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# In-Situ Gel-Free Plasmid Reassembling for Rapid Gene Subcloning and Truncation

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**HIGHLIGHTS**

- Two plasmids can be mixed and double-digested simultaneously
- DNA segment released from one plasmid can enter the other plasmid in the reaction
- A nucleotide sequence can be cut out from a plasmid by in-situ double digestion and ligation

**Abstract**: Gene subcloning, a process in which the nucleotide sequence of interest is excised from on plasmid and inserted into another, seems to be an easy task to done. However, not all subcloning attempts are successful, even when the insert sequence and the double digested target plasmid are successfully purified form agarose gel and thought to be ready for subsequent ligation. In the current study we introduce a reliable, easy, and time consuming method for gene subcloning and also truncation. The stages are all carried out in a single microtube without any running on a gel, making it possible to accomplish a successful gene subcloning or truncation even with low concentrations of DNA molecules. Summarily, subcloning is achieved by mixing the plasmids of interest in a microtube and subjecting to restriction enzymes whose restriction sites flank the segment that is going to be subcloned. Digestion mixture is precipitated in the same microtube using isopropanol and the resultant DNA molecules are allowed to take part in a ligation reaction. The recombinant plasmids of interest are screened by colony PCR. Truncation is achieved by double-digestion of the plasmid of interest using a restriction enzyme whose restriction site flanks the segment that is going to be cut out.

**Keywords:** plasmid mixing; concurrent digestion; simultaneous ligation; gel-free subcloning.

## INTRODUCTION

Restriction enzymes digest nucleotide sequences at specific restriction sites, making it possible to insert a gene of interest into a nucleotide sequence (e.g. genomic DNA, cloning or expression vectors) and produce a recombinant sequence. Digested DNA fragments are usually separated using electrophoresis in agarose gel, which then are purified from the gel either manually or by using commercially available kits. Large quantities of digested DNA molecules separated on the gel may be lost during gel purification steps, meaning

that researchers need to repeat plasmid extraction, digestion, and purification stages if the concentrations of starting DNA molecules are insufficient for subsequent ligation. Insertion of a gene of interest into a plasmid requires successful digestion and ligation reactions. Ligation reaction is not always successful, even when the insert and the digested plasmid carry similar sticky ends. Several reasons (e.g. high salt or EDTA concentration in the reaction, high DNA concentration, impurities, and so forth) and solutions have been proposed when ligation is unsuccessful 1. However, the main cause of ligation failure sometimes remains elusive. We had severe trouble with inserting NcoI/XhoI double-digested nucleotide sequence encoding for a scFv antibody into pET22b (+) expression vector double-digested with the same enzymes. Sometimes even, the extracted plasmids were resistant to enzymatic digestion. Methylation of restriction sites in plasmid within bacterium cytoplasm may be a reason why restriction enzymes are unable to digest the plasmid while it contains appropriate restriction sites 2,3. We did not consider methylation of restriction sites as a potential cause of plasmid digestion failure because bacterial Dam and Dcm methylases were unlikely at all to methylate nucleotides in the sticky ends of NcoI/XhoI digested sequences 3,4. UV radiation is also among the factors that interfere with DNA digestion and ligation 5,6. It usually takes both time and cost to discover what factor interferes with digestion and/or ligation reactions. So, we decided to set a gel-free protocol to by-pass all potentially interfering factors in these reactions in order to simplify gene sub-cloning and truncation. In summary, gene subcloning is achieved by mixing cloning and expression vectors within a microtube and double-digested at the same time. After ligation of DNA molecules in the mixture, a third enzyme is used to digest non-recombinant expression vectors. For gene truncation, plasmid is double digested with a single enzyme and allowed to re-assemble.

## MATERIALS AND METHODS

### Materials and reagents

pGH cloning vector containing the gene of interest (single chain anti-EGFR antibody-encoding sequence) was purchased from Nedaye Fan (Tehran, Iran). pET22b(+) expression vector was purchased from Novagen (Cat. No. 69744-3). Forward and Reverse primers (5´- CACTATAGGGGAATTGTGAGCG-3´ and 5´-CTAGTTATTGCTCAGCGGTGG-3, respectively, synthesized by BioBasic Co. Canada) were designed based on pET22b(+) cloning site. NcoI, XhoI, and Hind III restriction enzymes were purchased from Takara (Japan).

