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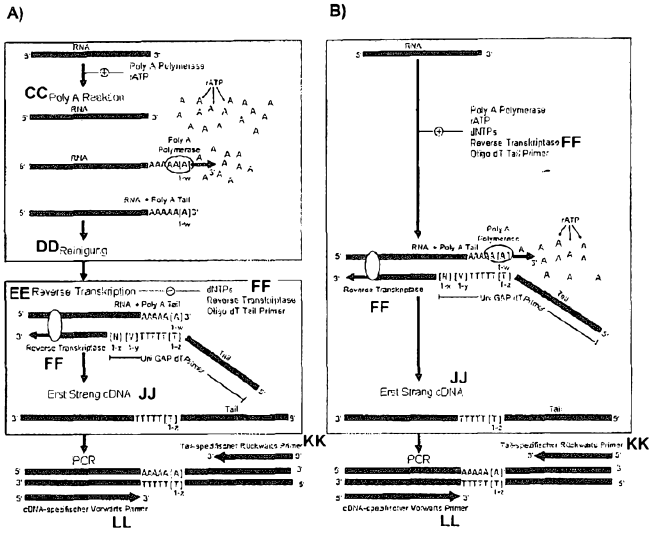
(54) **Title:** METHOD FOR SYNTHESIZING A CDNA IN A SAMPLE IN AN ENZYMATIC REACTION

(54) **Bezeichnung:** VERFAHREN ZUR SYNTHESE EINER CDNA IN EINER PROBE IN EINER ENZYMATISCHEN REAKTION

Fig. 1



CC Poly A Reaction
DD Cleaning
EE Reverse Transcription
FF Reverse Transcriptase
JJ First strand cDNA
KK Tail Specific Forward Primer
LL Primer cDNA Specific Reverse Primer

(57) Abstract: The present invention relates to a method for synthesizing a cDNA in a sample in an enzymatic reaction, the method comprising the steps of simultaneously preparing a first enzyme having polyadenylization activity, a second enzyme having reverse transcriptase activity, a buffer, at least one ribonucleotide, at least one deoxyribonucleotide, an anchor oligonucleotide; adding a sample comprising a ribonucleic acid and incubation of the agents of the preceding steps at one or more temperature steps selected such that the first and the second enzyme show activity, an amplification additionally occurring in the same reaction mixture. The invention further relates to a reaction mixture comprising a first enzyme having polyadenylization activity, a second enzyme having reverse transcriptase activity, optionally a buffer, optionally at least one ribonucleotide, optionally at least one deoxyribonucleotide, optionally an anchor oligonucleotide, and an enzyme having DNA synthesis activity.

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(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein Verfahren zur Synthese einer cDNA in einer Probe in einer enzymatischen Reaktion, wobei das Verfahren die Schritte umfasst, gleichzeitige Bereitstellung eines ersten Enzyms mit Polyadenylierungsaktivität, eines zweiten Enzyms mit reverser Transkriptaseaktivität, eines Puffers, mindestens eines Ribonukleotids, mindestens eines Desoxyribonukleotids, eines Anker Oligonukleotids, Zugabe einer Probe umfassend eine Ribonukleinsäure sowie Inkubation der Agenzien der vorhergehenden Schritte bei einem oder mehreren Temperaturschritten, welche so gewählt sind, dass das erste und das zweite Enzym Aktivität zeigen, wobei zusätzlich in dem selben Reaktionsgemisch eine Amplifikation erfolgt. Die Erfindung betrifft weiter einer Reaktionsgemisch umfassend ein erstes Enzym mit Polyadenylierungsaktivität, eines zweites Enzym mit reverser Transkriptaseaktivität, optional einen Puffer, optional mindestens ein Ribonukleotid, optional mindestens ein Desoxyribonukleotid, optional Anker Oligonukleotid und ein Enzym mit DNA Sytheseaktivität.

METHOD FOR SYNTHESIZING A CDNA IN A SAMPLE IN AN ENZYMATIC REACTION

BACKGROUND OF THE INVENTION

The invention relates to the field of molecular biology and research in this field, but also to human and nonhuman diagnostics.

The analysis of non-polyadenylated RNA molecules, for example bacterial RNAs or small RNAs, such as the so-called microRNAs (miRNAs), proves difficult and requires special methods. A possible method was described in the literature recently. This method comprises several successive enzymatic steps, i.e. "tailing" of the RNA with poly(A) polymerase and a suitable substrate, typically ATP, is carried out first. Then the poly(A) reaction is stopped and the reaction product is purified. Then the poly(A) RNA generated is submitted to a reverse transcriptase reaction and is transcribed with suitable primers to cDNA.

TECHNICAL FIELD

The execution of these two successive enzymatic reactions is complicated and there is a large number of sources of error, for example introduction of nucleases, loss of material or pipetting errors.

MicroRNAs (miRNAs) vary in size from about 20 to 25 nucleotides and represent a new class of non-coding RNAs.

They are processed by means of a so-called "hairpin precursor" and can play a role in gene expression as negative regulators. Thus, they down-regulate a large number of genes (Ambros, V., 2001, MicroRNAs: Tiny regulators with great potential, Cell 107, 823-826). MiRNAs are first transcribed as long "primary transcripts" (they are also called primary miRNAs) (Lee, Y., Jeon, K. et al., 2002, MicroRNA maturation: stepwise processing in subcellular localization, Embo J. 21, 4663-4670). These "primary transcripts" are then shortened, the resultant length being about 70 nucleotides. There is formation of so-called "stem-loop structures", which are also called "pre-miRNAs". Pre-miRNAs are exported into the cytoplasm. The exporting enzyme

is called exportin-5. Here, they are processed further and in this way there is formation of a mature miRNA molecule, about 22 nucleotides long (Lee, Y., et al., 2003, The nuclear RNA's III Drosha initiates microRNA processing, Nature 425, 415-419). Very recent studies have suggested that miRNAs play an important role in development and differentiation. Basically, microRNAs can exert a regulatory action in two different ways. In plants, miRNAs complement with their corresponding mRNAs through exact complementarity. This leads to destruction of the target mRNA by a mechanism that comprises RNA interference (RNAi). In animals, miRNAs prevent gene expression by a mechanism that comprises Lin-4 and Let-7. Here, the miRNAs are not exactly complementary to their corresponding mRNAs, but they prevent the synthesis and function of proteins (Ambros, V., 2004, The functions of animal microRNAs, Nature, 431, 350-355). In view of the decisive role that the only recently discovered miRNAs play, their detection and/or analysis also have a decisive role.

In eukaryotes, the synthesis of the 18 S, 5.8S and 25/28S rRNAs comprises processing in modifications of so-called precursor rRNAs (pre-rRNAs) in the nucleolus. This complex process of rRNA biogenesis comprises many small so-called "small nucleolar RNAs" (snoRNAs), which accumulate in the nucleolus. They do this in the form of so-called small nucleolar ribonucleo protein particles (snoRNPs) (Maxwell, E.S. et al., 1995, The small nucleolar RNAs, Annual Review Biochem., 35, 897-934).

All snoRNAs characterized to date, with the exception of the RNase MRP, can be divided into two families. These are the box c/D and box h/ACA slow RNAs, which can be distinguished by their common sequence motifs (Ballakin, A.D. et al., 1996, The RNA world of the nucleolus: two major families of small nucleolar RNAs defined by different box elements with related functions, Cell, 86, 823-834). The genomic organization of the snoRNA genes displays great diversity in different eukaryotes. In vertebrates, most snoRNAs are inserted within introns via "host genes". Exceptions such as U3 are transcribed independently. In yeast there are snoRNAs that are inserted in introns, but the majority of snoRNAs are transcribed as an individual gene with its own promoter. Clustered snoRNA genes are transcribed upstream through common promoters. Owing to the small sizes and the absence of polyadenylation, the detection and/or analysis of snoRNAs represent a molecular-biological challenge.

PCR is a frequently used tool for studying microbial organisms and is sometimes also used for analyzing 16S rRNA genes. However, the discovery of new genes in microbial samples is

restricted because synthesis of primers is only possible to a limited extent. Thus, primers for 16S RNA genes are derived from sequences that are already known from cultivated microbes (Olson, D.J., 1986, Microbial ecology and evolution: A ribosomal RNA approach, Annu. Rev. Microbial. 40: 337-365). Owing to the systematic way in which, for extracting 16S rRNA genes from organisms that are so far unknown, we resort to sequences that are already known, it is probable that microbial diversity is greatly underestimated and is not being isolated.

Just as the 16S rRNA molecules can only be isolated with difficulty, prokaryotic mRNA molecules can only be isolated with difficulty through lack of knowledge of the sequence and in particular lack of a poly(A) tail.

A two-stage process is known in the prior art. In this method, an RNA molecule is transformed, with the aid of the enzyme poly(A) polymerase and the substrate adenosine triphosphate, so that a polyadenylated ribonucleic acid molecule forms. The resultant polyadenylated ribonucleic acid molecule is purified in a further step, before reverse transcription takes place in a third step. The reverse transcription incorporates the polyadenylated tail, wherein a homopolymeric oligonucleotide as a rule adds on a poly(T) oligonucleotide complementary to the polyadenylated RNA tail. The 3'-end of the poly(T) oligonucleotide is now used by the polymerase to prepare a deoxyribonucleic acid strand, which is complementary to the existing ribonucleic acid strand. The resultant strand is called "first strand cDNA". This cDNA can be used in a PCR reaction, employing either random primers or alternatively specific primers, so as to generate an amplificate. Shi et al. teach in particular miRNA detection by means of an oligo-dT adapter-primer, using an adapter-specific primer in the PCR (Shi, R. and Chiang, V.L. (Shi, R. et al., Facile means for quantifying microRNA expression by real-time PCR, Biotechniques, 2005, 39, 519-25).

This method, published only recently, has decisive disadvantages with respect to the aforementioned special ribonucleic acid molecules.

Thus, the two-stage method in general possibly causes the introduction of contaminants. The purification step leads to losses of rare RNAs. The two-stage method requires inactivation of the first enzyme and an incubation time for the first and the second enzyme, which altogether leads to a very large expenditure of time. Furthermore, the two-stage method has the disadvantage that there is a risk of confusion of samples when two or more samples are processed simultaneously.

As is known from the prior art, ribonucleic acids are relatively sensitive as far as attack by nucleases is concerned. The two-stage method, especially the purification step after the first process, involves the risk of introducing nucleases. Finally, two or more steps always mean an increased risk of pipetting errors.

OBJECT OF THE INVENTION

The present invention provides a method that makes cDNA synthesis possible, largely prevents contamination, is less time-consuming, minimizes the risk of mix-up of the samples, minimizes the risk of introduction of nucleases and finally largely excludes the risk of pipetting errors.

In one embodiment the specification provides a method for synthesizing a cDNA in a sample, in an enzymatic reaction, wherein the method comprises the following steps:
(a) simultaneous providing of a first enzyme with polyadenylation activity, a second enzyme with reverse transcriptase activity, a buffer, at least one ribonucleotide, at least one deoxyribonucleotide, an anchor oligonucleotide, (b) adding a sample comprising a ribonucleic acid and (c) incubating of the agents from steps (a) and (b) in one or more temperature steps, which are selected so that the first enzyme and the second enzyme display activity.

Hitherto there were reservations that a combination of enzymatic polyadenylation and reverse transcription is technically possible. This can be seen from the fact that even very recently, i.e. after the discovery of microRNAs and snoRNAs, which represent a particular molecular-biological challenge with respect to analysis and isolation, enzymatic reactions have always been carried out successively (Want, J.F., et al., Identification of 20 microRNAs from Oryza sativa, Nucleic Acid Res, 2004, 32, 1688-95; Shi, R. and Chiang, V.L., Facile means for quantifying microRNA expression by real-time PCR, Biotechniques, 2005, 39, 519-25; Fu H. et al., Identification of human fetal liver miRNAs by a novel method; FEBS Lett, 2005, 579, 3849-54; Chen, C.L. et al., The high diversity of snoRNAs in plants: identification and comparative study of 120 snoRNA genes from Oryza sativa, Nucleic Acids Res, 2003, 31, 2601-13; Botero, L.M. et al., Poly(A) polymerase modification and reverse transcriptase PCR amplification of environmental RNA, Appl. Environ Microbiol, 2005, 71, 1267-75). Surprisingly, both methods, i.e. polyadenylation and reverse transcription, have already long been known by a person skilled in the art (Sano, H. and Feix, G., Terminal riboadenylate transferase from *Escherichia coli.* Characterization and application, Eur. J. Biochem., 1976,

71, 577-83). A person skilled in the art has, as a rule, after the poly(A) tailing step, purified the reaction product (Shi, R. et al., Facile means for quantifying microRNA expresssion by real-time PCR, Biotechniques, 2005, 39, 519-25). The reason for this lies both in the markedly differing compositions of the reaction buffers and the substrates required for the reaction.

Another embodiment of the present invention provides a simple method that makes cDNA synthesis possible, and optionally couples this reaction with a third enzymatic reaction, which permits the specific detection of the generated cDNA in the same reaction vessel. Owing to its very simple handling, this "3-in-1" method offers particular advantages when large numbers of samples are to be analyzed for one or a few analytes. The reason is that, for example coupled with real-time PCR, it represents a very simple and rapid method for analyzing large numbers of samples. Additional manipulations and contamination are thus largely avoided, so that it is less time-consuming, it minimizes the risk of mix-up of the samples, it minimizes the risk of introduction of nucleases and finally it largely excludes the risk of pipetting errors. These advantages are of great importance in diagnostics.

The problem of the "3-in-1" reaction is addressed by a method for synthesizing a cDNA in a sample, in an enzymatic reaction, followed by another enzymatic reaction, optionally an amplification, optionally coupled with detection, either in real-time during amplification or subsequently, wherein the method comprises the following steps:
(a) simultaneous preparation of a first enzyme with polyadenylation activity, a second enzyme with reverse transcriptase activity, a buffer, at least one ribonucleotide, at least one deoxyribonucleotide, an anchor oligonucleotide, at least one third enzyme with nucleic acid synthesis activity, at least one primer, optionally a probe (b) addition of a sample comprising a ribonucleic acid and (c) incubation of the agents from steps (a) and (b) in one or more temperature steps, which are selected so that the first enzyme and the second enzyme display activity and optionally the third enzyme is active or inactive. Optionally this is followed by one or more temperature steps, in which the first enzyme and the second enzyme have little activity or are inactive and the third enzyme is active.

The substrate of the poly(A) polymerase used *in vivo* is adenosine triphosphate (ATP). For some poly(A) polymerases it has been shown that the attachment of short tails with other NTPs as substrate may also be possible (Martin, G. and Keller, W., Tailing and 3'-end labeling of RNA

with yeast poly(A) polymerase and various nucleotides, RNA, 1998, 4, 226-30).

The inventors of the present invention found, surprisingly, that it is possible, with certain preconditions, to allow the two very different enzymatic reactions to take place simultaneously, in one reaction vessel. In a preferred embodiment of the invention, the sample is a ribonucleic acid, which is selected from the group comprising prokaryotic ribonucleic acids, eurkaryotic ribonucleic acids, viral ribonucleic acids, ribonucleic acids whose origin is an Archaeon, micro-ribonucleic acids (miRNAs), small nucleolar ribonucleic acids (snoRNAs), messenger ribonucleic acid (mRNA), transfer-ribonucleic acids (tRNAs), non-polyadenylated ribonucleic acids in general, and ribosomal ribonucleic acids (rRNAs); furthermore, a mixture of two or more of the aforementioned ribonucleic acids. The sample can naturally also already contain poly(A) RNA.

In an especially preferred embodiment of the present invention, the sample is a ribonucleic acid, which is selected from the group comprising prokaryotic ribonucleic acids, miRNA, snoRNA and rRNA. In the most preferred embodiment of the present invention, the sample comprises a ribonucleic acid, which is selected from the group comprising miRNA and snoRNA. Preferably, moreover, mixed samples from different amounts of ribonucleic acids of different kinds are accompanied by other substances.

Additionally, the inventors of the present invention found that it is possible, with certain preconditions, to allow the two very different enzymatic reactions to take place simultaneously, in one reaction vessel, and additionally couple this with a third enzymatic reaction for the specific detection of the cDNA generated, which is preferably a nucleic acid synthesis activity. In a preferred embodiment of the invention, the sample is a ribonucleic acid, which is selected from the group comprising prokaryotic ribonucleic acids, eukaryotic ribonucleic acids, viral ribonucleic acids, ribonucleic acids whose origin is an Archaeon, micro-ribonucleic acids (miRNAs), small nucleolar ribonucleic acids (snoRNAs), messenger ribonucleic acid (mRNA), transfer-ribonucleic acids (tRNAs), non-polyadenylated ribonucleic acids in general, and ribosomal ribonucleic acids (rRNAs); furthermore, a mixture of two or more of the aforementioned ribonucleic acids. The sample can of course also already contain poly(A) RNA.

In an especially preferred embodiment of the present invention, the sample is a ribonucleic acid, which is selected from the group comprising prokaryotic ribonucleic acids, miRNA, snoRNA

and rRNA. In the most preferred embodiment of the present invention, the sample comprises a ribonucleic acid, which is selected from the group comprising miRNA and snoRNA. Preferably, moreover, mixed samples from different amounts of ribonucleic acids of different kinds are accompanied by other substances.

Based on these advantages of the method according to the invention, the inventors were able to show that it is possible to prepare and characterize miRNAs efficiently and without contamination.

In one embodiment of the invention, the anchor oligonucleotide is a homopolymeric oligonucleotide, which is selected from the group comprising a poly(A) oligonucleotide, poly(C) oligonucleotide, poly(T) oligonucleotide, poly(G) oligonucleotide, poly(U) oligonucleotide, poly(A) oligonucleotide additionally comprising a 5'-tail, poly(C) oligonucleotide additionally comprising a 5'-tail, poly(T) oligonucleotide additionally comprising a 5'-tail, poly(G) oligonucleotide additionally comprising a 5'-tail and poly(U) oligonucleotide additionally comprising a 5'-tail. A poly(T) oligonucleotide, which optionally, as already mentioned above, can additionally have a 5'-tail, will be preferred.

The anchor oligonucleotide according to the invention is as a rule between 6 and 75 nucleotides long. It can, however, be up to approx. 150 nucleotides long. If the anchor oligonucleotide is synthetic, the maximum length follows from the technical limitations of DNA synthesis. Optionally the anchor oligonucleotide comprises a 5'-tail and/or an anchor sequence. A 5'-tail is an additional nucleotide sequence at the 5'-end of the oligonucleotide, which serves for example for introducing a cloning sequence, primer and/or probe binding sites or any other sequence. Identification of suitable sequences for the 5'-tail is possible for a person skilled in the art based on the requirements of the particular application.

An additional anchor sequence, typically with a length of one to five further nucleotides, can be contained at the 3'-end of the anchor oligonucleotide. The anchor sequence can have a length of at least one base, and in a preferred embodiment the first position is a degenerate base, which contains all bases except the base used in the homopolymeric moiety of the anchor oligonucleotide. This can be followed by further bases. These can also be degenerate. In a preferred embodiment, it is sensible here to use N wobbles, where N= A, C, G, T or corresponding analogs.

As a general rule the anchor oligonucleotide is a deoxyribonucleic acid (DNA). However, the anchor oligonucleotide can also be a peptide nucleic acid (PNA). Locked nucleic acids (LNA), phosphorothioate-deoxyribonucleic acids, cyclohexene-nucleic acids (CeNA), N3'-P5'-phosphoroamidites (NP), tricyclo-deoxyribonucleic acids (tcDNA), are also possible. However, an anchor oligonucleotide that is a deoxyribonucleic acid (DNA) is preferred. Mixtures of RNA and DNA or of one or more of the modified nucleic acids or analogs, and other modifications such as corresponding base analogs, which in the chosen conditions are able to hybridize to RNA or DNA, are possible. In an especially preferred embodiment, the anchor oligonucleotide is a poly(T) oligonucleotide, which additionally comprises a 5'-tail, is a deoxyribonucleic acid, is 15-150 nucleotides long and is in the form of a mixture. An additional anchor sequence typically with a length of one to five further nucleotides can be contained at the 3'-end of the anchor oligonucleotide. The anchor sequence can have a length of at least one base, and in a preferred embodiment the first position is a degenerate base, which contains all bases except the base used in the homopolymeric moiety of the anchor oligonucleotide. This can be followed by further bases. These can also be degenerate. In a preferred embodiment, the use of N wobbles is sensible, where N= A, C, G, T or corresponding analogs.

For example, we may mention the following anchor oligonucleotides according to the invention:

Example 1 (SEQ ID NO: 10): 5' TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC $(T)_x$VVN 3'

Example 2 (SEQ ID NO: 11): 5' AACGAGACGACGACAGAC$(T)_x$VN 3'

Example 3 (SEQ ID NO: 12): 5' AACGAGACGACGACAGAC$(T)_x$V 3'

Example 4 (SEQ ID NO: 13): 5' AACGAGACGACGACAGAC$(T)_x$N 3'

Example 5 (SEQ ID NO: 14): 5' AACGAGACGACGACAGAC$(T)_x$NN 3'

Example 6 (SEQ ID NO: 15): 5' AACGAGACGACGACAGAC$(T)_x$VNN 3'

Example 7 (SEQ ID NO: 16): 5' AACGAGACGACGACAGAC$(T)_x$VNNN 3'

Example 8 (SEQ ID NO: 17): 5' AACGAGACGACGACAGAC$(T)_x$NNN 3'

Example 9 (SEQ ID NO: 18): 5' TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC$(T)_x$VN 3'

Example 10 (SEQ ID NO: 19): 5' TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC$(T)_x$VNN 3'

X is preferably 10 to 30 bases.

V and N are from the single letter code for degenerate bases, V= A, C, G; N= A, C,

G, T.

A person skilled in the art will be able to identify other suitable 5'-tail sequences.

The optional 5'-tail comprises an additional 1-100 nucleotides, which can find application for subsequent analyses. Thus, in a preferred embodiment the 5'-tail can contain the binding sequence for an oligonucleotide, e.g. one or more DNA probes and/or one or more PCR primers. The sequences used for the 5'-tail are preferably selected to be compatible with the method according to the invention. This comprises for example the selection of sequences that do not cause any undesirable side reactions, both in the method according to the invention and in subsequent analyses.

Anchor oligonucleotides according to the invention are shown in Fig. 12.

Basically the enzymatic reaction according to the invention can take place on a carrier or in a container, i.e. the reaction can take place in a reaction vessel. Said reaction vessel can be a reactor tube or for example a microtiter plate. The reaction can take place on a chip. If it takes place on a chip, one or more components can be immobilized. The reaction can take place on a test strip or in a microfluidic system. Very varied embodiments with respect to the carrier or container are known by a person skilled in the art.

In a preferred embodiment the ribonucleotide is an adenosine-5'-triphosphate, a thymidine-5'-triphosphate, a cytosine-5'-triphosphate, a guanine-5'-triphosphate and/or a uracil-5'-triphosphate. The ribonucleotide can also be a base analog. The ribonucleotide can be modified or labeled. Basically it is important that the ribonucleotide can be transformed by the enzyme in the polyadenylation activity as substrate.

The deoxyribonucleotide according to the invention can be selected from the group comprising a deoxyadenosine-5'-triphosphate (dATP), a deoxythymine-5'-triphosphate (dTTP), a deoxycytosine-5'-triphosphate (dCTP), a deoxyguanosine-5'-triphosphate (dGTP), deoxyuracil-5'-triphosphate (dUTP) and modified deoxyribonucleotides and labeled deoxyribonucleotides. Applications are also conceivable in which one or more deoxyribonucleotides, which contain a universal base or a base analog, are used additionally or instead. It is important, for the carrying out of the invention, that the deoxyribonucleotides used permit cDNA synthesis.

According to the invention it is preferable for dATP, dCTP, dTTP and dGTP to be present together as a mixture.

According to the invention, deoxyuracil-5'-triphosphate can also be used in the mixture. This can be combined with an enzymatic reaction that takes place after the actual reaction, and uses uracil-DNA-glycosylase and is able to degrade enzymatically produced reaction product that is not used further.

If a deoxyribonucleotide is labeled, the labeling can be selected from the group comprising ^{32}P, ^{33}P, ^{35}S, ^{3}H, a fluorescent dye such as for example fluorescein-isothiocyanate (FITC), 6-carboxyfluorescein (FAM), xanthene, rhodamines, 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 6-carboxy-4',5'-dichloro-2',7'-dimethodyfluorescein (JOE), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 5-carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), rhodamine 110; coumarins, such as umbelliferones, benzimides, such as Hoechst 33258; phenanthridines, such as Texas Red, ethidium bromide, acridine dyes, carbazole dyes, phenoxazine dyes, porphyrin dyes, polymethine dyes, cyanin dyes, such as Cy3, Cy5, Cy7, BODIPY dyes, quinoline dyes and Alexa dyes. Other markers such as the insertion of biotin or one or more haptens, e.g. digoxigenin, which permit direct or indirect detection of nucleic acid. Indirect detections, e.g. via antibodies, which once again an e.g. enzymatic detection via an enzyme coupled to the antibody. Indirect detection is also possible by introducing nanoparticles, which are coupled for example to antibodies or an affinity ligand.

The deoxyribonucleotide can also be modified via the 5'-phosphate, which allows simpler cloning. By inserting reactive groups, e.g. an amino linker (also biotin), the deoxyribonucleotide can for example be immobilized or made accessible for direct or indirect detection.

Especially preferred modifications are selected from the group comprising fluorescent dyes, haptens, 5'-phosphate, 5'-biotin, 5'-amino linkers.

According to the invention the concentration of a deoxyribonucleotide in the reaction is at least 0.01 mM and at most 10 mM. This figure given for the concentration is the concentration of the individual deoxyribonucleotide. In one of the preferred embodiments, the deoxyribonucleotides,

in each case dATP, dCTP, dGTP and dTTP are present at a concentration from 0.2 mM to 2 mM. This figure given for the concentration is the concentration of the individual deoxyribonucleotide in the mixture. In an especially preferred embodiment of the invention, the individual deoxyribonucleotide, dATP, dCTP, dGTP and dTTP is present in each case at a concentration of 0.5 mM.

The inventors found, surprisingly, that the one-step enzyme reaction that is the object of the present invention can take place in a narrow buffer-pH range from 6 to 10 in the presence of magnesium ions (Mg^{2+}). Therefore in a preferred embodiment the pH is from 6 to 10.

In an especially preferred embodiment the buffer according to the invention has a pH from 6.8 to 9.

In another embodiment of the invention the buffer according to the invention additionally comprises ions, which can be selected from the group comprising Mn^{2+}, K^+, NH^{4+} and Na^+.

Buffers according to the invention comprise for example $MgCl_2$, $MgSO_4$, magnesium acetate, $MnCl_2$, KCl, $(NH_4)_2SO_4$, NH_4Cl, NaCl. The following may be considered as buffer substance: Tris, Tricine, Bicine, Hepes, and other buffer substances that are in the pH range according to the invention or mixtures of two or more suitable buffer substances.

A number of enzymes with polyadenylation activity are known by a person skilled in the art. According to the invention, these are selected from the group comprising enzymes of prokaryotic origin, of eukaryotic origin, of viral origin and of archaeal origin, as well as enzymes of vegetable origin.

A polyadenylation activity in the sense of this invention is an enzymatic activity that uses, as substrate, the 3'-end of a ribonucleic acid and is able, in a suitable buffer, to add on ribonucleotides to this 3'-end enzymatically, and preferably at least 10 to 20 ribonucleotides. In a preferred embodiment the enzyme is an enzyme that is able to use adenosine-5'-triphosphate as substrate. According to the invention this comprises enzymes and reaction conditions that have a polyadenylation activity in the sense of the invention when using single-stranded and double-stranded RNA, e.g. hairpin RNA, for example pre-miRNA. Depending on the RNA to be analyzed, a person skilled in the art will select the enzyme and reaction conditions so that either

single-stranded RNAs (e.g. mature miRNAs) or double-stranded RNAs (e.g. pre-miRNAs) or both are made accessible for analysis.

A polyadenylation activity in the sense of the invention is in general a transcriptase activity.

In a preferred embodiment the enzyme with polyadenylation activity is an enzyme that is selected from the group comprising poly(A) polymerase from *Escherichia coli*, poly(A) polymerase from yeast, poly(A) polymerase from cattle, poly(A) polymerase from frog, human poly(A) polymerase, and vegetable poly(A) polymerase. Others are known by a person skilled in the art or can be newly identified by analysis of homology with known poly(A) polymerases. In an especially preferred embodiment the enzyme with polyadenylation activity is a poly(A) polymerase from *Escherichia coli*.

The enzyme with reverse transcriptase activity according to the invention is selected according to the invention from the group comprising enzymes from viruses, bacteria, Archaebacteria and eukaryotes, in particular from thermostable organisms. This also includes, for example, enzymes from introns, retrotransposons or retroviruses. An enzyme with reverse transcriptase activity is according to the invention an enzyme that is able to incorporate deoxyribonucleotides complementarily, on a ribonucleic acid at the 3'-end of a deoxyoligonucleotide or ribooligonucleotide hybridized to the ribonucleic acid in suitable buffer conditions. This comprises enzymes that have this function naturally, as well as enzymes that only acquire such a function through alteration of their gene sequence e.g. through mutagenesis or corresponding buffer conditions.

Preferably the enzyme with reverse transcriptase activity is an enzyme that is selected from the group comprising HIV reverse transcriptase, M-MLV reverse transcriptase, EAIV reverse transcriptase, AMV reverse transcriptase, *Thermus thermophilus* DNA Polymerase I, M-MLV RNase H, Superscript, Superscript II, Superscript III, Monsterscript (Epicentre), Omniscript, Sensiscript Reverse Transcriptase (Qiagen), ThermoScript and Thermo-X (both Invitrogen). According to the invention it is also possible to use enzymes that only have reverse transcriptase activity after a modification of the gene sequence as enzyme. A reverse transcriptase activity that has an increased error accuracy can also be used. As an example we may mention e.g. AccuScript reverse transcriptase (Stratagene). It will be evident to a person skilled in the art that the use of mixtures of two or more enzymes with reverse transcriptase activity is also possible.

It is known by a person skilled in the art that most enzymes with reverse transcriptase activity require a divalent ion. Therefore in a preferred embodiment as already described above, for those enzymes that require a divalent ion, a divalent ion is present. Mg^{2+} and Mn^{2+} are preferred.

Preferred combinations of enzymes are HIV reverse transcriptase or M-MLV reverse transcriptase or EAIV reverse transcriptase or AMV reverse transcriptase or *Thermus thermophilus* DNA Polymerase I or M-MLV RNase H, Superscript, Superscript II, Superscript III or Monsterscript (Epicentre) or Omniscript Reverse Transcriptase (Qiagen) or Sensiscript Reverse Transcriptase (Qiagen), ThermoScript, Thermo-X (both Invitrogen) or a mixture of two or more enzymes with reverse transcriptase activity and poly(A) polymerase from *Escherichia coli*. In addition HIV reverse transcriptase or M-MLV reverse transcriptase or EAIV reverse transcriptase or AMV reverse transcriptase or *Thermus thermophilus* DNA Polymerase I or M-MLV RNase H, Superscript, Superscript II, Superscript I II or Monsterscript (Epicentre) or Omniscript Reverse Transcriptase (Qiagen) or Sensiscript Reverse Transcriptase (Qiagen), ThermoScript, Thermo-X (both Invitrogen) or a mixture of two or more enzymes with reverse transcriptase activity and poly(A) polymerase from yeast.

A person skilled in the art knows that high temperatures during reverse transcription mean that problems with secondary structures do not have such a decisive role. Moreover, high temperatures mean, with certain enzymes, that the specificity of reverse transcription increases because mispairings and incorrect priming are prevented. Therefore in one embodiment of the present invention, a reverse transcriptase that is thermophilic is used. An enzyme that has an optimal nucleic acid synthesis activity at between 45°C and 85°C, preferably between 55°C and 80°C and most preferably between 60°C and 75°C, will be preferred. *Thermus thermophilus* (Tth) DNA Polymerase I is preferred.

If the enzyme with polyadenylation activity is a nonthermophilic enzyme and the enzyme with reverse transcriptase activity is a thermophilic enzyme, according to the invention the method can take place in several temperature steps, where the first temperature step provides a temperature that is the optimal temperature for the enzyme with polyadenylation activity, and the second temperature step provides a temperature that is the optimal temperature for the enzyme with reverse transcriptase activity.

If for example AMV reverse transcriptase is used, the second temperature step occurs at 42°C, whereas the first temperature step, which is primarily suitable for the activity of the enzyme with polyadenylation activity, occurs at a temperature of 37°C.
However, it can also be carried out at constant temperature.

A person skilled in the art will be able to select the temperatures so that the respective enzyme activities are developed. If for example a combination of poly(A) polymerase from *Escherichia coli* together with DNA polymerase from *Thermus thermophilus* is used, the temperature sequence is as follows: incubation first at 37°C and then at 55 to 70°C. Thus, according to the invention a non-thermostable enzyme can be combined with a thermostable enzyme. In this case the temperature steps depend on which of the two enzymes has polyadenylation activity. According to the invention it is preferable for the enzyme with reverse transcriptase activity to be thermostable. Otherwise, as will be apparent to a person skilled in the art, it is possible that as a result of the incubation at high temperature in the polyadenylation step, the enzyme with reverse transcriptase activity will be inactivated partly or completely. Therefore it is also preferable if both enzymes are thermostable.

Furthermore, a person skilled in the art knows that the enzymes have very varied processivity, so that a person skilled in the art can combine enzymes with different processivity so that templates with different length are converted to cDNA with different efficiency. By using appropriate amounts of the respective enzymes, one or more suitable incubation temperatures and incubation times, it is possible for a person skilled in the art to achieve satisfactory results.

Preferably the method according to the invention additionally comprises poly(C) polynucleotides. Especially preferably, the method according to the invention additionally comprises poly(C) polyribonucleotides. Preferably 1 ng to 300 ng of poly(C) polyribonucleotides is used per 20 µl, preferably 10 ng to 150 ng of poly(C) polyribonucleotides is used per 20 µl reaction, especially preferably 25 ng to 100 ng of poly(C) polyribonucleotides is used per reaction and and most preferably 50 ng to 75 ng of poly(C) polyribonucleotides is used per 20 µl reaction.

The reaction according to the invention can comprise further reagents such as for example volume excluders, a single-strand binding protein, DTT and/or competitor nucleic acids.

If a volume excluder is used, this is selected from the group comprising dextran, polyethylene glycol, and volume excluders according to the invention are mentioned in EP1411133A1.

In a preferred embodiment the competitor nucleic acid is a homopolymeric ribonucleic acid and most preferably polyadenoribonucleic acid. Examples are disclosed in US 6,300,069.

Preferably the method according to the invention additionally comprises poly(C) polynucleotides. Especially preferably the method according to the invention additionally comprises poly(C) polyribonucleotides. Preferably 1 ng to 300 ng of poly(C) polyribonucleotides is used per 20 µl, preferably 10 ng to 150 ng of poly(C) polyribonucleotides is used per 20 µl reaction, especially preferably 25 ng to 100 ng of poly(C) polyribonucleotides is used per reaction and most preferably 50 ng to 75 ng of poly(C) polyribonucleotides is used per 20 µl reaction.

