Original Research Article

Augmenting Whole Recombinant Antibody Production in HEK293E Cells: Optimizing Transfection and Culture Parameters

Zealyn Shi-Lin Heng [1], Joshua Yi Yeo [1], Darius Wen-Shuo Koh [1], Samuel Ken-En Gan [1,2,*] and Wei-Li Ling [1,*]

[1] Antibody & Product Development Lab, EDDC-BII, Agency for Science, Technology and Research (A*STAR), Singapore 138672, Singapore, hengsl@sgut.bii.a-star.edu.sg (Z.S.-L.H.); joshua_yeo@eddc.a-star.edu.sg (J.Y.Y.); darius_koh@eddc.a-star.edu.sg (D.W.-S.K.)
[2]APD SKEG Pte Ltd., Singapore 439444, Singapore.

*Correspondence: samuel_gan@eddc.a-star.edu.sg; samgan@apdskeg.com (S.K.-E.G.); lingwl-eddc@eddc.a-star.edu.sg (W.-L.L.)

ABSTRACT

Optimizing recombinant antibody production is important for cost-effective therapeutics and diagnostics. With downstream impact on commercialization, higher productivity is highly sought after beyond laboratory scales, where efficient production can also accelerate antibody characterizations and investigations. Using HEK293E cells as the base model for mammalian antibody production, various transfection and culture parameters were systematically analyzed using antibody light chain production before applying them onto whole antibody production. Transfection parameters investigated include seeding cell density, the concentration of the transfection reagent and DNA, complexation time, temperature, and volume, as well as culture parameters such as media replacement, serum deprivation, media volume, post-transfection harvest, incubation temperature, and common nutrient supplements. Incorporating the most optimized parameters, Pertuzumab κ-chain only and whole recombinant Pertuzumab antibody production were found to increase by 452 % and 252 % respectively, that can be used to guide future cost-effective transient antibody production with the potential for further scaling up.

Statement of Significance

Recombinant antibody production is crucial for antibody research and development. Systematically investigating transfection and culture parameters such as PEI/DNA concentrations, complexation time, volume, and temperature, supplements, etc., we demonstrated a 452 % and 252 % increase for light chain and whole antibody production, relevant for research and downstream developmental processes.

Key words: Recombinant antibody production; Antibody therapeutics manufacturing; Transient transfection; Transfection parameters; HEK293E

INTRODUCTION

The efficient production of recombinant monoclonal antibodies underpins their functional and structural investigations (1) that includes toxicology and drug formulation studies (2) and thus, the successful development of both therapeutic and diagnostic antibodies. With direct impact on the production cost during development, enhancements at the already expensive mammalian production are highly desired.

Currently, whole antibodies are predominantly produced by mammalian cells (3, 4), commonly via permanent expressing stable cell lines (2), yet transient gene expression using Human Embryonic Kidney 293 (HEK293) cell lines (2) form the bulk of the smaller research scale investigations. With various HEK293 genetic variants such as those expressing the Epstein-Barr Virus Nuclear Antigen-1, HEK293E (6) and those expressing the SV40 T-antigen, HEK293FT and HEK 293T (5), HEK293 cell-based transfections are also shown to be highly scalable (3, 6, 7), and suitable for producing a wide range of recombinant proteins including whole viral particles (8). Nonetheless, transient transfection of HEK293 cells is dependent on a myriad of transfection and culture parameters, to which the optimization can guide its

scalability and efficiency. Such parameters include: seeding cell density (9), transfection reagent concentration (10), DNA concentration (11), complexation time, temperature, and volume (11), together with the culture conditions such as media replacements (6, 12), serum deprivation (13), culture volume, harvesting time (14–16), incubation temperature (17, 18), and nutrient supplementation (19–21).

Since optimization of the many mentioned parameters were performed in isolation (21, 22), we aim to investigate the combined optimized parameters using antibody light chains, specifically the common κ-chain which comprises two-thirds of the human antibody repertoire (23), before validating the parameters on the whole antibody production. The final combination of optimized parameters would complement our previous findings for production that discusses the role of heavy chain constant (24), Vκ-VH pairings (25), amino acid usage with leader selection (26), in a holistic manner (1, 27), to significantly boost the production of future antibody-based therapeutics and diagnostics.

MATERIALS & METHODS

Plasmid DNA

Pertuzumab and Trastuzumab light κ-chain (Vκ1) and heavy γ-chain (IgG1) plasmids, including their V-regions variants, designated Vκ2-6 and VH1-7 used were prepared as previously described (24–26, 28, 29). Light chain production optimizations were performed with the Pertuzumab κ-chain plasmid (Vκ1-Cκ), before combining with its corresponding heavy γ-chain plasmid (VH3-IgG1) to optimize whole antibody production. Usage of Pertuzumab and Trastuzumab plasmid variants are indicated in the respective sections.

Transfection Parameters

Linear 25 kDa Polyethyleneimine (PEI) (Polysciences, Cat no. 23966-1) at pH 7.0 was used in all the transfections. 2 mL and 40 mL scale transfections of HEK293E cells were performed in 6-well plates (Costar, Cat no. 3516) and T175 flasks (Greiner Bio-one, Cat no. 660175) respectively, with at least three independent replicates. The conditions tested for the transient gene expression in 6-well plate format were as follows: Cell seeding densities from 1 - 10×10^5 cells/mL; PEI concentrations from 1 - 10 μg/mL; DNA concentrations from 0.25 - 2 μg/mL; PEI-DNA complexation time from 5 - 120 minutes; PEI-DNA complexation temperatures of 18 - 45 °C; PEI-DNA complexation in 1 - 25 % serum free media (SFM) of total 2 mL volume; PEI-DNA complexation in light or dark environments; PEI-DNA complex addition to cultures with or without G418 antibiotic; culture media replacements; serum deprivation before transfection; culture volume from 2 - 5 mL; post-transfection harvest of 3 - 21 days, and culture incubation temperature at 32 or 37 °C. The experimental conditions were compared against a control parameter of : Seeding density at 2×10^5 cells/mL, DNA concentration at 1 μg/μL, PEI concentration at 2 μg/mL, PEI-DNA complexation time at 30 minutes in 18 °C, with 10 % SFM of total 2 mL volume, performed in the dark and added into cultures without G418. Harvest of the antibody produced was made on post-transfection day 14 with the cultures grown at 37 °C.

For cell culture conditions, 10 % FBS DMEM was used for κ-chain production, while 10 % Ultra Low IgG FBS DMEM was used for whole antibody production. Transfection and culture parameters were scaled up proportionately from the 6-well cultures to the T175 flasks (40 mL culture).

Supplement Preparation and Usage

The nutrient supplements tested were: OmniPur® Casamino acids (CA) (EMD Chemicals, Cat no. 65072-00-6), Casein from Bovine Milk (CS) (Sigma-Aldrich, Cat no. C3400), Bacto™ Peptone (PT) (Becton, Dickinson and Company, Cat no. 21167), Skim Milk (SM) (Millipore, Cat no. 70166), Tryptone (TT) (Bio basic, Cat no. TG217 (G211)), Bacto™ Yeast Extract (YE) (Becton, Dickinson and Company, Cat no. 212750) and Impact Essential Amino Acids (EA) (MYPROTEIN™)(26). Supplements were dissolved in water or PBS, filtered using 0.22 μM filters and stored at 4 °C before use.

The supplements were added to the Pertuzumab κ-chain only post-transfection, on day 7 at a final concentration of 1mg/mL. Further optimizations with peptone were performed at 1, 2, 4 and 6 mg/mL with various additions from post-transfection day 1 - 10, as well as dual additions on day 1 and day 7. Subsequently, using the set of combined optimized parameters without nutrient supplementation, peptone at 1 mg/mL, essential amino acids at 3.5 mg/mL (26) or both were added to whole antibody production cultures post-transfection day 7.

Recombinant Antibody Titer Quantification

κ-chain and whole antibody quantification on the cell culture supernatant were performed using Octet Red 96 instrument as previously described (24–26), with Protein L (PpL) and G (SpG) biosensors (ForteBio, Cat no. 18-5085 and 18-5082) respectively.

