Application Note

Influence of coating buffer and incubation conditions on ELISA performance

greiner bio-one

1. Introduction

ELISA (Enzyme-Linked Immunosorbent Assay) is one of the most widely used techniques in both basic immunology research and diagnostic analyses. Because ELISA enables peptides, proteins, antibodies and hormones to be selectively detected in small concentrations among a multitude of other substances with relatively low cost and high simplicity, the method provides an important and useful tool for disease monitoring, diagnostics and doping tests, as well as environmental and food analytics. ELISA methods yield both sensitive and accurate results, and employment of automated handling with a microplate platform allows rapid conduction of tests in a high-throughput manner.

Although ELISA methodology is used in a wide variety of different assays (e.g. direct, indirect, sandwich, competitive), all variants are based on the same principle: the binding of one assay component – antigen or specific antibody – to a solid surface by passive adsorption, and its selective interaction with subsequently added assay components. Molecules that do not specifically interact with the component bound to the solid surface are washed away during the assay process.

The specific binding of antigen and antibody can then be visualised via an appropriate detection system (e.g. radioactive, colorimetric, fluorescence, or luminescence) either by direct labelling or indirectly with an enzyme conjugate. In the latter case the assay is developed by adding an enzymatic substrate to produce a measurable signal. The strength of the signal is correlated to the amount of analyte present within the sample, enabling a means of quantitation.

Two enzyme labels that remain in widespread use today are horseradish peroxidase (HRP) and alkaline phosphatase (AP). When conjoined with a chemical substrate, a colorimetric reaction is produced and the resultant color can be measured by means of absorbance or optical density readings taken within a spectrophotometer.

There are a number of key elements to be considered when developing an ELISA: the nature and size of the analyte, number and localisation of epitopes, binding properties to the solid surface, and which or how many antibodies are available for use. Other factors that can have direct bearing on achieved results are substrate concentration, buffer composition and incubation conditions.

This application note discusses the influence of different coating buffers and incubation conditions on ELISA performance using three exemplary assays established in our laboratory:

(1) Biotin-labelled insulin (insulin-biotin) was bound by adsorption on the surface of a high binding microplate and detected with a streptavidin-horseradish peroxidase conjugate (Fig. 1). This assay is based on the specific non-covalent binding of biotin and streptavidin. The interaction between the 52.8 kDa protein streptavidin from *Streptomyces avidinii* and the vitamin biotin with a dissociation constant in the order of approx. 10-14 [1] is one of the strongest non-covalent bindings known in nature.

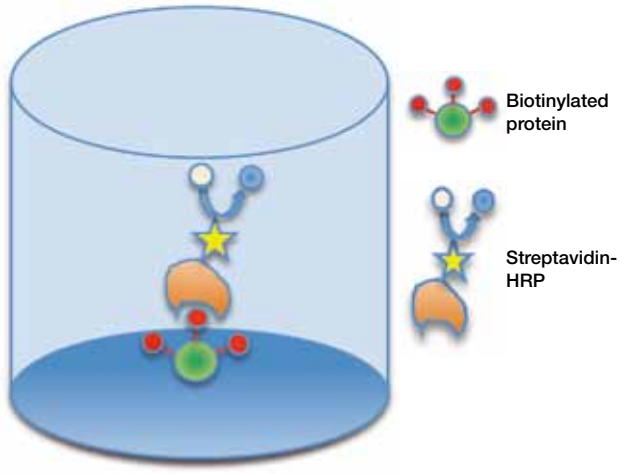

Figure 1: Biotin-Insulin assay. Biotin-labelled insulin is coated to the surface of a high binding microplate. A streptavidin-HRP conjugate interacts with the bound insulin-biotin and enables detection.

(2) The 10 AA peptide **Strep-tag II** was coated to the plate surface of a high binding microplate and detected with a StrepTactin® HRP conjugate (Fig. 2). The Strep-tag II peptide is usually used as affinity tag for the expression and purification of recombinant fusion proteins. Strep-Tactin® is a variation of streptavidin optimised for the binding of Strep-tag II fusion proteins [2].

