Vietnam Journal of Biotechnology **20**(2): 359-368, 2022

EXPRESSION OF A SYNTHETIC GENE ENCODING THE ENHANCED GREEN FLUORESCENT PROTEIN IN VARIOUS *ESCHERICHIA COLI* STRAINS

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Received: 04.11.2021
Accepted: 15.02.2022

SUMMARY

Enhanced Green Fluorescent Protein (eGFP) shows much stronger fluorescence than its ancestor, Green Fluorescent Protein (GFP), thus has been widely applied as a reporter for biomedical research. In this study, we reported the expression of a synthetic codon optimized gene encoding eGFP in *Escherichia coli* (*E. coli*). The gene was cloned into two expression vectors, pQE30 and pColdII and the resulting recombinant vectors were transformed into *E. coli* M15 and BL21 De3 RIL codon plus strains, respectively. The expression levels of functional eGFP showed a temperature dependent pattern, in which lowering the induction temperature increased the amount of functional eGFP. Surprisingly, eGFP showed a phenomenon called auto-induction when *E. coli* TOP10 cells carrying recombinant pQE30 and pColdII were grown on Luria Broth plates. The recombinant eGFP showed robust stability even at room temperature, thus greatly facilitated its purification and handling. Mouse polyclonal antibodies were conveniently generated against the protein. Besides its potential application as a reporter gene in *E. coli*, the gene and its expression systems reported here are extremely useful as models for teaching recombinant DNA technology at undergraduate level.

Keywords: eGFP, *E. coli*, cloning, expression, purification, polyclonal antibody, teaching

INTRODUCTION

Green Fluorescent Protein (GFP) was first described by Shimomura Osamu (Shimomura *et al*., 1962) as a companion of aequorin from *Aequorea victoria*. For a long time, the protein remained obscure to the scientific community, until a sudden surge of interest in its application as a novel reporter swept through the world around the middle of 1990s (Tsien, 1998). Chalfie et al were the first group to use GFP as a marker for gene expression (Chalfie *et al*., 1994), however, the native GFP was not as sensitive as other reporter genes at the time, such as alkaline phosphatase, β-galactosidase, firefly luciferase or chloramphenicol acetyltransferase (Zhang *et al*., 1996). A breakthrough came when Cormack et al created an enhanced version of the protein through directed evolution. Termed eGFP (enhanced Green Fluorescent Protein), the new protein was shown to be 35 times more fluorescent than the original GFP (Cormack *et al.*, 1996). Since its invention, eGFP has been applied in numerous studies as a reporter, with superior sensitivity compared with other reporters. Usually, eGFP is fused in tandem with other proteins to track their intracellular movement or presence. Some of the notable applications include tracking Cre/lox mediated excision in mice (Novak *et al*., 2000), gene expression tracking in yeasts (Cormack *et al*., 1997), tracking nuclear transfer in pigs (Park *et al*., 2001), or pH biosensor in plants (Gjetting *et al*., 2012).

eGFP is also extensively used as a reporter protein to study gene optimization, promoter and terminator selection, and expression and purification procedures. Lanza AM et al used eGFP as a model protein to study condition-specific codon optimization for protein expression in *S. cerevisiae* (Lanza *et al*., 2014). eGFP is often used as a reporter to screen promoters from various organisms, such as *Listeria monocytogenes* (Ji *et al*., 2021), *S. cerevisiae* (de Paiva *et al*., 2018) or both promoters and terminators from a novel yeast, *Kluyveromyces marxianus* (Kumar *et al*., 2021). Cedras G et al used eGFP as a reporter to detect unfolded protein response in *S. cerevisiae* (Cedras *et al*., 2020). Mukhopadhyay and Bagh studied the effect of microgravity on eGFP expression in *E. coli* as a biosensor for microgravity in space travel (Mukhopadhyay, Bagh, 2020). eGFP was also used as a model protein to study protein purification procedure, such as finding an alternative to 6xHis tag (Pan *et al*., 2019), finding an appropriate purification protocol (Song *et al*., 2020) or studying aqueous two-phase system purification (Lo *et al*., 2018).

