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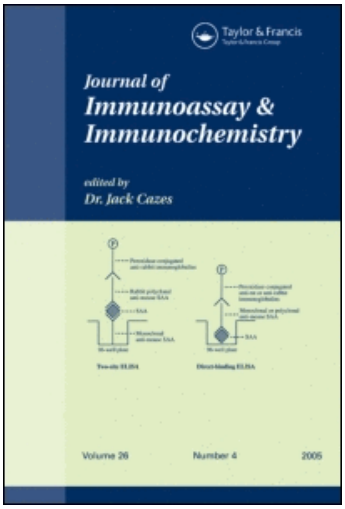


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# TIPS AND STEP-BY-STEP PROTOCOL FOR THE OPTIMIZATION OF IMPORTANT FACTORS AFFECTING CELLULAR ENZYME-LINKED IMMUNOSORBENT ASSAY (CELISA)

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# TIPS AND STEP-BY-STEP PROTOCOL FOR THE OPTIMIZATION OF IMPORTANT FACTORS AFFECTING CELLULAR ENZYME-LINKED IMMUNOSORBENT ASSAY (CELISA)

**R. Morandini,[1,*] J.-M. Boeynaems,[2] J. Wérenne,[3] and G. Ghanem[1]**

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## ABSTRACT

CELISA, or cellular enzyme-linked immunosorbent assay, is a powerful and easy to use technique to study cell surface antigens under different stimulations. Nevertheless, some factors (11) must be discussed and optimized prior to reaching a reproducible CELISA. These include the choice of cell density, fixative agent, blocking agent, culture medium, optimal antibody dilutions, and incubation time. In this paper, we first present a short review of some references devoted to CELISA by means of a comparison of these parameters, followed by their description. Then, we describe and study these different parameters using practical examples comparing TNF-induced

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ICAM-1 expression as an end point, on HBL melanoma and HUVEC. These cell lines were also chosen because they differ in their ability to grow as discontinuous and continuous layers, respectively. Furthermore, we designed a comprehensive flow chart, as well as a complete step-by-step protocol for CELISA optimization.

## INTRODUCTION

Cellular enzyme-linked immunosorbent assay (CELISA) was first described by Stocker,(1) Douillard,(2) and Morris,(3) and was developed for the large screening of hybridomas or immune complexes.(2,4,5,6) This technique was largely applied to detect cell surface antigens.(7,8,9,10,11,12)

The main steps of the technique include chemical fixation of cells followed by an incubation with specific antibodies and a colour generation through an enzyme-linked second antibody reaction with a suitable substrate. CELISA possesses some advantages as compared to other methods. It differs from conventional ELISA by the fact that intact cells are used instead of proteins. Its specificity and sensitivity are comparable to those of the FACS,(13) with the exception that, with the latter, the cells must be detached and used in suspension while, in CELISA, intact, adherent, and fixed cells are used as the targets. The maximal sensitivity is not only a function of the number of antigen binding sites per cell, but also of cell number, incubation time and activity of the enzyme-coupled to the antibody. In addition, proteins present in relatively large amounts can be easily and rapidly detected (within 30–45 minutes), while detecting antigens expressed in low amounts can be reached by longer incubations (2–3 h), thus increasing, further, the sensitivity of the method.

In practice, CELISA is an attractive, relatively simple, and efficient method for the detection and quantitation of cell surface antigens. However, its reliability will depend on a suitable optimization of: cell density, fixative agent, blocking agent, culture medium, optimal antibody dilutions, and incubation time. Although many authors reported CELISA methodologies (Table 1), none gave either guidelines for optimization or examples of results or a comprehensive and complete protocol within the same paper.

In this paper, we describe different critical steps to be considered for the design of a reliable CELISA by the independent measurement of ICAM-1 and E-Selectin as examples of target molecules in two types of cells, as well as a complete step-by-step protocol.

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***Table 1.*** Conditions of Some CELISA Parameters Found in Some Papers Relative to CELISA Optimization/Description and Three as an End Point (Oertli, Rothlein and Topley)

| Author | Antibodies Optimization | Cell Type | Adherent − No/+ Yes | Cell Density Optimi-zation | Cell Synchro-nization | Detergent Use | Fixative | Washing Buffer | Blocking Agent | Detection |
|---|---|---|---|---|---|---|---|---|---|---|
| Stocker (1979) (1) | N | Mouse thymocytes<br>Human lymphocytes | −<br>− | Y | N | N | 0.2% Glut. | PBS | BSA | $^{125}$I |
| Suter (1980) (5) | Y | Human melanoma<br>Human lymphocytes | +<br>− | N | N | 0.05% Tween 20 | 0.1% Glut.; 3 min. | PBS | Gelatine | AP |
| Morris (1982) (3) | Y | Human mononuclear | − | Y | N | N | N | PBS | BSA | AP |
| Posner (1982) (6) | Y | Mouse B lymphoblastoid | − | N | N | N | N | PBS Citrate | BSA | HRP |
| Morris (1983) (21) | N | Human mononuclear | + | Y | N | N | NN | VBS | BSA | AP |
| Effros (1985) (7) | N | Peripheral blood mononuclear | − | N | N | 0.2% Tween 20 | N | PBS Citrate | N | HRP |
| Epstein (1985) (19) | Y | Mouse spleen | + | N | N | N | Glut. Poly-L-Lysine | PBS | FCS | AP |
| Rothlein (1988) (8) | | Human fibroblasts, carcinoma, sarcoma | + | N | N | N | 1% Paraform.; 15 min. | RPMI | BSA | β-galactosidase |
| Arunachalam (1990) (23) | N | Human mononuclear<br>B lymphoblastoid | −<br>− | Y | N | 0.05% Tween 20 | N | PBS Citrate | Rabbit serum | HRP |
| Bishop (1992) (13) | N | Human lymphocytes<br>L66 | −<br>+ | N | N | N | 0.25% Glut. | PBS | N | Urease |
| Sánchez (1983) (24) | N | Human lymphocytes | − | N | N | 0.05% Tween 20 | 0.25% Glut. | TBS | BSA | AP |
| Topley (1996) (15) | N | HUVEC | + | − | − | N | 1% Glut.; 5 min. | PBS | N | HRP |

