

(19) **DANMARK**

(10) **DK/EP 3430373 T3**



(12) **Oversættelse af
europæisk patentskrift**

Patent- og
Varemærkestyrelsen

-
- (51) Int.Cl.: **G 01 N 1/34 (2006.01)** **G 01 N 1/40 (2006.01)**
- (45) Oversættelsen bekendtgjort den: **2023-10-02**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2023-07-05**
- (86) Europæisk ansøgning nr.: **17707850.8**
- (86) Europæisk indleveringsdag: **2017-02-28**
- (87) Den europæiske ansøgnings publiceringsdag: **2019-01-23**
- (86) International ansøgning nr.: **EP2017054582**
- (87) Internationalt publikationsnr.: **WO2017157650**
- (30) Prioritet: **2016-03-18 EP 16161077**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Hummingbird Diagnostics GmbH, Im Neuenheimer Feld 583, 69120 Heidelberg, Tyskland**
- (72) Opfinder: **BEIER, Markus, Marc-Aurel-Weg 20, 69469 Weinheim, Tyskland**
KELLER, Andreas, Albert-Schweitzer-Str. 6, 66346 Püttlingen, Tyskland
LAUFER, Thomas, Georg-Kieser-Weg 30, 68535 Edingen-Neckerhausen, Tyskland
KAHRAMAN, Mustafa, Nauwieserstr. 52, 66111 Saarbrücken, Tyskland
- (74) Fuldmægtig i Danmark: **NORDIC PATENT SERVICE A/S, Bredgade 30, 1260 København K, Danmark**
- (54) Benævnelse: **OPRENSNING AF RNA-FRAKTIONER VED HJÆLP AF ET HYDROFILT POLYMERMATERIALE**
- (56) Fremdragne publikationer:
WO-A1-2012/007569
WO-A1-2013/022342
WO-A1-2013/043562
WO-A1-2013/067520
WO-A1-2013/165870
WO-A1-2015/059065
WO-A2-02/072870
EDELBROEK PETER M ET AL: "Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls", THERAPEUTIC DRUG MONITORING, LIPPINCOTT WILLIAMS AND WILKINS, NEW YORK, NY, US, vol. 31, no. 3, 1 June 2009 (2009-06-01), pages 327-336, XP009126006, ISSN: 0163-4356
GOHRING K ET AL: "Influence of different extraction methods and PCR techniques on the sensitivity of HCMV-DNA detection in dried blood spot (DBS) filter cards", JOURNAL OF CLINICAL VIROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 48, no. 4, 1 August 2010 (2010-08-01), pages 278-281, XP027141568, ISSN: 1386-6532 [retrieved on 2010-07-13]
B. VOGEL ET AL: "Multivariate miRNA signatures as biomarkers for non-ischaemic systolic heart failure", EUROPEAN HEART JOURNAL, vol. 34, no. 36, 2 September 2013 (2013-09-02), pages 2812-2823, XP055145967,

Fortsættes ...

ISSN: 0195-668X, DOI: 10.1093/eurheartj/eh256

VOELLENKLE CHRISTINE ET AL: "MicroRNA signatures in peripheral blood mononuclear cells of chronic heart failure patients", PHYSIOLOGICAL GENOMICS, AMERICAN PHYSIOLOGICAL SOCIETY, US, vol. 42, no. 3, 1 August 2010 (2010-08-01) , pages 420-426, XP008133127, ISSN: 1094-8341, DOI: 10.1152/PHYSIOLGENOMICS.00211.2009 [retrieved on 2010-05-18]

DESCRIPTION

[0001] The present invention relates to a method of purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample. The present invention further relates to a method of determining the level of RNA molecules with < 200 nucleotides in length. In addition, the present invention relates to a method for diagnosing a disease in an individual. Moreover, the present invention relates to the use of a kit for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample.

BACKGROUND OF THE INVENTION

[0002] Whole blood samples are used for molecular biological analyses, e.g. in order to determine whether a patient suffers from a specific disease or not. These samples are often collected by sampling whole blood freezing and then processing the frozen blood later. Frozen blood requires a sample size of at least 200 µl. High costs are involved with the freezing transportation and processing of whole blood.

[0003] Therefore, the collection of whole blood samples using a bloodspot technique is preferred. It requires smaller sample volumes, typically 45-60 µl for humans, although evolving analytical techniques are using samples using 10-15 µl of human blood and smaller. Once sampling is complete, the blood spots are dried in air before transferring or mailing to labs for processing. Because the blood is dried, it is not considered hazardous. Thus no special precautions need be taken in handling or shipping. Once at the analysis site, the desired components, e.g. proteins or metabolites, are extracted from the dried blood spots into a supernatant which is then further analyzed, e.g. by liquid chromatography and/or mass spectrometry.

[0004] Today, biomarkers which are comprised in whole blood play a key role in early diagnosis, risk stratification, and therapeutic management of diseases. In particular, microRNAs (miRNAs) are biomarkers which are found in whole blood. They represent a group of regulatory elements that enable cells to fine-tune complex gene expression cascades in a wide range of biological processes, such as proliferation, differentiation, apoptosis, stress-response, and oncogenesis. They exist in blood as free circulating nucleic acids and in blood cells. This is due to the fact that miRNAs are expressed in blood cells or in diverse tissues which release the miRNAs into circulating blood.

[0005] At present, blood is collected using PaxGene blood RNA tubes in order to determine the level of RNA biomarkers such as miRNAs comprised therein. Great instrument-based and analytical effort is necessary to purify and isolate RNA biomarkers such as miRNAs comprised therein. In addition, this techniques are very time consuming and expensive.

[0006] WO 2013/165870 A1 discloses solid compositions for ambient extraction, stabilization,

and preservation of nucleic acids from a biological sample in a dry format.

[0007] There is, thus, a need for an improved method of blood preparation that reduces or eliminates one or more of the above errors and difficulties.

[0008] The inventors of the present invention assessed for the first time that the bloodspot technique is useful in order to reduce the complexity of a RNA fraction derived from a whole blood sample. In particular, the inventors of the present invention found that the bloodspot technique is valuable in order to remove RNA fractions with ≥ 200 nucleotides in length from a whole blood sample or to purify RNA fractions with < 200 nucleotides in length (including miRNA) from a whole blood sample. The purified and/or isolated RNA fractions comprise RNA molecules. The inventors of the present invention further found that expression profiles of such purified and/or isolated RNA molecules, e.g. miRNA molecules, can be determined and that said expression profiles are of great quality. In addition, the inventors of the present invention found that the blood spot technique provides sample stability under different environmental conditions (e.g. temperature and/or humidity), provides technical stability and allows the diagnosis or differential diagnosis of an individual suffering from a disease.

SUMMARY OF THE INVENTION

[0009] In a first aspect, the present invention relates to a method of purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample comprising the steps of:

1. (i) providing an absorbent probe comprising a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed,
2. (ii) contacting the absorbent probe with a fluid comprising a chaotropic agent (to reconstitute the whole blood), and
3. (iii) recovering the fluid comprising a chaotropic agent from step (ii), thereby removing the RNA fraction with ≥ 200 nucleotides in length, and .
4. (iv) purifying the RNA fraction with < 200 nucleotides in length from the recovered fluid by one or more separation techniques selected from the group consisting of centrifugation, evaporation/reconstitution, concentration, precipitation, liquid/liquid extraction, and solid phase extraction,

wherein the RNA fraction with ≥ 200 nucleotides in length does not enter the fluid and remains absorbed to the hydrophilic polymeric material,

wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester.

[0010] In a second aspect, the present invention relates to a method of determining the level

of RNA molecules with < 200 nucleotides in length comprising the steps of:

1. (i) carrying out the method according to the first aspect, and
2. (ii) determining the level of RNA molecules with < 200 nucleotides in length by a suitable technique.

[0011] In a third aspect, the present invention relates to a method for diagnosing a disease in an individual comprising the steps of:

1. (i) carrying out the method according to the first aspect, wherein the whole blood sample is from an individual,
2. (ii) determining the level of RNA molecules with < 200 nucleotides in length by a suitable technique,
3. (iii) comparing said level to one or more reference level(s), and
4. (iv) diagnosing or differentially diagnosing whether the individual is afflicted by the disease based on the comparison.

[0012] Said third aspect may alternatively be formulated as follows: A method for diagnosing a disease in an individual comprising the steps of:

1. (i) carrying out the method according to the second aspect, wherein the whole blood sample is from an individual,
2. (ii) comparing said level to one or more reference level(s), and
3. (iii) diagnosing or differentially diagnosing whether the individual is afflicted by the disease based on the comparison.

[0013] In a fourth aspect, the present invention relates to the use of a hydrophilic polymeric material for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample, wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester.

[0014] In a fifth aspect, the present invention relates to the use of a method according to the second aspect for diagnosing or differentially diagnosing a disease.

[0015] In a sixth aspect, the present invention relates to the use of a kit for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample, wherein said kit comprises

1. (i) an absorbent probe comprising a hydrophilic polymeric material, and
2. (ii) a fluid comprising a chaotropic agent to reconstitute whole blood absorbed in/to an

absorbent probe comprising a hydrophilic polymeric material,

wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester.

[0016] This summary of the invention does not necessarily describe all features of the present invention. Other embodiments will become apparent from a review of the ensuing detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0017] Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

[0018] Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

[0019] The term "comprise" or variations such as "comprises" or "comprising" according to the present invention means the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. The term "consisting essentially of" according to the present invention means the inclusion of a stated integer or group of integers, while excluding modifications or other integers which would materially affect or alter the stated integer. The term "consisting of" or variations such as "consists of" according to the present invention means the inclusion of a stated integer or group of integers and the exclusion of any other integer or group of integers.

[0020] The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

[0021] The term "nucleotides", as used herein, refers to organic molecules that serve as the monomers, or subunits, of nucleic acids like DNA (deoxyribonucleic acid) and RNA (ribonucleic

acid). The building blocks of nucleic acids, nucleotides are composed of a nitrogenous base, a five-carbon sugar (ribose or deoxyribose), and at least one phosphate group. Thus a nucleoside plus a phosphate group yields a nucleotide.

[0022] The term "RNA fraction with ≥ 200 nucleotides in length", as used herein, refers to an RNA portion which comprises large RNA molecules, namely one or more RNA molecules having a length of ≥ 200 nucleotides. The one or more RNA molecules encompass ribosomal RNA (rRNA) molecules. They may be selected from the group consisting of ribosomal 18S RNA and ribosomal 28S RNA.

[0023] In the context of the present invention, term "ribosomal ribonucleic acid (abbreviated rRNA)" refers to the RNA component of the ribosome, and is essential for protein synthesis in all living organisms. It constitutes the predominant material within the ribosome, which is approximately 60% rRNA and 40% protein by weight. Ribosomes contain two major rRNAs and 50 or more proteins. The ribosomal RNAs form two subunits, the large subunit (LSU) and small subunit (SSU). The large subunit encompasses the ribosomal 28S RNA and the small subunit encompasses the ribosomal 18S RNA.

[0024] The term "RNA fraction with < 200 nucleotides in length", as used herein, refers to an RNA portion which comprises small RNA molecules, namely one or more RNA molecules having a length of < 200 nucleotides in length. The one or more RNA molecules may be selected from the group consisting of miRNA, tRNA, siRNA, piRNA, and snorRNA.

[0025] The term "microRNA (abbreviated miRNA)" refers to single-stranded RNA molecules having a length of at least 10 nucleotides and of not more than 40 nucleotides. The nucleotides are covalently linked together. For example, the single-stranded RNA molecules have a length of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides. The miRNAs regulate gene expression and are encoded by genes from whose DNA they are transcribed but miRNAs are not translated into protein (i.e. miRNAs are non-coding RNAs). The genes encoding miRNAs are longer than the processed mature miRNA molecules. The miRNAs are first transcribed as primary transcripts or pri-miRNAs with a cap and poly-A tail and processed to short, 70 nucleotide stem-loop structures known as pre-miRNAs in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha. These pre-miRNAs are then processed to mature miRNAs in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC). When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC, on the basis of the stability of the 5' end. The remaining strand, known as the miRNA*, anti-guide (anti-strand), or passenger strand, is degraded as a RISC substrate. Therefore, the miRNA*s are derived from the same hairpin structure like the "normal" miRNAs. So if the "normal" miRNA is then later called the "mature miRNA" or "guide strand", the miRNA* is the "anti-guide strand" or "passenger strand".

[0026] The term "transfer RNA (abbreviated tRNA)", as used herein, refers to an adaptor molecule composed of RNA, typically 76 to 90 nucleotides in length that serves as the physical link between the mRNA and the amino acid sequence of proteins. It does this by carrying an amino acid to the protein synthetic machinery of a cell (ribosome) as directed by a three-nucleotide sequence (codon) in a messenger RNA (mRNA). As such, tRNAs are a necessary component of translation, the biological synthesis of new proteins according to the genetic code.

[0027] The term "small interfering RNA (abbreviated siRNA)", as used herein, refers to a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA plays many roles, but it is most notable in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequences. siRNA functions by causing mRNA to be broken down after transcription, resulting in no translation.

[0028] The term "piwi-interacting RNA (abbreviated piRNA)", as used herein, refer to the largest class of small non-coding RNA molecules expressed in animal cells. piRNAs form RNA-protein complexes through interactions with piwi proteins. These piRNA complexes have been linked to both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis. They are distinct from microRNA (miRNA) in size (26-31 nt rather than 21-24 nt), lack of sequence conservation, and increased complexity.

[0029] The term, "small nucleolar RNA (abbreviated snoRNAs)", as used herein, refers to a class of small RNA molecules that primarily guide chemical modifications of other RNAs, mainly ribosomal RNAs, transfer RNAs and small nuclear RNAs. There are two main classes of snoRNA, the C/D box snoRNAs, which are associated with methylation, and the H/ACA box snoRNAs, which are associated with pseudouridylation. SnoRNAs are commonly referred to as guide RNAs but should not be confused with the guide RNAs that direct RNA editing in trypanosomes.

[0030] The term "whole blood" as used herein, refers to non-fractionated blood. It, thus, comprises all blood components. Whole blood is made up of an acellular fluid (plasma or serum) and a mixture of multiple blood cell types. The three primary blood cell types are red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes). Each type contributes a different amount of RNA to the total RNA in blood.

[0031] The terms "red blood cells (RBC)" and "erythrocytes", as used herein, refer to the most abundant cells in whole blood, constituting 44% of the blood volume. Mature RBCs lose their nucleus and organelles during maturation and, thus, do not contribute any RNA to the total blood RNA pool. However, immature RBCs, also known as reticulocytes, may retain some residual nucleic acids. Because reticulocytes constitute about 1% of the RBC population, residual RNA from reticulocytes contributes up to 70% of the RNA in the total blood RNA pool.

[0032] The terms "white blood cells (WBC)" and "leukocytes", as used herein, refer to nucleated blood cells that carry out the immune functions of the body. Leukocytes are the most transcriptionally active cells in blood. Leukocytes are made up of granulocytes, lymphocytes, and monocytes. Granulocytes can be further divided into neutrophils, basophils, and eosinophils. Lymphocytes can be divided into B-cells, T-cells, and natural killer (NK) cells. The number of cells in each leukocyte subtype can change significantly during disease states such as inflammation and leukemia.

[0033] The terms "platelets" and "thrombocytes", as used herein, refer to blood cells playing a major role in blood clotting. Platelets enter the blood circulation by fragmentation of large bone marrow cells called megakaryocytes. Like red blood cells, platelets lack a nucleus and organelles. Immature platelets, called reticulated platelets, contain residual RNA from megakaryocytes. Up to 4.5% of platelet counts can be reticulated platelets. Platelet RNA, therefore, also contributes to the total RNA blood pool.

[0034] The term "plasma", as used herein, refers to the pale yellow liquid component of blood that normally holds the blood cells in whole blood in suspension. This makes plasma the extracellular matrix of blood cells. It is a fluid which is composed of about 92% water, 7% vital proteins such as albumin, gamma globulin, anti-hemophilic factor, and other clotting factors, and 1% mineral salts, sugars, fats, hormones and vitamins.

[0035] The term "serum", as used herein, refers to the blood component that is neither a blood cell (serum does not contain white or red blood cells) nor a clotting factor. It is the blood plasma not including the fibrinogens. Serum includes all proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances (e.g., drugs and microorganisms).

[0036] The term "whole blood sample", as used herein, refers to blood material which is not part of the body of an individual anymore. A whole blood sample may be provided by removing blood from an individual, but may also be provided by using previously isolated blood material. The whole blood sample may be removed from an individual using conventional blood collection techniques. For example, the whole blood may be extracted from a vein in the arm of an individual using a needle, or via finger prick. Thus, the whole blood sample may have the form of a blood drop. Said blood drop may be on an individual, e.g. on a fingertip of the individual. The whole blood sample may also be comprised in a blood collection tube, e.g. capillary tube, or in a pipette tip.

[0037] The term "blood spot" refers to a filter paper comprising one or more blood drops. Usually, the filter paper comprising the one or more blood drops is dried after sampling. The inventors of the present invention used a hydrophilic polymeric material to absorb the blood drops.

[0038] The term "blood spot technique" refers to a form of biosampling where blood samples are blotted on filter paper. After the biosampling, the filter paper comprising the blood is

preferably dried. The dried samples can easily be shipped to an analytical laboratory and analyzed using various methods such as RNA expression analysis. In particular, blood spot specimens are collected by applying a few drops of blood, drawn by lancet from the finger, heel or toe, onto absorbent filter paper. The blood is allowed to thoroughly saturate the paper and is preferably air dried. Specimens may be stored in low gas-permeability plastic bags with desiccant added to reduce humidity, and may be kept at ambient temperature, even in tropical climates. Once in the laboratory, technicians may separate a small disc of saturated paper from the sheet using an automated or manual hole punch for further analysis. As an alternative to punching out a paper disc, recent automation solutions may extract the sample by flushing an eluent through the filter without punching it out. The inventors of the present invention used a hydrophilic polymeric material onto which the blood is blotted/applied.

[0039] The term "individual" as used herein, refers to any subject from which a whole blood sample can be taken, obtained, or isolated.

[0040] The term "individual", as used herein, further refers to any subject for whom it is desired to know whether she or he suffers from a disease. In particular, the term "individual", as used herein, refers to a subject suspected to be affected by a disease. The individual may be diagnosed to be affected by the disease, i.e. diseased, or may be diagnosed to be not affected by the disease, i.e. healthy. The term "individual", as used herein, also refers to a subject which is affected by the disease, i.e. diseased. The patient may be retested for the disease and may be diagnosed to be still affected by the disease, i.e. diseased, or not affected by the disease anymore, i.e. healthy, for example after therapeutic intervention. It should be noted that a patient that is diagnosed as being healthy, i.e. not suffering from the disease, may possibly suffer from another disease not tested/known. The disease may be lung cancer. The individual for whom it is desired to know whether she or he suffers from a disease may also be designated as patient.

[0041] The individual may be a mammal, including both a human and another mammal, e.g. an animal such as a rodent, a rabbit, or a monkey. The rodent may be a mouse, rat, hamster, guinea pig, or chinchilla.

