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(54) **Title:** A METHOD FOR ENZYMATIC JOINING OF A DSRNA ADAPTER TO A DSRNA MOLECULE

(57) **Abstract:** The invention provides a method for enzymatic joining of a dsRNA-adapter to a dsRNA substrate thus forming a dsRNA-construct. The method requires no prior knowledge of the sequence of the dsRNA substrate. The method is based on the discovery that the enzyme T4 RNA ligase can successfully add a single-stranded RNA-oligomer to a blunt-ended dsRNA molecule.

A method for enzymatic joining of a dsRNA-adapter to a dsRNA molecule.

Technical background

Double-stranded RNA (dsRNA) is widespread in living organisms and plays structural and functional roles in various biological processes and pathways[1]. RNA higher order structures consist of dsRNA formed by cis and trans hybridization. Many viruses have dsRNA genomes or use dsRNA as intermediates during their life cycle. In prokaryotes, naturally occurring antisense RNAs form dsRNA complexes with mRNAs that regulate translation. In eukaryotes, dsRNA is involved in numerous processes, such as heterochromatin remodelling, RNA editing, interferon responses and the RNA interference/microRNA pathway[2,3]. Indeed approximately 5% of mammalian heterogeneous nuclear RNA (hnRNA) appears to be double-stranded[1].

Double-stranded RNA (dsRNA) is formed in cells as intra- and intermolecular RNA interactions and is involved in a range of biological pathways including RNA processing, RNA interference and translation control mediated by natural antisense RNA and microRNA. In vitro, dsRNA can be formed from single stranded RNA by addition of a complementary ssRNA sequence. Despite this breadth, few molecular tools are available to analyse dsRNA as native hybrids.

Although there is increasing information about the biogenesis and function of dsRNA in cells, methodology for the analyses and handling of RNA in its natural duplex form remains limited. Unlike DNA, dsRNA is not easily manipulated and analysed with current molecular tools. It is known that various adapters can be attached to DNA using restriction enzymes and ligases, but known analogous methods for dsRNA are limited.

For example it is disclosed that T4 RNA ligase can successfully add a single-stranded DNA-oligomer to the dsRNA molecule[5, 6], and also attach a short ribohomopolymers to the 5´-phosphoryl of blunt-ended DNA[7]. One known technique for cloning dsRNA involves the ligation of oligoribonucleotide adaptors to 3' ends of dsRNAs[12]. The adaptors were 3' modified to avoid concatenation, which makes it impossible to perform further ligations to the adaptors.

However, these known manipulation methods are not specific to dsRNA, as ssRNA is also tagged with even higher efficiency. Thus, there is a need for a method for specific labelling/tagging/manipulation of dsRNA.

The present invention describes a ligation method for enzymatic joining of dsRNA-adapters to any dsRNA molecule in its duplex form without a need for prior sequence or termini information. The method is specific for dsRNA and can be applied to cellular RNA.

Indeed, the new method provides the only available dsRNA-specific labelling/tagging reaction. The new method can ligate various adapters to label, map or amplify dsRNA sequences. When combined with RT-PCR, the method is sensitive and can be used to generate cDNA libraries of

cellular dsRNA. As examples, dsRNA regions within E. coli *hok* mRNA and the HIV TAR element using RNA prepared from bacteria and mammalian cells are mapped.

The an academic paper related to the method of the invention has been published after the priority date by the inventors and their co-workers[13].

Summary of the invention

The invention provides a method for enzymatically joining (ligating) dsRNA-adapters to a dsRNA substrate thus forming a dsRNA-construct.

Importantly, the method requires no prior knowledge of the sequence of the dsRNA substrate, although knowledge of the sequence can enable additional applications. The method of the invention can ligate RNA adaptors specifically to dsRNA substrates avoiding ligation to ssRNA substrates, which is not possible with known methods. In contrast, the method of the invention involves both strands in ligation; therefore, the ligation occurs only when a dsRNA end is provided. Previous methods only involve one strand in the ligation, with the effect that it is not possible to discriminate between ssRNA and dsRNA substrates.

The invented method can ligate various RNA adaptors (ssRNA, dsRNA, RNA hairpins) to a dsRNA substrate. This is an advantage when one wants to analyse or manipulate the dsRNA end. For example, the manipulation may be desirable in order to map both 5' and 3' ends (using known methods, mapping 5' end is not possible) or to connect the two strands of dsRNA from the native formed termini using a hairpin adaptor and a Y-adaptor. The application of the latter reaction can be used to analyse hybridised microRNA:target RNA and identify which target RNA is hybridised to a certain microRNA or to make a shRNA library of Dicer cleaved short dsRNAs.

Notably, the invented method can ligate to the ends of both strands of dsRNA in its native hybridised form, for further analysis.

The method is based on the discovery that the enzyme T4 RNA ligase can successfully add a single-stranded RNA-oligomer to a 5' end of a blunt-ended dsRNA molecule having a 5' phosphate group. Any dsRNA-molecule may be rendered blunt-ended by digestion with nucleases such as S1 prior to addition of the oligomers. The method for enzymatic joining of a dsRNA-adapter to a dsRNA substrate comprises the steps of

i) generating a sticky end sequence on a blunt-ended dsRNA substrate having a 5' phosphate group by enzymatically joining an oligoribonucleotide overhang-adapter to a 5' end of the blunt-ended dsRNA molecule;

ii) providing a dsRNA-adapter comprising a first strand comprising a sequence overhang capable of specifically annealing to the sticky end sequence generated in (i) on the dsRNA substrate so that said first strand can be subject to enzymatical joining to said dsRNA substrate and a second strand comprising a sequence capable of specifically annealing to the first strand such that said second strand can be subject to enzymatic joining to said dsRNA substrate; and

iii) enzymatically joining the dsRNA substrate to both first and second strands of the dsRNA-adapter to form a covalently joined dsRNA-construct.

In step (i), T4 RNA ligase attaches an overhang-adapter e.g. adenine six-mer to the dsRNA substrate producing overhangs (sticky termini). In steps (ii-iii) the sticky-ended dsRNA is joined, using T4 DNA ligase, to a dsRNA-adapter, which comprises an overhang complementary to the overhang-adapter.

In one embodiment, the method additionally includes the step of manipulating the dsRNA substrate, prior to step (i) above, to create blunt ends with enzymes, such as endonucleases, such as S1 nuclease (EC 3.1.30.1).

In one embodiment, the method additionally includes the step of manipulating the dsRNA substrate, prior to step (i) above, to artificially add a 5' phosphate to the dsRNA substrate, if such phosphate is not naturally present, e.g. with T4 polynucleotide kinase (EC 2.7.1.78) or via chemical modification.

In one embodiment, the method additionally includes the step of forming the dsRNA from a single-stranded RNA substrate by addition of synthetic single-stranded RNA molecule complementary to the single-stranded RNA substrate, prior to step (i) above.

In one embodiment, the dsRNA-adapter is a Y-adapter having non-complementary portions that provide at least one unique primer binding site on each strand of the dsRNA-construct.

In one embodiment, the overhang-adapter is not phosphorylated at 5'-end at step (i), and wherein a phosphorylation step is performed before or in conjunction with step (iii), preferably in conjunction with step (iii) using T4 polynucleotide kinase (EC 2.7.1.78).

In one embodiment, the dsRNA-adapter strand comprising the sequence overhang (overhang-strand) is modified such that ligation is prevented at the 3'-end and the other strand (opposite-strand) is modified such that ligation is prevented at 5'-end.

In one embodiment, T4 RNA ligase (EC 6.5.1.3) is used for the enzymatic joining step (i).

In one embodiment, T4 DNA ligase (EC 6.5.1.1) is used for the enzymatic joining step (iii).

The attached dsRNA-adapters, having a known sequence, may then be utilised in a number of manipulations known in the field of molecular biology, including analysing, profiling, labelling, mapping, tagging, cloning, amplification, or DNA-library construction of dsRNA substrates. Therefore methods are also provided for diagnosis of a microbial pathogen that contains dsRNAs, and for diagnosis of an altered physiological (e.g. pathological) state characterised by changes in endogenous dsRNAs.

