A method for amplifying low abundance nucleic acid sequences and means for performing said method

FIELD OF THE INVENTION

The present invention relates to methods as well as to nucleic acid primers and kits containing the same for performing efficiently an amplification of nucleic acid sequences from a sample, particularly of nucleic acid sequences that are initially poorly represented in said sample.

BACKGROUND OF THE INVENTION

DNA sequence information resulting from genome and expressed sequence tag (EST) sequencing projects is expected to provide the basis for further understanding of the control and mode of action of individual, and groups of gene products.

In this respect, analysis and comparison of when, where and to what degree genes are expressed, commonly known as expression profiling, is playing an essential role in the functional characterization of newly identified genes.

Many tissue cellular systems, such as the immune and nervous systems, are composed of highly heterogeneous cell populations. A key factor lies in understanding their physiology, and the role of specific gene products expressed with the ability to examine gene usage in the context of this cellular diversity.

In the past, low throughput and laborious methods such as Northern Blotting and nuclease protection assays were employed to study gene expression.

More recently, various methods have been developed for assessing simultaneously the expression of large numbers of genes.

All these techniques, however, require relatively large amounts of RNA and currently lack the sensitivity to analyze specimens derived from small populations of cells or indeed from an individual cell.

CONFIRMATION COPY

This is compounded by the fact that it is very difficult in the case of many cell types to obtain enough specific cellular material for RNA experimentation. Consequently many areas of investigation are frustrated by lack of starting material.

Thus in situ hybridization provides detailed information on the cellular expression pattern of a gene in intact tissue. However, this technique is laborious to perform, and does not allow the analysis of more than a very small number of transcripts in a single preparation, when performed in whole-mounts or tissue sections.

The polymerase chain reaction (PCR) has been used successfully to investigate gene expression in cytoplasmic samples, particularly with the nested-primer approach which provides good sensitivity, but restricts the analyses to a small number of closely related genes from specific gene families.

Some techniques allow detection of the expression of unrelated genes in a single cell, such as T7 RNA polymerase amplification of mRNA and PCR after prior homopolymeric tailing of the first strand cDNA. However, neither of these approaches have been demonstrated to allow the analysis of more than a small number of genes and are not widely used.

The former is technically difficult, whilst the latter may be biased against long transcripts and often requires subsequent cloning of the amplified products.

Alternatively, a method for expression profiling in single cells using 3' end amplification PCR has been developed by Dixon *et al.* (1998, Nucleic acids research, vol. 26 (n°19): 4426-4431). This method comprises a first step wherein mRNA species present in a cell are reversed transcribed using a first heeled primer, thereby providing a population of first strand cDNA species and a second step wherein partial 3' end second cDNA strand populations are synthesized using a second heeled primer population.

Using this technique of amplification, the authors have succeeded in detecting, from a mRNA population contained in the cytoplasm of a single cell, the presence of poorly expressed transcripts in cholinergic interneurons such as the neurokinin type 1 receptor.

However, one of the drawbacks of this technique is that it does not allow the detection of more than 40 low abundance genes from a single cell. This technique generates large amounts of high molecular weight cDNA in gene specific PCR reactions. This not only reduces the sensitivity of the PCR assay but means that much of the amplified product may not be assayed for gene sequence.

SUMMARY OF THE INVENTION.

The inventors have developed sensitive methods for amplifying mRNA species present in a sample that allows the detection and cloning of one or several mRNA species of interest, particularly mRNA species which are initially present at a low copy number in a sample to be assayed.

For instance, when applying the new method of the invention to mRNA samples obtained from cholinergic neurones, the inventors have succeeded in detecting the expression of low abundance A1 receptor mRNA at levels 50 fold lower than those possible using previous methods. In addition, when applying the method of the invention to 2.5 ng of total RNA (equivalent to that contained in approximately 250 cells), specific gene sequences could be detected using one millionth of the final product.

The present invention also relates to methods for increasing the number of nucleotide sequences corresponding to the mRNA species present initially at a low copy number in a sample to be assayed.

In addition, this technology allows high throughput analysis systems, e.g. arrays or gene chips to be used to analyse gene expression on extremely small samples, including analysing the expression of genes in a single cell.

The invention also pertains to various technical means that are necessary to perform these methods, and particularly to oligonucleotide primers that are required to perform the methods of the invention.

Additionally, other objects of the invention consist of kits that are specially designed to perform the disclosed methods, particularly kits containing the oligonucleotide primers mentioned above.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Detection of gene specific sequences after amplification of cDNA derived from 100 pg of total RNA using the first embodiment of the method of the present invention.

Figure 2: Detection of gene specific sequences after amplification of cDNA derived from total RNA using the second (I)and third (II) embodiments of the method of the present invention.

Figure 3: Diagram to illustrate product priming/product repair after amplification of small amounts of cDNA using the first and second embodiment of the method of the present invention.

Figure 4: Detection of gene specific sequences after high stringency amplification of cDNA derived from 1000pg of total RNA using the third embodiment of the method of the present invention.

Figure 5: Detection of gene specific sequences after *in vitro* transcription of RNA from amplified cDNA derived from liver total RNA using the third embodiment of the method of the present invention.

Figure 6: Size distribution of the RNA produced after incubating the amplification products obtained according to the third embodiment of the present invention in the presence of T7 polymerase (complementary RNA, left) or T3 polymerase (sense RNA right).

Figure 7A: Visualisation of the amplification products obtained according to the third embodiment to step d) after gene specific amplification with primers specific for tubulin, RL3, Synaptotagmin 1 and A2A receptor.

Figure 7B: Visualisation of the amplification products obtained according to the third embodiment of the method that have been

transcribed *in vitro* into the corresponding sense RNA using T3 RNA polymerase, and then reverse transcribed prior to gene specific PCR.

Throughout the specification, the following terms are defined as follows:

Low amounts of mRNA is intended to designate the amount of mRNA present in a maximum of 1000 cells, 1 to 100 cells being preferred, considering that in general, there are between 1 and 100 copies of any given mRNA present in a given cell.

Increase the number of nucleotide sequences corresponding to the mRNA species present in a sample is intended to designate an increase in nucleotide sequence to obtain a number of copies which is sufficient to allow at least one of the following methods:

(i) detection of the sequence of interest with specific oligonucleotide probes;

(ii) amplification of the sequence of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector, or

(iv) In vitro RNA transcription, either for hybridization assays of for further reverse transcription optionally using unlabelled or labeled substrates followed by gene specific PCR or hybridization.

Sample: is intended to designate material which contains the mRNA which is to be analyzed. For example a cellular extract obtained from 1 to 1000 cells.

High molecular weight DNA is intended to designate any nucleic acid species which is outside the expected range of molecular weight observed for natural mRNA species. Preferably any nucleic acid sequence with a size above 5kb.

DETAILED DESCRIPTION OF THE INVENTION

Three different ways of amplifying low amounts of mRNA present in a sample have been found, each of these methods being described in detail hereafter. Thus, these, ways of amplifying allow in several instances to amplify all mRMA species which are present in the sample of interest. Also included is a detailed description of reagents and oligonucleotide primers required for amplifying low amounts of mRNA

FIRST EMBODIMENT OF THE INVENTION.

The first embodiment of the amplification method takes advantage of the generation high molecular weight DNA molecules are formed following amplification of the cDNA species obtained through reverse transcription of the initial mRNA species present in the sample. The inventors have found that these high molecular weight DNA molecules or bridged products may result from the formation of partially duplexed DNA molecules during the annealing step. These partially duplexed DNA molecules would contain partially complementary sequences that hybridize with one another in the low stringency hybridization conditions used, thus forming bridges between two structurally related or unrelated amplified cDNA molecules contained in the amplification mixture. Repetitive amplification cycles result in large nucleic acid molecules. Furthermore, the first embodiment makes use of the high molecular weight DNA produced by this process to analyze amplified species of interest.

The first embodiment makes use of these findings by providing a process which by favoring an increase in the production of these high molecular weight DNA molecules in particular, allows to amplify mRNA species present in a low quantity in a sample to be analyzed.

More precisely, there is disclosed a method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;

c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method is characterized in that it comprises the steps of:

d') increasing the proportion of high molecular weight DNA molecules,

e') using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules,

PRIMERS

The term " heeled " primer will be readily understood in the art to be a primer comprising a hybridizing region and a non-hybridizing region, wherein the non-hybridizing region represents the " heel " of the primer.

The first heeled primer is actually a population of individual primer species. The first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which does not hybridize with the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iii) a nucleotide which should not be thymidine (A, C or G); and

(iv) a variable sequence of 2-4 nucleotides in length.

The components described in iii and iv being capable of hybridizing to a mRNA molecule at the 5' end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population .

In a specific embodiment of the first heeled primer, the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotides sequence:

5'-(A or G or C)-N_{1-3}-, wherein N is a nucleotide selected from A, T, C or G.

In a preferably advantageous alternative, the first heeled primer may also comprise an RNA polymerase binding site, such as the T7, T3 or SP6 promoter, located between the oligo dT sequence and the heel.

The second heeled primer is also a population of individual primer species. When the first strand cDNA population is contacted with the second heeled primer population under appropriate hybridizing conditions, each cDNA species will hybridize with at least one second heeled primer, (partly because of the selection of nucleotide sequences amongst the second heeled primers), second cDNA strand synthesis then proceeds in a 5' to 3' direction from the hybridized second primer.

The second heeled primer population may comprise primers differing by up to five nucleotide bases (differing in the hybridizing region),

the second heeled primer population preferably comprising a number of primers in the range 1000 to 100,000 primers, more preferably in the range 1024 to 65536 primers. In order to achieve this, the primers of the second heeled primer population preferably each comprise a first variable sequence of nucleotides in the range of 4 to 7 nucleotides 3' to the heel and a second variable sequence of at least 5 nucleotides contiguous 3' therewith. As will be appreciated, where there are 5 random nucleotides (which is preferred) there will be 4^5 (i.e. 1024) possible pentamer sequences.

The second variable sequence of this primer may be selected by sequence analysis of known sequences so as to promote the ability of

the second heeled primer as a whole to hybridize to the transcribed cDNA species. Sequence analysis can be carried out through databases of DNA or RNA sequences. In particular, known sequences of the organism of interest are preferably consulted. The second variable sequence of nucleotides preferably comprises a number of nucleotides in the range 2 to 10 nucleotides. In a particularly preferred embodiment, the second variable sequence of nucleotides may comprise a number of nucleotides equivalent to the number of nucleotides in the first variable sequence of this primer.

The second variable nucleotide sequence of the second heeled primers may be constant throughout the population of these primers and it is selected so as to stabilize the primers and to ensure optimal efficiency of hybridization to the target first strand cDNA species.

In a preferred embodiment, the second heeled primer from the population of second primers preferably hybridizes on average once in every 1kb portion of first strand cDNA species. This has been found to produce optimal amplification of mRNA in a sample.

Particularly preferred second variable sequences of nucleotides in the second primers are:

5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3'

The non hybridizing heel regions of the first and second heeled primers are preferably selected so that they lack the ability to hybridize to mRNA or first strand cDNA. The heel regions, like the hybridizing or variable sequence regions of the second primers, are selected by analysis of known nucleotide sequences. In particularly preferred embodiments, the heel regions preferably comprise sequences absent from the mRNA species in the sample. However, the heel regions may simply comprise sequences absent from the genome of the organism from which the sample is taken. The heel regions preferably comprise a number of nucleotides in the range 15 to 22, more preferably 18 to 20 nucleotides.

Preferably, the heel sequences are chosen among nucleic acid sequences having a GC content of about 50%, or for example from about 43% to about 55% of the heel sequence.

A particularly preferred heel sequence of the second heeled primer population is the following nucleic acid sequence of SEQ ID N°1:

5'-CTGCATCTATCTAATGCTCC-3'

PROCESS

The particular temperatures, enzymes and reagents (other than the first heeled primer) used in the process of reverse transcription in step a) may be those already known in the art.

Preferably, step a) is performed at 37°C in the presence of a reverse transcriptase.

The frequency with which an individual second heeled primer population species hybridizes along a given length of nucleic acid may be adjusted by employing suitable hybridizing conditions. Preferably, the hybridization conditions are of limited stringency so enabling efficient hybridization of the first variable sequence to target cDNA. The degree of stringency and the number of contiguous random bases in the second heeled primers may be varied according to routine trial and error in order to achieve the desired frequency of hybridization of second heeled primer species along a given length of nucleic acid material.

Most preferably, the conditions for the hybridization between the second heeled primer and the first cDNA strands obtained at step a) are of low stringency.

In step b), synthesis of the second cDNA strands is performed in the presence of DNA polymerase, preferably a Taq polymerase, in a suitable elongation buffer solution.

Preferably, the amount of second heeled primers added to the buffer solution vary from 0.01 ng to 10 ng in the elongation reaction buffer solution.

Particularly, the annealing buffer may comprise a concentration of magnesium, generally up to 6 mM magnesium, preferably between 1.5

mM and 6 mM magnesium and most preferably about 4.5 mM magnesium.

In the case wherein the concentration of magnesium in the elongation buffer has been adjusted to 4.5 mM, the temperature of annealing between the second heeled primer and the first cDNA strands is of about 50°C and the elongation temperature in the presence of the suitable DNA polymerase is of about 72°C.

The cDNA molecules that are generated at the end of step b) are highly representative of the spectrum of mRNA molecules in a sample, as mRNA species of low abundance are reverse-transcribed to the same level of efficiency as more abundant mRNA species.

Step c):

The amplification reaction of step c) is performed with a pair of oligonucleotide primers that respectively comprise at least a portion of the heel sequence of the first and second heeled primers that are defined above.

The first primer of step c) is preferably the heel of the first heeled primer. The second primer of step c) is preferably the heel of the second heeled primer.

The second primer of step c) may be the same as the second heeled primer and this can be advantageous in reducing the number of reagents needed to perform the first embodiment

A further alternative is to use the second heeled primer as the sole primer.

Preferably, the amplification reaction of step c) is performed using low stringency hybridization conditions. For example, amplification reactions are performed in the presence of a concentration of magnesium generally up to 5 mM, preferably between 4 mM and 5 mM magnesium and most preferably of about 4.5 mM magnesium. With the latter magnesium concentration, each amplification cycle comprises a denaturing step at 92°C, an annealing step at 60°C and an elongation step at 72°C.

Advantageously, the amplification reaction of step c) comprises from 30 to 50 amplification cycles, and most preferably comprises about 40 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

In a first series of further process steps, the cDNA may be submitted to *in vitro* transcription either immediately after step c) if the appropriate concentration of cDNA is present in the sample or after further amplification, such as through step d'), steps d) and e) which is/are discussed in more detail below. In this context, it is essential that at least one of the primers used in step a), b) and/or c) comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance by being labeled during reverse transcription and hybridized to DNA arrays. Alternatively, cDNA generated in the presence or absence of a labeled substrate can be used in gene-specific PCR experiments.

