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Cytokine Protocols

Edited by

Marc De Ley

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Preface

During the last 30 years there has been a growing interest in cytokines as biological molecules able to regulate the most diverse functions in living organisms, mainly at the level of cell–cell communication. Originally their definition was limited to the cells of the immune system (interleukins and lymphokines), but later that definition was extended to all cells, and their regulatory activity in such other processes as differentiation, apoptosis, angiogenesis, and wound healing has been now demonstrated. They comprise a group of small proteins (5–20 kDa) produced and released by cells in a tightly controlled fashion, active in the nano- or picomolar concentration range, and eliciting specific effects in neighboring cells; therefore, their action is said to be autocrine, paracrine, or juxtacrine. The latter property distinguishes them from hormones, which are produced by one tissue and are transported by the blood stream in order to act on a distant tissue. Chemokines are a subset of cytokines, but whether growth factors are included in the group is often a matter of discussion. The activity of several cytokines can be inhibited by other cytokines or by biological response modifiers; therefore, the latter are sometimes called "anti-cytokines." The biological response of a particular cell is usually the result of the sum of all interactions with cytokines present at a certain time and in a certain sequence in time—the "cytokine network." In order to understand the production and action of cytokines, experimental protocols at the DNA, RNA, protein, and (molecular) cell biological level are needed. This volume, *Cytokine Protocols*, in the Methods in Molecular Biology series describes a number of such protocols for specific cytokines, but most of them are broadly applicable and readily adaptable.

The first two chapters deal with DNA itself. Chapter 1 describes the large-scale generation and purification of plasmid DNA for use in such fields as gene therapy, DNA vaccination, transfection, and others, as well as the purity criteria that should be taken into account for such applications. Both the regulation of cytokine production and the expression of cytokine-induced protein synthesis are, at least in part, controlled by DNA–protein interactions, among which is the binding of transcription factors. One of the most direct and powerful methods, not only to demonstrate such interaction, but also to identify the interacting partners, is the gel mobility shift assay, as described in Chapter 2.

The next three chapters are concerned with phenomena at the RNA level. Again, both the induction of cytokine production and the sensitivity to their

action in terms of signal transduction, leading to the biological actions as a result of induced proteins, are in part the result of changes in RNA levels. Chapter 3 describes the generation of stable transfectants with antisense-RNA expression vectors, leading to cell lines with altered sensitivity for cytokines. This methodology allows the identification of signal transduction partners as well as that of alternative paths (leading to "cross-talk," see also Chapter 18). Both the precise quantification and the localization of such RNAs are needed in order to obtain a complete picture. The use of a competitive RT-PCR method in order to quantify small amounts of mRNA is described in Chapter 4, whereas the localization of cytokine-related mRNAs in human tissue biopsies by *in situ* hybridization is described in Chapter 5.

The next five chapters deal with the isolation and characterization of cytokines, cytokine-related proteins, and their interactions. Cytokines, their cellular receptors, and the components of their signal transduction chain are all proteins that are present only at very low abundance. Their isolation and characterization, as well as methods for demonstrating their interactions, are described. Starting from large volumes of conditioned culture media followed by initial concentration steps, Chapter 6 describes the purification of chemokines until homogeneity by consecutive affinity and ion exchange chromatography followed by RP-HPLC. Complete characterization is achieved by SDS-PAGE, sequence analysis, and mass spectrometry, and confirmed by chemical synthesis and determination of biological activity (*see* Chapter 13 for assays of chemotaxis). Similar methodology is described in Chapter 7 for the isolation and characterization of cytokine receptors, including soluble receptors, i.e., the extracellular part of membrane receptors, which occur free in biological fluids (often urine) and are an excellent, more soluble substitute for studying interactions with the corresponding cytokine. Establishment of the biological activity of cytokines is initiated by the formation of a complex with their receptor; hence, great efforts are devoted to the elucidation of this structure down to the atomic level. Chapter 8 describes the common principles and an overview of experimental conditions used for crystallization of cytokine–receptor complexes. Protein–protein interactions are instrumental in the establishment of the biological effects, not only at the receptor level, but also between the partners in the signal transduction chain. One of the most direct means of measuring such biomolecular interactions both in terms of affinity (K_d, dissociation constant) and kinetically (k_{on} and k_{off}) relies on the phenomenon of surface plasmon resonance. The principles and the practical elaboration of this method are the subjects of Chapter 9. The demonstration of such interactions within the signal transduction chain and the importance of posttranslational modifications are described in Chapter 10.