[0042] The term "(control) subject", as used herein, refers to a subject known to be affected by a disease (positive control), i.e. diseased. The term "(control) subject", as used herein, also refers to a subject known to be not affected by a disease (negative control), i.e. healthy. Thus, the term "healthy subject", as used herein, means a subject which is known to be not affected by a disease. It should be noted that a (control) subject which is known to be healthy, i.e. not suffering from the disease tested/known, may possibly suffer from another disease not tested/known. The disease may be lung cancer.

[0043] The (control) subject may be a mammal, including both a human and another mammal, e.g. an animal such as a rodent, a rabbit, or a monkey. The rodent may be a mouse, rat, hamster, guinea pig, or chinchilla.

[0044] The term "diagnosing a disease", as used herein, means determining whether an individual shows signs of or suffers from the disease. Preferably, the disease is lung cancer.

[0045] The term "differentially diagnosing a disease", as used herein, means determining whether an individual shows the signs of or suffers from a disease or shows the signs of or suffers from another disease. Preferably, the disease is lung cancer. The other disease is any other disease than lung cancer. "Differentially diagnosing a disease", as used in the context of the present invention, may also allow to distinguish differently treated diseases from each other. For example, the disease may be an adjuvant-treated disease, e.g. adjuvant-treated lung cancer, and the other disease may be a palliative-treated disease, e.g. palliative-treated lung cancer, or the disease may be a palliative-treated disease, e.g. palliative-treated lung cancer, and the other disease may be an adjuvant-treated disease, e.g. adjuvant-treated lung cancer.

[0046] The term "treatment", in particular "therapeutic treatment", as used herein, refers to any therapy which improves the health status and/or prolongs (increases) the lifespan of an individual. Said therapy may eliminate the disease in an individual, arrest or slow the development of a disease in an individual, inhibit or slow the development of a disease in an individual, decrease the frequency or severity of symptoms in an individual, and/or decrease the recurrence in an individual who currently has or who previously has had a disease. The therapeutic treatment may be a treatment of lung cancer. The therapeutic treatment of lung cancer is selected from the group consisting of chemotherapy, surgery, radiotherapy, adjuvant therapy, and palliative therapy.

[0047] The term "absorbent probe", as used herein, refers to any probe which can absorb whole blood comprised in the whole blood sample. The absorbent probe used herein comprises, essentially consists of, or consists of a hydrophilic polymeric material.

[0048] The term "hydrophilic polymeric material", as used herein, refers to any material which can absorb whole blood comprised in the whole blood sample. The term "hydrophilic polymeric material", as used herein, further refers to a polymeric material which contains polar or charged functional groups, rendering the material soluble in water. A hydrophilic polymeric material comprises hydrophilic polymers. A hydrophilic polymer is produced from two or more hydrophilic monomers. The term "hydrophilic polymeric material", as used herein, also encompasses a polymeric material which is not hydrophilic by nature, e.g. polyethylene, but which has been rendered hydrophilic. If the polymeric material is not initially hydrophilic, then there are numerous methods for converting the material into a hydrophilic state. Methods for creating hydrophilic surfaces include adsorptive treatment with surfactants such as Tween-40 or Tween-80 to create hydrophilic surfaces. Treatment may also occur with other molecules containing both hydrophilic and hydrophobic elements. The hydrophobic elements will interact strongly with the hydrophobic polymeric material and expose the hydrophilic elements creating hydrophilic surfaces. Additionally treatment with plasma (Corona, Air, Flame, or Chemical) is another well-known method of adding polar groups to the surfaces of such materials, including oxygen plasma treatments. Likewise, the grafting of hydrophilic polymers to the surface and

the chemical functionalization of active groups on the surface with polar or hydrophilic molecules such as sugars can be used to achieve a hydrophilic surface on a polymeric material. Covalent modification could also be used to add polar or hydrophilic functional groups to the surface of a polymeric material.

[0049] The hydrophilic polymeric material may be selected from the group consisting of a cellulose comprising material, e.g. cotton, a polysaccharide, a polyolefin, and a polyester, or modifications thereof. The polysaccharide may be cellulose, the polyolefin may be selected from the group consisting of polyethylene, polypropylene, polybutylene, polyisobutylene, and polymethylpentene, and the polyester may be selected from the group consisting of polycarbonate and polyethyleneterephthalate. Cotton, cellulose, and polyethylene as hydrophilic polymeric materials are particularly preferred.

[0050] Examples of devices/probes comprising a hydrophilic polymeric material include, but are not limited to, Mitra Microsampling Device (neoteryx, Torrance, CA, USA), Non-Indicating FTA Classic Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), Non-Indicating FTA Elute Micro Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), HemaSpot HF (Spot On Sciences, Austin, TX, USA), HemaSpot SE (Spot On Sciences, Austin, TX, USA), TNF (Munktell, Bärenstein, Germany), and TNF-Di (Munktell, Bärenstein, Germany).

[0051] The term "level", as used herein, refers to an amount (measured for example in grams, mole, or ion counts) or concentration (e.g. absolute or relative concentration) of RNA molecules with < 200 nucleotides in length. The term "level", as used herein, also comprises scaled, normalized, or scaled and normalized amounts or values. In one embodiment, the level is the expression level of RNA molecules with < 200 nucleotides in length. Said expression level may be indicated as (relative) RNA (e.g. miRNA) concentration, (relative) RNA (e.g. miRNA) amount, or (relative) RNA (e.g. miRNA) extinction units such as relative fluorescence units.

[0052] In the context of the present invention, the term "kit of parts (abbreviated kit)" is understood to be any combination of at least some of the components identified herein, which are combined, coexisting spatially, to a functional unit, and which can contain further components.

Embodiments of the invention

[0053] The present invention will now be further described. In the following passages different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous, unless clearly indicated to the contrary.

[0054] The inventors of the present invention assessed for the first time that the bloodspot technique is useful in order to reduce the complexity of a whole blood sample. In particular, the inventors of the present invention found that the bloodspot technique is valuable in order to remove RNA fractions with ≥ 200 nucleotides in length from a whole blood sample or to purify RNA fractions with < 200 nucleotides in length (including miRNA) from a whole blood sample. The purified and/or isolated RNA fractions comprise RNA molecules. The inventors of the present invention further found that expression profiles of such purified and/or isolated RNA molecules, e.g. miRNA molecules, can be determined and that said expression profiles are of great quality. In addition, the inventors of the present invention found that the blood spot technique provides sample stability under different environmental conditions (e.g. temperature and/or humidity), provides technical stability and allows the diagnosis or differential diagnosis of an individual suffering from a disease.

[0055] Described herein, but not encompassed by the present invention, is a method of removing an RNA fraction with ≥ 200 nucleotides in length from a whole blood sample comprising the steps of

1. (i) providing an absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed,
2. (ii) contacting the absorbent probe with a fluid (to reconstitute the whole blood), and
3. (iii) recovering the fluid from step (ii), thereby removing the RNA fraction with ≥ 200 nucleotides in length.

[0056] In step (i), an absorbent probe is provided, which comprises, essentially consists of, or consists of a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed. Therewith, RNA molecules with ≥ 200 nucleotides in length as well as RNA molecules with < 200 nucleotides in length which are comprised in the whole blood have been absorbed in/to the hydrophilic polymeric material.

[0057] The absorbent probe provided in step (i) may be obtained by placing an absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic polymeric material in (physical) contact with the whole blood sample, thereby absorbing whole blood in/to the hydrophilic polymeric material,

[0058] A (physical) contact between the absorbent probe and the whole blood sample may be achieved by dipping or immersing the absorbent probe into the whole blood sample. The whole blood sample may have the form of a blood drop. The whole blood may also be comprised in a blood collection tube. Alternatively, one or more drops of the whole blood sample may be applied to the absorbent probe, e.g. via a pipette. Due to the (physical) contact, the whole blood is soaked in/to the hydrophilic polymeric material and therewith the RNA molecules with ≥ 200 nucleotides in length as well as RNA molecules with < 200 nucleotides in length which are comprised in the whole blood.

[0059] Normally, it is sufficient to maintain the probe in (physical) contact with the whole blood sample for between 1 to 20 seconds, preferably for between 1 to 10 seconds, more preferably for between 1 to 5 seconds, e.g. for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 seconds. The contact time is desirably as short as possible. The probe absorbs a predetermined volume of blood during that time and, once saturated, does not absorb more blood. The size and shape of the probe can be varied to adjust the volume of absorbed blood and rate of absorption. Blood volumes of between 1 to 50 μ l, preferably of between 5 to 25 μ l, and even more preferably of between 10 to 20 μ l, e.g. of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 μ l, are believed suitable.

[0060] In step (ii), the absorbent probe is contacted with a fluid. The fluid may be a liquid or gas. Preferably, the fluid is a chaotropic fluid, e.g. a chaotropic liquid or gas. More preferably, the chaotropic fluid is selected from the group consisting of guanidinium thiocyanate, guanidinium isothiocyanate, guanidinium hydrochloride, guanidinium chloride, alkali thiocyanate, alkali isothiocyanate, alkali iodide, and alkali perchlorate. Even more preferably, the chaotropic fluid is guanidinium thiocyanate. Alternatively, the chaotropic fluid, e.g. the chaotropic liquid or gas, may comprise guanidinium thiocyanate, guanidinium isothiocyanate, guanidinium hydrochloride, guanidinium chloride, alkali thiocyanate, alkali isothiocyanate, alkali iodide, and alkali perchlorate. Preferably, the chaotropic fluid comprises guanidinium thiocyanate.

[0061] A contact between the absorbent probe and the fluid may be achieved by dipping or immersing the absorbent probe into the fluid. This can be done by hand or by a fluid handling robot. Step (ii) allows to reconstitute the whole blood. Physical agitation techniques such as sonication, mixing, or vortexing of the fluid and/or the absorbent probe can accelerate the reconstitution process. In particular, step (ii) allows the separation of molecules comprised in the whole blood from each other. Some molecules can be removed from the hydrophilic polymeric material, other molecules cannot. These molecules remain absorbed in/to the hydrophilic polymeric material. The inventors of the present invention surprisingly found that RNA molecules with ≥ 200 nucleotides in length remain absorbed in/to the hydrophilic polymeric material and do not enter the fluid, while RNA molecules with < 200 nucleotides in length can be extracted from the hydrophilic polymeric material by the fluid in step (ii).

[0062] In step (iii) the fluid is recovered from step (ii). Due to this process, the RNA fraction with ≥ 200 nucleotides in length is removed. In this respect, it should be noted that the removal of the RNA fraction with ≥ 200 nucleotides in length does not necessarily mean that the RNA fraction with ≥ 200 nucleotides in length is completely removed. Also embodiments are described, wherein at least a part of the RNA molecules with ≥ 200 nucleotides in length is removed. For example, in some embodiments, at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99%, or 100% of the RNA molecules with ≥ 200 nucleotides in length are removed. It is preferred that at least 90% of the RNA molecules with ≥ 200 nucleotides in length are removed. It is more preferred that at least 95% of the RNA molecules with ≥ 200 nucleotides in

length are removed. It is even more preferred that at least 98% or 99% of the RNA molecules with ≥ 200 nucleotides in length are removed. It is most preferred that 100% of the RNA molecules with ≥ 200 nucleotides in length, i.e. all RNA molecules with ≥ 200 nucleotides in length, are removed.

[0063] The recovering of the fluid from step (ii) may be achieved by removing the absorbent probe from the fluid.

[0064] Preferably, the RNA fraction with ≥ 200 nucleotides in length comprises one or more RNA molecules selected from the group consisting of ribosomal 18S RNA and ribosomal 28S RNA.

[0065] In a first aspect, the present invention relates to a method of purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample comprising the steps of:

1. (i) providing an absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed,
2. (ii) contacting the absorbent probe with a fluid comprising a chaotropic agent (to reconstitute the whole blood), and
3. (iii) recovering the fluid comprising a chaotropic agent from step (ii), thereby removing the RNA fraction with ≥ 200 nucleotides in length, and
4. (iv) purifying the RNA fraction with < 200 nucleotides in length from the recovered fluid by one or more separation techniques selected from the group consisting of centrifugation, evaporation/reconstitution, concentration, precipitation, liquid/liquid extraction, and solid phase extraction,

wherein the RNA fraction with ≥ 200 nucleotides in length does not enter the fluid and remains absorbed to the hydrophilic polymeric material

wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester.

[0066] In step (i), an absorbent probe is provided, which comprises, essentially consists of, or consists of a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed. Therewith, RNA molecules with ≥ 200 nucleotides in length as well as RNA molecules with < 200 nucleotides in length which are comprised in the whole blood has been absorbed in/to the hydrophilic polymeric material.

[0067] The absorbent probe provided in step (i) may be obtained by placing an absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic polymeric material in (physical) contact with the whole blood sample, thereby absorbing whole blood in/to the

hydrophilic polymeric material,

[0068] A (physical) contact between the absorbent probe and the whole blood sample may be achieved by dipping or immersing the absorbent probe into the whole blood sample. The whole blood sample may have the form of a blood drop. The whole blood may also be comprised in a blood collection tube. Alternatively, one or more drops of the whole blood sample may be applied to the absorbent probe, e.g. via a pipette. Due to the (physical) contact, the whole blood is soaked in/to the hydrophilic polymeric material and therewith the RNA molecules with ≥ 200 nucleotides in length as well as RNA molecules with < 200 nucleotides in length which are comprised in the whole blood.

[0069] Normally, it is sufficient to maintain the probe in (physical) contact with the whole blood sample for between 1 to 20 seconds, preferably for between 1 to 10 seconds, more preferably of between 1 to 5 seconds, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 seconds. The contact time is desirably as short as possible. The probe absorbs a predetermined volume of blood during that time and, once saturated, does not absorb more blood. The size and shape of the probe can be varied to adjust the volume of absorbed blood and rate of absorption. Blood volumes of between 1 to 50 μl , preferably of between 5 to 25 μl , even more preferably of between 10 to 20 μl , e.g. of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 μl , are believed suitable.

[0070] In step (ii), the absorbent probe is contacted with a fluid comprising a chaotropic agent. The fluid may be a liquid or gas. Preferably, the fluid is a liquid or gas comprising a chaotropic agent. More preferably, the chaotropic agent is selected from the group consisting of guanidinium thiocyanate, guanidinium isothiocyanate, guanidinium hydrochloride, guanidinium chloride, alkali thiocyanate, alkali isothiocyanate, alkali iodide, and alkali perchlorate. Even more preferably, the chaotropic agent is guanidinium thiocyanate.

[0071] A contact between the absorbent probe and the fluid comprising a chaotropic agent may be achieved by dipping or immersing the absorbent probe into the fluid comprising a chaotropic agent. This can be done by hand or by a fluid handling robot. Step (ii) allows to reconstitute the whole blood. Physical agitation techniques such as sonication or vortexing of the fluid and/or the absorbent probe can accelerate the reconstitution process. In particular, step (ii) allows the separation of molecules comprised in the whole blood from each other. Some molecules can be removed from the hydrophilic polymeric material, other molecules cannot. These molecules remain absorbed in/to the hydrophilic polymeric material. The inventors of the present invention surprisingly found that RNA molecules with ≥ 200 nucleotides in length remain absorbed in/to the hydrophilic polymeric material and does not enter the fluid comprising a chaotropic agent, while RNA molecules with < 200 nucleotides in length can be removed from the hydrophilic polymeric material by the fluid comprising a chaotropic agent in step (ii).

[0072] In step (iii), the fluid comprising a chaotropic agent is recovered from step (ii). Thereby,

the RNA fraction with ≥ 200 nucleotides in length is removed. The RNA fraction with < 200 nucleotides in length is recovered from the fluid comprising a chaotropic agent. It is particularly recovered from the aqueous phase of the reconstituted blood sample.

[0073] In step (iv), the RNA fraction with < 200 nucleotides in length is purified from the recovered fluid by one or more separation techniques selected from the group consisting of centrifugation, evaporation/reconstitution, concentration, precipitation, liquid/liquid extraction, and solid phase extraction.

[0074] The recovering of the fluid comprising a chaotropic agent from step (ii) may be achieved by removing the absorbent probe from the fluid comprising a chaotropic agent.

[0075] Preferably, the RNA fraction with < 200 nucleotides in length comprises one or more RNA molecules selected from the group consisting of miRNA, tRNA, siRNA, piRNA, and snorRNA.

[0076] In one embodiment of the first aspect of the present invention, the absorbent probe is contacted with the fluid comprising a chaotropic agent for ≤ 20 hours, preferably for ≤ 5 hours, e.g. for $\leq 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29$ minute(s), $0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19$, or 20 hour(s). More preferably, the absorbent probe is contacted with the fluid comprising a chaotropic agent for between 1 minute and 20 hours, even more preferably for between 1 minute and 5 hours, e.g. for $1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29$ minute(s), $0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19$, or 20 hour(s).

[0077] Preferably, the hydrophilic polymeric material has a density of $\leq 4 \text{ g/cm}^3$, e.g. of $0.5, 1.5, 2, 2.5, 3, 3.5$, or 4 g/cm^3 . More preferably, the hydrophilic polymeric material has a density of between 1 and 4 g/cm^3 , e.g. of $1.5, 2, 2.5, 3, 3.5$, or 4 g/cm^3 .

[0078] In one embodiment of the first aspect of the present invention, the hydrophilic polymeric material is porous.

[0079] In one embodiment of the first aspect of the present invention, the hydrophilic polymeric material has a pore volume of between 20 and 70%, preferably of between 30 and 50%, e.g. of $20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69$, or 70 % of the total volume of the material.

[0080] In one embodiment of the first aspect of the present invention, the hydrophilic polymeric material comprises pores that are $\leq 100 \text{ }\mu\text{m}$, preferably $\leq 50 \text{ }\mu\text{m}$, e.g. $\leq 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95$, or $100 \text{ }\mu\text{m}$, in diameter or largest cross-sectional dimension. More preferably, the hydrophilic polymeric material comprises pores that are

between 10 to 100 μm , even more preferably between 20 and 50 μm , e.g. 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 μm , in diameter or largest cross-sectional dimension.

[0081] In the first aspect of the present invention, the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester. Preferably,

1. (i) the polysaccharide is cellulose,
2. (ii) the polyolefin is selected from the group consisting of polyethylene, polypropylene, polybutylene, polyisobutylene, and polymethylpentene, or
3. (iii) the polyester is selected from the group consisting of polycarbonate and polyethyleneterephthalate.

[0082] More preferably, the hydrophilic polymeric material is cotton, cellulose or polyethylene.

[0083] In one embodiment of the first aspect of the present invention, the absorbent probe has absorbed a predetermined maximum volume of whole blood of between 1 to 50 μl , e.g. of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 μl .

[0084] In one embodiment of the first aspect of the present invention, the absorbent probe has a length of ≤ 10 mm, preferably of ≤ 5 mm, e.g. of $\leq 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5$, or 10 mm, and a cross-sectional area of ≤ 40 mm^2 , preferably of ≤ 20 mm^2 , e.g. of $\leq 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39$, or 40 mm^2 .

[0085] Such sizes are believed suitable when the whole blood sample is a blood drop, e.g. from a finger prick.

