ARTICLE

To Plate or to Simply Unfreeze, That Is the Question for Optimal Plasmid Extraction

Ryan Kye-Rhong Thean,[1] Debby Xin-Ying Ong,[1] Zealyn Shi-Lin Heng,[1] Samuel Ken-En Gan,[1,2,3,] and Joshua Yi Yeo[1,2,*]*

*[1]Antibody & Product Development Lab, A*STAR, Singapore 138671, Singapore; [2]Experimental Drug Development Centre, A*STAR, Singapore 138670, Singapore; and [3]p53 Laboratory, A*STAR, Singapore 138648, Singapore*

Many molecular biology applications require fast plasmid DNA extraction, spurring multiple studies on how to speed up the process. It is regularly instructed in standard laboratory protocols to plate out frozen glycerol bacterial stocks prior to bacteria incubation in liquid media and subsequent plasmid extraction, although the rationale for this is often unexplained (other than for the isolation of single colonies). Given the commonality and importance of this laboratory operation, such a practice is time-consuming and laborious. To study the impact of this practice and the alternative direct culturing method, we investigated the association between bacterial cell mass and its potential influence on plasmid yields from the 2 methods. Our results showed no difference with preplating for 7 out of 8 plasmid constructs used in the study, suggesting that direct glycerol recovery would not lead to poorer plasmid yields. The findings support the rationale for direct glycerol recovery for plasmid extraction, without the need of an intermediate preplating step.

Key Words: bacterial growth, glycerol stock, plasmid yield

INTRODUCTION

Large quantities of high-quality plasmid DNA are required for critical downstream molecular biology processes, from recombinant DNA manipulation [1] to gene therapy (requiring micrograms to milligrams of plasmid), [2] and DNA vaccines (requiring milligrams of DNA per dose). [2, 3]

Much research has focused on boosting DNA production through engineering bacterial host strains and plasmids. For instance, the down-regulation of pyruvate kinase, increased generation of NADPH, reduced production of acetate, and expression of antibiotic resistance markers encoded in plasmids were found to enhance plasmid production. [4–6] At the same time, altering fermentation factors such as temperature, nutrients, and oxygen can increase growth rate of host bacterial strains, typically *Escherichia coli*, improving plasmid yield and purity. [7] However, in most biological laboratories, researchers continue with established processes ("laboratory wisdom") of plasmid production without such vigorous and complex technical manipulations.

A relatively neglected research area for high plasmid yield is the effect of different bacterial stock recovery methods on plasmid extraction. It is typically instructed in standard laboratory protocols [8–10] to plate out frozen glycerol bacterial stocks prior to bacteria incubation in liquid media and subsequent plasmid extraction, although the rationale is often unexplained, other than for isolation of single colonies. Given that this is a common and important laboratory operation, such a practice involving an additional day of work (**Fig. 1**) is time-consuming and laborious. The obvious alternative, growing bacterial cultures directly from frozen glycerol bacterial stocks, is rarely seen in protocols due to the assumption that bacterial colonies on selective plates would be in the log phase of their growth curve for higher amounts of plasmid to be extracted [11,12]. In support of this, frozen bacterial glycerol stocks are often found to be in stress–induced dormancy, [13] requiring a longer lag phase, thereby resulting in lower plasmid yields.

When analyzing the impact of direct bacterial cultures on plasmid yields, it is also important to consider bacterial cell mass to optimize plasmid yields per round of extraction. At present, the association between final bacterial cell mass (measured by maximum optical density or OD) and plasmid yield is inconclusive. [3] Horn *et al.*[14] reported that they could merely extract 4 mg of plasmid per liter of culture when the cells were harvested at the late log phase with an OD_{600} of 30. In contrast, Diogo *et al.*[15] successfully extracted 38 mg of plasmid per liter of culture harvested at OD_{600} of about 14. Given that both studies employed simple batch cultivation with a similar cultivation medium and plasmid-extraction method, the discrepancy in plasmid yield remains multifactorial requiring more investigation into this area.

*Address correspondence to: Samuel Ken-En Gan, Antibody & Product Development Lab, EDDC-BII, A*STAR, 60 Biopolis Street, #B2 Genome, Singapore 138672, Singapore (Tel: +65 6407 0584; E-mail: samuel_gan@eddc.a-star.edu.sg).

