The TelN/tos-assisted precise targeting of chromosome segments (TAPE)

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Highlights

- We first apply the TelN/tos system to target genomic segments in E. coli host.
- We successfully cloned the targeted bacterial DNA fragment up to 156 kb.
- The TAPE method takes no more than five days to directly obtain the large DNA sequence.
- The TAPE method has no preferences on genome sequence.
- The results showed a considerable improvement of cloning efficiency.
- The TAPE method provides a powerful tool to support the study on synthetic biology.

Abstract

Introduction: Performing genomic large segmentation experiments will promote the annotation of complex genomic functions and contribute to the synthesis of designed genomes. It is challenging to obtain and manipulate large or complex DNA sequences with high efficiency.

Objectives: This study aims to develop an effective method for direct cloning of target genome sequences from different species.

Methods: The TelN/tos system and a linear plasmid vector were first used to directly clone the large genomic segments in E. coli. For the in vitro cloning reaction, two telomeric sites were developed using TelN protelomerase at the end of the linear plasmid vector. The target DNA sequence can be easily hooked with the homology arms and maintained as a linear artificial chromosome with arbitrary restriction sites in a specific E. coli strain.

Results: Using the linear cloning strategy, we successfully cloned the bacterial DNA fragment of 156 kb, a yeast genomic fragment of 124 kb and mammalian mitochondrial fragment of 16 kb. The results showed a considerable improvement in cloning efficiency and demonstrated the important role of vector ratio in the cloning process.

Conclusion: Due to the high efficiency and stability, TAPE is an effective technique for DNA cloning and fundamental molecular biotechnology method in synthetic biology.

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Introduction

Next-generation sequencing technologies help to broaden knowledge on the life of genomes [1,2]. The exponential growth of DNA sequence data facilitates the annotation of complex...
genomes, including non-coding DNA regions in a macroscopic view [3]. More novel biosynthetic gene clusters (BGCs) have also been uncovered for valuable bio-antibiotic, pharmaceutical and biofuel production [4–6]. Experts in synthetic biology need large DNA segments, even complete genomes, to study evolution or reprogramming [7–10]. To better understand the functions of genomic segments and facilitate the assembly of designed genomes, creative molecular techniques are needed to manipulate and engineer large genomes in vitro. Synthesis and cloning are the main methods to obtain target DNA sequences. However, long DNA synthesis is time-consuming as it uses yeast and bacteria hosts to assemble the designed sequence after multiple rounds [11–13]. Researchers always rely on BAC or YAC libraries to carry out studies on the functions of complete genes [14,15]. Nevertheless, it is difficult to acquire ideal genomic materials due to incomplete libraries [16]. Many cloning methods of genomic segments have emerged in recent years. However, transformation-associated recombination (TAR) is impeded by a low efficiency of chromosomal region capture [17]. For E. coli host, Cas9-Assisted Targeting of Chromosome Segments (CATCH), Cas12a-assisted precise targeted cloning using in vivo Cre-lox recombination (CAPTURE), Exonuclease Combined with RecET recombination (ExoCET) mainly focus on the excision of genome for target segments during cloning [18–20]. In addition, CAPTURE and CATCH technologies require an extra design of CRISPR/Cas system for a specific genomic segment that can clone multiple targets in one genomic library. The ExoCET takes advantage of the RecET homologous recombination’s capacity, indicating that this method is limited to some DNA fragments due to repetitive or similar sequences with the E. coli host’s genome [21].