[0086] In an alternative embodiment of the first aspect of the present invention the absorbent probe has a volume of ≤ 250 mm^3 , preferably of ≤ 200 mm^3 , e.g. of $\leq 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240$, or 250 mm^3 .

[0087] Such volumes are believed suitable when the whole blood sample is a blood drop, e.g. from a finger prick.

[0088] More preferably, the absorbent probe has a length of between 2 and 10 mm, preferably of between 2 and 5 mm, e.g. of 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10

mm, and a cross-sectional area of between 5 and 40 mm², preferably of between 5 and 20 mm², e.g. of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 mm².

[0089] In an alternative embodiment of the first aspect of the present invention, the absorbent probe has a volume of between 1 mm³ to 250 mm³, preferably of between 2.5 mm³ to 200 mm³, e.g. of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, or 250 mm³.

[0090] Flat ended cylinders or semicircular ends on cylindrical probes are believed desirable, but various configurations may be used.

[0091] In one embodiment of the first aspect of the present invention, the whole blood absorbed in/to the hydrophilic polymeric material has been dried. Drying may be carried out in the open air, e.g. at room temperature (20°C) or elevated temperatures (e.g. 30°C). Alternatively, the absorbent probe may be transferred to a special drying container configured to help drying while minimizing the contact between the absorbent probe and the walls of the drying container or other potential contaminant surfaces. The drying container may comprise a desiccant to facilitate drying. Drying may be carried out for between 0.5 and 5 hour(s) or for between 0.5 and 3 hour(s), e.g. for 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 hour(s). In this respect, it should be noted that the drying time may vary as it depends on humidity, temperature, volume to be dried and the shape and configuration of the absorbent probe. In addition, drying the blood quickly helps to slow down blood sample degradation. Drying a small sample is faster than drying a large sample. To reduce drying time, the hydrophilic material is preferably selected to be air permeable or gas permeable so that air can enter the absorbent probe and dry it faster.

[0092] It is particularly preferred that the purified RNA fraction with < 200 nucleotides in length has a purity of at least 70% or at least 75%, more preferably of at least 80%, even more preferably of at least 90%, and most preferably of at least 95%, e.g. at least 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.9%, or 100%.

[0093] In one embodiment of the first aspect of the present invention, the absorbent probe further comprises a substance selected from the group consisting of a reference standard, an anticoagulant, and a stabilizer.

[0094] The use of anticoagulants may be useful in maintaining the homogeneity of the blood as well as preventing unwanted degradation. An anticoagulant may be applied dry to the absorbent probe, but is preferably applied wet or in liquid form and allowed to dry before use. The most common anticoagulants fall into two categories polyanions (e.g. Heparin) or metal

chelators (e.g. EDTA, citrate). Suitable anticoagulants are believed to include acid citrate dextrose, citrate phosphate dextrose, citrate phosphate dextrose adenine, sodium citrate, K2 EDTA, K3 EDTA, sodium EDTA, lithium heparin, sodium heparin, potassium and oxalate. Any anticoagulant applied to the absorbent probe should be suitably matched with the fluid used and the downstream analysis so as not to adversely affect the accuracy of the analysis.

[0095] The use of a reference standard, e.g. internal and external standard, during analysis is common practice. Thus, a reference standard (wet or dried) may be applied to the absorbent probe during manufacture or sampling of the fluid, or it may be added to the reconstituting fluid when the (dried) blood is extracted from the absorbent probe. Many non-volatile materials which do not affect the analysis of the blood may be used as reference standards. Radiolabels, fluorescent labels, and deuterated labels may be used.

[0096] The use of stabilizers is also common practice. Stabilizers may be useful in stabilizing the blood. A stabilizer may be applied dry to the absorbent probe, but is preferably applied wet or in liquid form and allowed to dry before use. Examples of stabilizers include, but are not limited to, RNA-later, RNastable, PAXgene-reagent, RNAsin, and catrimox-14.

[0097] In one embodiment of the first aspect of the present invention, the absorbent probe is a probe as described in WO 2013/067520 A1 or US 2013116597 A1. In particular, the absorbent probe is part of a biological fluid sampling device as described in WO 2013/067520 A1 or US 2013116597 A1. In this respect, it is referred to page 10, line 28 to page 34, line 27, Figures 2, 3 and 8, and claims 1 to 19 of WO 2013/067520 A1 and to paragraphs [0068] to [0129], Figures 2, 3 and 8, and claims 1 to 19 of US 2013116597 A1.

[0098] In a second aspect, the present invention relates to a method of determining the level of RNA molecules with < 200 nucleotides in length comprising the steps of:

1. (i) carrying out the method according to the first aspect, and
2. (ii) determining the level of RNA molecules with < 200 nucleotides in length by a suitable technique.

[0099] The inventors of the present invention surprisingly found that the level of RNA molecules, e.g. miRNAs, purified according to the first aspect of the present invention, can be determined. In this way, sample processing times can be shortened, sample analysis (e.g. due to expression profiling) can be simplified, and costs can be saved.

[0100] Preferably, the RNA molecules with < 200 nucleotides in length are selected from the group consisting of miRNA, tRNA, siRNA, piRNA, and snorRNA.

[0101] In one embodiment of the second aspect of the present invention, the level which is determined in step (ii) is the expression level.

[0102] The determination in step (ii) may be carried out by any convenient means for determining the level of the RNA molecules, e.g. miRNAs, particularly the expression level of the RNA molecules, e.g. miRNAs. For example, qualitative, semi-quantitative, and/or quantitative detection methods may be used. A variety of techniques are well known to the person skilled in the art.

[0103] It is preferred that the level of the RNA molecules, e.g. miRNAs, particularly the expression level of the RNA molecules, e.g. miRNAs, is determined by nucleic acid hybridization, nucleic acid amplification, polymerase extension, sequencing, mass spectroscopy or any combination thereof. Nucleic acid amplification may be performed using real time polymerase chain reaction (RT-PCR) such as real time quantitative polymerase chain reaction (RT qPCR). The real time polymerase chain reaction (RT-PCR) is preferred for the analysis of a single RNA molecule, e.g. a miRNA, or a low number of RNA molecules, e.g. miRNAs. It is particularly suitable for detecting low abundance RNA molecules, e.g. miRNAs. The real time quantitative polymerase chain reaction (RT qPCR), however, allows the analysis of a single RNA molecule, e.g. a miRNA, and a high number of RNA molecules, miRNAs.

[0104] A variety of kits and protocols to determine the expression level of miRNAs by real time polymerase chain reaction (RT-PCR) such as real time quantitative polymerase chain reaction (RT qPCR) are available. For example, reverse transcription of miRNAs may be performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's recommendations. Briefly, miRNA may be combined with dNTPs, MultiScribe reverse transcriptase and the primer specific for the target miRNA. The resulting cDNA may be diluted and may be used for PCR reaction. The PCR may be performed according to the manufacturer's recommendation (Applied Biosystems). Briefly, cDNA may be combined with the TaqMan assay specific for the target miRNA and PCR reaction may be performed using ABI7300.

[0105] Nucleic acid hybridization may be performed using a microarray/biochip or *in situ* hybridization. *In situ* hybridization is preferred for the analysis of a single RNA molecule, e.g. a miRNA, or a low number of RNA molecules, miRNAs. The microarray/biochip, however, allows the analysis of a single RNA molecule, e.g. a miRNA, and a high number of RNA molecules, e.g. miRNAs. It is more preferred that (i) the nucleic acid hybridization is performed using a microarray/biochip or beads, or using *in situ* hybridization, and/or (ii) the nucleic acid amplification is performed using real-time PCR.

[0106] It is preferred that the suitable technique in step (ii) is selected from the group consisting of nucleic acid hybridization, nucleic acid amplification, polymerase extension, sequencing, and mass spectroscopy, or any combination thereof. It is more preferred that (i) the nucleic acid hybridization is performed using a microarray/biochip, beads, or *in situ* hybridization, and/or (ii) the nucleic acid amplification is performed using real-time PCR.

[0107] In a third aspect, the present invention relates to a method for diagnosing a disease in an individual comprising the steps of:

1. (i) carrying out the method according to the first aspect, wherein the whole blood sample is (taken, obtained, isolated) from an individual,
2. (ii) determining the level of RNA molecules with < 200 nucleotides in length by a suitable technique,
3. (iii) comparing said level to one or more reference level(s), and
4. (iv) diagnosing or differentially diagnosing whether the individual is afflicted by the disease based on the comparison.

[0108] The determination in step (ii) may be carried out by any convenient means for determining the level of the RNA molecules, e.g. miRNAs, particularly the expression level of the RNA molecules, e.g. miRNAs. For example, qualitative, semi-quantitative, and/or quantitative detection methods may be used. A variety of techniques are well known to the person skilled in the art.

[0109] It is preferred that the level of the RNA molecules, e.g. miRNAs, particularly the expression level of the RNA molecules, e.g. miRNAs, is determined by nucleic acid hybridization, nucleic acid amplification, polymerase extension, sequencing, mass spectroscopy or any combination thereof.

[0110] Nucleic acid amplification may be performed using real time polymerase chain reaction (RT-PCR) such as real time quantitative polymerase chain reaction (RT qPCR). The real time polymerase chain reaction (RT-PCR) is preferred for the analysis of a single RNA molecule, e.g. a miRNA, or a low number of RNA molecules, e.g. miRNAs. It is particularly suitable for detecting low abundance RNA molecules, e.g. miRNAs. The real time quantitative polymerase chain reaction (RT qPCR), however, allows the analysis of a single RNA molecule, e.g. a miRNA, and a high number of RNA molecules, miRNAs.

[0111] A variety of kits and protocols to determine the expression level of miRNAs by real time polymerase chain reaction (RT-PCR) such as real time quantitative polymerase chain reaction (RT qPCR) are available. For example, reverse transcription of miRNAs may be performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's recommendations. Briefly, miRNA may be combined with dNTPs, MultiScribe reverse transcriptase and the primer specific for the target miRNA. The resulting cDNA may be diluted and may be used for PCR reaction. The PCR may be performed according to the manufacturer's recommendation (Applied Biosystems). Briefly, cDNA may be combined with the TaqMan assay specific for the target miRNA and PCR reaction may be performed using ABI7300.

[0112] Nucleic acid hybridization may be performed using a microarray/biochip or *in situ* hybridization. *In situ* hybridization is preferred for the analysis of a single RNA molecule, e.g. a miRNA, or a low number of RNA molecules, miRNAs. The microarray/biochip, however, allows the analysis of a single RNA molecule, e.g. a miRNA, and a high number of RNA molecules,

e.g. miRNAs. It is more preferred that (i) the nucleic acid hybridization is performed using a microarray/biochip or beads, or using *in situ* hybridization, and/or (ii) the nucleic acid amplification is performed using real-time PCR.

[0113] It is preferred that the suitable technique in step (ii) is selected from the group consisting of nucleic acid hybridization, nucleic acid amplification, polymerase extension, sequencing, and mass spectroscopy, or any combination thereof. It is more preferred that (i) the nucleic acid hybridization is performed using a microarray/biochip, beads, or *in situ* hybridization, and/or (ii) the nucleic acid amplification is performed using real-time PCR.

[0114] Said third aspect may alternatively be formulated as follows: A method for diagnosing a disease in an individual comprising the steps of:

1. (i) carrying out the method according to the second aspect, wherein the whole blood sample is (taken, obtained, isolated) from an individual,
2. (ii) comparing said level to one or more reference level(s), and
3. (iii) diagnosing or differentially diagnosing whether the individual is afflicted by the disease based on the comparison.

[0115] The reference level may be any level which allows to determine whether an individual suffers from the disease or not. Preferably, the one or more reference level(s) (is) are determined by measuring one or more reference whole blood sample(s), e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 reference whole blood sample(s), from one or more healthy subject(s), e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 healthy subject(s),

one or more subject(s) suffering from a disease, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 subject(s) suffering from a disease, and/or

one or more subject(s) suffering from another disease e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 subject(s) suffering from another disease. In particular, the one or more reference level(s) is (are) determined by measuring one or more reference whole blood sample(s) from

one or more lung cancer subject(s), preferably lung cancer subjects subjected to lung cancer therapy, more preferably lung cancer subjects subjected to palliative or adjuvant lung cancer therapy, and/or

one or more subject(s) being healthy, preferably subjects not being subjected to lung cancer therapy, more preferably subjects not being subjected to palliative or adjuvant lung cancer therapy. It is practicable to take one reference whole blood sample per subject for analysis. If additional reference whole blood sample(s) are required, the same subject may be (re)tested.

[0116] Said reference level may be an average reference level. It may be determined by measuring reference levels and calculating the "average" value (e.g. mean, median or modal value) thereof. It is preferred that the reference level is from a subject of the same gender (e.g. female or male) and/or of a similar age/phase of life (e.g. adults or elderly) than the patient to be tested or diagnosed.

[0117] The disease may be any disease, e.g. lung cancer. The other disease may be any other disease than lung cancer.

[0118] In one embodiment, the method for diagnosing a disease in an individual comprises the steps of:

1. (i) carrying out the method according to the second aspect, wherein the whole blood sample is (taken, obtained, isolated) from an individual,
2. (ii) comparing said level to a reference level which is determined by measuring one or more reference whole blood sample(s) from one or more healthy subject(s), and
3. (iii) diagnosing whether the individual is afflicted by the disease based on the comparison.

[0119] Preferably, the disease is lung cancer.

[0120] In one another embodiment, the method for diagnosing a disease in an individual comprises the steps of:

1. (i) carrying out the method according to the second aspect, wherein the whole blood sample is (taken, obtained, isolated) from an individual,
2. (ii) comparing said level to a reference level which is determined by measuring one or more reference whole blood sample(s) from one or more subject(s) suffering from the disease, and
3. (iii) diagnosing whether the individual is afflicted by the disease based on the comparison.

[0121] Preferably, the disease is lung cancer.

[0122] In one another embodiment, the method for diagnosing a disease in an individual comprises the steps of:

1. (i) carrying out the method according to the second aspect, wherein the whole blood sample is (taken, obtained, isolated) from an individual,
2. (ii) comparing said level to a reference level which is determined by measuring one or more reference whole blood sample(s) from one or more subject(s) suffering from the

disease and to a reference level which is determined by measuring one or more reference whole blood sample(s) from one or more subject(s) suffering from another disease, and

3. (iii) diagnosing whether the individual is afflicted by the disease based on the comparison.

[0123] Preferably, the disease is lung cancer and the other disease is any other disease than lung cancer. The method may also allow to distinguish differently treated diseases from each other. For example, the disease may be an adjuvant-treated disease, e.g. adjuvant-treated lung cancer, and the other disease may be a palliative-treated disease, e.g. palliative-treated lung cancer, or the disease may be a palliative-treated disease, e.g. palliative-treated lung cancer, and the other disease may be an adjuvant-treated disease, e.g. adjuvant-treated lung cancer.

[0124] Described herein, but not encompassed by the present invention, is the use of a hydrophilic polymeric material for removing an RNA fraction with ≥ 200 nucleotides in length from a whole blood sample. Preferably, the RNA fraction with ≥ 200 nucleotides in length comprises one or more RNA molecules selected from the group consisting of ribosomal 18S RNA and ribosomal 28S RNA.

[0125] In a fourth aspect, the present invention relates to the use of a hydrophilic polymeric material for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample, wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester. Preferably, the RNA fraction with < 200 nucleotides in length comprises one or more RNA molecules selected from the group consisting of miRNA, tRNA, siRNA, piRNA, and snorRNA.

[0126] In one embodiment of the fourth aspect of the present invention, the hydrophilic polymeric material is comprised in an adsorbent probe (see first aspect of the present invention).

[0127] Preferably, the hydrophilic polymeric material has a density of $\leq 4 \text{ g/cm}^3$, e.g. of 0.5, 1.5, 2, 2.5, 3, 3.5, or 4 g/cm^3 . More preferably, the hydrophilic polymeric material has a density of between 1 and 4 g/cm^3 , e.g. of 1.5, 2, 2.5, 3, 3.5, or 4 g/cm^3 .

[0128] In one embodiment of the fourth aspect of the present invention, the hydrophilic polymeric material is porous.

[0129] In one embodiment of the fourth aspect of the present invention, the hydrophilic polymeric material has a pore volume of between 20 and 70%, preferably of between 30 and

50%, e.g. of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 % of the total volume of the material.

[0130] In one embodiment of the fourth aspect of the present invention, the hydrophilic polymeric material comprises pores that are $\leq 100 \mu\text{m}$, preferably $\leq 50 \mu\text{m}$, e.g. $\leq 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95$, or $100 \mu\text{m}$, in diameter or largest cross-sectional dimension. More preferably, the hydrophilic polymeric material comprises pores that are between 10 to $100 \mu\text{m}$, even more preferably between 20 and $50 \mu\text{m}$, e.g. 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or $100 \mu\text{m}$, in diameter or largest cross-sectional dimension.

[0131] As mentioned above, the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester. Preferably,

1. (i) the polysaccharide is cellulose,
2. (ii) the polyolefin is selected from the group consisting of polyethylene, polypropylene, polybutylene, polyisobutylene, and polymethylpentene, or
3. (iii) the polyester is selected from the group consisting of polycarbonate and polyethyleneterephthalate.

[0132] More preferably, the hydrophilic polymeric material is cotton, cellulose or polyethylene.

[0133] In one embodiment of the fourth aspect of the present invention, the hydrophilic polymeric material is a material as described in WO 2013/067520 A1 or US 2013116597 A1. In particular, the hydrophilic polymeric material is part of an absorbent probe which is comprised in a biological fluid sampling device as described in WO 2013/067520 A1 or US 2013116597 A1. In this respect, it is referred to page 10, line 28 to page 34, line 27, Figures 2, 3 and 8, and claims 1 to 19 of WO 2013/067520 A1 and to paragraphs [0068] to [0129], Figures 2, 3 and 8, and claims 1 to 19 of US 2013116597 A1.

[0134] With respect to other embodiments of the absorbent probe and/or hydrophilic polymeric material, it is referred to the first aspect of the present invention.

[0135] In a fifth aspect, the present invention relates to the use of a method according to the second aspect for diagnosing or differentially diagnosing a disease. The disease may be lung cancer. As to the diagnosis or differential diagnosis of a disease, it is referred to the third aspect of the present invention.

[0136] In a sixth aspect, the present invention relates to the use of a kit for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample, wherein said kit comprises

1. (i) an absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic

polymeric material, and

2. (ii) a fluid comprising a chaotropic agent to reconstitute whole blood absorbed in/to an absorbent probe comprising a hydrophilic polymeric material,

wherein the hydrophilic polymeric material has a density of $\leq 6 \text{ g/cm}^3$, and wherein the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester.

[0137] In one embodiment of the sixth aspect of the present invention, the kit further comprises

(ii) means to remove blood from an individual,

(iii) a container, and/or

(iv) a data carrier comprising the information that the absorbent probe with the whole blood from the whole blood sample absorbed in/to it is usable in the methods of the first to third aspect of the present invention.

[0138] In one embodiment of the sixth aspect of the present invention, the kit further comprises means for determining the level of RNA molecules with < 200 nucleotides in length.

