Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Journal Name

RSCPublishing

ARTICLE

Comparison of the methods for generating single-stranded DNA in SELEX

Cite this: DOI: 10.1039/x0xx00000x

Chao Liang,[#a-h] Defang Li, [#a,d-h] Guangxian Zhang, [#a] Hui Li,[i] Ningsheng Shao,[i] Zicai Liang,[d] Lingqiang Zhang,[*c] Aiping Lu,[*a,b,d-h] and Ge Zhang[*a,d-h]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

The generation of single-stranded DNA (ssDNA) from double-stranded PCR products is an essential step in selection of aptamers by systematic evolution of ligands by exponential enrichment (SELEX). Magnetic separation with streptavidin-coated beads is always the most commonly used method. Recently, two size separation methods derived from unequal primers with chemical or structural modification are raised in SELEX. In this report, we made a comparison between the magnetic separation and the two size separation methods in generation of ssDNA from double-stranded PCR products. Our results showed that all the methods produced good purity of ssDNA. Compared to the magnetic separation, size separation derived from unequal primers with chemical modification achieved an almost equivalent recovery rate of ssDNA, whereas size separation derived from unequal primers with structural modification showed a lower recovery rate of ssDNA. Considering the low cost, size separation derived from unequal primers with chemical modification could be a satisfactory alternative to the classic magnetic separation for generation of ssDNA in SELEX.

Introduction

Aptamers are ssDNA or RNA oligonucleotides, generally < 100-mer[1]. They can bind with high affinity and specificity to a wide range of targets, including peptides, proteins, drugs and even whole living cells[2-5]. Different with the conventional view on nucleic acids as carriers of genetic information, aptamers always function based on the specific three-dimensional structures and bind to targets with dissociation constants in the nanomolar to picomolar range, which is comparable and sometimes even superior to those of monoclonal antibodies[6]. Due to the nature of nucleic acids, aptamers also show low toxicity, increased stability, ease of regeneration and simple modification, making them as potential tools in disease diagnosis, drug development, targeted therapy and biosensing[7].

Aptamers are usually generated by an in vitro iterative selection and amplification technology called systematic evolution of ligands by exponential enrichment (SELEX)[8, 9]. From an ssDNA or RNA library composing a large sequence diversity and structural complexity, only those of oligonucleotides are selected and enriched, which can bind very tightly to the specific targets during multiple SELEX rounds[10-12]. Basic steps include the binding reaction between oligonucleotides and targets, washing steps to remove unbound oligonucleotides, enzymatic amplification of target-bound oligonucleotides and purification of the enriched oligonucleotide pools for the next round of selection10. Though RNA and ssDNA aptamers have similar binding affinity towards the targets, ssDNA is more stable than RNA owing to the absence of 2'-OH group, and requires only a single enzymatic step in comparison to the three-enzymatic steps of RNA (transcription, reverse transcription, and PCR amplification)[13]. Thus, ssDNA oligonucleotides are more suitable for aptamer selection and show greater potential in the subsequent clinical trial and other in vivo application[14].

During the SELEX process, an important and critical step is the generation of ssDNA from double-stranded PCR products. To date, several methods have been described, including asymmetric PCR[15], lambda exonuclease digestion[16], magnetic separation with streptavidin-coated beads[17] and two newly raised size separation derived from unequal primers with chemical or structural modification by denaturing urea-polyacrylamide gel[18, 19]. For asymmetric PCR, unequal molar ratio of forward and reverse primers is used in PCR reaction. The lower concentration primer is incorporated into dsDNA and the higher concentration primer is used to synthesize an excess of ssDNA in each cycle. However, asymmetric PCR products comprise not only single but also double stranded DNA (dsDNA), which may reduce the diversity of ssDNA in the enriched oligonucleotide pools[20]. For lambda exonuclease digestion, a 5´-phosphate group is introduced into one strand of dsDNA by performing PCR and the phosphorylated strand is then removed by digestion with lambda exonuclease. Nevertheless, incomplete digestion of the phosphorylated PCR products may also leads to the contamination of the dsDNA in reaction mixture[13]. Currently, the most commonly used method for isolation of ssDNA from dsDNA is magnetic separation with streptavidin-coated beads. Biotinylated PCR products are immobilized onto streptavidin-coated beads and the desired ssDNA is rapidly separated from biotinylated strand by alkaline denaturation[21]. Besides the methods described

above, another two promising methods based on size separation derived from unequal primers with chemical or structural modification have been raised in SELEX and attracts more and more attentions. With the introduction of a chemical terminator[18] or a GC-rich stem-loop structure[19] at the 5'-end of the reverse primer, unequal strands of DNA could be created and subsequently separated on denaturing urea-polyacrylamide gel.

