Strategies to prevent self-ligation of adapters used in enzyme mediated methylation sequencing

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Abstract

In our recent effort of developing methylation-dependent endonuclease mediated methylation sequencing technology, efficiency and reproducibility were restrained by adapters' self-ligation. In the present study, three strategies to prevent self-ligation of adapters used in enzyme mediated methylation sequencing have been developed. Our data have demonstrated that these strategies can either inhibit or eliminate the adapters' self-ligation. These strategies are crucial in enzyme-mediated methylation sequencing and may be useful in some other genomic sequencing technologies.

Biotech Methods

Strategies to prevent self-ligation of adapters used in enzyme mediated methylation sequencing

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Abstract In our recent effort of developing methylation-dependent endonuclease mediated methylation sequencing technology, efficiency and reproducibility were restrained by adapters' self-ligation. In the present study, three strategies to prevent self-ligation of adapters used in enzyme mediated methylation sequencing have been developed. Our data have demonstrated that these strategies can either inhibit or eliminate the adapters' self-ligation. These strategies are crucial in enzyme-mediated methylation sequencing and may be useful in some other genomic sequencing technologies.

Introduction

More than ten types of methylation assays have been developed for epigenetic study[1-5]. Predominantly, sodium bisulfite is used in genomic methylation sequencing[6, 7]. In either regular next generation sequencing or sodium bisulfite-mediated methylation sequencing, adapters with a 3' extruded T were widely used in library preparation with A-tailing target inserts. These adapters need to be removed before sequencing, which is easily accomplished based on size difference between adapters with and without inserts.

To eliminate the destruction of target DNA by sodium bisulfite [8], methylation-dependent endonuclease was chosen in our effort of developing sulfite-free methylation sequencing technology. We noticed that the efficiency and reproducibility of methylation-dependent endonuclease mediated methylation sequencing were restrained by self-ligation of adapters[9, 10]. Although adapters with 3' extruded T are still usable as the adapters ligated with inserts containing methylated CpG site, all other methylation site-independent adapters to be used can be self-ligated. Furthermore, methylation site-dependent adapters also can be self-ligated. These self-ligation of adapters has two potential shortages: to decrease the efficiency and reproducibility of methylation sequencing, and to substantially increase the cost[11]. When applied to single cell methylation sequencing in which the ratio of adapters to inserts is usually as high as 200, sequencing data will probably exclusively be consisted of the adapters from the self-ligation products. The latter is due to the sequencing chip competitively occupied by self-ligated adapters without insert. Different from conventional next generation sequencing, it is nearly impossible to physically separate self-ligated adapters and adapter-insert products as their similar sizes.

In addition to adapters with 3' extruded T, two more methylation site-independent adapters and one methylation site-dependent adapter are designed to the new methylation sequencing technology. These three types of adapters all can be self-ligated. The present study tested strategies to prevent their self-ligation and our data documented that their self-ligation can be either decreased or eliminated.

Materials and methods

2.1 Chemically synthesized templates and adapters

Nine oligonucleotides of the designed templates and adapters were synthesized from Sangon Biotech (Shanghai, China) as listed in Table 1. Oligonucleotides 1 and 2 are the artificially synthesized templates, which were based on the SDC2 gene with addition of two restriction cutting sites of HpaII (5'-CCGG-3') (New England Biolabs (Beijing) LTD)and MseI (5'-TTAA-3')(New England Biolabs (Beijing) LTD). The rest are the five double chain adapters to be tested. There are two methylation site-independent adapters with end of CGat (No.3) and TAat (No.4) respectively and their complementary oligonucleotides. The three methylation site-dependent adapters were with YNNY, RNNR, and NNNN in their 5' termini.

Each of oligonucleotides No. 3 and 4 is complementary paired with oligonucleotides No. 5 to form a double-chain with 2 bases protruding at their 5' ends (CGat and TAat). These two adapters were named as CGat adapter and TAat adapter. The oligos of No.6, 7 and 8 are respectively complimentary paired with the oligos No.9 to form a double-stranded adapters with 4 base protrusions at their 5' end with YNNY, RNNR and NNNN, named as YNNY, RNNR and NNNN adapters. Oligos were added to 1X annealing buffer (Beijing Solarbio Science & Technology Co., Ltd.) to gradually anneal the double-stranded templates and adapters with the following procedures: 95 °C 10 min, 85 °C 10 min, 75 °C 10 min, 65 °C 10 min, 55 °C 10 min, 45 °C 10 min, 35 °C 10 min, 25 °C 10 min, 15 °C 10 min ,and stored at 4 degC for use.

