Tips for selecting the "right" ELISPOT filter plate and making the best protocol choices

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☑ Nitrocellulose or PVDF?

There are two different types of membranes (in 96-Well plate formats) that are used in ELISPOT applications: Nitrocellulose (NC) and polyvinylidene fluoride (PVDF). The reason that these are the predominant membrane types used in ELISPOT is based largely on their fortuitous suitability in non-ELISPOT applications rather than on the development of optimized assay substrates to address the specific needs of the ELISPOT assay. NC and then PVDF membranes were developed to serve the needs of sterile filtration applications. As was later discovered, the porosity and binding properties of these two membrane types (NC and PVDF) enabled them to be used in two extremely important research applications; DNA and protein blotting. Finally, in response to the specific requirements of molecular biology and protein chemistry applications, NC and PVDF membrane-bottomed 96-well plates were developed and made commercially available. Independently, separately, and at about the same time, ELISPOT assays were developed on 96-well plastic plates and took advantage of enzyme-linked immunosorbent assay (ELISA) techniques that had been perfected in that format. Since fundamentally the immunodetection component of ELISPOT assays and protein (i.e., Western) blotting is essentially identical, it was only a matter of time until the overwhelming majority of ELISPOT assays were performed on membrane-bottomed, 96-well plates.

Shortly after NC-bottomed filter plates became available, the majority of ELISPOT assays were carried out in those plates. When PVDF filter plates were introduced, some investigators chose to use PVDF plates and some continued to use NC. The reasons for choosing one plate (membrane) over the other are highly varied and won't be addressed in detail here. The fact that some laboratories and individual researchers feel strongly that one membrane is superior to the other runs contrary to the large body of Western blotting experience: Despite some clear-cut differences in how each of the membranes can be used, there is essentially no reported difference in terms of detection sensitivity or signal to noise on NC versus PVDF in the Western Blotting application. This having been said, it is clear is that the two membranes and their properties are quite different. These properties and the differences between NC and PVDF membranes – especially in the ELISPOT application are highlighted in Table 1:

Table 1: *Attributes of Membranes Used in Elispot Applications*

Membrane Attribute	NC (used in ELISPOT) [nominal or average values]	PVDF (used in ELISPOT) [nominal or average values]
Pore Size[a]	*0.45 microns (µm)*	*0.45 microns (µm)*
Porosity[b]	*70 – 75%*	*65 – 70%*
Thickness	*150µm*	*135µm*
B.E.T. Surface Area[c]	*6.5m^2/gram*	*6m^2/gram*
Surface Area Ratio[d]	*250*	*350*
Saturation Binding Capacity (IgG)	*250µg/cm^2*	*350µg/cm^2*
(IgG) Binding Capacity of Top 1µm	*2µg*	*3µg*
Wettability	*Wettable due to the addition of surfactants or detergents to the membrane during membrane manufacture*	*Not directly wettable in water. Must be pre-wet with alcohol and then exchanged with water*
Additives	*Glycerin*	*None*
Solvent Compatibility	*Not compatible with methanol or ethanol*	*Broadly compatible with a wide range of aqueous and organic solvents. Avoid prolonged exposure to strong alkali (e.g., pH >12)*
Mechanism of Binding	*Electrostatic*	*Hydrophobic*
Things which will interfere with or destabilize binding of anti-cytokine antibodies	*Chaotropes (e.g., Tween-20, Triton-X 100, etc.). Water (if never dried), Proteins , especially larger molecular weight proteins*	*Detergents (e.g., SDS), low polarity solvents (e.g., dimethyl formamide, etc.)*
Compatibility with different detection modes	✓ *Colorimetric* × *Fluorescence* ✓ *Chemiluminescence*	✓ *Colorimetric* ✓ *Fluorescence (marginal)* ✓ *Chemiluminescence*

[a] *Pore size is nominal and corresponds to the diameter of the largest particle that can pass through the membrane structure. A 0.45µm pore size membrane is expected to retain 100% of particles whose diameter exceeds 0.45µm.*

[b] *Porosity is the portion of the membrane volume that is occupied by air (not occupied by polymer). 1cm^2 of membrane whose thickness is 140µm (i.e. 0.014cm) will have a volume of 0.014cm^3 (14µL). If the membrane is 70% porous, it will contain approximately 10µL of void space.*

[c] *B.E.T. surface area is the polymer surface area that is in direct contact with liquid or air in the membrane*

[d] *Surface area ratio is the ratio of internal to frontal surface area. A surface area ratio of 250 means that in a 1cm^2 piece of membrane, the polymer surface area is 250cm^2.*

☑ Pre-Wet in Alcohol or Not?

The major difference between NC and PVDF in ELISPOT applications is related to their mechanisms of binding and associated differences in handling or pretreatment. PVDF is very hydrophobic and will not wet out in water. In Western blotting applications, PVDF is always pre-wet in alcohol (typically 50 –

100% methanol), then rinsed in water, and ultimately equilibrated in a (transfer) buffer solution before the membrane is used in the protocol. The overwhelming majority of literature in Western blotting references the pre-wetting step, so it is not a surprise that many ELISPOT protocols also include an alcohol pre-wet step. What is surprising is that some ELISPOT protocols do not include a pre-wet step. At Millipore (Danvers, MA), two experiments were performed to determine the effect of alcohol pre-wetting on the performance of PVDF 96-well plates (catalog number: MSIPS4510). In the first experiment, which focused exclusively on antibody binding, 30 wells of a 96 well plate were pre-wet with 15µL of 35% v/v methanol and rinsed. A second set of 30 wells was pre-wet with 15µL of 70% v/v methanol in water and rinsed, and 30 wells were not pretreated at all. Immediately following the pre-wetting step, 100µL of antibody solution containing 10µg/mL IgG trace-labeled with ^{125}I-IgG was added to all (90) wells and incubated at 4°C overnight. All (90) wells were then washed 3 times in PBS and then dried. 100µL of the trace-labeled antibody solution was added to the remaining 6 wells and dried (used to determine counts per minute for 1µg antibody). Membranes were punched out of the MultiScreen® plates and assayed for radioactivity. The results are summarized in Table 2.