Data Analysis

The data were analyzed and plotted using Microsoft Excel 365 and GraphPad Prism Version 9.0.0. Antibody titers were normalized against the control and expressed as a percentage with the control at 100%. One-way ANOVA with Dunnett's comparison test against the control was used when examining three or more different parameters, while two-tailed unpaired parametric T-test were performed when comparing two parameters.

RESULTS

Optimizing Transfection Parameters

Cell seeding densities

Cell seeding densities ranging from 1 - 10 × 10^5 cells/mL in 6-well plate were evaluated with respect to their impact on Pertuzumab κ-chain production and normalized against that of the control parameter of 2 × 10^5 cells/mL set at 100 % in **Figure 1A**. The one-way ANOVA analysis showed no significant differences ($p > 0.05$) in the Pertuzumab κ-chain production across the seeding densities tested with a decreasing trend at cell densities above 6 × 10^5 cells/mL.

PEI and DNA concentrations

PEI concentrations ranging from 1 - 10 μg/mL in 6-well plate transfections were tested for Pertuzumab κ-chain production and normalised against that of the control parameter of 2 μg/mL set at 100 % in **Figure 1B**. The results showed a curvilinear relationship between PEI concentration and κ-chain production peaking at 145 % for 4 μg/mL of PEI. PEI concentrations lower or higher than 4 μg/mL negatively impacted κ-chain production with 1 μg/mL having the lowest production of 17 % that was significant at $p < 0.05$ when analysed with Dunnett's test against the control.

DNA concentrations ranging from 0.25 - 2 μg/mL, with intervals of 0.25 μg/mL in 6-well plate transfections were evaluated with respect to Pertuzumab κ-chain production and normalised against that of the control parameter of 1 μg/mL set at 100 % in **Figure 1C**. An inverse relationship between DNA concentration and κ-chain production was observed with the lowest and highest DNA concentration (0.25 and 2 μg/mL) corresponding to the best and poorest κ-chain production (190 % and 21 %) that were significant at $p < 0.05$ when analysed with Dunnett's test against the control.

PEI and DNA complexation

PEI-DNA complexation time from 5 - 120 minutes in 6-well plate transfections were evaluated with respect to Pertuzumab κ-chain production and normalized against that of the control parameter of 30 minutes, set at 100 % in **Figure 1D**. An inverse relationship between time and κ-chain production showed the shortest and longest incubation time (5 and 120 minutes) corresponding to the highest and lowest production (166 % and 31.5 %), which were both significant at $p < 0.05$ when analysed with Dunnett's test against the control.

PEI-DNA complexation temperature from 18 to 45 °C in 6-well plate transfections were evaluated with respect to Pertuzumab κ-chain production and normalized against that of the control parameter of 18 °C, set at 100 % in **Figure 1E**. An inverse relationship where the decline in κ-chain production was found with increasing temperatures. Analysis with Dunnett's test against the control for 25, 32 and 37 °C showed no significant decrease ($p > 0.05$) despite slight reductions. However, significant decreases ($p < 0.05$) were observed for 40 and 45 °C, at 54 % and 50 % of the control parameter production respectively.

PEI-DNA complexation volume using SFM from 1 to 25 % of total culture media in 6-well plate transfections were evaluated with respect to Pertuzumab κ-chain production and normalized against that of the control parameter of 10 % final cell culture volume (16) set at 100 % in **Figure 1F**. PEI-DNA complexation media volume was found to increase κ-chain production with the lowest 1 % SFM yielding the lowest production of 12.7 %, that of the control, significant at $p < 0.05$ analysed with Dunnett's test. While not significantly different ($p > 0.05$) from control, the highest SFM percentage at 25 % yielded 131 % κ-chain production. Production was observed to peak at 15 % transfection volume plateauing at higher complexation volumes.

To study photodegradation assumptions of transfection complexes, we performed PEI-DNA complexation incubation for κ-chain using the 6-well plate format under the biosafety

cabinet (BSC) light and without, in very dim external room light. We did not find any significant differences ($p > 0.05$) in κ-chain production between the two conditions (**Figure 1G**). Studying the effect of cell culture antibiotics, we also did not find G418 to have significant effects ($p > 0.05$) on the κ-chain production by T-test (**Figure 1H**).

Optimizing Culture Parameters

Culture media replacements

Fresh media replacement before (BT) or after (AT) transfection as well as both before and after (BAT) were tested for Pertuzumab κ-chain using the 6-well plates and normalized against that of the control parameter of no media replacement (NMR) set at 100 % in **Figure 2A**. Pertuzumab κ-chain production was found to increase by 36 % and 9 % for BT and BAT, respectively. A small reduction of 7 % was found for AT when compared to the control, but the One-way ANOVA analysis showed no significant difference ($p > 0.05$) for these increases and decreases.

We next studied serum deprivation by seeding cells in SFM prior to transfection, both with (SDRA) and without (SD) media change to 10% FBS supplemented DMEM after transfection in 6-well plate format for Pertuzumab κ-chain production. Normalization was performed against that of no serum deprivation (NSD) set at 100 % in **Figure 2B**. We found SD and SDRA to result in a non-significant ($p > 0.05$) 25 % and 37 % decrease in κ-chain production using One-way ANOVA analysis.

Culture media volumes

Media volumes varying from 2 - 5 mL in 6-well plate transfections were evaluated for Pertuzumab κ-chain production and normalized against that of the control parameter of 2 mL set at 100 % in **Figure 2C**). The one-way ANOVA analysis showed no significant difference ($p > 0.05$) in κ-chain production across the media volumes tested, although the highest media

volume of 5 mL showed an increase of 40 % when compared to the control and after volume normalization.

Culture supernatant harvest day

Post-transfection harvest of culture supernatants across 3 – 21 days from Pertuzumab κ-chain 6-well plate transfections were normalized against that of the control parameter of 14 days set at 100 % in **Figure 2D**. We found a direct relationship between the day of supernatant harvesting and κ-chain production. With analysis by Dunnett's test at day 14, there was an expected significantly ($p < 0.05$) lower κ-chain production on harvest days 3, 6 and 9. Although there was no significant difference ($p > 0.05$) detected from day 14 for harvest days 12, 15 and 18, there was a significant increase ($p < 0.05$) of 55 % for harvest day 21.

Culture incubation temperature

Culture incubation temperatures at 32 and 37 °C for Pertuzumab κ-chain 6-well transfections were shown with T-tests to reveal a significant production decrease at 32 °C ($p < 0.05$). Cultures grown in 32 °C showed 53% of the κ-chain produced at 37 °C, set at 100 % in **Figure 3A**. Further testing with a full panel of whole recombinant Pertuzumab (**Figure 3B**) and Trastuzumab (**Figure 3C**) variants (VH and Vκ combinations) transfected in T175 flask did not show significant difference ($p > 0.05$) between the two temperatures.

Nutrient Supplements

A variety of culture supplements commonly used to improve recombinant protein production were evaluated for Pertuzumab κ-light chains and normalized with respect to the control parameter of no supplement (NS) set at 100 % in **Figure 4A**. Adding the supplements to post-transfected cultures on day 7 at a final concentration of 1 mg/mL, a one-way ANOVA analysis showed no significant difference ($p < 0.05$) across the supplements tested. Given that peptone is the most common enhancer of mammalian recombinant protein production (30–33), further investigations were performed using a two-tailed unpaired T-test against the control NS,

revealing that it significantly boosted ($p < 0.05$) κ-chain production by 25 % (**Figure 4A**). Further optimizations of concentration and timing of peptone addition showed that 1 mg/mL, added on post-transfection days 5, 6 or 7, gave the highest boost to κ-chain production (**Figure 4B)** to over 190 %. Further streamlining the timing, we found that a single supplementation alone on day 1 or day 7 enhanced κ-chain production (**Figure 4C**).