Step d'):

In the preferred embodiment of step d'), step d') is a combination of the following steps d) and e).

Step d):

At the end of step c), the reaction mixture containing the population of amplified DNA molecules which include the "bridged products" can be diluted to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 x inferior to the cDNA concentration of the product of step c). Preferably the diluted cDNA concentration ranges between about 2 and 100 times less, and most preferably between 40 and 80 times less, than the initial cDNA concentration found in the reaction mixture obtained at the end of step c). This dilution step is essential for performing the further steps of the method as it results in the almost complete elimination of the primers initially added to the amplification mixture. The elimination of most of the primers, which are not part of the original gene sequence to be detected,

reduces the element of randomness which would be introduced in the further amplification steps. This element of randomness arises from the mis-hybridization occuring under the lower stringency conditions employed.

Step e):

Preferably step e consists of adding a thermoresistant DNA polymerase to the diluted cDNA solution of step d) and performing a further set of amplification reaction cycles without adding further primers.

Following dilution of DNA the amplification of step e) is performed without adding any primers to the diluted cDNA solution obtained at step d). Because no exogenous primers are added, the annealing step results in the hybridization between different amplified DNA molecules initially present in the diluted cDNA solution, which are then elongated before the resulting duplex elongated cDNA molecules are denatured at the end of each amplification cycle.

Without wishing to be bound by any particular theory, it appears that the "self priming" amplification of step e) also results in an increase of the number of bridged DNA molecules having a high molecular weight and therefore in an increase in the number of bridged but appropriately amplified genes from the sample.

Preferably, the amplification reaction of step e) is performed for a number of amplification cycles ranging from 20 to 40 amplification cycles, more preferably from 25 to 35 amplification cycles and is most preferably of about 30 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

Preferably, the amplification reaction of step e) is performed at hybridization conditions of low stringency, but with a greater stringency than the hybridization conditions used in the amplification reaction of step c). Typically, the magnesium concentration generally used is up to 4,5mM, preferably between 1.5 mM and 4.5 mM magnesium and most preferably about 3.5 mM. In these amplification conditions, each amplification cycle comprises the following steps of:

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75°C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles.

In a most preferred embodiment, the amplification reaction of step e) comprises a denaturation step at 92°C, an annealing step at a temperature comprised between 55°C and 72°C, for example 55°C, 60°C, 65°C or 72°C, and an elongation step at 72°C in the presence of a suitable DNA polymerase.

Step e'):

A preferred embodiment of step e') comprise a combination of the following steps f) and g).

Step f):

The amplification mixture which contains a population of amplified heterogeneous cDNA molecules is then submitted to a further step (step f) wherein the high molecular weight cDNA species, preferably those having a length of at least 4.5 kb, are separated.

Step g):

In a preferred embodiment step g) consists of confirming the presence of at least one nucleic acid sequence species contained in the high molecular weight cDNA separated at step f)

The high molecular weight cDNA species previously separated at step f) can readily be used, typically for detecting the presence of at least one nucleic acid sequence of interest.

Alternatively, the amplification method may comprise an additional amplification step following step f), which consists of

submitting at least a part of the high molecular weight DNA molecules separated at step f) to a further amplification reaction using a pair of primers, wherein a first primer comprises a portion of the first heel sequence and the second primer comprises a portion of the second heel sequence.

Step g) of the amplification method comprises anyone of the following methods:

(i) detection of the sequences of interest with specific oligonucleotide probes;

(ii) amplification of the sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

In part (iv) of step g), the resulting cDNA may also be submitted to *in vitro* transcription. In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and attached to DNA arrays for hybridization experiments or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting labeled cDNA can then be hybridized to a DNA array or used in gene-specific PCR experiments.

It is to be noted that the labeling of any of the reactants used in any one of the 3 embodiments of the invention, although optional, can be very useful in that it allows the skilled person to directly hybridize to a DNA array the products of the process of the present invention.

In conducting the series of experiments which lead to the first embodiment of the invention described above, the inventors came to the conclusion that even though the "bridged sequences" refered to previously contain useful and exploitable information on the genes present in the sample to be analyzed, it would be useful to reduce bridge formation in order to obtain individual gene sequences in better yields and which could then be analyzed more specifically. Thus a key element of embodiments II and III described below resides in preventing or at least reducing the formation of "bridge sequences" to the largest extent possible. Therefore, the methods of embodiments II and III are characterized in that they comprise a process step which allows either to prevent or to reduce the formation of "bridged sequences" following reverse transcription and amplification of the nucleic acid sequences present in the sample to be analyzed.

SECOND EMBODIMENT OF THE INVENTION

In the second embodiment of the amplification method, the generation of a large number of high molecular weight DNA molecules is prevented or reduced by inserting a nucleic acid sequence encoding a cleavage site, in particular a restriction endonuclease site, at least in the heel sequence of the second heeled primer

Consequently, another object of this invention consists of a method to increase the number of nucleic acid sequences corresponding to the mRNA species present in a low quantity in a sample, wherein said method comprises the steps of:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said first, and/or second heeled primer population optionally contains a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence;

c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;

d') cutting any large DNA molecules and preventing bridge formation in subsequent steps by suppressing the heel portions of at least one said first or second heeled primer

e') increasing the amount of long double strand products with sequences more 5' from the original mRNA.

Preferably, the synthesis of the first and second cDNA strands in steps a) and b) are performed under the same conditions defined for steps a) and b) of the first embodiment.

Step c):

Typically, the first amplification reaction of step c) of this second method is performed under low stringency hybridization conditions.

The low stringency hybridization conditions used at step c) increase the chances to elongate any sequence present initially in the sample containing the first and second cDNA strands population.

Preferably, the amplification reaction of step c) includes the following steps of:

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 95°C;

(ii) annealing the primers to the single stranded DNA molecules at a temperature comprised between 45°C and 65°C;

(iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C, preferably between 70°C and 75°C in the presence of a concentration of 4.5mM magnesium;

(iv) reiterating steps (i) to (iii) for the desired number of cycles.

In a most preferred embodiment, the amplification reaction of step c) includes a denaturation step at 92°C, an annealing step at 60°C and an elongation step at 72°C.

In another preferred embodiment, the amplification reaction step of the first and second cDNA strands comprises between 30 and 50

amplification cycles, more preferably between 35 and 45 amplification cycles, most preferably about 40 amplification cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

Step d'):

In a prefered embodiment of step d') consists of incubating the product obtained at step c with at least one restriction enzyme that specifically recognise the cleavage site in particular a rare restriction sites included in the primers.

Incubating the cDNA strands obtained at the end of the first amplification reaction step c) as shown in embodiment I with the corresponding cleavage agent such as a restriction endonuclease results in the cleavage of the high molecular weight cDNAs produced at this step. This prevents an increase in the number of the high molecular weight cDNA species that would have been generated during the second amplification reaction step e) (of embodiment 1). This step also serves to remove DNA sequences which can compete with the gene specific primers used later on in gene specific PCR. Furthermore, cutting and removing the resulting products increases the efficiency of *in vitro* RNA transcription from amplified DNA.

According to a specific variant of the second embodiment, the DNA molecules amplified in step c) are incubated in step d) with two restriction enzymes recognizing the rare cleavage site in particular a rare restriction sites of the first and the second heeled primers.

As a preferred variant, the rare cleavage site in particular a rare restriction site sequence present at the 3' end of the heel of at least the second heeled primer is a sequence recognized by the MluI restriction enzyme.

Restriction cleavage in step d) is performed using the conventional restriction cleavage techniques well known to those skilled in the art such as described for example in Sambrook, J., Fritsch, E.F. , and T. Maniatis, (1989, Molecular cloning: A laboratory Manual . 2nd ed.; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Step e'):
In a preferred embodiment step e') is a combination of the following steps e) and f).

Step e):

The cleavage step can be followed by step e) wherein the product of step d) is diluted by an order of magnitude of 2 to 100 times in order to almost completely eliminate the primers used for the first amplification of step d). This favours the phenomenon of self priming (as shown in figure 3) in the further set of amplification reaction cycles of step f).

As shown in Figure 3, in the absence of added primers, and after strand separation at 92°C, short strands (e.g. strand B) will be able to serve as primers on complementary longer strands (e.g. strand A), resulting in an increase in the amount of double stranded gene specific sequence 5' to the reverse transcription primer site. Note that removal of the second strand primer heel, facilitates this process since the heel primer sequence is not complementary to the gene sequence of strand A. Thick bars on the right side of the diagram represent the reverse transcription primer heel, while thick bars on the left represent the second strand cDNA primer heel.

More preferably, the product of step d) is diluted 10 to 80 times and is most preferably diluted about 40 times.

Step f):

In a preferred embodiment step f) consists of adding a thermoresistant DNA polymerase to the diluted sample of step e) and performing a further set of amplification cycles without adding further nucleic acid primer.

Subsequently to the dilutions of step e), a further set of amplification cycles without adding further nucleic acid primers can advantageously be performed in a step f).

In a preferred embodiment, each amplification cycle of step f) comprises the following steps of:

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a thermo-resistant DNA polymerase at a temperature comprised between 65°C and 75°C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles.

In a preferred variant of this embodiment, the denaturation step is performed at 92°C, the annealing step is performed at 55°C, 60°C, 65°C or 72°C and the elongation step is performed at 72°C.

In another preferred variant, the amplification cycles carried out in step f) compris between 10 and 40 cycles, more preferably between 25 and 35 cycles and most preferably about 30 cycles. However, other cycle numbers could be envisaged. One parameter of the optimum number of cycles required is determined by the polymerase used.

The set of amplification cycles carried out in step f) is preferably performed under low stringency hybridization conditions, the presence of about 3.5 mM magnesium.

In a specific variant of the second embodiment, the method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are discarded from the reaction mixture.

Step g):

Furthermore, step g) can comprises one or several of the following methods:

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled substrate followed by gene specific PCR or hybridization.

It is important to note that the amplified cDNA obtained from the reverse transcription and amplification of the nucleic acid sequences of the sample may be submitted to *in vitro* transcription either immediately after step d) if the appropriate concentration of cDNA is present in the sample or after further amplification such as through steps e), f) and g). In this context, it is essential that at least one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and hybridized to DNA arrays or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting labeled cDNA can then be hybridized to a DNA array or used in gene-specific PCR experiments.

It is to be noted that the labeling of any of the reactants used in the above method, although optional, can be very useful in that it allows the skilled person to directly hybridize on a DNA array the products of the process of the present invention.

PRIMERS

It is to be noted that although the presence of a cleavage site is an important feature of the second embodiment, this cleavage site can be located either on the first heeled primer, on the second heeled primer, on both primers or on primers used in step c). However, it is necessary that at least one primer comprise a cleavage site.

A) First heeled primers

For performing this second embodiment of the method, the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence optionally includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located at its 3' end;

(ii) An optional but preferably present RNA polymerase promoter sequence,

(iii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iv) a variable sequence of 2-4 nucleotides in length. This sequence is able to hybridize to a mRNA molecule at the 5' end of the poly-A tail thereof, wherein substantially every possible variable sequence combination is found in said first heeled primer population.

Typically, the variable sequence of 2-4 nucleotides in length of the first heeled primer is selected among the following variable nucleotide sequence: 5'-(A or G or C)-N_{1-3}-3', wherein N is a nucleotide selected from A, T, C or G.

In a specific embodiment of this second amplification method, the first heeled primer may therefore also include the sequence of a rare cleavagecleavage site in particular a rare restriction site. **cleavage site in particular a rare restriction sitecleavage site in particular a rare restriction sitecleavage site in particular a rare restriction site**

The sequence of a rare cleavage site in particular a rare restriction site is usually located within or close to the 3' end of its heel sequence. In the context of the present invention, 'close to the 3' end' is intended to designate that the cleavage site in particular a rare restriction site is to be positioned so as to leave as few bases as possible from the heel after restriction enzyme cutting so as to avoid subsequent aberrant hybridization between the remaining and generated sequences.

Preferably, the cleavage site in particular a rare restriction site is selected from the so-called 'rare cutter' the group which comprises, for example, Not1 BsshII, NarI, MluI, NruI and NaeI.

Preferably, the cleavage site in particular a rare restriction site of the first heeled primer is identical to the cleavage site in particular a rare restriction site of the second heeled primer.

Alternatively, the cleavage site in particular a rare restriction site of said first heeled primer may be different from the cleavage site in particular a rare restriction site of the second heeled primer.

B) Second heeled primer

The second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) wherein the heel optionally sequence includes the nucleotide sequence of a rare cleavage site, in particular a rarely used site, located at its 3' end;

(ii) An optional but preferably present RNA polymerase promoter sequence,

(iii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population; and

(iv) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

Preferably, the cleavage site is located within the heel sequence. More preferably, the cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence of the second heeled primer population, and is a rarely occurring cleavage site in particular a rare restriction site in the genome from which the initial mRNAs are expressed.

Most preferably, the cleavage site in particular a rare restriction site is selected among the cleavage site in particular a rare restriction sites that are found less than once every 20 kb in the genome of the organism from which the cDNA amplification is sought.

In mammals, and more particularly in the rat, such rare cleavage site in particular a rare restriction site is selected from the so-called 'rare cutter' group of cleavage site in particular a rare restriction sites which comprises, for example, Not1, BsshII, NarI, MluI, NruI and NaeI.

Preferably, the heel sequence of the second heeled primer consists of the nucleotide sequence of SEQ ID N°2, CTGCATCTATCTAGTACGCGT.

In a preferred embodiment of the second heeled sequence, said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

KITS

The invention further relates to a kits for the amplification of the mRNA species present in a sample, wherein said kit compris:

(i) a first heeled primer population; and

(ii) a second heeled primer population, as defined above for either embodiments I or II.

The invention also pertains to a kit for the amplification of the mRNA species presenting a sample wherein said kit further comprises:

(iii) a first primer consisting of the heel sequence of the first heeled primer;

(iv) a second primer consisting of the heel sequence of the second heeled primer.

In a specific embodiment, the mRNA amplification kit may further comprise one or more restriction enzymes that recognize the rare cleavage site in particular a rare restriction site sequence that may be present in the heel sequence of the heeled primers.

In another preferred embodiment, said mRNA amplification kit may further comprise a restriction enzyme that recognize the rare cleavage site in particular a rare restriction site sequence that may be present in the heel sequence of the first heeled primer.

In yet another preferred embodiment, the kit may also include a suitable RNA polymerase.

THIRD EMBODIMENT OF THE INVENTION

According to this third amplification method of the invention, higher stringency hybridization conditions are used to prevent the generation of bridged nucleic acids In the second embodiment, bridges were cleaved by using a primers containing a rare cleavage site in its heel sequence. This allowed cleavage by a cleaving agent, preferably a restriction endonuclease, of the long cDNA molecules formed during the first set of amplification cycles.

