The Biotage® PhyPrep Maxi Mega Giga Instrument and fully Automated Plasmid Purification for Transient Transfection and Mammalian Protein Production

Authors: *Fanny Huang, Jacqueline Nguyen, Lee Hoang, Sean Arabian*

Abstract

Life science researchers depend upon transient transfection to generate mammalian proteins with the full complement 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 they are endotoxin free, supercoiled, can be produced at three different scales, MaxiPrep, MegaPrep, and GigaPrep.

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 super coiled 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. The PhyPrep instrument utilizes the Växel columns to perform automated plasmid purification process, which is made possible by dual flow chromatography. Furthermore, the PhyPrep unit and Växel columns are capable of generating endotoxin free, super coiled plasmids that is optimal for many down-stream processes.

Methods

Reagents used in this study were purchased from VWR unless otherwise stated. The plasmid DNA used in this study, pFH001 and pFH002, is pUC cloned with EGFP and pCDNA3.1 cloned with EGFP, respectively. The plasmid was transformed into *E. coli* DH5α (ThermoFisher Scientific, 18265017) to generate strain BT02.

Culture growth conditions. BT02 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 10 uL of 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, mixed with 500 µL of 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 and 350 RPM 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 6,000 RPM at 4°C. The supernatant was decanted, and the bottles were left upside down on lab wipes to remove residual medium. Cell pellets were weighed to record the pellet wet weight.

PhyPrep Purification. Cell pellets were prepared and processed using PhyPrep MaxiPrep (DPM-16-05-72-KIT), MegaPrep (DPM-16-10-72-KIT), and GigaPrep kits (DPM-08-20-72-KIT) as per standard procedures. The kits were used in conjunction with the PhyPrep-Maxi, Mega, Giga instrument using the pre-installed procedures.

Analysis. Yield was measured by UV absorbance at 260 nm using a ThermoFisher Scientific NanoDrop OneC UV-VIS spectrophotometer. Plasmid DNA quality was assessed by gel electrophoresis. Either 5 µL of plasmid DNA or 100 ng of plasmid DNA was loaded into 1% agarose, TAE gels. 70 V was applied for 2 hours. Gels were stained in a solution of TAE and 0.5 µg/mL ethidum bromide for 10 minutes followed by destaining in TAE for 20 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 Cartridges with sensitivity of 0.05 – 5 Endotoxin Units/mL (Charles River, PTS5505F) and processed by the Charles River Endosafe nexgen-MCS reader.

Transient Transfection. Six purified plasmid samples from the Biotage PhyPrep and three samples from manual Endotoxin-free kits (Qiagen Cat. No. 12362, Cat. No. 12381, Cat. No.12391) of cell strain BT02 were used for transient transfection (Table. 8). Three samples from the Biotage PhyPrep (MX2, MG2, GG2) were additionally desalted and concentrated by alcohol precipitated. Alcohol precipitation of MX2 was performed by addition of 0.1 volume of 3M KOAc pH 5.3 and vortexing. Followed by addition of 0.7 volume of isopropyl alcohol and vortexing. The solution was then centrifuged (≥15,000 x g, 5 minutes, 4°C); the resulting pellet is washed with 5 mL of 70% ethyl alcohol and centrifuged again (≥15,000 x g, 3 minutes, 4°C). The resulting pellet is air dried for 10 minutes at room temperature and subsequently resuspended in 1 mL of endotoxin-free water.

Alcohol precipitation of MG2 was performed by addition of 0.1 volume of 3M KOAc pH 5.3 and vortexing. Followed by addition of 0.7 volume of isopropyl alcohol, vortexing, and a 15 minute incubation at room. The solution was then centrifuged (≥15,000 x g, 6 minutes, 4°C); the resulting pellet is washed with 5 mL of 70% ethyl alcohol and centrifuged again (≥15,000 x g, 5 minutes, 4°C). The resulting pellet is air dried for 10 minutes at room temperature and subsequently resuspended in 1 mL of endotoxin-free water. Alcohol precipitation of GG2 was performed by addition of 0.1 volume of 3M KOAc pH 5.3 and vortexing. Followed by addition of 0.7 volume of isopropyl alcohol, vortexing, and a 15 minute incubation at room. The solution was then centrifuged (≥15,000 x g, 15 minutes, 4°C); the resulting pellet is washed with 5 mL of 70% ethyl alcohol and centrifuged again (≥15,000 x g, 5 minutes, 4°C).
The resulting pellet is air dried for 10 minutes at room temperature and subsequently resuspended in 1 mL of endotoxin-free water. Transfection of mammalian cells were performed using CHO-K1 cells.

Results

I. Typical results from MaxiPrep, MegaPrep, and GigaPrep.

The PhyPrep instrument automates plasmid purifications at three different scales, which are MaxiPrep, MegaPrep, and GigaPrep. These three scales result in yields up to 1 mg for MaxiPrep, 5mg for MegaPrep, and 10 mg for GigaPrep. From these sample prep kits, it can be expected to obtain a certain level of concentrations, previously stated plasmid throughput of yields accordingly (Table 1 - Table 3), as well as maintenance of supercoiled plasmid quality (Figure 1 - Figure 3) when using high copy plasmid grown in rich media with the PhyPrep automated process .

V. Pellet wet weight and expected yield

In addition to transient transfection performance, the Biotage MaxiPrep, MegaPrep, and GigaPrep kits were designed to be versatile and robust to accommodate a range of different sample input. This would be a common situation in a core facility, such as when the operator would not have control of how the samples were grown. Therefore, identical cell cultures were prepared in a range of cell pellet weight according to each sample prep size to demonstrate the sample prep kit processing capability.

