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Development of a semi-automated high throughput transient transfection system

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ABSTRACT

Transient transfection of mammalian cells provides a rapid method of producing protein for research purposes. Combining the transient transfection protein expression system with new automation technologies developed for the biotechnology industry would enable a high throughput protein production platform that could be utilized to generate a variety of different proteins in a short amount of time. These proteins could be used for an assortment of studies including proof of concept, antibody development, and biological structure and function. Here we describe such a platform: a semi-automated process for PEI-mediated transient protein production in tubespins at a throughput of 96 transfections at a time using a Biomek FX^P liquid handling system. In one batch, 96 different proteins can be produced in milligram amounts by PEI transfection of HEK293 cells cultured in 50 mL tubespins. Methods were developed for the liquid handling system to automate the different processes associated with transient transfections such as initial cell seeding, DNA:PEI complex activation and DNA:PEI complex addition to the cells. Increasing DNA:PEI complex incubation time resulted in lower protein expression. To minimize protein production variability, the methods were further optimized to achieve consistent cell seeding, control the DNA:PEI incubation time and prevent cross-contamination among different tubespins. This semi-automated transfection process was applied to express 520 variants of a human IgG1 (hu IgG1) antibody.

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1. Introduction

Transient transfection of mammalian cells is a well-established method for protein production in the biotechnology industry. The use of mammalian cells for protein expression offers an advantage over prokaryotic or other eukaryotic systems due to its ability to promote correct folding and post-translational modifications for the expressed protein (Geisse and Fux, 2009). The benefit of mammalian transient over stable expression systems is the shorter timeline for material generation. CHO stable cell line generation may take 4–5 months whereas transient expression of secreted proteins requires only 7–14 days, depending on cell type and process (Baldi et al., 2007; Geisse and Fux, 2009; Pham et al., 2006). Suspension mammalian cell lines such as Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK) 293 cells have been widely used in industry for transient transfections both at larger scales up to 100 L in bioreactors and smaller scales in 50 mL tubespins (Baldi et al., 2005; Girard et al., 2002; Stettler et al., 2007). As such, transient transfections have been performed in a high throughput fashion for decades (Bennett et al., 1991).

Manual small scale transfections using tubespins, shake flasks and plates can be highly repetitive and executed in high quantities, making them an ideal candidate for automation. As such, 293 transfections have been automated for T flasks using a CompacT SelecT and 24 well plates with a Tecan Evo. The quality and quantity of secreted mammalian proteins produced from the automated and manual T flask transfection methods were found to be comparable (Nettleship et al., 2010; Zhao et al., 2011). However, thus far, there are no reports of an automated tubespin transfection system. Based on the optimal performance of transfections using tubespins (Stettler et al., 2007), we elected to automate the process.

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This process involves the batch transfection of 96 individual tubespins organized into a tray, 8 per column and 12 per row. Several approaches were taken to automate the manual tubespin transfection process and address the potential concerns associated with performing high throughput transient transfections. As with most high throughput processes that encompass large sample sizes, protein production consistency across the 96 individual tubespins is critical. Two factors that can influence transient protein production consistency are cell density and DNA:PEI complex addition. By utilizing a spinner bag agitated on a magnetic stir plate, we were able to prevent cell settling during the cell dispensing process and thus obtain comparable cell seeding densities among different tubes. Our data, along with previous studies (Bertschinger et al., 2008; Derouazi et al., 2004), shows that the incubation period for forming DNA:PEI complexes impacts DNA:PEI complex size and resulting transfection yields. This prompted us to program an automated complex addition method using the Biomek FX^P so that each tube received the DNA:PEI complex within a 9–10 min incubation range to minimize any decrease in protein expression due to increasing complex incubation time. Experiments were also conducted to confirm that no cross contamination occurred between tubespin columns during the DNA:PEI complex addition since the Biomek FX^P utilizes fixed, non-disposable tips for liquid transfer. This resulting semi-automated transient transfection process entails automated seeding and transfection and manual transfer of the tubespin rack from the incubator to the automated workstation. It is capable of performing 96 individual transfections in a single batch, making this a valuable tool for antibody development, expression screening and structure biology studies.

2. Materials and methods

2.1. Cell culture

The 293T cell line used for this study was a suspension adapted HEK293 cell line that was stably transfected with the SV40 large T-antigen. Cells were cultivated as a seed train in shake flasks under conditions of 37 °C, 5% CO_2, and 150 rpm agitation speed at a 25 mm throw diameter in an 80% humidified incubator before transient transfection. Gibco Freestyle 293 expression medium (Life Sciences, Carlsbad, CA) supplemented with 1% ultra-low IgG serum (Sigma, St. Louis, MO) was used as the seed train and production medium. Unless otherwise specified, all transient transfections were carried out in 50 mL tubespins (Stettler et al., 2007) with a 30 mL final working volume and processed in batches of 96. A Biomek FX^P liquid handling robot was used to bulk dispense cells into the 96 tubespins for efficiency. Post-transfection, cells were cultured for 7 days at 37 °C, 5% CO_2 and 225 rpm agitation speed at a 50 mm throw diameter in an 80% humidified Kuhner ISF1-X incubator.