Taking into account the high specific biological activity of most cytokines, they are often best detected and quantified with a very sensitive bioassay and compared to international standard preparations. These functional tests are used initially to detect a cytokine activity; often a cytokine's name refers to this original biological activity. Many cytokines were even first discovered in a functional test, which later proved to be a side activity of its true activity. Therefore, if cytokine concentrations are not expressed in "mass/volume," but in "units/volume," one should clearly describe the kind of assay that has been used and international standardization is an absolute requirement. Because it is not possible to cover all cytokine assays that are used at present, only the next four chapters are devoted to bioassays. The assays for antiviral activity (Chapter 11) and for cytotoxicity (Chapter 12) are rather similar, because, in fact, they both measure the number of cells remaining alive after impairing the viability of the cells. Three chemotaxis assays are described in Chapter 13: two in vitro assays, i.e., chemotaxis through micropores and chemotaxis under agarose, and one in vivo assay, i.e., the migration of ^{111}In-labeled granulocytes (in rabbit). Finally, taking the hematopoietic growth factor granulocyte colony-stimulating factor as an example, Chapter 14 demonstrates that the data obtained from in vitro bioassays must necessarily be complemented or confirmed by in vivo measurements, since the eventual activity of a cytokine may definitely be subjected to secondary factors.

The last five chapters deal with (molecular) cell biological techniques. Chapter 15 describes the generation of stable transfectants that are able to interfere in a controlled fashion with the signal transduction path of a cytokine (in this case TNF-α). Taking into account the low molecular mass (and hence their rapid diffusion out of cells) of most cytokines as well as the extremely low level of their expression, the detection of cytokines at the single cell level is a major challenge. In Chapter 16, the immunohistochemical detection, and even double labeling, of cytokines in tissue sections is described, using either fluorescent or chromogenic substrates. Fixation and permeabilization, though preserving the cellular morphology and the antigenicity of intracellular proteins, seem to be crucial steps in the preparation of biological material. Optimized staining protocols for cytoplasmic, indirect immunofluorescent, or immunocytochemical detection on slides are described in Chapter 17; these are, however, not suited for the detection of antigens in the nucleus. Cytokines are not only pleiotropic signaling molecules, endowed with partially overlapping activities, in part they also use common or synergistically acting intracellular signal transduction compounds for establishing their final biological action. This leads to a phenomenon that is known as "cross-talk," described in Chapter 18. Finally, the presence of noncytokine bioactive compounds, such as, for example, pros-

taglandins, may influence the outcome of cytokine stimulation by altering the ratio of producer or target cells, as described in Chapter 19.

These "cytokine protocols" are intended for use by researchers with knowledge of at least basic biochemistry, molecular biology, and cell culture techniques. It is also an absolute prerequisite to be familiar with biological safety regulations, particularly when working with human biological materials or with recombinant DNA harboring human sequences. Although each individual protocol is written for and applied on a certain cytokine, only minimal changes are required to adapt it to another. The chapters on assay systems may need more modification in order to be broadly applicable.

Marc De Ley

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Contents

1

Large-Scale Generation of Plasmids that Express Type I Interferon

Peter Härle and Daniel J. J. Carr

1. Introduction

Large-scale preparations of plasmid DNA encoding cytokine cassettes are continuously gaining in importance in many areas of gene-therapy *(1)* including DNA vaccinations with plasmid cytokine adjuvants *(2–4)*, tumor research *(5)*, and treatment of infectious diseases *(6,7)*. Small-scale plasmid preparations (approx 10–100 μg DNA) usually result in adequate yields, whereas large-scale (>1 mg DNA) preparations of plasmid DNA can result in disproportionally small yields of DNA. We have found that using Terrific broth (TB) rather than Luria-Bertani (LB) broth in available kits significantly (approx fivefold) increases plasmid DNA yields (*see* **Note 1**). This suggestion is especially true for low or very low copy plasmids. In this chapter, we give an overview of different plasmid purification techniques commonly used, and describe advantages and disadvantages for each method.