[0139] The means to remove blood from an individual may be selected from the group consisting of a syringe (with a needle), a needle, and a lancet. The syringe (with a needle) may be used to extract blood from a vein in the arm of an individual. The needle or lancet may be used to destroy the upper skin layer and remove a blood drop (e.g. via a finger prick) from an individual. The absorbent probe comprising, essentially consisting of, or consisting of a hydrophilic polymeric material is then placed in (physical) contact with the whole blood sample, thereby absorbing whole blood in/to the hydrophilic polymeric material. Afterwards, the absorbent probe may be dried, e.g. air dried. This can be done at room temperature (20°C) or at elevated temperatures (e.g. 30°C).

[0140] The absorbent probe comprising a hydrophilic polymeric material in/to which whole blood from the whole blood sample has been absorbed may then be sent in a laboratory for further analysis.

[0141] The means for determining the level of RNA molecules with < 200 nucleotides in length may comprise:

1. (i) polynucleotides for detecting RNA molecules with ≥ 200 nucleotides in length and/or of RNA molecules with < 200 nucleotides in length, and/or
2. (ii) a biochip, a RT-PCT system, a PCR-system, a flow cytometer or a next generation sequencing system.

[0142] Preferably, the RNA molecules with < 200 nucleotides in length are miRNA molecules.

[0143] Said polynucleotides may be attached to a biochip. Said polynucleotides may also be used as primers in a RT-PCT system, a PCR-system, or a next generation sequencing system.

[0144] The data carrier may be a non-electronical data carrier, e.g. a graphical data carrier such as an information leaflet (e.g. including details on how to employ said kit according to the first to third aspect of the present invention), an information sheet, a bar code or an access code, or an electronical data carrier such as a floppy disk, a compact disk (CD), a digital versatile disk (DVD), a microchip or another semiconductor-based electronical data carrier. The access code may allow the access to a database, e.g. an internet database, a centralized, or a decentralized database. The access code may also allow access to an application software that causes a computer to perform tasks for computer users or a mobile app which is a software designed to run on smartphones and other mobile devices.

[0145] Said data carrier may further comprise one or more reference level(s) of the level of the RNA molecules, e.g. miRNAs, determined herein. In case that the data carrier comprises an access code which allows the access to a database, said one or more reference level(s) may be deposited in this database.

[0146] The kit may optionally comprise materials desirable from a commercial and user standpoint including a buffer(s), a reagent(s) and/or a diluent(s) which may be useful in taking a whole blood sample.

[0147] The fluid comprising a chaotropic agent may be a liquid or gas. Preferably, the fluid comprising a chaotropic agent is a liquid or gas comprising a chaotropic agent. More preferably, the chaotropic agent is selected from the group consisting of guanidinium thiocyanate, guanidinium isothiocyanate, guanidinium hydrochloride, guanidinium chloride, alkali thiocyanate, alkali isothiocyanate, alkali iodide, and alkali perchlorate. Even more preferably, the chaotropic agent is guanidinium thiocyanate.

[0148] The kit is useful for carrying out the methods of the first to third aspect of the present invention, i.e. for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample, and for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample and (subsequently) determining the level of said RNA molecules.

[0149] The RNA fractions mentioned above comprise one or more RNA molecules, e.g. miRNA molecules. The RNA fraction with ≥ 200 nucleotides in length preferably comprises one or more RNA molecules selected from the group consisting of ribosomal 18S RNA and ribosomal 28S RNA and the RNA fraction with < 200 nucleotides in length preferably comprises one or more RNA molecules selected from the group consisting of miRNA, tRNA, siRNA, piRNA, and snorRNA.

[0150] If the kit is for purifying an RNA fraction with < 200 nucleotides in length from a whole blood sample and (subsequently) determining the level of said RNA molecules, it further comprises means for determining the level, particularly the expression level, of the RNA molecules (e.g. miRNA molecules). Preferably, said means comprise one or more polynucleotides for determining the (expression) level of one or more RNA molecules (e.g. miRNA molecules), and/or a microarray, a RT-PCT system, a PCR-system, a flow cytometer, a bead-based multiplex system or a next generation sequencing system.

[0151] In one embodiment of the sixth aspect of the present invention, the hydrophilic polymeric material has a density of $\leq 4 \text{ g/cm}^3$, e.g. of 0.5, 1.5, 2, 2.5, 3, 3.5, or 4 g/cm^3 . More preferably, the hydrophilic polymeric material has a density of between 1 and 4 g/cm^3 , e.g. of 1.5, 2, 2.5, 3, 3.5, or 4 g/cm^3 .

[0152] In one embodiment of the sixth aspect of the present invention, the hydrophilic polymeric material is porous.

[0153] In one embodiment of the sixth aspect of the present invention, the hydrophilic polymeric material has a pore volume of between 20 and 70%, preferably of between 30 and 50%, e.g. of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, or 70 % of the total volume of the material.

[0154] In one embodiment of the sixth aspect of the present invention, the hydrophilic polymeric material comprises pores that are $\leq 100 \mu\text{m}$, preferably $\leq 50 \mu\text{m}$, e.g. $\leq 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95$, or $100 \mu\text{m}$, in diameter or largest cross-sectional dimension. More preferably, the hydrophilic polymeric material comprises pores that are between 10 to $100 \mu\text{m}$, even more preferably between 20 and $50 \mu\text{m}$, e.g. 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or $100 \mu\text{m}$, in diameter or largest cross-sectional dimension.

[0155] As mentioned above, the hydrophilic polymeric material is selected from the group consisting of cotton, a polysaccharide, a polyolefin, and a polyester. Preferably,

1. (i) the polysaccharide is cellulose,
2. (ii) the polyolefin is selected from the group consisting of polyethylene, polypropylene, polybutylene, polyisobutylene, and polymethylpentene, or
3. (iii) the polyester is selected from the group consisting of polycarbonate and polyethyleneterephthalate.

[0156] More preferably, the hydrophilic polymeric material is cotton, cellulose or polyethylene.

[0157] In one embodiment of the sixth aspect of the present invention, the absorbent probe

has absorbed a predetermined maximum volume of whole blood of between 1 to 50 μl , e.g. of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 μl .

[0158] In one embodiment of the sixth aspect of the present invention, the absorbent probe has a length of ≤ 10 mm, preferably of ≤ 5 mm, e.g. of $\leq 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5$, or 10 mm, and a cross-sectional area of ≤ 40 mm^2 , preferably of ≤ 20 mm^2 , e.g. of $\leq 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39$, or 40 mm^2 .

[0159] Such sizes are believed suitable when the whole blood sample is a blood drop, e.g. from a finger prick.

[0160] In an alternative embodiment of the sixth aspect of the present invention the absorbent probe has a volume of ≤ 250 mm^3 , preferably of ≤ 200 mm^3 , e.g. of $\leq 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240$, or 250 mm^3 .

[0161] Such volumes are believed suitable when the whole blood sample is a blood drop, e.g. from a finger prick.

[0162] More preferably, the absorbent probe has a length of between 2 and 10 mm, preferably of between 2 and 5 mm, e.g. of 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 mm, and a cross-sectional area of between 5 and 40 mm^2 , preferably of between 5 and 20 mm^2 , e.g. of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 mm^2 .

[0163] In an alternative embodiment of the sixth aspect of the present invention, the absorbent probe has a volume of between 1 mm^3 to 250 mm^3 , preferably of between 2.5 mm^3 to 200 mm^3 , e.g. of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, or 250 mm^3 .

[0164] In one embodiment of the sixth aspect of the present invention, the absorbent probe is configured to absorb a predetermined maximum volume of whole blood of between 1 to 50 μl , preferably of between 5 to 25 μl , and even more preferably of between 10 to 20 μl , e.g. of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 μl .

[0165] With respect to other embodiments of the absorbent probe and/or hydrophilic polymeric material, it is referred to the first aspect of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

[0166] The following Figures are merely illustrative of the present invention and should not be construed to limit the scope of the invention as indicated by the appended claims in any way.

Figure 1: Depicted is the comparison of the Agilent Bioanalyzer Nano results of RNA isolated from whole blood samples i) with removal of the RNA-fraction with > 200 nt in length making use of an absorbent probe (Mitra Microsampling Device, lower curve) and ii) without removal of the RNA-fraction with >200 nt in length (whole blood collected in PAXgene Blood RNA Tube, upper curve) from a human individual. The upper curve clearly shows that the mayor RNA peaks (18S, 28S ribosomal RNA) is still present, while in the lower curve said 18S and 28S RNAs and other RNA species with >200 nt in length have been effectively removed by absorbing the whole blood sample to an absorbent probe of hydrophilic polymeric material.

Figure 2: Shown are the Agilent Bioanalyzer (Nano RNA kit) results of RNA isolated from whole blood samples collected from a human individual with removal of the RNA-fraction with > 200 nt in length making use of an absorbent probe of hydrophilic polymeric material (Mitra Microsampling Device): the >200 nt RNA-fractions, including -but not limited to- the most prominent ribosomal RNA peaks (18S fragment at -2,000 nt and 28S fragment at -4,000 nt) are removed from the RNA obtained from a whole blood sample by use of an absorbent probe of hydrophilic polymeric material (Mitra Microsampling Device).

Figure 3: Close-up of the small RNA fraction with <200 nt in length derived from a whole blood sample from which the RNA-fraction of >200 nt in length has been removed by use of a hydrophilic polymeric absorbent probe. Herein, said small RNA-fraction comprises various small-RNA species, such as miRNA (~ 15-35 nt), tRNA (-60-80 nt), siRNA (-20-25 nt), piRNA (-26-31 nt) or snorRNA.

Figure 4: Scanning electron microscope (SEM) images of hydrophilic polymeric absorbent probes: (A) hydrophilic cellulose comprising polymeric absorbent probe material; such as Non-Indicating FTA Classic Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), Non-Indicating FTA Elute Micro Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), HemaSpot HF (Spot On Sciences, Austin, TX, USA), HemaSpot SE (Spot On Sciences, Austin, TX, USA), TNF (Munktell, Bärenstein, Germany), TNF-Di (Munktell, Bärenstein, Germany); (B) hydrophilic organic polymeric absorbent probe material; such as Mitra microsampling device.

Figure 5: miRNA expression data: depicted are the miRNAs that were found to be expressed (on an Agilent dna-microarray, for details see Example 8) in the small RNA fraction that was obtained after removal of the >200 nt fraction from a whole blood sample that has been processed by use of an hydrophilic polymeric absorbent probe. Herein, as examples polymeric

absorbent probes such as the Mitra microsampling device or a hydrophilic cellulose comprising absorbent probe (such as Non-Indicating FTA Classic Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), Non-Indicating FTA Elute Micro Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), HemaSpot HF (Spot On Sciences, Austin, TX, USA), HemaSpot SE (Spot On Sciences, Austin, TX, USA), TNF (Munktell, Bärenstein, Germany), TNF-Di (Munktell, Bärenstein, Germany)) were used as the device comprising said hydrophilic polymeric absorbent probe to remove the >200 nt RNA fraction from the whole blood sample. With: SEQ ID NO = sequence identification number; miRNA = microRNA identifier according to miRBase; gTotalGeneSignal (A) = relative expression level of the corresponding miRNAs detected when employing a hydrophilic cellulose comprising absorbent material for >200 nt RNA removal; gTotalGeneSignal (B) = relative expression level of the corresponding miRNAs detected when employing a hydrophilic polymeric absorbent material (Mitra microsampling device) for >200 nt RNA removal.

Figure 6: Mitra Microsampling Device: the hydrophilic absorbent probe is comprised in the top tip of said device.

Figure 7: Depicted is the comparison of the Agilent Bioanalyzer Nano results of RNA isolated from whole blood samples (i) with removal of the RNA-fraction with > 200 nt in length making use of an absorbent probe (hydrophilic cellulose comprising absorbent device such as Non-Indicating FTA Classic Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), Non-Indicating FTA Elute Micro Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), HemaSpot HF (Spot On Sciences, Austin, TX, USA), HemaSpot SE (Spot On Sciences, Austin, TX, USA), TNF (Munktell, Bärenstein, Germany), TNF-Di (Munktell, Bärenstein, Germany)), lower curve) and (ii) without removal of the RNA-fraction with >200 nt in length (whole blood collected in PAXgene Blood RNA Tube, upper curve) from a human individual. The upper curve clearly shows that the major RNA peaks (18S, 28S ribosomal RNA) are still present, while in the lower curve said 18S and 28S RNAs and other RNA species with >200 nt in length have been effectively removed by absorbing the whole blood sample to an absorbent probe of hydrophilic polymeric material.

Figure 8: Stability of miRNomes from dried blood spots. **A.** Scatterplots and correlations of different conditions for each environmental factor. **B.** PVCA plot of influencing environmental factors.

Figure 9: Volcano plot of deregulated miRNAs regarding the comparison between adjuvant and palliative treated patients.

Figure 10: Biological variation depending on lung cancer therapy. Expression intensities, fold changes, p-values and the area under the receiver operator characteristics curve (AUC value) of deregulated miRNAs.

EXAMPLES

[0167] The examples given below are for illustrative purposes only and do not limit the invention described above in any way.

Part 1:

Example 1: Blood sample collection using hydrophilic polymeric absorbent device (Mitra Microsampling Device)

[0168] Blood was collected from different individuals (n=3) by puncture of the middle finger of the left hand using sterile safety lancets (Safety-Lancet Extra 18G, Sarstedt, Nümbrecht, Germany) and four Mitra Microsampling Devices (neoteryx, Torrance, CA, USA, Ordering No. 10005) per individual. Blood on Mitra Microsampling Device (**Figure 6**) was dried for 2 hours at ambient temperature. Four blood filled Sampler Tips per individual were removed from Sampler Body of the Mitra Microsampling Device and transferred to a 2 ml Tube (Eppendorf, Hamburg, Germany)

Example 2: Extraction of small RNA from Mitra Microsampling Device

[0169] Small RNA extraction with length <200 nt (including the microRNA-fraction) was carried out using Phenol-Chloroform extraction technique. Purification of the small RNA was performed by use of the miRNeasy Serum Plasma Kit (Qiagen GmbH, Hilden, Germany). Herein, 1 mL Qiazol reagent (Qiagen GmbH, Hilden, Germany, comprising guanidinium thiocyanate as a chaotropic reagent and phenol) was pipetted to the 2 mL tube containing four Mitra Sampler Tips (see Example 1, blood sample collection). The tube was then incubated at 4 °C on a shaker at 1,000 rpm for 16 hours. Afterwards, complete supernatant was transferred to a fresh 2 mL Eppendorf tube. After addition of 200 µl Chloroform, mixture was thoroughly vortexed for 15 sec and incubated for 2 min at room temperature, followed by centrifugation at 12,000 x g for 15 min at 4°C. Afterwards, the upper, aqueous phase was transferred to a new 1 mL tube without touching the other two phases. 1.5 volumes of 100% ethanol were added to the aqueous phase, thoroughly mixed by pipetting and incubated for 10 min at room temperature. 700 µl of the sample were then transferred into a Qiagen MinElute column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. The last two steps were repeated until complete sample volume was applied to the column. Afterwards, 700 µl of buffer RWT were added to each column, centrifuged again at 13,000 rpm for 15 sec at RT, discarding the flow-through. Then 500 µl Buffer RPE was added to the column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. Afterwards 500 µl 80% ethanol was added to the column and centrifuged at 13,000 rpm for 2 min at RT, discarding the flow-through. Then the column was placed into a new 2 ml collection tube, and centrifuged with open lid at 13,000 rpm for 5 min at RT to dry it. The column was transferred into a new 1.5 ml collection tube. For

elution of the total RNA incl. microRNA 14 µl RNase-free water was pipetted onto the column, incubated for 1 min and centrifuged at 13.000 rpm at RT for 1 min. Another 14 µl of RNase-free water was pipetted to the column, incubated for 1 min at RT and centrifuged at 13.000 rpm at RT for 1 min. The eluted small RNA fraction with length <200 nt (including the microRNA-fraction) was stored on ice until Quality Control and quantification.

Example 3: Blood sample collection using a hydrophilic cellulose comprising absorbent device

[0170] Blood was collected from different individuals (n=3) by puncture of the ring finger of the left hand using sterile safety lancets (Safety-Lancet Extra 18G, Sarstedt, Nümbrecht, Germany). Two blood drops per individual were added to the hydrophilic cellulose comprising absorbent device (cellulose comprising filter paper from various manufactures, including Non-Indicating FTA Classic Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), Non-Indicating FTA Elute Micro Card (GE Healthcare Life Science, Buckinghamshire, Great Britain), HemaSpot HF (Spot On Sciences, Austin, TX, USA), HemaSpot SE (Spot On Sciences, Austin, TX, USA), TNF (Munktel, Bärenstein, Germany), TNF-Di (Munktel, Bärenstein, Germany)) covering an area of approximate 0.5-4.0 square cm. The various hydrophilic cellulose comprising absorbent probes were dried for 2 hours and then stored for 1 week at ambient temperature. Blood absorbed area of the hydrophilic cellulose comprising absorbent probes were cut out and transferred to a 2 mL Tube (Eppendorf, Hamburg, Germany).

Example 4: Extraction of small RNA from a hydrophilic cellulose comprising absorbent device

[0171] Small RNA with a length < 200 nt (including the microRNA-fraction) extraction was carried out using Phenol-Chloroform extraction technique. Purification of small RNA was performed by use of the miRNeasy Serum Plasma Kit (Qiagen GmbH, Hilden, Germany). 1 mL Qiazol reagent (Qiagen GmbH, Hilden, Germany) was pipetted to the 2 mL tube containing the hydrophilic cellulose comprising absorbent device to which the whole blood sample was absorbed to by drying (see blood sample collection, example 3). The tube was then incubated at 4 °C on a shaker at 1,000 rpm for 16 hours. Afterwards, complete supernatant was transferred to a fresh 2 mL Eppendorf tube. After addition of 200 µl Chloroform, mixture was thoroughly vortexed for 15 sec and incubated for 2 min at room temperature, followed by centrifugation at 12,000 x g for 15 min at 4°C. Afterwards, the upper, aqueous phase was transferred to a new 2 mL tube without touching the other two phases. 1.5 volumes of 100% ethanol were added to the aqueous phase, thoroughly mixed by pipetting and incubated for 10 min at room temperature. 700 µl of the sample were then transferred into a Qiagen MinElute column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. The last two steps were repeated until complete sample volume was applied to the column. Afterwards,

700 µl of buffer RWT were added to each column, centrifuged again at 13,000 rpm for 15 sec at RT, discarding the flow-through. Then 500 µl Buffer RPE was added to the column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. Afterwards 500 µl 80% ethanol was added to the column and centrifuged at 13,000 rpm for 2 min at RT, discarding the flow-through. Then the column was placed into a new 2 ml collection tube, and centrifuged with open lid at 13,000 rpm for 5 min at RT to dry it. The column was transferred into a new 1.5 ml collection tube. For elution of the small RNA incl. microRNA 14 µl RNase-free water was pipetted onto the column, incubated for 1 min and centrifuged at 13,000 rpm at RT for 1 min. Another 14 µl of RNase-free water was pipetted to the column, incubated for 1 min at RT and centrifuged at 13,000 rpm at RT for 1 min. The eluted small RNA with <200 nt in length (incl. microRNA) was stored on ice until Quality Control and quantification.

