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# From Strain Selection to Purification – Key Factors for Successful Plasmid Production

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## Executive Summary

Plasmids serve as important tool in genetic engineering, gene therapy research or genetic vaccination. Recombinant plasmid DNA (pDNA) is produced in bacterial cultures, mostly in *Escherichia coli* (*E. coli*). Successful pDNA production, reaching high yield and quality, relies on multiple factors, including the type of vector and host strain, the insert, and the methodology chosen for cultivation and downstream purification. Here, we review the distinct steps in the plasmid production workflow and give tips on optimization: from vector and strain selection to cultivation, from shake flasks to fermenters up to downstream processing steps of harvesting and purification.

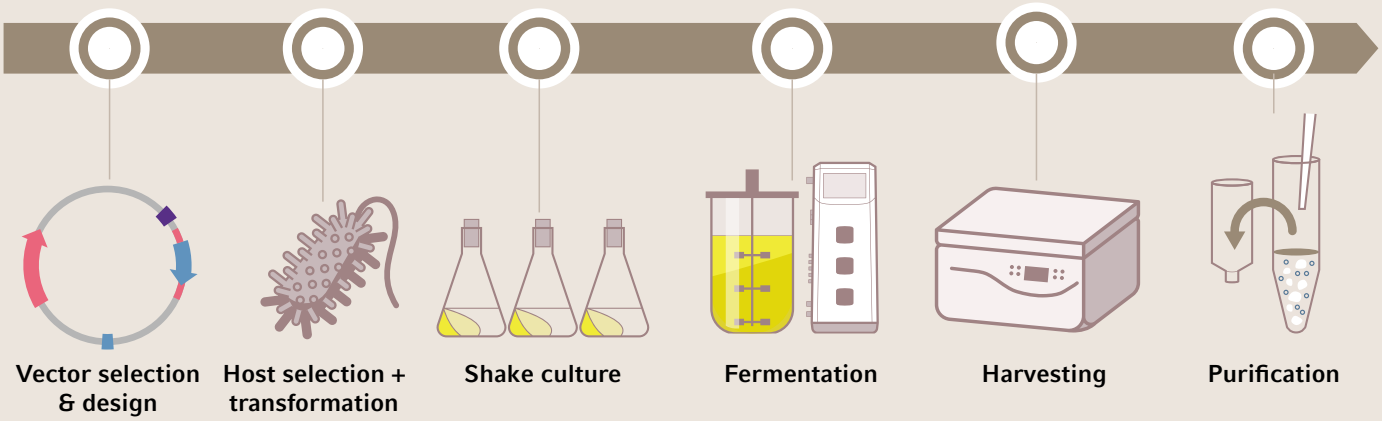


**Figure 1: Plasmid production workflow**

## Introduction

Plasmids serve as a tool in genetic engineering, either to clone and amplify DNA fragments, such as genes, express recombinant proteins or to serve as templates e.g. for *in vitro* mRNA production or transient CRISPR expression in gene therapy research and genetic vaccination [1,2]. Plasmid DNA (pDNA) can be easily genetically manipulated and produced in large amounts in bacteria, typically in *E. coli*. Furthermore, a variety of ready-to-use solutions allows easy subsequent downstream purification.

Depending on the application, pDNA production ranks from research laboratory scale (microgram range) to large industrial scale (milligram to gram range). The plasmid production includes multiple steps (Figure 1). In the following, we review the factors affecting plasmid production and give tips on optimization.

The production of pDNA can be separated in five parts:

## 1) Plasmid vector selection

Since the availability of the first commercial cloning plasmid vectors in the 1970s, the number of available products has increased substantially. Plasmids are small circular double stranded DNA molecules. The typical size ranks from thousand up to a few thousand base pairs. Minimal required components of plasmids used in the lab include the origin of replication (ori), which enables the independent replication from the host's chromosome, the multiple cloning site (MCS) or polylinker region with the restriction digestion sites, that allows to insert the gene of interest, and a selection marker to ensure that only bacteria containing the plasmid survive in culture. In expression plasmids, an additional promoter is inserted upstream to the multiple cloning site, which drives the transcription of the gene (Figure 2).

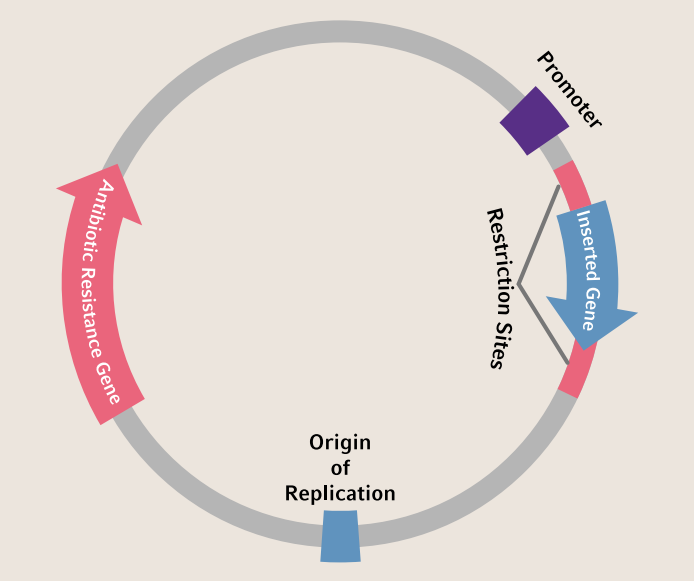


**Figure 2: Minimal components of a plasmid vector.** Important components include the origin of replication (ori), the multiple cloning site with the restriction sites (here shown with the inserted gene of interest) and the selection marker (here an antibiotic resistance gene). In expression plasmids an additional promoter is inserted upstream the multiple cloning site.

The following parameters are helpful for choosing the right plasmid for your needs.

### Replicon and copy number

One important factor affecting the yield of plasmids from a given system is the copy number, meaning the expected number of plasmids per host cell. A plasmid's copy number is determined primarily by the ori and the surrounding regulatory DNA sequences. This area, known as the replicon, controls the replication of plasmid DNA.

Plasmids can be grouped into low and high-copy plasmids (Figure 3). For example, plasmids derived from the plasmid pBR322 contain the ColE1 origin of replication from pMB1. This origin of replication is tightly controlled, resulting in only ~25 copies of the plasmid per bacterial cell (low copy number). On the other hand, pUC-derived plasmids contain a mutated version of the ColE1 origin of replication, which results in reduced replication control and ~200–700 plasmid copies per cell (high copy number).

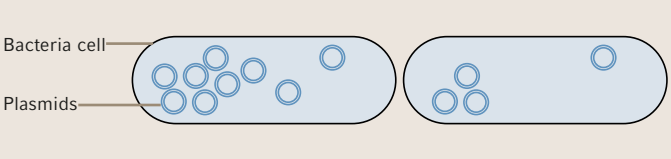


**Figure 3: High and low copy plasmids.** High copy plasmids (left) result in increased plasmid copies per cell, compared to low copy plasmid (right).

In most cases, a high-copy plasmid produces greater yields of plasmid DNA and is the preferred choice for gene cloning. Another advantage of the high copy number is higher plasmid stability when random segregation occurs during cell division [3]. However, high-copy plasmids pose a high metabolic burden on the host cell. That may lead to poor culture growth and can favor plasmid instability. Especially for ambitious cloning tasks, like amplifying plasmids with large inserts >8 kb or adenine and thymine rich ('AT-rich') sequences, a low copy plasmid may be considered to increase stability [4].

