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(57) Abrégé/Abstract:
Method for the determination of mRNA which is essentially characterized in that a hybrid formed in solution is coupled to a heat-stable solid phase; subsequently, a reverse transcriptase and PCR reaction are carried out in one single reaction vessel. It has proven to be particularly advantageous if the oligonucleotide necessary for the hybridization is labeled with biotin, the solid phase is coated with streptavidin or avidin, and the reaction is carried out without RNase. A corresponding kit is also described.

Abstract

Method for the determination of mRNA which is essentially characterized in that a hybrid formed in solution is coupled to a heat-stable solid phase; subsequently, a reverse transcriptase and PCR reaction are carried out in one single reaction vessel. It has proven to be particularly advantageous if the oligonucleotide necessary for the hybridization is labeled with biotin, the solid phase is coated with streptavidin or avidin, and the reaction is carried out without RNase.
A corresponding kit is also described.

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Method and reagent for the specific determination of mRNA

The invention addresses a method and reagent and a kit for the specific determination of mRNA, i.e. poly(A) sequences containing nucleic acids on coated solid phases. The method of the invention can be used to quantify and detect mRNA from mixtures of total RNA and extracts of cell cultures and tissue cells. The method does not require preceding enrichment or isolation of the mRNA.

Almost all the mRNAs present in eucaryotic cells have a terminal sequence of approximately 20 to 250 adenosine nucleotides, a fact that is used in most mRNA purification methods. Today, a standard method for the detection and quantification of mRNA is the combined use of reverse transcriptase (RT) and PCR (RT-PCR) (Larrick, J.W., Trends Biotechnol. 10, 146-152 (1992); Kawasaki, E.S., PCR Protocols: A Guide to Methods and Applications (eds. Innis, M.A. et al.), Academic Press, San Diego, California (1990)). The initial material for this method is purified total RNA (rRNA, tRNA, mRNA) or purified mRNA as the enzymes, particularly polymerases, used in the RT-PCR are easily inhibited by contamination. The purification of RNA and mRNA is known from several methods as, for example, extraction with organic solvents (e.g. phenol/chloroform), purification via oligo(dT)-cellulose, or isolation with the aid of oligo(dT)-coated magnetic particles (Sambrook, J., Fritsch, E.F. and T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2nd edition (1989); Farrell, R.E., RNA Modologies: A Laboratory Guide for Isolation and Characterization, Academic Press (1993)).

These methods are, hence, complex and difficult to automate. Moreover, purification can also be achieved via hybridization of the poly(A)-carrying 3'-end of the eucaryotic mRNA using biotinylated oligo(dT). The hybrid of biotin-oligo(dT)

and mRNA can then be bound to streptavidin-coated magnetic particles and isolated, for example. It is, however, a disadvantage that only larger amounts of mRNA hybrid can be isolated; the mixture is, hence, not sensitive enough for the detection of specific mRNAs. An RT-PCR at a solid phase, i.e. magnetic particles, was then developed and described. A drawback of this method is, however, that after binding and washing of the mRNA, the magnetic particles must be transferred into reaction vessels that are suitable for PCR, i.e. particularly heat-stable, which in turn requires another separation step.

It was, hence, an object of the invention to provide an improved method for the determination of mRNA which eliminates the drawbacks known from prior art.

This object is accomplished by a method for the specific determination of poly(A) sequence-containing nucleic acids, characterized in that

- a lysis and/or hybridization buffer is added to a suitable sample material and, if necessary, homogenized,

- the sample solution is brought into contact with a labeled poly or oligonucleotide probe, or labeled nucleotide derivative which carries at least one sequence that is complementary to poly(A) or an oligo- or polynucleotide or a corresponding derivative that is complementary to the nucleic acids to be determined,

- an aliquot of the mixture is transferred into the heat-stable, RNase-free reaction vessel coated with an organic chemical compound where it is incubated for at least 10 seconds in a temperature range of approximately 4 to 50°C,

- the supernatant is removed from the reaction vessel and optionally washed several times with a suitable buffer solution,

- and combined with a mixture which contains an enzyme with reverse transcriptase(RT) activity, or an enzyme with RT- and heat-stable DNA-polymerase activity, and incubated at approximately 30 to 75°C,

- if necessary, the mixture is removed and washed with washing buffer,

- if necessary, another mixture containing at least one heat-stable DNA polymerase and two specific DNA primers is added into the reaction vessel and incubated under conditions that support amplification, and

- if necessary, the DNA contained in the mixture is separated, particularly by means of electrophoretical procedures, and then detected.

