Title: SENSITIVE PREPARATION AND AMPLIFICATION OF EUKARYOTIC DERIVED NUCLEIC ACID FOR TRANSCRIPTION PROFILING

Abstract: A method for global amplification of c DNA from an RNA template is described. The c DNA produced retains the same relative abundance across the sample as the RNA transcripts from which it is produced. Embodiments of the method are sensitive enough to produce c DNA from samples equivalent to single cell amounts of RNA. The method comprises the steps of: a) reverse transcription of the template RNA population, to produce a global c DNA population; b) homopolymer tailing the c DNA population, to produce c DNA molecules having a homopolymer tail; and c) amplifying the tagged global c DNA; wherein at least one of steps (a) and (b), and preferably both, is/are effected in the presence of an acetate buffer. In preferred embodiments, step (a) is carried out in the presence of a non specific RNA template (NSRT) which does not comprise sequences found in the target; for example, the target may be mammalian RNA and the NSRT may be lambda phage RNA. Also in preferred embodiments, step (b) is carried out in the presence of Co Cl₂.
Sensitive preparation and amplification of eukaryotic derived nucleic acid for transcriptional profiling.

FIELD OF THE INVENTION

This invention relates to nucleic acid preparation and more particularly to improved conditions for global amplification of cDNA.

BACKGROUND TO THE INVENTION

By globally amplified cDNA we mean cDNA in which DNA molecules representing gene expression retain the same relative abundance across samples as the RNA transcripts from which they are derived.

Recent years have seen a growth in the realisation of the importance of gene expression in the control of biological activities. It is known that expression of specific subsets of genes regulate tissue formation and organogenesis during development and also the properties of adult tissues. Patterns of gene expression influence not only the structure and composition of specific tissues, but also the tissues' responses to various stimuli. These structures, composition and responses, and the patterns of gene expression encoding them, are distinctive markers for individual tissues and physiological conditions.

At a more complex level the pattern of genes expressed by whole organisms may be characteristic of specific individuals and provide an insight into their biological status. For instance, there is growing evidence that the pattern of genes expressed by an individual may influence factors such as the individual's predisposition to particular diseases or their responsiveness to certain therapeutic agents.

The current challenge to biologists is to learn how the products of the around 20-40,000 identified human genes interact to produce the complexity exhibited by higher eukaryotes. To a large extent the biological character of a cell can be inferred from the profile of genes it expresses. Although an examination of RNA or protein expression patterns alone does not directly address function, the knowledge of when and where a gene is expressed can provide valuable insights as to the potential role of a gene and has historically been instrumental in the discovery of developmentally regulated
genes. Recognition of the value of the examination of expression patterns led to the development of a plethora of advanced RNA profiling technologies such as cDNA microarrays (Duggan et al., 1999), SAGE (Velculescu et al., 1995), and cDNA display (Liang and Pardee, 1992) aimed at the simultaneous measurement of tens to several thousand genes in the target samples. Application of these profiling technologies to clinical diseases, such as cancer has confirmed the utility of profiling and provided useful diagnostic and prognostic assays (Shipp et al., 2002; Staunton et al., 2001; van ‘t Veer et al., 2002). More recently, the application of next generation sequencing approaches to measure RNA profiling (RNA-seq) has enabled the comprehensive identification and quantification of all expressed sequences (Sultan et al., 2008).

Despite the success of these approaches at the molecular level by identifying patterns of expression exhibited generally by relatively homogeneous cellular samples, the cellular complexity and sample preparation from higher eukaryotes still presents a major obstacle to expression profiling. Over the last 30 years a variety of molecular techniques have been developed for the analysis of gene-expression. In general, methods focussed either on the identification and characterisation of genes (either individual genes or networks of related genes) or the characterisation of the input tissue or cell based on a characteristic profile of expressed genes. Initial nucleic acid hybridization techniques (such as northern and dot blots) have been used for many years to analyse a small number of genes and samples and a variety of advanced RNA profiling technologies such as cDNA microarrays (Duggan et al., 1999), SAGE (Velculescu et al., 1995), cDNA display (Liang and Pardee, 1992) and RNA-seq (Sultan et al., 2008) have been developed to allow the simultaneous measurement of tens to many thousands of genes in the target samples. The ability to profile small samples including single-cells is a very important tool in a wide range of biological disciplines including both cancer and stem biology. For leukaemias, single cells can be readily identified by fluorescent-activated cell sorting (FACS) and for solid tumours, laser capture microdissection (LCM) offers one of the most promising means of isolating cells based on direct microscopic visualization of tissue sections (Betsuyaku, T., et al. (2001). Am J Respir Cell Mol Biol 25, 278-284). Methods of obtaining small sample inputs for global profiling methodologies such as the subject of this application are exemplified by, but not limited to, LCM, FACS, biopsy, FNA
(fine needle aspiration) or nucleic acid. More recently, the discovery of circulating
tumour cells (CTCs) and their relevance to treatment of the source tumour (Lin, H., et al. (2011). Crit Rev Oncol Hematol 77, 1-11) has highlighted the need for
methodologies, such as the subject of this application, which enable RNA profiling of
small input amounts from samples including single cells. Despite the advances in
assay methodologies there are few accurate, reliable and reproducible methods which
will generate amplified cDNA which faithfully represents the initial RNA profile of
small samples including single cells. This invention and methodologies addresses
these difficulties and limitations. It is therefore among the objects of the present
invention to provide a method suitable for generation of globally amplified cDNA
from small samples, including single cells.

Many of the techniques for analysis of gene-expression described above require the
use of labelled target DNA capable of binding to complementary DNA sequences in
reference samples. In order to take both full advantage of and to extend recent
improvements in gene-expression analysis it is important that the labelled target DNA
be sensitive, that is to say having a high binding affinity for complementary DNA
sequences. It is also beneficial to be able to produce labelled target DNA from small
samples, ideally single cells, since this allows a greater range of cell types to be used
(since it obviates a requirement for large numbers of cells), and improves confidence
that the starting population is "pure", rather than representing a mixed population of
cell types such as is found in many tissue samples. Furthermore, it is advantageous if
labelled target DNA can be produced rapidly, by cheap simple techniques which
generate representative cDNA from the sample of interest. Unfortunately many
known collections of labelled target DNA suffer from disadvantages in that they have
relatively low sensitivity, or are prepared by laborious, complicated or expensive
techniques.