**Excursus I: Considerations for protein expression plasmids**

For most protein expression applications, high-copy plasmids are well suited to produce high amounts of protein. However, when high-level expression results in protein aggregation, a slowed-down synthesis rate can have a positive impact on yields and a low copy plasmid may be considered [5]. Other measures, such as introducing additional backbone features into a protein-expression plasmid (for example protein-solubility increasing fusion tags), using weaker promotors, or co-expressing multiple protein components with a compatible dual expression plasmid system, are strategies to further improve solubility [6].

**Insert size**
Most commonly available plasmids like pUC and pBlueScript can take inserts of up to around 15 kb [6]. However, larger fragments can lead to decreased plasmid copy numbers or problems in plasmid DNA replication and stability [7]. There are special types of high-capacity vectors such as PACs, BACs, cosmids, which are suitable for cloning large DNA fragments [8].

**Selection marker**
Almost all plasmids have a selection marker, usually an antibiotic resistance gene, that ensures that only the cells that have incorporated the plasmid vector can survive in the presence of the specific antibiotic. Depending on the selection marker, the referring antibiotic must be added to the solid or liquid medium to keep the selection pressure and plasmid stability. To avoid degradation, antibiotics should be added to the medium after autoclaving at temperatures lower than 60 °C and always freshly prior use. For some therapeutic plasmid-based products, the presence of antibiotics is restricted or not recommended by regulatory authorities. Kanamycin is the most widely used and preferred selection agent for DNA vaccines, as it does not present a significant allergic risk [9]. Engineering more stable vectors and alternative selection strategies to antibiotics have been investigated [10].

## 2) Host strain selection and transformation

The choice of the *E. coli* host strain can have a significant impact on the quantity and quality of the produced pDNA. *E. coli* K-12 strains such as DH5α, JM109 and the slower growing XL1-Blue are common host strains for gene cloning applications generating high plasmid yields. They contain several gene mutations, including one in the endonuclease A (*endA*) gene to prevent plasmid degradation and one in the recombinase A (*recA*) gene to avoid homologous recombination. Constant genetic engineering efforts, such as metabolic engineering strategies targeting individual bacterial enzymes relevant to the cell's physiology, have been explored with the aim of increasing plasmid production [11,12].

**Excursus II: Host strain selection for protein expression**
Amongst protein expression strains *E. coli* B-strains, such as BL21 (DE3), are often chosen for high-level production of recombinant proteins. They are deficient in genes encoding certain proteases that can degrade proteins during purification. Several strain modifications for difficult protein expression have been engineered, such as BL21(DE3)-RIL for expression of rare codon-containing genes and BL21-AI for toxic proteins.

Two procedures are primarily used to transfer plasmids into bacterial cells: chemical transformation and electroporation. Chemical transformation or heat shock is cost-effective and well established for *E. coli* but is a more time-consuming and labor-intensive method compared to electroporation. Electroporation requires investment in a specific electroporation device and cuvettes but has been shown to increase transformation efficiency [13] and to be beneficial for larger insert sizes [14]. The transformation is followed by clonal selection on agar plates containing selection markers, such as antibiotics. Transformation efficiency can be calculated as the number of colony-forming units (CFU) per microgram of plasmid DNA used.

## 3) Production in shake flasks and fermenters

**Cultivation technique – choosing a shake flask or fermenter**
The growth environment plays a crucial role in pDNA production. Productivity is usually proportional to the final cell density and the specific productivity (amount of pDNA per unit cell mass). The pDNA yield is commonly indicated in milligrams of pDNA per liter of culture (mg/L). Typically, 0.5–1 g pDNA per kg of wet weight biomass is obtained from using high copy plasmids [15].

Besides existing lab resources, the application and yield demands determine the cultivation method of choice. Tube cultures and small shake flasks are suitable for gene cloning and laboratory scale protein production, where working volumes between 1 mL to 1 L per vessel and yields in the microgram range are usually sufficient. If higher plasmid yields are required, for example for transfection purposes, large-scale recombinant protein production, or if the plasmid is the final product itself in genetic vaccination or gene therapy applications, a controlled fermenter is usually the device of choice, being able to handle higher working volumes.

**Knowing the bacteria´s growth behavior**
Growth curve measurements based on optical density ($OD_{600}$) provide a simple tool to analyze the bacterial growth behavior, for example to compare different strains, analyze the influence of media composition, or determine the optimal point of harvesting. In a typical growth curve, one can differentiate four distinct phases of growth: the lag, exponential (log), stationary, and death phase (Figure 4).

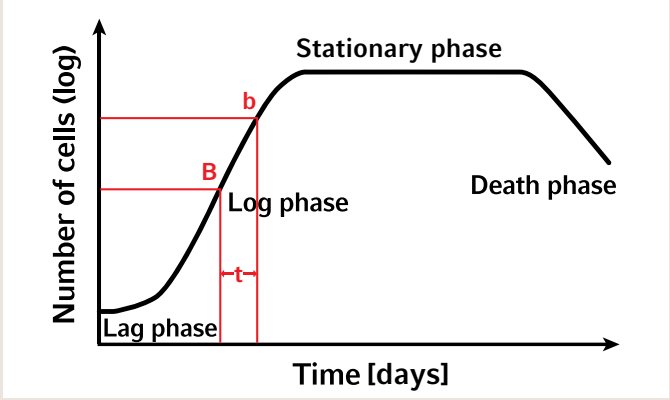


**Figure 4: Typical bacteria growth curve (batch culture).** In the initial lag phase, the cells are in a metabolically active state, but there is no or only little cell division. The cells accommodate to the new conditions. The subsequent logarithmic (log) phase is the phase of optimal growth with cell numbers increasing in a logarithmic fashion. In the stationary phase the cell growth rate slows upon nutrients depletion or toxic product accumulation and the rate of cell division and death is roughly equal. In the death phase the cell number decreases. **In red:** The generation or doubling time G of the cells is calculated during the exponential phase of growth. B is the number of bacteria present at the start of the observation, b is the number present after the time t.

From the growth curve, one can calculate the generation (=doubling) time (G) in minutes or hours using the following formula:

$$G = \frac{t}{n} = \frac{t}{3.3 \log b / B}$$

In the formula, B is the number of bacteria present at the start of the observation, b is the number present after the time t, and n is the number of generations. The generation time is calculated during the exponential growth phase (linear growth on a log-scale plot) (Figure 4).

### Inoculation strategies

The most frequently recommended method to inoculate a small liquid culture from a frozen stock, is to start from a single colony grown on a freshly streaked selective agar plate [16,17,18]. This ensures that the culture derives from one clone. However, a recent study comparing direct inoculation from a glycerol stock versus inoculation from a single colony did not identify significant differences between the two methods in terms of growth behavior and final plasmid yields [19].

