The Biotage® PhyPrep System and Fully Automated Maxiprep Plasmid Purification for Transient Transfection and Mammalian Protein Production

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Abstract

Life science researchers depend upon transient transfection to generate mammalian proteins with the full compliment of post translational modifications. In this process, plasmid DNA passes through the cell membrane of mammalian cells in liquid culture and the cells produce the protein coded by the gene on the plasmid. Since the plasmid does not recombine into the cell's genome, the duration of protein production is transient. If the researcher requires more protein, then additional transfection reactions are required. While advances in cell culture media, cell lines, transient transfection reagents, and knowledge have all contributed to routine production of mammalian proteins, plasmid purification technology has remained relatively unchanged for over 40 years. In this report, a new plasmid purification process designed specifically for mammalian protein production will be described. Using automation, the process is both simple and robust with the advantage of making a once tedious process easily integrated into a workflow. The plasmids produced by this automated method are especially suited for transient transfection because the plasmids are endotoxin-free, supercoiled, and of yield at the Maxiprep scale.

Introduction

Mammalian protein production using transient transfection of plasmid DNA has been a major breakthrough for therapeutic drug development and life science research. Prior to transient transfection, the standard procedure for generating mammalian IgG antibodies was through hybridoma technology, which is problematic both ethically and economically. To generate recombinant mammalian proteins besides antibodies, researchers relied upon *E. coli*, yeast and insect cell lines to produce mammalian proteins, even though these systems cannot generate the full complement of post translational modifications. Transient transfection of mammalian cells has now become a workhorse of recombinant mammalian protein expression because of new advances in protein quality, expression efficiency, speed and ease of use.

Mammalian protein production by transient transfection has become widely adopted by the life science community. The mammalian protein production process requires the desired plasmid DNA, which harbors the gene of interest, to enter the mammalian cells in liquid culture. Features of the plasmid direct it into the nucleus to be translated into proteins via mRNA. However, plasmid DNA is recognized as foreign DNA to be degraded by nucleases and excluded upon cell division. After a few days, the plasmid DNA is no longer present and protein production ceases. Thus, the quantity and quality of the DNA being used for protein production plays a significant role.

Since the foreign plasmid DNA being used will be degraded over time, higher transfection rates would be beneficial. Advances in transfection reagents have increased transfection efficiency along with evidence showing that the supercoiled form of the plasmid DNA has higher transfection efficiency. Conversely, the presences of endotoxins in the plasmid DNA preparation would decrease the efficiency of transient transfection. The amount of plasmid needed for transient transfection can depend on the reagents used for the process. For example, a common reagent used for transfection is calcium phosphate, which can use approximately 2.25 mg of plasmid DNA for the transient transfection. Therefore, large scale plasmid purifications, which are endotoxin-free and in supercoiled form, would be ideal for this workflow and throughput.

While advances in cell culture media, transfection reagents and cell line development have all contributed to significant improvements to transient transfection, plasmid purification technology has generally not kept pace. In this report, a plasmid purification technology designed specifically for mammalian protein production will be described. Using dual flow chromatography and PhyTip® columns, this procedure has the advantage of generating Maxiprep scale yields, that are endotoxin-free, that maintain plasmid in the supercoil form and that is fully automated using the Biotage® PhyPrep system. Here, highly reproducible plasmid purification results are demonstrated together with succesful transient transfection of mammalian cells.

Methods

Reagents used in this study were purchased from VWR unless otherwise stated. The plasmid DNA used in this study, pFH001, is pUC cloned with GFP. The plasmid was transformed into *E. coli* DH5α (ThermoFisher Scientific, 18265017) to generate strain BT01 and BT02.

