

Chapter 22

A DNA Vaccine Strategy for Effective Antibody Induction to Pathogen-Derived Antigens

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Abstract

DNA-based vaccines are currently being developed for treating a diversity of human diseases including cancers, autoimmune conditions, allergies, and microbial infections. In this chapter, we present a general protocol that can be used as a starting point for developing DNA vaccines to pathogen-derived antigens, using *Neisseria meningitidis* as an example. In addition, we describe a fusion gene-based vaccine protocol for increasing the potency of DNA vaccines that are based on poorly immunogenic antigens such as short pathogen-derived polypeptides. Finally, we provide a safe and effective protocol for delivery of DNA vaccines, based on intramuscular injection followed by electroporation.

Key words: DNA vaccine, Neutralizing humoral antibody, T cell help, Infectious disease

1. Introduction

DNA vaccines have been the subject of intense investigation over the last 2 decades and the technology provides an adaptable and powerful means for activating both innate and adaptive immune pathways and allowing multiple vaccines to be made and tested quickly and cost-effectively (1). The concept is very simple: DNA encoding the antigen of choice is inserted into a bacterial plasmid, with gene expression usually driven by a strong viral promoter. Delivery of the plasmid vaccine into muscle or skin cells leads to antigen production and presentation to the immune system, and both antibody and cell-mediated immune responses can be effectively induced. In addition, the plasmid also has excellent intrinsic adjuvant properties: the dsDNA acts as a pathogen-associated molecular pattern that can trigger a range of cellular receptors,

leading to type I interferon production and an inflammatory response by cells of the innate immune system. Thus, plasmid DNA not only delivers antigen, but engages multiple routes to activate innate immunity.

DNA-based vaccines are in development for infectious diseases and as therapies against autoimmune diseases, allergy and cancer, and several DNA vaccines are already licensed for veterinary use (2). Importantly, DNA vaccines can induce antibody responses against bacterial pathogens where humoral immunity is believed to be essential, e.g. against peptide mimotopes of meningococcal group B and group C polysaccharides (3, 4) and porin polypeptide (5) and *Streptococcus pneumoniae* capsular polysaccharide (6) and surface antigens (7), *Borrelia burgdorferi* outer surface proteins (8, 9), *Brucella* outer membranes (OM) (10) and OM porin OprF of *Pseudomonas aeruginosa* (11).

In this chapter we present a general protocol that can be used as a starting point for developing DNA vaccines to pathogen-derived antigens, using *Neisseria meningitidis* as an example. In addition, for antigens that can be considered as poorly immunogenic, such as short pathogen-derived polypeptides (5), we also provide a protocol for adopting a fusion gene-based vaccine design similar to the hapten-carrier system to increase the potency of our DNA vaccines (2). This strategy links highly immunostimulatory sequences, such as the fragment C (FrC) sequence of tetanus toxin, to weak target antigens within the DNA vaccine format. This fusion gene vaccine strategy can induce high-affinity antibodies to the linked target antigen through several mechanisms, the most important of which is the cognate CD4⁺ T-cell help provided by linked immunostimulatory elements. Finally, we provide a protocol for efficient delivery of DNA vaccine, based on intramuscular injection followed by electroporation, which delivers an immediate electrical current across the injection site. This causes an influx of inflammatory cells and improved cell transfection efficiency, leading to increased antigen production and presentation to the immune system. Importantly, this delivery system is safe and effective (12, 13) and appears to overcome the translational block to DNA vaccination in the clinic.

2. Materials

2.1. Bacterial Strains and Growth Conditions

1. 1% (w/v) proteose-peptone (Oxoid) and 8% (v/v) glycerol in water, for storage of *Neisseria meningitidis* under liquid nitrogen.
2. Luria Bertani (LB) medium (Oxoid) containing 10% (v/v) glycerol for storage of *Escherichia coli* strain JM109 competent cells (Promega) under liquid nitrogen. LB medium: 10 g bacto-tryptone (Sigma-Aldrich), 5 g yeast extract (Sigma-Aldrich) and

10 g NaCl per litre and autoclave (15 min at 6.8 kg per 0.4 cm²).