2.2. Automation instrumentation

A system for automated cell culture process development had been previously designed and was adapted for this process. A key design decision was made early on to use 50 mL tubes as the reactors and to not use a decapper. SeptaVent™ tubes (Optimum Processing, Greenbrae, CA) are similar to the more familiar TubeSpin® 50 bioreactors (TPP, Trasadingen, Switzerland) in that they are 50 mL conical tubes with 0.2 μm filters in the cap for gas exchange. However, the SeptaVent also has a pre-slit silicone septum in the cap. This septum allows a narrow pipette or cannula to pass through the cap into the bioreactor and the slit closes as the pipette is removed. These tubes are kept in an 8 × 12 tray which fits on the deck of a Biomek FX^P robot (Beckman Coulter, Brea, CA) with a 96-channel pipetting head utilizing single-use pipette tips (96-tip head) and an 8 channel pipettor (8-tip span). The 96-tip head is equipped with Beckman's Enhanced Selective Tip option, allowing it to load fewer than 96 tips at a time. The Biomek liquid handler is inside a class II biological safety cabinet (Baker, Sanford, ME).

To work efficiently with the SeptaVent tubes, the robot is equipped with an 8-tip span with extra-long fixed tips. These tips can reach to the bottoms of the tubes to access the full culture volume, and eight reactors are accessed in parallel. Modifications to the base FX^P robot were made to enable aseptic operation with fixed tips. A selection valve was connected to the system fluid line that allows cleaning fluids such as Steriplex® (sBioMed®, Orem, Utah) to be pumped through to sanitize the fluidics and tips. This valve also allows media and cell culture fluid to be pumped through the system and into the cell culture reactors. Procedures were developed to clean and sanitize the system before and after each batch of 96 tubespins. A shorter procedure was also developed to clean the tips between reactors.

2.3. Standard and direct transfection methods

Cells were seeded at 1.0e6 cells/mL for transfection and incubated at 37 °C, 5% CO_2 for 2 h prior to transfection. Plasmid DNA encoding either a standard hu IgG1 antibody, or murine IgG2a (mu IgG2a) antibody was purified at the giga prep scale (Sigma, St. Louis, MO). For the standard transient transfection method, 30 μg of DNA was diluted in a DMEM-based medium to a final volume of 3 mL. Then 60 μL of 7.5 mM 25 kDa linear PEI was added to the DNA solution, mixed and incubated at room temperature for the indicated times before being added to the cells. For the direct transfection method (Raymond et al., 2011), the DNA:PEI ratio used was equivalent to the ratio used in the standard transfection method. 30 μg of DNA was added to a DMEM-based medium to give a final volume of 3 mL and incubated for 5 min. The DNA-media mixture was then added directly to the cells. Lastly, 60 μL of PEI was added to the cells to complete the direct transfection method process. For the serum free vs serum containing direct transfection comparison, the seeded cells were centrifuged at 25G for 10 min, and resuspended in an equal volume of serum containing or serum free media.

2.4. Cell count and protein concentration measurement

Viable cell density and viability of 293T seed train (and spinner bag post-seeding counts) were measured using a NyOne 96-well imaging system by SynenTech (Elmshorn, Germany). The NyOne instrument has been validated to produce less than 10% variability among replicates. Samples from tubespin cultures were collected using the Biomek FX^P and sampled with the NyOne employing trypan blue exclusion to determine viability and cell density. For protein concentration determination, supernatant samples were harvested from the tubespin cultures on day 7. Hu IgG1 antibody expression levels in the supernatant were determined using a Protein A HPLC assay. For the cross contamination assessment, hu IgG1 expression levels in the supernatant were measured using an intact hu IgG1 ELISA and mu IgG2a antibody expression levels were measured using an intact mu IgG2a ELISA.

2.5. Particle size and zeta potential measurement

Both DNA:PEI complex particle size and zeta potential were measured using the Brookhaven Instruments (Holtsville, NY) ZetaPALS (Zeta Potential Analyzer Utilizing Phase Analysis Light Scattering). For particle size determination, a 3 mL DNA:PEI complex was prepared in a DMEM-based complex medium, as

described in the transfection section. Each sample ($n = 1$) was analyzed on ZetaPALS and particle size measured every min for 60 min. For zeta potential determination, 3 separate DNA:PEI complex samples were prepared and incubated for 10, 30 or 60 min at room temperature. Each zeta potential measurement was measured after the specified incubation period.

2.6. Plasmid DNA isolation from tip wash

Samples from various stages of the tip washing sequence (initial flush, end of tip wash, 1 mL dispense postwash and post system clean) were subjected to 70% ethanol precipitation and salt extraction to isolate plasmid DNA. The isolated DNA was either directly resolved on a 0.8% agarose gel or first PCR amplified using forward primer (5′-GCACCCCAGGCTTTACAC-3′) and reverse primer (5′-TGTTATCCGCTCACAATTCC-3′) and then run on an agarose gel. 2 ng of the transfection grade plasmid DNA was PCR amplified as the positive control.