Shake cultures and fermenter cultures are often inoculated from a liquid pre-culture to reduce the lag-phase (Figure 5). The pre-culture should be harvested within the logarithmic phase. Inoculation with a pre-culture does not only shorten the lag phase, but also allows a better synchronization of cultures by adjusting to a defined start $OD_{600}$. This is helpful when comparing strains. A typical starting $OD_{600}$ is around 0.1 for shake flasks (often higher for fermenters). However, the optimal inoculation density is dependent on the growth characteristics of the microorganism and the given growth environment and can be evaluated by performing initial growth curve comparisons. Unlike mammalian cell cultures, long, cost-intensive seed trains are not required, and, in most cases, a one-stage inoculum train is sufficient also in industrial settings [20].

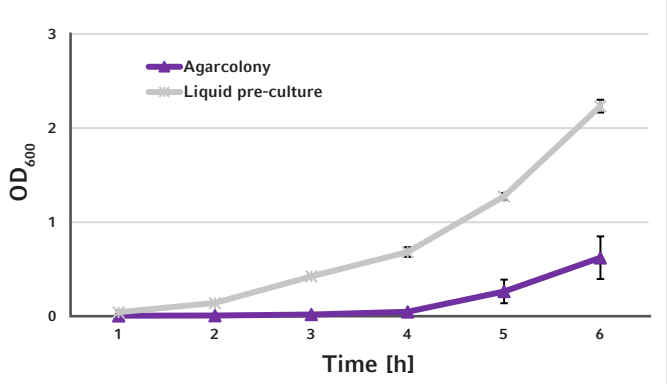


**Figure 5: Influence of inoculation method on bacteria growth.** Inoculation of 250 mL Erlenmeyer flasks filled with 20 % LB medium with either a single colony from an agar plate or from a liquid pre-culture, starting $OD_{600}$ ~0.04. (*E. coli* DH5α, incubation at 37 °C and 250 rpm)

### The optimal harvesting timepoint

The harvesting time point affects both the yield and quality of plasmid DNA. Bacterial cultures grown to insufficient density will yield reduced amounts of pDNA. Overgrown cultures may result in suboptimal yields and excessive chromosomal DNA contamination due to autolysis of bacterial cells after they have reached the stationary phase. Harvesting for plasmid preparation is usually recommended in the late logarithmic or early stationary phase [21]. By this time, the culture possesses high biomass, hence plasmid yield, but not too many dead cells. Growth times for cultures with high copy plasmids under standard conditions are typically between 12-16 hours, and for cultures with low copy plasmids ≥ 20 hours [18]. Due to the higher metabolic burden posed on the bacterial cell by high copy plasmids, the achievable maximum cell density of these cultures is usually lower compared to low copy plasmids, resulting in a shorter incubation period. The optimal cultivation time may vary for several reasons, such as the utilized strain, plasmid, temperature, agitation speed, flask type, media composition, and cultivation mode and should be evaluated based on growth curve analysis and sampling at various time points with subsequent plasmid preparations.

## Optimizing tube culture

For small-scale approaches culturing is typically carried out in 15 mL conical tubes. However, due to their small diameter, handling can be difficult. They are also not optimal for efficient aeration due to the small surface-to-volume ratio. A 25 mL conical tube such as the one provided by Eppendorf has the diameter of a 50 mL tube offering the advantage of a larger surface for oxygen transfer but is half the size of a standard 50 mL tube, thus better accessible with a pipette. A comparison between the 25 mL Eppendorf conical tube and a standard 15 mL tube, using the same inoculum amounts of *E. coli*, identified enhanced cell numbers and plasmid DNA yields in the 25 mL tube [22] (Figure 6). This indicates improved mixing and aeration due to the different shapes. As a rule of thumb, fill volumes in tubes should not exceed 20 % to facilitate adequate mixing. In addition, an increased liquid surface for oxygen supply can be achieved by cultivating the tube at an angle such as 30-45 °, especially when working at standard shaking speeds of around 200 rpm [23].

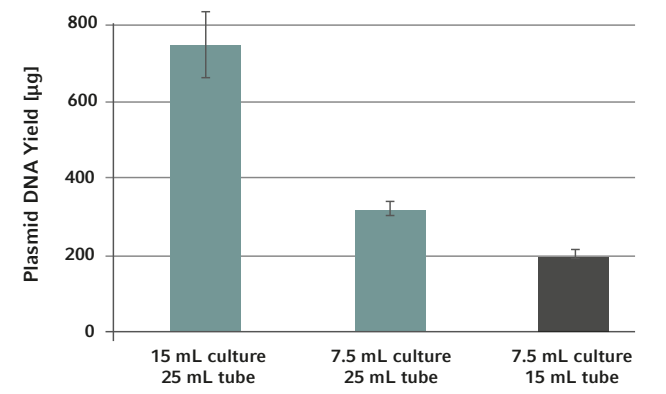


**Figure 6: Influence of tube design and size on plasmid yields.** Total yield of low copy plasmid DNA (PBR322) purified from bacterial cultures *E. coli* DH5α incubated in Eppendorf Conical Tubes 25 mL with snap cap and standard 15 mL conical tubes. Inoculated from one seed stock and grown under similar conditions.

## Optimizing shake flask culture

Shake flasks are the most used cultivation vessels for production of plasmids on laboratory scale. Also, early process development, such as initial strain and media selection, starts typically in shake flasks prior to further process development and expansion in fermenters. Shake flasks are inexpensive, easy to parallelize and simple to use. There are different parameters one can adjust in cultivation to increase biomass and product yields in a shake flask. Although *E. coli* is a facultative anaerobic microorganism, it grows best in the presence of oxygen [24]. In a shake flask, oxygen supply only occurs passively *via* the air-liquid interface. Oxygen limitation is one of the most frequent problems in shake flask culture.

Different shake flask designs are available to increase the oxygen transfer to the culture (Figure 7).

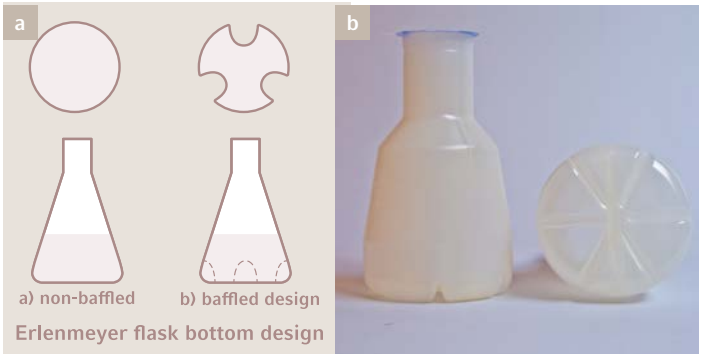


**Figure 7: DIfferent shake flasks**
**A)** Classic non-baffled and baffled Erlenmeyer flask designs. Baffled flasks have defined cavities in the bottom area **B)** Ultra Yield® flasks. Flasks with steep vertical walls and six baffles at the flask bottom in 2.5 L size (Thomson).

Baffles for example, disrupt the regular swirling liquid flow by the implemented cavities in the bottom area, thus improving the aeration of the culture [25]. Comparisons of different flask designs have shown that baffles can improve cell growth and maximum cell density drastically compared to unbaffled flasks (Figure 8).

