Journal of Experimental Microbiology and Immunology (JEMI) Vol. 20: 86 – 91

Initial Stages of Construction of a Plasmid to Study the Kinetics of Gene Expression at a Single Cell Level Following Uptake of DNA into *Escherichia coli*.

Ramy A. Slama and Adam S. Ziada
Department of Microbiology and Immunology, University of British Columbia

Bacterial transformation is a technical hallmark of molecular biology, and modifications that could expedite the process could translate into improvements in efficiency. Previous studies have introduced the idea of using Fluorescence Activated Cell Sorting (FACS), to detect and isolate transformed cells immediately after transformation with a plasmid bearing a gene coding for green fluorescence protein (GFP). Bennet *et al.* used GFP containing pGLO transformed into BL21 *E. coli* cells to visualize GFP expression through fluorescence microscopy and flow cytometry 1 hour following transformation. GFP expression was driven by the pBAD promoter on the plasmid induced with L-arabinose, however BL21 cells do not allow for regulation of gene expression. In this study we wanted to ask whether or not the kinetics of fluorescence detection following plasmid uptake are related to the level of transcription. To address this question, we aimed to clone a gene fragment coding for GFP into expression vector pET32a(+). Our goal was to transform this plasmid into in BL21 Tuner DE3 pLysS to measure the kinetics of expression using flow cytometry. We hypothesized that altering levels of transcription would decrease the time required to detect fluorescence following plasmid uptake into the cell. In this study, we report the parameters required to amplify a fragment of DNA corresponding to the expected size of the GFP gene from template plasmid pGLO using the polymerase chain reaction.

Bacterial transformation is an extensively used technique in molecular biology. Transformation involves the uptake of foreign, often plasmid, DNA by cells. Bacterial cells capable of DNA uptake are termed 'competent' and cells that have taken up DNA are termed 'transformants'. Plasmids often contain an antibiotic resistance gene for selection of transformants, a cloned gene of interest (GOI) and sometimes genes coding for proteins such as green fluorescent protein (GFP) which might be used as a molecular screening tool (1).

The process of transformation involves the following steps: (1) interaction of foreign DNA with a competent cell, (2) uptake of foreign DNA into cells, (2) stable replication and segregation in daughter cells during growth. Clones of identical genomic content can then be further grown in selective liquid medium to amplify the plasmid containing the GOI (1). The efficiency of plasmid uptake (i.e. transformation efficiency) can vary based on cell and DNA type.

Recent work has focused on accelerating the process of transformation confirmation by eliminating the overnight growth step. Using GFP as a molecular marker for transformation, flow cytometry has been used to visualize fluorescent cells following transformation. Furthermore, the additional use of fluorescence activated cell sorting (FACS) allows for fluorescent cells to be separated from non-fluorescent (thus non-transformed) cells (2). This process would remove 16-24 hours from the transformation protocol by directly amplifying the plasmid on selective liquid medium without the previous selection step.

Two groups, both using non-inducible *Escherichia coli* BL21 (BL21) cells have observed fluorescence after different time periods using flow cytometry: 30 min and 1 hour. The main difference between the two experimental approaches was the system of transcriptional control used; PrpoS promoter coupled with the RpoS transcription factor (TF) yielded earlier fluorescence detection than the pBAD promoter coupled with the AraC TF (2,3).

In prokaryotes such as *E. coli*, protein expression depends on transcriptional regulation, transcription, translation, and protein folding. In transcriptional regulation, proteins can bind to the promoter sequences upstream of genes and interact with RNA polymerase which increases or decreases transcription. This can be rate limiting when attempting to detect the function of a protein. After transcription, both the binding of the ribosome to the mRNA and rate at which the ribosome is able to translate the mRNA can have an effect on protein expression. Following translation, the amino acid sequence needs to fold properly to assume its functional three dimensional shape (4).

Our overarching research question is to address which aspects of protein expression (i.e. transcription, translation, folding, or post translational modification and subcellular targeting) impact the kinetics of fluorescence detection following bacterial transformation with a plasmid carrying a gene coding for GFP.

