

Immunoglobulin detection

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Today, more than 100 years since their discovery, it is difficult to overestimate the role of antibodies not only as a crucial part of our immune system but also as one of the most important and versatile tools in bioscience and biomedicine. With the 1984 Nobel Prize-awarded technique for making monoclonal antibodies, the use of antibodies has exploded and today they represent an integral part of most types of immunoassays.

To facilitate studies involving the use of antibodies, also referred to as immunoglobulins (Ig), we offer a series of carefully evaluated anti-Ig reagents, which allow detection and quantification of total as well as antigen-specific antibodies in a variety of situations. For example and as further described below, determination of antigen-specific antibodies in serum, plasma or cell culture supernatants can be measured by ELISA while the ELISpot and FluoroSpot techniques allow similar investigations being made at the single cell level. Access to isotype- as well as subclass-specific antibodies also enables a more qualitative analysis of the type of reactive antibodies.

ELISA and other immunoassays

The enzyme-linked immunosorbent assay (ELISA), developed in the early 1970's, remains the prototype for most immunoassays and is widely used in different variants in research and clinical laboratories and by manufacturers developing *in vitro* diagnostic

products. Whereas originally performed in test tubes, the assay today typically uses multiwell ELISA plates with 96 or 384 wells which allow simultaneous testing of multiple samples.

A standard ELISA application is where a sample (e.g. serum or plasma) is tested for the presence of specific antibodies using plates to which an antigen has been immobilized (Figure 1A). Binding of antibody to the antigen is then revealed by a secondary anti-Ig detection antibody that may be enzyme-labeled or biotinylated, the latter requiring an additional step with Streptavidin-enzyme. This is a format commonly used in diagnostic assays and can serve to e.g. confirm infections or allergies using microbial antigens or specific allergens, respectively.

Depending on the situation, the secondary antibodies can be broadly reactive to allow detection of all antigen-binding antibodies in a sample or be isotype-specific. For instance, separate analysis of IgG and IgM may give information about the stage of an infection with IgM often being the more prevalent antibody during early infection whereas allergies are typically detected using anti-IgE antibodies. More detailed information about the type of Ig response is provided by using IgG subclass-specific detection antibodies (as exemplified in Figure 2), which can also be of clinical relevance.

Anti-Ig antibodies can also be employed to measure the total amount of antibodies in a sample using sandwich ELISA (Figure 1B). Here a capture anti-Ig antibody is combined with an enzyme- or biotin-labeled detection antibody and depending on the

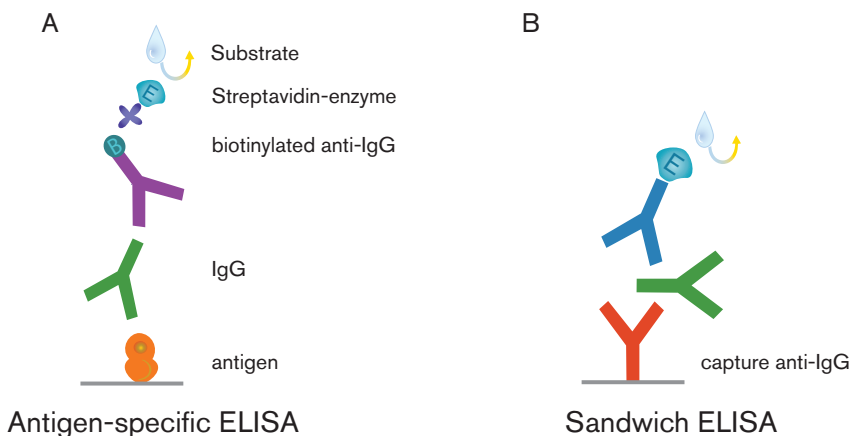


Figure 1. Ig ELISA assay principle. Antigen-specific antibodies can be detected in an antigen-specific ELISA where the antigen is coated to the ELISA plate (A). The total amount of Ig can be quantified in a sandwich ELISA by using Ig-specific antibodies for capture and detection (B). Detection antibodies directly conjugated to an enzyme can be used for detection of total IgG, IgA and IgM, while detection of total IgE is best performed using biotinylated detection antibody together with Streptavidin-enzyme.