2.7. Antibody expression screen

Amino acid variants of a hu IgG1 antibody were generated by using the PCR based QuikChange site directed mutagenesis kit (Stratagene). Samples were incubated in a thermal cycler (Applied Biosystems, Foster City, CA) for initial denaturation (4 min at 94 °C) followed by 20 cycles with 0.5 min denaturation (94 °C), 0.5 min annealing (52 °C) and 10 min chain elongation (68 °C). PCR amplified DNA samples were incubated with DpnI restriction enzyme for 4 h at 37 °C. Competent cells (Novablue Singles) were transformed with DpnI-treated PCR samples. Plasmid clones obtained were screened by DNA sequencing to identify the amino acid substitution. DNA for the transfections was prepared in a semi-automated method using the QIAwell 96 Ultra Plasmid Kit (Qiagen, Hilden, Germany) and a Hamilton STAR liquid handler (Reno, NV). Yields were 40–60 μg from a 5 mL DH5α culture grown in LB with A260/A280 and A260/A230 ratios between 1.8 and 2.2. Semi-automated tubespin transfections were performed as described above. Supernatants from transfected cultures were incubated with protein A resin (MabselectSure, GE Healthcare, Pittsburg, PA) overnight. The next day the resins were transferred to filter plates, washed with PBS and then bound material was eluted with sodium citrate pH 3.0. Samples were further neutralized with 3 M Tris pH 8.0, dialyzed vs. PBS, and filtered. The concentration of the final purified material was obtained by measuring the O.D at 280 nm using a Nano Drop (Thermo Scientific, Wilmington, DE).

3. Results and discussion

3.1. The impact of DNA:PEI complex incubation time on transfection yield

The effectiveness of a DNA:PEI complex for transfection is impacted by the incubation time of the DNA:PEI complex, DNA:PEI ratio and media (Bertschinger et al., 2008; Derouazi et al., 2004). Since the Biomek FX^P robot utilizes a fixed 8-tip span, liquid dispensing is performed in a column-wise manner, 8 tubespins at a time, until all 12 columns have been processed. A fixed amount of time (~3–4 min) for the tip washing step is needed between addition of the DNA:PEI complex to the columns of tubespins to remove any residual DNA:PEI complex from the tips and prevent cross-contamination between tubespin columns. The amount of time required to add the DNA:PEI complexes to the tubespins and wash the 8-tip span between each column extends the length of time the DNA is incubated with the PEI transfection reagent. In shake flask studies, we observed that longer DNA:PEI complex incubation time resulted in lower protein expression from 293T transfections using

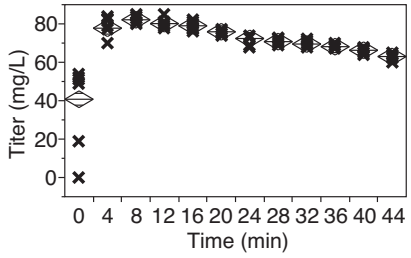


Fig. 1. The effect of DNA:PEI complex incubation time on protein expression from 293T transient transfections. 293T cells were transfected with DNA encoding for a hu IgG1 antibody. Transfection titers (mg/L) with increasing DNA:PEI complex incubation time (0–44 min). The plain text diamonds in the background represent the 95% confidence interval and mean (middle bar) for each time point.

PEI (data not shown). To assess the impact of DNA:PEI incubation times on titers from the Biomek FX^P semi-automated transfection process, we performed a DNA:PEI incubation time course experiment starting from 0 min (column 1, immediate transfer of DNA:PEI to the tubespin after PEI addition to the DNA plate) to 44 min (column 12) before addition to the tubespins. The time course data showed that the optimal DNA:PEI complex incubation time to maximize protein expression was between 8–12 min with a peak protein expression of approximately 80 mg/L (Fig. 1). Adding DNA:PEI to the tubespins without incubation (column 1) resulted in ~50% lower mean titers and greater variation than the optimal 8–12 min incubation (Fig. 1). Protein expression also decreased with longer incubation time; at 44 min there was approximately a 23% decrease in protein expression relative to tubespins in which the DNA:PEI complex was added within 8–12 min (Fig. 1).

