

DNA Vaccines for Allergy Treatment

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Summary

The ability of DNA vaccines to stimulate Th1 type reactions has rendered them a promising tool for immunotherapy of type I allergy. In this chapter, we describe strategies for up-to-date anti-allergic DNA-based immunization. This includes codon optimization of allergen genes, CpG-enrichment of plasmid vectors for enhanced Th1-bias, and the creation of hypoallergenic DNA vaccines either by gene fragmentation or by forced ubiquitination, both reducing the risk of side effects. Also, detailed protocols for plasmid DNA purification, intradermal immunization, and subcutaneous allergen sensitization are provided. Read-out systems presented in this chapter are focused on humoral immune responses and comprise measurement of mediator release from basophils induced by functional IgE and an ELISA protocol based on chemiluminescence technology.

Key Words: DNA vaccine; genetic immunization; type I allergy; immunotherapy; codon optimization; recoding; CpG enrichment; hypoallergenic fragments; ubiquitin; Th1/Th2 responses.

1. Introduction

The DNA vaccine revolution has opened a vast scope of novel approaches for protective and therapeutic treatments of type I allergy. In contrast to conventional immunotherapy (SIT) the anti-allergic effect of DNA vaccines can be clearly attributed to their Th1 inducing capacity (*1*). In this methodological review we will describe optimization strategies including (1) allergen recoding, (2) enhancement of the Th1 bias by using CpG-enriched vectors, (3) creation of hypoallergenic DNA vaccines by gene fragmentation, and (4) forced ubiquitination.

The pattern of codon usage differs between different genes and different organisms, and has been correlated with gene expression levels, tissue-specific patterns of expression, the degree of evolutionary conservation of proteins, and the overall or regional nucleotide composition of the genome. The codons of (plant)-allergen genes are usually suboptimal with regard to the codon usage

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of mammalian cells; expression levels of allergens encoded by DNA vaccines are therefore often poorly expressed in the mammalian host, thereby reducing the immunogenicity of the vaccine. Depending on the allergen, recoding of the cDNA sequence can drastically increase gene expression and efficacy of the vaccine (2).

The rationale of therapeutic DNA vaccines against allergies is based on the induction of a Th1 type cellular immune response, thereby suppressing or converting the allergic Th2 type response. For this purpose, Th1 promoting immunization strategies, such as the additional delivery of immunostimulatory DNA sequences (CpG motifs), or ubiquitination of the allergen are promising approaches (3,4). Here, we describe a simple polymerase chain reaction (PCR) method to create CpG-enriched vectors for DNA vaccination.

A major drawback of conventional SIT is the frequent occurrence of side effects, essentially anaphylactic reactions caused by cross-linking of pre-existing IgE antibodies on mast cells. Moreover, the injection of high doses of antigen can lead to anaphylaxis during the course of treatment via production of therapy-induced IgE antibodies. DNA vaccines in general avoid these unwanted side effects because of the minimal amount of translated antigen and the Th1-biased immune response. Nevertheless, before entering clinical trials, the safety of anti-allergic DNA vaccines has to be increased. Translation of native allergenic determinants must be avoided in order to prevent anaphylactic responses. However, in order to guarantee the recruitment of allergen-specific Th1 cells, T-cell epitopes of the encoded allergen must not be destroyed. Forced ubiquitination or fragmentation of allergens is a feasible approach to induce anti-allergic T-cell responses without the risk of exposure of B-cell epitopes (1,5).

The major birch pollen allergen Bet v 1a and the mugwort allergen Art v 1 will be used to illustrate the described methods in a preventive as well as a curative setting in a mouse model of type I allergy.

2. Materials

2.1. Construction of Vectors

1. pCI mammalian expression vector (Promega, Mannheim, Germany).
2. *Escherichia coli* strain XL1-blue competent cells (Stratagene, La Jolla, CA) and electroporator.
3. Standard Luria broth (LB) medium.
4. Ampicillin.
5. Oligonucleotide primers; deoxynucleotide triphosphate (dNTP) mix (25 mM each).
6. Pfu-polymerase, restriction enzymes; T4 DNA ligase; corresponding buffers.

7. Restriction Buffer D: 6 mM Tris-HCl, 6 mM MgCl₂, 150 mM NaCl, 1 mM DTT; Promega.
8. PCR-cycler with hotlid.
9. Agarose gel DNA electrophoresis equipment.
10. Gel extraction kit (e.g., NucleoSpin Extract; Macherey-Nagel, Easton, PA).
11. Genomic DNA preparation kit (e.g., NucleoSpin Tissue; Macherey-Nagel).

