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Full Length Research Paper

Quality control of enhanced hepatitis B plasmid DNA antibody

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DNA vaccination and gene therapy using plasmid DNA [pDNA] are emerging as the tools of choice for preventing and treating debilitating diseases. A crucial issue in manufacturing pharmaceutical grade pDNA is quality control to fulfill the requirements of regulatory agency for purity, potency, safety and efficacy. Among the criteria that pDNA used for vaccination needs to meet, in full integrity of the DNA sequence to be injected. To control this parameter, a number of validated analytical methods are currently available. In this paper, we describe the molecular biology techniques used to control the quality and integrity of a hepatitis B DNA vaccine plasmid gWizHBs. To carry out this work, we have sequentially propagated gWizHBs in *Escherichia coli* DH5α and analyzed the plasmid preparation, by agarose gel electrophoresis, DNA restriction profiling and DNA sequencing. In addition to this generic technical platform, we have also discussed the limitation of the current DNA sequencing technique used in quality control of plasmid DNA for vaccination and suggest the use of pyrosequencing as a surrogate to Sanger DNA sequencing technique in order to control pDNA-based vaccines. The quality of the DNA vaccine met the requirements for medical use.

Keywords: plasmid DNA [pDNA], gWizHBs, vaccines.

INTRODUCTION

In the last two decades, a significant leap forward has been made toward the development of safe and effective DNA vaccines and DNA-based therapies against human and animal diseases (Apostolopoulos and Weiner, 2009). Several plasmid-DNA (pDNA)-based therapies have entered phase III clinical trials for diseases such as metastatic melanoma, critical limb ischemia and non-small cell lung cancer in humans (Aventis, 2008; Vical, 2008; NovaRx, 2008). In the US, at least four DNA therapies have been licensed for veterinary uses in the last two years (Kutzler and Weiner, 2008). The DNA-based vaccine and therapeutic approaches demonstrated high efficiency in several viral infections such as West Nile virus infection and a DNA vaccine for use in horses was developed and licensed in 2005 by the United States Department of Agriculture (USDA). This vaccine has gone through a phase II trial in humans (Acambis, 2006).

Similarly, a DNA vaccine against H5N1 influenza was developed (Sharpe et al., 2007) and has gone through a phase I human clinical trial (NIAID, 2007).

These developments has spurred the production of high amount, high quality pharmaceutical grade pDNA. Large scale production of pDNA for medical use is however a new field in biopharmacy. Plasmids are close circular DNA molecule that are propagated in a host microorganism mainly bacteria. The produced material need to meet strict manufacturing guidelines and must satisfy stringent recommendations set forth by the health regulatory authorities (Schleef, 1999; FDA, 1996; 1998). Therefore naked DNA plasmid produced from bacteria

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[mainly *Escherichia coli*] culture needs to undergo a series of quality control tests prior to being used for genetic vaccination. pDNA needs to be highly purified, homogeneous preparations of circular covalently closed (ccc) (supercoiled) pDNA. It was indeed well established that optimal purity positively correlates with the average super coiled-plasmid copy number (Diogo et al., 2005). In addition, regulatory agencies recognize that the open circular and linear forms of pDNA are less effective for DNA vaccination than the supercoiled form (Shamlou, 2003). Therefore it became highly recommended to use supercoiled pDNA isoform for vaccination.

Since the spread of genetic engineering, several genetically engineered *E. coli* strains have been developed to enhance pDNA production. Among these strains *E. coli* DH5α [F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_K^- m_K^+), *λ*] was engineered to give high level of supercoiled (*gyrA96*) and more stable pDNA (*recA* = recombination deficient) and to avoid plasmid degradation (*end A1* = endonuclease I deficient) (Schoenfeld et al., 1995). These pDNA producing *E. coli* strains were reevaluated and are currently being redesigned to increase plasmid stability, enhance product yield and safety and facilitate down stream process and therefore be more suited to the production of biopharmaceutical-grade pDNA (Hodgson and Williams, 2006; Phue et al., 2008; Bower and Prather, 2009).

Quality control of pDNA needs to be carried out at two levels. The first involves checking for sterility and purity from bacterial contaminants essentially endotoxin (LPS). The first level of quality testing requires the use of a variety of chemical and microbiological sensitive analytical methods to detect different impurities (proteins, RNA, chromosomal DNA and endotoxins). Assay such as quantitative endotoxin test (Limulus, pyrogenicity), sterility testing via bioburden assay are routinely perfor-med (Stadler et al., 2004).

The second level of testing is about the intrinsic qualitative characteristics of the plasmid such as the plasmid size, the antibiotic resistance marker and the plasmid DNA isoforms (super coiled, open circle, linear) as well as the integrity of the sequence of the expression cassette for the gene carried by the plasmid and DNA sequence.

This quality testing level is exclusively based on molecular biology techniques such as DNA agarose gel electrophoresis, restriction digestion and analysis (Meyers et al., 1976; Elder and Southern, 1983) of the plasmid restriction profile along with plasmid DNA sequencing (Gluck et al., 2001).

