilization chemistry. Direct and indirect techniques are available, and numerous techniques have been published [51].

For the application of SPR, one fundamental element is to distinguish the active protein form from the inactive form. This feature is of great importance because it allows the quantification of the protein of interest not only in a pre-purified form but also in crude, complex matrices [52, 53].

The detection is based on measuring the internal reflection of plane-polarized light from a near-infrared LED with a diode array detector. If a change in mass occurs, the angle of light at which SPR occurs shifts due to a change in refractive index near the sensor surface. One RU corresponds to 0.0001° shift in SPR angle, or

Table 13
Summary of applicability and limitations of SPR technology

Advantages	Perceived limitations
Good dynamic range	Suitable for crude supernatants
Various surfaces available	Equipment costs
Label-free detection	Medium sensitivity without signal enhancement
Small sample volume Mass detection	Unspecific binding might be a challenge—increased number of references demanded
Qualitative/ quantitative	Sensor costs—regeneration recommended
Real time	Noise and drift optimization
Microfluidic	Interference of low-molecular-weight molecules
High sensitivity Automatization	Clogging due to the microfluidic system might occur
Accurate and precise	

1 pg/mm^2 of molecule on the surface. Since only the evanescent wave penetrates the sample, measurement of colored, turbid, and opaque solutions is possible.

SPR is a well-established technique for detection and monitoring biomolecular interactions in real time. Used orthogonally, it is powerful and complementary in basic research, drug discovery and development, and downstream bioprocessing. Advantages and perceived limitations are shown in Table 13.

3.3.2 *Example*

The example given below is applicable for proteins containing terminal his-tags, which can be attached to SPR-NTA sensor chips. Ligands are captured on the carboxymethylated dextran matrix which is pre-immobilized with nitrilotriacetic acid (NTA). NTA chelates present Ni^{2+}, creating coordination sites that bind to histidine residues in the ligand tag. Therefore, the NTA sensor chip has to be activated with Ni^{2+} ions (loading buffer), followed by binding of subsequent injected analyte (running buffer), which is finally detected (*see* **Note 25**). Thereafter, the chip surface is regenerated with EDTA solution (stripping buffer); after which a new analysis cycle with the Ni^{2+} solution pulse can be started [54–56].

Product: Single-chain Fv antibody fragment (ScFv4813; his-tagged) [54].

Host: Pichia pastoris.

Aim: Quantification.

Method: Surface plasmon resonance.

Sample matrix: Culture supernatant.

Equipment: Biacore 2000 system.

Sample preparation:

1. Clarify culture supernatants by centrifugation (2000 × *g*, 5 min at room temperature).

Method description:

1. Prepare the Biacore instrument with running buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) with a flow rate of 5 μL/min).
2. Each sample cycle consists of subsequent injections as follows:
3. 5 μL stripping buffer (running buffer containing 0.35 M EDTA at pH 8.3).
4. 10 μL of loading buffer (running buffer containing 500 μM $NiCl_2$).
5. 20 μL of the diluted sample (conc. Below 0.2 μM) in running buffer.

For quantification, the availability of a suitable standard is essential. An example for a concentration-dependent analyte binding is shown in Fig. 7 (*see* **Note 26**).

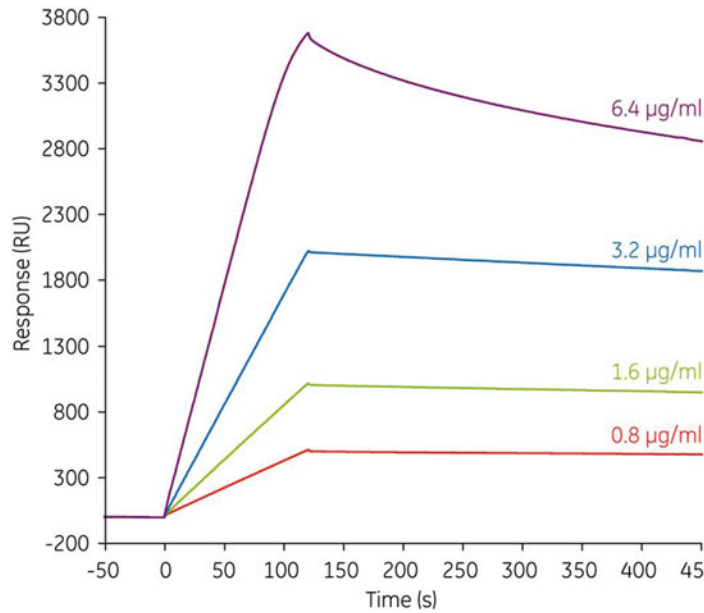

Fig. 7 Injection of different concentrations of histidine-tagged green fluorescent protein (0.8–6.4 μg/mL) over Sensor Chip NTA. Reproduced with permission from GE Healthcare Life Sciences

3.4 Electrochemiluminescence Assay

3.4.1 Background

The electrochemiluminescence technique, namely, the electrochemiluminescence immunoassay or ECLIA, has been established for drug development and clinical application but might be also used for biotechnological applications [57, 58].

