October 2021

Investigation of an Alternative Protocol for the Production of SARS-CoV-2 Antigenic Proteins

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Investigation of an Alternative Protocol for the Production of SARS-CoV-2 Antigenic Proteins

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
with a concentration in Global Communicable Disease
Department of Global Health
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University of South Florida

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Date of Approval:
October 28, 2021

Keywords: Spike Protein, Polyethylenimine, Transfection, HEK293, Protein Expression

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Abstract

With the COVID-19 pandemic showing no signs of slowing down, large-scale antigenic protein production is still needed for surveillance using serologic assays. From screening to vaccines to biotherapeutics, being able to produce the proteins for these assays is essential; however, the current gold standard method for producing SARS-CoV-2 spike proteins is prohibitively expensive for most research groups.

Alternative methods of transfecting mammalian cells to produce recombinant proteins that are relatively inexpensive have been used for years. Unlike the expensive, commercially available lipid-based methods, other established methods such as polyethyleneimine (PEI), are considerably easier, and cheaper to meet the needs of most laboratories.

This thesis research evaluated ExpiFectamine compared to PEI as methods to produce the antigenic receptor-binding domain (RBD) of SARS-CoV-2 Spike protein for standard serologic assays. The results obtained from the PEI method had consistently lower yields with different concentrations of PEI and different incubation periods, yielding 20-90% less protein than the ExpiFectamine method. In addition, the purity and functionality of the proteins in standard serologic assays were less than with the ExpiFectamine method.

Therefore, we could not conclude if the quality of the recombinant spike protein using the PEI method is adequate to serve as a substitute for ExpiFectamine product in standard serologic assays. While the significantly lower cost offers a viable alternative for many research

studies, the poorer yield and purity in our studies indicate further optimization is needed to assure reliable production of high-quality recombinant protein.

Introduction

SARS-CoV-2, a betacoroanvirus discovered in December 2019 [1] is responsible for the ongoing COVID-19 pandemic that has devastated the world for nearly two years. In that time, the virus has killed over 4.5 million people and infected over 200 million, costing trillions of dollars in lost life, health, and revenue [2] [3]. Though the case fatality rate varies from country to country, on average 2.5% of those infected with SARS-CoV-2 will die [4]. Infection rates in the United States continued to rise as of June 2021, with 75 infections per 100,000 people in a single week [5].

Even after the introduction of vaccines, the virus has continued to spread, mutate, and cause disease, showcasing a need to track immunity and prevalence in the population. There are many ways to do this, and many have a common factor: the need for antigenic proteins. However, the methods used for the CLIA approved diagnostic assays are prohibitively expensive for basic research and surveillance methods relatively to other standard laboratory methods. This thesis outlines a protocol for a cost-effective production of SARS-CoV-2 antigens using HEK293F cells that is comparable to one of the earliest standards of protein production outlined in Amanat et al [6].

COVID-19

COVID-19 is the common name for the disease caused by SARS-CoV-2. Typical manifestations of the disease include ground glass opacity in at least two lobes of the lungs [7] ,

cough, anosmia, and fever [8] In severe cases, pneumonia can progress to acute respiratory distress syndrome (ARDS), hypoxemia, cardiomyopathy, and death [8]. Severe COVID-19 is often associated with an influx in the lungs of proinflammatory cytokines (TNF-α, IL-1, IL-6, GM-CSF) which is often referred to as cytokine storm [9].

All age groups can have the disease. Most common are the adults aged 20-60 [10] with those in long-term care or with pre-existing conditions such as kidney disease, chronic lung disease, diabetes, and immunocompromising conditions at the greatest risk of severe disease and death [11]. COVID-19 usually causes only mild-to-moderate [12] disease in children although Kawasaki-like disease manifestations have been observed [8, 13].

SARS CoV-2 is a highly transmissible virus with an initial R_0 of 2-3 [4, 10], though the recent Delta variant is estimated to have a R_0 of 6-7 [ref]. Much of this transmission is driven by asymptomatic cases that can transmit virus as effectively as those with symptoms [14]. The proportion of asymptomatic cases has been difficult to determine and ranges from 30-65% [14-16] Since drivers of infection were invisible to the traditional symptoms-based screening techniques, the World Health Organization recommended universal face masks in public settings [16] which significantly reduced rates of transmission where implemented [17]. However, the national reporting of safety measures did not reflect what was being carried out at state level, and average utilization of such preventative public health measures peaked at 75% [18].

There are few treatment options available except in cases of severe or life-threatening disease [11] which makes prevention critical in stopping the pandemic. Currently there is one vaccine, produced by Pfizer and BioNTech that has FDA approval for adults 16 and over[19] and two additional vaccines are available in the United States under Emergency Use Authorization [20, 21]. Two, including Pfizer's, are mRNA-based multi-dose vaccines [22, 23] and one uses an

adenovirus vector [24]. As of June 2021, only 55% of the US population has been fully vaccinated [25] and this is considerably lower than 85-90% needed to break the continuous chain of transmission [26].

With the vaccination rates stalling out and asymptomatic cases driving infection, the need for monitoring systems that do not rely on symptoms alone is needed. One way to track the movement of the virus through a population, or past infections in the unvaccinated, is to screen for antibodies to the virus. Antibodies to the spike protein receptor binding domain (RBD; discussed in the next section) stay in the body for up to 8 months, making them a useful hallmark for past infection [27].

SARS- CoV-2 and the Spike protein Receptor Binding Domain

SARS-CoV-2 is a positive-sense single stranded RNA virus with a ~30 kilobase genome [28]. It is a member of the *Betacoronanvirus* genus, including SARS-CoV-1 and MERS-CoV, which shares a 80% genetic identity [28].

The virus infects cells of the alveoli and upper respiratory tract by way of its spike (S) protein; a homotrimeric class I fusion protein [29] of about 2,000 amino acids [6] present on the viral surface in one of three conformations: post-fusion, pre-fusion up, or pre-fusion down [30]. S is made up of two subunits: S1, which contains the signal peptide, N-terminal domain (NTD) and receptor binding domain (RBD), and S2, which contains the internal fusion peptide, heptad repeats, and transmembrane domains [28]. S is also heavily glycosylated, allowing for immune evasion by epitope shielding [31].

The host receptor that facilitates viral entry is angiotensin converting enzyme 2 (ACE2), a regulatory mediator of the circulatory system. The binding of RBD to ACE2 in alveolar and upper respiratory epithelia cells [31, 32] induces a conformational change in the S trimer from a

blunt flail-like structure to a thinner needle-like structure, that initiates fusion with the host cell membrane [33, 34].

While betacoronaviruses typically have slower rates of genetic mutation than other coronaviruses [35], new variants of SARS-CoV-2 have been common. These variants contain key mutations to the spike protein and RBD, (table 1). The CDC is currently monitoring ten variants [36].