The Biotage MaxiPrep kit was used to process cell pellets that were prepared and weighed to 3, 4, and 5 grams of pellet wet weights. The Biotage MegaPrep kit was used to process wet cell pellets weights that were weighed to 6, 7, 7.5, and 8 grams. The Biotage GigaPrep kit was used to process wet cell pellet weights of 13, 14, 15, and 16 grams.

Each pellet range and set were processed by the standard procedures of the respective sample kit sizes, MaxiPrep, MegaPrep, and GigaPrep. The resulting pure plasmid were analyzed by UV and by gel. The smallest sample prep kit scale is Biotage MaxiPrep kit, which can process the cell pellet weight range of 3 – 5 grams to produce 1 mg of plasmid that is supercoiled and endotoxin free. The efficiency of the Biotage MaxiPrep kit decreases with cell pellets above 5 grams as seen in lower concentrations. From the MaxiPrep cell pellet weight range, the optimal yield and concentration levels occurs when processing 3 - 5 gram pellets. The next scale of sample prep kit is the Biotage MegaPrep. This kit can process the cell pellet range of 6 – 8 grams to produce 5 mg of plasmid that is supercoiled and endotoxin free. However, the performance is reduced at cell pellets greater or equal to 7.5 grams, and the highest amount of plasmid was achieved at 7 grams of cell pellet. The largest sample prep kit scale is the Biotage GigaPrep kit that can be used to process a cell pellet weight range of 13 – 16 grams to purify 10 mg of plasmid that is supercoiled and endotoxin free. Sample prep kit performance decreases when cell pellet weights above 16 grams are used. For the best kit performance, cell pellet weight of 15 grams is the optimal weight to achieve 10 mg of plasmid (Table 7, Figures 8-9).

As the cell pellet ranges for the MegaPrep and GigaPrep were tested, it was found that using heavier cell pellets would have a reverse effect on plasmid yield and concentration across the board. In addition, using smaller pellets than the specified range for each kit would not achieve the sample prep kit yields of 1, 5, and 10 mg of plasmid for the MaxiPrep, MegaPrep, and GigaPrep. Therefore, using the optimal cell pellet weights of 3, 7, and 15 grams for MaxiPrep, MegaPrep, and GigaPrep, respectively, would have the most effective plasmid purification.

VI. Transient Transfection

Plasmid DNA was purified in preparation for protein expression analysis by a Contract Research Lab, Mirus Bio. Since plasmids purified on the PhyPrep system result in concentrations of about 200 ng/µL in a salt concentration of 0.5 M NaCl, an additional study as carried out to address how these factors impact protein expression. Two identical MaxiPrep samples were purified and then pooled to generate a homogenous sample. The sample was split and one half was subjected to alcohol precipitation to both desalt and concentrate the plasmid. For the MegaPrep, and GigaPrep samples, these were simply each split in half where one set was subjected to alcohol precipitation and the other set was not. Together with MaxiPrep, MegaPrep and GigaPrep samples purified by Qiagen Endo Free kits, quality was assed by UV absorbance and gel (Table 8 and Figure 10). Qiagen Maxi and Mega samples and alcohol precipitation samples from PhyPrep Maxi and Mega consistently show an increased relaxed form of the plasmid DNA. Qiagen GigaPrep plasmid DNA shows a smear in the background.

These plasmids, cloned with enhanced Green Fluorescent Protein (EGFP), were transfected into CHO cells using Trans-IT and transfection efficiency was measured. EGFP positive transfection rates ranged from around 94% to 98%. Overall, between alcohol precipitated samples from Växel MaxiPrep, MegaPrep and GigaPrep (MX2, MG2, GG2) compared to non-alcohol precipitated Växel MaxiPrep, MegaPrep and GigaPrep samples (MX1, MG1, GG1), the non-alcohol precipitated samples performed noticeably better across all scales. MX1 and MX2 had similar results; however, MX1 averaged 1% higher than MX2 for all three replications.

Conclusions

Biotage PhyPrep automates plasmid DNA purification at MaxiPrep, MegaPrep, and GigaPrep scales and produces 1, 5, and 10 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 which is the most important criteria for transfection efficiency. ERB buffer was developed to produce plasmids that are endotoxin free, <0.1 EU/µg. By utilizing an automated system, researchers can rely on the robustness of automation while freeing up time to carry out other experiments.

Figures and Tables

Sample	Concentration (ng/uL)	A260/A280	A260/ A230	A260	A280	Volume (mL)	Yield (mg)	Endotoxin Levels (EU/ug)
MaxiPrep 1	301.4	1.90	2.31	6.08	3.20	4.85	1.46	<0.0166
MaxiPrep 2	334.4	1.91	2.31	6.73	3.53	4.95	1.66	<0.0150
MaxiPrep 3	322.9	1.90	2.31	6.36	3.34	4.80	1.55	<0.0155
MaxiPrep 4	287 .0	1.92	2.31	5.74	2.99	5.57	1.60	<0.0174

Table 1. Typical MaxiPrep plasmid elution samples.