For this work, we have selected (as a hepatitis B DNA vaccine) the gWizHBs expression plasmid from "*Aldveron" Inc* USA, as the active pharmaceutical ingredient [API]. The gWiz expression vector was chosen on the basis that it has been designed to produce constitutively high level of transient gene expression in a wide variety of mammalian cells and tissues such as NIH-3T3, Cos 1, BHK 21, H2LA 33, jurkat and CV-1. In the gWizHBs plasmid, the transcription of the HBs antigen encoding sequence is driven by a modified CMV promoter followed by the CMV early gene [IE] intron A. The modified promoter is coupled with a high efficiency synthetic transcriptional terminator and a modified rabbit β-globin polyadenylation sequence. The modifications carried out to optimize the gene expression level in mammalian cells consisted in; i) the removal of the redundant sequences and those deleterious to high levels of expression while retaining the sequences of high transcriptional potency, ii) the modification and streamlining of the plasmid backbone, on which the expression cassette is con-structed, to accommodate both high-level gene expression in mammalian cells and high yield of plasmid production in *E. coli.*

In this paper we describe the molecular assays that we conducted to characterize and control the quality of gWizHBs hepatitis B DNA vaccine plasmid. In addition, we discuss the limitations of the Sanger DNA sequencing technique in quality control testing of pDNA and suggest using the more sensitive DNA pyrosequencing technique.

MATERIALS AND METHODS

Plasmid and bacterial strain

The bacterial host used for plasmid gWizHBs was *E. coli* DH5α strain, Genotype: F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ– , purchased from Promega Corp. (Madison, WI,USA). The gWiz high-Expression HBsAg plasmid (gWizHBsAg) 5 mg, was purchased from *Aldevron, USA* (http://www.aldevron.com). This plasmid was 6.435 kbp long and had a pUC origin of replication.

Chemical and other kits

Most chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were molecular biology grade. Kanamycin monosulfate from *Streptomyces kanamyceticus* was purchased from Sigma-Aldrich, catalog number K0879 (50 mg powder). Milliq Q grade water (18 ohm resistance) was used to make the buffers throughout the work. Good Microbiology laboratory practices were followed throughout the work.

Plasmid Mini Kit (25) with 25 QIAGEN-tip 20, reagents, buffers, reference 12123 was from QIAGEN (Germantown, Maryland, USA). Restriction enzymes EcoR1, BamH1, Hind III were from New England Biolabs (Boston, MA, USA), AluI, HaeIII and Sau3A were from Promega ((Madison, WI, USA)

Oligonucleotides and DNA sequencing

The oligonucleotides specific of the HBs Ag gene nucleotide sequence were designed and purchased from GenSynthCust (Luxembourg) and used as primers for plasmid DNA sequencing. These included HBs 1, 5'gctcagggcatattgcc 3'; HBs 2, 5'Ggcaatatgccctgagc 3'; HBs 3, 5'acccgtgtgtcttggcc 3' and HBs 4:

5'ccatcccatcatcttgg 3'.

The ABI PRISM® 310 Genetic Analyzer from applied Biosystem, (Palo-Alto, CA, USA) was used to sequence the gwizHBs plasmid. This instrument is an automated single-capillary genetic analyzer designed for a wide range of sequencing and fragment analysis applications. Auto-analysis with GeneMapper v3.5 and SeqScape v2.1 softwares, supplied with the instrument, were used for data collection. The following sequencing kit and reagents from ABI were also used: BigDye Terminator v3.1 Cycle Sequencing Kits, Running Buffer, 10X , Sequencing Buffer, 5X, Sequencing Standards and 310 Genetic Analyzer

Bacterial culture

Bacteria (*E. Coli* DH5α) were grown under standard conditions (37°C for 14 to 16 h under shaking at 250 rpm) in Luria Bertoni (LB) media (10 g bacto-tryptone., 5 g yeast extract, 10 g NaCl, pH adjusted to 7.5 with NaOH) in the presence of kanamycin at a concentration of 50 gr/ml.

***E. coli* DH5α competent cells**

E. coli DH5α was made competent using the $CaCl_2$ procedure (Ausubel et al., 1998); a single colony from a freshly grown *E. coli* DH5α plate was dispersed (briefly) in 100 ml of LB media in a 1 L flask. The culture was incubated at 37°C with vigorous shaking for approximately 3 h. Cell density was monitored by determining OD_{600} and was less than 10^8 cells / ml (log phase of growth). The culture was then centrifuged at 2,000 rpm for 10 min and the pellet resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ and centrifuged at 2,000 rpm for 10 min. The pellet was resuspended in 1.0 ml of ice cold 0.1 M $CaCl_2$.

Transformation with gWiz Hbs plasmid and isolation of recombinant strain

10 ng of the gWIZ HBs plasmid was added to 200 µL competent cells in ice cold Eppendorf tubes and placed at 42°C for exactly 90 s and returned to ice for 1 to 2 min. 1 ml of SOC media (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) was added and the bacteria were incubated for 2 h at 37°C. 200 ul of transformed bacteria was plated in LB/Kanamycin plate.

Preparation of pure pDNA

Plasmid gWiz DNA was prepared using the QIAGEN Plasmid Kits (Mini and midi size) according to the manufactures instructions. These kits are based on the alkaline denaturation method for the isolation of plasmid from bacterial culture described by Birnhoin (Birnboim and Doly, 1979; Rohde, 1995).

