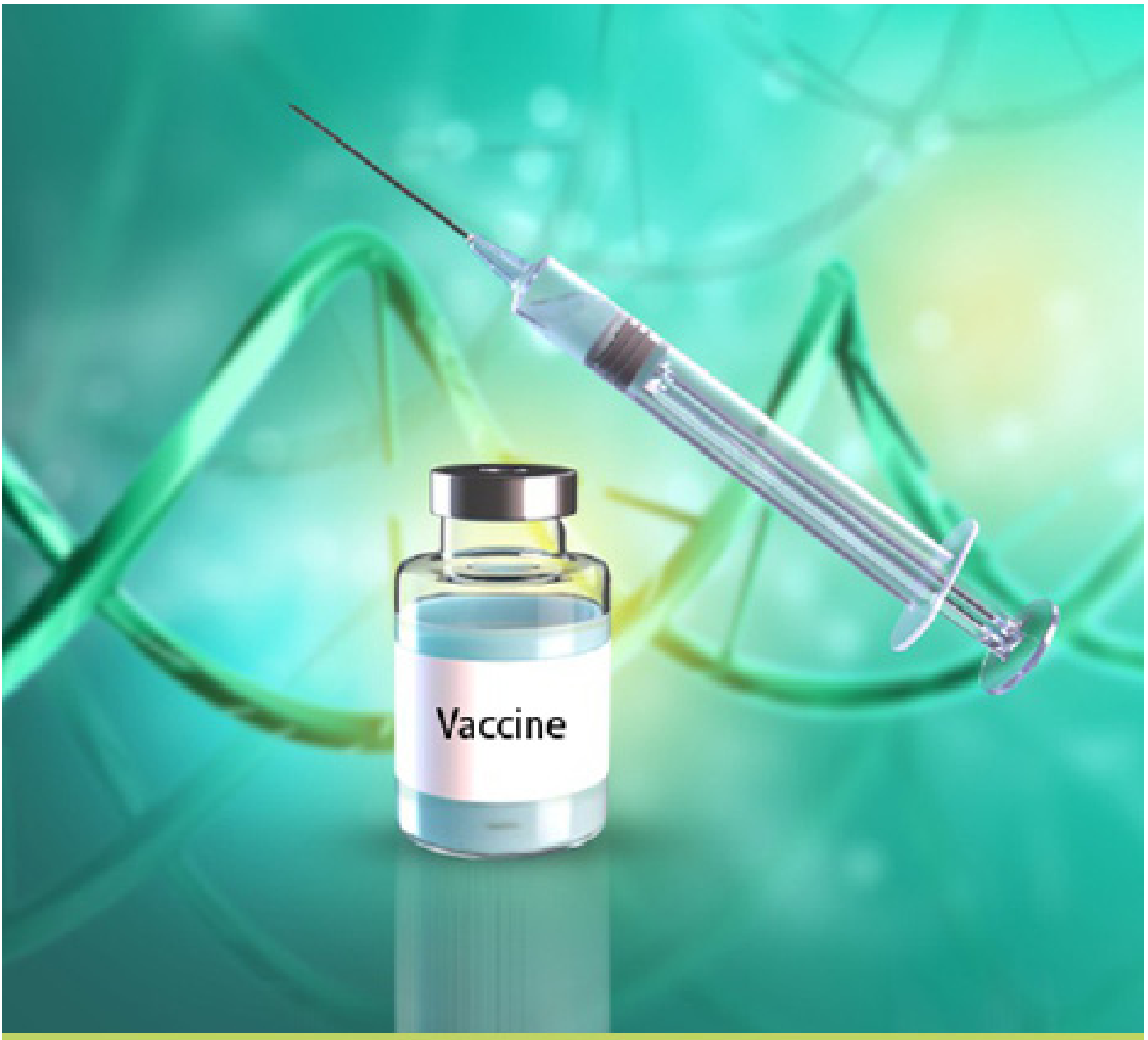

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DNA VACCINES

A Promising Vaccine for the Future

DNA Vaccines: A Promising Vaccine for the Future

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Published Online: Mar 08, 2021

eBook: DNA Vaccines: A Promising Vaccine for the Future

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Abbreviations: DNA: Deoxyribonucleic Acid; NA: Ribonucleic acid; PCR: Polymerase Chain Reaction; MHC: Major Histocompatibility Complex; MHC-I: Major Histocompatibility Complex-I; MHC-II: Major Histocompatibility Complex-II; CD: Cluster of Differentiation; CD4+: Cluster of Differentiation antigen 4; CD8+: Cluster of Differentiation antigen 8; CTL: Cytotoxic T Lymphocytes; MVA: Modified Vaccinia virus Ankara; TRAP: Thrombin Receptor Activating Peptide; rVv: recombinant Vaccinia virus; TNF: Tumor Necrosis Factor; TGF: Transforming Growth Factor or Tumor Growth Factor; TLRs: Toll-like receptors; CMV: Cytomegalovirus; dNTP: deoxy Nucleotide TriPhosphate; CHO: Chinese Hamster Ovary; DMEM: Dulbecco's Modified Eagle's Medium, Gibco; FCS: Fetal Calf Serum; RT: Reverse Transcriptase; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; ELISA: Enzyme Linked Immunosorbent Assay; SV40:Simian Virus 40.

Foreword

Microbial Pathogens cause much damage in the medical and veterinary fields every year, which in addition to a lot of material damage, in many cases lead to loss of life and in many others cause debilitating diseases.

The tendency not to use antibiotic drugs is increasing. In general, the use of drugs to treat microbial infections is not cost-effective in the long run because, in addition to causing microbial resistance, it remains in meat and animal products and adverse effects on the consumer. These drugs also have adverse effects on the environment.

Common vaccination strategies are all based on vaccine production using live and non-living agents, which have been successful in some cases. However, these strategies are not suitable for many microorganisms, and therefore the use of new methods such as strategy A molecule seems to be essential for advancing vaccine production. There is a need to provide useful and inexpensive vaccines against microbial pathogens. In recent years, advanced DNA vaccine technology has provided a promising future for developing multivalent vaccines and a powerful method for inducing specific humoral and cellular immune re-

sponses by injecting DNA plasmids into the host cells. The genes will express in the cells and will stimulate the immune system. Thus, gene vaccination induces effective and long-term immune responses. The antigen thus produced is processed through the MHC-I and MHC-II systems and stimulates immunological memory. Much research has been done to develop DNA vaccines against different diseases, which have opened up new horizons in this field.

In addition to designing DNA vaccines, the purpose of writing this book is to acquaint researchers and students with new generation vaccines and present practical methods for preparing DNA vaccines. This book is about DNA vaccines, how they are stimulated by the immune system and adjuvants' role in boosting vaccines. This book's contents can be useful for researchers and postgraduate and doctoral students in the fields of biotechnology, microbiology, laboratory sciences, and immunology and vaccine centers.

Molecular vaccines

Molecular vaccines are based on the production of recombinant antigens by microorganisms, and extensive research has begun. This research uses the organism's genetic agent, DNA, or RNA, which is translated into protein by the cells. Some types of these vaccines include DNA vaccines, vaccines with a viral vector, or a combination of the two. RNA's use to make vaccines has disadvantages, including challenging to extract and less stable than DNA. For this reason, most studies are performed using DNA.

In order to obtain the DNA vaccine after genome extraction, the gene fragment encoding the protective antigen is amplified by PCR, which is then cloned into a plasmid vector under the control of a strong eukaryotic promoter. This vector is injected into the host and expressed in the host, creating a resistance protein. This gene appears to remain extra-chromosomally within the nucleus. DNA vaccines elicit strong humoral and cellular responses and increase immunogenicity potential through vectors or adjuvants such as cytokine genes.

Vaccines and vaccination

A vaccine is a substance that originates from a microorganism or biological products and creates immunity related to the particular disease. The purpose of vaccination is to stimulate the immune system to produce sufficient amounts of antibodies and create cells that increase rapidly upon re-exposure to the antigen and provide significant immunity to the relevant infectious agent. In the development of vaccines, it is common to identify immunogenic antigens and parts of the molecule important in terms of immunogenicity.

Today, vaccinations are necessary to fight many diseases, especially infectious diseases. Vaccines reduce mortality from infectious diseases worldwide and play a key role in controlling infectious diseases. It is also a cost-effective way to control infectious diseases. Older vaccines, or the first generation of vaccines, are made from live microorganisms and reduced concentrations, which can cause several problems [1]. Second-generation vaccines are subunit vaccines that consist of several protein antigens or recombinant protein components. DNA vaccines are third generation vaccines. Recombinant DNA technology plays a vital role in the development of these types of vaccines. When the DNA vaccine enters the host cell, the intracellular transcription and translation systems transcribe the DNA vaccine and then make pathogen antigens from the mRNA. These antigens, or proteins, are recognized as foreign proteins by the immune system and produce a wide range of immune responses against them. Compared to older vaccines that required complex production processes, cell culture media, and enriched media, DNA vaccine is easy to prepare and process. The storage of DNA vaccine does not require a chain of cooling and storage in special conditions and is very resistant to environmental conditions [2].

History of DNA vaccines

The idea of gene therapy was first mooted in the 1950s and 1960s; When scientists found that injecting genetic material into the animals' bodies triggered an immune response (it was completely independent of vaccination). In the late 1980s, Robert Zwaga began a study to discover viruses' strategy to transmit DNA into cells. This leads to the production of antigens for vaccination. Wolff in 1990 showed that intramuscular injection of DNA stimulates the immune system. In this study, the induction of protein expression in intramuscular injection directly from plasmid DNA in myocytes has been proven. Direct transfect of the gene from the DNA plasmid into the mouse muscle occurs without the need for a special delivery system. Intramuscular inoculation with the gene encoding the DNA plasmid induces protein expression in muscle cells. These vaccines are new types of subunit vaccines that express protein after delivering plasmids or antigen-encoding viral vectors into mammalian cells. In these vaccines, the aim is to increase the uptake of DNA into the cell, its delivery to the nucleus, increase translation, increase the reaction between T cells and excitatory molecules on antigen-supplying cells, increase the activity of CD4 + cells, and induction of CTL and production of antibodies. DNA vaccines effectively activate and activate MHC-I and MHC-II pathways to induce CD4 +, CD8 +. However, soluble antigens, such as recombinant proteins, usually only induce the antibody response and weaken CD8 + induction. Genetic vaccines are administered into the host by several methods, such as syringe injection into muscle tissue and into the skin. Protective immunity by DNA vaccines has been described in many animal models for many diseases, including HIV, tuberculosis, cancer, and many other diseases, including parasites (Lee et al., 2004; Taracha et al., 2003; Pavlenko et al., 2004).

