



Short communication

pDNAVACCultra vector family: high throughput intracellular targeting DNA vaccine plasmids

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Abstract

DNA vaccines have the potential to provide a safe route for protective immunity to neoplasms and infectious agents. However, current DNA vaccine plasmids are not optimal with additional non-essential DNA, nor do they facilitate controlled or flexible targeting of antigens to various intracellular destinations. A family of DNA vaccine vectors, optimized and minimized to comply with FDA guidelines regarding content and elimination of extraneous materials, was constructed. The resulting vectors are much smaller than existing vectors, drive higher levels of target gene expression, facilitate high throughput cloning applications, and allow simultaneous cloning into multiple vectors that feature various intracellular targeting destinations for the protein product. The ability to control expression and trafficking is intended to provide a rapid, rational approach to cancer therapy and emerging infectious diseases.

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Keywords: DNA vaccine; Plasmid; Antigen presentation

1. Introduction

DNA vaccines offer a new way to immunize humans (or animals) with materials that are entirely gene-based and expressed by the organism's own cells, making an ideal mimic of intracellular antigens. This means there is greater control over the immunization and immune presentation processes, because the investigator determines which antigens to use, delivery (e.g. electroporation, nanoparticles, gene gun) where to elicit the response (e.g. mucosal, dendritic, or muscle), intracellular trafficking of antigen, and which immune stimulators (e.g. cytokines, CD80, CD86 immunostimulatory DNA sequences), if any, to be co-expressed to modulate the type of response (Th1 or Th2; reviewed in [1,2]).

Targeting heterologous proteins to various intracellular destinations including secreted (e.g. TPA) [3], membrane-

anchored (e.g. human alkaline phosphatase (PLAP) glycosylphosphatidylinositol (GPI)-anchor) [4], endosome (e.g. human Lamp1) [5–7] or proteasome (e.g. mouse Ubiquitin A76) [8,9] is a demonstrated method to alter and enhance immune responses often in an antigen specific manner [10–14]; reviewed in [15]. Endosomal targeting often promotes a MHC class II response [5], while the destabilizing ubiquitin molecule (UbiquitinA76 versus native UbiquitinG76) is utilized to enhance entry into proteosomal degradation pathway and MHC class I presentation, and shifts host response towards TH-1 type immunity [8,9].

Unfortunately, minor variations in vector backbone can alter expression levels [16], intracellular localization [17] and ultimately the immune response [18]. Consequently, using existing vectors to determine optimal antigen targeting destinations may generate misleading results, due to differences in expression, mRNA stability, plasmid isoforms, plasmid size or supercoiling, or immunostimulatory effects from plasmid encoded CpG. The controlled vectors described herein address this problem.

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2. Materials and methods

2.1. Cloning

DNA vaccine plasmids were created by single step assembly of six precloned modules using gene self assembly (GENSA) technology as described [19]. GENSA uses class IIS restriction enzymes to generate unique, non-palindromic overhanging termini that can ligate to only one other terminus in a complex mixture, thus assuring that each fragment ligates in the correct orientation to its correct partner.

The (GENSA) modules consisted of products representing:

- High copy number pUC prokaryotic replication origin.
- Prokaryotic selectable marker gene (kanamycin, *kanR*).
- Eukaryotic enhancer–promoter (CMV).
- Synthetic eukaryotic untranslated leader–intron–translational initiation sequence (Kozak sequence) cassette derived from rabbit β -globin leader and intron.
- High throughput seamless cloning site targeting gene leader cassette (basis for difference between pDNA-VACUltra1–7) or control module containing EGFP.
- Synthetic eukaryotic transcriptional terminator based on rabbit β -globin.

Additional features, such as prokaryotic terminators, were incorporated seamlessly into the modules through 5' extensions on the PCR primers.

Cloning and sequencing were performed using standard restriction enzyme cloning methodologies. PCR was performed using *Pfu* DNA polymerase.

2.2. Other techniques

Plasmid Copy Number was determined by quantification of plasmid obtained from Qiagen miniplasmid kit preparations. Plasmid quality was determined by resolving plasmid DNA on 1% agarose gels, and visualizing DNA by poststaining with SYBR green II (Sigma, St. Louis Missouri).

Expression of EGFP in vitro was determined using fluorescent microscopy 24–72 h post-transfection of fibroblast cell lines (either human HTam or mouse PA317) using either lipofectamine (Invitrogen, Carlsbad, CA, USA), Superfect (Qiagen, Valencia, CA, USA), or TROjane (Avanti Polar Lipids, Alabaster, AL, USA).