A method of diagnosis of an altered (e.g. pathological) state is provided comprising using the methods of the invention to analyse and compare the levels of endogenous dsRNAs of a patient to levels of corresponding dsRNAs in normal (e.g. healthy) individuals, wherein higher or lower levels than normal may be indicative of an altered (e.g. pathological) state.

A method of diagnosis of a microbial pathogen is provided comprising using the methods of the invention to detect dsRNA molecules specific to microbial pathogens, such as a virus, such as HIV, in which case an example of a pathogen-specific dsRNA molecule is TAR (appearance of TAR dsRNA is indicative of a HIV infection). Alternatively, the presence of a microbial pathogen (e.g. virus) in a host organism may be diagnosed by using the methods of the invention to detect changes in the levels of dsRNA molecules produced by the host, wherein the levels of said dsRNA molecules are either increased or decreased in a state of microbial infection.

Figure legends

Figure 1 | Ligation of a dsRNA-adapter to blunt-ended dsRNA substrate. (**a**) Illustration of the ligation method. The dsRNA substrate was 5-carboxyfluorescein (FAM)-conjugated (grey dsRNA with star) for easy tracking in gels. In step (I) (corresponding to step (i) of the method), T4 RNA ligase attaches an overhang-adapter [adenine six-mer (small bright grey oligo)] to the dsRNA substrate producing overhangs (sticky termini). In step (II) (corresponding to steps (ii) and (iii) of the method, which take place concomitantly), the sticky-ended dsRNA is phosphorylated by T4 polynucleotide kinase and joined to a dsRNA-adapter, which comprises an overhang complementary to the overhang-adapter, using T4 DNA ligase. (**b**) Native and (**c**) denaturing PAGE analyses of the steps (I) and (II). Each band is indicated with its corresponding cartoon.

Figure 2 | Specific adapter ligation to dsRNA. A sulforhodamine 101 acid chloride (Texas Red)-conjugated dsRNA-adapter (TxRed-adapter) was ligated to unmodified dsRNA and ssRNA substrates. Ligated product were fractionated with denaturing PAGE.

Figure 3 | RT-PCR amplification of dsRNA. (**a**) Y-adapter and its primer sites when attached to a dsRNA are illustrated. Y-adapters create two different primer sites in both ends of dsRNA. (**b**)

RT-PCR amplification of dsRNA as well as ssRNA substrates that were ligated to Y-adapter (expected PCR product is 62 bps).

Figure 4 | Trimming and adapter ligation of dsRNA with heterogeneous overhanging termini. (**a**) Illustration of overhang removal from dsRNA using S1 nuclease. (**b**) Native PAGE analysis showed that S1 nuclease cleaves overhangs but not the duplex in a range of enzyme concentrations. (**c**) Native PAGE analysis indicated that blunt ends generated by S1 nuclease were ligatable to dsRNA-adapters. (**d**) The overhanging dsRNA was spiked into *E. coli* total RNA in physiological amounts, ligated to Y-adapter and amplified with RT-PCR. The PCR product length is 63 bps.

Figure 5 | Mapping of dsRNA regions. Amplification of (a) a dsRNA region within *hok* mRNA (PCR product is 87 bps) and (b) a stem region of TAR RNA (PCR products are 55 and 62 bps). Gene-specific primer sites are highlighted and adaptor ligation sites (where S1 nuclease cleaves) are indicated with an arrow in the illustrations.

Figure 6 | S1 nuclease digestion (trimming) of overhanging dsRNA over a range of substrate concentrations and reaction times.

Definitions

"Sequence capable of annealing" and "specifically annealing" refer to the reaction where at least partially complementary single-stranded nucleotide sequences combine to form a double-stranded nucleotide. The degree of specificity of annealing depends on the length of the nucleotides, the sequence composition, concentrations of the molecules, temperature, salt ion concentration as well as other factors. A man skilled in the art is well familiar with methods of choosing and designing complementary sequences for annealing to each other under given conditions, and such selection will not require more than routine experimentation. For guidance, the skilled man may refer to textbooks in the technical field, for example Sambrook and Russell: Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press 2001) or Ausubel, Brent, Kingston, Moore, Seidman, Smith and Struhl (eds): Current Protocols in Molecular Biology (John Wiley & Sons 2008).

"dsRNA substrate" is a double-stranded RNA molecule to be manipulated by the method of the invention. The dsRNA substrate may be artificial or natural. For the method of the invention, the dsRNA substrate must have blunt ends, either naturally or though manipulation, e.g. digestion with S1 nuclease. For instance, the dsRNA substrate may also be derived though manipulation from one RNA-strand forming a local double-stranded secondary structure. For the method of the invention it is required that the 5'-ends of the dsRNA substrate are phosphorylated, either naturally, or through manipulation, e.g. with T4 polynucleotide kinase or via chemical modification.

"Sticky-end" in the context of the invention means an end of a poly- or oligonucleotide that comprises a single-stranded overhang. The overhang may be for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides long. An overhang should be at least 1, least 2, least 3, least 4, least 5 or least 6, such as 2-30, 3-20, 4-15, or 1-10 nucleotides long. An overhang 1-10 nucleotides long is preferred and an overhang of 6 nucleotides is most preferred.

"Overhang-adapter" is a single-stranded oligoribonucleotide that is ligated to 5'-ends of a blunt-ended dsRNA substrate to create a sticky-end overhang. The length of the overhang-adapter determines the length of the overhang. The overhang-adapter may be for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 ,13, 14, 15 or 16 nucleotides long. 6 nucleotides long polyadenine overhang-adapter is preferred (ref : 7). The sequence of the overhang-adapter may be freely selected as long as the annealing part of the corresponding dsRNA-adapter (see below) is modified accordingly (e.g. made complementary). In selecting the sequence of the overhang, sequences that form secondary structures are avoided. Such considerations are well known to the man skilled in the art (guidance can be found, for instance, in the textbooks mentioned above). Also, the sequence of the overhang should be a good substrate for T4 RNA ligase, the enzyme that is used for ligation[7]. The overhang-adapter is preferably not 5'-phosphorylated or is otherwise modified to avoid concatenation in the ligation reaction.

"dsRNA-adapter" comprises two strands of oligoribonucleotides that are ligated to the sticky-end created by the overhang-adapter on the dsRNA substrate. The "overhang-strand" or "first strand" of a dsRNA-adapter comprises a sequence capable of specifically annealing to the overhang-adapter such that the 5'-end of the overhang-strand can be ligated to the 3'-end of the dsRNA substrate. Preferably, said sequence is fully complementary to the overhang-adapter (e.g. six uridine nucleotides if an overhang-adapter of a six-mer oligoadenoribonucleotide is used). The strand not comprising the overhang ("opposite-strand" or "second strand") has a sequence capable of specifically annealing to the overhang-strand at least adjacent to the sequence annealing to the overhang-adapter such that the 5'-end of the dsRNA substrate (comprising the overhang-adapter) can be ligated to the 3'-end of the opposite strand. The length of the annealing part may be 1-100 nucleotides, preferably 3-40 nucleotides.

The 3'-end of the overhang-strand and/or the 5'-end of the opposite strand may preferably be modified to avoid ligation between two dsRNA-adapter molecules. Suitable modifications are basically any chemical groups that are attached to the ends that prevent ligation including but not limited to amination, biotinylations, attachment of a 3'-spacer or inverted ends. A preferred modification is amination. The 5'-end of the overhang-strand must be phosphorylated for use with the method of the invention, either during the process of the method or in beforehand.

”Y-adapter” is a special case of dsRNA-adapter which provides two different primer binding sites, one for each strand, when attached to both ends of a dsRNA substrate. Besides the characteristics of a dsRNA-adapter, a Y-adapter has the further characteristic that the overhang-strand and opposite-strand each possess a site for a primer. The sequences of the primer binding sites on the overhang-strand and the opposite strand are different from one another at least to such degree that primers can be selected that will anneal specifically to either one or the other under the selected conditions. Preferably, the primer binding sites show less than 50% homology.

“dsRNA-construct” is the dsRNA substrate molecule together with the enzymatically joined dsRNA-adapter or Y-adapter.