In this study, we determined the recovery rates and purity of ssDNA generated by magnetic separation with streptavidin-coated beads and two size separation methods derived from unequal primer with chemical or structural modification. The results demonstrated that size separation derived from unequal primer with chemical modification could be an alternative method to the classic magnetic separation for generation of ssDNA in SELEX.

Experimental

Chemical reagents

All chemical reagents were purchased from Sigma (The Woodland, TX) unless otherwise stated. Taq polymerase (5 U/μl) and dNTPs (2.5 mM) were obtained from Takara (Dalian, China). Streptavidin MagneSphere® Paramagnetic Particles, Agarose MS-6 and 50 bp DNA ladder were from Promega (Madison, WI). The SYBR® Safe dye was from Life Technologies.

ssDNA library and primers

An ssDNA library comprising a randomized region of 40 nucleotides (N) in the center flanked by fixed primer hybridization regions, forward primers and reverse primers[22] were synthesized in Sangon Biotech (Shanghai, China) and shown as follows. ssDNA Library: 5'-GCAATGGTACGGTACTTCC-40(N)-CAAAAGTGCACGCTACTTTGCTAA-3'. Forward primer: 5' -GCAATGGTACGGTACTTCC-3'. Reverse biotin-labeled primer: 5'-biotin-TTAGCAAAGTAGCGTGCACTTTTG-3'. Reverse primer comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20): 5'-AAAAAAAAAAAAAAAAAAAA-C18-TTAGCAAAGTAGCGTGCACTTTTG-3'. Reverse primer containing a GC-rich stem-loop structure at its 5'-end: 5'-GCTAAGCGGGTGGGACTTCCTAGTCCCACCCGCTTAGCAA AGTAGCGTGCACTTTTG-3'.

PCR reaction

100 ng of random ssDNA libraries were used as starting templates. 250 pM forward and reverse primers, 0.2 mM deoxynucleotide triphosphate (dNTPs) and 5 units of MyTaq HS DNA polymerase were added in per reaction. The volume of the PCR reaction was 100 μl. The thermocycling program comprised following basic steps, including the initial DNA denaturation step at 95 ℃ for 150 s, 20 cycles of DNA denaturation at 95 ℃ for 30 s, primer annealing and DNA polymerization at the appropriate temperature for 30 s. Final amplification at 72 ℃ for 180 s was performed in order to complete the polymerization process. For magnetic separation with streptavidin-coated beads or size separation derived from the unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20), gradient PCR was run at a series of annealing temperatures (52, 54, 56, 58, 60 and 62 ℃) and polymerization temperature at 72 ℃. For size separation derived from the unequal primers containing a GC-rich stem-loop structure, gradient PCR was run at annealing temperatures at 40 ℃ and a series of polymerization temperatures (45, 50, 55, 60, 65 and 70 ℃).

Agarose gel electrophoresis

The double stranded PCR products or ssDNA were examined by 2% agarose gel electrophoresis for comparison. For each method, 10 μl of the samples were added to the gel and the electrophoresis was carried out at 140 V for 30 min.

Magnetic separation with streptavidin-coated beads

After the PCR reaction, biotin-labeled dsDNA products were concentrated and ultra-filtered by an Amicon Ultra-15 centrifugal filter device with a membrane NMWL of 10 kDa (UFC901096, Merck KGaA, Darmstadt, Germany). The strand separation was performed with Streptavidin MagneSphere® Paramagnetic Particles. Before the incubation, the beads were pre-conditioned with 200 mM NaOH for 5 min and washed three times with PBS to remove the dissociated streptavidin. The purified PCR products were then incubated with the appropriate amounts of the pre-conditioned beads on a rotator for 2 h at room temperature. After the incubation, the beads with the immobilized biotin-labeled dsDNA were separated by a magnet stand and washed three times with PBS. An alkaline denaturation was performed with 100 μl freshly prepared 200 mM NaOH for 5 min. Elutes was neutralized by titration with 200 mM HCl and centrifuged to remove the beads that remain in the elute since the last magnetization step is not strong enough, the ssDNA was concentrated by ethanol precipitation.