### Combination and simultaneous digestion of plasmid pair- the first round of digestion

Simultaneous digestion of plasmids was carried out in a 0.5 mL microtube (total volume of 15 µL, Figure 1) containing 500 ng (2.5 µL) pGH cloning vector, 200 ng (1.5 µL) pET22b(+) expression, 5.5 µL distilled water (DW), 1 µL NcoI (10 u), 1 µL XhoI (10 u), and 3 µL Tango buffer 10 X. After 1 h incubation at room temperature, the restriction enzymes were inactivated by incubation at 85℃ for 15 min. Digestion product (combination of digested plasmids) was precipitated in the same microtube by addition of 15 µL isopropanol, 20 min incubation in refrigerator (-20℃), and 10 min centrifugation at 10000 g. The microtube was depleted and allowed to dry for 20 min. 100 µL ethanol 70% was added to the microtube and depleted again after 2 min centrifugation at 10000 g. After complete evaporation of ethanol 70%, 10 µL DW was added to the microtube to dissolve the precipitated DNA molecules in 20 min at room temperature.

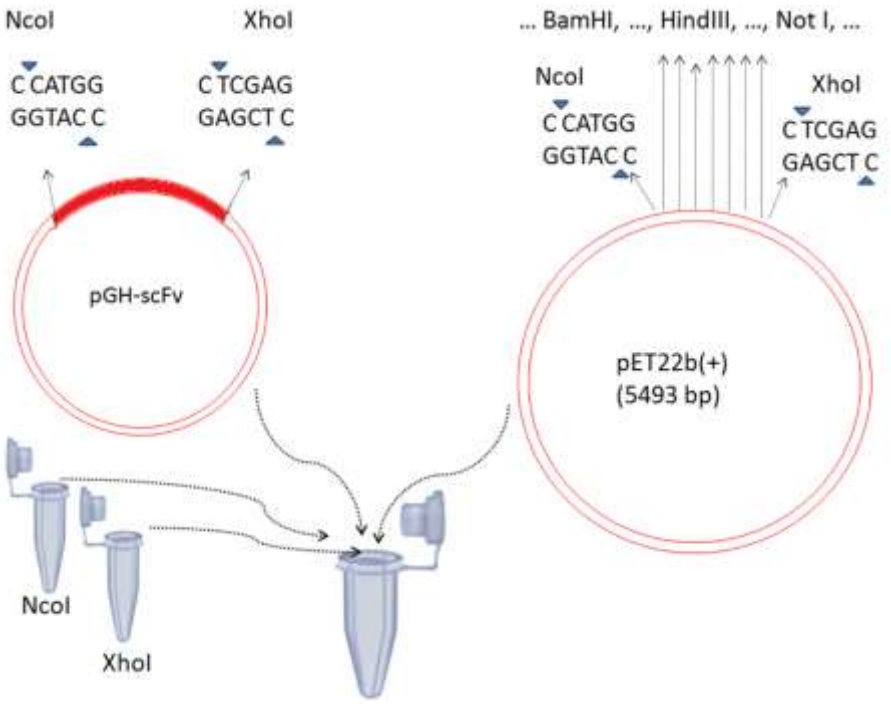


**Figure 1.** Schematic overview of simultaneous digestion of cloning and expression vectors. Both plasmids in company with both restriction enzymes (NcoI and XhoI) are added to a microtube at the same time.

## Ligation

Ligation of NcoI/XhoI double-digested plasmids was carried out in the same microtube. 1 µL ligase (10 u) and 1 µL of its 10X buffer were added to the microtube, which then incubated at room temperature for another 1 h. After this period, ligase was inactivated by 15 min incubation at 65 ºC and the ligation product was precipitated in the same way as already described. The precipitant was re-solubilized in 10 µL DW.

## Simultaneous digestion of ligation product with Hind III- the second round of digestion

Intact pET22b(+) contains several sites for restriction enzymes, including NcoI, BamHI, EcoRI, SacI, SalI, HidIII, NotI, and XhoI. So, all the restriction sites between NcoI and XhoI would be absent in recombinant pET22b(+) if the insert be devoid of these restriction sites. The scFv gene used in this study contains an EcoRI restriction site in its sequence between NcoI and XhoI restriction, so we did not consider this enzyme for the second round of digestion. Among the enzymes listed above, we used Hind III to digest unwanted plasmid constructs. 1 µL of the enzyme in company with 1.2 µL of its 10 X buffer was added to the microtube (containing 10 µL ligation product prepared in previous stage) and allowed to digest the ligation product for 1 h at 25℃. 5 µL of the final solution was used to transform competent DH5- Alpha bacterial cells.