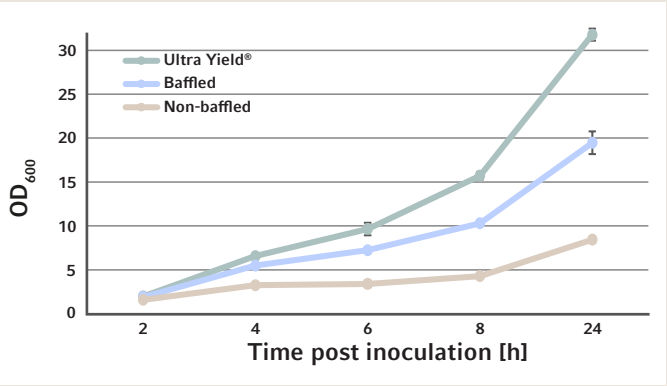


**Figure 8: Influence of flask design on bacterial growth.** Ultra Yield® flasks with the specialized baffled design resulted in the fastest growth and maximum cell density, followed by the standard baffled design and finally the non-baffled design (*E. coli* DH5α with pUC19 plasmid cultivated in modified TB media in 500 mL flasks with 25 % fill volume, 37 °C and 200 rpm).

The disadvantage of this design is a higher risk of foam formation that may lead to cross contamination by wetting the flask closure. However, this problem can be solved by using an antifoam agent. Unreproducible results caused by variances in baffle geometry and size have been described for glass flasks [26]. However, this has not been seen in disposable flasks [27], assuming these effects may be dependent on the manufacturing process in general.

Typical agitation speeds for flask cultures are 200-250 rpm. An increase in agitation speed supports the oxygen transfer to the liquid and increases both biomass and plasmid yields independently of flask size [28]. An increase from 250 to 350 or 400 rpm resulted in up to almost 2-fold higher biomass [data shown in original app note 449] and a ~30-90 % increase in pDNA yields in 500 mL and 2.5 L Ultra Yield® flasks, respectively [Figure 9A and B]. However, care must be taken, especially when using high agitation speeds with baffled flasks, to prevent cap wetting.

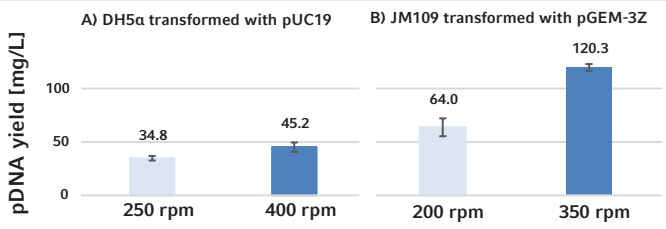


**Figure 9: Influence of agitation speed on pDNA yields.** An incubation at higher agitation speed resulted in higher pDNA yields for both *E. coli* strains.
**A:** Cultivation of *E. coli* DH5α transfected with pUC19 plasmid in 2.5 L Ultra Yield® flasks (20 % fill, 37 °C, modified TB media, harvest 8 h post inoculation).
**B:** *E. coli* JM109 with pGEM®-3Z plasmid in 500 mL Ultra Yield® flasks (25 % fill, 37 °C, modified TB media, harvest 8 h post inoculation).

As a rule of thumb, fill volumes in a shake flask should not exceed 20 % for microbial applications. In case of problems with oxygen supply, one can further reduce the fill volume to 10 or even 5 %. Some baffled designs, such as Thomson Ultra Yield®, may allow also higher fill volumes under certain conditions [28]. Again, when using higher fill volumes in combination with agitation and/or baffles, care must be taken to avoid cap wetting.

Flask size can also influence pDNA yields. A smaller flask will give higher maximum oxygen transfer rates compared to larger flasks (when similar conditions, same fill and agitation speed are applied) due to the relative volumetric surface area increase. Oxygen transfer rate (OTR) measurements with 2.5 L and 250 mL Ultra Yield flasks have been described [27]. Hence, when scaling up from small to large flasks, conditions may need optimization, such as agitation speed or fill volume to get similar yields per L.

### Influence of temperature

The standard cultivation temperature for *E. coli* is 37 °C. However, for some plasmids the copy number can be increased by a temperature up-shift to 42 °C. Temperature sensitivity has been described for high copy number pUC plasmids and explained with the existence of certain mutations [29]. This strategy of induced amplification is used primarily in fermenters. Typically, fermentation starts with a lower temperature at 30°C, at which the plasmid is maintained stably at low levels while biomass is accumulating. When cell density has reached a certain level, the plasmid amplification is induced by shifting the temperature to 42°C and by continued exponential nutrient feeding for a defined time [29]. It has also been reported that plasmid yield and quality can be improved by holding fermentation cells at a reduced temperature post the 42°C induction phase [30].

**Excursus III: Influence of temperature on protein expression**

A cultivation at reduced temperatures may reduce the problem of protein misfolding and aggregation and can be one factor to optimize when working with difficult to express proteins.

### Influence of media composition

The media should support cell growth as well as plasmid amplification and stability while minimizing other cell activities. Other than in mammalian culture, the media costs are relatively low due to the mostly simple formulations. One distinguishes minimal (defined) media and complex (semi-defined) media. In minimal media the exact chemical composition is known, whereas complex media contains components of unknown chemical composition and varying proportions, such as yeast extract or peptones for example. Minimal media allows for highly reproducible processes [31]. Complex media allows for high cell densities but may give more varying results and might interfere in downstream processing in industrial settings [32]. Formulations based on Lysogeny Broth (LB) or Terrific Broth (TB) are frequently used complex media.

Bacteria need as minimum set of nutrients to grow: water, a carbon source, a nitrogen source, and some mineral salts. However, typical bacterial complex components are protein hydrolysates like peptone or tryptone, yeast extract, carbohydrates, nitrogen, trace metals, and minerals.

Glucose is the most common carbon source added, as it is inexpensive and can be efficiently metabolized by *E. coli*, leading to good biomass and specific plasmid yields [33]. However, it might favor the undesirable accumulation of acidic by-products, such as acetate, due to metabolic overflows. Acetate accumulation is a major concern in aerobic fermentation known to inhibit cell growth and protein expression [34]. However, the effect of acetate on plasmid DNA production has not been investigated much. One study states that acetate formation can improve pDNA yields when present at low concentrations (3 g/L) [35]. Reduced growth rates have been linked to elevated copy numbers before [36]. Glycerol can be chosen as an alternative or complementary carbon source. It increases specific plasmid

productivity but may lower cell growth compared to Glucose [36]. The carbon sources of LB media are metabolizable amino acids in the form of tryptone and yeast extract with no additional carbon source added. Beside a higher amount of yeast extract and glycerol, TB media formulations contain an additional carbon source, which may increase pDNA yields compared to classic LB formulations (Figure 10) [27]. LB medium is excellent for routine molecular biology applications, but bacteria growth is limited due to the presence of the small amounts of utilizable carbon sources [37].

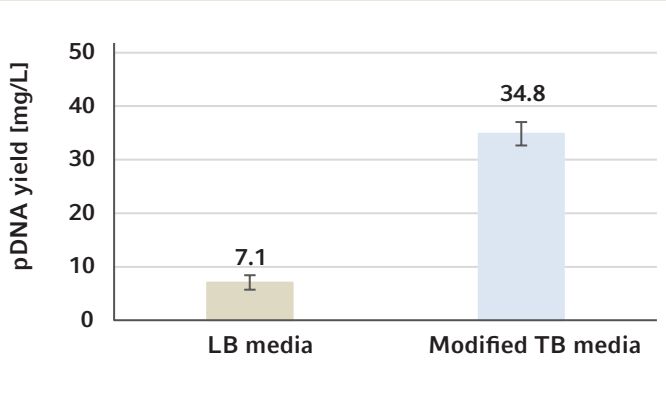


**Figure 10: Influence of media on pDNA yields.** The TB media formulation resulted in higher pDNA yields compared to LB media. Cultivation of *E. coli* DH5α with pUC19 plasmid in 2.5 L Ultra Yield® flasks in either LB or modified TB media (20 % fill, at 37 °C and 250 rpm).

