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Original Research Article

Replacing UV with Blue Light during DNA Purification Increases the Efficiency of Ligation-Independent Cloning

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HIGHLIGHTS

- Using blue light instead of UV light increases the efficiency of LIC.
- A simple LED blue light projector combined with a filter can be used for this purpose.
- This approach is not recommended for applications that require higher sensitivity.

ABSTRACT

Ligation-independent cloning is a simple method that provides several advantages over conventional cloning. However, the efficiency of ligation-independent cloning is considerably lower than that of conventional methods. Several studies have shown that competent cells used for ligation-independent cloning should preferably have a transformation efficiency of 10^6-10^7 cfu/µg DNA. Although such levels can be easily achieved using standard protocols with most *Escherichia coli* strains, some strains attain mush lower values. When such strains have to be used for ligation-independent cloning, certain measures need to be taken to avoid any situation that may further decrease the efficiency of the process. These measures, however, are usually time-consuming. This problem is exacerbated by the fact that some strains such as BL21 (DE3) appear to be intrinsically unsuited for ligation-independent cloning. Here we suggest that by avoiding DNA damage during purification simply by replacing UV transilluminators with blue light systems BL21 (DE3) cells with a transformation efficiency of 10^5 cfu/µg DNA can satisfactorily be used for ligation-independent cloning without any additional steps.

Introduction

Ligation-independent cloning (LIC), a cloning method that does not rely on restriction enzymes and ligases for the assembly of the recombinant expression construct, was first introduced in 1990 (Aslanidis and de Jong, 1990). In this method, a short complementary region is created on either end of both the target gene and the vector through PCR using carefully designed primers. In the original protocol, T4 DNA polymerase was used to create cohesive single-stranded tails to direct the assembly of the expression construct. Although this approach eliminated the need for time-consuming enzymatic cleavage and ligation steps, it included another form of enzymatic processing and relied on chemically modified primers and thus did not enjoy the popularity that one would have expected. However, it was later shown that PCR products containing short homology regions could be used for this process without any enzymatic pretreatment steps, a finding that greatly simplified the original LIC protocol (Klock et al., 2008). The authors argued that incomplete PCR extension resulted in the formation of single-

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stranded complementary ends and accordingly named their method Polymerase Incomplete Primer Extension (PIPE) cloning. This method was further improved by optimizing PCR conditions (Li et al., 2011). However, it has been proposed that the assembly of the construct takes place through homologous recombination and thus may not necessarily rely on the presence of cohesive single-stranded ends (Jacobus and Gross, 2015). Considering the fact that *recA* mutant *Escherichia coli* strains such as DH5α (Jacobus and Gross, 2015) and GeneHogs (Klock et al., 2008) have successfully been used for such experiments, it can be argued that different pathways including RecA-independent recombination (that requires single-stranded ends) (Dutra et al., 2007) may be involved in this process. Regardless of the underlying mechanisms, the fact that untreated PCR-amplified fragments can be used for LIC makes this method an attractive alternative to conventional cloning protocols. But there is a catch. The efficiency of unconventional cloning methods is usually considerably lower than that of the conventional approach (You et al., 2012; Beyer et al., 2015). LIC is no exception. This is not unexpected as additional processes such as recombination need to be carried out by the host's enzymatic machinery. The intrinsic instability of linear DNA molecules in *E. coli* may also play a role in this phenomenon (Kuzminov et al., 1994). To overcome this limitation, most researchers opt to use commercial competent cells with a transformation efficiency (TrE) of 10^9-10^{10} cfu/µg DNA (Stevenson et al., 2013; Kostylev et al., 2015). However, this is not an absolute requirement as a TrE of 10^6-10^7 cfu/µg DNA appears to be sufficient in most cases (Klock et al., 2008; Beyer et al., 2015). Such levels can be obtained with most *E. coli* strains using standard protocols available for the in-house preparation of competent cells. But some *E. coli* strains such as BL21 (DE3) and SCS110 attain much lower TrE (Chan et al., 2013). These low-TrE strains do not readily lend themselves to LIC unless certain precautions are taken to increase the overall efficiency of the process.

