

## Design of Plasmid DNA Constructs for Vaccines

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### Summary

For more than three decades, plasmids have been widely used in the biotechnology arena. Historically, they have been most often employed for the expression of heterologous proteins in a variety of microorganisms. More recently, plasmids have been used as vectors for the delivery of antigen encoding genes in order to elicit immune responses in higher order animals. In this chapter, we discuss methods for constructing vectors with this unique purpose. Considerations for choosing the replicon, antigen, expression elements, and host cells are discussed within the context of developing a commercially viable vaccine vector.

**Key Words:** Vaccine vector; pUC vectors; pVAX1; antigen.

### 1. Introduction

An ideal vector for DNA vaccines should be safe in humans and easily produced at commercial scale. The most obvious safety issue for a DNA vaccine vector is the possibility of the plasmid integrating into the human chromosome (1,2). To minimize the risk of integration, the vector should not replicate in mammalian cells. Therefore, a vector should be chosen that does not contain a mammalian origin of replication (ORI). To further minimize the possibility of the vector integrating into the human genome, the vector sequence should be blasted into the human genomic sequence to make sure there are few, if any, strong homologies to human genes (*see Note 1*).

Large numbers of molecules per dose are required for an effective DNA vaccine; therefore, a commercially viable vaccine must give high yields of plasmid, preferably through a simple production process. For this reason, bacterial plasmids propagated in the well-studied Gram-negative bacterium, *Escherichia coli*, is the most widely used production system. The bacterial replicon of a DNA vaccine vector has to allow high yield of plasmid molecules to meet the commercial needs, given that the issues of potential integration into the host

genome have been addressed. Because pUC-based vectors yield between 500 and 700 copies per bacterial cell and are readily available as a result of their widespread use for recombinant protein expression, they have been the basis of many DNA vaccine vectors (3–6). Unlike their ancestral ColE1-type vectors, pUC plasmids do not require amplification to achieve high yields from the fermentation process, although the copy number can be increased by manipulating the growth rate of the host cells (7–9). From a process development perspective, so-called “runaway replication” vectors also seem to be an attractive choice for DNA vaccines. Such plasmids are initially low-copy but loss of replication control can be induced to cause accumulation of plasmid DNA to high levels, up to copy numbers near 1000 (10,11). Induced amplification, usually through a temperature shift, would result in a slightly more complicated fermentation process to achieve the desired high DNA yields. These authors found no examples of such vectors being exploited for DNA vaccine production.

In addition to carrying the sequences necessary for replication in bacteria, the plasmid must also contain a selectable marker for growth in *E. coli*, which is usually a drug resistance gene. This gene cannot confer resistance to penicillin or other  $\beta$ -lactam antibiotics as these can cause severe allergic reactions in humans, and the use of such antibiotics in the manufacture of products for humans is not permitted by the Food and Drug Administration (FDA) (12). The selectable marker should be the only gene that is expressed in *E. coli*, because bacterial growth and plasmid production can be adversely affected by the expression of multiple genes, especially if the gene products are toxic. Taking all of these issues into consideration, a good DNA vaccine vector should be designed with minimal functions such that the only gene expressed in *E. coli* is the selectable marker and the only gene expressed in mammalian cells is the antigen. Any additional plasmid functions, such as an *f1* (+) origin or *lacZ* gene, should be removed.

The mammalian promoter and polyA termination signal also need to be addressed. The amount of plasmid that is internalized in vivo has been estimated to be in the picogram range (13) after injection into mouse muscle and in the picogram to femtogram range in tissues from 1 to 7 d after intravenous delivery of DNA (14). Because the plasmid will not replicate in the cells, the amount of plasmid available for expression is very low. For this reason, a strong mammalian promoter/terminator should be chosen to drive expression of the antigen gene. Attention should be paid to the transcription terminator used in conjunction with the promoter. We have found that the choice of the transcription terminator/polyA signal can have a dramatic effect on the strength of the promoter (unpublished data). The combination of the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) terminator provides a high level of transcription (3,4,15,16).

The vector that we designed consists of a pUC backbone, the CMV promoter with intron A, the BGH terminator, and a kanamycin resistance gene and has been described in previous papers (4,5,17). This vector can be obtained from Vical ([www.vical.com](http://www.vical.com)). There are two forms of the vector: one requires that all translation signals be cloned in with the gene of interest, and the other is a fusion vector where the gene can be fused in-frame to the signal sequence of the human tissue-specific plasminogen activator (tPA) gene for secretion. There are also commercially available vectors that have been designed for DNA vaccines. For example, Invitrogen sells pVAX1, which is similar to our vector except that it contains the CMV promoter without intron A. It is a nonfusion vector and requires that the inserted gene contains Kozak translation initiation sequence (Kozak), an initiation codon (ATG), and a termination codon (TAA, TGA, or TAG). Both the vector from Invitrogen and the vectors from Vical are designed to stimulate cellular as well as humoral immune responses.