3. GC agar medium for growth of meningococci (14).
4. LB agar medium for growth of *E. coli*, prepared by adding 15 g agar per litre of autoclaved LB medium.

2.2. Polymerase Chain Reaction (PCR)

1. Genomic template DNA.
2. Oligonucleotide primers. These are generally purchased as lyophilised pellets, which are resuspended in distilled, de-ionised water to prepare high concentration stocks (100 pmol/μL). Aliquots of working stocks (50 μL) are stored at -20°C to minimize freeze/thaw cycles.
3. HotStarTaq DNA polymerase (QIAGEN, supplied at 5 U/μL) and the proprietary 10× PCR buffer supplied with the enzyme. The enzyme is stored at -20°C in the supplied buffer and during use it is kept on ice in an insulated polystyrene cooler to protect it from temperature fluctuations (see Note 1).
4. 10× dNTP solution: 2 mM dATP, 2 mM dTTP, 2 mM dCTP, 2 mM dGTP in de-ionised water. We use a dNTP set supplied by Promega in which each dNTP is supplied separately at 100 mM. These are combined and diluted 1:50 using de-ionised water to produce a working stock containing all four dNTPs at 2 mM. Aliquots are stored at -20°C to minimize freeze/thaw cycles.
5. Suitable thin-walled microcentrifuge tubes (e.g. STARLAB 0.2 mL PCR tube).
6. Thermal cycler, e.g. GeneAmp PCR System 9700 (Applied Biosystems).

2.3. Agarose Gel Electrophoresis

1. Agarose (Fisher Scientific).
2. 1× TAE buffer: 40 mM Tris-acetate, 1 mM EDTA. A 50× TAE stock is made using 242 g Tris-HCl, 57.1 mL glacial acetic acid, and 200 mL 0.25 M Na₂EDTA (pH 8.0) per litre.
3. Agarose gel: 1% (w/v) agarose in 1× TAE buffer.
4. 10 mg/mL ethidium bromide (EtBr) stock solution in TE buffer: 10 mM Tris-HCl, pH 8.0, containing 0.1 mM Na₂EDTA.
5. 6× gel loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (v/v) xylene cyanol, 3 mL glycerol made up to 10 mL with TE buffer.
6. DNA molecular weight markers (Bioline HyperLadder I).

2.4. Purification of PCR Products and Digested Vector

1. QIAquick Gel Extraction kit (QIAGEN).
2. GeneClean II kit (BIO 101).
3. Elution buffer: 10 mM Tris-HCl, pH 8.5.

**2.5. Restriction
Digestion of PCR
Products and
Expression Vectors**

1. For the selected antigen, purchase the appropriate restriction enzymes (typically 10–20 U/ μ L) together with the appropriate 10 $\times$ reaction buffers from the same supplier (we usually use enzymes supplied by New England Biolabs or Promega) (see Note 1).
2. Elution buffer: 10 mM Tris–HCl, pH 8.5.

2.6. Vector Ligation

1. Purchase a commercial plasmid vector that promotes gene expression in mammalian cells. We use several vectors for our vaccine construction, including pcDNA3.1 (Invitrogen) and pCI (Promega). Both vectors are designed to promote constitutive expression of cloned DNA inserts in mammalian cells. The pCI vector includes a chimaeric intron that is reported to frequently increase the level of gene expression. These vectors are generally suitable for preclinical studies in animals; however, vector choice for clinical testing will be influenced by the appropriate regulatory authorities.
2. T4 DNA ligase (Promega, supplied at 1–3 U/ μ L) and the proprietary 10 $\times$ reaction buffer supplied with the enzyme. The enzyme is stored frozen at -20°C in aliquots to avoid freeze–thaw cycles (see Note 1).

**2.7. Transformation
of Vector into
Expression Hosts**

1. *E. coli* strain JM109 competent cells.
2. Ampicillin at 100 mg/mL stock concentration in de-ionised, sterile water.
3. LB medium (step 2, Subheading 2.1).
4. LB agar plates with ampicillin: 15 g agar per litre of LB medium; autoclave the mixture and add ampicillin when hand-cool to a final concentration of 100 μ g/mL; pour the plates and allow to set.