Example 5: Blood sample collection using PaxGene Blood RNA tubes

[0172] Blood was collected from different individuals (n=3). Herein, for each blood donor 2.5 ml of whole blood was collected by venous puncture into a PAXgene Blood RNA Tube (PreAnalytix, Hombrechticon, Switzerland). The blood cells were derived/obtained from processing the whole blood samples by centrifugation. Herein, the blood cells from the whole blood collected in said blood collection tubes were spun down by 10 min, 5000xg centrifugation. The blood cell pellet (the cellular blood fraction comprising red blood cells, white blood cells and platelets) was harvested for further processing, while the supernatant (including the extra-cellular blood fraction) was discarded. Total RNA, including the small RNA (<200nt including the miRNA-fraction), but also the >200 nt RNA fraction was extracted from the harvested blood cells using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany); for details see Example 6.

Example 6: Extraction of total RNA (incl. RNA-fraction with >200 nt) from PAXgene Blood RNA tubes

[0173] The isolation of total RNA, including the small RNA (<200nt including the miRNA-fraction) and the >200 nt RNA fraction was performed by use of the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Herein, the blood cell pellet (obtained as outlined in Example 5) was thoroughly resuspended in 700 µl QIAzol lysis reagent by pipetting up and down and immediately the suspension was transferred to a new 1.5 ml Eppendorf tube. Then 140 µl chloroform were added, vortexed thoroughly and incubated for 2-3 min at room temperature, followed by centrifugation at 12,000 g for 15 min at 4°C. Afterwards, the upper, aqueous phase was transferred to a new 2 ml tube with great care, without touching the other two phases. Then 1.5 volumes of 100% ethanol were added to the transferred aqueous phase and thoroughly mixing was done by pipetting. 700 µl of sample were then transferred into a column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. Afterwards 700 µl of Buffer RWT were added to each column, centrifuged again at 13,000 rpm

for 15 sec at RT, discarding the flow-through. Then 500 µl Buffer RPE was added to the column and centrifuged at 13,000 rpm for 15 sec at RT, discarding the flow-through. Afterwards another 500 µl Buffer RPE was added to the column and centrifuged at 13,000 rpm for 2 min at RT, discarding the flow-through. Then the column was placed into a new 2 ml collection tube and centrifuged at 13,000 rpm for 1 min at RT to dry it. The column was transferred into a new 1.5 ml collection tube. For elution of the total RNA incl. microRNA 40 µl RNase-free water was pipetted onto the column and incubated for 1 min, centrifuged at 13,000 rpm at RT for 1 min. Then the eluate was put back onto the same column, incubated for 1 min at RT and centrifuged again for 1 min. The eluted total RNA, including the small RNA (<200nt, including the miRNA-fraction) and the >200 nt RNA fraction was quantified using the NanoDrop 1000 and stored at -20°C before use in expression profiling experiments.

Example 7: Quality Control of small RNA fraction

[0174] Quality control and quantification of extracted RNA was performed by using Agilent's Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol and the Bioanalyzer Small and Nano Assay. The RNA was denatured for 2 min at 70°C, afterwards 1 µl was applied to the Bioanalyzer Chip for both Small and Nano Assay. Chips were vortexed at 2,400 rpm and run on Bioanalyzer Instrument within 5 min.

Example 8: Microarray-based determination of microRNA expression profiles

[0175] Total RNA sample including microRNA-fraction was analyzed on Agilent human miRNA 8x60k microarray (release v21) according to manufacturer's protocol. After enzymatic Cy3-labeling of microRNA and 20 hours of array hybridization at 55°C in a rotating hybridization oven, the microarray slide was washed twice (non-stringent and stringent) and afterwards scanned with Agilent's SureScan Microarray Scanner. Resulting image data was evaluated using Agilent Feature Extraction software (v11). Raw data files (GeneView) generated by Feature Extraction software were imported in Excel for present call analysis.

Part II:

[0176] It has further systematically be explored whether dried blood spots (DBS) facilitate stable miRNA measurements and compared technical stability to biological variability. First, the stability of DBS samples by generating whole-genome wide miRNA profiles of samples from the same individuals exposed to different environmental conditions (e.g. temperature and humidity) was tested. Second, technical reproducibility by performing seven replicates of samples taken from the same individual was investigated. Third, samples from 53 lung cancer patients undergoing different therapies were investigated. Across the three stages, 108 genome-wide miRNA profiles were generated and evaluated by biostatistical means.

Material and Methods

[0177] Sample collection: For the study, a total of 78 samples on dried blood spots were collected. TNF paper from (Munktell, Bärenstein, Germany) (Ahlstrom) was used. After arrival, dried blood spots were stored at -80 degrees Celsius until RNA extraction. For the stability analysis, one individual was included and exposed to different environmental conditions. The temperature was varied between 26 and 35 degrees Celsius, the humidity between 38.5 and 60%. To capture reproducibility across the whole workflow, i.e. between different extractions and also on different microarrays, we performed 7 technical replicates of another individual. The respective sample has been measured as process control along with the lung cancer study. The same individual has also been investigated using PAXGene Blood Tubes (BD) to compare the miRNA repertoire on dried blood spots and using the blood tubes. As clinical case, 53 lung cancer samples exposed to different therapy regimens were investigated. In detail, 17 samples belonged to patients with curative (adjuvant) therapy and 36 samples to patients with palliative care. The adjuvant therapy was additionally applied to the primary surgically therapy to improve the chances of cure, while the palliative care was used for improving quality of life in case of advanced cancer. The study has been approved by the local ethics committee and participants gave written informed consent. Blood samples have been collected on dried blood spots. All samples were measured on miRBase V21 microarrays (Agilent). A subset of 26 samples has also been measured on miRBase V19 microarrays (Agilent) in order to compare the different microarrays. In total, 30 whole miRNomes were profiled on V19 and 78 whole miRNomes on V21 microarrays.

[0178] miRNA extraction: Prior to RNA extraction, DBS samples were thawed at RT for one hour. Complete blood drop was cut out and transferred to a 2 mL Eppendorf tube. DBS papers were incubated for 16 hours in 1 mL Qiazol Lysis Reagent (Qiagen GmbH, Hilden, Germany) on a shaker at 1.000 rpm and 4 degrees Celsius. Supernatant was transferred to a fresh 2 mL tube for further processing. RNA extraction and purification was performed using Qiagen's miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany). RNA content and miRNA quality was checked (Quality Control and quantification of RNA eluates were performed) with Agilent 2100 Bioanalyzer using the Small RNA Kit according to manufacturer's instructions (Agilent Technologies, Santa Clara, USA).

[0179] miRNA measurement: For microRNA expression profiling, samples were analyzed on Agilent Sureprint G3 Human miRNA (8x60k) microarray slides with latest miRBase v21 content. Each array targets 2,549 microRNAs with 20 replicates per probe. Additionally, a part of the samples was measured on previous v19 version of Agilent's Sureprint miRNA Slides for technical evaluation. Extracted microRNA was labeled and hybridized using miRNA Complete Labeling and Hybridization Kit from Agilent according to manufacturer's protocol. After rotating hybridization for 20 hours at 55 degrees Celsius, slides were washed twice and scanned on Agilent's SureScan Microarray Scanner. Image files from the scanner were transformed to text raw data using Feature Extraction Software (Agilent Technologies).

[0180] Data preprocessing: For the preprocessing of the profiled samples, the Bioconductor library AgiMicroRna, that is designed to preprocess and analyze microRNA Agilent microarray data, was applied. The processed expression values were produced in the following three steps by the robust multiarray average (RMA) algorithm. The measured signal of a probe (one of 20 probe replicates for a miRNA) were first background corrected, then the arrays were normalized by quantile, and at last, the final signal of a miRNA was estimated by summarizing the corresponding probe signals. By applying an additional filtering, control features and miRNAs that are not detected in any experimental group are removed from the data set to obtain only expressed features for the bioinformatics analyses.

[0181] Bioinformatics analyses: To assess technical reproducibility, Pearson's correlation was computed for the process controls. Thereby, a comparison between chips belonging to one miRBase version and a comparison between chips belonging to different miRBase versions (v19 and v21) was made. At the same time, the coefficient of variation (CV) was estimated for relative miRNA expression values of the technical replicates. These miRNAs were also analyzed regarding their GC content and their first introduction to miRBase.

[0182] Furthermore, hierarchical clustering approach was applied for evaluation of the experimental groups and categories in terms of different environmental conditions like humidity and temperature. The measure of distance between pairs of observations was based on Euclidean distance.

[0183] For biostatistical evaluation to find candidates distinguishing between the three groups (adjuvant, palliative and process controls), pairwise comparisons have been performed using t-test. To control the false discovery rate, all p-values were adjusted by using Benjamin-Hochberg adjustment. Additional to the significance values, the Area Under the Receiver Operating Characteristics Curve (AUC) value was calculated.

Results

[0184] Aim of the study was to understand whether genome-wide miRNA profiles from dried blood spots can be reliably measured, facilitating the application in disease diagnosis. A three-staged approach was carried out. First, replicates under different environmental conditions were profiled. Second, technical replicates as process control were established and measured on a series of microarrays.

[0185] Third, clinical feasibility was tested using lung cancer patient samples. An overview of the number of measured miRNomes in the three stages is presented in **Table 1** below.

Table 1: Number of whole miRNomes measured in each stage

miRBase Version	Environmental factors [technical stability]	Process controls [technical stability]	Lung Cancer Therapy [biological variability]
V19	-	4	26
V21	18	7	53

Stability of miRNomes from dried blood spots

[0186] MiRNAs were extracted from a series of dried blood spot samples exposed to different environmental conditions. Combinations of three temperatures and three humidity levels were explored, while each combination was measured in triplicates. Thus, by having six combinations in form of triplicates, a total of 18 miRNomes has been profiled on microarrays. A cluster heat-map was established (data not shown). The different conditions were compared with each other. The results are shown in **Figure 8A**. The lowest correlation of 0.997 was calculated for the temperature. The highest correlation - corresponding to the lowest influence - was calculated for the humidity (correlation of 0.999). These results have been also confirmed by a principal variant component analysis (see **Figure 8B**). These results show that miRNAs measured from dried blood spots have an inherent stability and are technically suited as carriers of diagnostic information, even if they are exposed to varying conditions, e.g. induced by shipment of samples to a central lab.

Reproducibility of miRNAs over time

[0187] Next, it was explored how reproducible miRNomes from dried blood spots can be profiled on microarrays. From one individual, seven technical replicates on seven different Agilent slides (the Agilent technique allows for parallel processing of 8 samples on 8 physically separated arrays on one slide) were performed. In this regard, the sample to be processed can also be considered as a process control (= healthy control). The mean correlation of these process controls was as high as 0.993. The same measurements have been repeated with the previous version of the Agilent microarrays from miRBase version 19 instead of 21. Here, four process controls were included, leading to comparable correlation (data not shown). As the results in the previous analysis, the replicated process controls from one individual underline that miRNAs from dried blood spots are - from a technical perspective - suited as diagnostic tools.

Biological variation depending on lung cancer therapy

[0188] As last, the biological variation between the samples of lung cancer patients and the process controls were investigated. This was realized in two steps. First, 30 samples (26 lung

cancer and four process controls) on Agilent microarrays from both versions, V19 and V2, were profiled. 1. By applying hierarchical clustering, for each version two clusters with similar sample distribution were obtained (data not shown). Not only the samples with the same therapy form had a tendency to cluster together but also the process controls fell in one cluster. In the second step, 60 samples (53 lung cancer and the afore mentioned 7 process controls) were profiled on Agilent microarrays from miRBase V21. The average correlation of this analysis step was 0.974. While the average correlation for the process controls (0.993) and the lung cancer samples (0.976) exceeded this value, the lowest average correlation (0.967) was calculated between process controls and lung cancer samples.

[0189] Further of interest were the differences between two different lung cancer treatment cohorts. Therefore, statistical pairwise comparisons between the groups "adjuvant" and "palliative" treated patients were preformed next by using two-tailed t-test. Due to the exploratory nature of the study, miRNAs with raw p-values < 0.05 were considered as significantly deregulated. For the above-mentioned comparison (adjuvant vs. palliative), 51 significant deregulated miRNAs (8.6% of all 591 expressed miRNAs) were found based on two-tailed t-tests and the selected alpha-level of 0.05. Of these 51 miRNAs, 11 had higher expression in the adjuvant group (20%). The most up-regulated miRNA with a p-value of 0.019 was hsa-miR-150-5p, the most down-regulated marker with p-value of 0.014 was hsa-miR-642a-3p. All miRNAs are shown in the volcano plot in Figure 9, where up- and down-regulated miRNAs are highlighted in dark green (right upper corner) and light green (left upper corner) respectively. Detailed information on expression intensities, fold changes, p-values and the area under the receiver operator characteristics curve (AUC value) are provided in **Figure 10**.

Discussion

[0190] A growing number of studies dealt with miRNAs as non-invasive-biomarkers for various human diseases. In many cases, these miRNAs were derived from serum, plasma or whole blood. While preservation tubes like PAXgene and EDTA are commonly used, the investigation of miRNAs from DBS is still a new ground.

[0191] In the present study, the reliability of measurements of miRNA from DBS was analyzed by going after the following three aspects: (1) sample stability under different environmental conditions, (2) technical stability and reproducibility between chip versions, and (3) comparison of clinical samples using the example of lung cancer patients.

[0192] Regarding the first aspect, it has been shown that the measured miRNAs from DBS had high stability by considering the comparisons of the different environmental conditions (humidity and temperature) within one category.

[0193] In the second part of the study, it has been shown with the process controls on the one side that the equal and high correlations give evidences for technical stability across the different chip versions V19 and V21. On the other side, the clustering pattern of the clinical

samples and process controls from V21 could also be retrieved with similar structure from v19. These similar results indicate the high reproducibility of miRNAs measured in DBS despite different miRBase versions on the chips.

[0194] The third part of the study was about the clinical comparison between the three different groups: process controls, patients getting adjuvant and patients getting palliative therapy. In case of statistical significance of deregulated miRNAs, there was a very high number of significant miRNAs (adjusted p-value < 0.05) between lung cancer patients and process controls. This high number could be caused by the lung cancer disease itself and the application of the therapies.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [WO2013165870A1 \[0006\]](#)
- [WO2013067520A1 \[0097\] \[0097\] \[0097\] \[0133\] \[0133\] \[0133\]](#)
- [US2013116597A1 \[0097\] \[0097\] \[0097\] \[0133\] \[0133\] \[0133\]](#)

Non-patent literature cited in the description

- A multilingual glossary of biotechnological terms: (IUPAC Recommendations)Helvetica Chimica Acta19950000 [\[0018\]](#)

PATENTKRAV

1. Fremgangsmåde til oprensning af en RNA-fraktion med < 200 nukleotider i længden fra en fuldblodsprøve omfattende følgende trin:

(i) tilvejebringelse af en absorberende sonde omfattende et hydrofilt polymermateriale, hvori/hvortil fuldblod fra fuldblodsprøven er absorberet,

(ii) at bringe den absorberende sonde i kontakt med et fluid omfattende et chaotroisk middel, og

(iii) genvinding af fluidet omfattende et chaotroisk middel fra trin (ii), hvorved RNA-fraktionen med ≥ 200 nukleotider i længden fjernes, og

(iv) oprensning af RNA-fraktionen med < 200 nukleotider i længden fra det genvundne fluid ved én eller flere separationsteknikker valgt fra gruppen bestående af centrifugering, fordampning/rekonstitution, opkoncentration, præcipitation, væske/væske-ekstraktion og fastfaseekstraktion,

hvor RNA-fraktionen med ≥ 200 nukleotider i længden ikke trænger ind i fluidet og forbliver absorberet til det hydrofile polymermateriale,

hvor det hydrofile polymermateriale har en densitet på $\leq 6 \text{ g/cm}^3$, og

hvor det hydrofile polymermateriale vælges fra gruppen bestående af bomuld, et polysaccharid, en polyolefin og en polyester.

2. Fremgangsmåde ifølge krav 1, hvor

(i) polysaccharidet er cellulose,

(ii) polyolefinen vælges fra gruppen bestående af polyethylen, polypropylen, polybutylen, polyisobutylen og polymethylpenten, eller

(iii) polyesteren vælges fra gruppen bestående af polycarbonat og polyethylenterephthalat.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor det chaotropiske middel vælges fra gruppen bestående af guanidiniumthiocyanat, guanidiniumisothiocyanat, guanidiniumhydrochlorid, guanidiniumchlorid, alkali-thiocyanat, alkali-isothiocyanat, alkali-iodid og alkali-perchlorat.

4. Fremgangsmåde til bestemmelse af niveauet af RNA-molekyler med < 200 nukleotider i længden omfattende følgende trin:

(i) udførelse af fremgangsmåden ifølge et hvilket som helst af kravene 1 til 3 og

(ii) bestemmelse af niveauet af RNA-molekyler med < 200 nukleotider i længden ved en egnet teknik.

5. Fremgangsmåde til diagnosticering af en sygdom hos et individ omfattende følgende trin:

5 (i) udførelse af fremgangsmåden ifølge et hvilket som helst af kravene 1 til 3, hvor fuldblodsprøven er fra et individ,

(ii) bestemmelse af niveauet af RNA-molekyler med < 200 nukleotider i længden ved en egnet teknik,

(iii) sammenligning af niveauet med ét eller flere referenceniveauer og

10 (iv) diagnosticering eller differentiell diagnosticering af, hvorvidt individet er ramt af sygdommen baseret på sammenligningen.

6. Fremgangsmåde til diagnosticering af en sygdom hos et individ omfattende følgende trin:

(i) udførelse af fremgangsmåden ifølge krav 4, hvor fuldblodsprøven er fra et individ,

15 (ii) sammenligning af niveauet med ét eller flere referenceniveauer og

(iii) diagnosticering eller differentiell diagnosticering af, hvorvidt individet er ramt af sygdommen baseret på sammenligningen.

7. Fremgangsmåde ifølge krav 5 eller 6, hvor det ene eller flere referenceniveauer

20 bestemmes ved måling af én eller flere referencefuldblodsprøver fra

ét eller flere raske individer,

ét eller flere individer, der lider af en sygdom, og/eller

ét eller flere individer, der lider af en anden sygdom.

25 **8.** Anvendelse af et hydrofilt polymermateriale til oprensning af en RNA-fraktion med < 200 nukleotider i længden fra en fuldblodsprøve, hvor det hydrofile polymermateriale har en densitet på $\leq 6 \text{ g/cm}^3$, og hvor det hydrofile polymermateriale vælges fra gruppen bestående af bomuld, et polysaccharid, en polyolefin og en polyester.

30 **9.** Anvendelse ifølge krav 8, hvor

(i) polysaccharidet er cellulose,

(ii) polyolefinen vælges fra gruppen bestående af polyethylen, polypropylen, polybutylen, polyisobutylen og polymethylpenten, eller

(iii) polyesteren vælges fra gruppen bestående af polycarbonat og polyethylenterephthalat.

