

Plasmid DNA Manufacturing Technology

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Abstract: Today, plasmid DNA is becoming increasingly important as the next generation of biotechnology products (gene medicines and DNA vaccines) make their way into clinical trials, and eventually into the pharmaceutical marketplace. This review summarizes recent patents and patent applications relating to plasmid manufacturing, in the context of a comprehensive description of the plasmid manufacturing intellectual property landscape. Strategies for plasmid manufacturers to develop or in-license key plasmid manufacturing technologies are described with the endpoint of efficiently producing kg quantities of plasmid DNA of a quality that meets anticipated European and FDA quality specifications for commercial plasmid products.

Keywords: Plasmid, DNA vaccine, gene therapy, fermentation, purification, alkaline lysis, downstream processing.

INTRODUCTION

DNA vaccines and medicines are potential disruptive technologies that offer the promise of a new way to treat humans and animals with materials that are entirely gene-based. Plasmid DNA (DNA vaccines, DNA replicon vaccines, shRNA vectors, AAV helper plasmids, therapeutic plasmids, gene targeting plasmids) may find application as preventive vaccines (for viral, bacterial, or parasitic diseases), immunizing agents (for preparation of hyper immune globulin products), therapeutic vaccines (infectious diseases, allergy, autoimmune), cancer vaccines, or gene therapy vectors.

Rapid Deployment Vaccines

The ability to make DNA molecules strictly by rational design makes it possible to bypass years of development for the production of efficacious vaccines. Literally, new vaccine entities can be created in days and mass produced in 2-3 weeks for rapid deployment against new biological agents. By contrast, a new influenza H5N1 'bird flu' vaccine, using existing technology, would require at least 6 months for production, and only a few countries have the manufacturing infrastructure to accomplish this [1]. Thus, DNA vaccines have the potential to be the most rapidly deployed vaccine platform for pandemic application [2].

H5N1 DNA vaccines are under development by PowderMed/Pfizer, the NIH Vaccine Research Center, CytoDyn and Vical. Clinical trial safety and efficacy data with the PowderMed gene gun delivered DNA vaccine has been encouraging, with responses observed with a 4 µg dose [3]. Considerations to manufacture a DNA vaccine for an influenza pandemic at this dose level have been recently reviewed [4]. Ideally, DNA vaccines would be delivered without specialized unapproved devices (such as the gene gun) or adjuvants that complicate manufacturing and regulatory approval. However, doses of 0.5-4 mg of plasmid are used in studies using simple intramuscular injection of plasmid DNA. Such high doses are considered not attainable for mass vaccination strategies using current plasmid production technologies [4].

Optimization of Plasmid Vectors

Before developing a manufacturing process, a critical evaluation of the plasmid vector is recommended, to ensure that 1) the fermentation production yield and quality is acceptable and 2) the plasmid composition, and final product purity after downstream processing is acceptable for regulatory approval and marketing licensure.

Production Yield/Quality

Shake Flask Versus Fermentation

A common misconception is that high shake flask yields project high fermentation yields. In fact, many plasmids optimized for high yield in shake flasks are poor fermentation performers [5]. The biologic basis for this phenomenon is unknown, but it is critical that vector changes to increase yield be driven by fermentation rather than shake flask evaluation.

Composition and Orientation of Elements

The DNA quality and yield are affected by the organization of elements (*e.g.* kanamycin resistance gene), as well as the specific elements included (*e.g.* IRES, CMV promoter).

Supercoiling: Certain sequences promote poor supercoiled content (*i.e.* large percent of total plasmid is open circular). Nicking is associated with AT rich regions that 'breathe' and are susceptible to endogenous single stranded nucleases.

Stability: Palindrome sequences are unstable, as are direct or inverted repeats. Plasmids containing Z DNA-forming sequences (*e.g.* alternating pyrimidine/purine sequences such as CpG repeat sequences [6]) are unstable in batch fermentation [7]. Oligo-pyrimidine or oligo-purine sequences have been shown to increase dimer formation in a pUC plasmid, presumably through formation of unusual DNA structures such as a triple helix [8].

Replication intermediates: Replication tends to terminate when the orientation of the origin is close to and parallel with the CMV promoter, resulting in production of linear fragments called replication intermediates. This phenomenon is only seen in high copy plasmids. The presence of the difficult to remove replication intermediate is readily observed as an extra small band in purified plasmid preparations; redesigning the plasmid to eliminate replication intermediates is the subject of a Patent [9].

Eukaryotic expression: The orientation of components also affects plasmid function in eukaryotic cells. A number of prokaryotic sequences have been shown to negatively affect gene expression in eukaryotic cells [10,11] or bind eukaryotic transcription factors [12-14]. The orientation of the kanamycin resistance gene has been demonstrated to dramatically affect expression from the adjacent CMV promoter, as well as overall plasmid yield [15,16].

Clearly, the sequences in the plasmid backbone need to be carefully optimized to eliminate sequences or orientations that might affect the intended purpose of the vector.

Regulatory Issues

DNA vaccines have been licensed for animals [*e.g.* Aqua Health (Novartis Animal Health) in Canada for an infectious

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hematopoietic necrosis virus DNA vaccine for farmed salmon; Fort Dodge Animal Health (Wyeth) for a West Nile DNA vaccine for horses; Merial for a therapeutic vaccine for canine melanoma]. However, no DNA vaccine for human use is currently approved. Consequently, the final specifications for a commercial human DNA vaccine have not been clearly established.

The introduction of plasmid DNA into humans presents some special considerations and challenges, which have been addressed in several recent World Health Organization (WHO), US Food and Drug Administration (FDA), or European Agency for the Evaluation of Medicinal Products (EMA) regulatory draft guidances [17-20, 39]; [reviewed 21,22]. Key issues are outlined below.

Selectable Markers

Antibiotic resistance markers are the most commonly utilized selectable markers. Of these, kanamycin resistance is the marker of choice in most vectors. To improve safety, a less reactive chimeric kanamycin resistance gene resistant to a much more limited number of clinically relevant aminoglycosides was developed and patented [23]. Ampicillin resistance is not acceptable due to concerns with hyper reactivity of some patients to β lactam antibiotics. The tetracycline resistance marker is toxic to the *E. coli* host at high copy number and/or stationary phase [24-26].

However, the EMA guidance [20] expresses the following concern regarding the inclusion of antibiotic resistance markers in DNA vaccine plasmids: "the use of certain selection markers, such as resistance to antibiotics, which may adversely impact on other clinical therapies in the target population" "consideration should be given to avoiding their use, where feasible". Alternative selection strategies, to address such concerns, are described below.

Balanced lethals: An essential gene is maintained on the plasmid, with a corresponding chromosomal deletion or suppressible mutation. Numerous examples exist; these systems eliminate the need for antibiotic resistance markers on the plasmid, and are often very small (*i.e.* for suppressor tRNA genes). Many such systems are patented.

Operator titration vector systems: In this case, an operator sequence, placed on a multicopy plasmid, derepresses a chromosomal gene. A number of repressor-operator systems that could be utilized are available [reviewed in 27]. For example, the *lac* operator or *tet* operator may be on the plasmid, and an antibiotic gene, regulated by the relevant operator is on the chromosome. Titration of the repressor by the operator leads to expression of the chromosomal gene, and antibiotic resistance. Valentis (formed by merger of Genemedicine and Megabios) has developed optimized plasmid backbones that can be utilized for repressor titration selection (*e.g.* pGM509 [27]). While antibiotics may still be used in fermentation, their removal during downstream processing can be validated, as is currently performed for numerous protein products. An alternative system patented by Cobra Biomanufacturing is the Operator Repressor Titration (ORT) system [28]. This utilizes an engineered production strain that has the *dapD* essential chromosomal gene under the control of the *lac* operator/promoter. Three copies of the operator are on the plasmid, and titrate the *lac* repressor, allowing expression of the essential chromosomal gene. In the absence of the multicopy plasmid, *dap* expression is repressed, and the cell dies [29]. This system eliminates antibiotic resistance genes from the vector and, less critically, from the growth media.

Chromosomal DNA (gDNA)

Regulatory guidances indicate concern about various contaminating substances, and suggest tests that can be used to assess the levels of each contaminant. The guidance documents stop short, however, of suggesting maximum acceptable levels of contaminating host cell RNA, DNA, or proteins, as these are not known. In general, removal of host cell protein, endotoxin and RNA is

accomplished effectively with a wide variety of downstream purification strategies. The allowable limit for gDNA is 100 pg gDNA/dose [30]. This was originally established for protein products from transformed mammalian cell lines to limit the chance of oncogene transfer (or the transfer of an integrated or extrachromosomal genome of an infectious virus) to the patient [31]. More recently, a limit of 10 ng/dose for DNA from continuous cell line substrates has been suggested [32]; this corresponds to a 10 μ g dose of a plasmid containing 0.1% residual gDNA. This dose is well below that required for non gene gun delivery vaccines. Since other impurities are relatively easily removed, we recommend that processes be selected based on superior gDNA removal. Combining the purification process with fermentation optimized vectors and a high yield fermentation process (that dramatically increase the plasmid DNA/genomic DNA ratio; see below) may be critical to reduce final product residual gDNA to acceptable levels.

Insertion Elements

Insertion of transposable elements into plasmids during production may be an issue with some processes and plasmids. Prather and colleagues [33] report high frequency transposition of insertion element 1 (IS1) into the neo selectable marker during cell line creation in defined media while [34] identified IS1 transposition into plasmids propagated in standard *E. coli* strains. Engineered reduced genome (transposon free) *E. coli* strains have been created and their application for plasmid production is included in a patent application [35].