Nitrogen is important to produce proteins and nucleic acids and can be either supplemented from organic sources, such as yeast extract or hydrolysates like tryptone/peptone or from inorganic supplemented nitrate.

Beside the availability of nutrients, the pH of the medium affects the culture. The optimal pH range for *E. coli* is between 6.5 and 7.5, depending on temperature [38]. During growth, the pH is drifting as consequence of production of substrates and metabolic compounds. A decrease of pH occurs for example by acetate accumulation due to metabolic overflow when cultivating with glucose as a carbon source. However, after glucose depletion consumption of acetate by the bacterial cells causes the pH to increase again [39]. Hence, pH control is important, especially when the medium contains additional carbon sources like glucose. Phosphates are commonly used buffer systems. Alternative buffer solutions are zwitterionic buffers like MOPS [40]. An overview of commonly used media with a short description of their properties can be found in online sources of suppliers [41]

There are also commercially available ready-to-use media optimized for plasmid production that support high-density growth and high plasmid yields (Table 2) [42].

**Table 2:** Plasmid+® Media yields higher plasmid concentrations over longer time periods compared to other complex media. Shown are the plasmid yields of Plasmid+ versus commonly used TB medium cultures in 2.5 L Ultra Yield flasks. Plasmid concentrations are given in ng/µL. Yield differences between Plasmid+ and TB medium cultures are given in percent. Experiments were done at 310 rpm and 37 °C (Source: Thomson)

| | Yield [ng/µL] | | | % increase vs TB | | |
|---|---|---|---|---|---|---|
| **Incubation time [h]** | **20 hr** | **22 hr** | **24 hr** | **20 hr** | **22 hr** | **24 hr** |
| Plasmid+, at 310 rpm | 94.9 | 103.3 | 142.6 | 190.2 | 274.3 | 354.1 |
| Terrific Broth, at 310 rpm | 32.7 | 27.6 | 31.4 | – | – | – |

Traceability and source documentation for media components, such as batch certificates of analysis, are especially important in regulated areas. The trend in the industry towards eliminating the use of any animal-derived components or even completely chemically defined medium to alleviate potential regulatory concerns has been described [43].

## Optimizing fermenter culture

A bioreactor or fermenter in microbial settings is a vessel that provides a biological growth environment for the (micro)organism of choice and is usually used together with a bioprocess control station and software to monitor, adjust and maintain this environment (Figure 11).


**Figure 11: Composition of a bioreactor.** Components of a stirred tank-bioreactor (left) and a connected control station (right).
**a:** bioprocess control software
**b:** motor
**c:** pump
**d:** head plate
**e:** sensor
**f:** bioreactor
**g:** bioprocess control station
**h:** impeller

The biggest advantage of bioreactors is a stable and adjustable growth environment due to precise control of various incubation parameters, such as temperature, agitation speed, gas supply and pH. Furthermore, it offers the possibility to scale up the bioprocess which enables reaching increased cell numbers and plasmid yields [44].

However, also bioreactors offer certain challenges that can influence process efficiency. This section aims towards overcoming them and giving insights into the most common considerations for the use of bioreactors. For this, we will look at the example of the stirred-tank bioreactor type which utilizes a motorized impeller to swirl the bacterial cell suspension to support gas exchange, nutrient distribution, and culture homogeneity (Figure 12).

The topics of this section include the method or mode of fermentation, considerations concerning culture conditions and equipment, as well as bioprocess scale-up.

**Fermentation mode**

When starting fermentation in a bioreactor, one has the choice between three commonly used fermentation modes, namely batch, fed-batch and continuous fermentation.

In batch fermentation, inoculation is carried out in a fixed volume without the addition, removal, or exchange of medium or feeding solution. In the process of nutrient consumption, the culture will undergo the typical cellular growth phases: first a lag phase in which the microorganisms adapt to the new environment, then an exponential phase marked by rapid cell division and cell density increase, followed by a stationary phase where growth is halted due to nutrient limitations and lastly a death phase in which the cells start to die [45]. Batch fermentation is easy to set up and bears a low contamination risk. However, the microorganisms experience a constantly changing environment due to the ongoing nutrient depletion and accumulation of potentially toxic by-products within the medium [45]. This might affect the growth of the culture resulting in lower cell density and ultimately decrease plasmid yields.

Therefore, fed-batch fermentation is an advancement of the batch mode to overcome its disadvantages. Here, nutrients are replenished during the incubation creating a more stable environment for the cells. Usually, the feeding is initiated during the mid-exponential growth phase ($OD_{600}$ of 8-12) to prevent nutrient deprivation [46]. Thus, fed-batch not only enables higher cell density and biomass production than batch fermentation, but it also allows for longer cultivation periods and limits by-product accumulation [45, 47]. Consequently, higher plasmid yields were observed for fed-batch over batch fermentation [48, 49]. However, the more complex structure of the fed-batch process also increases the risk of contamination [45]. Thus, care needs to be taken when preparing and adding the required feeding solutions.

The last fermentation method we look at, continuous fermentation, is the most complex operation mode. However, once it is ideally adjusted long cultivation of days, weeks and even months are possible. This main advantage is achieved by continuous removal of used medium and cells while fresh medium and nutrients are added simultaneously [45]. The resulting steady state ideally keeps the volume and nutrient concentrations constant while simultaneously removing toxic by-products, thus creating the most stable cell environment yet. Furthermore, the long run times reduce process costs and downtimes compared to batch and fed-batch, for example, due to fewer cleaning procedures [45, 50]. However, the greatest strength of continuous fermentation also causes a lot of its weaknesses. Long-term incubation makes maintaining sterile and stable growth conditions more challenging. As a result, product yields are usually lower than for batch and fed-batch fermentation which increases downstream costs [50].

Therefore, if long-term production of plasmids in a bioreactor is not your main concern, fed-batch fermentation is recommended as the best synthesis in terms of productivity, downtime, and contamination risk. Table 3 summarizes the advantages and disadvantages of each fermentation mode.