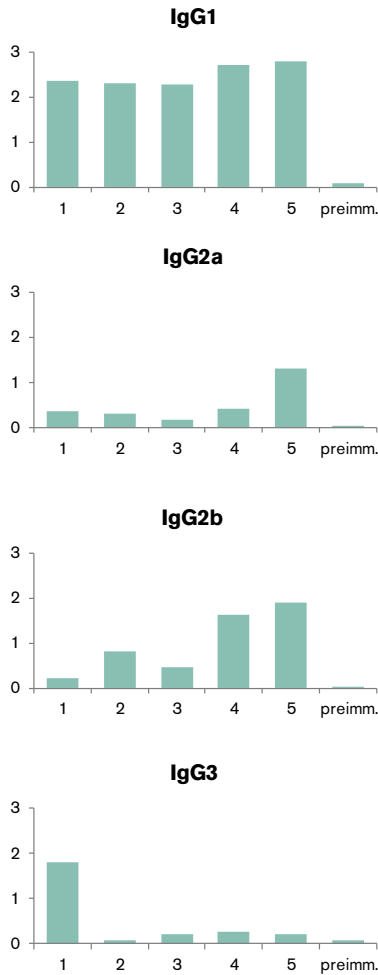


Figure 2. Ag-specific IgG-subclass ELISA. Antigen-specific IgG1, IgG2a, IgG2b and IgG3 antibodies in serum from five immunized mice. Human IL-2 was used as antigen.

specificity of the antibodies, the test will allow the determination of a specific isotype or IgG subclass. This type of assay can be used for establishing the antibody content in basically any type of sample, and is used both in research and for clinical assessment of immunodeficiencies characterized by a low production of one or more immunoglobulins.

In addition to the many variants of ELISA, there are a multitude of other immunoassays designed to measure antibody reactivity. Many of these are in a similar way dependent on the availability of suitable anti-Ig reagents for detection. These include immunoblotting (also referred to as western blot), immunostaining of cells or tissue sections as well as different types of microarray assays in which a sample is tested against multiple antigens immobilized to a solid matrix.

Immunoglobulin ELISA:

- Quantify antibodies in samples
- Determine antigen-specific antibodies
- Reveal details of immune responses by determining antibody subclasses

B-cell ELISpot

In contrast to the many assays designed to measure antibody reactivity in solutions, only a few focus directly on the antibody-secreting cell (ASC). One of these is the B-cell ELISpot, first described in 1983^{1,2}. With this method one can, in a highly sensitive manner, identify and enumerate both the total number of ASC in a sample and those secreting antibodies to a specific antigen (Figure 3). For example, it has been used to demonstrate

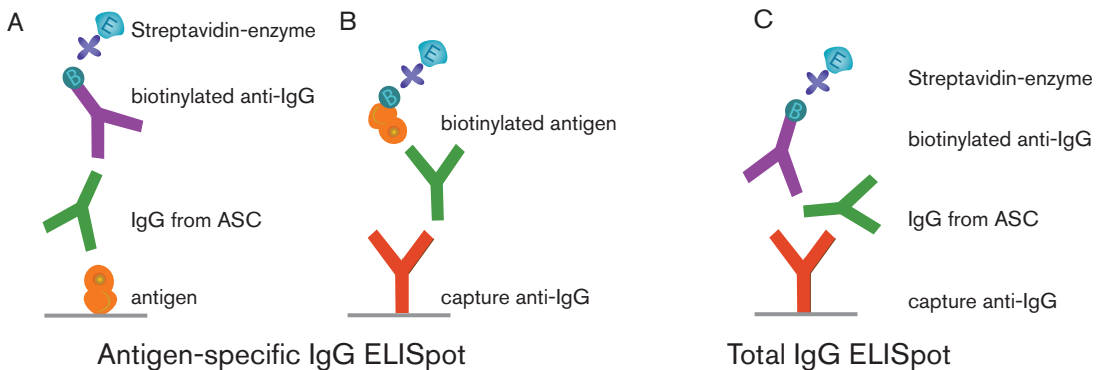


Figure 3. Ig ELISpot. Schematic illustration of the two ways of making antigen-specific B-cell ELISpot using antigen for coating (A) or biotinylated antigen for detection (B). In the example, analysis is restricted to IgG-producing cells. Total Ig ELISpot is depicted in (C). IgG is used as an example in this figure and analysis of other isotypes or IgG subclasses can be done in a similar way.

the presence and frequencies of long-term memory B cells in the blood ³, information not easily obtained using other methods.

To meet the need for optimized and validated reagents we have developed ELISpot kits to be used for analyzing B cells secreting antibodies of different isotypes in human, monkey and mouse samples as well as systems for detecting antigen-specific ASC of different IgG subclasses.