Plasmid DNA Production Overview

Plasmid DNA is produced by cultivation of recombinant *Escherichia coli*. While experimental new methods for *in vitro* synthesis of plasmid DNA, using isothermal rolling circle amplification, have been reported (WO2006063355 [36]), these methods require specialized equipment and several costly enzymes and reagents to make supercoiled plasmid (*e.g.* Phi29 DNA polymerase, dNTPs, DNA primers, restriction enzymes, DNA ligase, DNA gyrase; see Fig. 3 of WO2006063355). The feasibility of *in vitro* synthesis methods also need to be demonstrated at large scale.

The purification of plasmid DNA from *E. coli* has posed unique challenges in comparison with protein purification. These challenges arise due to plasmid DNA's chemical and physical similarities with host cell nucleic acids. Several excellent reviews on plasmid DNA fermentation and purification have been recently published [4,37,38].

An overview of general steps in plasmid processing is shown in Fig. (1). There are three critical base unit operations: Fermentation, Cell Lysis, and Downstream processing. Each of these steps is reviewed separately below.

Intellectual Property in Plasmid DNA Production

Plasmid DNA manufacturing is a new technology in which virtually all steps outlined in Fig. (1) are the subject of issued or pending patents. Most of these patents stem from work to scale up processes well known in the art, and as such are relatively narrowly focused and restricted by prior art. For example, alkaline and heat lysis methodologies date to the 1970s. Hydroxyapatite (HAP) and diatomaceous earth have long been used in purification (*e.g.* HAP used to separate single from double stranded DNA since the 1970s) as has PEG or CTAB for precipitation of DNA. Thus the multitude of patents using methodologies such as PEG, CTAB, or diatomaceous earth and silicate sorbents (*e.g.* LRA®) must demonstrate novelty and an inventive step beyond the known use of these reagents/methodologies in small scale purifications.

In general, these patents teach new methodologies, applications, or combinations with other purification modalities. The later is the case with CTAB, PEG, and silicate sorbent applications, while

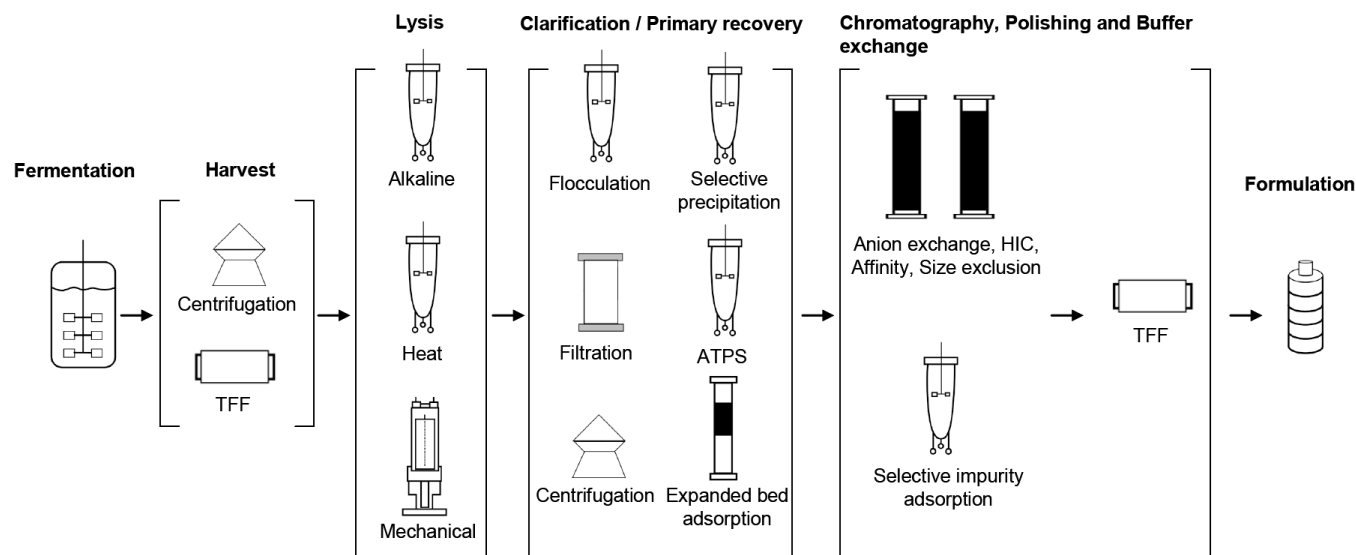


Fig. (1). Typical plasmid production process alternatives. Commonly used unit operations for each phase of the process may be combined to make a variety of processing routes.

many of the chromatography patents claim a “new use” or novel elimination of a contaminant not previously attributed to the separation. Thus, while these patents are individually narrow, the sheer number of patents broadly prevents utilization of so called “obvious” purification steps. Multitudes of narrow focus patents is the norm for this field. Two examples are discussed below:

Static Mixers

This technology, developed by Genzyme (US5837529 [73]), solved a scaleup issue with alkaline lysis, mainly control of mixing to prevent plasmid damage and shearing of gDNA. While static mixers were not new, the application to plasmid purification was considered novel and inventive. The value of the US5837529 patent is illustrated by the cross license agreement with Valantis in 2002 for production of plasmids. However, other companies created new solutions, or reverse engineered around the issued claims on the use of static mixers, resulting at least seven alternative methods to accomplish the same end (*e.g.* T tubes, vortex mixers; see Table 2). Thus, the use of alkaline lysis now appears to require licensing one of these technologies, since many of the potential optimal methods of mixing at scale are the subject of this patent group.

Hydrophobic Interaction Chromatography (HIC)

Multiple recent patents and patent applications (see Table 5) claim use of hydrophobic interaction chromatography (HIC). In general, these patents find a new use for, or combine different purification modalities with HIC into a feasible large scale process. Thus, theoretically, several companies may be able to use HIC, since each may obtain protection for different combinations of HIC with other purifications. Again, use of HIC appears to require licensing, since many of the potential combinations of HIC with other purifications are tied up in the pending applications.

We do not attempt herein to determine priority or investigate inventiveness or novelty of the multiple pending patents for similar technologies; rather, we report both issued and critical pending applications for which we project the applicants will obtain some measure of intellectual property protection for their inventions.

FERMENTATION

The primary goal when designing a fermentation process for plasmids is to maximize both the volumetric yield (mg/L) and specific yield (mg/g) of supercoiled plasmid. Optimization of the

volumetric yield allows for smaller and more economical fermentations, while optimization of the specific yield improves the purity of the plasmid in downstream processing. It is also critical that the fermentation produces high quality plasmid. The FDA recognizes that open circle, linear, and nicked forms may be less effective therapeutically than the supercoiled form [39]. These other forms of plasmid can be very difficult to separate from the supercoiled plasmid during purification; therefore the fermentation process should also be optimized to retain a high percentage of supercoiled plasmid.

Media composition can drastically affect plasmid quality and yield. High cell density fermentation requires a balanced medium that supplies adequate amounts of the nutrients needed for energy, biomass, and cell maintenance. Until recently, most media optimization efforts have focused on production of recombinant proteins. Media for plasmid production should support high nucleotide pools in the cell and supply energy for replication, while minimizing other cell activity. The following factors should be considered when formulating media for therapeutic plasmid production.

- Effect of components on plasmid yield and quality
- Biomass yield
- Lot to lot consistency
- Potential interference with downstream purification
- Regulatory concerns (*e.g.* animal product free)

High cell density fermentation media contains sources of carbon and nitrogen, various salts, and trace metals. Additionally, vitamins and/or amino acids are supplemented as growth factors or to satisfy the needs of auxotrophic host strains.

Vector Backbones

Therapeutic plasmids typically contain a ColE1 or pBR322 derived replication origin. Common high copy number derivatives have mutations affecting copy number regulation, such as rop (Repressor of primer gene) deletion, with a second site mutation that increases copy number (*e.g.* pUC G to A point mutation, or ColE1 pMM1). Higher temperature (42°C) can be employed to induce selective plasmid amplification with pUC and pMM1 replication origins [reviewed in 38].

By contrast, the pCOR vector, developed by Gencell, utilizes the R6K origin, with the pir-116 high copy activator integrated into the host chromosome [40]. This design restricts the host range of the plasmid to the specific production strain. In addition, the vector contains multiple copies of the trinucleotide GAA, which is capable of forming a DNA triple helix. This sequence facilitates purification on triple helix affinity resin using the complementary oligonucleotide. The pCOR vectors use a suppressor tRNA selectable marker that suppresses an argE TAG amber mutation in the host strain. The suppression is required for growth in minimal media. Mutagenesis of the pir-116 replication protein and selection for increased copy number has been used to make new production strains with reported yields of up to 500 mg/L [41].

Herein, we limit our discussion to fermentation conditions for ColE1 or pBR322 derived replication origin containing plasmids.

Growth Conditions

The use of reduced growth rate coupled with these high copy replication origins is the unifying principle in high quality, high yield plasmid fermentations [Reviewed in 38]. Generally, lower growth rates favor increased plasmid copy numbers [42]. In a study to determine the effects of fermentation strategy on plasmid quality, O'Kennedy *et al.* [43] found that higher growth rates in batch and fed-batch fermentations were associated with lower percentages of supercoiled plasmid.