Table 1. Comparison of SARS-CoV-2 variants Alpha and Delta

SARS-COV-2 VARIANT	**ALPHA**	**DELTA**
Pango Lineage [36]	B.1.1.7, Q.1-Q.8	B.1.617.2 and AY lineages
Origin [36]	United Kingdom	India
Date of Variant of Concern designation [36]	12-29-2020	5-7-2021
Characteristics	Increased Binding affinity to ACE2 [37]	Increased Transmissibility [39]
	Increased Resistance to NTD antibody [38]	Decreased mAb treatment efficacy [39]
	Decreased Transmission rate [36]	Decreased Neutralization by post-vaccine sera [36]
RBD mutations of note	N501Y [40]	L452R, E484Q [39]

Protein Expression in Mammalian Cells

Many biopharmaceuticals require recombinant proteins from the pathogen of interest, usually these are the antigens that can elicit an immune response. In the case of SARS-CoV-2, antigenic proteins serve two important applications, antibody screening and vaccine development.

Antibody screening can more accurately determine rates of exposure and convalescence than symptom-based approaches, particularly in populations with mild-to-no symptoms like children [41]. Levels of antibodies to S, and RBD, can remain stable for up to 240 days providing an extended window for such screens to be effective. [42].

Protein subunit vaccines are not a new technology and safer than attenuated whole virus vaccines [43] A protein-based vaccine for Herpes Zoster is currently in circulation [43] and has been working effectively for years. S was the antigen of choice for SARS-CoV-2 subunit vaccine development, with Novavax [44] and others. Early trials in animal models showed not only promising immune responses, but cross-reactive glycosylation sites with convalescent plasma [45].

Production of these antigens depends on mammalian protein expression. Heavily glycosylated and multi-subunit proteins like S require more elaborate folding and post-translational modification provided by a eukaryotic expression system, as opposed to bacterial system employed for simpler polypeptides [43, 46, 47]

This research project utilized Human Embryonic Kidney-293 (HEK293) cells grown in suspension for transient gene expression of the SARS-CoV-2 RBD. HEK293 cells are derived from a cell line transformed by an adenovirus (Ad5) partial genome insertion on chromosome 19 to increase proliferation and prevent auto-apoptosis [48] allowing them to grow at high densities.

They can be used in either adherent or suspension culture, but suspension with volumetric growth is better for producing proteins in large quantities [47].

Transient gene expression, hereafter termed ‘transfection’, is a faster method of producing antigenic proteins than attempting to integrate whole transgene expression cassettes into a genome [49]. This project was focused on evaluating two transfection methods, lipid-based and polymer based, for expression of recombinant SARS-CoV-2 proteins.

Lipid-based transfection involves the use of an amphiphilic cationic lipid that binds and compacts the negatively charged DNA of the plasmid of interest to form a positively charged lipocomplex. This is often accompanied by secondary lipids such as cholesterol which help facilitate the binding of the lipocomplex to the negatively charged cell surface, facilitating uptake into the cell by endosomal transport [50]. Lipid-based transfection have efficacy rates of 40-90% after 48 hours [52, 53]. Of particular interest in this project was the commercial reagent ExpiFectamine293 used in one of the first SARS-CoV-2 diagnostic protocols (Amanat et al (2021) that received FDA emergency approval for clinical diagnosis of COVID-19. ExpiFectamine293 can also transfect at high densities, its standard protocol calling for $3x10^6$ cells/mL, nearly 3 times that of other protocols [53]. Although the ExpiFectamine293 method is capable of high yields, it is very expensive at over $500 per liter of cell culture [54].

Polymer-based transfection, specifically that of polyethylenimine (PEI) operates under a similar principle to the lipid-based. The polymer and plasmid DNA complex form a large positively charged molecule that interacts with the anionic elements of the extracellular matrix of the target cell facilitating entry into a cell via endosomal transport [55]. Unlike ExpiFectamine, PEI is not limited to one specific type of mammalian cell line [46]. PEI has a transfection

efficacy of 55-73% after 48 hours [56, 57]. PEI uses exceptionally small quantities of only 0.5-3 μg/mL culture [46, 57, 58] to lower the cost per liter transfected cell culture to <$1.

Experimental Design

The objective of this study was to evaluate the PEI transfection protocol by Von Boehmer et al (2003) [58] as a cost-effective alternate transfection method for expression of recombinant SARS-CoV-2 RBD proteins compared to the lipid-based ExpiFectamine293 protocol [6]. If PEI can render comparable yields and quality of diagnostic proteins to ExpiFectamine this could be an invaluable resource to laboratories with limited funding. The evaluation process had two stages of evaluation.

The first stage involved a direct comparison of the two methods to control for cell culture viability and plasmid purity. Two batches of cell culture were established and transfected at the same cell density, using the two different protocols, and harvested after the same incubation period. Then, if the results were comparable, a series of subsequent transfections optimized the PEI method with respect to incubation period and a reagent concentration.

The second stage was functional validation of the recombinant protein products produced by the two methods. SDS-PAGE and western blot were used to confirm protein purity. Then an enzyme-linked immunosorbent assay (ELISA) [6] using commercial antibodies were used to confirm functionality and correct protein folding of the recombinant RBD proteins.

The RBD produced by both methods were compared using a selection of COVID-convalescent samples provided by Tampa General Hospital (TGH). My hypothesis was if the resultant antibody titers using the RBD products from the two expression methods were not significantly different, then the PEI method is a viable alternative.

Methods

Generation of Plasmid DNA Stocks

The plasmid constructs for RBD were provided by the Krammer Lab (Mt. Sinai) (GenBank NC_045512.2) (Genescript) and were transformed into separate stocks of competent DH5α *E. coli* cells. Plasmid DNA was purified from 6-8 colonies from each construct using a Promega mini prep kit (Cat # A1330). Gene sequences were confirmed by Sanger DNA sequencing (Eton Biosciences). Once confirmed, large scale plasmid stocks were made using Qiagen Maxi Prep kits (Cat# 12162) following manufacturer protocol. Plasmid stocks were stored in nuclease free water at -20°C until needed.

Transfection of HEK293 Cells with ExpiFectamine

HEK293 cells (Gibco; Cat# A14527) were maintained in suspension using either Expi293 (Gibco; Cat# A1435101) or Freestyle293 (Gibco; Cat# 12338018). Samples of 10 µL were taken at 3-5 day intervals for cell density determination and cell viability evaluation checked using Trypan Blue. Cultures at 3-5 $x10^6$ cells/mL were passaged down to 0.3 x 10^6 cells/mL and incubated for 3-5 days at 37°C, 8% CO_2 and >80% humidity with shaking at 100 rpm. Transfections were performed after 5 passages.

At time of transfection, cultures that were ≥90% viable were diluted to a density of 2.5-3.5 x 10^6 cells/ mL. Then cells were transfected with one of the plasmid DNA stocks described above, using a commercial ExpiFectamine kit (Cat# A14525; Gibco) per manufacturer

instruction. The transfected cells incubated undisturbed, at 37°C, shaking at 90-100 rpm, 8% CO_2 and 90% humidity for 3 days.