Moreover, we have established an improved protocol in which the antigen is used for detection instead of immobilized to the ELISpot plate as in the conventional assay.

Applications

The B-cell ELISpot represents a powerful tool to analyze various aspects of the antibody immune response. It may be particularly suitable in situations where a high degree of sensitivity is required or when the response is best studied at the cellular level ^{4,5}.

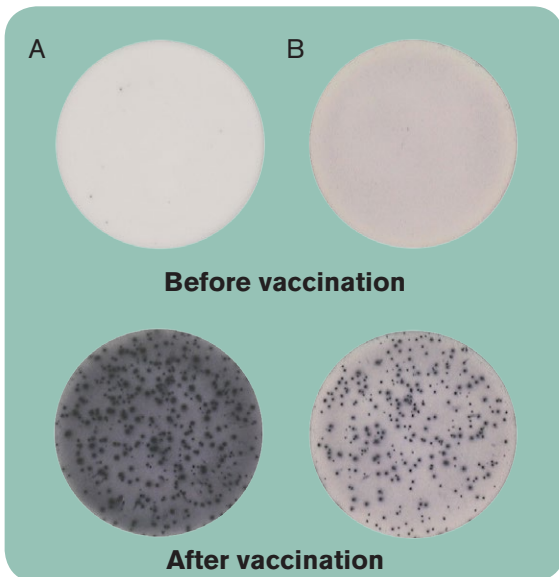


Figure 4. Swine flu-specific IgG ELISpot. Human PBMC were collected before and seven days after vaccination with the intramuscularly administered influenza vaccine Pandemrix®. Ag-specific IgG-producing cells were analyzed using Pandemrix® at 7.5 µg/well for coating (A) or at 0.03 µg/well as a biotinylated detection reagent (B).

Two major application areas have been in the detection of B-cell responses to natural infections and those elicited by vaccination ⁶⁻⁸. The assay can be used for analyzing B cells in peripheral blood as well as B cells present in various lymphoid tissues and other sites harboring immune cells such as mucosal layers and synovial fluid. For instance, it has been used to determine the distribution of antigen-specific B cells in lymphoid tissues after vaccination and to demonstrate the primary confinement of long-lived memory B cells to the spleen ^{9,10}.

Two ways to perform the B-cell ELISpot

Since first described, the B-cell ELISpot has been performed in essentially the same way with only minor modifications. As outlined in Figure 3A, the antigen is first immobilized to a membrane-based 96-well ELISpot plate, followed by the addition of a cell preparation containing the B cells to be tested. After suitable incubation time, allowing sufficient secretion and binding of antibodies to the coated antigen, bound antibodies are detected by the sequential addition of a biotinylated anti-Ig antibody followed by enzyme-conjugated Streptavidin. Finally, a precipitating substrate will form a colored spot at the location of the secreting cell, which can be viewed and counted in a microscope or, preferably, in an ELISpot reader.

While this procedure is simple and straightforward, it does have certain weaknesses, which are related to the antigen. To obtain distinct spots, the antigen needs to be coated at a relatively high concentration and in a way that leaves the relevant epitope/s accessible for binding, the latter of which is difficult to control. Furthermore, the antigen needs to be resistant to the potentially proteolytic environment encountered during cell incubation.

To avoid some of these problems, we have developed a novel B-cell ELISpot format based on using the antigen for detection. As shown in Figure 3B, the assay starts by coating the ELISpot plate with capture anti-Ig antibodies. These antibodies can be chosen to bind a specific isotype or IgG-subclass. During the cultivation with cells, antibodies from the ASC are bound by the capture antibody independent of their antigen specificity. To detect only those cells that secrete antibodies with the appropriate antigen specificity, biotinylated antigen is added followed by incubation with Streptavidin-enzyme and finally