*Address correspondence to: Joshua Yi Yeo, Antibody & Product Development Lab, EDDC-BII, A*STAR, 60 Biopolis Street, #B2 Genome, Singapore 138672, Singapore (Tel: +65 6407 0584; E-mail: yeoyj@bii.a-star.edu.sg).

https://doi.org/10.7171/jbt.20-3203-001

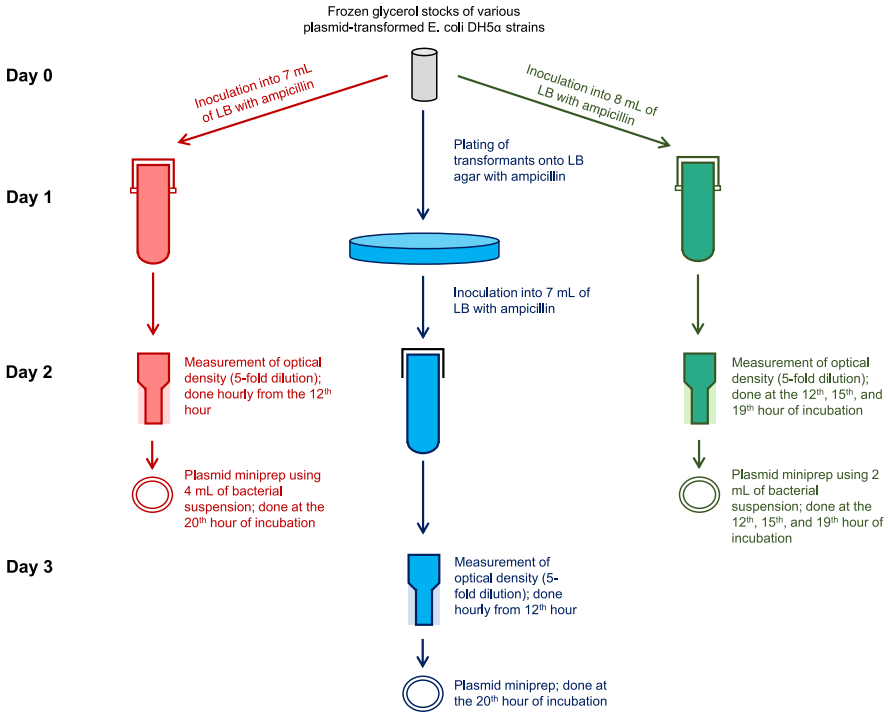

FIGURE 1

Schematic diagram outlining the processes of the plasmid extraction study and their timelines. Experiments were conducted in sextuplicate.

In the midst of the assumptions and contradicting findings, we have yet to find an in-depth study comparing the plasmid yields of bacteria from preplated colonies and frozen stocks. To fill this gap, we investigated the different effects of pre-plated and direct glycerol recovery methods on bacterial growth and plasmid yields while also deciphering the contributions of plasmid backbones and gene inserts on bacterial growth, plasmid yields and incubation time.

MATERIALS AND METHODS

Bacterial strains and plasmids

In-house competent *E. coli* DH5α cells were chemically transformed [16] with two ampicillin-resistant plasmids, pTT5 [17] and pET21d (Thermo Fisher Scientific, Singapore). These plasmids hold the following gene inserts: HIV-1 protease (Hprot, accession number: AY622223.1), HIV-1 Gag (Hgag [18]), human CD89 (Fc fragment of IgA receptor FCAR, accession number: NM_002000.4, [19, 20]), and human CD32 (Fc fragment of IgG receptor IIa FCG2A, accession number: NM_001136219.3 [21]).

Colony-standardized glycerol stock preparation

Transformed *E. coli* cells were streaked onto Luria-Bertani (LB) agar plates supplemented with 100 μg/ml of ampicillin and incubated at 37°C overnight. A single clone was picked from each plate, inoculated into 5 ml of ampicillin added to LB broth, and incubated with shaking at 250 rpm for 8 h. Glycerol stocks were prepared in sterile 2-ml microcentrifuge tubes by adding presterilized glycerol to a final concentration of 10% and stored at −80°C.