Since the quality of substrate DNA is one of the most important factors that affect the overall efficiency of cloning experiments (Grundemann and Schomig, 1996), procedures that may result in DNA damage should be avoided or at least minimized when LIC has to be carried out using low-TrE strains. A crucial step during which significant DNA damage may occur is the purification step. The template used for the amplification of the vector backbone for LIC should be removed before transformation as it may result in the appearance of false positive colonies (Jacobus and Gross, 2015). This may be achieved by using endonucleases that selectively digest the methylated template while leaving the unmethylated PCR-amplified fragments intact (Stevenson et al., 2013). However, even when the template DNA is completely digested, there is still a possibility that the amplification process itself results in the generation of complete plasmids that may produce a significant background of empty vectors (Stevenson et al., 2013). An alternative approach is to purify the PCR-amplified fragments by gel electrophoresis. But, in order to protect the DNA molecules from the negative effect of UV exposure during gel-purification, some protective measures are usually called for. Arguably, the most commonly used approach is to run two parallel lanes during electrophoresis and expose only one to UV light (the reference lane) while covering the other (the target lane) and then using the reference lane to determine the location of the desired band in the target lane for DNA recovery (Jacobus and Gross, 2015). This, of course, increases the complexity of the process especially when large numbers of samples need to be processed. In such cases, using blue light epi- or transilluminators may offer a more convenient alternative.

Both UV and blue light can damage DNA molecules. Nevertheless, unlike UV light that directly alters DNA molecules (Cadet and Wagner, 2013), blue light appears to affect DNA molecules indirectly through the action of reactive oxygen species formed *in vivo* (Godley et al., 2005). Thus, using blue light instead of UV during *in vitro* DNA manipulation may reduce the extent of DNA damage. According to some blue light illuminator manufacturers this is a feature that represents a practical advantage compared to UV instruments (for example please see Application Note 2014(2), NIPPON Genetics EUROPE, Duren, Germany and Invitrogen Quick Reference MAN0007581 Rev.3). Some studies involving conventional cloning procedures appear to confirm those claims (Robin and Martineau, 2012). In order to evaluate whether using blue light instead of UV light can increase the efficiency of LIC when working with low-TrE strains, we conducted a series of LIC experiments with *E. coli* BL21 (DE3) as a host using both blue light and UV. The results are reported in this paper.

Materials and Methods

Amplification of bla and vector backbone

The sequences of pET-22 and pET-28 plasmids were analyzed using Ape (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and primers were designed using Primer-BLAST (Ye et al., 2012). Primers FA (5′-TTCTACGGGGTCTGACGCTC-3′) and RA (5′-TTCCCTTCCTTTCTCGCCAC-3′) were used to amplify the *bla* gene from pET-22 using a peqSTAR thermocycler (PEQLAB biotechnologie, Germany) with a PCR program consisting of an initial denaturation at 94 °C for 120 s, 25 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 40 s, extension at 72 °C for 240 s, and a final extension at 72 °C for 420 s. The backbone region was amplified using the pET-28 plasmid as template

with primers FB (5′-AGCCCCCGATTTAGAGCTTG-3′) and RB (5′-TGACCAAAATCCCTTAACGTGAG-3′) and the same PCR program. All PCR amplifications were performed using *Pfu* DNA Polymerase (Vivantis Technologies, Malaysia) according to the manufacturer's instructions.

Gel purification

Electrophoresis was performed according to standard protocols (Sambrook and Russell, 2001). Gels were stained using RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Republic of Korea). Gel extraction was carried out using either UV or blue light. For UV experiments a TFX 20 MC UV transilluminator (Vilber Lourmat, France) set at 312 nm was used. Blue light experiments were carried out using two 12 W blue light LED projectors (ZFR LED Lighting, China) positioned at a 15-cm distance from the gel and a pair of dental blue light protection wraparound glasses (Golden Max, Taiwan). Exposure time was kept at minimum and a GF-1 Gel DNA Recovery kit (Vivantis Technologies, Malaysia) was used to recover DNA fragments following the manufacturer's instructions. Only the *bla* region was gel purified. As for the backbone region, only a buffer exchange step was performed. A single batch of the buffer-exchanged backbone region was used for all experiments. In all cases elution was carried out using water instead of elution buffer.

