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Monica Rinaldi
Daniela Fioretti
Sandra Iurescia *Editors*

# DNA Vaccines

## Methods and Protocols

*Third Edition*

# Methods in Molecular Biology

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## Third Edition

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# Preface

Vaccination has had a profound positive effect on the quality of public health. Vaccines have long been used to combat infectious disease; however, the last decade has witnessed a revolution in the approach to vaccine design and development. Several groundbreaking studies demonstrated that immunological responses could be generated against antigenic transgenes delivered via DNA vaccination. Since then new sophisticated technologies, advances in molecular biology techniques, and new bioinformatics analysis tools to study and manipulate the basic elements of an organism's genome have been used for the rational design and production of DNA vaccines.

Nowadays, DNA vaccination is the most important early application of nonviral gene therapy, and it seems clear that the route of DNA vaccine and the methods of vaccine preparation have strong effects on the immune response and the effectiveness of that response in preventing or treating disease.

*DNA Vaccines: Methods and Protocols, Third Edition* reviews innovative approaches and technologies used to design, deliver, and enhance the efficacy of DNA vaccines. In this book, expert international authors critically review the current cutting-edge research in DNA vaccine design and development. Topics also include methods of production and purification. The book also has chapters on recent DNA vaccine applications which should be of great value in moving vaccines from research to clinic. All of these chapters, as well as the others presented in the previous *DNA Vaccines* editions, have the important role of further documenting the potential of the DNA vaccination as a platform technology for treatment and prevention of human diseases suitable also for developing nations. Several peculiar features of DNA vaccines (i.e., preparation and purification, stability, cost-effectiveness and non-requirement of cold chain) emphasize this prospect.

The current status of three gene vaccines licensed for veterinary use (i.e., the West Nile virus DNA vaccine for horses, a fish DNA vaccine against the Infectious Haematopoietic Necrosis virus, and the Canine Malignant Melanoma vaccine (ONCEPT™)) will pave the way for future application in humans.

To date, no human DNA vaccine has been licensed; however, during recent years, more than 100 clinical trials have been undertaken worldwide on DNA vaccines covering the full range of prophylactic through to therapeutic vaccines against infections, cancers, and a range of other disorders (details at: http://www.dnavaccine.com/; http://clinicaltrials.gov; http://www.cancer.gov/clinicaltrials).

DNA-based vaccine technology has moved from pioneering animal studies to clinical testing quite rapidly. However, more work is still required on design and delivery to lift the immunogenicity of DNA vaccines to the levels required for human regulatory approval and commercial exploitation.

Consistent with the approach of the *Methods in Molecular Biology* series, *DNA Vaccines, Third Edition* contains detailed practical procedures on the latest DNA vaccine technology and is recommended to microbiologists and vaccinologists, immunologists, infectious diseases and public health physicians, and to the many scientists working on vaccine development (e.g., biochemists, and molecular biologists).

In conclusion, we hope that this book will be a productive opportunity to further push the recent improvements in DNA vaccine technology to their full clinical potential, moving from the benchtop to the patient.

***Rome, Italy***

***Monica Rinaldi***
***Daniela Fioretti***
***Sandra Iurescia***

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# Part I

## DNA Vaccine Design and Enhancement

# Chapter 1

## A Blueprint for DNA Vaccine Design

**Sandra Iurescia, Daniela Fioretti, and Monica Rinaldi**

### Abstract

Although safety concerns have been overcome, lower immunogenicity profiles of DNA vaccines have hindered their progress in humans. DNA vaccines need to make up for this limitation by altering plasmid construction through vector design innovations intended for enhancement of transgene expression and immunogenicity. The next-generation vectors also address safety issues such as selection markers. This chapter discusses (a) plasmid backbone design, (b) enhancement of antigenic protein expression and immunogenicity, and (c) vector modification to increase innate immunity. Modifications of the basic design, when combined with improved delivery devices and/or prime/boost regimens, may enhance DNA vaccine performance and clinical outcomes.

**Key words** DNA vaccines, Plasmid design, Regulatory elements, Antigen, Immunogenicity, Transgene expression, Marker selection, DNA uptake, Innate immunity

## 1 Introduction

DNA vaccine efficacy is hampered by low level of transgene expression. Basically, a DNA vaccine consists in a self-replicating, circular double-stranded DNA molecule (the plasmid) joining bacterial regions necessary for selection and replication in *E. coli* host with eukaryotic sequences that regulate expression of the encoded antigen in the target tissue.

Ongoing efforts are focused to optimize the DNA vaccine platform to improve antigen (Ag) expression and immunogenicity as increased antigen expression correlates with improved immunogenicity and augmented levels of immune response in humans and large animal models [1].

This blueprint presents an overview of the issues facing the development of a platform technology for the production of improved DNA vaccines. Specifically, this chapter deals with strategies relevant to (a) engineering the plasmid backbone to obtain a

Sandra Iurescia and Daniela Fioretti contributed equally to this work.