Electrochemiluminescence labels generate light when stimulated by electricity. In the appropriate chemical environment, this light signal is used to measure proteins and other biomedical molecules. Analogous to chemiluminescence it does not require the use of external light sources. The combination of chemiluminescence and electrochemistry implies potential advantages. Meanwhile, various systems are launched, which can be easily adapted to biotechnological applications.

The first commercial instrument was produced by IGEN International, Inc. (technology later acquired by Roche Diagnostics Corp.) in 1994. This system uses magnetic microbeads modified with probe molecules, such as antibodies or nucleic acids, to capture analyte molecules in a sample labeled with $Ru(bpy)_3^{2+}$. Before the measurement is performed, the unbound $Ru(bpy)_3^{2+}$ labels are flushed from the cell. The bound $Ru(bpy)_3^{2+}$ labels are measured in tripropylamine (TPrA) solution. Both $Ru(bpy)_3^{2+}$ and TPrA are oxidized at the electrode surface, and $Ru(bpy)_3^{3+}$ is reduced by TPrA to produce the excited state. However, TPrA has several disadvantages. It is toxic and volatile and needs to be used in high concentrations (usually up to 100 mM) to reach good sensitivity.

Therefore, a more environmentally friendly co-reactant, the 2-(dibutylamino)ethanol (DBAE), was introduced. The intensities of the $Ru(bpy)_3^{2+}$/DBAE system at Au and Pt electrodes were approximately 10–100 times greater compared to the previously used system, when 25 mM of each co-reactant was used. The sensitivity is about an order of magnitude better. DBAE is much less toxic and less volatile and better soluble in aqueous solutions. Alternatively, "reductive-oxidative" co-reactants are used. The $Ru(bpy)_3^{2+}/S_2O_8^{2-}$ system has a very negative potential. Unfortunately, it is difficult to create stable signals in aqueous systems because of serious hydrogen evolution upon applying very negative potentials. To overcome this problem, bismuth electrode with a high hydrogen evolution potential is provided as a sufficient solution.

Another prominent example of a well-recognized microwell plate technique has been launched by Meso Scale Discovery (MSD), which is available in a classical 96-well but also 384-well formats, equipped with electrodes at the bottom of each well (Fig. 8). The excitation caused by a SULFO-TAG label is measured through a CCD camera. The label is either conjugated to streptavidin or to the detection antibody. This technology is meanwhile well recognized due to the advantages of the broad dynamic range (3–4 logs), low detection limits (1.0–10,000 pg/mL), low sample volumes (10–25 μL), and the suitability in complex matrices. Advantages and perceived limitations are shown in Table 14.