**2.8. Screening
and Validation of
Expression Constructs**

1. QIAprep Spin Miniprep kit (QIAGEN) with supplied elution buffer: 10 mM Tris–HCl, pH 8.5.
2. Inoculation loops, 1 μ L (Greiner Bio One).
3. Appropriate restriction enzymes and suitable 10 $\times$ reaction buffer (e.g. New England Biolabs or Promega).
4. ABI Prism Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).
5. Oligonucleotide sequencing primers: 1.6 pmol/ μ L working stock.
6. Suitable thin-walled microcentrifuge tubes (e.g. STARLAB 0.2 mL PCR tube).
7. Hi-Di formamide (Applied Biosystems).
8. 3 M sodium acetate buffer: 24.61 g in 50 mL de-ionised water, adjust pH to 5.2 with glacial acetic acid and make up to 100 mL with water.

9. 3130xl Genetic Analyzer (Applied Biosystems) for DNA sequencing.
10. MacVector (MacVector, Inc.) and Lasergene (DNASTAR) DNA sequencing software.

2.9. Large Scale DNA Vaccine Production and Storage

1. QIAfilter Plasmid Giga kit (QIAGEN).
2. Alcohols: isopropanol, 70% (v/v) ethanol.
3. 50% (v/v) glycerol in de-ionised water: aliquot 0.5 mL of the solution into sterile cryo-tubes (e.g. Cryo.s tubes, Greiner Bio-One), re-cap them and autoclave (15 min at 6.8 kg per 0.4 cm²).
4. BioPhotometer (Eppendorf AG).

2.10. DNA Vaccine Preparation and Recommended Vaccination Schedule

1. 70% (v/v) ethanol.
2. Sterile sodium chloride 0.9% (w/v) for in vivo use. We use “NaCl 0.9% for intravenous infusion BP” (Baxter).
3. The appropriate animal model (e.g. an inbred strain of mouse, usually BALB/c) and the necessary permissions to conduct animal research.
4. Myjector 27 G× ½” 0.5 mL syringe (Terumo).
5. Elgen pulse generator and associated software (Inovio Pharmaceuticals, Inc.) for electroporation.
6. Conductance gel: 0.57 g NaCl, 2.3 g methyl cellulose, 1 M NaOH, make up to 100 mL with de-ionized water.

3. Methods

3.1. Bacterial Strains and Growth Conditions

Grow *Neisseria meningitidis* on GC agar medium plates at 37°C for 18 h in an atmosphere of 5% v/v CO₂ (14) and *E. coli* strains on LB agar medium plates and in LB liquid medium in a dry 37°C incubator.

3.2. Polymerase Chain Reaction (PCR)

1. Genomic DNA template: single colonies of overnight growth of *N. meningitidis* are picked and suspended in 10 µL distilled, de-ionised water in a sterile tube. To extract DNA, the bacteria are lysed with the addition of 10 µL 0.25 M KOH with boiling for 5 min. To adjust the pH for PCR, 10 µL of 0.5 M Tris-HCl, pH 7.5 buffer is then added. The lysed samples are then diluted to 300 µL with water, briefly centrifuged to remove any particulate material, and stored at -20°C. A negative control is also prepared from 10 µL de-ionised water treated similarly.
2. In thin-walled PCR tubes assemble the PCR reaction components consisting of: 10–500 ng template DNA (or de-ionised

water for control reaction), 10 μL 10 $\times$ reaction buffer, 10 μL dNTP solution (2 mM of each; final concentration in reaction: 200 μM each), 1 μL of each forward and reverse primer (100 pmol each), 1 μL HotStarTaq DNA polymerase (5 U) and de-ionised water to make a final volume of 100 μL (see Note 1).