In the DNA vaccine, a naked plasmid is injected into the host, and the host cells express the encoded protein. It is a powerful method for inducing specific humoral and cellular immune responses.

Intramuscular administration of plasmids encoding influenza viral proteins stimulates cytotoxic lymphocytes' responses specifically to this antigen, which may induce immunity in Exposure to the flu virus [3,4]. These findings were the first evidence that DNA administration could stimulate immune responses to antigens expressed by plasmid DNA. DNA vaccines are also used for gene therapy and vaccination [5].

DNA structure of vaccines

A DNA vaccine is made up of a plasmid, a small circular DNA that can multiply inside a cell. The DNA vaccine is genetically engineered and can be used to produce and express one or more specific antigens from different types of infectious agents and pathogens in the host cell. When the DNA vaccine is injected into the host cell, the host cell begins to read from this plasmid, and foreign proteins and antigens are produced inside the host cell and these antigens are processed by the host cell and

are delivered at the cell surface, thus activating immune cells. When the immune system prepares for the initial response to a foreign antigen, protective and memory immunity against that pathogen is produced and leads to immunity against the pathogen [4,6]. The circular plasmid is isolated from chromosomal DNA and can reproduce alone. Plasmids are naturally present in bacteria but are sometimes also found in eukaryotic organisms [7].

Plasmid size for clinical purposes is also an issue that should be considered and is usually between 3-12 kg bp. DNA vaccine prepared for clinical purposes must be free of any bacterial genomic DNA, protein, or endotoxin. The DNA vaccine is based on recombinant DNA technology. This technology is used to provide genes that are not naturally present in the body. In this technology, a series of specific genes are transferred from an organism to an organism that lacks these genes [8,9]. The instrument used to clone the genes into the plasmid also consists of nucleases, ligases, DNA-modifying enzymes, and topoisomerases [9,10].

Nucleases: Nucleases (exonuclease and endonuclease) destroy DNA molecules by breaking phosphodiesterase bonds [9,10].

Modifying Enzymes: Several DNA molecule-modifying enzymes cause DNA mutation by adding or removing specific chemical groups. Such as: Alkaline phosphatase, polynucleotide kinase, terminal nucleotidyltransferase, topoisomerases, etc [10].

Ligases: The enzyme ligase repairs a broken strand of double-stranded DNA and binding two strands of double-stranded DNA [10].

DNA components of the vaccine

Gene Selection

Different genes from different sources can be cloned into a DNA vaccine [11].

Promoters

Many designed vectors use viral promoters. Viral promoters are typically isolated from cytomegalovirus and SV40 virus. In general, viral promoters provide higher levels of gene expression than eukaryotic promoters. There are other promoters such as Creatine kinase and Metallothionein promoters that are specific for muscle cells and keratinocytes [10-12].

The origin of replication

This part is isolated from E. coli bacteria and is used in the DNA of vaccines. This section leads to an increase in the number of plasmid copies within the bacterium, which allows the production of large amounts of plasmid DNA [10-12].

Selected marker

Selective markers such as antibiotic-resistant genes, triclosan, and selective RNA markers facilitate the selection of transformed bacteria. The most commonly used markers are antibiotic resistance genes, such as the ampicillin-resistant gene. However, because the ampicillin-resistant gene cannot be used in eukaryotic cells, the kanamycin-resistant or neomycin-resistant gene is used instead of the ampicillin-resistant gene [11,13].

Polyadenylated signal sequence

There is a polyadenylated sequence downstream of the promoter, such as bovine growth hormone polyadenylated sequence and SV40 polyadenylated sequence, which results in the transcribed stability mRNA. Their role is to provide a consistent and effective expression [11].

Kozak sequence

Sequences around the AUG initiation codon within mRNA affect its detection by eukaryotic ribosomes. Studies have shown that the Kozak sequence plays a crucial role in maximal protein expression. By using this Kozak sequence, the expression level of these genes can be increased. Prokaryotic genes and some eukaryotic genes do not have this Kozak sequence, so using this Kozak sequence can increase the expression level of these genes [10,11].

Mechanism of action of DNA vaccine

The DNA vaccine gene is expressed inside the host cell, then processed and delivered by the MHC to the antigen-supplying cells. A DNA vaccine is like a viral infection and can activate cell-mediated immunity and humoral immunity. When a foreign protein is produced, the produced protein is converted to antigenic peptides by the host proteases. These antigenic peptides enter the endoplasmic reticulum and bind to MHC-I molecules, which are then delivered to the cell surface along with MHC-I and can become T-cells. Stimulating CD8 + Cytotoxic cells activated by two mechanisms (disintegration of infected cells and chemokine production stimulation) leads to a disintegration of infected cells. Antigenic proteins are delivered to the cell surface via MHC-II. In this way, T-CD4 + cells are stimulated and can detect exogenous proteins endocytosed by APC cells. After activation of T-CD4 + cells, these cells are also able to stimulate B cells, which eventually produce activated B cells that produce specific antibodies against the antigen [6,12,14].

Benefits of DNA vaccines

- Along with the DNA vaccine, oligonucleotides can be inserted into the host cell, altering gene processing and gene expression within the cell. One of these oligonucleotides is siRNA. siRNAs are double-stranded RNA molecules that are involved in controlling the transcription and expression of genes. Their specific function has also caused them to be considered today in the fight against cDNA vaccine has the power of immunization and protection against infection and at the same time is not infectious [2,12].
- DNA vaccine stimulates all three immune systems: Humoral, cellular, and mucosal DNA Vaccines cause long-term expression of antigens, thus creating an excellent immune memory [2,6,12].
- Administration of small amounts of DNA vaccine can induce a very strong and stable immune response [2,4,16].
- Lack of immune response to vector injection and lack of antibody production against vector receptor cells [4].
- DNA vaccines are not as high risk as traditional vaccines [4].
- MHC-I and MHC-II are stimulated by DNA vaccines, which is a critical feature for these vaccines [4,6,12].

- A DNA vaccine can be designed to encode several antigens from several different pathogens at the same time, and thus by designing a DNA vaccine, immunity against multiple pathogens can be stimulated [4,6,12].

- DNA vaccines are much safer than attenuated vaccines [4].Unlike protein immunization, the protein produced by the DNA vaccine inside the eukaryotic cells has a better structure and is closer to the original protein. It is appropriately glycosylated and undergoes post-translational changes similar to the original protein. In this way, because the produced protein has a better spatial structure, a higher quality of neutralizing antibody [4,17]. Antigens are produced directly in the vaccinated animal (such as a eukaryotic system), thus avoiding incorrect folding or the lack of some post-translational corrections resulting from the prokaryotic expression of recombinant proteins.The DNA vaccine is more resistant to temperature than older vaccines and does not require the preparation of a cooling chain [4].

- Do not need to produce and purify protein.

- Concomitant use of these vaccines with plasmids encoding cytokines or immune-stimulating molecules (adjuvants) increases or modulates immune responses against the encoded antigen.

- In this type of vaccine, DNA itself acts as an adjuvant, and the presence of specific sequences in DNA, and the presence of an unmethylated CpG motif-rich agent in the vector or its addition to the vaccine formula, strongly enhances the Th1 response and IFN-γ activity.

Limitations of DNA vaccine

- It can only be used to supply protein antigens and cannot produce non-protein antigens [4,17].

- These vaccines can affect genes that control cell growth. It is also possible to develop tolerance to the antigens produced by the DNA vaccine, although it is suggested that the DNA vaccine may be tumorigenic and inserted into the host cell genome, leading to a response. Autoimmune diseases, but in these cases, especially in humans and primates, there is little evidence [4].

DNA vaccines for todays and future

In recent years, strategies have been developed to increase the DNA potency of vaccines, including electroporation, concomitant use of cytokines, viral vectors, liposomes, or microparticles Pointed out. Although some of these can significantly enhance the immune responses of DNA vaccines, there are limitations to their general use as a vaccine adjuvant. For example, electroporation is very effective but not readily available in humans, and cytokines and viral vectors also have side effects and endanger human health.

In general, DNA vaccination induces a Th1-dependent immune response more than a Th2-dependent immune response. At the same time, the efficiency of vaccination depends more on the way of immunization. DNA vaccines are highly immunogenic and induce immune responses different from the normal response. as regards DNA is more comfortable to obtain and heat-resistant, it seems that the use of DNA vaccines in tropical countries where protein vaccines require cold-chain transmissions may be more appropriate.

Mechanism of DNA vaccines according to plasmid structure, plasmid content, antigen expression level, immunization method, immunization route, target tissue, number of immunizations, presence or absence of introns in the gene, vaccine strain, and animal age depends. There are different ways of getting the DNA vaccine into the host body, which differs in terms of ease of operation, cost, and type of immune response. Syringe injection into muscle or skin tissue is the most commonly used method. Different spleens and mucosal surfaces, such as the nose and gastrointestinal tract, are also targets for the genetic vaccine. The first two methods induce an extraordinary immune response.

Intracellularly produced antigens (such as muscle cells) are delivered to MHC-I by antigen-supplying cells. Once released, proteins are ingested by macrophages that deliver immune peptides alongside MHC-II, which in turn induce Th1,2 immune responses.

In molecular vaccines, the immune responses are strong despite the small amount of antigen produced (in nanograms or picograms) by the transfected cells.

Extensive efforts have been made to improve the immunization of these vaccines, including using genes related to stimulant molecules, cytokines, and various methods as a vaccine supply. For example, the IL-12 gene in a vaccine DNA causes IL-12 expression and TH1-type immune stimulation by the vaccine. Today, the Prime-Boost strategy is used to increase the immunogenicity of vaccine DNA. In this procedure, the vaccine DNA is injected with a secondary antigen. This secondary antigen can be a subunit protein, an inactivated microorganism, or a recombinant viral vector such as adenovirus or recombinant Pax virus. This method is much more effective in increasing the vaccine's immunization than when the vaccine is given alone; Because vaccine injection techniques and antigen delivery systems in DNA, vaccination can play an essential role in stimulating immune responses [12,18].

Reinforcement the efficacy of DNA vaccines

In the future, DNA vaccines will focus on increase the efficacy of DNA vaccines.

Prime-boost immunization: In intracellular pathogens that cell-mediated immunity is the main route for protection, and increase the response of T cells to vaccination is based on an immune-driven heterologous prime-boost strategy [19,20]. This method is a combination of primary immunization, or it starts with a DNA (prime) vaccine by amplifying the various viral vectors that are encoded for them the same antigen (booster) [21].