(*continued*)

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***Table 1.*** Continued

| Author | Antibodies Optimization | Cell Type | Adherent − No/+ Yes | Cell Density Optimi-zation | Cell Synchro-nization | Detergent Use | Fixative | Washing Buffer | Blocking Agent | Detection |
|---|---|---|---|---|---|---|---|---|---|---|
| Balkian (1997) (25) | Y | Splenocytes | − | Y | Y | 0.05% Tween 20 | 0.5% formalin | PBS | BSA | HRP |
| Smith (1997) (20) | Y (?) | Human synoviocyte<br>Human mononuclear | +<br>− | N | N | N | 1% Paraform.; 30 min. | PBS | BSA or Non fat dry milk | HRP |
| Oertli (1998) (14) | N | Fibroblast 3T3<br>Rat lung epithelial<br>Mouse endothelial | +<br>+<br>+ | N | N | N | 3% Paraform. | PBS | N | HRP |
| Schmidt (1999) (26) | N | Hybridoma | − | N | N | 0.05% Tween 20 | 0.01% Glut. | PBS | N | HRP |

Abbreviations: PBS: Phosphate Buffer Saline; TBS: Tris Buffer Saline; VBS: Veronal Buffer Saline; Glit.: Glutaraldehyde; Paraform: Paraformaldehyde; N: No or none; NN: Not necessary; AP: Alkaline Phosphatase; HRP: Horseradish peroxydase.

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## EXPERIMENTAL

### Materials

Culture media, FCS (Foetal Calf Serum), NCS (Newborn Calf Serum), antibiotics, L-glutamine, and trypsin, were purchased from GIBCO Life Technologies. TNF-α was a gift from Boehringer Ingelheim. The monoclonal antibodies against ICAM-1 (BBA3) and E-Selectin were purchased from R & D Systems Europe. Anti-[mouse IgG]-alkaline phosphatase, was from Chemicon. Paraformaldehyde, p-Nitrophenyl Phosphate substrate tablets (pNPP) and MTT were from Sigma. BSA (fraction V, Protease-, Peroxidase-, Alkaline Phosphatase-free) were from Boehringer Ingelheim. 96-Well, flat-bottomed microtiter plates were from Nunc. Mycoplasma Removal Agent (MRA) was from ICN Flow.

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### Cells

HBL (a human melanoma cell lines) and HUVEC (Human Umbilical Vein Cells) were established in our Laboratory.(10) HBL were use in the present study to test the described different conditions below and HUVEC were used as controls. This choice was also guided by the fact that these two types of cells differ in a growth property to give continuous (HUVEC) or discontinuous (HBL) layers.

### Melanoma Cell Culture

Melanoma cells were cultured in the following medium: HAM F-10 supplemented with 5% FCS (Foetal Calf Serum), 5% NCS (Newborn Calf Serum), 2 mM l-glutamine and antibiotics: kanamycin sulphate 100 μg/mL, penicillin 100 U/mL and streptomycin sulphate 100 μg/mL referenced to as melanoma medium.

### Culture of HUVEC

HUVEC were obtained by trypsin-EDTA (0.5 g/L) digestion of the lumen of human umbilical veins. They were grown on gelatine-coated dishes, at 37°C, in a humidified 95% air–5% $CO_2$ incubator. The culture medium used was M199 medium supplemented with 20% foetal calf serum (FCS), penicillin G (100 U/mL), kanamycin sulphate (100 μg/mL),

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streptomycin (100 μg/mL), L-glutamine (2 mM), hepes (6 mM), Ultroser G (0.5%), heparin (55 μg/mL) and a bovine pituitary extract (20 μg/mL). Referenced to as HUVEC medium. For 1 week before the 1st passage, the cells were treated with Mycoplasma Removal Agent (0.5 μg/mL, Flow). At each passage, HUVEC were incubated for 5 min at 37°C in a calcium- and magnesium-free phosphosaline buffer containing trypsin (0.25 g/L) and EDTA (0.1 g/L). After the first passage, they were grown on uncoated dishes. Experiments were performed with cells between passage 3 and 9. For the assay, HUVEC were seeded with Dulbecco modified essential medium (DMEM) supplemented with 10% FCS, glutamine (2 mM), and antibiotics referenced to as HUVEC assay medium.

All cell lines were routinely checked for mycoplasma contamination (Mycoplasma T.C., Gen-Probe, San Diego, CA).

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## Seeding of Cells

Cells were seeded in 96-well, flat-bottomed microtiter plates. Wells located at the periphery of each plate usually give non-reproducible results, compared to replicate wells elsewhere on the plate; so, edge wells were routinely excluded from use and simply filled with sterile water. For the same reasons, the plates were not stacked inside the incubator. The cells were seeded at a density of $25 \times 10^3$ cells/well for HBL in 100 μL melanoma medium and $50 \times 10^3$ cells for HUVEC (this cell density give a continuous monolayer at "day 0" ) in 100 μL HUVEC assay medium unless otherwise indicated. The seedings were done at "day −1" of the assay. At the "day 0", 100 μL of each of the different effectors or medium were added to the appropriate wells.