Culture Growth Conditions

BTo1 and BTo2 was streaked onto LB agar plates containing 100 µg/mL carbenicillin (Teknova, L1010) from a frozen glycerol stock. The plate was incubated at 37 °C for 24 hours. Single colonies were used to inoculate a starter culture of 10 mL Terrific Broth (Teknova, T7000) and 100 µg/mL carbenicillin (Teknova, C2135). The starter culture was shaken at 38 °C at 350 RPM in a New Brunswick Scientific I26 incubator shaker for 8 hours. 500 µL was used to inoculate 500 mL of TB, 100 µg/mL carbenicillin in 2.5 L baffled flasks (Thomsom, 931136-B) and sealed with AirOtop enhanced seals (Thomson, 899425). The cultures were shaken at 38 °C for 16 hours. Cells were harvested in 250 mL Fiberlite centrifuge bottles (ThermoFisher Scientific, 001-0303) by centrifugation using a ThermoFisher Sorvall Lynx 400 Superspeed Centrifuge fitted with a Fiberlite F10-6x250y Rotor for 15 minutes at 5,000 RPM. The supernatent was decanted and the bottles were left upside down on lab wipes to remove residual medium. Cell pellets were weight to record the pellet wet weight.

Biotage® PhyPrep Purification

5 grams of pellet wet weight are used unless otherwise stated. Cell pellets were prepared using Biotage® plasmid maxiprep kit endotoxin-free (Biotage, DMP-16-03-72-01) as per standard procedures. The kit was used in conjunction with the PhyPrep system using the pre-installed procedures with final, pure plasmid eluted in 2, 3, or 5 mL of elution buffer.

As a reference to the automated protocol and gel loading control, plasmid purification on 1 mL of an identical *E.coli* culture was performed using a manual plasmid miniprep kit from Qiagen according to manufacturers' instructions.

Analysis

Yield was measured by UV absorbance at 260 nm using a ThermoFisher Scientific NanoDrop One^{C} UV-VIS spectrophotometer. Plasmid DNA quality was assessed by gel electrophoresis. Either 5 µL of plasmid DNA or 100 ng DNA was loaded into 1% agarose, TAE gels. 70 V was applied for 1 hour. Gels were stained in a solution of TAE, 0.5 µg/mL ethidum bromide for 15 minutes followed by destaining in water for 10 minutes. Gels were visualized using Bio-Rad Gel Doc EZ System. Endotoxin was measured by diluting the plasmid DNA 1:100 using endotoxin free water (Growcells, CCPW-1000) by sterile technique. Samples were loaded onto Charles River Endosafe LAL Cartidges with sensitivity of 0.05 Endotoxin Units/mL (Charles River, PTS5505F) and processed by the Charles River Endosafe nexgen-MCS reader.

Results

I. Purification of >1 mg Endotoxin-free Plasmid DNA

Plasmid purification in Maxi-scale by the automated protocol by Biotage® PhyPrep and Biotage plasmid maxiprep kit endotoxin-free generates highly reproducible results in terms of purity and yield. Endotoxin-levels were consistently below 0.1 EU/µg (Table 1, Figure 1). This yield is from purification of 5 g cell pellet and a high copy plasmid. For low-copy plasmids, the yield is expected to be lower.