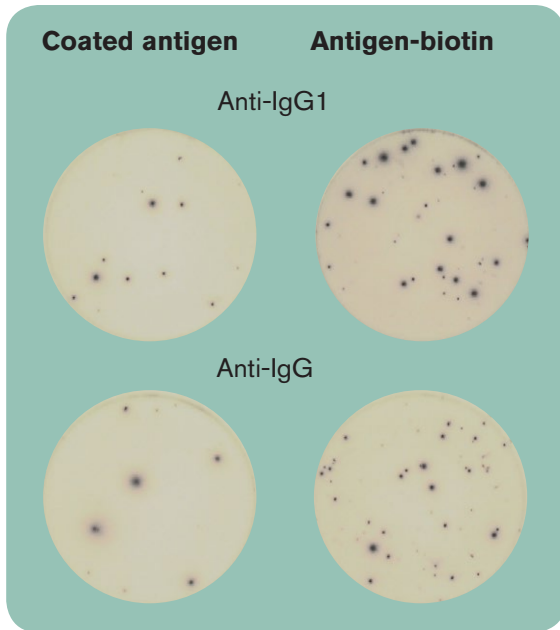


Figure 5. Coated vs. biotinylated antigen in IgG1/IgG ELISpot. Spleen cells (200 000 cells/well) were incubated for 20h in plates coated either with antigen followed by biotinylated detection antibodies (left wells) or with IgG1/IgG-specific antibodies followed by biotinylated antigen (right wells). Human IL-29 was used as antigen.

the addition of substrate. In our experience with a number of antigen systems, this alternative way of performing the B-cell ELISpot may result in more distinct and easily evaluated spots, lower background staining of the membrane and, in many cases, improved sensitivity (see example in Figures 4 and 5). In addition to these advantages, approximately 20 to 200 times less antigen is needed and the antigen is not subject to the potentially degrading conditions during cell cultivation^{11,12}.

Sources of B cells

While most B cells can be tested in the ELISpot assay, conditions will differ depending on the source and state of the cells. For instance, when using samples from blood or lymphoid tissue, cells may be in an activated form as a consequence of an acute infection or a recent immunization/vaccination or in the form of memory cells.

If cells have been activated *in vivo*, they can be added to the plates and incubated in cell culture medium without any further stimulation. However, studies have shown that the time window in which one can

detect ASC in blood after e.g. vaccination is narrow¹³. Typically cells need to be collected 5 to 10 days after the administration of antigen. After this, a sharp drop in the number of circulating ASC occurs⁴. Memory B cells, on the other hand, need to be stimulated before they produce detectable amounts of antibody. This is typically done in a pre-incubation step where the cells are cultured in the presence of factors that promote proliferation and maturation of the B cells. There exist many protocols of stimulation often including a combination of different activating signals, like R848 together with IL-2 (included in Mabtech's kits) (see Figure 6). The period of stimulation varies but is normally 3 to 8 days^{4,5}. After the pre-incubation, before addition to the ELISpot plates, cells are washed to remove any antibodies present in the culture medium that could bind and cause darkening of the ELISpot membrane.

Cell samples from humans are usually in the form of peripheral blood mononuclear cells (PBMC) but may also be obtained from lymphoid organs or mucosal sites. As the B cells normally constitute only a minor fraction of the cells (5-12% of PBMC), they may be enriched using e.g. negative selection by depleting non-B cells or by cell sorting. Enrichment leads to increased sensitivity simply due to more B cells being analyzed in the individual wells.

Mouse B cells are usually obtained from the spleen or other lymphoid organs but can also come from peripheral blood. In the latter case, it may be difficult to obtain sufficient amounts of cells whereas spleen cells are easily obtained in great numbers and also contain a larger proportion of B cells compared to the blood.



Figure 6. Detection of total number IgG producing cells using in vitro stimulated ASC. Human PBMC were stimulated with R848 and IL-2 for 3 days. The cells were washed, added to the ELISpot plate (50 000 cells/well) and incubated for 20h without further stimulation.

Technical aspects

Requirements for a successful B-cell ELISpot include the use of PVDF membrane plates, pre-activation of the membrane with ethanol and coating with a relatively high concentration of antibodies or antigen. These measures combine to give well-defined spots of high intensity, which will not only facilitate spot counting and evaluation but also increase the sensitivity of the assay.

As in all immunological assays, reagents should be of high quality and validated for optimal performance. Apart from the capture antibodies and detection reagents, the medium and serum used for culturing the cells also need to be of high quality. To assure this, multiple serum batches should be pre-tested. Fetal calf serum is typically used for the assay and one should avoid using autologous serum since antibodies present in this serum will interfere with the assay.

As mentioned earlier, the number of cells added to the wells is highly dependent on the expected frequency of positive cells. However, even in cases where antigen-specific cells are expected to be present at very low frequencies, cell numbers over 5×10^5 cells/well should be avoided as this may result in multiple layers of cells where cells in the upper layer/s tend to produce weaker and less focused spots.