Previous studies using flow cytometry to detect transformants used BL21 cells whose gene expression levels cannot be regulated; presence of L-arabinose disrupts the interaction of the AraC repressor with DNA, thus activating gene expression in an "all" or "none" fashion (2). We decided to use BL21 Tuner DE3 pLysS cells, which are *lacZY* deletion mutants, in which the lac permease (*lacY*) mutation allows for consistent entry of IPTG into cells. The DE3 designation indicates that the cell contains a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter, which is induced by IPTG. Differential levels of IPTG concentrations would

allow for varying levels of T7 polymerase to be expressed, thus altering the level of gene expression of GOIs under the control of T7 promoters (5).

In this study, we attempted to construct a GFP containing pET32a(+) plasmid under the control of a T7 promoter. Our goal was to transform *E. coli* BL21 Tuner DE3 pLysS cells with the resulting plasmid in order to study the kinetics of gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. pGLO was used as a template to amplify the GFP gene. pUC19 was used for test transformations, pET32a(+) was used as a template for the second Quick Change PCR reaction. *E. coli* DH5α cells (genotype F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1) were used for test transformations and amplification of plasmids. *E. coli* BL21 (*genotype fhuA2 [lon] ompT gal [dcm] ΔhsdS),* as well as DH5α cells were used to transform putatively recombinant pET32a(+). All plasmids and bacterial strains were obtained in-house.

Primers. See Table 1 for primer sequences and T_m. Primers were suspended in sterile dH_2O, aliquoted into 100 µl aliquots and stored at -20°C. Forward primer binds to positions 1262-1290 and reverse primer binds to positions 2233-2255 of pGLO, corresponding to the GFP gene. The overhang (in lower case notation) of the forward primer binds to positions 727-750 of pET32a(+) and the overhang of the reverse primer binds to positions 365-379 of pET32a(+). Sequences and positions are available on Addgene (6,7).

Media and stock solutions. Liquid Lysogeny broth (LB) was prepared from in-house components (5 g Tryptone, 2.5 g yeast extract, 5 g NaCl, dH_2O to 500 ml, pH adjusted to 7.5) and autoclaved on wet cycle. LB plates were made with LB agar (5 g Tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g Bacto Agar, dH2O to 500 ml, pH adjusted to 7.5), autoclaved on wet cycle and poured into dishes aseptically. Ampicillin (Sigma-Aldrich®) was prepared at 50 mg/ml in dH_20, filter sterilized with a 0.45 µm nitrocellulose filter (EMD Millipore™), aliquoted into 1 ml aliquots and stored at -20°C. LB-Amp plates and broth were prepared and used at 50 µg/ml ampicillin. TSS (10 ml LB broth, 1 g PEG 8000, 0.5 ml DMSO, 0.048 g $MgCl_2$, pH adjusted to 6.5) and TSS+20mM glucose (10 ml LB broth, 1 g PEG 8000, 0.5 ml DMSO, 0.048 g $MgCl_2$, 0.036 g glucose, pH adjusted to 6.5) were filter sterilized with a 0.45 µm nitrocellulose filter (EMD Millipore™) and stored at -20°C.

Plasmid purification. DH5α cells containing pGLO, pET32a(+) and pUC19 were streaked on LB plates. Overnight growths of 5 ml were used to make 1ml aliquots for purification. Plasmids were purified using the PureLink® Plasmid Miniprep Kit (cat. no. K2100-10). The PureLink® HiPure Plasmid Maxiprep Kit (cat. no. K2100-06) was used for an additional pET32a(+) purification from a 400 ml overnight culture. The Beckman Coulter Avanti J-301 centrifuge (cat. no. 363118) was used to spin down the overnight culture. The NanoDrop 2000 (ThermoScientific, cat.no. ND-2000) was used for quantifying purified plasmids.