5 **10.** Anvendelse af en fremgangsmåde ifølge krav 4 til diagnosticering eller differentiell diagnosticering af en sygdom.

11. Anvendelse af et kit til oprensning af en RNA-fraktion med < 200 nukleotider i længden fra en fuldblodsprøve, hvor kittet omfatter:

10 (i) en absorberende sonde omfattende et hydrofilt polymermateriale og
(ii) et fluid omfattende et chaotroisk middel til at rekonstituere fuldblod absorberet i/til en absorberende sonde omfattende et hydrofilt polymermateriale,

hvor det hydrofile polymermateriale har en densitet på $\leq 6 \text{ g/cm}^3$, og hvor det hydrofile polymermateriale vælges fra gruppen bestående af bomuld, et polysaccharid, en polyolefin og en polyester.

15 **12.** Anvendelse ifølge krav 11, hvor kittet anvendes til udførelse af fremgangsmåderne ifølge et hvilket som helst af kravene 1 til 7.

20 **13.** Anvendelse ifølge krav 11 eller 12, hvor det chaotropiske middel vælges fra gruppen bestående af guanidiniumthiocyanat, guanidiniumisothiocyanat, guanidiniumhydrochlorid, guanidiniumchlorid, alkali-thiocyanat, alkali-isothiocyanat, alkali-iodid og alkali-perchlorat.

DRAWINGS

FIGURE 1

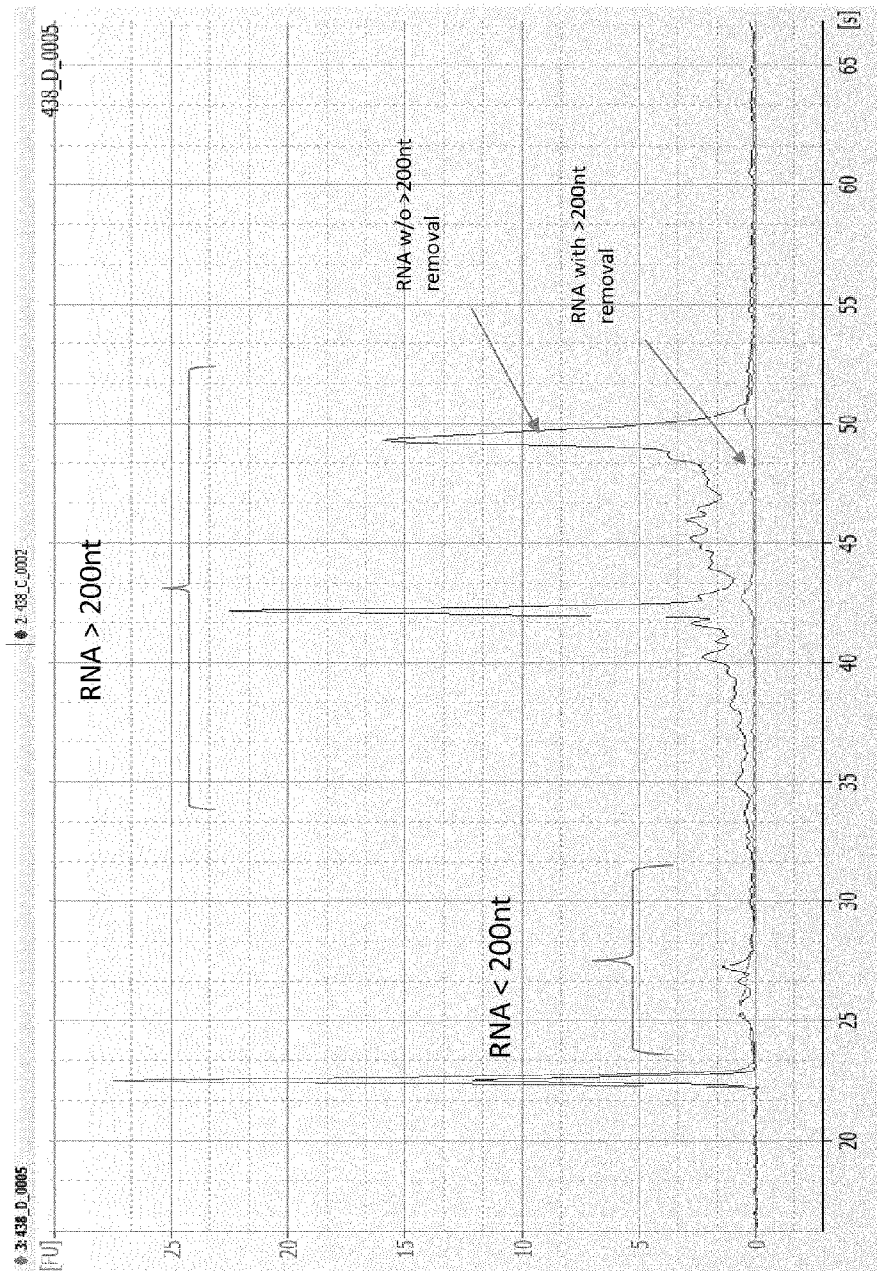


FIGURE 2

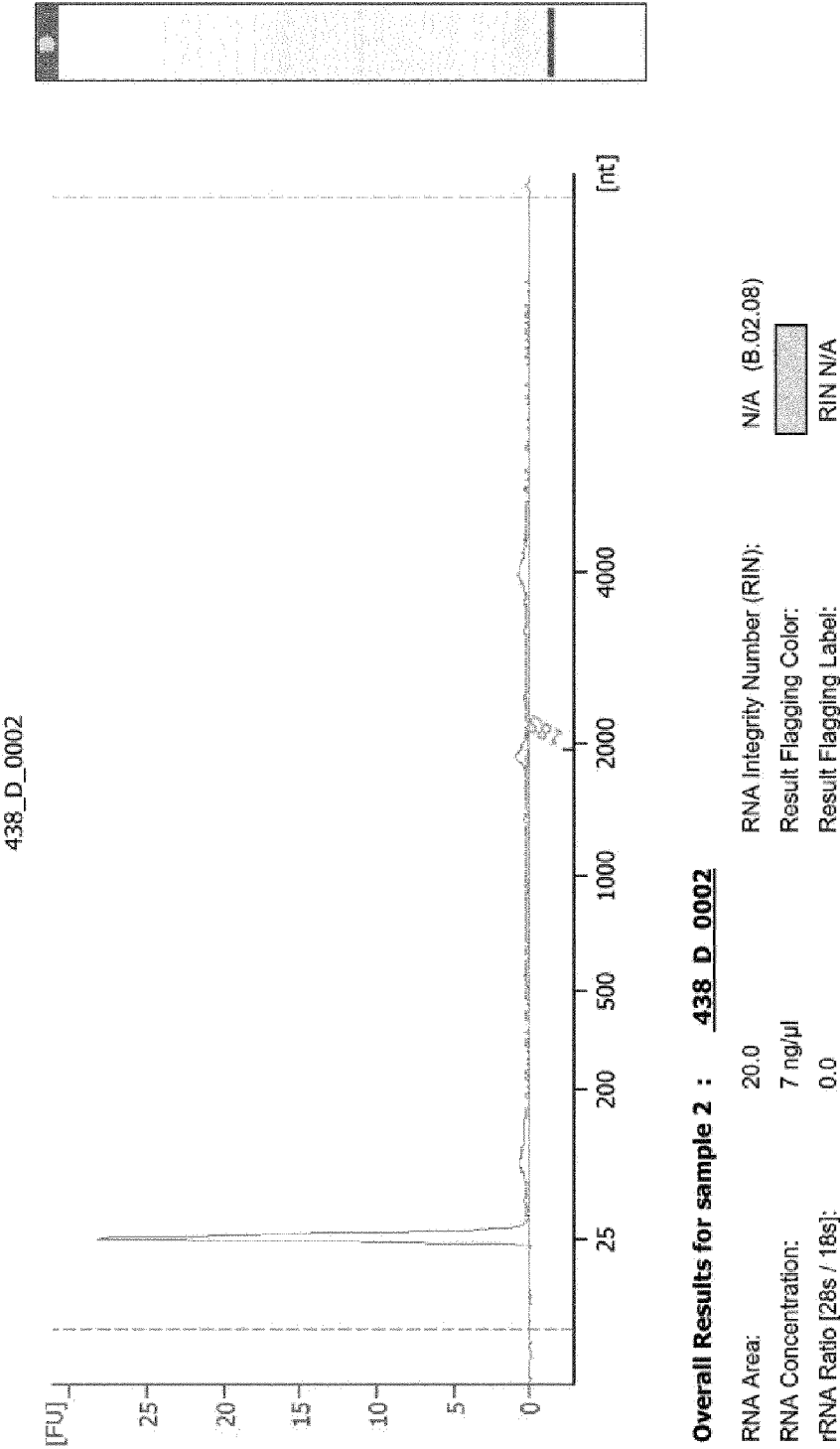


FIGURE 3

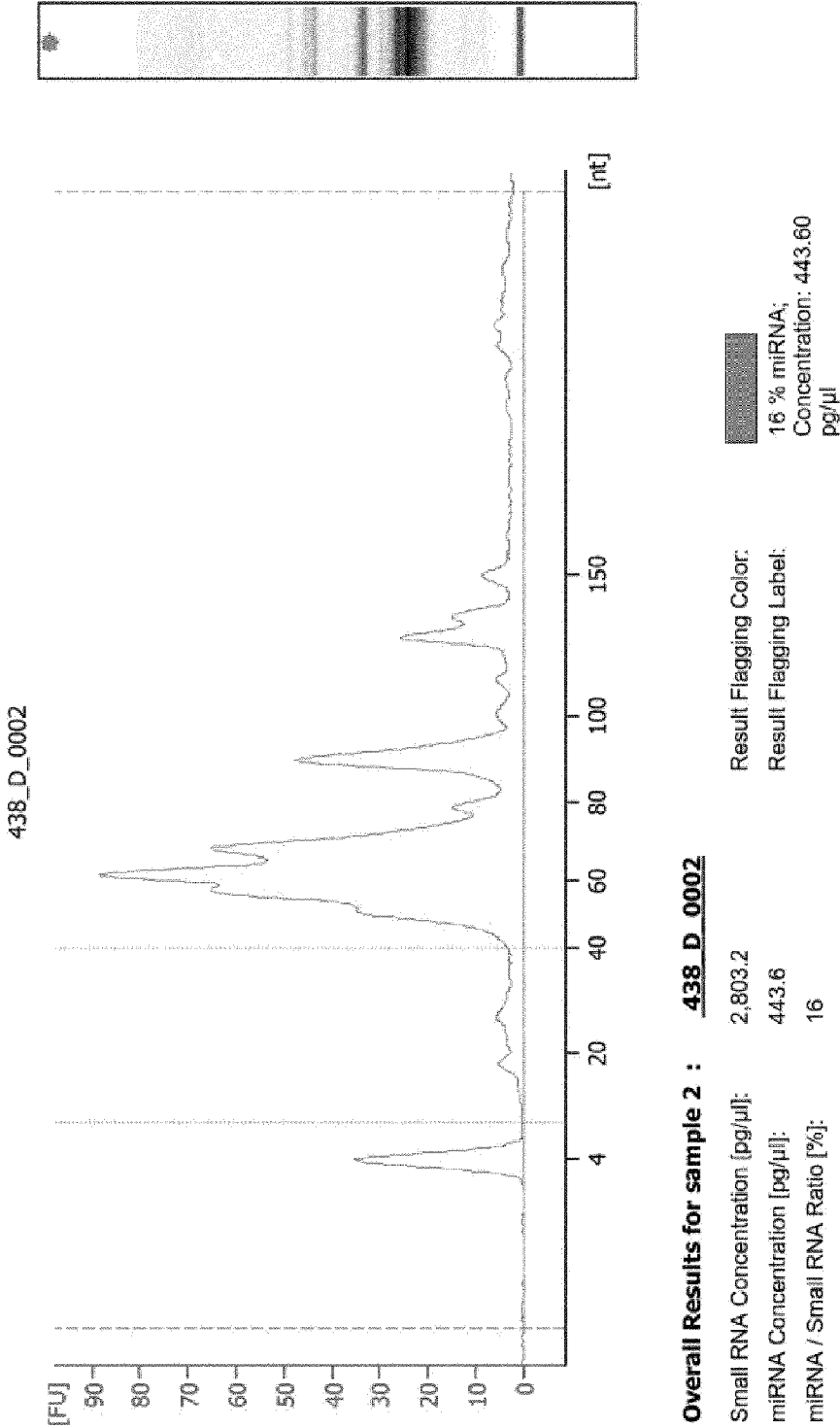


FIGURE 4

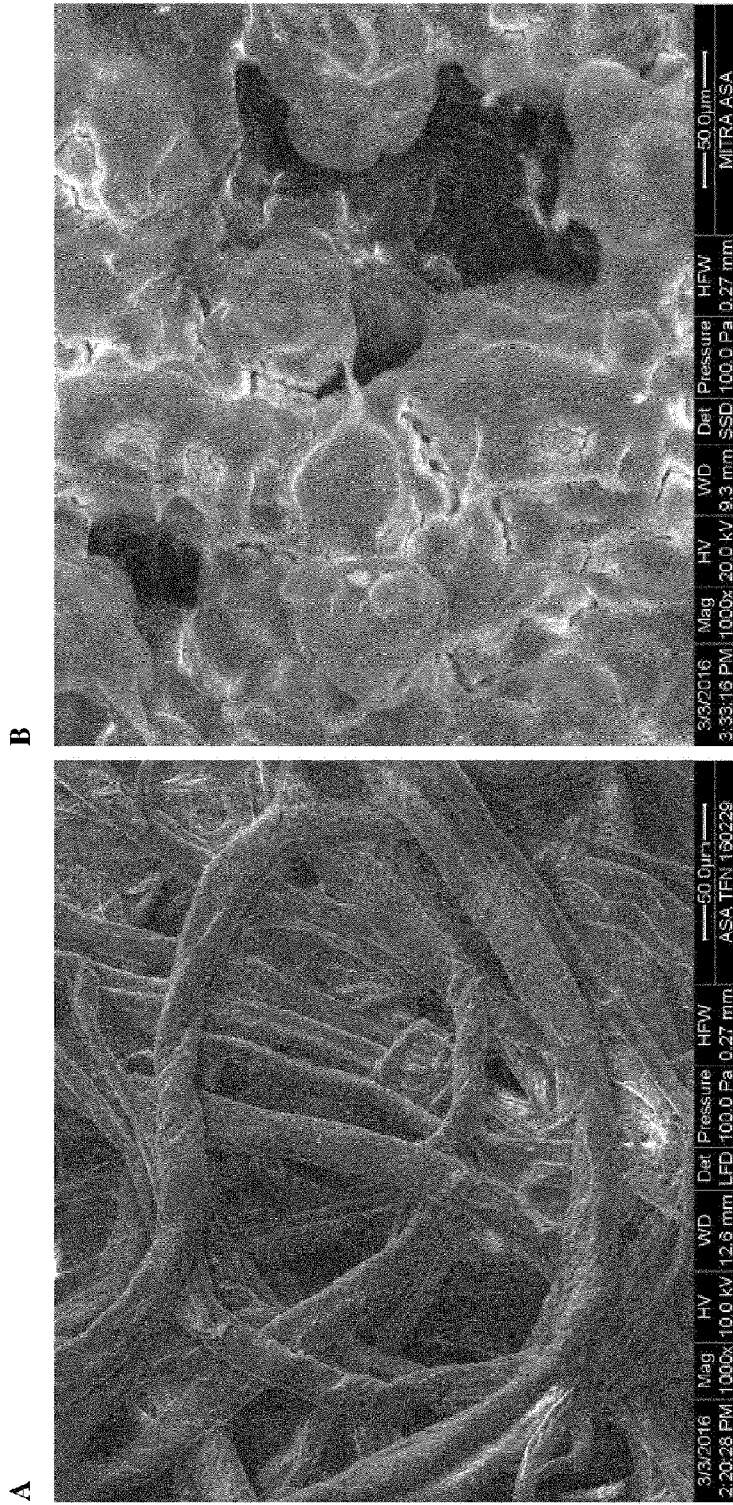


FIGURE 5

SEQ ID NO:	miRNA	gTotal Gene Signal (A)	gTotal GeneSignal (B)
1	hsa-miR-451a	163371	142265
2	hsa-miR-16-5p	139870	120550
3	hsa-miR-15b-5p	112389	75044
4	hsa-miR-144-3p	111793	48692
5	hsa-let-7a-5p	85577	67800
6	hsa-miR-19b-3p	79045	35603
7	hsa-let-7f-5p	72429	46812
8	hsa-let-7b-5p	71346	67652
9	hsa-miR-20a-5p	70079	37010
10	hsa-miR-486-5p	66439	54350
11	hsa-miR-15a-5p	64365	33946
12	hsa-miR-26b-5p	64186	32948
13	hsa-miR-142-3p	63789	17166
14	hsa-let-7g-5p	63079	34930
15	hsa-let-7i-5p	61276	36081
16	hsa-miR-106b-5p	60205	25911
17	hsa-miR-107	52623	40784
18	hsa-miR-25-3p	45550	29761
19	hsa-miR-19a-3p	41505	9967
20	hsa-miR-223-3p	39792	19567
21	hsa-miR-4306	37357	21482
22	hsa-miR-92a-3p	36912	31901
23	hsa-miR-144-5p	34077	22262
24	hsa-miR-103a-3p	33567	27524
25	hsa-miR-20b-5p	32324	16603
26	hsa-miR-21-5p	30748	13628
27	hsa-let-7d-5p	29179	21396
28	hsa-miR-17-5p	28916	17407
29	hsa-miR-101-3p	28060	6040
30	hsa-miR-185-5p	27711	12351
31	hsa-miR-7977	25257	25273
32	hsa-let-7c-5p	24313	22411
33	hsa-miR-126-3p	23566	12387
34	hsa-miR-93-5p	22857	15302
35	hsa-miR-22-3p	21352	15362
36	hsa-miR-363-3p	18685	10983
37	hsa-miR-374a-5p	17002	3956
38	hsa-miR-142-5p	16124	2720
39	hsa-miR-29c-3p	14117	3887
40	hsa-miR-5100	12321	15857
41	hsa-miR-30b-5p	11134	6402
42	hsa-miR-425-5p	10549	7636
43	hsa-miR-26a-5p	10358	6690
44	hsa-miR-140-3p	9299	5248
45	hsa-miR-130a-3p	7797	5272
46	hsa-miR-30e-5p	7670	2481
47	hsa-miR-320d	7402	7055
48	hsa-miR-320b	6417	6025
49	hsa-miR-96-5p	6320	1921
50	hsa-miR-8069	6305	3660
51	hsa-miR-320e	5943	5683
52	hsa-miR-151a-5p	5201	3192
53	hsa-miR-320c	5013	4726
54	hsa-miR-29b-3p	4891	1072
55	hsa-miR-18a-5p	4287	1822
56	hsa-miR-454-3p	4086	2339
57	hsa-miR-374b-5p	3994	2281
58	hsa-miR-7641	3820	6761
59	hsa-miR-194-5p	3707	1908
60	hsa-miR-301a-3p	3654	1051
61	hsa-miR-320a	3653	3603
62	hsa-miR-192-5p	3597	1366
63	hsa-miR-4516	3507	2301
64	hsa-miR-331-3p	3444	1677
65	hsa-miR-660-5p	3431	1182
66	hsa-miR-150-5p	3430	2461
67	hsa-miR-24-3p	3224	2503
68	hsa-miR-23a-3p	2712	2086
69	hsa-miR-18b-5p	2700	1127
70	hsa-miR-29a-3p	2684	1111
71	hsa-miR-30c-5p	2649	1669
72	hsa-miR-183-5p	2477	1714
73	hsa-miR-3960	2471	2226
74	hsa-miR-590-5p	2465	324
75	hsa-miR-27a-3p	2440	782
76	hsa-miR-324-3p	2431	2076
77	hsa-miR-30d-5p	2384	1796
78	hsa-miR-6089	2295	2557
79	hsa-miR-186-5p	2138	1100
80	hsa-miR-7-5p	2137	749
81	hsa-miR-151b	2134	1427
82	hsa-miR-652-3p	2078	1658
83	hsa-miR-215-5p	2008	763
84	hsa-miR-181a-5p	1913	924
85	hsa-miR-424-5p	1872	305
86	hsa-miR-4505	1735	402
87	hsa-miR-148a-3p	1680	339
88	hsa-miR-1273g-3p	1663	3600
89	hsa-miR-6090	1620	2225
90	hsa-miR-17-3p	1595	252
91	hsa-miR-1260a	1544	2453
92	hsa-miR-98-5p	1508	779
93	hsa-miR-130b-3p	1366	1021
94	hsa-miR-4286	1355	1834
95	hsa-miR-6803-5p	1309	354
96	hsa-miR-140-5p	1250	467
97	hsa-miR-148b-3p	1215	420
98	hsa-miR-195-5p	1194	779