2.2. Endofree Plasmid Purification

1. *E. coli* strain XL1-blue.
2. EndoFree Plasmid Giga Kit (Qiagen; Hilden, Germany).
3. Standard LB medium.
4. Ampicillin.
5. 1-L, 45-mm neck, vacuum-resistant glass bottle (e.g., Schott or Corning).
6. Vacuum source generating pressures between -200 and -600 millibars (e.g., house vacuum, vacuum pump, or water aspirator).
7. Ethanol.
8. Isopropanol.
9. Ultraviolet (UV) spectrophotometer.
10. Agarose gel DNA electrophoresis equipment.

2.3. Immunization/Sensitization of Mice

1. Female BALB/c mice (5–8 wk of age)
2. 27-Gauge needles.
3. 1-mL syringes.
4. Al(OH)₃ (Serva, Heidelberg, Germany).
5. Recombinant protein.
6. Purified plasmid DNA.
7. Standard phosphate buffered saline (PBS), pH 7.5.
8. Endotoxin-free H₂O.
9. Curved forceps with fine serrated tips.
10. Ether.
11. Hair-clipper.

2.4. RBL Release Assay

1. Rat basophil leukemia (RBL)-2H3 cell line (ATCC no. CRL-2256).
2. RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 100 μM 2-mercaptoethanol, 1% penicillin/streptomycin (stable at 4°C for up to 3 wk).
3. Dulbecco's PBS (Sigma, Deisenhofen, Germany): 0.20 g KH₂PO₄, 1.15 g NaHPO₄·2H₂O, 8.00 g NaCl, 0.20 g KCl dissolved in L H₂O. Adjust to pH 7.2 with 1 N NaOH or 1 N HCl.
4. Trypsin/ethylene-diamine tetraacetic acid (EDTA): 5 g trypsin, 2 g EDTA in 1 L PBS.

5. Cell culture equipment (96-well flat-bottom culture plates).
6. Tyrode's buffer: 9.6 g Tyrode's salts (Sigma), 2.38 g HEPES, 1 g NaHCO_3/L , pH adjusted to 7.2 with NaOH or HCl; add 0.1% bovine serum albumin (BSA) just before use.
7. Glycine buffer: 15 g glycine, 11.7 g NaCl/L ; adjust pH to 10.7 with NaOH.
8. Citrate buffer: 0.1 M citric acid or sodium citrate in H_2O , adjust pH to 4.5 with NaOH.
9. 4-MUG (4-methyl umbelliferyl-*N*-acetyl- β -D-glucosaminide) (Sigma): 80 μL aliquots of 10 mM stock solution in dimethylsulfoxide (DMSO) stored at -70°C .
10. Triton X-100.
11. 96-well flat-bottom microtiter plates (nonsterile).
12. Fluorescence microplate reader.

2.5. Analysis of Antibody Subclass Distribution by Enzyme-Linked Immunosorbent Assay

1. Black 96-well high-bind flat-bottom immunoplates (Greiner, Kremsmuenster, Austria).
2. Recombinant allergen.
3. Standard PBS, pH 7.5.
4. Tween-20.
5. Dried skim milk (blotting grade).
6. Detection antibodies: anti-mouse IgG1 and IgG2a, horseradish peroxidase (HRP)-conjugated.
7. Chemiluminescence enzyme-linked immunosorbent assay (ELISA) substrate (Roche, Mannheim, Germany).
8. Plate luminometer with photo-multiplier.

3. Methods

3.1. Construction of Vectors

The basic eukaryotic expression vector pCI utilizes the cytomegalovirus (CMV) major immediate-early enhancer/promoter region, which allows strong, constitutive expression in mammalian cells. A β -globin/IgG chimeric intron region can further increase protein expression and an SV40 late adenylation site increases the steady-state level of transcribed mRNA, making this vector an excellent tool for DNA vaccination. DNA manipulations were performed by standard recombinant DNA methods (6) and are not described in detail because of space limitations.

