(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 June 2014 (26.06.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/100216 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C12N 15/11* (2006.01)
G01N 33/574 (2006.01)

(21) International Application Number:
PCT/US2013/076193

(22) International Filing Date:
18 December 2013 (18.12.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/739,114 19 December 2012 (19.12.2012) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

WO 2014/100216 A1

(54) **Title**: Cancer-Associated MicroRNAs and Related Antibodies

(57) **Abstract**: Disclosed herein are antibodies and autoAbs which specifically bind cancer-associated miRNAs, assay methods, and methods of immunizing subjects against miRNAs.

CANCER-ASSOCIATED MICRORNAS AND RELATED ANTIBODIES

[01] CROSS-REFERENCE TO RELATED APPLICATIONS

[02] This application claims the benefit of U.S. Patent Application No. 61/739,114, filed 19 December 2012, which is herein incorporated by reference in its entirety.

[03] ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[04] This invention was made with Government support of Grant No. CA137651 and CA164388, awarded by the National Institutes of Health. The Government has certain rights in this invention.

[05] BACKGROUND OF THE INVENTION

[06] 1. FIELD OF THE INVENTION

[07] This invention generally relates to the field of microRNA (miRNA) and cancer.

[08] 2. DESCRIPTION OF THE RELATED ART

[09] miRNAs are single-stranded, non-coding 21-23 oligoribonucleotides and represent an entirely independent dimension of the human genome. As nearly 50% of human miRNAs are localized in fragile chromosomal regions, which may exhibit DNA amplifications, deletions or translocations during tumor development, their expression is frequently deregulated in cancer (1, 2). Several studies have shown that the miRNA 17-92 cluster, which is under the control of the myelocytomatosis viral oncogene homolog (MYC) oncogene (3), is overexpressed in multiple cancer cells, including prostate cancer and lung cancer (4, 5, 6). Of which, miR-17 was found to be an important member of a 34-miRNA signature for non-small cell lung cancer (NSCLC) (7) and was significantly upregulated in metastatic prostate cancer versus localized prostate cancer (8). High expression levels of miR-21 have been observed in multiple cancers, including NSCLC (45), glioblastoma, breast cancer, hepatocellular cancer, gastric cancer, prostate cancer and colon cancer, when compared to the corresponding normal tissues (9). In addition, miR-21 has been reported to counteract the expression of putative tumor-suppressive targets, such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and programmed cell death 4 (PDCD4). More recently, *in vivo* evidence of tumor dependency to miR-21 showed that upregulation of miR-21 was the initial and causal event in a mouse model of lymphoma (10). Another cancer-associated miRNA shared among multiple solid tumors in a global evaluation of miRNA expression

patterns was miR-191 (11), although no specific functions were associated in studies using cell lines (12). More recent findings suggest that miR-191 is associated with a variety of cancers. Higher expression levels of miR-191 were found in hepatocellular carcinoma (HCC) tissues than in adjacent noncancerous tissues. Interestingly, hypomethylation of the miR-191 locus was associated with increased miR-191 expression in HCC tissues and induced the transition of HCC cells into mesenchymal-like cells (47). Similarly, another study found inhibition of miR-191 decreased cell proliferation and induced apoptosis *in vitro*, and significantly reduced tumor masses *in vivo* using a mouse model of HCC (46). On the basis of the above evidence, cancer-associated miR-17, miR-21, and miR-191 are regarded as onco-microRNAs.

[10] In addition to the many roles miRNAs play in regulating physiological processes ranging from apoptosis, metabolism, development, cell cycle, oncogenesis and so on (1, 5), miRNAs are also known to play regulatory roles for the immune system, and can profoundly alter the phenotype and outcome of immune responses (13), including alleviation of immune suppression by miR-17 (14). For example, miR-155 plays the role of a proinflammatory regulator in clinical and experimental arthritis. In an animal model of arthritis, wild-type mice produced higher titre autoAb against collagen as compared to miRNA-155 knockout mice (15). miRNAs are generally around 6.9 to 7.6 kDa and often found in association with large RNA-binding proteins. In blood, miRNAs seem to be stable because most of them are included in apoptotic bodies, microvesicles or exosomes and protected from known mRNA degradation factors (16). In addition, miRNA folds into hairpin structures via complementary base pairing to form a secondary structure thereby further stabilizing its bioavailability and function in post-transcriptional regulation as well as promoting its potential interaction with the immune system to elicit an immunogenic response.