## Double digestion for truncation of a nucleotide sequence

There are lots of engineered plasmids whose nucleotide sequences encode for fusion proteins [e.g. proteins fused to nuclear localization signals (NLS) or cell penetrating peptides] or multi-domain proteins. If the nucleotide sequence encoding for a protein subunit is flanked by an enzyme restriction site, it would be easily cut out of the plasmid. The in-situ digestion-ligation gel-free method described in this study (Figure 2) will be also useful for truncation of such multi- subunit genes. Using this method, we cut out NLS-encoding sequences form Herceptin VH-$(NLS)_4$ fusion protein- encoding sequence. pET22b(+)-Herceptin VH-$(NLS)_4$ recombinant plasmid (150 ng) was digested using Hind III restriction enzyme within a 250 µL microtube and precipitated in the same way as described NcoI/XhoI double digested plasmids. The precipitant was dissolved in 8 µL DW and subjected to ligation reaction by addition of 1 µL T4 DNA ligase and 1 µL of its 10X buffer. 5 µL of the solution was used to transform competent *E. coli* cells. Similarly, we cut "C-terminal signal peptide-encoding segment" out from "pET22b(+)-Cetuximab VH- C. terminal signal peptide" recombinant plasmid using Hind III whose restriction site flanks the segment. Precipitation and ligation stages were carried out in the same way as described for pET22b (+)-Herceptin VH-$(NLS)_4$ plasmid.