In this study, we reported the cloning, expression, purification and characterization of a synthetic mammalian codon optimized gene encoding eGFP in *E. coli* strains. We aimed to produce eGFP in purified form for various use such as positive control for other host expression, antibody production, educational tools, as well as establishing a system for studying codon optimization strategies in *E. coli*.

MATERIALS AND METHODS

Strains and culture conditions

E. coli TOP10 strain (Invitrogen) was used for the cloning work. *E. coli* M15 (Qiagen, F-Φ80ΔlacM15, thi, lac- mtl-, recA+, KmR) and BL21 De3 RIL codon plus (Agilent) were used for the expression of eGFP. *E. coli* TOP10 was maintained on Luria Broth (LB) plates and liquid medium at 37ºC while M15 and BL21 were maintained in the same media but supplemented with Kanamycin, Streptomycin and Chloramphenicol, respectively. Appropriate antibiotics (Ampicillin for TOP10, Ampicillin and Kanamycin for M15, Ampicillin, Streptomycin and Chloramphenicol for BL21 De3 RIL) were added to the media whenever these strains were transformed with plasmids. The working concentrations of Ampicillin, Streptomycin and Kanamycin are 50 µg/ml while the working concentration of Chloramphenicol is 34 µg/ml. All *E. coli* cells were stored long term in liquid LB supplemented with 20% Glycerol at -80ºC.

Construction of expression plasmids

The synthetic, mammalian codon optimized gene encoding eGFP, termed *eGFP* (MT891343.1) was obtained from Addgene (#58855). The gene was cloned by PCR using *Pfu* DNA Polymerase (Thermofisher) to incorporate a *Bam*HI site at the 5' terminus and *Sal*I site at the 3' terminus. The obtained PCR product was cleaned up using MEGAquick-spin™ DNA purification kit (iNtRON). Then, its product was subjected to A tailing using GoTaq® Green Master Mix (Promega), cleaned up again with MEGAquick-spin™ kit and TA cloned into pGEM-T Easy vector (Promega) with T4 ligase (Thermofisher). The sequence of *eGFP* was determined by Sanger sequencing (1st BASE DNA Sequencing), and aligned with the theoretical sequence to confirm its accuracy. Subsequently, *eGFP* was released from pGEM-T Easy using Fastdigest *Bam*HI/*Sal*I (Thermofisher) double digestion. It was then cloned into pQE30 and pColdII vectors which were opened using the same pair of restriction enzymes. The resulting recombinant vectors, termed pQE30-eGFP and pColdII-eGFP.

Transformation and screening of transformants

pQE30-eGFP and pColdII-eGFP were maintained in *E. coli* TOP10 for long-term storage. pQE30-eGFP was subsequently transformed into *E. coli* M15 strain while pColdII-eGFP was transformed into BL21 De3 RIL strain using chemical transformation. Eight random colonies for each construct were selected, cultured, expression induced, cell harvested and

subjected to SDS-PAGE analysis to select one colony with the highest expression level.

To screen the transformants, equal amounts of cells were harvested before and at the end of induction period. The cells were lysed by heating at 100^{o}C in 6x loading buffer for 10 minutes, spun for 1 minute and the clear lysates were loaded on SDS-PAGE gels side by side (before induction vs after induction). This cell lysate is conveniently referred to as the total protein in this study as opposed to the total soluble protein, which refers to the soluble fraction obtained from cell enzymatic lysis or freeze/thaw lysis. The selection is based on the intensity of a band at approximately 27 kDa in the induced samples, but is absent in the non-induced samples.