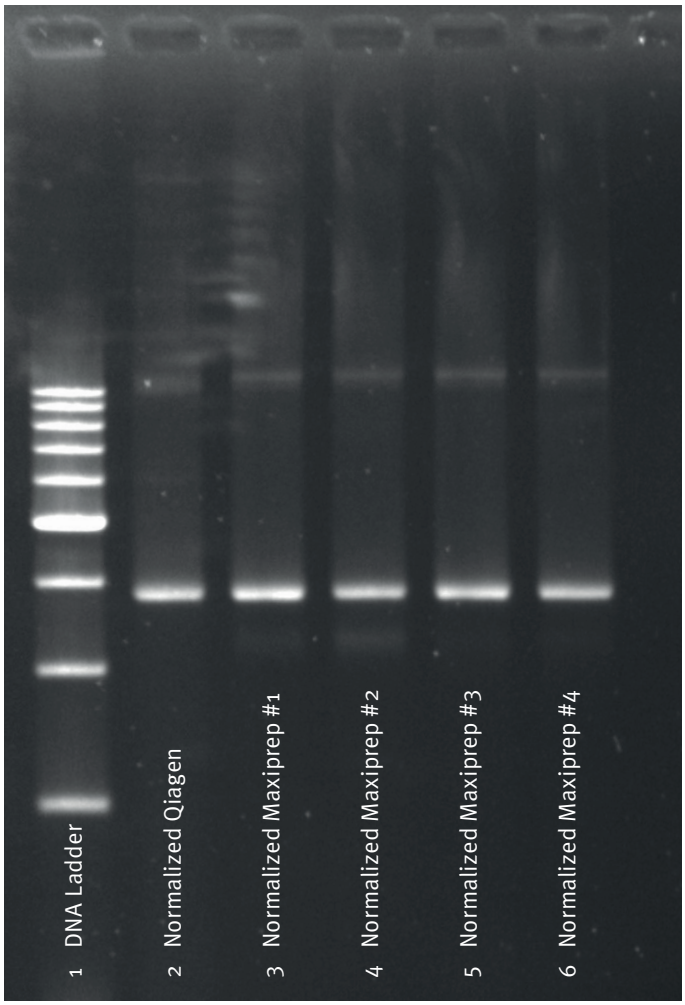

Figure 1. Agarose gel electrophoresis analysis of plasmid DNA purified by the Biotage® plasmid maxiprep kit endotoxin-free.

Table 1. Purification of 4 plasmid samples in one run on Biotage® PhyPrep eluted in an elution volume of 5 mL elution buffer.

	A260/A280	A260/A230	Plasmid Concentration (mg/mL)	Volume (mL)	Yield (mg)	Endotoxin Level	
						EU/mL	EU/µg
Sample 1	1.88	2.47	0.25	4.6	1.15	< 5.0	< 0.02
Sample 2	1.88	2.28	0.29	4,7	1.39	< 5.0	< 0.02
Sample 3	1.89	2.29	0.29	4.8	1.39	< 5.0	< 0.02
Sample 4	1.88	2.27	0.32	4.9	1.58	< 5.0	< 0.02

II. Plasmid yield and concentration vary with eluted volume

During setup of a new run, the graphical user interface of Biotage® PhyPrep provides the user with the option to select between 2 mL, 3 mL or 5 mL elution volume to be used during elution of the purified plasmid DNA from the PhyTip column.

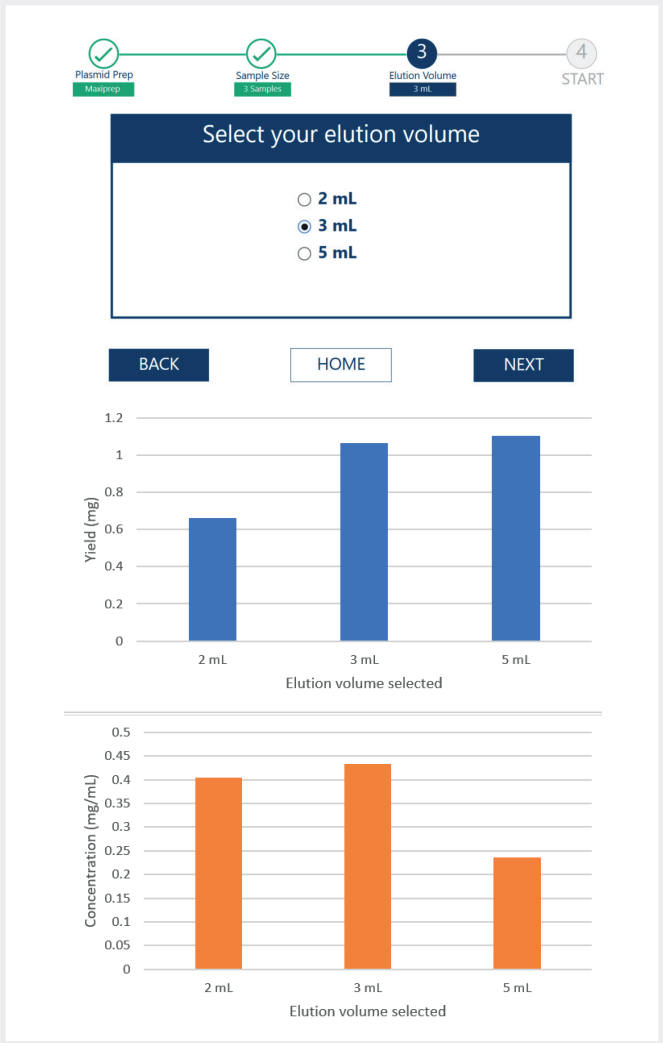

Figure 2. Plasmid DNA yield and sample concentration using various elution volumes during a run of four samples.