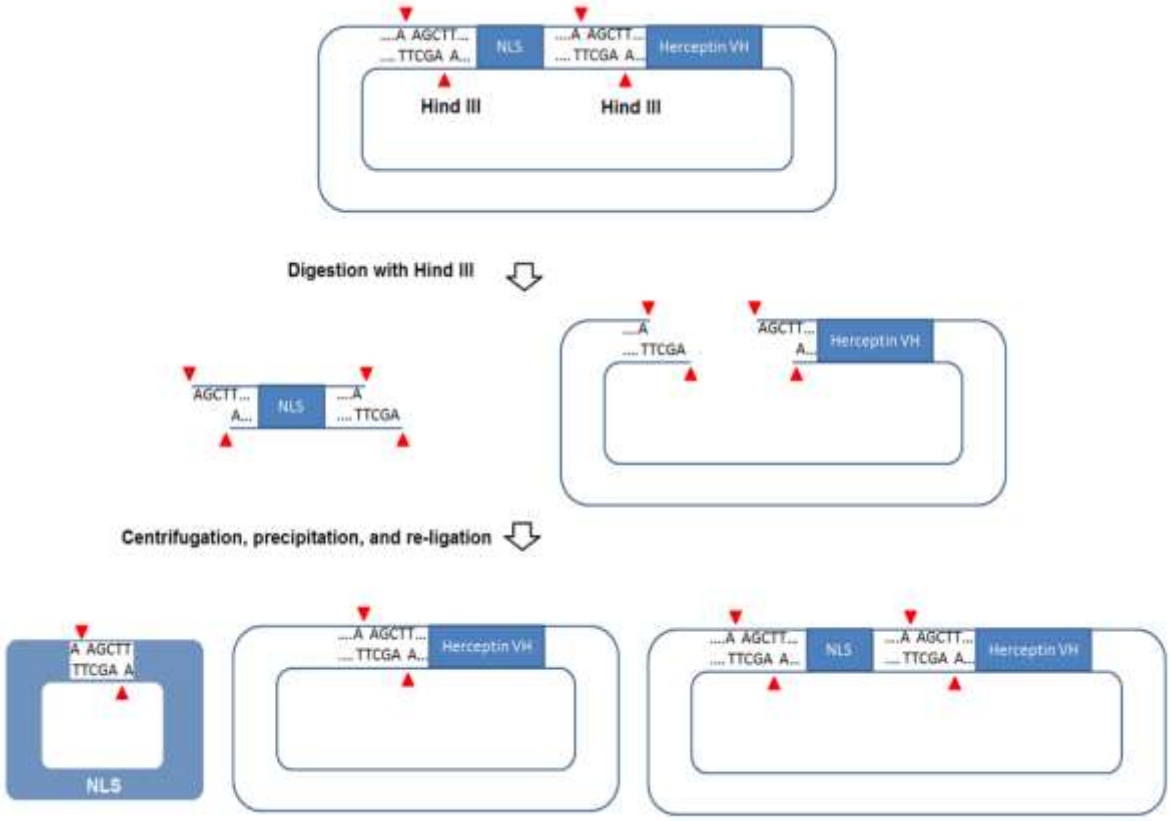


**Figure 2.** In-situ double digestion of Herceptin VH-$(NLS)_4$ encoding plasmid using Hind III for gene truncation. Hind III double digestion results in formation of two DNA molecules, $(NLS)_2$-containing fragment and a linearized plasmid containing Herceptin VH-$(NLS)_2$. Re-ligation of this mixture may result in formation of three types of circular DNA molecules. Circular NLS does not contain Ori sequence and therefore will be degraded in bacterial cytoplasm.

## Colony PCR

Colony PCR was carried out to analyze the digestion-ligation products. Bacterial colonies were suspended in 50 µL distilled water and boiled for 5 min to release their plasmids. 2 µL of the prepared solution of each colony was added to a microtube containing 1 µL forward primer, 1 µL reverse primer, 10 µL of 2X PCR master mix, and 6 µL distilled water (DW). PCR was done for 30 cycles at the condition described in Table 1.

**Table 1.** Temperature and time table of colony PCRstages

| Cycle (s) | Step | Temperature | Time |
|---|---|---|---|
| 1 | Initial denaturation | 94 ℃ | 1 min |
| 30 | Denaturation | 94 ℃ | 1 min |
| | Annealing | 51 ℃ | 45 s |
| | Extension | 72 ℃ | 1 min |
| 1 | Final extension | 72 ℃ | 5 min |

## RESULTS

Simultaneous digestion of pGH-scFv and empty pET22b(+) vectors in a single microtube and subsequent ligation of digested DNA molecules gives rise to four potential plasmid constructs, one of which is recombinant pET22b(+) containing our gene of interest (Figure 3).

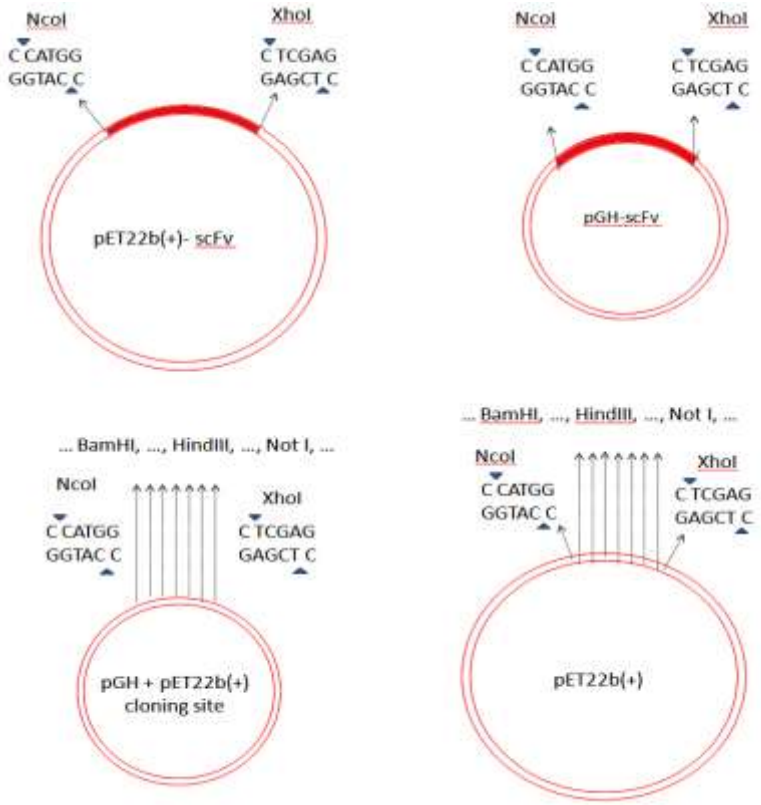


**Figure 3.** Potential plasmid constructs following NcoI/XhoI double digestion, centrifugation, and re-ligation of digestion products. (A) The gene of interest may exit pGH and enter pET22b(+) expression vector. (B) The gene of interest may re-conjugate to pGH. (C): Cloning region of pET22b(+) which carries the NcoI/XhoI sticky ends enters NcoI/XhoI double digested pGH. (D): Cloning region of pET22b(+) may re-conjugate to NcoI/XhoI double digested pET22b(+), making an re-assembled full-length plasmid.