Table 2*: The Effect of Pre-Wetting on Antibody Binding*

Pre-Wetting Condition	Bound Antibody (µg/well) Average ± Std. Dev.
No Pre-Wetting	*0.04 ± 0.001*
15µL 35% MeOH	*0.91 ± 0.011*
15µL 70% MeOH	*0.89 ± 0.016*

The results of the pre-wetting experiment clearly show that the amount of protein binding is very much affected by the membrane's ability to wet out completely. These data are consistent with what has been published in the context of Western blotting performance. Many laboratories have reported that even without blocking, non-wet out areas of PVDF membrane (Immobilon-P) exhibit low non-specific binding. The second set of experiments focused on the effect of pre-wetting with 15µL of 70% ethanol on Elispot assay performance. In these experiments, following the alcohol pretreatment (or no pretreatment), plates were coated with 1µg of anti-human interferon-gamma (Mabtech, Stockholm, Sweden), and blocked for 2 hours in tissue culture media (RPMI, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen). 50,000 peripheral blood mononuclear cells were added per well to 16 wells per plate, stimulated with 0.5µg Phytohemagglutinin (PHA-L, Sigma, St. Louis MO) and the plates were incubated overnight in a humidified, 37°C, 5% CO_2 tissue culture incubator. ELISPOTs were visualized using biotinylated anti-human interferon-gamma (Mabtech), conjugated avidin-alkaline phosphatase (Mabtech) and BCIP/NBT Plus (Moss, Inc., Pasadena, CA) and then enumerated using an automated microscope (KS Elispot, Zeiss, Thornwood, New York) and its associated software. The results of 4 different experiments are summarized in Table 3.

Table 3. *The Effect of Alcohol Pretreatment of PVDF on ELISPOT Assay Performance*

Experiment Number	Pre-Wet Spot Number Mean ± Std. Dev*.	Non Pre-Wet Spot Number Mean ± Std. Dev*.	Non Pre-Wet As A Percent of Pre-Wet
1	*606 ± 46*	*413 ± 37*	*68%*
2	*577 ± 37*	*416 ± 34*	*72%*
3	*604 ± 35*	*440 ± 42*	*73%*
4	*609 ± 40*	*391 ± 28*	*64%*

**n = 12 – 16*

In these experiments, the cells in the untreated (non pre-wet) plates produced approximately 30% fewer detectible spots. However, the consistency well to well and plate to plate was equivalent. Spot quality (intensity, uniformity and size) and overall assay background were comparable in both plate types. Considering that half these results were obtained without pre-wetting and that non pre-wet membranes bind a great deal less capture antibody, the comparable, side by side performance is quite remarkable. Based on these results, and more importantly, based on the compelling results of many laboratories that don't pre-wet their PVDF plates, it would appear that the determination to pre-wet with alcohol or not can be made by individual laboratories based on their specific reagents and particular assay requirements.

☑ How Many Cells to Add?

It is frequently reported that to monitor weak antigen responses or detect changes in low-responding patients, it may be a good idea to add 250,000 or more target cells per well. This recommendation may be at odds with geometry and cell biology. The membrane frontal surface area in a typical 96-well plate is approximately 0.3cm^2. If the responding T-cell is assumed to be round and estimated to have a nominal diameter of 10 – 15µm, then approximately 150,000 cells would constitute a monolayer (i.e., a single layer of cells covering the entire surface). Adding more than approximately 100,000 cells creates the risk of some of these cells not being in intimate contact with the membrane. If the cell that is secreting cytokine in response to antigen stimulation is not in direct contact with the antibody-coated membrane, it is possible that the shape and intensity of its corresponding ELISPOT may be so irregular or diffuse as to disqualify it from being enumerated. In instances when the response rate to antigen is anticipated to be so low that it is advisable to stimulate 250,000, 500,000 or 1,000,000 cells to get a significant response above background, it might be best to add 100,000 cells per well to 5 or 10 wells and then to determine the aggregate response (the sum of the ELISPOTs in the 5 or 10 wells), not the average response per well.

☑ **What's the Best Membrane for Fluorescence Detection of ELISPOTS?**

As has been noted earlier, membranes are highly porous structures. Their surfaces are very rough as is evidenced by the scanning electron micrograph of a cross section of PVDF membrane. Consequently, they scatter light (i.e. reflect incident light at higher wavelengths) and therefore exhibit high fluorescence background. PVDF is a better membrane to use than nitrocellulose in fluorescence-based ELISPOT applications and there are reports in the literature of successful, dual-label ELISPOT assays based on the use of fluorescein and rhodamine-labeled antibodies. Millipore has recently made available a PVDF membrane (Immobilon-FL) that has been developed specifically for fluorescence detection in Western blotting applications. Its fluorescence background is 10 to 100 times lower than standard PVDF (Immobilon-P) membrane and has been shown to be compatible with high sensitivity fluorescence detection using organic and nanoparticle fluorescent dyes. It is anticipated that this membrane will be available in MultiScreen plates sometime in the future. Please check with your Millipore applications specialist for additional information.