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(54) **METHOD FOR THE NON-SPECIFIC AMPLIFICATION OF NUCLEIC ACID**
VERFAHREN ZUR UNSPEZIFISCHEN AMPLIFIZIERUNG EINER NUKLEINSÄURE
PROCEDE D'AMPLIFICATION NON SPECIFIQUE D'ACIDE NUCLEIQUE

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Description

[0001] The present invention is concerned with a method for generating, in a non specific manner, multiple copies of RNA from a pool of mRNA's. Such a method is of particular importance in techniques for screening the differences in expression in given cell types or in cells under specific conditions.

[0002] In cells of higher organisms only some 15% of the genes present (each cell contains about 100,000 genes) is expressed. Gene expression varies between different cell types and between different stages of development of a given cell and is crucial to all biological processes, such as aging, cell differentiation, and infectious or other disease states. Thus the identification of genes that are differentially expressed in cells under different conditions is of prime interest in cellular biology.

[0003] To be able to analyze the mRNA content derived from only a few cells a method is needed to amplify the mRNA present in the cell(s) under investigation. Much effort has already been put in methods to examine the mRNA population of a cell. This has lead to the development of techniques to label nucleic acid material starting from the mRNA population of a cell aimed at the identification of genes that are differentially expressed in cells under various conditions.

[0004] One method for screening differences in gene expression is a method known as Differential Display (Liang and Pardee, Science, Vol 257, 967-971, 1992; US 5,262,311 which issued on Nov. 16, 1993). With the method of Liang and Pardee mRNA is first transcribed into cDNA and amplified using the Polymerase Chain Reaction (PCR). A set of oligonucleotide primers is used, the first of which is anchored to the polyadenylated tail of a subset of mRNA's, the other being short and arbitrary in sequence so that it anneals at different positions relative to the first primer. The method is used with different pairs of alterable sequences aiming at the amplification of as many mRNA's as possible from the cells under investigation. The PCR products are labeled using tracer amounts of labeled (radioactive) nucleotides.

[0005] An improvement on the Differential Display method of Liang and Pardee was disclosed in US 5,589,726. The method described in US 5,589,726 differs from the method of Liang and Pardee in that it uses longer primers (22-30 nucleotides compared to the 9-14 base primers originally described by Liang and Pardee).

[0006] Another alleged improvement over the Differential Display technique as originally disclosed by Liang and Pardee is disclosed in WO 97/37045. In this application a method is disclosed that, again, is based on PCR: This method uses an oligo-dT primer rather than an anchored primer. Thus, after the reverse transcription step only one cDNA population covering all possible mRNA sequences is created. The cDNA thus obtained is titrated into the PCR process by running several PCR reactions at decreasing concentrations of cDNA. This serves to calibrate the method and to protect it against false negatives. The PCR reaction may be performed with anchored primers again.

[0007] Yet another method for "expression profiling" of mRNA's is disclosed in US 5.514,545. With this method mRNA in a single cell can be characterized by microinjecting into a cell a first amplification oligonucleotide comprising oligo-dT and the sequence of a bacteriophage promoter such as T7, T3 or SP6, reverse transcriptase and nucleotides to synthesize a first strand of cDNA from the mRNA in the cell. From the first strand of cDNA double stranded cDNA is synthesized. Since this double stranded cDNA includes a functional promoter aRNA (anti-sense RNA) can now be synthesized therefrom using an RNA polymerase. The aRNA is now reamplified using random hexanucleotide primers with a reverse transcriptase to form first strand cDNA.

[0008] US 5,545,522 discloses a method of non-specifically generating multiple RNA copies of a target mRNA by using RNA polymerase. In this method, cDNA is synthesized from a RNA sequence using a complementary primer linked to an RNA polymerase promoter region complement and then anti-sense RNA (aRNA) is transcribed from the cDNA by introducing an RNA polymerase capable of binding to the promoter region.

[0009] With all the above techniques cDNA is made starting with a primer using the mRNA as a template. However, the enzyme that is used for this reaction (reverse transcriptase) is hampered in the cDNA synthesis by structures in the mRNA. As a result the prior art methods are selective for mRNA's with little or no structure. This effect is further enhanced if the synthesized cDNA is amplified further, for instance by PCR. Due to the aforementioned it is common practice to use a large sample amount in these type of expression profiling analysis. Thus this technical threshold does not allow the analysis of only a few cells isolated on a cell sorter or a few cells isolated via micro dissection from a glass slide after microscope identification and selection.

[0010] The solution to the problem is the use of a non-selective poly A mRNA labeling and amplification method, i.e. a method not encompassing cDNA synthesis.

[0011] The present invention provides such a method. The present invention is directed to a method for amplifying RNA by creating, in a non specific manner, multiple RNA copies starting from nucleic acid containing starting material comprising a pool of mRNA's each mRNA comprising a poly-A tail, wherein the material is contacted simultaneously with an oligonucleotide comprising an oligo-dT sequence at its 3' end, the sequence of a promoter recognized by a RNA polymerase at its 5' end and a transcription initiation region which is located between the oligo-dT sequence and the sequence of the promoter wherein the oligonucleotide is blocked at its 3' end in such a way that extension therefrom is prohibited, and further with an enzyme having reverse transcriptase activity, an enzyme having RNase H activity and an enzyme having RNA polymerase activity and the necessary nucleotides and the resulting reaction mixture is maintained under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

[0012] This will lead to the formation of multiple anti-sense RNA copies of the mRNA's present in the reaction mixture. The method of the present invention does not involve the production of cDNA intermediates; RNA is copied directly from the mRNA present in the material under investigation. The method of the present invention does not need a cDNA as a basis for the amplification of the RNA. The RNA is synthesized by an RNA polymerase, directly from the mRNA template. The activity of the RNA polymerase is independent from any secondary structures present in the mRNA and thus there are no differences in the way the different mRNA's are amplified depending on structures in the mRNA's. The copies made represent the original mRNA population as present in the starting material.