The importance of vectors is often neglected in the cloning process. The circular plasmid is usually used to carry the cloned gene as the conventional vector, and it is widely believed that circular plasmid supercoiling produces cruciforms and other secondary structures that might cause DNA sequence deletions or rearrangements [22]. Linear plasmid can stably maintain a variety of inserts that are difficult to clone through the conventional circular plasmid, such as the skewed sequence, repetitive and AT-rich sequences that are difficult to clone through the conventional circular plasmid, even complete genomes, to study evolution or reprogramming [7–10]. To better understand the functions of genomic segments and facilitate the assembly of designed genomes, creative molecular techniques are needed to manipulate and engineer large genomes in vitro. Synthesis and cloning are the main methods to obtain target DNA sequences. However, long DNA synthesis is time-consuming as it uses yeast and bacteria hosts to assemble the designed sequence after multiple rounds [11–13]. Researchers always rely on BAC or YAC libraries to carry out studies on the functions of complete genes [14,15]. Nevertheless, it is difficult to acquire ideal genomic materials due to incomplete libraries [16]. Many cloning methods of genomic segments have emerged in recent years. However, transformation-associated recombination (TAR) is impeded by a low efficiency of chromosomal region capture [17]. For E. coli host, Cas9-Assisted Targeting of Chromosome Segments (CATCH), Cas12a-assisted precise targeted cloning using in vivo Cre-lox recombination (CAPTURE), Exonuclease Combined with RecET recombination (ExoCET) mainly focus on the excision of genome for target segments during cloning [18–20]. In addition, CAPTURE and CATCH technologies require an extra design of CRISPR/Cas system for a specific genomic segment that can clone multiple targets in one genomic library. The ExoCET takes advantage of the RecET homologous recombination’s capacity, indicating that this method is limited to some DNA fragments due to repetitive or similar sequences with the E. coli host’s genome [21].

In this study, we developed a facile cloning method named TelN/tos-assisted precise targeting of chromosome segments (TAPE). We used the TelN/tos system and flexible linear plasmid vector to target the DNA sequence from genomic libraries in vitro. A linear vector is designed and constructed with tos sequence to track the target DNA sequence in vitro, which was stably maintained with a linear chromosome in E. coli. Two primers were used with the target segments for the 40 bp homologous sequence in vectors. In a single step, we cloned the target segment of bacterial genome of up to 156 kb, a 124 kb genomic fragment containing yeast centromere and mammalian mitochondrial sequence in F9 cells from excised genomic libraries. With its stability, easy operability and high efficiency, the TAPE platform can be a practical tool in obtaining the target genome sequence without wasting time purchasing from companies or conducting lab synthesis.

Materials and methods

Materials

TelN protelomerase, β-Agarase I, SfiI, MluI, NotI and 2 × NEBuilder HiFi DNA Assembly Master Mix were purchased from NEB (Ipswich, MA, USA). The zymolysase-20 T was bought from Nacalai Tesque (Kyoto, Japan). The Low Melting Point Agarose, Micropuler and CHEF Mapper XA System were purchased from Bio-Rad (Hercules, USA), Dulbecco’s modified Eagle’s medium (DMEM) was bought from Corning (NY, USA) and the 10% fetal bovine serum was bought from BI (Kibbutz Beit-Haemek, Israel). The phenylmethyl sulphonyl fluoride (PMSF), Proteinate K, Kana- mycin, Spectinomycin and GlycoBlue™ Coprecipitant were purchased from Sangon Biotech (Shanghai, China). The Azure Biosystems C150 was bought from Azure Biosystems (Dublin, CA, USA), The bacterial genomic segments for targeted cloning were from E. coli str. MG1655. The E. coli str. GB05RedtrfA (DH10B, fluh::IS2, AybCcc. ArcrET, Pmads-ap) was obtained from the laboratory of Professor Youming Zhang of Shandong University [29]. The tos sequence (519 bp) and TelN protelomerase expression gene (1896 bp), primers were synthesized from GENEWIZ (Suzhou, China) (Supplementary Table S1 and Table S2).

Construction of the E. coli host strain and plasmid

The TelN protelomerase expression cassette was constructed with the J23100 promoter and RBS B0034, integrating in the poxB gene site (910849 to 910888) in the genome of GB05RedtrfA via CRISPR-Cas9 System [30]. The GB05RedtrfA::TelN-positive colonies were verified using PCR and sequencing, as well as analyzing the plasmid linearization after transforming the plasmid carrying the tos sequence. The pBAC-tos plasmid was constructed by adding the tos sequence to the designated site based on pBAC plasmid. The plasmid backbone was amplified from pBAC plasmid flanking 40 bp homology with the tos sequence and transformed to the E. coli str. DH5a host after cloning with the 2 × NEBuilder® HiFi DNA Assembly Master Mix. The pBAC-tos plasmid was inserted with the tos sequence between the incC and sopA elements in pBAC plasmid.