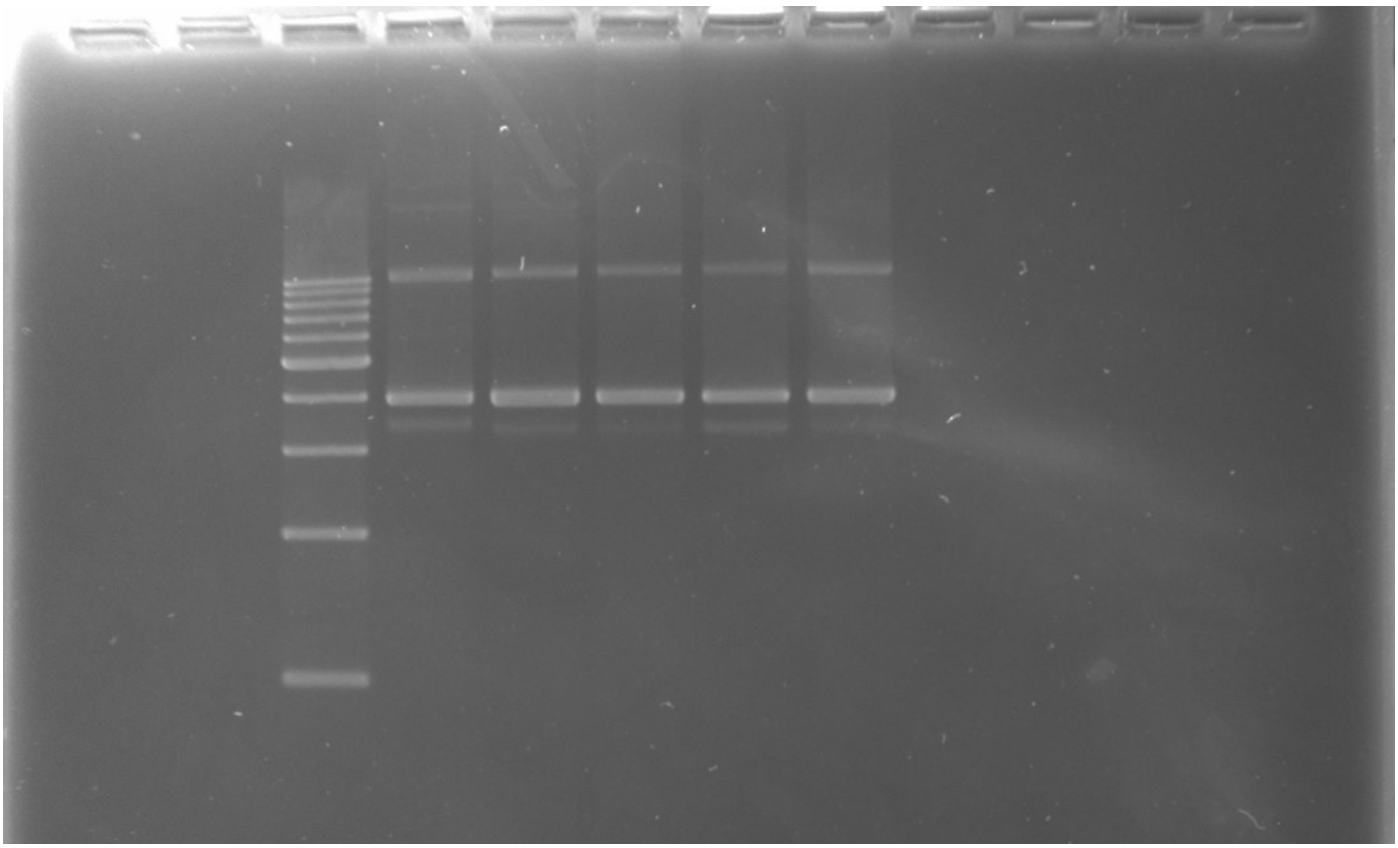

Figure 1. The MaxiPrep plasmid elution samples from Table 1 were processed through gel electrophoresis where gel lanes 1 – 6 from left to right is the DNA Ladder, Normalized Qiagen MaxiPrep plasmid sample, Normalized MaxiPrep 1, Normalized MaxiPrep 2, Normalized MaxiPrep 3, and Normalized MaxiPrep 4.

Sample	Concentration (ng/uL)	A260/ A280	A260/ A230	A260	A280	Volume (mL)	Yield (mg)	Endotoxin Levels (EU/ug)
MegaPrep E1	326	1.92	2.42	6.53	3.40	18.09	5.91	< 0.015
MegaPrep E2	330	1.92	2.43	6.61	3.44	17.88	5.9	< 0.015
MegaPrep E3	270	1.91	2.39	5.40	2.82	18.61	5.0	0.023
MegaPrep E4	301	1.91	2.35	6.02	3.15	19.97	6.0	< 0.017

Table 2. Typical MegaPrep plasmid elution samples.