Growing of agar-plated colonies and glycerol-recovered cultures

Transformed clones of each plasmid were streaked from frozen glycerol stocks onto selective agar plates supplemented with ampicillin and incubated at 37°C overnight. Independent bacteria colonies were inoculated into 7 ml of LB broth with ampicillin in 14-ml round-bottom tubes and incubated at 37°C, 250 rpm. Bacterial growth of glycerol-recovered cultures was initiated by directly inoculating frozen glycerol stocks in a similar manner.

OD measurement, plasmid miniprep, and plasmid quantification

The bacterial cultures were diluted 5-fold at required time intervals before OD_{600} measurements were taken using IMPLEN Nanophotometer P330 and APD SpectBT. [22] LB with ampicillin was used as blank. Plasmids were extracted at the end of required incubation periods using spin columns according to BioBasic's Plasmid DNA Miniprep Kit, as previously described, [23, 24] and quantified using Thermo Scientific Nanodrop 1000. The number of plasmid copies extracted was calculated using the equation presented in Lee *et al.*[25] to account for differences in molecular

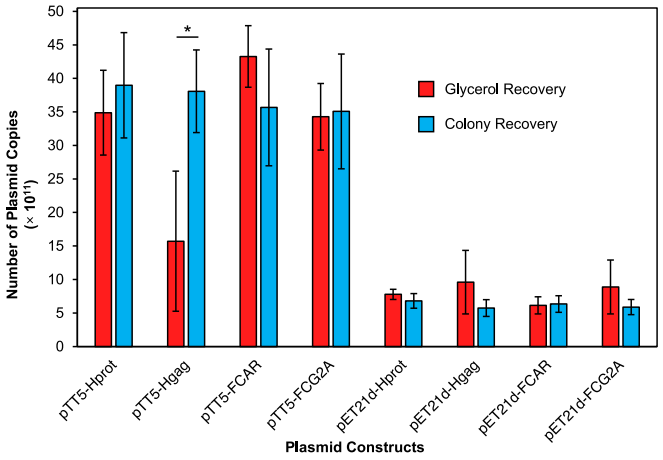

FIGURE 2

Comparison of bacterial stock recovery methods on plasmid yields of various pTT5 and pET21d plasmid constructs. Plasmid extraction and calculation of number of plasmid copies was performed after 20 h of incubation. Unpaired *t* test was performed using MiniTab 18. Data represent the means of sextuplicates, with error bars representing the SD. $^{*}P < 0.05$.

weight (MW). An overview of the experiment is illustrated, and all experiments were performed in sextuplicates (**Fig. 1**).

Plasmid DNA sequence analysis

For verification, plasmids were sequenced with Human cytomegalovirus (CMV) immediate early promoter forward (5′ CGCAAATGGGCGGTAGGCGTG 3′) and T7 terminator (5′ GCTAGTTATTGCTCAGCGG 3′) primers for pTT5 and pET21d constructs respectively. Sequencing results were analyzed with DNAapp [26] and DNA2app [27] and compared with a reference sequence [28] using MAFFT (Multiple Alignment using Fast Fourier Transform). [29]

Statistical analysis using Minitab 18

For the comparison of plasmid yields between the two recovery methods, unpaired 2-sample *t* test was performed, while the comparison of plasmid yields across 3 time points were performed using paired 2-sample *t* test, 12th hour *vs.* 15th hour and 15th hour *vs.* 19th hour.

RESULTS AND DISCUSSION

In this study, we aim to investigate plasmid yields between colony and frozen glycerol stock–recovered bacteria and found no significant difference in plasmid yields at the 20th hour of liquid media incubation for 7 out of 8 plasmid constructs tested (**Fig. 2**). The single exception was pTT5-Hgag, with a significantly lower plasmid yield from frozen glycerol stock–recovered bacteria in comparison to colony-recovered bacteria. Examining the OD_{600} measurements taken at the 20th hour prior to plasmid extraction, the average total cell mass was comparable or marginally higher for frozen stock–recovered bacteria, ruling out overall bacteria counts as the reason for the decreased plasmid yield. Given that Gag is a viral protein, we speculate that leaky promoters causing plasmid instabilities [30] may be a factor requiring further bacterial physiological investigations.