Multi-epitope DNA vaccines: In this method, the efficacy of DNA vaccines increased by isolating the efficient epitopes from the whole genes. In this technology, the researchers get help from bioinformatics tools to design efficient epitopes.

Multi-epitope DNA vaccination is based on the isolation of efficient epitopes from whole genes. This technology requires the use of bioinformatics tools for designing the epitope mapping and selecting the protective sites [21,22].

Preparation of DNA vaccines with viral vectors: Agaves are used to enhance the immune response of the vaccine, and their use is essential in the development of modern vaccines. Genetic aggregates are expression vectors that encode biologically active molecules such as cytokines and chemokines. These aggregates can be in the same vector that encodes the antigen or

can be used in a separate vector with the vaccine. This method induces agonist activity at the antigen delivery site with a long-lasting effect on transfected cells. Cytokines have been selected as genetic aggregates because they alter the host defense cells and use them to induce immune responses. The use of cytokines in the DNA formulation of vaccines in many infectious and parasitic diseases is done to enhance the protective responses of the T cell subset [23].

Conventional and genetic adjuvants: DNA vaccines can stimulate immune responses, and the plasmid of DNA vaccine acts as an adjuvant. In cases that the responses are weak, both adjuvants, genetic and conventional, can be helpful [21,24,25].

Preparation of polyvalent vaccines: One of the advantages of DNA vaccines is the possibility of combining several antigens in one plasmid or administering a mixture of plasmid vectors simultaneously. The production of multivalent vaccines that contain several antigens is a new way to build resistance in the host against different strains of parasites and different stages of the parasite life cycle. Parasites are complex organisms with a multistage evolution and have been identified as antigen change mechanisms to escape the immune system. Multivalent vaccines have a higher number of protective epitopes and are useful in a large population percentage. In multivariate vaccines, the composition and extent of the combination of vaccines and antigens should be evaluated and optimized to determine their synergistic effects. In studies on vaccination against leishmaniasis in mice, different parasitic antigens with different degrees of resistance were identified as polyvalent DNA vaccines. When these antigens are used in combination, they can create complete resistance or increase resistance [6].

Codon optimization: The most significant barrier to making DNA vaccines is the intraspecific difference in the codon used. Because vaccine DNA uses host cells for transcription and translation, and each has a bias codon, most genes are encoded, and the use of these selected codons is related to the adequacy of gene expression. Very close species use the same codon. In cases where there are considerable differences in the codon used, for example, between the pathogen and the mammal, codon optimization may be required. In this coding strategy to encode genes in The vaccine, DNA is used to modify the biased codon to increase expression in mammals [6].

Immunization of Prime-Boost (primary and enhancer) by DNA vaccines

Conventional subunit vaccines often produce strong antibody responses and weak cellular immune responses. DNA vaccines in animal models can elicit strong humoral and cellular responses, but although these vaccines are safe for humans, they cannot produce cellular immune responses similar to animal models in humans. In cases where the pathogen is intracellular, the antibody response is insufficient to protect because cell-mediated responses are also required. Like malaria, a parasite of hepatocytes and infects erythrocytes, cytotoxic T cells play an essential role. Therefore, it is important to adopt measures for the vaccination strategy that increase T cells' immunogenicity and the protective immune responses against intracellular pathogens. Research to increase T cell responses to vaccination is an inconsistent Prime-boost-based immunization strategy [19]. This method combines primary and booster vaccination with different vectors that encode the same antigen. This strategy's basis is that in primary vaccination (prime), some specific T cells act antigen, and in booster injections, they cause T cells to expand and respond quickly. DNA plasmids are good agents for priming, so they enter antigen-supplying cells and are supplied by MHC classes I and II. DNA plasmids induce cell-mediated immune responses due to the presence of the methylated CpG motif, which induces the expression of Th1 cytokines. Recombinant viral vectors that are safe are suitable for booster injections. Viral vectors cause high protein expression and delivery by MHC class I, leading to specific T cell responses [19]. Viral vectors cause high production of proinflammatory cytokines, resulting in increased cellular immune activity. In addition, the immunogenicity of viruses is greater than that of DNA plasmids, and if used alone, immune responses to vector components (viruses) are generally targeted. That is why primary heterogeneous and booster vaccination performed with different vectors results in a more significant increase in antigen-specific responses than vector-specific responses. Using this technique is the production of memory T cell antigens in primary vaccination and their amplification in booster vaccination.

Common vectors for booster vaccines include Ankara Modified Vaccine Virus (MVA), recombinant Vaccine virus (rVv), Diluted Adenovirus, and smallpox viruses such as chickenpox (FP9). These highly diluted viruses are not able to transcribe but have the ability to produce proteins. The MVA vector was passaged for preparation 500 times in egg embryo fibroblasts. This vector was used for the smallpox vaccine in 1970, which is characterized by safety and high immunogenicity.

The immunogenicity induced by viruses is greater than that of plasmids, although there will be immune responses against viral components when used alone. For this reason, primary and booster vaccination with different vectors increases antigen-specific responses relative to vector-specific responses. This result shows that when a heterogeneous prime-boost technique is used, it produces antigen-memory T cells by amplifying these cells by a booster dose [26-28].

In cases such as malaria, which infects hepatocytes and erythrocytes, cytotoxic T cells play an important role in resistance. One of the new methods that increase T cell response is the heterogeneous prime-boost strategy. In this method, both prime and boost injections have different vectors, but both encode the same antigen. In this strategy, after the initial vaccination, some T cells become specifically sensitive to some antigens, and after booster vaccination, T cells develop rapidly after repeated contact with the specific antigen. This method is widely used in immunization against malaria and several other parasites [6].

To enhance the efficacy of Plasmodium urea DNA vaccine, the initial injection of DNA vaccine intramuscularly and a plasmid containing Granulocyte and Macrophage Colony-Stimulating Factor gene (GM-CSF) by rVv injection of Sporozoite Cyclic Protein (CSP) is a booster [28]. This mixed strategy with genetic aggregation and immunization in the form of prime-boost increases protective responses while reducing the original antigen DNA vaccine dose. In chimpanzees, initial DNA injection and MVA amplifier encoding the Thrombin-Associated Adhesive Protein (TRAP) together with the GM-CSF protein as an adjuvant, it induces a specific response of T cells and antibodies' production, leading to a long-lasting and persistent response against Plasmodium falciparum [29]. Complete protection against leaf Plasmodium is characterized by strong CD8 + T cell responses and develops in mice after initial intradermal injection of adenovirus and MVA booster CSP encoding the sporozoite cyclic protein [30]. These studies led to human immunization of prime-

boost in volunteers and field experience in endemic areas. The DNA-Prime-MVA-boost codon of Plasmodium falciparum TRAP-erythrocyte fusion encoding polypapyotope antigen used by gene gun method had no adverse effects on volunteers [31]. In fact, this heterogeneous prime-boost immunization leads to interferon-gamma secretion, an antigen-specific T cell in humans that acts significantly more than the vector alone [32]. Relative protection is designed to reduce parasitism after challenge with different strains of Plasmodium falciparum. In another group, the initial injection of DNA vaccine for CSP gene of Plasmodium falciparum and booster with recombinant protein with adjuvant (RTS, S / AS02A) resulted in a significant increase in antibody and T cell responses in volunteers [33]. Phase 1 Clinical experience in the Gambia in semi-safe adults with the injection of primary DNA (MVA primer) and DNA booster of Plasmodium falciparum antigen encoding TRAP and resulted in more responses than unvaccinated individuals. Booster with MVA vaccine 12 months after initial injection in clinical experience successfully activated T cells and showed that the MVA booster injection at different periods leads to the renewal of T cell immunity.

Multivalent DNA vaccines

Another advantage of DNA vaccines is the possibility of using several genes in one plasmid or in several plasmids and their simultaneous use, which leads to increased protection against parasitic infections. Parasites are complex organisms with different life stages and antigenic changes to attack the host immune system. In addition, in natural contaminants, individuals' immune responses to similar antigens are not the same. Multivalent vaccines have more protective epitopes and are therefore more effective. However, in multidrug vaccines, the antigen composition used must have a synergistic effect.

Vaccination studies against leishmaniasis in mice with different protein antigens lead to varying degrees of protection. When combined with multivalent DNA vaccines, they result in complete protection or increased protection. In fact, a DNA vaccine that encodes three different Leishmania antigens, LACK (Leishmania major-activated C kinase), TSA (thio-specific antioxidant), and LmST11 (L. major stress-inducible protein), can provide complete protection against Leishmania major. Compared to killed parasites. This protection reduces the parasite load and uses T cells of both CD8 + and CD4 + [34].

In addition, intradermal injection of the plasmid compound is more effective than intramuscular injection or subcutaneous injection and reduces the dose by up to 5 times. In another study, the prime-boost method was protective with Cpa / Cpb genes (L. major cysteine proteinase genes) and increased IFN-γ production by spleen cells, but was not protective when injected separately. Cysteine proteinase is produced in varying amounts during the parasite evolution period and is thought to modulate host responses for parasite survival. Cysteine proteinase only triggers Th1-type immune responses when used in combination. Just as natural antigens have an important effect on vaccine efficacy, a comparative evaluation of antigens' protective potential is necessary to determine the optimal DNA vaccine design. In order to increase the efficacy of DNA vaccines against parasites, the Prime-Boost immunization method, genetic agonists, and multivalent vaccines are used [35,36].

Steps of gene cloning

A. Preparation of DNA fragment for cPreparation of the desired DNA fragment

There are several methods for isolating a gene from the source genome, which are briefly described below.

1. Using enzymatic digestion of the source genome with cutting enzymes to cut the desired fragment.
2. Using mechanical methods to fragment the source genome and then cloning the fragments whose nature is unknown and finally select the clone containing the desired gene.
3. Duplicate cDNA is synthesized from mRNA extracted from the target cell, and RT-PCR amplifies the fragment.
4. Chemical synthesis of the desired component in the test tube DNTPs are polymerized in the desired order using chemical methods.
5. After designing the primer for the gene in question, the sequence of which has already been determined, PCR allows the gene to be explicitly amplified millions of times and used for cloning. If the gene sequence is unknown, a random primer can be used to amplify it by PCR.

B. Create a suitable end to connect to the carrier

These cut-offs are made using cutting enzymes that make pieces with sticky or smooth ends. Different types of these enzymes, which are able to break phosphodiesterase bonds inside a DNA molecule and identify their specific sequence, have been isolated from different bacteria. The distinguishing feature of these enzymes is the specific sequences that each identifies and only cut that site. Table (1-2) lists the different types of these enzymes and their identified sequences. Most of the identified sequences of these enzymes are either symmetric or palindromic.