Conveniently labelled double-stranded DNA representative of gene expression in a
sample of interest may be prepared using primers comprising a homopolymer T tract
(for example CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTT). When
combined with homopolymer tailing (for example using terminal transferase) PCR
using such primers produces a population of DNA molecules, in which all molecules
have a poly-T region at one end and a poly-A region at the other. This technique has
the advantage that a single oligonucleotide can be used for the initial and all subsequent PCR amplifications. The technique also obviates the need to create new priming sites within the molecules to be amplified, since each molecule produced by amplification contains a poly-A region that can anneal to a poly-T region in the primer allowing further rounds of amplification.

Primers comprising a poly-T tail (as described above) may also comprise a further sequence of nucleotides in addition to the tail region. Such further nucleotides may be selected to allow the incorporation into DNA molecules, produced by PCR using these primers, of regions that may be advantageous for the further amplification or subsequent use of molecules produced. For example primers may be designed such that they will incorporate "anchor" sequences (thereby enabling improved specificity of subsequent PCR) or cloning sites (allowing subsequent manipulation of amplified DNA products). Suitable sequences for incorporation into such primers to achieve these purposes would be immediately appreciated by one skilled in the art.

The success of the entire amplification process relies on three successive enzymatic steps which are carried out by simple addition of a buffer/enzyme mixture to the preceding step. Maintaining effective enzymatic performance for each step is problematic when the standard reverse transcriptase, terminal transferase and Taq polymerase buffers are used because they are very different from one-another. For effective sampling and representative amplification of low RNA input such as a directly lysed single cell or 10 pg total RNA for example, it is essential that initial RNA priming and cDNA production is representative and efficient. Since low concentrations of input RNA can lead to inefficient RNA priming and cDNA production this application directly address this issue by use of agents which increase the effective concentration of reagents and input RNA. For molecular biology kits capable of amplifying small amounts of input RNA it is essential to minimise potential sources of nucleic acid contamination in the kit reagents themselves. Proteinaceous agents such as glycogen and bovine serum albumin (BSA) are essential for efficient generation of representative cDNA, however, both glycogen and BSA are derived from organisms which express RNA and therefore present a potential contamination risk. Here we propose to avoid the potential of contamination by replacing glycogen and/or BSA with chemically derived synthetic peptides thereby
enabling a method that delivers a more specific and accurate cDNA product which is devoid of contamination from buffer components.

SUMMARY OF THE INVENTION

The invention proposes a method of producing globally amplified cDNA comprising the steps of

i) reverse transcription (to produce a global cDNA population);

ii) terminal transferase "tailing" (to extend the molecules of the global cDNA); and

iii) polymerase chain reaction (to amplify the tailed global cDNA)

wherein at least one of steps (i) and (ii), and preferably both, is/are effected in the presence of an acetate buffer.

The global amplified cDNA is prepared from RNA using limiting concentrations of nucleotides and a relatively short incubation time in order to limit cDNA synthesis. This ensures that, no matter what the length of the original RNA transcript, all cDNA molecules produced are of approximately the same relatively small size. Since all the cDNA molecules are of approximately equal size subsequent amplification of the cDNA results in equal reproduction of all the cDNA molecules present. This ensures that the amplified cDNA produced reflects the original relative abundance of the RNA present in the biological sample. In addition to the advantage of allowing the production of amplified populations of cDNA that maintain the relative abundance of the original RNA the use of global amplified cDNA also provides other advantages. For example, global amplified cDNA can be derived either directly from one or more freshly isolated living cells without the need for RNA isolation, or from RNA purified from a biological sample. Additionally, the production of global cDNA is well suited to automation, providing advantages in terms of ease and speed of use and is also compatible with assays such as, but not limited to, quantitative PCR (qPCR), DNA arrays and DNA sequencing methodologies such as, but not limited to, RNA-seq.

A first advantage of the invention arises from the fact that globally amplified cDNA can be produced from samples as small as a single cell, which may typically contain in the region of 10-20 pg total RNA. Since conventional techniques for the production of collections of target DNA typically require starting quantities of RNA
in the region of 1-20 µg the ability to work with single cells represents up to a million fold increase in template sensitivity. Although methods have been improved in the last 10 years and allow routine RNA profiling using an input of 10-100 ng total RNA there is still a clear need for reliable methods capable of profiling sub ng total RNA levels. A second advantage of the use of globally amplified cDNA is that large amounts of DNA can be made, which can be readily and simply checked by methods such as gel electrophoresis and/or real-time quantitative PCR for example prior to and/or following incorporation of label. This provides advantages not only in terms of ease of production, but also in that it avoids the costs associated with inefficient labelling of target DNA molecules and ineffective use or wastage of arrays. A third advantage of the use of globally amplified cDNA is that it can be readily incorporated into downstream readouts such as, but not limited to, qPCR, DNA array and sequencing.

One method by which global amplified cDNA for use in accordance with the invention may be prepared comprises the following steps:

a) preparing a global cDNA population representative of gene expression in a biological sample of interest from RNA of the sample by using primers and limiting concentrations of nucleotides;

b) homopolymer tailing the global cDNA population; and

c) amplifying the tailed global cDNA population.

Preferably step a) comprises the reverse transcription of RNA from the biological sample of interest using primers capable of binding to the poly A tail of the RNA. As described above, the reverse transcription is preferably carried out in the presence of limiting concentrations of nucleotides in order to limit the length of the transcripts produced.

Preferably within step a) which comprises the reverse transcription of RNA from the biological sample of interest using primers capable of binding to the poly A tail of the RNA. As described above, the reverse transcription is preferably carried out in the presence of limiting concentrations of nucleotides in order to limit the length of the transcripts produced in the presence of volume excluding agents such as, but not
limited to, polyethylene glycol (PEG) or dextran sulphate for example, which will enhance primer annealing and subsequent cDNA production.