[0013] The oligonucleotides used with the method of the invention comprise an oligo-dT sequence which will hybridize to the poly-adenylated tail at the 3' end of the mRNA's. The oligonucleotides further comprise the sequence of a promoter recognized by an RNA polymerase and a transcription initiation region which is located between the oligo-dT sequence and the sequence of the promoter. The promoter may be the promoter for any suitable RNA polymerase. Examples of RNA polymerases are polymerases from E. coli and bacteriophages T7, T3 and SP6. Preferably the RNA polymerase is a bacteriophage-derived RNA polymerase, in particular the T7 polymerase.

[0014] The oligonucleotide is blocked at its 3' end to prevent any extension therefrom by the reverse transcriptase along the RNA template; the reverse transcriptase will not be able to start extension of the 3' end of the oligonucleotide and no cDNA is synthesized. The reverse transcriptase does synthesize a complementary strand of the promoter sequence present in the template. The use of the oligonucleotide is depicted schematically in figure 1. Upon hybridization of the oligonucleotide the poly-A sequence of the mRNA is cut by an enzyme having RNase H activity. This activity may be the RNase H activity of the reverse transcriptase or the RNaseH activity of a separate enzyme like, for example, E.coli RNaseH, or both. In that respect preferred transcriptases used with the method of the invention are transcriptases having RNaseH activity, such as AMV-RT or MMLV-RT. The newly generated 3' end of the RNA is extended on the oligonucleotide template to generate a double stranded promoter sequence. By application of the RNA polymerase new RNA copies of the original mRNA are made. During this transcription step labels may be incorporated and typically 100-1000 copies of each RNA are being made. The copies made are antisense RNA and thus comprise a poly-T stretch at the 5' end.

[0015] Since the RNA polymerase normally uses a double stranded template for the transcription the enzymes is not likely to be hampered by structures in the mRNA. Furthermore, the processivity of, for example, the T7 RNA polymerase is very high, usually more than 250 nucleotides per second on a DNA template. This means that the amplification rate is determined by the number of initiation events per promoter, per time unit. Since the promoter is identical for each mRNA there is no selectivity in the amplification.

[0016] The conditions under which the reactions should be performed are the normal conditions, i.e. buffer constitutions and temperatures, known to be optimal for the mix of enzymes used.

[0017] If the interest exists to make an expression profile of just a few cells the above described amplification may not yield enough copies of the RNA, for example to generate a signal if the copies are labeled. In certain special cases the RNA may need to be amplified further without introducing selectivity, thus again avoiding i.e. cDNA synthesis. There are multiple solutions to this problem, all transcription based. An elegant solution is depicted in figure 2. The newly synthesized RNA may now be further amplified by the following method. To the 3' end of every RNA molecule a double stranded promoter sequence is ligated by using RNA ligase. Since all 3' ends are chemically identical there is no selectivity. The ligated promoter is used to initiate a second round of transcription generating more (labeled) RNA. This is illustrated in figure 2.

[0018] Thus in a preferred method of the invention the generated RNA copies made as described above are contacted with an RNA ligase, a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by a RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands, an enzyme having RNA polymerase activity, and the necessary nucleotides. The resulting reaction mixture is maintained under the appropriate conditions for a sufficient amount of time for the amplification to take place

[0019] Again, one or more of the nucleotides used may be labeled.

[0020] Due to the orientation of the RNA polymerase promoter sequence the RNA template is used to generate new sense strand RNA molecules. Typically 100-1000 copies of each RNA is being made in the transcription reaction by the RNA polymerase.

[0021] Preferably the stretch of RNA attached to the 5' end of one of the DNA strands is phosphorylated at the 5' end. Phosporylation enables the 5' end to be ligated.

[0022] The promoter may be the same as in the first part of the procedure for example, the T7-promoter sequence may be used and the RNA polymerase than is T7 RNA polymerase.

[0023] Interestingly the sense RNA made in this second round of transcription contains again a poly A stretch at the

3' end making is possible to perform multiple cycles of amplification by repeatedly performing the method as illustrated by figure 1 and the method using the ligase as illustrated in figure 2.

[0024] The procedure wherein the ligase is used may be performed as a separate reaction. That is, after RNA copies have been generated in a procedure like the one depicted in figure 1, the RNA copies may be transferred to another reaction medium and subjected to the second reaction.

[0025] When all enzymes and the oligonucleotide and the promoter construct are combined with the initial reaction mixture a continuous process may even be obtained. Another elegant method to further enhance the amplification factor of the non-biased mRNA amplification method is by adding a poly A nucleotide stretch to the 3' end of the newly synthesized RNA. The poly nucleotide stretch is added by the enzyme poly A polymerase. To this added poly A sequence the oligonucleotide, encompassing an oligo T stretch and T7 promoter can hybridize again and the previously described process may take place again. As a result again RNA will be made by the transcription process and this newly synthesized RNA will be identical (for the large part) to the original mRNA that the whole reaction started with in the first place. One skilled in the art understands that the oligonucleotide, encompassing an oligo T stretch and T7 promoter can also hybridize again to this RNA and now the process has entered in a continuous process of RNA synthesis by transcription, oligonucleotide annealing and double strand promoter synthesis. Thus in a preferred method of the invention the generated RNA copies made as described before in the basic method are contacted with a poly A polymerase, an oligonucleotide, encompassing an oligo T stretch and T7 promoter, a reverse transcriptase, a RNase H, a RNA polymerase and the necessary nucleotides. The resulting reaction mixture is maintained under appropriate conditions for a sufficient amount of time for the amplification to take place. In the mix one or more nucleotides used may be labeled.