Separation of ssDNA by size separation derived from unequal primers

For size separation derived from unequal primers (a reverse primer comprised of an 18-carbon ethylene glycol spacer as an terminator and an extension of PolyA (20) or a reverse primer with a GC-rich stem-loop structure in conjunction with unmodified forward primer), 10 μl PCR products with unequal strands were analyzed by electrophoresis in a 15 % polyacrylamide-8 M urea gel and stained with SYBR® Safe dye in 1 x TBE buffer for 20 min. The band of interest was purified and extracted from the gel for the next round of selection. Following the addition of elution buffer [0.5 M ammonium acetate, 0.2% sodium dodecyl sulfate (SDS), 1 M EDTA (pH 8.0)], ssDNA was recovered from the solution by ethanol precipitation (3 M sodium acetate, 1 M $MgCl_2$ in 100% ethanol) and allowed to settle for 24 h at -20 ℃. The resulting sample was centrifuged, and the pellet was rinsed twice with 70% ethanol and allowed to dry.

Statistical Analysis

All the variables were expressed as mean ± standard deviation. Student's t-test was conducted to analyze the recovery rate of ssDNA, which was calculated by the amounts of separated ssDNA divided by the half amounts of dsDNA products. A statistical software SPSS (version 22.0) was used and $P<0.05$ was considered to be statistically significant.

Results and Discussion

Technological process for generation of ssDNA

For magnetic separation with streptavidin-coated beads, a biotin-labeled reverse primer is used in conjunction with unmodified forward primer in PCR amplification and the biotinylated dsDNA products are concentrated and ultra-filtered to remove the excessive primers and salt ions. Tetrameric streptavidin is covalently attached to the bead's surface, whereas monomeric streptavidin is non-covalently attached to the bead's surface. It has been reported that the non-covalent-bonded streptavidin could be dissociated from the

bead's surface by alkaline treatment and then cause the re-annealing between the biotinylated strand and the desired ssDNA strand[23]. According to previously reported solution[21], we pre-conditioned the beads with alkaline and then incubated them with the concentrated PCR products. Following the attachment of the biotinylated PCR products onto the pre-conditioned streptavidin-coated beads, separation of desired ssDNA is conducted by the second alkaline denaturation (**Fig. 1a**). For size separation derived from unequal primers with chemical modification, an 18-carbon ethylene glycol spacer is inserted at the 5'-end of the reverse primer as a terminator to impede the elongation of sense strand and an extension of 20-nucleotides length of a string of adenosine (PolyA (20) is designed to create a size difference of the amplicon strands (**Fig. 1b**). For size separation derived from unequal primers with structural modification, a GC-rich stem-loop region is constructed at the 5'-end of the reverse primer, which is supposed to keep the advanced structure during the PCR reaction owning to its high melting temperature and thus create a size difference between the two strands of dsDNA products (**Fig. 1c**). After the PCR reaction, the resulting strands of different sizes are separated on denaturing urea-polyacrylamide gel electrophoresis and the desired ssDNA is purified from the gel (**Fig. 1b and 1c**).