The conditions for performing the third embodiment have been chosen to further reduce bridge formation. Such conditions include for example, (apart from the optional presence of a restriction site on the primer) increasing the stringency of hybridization with respect to the stringency used in embodiment 1 or 2, for example by optimizing buffer conditions which will in turn decrease mis-hybridizations and/or increasing the GC content of the primers which allows elevated annealing temperatures, which also reduces mis-hybridization and increases the distance between hybridized paired oligonucleotides.

These higher stringency hybridization conditions may be met according to two alternatives of this third embodiment which are described hereunder.

The method of embodiment III is a method to increase the number of nucleotide sequences corresponding to an mRNA species present in a sample in a low quantity comprising the steps of:

a) reverse transcribing the mRNA species using first heeled primer population to provide first strand cDNA species;

b) synthesizing second cDNA strands using a second heeled primer population;

c) amplifying said second cDNA strands resulting from step b) over a number of amplification cycles in order to generate second cDNA

strands comprising heels at both ends and increasing the number of second cDNA strands corresponding to long mRNA species present initially in the sample to be assayed;

d) amplifying the DNA molecules resulting from step c) under hybridization conditions which are of a higher stringency than those of step c) and which enable reduction of the synthesis of high molecular weight cDNA molecules; and

e) recovering the population of DNA molecules obtained at step d).

PROCESS

Step a)

Preferably, step a) is the same as for the first and the second embodiments, except that the first heeled primer population comprises a heel sequence that must have a GC content ranging from 60% to 80% and which is most preferably of about 75%, in order to permit an increase in the stringency of the hybridization conditions used in the first set of amplification cycles of step c), thereby reducing the formation of nucleic acid bridges inside the amplified cDNA molecule.

Step b)

Step b) of synthesis of the second cDNA strands is also performed at hybridization conditions of a higher stringency than the hybridization conditions used in step b) as described for the first and the second embodimentsof the invention.

Step b) is preferably performed at high stringency conditions.

A preferred example of high stringency conditions is as follows:

synthesizing second cDNA strands using a second heeled primer population preferably at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions;

(i) adding the primers to the cDNA product obtained at step a);

(ii) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 95°C preferablyfor a period of time which ranges from 2 to 5 min,;

(iii) adding a thermoresistant DNA polymerase and optionally a thermoresistant proof reading enzyme to the mixture obtained at step (ii);

(iv) Optionally maintaining the temperature of the mixture at approximately 94°C during a period of time up to 5 min

(v) annealing the healed primers to said single stranded DNA at a temperature comprised between 40°C and 72 °C;

(vi) elongating the annealed DNA molecules at a temperature comprised between 60°C and 75 °C;

Hgh stringency hybridization conditions are notably obtained according to the specific structural features of the second heeled primer used.

Preferably, step b) is performed in the presence of a magnesium concentration generally up to 5 mM magnesium, preferably between 3 and 5mM magnesium, most preferably of 3.5 mM.

The thermoresistant DNA polymerase is preferably added at step (b) in an amount that rangesfrom 3U to 5U, most preferably 4.5U DNA polymerase in a volume of 1µl. Optionally step (b) is performed in the presence of both a thermoresistant DNA polymerase and a proof reading enzyme.

This enzyme being added at the same time as the DNA polymerase and in an amount which preferably ranges from 0.1 U to 0.5U, most preferably 0.25 U and is admixed with the DNA polymerase in a volume of 1µl.

With regard to step (b) (iv), it is performed for a period of time preferably from 1 min to 3 min, most preferably during 2 min.

With regard to step (b) (v), it is preferably performed at a temperature of 50°C for a period of time generally up to10 min, preferably between 4 min and 10 min, more preferably 6 min and 8 min and most preferably 7.5 min.

With regard to, step (b) (vi), it is preferably performed at a temperature of 72°C for a period of time comprised between 1 min and 5 min, preferably between 2 min and 4 min and most preferably during 2.5 min.

High amounts of second heeled primer population used in steps b) and c) increases the probability of annealing of at least one primer to

every sequence contained in the first cDNA strands previously synthesized at step a).

Step c)

Although the inventors do not wish to be bound to any particular theory, it appears that through the successive cycles of the amplification reaction of step c), the sequences that contain at their 5'-end the heel sequence of the second heeled primer will anneal to the first strand cDNA in order to generate second cDNA strands comprising heels at both ends. These repetitive cycles of step c) increase the chances of detecting every first strand present in the reaction mixture of step b).

As a consequence, complementary sequences to the 5'end of the gene sequences present in the sample are generated.

In a first preferred embodiment of step c), no denaturation is performed during the successive cycles. This situation permits an increased efficiency in long sequence elongation by allowing the polymerase to work through several cycles without removing the primers or short DNA sequences hybridized to the first strand. Furthermore, the inventors believe that the polymerase may actually displace small sequences hybridized to the first strand during the elongation to favor the extension of longer sequences already hybridized to this first strand.

In a second preferred embodiment of step c), denaturation is performed under mild temperature conditions, preferably in the range of 80 to 85°C. In these conditions, small mismatched sequences, generally of less than 50 bp in length and preferably at least the second heel primers, are removed from their hybridization site on the first strand and are thus available for further priming in subsequent reactions. The further increases the yield in the amplification of the second strand cDNA.

In a third preferred embodiment of step c), denaturation is performed under usual temperature conditions, preferably in the range of 85 to 95°C.

The first and second cDNA strands previously synthesized are preferably amplified over a number of amplification cycles with the

second heeled primer at a concentration ranging between 0.02 to 200 ng per reaction, preferably 0.02 to 100 ng, more preferably between 1 and 50 ng and most preferably between 1 and 10 ng.

The preferred amount of second heeled primer population used in step c) increases the probability of annealing of at least one primer to every sequence contained in the first and second cDNA strands previously synthesized at steps a) and b).

Preferably, a population of approximately 4^{17} primers is used during the amplification reaction of step c). This increases the chances of each gene sequence annealing to at least one primer.

The availability of each primer is increased by multiplying the number of cycles in the amplification reaction of step c).Preferably, step (c) is performed in the presence of 4.5 mM magnesium between 30 and 50 amplification cycles, more preferably between 35 and 45 amplification cycles and most preferably about 40 amplification cycles.

Advantageously, the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

The presence of a thermoresistant proof reading enzyme in the amplification buffer allows a significant increase in the quality of the sequences that are synthesized during the elongation step of each amplification reaction cycle.

Most preferably, step c) comprises a step wherein the heeled primers are elongated in the presence of the DNA polymerase and optionally the proof reading enzyme at a temperature ranging between 40 and 72°C.

Optionally, an annealing step may be performed between the denaturation step and the elongation step, at 40°C, a temperature wherein the DNA polymerase is almost prevented to synthesize DNA.

Preferably, step c) comprises an elongation step wherein the annealed DNA molecules are elongated at a temperature comprised between 65 and 75°C in the presence of a thermoresistant DNA polymerase.

In a preferred alternative variant, step c) can comprise the step of amplifying second cDNA strands resulting from step b) over a

number of amplification cycles with said second heeled primer preferably at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions;

(i) optionally obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C, in the presence of a thermoresistant DNA polymerase,

(ii) annealing the second strand primers to the first strand (single stranded) DNA molecules at a temperature comprised between 40°C and 72°C; preferably between 40°C and 60°C,

(iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C optionally in the presence of a thermoresistant DNA polymerase;

(iv) repeating steps (ii) and (iii) (with (i) as an option) for the desired number of cycles.

Preferably, steps (c) (ii) to (iv) are repeated for 10 to 60 cycles, preferably from 20 to 50 cycles and most preferably about 20 or about 40 cycles.

In a preferred variant of step (c), a population of second heeled primers is added at step (b).

Preferably, step c) is performed in the presence of a magnesium concentration up to 5 mM and most preferably of 3.5 mM.

In a most preferred variant, step (c) is performed in the presence of both a thermoresistant DNA polymerase and a proof reading enzyme.

Advantageously, the proof reading enzyme is added at the same time as the DNA polymerase and in an amount which ranges from 0.1 U to 0.5U, most preferably 0.25 U and is admixed with the DNA polymerase in the volume of 1µl.

Step d)

The third embodiment further comprises a second set of amplification cycles which are performed at step d) (referred to above) under more stringent hybridization conditions. This serves to amplify all the cDNAs bearing heel sequences with minimum bridge formation and,

due to the high stringency conditions used, at high efficiency, thus increasing the yield of sequences initially present in the sample (when compared to embodiments 1 and 2 above).

Preferably, each amplification reaction cycle of step d) comprises the following steps of:

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C;

(ii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75°C;

(iii) reiterating steps (i) and (ii) for the desired number of reaction cycles.

Preferably, the amplification reaction of step d) is performed in the presence of 2.5mM magnesium, between 30 and 50 amplification cycles, more preferably between 35 and 45 amplification cycles and most preferably about 40 amplification cycles. However, other magnesium concentrations could be used, depending on the choice of polymerase.

The denaturation temperature is preferably 95°C and the elongation temperature is preferably 72°C.

Most preferably, the annealing and elongation step is performed during a period of time which is sufficient for maximizing the annealing of the primers to the single stranded cDNA molecules.

Typically, such annealing and elongation step ranges from 2.5 to 3.5 minutes and is most preferably about 3 minutes.

In another preferred embodiment, step d) can be performed as follows:

amplifying said first and second cDNA strands resulting from step c) over a number of amplification cycles with primers selected from the group consisting of (1) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (2) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the

hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers preferably ranges between 0.02 and 500 ng per reaction in the following conditions:

(i) adding the primers to the cDNA product obtained at step c);

(ii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C;

(iii) adding a thermoresistant DNA polymerase;

(iv) maintaining the temperature at a range from 80°C to 95°C for a period of time preferably comprised between 5 sec to 15 min;

(v) annealing the primers to the said single stranded DNA and elongating the annealed DNA molecules at a temperature comprised between 65°C and 75 °C;

(vi) carrying out steps (iv) and (v) for a desired number of cycles.

With regard to step d), in a preferred embodiment, step d) (iv) is performed at a temperature of 94°C. According to these conditions, the reaction mixture contains essentially the single stranded cDNA products obtained at step c), the amplification primers as well as the thermoresistant DNA polymerase which is not active at this high temperature.

Preferably, at the first occurrence of step d) (iv) in the amplification method, the temperature is maintained in the range from 80°C to 95°C, most preferably 94°C, for a period of time up to 3 minutes, most preferably 2 minutes. For the further occurrences of step d) (iv),, then the temperature ranges from 80°C to 95°C, most preferably 94°C, and is maintained up to 60 sec, most preferably 20 sec.

At step d) (v) the primers are annealed to the single stranded DNA molecules at a temperature wherein the thermoresistant DNA polymerase is able to elongate the primers using the cDNA molecules as templates.

Preferably, step d) (v) is performed at a temperature comprised between 68°C and 74°C, most preferably 72°C.

In a preferred embodiment, step d) (v) is performed during a period of time comprised between 1 min and 10 min, most preferably 5 min.

In a preferred embodiment, the last occurrence of step d) (v),, is performed during a period of time comprised between 10 and 60 min, preferably between 25 and 40 min, most preferably during 35 min.

Step d), (vi) preferably comprises between 10 and 50 amplification cycles.

Preferably, the amplification reaction of step d) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme, also step d) is preferably performed in the presence of a concentration of magnesium comprised between 2 and 5 mM.

In a preferred expression of the second alternative of the third embodiment, the respective concentration of primers at step (d) ranges from 0.02 to 500 ng.

Step d) which includes steps (i) to (vi) as described above, is preferably performed when the initial sample contains a large amount of mRNA, such as for example an amount of mRNA corresponding to the whole mRNA that can be found after extraction from about 100 cells (e.g. 100 mammalian cells).

In this situation step d) is preferably performed in the presence of a concentration of magnesium up to 3 mM, most preferably 2 mM.

If the initial sample contained a small amount of mRNA, such as the amount of mRNA that may be found after extraction from 1 to 10 cells, step d) above will preferably comprise further steps of amplifying the products obtained at step d) (vi) of the second alternative.

In this situation, step d) further comprises the steps of amplifying the DNA molecules obtained at step d) (vi) over a number of amplification cycles with primers selected from the group consisting of (1) a primer comprising a portion of the heel sequence of the first heeled

primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (2) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers preferably ranges between 0.02 and 200 ng per reaction in the following conditions:

(vii) obtaining single stranded DNA molecule at a temperature comprised between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature comprised between 65°C and 75°C;

(x) carrying out steps (vii) and (ix) for a desired number of cycles.

With regard to the magnesium concentration used at step (d), these are preferably of (a) 2.5 mM magnesium at steps (d) (i) to (vi) and (b) 2mM magnesium at steps (d) (vii) to (x).

As for amplification reaction steps (d) (i) to (vi) these are performed with a respective concentration of primers which ranges from 0.02 to 90 ng, preferably from 10 to 50 ng, most preferably about 30 ng, then step (d) (vii) to (x) is performed with a respective concentration of primers that ranges from 50 ng to 300 ng, preferably from 65 ng to 200 ng and most preferably about 100 ng.

Thus, when the initial sample contained a small amount of mRNA species, step (d) (i) to (x) are preferably performed, using a total amount of primers ranging from 0.02 to 500 ng, preferably from 60 to 300 ng and most preferably about 130 ng.

Preferably, step (d) (x) of the second alternative of the third amplification method described above comprises between 20 and 60 amplification cycles, preferably between 30 and 50 amplification cycles and most preferably about 40 amplification cycles.

As it is detailed above, when step (d) is performed by carrying out steps (i) to (x), the first set of amplification reactions of steps (i) to (vi)

is performed with a smaller amount of primers than when step (d) is performed by carrying out solely steps (i) to (vi). This lower amount of primers added at step (i) in this specific situation will permit a reduction in the level of mis-hybridizations in the first set of amplification reactions. Thus the products obtained at step d) (vi) are fully representative of the mRNA species initially contained in the sample. According to this variant of the embodiment, the second set of amplification reactions, namely steps (d) (vii) to (x) will increase the amount of material initially amplified at steps (d) (i) to (vi).

According to the method above, the amplification reaction steps (d) (viii) to (x) are preferably performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

Preferably, the amplification reactions steps (d) (viii) to (x) are performed in the presence of a concentration of magnesium up to 4 mM, preferably between 1.6 and 2.5 mM and most preferably at a magnesium concentration of 2.0 mM.

In a preferred embodiment, the respective concentration of primers used at steps (d) (vii) to (x) ranges from 10 to 500 ng, and most preferably from 30 to 300 ng.

Step e)

With regard to step e) (referred to above) the method may comprise a further step wherein the DNA molecules obtained at step e) having a length of less than 50 bp are discarded from the reaction mixture.

Step e')

In an advantageous alternative variant of this embodiment, step d) is followed by the following steps :

i) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes a restriction site included in the heeled sequence of the first and/or second heeled primer; and/or

ii) diluting the product of step i) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step i); and

adding a thermoresistant DNA polymerase to the diluted sample and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and/or

iii) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step i) and/or ii).

