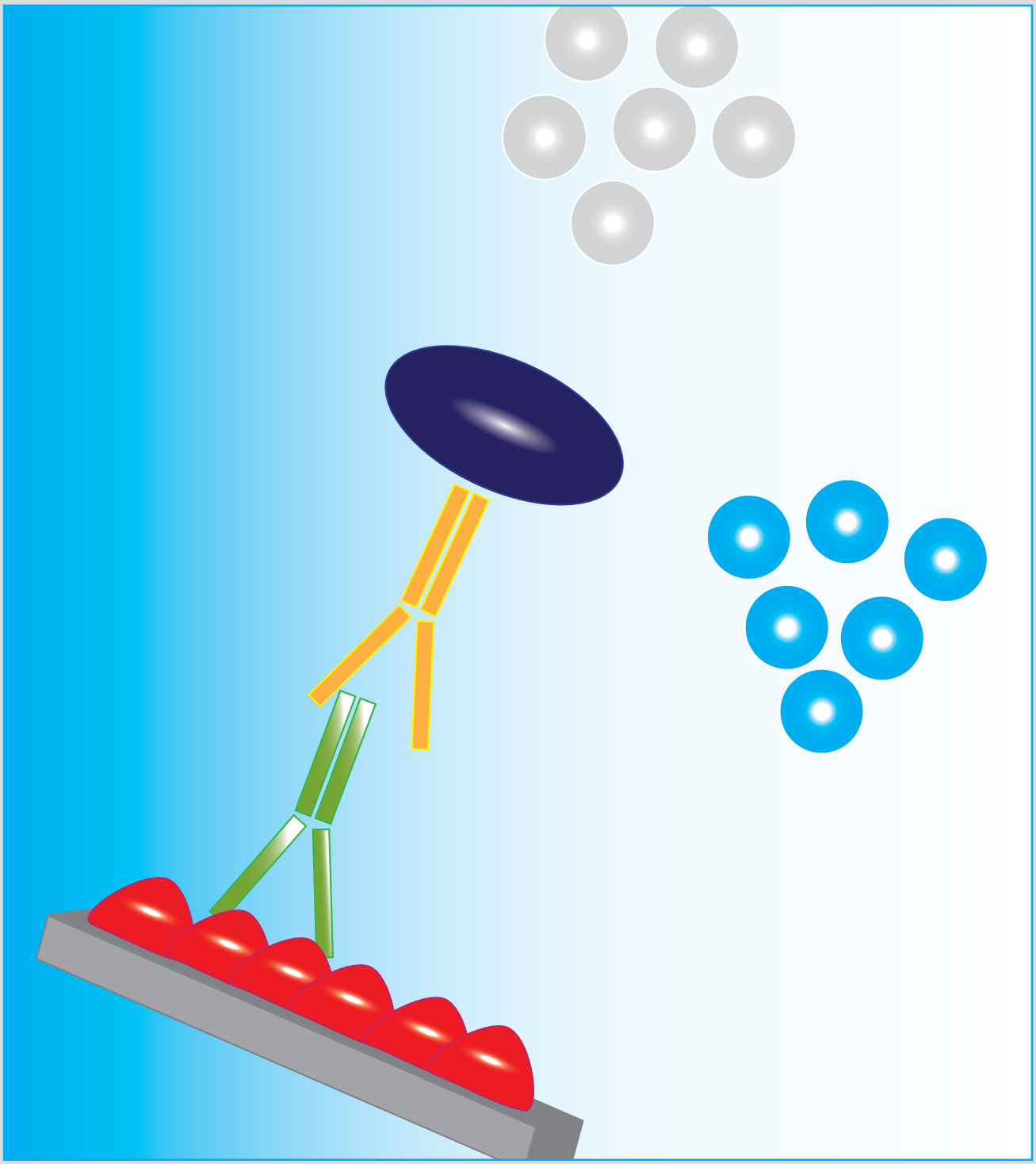
Application Note

Insulin ELISA on high binding MICROLON® 600 and CELLSTAR® microplates



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1 Introduction

ELISA (enzyme-linked immunosorbent assay) is one of the most widely used laboratory techniques in analysis and diagnostics. Analytes such as peptides, proteins, antibodies and hormones can be selectively detected and quantified in small concentrations among a multitude of other substances with relatively low cost and high simplicity.
Although ELISA methodology is used in a wide variety of different types of assays (e.g. direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA), all ELISA variants are based on the same principle: the binding of one assay component – antigen or specific antibody – to a solid surface, and its selective interaction with a subsequently added assay component. Molecules that do not specifically interact with the solid surface bound assay component are washed away during the assay. ELISA is typically carried out in microplates or in tubes (Fig.1).