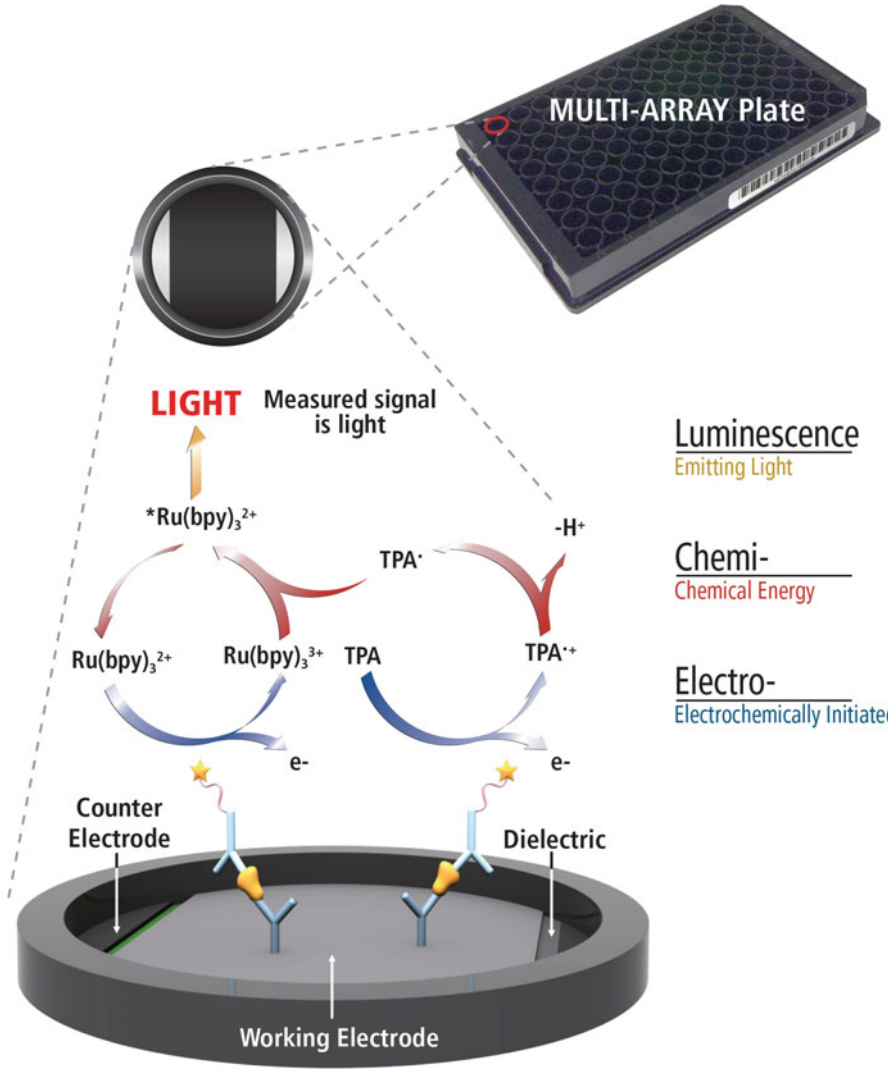

Fig. 8 Principle of electrochemiluminescence assay provided by MSD. Reproduced with permission from ©2006, 2009 Meso Scale Discovery, a division of Meso Scale Diagnostics, LLC. All rights reserved

3.4.2 Example

Meso Scale Discovery (MSD) provides a sandwich immunoassay for human insulin that detects insulin in cell supernatant, human serum, and plasma samples. In principle, in this single-plex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. The assay kit consists of a multi-array 96-well plate that has been pre-coated with insulin antibody (capture antibody) and an anti-insulin antibody, labeled with an electrochemiluminescent compound, MSD SULFO-TAG label (detection antibody). Insulin in the sample binds to the immobilized capture antibody and the added detection antibody as schematically demonstrated in Fig. 9. Adding the MSD Read Buffer ensures the appropriate chemical environment for electrochemiluminescence measurement. This Tris-based buffer contains tripropylamine

Table 14
Summary of applicability and limitations of electrochemiluminescence assay

Advantages	Perceived limitations
Various assays available	Equipment costs
Suitable for crude supernatants	Some reagents are toxic, volatile
Small sample volume	System related instable ECL signals
Qualitative/quantitative Accurate and precise	Sensor costs—regeneration Noise and drift optimization
Broad dynamic range	Detection co-reactants need to be optimized/individually selected
Non-fluidic	
Significant throughput and flexibility	

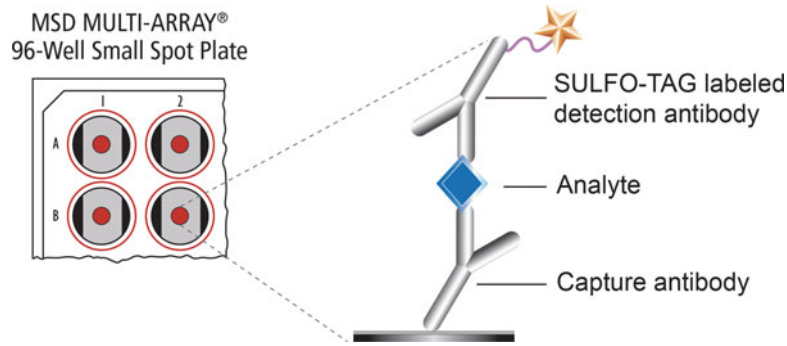

Fig. 9 Principle of insulin sandwich immunoassay established by MSD platform. Reproduced with permission from ©2006, 2009 Meso Scale Discovery, a division of Meso Scale Diagnostics, LLC. All rights reserved

(TPA) which should facilitate higher signals and sensitivity as well as lower background. After loading the plate in the instrument, a voltage is applied to the plate electrodes, which causes the labels bound to the electrode surface to emit light. The intensity of the emitted light is a quantitative measure of insulin present in the sample. Furthermore, an insulin calibrator and all buffer solutions, required for the assay, are provided in the kit [59].

Product: Insulin [59].

Host: Not specified.

Aim: Quantification.

Method: ECLIA.

Sample matrix: Cell supernatant, human serum, and plasma samples.

Equipment: MSD-SECTOR Imager.

Sample preparation: 25 μL sample.

Method description:

1. All buffer stock solutions delivered in the assay kit have to be diluted according to the manufacturer's recommendations.
2. The MSD plate is pre-coated with insulin antibody and is ready to use.
3. Dispense 150 μL of Blocker A solution into each well (mixture of proteins in a PBS buffer preventing non-specific binding, improving sensitivity, and reducing background signals).
4. Incubate at room temperature at 300–1000 rpm for 1 h.
5. Wash plate three times with PBS-T (0.05% Tween 20).
6. Dispense 25 μL/well detection antibody solution.
7. Immediately dispense 25 μL/well sample or calibrator.
8. Incubate the sealed plate at room temperature at 300–1000 rpm for 2 h.
9. Wash plate three times with PBS-T.
10. Dispense 150 μL/well Read Buffer.
11. Analyze the plate immediately after Read Buffer addition on SECTOR Imager.