2- Connection to the carrier

1. A piece of DNA containing a gene to be cloned is inserted into a circular DNA molecule called a vector or DNA vector. Once the fragment is placed, a recombinant DNA molecule is produced. DNA ligase enzyme is used to bind two DNAs together. This enzyme forms a phosphodiesterase bond between two nucleotides. There are two types of DNA ligase: T4, which is extracted from phage T4 and provides the energy needed for its ATP activity. This enzyme connects two smooth ends as well as two sticky ends. Another enzyme is the DNA ligase bacterium E. coli, which derives its energy from NAD and is mostly used to bind adhesive ends.
2. A vector is a means of transport that carries a gene to a host cell. Usually, the host cell is a bacterium, although different types of eukaryotic cells can also be used.
3. The vector is amplified inside the host cell. Several copies of it are made inside the host cell, each containing the transferred gene.
4. When the host cell divides, copies of the recombinant DNA molecule are transferred to the daughter cells, and its replication inside the cells continues.
5. After many clonal cell divisions are formed from the same host cells containing the vector with the desired gene, Figure 1shows the steps of gene cloning.

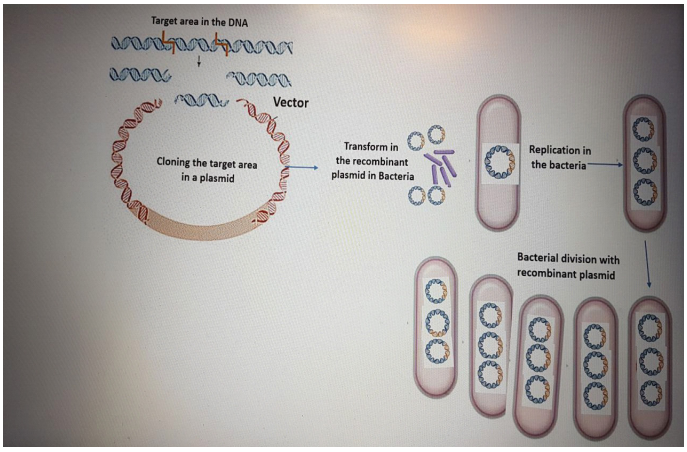

Figure 1: Cloning gene steps including the insertion of the desired fragment into the vector, transformation of the recombinant vector into the bacterium, and bacterial proliferation.

In order for a carrier to be usable, it must have properties that are the criteria for selecting that carrier. The first is to have replication independent of the host cell so that it can proliferate faster than cell division and then transfer to new cells after cell division. It should be small in size (up to 10 kg open) to not be crushed during the cloning and purification steps. The size of the desired piece is one of the factors that limit the choice of the carrier. Another criterion for selecting a vector is the type of host cell. According to the different purposes of cloning genes, the appropriate vector should be selected. For example, for an expression gene, it sought an expression vector with a strong promoter that was compatible with the host cell.

After cutting the vector that has the desired enzymatic cutting sites in the Multiple Cloning Site (MCS) region, at this stage, considering that the DNA fragments are prepared, and also the vector has a sticky or smooth end, the following methods Are used.

1. Connecting the sticky ends
2. Bonding of smooth ends, produced by the homopolymer method at the end of which a single-stranded homopolymer and therefore the sticky end is produced.
3. Connecting smooth ends by connecting linker molecules or adapters and creating sticky terminals.

Success in cloning produced PCR fragments depends on several factors, including the purity of the PCR product, the selection of appropriate cutting enzymes, the primer's design, and the selection of the plasmid. Although cloning of amplified products by PCR is sometimes difficult, new and evolved carriers and modern methods to increase cloning efficiency have now been developed.

A number of vectors are designed to clone high-performance PCR products. These vectors can select and evaluate recombinant plasmids.

The activity of Taq DNA polymerase will be to design and produce vectors that will enable us to directly clone the highly efficient PCR product produced within these vectors.

This method is often referred to as T A cloning. The advantages of this method are:

1) It is not necessary to have information from the DNA sequence.
2) Enzymatic digestion is not necessary after PCR.
3) No enzyme treatment is required for smooth ends of PCR product.
4) Reliable and simple.

The genetic elements embedded in the pTZ57R / T vector, their function and position, and the position of the multiple cloning site of the pTZ 57 RT vector are shown in Figure 2.

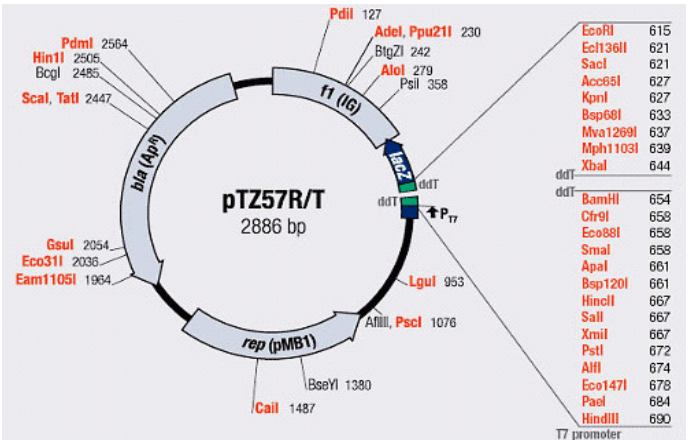

Figure 2: Position of multiple cloning vector site pTZ 57 RT (Adapted from https://tools.lifetechnologies.com/content/sfs/gallery/high/k121_2.jpg)

Table 1: Genetic elements embedded in pTZ57R / T vector, their function and location.

Genetic elements of pTZ57R/T cloning vector Element	Function	Position (bp)
rep (pMB1)	A replicon (rep) from the pMBI plasmid is responsible for the replication.	1122-1736
Replication start	Initiation of the replication.	1136 (±1)
bla (ApR)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells.	1896-2756
LacZ α-peptide	Blue/white screening of recombinant clones	449-739
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	615-695
Cloning site	3'-ddT tailed DNA ends for ligation with insert	650-651
Phage f1 origin	Synthesis of a single-stranded DNA	2-457
T7 promoter	*In vitro* transcription of insert DNA with T7 RNA polymerase	697-716

M13/pUC forward sequencing primer (-20)	Sequencing of insert, colony PCR	599-614
M13/pUC reverse sequencing primer (-26)	Sequencing of insert, colony PCR	735-751
T7 promoter sequencing primer	Sequencing of insert, colony PCR	697-716

Table 2: Identification sequence of some cleavage enzymes commonly used in cloning genes.

Sequence and place of cutting the enzyme from the direction 5' → 3'	Kind of restriction	Origin of enzyme	Enzyme
G* G A T C C	Sticky	*Bacillus amyloliquefaciens*	Bam HI
G* A A T T C	Sticky	*Escherichia coli RY 13*	Eco RI
A* A G C T T	Sticky	*Haemophilus inflenzae Rd*	Hind III
*G A T C	Sticky	*Moraxella bovis*	Mbo I
C T G C A * G	Sticky	*Providencia stuartii*	Pst I
C C C * G G G	Blunt	*Serratia marcescens*	Sma I
T * C G A	Sticky	*Thermophilus aquaticus*	Taq I
C * C C G G G	Sticky	*Xanthamonas malvacearum*	Xma I
GG*CC	Blunt	*Haemophilus aegyptius*	Hae III
AG*CT	Blunt	*Arthrobacter luteus*	Alu I
CGATCG	Sticky	*Proteus vulgaris*	Pvu I
GANTC	Sticky	*Haemophilus inflenzae*	Hinf I
GATC	Sticky	*Staphylococcus aureus*	Sau 3A
GCGGCCGC	Sticky	*Nocardia otitidis*	Not I
GGCCNNNNGGCC	Sticky	*Streptomyces fimberiatus*	Sfi I

-N-N-A-G-C-T-N-N- -N-N-A-G C-T-N-N-

Alu I

-N-N-T-C-G-A-N-N- -N-N-T-C G-A-N-N-

N= A, G, C or T Blunt ends

-N-N-G-A-A-T-T-C-N-N- -N-N-G A-A-T-T-C-N-N-

EcoR I

N-N-C-T-T-A-A-G-N-N- -N-N-C-T-T-A-A G-N-N-

Sticky ends

Mechanism of action of DNA ligase enzyme

Escherichia coli and phage T4 stagnate an enzyme called DNA ligase, filling the gaps in a strand of double-stranded DNA and creating a phosphodiesterase bond between them. The enzyme first binds to an ATP, and when a pyrophosphate group is released, the AMP enzyme complex is formed, which binds to it through a 5-P slit to form a new phosphodiester bond.

1. In the case of parts with smooth ends, to increase the possibility of connection with the carrier, it is possible to create a sticky end in the terminals of the desired part. Using short linker parts
2. Using short adapter parts
3. Using the homopolymer method to create a sticky end

Linkers are short pieces of double-stranded DNA with a specific sequence and are synthesized synthetically in a test tube. The linkers are smooth-ended but have a position of identification for the desired limited-effect enzyme because a large number of them can be used in the binding reaction. It increases, and the smooth end can easily be turned into a sticky end.

Adapters are like linkers, the only difference being that they have a sticky end and a smooth end.

In the homopolymer sequence, the terminal enzyme deoxyribonucleotide transferase is used, and similar nucleotides are added to the fragment's end. Like polymerases, this enzyme needs an OH3 to perform its polymerization action.

DNA transform to bacteria or transformation

After generating the recombinant DNA molecule using the methods discussed above, the next step in cloning the gene is to transfer it into a living cell.

Most species of E. coli only receive limited amounts of DNA under normal conditions, so the bacteria must be physically or chemically treated to increase their ability to receive DNA. The cells on which this treatment is performed are called susceptible cells.

1. After generating the recombinant DNA molecule using the methods discussed above, the next step in cloning the gene is to transfer it into a living cell. Cloning has two main purposes:Allows large amounts of recombinant DNA molecules to be produced from a limited number of raw materials.
2. Purification of the desired gene that has been cloned.

The types of transformation methods can be summarized as follows:

1. Transformation with recombinant plasmid DNA
2. Transformation with recombinant phage DNA

3. Packaging inside phage coating in vitro and transformation by recombinant phage or cosmid

The first method is commonly used in the laboratory and can be done as follows:

1- Chemical method of heat and cold shock and use of calcium chloride or rubidium chloride salt

The physical method of electroporation with electrical pulses creates pores in the cell surface, and recombinant DNA enters the cell.