Preparation of competent DH5α and BL21 cells. TSS was thawed and maintained on ice. A 10 ml overnight culture in LB was prepared and its optical density at 600 nm ($O.D._{600}$) was measured using a Pharmacia Biotech Ultrospec 3000 spectrophotometer (cat. no. 80-2106-20). LB was added to a final $O.D._{600}$ reading of 0.075. Cell density was measured every 15-20 min until $O.D._{600}$ reached 0.3-0.4. 1ml aliquots were centrifuged in cold microfuge tubes at 12,000 rpm for 5 min and the supernatants discarded. Samples were suspended in 100 µl ice cold TSS. Competent cells were then flash frozen in a liquid nitrogen and ethanol bath and stored at -80°C.

Bacterial transformation. DH5α and BL21 competent cells were thawed on ice. Plasmid was added (1 ng and 100 ng) and the samples were incubated at 4°C for 30 min. 0.9 ml TSS+20 mM glucose was added and the samples were incubated in a 37°C shaker for 1 hr at 150 rpm. Following recovery, samples were plated at a 10^{-3} dilution on non-selective (LB) plates and at a 10^{-1} dilution on selective (LB-Amp) plates. Colonies were counted after an overnight growth in a 37°C oven. The transformation with digested and undigested long PCR products were performed using the Invitrogen Subcloning Efficiency DH5α Competent Cells kit (cat. no. 18265-017). With the exception of using LB-Amp plates at 50 µg/ml ampicillin instead of 100 µg/ml ampicillin to plate the provided pUC19 control, no further changes to the protocol were made.

PCR conditions, gel electrophoresis and DpnI digestion. The Agilent Pfu Ultra II Fusion HS DNA Polymerase kit (cat. no. 600670) and the Biometra TGradient 96 (cat. no. 050-801) thermocycler were used in all PCRs. The first, GFP-amplifying PCR followed the following cycle: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of a 95°C denaturation for 20 s, annealing at a 52°C-66°C gradient for 20 s, extension at 72°C for 30 s, with a last extension step at 72°C for 3 min. Final reaction volumes (per reaction) were 18.25 µl dH_2O, 2.5 µl 10X PfuUltra II reaction buffer, 0.25 µl dNTPs (25 mM), 0.5 µl each forward and reverse primers (10 µM), 0.5 µl PfuUltra II polymerase, 2.5 µl template DNA (4 ng/µl) for a total volume of 25 µl. The second, long PCR followed the following cycle: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of a 95°C denaturation for 20 s, annealing at a 45°C-59°C gradient for 20 s, extension at 72°C for 2 min, with a last extension step at 72°C for 3 min. Final reaction volumes (per reaction) were 11.16 µl dH_2O, 2.5 µl 10X PfuUltra II reaction buffer, 0.25 µl dNTPs (25 mM), 8.9 µl purified PCR product from the first PCR reaction, 0.5 µl PfuUltra II polymerase, 2.5 µl template DNA (20 ng/µl) for a total volume of 25 µl. Gels were prepared at 1.2% agarose, run at 120 V for 2 hr with TAE buffer. Both 1 kb plus and 100 bp (ThermoFisher, cat. no.s 10787-018 and 15628-019, respectively) were consistently loaded. DpnI digestions were performed with the ThermoFisher DpnI kit (cat. no. 15242-019).

Table 1. Primer sequences and annealing characteristics used in this project

Primer name	P1F-2alpha7-15	P2R-2alpha7-15
Primer sequence (5'-3')	cggataacaattcccctct agaaaCGCAACTC TCTACTGTTTCT CCATACCCG	cggccaggttagcgtCT TCTGAGTTCGG CATGGGGTCA
Tm (whole primer)	67.4°C	71.7°C
Tm (GFP specific region)	61.6°C	61.2°C
Tm (pET32a(+) specific region)	54.3°C	55.2°C
Homodimer	-8.4 kcal/mole	-9.28 kcal/mole
Heterodimer	-9.21 kcal/mole	-9.21 kcal/mole
Tm (secondary structure)	45°C	37.4°C