The global cDNA population produced in step a) is preferably homopolymer such that a population of double-stranded DNA molecules that have both homopolymer A and homopolymer T tracts is produced. Homopolymer tailing may be effected using terminal transferase enzymes.

At least one of steps a) and b) is effected in the presence of an acetate buffer. Preferably both steps a) and b) are effected in the presence of an acetate buffer. The use of acetate buffers produces conditions that more closely approximate physiological conditions, and thereby improves the sensitivity and yield of the reaction. A preferred acetate buffer suitable for use in the preparation of cDNA for use according to the invention comprises Tris acetate incorporating potassium acetate and/or magnesium acetate. Preferably the acetate buffer comprises 2-200 mM Tris Acetate, 5-500 mM potassium acetate.

**Denature Buffer** (160mM Tris HCl, pH8.0, 270mM KC1, 23mM MgCl2, 282µg/ml glycogen, 282µg/ml BSA, 28mM Tris Acetate pH7.9, 70mM KAc)

**RNase Inhibitor Buffer** (1.25µM each dNTP, 12.5µM dT24, 10U/µl total RNase Inhibitors, 17.5pg/µl NSRT)

**RT Buffer** (40mM Tris HCL, pH8.0, 70mM KC1, 5.8mM MgCl12, 72µg/ml glycogen, 72µg/ml BSA, 7.2mM Tris Acetate pH7.9, 17.6mM KAc)

It is also preferred that at least one of steps a) and b) is effected in the presence of either BSA or preferably chemically derived synthetic peptide(s). The presence of BSA in the reaction mixture during the reverse transcription (step a) and homopolymer tailing (step b) steps significantly, and surprisingly, increases the efficiency of the reaction. However, the BSA used is a naturally occurring protein and may contain contaminating nucleic acids including RNA. In contrast, synthetic peptides are chemically synthesised and therefore do not suffer from the potential contamination problems associated with use of a purified naturally occurring protein.
Substitution of BSA and/or glycogen by synthetic peptides in the cDNA amplification process provides a means of maintaining accurate, efficient and reproducible amplification without risk of biological contamination from undesired sources. Furthermore, since the amino acid composition of each synthetic peptide will influence the chemical/biochemical balance (eg pH) of any solution they are part of it is possible to select individual or combinations of peptides which provide a chemical/biochemical balance best suited to each step in the cDNA amplification process. Given the complex nature of the cDNA amplification process where each of the 3 biochemical steps is facilitated by addition of a new buffer the flexibility of using individual or combinations of peptides will both simplify the production of the buffers used and increase the efficiency of the overall process.

It is also preferred that in the reverse transcription step an artificial nucleic acid-based template known as non-specific suppression RNA templates (NSRT), such as but not limited to, synthetic or natural nucleic acid sequences derived from phages such as Lambda phage, is added which serves to suppress any accumulation of non-specific reaction products produced via non-specific side products accumulating during the multiple PCR reactions or via inclusion of trace contaminants accompanying the enzymes used for the entire process. For preferred embodiments of the invention, the starting template to be amplified is mammalian RNA, and the NSRT is non-mammalian RNA, preferably non-vertebrate RNA, more preferably non-animal RNA, and most preferably non-eukaryotic or phage RNA. The NSRT may in preferred embodiments be present in the reaction mix at 1 - 1000 pg RNA. Also steps a) and b) should be effected in the presence of either BSA or preferably chemically derived synthetic peptide(s).

In a further preferred embodiment of the method, the homopolymer tailing step, step b), is carried out in the presence of CoCl$_2$. It has been found that the presence of CoCl$_2$ causes a surprising increase in the efficiency of cDNA production and thus significantly increases cDNA yield per unit starting RNA. Preferably the concentration of CoCl$_2$ is between 0.5-1.5mM, more preferably between 0.8-1.2mM, and most preferably between 0.9-1.1mM (e.g. 1mM).
In a most preferred embodiment, step a) is carried out in the presence of NSRT as described above, and step b) is carried out in the presence of CoCl$_2$.

It is preferred that homopolymer tailing, step b), is performed in the absence of dithiothreitol (DTT). Surprisingly, the absence of DTT increases the efficiency, and so yield, of the reaction. This finding is surprising since it has previously been believed that DTT confers an advantage in the production of cDNA.

Preferably the buffer for step (a) comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-500 mM</td>
<td>Tris pH 8.3;</td>
</tr>
<tr>
<td>10-300 mM</td>
<td>KCl;</td>
</tr>
<tr>
<td>1-20 mM</td>
<td>MgCl$_2$;</td>
</tr>
<tr>
<td>2-200 mM</td>
<td>Tris Acetate pH 7.9;</td>
</tr>
<tr>
<td>5-500 mM</td>
<td>Potassium Acetate;</td>
</tr>
<tr>
<td>0-10 mM</td>
<td>Mg Acetate.</td>
</tr>
</tbody>
</table>

Preferably the reaction mixture for step (a) further comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-500 µg/ml</td>
<td>Glycogen;</td>
</tr>
<tr>
<td>0-5 %</td>
<td>NP-40;</td>
</tr>
<tr>
<td>0.02-10 u/ml</td>
<td>RNase Inhibitor</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>BSA.</td>
</tr>
<tr>
<td>2-200 µM</td>
<td>dNTPs;</td>
</tr>
<tr>
<td>0.01-100 µM</td>
<td>oligonucleotide.</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>peptide(s)</td>
</tr>
<tr>
<td>1-lOOOpG</td>
<td>Non-specific suppression RNA templates (NSRT).</td>
</tr>
</tbody>
</table>