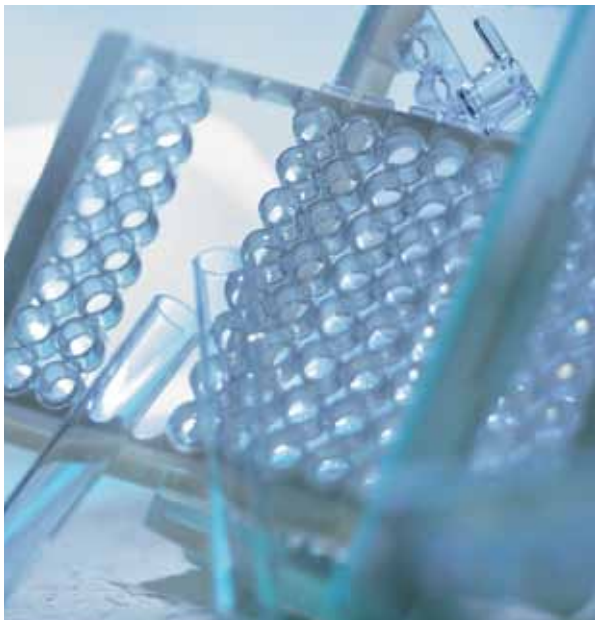

Figure 1: 96 well ELISA strip plate and immuno tubes

This Application Note describes the development of an indirect ELISA for quality assurance applications in our lab. Insulin, a peptide hormone of about 5,8 kDa, is bound to the plate surface and detected using a monoclonal mouse anti-insulin antibody as primary antibody and an anti-mouse-IgG-horseradish peroxidase (HRP) conjugate as secondary antibody (Fig.2). The clinical application of an ELISA based on the binding of insulin to a solid surface is to monitor the presence of insulin-binding antibodies in the sera of patients with type I diabetes mellitus and of patients with administered insulin [1, 2]. An additional aspect of this Application Note is to compare the performance of high binding plates with CELLSTAR® adherent cell culture plates for ELISA methods, as the suitability of CELLSTAR® plates for the purpose of ELISA is a frequently asked question.

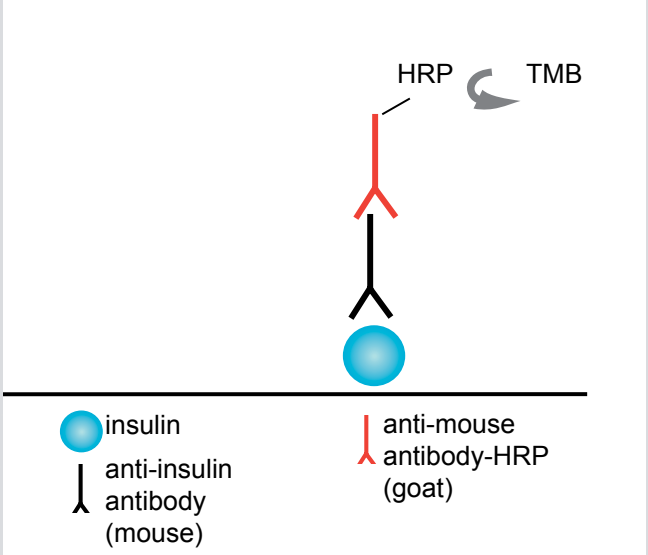

Figure 2: Insulin ELISA: Insulin is coated to the surface of a microplate. A monoclonal mouse anti-insulin antibody as primary antibody interacts with the bound insulin. In a second incubation step a labelled secondary antibody (anti-mouse-IgG-HRP) that recognizes the primary antibody is employed to bind the primary antibody/Insulin complex and enable detection.