Preparation of competent cells and transformation

Escherichia coli BL21 (DE3) competent cells were prepared using standard protocols (Seidman et al., 2001) with minor modifications. In brief, 250 µL of an overnight *E. coli* BL21 (DE3) culture was used to inoculate 25 mL lysogeny broth (LB) medium which was then incubated at 37 °C and 250 rpm until the OD_{600} reached 0.35. Cells were harvested by centrifugation at 4 °C, washed three times with cold $CaCl_2$ solution (Tris –HCl 10 mM, pH 7.0, $CaCl_2$ 60 mM, glycerol 15%) and stored at -80 °C in 100 µL aliquots until used. Transformation was carried out using standard protocols (Seidman et al., 2001) except that 100 ng of 1:1 molar ratio of backbone:*bla* DNA was used for each transformation experiment. Transformants were selected on LB agar plates containing ampicillin at a final concentration of 100 µg/mL.

Verification of recombinant plasmids

In order to verify the correct assembly of the resulting plasmids, primers ConF (5′-TTGTTGCCGGGAAGCTAGAG-3′) and ConR (5′-GGCTAGCATGACTGGTGGAC-3′) were used to amplify a 1447 bp region of plasmids extracted from a number of randomly selected colonies formed on ampicillin selection plates with a PCR program comprising an initial denaturation at 94 °C for 120 s, 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 300 s.

Results and Discussion

The LIC strategy used in this study is shown in Fig.1. The pET-22 and pET-28 vectors are identical except for two regions. The first is an approximately 100 bp-long stretch that contains the *pelB* periplasmic localization signal sequence in pET-22 and the T7•Tag detection and purification fusion tag in pET-28. The second region is relatively longer (approximately 1000 bp) and encompasses the *bla* (ampicillin resistance) gene in pET-22 and the *kan* (kanamycin resistance) gene in pET-28. In order to replace the *kan* gene of pET-28 with the *bla* gene from pET-22, the *bla* gene from pET-22 and the backbone region of pET-28 were amplified using primer pairs FA/RA and FB/RB, respectively. These primer pairs are designed in a manner that results in the formation of an approximately 50 bp homology region on either ends of the amplified fragments. Thus the correctly assembled recombinant plasmid should contain the *bla* gene from pET-22 and the backbone region of pET-28. Accordingly, transformants containing this plasmid should be selected on ampicillin selection plates. Fig. 2 shows the results of PCR amplification of *bla* and backbone regions. Bands of desired length (1500 bp for *bla* and 4117 bp for backbone) can clearly be seen. Since the pET-22 plasmid used as template for the amplification of the *bla* region is also capable of conferring ampicillin resistance, it was necessary to remove this template from PCR products before LIC. The pET-28 template plasmid used for the amplification of the backbone region, on the other hand, does not contain ampicillin resistance genes and thus need not be removed.

Factors such as the insert:vector molar ratio and the total amount of DNA used for transformation can affect the overall efficiency of LIC experiments. However, there appears to be no consensus regarding the optimum levels for these factors as different research teams have suggested different combinations (Aslanidis and de Jong, 1990; Beyer et al., 2015; Jacobus and Gross, 2015; Kostylev et al., 2015). Our preliminary studies performed using 100 to 300 ng of total DNA and insert:vector molar ratios of 1:1 to 3:1 (data not shown) revealed that in these range results were not significantly affected by the above factors which is in accordance with the results reported by Jacobus and Gross (2015). We thus decided to use a total DNA amount of 100 ng and an insert:vector ratio of 1:1 in all experiments.