Optimization of PEI transfection protocol

A 1% stock of PEI was prepared by dissolving 1 mg of branched PEI solution (Sigma-Aldrich; Cat# 408727) in 10 mL of sterile water, filtered through a 0.22 um membrane filter and were stored at -80°C until needed. The 1% stock solution was further diluted down to the 0.045% with sterile water before use. For the initial transfections against ExpiFectamine, freshly made PEI was used.

Cell cultures were diluted down to 2.5-3.5 x 10^6 cells/mL. The DNA complex solution was made using 4% cell culture volume of Opti-MEM, 0.34% cell culture volume of PEI solution, and 1000 ng/mL of cell culture of plasmid DNA. DNA complexes were incubated at room temperature for 20 minutes before being added to the cultures.

Initial transfection with PEI was done at the same time as the ExpiFectamine kit to control for cell stock and DNA stock variation, this was done using the 0.045% PEI solution and the supernatants for both transfections were harvested after 3 days. Subsequent transfections used either 0.045% or 1% w/v PEI and supernatants were harvested on days 3, 4, or 5 post-transfection.

Protein Purification

The cell cultures were centrifuged at 400 x *g* for 5 minutes. The supernatant was further centrifuged at 3000 x *g* for 30 minutes. The resulting supernatant was filtered through a 0.22 um vacuum filter and diluted 1:2 (v/v) with pre-filtered, pre-chilled binding buffer (500 mM Na_2HCO_3, 300 mM NaCl, 20 mM imidazole; pH 8).

The proteins were purified by affinity chromatography on a 5mL nickel sepharose column (GE Lifesciences; Cat# 17524802) using Äkta Pure system. The column was washed with binding buffer. Protein was eluted into 2-4 mL aliquots with elution buffer (50mM Na_2HCO_3, 300 mM NaCl, 300 mM imidazole; pH 8).

Purified proteins were concentrated using Amicon Millipore centrifuge filters and buffer exchanged to phosphate buffered saline (PBS) for storage. Concentrations were determined by Nanodrop spectrophotometer.

Validation of Expression

For purity confirmation, 6-10 µg of protein was diluted 1:1 with 2x Laemmli buffer (for an end protein/well of 3-5 ug), heated at 80°C for 3-5 minutes, then analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or a commercial 4-20% gradient gel (Bio-Rad; Cat# 5678095) at 100 V for 70 minutes, and then stained with Coomassie blue to visualize the protein bands.

For western blot analysis, proteins were separated on a 10% SDS gel, run as described above, and transferred onto a PVDF membrane previously soaked in methanol for 3 minutes. The proteins were electro transferred using 175 mA for 90 minutes. After transfer, the membrane was blocked with 10% milk in PBS and 0.05% tween (PBST) for two hours, rinsed with PBST, then incubated with a 1:5000 solution of HRP conjugated 6x-His antibody (Invitrogen Cat# R931-25) in 1% milk in PBST overnight at 4C. After three washes with PBST, the membrane was incubated with Amersham ECL start western blotting detection reagent (GE Healthcare; Cat# RPN3244) and imaged after 3 minutes using a GeneSys system.

For confirmation ELISA, 96 well MaxiSorp microtiter plates were coated overnight at 4°C with 50 µL/well of purified protein diluted to 2 µg/mL in PBS. Blank wells were coated

with PBS only and previously confirmed proteins were used as positive and negative controls. The plates were blocked with 10% milk in PBST for 2 hours at room temperature, then incubated with 50 µL of 0.4 µg/mL of commercial anti-RBD antibody (Cat# A2103-200; BioVision) and for 2 hours at room temperature. The plates were then incubated with 50µL of 0.4µg/mL concentration of AP-conjugated secondary antibodies for 2 hours at room temperature (Goat anti-human IgG, Reserve AP Labeled, SeraCare: 0751-1006). The plates were developed using 50-100 µL/ well KPL BluePhos Microwell Phosphatase substrate system (SeraCare, Cat# 5120-0059) for ten minutes and read at 650 nm using a spectrophotometer.

Quality Assurance: RBD Screening ELISA

96 well MaxiSorp microtiter plates were coated with 50 µL of RBD protein (either ExpiFectamine derived, 0.045% PEI derived, 1% PEI, or total pooled PEI) at a concentration of 2 µg/mL. Controls included blank wells coated with PBS and a non-specific antigen (EBP2) at the same concentration as RBD. Assay controls consisted of a normal human serum sample, either known negative or pre-immune plasma at a 1:100 dilution, a nonspecific human antibody (mAb 092096, a Duffy Binding Protein specific antibody), and a positive SARS-CoV-2 Spike S1 antibody (BioVision; Cat# A2103-200) at a concentration of 0.5 µg/mL each. The coated plates were washed 3 times with 1x PBS and 0.05% Tween (PBST). The plates were blocked with 200µL/well with 5% milk in PBST for 2 hours at room temperature and washed 3 times with PBST. To the control wells, 50µL volumes of PBS, the normal human sera, specific, and nonspecific antibodies were added. A 1:2 dilution series of COVID-19 convalescent human serum samples (n=25) provided by TGH, starting at a 1:100 dilution, to a final volume of 50 µL/well was added to the plates in duplicates and incubated for 2 hours at room temperature and then washed 3x with PBST. 50 µL of secondary antibody (Goat anti-human IgG, Reserve AP

Labeled, SeraCare: 0751-1006) was added to all wells at a concentration of 0.5 µg/mL. Plates were incubated for 2 hours at room temperature, then washed 3x with PBST. 50µL of mixed, room temperature, developing reagents (KPL BluePhos Microwell Phosphatase substrate system) were added to all wells and absorbance was measured at 650 nm after 10-20 minutes of development.

Statistical Analyses

Data analysis was performed using Microsoft Excel 2010 and GraphPad Prism 8.1. For the optimization of PEI groups were compared using nonparametric T tests when there were two groups or 1-way ANOVA when there were three. The overall comparison of PEI versus ExpiFectamine yields was done using a 1 sample T-test for the PEI groups, using the ExpiFectamine yield as the hypothetical mean. For the end point-titers, data was blanked and any values above the average reading for the NHS wells were considered a positive result, and the divisions between high and low titers are described in the results section. Comparisons between the titers were done using a mixed-methods analysis.

Results

Cell Growth and Viability

HEK293 grown in Expi-293 media had a healthier appearance than the same cells grown in Freestyle 293 media under the similar conditions (Figure 1). Freestyle 293 media required 2-3 days longer to reach a cell density of 3-5 x 10^6 cells/ mL (Figure 2). Though viabilities of both groups ranged between 85-99% for the first 5 passages, the Freestyle 293 cultured cells dropped to 50% viability after the sixth passage (P6) and had to be discontinued.