3.1.1. Construction of pCI-Bet v 1a

A plasmid containing the cDNA of Bet v 1a was kindly donated by Dr. Ferreira (University of Salzburg). The coding region of Bet v 1a was amplified by PCR. A forward (fw) and reverse (rv) primer was designed encompassing

20 nucleotides on the 5'- and 3'-end, respectively. At the 5'-end of the primers the restriction sites for *EcoRI* (sense) and *XbaI* (antisense) were added. A random nucleotide was added to 5'-end of each restriction site to allow cleavage of the respective PCR fragments (see **Note 1**). So the length of the final primers was 27 nucleotides (20 + 6 + 1). The gene was amplified with Pfu DNA Polymerase: 1 μ L of Pfu (2–3 U) was added to 5 ng of template plasmid with 10 pg of each primer and a final dNTP concentration of 0.2 mM each, in a total volume of 50 μ L in the reaction buffer supplied by the manufacturer. PCR was performed with one cycle (4 min at 95°C), 35 cycles (1 min at 95°C; 1 min at 55°C; 1 min at 72°C), one cycle (5 min 30 s at 72°C). The corresponding band was excised from 1% agarose gel and purified with NucleoSpin gel extract columns. After digestion with *XhoI/XbaI* and ligation into the respective restriction sites of pCI, the DNA was transformed into *E. coli* XL1-blue by standard electroporation. The bacteria were plated on LB plates containing ampicillin (50–100 μ g/mL) and incubated overnight at 37°C. Single colonies were selected and grown overnight in LB containing ampicillin. The plasmid DNA was then isolated and checked by restriction enzyme digestion and DNA sequencing (MWG-Biotech).

3.1.2. Recoding of Allergens (see **Fig. 1**)

A synthetic gene with optimized human codon usage can be constructed from synthesized oligonucleotides as demonstrated with Art v 1 (**2**). A complete database on codon usage is available on <http://www.kazusa.or.jp/codon/>. It is convenient to synthesize high-pressure liquid chromatography (HPLC)-purified oligos with a length of approx 150 bp and an overlap of about 20 bp between individual oligos. Additionally, 20-mer oligos corresponding to the 5'- and 3'-end of the synthesized gene have to be constructed to amplify the assembled gene (fw and rv primers). Appropriate restriction sites for further cloning can be attached to these primers as described above.

The main pitfall in designing suitable oligos are inter- or intra-oligonucleotide complementarity, therefore, each oligosequence has to be checked for potential hairpins or matches to other oligonucleotides.

Also even HPLC purified oligos frequently contain synthesis errors (most often one-base deletions), therefore we strongly recommend sequencing of several clones.

The assembly is performed as follows:

1. Mix all oligos with a final concentration of 25 μ M (total).
2. Assemble oligos in a 50- μ L PCR reaction: 1 μ L Pfu polymerase, 0.4 μ L dNTPs (25 mM each), 5 μ L 10X Pfu-Buffer, 2 μ L 25 μ M oligomix, 41.6 μ L H₂O.
3. PCR 1 cycle (4 min at 95°C), 35 cycles (1 min at 95°C; 1 min at 55°C; 1 min at 72°C), 1 cycle (5 min 30 s at 72°C) (see **Note 2**).

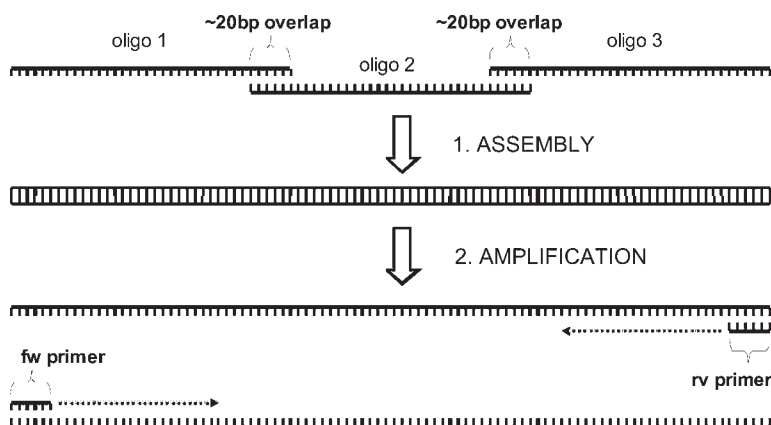


Fig. 1. Construction of a recoded synthetic gene.

4. Amplify the full-length product with the sense- and anti-sense primers in a 50- μ L PCR reaction: 1 μ L Pfu polymerase, 0.4 μ L dNTPs (25 mM each), 5 μ L 10X Pfu-Buffer, 1 μ L fw primer (25 μ M stock), 1 μ L rv primer (25 μ M stock), 1 μ L of PCR reaction from point 3 as template, 40.6 μ L H₂O.
5. PCR as in point 3.
6. Run PCR reaction on a 1% agarose gel and purify the full-length gene product with a gel extraction kit

The purified synthetic gene can then be restriction digested with the respective enzymes and cloned into pCI as described.