InvivoGen also sells pVAC1-mcs and pVAC2-mcs, which are designed to elicit a humoral immune response, with the antigen targeted and anchored to the muscle cell surface for immune processing. In pVAC1-mcs, the antigen gene is fused to the IL2 signal sequence for secretion and to the C-terminal transmembrane anchoring domain of the human placental alkaline phosphatase gene. pVAC2-mcs was designed for secretory antigens which have their own signal sequences but these antigen genes are still fused to the transmembrane anchoring domain. These vectors also contain a reduced number of CpG motifs to keep the response focused on humoral immunity (18–20).

The choice of vector for a DNA vaccine depends on the type of immune response desired. This chapter will use pVAX1 from Invitrogen as an example for cloning the desired antigen. Modifications would need to be done to put a gene into other vectors.

## **2. Materials**

### **2.1. Generation of the Antigen Insert**

1. *Pfu* DNA polymerase (Stratagene).
2. QIAquick Gel Extraction Kit (Qiagen).
3. Agarose gel electrophoresis equipment.
4. Restriction enzymes.

### **2.2. Insertion of the Antigen Gene Into the DNA Vaccine Vector**

1. pVAX1 (Invitrogen).
2. Agarose gel electrophoresis equipment.
3. Rapid Ligation Kit (Roche).
4. DH5 competent cells (Invitrogen).
5. Luria broth and agar plates (Teknova).

6. Kanamycin (Teknova).
7. QIAprep Miniprep Kit (Qiagen).

### **2.3. Verify Clones by Sequence and Expression**

1. T7 promoter and BGH reverse sequencing primers (Invitrogen).
2. DNA sequencing equipment (or service).
3. Rhabdomyosarcoma (RD) cell (ATCC CCL136).
4. Dulbecco's modified Eagle's medium (DMEM) (Cellgro).
5. Fetal calf serum (FCS) (Cellgro).
6. Penicillin/streptomycin (Cellgro).
7. L-glutamine (Cellgro).
8. Lysis buffer: 1% Nonidet-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NaN<sub>3</sub> supplemented with complete, ethylene-diamine tetraacetic acid (EDTA)-free protease inhibitor cocktail tablets (Roche).
9. Tissue culture facilities.
10. Glycerol.
11. Sonicator.
12. Coomassie protein assay reagent (Pierce).
13. 12% Tris-glycine gels (Novex).
14. Immobilon-P membranes (Millipore).
15. Western Breeze (Invitrogen).
16. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transfer equipment.

### **2.4. Choosing the Best *E. coli* Strain for Plasmid Production**

1. Several strains of competent *E. coli*.
2. NaOH, SDS, potassium acetate, ethanol, and isopropanol (for plasmid isolation).
3. Agarose gel electrophoresis equipment and/or spectrophotometer.

### **2.5. Preparation of Seed Stocks**

1. L-Broth and agar plates with kanamycin broth and agar plates, or
2. Defined medium broth and agar plates.
3. Sterile 40% glycerol (in water).
4. 2-mL cryotubes.

## **3. Methods**

The methods described next include the steps needed to (1) generate the antigen gene, (2) insert the gene into pVAX1, (3) verify clones by sequencing and checking expression in transiently transfected cell lines, (4) choose the *E. coli* strain for production in fermentation, and (5) prepare a seed stock for fermentation. Standard molecular biology procedures used for DNA manipulations can be found in **ref. 21**.