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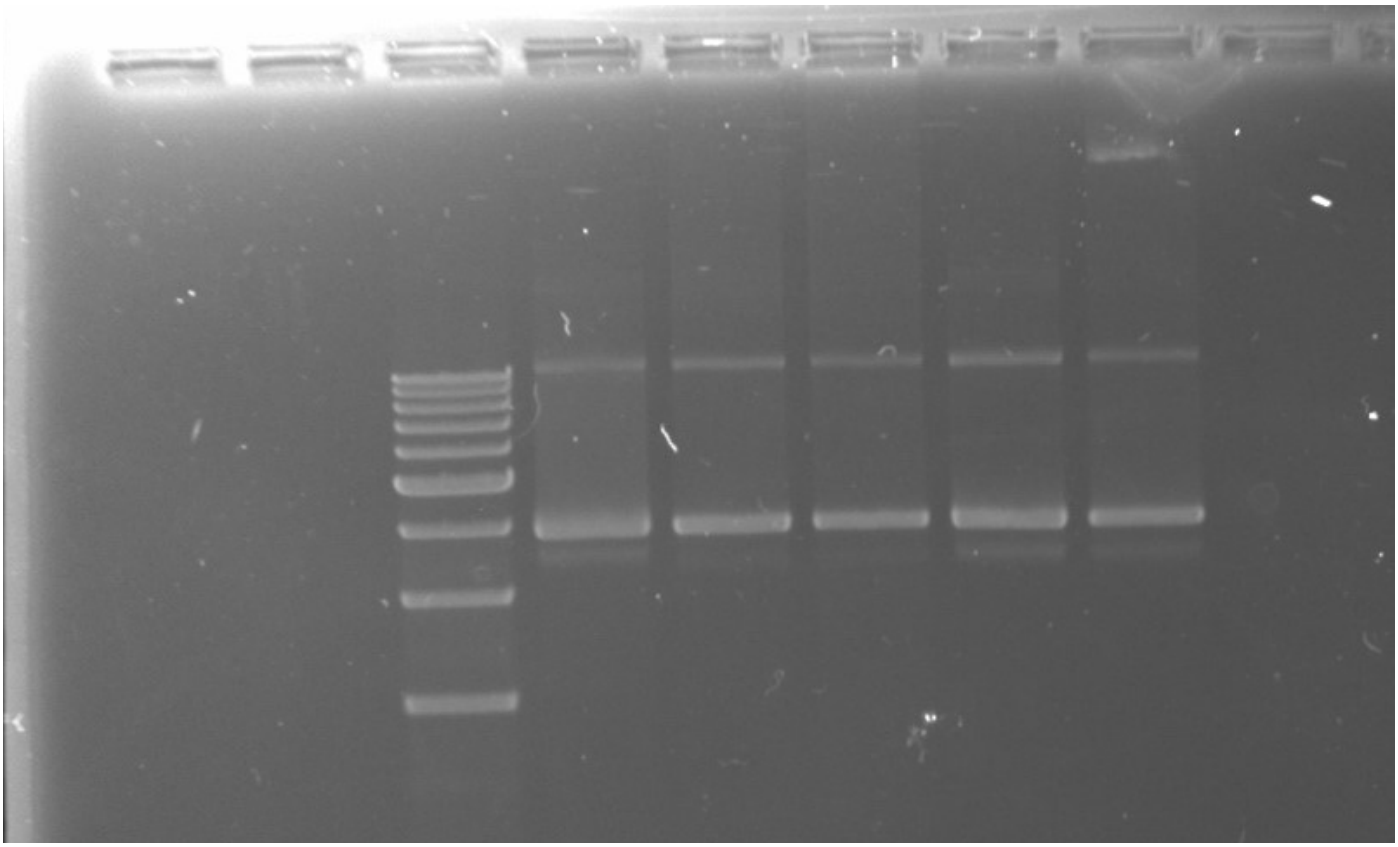

Figure 2. The MegaPrep plasmid elution samples from Table 2 were processed through gel electrophoresis where gel lane 1 – 6 from left to right is the Figure 2. The Megaprep plasmid elution samples from Table 2 were processed through gel electrophoresis where gel lane 1 – 6 from left to right is the DNA Ladder, Normalized Qiagen Megaprep plasmid sample, Normalized Megaprep 1, Normalized Megaprep 2, Normalized Megaprep 3, and Normalized Megaprep 4.

Sample	Concentration (ng/uL)	A260/A280	A260/A230	A260	A280	Volume (mL)	Yield (mg)	Endotoxin Levels (EU/ug)
GigaPrep E1	537.0	1.98	2.38	10.74	5.42	30.82	16.55	< 0.009
GigaPrep E2	378.7	1.94	2.34	5.57	3.90	31.53	11.94	0.070
GigaPrep E3	362.6	1.92	2.35	7.25	3.77	28.71	10.41	0.014
GigaPrep E4	529.8	2.01	2.41	10.60	5.28	25.96	13.75	0.030

Table 3. Typical GigaPrep plasmid elution samples.