Genome digestion and isolation

The E. coli str. MG1655 was cultured overnight in 5 ml LB medium. The next morning, 1% bacteria liquid was transferred to fresh LB medium of up to OD 0.8–1.0 at a concentration of 5 × 10⁸ cells ml⁻¹. Later, 3 ml bacteria liquid was centrifuged and resuspended in 250 ul suspension buffer, then mixed and embedded in 250 ul 2% low melting point agarose gel plugs [31]. These plugs were directly treated with Proteinase K solution for two days and washed four times using 1 × wash buffer. During the third wash, 1 mM of phenylmethyl sulphonyl fluoride (PMSF) was added to inactivate the residual proteinase K in 1 × wash buffer. The plugs were first washed with 0.1 × wash buffer and 1 × cutsmart buffer for 1 h. The bacterial genomic DNA in plugs were then immersed in 600 ul 1 × cutsmart buffer for 1 h with 25 units of the restriction enzyme NotI in ice bath. After digesting in 37 °C water bath for 2 h, about a quarter of the plugs were used to analyze the quality of genomic segments through PFGE. The remaining plugs were washed with 0.1 × wash buffer for 10–30 mins and melted with 1/10 vol of 10 × β-Agarase Buffer at 65 °C for 10 min. The molten agarose plugs were cooled for 10 min in 42 °C water bath and incubated overnight at the same temperature with 6 units of β-Agarase I per 250 ul of plug. The digested genomic segments were added with 0.3 M sodium acetate and 5 ul GlycoBlueCoprecipitant for 10 min, precipitated by 4 volumes of ethanol at room temperature for 2 h and resuspended in 20 ul sterile water overnight. The genomic libraries can be stored at 4 °C for a week.

F9 cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium and supplemented with 10% fetal bovine serum. The 2 × 10⁶ F9 cells adopted the same procedures with E. coli to make low melting point agarose for constructing genomic
segments libraries, which use MluI for digestion. The genomic plugs of Saccharomyces cerevisiae BY4741 containing 1 × 10^7 cells were digested using SfiI to obtain the target segments. An additional procedure was performed to remove the yeast's cell wall. Before washing the plugs, the yeast genomic plugs were digested in 1 ml lytase solution (zymolyase-20 T, 2 mg/ml) at 37 °C for 2 h [32].

PFGE was used to verify the linear plasmid carrying the large inserts and quality of genomic segments with 1% agarose gel in 0.5 × TBE using the CHEF Mapper XA System with fixed parameters (6 V cm^-1, 1–25 s, 16–18 h, 120°) and buffer circulation at 14 °C. After PFGE, the gel was stained with ethidium bromide and the plugs of segments libraries, which use MluI for digestion, were visualized using Azure Biosystems C150.

Targeted cloning using linear plasmid vectors in vitro

In this study, a kanamycin resistance expression cassette (Kana cassette) was amplified from the pCas plasmid [30]. The complete vectors were first amplified using PCR from pBAC-tos plasmid flanking 40 bp homology with the target genomic terminal sequences introduced by primers (Supplementary Table S3). To obtain the linear plasmid vectors in vitro, three parallel tubes containing 3 ug of the recovery vectors were separately digested using 2 U TelN protelomerase. Three tubes of digestion production were collected and recovered using one recovery column to obtain a high concentration of the linear plasmid vectors. The final linear plasmid vectors and prepared genomic segments were mixed with 2 × NEBuilder HiFi DNA Assembly Master Mix at a temperature of 50 °C for 1 h in the thermocycle instrument. During the cloning process, 3 ul was taken out and mixed with 100 ul electrocompetent E. coli host cells by end-cut wide bore tips in the 2 mm cuvette at 1250 V using Biorad Micropulser (Auto Ec2 Mode). The cells were recovered at 37 °C for 2 h in 900 ul LB medium without antibiotics and plated on LB agar medium containing antibiotics. The LB agar medium for cloning the Kana cassette had a final concentration of 100 ng/ul spectinomycin and 50 ng/ul kanamycin. The colonies carrying the target segments were screened on the LB agar plate with 100 ng/ul spectinomycin and further validated by PCR covering the two intersections between vector and target genomic segments.