It is particularly advantageous that in the method of the invention, isolation and purification of mRNA from the corresponding sample materials are already carried out in a vessel that can be used for the reverse transcriptase (RT) reaction and the subsequent PCR. Sample preparation (isolation and purification) and transcription and/or amplification (RT-PCR) are then carried out in one single reaction vessel.

In addition to pure mRNA fractions, the sample material to be analyzed also includes natural and artificial mixtures of total RNA (rRNA, tRNA, mRNA), but also fractions (cell lysates) obtained from cell cultures and tissue cell extracts, and/or tissue homogenates of human or animal origin, and plant extracts.

Suitable lysis and/or hybridization buffer are based on buffering substances which exhibit good buffering capacity at a pH value between approximately 5 - 10, preferably between pH 7.0 and 8.5. Corresponding buffering substances are, for example, Tris·HCl, HEPES, MOPS, or Tris·borate.

Moreover, buffers that are suitable for the method of the invention can contain a disulfide-reducing reagent, such as dithioerythritol, dithiothreitol, mercaptoethanol, preferably at a concentration between 0.01 and 1 % (w/v). Moreover, it has proven to be advantageous when the buffering system contains a denaturing substance such as detergents in relatively high concentrations. Dodecylsulfate salts or corresponding derivatives at a concentration of 0.1 to 15 % (w/v) have proven to be particularly advantageous; guanidinium thiocyanate salts and the corresponding derivatives at a concentration range between approximately 1 to 7

mM, particularly between 1 and 5 mM are particularly useful when dealing with RNase-rich tissue. Experience has shown that it is particularly advantageous when the solution used for the determination is almost free of RNase activities. This means the maximum residual activity of RNase must not exceed 5 %.

Moreover, the buffering systems used in a method of the invention can also contain salts, such as lithium chloride or other additives. It has proven to be particularly advantageous when an additional RNase inhibitor such as one obtained from placenta is present, and/or the buffering solution is sterilized and/or decontaminated with dimethyldicarbonate or diethylpyrocarbonate prior to use.

Possible labeled poly and/or oligonucleotide probes or labeled nucleotide derivatives which carry a poly(A)-complementary sequence are especially oligo(dT)- or oligo(U)-nucleotide probes or corresponding peptide nucleic acid derivatives such as PNA, i.e. nucleic acids with a peptide backbone of any desired length. Preferred are 15 to 30mers, frequently a 20mer is sufficient.

Possible labeling groups for the nucleotide probe are all radicals that can be coupled or bound to a certain matrix, including haptens, protein with antigen and/or antibody structure. Biotin bound to the oligo(dT) sequence has proven to be particularly suitable as it can be linked to the solid phase when bound via streptavidin or avidin; this requires that the organic chemical "coating substance" is streptavidin. Alternatively, the coating substance can also be oligo(dT) nucleotides or suitable chelate compounds that are suitable for fixing corresponding probes via hybridization or complex formation. Immobilization of mRNA to streptavidin-coated solid phases and/or reaction vessels is, hence, a very comfortable and reliable method for mRNA extraction for the subsequent transcription or amplification. Moreover, it is also possible to use other labeling groups for fixation to the solid phase. The nucleotide probes such as, for example, oligo(dT) probes can be obtained according to known methods or be purchased from commonly known suppliers.

The polyadenylated mRNA-containing sample material is, for example, combined with the aqueous solution that contains the labeled oligo(dT) probe. Hybridization is preferably carried out at approximately 37°C and usually completed after only a few seconds. A period of 10 seconds to 10 minutes, preferably 5 minutes has in most cases proven to be sufficient for hybridization.

An aliquot of the biotin-oligo(dT)-mRNA hybrid solution is then directly added into the coated, heat-stable reaction vessel. Under optimal conditions, the volume of the coated aliquot is in a magnitude of the respective vessel volume and should not contain more than approximately 5 pmol of the hybrid. A sample of approximately 20 to 200 µl lysate is usually sufficient. For immobilization on the coated solid phase, only a few seconds of incubation time, i.e. appr. 10 seconds and a temperature of approximately 4 to 50°C, preferably approximately 37°C, are usually sufficient; to be on the safe side, the process is usually continued for 1 to 10 minutes.