[0026] Due to the position of the newly added poly A stretch (3' end of the RNA molecules) the RNA polymerase will generate RNA of the opposite polarity. The oligonucleotide, encompassing an oligo T stretch and T7 promoter may be the same as in the first part of the procedure. The procedure in which the poly A polymerase is added may be performed as a separate reaction. That is, after RNA copies have been made in a procedure like the one depicted in figure 1, the RNA copies may be transferred to another reaction medium and subjected to the reaction with poly A polymerase. Starting a continuous amplification process. When the poly A polymerase is added to the initial reaction mixture the continuous amplification process may even start immediately from the original mRNA template.

BRIEF DESCRIPTION OF THE FIGURES:

[0027]

Figure 1: Schematic presentation of non-selective poly A mRNA amplification based on transcription. RNase H activity necessary for cutting the RNA can be associated with the AMV-RT.

Figure 2. Schematic presentation of the second round of non-selective amplification of the RNA products resulting from the first described non-selective amplification scheme (such as the one depicted in figure 1).

Figure 3. Silver stained Cleangel analysis of Tyras reactions using different dilutions of in vitro transcribed RNA form example 1 as template.

1) 50 x dilution
2) 100 x dilution
3) 500 x dilution
4) 1000 x dilution
5) no template
M) 100-400 nt marker
i) input

Figure 4. Autoradiograph of Cleangel showing the radiolabeled Tyras amplification products. Lane 1, input poly A+ RNA; lane 2, 5 minute labeling; lane 3, 10 minute labeling; lane 4, 20 minute labeling; lane 5, no template reaction; lane M, non-labeled marker.

Figure 5. Autoradiograph of the probe array filer described in table 1 interrogated with the Tyras reaction mixture of example 3.

Figure 6. Autoradiograph of the filter with the "E quadrant" of an Atlas Human cDNA Expression Array. The visible spots dearly indicate hybridization of labeled poly A+ RNA by Tyras. The arrow points to a control spot of G3PDH

probe OT1446 that was also positive in example 4 (see figure 5).

Figure 7. Silverstained Cleangel analysis of Tyras reactions using the poly A polymerase added poly A tail as target for hybridization of the T stretch encompassing oligonucleotide.

Lane 1, Tyras reaction using 5 microliter of reaction A as input: lane 2, Tyras reaction using 5 microliter of reaction B as input; lane 3 and lane 4, negative control lanes; M is marker lane. The arrow indicates the position of the specific amplification product.

EXAMPLES:

Introduction

[0028] The method used in the examples is an embodiment of the method of the invention and referred to in the examples as "Tyras". The method referred to as Tyras comprises the hybridization of an oligonucleotide, encompassing an oligo T stretch, to the poly A tail of the mRNA followed by RNase H digestion opposite the oligonucleotide and extension of the newly formed 3' end of the mRNA with reverse transcriptase. In this way the T7 RNA polymerase recognition sequence (i.e. T7 promoter) that is part of the oligonucleotide encompassing an oligo T stretch is made doubts stranded. Upon binding of the T7 RNA polymerase to the promoter the original mRNA molecules are transcribed in multiple RNA copies of the opposite polarity ***(see figure 1).***

Materials

[0029] Most enzymes, radiolabeled nucleotides, acrylamide Cleangels and oligonucleotides were purchased from Amersham Pharmacia, Bergrand 230, 4707 AT Roosendaal, The Netherlands. AMV-reverse transcriptase was purchased from Seikagaku, Rockville, MD 120248,USA. Human poly A+ RNA was purchase from ClonTech/Westburg, PO Box 214, 3830 AE Leiden, The Netherlands.

Example 1.

[0030] Plasmid pG30 containing part of the genomic hepatitis B virus (HBV) sequence (nucleotide numbers 1662-1914, reference: Lai,M.E et al. (1991). Sequence analysis of hepatitis B virus genome of a new mutant of ayw subtype isolated in Sardinia **Nucleic Acids Res. 19 (18), 5078)** cloned in the Eco RI site behind the T7 promoter was used to generate in vitro transcribed RNA containing a poly A stretch (25 nts) adjacent to the HBV sequence (nucleotide numbers 1662-1914). The plasmid pG30 was digested with restriction enzyme Hind III according to a standard protocol as known by persons skilled in the art. The linearized plasmid was transcribed in a standard T7 RNA polymerase in vitro transcription reaction (composition: tris-HCl 40 mM, pH=7.5, $MgCl_2$ 6 mM, spermidine 2 mM, NaCl 10 mM, DTT 10 mM, rNTP's 0.5 mM each, RNA Guard 20 units and 46.5 units T7 RNA polymerase) for 3 hours at 37 °C. The length of the in vitro transcribed RNA is 306 nucleotides. The in vitro transcribed RNA was DNase I treated (1 µl, 10 units) for 30 minutes at 37°C. Following the DNase I treatment the in vitro transcribed RNA was phenol/chloroform purified and ethanol precipitated with standard protocols known to persons skilled in the art. The pelleted in vitro transcribed RNA was dissolved in 20 µl water and dilutions in water used for subsequent experiments.

Example 2

[0031] The in vitro transcribed RNA (APPROXIMATELY 1 µg/µl) generated in example 1 was used as template to generate new RNA in a Tyras reaction. The in vitro transcribed RNA from example 1 was diluted 50, 100, 500 and 1000 times, respectively, in water. The Tyras reactions contained: 2 µl water, 4 µl 5x NN buffer (Tris-HCl 200 mM, pH 8.5, MgCl2 60 mM, KCL 350 mM, DTT 25 mM, dNTP's 5 mM each, rATP 10mM, rUTP 10mM, rCTP 10mM, rGTP 7.5 mM, ITP 2.5 mM), 4 µl primer mix (76.9 µl 100% DMSO, 11.6 µl oligonucleotide PH26 [42.9µM, sequence 5' AAT TCT AAT ACG ACT CAC TAT AGG GAG AGA AGG ATA CCA CTA GCT AGC GTT TTT TTT TTT TTT TTT TTT TTT 3'-biotine] and 11.5 µl water for total volume of 100 µl) and 5 µl of the appropriate dilution of in vitro transcribed RNA from example 1. The reaction was incubated at 65°C for 5 minutes and subsequently at 41°C for 5 minutes. Than 5 µl enzyme mix (sorbitol 1.5M, BSA 2.1 µg, RNase H 0.08 units, T7 RNA polymerase 32units and AMV-Reverse Transcriptase 25.3 units) was added to the reaction and gently mixed by tapping the tube. After a short incubation of 5 minutes 41°C the tubes were briefly spun in a centrifuge to collect all the droplets on the bottom of the tube. The reactions were than incubated for 90 minutes at 41°C. After the reaction the tubes were stored at -20°C.