Optimization of PCR condition

For magnetic separation with streptavidin-coated beads or size separation derived from the unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20), optimization of annealing temperature was conducted for the generation of optimum yield of dsDNA products. With 100 ng of ssDNA library containing 83 oligonucleotides as template, gradient PCR was run at annealing temperature ranging from 52 ℃ to 62 ℃ and polymerization temperature at 72 ℃ in 100 ℃ reaction systems. After 20 cycles, agarose gel electrophoresis showed that annealing temperature at 60 ℃ (Lane 11) produced the maximum double-stranded products at the correct size (less than 100 bp) when using the biotin-labeled primer as the reverse primer (**Fig. 2a**). With the primer comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) as the reverse primer, the best amplification was also obtained at the annealing temperature of 60 ℃ (Lane 11) (**Fig. 2b**). For size separation derived from unequal primers containing a GC-rich stem-loop structure, optimization of polymerization temperature was conducted for the generation of optimum yield of dsDNA products. With 100 ng of ssDNA library containing 83 oligonucleotides as template, gradient PCR was run at annealing temperature of 40 ℃ and polymerization temperature ranging from 45 ℃ to 70 ℃. Surprisingly, our results showed that two dsDNA bands were detected at each temperature after 20 cycles of amplification. The lower band showed the correct molecular weight of less than 100 bp and the strongest intensity at 45 ℃ (Lane 3). The higher band showed an unexpected molecular weight of around 120 bp and the strongest intensity at 70 ℃ (Lane 13). Furthermore, with the increase of polymerization temperature from 45 ℃ to 70 ℃, the intensity of the lower band gradually decreased, whereas the intensity of the higher band gradually increased (**Fig. 2c**). These results implied that the advanced structure of GC-rich stem-loop region might be partly disrupted and the sense strand continued to be elongated to generate a portion of equal strands of DNA (the higher band) by Taq DNA polymerase, as the increased polymerization temperature approached the melting temperature of GC-rich stem-loop region.

Purity of ssDNA after the separation

After generation of ssDNA by magnetic separation with streptavidin-coated beads in combination with alkaline pre-condition, agarose gel electrophoresis showed a unique ssDNA band at the correct molecular weight (Lane 5). However, without the alkaline pre-condition, there were two bands appeared on agarase gel, including a lower band at the correct molecular weight of ssDNA (less than 50 bp) and a higher band at a molecular weight of dsDNA (less than 100 bp) (Lane 4) (**Fig. 3a**). These results demonstrated that the introduction of pre-condition might remove the unstable interaction between the streptavidin and the beads and eliminated the re-annealing between the biotinylated strand and the desired ssDNA strand. After generation of ssDNA by size separation derived from unequal primers, including a reverse primer comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) and a reverse primer containing a GC-rich stem-loop structure, results from denaturing urea-polyacrylamide gel showed that the desired ssDNA bands at the correct molecular weight (less than 100 nt) and the complementary ssDNA with a larger molecular weight (greater than 100 nt) were clearly distinguishable (Lane 3 and 4), which enabled selective excision of the lower desired strand with the aid of UV shadowing (**Fig. 3b**).

Recovery rate of ssDNA

After generation of ssDNA from dsDNA products by magnetic separation with streptavidin-coated beads and size separation derived from unequal primers, respectively, we compared the recovery rate of ssDNA. PCR reaction was conducted with the same amount of ssDNA template (100 ng), primers (250 pM) and other components (DNase-free water, dNTP and Taq DNA polymerase). Quantitative data from spectrophotometer analysis showed that size separation derived from unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) produced an almost equivalent recovery rate of ssDNA when compared to magnetic separation with streptavidin-coated beads (**Fig. 4**). However, size separation derived from unequal primers containing a GC-rich stem-loop structure generated a significantly lower recovery rate of ssDNA ($P < 0.05$) (**Fig. 4**). This could be explained by the occurrence that PCR reaction with structural modified unequal primer generated not only desired unequal strands of the products but also undesired equal strands of the products with a larger molecular weight, as the stem-loop structure might be partly disrupted at high polymerization temperatures, thus leading to the waste of PCR components, inefficient PCR amplification and low recovery rate for ssDNA. In summary, considering the low cost for large scale application, size separation derived from unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) could be a satisfactory alternative to magnetic separation with streptavidin-coated beads for generation of ssDNA from dsDNA products.

Conclusions

In Summary, size separation derived from unequal primers with chemical modification achieved an almost equivalent recovery rate and purity of ssDNA when compared to the magnetic separation. Considering the low cost for large scale application, it could be a satisfactory alternative to magnetic separation with streptavidin-coated beads for generation of ssDNA from dsDNA products.