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more efficient expression plasmid, (b) enhancement of antigenic protein expression and immunogenicity, and (c) complementary plasmid design modifications to increase innate immunity. All modifications outlined herein are made at the level of molecular cloning (*see* **Note 1**).

The aspects next described depict potential design strategies and technologies necessary to carry out the development of improved DNA vaccines. A selection of relevant patents employed to improve DNA vaccine immunogenicity through several strategies such as the use of tissue-specific transcriptional elements, nuclear localization signaling, and codon optimization are reported in Fioretti et al. [2].

## 2 Materials

1. Plasmid DNA backbone.
2. DNA coding for the transgene.
3. Oligonucleotide primers to amplify target transgene from the DNA template by polymerase chain reaction (PCR) (*see* **Note 2**).
4. Regulatory elements addressing antigen expression and immunogenicity augmentation.

## 3 Methods

### 3.1 Plasmid DNA Backbone

DNA vaccine design is relatively simple. Basically, the construction of a DNA vaccine requires a plasmid backbone such as pcDNA™3.1 or pVAX1© (Invitrogen, Life Technologies Corporation). An important component of the plasmid backbone is the promoter that drives expression of the transgene of interest.

For most expression plasmids, the human cytomegalovirus (CMV) promoter (reviewed in ref. 3) is the usual choice as it promotes high-level constitutive expression in a wide range of mammalian cells. Some early investigations reported that CMV immediate/early enhancer/promoter activity was consistently the highest among several constructs tested in mammalian somatic tissues [4, 5]. Otherwise, the use of host tissue-specific promoters prevents antigen expression in inappropriate tissues yet leading to sufficient stimulation of immune responses [6]. A Kozak sequence conforming to the consensus gccgccRcc<u>AUG</u>G (R = G or A, AUG start codon underlined, critical residues in caps) is included immediately prior to the ATG start codon ensuring efficient translation. Approaches to increase transcription and translation thereby improve DNA vaccine immunogenicity. This can be achieved by optimization of regulatory elements in the plasmid backbone [2]. The inclusion of an intron (i.e., the intronA of the CMV immediate–early

gene) in the vector backbone downstream of the promoter can enhance the stability of mRNA and may improve gene expression. Furthermore, the presence of the polyadenylation (polyA) signal site, which contains accessory sequences upstream and downstream of the polyA tail, ensures proper termination of transcription, increased mRNA levels, and export of the mRNA from the nucleus resulting in improved transgene expression. Both enhancer elements and transcriptional transactivators may increase promoter efficiency. For example, incorporation of the human T-cell leukemia virus type I R 5′ untranslated region (UTR) (HTLV-I R-U5) downstream of the CMV promoter increased mRNA translation efficiency [7] and immunogenicity of DNA vaccines encoding multiple antigens in small animals and in nonhuman primates [8].

The transcribed 3′ and 5′ UTRs should not contain cryptic open reading frames (ORFs) since unforeseen immunogenic epitopes could be generated able to elicit a cytotoxic T lymphocyte (CTL) response [9].

A critical step of the DNA vaccine design is a careful selection and assembly of bacterial regions that provide both replication origin and selection marker necessary for propagation in different *E. coli* host strains. The compositions and orientations of the so-called relaxed origins of replication (i.e., pUC origin) can interfere with the transgene expression, manufacturing yields, and plasmid quality. Reduced expression may in part be due to the presence of TATA-containing cryptic promoter within the replication origin or the selectable marker generating spurious transcripts that triggers protein kinase R (PKR)-mediated selective translational shutdown or RNA interference (*see* Williams J.A. for a review) [10].

The use of antibiotic-resistance selection markers in DNA vaccines has safety issues and represents a key aspect for high-scale plasmid production. The European Medicines Agency (EMA) stated that “neomycin and kanamycin are of importance for veterinary and human use and that their current and potential future use cannot be classified as of no or only minor therapeutic relevance” due to current use in critical clinical settings [11]. To address these regulatory concerns, alternative non-antibiotic selection methods are being developed [10, 12]. In Chapter 6 of this book, Williams and colleagues describe the development of RNA-based antibiotic-free selection system for safer DNA vaccination.

In vector development, an important objective is plasmid backbone shortening and removal of bacterial elements. Plasmid size reduction improves pDNA structural stability and enhances the transfection efficiency, leading to increased duration of antigen expression [10, 12]. Overall, smaller plasmid size is therefore beneficial for gene delivery efficiency. Minicircle DNA technology was developed to obtain a plasmid backbone nearly devoid of any prokaryotic sequence by using site-specific recombination. The resulting miniplasmid contains almost exclusively the gene of interest

and its regulating sequence motifs. The most recent developments in the field of minicircles are reviewed elsewhere [13]. Minicircle DNA-based gene therapy has been successfully exploited in mice [14] and in generation of adult human induced pluripotent stem cells (hiPSCs) [15].