3. Place the reaction tubes in a thermal cycler and subject to PCR using a typical PCR program as follows: an initial denaturation step at 95°C for 15 min (required to activate the HotStarTaq enzyme), followed by 30 cycles each at 94°C for 0.5 min (denaturation), 50–68°C for 0.5 min (annealing), 72°C for 1 min (extension). Finish the reaction with a final extension step at 72°C for 10 min (see Notes 5 and 6).
4. Several techniques can be used to incorporate a leader sequence or immunostimulatory sequence, such as FrC of tetanus toxin, into the final vaccine if required. One method involves serial restriction digestion and ligation of each PCR component into the vector backbone, building up the insert in stages, although careful planning is required to ensure that unwanted coding sequence does not remain from restriction enzyme sites. We recommend the technique of PCR SOEing (15), which links separate PCR products together in frame and amplifies the resulting product. To achieve this, perform a separate PCR reaction for each component (e.g. leader, antigen, FrC) using PCR primers that provide an overlap with the adjacent vaccine component. To link, e.g. the PCR product encoding the antigen sequence with that encoding FrC:
 - (a) Visualize and purify each separate PCR fragment (Subheadings 3.3 and 3.4).
 - (b) Set up a new PCR reaction (step 2, Subheading 3.2) but using 1–5 μL of each purified PCR product as template DNA and the two primers from the original PCR reactions that will anneal at the extreme 5' and 3' termini of the final bonded PCR product.
 - (c) Subject to PCR using a typical program (step 3, Subheading 3.2), but with slight modification: after the initial denaturation step run the program for five cycles with a low annealing temperature (e.g. 5°C below the lowest that was used in the original PCR reactions) followed by 25 cycles with the annealing temperature identical to the lowest used in the original PCR reactions.
 - (d) Visualize and purify the larger PCR product (Subheadings 3.3 and 3.4). Now repeat step 4, Subheading 3.2 to add a leader sequence, if required.

3.3. Agarose Gel Electrophoresis

1. Mix 5 μL aliquots of the PCR reactions with 1 μL of 6 $\times$ loading buffer and load onto a 1% (w/v) agarose gel containing 0.5 $\mu\text{g}/$

mL EtBr for electrophoresis; load an additional lane with DNA molecular weight markers (see Note 4).

2. Carry out electrophoresis at ~ 5 V/cm (measured as the distance between the electrodes) until the dyes in the loading buffer have separated 2–4 cm. The distance travelled by the PCR product is size-dependent, so check the gel regularly to ensure the product does not travel too far in the gel.
3. View the gel on a UV light box and gauge the size of the resulting PCR product; there should be no corresponding band in the negative control lane.

3.4. Purification of PCR Products and Digested Vector

1. Following UV visualisation of the PCR product, purify the remaining PCR product using one of a variety of commercially available kits. We use the QIAquick Gel Extraction kit in two ways:
 - (a) If the PCR reaction produces a single DNA band of the expected size, purify this directly from the PCR reaction liquid. Elute the purified PCR product in 50 μ L elution buffer.
 - (b) If the PCR reaction produces several discrete DNA products, then subject some/all of the remaining PCR reaction to electrophoresis on an agarose gel and view the DNA products using a UV lamp. Use a clean scalpel blade to cut the DNA band of the expected size from the gel and transfer it to a sterile 1.5 mL microcentrifuge tube. Take care to excise only the required band with minimal excess gel. Purify the DNA using the Gel Extraction kit and elute the PCR product in 50 μ L elution buffer.

3.5. Restriction Digestion of PCR Products and Expression Vectors

Digest the PCR product and the vaccine expression vector with the same restriction enzymes to enable cloning of the product into the vector.

1. Set up a restriction digestion reaction in a 1.5-mL microcentrifuge tube containing 0.5–2 μ g PCR product, 10–20 U restriction enzyme, 10 $\times$ reaction buffer (final concentration 1 $\times$), and de-ionised water. It may be possible to use two restriction enzymes in one reaction if they are compatible in the same 10 $\times$ reaction buffer (see Note 2). Ensure that enzyme is the final ingredient added to the reaction and that the total volume of enzyme does not exceed 10% of the reaction volume.
2. Incubate the reaction in a heating block or water bath at 37°C for 2–3 h.
3. Purify the digested PCR product using a commercial kit (Subheading 3.4) and elute the DNA in 50 μ L elution buffer.
4. If digestion with a second restriction enzyme is required then repeat the above steps 1–3 (see Note 2).
5. Digest the expression vector using the method given above (steps 1–4).