Supercoiling is known to be affected by oxygen and temperature [44,45]. Oxygen has been shown to play a role in plasmid stability. One study found that a single drop in dissolved oxygen concentration to 5% of air saturation led to rapid loss in plasmid stability [46]. Another study showed that fluctuations in oxygen input lead to plasmid instability [47]. Furthermore, the formation of nicked plasmids and multimers can be affected by many parameters, including temperature, pH, dissolved oxygen, nutrient concentration, and growth rate [48]. The optimal temperature for *E. coli* growth is 37°C. However, lower temperatures (30-37°C) may be used in batch fermentation to cause a reduced maximum specific growth rate. Temperature can also be employed to induce selective pUC plasmid amplification [49].

Batch Fermentation

Batch fermentation (Fig. (2)) has the main advantage of simplicity. All nutrients that will be utilized for cell growth and plasmid production throughout the culture period are present at the time of inoculation. The use of a suitable inoculum (1-5% of the culture volume) is recommended to minimize lag. During the exponential phase all nutrients are in excess; thus the specific growth rate will be essentially the maximum specific growth rate, μ_{max} , as predicted by Monod kinetics. As discussed previously, reduced growth rates are desirable for plasmid production. In batch fermentation the growth rate can only be reduced by reducing μ_{max} . This has been achieved by growth at lower temperatures and by growth on different types of media. Batch fermentation at 30°C using glycerol will typically result in $\mu_{max} \leq 0.3 \text{ h}^{-1}$, which is sufficient to prevent deleterious acetate accumulation and growth rate associated plasmid instability [38]. Glycerol can also be used at much higher concentrations than glucose without being inhibitory, leading to higher biomass yields. Durland and Eastman [48] report a batch fermentation at 37°C in a proprietary medium with typical yields of 130 mg/L and as high as 250 mg/L.

Fed-Batch Fermentation

Fed-batch fermentation (Fig. (3)) is especially useful for plasmid production. Controlled addition of a limiting nutrient allows for control of growth rate $< \mu_{max}$. Also, fed-batch fermentation results in higher biomass yields because substrate is supplied at a rate such that it is nearly completely consumed. As a result, conversion of

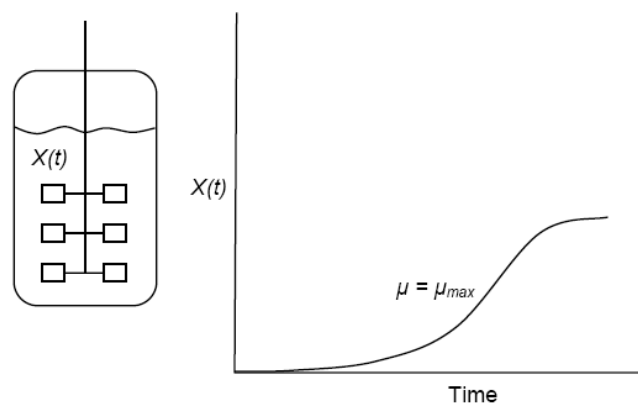


Fig. (2). Typical batch fermentation. X is biomass concentration, t is time, μ is the specific growth rate.

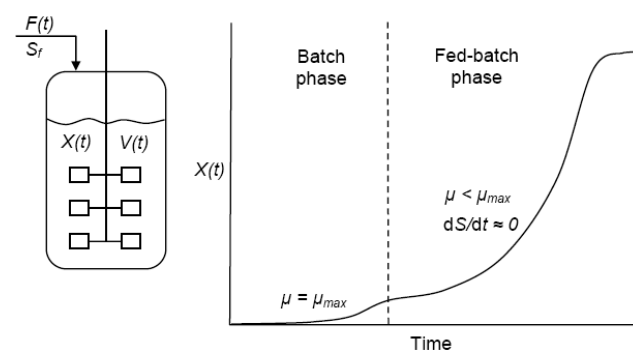


Fig. (3). Typical fed-batch fermentation. F is the feed rate, S is limiting substrate concentration, S_r is the limiting substrate concentration in the feed, V is culture volume.

substrate to biomass is very efficient and residual substrate concentration is approximately zero, never reaching inhibitory concentrations. Metabolic overflow from excess substrate is reduced, avoiding excessive formation of inhibitory acetate.

Fed-batch fermentation starts with a batch phase. Cells are inoculated into an initial volume of medium that contains all non-limiting nutrients and an initial concentration of the limiting substrate. Controlled feeding of the limiting nutrient begins once the cells have consumed the initial amount of substrate. At the industrial level, feeding strategies are either feedback controlled (*e.g.* DO-stat, pH stat, metabolic activity, biomass concentration, substrate concentration), or predetermined (*e.g.* constant, linear, stepwise, or exponential feeding).

Several fed-batch plasmid fermentation processes have been described that provide yields between 100-250 mg/L plasmid.

Lahijani *et al.* [49] have reported using a pUC origin plasmid in a fermentation with exponential feeding and a temperature shift from 37°C to 42-45°C.

US5955323 [50] discloses a feed-back fed-batch process that uses an automated feeding scheme based on dissolved oxygen concentration (DO) and pH (*i.e.* DO-stat and pH-stat) to maintain a controlled slow growth rate by triggering on the nutrient feeding whenever DO or pH rises above threshold setpoints. A plasmid yield of about 98 mg/L after 22 hours of fermentation was achieved in an experiment described in this patent.

US6664078 [51] describes a fed-batch process that also utilizes the DO-stat strategy. Additionally, US6664078 includes semi-defined and defined media compositions and reports that high levels of MgSO_4 results in high homogeneity of supercoiled plasmid

monomers. One example used plasmid pUT649 (4618 bp) in *E. coli* DH5 α with glycerol yeast medium. A plasmid yield of 230 mg/L and a biomass concentration of 60 g/L DCW (dry cell weight) was achieved after 41 hours of fermentation.

High Yield Fermentation Processes

Three fermentation processes have recently been described that provide >500 mg/L pUC plasmid yields.

Merck Process

WO2005078115 [52] describes a method for plasmid DNA production that involves selecting a highly productive clonal subtype of the transformed *E. coli* and cultivation with a fed-batch process. The high producer clonal subtype selection includes plating on blood agar, on which highly productive subtypes are phenotypically grey, and plating on chemically-defined agar medium, on which highly productive subtypes are phenotypically cream colored in a population of both cream-colored colonies and cream-colored colonies with brown centers. *E. coli* strain DH5 is used in the description.

The highly productive clonal subtypes isolated by the selection process are cultivated by a fed-batch fermentation process in chemically defined medium with a continuous feeding regime of a solution of 60% glycerol or 50% glycerol and 25% monosodium glutamate.

An example describes 18 fed-batch fermentations of 15L batch volume of a defined medium. The nutrient feed solution was 60% glycerol. Nutrient feeding was at constant feed rates and was started when the carbon evolution rate (CER) reached 35 mmol/L/hour. The feeding rates ranged from 2-12 g/L/hour. Maximum specific plasmid yields of 30-32 μ g plasmid / mg DCW were achieved with feed rates from 8-12 g/L/hour. The volumetric plasmid yields ranged from 0.2 g/L to 1.3 g/L and were dependent on the feed rate. The fermentation process of the invention is also described in [53,54], and a yield as high as 1.6 g/L, 39mg plasmid/g DCW with a 7.3 kb plasmid is reported [54].

Boehringer Ingelheim Process

WO2005097990 [55] discloses fed-batch fermentation and chemically defined media that uses an exponential feeding profile for at least part of the fed-batch phase for biomass growth, followed by linear constant feeding to cause lower specific growth rates for increased plasmid accumulation. The preferred host strain is *E. coli* JM108; the use of which is also described in WO2005098002 [56] to result in higher plasmid yields and homogeneity of supercoiled plasmid (>90%), compared to DH5 α and DH10B.

WO2005097990 also reports the presence of isoleucine at a concentration of 0.2 g/L in the medium as a preferred embodiment to overcome problems associated with valine toxicity, which is the phenomenon of repressed isoleucine synthesis in the presence of valine in *E. coli* K-12 strains [57,58]. While isoleucine is not necessary for biomass growth, the plasmid yield was significantly improved with the isoleucine addition. An example is included describing two fed-batch fermentations with the only difference being that one medium contained isoleucine and the other did not. Both fermentations had almost identical biomass growth. The fermentation with isoleucine reached 633 mg/L and the one without isoleucine reached 398 mg/L.

Plasmid yield is generally improved in *relA*- strains due to the relaxed response to amino acid starvation that allows plasmid replication to continue [reviewed in 59]. The isoleucine addition seems to enhance this effect in *relA*- strains.

Another example describes dividing the fed-batch phase into two parts. First, an exponential feeding regime is used to maintain a specific growth rate of $\mu = 0.25\text{h}^{-1}$. After 10 hours of exponential feeding, a phase of linear constant feeding is maintained for another

10 hours. Volumetric and specific plasmid yields ranging from 500-800 mg pDNA/L and 20-30 mg plasmid/g DCW are reported with this process.

Nature Technology Corporation Process

WO2006023546 [60] discloses methods fed-batch fermentation, in which plasmid-containing *E. coli* cells are grown at a reduced temperature during part of the fed-batch phase, during which growth rate is restricted, followed by a temperature up-shift and continued growth at elevated temperature in order to accumulate plasmid; the temperature shift at restricted growth rate improves yield and purity of plasmid.

This process takes advantage of the temperature sensitivity of high copy number plasmids. In the preferred process, the initial temperature setpoint is 30°C, at which the plasmid is maintained stably at low levels while biomass can accumulate efficiently. During this period, the specific growth rate is controlled at approximately $\mu = 0.12\text{h}^{-1}$ by an exponential feeding strategy. Induction of plasmid accumulation is performed when the cell density is in the range of 25-60 OD₆₀₀ by shifting the temperature to 42°C and continued exponential nutrient feeding for up to 15 hours.