3.1.3. Construction of a CpG-Enriched Vector (see [Fig. 2](#))

A CpG-enriched vector can be easily constructed by a similar method as described by Liu et al. (7) using the compatible restriction sites *NotI* and *Bsp120I*:

1. Synthesize an artificial DNA by annealing two oligos (see **Note 3**). These two oligos build a *XbaI* compatible overhang on the 5'-end, a *Bsp120I* site (gggccc), five copies of a CpG motif (gacgtt), and a *NotI*-compatible overhang on the 3'-prime end:
 CpG fw: 5'-ctagagggcccgacgttgacgttgacgttgacgttgacgttgac-3'
 CpG rv: 5'-ggccggaacgtcaacgtcaacgtcaacgtcaacgtcgggcct-3'
2. Mix 4 μ L CpG fw (100 pmol μ L) + 4 μ L CpG rv (100 pmol/ μ L) + 2 μ L restriction Buffer D.
3. Heat up to 95°C in a PCR cycler with hotlid (to avoid evaporation); turn off the machine and let slowly cool down to room temperature; the resulting product will be in stable, double-stranded form and can be stored at 4°C or frozen.
4. Ligate the product into the *XbaI/NotI* sites of pCI-Bet v 1a (the artificial DNA has already a *XbaI/NotI* overhang). This results in the vector pCI-Bet v 1a/5X CpG.

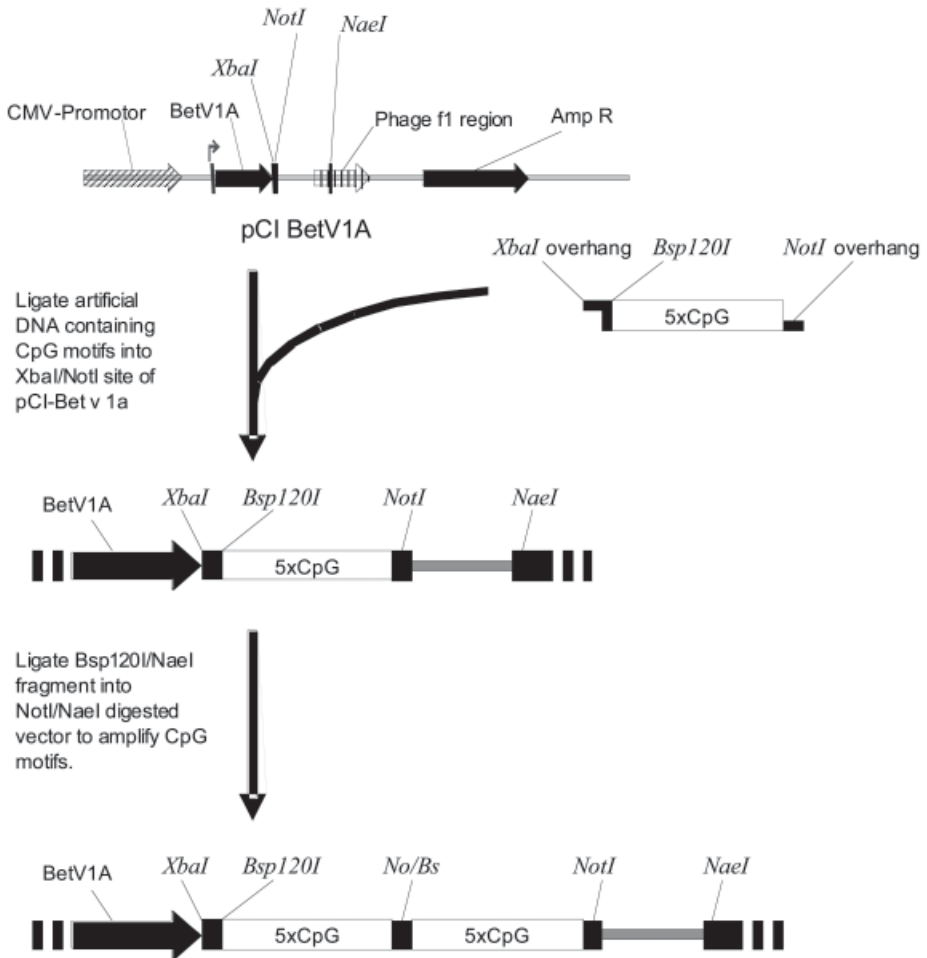


Fig. 2. Generation of a CpG enriched vector. No/Bs, the fusion site produced by ligation of compatible ends of *NotI* and *Bsp120I*.