DNA quantification by spectrophotometry

Estimation of the amount of plasmid DNA was carried out by measuring the optical density (OD) at 260 nm in a spectro-photometer (Ausubel et al., 1998). The Beer-Lambert law relates the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the extinction coefficient for double-stranded DNA is 50 $(\mu g/ml)^{-1}$ cm^{-1}; for single-stranded DNA and RNA it is 38 $(\mu g/ml)^{-1}$ cm^{-1}. Thus, an OD of one corresponds to 50 µg/ml of double-stranded DNA. This method of calculation is valid for up to an OD of two.

Concentration of double stranded DNA = Absorbance at 260 nm × 50 × dilution

Agarose gel electrophoresis

0.8 to 2% agarose gels containing 0.5 gr/ml ethidium bromide were run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The running buffer was TAE [40 mM Tris, 20 mM acetic acid, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0]. Electrophoresis was carried out at 100 V for 1 h on an Amersham-Pharmacia Biotech (Uppsala, Sweden) power supplier unit ECPS3000/150.

Digestion of pDNA with restriction enzymes

1 µg of plasmid DNA was digested or double digested with the enzymes Hind III, BamH1 and Alu1 and Hae III and Sau3A in a single digestion. The reaction was carried out in the presence of the respective enzyme buffer 5 ul [1X] and 10 units of enzyme resulting in a final volume of 50 µl. The digestion was carried out for 3 h at 37°C. Then 20 µl of each reaction was controlled by electrophoresis in a 0.8% agarose gel run at 80 V for 2.5 h along side with uncut plasmid DNA prediction of the restriction profile with the expected sizes of the DNA fragments that plasmid gWiz HBs and control plasmids would generate after complete digestion by the various endonclases was performed using New England Biolabs software NEB cutterV2.0 (http://tools.neb.com/NEBcutter2/).

Sequencing of pDNA

1 µg of close circular super coiled gWizHbs DNA plasmid was used to sequence the HBs antigen cDNA inserted in the Bam H1 site of the gWiz plasmid using the Sanger and Coulson dideoxy DNA sequencing method (Sanger et al., 1977). Four different forward sequencing primers were used. The sequencing reactions were run into an ABI prism automated sequencing machine and the sequence were retrieved using the Chromass Pro 2.33 DNA sequence retrieval software (http://en.bio-soft.net/dna/chromas.html).

RESULTS

Table 1 summarizes the different tests carried out for the quality control of plasmid gWizHBs and indicates the sequence at which these tests need to be carried out.

Plasmid gWIZ HBs restriction map

The DNA sequence of the gWizHBs plasmid was fed into the restriction profiler software NEB cutter and the Hind III, BamH1 restriction profile as well as the Alu1, HaeII and Sau3A DNA fingerprints were determined. Figure 1a shows the gWizHBs plasmid restriction map with the enzyme' cut sites and Figures 1b, c and d provides the restriction pattern for each enzyme.

Table 1. Summary of the different tests carried out for the quality control of plasmid gWizHBs and indication of the sequence at which these tests need to be carried out.

Test	Test description
Control of the antibiotic selection marker	Growth of the bacterial clone on agar plate supplemented with the ATB
Overall quality of the pDNA	Agarose gel electrophoresis and estimation of the ccc form and matching of the size with depository data
Control of the restriction profile	Digestion of the pDNA with a single endonuclease cutter and estimation of the band size. Double digestion with two endonucleases that release the inserted DNA sequence of interest [cDNA] and size estimation
Control of the DNA sequence	DNA sequencing of the entire pDNA or the inserted cDNA of interest by automated dedioxy-DNA sequencing or any other sensitive method

Propagation of gWiz plasmids in *E. coli* DH5α and control of plasmid antibiotic selection

Following transformation of competent *E. coli* DH5α cell with 100 pg of gWizHBs plasmid DNA, the uptake of the pDNA was estimated by counting the number of bacteria that grew in presence of kanamycin and the competency of the DH5α was calculated using the following formula:

$$\frac{\#\text{ of colonies}}{100\text{ pg transformed}} \times \frac{10^6\text{ pg}}{\mu\text{g}} \times \frac{300\mu l\ total\ transformation\ volume}{?\ \mu\text{l plated}} = \frac{\#\text{ of transformants}}{\mu\text{g plasmid DNA}}$$

The data in Table 2 shows that in the transformed clone, the kanamycin resistance gene was expressed to confer the resistance phenotype to the transformed bacteria and allowed clone selection. Growing the bacteria harboring the gWiz plasmid on kanamycin plate is a functional test for the antibiotic selection encoding sequence.

Analytical plasmid preparation

The gWizHBs plasmid was prepared from a fresh single colony at both small analytical scales, from a 3 ml *E. coli* gWizHBs culture as well as at medium preparative scale in 50 ml culture in LB media supplemented with Kanamycin as shown in the materials and methods section. The purified plasmid was resuspended in TE buffer and kept at 4°C. The gWiz plasmid that did not carry the Hbs encoding cDNA was prepared similarly and used as an internal control in all the quality testing assays.