3. Results

3.1. DNA vaccine plasmid design and construction

The DNA vaccine vectors were constructed by simultaneously joining six fragments, using GENSA technology [19]. A pDNAVACUltra vector is shown in Fig. 1A. The vector facilitates cloning of genes or epitopes of interest seamlessly

downstream of the desired intracellular targeting gene leader cassette. The cloning site is designed for high throughput cloning applications, and is compatible between multiple vectors, allowing several different intracellular targeted gene constructs to be made in one step. The prokaryotic region (origin and Kanamycin resistance [*kanR*] gene) is flanked by prokaryotic transcriptional terminators to improve stability and yield with a broad range of target genes. Unique restriction sites flank the prokaryotic modules, to allow easy modifications (i.e. replacement of KanR cassette with repressor titration cassette [20]). All plasmid elements have been optimized and minimized to comply with FDA guidelines [21] regarding content and elimination of extraneous materials. The resulting vector is much smaller than existing vectors such as gWiz (5 kb versus 3–3.5 kb for the pDNAVACC vectors) yet drives higher levels of expression in vitro (see below).

3.2. Vector backbone alterations dramatically affect copy number and expression

The yield of plasmid is dramatically altered by orientation of the prokaryotic origin and *kanR* gene relative to each other in the same vector backbone (Table 1). In general, the replication origin needs to be protected from read-through transcription from adjacent genes to prevent plasmid destabilization or reduced copy number [22]. The optimal orientation determined herein protects the origin from transcriptional readthrough, by divergent transcription of the origin and *kanR* gene, as well as inclusion of dual transcriptional terminators after the *kanR* gene.

The pDNAVACUltra plasmid has the optimized *kanR* gene-origin orientation, as well as a rationally designed RNAII mutation. This increases plasmid copy number two-fold versus pDNAVACC (Table 1).

DNA quality is also affected by the organization of elements, as well as the specific elements included (e.g. CMV promoter) [23]. The pDNAVACUltra vector developed herein has a high degree of stability and quality with respect to: (1) replication intermediates; (2) dimerization; and (3) percent supercoiling. In contrast, reversal of the *kanR* gene in pDNAVACC leads to low-level replication intermediate formation (Table 1).

Transfection with pDNAVACUltra–EGFP results in high level of expression with close to 100% transfection. Expression is improved relative to the control gWIZ–GFP plasmid (Fig. 2) and the pDNAVACC–EGFP vector (*kanR* gene in opposite orientation; Table 1).

3.3. Intracellular targeting

High throughput seamless cloning site targeting gene leader cassette incorporating targeting leader signals (human TPA, mouse ubiquitin A76) and/or C terminal membrane anchoring tags (human PLAP GPI-Anchor or human Lamp1 endosome), as necessary, were created, and incorporated

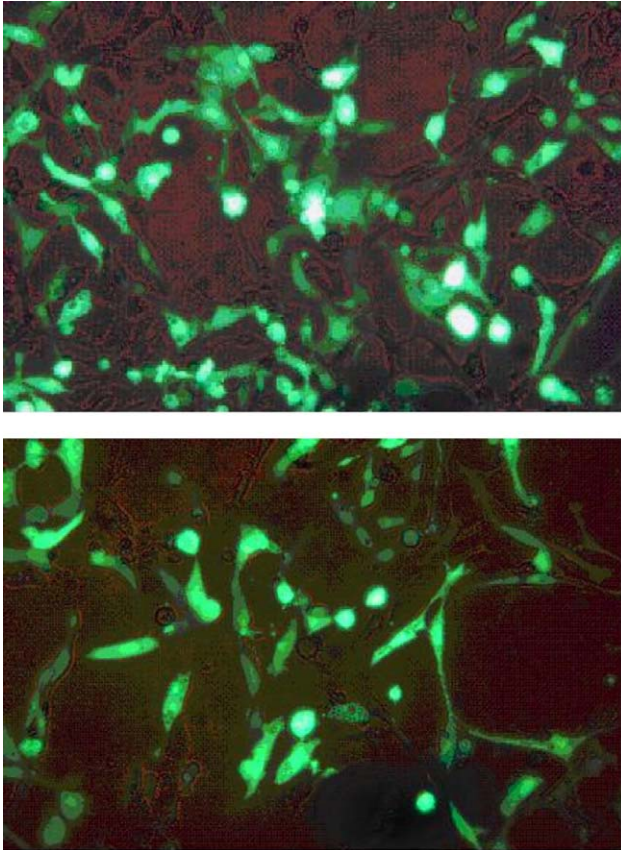


Fig. 2. In vitro expression from pDNAVACC plasmids. In vitro expression of EGFP driven by pDNAVACCultra-EGFP vector (top) or gWIZ-GFP (bottom).

into a family of pDNAVACCultra vectors, pDNAVACCultra1 (secreted-endosome; TPA-Lamp1), pDNAVACCultra2 (secreted; TPA), pDNAVACCultra3 (secreted-membrane-anchored; TPA-PLAP; Fig. 1A), pDNAVACCultra4 (proteasome; ubiquitin), pDNAVACCultra5 (native; Fig. 1B), pDNAVACCultra6 (membrane-anchored; PLAP), pDNAVACCultra7 (endosome; Lamp1). All cloning sites are designed for high throughput cloning applications (Fig. 1B; see below), and are compatible between multiple vectors, allowing several different intracellular targeted gene constructs to be made in one step. The sequences of the vectors are available upon request.

