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Preparation, Separation,
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Application Note

Cell Harvest, Lysis, Neutralization & Clarification of Plasmid DNA

1. Recommendations

Plasmid DNA (pDNA) is typically produced via fermentation using a microbial source. Following *E. coli* fermentation, the primary downstream purification begins with harvesting of the cells, lysis, and clarification. During cell harvest, cells are concentrated, and the fermentation broth is removed via centrifugation or microfiltration tangential flow filtration (MF-TFF).

For MF-TFF, open-channel, flat-sheet TFF devices such as Prostak™ cassettes with Durapore® 0.1 or 0.2 µm microfiltration membranes or Pellicon® cassettes with Durapore® V screen or Biomax® 1,000 kD V screen ultrafiltration (UF) membranes are recommended (Table 1).

The harvested *E. coli* cells are then disrupted to release the plasmid DNA. Lysis is most often performed via an alkaline method. Alkaline lysis with 0.1–0.5 N NaOH with 0.1–0.2% SDS or Triton® X-100 is commonly used. Lysis time and mixing should be optimized.

Precipitation/flocculation is the first step to separate the supercoiled pDNA by selectively precipitating and removing impurities (high molecular weight RNA and genomic DNA, proteins and endotoxins) typically by use of 0.7–3 M potassium acetate with or without $CaCl_2$ (1.0–1.5%), pH range 5.0–7.5.

Lysate can be clarified using depth filtration, such as Clarisolve® filter or Millistak+® HC and Millistak+® HC Pro filters, to achieve high filtration capacity and yield. These filters are available in a wide range of formats with sizes from 0.014 m^2 to 1.1 m^2. Preclarification/pretreatment significantly affects the capacity of the depth filter and process development should therefore be carefully considered for optimization of the step. Yield from the clarification step is generally >90%. Table 2 summarizes the recommended filters for clarification.

Table 1. Recommended modules for harvest step.

Option	Harvest	Bioburden
Option 1	Prostak™ 0.1 or 0.2 or 0.45 µm filter	Millipore Express® SHC 0.5/0.2 µm filter
Option 2	Pellicon® cassette with Biomax® or Ultracel® 1,000 kD membrane, V screen	

Table 2. Recommended filters for the clarification step.

Option	Primary	Secondary (if needed)	Bioburden
Option 1	Clarisolve® 60 HX filter	Milligard® PES 1.2/0.45 µm filter	Millipore Express® SHC 0.5/0.2 µm filter
Option 2	Millistak+® HC D0HC filter		
Option 3	Millistak+® CE20 filter	Millistak+® CE50 or Polysep™ II 1.0/0.5 µm filter	

Note – Filter selection and capacity depend on whether feed is pretreated/untreated. Pre-treatments will have a significant impact on performance.

2. Overview

When harvesting pDNA, using MF-TFF and normal flow filtration (NFF) attributes, parameters, and considerations outlined in Table 3 are important.

Table 3. Overview of MF-TFF and NFF step.

Attributes	Parameters	Key Considerations
Filtration capacity	Filter selection (chemistry and pore sizing)	Viscosity of pDNA solution
Filtration flux	Filtration endpoint	Shear sensitivity of pDNA
pDNA yield	Driving force	High pH of lysis – near denaturization point of pDNA
Impurity reduction (gDNA, protein, and RNA)	Feed treatments	High solids content from fermenter
Bioburden protection and reduction	Mixing formulation (pH, conductivity, buffer components)	

2.1. Cell harvest

2.1.1. Attributes

Bacterial cells containing the plasmid of interest are typically harvested by either centrifugation or tangential flow filtration (TFF). Centrifugation is often more cost-effective for the harvest step when smaller batch volumes (<10 L) or larger batch volumes (>1,000 L) need to be processed.