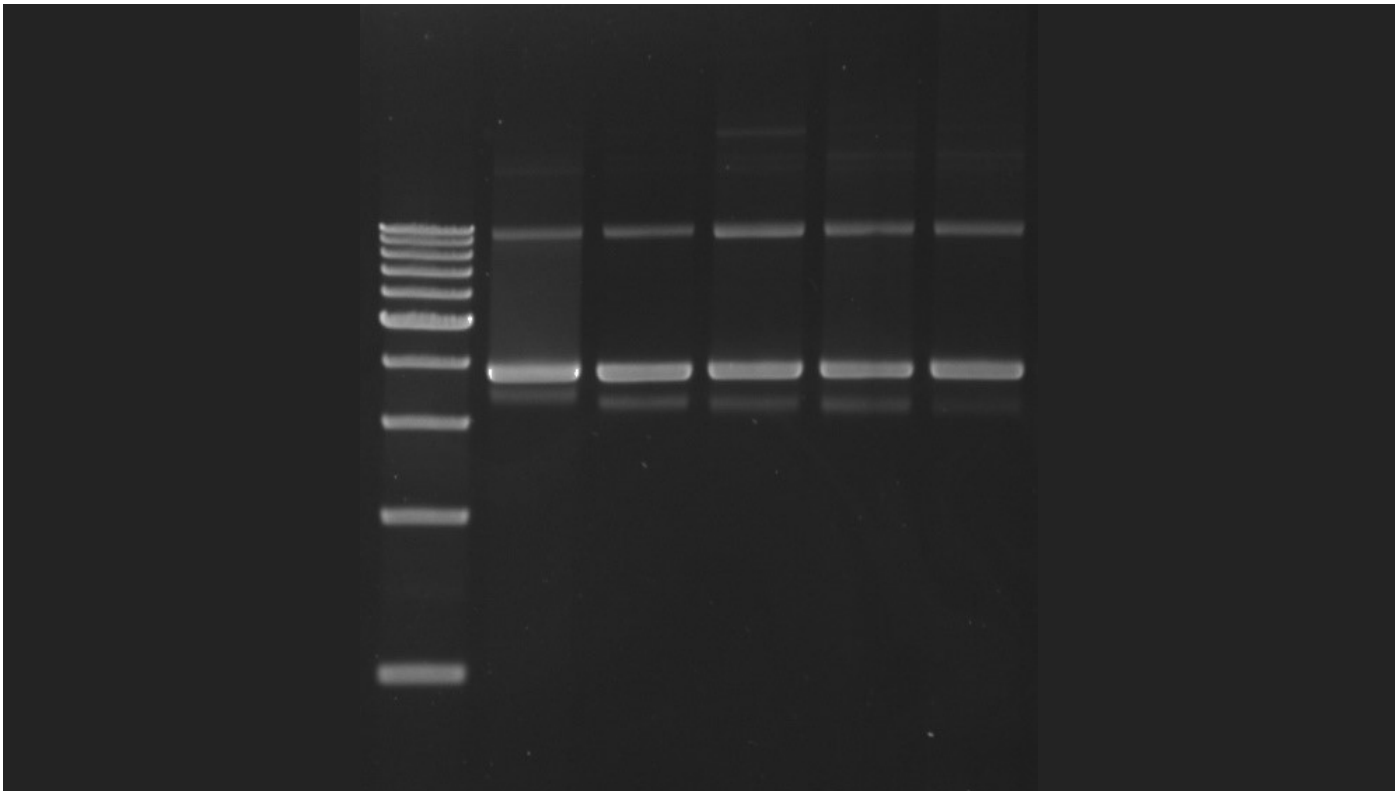

Figure 3. The GigaPrep plasmid elution samples from Table 2 were processed through gel electrophoresis where gel lane 1 – 6 from left to right is the DNA Ladder, Normalized Qiagen GigaPrep plasmid sample, Normalized GigaPrep 1, Normalized GigaPrep 2, Normalized GigaPrep 3, and Normalized GigaPrep 4.

Note: Cells are usually cultured and acquiring one cell pellet weight can be difficult to prepare depending on the cell culture, which is a common situation in the wet lab. Therefore, the sample prep kit processing capabilities is seen from using identical cell pellets at different ranges. For MaxiPrep, MegaPrep, and GigaPrep, 3 – 5 grams, 6 – 8 grams, and 13 – 16 grams of cell pellets were processed, respectively.

Sample Prep Kit	Cell Pellet Weight (g)	Conc. [ng/uL]	A260/ A280	A260/ A230	A260	A280	Volume [mL]	Yield [mg]	Endotoxin Lvl [EU/ug]
Maxi	1	133	1.890	2.510	2.660	1.410	5.45	0.7	< 0.038
Maxi	3	287	1.920	2.310	5.740	2.990	5.57	1.6	< 0.017
Maxi	4	339	1.900	2.300	6.780	3.560	5.03	1.7	< 0.015
Maxi	5	325	1.910	2.310	6.380	3.330	5.84	1.9	< 0.015
Mega	6	269	1.900	2.340	5.380	2.830	19.56	5.3	< 0.019
Mega	7	301	1.910	2.350	6.020	3.150	19.97	6.0	< 0.017
Mega	7.5	291	1.910	2.480	5.740	3.010	18.53	5.4	< 0.017
Mega	8	281	1.840	1.810	5.580	3.040	19.85	5.6	< 0.018
Giga	13	453	1.980	2.420	9.060	4.580	31.99	14.5	0.083
Giga	14	523	1.960	2.420	10.470	5.350	29.16	15.2	< 0.010
Giga	15	529	1.980	2.350	5.820	3.100	31.53	16.7	0.050
Giga	16	331	1.940	2.360	6.670	3.430	30.83	10.2	0.072

Table 4. Samples processed in a range of cell pellet weights for MaxiPrep, MegaPrep, and GigaPrep.