Preferably the buffer for step (b) comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-250 mM</td>
<td>Tris pH 7.5 - 8.5;</td>
</tr>
<tr>
<td>5-150 mM</td>
<td>KCl;</td>
</tr>
<tr>
<td>0.5-10 mM</td>
<td>MgCl$_2$;</td>
</tr>
<tr>
<td>2-200 mM</td>
<td>Tris Acetate pH 7.5 - 8.5;</td>
</tr>
<tr>
<td>5-500 mM</td>
<td>Potassium Acetate;</td>
</tr>
<tr>
<td>0-10 mM</td>
<td>Mg Acetate.</td>
</tr>
</tbody>
</table>
Preferably the reaction mixture of step (b) further comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5000 µg/ml</td>
<td>Glycogen</td>
</tr>
<tr>
<td>0-2.5 %</td>
<td>NP-40</td>
</tr>
<tr>
<td>0.1-10 mM</td>
<td>CoCl$_{2}$</td>
</tr>
<tr>
<td>5 1-100 µM</td>
<td>dNTPs</td>
</tr>
<tr>
<td>0.005-2500 µM</td>
<td>dT24</td>
</tr>
<tr>
<td>0.01-5 u/ml</td>
<td>RNase Inhibitor</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>BSA</td>
</tr>
<tr>
<td>0.05-5 mM</td>
<td>dATP</td>
</tr>
<tr>
<td>10 1-500 u/ml</td>
<td>TdT enzyme.</td>
</tr>
<tr>
<td>0.5 - 3.5 u/µl</td>
<td>Reverse Transcriptases</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>peptide(s)</td>
</tr>
</tbody>
</table>

Preferably the buffer for step (c) comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-500 mM</td>
<td>Tris pH 7.5 - 8.5</td>
</tr>
<tr>
<td>10-300 mM</td>
<td>KCl</td>
</tr>
<tr>
<td>1-20 mM</td>
<td>MgCl$_{2}$</td>
</tr>
<tr>
<td>2-200 mM</td>
<td>Tris Acetate pH 7.5 - 8.5</td>
</tr>
<tr>
<td>5-500 mM</td>
<td>Potassium Acetate; and</td>
</tr>
<tr>
<td>0-10 mM</td>
<td>Mg Acetate</td>
</tr>
</tbody>
</table>

Preferably the reaction mixture of step (c) further comprises:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7 µM</td>
<td>Oligonucleotide;</td>
</tr>
<tr>
<td>0.1-10 mM</td>
<td>dNTPs</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>Glycogen</td>
</tr>
<tr>
<td>0.03-3.3 mM</td>
<td>CoCl$_{2}$</td>
</tr>
<tr>
<td>0.02-1 %</td>
<td>Triton X-100;</td>
</tr>
<tr>
<td>0-2.5 %</td>
<td>NP-40</td>
</tr>
<tr>
<td>0-500 µg/ml</td>
<td>BSA</td>
</tr>
<tr>
<td>0.05-5 mM</td>
<td>dATP</td>
</tr>
<tr>
<td>0.005-2500 µM</td>
<td>dT24</td>
</tr>
<tr>
<td>0.01-5 u/µl</td>
<td>DNA Polymerase;</td>
</tr>
<tr>
<td>0.01-5 u/ml</td>
<td>RNase Inhibitor; and</td>
</tr>
</tbody>
</table>
Here we have developed a set of buffers which increase stability, reliability and efficiency of the amplification method while maintaining a desirable and simple "single"-tube workflow method. In essence we have an overall buffer base which includes acetate buffers since they are more representative of cellular conditions and have provided a broad buffer system for restriction enzymes (McClellan et al 1988).

We have also included agents which increase the efficiency of the initial conversion of RNA to cDNA from target RNA of interest, whilst including reagents that suppress amplification from non-desired sources. In addition we have optimised the concentrations of dNTPs, C0Cl₂, DTT and propose the replacement of biologically derived reagents (BSA and glycogen) with chemically synthesised peptides to provide a radically new set of buffers which together provide a dramatic improvement over the previous methods. Chemically synthesised peptides can be included as single peptides or mixtures of two or more peptides and will be chosen on the basis of their ability to support effective amplification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 compares the sensitivity of the methods of the present invention with data from published descriptions of prior art methods.

Figure 2 compares unamplified data from prior art methods with amplified data obtained using methods of the invention.

Figure 3 compares global amplified cDNA produced in the presence of C0Cl₂ with cDNA produced in the absence of this reagent.

Figure 4 compares global amplified cDNA produced in the presence and absence of NSRT (non-specific RNA template).

Figure 5 shows transcriptional profiling by quantitative PCR of amplified cDNA using an assay targeted to a transcript known to be of higher abundance in SUHRR samples relative to HBRR.

Figure 6 shows transcriptional profiling by quantitative PCR of amplified cDNA, using an assay targeted to a transcript known to be of higher abundance in HBRR samples relative to SUHRR.
Figure 7 shows the proportion of targets that are calculated as being detected, or present (\( \%P \)) when performing transcriptional profiling of the amplified cDNA.

Figure 8 shows Principal Components Analysis visualisation of microarray data generated from application of the amplified cDNA to Affymetrix microarrays.

DETAILED DESCRIPTION OF THE INVENTION

Protocols.
The following Protocols are non-restrictive examples that are suitable for effecting the method of preparation of global amplified cDNA from small initial samples (such as single cell equivalent amounts of RNA).

(a) Preparation of global amplified cDNA
   (i) Reverse transcription - Preparation of cDNA
   (ii) Terminal transferase - "Tailing"
   (iii) Global cDNA amplification

(b) Quantitative PCR

(a) Preparation of global amplified cDNA.

Suitable starting materials include total RNAs, which may be prepared from biological tissues of interest (using commercially available kits such as those manufactured by Clontech), or RNA present in biological cells ("direct amplification").

The following reagents and protocol increase the efficiency, accuracy and yield of cDNA produced. A kit for carrying out the method of the invention may comprise reagents/compositions as defined in A1 to A9 and E1 to E3 below.