Expression and purification

Normal looking transformants were inoculated into 5 ml liquid LB supplemented with appropriate antibiotics and cultured overnight (ON) at 37^{o}C on a shaking incubator at 220 rpm. The next day, 2.5 ml of ON cultures were transferred to 250 ml LB containing appropriate antibiotics, and the cultures were mounted on a shaking incubator and grown at 37^{o}C for another 3-5 hours until optical densities (OD) reached the desirable values. For the M15 transformant, the culture was induced at OD 0.7-0.8 with 0.5 µM isopropylthio-β-galactoside (IPTG) for 4 hours at either 37^{o}C (recommended temperature by the manufacturer) or for 6 hours at 30^{o}C, 20^{o}C and 15^{o}C. For the BL21 transformant, when the OD reached 0.4-0.5, the culture was first transferred to a refrigerator for 10 minutes, then returned to the incubator set at 15^{o}C and left for another 20 minutes without shaking (based on the manufacturer's recommendation). IPTG was added to a final concentration of 0.5 µM and the culture was induced for 24 hours at 15^{o}C, which is the recommended induction temperature for pColdII. Cells were harvested, washed with distilled H_2O, weighed and stored at -80^{o}C. To recover soluble eGFP, cells were first lysed by freezing and thawing repeatedly for 10 rounds, and phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0) was added to resuspend the released proteins (Johnson BH, Hecht MH, 1994). The lysates were centrifuged at 13,000 rpm, 4^{o}C for 15 minutes to separate cell debris from soluble proteins. Cell pellets were resuspended in 8 M Urea buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, 5 mM imidazole, pH 8.0) to recover inclusion bodies.

To purify soluble eGFP, the soluble fractions were subjected to metal affinity chromatography. One ml of Ni-NTA agarose (Qiagen) was packed on a polyprep column (Biorad). The column was first equilibrated with 5 mM imidazole (Sigma), and protein mixtures were loaded onto the column 3-4 times until most eGFP bind to the column, which was indicated by the loss of greenness of the flowthrough fraction. The column was washed 3 times with 5 ml of the phosphate buffer containing 25 mM imidazole and eluted with 2 ml of the same buffer containing 250 mM imidazole.

To purify eGFP inclusion bodies, the clear lysate from 8M urea lysis was loaded onto a Ni-NTA agarose column pre-equilibrated with 8 M urea, 5 mM imidazole, pH 8 buffer. Washing and elution were carried out in the same manner described for purification of soluble eGFP, except for the buffer (8 M urea instead of phosphate buffer).

SDS-PAGE and Western blot analysis

Protein samples were mixed with 6x SDS-PAGE loading buffer and loaded onto a discontinuous SDS-PAGE gel consisting of 5% stacking gel and 12% resolution gel. The proteins were subsequently separated at 60 mV for 30 minutes followed by 80 mV until the front dye run off the gels. Samples were analyzed in twin gels, in which one gel was used to visualize separated proteins by Coomassie staining and the other gel was used for blotting. The gel was blotted onto a nitrocellulose membrane (Hybond^{TM}, GE Healthcare), probed with rabbit anti-His tag antibody and developed with goat anti-rabbit AP-conjugated antibodies and NBT/BCIP substrate solution (Thermofisher).

Protein quantification and expression level comparison

Protein concentrations are determined in absolute terms by Bradford assay. Western blot band intensities were used to compare eGFP expression levels among samples in relative terms. To roughly determine the yields of eGFP, samples were analyzed together with standards (purified eGFP) and Western blot band intensities were plotted on a graph containing the standard curve of various pre-determined amounts of purified eGFP against their Western blot band intensities. The intensities of Western blot bands were determined as the area under the curve using imageJ program (Rueden *et al*., 2017).

Mouse immunization and anti-eGFP polyclonal antibody production

Purified eGFP in denatured form (in 8 M urea) was dialyzed in PBS buffer pH 7.4, quantified by Bradford assay and purity checked by SDS-PAGE. Subsequently, eGFP was mixed with Freund's complete adjuvant at 1:1 ratio (volume to volume), emulsified and vaccinated to 6 week old female Balb/c mice at 100 µg dose per mouse. This priming was followed by 2 boost immunizations in which Freund's complete adjuvant was replaced by Freund's incomplete adjuvant. Mice were bled retro-orbitally using micro-hematocrit tubes, and sera were collected according to the method described previously (Nguyen *et al.*, 2018).

RESULTS AND DISCUSSION

Construction of expression plasmids

From three TOP10 strains carrying eGFP cloned into pGEMT Easy that were sequenced, all strains yielded the result of 100% sequence identity with the theoretical *eGFP* sequence (data not shown).