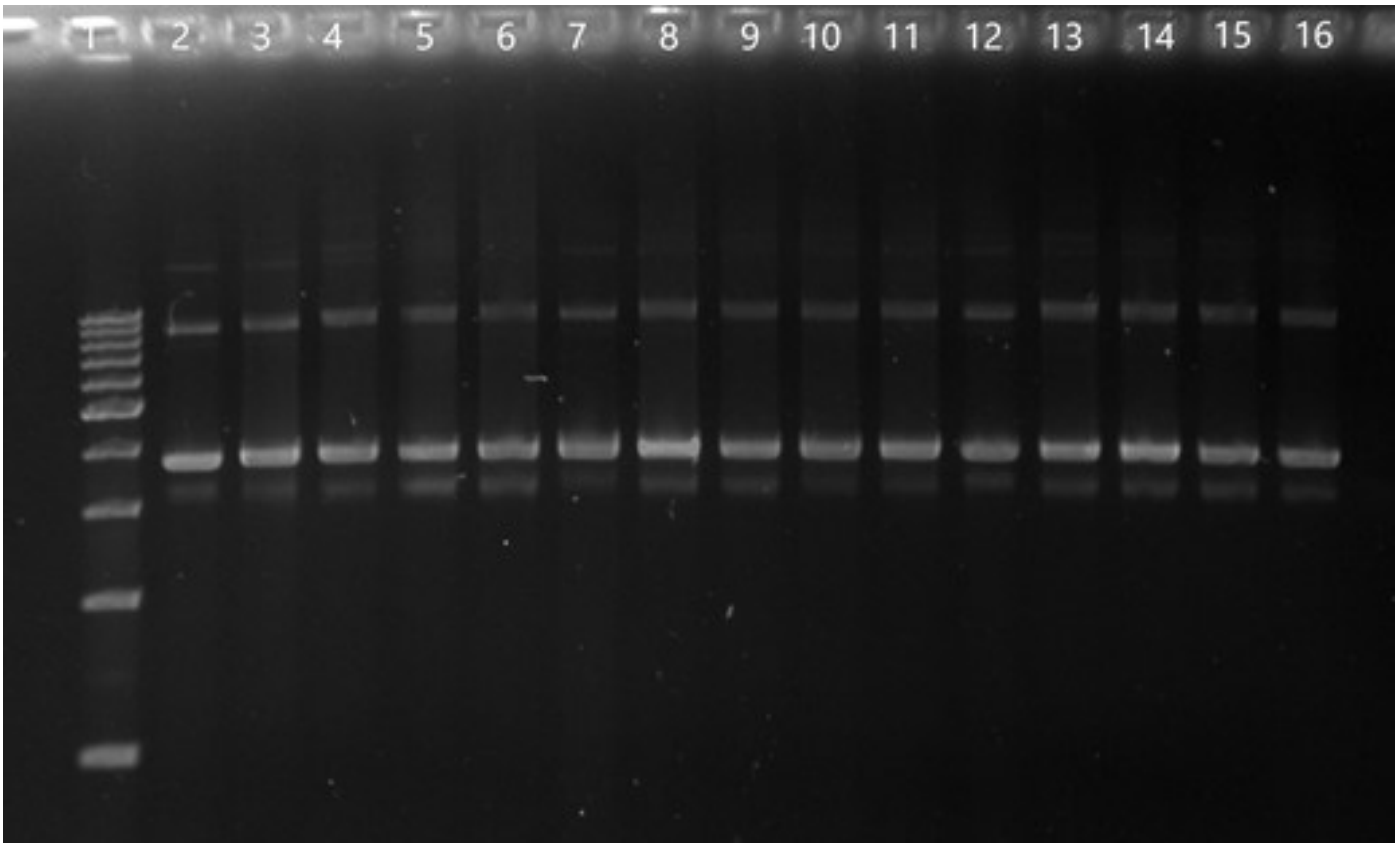

Figure 4. Samples processed using a range of cell pellet ranges for MaxiPrep, MegaPrep, and GigaPrep.

Lane	Sample
1	1kb Ladder
2	Qiagen Maxi
3	Maxi - 1g #1
4	Maxi - 3g
5	Maxi - 4g
6	Maxi - 5g
7	Qiagen Mega
8	Mega - 6g
9	Mega - 7g
10	Mega - 7.5g
11	Mega - 8g
12	Qiagen Giga
13	Giga - 13g
14	Giga - 14g
15	Giga - 15g
16	Giga - 16g

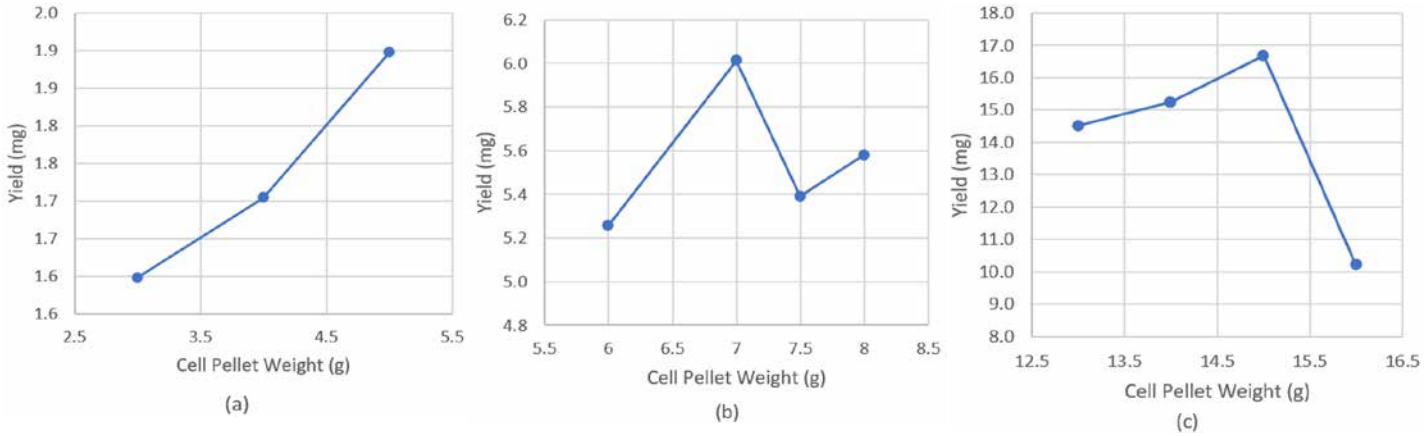

Figure 5. Purified plasmid yields from the corresponding cell pellet weights for (a) MaxiPrep, (b) MegaPrep, and (c) GigaPrep.