Reagents:

A1 
RT Buffer 1
157 mM Tris HCl pH 8.3
273 mM KC1
23 mM MgCl₂
260 µg/ml Glycogen
260 µg/ml BSA
2.6% NP40
5 28 mM Tris Acetate pH7.9
69 mM K Ac

A2 RNase Inhibitors
1.25 mM dNTPs
10 12.5 µM dT
2.5 U/µl SUPERase (Ambion)
7.5 U/µl RNase Inhibitor+ (Promega)
17.5 pg Non-specific Suppression RNA templates (NSRT)
22.2% PEG

A3 RT Buffer
40 mM Tris Acetate pH 7.9
70 mM K Acetate
5.8 mM Mg Acetate
20 72 µg/ml Glycogen
72 µg/ml BSA
0.66% NP40
7.2 mM Tris Acetate pH7.9
17.6 mM K Ac

A4 TdT Buffer
22.2 mM Tris Acetate pH7.9
55.5 mM K Ac

A5 CoCl₂
24 mM CoCl₂

A6 dATP
100mM dATP

**A7 Taq Buffer**

143mM KC1
30mM Tris HCl pH ~8.5
10mM MgC12
0.5mg/ml Glycogen
0.03% Triton X-100

**A8 dNTPs**

dNTPs 25mM each

**A9 Oligo**

297 µM NotldT

(CATCTCGAGCGGCCGCT<sub>24</sub>)

**E1 RT**

200/µl AMV reverse transcriptase
175U/µl M-MLV reverse transcriptase

**E2 TdT**

200/µl terminal transferase

**E3 Taq Polymerase**

5 u/µl Taq polymerase

**Protocol:**

Prior to starting thaw all reagents on ice, mix well and briefly spin.

*Reverse Transcriptase*

1. Make up fresh *Denaturation Mix* as follows:

2.36 µl A1 Denaturation Buffer

0.196 µl A2 RNAse Inhib+
2. Mix 1 µl RNA sample with 5.75 µl water and 2.25 µl Denaturation Mix, heat 65°C for 1 minute and cool at room temperature for 3 minutes then cool to 4°C.

3. Make up fresh RT Solution as follows:
   - 1.57 µl RT Buffer
   - 0.196 µl E1 RT

4. Add 1.5 µl RT Solution to each sample and incubate 15 minutes at 37°C, heat inactivate at 65°C for 10 minutes then cool to 4°C.

**Tailing**

5. Prepare Tailing Mix as follows:
   - 9.33 µl A4 TdT Buffer
   - 0.8 µl A5 CoCl₂
   - 0.17 µl A6 dATP
   - 0.33 µl E2 TdT

6. Add 7.5 µl Tailing Mix to all samples, incubate 15 minutes at 37°C, heat inactivate at 65°C for 10 minutes then cool to 4°C.

**PCR**

7. Prepare PCR Mix as follows:
   - 36.0 µl A7 Taq Buffer
   - 3.33 µl A8 dNTPs
   - 1.17 µl A9 Oligo
   - 1.17 µl E3 Taq Polymerase
8. Add 42 µL *PCR Mix* to all samples, mix and subject to the following PCR protocol:

- 95 °C - 30 seconds
- 42 °C - 2 minutes
- 72 °C - 6 minutes X 24-50 cycles

**NOTE:** Use a PCR machine fitted with a heated lid to avoid evaporation.

9. Purify using either the Millipore 96 well purification system (Millipore MANU 03050) or the GFX™ PCR DNA and Gel Band Purification Kit (Amersham).

 *(b) Quantitative PCR measurements.*

Quantitative PCR measurements were made using the "qPCR™ core kit for Sybr® Green I" (Eurogentech) as recommended by the manufacturer.

**Experimental protocols:**

1. **Reverse transcription - preparation of cDNA.**

cDNA was prepared from RNA from biological tissues according to the following protocol:

- 1. RNAs are adjusted to the desired concentration in 10 mM Tris pH 7.5, 1 mM EDTA
- 2. 1 µL of each RNA is added to 5.75 µL RNAse/DNase-free water
- 3. *Denaturation Mix* is prepared by mixing 2.36 µL A1 Denaturation Buffer with 0.196 µL A2 RNAse Inhibitor-i- Buffer (volumes are multiplied depending by the total number of samples if more than one sample is to be prepared).
- 4. 2.25 µL *Denaturation Mix* is added to each sample, and mixed well
5. Samples are heated to 65°C for 1 minute allowed to cool to 25°C for 3 minutes then held at 4°C.

6. *RT Solution* is prepared by mixing 1.57 µl A3 RT Buffer with 0.196 µl E1 RT enzymes (volumes are multiplied depending by the total number of samples if more than one sample is to be prepared).

7. 1.5 µl of *RT Solution* is added to each sample and mixed well.

8. Samples are incubated 15 minutes at 37°C, heat inactivated at 65°C for 10 minutes then cooled to 4°C.

**a (ii) Terminal Transferase - 'Tailing'**

1. *Tailing Mix* is prepared by mixing 9.33 µl A4 TdT Buffer, 0.8 µl A5 CoCl₂, 0.17 µl A6 dATP, and 0.33 µl E2 TdT (volumes are multiplied depending by the total number of samples if more than one sample is to be prepared).

2. 7.5 µl of *Tailing Mix* is added to each sample and mixed well.

3. Samples are then incubated 15 minutes at 37°C, 10 minutes at 65°C and cooled to 4°C.

**a (iii) Global cDNA amplification.**

Global cDNA prepared from biological tissues according to the preceding protocols may be amplified according to the following protocol:

1. *PCR Mix* is prepared by mixing 36 µl A7 Taq Buffer, 3.33 µl A8 dNTPs, 1.17 µl A9 oligo and 1.17 µl E3 polymerase.

2. 42 µl *PCR Mix* is added to each sample and mixed well.

3. Samples are then placed into a PCR machine and subjected to:

   - 25-50 cycles
   - 30 seconds at 95°C
   - 2 minutes at 42°C
   - 6 minutes at 72°C

4. Following completion of PCR samples are purified using the Millipore 96 well purification system (Millipore MANU 03050) following instructions provided by the manufacturer.
Experimental Results:
(i) Output of cDNA amplification is sensitive, reproducible and linear.

Here we have used FDA recognised MicroArray Quality Control (MAQC) reference RNA samples to compare data produced using the amplification methods described in this patent to published data obtained from Agilent and other array platforms (Shi et al. 2006, Chen et al. 2007).

Reproducibility, sensitivity and linearity evaluations were conducted on the following samples:

- Sample A - 100% Stratagene Universal Human Reference RNA - SUHRR
- Sample B - 100% Ambion First Choice Human Brain Reference RNA - HBRR
- Sample C - Mixture of: 75% SUHRR / 25% HBRR
- Sample D - Mixture of: 25% SUHRR / 75% HBRR

Figure 1 compares unamplified data reproduced from Shi et al. (2006) (left hand graph) to amplified data (right hand graph, labelled "Epistem") where blue bars show replicate Coefficient of Variation (CV) of signals. The results obtained demonstrate that amplified material is reproducible with CV's below 10% and is also very sensitive (8,000-12,000 genes detected/sample) equivalent to the data obtained by Shi et al. (2006).