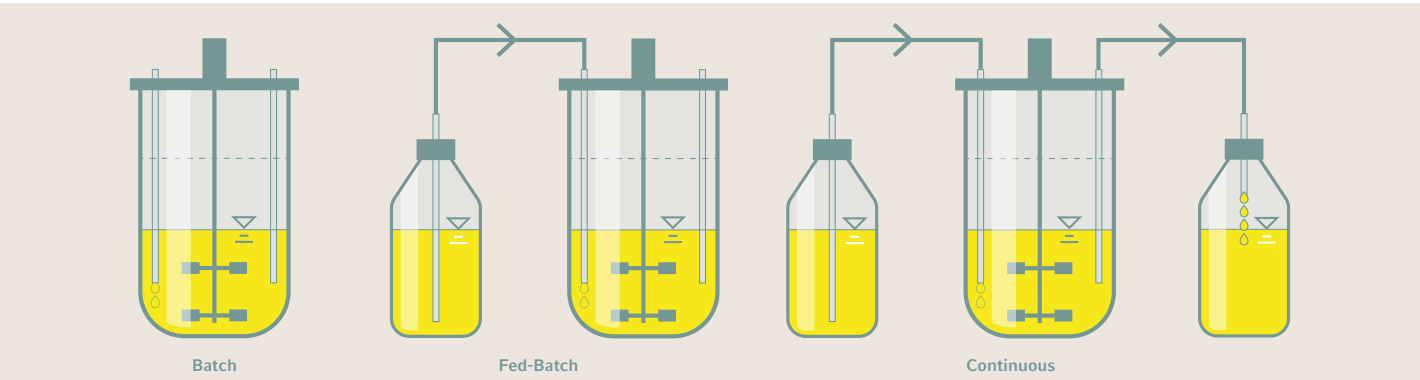


**Figure 12: Bioreactor operation modes.** Batch cultivation carried out in a fixed volume, fed-batch with medium and nutrient addition after the initial lag-phase until the end of the process, and continuous cultivation with constant medium/nutrient replenishment to ensure a stable growth environment.

Table 3: Pros and cons of common fermentation modes

| MODE | PROS | CONS |
|---|---|---|
| **Batch** | > Ease of use<br>> Low contamination risk | > Constantly changing environment<br>> By-product accumulation<br>> Low cell density<br>> High downtime for cleaning due to short run-time |
| **Fed-batch** | > Longer cultivation time compared to fermentation and thus less downtime<br>> More stable environment than batch fermentation due to feeding<br>> Higher cell density possible compared to batch fermentation<br>> Consequently, higher plasmid yield than batch fermentation<br>> By-product accumulation limited | > More complex structure than batch fermentation<br>> Higher contamination risk than batch fermentation due to feeding |
| **Continuous** | > Longest possible cultivation period (weeks to months)<br>> Ideally a constant environment<br>> Growth rate constant<br>> Possibility to remove by-products and prevent accumulation in the culture<br>> Strongly reduced downtime | > Most complex process structure<br>> Highest contamination risk of all operation modes<br>> Compared to fed-batch lower productivity (cell density, plasmid yield) |

## Culture conditions and equipment

Once the fermentation mode is selected, the culture needs to be maintained. Many parameters impact the efficiency of cultivation and most of them can be precisely controlled in a bioreactor.

The first and foremost is the optimal growing temperature which is 37°C for most bacteria. As opposed to shake flasks that need to be transferred to an incubator, the temperature of a bioreactor can be set, controlled, and automatically adjusted in place [51]. This is achieved by temperature probes, as well as heating blankets, thermowells, water jackets, and cooling fingers which can provide heat to or remove heat from the medium.

Another factor important for bacterial growth and plasmid yield is the pH value. Of course, the optimal value varies from microorganism to microorganism. However, in most cases, *Escherichia coli* is used for plasmid production. Here, we will look exemplarily at *E. coli* DH5α, one of the most commonly used strains for plasmid production. Studies in tubes and shake flasks identified its optimal pH range for plasmid production to be between 6.7 and 7.5, depending on the plasmid and the medium type [52, 53]. However, it is worth mentioning that the pH changes over the course of nutrient consumption and growth. Studies showed that the pH usually drops over the course of incubation [53,54]. As pH shifts can impact the resulting plasmid yields, it is desirable to monitor and adjust the pH accordingly, something that is more difficult to achieve in shake flask cultures. Bioreactors on the other hand offer precise and continuous pH measurements through sensors with the option of automatic adjustments by adding either acid or base solutions to the medium [51].

Ensuring efficient mixing and aeration of the culture is also critical for the bioprocess. Therefore, agitation speed and the right impeller type need to be selected for optimal culture growth. Both parameters are usually a tradeoff between sufficient aeration, mixing, and prevention of shear stress on the cells. However, as most bacteria have a relatively low shear sensitivity, a commonly used Rushton or Rushton-like impeller (Figure 13) with high agitation speed can be recommended for bacterial fermentation [55].

Figure 13: Rushton-type impeller used for bacterial bioreactor cultures with low shear-sensitivity.

If the impeller is not sufficient to aerate the culture efficiently, additional gassing can help. Oxygenation is reached by addition of $O_2$, while anaerobic conditions can be achieved by gassing with nitrogen or other anaerobic gasses [51]. Gas supply can be provided by surface gassing as well as submerged dip or sparging tubes which directly transfer the gas into the medium, thereby increasing the surface for gas exchange by introducing bubbles [51].

However, introducing bubbles combined with agitation and the protein composition of the medium can facilitate foam formation [56]. Amongst others, foam formation in bioreactors is associated with reduced working volumes, loss of medium and cells, as well as increased contamination risk [57]. Thus, it is important to detect and control foam. Detection of foam is usually achieved by using a probe located in the bioreactor's top part. It measures the electric resistance, that is changing once the foam is reaching the probe [56]. It can also be used to release surface-active antifoam substances. However, these newly introduced components within the medium might change the microbe's metabolism, reduce oxygen transfer, or impact downstream processes and upscaling [56]. Therefore, testing the antifoam with the target cell and medium of choice beforehand or using mechanical antifoam solutions is recommendable. Furthermore, to reduce foam formation in the first place, agitation and gassing should be balanced with the culture's aeration needs.

Once the culture conditions are set, further steps to support and monitor optimal culture growth can be undertaken. One of them is shortening the lag-phase after inoculating the main culture. This is achieved by (i) the right growth phase indicated by the optical density at 600 nm ($OD_{600}$) within the pre-culture, (ii) the correct initial $OD_{600}$ in the main culture after inoculation with the pre-culture and (iii) the right volumetric amount of bacterial pre-culture within the main culture. According to the literature, *E. coli* inoculation pre-cultures from shake flasks are grown to $OD_{600}$ of 6-15 [58, 59]. As mentioned earlier, these values are within the range of exponential growth and ensure actively expanding bacteria [46]. When inoculating the main culture within the bioreactor, the pre-culture gets diluted to initial $OD_{600}$ between 0.04 and 0.4, as described for *E. coli* in various settings [58, 59, 60, 61, 62, 63]. At this point, it is also important to keep the inoculation volume relatively high. 0.1-10 % of the total working volume is described in the literature but the ideal ratio should be determined by testing [45, 46, 58, 64]. After preparing the main culture this way, regular $OD_{600}$ measurements should be carried out during cultivation, for example, to identify the right feeding or termination time points. Sample-taking for $OD_{600}$ measurement can be performed by special sampling valves within the bioreactor and even non-invasive options exist to reduce the contamination risk even more. These tips should enable an efficient start for bioreactor runs for small-scale and scale-up processes.

**Scale-up**

Scale-up is the process of increasing the fermentation size from a small scale consisting of several milliliters to several liters to an industrial production scale with thousands or even millions of liters [65]. In principle, the same parameters important for small-scale approaches also apply to large-scale, such as consistent temperature, pressure, dissolved oxygen set point, or feeding solution sterilization [64].

However, scaling up is not a one-step process of simply enlarging bioreactors and volumes. Many parameters are influenced simultaneously during scale-up which can result in inhomogeneous culture conditions.