3.5 Microchip-Based Assays

3.5.1 Background

Automatization of electrophoretic techniques is also an alternative issue to minimize manual manipulation. Basically, for product development, the analysis of proteins and related posttranslational modifications, such as glycan profiling, can be performed [60, 61]. A meanwhile well-established technology has been launched by PerkinElmer, Inc. (through its Caliper Life Sciences, Inc. division), but also comparable systems from other suppliers are on the market (Fig. 10).

To achieve high-throughput performance, chip-based microfluidic assays have been developed, which are proposed for the qualitative and quantitative analysis of proteins directly performed from supernatants, cell lysates, and column fractions, purified and partially purified samples. The commercially available systems enable the separation of proteins sizing from 14 to 200 kDa, applying samples directly from 96- and/or 384-well plates. Assays can be completed within a very short time, faster than 1 min, and repeated runs can be performed without changing the used chip. This can be achieved by sample preparation within tiny chambers and channels that are fabricated in quartz, glass, or plastic. Under the computer-controlled instrumentation for process control and data processing, chips have been optimized to achieve precise microfluidic transport of liquids and samples through the micro-channels. A typical workflow consists of the following steps: separation, staining, destaining, imaging, band detection, quantification, and basic data analysis.

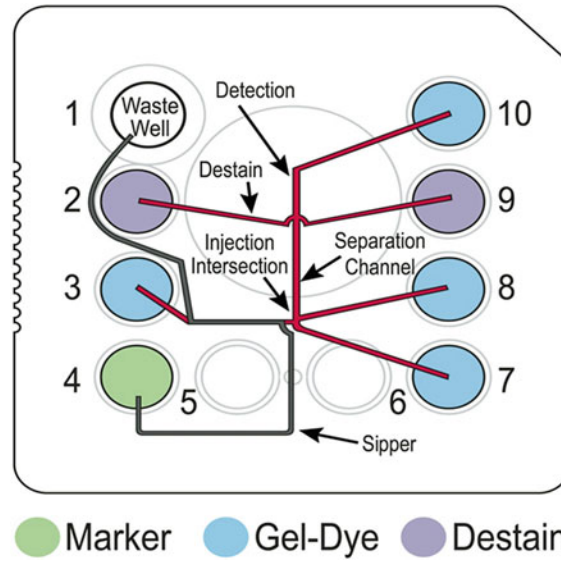

Fig. 10 Schematic diagram of the sipper LabChip® GXII Touch™ protein chip.

Staining is preferentially performed with fluorescent dyes, which are directly incorporated into the sample components during separation. As the sample components pass through the microchannels, they are detected by laser-induced fluorescence as they pass by the position of the laser. Both the fluorescence signal and migration time are processed. Molecular weight sizing determination as well as quantification of individual compounds is aimed.

In advance to the protein quantification, glycan profiling assays have been developed (application notes are provided). The glycan profiling concept is based on a typical workflow: (1) glycan cleavage from purified proteins, (2) fluorophore labeling, (3) separation, and (4) identification. This technique can be used as a competitive assay for the determination of glycan binding sites and investigation of the core structures prior to the analysis of detailed glycan structures. Advantages and perceived limitations of microchip-based assays are shown in Table 15.

3.5.2 Example

The LabChip® GXII system is an automated platform for rapid analysis of proteins and nucleic acids. Each step (sample loading, electrophoresis, staining, destaining, and detection) is integrated in a microfluidic device. The heat-denatured proteins in high SDS concentration are loaded directly out of a 96- or 384-well plate through a capillary sipper. Protein chips are prepared by priming the channels with gel-dye and destaining solution, whereas the gel-dye solution serves as matrix for separation and as staining solution. Then a marker solution is pipetted onto the chip. Subsequent 170 nL of sample from the well plate are applied into the microfluidic channels. Simultaneously, the sample is diluted 2:1

Table 15
Summary of applicability and limitations of microchip-based assays

Advantages	Perceived limitations
Rapid, automated results	Changing pH gradient due to electrolysis
Detection of 0.8–1 μg/mL Small sample volumes (1–4 μL)	Streaming currents which counteract the external electric field or gas bubbles Quantification 5–10 μg/mL
Good reproducibility	Massively parallel setup is problematic due to the heat generated
Qualitative/quantitative	Handheld devices are challenging
Microfluidic	Separation distance—separation efficiency
Automatization	Instrumentation costs
Real time	

with a marker solution, which is used as a reference for migration time and determination of relative concentration of samples. A 40 pL sample plug of the marker-protein mixture is then automatically injected into the separation channel. Proteins are stained with a fluorescence dye, which is mixed with the gel and separated with resolution comparable to a 4–20% SDS-PAGE. Destaining is carried out by a dilution step, flushing SDS-free ions into the separation channel. Thus, free SDS micelles diffuse into the SDS-free fluid, which results in breakup of the micelles and a significant drop in the background fluorescence. SDS micelles bound to the protein remain intact, and protein bands are detected by using laser-induced fluorescence.