6. Run the digested vector on an agarose gel as described above (Subheading 3.3).
7. Visualise the band representing the linearised vector using a UV lamp and cut the band from the gel using a scalpel blade.
8. Purify the linearised vector using a commercial kit (Subheading 3.4) and elute the DNA in 50 μL elution buffer. Perform a second digestion if required and re-purify the digested vector.

3.6. Vector Ligation

1. Set up a ligation reaction for the vaccine construct in a sterile 1.5 mL microcentrifuge tube. This should include 1 μL of linearised plasmid vector (~ 100 ng/ μL), 5 μL of purified, restriction-digested PCR product (10–20 ng/ μL), 1 μL of 10 $\times$ ligase reaction buffer, 1 μL of T4 DNA ligase (1–3 U), and 2 μL of de-ionised water.
2. Set up a control reaction containing no PCR product, instead replacing it with de-ionised water. This can be used to determine background levels of vector re-ligation or incomplete linearization.
3. Keep the ligation reactions overnight at 4°C.

3.7. Transformation of Vector into Expression Hosts

1. Gently thaw the *E. coli* JM109 competent cells on ice (see Note 10).
2. Using separate, pre-chilled 1.5 mL microcentrifuge tubes mix aliquots (50 μL) of the JM109 cells with aliquots (2–5 μL) of the ligation reactions described above (Subheading 3.6); flick the tubes gently to mix the contents and keep on ice for 20 min.
3. Heat shock the cells by placing the reaction tubes in a water bath for 45–50 s at exactly 42°C, then return them to ice for 2 min.
4. Add 0.5 mL LB medium to each transformation reaction, gently invert the tubes to mix and incubate for 1 h at 37°C on a shaker (200 rpm).
5. Gently pellet the cells using a microcentrifuge ($\sim 955\times g$ for 10 min), resuspend in 50–100 μL LB medium and plate them out onto LB agar containing 100 $\mu\text{g}/\text{mL}$ ampicillin. Incubate the plates overnight at 37°C.
6. Following incubation (~ 16 h) each agar plate containing bacteria transformed with PCR product should have numerous colonies (50–500) (see Note 3). The control plate should have few or no colonies.

3.8. Screening and Validation of Expression Constructs

1. Select single colonies, using individual sterile micropipette tips or sterile inoculation loops, and inoculate each into separate 25 mL sterile plastic universal tubes containing 5 mL LB medium supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin; select up

to 12 colonies per plate. Plates secured with Saran wrap can be stored upside down at 4°C for ~1 month.