Detailed description of the invention

The invention provides a method for enzymatically joining (ligating) dsRNA-adapters to dsRNA substrate thus forming a dsRNA-construct. The method requires no prior knowledge of the sequence of the dsRNA substrate and can be used for a wide variety of purposes, although prior sequence knowledge can enable sequence-directed applications. The method is also specific to dsRNA and avoids ligation to ssRNA.

According to one embodiment of the invention, the dsRNA substrate to be manipulated by the method is blunt-ended. If the dsRNA substrate is not blunt-ended to begin with, it may be treated to create blunt end (trimmed). Examples of such treatments include enzymatic treatment e.g. by endonucleases such as S1 nuclease and Mung bean nuclease. According to one embodiment of the invention, any nucleases used for creating a blunt end are inactivated or removed from the dsRNA-substrate before proceeding with the following steps. Methods for such inactivation/removal include extraction with Trizol® reagent or phenol/chloroform.

To generate a sticky-end on the dsRNA-substrate, an overhang-adapter is enzymatically joined to 5’-phosphoryl the dsRNA-substrate. To avoid concatenation of the overhang-adapter, it is preferred that the overhang-adapter does not have a 5’-phosphoryl, or is otherwise modified to avoid concatenation. The enzymatic joining reaction may be preferably performed using T4 RNA ligase (EC 6.5.1.3) but other enzymes having the same or similar activity may also be used. High concentrations of overhang-adapter leading to molecular excess are preferred (preferable concentration range of 0.5 µM- 50 µM), as well as using a molecular crowding agent such as PEG6000 at 0.5-15% (w/v). Other molecular crowding agents suitable for use with the method of the invention are known in the art and include but are not limited to polyethylene glycols of various molecular weights, hexammine cobalt chloride, dextrans and ficolls. ATP is a necessary co-factor for the ligase and is present preferably in the range of 0.5 µM-2 µM. After the reaction,

the T4 RNA ligase is preferably inactivated, e.g. by phenol/chloroform extraction followed by ethanol precipitation. See Fig 1 for illustration.

In the next step, the dsRNA-adapters are enzymatically joined to the sticky-ended dsRNA-substrate. A phosphorylation in the 5'-position of the sticky-ended dsRNA-substrate and overhang-adapters (in case overhang-adapters without 5'-phosphoryl were used) is necessary prior to or concomitantly with the enzymatic joining reaction. According to one embodiment, the enzymatic joining and phosphorylation reactions are preferentially performed simultaneously. Preferably, T4 polynucleotide kinase (EC 2.7.1.78) can be used for phosphorylation while using T4 DNA ligase (EC 6.5.1.1) for the joining reaction, but other enzymes having the same or similar activities can also be used. A crowding agent such as PEG 4000 may preferably be used, and ATP is a required co-factor for the ligase. Preferred range for PEG 4000 is 0.5%- 15%, preferred range for ATP is 0.5 µM -2 µM. At the end of the reaction, the enzymes may be heat-inactivated at 70ºC for 10 minutes. See Fig 1 for illustration. As shown in example 3, the above procedure is specific for dsRNA.

By utilising dsRNA-adapter-specific primer(s), the sequence of the dsRNA substrate may be reverse-transcribed into DNA. At least one primer specific to the overhang-strand of the dsRNA-adapter is added to a solution comprising dsRNA-construct. Reverse-transcriptase enzyme (e.g. ThermoScript) and deoxynucleotides are further added, and the reaction is incubated to carry out the reverse transcription. To avoid problems that RNA secondary structures may cause, thermostable reverse-transcriptase and a high reaction temperature (e.g. 60ºC) may be used. Thus, the overhang-strand to which the primer is bound is used as a template to reverse-transcribe a single-stranded DNA-molecule with a sequence complementary to the dsRNA-construct comprising the dsRNA substrate. After reverse transcription, any of the various techniques known in the art may be used to analyse and/or manipulate the sequence. Examples of such known techniques include sequencing, profiling, labelling, mapping, tagging, cloning, amplification, and DNA-library construction.

Labelled dsRNA-adapters may be used to provide labelled dsRNA-constructs. Examples of such labels include but are not limited to fluorescent labels such as sulforhodamine 101 acid chloride (Texas Red or TxRed), fluorescein or 5-carboxyfluorescein (FAM or 6-FAM), labels with binding affinity such as streptavidin, biotin, glutathione or digoxigenin, enzymatic labels such as alkaline phosphatase or horseradish peroxidise and radioactive labels such as ^{32}P, ^{35}S, ^{125}I or ^{131}I. It will be recognized by the man skilled in the art that labelling of the dsRNA-constructs is useful for many purposes. One purpose may be following the course of a ligation reaction as shown in the Examples. Labelling of dsRNA may also be used for e.g. detection of dsRNA in total RNA, separating one strand from another, immobilization of dsRNA and isolation of dsRNA binding proteins.

The method may also be applied on RNA that is single-stranded (ss) to begin with, if a complementaty RNA oligonucleotide (or polynucleotide) is added to the sample prior any nuclease digestion and the ligation of overhang-adaptors, The added complementary RNA will hybridise with the ssRNA forming dsRNA, which can be manipulated using the method. However, the creation of the complementary RNA oligonucleotide (or polynucleotide) may require some prior knowledge of the sequence to be analysed.

One application of the method of the invention is to enable diagnosis of a microbial pathogen that contains dsRNAs. Double-stranded (ds) RNA viruses are a diverse group of viruses that vary widely in host range (humans, animals, plants, fungi, and bacteria). Members of this group include the rotaviruses, renowned globally as the commonest cause of gastroenteritis in young children, and bluetongue virus, an economically important pathogen of cattle and sheep. Viruses with dsRNA genomes are currently grouped into six families: Reoviridae, Birnaviridae, Totiviridae, Partitiviridae, Hypoviridae, and Cystoviridae. Single-standed (ss) RNA viruses can be classified according to the sense or polarity of their RNA into negative-sense and positive-sense, or ambisense RNA viruses. Examples of positive-sense ssRNA viruses include but are not limited to the families: Flaviviridae - includes Yellow fever virus, West Nile virus, Hepatitis C virus, Dengue fever virus; Picornaviridae - includes Poliovirus, the common cold virus, Hepatitis A virus; Filoviridae - includes Ebola virus, Marburg virus; and Paramyxoviridae - includes Measles virus, Mumps virus, Nipah virus, Hendra virus. The method of the invention may be used to detect the presence of any RNA-virus in a biological sample facilitating diagnosis.

Another application is to enable diagnosis of an altered physiological (e.g. pathological) state characterised by changes in endogenous dsRNAs.

By utilising a particular type of dsRNA-adapters namely Y-adapters, the dsRNA substrate may be PCR-amplified without need of knowledge of the sequence of the dsRNA substrate. Reverse transcription may be carried out as above. Thanks to the special construction of the Y-adapters, the ends of the reverse-transcribed DNA-molecule will have different and known sequences. The single-stranded DNA-molecule can then be used as a template for PCR using primers specific to the known ends to amplify the dsRNA substrate sequences in vitro. See figure 3 for illustration.

In another embodiment, the PCR-amplification may also be carried out using one dsRNA-adapter-specific primer and one gene-specific primer. As exemplified in Example 6, such methods may be used for mapping the secondary structure of dsRNA-molecules of known sequence.

The invention further provides a kit for carrying out the method of the invention. Minimally the kit includes instructions for carrying out the method and at least one of the following: overhang-adapter, dsRNA-adapter, labelled dsRNA-adapter, control dsRNA substrate, Y-adapter.

The details and particulars described above and in the claims and relating to the methods and kit according to the invention apply mutatis mutandis to the other aspects of the invention.

While the invention has been described in relation to certain disclosed embodiments, the skilled person may foresee other embodiments, variations, or combinations which are not specifically mentioned but are nonetheless within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

The expression "comprising" as used herein should be understood to include, but not be limited to, the stated items.

The invention will now be described by way of the following non-limiting examples.

Examples

Oligoribonucleotides and enzymes.

All oligoribonucleotides and oligonucleotides (see SEQ ID NO: 1-16) used in this study were purchased from Biomers (biomers.net GmbH. Söflinger Str. 100
D-89077 Ulm. Germany; www.biomers.net**).** All enzymes were purchased from Fermentas (FERMENTAS INTERNATIONAL INC., CANADA, 830 Harrington Court, Burlington, Ontario L7N 3N4; www.fermentas.com) unless specified otherwise.