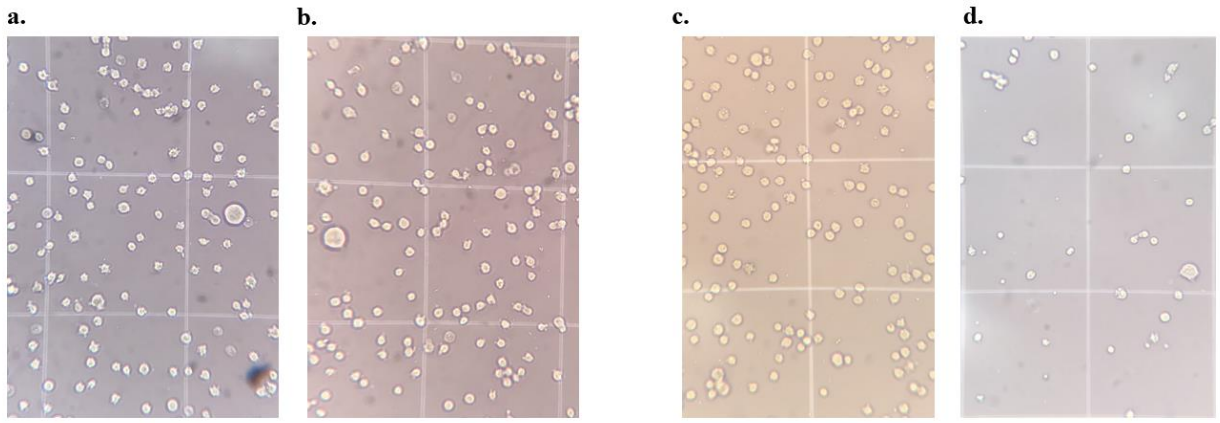

Figure 1. Cell Health in Media. Photos of HEK293 cells at P1 (a and b) and P4 (c and d) showing the size, health, and confluence of cells. Expi293 media (a and c) showed consistent cell sizes and higher growth densities at both timepoints. Freestyle293 media was consistent in cell size at early passages (b) but showed lower density and more size variation at P4 (d).

Transfection Yields

The initial transfection established a baseline comparison between ExpiFectamine and the lowest concentration and incubation time being tested for PEI, controlling for DNA

concentration, and cell health. The ExpiFectamine transfected cells yielded 21 mg/L of RBD and the PEI cells yielded 16 mg/L.

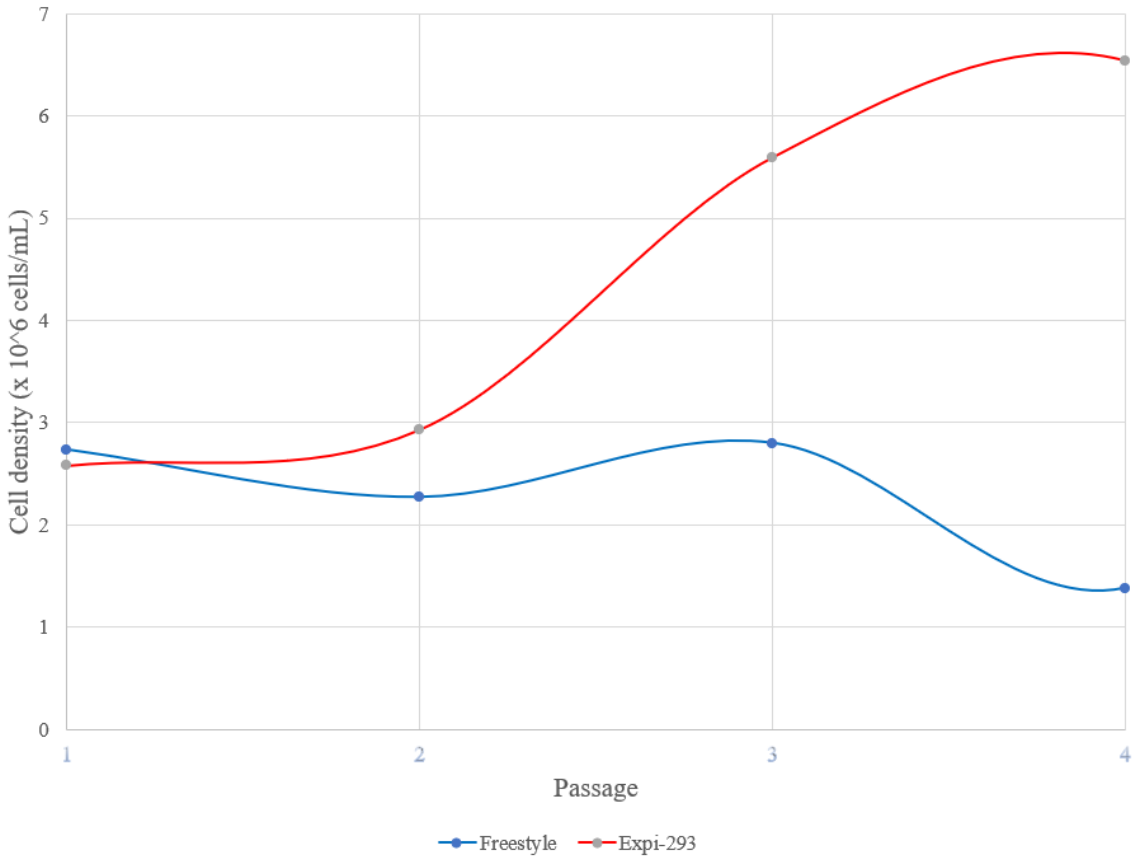

Figure 2. Comparison of Growth Rates by Media. A graph comparing the cell densities of HEK293 cells across five passages in Expi293 media (red) and Freestyle293 media (blue). Passages were diluted to $3x10^5$ cells/mL every 4 days. Densities in Freestyle were generally lower and took longer to come up to passage density than Expi293 and continued to decrease over time.

Subsequent transfections performed to optimize the PEI protocol and see if the method could yield higher volumes of RBD with longer incubation times or higher concentrations of reagent, expressed considerably less protein than ExpiFectamine and the initial transfection with PEI (Table 2) at 3.36 mg/L for 0.045% PEI and 4.22 mg/L for the 1% concentration. 1% PEI appeared to yield more protein, with highest yield being four days post transfection (Table 3), but there was no significant difference in yield between either the 0.045% and 1% PEI concentrations ($p=0.8152$; Figure 3a) or the days post-transfection ($p= 0.7$; Figure 3b). Overall, PEI transfection yields were lower than ExpiFectamine in all transfections (Figure 4).