## Cell Growth and MTT Assay

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to monitor cell growth. At "day −1", the cells were seeded in microplates at the density of $5 \times 10^3$ to $25 \times 10^3$ cells/well. Each day (beginning after 4 hours seeding at "day −1" over a period of one week) the supernatants were removed and 100 μL of MTT solution (1 mg/mL in PBS) were added to each well. After 3 hours incubation at 37°C, the supernatants were replaced by 100 μL of DMSO. After a brief mixing, the OD was measured at 515 nm.

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### ICAM-1 Expression on HBL Melanoma Cells and HUVEC Under TNF Challenge

At "day 0", TNF (1 to 500 U/mL) was added to each well, except for background and control wells where 100 µL of the adequate culture medium was added. At "day 1" the plates were submitted to CELISA as described in Flow-Chart 2.

### E-Selectin Expression on HUVEC Under TNF Challenge

At "day 0", TNF (1 to 500 U/mL) was added to each well, except for background wells and control wells, where 100 µL of the adequate culture medium was added. 6 h after TNF addition plates were either submitted to CELISA or fixed in 1% paraformaldehyde for 15 min and further incubated overnight at 4°C prior to CELISA, as described in Flow-Chart 2.

### Solutions

Assay Buffer

10 mM phosphate buffer (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.5 g/dl sodium azide.

1% Paraformaldehyde

Dissolved in assay buffer at 70°C during 10 minutes and then filtered through 0.45 µm filter. The temperature was kept below 80°C to avoid paraformaldehyde degradation.

Blocking Buffer

2% BSA (Protease-, Peroxidase-, Alkaline Phosphatase-free), dissolved in assay buffer.

Antibody Dilution Buffer

0.1% BSA (Protease-, Peroxidase-, Alkaline Phosphatase-free) in assay buffer.

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All solutions were kept at 4°C and brought to room temperature before use.

### Expression of Results

CELISA results are presented as mean ± SD. One representative of four independent assays is shown, and each experimental condition was performed in sextuplicate.

### Background Measurement

Background was measured by replacing the first antibody with the dilution buffer.

## RESULTS AND DISCUSSION

CELISA is a powerful and easy to use technique to study cell surface antigen expression under different stimulatory conditions. Nevertheless, some parameters are critical and must be optimised to have, not only reproducible results, but also the highest signal to background ratio. To this aim, we will describe and discuss below the important steps and parameters to be considered.

We have chosen ICAM-1 and E-selectin expression under TNF stimulation as an end-point to study the effect of the antibodies dilution on the sensitivity of the assay. In the same way, we have chosen HBL, which give a discontinuous monolayer, and HUVEC, which give a continuous monolayer to study the importance of the blocking step.

In Table 1, we made a comparison of the most important parameters to be considered in CELISA, based on some publications reporting parameter optimization, while three(8,14,15) are papers where CELISA are used as a technique to perform an assay. It is suprising to see that few authors (see Table 1) take into consideration antibody dilution, cell growth curve, the effect of the detergent and the fixative used altogether. In addition, none give neither extensive results nor a complete protocol, although these parameters are of particular importance in CELISA and can affect sensitivity of the assay.

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## Parameters to Be Considered in CELISA

### Assay Buffer and Detergent

First, we have tested different assay buffers (Tris, Citrate, Phosphate) and found no significant difference in target molecule expression. However, in accordance with some authors (see Table 1) we have found that the use of a phosphate buffer is the most convenient, the most used, and the cheapest.

Adding a detergent like Tween-20 gave non reproducible results, probably due to the presence of intact cells; also, the detergents may disturb the cell monolayer by detaching cells and opposing the effect of the blocking agent, thus affecting the background. Detergents were then omitted from the assay buffer.

### Mycoplasma Contamination

It is well known that mycoplasma can affect protein expression(16) and epitope recognition by the antibody.(17) To avoid this effect, all cell lines were treated with MRA 2 weeks before use (one-week treatment and one week without treatment) and routinely checked for mycoplasma contamination. In papers reviewed in Table 1, none discussed mycoplasma effect on CELISA.

### Trypsin and Cell Synchronisation

Cell seeding was done 24 hours prior to adding the effectors to allow the recovery of cell integrity, as well as protein synthesis, after trypsin treatment (to detach cells). This step is critical, since synchronised cell cycle allows to yield optimal growth and protein metabolism resulting in constant protein expression and, consequently, reproducible results in CELISA. A simple trypsin detachment is sufficient to allow cells to quit G0 phase, as shown by Merrill.(18)

### Well Washing

Washing the wells allows the elimination of the fixative, the blocking agent, and the two (the first and second) antibodies. It must be cautiously done to avoid disrupting the cell layer. However, extensive washings may give non-reproducible results. In addition, a careful removal of supernatants

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at each step is critical. To avoid cell monolayer disruption, the use of a light vacuum and needles of $0.6 \times 30$ mm, with the bevelled edge down and the plate at a 45° angle is necessary. Washing solution was always added when the plate is sitting flat on the working bench, with a micropipette bent at a 45° angle touching the first third of the wall. We do not recommend the use of a semiautomatic or automatic washer because they may substantially disturb the cell layer due to high pressure/flow, or/and to scraping the bottom of the well.

## Cell Fixation

24 h after seeding, adherent cells naturally attach to the bottom of the plate wells; however, the stronger they adhere, the smaller is the cell loss during repetitive washings. The use of a good fixative is also of importance; it must not affect cell morphology, so, at least theoretically, cell surface ligand structure would be better preserved.