To evaluate whether the efficiency of LIC with low-

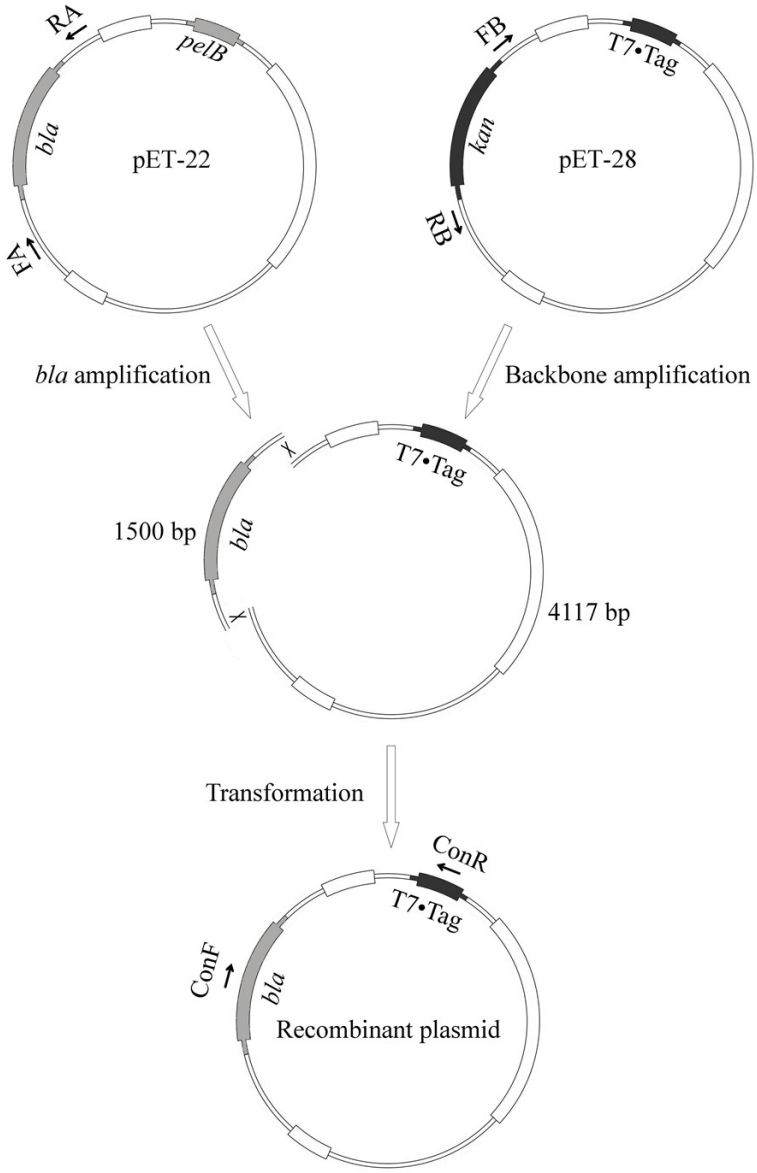

Figure 1. The LIC strategy used in this study. pET-22-specific regions are shown in gray, pET-28-specific regions in black and regions common to both vectors in white. Solid arrows represent primers. The size of resulting PCR products in bp are shown next to their corresponding fragment. Features are not drawn to exact scale.

TrE strains can be improved by avoiding UV exposure, BL21 (DE3) competent cells with a TrE of approximately 10^5 cfu/µg DNA were transformed by substrate DNA exposed to either UV or blue light during gel purification. For each experiment, 3 independent extractions were performed and each extracted product was used for 3 transformations (i.e. a total of 9 replicates for each method, supplementary Table S1). Results are shown in Fig. 3. The number of colonies formed using blue light was almost ten times higher than that obtained with UV light.

In theory, only transformants harboring recombinant plasmids should be able to grow on ampicillin selection plates. Nevertheless, we designed verification primers ConF and ConR as a safeguard to ascertain that no unpredicted events that may affect the results or interfere with their interpretation occur. ConF anneals to a region inside the *bla* gene while ConR is complementary to a portion of the T7 leader sequence (Fig. 1). Amplification of the recombinant plasmid using these primers is expected to result in the appearance of a 1447 bp product

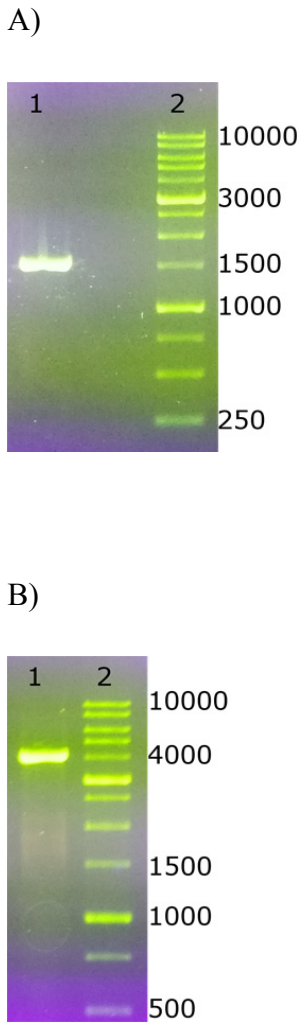

Figure 2. Electrophoresis gels of PCR-amplified target gene and vector backbone. A) Lane 1: the 1500 bp region of pET-22 containing the *bla* gene; Lane 2: DNA ladder (bp). B) Lane 1: the 4117 bp pET-28 backbone; Lane 2: DNA ladder (bp).

while neither pET-22 nor pET-28 would produce any products as only one of the two primers can bind to these plasmids. Four colonies from blue light experiments were randomly selected and used for verification. Results are shown in Fig. 4. As expected, all four colonies produced a PCR product of the anticipated size while none of the parent vectors were amplified.