RESULTS

In this paper, we attempted to construct a GFP containing pET32a(+) plasmid via Quikchange Mutagenesis, which would then be cloned into BL21 DE3 pLysS for expression and future testing. In this strategy, GFP would have been amplified with primers containing pET32a(+) specific

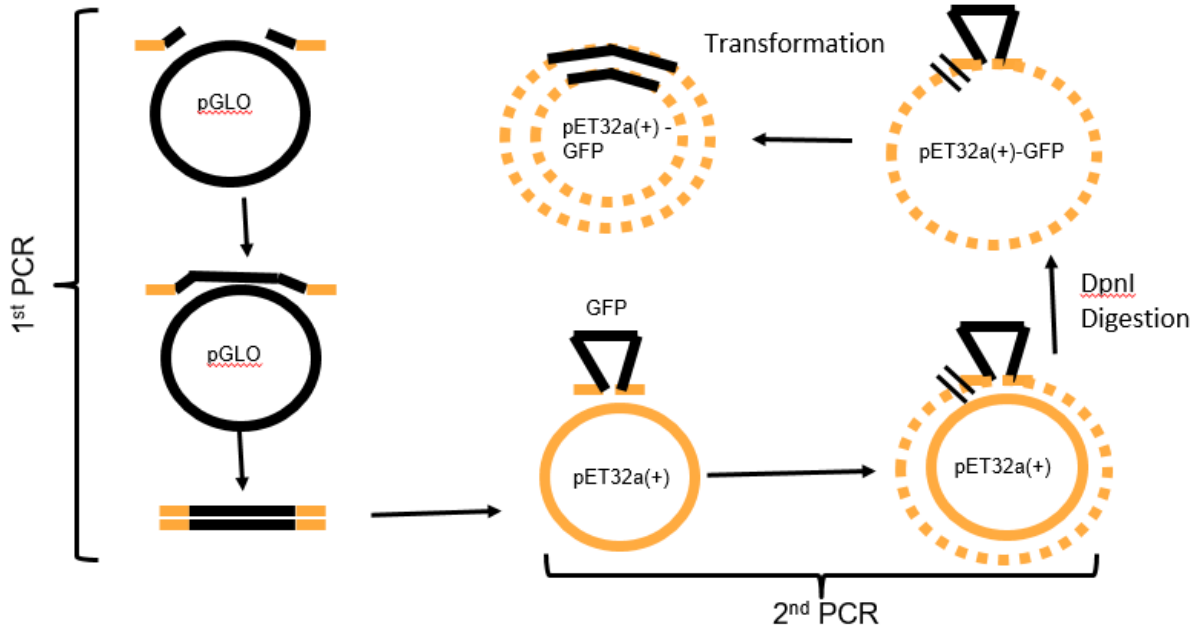

FIG 1 Quick Change PCR cloning strategy. GFP-specific primers contain 5' overhangs specific to pET32a(+). Amplified GFP strands act as primers for the second, long PCR, where the gene is inserted downstream of the T7 polymerase. DpnI digestion degrades methylated template pET32a(+). Transformation into a bacterial host re-circularizes the recombinant plasmid.

overhangs (FIG 1). These overhangs would allow the PCR products to be used as primers for a subsequent long PCR using pET32a(+) as template, and thus generating a nicked double stranded pET32a(+) sequence with a GFP insert downstream of the T7 promoter (FIG 1). Dpn1 would then be used to digest the parent plasmid DNA, and the remaining GFP containing pET32a(+) would be cloned into BL21 DE3 pLysS and tested for expression of GFP.

Primer design for Quick Change Mutagenesis. Two primers were designed to amplify GFP, as well as its ribosomal binding site, from pGLO, with 5' over hangs that would allow the resulting GFP amplicon to bind to and potentially amplify pET32a(+) (Fig 1). The primers were designed to amplify the GFP ribosomal binding site, as well as the GFP gene, and the resulting amplicon would bind to pET32a(+) just downstream of the T7 promoter, but upstream of the pET32a(+) ribosomal binding site, effectively deleting it from the plasmid - replacing it with the GFP gene and its native ribosomal binding site. The melting temperature of both GFP specific, and pET32a(+) specific regions of the QuickChange Mutagenesis primers were designed to be within 5°C of each other (Table 1). In addition, to prevent dimerisation and promote amplification of the intended products, both primers were screened for potential homodimers or heterodimers, and only primer designs with a change in Gibbs free energy above -9.3 kcal/mole were kept (Table 1).