With no other significant differences in plasmid yields between both bacterial recovery methods, we examined the effects of different plasmid constructs on bacterial growth rates and plasmid yields. Bacterial growth curves of different plasmids were plotted using hourly measurements of OD_{600} from the 12th hour to the 20th hour of incubation (**Fig. 3**), which flank the recommended incubation periods for plasmid harvesting in many protocols. [8, 10] Categorizing our 8 plasmids into its two distinct plasmid backbones (pTT5 and pET21d), we found that bacterial transformants containing the high plasmid copy number pTT5 backbones [31] had significantly lower OD_{600} values throughout the measured time course and maximum OD_{600} (Fig. 3) when compared with those of the low plasmid copy number pET21d backbones [32] (Fig. 3) in both bacterial recovery methods. This is in line with previous findings which demonstrated an inverse relationship between growth rates and plasmid copy number. [33–36] High–copy-number plasmids were proposed to impose a higher metabolic burden on host bacteria, [37, 38] in which more energy, reducing power, and precursor metabolites would be utilized for production of antibiotic-resistant proteins and maintaining high copy numbers. [4, 5, 39, 40]

In the same line of thought, larger plasmids were found to limit maximum OD_{600}. [40] However, we did not find such correlations in our lengths (4698–6829 bp) and MWs (2903–4220 kDA) of our tested plasmids (**Table 1**) to support this. As such, we next examined differences in plasmid nucleotide composition on maximum OD_{600} attained, given that higher guanine and cytosine composition was previously shown to increase cell growth rates. [41]However, this was not supported by our findings (Table 1), possibly because the differences between our various plasmids were not sufficiently distinct.

Plasmid yields at the 20th hour of incubation for both bacterial recovery methods across plasmid types and gene inserts showed significantly higher yields for pTT5 than pET21d plasmid constructs, with the exception of pTT5-Hgag recovered from frozen glycerol bacterial stocks. The higher pTT5 and lower pET21d constructs can be best explained by their copy number differences. pTT5 has a high–copy-number origin (pMB1), derived from the *E. coli* cloning vector (pBR332), [31] allowing pTT5 constructs to be highly amplified. Meanwhile, the *rop* gene in

