

Chemistry and Biology of the ELISPOT Assay

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Summary

Enzyme-linked immunospot, or ELISPOT, assay allows the detection of low frequencies of cells secreting various molecules. ELISPOT can be used in many areas of research and, because of its high sensitivity, has the potential to become a valuable diagnostic tool. Based on the same “sandwich” immunochemical principles as enzyme-linked immunosorbent assay, ELISPOT is easy to perform and quantify the results. At the same time ELISPOT remains a state-of-the-art technique that requires accuracy, thorough selection of antibodies and detection reagents, and an understanding of the principles of data analysis. This review covers various technical aspects of the ELISPOT assay, including immunochemical principles of the assay, selection of reagents and plates, and troubleshooting recommendations.

Key Words: ELISPOT; detection antibodies; capture antibodies; spot-forming cells; quantification of spots; spot artifacts.

1. Historic Overview

In 1983, Sedgewick and Holt (1) published a paper in the *Journal of Immunological Methods* describing a novel technique for the enumeration of antibody-secreting cells. The new technique was built on the same solid-phase immunoenzymatic principles as the enzyme-linked immunosorbent assay (ELISA): antigen was immobilized to a solid support (plastic dish) to bind antibodies released by cultured splenocytes. Later, in 1983, another article describing a similar antibody detection technique was published in the same journal by Czerkinsky and colleagues (2), who coined the name for this assay “enzyme-linked immunospot,” or ELISPOT. Later, the original ELISPOT technique was modified in that the solid phase was coated with antibodies (rather than the antigen) to capture antigens (for example, cytokines) released by cultured cells (3). As modified, reversed ELISPOT has become very popular and appears to be used more frequently than its predecessor. Some researchers call it “reversed

ELISPOT,” whereas most truncated this name to just “ELISPOT,” In this chapter, I will cover various technical aspects of the reversed ELISPOT assay and, like most researchers, also will call it simply ELISPOT.

2. Fields of Application of ELISPOT Assay

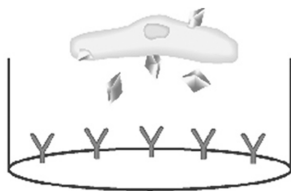
As it has been reported by Tanguay and Killion, ELISPOT appears to be 200 times more sensitive than ELISA in detecting secreted cytokines (4). These authors have shown that it was below delectability level of ELISA to detect cytokines released by less than 10^4 cells, whereas as many as 10–100 cells per well was sufficient for the detection of cytokine-releasing cells. Such a high sensitivity makes ELISPOT a technique of choice for the detection of spontaneous and antigen-induced secretion of cytokines (e.g., interferon [IFN]- γ , tumor necrosis factor [TNF]- α , interleukin [IL]-2, IL-4) from peripheral blood lymphocytes (5,6). ELISPOT is widely used for vaccine development (7–9), AIDS research (10,11), cancer research (for review. *see* ref. 12), infectious diseases monitoring (13), autoimmune disease studies (14), and allergy and transplantation research (15,16).

3. Immunochemical Principles of ELISPOT Assay

Even though ELISPOT uses the same immunochemical “sandwich” principles as ELISA (Fig. 1) there are two main differences between these two assays. First, ELISA measures the real concentration of the cytokine (17) and thus answers the question “how much is secreted?”, whereas ELISPOT enumerates secreting cells answering the question “what is the frequency of secreting cells?” (1,2). Therefore, one assay should be used not “instead of,” but rather “in addition to” the other. Second, ELISA is an immunoassay designed to analyze mostly cell-free media (17), whereas ELISPOT is a combination of both immunoassay and bioassay because live cells are cultured directly in ELISPOT plates. It appears that the quality of spots depends on both immunoassay and bioassay components (*see* examples in troubleshooting in Subheading 7.1.).

