method (Patent Document 1, Non-Patent Document 5, Non-Patent Document 6). In this method, a primer of several ten base pairs called template-switching oligo, which has a base sequence such as [guanosine]-[guanosine]-[guanosine] at the 3'-end, is added during the cDNA synthesis from mRNA, thereby appending a desired base sequence at the cDNA terminus and synthesizing the second strand. The second is the TdT method (Non-Patent Documents 7 to 9). In this method, a polynucleotide sequence composed of identical bases is added to the terminus of the synthesized cDNA using terminal deoxynucleotidyl transferase (TdT), and the second strand is synthesized using a primer that has the sequence complementary to this polynucleotide at its 3'-end and a known adapter sequence at its 5'-end.

[0006]

The TS method has the advantages that the loss of cDNA associated with purification is low since the method requires only a small number of reaction steps, and that full-length cDNA is readily generated due to its nature that the TS reaction occurs predominantly on mRNA having the 5' cap structure. On the other hand, the method has the disadvantage that the efficiency of the TS reaction is highly dependent on the terminal structure of the mRNA. For example, the efficiency decreases depending on the terminal base in the order of G>A>C>U, and, while relatively high efficiency (within the range of about 20% to 60%) is achieved for full-length mRNA having a cap structure at the 5'-end, the efficiency is low (within the range of several percent to 40%) when mRNA without the cap structure such as non-full-length mRNA, immature mRNA, or partially degraded mRNA is involved. Such uneven efficiency leads to an amplification bias (Non-Patent Document 6). Another disadvantage is that the polynucleotide addition efficiency of the TS method is lower than that of the TdT method (92 to 95%, Non-Patent Document 10).

[0007]

On the other hand, although the TdT method achieves higher efficiency than

a predetermined amount of terminator nucleotides is added in this method, cDNA molecules to which a polynucleotide with a length sufficient to form a stable complementary strand with a primer used in the second-strand synthesis is not appended are inevitably produced at a certain amount. Since the TAS-Seq method is a type of the TdT method, it is similar to the common TdT method in terms of the facts that more steps are required than the TS method, and that long-chain full-length cDNA molecules are less likely to be synthesized compared to the TS reaction. Further, since the known TAS-Seq method is a solid-phase cDNA amplification method, its application to a liquid-phase system has not been known.

[0012]

An object of the present invention is to provide a cDNA amplification method capable of highly efficiently and comprehensively amplifying mRNA with a low bias even from a very small amount of cell sample.

MEANS FOR SOLVING THE PROBLEMS

[0013]

When the TS method and the TdT method are combined, it is expected that the increase in the cDNA yield because of the smaller number of reaction steps, which is an advantage of the TS method, cannot be achieved, and that the high reaction efficiency, which is an advantage of the TdT method, cannot be achieved since formation of a protruding end, which is a preferred end structure for the TdT reaction, may be prevented due to exposure of the 3'-end of cDNA during the template-switching reaction in the TS method. Further, in the liquid-phase TS method, in order to avoid loss of the product during the purification process, removal of unreacted target RNA-capture oligos (i.e., target RNA-capture oligos that have not captured the target) is usually not performed after the cDNA synthesis (Non-Patent Document 15). That means, there is a common technical knowledge that, if a purification step is carried out for removing unreacted target RNA-capture oligos

whose homopolymer portion (especially, poly(C)) is too short can be decreased due to addition of bases such as CCC to the cDNA terminus, so that the TAS-Seq method can be efficiently carried out even in a liquid-phase system that does not use a solid phase.

[0016]

The present invention, completed by the above intensive study, is a method of amplifying a complementary DNA strand, the method comprising steps of capturing a target RNA and synthesizing a complementary DNA strand using a reverse transcriptase having terminal transferase activity in a liquid-phase system or a solid-phase system to add bases such as CCC to the terminus of the complementary DNA strand, and then carrying out homopolymer tailing by the TdT reaction in the presence of a chain-terminating nucleotide triphosphate. When a TS reaction using a template-switching oligo is further combined with this method, more efficient and lower-biased amplification of the complementary DNA strand can be achieved. The present invention includes the following modes.

[0016a] In one aspect, provided herein is a method of amplifying a complementary DNA strand, the method comprising: a target RNA-capturing step of capturing a target RNA with a target RNA-capture oligo comprising a target capture portion, said target RNA-capture oligo being a free oligo not bound to a solid-phase carrier; a complementary-strand synthesis step of carrying out reverse transcription reaction using a reverse transcriptase having terminal transferase activity, to synthesize a complementary DNA strand whose sequence is complementary to the captured RNA, and to add an additional sequence consisting of several arbitrary bases to the 3'-end of at least part of the complementary DNA molecules; an unreacted-oligo removal step of removing a target RNA-capture oligo that has not captured the target RNA by nucleic acid size fractionation; an RNA degradation step of degrading RNA using a ribonuclease; a homopolymer addition step of carrying

out a reaction using terminal deoxynucleotidyl transferase in the presence of dATP, dTTP, dCTP or dGTP and a chain-terminating nucleotide triphosphate, to add a nucleotide homopolymer to the 3'-end of the complementary DNA strand; a second-strand synthesis step of synthesizing a second strand for the complementary DNA strand using a second-strand-synthesizing primer which comprises a primer comprising a complementary homopolymer portion whose sequence is complementary to the nucleotide homopolymer, to generate a DNA double strand composed of the complementary DNA strand and the second strand; and a nucleic acid amplification step of carrying out nucleic acid amplification reaction using the DNA double strand as a template.