Figure 2 compares unamplified data reproduced from Chen et al. (2007) to amplified data with the amplified results being given in the final row of the table (labelled "Epistem"). Results show linearity of the amplified data is equivalent to, or better, than the results obtained with conventional platforms.

(ii) Optimised reagent formulation is necessary for performance

Figures 3 and 4 compare global amplified cDNA produced using all optimised buffers (as described in the Reagents section above) to amplification using suboptimal reagents. Figure 3 shows an evaluation of test reagents carried out using reducing input amounts of total human RNA from either Ambion First Choice Human Brain Reference RNA (HBRR)) or Stratagene Universal Human Reference RNA (SUHRR) down to levels equivalent to single cell input amounts. Omission of CoCl$_2$ is detrimental both qualitatively and quantitatively, and requirement for presence of it is
particularly evident at low RNA input amounts equivalent to single cell input. Figure 4 shows that inclusion of NSRT non-mammalian template RNA is beneficial for abrogating non-specific amplification of target mammalian RNA.

(Hi) *The output of the cDNA amplification is applicable to a wide variety of platforms for transcriptional profiling, and faithfully retains biological differences between samples of interest.*

Figure 5 shows transcriptional profiling by quantitative PCR of amplified cDNA, using an assay targeted to a transcript known to be of higher abundance in SUHRR samples relative to HBRR. Identification of this differential expression is evident through to RNA input amounts equivalent to single cell levels.

Figure 6 shows transcriptional profiling by quantitative PCR of amplified cDNA, using an assay targeted to a transcript known to be of higher abundance in HBRR samples relative to SUHRR. Identification of this differential expression is evident through to RNA input amounts equivalent to single cell levels.

Figure 7 shows the proportion of targets that are calculated as being detected, or present (%P) when performing transcriptional profiling of the amplified cDNA using Affymetrix microarray technology. Using either SUHRR or HBRR as example input RNA results in detection of significant number of transcripts down to RNA input levels equivalent to single cell input.

Figure 8 shows Principal Components Analysis visualisation of the microarray data generated from application of the amplified to Affymetrix microarrays. Retention of biological differences between SUHRR and HBRR samples through to RNA input amounts equivalent to single cell input are evident.

**Further optimisation**

Further optimisation of the methods and reaction conditions may be carried out along the following lines.

1) **Optimisation of initial RNA priming and cDNA production.**

Reverse transcriptase and terminal transferase reactions as described in the global amplification protocol (Experimental Protocols section) are carried out using buffers
containing a range of concentrations of the volume excluder PEG, acetate buffers, 
CoCl$_2$. Buffers with and without these added reagents are applied to duplicate sets of 
total RNA samples added at a variety of input amounts (eg. 12.5 - 1000 pg). Analysis 
of cDNA yields by gel and qPCR especially at lower RNA inputs will establish the 
benefit of including volume excluding agents.

**ii) Inclusion of synthetic peptides.**

Buffers are prepared using either BSA, glycogen, individual peptides or combinations 
of peptides. Test buffers are applied to duplicate sets of total RNA samples added at 
either 10, 100 or 1000 pg input amounts. Analysis of cDNA yields by gel and qPCR 
especially at lower RNA inputs will establish the benefit of the use of synthetic 
peptides.

**iii) Inclusion of non-specific suppression RNA templates (NSRT).**

Reverse transcriptase and terminal transferase reactions as described in the global 
amplification protocol (Experimental Protocols section) will be carried out using 
buffers containing added artificial RNA NSRT templates selected on the basis that the 
added RNAs do not comprise any sequences found in the mammalian genome. 
Buffers either containing or not containing NSRT templates will be applied to 
duplicate sets of total RNA samples added at a range of input amounts (eg. 12.5 - 
1000 pg). Analysis of cDNA yields by gel and qPCR especially at lower RNA inputs 
will establish the benefit of including NSRT in the initial reverse transcriptase step.

**iv) Combining all optimised reagents.**

Buffers will be prepared incorporating the optimum buffers described in sections i -Hi. 
Buffers either containing or not containing the optimum buffers described in sections i 
-iii will be applied to duplicate sets of total RNA samples added at a range of input 
amounts (eg. 12.5 - 1000 pg). Analysis of cDNA yields by gel and qPCR especially 
at lower RNA inputs will establish the benefit of including all optimised reagents.
References:


Kuribayashi-Ohta, K., Tamatsukuri, S., Hikata, M., Miyamoto, C., and Furuichi, Y. "Application of oligo(dT)30-latex for rapid purification of poly(A)+ mRNA and for hybrid subtraction with the in situ reverse transcribed cDNA." Biochim Biophys Acta 1993, 1156: 204-12.


CLAIMS:

1. A method of producing globally amplified cDNA from a template RNA population, the method comprising the steps of
   a) reverse transcription of the template RNA population, to produce a global cDNA population;
   b) homopolymer tailing the cDNA population, to produce cDNA molecules having a homopolymer tail; and
   c) amplifying the tailed global cDNA

2. A method according to claim 1, wherein the reverse transcription step is carried out in the presence of non specific RNA template (NSRT).

3. A method according to claim 1 or claim 2, wherein the tailing step is carried out in the presence of CoCl₂.

4. A method according to any preceding claim, wherein the reverse transcription step is carried out with limiting concentration of nucleotides; and/or for a restricted time; such that the length of cDNA molecules produced is restricted.

5. A method according to any preceding claim, wherein the homopolymer tailing step is carried out by terminal transferase.

6. A method according to any preceding claim, wherein the amplification step is carried out by polymerase chain reaction (PCR).

7. A method according to any preceding claim, wherein the PCR reaction is carried out using primers which bind to the homopolymer tail of the tailed cDNA produced in step (b).