3.2. Zeta potential and particle size of DNA:PEI complexes over time

The decrease in protein expression from DNA:PEI complexes with extended incubation times could be due to changes in the physical properties of the DNA:PEI complex during incubation. An overall positive DNA:PEI complex charge may be advantageous for promoting interaction with the negatively charged plasma membrane to facilitate uptake into the cell (Patino et al., 2012). Smaller DNA:PEI complex sizes could also lead to higher transfection efficiency (Putnam, 2006). However, particle size or charge (zeta potential) of DNA:PEI complexes have been shown to change over time (Sharma et al., 2005). To determine whether a change in DNA:PEI complex size was responsible for the decreased titer with longer DNA:PEI complex incubation times, we measured DNA:PEI complex particle size and zeta potential over time. For particle size measurement, a single DNA:PEI complex was prepared according to the standard DNA:PEI complex preparation protocol. The sample was then placed in ZetaPALS and particle size measurements were taken from 0 to 60 min. DNA:PEI complex particle size increased over the range of 0–60 min from 200 nm to 800 nm (Fig. 2A), with DNA:PEI complexes formed at 8 min (400 nm) correlating with the highest transfection yield. The increased particle size could be due to the DNA:PEI complexes aggregating to form larger complexes over time. This suggests that the larger DNA:PEI complexes are less efficiently internalized into the cell, resulting in lower protein expression. The zeta potential of DNA:PEI complexes (Fig. 2B) did not vary significantly at the time points tested and is an unlikely influencing factor on the protein expression decrease over time. Therefore, it appears that the decrease in protein expression with longer DNA:PEI complex incubation time is associated with increased DNA:PEI size.

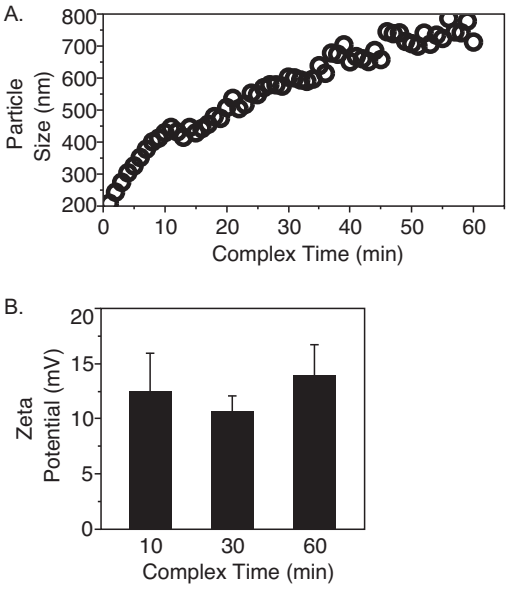


Fig. 2. The effect of incubation time on DNA:PEI complex size and zeta potential. Particle size and zeta potential measurements determined using Brookhaven Instruments ZetaPALS. (A) Particle size by time (B) Zeta potential over time. Mean values ± SD of 10 separate measurements of one DNA:PEI complex sample.

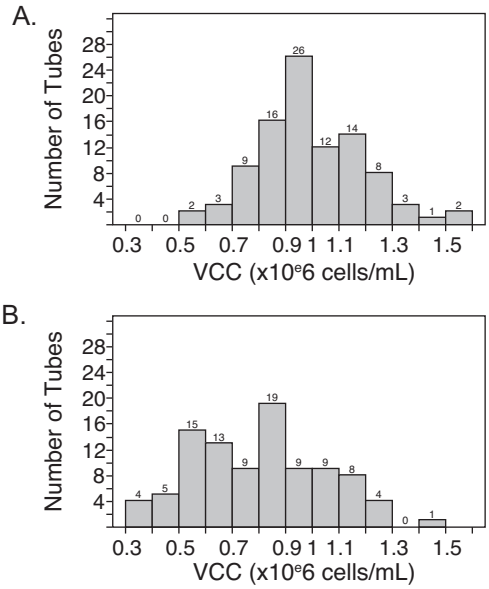


Fig. 3. Assessing the consistency of bulk cell dispensing for automated tubespin transient transfections. Cells were dispensed from a 5 L spinner bag containing 4.5 L of 293T cells at a target seeding density of 1.0e6 cells/mL into 96 individual tubespins using a Biomek FX^P robot. Cells were dispensed into tubespins by column (n = 8) and viable cell counts and cell viability were determined using a Synentec NyOne. (A) Histogram of viable cell count for tubespin tray (n = 96) seeded with agitation by magnetic rod. The values above each bar represent the number of tubes for the viable cell count range displayed on the x-axis. The mean viable cell count was 1.0e6 cells/mL with a % CV of 20.2. The mean viability count was 92.0% viable with a SD of 1.9% and a % CV of 3.6. (B) Histogram of viable cell count for tubespin tray (n = 96) seeded without agitation by magnetic rod. The values above each bar represent the number of tubes for the viable cell count range displayed on the x-axis. The mean viable cell count was 0.8e6 cells/mL with a % CV of 30.3. The mean viability count was 94.0% viable with a SD of 2.2% and a % CV of 2.4.