In the above variants and especially in the variant wherein step i) is performed, the primers preferably each comprise at least one rare restriction site.

These variants may also comprise a further step wherein the DNA molecules obtained at step i) having a length of less than 50 bp are discarded from the reaction mixture.

Preferably , the number of amplification reaction cycles performed in step ii) is comprised between 20 and 40, more preferably between 25 and 40 and most preferably between 30 and 40.

As for step iii), it can comprise anyone of the following methods:

(i) detection of sequences of interest with specific oligonucleotides probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers; and

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

The conditions for performing step i) to iii) of this preferred variant are the same as those described for performing steps d) to g) of the first and second embodiments.

Step f)

One specific variant of this embodiment comprises the additional step of:

f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e) or e').

Step f) comprises any one of the following methods:

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) *in vitro* RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled substrate followed by gene specific PCR or hybridization.

It is important to note that, the resulting cDNA may be submitted to *in vitro* transcription, either immediately after step c) if the appropriate concentration of cDNA is present in the sample or after further amplification such as through step d) or through optional steps of e') described above. In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and hybridized to DNA arrays or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting product can then be hybridized to a DNA array or used in gene-specific PCR experiments. If unlabelled the products can be attached to a microarray base and be hybridized to labeled oligonucleotides.

It is to be noted that the labeling of any of the reactants used in this embodiment of the invention, although optional, can be very useful in that it allows the skilled person to directly attach or hybridize to a DNA array the products of the process of the present invention.

Alternatively, after step e) or e' RNA transcription can be carried out by first optionally removing low molecular weight DNA, including heel primers, to provide a 'cleaner' environment for subsequent RNA

polymerase reactions to take place. This 'cleaning up' also allows the skilled person to change the buffer solution to a buffer that would be more appropriate for subsequent RNA polymerase reactions.

It is important to note that the resulting cDNA may be submitted to *in vitro* transcription It should be noted that inclusion of the RNA polymerase promoter in the primer allows synthesis of complementary RNA, suitable for hybridising to Gene Chips or arrays bearing sense gene specific oligonucleotides, or for subsequent reverse transcription and hybridising of the resultant cDNA to antisense gene specific oligonucleotides. In contrast, inclusion of the RNA polymerase promoter in the second heeled primer allows synthesis of sense RNA, suitable for hybridising to arrays bearing antisense oligonucleotides, or for subsequent reverse transcription and hybridization of the resultant cDNA to GeneChips or arrays bearing sense gene specfic oligonucleotides.
In this context, it is essential that one of the primers comprises a RNA polymerase binding site such as the T7 RNA polymerase promoter. The RNA generated can then be subjected to further process steps, for instance either by being labeled and hybrdized to DNA on arrays or by being reverse transcribed, optionally using a fluorescent, radioactive or otherwise labeled substrate, to generate labeled cDNA strands. The resulting product can then be hybridised to a DNA array, or attached to a support (e.g. glass, nylon, silcon etc) for subsequent hybridisation with other nucleic acids, or used in gene-specific PCR experiments.

.

PRIMERS

The specific structural features of the primers used in this embodiment (first and second heeled primers, primers used in step c) include an increase in GC content as compared to primers of embodiments I and II.

Thus, the primers used in this embodiment comprise a heel sequence having a GC content ranging from 60% to 80%, most preferably of about 75%. This increase in the GC content permits an increase in the stringency of the hybridization conditions used in the first

set of amplification cycles of step c), thereby preventing the generation of nucleic acid bridges inside the amplified cDNA molecules and thus preventing the synthesis of the high molecular weight cDNA species observed during step c) of the first and second embodiments.

In a preferred embodiment, the above primers comprise at least one cleavage site in their heel sequences or at the 3' end of their heel sequence.

In another aspect of the third embodiment of the present invention, the first heeled primer population consists of a population of nucleic acids comprising, from 5' end to 3' end:

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the first strand cDNA nor the mRNA molecules initially present in the sample;

(ii) An option but preferably present RNA polymerase promoter site;

(iii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iv) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

According to this specific embodiment of the method, the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N-$_{1-3}$, wherein N is a nucleotide selected from A, T, C or G.

Preferably, the first heeled primer includes the sequence of a rare restriction site which may be located at any position within the heel sequence and preferably at the 5' end or at the 3'end of the heel sequence of said first heeled primer.

Preferably, the oligo dT sequence has a length comprised between 20 and 35, more preferably between 25 and 35 and is most preferably of about 30 nucleotides in length.

In a preferred variant, the variable sequence of 2 to 4 nucleotides of the first heeled primer is selected among the following variable nucleotide sequences: 5'-(A or G or C)-N_{1-3}-3', wherein N is a nucleotide selected from A, T, C or G.

As already described, the GC content of the heel sequence of the first heeled primer is comprised between 50 and 80%, more preferably between 60 and 80% and is most preferably of about 75% . The high GC content of the heel sequence of the first heeled primer allows a good annealing of said primer to the corresponding complementary sequence, even at the medium stringency hybridization conditions that are used notably at step d) of the present third cDNA amplification.

In another aspect of the third embodiment, the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end:

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a); and

(ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

Preferably, the heel sequence of said second heeled primer comprises the sequence of a rare restriction site, which may be located at any location within the heel sequence, but is preferably located at the 3'end or at the 5'end of the heel sequence of said second heeled primer.

In a specific embodiment, the heel sequence of the second heeled primer ranges from 25 to 35 nucleotides in length.

In another specific variant, the heel sequence of the second heeled primer ranges from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

In a further specific variant, the heel sequence of the second heeled primer ranges from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site located at the 3' end of the heel sequence.

The first variable sequence of the second heeled primer population has preferably 15 to 20 nucleotides in length and is most preferably of about 17 nucleotides in length. The first variable sequence of the second heeled primer population is longer the first variable sequence of the second heeled primer used to perform the first and second embodiments described above and are thus suitable to stabilize every second heeled primer of the population to its corresponding complementary DNA sequence during the annealing and the elongation step of the first and second set of amplifications cycles of steps c) and d).

A longer first variable sequence for stabilizing the primers belonging to the second heeled primer population was required, particularly due to the greater length of the heel sequence, which is preferably comprised between 25 and 30 nucleotides in length and is most preferably of about 27 nucleotides in length.

In a first preferred embodiment of the second heeled primer population, each nucleic acid sequence also comprises a second variable nucleotide sequence preferably selected according to the criteria set forth in the first embodiment. Preferably, the second variable sequence of the second heeled primer is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of second said variable sequence is found in said second heeled primer population.

In a preferred variant, the heel sequence has 28 nucleotides in length.

In a preferred variant of the second heeled primer population for performing the third embodiment, said second heeled primer population comprises a heel sequence of 25 to 30 nucleotides in length, more preferably about 28 nucleotides in length and having a GC content comprised between 50 and 70%, more preferably between 60 and 70% and is most preferably of about 68%.

In one specific variant of the second alternative of the third embodiment, the heel sequences of the first heeled primer and the second heeled primer are identical.

Alternatively, the heel sequences of the first heeled primer and the second heeled primer share a sequence of at least 15 consecutive nucleotides, preferably at least 20 or 25 consecutive nucleotides.

According to this specific embodiment, the cDNA strands present in the mixture obtained at the end of step (c) of the present method comprise a sequence in their 5' end of at least 15 nucleotides which are complementary to a sequence comprised in their 3' end. In this context, second cDNA strands of a short nucleic acid length that are regenerated during step c) have a high tendency to self-anneal and thus be no longer available for the set(s) of amplification reactions of step (d). Accordingly, the first and second cDNA strands that are amplified when carrying out step (d) of the present method are mainly large cDNA molecules, including cDNA molecules comprising a sequence which is identical or which is complementary to the full length mRNA species initially present in the sample.

The heel sequences of the first and second heeled primers preferably comprised the sequence of a rare restriction site located at the 3'-end or at the 5'end of their respective heel sequence, as well as a RNA polymerase binding site, preferably located downstream from the restriction site.

In a first variant, the restriction site sequence of the first heeled primer is identical to the restriction site sequence present in the heel of the second heeled primer.

In a second variant, the restriction site sequence of the first heeled primer is different from the restriction site sequence present in the heel of the second heeled primer.

Advantageously, the restriction site sequence included in the heel sequence of the first heeled primer or the second heeled primer is selected from the group of so-called 'rare cutters' which comprises for example Not1, BsshII, NarI, MluI, NruI and NaeI.

KITS

The present invention further relates to kits for the amplification of the mRNA species present in a sample, said kit being specifically designed for performing the third cDNA amplification method described above.

Thus, another object of the invention consists of a kit for the amplification of the mRNA species present in a sample, wherein said kit comprises:

(i) a first heeled primer population; and

(ii) a second heeled primer population,

the first and second heeled primer populations being defined above.

Said amplification kits may further comprises:

(iii) a first primer consisting of at least a portion of the heel sequence of the first heeled primer; and

(iv) a second primer consisting of at least a portion of the heel sequence of the second heeled primer.

In a specific embodiment of the kit above, the heel sequences of the first heeled primer and of the second heeled primers are identical or alternatively share a common sequence of at least 15, preferably at least 20, most preferably at least 25 consecutive nucleotides in length.

In a specific embodiment, said amplification kit may further comprises a restriction enzyme that recognizes the rare restriction site sequence present in the heeled sequence of the second heeled primer.

In another specific embodiment, said amplification kit may further comprises a restriction enzyme that recognizes the rare restriction site sequence present in the heeled sequence of the first heeled primer.

In yet another embodiment, the kit further comprises a suitable RNA polymerase.

The three mRNA amplification methods of the invention make it possible to amplify large numbers of samples easily and with high sensitivity.

The ability to analyze subsequently the expression of many genes of annealed sequences, both at high and low abundance, in samples taken from as little as a single cell, potentially allows it to be used in high throughput screening systems.

Various kinds of mRNA containing samples may be used as starting materials for performing the cDNA amplification methods of the invention, such as the whole content of a cell cytoplasm or even a portion of the cell cytoplasm such a portion of cytoplasm of neuronal cells and also mRNA molecules extracted from a desired tissue.

cDNA molecules obtained at the end of any one of the three cDNA amplification methods described above can be used for many purposes including:

a) cloning and production of cDNA libraries from small amounts of tissue;

b) sequencing analysis of gene expression in small tissue samples;

c) subtracting the amplified product from two different samples and analysing genes differentially expressed between them such as described by Diatchenko et al. (1996, Proc. Natl. Acad. Sci. USA, vol. 93: 6025-6030), and then by cloning and sequencing the sequences expressed only in one or several tissues;

d) transcribing mRNA using labelled precursors for use on hybridisation arrays, such as described by Duggan , D. J. et al., (1999, Nature Genetics, vol.21: S10-S14);

e) transcribing RNA and reverse transcribing in the presence of labelled precursors for use on hybridising arrays;

f) labelling the cDNA obtained by anyone of the cDNA amplification methods of the invention during the amplification for use on hybridization arrays;

g) diagnosis of aberrant gene expression in small tissue samples from humans, animals and plants.

h) analysis of the effects of drugs and other agents (e.g. infectious agents, carcinogens) on gene expression in vivo and *in vitro*.

In the latter case, small tissue samples or cultured cells may be used. In the former case, small tissue samples can be taken from living organisms without any disadvantages since only very small samples are needed;

i) analysis of gene expression in single cells using anyone of the cDNA amplification methods described above.

j) amplification of full length RNA samples from single cells and small samples, for subsequent library making or expression in suitable expression systems.

Preferred embodiments of the invention will now be illustrated, but not limited to, the examples presented hereafter.

EXAMPLE I - Rat brain mRNA amplification using the first embodiment.

mRNA isolated from whole rat brain was reverse transcribed. cDNA derived from 100 pg total RNA (equivalent to the RNA content of between 5 and 10 cells) was amplified according to the first embodiment. After reverse transcription only, gene specific PCR assays were positive when cDNA derived from more than 10 pg of total RNA was used in each assay, as shown in figure 1A. After the first amplification step (c), the majority of the genes were detected using 2.5% of the amplified product in each gene specific assay (i.e. each sample contained material derived from 2.5 pg of the original RNA), with some gene sequences detectable at lower levels, as shown in figure 1B. After step (f) a further increase in sensitivity was observed with all the genes assayed being positive using as little as 0.1% of the amplified product (i.e. amplified cDNA derived from 0.1 pg of the initial total mRNA sample), as shown in figure 1C. Therefore, using this approach, the expression of up to a 1000 genes could be assayed using 0.1% of the final product in each gene specific PCR reaction.

Reverse transcription

Total mRNA was prepared from rat whole brain using the total mRNA isolation system from Promega according to the manufacturer's instructions. Reverse transcription was performed using thermoscript reverse transcriptase or MMLV reverse transcriptase according to the manufacturer's (GIBCO-BRL,Paisley, Scotland) instructions. The reverse transcription primers used were composed of an anchored oligo-dT primer with a specific 5' heel sequence absent from the mammalian data bases. In some instances a RNA polymerase (T7) promoter site

was incorporated at the 3' end of the heel sequence. The primers used were as shown in SEQ ID N°3:

SEQ ID N°3: CTCTCAAGGATCTTACCGCTTTTTTTTTTTTTTTT (A,G,C)(A,G,C,T)

Second strand cDNA synthesis was initiated by incubating cDNA derived from 100 pg of total RNA with 25 pg of a mixed primer population consisting of (5'-3'): a 5' heel sequence absent from the mammalian data bases (CTGCATCTATCTAATGCTCC), a stretch of 5 random nucleotides (NNNNN, where N represents A, C, G or T) and a variable pentameric sequence chosen from CGAGA, CGACA, CGTAC and ATGCG, as shown in SEQ ID N°4, 5, 6 and 7. These primers will bind at multiple sites on the first strand cDNA and prime second strand synthesis from such priming sites. After annealing (7.5 mins at 50°C), primer extension was performed for 8 mins at 72°C using ampliTaq DNA polymerase (0.35 units, Applied Biosystems, Warrington, UK) in PCR-1 buffer containing 67 mM TrisHCl (pH 8.3) 4.5 mM $MgCl_2$, 6 mM beta-mercaptoethanol, 0.16% bovine serum albumin and 0.5 mM dNTPs.

Subsequently 1 ng (each) of the reverse transcription primer heel and second strand primer heel were added in 5 µl of PCR-1 buffer and the reaction subjected to 10 cycles of 92°C for 0.5 min, 60°C for 1.5 min and 72°C extension of 1 min, followed by a final l0 min extension. A further 10 ng of each heel primer were then added in 20 µl of PCR-1 buffer and subjected to a further 40 cycles (as before). The final product was then diluted to 100 µl with water and samples (2.5 or 5 µl) used for subsequent gene specific PCR assays, or subjected to a further 40 cycles (of 92oC for 0.5 min, 60°C for 1.5 min and 72°C for 1 min, followed by a final 10 min extension) in the absence of added primers. This was performed in a PCR-2 buffer containing 3.5 mM $MgCl_2$, 45 mM Tris HCl pH 8.8 and, 12.5% sucrose, 0.1 mM cresol red, 12 mM beta-mercaptoethanol, 0.5 mM dNTPs (Pharmacia), with 0.6 U AmpliTaq DNA polymerase (Applied Biosystems). This product was electrophoresed in a 2% agarose gel (E-gel, Invitrogen) and the high molecular weight products isolated from the gel using the Qiagen Gel extraction kit according to the manufacturer's instructions.