[0032] The reactions were analyzed on a 10% acrylamide Cleangel, 0.5 µl of the Tyras reaction was mixed with 7.5 µl formamide loading dye (Ambion, 2130 woodward St. #200, Austin, Texas 78744-1832, USA) and run on the Cleangel according to the manufacturers protocol. The result is depicted in figure 3.

Example 3

[0033] In this example the Tyras reaction was used to generate ^{32}P radiolabeled RNA from human placenta poly A+ RNA template. The Tyras reaction contained 4 µl 5x NN* buffer (Tris-HCl 200 mM, pH 8.5, MgCl2 60 mM, KCL 350 mM, DTT 25 mM, dNTP's 5 mM each, rGTP 10mM, rUTP 10mM, rCTP 10mM), 4 µl primer mix PH26 (see example 2), 6.5 µl α-^{32}P-ATP and 0.5 µl human poly A+ RNA (1 µg/µl, Clontech lot NR. 7050106, Cat.6518-1). The ingredients were mixed tapping the tube and incubated at 65°C for 5 minutes and subsequently at 41°C for 5 minutes. Than 5 µl enzyme mix (sorbitol 1.5M, BSA 2,1 µg, RNase H 0.08 units, T7 RNA polymerase 32 units and AMV-Reverse Transcriptase 25.3 units) was added to the reaction and gently mixed by tapping the tube. After a short incubation of 5 minutes 41°C the tubes were briefly spun in centrifuge to collect all the droplets on the bottom of the tube. In three tubes labeled A, B and C , respectively, 0.4 µl rATP (100 mM) was added after 0, 5 and 15 minutes incubation at 41°C, respectively. After the addition of the rATP the reactions were incubated for 90 minutes at 41°C. After the reaction the tubes were stored at -20°C.

[0034] The reactions were analyzed on a 10% acrylamide Cleangel, 0.5 µl of the Tyras reaction was mixed with 7.5 µl formamide loading dye (Ambion) and run on the Cleangel according to the manufacturers protocol. The result is depicted in figure 4.

Example 4

[0035] The Tyras reactions of example 3 were pooled and used to interrogate a filter probe array. The composition of the probe array is shown in table 1, the oligonucleotides were spotted on a zeta-probe membrane (BioRad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA).

[0036] The pooled Tyras reactions from example 3, in total 58.5 µl were added to 25 ml hybridization mix (5x SSC [20x SSC is NaCl 3M, Na-citrate0.3M], 7% SDS, 20 mM NaPi, 10x Denhardts [100x Denhardts is Polyvinylpyrolidone 2%, BSA 2% and Ficol 2%]). The filter with the probe array was incubated in the hybridization mix for 16 hours (O/N) in a shaking incubator at 42°C.

Table 1.

Composition of the oligonucieotide probe array interrogated with the labeled Tyras reactions of example 3.			
gamma-actin (+)probe: OT858 3.0^13 molecules	gamma-actin (+)probe: OT858 6.0^11 molecules	gamma-actin (+)probe: OT859 3.0^13 molecules	gamma-actin (+)probe: OT859 6.0^11 molecules
gamma-actin (+)prabs: OT860 3.0*13 molecules	gamma-actin (+)probe: OT860 6.0^11 molecules	G3PDH P2: OT1446 3.0^13 molecules	G3PDH P2: OT1446 6.0^11 molecules
G3PDH P2: OT1447 3.0^13 molecules	G3PDH P2: OT1447 6.0^11 molecules	G3PDH P2: OT1448 3.0^13 molecules	G3PDH P2: OT1448 6.0^11 molecules
Factor V P1: OT1915 P1: OT1915 3.0^13 molecules	Factor V P1: OT1915 P1: OT1915 6.0^11 molecules		

[0037] The probe array filter was washed two times in 3x SSC/1% SDS for 7 minutes at room temperature. After the washes the damp filter was wrapped in foil and exposed to an X-ray film O/N at -70°C. The result of the exposure is shown in figure 5. The autoradiograph clearly shows a positive signal at the position of the G3PDH probe OT1446 on the array.

***Example** 5*

[0038] In this example the Tyras reaction was used to generate ^{32}P radiolabeled RNA from human poly A+ RNA template. The Tyras reaction contained 4 µl 5x NN* buffer (Tris-HCl 200 mM, pH 8.5, MgCl2 60 mM, KCL 350 mM, DTT 25 mM, dNTP's 5 mM each, rGTP 10mM, rUTP 10mM, rCTP 10mM), 4 µl primer mix PH26 (see example 2), 6.5 µl α-^{32}P-ATP (reactions A and B) or 5.5 µl α-^{32}P-ATP (reactions D, E and C) and 0.5 µl human poly A+ RNA (Clontech lot NR. 7050106, Cal.6518-1), in reaction C only water was added since this was the negative control. The ingredients were mixed by pipetting up and down 5 times and incubated at 65°C for 5 minutes and subsequently at 41°C for 5 minutes. Than 5 µl enzyme mix (sorbitol 1.5M, BSA 2.1 µg, RNase H 0.08 units, T7 RNA polymerase 32units and