If plasmid DNA is used for transfection purposes, it is advantageous to use a method yielding low lipopolysaccharide (LPS) contamination. It is well established that LPS contamination negatively influences transfection efficiency *(8)*. In addition, LPS alone is a strong inducer of chemokine and cytokine synthesis *(9)*. LPS contamination of DNA obtained from commercially available kits is reportedly much lower compared to DNA using a single CsCl gradient purification scheme *(8)*. Another advantage of commercially available kits is the reduced time needed, and the use of less health-hazardous chemicals. Ready-to-use DNA can be obtained with kits in less than 1 d compared to approx 5–7 working days needed for the CsCl preparation. In our laboratory, we transfect murine eyes/vaginas with 100 μg type I interferon (IFN) plasmid DNA per eye and per vagina to protect animals from otherwise lethal herpes simplex virus type 1 (HSV-1) and HSV-2 infections *(6,7)* in the

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hopes of utilizing this technique for the human condition. We have also initiated the generation of human IFN-β plasmid DNA for use in higher vertebrates. In these experiments between 2–4 mg of high-purity (260:280 ratio = 1.7–2.0), supercoiled plasmid DNA is needed.

Figure 1 gives an overview of the process from cloning the type I IFN gene into the desired plasmid vector, isolating the plasmid, and finally using the plasmid constructs in in vitro and in vivo studies.