Screening and isolation of the desired clone

1. Different methods are used for screening:Direct selection: In this method, an antibiotic marker is used to isolate the plasmid containing the resistance gene. Only colonies containing this plasmid can grow on the medium containing antibiotics.

2. Indirect selection: This method uses exotrophic bacteria that will grow in hosts that use and grow the gene product inserted into the plasmid.

Clone analysis is performed to confirm the cloning accuracy and methods such as enzymatic digestion with enzymes used in the cloning process on the plasmids purified from the clones and examined on gel electrophoresis of the clones. They are analyzed to confirm their accuracy. Finally, by determining the recombinant plasmid sequence, the presence of the mutation in the inserted fragment can be investigated.

Screening by inactivating the LacZ gene

Although resistance to antibiotics after cloning is a common screening method, some plasmid vectors use other systems. For example, some plasmids have an ampicillin resistance gene and the LacZ gene, which stagnates beta-galactosidase. Beta-galactosidase is one of the enzymes involved in breaking down lactose. Typically, this enzyme is encoded by LacZ on the E. coli chromosome. Some strains lack the LacZ` gene. This mutant synthesizes the enzyme only if it contains a plasmid with the LacZ` gene.

After transformation, the cells are cultured in a medium containing IPTG and X-GAL. The cells that carry the normal plasmid are ampR, B-gal +. The cells that carry the recombinant plasmid are -ampR, B-gal. X-GAL is a lactose analogue (5-bromo-4-chloro and 3-indolel D-B-gala-ketopyranoside) broken down by beta-galactosidase like lactose, the product of which is a dark blue color. Therefore, in the culture medium, aquatic colonies containing empty plasmids can be easily distinguished from the recombinant white colony.

Multiple cloning of the Bluescript plasmid site is embedded in the LacZ` gene. The sequence of this gene is part of the gene sequence of the enzyme beta-galactosidase, which breaks down beta-galactoside bonds of lactose and converts lactose to glucose and galactose. Produces blue. Bacteria that are deficient in the beta-galactosidase enzyme, if transformed into a plasmid with a LacZ` gene sequence, complete the beta-galactosidase enzyme and can break down lactose or X-gal, thus producing blue colonies. If insert DNA is cloned into the multiple cloning site of this plasmid, the gene is inactivated and the bacterium cannot degrade the X-gal after being transformed with this plasmid, resulting in no blue dye and colorless colonies.

Types of vectors used in cloning gene

The term carrier is used for all the means of transport that carry DNA into the cell. Some types are as follows:

Plasmid

An extra-chromosomal loop DNA fragment that replicates itself independently of the bacterial genome and is used as the primary tool for inserting genetic information into microorganisms, plants, etc., and is easily extracted from bacteria. Plasmids usually contain one or more genes that cause specific characteristics in the host cell. Each plasmid has an origin of replication (ori) from which the replication of the plasmid begins. A portion of the plasmid sequence has a cleavage site for a number of cleavage enzymes, this region is called the multiple cloning site, and this region is used to clone genes in the plasmid. For work

Genetic engineering Special plasmids have been designed and fabricated and have certain traits embedded in them. Plasmids contain the antibiotic resistance gene and are used for screening. If the origin of the replication of two plasmids is the same, they compete with each other during transformation, and only one of them enters the bacterium. However, suppose the origin of their replication is different. In that case, two plasmids can also enter the same bacterium during transformation. In block script plasmids, the Lacz gene (the terminal portion of the beta-galactosidase gene) is embedded in the plasmid so that the multiple cloning site is part of the gene sequence, as previously described, is destroyed when the outer part of the clone is cloned. It becomes. This gene is active when the foreign component does not enter this part, and if it is present in the X-Gal environment, this enzyme changes color and creates a blue color. In genetic engineering, this feature is used to screen recombinant colonies (white colonies) from non-recombinant colonies (blue colonies). The size of the cloned piece in the plasmid is between 2 and 10 kg. Plasmids are most commonly used in the preparation of DNA vaccines against parasites.

Bacteriophage

Bacteriophages are another class of DNA molecules that are used as cloning vectors. Bacteriophages are viruses that can infect bacteria. In principle, cloning genes mostly use phages with lytic and lyrogenic cycles, such as Landa phage.

Like other viruses, phages contain DNA or RNA molecules and a protective protein coating called a capsid.

Generally, to infect a bacterium, a phage particle attaches to the bacterial wall and injects its genetic material into the bacterial cell. The phage DNA molecule then replicates, and after the bacterial components are made, new phage particles are formed.

Lambda phage

Carriers made from Landa bacteriophage are more suitable than plasmid carriers for cloning DNA fragments larger than 25 kb. Hence the use of carriers derived from the Landa phage makes it more common to build a genomic library (a comprehensive set of DNA or mRNA fragments transcribed from them, which represents the genome of organisms).

In order for the recombinant Lambda genome to be packaged in the phage head structure, its total size must not exceed 52 kg. Otherwise, no packaging is done, and no contaminating phage particles are formed.

To address the shortcomings of this type of vector and increase its efficiency, genetic engineers have made various modifications to it by making changes.

Phage M13

Phage M13 is a filamentous bacteriophage composed of single-stranded DNA with a length of 6.4 (6404 nucleotides). This phage infects Escherichia coli bacteria. How M13 enters the host cell is via a battery. The P3 protein binds to receptors at the pill's tip and injects virus DNA into the host. The M13 infection cycle is non-lytic, and therefore infection with it is not fatal to the host. On the other hand, M13 single-stranded DNA does not enter the bacterial chromosome and does not follow the lysogenic cycle.

Casmid

The plasmids contain a fragment of Landa's DNA containing the Cos locus and are used as vectors to clone fragments of 40-45 kg bp.

Vectors such as PAC, BAC, and YAC are used to clone larger components. They can also be used to create a gene library of the desired genes.

1-6- How to choose the right carrier

The maximum part size inserted in different carriers is shown in Table (1-3). Therefore, the size of the desired piece can be one of the things that limit vector choice.

Piece size is not the only important factor in choosing a carrier. Instead, the type of host cell and the appropriate vectors should be selected according to the cloning gene's different purposes. For example, for an expression gene looking for an expression vector with a strong rbs promoter that is compatible with the host cell.

Table 3: Maximum part size entered, which is possible for different carriers.

Inserted size of DNA	Host	Vector
5-25 kb	E. coli	phage
35-46 kb	E. coli	cosmid
70-100 kb	E. coli	P1 phage
100-300 kb	E. coli	PACs
≤ 300 kb	E. coli	BACs
200-2000 kb	Sarcomyces cervisiae	YACs

Promoters used in the carrier

Transcriptional synthesis is initiated by binding the RNA polymerase enzyme to a DNA site called a promoter. In the beginning, bacterial promoters such as E. coli were used, which did not have enough expressive power. Nevertheless, today they use viral promoters that have high copying power.

The lac operator tunes these promoters. This is done by an inducing agent, IPTG.

The promoter causes the expression of genes in different cells:

1. Expression in mammalian cells In this method, a human or hamster cell line is used, and expression in these cells has a higher percentage of purity.
2. Expression in Insect Cells In this method, insect cells are used, which are usually carried by baculovirus, which is a backpack.
3. The use of the expression in living cells of animals, in this case, is the creation of transgenic organisms. For example, sheep that produce the beta-lactoglobulin gene in their embryos, and these sheep are in the milk of beta-lactoglobulin after lactation.
4. Expression in plant cells To control insects and produce toxins, plant cells themselves are commonly used.

1-8- Types of promoters

In eukaryotic expression plasmids, a promoter is embedded, which are listed in different types.

CMV promoter cytomegalovirus for high expression in many mammalian eukaryotic cells.

EF1-1α Human elongation factor 1α-subunit promoter for high expression in mammalian eukaryotic cells.

Ubc promoter Human ubiquitin C for high expression found in many species.

SV40 Simian 40 virus promoter for high expression and replication license in cell lines.

PGK promoter of rodent phosphoglycerate kinase 1 for long-term expression in cells

The types of promoters, select markers, and express conjugates are listed in Table 1-4.

Table 4: Types of promoters, selective markers and expressive conjugates.

Promotor	Selected marker	Express conjugate
pcDNA=CMV	Gentamicin resistance	His = N-terminal His tag
pEF= EF-1a	Zeosin resistance	Myc-His= C-terminal myc-His tag
pUB= Ubiquitin	Blasticidin resistance	V5-His=C terminal
		V5-His tag

Expressive plasmids in eukaryotic cells

These plasmids have characteristics such as high expression power in mammalian eukaryotic cells, have a suitable promoter, have a selective epitope tag for proper tracing such as polyhistidine (6% His) or GST. Tags for rapid purification are used to express several genes in a cell line (except for non-tag expression vectors that contain the pCDNA3.1 expression vector). There are also three types of frameworks for tag carriers. These plasmids are also used to study transfection-induced expression temporarily or by integration into the genome. The translational modification system not found in E. coli, yeast, and insect cells is embedded in these plasmids.

Types of expression plasmids in eukaryotic cells

Vector specific information

pCDM8

pcDNA1, pcDNA1.1, pcDNA1 / Amp, pcDNA1.1 / Amp and pcDNA1 / Neo

pcDNA3 and pcDNA3.1 Difference between pcDNA3 and pcDNA3.1

pCDNA3.2 and pCDNA6.2

pDEST26

pDEST27

pCR3.1 pCDNA3.1 His

pCDNA3.1 myc-His

pcDNA3.1/V5-His pcDNA3.1/V5-His-TOPO pcDNA3.1D/V5-His-TOPO

pCDNA3.2/V5/GW/D-TOPO and pCDNA6.2/V5/GW/D-TOPO vectors

pcDNA6/BioEase-DEST

pcDNA4/HisMax

pcDNA6.2/nGeneBLAzer-DEST

pcDNA6.2/nGeneBLAzer-GW/D-TOPO

pRc/CMV and pRc/CMV2

pRc/RSV pZeoSV

pEF

pBudCE4.1

PVAX1

Expressive plasmids that have more applications

Mechanism of action of DNA vaccination

The discovery of immunization with plasmid DNA, which results in strong and stable humoral and cellular immune responses to the coded antigen, is a fascinating method. DNA vaccines provide a mechanism for intracellular antigen synthesis to stimulate the immune system, similar to a viral infection. However, soluble antigens, such as recombinant proteins, usually induce only a humoral response. Recombinant protein vaccines have not received much attention due to their high cost during the manufacturing process, difficult purification, incorrect folding, and poor induction of TCD8 + cells.