[11] The above factors make miRNAs potentially immunogenic when they are encountered by the immune system alone, as foreign or neo-antigens, or as a complex in association with large miRNA binding proteins under some pathological conditions. Indeed, it has been known that miRNA-associated proteins, such as Ago2, other Ago proteins and DICER were recognized by autoAb in patients with rheumatoid diseases (17). Ago2 autoAb was also found in patients with hepatitis C virus infections (18). However, whether immune responses, particularly class-switched IgG Ab responses, are specifically induced against miRNAs are unknown in the art.

[12] SUMMARY OF THE INVENTION

[13] In some embodiments, the present invention provides methods for detecting the presence of and/or diagnosing a cancer in a subject which comprise directly or indirectly detecting and/or quantifying one or more cancer-associated miRNAs in a sample from the subject. In some embodiments, BLItz interferometry is used to detect and/or quantify the one or more cancer-associated miRNAs. In some embodiments, the miRNAs are selected from the group consisting of miR-17, miR-21, and miR-191. In some embodiments, one or more antibodies that specifically bind the one or more cancer-associated miRNAs are used as the capture reagent. In some embodiments, one or more autoAbs are used to indirectly detect the one or more cancer-associated miRNAs. In some embodiments, the one or more autoAbs specifically bind one or more nucleic acid molecules which have the same or substantially the same sequence as the one or more cancer-associated miRNAs. In some embodiments, the capture reagents may be immobilized on a substrate. In some embodiments, the methods further comprise detecting and/or quantifying one or more additional biomarkers in the sample. In some embodiments, the cancer is selected from the group consisting of lung cancers (such as NSCLC), glioblastomas, breast cancer, hepatocellular cancers, gastric cancers, prostate cancer, and colon cancers. In some embodiments, the subject is mammalian, preferably human. In some embodiments, the subject is a test animal, such as a mouse. In some embodiments, the subject is suspected of having cancer or at risk for cancer.

[14] In some embodiments, the present invention provides methods for detecting the presence of and/or diagnosing a cancer in a subject which comprise directly or indirectly detecting and/or quantifying one or more autoantibodies against cancer-associated miRNAs in a sample from the subject. In some embodiments, BLItz interferometry is used to detect and/or quantify the one or more autoantibodies. In some embodiments, the miRNAs are selected from the group consisting of miR-17, miR-21, and miR-191. In some embodiments, one or more antibodies and/or one or more cancer-associated miRNAs that specifically bind the one or more autoantibodies are used as the capture reagent. In some embodiments, the one or more autoAbs specifically bind one or more nucleic acid molecules which have the same or substantially the same sequence as the one or more cancer-associated miRNAs. In some embodiments, the capture reagents may be immobilized on a substrate. In some embodiments, the methods further comprise detecting and/or quantifying one or more

additional biomarkers in the sample. In some embodiments, the cancer is selected from the group consisting of lung cancers (such as NSCLC), glioblastomas, breast cancer, hepatocellular cancers, gastric cancers, prostate cancer, and colon cancers. In some embodiments, the subject is mammalian, preferably human. In some embodiments, the subject is a test animal, such as a mouse. In some embodiments, the subject is suspected of having cancer or at risk for cancer.

[15] In some embodiments, the present invention is directed to methods of immunizing a subject against one or more miRNAs. In some embodiments, the miRNAs are cancer-associated miRNAs. In some embodiments, the miRNAs are selected from the group consisting of miR-17, miR-21, and miR-191. In some embodiments, the methods comprise administering to the subject an immunogenic amount of miRNA in the form of being bound to streptavidin and/or a substrate particle such as a bead. In some embodiments, the miRNA is bound to a streptavidin coated particle. In some embodiments, the miRNA is bound to a magnetic particle. In some embodiments, the miRNA is bound to a streptavidin coated magnetic particle such as a streptavidin coated magnetic bead. In some embodiments, the miRNA is biotinylated. In some embodiments, the method comprises administering to the subject a second immunogenic amount of the miRNA a given period of time, e.g., two weeks, after the first administration. In some embodiments, the miRNA is administered to the subject by intraperitoneal injection. As used herein, an immunogenic amount is an amount that elicits an immune response in a subject. In some embodiments, the subject is mammalian, preferably human. In some embodiments, the subject is a test animal, such as a mouse. In some embodiments, the antibodies generated are used in assays to detect and/or measure the miRNA in test samples, e.g., samples obtained from a subject.