PCR amplification of a 1kb product from pGLO. To amplify GFP from pGLO we first designed GFP specific primers. A PCR gradient was performed using annealing temperatures from 52°C to 66°C. A negative control without pGLO plasmid DNA template was added (Fig 2). PCR products were resolved on an agarose gel. Bands were observed that migrated at approximately 100 bp (Fig 2). This is consistent with our prediction that the amplified GFP with pET32a(+) specific overhangs would be 1016 bp, and suggests that the GFP gene from pGLO had been amplified (Fig 2).

Attempted construction of GFP-pET32a(+) plasmid using Quick Change Mutagenesis. Long range gradient PCR was performed using our PCR product as a primer, and pET32a(+) as template. This was followed by PCR purification and DpnI digestion of the PCR products. A band of the expected size (6916 bp) was not observed when the reaction mixture was resolved with agarose gel electrophoresis at any of the annealing temperatures. Further tests demonstrated that a minimum of 5.5 ng pET32a(+) is required for visualization on gel electrophoresis, however band sizes were above the 5000 bp mark on our 1 kb plus DNA ladder. From this data we cannot conclude that any annealing temperature used for the long range PCR produced recombinant GFP containing pET32a(+) plasmid.

***E. coli* DH5α transformed with a putative GFP containing pET32a(+) construct did not yield ampicillin resistant colonies.** To determine if any long PCR condition produced recombinant GFP containing pET32a(+) construct with concentrations of our desired product we transformed *E.coli* strain DH5α with the products of each PCR reaction. With a reported transformation efficiency of $1x10^6$ transformants/µg DNA (reference), these DH5α cells were expected to yield colonies with as little as 1 pg of DNA.

DpnI digested long PCR products generated with annealing temperatures ranging from 45°C to 59°C were transformed into commercially competent DH5α. Plasmid

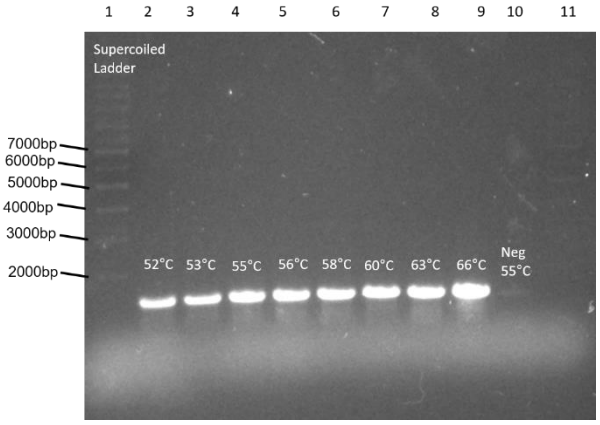

FIG 2 Gel electrophoresis of PCR product using pGLO template and GFP specific primers. Gel prepared at a 1.2% agarose concentration, ran at 120V for 2 hours in TAE buffer. Lane 1 contains a 1kb supercoiled ladder. Lanes 2-9 contain PCR products on GFP amplification from pGLO generated at various annealing temperatures. Lane 10 contains a negative dH2O control.

pUC19 was used as a positive control and buffer (containing no DNA) was used as a negative control. pET32a(+) plasmid and undigested long PCR products to control for any failure of the DpnI digestion to degrade leftover parental plasmid DNA. The transformation using pUC19 and pET32a(+) controls produced colonies on LB plates supplemented with ampicillin. The buffer control, digested DpnI reaction mixture, and undigested reaction mixture did not yield colonies. The pUC19 positive control showed that the DH5α cells were competent. The TE buffer control demonstrated that background contamination with an ampicillin resistant strain or plasmid in either the cells, TE buffer, or reaction mixture had not occurred.