5. Restriction digest *pCI-Bet v 1a/5X CpG* once with *Bsp120I/NaeI* and once with *NotI/NaeI*.
6. Ligate the *Bsp120I/NaeI* fragment containing the CpG motifs into the open vector from the *NotI/NaeI* digest. This will result in the vector *pCI-Bet v 1a/10X CpG*.

This process can be repeated ad libitum, each time doubling the amount of CpG motifs in the vector.

3.1.4. Construction of Bet v 1a Fragments

Similar to the cloning of Bet v 1a into vector pCI (see **Subheading 3.1.1.**) we cloned the C-terminal half (AA 1-74) and the N-terminal half (AA 75-160) of Bet v 1a into pCI separately, thereby destroying the conformational epitopes of the protein (**1**), while keeping the T-cell epitopes. However, potential T-cell epitopes may be lost at the cutting site. This may be avoided by using overlapping fragments.

It has to be kept in mind that fragmenting a protein not necessarily results in loss of conformational epitopes and every protein has to be tested on a case-to-case basis. A more generalized approach to destroy B-cell epitopes is described in **Subheading 3.1.5.**

3.1.5. Construction of a Vector Encoding Ubiquitinated Bet v 1a

The sequence encoding ubiquitin (ubi) was amplified from the mouse genome via PCR. As a template, genomic DNA was prepared from mouse spleen with a NucleoSpin Tissue kit according to the protocol of the manufacturer. Ubi was amplified with the fw primer ccc**GCTAGC**caccatgcagatcttcgtaagaccctg and the rv primer ccc**GAATTC**ggcacctctcaggcgaaggaccagg. The fw primer introduced a *NheI* site (**bold caps**) and a Kozak consensus sequence (underlined), which is crucial for efficient initiation of translation (**8**). The rv primer introduced an *EcoRI* site (**bold caps**) and a point mutation (gcc -> ggc; anti-sense strand, underlined) that changed Gly76 to Ala76. This mutation diminishes the rate of cleavage of the fusion protein and thus enhances degradation of the protein (**9**).

A PCR reaction was setup as described for Bet v 1a with 10 ng of the purified genomic DNA used as a template. The resulting 250-bp fragment was purified from a 1% agarose gel and cloned into the *NheI/EcoRI* restriction sites of pCI-Bet v 1a thereby generating pCI-ubi/Bet v 1a. When constructing an ubi-fusion protein, the sequence of the fusion partner has to be in-frame with the start-codon of ubi.

3.2. Endofree Plasmid Purification

Endotoxins, also known as lipopolysaccharides (LPS), are cell membrane components of Gram-negative bacteria (e.g., *E. coli*). A single *E. coli* cell contains about 2×10^6 LPS molecules, which are released from the outer membrane into the lysate during plasmid preparation from bacterial cells. The level of endotoxin contamination in plasmid DNA depends on the purification method used. DNA purified with EndoFree Plasmid kits contains only negligible amounts of endotoxin (<0.06 ng/ μ g DNA). For gene therapy applications, endotoxin-free DNA has to be used, because endotoxins cause fever,

endotoxic shock syndrome, and activation of the complement cascade in animals and humans (10). Removal of endotoxin contamination of plasmid DNA is necessary to avoid misinterpretations of experimental results in DNA-based immunization studies.

Up to 10 mg of a high-copy plasmid such as pCI can be obtained with EndoFree Plasmid Giga Kits. The purification protocol is based on a modified alkaline lysis, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

Use endotoxin-free or pyrogen-free plastic pipet tips and tubes for elution and subsequent steps. Alternatively, glass tubes may be used if they are baked overnight at 180°C to destroy endotoxins.

Before starting, add one vial of the provided RNase A solution to one bottle of resuspension buffer P1, to give a final concentration of 100 µg/mL. Prepare endotoxin-free 70% ethanol by adding 40 mL of 96–100% ethanol to the endotoxin-free water from the kit. Check buffer lysis P2 for sodium dodecyl sulfate (SDS) precipitation due to low storage temperatures and, if necessary, dissolve the SDS by warming to 37°C. Prechill buffer neutralization P3 to 4°C.

