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Full Length Research Paper

A new screening method for selection of desired recombinant plasmids in molecular cloning

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One of the problems in cloning process is the low concentration of gene fragment and vector following gel extraction stage which may lead to decreased likelihood of effective ligation. Regarding the facts of this study, after digestion process, the products directly were subjected to ligation. Due to the presence of three antibiotic resistance genes in the reaction, a new strategy based on design of selective media containing different antibiotics was used for selecting the desired colonies. The results of this study indicated that skipping gel extraction process could result in a successful, simple and quick cloning process with high efficiency.

Key words: Cloning, gel extraction, vector, screening, antibiotic resistance genes.

INTRODUCTION

In recombinant protein production and in other molecular biology procedures, one of the most important steps is gene cloning in expression vector. Various methods are used for this purpose, briefly in which the desired gene is incorporated into a T-vector. T-vector containing desired insert is digested with one or two restriction enzyme(s) and the vector is also nicked with the same enzyme(s). After gel agarose electrophoresis and gel extraction of desired bands, both fragments are ligated (Sambrook and Russell, 2001). However, the important problem with this process is the considerable decrease in gene fragments concentration. This collateral effect directly influences the ligation procedure and could be the reason for failed ligation in many cases. Hence, in most textbooks and protocols, it is recommended that ligation reaction should start with either the highest concentration of the gene fragment or a large volume of digestion reaction in order to yield a high concentration of gene fragments after gel extraction (Spackman, 2008; Kontermann, 2010).

pPICZalphaA is one of the expression vectors for which the ligation process is dependent on concentrations of both gene and expression vector. Necessary components for certain amplification in both *Escherichia coli* and yeast (*Pichia pastoris*) are accommodated in this expression vector (Cregg, 1985).

In this study, we used tissue-type plasminogen activator (t-PA) gene cDNA as an insert (Majidzadeh-A, 2010; Soleimani, 2006). The gene is transcribed into a 1.7 Kbp mRNA and belongs to human fibrinolytic system that causes digestion of blood clots through its effect on plasminogen (Loscalzo, 1988; Majidzadeh-A, 2010a, b; Soleimani, 2007). In order to tackle the low concentration defect in cloning process, we incorporated this gene into the expression vector by means of a new method.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain TOP10F´ which was grown overnight at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) containing 1.5% agar. pPICZalphaA expression vector (Invitrogen, USA) and pTZ57R/T vector (T-vector) containing cDNA

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Abbreviation: t-PA, Tissue-type plasminogen activator.

of the t-PA gene were used in this study.

Enzymatic digestion and purification

Due to the restriction sites that existed on either ends of the gene in T-vector and multiple cloning site of the expression vector, they were digested by *XhoI* enzyme (Fermentas, Vinius and Lithuania) at starting concentration of 1 µg in 20 µl volume. To remove the salts in reaction solution, digestion product was purified by means of a polymerase chain reaction (PCR) purification kit (Bioneer, Korea) without gel extraction process.

Cloning process

After purification, a ligation reaction was imposed between digestion products of the shuttle and T-vectors with the same concentration (1 µg). Ligation product was transformed into host *E. coli* strain TOP10F´ (Fermentas, Vinius and Lithuania). Regarding the presence of tetracycline resistance gene in TOP10F´ strain, ampicillin resistance gene in pTZ57R/T-tPA and zeocin resistance gene in pPICZalphaA expression vector, at first a low salt LB agar medium (Merk, Germany) as the primary medium containing zeocin (100 mg/ml) (Invitrogen, USA) and tetracycline (10 mg/ml) (Roche, USA) was prepared and transformed cells were propagated into it for 16 h.

Matrix preparation from grown colonies

Since ligation process was carried out without gel extraction, therefore, the presence of pPICZalphaA expression vector contributed to resistance to zeocin. After ligation process, four types of plasmids were expected: (1) the construct containing pPICZalphaA expression vector fragment and T-vector fragment and t-PA gene, (2) The construct containing pPICZalphaA expression vector fragment and T-vector fragment (3) The construct containing pPICZalphaA expression vector fragment (4) The construct containing pPICZalphaA expression vector fragment and t-PA gene. To screen the zeocin resistant colonies, two kinds of LB agar media were prepared, one containing zeocin (100 mg/ml) and tetracylin (10 mg/ml) and the other containing zeocin (100 mg/ml), tetracycline (10 mg/ml) and ampicillin (50 mg/ml) (Roch, USA). (Crueger., 1990). After growth of the transformed colonies, a matrix was made. The 10 grown colonies were cultured on both type media plates simultaneously for 16 h.