We did not observe positive transformants for any of our long range PCR products, regardless of Dpn1 digestion. This suggests that either the long range PCR failed to produce any GFP containing pET32a(+) recombinant DNA, or that the long range PCR produced recombinant plasmid but at a concentration too low to detect via gel electrophoresis or DH5α transformation, or that something in the reaction mixture is inhibiting the transformation procedure.

DISCUSSION

The goal of this paper was to build a plasmid that could be used to regulate GFP expression, in BL21 Tuner DE3 pLysS, in order to study gene expression following plasmid uptake into a bacterial cell. We hypothesized that adjusting the level of transcriptional activation would affect the kinetics of fluorescence detection via flow cytometry. Our experimental aims were to amplify GFP, clone it into pET32 via QuickChange Mutagensis, transform BL21 Tuner and measure fluorescence at a single cell level over time using flow cytometry. In this study we developed a cloning strategy based on QuickChange Mutagenesis and established conditions to amplify GFP from the pGLO plasmid.

Our results showed that GFP-amplifying PCR products of the correct size were produced under all annealing temperatures (Fig 2). These result were expected, as the primers used to produce these PCR products had low specificity to other regions of the pGLO plasmid, low change in Gibbs free energy for both heterodimers and homodimers, and the highest Tm of any secondary structures was below any annealing temperature tested (Table 1). In addition, due to the 5' overhangs, PCR products could be produced at temperatures above the melting temperature (Tm) of their GFP specific regions. Since the Tm represents the temperature at which 50% of the primer is associated with template DNA, higher temperatures still yield a fraction of primer annealing to the template. The products of these rare reactions would produce template DNA that is homologous to the full length of the primers, including the 5' overhang, increasing the Tm of the primers from 61.6°C and 61.2°C to 67.4°C and 71.7°C (Table 1).

When running the products of our long PCR using GFP with pET32a(+) overhangs as primers and pET32a(+) as template, it was expected that no bands corresponding to a pET32a(+) GFP containing construct would appear on gel (Fig 4), as quick change PCR is not known to produce high product concentrations (8). Although the GFP sequence was flanked by pET32a(+) specific sequences, which did not form secondary structures with a melting temperature above 45°C, it is possible that the GFP coding region could have formed a secondary structure that prevented the pET32a(+) specific sequences from binding to pET32a(+). In addition, the 1000bp band was expected as the primers were used at a concentration of 10ng/µL, which is above the 5.5ng/µL we observed to be the minimum concentration of DNA required to be visualized via agarose gel electrophoresis.

We did not observe ampicillin resistant colonies from transformation of DH5α with the long range PCR products. Failure of any of the Dpn1 digested long PCR products to produce transformants suggests that any putative GFP containing pET32a(+) recombinant plasmid is at an extremely low concentration, or that something in the Dpn1 digested PCR products is inhibiting the transformation reaction. One possibility is that a component of the Dpn1 Buffer T is inhibiting the transformation reaction, however further tests and disclosure of the buffer's components are required before any conclusions can be drawn.

In addition it was not expected that the non-Dpn1 digested long PCR products - which should have contained the original pET32a(+) template from the preceding long PCR - would not yield transformants. Similarly, it was not expect that pET32a(+) plasmid - diluted to the same concentration that should have been present in the non-Dpn1 digested long PCR products - would yield transformants. The calculation to determine how much pET32a(+) plasmid DNA would be left over

in the non-Dpn1 digested samples assumed that the PCR purification kit had a yield close to 100%. Since only the Dpn1 and non-Dpn1 digested samples were purified, it is possible that factors associated with the PCR purification kit could have resulted in a more pronounced loss of the DNA than expected. For example, It is possible that an issue with the DNA purification after the long PCR resulting in substantial loss of initial pET32a(+) template, or that a reagent associated with the DNA purification could have resulted in inhibition of the transformation reactions. It is also possible that because the PCR purification kit was designed to purify double stranded DNA products from single stranded DNA primers (9), it may have also removed the partially single stranded versions of Quick Change PCR products. The ethanol in buffer W1 may not have been completely removed by the end of the purification, thus inhibiting the subsequent transformation reactions.