2. Incubate overnight (~16 h) at 37°C on a shaker (200 rpm).
3. Purify plasmid DNA from 1.5 mL of each culture using a suitable commercial kit. Elute the purified DNA in 100 µL elution buffer supplied. Excess culture volume can be stored for the short term at 4°C for subsequent use in large-scale plasmid preparation (Subheading 3.9).
4. Run 5 µL of each purified DNA sample on a 1% (w/v) agarose gel to ensure sufficient yield, together with a DNA size marker.
5. This small-scale DNA preparation is suitable for screening by restriction digestion and DNA sequencing to identify plasmids containing the correct DNA insert.
 - (a) Use the appropriate restriction enzymes to digest the purified plasmids, using 5 µL of each DNA preparation; if the two desired restriction enzymes cannot be used together then two digestion reactions should be performed as described above (Subheading 3.5).
 - (b) Run the digested plasmids on a 1% (w/v) agarose gel, together with a separate molecular weight marker; the presence of a band of the correct size corresponding to the excised DNA insert provides initial confirmation that the undigested plasmid may contain the PCR product insert.
6. Using a commercially available kit, sequence the DNA insert to confirm its integrity and orientation/location within the plasmid vector, as follows (see Notes 7 and 11):
 - (a) Set up sequencing reactions in 0.2 mL PCR tubes that contain 2 µL Big Dye Terminator Ready Reaction Mix (labelled A-dye, C-dye, G-dye and T-dye terminators, dNTPs, AmpliTaq DNA polymerase), 2 µL 5× sequencing buffer, 5 µL miniprep DNA, 1 µL sequencing primer (1.6 pmol/µL).
 - (b) Perform sequencing reactions on a thermal cycler (step 6, Subheading 2.2) using the following conditions: 94°C for 10 s (denaturation), 50°C for 5 s (annealing), and 60°C for 4 min (extension) for 25 cycles.
 - (c) Precipitate the DNA from each sequencing reaction by adding the 10 µL reaction volume to a 1.5-mL microcentrifuge tube containing 50 µL ethanol (100%) and 2 µL 3 M sodium acetate buffer, pH 5.2 and keep on ice for 30 min.
 - (d) Pellet the DNA precipitate in a microcentrifuge at 20,817×*g* for 30 min at 4°C; remove the liquid carefully with a micropipette tip (care! the pellet will not be visible).
 - (e) Wash the DNA pellet by adding 150 µL of 70% (v/v) ethanol and microcentrifuge at 20,817×*g* for 5 min at 4°C; remove the liquid carefully and air dry the pellet for 5–10 min.

- (f) Resuspend the DNA pellet in 10 μ L formamide and run the reaction products on a DNA sequencing machine.
- (g) Analyze the sequencing data using the appropriate software; to achieve this, first produce a word processing file containing a template of the single-letter nucleotide sequence for the DNA insert that you are trying to create; the published DNA sequence of known proteins can be found by searching a suitable nucleotide database (e.g. <http://www.ncbi.nlm.nih.gov/nuccore>). Use the analysis software (according to the manufacturer's instructions) to align and compare your expected template sequence with the data generated from the sequencing reactions to determine whether your DNA inserts are correct (see Notes 8 and 9).

3.9. Large-Scale DNA Vaccine Production and Storage

1. Once the correct vaccine vector has been identified, plate out 50 μ L of the corresponding bacterial culture (from step 3, Subheading 3.8 above) onto a fresh LB agar plate supplemented with 100 μ g/mL ampicillin. Incubate overnight (~16 h) at 37°C.
2. Pick a single bacterial colony and inoculate into a 25-mL universal tube containing 10 mL LB plus 100 μ g/mL ampicillin. Incubate this starter culture during the day (~8 h) at 37°C on a shaker (200 rpm). Culture a separate colony as insurance against failure of the first to grow sufficiently. Colony growth should be evident after 8 h with the LB medium beginning to look cloudy.
3. Use the 10 mL starter culture to inoculate 1.2 L of LB medium supplemented with 100 μ g/mL ampicillin. Incubate this large-scale culture overnight at 37°C on a shaker (200 rpm).
4. Reserve 2 mL of the overnight grown culture for long-term storage of this vaccine-containing host. Add 0.5 mL of this to a sterile cryotube containing 0.5 mL 50% (v/v) glycerol and mix gently by inversion. We recommend preparing several glycerol stocks of each vaccine strain and storing them separately at -20 and -80°C. Colonies can be recovered by using a sterile loop to streak out a small volume of the stock onto a fresh LB agar plate containing 100 μ g/mL ampicillin.
5. Purify the DNA vaccine construct using the suggested commercial kit, which will purify up to 10 mg plasmid DNA (see Note 11).
6. Resuspend the resulting purified DNA pellet in 5 mL of 10 mM Tris-HCl buffer, pH 8.0.
7. Determine the DNA yield and concentration by measuring the absorbance at 260 nm wavelength. The ratio of absorbance at 260 and 280 nm wavelength (A_{260}/A_{280}) should be above 1.8 to ensure minimal contamination with protein and RNA.