99	hsa-miR-210-3p	1173	747	151	hsa-miR-584-5p	283	247
100	hsa-miR-423-5p	1130	1255	152	hsa-miR-29c-5p	281	159
101	hsa-miR-4507	1102	272	153	hsa-miR-4459	279	597
102	hsa-miR-32-5p	1044	87	154	hsa-miR-221-3p	274	269
103	hsa-miR-532-5p	991	599	155	hsa-miR-338-3p	269	107
104	hsa-miR-199a-3p	874	396	156	hsa-miR-1915-3p	267	204
105	hsa-miR-6125	866	805	157	hsa-miR-4318	255	189
106	hsa-miR-484	828	588	158	hsa-miR-6749-5p	251	527
107	hsa-miR-342-3p	721	566	159	hsa-miR-629-5p	251	169
108	hsa-miR-362-5p	696	430	160	hsa-miR-199a-5p	235	84
109	hsa-miR-550a-3p	656	297	161	hsa-miR-502-5p	235	70
110	hsa-miR-6869-5p	638	742	162	hsa-miR-6085	228	393
111	hsa-miR-6875-5p	629	1159	163	hsa-miR-190a-5p	227	54
112	hsa-miR-27b-3p	613	318	164	hsa-miR-6794-5p	216	166
113	hsa-miR-1587	582	114	165	hsa-miR-361-3p	215	154
114	hsa-miR-126-5p	579	223	166	hsa-miR-625-5p	214	176
115	hsa-miR-4443	576	2918	167	hsa-miR-15b-3p	204	93
116	hsa-miR-151a-3p	570	361	168	hsa-miR-374c-5p	200	127
117	hsa-miR-146b-5p	562	244	169	hsa-miR-4763-3p	194	156
118	hsa-miR-324-5p	553	305	170	hsa-miR-361-5p	192	154
119	hsa-miR-6821-5p	523	653	171	hsa-miR-4530	185	193
120	hsa-miR-128-3p	499	259	172	hsa-let-7e-5p	182	118
121	hsa-miR-500a-3p	478	268	173	hsa-miR-6779-5p	178	31
122	hsa-miR-4281	467	456	174	hsa-miR-638	177	283
123	hsa-miR-6127	462	793	175	hsa-miR-196b-5p	173	71
124	hsa-miR-362-3p	450	65	176	hsa-miR-5001-5p	169	121
125	hsa-miR-340-5p	449	99	177	hsa-miR-5739	169	482
126	hsa-miR-378i	445	317	178	hsa-miR-505-5p	167	151
127	hsa-miR-550a-3-5p	438	264	179	hsa-miR-1202	166	286
128	hsa-miR-146a-5p	433	429	180	hsa-miR-30e-3p	161	91
129	hsa-miR-3665	414	417	181	hsa-miR-1207-5p	161	186
130	hsa-miR-532-3p	413	270	182	hsa-miR-6879-5p	157	245
131	hsa-miR-4687-3p	406	426	183	hsa-miR-28-5p	156	72
132	hsa-miR-6087	402	547	184	hsa-miR-7-1-3p	154	62
133	hsa-miR-502-3p	402	254	185	hsa-miR-197-3p	151	118
134	hsa-miR-3656	389	277	186	hsa-miR-6756-5p	148	94
135	hsa-miR-182-5p	389	377	187	hsa-miR-219a-5p	147	14
136	hsa-miR-16-2-3p	386	423	188	hsa-miR-3162-5p	141	209
137	hsa-miR-20a-3p	368	161	189	hsa-miR-342-5p	140	143
138	hsa-miR-6724-5p	354	164	190	hsa-miR-627-5p	140	70
139	hsa-miR-1260b	353	534	191	hsa-miR-335-5p	137	54
140	hsa-miR-23b-3p	341	243	192	hsa-miR-671-5p	129	33
141	hsa-miR-6088	327	315	193	hsa-miR-4788	128	107
142	hsa-miR-4466	322	354	194	hsa-miR-4463	125	92
143	hsa-miR-4284	319	403	195	hsa-miR-501-5p	119	50
144	hsa-miR-6800-5p	313	416	196	hsa-miR-4787-5p	117	101
145	hsa-miR-7704	313	278	197	hsa-miR-6780b-5p	115	178
146	hsa-miR-6727-5p	306	68	198	hsa-miR-378d	115	65
147	hsa-miR-2861	305	357	199	hsa-miR-574-3p	114	106
148	hsa-miR-378a-3p	299	225	200	hsa-miR-545-3p	111	15
149	hsa-miR-222-3p	294	263	201	hsa-miR-500a-5p	110	65
150	hsa-miR-624-5p	284	64	202	hsa-miR-132-3p	108	77

203	hsa-miR-4713-3p	108	164	255	hsa-miR-6723-5p	59	85
204	hsa-miR-598-3p	106	32	256	hsa-miR-365a-3p	58	50
205	hsa-miR-744-5p	105	98	257	hsa-miR-4291	57	57
206	hsa-miR-7114-5p	105	189	258	hsa-miR-6850-5p	57	81
207	hsa-miR-155-5p	104	120	259	hsa-miR-125a-5p	55	58
208	hsa-miR-6740-5p	104	153	260	hsa-miR-1268a	55	48
209	hsa-miR-101-5p	103	43	261	hsa-miR-7847-3p	54	40
210	hsa-miR-4739	100	73	262	hsa-miR-6791-5p	54	59
211	hsa-miR-6165	100	213	263	hsa-miR-4298	53	32
212	hsa-miR-371b-5p	98	87	264	hsa-miR-664a-3p	52	56
213	hsa-miR-6126	98	90	265	hsa-miR-3195	52	105
214	hsa-miR-6124	96	129	266	hsa-miR-1268b	51	54
215	hsa-miR-3200-5p	96	77	267	hsa-miR-1305	51	77
216	hsa-miR-1225-5p	94	119	268	hsa-miR-4442	51	64
217	hsa-miR-1246	92	74	269	hsa-miR-1285-3p	51	28
218	hsa-miR-4732-5p	92	89	270	hsa-miR-340-3p	51	23
219	hsa-miR-6068	91	117	271	hsa-miR-3620-5p	49	22
220	hsa-miR-15a-3p	90	22	272	hsa-miR-642a-3p	49	41
221	hsa-miR-7107-5p	89	187	273	hsa-miR-3135b	49	102
222	hsa-miR-197-5p	89	99	274	hsa-miR-7108-5p	49	46
223	hsa-miR-181b-5p	87	56	275	hsa-miR-3141	49	38
224	hsa-miR-1229-5p	87	45	276	hsa-miR-223-5p	48	36
225	hsa-miR-6826-5p	86	129	277	hsa-miR-378g	48	24
226	hsa-miR-939-5p	85	127	278	hsa-miR-145-5p	48	34
227	hsa-miR-3940-5p	85	75	279	hsa-miR-8063	48	36
228	hsa-miR-937-5p	82	53	280	hsa-miR-5787	47	77
229	hsa-miR-181c-5p	81	49	281	hsa-miR-4299	47	47
230	hsa-miR-376c-3p	80	25	282	hsa-miR-3679-5p	45	63
231	hsa-miR-6717-5p	79	130	283	hsa-miR-542-5p	45	21
232	hsa-miR-505-3p	77	38	284	hsa-miR-4690-5p	45	34
233	hsa-miR-4685-5p	76	108	285	hsa-miR-21-3p	45	24
234	hsa-miR-494-3p	75	118	286	hsa-miR-1914-3p	44	61
235	hsa-miR-22-5p	73	64	287	hsa-miR-4270	44	44
236	hsa-miR-550b-2-5p	73	50	288	hsa-miR-6763-5p	44	70
237	hsa-miR-582-5p	72	44	289	hsa-miR-542-3p	44	8
238	hsa-miR-30a-5p	72	46	290	hsa-miR-6831-5p	43	14
239	hsa-miR-6131	71	117	291	hsa-miR-1255b-5p	42	31
240	hsa-miR-664b-3p	70	78	292	hsa-miR-378a-5p	42	26
241	hsa-miR-3200-3p	69	39	293	hsa-miR-6820-5p	41	24
242	hsa-miR-193a-3p	69	6	294	hsa-miR-4486	41	13
243	hsa-miR-6891-5p	65	82	295	hsa-miR-6799-5p	40	13
244	hsa-miR-199b-5p	64	23	296	hsa-miR-628-5p	40	17
245	hsa-miR-4433b-3p	64	45	297	hsa-let-7d-3p	40	36
246	hsa-miR-6073	63	77	298	hsa-miR-557	39	28
247	hsa-miR-152-3p	63	15	299	hsa-miR-188-5p	39	19
248	hsa-miR-6734-5p	62	79	300	hsa-miR-500b-5p	39	20
249	hsa-miR-376a-3p	62	21	301	hsa-miR-3663-3p	39	44
250	hsa-miR-93-3p	61	44	302	hsa-miR-6789-5p	39	29
251	hsa-miR-3196	61	74	303	hsa-miR-377-3p	38	12
252	hsa-miR-501-3p	60	41	304	hsa-miR-3652	38	86
253	hsa-miR-3198	60	89	305	hsa-miR-6767-5p	38	53
254	hsa-miR-4732-3p	60	52	306	hsa-miR-1307-5p	37	3

307	hsa-miR-5581-5p	37	53	359	hsa-miR-339-3p	22	17
308	hsa-miR-99a-5p	36	46	360	hsa-miR-6512-5p	21	29
309	hsa-miR-4716-3p	36	53	361	hsa-miR-7150	21	84
310	hsa-miR-1343-5p	36	23	362	hsa-miR-191-5p	20	19
311	hsa-miR-486-3p	36	39	363	hsa-miR-1275	20	23
312	hsa-miR-766-3p	36	26	364	hsa-miR-339-5p	20	26
313	hsa-miR-4669	35	27	365	hsa-miR-7106-5p	20	3
314	hsa-miR-141-3p	35	7	366	hsa-miR-5690	20	6
315	hsa-miR-125b-5p	35	197	367	hsa-miR-410-3p	19	10
316	hsa-miR-6076	35	30	368	hsa-miR-454-5p	19	10
317	hsa-miR-4695-5p	34	17	369	hsa-miR-4737	19	4
318	hsa-miR-3651	34	39	370	hsa-miR-7110-5p	19	31
319	hsa-miR-942-5p	34	19	371	hsa-miR-1255a	19	11
320	hsa-miR-5194	33	47	372	hsa-miR-193a-5p	19	14
321	hsa-miR-450a-5p	33	8	373	hsa-miR-5088-5p	18	27
322	hsa-miR-1227-5p	32	46	374	hsa-miR-4651	18	14
323	hsa-miR-4271	32	20	375	hsa-miR-6812-5p	18	12
324	hsa-miR-34a-5p	32	24	376	hsa-miR-664a-5p	18	28
325	hsa-miR-874-3p	32	21	377	hsa-miR-99b-5p	18	10
326	hsa-miR-4653-3p	31	42	378	hsa-miR-503-5p	17	6
327	hsa-miR-769-5p	31	21	379	hsa-miR-548e-3p	17	4
328	hsa-miR-328-3p	31	22	380	hsa-miR-5010-5p	17	18
329	hsa-miR-183-3p	31	26	381	hsa-miR-1307-3p	17	12
330	hsa-miR-192-3p	31	9	382	hsa-miR-548am-5p	17	10
331	hsa-miR-6775-5p	30	41	383	hsa-miR-6893-5p	17	17
332	hsa-miR-487b-3p	29	22	384	hsa-miR-3127-5p	17	23
333	hsa-miR-136-5p	28	8	385	hsa-miR-762	17	33
334	hsa-miR-326	28	13	386	hsa-miR-4497	17	29
335	hsa-miR-4741	28	32	387	hsa-miR-409-3p	16	16
336	hsa-miR-6722-3p	28	28	388	hsa-miR-3188	16	18
337	hsa-miR-221-5p	28	18	389	hsa-miR-4659a-3p	16	7
338	hsa-miR-296-5p	28	24	390	hsa-miR-4745-5p	15	20
339	hsa-miR-575	26	57	391	hsa-miR-4665-3p	15	15
340	hsa-miR-6807-5p	25	30	392	hsa-miR-497-5p	15	6
341	hsa-miR-3667-5p	25	4	393	hsa-miR-3156-5p	15	19
342	hsa-miR-6785-5p	25	69	394	hsa-miR-421	15	12
343	hsa-miR-628-3p	24	22	395	hsa-miR-654-3p	15	7
344	hsa-miR-574-5p	24	81	396	hsa-miR-103a-2-5p	15	17
345	hsa-miR-200c-3p	24	19	397	hsa-miR-1271-5p	14	6
346	hsa-miR-940	24	26	398	hsa-miR-4317	14	10
347	hsa-miR-10a-5p	24	26	399	hsa-miR-3136-5p	14	9
348	hsa-miR-378f	24	12	400	hsa-miR-6840-3p	14	28
349	hsa-miR-423-3p	23	17	401	hsa-miR-579-3p	14	3
350	hsa-miR-1288-3p	23	29	402	hsa-miR-19b-1-5p	14	6
351	hsa-miR-3125	23	33	403	hsa-miR-1537-3p	14	3
352	hsa-miR-3907	23	96	404	hsa-miR-4455	14	21
353	hsa-let-7i-3p	23	5	405	hsa-miR-499a-5p	14	3
354	hsa-miR-7152-3p	22	37	406	hsa-miR-548q	14	13
355	hsa-miR-4672	22	19	407	hsa-miR-495-3p	14	5
356	hsa-miR-6780a-5p	22	32	408	hsa-miR-1233-5p	14	40
357	hsa-miR-211-3p	22	62	409	hsa-miR-4534	13	14
358	hsa-miR-572	22	35	410	hsa-miR-378e	13	8

411	hsa-miR-4313	13	9
412	hsa-miR-148b-5p	13	7
413	hsa-miR-4323	13	10
414	hsa-miR-610	13	7
415	hsa-miR-4634	13	18
416	hsa-miR-328-5p	13	67
417	hsa-miR-381-3p	13	3
418	hsa-miR-4721	13	20
419	hsa-miR-6516-5p	12	10
420	hsa-miR-31-5p	12	5
421	hsa-miR-7845-5p	12	16
422	hsa-miR-892b	12	17
423	hsa-miR-6786-5p	12	14
424	hsa-miR-3163	12	6
425	hsa-miR-1270	12	12
426	hsa-miR-642b-3p	12	11
427	hsa-miR-106a-3p	12	4
428	hsa-miR-6728-5p	12	17
429	hsa-miR-143-3p	12	3
430	hsa-miR-4499	11	13
431	hsa-miR-4632-5p	11	12
432	hsa-miR-548d-5p	11	2
433	hsa-miR-3911	11	8
434	hsa-miR-6075	11	7
435	hsa-miR-1228-3p	11	12
436	hsa-miR-134-5p	11	18
437	hsa-miR-6515-3p	11	13
438	hsa-miR-1234-3p	11	11
439	hsa-miR-6833-5p	10	8
440	hsa-miR-224-5p	10	12
441	hsa-miR-6829-5p	10	15
442	hsa-miR-4742-5p	10	14
443	hsa-miR-2110	10	12
444	hsa-miR-100-5p	10	410
445	hsa-miR-1973	10	28
446	hsa-miR-5006-5p	10	12
447	hsa-miR-6872-3p	10	12
448	hsa-miR-4465	9	18
449	hsa-miR-1281	9	11
450	hsa-miR-8072	9	18
451	hsa-miR-6752-5p	9	14
452	hsa-miR-630	8	42
453	hsa-miR-212-3p	8	23
454	hsa-miR-6510-5p	8	22
455	hsa-miR-2392	8	13
456	hsa-miR-181d-5p	8	13
457	hsa-miR-6849-5p	8	31
458	hsa-miR-150-3p	7	14
459	hsa-miR-29b-2-5p	7	11
460	hsa-miR-4800-5p	7	13
461	hsa-miR-432-5p	6	11
462	hsa-miR-127-3p	6	11

463	hsa-miR-4261	6	10
464	hsa-miR-4327	4	13
465	hsa-miR-6769b-5p	4	18
466	hsa-miR-483-5p	3	10
467	hsa-miR-623	3	13

hsa-miR-1250-3p (SEQ ID NO: 468)

hsa-miR-3138 (SEQ ID NO: 469)

hsa-miR-4767 (SEQ ID NO: 470)

hsa-miR-551b-3p (SEQ ID NO: 471)

hsa-miR-5703 (SEQ ID NO: 472)

hsa-miR-6716-3p (SEQ ID NO: 473)

hsa-miR-718 (SEQ ID NO: 474)

hsa-miR-8071 (SEQ ID NO: 475)