Results

Design and construction of TAPE cloning platform

The design of the TelN/tos-assisted precise targeting of chromosome segments (TAPE) is shown in Fig. 1. The TAPE platform used the TelN/tos system and a linear plasmid vector to improve the construction of BAC. The cloning vector constitutes two parts. One part was mainly constructed with replication elements and the other carried the partitioning protein genes. Both parts carried the homologous sequence of target genomic segments on one end while the other end was sealed with covalently closed end through TelN protelomerase, similar to two matchsticks. The target segment in the genomic libraries were cloned with homologous sequence using assembly mix in vitro. The target DNA sequence was then transformed and maintained in the E. coli host with a constitutive expression of TelN protelomerase protein.

In our experiment named pBAC-tos, the tos sequence was added to the pBAC plasmid between elements incC and sopA. To get the cloning vectors for TAPE, the vector was amplified from pBAC-tos and further digested into two parts where each end had a tos sequence with covalently closed ends through TelN protelomerase in vitro (Fig. 1A). Meanwhile, the E. coli str. GB05RedTrfA::TelN host was constructed by integrating the TelN expression cassette in the poxB gene site. The TelN protelomerase cleaved the tos sequence and maintained the plasmid as a linear chromosome in vivo when the circular or linear plasmids carrying tos sequence were transformed into the E. coli (Fig. 1B). In this cloning system, the target genomic segment and cloning vectors sharing 40 bp overlap sequences were ligated in 2 × NEBuilder HiFi DNA Assembly Master Mix, and electro-transformed into the E. coli host. The genomic libraries for different species were constructed through restriction enzyme digestion of the genome set in low melting point agarose. To reduce the tedious steps and DNA damage, extra steps were not taken to remove the background plasmid in recovery vectors or
procedures such as DNA desalt and high voltage for electrotransformation [33].

Effects of different cloning strategies for TAPE

To adopt a good vector strategy for constructing the linear plasmid with inserts, we designed and evaluated three vectors through two technical routes (Fig. 2). The first strategy used the untreated linear vector directly amplified from pBAC-tos plasmid to clone the DNA sequence, which was linearized in specific E. coli host in vivo (Fig. 2A). The second approach used the linear plasmid vector derived from the digestion of the PCR-amplified linear vector by TelN protelomerase in tos sequence site with covalent closed sequence in vitro on each end (Fig. 2B). Conventional vector control amplified from pBAC plasmid was used (Fig. 2C).

To test the cloning ability of the three vectors, a kanamycin resistance expression cassette was ligated with these vectors sharing 40 bp homology arms in the 2 × NEBuilder® HiFi DNA Assembly Master Mix. Through chemical transformation, 5 ul was taken out from the reaction mixtures separately to the E. coli strain GB05RedTrfA::TelN. PCR was used to check the colonies grown on the double selection LB plates of spectinomycin and kanamycin (Supplementary Fig. S1A-C). The positive colonies were counted and some were randomly chosen to extract plasmids for further verification using restriction enzyme. The results showed that the different strategies had contrasting effects on cloning ability (Fig. 2D). The pBAC-tos-PCR vector was amplified from pBAC-tos plasmid and had low cloning efficiency compared to the other two vectors. This suggests that damage might have decreased the cloning efficiency during the plasmid linearization by TelN protelomerase in vivo. The pBAC-PCR vector (amplified from pBAC plasmid) and pBAC-tos-TelN vector (amplified from pBAC-tos plasmid and digested using TelN protelomerase) both reported efficient cloning with the addition of 20 ng Kana cassette. The two vectors had similar cloning ability with the same number of positive colonies following the addition of 0.5 ng Kana cassette. The restriction endonuclease pattern demonstrated that the linear plasmids were cleaved and maintained in the constructed E. coli host (Supplementary Fig. S1D). Thus, cloning large DNA sequences is possible using the TelN protelomerase digested vectors based on pBAC-tos plasmid in a specific host.