Gene specific PCR was performed on samples (2.5 to 10 µl) of amplified cDNA in PCR-2 buffer with gene specific primers at 100 ng/reaction. Following an initial 2 min denaturing step (92°C), each PCR cycle consisted of 0.5 min denaturing (92°C), 1.5 min annealing (55°C), and 1 min elongation (72°C). with a final extension for 10 min at 72°C. The PCR products were then separated by electrophoresis in a 2.5% agarose gel, stained with ethidium bromide and the image recorded. The gene-specific primers used were as follows: α Tubulin, (accession number, V01226, SEQ ID N°8 and 9), β-actin, (accession number, V01217, SEQ ID N°10 and 11), Cyclophilin, (accession number, M25637, SEQ ID N°12 and 13), Adenosine A1 receptor, (accession number, Y12519, SEQ ID N°14 and 15), Adenosine A2A receptor, (accession number, L08102, SEQ ID N°16 and 17), Adenosine A2B receptor (accession number, M91466, SEQ ID N°18 and 19), Adenosine A3 receptor, (accession number, M94152, SEQ ID N°20 and 21), NK1 receptor (accession number, J05097, SEQ ID N°22 and 23), NK2 receptor (accession number, M31838, SEQ ID N°24 and 25), trkA receptor (accession number, M85214, SEQ ID N°26 and 27), trkB receptor (accession number, M55291, SEQ ID N°28 and 29), proenkephalin, (accession number, S49491, SEQ ID N°30 and 31), synaptotagmin 1 (accession number, X52772, SEQ ID N°32 and 33), synaptotagmin 5 (accession number, X84884, SEQ ID N° 34 and 35), mammalian degenerin, (accession number, U53211, SEQ ID N°36 and 37), Glutamate decarboxylase (GAD67, accession number, X57573, SEQ ID N°38 and 39), choline acetyltransferase (not in GenBank/EMBL data bases, see Brice et al., (1989) J. Neursoci. Res., 23, 266-273, SEQ ID N°40 and 41),

Without wishing to be bound by any particular theory, the inventors believe that the increased sensitivity seen after step (e) is due to the removal of products formed during step (c) which compete in the gene specific PCR amplification. These products contain repetitive primer sequences which arecapable of priming on the amplified cDNA molecules and thus reduce theefficiency of the gene specific reaction. These products are removed

during step (f), while the amplified gene sequences which have been incorporated into the high molecular weight products during step (e) are retained.

As shown in Figure 1, amplification of cDNA derived from 100 pg of total RNA permits the detection of specific gene sequences by PCR at levels lower than those of unamplified cDNA. (A) dilutions of unamplified cDNA; B) dilutions of the amplified cDNA (step c); C) dilutions of the amplified cDNA (step e). The scale in (A) indicates the amount of total RNA from which the cDNA used in each gene specific assay was synthesised. In (B) and (C), the scale indicates the amount of total RNA from which the gene specific assay sample was amplified (i.e. 0.1 pg represents one thousandth of the final product obtained after amplification of cDNA derived from 100 pg of total RNA). Gene sequences were detected after amplification (as described in steps (a) to (c) of the first embodiment of the invention when using amplified product containing as little 1 pg of the initial RNA. After step (e) a further increase in sensitivity can be seen with detection at levels as low as 0.05 pg.

EXAMPLE II: The effect of restriction digestion on the detection of specific sequences after rat brain mRNA amplification using the second and third embodiments.

I. mRNA isolated from whole rat brain was reverse transcribed, and the cDNA derived from 25 pg total mRNA (equivalent to the mRNA content of between 2 and 5 cells) amplified according to Example 1, with (A) or without (B) cutting with Mlu1 as described in step d), followed by steps e) to g), figure 2. Each gene specific assay contained amplified product derived from 0.6 pg of total RNA. Note the detection of the adenosine A1 and A3 receptor after cutting (A) which were not detected without cutting (B).

II. mRNA isolated from whole rat brain was reverse transcribed, and the cDNA derived from 25 pg total mRNA (equivalent to the mRNA content of between 2 and 5 cells) amplified according to Example III, with

(A) or without (B) cutting with Mlu1 as described in step g), followed by steps h) to j), figure 2. Each gene specific assay contained amplified product derived from 0.6 pg of total RNA. Note the increased frequency of detection of the low abundance mRNAs (mammalian degenerin (MDEG), A2B receptor) as well as those of medium (GAD67, (glutamate decarboxylase; ChAT, choline acetyltransferase) and high (Synaptotagmin 1) abundance.

cDNA (derived from 25 pg total RNA from rat brain) was prepared and subjected to second strand synthesis as described in Example 1, except the heel of the second strand primers as with SEQ ID N°2,which contains the MluI cleavage site in particular a rare restriction site (ACGCGT) at the 3' end. After amplification to step c) as described in Example 1 (I) or Example 3 (II)), 10 µl of the diluted product was incubated in a total of 20 µl at 37°C for 60 min with 2 units of MluI in 6.0 mM Mg^{2+}, according to the manufacturer's instructions (Promega). After addition of EDTA to chelate the Mg^{2+} and incubation at 65°C for 5 mins to inactivate the enzyme, 10 µl aliquots were reamplified in PCR-2 buffer containing 0.625 units of AmpliTaq (and 0.05 units of pfu) DNA polymerases for 40 cycles of 92°C for 0.5 min, 60°C for 1.5 min and 72°C extension of 1 min, followed by a final 10 min extension, in the absence of added primers (I). In (II), 10 µl aliquots were subjected to a further 40 cycles at 92°C for 1.0 min, 95°C for 0.33 min, 72°C for 3 min, followed by a final 15 min. extension. The product was diluted to a final volume of 50 µl or 100 µl and samples subjected to gene specific PCR assays as described in Example 1.

In this second embodiment of the method of the invention, removal of the heel sequence of the second strand primer was designed to increase the sensitivity of the gene specific PCR by cutting of the competitor products described in Example I (so that they no longer compete in any of the subsequent PCR reactions), and also to promote the detection of gene sequences upstream from the reverse transcription primer site. The increased sensitivity due to removal of the primer sequences, is apparent in the increased sensitivity of detection of the gene sequences indicated. It is believed (but the applicants do not wish

to be bound by any theory) that short amplified products of gene sequence generated during step (c) can, after strand separation at 92°C, be extended after annealing to longer complementary products. Removal of the second strand primer heel, and amplification with the proof reading DNA polymerase pfu can assist this process. In this way the amount of amplified material containing sequence upstream from the reverse transcription primer site can be increased.

As shown in Figure 2, part (I), gene specific PCR after step (f), without (B) and with (A) cutting with the rare cutter restriction enzyme Mlu1 shows that cutting increases the detection of low abundance gene sequences such as the adenosine A1 and A3 receptors.

EXAMPLE III - Amplification of rat brain mRNA using the third embodiment.

In order to increase the sensitivity and specificity of the amplification process, two heel primers were designed for use at high stringency which were able to amplify single copies of lambda bacteriophage DNA in the presence of a 1000-fold excess of rat genomic DNA i.e. they were highly specific for the complementary sequences and able to amplify single copies (data not shown). Using these primers in the third embodiment of the method of the present invention, amplified product derived from as little as 0.01 pg of the initial RNA were positive in the gene specific PCR assays. This amount of RNA represents approximately 0.1% of that contained in a single cell.

Reverse transcription: Total mRNA prepared from rat whole brain using the total mRNA isolation system from Promega according to the manufacturer's instructions. Reverse transcription was performed using MMLV reverse transcriptase again according to the manufacturer's (GIBCO-BRL, Paisley, Scotland) instructions. The reverse transcription primers used were composed of an anchored oligo-dT primer with a specific 5' heel sequence absent from the mammalian data bases. The primer used SEQ ID N°42 is indicated below:

ACTGCCAGACCGCGCGCCTGAATTTTTTTTTTTTTTTTTTTTTTTTT
TTTT(A, C, G) (A, C, G, T) .

Second strand synthesis was initiated by incubating cDNA derived from 1000 pg of total RNA with 20 ng of second strand primers which were composed of (5' to 3'): a heel sequence absent from the mammalian data bases (TGTCCGTTTGCCGGTCGTGGGC) an Mlu1 site (ACGCGT), 17 random nucleotides (NNNNNNNNNNNNNNNNN) and three arbitrary bases (G, T) (A, G, T) (A, C, G), as shown in SEQ ID N°43.

These primers were added to give a final volume of 20 µl of PCR-1 buffer containing 4.5 mM Mg^{2+}, 1 unit of AmpliTaq DNA polymerase and 0.05 units of pfu DNA polymerase (Stratagene) and annealed and extended over 40 cycles under the following conditions: 92°C for 0.5 min, 40°C for 0.5 min (optional) and 72°C for 5 min, followed by a final 30 min extension. The repetitive annealing of these primers serves to increase the probability that all the gene sequences present in the initial cDNA population are copied into double stranded products.

Subsequently 300 ng of the reverse transcription and second strand heel primers were added with a further 1.25 units of AmpliTaq DNA polymerase in the presence of the Taqstart antibody (ClonTech) and 0.25 units of pfu DNA polymerase in 35 µl of buffer containing 1.5 mM Mg^{2+}, 67 mM Tris HCl (pH8.3) and 0.17 mM dNTPs. In some cases the amplification products were then subjected to restriction digestion with Mlu1 as described in Example 2, the low molecular weight products (including the heel of the second strand primers) removed through a Nanosep 10K column (double stranded DNA less than 100bp in length pass through the filter of these Nanosep columns) and the larger products either subjected to gene specific PCR, or subjected to a further 40 cycles of 92°C for 1.0 min, 95°C for 0.33 min, 72°C for 3 min, followed by a final l5 min extension. Removal of the second strand heel sequences was designed to both reduce the influence of any competing products and primers in the gene specific PCR and to permit product priming/repair as described in Embodiment 2.

The outcome of this procedure was the ability to detect genes by gene specific PCR (as described in Example 1) at dilutions of the amplified cDNA derived from as little as 0.01 pg of total RNA, illustrating that approximately 100,000 gene specific PCR assays could successfully be performed after amplification of cDNA derived from 1ng of total RNA (i.e. the content of approximately 100 cells). In addition product priming/product repair was shown to occur with the detection of a gene sequences 2.4 kb 5' to the reverse transcription priming site (Figure 4).

As shown in Figure 4, high stringency amplification cDNA derived from 1000 pg of total RNA as described in embodiment 3 permits increased detection of gene sequences. (I) The scale indicates the amount of total RNA from which the cDNA used in each gene specific assay was synthesised (A), or the amount of RNA from which the gene specific assay sample was amplified (B).

Specific sequences were detected when product amplified from as little as 0.01 pg of the initial RNA was used in the gene specific PCR. (II) Gene specific PCR using cDNA amplified from 2.5 pg of total RNA per gene specific assay. Inclusion of steps g) to i) of Amplification Method 3 also resulted in the detection of NK2 receptor gene sequence located 2350 bp upstream of the polyA splice site (A). This sequence was not detected if step g) was omitted (B). III Amplification of cDNA derived from 1 ng total whole brain RNA by Amplification Method 3 permits the detection of gene specific sequences in 0.006%of the product (equivalent to cDNA amplified from 0.06 pg of total RNA). Control: no amplification.

Furthermore, as shown in (II) of Figure 2, including steps g) to i) in embodiment 3 (A) increases the detection of low abundance messages such as those encoding the adenosine A2B receptor and the mammalian degenerin MDEG, when compared to an amplification which omitted step g) (B). In addition the detection of abundant mRNA species such as that encoding synaptotagmin 1 was also increased.

EXAMPLE IV *In vitro* transcription of RNA from cDNA amplified according to embodiments 1 and 3

Incorporation of the T7 promoter into the reverse transcription primer heel was performed so that RNA could be produced for subsequent analysis by hybridisation methods, for instance on oligo arrays. The yield of RNA from the amplified cDNA was estimated by running two parallel transcriptions, one for RNA synthesis and the other containing 35S-UTP as a substrate, so that the incorporation of the radioactivity into RNA could be used used as an index of RNA synthesis. After amplification of rat liver cDNA derived from 500 pg of total RNA by Amplification Procedure 1 to step (c), *in vitro* transcription resulted in a yield of 12.5 micrograms of RNA. Using Embodiment 3 to step (c), the yield of RNA from liver cDNA (derived from 500 pg of total RNA) was 34 micrograms (mean of 5 experiments). Similarly the yield from cDNA derived from 2500 pg was 90 micrograms (mean of 5 experiments).. Inclusion of step g) of Embodiment 3 prior to in vitro transcription increased the yield of RNA 1.7-fold (mean of two experiments). In order to examine the sequence content of the RNA transcribed from cDNA amplified according to Embodiment 3 (to step c) the RNA was reverse transcribed using the heel of the second strand heeled primer. Figure 5 illustrates that the cDNA derived from the transcribed RNA contained abundant gene sequence with actin tubulin and cyclophilin sequences being detected in aliquots representing 0.0001% of the RNA so produced. Therefore it appears that the expression of up to 1,000,000 genes may be assessed in amplified samples derived from 2500 pg of total RNA i.e. RNA derived from approximately 250 cells.

Reverse transcription of liver RNA was performed essentially as described in Examples 1 and 3, using primers containing a T7 RNA polymerase promoter site. The primers usedwere, SEQ ID N°44,:

CTCTCAAGGATCTTACCGCTAATACGACTCACTATAGGCGCTTTTTT
TTTTTTTTTTTTTTTTT
(A,G,C)(A,G,C,T) and SEQ ID N°45

GACTGCCAGACCGCGCGCCTGACGCGTAATACGACTCACTATAGG
GTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
(A, C, G) (A, C, G, T)

Subsequent second strand synthesis and amplification was carried out as described in the relevant examples. After step (c) of each Embodiment the amplified cDNA was isolated using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions to remove primers and other low molecular weight products, and 5 µl aliquots subjected to in vitro RNA transcription using the T7 Megascript kit (Ambion) according to the manufacturer's instructions. After DNase treatment to remove cDNA (DNase 1, 30 min, 37°C), the RNA was isolated using the Rneasy kit (Qiagen). One of the aliquots was transcribed in the presence of 35S-UTP to determine the yield of RNA. The RNA was subsequently ethanol precipitated (75% ethanol, 5% sodium acetate at -20°C for 30 mins), before reverse transcription with MMLV reverse transcriptase according to the manufacturer's instructions. The reverse transcription primers used were part or the whole of the heel sequences of the second strand primers, SEQ ID N°1 and 44

Subsequent gene specific PCR using this cDNA as substrate revealed that as little as 0.0001% of the product could be used in gene specific PCR and result in the detection of gene sequence. The data presented show that embodiment 3 with *in vitro* RNA transcription generates sufficient RNA of good quality for application to cDNA and oligonucleotide arrays. This will permit the analysis of the expression of thousands of genes from tissue samples containing approximately 250 cells and with further improvements perhaps from samples as small as single cells.