Plasmid yields prior to the temperature shift remain low. The specific plasmid yields after temperature shift are very high. Interestingly, after the temperature shift, the cells are able to tolerate significantly higher quantities of plasmid than growing cells at a constant temperature of 37°C with the same media and feeding strategy.

The examples in the patent report yields up to 1.1 g/L achieved when the disclosed process was used with a temperature shift from 30°C to 42°C. The preferred process of WO2006023546 is also described in [38,61,62] where volumetric yields of 1.5-2.1 g/L, and specific yields as high as 43 mg plasmid/g DCW are reported. The plasmid DNA produced with the process is high quality, being 96% supercoiled [62] or greater with no detectable deletion or other rearrangement. The method is simple, can be used with multiple pUC based backbones, and does not require prescreening of individual colonies for high producing cell lines. A key advantage of the inducible fed-batch process is that amplification of plasmid copy number after suitable biomass accumulation helps preserve quality and stabilize toxic plasmids, while maximizing yield. This is because selection pressure at the cellular level is reduced during the biomass accumulation phase by minimizing the growth rate difference between monomer or dimer or rearranged plasmid-bearing and plasmid-free cells.

Fermentation Summary

Fed batch production processes, using processes described in the public domain, plateau at about 100-250 mg plasmid/L. Three patent pending processes have been described, use of which will increase volumetric and specific yields up to 10 fold (Table 1). High specific yields are very desirable since increased plasmid yield per gram of bacteria, or increased plasmid relative to genomic DNA (utilizing the WO2006023546 process, up to 75% of the total DNA in the cell at harvest is plasmid DNA) leads directly to higher final product purities. High yield fermentation processes can generate far more plasmid than can be processed in a single lot in a typical production facility (*i.e.* a 500 L fermentor at 2 g/L productivity would generate 1 kg of plasmid in a 40 hour process); improvements in the bottleneck cell lysis technologies are needed to solve this mismatch (see below).

CELL DISRUPTION—PLASMID RELEASE

The *E. coli* biomass generated by a fermentation process must be lysed to release the plasmid DNA. Cell disruption methods fall into two main categories [63]:

Table 1. Fermentation Key Patents

Patent Title	Patent No.	Summary
Process for plasmid DNA fermentation	WO2006023546	Nature Technology Corporation inducible fed-batch process
Method for the isolation of ccc plasmid DNA	US6664078	Qiagen fed-batch process
Automated high-yield fermentation of plasmid DNA in <i>Escherichia coli</i>	US5955323	American Home Products (Wyeth) fed-batch process
Fed-batch fermentation process and culture medium for the production of plasmid DNA in <i>E. coli</i> on a manufacturing scale	WO2005097990	Boehringer Ingelheim fed-batch process
Method for producing plasmid DNA on a manufacturing scale by fermentation of the <i>Escherichia coli</i> K-12 strain JM108	WO2005098002	Boehringer Ingelheim fermentation with <i>E. coli</i> JM108
Process for large scale production of plasmid DNA by <i>E. coli</i> fermentation	WO2005078115	Merck high producer clonal selection and fed-batch process

Physico-mechanical	Chemical
<ul style="list-style-type: none"> • liquid shear • solid shear • agitation with abrasives • freeze-thawing • ultrasonication • heat 	<ul style="list-style-type: none"> • detergents • osmotic shock • alkali treatment • enzyme treatment

The cell disruption method for plasmid isolation must be chosen such that minimal damage is inflicted on the plasmid DNA product, and in most cases, it is also desired to avoid shearing of the host cell chromosomal DNA into smaller fragments that are more difficult to separate from plasmid DNA. Thus, the methods available for plasmid purification are more limited compared to the harsher methods that are often used for purifying smaller molecules such as proteins. Ideally the method releases a high yield of intact plasmid, while limiting release of difficult to remove impurities such as gDNA.

Most of the above methods have been applied, either alone or combined, to plasmid DNA purification. To date, the two most commonly used methods for plasmid DNA recovery are alkaline lysis and heat lysis; additionally, detergents and enzyme (*i.e.* lysozyme) treatment are often used to aid lysis in these methods.

Alkaline Lysis

The standard alkaline lysis method of Birnboim and Doly [64] is well known and is widely used without restriction in molecular biology laboratories. The details of alkaline lysis, its modifications, and its use at larger scales have been carefully discussed by a number of review articles [4,48,65-69].

Generally, a lysis time of five minutes has been used with the standard alkaline lysis method; longer times have been known to cause irreversible denaturation of plasmid DNA [64, Qiagen plasmid handbook]. A study on the time course of standard alkaline lysis was performed by measuring viscosity of a lysis mixture as a function of time and by performing cell counts over a range of lysis times [70]. The results indicate that for *E. coli* DH5 α , complete cell lysis occurs after about 40 seconds and complete denaturation of chromosomal DNA takes 80-120 seconds after mixing with the lysis buffer. Longer reaction times were reported to lead to shear degradation of chromosomal DNA.

US6503738 [71] describes a method to determine the optimum lysis pH value, which is about 0.2 pH below the "irreversible alkaline denaturation value", defined as "the pH value at which no

more than about 50% of the alkaline denatured plasmid DNA fails to renature as determined by standard agarose gel electrophoresis". The optimum lysis value can be different for various plasmid/host strain combinations.

The patent landscape includes various methods and devices aimed at performing alkaline lysis at large scale. Insufficient mixing will result in local pH extremes, causing irreversibly denatured plasmid. Mixing that is too aggressive can damage the plasmid DNA and fragment chromosomal DNA. At the laboratory scale, mixing is performed gently by hand. Hand mixing at larger scales is not possible because of the large volumes and lack of reproducibility from person to person. Thus, batch mixing in a mechanically agitated vessel is often used, but the viscos, non-Newtonian properties of the lysis mixture require some consideration. US6395516 [72] discloses a specialized vessel design for mixing cell lysate that utilizes baffles, low power number impellers, feed lines, and monitoring the degree of lysis by measuring viscosity.

Continuous flow through devices have been employed as alternatives to the challenge of achieving complete, but gentle mixing of large lysis volumes in stirred tanks, and are perhaps more easily implemented in facilities that do not already contain specialized batch mixing equipment. Additionally, the lysis reaction time can be closely controlled by the residence time of tubing or pipe (*e.g.* as in US5837529 [73], US6664049 [74], WO2006060282 [75]), or of the holding vessel (*e.g.* as in US6699706 [76]) before the neutralization step. An example flow through lysis setup is shown in Fig. (4).

Inline static mixers (motionless mixers) have long been used in industry and more recently have been applied for cell lysis. A static mixer is a cylindrical tube containing stationary mixing elements. The mixing elements are shaped and positioned to combine materials as they flow through the mixer. US5837529 describes the use of static mixers to achieve gentle mixing of a cell suspension with a lysis solution. Mixing of the cell suspension stream with the lysis buffer stream is completed rapidly and the degree of mixing and lysis time can be adjusted by the number of mixing elements, flowrate, and length. Neutralization may occur in a second static mixer.

US6664049 claims mixing methods that use only tubing, without the static mixers; instead, smaller diameter tubing is used and flowrates are adjusted to cause homogeneous mixing for the desired contact time.

WO2006060282 discloses flow-through mixing devices consisting of a conduit through which the lysis solution flows, and an inlet, such as a nozzle, into the conduit in which the cell suspension is injected in either a counter-flow or co-flow direction.

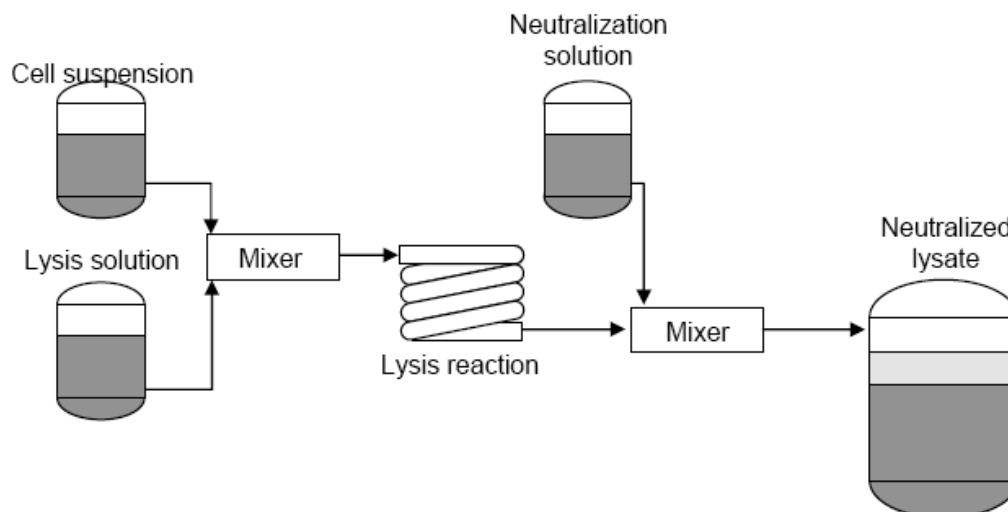


Fig. (4). Example of a flow through alkaline lysis process. The cell suspension is continually mixed with lysis solution in a flow through mixing device, then proceeds through a specific length of tubing at a flow rate designed to give a desired reaction time for cell lysis. The lysis mixture is then continually neutralized in a second mixing device.