Example 1: Generating sticky-ended dsRNA (Step (I), equal to step (i) of the method of the invention)

dsRNA extraction and S1 nuclease digestion (trimming)

For bacterial dsRNA, *Escherichia coli* cells from 1.5 ml overnight cultures **(**Strain K12**)** were harvested with centrifugation **(**7000×g for 5 minutes**)** and resuspended in 100 µl of digestion reaction (1X S1 reaction buffer [40 mM sodium-acetate (pH 4.5 at 25°C), 0.3 M NaCl and 2 mM $ZnSO_4$], 1 unit/µl S1 nuclease). To break cells, 700 mg of 0.1 mm Zirconia/Silica beads (Biospec Products Inc, Bartlesville, OK) was added to the cell suspension and the mixture was vigorously vortexed in a 1.5 ml microtube for 10 min at room temperature. dsRNA was extracted from cell lysate/beads mixture using one ml of Trizol (Invitrogen [Invitrogen, Carlsbad, CA, www.invitrogen.com]) according to the manufacturer's protocol.

For mammalian cell-derived dsRNA, Human Embryonic Kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine and penicillin-streptomycin (Invitrogen). Cells in 35mm dishes were transfected using the calcium phosphate method with the plasmids pSVIII-HXB2 (containing TAR RNA of HIV) (received from J. Sodroski) and pcDNA3.1-Tat (containing no TAR RNA), plated for

24h. Then, the cells were resuspended in 300 µl of digestion buffer (1X S1 reaction buffer, 1 unit/µl S1 nuclease, 0.1% (v/v) Triton X-100) and incubated in room temperature for 30 min. Cell lysate was centrifuged to separate nuclear and cytoplasmic fractions. Nuclear fraction was frozen and thawed three times. Finally, dsRNA was extracted from both fractions using Trizol (Invitrogen).

Attachment of overhang-adapters by T4 RNA ligase reaction

To attach short ssRNA to blunt-ended dsRNA for generating sticky-ends, 20 pmol of synthetic dsRNA substrate (comprised of annealed SEQ ID NO 1 and 2 obtained by mixing 2 oligo RNAs in distilled H_2O and incubation in room temperature for 5 minutes) or 5 µl of trimmed dsRNA from the bacterial dsRNA was incubated in 50 µl of the ligation reaction (1X T4 RNA ligase buffer [50 mM HEPES-NaOH (pH 8.0 at 25°C), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP], 10 µM adenine six-mer (3'-aaaaaa-5'), 2.5 units/µl T4 RNA ligase, 5 % PEG 6000 [Fermentas]) at 16 ºC for 15 hours. Sticky-ended dsRNA was phenol/chloroform extracted (phenol/chloroform/isoamyl alcohol (125:24:1 mixture, pH ≤6) used in 4 volumes of the reaction, aqueous phase was recovered), and precipitated with NaOAc/Ethanol. Briefly, 0.3 M NaOAc pH 4.5 was used. Ethanol was used in 3 volumes of the reaction. The mixture of reaction, NaOAc and Ethanol was incubated in -20 for 1 hour and then centrifuged for 14000 ×g for 15 minutes. Then, supernatant was removed and the pellet was washed with Ethanol 70% and air-dried and dissolved in 10 µl H_2O.

Discussion

To generate sticky termini from blunt-ended dsRNA, an adenine six-mer oligoribonucleotide (3'-aaaaaa-5') was ligated to the 5' phosphoryl of dsRNA substrate using T4 RNA ligase. The adenine six-mer was not phosphorylated to avoid concatenation. One strand of dsRNA substrate (SEQ ID NO 2) was conjugated to FAM fluorophore to track the molecule in gels. Polyacrylamide gel electrophoresis (PAGE) showed that the adenine six-mer was joined to dsRNA substrate with high efficiency (**Fig. 1b**). The addition of RNA oligomers to dsRNA substrate in this way provides a new biochemical reaction for molecular biology.

Example 2: Phosphorylation and T4 DNA ligation (Step (II), comprising adapter joining in steps (ii) and (iii) of the method of the invention).

In the second and third steps, enzymatic joining (ligation) between dsRNA substrate (comprised of annealed SEQ ID NO 1 and 2,) and dsRNA-adapter (comprised of annealed SEQ ID NO 4 and 5, obtained by mixing 2 oligo RNAs in distilled H_2O and incubation in room temperature for 5 minutes) is completed. A schematic outline of the ligation method of the invention is provided in

Figure 1a. For joining dsRNA-adapter, 5 µl of sticky-ended RNA (from Example 1) was incubated in 10 µl of the coupled phosphorylation and ligation reaction [1X T4 PNK forward reaction buffer (50 mM Tris-HCl pH 7.6 at 25°C, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA), 1 mM ATP, 5 µM RNA adapters, 0.5 units/µl T4 polynucleotide kinase, 1.5 units/µl T4 DNA ligase and 5 % PEG 4000 (Fermentas)] at 30 ºC for 15 h. The reaction was heat inactivated at 70 ºC for 10 minutes.

Analysis by Polyacrylamid Gel Electrophoresis (PAGE).

Samples were applied to polyacrylamide (Bio-Rad Laboratories Headquarters 1000 Alfred Nobel Drive,Hercules, CA 94547)/TBE gels (20 %) for native PAGE and (12 %) containing 7 M urea (run in 60 ºC water bath) for denaturing PAGE. All gels were scanned for FAM or Texas Red dyes using Typhoon 9400 scanner 5 (Amersham Biosciences, Piscataway, NJ) and the images were analysed by ImageQuant, version 5 (Amersham Biosciences, Piscataway, NJ).

Discussion

In the subsequent step(s), dsRNA substrate with the added adenine six-mer overhang was incubated along with a dsRNA-adapter having an extension of six uridines at one end (dsRNA-adapter). These molecules were then phosphorylated using T4 polynucleotide kinase and ligated using a high concentration of T4 DNA ligase. PAGE analysis and FAM fluorophore quantification showed that adapter ligation was moderately efficient (~ 50%) (**Fig. 1b**). To ensure that the gel shift was not due to hybridization of short complementary stretches, the reactions were rerun in a denaturing urea PAGE. Migration of the ligated product was retarded as expected, suggesting that the two molecules were joined covalently (**Fig. 1c**). Therefore, the ligation method of the invention can attach a dsRNA-adapter to a blunt-ended dsRNA substrate without a need for prior sequence information.

Example 3: The ligation method of the invention is specific for dsRNA.

To examine the specificity of ligation to dsRNA using the ligation method of the invention, an unmodified blunt-ended dsRNA (same as in Example 1) and an unmodified ssRNA **(**SEQ ID NO 1**)** were used as substrates. A dsRNA-adapter conjugated to Texas Red (TxRed-adapter, comprising annealed SEQ ID NO 4 and 9) was ligated to the substrates. The results show that the TxRed-adapter was only attached to dsRNA but not to ssRNA (**Fig. 2**). Therefore the method enables specific tagging or labelling of blunt-ended dsRNA.

Example 4: Reverse transcription of dsRNA-construct followed by PCR.

Y-adapter (comprising SEQ ID NO:s 10 and 11)-ligated dsRNA was converted to cDNA using ThermoScript Reverse Transcriptase (Invitrogen, Carlsbad, CA) and adapter-specific primer (SEQ ID NO 14). Two µl of ligation reaction (from Example 2) was directly transferred to a 20µl of RT reaction and incubated at 60 ºC for 1 h according to the manufacturer's protocol. PCR was carried out with adapter-specific (SEQ ID NO:s 12 and 13) or gene-specific primers (O-dsRNA-p: SEQ ID NO 15, Hok-p: SEQ ID NO 16 or TAR-p: SEQ ID NO 3) and products were run in 4% agarose/TBE gels.

Amplification of dsRNA using ligated adapters

The ligation method of the invention can be combined with RT-PCR to amplify dsRNA sequences using the primer sites provided by ligated adapters. However, as the same adapters are ligated to both ends of dsRNA, their primer sites are the same and this causes amplification from molecules that do not contain the desired sequence. Such procedures are known in the art[8]. Therefore, the inventors designed a "Y" shaped adapter (Y-adapter) that can provide two different primer sites when attached to both ends of dsRNA (**Fig. 3a**).