2.2. Strategies to prevent adapters' self-ligation

2.2.1 Prevention of CGat adapters' self-ligation

The self-ligation of CGAT adapters were tested in two groups, the adapters with T4 ligase alone, and adapters with the combination of T4 ligase and ClaI enzymes. The T4 DNA ligase group contains 200 ng of CGat adapters, 5 μL of 10X CutSmart buffer , ATP (10 μM) 5 μL, T4 DNA Ligase (New England Biolabs (Beijing) LTD)1 μL(400uints) (last added) in a 50 μL reaction system. The components in ClaI group is the same as the T4 ligase control group except for the addition of Cla I restriction enzyme(New England Biolabs (Beijing) LTD) 2.5 μL (25uints). The reactions were performed overnight in a 37-degree water bath. After the reaction completed, 5 μL of the reaction products was visualized by electrophoresis under a 2.5% agarose gel under 100 V 30 min.

The ClaI was further tested in promoting the formation of CGat adapters and insert ligation. As tabulated in Table 2, the effect of ClaI was evaluated in the groups 4 and 5, one without and one with ClaI. The restriction enzyme HpaII was used to digest the artificial template and added in groups 2, 4, and 5.

The ligation product of the insert and adapters was then used as template for PCR amplification. The primer sequence is: 5-ccatccagcatccaactcggtgggggtggggat-3,5-gtcagctccacgcatacattacg-3. PCR reaction system: 2× taq Master Mix (Vazyme Biotech Co.,Ltd)10 μL, junction primer 1 μL, destination fragment primer 1 μL, ligation product 3 μL, replenish water to a total volume of 20 μL. PCR was set with 95 °C 2min 1cycle, 95 10s,58 20s,72 30s,20 cycle, and a final cycle at 72 1min. The PCR product was sent for Sanger's sequencing.

2.2.2 Prevention of TAat adapters' self-ligation

The self-ligation of TAAT adapters was tested with restriction enzyme AseI(New England Biolabs (Beijing) LTD). This experiment had two groups: adapters without and with AseI. For T4 DNA ligase group, 200 ng of TAat adapters, 5 μL 10X T4 DNA ligase buffer, and 1 μL of T4 DNA ligase (400 uints) were added to a total of 50 μL reaction system. The composition of the AseI group was the same as that of the T4 ligase control group, except that 2.5 μL (25uints) of AseI restriction endonuclease was added. The reaction was carried out overnight in a 37°water bath. After the reaction was complete, 5 μL of the reaction products were electrophoresed for each group.

The combination of MseI and AseI was tested in promoting the ligation of insert and adapters in addition to the decrease of adapters' self-ligation. Five groups were set up for this experiment: artificial template alone, template with MseI, the TAat adapter self-ligation group, template plus adapters with addition of MseI and T4 ligase, and template plus adapters with addition of MseI, T4 ligase and AseI group as summarized in Table 3.

The ligation products of the fifth group were amplified by PCR, and the PCR products were subjected to Sanger sequencing in Sangon Biotech. The primer pairs were with the sequences 5-ccatccagcatccaactcggtgggggtggggat-3, and 5-gtcagctccacgcatacattatacg-3. PCR reaction performed in a total volume of 20 μL system containing 2× Taq Master Mix 10μL, adapter primer 1μL, target fragment primer 1μL, ligation product 3μL. The PCR program consists of 95°C 2min 1cycle, 95°C 10s, 58°C 20s, 72°C 30s, 20cycles, and a final cycle at 72°C 1min.

2.2.3 Prevention of methylation site specific adapters' self-ligation

The enzymatic products with methylation dependent restriction endonucleases yield highly viscous end with four-base extrusion. To use these enzymatic products for gnomic sequencing, adapters with four wobble bases of NNNN is a reasonable option. To minimize or eliminate the potential self-ligation of the NNNN adapters, the YNNY and RNNR adapters were compared with NNNN adapters in the condition of T4 ligase. The ligation reaction was set in conditions as shown in the Table 4. After ligation reaction completed, 5 μL of each ligation reactions was visualized by agarose gel electrophoresis under 100 V for 30 min.