Confirmation of the desired colony and plasmid

To confirm the presence of t-PA gene in pPICZalphaA expression vector, colony PCR was performed on grown colonies. The following PCR reaction conditions were used: 2 min at 95°C for 1 cycle, 30 cycles of 1 min at 95°C, 45 s at 68°C, 2 min at 72°C and a final extension cycle of 10 min at 72°C. Accordingly, after colony PCR, for confirmation of the desired plasmid construct, plasmid was extracted from the mentioned colonies, and then enzymatic digestion was performed by the enzyme *XhoI*. Finally, the desired plasmid was subjected to the sequencing process.

RESULTS AND DISCUSSION

Enzymatic digestion

XhoI digestion of pPICZalphaA expression vector and T-vector containing t-PA gene resulted in linear pPICZalphaA, and also, t-PA gene was separated from the T-vector. The length of fragments was 3600 base pair for pPICZalphaA expression vector, 1628 base pair for t-PA gene and 2886 base pair for back-bone of T-vector.

Growth in selective medium

After the preparation of a matrix from the starting grown colonies, some colonies only grew on media containing zeocin and tetracycline (colonies number. 1, 2, 3, 6, 7 and 8). Others showed growth both on mentioned medium and the medium containing zeocin, tetracycline and ampicillin (colonies number 5 and 9). Such a growth means that grown colonies on the subcultured media that contained zeocin and tetracycline, either had one or more of the four plasmid constructs or all of the plasmid constructs. On the other hand the colonies that grew on both media were those which contained either the first plasmid construct or the second plasmid construct or both. Such a growth implied that the colonies being able to grow on both media were devoid of our desired plasmid construct; however, the colonies that were only able to grow on medium containing zeocin and tetracycline, were the colonies that contained our desired plasmid construct.

Colony PCR

The basis of this study was the hypothesis that the consequence of gel extraction omission would result in occurrence of four plasmid construct shuffling species, because of the presence of the excessive fragments in the reaction tube. The screening of the desired plasmid construct was done through two stages (a selective system containing various antibiotics accompanied with colony PCR). In other words, designing a selective system on the basis of using various antibiotics, two species of the undesired plasmids were omitted. Finally, in order to select the desired plasmid construct (pPICZalphaA-tPA), the colony PCR technique was used. The colonies, whose colony PCR tests turned out to be positive, contained our desired plasmid constructs. After choosing some of these colonies and doing colony PCR with t-PA gene specific primers, some were positive and the rest were negative. The colony number 9 grew on both media. On the other hand, its colony PCR result was positive, suggesting that this colony contained the plasmid construct number 1. The colony number 5 grew on both media. On the other hand, its colony PCR result was negative, therefore, it is determined that this colony contained the plasmid construct 2. The colonies number 1 and 2 only grew on media containing zeocin and tetracycline. Moreover, its colony PCR result was negative; thereby, it showed that these colonies contained the plasmid construct 3. The colonies number 3, 6, 7 and 8 only grew on media containing zeocin and tetracycline;