It will be obvious to a person skilled in the art that it may be advantageous to prevent the competitor nucleic acid itself serving as a substrate for the poly(A) polymerase activity. A possible solution is blocking of the 3' OH group of the competitor nucleic acid. Appropriate solutions, e.g. the use of a 3' phosphate, incorporation of a dideoxynucleotide or inverse bases are known by a person skilled in the art.

It will also be obvious to a person skilled in the art that it may be advantageous to prevent the competitor nucleic acid itself serving as substrate for the reverse transcriptase activity. This can be ensured by selecting a competitor nucleic acid that cannot be transcribed to cDNA under the given reaction conditions, e.g. because the primers used cannot hybridize to these. Another possible solution is to block the 3' OH group of the competitor nucleic acid. Appropriate solutions, e.g. the use of a 3' phosphate, incorporation of a dideoxynucleotide or inverse bases are known by a person skilled in the art.

The invention further relates to a reaction mixture comprising a first enzyme with polyadenylation activity, a second enzyme with reverse transcriptase activity, optionally a buffer, optionally at least one ribonucleotide, optionally at least one deoxyribonucleotide and optionally an anchor oligonucleotide. Preferably the anchor oligonucleotide comprises a homopolymeric moiety, an anchor sequence and/or a tail. Preferably the reaction mixture additionally comprises random primers. The additional use of random primers has the advantage

that 5' ends of long transcripts are also transcribed efficiently, which is important in quantitative analyses. The reaction mixture can contain the same agents as are used for the method according to the invention.

In one embodiment of the invention, the anchor oligonucleotide is a homopolymeric oligonucleotide, which is selected from the group comprising a poly(A) oligonucleotide, poly(C) oligonucleotide, poly(T) oligonucleotide, poly(G) oligonucleotide, poly(U) oligonucleotide, poly(A) oligonucleotide additionally comprising a 5'-tail, poly(C) oligonucleotide additionally comprising a 5'-tail, poly(T) oligonucleotide additionally comprising a 5'-tail, poly(G) oligonucleotide additionally comprising a 5'-tail and poly(U) oligonucleotide additionally comprising a 5'-tail. A poly(T) oligonucleotide, which optionally, as already mentioned above, can additionally have a 5'-tail, is preferred.

The anchor oligonucleotide according to the invention is as a rule between 6 and 75 nucleotides long. It can, however, be up to approx. 150 nucleotides long. If the anchor oligonucleotide is synthetic, the maximum length depends on the technical limitations of the DNA synthesis. Optionally the anchor oligonucleotide comprises a 5'-tail and/or an anchor sequence. A 5'-tail is an additional nucleotide sequence at the 5'-end of the oligonucleotide, which for example serves for inserting a cloning sequence, primer and/or probe binding sites or any other sequence. Identification of suitable sequences for the 5'-tail is possible for a person skilled in the art on the basis of the requirements of the particular application.

At the 3'-end of the anchor oligonucleotide, an additional anchor sequence typically with a length of one to five further nucleotides can be contained. The anchor sequence can have a length of at least one base, and in a preferred embodiment the first position is a degenerate base, which contains all bases except the base used in the homopolymeric moiety of the anchor oligonucleotide. This can be followed by further bases. These can also be degenerate. In a preferred embodiment the use of N wobbles is sensible, where N= A, C, G, T or corresponding analogs.

The optional 5'-tail comprises an additional 1-100 nucleotides, which can be used for subsequent analyses. Thus, in a preferred embodiment the 5'-tail can contain the binding sequence for an oligonucleotide, e.g. one or more DNA probes and/or one or more PCR primers. The sequences used for the 5'-tail are preferably selected so as to be compatible with the method according to

the invention. This comprises for example the selection of sequences that do not cause any undesirable side reactions, both in the method according to the invention and in subsequent analyses.

The reaction mixture according to the invention comprises the anchor oligonucleotide according to the invention, which has a length between 10 and 150 nucleotides, and optionally bears, at the 3'-end, an anchor sequence according to the invention, with a length of one to five nucleotides. The reaction mixture according to the invention comprises the anchor oligonucleotide, which as described above is for example a deoxyribonucleic acid (DNA), a peptide nucleic acid (PNA) or a locked-nucleic acid (LNA). The reaction mixture according to the invention comprises, in a preferred embodiment, an anchor oligonucleotide according to the invention, which is a poly(T) oligonucleotide and additionally bears a 5'-tail, and the oligonucleotide is a deoxyribonucleic acid that is 10 to 75 nucleotides long and is in the form of a mixture, and an anchor sequence is present at the 3'-end, in each case consisting of a nucleotide, which is selected from the group comprising A, G and C, optionally followed by one to five further nucleotides comprising all four bases A, C, G, and T or corresponding analogs.

Anchor oligonucleotides of the reaction mixture according to the invention are shown in Fig. 12.

The reaction mixture according to the invention further comprises at least one ribonucleotide, as described above for the method according to the invention. In particular the reaction mixture according to the invention comprises at least one ribonucleotide selected from ATP, TTP, CTP, GTP, UTP or corresponding base analogs. The ribonucleotides can, as described above, optionally be modified or labeled. The reaction mixture according to the invention comprises deoxyribonucleotides, as was described for the method according to the invention. In particular the reaction mixture according to the invention comprises one or more deoxyribonucleotides, for example dATP, dCTP, dGTP, dUTP and/or dTTP. In a preferred embodiment a mixture of deoxyribonucleotides is used, which permits a cDNA synthesis. These deoxyribonucleotides can optionally be modified or labeled.

If a deoxyribonucleotide of the reaction mixture according to the invention is labeled, the marker can be selected from the group comprising ^{32}P, ^{33}P, ^{35}S, ^{3}H, a fluorescent dye such as for example fluorescein-isothiocyanate (FITC), 6-carboxyfluorescein (FAM), xanthene, rhodamines, 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 6-carboxy-4',5'-dichloro-2',7'-

dimethodyfluorescein (JOE), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 5-carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), rhodamine 110; Cy3, Cy5, Cy7, coumarins, such as umbelliferones, benzimides, such as Hoechst 33258; phenanthridines, such as Texas Red, ethidium bromide, acridine dyes, carbazole dyes, phenoxazine dyes, porphyrin dyes, polymethine dyes, cyanin dyes, such as Cy3, Cy5, BODIPY dyes, quinoline dyes and Alexa dyes. Other markers such as the insertion of biotin or one or more haptens, e.g. digoxigenin, which permit direct or indirect detection of the nucleic acid. Indirect detections, e.g. via antibodies, which once again e.g. enzymatic detection via an enzyme coupled to the antibody. An indirect detection is also possible by introducing nanoparticles, which are for example coupled to antibodies or an affinity ligand.

The reaction mixture according to the invention comprises in each case a deoxyribonucleotide at a concentration from 0.01 mM to 10 mM. Preferably the individual deoxyribonucleotide A, C, G and T is present at a concentration in each case from 0.2 mM to 2 mM. It is especially preferable if the deoxyribonucleotides A, C, G and T are present together. Each individual one is present in this preferred embodiment at a concentration of 0.5 mM.

Furthermore, the reaction mixture according to the invention comprises a buffer. This buffer has a pH from 6 to 10. The reaction mixture according to the invention additionally contains Mg^{2+} ions. In an especially preferred embodiment the reaction mixture according to the invention has a buffer with a pH from 6.8 to 9. The reaction mixture can additionally also comprise ions, which can be selected from the group comprising Mn^{2+}, K^{+}, NH^{4+} and Na^{+}. The presence of two different enzyme activities is important for the reaction mixture according to the invention. The reaction mixture according to the invention comprises at least one first enzyme activity with polyadenylation activity and secondly a second enzyme activity with reverse transcriptase activity. The preferred embodiments of these activities have already been described above for the method. The reaction mixture can, like the method above, also comprise additional substances, for example a volume excluder, a single-strand binding protein, DTT or one or more competitor nucleic acids.

If a volume excluder is used, it is preferable for this to be selected from the group comprising dextran, polyethylene glycol. Other volume excluders according to the present invention will be found in EP1411133A1.

If the reaction mixture optionally comprises a competitor nucleic acid, this is selected from the group comprising homopolymeric ribonucleic acids and polyadenoribonucleic acid. Other competitor nucleic acids according to the invention are disclosed in US 6,300,069.

Preferably the reaction mixture according to the invention additionally comprises poly(C) polynucleotides. Especially preferably, the reaction mixture according to the invention additionally comprises poly(C) p olyribonucleotides. Preferably, 1 ng to 300 ng of poly(C) polyribonucleotides is used per 20 µl, preferably 10 ng to 150 ng of poly(C) polyribonucleotides is used per 20 µl reaction, especially preferably 25 ng to 100 ng of poly(C) polyribonucleotides is used per reaction and most preferably 50 ng to 75 ng of poly(C) polyribonucleotides is used per 20 µl reaction.

The invention further relates to a kit, comprising a reaction mixture, as was described above. In a preferred embodiment the reaction mixture is in a single reaction vessel. In another embodiment the kit comprises a reaction vessel, comprising the enzyme with polyadenylation activity, the enzyme with reverse transcriptase activity, optionally the deoxyribonucleotides, optionally at least one ribonucleotide, optionally a buffer containing Mg^{2+} and optionally one or more oligodeoxyribonucleotides in the sense of the invention. Optionally the reaction vessel in the kit according to the invention can contain further constituents, as were stated for the reaction mixture according to the invention. The kit can moreover comprise a probe for the 5'-tail of the anchor oligonucleotide according to the invention. Furthermore, the kit can contain one or more further deoxyribonucleotides, e.g. a generic primer for detecting the tail sequence introduced by the reverse transcription. The reaction mixture can be in "pellet form", for example lyophilized. Other methods of preparation, for example that do not comprise liquid forms, are known by a person skilled in the art.

Furthermore, the kit can optionally be combined with reagents that are required for the PCR reaction or real-time PCR reaction. Preferably these are reagents for at least one PCR reaction, which permits the detection of at least one of the cDNAs generated in the method according to the invention.

The kit can moreover optionally comprise random primers and optionally one or more primers or primer/probes for the detection of further target genes in singleplex or multiplex PCR-reactions and/or real-time singleplex or multiplex PCR reactions.

The reaction mixture, the method according to the invention or the kit, can moreover contain target-specific primers. The length of the target-specific primers should be selected so that a specific detection is possible in a PCR reaction, the sequence of the target primer should be so specific that binding is only possible on one site in the generated cDNA sequence. As a general rule said primer has a length of 15 to 30 nucleotides, preferably 17 to 25 nucleotides.

In an especially preferred embodiment the reaction mixture comprises the enzyme with polyadenylation activity and the enzyme with reverse transcriptase activity as a two-enzyme pre-mix. The kit comprises, in this especially preferred embodiment, the two-enzyme pre-mix reaction mixture in one reaction vessel and, in a separate vessel, a buffer, Mg^{2+}, rNTP(s), dNTP(s), optionally an anchor oligonucleotide according to the invention and optionally random primers and furthermore optionally a volume excluding reagent and/or a competitor nucleic acid.

The method according to the invention can, as already mentioned above, take place in one or more temperature steps. In a preferred embodiment the method according to the invention takes place in a single temperature step for the incubation and another temperature step for the inactivation for the enzymes. Thus, in a preferred embodiment the incubation step in which the enzymes display activity is about 37°C. The incubation time is about 1 to 120 minutes, preferably 5 to 90 minutes, more preferably 10 to 75 minutes, even more preferably 15 to 60 minutes, even more preferably 20 to 60 minutes and most preferably 50 to 70 minutes. As a general rule an incubation time beyond this is not harmful. In another step, which serves for denaturation of the enzymes, a temperature of at least 65°C and at most 100°C is used. Preferably a temperature of about 80-95°C is used. The denaturation takes place for a period of at least 1 minute and at most for 30 minutes. In a preferred embodiment denaturation is carried out for a period of 5 minutes.

The method according to the invention for generating cDNA can subsequently comprise a polymerase chain reaction. If this is the case, preferably a primer specific for the tail introduced during cDNA synthesis and/or a specific primer is added to the reaction mixture according to the invention. The reaction mixture then also contains a thermostable DNA polymerase.

In a preferred embodiment, all reaction components are already present in the tube, before the

first reaction is started (see example 9). To achieve an optimal signal-noise ratio, which is displayed e.g. by high Ct values in reactions without templates (so-called "no template controls") in a real-time PCR, it may be advantageous for one or more components that are critical for the third reaction to be offered in an inactive form or to be separated physically. In example 8, for example, the primers were "fixed" in the cover.

If the third reaction is a PCR, it is preferably a real-time PCR. A number of methods that allow reversible inactivation of the thermostable DNA polymerase used are known by a person skilled in the art. The method known as hot-start PCR offers a number of technical solutions. Known hot-start methods for PCR enzymes are: chemical (cf. DE69928169T), antibodies, aptamers, DNA oligos, Affybodies, microencapsulation (see also DE69930765T).

Furthermore, when using PCR as the third reaction, it has proved advantageous if the oligonucleotides that are not required until the PCR, i.e. one or more target-specific primers, the tail-specific primer, and optionally one or more fluorescence-labeled probes, are only available at the start of the third reaction. As technical transformation for preparing the oligonucleotides in initially inactive form followed by activation or reactivation of these primers that are relevant to the PCR reaction, various technical transformation forms are conceivable. A preferred possibility is the temporal compartmentation of individual reagents. These are then released as required. It is especially preferred to separate the amplification enzyme or the amplification primers.

This comprises chemical modifications of the relevant primers for the PCR reaction, e.g. by inserting sides groups on the backbone, or the bases or a combination thereof or packaging in complexes, which reduce or completely inhibit the interaction with the target nucleic acid and/or one or more participating enzymes (see also families to DE69930765T, US6274353, EP0866071), and/or a physical barrier. For removing these sides groups or complexes with the aim of providing the relevant oligonucleotides for the third reaction, preferably a PCR or real-time PCR, a number of methods are conceivable, or are known by a person skilled in the art. This activation can take place chemically or enzymatically by means of a number of physical parameters such as temperature, pH, ionic strength, mechanical effects, or a combination of two or more of the parameters. As examples, possible embodiments of the Hot Start primers are presented in the examples. The prior art describes various embodiments for a Primer Hot Start (cf. DE69930765T, US6274353, EP0866071).

In the case of enzymatic activation, the enzyme used for activation can also be provided in an inactive form. Suitable Hot Start methods are known by a person skilled in the art and have already been described above (among others, EP0962526). In this connection it is obvious to a person skilled in the art that by using a thermostable enzyme, which was isolated e.g. from a thermophilic or hyperthermophilic organism, in combination with a suitable Hot Start method, e.g. EP0962526, it is possible to adapt the enzymatic activation to the requirements of the third reaction. The activation can also take place chemically or enzymatically by means of a number of physical parameters such as temperature, pH, ionic strength, mechanical effects, or a combination of two or more of the parameters.

As a further preferred embodiment, the aforesaid compartmentation of the reactions is conceivable. First, conditions are provided in which the 2-in-1 PAP-RT reaction proceeds. Then reactants for a third reaction are prepared without handling steps on the part of the experimenter, preferably without opening the vessel, e.g. by lifting a physical block (e.g. thermal, mechanical). In a possible embodiment, a thermolabile barrier is used. Preferably this employs a clear polymer e.g. wax with a defined melting point. This is mixed under suitable conditions with the relevant oligonucleotides for the third reaction, producing a phase that is solid under the reaction conditions of the first and second reaction. The solid phase can be obtained in many different ways. It can be, for example, a solid drop on the bottom of the vessel, one or more particles, a coating on the surfaces of the reaction compartments or some other form. By changing the temperature for the third reaction, the relevant oligonucleotides are released and become available to the third reaction.

Another form of compartmentation of the reactions is a physical barrier, by which contact of the reactants is prevented or at least minimized uncritically. This can be a septum, e.g. of wax or polymer, the permeability of which can then be influenced chemically or enzymatically by means of a number of physical parameters such as temperature, pH, ionic strength, mechanical effects. In one possible embodiment, one or more components of the third reaction are separated by a wax barrier from the components of the 2-in-1 PAP-RT reaction. For this, in a special embodiment in conventional reaction vessels for PCR or real-time PCR, one or more components of the third reaction are present on the bottom of the reaction vessel, covered with at least one septum in the sense of this invention, and then on top of that, the components of the 2-in-1 PAP-RT reaction. It is obvious to a person skilled in the art that the components of the third reaction can be either in liquid form or dried or otherwise stabilized, or as multiple

concentrates, or combinations of one or more formats.

In one embodiment, which is also preferred, the primers were used in a modified form, as so-called "hot-start" primers.

These are then so-called "CleanAmp" primers, obtainable from Trilinkbiotech.com. "CleanAmp" primers possess the property that they only become functional by the initial heating step at the start of the PCR and therefore are not available in functional form until the PCR. In a preferred embodiment, the primers are inactivated as described in WO2007/139723 (applicant: Trilink), in particular in claim 1 and the subsequent claims.

The method in which the primers are inactive at first was carried out with the following primers:

SEQ ID NO. 3	Hum Uni	5'-AAC GAG ACG ACG ACA GAC-3'
SEQ ID NO. 4	Let 7short	5'-GAG GTA GTA GGT TGT ATA G-3'
SEQ ID NO. 5	hsa-miR-24	5'-TGG CTC AGT TCA GCA GGA-3'
SEQ ID NO. 6	hsa-miR-15a	5'-TAG CAG CAC ATA ATG GTT T-3'
SEQ ID NO. 7	hsa-miR-16	5'-TAG CAG CAC GTA AAT ATT G-3'

The PCR reaction that takes place next can also be a quantitative PCR reaction. It can take place on an array, take place in a microfluidic system, take place in a capillary or it can be a real-time PCR. Other variants of the PCR are known by a person skilled in the art and are also included in the method according to the invention.

The DNA polymerase is preferably thermostable. It can be selected from the group comprising enzymes derived from thermophilic bacteria or from thermophilic Archaea. Preferably it is selected from the group comprising polymerases from *Thermus aquaticus, Thermus pacificus, Thermus thermophilus, Pyrococcus furiosus, Thermus brockianus, Aquifex aeolicus* and other DNA polymerases that are thermostable.

Other methods of amplifaction can also be used, and these can be selected from the group comprising "rolling circle amplification" (as de scribed in Liu, et al., "Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerases." J. Am. Chem. Soc. 118:1587-1594 (1996)), "isothermal amplification" (as described in Walker, et al., "Strand displacement amplification – an isothermal, in vitro DNA amplification technique" Nucleic Acids Res. 20(7):1691-6 (1992)), "ligase chain reaction" (as described in Landegren, et

al., "A Ligase-Mediated Gene Detection Technique" Science 241:1077-1080, 1988, or in Wiedmann, et al., "Ligase Chain Reaction (LCR) – Overview and Applications," PCR Methods and Applications (Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY, 1994) pp. S51-S64.)). However, the polymerase chain reaction is preferred.

The invention also relates to a method for reverse transcription of RNA to DNA, said method comprising the following steps: preparation of a sample comprising RNA, addition of a first enzyme with reverse transcriptase activity, a buffer, at least one deoxyribonucleotide, an oligonucleotide, incubation of the agents in one or more temperature steps, which are selected so that the enzyme displays activity, with the reaction additionally comprising poly(C) polynucleotides.

Preferably the enzyme with reverse transcriptase activity is HIV reverse transcriptase, M-MLV reverse transcriptase, EAIV reverse transcriptase, AMV reverse transcriptase, *Thermus thermophilus* DNA Polymerase I, M-MLV RNase H$^-$ (Superscript, Superscript II, Superscript III), Monsterscript (Epicentre), Omniscript Reverse Transcriptase (Qiagen), Sensiscript Reverse Transcriptase (Qiagen), ThermoScript, Thermo-X (both Invitrogen) or a mixture of two or more enzymes with reverse transcriptase activity and poly(A) polymerase from *Escherichia coli.* HIV reverse transcriptase is especially preferred.

Preferably the reaction comprises poly(C) polyribonucleotides. Preferably 1 ng to 300 ng of poly(C) polyribonucleotides is used per 20 µl, preferably 10 ng to 150 ng of poly(C) polyribonucleotides is used per 20 µl reaction, especially preferably 25 ng to 100 ng of poly(C) polyribonucleotides is used per reaction and most preferably 50 ng to 75 ng of poly(C) polyribonucleotides is used per 20 µl reaction.

A hompolymeric nucleic acid can be used in the 3-in-1 reaction.

In a preferred embodiment of the invention, the sample is a ribonucleic acid, which is selected from the group comprising prokaryotic ribonucleic acids, eukaryotic ribonucleic acids, viral ribonucleic acids, ribonucleic acids whose origin is an Archaeon, micro-ribonucleic acids (miRNAs), small nucleolar ribonucleic acids (snoRNAs), messenger ribonucleic acid (mRNA), transfer-ribonucleic acids (tRNAs), non-polyadenylated ribonucleic acids in general, and ribosomal ribonucleic acids (rRNAs); furthermore, a mixture of two or more of the

aforementioned ribonucleic acids. Of course, poly(A) RNA can also be contained already in the sample.

An RNA can serve as template that is selected from the group comprising eukaryotic ribonucleic acids, mRNA, prokaryotic ribonucleic acids, miRNA, snoRNA and rRNA. In the most preferred embodiment of the present invention, the sample comprises a ribonucleic acid, which is selected from the group comprising miRNA and snoRNA. Moreover, mixed samples of different amounts of ribonucleic acids of different kinds, accompanied by other substances, are preferred.

Based on these advantages of the method according to the invention, the inventors were able to show that it is possible to prepare and characterize miRNAs efficiently and without contamination. Generally the method provides good reverse transcription of small amounts of RNA.

The invention further relates to a kit for reverse transcription, comprising an enzyme with reverse transcriptase activity and poly(C) polynucleotides, preferably poly(C) polyribonucleotides.

In one embodiment the RNA is first polyadenylated before the sample is reverse-transcribed.

EXAMPLES

EXAMPLE 1

Demonstration of the feasibility of a coupled one-stage process of poly(A) reaction and reverse transcription in the same reaction vessel; effect of different buffers on the efficiency of detection of a 22-mer RNA oligonucleotide

In this experiment the feasibility of a coupled one-stage process of poly(A) polymerase reaction and reverse transcription in the same reaction vessel was to be demonstrated. For this purpose the coupled one-stage process was carried out under various conditions. These were on the one hand the buffer supplied with the poly(A) polymerase, and on the other hand the buffer supplied with the reverse transcriptase. In addition, a mixture of poly(A) polymerase buffers and reverse transcriptase buffers was tested. As control, the reaction was carried out in a two-stage method

on the basis of Fig. 1 A.

The reactions were set up as shown in Table 1.

Table 1: Poly(A) reaction and reverse transcription

	Assay 1 a/b	Assay 2 a/b	Assay 3 a/b	Assay 4 a/b	
	Final concentration figures			two-stage method	
Reagents	PAP buffer	RT buffer	Mixture of both buffers	1.) PAP reaction	2.) RT reaction
5x PAP buffer	1 x		1 x	1 x	
10x RT buffer		1 x	1 x		1x
$MnCl_2$ solution 25 mM	2.5 mM		2.5 mM	2.5 mM	
rATP, 10 mM	1 mM	1 mM	1 mM	1 mM	
Poly(A) polymerase 2U/µl	4 U (0.2U/µl)	2 U (0.1U/µl)	4 U (0.2U/µl)	2 U (0.2U/µl)	
dNTP mix (dA, dT, dG, dC per 5 mM)	0.5 mM	0.5 mM	0.5 mM		0.5 mM
UniGAPdT primer 10 µM	1 µM	1 µM	1 µM		1 µM
RNase inhibitor 10 U/µl	10 U	10 U	10 U		10 U
Sensiscript reverse transcriptase	1 µl	1 µl	1 µl		1 µl
RNase-free water	variable	variable	variable	variable	variable
a) mleu7a	2x10E9 copies	2x10E9 copies	2x10E9 copies	2x10E9 copies	10 µl PAP reaction 1.)
b) neg control (H_2O instead of mleu7a)	H_2O	H_2O	H_2O	H_2O	
a) maize RNA	50 ng	50 ng	50 ng	50 ng	
b) neg control (H_2O instead of maize RNA)	H_2O	H_2O	H_2O	H_2O	
Total volume	20 µl	20 µl	20 µl	10 µl	20 µl

Incubation	1 h 37°C 5 min 93°C			1 h 37°C, then continue at 2.) RT reaction	1 h 37°C 5 min 93°C

PAP: poly A polymerase
RT: reverse transcription
rATP: adenosine 5'-triphosphate

The reagents shown in Table 2 were used.

Table 2: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106); Plant Protocol with 10-fold upscale (Maxi shredder and column)
mleu7a RNA oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

In each case reactions were carried out with template (1a, 2a, 3a, 4a, all with synthetic RNA oligonucleotide against a background of maize RNA) or without template (1b, 2b, 3b, 4b, all with H_2O added instead of template). The reactions without template were carried out as control for possible appearance of nonspecific background. Maize RNA was selected as background RNA, because the sequence of the 22-mer RNA oligonucleotide to be detected does not occur in maize.

After inactivation of the enzymes (see Table: 5 min at 93°C), 2 µl of each assays 1 a/b to 4 a/b was used as template in a real-time PCR. The real-time PCR assay was carried out in triple assays as shown in Table 3 with QuantiTect SYBR Green PCR Kit (catalog No. 204143) and the primers shown in Table 4.

Table 3: Components for SYBR Green real-time PCR

	Final concentration
2x SYBR Green PCR Master Mix	1x
Hum Uni Primer 10 µM	0.5 µM
miRNA primer let7short 10µM	0.5 µM
RNase-free water	variable
PAP/RT reaction	2 µl
Final reaction volume	20 µl

Table 4: Materials for SYBR Green PCR

QuantiTect SYBR Green PCR Kit (200)	Qiagen; Material Number: 204143

Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'

The sequence 5'-AAC GAG ACG ACG ACA GAC-3' contained in the universal tail-primer Hum Uni Primer was described in US2003/0186288A1.

The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in the QuantiTect SYBR Green PCR Master Mix for 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 52°C and 30 s at 72°C (see Table 5).

Table 5: PCR protocol for SYBR Green real-time PCR

PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	40x
Annealing	30 s 52°C	
Extension (data acquisition)	30 s 72°C	
Melting curve		

The fluorescence data were collected during the 72°C extension step. The PCR analyses were carried out with an ABI PRISM 7700 (Applied Biosystems) in a reaction volume of 20 µl.

The PCR products then underwent a melting curve analysis. This was carried out on an ABI PRISM 7000 real-time PCR instrument.

Table 6: Results of real-time PCR analysis of the assays from Table 1

Detector	PAP/RT	Template	Ct	Ct mean	CV, %
SYBR Green	1) PAP buffer	a) mleu7a 2x10^8 copies + maize cDNA 5 ng	25.64		
			25.22	25.50	0.95
			25.64		
		b) H_2O in PAP - RT reaction	No Ct		
			No Ct	No Ct	
			No Ct		
	2) RT buffer	a) mleu7a 2x10^8 copies + maize cDNA 5 ng	17.43		
			17.31	17.32	0.58
			17.23		
		b) H_2O in PAP - RT reaction	No Ct		
			No Ct	No Ct	
			No Ct		
	3) mixture of	a) mleu7a	30.41		

	both buffers	2x10^8 copies + maize cDNA 5 ng	30.23	30.28	0.36
			30.21		
		b) H_2O in PAP - RT reaction	No Ct		
			No Ct	No Ct	
			No Ct		
	4) two-stage method	a) mleu7a 2x10^8 copies + maize cDNA 5 ng	25.54		
			25.74	25.64	0.39
			25.65		
		b) H_2O in PAP - RT reaction	No Ct		
			No Ct	No Ct	
			No Ct		

The identity of the PCR products was then verified by agarose-gel electrophoresis. For this, 10 µl of each PCR reaction was loaded on 2% agarose gel stained with ethidium bromide and separated. 100bp Ladders (Invitrogen, catalog number 15628-050) were used as the size standard. The results are shown in Fig. 3.

EXAMPLE 2

Demonstration of the reproducibility and specificity of a coupled one-stage process of poly(A) polymerase reaction and reverse transcription in the same reaction vessel

In this experiment the aim was to reproduce the feasibility of a coupled one-stage process of poly(A) polymerase reaction and reverse transcription in the same reaction vessel. For this, the efficiency of the one-stage process of poly(A) polymerase reaction and reverse transcription in the same reaction vessel was analyzed for the example of detection of a 22-mer RNA oligonucleotide. A reverse transcription reaction for the template used according to standard conditions served as control for the specificity of detection.

For this purpose the coupled one-stage process was carried out under various conditions. These were on the one hand the buffer supplied with poly(A) polymerase, and on the other hand the buffer supplied with the reverse transcriptase (Tables 7, 8).

Table 7: Designation and components of the reactions carried out

Designation	Buffer	Template
Reaction 1	1 step method in PAP buffer	RNA 50 ng + mleu7a 2x10E9 copies
Reaction 2		RNA 50 ng

Reaction 3		negative control: H_2O
Reaction 4	1 step method in RT buffer	RNA 50 ng + mleu7a 2x10E9 copies
Reaction 5		RNA 50 ng
Reaction 6		negative control: H_2O
Reaction 7	Standard RT	RNA 50 ng + mleu7a 2x10E9 copies
Reaction 8		RNA 50 ng

Table 8: Composition of the combined poly(A) polymerase/reverse transcription reaction and the standard reverse transcription reaction

Figures for final concentration

Reagents	Reaction 1, 2, 3 PAP buffer	Reaction 4, 5, 6 RT buffer	Reaction 7, 8 standard RT
5x PAP buffer	1 x		
10x RT buffer		1 x	1x
$MnCl_2$ 25 mM	2.5 mM		
rATP 10 mM	1 mM	1 mM	
Poly(A) polymerase 2U/µl	2 U (0.1U/µl)	2 U (0.1U/µl)	
dNTP mix (dA, dT, dG, dC, per 5 mM)	0.5 mM	0.5 mM	0.5 mM
UniGAPdT primer 10 µM	1 µM	1 µM	1 µM
RNase inhibitor 10 U/µl	10 U	10 U	10 U
Sensiscript reverse transcriptase	1 µl	1 µl	1 µl
RNase-free water	variable	variable	variable
mleu7a: reaction 1, 4, 7	2x10E9 copies	2x10E9 copies	2x10E9 copies
RNA: reaction 1, 2, 4, 5, 7, 8	50 ng	50 ng	50 ng
Neg. control (H_2O instead of RNA) in reaction 3, 6	H_2O	H_2O	
Total volume	20 µl	20 µl	20 µl

Incubation	1 h 37°C 5 min 93°C

All reaction mixtures were then divided up:
Assays a) 10 µl was taken and stored at 4°C;
For all assays b): Uni GAP dT primer [1 µM] and 0.5 µl Sensiscript Reverse Transcriptase were added again to 10 µl and a reverse transcription was carried out again (1 h 37°C), then the reverse transcriptase was inactivated (5 min 93°C).

Furthermore, a standard reverse transcription reaction was carried out, for the purpose of verifying the specificity of detection in the subsequent PCR (Table 7, 8 see above). After the poly(A) polymerase reaction and reverse transcription, the samples were divided up. In each case, Uni GAP dT primer and reverse transcriptase were added again to half of a sample (see

Table 7, above). The aim of this was to rule out the possibility that, to a slight extent, false-positive signals arise through unwanted attachment of an A-tail on the Uni GAP dT primer. Total RNA, which was isolated from human blood with an RNeasy Midi Kit (Qiagen, Hilden, Germany, Cat. No. 75144), was added as template.

The components used in the individual reactions and their designations are presented in Table 7, see above. The sequence of the 22-mer RNA detected corresponds to human leu7a miRNA (EMBL Acc#: AJ421724) and is possibly expressed in human blood cells such as leukocytes. However, owing to the purification technology of the RNeasy method used for RNA isolation, such small RNAs are only purified very inefficiently. The RNeasy method only guarantees efficient binding of RNAs with a size greater than 200 bases on the silica membrane of the RNeasy column (QIAGEN RNeasy Midi/Maxi Handbook, 06/2001, p. 9) and therefore there is severe depletion of small RNAs such as miRNAs.

In addition, the synthetic 22-mer RNA was used at a concentration that is far higher than the expected endogenous copy number. The reactions were set up as shown in Table 8 (see above).

The reagents shown in Table 9 were used.

Table 9: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV V(N-Q)-3'
RNA	Leukocyte RNA isolated from human blood with an RNeasy Midi Kit (Qiagen, Cat. No. 75144)
mleu7a RNA oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

After inactivation of the enzymes (5 min at 93°C) the assays were diluted 1:2 with water and 2 µl of each of the assays 1a/b to 8a/b was used as template in a real-time SYBR Green PCR. The assay in the real-time PCR was carried out in double assays as shown in Table 9 (above) with QuantiTect SYBR Green PCR Kit (catalog No. 204143) and the primers shown in Table 10.

Table 10: Components for SYBR Green real-time PCR

	Final concentration
2x SYBR Green PCR Master Mix	1x
Hum Uni Primer 10 µM	0.5 µM
miRNA primer let7short 10µM	0.5 µM
RNase-free water	variable
PAP/RT reaction prediluted 1:2	2 µl
Final reaction volume	20 µl

The sequence 5'-AAC GAG ACG ACG ACA GAC-3' contained in the universal tail-primer Hum Uni Primer was described in US 2003/0186288A1. The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in the QuantiTect SYBR Green PCR Master Mix for 15 min at 95°C, followed by 40 cycles with 15 s at 94°C, 30 s at 52°C and 30 s at 72°C (see Table 11).

Table 11: Materials for SYBR Green PCR

QuantiTect SYBR Green PCR Kit (200)	Qiagen; Material Number: 204143
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'

The fluorescence data were recorded during the 72°C extension step. The PCR analyses were carried out with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl and then a melting curve analysis was carried out.

The coupled one-stage poly(A) reaction and reverse transcription are possible both in poly(A) polymerase buffer and in RT buffer, which is clear from the real-time PCR analyses. Preferred buffer conditions have already been given in the text (see above). There are large differences in the Ct values obtained using the different buffers.

The standard reverse transcription reactions (reactions 3, 6) without poly(A) reactions, carried out for checking the specificity, are all negative (no Ct). It can therefore be concluded that without polyadenylation of the 22-mer RNA (1, 4, 7) or of the naturally occurring miRNA (2, 5, 8) as expected there is no template for PCR amplification and therefore no signal can be generated ("no Ct").

In the assays "RT doubled", after the first incubation further RT enzyme and Uni GAP dT

primers were added with the aim of making UniGap dT primer, possibly poly(A)-tailed in the first reaction, detectable by an RT reaction. All these assays showed no Ct, i.e. unwanted artifacts are not detectable.

Unwanted artifacts such as poly(A) tailing of the primer used for the cDNA synthesis are also not detectable.

EXAMPLE 3

Detection of various miRNAs by the method according to the invention

The aim of this experiment was to demonstrate that from a template cDNA, which was synthesized with the method according to the invention with poly(A) reac tion of common reverse transcription, several targets can be detected by miRNA specific PCR primers. For this, a method with 293 RNA as templates was carried out. A reverse transcription reaction for the template used according to standard conditions served as control for the specificity of detection.

In the subsequent SYBR Green PCR, altogether in each case one of 4 different specific primers for miRNAs was used together with the tail-specific primer. In addition, a primer localized at the 3'-end of the human β-actin transcript was used together with a tail-specific primer, to test the efficiency of the poly(A) reaction and reverse transcription.