Combination of Transfection and Culture Parameters

From the optimization of the individual transfection and culture parameters in **Table 1**, the combined methods for Pertuzumab κ-chain production led to a 217 % increase, that was further enhanced 2-fold to 452 % after peptone supplementation (**Figure 5A**). Validating this on whole antibody production, an increase of 236 % was elicited, with a subtle boost to 252 % with peptone supplementation (**Figure 5A**).

One-way ANOVA analysis showed no significant difference ($p > 0.05$) in whole antibody titers with PEI-DNA complexation incubation times of 5, 15 and 30 minutes in **Figure 5B**. Following on from our previous work where 7 mg essential amino acids showed improvements in whole wild-type Pertuzumab antibody production (26), we attempted to merge the parameters but found an unexpected significant decrease ($p < 0.05$) of 13 % by Dunnett's test. However, the dual supplementations of both peptone and EAA gave a significant increase ($p < 0.05$) of 23 % (**Figure 5C**). Compared to the addition of peptone or essential amino acids alone or together to boost whole antibodies that we previously determined to be limited in production by the light chain (e.g. Vκ5 (25)), using Pertuzumab Vκ5|VH3 plasmids we found peptone alone to also significantly ($p < 0.05$) improve whole antibody production by 28 % but not for the other types of supplements using Dunnett's test.

DISCUSSION

The cost-effective and efficient scalable production of recombinant monoclonal antibodies is key to the commercial success of both therapeutic and diagnostic antibodies. For this purpose, we set out to optimize transfection and culture parameters with relevance beyond antibody production to that of recombinant proteins using mammalian cell culture. Given that whole recombinant antibody production is affected by many factors such as the constant (24) and complementarity determining (25) regions of Vκ and VH chains, deletions in the Vκ chain region (34), types of signal peptide and usage of amino acids (26), as well as the nature of Vκ-VH co-transfections, we simplified the optimization by first examining wild-type Pertuzumab κ-chain (Vκ1-Cκ) production detected using PpL biosensors. Thereafter, we applied the combined optimized parameters to produce the corresponding wild-type whole Pertuzumab (Vκ1|VH3) detected using SpG biosensors.

Counter-intuitively to the assumption where higher cell seeding density could increase antibody production (35), we did not find significant difference ($p > 0.05$) in κ-chain production levels over the range of tested seeding densities (**Figure 1A**), hinting on the influence of other factors. In fact, high seeding cell densities significantly decreased production and this was consistent with previous literature (31), attributed to the reduction in transfection efficiency. For this, we found our typical usage of cells in the range of 2×10^5 cells/mL to be suitable as a baseline for further optimizations.

With linear PEI previously reported to be a cost effective (16), scalable (36), and efficient (37, 38) as a transfection reagent, we found the κ-chain production to peak at a PEI concentration of 4 μg/mL with diminishing returns at higher concentrations. While increasing PEI can improve transfection efficiency, higher levels can be toxic to cells leading to cell death, resulting in poorer transfection efficiency and eventual decreased recombinant protein production (11, 31). Although the Dunnett's test did not show significant increase ($p > 0.05$)

in κ-chain production between our control of 2 μg/mL and 4 μg/mL (**Figure 1B**), the T-test showed a significant increase of 45 % ($p < 0.05$), suggesting its use at 4 μg/mL for subsequent optimizations. Interestingly, DNA concentration as low as 0.25 μg/mL augmented κ-chain production (**Figure 1C**) and this was thus incorporated into the optimized parameters, given that high amounts were likely toxic to result in decreased production (11).

Next, we optimized PEI-DNA complexation incubation parameters, finding good agreement with other studies (11, 22, 39), where longer complexation incubation decreased protein production (**Figure 1D**). This is likely explained by the increase of PEI-DNA complex size with time (40, 41), reducing cell penetration and lowering transfection efficiency. From these findings, we reduced our complexation incubation time to 5 minutes. With temperature affecting molecular kinetics, we also investigated complexation temperature, finding 18 °C, our default air-con maintained room temperature to be optimal. Higher temperatures reduced κ-chain production (**Figure 1E**), possibly due to higher reaction rate resulting in larger complexes. While these effects may not be the same for co-transfections of more than one type of plasmid, smaller complexes are likely to improve transfection efficiency for single plasmid transfections.

Further examining PEI-DNA complexation volume, where complexation typically occurs in a volume of SFM (42) at 10 % of the total media volume (16). Our results were in agreement with a previous study not finding significant differences at 5 and 10 % (11), and even at higher SFM percentages up to 25 %. Based on this, we retained the recommended 10 %. Nonetheless, since the overall direct relationship between SFM percentage used and κ-chain production suggests that reducing complexation volume increases the proximity of PEI and DNA due to increasing concentration, enhancing its complexation to form larger complexes can lead to poorer transfection efficiency (11).

As PEI-DNA complex is the key element of the transfection process, we explored certain lab-culture practices with regards to complexation under light to explore if nucleic acid photodegradation (43) or if the presence of mammalian G418 antibiotics would have any effect. Given that our results show no significant difference ($p > 0.05$) in κ-chain production with the controls (in the dark and without G418) the process can be made operationally easier, with the presence of light of G418 irrelevant. While appearing insignificant, this has impact on the cost and convenience of large-scale protein production.

Turning to optimize culture conditions, we studied the effect of media replacements, serum deprivation, culture media volume, post-transfection harvest time, culture incubation temperature and the effect of supplements. Interestingly, we did not find that media replacements showed significant difference ($p > 0.05$) by one-way ANOVA even though the T-test showed significant increase ($p < 0.05$) in κ-chain production with fresh media replacement performed just prior to transfection (**Figure 2A**). This could be explained by transfection efficiency improving with waste product removal (6, 15). While such a practice is reasonable in laboratories, for large-scale antibody production, such a need would be costly and burdensome (6). Interestingly, we found that media replacements after transfection, conventionally performed with the aim of reducing transfection reagent toxicity, to not only be unnecessary, but also negatively impacting κ-chain production (**Figure 2A**). Thus, from our results, there should be no need for media replacements prior or after transfection, with clear impact on the cost of large-scale production.

With serum deprivation reported to improve transfection efficiency in some cell lines for purposes other than recombinant antibody production (13), we examined if serum deprivation could improve transfection efficiency, hence enhancing κ-chain production. We found a decline in κ-chain production (**Figure 2B**) for the easily transfected HEK293E cells (32) even though it was not significant ($p > 0.05$) by one-way ANOVA. The likely decrease

could be due to the mechanism found in our previous work where the diversity of antibody signal peptides were a natural mitigation method for more distributed usage of essential amino acids that would otherwise be a limiting factor for antibody production (26). It is thus perhaps from the limitation of specific amino acids that serum deprivation naturally affected protein production (44).

On the reasonable assumption that the culture media would provide the necessary nutrients for protein production, we investigated if increasing the culture medium would overcome nutrition bottlenecks and improve κ-chain production. Although a 40 % increase of κ-chain was detected with the use of 5 mL total culture volume, the one-way ANOVA showed no significant difference ($p > 0.05$) across the media volumes tested. Any media volume increases were not feasible in 6-well plates with possible spillage, making the gain not worth the trade off in a larger scale practice.

Following this, we explored post-transfection harvest, finding a direct relationship between post-transfection harvest time and κ-chain production, with the highest κ-chain production equivalent to 155 % of the control achieved on day 21. Depending on the downstream application and its associated quantity of antibody needed, our results suggest that culture supernatant can be reasonably harvested anytime between post-transfection day 3 to 21 (**Figure 2D**), with the typical cell supernatant harvest between days 5 to 14 (45, 46), The additional significant production increase of 55 % on day 21 may hence come at the expense of time and operational costs, that would need to be separately assessed on the trade-offs.

Based on the assumption that growth rate retardation would retain higher cell viability, lower nutrient consumption rate and waste accumulation reduction, amongst better mRNA stability, improved protein folding and fidelity of post-translational events (47), attributing to increase protein production, cell culture incubation temperature was investigated. Notably, the literature in this area has yet to propose a unified solution with varying results on different cell

types and proteins. Some were reportedly better (48), having no effect (49) or decreasing production (50) at lower temperatures, with no recent studies performed on HEK293E cells other than one on HEK293S suspension cells (12) finding better production of non-antibody recombinant proteins at lower temperatures. From our experiments, we found that lower temperatures were in general detrimental to κ-chain production (**Figure 3A**) with no clear consistent trends for whole antibody production (**Figure 3B and 3C**). Thus, from our findings, we did not depart from the optimal growth temperature of 37 °C.