Successful vaccines can produce strong, long-lasting immune responses and protect against pathogens in many cases. Advances in the production of DNA vaccines against viruses and pathogenic bacteria have led to lasting protection.

In the DNA vaccination process, the immunogenic antigen gene is first cloned into the expression eukaryotic plasmid. The resulting recombinant plasmid is then transformed and amplified into susceptible bacteria. Finally, the recombinant plasmids are injected into a suitable laboratory animal after extraction and purification to evaluate immunization. Strong eukaryotic promoters such as CMV or SV40 promoters.

The main building blocks of a plasmid DNA vector are:

1. Strong eukaryotic promoters such as CMV or SV40 promoters.
2. Cloning site.
3. Polyadenyl sequence.
4. Selectable markers such as bacterial antibiotic resistance genes (ampicillin or kanamycin).
5. Origin of bacterial copying.

Prospects for the development of genetic parasitic vaccines

There have been many advances in parasitic vaccines' development in the last decade, but few commercial vaccines are available. In addition, no commercial parasitic vaccine has been developed for humans. The most critical challenge in designing DNA for human parasitic vaccines is to adapt the vaccine to humans in a way that results in strong, long-lasting responses. Many studies in laboratory animals have been successful but not feasible in humans. The Prime-Boost strategy has been more successful in large animals and humans than other methods [36]. Several types of antiparasitic vaccines are currently being tested in clinical trials, some of which have been developed using vaccine DNA technology or viral vectors, most of which are malaria vaccines.

DNA vaccination against parasitic diseases such as malaria, leishmaniasis, toxoplasmosis, cryptosporidiosis, schistosomiasis, and fascioliasis has already created new opportunities.

This type of vaccine technology for parasitic infections has opened new hopes in vaccine research against parasites. What is being considered about the parasite vaccine is the complexity of the parasitic diseases. Unlike viral and microbial diseases, parasitic diseases are chronic and are associated with immunosuppression or inadequate immune responses. Parasites have a complex life cycle, and host immunity is active against antigens at a particular stage of the parasite's life. Antigenic changes and other systems evading immune responses add to the complexity of producing a vaccine against parasites. However, with recombinant DNA technology and the ability to modify DNA vaccination, the possibility of a parasitic vaccine design strategy now seems plausible. Improving the efficacy of DNA vaccines against parasitic diseases is possible with booster injections, genetic and non-genetic agents, and multivalent vaccines.

Practical methods of designing a parasitic DNA vaccine

First, choosing the right gene is very important. An appropriate gene is a gene that can be expressed, express an immunogenic antigen, and expressed at an important stage or stages in the life of the pathogen. Sequences of this gene can be obtained from gene banks such as the NCBI. For example, if we consider the toxoplasma GRA5 gene as the target gene, we can first search for this gene in the gene bank.

Toxoplasma gondii ME49 dense granule protein GRA5 (GRA5), mRNA

NCBI Reference Sequence: XM_002369230.2

FASTA Graphics

Go to:

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LOCUS       XM_002369230            2012 bp    mRNA    linear   INV 20-OCT-2016
DEFINITION  Toxoplasma gondii ME49 dense granule protein GRA5 (GRA5), mRNA.
ACCESSION   XM_002369230
VERSION     XM_002369230.2
DBLINK      BioProject: PRJNA32719
            BioSample: SAMN00255192
KEYWORDS    RefSeq.
SOURCE      Toxoplasma gondii ME49
  ORGANISM  Toxoplasma gondii ME49
            Eukaryota; Alveolata; Apicomplexa; Conoidasida; Coccidia;
            Eucoccidiorida; Eimeriorina; Sarcocystidae; Toxoplasma.
REFERENCE   1  (bases 1 to 2012)
  CONSRTM   NCBI Genome Project
  TITLE     Direct Submission
  JOURNAL   Submitted (18-OCT-2016) National Center for Biotechnology
            Information, NIH, Bethesda, MD 20894, USA
REFERENCE   2  (bases 1 to 2012)
  AUTHORS   Sibley,D., Venepally,P., Karamycheva,S., Hadjithomas,M., Khan,A.,
            Brunk,B., Roos,D., Caler,E. and Lorenzi,H.
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     1 ttgctgcaaa gtgcccccct acatactctt catacttcag tgatgcaatc tatgtctgat
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   121 cggcattcgt gctgactgcc gcaacatcgc atatgaaaaa aacaatgcaa cccccccttt
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   481 aggtgccgaa atgatgatac agtacgccat gcagcaatca tcgagacagg atttgggacg
   541 catgactccc tcaggtggtt agcggagaaa cctcagatcc ctcggcgcgc gacgcgtgcc
   601 agagcgcggg acggggtggc aacgagacac gtttggataa aggtcctgcc aggttgtgga
   661 atcagacgtg tgggctgttc cgtgtcggtt tggtttgtgc agagacgcac tgacggttga
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   781 cgcgcgtgtg ttcactctaa ctgtgtgtat ggttcactgt tttttattgc gattttcgtg
   841 aagtaacaaa atggcgtctg taaaacgcgt cgttgtggcg gtaatgatcg tgaacgtgct
   901 ggctttaatt tttgtgggcg ttgccggttc aacgcgtgac acagggtcag gcggggatga
   961 ctccgaaggt gcttgggggg gtgaacaaca acaggtacaa caacacggac aaagtgaaga
  1021 ccgatcgtta ttcgaaaggg gaagagcagc ggtgactgga catccagtga ggactgcagt
  1081 gggacttgct gcagctgtgg tggccgttgt gtcactactg cgattgttga gaaggaggag
  1141 gagacgcgcg attcaagaag agagcaagga gtctgcaacc gcggaagagg aagaagttgc
  1201 cgaggaagag taaggggcac tgtgttgctc ggctctttgt tgtctcagcg tgaggattta
  1261 gtgcgtgtag cggagcatgt atcgatcgat acaggcacgg ttggacgtgt cgtctgtatc
  1321 ccttgtggca gacggcagac gtcattgtca gagcgtgttg cacgttggaa gaaaatgtgt
  1381 tggtgtaatc gctcgtcgga cagataccga gggaggttgc gtgatgatcg ttgtggtgcg
  1441 tagaggtgtg cctcgtgata acatgaaggg caaggacctt ttttgtcgag cacatactca
  1501 aaccagtgat tgtgcgaggc gggttgcacg cgactttgat ccattacagt taaatatgcc
  1561 gaaggccgtg gcctgattcg cacacaaggc gcacagacgt accgttgatg agaggaaacc
  1621 ggccacgtcg gcccacccat atcatatgca ggagaccaat cgaaataagt ttctacgagc
  1681 agattcaaag agcagaaact tataataccg gaatagtgat gtgtttttgt acgacggttt
  1741 caacaaacac gctgaagatg ctccgtagtc gcggagtcac cggatcgtgg tgtacgcagt
  1801 gcattagttg tcacttgttc agggtgatgt atgagtttag gctttttttat gctgcctgtg
  1861 ggttgcccac gtttagtaat ttaccgcaat ctgtcttgca ccagtaagat gctcagaagt
  1921 gaacgccact acctacgctt aagagacgtc acgagcgtct tgcgcagaag acggtaatgc
  1981 ttttctcctg caggtctatg tacgcgtaac tc
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Sequence of GRA5 gene of Toxoplasma gondii

(Adapted from NCBI website https://www.ncbi.nlm.nih.gov/nuccore/XM_002369230.2)

The number of sequences of this gene is open in 2012. It does not take the whole gene to make a DNA vaccine because many of these sequences are untranslatable. The beginning of protein-making and gene translation begins with the protein-building codon, ATG, which encodes the amino acid methionine.

translation= "MASVKRVVVAVMIVNVLALIFVGVAGSTRDTGSGGDDSEGAWGGEQQQVQQHGQSEDRSLFERGRAAVTGHPVRTAVGLAAAVVAVVSLLRLLRRRRRRAIQEESKESATAEEEEVAEEE"

In the translatable regions after the amino acid methionine (M), the amino acid alanine (A) is mentioned, so after ATG, we should look for one of the four codons encoding alanine, namely GCT, GCC, GCA, GCG (codons related to each amino acid in Attached is the book). For ease of use, the FASTA section of this gene sequence can be used:

TTGCTGCAAAGTGCCCCCCTACATACTCTTCATACTTCAGTGATGCAATCTATGTCTGATGAACACCAGTAATTTTACAGCCTGTAACTCTATGTGGCGGAGTCTTGGGCGCATGTACCGCGGCATTCGTGCTGACTGCCGCAACATCGCATATGAAAAAAACAATGCAACCCCCCCTTTCCCACCCCCACCAGGTTTTGGGACGCACTCAAACATGTTACATGAAAAATCCTGGCCTTGCCTCCCTGTTCACGGACTGGTGAAAAAACTGTACAAGTTGCTAACAAATTCCAAGCTGTGAGAAACTGATGCTGCTATATAGAAGGGGAATTTCTGTCTCTCAGCCCTGCCACCAAACGACGTTACATGTAACACGAGACAAGGTATCCAGCCAGTACTTCAAGATGGAACCAGTTTCCTTAAGCCGCCACTACAACTAAGCAGGATCAGGCTGCCACCATCCGCTTGTTCCGGCAAGAAAGGTGCCGAAATGATGATACAGTACGCCATGCAGCAATCATCGAGACAGGATTTGGGACGCATGACTCCCTCAGGTGGTTAGCGGAGAAACCTCAGATCCCTCGGCGCGCGACGCGTGCCAGAGCGCGGGACGGGGTGGCAACGAGACACGTTTGGATAAAGGTCCTGCCAGGTTGTGGAATCAGACGTGTGGGCTGTTCCGTGTCGGTTTGGTTTGTGCAGAGACGCACTGACGGTTGACGTCGATCGGCACTCGATCCTACCGTCAGTCAATTTTATTTTGGTTTTTGCAGATATCATCGCGCGTGTGTTCACTCTAACTGTGTGTATGGTTCACTGTTTTTTATTGCGATTTTCGTGAAGTAACAAA**ATGGCG**TCTGTAAAACGCGTCGTTGTGGCGGTAATGATCGTGAACGTGCTGGCTTTAATTTTTGTGGGCGTTGCCGGTTCAACGCGTGACACAGGGTCAGGCGGGGATGACTCCGAAGGTGCTTGGGGGGGTGAACAACAACAGGTACAACAACACGGACAAAGTGAAGACCGATCGTTATTCGAAAGGGGAAGAGCAGCGGTGACTGGACATCCAGTGAGGACTGCAGTGGGACTTGCTGCAGCTGTGGTGGCCGTTGTGTCACTACTGCGATTGTTGAGAAGGAGGAGGAGACGCGCGATTCAAGAAGAGAGCAAGGAGTCTGCAACCGCGGAAGAGGAAGAAGTTGCC**GAGGAAGAGTAA**GGGGCACTGTGTTGCTCGGCTCTTTGTTGTCTCAGCGTGAGGATTTAGTGCGTGTAGCGGAGCATGTATCGATCGATACAGGCACGGTTGGACGTGTCGTCTGTATCCCTTGTGGCAGACGGCAGACGTCATTGTCAGAGCGTGTTGCACGTTGGAAGAAAATGTGTTGGTGTAATCGCTCGTCGGACAGATACCGAGGGAGGTTGCGTGATGATCGTTGTGGTGCGTAGAGGTGTGCCTCGTGATAACATGAAGGGCAAGGACCTTTTTTGTCGAGCACATACTCAAACCAGTGATTGTGCGAGGCGGGTTGCACGCGACTTTGATCCATTACAGTTAAATATGCCGAAGGCCGTGGCCTGATTCGCACACAAGGCGCACAGACGTACCGTTGATGAGAGGAAACCGGCCACGTCGGCCCACCCATATCATATGCAGGAGACCAATCGAAATAAGTTTCTACGAGCAGATTCAAAGAGCAGAAACTTATAATACCGGAATAGTGATGTGTTTTTGTACGACGGTTTCAACAAACACGCTGAAGATGCTCCGTAGTCGCGGAGTCACCGGATCGTGGTGTACGCAGTGCATTAGTTGTCACTTGTTCAGGGTGATGTATGAGTTTAGGCTTTTTTATGCTGCCTGTGGGTTGCCCACGTTTAGTAATTTACCGCAATCTGTCTTGCACCAGTAAGATGCTCAGAAGTGAACGCCACTACCTACGCTTAAGAGACGTCACGAGCGTCTTGCGCAGAAGACGGTAATGCTTTTCTCCTGCAGGTCTATGTACGCGTAACTC