Depending on the analyte, good linearity can be achieved over the concentration range 8–2000 μg/mL as well as the ability to detect low-level impurities with a signal-to-noise ratio of 9:1 [62, 63].

Product: mAb [63].

Host: Not specified.

Aim: Purity and quantification.

Method: Microchip-based SDS electrophoresis.

Sample matrix: Crude samples.

Equipment: Caliper's LabChip GXII system.

Chip preparation:

1. Rinse wells of the chip three times by repeatedly pipetting and aspirating deionized water.
2. Prepare gel-dye: mix 520 μL gel matrix and 18 μL dye solution (mix for at least 20 s before use, and avoid light exposure) in a

sample tube; then centrifuge mixture in a spin filter for 5 min at 9300 rcf at room temperature.

3. Destain: centrifuge 250 μL gel matrix in a spin filter for 5 min at 9300 rcf at room temperature.
4. Marker is ready to use.
5. Prepare chip according to the schema shown in Fig. 10:

   1 × 120 μL + 3 × 75 μL gel-dye (positions 3, 7, 8, and 10)

   2 × 75 μL destain (positions 2 and 9).
6. 50 μL marker are adequate for 96 samples, and 120 μL marker are adequate for 384 samples (position 4).

Ladder and buffer preparation:

1. Spin 12 μL ladder in a sample tube.
2. Heat for 5 min at 100 °C or 10 min at 70 °C.
3. Add 108 μL deionized water directly after heating.
4. Transfer to ladder tube.
5. Transfer 750 μL wash buffer to buffer tube.
6. Put the tubes in the designated position.

Sample preparation:

1. Pipette 8 μL sample + 28 μL sample buffer in a 96-well plate.
2. Cover plate and spin down.
3. Heat plate 10 min at 70 °C or 5 min at 100 °C.
4. Centrifuge 5 min at 3000 rcf at room temperature.
5. After cooling, add 140 μL deionized water to denatured sample.
6. Centrifuge for 10 min at 3000 rcf at room temperature.
7. Ensure measurement within 1 h after water addition.
8. Data evaluation with the software of the specific instrumentation.

4 Notes

1. The individual application of these agents is strongly related with the characteristics of the protein, the accompanying proteins, and the aim of the analysis, which cannot be generalized.
2. For subsequent MS analysis, use fresh, previously unused containers for staining; otherwise you may detect false-positive proteins from previous uses.
3. In case of unacceptable high background it is expected that conventional wash steps are insufficient, additional washes should be performed, adding detergent or protein to the

wash buffer if appropriate or soaking for a few minutes between washes.

4. Carbonate/bicarbonate buffer at pH >9 is suggested for antigens with an isoelectric range narrow to this pH value, and alternative buffer systems should be evaluated.
5. For sample matrices, containing yeast extract and peptone, direct quantitative coating is only possible if a pre-dilution of 1:50 or more is applicable. Otherwise only a qualitative analysis can be performed.
6. The selectivity of commercially available antibodies needs to be carefully evaluated prior assay development. The selectivity is often not given in complex matrices.
7. Sandwich ELISAs sometimes require more optimization than traditional ELISAs, but usually the signal-to-noise (S/N) ratio is higher. If a monoclonal and a polyclonal antibody is available, the monoclonal should be coated, and the polyclonal should be used for the detection. The fine-tuning for the antibody selection can be performed with alternative methods, such as electrophoresis combined with Western blot to avoid false positive or negative results.
8. If cross-reactivity is observed, a different blocker should be tested, and if repeated cross-reactivity is observed, it may be advisable to switch to a nonmammalian protein blocker such as salmon serum or a protein-free blocking solution.
9. It needs to be evaluated whether the surfactants affect intramolecular hydrophobic interactions, which hamper correct quantification. For this approach different Tween variants with distinct properties are commercially available.
10. In principle, it is important to ensure that there is no reactivity or cross-reactivity between secondary antibody and coating antibody.
11. The individual time-dependent signal generation needs to be carefully adjusted to the applied standard for quantification and calibration purposes.
12. Fluorescent detection requires the use of an opaque black or white plate. Chemiluminescence detection requires the use of a black or white opaque plate and may also be measured from the top or the bottom.
13. Nickel or copper pre-coated plates can also be used to bind and orient capture antibodies as IgG molecules with histidine-rich sequence in their Fc domain.
14. Pre-coated plates may be stored at room temperature or at 4 °C overnight maximally. Longer periods will reduce OD values.
15. For each HSA ELISA plate, you need a 400 µL HSA standard aliquot, and therefore it is recommended to prepare ready-to-

use aliquots and store them at −20 °C. As described in the manufacturer's protocol, dilute 5 μL of HSA standard (22 mg/mL) in 11 mL dilution buffer, and perform another 1:25 dilution step (e.g., mix 2 mL of the first dilution with 48 mL dilution buffer) to get a HSA concentration of 400 ng/mL.