2.1.2. Parameters

Bacterial cells containing the plasmid of interest are typically harvested based on OD600nm. Harvest OD600 depends on the type of media used in fermentation and the type of fermentation. OD-based harvest parameters are outlined in Table 4.

Table 4. Harvest OD values for different types of fermentation media.

Fermentation media	Harvest OD600
LB media	3–5
Super broth media	Up to 8
Super broth media with glycerol	25–35

(Reference: based on Input from Industry)

High cell density fermentation techniques for culturing *E. coli* have been developed to improve productivity and obtain high cell density.[2]

The goal of fermentation is to maximize cell density of dry cell weight at approximately 40–60 g/L and pDNA titers of approximately 1 g/L. It was possible to reach 2.2 g/L with use of optimized vectors and optimization of the fermentation process.

TFF devices used in a harvest step include MF membranes such as Durapore® (PVDF) 0.1 µm or 0.22 µm or 0.45 µm V screen (suspended screen) membranes and open grade UF such as Biomax® (PES) or Ultracel® (Regenerated Cellulose) 1,000 kDa V screen membranes. When using membrane cut-offs such as these, it is important to utilize a two-pump (permeate-controlled) TFF system.[3] The TFF harvest step typically involves a 2–5X volumetric concentration followed by a 3–5 volume diafiltration for washing out spent media components and extracellular impurities prior to further downstream purification. TFF harvest is typically operated at low transmembrane pressure (TMP; 3–5 psi) and ΔP (<7 psi) with a control on the permeate flux (Table 5).

Table 5. Operating parameters for MF-TFF.

Parameters	Value
Device	Durapore® 0.1 µm or Durapore® 0.22 µm or Durapore® 0.45 µm or Biomax® 1,000 kDa, V screen or Ultracel® 1,000 kDa, V screen membranes
Volumetric loading	10–60 L/m^2
Feed flow	7–9 L/min/m^2
TMP	<0.5 bar
Average flux	20–30 LMH
Volumetric concentration factor	2 to 5
Diafiltration volume	3 to 5

(Reference: Internal data)

E. coli cells could be harvested into a pellet by batch centrifugation using 4,500–6,000 g for ~15–20 min (at room temperature or ~4 ºC). Other types of centrifuges such as continuous-feed, intermittent-solids-discharge, disc-stack, batch-discharge or solid-bowl could also be used on the harvest step.

2.2. Cell lysis

2.2.1. Attributes

The methods used for cell disruption can be divided into two main categories – chemical (alkali, detergents, enzymes, osmotic shock) and physio mechanical (heat, shear, agitation, ultra-sonification, and freeze-thawing) lysis.

Alkaline lysis (NaOH at pH ~12) accompanied by detergents such as sodium dodecyl sulfate (SDS) and Triton® X-100 is the most common approach. The detergent solubilizes the cell walls and the alkaline environment denatures genomic DNA. It is important to optimize the lysis incubation time as it directly impacts the quality and quantity of plasmid DNA. Longer incubation time could lead to irreversible denaturation of plasmid DNA and shear degradation of genomic DNA. It is critical to have efficient but not too aggressive mixing employed on the alkaline lysis step to ensure there are no pH extremes causing irreversible denaturing of the plasmids or degrading it due to excessive shear.

A completely different method for cell lysis involves the use of newly developed autolytic *E. coli* strains. The pDNA is recovered by autolytic extraction under slightly acidic, low-salt buffer conditions and treatment with a low concentration of nonionic detergent. Genomic DNA remains associated with the insoluble cell debris and is removed by solid-liquid separation using a thermal flocculation followed by coarse filtration.[1]

2.2.2. Parameters

During the alkaline lysis method, cells are treated at specific, narrow range of pH (typically around pH 12) at which the genomic DNA will be irreversibly denatured, while the pDNA double chain remains intact (pH range of 12.0 to 12.5). The optimum pH value varies depending on the type of plasmid and host strain. A deviation of more than 0.1 pH unit from the optimum value may affect the yield and it is therefore critical to maintain a tight control of the pH range during alkaline lysis; at a pH >12.5, pDNA becomes irreversibly denatured and if the pH is too low, genomic DNA won't be completely denatured and could complicate further downstream purification process.