Qualitative plasmid DNA control by agarose gel electrophoresis

As shown in Figure 2, our preparation of uncut plasmids DNA appears in three conformations: i) supercoiled or covalently closed-circular DNA [ccc] that is, fully intact plasmid with both strands uncut and with a twist built in, resulting in a compact form, (ii) Nicked open-circular DNA that has one strand cut and; (iii) Linear" DNA that has free ends.

Supercoild plasmid has a certain shape in the gel as it interacts with more molecules of stain such as ethidium bromide, tending to make it more fluorescent than other DNA forms. Figure 2 also shows that of the two plasmid preparations, the majority (over 90%) of the DNA is under the supercoiled form which shows high quality plasmid DNA preparation. In addition, the plasmid size estimated from the molecular weight of the ccc DNA matches the depositor's data.

Plasmid DNA quantitation by spectrophotometry

DNA concentration was calculated by optical density, UV absorbance at 260 nm using a 1 cm quartz cuvette (optical path length = 1cm) according to Beer-Lambert law.

ng/ul dsDNA = OD260 × dilution factor × 50

Typical results were: gWiz HBsAg = 0.212 × 500 × 50 = 5300 ng/µL = 5.33 µg/µL

Control of plasmid integrity

Plasmid gWizHBs HindIII and BamH1 restriction profile

In plasmid DNA profiling, the presence and the location of at least two restriction sites must be confirmed. The expected sizes for the linear forms of plasmids gWiz-

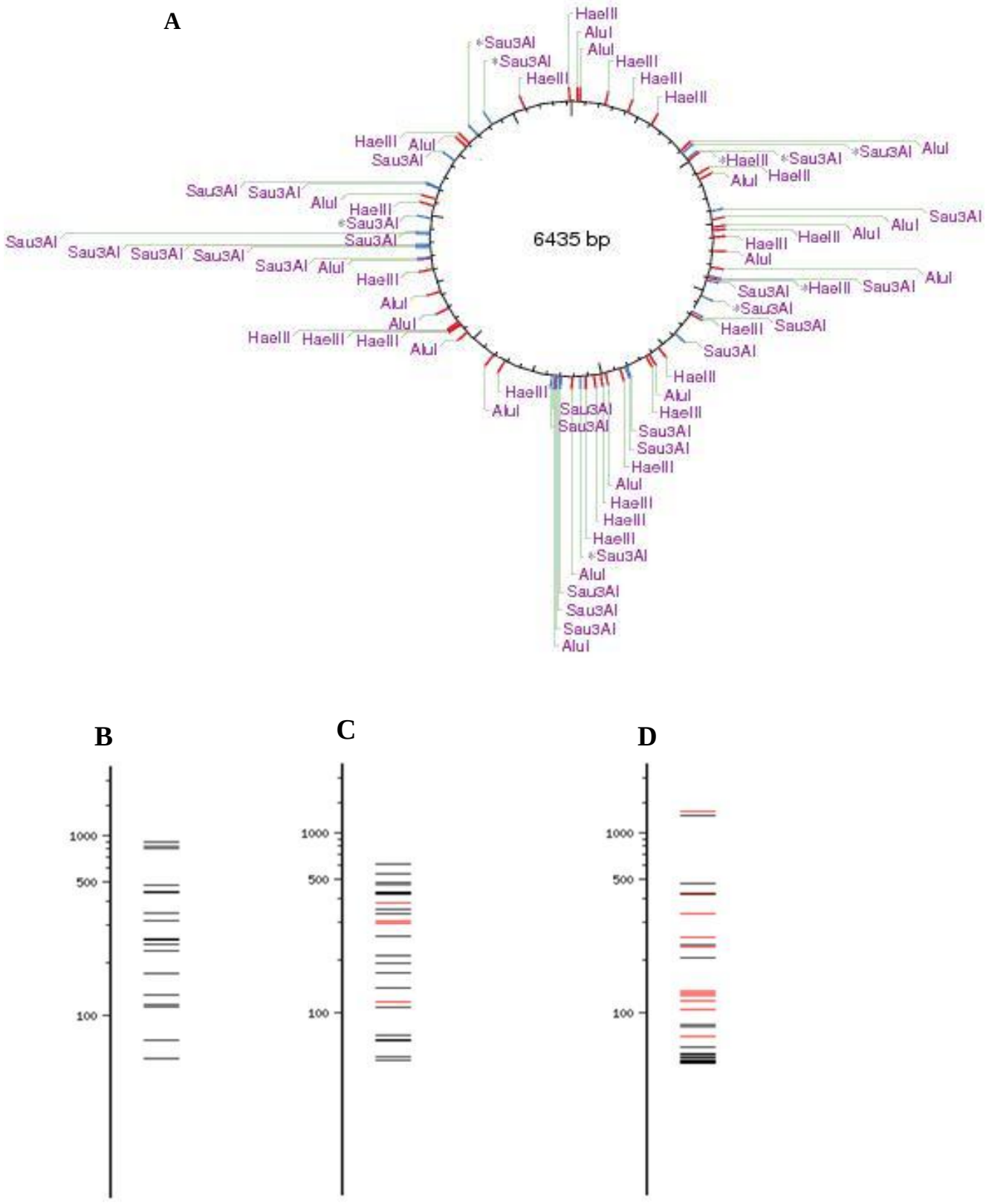


Figure 1. *In silico* prediction of plasmid gWiz restriction map and profiles. A, Restriction map of plasmid gWizHBs showing the position of the endonuclease Alu1, Sau3AI and HaeIII; B, C, D, predicted restriction profile for endonuclease AluI, HaeIII and Sau3A, respectively.