AMV-Reverse Transcriptase 25.3 units) was added to the reaction and gently mixed by tapping the tube. To reaction C, D and E 1µl of T7 mix (31 units T7 RNA polymerase and 0.6 units RNase H) was added and gently mixed by tapping the tube. After a short Incubation of 5 minutes 41°C the tubes were briefly spun in centrifuge to collect all the droplets on the bottom of the tube. in all tubes 0.4 µl rATP (100 mM) was added. After the addition of the rATP the reactions A and B were incubated for 90 minutes at 41°C and reaction C, D and E for 150 minutes at 41°C. After the reaction the tubes were stored at -20°C. The reactions A, B and D were pooled and used to interrogate the "E quadrant" of an Atlas Human cDNA Expression Array (Clontech Laboratories, 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA, lot number 7090625). The filter with the "E quadrant" of an Atlas Human cDNA Expression Array was incubated in hybridization mix (see example 4) for 15 minutes at 50°C. The pooled reactions A, B and D were added to the hybridization mix on the filter and further incubated for 16 hours (O/N). After the hybridization the filter with "E quadrant" of an Atlas Human cDNA Expression Array was washed 4 times with 3x SSC/1% SDS at room temperature. The damp filter was wrapped In foil and exposed to an X-ray film at -70°C for 65 hours. The result is depicted in figure 6 below and clearly shows positive spots on the array indicating good labeling of the poly A+ RNA with the Tyras method.

Example 6

[0039] In this example the addition of poly A polymerase to enhance the amplification of the Tyras reaction was investigated. The reaction consisted of model RNA not containing a poly A tail (see also example 1) ATP 1mM, tris 50 mM, pH=7.9, NaCl 250 mM, $MgCl_2$ 10 mM, BSA 2.5 mg/ml and poly A polymerase (Gibco BRL, catalogue number 18032-011) 1.3 units in a total volume of 30µl. The reactions were incubated at 37°C for 20 minutes (reaction A) or 60 minutes (reaction B). Subsequentiy the products of the reaction that now have a newly added poly A stretch to the 3' end were used in a Tyras reaction as described in example 2. After the 90-minute incubation at 41°C the Tyras reaction products were analyzed on a 10% acrylamide Cleangel. For loading on the gel 0.5 µl of the Tyras reaction was mixed with 7.5 µl formamide loading dye (Ambion, 2130 woodward St. #200, Austin, Texas 78744-1832, USA) and run on the Cleangel according to the manufacturers protocol. The result is depicted in figure 7.

[0040] Although many of the visible bands on the gel seem to be the result of ingredients in the reaction (see lane 3 and 4) at the position of the arrow a specific band can be observed in lanes 1 and 2. This result cleariy indicates that it is possible to add a poly A stretch to RNA and subsequently use this newly added poly A stretch as start for the Tyras amplification.

Claims

1. Method for creating, in a non specific manner; multiple RNA copies starting from nucleic acid containing starting material comprising a pool of mRNA's each mRNA comprising a poly-A tail wherein the material is simultaneously contacted with:

   - an oligonucleotide comprising an oligo-dT sequence *at its 3' end,* the sequence of a promoter recognized by a RNA polymerase *at its 5' end* and a transcription initiation region which is located between the oligo-dT sequence and the sequence of the promoter *wherein the oligonucleotide is blocked at its 3' end in such a way that extension therefrom is prohibited,*
   - an enzyme having reverse transcriptase activity
   - an enzyme having RNase H activity and
   - an enzyme having RNA polymerase activity,
   - *sufficient amounts of dNTP's and rNTP's* and the resulting reaction mixture is maintained under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

2. Method according to claim 1, wherein the promoter sequence is the T7-promoter sequence and the RNA polymerase is T7 RNA polymerase.

3. Method according to any of the preceding claims wherein the enzyme having reverse transcriptase activity is AMV-RT or MMLV-RT.

4. Method according to any of the preceding claims wherein (one of) the enzyme(s) having RNase H activity is *E. coli* RNase H.

5. Method according to claim 1 wherein the enzyme having RNase H activity is a reverse transcriptase.

6. Method according to claim 5 wherein the enzyme having RNase H activity is AMV-RT or MMLV-RT.

7. Method according to any of the preceding claims, wherein at least one of the nucleotides is provided with a label.

8. Method according to any of the preceding claims wherein the generated RNA is used as input material for further amplification.

9. Method according to claim 8 wherein the generated RNA copies made by the method described in claim 1 are contacted with

- a RNA ligase,
- a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by a RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands,
- an enzyme having RNA polymerase activity,
- sufficient amounts of dNTP's and NTP's,

and the resulting reaction mixture is maintained under the appropriate conditions for a sufficient amount of time for the enzymatic processes amplification to take place

10. Method according to claim 9, wherein the stretch of RNA attached to the 5' end of one of the DNA strands is phosphorylated at the 5' end

11. Method according to claim 10, wherein the promoter sequence is the T7-promoter sequence and the RNA polymerase is T7 RNA polymerase.

12. Method according to claim 10, wherein at least one of the nucleotides is provided with a label.

13. Method according to claim 1, wherein the reaction mixture further comprises an RNA ligase and a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by the RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands.

14. Method according to claim 1, wherein the generated RNA copies made by the method described in claim 1 are contacted with a poly A polymerase.