Subsequently, the rest of the lysate is removed and the bound RNA is thoroughly washed (approx. 5 times) and the washing buffer is then quantitatively removed such that the reverse transcriptase reaction can be carried out. In addition to the conventional buffer substances and salt, a typical mixture for the RT reaction also contains magnesium ions, all four deoxynucleotide triphosphates (dNTPs), an enzyme with RT activity, or an enzyme with RT and heat-stable DNA-polymerase activity such as DNA polymerase from Thermus thermophilus (Tth), and advantageously an RNase inhibitor. An aliquot of such a mixture of approximately 20 to 200 µl is then added into the reaction vessel. AMV- or M-MuLV-RT, the latter particularly in its RNase H^- form (US 5.244.797), are particularly used as enzymes with RT activity, preferably at a concentration between 0.01 to 5 U/µl. The preferred final RT concentration is between 0.1 and 1.0 U/µl. Moreover, experience has shown that RTs of other virus types and sources can also be used in accordance with the invention.

Incubation for the cDNA synthesis can be carried out at appr. 30 to 75°C, preferably between 35 and 45°C and usually takes 30 to 120 minutes. In many cases, an incubation temperature of approximately 42°C has proven to be particularly advantageous.

After completion of the reaction, the reaction solution is removed and the remainder is again washed thoroughly with washing buffer. Subsequently, a defined volume of the PCR mix is given into the coated reaction vessel, usually 20 - 200 µl and incubated under less stringent conditions. An optimum PCR mix contains a suitable buffer system, e.g. the buffer components given in Maniatis et al. (2nd ed., 1989, s.o., pages 14.15 to 14.17), magnesium ions in a concentration range of approximately 0.5 to 5.0 mM, a defined amount of heat-stable DNA polymerase, preferably between 5 to 300 mU/µl, all four dNTPs, each at a concentration of 0.05 to 0.6 mM, preferably each approximately 0.2 mM and at least two DNA primers between 0.1 and 1.0 pmol/µl, preferably each at approximately 0.4 pmol/µl.

The PCR reaction itself is carried out according to measures known to the expert (so-called standard PCR), such as a first cycle at 95°C (5 minutes), 50°C (2 minutes) and 72°C (3 minutes); the subsequent cycles (a total of approximately 40 cycles) can then be carried out according to the following scheme: 95°C (1 minute), 50°C (2 minutes) and 72°C (3 minutes and, in the last run, 10 minutes).

After completion of the PCR reaction, the solution containing the transcribed and amplified DNA is removed from the reaction vessel and separated, for example electrophoretically, and detected with ethidium bromide, for example.

Another advantageous embodiment is that the RT- and PCR reactions are not carried out sequentially, but in a coupled reaction without an intermediate washing step. In such a simultaneous process, the RT solution must not be removed after completion of the RT reaction. In a particularly advantageous embodiment, a bifunctional enzyme is used which exhibits both RT and DNA polymerase activity and is heat-stable. In such a 1 vessel/1 buffer embodiment, an incubation time of

approximately 5 minutes up to 1 hour have proven to be particularly advantageous.

Principally, the conditions for an RT and/or PCR reaction described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2nd edition (1989) has proven to be particularly suitable.

The DNA mixture that is then obtained in accordance with the invention, is usually detected following electrophoretic separation and using suitable hybridization methods (Southern Blot, ELISA). It is, however, also possible to directly detect the DNA mixture without prior separation. The expert is familiar with corresponding methods. It was thus possible to detect in accordance with the invention mRNA in less than 1 cell.

The preparation of coated solid phases and/or reaction vessels is known to the expert (e.g. EP 0331127). When streptavidin and/or avidin-coated carrier materials are used, the reaction vessels disclosed in EP 0344578 have proven to be particularly advantageous.

Another subject matter of the invention is a reagent and/or a kit for the determination of mRNA which includes the following components:

(a) lysis and hybridization buffer,
(b) buffer solution with a labeled nucleotide probe that carries a poly(A) complementary sequence,
(c) washing buffer,
(d) a mixture containing an enzyme with reverse transcriptase activity,
(e) a mixture containing at least one heat-stable DNA polymerase, and
(f) a device having at least one coated, RNase-free reaction vessel.