8. Validate DNA vaccine integrity by DNA sequencing as described above (step 6, Subheading 3.8).
9. Mix 1 mg aliquots of the DNA vaccine vector with 0.8× volume of isopropanol (100%) and 0.1× volume of 3 M sodium acetate buffer, pH 5.2 in 1.5 mL microcentrifuge tubes. Mix the contents by inversion and store the 1 mg DNA aliquots long-term as isopropanol precipitates at -20°C .

**3.10. DNA Vaccine
Preparation and
Recommended
Vaccination Schedule**

1. Pellet the 1 mg DNA vaccine aliquot in a microcentrifuge at $20,817\times g$ for 10 min at 4°C .
2. Decant the liquid, add 1 mL 70% (v/v) ethanol and microcentrifuge for a further 5 min.
3. Decant the liquid, microcentrifuge at $20,817\times g$ for 5 s at 4°C and remove all remaining liquid with a pipette tip. Allow the pellet to air dry for no longer than 5 min.
4. Resuspend the pellet in 1 mL sterile 0.9% (w/v) NaCl (saline) for injections. The pellet will usually dissolve with gentle agitation at 37°C within no more than 30 min.
5. Check the DNA vaccine concentration (absorbance at 260 nm wavelength) and prepare samples for injection by dilution with sterile saline.
6. For the generation of antibody responses in murine models (see Note 12), we recommend either of the following two schedules:
 - (a) Single injection into each tibialis anterior muscle in the hind limbs of 50 μL DNA vaccine (0.5 mg/mL) in sterile saline using a 27-G needle. Repeat injections on days 21 and 42.
 - (b) Single injection into each rear tibialis anterior muscle as above. Give booster injections at day 21 in combination with electroporation, using a commercial pulse generator as described below.
7. To perform DNA vaccination with electroporation first anaesthetize the mice using an appropriate anaesthetic, then shave the fur overlaying the quadriceps muscle at the front of each rear limb exposing bare skin.
8. Inject the DNA vaccine directly into the quadriceps muscle of one limb and then immediately apply conductance gel to the skin overlaying this muscle and the surrounding area.
9. Immediately place the silver electrodes of the Elgen pulse generator on the skin on either side of the vaccine injection site and apply a local electrical field using the pulse generator and associated software according to the manufacturer's instructions. We use an electrical field comprising 10 trains of 1,000 square wave pulses delivered at a frequency of 1,000 Hz, with each pulse lasting 400 μs (200 μs positive and 200 μs negative).

Each train is delivered at 1 s intervals with the electric pulse kept constant at ± 50 mAmp.

10. As the electrical pulses are delivered, move the electrodes slowly up and down the muscle length to ensure that the whole muscle receives electroporation. Make sure that skin contact is maintained and that conductance gel fully covers the area to be electroporated.
11. Repeat the DNA vaccine injection and electroporation procedure into the second rear quadriceps muscle and allow the mice to recover from anaesthesia; no adverse effects should be observed following electroporation. Excluding the induction and recovery from anaesthesia, the whole procedure (steps 7–11, above) takes approximately 2 min per animal.
12. Collect blood/tissues for immune screening according to your model (e.g. day 42/56) (see Notes 13, 14 and 15).

4. Notes

1. Store all enzymes at -20°C in the buffer in which they are supplied. For use, keep the enzyme(s) in an insulated bench top cooler or ice box to protect it from temperature fluctuations which could inactivate it.
2. For “double” restriction digestion reactions (i.e. when two restriction enzymes are used together in one digestion reaction) ensure that both enzymes retain an appropriate level of activity in the reaction buffer; this may not be possible with certain enzyme combinations and two separate digestion reactions may need to be performed, with the template DNA cleaned up between each reaction to remove enzyme and buffer (e.g. using the QIAquick Gel Extraction kit). Commercial suppliers will provide information on reaction buffer compatibility.
3. If difficulty is experienced in cloning the PCR product into the desired vaccine vector then we recommend the use of pGEM-T Easy Vector System I (Promega), if appropriate. PCR amplification with Taq DNA polymerase introduces a template-independent single A nucleotide overhang at the 3' end of the DNA strand. This allows relatively straightforward cloning of the purified PCR product directly into the T vector which has a complementary 3' single T residue overhang. Once cloned into this plasmid, the DNA insert may then be excised by restriction digestion and inserted into the vaccine vector of choice, assuming that the appropriate restriction enzyme sites occur in both vectors.
4. When preparing agarose gels, ensure that the melted gel is hand cool and no longer giving off steam before adding the EtBr