Fig. 2. Efficiency of cloning Kana cassette using different vectors. (A) The pBAC-tos-PCR cloning vector was directly amplified from pBAC-tos plasmid. (B) The pBAC-tos-TelN vector for TAPE was constructed by digestion of pBAC-tos-PCR vectors using TelN protelomerase. (C) The pBAC-PCR vector was amplified from pBAC plasmid. (D) Direct cloning of Kana cassette using different vectors. Vectors with 50 ng and Kana cassette fragments of 0.5 ng and 20 ng were added into the cloning mix. The error bars indicate the standard deviations of all (n = 3) biological replicates.
We also targeted the chromosome segments using the vector control amplified from pBAC. The results showed that our method had a higher positive rate in cloning large segments compared to conventional vectors (Table 1). The vector amplified from pBAC plasmid had a low positive rate for cloning the large segments in our experiments. Through PCR validation, the negative colonies were found to be self-ligated vectors and plasmid residues (Supplementary Fig. S3). The cloning positive rate showed that it was hard to clone segments larger than 98 kb using both vectors, but the linear plasmid vector achieved more efficient cloning. The linear plasmids with 156 kb insert were successfully obtained using the TAPE method at 5.5%-11.5% positive rate. No positive colonies for 156 kb insert were obtained using the conventional vector. The cloning vectors were found to be far shorter than target genomic segments, while linear plasmid vectors came into contact with target sequence terminals more easily and ligated to the target sequence. Moreover, the conventional vector had the same size as the template plasmid, which was difficult to eliminate during the recovery of vectors with high concentration. The failed cloning using circular plasmid vectors was repaired through self-ligation in the host, further improving the negative rate. Our linear plasmid vectors were obtained from the digestion of PCR-amplified vector and contained two parts with a lower background plasmid size, which significantly decreased plasmid residue.

For targeted cloning of large gene clusters, we believe that the most valuable parameter is the success rate, which is positive correlation with the number of positive colonies. In this experiment, different variables were taken into consideration to study their relationship with the cloning success rate (Fig. 4). The x-axis was set as the ratio of genomic segments, while the y-axis represented the number of positive colonies based on PCR screening, which offers a more realistic guide to achieve cloning (Fig. 4A). According to the cloning results in target segments of 15 kb and 40 kb, the
Table 1
Effects of different vectors on cloning bacterial segments.

| Vector a | Target | Repeat | GB05redTrfA::TelN | Positive rate |
|----------|--------|--------|-------------------|---------------|
| pBAC-15 kb | E. coli-14,962 bp | Repeat 1 | 849 | 5/24 |
| pBAC-40 kb | E. coli-40,597 bp | Repeat 1 | 177 | 24/24 |
| pBAC-98 kb | E. coli-98,648 bp | Repeat 1 | 11 | 6/11 |
| pBAC-156 kb | E. coli-156,067 bp | Repeat 1 | 102 | 0/102 |
| pBAC-tos-TelN-15 kb | E. coli-14,962 bp | Repeat 1 | 177 | 18/18 |
| pBAC-tos-TelN-40 kb | E. coli-40,597 bp | Repeat 1 | 875 | 0/0 |
| pBAC-tos-TelN-98 kb | E. coli-98,648 bp | Repeat 1 | 582 | 0/24 |
| pBAC-tos-TelN-156 kb | E. coli-156,067 bp | Repeat 1 | 483 | 1/24 |

* a The pBAC-15 kb, pBAC-40 kb, pBAC-98 kb, pBAC-156 kb represented the vectors that were amplified from pBAC plasmid and targeted the bacterial segments of 14,962 bp, 40,597 bp, 98,648 bp, 156,067 bp, respectively. pBAC-tos-TelN-15 kb, pBAC-tos-TelN-40 kb, pBAC-tos-TelN-98 kb, pBAC-tos-TelN-156 kb were the linear plasmid vectors obtained from the digestion of amplified vectors based on pBAC-tos plasmid using TelN protelomerase that targeted the bacterial segments of 14,962 bp, 40,597 bp, 98,648 bp, 156,067 bp.