Hind III restriction enzyme used in the second round of digestion makes a nick in the plasmids that carry at least one restriction site for this enzyme. So, re-assembled pET22b(+) and recombinant pGH [pGH containing cloning site of pET22b(+)] plasmids are exposed to Hind III digestion. Linear plasmids are degraded in the cytoplasm and therefore their host would not survive on selective culture medium. Transformation of Hind III-treated plasmid constructs into *E. coli* DH5-Alpha cells resulted in formation of 44 bacterial colonies on $Amp^+$ LB medium, 19 of which were randomly selected for analysis by colony PCR.

4 out of the 19 colonies were identified to be pET22b(+)-scFv (lanes D, M, U and V) (Figure 4). To insure that the colonies were identified correctly, two clones were tested again and confirmed to have recombinant pET22b(+). Since the primers do not match with the strands pGH cloning vector, the negative colonies should be of pGH nature, recombinant pGH [pGH containing cloning site of pET22b(+)] or re-assembled pGH. The first contains a Hind III restriction site in its sequence (Figure 3) and therefore it should be sensitive to Hind III digestion, meaning that it does not contribute to colony formation.

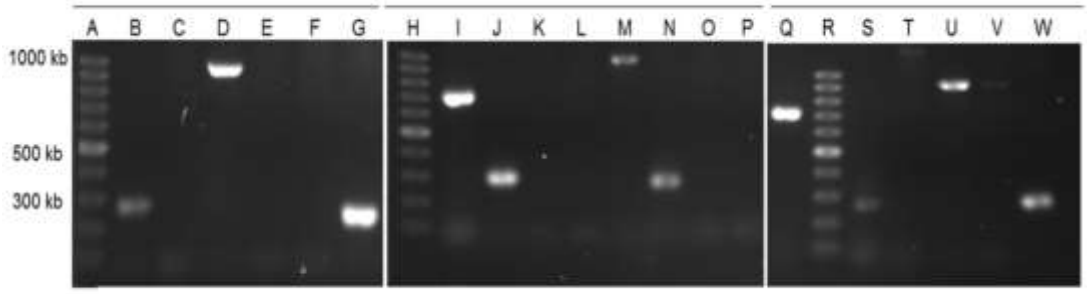


**Figure 4.** Colony PCR with pET22b(+) specific primer pair for selection of bacterial colonies hosting scFv gene. Lanes A, H, and R: 100 bp DNA ladder. Lanes B, J, N, S and W: the size of PCR products indicates that the colonies contain empty pET22b(+) vector. Lane G: empty pET22b(+) vector (control). The primers replicate a 298 bp segment, extending from T-7 promoter region to T-7 terminator region. Lanes D, M, U and V: recombinant pET22b(+)-scFv. DNA bands of 973 bp appear in these lanes. Lanes I and Q: unknown. Lanes C, E, F, K, L, O, P and T: the bacterial colonies should contain pGH vector since the pET22b(+) specific primers do not recognize pGH strands.

### Truncated plasmids

We expected to have DNA bands of 889 and 760 bp in PCR products of Herceptin VH-$(NLS)_4$ and Herceptin VH-$(NLS)_2$, respectively. We tested 8 bacterial colonies using PCR colony, 6 of which were found to be Herceptin VH-$(NLS)_2$ (Figure 5-A). We also tested the method for truncation of "Cetuximab VH-C.terminal signal peptide fusion protein" encoding plasmid (schematic overview not shown). We examined eight bacterial colonies using colony PCR. The colonies all produced a DNA band of 613 bp in PCR (Figure 5-B), indicating that the nucleotide sequence encoding for C. terminal signal peptide (210 bp) has been removed from the whole plasmid, which produces a DNA band of 823 bp in PCR.