Table 2. Result of transformed cell.

Parameter	Number of clones	Competency CFU*/µg plasmid DNA
E. coli DH5α that grew on kanamycin plate	310	9.1×10^{7}

*CFU, Colony forming unit.

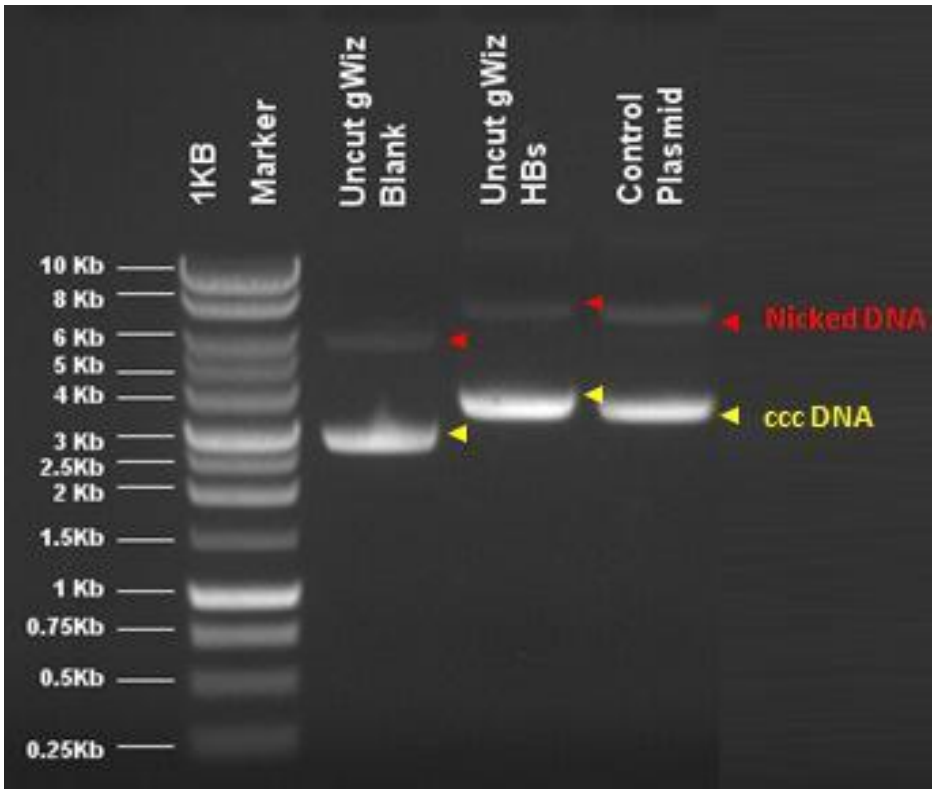


Figure 2. Quality control of plasmid preparations in agarose gel; evaluation of the ratio of the gWiz plasmid ccc form versus the other form is carried out through the visual estimation of the fluoresecence due to ethidium bromide bound to the plasmid DNA. Here >90% of plasmid gWiz HBs is under the ccc form.

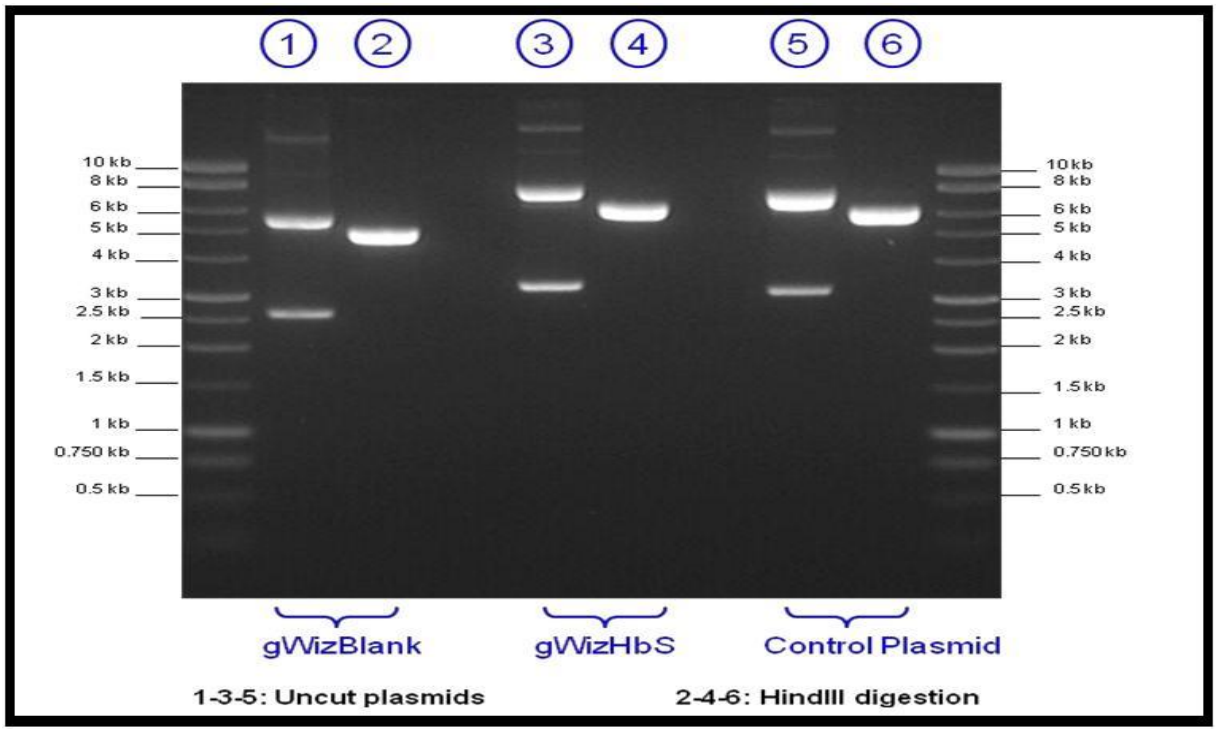
blank and gWizHbs after complete digestion by the endonuclease Hind III are respectively 5060 and 6435 bp. We carried out single Hind III (Figure 3a) and double HindIII/BamH1 (Figure 3b) digestions using 1 µg of each plasmid. A control of the digestion was carried out with a different plasmid preparation. As shown in Figure 3, all the plasmids were fully digested and exhibited a single linear fragment of the expected size. The plasmids' sizes estimated from linearized DNA matched the theoretical data and the double digestion plasmids profiles were as expected.