Discussion

With the development of molecular biology, a simple and easy method is urgently needed to overcome gene size limitations [35]. To clone large DNA sequences, we developed a facile method named TAPE to directly hook the long DNA sequence from genomic libraries. The linear plasmid vectors were mixed and ligated with genomic fragments using 2 × NEBuilder® HiFi DNA Assembly Master Mix in vitro, while the DNA fragments were cloned and maintained in E. coli with linear chromosome. We used NEBuilder® HiFi DNA Assembly Master Mix as the cloning regent, which circumvented the homologous recombination of E. coli. This is an ideal method for cloning repetitive sequences or high GC-content sequences that contribute to the synthesis of complex genomes. The linear cloning method showed an obvious improvement in efficiency and large capacity to carry and clone at least several hundred kilobase pairs. The genomic fragments from different species were successfully cloned in a linear chromosome. This means our method can clone all types of genome sequences. The cloning results in our experiments further demonstrated that the high ratio of cloning vectors increased the success rate when the genomic libraries are of high quality and at a suitable concentration. In the cloning reaction, the vector and genomic segment concentrations were the key factors that contributed to successful cloning. However, the high concentration of recovery genome was too viscous to pipette and affected ligation. Thus, the genomic libraries need to be optimized based on the characteristics of different species.
The success rate of cloning decreased when the plasmid size went beyond 98 kb, which is consistent with previous research findings [18,20]. In terms of reduced efficiency in cloning of large DNA sequences, we deduced that long DNA fragments in vitro are easily damaged and the large plasmids have low efficiency for electro-transformation. In addition, the mutual contact between linear plasmid vectors and large target segments is higher than conventional vectors, suggesting that it is easier to clone the target sequence. The unsuccessful cloning using circular plasmid vectors can be addressed by self-ligation to improve the negative rate. As a result, we managed to obtain the genomic segment of 156 kb, which is reported to be the largest DNA sequence directly cloned in E. coli host to date (Supplementary Table S4). The direct cloning segments of 124 kb and 156 kb reflect the high efficiency of our technique compared to other cloning methods [18,20,29].

Furthermore, the genomic libraries contain various segments, which is cost-efficient and takes a shorter time to clone numerous target DNA sequences from one species. The linear plasmids can be purified in low melting point agarose or extracted using plasmid kit, which can be recovered and transformed to yeast that assemble genomes in megabase pairs. Meanwhile, the bacteria host also bypasses the yeast host to clone yeast genome. This further

![Fig. 4. Cloning efficiencies of different target sequences using TAPE method. (A) Parameters in the cloning process in vitro. (B) Results of cloning the bacterial DNA segments with different lengths in E. coli str. GB05RedTrfA::TelN host. The L15, L40, L98, L156 plasmids represented the linear plasmid carrying the bacterial segments of 14,962 bp, 40,957 bp, 98,648 bp, 156,067 bp, respectively. PCR was used to validate the positive colonies.]
demonstrates that it can assemble or edit the yeast genome in E. coli. In addition, arbitrary restriction sites can be introduced in vectors using primers. This means that our method can be applied to improve the conventional construction of BAC libraries to facilitate next-generation sequencing of complex genomes. The linear plasmids also contribute to functional expression of large genomic loci and linear viral genomes in natural linear forms.

Conclusion

In conclusion, an efficient method was developed to obtain the target genomic segments using the TelN/tos system and a linear plasmid vector. The TAPE method had a considerable improvement in cloning efficiency and could clone different types of genome sequences. This method took no more than five days to directly obtain the target DNA sequence of up to hundreds of thousands of base pairs. This cloning method is useful in DNA sequencing and cloning, and the annotation of complex genomes.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

You-Zhi Cui: Methodology, Validation, Formal analysis, Investigation, Writing – original draft. Jian-Ting Zhou: Validation, Formal analysis, Investigation, Writing – review & editing, Resources. Bing-Zhi Li: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing. Ying-Jin Yuan: Conceptualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Professor Youming Zhang from Shandong University for providing the bacteria strains. This work was supported by grants from National Key Research and Development Program of China (2018YFA0900100), Tianjin Fund for Distinguished Young Scholars (19JCQJC63300) and the National Natural Science Foundation of China (21621004).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.01.017.

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Fig. 5. Targeting genomic sequences of yeast and mammalian mitochondria. (A) Genome location and cleavage sites for target segments of yeast and mammalian mitochondria. The genome of Saccharomyces cerevisiae BY4741 was digested by SbfI enzyme, while a fragment of 39,945 bp in chromosome I and a fragment of 124,641 bp in chromosome XV were tested as the target DNA sequences. Mitochondria in F9 cells was also selected as the cloning target by digesting the genome using MluI enzyme. (B) Verification of the positive colonies through PFGE with bacterial plugs. XLT16: the linear plasmid with 16 kb mitochondria DNA sequence from F9 cells. Y40 and Y124 were the linear plasmids carrying 39,945 bp, 124,641 bp genomic segments of yeast, respectively.
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