1. Purification procedure: transform XL1-blue *E. coli* cells with plasmid pCI-Bet v 1a using standard molecular biology methods (6).
2. Plate cells on LB plates containing ampicillin and incubate overnight at 37°C.
3. Pick a single colony from the plate and inoculate a starter culture of between 5 and 10 mL LB containing ampicillin (see Note 4).
4. Grow overnight at 37°C with vigorous shaking in a flask with a volume of at least four times the volume of the culture.
5. Dilute the starter culture 1:500 into LB medium with ampicillin. Inoculate 2 L of medium, again using a vessel with a volume of least 4 times the volume of the culture. Grow at 37°C for 16–18 h with vigorous shaking.
6. Harvest the bacteria by centrifugation at 2900g or higher for 20 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge bottle until all medium has been drained (see Note 5).
7. Screw the QIAfilter Giga Cartridge onto a 45-mm-neck glass bottle and connect it to a vacuum source.
8. Resuspend the bacterial pellet completely by vortexing or pipetting up and down in 125 mL buffer P1. Use a 1000-mL bottle to ensure complete mixing of the lysis buffers, which is important for efficient lysis.
9. Add 125 mL buffer P2, mix by inverting four to six times and incubate at room temperature for exactly 5 min. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Immediately close the bottle containing P2 to avoid acidification from CO₂.

10. Add 125 mL chilled buffer P3, mix immediately by inverting four to six times until a white fluffy material has formed and the lysate is no longer viscous. The white precipitate contains genomic DNA, proteins, cell debris, and SDS.
11. To avoid clogging of the QIAfilter cartridge, remove most of the precipitate by centrifuging the lysate for 10 min at 2900g.
12. Pour the supernatant into the QIAfilter Giga cartridge.
13. Switch on the vacuum source. After all liquid has been pulled through, switch it off and leave the filter cartridge attached.
14. Add 50 mL QIAfilter wash buffer FWB to the cartridge and gently stir the precipitate with a sterile spatula. Switch on the vacuum source until the liquid has been pulled through completely.
15. Add 30 mL endotoxin removal buffer ER to the filtered lysate in the bottle, mix by inverting approx 10 times, and incubate on ice for 30 min. The lysate will become turbid, but will clear again during the incubation on ice.
16. Equilibrate the QIAGEN-tip 10,000 by applying 75 mL equilibration buffer QBT and allow the column to empty by gravity flow. The flow of buffer will stop when the meniscus reaches the upper frit in the column.
17. Apply one-half of the filtered lysate onto the tip and allow it to enter the resin by gravity flow (*see Note 5*). The lysate may become turbid again owing to the presence of buffer ER, but this does not affect performance (*see Note 6*).
18. Wash the tip with a total of 300 mL washing buffer QC. Allow the buffer to move through the tip by gravity flow.
19. Elute DNA into a new polypropylene centrifuge tube with 100 mL elution buffer QN. Drain the tip by allowing it to empty by gravity flow.
20. Equilibrate the tip again by applying 75 mL buffer QBT as described in **step 16** (*see Note 7*).
21. Proceed with the second half of the filtered lysate as described in **steps 17–19**.
22. Pool eluate from both runs.
23. Precipitate DNA by adding 140 mL room-temperature isopropanol (0.7 vol) to the eluted DNA. Mix, centrifuge immediately in conical tubes at 2900g or higher for 60 min at 4°C, and carefully decant the supernatant.
24. Wash DNA pellet with 10 mL of endotoxin-free room-temperature 70% ethanol and centrifuge as in **step 23**. Carefully decant the supernatant without disturbing the pellet.
25. Air-dry the pellet for about 10–20 min and redissolve the DNA in a suitable volume of endotoxin-free H₂O by rinsing the walls of the tube. Avoid pipetting the DNA up and down as this may cause shearing. Overdrying the pellet will make the DNA difficult to redissolve.
26. Determine the DNA concentration by standard UV spectrophotometry.
27. Digest the plasmid with suitable restriction enzymes and analyze the DNA on an agarose gel.
28. Store aliquots of the plasmid at –20°C.

3.3. Immunization/Sensitization of Mice

BALB/c mice are chosen for allergy model systems because they produce high amounts of IgE and exhibit a strong Th2 type response, resulting in elevated levels of IL-4 and IL-5. Take blood samples for preparation of sera from the tail vein prior to each immunization / sensitization and at regular intervals thereafter. Incubate samples for 30 min at 37°C for coagulation and centrifuge at 16,000g for 30 min. Transfer clear supernatants into fresh tubes. Sera can be stored at 4°C with 0.2% NaN₃. The skin is the preferable target tissue for DNA-based anti-allergy immunization because of resident professional antigen presenting cells, the Langerhans cells. Methods different from delivery via needle and syringe, such as gene gun immunization, have been demonstrated to be unsuitable for anti-allergic vaccination due to their inherent Th2 bias (11).