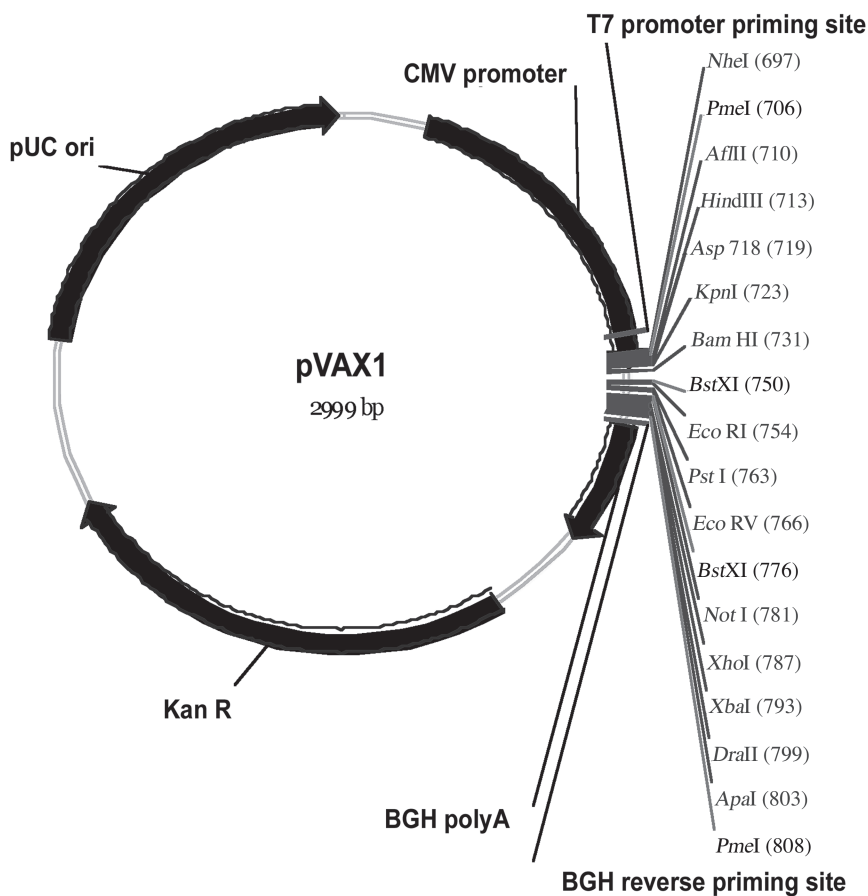


Fig. 1. Map of plasmid pVAX1 (Invitrogen).

### 3.1. Generation of the Antigen Insert

1. Design primers: prepare a restriction map of the antigen sequence and chose two restriction sites in the pVAX1 multiple cloning site (see **Fig. 1**) that do not appear in the antigen (see **Note 2**). These sites will be added to the gene of interest as it is generated by polymerase chain reaction (PCR) so that the antigen gene can be directionally cloned (see **Note 3**). For example, if *NheI* and *XhoI* are chosen for the cloning sites in pVAX1, the *NheI* site will be added to the 5'-end of the antigen gene and the *XhoI* site will be added to the 3'-end of the gene. The 5'-end of the gene will also need to contain the Kozak sequence and an ATG from the gene (see **Notes 4** and **5**), and the 3'-end will need to contain a termination codon. An example of PCR primers for cloning a viral gene into pVAX1 is given in **Fig. 2**.

1. Clones that are identified by restriction mapping should be further verified by sequencing the insert. This can be done using the T7 promoter-priming site and

the BGH reverse priming site present in the pVAX1 vector (*see Fig. 1*). Both of these primers are available from Invitrogen. If the inserted gene cannot be completely sequenced from a single read with these two primers, additional sequencing primers will need to be used. The primers can be designed from the antigen sequence to be complementary to the cloned gene. These internal primers should be selected to overlap at least 20–50 bases of the sequence that will be obtained from the first two primers to ensure complete coverage.

2. Check for expression of sequence-verified clones in a transiently transfected mammalian cell line before using the DNA vaccine in animal experiments. A cell line such as rhabdomyosarcoma (RD) cells (ATCC CCL136) is a good choice because it is easily maintained and transfected. Seed the cells at  $8 \times 10^5$  cells/100-cm plate in DMEM supplemented with 10% FCS, 4 mM L-glutamine, and 100  $\mu$ g/mL each of penicillin and streptomycin. Grow the cells at 37°C, 6% CO<sub>2</sub> for 24 h.
3. Transfect the cells with 10  $\mu$ g of plasmid using a CellPfect transformation kit (Amersham Bioscience), following the manufacturer's procedure. Glycerol shock the cells 5 h after transfection by treating them with 15% glycerol in phosphate-buffered saline (PBS), pH 7.2, for 2.5 min. The glycerol shock is needed to enhance gene transfection. Refeed the cells and let them grow for 48–72 h.
4. Harvest cells and lyse them in lysis buffer. Sonicate briefly to reduce viscosity.
5. Determine the protein concentration of the cell lysates by Bradford analysis (22) using Coomassie Protein Assay Reagent (Pierce) and load equal amounts of total cell protein on 12% Tris-glycine gels (Novex).
6. Transfer to Immobilon-P membranes (Millipore) and detect the antigen expression using Western Breeze (Invitrogen) kit, following the manufacturer's procedure (*see Note 9*).