Table 2. Plasmid DNA purification using various elution volumes during run of four samples producing highly reproducible yield, concentration and purity.

Elution volume selected in GUI	2 mL	3 mL	5 mL
Average plasmid DNA yield (CV%)	0.66 mg (9%)	1.06 mg (5%)	1.10 mg (5%)
Average Plasmid DNA concentration (CV%)	0.40 mg/mL (6%)	0.43 mg/mL (6%)	0.24 mg/mL (5%)
Endotoxin-levels		<0.1 EU/µg	
Average A260/A280 (CV%)	1.90 (0.2%)	1.90 (0.4%)	2.31 (1.7%)
Average A260/A230 (CV%)	2.28 (0.9%)	1.88 (0.2%)	2.35 (0.6%)

As the yield and plasmid concentration of the purified sample vary with the elution volume, this enables the user to select the elution volume depending what is most suitable for downstream assays. For example, is the user wants to use ethanol precipitation after plasmid purification and have maximal yield, 5 mL elution volume can be selected as concentration is less important as the sample will be concentrated during the ethanol precipitation step. Alternatively, as the elution buffer is 50 mM Tris, 500 mM NaCl, transfection may also be carried out without ethanol precipitation, 3 mL elution volume can be selected to achieve the highest concentration of plasmid DNA.

III. Over incubation lysis buffer results in high endotoxin

The Biotage plasmid maxiprep kit endotoxin-free was optimized for yield and quality. In addition to the pre-aliquoted buffers, automation and user-friendly User Interface, the procedures for preparation of the cell pellets was also optimized. Four identical MaxiPrep purifications were prepared from 5 grams of cell pellet. The cells were prepared using the MaxiPrep kits either as per recommended procedures, by vigorous shaking of the lysis mixing step, or by extended incubation of the lysis step. In all cases, the yields were above 1 mg. However, when the lysis buffer is incubated from 10 minutes compared to the recommendation of 3 minutes, a greater amount of endotoxin contamination was observed (Table 3). The acceptable amount of endotoxin contamination of plasmid DNA for a transient transfection reaction is ‹ 0.1 EU/µg. Both low endotoxin and higher supercoiled DNA is required for efficient transient transfection. No change in plasmid DNA quality due to overlysis of samples was observed (Figure 3 and Figure 4).

Table 3. Over lysis of the samples can create issues in the quality of the plasmid, thus, over shaking and over incubating were evaluated. Two samples of 5 g of *E. Coli* were ran parallel on Biotage® PhyPrep where one sample was prepared as normal conditions, and the other sample was shaken vigorously during the lysis step. In addition to the shaking factor, over incubation of lysis buffer can affect the plasmid, which was studied by running parallel runs where one maxiprep was prepared as normal and the other sample had incubated in lysis buffer for 10 minutes.

Sample	Conc. [ng/uL]	Volume [mL]	Yield [mg]	Endotoxin Lvl [EU/mL]	Endotoxin Lvl [EU µg]
Regular sample preparation	228.6	5.51	1.26	12.9	0.06
Over shaking lysis	263.6	4.72	1.24	5.58	0.02
Regular sample preparation	184.4	5.56	1.03	5.00	0.03
Over incubating lysis	214.2	5.59	1.20	48.9	0.23

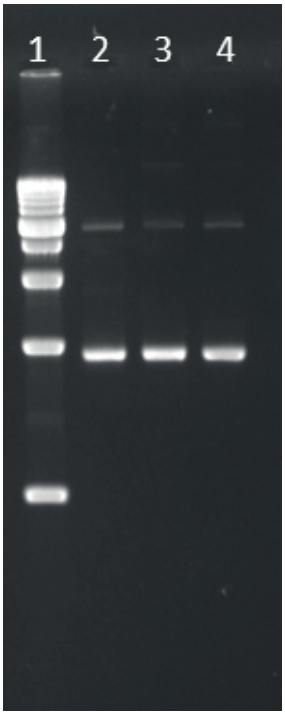

Figure 3. Gel image of the Maxi Prep plasmid elution when sample was over shaken during lysis step.