Despite the apparent simplicity of LIC many researchers still prefer to use conventional cloning methods. Arguably, one of the most important factors that have contributed to this situation is the problems associated with setting up a new method in a cloning laboratory. Many researchers have adopted different variations of existing protocols that have been optimized and validated over time through extensive work in their laboratories. If introducing a new method requires substantial revisions and modifications in upstream protocols, it is usually more convenient to adhere to the original method even if includes a few additional steps. In other words, the compatibility of the new method with other components of the whole process is a major determinant of its acceptance by researchers. As stated above, some *E. coli* strains such as BL21 (DE3) normally yield low-TrE competent cells when common protocols are used (Chan et al., 2013). In our laboratory we routinely use a modified $CaCl_2$ method that typically produces BL21 (DE3) competent cells with a TrE of ~10^5 cfu/µg DNA. Although these cells can easily be used in conventional cloning experiments, they are not well suited for LIC as the overall efficiency of the latter is considerably lower. In addition, BL21 (DE3) cells appear to have a limited intrinsic capacity for LIC. A recent study that explored the performance of different *E. coli* strains in LIC revealed that commercial BL21 (DE3) competent cells with a TrE of 1-5×10^7 cfu/µg DNA produced on average only 9 colonies per plate while TOP10 cells with a much lower TrE (6×10^6 cfu/µg DNA) gave rise to over 1000 colonies under equal conditions (Beyer et al., 2015). In our UV experiments we were unable to obtain any colonies in 2 out of 9 plates (Fig. 3 and supplementary Table S1). However, this does not necessarily mean that low-TrE strains such as BL21 (DE3) cannot be used in LIC. Our results suggest that by using blue light instead of UV light it is possible to reproducibly obtain recombinant clones even with such strains. Blue light illuminators are also safer and usually less expensive than their UV counterparts. In fact, a simple blue light LED projector combined with an appropriate filter can satisfactorily be used for this purpose as described in this paper. Nevertheless, this simple system has its drawbacks. In supplementary Fig. S1 two images of a typical gel visualized under UV and blue light are presented. The sensitivity of the blue light system is clearly lower. This image was captured using a Wratten 15 filter (Rodenstock, Germany). Considering the excitation and emission maxima of RedSafe (514 and 537 nm, respectively), using a Wratten 16 or even 21 filter may have resulted in slightly improved contrast. It should be noted, however, that the objective of this study was not to develop a perfect blue light system as commercial blue light illuminators with detection and imaging performance equal to or even higher than that of UV systems are available. We only sought to show that LIC can easily be used instead of conventional cloning methods even with "unsuited" strains using a simple approach. In cases where sensitivity is of prime importance, e.g. when the electrophoresis pattern is used for identification such as in random amplified polymorphic DNA-PCR (RAPD-

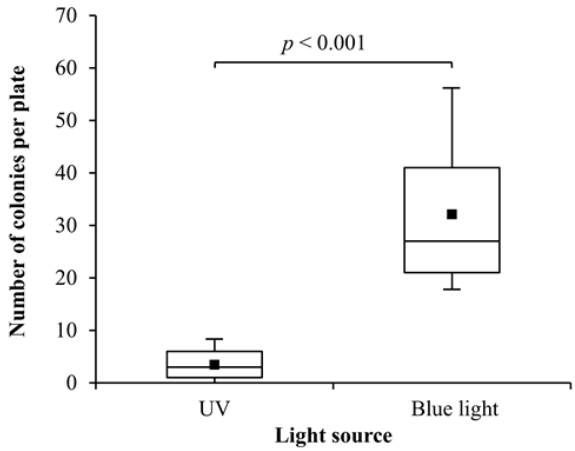

Figure 3. A schematic box plot showing the number of colonies formed on selection plates after LIC using UV and blue light. No outliers were detected. Black squares represent the mean and the *p*-value was calculated through two-sample *t*-test using the IBM SPSS Statistics 22 software (IBM Corp., USA).