US6699706 claims the use of fluidic vortex mixers for continuous flow-through lysis and neutralization. A vortex mixer is described by the patent as a cylindrical chamber with an axial outlet at the center of one end wall with two tangential inlets along the periphery. The dimensions of the mixer and the flowrates used are chosen so that the residence time in the mixers is much less than the time required for lysis (about 0.01-0.1 seconds) so that the cell suspension and lysis solution are mixed completely. The cells can then react with the lysis solution after exiting the vortex mixer. In an example, the cells and lysis solution are mixed in a first vortex mixer, flow into a tank for completion of lysis, then the mixture flows through an outlet of the tank where it is mixed with neutralization buffer in a second vortex mixer.

WO2005026331 [77] discloses continuous alkaline lysis through the use of T tubes with lengths of turbulent flow (achieved by small diameter tubing) to rapidly mix the cell suspension and lysis solution, followed by a length of laminar flow (in larger diameter tubing) for incubation and time for lysis and denaturation without substantial agitation which would damage the plasmid and fragment gDNA. Neutralization solution may then be introduced continuously in a second T tube.

The above flow-through mixing devices are described to cause low shear mixing. It has been generally recognized that shear forces created by mixing too intensely may cause damage to plasmid DNA and fragmentation of chromosomal DNA, leading to co-purification of chromosomal DNA with plasmid DNA [68,78]. Chamsart *et al.* [79] have reported that shear induced degradation of chromosomal DNA may not be as critical as previously thought. They demonstrated that chromosomal DNA contamination in the neutralized lysate was < 2% over a wide range of shear rates and agitation speeds, even speeds greatly in excess of those required for effective mixing. Another review of shear-induced DNA degradation [80] reports that plasmids smaller than 5 kb are not very sensitive to shear-induced degradation, but plasmids larger than 5 kb become increasingly sensitive with size to shear-induced degradation.

WO2004108260 [81] discloses a method and device for mixing the cell suspension and lysis solution using a low residence time, high shear mixing device. However, it notes that gentle, low shear mixing is still necessary during the neutralization and flocculation step to avoid releasing excessive amounts of cellular impurities into the plasmid-containing liquid lysate. Thus, this patent application also claims the use of a bubble mixer for gentle mixing of the lysis

mixture with the neutralization solution. This method has the added advantage of a high degree of flocculant flotation due to the air bubbles trapped in the floc.

WO2004085643 [82] discloses methods and devices for continuous flow lysis that utilize glass bead columns to act as lysis and clarification reactors. A detailed description of the development and automation of this system is presented in [83].

Alternative Lysis Methods

Heat Lysis

Plasmid isolation using heat lysis was first reported by Holmes and Quigley [84], and is perhaps the most widely used method after alkaline lysis.

Merck has developed and patented processes to adapt heat lysis to large scale processing. In US6197553 [85], a bacterial suspension in modified STET buffer (*e.g.* 50 mM Tris, 50-100 mM EDTA, 8% sucrose, 2% Triton X-100, pH 8.0-8.5) is pumped through a heat exchanger at such a rate that the suspension exits with a temperature of 70-100°C, resulting in lysis. The lysate is then centrifuged to remove cell debris, protein, and chromosomal DNA, leaving RNA and plasmid in solution. The optional use of lysozyme is reported to increase the plasmid concentration in the lysate by 4-5 times. It was also found that the formation of undesirable open circle plasmid by endogenous DNase during this lysis process could be reduced by increasing the EDTA concentration from 50mM to 100mM. They report higher plasmid recovery by heat lysis than by chemical lysis.

Mechanical Disruption

Generally, mechanical disruption of bacteria (*e.g.* french press, sonication, homogenization, nebulization) for plasmid isolation is seen as unfeasible due to the damage it would cause to the DNA. US6455287 [86] reports that sonication, nebulization, and Gaulin Mill homogenization resulted in almost complete destruction of plasmid DNA in their experiments. However, disruption with a bead mill device under optimized conditions resulted in over 90% of the plasmid solubilized without destruction. They also report that an impinging-jet homogenizer released up to 50% of the plasmid DNA intact.

Another method used to overcome destruction of DNA during mechanical disruption is the use of DNA compaction agents. The process of US2002197637 [87], also described in [88], discloses the

Table 2. Alkaline Lysis Key Patents

Patent Title	Patent No.	Summary
Method for lysing cells	US5837529	Genzyme static mixers
Mixing devices for chemical lysis of cells	WO2006060282	Merial flow through mixing device
Method of preparation of pharmaceutically grade plasmid DNA	WO2005026331	Gencell T tube mixer
Devices and methods for biomaterial production	WO2004108260	Advisys high shear, low residence time lysis mixer, bubble mixer for neutralization
Methods and devices for producing biomolecules	WO2004085643	Boehringer Ingelheim glass bead lysis and clarification reactors
Method of plasmid DNA production and purification	US6503738	Cobra Biomanufacturing method to determine the optimum lysis pH
Vessel for mixing a cell lysate	US6395516	Cobra Biomanufacturing vessel design for mixing cell lysate
Method and device for cell lysis	US6664049	Aventis Tubing only mixing
Cell lysis method using a vortex mixer	US6699706	Accentus Fluidic vortex mixers

use of polycationic compaction agents (*e.g.* polylysine, spermine, spermidine) to protect DNA from shear damage during mechanical lysis. The compaction agents cause the DNA to be pelleted with the insoluble cell debris. The pellet is washed, and the plasmid DNA is resolubilized to give an enriched solution. The use of compaction agents also results in reduced lysate viscosity.

Lysozyme Lysis

A process developed by Merck (WO2006083721 [89]) describes a STET/lysozyme lysis performed at 20°C or 37°C, preferably with an additional alkaline pH shift to denature genomic DNA. To reduce viscosity, PEG is added to flocculate genomic DNA and host cell debris. This allows debris removal with low speed centrifugation. While the process retains the pH shifting of alkaline lysis, shear forces are reduced by performing the shift after lysis. Therefore this process does eliminate many of the difficult processing and equipment needs of alkaline or heat lysis. The limitation of this reduced temperature lysis method is the need for large amounts of recombinant lysozyme and potentially high levels of gDNA in the clarified lysate (levels of genomic DNA in the clarified lysate or after downstream plasmid purification are not reported).

Autolysis

Autolytic strains using phage T4 lysis proteins have been patented for protein production as in U56258560 [90]. In this system, lysozyme (endolysin) is expressed by the cell in the cytoplasm and released to the periplasm at the desired time by co-expression of a holin (membrane spanning peptide or protein) that creates a channel, allowing leakage of lysozyme from the cytoplasm to the periplasm. Other autolytic *E. coli* strains that are described in US2006040393 [91] contain the bacteriophage λ R lytic endolysin gene. The endolysin is induced by arabinose, which then causes the *E. coli* to be lysed after a freeze-thaw cycle.

Autolysis conditions, as opposed to alkaline or heat lysis, do not selectively denature gDNA. The product of lysis is very viscous due to high levels of residual gDNA, creating processing problems. For protein production, non specific nucleases are added, or expressed periplasmically in the strain (*e.g.*, *endA* nuclease [90]; *Staphylococcus* nuclease [92-94]) to reduce viscosity after cell lysis. Such systems could not be utilized for plasmid production, since the plasmid would be degraded or damaged by the nuclease. While autolysis is not an essential design improvement for protein production (since cell lysis is performed at high density using generally available equipment) it has tremendous potential for

plasmid purification since alkaline or heat lysis steps are key bottlenecks in plasmid processing.

Plasmid purification by autolysis has been facilitated by WO2006026125 [95], which combines novel autolytic *E. coli* strains with plasmid-safe nuclease. Specifically, these strains include chromosomally encoded endolysin and chimeric ribonuclease-T5 exonuclease genes that are expressed during the fermentation process (Fig. (5)).

Bacteriophage T5 exonuclease is an ideal DNase to use in plasmid processing. T5 exonuclease does not digest supercoiled plasmid, but is able to digest linear single- and double-stranded DNA (ssDNA, dsDNA) [96]. It will also digest irreversible denatured plasmid, such as 'ghost' or 'shadow band' DNA, which, while similar to supercoiled plasmid, is refractile to restriction enzyme digestion [97].

These strains have been successfully fermented in the high yielding process of [60] without any deleterious effects to plasmid or biomass yield, and the harvested biomass readily lyses upon resuspension in simple buffer solutions [Williams, Carnes unpublished 2006, 2007]. The elimination of a significant amount of contaminating nucleic acids by the nuclease would simplify and increase the purification efficiency for any of the downstream purification operations discussed later in this review. As well, application of autolytic cells to the lysozyme lysis process of WO2006083721 would eliminate the need for costly recombinant lysozyme.

Cell Disruption Summary

Use of alkaline or heat lysis in large scale is highly restricted, due to a picket fence of IP created by various companies to deal with mixing, shear force and flocculation issues (Table 2). However, alkaline and heat lysis methodologies are by nature problematic and are a major process bottleneck; most facilities cannot process large amounts of fermentation paste due to the extreme process volumes and or flow through times required for alkaline or heat lysis. As well, costly chemicals and large waste streams (SDS, NaOH, acetate, EDTA) create environmental problems at scale for both these processes.

A new technology is needed to eliminate this critical processing bottleneck. Several early stage alternative mechanical, lysozyme or autolysis methods have been developed and patented (Table 3); these new approaches may ultimately result in greatly improved industrial lysis technologies.