Subsequently, *eGFP* was released from pGEM-T Easy and cloned into pQE30 and pColdII expression vectors. The rationale for using these two vectors is that soluble proteins are normally obtained by induction at low temperatures (Arya *et al*., 2015), and pColdII vector requires to be expressed at 15^{o}C (Qing *et al*., 2004). The maps of the recombinant pColdII-eGFP and pQE30-eGFP were shown in Figure 1. The cloning results were confirmed by digesting putative recombinant plasmids with *Bam*HI/*Sal*I and analyzed on 1% Agarose gel (data not shown).

Transformation and screening of putative transformants for eGFP expression

The transformation efficiency for both constructs was high, yielded several hundred transformants each. SDS-PAGE analysis of 8 randomly selected showed that all candidates expressed a distinct band at approximately 25-30 kDa, corresponding to the expected eGFP size (data not shown). Based on the result we selected two highly expressed transformants for further analysis.

Expression and purification of eGFP

At first, we expressed eGFP at the temperatures recommended for each *E. coli* strain as described in the method section. We observed that the M15 cells did not show the typical greenness of eGFP while the BL21 cells showed intense greenness (Figure 2C). Upon SDS-PAGE analysis of soluble and insoluble fractions from these two samples, it showed that in the M15 strain expressed at 37^{o}C, most of the target protein ended up in the insoluble fraction as inclusion bodies. On the other hand, in the BL21 strain, a significant proportion of expressed eGFP was in soluble form. (Figure 2A, B). Based on this observation, the greenness of *E. coli* cells can be used as indirect evidence to show whether eGFP is expressed in functional/soluble form or in inclusion bodies.

Although the *eGFP* gene was originally optimized for mammalian cell expression, we observed that it was equally well expressed in *E. coli*. Such "universally optimized genes", where expression levels are consistently high across different hosts, have been observed in our laboratory (Nguyen *et al*., 2021; Nguyen *et al*., 2021).

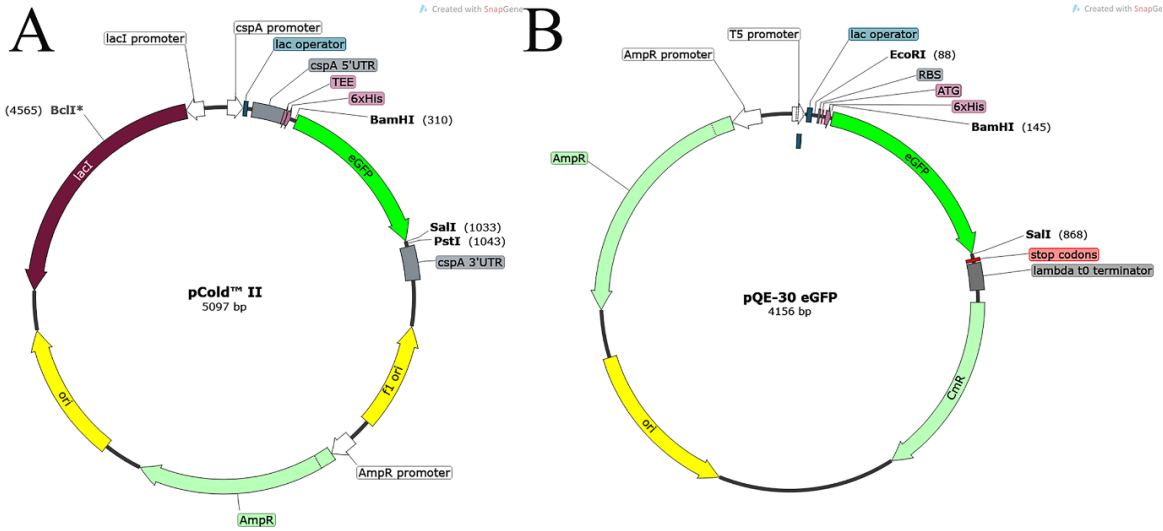

Figure 1. Vector maps of pColdII-eGFP (A) and pQE30-eGFP (B). The size of eGFP is 723 bp. Due to the size difference between pColdII and pQE30 backbones, eGFP looks a little different in sizes in the A and B.