Following the finding that the addition of media may slightly improve the recombinant protein production, we explored the addition of supplements. Given that peptone (30–33), tryptone (33), casamino acids (51), yeast extract (52), casein (53), and skim milk (54) were reportedly useful, there were tested. From our panel, we found only peptone to significantly boost ($p < 0.05$) κ-chain production by T-test (**Figure 4A**), in agreement to previous reports (30–33). Optimizing the time of the addition, we further demonstrated that single small amounts of peptone added on post-transfection days 5 to 7 were most useful in boosting κ-chain production (**Figure 4B**) and repeated additions did not yield significant improvements (**Figure 4C**). With no further increase from repeated additions, the accumulation of waste is likely the limiting factor (55) for such cultures.

Combining the optimized parameters, we improved Pertuzumab κ-chain production by 217 % which was further augmented to 452 % by the addition of peptone (**Figure 5A**). Applied to whole Pertuzumab antibody production, a similar increase to 236 % was observed but with a small augmentation by peptone at 252 % (**Figure 5A**). With some clear differences where PEI-DNA complexation times did not augment whole antibody production as for κ-light chain, likely due to the use of co-transfections requiring more complexation time (**Figure 5B**). From a possible lead in our previous paper (26), where 3.5 mg/mL essential amino acids equivalent of essential amino acid supplements showed improvements in whole antibody production, we

explored a dual supplementation of both essential amino acids and peptone with each supplement added separately. We found an unexpected significant whole antibody production decrease upon essential amino acids addition which was rescued by the addition of peptone (**Figure 5C**) for the Pertuzumab antibody.

Considering that some whole antibody productions are limited by Vκ-chain (e.g. Pertuzumab Vκ5 from our previous work (25) and that peptone appeared to benefit light chain production, we performed similar experiments using Vκ5|VH3 whole antibodies. We boosted the whole Vκ5|VH3 antibodies production (Figure **5D**) by 28 %, supporting the use of peptone supplements for antibodies limited by light chains in transient production.

Through a detailed systematic selection of antibodies in a holistic (1, 27) manner, the selection of previously published factors of best heavy chain constant (24), Vκ-VH pairings, amino acid usage with leader selection (26), antibody production could be made more efficient, mitigating commercial failures due to production costs.

CONCLUSION

Through the optimization of transfection and culture parameters, we demonstrated a ~2-fold increase of κ-chain only and whole antibody production from HEK293E using 25 kDa linear PEI in a cost-effective manner. With peptone supplements, κ-chain increased by ~4-fold while whole antibody increased to a lesser extent, with most benefits found for antibodies limited by their light chains in production. The findings of which can make antibody production more efficient and convenient with potential application to other mammalian-based recombinant protein production.

DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, W.-L.L and S.K.-E.G.; methodology, W.-L.L. and Z.S.-L.H.; formal analysis, W.-L.L., Z.S.-L.H.; S.K.-E.G; investigation, W.-L.L. and Z.S.-L.H.; validation, D.W.-S.K., J.Y.Y., writing—original draft preparation, Z.S.-L.H; writing—review and editing, D.W.-S.K., J.Y.Y., S.K.-E.G., W.-L.L. and Z.S.-L.H; visualization, S.K.-E.G., W.-L.L. and Z.S.-L.H; supervision, S.K.-E.G. and W.-L.L; funding acquisition, S.K.-E.G. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENTS

This work was partially supported by the Joint Council Office, Agency for Science, Technology, and Research, Singapore, under Grant number JCO1334i00050 and the National Research Foundation (NRF) Singapore grant to Experimental Drug Development Centre (EDDC) for the platform AMDED, acquired by S.K.-E.G. The authors would also like to thank Jun-Jie Poh for his large-scale transfection data contribution.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Ling,W.-L., Lua,W.-H. and Gan,S.K.-E. (2020) Sagacity in antibody humanization for therapeutics, diagnostics and research purposes: considerations of antibody elements and their roles. *Antib. Ther.*, **3**, 71–79.

2. Pham,P.L., Kamen,A. and Durocher,Y. (2006) Large-Scale Transfection of Mammalian Cells for the Fast Production of Recombinant Protein. *Mol. Biotechnol.*, **34**, 225–238.

3. Geisse,S. and Henke,M. (2005) Large-scale Transient Transfection of Mammalian Cells: A Newly Emerging Attractive Option for Recombinant Protein Production. *J. Struct. Funct. Genomics*, **6**, 165–170.

4. Wurm,F.M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.*, **22**, 1393–1398.

5. Yuan,J., Xu,W.W., Jiang,S., Yu,H. and Poon,H.F. (2018) The Scattered Twelve Tribes of HEK293. *Biomed. Pharmacol. J.*, **11**, 621–623.

6. Durocher,Y. (2002) High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.*, **30**, 9e–99.

7. Meissner,P., Pick,H., Kulangara,A., Chatellard,P., Friedrich,K. and Wurm,F.M. (2001) Transient gene expression: Recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol. Bioeng.*, **75**, 197–203.

8. Ho,Y.K. and Too,H.P. (2020) Development of a laboratory scalable process for enhancing lentivirus production by transient transfection of HEK293 adherent cultures. *Gene Ther.*, **27**, 482–494.

9. Bollin,F., Dechavanne,V. and Chevalet,L. (2011) Design of Experiment in CHO and HEK transient transfection condition optimization. *Protein Expr. Purif.*, **78**, 61–68.

10. Sou,S.N., Polizzi,K.M. and Kontoravdi,C. (2013) Evaluation of transfection methods for transient gene expression in Chinese hamster ovary cells. *Adv. Biosci. Biotechnol.*, **04**, 1013–1019.

11. Greene,E., Cazacu,D., Tamot,N., Castellano,S., Datar,A., Kronkaitis,A., Gebhard,D., Reed,J., Mawson,P., Florin,L., *et al.* (2021) Optimization of a transient antibody expression platform towards high titer and efficiency. *Biotechnol. J.*, **16**, e2000251.

12. Lin,C.-Y., Huang,Z., Wen,W., Wu,A., Wang,C. and Niu,L. (2015) Enhancing Protein Expression in HEK-293 Cells by Lowering Culture Temperature. *PLOS ONE*, **10**, e0123562.

13. Wallenstein,E.J., Barminko,J., Schloss,R.S. and Yarmush,M.L. (2010) Serum starvation improves transient transfection efficiency in differentiating embryonic stem cells. *Biotechnol. Prog.*, **26**, 1714–1723.

14. Baldi,L., Hacker,D.L., Adam,M. and Wurm,F.M. (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnol. Lett.*, **29**, 677–684.

15. Fang,X.T., Sehlin,D., Lannfelt,L., Syvänen,S. and Hultqvist,G. (2017) Efficient and inexpensive transient expression of multispecific multivalent antibodies in Expi293 cells. *Biol. Proced. Online*, **19**, 11.

16. Schlaeger,E.-J. and Christensen,K. (1999) Transient gene expression in mammalian cells grown in serum-free suspension culture. *Cytotechnology*, **30**, 71–83.

17. Mason,M., Sweeney,B., Cain,K., Stephens,P. and Sharfstein,S. (2014) Reduced Culture Temperature Differentially Affects Expression and Biophysical Properties of Monoclonal Antibody Variants. *Antibodies*, **3**, 253–271.

18. Xu,J., Tang,P., Yongky,A., Drew,B., Borys,M.C., Liu,S. and Li,Z.J. (2019) Systematic development of temperature shift strategies for Chinese hamster ovary cells based on short duration cultures and kinetic modeling. *mAbs*, **11**, 191–204.