8. A method according to any preceding claim, wherein each of steps a) and b) is effected in the presence of an acetate buffer.
9. A method according to any preceding claim, wherein the acetate buffer comprises Tris acetate incorporating potassium acetate and/or magnesium acetate.

10. A method according to claim 9, wherein the acetate buffer comprises 2-200 mM Tris acetate, 5-500 mM potassium acetate, and 1-10 mM magnesium acetate.

11. A method according to any preceding claim, wherein step a) comprises reverse transcription of RNA using primers capable of binding to the poly A tail of RNA, the reverse transcription being performed in the presence of limiting concentrations of nucleotides.

12. A method according to claim 11, wherein the primers comprise a homopolymer T tract capable of binding to the poly A tail of RNA.

13. A method according to claim 11 or 12, wherein the primers comprise additional sequences, such that the additional sequences are incorporated into the global cDNA population.

14. A method according to any preceding claim, wherein step a) is effected in the presence of a buffer comprising:

- 20-500 mM Tris pH 8.3;
- 10-300 mM KCl;
- 1-20 mM MgCl$_2$;
- 2-200 mM Tris Acetate pH 7.9;
- 5-500 mM Potassium Acetate;
- 1-10 mM Mg Acetate.

15. A method according to any preceding claim, wherein the reaction mixture of step a) further comprises:

- 5-500 µg/ml Glycogen;
- 0.01-5 % NP-40;
- 0.02-10 u/ml RNase Inhibitor; and
- 70-80 µg/ml BSA.
2 -200 μM dNTPs;
0.01-100 μM oligonucleotide.
1 - 1000 pg Non-specific suppression RNA templates (NSRTs)

16. A method according to claim 15, wherein the reaction mixture comprises 0.1-5 μM oligonucleotide

17. A method according to any preceding claim, wherein step b) comprises homopolymer tailing the global cDNA population, produced in step a), to produce a population of double-stranded DNA comprising both homopolymer A and homopolymer T tracts.

18. A method according to any preceding claim, wherein step b) is effected in the presence of a buffer comprising:

15 10-250 mM Tris pH 7.5-8.5;
14 5-150 mM KCl;
13 0.5-10 mM MgCl₂;
12 2-200 mM Tris Acetate pH 7.5-8.5;
11 5-500 mM Potassium Acetate; and
10 1-10 mM Mg Acetate.

19. A method according to any preceding claim, wherein step b) is performed in the presence of bovine serum albumin.

20. A method according to claim 19, wherein the bovine serum albumin is synthetically produced.

21. A method according to any preceding claim, wherein step b) is performed in the absence of DTT.

22. A method according to any preceding claim, wherein the reaction mixture of step b) comprises:

2.5-250 μg/ml Glycogen
0.005-2.5 % NP-40;
0.1-10 mM CoCl$_{34}$
1-100 µM dNTPs;
0.005-2500 µM dT24;
0.01-5 u/ml RNase Inhibitor;
35-40 µg/ml BSA;
0.05-5 mM additional dATP; and
1-500 u/ml TdT enzyme.

23. A method according to any preceding claim, wherein step b) is performed in the presence of 0.5-2 mM CoCl$_2$.

24. A method according to either claim 22 or claim 23, wherein step b) is performed in the presence of ImM CoCl$_2$.

25. A method according to any preceding claim, wherein step c) comprises amplifying the tailed double-stranded DNA by performing the polymerase chain reaction using the primers employed in step a).

26. A method according to any preceding claim, wherein step c) is effected in the presence of a buffer comprising:

20-500 mM Tris pH 8.3;
10-300 mM KCl;
1-20 mM MgCl$_2$;
2-200 mM Tris Acetate pH 7.9;
5-500 mM Potassium Acetate; and
1-10 mM Mg Acetate.

27. A method according to any preceding claim, wherein the reaction mixture of step c) comprises:

6-7 µM Oligonucleotide;
0.1-10 mM dNTPs;
2.5-250 µg/ml Glycogen;
0.03-3.3 mM CoCl$_2$;
0.02-1% Triton X-100;
0.005-2.5% NP-40;
35-40 µg/ml BSA;
0.05-5 mM additional dATP;
0.005 - 2500 µM dT24;
0.01-5 u/µl DNA Polymerase;
0.01-5 u/ml RNase Inhibitor; and
1-500 u/ml TdT enzyme.

28. A method according to any preceding claim, wherein either or both of steps a) and c) are carried out in the presence of a volume excluding agent.

29. The method of claim 28, wherein the volume excluding agent is PEG.

30. A method according to any preceding claim, wherein either or both of steps a) and c) are carried out in the presence of an additional foreign template nucleic acid, which has a sequence not found in the template RNA population.

31. The method of claim 30, wherein the foreign template nucleic acid is a non-eukaryotic nucleic acid, and the template RNA is eukaryotic, preferably mammalian, RNA.

32. The method of claim 30 or 31, wherein the template RNA is mammalian RNA, and the foreign template nucleic acid is synthetic or native lambda phage genome nucleic acid.

33. A kit for producing globally amplified cDNA, the kit comprising:
   (i) an exonuclease;
   (ii) terminal transferase, and/or primers; and
   (iii) labelled nucleotides.

34. A kit according to claim 33, further comprising reagents for PCR.
35. A kit according to any one of claims 33 to 34, wherein the labelled nucleotides are labelled with a reporter molecule, and are preferably fluorescently labelled or biotin labelled.

36. A kit according to any one of claims 33 to 35, wherein the nuclease is an endonuclease, exonuclease or is a DNA polymerase.

37. A kit according to any one of claims 33 to 36, further comprising one or more acetate buffers.

38. The kit according to any one of claims 33 to 37, for use in carrying out the method of any one of claims 1 to 32.

39. A kit according to any one of claims 33 to 38, comprising one or more buffers comprising:
   Tris HCl;
   KCl;
   MgCl₂;
   Tris acetate;
   K acetate; and
   Mg acetate.

40. A kit according to claim 39, wherein a first buffer comprises:
   20-500 mM Tris HCl;
   10-300 mM KCl;
   1-20 mM MgCl₂;
   2-200 mM Tris acetate;
   5-500 mM K acetate; and
   1-10 mM Mg acetate.