3.3. Bulk cell dispensing consistency

Both initial seeding density and cell viability are key factors that can potentially affect protein expression from transient transfections (Liu et al., 2008). Variation in initial cell seeding density, which alters the proportion of cells to DNA:PEI complexes, could result in variable protein production from the 96 separate transfections within a tubespin tray. Additionally, cell viability lower than 90% has been observed to be detrimental to protein expression (data not shown). To establish a method for consistent cell seeding across the 96 tubespin tray, we compared cell counts from two full trays seeded utilizing an agitated or non-agitated spinner bag. 5 L single use plastic bags (Parker, Inc.) containing magnetic stir bars were filled with 4.5 L of cells from the seed train (~4.0e6 cells/mL) diluted in fresh media to the transfection density of 1.0e6 cells/mL. The agitated spinner bag was placed on a magnetic stir platform to promote homogenous cell mixing prior to dispensing into the 96 tubespins. Cells from the spinner bag were added column-wise into the 96 tubespin tray using the 8-tip span on the Biomek FX^P liquid handling robot. After seeding, the tubespins were immediately sampled and arrayed to a 96-well microplate for viability and cell density measurements. The same method was then repeated to dispense and sample 96 tubespins seeded with a non-agitated spinner bag. Both agitated and non-agitated tubespin seeding cases had cell viabilities higher than 90%. Tubespins with agitated seeding had a mean viable density of 1.0e6 cells/mL and showed a normal distribution which indicates that the target seeding was consistently achieved across the entire 96 tubespin tray (Fig. 3A). Tubespins seeded in conjunction with a non-agitated spinner bag had a mean viable density of 0.8 × 10e6 cells/mL and showed an abnormal distribution (Fig. 3B). The high proportion of below-target seeding density was likely attributed to the non-homogenous cell culture mixture that developed over time due to cell settling. The agitated spinner bag has a significant effect on consistent cell seeding and is therefore critical to include in the implementation of the semi-automated transfection process to reduce variability and maximize transfection yields.

3.4. Automating the standard transfection method

The direct transfection method, which entails separately adding DNA and PEI directly to cell culture (Raymond et al., 2011; Schlaeger and Christensen, 1999), was tested as a potential way to automate the activation of the DNA:PEI complex and transfer the complex to the cells. However, the direct transfection method resulted in 70% lower titers than the standard transfection method (Fig. 4A). One possible reason for this may be that the direct transfection method was previously optimized for a serum free transfection process and our process contains 1% serum in both the seed train and production media. The 293T cell line used in our transfection process achieves peak productivity with serum containing media. Removing serum decreased titers significantly and experiments to optimize DNA and PEI ratios in the absence of serum did not produce further yield improvement (data not shown). Components in the serum could interfere with formation of the DNA:PEI complex, resulting in the lower protein expression observed with the direct transfection method. In order to test this hypothesis, we performed transfections with cells that were medium exchanged into serum containing or serum free media. The direct serum free transfection (Direct S−) yielded approximately 30% lower titer than the serum containing direct transfection (Direct S+) (Fig. 4B). This is similar to the titer differences we have observed in the past with serum free vs serum containing 293T transfections (data not shown). It also indicates that the lower yield from the direct transfection method relative to the standard transfection method was not due to the presence of serum. It has been shown that optimization of cell density, PEI and DNA concentrations for the direct transfection method improves the yield (Raymond et al.,