For the poly(A) reaction and reverse transcription (PAP + RT reaction), the reagents from Table 13 were pipetted together, as shown in Table 16.

Table 13: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25μmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
293 RNA: from human cell line 293 (ATCC number: CRL-1573)	Isolated with Qiagen RNeasy Midi Kit

Table 16: PAP + RT reaction

Reagents	Final concentration
10x Buffer RT	1 x
rATP 10 mM	1 mM
Poly(A) polymerase 2U/µl	2 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U
Sensiscript reverse transcriptase	1 µl
RNase-free water	variable
a) 293 RNA 20 ng/µl b) H_2O for neg control	5 µl (100 ng) 5 µl
Total volume	20 µl
Incubation	1 h 37°C 5 min 93°C

In reaction a) 293 RNA and in reaction b) water was added as negative control (neg control). As control for the specificity of detection, a standard reverse transcription reaction was carried out with the reagents stated in Table 14 based on the schemes in Table 17.

Table 14: Materials for the reverse transcription

Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
293 RNA	Isolated with Qiagen RNeasy Midi Kit

Table 17: Standard RT reaction

Reagents	2.) RT reaction
10x Buffer RT	1x
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U
Sensiscript reverse transcriptase	1 µl
RNase-free water	variable
c) 293 RNA 20 ng/µl d) H_2O for neg control	5 µl (100 ng) 5 µl
Total volume	20 µl
Incubation	1 h 37°C 5 min 93°C

In reaction c) 293 RNA and in reaction d) water was added as negative control (neg control). The samples were then incubated for one hour at 37°C. To stop the reaction, the reactions were

incubated for 5 min at 93°C; this temperature step inactivates the enzymes.

After inactivation of the enzymes, the assays were diluted 1:2 with water and 2 µl of each of the assays a) to d) was used as template in a real-time SYBR Green PCR. The materials for the PCR are shown in Table 15.

Table 15: Materials for SYBR Green PCR

QuantiTect SYBR Green PCR Kit (200)	Qiagen; Material Number: 204143
let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
hsa-miR-24 (specific miRNA primer)	5'-TGG CTC AGT TCA GCA GGA-3'
hsa-miR-15a (specific miRNA primer)	5'-TAG CAG CAC ATA ATG GTT T-3'
hsa-miR-16 (specific miRNA primer)	5'-TAG CAG CAC GTA AAT ATT G-3'
β-actin 3' primer	5'-GTA CAC TGA CTT GAG ACC AGT TGA ATA AA-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'

Ten different reaction mixtures were pipetted. In reaction 1-5 (Table 18), in each case the miRNA specific or β-actin 3' primer and the tail-primer (Hum Uni) were used.

Table 18: SYBR Green PCR reaction 1-5

Components for SYBR Green PCR	Final concentration
2x SYBR Green PCR Master Mix	1x
Hum Uni Primer 10 µM	0.5 µM
one specific miRNA primer (10 µM) for each let 7short hsa-miR-24 hsa-miR-15a hsa-miR-16 or β-actin 3' primer	0.5 µM
RNase-free water	variable
PAP + RT reaction a) b) prediluted 1:2 Standard RT reaction c) d) prediluted 1:2 or H_2O as neg control	2 µl (5 ng) 2 µl (5 ng) 2 µl
Final reaction volume	20 µl

In reaction 6-10 (Table 19), in each case only one primer was used, either the miRNA specific primer or the tail-specific primer.

In reaction 6-10 (Table 19), in each case only one primer was used, either the miRNA specific primer or the tail-specific primer.

Table 19: Control with only one primer reaction 6-10

Components for SYBR Green PCR	Final concentration
2x SYBR Green PCR Master Mix	1x
one specific miRNA primer (10 µM) each let 7short hsa-miR-24 hsa-miR-15a hsa-miR-16 in each case with 3' Hum Uni primer	0.5 µM
RNase-free water	variable
PAP + RT reaction a) b) prediluted 1:2 Standard RT reaction c) d) prediluted 1:2 or H_2O as neg control	2 µl (5 ng) 2 µl (5 ng) 2 µl
Final reaction volume	20 µl

The order in which the primers were introduced into the reaction mixtures can be seen from Table 20. The assay in the real-time PCR was carried out in double assays.

Table 20: Primers

	Primer
Reaction 1	β-actin 3' primer + Hum Uni
Reaction 2	let 7 short + Hum Uni
Reaction 3	hsa-miR-24 + Hum Uni
Reaction 4	hsa-miR-15[a] + Hum Uni
Reaction 5	hsa-miR-16 + Hum Uni
Reaction 6	Hum Uni
Reaction 7	let 7short
Reaction 8	hsa-miR-24
Reaction 9	hsa-miR-15a
Reaction 10	hsa-miR-16

The sequence AAC GAG ACG ACG ACA GAC contained in the universal tail-primer Hum Uni Primer was described in US2003/0186288A1.

The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in the QuantiTect SYBR Green PCR Master Mix for 15 min at 95°C, followed by 40 cycles with 15 s at 94°C, 30 s at 52°C and 30 s at 72°C (see Table 21). The fluorescence data were recorded during the 72°C extension step. The PCR analyses were carried out with an Applied Biosystems 7000 Fast Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl and then a melting curve analysis was carried out.

Table 21: 3-step PCR protocol

PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	40x
Annealing	30 s 52°C	
Extension	30 s 72°C	
Melting curve		

It can be seen that the efficiency of the reverse transcription carried out under standard conditions and of the method according to the invention for the β-actin system chosen as an example is comparable, as is clear from comparable Ct values in the real-time PCR (Fig. 6).

Real-time PCR gives, when using the cDNA produced under standard conditions, very high Ct values of over 38, which mean, in a real-time PCR carried out with SybrGreen, a very good specificity of detection (Fig. 6). In the agarose-gel analysis of the PCR products, PCR products of the expected size were detected (Fig. 8), or no product was detected when using the cDNA produced under standard conditions.

An exception is the miR24 product. Here, when using the cDNA produced under standard conditions, a PCR product of the wrong size is obtained (Fig. 8, see also Fig. 6). This product is not detectable, provided a cDNA that was produced by the method according to the invention is used (Fig. 8).

All control reactions in which water was used instead of template RNA in the reverse transcription or the method according to the invention, show no signal, i.e. no Ct value was obtained in the relevant real-time PCRs (Fig. 7, top part). The same also applies to reactions in which only one primer was used (Fig. 7, top part). Also, no Ct value was obtained for negative controls, in which water was used instead of cDNA in the PCR (Fig. 7).

EXAMPLE 4

Detection of miRNA by the coupled, one-stage process of poly(A) reaction and reverse transcription and subsequent detection of the generated cDNA by real-time PCR with tail-specific probe.

In this experiment, a real-time PCR was carried out, using a Taqman probe that has a specific

binding site on the tail-primer (Uni GAP dT). Detection via a probe represents a conceivable alternative to detection by SYBR Green real-time PCR. Use of the probe offers the additional possibility of a multiplex PCR, i.e. co-amplification of one or more additional target nucleic acids, such as an internal control, which can for example be a house-keeping gene.

For the poly(A) reaction and reverse transcription (PAP + RT reaction), the reagents from Table 22 were pipetted together, as shown in Table 24.

Table 22: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
mleu7a oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

Table 24: PAP + RT reaction

Reagents	Final concentration
10x Buffer RT	1 x
rATP 10 mM	1 mM
Poly(A) polymerase 2U/µl	2 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U
Sensiscript reverse transcriptase	1 µl
RNase-free water	variable
mleu7 (10^9 copies/µl)	2 µl (2x10^9 copies)
Total volume	20 µl
Incubation	1 h 37°C 5 min 93°C

Then the reaction mixture was incubated at 37°C, followed by inactivation of the enzymes for 5 min at 93°C.

After inactivation of the enzymes, 2 µl of the undiluted mixture was used as template in a real-time PCR, which contained a Taqman probe for detection. The materials for the PCR are shown

in Table 23, and were pipetted together as stated in Table 25.

Table 23: Materials for the QT Probe PCR

QuantiTect Probe PCR kit (200)	Qiagen Material No.: 204343
let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni primer	5'-AAC GAG ACG ACG ACA GAC-3'
Hum Uni probe	5'- HEX-CAA GCT TCC CGT TCT CAG CC-BHQ-3' 5' reporter dye: HEX 3' quencher: Black Hole Quencher 1

Table 25: QuantiTect Probe PCR

Components for QuantiTect Probe PCR	Final concentration
2x QuantiTect Probe PCR master mix	1x
Hum Uni Primer 10 µM	0.5 µM
let 7short (specific miRNA primer)	0.5 µM
RNase-free water	Variable
PAP + RT reaction undiluted	2 µl (2x10^8 copies)
Final reaction volume	20 µl

The assay in real-time PCR was carried out in double assays.

The sequence AAC GAG ACG ACG ACA GAC contained in the universal tail-primer Hum Uni Primer was described in US2003/0186288A1. The sequence of the Taqman probe was taken from the human GAPDH gene locus; it is not contained in US2003/0186288A1.

The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in the QuantiTect Probe PCR Master Mix for 15 min at 95°C, followed by 45 cycles of 15 s at 94°C and 30 s at 52°C (see Table 26).

Table 26: 2-step PCR protocol

PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	45x
Annealing	30 s 52°C	

The fluorescence data were recorded during the 52°C annealing step. The PCR analyses were carried out with a 7700 Sequence Detection System (Applied Biosystems) in a reaction volume of 20 µl. The PCR results are shown in Table 27.

Table 27: PCR result

mleu7a	Ct	Ct mean	CV, %
undiluted 2x10^8	20.24	20.31	0.45
	20.37		

Detection by means of a tail-specific probe is possible and gives the expected result.

EXAMPLE 5

Effect of poly(A) polymerase concentration and incubation time on the coupled, one-stage process of poly(A) reaction and reverse transcription.

In this experiment, two concentrations of poly(A) polymerase (2 U or 0.5 U) were used, in each case for 15 min or 1 h in the method according to the invention. All conditions were tested in each case in RT buffer (Qiagen) and poly(A) polymerase buffer.

For the poly(A) reaction and reverse transcription (PAP + RT reaction), the reagents from Table 28 were pipetted together as stated in Table 30 (reaction a) 2 U poly(A) polymerase, reaction b) 0.5 U poly(A) polymerase).

Table 28: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
Sensiscript RT Kit	Qiagen; Material Number 50rxn: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106) according to Plant Protocol.
mleu7a oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

Table 30: PAP + RT reaction

Reagents	Final concentration
10x buffer RT	1 x
rATP 10 mM	1 mM
Poly(A) polymerase 2U/µl	a) 2 U b) 0.5 U
dNTP mix (ATGC per 5 mM)	0.5 mM

Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U
Sensiscript reverse transcriptase	1 µl
RNase-free water	variable
mleu7a 10^9 copies/µl	2 µl (2x10^9 copies)
Maize RNA 25 ng/µl	2 µl (50 ng)
Total volume	20 µl
1.) Incubation	1 h 37°C 5 min 93°C
2.) Incubation	15 min 37°C 5 min 93°C

Then the samples were incubated at 37°C (1. 1 h/2. 15 min). Then the reactions were heated for 5 min at 93°C, so that the enzymes were inactivated.

Then 2 µl from each reaction was used undiluted in a SYBR Green PCR. The reactions were tested in double determinations. For this, the reagents from Table 29 were pipetted together as stated in Table 31 and then the PCR was carried out as shown in Table 32.

Table 29: Materials for SYBR Green PCR

QuantiTect SYBR Green PCR Kit (200)	Qiagen; Material Number: 204143
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'

Table 31: SYBR Green PCR

Components for SYBR Green PCR	Final concentration
2x SYBR Green PCR Master Mix	1x
Hum Uni Primer 10 µM	0.5 µM
let 7short (specific miRNA primer)	0.5 µM
RNase-free water	variable
PAP + RT reaction 1a) b)/2a) b)	2 µl (2x10^8 copies)
Final reaction volume	20 µl

Table 32: 3-step PCR protocol

PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	45x
Annealing	30 s 52°C	
Extension	30 s 70°C	
Melting curve		

The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in the QuantiTect SYBR Green PCR Master Mix for 15 min at 95°C, followed by 40 cycles with 15 s at 94°C, 30 s at 52°C and 30 s at 72°C (see Table 32 above). The fluorescence data were recorded during the 72°C extension step. The PCR analyses were carried out with an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl and then a melting curve analysis was carried out.

It can be seen that the efficiency of the one-stage process of poly(A) reaction and reverse transcription depends both on the concentration of poly(A) polymerase and on the incubation time (see Fig. 9).

EXAMPLE 6

Carrying out of the method according to the invention with various reverse transcriptases

The method according to the invention was carried out with a total of five different reverse transcriptases (see Table 35) in Buffer RT (Qiagen) (reaction 1-5), or additionally for comparison in each case in the buffer supplied with the reverse transcriptase (reaction 6-9).

Table 35: Reverse transcriptases and buffers used

1.) AMV Reverse Transcriptase	AMV Reverse Transcriptase 5 x reaction buffer
2.) Superscript III Reverse Transcriptase	5 x first-strand buffer
3.) HIV Reverse Transcriptase	10 x first-strand synthesis buffer
4.) M-MuLV Reverse Transcriptase	10 x Reverse Transcriptase reaction buffer
5.) Sensiscript Reverse Transcriptase	10x Buffer RT

For the one-stage process of poly(A) reaction and reverse transcription (PAP + RT reaction), the reagents from Table 33 were pipetted together for reaction 1-5 (reaction buffer: Buffer RT (Qiagen) as given in Table 36, and for reaction 6-9 (further reverse transcriptases, in each case in the buffer supplied) as given in Table 37.

Table 33: Materials for the poly(A) reaction and reverse transcription

Poly(A) polymerase	Ambion; Material Number 80U: 2030
AMV reverse transcriptase	Promega; Material Number 300 U: m5101
Superscript III reverse transcriptase	Invitrogen; Material Number 10 000 U: 18080-044
HIV reverse transcriptase	Ambion; Material Number 500 U: #2045

M-MuLV reverse transcriptase	BioLabs; Material Number 10 000 U: m0253S
Sensiscript RT Kit	Qiagen; Material Number 50 rxns: 205211
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV V-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106) Plant Protocol, 10-fold upscale (Maxi shredder and column) see above
mleu7a oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'

Table 36: PAP + RT reaction in Buffer RT (Qiagen)

Reagents	Final concentration
10x Buffer RT	1 x
rATP 10 mM	1 mM
Poly(A) polymerase 2U/µl	1 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U
1.) AMV Reverse Transcriptase 2.) Superscript III Reverse Transcriptase 3.) HIV Reverse Transcriptase 4.) M-MuLV Reverse Transcriptase 5.) Sensiscript Reverse Transcriptase	24 U 10 U 1 U 10 U 1 µl
RNase-free water	variable
mleu7a 10^9 copies/µl	2 µl (2x10^9 copies)
Maize RNA 20 ng/µl	2 µl (40 ng)
Total volume	20 µl
Incubation	1 h 37°C 5 min 93°C

Table 37: PAP + RT reaction in the buffer supplied with the Reverse Transcriptase

Reagents	Final concentration
6.) AMV Reverse Transcriptase 5 x reaction buffer 7.) 5 x First-strand Buffer 8.) 10 x First-strand Synthesis Buffer 9.) 10 x Reverse Transcriptase reaction buffer	1 x 1 x 1 x 1 x
rATP 10 mM	1 mM
Poly(A) polymerase 2U/µl	1 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	1 µM
RNase inhibitor 10 U/µl	10 U

6.) AMV Reverse Transcriptase 7.) Superscript III Reverse Transcriptase 8.) HIV Reverse Transcriptase 9.) M-MuLV Reverse Transcriptase	24 U 10 U 1 U 10 U
RNase-free water	variable
mleu7a 10^9 copies/µl	2 µl (2x10^9 copies)
Maize RNA 20 ng/µl	2 µl (40 ng)
Total volume	20 µl
Incubation	1 h 37°C 5 min 93°C

Then the samples were incubated for 1 h at 37°C. Then the reactions were heated for 5 min at 93°C, so that the enzymes were inactivated.

Next, 2 µl from each reaction was used in a SYBR Green PCR. The reactions were tested in double determinations. For this, the reagents from Table 34 were pipetted together as stated in Table 38, and the PCR was carried out as shown in Table 39.

Table 34: Materials for SYBR Green PCR

QuantiTect SYBR Green PCR Kit (200)	Qiagen; Material Number: 204143
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'

Table 38: SYBR GREEN PCR

Components for SYBR Green PCR	Final concentration
2x SYBR Green PCR Master Mix	1x
Hum Uni Primer 10 µM	0.5 µM
let 7short (specific miRNA primer)	0.5 µM
RNase-free water	variable
PAP + RT reaction undiluted	2 µl (2x10^8 copies)
Final reaction volume	20 µl

Table 39: 3-step PCR protocol

PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	45x
Annealing	30 s 52°C	
Extension	30 s 70°C	
Melting curve		

The PCR protocol consisted of an initial reactivation of the HotStarTaq polymerase contained in

the QuantiTect SYBR Green PCR Master Mix for 15 min at 95°C, followed by 40 cycles with 15 s at 94°C, 30 s at 52°C and 30 s at 72°C (see Table 39). The fluorescence data were recorded during the 72°C extension step. The PCR analyses were carried out with an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl and then a melting curve analysis was carried out.

The amounts of the reverse transcriptases used were optimized for standard reverse transcription reactions, which may possibly explain the differences observed in the Ct value.

EXAMPLE 7

Demonstration of the feasibility of a coupled three-stage process of poly(A) reaction, reverse transcription and PCR in the same reaction vessel; effect of various additives on the efficiency of detection of a 22-mer RNA oligonucleotide

The purpose of this experiment was to demonstrate the feasibility of a coupled three-stage process of poly(A) polymerase reaction, reverse transcription and PCR in the same reaction vessel. For this purpose, the coupled three-stage process was carried out under the following conditions with the stated additions.

As a control, the reaction was carried out in a two-stage method based on Fig. 1 B.

For the three-stage process of poly(A) reaction, reverse transcription and PCR (PAP + RT reaction + PCR), the materials from Table 40 were used together, as stated in Table 41.

Table 40: Materials for the poly(A) reaction, reverse transcription and PCR

Poly(A) polymerase	Epicentre Biotechnologies; Material Number 400U: PAP5104
QuantiTect Multiplex RT-PCR kit	Qiagen; Material Number 200 rxns: 204643
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
dNTP mix (ATGC per 10 mM)	Amersham; Material Number Qiagen internal 1007430
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy

	Mini Kit (Cat. No. 74106) Plant Protocol, 10-fold upscale (Maxi shredder and column) see above
mleu7a oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'
GAPDH-TM-HEX_BHQ	5' HEX-CAA GCT TCC CGT TCT CAG CC-BHQ 3'
Poly A RNA	Amersham Biosciences Material Number 27-4110-01, dissolved at 25µg/µl in RNase-free water
Random N8 Primer	NNNNNNNN
Oligo dT 12	TTTTTTTTTTTT-3' phosphate

Table 41: PAP + RT reaction + PCR with additives in QuantiTect Multiplex RT-PCR Master Mix (Qiagen)

Reagents	Final concentration
2x QuantiTect Multiplex RT-PCR Master Mix	1 x
rATP 10 mM	100 µM
Poly(A) polymerase 4U/µl	1 U
dNTP mix (ATGC per 10 mM)	0.5 mM
Uni GAP dT primer 10 µM	0.05 µM
RNase inhibitor 40 U/µl	10 U
QuantiTect Multiplex RT mix	0.2 µl
Hum Uni Primer 10 µM	0.5 µM
let 7short (specific miRNA primer)	0.5 µM
GAPDH-TM-HEX_BHQ	0.2µM
RNase-free water	variable
Poly A RNA 25µg/µl	10ng/µl
N8 random primer	0.05µM
Oligo dT12	5µM
mleu7a 10^9 copies/µl	2 µl (2x10^9 copies)
Maize RNA 20 ng/µl	2 µl (40 ng)
Total volume	20 µl

The reactions were tested in triple determinations. For this, the reagents from Table 40 were pipetted together as shown in Table 41, and the reaction was carried out as shown in Table 42.

Table 42: Reaction protocol of the "3-in-1" reaction

Poly(A) reaction and reverse	45 min 37°C	

transcription	15 min 50°C	
PCR initial reactivation	15 min 95°C	
Denaturation	15 s 94°C	45x
Annealing/ Extension	30 s 52°C	

The reaction protocol consisted first of conditions for the combined reaction of poly-A polymerase and reverse transcription with the QuantiTect Multiplex Reverse Transcriptase Mix (45 min 37°C and 15 min 50°C). This was followed by incubation for 15 min at 95°C, for the purpose of inactivating the poly(A) polymerase and reverse transcriptase, and of activating the HotStarTaq DNA polymerase contained in the QuantiTect Multiplex RT-PCR Master Mix. This was followed by 45 PCR cycles of 15 s at 94°C and 30 s at 52°C (see Table 43) in order to amplify the generated let7a-cDNA in a real-time PCR. A fluorescence-labeled probe specific to the 5'-tail of the Uni GAP dT primer was used for detection. The fluorescence data were recorded during the 52°C annealing/extension step. The "3-in-1" reaction was carried out with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl.

As shown in Fig. 14, the "3-in-1" reaction permits the specific detection of the synthetic 22-mer RNA oligonucleotide against a background of maize RNA. The sample that contained the synthetic 22-mer RNA oligonucleotide against a background of maize RNA gave a Ct value of 20.61, and the control reaction with maize RNA gave a Ct value of 30.65. This experiment shows that the "3-in-1" reaction, in the given conditions, is technically convertible.

EXAMPLE 8

Carrying out of the "3-in-1" method according to the invention using a manual PCR Primer "Hot-Start", with the aim of promoting the reaction of the coupled three-stage process. As a control, the reaction was carried out in a two-stage method on the basis of Fig. 1 B. The reactions were set up with the materials stated in Table 43, as shown in Table 44.

Table 43: Materials for the poly(A) reaction, reverse transcription and PCR

Poly(A) polymerase	Epicentre Biotechnologies; Material Number 400U: PAP5104
QuantiTect Multiplex RT-PCR kit	Qiagen; Material Number 200 rxns: 204643
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
dNTP mix (ATGC per 10 mM)	Amersham; Material Number Qiagen internal 1007430

RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106) Plant Protocol, 10-fold upscale (Maxi shredder and column) see above
mleu7a oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
PCR primer:	
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni Primer	5'-AAC GAG ACG ACG ACA GAC-3'
GAPDH-TM-HEX_BHQ	5'HEX-CAA GCT TCC CGT TCT CAG CC-BHQ 3'
Additives:	
Poly A RNA	Amersham Biosciences Material Number 27-4110-01, dissolved at 25µg/µl in RNase-free water
Random N8 Primer	NNNNNNNN
Oligo dT 12	TTTTTTTTTTTT-3' phosphate

Table 44: PAP + RT reaction + PCR in QuantiTect Multiplex RT-PCR Master Mix (Qiagen)

Reagents	Final concentration
2x QuantiTect Multiplex RT-PCR Master Mix	1 x
rATP 10 mM	100 µM
Poly(A) polymerase 4U/µl	1 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	0.05 µM
RNase inhibitor 40 U/µl	10 U
QuantiTect Multiplex RT mix	0.2µl
RNase-free water	variable
Poly A 25µg/µl	10ng/µl
N8 random primer	0.05µM
Oligo dT12	5µM
mleu7a 10^9 copies/µl	2 µl ($2x10^9$ copies)
Maize RNA 20 ng/µl	2 µl (40 ng)
Total volume	20 µl

A primer mix is prepared with the PCR primers from Table 45 and the required amount in

each case for one reaction is pipetted in each case into a cover of an Optical Cap (cover for real-time PCR vessels, Applied Biosystems; Material Number 4323032). Then the covers were incubated on a heating block at 37°C for approx. 20 min, until the liquid evaporated and therefore the primers had been dried.

After complete drying of the PCR primers in the cover, the reagents are pipetted together as in Table 44 and are put in Optical Tubes (real-time PCR vessels, Applied Biosystems; Material Number 4316567), sealed with the pretreated Optical Caps and the PCR is carried out as shown in Table 46.

The reactions were tested in triple determinations.

Table 45: Composition of dried oligo-mix in the PCR cover

	Amount of oligo/rxn	**Final concentration in PCR after redissolving**
Hum Uni Primer	10 pmol	0.5 µM
let 7short (specific miRNA primer)	10 pmol	0.5 µM
GAPDH-TM-HEX_BHQ	4 pmol	0.2 µM

Table 46: Reaction protocol of the "3-in-1" reaction

Poly(A) reaction and reverse transcription	45 min 37°C	-
	15 min 50°C	
Incubation	95°C 3 min	
Briefly invert the 8-strip reaction vessels, so that the dried primer in the cover dissolves in the reaction mix		
PCR initial reactivation	12 min 95°C	
Denaturation	15 s 94°C	45x
Annealing/ Extension	30 s 52°C	

The reaction protocol consisted first of conditions for the combined reaction of poly-A polymerase and reverse transcription with the QuantiTect Multiplex Reverse Transcriptase Mix (45 min 37°C and 15 min 50°C). Then the reactions were heated for 3 min at 95°C, for the purpose of inactivating the poly(A) polymerase and reverse transcriptase enzymes. Then the PCR tubes were briefly taken out of the equipment and inverted, for the purpose of redissolving the dried primers contained in the covers and to make them available for the subsequent PCR reaction. This was followed by incubation for 12 min at 95°C, in order to

activate the HotStarTaq DNA polymerase contained in the QuantiTect Multiplex RT-PCR Master Mix. A 3 min shorter reactivation was selected here, as the reaction mix had already been heated for 3 min at 95°C to inactivate the poly(A) polymerase and reverse transcriptase enzymes. This was followed by 45 PCR cycles of 15 s at 94°C and 30 s at 52°C (see Table 46), to amplify the generated let7a-cDNA in a real-time PCR. A fluorescence-labeled probe specific to the 5'-tail of the Uni GAP dT primer was used for detection. The fluorescence data were recorded during the 52°C annealing/extension step. The "3-in-1" reaction was carried out with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl.

EXAMPLE 9

Carrying out of the "3-in-1" method according to the invention using PCR "Hot-start" primer, with the aim of promoting the specificity of the reaction of the coupled three-stage process. As a control, the reaction was carried out in a two-stage method on the basis of Fig. 1 B. The reactions were set up with the materials given in Table 47, as shown in Table 48.

Table 47: Materials for the poly(A) reaction, reverse transcription and PCR

Poly(A) polymerase	Epicentre Biotechnologies; Material Number 400U: PAP5104
QuantiTect Multiplex RT-PCR kit	Qiagen; Material Number 200 rxns: 204643
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
dNTP mix (ATGC per 10 mM)	QIAGEN, **dNTP mix, PCR grade (200 µl)** Cat # 201900
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106) Plant Protocol, 10-fold upscale (Maxi shredder and column) see above
mleu7a RNA oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
Poly A RNA	Amersham Biosciences Material Number 27-4110-01, dissolved at 25µg/µl in RNase-free water
Random N8 Primer	NNNNNNNN
Oligo dT 12	TTTTTTTTTTTT-3' phosphate

PCR Hot Start primer	
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni primer	5'-AAC GAG ACG ACG ACA GAC-3'
All primers listed under PCR Hot Start Primers are so-called "CleanAmp" primers, obtainable from Trilinkbiotech.com. "CleanAmp" primers possess the property of only becoming functional as a result of the initial heating step at the start of the PCR and therefore are not available in functional form until the PCR.	
Fluorescence-labeled probe GAPDH-TM-HEX_BHQ	5' HEX-CAA GCT TCC CGT TCT CAG CC-BHQ 3'

Table 48: PAP + RT reaction + PCR in QuantiTect Multiplex RT-PCR Master Mix (Qiagen)

Reagents	Final concentration
2x QuantiTect Multiplex RT-PCR Master Mix	1 x
rATP 10 mM	100 µM
Poly(A) polymerase 4U/µl	1 U
dNTP mix (ATGC per 5 mM)	0.5 mM
Uni GAP dT primer 10 µM	0.05 µM
RNase inhibitor 40 U/µl	10 U
QuantiTect Multiplex RT mix	0.2µl
Hum Uni Primer (Hot Start primer)	0.5µM
let 7short (specific miRNA Hot Start Primer)	0.5µM
GAPDH-TM-HEX_BHQ	0.2µM
RNase-free water	variable
Poly A RNA 25µg/µl	10ng/µl
N8 random primer	0.05µM
Oligo dT12	5µM
mleu7a 10^9 copies/µl	2 µl (2x10^9 copies)
Maize RNA 20 ng/µl	2 µl (40 ng)
Total volume	20 µl

Table 49: Reaction protocol of the "3-in-1" reaction

Poly(A) reaction and reverse transcription	45 min 37°C	-
	15 min 50°C	
PCR initial reactivation	95°C 15 min	
Denaturation	15 s 94°C	45x
Annealing/ Extension	30 s 52°C	

The model sample consisted of an RNA oligonucleotide with the sequence of the mleu7a miRNA. This was inoculated against a background of maize RNA. The reactions were tested in triple determinations. For this, the reagents from Table 47 were pipetted together as shown in Table 48, and the reaction was carried out as shown in Table 49.

The reaction protocol consisted first of conditions for the combined reaction of poly-A polymerase and reverse transcription with the QuantiTect Multiplex Reverse Transcriptase Mix (45 min 37°C and 15 min 50°C). This was followed by incubation for 15 min at 95°C, to activate the HotStarTaq DNA polymerase contained in the QuantiTect Multiplex RT-PCR Master Mix. This was followed by 45 PCR cycles of 15 s at 94°C and 30 s at 52°C (see Table 49) in order to amplify the generated let7a-cDNA in a real-time PCR. A fluorescence-labeled probe specific to the 5'-tail of the Uni GAP dT primer was used for detection. The fluorescence data were recorded during the 52°C annealing/extension step. The "3-in-1" reaction was carried out with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl.

EXAMPLE 10

Carrying out of the "3-in-1" method according to the invention using PCR "Hot-start" primer, for the purpose of promoting the specificity of the reaction of the coupled three-stage process. As a control, the reaction was carried out in a two-stage method on the basis of Fig. 1 B.

The reactions were set up with the materials given in Table 50, as shown in Table 51.

Table 50: Materials for the poly(A) reaction, reverse transcription and PCR

Poly(A) polymerase	Epicentre Biotechnologies; Material Number 400U: PAP5104
QuantiTect Multiplex RT-PCR	Qiagen; Material Number 200 rxns:

kit	204643
Adenosine 5'-triphosphate (rATP)	Amersham; Material Number 25µmol: 27-2056-01
RNase inhibitor	Promega; Material Number: N2511
Uni GAP dT primer	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
Maize RNA	From 1g triturated maize leaves with Qiagen RNeasy Mini Kit (Cat. No. 74106) Plant Protocol, 10-fold upscale (Maxi shredder and column) see above
mleu7a RNA oligonucleotide	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
Poly A RNA	Amersham Biosciences Material Number 27-4110-01, dissolved at 25µg/µl in RNase-free water
Random N8 Primer	NNNNNNNN
Oligo dT 12	TTTTTTTTTTTT-3' phosphate
PCR Hot Start Primer	
Let 7short (specific miRNA primer)	5'-GAG GTA GTA GGT TGT ATA G-3'
Hum Uni primer	5'-AAC GAG ACG ACG ACA GAC-3'
All primers listed under PCR Hot Start Primers are so-called "CleanAmp" primers, obtainable from Trilinkbiotech.com. "CleanAmp" primers possess the property of only becoming functional as a result of the initial heating step at the start of the PCR and therefore are not available in functional form until the PCR.	
Fluorescence-labeled probe	
GAPDH-TM-HEX_BHQ	5' HEX-CAA GCT TCC CGT TCT CAG CC-BHQ 3'

Table 51: PAP + RT reaction + PCR in QuantiTect Multiplex RT-PCR Master Mix (Qiagen)

Reagents	Final concentration
2x QuantiTect Multiplex RT-PCR Master Mix	1 x
rATP 10 mM	100 µM
Poly(A) polymerase 4U/µl	1 U
Uni GAP dT primer 10 µM	0.05 µM

RNase inhibitor 40 U/µl	10 U
QuantiTect Multiplex RT mix	0.2µl
RNase-free water	variable
Hum Uni Primer (Hot Start primer)	0.5µM
(specific miRNA Hot Start primer) (for each of the 4 primers per separate reaction mixture in replicates + controls) Hum Uni Primer (Hot Start Primer)	0.5µM
GAPDH-TM-HEX_BHQ (specific miRNA Hot Start primer) (for each of the 4 primers per separate reaction mixture in replicates + controls)	0.2µM
GAPDH-TM-HEX_BHQ	0.2µM
Template:	
leu7aTemplate:	
mleu7a 10^9 copies/µl for leu7a	2 µl (2x10^9 copies)
Maize RNA 20 ng/µl mleu7a 10^9 copies/µl	2 µl (40 ng)
Total volume	20 µl

Table 52: Reaction protocol of the "3-in-1" reaction

Poly(A) reaction and reverse transcription	45 min 37°C	-
PCR initial reactivation	95°C 15 min	
Denaturation	15 s 94°C	40x
Annealing/ Extension	30 s 52°C	

The model sample consisted of an RNA oligonucleotide with the sequence of the mleu7a miRNA. This was spiked in against a background of maize RNA.

The reactions were tested in triple determinations. For this, the reagents from Table 50 were pipetted together as shown in Table 51, and the reaction was carried out as shown in Table 52. The reaction protocol consisted first of conditions for the combined reaction of poly-A polymerase and reverse transcription with the QuantiTect Multiplex Reverse Transcriptase Mix (45 min 37°C). This was followed by incubation for 15 min at 95°C, to activate the HotStarTaq DNA polymerase contained in the QuantiTect Multiplex RT-PCR Master Mix. This was followed by 40 PCR cycles of 15 s at 94°C and 30 s at 52°C (see Table 52) in order

to amplify the generated let7a-cDNA in a real-time PCR. A fluorescence-labeled probe specific to the 5'-tail of the Uni GAP dT primer was used for detection. The fluorescence data were recorded during the 52°C annealing/extension step. The "3-in-1" reaction was carried out with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) in a reaction volume of 20 µl.

DESCRIPTION OF DRAWINGS

Fig. 1 shows a comparison between the one-stage methods according to the present invention (B) and the two-stage process as is known in the prior art (A). Pol A polymerase: enzyme with polyadenylation activity in the sense of the invention; reverse transcriptase: enzyme with reverse transcriptase activity in the sense of the invention; rATP: ribonucleotide, here for example adenosine-5'-triphosphate; dNTPs: deoxyribonucleotides; oligo dT tail primer: anchor oligonucleotide with various possible embodiments in the sense of the invention; Uni GAP dT primer: special embodiment of the anchor oligonucleotide; tail: 5'-tail as optional part of the anchor oligonucleotide; w: defines the length according to the invention of the homopolymeric tail added on by the polyadenylation activity (larger than 10-20 bases); x,y: defines the type and length according to the invention of the 3' anchor sequence of the anchor oligonucleotide according to the invention; z: defines the length of the homopolymeric moiety of the anchor oligonucleotide according to the invention.

Fig. 2 shows a graphical representation of the Ct values from Table 6: condition a) contained template in each case, in condition b) instead of template, only H_2O was added (H_2O in PAP reaction). In b), no signal was obtained up to PCR cycle 40 (maximum number of cycles carried out), therefore "No Ct" is shown.