The incubation time for a standard alkaline lysis is fairly short and the step is usually completed typically within 5 minutes. The degree of lysis could be controlled by measuring viscosity/residence time in a vessel.

In a laboratory setting, mixing is often performed gently by hand, which is not feasible at larger scales.

For achieving complete but gentle mixing of large lysis volumes, batch mixing in a mechanically agitated vessel (specialized vessel design with utilizing baffles, low power number impellers, feed lines) and/or continuous flow-through devices/in-line static mixers have been used, taking into consideration viscous non-Newtonian properties of the lysate. Mobius® single-use mixers can be very effective for batch lysis.

2.3. Precipitation/flocculation

2.3.1. Attributes

Precipitation/flocculation is the first step in removing host cell contaminants in a pDNA manufacturing process. Neutralization can be done using a high concentration of sodium or potassium acetate with or without surfactant, RNAse, or $CaCl_2$. This step causes precipitation of detergent solubilized proteins including high molecular weight genomic DNA. Smaller, covalently closed circular pDNA renatures into double stranded molecules and remains in a soluble state. RNAse can be added into the neutralization buffer for degradation of high molecular weight RNA impurities (RNA could be present at least 20X amount of pDNA). Some chaotropic salts, such as lithium chloride, ammonium acetate, and calcium chloride have the additional advantage of precipitating high molecular weight RNA together with the proteins. Polyethylene glycol (PEG) and polyethylenimine (PEI) can also be used for precipitation of genomic DNA.

2.3.2. Parameters

Rapid neutralization occurs with high-salt buffer (such as sodium or potassium acetate at concentration of 0.7 M–3.0 M and pH ~5–7.5, with/without 1.0–1.5% $CaCl_2$) in the presence of a detergent (1% SDS).

A low-cut off PEG precipitation (at 4% w/v) can also be used for precipitation of genomic DNA with up to 20% (w/v) of the precipitate formed during the step. Homogenous mixing during neutralization and precipitation is critical to maintain pDNA quality.

Based on our internal data, impurities such as high molecular weight RNA and genomic DNA, proteins and endotoxins can be selectively precipitated using high salt buffer, PEG and PEI. Proper optimization is recommended.

To separate the precipitated solids, typical clarification methods such as settling with decanting, depth filtration and centrifugation are used. Product loss has been observed occasionally with filtration, and therefore filters with low adsorption are preferred.

2.4. Clarification

2.4.1. Attributes

Clarification unit operations for pDNA processes should enable removal of solid content from the feed stream. Feed streams can either be untreated, pretreated or preclarified. Post chemical lysis and neutralization with sodium or potassium acetate leads to development of large floccules/precipitates.

Pretreatment has a major impact on the clarification filter capacity and must be selected carefully along with a consideration of the scalability of the process. Pretreatment options include use of gravity settling and separation, PEG, PEI, bag filters stainless steel screen filters, paper filters, and centrifugation.

2.4.2. Parameters

To achieve the desired attributes, clarification operations should ensure proper filter selection to handle the solids load of the lysate. Depth filters are ideal, as capacity can be high and adsorptive interactions are masked by the high salt concentration of lysate feed allowing high yield. Feed flux and filtration endpoints can be optimized to ensure minimal

filter area is used and high yield of pDNA is achieved. Additionally, product recovery operation such as blow down and buffer flushing should be considered.

3. Technical Data

The data presented in this section is derived from our internal database.

3.1. Harvest

Biomax® or Ultracel® 1,000 kDa V screen membranes or Durapore® V screen MF TFF membranes are used for harvest at low TMP and permeate control. Normalized water permeability (NWP) recovery post use is >90%. The load challenge reported for Biomax® 1,000 kDa membrane ranges from 10–60 L/m^2 with an optimum permeate flux around 25–30 LMH.