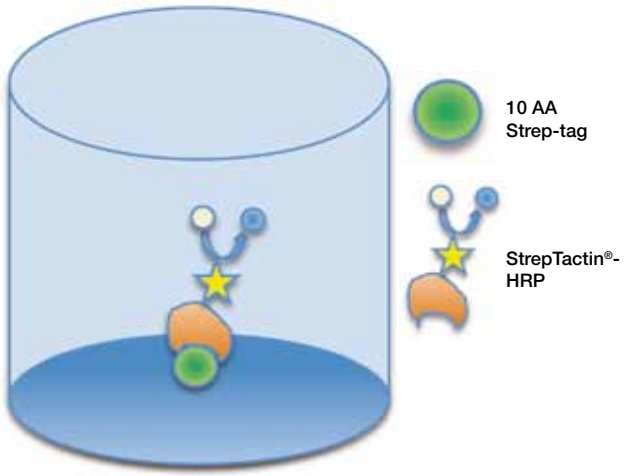

Figure 2: Strep-tag II assay. A high binding microplate is coated with Strep-tag II. In a second incubation step a Strep-Tactin® HRP conjugate binds to the Strep-tag II peptide and enables detection.

(3) Insulin, a peptide hormone of about 5,8 kDa, was 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. 3).

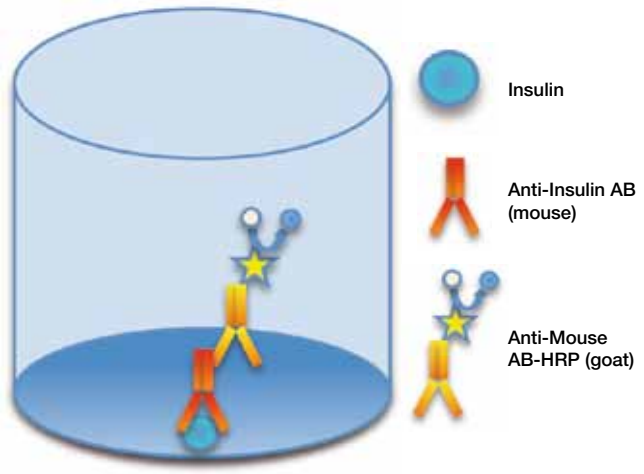

Figure 3: Insulin ELISA. In an indirect ELISA, insulin is coated to the surface of a microplate. A monoclonal mouse anti-insulin antibody as primary antibody interacts with the bound insulin. In a second incubation step a labelled secondary antibody (anti-mouse-IgG-HRP) that recognises the primary antibody is employed to bind the primary antibody-insulin complex and enables detection.

2. Material and Methods

2.1 Material and Reagents

Item	Supplier	Cat.-No.
Strep-tag II peptide	IBA GmbH	2-1018-002
Strep-Tactin®-HRP	IBA GmbH	2-1502-001
Insulin-biotin	Sigma-Aldrich	I2258-2MG
Streptavidin-HRP	Sigma-Aldrich	S5512-1MG
Insulin (bovine)	Sigma-Aldrich	I5500
Anti-insulin antibody (mouse)	Sigma-Aldrich	I2018
Anti-mouse-IgG-HRP (goat)	Sigma-Aldrich	A9917
EASYseal™ sealing tape	Greiner Bio-One GmbH	676 001
96 well F8 Strip high binding ELISA (MICROLON® 600) microplate	Greiner Bio-One GmbH	762 071
Carbonate coating buffer pH 9.6 (0.05 M carbonate, pH 9.6)	Sigma-Aldrich	C3041
PBS coating buffer pH 7.4	Biochrom	L1825
Acetate coating buffer pH 5.5 (0.2 M Na-Acetate, pH 5.5)		
Washing buffer PBS-T (0.14M 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 Influence of coating buffer on binding