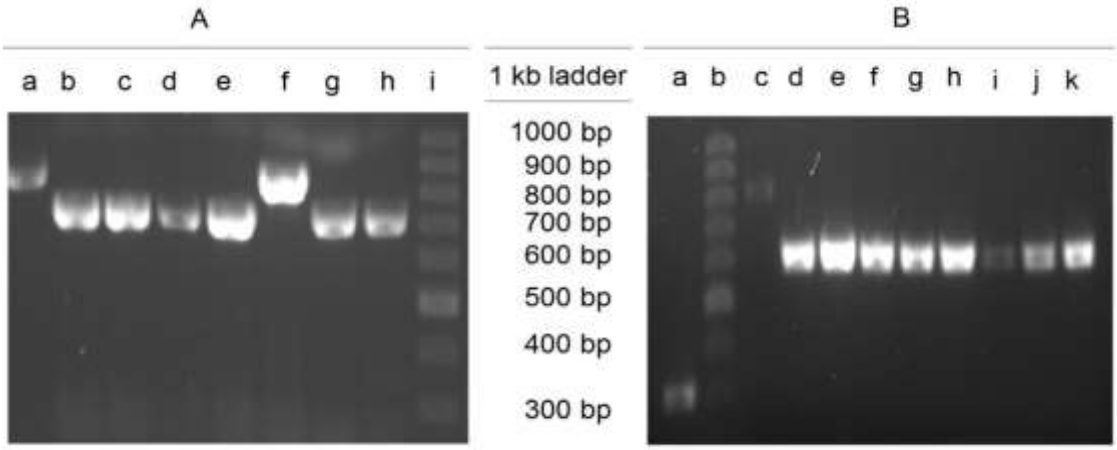


**Figure 5.** Colony PCR for identification of bacterial colonies hosting plasmids of interest. (A) line “a” indicates the control vector, (pET22b(+)-Herceptin VH-$(NLS)_4$. 6 colonies (b, c, d, e, g and h) were found to contain pET22b(+)-Herceptin VH-$(NLS)_2$ vector, which produces a DNA band of 760 bp in PCR. One colony (f) was found to contain intact plasmids, which produces DNA bands of 889 bp using pET22b(+) specific primer pair. Appearance of this band indicate that a number of (pET22b(+)-Herceptin VH-$(NLS)_4$ vectors remains intact during incubation with Hind III. (B) Colony PCR of *E. coli* BL21 (DE3) cells transformed with in-situ double-digested "pET22b(+)-Cetuximab VH-C.terminal signal peptide" vector. All the eight colonies tested (c-l) were found to have a 210 bp deletion (C.terminal signal peptide encoding segment) when compared to parental undigested vector, which produces a DNA band of 833 bp in PCR (b).

## DISCUSSION

In the current study, we introduced a gel-free in-situ method for subcloning and truncation of nucleotide sequences of interest. In regular subcloning procedures, the molar ratio of insert: plasmid is often adjusted to 1:1 to 10:1 [7]. When the same number of two circular plasmids are mixed together in a microtube and subjected to simultaneous digestion, this ration is about 1:1. As shown in Figure 2, four different types of plasmids are expected to form when two plasmids are mixed and digested. Since we used Hind III restriction enzyme in the second round of digestion, we did not expect to have bacterial colonies hosting re-assembled pET22b(+). Emergence of a bacterial colony hosting empty pET22b(+) vector (Figure 3) indicates that a number re-assembled pET22b(+) plasmids have not been digested by Hind III, while carrying Hind III restriction site in their sequence. Presence of a number of undigested plasmids in digestion mixture is not surprising; they may occur even in a good digestion [8]. When a circular plasmid is double-digested with a single enzyme, two DNA molecules with identical sticky ends are formed (Figure 4). In this condition, two independent ligations between the linearized plasmid and the released segment are required to obtain the original plasmids. While, each of the DNA molecules can form a circular molecule by only one ligation reaction. It may justify why the majority of bacterial colonies host truncated plasmids rather that re-assembled full-length plasmids.

## CONCLUSION

Parental cloning vector and the expression vector to which the gene of interest is going to be inserted can be mixed and subjected to defined restriction enzymes to exchange nucleotide sequences standing between the restriction sites. Bacterial colonies containing the recombinant expression plasmid of interest can be identified using colony PCR. Plasmids encoding for multi-subunit proteins can be easily truncated by the gel-free method described in this study if the nucleotide sequence encoding for a subunit is flanked by specific restriction site of a restriction enzyme. In such condition, truncated plasmid can be obtained by in-situ digestion and re-ligation of the digestion product in a single microtube. The method is reliable, easy, and time consuming.

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**Conflicts of interests:** There are no conflicts of interests to declare.

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