4. Nuts and Bolts of ELISPOT Assay

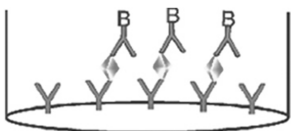
The performance of ELISPOT assay depends on the quality of four major components: (1) antibodies (both capture and detection), (2) enzyme conjugates, (3) chromogenic substrates, and (4) membrane-backed plates. Because the secretion activity of cells in ELISPOT is determined by the number of spots on the bottom of the plate (1,2), it appears that all four components should be optimized to facilitate the formation of detectable spots. Spots should have strong staining intensity (high signal-to-noise ratio) and have well-defined edges. It also is desirable that spots have a small diameter to



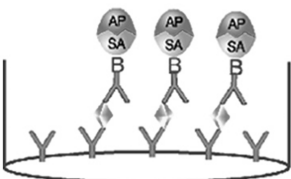
Step 1
Incubation of cells in wells coated with capture antibodies



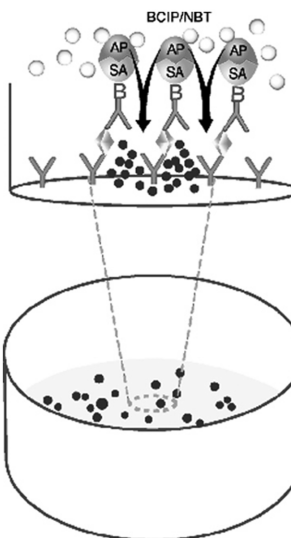
Step 2
Removal of cultured cells by washing



Step 3
Incubation with biotinylated detection antibodies



Step 4
Incubation with alkaline phosphatase conjugated streptavidin



Step 5
Addition of BCIP/NBT chromogen which turns into colored spots.

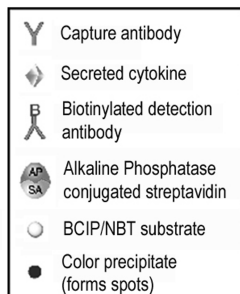


Fig. 1. Typical ELISPOT assay procedure.

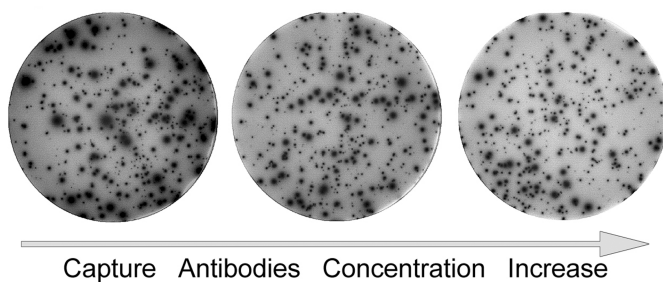


Fig. 2. Effect of capture antibodies' concentration on the size of spots and background staining (human IL-8 ELISPOT kit; R&D Systems).

avoid their merging with each other: a few merged spots may be erroneously counted as a single spot.

4.1. Antibodies

Both monoclonal and polyclonal antibodies can be used in ELISPOT assays for either antigen capture or antigen detection. ELISPOT can use capture and detection antibodies that were raised either against the entire antigen molecule (e.g., antirecombinant protein antibodies) or against a portion of the antigen (e.g., antipeptide antibodies). The critical factor in choosing capture and detection antibodies is their ability to recognize nonoverlapping epitopes of the target antigen (17). For these reasons it is not recommended to use the same monoclonal antibody for both capture and detection in the same ELISPOT assay. Suitability of antibodies for such applications as immunohistochemistry and western blotting and even ELISA does not necessarily guarantee that these antibodies will also work in ELISPOT (A. Kalyuzhny, personal observations). The only reliable method to identify the best capture and detection antibody combinations is to test antibodies directly in an ELISPOT assay. The concentration of capture antibodies has to be optimized to obtain intensely stained spots with well-defined edges: **Fig. 2** illustrates the effect of coating antibody concentration on the size of spots, intensity of their staining, and the background. Detection antibodies used in ELISPOT need to be conjugated to biotin to make possible their reaction with streptavidin-enzyme conjugates (18). The reason detection antibodies need to be biotinylated is to avoid crossreactivity: if both capture and detection antibodies are raised in the same species (e.g., mouse), antibodies (e.g., anti-mouse) conjugated to enzyme will bind to both capture and detection antibodies rather binding to detection antibodies only. Alternatively, detection system may use detection antibodies directly conjugated to enzyme (so-called direct conjugate). Unfortunately the sensitivity of