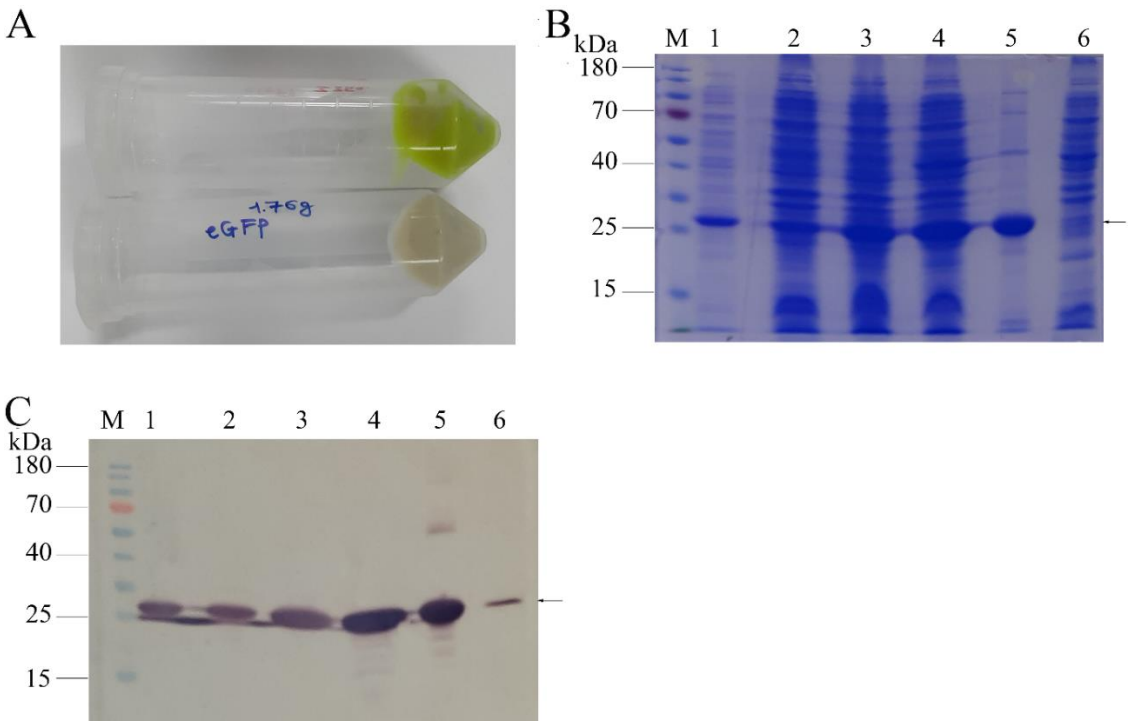

Figure 2. SDS-PAGE analysis of M15 and BL21 strains expressing eGFP at recommended temperatures. A: cells harvested at the end of induction at recommended temperatures. The green cell mass was BL21 while the whitish cell mass was M15; B: SDS-PAGE; C: Western blot of the twin gel probed with anti-HisTag monoclonal antibody (Biorad). Lane 1: BL21 inclusion bodies solubilized in 8M urea; Lane 2: BL21 total soluble protein; Lane 3: BL21 total protein after induction (see Materials and Methods for more details); Lane 4: M15 total protein after induction; Lane 5: M15 inclusion bodies solubilized in 8M urea; Lane 6: M15 total soluble protein; M: Thermofisher scientific prestained protein ladder. The arrows indicated the position of eGFP bands.