Two assays were established to investigate the influence of the coating buffer on binding properties to the high binding surface. For the insulin-biotin assay, a 96 well F8 Strip high binding ELISA microplate (Cat.-No. 762 071) was coated with 100 µl per well biotin-labelled insulin (serial dilution from 1 mg/ml to 0 mg/ml) in carbonate, PBS or acetate coating buffer, respectively, and incubated overnight. The plate was washed three times with 350 µl per well washing buffer (BioTek, Elx405Select), blocked with 150 µl blocking buffer per well and incubated for one hour. Blocking buffer was removed and 100 µl per well streptavidin-HRP (8 ng/ml) in blocking buffer was added. After incubation for one hour and subsequent washing, 100 µl per well substrate solution was added. The reaction was stopped by adding 100 µl per well 0.5 M sulphuric acid. The optical density was measured in a Tecan SpectraFluorPlus at 450 nm. All incubation steps were performed at 23 °C in a humidified chamber.

The Strep-tag II assay was performed according to the insulin-biotin assay procedure. Coating was done with Strep-tag II peptide (serial dilution from 2 mg/ml to 0 mg/ml) instead of biotin-labelled insulin. For detection a 1:10.000 dilution of Strep-Tactin®- HRP in blocking buffer was used.

2.3 Influence of incubation conditions on assay performance

The influence of temperature and incubation conditions on assay performance was studied using an insulin ELISA established in our lab [3]. 96 well F8 high binding ELISA strip plates (Cat.-No. 762 071) were coated with 100 µl per well insulin (800 ng/ml) in carbonate coating buffer and incubated overnight. The plates were blocked with 3 % BSA in washing buffer (150 µl per well) and incubated for one hour. After removal of the blocking buffer 80 ng/ml anti-insulin antibody in washing buffer (100 µl per well) was added. After incubation for one hour and subsequent washing, an anti-mouse-IgG-HRP secondary antibody was added (300 ng/ml in washing buffer, 100 µl/well). The plate was incubated for one hour 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.

For all incubation steps the plates were either stored stacked (5 plates per stack) on the lab bench at room temperature (approx. 20 °C) or in a humidified chamber at 23 °C. The upper most plates of the stacks were each sealed with an EASYseal™ sealing tape. Incubation with the substrate solution was done for all plates non-stacked, without a sealing tape and in a humidified chamber at 23 °C.

3. Results and discussion

3.1 Influence of coating buffer on binding

During the coating procedure ionic strength and pH value of the buffer can play an important role. The most widely used coating buffer for ELISA is 0.05 M Carbonate buffer at pH 9.6. Nevertheless, for some molecules a different pH value may result in a more favourable structure and charge for an adsorptive binding to the microplate surface. Alternative coating buffers are 10 mM PBS at pH 7.4 and acetate buffer at pH 5.5 [4]. PBS is often used in sandwich ELISA applications to coat the capture antibody to the plate surface. Cuvelier *et al.* describe the positive effect of substituting carbonate by acetate buffer for some IgG species for coating in sandwich ELISA [5].

The influence of different coating buffers was first tested with an assay where biotinylated insulin was bound to the plate surface and then detected with a streptavidin-HRP conjugate. With this assay no significant impact of the coating buffer was detectable (Fig. 4).

In a second assay the 10 amino acid (AA) peptide Strep-tag II was bound by passive adsorption to the surface and detected with a StrepTactin®-HRP conjugate. Adsorptive binding of small molecules like a 10 AA peptide is generally difficult to achieve [6]. Often direct binding is not possible and instead a sandwich or a competitive ELISA format, which involves the initial binding of a capture antibody to the plate surface, is applied. Biotin labelling of the molecule and subsequent binding to a streptavidin coated surface is another alternative. Results from this experiment did not exhibit significant binding of Strep-tag II to the plate surface when carbonate or acetate coating buffer were used. A dramatic increase in binding was apparent when PBS buffer was employed (Fig. 5), demonstrating that the choice of coating buffer can have a major influence on binding properties.