solution. EtBr is a *mutagen* that is stored at room temperature in the dark; ensure that suitable protective clothing and containment procedures are used and that the “spent” gel is disposed of in a suitable manner.

5. For optimal PCR specificity and product yield, temperatures and cycling times should be optimized for each new template sequence or primer set. Generally, the annealing temperature should be 5°C below the lowest melting temperature (T_m) of the primers used.
6. For PCR products larger than 1 kb, use an extension time of 1 min per kb DNA product. If using a different DNA polymerase then check the manufacturer’s instructions for recommended extension times.
7. For complete DNA sequencing of vaccine inserts, design and purchase sequencing primers in the forward and reverse orientations that anneal at ~300 base intervals covering the whole of the DNA insert plus the insertional junction with the vector sequence.
8. Bacterial clones that do not contain the correct plasmid insert should be discarded: e.g. the insert may be missing, certain sequences may be deleted/inserted or DNA sequencing may reveal nucleotide mutations leading to single amino acid changes. For these cases, there are several options: (a) screen more bacterial colonies; (b) repeat the restriction enzyme digestion/ligation/cloning process using stored PCR product; and/or (c) begin the PCR construction process of the insert DNA from scratch.
9. Although we perform our own sequencing reactions, various commercial enterprises offer DNA sequencing as a service. The exact requirements may vary, but many commercial services simply require a sample of the plasmid DNA and sequencing primers; they will carry out the reactions and subsequent gel/capillary analysis. We routinely use Geneservice (<http://www.geneservice.co.uk/>).
10. *E. coli* competent cells: we prefer to buy ready-competent bacteria for transformations, for reasons of reproducibility and convenience. Alternatively, standard methods can be used to make *E. coli* competent for DNA uptake (16).
11. Whilst we recommend various commercial kits, reagents, services, equipment and software that we routinely use in our laboratories, we do not endorse these; alternatives are usually available from other sources and all should be used according to the manufacturer’s instructions.
12. We routinely include a signal peptide/leader sequence at the N-terminus of our DNA vaccine-encoded antigen sequence. This guides the encoded antigen into the endoplasmic reticulum

where folding and initial glycosylation occur. We have found that this can promote the induction of both antibody and T-cell responses. We routinely use the leader sequence derived from the V_H gene utilized by the IgM of the murine BCL1 tumour (17). The leader sequence is usually amplified by PCR using a reverse primer that overlaps with the 5'-terminus of the antigen of choice. The leader and antigen sequences can be separately amplified by PCR and subsequently linked by PCR SOEing (15). Alternatively, the target protein may contain its own leader sequence.

13. Analysis of humoral immune response is largely dependent on the meningococcal antigens(s) of interest, but should include enzyme-linked immunosorbent assays against native protein, OM and vesicles and whole bacteria; Western immunoblotting; immuno-fluorescence assays; serum bactericidal activity and opsonophagocytosis assays. Detailed protocols for these assays can be found in a previous series volume (18).
14. If the resulting immune response is weak, there is an option to introduce an in-built immunostimulatory sequence. We recommend the FrC portion of tetanus toxin protein as a non-toxic, immunogenic fusion partner for the generation of a DNA fusion vaccine.
15. Ensuring the correct tertiary structure of the antigen encoded by a DNA vaccine is crucial for the induction of an appropriate and effective humoral antibody response. When incorporating FrC as a fusion partner for the pathogen-derived target antigen of choice we routinely include a short "linker" between the two sequences, allowing each to fold into its natural conformation following protein expression in vivo. Previously, we have used the linker sequence AAAGPGP (5).

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