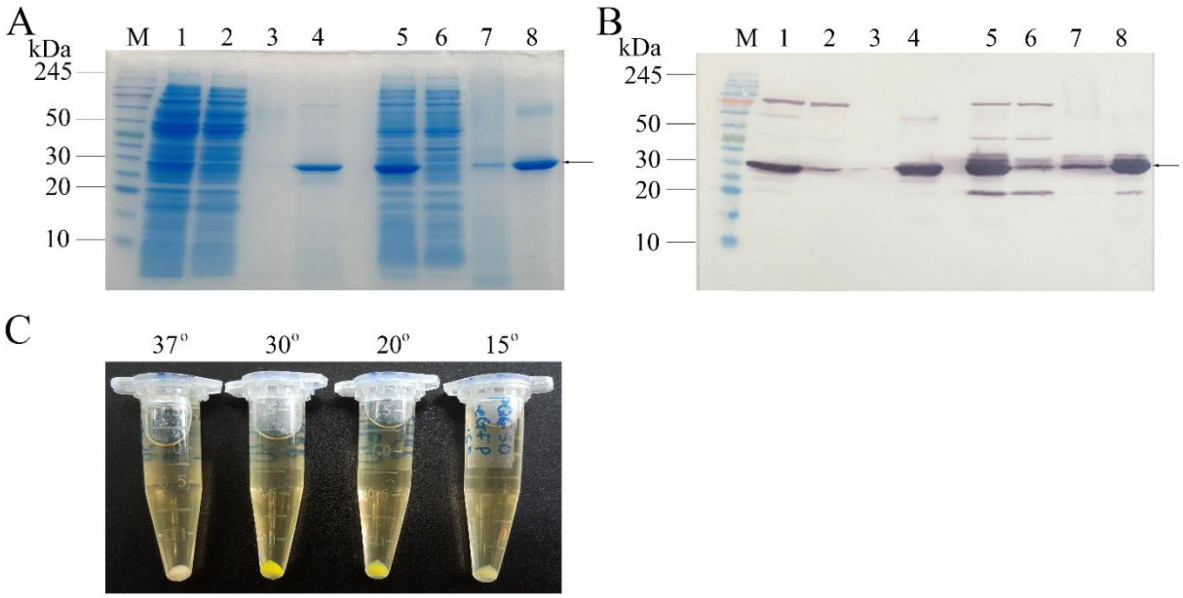

Figure 3. Comparing the expression of functional eGFP at different temperatures as well as between M15 strain vs BL21 strain. A: SDS-PAGE; B: Western blot of a twin gel probed with anti-eGFP polyclonal antibodies. Lane 1: Total soluble protein from M15; Lane 2: flowthrough fraction of that protein sample when loaded on a Ni-NTA column; Lane 3: Washing fraction; Lane 4: Elution fraction; Lane 5: Total soluble protein from BL21; Lane 6: Flowthrough fraction of that protein sample; Lane 7: Washing fraction; Lane 8: Elution fraction; M: SMOBIO (PM5100) prestained protein ladder. The arrows indicated the position of eGFP bands. C: M15 cell masses collected at the end of induction period for 4 different induction conditions: 37ºC, 30ºC, 20ºC and 15ºC. Functional eGFP was present at 30ºC and 20ºC, but virtually absent at 37ºC, due to most expressed eGFP ending up in inclusion bodies, and only slightly present at 15ºC, probably due to substantial decrease in protein translation rate rather than eGFP ending up in inclusion bodies.

In an attempt to improve the solubility of eGFP in the M15 transformant, we lowered the induction temperature to 30ºC. At this temperature, the greenness of M15 cells significantly improved and we were able to purify soluble eGFP from these cells. However, comparing with the BL21 strain, the amount of soluble eGFP in the M15 strain was only half as much (Figure 3A, B), based on their relative intensities determined by imageJ. Furthermore, when the M15 strain was induced at progressively low temperatures (30ºC, 20ºC and 15ºC), cells showed more greenness compared with the induction at 37ºC (Figure 3C). This showed that eGFP expression is temperature dependent, and more functional target proteins is formed when cells are induced at low temperatures. This is consistent with previous observations that induction at low temperatures not only improves yields but also solubility of *E. coli* expressed proteins. And the less greenness observed in 20ºC and 15ºC samples compared with that of 30ºC could be the result of lower translation rates instead of more eGFP ending up in the inclusion bodies (Arya *et al*., 2015). A notable observation of eGFP expression is that when TOP10 cells were transformed with pQE30-eGFP/pColdII-eGFP and cultured for 18-22 hours at 37ºC, transformants started showing greenness even without any IPTG added to the media. The greenness increased when these transformed cells were kept at 4ºC (Figure 4A, B). A similar phenomenon has previously been reported for various kinds of fluorescent proteins expressed in *E. coli*. However, in this study the author reported that the key for this un-induced expression is the BL21-Gold (DE3) strain (Sarabipour *et al*., 2015). The mechanism for this phenomenon is still unresolved but a form of auto-induction has probably been involved (Studier, 2005).