As shown in Figure 5, *in vitro* transcription of RNA from amplified cDNA contains large amounts of bona fide gene sequence. cDNA derived from 125, 500 and 2500 pg of liver total RNA was

amplified using Amplification Method 3 as far step e) and RNA transcribed. Cutting with the restriction enzyme (step g) was omitted so that the RNA so produced would contain the heel sequence of the second strand primer. This heel primer was then used to prime reverse transcription of the RNA, and the resulting cDNA analysed for the presence of 3 gene sequences. Note that all 3 gene sequences were detected even after 10^6 fold dilution of the product.

Example V. Amplification of rat spinal cord cDNA derived from 1 ng total RNA (equivalent to approximately 100 cells) using the third embodiment.

In example IV it was shown that antisense RNA could be *in vitro* transcribed from cDNA amplified by embodiment 3, this RNA could be applied to gene chips bearing sense probes, or reverse transcribed and applied to microarrays bearing antisense probes. However many microarrays bear sense probes (i.e. they recognise antisense DNA), but are not suitable for the hybridization of labelled RNA samples. In order to maximise the utility of embodiment 3, sense RNA was also transcribed from the amplified cDNA *in vitro,* reverse transcribed and the gene sequence content assessed by gene specific PCR.

Reverse transcription

Reverse transcription (RT) was performed in 10 µl containing; 1x first-strand buffer, 200 Units MMLV reverse transcriptase (BRL), and 0.5 ng first strand primer for 60 min at 37°C. The first strand primer (SEQ ID N°46),ACTGCCAGACCGCGCGCCTGAACGCG TAATACGACTCACTATAGGGTTTTTTTTTTTTTTTTTTT(A, C, G) (A, C, G, T), contained (5' to 3') a 26 base sequence absent from the mammalian data bases capable of hybridising to its complement at 72°C in the presence of 2mM Mg^{2+} with an MluI site at its 3' end, the T7 RNA polymerase promoter sequence, and an anchored stretch of oligodTs for hybridising to the 5' end of the polyA sequence of mRNA.

Second strand synthesis

Second strand synthesis was performed by adding an excess of second strand primer (1 ng) (to increase chances of annealing to every first strand sequence) in 4 microlitres of buffer giving a final Mg^{2+} concentration of 3.5mM.

After heating to 80°C, 5 units of Taq (Applied Biosystems, Warrington, UK) was added with 0.25 units of the proofreading enzyme pfu (Stratagene). Adding the Taq at high temperature ("hot start") prevents the enzyme copying mishybridised sequences in the mixture, such mishybridization tending to occur at the low temperatures encountered when setting up the reaction.

Primer annealing occurred at 50°C (7.5 mins decreasing by 10 secs per cycle) and extension at 72°C for 2.5mins. The temperature was cycled between 50°C and 72°C 40 times.

Although not wishing to be bound by theory, is the inventors believe that under these conditions each first strand cDNA will be annealed in multiple positions by the second strand primer. Each cycle permits further annealing by the primer. However, unlike normal PCR, the second strands are not dissociated from the first strand by melting in each cycle, consequently each primer has an equal chance of being extended to the 5' end of the first strand (which bears one of the heels), thus increasing the efficiency of subsequent PCR. It is envisaged that extension of primers at the 3' end of the first strand will displace those nearer the 5' end producing multiple copies of each second strand. The second strand primer contained (from 5' to 3'): a sequence absent from the mammalian data bases which is capable of hybridising to its complement and 72oC in the presence of 2mM Mg^{2+} and standard PCR buffers, an MluI site (ACGCG), the T3 RNA polymerase promoter and a random sequence of 15 bases, SEQ ID N°47:

AAAACTGCCAGACCGCGCGCCTGAACGCGTCGTATTAACCCTCACT AAAGGGN15

Amplification reactions

Subsequent PCR was performed by adding 4 microliters in AmpliTaq buffer (Applied Biosystems, Warrington, UK) containing1.25

mM dNTPs and 33ng of primers (the sequence absent from the mammalian data bases which is capable of hybridising to its complement and 72°C in the presence of 2mM Mg^{2+} with an MluI site) to give a final Mg^{2+} concentration of 2.6 mM.

In this example, this primer was common to both the first and second strand primers). After heating to 80°C, 5 units of Taq (Applied Biosystems, Warrington, UK) was added with 0.25 unit of the proofreading enzyme pfu (Startagene).

The reaction was then subjected to 20 cycles of denaturation (94°C, 20 secs), and annealing with extension (72°C, 5mins). 19 microlitres of AmpliTaq buffer were then added (at 80°C) containing and 0.2 mM dNTPS, 100 ng of primers and giving a final Mg^{2+} concentration of 2.1 mM. 5 units of Taq (Applied Biosystems, Warrington, UK) was then added with 0.25 units of the proofreading enzyme pfu (Stratagene). The reaction was then cycled 40 times as described above, with a final extension at 72°C for 30 min.

After amplification small MW primers and products were removed by passage through a Qiaquick PCR purification kit (Qiagen). The amplified cDNA was then cut with MluI and recleaned using the same kit same prior to subsequent gene specific PCR assays or in vitro transcription. Gene specific PCR was performed as previously described. In vitro transcription of RNA was performed using the Ambion Megascript Kit according to the manufacturers instructions.

After DNase treatment, some of the resulting RNA was reverse transcribed for gene specific PCR. Figure 6 shows the size distribution of the RNA produced from both the T3 and T7 RNA polymerase promoters (i.e. most between 200 and 600 bp with detectable higher molecular weight material). 10% of the product obtained after amplification of cDNA derived from 1 ng of total RNA was in vitro transcribed with T7 polymerase or T3 polymerase and 30% of each RNA applied to a gel. The estimated yields from the two RNA polymerases were 0.5 and 1.5 micrograms respectively. Figure 7A shows that the amplified cDNA contained both rare (A2A receptor) and abundant (e.g. tubulin) gene sequences detectable by gene specific PCR. I: amplification with a second strand primer lacking the T3 promoter, II

amplification with a second strand primer bearing the T3 promoter. Samples were diluted up to 1/3,000 prior to gene specific PCR. Figure 7B shows that the in vitro transcribed sense RNA generated using the T3 RNA polymerase (after reverse transcription to cDNA) also contains abundant gene sequence.

Example VI. Single cell expression analysis using microarrays after cDNA amplification of striatal cholinergic neuron al mRNA using embodiment 3.

In order to assess single cell gene expression, mRNA was amplified by the third embodiment using the primers and conditions described in Example V. T3 RNA polymerase was used to generate sense RNA which was then reverse transcribed using fluorescently labelled dCTP (Cy3 or Cy5) for application to glass microarrays bearing sense DNA probes

Harvesting of single cell mRNA

Striatal cholinergic neurons were identified on the basis of their size and electrophysiological characteristics in 300 µm coronal slices from 14-28 day-old male Sprague Dawley rats containing the striatum were viewed with a Zeiss Axioskop microscope (Carl Zeiss Ltd., Welwyn Garden City, U.K.) fitted with a x64 water-immersion objective lens.

Light in the infrared range (>740nm) was used in conjunction with a contrast-enhancing Newvicon camera (Hamamatsu, Hamamatsu City, Japan) to resolve individual neurones within slices (Lee et al., 1998).

The physiological saline bathing the slices contained (mM) 125 NaCl, 25 NaHCO3, 10 glucose, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 $MgCl_2$ and was bubbled with 95%/5% O_2/CO_2. The electrode buffer contained 120 K gluconate, 10 NaCl, 2 $MgCl_2$, 0.5 EGTA, 10 HEPES, 1-4 mM Na2ATP, 0.3 Na_2GTP, pH adjusted to 7.2 with KOH. 0.5 µg/ml glycogen (Boehringer) and RNase inhibitor (Pharmacia, 0.1 units/µl) were included to facilitate harvesting of RNA from the cells. This buffer also contained 10 fg each of bacterial sequences derived from the trp, thr and lys codons of E. coli. These mRNAs had polyA sequences

attached to the 3' end so that they could be amplified by XTPEA. All solutions were made up in diethylpyrocarbonate (DEPC) treated water. Borosilicate recording electrodes were baked (2h, 250°C) before being pulled to a resistance of between 3 and 5 M‡. Electrophysiological signals were detected using an Axopatch-1D patch-clamp amplifier (Axon Instruments, CA, USA) and were recorded onto digital audiotape. Following formation of the whole cell configuration, series resistance was partially compensated using the amplifier, and cellular conductance continuously monitored via the injection of hyperpolarising current or voltage. Membrane signals were filtered at 1 kHz and were digitized at 5kHz through a Digidata 1200A/D converter using pClamp 6.0 software (Axon Instruments Inc, CA, USA).

Extraction of Neuronal contents and amplification

The cytoplasm from large cells (>30µm in one dimension) was aspirated under visual control into a patch-clamp recording electrode until approximately 40% of the somatic cytoplasm had been collected. Usually the nucleus was sucked onto the end of the electrode until an electrical seal (>0.5G‡) was formed prior to withdrawal of the electrode to prevent contamination from the slice. Since withdrawal of the nucleus from the cells caused structural damage, outside-out patches were used to seal the electrodes if the cells were to be subsequently examined immunohistochemically. The contents of the electrode were forced into a microtube and reverse transcribed, amplified, low molecular weight components removed and all of the product *in vitro* transcribed as described for Example V. After Dnase treatment the RNA was reverse transcribed using Cy3 or Cy5 labelled dCTP prior to application to the microarrays.

Microarray Synthesis

Custom synthesised amine-modified oligonucleotide probes (probes) were purified in desalting columns to remove amine contaminants. The probes were prepared to a final concentration of 10-25 nmole/ml in 1X Surmodics Printing Buffer, containing 150 mM sodium phosphate, pH 8.5 (SurModics Inc, USA). The probe solution was printed

on 3D-Link Activated Slides (SurModics Inc, USA), and stored overnight in a saturated NaCl chamber. Printed slides were stored at room temperature. The microarrays contained probes capabale of recognising the bacterial sequences which were included in the patch electrode buffer. These served to ensure that successful amplification had occurred. In addition 3 probes from the Dengue virus genome were included as negative controls. The arrays contained a total of 510 oligonucleotide probes, recognising 141 different transcripts, each transcript being recognised by 3 or more separate probes.

Microarray hybridisation

Slides were exposed to 15 ml SurModics Blocking Solution (50 mM ethanolamine, 0.1 M Tris, pH 9) with 0.1 % SDS at 50°C for 15 minutes. Slides were rinsed twice with water, and washed with 15 ml 4X SSC / 0.1 % SDS prewarmed to 50°C for 40 minutes on a shaker. Slides were washed with water, and centrifuged at 800 rpm for 3 minutes. Labelled cDNA (target) hybridisation mixture was heated for 2 minutes in a boiling water bath, spun briefly to cool, and 2.5 l of target added per cm^2 of coverslip. Slides were placed in a humidified incubator overnight. Slides were removed from the incubation chamber and successively washed with 4X SSC for 30 seconds, 2X SSC / 0.1 % SDS for 5 minutes, 0.2X SSC for 1 minute and 0.1X SSC for 1 minute. Slides were spun to dry and scanned.

Gene expression analysis of single cells after cDNA amplification using microarrays.

cDNA amplification by embodiment 3 was used to assess the expression of a large number of genes in 4 striatal cholinergic neurons, the aim being the detection of both low and high abundance transcripts. In any analysis of gene expression at the single cell level problems are encountered with low abundance transcripts and with the non detection of some mRNAs in subpopulations of cells. This has been discussed in Surmeier et al (1996) J. Neurosci. 16, 6579-6591 and Richardson et al. (2000) J. Neurochem 74, 839-846.

Currently it is accepted that the number of cells in which a transcript is detected is related to the abundance of the transcript i.e. the more often a transcript is detected in individual cells the more abundant is the mRNA. Thus in any study of an apparently homogeneous population of cells, some low abundance transcripts may be detected in only a subpopulation of cells. For example, many $GABA_A$ receptor subunit mRNAs were detected in less than 100% of the cholinergic neurons tested by Yan and Surmeier (1997), suggesting that these transcripts were expressed either at low abundance in all the cells, or only in a specific subpopulation of cells. In the former case, more sensitive techniques will reveal a higher proportion of cells as positive for given transcripts, whereas in the latter there will be little change in the % of cells positive for a given transcript.

Table 1 shows some of the genes detected i.e. those whose expression in these cells had been previously characterised, and that the bacterial positive controls were detected but not the viral negative controls. All the housekeeping mRNAs are expected to be expressed in all cholinergic neurons. The neuronal markers dynorphin, enkephalin and PPTA are markers for non cholinergic neurons in the striatum, and lipoprotein lipase for endothelial cells.

Table 1 also shows that the use of embodiment 3 increased the number of cells (compared to previous estimates) in which the Voltage sensitive Na channel α6, trkC and NK3R mRNAs were detected, showing the ability of this method to detect low abundance transcripts in single cells.

In addition a number of mRNAs not previously suspected to be expressed in these cells were detected including somatostatin (SST), mAChR3 and 5, SUR2 and the D3 and D4 receptors, again showing the high sensitivity of this method.

In contrast, many of the GABA receptor subunit mRNAs were only detected in a proportion of the cells, suggesting that subpopulations of these cells may exist which express different complements of GABA receptor subunits, as suggested by Yan and Surmeier (1997). Other references in table 1 are: Yan & Surmeier (1996), Yan et al., (1997) and Tallaksen-Greene et al (1998).

Table 1: List of mRNAs detected in 4 single cholinergic neurons using embodiment 3 followed by hybridization to microarrays. The percentage of cells expected (e.g. housekeeping mRNAs are expected to be expressed in all cells) or previously shown by other methods, is shown in the third column (% positive cells) with the appropriate reference in the second column. The percentage of cholinergic cells in which the corresponding mRNAs were detected after embodiment 3 and microarray analysis is shown in column 4 (% positive cells by embodiment III).