As can be seen in the above sequence, after ATG, the GCG codon can be found, which is related to the amino acid alanine. You can also check the codons for the next amino acids, serine, and valine for more certainty. The end of the gene ends with the three amino acids glutamate, which have two codons, GAG, and GAA, and finally ends with one of the three protein codons, TAG, TAA, and TGA, which is TAA. That is how we find the translatable part of the gene from the whole gene, which is as follows. This sequence must be a multiple of three because three base numbers encode each amino acid.

ATGGCGTCTGTAAAACGCGTCGTTGTGGCGGTAATGATCGTGAACGTGCTGGCTTTAATTTTTGTGGGCGTTGCCGGTTCAACGCGTGACGTAGGGTCAGGCGGGGATGACTCCGAAGGTGCTAGGGGGCGTGAACAACAACAGGTACAACAACACGAACAAAATGAAGACCGATCGTTATTCGAAAGGGGAAGAGCAGCGGTGACTGGACATCCAGTGAGGACTGCAGTGGGACTTGCTGCAGCTGTGGTGGCCGTTGTGTCACTACTGCGATTGTTGAAAAGGAGGAGAAGACGCGCGATTCAAGAAGAGAGCAAGGAGTCTGCAACCGCGGAAGAGGAAGAAGTTGCCGAGGAAGAG**TAA**

Primers are oligonucleotides designed and synthesized based on the two ends of the sequence, the region to be amplified. To design primers for this gene that are used for PCR amplification, we do the following:

For the left starter or forward, we start from the ATG. The right starter or reverse also starts with the completion of the end codon. The length of the primers varies and depends on the type of test. The minimum length of the fragment is 15 open, but it is better to be between 17 and 28 open, and for a stronger connection to the template DNA, it is better than the end of the primer in region 3 ends in the C or G bases.

The left and right primers should be designed so that their connection or annealing temperatures are close to each other. The temperature and time required for the primer to bind to the template DNA depends on the available composition, length, and concentration of the primer and is usually lower than the melting temperature. For small pieces of DNA, Tm is calculated from the relation Tm = {2 (A + T) +4 (G + C). The best Tm temperature for each primer is between 52 and 65 ° C and the relation Ta = Tm-4 is used to calculate the connection temperature (annealing temperature).

The primers should be designed in such a way that it is possible to create an inductor (connecting two identical primers) and a heterodimer (connecting two dissimilar primers) and a hairpin (pin model due to the rotation of a primer around itself and creating a connection in Supplementary bases) should be avoided. Having at least three consecutive open bases can lead to dimer and herpin. The types of these adverse connections are shown in the Figure 3.

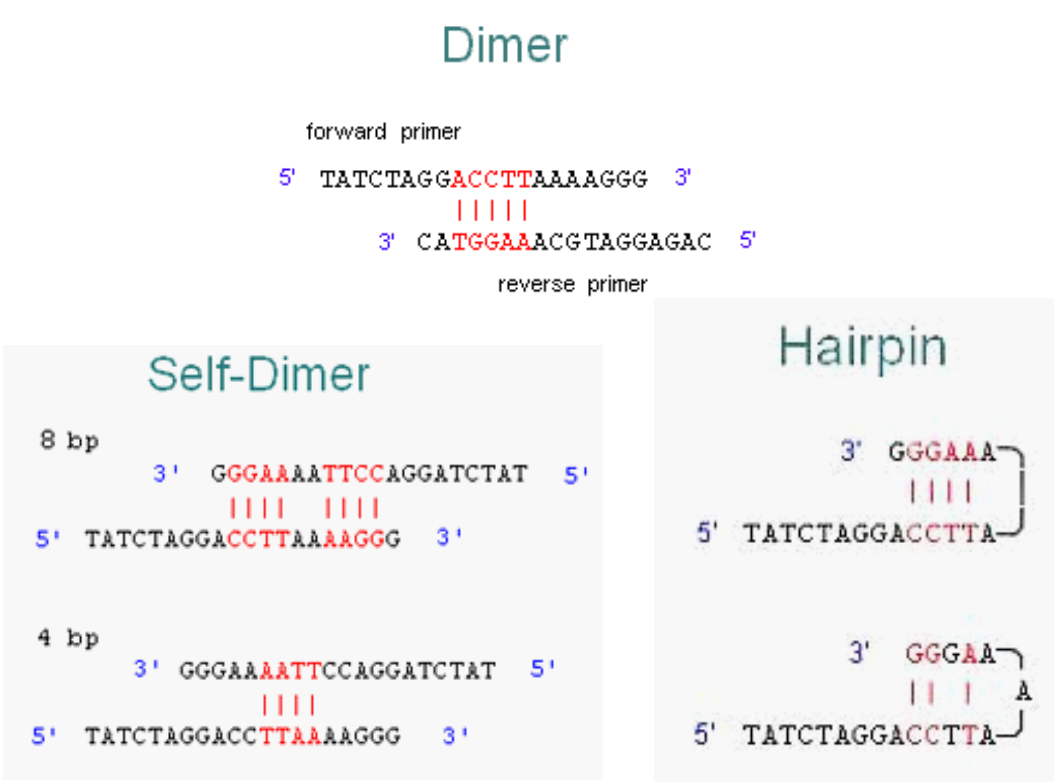

Figure 3: How to connect dimer, inductor dimmer and herpin in primers (adapted from http://bioweb.uwlax.edu/genweb/molecular/seq_anal/primer_design/primer_design.htm).

The ratio of G and C bases to total bases should be between 40 and 60%. The design of the primers is designed for the above sequences and according to the mentioned characteristics.

• ATGGCGTCTGTAAAACGCGTCGTTGTGGCGGTAATGATCGTGAACGTGCTGGCTT

TAATTTTTGTGGGCGTTGCCGGTTCAACGCGTGACGTAGGGTCAGGCGGGGATG

ACTCCGAAGGTGCTAGGGGGCGTGAACAACAACAGGTACAACAACACGAACAA

AATGAAGACCGATCGTTATTCGAAAGGGGAAGAGCAGCGGTGACTGGACATCCA

GTGAGGACTGCAGTGGGACTTGCTGCAGCTGTGGTGGCCGTTGTGTCACTACTG

CGATTGTTGAAAAGGAGGAGAAGACGCGCGATTCAAGAAGAGAGCAAGGAGT

CTGCAACCGCGGAAGAGGAAGAAGTTGCCGAGGAAGAGTAA

TT ACTCT TCCTCGGCAACT TC

Explicit sequence of the Toxoplasma toxin GRA5 gene with designed primers

- Left starter: 5′- ATGGCGTCTGTAAAACGCG- 3′
- Right starter: 5′- TTACTCTTCCTCGGCAACTTC- 3′

It should be noted that the left primer is like a gene sequence, but the right primer must complement and reverse the gene sequence.

Since this amplified fragment must be able to be stored in the plasmid and, if necessary, it can be extracted from the plasmid and inserted into another plasmid, a sequence of cleavage enzymes can be added to the beginning of these primers. Care must be taken to select the enzyme sequence so that the selected enzyme should not have a place in the gene itself, as this would cause the gene to be cut and destroyed. It must also be consistent with the sequence of enzymes in the plasmid and vector site's multiple cloning region to prevent the fragment from binding to the plasmid in reverse.

•: 5′- AAGCTTATGGCGTCTGTAAAACGCG- 3′ Left starter

•: 5′- GAATTCTTACTCTTCCTCGGCAACTTC- 3′ right starter

This design uses Hind III enzymes with AAGCTT sequence for left primer and Eco RI with GAATTC sequence for right primer. Both of these enzymes form a sticky end. Here are some of the more commonly used cutting enzymes.

After designing the primers, we extract the DNA from the pathogen and apply PCR to amplify the desired fragment.

PCR cycles

The PCR reaction consists of three cycles. Each cycle has its own specific time and temperature that must be determined experimentally for PCR work. The appropriate temperature and time of each cycle depend on the type and efficiency of the thermocycler. The steps of a PCR cycle are as follows:

1. Initial denaturation stage: Normally for initial denaturation, the PCR mixture is heated for 2-5 minutes at 94-95 ° C so that the target DNA is single-stranded due to heat.
2. Denaturation step: In each PCR cycle, DNA strands are denatured at 94-95 oC for 30 seconds to one minute.
3. Binding step: In this step, by reducing the system temperature, the primers are attached to the template DNA in a suitable place on the complementary strand. Pairing is done at a temperature between 50-75 oC. The choice of this temperature is the most important factor in the PCR reaction that must be optimized for each reaction. If the temperatures are too high, the primer will not be bonded to the pattern string, and at very low temperatures we will have a non-specific band.
4. DNA strand expansion step At this stage, when the temperature is favorable for the Taq DNA-polymerase en-

zyme, it promotes the development of primers and replication of the target DNA. This step is performed at a temperature of 72 ° C and the time required for this cycle is less than two minutes. Steps 2, 3 and 4 are repeated 25-25 times in the same way.