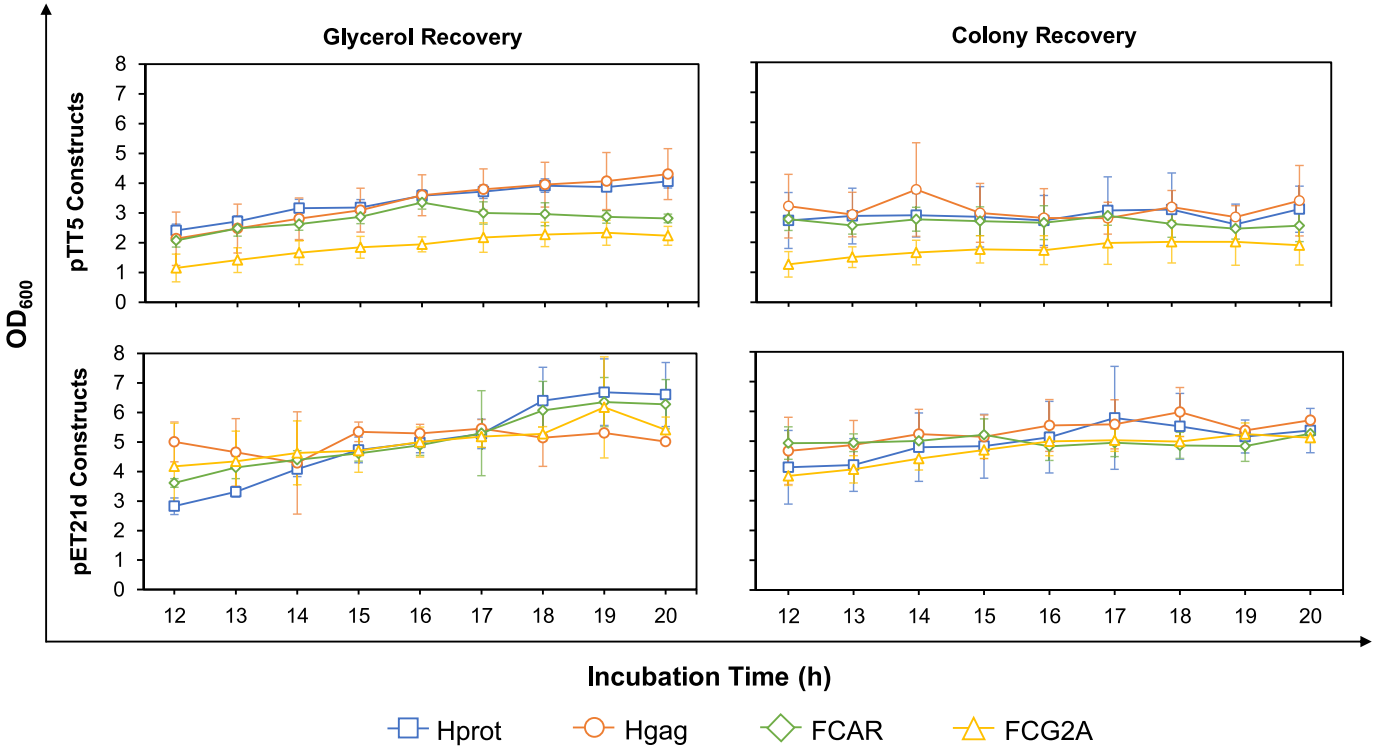

FIGURE 3

Bacterial growth curves of various pTT5 and pET21d plasmid constructs and bacterial stock recovery methods. OD_{600} measurements were taken hourly from the 12th to 20th hour of incubation. Data shown represent the means of sextuplicates, with error bars representing the SD.

pET21d controls the copy number of pET21d constructs *via* complementary RNA binding, [32] reducing synthesis of pET21d constructs. Our results reflect the role of the *rop* gene in controlling plasmid copy number of the pET21d constructs in *E. coli* DH5α (Fig. 2).

To determine the incubation time necessary for bacterial cultures to reach a sufficiently high OD for optimal plasmid extractions, we repeated the experimental setups using frozen stock–recovered bacteria with slight modifications (Fig. 1) to measure OD_{600} and quantify plasmid yields at three selected time points: 12th, 15th, and 19th hour of incubation. By lengthening the bacterial incubation time to increase OD_{600} values, we could extract higher yields for all plasmid constructs (Fig. 4*A, B*). Although the increase in plasmid yields may be significantly different for certain plasmid constructs with prolonged incubation, there is little reason to extend incubation time for some plasmids, *e.g.*, pTT5-Hprot ($P < 0.01$)

TABLE 1

Length, MW, and nucleotide composition of plasmid constructs used in this study

			Nucleotide composition (no. and %)			
Plasmid construct	**Length (bp)**	**MW (kDa)**	**Adenine**	**Guanine**	**Thymine**	**Cytosine**
pTT5-Hprot	4698	2903	1207 (25.7%)	1155 (24.6%)	1253 (26.7%)	1083 (23.1%)
pET21d-Hprot	5626	3476	1343 (23.9%)	1484 (26.3%)	1334 (23.7%)	1465 (26.0%)
pTT5-Hgag	5901	3646	1651 (28.0%)	1454 (24.6%)	1461 (24.8%)	1335 (22.6%)
pET21d-Hgag	6829	4220	1811 (26.5%)	1788 (26.2%)	1518 (22.2%)	1712 (25.1%)
pTT5-FCAR	5262	3251	1311 (24.9%)	1312 (24.9%)	1374 (26.1%)	1265 (24.0%)
pET21d-FCAR	6190	3825	1447 (23.4%)	1641 (26.5%)	1455 (23.5%)	1647 (26.6%)
pTT5-FCG2A	5352	3307	1349 (25.2%)	1308 (24.4%)	1381 (25.8%)	1314 (24.6%)
pET21d-FCG2A	6286	3884	1486 (23.6%)	1638 (26.1%)	1463 (23.3%)	1699 (27.0%)