Table 2. Transfection Yields

	INITIAL TRANSFECTION			**SUBSEQUENT TRANSFECTIONS**			
Method	Total Culture Volume (mL)	Yield (mg)	Yield (mg/L)	PEI Concentration	Total Culture Volume (mL)	Yield (mg)	Yield (mg/L)
ExpiFectamine	620	13	21	0.045%	660	2.219	3.362
PEI (0.045%)	660	10.6	16	1%	750	3.162	4.216

Table 3. Transfection Yields for PEI Optimization

0.045% W/V PEI			1% W/V PEI		
Days Post Transfection	**Vol of cell culture**	**Yield**	**Days Post Transfection**	**Vol of cell culture**	**Yield**
3	220 mL	1.169 mg	3	250 mL	0.51 mg
4	220 mL	0.6 mg	4	250 mL	1.602 mg
5	220 mL	0.45 mg	5	250 mL	1.05 mg

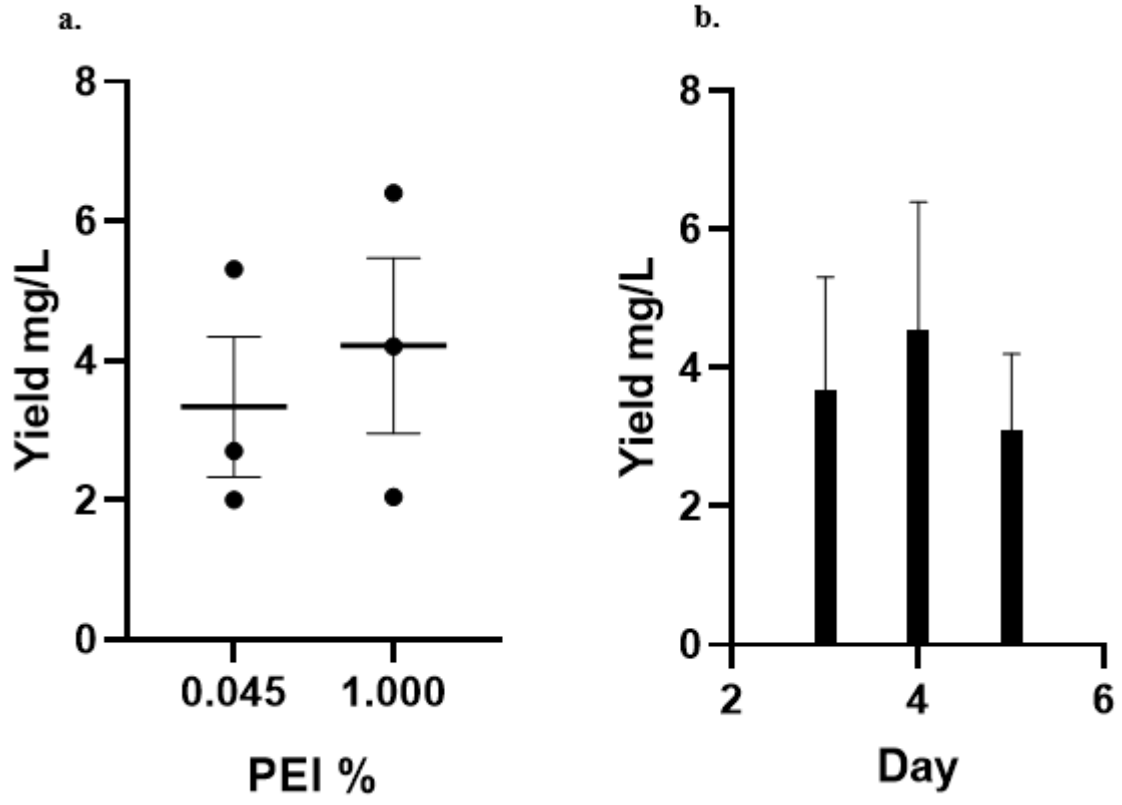

Figure 3. Optimization Yields. Graph a compares yields by transfection reagent concentration graphed as mean ± SEM; 0.045% showed an average of 3.3 mg/L (SD= 1.74). 1% yielded an average of 4.2 mg/L (SD=2.18). A Mann-Whitney test of the concentrations showed no

Figure 3. (Continued) significant difference (p=0.7). Graph b compares yields by days between transfection and supernatant harvest graphed as mean ± SEM. Day 4 showed the highest average of 4.5 mg/L (SD=2.62), followed by day 3 (mean=3.68; SD= 2.312) and then day 5 (mean=3.1; SD=1.56). A one-way ANOVA showed no significant difference in the yields (p=0.815)

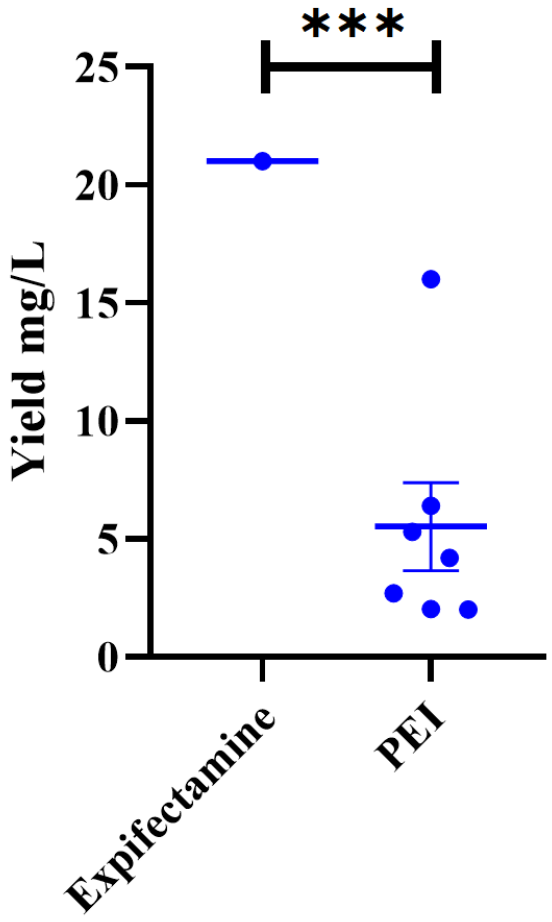

Figure 4. Overall Comparison of Transfection Methods. A graph comparing Expifectamine (single point, left) and all PEI yields (right; graphed as mean ± SEM; mean= 5.52, SD=4.91). A one-sample t-test of the PEI values using the ExpiFectamine yield (21 mg/L) as the theoretical mean showed a significant difference (p=0.0002).

Protein Quality

Purity of the purified proteins were confirmed by SDS-PAGE and western blot using an anti-His tag antibody. The initial results for ExpiFectamine and PEI transfections produced single protein bands at 32-35 kDa by SDS-PAGE and western blot analyses (Figure 5). However, results from subsequent transfections produced lower yields and had additional non-specific protein bands by SDS-AGE analysis, especially in the 0.045% PEI derived samples on day 5 post transfection (Figure 6a). These other proteins were not reactive with antibody to the 6x-His tag, while the RBD His-tagged products were considerably fainter in the subsequent transfections (Figure 6b).