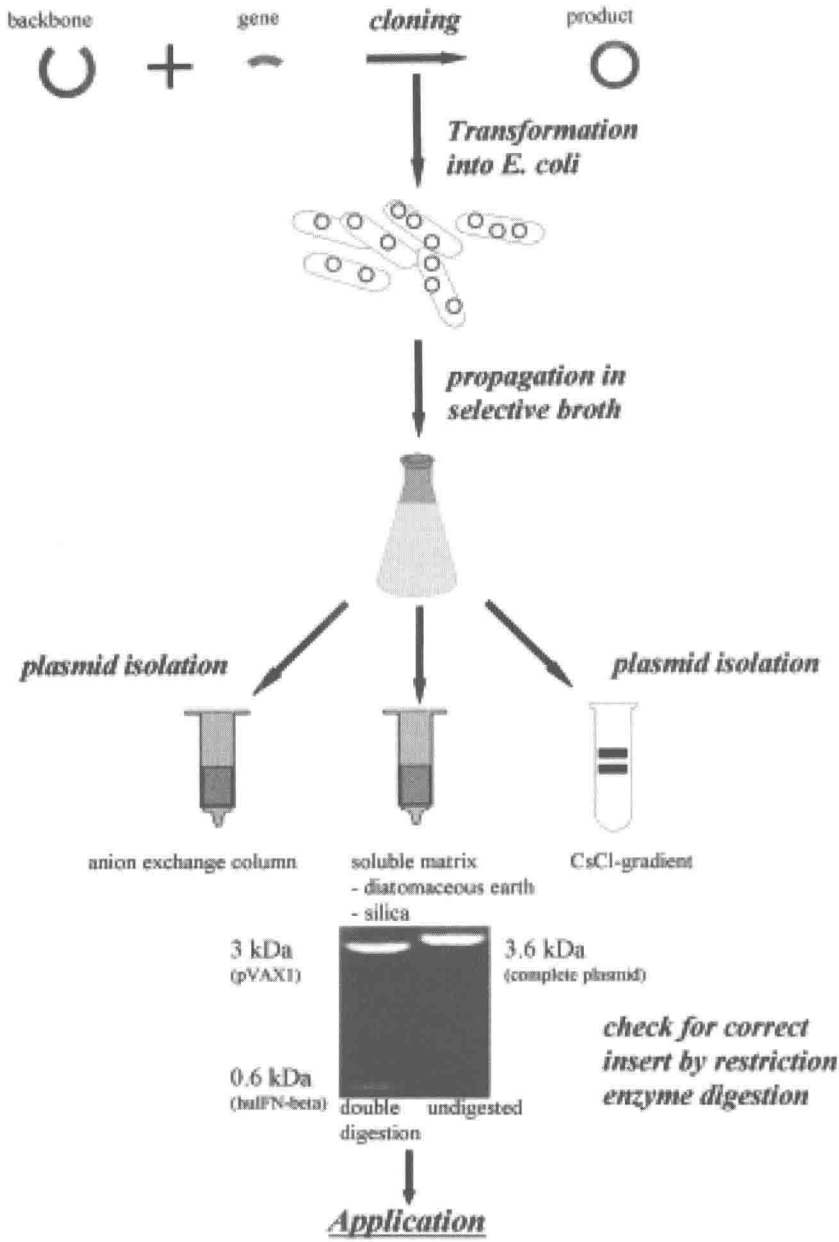
2. Materials

1. Luria-Bertani broth: 10 g/L tryptone peptone and 5 g/L yeast extract (both from Becton Dickinson, Sparks, MD), and 10 g/L NaCl (Fischer, NJ). After all ingredients are dissolved, autoclave broth immediately and let it cool before inoculating with bacteria. We use 1 mL of frozen bacterial stock (1 mL bacterial culture gently mixed with 480 µL of 50% sterile glycerol, stocks frozen at –80°C) per L TB (*see* **Note 2**).
2. TB: Prepare a stock solution of 0.17 *M* KH_2PO_4 (11.5 g/500 mL), 0.72 *M* K_2HPO_4 (62.7 g/500 mL) (J.T. Backer Inc., Phillipsburg, NJ) and autoclave. Mix 12 g/L trytone peptone and 24 g/L yeast extract (both from Becton Dickinson), and 4 mL glycerol (Fischer), adjust with 900 mL deionized water, and autoclave. When the broth has cooled, add 100 mL of your sterile stock potassium phosphate solution and inoculate broth (for bacterial inoculum, *see* **item 1**).
3. Agar plates: To prepare the plates, use the same broth as described above and add 15 g/L agar (DIFCO Laboratories, Detroit, MI) and pour into sterile dishes after adding the selective antibiotic.
4. Miniprep kits: Qiaprep® Miniprep (Qiagen, Valencia, CA) or Wizard® Minipreps (Promega, Madison, WI).
5. TE buffer, LPS free (GIBCO, NY).

3. Methods

1. Take three agar plates containing the recommended concentration of selective antibiotics. Transfer 25 µL, 50 µL, and 100 µL of the transformed bacteria suspension onto the agar plates, spread the liquid evenly, and incubate plates at 37°C for 24 h.
2. Using a sterile wire loop, select five typical, single colonies (2–3 mm diameter) and inoculate them separately into 6 mL of TB. Incubate in a shaking incubator at 37°C, 250 rpm for about 8 h.
3. Isolate DNA from the 8 h cultures, using plasmid miniprep kits for each day culture (5 mL) to find the sample with the highest DNA yield (minipreps take about 1 h).

Fig. 1. *(opposite page)* The process from cloning the type I IFN gene (we used human/macaque type I IFN) into the FDA-approved pVAX1 plasmid vector (Invitrogen®, Carlsbad, CA) (3000 bp size) to the application. The plasmid is transformed into the *Escherichia coli* strain INVαF' One shot® (Invitrogen®) and grown up



in TB followed by purification using different methods. The insert, in this case human IFN-β (600 bp), was verified using restriction enzyme digestion (*Kpn*I and *Xho*I, New England Biolabs, Beverly, MA), yielding a 600 bp and 3000 bp band on a 1% agarose gel, containing 0.5 μg/mL ethidium bromide. Freshly isolated high-grade DNA is then used for in vitro and in vivo studies.