In conclusion, we have established conditions to PCR amplify a 1kbp fragment from pGLO using primers designed to bind to the region coding for GFP. We have also described a ligase–free strategy to clone this PCR product into pET32a(+) using a the procedure published by Bok *et al.* (8). This strategy and PCR product may prove useful in future studies looking at the kinetics of gene expression following plasmid uptake into *E. coli*.

FUTURE DIRECTIONS

The next step in our project would be to clone our GFP PCR products into pET32a(+). Given the lack of positive transformants using our long range PCR products, further optimization of the long range PCR, or investigation of alternate means of cloning, are both very realistic future directions. As mentioned earlier, it is also possible that the QuickChange Mutagenesis is working, just not producing enough DNA for detection or that something in the transformation mix is inhibiting the transformation. In that case, it might be beneficial to explore potential ways of working with such low concentrations of DNA, or increasing transformation efficiency.

Optimization of the long range PCR. In addition, further optimization of long PCR conditions may potentially yield higher product concentrations. It is possible that increasing the PCR extension time from 2 minutes to 3 or 4 minutes could potentially allow the polymerase more time to generate the full Quick Change PCR product. It is also possible that varying template DNA or primer concentration could also affect the efficiency of the Quick Change long PCR.

Without sequencing data, we were unable to confirm if our putative amplified GFP gene with pET32a(+) specific overhangs was anything more than a band of DNA that was roughly 1000bp long. Cloning this amplicon into a sequencing vector, and sequencing it would eliminate the possibility that the amplicon is merely a 1000bp non-specific product.

Alternative means of cloning GFP in pET32a(+). Finally it may be beneficial to consider alternative means of cloning GFP into pET32a(+), such as traditional cloning with restriction enzymes or Gibson assembly. Both techniques have their benefits and drawbacks, however if Quikchange Mutagenesis cannot be easily optimized, it may be worthwhile to investigate other means of creating a GFP containing pET32a(+) plasmid.

Working with low concentration of recombinant DNA. Developing a simple PCR assay to detect, or qPCR assay to quantify, the presence of a pET32a(+) plasmid containing a GFP insert would be beneficial. This would require the use one GFP specific primer with one pET32a(+) specific primer that could be used for detection or quantification. Since all of our Quick Change long PCR products do not show up on gel, this would allow for more accurate quantifications and comparisons of different Quick Change long PCR conditions that produced products below the limit of detection of SYBR Safe stained agarose gel electrophoresis, and allow the expedition of optimizing the Quick Change long PCR conditions.

Increasing transformation efficiency. This study examined the minimum concentration of pET32a(+) DNA needed to transform BL21 DE3 pLysS cells, and attempted to determine if Quick Change PCR could produce enough putative GFP containing pET32a(+) recombinant plasmid to transform TSS competent BL21 DE3 pLysS cells. Although we were able to use TSS based transformation to transform BL21 DE3 pLysS cells with pET32a(+), the transformation efficiency was very low. One possible future direction might be to optimize aspects of the TSS transformation, such as the percentage and molecular weight of Poly Ethylene Glycol used to make the TSS, or concentration of magnesium (10).

It was noted earlier that the Dpn1 Buffer T may be inhibiting the transformation reaction. A future direction could be to test how Dpn1 Buffer T affects transformation efficiency. This could be done by transforming a series of competent cells with samples containing the same concentration of DNA, but different concentrations of Dpn1 Buffer T. Such an experiment could shed light on any potential inhibitory properties Dpn1 Buffer T may display in different transformation reactions.

ACKNOWLEDGEMENTS

The Department of Microbiology and Immunology at the University of British Columbia provided the financial support and laboratory space needed for the project. We would like to thank Dr. David Oliver and Christoph Deeg for their support and advice. We also thank the media room of the Wesbrook building for providing the necessary equipment for the study.

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