Most protocols suggest glutaraldehyde as the fixative during the preparation of cell-coated plates (Table 1). Nevertheless, the use of glutaraldehyde has two drawbacks: it can cause antibodies to bind non-specifically to the cells and also it can alter the targeted antigenic determinants and decrease the binding (Data not shown and see ref. 19). Other fixatives, such as methanol or acetone, may give bad results due to a poor antibody binding or a non-specific decrease of the recorded signal.(20) In our hands (see also Table 1), the best reproducible results were obtained using 1 h fixation with 1% paraformaldehyde. Monolayers of fixed cells may be stored for at least 48 h at 4°C without a significant loss of antigenic reactivity or background increase (Data not shown and see ref. 20). Nevertheless, the choice of a fixative remains epitope- and antibody-dependent.

With non-adherent cells, it is possible to avoid cell fixation by using V-bottom microplates.(21) This technique cannot be applied to adherent cells.

## Optimization of Cell Density

Cell density is a critical parameter to take into consideration. The cell number to be seeded per well depends on the surface offered for the exponential growth, over the time period needed to perform the assay. In addition, some medium components may be present at concentrations that affect cell metabolism or even survival. If cell density is too high, cells can detach from the bottom of the wells. If cells are not in an adequate growth phase,

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proteins are not expressed in an optimal manner. In contrast, when cell density is too low, a lag in the growth phase occurs, resulting in low protein expression and a poor sensitivity. Nevertheless, some cell types, such as fibroblasts, keratinocytes, and HUVEC need to be seeded at a cell density that gives a continuous monolayer in order to mimic their phenotype *in vivo*. Thus, cell density is dependent not only on cell growth characteristics, but also on cell type.

Considering the above, cell growth was monitored using MTT(22) and an example is given in Figure 1 with HBL cells seeded at different cell densities. The latter shows that, in order to study an effector effect over 24 h, HBL cell seeding had to be done at $25 \times 10^3$ cells/well while, for a 48 h period, only $20 \times 10^3$ cells/well were needed. In comparison, to obtain a continuous monolayer (without affecting cell adhesion), HUVEC had to be seeded at $50 \times 10^3$ cells/well for the 24 h period and only $35 \times 10^3$ cells/well for 48 h (Data not shown). It is, therefore, important to know growth characteristics in order to seed cells at the beginning of their exponential growth. Nevertheless, some authors did not or do not mention the importance of this step (Table 1).

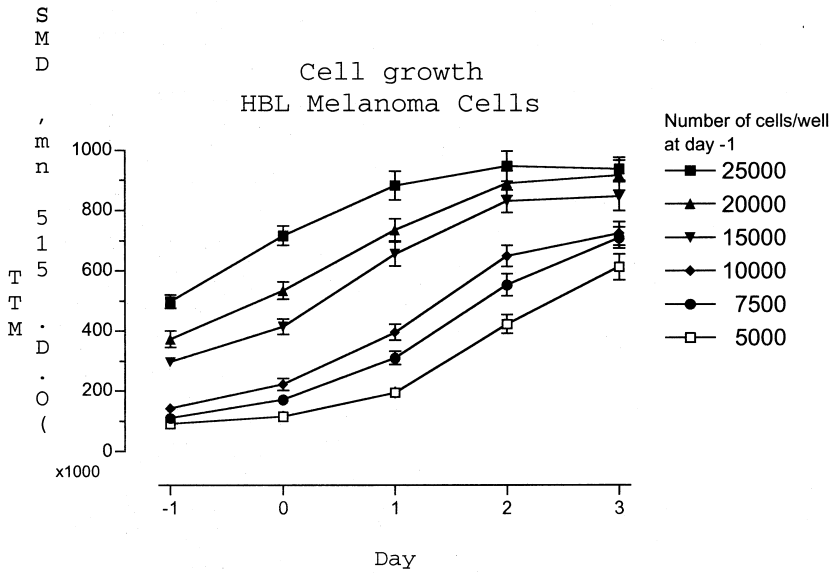


***Figure 1.*** HBL human melanoma cell growth curves at different cell densities. The cells were seeded (day −1) at the density indicated on the graph. After the indicated time, the cell number was measured by MTT assay as described in Experimental.

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## Well Blocking

The higher the needed sensitivity of CELISA, the better it is to keep a low background signal. This is best achieved by "blocking" the wells with an inert or an unrelated protein like BSA, casein, or other commercially available blocking agents, such as those from Roche (Blocking Agent) or from Pierce (SuperBlock). As they all proved equally efficient, we used BSA Protease-, Peroxidase-, and Alkaline Phosphatase-free.

The blocking step is crucial. Without blocking, the increase in background can be substantial, depending on the cell type used. An example is illustrated in Figure 2, panels A and B. This increase was of 115% for HBL and only 58% for HUVEC. The difference between the two cell types is probably due to the fact that the antibody has more places to attach to the plastic in a non-confluent cell line such as HBL.

## Choice of the Enzyme Coupled to the Antibody

Many enzymes are proposed (Table 1): horseradish peroxidase, alkaline phosphatase, urease, and galactosidase. However, the choice of the enzyme should take into account the presence of the same catalytic activity in the cells being used. For example, unlike peroxidases, the use of alkaline phosphatase gave, in our hands, much better results when working with cells like melanoma, melanocytes, fibroblasts, keratinocytes, and HUVEC (Data not shown). If cells with high peroxidase or alkaline phosphatase are to be used, then it is necessary to lower these activities by the use of a specific competitive inhibitor like levamisole (for alkaline phosphatase) and hydrogen peroxide (for peroxidase) prior to the assay.

## Incubation Time

Maximum sensitivity can be reached by defining the minimum necessary time to obtain the best specific binding with the optimal antibody dilution. Generally, 1 hour incubation for each antibody seems to be the right choice/compromise to perform all the necessary steps over a reasonable period of time.

## Antibody Dilution

The choice of the optimal first and second antibody dilutions/concentrations to be used depends on the highest signal to background ratio. This also implies the use of the highest expression of the targeted molecule.