PCR), it may be necessary to use a commercial system as closely related strains may produce similar patterns that differ only in the relative intensity of individual bands (for an example see Sharafi et al., 2017). Such subtle differences may go undetected using the simple blue light system described in this paper.

Conclusion

The results presented in this paper suggest that, by avoiding DNA damage during gel-purification by using blue light instead of UV light, it is possible to obtain recombinant clones in a reproducible manner through LIC, even with *E. coli* strains that appear to be unsuited for this method. A conventional blue light LED projector and a pair of blue light protection glasses can conveniently be used for this purpose. For imaging purposes, however, the photographic filter should be carefully selected according to the excitation and emission maxima of the employed DNA stain.

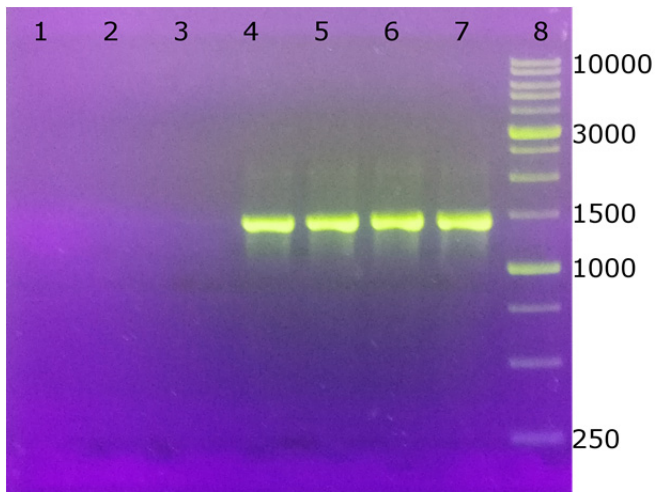

Figure 4. Electrophoresis gel of verification PCR experiments. Lane 1: negative control; Lane 2: pET-22; Lane 3: pET-28; Lanes 4-7: purified plasmids from randomly selected colonies; Lane 8: DNA ladder (bp).

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Competing Interests

The authors have no competing interests to declare.

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Supplement

Table S1. Number of colonies obtained in individual UV and blue light LIC experiments.

Extraction	Repeat	Number of colonies per plate	
		UV	Blue light
1	1	4	25
	2	8	45
	3	2	41
2	1	7	56
	2	6	21
	3	3	19
3	1	0	37
	2	1	27
	3	0	18
Average		3.4	32.1

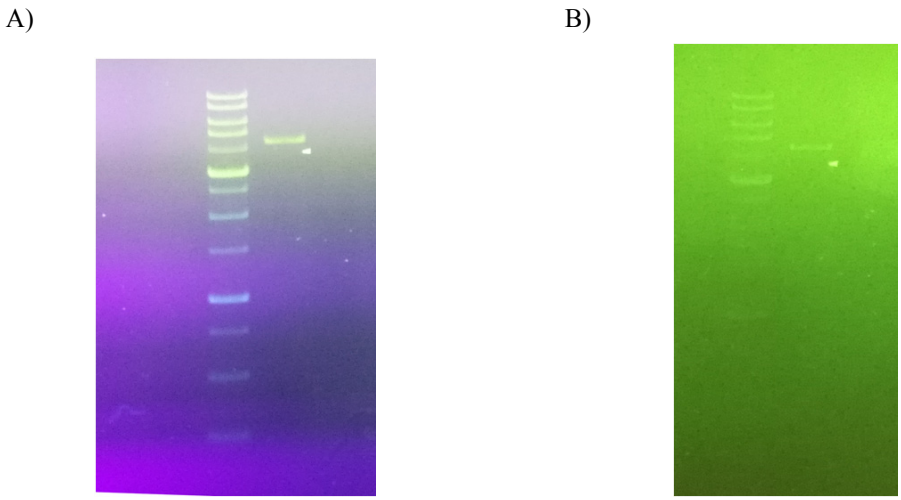

Figure S1. Typical photographs of agarose gels visualized under UV (A) and blue light (B). In order to provide a means for direct comparison, an agarose gel loaded with a DNA ladder and the PCR-amplified vector backbone (4117 bp) and stained with RedSafe was photographed using both systems.