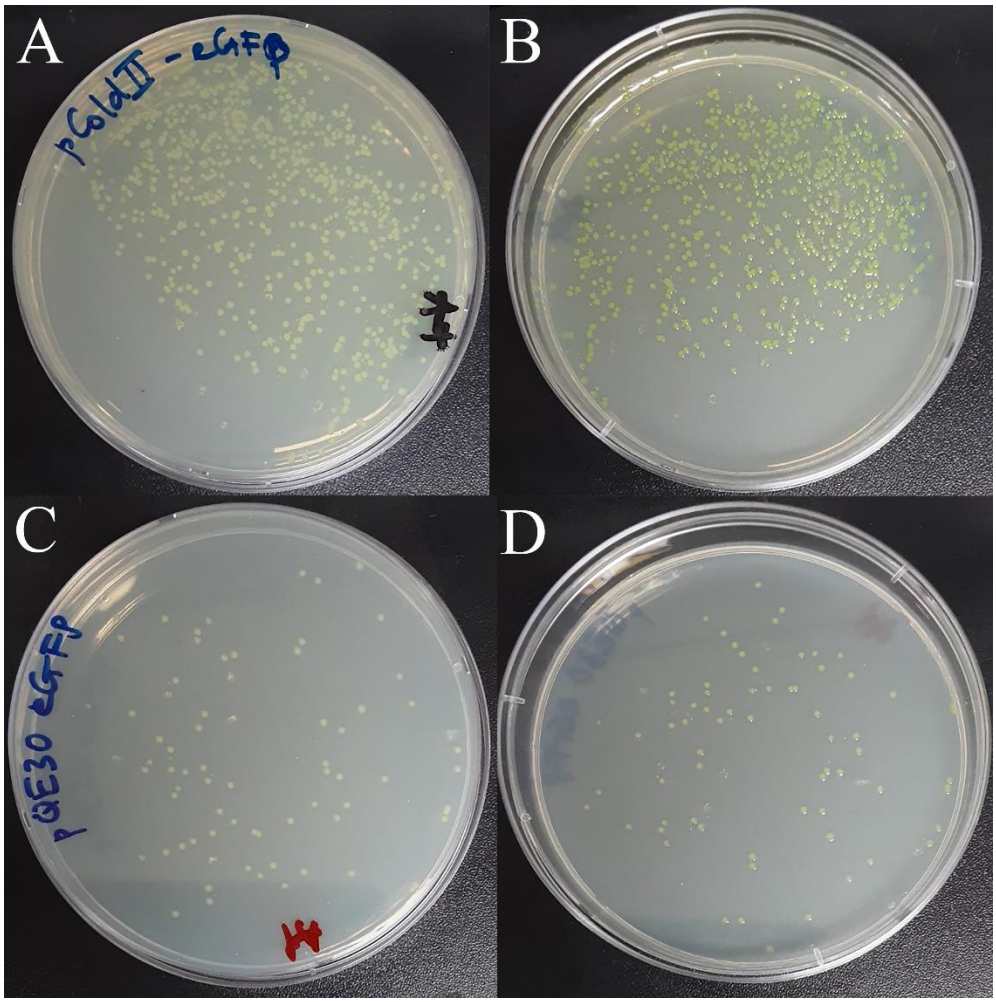

Figure 4. Auto-induction of eGFP in *E. coli* TOP10 strain when cells were grown on LB plates at 37ºC for 18-22 hours and subsequently stored at 4ºC. A & B are the back and front views of TOP10 carrying pColdII-eGFP; C & D are the back and front views of TOP10 carrying pQE30-eGFP.

Table 1. Yields of eGFP as estimated by imageJ combining with Bradford assay (see Materials and methods for more details).