Larger bioreactors with higher volumes lead to longer mixing times with the risk of impaired oxygen and substrate distribution. Additionally, the stronger hydraulic pressure can influence the oxygen transfer rate, a critical parameter for air-to-liquid oxygen delivery. Such $O_2$ and nutrient gradients can induce metabolic shifts within the cells, resulting in the accumulation of unwanted by-products, such as lactate or succinate which in turn affect the medium's pH value [66]. Furthermore, high-density microbial cultures can produce significant amounts of heat which combined with inefficient mixing can lead to zonal overheating and further stress on the cells [64,66].

Thus, instead of moving immediately from small volumes to the endpoint, intermediate steps are introduced before the production scale is reached (Figure 14). Small and bench scale with volumes up to about 10 L are translated into so-called pilot scales with 1-10% of the full production scale volume [65]. Once sufficient yields are achieved in the pilot scale, translation into the manufacturing scale can be initiated.

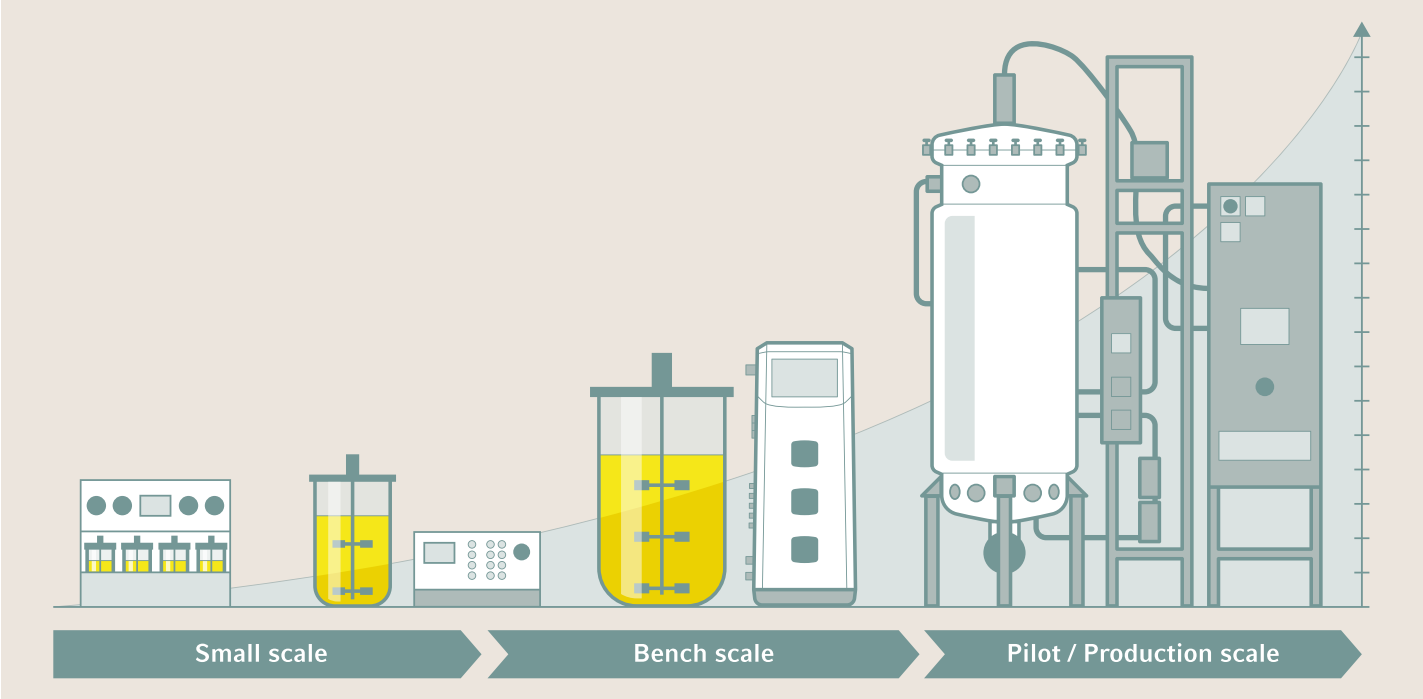


**Figure 14: Illustration of a scale-up process in bioreactors.** In the process of translating small (left) into production scale (right), intermediate steps, such as bench scale and pilot scale approaches, are introduced to ensure consistent bioprocess performance between the different bioreactor dimensions.

During this process it is crucial to ensure reproducibility between the different scales. To this end, different scale-up parameters were defined over time, all with the goal of overall consistent culture conditions for the different scales. Such parameters include constant agitation power input per volume, oxygen transfer rate and oxygen transfer coefficient (kLa), heat transfer rate per volume, mixing time, impeller tip speed, gas volumetric flow rate, and gas superficial velocity, to name a few [64, 67].

However, due to the bioreactor's physical properties it is not possible to keep all the parameters constant at the same time, as they can influence one another. Therefore, a selection of these parameters should be tested over all scale sizes. Out of the above, constant agitation power input per volume, constant oxygen transfer rate and constant heat transfer rate per volume are among the commonly used ones in large-scale microbial fermentation [64].

The agitation power consumption of an impeller is influenced by the agitation speed (rpm), the medium's viscosity ($kg/m^3$), the impeller's outer diameter (m), and a dimensionless power number [64]. The power or Newton number is experimentally determined for a given impeller by measuring its torque but can also be obtained from standard impeller references or vendors [64,67,68]. Once all these variables are obtained, the final power consumption per volume given in $W/m^3$ can be calculated by a specific mathematical formula [64, 68].

The oxygen transfer rate (OTR), given in mmol $O_2$/(L x h), is a measure of the gas-to-liquid transfer efficiency of oxygen during gassing. It is influenced by the agitation speed, airflow rate, pressure, and pure oxygen supply [64]. Just as the power consumption per volume, the OTR requires experimental determination as well. Different methods exist, for example the sulfite depletion method. Here, the oxygen-driven reaction of $Na_2SO_3$ to $Na_2SO_4$ is used to determine how fast oxygen is replenished after it was absorbed by sulfite within the medium [69]. With these results the oxygen transfer rate for a specific bioreactor can be calculated. Furthermore, oxygen transfer rates can be obtained from equipment manufacturers. However, as these values often represent the possible maximum capability of a bioreactor it should be considered to perform own experiments for a given setup [69].

As mentioned already, high-density cell cultures can produce large amounts of heat which make the heat transfer rate an important factor for keeping temperatures controllable. It is influenced by the area covered by the cooling device, as well as the culture's and cooling agent's temperature [64].

Besides parameters directly linked to the bioreactor, also other things need to be considered when planning a scale-up process. Deep freeze cell banks for storing backups of bacterial strains as well as the necessity of seed trains or bioreactor plant stress tests need to be considered [65, 64].

As shown by these examples, scale-up processes are complex and lengthy. Transitioning from lab to production scale

can take several years [65]. Nevertheless, it is important not to rush it. The scale-up should be planned out with the final goal in mind and all your actions should be positioned towards this goal. A detailed road map should be created beforehand, containing considerations like timelines, costs, unit operation design, energy-balance, risk assessment and mitigation plans [65]. Especially during this early stage of planning, input from experts with scale-up experience and inclusion of such expertise within your team are valuable [65, 64]. Once the foundation is laid, the initial ideas should be regularly adapted according to small, bench and pilot scale experimental results. Furthermore, the smaller scale approaches should always be designed in a way to represent the larger scales as closely as possible to make the larger scales as reproducible as possible [65].