[0016b] In another aspect, provided herein is a method of amplifying a complementary DNA strand, the method comprising: a target RNA-capturing step of capturing a target RNA with a target RNA-capture oligo comprising a target capture portion; a complementary-strand synthesis step of carrying out reverse transcription reaction using a reverse transcriptase having terminal transferase activity, to synthesize a complementary DNA strand whose sequence is complementary to the captured RNA, and to add an additional sequence consisting of several arbitrary bases to the 3'-end of at least part of the complementary DNA molecules; an unreacted-oligo removal step of removing a target RNA-capture oligo that has not captured the target RNA; an RNA degradation step of degrading RNA using a ribonuclease; a homopolymer addition step of carrying out a reaction using terminal deoxynucleotidyl transferase in the presence of dATP, dTTP, dCTP or dGTP and a chain-terminating nucleotide triphosphate, to add a nucleotide homopolymer to the 3'-end of the complementary DNA strand; a second-strand synthesis step of synthesizing a second strand for the complementary DNA strand using a second-strand-synthesizing primer which comprises a primer comprising a complementary homopolymer portion whose sequence is complementary to the nucleotide

homopolymer, to generate a DNA double strand composed of the complementary DNA strand and the second strand; and a nucleic acid amplification step of carrying out nucleic acid amplification reaction using the DNA double strand as a template, wherein: the target RNA-capture oligo comprises a target poly(A) RNA-capture oligo comprising a poly(T) portion as a target capture portion; and the target RNA comprises a poly(A) RNA; and wherein in the complementary-strand synthesis step, reverse transcription reaction is carried out in the presence of a template-switching oligo comprising: a sequence capable of hybridizing with the additional sequence, at its 3'-end; and a switching sequence on the 5'-side of said sequence capable of hybridizing with the additional sequence; to hybridize the template-switching oligo with the complementary DNA strand having the additional sequence added thereto, and to further add a sequence complementary to the switching sequence to the 3'-end of the complementary DNA strand.

[0017]

[1] A method of amplifying a complementary DNA strand, the method comprising:

a target RNA-capturing step of capturing a target RNA with a target RNA-capture oligo comprising a target capture portion;

a complementary-strand synthesis step of carrying out reverse transcription reaction using a reverse transcriptase having terminal transferase activity, to synthesize a complementary DNA strand whose sequence is complementary to the captured RNA, and to add an additional sequence consisting of several arbitrary bases to the 3'-end of at least part of the complementary DNA molecules;

an unreacted-oligo removal step of removing a target RNA-capture oligo that has not captured the target RNA;

each cell according to single-cell RNA sequence analysis data, between a template-switch method and a template-switch + TdT method in a liquid-phase system (10X Chromium single-cell analysis system). The template-switch + TdT method was able to detect a larger number of genes under conditions with a similar distribution of the number of reads per cell.

[Fig. 13] Results of single-cell clustering analysis of single-cell RNA sequence analysis data by a template-switch method and a template-switch + TdT method in a liquid-phase system (10X Chromium single-cell analysis system). The template-switch + TdT method has an improved separation capacity for cell populations.

[Fig. 14] Comparison of the number of detected genes as observed for each cell subset when the number of reads was changed, according to single-cell RNA sequence analysis data by a template-switch method and a template-switch + TdT method in a liquid-phase system (10X Chromium single-cell analysis system). The template-switch + TdT method was able to detect a larger number of genes for all cell populations.

MODE FOR CARRYING OUT THE INVENTION

[0020]

In the present invention, A, T, G, and C constituting the base sequence of a target RNA-capture oligo, a template-switching oligo, a primer, an adapter, a nucleotide homopolymer, or the like include not only deoxyribonucleotides constituting DNA (A, T, G, and C of DNA), but also ribonucleotides constituting RNA (A, T, G, and C of RNA), and further, their corresponding nucleotide analogues (including bridged nucleic acids such as LNA, ENA, and PNA; and modified bases such as Super T (5-hydroxybutynyl-2'-deoxyuridine), Super G (8-aza-7-deazaguanosine), deoxyinosine, 5-methyl dC, deoxyuridine, 2,6-diaminopurine, 2-aminopurine, and 2-Amino-dATP). Thus, the target RNA-capture oligo (for example, any of the target capture portion, the first adapter portion, the barcode

partially degraded mRNAs that have no cap structure are captured by a target poly(A) RNA-capture oligo having a poly(T) portion as a target capture portion, as long as they have poly(A).

[0076]

The target RNA-capture oligo has a target capture portion at its 3'-end. On the 5'-side of the target capture portion, a first adapter portion that can be used as a primer setting region in the nucleic acid amplification step (represented as Universal 1 in Fig. 4 and Fig. 5) may be contained. Between the first adapter portion and the target capture portion, a cell identification barcode portion for identifying each cell from which the target RNA is derived may be contained. The cell identification barcode is a barcode corresponding to the bead identification barcode portion and the compartment identification barcode portion in the solid-phase system. The target RNA-capture oligos to be encapsulated together with one cell in the same microwell or microdroplet have a cell identification barcode consisting of the same sequence, and the cell identification barcode sequence is different among microwells or microdroplets. Therefore, cDNAs derived from mRNAs captured by the capture oligos in the same microwell or microdroplet can be distinguished from cDNAs derived from mRNAs captured in other microwells or microdroplets, and hence cDNAs derived from the same cell can be distinguished from cDNAs derived from other cells.

[0077]

B-2. Complementary-Strand Synthesis Step (Step 2 in Fig. 4 and Fig. 5)

In a liquid-phase system, the target RNA-capturing step is directly followed by the complementary-strand synthesis step, without carrying out purification and the like. To the reaction liquid after the target RNA-capturing step, a reverse transcriptase having terminal transferase activity and template-switching oligos are added to carry out the reverse transcription reaction and the TS reaction in one step.