### 3.4. Selection of *E. coli* Strain for Plasmid Production

Many *E. coli* strains are commercially available as competent cells and have been developed for specific needs in molecular biology. Most strains have been characterized for protein expression, not for plasmid stability and production; therefore, several strains should be tested prior to choosing a host strain. The desired fermentation medium should be carefully considered when deciding which strains to survey. A chemically defined medium, consisting primarily of salts and a carbohydrate carbon source such as glucose, may be desirable for use in the final production process. This provides process robustness because of the ability to chemically characterize each of the medium components (23). If a chemically defined medium is desired, candidate strains should be chosen with few auxotrophies to minimize the additional expense and labor associated with medium preparation (*see Note 10*). Once several candidate strains are selected, each should be tested with the final vaccine clone to determine which will grow at reasonable rates (2 h or less doubling time), achieve a high cell density, and give the highest plasmid DNA yields in the fermentation medium in large-scale production.

1. Transform multiple strains of *E. coli* with a 1/100 dilution of a QIAprep miniprep of the final plasmid construct. This low concentration of plasmid DNA should ensure that each cell is likely to receive only one plasmid copy. This will allow detection of low-level contamination of the prep with empty vector.
2. Pick several colonies from each transformation, make minipreps of each, and verify the plasmid by restriction mapping.
3. Prepare a large-scale plasmid preparation of one to five clones from each of the *E. coli* strains. Inoculate a 1-mL L-broth with kanamycin culture with a colony and grow at 37°C, 250 rpm for 5–8 h. Use this fresh culture to inoculate 500 mL of the desired fermentation medium (e.g., a chemically defined medium) in a 2-L flask, and grow the large cultures overnight at 37°C, 250 rpm. Samples can be collected hourly for 4–5 h for measurement of the optical density at 600 nm if calculation of a growth rate is desired.
4. Harvest the cells by centrifugation and lyse by a modification of the alkaline SDS procedure (21). The modification consists of increasing the volumes threefold for cell lysis and DNA extraction. At this step, differences in cell growth rates, maximum cell densities, and ease of cell lysis can be determined.
5. Determine the yield of plasmid DNA from each set of strains. This can be done qualitatively by comparing the band intensities of plasmid DNA minipreps isolated from each strain following agarose gel electrophoresis. Alternatively, the DNA concentration in each prep can be quantified by measuring the absorbance at 260 nm, where  $A_{260}$  of one corresponds to a concentration of approx 50 µg/mL double-stranded DNA. With either method, the specific yield (i.e., the amount of plasmid DNA per unit of biomass) should be used to determine the most productive strain since cell density can be optimized in the fermentation step. At this step, any significant heterogeneity in productivity among various clones of the same strain can be identified. The plasmid prep(s) can be further purified through a CsCl-ethidium bromide gradient for use in animal studies as needed (21) (see **Note 11**). The plasmid prep should be double-banded, then treated with *n*-butanol to remove the ethidium bromide (EB), phenol/chloroform extracted to remove any traces of endotoxin, and ethanol precipitated. Special attention should be paid to removing endotoxin because it can induce inflammation, and give a false sense of immunogenicity.

### 3.5. Preparation of Seed Stocks

Once the appropriate *E. coli* strain has been identified, seed stocks need to be generated and stored at –80°C. The stocks are prepared from mid-exponential phase cells so that the thawed vials can be used to directly inoculate a seed flask with minimal lag time.

1. Streak the selected culture (see **Subheading 3.4.**) onto an L-agar with kanamycin agar plate and let it grow overnight at 37°C. If a defined medium is being used for production, the culture should be streaked on a corresponding agar plate.
2. Inoculate 5–25 mL of L-broth with kanamycin (50 µg/mL) in chosen defined culture medium with 5–10 single colonies and let the cultures grow overnight.



3. Isolate plasmid DNA from an aliquot of each culture and evaluate qualitatively or quantitatively as described in **Subheading 3.4., step 5** to ensure that there are no obvious differences in clonal productivity within each group. Verify plasmid identity by restriction mapping and select a clone to carry forward as a stock.
4. Using an aliquot of the remaining culture from **step 3**, inoculate 50 mL L-broth with kanamycin (50  $\mu\text{g/mL}$ ) with a 1% inoculum and allow the culture to grow to mid-exponential phase at 37°C with shaking at 250 rpm. Mid-exponential phase must be determined based on the specific strain and growth medium being used. As a general guideline, cells can be harvested when the optical density measured at 600 nm is one-half of the maximum (stationary phase) cell density. A full growth curve can also be measured to determine the range of densities for mid-exponential phase.
5. Add an equal volume of 40% sterile glycerol to a final concentration of 20% and mix well.
6. Transfer 1 mL to each of about 50 cryotubes and freeze the tubes on dry ice.
7. Transfer the tubes to -80°C for long-term storage.