Acknowledgements

We thank the technical staff (Cheung Yeuk Siu) from Institute for Advancing Translational Medicine in Bone & Joint

Diseases, Hong Kong Baptist University for providing technical support. This study was supported by the Ministry of Science and Technology of China (2013ZX09301307), the Hong Kong General Research Fund (HKBU479111, HKBU478312, HKBU12102914 and HKBU261113), the Natural Science Foundation Council (81228013), the Research Grants Council & Natural Science Foundation Council (N_HKBU435/12), the Interdisciplinary Research Matching Scheme (IRMS) of Hong Kong Baptist University (RC-IRMS/12-13/02 and RC-IRMS/13-14/02), the Hong Kong Baptist University Strategic Development Fund (SDF) (SDF13-1209-P01), the Hong Kong Research Grants Council (RGC) Early Career Scheme (ECS) (489213) and the China Academy of Chinese Medical Sciences (Z0252 and Z0293).

Notes

[#] Co-First Authors

[*] Corresponding authors: Ge Zhang (zhangge@hkbu.edu.hk), Aiping Lu (aipinglu@hkbu.edu.hk) and Lingqiang Zhang. (zhanglq@nic.bmi.ac.cn)

[a] Institute for Advancing Translational Medicine in Bone & Joint Diseases, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China.

[b] Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing, China.

[c] State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing, China.

[d] Academician Chen Xinzi Workroom for Advancing Translational Medicine in Bone & Joint Diseases, Kunshan RNAi Institute, Kunshan Industrial Technology Research Institute, Kunshan, Jiangsu, China.

[e] Institute of Integrated Bioinfomedicine & Translational Science, Hong Kong Baptist University Shenzhen Research Institute and Continuing Education, Shenzhen, China.

[f] Shum Yiu Foon Shum Bik Chuen Memorial Centre for Cancer and Inflammation Research, Hong Kong Baptist University Shenzhen Research Institute and Continuing Education, Shenzhen, China.

[g] Hong Kong Baptist University Branch of State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University, Hong Kong SAR, China.

[h] Hong Kong Baptist University–Northwestern Polytechnical University Joint Research Centre for Translational Medicine on Musculoskeletal Health in Space, Shenzhen, China.

[i] Department of Biochemistry and Molecular Biology, Beijing Institute of Basic Medical Science, Beijing, China.

References

1. G. R. Mazars and C. Theillet, *Methods Mol Biol*, 2003. **226**: p. 355-60.
2. B. J. Hicke, C. Marion, Y. F. Chang, T. Gould, C. K. Lynott, D. Parma, P. G. Schmidt, and S. Warren, *J Biol Chem*, 2001. **276**(52): p. 48644-54.
3. H. Ulrich, M. H. Magdesian, M. J. Alves, and W. Colli, *J Biol Chem*, 2002. **277**(23): p. 20756-62.
4. L. Cerchia, F. Duconge, C. Pestourie, J. Boulay, Y. Aissouni, K. Gombert, B. Tavitian, V. de Franciscis, and D. Libri, *PLoS Biol*, 2005. **3**(4): p. e123.
5. K. Sefah, D. Shangguan, X. Xiong, M. B. O'Donoghue, and W. Tan, *Nat Protoc*, 2010. **5**(6): p. 1169-85.
6. S. M. Nimjee, C. P. Rusconi, and B. A. Sullenger, *Annu Rev Med*, 2005. **56**: p. 555-83.
7. K. M. Song, S. Lee, and C. Ban, *Sensors (Basel)*, 2012. **12**(1): p. 612-31.
8. A. D. Ellington and J. W. Szostak, *Nature*, 1990. **346**(6287): p. 818-22.
9. C. Tuerk and L. Gold, *Science*, 1990. **249**(4968): p. 505-10.
10. R. Stoltenburg, N. Nikolaus, and B. Strehlitz, *J Anal Methods Chem*, 2012. **2012**: p. 415697.
11. G. Aquino-Jarquin and J. D. Toscano-Garibay, *Int J Mol Sci*, 2011. **12**(12): p. 9155-71.
12. R. Stoltenburg, C. Reinemann, and B. Strehlitz, *Biomol Eng*, 2007. **24**(4): p. 381-403.
13. C. Marimuthu, T. H. Tang, J. Tominaga, S. C. Tan, and S. C. Gopinath, *Analyst*, 2012. **137**(6): p. 1307-15.
14. A. P. Null, J. C. Hannis, and D. C. Muddiman, *Analyst*, 2000. **125**(4): p. 619-26.
15. U. B. Gyllensten and H. A. Erlich, *Proc Natl Acad Sci U S A*, 1988. **85**(20): p. 7652-6.
16. R. G. Higuchi and H. Ochman, *Nucleic Acids Res*, 1989. **17**(14): p. 5865.
17. T. Hultman, S. Stahl, E. Hornes, and M. Uhlen, *Nucleic Acids Res*, 1989. **17**(13): p. 4937-46.
18. K. P. Williams and D. P. Bartel, *Nucleic Acids Res*, 1995. **23**(20): p. 4220-1.
19. X. Cao, S. Li, L. Chen, H. Ding, H. Xu, Y. Huang, J. Li, N. Liu, W. Cao, Y. Zhu, B. Shen, and N. Shao, *Nucleic Acids Res*, 2009. **37**(14): p. 4621-8.
20. M. Avci-Adali, A. Paul, N. Wilhelm, G. Ziemer, and H. P. Wendel, *Molecules*, 2010. **15**(1): p. 1-11.
21. R. Wilson, *Nucleic Acid Ther*, 2011. **21**(6): p. 437-40.
22. Y. Tan, Y. S. Shi, X. D. Wu, H. Y. Liang, Y. B. Gao, S. J. Li, X. M. Zhang, F. Wang, and T. M. Gao, *Acta Pharmacol Sin*, 2013. **34**(12): p. 1491-8.
23. A. Paul, M. Avci-Adali, G. Ziemer, and H. P. Wendel, *Oligonucleotides*, 2009. **19**(3): p. 243-54.