2 Material and Methods

2.1 Material and reagents

Item	Supplier	Cat.-No.
Insulin (bovine)	Sigma-Aldrich	I5500
Anti-insulin antibody (mouse)	Sigma-Aldrich	I2018
Anti-mouse-IgG-HRP (goat)	Sigma-Aldrich	A9917
Human IgG	Dianova	009-000-003
Peroxidase AffiniPure Goat anti-human-IgG	Dianova	109-035-003
96 well high binding ELISA (MICROLON® 600) plate	Greiner Bio-One GmbH	655 061
96 well CELLSTAR® plate	Greiner Bio-One GmbH	655 160
Coating buffer (0.05 M carbonate, pH 9.6)	Sigma-Aldrich	C3041
Washing buffer PBS-T (0.14 M NaCl, 0.01 M phosphate, 0.05 % Tween 20)	Sigma-Aldrich	P3563
Blocking buffer (3 % BSA in washing buffer)		
BSA Fraction V	Roth	8076.3
Substrate solution (1 part of TMB to three parts of citric acid/acetate, each 0.1 M, pH 4.9)		
TMB	Sigma-Aldrich	T0440
Citric Acid	Sigma-Aldrich	C0759
Sodium Acetate	Sigma-Aldrich	71183
Sulphuric acid 0.5 M	Roth	K027.1

2.2 Determination of optimal working concentrations

2.2.1 Checkerboard titration of insulin and anti-insulin antibody

A 96 well flat bottom high binding ELISA MICROLON® 600 plate (cat. no. 655 061) was coated with 100 µl per well insulin (0 ng/ml to 8 µg/ml) in coating buffer and incubated overnight. The plate was washed three times (BioTek, Elx405Select) with 350 µl washing buffer per well, blocked with 3 % BSA in washing buffer (150 µl per well) and incubated for 60 min. Anti-insulin antibody in blocking buffer (100 µl per well) was added in a concentration from 19.5 ng/ml to 2.5 µg/ml. After incubation for 1 h and subsequent washing, an anti-mouse antibody horseradish peroxidase-conjugate (anti-mouse-HRP) was added (5 µg/ml in blocking buffer, 100 µl/well). The plate was incubated for 1 h and washed before addition of 100 µl/well substrate solution for a 20 min incubation. The reaction was stopped by adding 100 µl per well 0.5 M sulphuric acid, after which optical density was measured in a Tecan SpectraFluorPlus at 450 nm. All incubation steps were performed at 23 °C in a humidified chamber.

2.2.2 Checkerboard titration of anti-insulin antibody and anti-mouse-IgG-HRP

A 96 well flat bottom high binding ELISA MICROLON® 600 plate (cat.-no. 655 061) was coated with 800 ng/ml insulin and processed as described above (Section 2.2.1). Anti-insulin antibody was added in concentrations from 19.5 ng/ml to 2.5 µg/ml. Anti-mouse-IgG-HRP was added in a concentration from 2.4 ng/ml up to 5 µg/ml.

2.3 Comparison of the performance of a high binding 96 well ELISA MICROLON® 600 plate with a CELLSTAR® plate

Two different assays were performed to compare binding of insulin and IgG within a high binding 96 well flat bottom ELISA MICROLON® 600 plate (cat.-no. 655 061) and a corresponding CELLSTAR® plate for adherent cell culture (cat.-no. 655 160). For the insulin assay, both plate types were coated with 800 ng/ml insulin. For the immunological reactions 80 ng/ml anti-insulin and 300 ng/ml anti-mouse IgG-HRP were used, respectively.
For the IgG assay, both plates were coated with 100 µl/well human IgG (5 µg/ml) in coating buffer. For detection goat anti-human IgG-HRP was added in a concentration of 3.3 ng/ml in blocking buffer. Both assay procedures were conducted according to the insulin assay protocol as described in Section 2.2.1.

3 Results and Discussion

3.1 Determination of optimal working concentrations

3.1.1 Checkerboard titration of insulin and anti-insulin antibody

When establishing a new assay optimal working concentrations for all components must be determined empirically. This is done by performing a so-called checkerboard titration. In such an experiment serial dilutions of two assay components, e.g. coating concentration and primary antibody, are screened in a matrix (Fig. 3).