Plasmid DNA sequencing

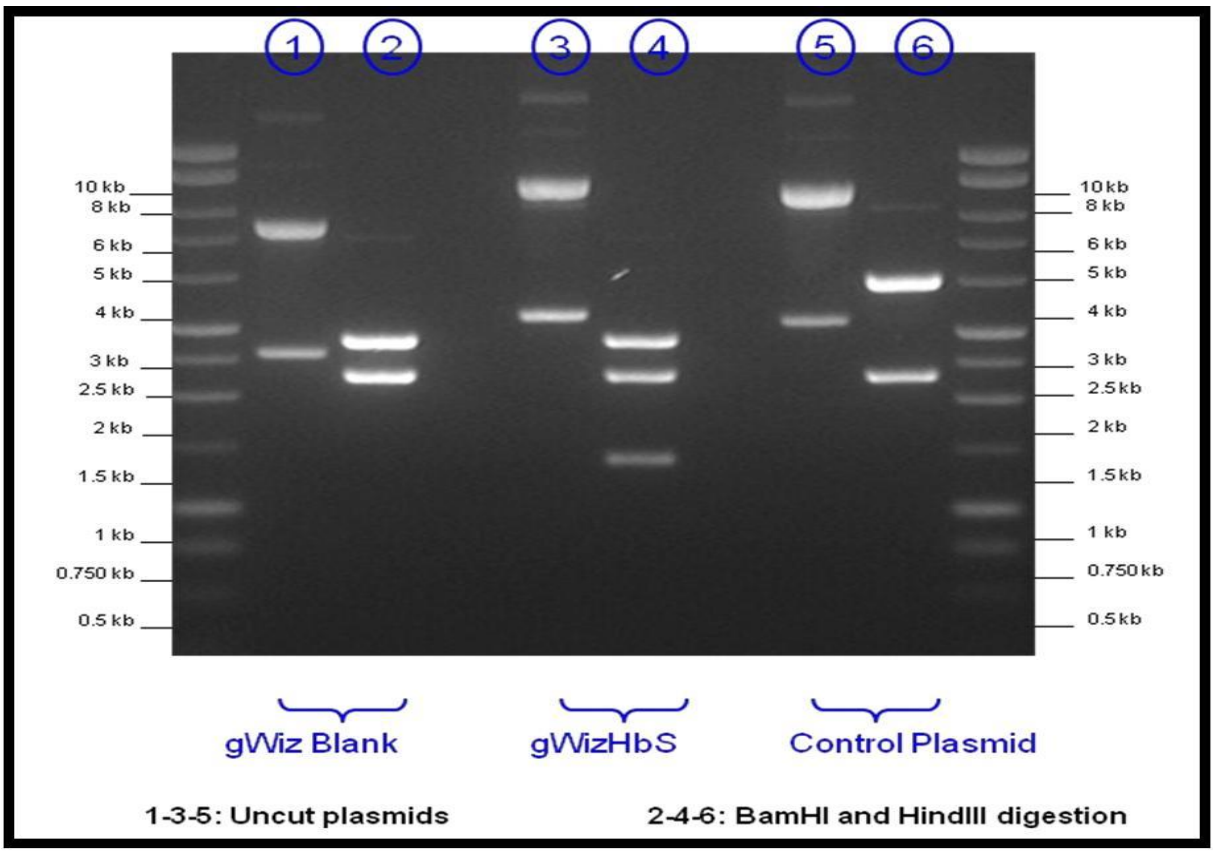
Control of the integrity of the nucleotide sequence of the plasmids' DNA was focused on the expression cassette encoding the hepatitis B surface antigen. An average reading of 800 bp was repeatedly achieved. Each sequencing reaction was carried out in triplicates. Analysis of the plasmid DNA sequences (data not shown) showed full conformity to the nucleotide sequence of the native HBs antigen coding sequence and no mutation was observed. Within the limit of the Sanger method for DNA sequencing, the result of this plasmid quality control test confirms the integrity of the HBs cDNA sequence inserted in the gWiz blank plasmid, amplified and characterized as described earlier.