19. Davami,F., Baldi,L., Rajendra,Y. and Wurm,F.M. (2014) Peptone Supplementation of Culture Medium Has Variable Effects on the Productivity of CHO Cells. *Int. J. Mol. Cell Med*, **3**, 146–156.

20. Pérez-Rodriguez,S., Ramírez-Lira,M. de J., Trujillo-Roldán,M.A. and Valdez-Cruz,N.A. (2020) Nutrient supplementation strategy improves cell concentration and longevity, monoclonal antibody production and lactate metabolism of Chinese hamster ovary cells. *Bioengineered*, **11**, 463–471.

21. You,M., Liu,Y., Chen,Y., Guo,J., Wu,J., Fu,Y., Shen,R., Qi,R., Luo,W. and Xia,N. (2013) Maximizing Antibody Production in Suspension-Cultured Mammalian Cells by the Customized Transient Gene Expression Method. *Biosci. Biotechnol. Biochem.*, **77**, 1207–1213.

22. Liu,C., Dalby,B., Chen,W., Kilzer,J.M. and Chiou,H.C. (2008) Transient Transfection Factors for High-Level Recombinant Protein Production in Suspension Cultured Mammalian Cells. *Mol. Biotechnol.*, **39**, 141–153.

23. Rees,A.R. (2020) Understanding the human antibody repertoire. *mAbs*, **12**, 1729683.

24. Lua,W.-H., Ling,W.-L., Yeo,J.Y., Poh,J.-J., Lane,D.P. and Gan,S.K.-E. (2018) The effects of Antibody Engineering CH and CL in Trastuzumab and Pertuzumab recombinant models: Impact on antibody production and antigen-binding. *Sci. Rep.*, **8**, 718.

25. Ling,W.-L., Lua,W.-H., Poh,J.-J., Yeo,J.Y., Lane,D.P. and Gan,S.K.-E. (2018) Effect of VH–VL Families in Pertuzumab and Trastuzumab Recombinant Production, Her2 and FcγIIA Binding. *Front. Immunol.*, **9**, 469.

26. Ling,W.-L., Su,C.T.-T., Lua,W.-H., Poh,J.-J., Ng,Y.-L., Wipat,A. and Gan,S.K.-E. (2020) Essentially Leading Antibody Production: An Investigation of Amino Acids, Myeloma, and Natural V-Region Signal Peptides in Producing Pertuzumab and Trastuzumab Variants. *Front. Immunol.*, **11**, 604318.

27. Phua,S.-X., Chan,K.-F., Su,C.T.-T., Poh,J.-J. and Gan,S.K.-E. (2019) Perspective: The promises of a holistic view of proteins—impact on antibody engineering and drug discovery. *Biosci. Rep.*, **39**, BSR20181958.

28. Chan,W.-T., Verma,C.S., Lane,D.P. and Gan,S.K.-E. (2013) A comparison and optimization of methods and factors affecting the transformation of Escherichia coli. *Biosci Rep.*, **33**, e00086.

29. Poh,J.-J. and Gan,S.K.-E. (2014) The Determination of Factors involved in Column-Based Nucleic Acid Extraction and Purification. *J. Bioprocess. Biotech.*, **4**, 157.

30. Heidemann,R., Zhang,C., Qi,H., Larrick Rule,J., Rozales,C., Park,S., Chuppa,S., Ray,M., Michaels,J., Konstantinov,K., *et al.* (2000) The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density perfusion cultures of mammalian cells. *Cytotechnology*, **32**, 157–167.

31. Davami,F., Eghbalpour,F., Barkhordari,F. and Mahboudi,F. (2014) Effect of Peptone Feeding on Transient Gene Expression Process in CHO DG44. *Avicenna J. Med. Biotechnol*, **6**, 147–155.

32. Pham,P.L., Perret,S., Doan,H.C., Cass,B., St-Laurent,G., Kamen,A. and Durocher,Y. (2003) Large-Scale Transient Transfection of Serum-Free Suspension-Growing HEK293 EBNA1 Cells: Peptone Additives Improve Cell Growth and Transfection Efficiency. *Biotechnol. Bioprocess Eng.*, **84**.

33. Pham,P.L., Perret,S., Cass,B., Carpentier,E., St-Laurent,G., Bisson,L., Kamen,A. and Durocher,Y. (2005) Transient gene expression in HEK293 cells: Peptone addition posttransfection improves recombinant protein synthesis. *Biotechnol. Bioeng.*, **90**, 332–344.

34. Su,C.T.-T., Ling,W.-L., Lua,W.-H., Poh,J.-J. and Gan,S.K.-E. (2017) The role of Antibody Vκ Framework 3 region towards Antigen binding: Effects on recombinant production and Protein L binding. *Sci. Rep.*, **7**, 3766.

35. Backliwal,G., Hildinger,M., Hasija,V. and Wurm,F.M. (2008) High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. *Biotechnol. Bioeng.*, **99**, 721–727.

36. Nyamay'Antu,A., Kedinger,V. and Erbacher,P. (2018) Addressing scaling-up limitations: optimized PEI-mediated production of clinical grade viral vectors. *Cell Gene Ther. Insights*, **4**, 71–79.

37. Wiseman,J.W., Goddard,C.A., McLelland,D. and Colledge,W.H. (2003) A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. *Gene Ther.*, **10**, 1654–1662.

38. Hanzlíková,M., Ruponen,M., Galli,E., Raasmaja,A., Aseyev,V., Tenhu,H., Urtti,A. and Yliperttula,M. (2011) Mechanisms of polyethylenimine-mediated DNA delivery: free carrier helps to overcome the barrier of cell-surface glycosaminoglycans: Role of free PEI in transfection. *J. Gene Med.*, **13**, 402–409.

39. Bos,A.B., Duque,J.N., Bhakta,S., Farahi,F., Chirdon,L.A., Junutula,J.R., Harms,P.D. and Wong,A.W. (2014) Development of a semi-automated high throughput transient transfection system. *J. Biotechnol.*, **180**, 10–16.

40. Han,X., Fang,Q., Yao,F., Wang,X., Wang,J., Yang,S. and Shen,B.Q. (2009) The heterogeneous nature of polyethylenimine-DNA complex formation affects transient gene expression. *Cytotechnology*, **60**, 63–75.

41. Sang,Y., Xie,K., Mu,Y., Lei,Y., Zhang,B., Xiong,S., Chen,Y. and Qi,N. (2015) Salt ions and related parameters affect PEI–DNA particle size and transfection efficiency in Chinese hamster ovary cells. *Cytotechnology*, **67**, 67–74.

42. Yang,J.-P. and Huang,L. (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther.*, **4**, 950–960.

43. Korycka-Dahl,M. and Richardson,T. (1980) Photodegradation of DNA with fluorescent light in the presence of riboflavin, and photoprotection by flavin triplet-state quenchers. *Biochim. Biophys. Acta BBA - Nucleic Acids Protein Synth.*, **610**, 229–234.

44. Oya,N., Zölzer,F., Werner,F. and Streffer,C. (2003) Effects of Serum Starvation on Radiosensitivity, Proliferation and Apoptosis in Four Human Tumor Cell Lines with Different p53 Status. *Strahlenther. Onkol.*, **179**, 99–106.

45. Shatz,W., Ng,D., Dutina,G., Wong,A.W., Dunshee,D.R., Sonoda,J., Shen,A. and Scheer,J.M. (2016) An efficient route to bispecific antibody production using single-reactor mammalian co-culture. *mAbs*, **8**, 1487–1497.

46. Li,F., Vijayasankaran,N., Shen,A. (Yijuan), Kiss,R. and Amanullah,A. (2010) Cell culture processes for monoclonal antibody production. *mAbs*, **2**, 466–479.

47. Masterton,R.J., Roobol,A., Al-Fageeh,M.B., Carden,M.J. and Smales,C.M. (2010) Post-translational events of a model reporter protein proceed with higher fidelity and accuracy upon mild hypothermic culturing of Chinese hamster ovary cells. *Biotechnol. Bioeng.*, **105**, 215–220.