Fig. 3 shows an agarose-gel analysis of the real-time PCR products from example 1. M: 100 bp ladder (Invitrogen, Catalog No. 15628-050). 2% agarose, stained with ethidium bromide in TAE as running buffer. Loading scheme: Tr ack 1: marker; then in each case the triple determinations were applied next to one another; 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b.

Fig. 4 shows a tabulation of Ct values, which were obtained by real-time PCR analysis of the reaction products of the mixtures described in Table 8.

For 3 and 6, no signal was obtained up to PCR cycle 40 (maximum number of cycles carried

out), therefore "no Ct" is shown.

Fig. 5 shows a graphical representation of Ct values, which were obtained by real-time PCR analysis of the reaction products of the mixtures described in Table 8.

Fig. 6 shows a tabulation of Ct mean values, which were obtained by real-time PCR analysis of the reaction products of mixtures b) and e) described in Table 18.

For 2, 4 and 5, with standard RT a signal was detected after cycle 38 at the earliest, or for 2) no signal, therefore "no Ct" is shown.

Fig. 7 shows a tabulation of the real-time PCR results of mixtures b) and d) from Table 18, and of the controls with only one primer from Table 19, mixtures a) - d). No signal was obtained up to PCR cycle 40 (maximum number of cycles carried out), therefore "no Ct" is shown.

Fig. 8 shows an agarose-gel analysis of the real-time PCR products from example 3. M: 100 bp ladder (Invitrogen, Catalog No. 15628-050). 2% agarose, stained with ethidium bromide in TAE as running buffer.

Fig. 9 shows a tabulation of Ct mean values, which were obtained by real-time PCR analysis of the reaction products of mixtures 1a) b) and 2 a) b) described in Table 30.
Bottom part: Graphical representation of Ct mean values, which were obtained by real-time PCR analysis of the reaction products of the mixtures described in Table 30.

Fig. 10 shows a tabulation of Ct mean values, which were obtained by real-time PCR analysis of the reaction products of the mixtures described in Table 36 1-5 and in Table 37 6-9.
Bottom part: Graphical representation of Ct mean values, which were obtained by real-time PCR analysis of the reaction products of the mixtures described in Table 36 1-5 and in Table 37 6-9.

Fig. 11 shows a list of the nucleic acid sequences used.

Fig. 12 shows anchor oligonucleotides according to the invention.

Fig. 13 shows a comparison between the one-stage "3-in-1" methods according to the present invention (B) and the three-stage process as is known in the prior art (A). Pol A polymerase: enzyme with polyadenylation activity in the sense of the invention;
Reverse transcriptase: enzyme with reverse transcriptase activity in the sense of the invention; rATP: ribonucleotide, here for example adenosine-5'-triphosphate; dNTPs: deoxyribonucleotides; oligo dT tail primer: anchor oligonucleotide with various possible embodiments in the sense of the invention; Uni GAP dT primer: special embodiment of the anchor oligonucleotide; tail: 5'-tail as optional part of the anchor oligonucleotide; w: defines the length according to the invention of the homopolymer tail added on by the polyadenylation activity (larger than 10-20 bases); x,y: defines the type and length according to the invention of the 3' anchor sequence of the anchor oligonucleotide according to the invention; z: defines the length of the homopolymeric moiety of the anchor oligonucleotide according to the invention. PCR primer: at least one oligonucleotide for the specific detection of cDNA species, optionally at least one probe; PCR enzyme: enzymatic activity that permits the specific detection of cDNA species contained in the sample.

Fig. 14 shows a tabulation of Ct mean values of a "3-in-1" reaction, i.e. of the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel, according to reaction mixture from example 7 corresponding to Table 41 and the reaction mixture from Table 42.

Bottom part: Graphical representation of Ct mean values of a "3-in-1" reaction, i.e. of the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel, according to reaction mixture from example 7 corresponding to Table 41 and the reaction mixture from Table 42.

Fig. 15 shows a tabulation of Ct mean values of a "3-in-1" reaction, i.e. of the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel, according to reaction mixture from example 8 corresponding to Table 44 and the reaction mixture from Table 46.

Bottom part: Graphical representation of Ct mean values of a "3-in-1" reaction, i.e. of the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel.

Fig. 16 shows various amounts (10 pg to 1 µg) of miRNAeasy RNA, which were reverse-transcribed using miScript in the presence or absence of 100 ng of poly(A) or poly(C). The resultant cDNA was used in a real-time PCR; in this case miR-16 and let-7a were tested.

Fig. 17 shows various amounts (10 pg to 1 µg) of miRNAeasy RNA, which were reverse-transcribed using miScript in the presence or absence of various amounts of poly(A). The resultant cDNA was used in a real-time PCR; in this case miR-16 was tested.

Fig. 18 shows various amounts (10 pg to 1 µg) of miRNAeasy RNA, which were reverse-transcribed using miScript in the presence or absence of various amounts of poly(C). The resultant cDNA was used in a real-time PCR; in this case miR-16 was tested.

Fig. 19 shows the use of 10 pg of miRNeasy RNA, which was reverse-transcribed in the presence or absence of 50 ng of poly(C) using the miScript RT kit. The resultant cDNA was used in a real-time PCR to detect GAPDH.

Fig. 20 shows various amounts (1 – 100 ng) of miRNeasy RNA, which were reverse-transcribed using miScript in the presence or absence of various amounts of poly(C). The resultant cDNA was used in a real-time PCR; in this case to test GADPH.

Fig. 21 shows the use of 10 and 100 pg of miRNeasy RNA, which was reverse-transcribed in the presence or absence of 50 ng of poly(C) and using the miScript RT kit. The resultant cDNA was tested in a real-time PCR to detect GAPDH.

Fig. 22 shows various amounts (1 – 100 ng) of miRNeasy RNA, which were reverse-transcribed using miScript in the presence or absence of various amounts of poly(C). The resultant cDNA was used in a real-time PCR; in this case to test CDC2.

Fig. 23 shows the use of 1 ng of miRNeasy RNA, which was reverse-transcribed in the presence or absence of 50 ng of poly(C) and using the miScript RT kit. The resultant cDNA was tested in a real-time PCR to detect CDC2.

Fig. 24 shows a tabulation of Ct mean values of a "3-in-1" reaction, i.e. the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis with Hot Start primer

coupled in a reaction vessel, according to reaction mixture from example 9 corresponding to Table 48 and the reaction mixture from Table 49.

Bottom part: Graphical representation of Ct mean values of a "3-in-1" reaction, i.e. the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel, according to reaction mixture as in Table 48.

Fig. 25 shows a tabulation of Ct mean values of a "3-in-1" reaction, i.e. the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis with Hot Start primer coupled in a reaction vessel, according to reaction mixture from example 10 corresponding to Table 51 and the reaction mixture from Table 52.

Bottom part: Graphical representation of Ct mean values of a "3-in-1" reaction, i.e. the combined poly(A) polymerase reaction, reverse transcription and real-time PCR analysis coupled in a reaction vessel, according to reaction mixture as in Table 51.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for synthesizing a cDNA in a sample in an enzymatic reaction, characterized in that the method comprises the following steps:

   a. simultaneously providing of a first enzyme with polyadenylation activity, a second enzyme with reverse transcriptase activity, a buffer, at least one ribonucleotide, at least one deoxyribonucleotide, an anchor oligonucleotide comprising a poly(T) sequence or a poly(U) sequence and a homopolymeric competitor-ribonucleic acid, and an enzyme and reagents for the amplification of cDNA generated,

   b. adding of a sample comprising a nucleic acid and

   c. incubating the agents from steps a) and b) in one or more temperature steps, which are selected so that the first enzyme and the second enzyme display activity, and the cDNA thus generated in the method is then amplified.

2. The method as claimed in claim 1, characterized in that the reaction additionally comprises at least one temperature step at a higher temperature of about 65°C to 95°C.

3. The method as claimed in claim 1 or 2, characterized in that the cDNA generated in the method is then amplified in a polymerase chain reaction and the reaction comprises random primers and/or comprieses specific primers and/or one or more probes.

4. The method as claimed in one of claims 1 to 3, characterized in that the sample comprises a ribonucleic acid, which is selected from the group comprising prokaryotic RNA, eukaryotic RNA, viral RNA, Archaea-RNA, miRNA, snoRNA, mRNA, non-polyadenylated RNA, and rRNA, and mixtures thereof.

5. The method as claimed in one of claims 1 to 4, characterized in that the anchor oligonucleotide is selected from the group comprising a poly(T) oligonucleotide comprising a 5'-tail, and a poly(U) oligonucleotide comprising a 5'-tail.

6. The method as claimed in claim 5, characterized in that the anchor oligonucleotide has a length between 6 and 150 nucleotides.

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7. The method of claim 6, wherein the anchor oligonucleotide includes an anchor sequence at the 3'-end.

8. The method as claimed in one of claims 5 to 7, characterized in that the anchor oligonucleotide is a deoxyribonucleic acid (DNA), a peptide-nucleic acid (PNA) a locked-nucleic acid (LNA), a phosphorothioate-deoxyribonucleic acid, a cyclohexene-nucleic acid (CeNA), an N3'-P5'-phosphoroamidate (NP), or a tricyclo-deoxyribonucleic acid (tcDNA).

9. The method as claimed in one of claims 1 to 8, characterized in that the deoxyribonucleotide is selected from the group comprising deoxyadenosine-5'-triphosphate (dATP), deoxythymine-5'-triphosphate (dTTP), deoxycytosine-5'-triphosphate (dCTP), deoxyguanine-5'-triphosphate (dGTP), deoxyuracil-5'-triphosphate (dUTP).

10. The method according to one of claims 1 to 9, wherein the deoxyribonucleotide is modified or labeled.

11. The method as claimed in claim 10, characterized in that the label to be selected from the group comprising a radioactive marker ^{32}P, ^{33}P, ^{35}S, ^{3}H, a fluorescent dye, fluorescein-isothiocyanate (FITC), 6-carboxyfluorescein (FAM), xanthene, rhodamines, 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 6-carboxy-4',5'-dichloro-2',7'-dimethodyfluorescein (JOE), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 5-carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), rhodamine 110; coumarins, umbelliferones, benzimides, Hoechst 33258; phenanthridine, Texas Red, ethidium bromide, acridine dyes, carbazole dyes, phenoxazine dyes, porphyrin dyes, polymethine dyes, cyanin dyes, such as Cy3, Cy5, Cy7, BODIPY dyes, quinoline dyes and Alexa dyes.

12. A reaction mixture comprising a first enzyme with polyadenylation activity, a second enzyme with reverse transcriptase activity, a buffer, at least one ribonucleotide, at least one deoxyribonucleotide, an anchor oligonucleotide comprising a poly(T) sequence or

a poly(U) sequence and a homopolymeric competitor-ribonucleic acid, a random primer, and an enzyme for cDNA amplification.

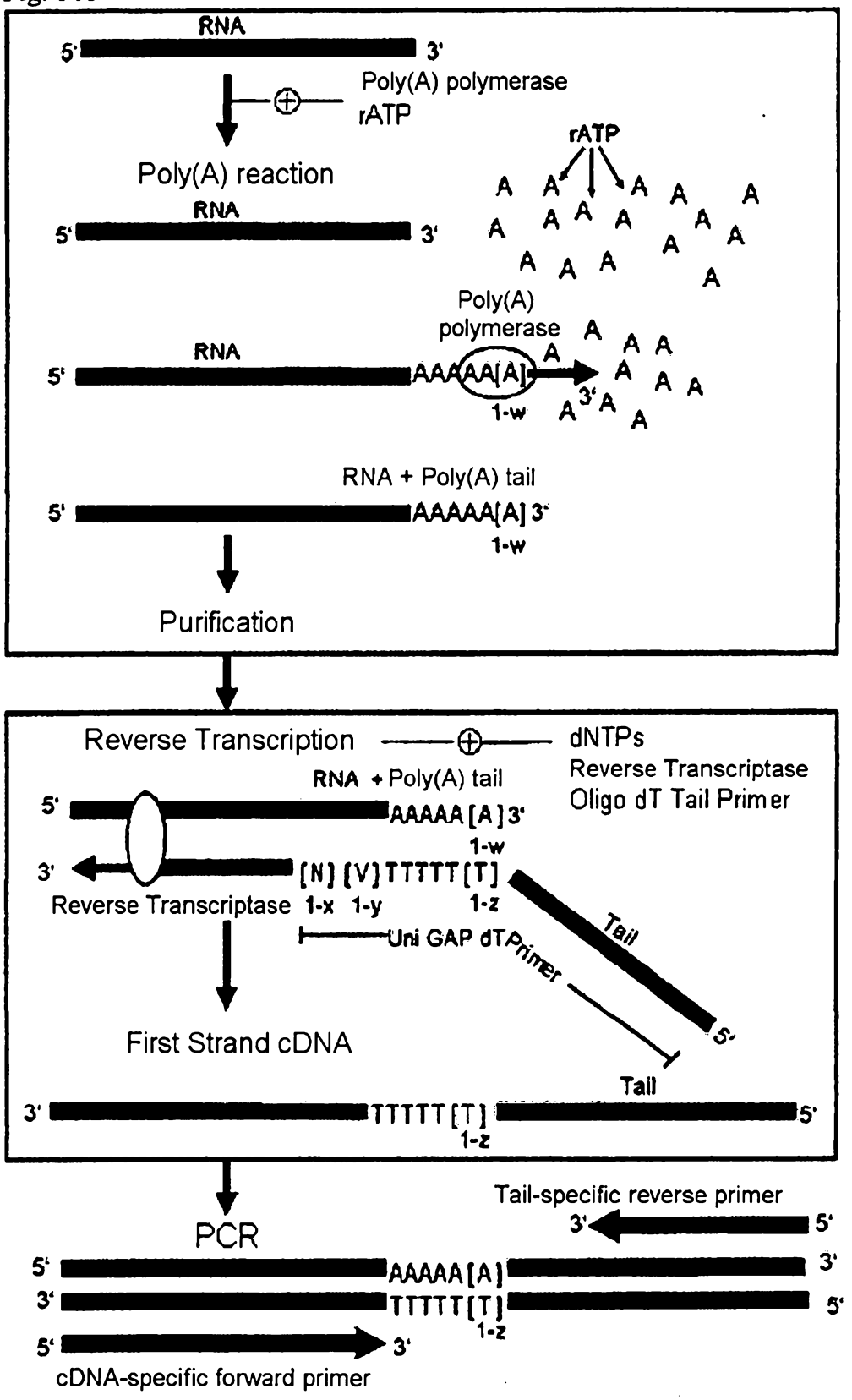
13. The reaction mixture as claimed in claim 12, comprising at least one specific primer, wherein in the primer or the enzyme for cDNA amplification are either reversibly inactivated or physically separated from other reagents.

14. Reaction mixture according to claim 13, wherein the primer or enzyme for cDNA amplification are either Hot-start Primer or a Hot-start DNA-polymerase.

15. A kit comprising a reaction mixture according to any one of claims 12 to 14.

16. A method for synthesizing a cDNA according to any one of claims 1 to 11, a reaction mixture according to any one of claims 12 to 14 or a kit according to claim 15, substantially as hereinbefore described with reference to the Examples and/or Figures.

Fig. 1 A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3'
Poly(A) polymerase
rATP
Poly(A) reaction
rATP
RNA
5'
3'
Poly(A)
polymerase
RNA
5'
AAAAA[A]
1-w
3'
RNA + Poly(A) tail
5'
AAAAA[A] 3'
1-w
Purification
Reverse Transcription
dNTPs
Reverse Transcriptase
Oligo dT Tail Primer
RNA + Poly(A) tail
5'
AAAAA[A]3'
1-w
3'
[N][V]TTTTT[T]
Reverse Transcriptase 1-x 1-y 1-z
Uni GAP dT Primer
Tail
5'
First Strand cDNA
Tail
3'
TTTTT[T]
1-z
5'
PCR
Tail-specific reverse primer
3'
5'
5'
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3'
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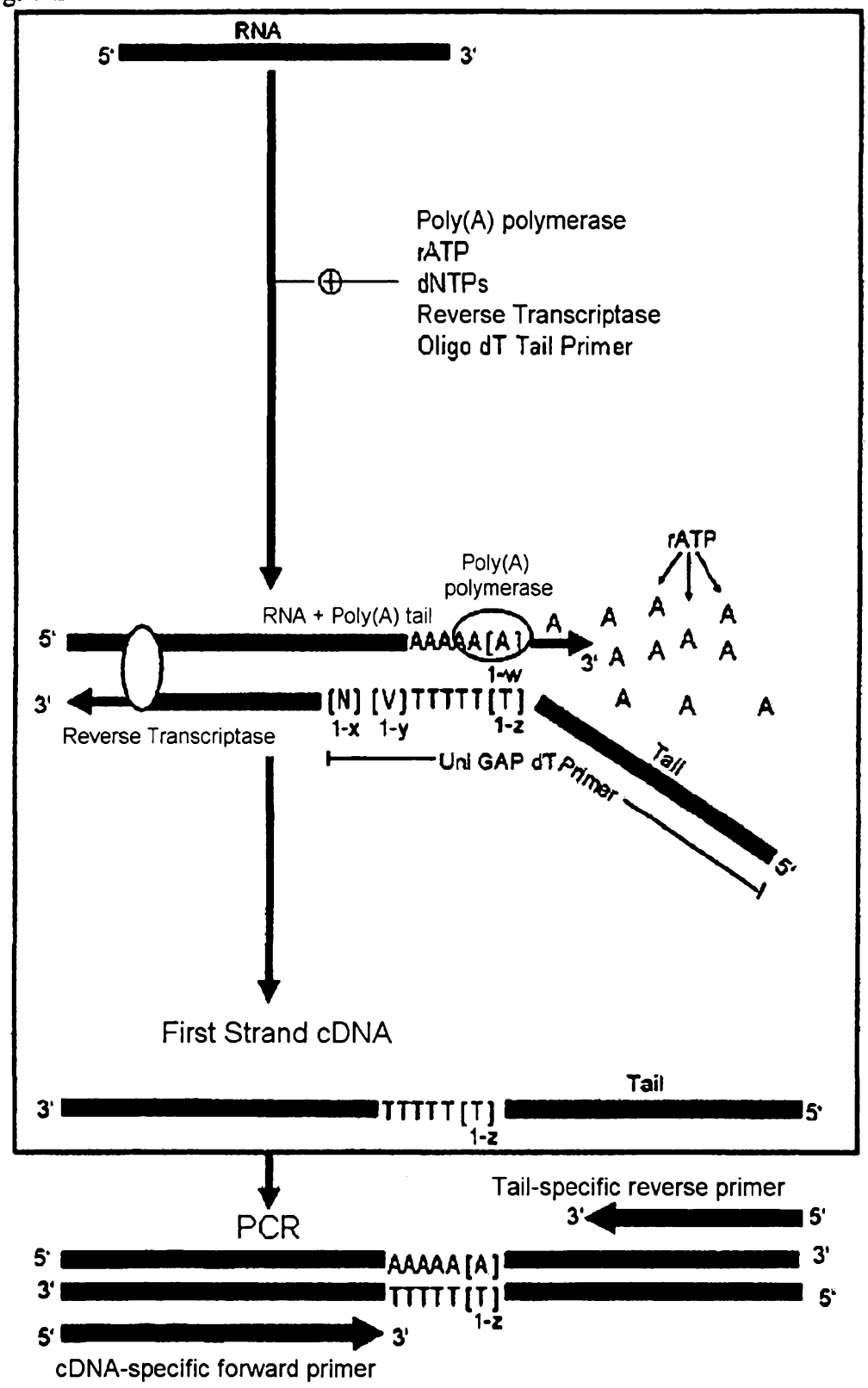
1-z
5'
5'
3'
cDNA-specific forward primer

Fig. 1 B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5'
3'
Poly(A) polymerase
rATP
dNTPs
Reverse Transcriptase
Oligo dT Tail Primer
rATP
Poly(A) polymerase
RNA + Poly(A) tail
AAAAA[A]
1-w
[N] [V]TTTTT[T]
1-x 1-y 1-z
Reverse Transcriptase
Uni GAP dT Primer
Tail
First Strand cDNA
TTTTT[T]
Tail
PCR
Tail-specific reverse primer
cDNA-specific forward primer

Fig. 2

	a	b
1) PAP buffer	25,50	no Ct
2) RT buffer	17,32	no Ct
3) Mixture PAP RT buffers	30,28	no Ct
4) two-stage method	25,64	no Ct

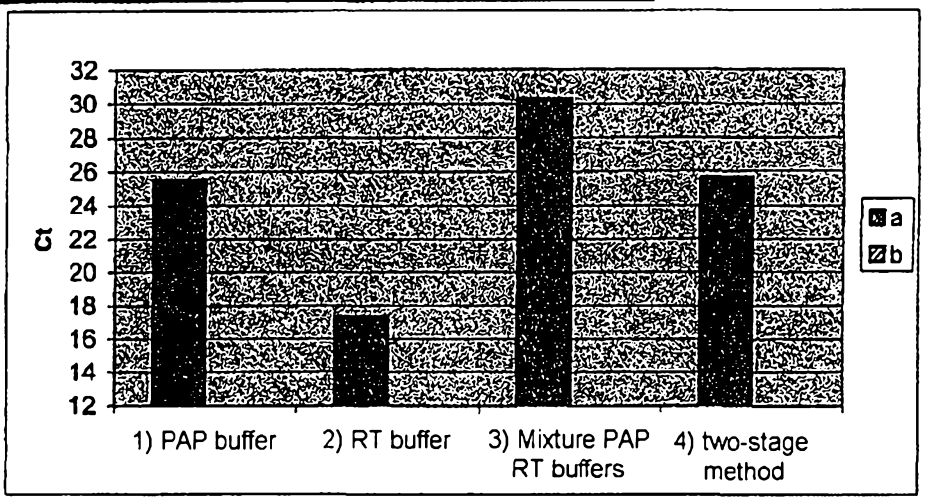


Fig. 3

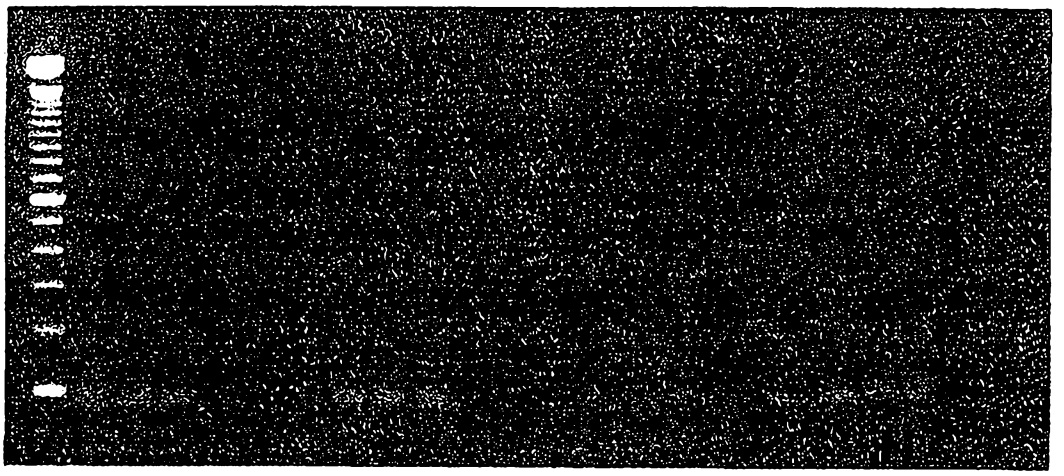


Fig. 4 (table) and Fig. 5 (diagram)

		PAP Buffer	RT Buffer	Standard RT
Reaction: 1, 4, 7 RNA 2,5 ng + mleu7a	a)	22,62	19,69	no Ct
	b) RT reaction doubled	24,30	19,76	no Ct
Reaction: 2, 5, 8 RNA 2,5 ng	a)	34,69	31,41	no Ct
	b) RT reaction doubled	34,20	32,77	no Ct
Reaction 3, 6 H2O in PAP/ RT	a)	no Ct	no Ct	
	b) RT reaction doubled	no Ct	no Ct	

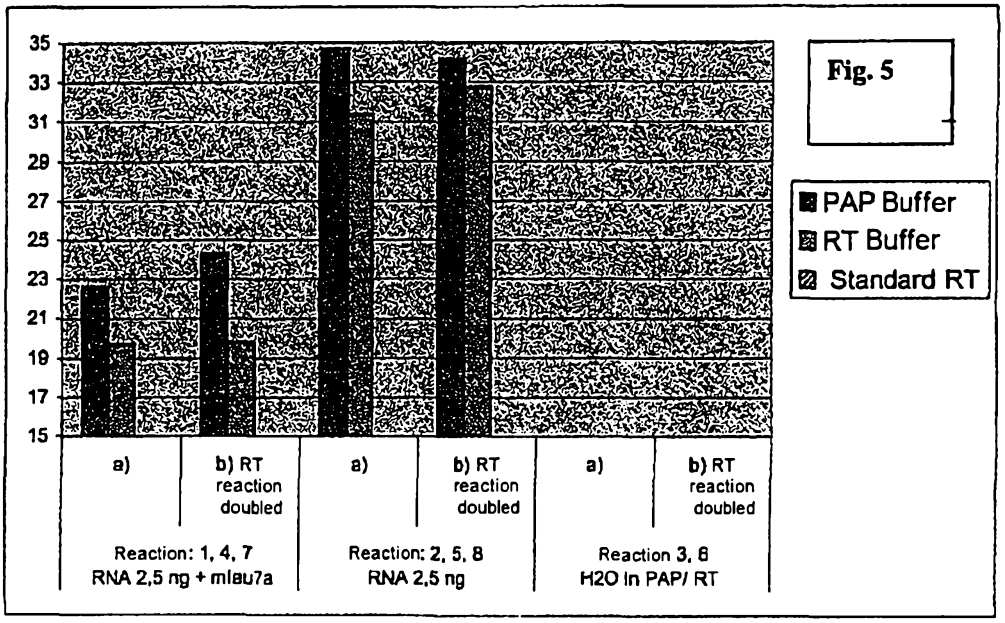


Fig. 5

Fig. 6

	1.) ß-Actin 3' Primer	2.) leu7short	3.) miR24	4.) miR15a	5.) miR16
b) PAP + RT	23,16	25,30	28,47	25,90	23,83
e) Standard RT	22,75	no Ct	30,95	39,81	38,78

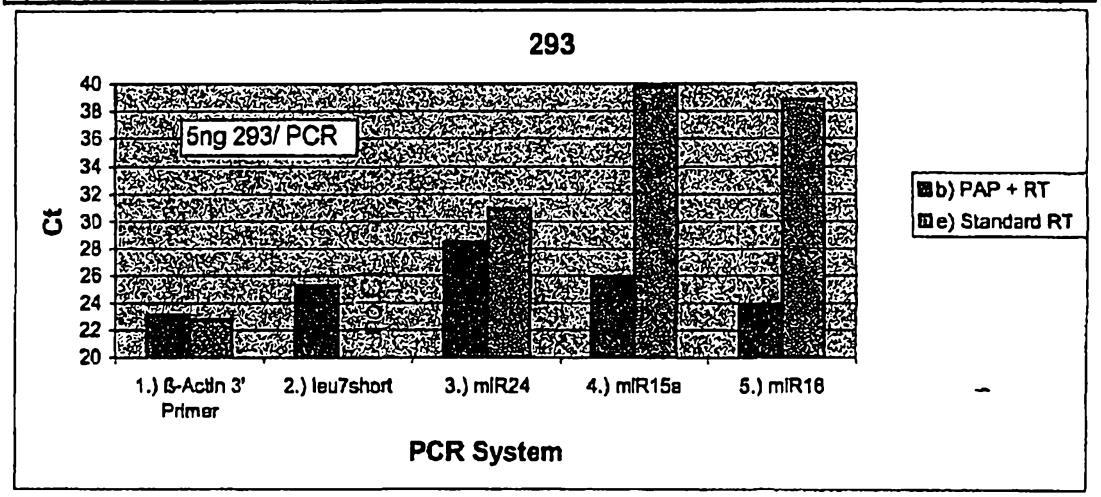


Fig. 7

HumUni +	1.) ß-Aktin 3' Primer	2.) leu7short	3.) miR24	4.) miR15a	5.) miR16
b) H_2O PAP + RT	No Ct				
d) H_2O Standard RT					
H_2O in PCR					

	6.) Hum Uni	7.) leu7short	8.) miR24	9.) miR15a	10.) miR16
a) 293 PAP + RT	No Ct				
b) H_2O PAP + RT					
c) 293 Standard RT					
d) H_2O Standard RT					
H_2O in PCR					

Fig. 8

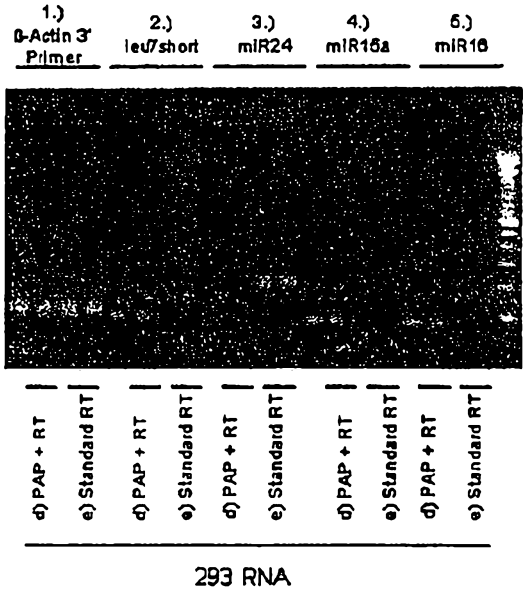


Fig. 9

PAP + RT reaction	1.) 1 h incubation time		2.) 15 min incubation time	
	a) 2 U PAP enzyme	b) 0,5 U PAP enzyme	a) 2 U PAP enzyme	b) 0,5 U PAP enzyme
2x10^8 copies mleu7a + maize RNA 5ng	21,14	22,70	22,06	24,82

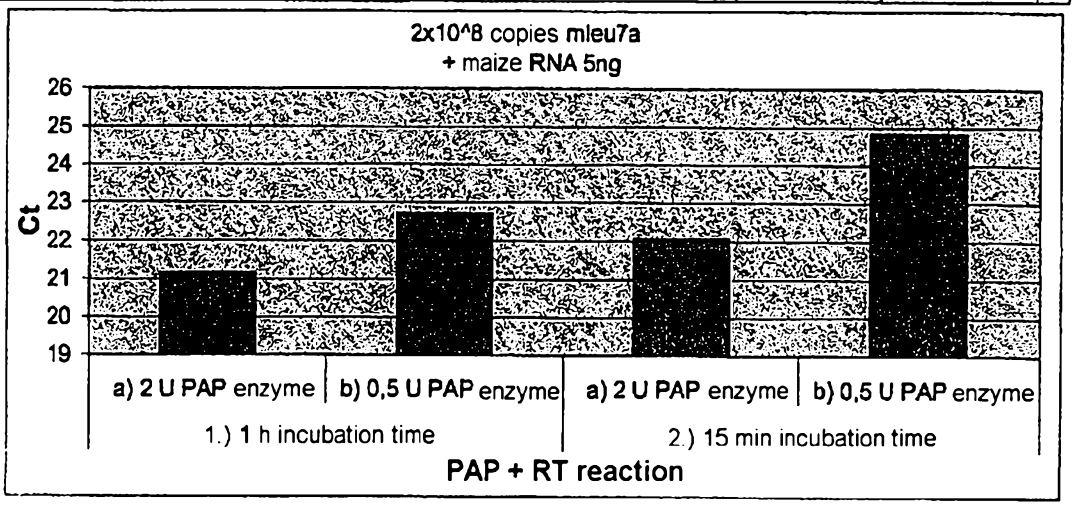


Fig. 10

	Reaction	10x buffer RT (Qiagen)	Reaction	Buffer suitable for enzyme
AMV Reverse transcriptase 24 U	1.)	19,95	6.)	20,23
SuperScript III Reverse transcriptase 10 U	2.)	16,78	7.)	15,80
HIV Reverse transcriptase 1 U	3.)	18,65	8.)	18,88
M-MuLV Reverse transcriptase 10 U	4.)	17,28	9.)	14,86
Sensiscript Reverse transcriptase	5.)	20,75		