Gel Lane Sample
1 DNA Ladder
2 Qiagen (normalized)
3 Regular sample preparation (normalized)
4 Over shaking lysis buffer (normalized)

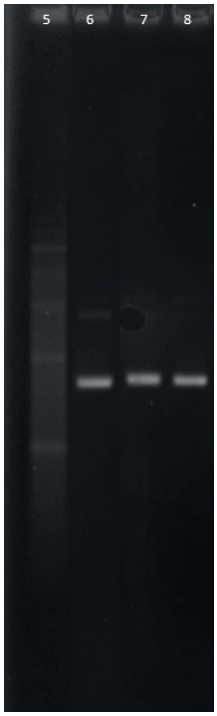

Figure 4. Gel image of the Maxi Prep plasmid elution over incubating during lysis step.

Gel Lane Sample
1 DNA Ladder
2 Qiagen (normalized)
3 Regular sample preparation (normalized)
4 Over shaking lysis buffer (normalized)

IV. Prepared plasmid samples are stable for up to 3 hours

The Biotage® PhyPrep system is a dual channel system with capacity for 4 samples to be processed in a walk-away fashion. In such a configuration, 2 samples will be processed, while 2 samples are on stand-by. To determine if the processing time of samples 1 and 2 affects samples 3 and 4, an extended incubation study was carried out. 4 identical samples were prepared for processing. Two samples were processed, immediately, and 2 samples were held for 3 hours before processing. In all cases, yields were at or close to 1 mg and endotoxin were measured to be less than 0.1 EU/µg (Table 4). In summary, there was no change in yields or endotoxin levels for samples regardless if they were purified in the beginning or in the end of the run. Also, there was no notable difference in the level of supercoiled plasmid DNA between the samples (Figure 5).

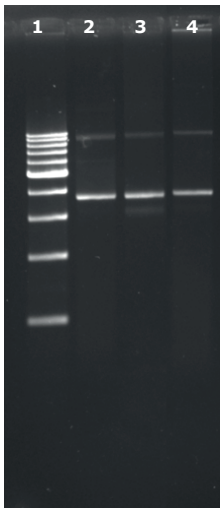

Figure 5. Gel image of the Maxi Prep plasmid elution purified 3 hours after precipitation.

Gel Lane Sample
1 DNA Ladder
2 Qiagen (normalized)
3 Regular sample preparation (normalized)
4 Sample preparation post 3 hours (normalized)

Table 4. Since Biotage® PhyPrep has the capability to purify up to 4 samples with set methods, the stability of the lysed samples and elutions during the time gap of purifying the first 2 samples and the second 2 samples were evaluated. The 2 samples of 5 g of *E. Coli* cells each (wet pellet weight) were prepared and lysed using buffers from the MaxiPrep kit at the same time, but only one sample was purified right away. The second sample was purified 3 hours after the sample preparation, which is longer than the 4 sample method run time.

Sample	Concentration (ng/uL)	Volume (mL)	Yield (mg)	Endotoxin Level (EU/mL)	Endotoxin Level (EU/µg)
Regular sample preparation	277.4	4.67	1.3	< 5	< 0.02
Sample preparation post 3 hrs	181.9	6.59	1.2	< 5	< 0.03

V. Endotoxin removal buffer (ERB) is critical to achieve endotoxin-free plasmid DNA

The Biotage® plasmid maxiprep kit endotoxin-free was designed specifically for mammalian protein production. To ensure optimal and efficient transient transfection, the plasmid DNA must be both in the supercoiled form and be endotoxin free. The ERB buffer was developed with the latter in mind. Samples were prepared in triplicate, with and without ERB. When ERB is omitted from the purification, there is 6,000 times more endotoxin contamination in solution, which corresponds to 13 times more endotoxin per unit of plasmid DNA (Table 3).

