Benchmarks

Cost-Efficient Quantification of Enzyme-Linked Immunospot

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Enzyme-linked immunospot (ELISPOT) analysis is a sensitive technique for the detection and quantification of antigen-specific T cells (5,7) and also for the detection of various cytokines released by immune cells (2). It is used extensively to monitor peptide-specific T cell responses in vaccination protocols (3), melanoma patients (6), and adoptive cellular immunotherapy (9) because it can detect and quantify low numbers of antigen-specific T cells in freshly isolated blood lymphocytes with no need for prior *in vitro* expansion (9). The assay is based on the visualization of cytokine secretion by individual T lymphocytes after *in vitro* stimulation by an antigen. Briefly, the ELISPOT plates are coated with antibody that is specific for the cytokine being assayed. The antibody binds to the nitrocellulose base of the ELISPOT plate. The activated cells are then transferred to the plate, and cytokines are released during the incubation period. Cytokines released locally around each cell are captured by the specific antibody, and any excess cytokine is washed off. A second antibody that is also specific for the cytokine of interest is then added; this antibody is coupled to an enzyme that is capable of converting a substrate into an insoluble colored product. The plate is then washed again, and the enzyme substrate is added. The substrate gets converted into the insoluble product, forming colored spots representing the areas of captured cytokines secreted by activated cells. These spots are then counted for the quantification of cytokines or activated cells.

Even though it is a widely used technique, most laboratories still rely on the manual quantification of the colored spots using a stereomicroscope, which limits the great potential of this otherwise powerful technique. A commercially available digital quantification system utilizing the AlphaImager® System (Alpha Innotech, San Leandro, CA, USA) incorporating a charge-cou-

pled device (CCD) camera and the software to quantify the spots has been recently reported (8). However, it is impractical for most laboratories to invest in such expensive systems (approximately AUS \$80000 from Carl Zeiss, Thornwood, NY, USA) with a limited area of application. An alternative approach currently being used involves removal of the nitrocellulose membrane from each well of the microplate for enumeration. This is, however, very tedious and also restricts the possibility of storing the plates for future reference. We have found that the test plates can be stored wrapped in aluminium foil at 4°C for years without any effect on the quality of spots.

We suggest an accurate and more cost-efficient approach for the enumeration of these spots using equipment that can be assembled in most laboratories. The ELISPOT plates are stained

after culture, dried at room temperature, and illuminated from the top. The image of each well is captured by a CCD video camera (DAGE; AUS \$1500; Maryland Telecommunications Inc., Michigan, IN, USA) directly attached to a microscope lens (25 mm, 1:1.3; AUS \$200) and held by a clamp on a support stand approximately 10 cm from the plate. The lens magnifies the image of each well of the microplate to the full screen size. Optimum resolution can then be achieved by adjusting the contrast from the lens. Thereafter, the distance of the camera and the aperture size of the lens are kept constant for each experiment. The parameters set for the enumeration of the spots of interest are the intensity threshold and the diameter of spots. The intensity threshold is thus set optimally so as to eliminate any small artifacts. The image is then captured and the spots are counted automatically (Figure 1) using the Image-Pro Plus imaging software (AUS \$5500; Media Cybernetics, Silver Spring, MD, USA). The data (number of spots and the area of each spot) is transferred to a Microsoft® Excel® spreadsheet, and the means and standard deviations are cal-

Figure 1. Enumeration of ELISPOT by the macroscopic approach. This figure depicts images from an interferon (IFN)- γ -ELISPOT assay captured by the CCD camera attached directly to a microscope lens. The control (no peptide) well contains 10^5 peripheral blood mononuclear cells (PBMCs) from an Epstein-Barr virus (EBV) seropositive donor, while the test (with peptide) contains 10^5 PBMCs from the same donor stimulated with an EBV latent antigen peptide. Briefly, 96-well multiscreen HA filtration plates (Millipore, Bedford, MA, USA) were coated with an anti-IFN- γ mAb, 1-DIK (MABTECH, Stockholm, Sweden) and were incubated overnight at 4°C. The next day, PBMCs were incubated in triplicate with or without peptide at 37°C for 14–20 h. The following day, the cells were discarded, and the second biotinylated anti-IFN- γ mAb, 7-B6-1 b10 (MABTECH) was added, and the plate was kept at room temperature for 2–4 h. This was followed by the addition of streptavidin-conjugated alkaline phosphatase (Sigma, St. Louis, MO, USA) for another 2 h. Cytokine-producing cells were detected as purple spots after a 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. These spots were counted, and the number of peptide-specific T cells was calculated after subtracting spots in control wells (cells only). The graphic representation of this data (bottom panel) depicts the area (in pixels) and number of peptide-specific spots counted by the lens-assisted computer-based system.

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culated. During analysis, a cut-off value is set up for the spot size at a minimum of six pixels to eliminate any small nonspecific background spots, and this value is kept constant.

Once the system is set up, a macro is written with the optimal settings. This makes the system more efficient and reproducible. It takes around 20 s to count each well of the microplate, and up to 400 spots/well can be counted quite accurately compared to the exhausting exercise of counting as many spots manually under a microscope. Occasional clusters of cells can be easily and accurately counted by using the "split clustered objects" function of the Image-Pro software. The whole system can be set up at less than one-tenth the cost of the commercially available digital quantification systems. The results are more consistent than manual counts in which the counts can vary between two investigators, especially when there are too many spots/well. It is difficult to manually count and also to differentiate between specific and nonspecific spots if there are more than 200 spots/well, and this can affect the consistency. As reported earlier (8), we also observed that, although the results of manual reading correlate with those counted by the computer, there is a greater standard deviation in manual counts and that the counts are always lower, especially in wells having more than 100 spots. For example, the mean $\pm$ standard deviation of four counts of a particular well was 200 ± 1.6 by the lens-assisted computer-based counting as compared to 188 ± 23 spots counted manually under a microscope by four individuals. Other image analysis software available in the laboratory can also be used. The National Institutes of Health (NIH) Image Version 1.55 shareware (<http://rsb.info.nih.gov/niimage>) for ELISPOT quantification can further cut down costs. This software has been used earlier by Cui and Chang (1); however, their overall set up is very complicated: the image is captured, optimized by the computer, saved in a picture file format, and then processed after improving the resolution by the help of a computer. A modified version of this setup was later reported (4) with a fully automated system that, besides being more expensive, also requires the

removal of the nitrocellulose membrane from the plates. Recently, the AlphaImager imaging system has been introduced and is commercially available (8). However, our system based on the same principle can be set up in-house at a fraction of the cost and is equally efficient. Overall, the advantages of our system over those previously published (1,4) are: (i) the direct attachment of a microscope lens to the CCD camera eliminates the requirement for a microscope; (ii) the complicated and time-consuming steps of saving each image in a picture file format followed by image resolution by the computer are also eliminated because the microscope lens magnifies and helps in transporting a highly resolved image of the spots on to the computer screen, which is instantly counted; and (iii) the automatic quantification of these spots by the Image-Pro Plus, or any other available standard imaging software, ensures reproducible and efficient quantification and can also, if required, supply information on other parameters, for example, the area of each spot (Figure 1).

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Microplate Assay for the Measurement of Hydroxyproline in Acid-Hydrolyzed Tissue Samples

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Collagens are major constituents of connective tissues. The organic matrix of bone, for example, consists of approximately 90% type I collagen (2) with a small amount of type III and other minor collagens. Typically, the fibrillar collagens contain approximately 100 hydroxyproline residues per 1000 amino acid residues (3), and measure-