Results:

3.1 CGat adapters' self-ligation prevented by enzyme ClaI

As shown in Fig. 2, with T4 DNA ligase, CAat adapters were completely self-ligated (lane 2). The addition of restriction enzyme ClaI efficiently prevented the formation of self-ligated products. Fig. 3 illustrated the strategy of preventing CGat adapters' self-ligation and promoting adapter-insert ligation. Lanes 1 and 2 show the artificial template and its enzymatic product by HpaII digestion. Without ClaI, both adapters' self-ligated products and the products from insert and adapters ligation were observable, but adapters' self-ligated products are predominant (lane 4). Three observable bands in lane 5 were the un-ligated adapters, self-ligated adapters, and the ligation products of insert and adapters, in which the ligation products of insert and adapters are predominant instead. The ligation products of insert and adapters were confirmed by Sanger sequencing, with characteristic nucleotides CGAT in the sense strand (Fig.3 b).

3.2 TAat adapters' self-ligation decreased by enzyme AseI

Fig. 4 showed the effects of restriction enzyme AseI in decreasing the self-ligation of TAat adapters. TAat adapters were largely self-ligated with T4 ligase, which was virtually eliminated with the addition of restriction enzyme AseI. Different form the ClaI in prevention of adapter's ligation, the enzyme of AseI efficiently decreased adapters' ligation and increased the products of insert-adapter ligation (Fig. 5 lane 5). Sanger sequencing confirmed the ligation products of insert and adapter were as expected.

3.3 No self-ligation products from YNNY and RNNR adapters

To prevent self-ligation NNNN adapters, adapters YNNY and RNNR were designed. Adapters ending with YNNY and RNNR can theoretically avoided self-ligation occurred with NNNN adapters. The methylation site dependent adapters have four nucleotides at 5' extrusion. Their self-ligation between the four wobble NNNN are complementarily paired with 256 types of combinations. When YNNY and RNNR are designed, the Y(C or T base) and Y is not complementary and neither the R and R.

Figure 6 illustrated the results for the three adapters ending with YNNY, RNNR, and NNN with and without T4 ligase. Self-ligated products from NNNN adapters were observed (lane 6). No self-ligation products were observed from the YNNY and RNNR adapters as expected.

Discussion:

The present study developed and tested three strategies to prevent the self-ligation of adapters used in enzyme-mediated methylation sequencing technology. The self-ligation of the two types of methylation site independent adapters have been largely prevented, and more required ligation products with inserts were yielded. The self-ligation of methylation site dependent adapters were completed avoided based on their design.

Adapters targeting the ends yielded from either methylation-sensitive or dependent restriction endonuclease digestion were able to be self-ligated because of their sticking termini designed for ligation with related ends from restriction digestion. These two types of restriction enzymes, particularly the methylation-dependent endonuclease is essential in methylation-dependent endonuclease mediated methylation sequencing. Strategies to prevent adapters' self-ligation is thus crucial in the development of this new methylation sequencing method (Chinese patent application number 202210881697.2). This new methylation sequencing method may employ four types of adapters, one methylation site-dependent and three methylation site-independent adapters. The self-ligation of methylation site-dependent adapters are completely prevented by eliminating any possible complementary base paring within the four wobble nucleotides in the adapters tested. For the three types of methylation site independent adapters, adapter with 3' extruded T has no self-ligation issue, but its efficiency in insert coverage of less than 30% [12, 13] prompted us to develop the other two types of adapters tested in the present study. As illustrated in Fig. 1 and Fig. 2, adapters with CGat at its 5' end together with the enzyme ClaI virtually changed an reversible reaction to unidirectional reaction of insert-adapter ligation. For the adapter with terminal TAat string, the combination of enzymes MseI and AseI substantially decreased adapters' self-ligation (Fig. 3).