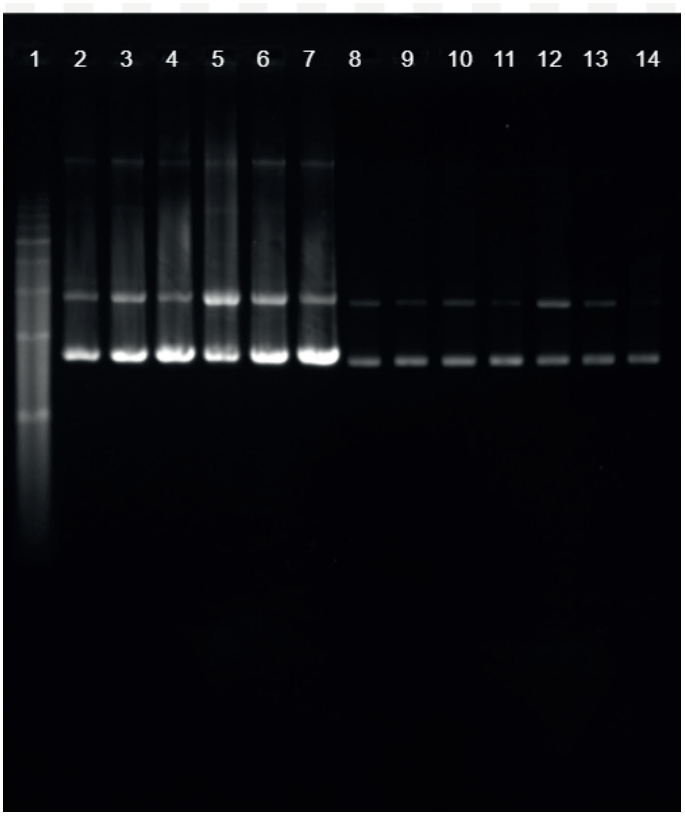

Figure 6. Gel image of the MaxiPrep plasmid elution purified with and without ERB.

Gel Lane	Sample
1	DNA Ladder
2	Sample with ERB #1
3	Sample with ERB #2
4	Sample with ERB #3
5	Sample without ERB #1
6	Sample without ERB #2
7	Sample without ERB #3
8	Qiagen - Normalized (100 ng)
9	Sample with ERB #1 - Normalized (100 ng)
10	Sample with ERB #2 - Normalized (100 ng)
11	Sample with ERB #3 - Normalized (100 ng)
12	Sample without ERB #1 - Normalized (100 ng)
13	Sample without ERB #2 - Normalized (100 ng)
14	Sample without ERB #3 - Normalized (100 ng)

Table 5. To evaluate the function of the Endotoxin Removal Buffer, two samples of 5 g of *E. Coli* cells (wet pellet weight) were prepared and lysed using buffers from the MaxiPrep kit. The preset method, Maxiprep 2 sample 5 mL elution, was used to purify the two samples where one sample had Endotoxin Removal Buffer, whereas the other did not. This was repeated two more times for a total of 3 samples purified with ERB and 3 samples without ERB. The plasmid that eluted from the column was analyzed for yield, purity, and endotoxin levels.