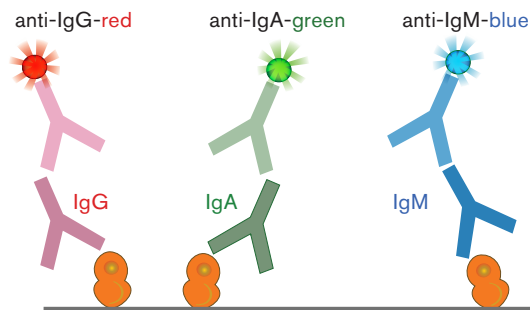


Figure 7. Schematic illustration of antigen specific B-cell FluoroSpot using coated antigen. Antibodies from B cells secreting antigen-specific IgG, IgA or IgM are detected using differently labeled, isotype-specific detection antibodies, making spots appear as either red (IgG), green (IgA) or blue (IgM). By coating with anti-Ig antibodies instead of antigen the total number B cells secreting different isotypes or IgG subclasses can be determined in a similar manner.

B-cell FluoroSpot

As an attractive alternative to the ELISpot, antibody-secreting cells may also be analyzed by FluoroSpot using fluorophore-conjugated secondary reagents for detection. This technique is as sensitive as the ELISpot but offers the additional possibility to, in the same well, separately define and enumerate B cells secreting antibodies of different isotypes or subclasses. This can be done in an antigen-specific manner as depicted in Figure 7 or with the purpose to determine the total number of B cells producing different immunoglobulin isotypes. Apart from an easy procedure, the fact that more than one type of antibody response can be measured in the same well makes the technique particularly suitable in situations where the source of cells or the amount of antigen is limited¹⁴.

An example of a vaccine-induced antibody response is shown in Figure 8. Here the number of antigen-specific IgG and IgA secreting cells were investigated before and after vaccination with Dukoral (cholera). There were no detectable spots before vaccination.

Using three-color FluoroSpot, antigen-specific as well as total numbers of IgG, IgA or IgM producing B cells can be enumerated simultaneously in a single FluoroSpot well. Specific vaccine-induced IgG, IgA and IgM responses can also be monitored (Figure 9).

We believe that the many beneficial properties of the B-cell ELISpot and FluoroSpot make them excellent assays to be used in studies aiming to provide further knowledge about B-cell immunity in general as well as in specific applications where B cells play either a protective role (e.g. infection and cancer) or may be linked to some diseases.

ELISpot/FluoroSpot enables studies of:

- Humoral immunity, e.g. in infection, allergy and autoimmunity
- Vaccine-induced antibody responses
- Antigen-specific memory B cells
- Definition and enumeration of B cells secreting antibodies of different isotypes/subclasses in the same well

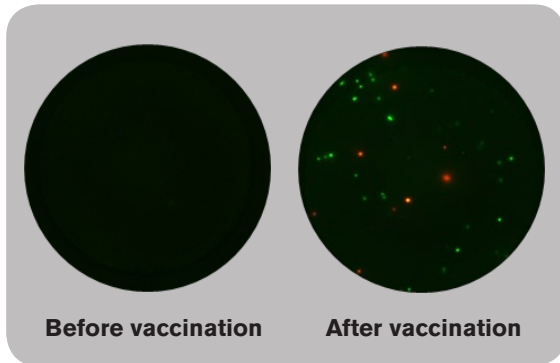


Figure 8. Detection and enumeration of cholera-specific IgG and IgA secreting B cells. Human PBMC were collected before and seven days after vaccination with the oral vaccine Dukoral. Antigen-specific IgG (red) and IgA-(green) secreting cells were analyzed using wells coated with the cholera toxin B subunit (1 μ g/well).

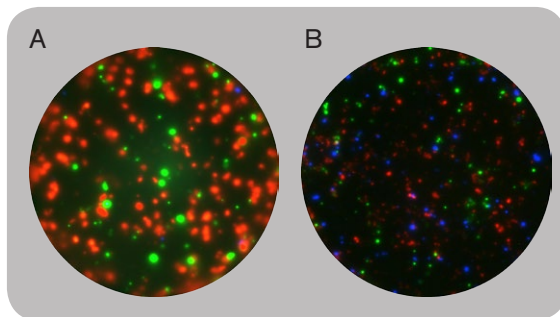


Figure 9. (A) FluoroSpot analysis of in vivo-activated human B cells (200 000 cells/well) secreting seasonal flu-specific IgG, IgA and IgM. (B) Detection of total IgG, IgA and IgM-secreting B cells (25 000 cells/well) stimulated in vitro for three days with R848 and IL-2. Spots were detected by adding a mixture of anti-IgG-red, anti-IgA-green and anti IgM-blue.

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