3.3.1. Intradermal DNA Immunization

Prepare 100 µg of plasmid DNA in a volume of 200 µL sterile PBS/animal. Shave mice at the back with an electric shaver and anesthetize them with ether or other reagents. Intradermal injections are applied to several sites with the aid of forceps (*see Note 8*). Perform this immunization once a week for three consecutive weeks. For a preventive approach, 4 wk after the last immunization mice are sensitized as described in **Subheading 3.2.2**. A therapeutic setting includes presensitization with allergen followed by treatment via DNA-based immunization 4 wk after the final sensitization.

3.3.2. Subcutaneous Sensitization

For each animal, 5 µg of recombinant allergen adsorbed to 100 µL Al(OH)₃ (1.3% suspension in water, measured as Al₂O₃, pyrogen-free, salt-free in a total volume of 200 µL sterile PBS) are prepared. Let adjuvant–protein complex formation take place by shaking the solution for about 2 h at room temperature prior to injection. The solution is subcutaneously injected into two sites while lifting up the skin with your fingers or a forceps (*see Note 9*). Sensitizations are performed on days 0, 14, and 21 (*see Note 10*).

3.4. Assays for Monitoring Humoral Immune Responses

Methods for evaluating antibody-mediated immune reactions following DNA-based immunization are described in **Subheadings 3.3.1.–3.3.3.**

Together with other mediators such as histamine, β-hexosaminidase is an enzyme, which is released during degranulation of mast cells and basophils.

Therefore, the rat basophil leukemia cell release assay provides a functional read-out for IgE mediated degranulation, which is to be preferred compared to IgE-ELISA. 4-methyl-umbelliferyl-*N*-acetyl- β -D-glucosaminide (4-MUG) serves as substrate for β -hexosaminidase and forms stable complexes with the enzyme, which can be detected by fluorescence spectroscopy.

To analyze allergen-specific subclass antibody production, sera are investigated for IgG1 and IgG2a antibodies by ELISA. The IgG1:IgG2a ratio provides an indication of the Th1/Th2 bias of the ongoing immune response.

A detailed description of assays for investigation of cellular immune responses following DNA-based immunization can be found in Chapter 16 in this book.

3.4.1. Culture of RBL Cells

Culture basophils in RPMI with supplements at 37°C, 95% RH, 7% CO₂ in culture flasks (25 cm² with 5 mL or 75 cm² with 20 mL) until they become confluent. Remove culture medium, wash cells three times with DPBS and cover the cell layer with trypsin/EDTA. Incubate culture flask for 5 min in incubator and then add warm culture medium. Rinse off the cells with a serological pipet, centrifuge the suspension at 300g for 10 min, discard the supernatant, and dissolve the cell pellet in culture medium. Dilute cells 1:10 for 2 d in culture, 1:30 for 3 d.

3.4.2. β -Hexosaminidase Release

Plate RBL cells at 4×10^5 cells/mL in 100 μ L/well of a 96-well culture plate overnight. Add serial dilutions of sera and incubate for 2 h at 37°C. Background wells and wells for maximum release are left untreated. Discard the supernatant and tap the plate dry on a paper towel. Wash cells twice with 200 μ L Tyrode's buffer and add 100 μ L of 0.3 μ g/mL recombinant allergen or allergen extract for sensitization. Incubate the plate for 30 min at 37°C, add 10 μ L 10% Triton X-100 for maximum release to some wells and resuspend their contents. Centrifuge for 5 min at 300g, remove 50 μ L supernatant and transfer it to a fresh, nonsterile 96-well plate. Supernatants can be frozen at -20°C for later examination. Prepare assay solution by adding 80 μ L 4-MUG per 5 mL citrate buffer. Add 50 μ L assay solution to 50 μ L supernatant and incubate for 1 h at 37°C. Stop reaction with 100 μ L glycine buffer and measure fluorescence (in relative fluorescence units, rfu) at λ_{ex} (360 nm)/ λ_{em} (465 nm) using a fluorescence microplate reader. Calculate percentage of specific release according to the equation $[(\text{experimental}_{rfu} - \text{background}_{rfu})/(\text{maximum}_{rfu} - \text{background}_{rfu})] \times 100$ (see **Note 11**).