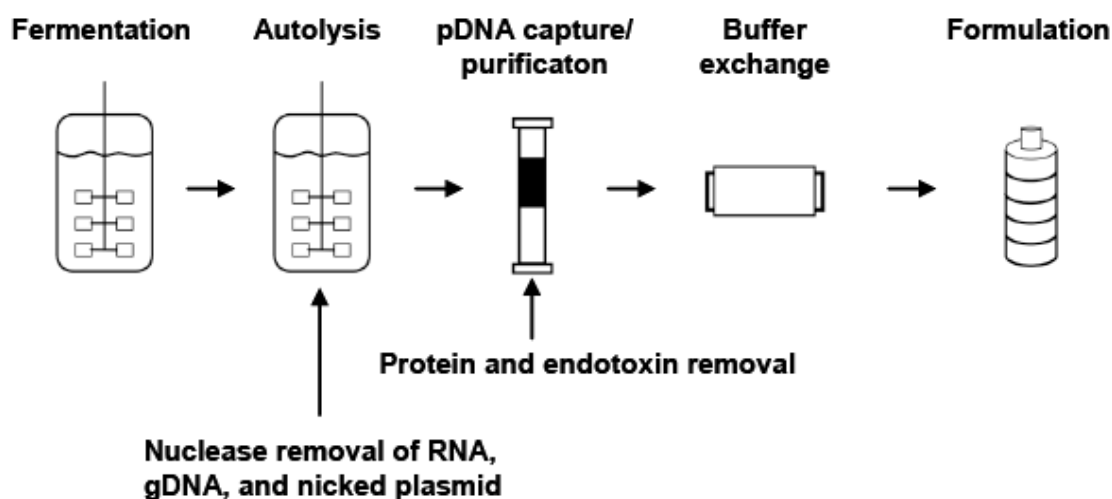


Fig. (5). Example process flow diagram for autolytic plasmid processing. Autolytic host strains that express RNase and plasmid-safe DNase may simplify lysis and downstream purification.

Table 3. Alternative Lysis Key Patents

Patent Title	Patent No.	Summary
Upstream and a downstream purification process for large scale production of plasmid DNA	WO2006083721	Merck STET/lysozyme lysis, alkaline pH shift, PEG flocculation
Mechanical disruption of bacterial cells for plasmid recovery	US6455287	Wyeth mechanical lysis
Process and compositions for protection of nucleic acids	US2002197637	Mechanical lysis in the presence of compaction agents
Improved strains of <i>E. coli</i> for plasmid DNA production	WO2006026125	Nature Technology Corporation autolysis
Controlled lysis of bacteria	US2006040393	Zymo Research autolytic strains for Freeze thaw lysis
Process for bacterial production of polypeptides	US6258560	Genentech autolytic strains for protein production

DOWNSTREAM PURIFICATION

Overview

The goal of downstream processing is to remove all impurities present in the lysate, leaving highly purified plasmid DNA. Plasmid isolation usually accounts for the majority of manufacturing costs. The high cost of plasmid isolation stems mainly from two factors 1) plasmid DNA makes up a small fraction of the cell mass produced by fermentation, and 2) separation of plasmid from host nucleic acids is difficult. Thus, the primary objective is to design a process that maximizes yield, purity, and safety, while minimizing cost.

Plasmid size must be considered when selecting a process. For example, terminal 0.22 μm filtration of a large plasmids (>11 kb) was shown to be dramatically less efficient compared to smaller plasmids, with excessive fouling and yield losses [98]. Larger plasmids, such as 20 kb AAV helper plasmids often cannot be sterile filtered without unacceptable membrane fouling and yield loss. Thus the selected process must be customized to the plasmid size, and flexibility built in if a single process is to be utilized to purify plasmids spanning a broad range of sizes.

Furthermore, the formation of undesirable plasmid isoforms can occur during fermentation and downstream processing. These include linear plasmid, multimers, relaxed (open circle) plasmid, and irreversibly denatured plasmid. During downstream processing damaged plasmid can be formed at high and low pHs and by excessive shear forces. The formation rate will be plasmid size dependent. The process must be optimized to both limit their

formation, and to remove (or repair *in vitro*, WO2005113808 [99]) the specific isoforms that arise during fermentation or cell lysis.

Start with the End in Mind

A crucial starting point in developing a purification process is to define the purity requirements and acceptable levels of impurities. The major impurities are protein, chromosomal DNA, RNA, and endotoxin. Current specifications for clinical grade plasmid DNA are outlined in Table 4. FDA guidance recommends that the upper limit for endotoxin be 5 EU/kg body weight/dose [100]; this is obtainable with current manufacturing methodologies. As discussed previously, protein and gDNA specifications may need to be considerably lower for commercial plasmid products; licensed protein products have much lower residual host protein (typically <100 ppm) and gDNA (typically <100 pg/dose) limits [specifications and assay formats are reviewed in 101].

Process Options

A variety of operations are used in downstream purification of plasmid DNA, including:

- Nuclease treatment
- Selective precipitation
- Selective impurity adsorption
- Tangential flow filtration (TFF)
- Chromatography
 - Anion exchange chromatography

Table 4. Quality Criteria for Plasmid DNA (Clinical)

Impurity	Recommended assay	Approval specification
Proteins	BCA protein assay	Undetectable (<0.5%)
RNA	Agarose gel electrophoresis	Undetectable (<1%)
Chromosomal DNA	Agarose gel electrophoresis	undetectable
	Southern blot, qPCR	< 0.01 µg/µg plasmid (<1%)
Endotoxins	LAL assay	< 10 EU/mg plasmid
Plasmid isoforms (linear, relaxed, denatured)	Agarose gel electrophoresis	< 5%
Percentage of covalently closed plasmid DNA	Agarose gel electrophoresis	>90%
Biological activity and identity	Restriction endonucleases	Coherent fragments with the plasmid restriction map
	Agarose gel electrophoresis	Expected migration for size and supercoiling
	Transformation efficiency	Comparable with plasmid standards

Abbreviations: BCA, bicinchoninic acid; LAL, Lymulus ameobocyte lysate; EU, endotoxin units

- Hydrophobic interaction chromatography (HIC)
- Affinity chromatography
- Size exclusion chromatography (SEC)

Aqueous two phase systems (ATPS)

The final scale of manufacture must be considered. gDNA in early clinical supplies were kept <0.01% by incorporating size exclusion or triple helix chromatography steps into the process [67]. The combination of anion exchange, triple helix chromatography, and HIC was reported by Centelion (Gencell) to reduce genomic DNA to < 0.00008% (see Fig. 4 in WO2005100542 [102]) which clearly meets the criteria for pharmaceutical grade DNA. While combinations of chromatography steps dramatically reduce gDNA and can be used for clinical production, due to cost and poor capacity such processes cannot be utilized for cost effective commercial scale purification.

Some downstream plasmid purification processes (after cell lysis) that reduce genomic DNA levels to 0.01-1% or less have been reported and should be considered. The following processes include the specified genomic DNA reduction steps:

- 0.01% with hydroxyapatite [103],
- 0.05% with hydrophobic interaction chromatography [104,105]
- 1% with ammonium sulfate precipitation [106],
- 0.2% with group selection size exclusion chromatography [107],
- <1% with tangential flow ultrafiltration [108],
- <1% with differential polyethylene glycol precipitation [109],
- 0.1% with CTAB precipitation and gyrolite LRA absorption [110],
- 0.1% with triple helix chromatography [111].
- < 0.00008% with anion exchange, triple helix chromatography, and HIC [102].
- < 0.000008% with heat denaturation, immobilized metal chelate chromatography [168].

Nuclease Treatment

Nuclease Elimination of RNA

A relatively common practice, until recently, was the use of bovine pancreatic ribonuclease (RNase A) in the lysis buffer, to degrade RNA. Although it was reasonably effective in reducing the

quantity and size of RNA, it also introduced the bovine-sourced RNase, which is undesirable from a regulatory standpoint, as it could be contaminated with prion agents, notably with the bovine spongiform encephalitis (BSE) agent. Indeed, there is a growing desire to perform fermentations and purifications of bacterial products (intended for human or animal use) entirely under animal product free (APF) conditions.

In order to overcome this obstacle, specialized *E. coli* strains have been developed which express recombinant bovine pancreatic RNase (US6780632 [112]; Cooke *et al.* 2001 [113]). The RNase is directed to the periplasmic space by means of a secretion signal, in order not to disrupt *E. coli* gene expression during cell growth. Upon lysis, the RNase becomes mixed with the RNA, degrading it.

Nuclease Elimination of gDNA

Enzymes for specifically degrading *E. coli* gDNA while leaving supercoiled plasmid, such as ATP-dependent exonuclease enzymes (*e.g.*, RecBCD exonuclease), can be added to partially purified plasmid DNA preparations (US6242220) [114]). The DNase enzymes cannot be added directly to alkaline or heat lysis (as with RNase) because these enzymes are generally more fragile than RNase, and would be inactivated in an alkaline/SDS environment. Thus, this is not a commercially viable method to remove gDNA from large scale alkaline or heat lysis plasmid preparations.

Alternative approaches have been developed that utilize endogenous nucleases to remove gDNA. Early methods induced general DNA damage (*e.g.*, ultraviolet radiation in repair deficient hosts [115] or ionizing irradiation in US4755464 [116]) in which plasmids survive due to a lower probability of damage relative to the chromosome (*i.e.*, smaller target than the genome); degradation is mediated by endogenous nucleases (*e.g.* RecBCD) that proceed from the DNA breakage sites in the genome. A more specific system utilizes restriction endonucleases to cleave gDNA, wherein restriction endonuclease activity is controlled by a thermosensitive methylase. Shifting to the restrictive temperature inactivates the methylase, leading to cleavage of gDNA, and subsequent endogenous exonuclease digestion (WO0129209 [117]). However, gDNA reduction is modest, and this method does not have general utility since existing plasmids may need to be reengineered to lack the relevant restriction sites.