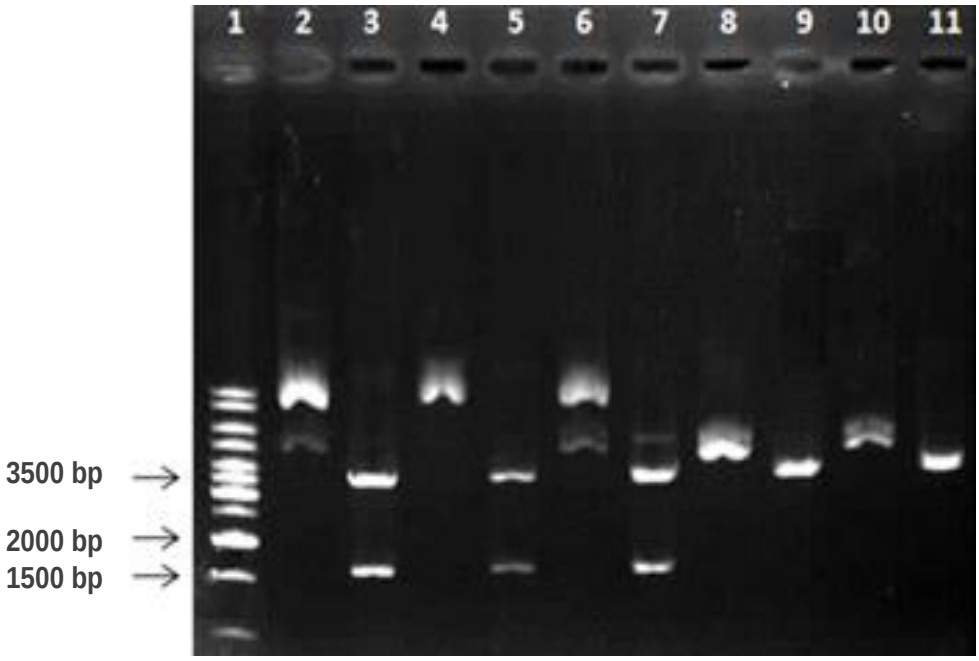

Figure 1. Results related to restriction map preparation from the plasmid constructs using *XhoI* enzyme. Line 1, 1 kb DNA marker; lines 2, 4, 6, 8 and 10 correspond to undigested plasmids related to colonies number 3, 6, 8, 1 and 2, respectively; lines 3, 5, 7, 9 and 11 correspond to digested plasmids related to colonies number 3, 6, 8, 1 and 2, respectively. As evidence, two bonds appear in lines 3, 5 and 7 after digestions, whereas one of the bonds (1628 bp) is related to t-PA gene and the other bond (3600 bp) is related to pPICZalphaA expression vector. This figure proves t-PA gene presence incorporated in to pPICZalphaA expression vector in 3 first plasmids.

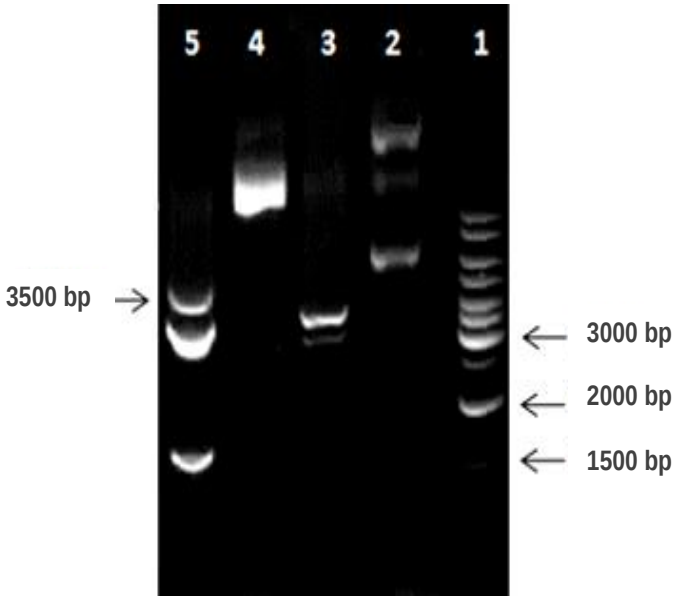

Figure 2. Results related to restriction map preparation from plasmid constructs via *XhoI* enzyme. Line 1, 1 kb DNA marker; lines 2 and 4 correspond to undigested plasmids related to colonies number 5 and 9, respectively; lines 3 and 5 correspond to digested plasmids related to colonies number 5 and 9, respectively. The figure depicts other combinations of plasmid constructs in ligation reaction.

likewise their colony PCR results were positive. Consequently, it is settled that these colonies contained the plasmid construct 4; therefore, the last one contains our favorable colony.