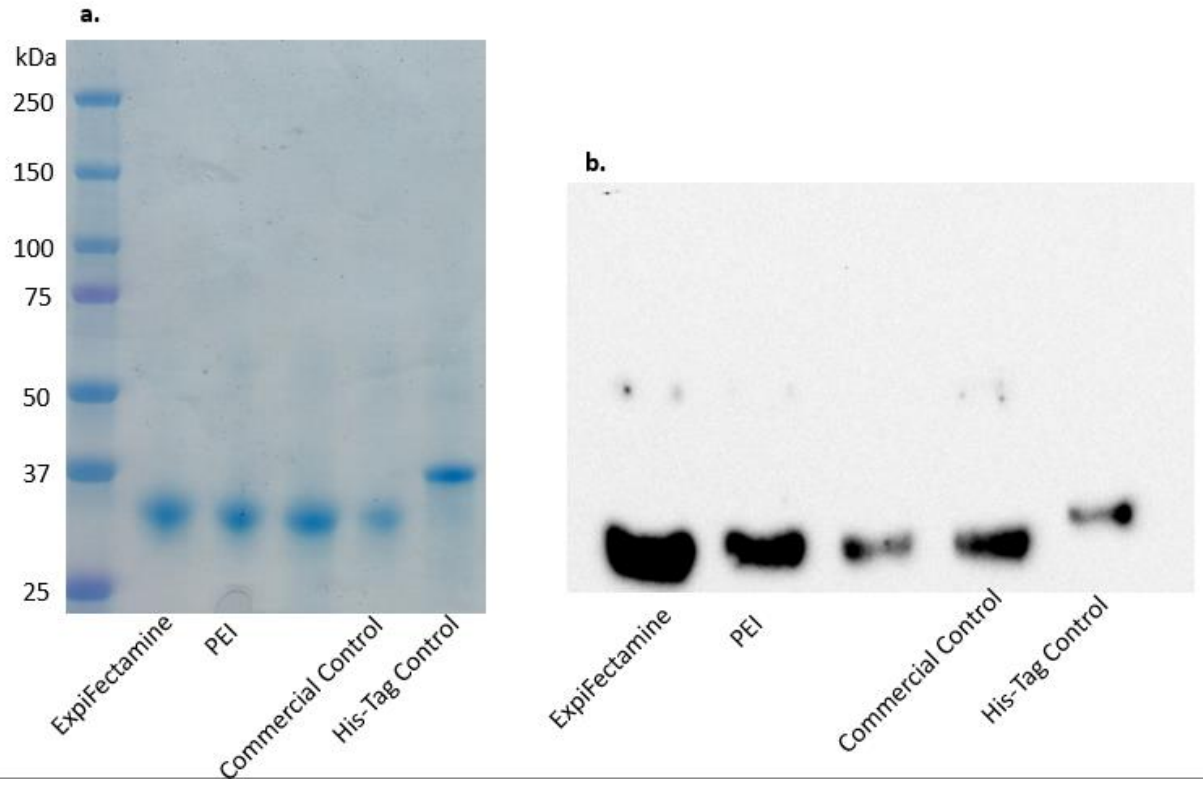

Figure 5. Qualitative Results for Initial Transfection. Image A shows the SDS-PAGE results for the initial transfection. Both PEI and ExpiFectamine had pure yields, evidenced by the single, distinct bands at the expected weight (32-35 kDa) in line with the control. Image B shows the results of the western blot performed using HRP-conjugated anti-His Tag antibody. All samples showed clear, distinct, single bands consistent with the positive controls.

Once confirmed for purity, the proteins were further assessed for binding specificity and conformation by ELISA using an anti-SARS-CoV-2 RBD antibody (BioVision; Cat# A2103-200). The proteins purified from the 1% PEI transfected cells had similar levels of reactivity with the ExpiFectamine cells, while the proteins purified from the 0.045% PEI transfected cells had very low levels of reactivity with the RBD antibody (Figure 6c).

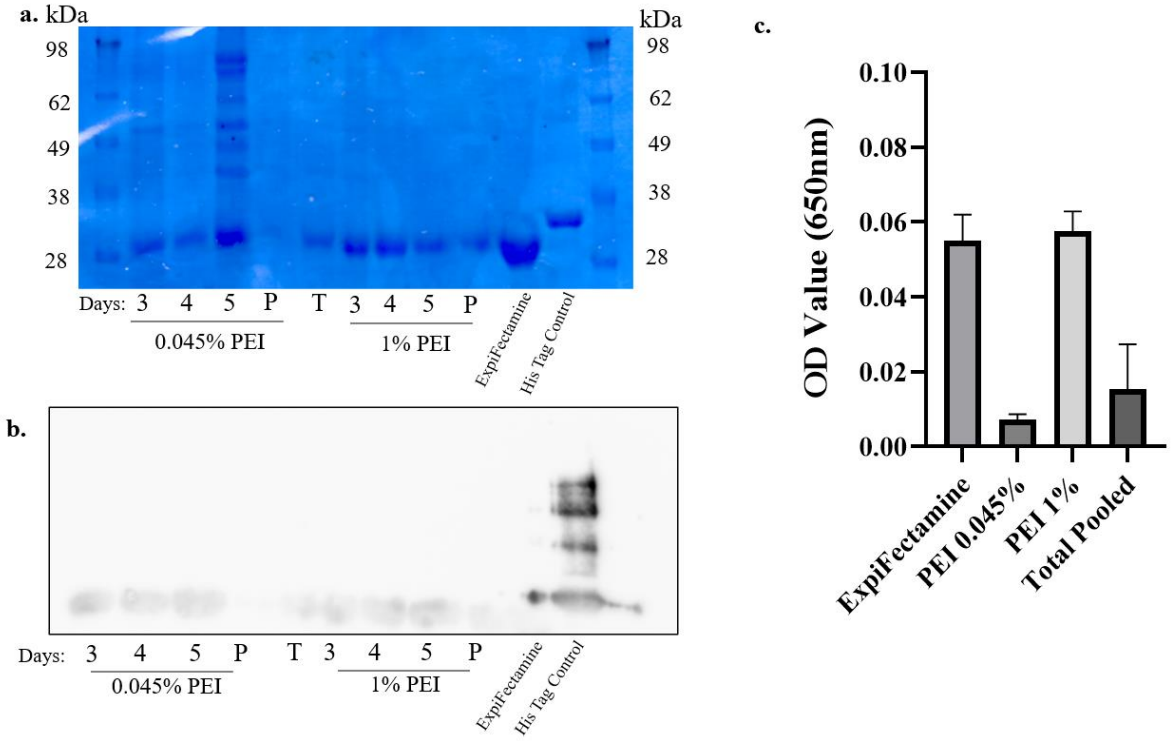

Figure 6. Qualitative Results for PEI Optimization. Image A shows SDS-PAGE using a 10% SDS gel made in-house. Where 3, 4, and 5 denote days post-transfection, p denotes the pooled sample (1:1:1) for that concentration and T at the center denotes the total pool of all PEI samples. The 0.045% samples were the least pure showing pronounced non-specific banding, especially on day 5. Image B shows western blot for detecting C-terminal His-Tag results using the same labelling as the SDS-PAGE. All but two lanes (0.045% pooled and the his-tag positive control) showed faint, singular bands. Image C shows The OD values for the confirmatory ELISA graphed as mean ± SEM, where 1% had comparable results to the ExpiFectamine group, but 0.045% and the total pooled groups were lower.