48. Yoon,S.K., Hwang,S.O. and Lee,G.M. (2004) Enhancing Effect of Low Culture Temperature on Specific Antibody Productivity of Recombinant Chinese Hamster Ovary Cells: Clonal Variation. *Biotechnol. Prog.*, **20**, 1683–1688.

49. Bloemkolk,J.-W., Gray,M.R., Merchant,F. and Mosmann,T.R. (1992) Effect of temperature on hybridoma cell cycle and MAb production. *Biotechnol. Bioeng.*, **40**, 427–431.

50. Barnabé,N. and Butler,M. (1994) Effect of temperature on nucleotide pools and monoclonal antibody production in a mouse hybridoma. *Biotechnol. Bioeng.*, **44**, 1235–1245.

51. Kaushik,N., Rohila,D., Arora,U., Raut,R., Lamminmäki,U., Khanna,N. and Batra,G. (2016) Casamino acids facilitate the secretion of recombinant dengue virus serotype-3 envelope domain III in Pichia pastoris. *BMC Biotechnol.*, **16**, 12.

52. Li,M., Liao,X., Zhang,D., Du,G. and Chen,J. (2011) Yeast Extract Promotes Cell Growth and Induces Production of Polyvinyl Alcohol-Degrading Enzymes. *Enzyme Res.*, **2011**, 1–8.

53. Russell,J.B., Sniffen,C.J. and Van Soest,P.J. (1983) Effect of Carbohydrate Limitation on Degradation and Utilization of Casein by Mixed Rumen Bacteria. *J. Dairy Sci.*, **66**, 763–775.

54. Khani,M.-H. and Bagheri,M. (2020) Skimmed milk as an alternative for IPTG in induction of recombinant protein expression. *Protein Expr. Purif.*, **170**, 105593.

55. Cudna,R.E. and Dickson,A.J. (2003) Endoplasmic reticulum signaling as a determinant of recombinant protein expression. *Biotechnol. Bioeng.*, **81**, 56–65.

FIGURES

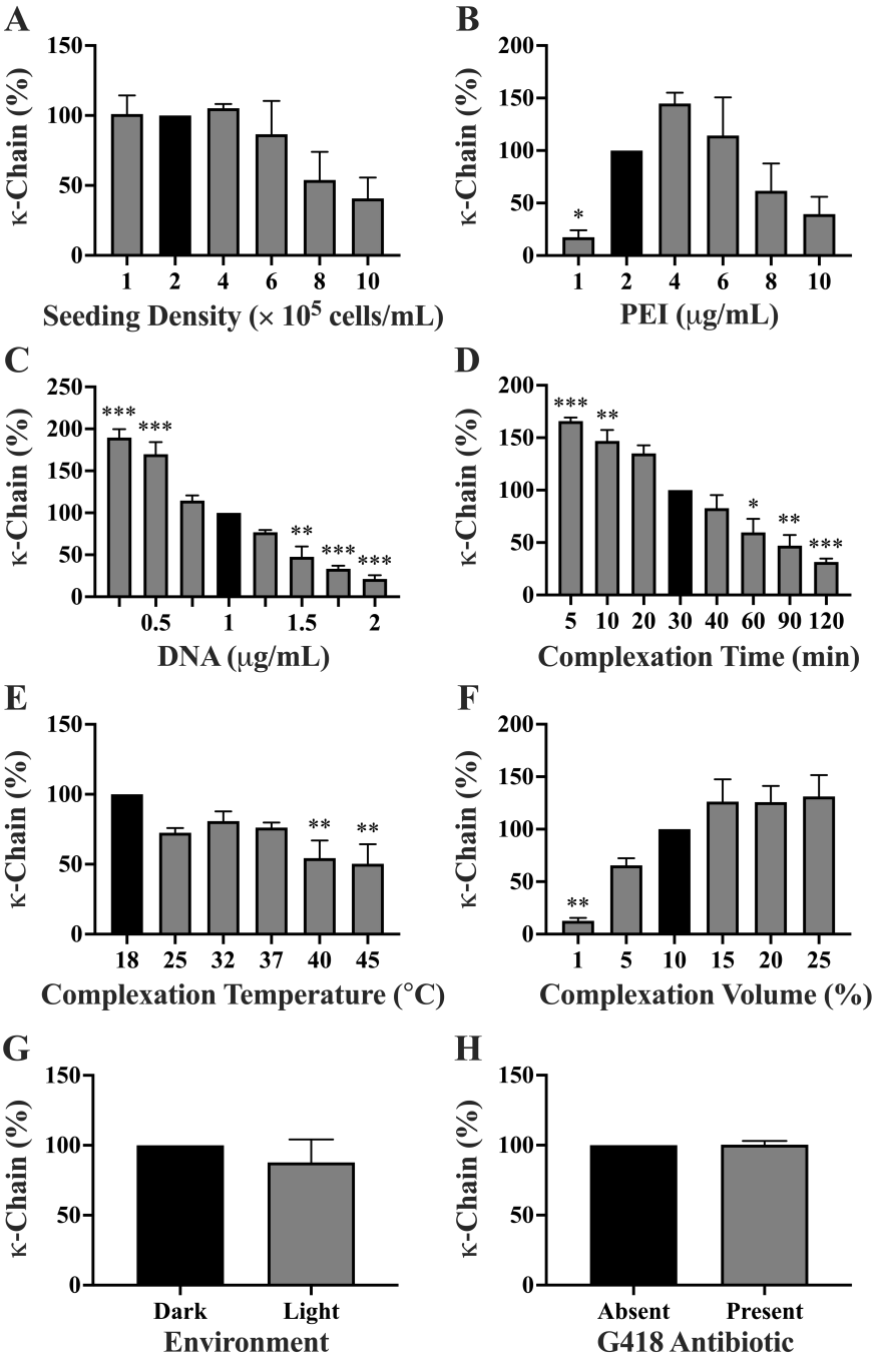

Figure 1: Pertuzumab κ-chain production from optimizing the transfection parameters of (A) Seeding density; (B) PEI concentration; (C) DNA concentration; (D) PEI-DNA complexation time (E); PEI-DNA complexation temperature; (F) PEI-DNA complexation volume; (G) PEI-DNA complexation in light and dark environments (H) PEI-DNA complex in the presence or absence of G418 antibiotic. Black colored bars represent the controls to which the experimental parameters were compared against, one-way ANOVA (A-F), T-test (G and H). *, **, *** denote significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