Discussion

Accordingly, Y-adapters were ligated to an unmodified blunt-ended dsRNA. Next, dsRNA was reverse transcribed to a full length cDNA using an adapter-specific primer. The inventors used thermo-stable reverse transcriptase to overcome secondary structure hindrance of duplex RNA. Consequently, cDNA was amplified using adapter-specific primers (Y-adapter-a & b primers). The results indicate that dsRNA ligated to adapters can be amplified by PCR and confirmed that the dsRNA substrate and adapters were covalently linked (**Fig. 3b**). Therefore, the full length dsRNA can be amplified regardless of its sequence information using primers that are specific for two arms of the Y-adapter. Again to verify the specificity of the ligation method for dsRNA, an ssRNA was included in the amplification experiment which resulted in no product (**Fig. 3b**). Therefore the ligation method combined with RT-PCR only amplifies dsRNA but not ssRNA sequences.

Example 5: Trimming of dsRNA to make blunt ends by S1 nuclease digestion

In the above ligation reactions, a purified blunt-ended dsRNA was used as substrate; however, total RNA derived from cells contains dsRNA that is rarely blunt-ended. Certain single-strand-specific nucleases, such as S1 nuclease, are known to remove single-stranded overhangs from DNA fragments leaving ligatable blunt ends [9, 10]. Therefore, the inventors tested whether S1 nuclease is capable of reliably trimming dsRNA ends in the same way.

The inventors designed a dsRNA substrate with heterogeneous overhangs (overhanging dsRNA: SEQ ID NO 6 and 2) and performed trimming reactions with S1 nuclease (**Fig. 4a**). To generate blunt ends, 40 pmol of overhanging dsRNA or 2.5 µg cellular RNA was incubated in 50 µl of the S1 digestion reaction [1X S1 reaction buffer (40 mM sodium-acetate (pH 4.5 at 25°C), 0.3 M NaCl and 2 mM $ZnSO_4$), 1 unit/µl S1 nuclease] at 30 ºC for 1 h. Trimmed dsRNA was phenol/chloroform extracted [phenol/chloroform/isoamyl alcohol (125:24:1 mixture, pH ≤6) used in 4 volumes of the reaction. Aqueous phase was recovered], precipitated with NaOAc/Ethanol/GlycoBlue® (Ambion Inc, Foster City, CA) [0.3 M NaOAc pH 4.5 was used. Ethanol was used in 3 volumes of the reaction. The mixture of the reaction, NaOAc and Ethanol and 1 µl of GlycoBlue® were incubated in -20 for 1 hour and then centrifuged for 14000 ×g for 15 minutes. Then, supernatant was removed and pellet was washed with Ethanol 70% and air-dried and dissolved in 10 µl H_2O.

PAGE analysis showed that dsRNA was trimmed effectively over a range of reaction times as well as enzyme and substrate concentrations (**Fig. 4b** and **Fig. 6**). Also, ligation of dsRNA-adapters followed by PAGE fractionation showed that the S1 treated dsRNA was blunt-ended and ligatable (**Fig. 4c**). Ligation of the trimmed dsRNA was less efficient relative to the defined blunt-ended molecules, possibly due to incomplete trimming activity, as reported for DNA trimming [11]. However, the overall efficiency appears to be sufficient for downstream applications. Therefore, the new ligation method together with S1 nuclease treatment is applicable to dsRNA with unpaired termini.

To further evaluate whether the method could be applied to cellular RNA, the inventors aimed to detect small amounts of dsRNA in a complex pool of RNA. Low nanomolar concentrations of the unmodified overhanging dsRNA (comprising annealed SEQ ID 6 and 2) were spiked into *E. coli* total RNA and subjected to S1 nuclease digestion, Y-adapter ligation and RT-PCR using an overhanging dsRNA-specific primer (O-dsRNA-p, SEQ ID NO 15) and an adapter-specific primer (Y-adapter-b, SEQ ID NO 13). PCR produced an amplicon corresponding to dsRNA sequences in agarose gel (**Fig. 4d**). Therefore, the sensitivity is sufficient to detect specific dsRNA sequences in cellular RNA present at physiological concentrations.

Example 6: Mapping a dsRNA region of *hok* mRNA and HIV TAR using the ligation method of the invention

Given the specificity and sensitivity demonstrated in the preceding examples, the inventors considered that it should be possible to use the ligation method of the invention to map dsRNA regions within RNA in total cellular preparations. The inventors chose to map RNAs that contain well studied duplex regions. In bacteria the inventors examined the hok mRNA of R1 plasmid in

E. coli and in mammalian cells the inventors examined the HIV TAR RNA. Because dsRNA may arise artificially during RNA preparation (as the consequence of hybridization in concentration steps or phenol extraction), cells were broken in a non-denaturing condition and in the presence of S1 nuclease to remove ssRNA from native riboproteins. After RNA purification, a second S1 nuclease digestion was performed to trim dsRNA and Y-adaptors were ligated using the ligation method of the invention. RT-PCR amplification using gene-specific primers (Hok-p SEQ ID NO 16 or TAR-p SEQ ID NO 3) and adaptor-specific primer (Y-adaptor-b SEQ ID NO 13) produced amplicons that corresponded in size to the dsRNA region in the predicted RNA secondary structure (**Fig. 5**). Sequencing of the amplicons confirmed ligation to the hok mRNA and TAR RNA and specified the ligation site adjacent to a dsRNA stem. In the case of TAR RNA, a three base bulge in the stem was tagged by ligation site and mapped by sequencing. Furthermore, TAR dsRNA regions were detected in the nuclear fraction but not the cytoplasmic fraction. Therefore, the new method is useful for studying dsRNA structures in cellular RNA preparations from both bacterial and mammalian cells.

SEQUENCE LISTING

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Faridani, Omid R
Good, Liam

<120> A method for enzymatic joining of a dsRNA-adaptor to a dsRNA molecule

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References

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Claims

1. A method for enzymatic joining of a dsRNA-adapter to a dsRNA substrate having a 5'-phosphate group comprising the steps of

i) generating a sticky end sequence on a blunt-ended dsRNA substrate having a 5'-phosphate group by enzymatically joining an oligoribonucleotide overhang-adapter to a 5' end of the blunt-ended dsRNA molecule;

ii) providing a dsRNA-adapter comprising a first strand comprising a sequence overhang capable of specifically annealing to the sticky end sequence generated in (i) on the dsRNA substrate so that said first strand can be subject to enzymatic joining to said dsRNA substrate and a second strand comprising a sequence capable of specifically annealing to the first strand such that said second strand can be subject to enzymatic joining to said dsRNA substrate; and

iii) enzymatically joining the dsRNA substrate to both first and second strands of the dsRNA-adapter to form a covalently joined dsRNA-construct.

2. The method according to claim 1, wherein the dsRNA substrate has been manipulated to create blunt ends with enzymes, such as endonucleases, such as S1 nuclease (EC 3.1.30.1).

3. The method according to claims 1-2 wherein a dsRNA substrate has been formed from a single-stranded RNA substrate by addition of synthetic single-stranded RNA molecule complementary to the single-stranded RNA substrate prior to any of the steps of claims 1 or 2.

4. The method according to claims 1-3, wherein the dsRNA-adapter is an Y-adapter having non-complementary portions that provide at least one unique primer binding site on each strand of the dsRNA-construct.

5. The method according to claim 1-4 wherein the overhang-adapter is not phosphorylated at 5'-end at step (i), and wherein a phosphorylation step is performed before or in conjunction with step (iii).

6. The method according to claim 5, wherein the phosphorylation step is performed enzymatically in conjunction with step (iii) using T4 polynucleotide kinase (EC 2.7.1.78).

7. The method according to claim 1-6, wherein the dsRNA-adapter strand comprising the sequence overhang (overhang-strand) is modified such that ligation is prevented at the 3'-end and the other strand (opposite-strand) is modified such that ligation is prevented at 5'-end.