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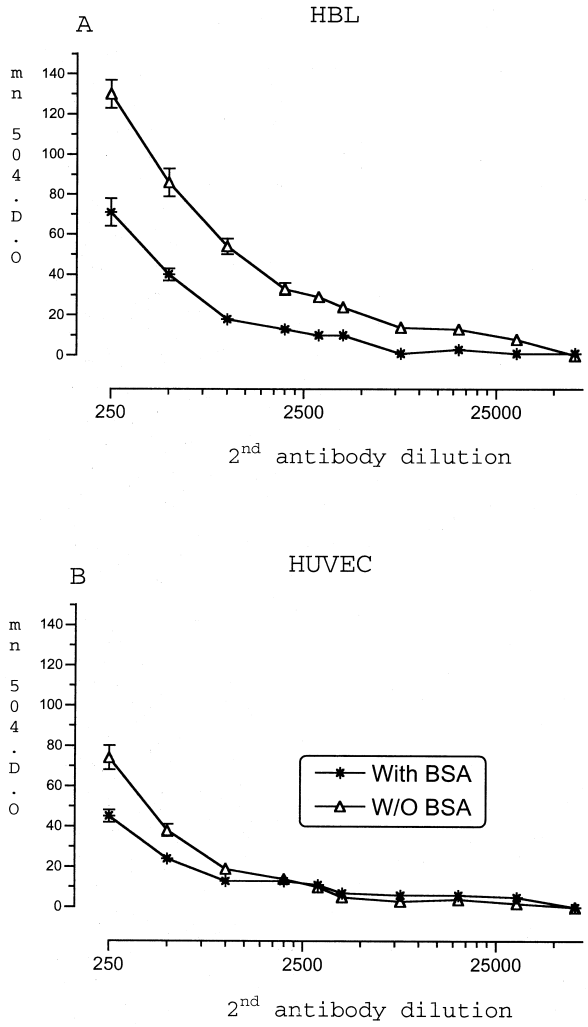


***Figures 2A and B.*** Effect of the 2nd antibody dilution on the background, in presence and absence of blocking agent (BSA). Cells were seeded at day −1 at the density of 25,000 cells/well (HBL, melanoma medium, panel A) or 50,000 cells/well (HUVEC, HUVEC assay medium, panel B). At day 0, 100 µL of the adequate medium were added. At day +1, different dilutions of the 2nd antibody (enzyme-linked) were added following the scheme in Flow-Chart 2. Measurement of background consists of adding all the solutions, except the 2nd antibody, which is replaced by 100 µL of assay buffer (absence of BSA) or 100 µL of BSA 0.1% (presence of BSA).

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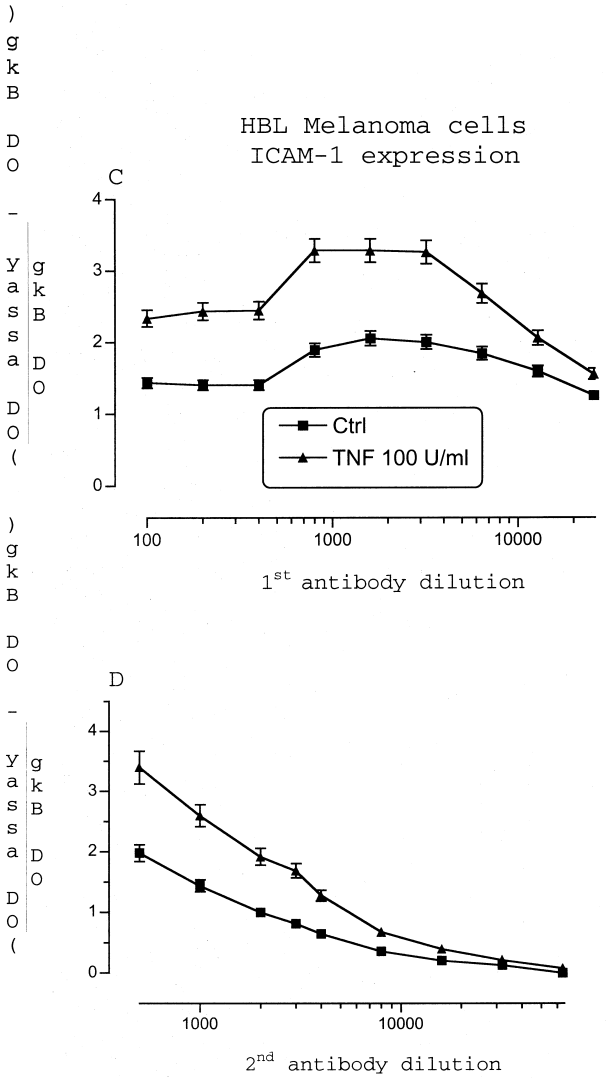


***Figures 2C and D.*** Panel C: Effect of the 1st antibody dilution on ICAM-1 signal while maintaining constant the 2nd antibody dilution (500-fold). Panel D: Effect of the 2nd antibody dilution on ICAM-1 signal while maintaining constant the 1st antibody dilution (1,000-fold). For Panel C and D, cells were seeded at "day −1" at the density of 25,000 cells/well (HBL, melanoma medium). At "day 0", the effectors were added (TNF 100 U/mL) or the medium for the control and background wells. 24 h later; CELISA was performed as decribed in Flow-Chart 2.

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Assuming that all antibodies have high affinities for the ligands, antibody dilution not only depends on incubation time, but also on an acceptable background. The latter should ideally not exceed two- or three-fold the mean STD of the assay value. Lower antibody dilutions usually result in too high backgrounds and higher dilutions give a low signal and low association constants.

