Detection of a Membrane-Associated Cytokine (Macrophage Colony Stimulating Factor) Using a Modified Commercially Available ELISA

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Many uses of flow cytometry are based on analyzing the exterior membrane antigens found on cells. The percentage of fluorescent cells is calculated by subtracting out the isotypic control value from the experimental values. The intensity of cellular staining is directly proportional to the antigen concentration. However, somewhat cumbersome methodologies are used to quantitate the actual amount of antigen found on these cells by using the flow cytometer (3).

Several cytokines are known to exist either as soluble or membrane anchored form (2). Such cytokines include macrophage colony stimulating factor (M-CSF) also known as colony stimulating factor-1, kit ligand also known as stem cell factor or steel factor, tumor necrosis factor-α, transforming growth factor-α and epidermal growth factor. There are no simple ways to quantitate the amount of these membrane-attached cytokines as there are for their soluble counterparts other than first lysing the cells. Several commercial enzyme-linked immunosorbent assay (ELISA) kits now exist to measure the concentration of these soluble cytokines. Recently, we needed to measure the amount of the membrane isoform of macrophage colony stimulating factor (mM-CSF) present on transfected tumor cells (1). In this report, we describe how we modified a commercially available ELISA to readily measure the amount of mM-CSF found on transfected cells bearing this unique form of a cytokine.

ELISA is based upon the ability of the primary antibody immobilized to the bottom of a 96-well microplate to capture the antigen of choice. The secondary antibody then reacts to those captured antigens. This antibody is conjugated with a colorimetric detection enzyme, such as alkaline phosphatase or horseradish peroxidase, which allows a quantitative analysis to be performed.

In our modification, we took standard 96-well flat-bottom microplates (Corning Costar, Cambridge, MA USA) and treated them first with 100 µL of 1 mg/mL poly-L-lysine (PLL)/phosphate-buffered saline (PBS) solution for 2 h. The unbound PLL was removed by 3 PBS washes before adding the cells. PLL was used because it bound to the bottom of the plate and allowed the cells to stay attached to the bottom of the well, despite the many wash steps needed in the ELISA protocol. Normal adherent cells are removed after the wash cycles, necessitating the need of PLL. The PLL immobilized the entire cell, which allowed the binding of the detection antibody to the external antigens.

We used the human M-CSF Quantikine™ kit (R&D Systems, Minneapolis, MN, USA) because our transfected rat T9 glioma cells were expressing the human mM-CSF gene. This M-CSF ELISA kit is a species-specific test and is not influenced by the host cell’s ability to make rat M-CSF. T9 cells were infected with retroviruses designed to transfect stably either the secreted isoform (sM-CSF) or the membrane isoform (mM-CSF) (1). Both cell types were cloned. The sM-CSF transfected clone, H1, was selected because it produced the highest amount of sM-CSF >2000 pg/mL (10^5 cells were cultured for 3 days). The mM-CSF transfected clone, C2, was selected because it gave the highest fluorescence by flow cytometry and was greater than 95% positive (Figure 1). Both the parental T9 and the sM-CSF transfected clone, H1, were less than 0.5% positive and were considered negative.

The T9 glioma cells and their M-CSF transfected clones were grown in RPMI 1640 media supplemented with 10% fetal bovine serum along with standard antibiotics. These cells were detached by using a collagenase solution (0.5 mg/mL). Afterwards, 10 000 cells in complete RPMI media were allowed to attach themselves to the bottoms of the PLL-coated microplates. Cells were allowed to attach to the PLL at 37°C for 2–3 h. The supernatant was replaced with 100 µL of PBS solution containing 0.3% paraformaldehyde, and the cells were fixed overnight. The cells were washed 3 times with PBS.
followed by 2 washes with the M-CSF ELISA kit wash buffer. The ELISA kit’s horseradish peroxidase-conjugated secondary anti-M-CSF antibody was then added to the cells, and the rest of the procedure was then followed according to the manufacturer’s directions. The Model EL307 ELISA plate reader (Bio-Tek, Winooski, VT, USA) was used to measure the optical density at 450 nm for each well. The calibration curve obtained from the M-CSF standard curve, ranging from 31 pg/mL to 2000 pg/mL, was then applied to the detection of M-CSF found on the cells.

Data from 3 separate experiments consistently showed that no signal was detected from the T9 parental cells, indicating that no endogenous cellular reactions gave false-positive readings. The paraformaldehyde-fixed sM-CSF producing cells, H1, also did not generate any signal, even though these cells produced >2000 pg/mL M-CSF by the M-CSF ELISA. This indicated that the intracellular M-CSF was not detected by the secondary antibody. Reproducible positive results have been generated with the mM-CSF transfected C2 cells. These cells gave 1002 pg ± 61 pg (SD)/10,000 cells. When 5000 C2 cells were tested, they yielded 396 ± 83 pg, while 2500 C2 cells yielded 243 ± 75 pg M-CSF. Thus, only cells bearing the mM-CSF gave a positive signal.