8. The method according to claims 1-7, wherein T4 RNA ligase (EC 6.5.1.3) is used for the enzymatic joining step (i).

9. The method according to claims 1-8, wherein T4 DNA ligase (EC 6.5.1.1) is used for the enzymatic joining step (iii).

10. The method according to claims 1-9, wherein the dsRNA-adapter is a Y-adapter according to claim 4, further comprising a step iv) wherein a RT-PCR reaction is performed to amplify the dsRNA-construct using the primer sites provided by the enzymatically joined adapters.

11. The method according to claims 1-10, to enable analysing, profiling, labelling, mapping, tagging, cloning, amplification, or DNA-library construction of dsRNA substrates.

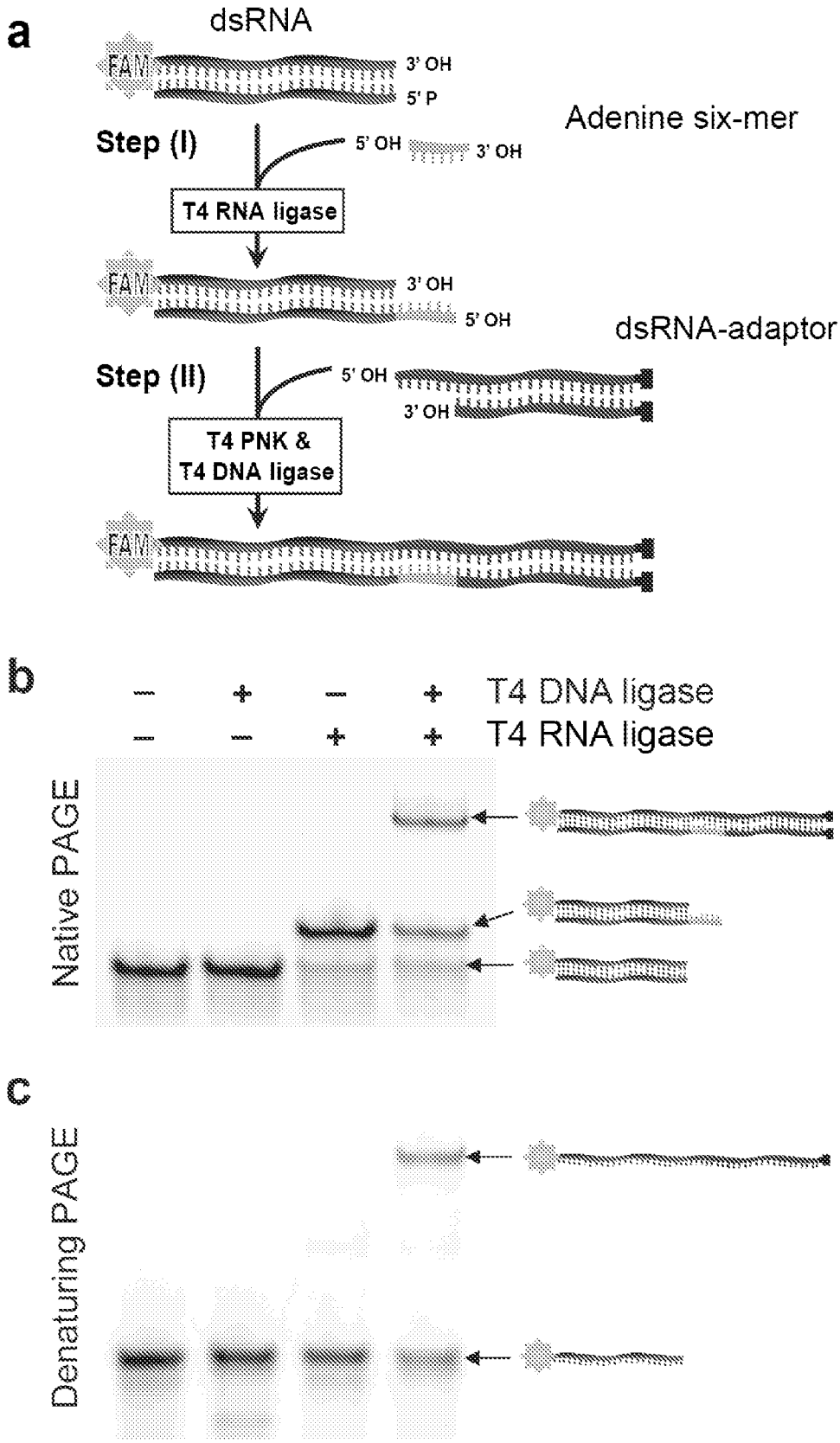
12. The method according to claims 1-11, to enable diagnosis of a microbial pathogen that contains dsRNAs.

13. The method according to claims 1-11, to enable diagnosis of an altered pathological state characterised by changes in endogenous dsRNAs.

14. The method according to claims 1-13, wherein the dsRNA substrate is synthetic dsRNAs, natural dsRNAs, siRNAs, microRNAs, derived from tissues, derived from biological fluids or derived from cells.

15. The method according to claims 1-14, wherein libraries of dsRNAs are constructed.

16. Kit for use with methods according to claims 1-15 comprising instructions for performing the method according to claims 1-15 and at least one of the following: overhang-adapter, dsRNA-adapter, labelled dsRNA-adapter, control dsRNA substrate, Y-adapter..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Adenine six-mer
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5' OH
dsRNA-adaptor
Step (II)
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Denaturing PAGE