Figure Legends

Fig. 1 Schematic illustration of ssDNA seperation. (a) Following the attachment of the biotinylated PCR product onto streptavidin, treatment with NaOH breaks the hydrogen bonds between the strands, releasing the unmodified ssDNA strand. **(b, c)** The inclusion of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) **(b)** or GC-rich stem-loop structure **(c)** in the primers creates a size difference of the two strands of the PCR products, which migrate at different rates upon resolving on the denaturing urea-polyacrylamide gel.

Fig. 2 Agarose gel electrophoresis of dsDNA products after PCR reaction. (a) PCR products generated by an unmodified forward primer and an biotin-labeled reverse primer at a series of annealing temperatures (52, 54, 56, 58, 60 and 62 ℃). Lane 1: 50 bp DNA ladder; Lane 2, 4, 6, 8, 10 and 12: Negative control with no template DNA in PCR reaction systems at the above annealing temperatures; Lane 3, 5, 7, 9, 11 and 13: dsDNA products generated by PCR reaction at the above annealing temperatures. **(b)** PCR products generated by an unmodified forward primer and a reverse primer comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20) at a series of annealing temperatures (52, 54, 56, 58, 60 and 62 ℃). Lane 1: 50 bp DNA ladder; Lane 2, 4, 6, 8, 10 and 12: Negative control with no template DNA in PCR reaction systems at the above annealing temperatures; Lane 3, 5, 7, 9, 11 and 13: dsDNA products generated by PCR reaction at the above annealing temperatures. **(c)** PCR products generated by an unmodified forward primer and a reverse primer containing a GC-rich stem-loop structure at a series of polymerization temperatures (45, 50, 55, 60, 65 and 70 ℃). Lane 1: 50 bp DNA ladder; Lane 2, 4, 6, 8, 10 and 12: Negative control with no template DNA in PCR reaction systems at the above polymerization temperature; Lane 3, 5, 7, 9, 11 and 13: dsDNA products generated by PCR reaction at the above polymerization temperatures. The arrow indicated the DNA marker with a molecular weight of 100 bp.