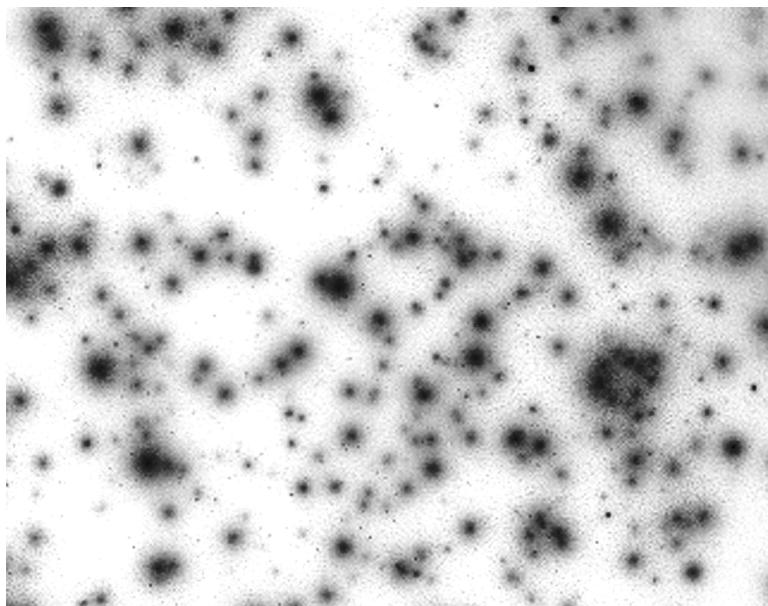


Fig. 3. Two-cytokine ELISPOT assay (custom-made kit; R&D Systems). IL-2 release from human peripheral blood lymphocytes is detected using Alkaline phosphatase-BCIP/NBT reagents, whereas IFN- γ is detected using HRP-AEC detection system. See **Color Plate 1** following page 50.

ELISPOT assays that use direct conjugates may be lower than that of avidin-biotin ones.

4.2. Enzyme Conjugates

Horseradish peroxidase (HRP) or alkaline phosphatase (AP) can be used as streptavidin conjugates (**18**). HRP (optimum pH 7.6) in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of substrates, which change color with the loss of electrons. The advantage of using HRP is its high turnover rate (spots develop faster), whereas the drawback is increased background. Unlike HRP, AP (optimum pH 9.0–9.6) has a linear reaction rate (spots develop slower), allowing for longer incubations with chromogenic substrates (**18**) without a risk of developing background staining. Longer incubation may be performed if it is necessary to increase the sensitivity of AP-based assay. By combining HRP and AP, it is possible to develop an ELISPOT assay for simultaneous detection of two different cell-secreted molecules (**Fig. 3**; **refs. 19** and **20**). The major drawback of mulianalyte systems is the loss of sensitivity for each of the antigens. I have observed that a number of spots formed by IL-2 and IFN- γ

secreted from peripheral blood mononuclear cells in the plate coated with anti-IL-2 and anti-IFN- γ antibodies was noticeably lower in comparison with corresponding single-cytokine assays (A. Kalyuzhny, personal observation). I have found that the drop in sensitivity becomes even more profound if ELISPOT plate is coated with more than two capture antibodies (*see* Chapter 18). The mechanism underlying this phenomenon is not known, and additional research is needed to find the ways of building high-sensitivity multianalyte ELISPOT assays.

4.3. Enzyme Substrates

Regardless of which enzyme conjugate is used, their corresponding substrates should produce intense and stable colors. HRP substrate such as AEC (3-amino-9-ethylcarbazole, $C_{14}H_{14}N_2$) forms intense red color spots (**18**). However, AEC is unstable (**18**), and spots will bleach in a short period of time. This, in turn, will result in irrecoverable loss of primary data. Another HRP substrate, DAB (3,3'-diaminobenzidine, $C_{12}H_{14}N_4$), produces brown color spots that are less intense than their AEC counterparts (**18**) and, although stable, DAB is poisonous and potentially carcinogenic. One of the most frequently used substrates for AP is a mixture of BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt) and NBT (Nitroblue tetrazolium chloride) which forms intense black-blue spots (**18**). Because of the high stability of BCIP/NBT, spots do not fade, and ELISPOT plates can be re-analyzed after being stored for several years.

4.4. Assay-Developing Procedures

The secretion capacity of cells may be tested in two ways: (1) cells are cultured in a designated plate and then transferred into ELISPOT plates (**21–23**), or (2) cells are stimulated and cultured directly in ELISPOT plates (**24**). Depending on the research project, cells may be stimulated one way or the other, but it should be kept in mind that cells cultured and stimulated outside ELISPOT plate need to be transferred into a fresh culture medium before being plated into an ELISPOT plate to avoid background staining.