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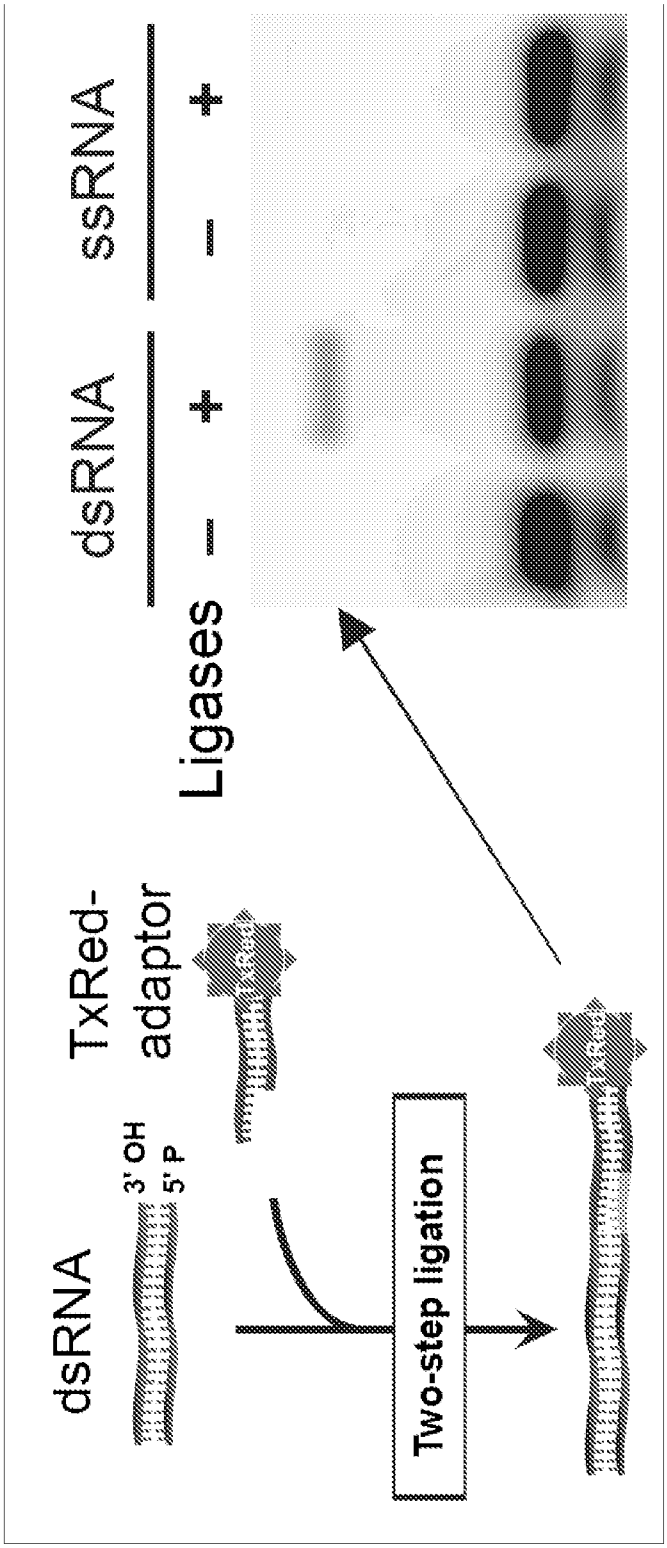
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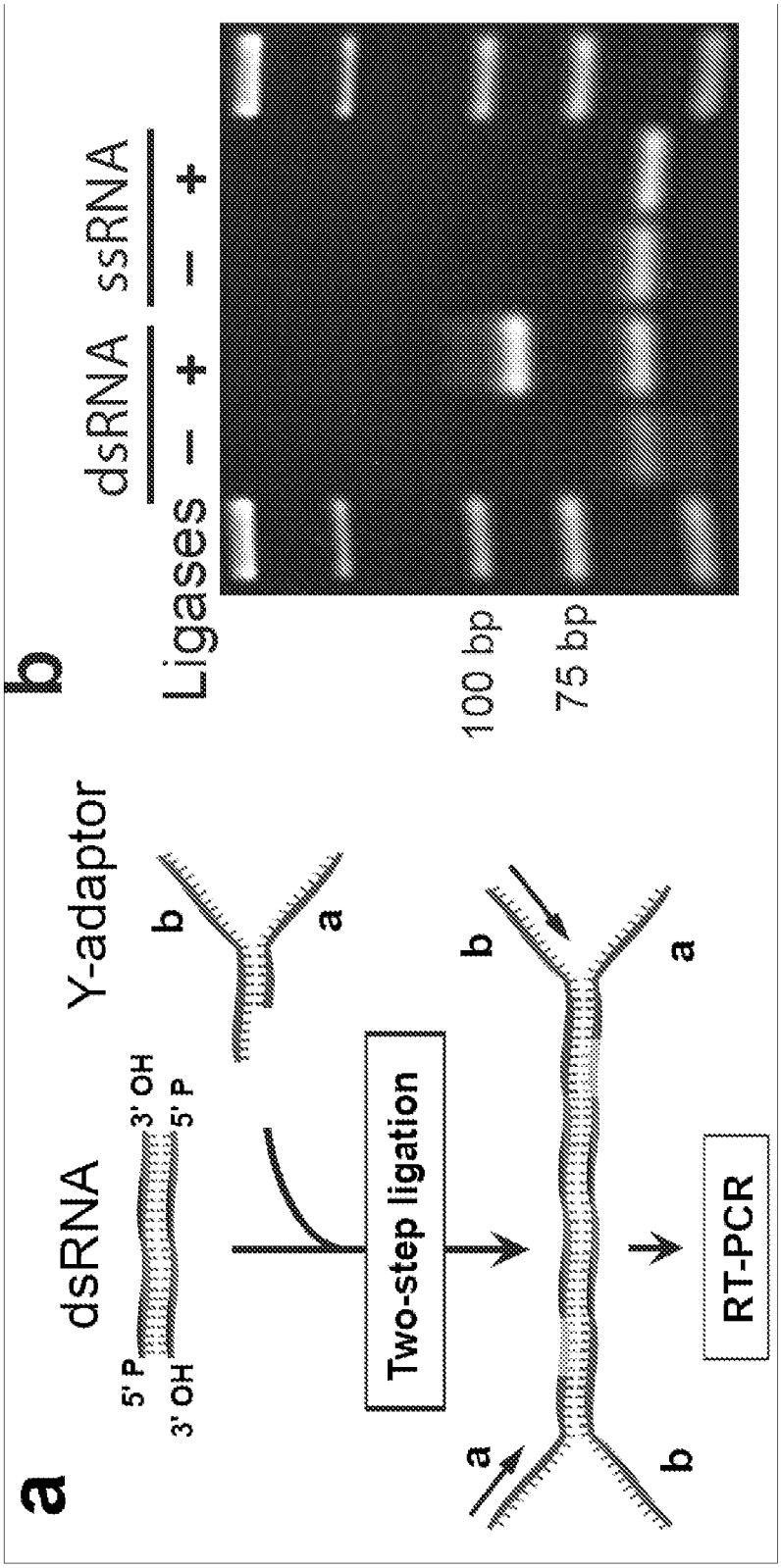
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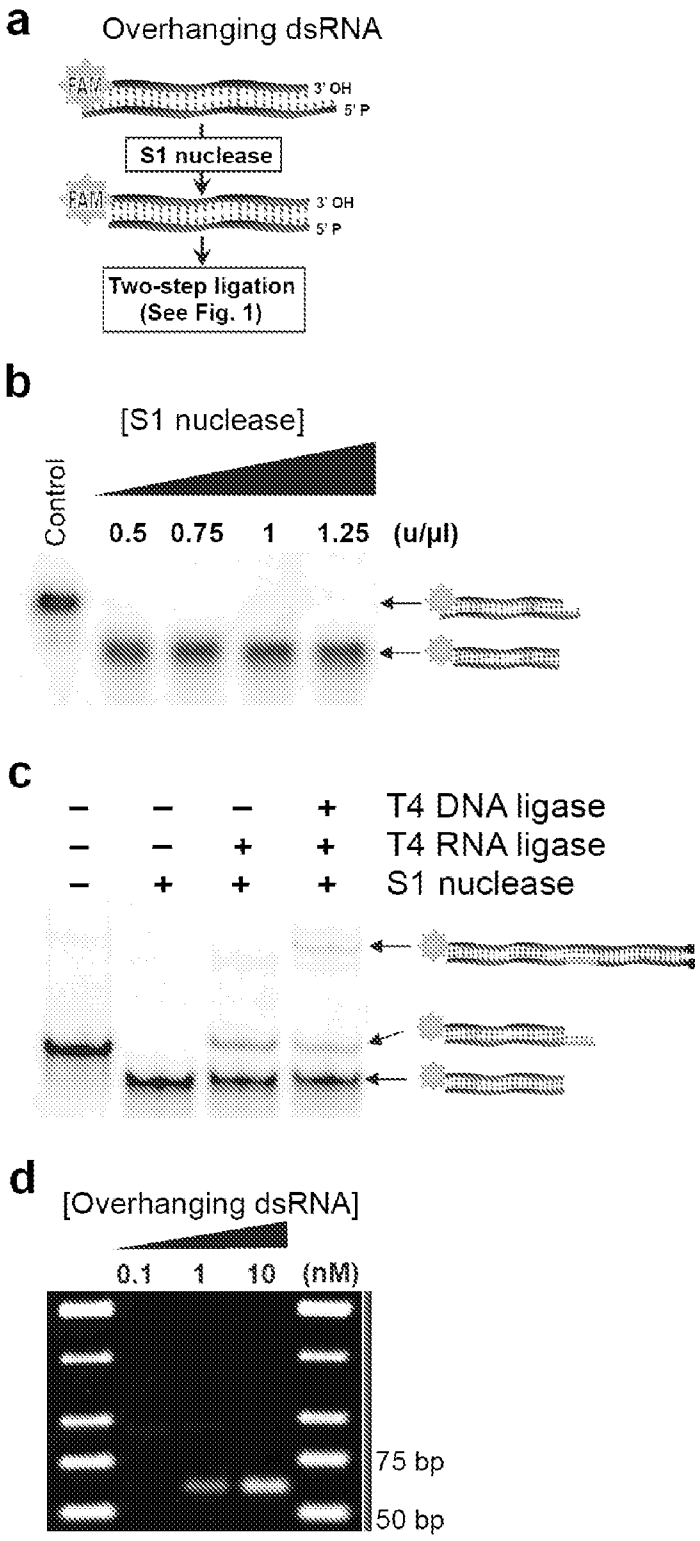
Figure 3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5' P
S1 nuclease
FAM
3' OH
5' P
Two-step ligation
(See Fig. 1)
b
[S1 nuclease]
Control
0.5 0.75 1 1.25 (u/µl)
c
– – – + T4 DNA ligase
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– + + + S1 nuclease
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Figure 4