Table 1

Housekeeping genes	**Reference**	**% +ve predicted**	**% +ve by embodiment 3**
Ribosomal L18	Expected	**100**	**100**
GAPDH	Expected	**100**	**75**
G6PDH	Expected	**100**	**75**
NS Enolase	Expected	**100**	**100**
α tubulin	Expected	**100**	**75**
Heavy Neurofil	Expected	**100**	**100**
Neurofil 68	Expected	**100**	**100**
β actin	Expected	**100**	**75**
MAP2	Expected	**100**	**75**
Neuronal cell markers			
Dynorphin	Expected	**0**	**0**
Enkephalin	Expected	**0**	**0**
SST	Expected	**0**	**50**
Parvalbumin		Unknown	**50**
Calretinin		Unknown	**75**
PPTA	Expected	**0**	**0**
GAD67	Richardson et al (2000)	**28**	**75**
GAD65	Expected	**28**	**25**
Lipoprotein lipase	Expected	**0**	**0**
Glutamate receptor subunits			
NR1	Richardson et al (2000)	**100**	**100**

NR2A	Richardson et al (2000)	50	75
NR2B	Richardson et al (2000)	20	50
NR2C	Richardson et al (2000)	0	100
NR2D	Richardson et al (2000)	80	100
GluR1	Richardson et al (2000)	30	75
GluR2	Richardson et al (2000)	40	50
GluR3	Richardson et al (2000)	30	50
GluR4	Richardson et al (2000)	20	25
mGluR1	Tallaksen-Greene et al (1998)	89	25
mGluR5	Tallaksen-Greene et al (1998)	65	50
GABA receptor subunits			
GABA α1	Yan & Surmeier (1997)	Present	50
GABA α2	Yan & Surmeier (1997)	Present	25
GABA α3	Yan & Surmeier (1997)	73	100
GABA α4	Yan & Surmeier (1997)	92	75
GABA β1	Yan & Surmeier (1997)	100	50
GABA β2	Yan & Surmeier (1997)	39	25
GABA β3	Yan & Surmeier (1997)	77	50
GABA γ1	Yan & Surmeier (1997)	62	0
GABA γ2	Yan & Surmeier (1997)	100	75
GABA γ3	Yan & Surmeier (1997)	77	50
Growth factor receptors			
trkA	Richardson et al (2000)	100	75
trkB	Richardson et al (2000)	70	100

trkC	Richardson et al (2000)	10	75
Sulphonylurea receptors			
SUR1	Lee et al (1998)	100	75
SUR2	Lee et al (1998)	0	100
Voltage sensitive Ca channel subunits			
α1 A	Yan & Surmeier (1996)	60	75
α1 B	Yan & Surmeier (1996)	90	25
α1 C	Yan & Surmeier (1996)	50	100
α1 D	Yan & Surmeier (1996)	50	100
α1 E	Yan & Surmeier (1996)	50	50
G protein coupled receptorss			
mAChR1	Yan & Surmeier (1996)	30	100
mAChR3	Yan & Surmeier (1996)	0	25
mAChR4	Yan & Surmeier (1996)	90	100
mAChR5	Yan & Surmeier (1996)	0	100
Adenosine A1R	Richardson et al (2000)	60	100
Adenosine A2AR	Richardson et al (2000)	45	75
Adenosine A2BR	Richardson et al (2000)	30	50
Adenosine A3R	Richardson et al (2000)	65	0
Dopamine D1R	Yan et al (1997)	20	25

Dopamine D2R	Yan et al (1997)	100	75
Dopamine D3R	Yan et al (1997)	0	50
Dopamine D4R	Yan et al (1997)	0	50
Dopamine D5R	Yan et al (1997)	100	50
NK1R	Richardson et al (2000)	80	100
NK2R	Richardson et al (2000)	0	50
NK3R	Richardson et al (2000)	5	75
Voltage sensitive Na channel subunits			
α1	Richardson et al (2000)	45	0
α2	Richardson et al (2000)	50	25
α3	Richardson et al (2000)	20	25
α4		Unknown	100
α5		Unknown	25
α6	Richardson et al (2000)	5	100
Bacterial positive controls			
lys	Expected	100	75
thr	Expected	100	100
trp	Expected	100	100
Viral negative controls			
Dengue	Expected	0	0

What is claimed is:

1. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a sample, said method comprising the steps of :

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;

c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with :

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method further comprises the steps of :

d) diluting the product of step c) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step c);

e) adding a thermoresistant DNA polymerase to the diluted cDNA solution of step d) and performing a further set of amplification reaction cycles without adding further nucleic acid primers;

f) separating the high molecular weight cDNA species, preferably those having a length of at least 4.5 kb, from the product obtained at step e); and

g) confirming the presence of at least one nucleotide acid sequence contained in the high molecular weight cDNA species separated at step f).

2. The method according to claim 1, wherein the amplification reaction of step c) is performed under low stringency hybridization conditions.

3. The method of claims 1 or 2, wherein the amplification reaction of step c) includes the steps of :

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 97°C;

(ii) annealing the single stranded DNA molecules at a temperature comprised between 55°C and 65°C; in the presence of a concentration of magnesium ranging from 1.5-6 mM.

(iii) elongating the annealed DNA molecules at a temperature comprised between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium;

(iv) reiterating steps (i) to (iii) for the desired number of cycles.

4. The method of any one of claims 1 to 3, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

5. The method according to any one of claims 1 to 4, wherein each amplification reaction cycle of step e) comprises the following steps of :

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C ;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles desired.

6. The method of any one of claims 1 to 5, wherein the further set of amplification reaction cycles carried out in step e) comprises between 10 and 40 cycles.

7. The method of claim 5 or 6, wherein the amplification reaction of step e) is performed in the presence of magnesium concentration rangi:ng from 1,5 to 4,5 mM.



8. The method according to any one of claims 1 to 7, wherein said method comprises an additional amplification step following step f) which comprises submitting at least a part of the high molecular weight DNA molecules separated at step f) to a further amplification reaction using at least a portion of the first heel sequence and at least a portion of the second heel sequence as the primers.

9. The method of any one of claims 1 to 8, wherein step g) comprises any one of the following methods :

(i) detection of the sequences of interest with specific oligonucleotide probes;

(ii) amplification of the sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription optionally using unlabelled or labeled substrate followed by gene specific PCR or hybridization.

10. The method according to any one of claims 1 to 9, wherein the first heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3'end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample or to the first strand cDNA molecules obtained at step a);

(ii) an oligo dT sequence of 15 to 25 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

11. The method according to claim 10, wherein said first heeled primer comprises a RNA polymerase binding site such as the T7 promoter.

* C L M S *

12. The method according to any one of claims 1 to 11, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

13. The method according to claim 11, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAATGCTCC-3'.

14. The method according to claim 12 or 13, wherein said second heeled primer comprises a RNA polymerase binding site such as the T7 promoter.

15. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said second heeled primer population contains a rare cleavage site in particular a rare restriction site located at or close to the 3'end of its heel sequence;

c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with :

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

d) incubating the product obtained at step c) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

e) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step c);

f) adding a thermoresistant DNA polymerase to the diluted sample of step e) and performing a further set of amplification reaction cycles without adding further nucleic acid primer; and

g) confirming the presence of at least one nucleic acid sequence contained in the reaction mixture obtained at step f).

16. The method according to claim 15, wherein the amplification reaction of step c) is performed under low stringency hybridization conditions.

17. The method of claim 15 or 16, wherein the amplification reaction of step c) includes the following steps of :

(i) obtaining single stranded DNA molecules at a temperature comprised between 85°C and 97°C ;

(ii) annealing the single stranded DNA molecules at a temperature comprised between 45°C and 65°C ;

(iii) elongating the annealed DNA molecules at a temperature comprised between 70°C and 75°C in the presence of a concentration of 4.5 mM Magnesium;

(iv) reiterating steps (i) to (iii) for the desired number of cycles.

18. The method of any one of claims 15 to 17, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

19. The method according to any one of claims 15 to 18, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5'end to 3'end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

20. The method according to claim 19, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N_{1-3}, wherein N is a nucleotide selected from A, T, C or G.

21. The method according to any one of claims 15 to 20, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located at its 3'end;

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

23. The method according to claim 21, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.



23. The method according to claim 21 or 22, wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

24. The method according to any one of claims 15 to 23, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of its heel sequence.

25. The method according to claim 24, wherein the cleavage site in particular a rare restriction site of said first heeled primer is identical to the cleavage site in particular a rare restriction site of the second heeled primer.

26. The method according to claim 24, wherein the cleavage site in particular a rare restriction site of said first heeled primer is different from the cleavage site in particular a rare restriction site of the second heeled primer.

27. The method according to claim 26, wherein in step d) the DNA molecules amplified at step c) are incubated with two restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction site of the first and the second heeled primer.

28. The method according to any one of claims 15 to 27, wherein each amplification reaction cycle of step f) comprises the following steps of :

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 85°C and 95°C ;

(ii) annealing the single stranded DNA molecules obtained at step (i) at a temperature comprised between 55°C and 75°C;

(iii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

(iv) reiterating steps (i) through (iii) for the desired number of cycles.

29. The method of any one of claims 15 to 28, wherein the further set of amplification reaction cycles carried out in step (f) comprises between 20 and 40 cycles.

30. The method of claim 28 or 29, wherein the amplification reaction of step f) is performed in the presence of a 3.5 mM Magnesium concentration.

31. The method according to any one of claims 15 to 30, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having a length of less than 50 base pairs are separated from the reaction mixture.

32. The method of any one of claims 15 to 31, wherein step g) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) in vitro RNA transcription, either for hybridization assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridization.

33. A heeled primer population comprising :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 25 nucleotides in length;

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

34. The heeled primer population according to claim 33, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N_{1-3}, wherein N is a nucleotide selected from A, T, C or G.

35. The heeled primer population of claim 33 or 34, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site located at its 3' end.

36. The heeled primer population of claim 35, wherein the cleavage site in particular a rare restriction site is selected from the rare cutter group of enzymes which comprises Not1, BsshII, NarI, MluI, NruI and NaeI.

37. A heeled primer population comprising :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a) and wherein the heel sequence includes the nucleotide sequence of a rare cleavage site in particular a rare restriction site located within or close to its 3'end;

(ii) a first variable sequence of 4 to 7 nucleotides in length selected such that substantially every possible sequence combination of 4 to 7 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence.

38. The heeled primer population of claim 37, wherein the cleavage site in particular a rare restriction site is selected from Not1, BsshII, NarI, MluI, NruI and NaeI.

39. The heeled primer population according to claim 37 or 38, wherein the heel sequence consists of the nucleic acid sequence 5'-CTGCATCTATCTAGTACGCGT-3'.

40. The heeled primer population according to any one of claims 37 to 39, which further comprises a RNA polymerase promoter site.

41. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises :

(i) a first heeled primer population according to any one of claims 33 to 36; and

(ii) a second heeled primer population according to any one of claims 37 to 40.

42. The kit of claim 41, which further comprises :

(iii) a first primer consisting of the heel sequence of the first heeled primer;

(iv) a second primer consisting of the heel sequence of the second heeled primer.

43. The kit according to claim 41 or 42, which further comprises one or several restriction enzymes that recognize the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

44. The kit according to any one of claims 41 to 43 which further comprises a RNA polymerase.

45. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;

b) synthesizing second cDNA strands using a second heeled primer population;

c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with said second heeled primer at a concentration ranging between 0.02 to 200 ng per reaction in the following conditions :

(i) obtaining single stranded DNA molecules at a temperature comprised between 78°C and 95°C ;

(ii) optionally annealing said single stranded DNA molecules at a temperature comprised between 40°C and 72°C;

(iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C in the presence of a thermoresistant DNA polymerase;

d) amplifying the DNA molecules resulting from step c) over a number of further amplification cycles with :

(i) a first primer comprising the heel sequence of the first heeled primer; and

(ii) a second primer comprising the heel sequence of the second heeled primer,

wherein the respective concentration of each primer ranges between 10 and 500 ng per reaction, whereby a population of amplified DNA molecules is obtained; and

e) recovering the population of DNA molecules obtained at step d).

46. The method of claim 45, wherein the amplification of said first and second cDNA strands carried out in step c) comprises between 30 and 50 amplification cycles.

47 The method of claim 45 or 46, wherein the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

48. The method of any one of claims 45 to 47, wherein the amplification reaction of step c) is performed in the presence of a concentration of 4.5 mM Magnesium.

49. The method of any one of claims 45 to 48, wherein the further set of amplification reaction cycles carried out in step d) comprises between 30 and 50 cycles.

50. The method of any one of claims 45 to 49, wherein each amplification reaction cycle of step d) comprises the following steps of :

(i) obtaining single stranded DNA molecules by incubating the sample at a temperature comprised between 78°C and 95°C;

(ii) elongating the annealed DNA molecules using a thermoresistant DNA polymerase at a temperature comprised between 65°C and 75 °C;

(iv) reiterating steps (i) through (iii) for the desired number of reaction cycles.

51. The method of any one of claims 45 to 50, wherein the amplification reaction of step d) is performed in the presence of 2.5 mM Magnesium concentration.

52. The method of any one of claims 45 to 51, wherein said method comprises the additional step of :

f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

53. The method according to claim 52, wherein said method comprises a further step wherein the DNA molecules obtained at step f) having length of less than 50 base pairs are discarded from the reaction mixture.

54. The method of claim 52 or 53, wherein step f) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) in vitro RNA transcription, either for hybridisation assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridisation.

55. The method according to any one of claims 45 to 54, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5'end to 3'end :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

56. The method according to claim 55, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N_{1-3}, wherein N is a nucleotide selected from A, T, C or G.

57. The method according to any one of claims 45 to 56, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of its heel sequence.

58. The method according to any one of claims 45 to 57, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

(i) a heel sequence of 25 to 30 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a);

(ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population ; and

(iii) a second variable nucleotide sequence.

59. The method of any one of claims 45 to 58, wherein the heel sequence of the second heeled primer comprises the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of its heel sequence.

60. The method according to claim 59, wherein said second heeled primer comprises a RNA polymerase binding site located downstream from said cleavage site in particular a rare restriction site.

61. The method of any one of claims 45 to 60, wherein the heel sequences of the first and second heeled primers comprise the sequence of a rare cleavage site in particular a rare restriction site located at the 3'end of their respective heel sequence.

62. The method according to claim 58, wherein the cleavage site in particular a rare restriction site sequence of the first heeled primer is identical to the cleavage site in particular a rare restriction site sequence present in the heel of the second heeled primer.

63. The method according to claim 61, wherein the cleavage site in particular a rare restriction site sequence of the first heeled primer is different from the cleavage site in particular a rare restriction site sequence present in the heel of the second heeled primer.

64. The method of any one of claims 60 to 63, wherein step e) is followed by the additional steps of :

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

h) diluting the product of step d) to obtain a diluted cDNA solution containing a cDNA concentration which is between about 2 and 100 times inferior to the cDNA concentration of the product of step d);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

65. The method according to claim 64, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction sites present in the heel sequence of the first and the second heeled primer.

66. The method according to claim 64 or 65, wherein said method comprises a further step wherein the DNA molecules obtained at step g) having length of less than 50 base pairs are separated from the reaction mixture.