As illustrated in Fig. 1, an ideal strategy should have three effects: i. to inhibit adapters' self-ligation, ii. to promote the production of insert-adapter ligation, and iii. to regenerate adapters from adapters' self-ligation products. The present study demonstrated three types of strategies in decreasing or preventing adapters' self-ligation, with different advantages and disadvantages. The strategy in preventing CGat adapters' self-ligation is overall better than the other two as it is fulfill all the three standards as discussed.

The insert-adapter ligated products and adapters' self-ligated product in methylation dependent endonuclease mediated methylation sequencing technology are hardly separated based on their sizes as they may be predominantly between 100 and 200 base pairs[14-16]. In real methylation sequencing library, the size difference between a large portion of ligation products of insert and adapters and adapters' self-ligation is about 30-34 bp, and some are as less as 17 bp. For example, when a palindromic methylation site is cut by MspJI ((N)13mCGmCG(N)9), the insert in the library is only 30 bp. Application of more types of adapters to decrease or eliminate self-ligated adapters has at least four advantages. Firstly, the three types of methylation site-independent adapters can be combined to increase the coverage of inserts to be assayed as more inserts are ligated with adapters. Secondly, insert-adapter ligation is preferred over insert-insert ligation and thus coverage of insert is further increased. Thirdly, less self-ligated adapters in the final sequencing library will minimize sequencing noise. Fourthly, decrease self-ligated adapters will greatly cut the cost of methylation sequencing by maximally using the sequencing chip with most of the clusters are formed from insert-adapter products. These advantages are precious in methylation sequencing of single cell and circulating free DNA samples.

In conclusion, three strategies were introduced in our newly developed methylation-dependent endonuclease mediated methylation sequencing method. Self-ligation of the methylation site-dependent adapters could be theoretically eliminated by design. For the two methylation site-independent adapters, the one with methylation sensitive enzymes is virtually abolished by forcing the reversible reaction to a unidirectional reaction of insert-adapter ligation. The third type of adapter with methylation unrelated endonuclease, the combination of enzymes MseI and AseI as an example showed its usefulness in decreasing adapters' self-ligation. Although these three strategies are crucial for the development of methylation-dependent endonuclease mediated methylation sequencing, they are not equally efficient and improvement is expected whenever new suitable endonucleases are available. These strategies may find more applications in the development of other enzyme-mediated genomic sequencing technologies.

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CONFLICT of INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

KL and DFL designed the research. PQ, YLZ, XY,and ML completed the experiments. PQ, LX, and GW analyzed the data. DFL and KL wrote the manuscript. JZ and SP reviewed the manuscript.

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Figures and legends

Table 1. The sequences of chemically synthesized templates and adapters

No.	sequence
1	5-CAATCGCTCCGGTTAACTGCTCCGCATTCGTGTGCGCGGGC AACTTCGTATAATGTATGCGTGGAGCTGAC-3
2	5-GTCAGCTCCACGCATACATTATACGAAGTTTGCCCAGCGCTC CACACGAATGCGGAGCAGTTAACCGGAGCGATTG-3

No.	sequence
3	5-P-*CGAT*CCCCACCCCACCGAGTTGGATGCTGGATGG-3
4	5-P-*TAAT*CCCCACCCCACCGAGTTGGATGCTGGATGG-3
5	5-CCATCCAGCATCCAACTCGGTGGGGTGGGGAT-3
6	5-P-*YNNY*TTCCCACCATCCAGCACATATATCTACTCCTGAGCGCA 3
7	5-P-*RNNR*TTCCCACCATCCAGCACATATATCTACTCCTGAGCGCA 3
8	5-P-*NNNN*TTCCCACCATCCAGCACATATATCTACTCCTGAGCGCA 3
9	5-ATCACATAGACAACAGGTGCGCTCAGGAGTAGATATATGTGC 3

Table 2 The connection system and reaction procedure of CGat adapter to the fragment of artificial template under test

	template	HpaII	CGat adapter	CGat adapter+template+HpaII	CGat adapter +template+HpaII+ClaI
template	200ng	200ng	0	200ng	200ng
CGatadapter	0	0	300ng	300ng	300ng
10X CutSmart Buffer	5	5	5	5	5
ATP(10μM)	5	5	5	5	5
T4DNA Ligase	0	0	1	1	1
HpaII	0	1.5	0	1.5	1.5
ClaI	0	0	0	0	2.5
Total volume	50	50	50	50	50
Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min