5. Final expansion step: To complete the synthesized filaments, the polymerization process is continued for 10 minutes at 72 oC.

At the end of the work, the PCR product is removed from the device and electrophoresed on the agarose gel (Figure 4).

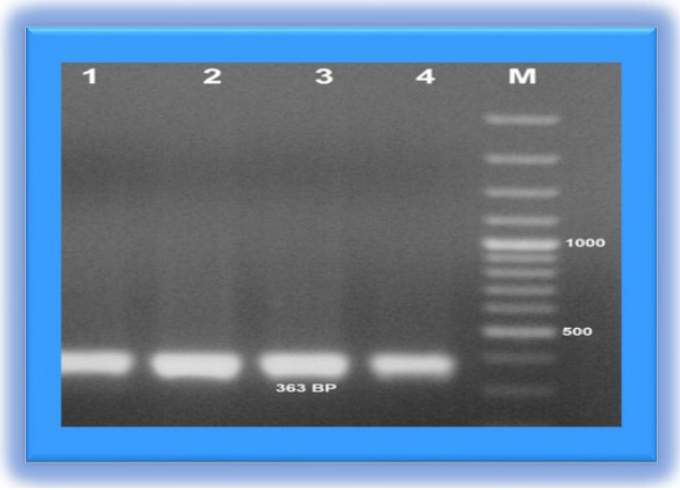

Figure 4: PCR result obtained from GRA5 fragment with a length of 363 bp in comparison with standard DNA.

The amplified fragment is cloned into pTZ57R / T vector plasmid by PCR. The cloned plasmid is transformed into bacteria. To transform E. coli with the recombinant plasmid pcGRA5, the recombinant plasmid is inserted into the E. coli bacterium using heat shock. After transformation, the bacteria are cultured on LB medium containing IPTG, X-GAL and ampicillin. Plasmid-free bacteria could not grow in the ampicillin-containing medium. The bacteria that carry the empty plasmid are ampR, B-gal + and formed an aqueous colony. The bacteria that carry the recombinant plasmid are ampR, B-gal, and formed a white colony (Figure 5). To confirm the work, the plasmid was extracted from both white and blue colonies and compared after electrophoresis (Figure 6).

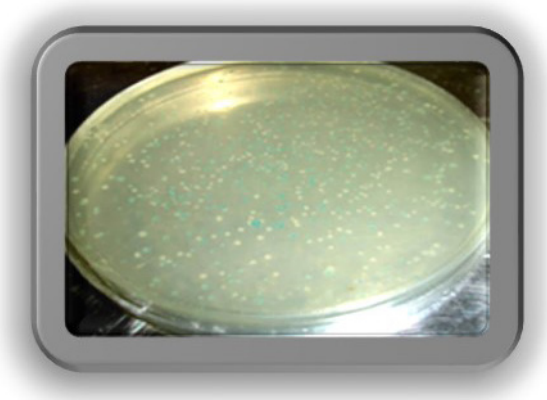

Figure 5: Culture plate of plasmid-transformed bacteria containing white colonies containing plasmid-transformed bacteria containing the desired gene and aqueous colonies containing empty plasmid-transformed bacteria and non-transformed bacteria due to lack of ampicillin-resistant gene-Growth in an environment that does not contain ampicillin.

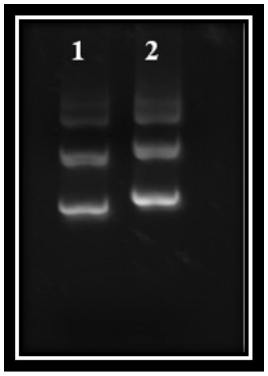

Figure 6: Comparison of bands of plasmids extracted from white colonies and aquatic colonies in 0.8% agarose gel in column 1 is related to plasmids extracted from aquatic colonies where the plasmid lacks genes and is lighter so it moves faster and the column 2 refers to plasmids extracted from white colonies in which the plasmid has a gene and is lighter, so it moves faster.

Design of multi-epitope for DNA vaccine

Sequence detection: For Amino acid sequence, at first, the accession number of the special gene needs to find from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/protein/) in FASTA form [37].

B cell epitope prediction: BCPREDS (http://ailab.ist.psu.edu/bcpred/predict.html) is an online website for B cell epitopes prediction. The server employs a combination of the two approaches with 74.57% performance accuracy [38].

Prediction of major histocompatibility complex I and II binding epitopes

The common database that used for this prediction is IEDB (Immune Epitope Database). This database is a website for computing and estimate IC50 values of sequences to binding to MHC I (http://tools.iedb.org/mhci/) and MHC II (http://tools.immuneepitope.org/mhcii). In IEDB the recommended number is ten amino acids. The prediction by IEDB recommended the length of 15-mer and output of percentile rank [39].

Physico-chemical parameters: Physico-chemical parameters can predicted by using Expasy ProtParam online website (https://web.expasy.org/protparam/) [40]. The parameters include, the number of amino acid residues, average Molecular Weight (MW), theoretical isoelectric point (pI), extinction coefficients, total number of positive and negative charged residues, estimated in vitro and in vivo half-life, instability index, aliphatic index, and Grand Average of Hydropathicity (GRAVY).

Post-translational modifications (PTMs): For Post-translational modifications, there are several tools such as http://www.cbs.dtu.dk/services/SignalP/ that used for analyze of signal peptide [41]. Moreover, NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) [42], NetAcet 1.0 (http://www.cbs.dtu.dk/services/NetAcet/) [43], and NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) [44] can used for predict Post-translational modifications in vaccine construct, N-glycosylation, acylation, and phosphorylation sites, respectively.

Secondary and tertiary structure prediction: In this regard, the online server https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html can used to predict secondary structure of the peptide [45]. SWISS-MODEL with online server (https://swissmodel.expasy.org/) used To construct the 3 dimensional models [46].

Tertiary structure refinement and validation: The best model that selected by SWISS-MODEL is an online server (https://zhanglab.ccmb.med.umich.edu/ModRefiner/) [47]. After refine by this model, the assessment of validation for tertiary structure will done by RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) [48] that develop Ramachandran plot.

B cell epitope prediction of the multi-epitope vaccine: There are a lot of characters for B cell epitope prediction that according to our goals we can check them as multi-epitope prediction for vaccines. Linear epitope prediction (Bepipred) [49], surface accessibility prediction (Emini) [50], beta-turn prediction [51], flexibility prediction [52], Parker hydrophilicity prediction [53], and antigenicity [54] that for prediction we can use the IEDB tool (http://tools.iedb.org/bcell/). For prediction of B-cell epitopes we can use ElliPro (http://tools.iedb.org/ellipro/) [55]. The default of range score from 0.5 to 6 Angstrom will used for each prediction.

Antigenicity, allergenicity, and solubility evaluation: For antigenicity prediction, the online websites such as ANTIGENpro http://scratch.proteomics.ics.uci.edu/ [56], VaxiJen version 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) [57] can used. AlgPred server (http://www.imtech.res.in/raghava/algpred/) [58] to predict the allergenicity of epitopes for vaccines. This online server can predict the allergenicity by 6 different approaches:

SVMc, SVMd , MAST (ev100) , MAST (ev 0.1) , BLAST (ARP) and IgE epitope.

Protein solubility for constructed vaccine can predicted by using SOLpro server at http://scratch.proteomics.ics.uci.edu/ [59].

Codon optimization and *in silico* cloning: Since codon usage frequency is different among various organisms, thus, codon optimization is a fundamental strategy to improve the protein expression in the host's cell system of interest. In order to cloning and expression of constructed vaccine in a suitable expression vector, we recruited Java Codon Adaptation Tool (JCAT) (http://www.jcat.de/) for codon optimization [60]. This server is specialized to predict the optimized coding sequence for both protein/DNA input sequence and gives the following output: optimized gene sequence, Codon Adaptive Index (CAI), and percentage GC content. We used the designed vaccine as the input sequence. Then, Escherichia coli (E. coli) K12 strain was selected as host microorganism to warrant a high expression rate. For the analysis, the following options were also selected: Avoid rho-independent transcription terminators, avoid prokaryotic ribosome binding sites, and avoid cleavage sites of restriction enzymes. Output result of JCAT was further analyzed by Webcutter (http://heimanlab.com/cut2.html) online server to evaluate the presence of all restriction enzyme cleavage sites. In silico cloning procedure was done using SnapGene software and the optimized codon sequence was ligated into pcDNA3.1 (as an expression vector) plasmids to make a clone and ensure its expression.

Human diseases and DNA vaccines

The first trial of human DNA immunization evaluated the immunity and immune response of a host to an AIDS vaccine. Other DNA vaccine trials were followed by prevention and treatment, including tested the DNA vaccine against cancer, influenza, hepatitis B, and malaria. These tests showed that the DNA vaccines were well-tolerated and safe, with no side effects were reported, and all studies were completed [21,61-64].

COVID-19 DNA vaccines

Among the vaccines that developed against SARS-CoV-2, DNA vaccine is a novel approach to deal with this virus. In DNA vaccine, the host cells that transfected with spike gene, the cells started to produce the protein of SARS-CoV-2 spike. This protein can recognize by host immune system as well as antibody production by B cells. This antibody capable of neutralizing this virus.

The pandemic of COVID-19 that has recently hit the world demonstrates the need to develop an effective vaccine with minimal side effects. A wide range of vaccines categorized into three categories include I. attenuated live viruses and inactivated viruses, II. subunits of proteins and peptides, III. and recombinant vaccines contain viral vector delivery system, DNA plasmids, and synthetic mRNA. Clinical studies have demonstrated strong immune responses, reduced viral load, and protection against SARS-CoV-2 challenges in rodents and mammals. Vaccine candidates have undergone clinical trials under healthy volunteers under all of the above systems. Phase I clinical trials have shown good safety and tolerability in the initial findings. Evaluation of immune responses in a small number of subjects compared to the immunogenicity detected in COVID-19 patients showed similar or superior levels of neutralizing antibodies Adenovirus and mRNA vaccines had entered Phase II, and protocols for testing Phase III trials have been finalized with 30,000 participants [65].

Acknowledgments

The author sincerely appreciates the assistance of Mhran Shahpari and Dr. Ghazal Shahpari for editing this eBook.

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