Patentansprüche

1. Verfahren zum Erzeugen, auf nicht spezifische Weise, von zahlreichen RNA-Kopien ausgehend von einem Nukleinsäure-enthaltenden Ausgangsmaterial, das einen Pool von mRNAs umfaßt, wobei jede mRNA einen PolyA-Schwanz umfaßt, wobei das Material gleichzeitig in Kontakt gebracht wird mit:

- einem Oligonukleotid umfassend eine Oligo-dT-Sequenz an seinem 3'-Ende, eine Promotor-Sequenz, die von einer RNA Polymerase erkannt wird, an seinem 5'-Ende und eine Transkriptionsinitiationsregion, die zwischen der OligodT-Sequenz und der Promotor-Sequenz liegt, wobei das Oligonukleotid an seinem 3'-Ende derart blockiert ist, daß die Extension vom 3'-Ende aus verhindert wird,
- einem Enzym mit reverser Transkriptase-Aktivität,
- einem Enzym mit RNaseH-Aktivität und
- einem Enzym mit RNA Polymerase-Aktivität,
- genügend großen Mengen an dNTPs und rNTPs;

und das resultierende Reaktionsgemisch genügend lang unter geeigneten Bedingungen gehalten wird, so daß die enzymatischen Reaktionen ablaufen können.

2. Verfahren nach Anspruch 1, wobei die Promotor-Sequenz die T7 Promotor-Sequenz und die RNA Polymerase die T7 RNA Polymerase ist.

3. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Enzym mit reverser Transkriptase-Aktivität die AMV-RT oder die MMLV-RT ist.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Enzym/eines der Enzyme mit RNaseH-Aktivität die RNaseH von *E. coli* ist.

5. Verfahren nach Anspruch 1, wobei das Enzym mit RNaseH-Aktivität eine reverse Transkriptase ist.

6. Verfahren nach Anspruch 5, wobei das Enzym mit RNaseH-Aktivität die AMV-RT oder die MMLV-RT ist.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei zumindest eines der Nukleötide mit einer Markierung versehen ist.

8. Verfahren nach einem der vorhergehenden Ansprüche, wobei die erzeugte RNA als Ausgangsmaterial für die weitere Amplifikation verwendet wird.

9. Verfahren nach Anspruch 8, wobei die erzeugten RNA-Kopien, die mittels des in Anspruch 1 beschriebenen Verfahrens hergestellt worden sind, in Kontakt gebracht werden mit:

- einer RNA Ligase,
- einem doppelsträngigen Nukleinsäure-Komplex umfassend eine doppelsträngige DNA Promotor-Sequenz, die von einer RNA Polymerase erkannt werden kann, wobei ein Strang des Komplexes einen Abschnitt RNA aufweist, der an das 5'-Ende von einem der DNA-Stränge gebunden ist,
- einem Enzym mit RNA Polymerase-Aktivität,
- genügend großen Mengen an dNTPs und rNTPs;

und das resultierende Reaktionsgemisch genügend lang unter geeigneten Bedingungen gehalten wird, so daß die enzymatischen Reaktionen der Amplifikation ablaufen können.

10. Verfahren nach Anspruch 9, wobei der Abschnitt RNA, der an das 5'-Ende von einem der DNA-Stränge gebunden ist, an seinem 5'-Ende phosphoryliert ist.

11. Verfahren nach Anspruch 10, wobei die Promotor-Sequenz die T7 Promotor-Sequenz und die RNA Polymerase die T7 RNA Polymerase ist.

12. Verfahren nach Anspruch 10, wobei zumindest eines der Nukleotide mit einer Markierung versehen ist.

13. Verfahren nach Anspruch 1, wobei das Reaktionsgemisch außerdem eine RNA Ligase und einen doppelsträngigen Nukleinsäure-Komplex umfassend eine doppelsträngige DNA Promotor-Sequenz, die von der RNA Polymerase erkannt werden kann, umfaßt, wobei ein Strang des Komplexes einen Abschnitt RNA aufweist, der an das 5'-Ende von einem der DNA-Stränge gebunden ist.

14. Verfahren nach Anspruch 1, wobei die erzeugten RNA-Kopien, die mittels des in Anspruch 1 beschriebenen Verfahrens hergestellt worden sind, in Kontakt gebracht werden mit einer PolyA Polymerase.

Revendications

1. Méthode pour créer, de manière non spécifique, de multiples copies d'ARN à partir d'acide nucléique contenant un matériel de départ comprenant un ensemble d'ARNm's, chaque ARNm comprenant une queue polyA, selon laquelle le matériel est mis en contact simultanément avec :

- un oligonucléotide comprenant une séquence oligo-dT à son extrémité 3', la séquence d'un promoteur reconnu par une ARN polymérase à son extrémité 5' et une région d'initiation de la transcription localisée entre la séquence oligo-dT et la séquence du promoteur, l'oligonucléotide étant bloqué à son extrémité 3' de telle sorte qu'une extension à partir de celle-ci soit empêchée,
- une enzyme ayant une activité de transcriptase inverse,
- une enzyme ayant une activité RNase H, et

- une enzyme ayant une activité ARN polymérase,
- des quantités suffisantes de dNTP's et de rNTP's

et le mélange réactionnel résultant est maintenu en conditions appropriées pendant une période de temps suffisante pour permettre la réalisation des procédés enzymatiques.

2. Méthode selon la revendication 1, selon laquelle la séquence de promoteur est la séquence du promoteur T7 et l'ARN polymérase est l'ARN polymérase T7.

3. Méthode selon l'une quelconque des revendications précédentes, selon laquelle l'enzyme ayant une activité de transcriptase inverse est AMV-RT ou MMLV-RT.

4. Méthode selon l'une quelconque des revendications précédentes, selon laquelle l'enzyme (ou l'une des enzymes) ayant une activité RNase H est la RNase H de E. coli.

5. Méthode selon la revendication 1, selon laquelle l'enzyme ayant une activité RNase H est une transcriptase inverse.

6. Méthode selon la revendication 5, selon laquelle l'enzyme ayant une activité RNase H est AMV-RT ou MMLV-RT.

7. Méthode selon l'une quelconque des revendications précédentes, selon laquelle au moins l'un des nucléotides comporte un marqueur.