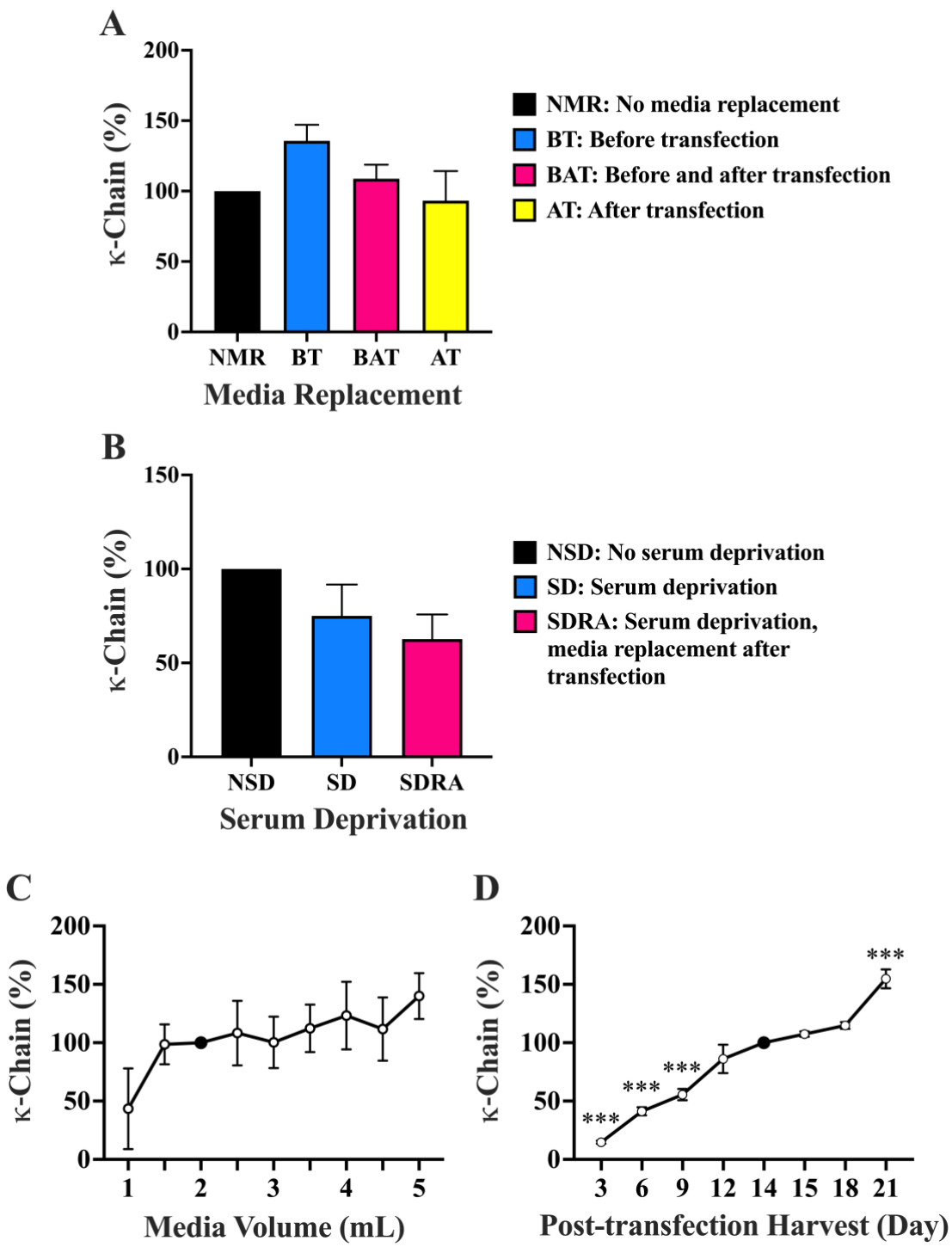

Figure 2: Pertuzumab κ-chain production from optimizing culture parameters with respect to (A) media replacement timing; (B) serum deprivation; (C) culture media volume; and (D) post-transfection harvest day. Black colored bars in A and B show the controls, whereas the black colored dots in C and D represent the controls to which the respective experimental parameters were compared against using one-way ANOVA. *** denote significance at $p < 0.001$.

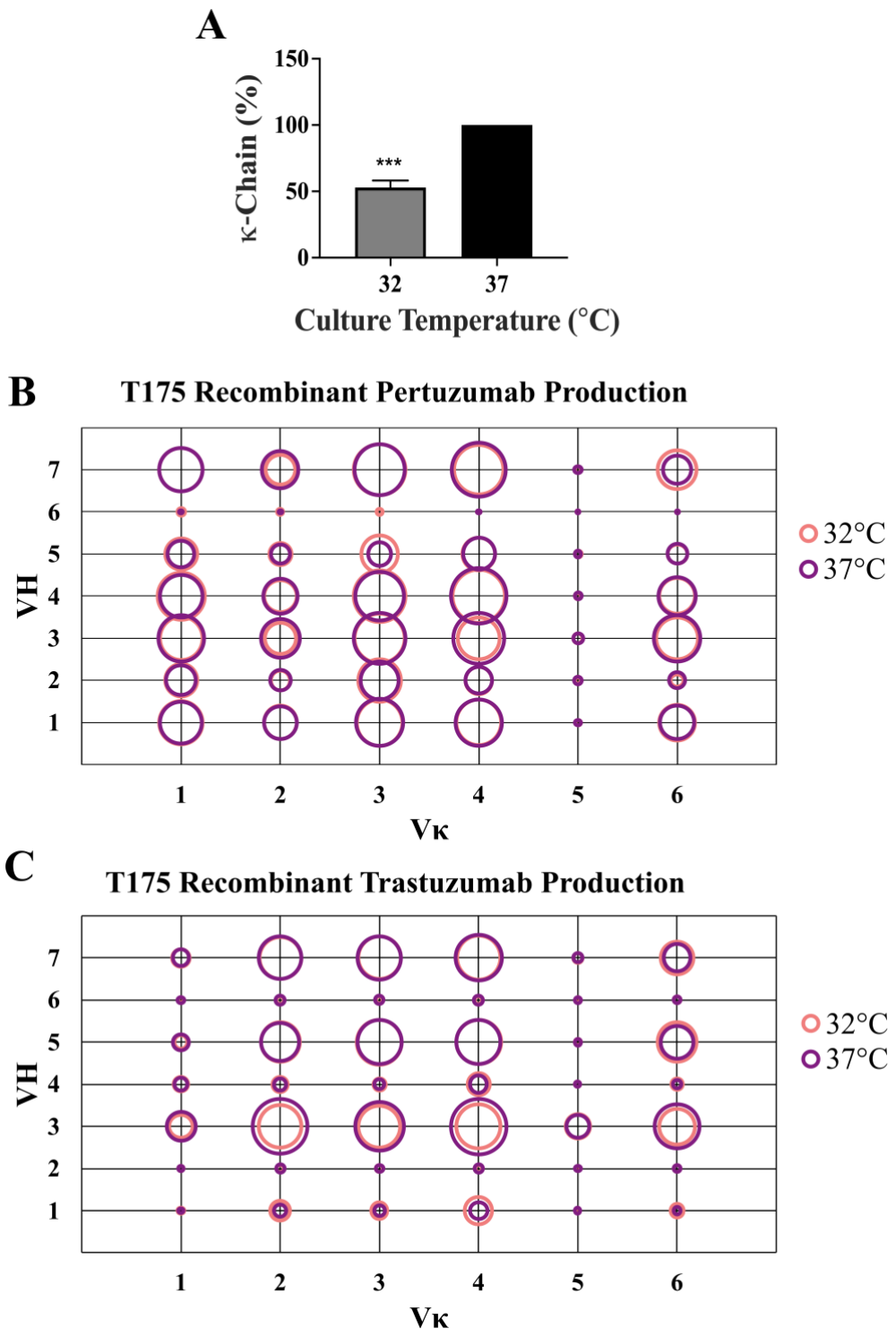

Figure 3: Antibody production from cultures at 32 and 37 °C. (A) Antibody κ-chain production in 2 mL, 6-well plates using the recombinant wild-type Pertuzumab Vκ1. Black colored bars in A represent the control to which experimental parameters were compared against by T-test. *** denotes $p < 0.001$. Bubble chart representations of T175 flasks 40 mL transfections of a panel of recombinant Pertuzumab (B) and Trastuzumab (C) variants (VH and Vκ) previously described (25, 26). Each circle in the respective combination shows the production level of the antibody.