In summary, there is no one-fits-all solution for upscaling. Many parameters need to be evaluated for your individual approach. However, this extra work will pay by receiving consistent yields from benchtop to production scale.

## 4) Culture harvesting

Small scale cultures from shake flasks or small fermenters are typically harvested by centrifugation. A benchtop multipurpose centrifuge with swing-bucket rotor is typically used for harvesting from conical tubes or conical bioreactors. A typical *E. coli* pelleting run is performed at around 5,000 g for ~10–20 min at room temperature or 4 °C. If higher g-forces are required to reduce the harvesting time or for harvesting larger volumes, fixed-angle rotors are the appropriate choice. The available purification system protocol usually gives a good guideline on the centrifugation settings. If the recommended centrifugation time or speed is exceeded, the cell pellet may be more difficult to resuspend. On the other hand, insufficient centrifugation time or speed may result in incomplete pelleting of cells and loss of starting material. After pelleting the cells and removal of the supernatant, the cells can be stored at -20°C for subsequent plasmid isolation.

Larger industrial-scale fermenter cultures are typically harvested by either centrifugation or by tangential flow filtration (TFF) [70]. Besides conventional centrifuges, other types such as continuous-feed centrifuges can be used for the harvesting step [71].

## 5) Purification

The process of DNA extraction and purification consists of five basic steps: 1) cell lysis, the disruption of the cellular structure to create a lysate 2) separation of the soluble DNA from cell debris and other insoluble material, 3) binding of desired the DNA to a purification matrix, 4) washing step to remove cellular contaminants from the matrix, 5) elution of the DNA.

The primary consideration for plasmid purification is separation of plasmid DNA from the host bacterium's chromosomal DNA and cellular RNA. Besides these components, other contaminants such as proteins, salts, and endotoxins are removed as well. Furthermore, the process aims towards eliminating undesired plasmid isoforms in the final product, as plasmids may exist in supercoiled, open circular and linear configuration, resulting from conformational changes within the bacterial host cell, or during the purification process [72]. However, the supercoiled structure is considered the native and active plasmid form and mostly the desired conformation.

To generate a cleared lysate with free plasmid DNA, several methods have been developed, including SDS-alkaline denaturation [73,74], salt-SDS precipitation [75], or rapid boiling [76]. SDS-alkaline denaturation is a popular procedure for purifying plasmid DNA because of its overall versatility and consistency. This technique exploits the difference in denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA fragments. Under alkaline conditions (pH 11.5-12.5) [77], both plasmid and chromosomal DNA are efficiently denatured. Rapid neutralization with a high-salt buffer, such as potassium acetate, in the presence of SDS has two effects that contribute to the overall effectiveness of the method. First, the rapid neutralization causes the chromosomal DNA to base-pair in an intrastrand manner, forming an insoluble aggregate that precipitates out of solution. The covalently closed nature of the circular plasmid DNA promotes interstrand rehybridization, allowing the plasmid to remain in solution. Second, the potassium salt of SDS is insoluble, so the proteins and detergent precipitate and aggregate, which assists in the entrapment of the high-molecular-weight chromosomal DNA.

Accurate execution of SDS-Alkaline lysis is critical for sufficient plasmid yields. First, the lysis pH of ~11.5-12.5 is close to the denaturation pH of pDNA. Thus, small pH deviations can favor the formation of undesired circular, single stranded plasmid DNA isoforms. Lysis time should be long enough for complete cell lysis but short enough to not denature pDNA. Second, aggressive mixing may lead to degrading of pDNA due to excessive shear forces, while insufficient mixing may lead to incomplete cell lysis. Also, ensuring complete neutralization of the solution during the neutralization step is critical. For research scale kit-based purification, adhere to the given times in the supplier's protocol.

The separation of soluble and insoluble material is accomplished by a clearing method (e.g. filtration, magnetic clearing, or centrifugation). The soluble plasmid DNA is ready to be further purified.

There are several methods available to purify plasmid DNA from the cleared lysate, including:

> binding plasmid to silica in the presence of high concentrations of chaotropic salts [78,79,80]
> differential precipitation of plasmid DNA from aqueous chaotropic salt/ethanol solutions [81,82,83]
> ion exchange chromatography over DEAE-modified cellulose membranes [84]
> precipitation with polyethylene glycol (85,86) organic extraction using phenol [87]

These methods can be adapted to various scales, from centrifugation-based minipreps to large-scale automated and semi-automated methods.

The volume of the bacterial culture should match the distinct isolation system. For research scales, spin column based mini, midi or maxi preps provide fast and easy purification usually together usually with a fixed-angle microcentrifuge. Typical volume ranges and yields from Promega [88] plasmid preparation kits are listed (Table 4). Some suppliers provide Mega and Giga preps, that allow purification from even larger volumes up to 5 L with yields up to 10 mg [16].

**Table 4: Typical volume ranges and yields with Promega plasmid purification kits for laboratory scale purifications** (Source: Promega). Please note that kits from other suppliers can vary.

| Culture size | Sample volume | Isolation system | Average pDNA yield high copy plasmid |
|---|---|---|---|
| **Small** | 0.6-3 mL | Miniprep | 1.5–15.0 µg |
| **Medium** | 50–100 mL | Midiprep | 100–200 µg |
| **Large** | 250 mL | Maxiprep | Up to 1 mg |

For low volume, high-throughput processing, isolation systems based on a 96-well format are available based on vacuum or magnetic beads.

Methods used at small scale are not readily scalable for large scale. For large-scale pDNA production, the steps after alkaline lysis are followed typically by different filtration techniques or centrifugation with a final chromatography to separate pDNA from residual impurities [72].

The biomass should be kept in a range that is acceptable for the plasmid isolation system used. Most research scale spin column based purification systems are optimized for use with LB media [88,89]. Using enriched media like TB that leads to higher biomasses per liter, that may cause an overloading of the purification system and insufficient lysis [16,88]. The culture volume per column should be reduced in these cases to match the recommended biomass. That also applies to extremely high copy plasmids and host strains that show high growth rates. Furthermore, the potential higher viscosity of the lysate will require more vigorous mixing, which may result in shearing of bacterial genomic DNA and contamination of the plasmid DNA [16].

For very high-sized plasmids (> 20,000 bps) a reduced efficiency in elution from the column is noted, the limitation in size should be checked in the manual of the used supplier [16,88].

pDNA concentration in a laboratory scale is typically determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer, for example using a quartz cuvette. Standard spectrometers allow measurement of the A260/230 and the A260/280 ratios to provide valuable information on the plasmid DNA's purity.

1) Determination of the purity ratios A260/A280 reveal nucleic acid sample quality and possible contamination with protein, phenols or other aromatic compounds. A ratio of 1.8-2.0 indicates sufficient pDNA quality [89]
2) The A260/A230 ratio reveals nucleic acid sample quality and possible contamination with organic compounds. This ratio should be > 2.0 [89].

To check the quality and presence of plasmid isoforms in the eluate, agarose gel electrophoresis can be performed to separate the plasmids according to their conformation.

## Appendix

Methods are intended for research applications. They are not intended, verified or validated for use in the diagnosis of disease or other human health conditions.

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