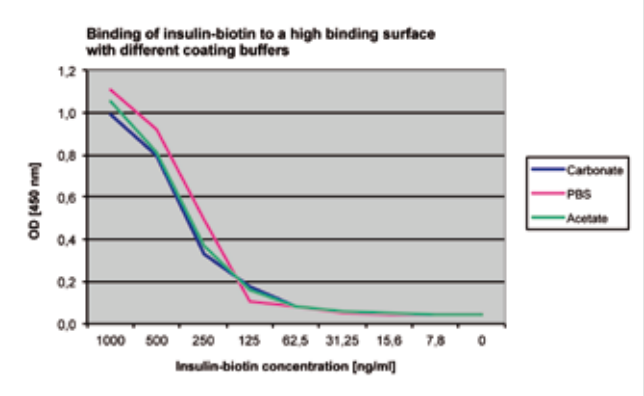

Figure 4: Choice of coating buffer has no influence on the binding of biotinylated insulin to the surface of a high binding F8 strip plate

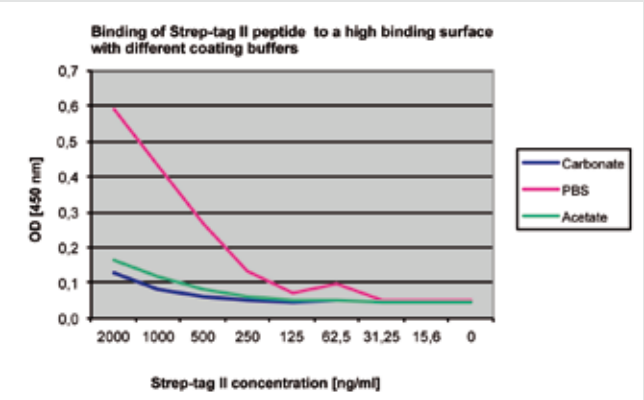

Figure 5: Usage of PBS buffer enhances binding of the Strep-tag II peptide significantly in comparison to the usage of carbonate or acetate as coating buffer.

3.2 Influence of incubation conditions

Adsorptive binding of molecules to a solid surface, the specific interaction between antigen and antibody or primary and secondary antibody, as well as the enzyme-substrate reaction, are kinetic reactions and therefore sensitive to incubation time and temperature. Hence, coating is often done overnight to allow maximum saturation of the available binding sites on the plate surface. For the ELISA steps subsequent to coating, shorter incubation times are frequently used. In this context consideration should be given that incubation temperature has a major influence on the resulting signal intensity. In general, a higher incubation temperature with a consistent incubation time will result in higher OD values. Therefore, an increase of incubation temperature will allow a shorter incubation time to achieve the same signal intensity as those obtained with longer incubations at lower temperatures. Incubation temperatures can typically range from 4 °C to room temperature and even be as high as 37 °C. When selecting the appropriate incubation temperature, it is imperative to take into account the temperature stability of the molecules involved. In the depicted insulin ELISA, an increase of incubation temperature from about 20 °C (RT) to 23 °C for the incubation steps with the primary and secondary antibody resulted in an increase of OD values from about 0.87 to 1.08 units (Fig. 6).

Not only does temperature play an important role for signal intensity, it can also, besides evaporation, significantly affect consistency of ELISA results. A temperature and evaporation gradient between the inner and outer wells of a microplate may result in a significant difference in standard readings between outer and central wells, known as edge effect [6]. The edge effect is based on the fact that a temperature adaption is taking place more rapidly in outer vs. inner microplate wells. This effect is often enhanced if non-covered microplates are stored in stacked formation, when, in addition to the temperature gradient, a gradient caused by evaporation from the inner to the outer area of a microplate may become relevant. For this reason, the edge effect is most pronounced when non-covered microplates are stored stacked in an environment lacking temperature and humidity control. To overcome edge effect, use of an appropriate sealing tape like EASYseal™ (Fig. 7), storage of openly stacked plates within a humidified chamber (Fig. 8), or implementation of a storage system with temperature and humidity control can dramatically reduce data inconsistency.