The PhyPrep results in highly pure, endotoxin-free, supercoiled plasmid DNA for downstream applications such as transient transfection and gene therapy.

To verify this, MaxiPrep, MegaPrep, and GigaPrep samples were outsourced for transient transfection to MirusBio.

Sample	Conc. [ng/uL]	A260/A280	A260/ A230	A260	A280	Elute Volume [mL]	Yield [mg]	Endotoxin Lvl [EU/ug]
QMX	1274.6	1.89	2.31	25.50	13.56	1.14	1.46	0.006
MX 1 -Before Alcohol Precip.	344.1	1.93	2.39	6.48	3.36	5.75	1.98	0.026
MX 2 - After Alcohol Precip.	1148.2	1.90	2.43	22.96	12.06	1.00	1.15	0.004
QMG	4746.5	1.91	2.30	94.93	49.66	1.06	5.03	0.007
MG 1 - Before Alcohol Precip.	313.2	1.90	2.34	6.26	3.29	17.94	5.62	0.016
MG 2 – After Alcohol Precip.	2450.0	1.89	2.40	49.00	26.86	1.02	2.50	0.014
QGG	4256	1.93	2.33	85.12	44.13	3.68	15.66	0.003
GG 1 - Before Alcohol Precip.	2375.6	1.94	2.35	7.35	3.80	28.71	10.78	0.014
GG 2 – After Alcohol Precip.	4223.6	1.91	2.39	84.47	44.14	1.13	4.77	0.060

Table 5. Nanodrop and endotoxin testing results of samples. Samples MX1 , MG1, and GG1 are from MaxiPrep, MegaPrep and GigPrep purifications, respectively, that did not undergo alcohol precipitation. The NanoDrop and endotoxin testing followed immediately after PhyPrep purification. Samples MX2, MG2, and GG2 are from MaxiPrep, MegaPrep and GigPrep purifications, respectively, that underwent alcohol precipitation and were resuspended in 1 mL of endotoxin-free water.

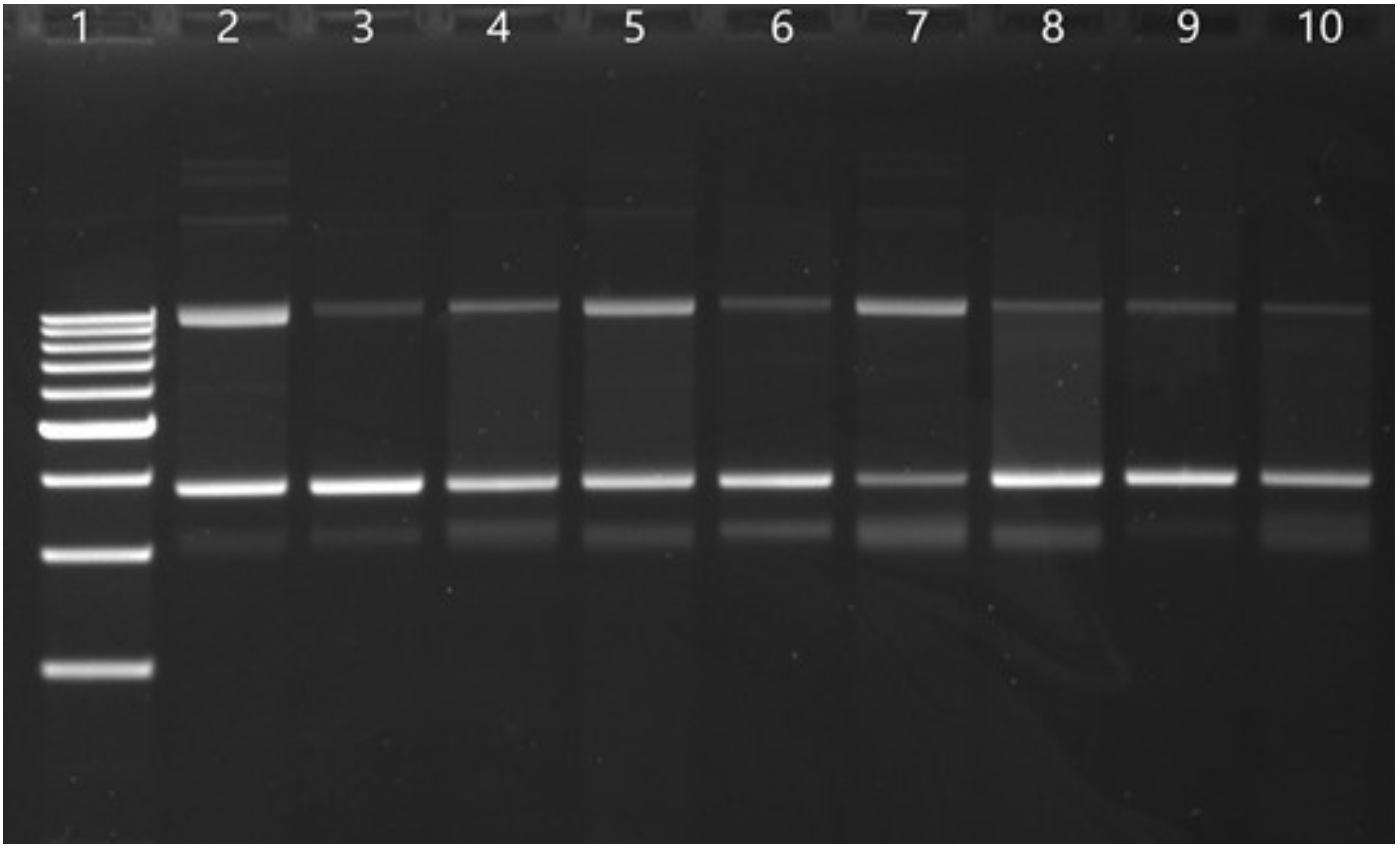

Figure 6. Plasmids processed by PhyPrep and Qiagen kits were submitted for transient transfection efficiency analysis. 100 ng of each sample were loaded to assess quality of the plasmid prior to transient transfection.