4.5. Membrane-Backed Microplates

ELISPOT assays can be performed using either 96-well clear plastic plates (**4,25**) or plates backed with membranes such as polyvinylidene difluoride (**26,27**) and nitrocellulose (**25,28**). Unlike lateral flow and flow-through assays, membranes in ELISPOT assay are used for other reasons: they support the growth of cells and have a much higher retaining capacity for capture antibodies (because higher surface area) than conventional plastic plates. In ELISPOT assay the flow of reagents through or across the membrane is not required, but rather a diffusion

of cell-secreted molecules towards capture antibodies immobilized on the membrane. Membrane plates are manufactured by different vendors, including the Millipore Corporation, Pall Corporation, and Whatman. All vendors manufacture comparable plates, but it appears that Millipore plates are more popular for ELISPOT assay. This may be attributed to the fact that membranes with spots can be easily removed from Millipore plates for compact filing and protection purposes (*see* membrane removal systems in **Subheading 6.**).

4.6. Types of ELISPOT Assays

There are two major commercial formats of ELISPOT assay: (1) fully developed and optimized ready-to-use kits (RTU) and (2) so-called do-it-yourself (DIY) kits which, include reagents and uncoated 96-well plates to develop an assay. RTU kits may include precoated 96-well plates and all necessary reagents to run the assay. DIY kits need to be optimized by the researcher, which can be a very laborious procedure. RTU kits are more expensive than DIY ones, but RTU kits are the best choice for large-scale clinical trial experiments requiring convenience and a high degree of accuracy (29). R&D Systems, Inc. was the first company to design and introduce completely optimized RTU ELISPOT kits, which include dry precoated membrane microplates, wash buffers, detection antibodies, and AP-BCIP/NBT detection reagents.

5. ELISPOT Data Analysis

In ELISA assays, the concentration of the molecules in the sample is determined by measuring the optical density of the color substrate solution filling the wells (17), whereas in ELISPOT, cell-secretion capacity is measured by counting colored spots on the bottom of the well (1,2). The term “spot-forming cells,” or SFC, is used as a quantitative measure of the cell secretion activity in ELISPOT assay (30,31).

5.1. Quantification of Spots

After finishing the assay, spots can be counted either manually or by using computer-aided image analysis (32). Manual counting is performed under the stereomicroscope using, for example, a hand-held tally counter. Manual counting is very tedious and time-consuming but appears to be of higher sensitivity, allowing investigator to identify faint spots of smaller sizes and decide whether spot is “real” or an artifact. Computer-aided quantification can be performed using either inexpensive semiautomated (MVS Pacific; www.mvspacific.com) or more expensive but fully automated systems offered by such vendors as Zeiss (www.zeiss.com; *see* Chapter 8 in this book), Cellular Technology (www.immunospot.com; *see* Chapter 7 in this book), and AutoImmun Diagnostika (www.elispot.com)

Regardless of the system used, a 96-well ELISPOT plate is mounted onto microscopy stage and moved in front of the lens to capture images of individual wells. When using semiautomated systems, the operator moves the ELISPOT plate either by hand or by using a joystick connected to the stage, whereas on fully automated readers the plate is moved automatically according to the image collection sequence set by the operator. The main component of each ELISPOT reader is its software allowing capture of images and quantification of spots: the better the design of the spot-recognition and image-processing algorithm, the higher the value of the software. Fully automated systems are faster and more expensive but not necessarily more accurate than semi-automated ones. It appears that customers are paying more for convenience of automation rather than for higher accuracy of quantification.

5.1.1. Manual Quantification of Spots

The typical set-up for manual spot counting would be a 4X objective lens with 10X eyepieces. The main concern with manual counting is the human error and subjective bias, for example, very small spots may go unnoticed, whereas two close spots may be counted as a single spot. Interestingly, that even with these limitations, the human visual system has higher resolution than the existing computerized analyzers.

5.1.2. Computer-Aided Quantification of Spots

Computer-aided quantification of spots is thought to be more reliable than the manual counting (32). Computerized systems use a charged-coupled device (CCD) camera to visualize and capture digital images of each well. Many image-processing algorithms are designed to detect and count spots in each captured image automatically. Unfortunately, the finite pixel size of the CCD camera poses serious limitations on both resolution and detectability for both smaller spots and for clusters of closely-spaced spots of all sizes. These limitations are usually summarized by quoting a Nyquist-limited resolution of two-pixels' width (33). Pixel size is an important consideration in computerized ELISPOT readers. For accurate quantification pixels need to be at least half the size of the smallest spots.