Lastly, the proteins were tested for their ability to react with naturally acquired human antibodies of convalescent serum from SARS-CoV-2 infected individuals. The ExpiFectamine purified protein group reacted with all but 4 serum samples (Figure 7a, c, and e). The levels of antibody reactivity were divided into two groups based on their end-point titers; values above the mean (1: 7571) were considered high and values below excluding 0 were considered low. About half (n=12) of serum samples had high titer antibodies for the ExpiFectamine group.

For the PEI 0.045% purified proteins of the ten samples tested only 1 showed high titer reactivity (Figure 7b). The 1% (n=10) and pooled (n=5) groups displayed antibody recognition similar to the ExpiFectamine group (Figure 7d and f). Using the same criteria to divide the end-point titers as described above: the PEI groups showed no titer in more samples, 4 of which were in the 0.045% group, and low titers in half (n=13) most of which were in the 1% group (Table 4).

Table 4. Reactivity of Sera with Recombinant Proteins: End-Point Titer Groupings*

	ExpiFectamine	PEI 0.045%	PEI 1%	PEI Pooled	PEI (All Concentrations)
High Titer	12	1	4	2	7
Low Titer	9	5	5	3	13
No titer	4	4	1	0	5
Total	25	10	10	5	25

**Where: High Titer is any value greater that 1:7024, low is any value between 0 and 1:7024, and no titer is all 0 values.*

Because the same samples were used for ExpiFectamine and the PEI groups, a comparison of titers overall was performed to see if PEI-derived proteins would yield similar titers for identical samples. Across all concentrations, the titers for PEI were lower than ExpiFectamine with only 20% of the samples having equal results (Table 5) and these were at the extremes of the assay; either no titer at all or the highest possible (1:12800). Overall, the proteins purified from the PEI transfected cells had lower antibody titers, but this was only significant when ExpiFectamine was compared to 0.045% PEI ($p=0.0265$) and all other pairings had a p value greater that 0.05 (Figure 8).

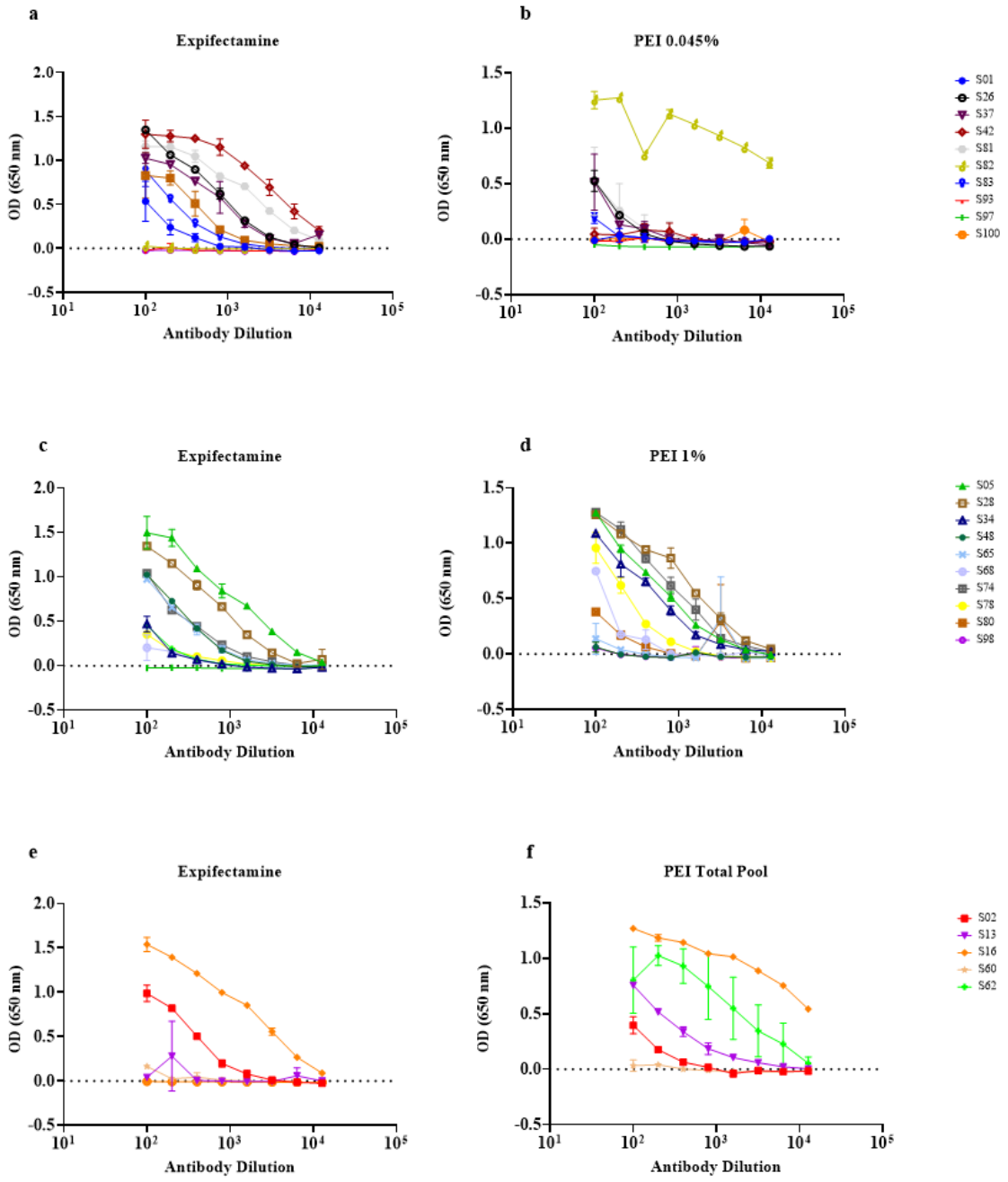

Figure 7. OD values for anti-RBD Antibody Screening ELISA. Results for the anti-RBD screen expressed as mean OD value ± SEM and each color denotes an individual sample. Each grouping (a&b, c&d, e&f) used identical sera samples, with ExpiFectamine serving as a control group. Graphs a and b compare the first group (n=10); only one sample showed pronounced reactivity in 0.045% set (S82). Graphs c and d compare the second group (n=10); where 1% PEI shows comparable reactivity to human sera to the ExpiFectamine control. Graphs e and f compare the final group where the total pooled PEI showed more reactivity with human sera than the control, suggesting non-specific binding of human sera to the recombinant protein.