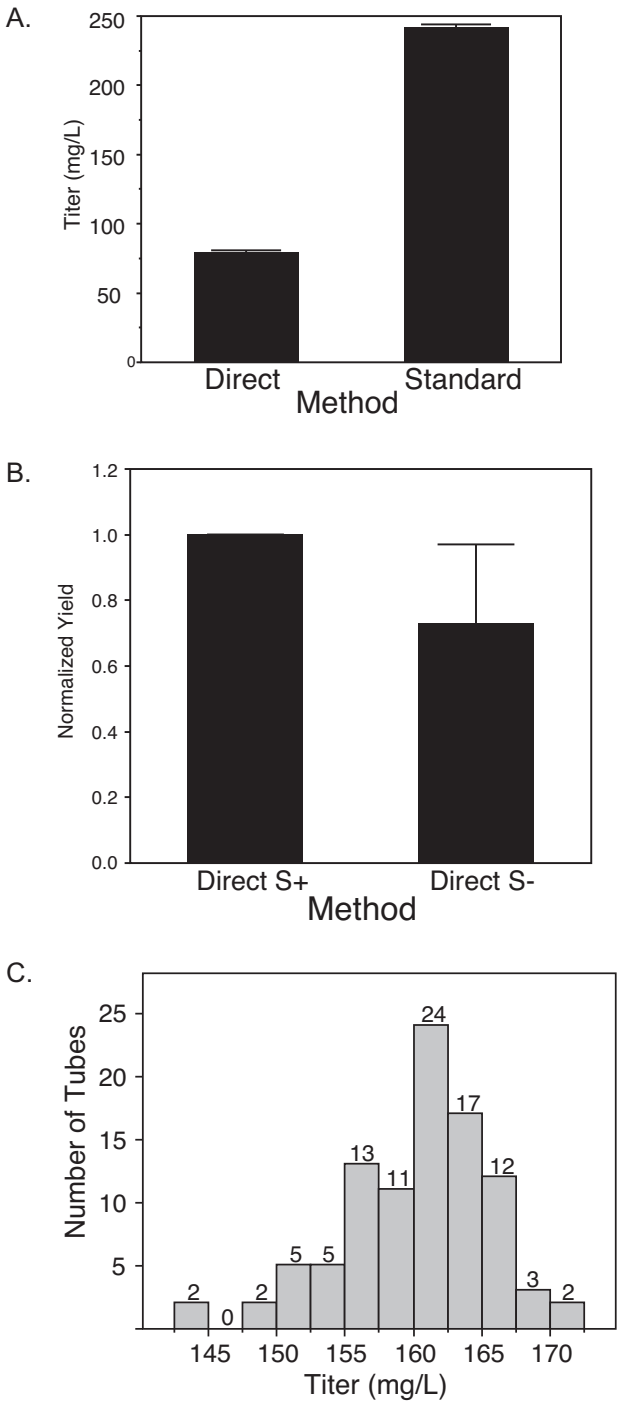


Fig. 4. Automation of the tubespin transfection method. 293T cells were transfected with DNA encoding for a hu IgG1 antibody. (A) Comparison of antibody expression titers (mg/L) for direct vs standard transfection method. (B) Comparison of the direct transfection method performed in either serum containing medium (Direct S+) or serum free media medium (Direct S−). Mean Direct S− yields were normalized to the Direct S+ transfection yields ($n = 2$ for each case). (C) Antibody expression (mg/L) using the automated standard transfection method. The DNA:PEI complex incubation time range for columns 1 through 12 was approximately 9–10 min. The DNA:PEI complex addition method yielded a mean of 160 mg/L, a SD of 5 mg/L and a % CV of 3. The values above each bar represent the number of tubes for the titer range displayed on the *x*-axis.

2011). Thus, expression levels from our direct serum containing transfection method could be higher if additional optimization was performed. Rather than optimize the direct transfection method, we elected to use an automation approach with the standard transfection method to address the decrease in titers observed with increased complex incubation time. Previous findings from direct transfection optimization demonstrated that the ratio of DNA:PEI for direct transfections (1:3) was higher compared to the standard transfection method (1:2.5) (Raymond et al., 2011). An automated standard transfection approach could mitigate potential cost increase associated with higher reagent volumes.

The potential for longer complex incubation times associated with the standard transfection method can be mitigated through the use of skillfully timed and stepwise automation. The standard transfection method was implemented by utilizing scripting that controls both pipetting heads of the Biomek FX^P simultaneously for activation of the DNA:PEI complex and addition of the complex to the cells. The 96-head with Enhanced Selective Tips was used to add PEI to DNA one column (8 wells) at a time. The 8-tip span head was used to transfer the DNA:PEI complexes to the tubespins containing 293T cultures, 8 tubes at a time. For each column, the PEI was added to the DNA, the complex was incubated, the DNA:PEI complex was transferred to the cultures, and the 8-tip span fixed tips were washed to prevent cross-contamination. The columns were staggered to start a column every 180 s and a series of timers were used to keep the DNA:PEI complex incubation time between 9 and 10 min for all cultures. 180 s was empirically derived as being sufficient for the 8-tip span head to transfer the DNA:PEI complex and go through the tip washing sequence, so both pipetting heads could remain synchronized. Using this methodology on the Biomek FX^P, high consistency of protein expression was observed from 96 separate tubespin transient transfections following the standard transfection method (Fig. 4C). While we have observed titers as high as 280 mg/L using the 293T expression system, the lower range of titers seen in this set of transfections may be the result of extended seed train cell age. The cells transfected in Fig. 4A were 74 days old whereas cells used for transfections in Fig. 4C were a separate thaw that was 96 days old. Cell age of HEK293 cells has been shown to impact transfection titers (de Los Milagros Bassani Molinas et al., 2013). Overall, the semi-automated transfection process was successfully developed and optimized for timing efficiencies around the requirements of a serum containing 293T transfection process to maximize productivity. Furthermore, due to the high throughput nature of the system it was necessary to employ a transfection system that was economical. Other commercially available serum free 293 expression systems require the use of costly liposomes and/or media which were not considered for our use but can be easily adapted to this semi-automated process.