5.1.3. Types of ELISPOT Readers

Current systems can be further grouped as “macro-imagers” or “image-tiling systems.”

5.1.3.1. MACRO-IMAGERS

These readers capture an image from an entire well. The camera is moved from well to well, either manually or automatically. The limitation of the

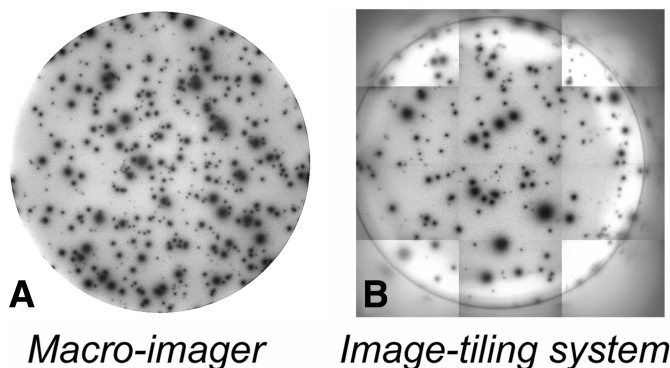


Fig. 4. Typical single well ELISPOT images. (A) image captured with macro-imager (ImageHub; MVS Pacific: www.mvspacific.com). (B) image captured using the image-tiling system (KS ELISPOT reader; Carl Zeiss).

macro approach is the number of pixels in the CCD's focal plane array. A typical 1000×1000 pixel CCD imager would have $6\text{-}\mu\text{m}$ pixels when focused on a 6-mm diameter membrane on the bottom of the well. As a result, small spots may go undetected, whereas clusters of spots will be counted as a single spot. **Figure 4A** shows the typical image of an ELISPOT well captured using a macro-imager.

5.1.3.2. IMAGE-TILING SYSTEMS

Higher resolution can be achieved by using higher magnification and capturing multiple image “tiles,” each from a small portion of a single membrane. These individual image tiles are then “stitched” or “seamed” together into a larger image that can be analyzed (for example, U.S. Patent 4,760,385 discloses the principles of image tiling). Image-tiling systems are more expensive than macro-imagers because they require a fully automated microscope that moves the 96-well plate while a computer and a video-formatted CCD camera automatically coordinate the capture of many individual images or tiles. **Figure 4B** shows the typical image collected by such a tiling system, the KS Elispot reader (Zeiss). Tiling systems are not only expensive, but they become prohibitively slow at higher resolutions. An additional drawback of tiling systems is that they often sacrifice image quality at tile boundaries where the combination of imperfect tile alignment and optical distortion may result in image artifacts (refer to **Fig. 4B**).

6. Archiving of Primary ELISPOT Data

After finishing the experiment, stained 96-well plates become primary experimental data, and it may be required to store them in a safe place. Unfortunately

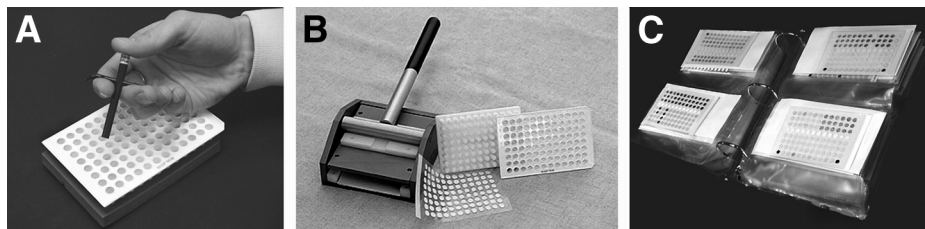


Fig. 5. Archiving stained membranes from ELISPOT plates. (A) single-membrane removal tool (Zellnet; www.zellnet.com). (B) membrane-removal device for simultaneous removal of all 96 membranes from the plate and their transfer onto adhesive film (MVS Pacific; www.mvspacific.com). (C) removed membranes can be stored in a regular photo album and reanalyzed when needed.