In a recent review of bioprocessing issues of producing a DNA vaccine for an influenza pandemic [4], Hoare and colleagues suggest that all plasmid purification processes would benefit from RNase degradation of high molecular weight RNA, and they

recommend the use of RNase-expressing *E. coli* strains. They also note that the availability of recombinant lysozyme, needed for efficient recovery from the heat lysis process of [85], would be an issue. The autolytic nuclease *E. coli* strains of WO2006026125 are an attractive solution to these considerations.

Selective Precipitation

One form of selective precipitation has already been discussed above regarding the use of compaction agents to protect the DNA during mechanical cell disruption (US2002197637). After clarification, the lysate may be subjected to precipitation to concentrate the plasmid. At the laboratory scale, precipitation with isopropanol or ethanol is often used, but large amounts would be required at an industrial scale and constitute a fire hazard. US6617108 [118] discloses methods, also described in [119-121], for separation of RNA and DNA by selective precipitation of DNA using polycationic compaction agents.

The use of the detergent cetyltrimethylammonium bromide (CTAB) for recovery and partial purification of plasmid from a clarified STET lysate (prepared according to the heat/lysozyme process of [85]) is described in US6797476 [122], and also in a paper of its inventors [123]. Briefly, genomic DNA, open circle plasmid DNA, and supercoiled plasmid DNA are fractionally precipitated away from RNA, protein, and endotoxin by stepwise CTAB addition. Plasmid DNA is then selectively solubilized under controlled salt conditions. The fractional precipitation of genomic DNA, open circle plasmid, and linear plasmid from supercoiled plasmid occurs within a narrow range of CTAB concentration and is subject to batch-to-batch variability; therefore, a probing assay and real-time monitoring methods (turbidity and viscosity) are used.

Selective precipitation using polyethylene glycol (PEG) precipitation is described in similar and related processes of US5561064 [109] and WO2004060277 [124]. A high molecular weight polyol, preferably PEG-8000, at about 10% (weight/volume) is used to precipitate plasmid from lysate. Alternatively, US5561064 describes a low-cut PEG precipitation (4% PEG) to precipitate and remove impurities (e.g. chromosomal DNA), followed by a high cut (10% PEG) to precipitate the plasmid DNA. A high salt precipitation of impurities is described in WO 2004060277 to remove some of the RNA and much of the endotoxin. This is performed after redissolving the partially purified plasmid DNA from the PEG precipitation.

Various systems for affinity absorption/precipitation based on polymers or proteins have been developed. PolyTag Technology SA has developed temperature triggered affinity macroligands by coupling oligonucleotides for triple helix affinity interaction to water soluble poly-N-alkylacrylamides with defined transformation temperature. After binding, plasmid is precipitated by heating. A similar temperature triggered reversible phase transition system using elastin-like proteins fused to a DNA binding protein has been developed for affinity purification of plasmid [125]. These systems are restricted to plasmids for which the DNA recognition sequences are present.

Selective Adsorption

Hydrated calcium silicate (gyrolite) may be used to selectively adsorb chromosomal DNA, open-circular plasmid, and other impurities and contaminants. The use of synthetic hydrated calcium silicate for endotoxin removal is discussed by [126,127]. Synthetic hydrated calcium silicate adsorbents LRA® and LRAII® are manufactured by Advanced Minerals Corporation and in their bulk form resemble diatomaceous earth.

Hydrated calcium silicate adsorption is used after the CTAB recovered plasmid in the process of US6797476 and is also described by the inventors in [128]. The hydrated calcium silicate adsorbs the primary impurities: CTAB, endotoxin, genomic DNA,

plasmid degradation products. Other residual impurities adsorbed include proteins, RNA, and detergent. The patent describes using up to 200g/L of hydrated calcium silicate. Genomic DNA and open circle plasmid DNA adsorb with greater affinity than supercoiled plasmid. Adsorption is highly dependent on ionic strength, especially with respect to divalent cation concentration, and the importance and effects of salt conditions that promote removal of impurities with minimal adsorption of supercoiled plasmid DNA are discussed.

Systems for protein-based selective affinity absorption of plasmid have been developed using GST-Zinc finger protein [129] or His-tagged LacI repressor [130]. DNA binding proteins that recognize sequences present naturally or engineered onto the plasmid.

Tangential Flow Filtration

The patent literature contains numerous examples that use crossflow or tangential flow filtration (TFF) for cell harvest, concentration, buffer exchange, and to selectively remove smaller impurities. Alternative uses of TFF as a plasmid purification operation are described in [131-133].

Plasmid purification using tangential flow filtration (TFF) as a major purification operation is claimed in WO0107599 [134] and also described by the inventors in [133]. An example in this process used 0.5 ft² (0.046 m²) of a polyethersulfone membrane per 10-15 g of cells processed, with 500-1000kDa molecular weight cut-off (MWCO) after alkaline lysis and clarification. This is reported to give purified plasmid without the use of RNase. A preferred method of the process includes incubation of the lysate for 20-24 hours for sufficient degradation of RNA so that it can be removed by TFF. Recirculation of the filtrate stream for 10-15 minutes upon startup of the filtration process was found to minimize plasmid loss through the filtrate, likely due to the formation of a gel layer on the retentate side.

The descriptions of this process interestingly note that the use of an anionic detergent, as opposed to cationic, non-ionic, or zwitterionic detergents, during lysis resulted in lysates with much lower endotoxin levels. WO0107599 also states that endotoxin levels were higher with this process than with alternative processes, but that the ability of the plasmid prepared with this process to transfect cells and express protein was not adversely affected.

US6011148 [108] also describes the use of TFF (referred to as "tangential flow ultrafiltration" or TFU in this patent) for plasmid DNA purification. This patent recommends membranes with a MWCO of 300 kDa for plasmids in the 2-15 kb size range, and a MWCO of 500 kDa for plasmids in the size range of 15-50 kb. It also recommends about 5 ft² (0.46 m²) of membrane area per 200-400 mg of DNA. Because the specific plasmid yield (mg pDNA/g cell weight) can be variable, membrane sizing based on amount of plasmid, rather than on the amount of biomass processed, may give more reliable performance. This is especially relevant when considering the high yielding processes discussed earlier, for which sizing based solely on biomass might result in too little membrane area for the amount of plasmid present.

US6011148 also reports the finding that open-channel membrane devices minimize shearing of the DNA, whereas shearing and plasmid loss is increased in screened channel membrane devices. Screened channel membranes contain screens in the retentate side that are meant to create a gentle turbulent flow across the membrane surface to minimize gel layer formation. Thus, the use of screened channel membranes is also not advisable with respect to gel layer formation; like the process description of WO0107599 and [133], the description of US6011148 also states the importance of an initial recirculation of the filtrate for at least 10-15 minutes to allow a gel layer to form for minimal plasmid loss to the filtrate.

Chromatography

While several existing chromatography methodologies (*e.g.* size exclusion, triple helix chromatography, HIC) can be combined in clinical production processes to dramatically reduce gDNA (<0.00008% gDNA in WO2005100542 [102]), a general limitation of such chromatography methods is low binding capacity for plasmid DNA. The resins were originally developed and optimized for binding much smaller molecules like proteins, and low binding limits application to large scale plasmid manufacture. New ion exchange materials are being developed specifically for DNA applications, some of which can be used for plasmid purification (*e.g.* Convective Interaction Media®, Mustang™ Q membrane devices, see ion exchange chromatography section below). However these are not optimal for removal of gDNA.

Ion Exchange Chromatography

Anion exchange chromatography is particularly well suited to the capture of plasmid DNA due to the polyanionic structure of DNA. The positively charged groups on the anion exchange resins interact with the negatively charged phosphates of DNA. Selective elution with increasing ionic strength is used to remove impurities and recover the plasmid DNA. Anion exchange chromatography is capable of separating plasmid from proteins and small nucleic acid fragments. However, size resolution of larger DNA, including plasmid and chromosomal DNA, is difficult.

A wide variety of anion exchange resins are commercially available with functional groups generally classified as either strong or weak anion exchangers. Strong anion exchangers contain a strong base and therefore remain positively charged over a wide pH range. Quaternary ammonium (Q) is a common functional group for strong anion exchangers. A weak anion exchanger, such as diethylaminoethyl (DEAE), contains a weak base and is deprotonated at high pH, causing it to lose its positive charge.

Numerous detailed discussions on the theory and application of anion exchange chromatography to plasmid purification can be found in the scientific literature [135-141].

Binding of DNA to anion exchange materials is favored by optimal interaction of the DNA with the surface of the anion exchange material. Because of the relatively large size of plasmid DNA, diffusion is slow and binding occurs only on the surface when using traditional resins. This limits throughput and binding capacity. Thus, much of the patent literature focusing on anion exchange for plasmid purification describes improvements in binding capacities and throughput of anion exchange chromatography.

US7026468 [142] describes improved anion exchange binding capacities, relative to conventional anion exchange resins, with tentacle anion exchange resins (*e.g.* 1.5 mg/ml using Fractogel® EMD TMAE).

US5707812 [143] describes the addition of a compaction agent, specifically PEG-8000 at a concentration of 1%, to a plasmid DNA solution before anion exchange chromatography. In an example, the plasmid recovery from anion exchange chromatography increased from about 20% to about 80% when PEG-8000 was used. The use of compaction agents in enhancing anion exchange chromatography is also discussed in [144].