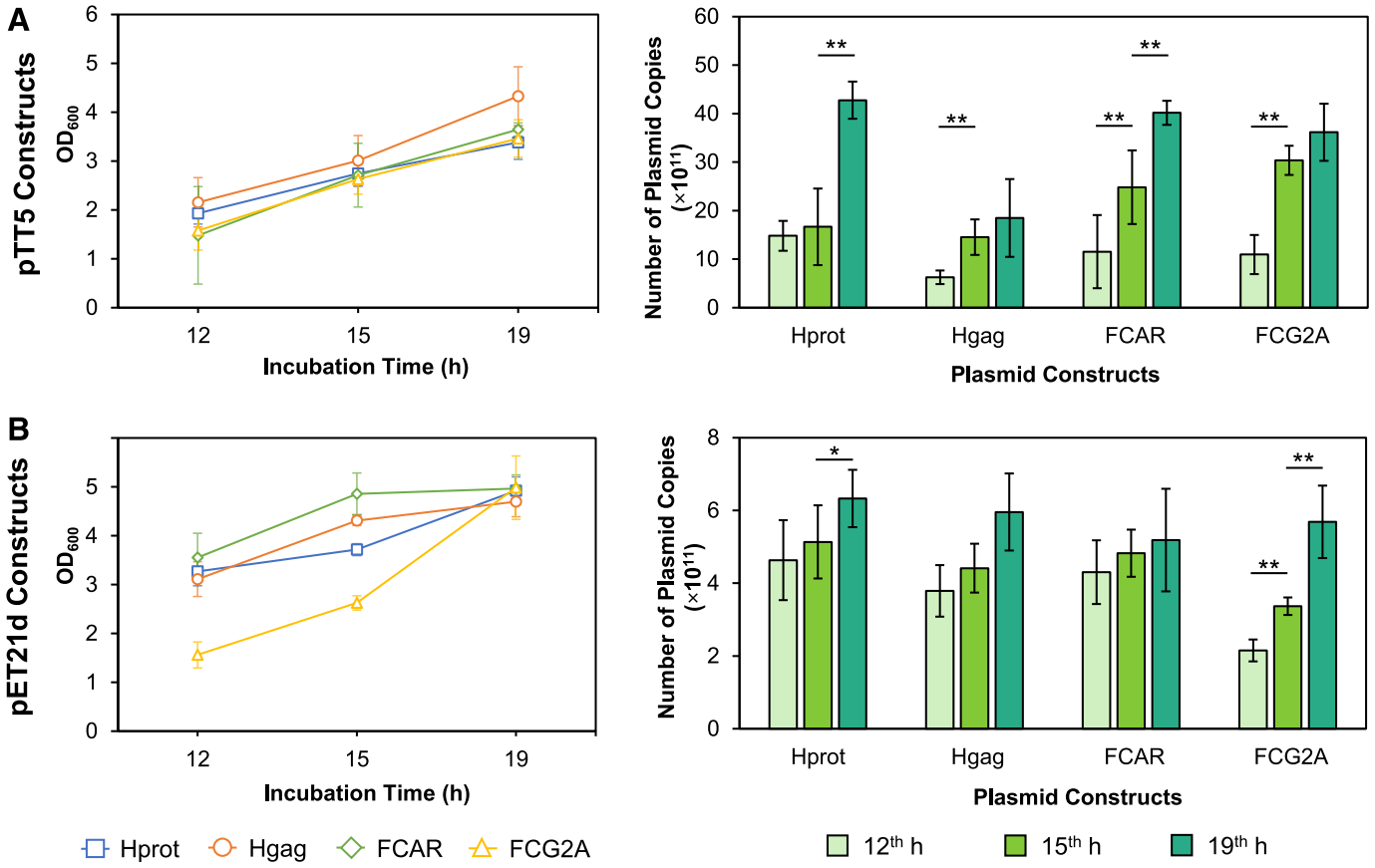

FIGURE 4

OD_{600} measurements and corresponding number of plasmid copies of various pTT5 and pET21d constructs recovered from frozen glycerol stocks at 12th, 15th, and 19th hour of incubation. *A*) pTT5 constructs. *B*) pET21d constructs. Paired *t* test was performed using MiniTab 18, comparing the number of plasmid copies at the 12th and 15th hour of incubation, as well as the 15th and 19th hour. Data shown represent the means of sextuplicates, with error bars representing the SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

and pET21d-Hprot ($P<0.05$), which can be extracted at the 19th hour, whereas pTT5-Hgag ($P < 0.01$) and pTT5-FCG2A ($P < 0.01$) constructs could be extracted earlier at the 15th hour for optimum yields (see Fig. 4*A* for pTT5 constructs and Fig. 4*B* for pET21d constructs). As for pTT5-FCAR (Fig. 4*A*) and pET21d-FCG2A (Fig. 4*B*) constructs, depending on the plasmid yield desired and available waiting time, the plasmids can be extracted at either the 15th or the 19th hour ($P < 0.01$). The remaining 2 constructs, pET21d-Hgag and pET21d-FCAR, could be extracted at the 12th hour, which would have likely reached saturation. It should be noted that the length and MW also had no apparent effect on when such plasmid yield saturations would be reached.