Fig. 3 Purity of ssDNA after the separation. (a) Agarose electrophoresis showed the ssDNA generated by magnetic separation with streptavidin-coated beads. Lane 1: 50 bp DNA ladder; Lane 2: ssDNA library; Lane 3: dsDNA PCR products; Lane 4: ssDNA generated by magnetic separation without the alkaline pre-condition; Lane 5: ssDNA generated by magnetic separation with the introduction of alkaline pre-condition. The black arrow indicated the DNA marker with a molecular weight of 50 bp. **(b)** Urea denaturing-PAGE showed the generation of ssDNA by size separation derived from unequal primers. Lane 1: DNA ladder; Lane 2: ssDNA library; Lane 3: separation of the unequal DNA strands generated by unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20); Lane 4: Separation of the unequal DNA strands generated by unequal primers containing a GC-rich stem-loop structure; Lane 5: DNA ladder. The black arrow indicated the DNA marker with a molecular weight of 100 nt.

Fig. 4 Recovery rate of ssDNA. The recovery rate of ssDNA was expressed by the amounts of separated ssDNA divided by the half amounts of dsDNA products. Magnetic: magnetic separation with streptavidin-coated beads; Chemical: size separation derived from unequal primers comprised of an 18-carbon ethylene glycol spacer and an extension of PolyA (20); Structural: size separation derived from unequal primers containing a GC-rich stem-loop structure. The experiments were repeated three time (n = 3). Student's t-test was conducted to analyze the recovery rate of ssDNA. The data were presented as the means ± standard deviation. * $P < 0.05$.