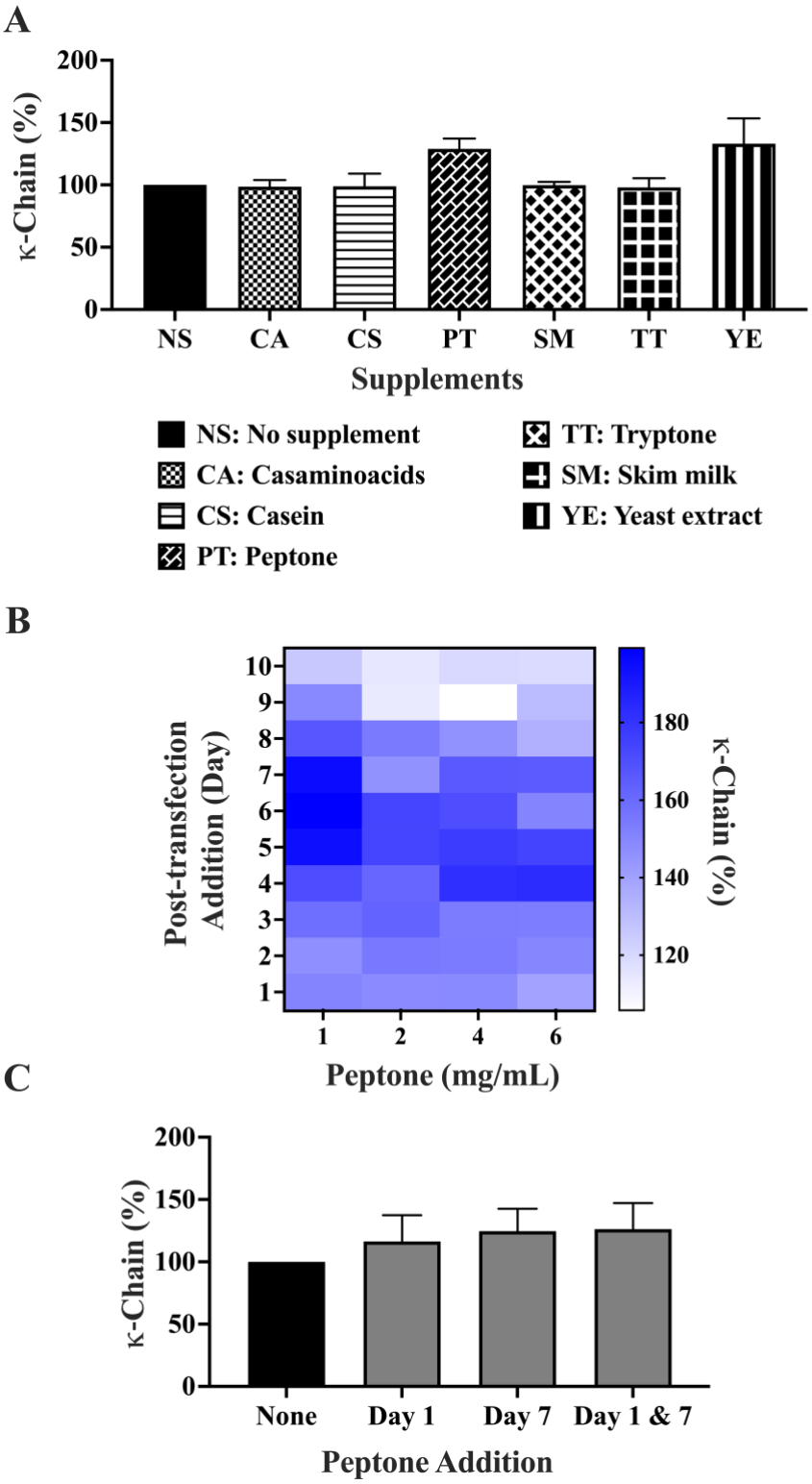

Figure 4: Antibody κ-chain production with (A) nutrient supplements; (B) Heat map of one-time additions of different peptone concentrations; (C) Repeated peptone additions of 1 mg/mL. Black colored bars in A and C represent the control to which the respective experimental parameters were compared against.

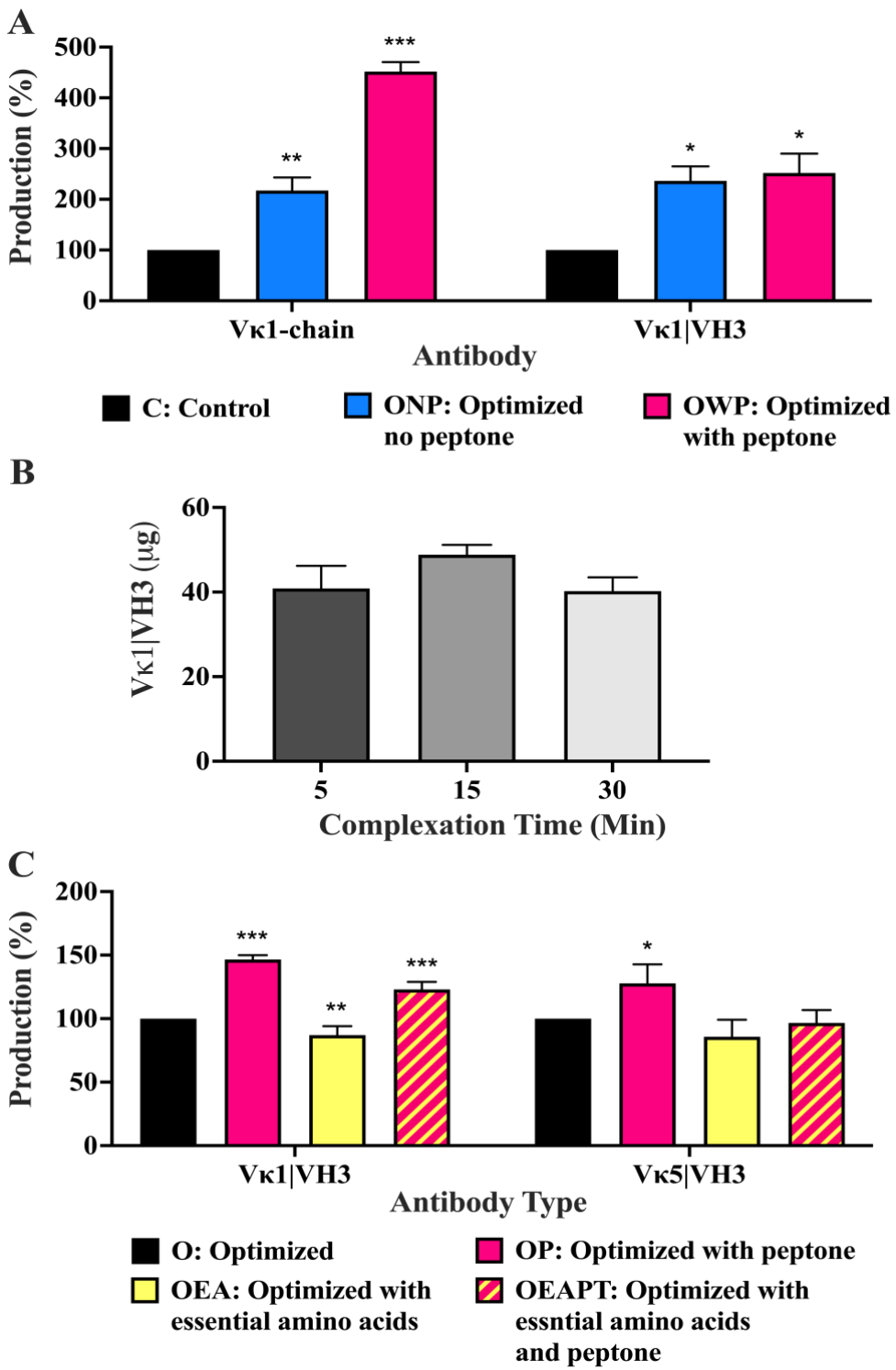

Figure 5: Production of wild-type Pertuzumab (A) κ-chain (Vκ1) and whole Vκ1|VH3 antibody among standard, optimized and with peptone supplementation conditions; (B) Whole Vκ1|VH3 antibody production at optimized conditions with PEI-DNA complex time variations; (C) Vκ1|VH3 and Vκ5|VH3 antibodies among optimized conditions with peptone and/or essential amino acids supplements. Black colored bars in A and C represent the control to which the respective experiment parameters were compared against by one-way ANOVA. *, **. *** denote significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

TABLE

Table 1: Control and optimized conditions for transient recombinant production in 2 mL, 6-well plate cultures.

Conditions	**Control**	**Optimal**
Cell Density (cells/mL)	2×10^5	2×10^5
PEI Concentration (μg/mL)	2	4
Plasmid DNA Concentration (μg/mL)	1	0.25
Complexation Time (min)	30	5
Complexation Temperature (°C)	18	18
Complexation in SFM (% of transfection volume)	10	10
Culture Media Volume (mL)	2	2
Post-transfection Harvest (day)	14	14
Culture Incubation Temperature (°C)	37	37
Supplements (Post-transfection)	-	1 mg/mL Peptone on Day 7