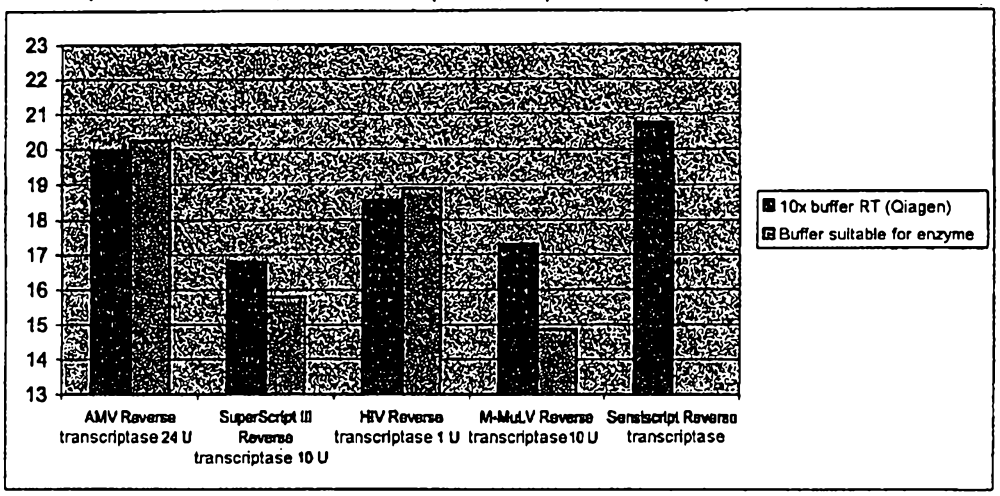


Fig. 11

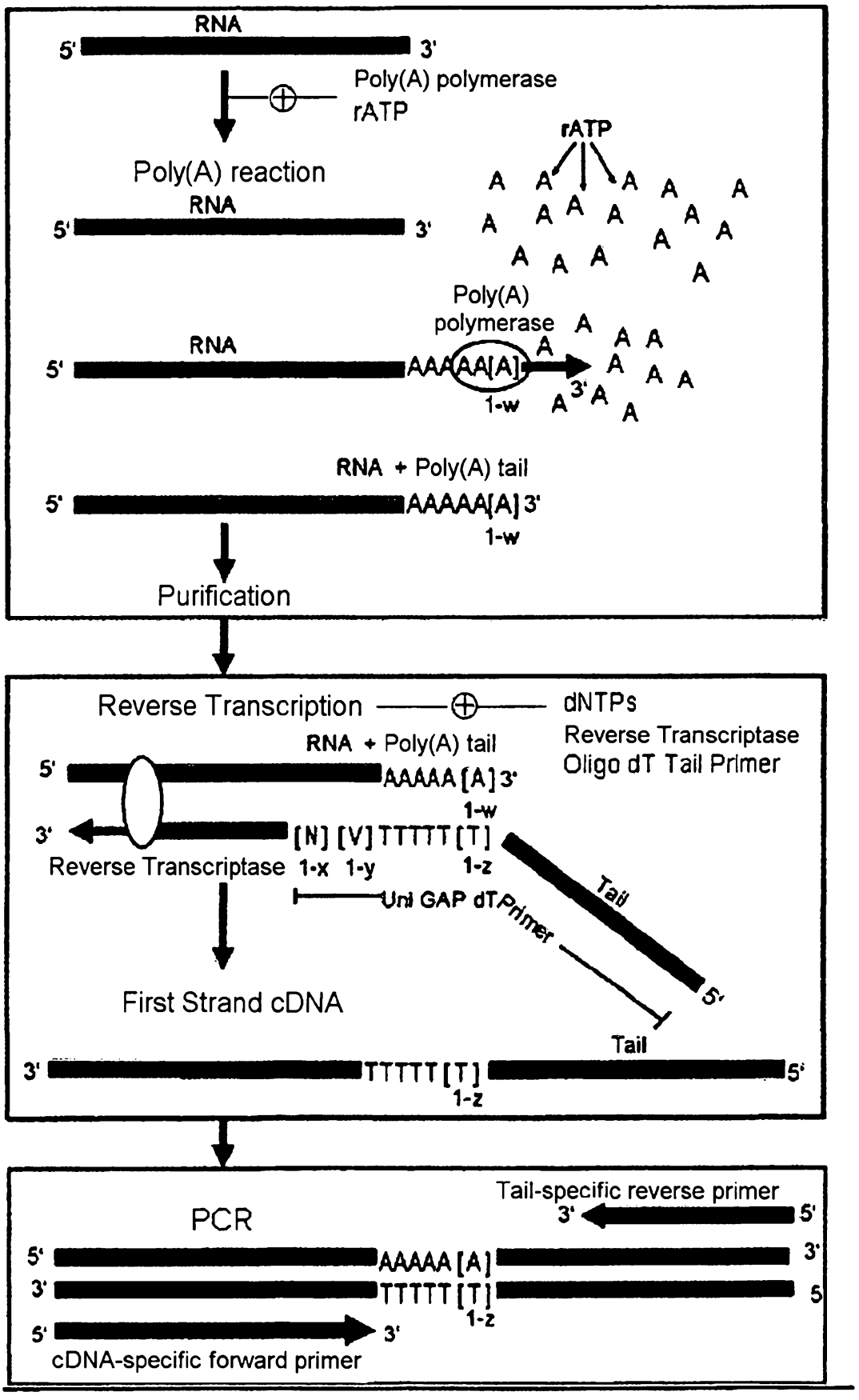
	Synthetic miRNA	
SEQ ID NO. 1	mleu7a RNA	5'-UGA GGU AGU AGG UUG UAU AGU U-3'
	RT (Tail) Primer	
SEQ ID NO. 2	Uni GAP dT	5'-TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CCT TTT TTT TTT TTT TTT TTT TTV VN-3'
	PCR Primer	
SEQ ID NO. 3	Hum Uni	5'-AAC GAG ACG ACG ACA GAC-3'
SEQ ID NO. 4	Let 7short	5'-GAG GTA GTA GGT TGT ATA G-3'
SEQ ID NO. 5	hsa-miR-24	5'-TGG CTC AGT TCA GCA GGA-3'
SEQ ID NO. 6	hsa-miR-15a	5'-TAG CAG CAC ATA ATG GTT T-3'
SEQ ID NO. 7	hsa-miR-16	5'-TAG CAG CAC GTA AAT ATT G-3'
SEQ ID NO. 8	ß-Actin 3'	5'-GTA CAC TGA CTT GAG ACC AGT TGA ATA AA-3'
	PCR Probe	
SEQ ID NO. 9	Hum Uni	5'- HEX-CAA GCT TCC CGT TCT CAG CC-BHQ-3' 5' Reporter Dye: HEX 3' Quencher: Black Hole Quencher 1

Fig. 12

	5'-end (5'-tail)	Middle: Poly (A, C, G or T)	3'-end (priming nucleo-tides)
General description	Absent, or random, approx. 1-100 nucleotides long, preferably with binding sequence for an oligonucleotide, or mixtures thereof	Preferably Poly(T); as a general rule 15-50 nucleotides long, if Poly (T) then preferably 10-30 nucleotides long or mixtures thereof	Absent, or preferably VVN-3', VN-3' or V-3', where V = A, C or G and N = A, C, G or T
Anchor oligo-nucleotide 1	$N_{(n=14-34)}$	Poly $(T)_{(n=10-30)}$	VVN-3'
Anchor oligo-nucleotide 2	$N_{(n=14-34)}$	Poly $(T)_{(n=10-30)}$	VN-3'
Anchor oligo-nucleotide 3	$N_{(n=14-34)}$	Poly $(T)_{(n=10-30)}$	V-3'
Anchor oligo-nucleotide 4	Binding sequence for oligonucleotide	Poly $(T)_{(n=10-30)}$	VVN-3
Anchor oligo-nucleotide 5	Binding sequence for oligonucleotide comprising a restriction site	Poly $(T)_{(n=10-30)}$	VVN-3'
Anchor oligo-nucleotide 6	$N_{(n=14-34)}$	Poly $(T)_{(n=10-30)}$	VNN-3'
Anchor oligo-nucleotide 7 (Example 1)	TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC $(T)_x$	Poly $(T)_{(n=10-30)}$	VVN-3'
Anchor oligo-nucleotide 8 (Example 2)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	VN 3'
Anchor oligo-nucleotide 9 (Example 3)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	V 3'
Anchor oligo-nucleotide 10 (Example 4)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	N 3'
Anchor oligo-nucleotide 11 (Example 5)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	NN 3'
Anchor oligo-nucleotide 12 (Example 6)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	VNN 3'
Anchor oligo-nucleotide 13	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	VNNN 3'

(Example 7)			
Anchor oligo-nucleotide 14 (Example 8)	AACGAGACGACGACAGAC$(T)_x$	Poly $(T)_{(n=10-30)}$	NNN 3'
Anchor oligo-nucleotide 15 (Example 9)	TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC$(T)_x$	Poly $(T)_{(n=10-30)}$	VN 3'
Anchor oligo-nucleotide 16 (Example 10)	TGG AAC GAG ACG ACG ACA GAC CAA GCT TCC CGT TCT CAG CC$(T)_x$	Poly $(T)_{(n=10-30)}$	VNN 3'

Fig. 13 A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5'
3'
Poly(A) polymerase
rATP
Poly(A) reaction
RNA
5'
3'
rATP
A
Poly(A)
polymerase
RNA
5'
AAAAA[A]
1-w
3'
RNA + Poly(A) tail
5'
AAAAA[A] 3'
1-w
Purification
Reverse Transcription
dNTPs
Reverse Transcriptase
Oligo dT Tail Primer
RNA + Poly(A) tail
5'
AAAAA [A] 3'
1-w
3'
[N] [V] TTTTT [T]
Reverse Transcriptase
1-x 1-y
1-z
Uni GAP dT Primer
Tail
5'
First Strand cDNA
Tail
3'
TTTTT [T]
1-z
5'
PCR
Tail-specific reverse primer
3'
5'
5'
AAAAA [A]
3'
3'
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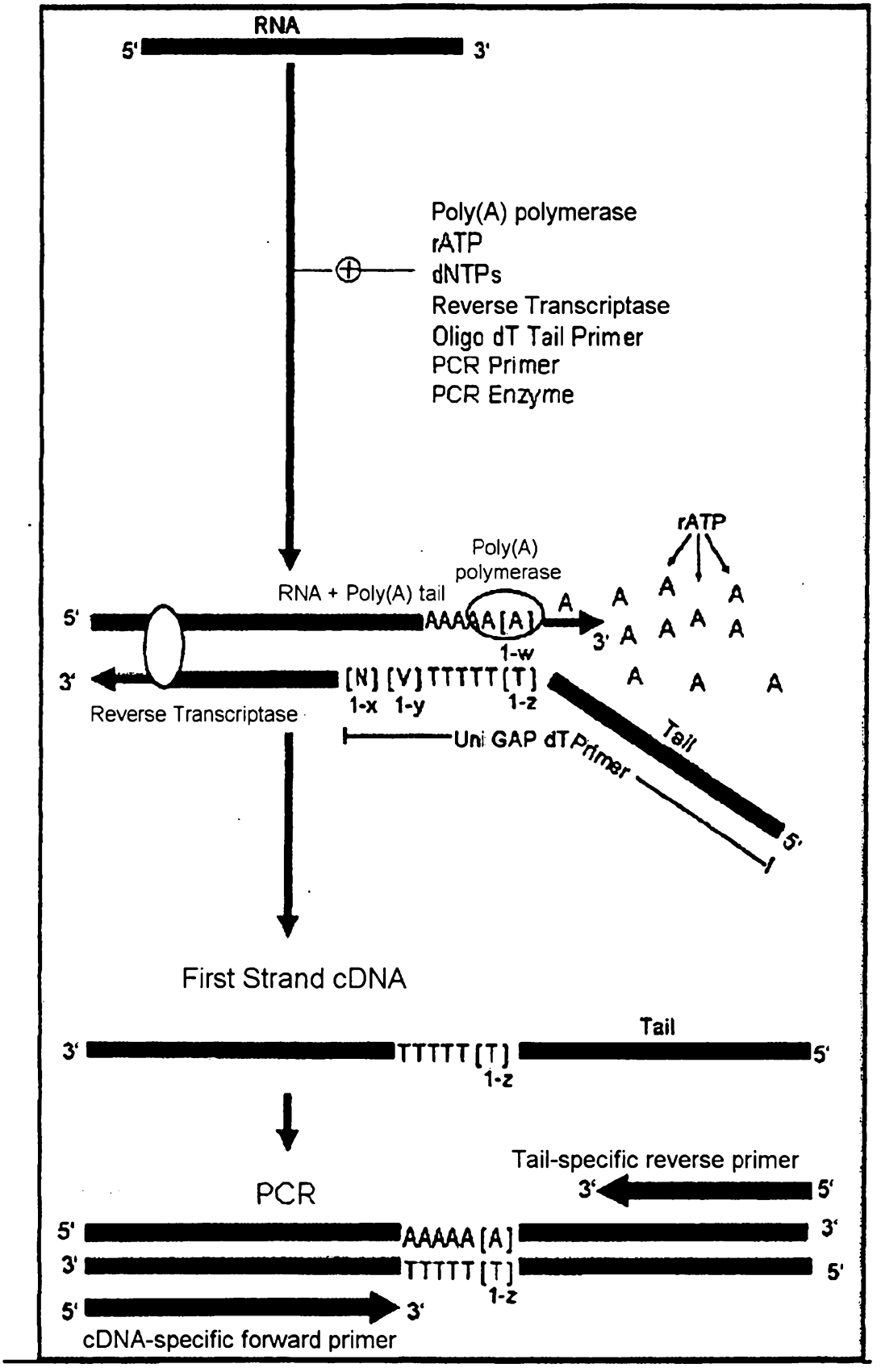
1-z
5
5'
3'
cDNA-specific forward primer

Fig. 13 B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3'
Poly(A) polymerase
rATP
dNTPs
Reverse Transcriptase
Oligo dT Tail Primer
PCR Primer
PCR Enzyme
rATP
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RNA + Poly(A) tail
5'
AAAAA[A]
1-w
3'
[N][V]TTTTT[T]
1-x 1-y
1-z
Reverse Transcriptase
Uni GAP dT Primer
Tail
5'
First Strand cDNA
Tail
3'
TTTTT[T]
1-z
5'
PCR
Tail-specific reverse primer
3'
5'
5'
AAAAA[A]
3'
3'
TTTTT[T]
1-z
5'
5'
3'
cDNA-specific forward primer

Fig. 14:

	Uni Gap dT-VVN
Maize RNA	30,65
Maize RNA/ miRNA	20,61

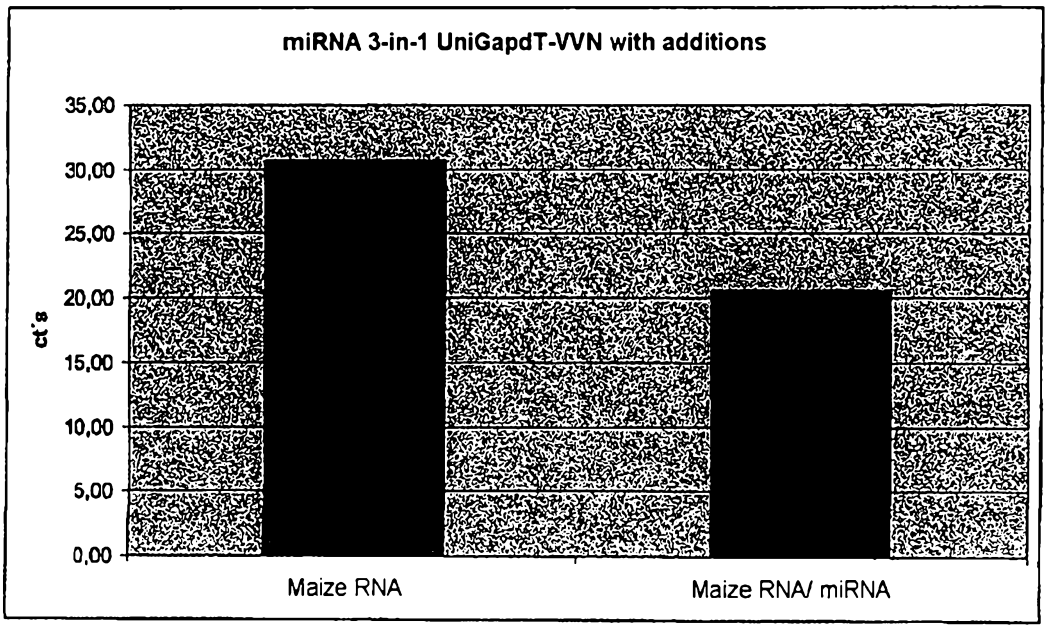

Fig. 15 :

	UniGap dT-VVN		UniGap dT-VN		2-step control
	+ additions	- additions	+ additions	- additions	
Maize RNA	No Ct	No Ct	No Ct	No Ct	N.D.
miRNA/Maize RNA	33.23	28.44	25.54	29.30	29.67
No Ct: No signal detectable before PCR cycle 40; N.D.: not done					

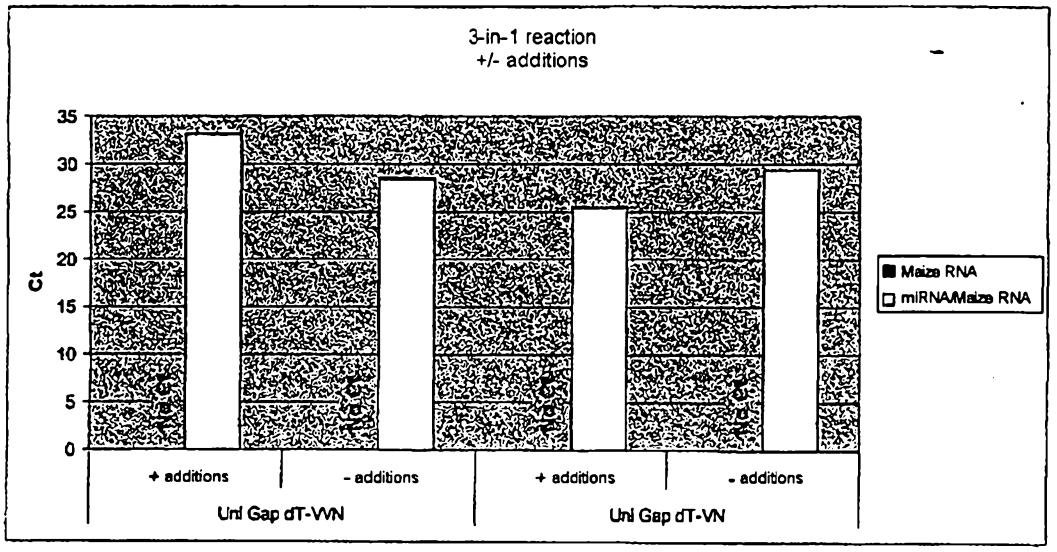

Fig. 16 A

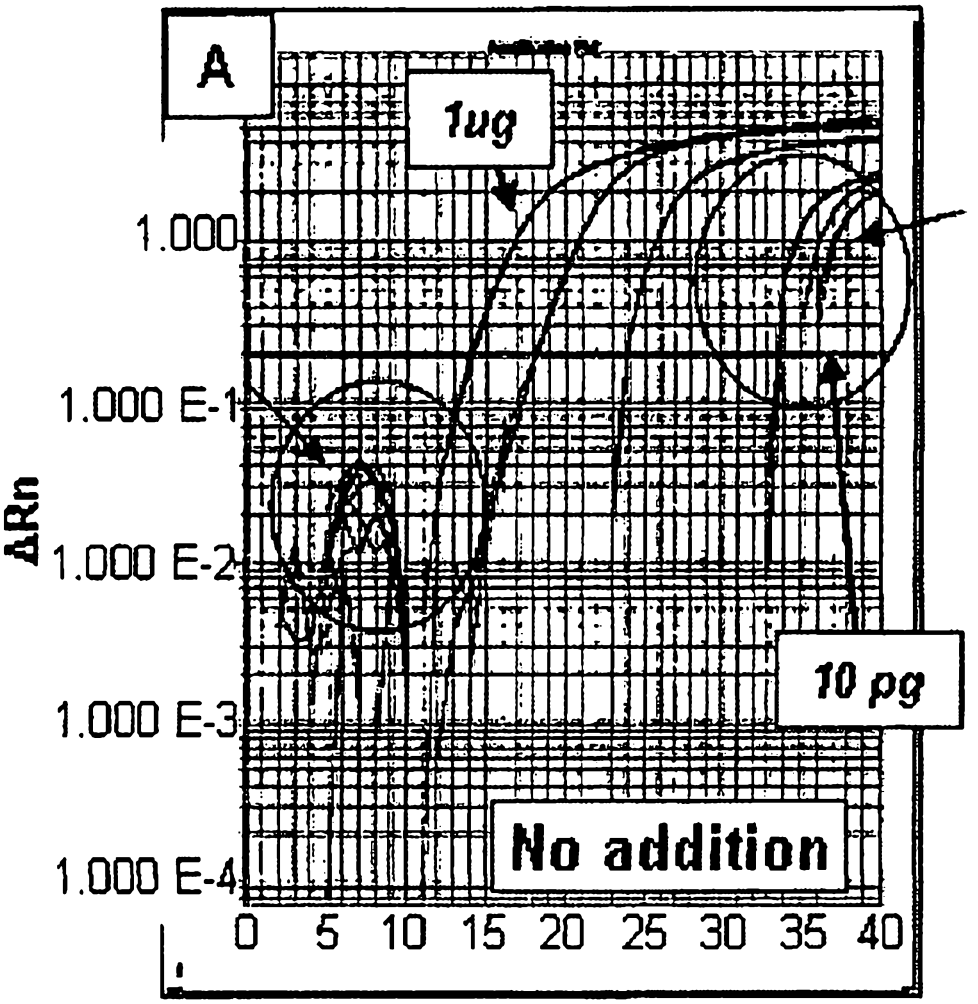
A
1ug
10 pg
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40

Fig. 16 B

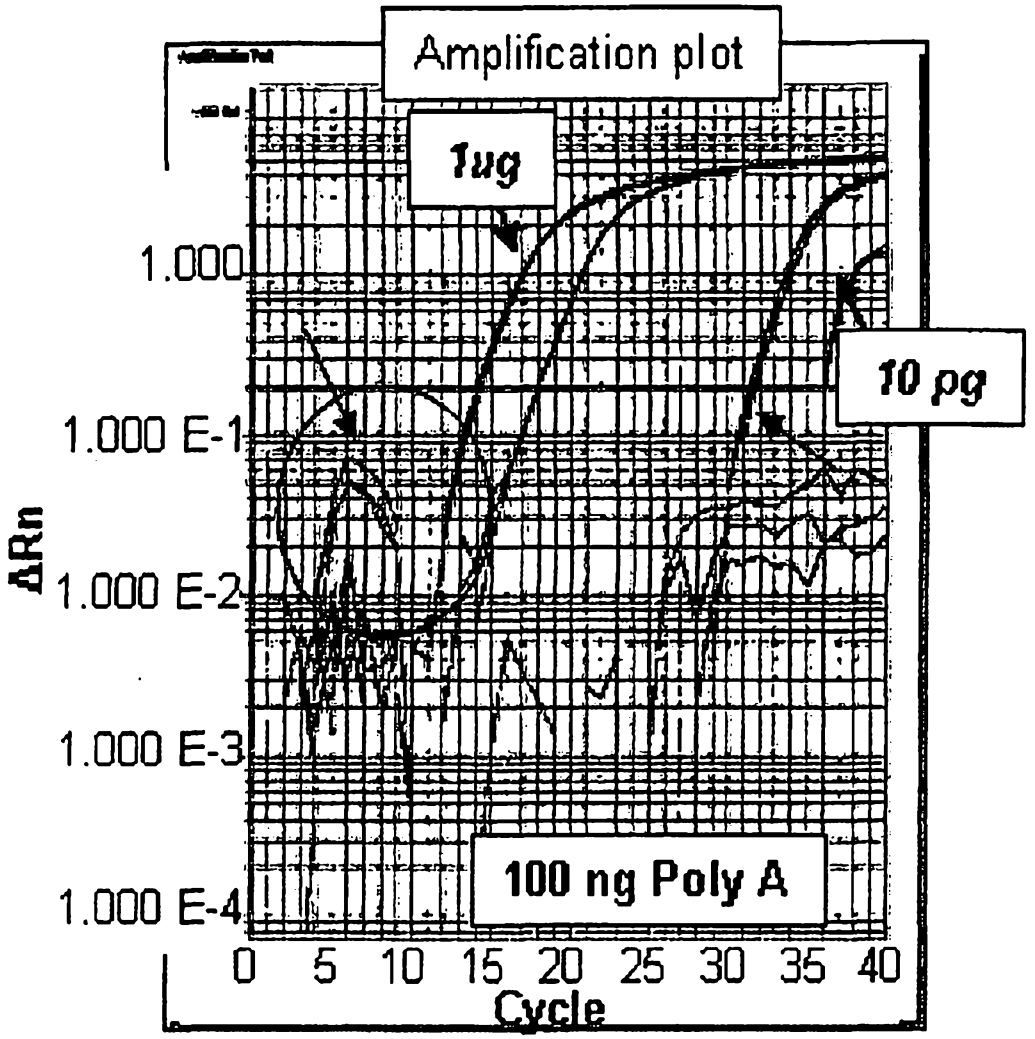
Amplification plot
1ug
10 pg
100 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 16 C

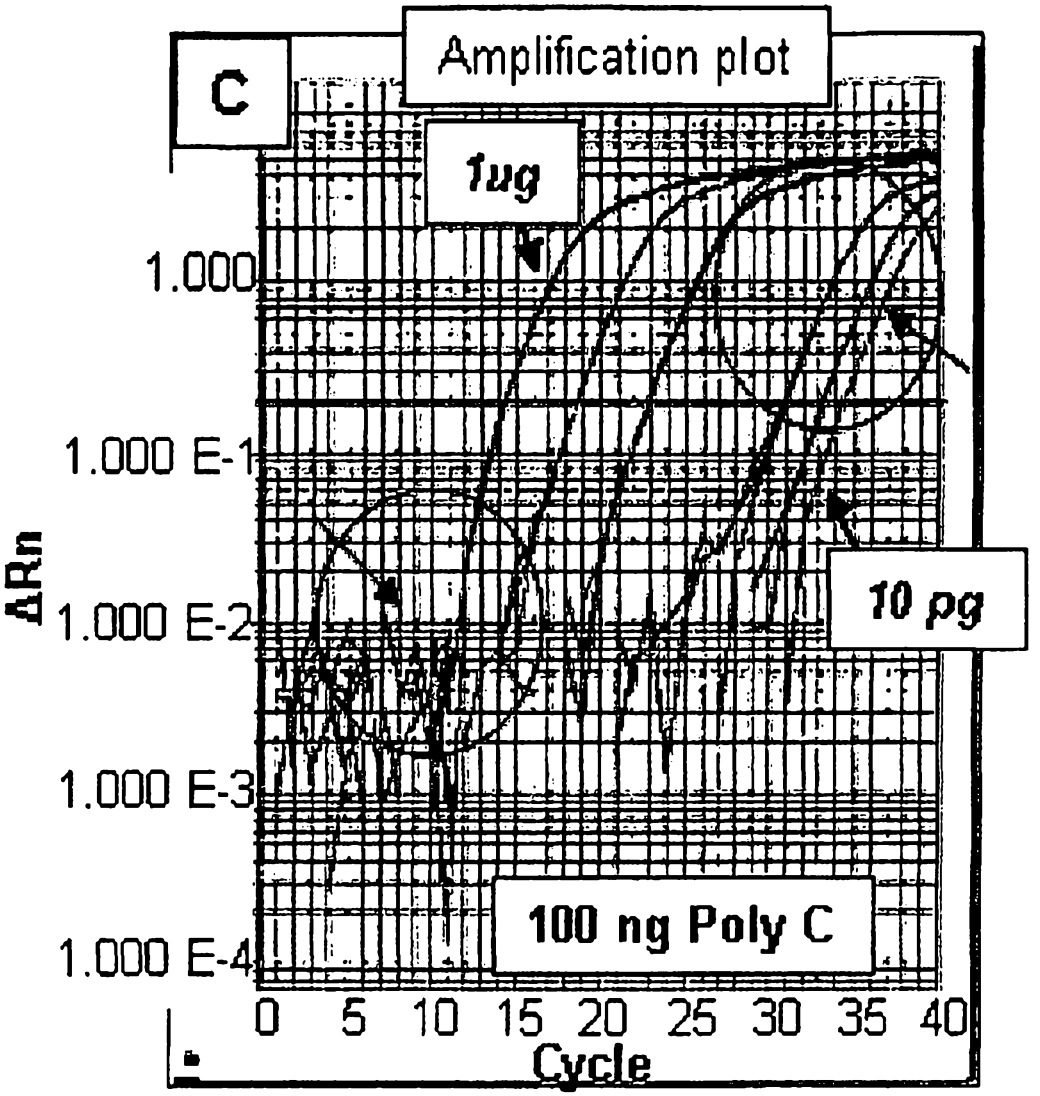
C
Amplification plot
1ug
10 pg
100 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 16 D

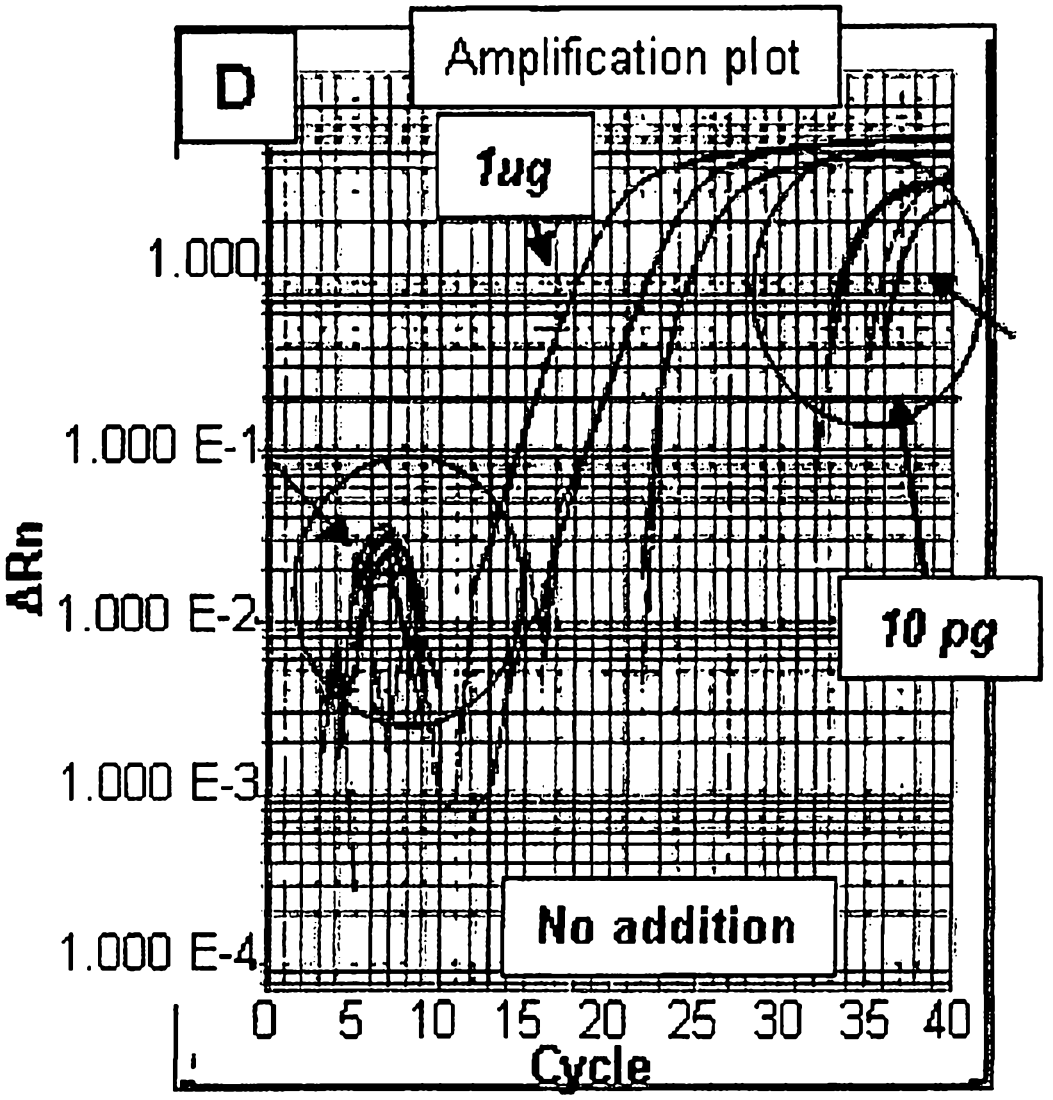
D
Amplification plot
1ug
10 pg
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 16 E

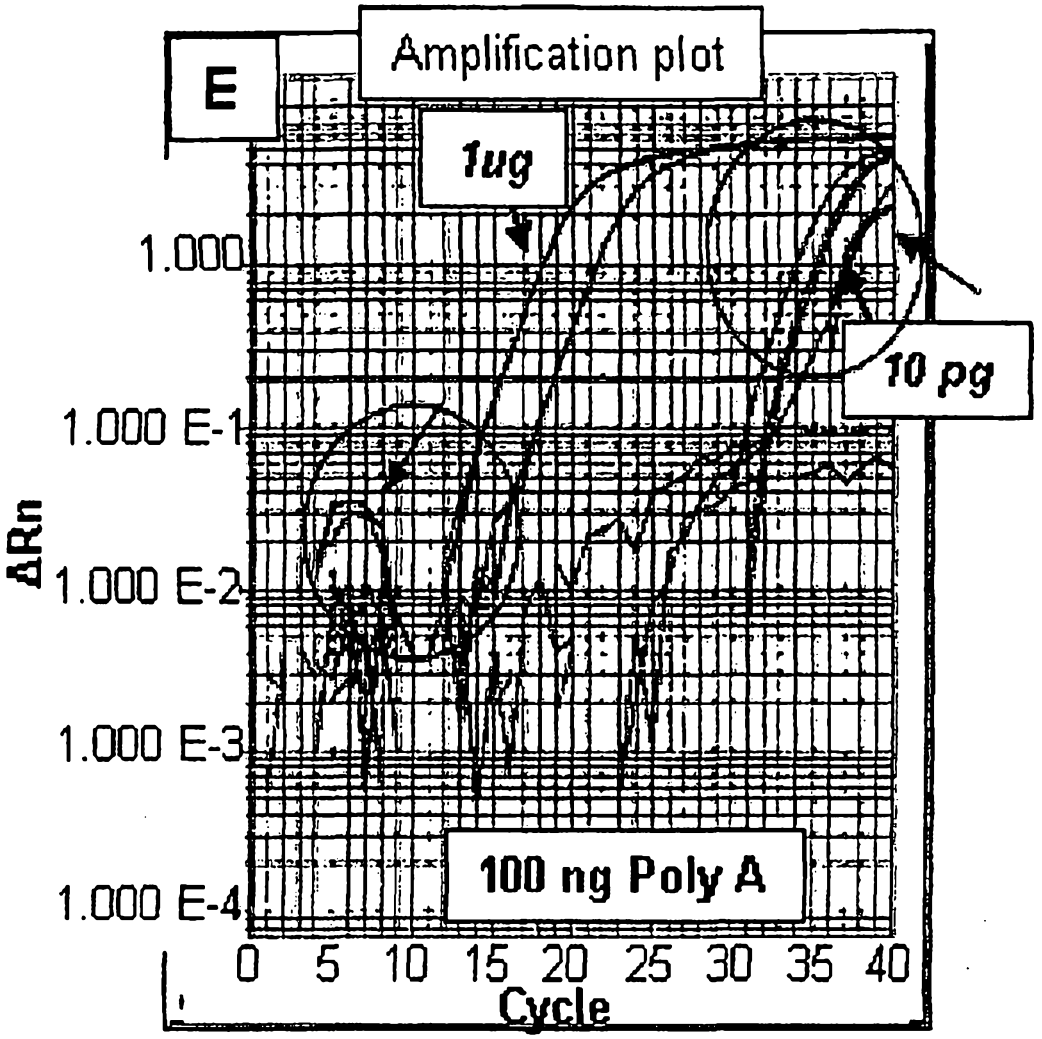
E
Amplification plot
1ug
10 pg
100 ng Poly A
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
ΔRn
0 5 10 15 20 25 30 35 40
Cycle

Fig. 16 F

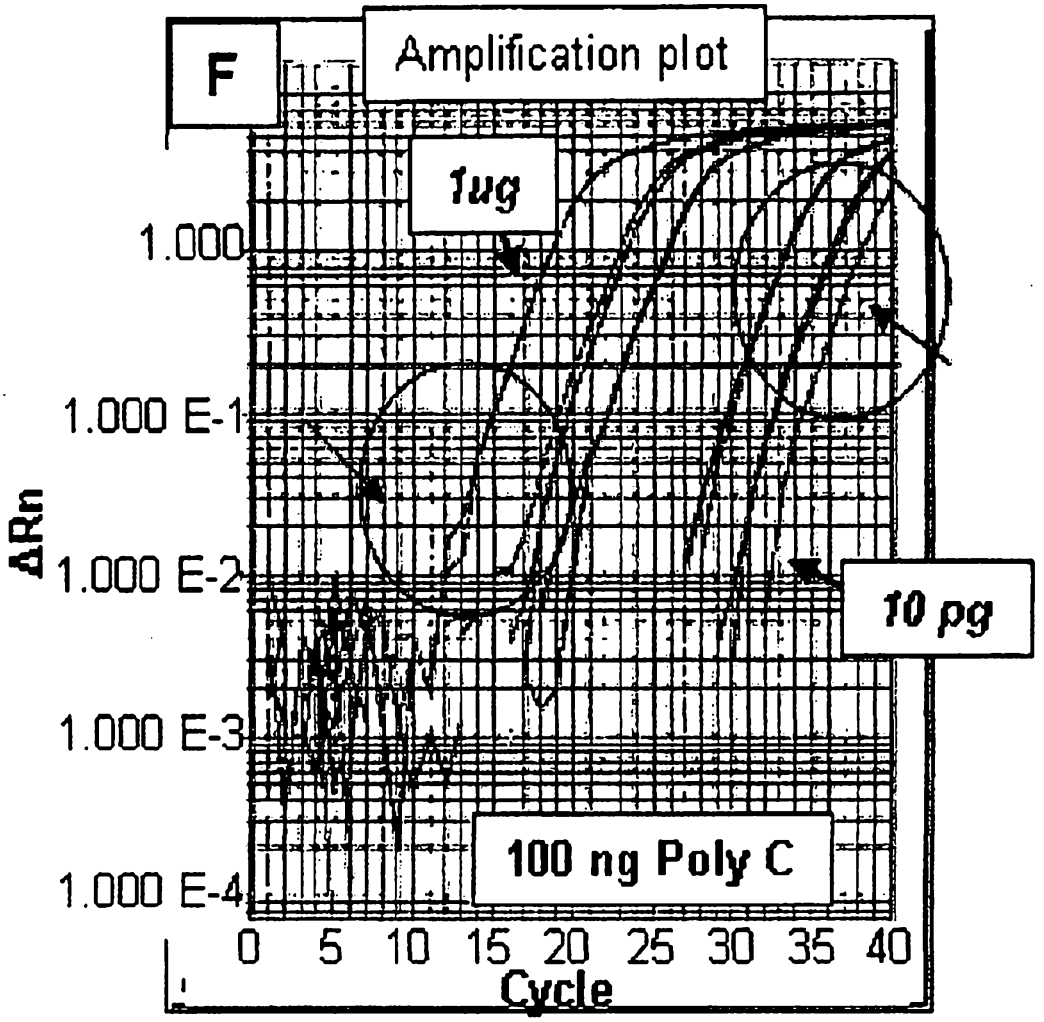
F
Amplification plot
1ug
10 pg
100 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 A