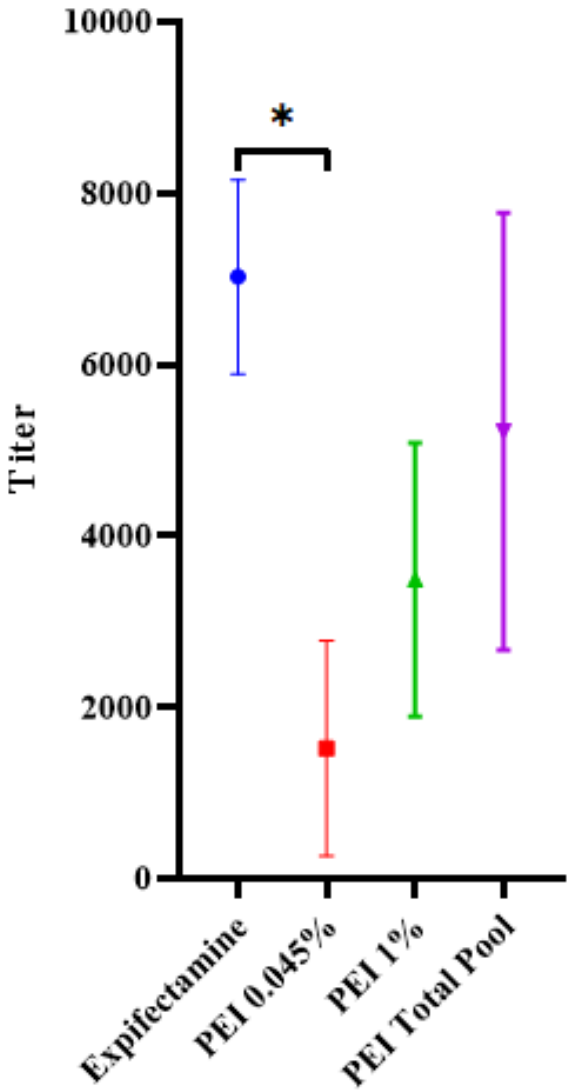

Figure 8. Comparison of End-point Titers. Titers are expressed as mean ± SEM. The ExpiFectamine control group showed the highest average titer (mean= 7024; SD=5680). 0.045% PEI derived proteins (n=10) showed the lowest titers of the group (mean= 1520; SD=3977). 1% (n=10) showed more reactivity (mean=3490; SD=5048). And the Total Pool (n=5) had the highest deviation from the mean of all four groups (mean= 5220, SD=5705). A mixed-effects analysis only showed significant difference between the ExpiFectamine and 0.045% PEI group (p=0.0265)

Discussion

This goal of this research project was to determine if an established protocol for expression of recombinant protein in HEK293 cells was a suitable alternative to produce SARS-CoV-2 spike RBD instead of a recently published protocol using very expensive commercial reagents. If successful, this would provide a protocol more easily accessible to labs with less financial resources to devote to COVID-19 research . The first aim of the methods described here were intended to compare the methods using PEI to ExpiFectamine transfection directly to determine if they were equivalent in efficiency. The second aim was to optimize the culture method comparing Expi293 and Freestyle293 media to match or exceed the standard by adjusting the concentration of the reagent and the days it was allowed to incubate post-transfection, still staying within the 3-5 day timeframe described by the ExpiFectamine Protocol [53]. Qualitative testing sought to demonstrate if the protein produced by the alternative PEI method had equal purity and provided similar results as measured by reactivity to a panel of commercial and naturally acquired antibodies to SARS-CoV-2.

The yield of RBD from the ExpiFectamine culture was within expected limits of 20-22 mg as previously reported [6]. The initial PEI transfection done alongside produced similar yields, with only 5 mg less overall. Though 1% PEI showed slightly higher yields, there was no significant difference ($p= 0.817$) between 0.045% and 1% PEI during the optimization transfections (Figure 3a). It is possible to go higher than 1%, however PEI concentrations greater than 3% have been shown to be toxic to the cells [49]. Four days post-transfection appeared to

generate slightly better yields than days 3 and 5 though the difference was not significant (p=0.7) (Figure 3b). This is interesting as most PEI protocols call for 5-10 days harvesting post transfection, albeit with lower seeding densities at the time of transfection [58].

Both sets of RBD from the initial transfection were pure, as shown by the SDS-PAGE and Western Blot (Figure 5). The optimization yield RBD all showed bands at the expected region of about 34 kDa, however there were non-specific bands for all samples, with Day 5 of the 0.045% transfection being the least pure. This lack of purity is further confirmed by the lack of distinct bands on the Western Blot, and the low readings on the ELISA when compared to the control (Figure 5). Therefore, we concluded longer time periods are not useful to increase high quality RBD when using the PEI method. [you might want to comment on the media and cell viability here as well]

As expected, based on the qualitative results described above, the PEI screening results were consistently lower than the purer ExpiFectamine controls. Comparisons between each group (0.045%, 1%, and pooled PEI total) showed no significant difference between them. Interestingly, the total pooled PEI samples showed generally higher titers compared to the expected. Overall, only 20% of the samples screened had the same titer as their ExpiFectamine counterparts and those were either high titers greater than the upper limit of the assay (12,800) or 0, likely due to low purity. The upper threshold of the assay is only 1:12800, leaving a range of high titers to be lumped together and a smaller group for mid and low range titers. This was not a large source of error, as only 3 samples would be impacted by this, but should still be considered.

The major challenge for the optimization of the protocol was dramatic decrease protein production after the initial transfections (Table 2 & 3) (Figure 4), which suggested a problem with the reagents. To investigate the loss, subsequent transfections with different batches of cells

and fresh-made PEI stocks were tested as well as new buffers were made and filtered fresh at various timepoints, but none were identified as the problem. The conclusion was the plasmid DNA degraded or precipitated out of solution, resulting in a lower concentration than what was initially measured, making transfection less efficient and thus yields lower. This occurred with other proteins yielded from similar transfection methods, specifically SARS-CoV-2 spike and an avi-tagged version of RBD, that also showed diminishing yields with subsequent transfections (data not shown), and it was discovered in one case that the DNA concentration of the plasmid stocks had decreased by half over the storage period.

Going forward, a different storage method for plasmid DNA such as in TE buffer is recommended in addition to using fresh plasmid stocks to improve PEI yields. Secondly, lower cell densities at the time of transfection [46, 58] are recommended, since other studies have shown that higher cell densities negatively impact transfection efficacy [37]. In addition, additives such as 20mM lithium acetate 3 hours prior to media exchange for transfection and 3.36mM valproic acid 5.04 mM caffeine 4 hours post-transfection can be added to enhance expression levels. [57]

Conclusion

Based on purity and yield alone, using the methods of this study PEI did not produce comparable results as with ExpiFectamine as a transfection reagent for producing large quantities of RBD. PEI works best when made fresh and when using fresh plasmid stocks. Practically, PEI also does not appear to compare with ExpiFectamine in terms of recombinant protein purity. High-concentration PEI did produce comparable results when tested with commercial antibodies, but when tested using human sera, the results were significantly different. Although the PEI method as tested could potentially be optimized to generate more consistently high yields, the current protocol was not determined to be a suitable substitute to the expensive commercial reagents.

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