3.4.3. Analysis of Antibody Subclass Distribution by ELISA

Coat ELISA-plates overnight at 4°C with recombinant allergen at a concentration of 1 µg/mL in PBS in a volume of 50 µL/well. Wash plates three times with PBS/0.1% Tween-20 and block for 1 h at room temperature with 200 µL/well blocking buffer (PBS/0.1% Tween-20/2% skim milk) (*see Note 12*). After washing three times, incubate wells for 1 h at room temperature with serial dilutions of individual serum samples in blocking buffer (50 µL/well). Each plate has to contain between 8 and 10 wells of a serially diluted standard serum. This standard is prepared from pooled high titered antisera against the plate antigen. Standard serum can be stored in 50% glycerol, 0.2% NaN₃ at -20°C. Wash the plates five times (*see Note 13*), add 50 µL/well of the detection antibodies diluted 1:1000 in blocking buffer, and incubate for 1 h at room temperature. After washing five times, the assay is developed with 50 µL/well of chemiluminescence substrate diluted 1:2 in H₂O (*see Note 14*). Incubate for 3 min and determine chemiluminescence in photon counts/s in a luminometer (*see Note 15*). End-point titer of individual serum antibody bound to the plates can be calculated by interpolating into a standard curve generated with known dilutions of the standard serum. For end-point titer determination of the standard serum any well with a luminescence greater than three standard deviations above background (calculated using more than 20 wells containing no primary antibody) is scored as positive.

4. Notes

1. Cleavage efficiency close to the end of linear DNA fragments (PCR fragments) depends on the restriction enzyme used. Several enzymes have been tested by Moreira and Noren (*12*): “base pairs from end” refers to the number of double-stranded base pairs between the recognition site and the terminus of the fragment; this number does not include the single-stranded overhang from the initial cut. Because it has not been demonstrated whether these single-stranded nucleotides contribute to cleavage efficiency, some authors suggest adding four additional bases to the number indicated when designing PCR primers. However, in our experience the indicated numbers are usually sufficient.
2. For genes larger than 1 kb, extension time (72°C) should be increased 1 min per kilobase. Longer extension time may also help when no complete gene assembly is achieved.
3. To generate sticky ends, only full-length oligos can be used for the annealing process. Therefore HPLC-purified oligos must be used. Slow cooling to room temperature should take between 45 and 60 min to increase annealing efficacy. Although not necessary, using 5'-phosphorylated oligos may increase the ligation efficacy in the following steps.

4. A number of slightly different LB culture broths, containing different NaCl concentrations, are commonly used. To obtain highest plasmid yields, cultures should be grown in LB containing 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter.
5. The pellet wet weight per liter LB culture in the handbook for QIAGEN plasmid purification is estimated as 3 g/L. However, in our hands the pellet weight is up to 7 g/L culture as reproduced with several different constructs.
6. In the handbook for Qiagen plasmid purification, it is recommended to start with a culture volume of 2.5 L and to apply the whole filtered lysate onto the tip. In our experience this leads to extremely low DNA yields because of overloading of the column. We therefore suggest to use only 2 L of culture and to perform binding, washing, and elution steps twice, with half of the lysate, respectively.
7. Composition of all buffers needed is provided with the handbook. If you are running out of buffers, prepare them using endotoxin-free H₂O and glassware baked overnight at 180°C to destroy endotoxins. Chemical components are usually endotoxin-free if you do not use contaminated spatulas. Endotoxin-content of buffers and of purified plasmid DNA can be measured by Limulus amoebocyte assay (Pyroquant, Walldorf, Germany).
8. If the plasmid solution is properly administered, blisters will form at the injection sites, which should persist for minutes to hours. If they disappear immediately or never form, a subcutaneous injection has been given, most likely resulting in a suboptimal immune reaction.
9. For untrained researches subcutaneous sensitization with protein can be facilitated by shaving the back of the animals as described for DNA immunization.
10. For a therapeutic approach it is recommended to perform several rounds of sensitization/therapy, reflecting a stringent therapeutic situation in the presence of continuous allergen pressure.
11. Because the outcome of RBL release assays is variable depending on the condition of the cells seeded, all serum samples of an animal study should be measured at once.
12. Alternative formulations of blocking buffer for ELISA include the use of BSA, serum, casein, or hydrophobized proteins instead of skim milk.
13. Most interactions that contribute to nonspecific binding are of low or intermediate affinity and reversible in character. Therefore, you can markedly reduce background levels by leaving the buffer in the wells for up to 1 min between each individual wash. An automated washer has been shown to be superior to manual washing.
14. Add the chemiluminescence substrate to the wells with a multichannel pipet because all wells should be started within a minimum period of time. To avoid "burning out" of substrate, samples should be quantitated within 30 min after adding the reagent.
15. The advantages of chemiluminescence compared to commonly used chromogenic substrates are improved sensitivity, a large dynamic range, and a rapid and constant signal. However, if your lab is not equipped with a luminometer, a stan-

standard colorimetric development can be performed with commercially available substrate kits for HRP.

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