96-well plates are bulky, and their storage requires a lot of space, especially during large-scale clinical trials. To solve this problem, membranes with spots can be punched out of the plates, laminated, barcoded, and stored in a regular photo album. **Figure 5** depicts two types of membrane removal systems: single-well puncher made by Zellnet (**Fig. 5A**; www.zellnet.com) and 96-well membrane removal device (**Fig. 5B**) designed by MVS Pacific (US Patent 6,631,649; www.mvspacific.com). The latter device allows for simultaneous removal and transferring of all 96 membranes from the plate onto adhesive film in less than a minute. Adhesive film with attached membranes can be laminated to protect membranes with spots from damage during their handling. Removed and laminated membranes can be stored in a regular photo album as shown on **Fig. 5C**. If needed, removed membranes can be reanalyzed using ELISPOT readers.

7. Troubleshooting ELISPOT Assays

7.1. Staining

The quality of staining has a strongest impact on the accuracy of the quantification of spots in an ELISPOT assay. There are two major staining problems that require troubleshooting: background staining and staining of spots. Background in an ELISPOT assay is defined as a staining that covers either a part of or the entire membrane. Backgrounds may be further categorized as either specific or nonspecific. Specific background is formed as the result of specific binding of cell-secreted molecules by capture antibodies: molecules that are released from the cell dissociate from capture antibodies surrounding the releasing cell, diffuse, and bind to capture antibodies in the cell-free zone. A specific background may occur, for example, if an ELISPOT plate with cells is disturbed during the incubation. Once an ELISPOT plate is placed into the incubator, it should not be touched or moved for the entire incubation period.

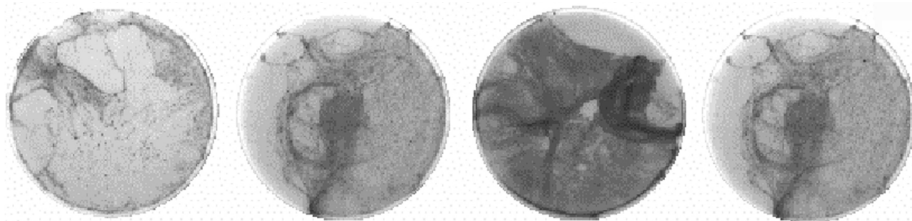


Fig. 6. Variations of high background staining and spot-looking artifacts, which can be caused by high number of dead cells added into the ELISPOT plate.

Frequent opening and closing of the incubator's door also may disturb cells in the plate. Nonspecific background is caused by the adsorption of detecting components (detection antibodies, enzyme conjugate, and precipitating substrate) onto the membrane. Both specific and nonspecific backgrounds hinder the detection and counting of spots. It is easier to troubleshoot one rather than both types of background. It is more difficult to identify the source of nonspecific background because of multiple factors contributing to it. We have found that one of the universal remedies against both specific and nonspecific backgrounds is aluminum foil. Wrapping ELISPOT plates into aluminum foil reduces background staining and improves contrast. It also produces a more uniform distribution of specific spots across the filter membrane (34). In addition, application of foil appears to improve well-to-well reproducibility. The reason aluminum foil reduces the background staining is not known, but it is tempting to speculate that aluminum foil facilitates even distribution of heat over the bottom of ELISPOT plate during its incubation in CO₂ incubator.

7.2. Cells

The quality of staining also depends on the quality of cultured cells. It is of critical importance to determine the percent of dead cells because we have found that a high number of dead cells (30–50% and more) may be a reason for a high background staining and even lack of specific spots (**Fig. 6**). In some cases, even though the number of dead cells is low (e.g., approx 5%), there may be no spots formed at all because of apoptosis (**Fig. 7**). Intensity of staining also depends on the number of cells plated into the well—the addition of excessive number of cells per well may result in overstaining due to a specific background. Because the secretion capacity of cells is not known in advance, it is always recommended to test serial dilution of cells from each individual donor (e.g., 10³, 10⁴, 10⁵, 10⁶ cells per well) in the same ELISPOT plate. This ensures having enough data points to choose from in case over- or underdevelopment occurs. Using cells of unknown secretion capacity requires dedicating many