Sample	Concentration (ng/uL)	Volume (mL)	Yield (mg)	Endotoxin Lvl (EU/mL)	Endotoxin Lvl (EU/µg)	Total Yield (mg)
Samples with ERB						
Sample 1	124.2	9.02	1.12	8.2	0.07	1.25
Sample 2	163.5	5.58	0.91	5.0	0.03	1.06
Sample 3	228.6	4.66	1.07	5.0	0.02	1.07
Samples without ERB						
Sample 1	178.8	5.13	0.92	1150.0	6.43	0.99
Sample 2	194.4	4.81	0.94	1270.0	6.53	0.98
Sample 3	222.5	4.58	1.02	500.0	2.25	1.02

VI. Pellet wet weight and expected yield

The Biotage® plasmid maxiprep kit endotoxin-free is optimized to purify plasmid DNA from 5 g cell wet weight. In addition to transient transfection performance, the kit was designed to be versatile and robust to accommodate a range of different sample input. This would be a common situation in a core facility, for example, where the operator would not have control of how the samples were grown. Identical cell cultures were prepared. The cell pellets were prepared and weighed to 3, 5 and 7 grams of pellet wet weight. Each pellet was processed by the standard procedures of the kit. The resulting pure plasmid were analyzed by UV (Figure 7).

These results with varying cell wet weights and a high copy plasmid show that when too low cell wet weight is used, there is a risk that the plasmid yield will be lower than specified. As the lysis buffer of the kit is optimized for 5 g cell wet weight, if too much cell pellet is used there is a risk that the lysis buffer is not able to effectively lyse all cells. The ideal wet pellet weight to achieve around 1 mg of plasmid more consistently would be from 5 g as specified for the kit.

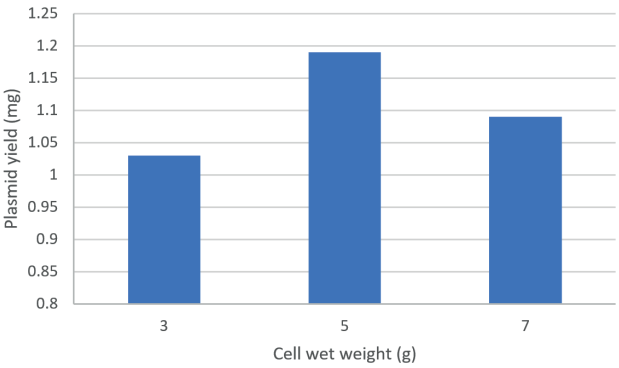

Figure 7. Plasmid yield from purification of various cell wet weights on Biotage® PhyPrep. The recommended cell wet weight for the Biotage plasmid maxiprep kit endotoxin-free is 5 g.

Materials and Methods

Results

VII. Transient Transfection of Cells

Two purified plasmid samples from the Biotage® PhyPrep (eluted in 5 mL volume) and a manual Endotoxin-free MaxiPrep kit (Qiagen) (2.9 g cell wet weight) of cell strain BTo2 was used for transient transfection.

Ethanol Precipitation

Ethanol precipitation of the purified plasmid samples was performed by addition of 450 µL 3M KOAc and vortexing. 13.5 mL 200 proof ethanol was added followed by vortexing and 30 min. incubation at room temperature. The samples were centrifuged (14,000 g, 30 min.) and the pellets were rinsed with 5 mL 70% ethanol and centrifuged again (14,000 g, 30 min). The pellets were resuspended in 1 mL of endotoxin-free water.

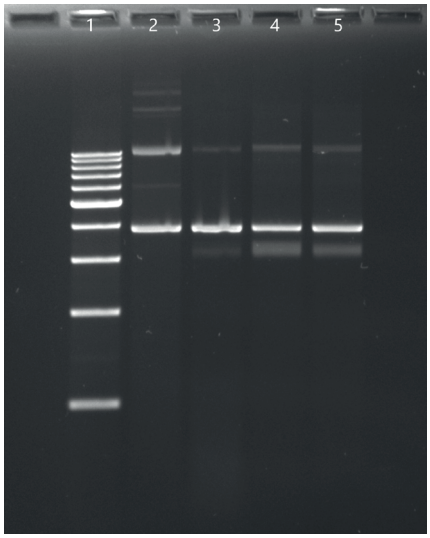

Figure 8. Agarose gel electrophoresis of ethanol precipitated plasmid DNA purified by Biotage® PhyPrep and a manual MaxiPrep kit.

Gel Lane	Sample
1	DNA Ladder
2	MiniPrep control
3	Manual MaxiPrep kit
4	Biotage® PhyPrep – Sample A
5	Biotage® PhyPrep – Sample B

Table 5. Plasmid samples used for transient transfection after ethanol precipitation.