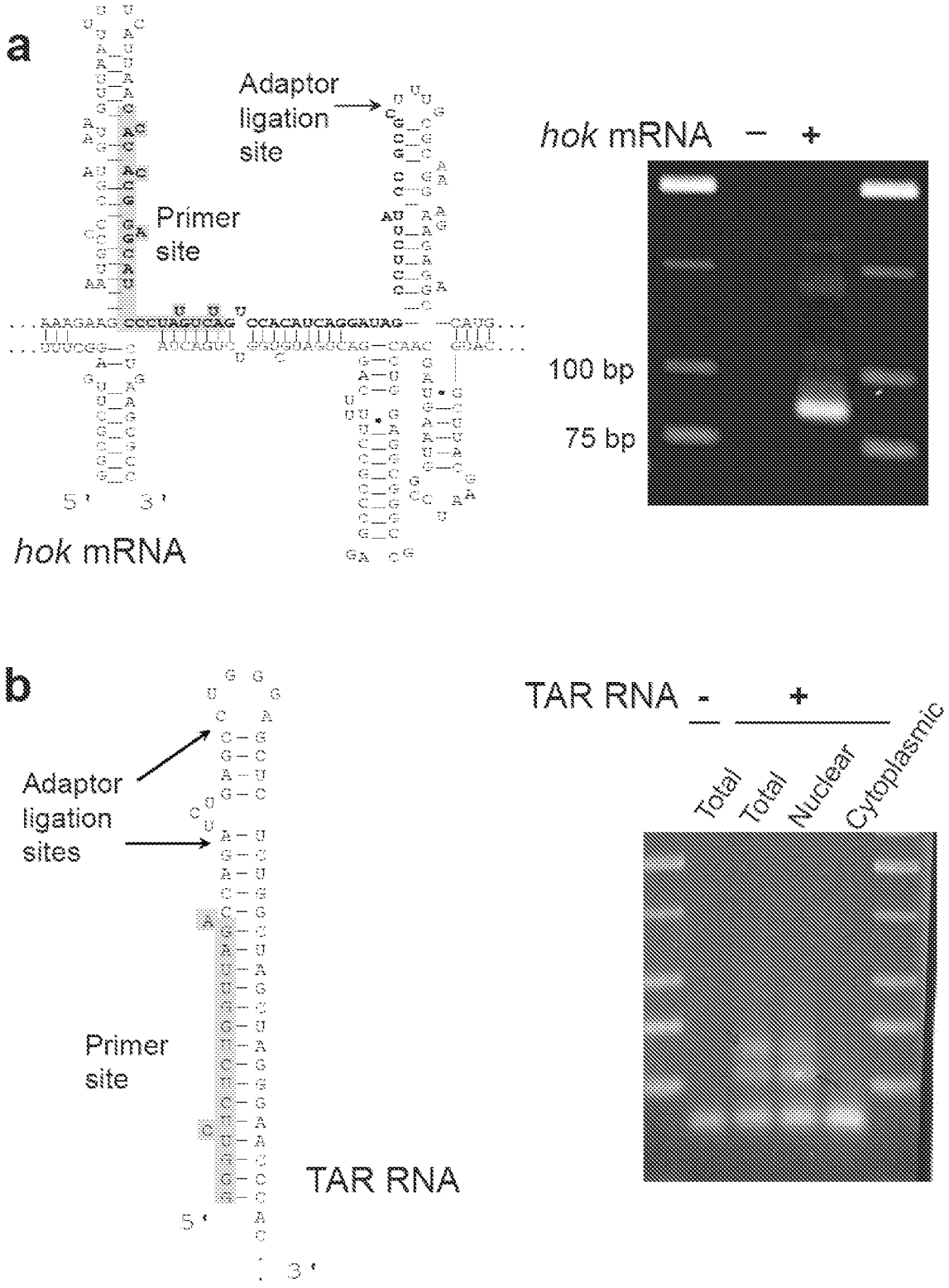
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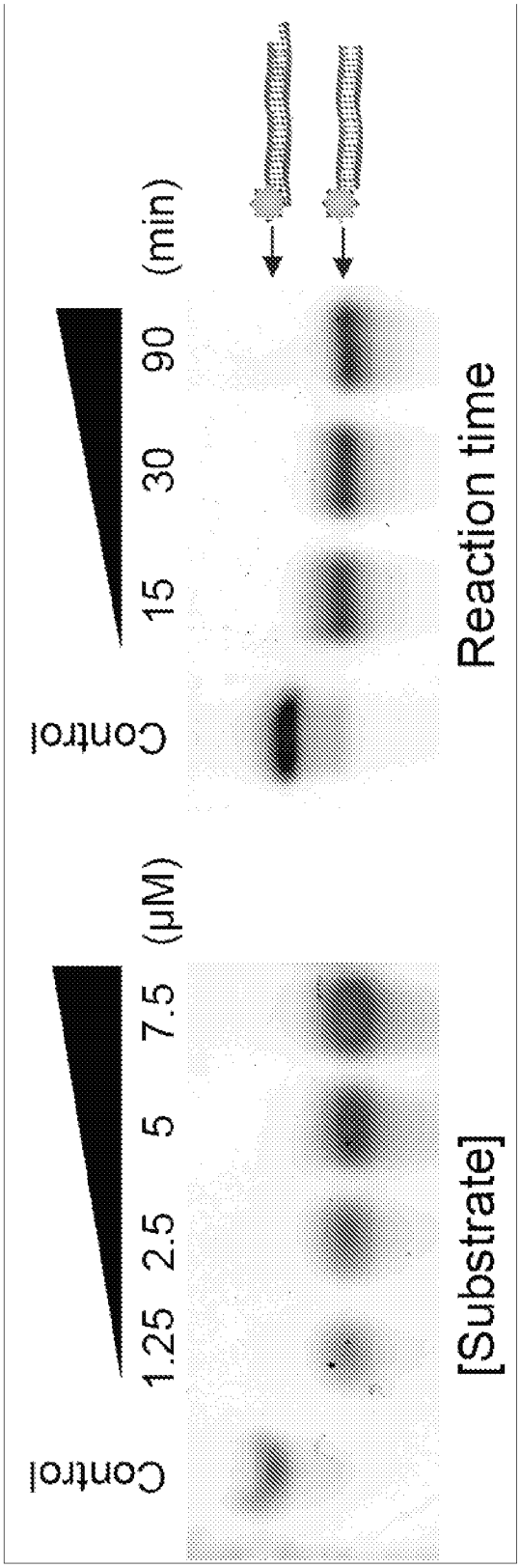
Figure 5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(min)
Reaction time

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/051396

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEKKER, N. H.et al: "Joining of longdouble-stranded RNA molecules through controlledoverhangs", Nucleic Acids Research2004. Vol. 32,No. 18, ISSN 1362-4962, p. 1-8 page 1, column 1, line 14 - page 1, column 2, line 11, page 5, column 2, line 18 - page 6, column 1, line 1, page 8, column 2,line 21 - line 28, abstract ---	1-16
Y	IMAI, M. et al, "Molecular cloning of double-stranded RNA virus genomes",Proc.Natl.Acad.Sci., January 1983,Vol. 80, No. 2,pages 373-377, ISSN 0027-8424, page 373, column 1, line 27 - line 31; page 347, column 1, line 20, column 2, line 11, abstract ---	1-16

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2009	05 -03- 2009
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Magnus Hallberg/ELY Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2008/051396

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 2006007569 A2 (SOMAGENICS, INC.), 19 January 2006 (19.01.2006), abstract, example 1	1-16
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Y	SMITH, R. E.ET AL, "Separation of the plus and minus strands of cytoplasmic polyhedrosis virus and human reovirus double-stranded genomeRNAs by gel electrophoresis", Nucleic Acids Res., October 1981, Vol. 9, No. 20, pages 5269-5286, ISSN 0305-1048, page 5270, line 7 - line 12	1-16
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A	ALI, A., ET AL,"Sequenceanalysis of TnphoA insertion sites in Vibriocholerae mutants defective in rugose polysaccharide production", Infection and immunity December 2000, Vol. 68, No. 12, pages 6857 - 6864, ISSN 0019-9567 page 6858 - 6859, figure 1	4,10
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INTERNATIONAL SEARCH REPORT

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