From this experimental result, we calculated how much recombinant M-CSF was required to achieve a 100-fold excess of M-CSF to block macrophage-mediated killing of the C2 clone. Data in Table 1 show that this concentration of recombinant M-CSF significantly blocks the macrophage-mediated killing of the C2 clone in a 24-h assay at each macrophage:tumor cell ratio used. This method to measure mM-CSF required only an ELISA plate reader and an ELISA kit, but did not require a flow cytometer and its calibration beads. Consequently, this technique is available to others who wish to quantify their membrane cytokines, but who do not have access to a flow cytometer. This modification neither required lysing the cells nor required performing complicated mathematics to calculate the concentration using flow cytometry (3), but it simply used the same calcula-

| Macrophage:C2 Ratio | % Specific Release ± SD<sup>a</sup> | Without M-CSF | With M-CSF<sup>b</sup> |
|---------------------|----------------------------------|--------------|----------------|---|
| 20:1                | 19 ± 2                           | 3 ± 1        |               |   |
| 10:1                | 16 ± 2                           | 5 ± 3        |               |   |

<sup>a</sup>Murine bone marrow-derived macrophages were grown in 25% M-CSF-containing supernatant derived from the M-CSF-transfected cells for 3 weeks. After the first week, the macrophages were fed with fresh media containing 25% M-CSF supernatant every 3–4 days. The C2 target cells were cultured overnight in the presence of 8 µCi of [3H]thymidine. Macrophage-mediated cytotoxicity was measured after a 24-h incubation. Each well had 10⁴ C2 cells along with or without various amounts of macrophages. More specific details for macrophage-mediated cytotoxicity were described by Jadus et al. (1).

<sup>b</sup>As identified by our ELISA test, 10⁴ C2 cells possessed 1002 pg mM-CSF, and endotoxin-free recombinant M-CSF (100,000 pg) (Chiron, Emeryville, CA, USA) was added initially to the reaction to achieve a 100-fold excess. At each macrophage:C2 ratio used, the blocked killing was significantly different from that of cells without any recombinant M-CSF (P <0.05 by a Student’s t test).

Figure 1. mM-CSF flow cytometric profile of cloned transfected T9 glioma cells. Cloned transfected T9 glioma cells (H1: sM-CSF; C2: mM-CSF) were incubated with either an IgG1 isotypic or an anti-M-CSF antibody (IgG1; Oncogene Research Products, Cambridge, MA, USA). The surface fluorescence of 10,000 cells was collected. The values of the isotypic controls were subtracted from the anti-M-CSF fluorescence values. The percent positive cells are: 0.23% for T9; 0.19% for the sM-CSF clone, H1, and 95.12% for the mM-CSF clone, C2.
Benchmarks


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Techniques for Colony DNA Hybridization and Protein Immunoassays for a Broad Spectrum of Yeast Genera


Both filter techniques presented here are based on the new principle of disruption of yeast cells. This appears to be the limiting step of all current techniques, where yeast cell walls must be degraded by action of enzymes specific for particular yeast species. Degradation of cell walls is efficient only in exponentially growing cells, and there are some yeasts for which such enzymes are not available. In our approach, we used autolysis for disruption of yeast cells followed by any standard hybridization (Table 1) or immunodetection (Table 2) procedure. The advantages of our methods are as follows: (i) it is possible to induce the autolysis of cells of a broad spectrum of yeast genera by the same procedure (7) without addition of any lytic enzymes; (ii) yeast clones can be autolyzed on membranes in any phase of their growth; (iii) a great number of yeast clones can be analyzed in parallel; and (iv) both methods are cheap and simple.

We demonstrate our filter techniques on the detection of specific DNA fragments in five different yeast genera (Saccharomyces, Schizosaccharomyces, Kluyveromyces, Endomycopsis and Schwanniomyces) and on immunodetection of human MN-protein (10) intracellularly expressed in Saccharomyces cerevisiae.

We applied the described protocol for the detection of the autonomously replicating plasmid pJBt0CHE, both in S. cerevisiae GRF18/pJBt0CHE (4) (Figure 1, lane 1) and in Schizosaccharomyces pombe LP36/pJBt0CHE (5) (Figure 1, lane 4), and for the detection of the integrative plasmid pUCL (pUC18 with the LEU2 gene inserted in the Ps1 site) in S. cerevisiae GRF18/ pUCL (Figure 1, lane 3). The plasmid pHC624 (1), digested with EcoRI and labeled by using the random prime extension technique with [α-32P]dCTP, was used as the specific probe against bacterial sequences (ori, AmpR) present in both tested plasmids. The parental strains, S. cerevisiae GRF18 (α, his3-II, his3-I5, leu2-3, leu2-II2) and S. pombe LP36 (h+, leu1-32), were used as the negative controls (Figure 1, lanes 2 and 5). The presence of specific DNA sequences in three more different yeast genera was analyzed to prove the universality of the technique: cytoplasmic linear double-stranded (ds)DNA plasmids pGKL1 and pGKL2 (2) were detected in Kluyveromyces lactis IFO1267 using the agarose-gel-purified [α-32P]dCTP labeled pGKL DNA as the probe (Figure 1, lane 6). The genes for glucoamylase (GLU1) and α-amy lase (Soa) were detected in Endomycopsis fibuligera CCY42-3-2 (Figure 1, lane 8) and Schwanniomyces occidentalis CCY47-1-1 (Figure 1, lane 10) genomes, respectively. Agarose purified and radioactively labeled DNA fragments, containing either GLU1 from plasmid pSfGlu1 (9) or Soa from plasmid pRS306-Soa (3), were used for hybridization. The strain, S. cerevisiae N247 (a, leu2, ura3) (Figure 1, lanes 7, 9 and 11), in which pGKL, GLU1 and Soa sequences were absent, was used as the negative control.

Intracellular production of human MN protein in S. cerevisiae GRF18 from an inducible GAL7 promoter placed on plasmid pWYGLMN (11)

Figure 1. Detection of specific DNA sequences in yeast. Lane 1, S. cerevisiae GRF18/pJBt0CHE; lane 2, S. cerevisiae GRF18; lane 3, S. cerevisiae GRF18/pUCL; lane 4, S. pombe LP36/pJBt0CHE; lane 5, S. pombe LP36; lane 6, K. lactis IFO1267; lanes 7, 9 and 11, S. cerevisiae N247; lane 8, E. fibuligera CCY42-3-2; and lane 10, S. occidentalis CCY47-1-1.