DISCUSSION

DNA vaccines have a number of advantages over conventional vaccines, including the ability to induce a



A



B

Figure 3. Restriction profiling of plasmids gWiz-blank and gWizHbs preparations following; A, digestion with restriction enzyme Hind III; B, double digestion with restriction enzyme Bam H I and Hind III.

wider range of immune response types. The active pharmaceutical ingredient, API is made up of a small, circular piece of bacterial DNA (plasmid) that is genetically engineered to produce a specific protein (antigens). The methods currently used to manufacture and characterize DNA plasmid for biopharmaceuticals, and typically for clinical trials require the plasmid preparation to pass specific quality control procedures.

In this work we have developed the technical platform with the necessary molecular biology techniques needed to characterize the plasmid and control its quality with a focus on the integrity of the DNA sequence. We have sequentially propagated pDNA gWizHB that encodes for the Hepatitis B virus' antigen and used a version of this plasmid that does not contain a sequence encoding a hepatitis B s-antigen as control. The propagation of these two plasmids was done through the transfer of the plasmids DNA to *E. coli* DH5α strain. This strain was chosen because of its genotype that includes the endA1 and the recA1 relA1 mutations. These mutations respectively prevent plasmid lost through endonuclease activity and plasmid DNA recombination. We have then produced the plasmid DNA from the corresponding recombinant *E. coli* strain, and quantified the preparations by spectrophotometry. Thus, we have controlled the quality on the plasmid DNA by agarose gel electrophoresis. We have also controlled the integrity of the plasmids DNA by generating restriction enzyme profiles for each version of the plasmid and analyzed them on agarose gel. The profiles we obtained for the two plasmids were fully conforming to the theoretical one. pDNA sequencing is also recommended (Gluck et al., 2001) as a means for quality control of plasmid preparations intended for clinical applications (that is, gene therapy or DNA vaccination). Therefore we used the automated Sanger Dideoxy method to control plasmid gWizHBs nucleotide sequence with focus on the sequence encoding for the hepatitis B s-antigen. This test is of paramount importance. Indeed the vaccination depends on the expression of the hepatitis B s-antigen. If the sequences driving the transcription of the cDNA (promoter) encoding for this antigen or the cDNA sequence itself are altered, no expression of the antigen can occur and consequently no subsequent genetic immunization (vaccination) can be achieved. Our quality control sequencing test showed no alteration of the HBs encoding cDNA. However, it should be emphasized that the plasmid preparation controlled by sequencing is a plasmid population obtained after propagation and amplification in *E. coli*. Considering that during plasmid amplification in *E. coli*, the rate of spontaneous mutation is estimated at 10^{-9} mutations/cell divisions, the pDNA manufacturing step leads to the presence of mutated molecules. These mutations could not be detected using the Sanger dideoxy method for DNA because their number in the plasmid preparation is below the threshold of the methods sensitivity. To be able to detect and identify such mutations and to evaluate their effect on vaccination or therapeutic use, a more sensitive DNA technique need to be used. We suggest DNA pyrosequencing as an alternative to the Sanger dedioxy method.

Pyrosequencing is a real-time DNA sequencing technique (Ronaghi et al., 1998) which via a cascade of enzymatic reactions detects pyrophosphate (PPi) released during DNA synthesis as visible light. The light released is quantitative and enables the rapid generation of sequence information. This technique has the advantage over current methodologies to be less time consuming and to have the sensitivity to detect mutations in mixed DNA populations (Harrison et al., 2006; Wen et al., 2008; Dufort et al., 2009). This may suggest that pyrosequencing could find those minor mutations probably missed by Sanger sequencing in pDNA to be used for vaccination or therapy. However, the use of this rapid technique is not yet widespread due to some limitations such as equipment and high costs of reagents.

Production of multi-gram quantities of pDNA at purities of 95% or more is currently possible and the recent advances in delivery vehicles and adjuvant are increasing the potency of naked pDNA (Green et al., 2007; Pan et al., 2008). However, further development of pDNA quality control testing approaches is needed to strengthen the control processes and enhance safety. The biophar-maceutical industry in this field depends heavily on such technical platform and so do the future of pDNA-based vaccine and therapy.

REFERENCES

Acambis (2006). in Acambis company news (September 12, 2006) http://www.acambis.co.uk/default.asp?id¼1728.

Apostolopoulos V, Weiner DB (2009). Development of more efficient and effective DNA vaccines Expert Review of Vaccines September, 9: 1133-1134.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidmann JG, Smith JA, Struhl K (1998). Current Protocols in Molecular Biology. Whiley & Sons, Volumes 0-471-50338-X-Looseleaf or 0-471-30661-4-CD-Rom. Florida, USA.

Aventis S (2008). Efficacy and safety of XRP0038/NV1FGF in critical limb ischemia patients with skin lesions (TAMARIS). In: Clinical Trials.gov. U.S. National Library of Medicine. http://clinicaltrials.gov/ct2/show/NCT00566657.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7: 1513-1519.