Restriction map and sequencing data analysis

Since all fragments used in the ligation reaction had sticky ends related to *XhoI* enzyme, to demonstrate the formation of the four constructs, the extracted plasmids were digested with *XhoI* enzyme, and the presence of each fragment in plasmid constructs was confirmed as explained earlier (Figures 1 and 2). Sequencing results showed and proved the presence of desired gene in the expression vector. The results are in full compliance with the results of the colony screening on both plates with different antibiotics. Also, the results show that in case of the gel extraction omission, although, the mentioned excessive fragments were present in the ligation reaction tube, the ligation process could be successful so that the desired plasmid construct could be easily selected. The advantage of the method posed earlier is sustaining the initial high gene fragments concentration leading to a highly successful ligation.

In the study, we used one enzyme for enzymatic

digestion and for this reason we had four forms of plasmid constructions after ligation reaction, however, when two sites were used for enzymatic digestion leading to two different sticky ends, we had three forms of plasmid construction after ligation reaction. Accordingly, in this case, we could have easier selections after molecular cloning.

To the best of our knowledge, there were few reports about application of novel strategies in molecular cloning, especially cloning carried out without gel extraction procedure (Balganesh, 1984; Chen, 2006; Busso, 2005). For example, Misra cloned an insert into one of the unique restriction enzyme sites, adjacent to the region which was complementary to a commercially available primer, in one of the Ml3 vectors. This method does not involve isolation and purification of enzymatically digested target DNA from the gels (Misra, 1985). No more details from the mentioned study were described.

The advantage of this strategy is the omission of the gel extraction following digestion that prevents severe decrease of the gene and expression vector concentration. With this approach, it is not necessary to carry out digestion in too high volumes for increasing the gene and expression vector concentration. Doing the digestion for one time will be enough in a volume just as little as 20 µl. In conclusion, the cloning procedure via the proposed strategy will lead to rapid, high efficiency and convenient transfer of the desired gene into the expression vector.

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REFERENCES

Balganesh TS, Sanford AL (1984). Plasmid vector for cloning in Streptococcus pneumoniae and strategies for enrichment for recombinant plasmids. Gene, 29: 221-230.

Busso D, Delagoutte-Busso B, Moras D (2005). Construction of a set of Gateway- based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*. Anal. Biochem. 343: 313-321.

Chen QJ, Zhou HM, Chen J, Wang XC (2006). Using a modified TA cloning method to create entry clones. Anal. Biochem. 358: 120-125.

Cregg JM, Barringer K, Hessler AY (1985). *Pichia pastoris* as a host System for transformations. Mol. Cell. Biol . 5: 3376-3385.

Crueger W, Crueger A (1990). Biotechnology: A Textbook of Industrial Microbiology. 2nd ed. Sinauer Associates Inc.

Kontermann R, dubel S (2010). Antibody Engineering. 2nd ed. 1. Springer, New York.

Loscalzo J (1988). Tissue plasminogen activator. New Eng. 319: 925-931.

Majidzadeh AK, Khalaj V, Davami F, Hemayatkar M, Barkhordari F, Adeli A, Mahboudi F (2010) a. Cloning and Expression of Functional Full-Length Human Tissue Plasminogen Activator in Pichia pastoris. Appl. Biochem. Biotechnol. 162(7): 2037-2048

Majidzadeh AK, Mahboudi F, Hemayatkar M, Davami F, Barkhordary F, Adeli A, Soleimani M, Davoudi N, Khalaj V (2010) b. Human Tissue Plasminogen Activator Expression in *Escherichia coli* using Cytoplasmic and Periplasmic Cumulative Power. Avicenna. J. Med. Biotech. 2(3): 131-136.

Misra TK (1985). A new strategy to create ordered deletions for rapid nucleotide sequencing. Gene, 34: 263-268.

Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, New York.

Soleimani M, Davudi N, Fallahian F, Mahboudi F (2006). Cloning of Tissue Plasminogen Activator cDNA in Nonpathogenic Leishmania. Yakhteh Medical J. 8: 196-203.

Soleimani M, Mahboudi F, Davoudi F, Amanzadeh A, Azizi M, Adeli A, Rastegar H, Barkhordari F, Mohajer-Maghari B (2007). Expression of human tissue plasminogen activator in the trypanosomatid protozoan Leishmania tarentolae. Biotechnol. Appl. Biochem. 48: 55-61.

Spackman E (2008). Avian influenza virus (Methods in Molecular Biology), Erica Spackman ed. 436. Springer, New York.