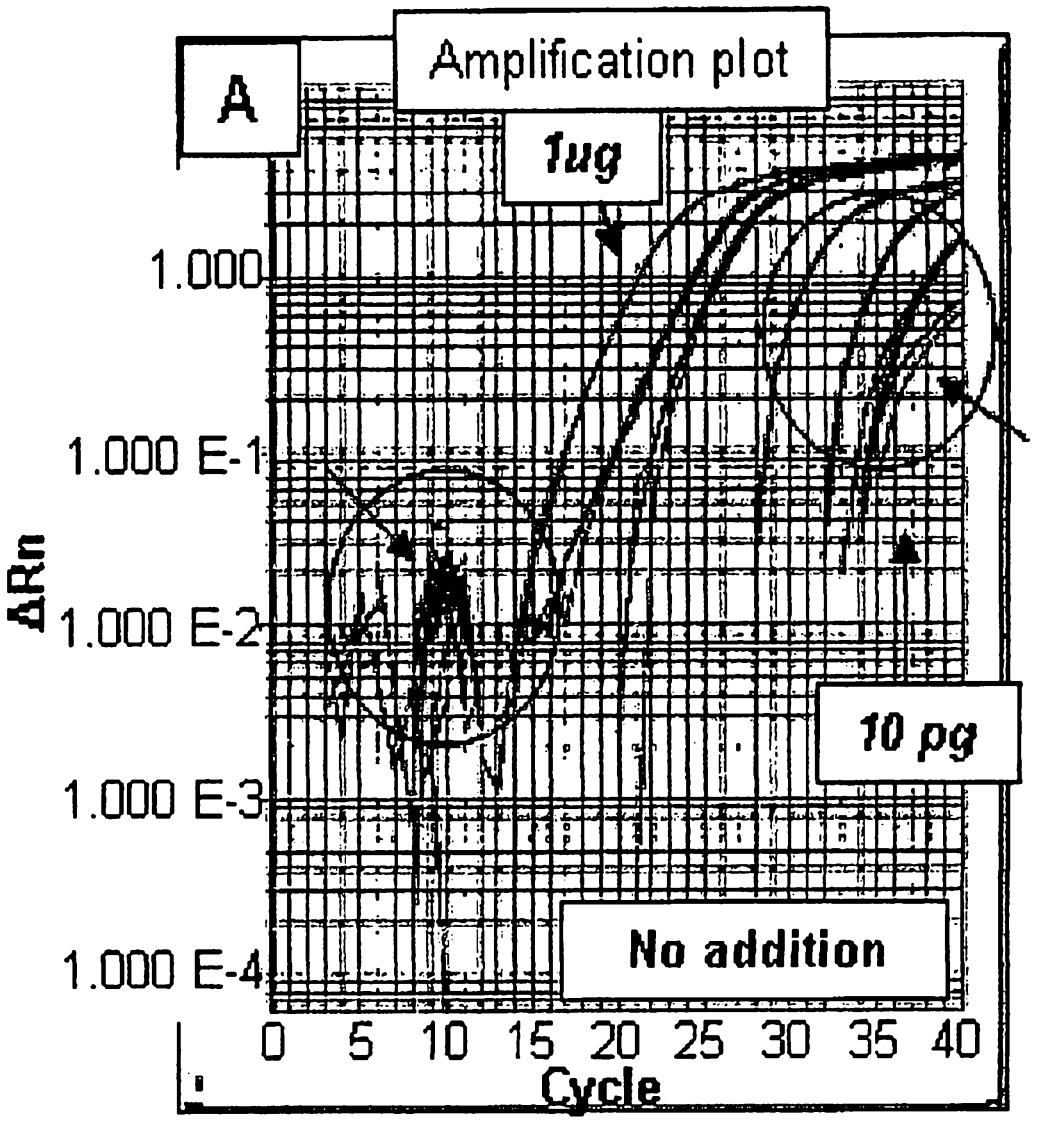
A
Amplification plot
1ug
10 pg
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 B

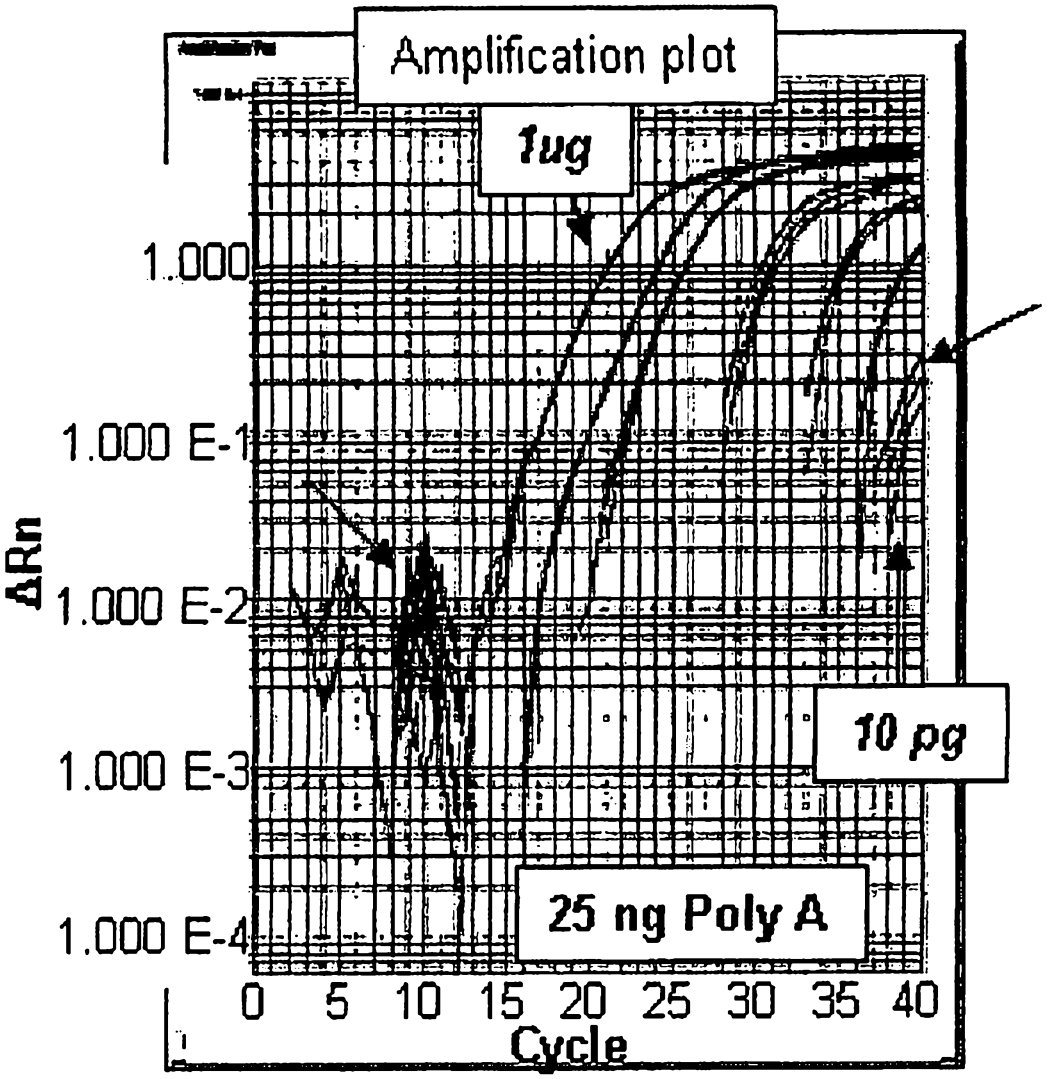
Amplification plot
1ug
10 pg
25 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 C

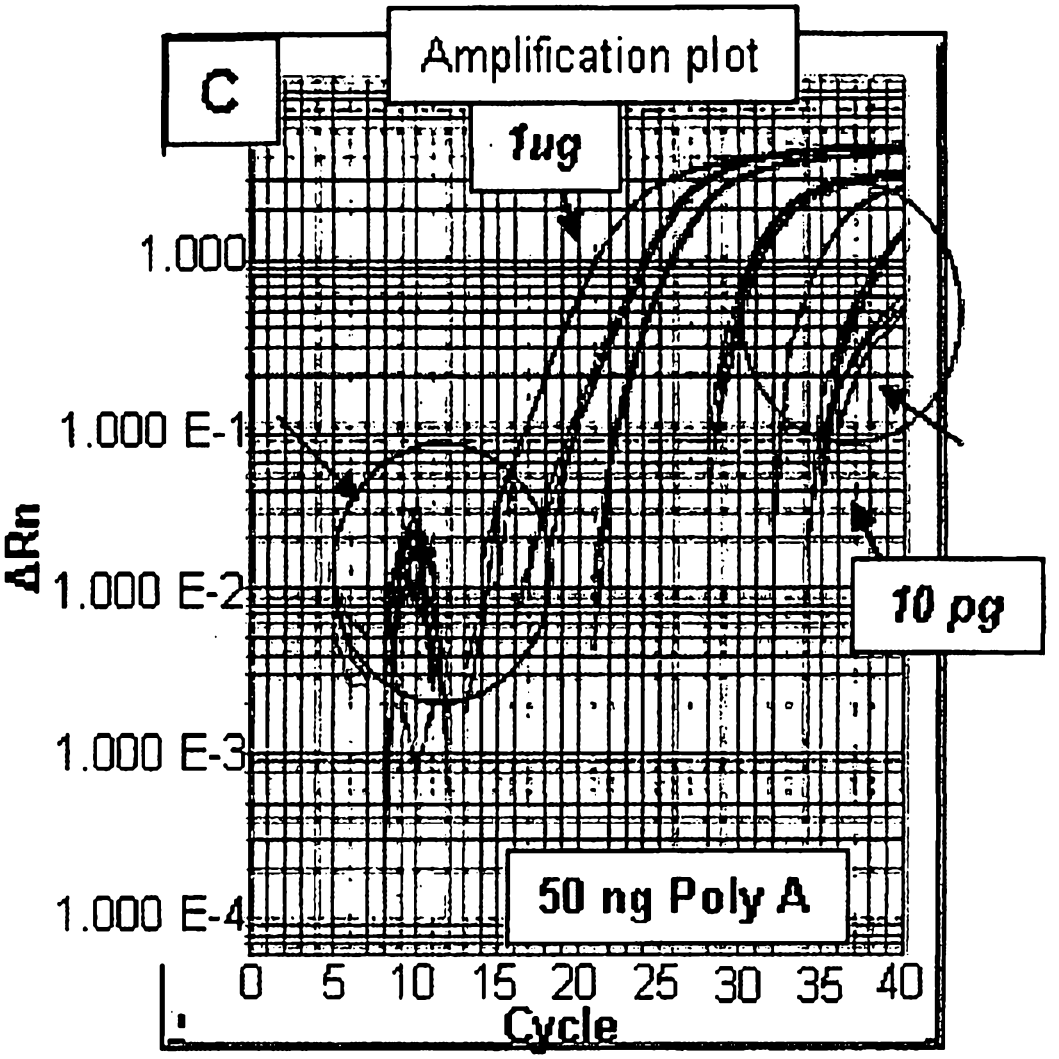
C
Amplification plot
1ug
10 pg
50 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 D

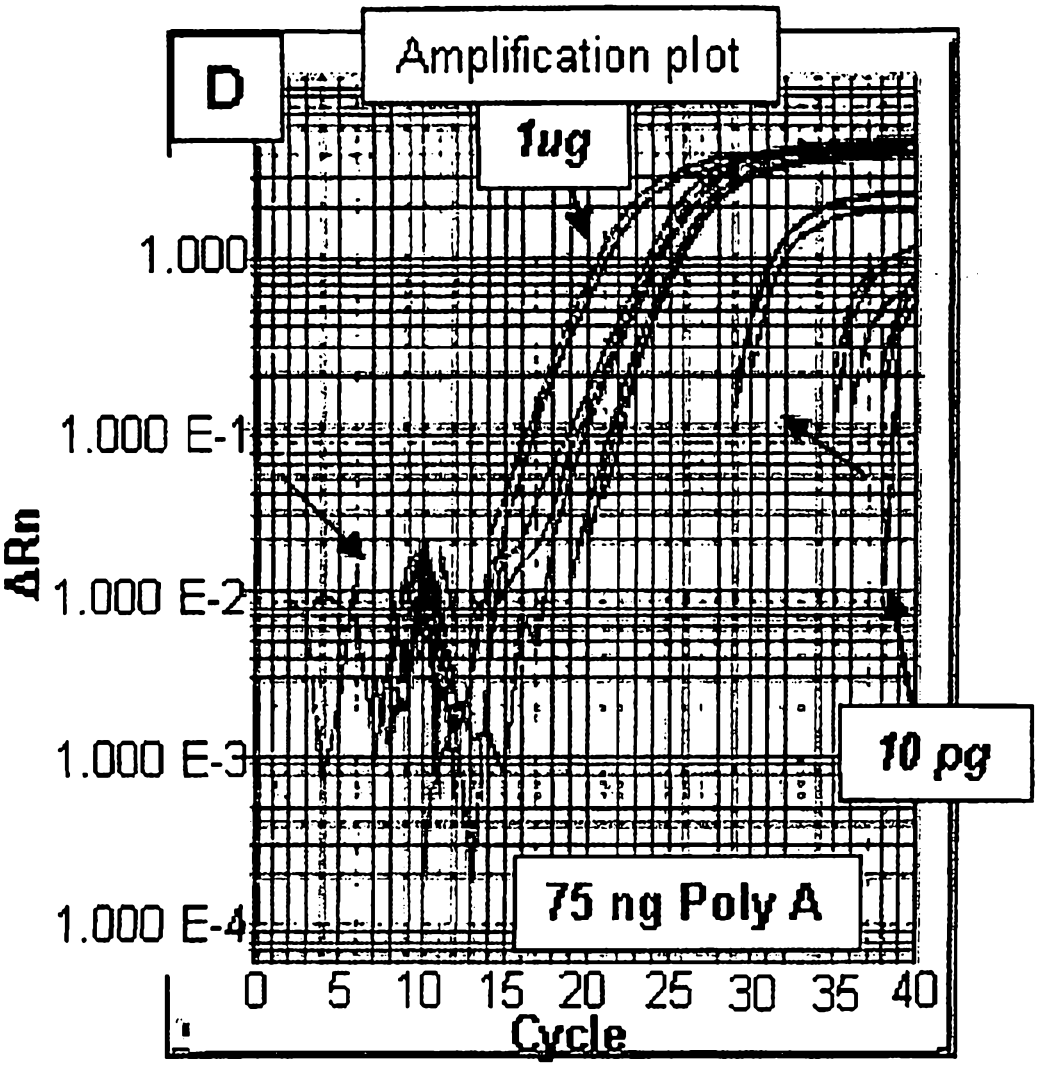
D
Amplification plot
1ug
10 pg
75 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 E

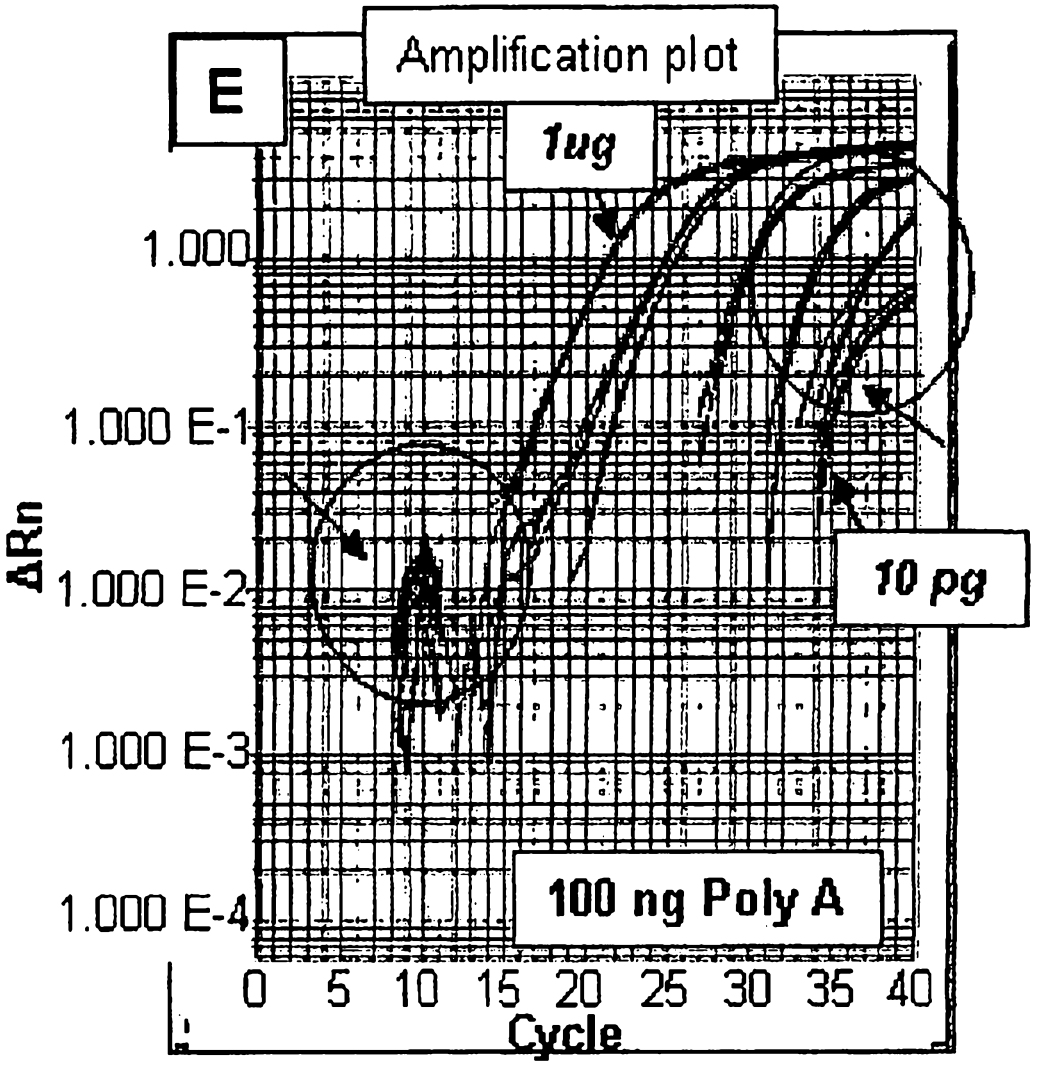
E
Amplification plot
1ug
10 pg
100 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 17 F

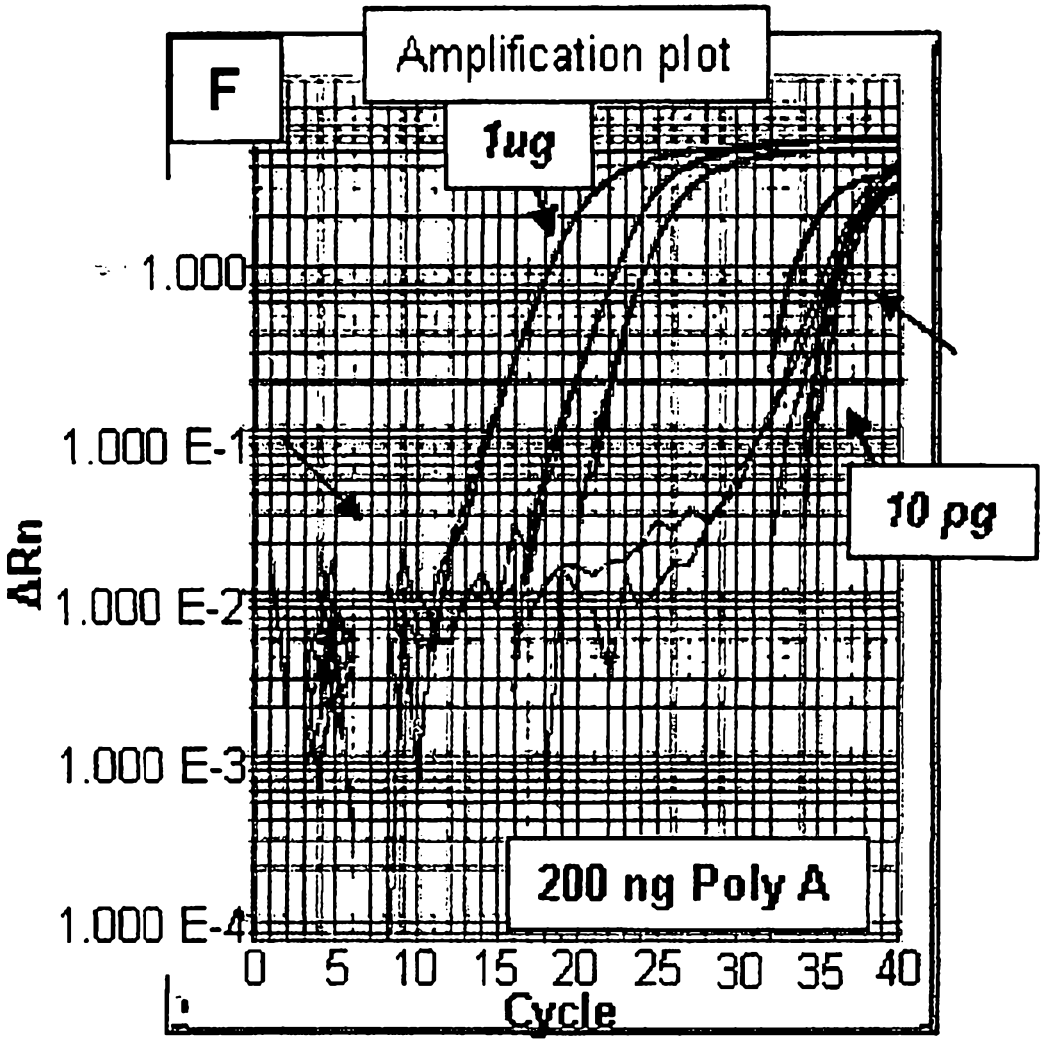
F
Amplification plot
1ug
10 pg
200 ng Poly A
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 18 A

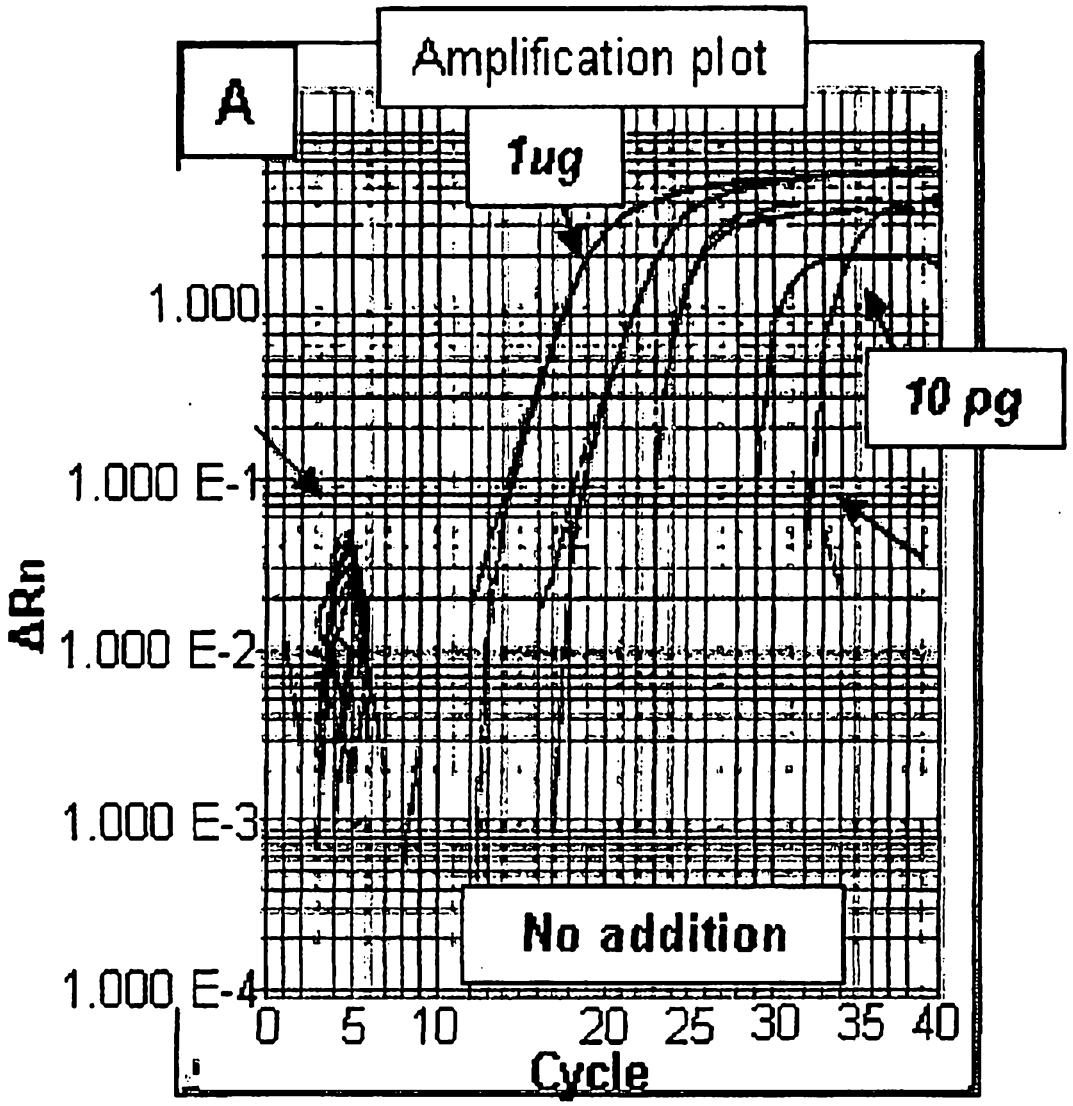
A
Amplification plot
1ug
10 pg
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 20 25 30 35 40
Cycle

Fig. 18 B

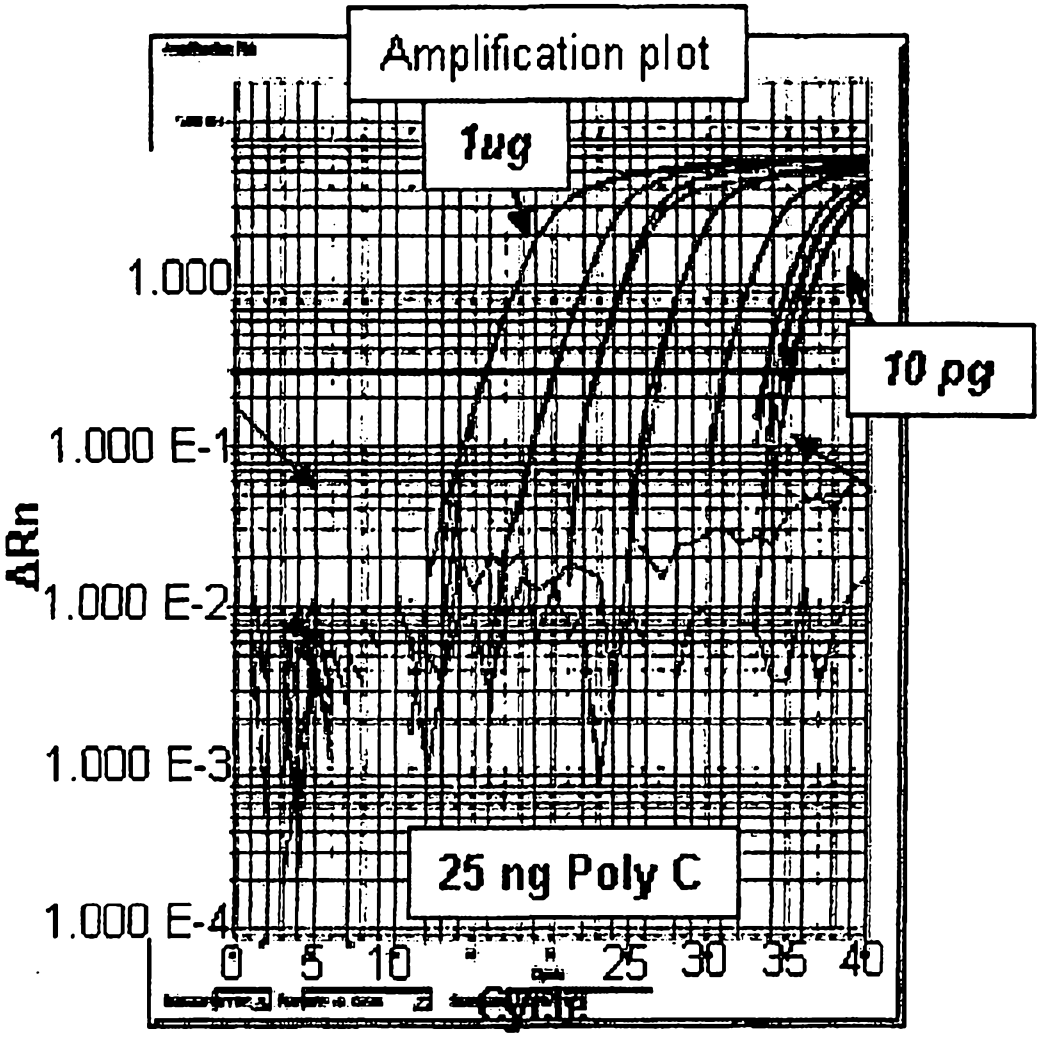
Amplification plot
1ug
10 pg
25 ng Poly C
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
ΔRn
0
5
10
25
30
35
40
Cycle

Fig. 18 C

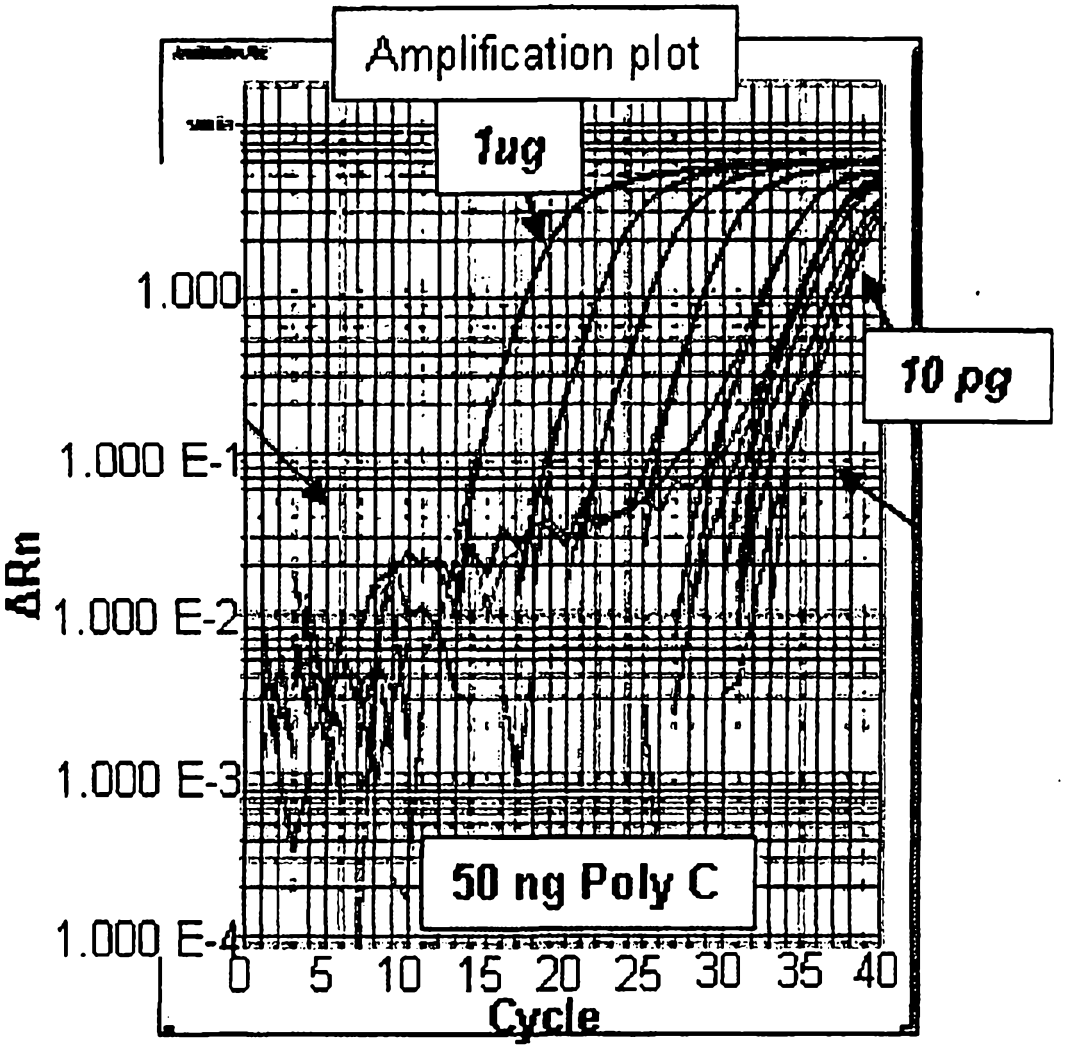
Amplification plot
1ug
10 pg
50 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 18 D