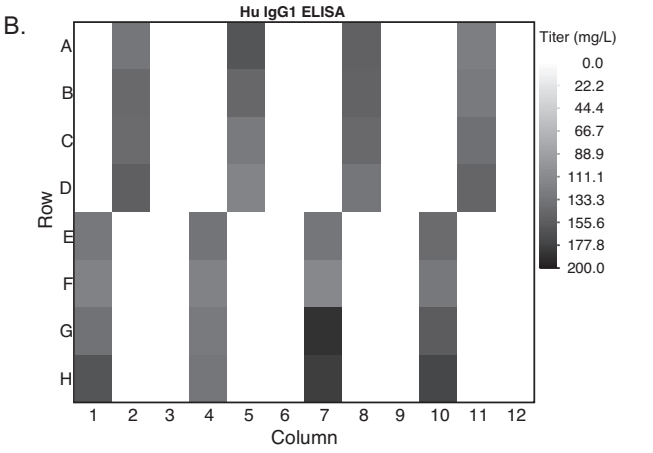
3.5. Cross-contamination prevention

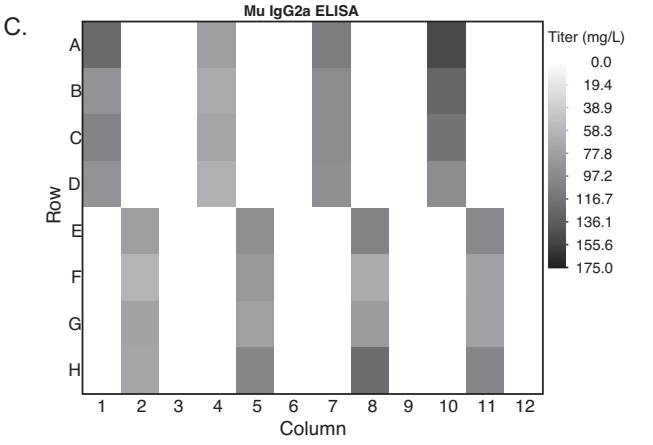
Since the Biomek FX^P utilizes a fixed 8-tip span to transfer the DNA:PEI complex into a column of tubespins containing the cells it was important to maintain sterility and prevent cross-contamination between the columns. Any residual DNA:PEI complex carried over from column to column could lead to cells expressing proteins other than the protein of interest. To prevent

Fig. 5. Analysis of DNA contamination after standard wash steps. DNA was precipitated by ethanol and salt after the wash steps, PCR-amplified and run on a 0.8% agarose gel. Initial PBS flush (lane 1), end of tip wash (lane 2), dispense postwash (lane 3), postsystem clean (lane 4).

A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.
B	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.
C	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.
D	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.	Mu	Hu	V.O.
E	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.
F	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.
G	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.
H	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.	Hu	Mu	V.O.





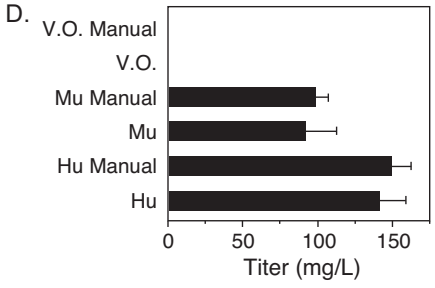


Fig. 6. Assessment of cross contamination due to the Biomek FX^P robot utilizing non-disposable tips for liquid transfer. (A) Layout of DNA plate prepared in a staggered fashion using human (Hu) antibody construct, murine (Mu) antibody construct or vector only (V.O.). (B) Hu IgG1 ELISA titer results from the automated transfections. The tubespins with detectable hu IgG1 had a mean titer of 141.2 mg/L with a SD of 17.9 mg/L. The hu IgG1 ELISA minimum and maximum detectable limits are 0.082 mg/L and 1000 mg/L, respectively. (C) Mu IgG2a ELISA titer results from the automated transfections. The tubespins with detectable mu IgG2a expression had a mean titer of 91.8 mg/L with a SD of 20.8 mg/L. The mu IgG2a ELISA minimum and maximum detectable limits are 0.156 mg/L and 1250 mg/L, respectively. (D) Expression levels of hu IgG1, mu IgG2a and V.O. from automated vs manual transfections. Mean values ± SD were obtained from $n = 32$ (automated) and $n = 2$ (manual) transfections.

cross contamination between columns of transient transfections on a 96 tubespin tray, a tip washing method was developed to remove any residual DNA:PEI complex from the tips. The tip washing steps include first flushing 5 mL of Phosphate Buffered Saline (PBS) through the fluid lines (initial flush), then rinsing the tips with a series of different reagents in the following order: Steriplex®, deionized water, 70% alcohol (end of tip wash), deionized water, and PBS (1 mL dispense postwash), then a second 5 mL PBS flush of the fluid lines (postsystem clean). The samples were collected after the initial flush, after the 70% alcohol wash, after the PBS tip wash, and after the second PBS flush. After the series of flushes, the 8-tip span pulls up the next set of DNA:PEI complexes from the deep well plate and dispenses them to the next column of tubespins containing cells. DNA was precipitated from samples collected from the 4 wash steps, PCR amplified and resolved on an agarose gel. While DNA was present in the initial flush, it was not present after the tip wash sequence was completed (Fig. 5).