Monolithic chromatographic supports are described in [145-150]. A monolith is a bed consisting of a single piece of highly porous solid material. The large size of the convective pores of the surface allows maximum interaction of the plasmid DNA with the anion exchange surface. Reported advantages of plasmid purification with anion exchange monolithic columns include increased mass transfer properties, high binding capacities, and low void volumes. Boehringer Ingelheim and BIA separations collaborated to develop and patent the use of monolithic support chromatography (Convective Interaction Media®, CIM®) for plasmid purification in WO03051483 [151].

Anion exchange membranes, such as the Pall Mustang™ Q membrane devices, also offer improved binding capacities and flowrates. Like monolithic supports, the membranes have large convective pores with an anion exchange surface, allowing maximum interaction with plasmid and high throughput. One study reports a 20-25 times greater dynamic binding capacity and a 55-550 times greater flow rate when compared to anion exchange beads [152]. Other studies describing plasmid purification with anion exchange membranes have been published [153-157]. Plasmid purification using anion exchange membranes is disclosed in patent applications WO0194573 [158] and WO2004108260 [81]. WO2004108260 additionally discloses the use of a second charged membrane to selectively bind endotoxins.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) exploits the difference in surface hydrophobicity of supercoiled plasmid from RNA, endotoxin, single stranded DNA, relaxed double stranded DNA and proteins. HIC works by salt promoted binding; thus it can be more conveniently used after anion exchange chromatography, or when the plasmid pool has a high salt content.

Ammonium sulfate is most commonly used. Increasing ammonium sulfate concentrations increase hydrophobic interactions between hydrophobic regions of solutes and hydrophobic functional groups on the resin (*e.g.* phenyl, octyl). Removal of RNA, single stranded DNA, and endotoxin is achieved by an ammonium sulfate concentration that promotes binding of the nucleic acid bases and the lipid A group of endotoxins to the resin. The bases of double stranded DNA are protected from binding and the plasmid elutes in the flow through. At even higher ammonium sulfate concentrations the more exposed bases of supercoiled plasmid cause it to bind, leaving double stranded chromosomal DNA and open circle plasmid to elute in the flow through. However, the plasmid binding capacities of HIC resins are low, so it might be most efficiently used as a polishing step in the plasmid flow through mode. See [105,141,159-161] for more detailed reviews of plasmid purification with HIC. The application of HIC for plasmid purification is the main subject of patents US6953686 [162]/WO0073318 [163] and US7169917 [164]/WO0204027 [165].

Immobilized Metal Chelate Affinity Chromatography (IMAC)

IMAC has been utilized to selectively remove nucleic acid impurities from plasmid DNA. Specifically, Cu(II)- iminodiacetic acid (IDA) resins (IDA chelating sepharose resins are commercially available from GE Healthcare) bind plasmid and gDNA with low binding affinity, while single stranded RNA and DNA are strongly bound [166,167]. Incorporation of a heat denaturation step to generate single stranded ends on renatured gDNA allowed removal of spiked gDNA from a plasmid preparation to <0.00008% gDNA by weight [168].

Aqueous Two Phase Systems

Separation of nucleic acids and other biomolecules by aqueous two phase systems (ATPS) was first described by Albertsson in 1962 [169]. More recently, the use of ATPS specifically for plasmid purification has been described using polymer-salt systems (*e.g.* PEG/potassium phosphate) [170-174] and polymer-polymer systems (*e.g.* thermoseparating EO-PO/Dextran) [175,176]. Plasmid DNA can be made to partition to one phase or the other by adjustment of the system properties (*e.g.* polymer MW, ionic strength) to separate the plasmid from impurities. ATPSs are most commonly used as a primary recovery/enrichment step after cell lysis.

WO2004020629 [177] discloses a process, also described in [175,176], for plasmid purification using a polymer-polymer ATPS in which the plasmid is first recovered in the top polymer phase. Upon heating of the isolated top phase, the polymer clouds out, leaving an enriched plasmid solution.

Table 5. Downstream Purification Key Patents

Patent Title	Patent No.	Summary
Purification of cellular components that are substantially RNA free	US6780632	Cobra Biomanufacturing Periplasmic RNase in host strain for RNA removal in alkaline lysis
Process for the scaleable purification of plasmid DNA	US6797476 WO0146215	Merck STET/lysozyme heat lysis, Selective precipitation (CTAB), Selective impurity adsorption (LRA)
Upstream and a downstream purification process for large scale production of plasmid DNA	WO2006083721	Merck STET/lysozyme lysis, Alkaline pH shift, Selective precipitation (PEG)
Process for purification of plasmid DNA	WO2004060277	Vical Lysis, TFF, Selective precipitation (PEG), AEX, HIC
Production of pharmaceutical-grade plasmid DNA	US5561064	Vical Size exclusion or anion exchange chromatography
Purification of plasmid DNA during column chromatography	US5707812	Vical Diatomaceous earth clarification, PEG precipitation prior to column chromatography
Method for RNase- and organic solvent-free plasmid DNA purification using tangential flow filtration	WO0107599	Genentech TFF on clarified lysate
Methods for purifying nucleic acids	US6011148	Megabios/Valentis Ultrafiltration
Recovery of plasmids in an aqueous two-phase system	WO2004020629	Amersham/GE Healthcare Thermoseparating ATPS with chromatography
Method for obtaining plasmid-DNA by means of an aqueous biphasic system	US2006286080	Qiagen PEG/salt ATPS
Isolation of DNA molecules	US6916919	Amersham/GE Healthcare S-aryl ligands for plasmid purification
Method for purifying plasmid DNA	WO2005100542	Gencell/Centelion Alkaline lysis, AEX, triple helix, HIC chromatography process with gDNA <0.00008%
Method and device for isolating and purifying a polynucleotide of interest on a manufacturing scale	WO03051483	Boehringer Ingelheim-BIA separations Combination of 2 chromatography steps with different principles wherein at least one is a monolithic column
Purification of plasmid DNA by hydrophobic interaction chromatography	US7169917 WO0204027	Pre-purification steps linked to HIC Removal of specified impurities by HIC
Methods of DNA purification and purified DNA	US6953686 WO0073318	Cambrex Bioscience HIC with salt. In flow through mode or binding mode removes impurities
Process and equipment for plasmid purification	US2006106208 US7026468	Valentis HIC in variety of separation modes Valentis Tentacle anion exchange resin and/or HIC
Processing of plasmid-containing fluids	WO0194573	Pall Variety of process combinations using membranes
Nucleic acid separation using immobilized metal affinity chromatography	WO0246398	Plasmid and gDNA low binding affinity, resin removes single stranded RNA and DNA

The process of US2006286080 [178], also described in [172], uses a PEG-salt ATPS to recover and purify plasmid directly from an unclarified alkaline lysate. After isolation of the plasmid phase, the ATPS may be repeated for further purification.

Purification Summary

Chromatographic and nonchromatographic large scale processes have been developed and patented (Table 5). Modifications are needed to further reduce genomic DNA levels to that acceptable for commercial high dose plasmid products. While combinations of HIC, SEC, or triple helix chromatography that dramatically reduce gDNA can be used for clinical production, current resins are not contemplated for commercial scale use due to low binding capacity or expense. The new higher capacity ion exchange membranes are

not optimal for removal of gDNA. A great variety of alternative purification approaches (e.g. ATPS, affinity absorption) have been developed at the bench scale and patented; most of these have not yet been tested for application at large scale. Immobilized metal affinity chromatography may have utility as an absorption step to remove single stranded impurities.

CURRENT & FUTURE DEVELOPMENTS

Large scale plasmid purification processes have been recently developed by various companies, and are the subject of many issued and pending patents. For a company looking to develop a plasmid manufacturing process for clinical plasmid production, there are a number of excellent in-license or subcontract options available since a number of processes can be utilized to produce a highly pure plasmid.

Boehringer Ingelheim and Merck have combination process platforms that couple high yield fermentation, cell lysis, and purification patent portfolios. Various other companies have optimized individual components. An off-the-shelf robust purification process can be combined with high yield fermentation and cell lysis methods to create superior hybrid processes. For example, GE Healthcare has developed the PlasmidSelect Xtra downstream purification package, utilizing proprietary thiophilic aromatic chromatography media (S-aryl ligands for plasmid purification are claimed in US6916919 [179]) that are made available to customers [180]; this has been packaged with Nature Technology Corporation's high yield fermentation process [62]. Such a process delivers highly pure plasmid DNA that is ideal for clinical plasmid manufacturing needs.

A number of issues must be addressed before any existing process can be utilized for cost-effective commercial plasmid manufacture. While yields from current high yield fermentation processes are acceptable, cell lysis methodologies based on alkaline or heat lysis are a bottleneck and current cost-effective large scale downstream processes retain excessive gDNA in the final product for high dose human vaccines or therapeutics. Clearly, increasing the purity of the starting material and achieving better downstream purification are essential goals for manufacturing licensable plasmid DNA on an industrial scale.

Future critical developments include:

1. Development of alternative cell lysis methodologies. Currently available plasmid production processes are cost- and facility-limited by use of complex alkaline or heat lysis steps; extreme process volumes are required for alkaline lysis, while heat lysis with lysozyme requires tight control and represents 25% of the total processing costs [reviewed in 4]. In this context, it is recommended that emerging new cell lysis methods be closely monitored as replacements for the key bottleneck alkaline or heat lysis steps to create an optimal hybrid process.
2. Improved methodologies for removal of genomic DNA. gDNA is the key contaminant that must continue to be reduced prior to commercialization of high dose (e.g. 1 mg) plasmid DNA vaccines or therapeutics.

CONFLICT OF INTEREST STATEMENT

AEC and JAW have an equity interest in Nature Technology Corporation.

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