Centrifugation is one of the preferred methods for harvesting at lab scale; at large scale, centrifugation process can be cumbersome and provide low yield. Disk stack centrifuges operating at high speed with intermittent ejection gave supercoiled plasmid yields as low as 40% because of shear damage during discharge.[4]

3.2. Lysis and neutralization

Cell lysis is typically carried out at pH 12–12.5 with 0.2% SDS, followed by neutralization using potassium acetate (0.7–3 M). Typically, neutralization is carried out at approximately 5.0. but has been reported at pH 6.0 and pH 7.5. Use of $CaCl_2$ is common for RNA precipitation during neutralization.

Floccules generated during the neutralization step after undisturbed incubation commonly float on top of the liquid.

Preclarification methods reported in our internal database show pretreatment by use of a range of approaches were used approximately 75% of the time; in 25% of the studies, no treatment or prefiltration was used (Figure 1).

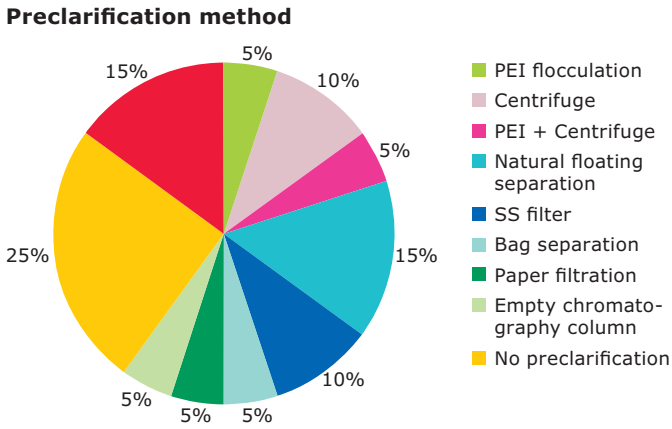

Figure 1. Various reported pretreatment/prefiltration conditions.

3.3. Clarification

A review of internal data for clarification filtration of post lysis and neutralization feeds showed that filtration capacity varies significantly based on whether the feed is pretreated or untreated.

Feed quality impacts the NFF operation. Our internal database shows two kinds of feed, either pretreated (feed turbidity 20 to <500 NTU) or untreated feed (feed turbidity >1,000 NTU).

The pretreatment condition reported in the majority of studies in our database was gravity separation of floccules and solution; solutions were carefully filtered without disturbing floccules/sediments and a product loss of approximately 20% was reported in floccules.

Another pretreatment method includes use of stainless-steel filter, bag filter, empty column, paper filter, centrifuge, PEI flocculation and centrifugation, and use of Polygard® CR 1 µm/Polygard® CR 50 µm filters. Capacity of the Polygard® CR filters were in range of 0.55–8 L/inch.

Filters commonly used for pretreated or untreated feed are listed in Table 6. Average capacity of the filters is shown in Figure 2.

Table 6. Recommended filters, conditions and capacity expected capacity ranges.

			Pretreated		Untreated	
Filter	**Media**	**Pore rating**	**Operating flux (LMH)**	**Avg. capacity range (L/m²)**	**Operating flux (LMH)**	**Avg. capacity range (L/m²)**
Clarisolve® 60 HX filter	Polypropylene	7.5–60 µm	100–150	150–300	100–150	50–300
Clarisolve® 40 MS filter	Polypropylene + cellulosic + inorganic filter aid	0.6–40 µm	100–150	190–460	100–150	50–250
Millistak+® HC D0HC filter	Diatomaceous earth and cellulose	0.6–8.0 µm	90–150	115–200	90–150	25–100
Millistak+® HC C0HC filter	Diatomaceous earth and cellulose	0.2–2.0 µm	100–150	85–300	100–150	30–100
Millistak+® CE20 filter	Cellulose	5.0–10.5 µm	60–200	100–400	50–100	50–100
Millistak+® CE50 filter	Cellulose	0.6–1.0 µm	60–200	100–285		
Millistak+® HC Pro-DOSP filter	Polyacrylic + Silica	0.6–8.0 µm	100–150	100–275	100–150	150–200