67. The method according to any one of claims 64 to 66, wherein the number of amplification reaction cycles performed in step i) is comprised between 20 and 40.

68. The method of any one of claims 61 to 64, wherein step j) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers; and

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

69. A method to increase the number of sequences corresponding to the mRNA species present in a sample, wherein said method comprises the steps of :

a) reverse transcribing the mRNA species using a first heeled primer population to provide first strand cDNA species;

b) synthesizing second cDNA strands using a second heeled primer population;

c) amplifying said second cDNA strands resulting from step b) over a number of amplification cycles with second heeled primers

d) amplifying the first and second strands resulting from step c) using primers selected from the group consisting of (1) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (2) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (3) a mixture of the primers (1) and (2), wherein the total concentration of primers ranges between 0.02 and 500 ng per reaction in the following conditions :

(i) adding the primers to the cDNA product obtained at step c);

(ii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C;

(iii) adding a thermoresistant DNA polymerase;

(iv) maintaining the temperature at a range from 80°C to 95°C for a period of time comprised between 5 sec to 15 min;

(v) annealing said single stranded DNA and elongating the annealed DNA molecules at a temperature comprised between 65°C and 75 °C;

(vi) carrying out steps (iv) and (v) for a desired number of cycles.

e) recovering the population of DNA molecules obtained at step d)

70. The method of claim 69, wherein step d) further comprises the steps of amplifying the DNA molecules obtained at step d) (vi) over a number of amplification cycles with primers selected from the group consisting of (a) a primer comprising a portion of the heel sequence of the first heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, (b) a primer comprising a portion of the heel sequence of the second heeled primer which portion is of a nucleotide length sufficient to hybridize with its complementary sequence under the hybridization conditions specified, and (c) a mixture of the primers (a) and (b), wherein the total concentration of primers ranges between 0.02 and 200 ng per reaction in the following conditions :

(vii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C;

(viii) adding a thermoresistant DNA polymerase to the single stranded DNA molecules obtained at step (vii);

(ix) annealing and elongating the single stranded DNA molecules at a temperature comprised between 65°C and 75°C.

(x) carrying out steps (vii) and (ix) for a desired number of cycles.

71. The method of claim 69, wherein step c) (vi) comprises between 10 and 50 amplification cycles.

72. The method of claim 69, wherein the amplification reaction of step c) is performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

73. The method of claim 69, wherein the amplification steps b) and c) are performed in the presence of a concentration of Magnesium comprised between 2 and 5 mM.

74. The method of claim 88, wherein the amplification step d) is performed in the presence of a concentration of Magnesium comprised between 2 and 3 mM.

75. The method of claim 69, wherein the respective concentration of primers at steps b) and c) range from 0.02 to 500 ng.

76. The method of claim 69, wherein the respective concentration of primers ranges from 0.02 to 90 ng.

77. The method of claim 69, wherein the respective concentration of primers at step d) ranges from 100 to 500 ng.

78. The method of claim 70, wherein step d) (x) comprises between 20 and 60 amplification cycles.

79. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of both a thermoresistant DNA polymerase and a thermoresistant proof reading enzyme.

80. The method of claim 70, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium comprised between 1.5 and 4 mM.

81. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium comprised between 1.6 and 2.5 mM.

* C L M S *

82. The method of claim 80, wherein the amplification reaction steps d) (viii) to (x) are performed in the presence of a concentration of Magnesium of 2.0 nM.

83. The method of claim 70, wherein the respective concentration of primers at steps d) (vii) to (x) ranges from 10 to 500 ng.

84. The method of claim 83, wherein the respective concentration of primers at steps d) (vii) to (x) ranges from 30 to 300 ng.

85. The method of claim 69, wherein step b) of synthesizing second cDNA strands is performed in the presence of a Magnesium concentration ranging from 3 to 5 nM.

86. The method of claim 85 wherein the Magnesium concentration is of 4.5mM.

87. The method of claim 69 or 70, wherein step b) of synthesizing second cDNA strands comprises the steps of :

(i) adding the primers to the cDNA product obtained at step a);

(ii) obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C

(iii) adding the DNA polymerase and the proof reading enzyme to the mixture obtained at step (ii);

(iv) maintaining the temperature of the mixture at approximately 94°C during a period of time comprised between 30 sec to 5 min

(v) annealing said single stranded DNA at a temperature comprised between 40°C and 72 °C;

(vi) elongating the annealed DNA molecules at a temperature comprised between 60°C and 75 °C;

88. The method of claim 69 or 70, wherein step c) of synthesizing second cDNA strands comprises the steps of :

(i) optionally obtaining single stranded DNA molecules at a temperature comprised between 80°C and 95°C in the presence of a thermoresistant DNA polymerase;

(ii) annealing the single stranded DNA molecules obtained at step (i) with the second heeled primer population at a temperature comprised between 40°C and 60°C;

(iii) elongating the annealed DNA molecules at a temperature comprised between 65°C and 75°C;

(iv) repeating steps (ii) to (iii) for a desired number of cycles.

89. The method of claim 88, wherein in step (iv), steps (ii) to (iii) are repeated for 10 to 60 cycles.

90. The method of claim 69 or 70, wherein said method comprises the additional step of :

f) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at step e).

91. The method according to claim 90, wherein said method comprises a further step wherein the DNA molecules obtained at step e) having a length of less than 50 base pairs are discarded from the reaction mixture.

92. The method of claim 69 or 70, wherein step f) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers;

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector; or

(iv) in vitro RNA transcription, either for hybridisation assays or for further reverse transcription using unlabeled or labeled primers or substrates followed by gene specific PCR or hybridisation.

93. The method according to any one of claims 69 to 92, wherein the first heeled primer population consists of a population of nucleic acids comprising, from 5'end to 3' end :

* C L M S *

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

94. The method according to claim 93, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable nucleotide sequence : 5'-(A or G or C)-N_{1-3}, wherein N is a nucleotide selected from A, T, C or G.

95. The method according to any one of claims 69 to 94, wherein the first heeled primer includes the sequence of a rare cleavage site in particular a rare restriction site.

96. The method according to claim 95, wherein the rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence of said first heeled primer.

97. The method according to claim 95, wherein the rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence of said first heeled primer.

98. The method according to any one of claims 69 to 97, wherein the second heeled primer population consists of a population of nucleic acid sequences each comprising, from 5'end to 3' end :

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules present in the sample or with the first strand cDNA molecules synthesized at step a); and

(ii) a variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population;



99. The method of claim 98, wherein the heel sequence of said second heeled primer comprises the sequence of a rare cleavage site in particular a rare restriction site.

100. The method of claim 99, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.

101. The method of claim 100, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

102. The method of claim 99, wherein the heel sequence of the second heel primer ranges from 25 to 35 nucleotides in length.

103. The method of claim 98 wherein the heel sequence of the second heeled primer ranges from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site.

104. The method of claim 99, wherein the heel sequence of the second heeled primer ranges from 45 and 75 nucleotides in length and comprises a RNA polymerase binding site located at the 3' end of the heel sequence.

105.The method according to any one of claims 99 to 104, wherein step e) is followed by the additional steps of :

g) incubating the DNA molecules obtained at step e) with at least one restriction enzyme that specifically recognizes the cleavage site in particular a rare restriction site included in the heel sequence of the second heeled primer;

h) diluting the product obtained at the end of steps d) and g) to obtain a diluted cDNA solution containing a cDNA concentration which is between 2 and 100 times inferior to the cDNA concentration of the product of step d) or g);

i) adding a thermoresistant DNA polymerase to the diluted sample of step h) and performing a further set of amplification reaction cycles without adding any nucleic acid primer; and

j) confirming the presence of at least one nucleic acid sequence contained in the population of DNA molecules obtained at steps g), h) and i).

106. The method of claim 105, wherein when the heels of the first and second heeled primers each comprise a rare cleavage site in particular a rare restriction site, the method is characterized in that in step g), the DNA molecules are incubated with restriction enzymes recognizing respectively the rare cleavage site in particular a rare restriction sites present in the heel sequence of the first and the second heeled primer.

107. The method according to claim 105 or 106, wherein said method comprises a further step wherein the DNA molecules obtained at steps e) and g) having length of less than 50 base pairs are separated from the reaction mixture.

108. The method according to any one of claims 105 to 107, wherein the number of amplification reaction cycles performed in step i) is comprised between 20 and 40.

109. The method of any one of claims 105 to 108, wherein step j) comprises any one of the following methods :

(i) detection of sequences of interest with specific oligonucleotide probes;

(ii) amplification of sequences of interest with specific oligonucleotide primers; and

(iii) cloning of the DNA molecules obtained in a replication and/or expression vector.

110. The method according to any one of claims 69 to 109, wherein the heel sequences of the first heeled primer and the second heeled primer are identical.

111. A heeled primer population comprising :

(i) a heel sequence of 15 to 22 nucleotides in length which is not complementary to the mRNA molecules initially present in the sample;

(ii) an oligo dT sequence of 15 to 35 nucleotides in length; and

(iii) a variable sequence of 2-4 nucleotides in length that can hybridize to a mRNA molecule at the 5'end of the poly-A tail thereof,

wherein substantially every possible variable sequence combination is found in said first heeled primer population.

112. The heeled primer population according to claim 111, wherein the variable sequence of 2 to 4 nucleotides is selected among the following variable dinucleotide sequence : 5'-(A or G or C)-N_{1-3}, wherein N is a nucleotide selected from A, T, C or G.

113. The heeled primer population of claim 111 or 112, wherein the heel sequence comprises the sequence of a rare cleavage site in particular a rare restriction site.

114. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

115. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.

116. The heeled primer population of claim 113, wherein the cleavage site in particular a rare restriction site is selected from the rare cutter group of enzymes which comprises Not1, BsshII, NarI, MluI, NruI and NaeI.

117. The heeled primer population of any one of claims 111 to 116, wherein the heel sequence has a GC content comprised between 50 and 80%.

118. A heeled primer population comprising :

(i) a heel sequence of 25 to 75 nucleotides in length which is not complementary to the mRNA molecules or with first strand cDNA molecules present in a sample;

(ii) a first variable sequence of 15 to 25 nucleotides in length selected such that substantially every possible sequence combination of 15 to 25 nucleotides is found in said second heeled primer population.

119. The heeled primer population of claim 118, wherein each primer further comprises :

(iii) a second variable nucleotide sequence that was calculated to hybridize on average once in every 1 kb portion of said first strand cDNA molecules under low stringency hybridization conditions.

120. The heeled primer population according to claim 119, wherein said second variable sequence is chosen from the group of sequences consisting of 5'-CGAGA-3', 5'-CGACA-3', 5'-CGTAC-3' and 5'-ATGCG-3', such that each of said second variable sequence is found in said second heeled primer population.

121. The heeled primer population of claim 118, which contains the sequence of a rare cleavage site in particular a rare restriction site.

122. The heeled primer population of claim 1201, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 3' end of the heel sequence.

123. The heeled primer population of claim 121, wherein the sequence of a rare cleavage site in particular a rare restriction site is located at the 5' end of the heel sequence.



124. The heeled primer population of claim 121, wherein the cleavage site in particular a rare restriction site is selected from Not1, Bsshll, Narl, Mlul, Nrul and Nael.

124. The heeled primer population of any one of claims 111 to 124, wherein the heel sequence has a GC content comprised between 50 and 70%.

126. The heeled primer population of claim 115 wherein the heel sequence ranges from 25 to 35 nucleotides in length.

127. The heeled primer population of claim 115 wherein the heel sequence ranges from 45 to 75 nucleotides in length and comprises a RNA polymerase binding site.

128. The heeled primer population of claim 127 wherein the RNA polymerase binding site is located at the 3' end of the heel sequence.

129. A kit for the amplification of the mRNA species present in a sample, wherein said kit comprises :

(i) a first heeled primer according to any one of claims 11 to 116;

(ii) a second heeled primer population according to claims 117 to 127.

130. The kit of claim 126, which further comprises :

(iii) a first primer selected form the group consisting of (a) the heel sequence of the first heeled primer and (b) a primer comprising at least 15 consecutive nucleotides of the heel sequence of the first heeled primer.;

(iv) a second primer selected form the group consisting of (a) the heel sequence of the second heeled primer and (b) a primer

comprising at least 15 consecutive nucleotides of the heel sequence of the second heeled primer.;

131. The kit of claim 129, wherein the heel sequences of the first heeled primer and of the second heeled primer are identical.

132. The kit of claim 130, wherein the sequences of the first – and second primers are identical.

133. The kit according to anyone of claims 129 to 132, which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the second heeled primer.

134. The kit according to any one of claims 129 to 133 which further comprises a restriction enzyme that recognizes the rare cleavage site in particular a rare restriction site sequence present in the heel sequence of the first heeled primer.

135. The kit according to any one of claims 129 to 134, which further comprises a RNA polymerase.

136. A method to increase the number of nucleotide sequences corresponding to the mRNA species present in a low quantity in a sample, comprising:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population;

c) amplifying said first and second cDNA strands resulting from step b) over a number of amplification cycles with the aid of a thermoresistant DNA polymerase(s) with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer,

wherein said method is characterized in that it comprises the steps of:

d') increasing the proportion of high molecular weight DNA molecules,

e') using or analyzing specific nucleic acid sequences present in the high molecular weight DNA molecules,

137. A method to increase the number of nucleic acid sequences corresponding to the mRNA species present in a low quantity in a sample, wherein said method comprises the steps of:

a) reverse transcribing said mRNA species using a first heeled primer population to provide first strand cDNA sequences;

b) synthesizing second cDNA strands from said first strand cDNA sequences using a second heeled primer population, wherein each of the primers of said first, and/or second heeled primer population optionally contains a rare cleavage site in particular a rare restriction site located at the 3' end of its heel sequence;

c) amplifying the first and second cDNA strands resulting from step b) over a number of amplification cycles with:

(i) a first primer comprising at least a portion of the heel sequence of the first heeled primer; and

(ii) a second primer comprising at least a portion of the heel sequence of the second heeled primer;

d') cutting any large DNA molecules and preventing bridge formation in subsequent steps by suppressing the heel portions of at least one said first or second heeled primer

e') increasing the amount of long double strand products with sequences more 5' from the original mRNA.

138. A method to increase the number of nucleotide sequences corresponding to an mRNA species present in a sample in a low quantity comprising the steps of:

a) reverse transcribing the mRNA species using first heeled primer population to provide first strand cDNA species;

b) synthesizing second cDNA strands using a second heeled primer population;

c) amplifying said second cDNA strands resulting from step b) over a number of amplification cycles in order to generate second cDNA strands comprising heels at both ends and increasing the number of second cDNA strands corresponding to long mRNA species present initially in the sample to be assayed;

d) amplifying the DNA molecules resulting from step c) under hybridization conditions which are of a higher stringency than those of step c) and which enable reduction of the synthesis of high molecular weight cDNA molecules; and

e) recovering the population of DNA molecules obtained at step d).