When developing and performing Enzyme-Linked Immunosorbent Assays, there are a multitude of parameters that should be considered to achieve optimal accuracy, reliability and sensitivity of the test. Optimisation of coating buffer and incubation conditions, such as temperature, incubation time and environment, are fundamental steps to establish an ELISA successfully.

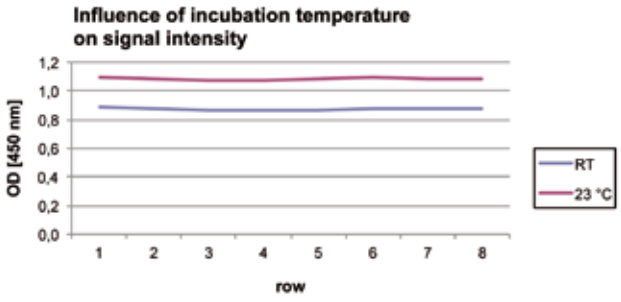

Figure 6: An increase in incubation temperature from about 20 °C up to 23 °C results in significantly higher OD values with an identical experimental set-up. The graph shows mean OD values of microplate wells per row. Microplates were sealed with EASYseal™ for incubation.

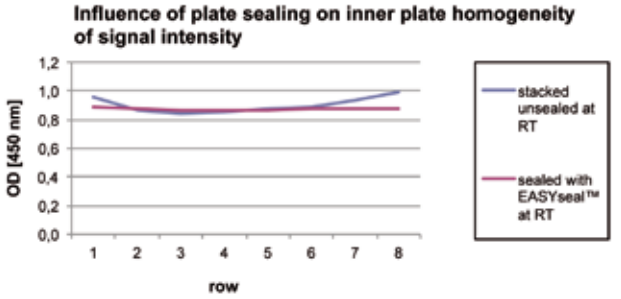

Figure 7: Sealing of microplates during incubation steps with primary and secondary antibody in an environment without humidity control prevents the edge effect. The graph displays mean OD values of microplate wells per row.

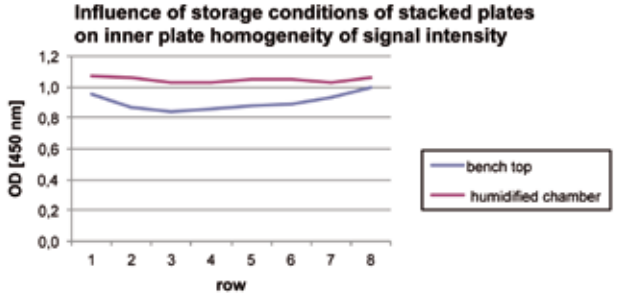

Figure 8: Storage of openly stacked microplates during incubation with primary and secondary antibody in a humidified chamber significantly reduces differences in OD values due to the edge effect. The graph depicts mean OD values of microplate wells per row.

4. Literature

[1] Green,N.M. 1975. Avidin. Adv. Protein Chem. 29 pp 85-133

[2] Voss S., Skerra A. (1997) Mutagenesis of a flexible loop in streptavidin leads to higher affinity for the Strep-tag II peptide and improved performance in recombinant protein purification. Protein Engineering vol.10 no.8 pp.975–982.

[3] Application Note Greiner Bio-One: „Insulin ELISA on high binding MICROLON® 600 and CELLSTAR® microplates“ (Cat.-No. F073 106) www.gbo.com/bioscience/application_notes

[4] Raem A. M, Rauch P (2007) Immunoassays. Spektrum Akademischer Verlag, Elsevier GmbH, München.

[5] Cuvelier A. et al. (1996) Substitution of carbonate by acetate buffer for IgG coating in sandwich ELISA. J Immunoassay. 17(4):371-82.

[6] Gosling J.P. (2000) Immunoassays. Practical approach. Oxford University Press, Oxford.

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
762 070	96 well strip plate, 12 x F8 strips, F-bottom, MICROLON® 200, med. binding, clear	5	100
762 071	96 well strip plate, 12 x F8 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

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Revision: July 2012 - F073 118

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