16. Best results are obtained when samples have a very similar concentration to the standard (400 ng/mL).
17. The incubation time of the TMB substrate depends on the intensity of color development. The highest standard should have an OD value of 2.0–2.5, and the lowest standard should be above background.
18. Standard curve can also be constructed by plotting the mean absorbance for each standard against concentration and drawing a best-fit curve through the obtained points. Linearization can be achieved by plotting the log of HSA concentrations versus the log of OD values, and best-fit line can be determined by regression analysis. This approach provides an adequate but less precise fit of the data.
19. For analytical application it is suggested to inject several μL up to 100 μL.
20. The use of guard columns is advantageous for crude samples but also samples with other complex matrices. They protect altering of the separation material and spend therefore costs and time; however it is essential that the guard columns have no separation capability.
21. The selection of a suitable detector depends on the detectability of the molecule, the choice of mobile phase, as well as the individual sensitivity. It needs to be mentioned that not all detection systems have a linear molecule response, which is important for quantification and validation aspects.
22. The following steps should be kept in mind for proper method development:
    (a) Define method objectives and understand the chemistry.
    (b) Determine the goals of the intended use of the method.
    (c) Understand the chemistry of the analytes and the drug product.
    (d) Develop preliminary HPLC conditions to achieve acceptable separations.
    (e) Develop a suitable sample preparation scheme for the drug product.
    (f) Appropriately standardize and use the relative response factors in calculations.
    (g) Optimize final method for robustness.

(h) Identify the "weaknesses" and optimize the method through experimental design.

(i) Perform method validation.

23. 280 nm is corresponding to absorbance of the aromatic amino acid residues and 214 nm for peptide bonds, respectively. Protein detection at 214 nm is more sensitive, but any organic compounds (any organic salts, amino acids, organic solvents, or detergents present in the running buffer) will also be detected at this absorbance wavelength. Those interference affects the baseline noise but also the quantification.
24. Stepwise performance is important to allow complete pH transition and stabilization of UV baseline. The elution is performed isocratic with 100% buffer B. Injection volumes for standards and samples should range between 20 and 100 µL, depending on sample concentration.
25. There are some aspects which should be considered when selecting the running buffer for SPR, such as avoidance of imidazole or other chelating agents and bivalent metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+}) which can interfere with binding of Ni^{2+} to NTA.
26. When analyzing crude samples, it has to be evaluated, if non-analyte proteins bind to free Ni^{2+} on the chip surface, resulting in unwanted background responses.

References

1. Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98:5301–5317. https://doi.org/10.1007/s00253-014-5732-5
2. Liu Z, Tyo KEJ, Martinez JL et al (2012) Different expression systems for production of recombinant proteins in *Saccharomyces cerevisiae*. Biotechnol Bioeng 109:1259–1268. https://doi.org/10.1002/bit.24409
3. Weinacker D, Rabert C, Zepeda AB et al (2013) Applications of recombinant *Pichia pastoris* in the healthcare industry. Brazilian J Microbiol 44:1043–1048. https://doi.org/10.1590/S1517-83822013000400004
4. Porro D, Sauer M, Branduardi P, Mattanovich D (2005) Recombinant protein production in yeasts. Mol Biotechnol 31:1–15
5. Nielsen KH (2014) Protein expression-yeast. Methods Enzymol 536:133–147. https://doi.org/10.1016/B978-0-12-420070-8.00012-X
6. Garfin DE (2009) Chapter 29: One-dimensional gel electrophoresis. Methods Enzymol 463:497–513. https://doi.org/10.1016/S0076-6879(09)63029-9
7. Garfin DE (1990) One-dimensional gel electrophoresis. Methods Enzymol 182:425–441
8. Nowakowski AB, Wobig WJ, Petering DH (2014) Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. Metallomics 6:1068–1078. https://doi.org/10.1039/c4mt00033a
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
10. Zhou J, Dann GP, Shi T et al (2013) A simple sodium dodecyl sulfate-assisted sample preparation method for LC-MS-based proteomics. Anal Chem 84:2862–2867. https://doi.org/10.1021/ac203394r
11. Goldberg M, Expert-Bezancon N, Vuillard L et al (1996) Non-detergent sulphobetaines: a new class of molecules that facilitate in vitro