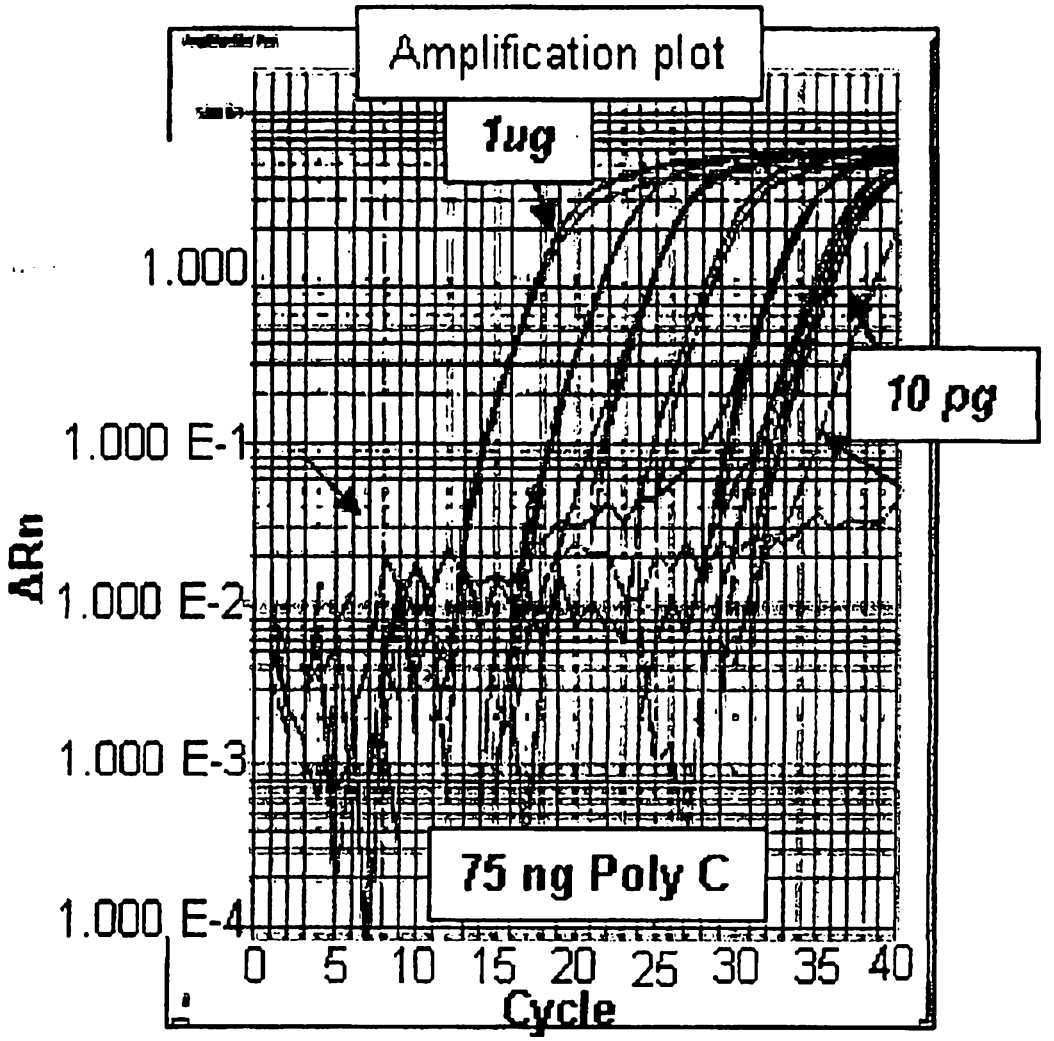
Amplification plot
1ug
10 pg
75 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 18 E

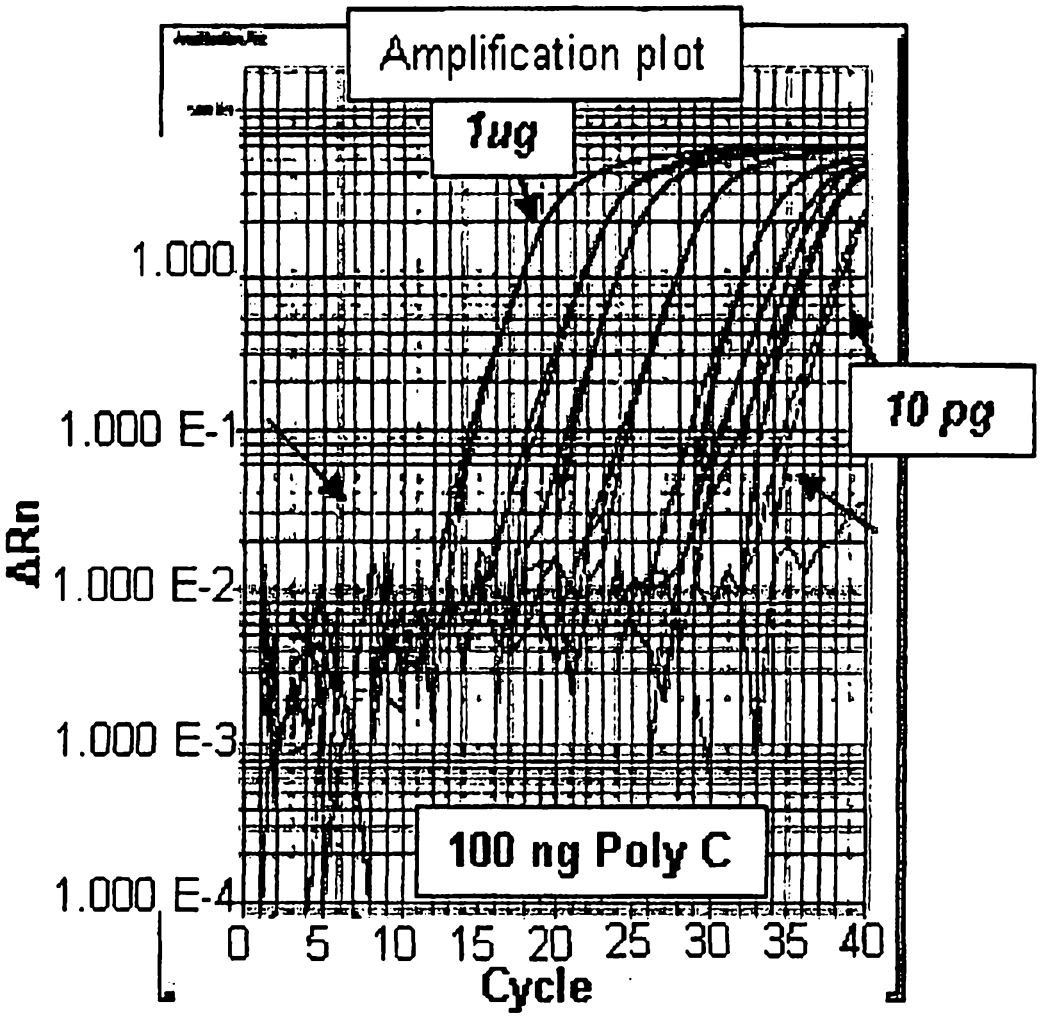
Amplification plot
1ug
10 pg
100 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 18 F

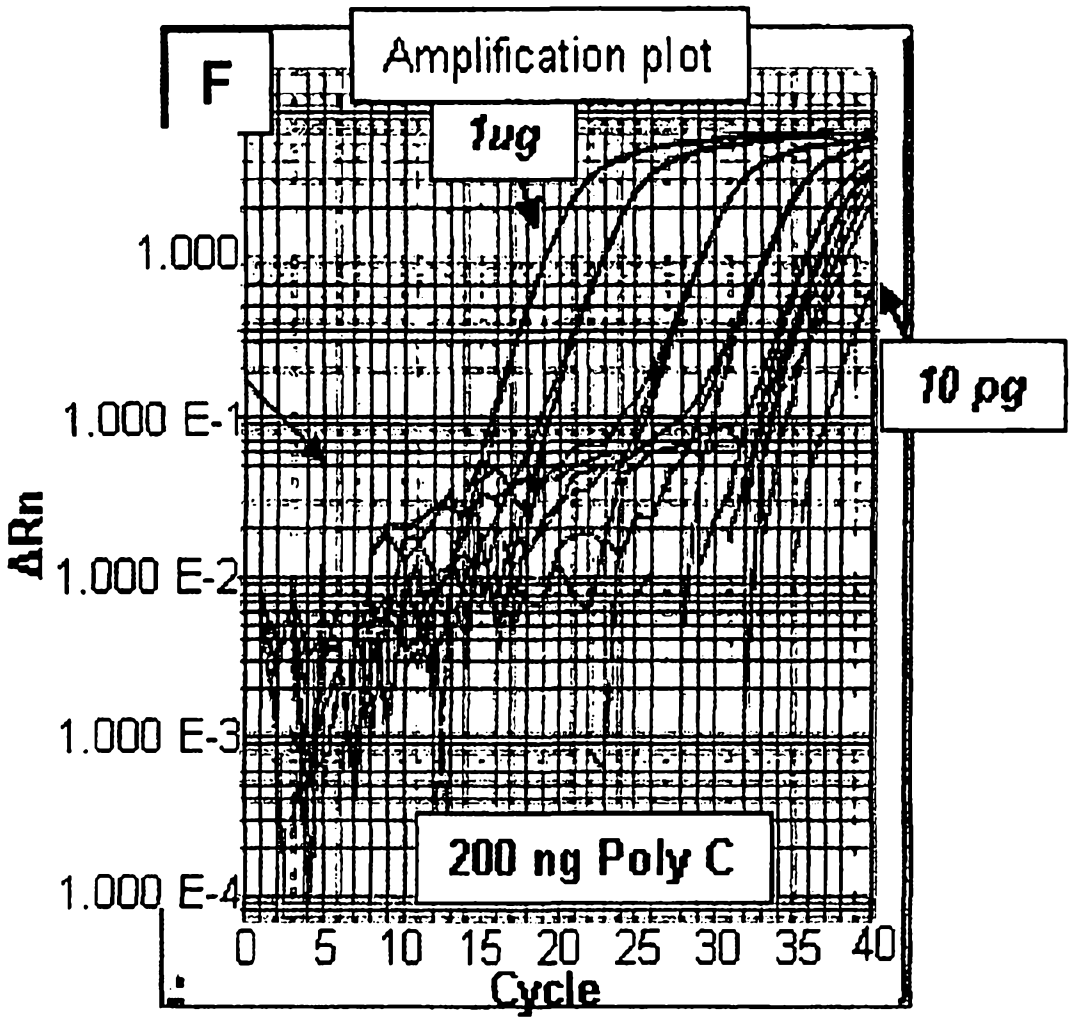
F
Amplification plot
1ug
10 pg
200 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 19 A

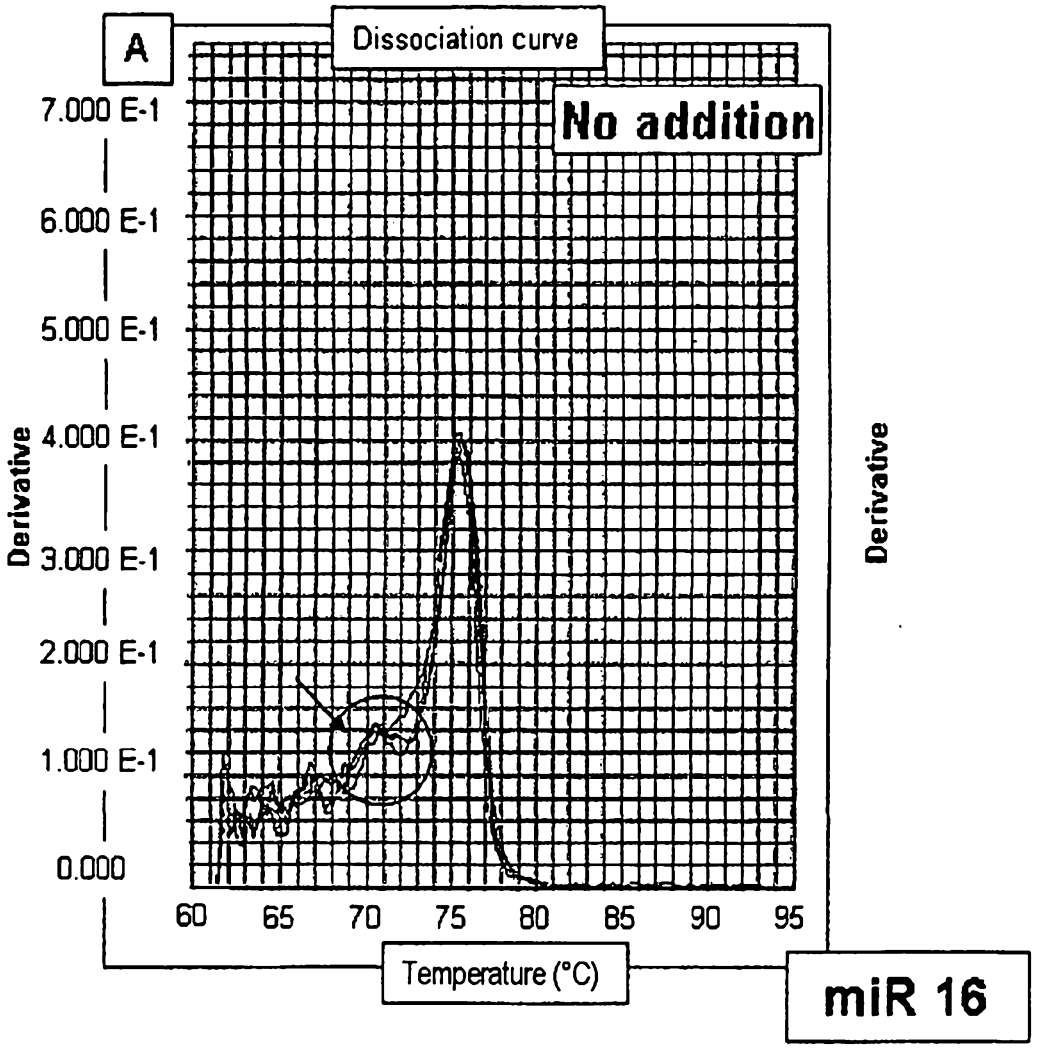
A
Dissociation curve
No addition
7.000 E-1
6.000 E-1
5.000 E-1
4.000 E-1
3.000 E-1
2.000 E-1
1.000 E-1
0.000
Derivative
Derivative
60
65
70
75
80
85
90
95
Temperature (°C)
miR 16

Fig. 19 B

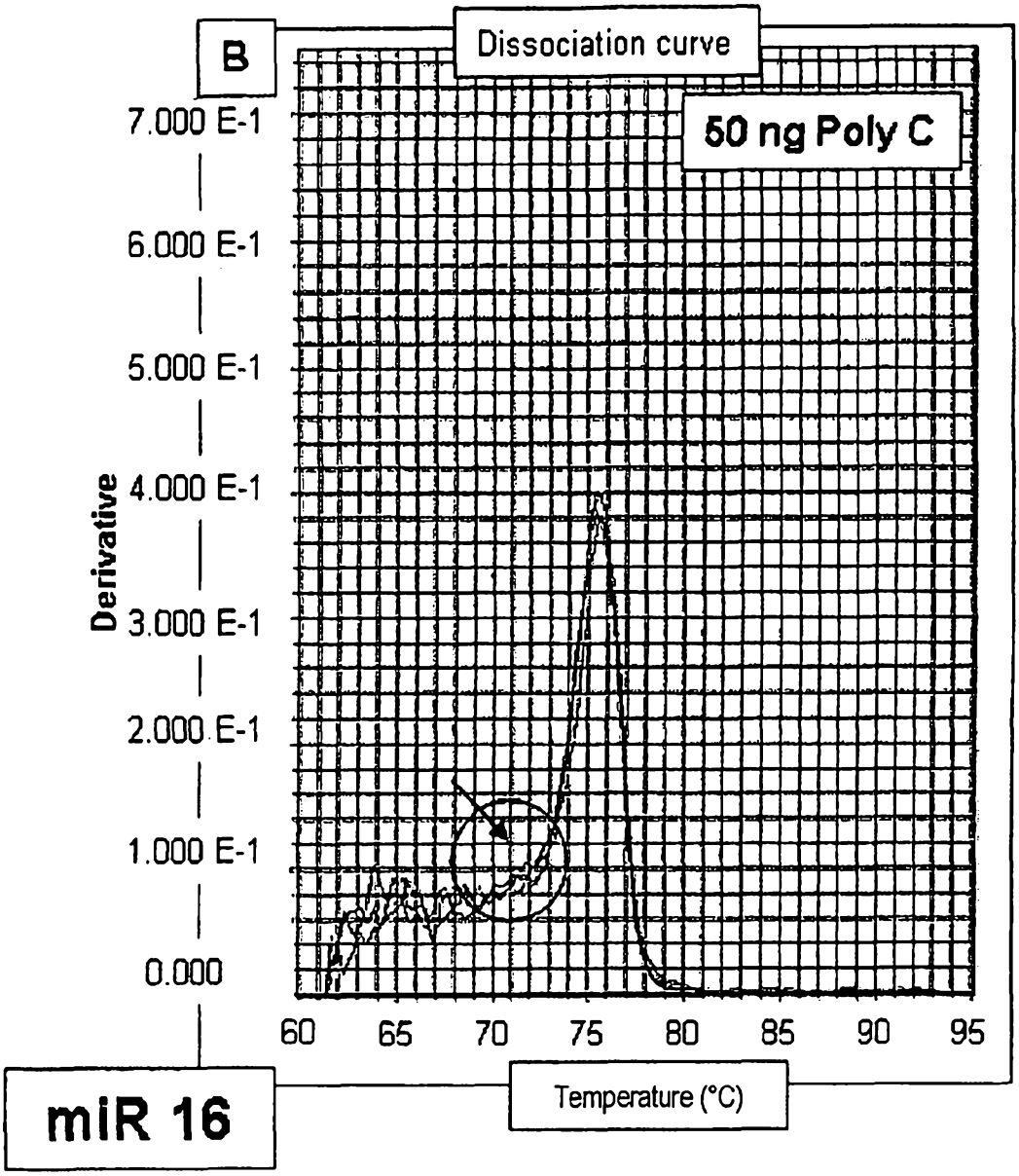
B
Dissociation curve
50 ng Poly C
7.000 E-1
6.000 E-1
5.000 E-1
4.000 E-1
3.000 E-1
2.000 E-1
1.000 E-1
0.000
Derivative
60
65
70
75
80
85
90
95
Temperature (°C)
miR 16

Fig. 20 A

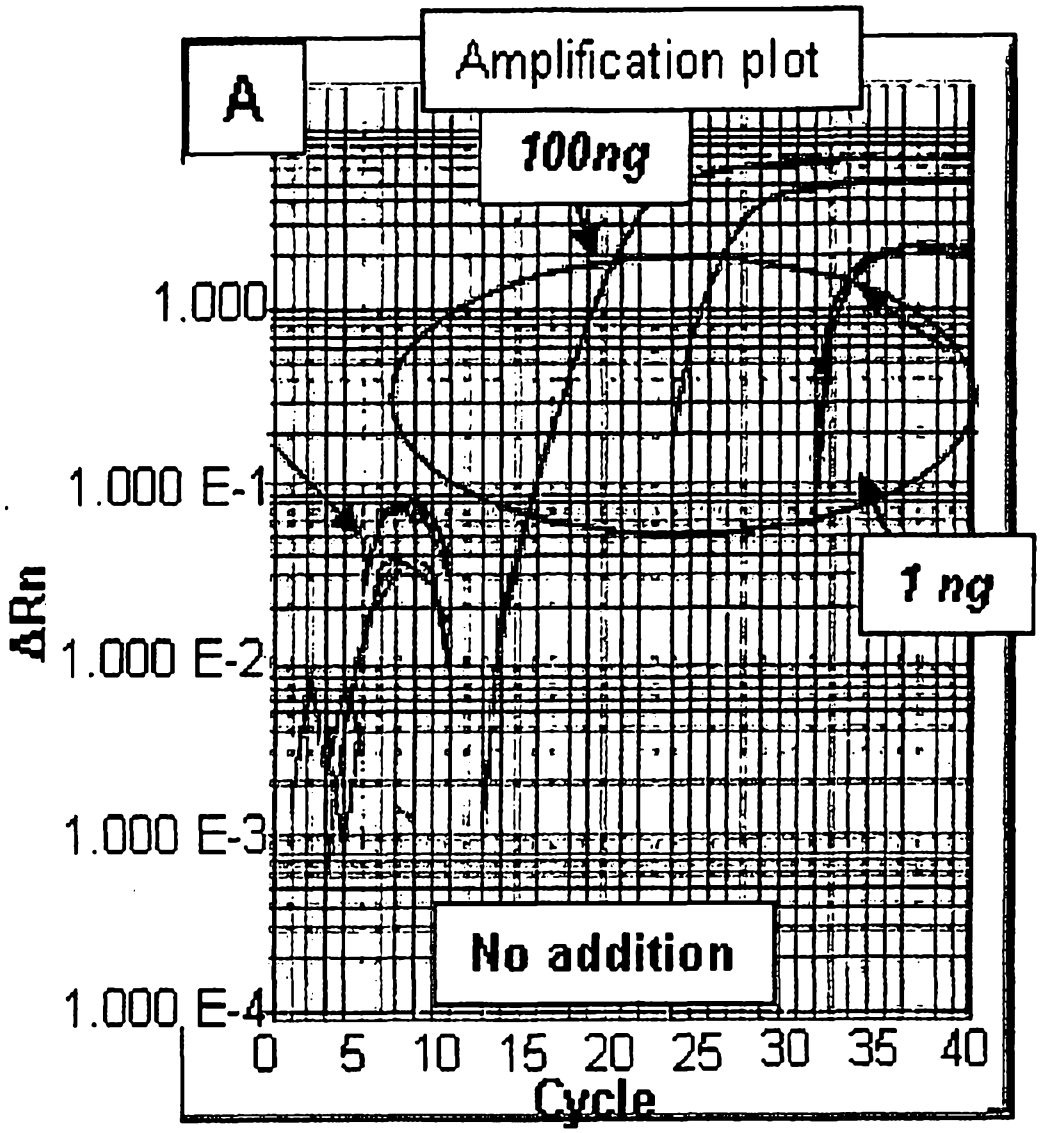
A
Amplification plot
100ng
1 ng
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 20 B

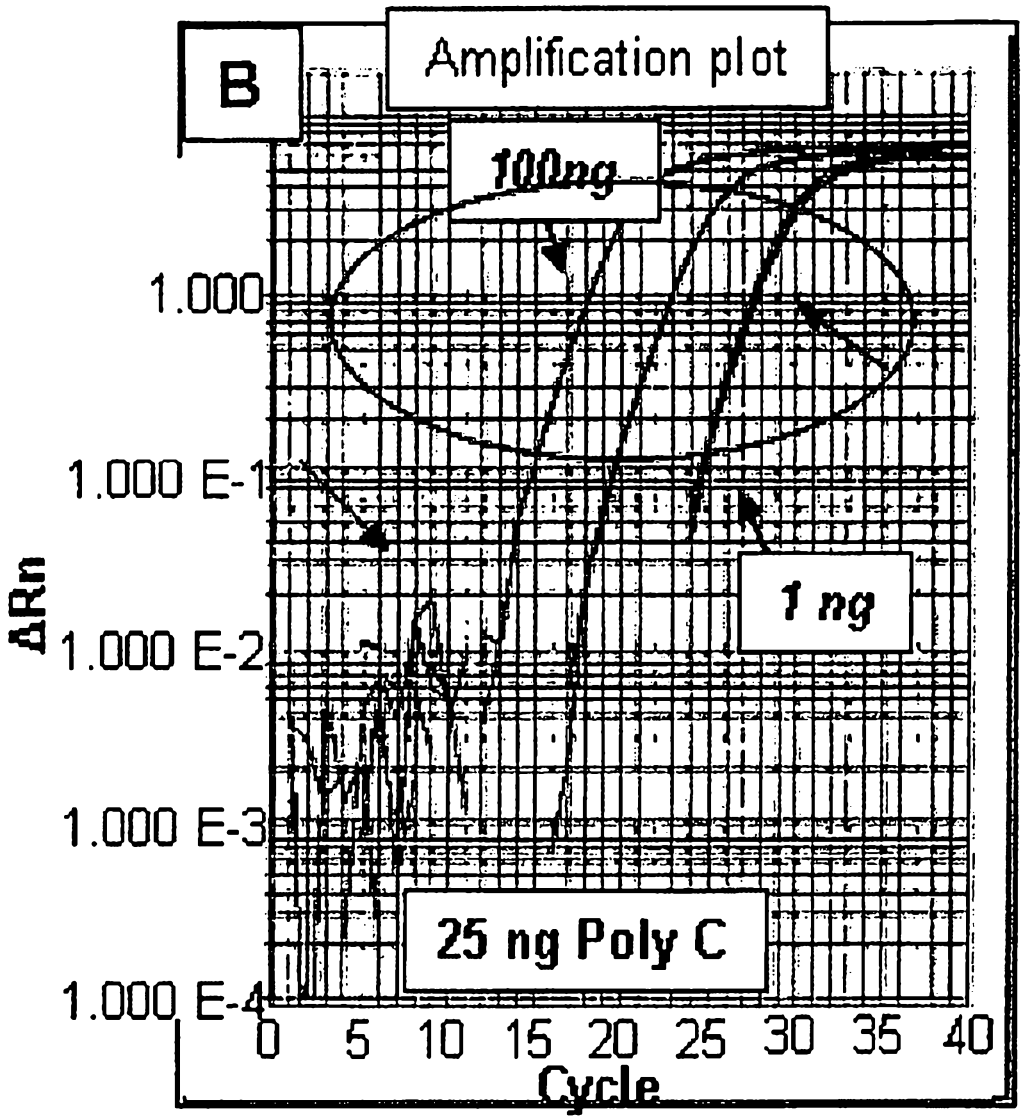
B
Amplification plot
100ng
1 ng
25 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 20 C

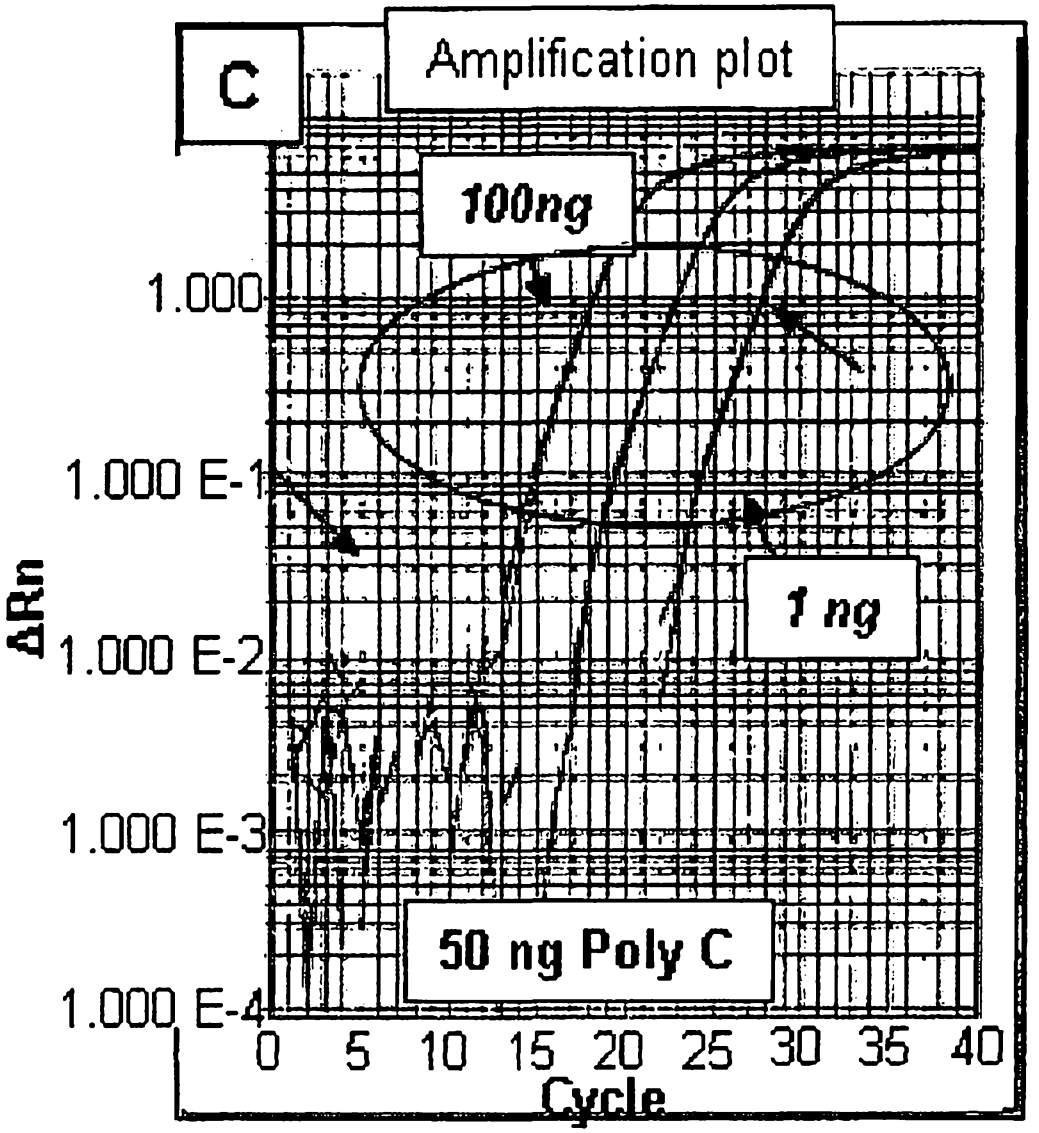
C
Amplification plot
100ng
1 ng
50 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 20 D

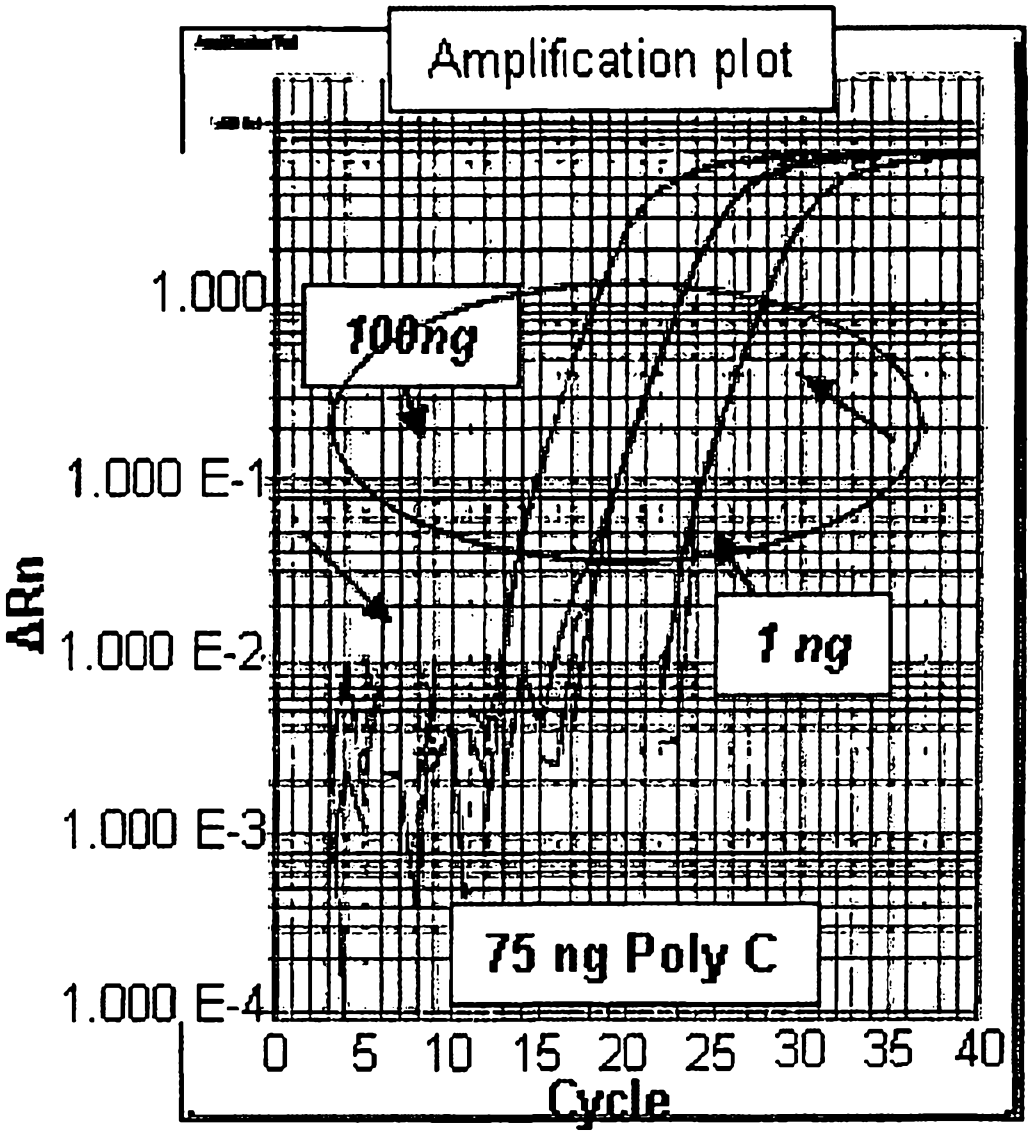
Amplification plot
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
ΔRn
100ng
1 ng
75 ng Poly C
0 5 10 15 20 25 30 35 40
Cycle

Fig. 20 E

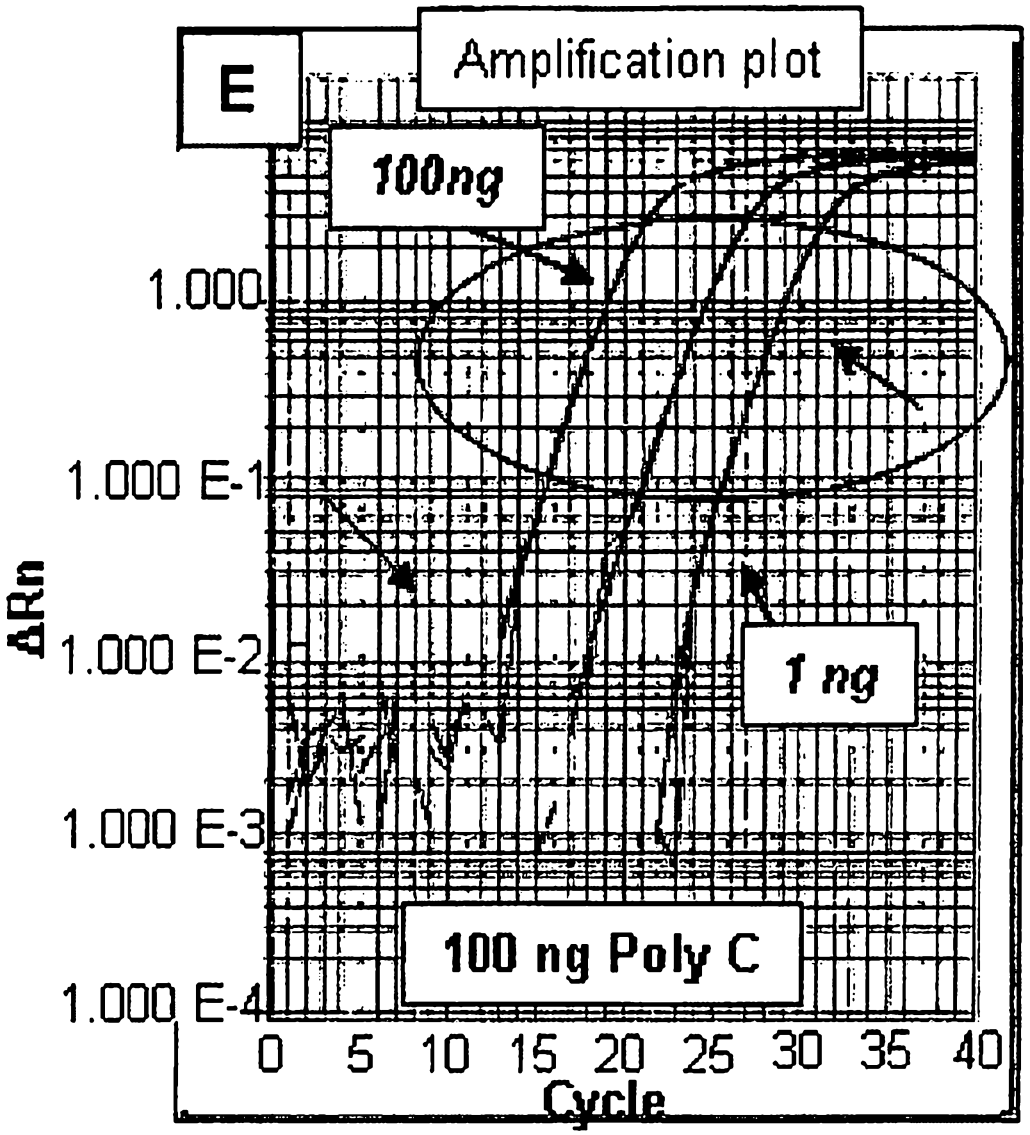
E
Amplification plot
100ng
1 ng
100 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 21 A