Further confirmation of adequate tip washing was obtained via analysis of the transfection product. Two separate deep well plates each containing 48 separate DNA:PEI complexes in columns 1–6, were prepared with media and DNA and added to the 96 tube spins for 30 mL transfections. Plates were prepared with DNA encoding a murine antibody, human antibody or vector only negative control, resulting in the transfected tubespin tray pattern shown in Fig. 6A. After 7 days of transfection, human and murine antibody expression was quantitated using isotype-specific human and murine ELISAs. Since the fixed 8-tip span on the Biomek FX^P adds the DNA:PEI complexes by column, if any cross contamination occurred between tubes due to residual DNA:PEI complex, both human and murine antibodies could be detected via ELISA in samples from a tube. The hu IgG1 ELISA detected expression only in tubespins transfected with DNA encoding the human antibody (Fig. 6B). The tubespins with detectable hu IgG1 had a mean titer of 141.2 mg/L with a SD of 17.9 mg/L. The mu IgG2a ELISA detected expression only in tubespins transfected with DNA encoding a murine antibody (Fig. 6C). The tubespins with detectable mu IgG2a expression had a mean titer of 91.8 mg/L with a SD of 20.8 mg/L. Thus both the PCR analysis of wash steps (Fig. 5) and ELISA quantitation of the supernatant samples (Fig. 6) demonstrate that the series of tip washes successfully remove any residual DNA:PEI complex from the tips, minimizing the risk of sample contamination between columns. Additionally, the expression levels from cultures transfected using the Biomek FX^P semi-automated system were comparable to the expression levels from manually transfected cultures (Fig. 6D).

3.6. Antibody expression screen

The semi-automated transfection method was successfully used as an approach to screen a large number of hu IgG1 antibody mutants (Fig. 7). A total of 520 hu IgG1 variants including wild-type (done in triplicate), were expressed over multiple runs of the semi-automated process and purified with protein A resin. Overall, the total amount of purified protein was sufficient for downstream characterization and binding studies and other screening applications. Furthermore, the success and reliability of the high throughput semi-automated transfection system, demonstrated by the generation of 520 IgG1 variants, can be easily adapted for various other projects and cell types if desired.

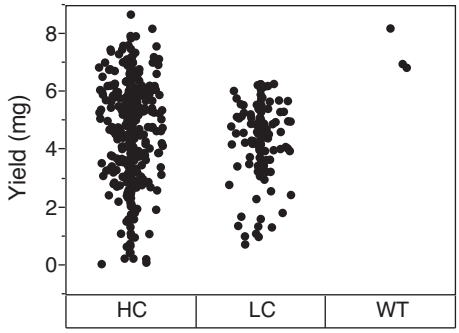


Fig. 7. Total purified yield from transfections to express hu IgG1 antibody variants. Cultures expressing heavy chain (HC) mutation variants ($n = 390$) had a mean yield of 4.7 mg with a SD of 1.7 mg. Cultures expressing light chain (LC) mutation variants ($n = 127$) had a mean yield of 4.4 mg with an SD of 1.3 mg. Cultures expressing wild type (WT) mutation variants ($n = 3$) had a mean yield of 7.3 mg with a SD of 0.75 mg.

4. Conclusions

In the present work we describe the development of a semi-automated high throughput PEI-mediated 293T transient transfection process using a Biomek FX^P liquid handler with tubespins. It should be noted that although our 293 transfection process utilizes serum, serum free 293 transfections are prevalent across the industry. We have observed that our 293T transfection process is more robust, exhibiting less variability across different thaws and cell ages, compared to commercial serum free 293 transfection systems (data not shown). Nevertheless, the objective of this report is to describe the establishment of an automated transfection process in tubespin vessels which can be applied to various cell lines grown in different types of media. This process offers a way to express a wide array of different proteins for research purposes in a short amount of time. The popularity of genome-scale technologies, such as transcriptomics and proteomics, makes high throughput protein expression systems a valuable tool to perform follow up expression screening and structure biology experiments. In addition, this system can be employed to produce proteins for initial panning and screening studies associated with therapeutic antibody discovery.

Various steps were optimized to utilize a Biomek FX^P liquid handler to perform semi-automated transfections. To develop a consistent, high throughput transient transfection system, we investigated several key factors including consistency in seeding density, controlling DNA:PEI complex incubation timing and preventing cross-contamination. An agitated 5 L spinner bag containing the cells to be transfected at the target seeding density was implemented to assure consistent cell dispensing and cell viability across a 96 tubespin tray. A decrease in protein expression was observed from 293T PEI-mediated transient transfections with longer DNA:PEI complex incubation times. This posed a potential concern when using the Biomek FX^P liquid handler due to the amount of time needed to add the DNA:PEI complexes to the cells combined with the time needed to wash the 8-tip span to prevent cross contamination. A staggered DNA:PEI complex addition method using both tip heads on the Biomek FX^P was developed to maximize the protein expression from a tray of 96 tubespin transfections by keeping the DNA:PEI complex incubation time within 9–10 min. Finally, a series of wash steps designed to remove any residual DNA:PEI from the 8-tip span was validated to prevent cross contamination between tubespin cultures. This semi-automated system, which yields comparable titers to manual transfections, provides substantial advantages in throughput capacity and ergonomic savings over manual methods. It was used to efficiently express over 500 antibody variants and has been applied to CHO and other 293 cell lines with similar success. Using this instrument, one could rapidly express thousands of different recombinant proteins or antibody variants to support discovery research studies.

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