In conclusion, our results demonstrate that it is not necessary to preplate bacterial stock cultures for optimum plasmid yields, saving time and cost, because comparable yields were obtained from frozen stock–recovered bacteria. Differences in the maximum OD and plasmid yields from bacterial cultures were more likely to be affected by genetic elements present in plasmid constructs rather than differences in recovery methods or plasmid length or MWs.

ACKNOWLEDGMENTS

The study was funded by Experimental Drug Development Centre, A*STAR. The authors declare no conflicts of interest.

REFERENCES

1. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol.* 1987;7:2745–2752.
2. Voss C. Production of plasmid DNA for pharmaceutical use. *Biotechnol Annu Rev.* 2007;13:201–222.
3. Prather KJ, Sagar S, Murphy J, Chartrain M. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. *Enzyme Microb Technol.* 2003;33:865–883.
4. Cunningham DS, Koepsel RR, Ataai MM, Domach MM. Factors affecting plasmid production in *Escherichia coli* from a resource allocation standpoint. *Microb Cell Fact.* 2009;8:27.
5. Mairhofer J, Cserjan-Puschmann M, Striedner G, Nöbauer K, Razzazi-Fazeli E, Grabherr R. Marker-free plasmids for gene

therapeutic applications–lack of antibiotic resistance gene substantially improves the manufacturing process. *J Biotechnol.* 2010;146:130–137.
6. Wunderlich M, Taymaz-Nikerel H, Gosset G, Ramírez OT, Lara AR. Effect of growth rate on plasmid DNA production and metabolic performance of engineered *Escherichia coli* strains. *J Biosci Bioeng.* 2014;117:336–342.
7. Carnes AE. Fermentation design for the manufacture of therapeutic plasmid DNA. *Bioprocess Int.* 2005;3:36–44.
8. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 1979;7:1513–1523.
9. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol.* 1981;145:1365–1373.
10. Qiagen. Growth of bacterial cultures website. Available at: https://www.qiagen.com/au/service-and-support/learning-hub/technologies-and-research-topics/plasmid-resource-center/growth-of-bacterial-cultures/. Accessed December 8, 2020.
11. Reinikainen P, Virkajärvi I. *Escherichia coli* growth and plasmid copy numbers in continuous cultivations. *Biotechnol Lett.* 1989;11:225–230.
12. Singer A, Eiteman MA, Altman E. DNA plasmid production in different host strains of *Escherichia coli*. *J Ind Microbiol. Biotechnol.* 2009;36:521–530.
13. Tashiro Y, Kawata K, Taniuchi A, Kakinuma K, May T, Okabe S. RelE-mediated dormancy is enhanced at high cell density in *Escherichia coli*. *J Bacteriol.* 2012;194:1169–1176.
14. Horn NA, Meek JA, Budahazi G, Marquet M. Cancer gene therapy using plasmid DNA: purification of DNA for human clinical trials. *Hum Gene Ther.* 1995;6:565–573.
15. Diogo MM, Ribeiro SC, Queiroz JA, Monteiro GA, Tordo N, Perrin P, Prazeres DM. Production, purification and analysis of an experimental DNA vaccine against rabies. *J Gene Med.* 2001;3:577–584.
16. Chan WT, Verma CS, Lane DP, Gan SK-E. A comparison and optimization of methods and factors affecting the transformation of *Escherichia coli*. *Biosci Rep.* 2013;33:e00086.
17. Ling W-L, Su CT-T, Lua W-H, Poh JJ, Ng YL, Wipat A, Gan SK. Essentially leading antibody production: an investigation of amino acids, myeloma, and natural V-region signal peptides in producing pertuzumab and trastuzumab variants. *Front Immunol.* 2020;11:604318.
18. Yeo JY, Koh DW, Yap P, Goh G-R, Gan SK-E. Spontaneous mutations in HIV-1 gag, protease, RT p66 in the first replication cycle and how they appear: insights from an in vitro assay on mutation rates and types. *Int J Mol Sci.* 2020;22:370.
19. Phua S-X, Lua W-H, Gan SK-E. Role of FcαR EC2 region in extracellular membrane localization. *Cell Cycle* 2018;17:669–670.