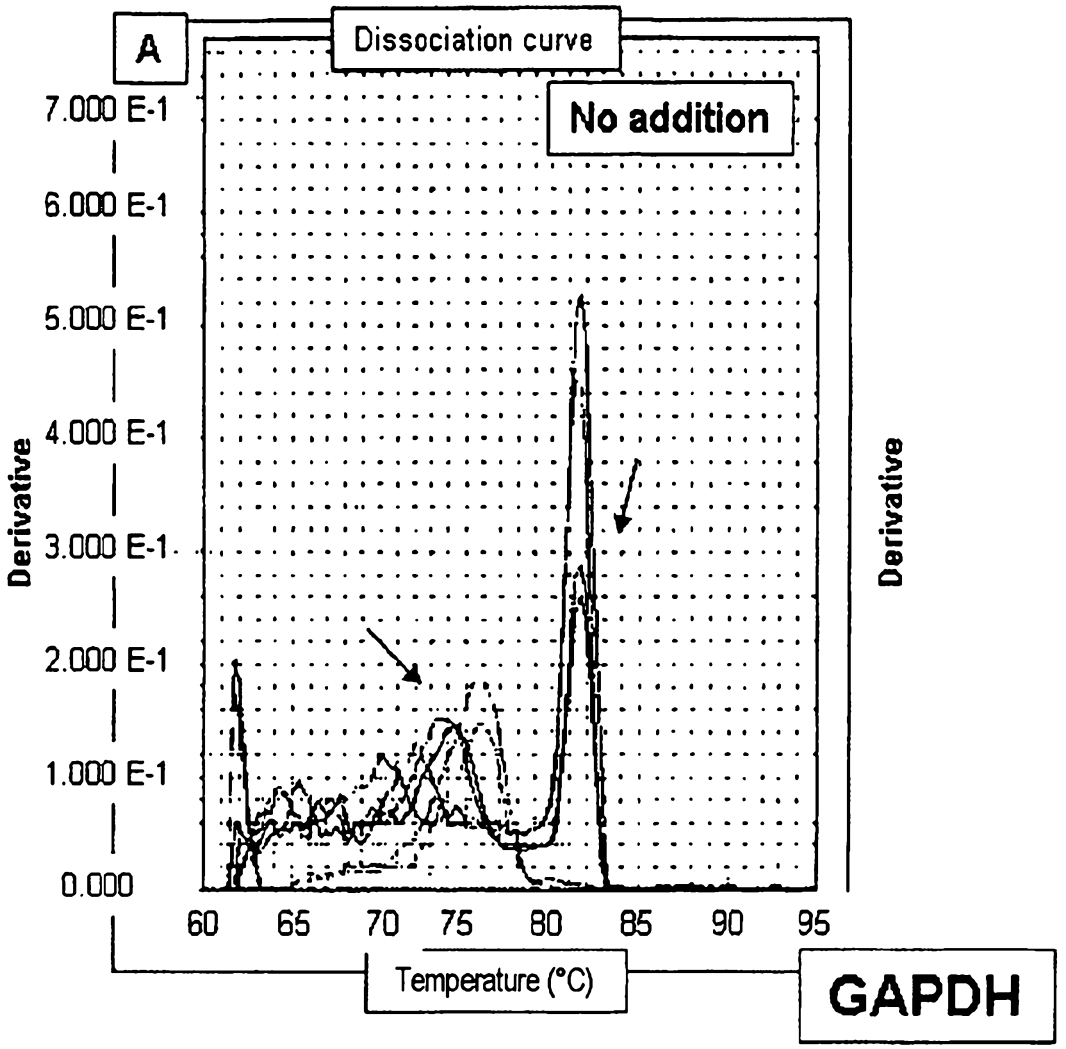
A
Dissociation curve
No addition
7.000 E-1
6.000 E-1
5.000 E-1
4.000 E-1
3.000 E-1
2.000 E-1
1.000 E-1
0.000
Derivative
Derivative
60
65
70
75
80
85
90
95
Temperature (°C)
GAPDH

Fig. 21 B

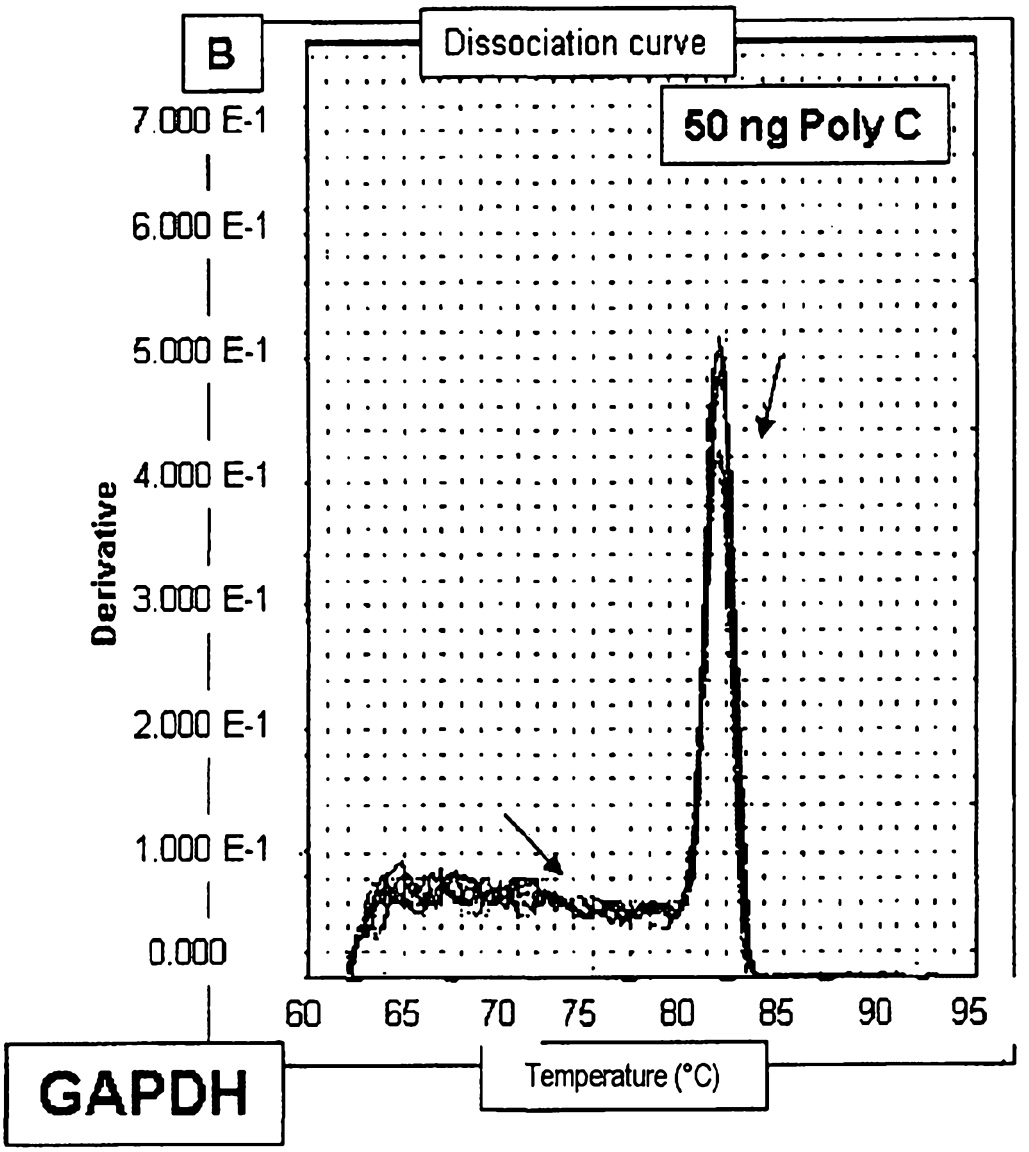
B
Dissociation curve
50 ng Poly C
7.000 E-1
6.000 E-1
5.000 E-1
4.000 E-1
3.000 E-1
2.000 E-1
1.000 E-1
0.000
Derivative
60
65
70
75
80
85
90
95
Temperature (°C)
GAPDH

Fig. 22 A

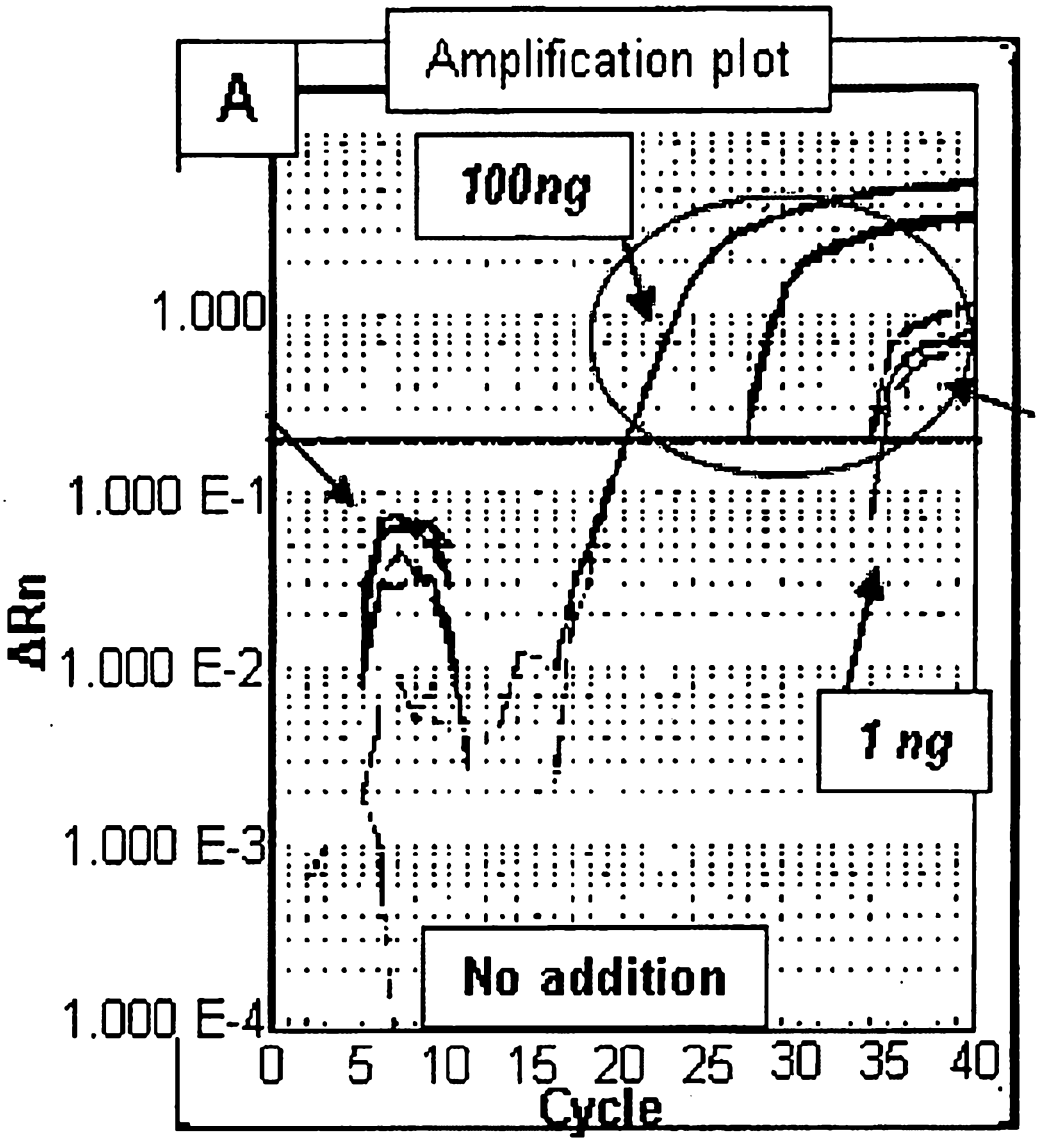
A
Amplification plot
100ng
1 ng
No addition
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 22 B

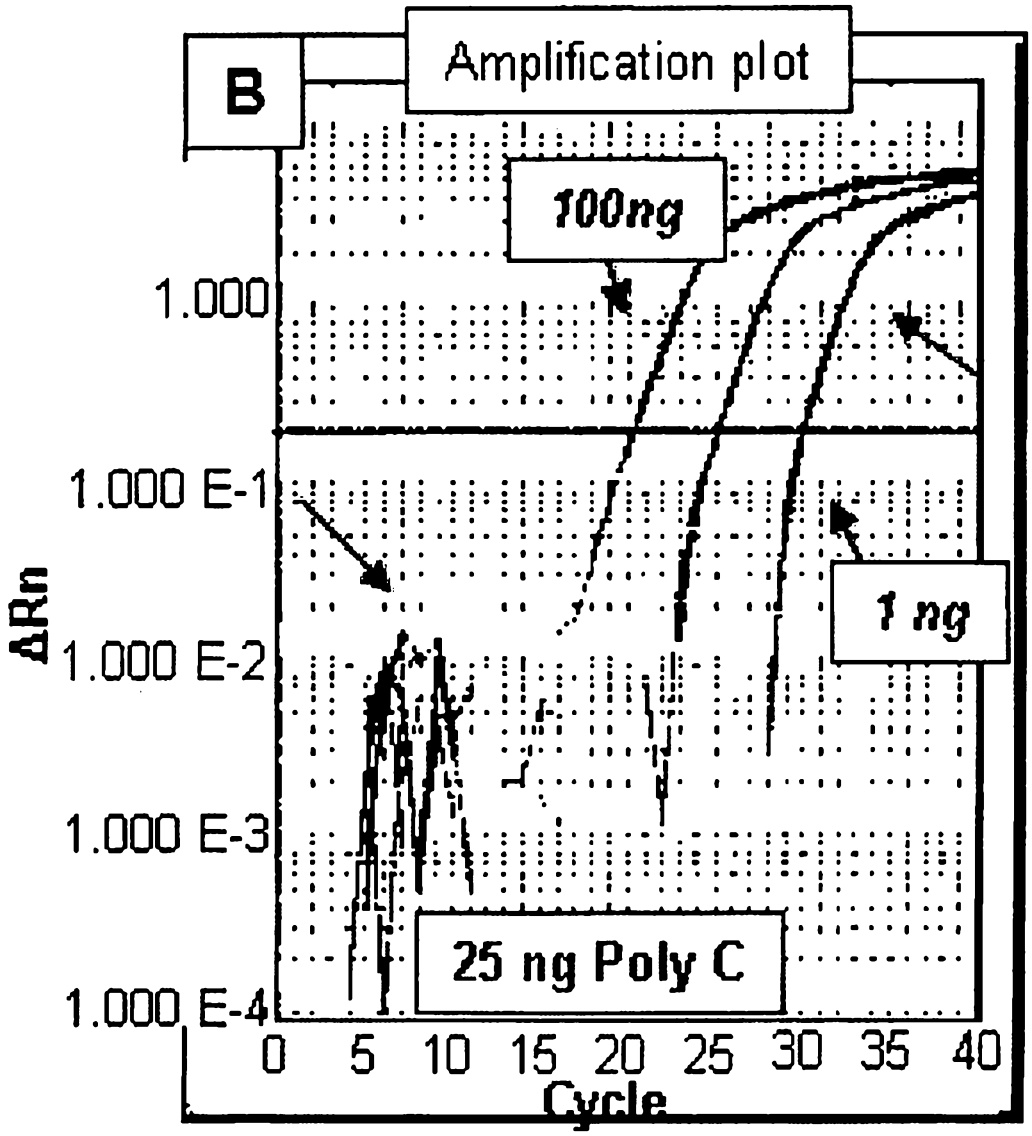
B
Amplification plot
100ng
1 ng
25 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 22 C

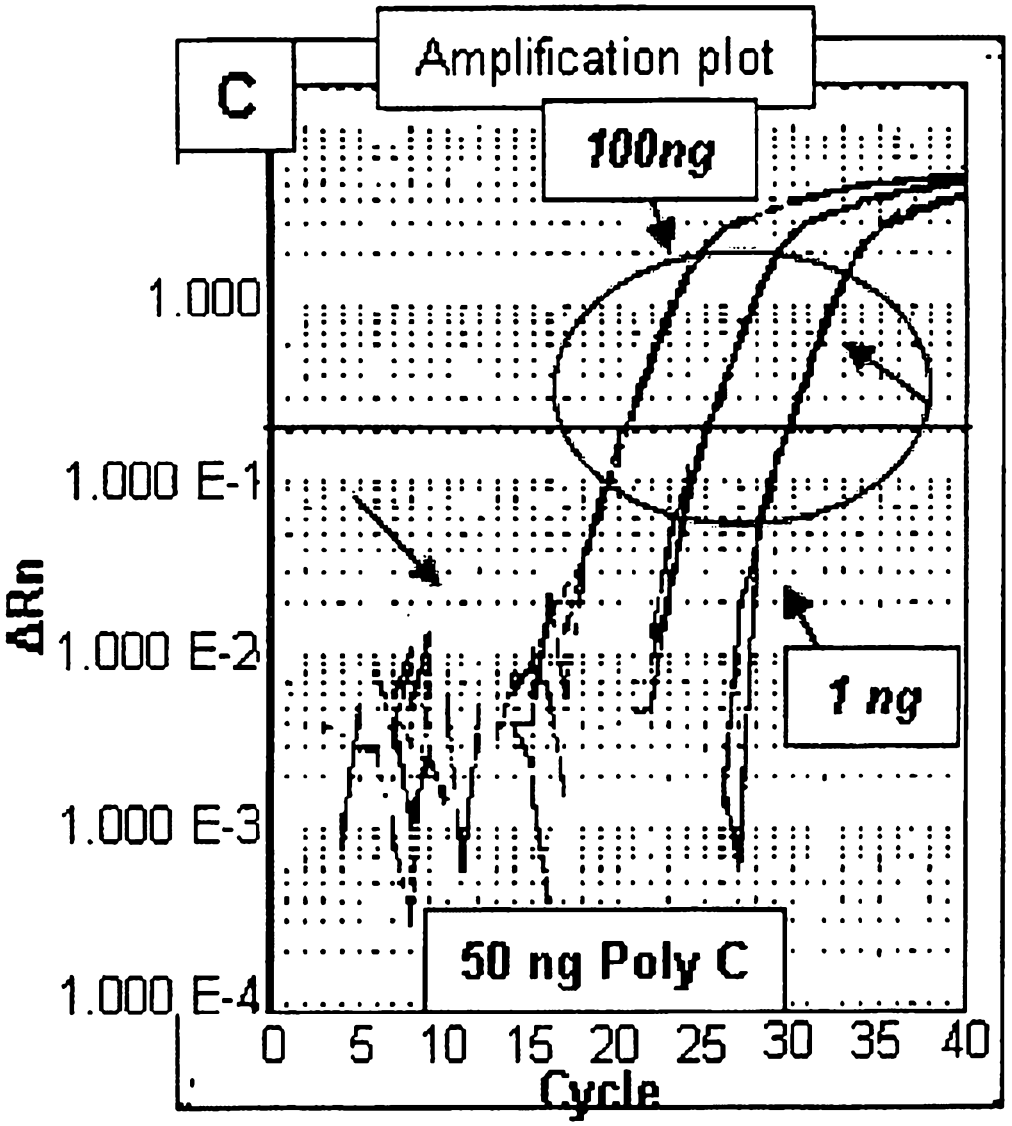
C
Amplification plot
100ng
1 ng
50 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0 5 10 15 20 25 30 35 40
Cycle

Fig. 22 D

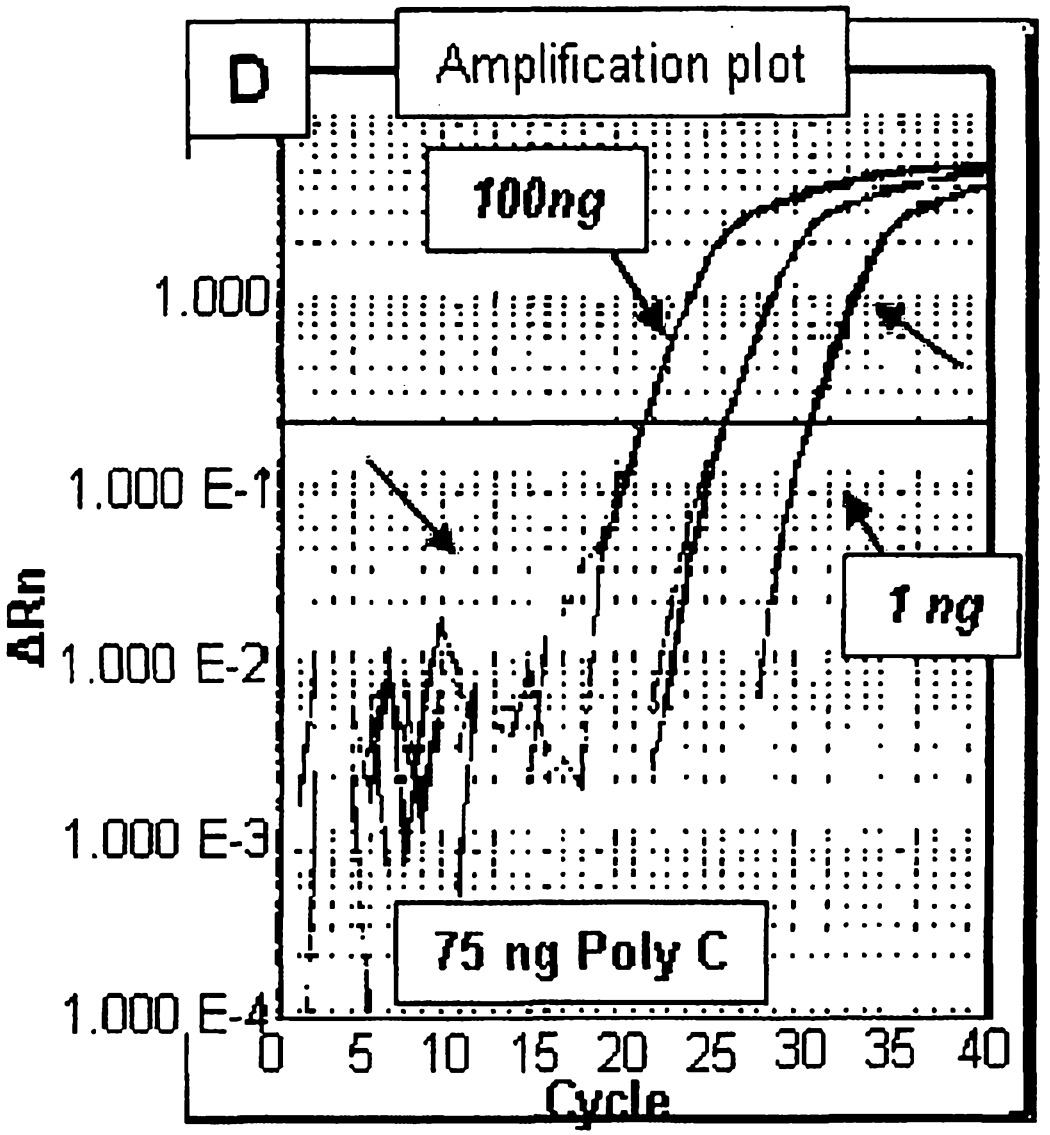
D
Amplification plot
100ng
1 ng
75 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0
5
10
15
20
25
30
35
40
Cycle

Fig. 22 E

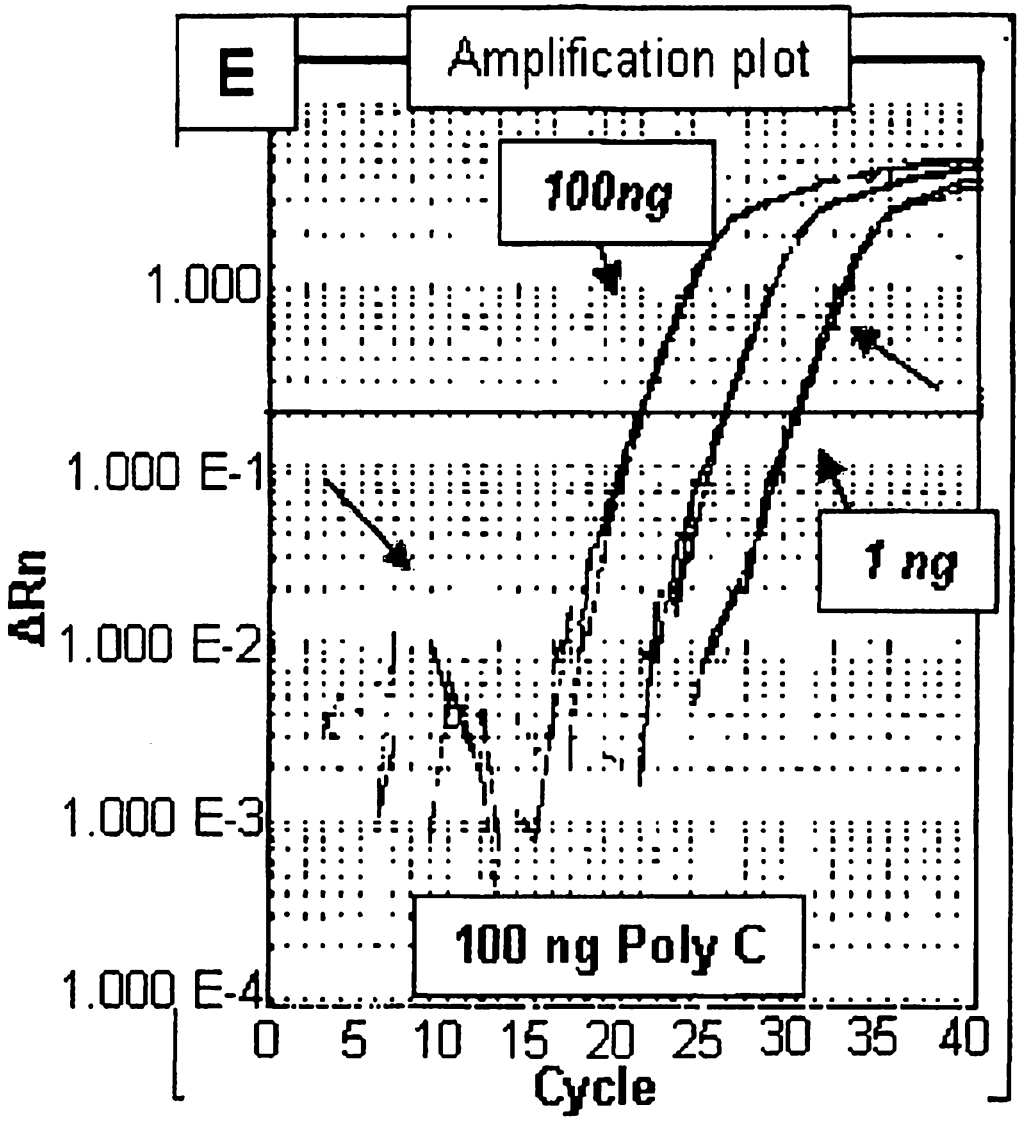
E
Amplification plot
100ng
1 ng
100 ng Poly C
ΔRn
1.000
1.000 E-1
1.000 E-2
1.000 E-3
1.000 E-4
0
5
10
15
20
25
30
35
40
Cycle

Fig. 23

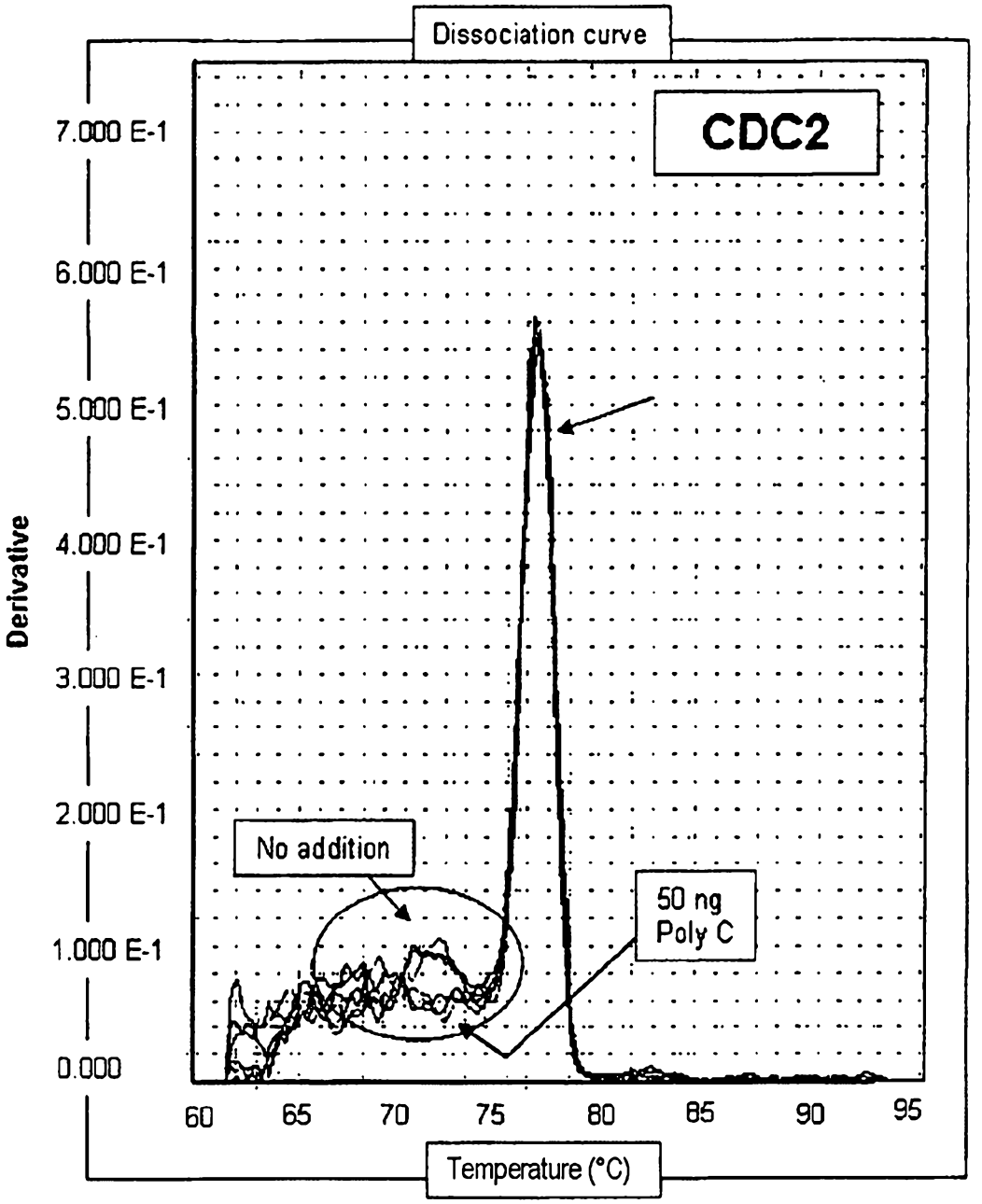
Dissociation curve
CDC2
7.000 E-1
6.000 E-1
5.000 E-1
4.000 E-1
3.000 E-1
2.000 E-1
1.000 E-1
0.000
Derivative
No addition
50 ng
Poly C
60
65
70
75
80
85
90
95
Temperature (°C)

Fig. 24

	HotStart Primer Prec1: HUM Uni/ leu7short
No template	No Ct
RNA	33,06

No Ct: No signal detectable before PCR cycle 40;

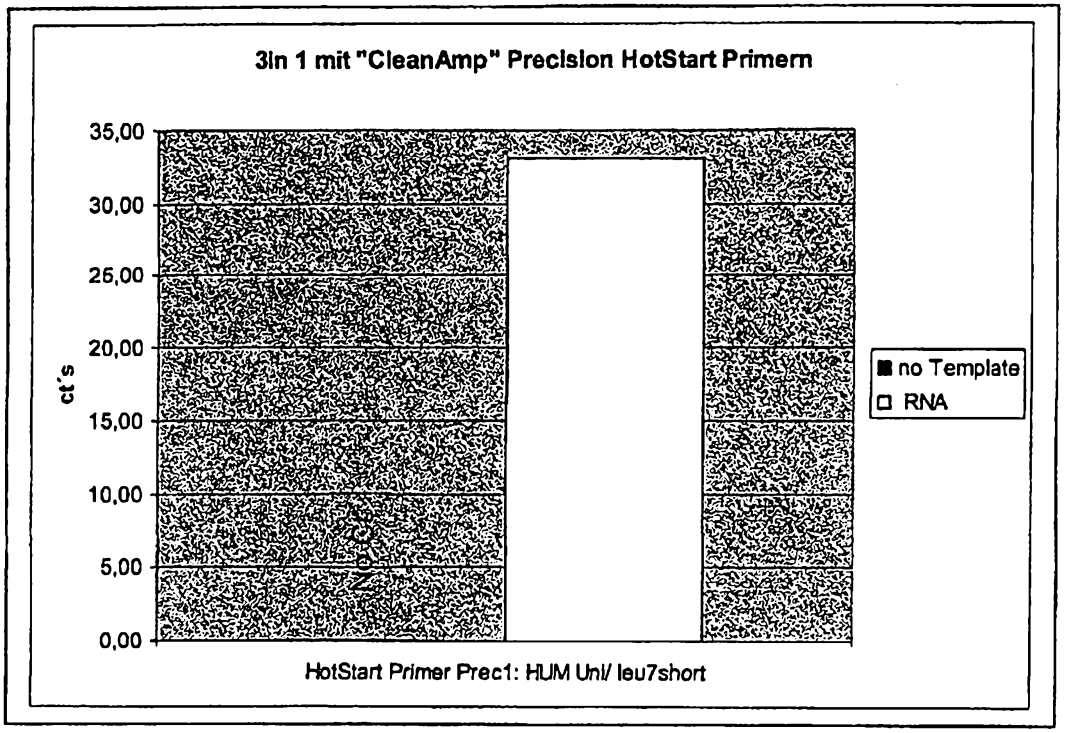

Fig. 25

	Prec1 Primer: HUM Uni/ leu7short
No template	34,51
RNA	28,41