8. Méthode selon l'une quelconque des revendications précédentes, selon laquelle l'ARN généré est utilisé comme matériel de départ pour une amplification supplémentaire.

9. Méthode selon la revendication 8, selon laquelle les copies d'ARN générées par la méthode décrite dans la revendication 1 sont mises en contact avec :

- une ligase ARN,
- un complexe d'acide nucléique double-brin comprenant la séquence d'ADN double-brin d'un promoteur qui peut être reconnue par une ARN polymérase, un des brins dudit complexe possédant une portion d'ARN liée à l'extrémité 5' d'un des brins d'ADN,
- une enzyme ayant une activité ARN polymérase,
- des quantités suffisantes de dNTP's et de rNTP's,

et le mélange réactionnel résultant est maintenu en conditions appropriées pendant une période de temps suffisante pour permettre la réalisation des procédés d'amplification enzymatiques.

10. Méthode selon la revendication 9, selon laquelle la portion d'ARN liée à l'extrémité 5' d'un des brins d'ADN est phosphorylée à l'extrémité 5'.

11. Méthode selon la revendication 10, selon laquelle la séquence de promoteur est la séquence du promoteur T7 et l'ARN polymérase est l'ARN polymérase T7.

12. Méthode selon la revendication 10, selon laquelle au moins l'un des nucléotides comporte un marqueur.

13. Méthode selon la revendication 1, selon laquelle le mélange réactionnel comprend en outre une ligase ARN et un complexe d'acide nucléique double-brin comprenant la séquence d'ADN double-brin d'un promoteur qui peut être i reconnue par l'ARN polymérase, un des brins dudit complexe possédant une portion d'ARN liée à l'extrémité 5' d'un des brins d'ADN.

14. Méthode selon la revendication 1, selon laquelle les copies d'ARN générées par la méthode décrite dans la revendication 1 sont mises en contact avec une polymérase poly A.

FIGURE 1

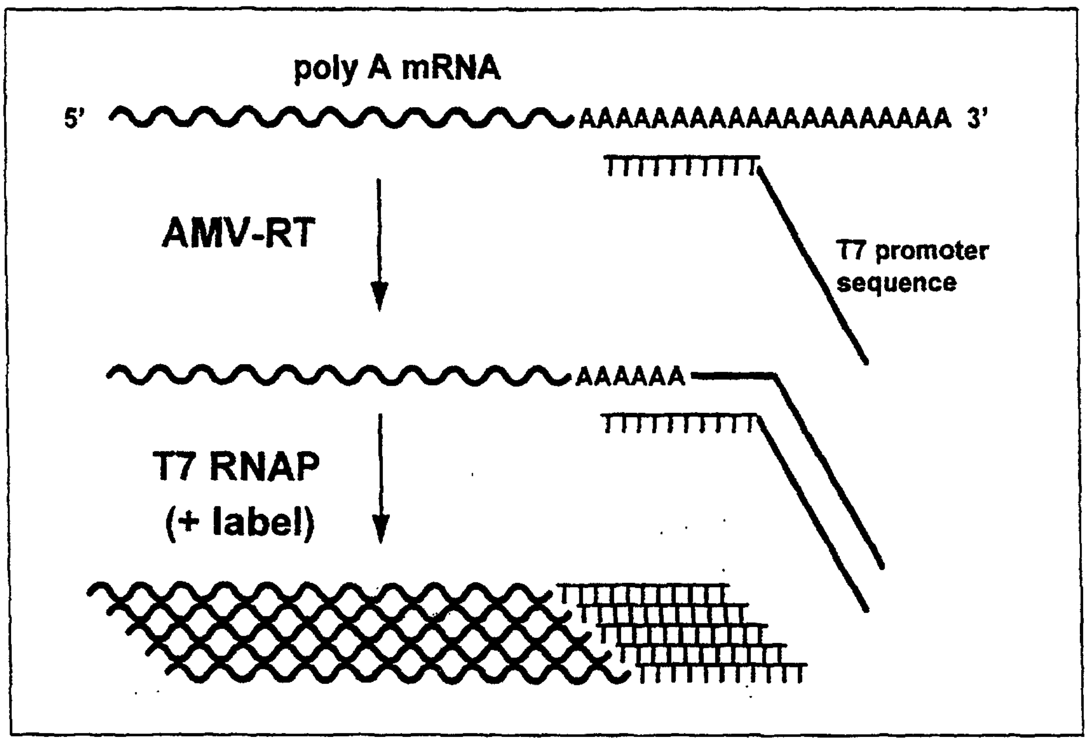
poly A mRNA
5'
AAAAAAAAAAAAAAAAAAAAA 3'
TTTTTTTTTT
AMV-RT
T7 promoter
sequence
AAAAAA
TTTTTTTTTT
T7 RNAP
(+ label)