Accordingly, both the first and enzyme-linked (second) antibodies should be tested at different dilutions, starting at the one recommended by the manufacturer. A first step consists of choosing the right dilution for the 2nd antibody (enzyme-linked) (or, in some cases, the enzyme-linked antibody when only one antibody is used) and perform the assay without the 1st antibody. In a first approximation, a dilution between 500 (O.D. is 0.045 for HBL and 0.024 for HUVEC) and 1000 (O.D. is 0.018 for HBL and 0.013 for HUVEC) seems to be adequate to perform the second step (Figure 2 panels A and B).

In a second step, the optimal dilution of the 1st antibody (against ICAM-1 or E-Selectin in our example) is measured after addition of TNF to boost ICAM-1 expression in HUVEC and HBL cells 10,11). Changing the dilutions of the 1st antibody, while maintaining the 2nd constant (at 500-fold) gave a plateau ranging between 800- and 3200-fold dilution (similar results are obtained using HUVEC and E-Selectin or ICAM-1) with the highest $[(OD\ answer - OD\ Bkg)/OD\ Bkg]$ ratio (Figure 2, panel C). The best 1st antibody dilution can be obtained from the first half dilution range, following the formula:

$$[(End\ value\ of\ the\ plateau - First\ value\ of\ the\ plateau)/2]$$

giving for ICAM-1:

$$[(3200–800)/2] = 1200\text{-}fold$$

The suitable dilution range could then be between 800- and 1200-fold, 1000-fold was our choice for the assay.

In a third step, the choice of the 2nd antibody dilution (found in the first step) is checked and confirmed by testing a range of dilutions of the 2nd antibody (500- to 64,000-fold) and by maintaining constant the first (1000-fold; Figure 2, panel D). Our data show that the highest difference between: 1) the control and the background, and 2) TNF stimulated ICAM-1 expression and the control, is obtained at 500-fold dilution of the second antibody.

To confirm the choice of the right dilutions for both antibodies, we combined $2 \times 2$ dilutions of these antibodies with different concentrations of TNF to test ICAM-1 expression (Figure 3). We observed that the highest value for $[(OD\ answer - OD\ Bkg)/OD\ Bkg]$ and the highest difference between TNF 500 U/mL and the control is obtained by using

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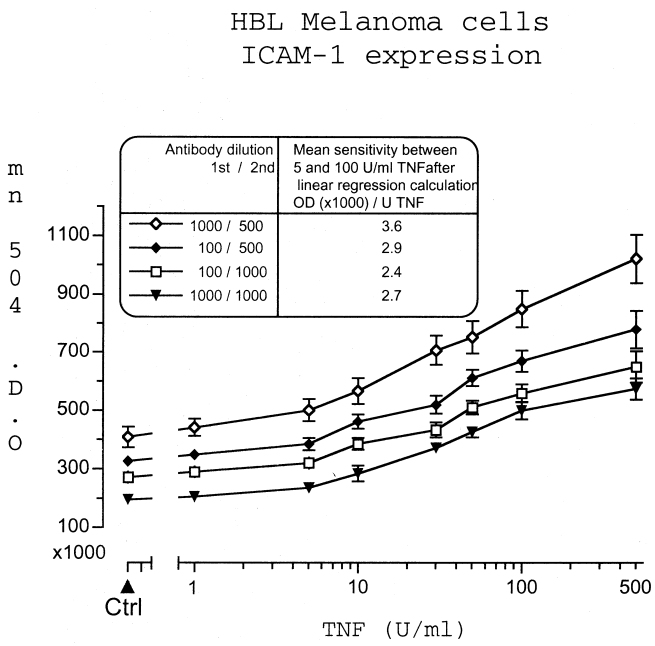


***Figure 3.*** Effect of both the antibody dilution and TNF concentration on ICAM-1 expression and sensitivity of the assay. At "day 0", TNF was added at 100 U/mL or medium for the control and background wells. 24 h later CELISA was performed as decribed in Flow-Chart 2.

the 1st antibody at 1000-fold and the 2nd at 500-fold dilutions. These results show that antibody dilution is of critical importance to reach both the highest signal to background ratio and the sensitivity (Figure 3 insert).

In a 4th step, cell density was examined because it is not only important to have a suitable growth curve, but also to obtain the highest recorded signal. Effectively, the signal to background ratio [$(OD\ answer - OD\ Bkg) / OD\ Bkg$] linearly decreases along with the cell number counted at "Day −1" both for Control and TNF-treated cells. Accordingly, the O.D. ratios (Control to TNF) decrease as well, resulting in a significant loss in sensitivity (Figure 4, panel A). To choose the right cell density, it is, therefore, important to perform a cell growth curve (Figure 1); this can give the highest cell density to use over the time needed for the assay and the cell type.

Optimization of antibody dilution is evident; nevertheless, in some papers, it is not really clear if they do perform such a step before a CELISA (Table 1).(15)