20. Lua W-H, Ling W-L, Su CT-T, et al. Discovery of a novel splice variant of Fcar (CD89) unravels sequence segments necessary for efficient secretion: a story of bad signal peptides and good ones that nevertheless do not make it. *Cell Cycle.* 2017;16:457–467.
21. Ling W-L, Lua W-H, Poh J-J, Yeo JY, Lane DP, Gan SK-E. Effect of VH-vl families in pertuzumab and trastuzumab recombinant production, Her2 and FcγIIA binding. *Front Immunol.* 2018;9:469.
22. Ng KM, Wong C-F, Liang AX, et al. Republication – APD SpectBt: arduino-based mobile vis-spectrophotometer. *Scientific Phone Apps and Mobile Devices.* 2019;5:6.
23. Poh JJ, Gan SK-E. Comparison of customized spin-column and salt-precipitation finger-prick blood DNA extraction. *Biosci Rep.* 2014;34:e00145.
24. Poh JJ, Gan SK-E. The determination of factors involved in column-based nucleic acid extraction and purification. *J Bioprocess Biotech.* 2014;4:157.
25. Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J Biotechnol.* 2006;123:273–280.
26. Nguyen PV, Verma CS, Gan SK-E. DNAApp: a mobile application for sequencing data analysis. *Bioinformatics.* 2014;30:3270–3271.
27. Sim J-Z, Nguyen P-V, Zang Y, Gan SK-E. DNA2App: mobile sequence analyser. *Scientific Phone Apps and Mobile Devices.* 2016;2:2.
28. Pruitt KD, Tatusova T, Brown GR, Maglott DR. NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. *Nucleic Acids Res.* 2012;40(D1):D130–D135.
29. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 2002;30:3059–3066.
30. Mertens N, Remaut E, Fiers W. Tight transcriptional control mechanism ensures stable high-level expression from T7 promoter-based expression plasmids. *Biotechnology (N Y).* 1995;13:175–179.
31. Balbás P, Soberón X, Merino E, et al. Plasmid vector pBR322 and its special-purpose derivatives–a review. *Gene.* 1986;50:3–40.
32. Castagnoli L, Scarpa M, Kokkinidis M, Banner DW, Tsernoglou D, Cesareni G. Genetic and structural analysis of the ColE1 rop (rom) protein. *EMBO J.* 1989;8:621–629.
33. Zabriskie DW, Arcuri EJ. Factors influencing productivity of fermentations employing recombinant microorganisms. *Enzyme Microb Technol.* 1986;8:706–717.
34. Seo JH, Bailey JE. Effects of recombinant plasmid content on growth properties and cloned gene product formation in *Escherichia coli*. *Biotechnol Bioeng.* 1985;27:1668–1674.
35. Seo JH, Bailey JE. Continuous cultivation of recombinant *Escherichia coli*: existence of an optimum dilution rate for maximum plasmid and gene product concentration. *Biotechnol Bioeng.* 1986;28:1590–1594.
36. Lara AR, Ramírez OT. Plasmid DNA production for therapeutic applications. In Lorence A, (ed): *Recombinant Gene Expression*, 3rd Ed. Totowa, NJ: Humana Press, 2012:271–303.
37. Jones KL, Kim SW, Keasling JD. Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metab Eng.* 2000;2:328–338.
38. Williams JA, Luke J, Langtry S, Anderson S, Hodgson CP, Carnes AE. Generic plasmid DNA production platform incorporating low metabolic burden seed-stock and fed-batch fermentation processes. *Biotechnol Bioeng.* 2009;103:1129–1143.
39. Ow DSW, Nissom PM, Philp R, Oh SKW, Yap MGS. Global transcriptional analysis of metabolic burden due to plasmid maintenance in *Escherichia coli* DH5α during batch fermentation. *Enzyme Microb Technol.* 2006;39:391–398.
40. Rozkov A, Avignone Rossa CA, Ertl PF, et al. Characterization of the metabolic burden on Escherichia coli DH1 cells imposed by the presence of a plasmid containing a gene therapy sequence. *Biotechnol Bioeng.* 2004;88:909–915.
41. Raghavan R, Kelkar YD, Ochman H. A selective force favoring increased G+C content in bacterial genes. *Proc Natl Acad Sci USA.* 2012;109:14504–14507.