The method of the invention has proven to be particularly preferred when a biotin-labeled oligo(dT) probe is used and when the solid phase suitable for the immobilization of the hybrid is coated with streptavidin and/or avidin. Moreover, a

preferred kit is one which contains one mixture (d') instead of two mixtures (d) and (e); said mixture (d') includes an enzyme with RT and heat-stable DNA-polymerase activity. Moreover, the features that are disclosed in connection with the method of the invention also apply to the kit.

The following examples illustrate the invention in greater detail:

Example 1

The streptavidin-coated PCR vessels are used to carry out the RT-PCR directly from cells without prior isolation of the RNA.

The cells are lysed in lysis and/or hybridization buffer, the DNA is separated, the mRNA immobilized by hybridizing the poly A^+ tale with $BidT_{20}$ and binding it to the streptavidin matrix in the reaction vessel. Synthesis of the corresponding cDNA is then achieved with the aid of a reverse transcriptase; using specific primers, the amplification of the specific transcript is achieved via PCR followed by quantification, e.g. in agarose gel via ethidium bromide staining.

This example describes an RT-PCR directly from human K562 cells for the detection of mRNA of G3PDH.

All components used are manufactured by Boehringer Mannheim GmbH, except the PCR primers for human G3PDH-mRNA (983 bp), which were obtained from Clontech (Art. No. 5406-1). The buffer (lysis/hybridization buffer) and the RT and PCR mixtures were prepared as follows:

1) Lysis/hybridization buffer

a) Detergent buffer (volume for the manufacture of 250 ml):

25.0 ml Tris·HCl, 1M, pH 7.5 (4°C)

15.0 ml LiCl, 5M

5.0 ml EDTA, 0.5M, pH 8.0

25.0 ml lithium-dodecylsulfate

193.0 ml 1,4-dithioerythritol

180.0 ml redist. water

(The autoclaved stock solutions are stable at RT).

b) GTC buffer

4.0 ml guanidinium thiocyanate (GTC)

0.1M Tris·HCl, pH 8.0

1.0 % 1,4-dithiothreitol (w/v)

0.5 % laurosylsarcosin (w/v)

(store at -20°C)

2) Biotin-labeled oligo(dT) probe

(volume for 20 µl)

19.0 µl redist. water

1.0 µl biotin-oligo$(dT)_{20}$ sample (^ 100 pmol)

3) Washing solution

(volume for 250 ml)

2.5 ml Tris·HCl, 1M, pH 78.5 (4°C)

10.0 ml LiCl, 5M

0.5 ml EDTA, 0.5M, pH 8.0

237.0 ml redist. water

4) RT mix

(volume for 1 ml)

204.1 µl Tris·HCl, 10 mM, pH 7.4

51.0 µl Tris·HCl, 1M, pH 8.3 (42°C)

142.0 µl KCl, 1M

40.8 µl $MgCl_2$, 250 mM

102.0 µl dNTPs, 10 mM each

40.8 µl 1,4-dithioerythritol, 100 mM

20.0 µl RNase inhibitor (40 U/µl)

398.4 µl redist. water

32.0 µl AMV reverse transcriptase (25 U/µl)

5) PCR mix

(volume for 1 ml)

100.0 µl Taq buffer (10 times)

20.0 µl dNTPs, 10 mM each

4.0 µl primer, 100 pmol/µl each

867.0 µl redist. water

5.0 µl taq polymerase (5 U/µl)

All solutions and/or the redistilled water used for their preparation should be treated with dimethyldicarbonate (DMDC) or diethylpyrocarbonate (DEPC) to eliminate possibly present RNases.

Cell lysis and immobilization of the mRNA in the SA-coated reaction vessel

K562 cells exhibited a logarithmic growth with 3×10^5 cells/ml. 1 ml cell suspension was removed by centrifugation at 300 g for 5 minutes, washed once with ice-cold PBS; the pellet was stored in liquid nitrogen. The frozen pellet was then resuspended in 200 µl lysis buffer, the DNA was separated by drawing it in 6 times in a 0.8 ml syringe; a $\log_{10}$ dilution series was prepared from the lysate by pipetting portions of 20 µl onto the prepared 180 µl lysis buffer. Portions of 5 pmol

$BidT_{20}$ were pipetted into the mixtures and hybridized for 5 minutes at 37°C. Portions of 50 µl of the mixtures were pipetted into streptavidin-coated PCR vessels and bound for 3 minutes at 37°C. Subsequently, the solution was removed by means of pipetting, and the vessels were washed on ice 4 times with 250 µl of washing buffer each time.

cDNA synthesis

Portions of 50 µl 1 time RT mix are pipetted into the individual vessel segments and incubated approximately 2 hours at 42°C. As a control, the highest RNA concentration with RT mix without the reverse transcriptase was given into one segment and there was no RNA with RT mix with RT as another control in another segment. Subsequently, the RT was thermally denatured for 10 minutes at 65°C and washed 4 times with 250 µl of washing buffer each time.