protein renaturation. Fold Des 1(1):21–27 Current Biology Ltd ISSN 1359-0278

12. Sundaram RK, Balasubramaniyan N, Sundaram P (2012) Protein stains and applications. Methods Mol Biol 869:451–464. https://doi.org/10.1007/978-1-61779-821-4_39
13. Kurien BT, Scofield RH (2012) A brief review of other notable protein detection methods on acrylamide gels. Methods Mol Biol 869:617–620. https://doi.org/10.1007/978-1-61779-821-4_56
14. Karlinsey JM (2012) Sample introduction techniques for microchip electrophoresis: a review. Anal Chim Acta 725:1–13. https://doi.org/10.1016/j.aca.2012.02.052
15. Amersham™ (2014) Automated Western blotting systems. Protein labeling and detection. Application note 29-1138-92 AB 09/2014
16. Amersham™ (2014) Analysis of therapeutic antibodies using Amersham™. WB system. Protein labeling and detection. Application note 29-1140-27
17. Amersham™ (2014) Quantitative fluorescence Western blot using Amersham™. WB system. Protein labeling and detection. Application note 29-1138-93
18. Engvall E, Perlmann P (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871–874
19. Engvall E, Jonsson K, Perlmann P (1971) Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim Biophys Acta 251:427–434
20. Koehler G, Milstein C (2005) Continuous cultures of fused cells secreting antibody of predefined specificity. J Immunol 174:2453–2465
21. Lequin RM (2005) Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem 51:2415–2418. https://doi.org/10.1373/clinchem.2005.051532
22. Aydin S (2015) A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides 72:4–15. https://doi.org/10.1016/j.peptides.2015.04.012
23. Cheng HM (1996) Tween 20 selectively enhances naturally occurring anticardiolipin antibody binding in ELISA procedures. J Immunol Methods 191:87–88. https://doi.org/10.1016/0022-1759(96)00016-6
24. Cabral AR, Cabiedes J, Alarcón-Segovia D (1994) Tween 20 detaches cardiolipin from ELISA plates and makes anticardiolipin antibodies undetectable regardless of the presence of beta 2-glycoprotein-I. J Immunol Methods 175:107–114
25. Nielsen UB, Geierstanger BH (2004) Multiplexed sandwich assays in microarray format. J Immunol Methods 290:107–120. https://doi.org/10.1016/j.jim.2004.04.012
26. Zhang QY, Chen H, Lin Z, Lin JM (2012) Comparison of chemiluminescence enzyme immunoassay based on magnetic microparticles with traditional colorimetric ELISA for the detection of serum α-fetoprotein. J Pharm Anal 2:130–135. https://doi.org/10.1016/j.jpha.2011.10.001
27. Gao Z, Hou L, Xu M, Tang D (2015) Enhanced colorimetric immunoassay accompanying with enzyme cascade amplification strategy for ultrasensitive detection of low-abundance protein. Sci Rep 4:1–8. https://doi.org/10.1038/srep03966
28. Larsson A, Holmdahl R (1990) A microELISA useful for determination of protein A-binding monoclonal antibodies. Hybridoma 9:289–294. https://doi.org/10.1089/hyb.1990.9.289
29. Maccani A, Landes N, Stadlmayr G et al (2014) *Pichia pastoris* secretes recombinant proteins less efficiently than Chinese hamster ovary cells but allows higher space-time yields for less complex proteins. Biotechnol J 9:526–537. https://doi.org/10.1002/biot.201300305
30. Liu R, Lin Q, Sun Y et al (2009) Expression, purification, and characterization of hepatitis B virus surface antigens (HBsAg) in yeast *Pichia pastoris*. Appl Biochem Biotechnol 158:432–444. https://doi.org/10.1007/s12010-009-8527-x
31. Howard JW, Kay RG, Pleasance S, Creaser CS (2012) UHPLC for the separation of proteins and peptides. Bioanalysis 4:2971–2988. https://doi.org/10.4155/bio.12.283
32. Nováková L, Solichová D, Solich P (2006) Advantages of ultra performance liquid chromatography over high-performance liquid chromatography: comparison of different analytical approaches during analysis of diclofenac gel. J Sep Sci 29:2433–2443
33. Andrés A, Broeckhoven K, Desmet G (2015) Methods for the experimental characterization and analysis of the efficiency and speed of chromatographic columns: a step-by-step tutorial. Anal Chim Acta 894:20–34. https://doi.org/10.1016/j.aca.2015.08.030
34. Vehovec T, Obreza A (2010) Review of operating principle and applications of the

charged aerosol detector. J Chromatogr A 1217:1549–1556. https://doi.org/10.1016/j.chroma.2010.01.007