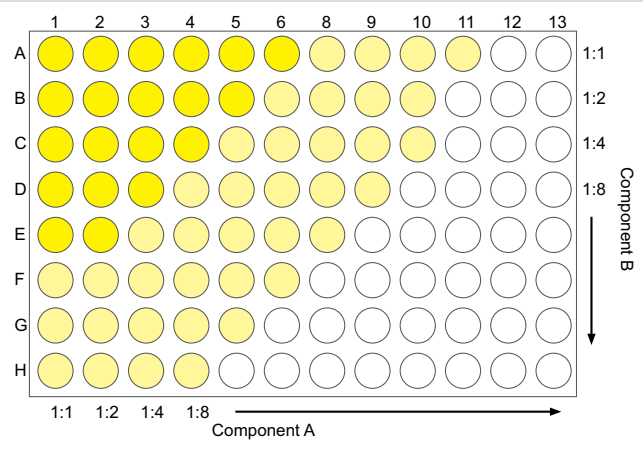

Figure 3: Checkerboard titration

In a first step the insulin coating concentration was titrated against the concentration of the primary (anti-insulin) antibody to determine the optimal coating concentration. Carbonate-buffer was used for coating as described in the literature [1, 2]. In case the ideal coating buffer is not known it is recommendable to try coating buffers with differing pH-values, e.g. carbonate buffer at pH 9.6, phosphate buffer at pH 7.4 and acetate-buffer at pH 5.5 as some proteins or peptides bind better to the plastic surface at neutral or acidic pH [3]. The plates were incubated overnight at room temperature in a humidified chamber. Incubation in a humidified chamber prevents the formation of a temperature gradient and water evaporation within the plate which may result in a shift of OD values between outer and central wells. Alternatively the plates can be covered with a sealing tape (EASYseal™, cat.-no. 676 001). Incubation times and temperature were adopted from a protocol already established in our lab. Even though incubation time and temperature can be optimized to improve assay performance, because existing protocol incubation times and temperature worked well to achieve results, these parameters were not modified.

After coating, the plates were blocked with 150 µl/well of 3 % BSA in washing buffer. It is essential to block any remaining unoccupied binding sites. Blocking with an appropriate blocking reagent prevents non-specific binding of assay components in a later step of the assay that could lead to increased background signals. BSA is the most commonly used reagent for blocking. Alternative blocking reagents are e.g. gelatine, casein or commercially available solutions. After blocking, the primary antibody – a monoclonal mouse anti-insulin antibody – was added in a concentration ranging from 19.5 ng/ml to 2.5 µg/ml.

The plates were washed with a washing buffer containing the detergent Tween 20. The addition of a detergent enhances the removal of non-specifically bound antibody molecules and therefore helps to minimise background.

The secondary antibody, an anti-mouse-IgG-HRP conjugate, was added in excess to eliminate any influence on the test results. A colorimetric detection was performed using 3, 3', 5, 5' tetramethylbenzidine (TMB) as the substrate. Another frequently used enzyme label for colorimetric assay is alkaline phosphatase (AP) with p-nitrophenyl phosphate (pNPP) as the substrate.

The enzymatic reaction was stopped after 20 minutes by inactivating HRP with sulphuric acid. The optical density at 450 nm was plotted against the serial dilution of insulin resulting in a saturation curve showing the maximum concentration of insulin bound to the plate surface under the chosen conditions (Fig. 4). Because saturation of the high binding plate surface occurred at approximately 800 ng/ml, this value was chosen as coating concentration for further proceedings.

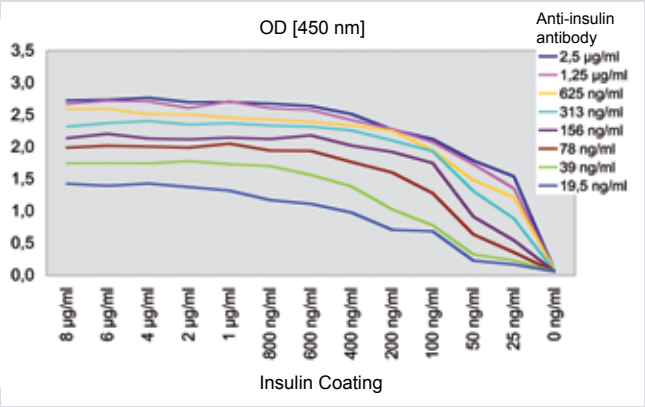

Figure 4: Checkerboard titration of the insulin coating concentration against the anti-insulin antibody concentration

3.1.2 Checkerboard titration of anti-insulin antibody and anti-mouse-IgG-HRP

In a second step the high binding microplate was coated with 800 ng/ml insulin and the primary antibody (anti-insulin antibody) concentration was screened against the concentration of the secondary antibody (anti-mouse-IgG-HRP) to determine appropriate antibody concentrations. The criteria for a suitable combination were (1) an optical density of about 0.8 to 1.5 units, (2) a high signal to noise ratio, and (3) the location in the linear part of the curve to assure assay sensitivity. A combination of an anti-insulin concentration of 80 ng/ml and an anti-mouse-IgG-HRP concentration of 300 ng/ml was chosen for further experiments (Fig. 5). The resulting protocol is shown in Fig.6.