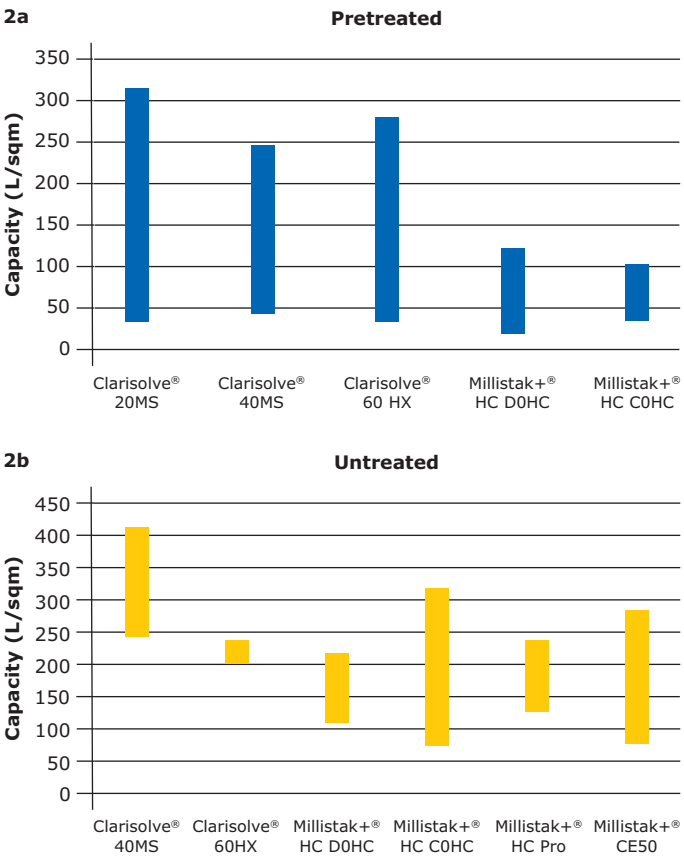

Figure 2a/b. Average capacity range of Clarisolve®, Millistak+® HC, and Millistak+® HC Pro filters for pretreated and untreated feed.

Milligard® PES 1.2/0.45 µm filter can be used as a secondary filter for the Clarisolve® filter. Reported capacity for the Milligard® PES 1.2/0.45 µm filter after Clarisolve® filter is >150 L/m^2. A secondary filter such as Millistak+® HC XOHC and Millistak+® HC Pro XOSP filters, can also be evaluated if required but recovery needs to be monitored.

Millistak+® CE 50 filter is generally reported to be used as primary or secondary filter based on feed conditions.

A combination of Millistak+® CE20 or CE30 or CE40 filter as primary filter with Millistak+® CE50 filter as secondary filter can also be evaluated. The reported capacity for Millistak+® CE20 filter is >300 L/m^2; Millistak+® CE30 filter is >150 L/m^2, Millistak+® CE40 filter is >100 L/m^2; whereas for Millistak+® CE50 filter reported capacity ranged from 80–320 L/m^2.

Recovery of >90% is reported with Clarisolve® and Millistak+® filters. Clarification recovery for Millistak+® filters can be increased using a chase with salt containing buffer.

It is observed that the clarification unit operation is run at low flux considering viscosity of feed. Typical operation flow was in range of 60–150 LMH.

Use of Millipore Express® SHC filter has been reported as a bioburden reduction filter after clarification with average capacity range of 400–650 L/m^2 based on feed quality.

4. References

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