	A260/A280	A260/A230	Plasmid Concentration (mg/mL)	Volume (mL)	Yield (mg)	Endotoxin Level	
						EU/mL	EU/µ
Manual kit	1.885	2.289	1.24	1	1.24	5	0.004
Biotage® PhyPrep – Sample A	1.835	2.320	1.4	1	1.40	5	0.004
Biotage® PhyPrep – Sample B	1.857	2.338	1.31	1	1.31	5	0.004

Transient Transfection of Mammalian Cells

The plasmid samples were used for transient transfection of CHO-K1 cells. CHO-K1 cells were seeded 24 hours prior to transfection at 100,000 cells per mL into a standard, tissue culture treated 24-well plate. Cells were 50–70% confluent at transfection. 2 µg of pDNA was mixed with 6 µL of TransIT LT1 in Opti-MEM (Life Tech) in a final volume of 200 µL. This mixture was incubated at room temperature for 15 minutes. Triplicate complexes were formed for each pDNA preparation and applied to three separate wells. CHO-K1 cells were transfected as 50 µL of each complex was added drop-wise onto the cells in their full media and gently swirled. For cell harvest 48 hours post-transfection, the media and complexes were removed from the cells. The cells were washed once with PBS and trypsinized with 100 µL trypsin for 5 min. at 37 °C.Trypsin was inactivated by adding 300 µL of full media (which contains 10% fetal bovine serum). 60 µL of trypsinized (suspended) cells was added to 150 µL of full media in an untreated 96-well plate and mixed. Flow cytometry analysis was performed using a Guava easyCyte™ 5HT flow cytometer (EMD Millipore). Untransfected cells were used to determine the gain settings for forward and side scatter, and GFP positivity. Cells were gated based on forward and side scatter of untransfected cells, and GFP positive cells were gated such that the untransfected cells were under 0.15%. 5,000 events were collected.

The results from the transfection of CHO-K1 cells shows that the automated plasmid DNA purification protocol by Biotage® PhyPrep results in a similar transfection results as the manual maxiprep kit.

Conclusions

Biotage® PhyPrep automated for Plasmid DNA Maxiprep purification produces 1 mg of plasmid DNA that is pure, endotoxin free and supercoiled. The system was designed for mammalian protein production so transient transfection efficiency was of the highest consideration. The Anion Exchange chemistry produces plasmid DNA that is maintained in the supercoiled form. This is the most important criteria for transfection efficiency. Secondly, ERB buffer was developed to produce plasmid that are endotoxin free, <0.1 EU/µg. Succesful transient transfection of mammalian cells by the purified plasmid DNA by Biotage® PhyPrep was also demonstrated by transfection of CHO-K1 cells with very similar results to plasmid DNA that was purified with a manual kit. By utilizing a fully automated system, the researcher can rely on the robustness of automation while freeing up time to carry out other experiments.

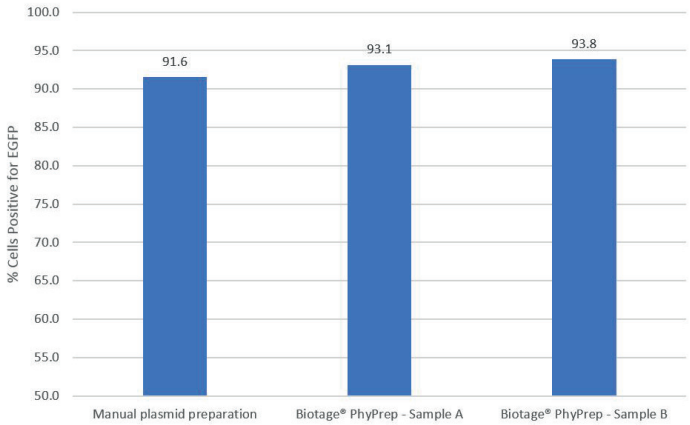

Figure 9. Transfection results from transient transfection of CHO-K1 cells (n=3).

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