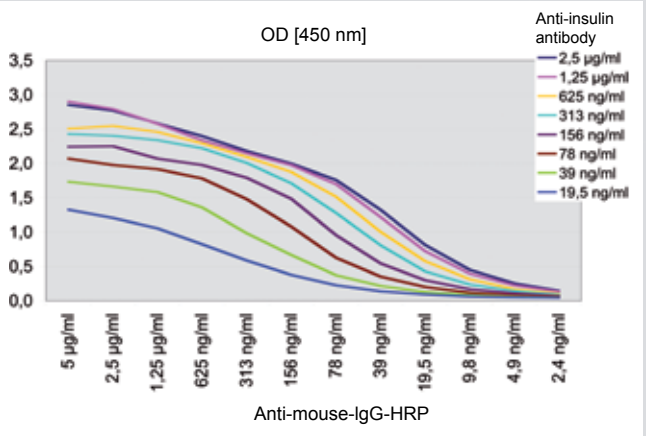

Figure 5: Checkerboard titration of the anti-insulin antibody concentration against anti-mouse-IgG-HRP

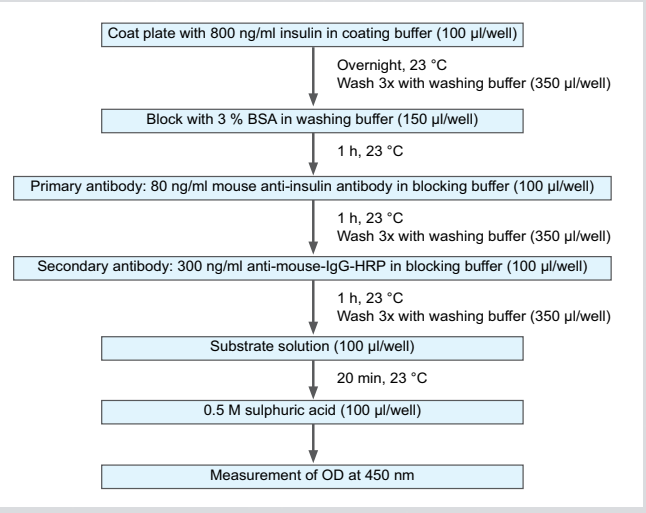

Figure 6: Insulin ELISA protocol

3.2 Comparison of the performance of a high binding ELISA MICROLON® 600 plate and a CELLSTAR® plate

The attachment of biomolecules to a solid surface is based upon passive adsorption. Therefore physicochemical forces such as hydrophobic bonds, hydrophilic interactions and H-bonding are relevant. Polystyrene, the most widely used material for immunological microplates, has hydrophobic characteristics. Because for most ELISA applications the presence of hydrophilic groups is beneficial, high binding ELISA MICROLON® 600 plates are physically treated to introduce a defined number of hydrophilic groups.
CELLSTAR® plates, with intended application for adherent cell culture, undergo a different kind of physical treatment to improve cell adhesion. This type of treatment renders the plate surface more hydrophilic, similar to the treatment of high binding plates, but the number of polar groups introduced on the CELLSTAR® surface is much higher than on a high binding surface intended for ELISA application. As the usability of CELLSTAR® plates for ELISA is a frequently asked question, high binding ELISA and CELLSTAR® plates were compared with the insulin assay described above as well as another assay based on the adsorption of human IgG to the plate surface.