Strains	Wet weight	Total eGFP	Total soluble eGFP
BL21 pColdII-eGFP	3.5 g/L	21 mg	10 mg
M15 pQE30-eGFP 37ºC	5g/L	200 mg	negligible
M15 pQE30-eGFP 30ºC	4g/L	120 mg	4 mg

Soluble eGFP could be purified to relatively high homogeneity by Ni-NTA column as shown in Figure 3. More importantly, the whole purification process could be carried out at room temperature for hours without affecting the biochemistry of eGFP. When purified eGFP was placed under long wavelength UV light, the solution glowed in a very brilliant color (Figure 5). We also observed that with careful adjustment of the number of freeze and thaw cycles, eGFP could be obtained in relatively pure form without column chromatography step (data

not shown). Based on our estimation, 1 L of BL21 culture yielded approximately 10 mg of soluble eGFP while 1 L of M15 culture yielded approximately 200 mg of eGFP in the form of inclusion bodies. For recombinant BL21 strains, eGFP accounted for approximately 8-10% of total soluble protein (Table 1).

Production of eGFP polyclonal antibodies in Balb/c mice

eGFP is such a popular reporter that the production of antibodies against it warrants. We proceeded with the production of polyclonal antibodies against eGFP in mice based on our previously established procedure (Nguyen NL, Phan TMP, 2018). eGFP was purified to the highest possible homogeneity in denatured condition (data not shown), formulated with Freund's adjuvants, and immunized to three 6 week old female Balb/c mice. After the second boost, anti-sera were collected. Each mouse yielded approximately 200-250 µL of anti-serum. The anti-sera were stored at 4ºC in 50% glycerol and 0.2% sodium azide. When testing with Western blot and ELISA, the antisera from all three mice showed high titers and specificity to eGFP (data not shown). The antibodies could be used for ELISA or Western blot at 1:2000 to 1:4000 dilution factors, respectively. When the antibodies were tested against eGFP expressed in *S. cerevisiae*, they could detect a specific band of yeast expressed eGFP (Figure 6).

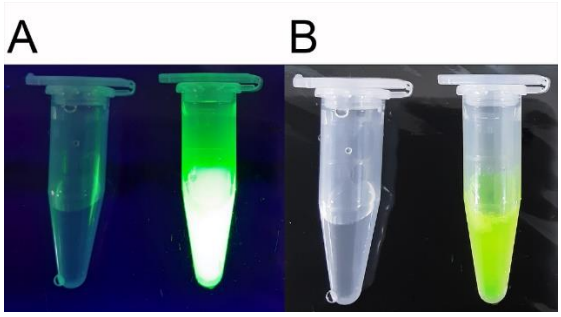

Figure 5. Purified eGFP viewed under long wavelength UV light (A) and visible light (B). The tube on the left side contained PBS and was used as a negative control. The tube on the right side contained purified eGFP in PBS buffer.

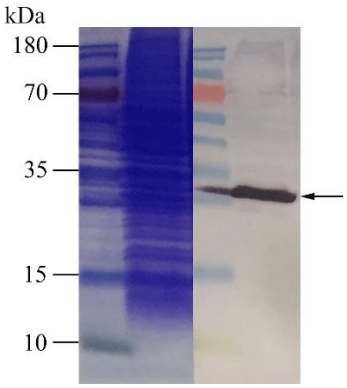

Figure 6. Anti-eGFP polyclonal antibodies could detect yeast expressed eGFP, indicating the specificity of the antibodies. The expressed eGFP could be visualized under UV light in the SDS-PAGE gel before staining.

CONCLUSION

A synthetic gene encoding eGFP was cloned and expressed in various *E. coli* strains. The expression of the target protein showed a temperature dependent pattern, where more functional protein was produced at low temperatures. *E. coli* cells expressed functional eGFP showed intense green color at visible light, indicating high expression levels. The target protein could be expeditiously purified by Ni-NTA affinity chromatography at room temperature without losing its functionality. Polyclonal antibodies against eGFP were successfully produced in Balb/c mice and showed high specificity and titers. eGFP cloning, expression and purification could be adapted into curriculums as a practical course to teach basic molecular biology and biotechnology concepts to undergraduate and high school students.

Acknowledgement: *This work was supported by NAFOSTED grant number 106.02-2018.49. The authors declared no conflict of interest*.

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