Bower DM, Prather KL (2009). Engineering of bacterial strains and vectors for the production of plasmid DNA. Appl. Microbiol. Biotechnol. 82: 805-813.

Diogo MM, Queiroz JA, Prazeres DM (2005). Chromatography of plasmid DNA. J. Chromatogr. A. 1069: 3-22.

Dufort S, Richard MJ, Fraipon F (2009). Pyrosequencing method to detect *KRAS* mutation in formalin-fixed and paraffin-embedded tumor tissues. Anal. Biochem. 391: 166-168.

Elder JK, Southern EM (1983). Measurement of DNA length by gel electrophoresis II: Comparison of methods for relating mobility to fragment length. Anal. Biochem. 128: 227-231.

FDA (1996). US Food and Drug Administration, Points to consider on plasmid DNA vaccines for preventive infectious disease indications, US Food Drug Adm. Rockville, MD, USA.

FDA (1998). US Food and Drug Administration, Guidance for industry: guidance for human somatic cell therapy and gene therapy, US Food Drug Adm. Rockville, MD, USA.

Gluck L, Alazay L, Ratel D, Puget S, Wion D (2001). Quality control of plasmid preparations. Nat. Biotech. 19: 715-721.

Green JJ, Zugates GT, Tedford NC, Huang YH, Griffith LG, Lauffenburger DA, Sawicki JA, Langer R, Anderson DG (2007). Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus. Adv. Mater. 19: 2836-2842.

Harrison C, Musgrave-Brown E, Bender K, Carracedo A, Morling N, Schneider P, Syndercombe-Court D (2006). The SNP*for*ID Consortium. A sensitive issue: Pyrosequencing as a valuable forensic SNP typing platform. Int. Congress Series. 1288: 52-54.

Hodgson CP, Williams JA (2006). Improved strains of *E. coli* for plasmid DNA production. WO 2006/026125 A2. http://www3.niaid.nih.gov/news/newsreleases/2007/FluDNAVax.htm.

Kutzler MA, Weiner DB (2008). DNA vaccines: ready for prime time? Nat. Rev. Genet. 9: 776-788.

Meyers JA, Sanchez D, Elwell LP, Falkow S (1976). Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bact. 127: 1529-1537.

NIAID (2007). NIH news NIAID DNA Vaccine for H5N1 Avian Influenza Enters human Trial. http://www.nih.gov/news/pr/jan2007/niaid-02.htm.

NovaRx Corporation (2008). Phase III LucanixTM vaccine therapy in advanced non-small cell lung cancer (NSCLC) following frontline chemotherapy (STOP). In: ClinicalTrials.gov.U.S.NationalLibrary of Medicine. http://clinicaltrials.gov/ct2/show/NCT00676507.

Pan CH, Jimenez GS, Nair N, Wei Q, Adams RJ, Polack FP, Rolland A, Vilalta A, Griffin DE (2008). Use of Vaxfectin adjuvant with DNA vaccine encoding the measles virus hemagglutinin and fusion proteins protects juvenile and infant rhesus macaques against measles virus. Clin Vaccine Immunol. 15: 1214-1221.

Phue JN, Lee SJ, Trinh L, Shiloach J (2008). Modified *Escherichia coli* B (BL21), a superior producer of plasmid DNA compared with *Escherichia coli* K (DH5alpha). Biotechnol. Bioeng. 101: 831-836.

Rohde C (1995). Technical Information Sheet Plasmid isolation from Bacteria: some fast procedures. World J. Microbiol. Biotech. 2(11): 367-369.

Ronaghi M, Uhlén M, Nyrén P (1998). A sequencing method based on real-time pyrophosphate. Science, 281: 363-365.

Sanger F, Nicklen S, Coulson AR (1977). Proc. Natl. Acad. Sci. USA, 74: 5463-5467.

Schleef M (1999). Biotechnology, Wiley, Weinheim, USA. p. 5a.

Schoenfeld T, Mendez J, Storts DR, Portman E, Patterson B, Frederiksen J, Smith C (1995). Effects of Bacterial Strains Carrying the *endA1* Genotype on DNA Quality Isolated with Wizard(TM) Plasmid Purification. Systems Promega Notes Magazine. 53: 12-16.

Shamlou PA (2003). Scaleable processes for the manufacture of therapeutic quantities of plasmid DNA. Biotechnol. Appl. Biochem. 37: 207-218.

Sharpe M, Lynch D, Topham S, Major D, Wood J, Loudon P (2007). Protection of mice from H5N1 influenza challenge by prophylactic DNA vaccination using particle mediated epidermal delivery. Vaccine. 25: 6392-6398.

Stadler J, Lemmens R, Nyhammar T (2004). Plasmid DNA purification. J. Gene Med. 6: 54-66.

Vical (2008). A Phase 3 pivotal trial comparing Allovectin-7 alone vs. chemotherapy alone in patients with stage 3 or stage 4 melanoma. In: ClinicalTrials.gov. U.S. National Library of Medicine. http://clinicaltrials.gov/ct2/show/NCT00395070.

Wen J, Zhao W, Jiang S, Fang Z, (2008). Clinical Application of Pyrosequencing in Detection of YMDD Mutations. Lab. Med. 39: 218-222.