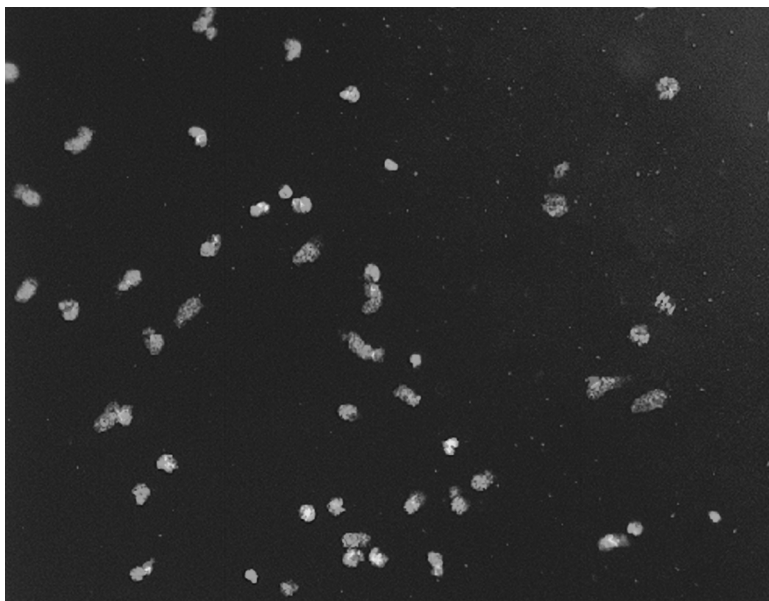


Fig. 7. High degree of programmed cell death (apoptosis) in cells plated into the ELISPOT plate may result in lack of spots. Cells attached to membranes (green fluorescence) were labeled immunocytochemically for an apoptosis marker active Caspase-3 using R&D Systems anticaspase-3 antibodies (red color). Note the high number of apoptotic cells. See **Color Plate 2** following page 50.

wells in the plate for cell optimization rather than for experimental groups. The solution to this problem is to preserve cell suspensions, freeze them, and store them in liquid nitrogen. It was reported that freezing of peripheral blood lymphocytes did not significantly affect their rosette formation (35) and that freezing of dendritic cells did not impair their ability to respond to maturation signals (36). In the ELISPOT assay, cryopreserved peripheral blood mononuclear cells were shown to be similar to freshly isolated cells in their capacity to release IFN- γ (37–39) or even exceeded the latter (27). A stock of cryopreserved cells with a known secretion capacity may be used in a single predetermined concentration in the entire ELISPOT plate. We have reported previously that the same cryopreserved peripheral blood lymphocytes can be used to study release of different cytokines (40). Cryopreservation of cells for ELISPOT is advantageous for clinical trial studies because it helps to avoid variations in biological samples collected from the same donor but on different dates. Interestingly, cryopreserved cells are more active in secreting some cytokines (41,42), which is thought to be caused by elimination of inhibitory platelets, which do not withstand freezing (27).

7.3. Washing Procedures

The ultimate purpose of washing ELISPOT plates is to remove cultured cells and unbound reagents (detection antibodies, enzyme conjugate, enzyme substrate) from ELISPOT plates to minimize background staining. Plates can be washed, for example, with phosphate-buffered saline of various pH and molarity. It is necessary to remove as many cells as possible by washing since stained cells may be confused with specific spots and thus affect the accuracy of quantification. In some cases stimulated cells become very sticky and their complete removal may require incubation with enzymatic cell-detachment solutions (*see* Chapter 5).

8. ELISPOT Assay as a Tool for In Vitro Diagnostics

ELISPOT is widely used for research purpose but has a great potential as a diagnostic tool. For example, it was reported that the ESAT-6/CFP-10-based ELISPOT assay can be used to detect active tuberculosis in HIV-positive individuals with high sensitivity (43). Authors of this study suggested that ELISPOT was more specific and more sensitive than PPD-based methods to detect latent *Mycobacterium tuberculosis* infection. ELISPOT may be also used for allergy diagnostics: it was reported that peripheral blood mononuclear cells from nickel-allergic individuals responded to Ni^{2+} with significantly greater production of IL-4, IL-5, IL-13, and IFN- γ , compared with the healthy controls (44). It appears that the format of 96-well-based ELISPOT assay needs to be modified for diagnostic applications. First, the assay should be miniaturized to reduce the volume of samples needed for analysis: this is particularly important in pediatrics. Second, a fast and easy-to-operate turnkey ELISPOT reading system/scanner should be available to analyze staining and creating a report. Third, matrix with stained spots (e.g., membranes, plastics, etc.) should be both small enough for compact filing and have enough room for bar code labeling. Fourth, dyes used to stain spots should be stable to allow their re-evaluation after an extended period of time.

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