41. A kit according to either claim 39 or claim 40, wherein a second buffer comprises:
   10-250 mM Tris pH 8.3;
   5-150 mM KCl;
0.5-10 mM \( \text{MgCl}_2 \);
2-200 mM Tris Acetate pH 7.9;
5-500 mM Potassium Acetate; and
1-10 mM Mg Acetate.

42. A kit according to any one of claims 33 to 41, further comprising:
Glycogen;
\( \text{CoCl}_2 \);
NP-40;
dNTPs;
dT;
RNase inhibitors;
bovine serum albumin;
dATP; and
Triton X-100.

43. A kit according to claim 42 comprising:
2.5-250 \( \mu \text{M} \) Glycogen;
0.1-10 mM \( \text{CoCl}_2 \);
0.005-2.5% NP-40;
1-100 \( \mu \text{M} \) dNTPs;
0.005-2500 \( \mu \text{M} \) dT;
0.01-5u/ml RNase inhibitors;
70-80 \( \mu \text{g/ml} \) bovine serum albumin;
0.05-5 mM dATP; and
0.02-1% Triton X-100.

44. A kit according to claim 42 or 43 wherein the glycogen and/or the BSA or a functional equivalent are synthetically produced.

45. A kit according to any one of claims 33 to 44, further comprising reverse transcriptase.
46. A kit according to any one of claims 33 to 45, further comprising a DNA polymerase.

47. A kit according to any one of claims 33 to 46, comprising terminal transferase.

48. A kit according to any one of claims 33 to 47, wherein the primers comprise a homopolymer sequence; preferably poly-T.

49. A kit according to any one of claims 33 to 48, comprising a volume excluding agent.

50. A kit according to any one of claims 33 to 49, comprising native or synthetic lambda phage genome nucleic acid.

51. The use of amplified DNA prepared according to the method of any one of claims 1 to 32 for subsequent sequencing.
Figure 1

Figure 2

<table>
<thead>
<tr>
<th>Platform</th>
<th>Sample C</th>
<th>Sample D</th>
<th>Sample C</th>
<th>Sample D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affymetrix</td>
<td>90.9</td>
<td>91.1</td>
<td>99.7</td>
<td>99.3</td>
</tr>
<tr>
<td>ABI</td>
<td>91.6</td>
<td>92.8</td>
<td>88.0</td>
<td>89.7</td>
</tr>
<tr>
<td>Agilent</td>
<td>93.0</td>
<td>93.9</td>
<td>94.7</td>
<td>92.8</td>
</tr>
<tr>
<td>Illumina</td>
<td>93.0</td>
<td>93.6</td>
<td>96.3</td>
<td>97.2</td>
</tr>
<tr>
<td>GE Healthcare</td>
<td>92.3</td>
<td>93.4</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td>Epistem agilent</td>
<td>97.1</td>
<td>93.3</td>
<td>96.9</td>
<td>99.7</td>
</tr>
</tbody>
</table>
Figure 3

CoCl₂: - - + + + + + + + +

100pg RNA input

25pg RNA input

12.5pg RNA input

Figure 4

NSRT in NTC: + - + - + - + + + + + +
Figure 5

**BIRC5 (Survivin)**

- **SUHRR**
- **HBRR**

40-Ct

Figure 6

**ERMIN**

- **SUHRR**
- **HBRR**

40-Ct

Figure 7

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>Amount RNA (pg)</th>
<th>Average %P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBRR</td>
<td>100</td>
<td>42.02</td>
</tr>
<tr>
<td>SUHRR</td>
<td>100</td>
<td>45.21</td>
</tr>
<tr>
<td>HBRR</td>
<td>25</td>
<td>30.78</td>
</tr>
<tr>
<td>SUHRR</td>
<td>25</td>
<td>35.04</td>
</tr>
<tr>
<td>HBRR</td>
<td>12.5</td>
<td>24.94</td>
</tr>
<tr>
<td>SUHRR</td>
<td>12.5</td>
<td>25.4</td>
</tr>
</tbody>
</table>
Figure 8

PCA Mapping (60.5%)

RNA Sample Type
- HBRR
- SUNRR

Amount RNA pg
- 12.5
- 25
- 100

PC #1 37.2%
PC #2 23.3%

PC #3 17.8%

PC #4 14.0%

PC #5 13.6%

PC #6 12.2%
According to International Patent Classification (IPC) or to both national classification and IPC

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/10 C12Q 1/69

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, COMPENDEX, Sequence Search, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WO 2004/013351 A2 (EPITEM LTD [GB]; BRADY GERARD [GB]) 12 February 2004 (2004-02-12) the whole document, in particular par. 5, 3rd par.; p. 7, last par.; p. 8-9; p. 18; p. 20-23 and claims, especially claims 22-57</td>
<td>1-51</td>
</tr>
<tr>
<td>Y</td>
<td>the whole document, in particular [0041] and [0061-0065] and claims 13 and 14</td>
<td>1-51</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search
18 June 2012

Date of mailing of the international search report
02/07/2012

Bassi as, Ioannis

Authorized officer
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>EP 1 845 160 Al (RIKEN [JP])</td>
<td>1-51</td>
</tr>
<tr>
<td></td>
<td>17 October 2007 (2007-10-17) the whole document, in particular the claims</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>EP 1 241 268 A2 (NISSHIN SPINNING [JP])</td>
<td>1-51</td>
</tr>
<tr>
<td></td>
<td>18 September 2002 (2002-09-18) the whole document</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>EP 1 469 069 Al (RI KAGAKU KENKYUSHO [JP])</td>
<td>1-51</td>
</tr>
<tr>
<td></td>
<td>20 October 2004 (2004-10-20) the whole document, in particular [0038] and [0041]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-51</td>
<td></td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1521829 A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2005244831 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2004013351 A2</td>
</tr>
<tr>
<td>US 2007218478 A1</td>
<td>20-09-2007</td>
<td>NONE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2009291852 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2006085616 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2002262882 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2002127592 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2005010159 A2</td>
</tr>
<tr>
<td>EP 1469069 A1</td>
<td>20-10-2004</td>
<td>NONE</td>
</tr>
</tbody>
</table>