35. Fekete S, Veuthey JL, Guillarme D (2015) Comparison of the most recent chromatographic approaches applied for fast and high resolution separations: theory and practice. J Chromatogr A 1408:1–14. https://doi.org/10.1016/j.chroma.2015.07.014
36. Gurramkonda C, Polez S, Skoko N et al (2010) Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. Microb Cell Factories 9(31):1–11. https://doi.org/10.1186/1475-2859-9-31
37. Polez S, Origi D, Zahariev S et al (2016) A simplified and efficient process for insulin production in *Pichia pastoris*. PLoS One 11:1–15. https://doi.org/10.1371/journal.pone.0167207
38. Gurramkonda C, Adnan A, Gäbel T et al (2009) Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: Application to intracellular production of Hepatitis B surface antigen. Microb Cell Factories 8(13):1–8. https://doi.org/10.1186/1475-2859-8-13
39. Heo J-H, Won HS, Kang HA et al (2002) Purification of recombinant human epidermal growth factor secreted from the methylotrophic yeast *Hansenula polymorpha*. Protein Expr Purif 24:117–122. https://doi.org/10.1006/prep.2001.1527
40. Ye J, Ly J, Watts K et al (2011) Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. Biotechnol Prog 27:1744–1750. https://doi.org/10.1002/btpr.695
41. Applied Biosystems (2010) POROS A20 Analytical HPLC columns for the Quantitation of Monoclonal antibodies. Application Note 1-13
42. Moore JD, Perez-Pardo MA, Popplewell JF et al (2011) Chemical and biological characterisation of a sensor surface for bioprocess monitoring. Biosens Bioelectron 26:2940–2947. https://doi.org/10.1016/j.bios.2010.11.043
43. Petersen R (2017) Strategies using bio-layer interferometry biosensor technology for vaccine research and development. Biosensors 7 (49):1–15. https://doi.org/10.3390/bios7040049
44. Fortebio P (2009) MAb quantitation: protein A HPLC vs. protein A bio-layer interferometry. Application Note 15:1–21
45. Fortebio P (2009) High sensitivity detection of human IgG using protein A biosensors. Technical Note 15:1–9
46. Gilmore J, Islam M, Martinez-Duarte R (2016) Challenges in the use of compact disc-based centrifugal microfluidics for healthcare diagnostics at the extreme point of care. Micromachines 7:1–26. https://doi.org/10.3390/mi7040052
47. Gyros (2015) Instruction For Use Gyrolab™ huIgG Kit, D0024808/C:1-10
48. Liedberg B, Nylander C, Lundström I (1995) Biosensing with surface plasmon resonance—how it all started. Biosens Bioelectron 10:i–ix
49. Karlsson R, Larsson A (2004) Affinity measurement using surface plasmon resonance. Methods Mol Biol 248:389–415
50. Reinartz HW, Quinn JG, Zänker K, O'Kennedy R (1996) Bispecific multivalent antibody studied by real-time interaction analysis for the development of an antigen-inhibition enzyme-linked immunosorbent assay. Analyst 121:767–771
51. Mcdonnell JM (2001) Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. Curr Opin Chem Biol 5:572–577
52. Nelson RW, Nedelkov D, Tubbs KA (2000) Biosensor chip mass spectrometry: a chip-based proteomics approach. Electrophoresis 21:1155–1163. https://doi.org/10.1002/(SICI)1522-2683(20000401)21:6<1155::AID-ELPS1155>3.0.CO;2-X
53. Hartmann-Petersen R, Gordon C (2005) Quantifying protein-protein interactions in the ubiquitin pathway by surface plasmon resonance. Methods Enzymol 399:164–177. https://doi.org/10.1016/S0076-6879(05)99011-3
54. Hellwig S, Emde F, Raven NPG et al (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. Biotechnol Bioeng 74:344–352. https://doi.org/10.1002/bit.1125
55. GE Healthcare Life Sciences (2008) Sensor Chip NTA. Instruction 22-0519-97:1–11
56. GE Healthcare Life Sciences (2011) Sensor Chip NTA and NTA Reagent Kit Data file 29-0079-27 AA:14
57. Namba Y, Usami M, Suzuki O (1999) Highly sensitive electrochemiluminescence immunoassay using the ruthenium chelate-labeled antibody bound on the magnetic micro beads. Anal Sci 15:1087–1093. https://doi.org/10.2116/analsci.15.1087

58. Sanchez-Carbayo M, Espasa A, Chinchilla V et al (1999) New electrochemiluminescent immunoassay for the determination of CYFRA: analytical evaluation and clinical diagnostic performance in urine samples of patients with bladder cancer. Clin Chem 45:1944–1953
59. Meso Scale Discovery (2012) MULTI-ARRAY® Assay System Human Insulin Kit 17099-v5-2012May:1-15
60. Buhlmann C, Preckel T, Chan S et al (2003) A new tool for routine testing of cellular protein expression: integration of cell staining and analysis of protein expression on a microfluidic chip-based system. J Biomol Techniques 14:119–127
61. Pandey S, Lu CM, Herold DA (2008) Measurement of microalbuminuria using protein chip electrophoresis. Am J Clin Pathol 129:432–438. https://doi.org/10.1309/4JU0XQH62D3YLTGK
62. Caliper Life Sciences (2009) Antibody analysis using Caliper's LabChip GXII system. Application Note 400:1–4
63. Caliper Life Sciences (2009) Automated analysis of proteins using the LabChip 90 System. Application Note 100:1–4