FIGURE 2

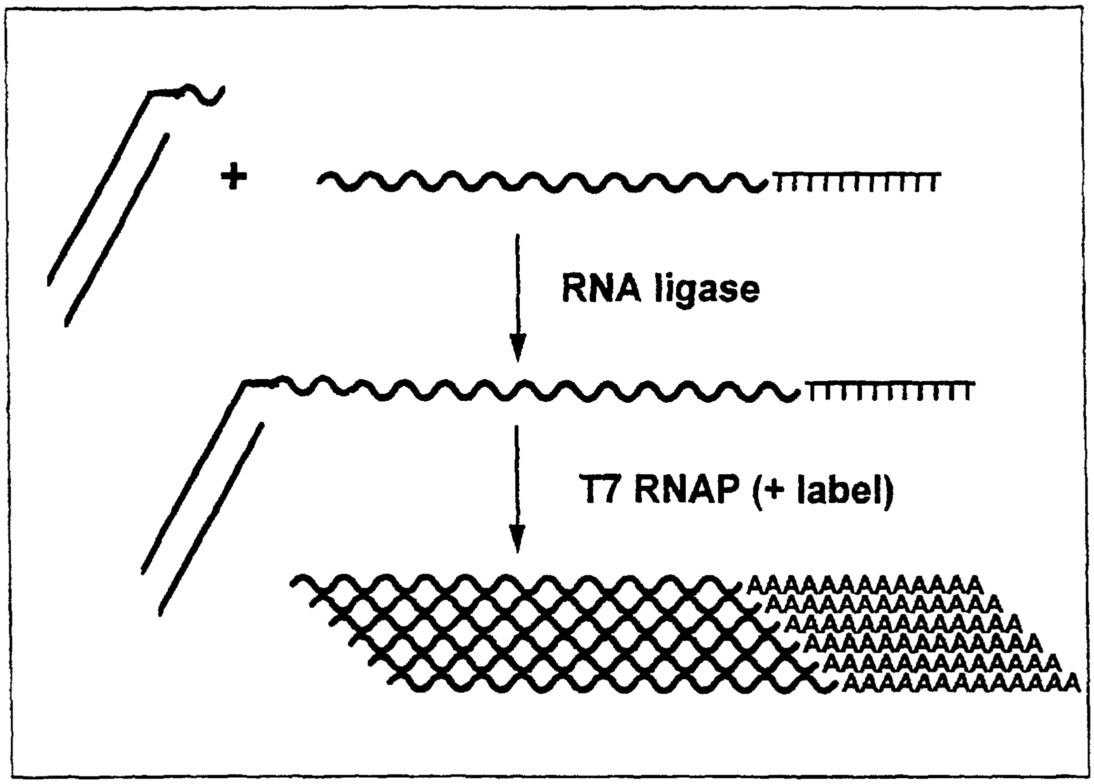
+
TTTTTTTTTT
RNA ligase
TTTTTTTTTT
T7 RNAP (+ label)
AAAAAAAAAAAAA
AAAAAAAAAAAAA
AAAAAAAAAAAAA
AAAAAAAAAAAAA
AAAAAAAAAAAAA
AAAAAAAAAAAAA

FIGURE 3

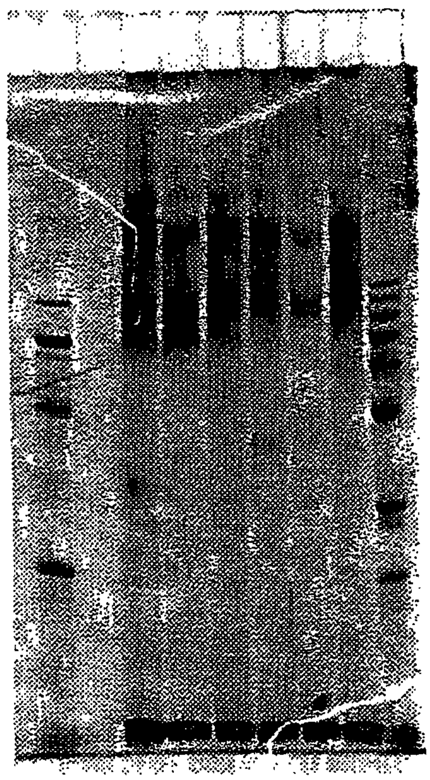

FIGURE 4

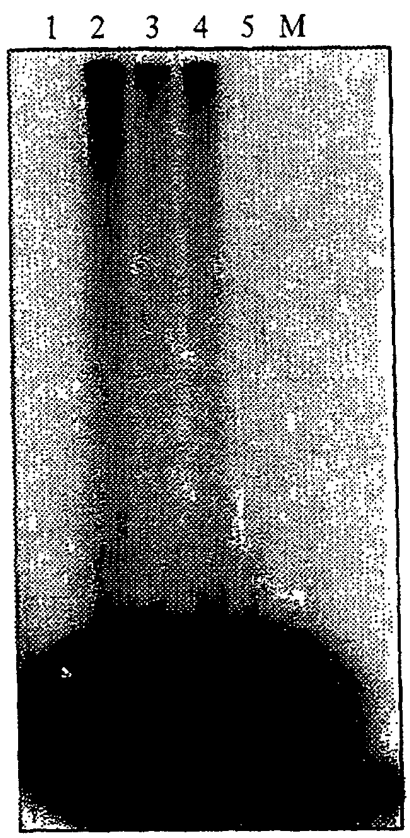

FIGURE 5

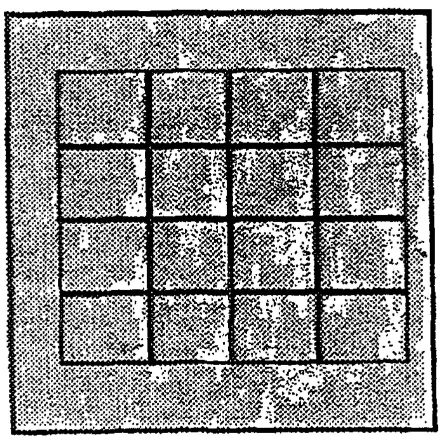

FIGURE 6

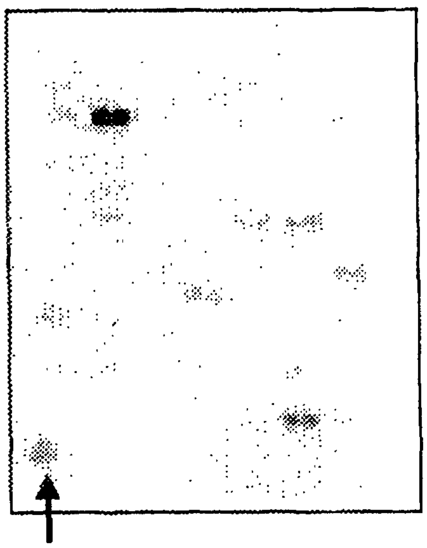

FIGURE 7