Fig. 1

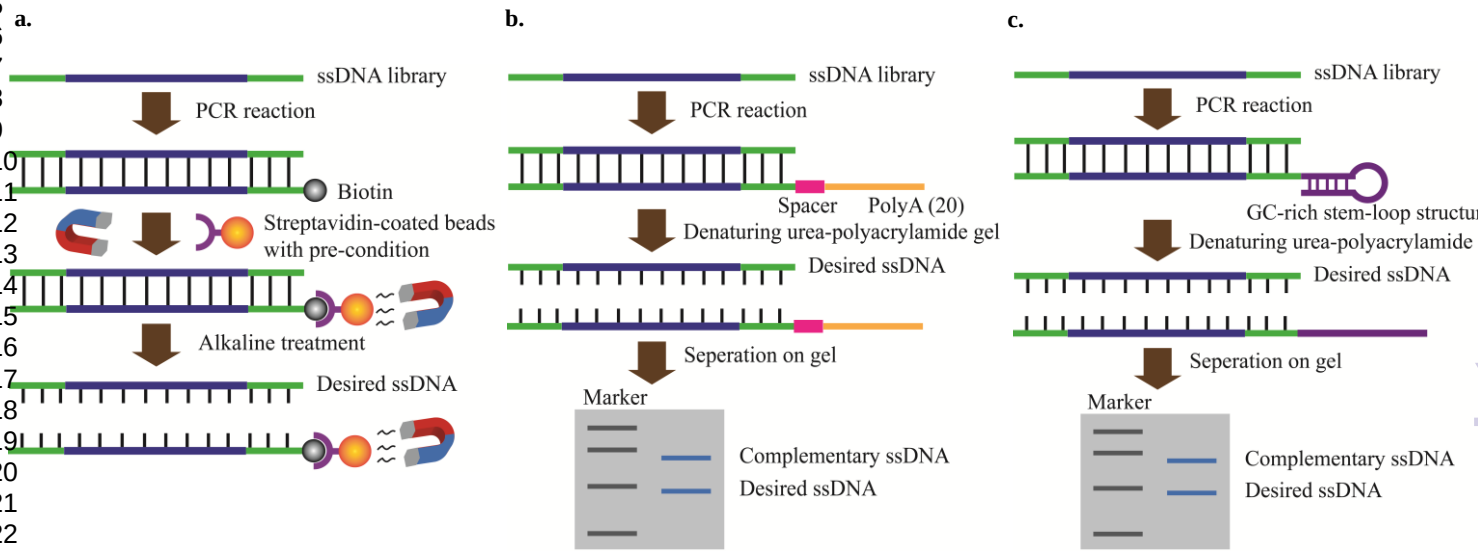


Fig. 2

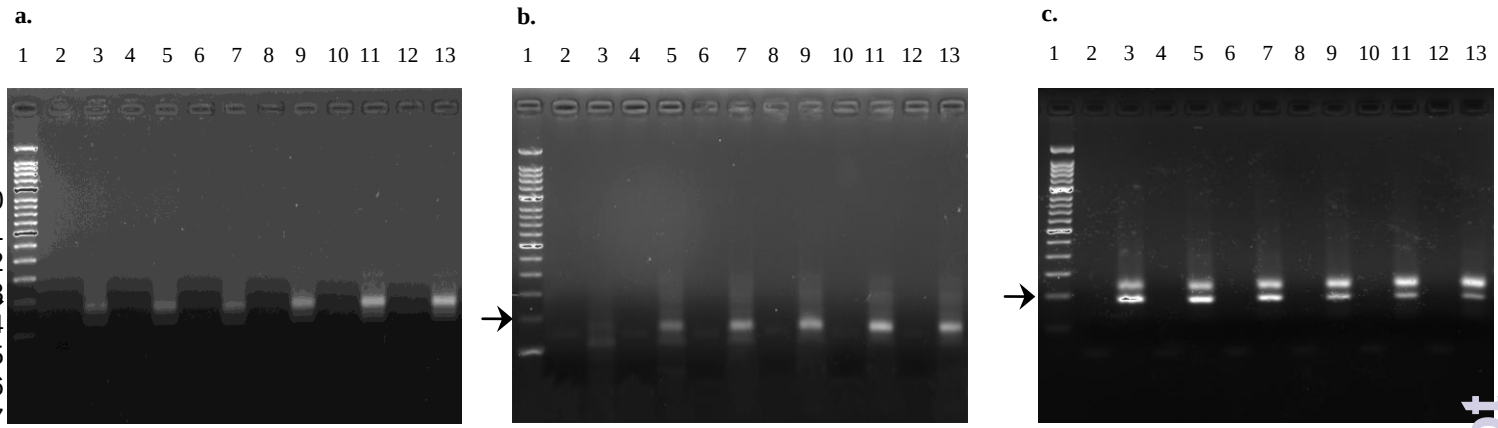
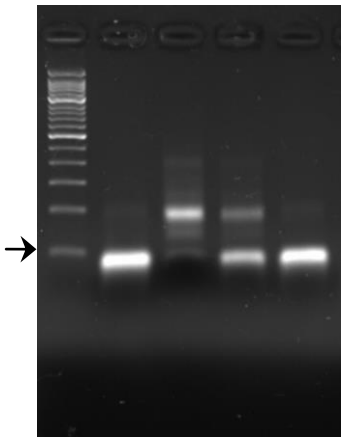


Fig. 3

a.



b.

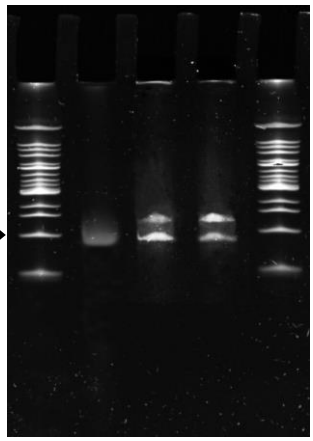
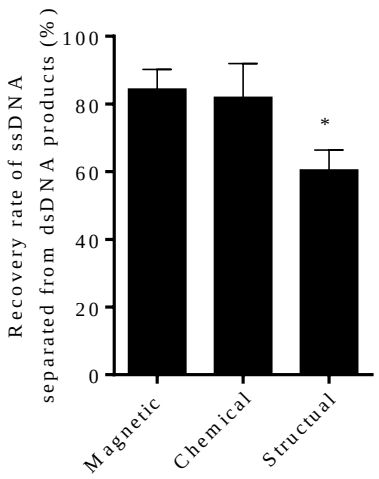
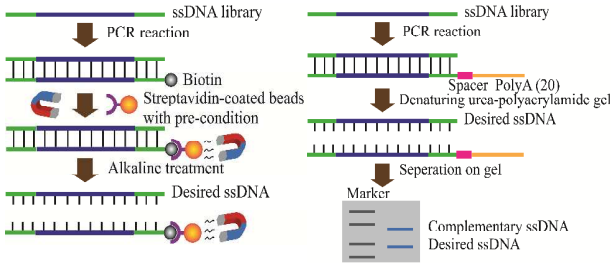


Fig. 4





Size separation derived from unequal primers with chemical modification (right) achieved an almost equivalent recovery rate as magnetic separation (left).