Gel Lane	Sample
1	1 kb Ladder
2	QMX
3	MX 1 – Before alcohol precip.
4	MX 2 – After alcohol precip.
5	QMG
6	MG 1 – Before alcohol precip.
7	MG 2 – After alcohol precip.
8	QGG
9	GG 1 – Before alcohol precip.
10	GG 2 – After alcohol precip.

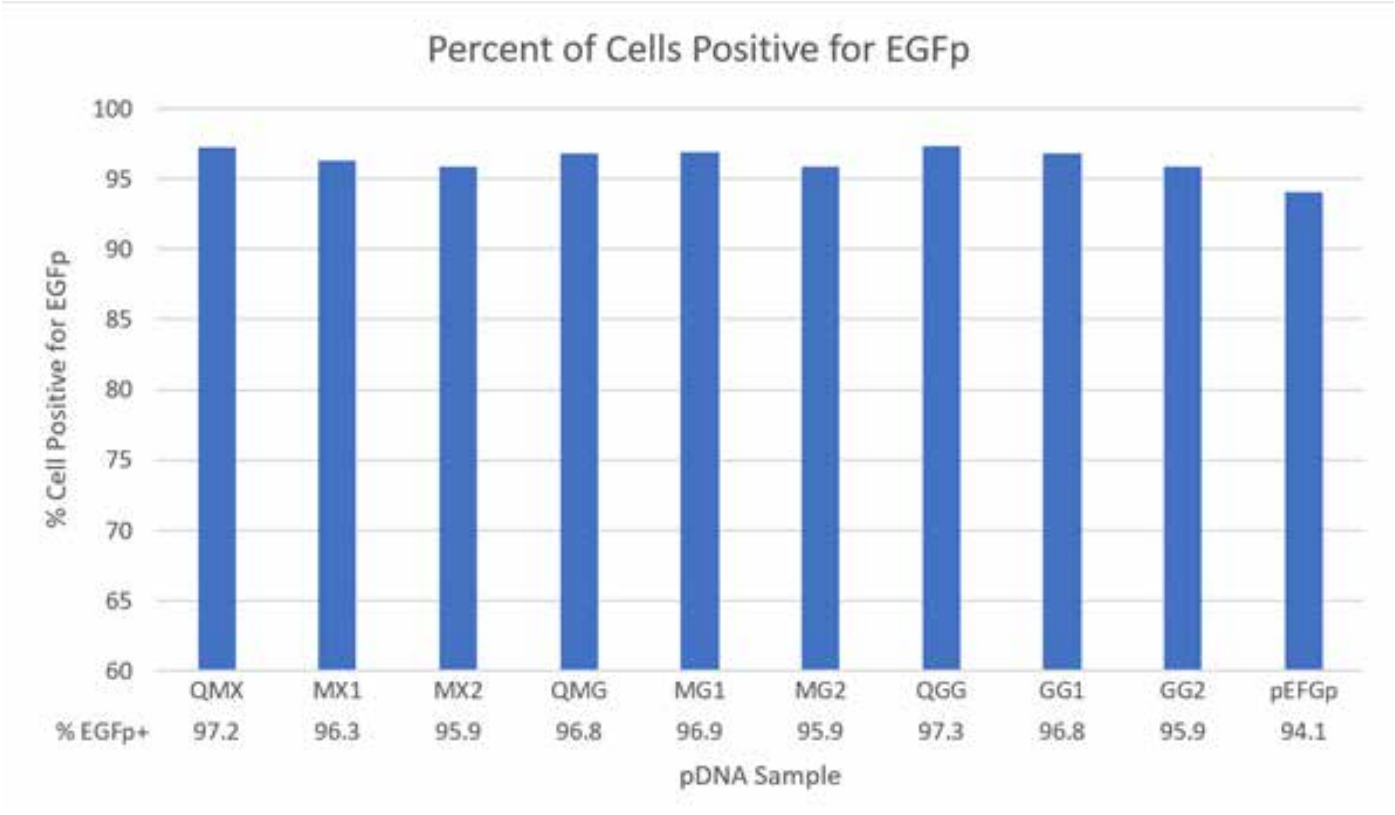

Figure 7. Results of transient transfection with samples from Table 8. Successful transient transfection is determined via the percent of cells positive for EGFP. In all cases, plasmids sucessfully transfected over 95% of the mammalian cells. pEFGp is an external plasmid used as a standard control for the transient transfection reagent.

EUROPE
Main Office: +46 18 565900
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 800 446 4752
Order Fax: +1 704 654 4917
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 68162810
Fax: +86 21 68162829
cn_order@biotage.com
cn-1-pointsupport@biotage.com

KOREA
Tel: +82 31 706 8500
Fax: +82 31 706 8510
korea_info@biotage.com
kr-1-pointsupport@biotage.com

INDIA
Tel: +91 11 45653772
india@biotage.com

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