To permit insertion of the sequence coding for the antigen, the plasmid should also contain a synthetic 100-base pairs DNA sequence (the multiple cloning site, MCS). The target transgene is often available in another construct but rarely presents useful restriction sites to allow directional cloning of the insert into the plasmid vector. Therefore, oligonucleotide primers are designed to contain appropriate restriction sites upstream of the ATG codon and downstream of the stop codon to amplify target transgene from the DNA template by PCR (*see* **Notes 1** and **2**). Selection of different restriction sites guarantees the insertion of the fragment in the MCS in the correct orientation for the transcription and translation.

### 3.2 Enhancement of Transgene Expression

Gene inserts traditionally are transferred from a genomic DNA construct directly into a DNA vaccine vectors. Notably, following the gene delivery the level of antigen expression is affected by the rate of transcription and translation. Therefore, some approaches have proved to be very helpful in enhancing antigen immunogenicity such as the effect of codon bias on rate of expression. To increase the expression of the encoded transgene, synthetic genes can be constructed such that the codons employed are those with a higher frequency of mammalian tRNA than codons present in the native sequence (i.e., codon optimization) [16]. Codon optimization matching high-use codons for the target species has been shown to dramatically increase transgene expression and immunogenicity of the DNA vaccines [17]. An optimal coding sequence is back translated from the amino acid sequence of the antigen by algorithms (*see* **Note 3**) that take into account the abundance of specific tRNAs in the cytosol of human cells and the predicted structure of the mRNA. Thereafter the synthetic gene sequence is designed and synthesized in vitro. Adverse rare codons are avoided, and secondary structures in the mRNA are minimized [18]. Thereby, the target transgene codons can benefit from codon reengineering to correspond to the available pool of tRNAs at the vaccination site leading to the induction of a specific immune response.

It is recommended, for DNA vaccines intended for potential licensure, that new gene inserts should be designed *de novo* for compatibility, regulatory compliance, and improved eukaryotic expression and then made synthetically. Gene optimization is an important factor for a successful protein expression, and synthetic transgene design is a critical step to maximize the expression of synthetic genes. Some multiparameter DNA sequence optimization procedures such as the GeneArt GeneOptimizer® software take codon usage, GC content, mRNA structure, and species-specific

sequence motifs into account [17] (*see* **Note 3**). For pathogens containing various serotypes or amino acid variations, determination of amino acid sequence may involve defining a "consensus immunogen" to engineer a single broadly cross-neutralizing antigen [1, 19]. Consensus vaccines offer novel means of inducing cross-reactive cellular and humoral immune responses in humans. The ability to induce such responses would be a significant advance in the development of next-generation vaccines, especially for seasonal and pathogenic H5N1 influenza viruses, which have become endemic in many countries. These vaccines are designed using a large number of primary viral sequences and as such contain the most highly conserved characteristics of each. These synthetic constructs, with in vivo electroporation, have the ability to induce strong $CD8^+$ and $CD4^+$ cellular immune responses in small and large animal models of vaccination. Furthermore, the synthetic consensus antigens result to be more cross-reactive than their individual component antigens as showed by the ability of inhibiting divergent viruses of the H5N1 subtype [20].

A flow chart for synthetic transgene design to generate a codon-optimized antigen gene is presented in Chapter 6 of this book.

Given that plasmid DNA nuclear import is coupled to active transcription, DNA nuclear targeting sequence (DTS) could be introduced to increase the efficiency of nuclear plasmid uptake from cytoplasm and to selectively improve extrachromosomal transgene expression especially in nondividing cells, e.g., after intramuscular injection [7, 21, 22]. The sequence of Simian virus 40 (SV40) enhancer that is known to bind to distinct, ubiquitously expressed, transcription factors and to mediate plasmid nuclear entry in all cell types tested was mapped to a 372 bp region of the DNA (*see* Lam and Dean for a review) [22].

Chapter 2, "Enhancement of plasmid-mediated transgene expression," of this book elaborates on the basic concept described here.

Long-term tissue Ag expression may be achieved using scaffold/matrix attachment region (S/MAR) as *cis*-acting elements to maintain the episomal status of the circular vector [23]. Actually, Argyros et al. demonstrated prolonged expression of such vector-encoded transgenes (i.e., 6 months) in mouse tissues. Development of S/MAR minicircles led to higher and more sustained expression of transgene in vitro and in vivo [24].

### 3.3 Vector Modifications to Increase Innate Immunity

Recent advances provide insights into molecular and cellular mechanisms by which double-stranded structure is essential for DNA vaccine-induced immunogenicity. After transfection, DNA is sensed in the cytoplasm through nucleic acid-sensing immune machinery that uses mainly two types of immune triggers. First, immunostimulatory elements in the plasmid backbone such as