#### 4. Notes

1. Wolff et al. (13,24) demonstrated that vectors such as those derived from pUC or pBR322 do not replicate in mammalian cells *in vivo* at a detectable level. Ledwith et al. (25) and Manam (26) reported a sensitive and systematic analysis of the fate of DNA after being injected into mouse quadriceps. They did not observe any evidence of integration at the level of sensitivity of 1–7.5 plasmids/150,000 nuclei, which is  $10^{-3}$  that of the spontaneous rate of gene-inactivating mutations.
2. Knowing the sequence of the antigen insert facilitates generating the gene by PCR. Sites cannot be added to the ends of the gene if they exist within the gene, because the PCR fragment will need to be cut with those enzymes before cloning. If the sequence is not known, PCR primers can be designed to generate the gene for sequence determination before generating it for cloning.
3. Cloning into two different sites forces the antigen gene into the vector in the correct orientation for expression, prevents ligation of empty vector and thus minimizes the number of clones to be screened. If the sequence of the gene contains most of the sites within the multiple cloning site, a different enzyme with the same four-base cohesive end as one in the multiple cloning site can be used. For example, a *Bgl*II (AGATCT) cut end can be cloned into a *Bam*HI (GGATCC) cut vector, or an *Mfe*I (CAATTG) cut end can be cloned into an *Eco*RI (GAATTC) cut vector.
4. A Kozak translation initiation sequence is important for efficient translation of a gene from a mammalian promoter (27–29). An example of a Kozak consensus sequence is ANNATGG. The A in position -3 and the G in position +4 are the most critical sequences. If the sequence of the antigen does not have a codon starting with a G right after the ATG, it may help improve expression by inserting an extra codon to provide the G.
5. If the antigen is a mammalian gene, you may be able to include the Kozak signal, ATG, and termination codon from the antigen gene itself. However, if the anti-

gen is a viral or bacterial gene, it is best to only keep the coding region and add the Kozak signal, ATG, and the termination codon to the PCR primers. Signals from bacterial or viral genes can be detrimental to expression.

6. The cleavage of PCR generated fragments with restriction sites close to the ends is not very efficient. Cleavage can be improved if the fragment is purified by agarose gel electrophoresis before cutting and if the cleavage time is extended. Cleavage may also be improved by adding additional nucleotides to the 5'-end of the primers to provide a larger binding footprint for the restriction enzymes.
7. Gel purification of a pUC-based vector is required because of the high transformation efficiency of pUC vectors. Even a small amount (1%) of uncut vector contaminating a ligation reaction can yield more than 50% of the resulting clones being vector without insert. Running the gels slowly (10–18 h at 10 V) enhances the separation from uncut bands because the DNA bands are very tight under these conditions.
8. Ligation reactions done at molar ratios of insert:vector of 3:1 do not always yield adequate clones. It may result from the inefficient cleavage of PCR fragments by restriction enzymes. Increasing the ratio to 6–10:1 usually yields adequate clones without causing double inserts.
9. Western blot analysis requires an antibody for detecting the antigen being expressed. If there is no antibody reagent available for the antigen being cloned, an antigenic tag (such as His-tag) should be added to the gene when it is inserted into pVAX1.
10. Auxotrophies should also be considered in terms of the ability of the strain to grow at reasonable rates (doubling time of 2 h or less) and to high maximum cell densities (optical density at 600 nm of 3.0 or higher at stationary phase) even when the required nutrient is supplied. For example, leucine mutations are commonly present in commercially available strains designed for molecular biology applications; however, such strains can be either difficult to grow when only leucine is supplemented, or they may require prohibitively large amounts of leucine supplementation to achieve high cell density. Amino acids with low aqueous solubilities should be avoided because delivery of a sufficient amount to achieve the desired cell density for maximum productivity may be difficult. One should also consider the cost of any required nutrients for large-scale processes.
11. In our experience, DH5 cells have given good yields of plasmid DNA and grow at reasonable rates and to high densities in chemically defined medium in fermentors. Some other strains, such as DH5 $\alpha$ , did not grow as well in the fermentor. We have also found that some cells, such as TOP10, produce good yields of plasmid DNA at mini-prep scale but cannot be lysed well in large-scale preps. These properties are not obvious when only comparing small-scale preps using minipreps kits.

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