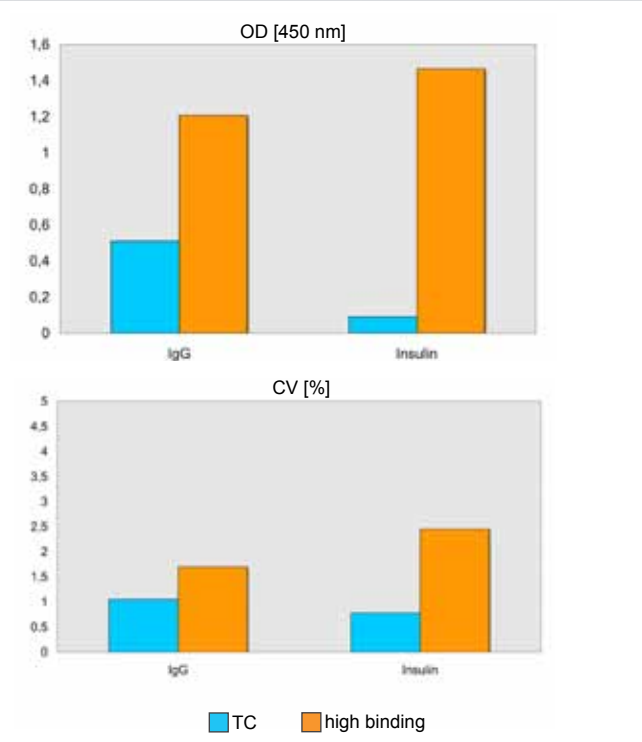

Figure 7: Optical density values and coefficients of variations for the insulin and the IgG ELISA on high binding and CELLSTAR® microplates.

Both IgG and insulin bound very well on the high binding ELISA MICROLON® 600 surface (Fig. 7). However, insulin did not bind significantly to the CELLSTAR® surface, resulting in a very low OD value, and the binding of IgG to the CELLSTAR® surface was much weaker in comparison to the high binding ELISA MICROLON® 600 surface. Coefficients of variation (CV) for both assays were well below 5 % in each plate type. Non-specific binding on the CELLSTAR® surface, as is often discussed for tissue culture plates, was not detected under the tested conditions (data not shown).
These results demonstrate that the performance of ELISA applications on the CELLSTAR® surface is highly dependent on the nature of the molecule being bound to the plate. It is likely the high charge density on the CELLSTAR® surface is not beneficial for binding of insulin and IgG, but rather prevents binding by rejection. This effect is more pronounced with the smaller insulin molecule than with the significantly larger IgG molecule.
It is important to note that, due to intended application for use, CELLSTAR® plates are not subject to the same quality assurance procedure that is implemented for immunological plates. For production of Greiner Bio-One immunological products, raw material batches are checked as a part of an integrated quality control process to ensure specific criteria are satisfied prior to the release of the finished goods. To guarantee consistent binding properties the ELISA products are continuously monitored for inter and intra plate homogeneity. Quality testing is performed to assure that the coefficient of variation in one plate does not exceed 5 % for colorimetric assays and the ratio of OD values of new plates to reference plates is in the range of 100 +/- 10 %. As CELLSTAR® plates have a designated intent for cell culture, the quality control procedures for these products are vastly different and involve comprehensive monitoring and testing surrounding that intended purpose. In addition to the superior comparative results achieved using ELISA vs. CELLSTAR® plates within this Application Note, for the quality testing reasons outlined above ELISA plates are infinitely preferable over CELLSTAR® plates for ELISA applications.

4 Literature

[1] Wilkin T. et al. (1984), A Micro Enzyme-Linked Immunosorbent Assay for Insulin Antibodies in Serum, Journal of Immunological Methods, 76: 185-194

[2] Kobayashi N. et al. (1985), A Solid-Phase Enzyme Immunoassay for Anti-Insulin Antibody in Diabetes Mellitus Patiens, Journal of Immunological Methods, 84: 245-250

[3] Raem A.M., Rauch P. (2007) Immunoassays, Spektrum Akademischer Verlag

5 Ordering Information

Cat.-No.	Description	Qty./ bag	Qty./ case
655 001	Microplate, 96 well, PS, F-bottom/standard, MICROLON® 200, med. binding, clear	5	40
655 061	Microplate, 96 well, PS, F-bottom/standard, MICROLON® 600, high binding, clear	5	40
655 080	Microplate, 96 well, PS, F-bottom/chimney well, MICROLON® 200, med. binding, clear	5	40
655 081	Microplate, 96 well, PS, F-bottom/chimney well, MICROLON® 600, high binding, clear	5	40
675 001	Microplate, 96 well, PS, half area, MICROLON® 200, med. binding, clear	10	40
675 061	Microplate, 96 well, PS, half area, MICROLON® 600, high binding, clear	10	40
701 070	96 well strip plate, 8 x F12 strips, F-bottom, MICROLON® 200, med. binding, clear	5	100
701 071	96 well strip plate, 8 x F12 strips, F-bottom, MICROLON® 600, high binding, clear	5	100
705 070	Single-break strip plate, 12 x C8 strips. PS, C-bottom, MICROLON® 200, med. binding, clear	5	100
705 071	Single-break strip plate, 12 x C8 strips. PS, C-bottom, MICROLON® 600, high binding, clear	5	100

Please note this is only an extract of immunological products. You can find more information in our catalogue or on our website: www.gbo.com/bioscience.

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