Amplification via PCR

Portions of 50 µl of 1 time PCR mix were pipetted into the individual vessel segments and the PCR runs were carried out in a thermocycler according to the manufacturer's instructions. The PCR products were then separated in 2 % agarose gel (with ethidium bromide) at 100 V for 35 minutes and then analyzed on the screen.

Example 2

Corresponding to the data and solutions given for example 1, β-actin-mRNA was determined. Summarizing, β-active mRNA could be detected in less than one cell.

Figure 1

Result of example 1, wherein lanes 1 to 7 of the electrophoresis gel give the following samples:

Lane 1: Control with cells, without RT

Lanes 2-5: Dilution series of the cell lysate after the methods of the invention has been carried out (in lane 4 of the original, only one lane is visible), the following quantities were applied: lane 2: 7500 cells; lane 3: 750 cells; lane 4: 75 cells; lane 5: 7.5 cells.

Lane 6: Control without cells, with 1 x RT mix

Lane 7: DNA molecular weight standard No. 4 (Boehringer Mannheim).

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. Method for the specific determination of poly(A) sequence containing nucleic acids, characterized in that

- a lysis and/or hybridization buffer is added to a suitable sample material
and, if necessary, homogenized,

- the sample solution is brought into contact with a labeled poly or oligonucleotide probe, or labeled nucleotide derivative which carries at least one sequence that is complementary to poly(A) or an oligo- or polynucleotide or a corresponding derivative that is complementary to the nucleic acids to be determined,

- an aliquot of the mixture is transferred into a heat-stable, RNase-free reaction vessel coated with an organic chemical compound which binds to the label on the labeled poly or oligo-nucleotide probe, or labeled nucleotide derivative where it is incubated for at least 10 seconds in a temperature range of approximately 4 to 50°C,

- the supernatant is removed from the reaction vessel and washed several times with a suitable buffer solution,

- and combined with a mixture which contains an enzyme with reverse transcriptase(RT) activity, or an enzyme with RT- and heat-stable DNA-polymerase activity, and incubated at approximately 30 to 75°C,

- if necessary, the mixture is removed and washed with washing buffer,

- another mixture containing at least one heat-stable DNA polymerase and two specific DNA primers is added into the reaction vessel and is incubated under conditions that support amplification, and

- if necessary, the DNA contained in the mixture is separated, particularly by means of electrophoretical procedures, and then detected.

2. Method according to claim 1, characterized in that the sample material is a mixture consisting of total RNA, cultivated cells, tissue cells of animal or human origin or plant extracts.

3. Method according to claim 1 or 2, characterized in that the lysis and/or hybridization buffer contains a disulfide group reducing substance, a substance buffering between pH 7.0 and pH 8.5 and a detergent or a denaturing agent, and, if necessary an RNase inhibitor.

4. Method according to claim 3, characterized in that the buffer contains 0.5 to 15.0 % (w/v) of a dodecylsulfate salts or 1 to 7 mM guanidinium thiocyanate.

5. Method according to any one of claims 1 to 4, characterized in that the nucleotide probe is labeled with biotin and the reaction vessel is coated with streptavidin or avidin.

6. Method according to any one of claims 1 to 5, characterized in that an oligo(dT)- or an oligo(U)- or a PNA derivative is used as the labeled nucleotide probe.

7. Method according to any one of claims 1 to 6, characterized in that a 15 to 30mer nucleotide probe is used and/or hybridization is carried out within 1 to 10 minutes at approximately 37°C.

8. Method according to claim 5, characterized in that an aliquot which contains 5 pmol poly(A/dT) hybrid or less is added into streptavidin-coated reaction vessel of the corresponding size.

9. Method according to any one of claims 1 to 8, characterized in that for the reverse transcription, the mixture contains 0.01 to 5.0 U/µl AMV- or M-MuLV-reverse transcriptase, an SH reagent, an RNase inhibitor, potassium and magnesium ions and a suitable buffering substance.