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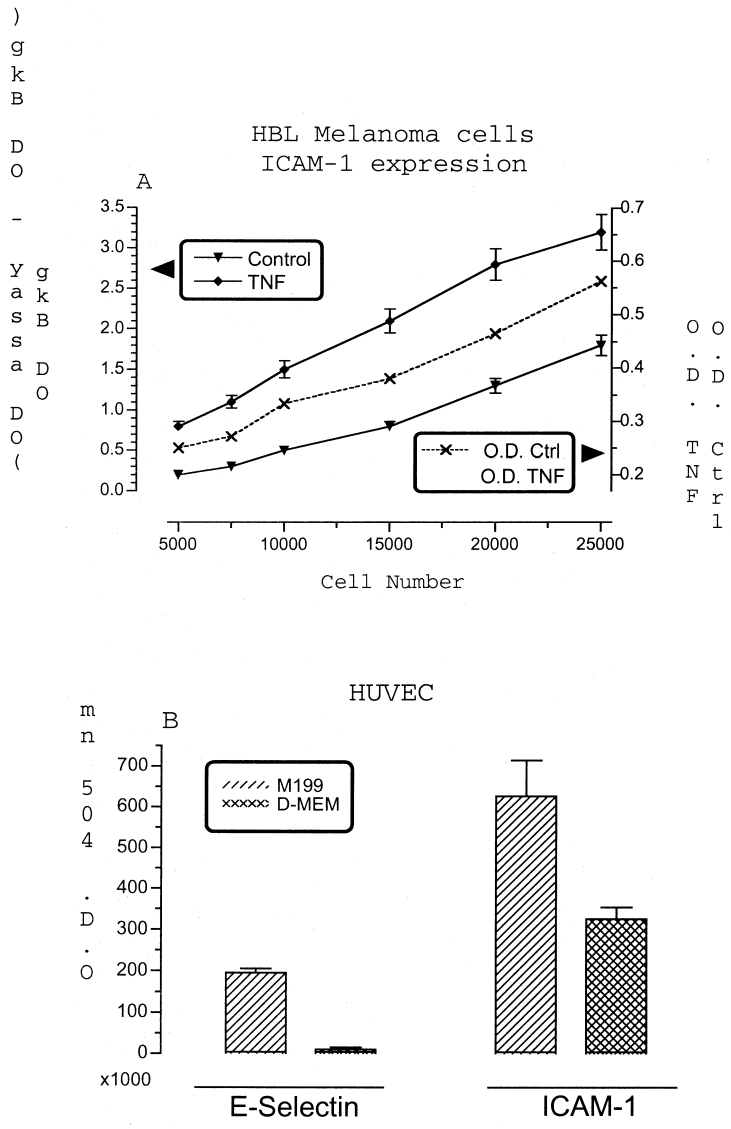


***Figure 4.*** Panel A: Effect of cell number and TNF (100 U/mL) on ICAM-1 (antibody dilution 1,000-fold) measured signal. Panel B: Effect of medium and TNF (100 U/mL) on ICAM-1 expression. At "day 0", TNF was added at 100 U/mL or the medium alone (for the control and background wells) and then assayed for ICAM-1 (antibody dilution 1000 folds) and E-Selectin (antibody dilution 800-fold), as described in Experimental.

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In conclusion, the recorded signal is not only dependent on the nature and density of the epitope present at the cell surface, but also on the antibody dilutions themselves, depending on their affinity and specificity.

### Choice of the Best Culture Medium

It is known that the selection of the best culture medium to use in CELISA should be guided by the right balance between optimal conditions needed for cell growth and/or maintenance of the adherent cell phenotype, and presentation of target surface antigens. The nature of the medium and additives are crucial for cell growth and, therefore, for protein expression. Nevertheless, it is possible to add effectors to the most simple culture medium that can allow adequate cell growth over the period of time needed to perform the assay, and to consider adding fresh medium if the lack of a component is known to affect protein expression. By switching from M-199 to D-MEM in HUVEC,(10) we avoided the effect of some constituents like ATP and glutathione on the expression of the adhesion molecules studied. Thus, the basal expression of E-Selectin was different in HUVEC medium, in comparison to HUVEC assay medium; the same is true for ICAM-1 expression (Figure 4, Panel B). Two explanations can be given: first, the cell growth is higher in HUVEC medium, rapidly leading to confluency where oxidative stress is increased. This leads to the activation of specific transcription factors, a continuous expression of E-Selectin, and an increased expression of ICAM-1.(12) Secondly, the presence of unknown constituents in HUVEC medium, particularly in BPE, can give the same kind of effect or, of course, a combination of the two phenomena.

## GENERAL CONCLUSION

We have reviewed, here, different parameters influencing CELISA, of which some are of a particular importance and can affect not only reproducibility but also signal to background ratio, such as the choice of: cell density, a good fixative and blocking agent, the culture medium, and antibody dilutions. The choice of the latter is one of the critical parameters that can affect the measured signal and, consequently, sensitivity.

Flow Chart 1 summarises a five-step protocol to optimise a CELISA. After choosing the antibodies, the blocking agent and the cell line (Step 1), the cell density is calculated according to the assay duration, the growth curve and the cell type (continuous monolayer or not) (Step 2). In the third step, the best second antibody dilution is defined by performing