Table 3. The connection system and reaction procedure of TAat adapter to the fragment of artificial template under test

	template
template	200ng
TAatadapter	0
10X T4 DNA Ligase Buffer	5
T4DNA Ligase	0
MseI	0
AseI	0
Total volume	50
Reacted overnight at 37 °C, T4 ligase was inactivated at 65 degC for 20 min	Reacted overnight at 37 °C, T4 ligase was in

Table 4 The connection system and reaction procedure of adapters with YNNY, RNNR and NNNN to the fragment of artificial template under test

	YNNY	YNNY +T4	RNNR	RNNR +T4	NNNN	NNNN +T4
T4DNA Ligase	0	1	0	1	0	1
10X CutSmart Buffer	5	5	5	5	5	5
ATP(10μM)	5	5	5	5	5	5
adapter	200ng	200ng	200ng	200ng	200ng	200ng
Total volume	50	50	50	50	50	50
Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min	Reacted at room temperature for 1h,and T4 ligase was inactivated at 65 °C for 20 min

Fig. 1. Strategy (Strategic diagram) of preventing adapters self-ligation using methylation-sensitive endonucleases (with CGat adapter as an example).

Three enzymatic sites harboring CGc, CGg and CGt strings are yielded and their compatible ends in the adapters is CGa. Two of CGa with a following T composes a ClaI site when the adapters are self-ligated. However, the ligated products between CGc, CGg and CGt from target nucleic acids and CGa from the adapters form no restriction site and thus the combination of these enzymes ensures a virtually unidirectional reaction of producing ligation between target inserts and their adapters. Here MSRE represents methylation sensitive restriction endonuclease

Fig. 2. Self-ligation of CGat adapters prevented by the addition of endonuclease ClaI.

Fig. 3. Endonuclease ClaI prevented CGat adapters from self-ligation and promoted its ligation with insert

(A) The expected product from the ligation of insert and adapters in lane 5 is obviously more than that observed in lane 4. (B)The ligated products were confirmed by Sanger sequencing.

Fig. 4. Self-ligation of the TAat adapters were inhibited by adding restriction endonuclease AseI.

Fig. 5. Endonuclease AseI and MseI together prevented TAat adapters from self-ligation and promoted its ligation with insert.

(A)The expected band of the insert-adapter ligated product in lane 5 was more than that in lane 4. (B) Sequencing chromatographs of the ligated products from insert and adapters.

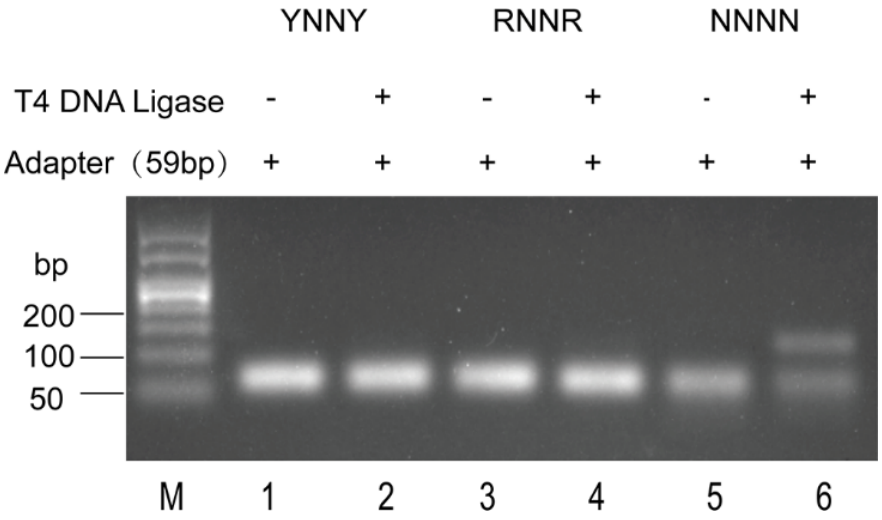

Fig. 6. Self-ligation of methylation site specific adapter was avoided by eliminated self-complementary pairing of the extruded 4 wobble bases.