10. Method according to claim 1 or 9, characterized in that the RT reaction is carried

out at approximately 35° to 45°C.

11. Method according to any one of claims 1 to 10, characterized in that a PCR is carried out in the presence of 5 to 300 mU/µl of the heat-stable DNA polymerase, approximately 0.1 to 1.0 pmol/l of each of the specific primers, approximately 0.05 to 0.6 mM dNTPs, a suitable PCR buffering system and, if necessary, in the presence of magnesium ions.

12. Method according to any one of claims 1 to 8, characterized in that an enzyme which exhibits RT and DNA polymerase activity and is heat-stable is used, and incubated for approximately 5 minutes to 1 hour under conditions that support a PCR reaction.

13. Kit for the determination of mRNA consisting of the following components:

(a) lysis and hybridization buffer,

(b) buffer solution with labeled nucleotide probe that carries a poly(A) complementary sequence,

(c) washing buffer,

(d) a mixture containing an enzyme with reverse transcriptase activity,

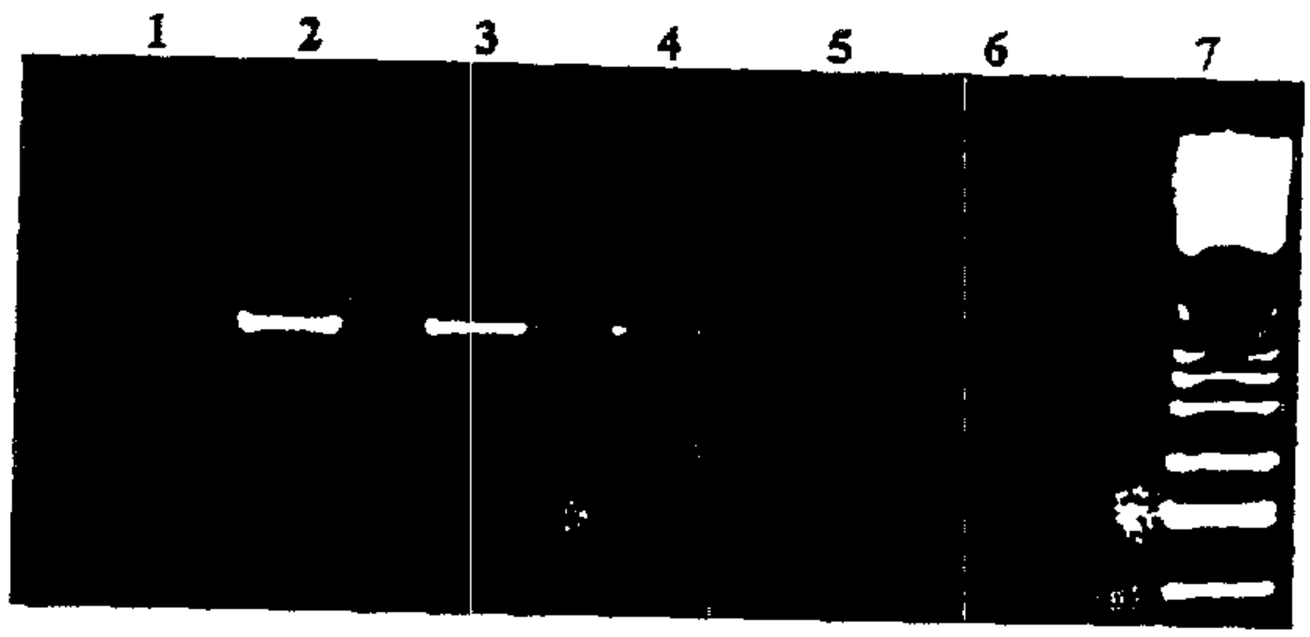
(e) a mixture containing at least one heat-stable DNA polymerase, and at least two DNA primers,

(f) a device having at least one coated, heat-stable RNase-free reaction vessel; the reaction vessel being coated with an organic chemical compound which binds to label on the labeled nucleotide probe.

14. Kit according to claim 13, characterized in that an enzyme mixture containing an enzyme with RT and heat-stable DNA polymerase activity is used.

15. Kit according to claim 13 or 14, characterized in that an oligo(dT) probe is labeled with biotin, and the reaction vessel is coated with streptavidin or avidin.

Fig. 1



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