FIGURE 6

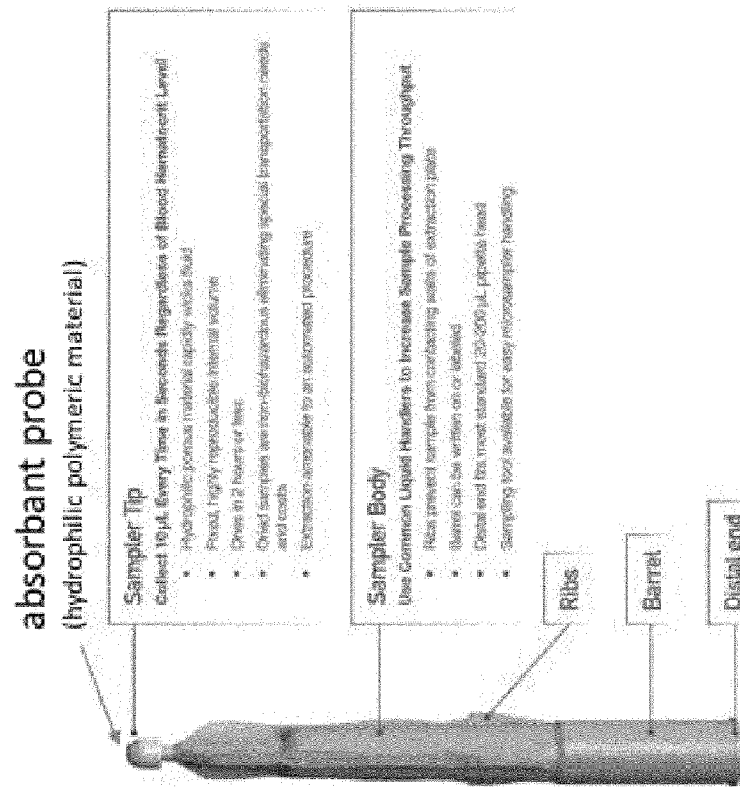


FIGURE 7

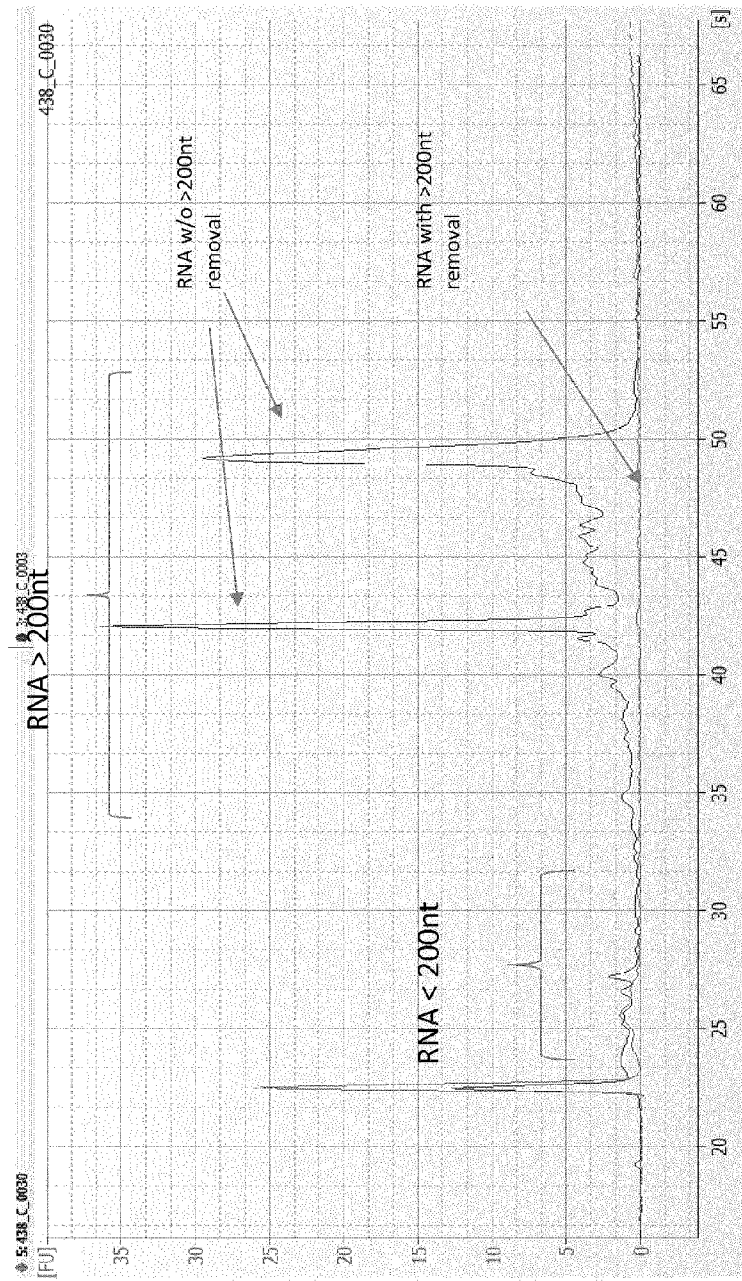


FIGURE 8A

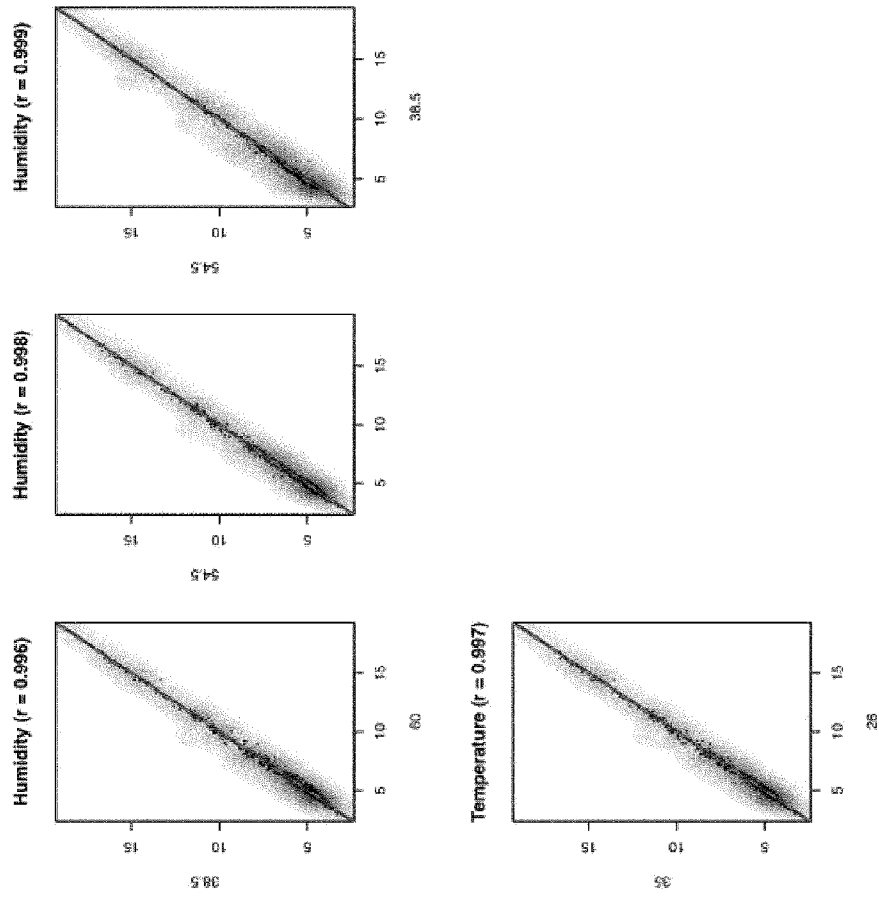


FIGURE 8B

PVCA: influence of the factors to the variance

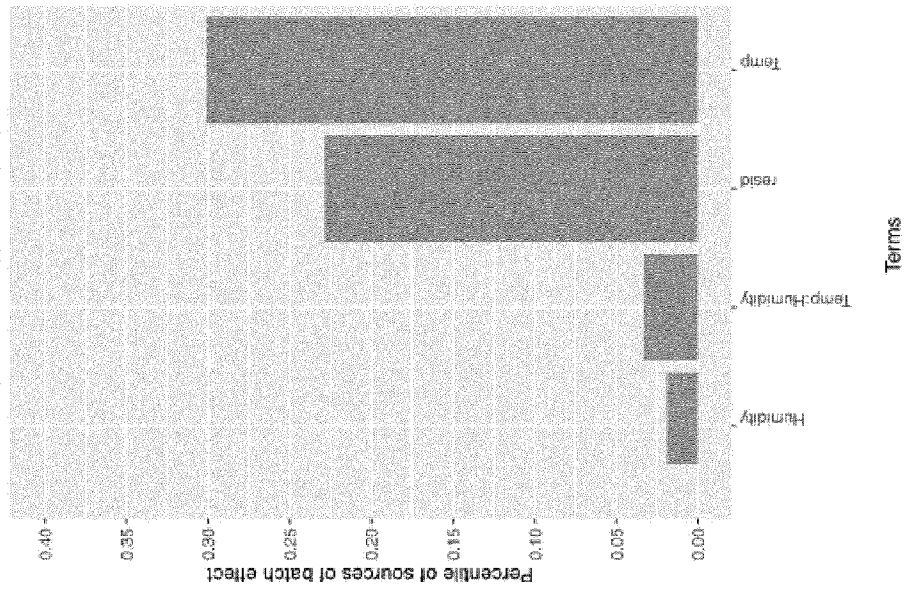


FIGURE 9

Left: palliative , right: adjuvant (t-test raw p-val)

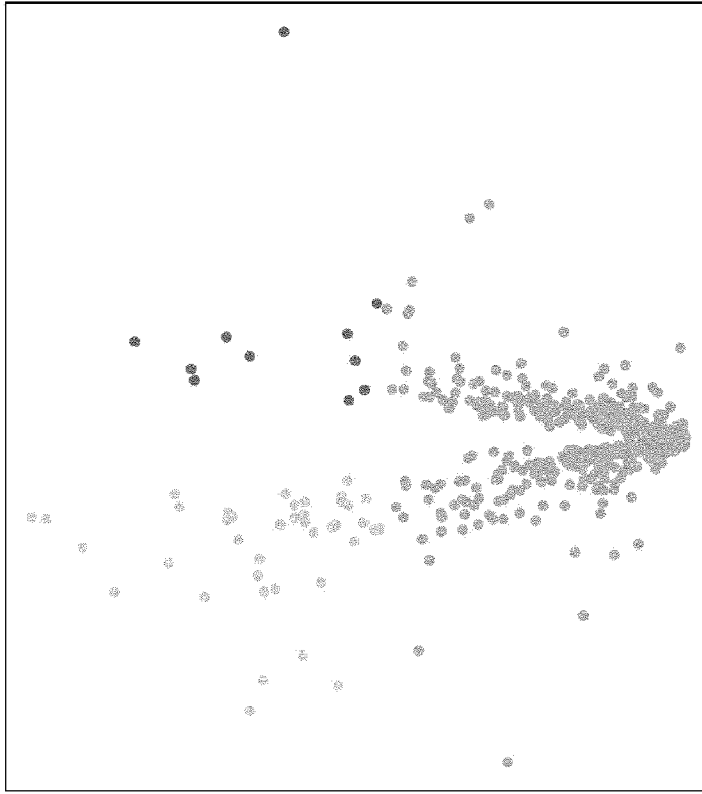


Figure 10

miRNA	limma.rawp	limma.adj.p	ttest.rawp	ttest.adj.p	AUC	mean.adjuvant	mean.palliative	fold_change	log2_fold_change
hsa-miR-1202	0.029817521	0.608516285	0.027447818	0.477107663	0.681372549	597.0639992	873.0397815	0.683890938	-0.548161822
hsa-miR-1250-3p	0.019778769	0.608516285	0.035582751	0.496147911	0.261437908	28.3706511	21.50904813	1.319010071	0.39945558
hsa-miR-140-5p	0.014325763	0.608516285	0.013557299	0.419479035	0.264705882	646.6693735	520.6639018	1.242009233	0.312675899
hsa-miR-146b-5p	0.014028998	0.608516285	0.010703438	0.419479035	0.289215886	209.8421757	160.5142154	1.307312098	0.386603601
hsa-miR-150-3p	0.043202265	0.608516285	0.010749976	0.419479035	0.691176471	24.23207734	30.06110828	0.806093944	-0.310980111
hsa-miR-150-5p	0.003318936	0.608516285	0.019034207	0.43119737	0.308823529	1955.064781	668.2694351	2.925563671	1.548714617
hsa-miR-1915-3p	0.0582736	0.624382729	0.042509788	0.523401761	0.661764706	114.6648605	134.5660703	0.852108263	-0.230891353
hsa-miR-2861	0.019345011	0.608516285	0.010864012	0.419479035	0.685542484	135.8860881	165.468412	0.821220718	-0.284158071
hsa-miR-296-5p	0.030303925	0.608516285	0.035453444	0.496147911	0.66503268	27.00515256	30.22588154	0.893444884	-0.182549718
hsa-miR-3139	0.076201354	0.68234849	0.023347404	0.43119737	0.691176471	23.08891757	27.30473184	0.84560133	-0.241950448
hsa-miR-328-5p	0.028075461	0.608516285	0.017397433	0.43119737	0.684640523	37.74116241	56.12899723	0.67240044	-0.572607427
hsa-miR-331-3p	0.028013859	0.608516285	0.007817933	0.419479035	0.300653595	1459.120362	1250.814424	1.166536246	0.222231134
hsa-miR-342-3p	0.008489608	0.608516285	0.004353233	0.419479035	0.282679739	251.8517796	195.0531564	1.291195545	0.368707506
hsa-miR-342-5p	0.049065548	0.608516285	0.038424755	0.504645122	0.326797386	57.05980733	46.4786392	1.227651299	0.295900837
hsa-miR-3679-5p	0.029282091	0.608516285	0.014557504	0.419479035	0.656862745	148.5992996	213.3654946	0.696454418	-0.521899161
hsa-miR-4270	0.028741569	0.608516285	0.01496353	0.419479035	0.668300654	61.01227575	83.77119061	0.728320504	-0.457354634
hsa-miR-4327	0.06182773	0.624382729	0.018331141	0.43119737	0.647058824	20.47150825	25.72606435	0.79574971	-0.329613389
hsa-miR-4459	0.068110204	0.624382729	0.032292938	0.496147911	0.617647059	676.8462427	1298.790012	0.52073507	-0.941378524
hsa-miR-4466	0.062775291	0.624382729	0.035633156	0.496147911	0.645424837	185.1573635	221.0290968	0.837705832	-0.255484377
hsa-miR-4530	0.064598238	0.624382729	0.038140222	0.504645122	0.666666667	250.9580914	329.5938549	0.761416172	-0.393242882
hsa-miR-4634	0.04089815	0.608516285	0.046160141	0.556747827	0.673202614	115.8567308	147.377253	0.78612356	-0.347172008
hsa-miR-4672	0.039478591	0.608516285	0.033534918	0.496147911	0.683006536	41.77141326	48.71698667	0.857430151	-0.221908945
hsa-miR-4745-5p	0.013956618	0.608516285	0.006766681	0.419479035	0.676470688	23.94985284	28.67755317	0.835142827	-0.259905144
hsa-miR-4763-3p	0.031838583	0.608516285	0.025488464	0.456475212	0.68627451	126.4012197	162.1244303	0.779655598	-0.359091121
hsa-miR-4767	0.049526793	0.608516285	0.033417537	0.496147911	0.678104575	36.75234435	43.45202917	0.845814224	-0.241587272
hsa-miR-551b-3p	0.00269497	0.608516285	0.007570367	0.419479035	0.269607843	20.31119751	16.90318341	1.201192856	0.264467799
hsa-miR-5703	0.017864211	0.608516285	0.015402228	0.419479035	0.681372549	216.7224571	410.8403815	0.527510115	-0.922729336
hsa-miR-575	0.010590652	0.608516285	0.006061606	0.419479035	0.709150327	84.34844129	117.0417951	0.720669409	-0.472590488

hsa-miR-6087	0.020992589	0.608516285	0.015615125	0.419479035	0.679738562	694.5589831	1040.775772	0.667347378	-0.583490163
hsa-miR-630	0.029456907	0.608516285	0.022811749	0.43119737	0.668668667	182.3812212	322.8984684	0.564825291	-0.824123407
hsa-miR-638	0.009717849	0.608516285	0.002590664	0.419479035	0.722222222	72.08661638	96.1318202	0.749874144	-0.415279616
hsa-miR-642a-3p	0.038543523	0.608516285	0.013503949	0.419479035	0.619281046	144.0000965	295.9648622	0.486544898	-1.039395152
hsa-miR-642b-3p	0.044664051	0.608516285	0.012014663	0.419479035	0.612745098	30.95796752	40.399639	0.766293172	-0.384031644
hsa-miR-6512-5p	0.035252922	0.608516285	0.041939948	0.523401761	0.328431373	33.24701485	29.26072917	1.136233299	0.18425909
hsa-miR-6716-3p	0.036880867	0.608516285	0.047268892	0.558715941	0.31372549	62.74897117	43.92181554	1.42865158	0.514654114
hsa-miR-6752-5p	0.048488949	0.608516285	0.019268544	0.43119737	0.668300654	30.12179058	34.87045608	0.863819805	-0.21111977
hsa-miR-6786-5p	0.008499207	0.608516285	0.001565159	0.419479035	0.715686275	27.88426612	34.36232102	0.811477969	-0.301376186
hsa-miR-6789-5p	0.035059896	0.608516285	0.023345503	0.43119737	0.676470588	43.89483804	54.71030855	0.802313809	-0.317761487
hsa-miR-6791-5p	0.018415696	0.608516285	0.006454289	0.419479035	0.72875817	29.96471892	34.74218485	0.86248804	-0.213423644
hsa-miR-6831-5p	0.048456967	0.608516285	0.021094283	0.43119737	0.64669281	48.87471308	58.33658517	0.83780552	-0.255312705
hsa-miR-6850-5p	0.027764277	0.608516285	0.011317232	0.419479035	0.668300654	52.11133631	64.1649768	0.81214611	-0.300188794
hsa-miR-6891-5p	0.010960068	0.608516285	0.001812554	0.419479035	0.709150327	41.37455602	51.19489556	0.808179321	-0.307252657
hsa-miR-7107-5p	0.062098907	0.624382729	0.030540288	0.496147911	0.650326797	155.486494	196.6454463	0.790594808	-0.338807509
hsa-miR-7110-5p	0.029358816	0.608516285	0.008609868	0.419479035	0.650326797	77.00612265	116.9960397	0.658194268	-0.503414633
hsa-miR-7150	0.007999271	0.608516285	0.003548561	0.419479035	0.70751634	74.89441412	112.3330749	0.866717386	-0.584852745
hsa-miR-718	0.046823041	0.608516285	0.021056295	0.43119737	0.660130719	20.40825361	25.17934604	0.810515634	-0.303088081
hsa-miR-762	0.054968372	0.615398223	0.048840067	0.565970192	0.689542484	54.15375937	68.78024245	0.7873447	-0.344992708
hsa-miR-7704	0.065641102	0.624382729	0.031674408	0.496147911	0.630718954	92.61822591	116.5360963	0.794759983	-0.331408862
hsa-miR-8071	0.032852994	0.608516285	0.036098748	0.496147911	0.336601307	16.3416183	14.779708	1.105679375	0.144933093
hsa-miR-8072	0.090880859	0.716141171	0.02313643	0.43119737	0.593137255	22.61903149	27.70214424	0.816508329	-0.292460493
hsa-miR-940	0.03685856	0.608516285	0.041605891	0.523401761	0.668300654	146.6708879	183.394924	0.799754349	-0.322371163