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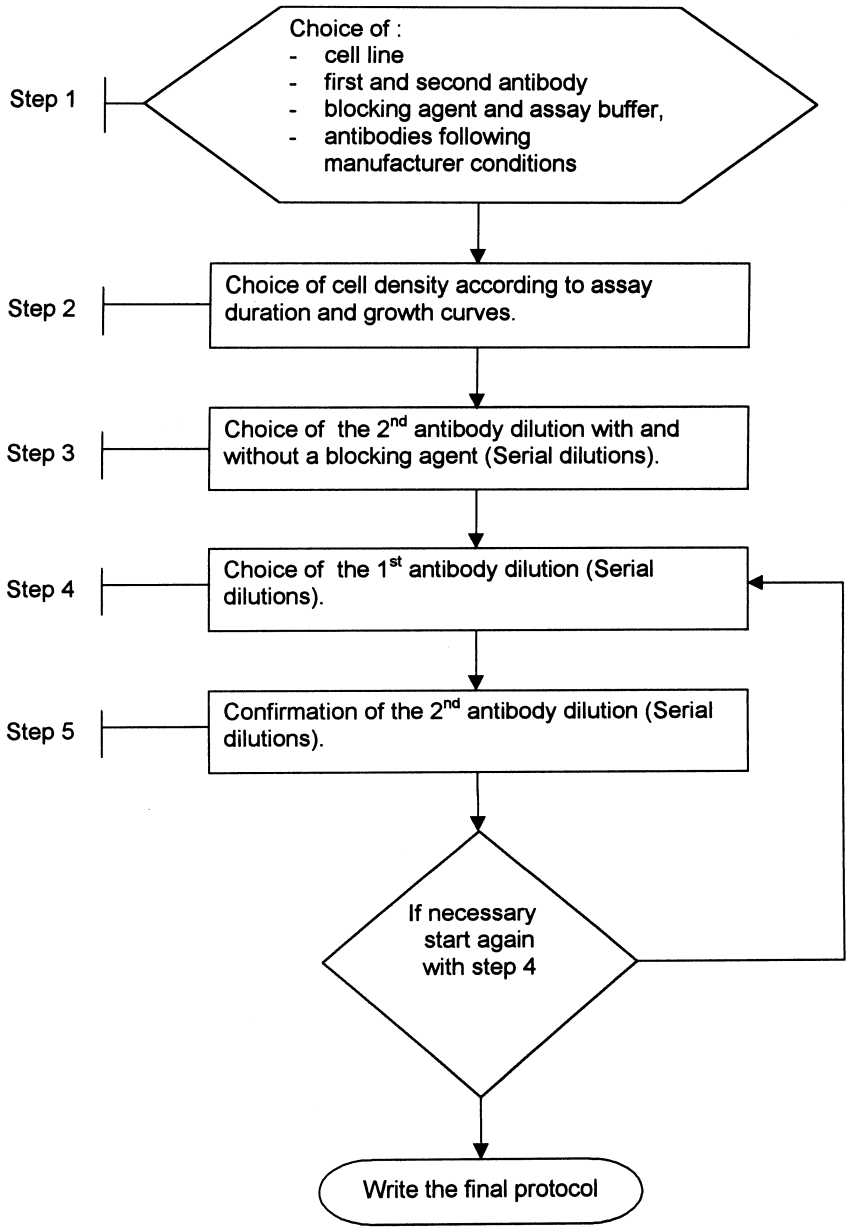


***Flow Chart 1.*** Summary of a five-step protocol to initiate a CELISA optimization.

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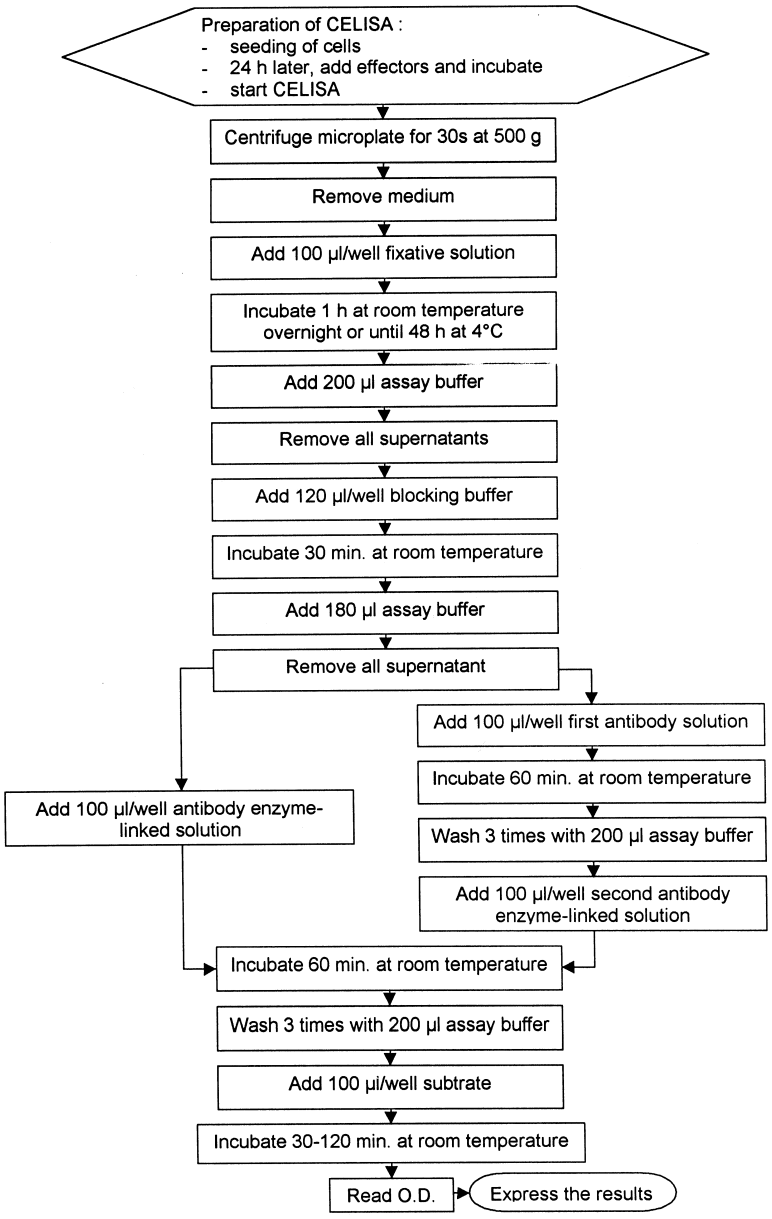


***Flow Chart 2.*** Summary of a step-by-step protocol to design a CELISA.

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serial dilutions in presence or absence of a blocking agent. In the same way, in Step 4, the best first antibody dilution is calculated, from the first half of the plateau (see above). Finally, in Step 5, confirmation of the defined second antibody dilution is done by performing serial dilutions of the latter and by maintaining the first constant. If the result is different, then start again at Step 4. Having all these parameters, the final protocol can be written.

In Flow Chart 2, a detailed step-by-step protocol is given as routinely and currently used in our laboratory to perform a CELISA over an overall 4 to 5 hours time.

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