3.4. High throughput cloning

An example cloning application of the pDNAVACC vectors is outlined in Fig. 3. Genes are copied by amplification from clones or genomic DNA using primers incorporating *SapI* sites into termini to generate either 5'-ATG and 3'-TAA or 5'-ATG and 3'-GGC 3 bp sticky ends upon digestion with *SapI* (New England Biolabs, Beverly, MA, USA). For the stuffers that do not have a C terminal extension (e.g. Ubiquitin, native and secreted) the address tags correspond precisely to the start (ATG) and stop (TAA) codons of the gene (Fig. 1B;

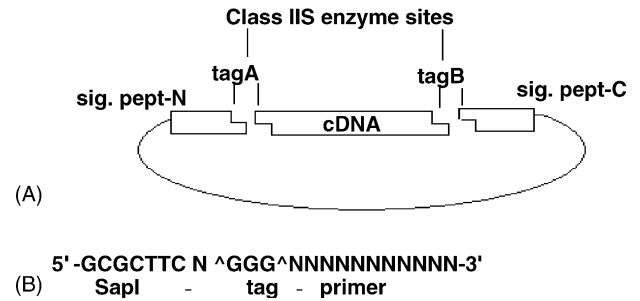


Fig. 3. Method for directional amplification and cloning of cDNA sequences into pDNAVACC vectors. (A) Plasmid containing two unique address tags, created by digestion with class IIS enzymes located between cuts. (B) Typical primer, containing a class IIS enzyme recognition signal (*SapI*), at least one intervening nucleotide, and an overlapping region with a unique, non-palindromic sequence (GGG, the address tag in this example).

the ubiquitin and secreted clones contain targeting peptide immediately upstream of the ATG). For membrane or endosome anchored vectors, a GGC glycine linker is used instead of TAA stop, to facilitate the C terminal extensions needed for trafficking (i.e. GPI or endosomal targeting) to be inserted between the GGC and the TAA stop codon). Cleavage of the vectors with *SapI* generates sticky ends compatible with the cleaved PCR product. The insert is thus directionally and precisely cloned into the vector. The vector and PCR product *SapI* sites are not incorporated into the final vector. *SapI* sites within the target gene are generally not detrimental since there is only a 1/16 chance that an internal *SapI* site would match one of the address tags. Cloning of several independent antigens into the vector family has been performed; in all cases the majority of recovered colonies were correct recombinants (data not shown). An 8–96 well (PCR [96-well gradient block]) format can be used for high throughput applications (PCR, purification, digestion, ligation to *SapI* digested vector). This method is superior to recombination mediated cloning for this application, since with class IIS cloning a single primer pair facilitates cloning into three vectors, whereas sets of longer primers with vector specific sequences would be needed for seamless recombination cloning into trafficking vectors.

4. Discussion

4.1. Intracellular targeting

A DNA vaccine vector family, with an identical backbone to limit variability, and seamless cloning cassette, has been developed, such that a gene product can be targeted to multiple intracellular destinations, without alteration of flanking vector or gene sequences. These vectors are designed to allow simultaneous cloning into multiple vectors that feature various intracellular targeting destinations for the protein product. The cloning requires no additional bases, such as a

restriction enzyme site, to be present in the final vector. Thus, the variability between vectors imposed by additional bases required for traditional cloning is eliminated.

As well, the vectors facilitate “mixed presentation immunization” wherein an immune response to a target antigen is enhanced by immunization with a plasmid cocktail [24,25]. pDNAVACCultra plasmids allow testing all combinations of antigen targeting for up to five different intracellular destinations.

The vectors are also suited for high throughput cloning and expression of putative genes from sequenced genomes such as malaria [26]. This so called “reverse vaccinology” approach is a time and cost effective method for applying genome data to vaccine development (reviewed in [27]).

4.2. Vector design considerations

Expression levels from the CMV promoter is affected by the orientation of the kanamycin resistance gene, with dramatically higher expression when the kanamycin gene promoter is distal to this eukaryotic promoter. This may reflect the presence of spurious binding sites for eukaryotic repressor or activator proteins. Indeed, a number of elements from prokaryotic plasmids have been shown to negatively affect gene expression in eukaryotic cells [28,29] or bind eukaryotic transcription factors [30–32]. Our observations of effects of *kanR* gene orientation on eukaryotic expression, prokaryotic copy number and replication intermediate formation, further demonstrate that sequences in the plasmid backbone need to be consistent between targeting constructs, and be carefully optimized for therapeutic applications.

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