[16] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

[17] DESCRIPTION OF THE DRAWINGS

[18] This invention is further understood by reference to the drawings wherein:

[19] Figure 1A shows the primary structures of the cancer-associated miRNA involved in the study. Modification of the RNA backbone with 2'-O-methylation, represented by m, was introduced to minimize hydrolysis of the RNA backbone by RNases. A control miRNA was also synthesized as a randomized sequence and searched against the miRNA database to confirm that there is no identity or homology to any known miRNA. The control miRNA was designed and used as a negative control to ensure specificity of binding.

[20] Figure 1B schematically shows a 96-well plate for immunoassays. An enlarged well depicts 4 differently colored beads, 1 coated with human albumin or a randomized miRNA as negative control, 3 coated with targets of interest, miR-17, miR-21, and miR-191 for measuring reactive autoAb.

[21] Figures 1C1-1E2 are graphs summarizing the results of various autoAb assays. Prostate cancer patient serum (n=30) has high frequency of autoAb against miR-17 (n=3/30; 10%, Figure 1C1), miR-21 (n= 3/30; 10%, Figure 1D1), and miR-191 (n=2/30; about 7%, Figure 1E1) compared to the same number of healthy male donors (n=0/30; 0%) screened. Bars represent the cutoff at 99% confidence level based on RFI values from HD. Other labels (triangles, squares, and large dots) represent seropositive patients, who are different individuals found reactive to the miRNA targets in at least 2 of 3 dilutions measured with at least a 99% confidence level. Similarly, lung cancer patient plasma (n=27) has high frequency of autoAb against miR-17 (n=2/27; about7%, Figure 1C2), miR-21 (n=2/27; about 7%, Figure 1D2), and miR-191 (n=3/27; 11%, Figure 1E2) at 99% confidence levels or higher compared to the number of healthy donor plasma (n=0/15; 0%) screened.

[22] Figures 2A-2D graphically show the characterization of the humoral immune responses against cancer-associated miRNA. Binding kinetics to miR-191 or miR-17 ligand using BLItz interferometry (ForteBio) with (Figure 2A) seropositive lung cancer patient plasma, (Figure 2B) BSA negative controls for binding specificity and affinity, (Figure 2C) healthy patient plasma, and (Figure 2D) human serum albumin (HSA) non-specific binding controls. BSA and HSA lacked binding specificity and affinity to miR-191 or miR-17 even at or above the highest concentrations of autoantibody used (shown in nM). Estimated binding affinity of autoAb is 20 pM using BLItz software data analysis with the assumption that the specific Ab accounted for about 0.1% of the entire serum protein components of 20 mg/ml. HSA estimated binding affinity to miR-191 or miR-17 is 5 mM using BLItz software data analysis.

[23] Figure 3A-3L are pictures showing the morphological changes and upregulation of co-stimulatory and maturation molecules on the surface of immature human DC induced by miRNA. Day 6 human monocyte-derived immature DC (Figure 3A) were grown in the presence of 6 μg/ml of miR-control (Figure 3B), miR-17 (Figure 3C), miR-21 (Figure 3D), miR-191 (Figure 3E), 100 ng/ml of lipopolysaccharide (LPS) (Figure 3F), and 100 μg/ml of polyI:C (Figure 3G) and imaged by light microscopy at 200X magnification. DC morphological changes such as elongation and attachment to the culture dish were correlated with the re-arrangement of filamentous actin, reproduced in at least 3 donors, imaged by fluorescence microscopy, 200X magnification (Figures 3H-3L).

[24] Figure 3M are graphs showing that extracellular miRNA induced upregulation of maturation and co-stimulatory molecules on the surface of DC. Human monocyte-derived DC were incubated with synthesized miR-control, -17, -21, and -191 (6 μg/mL) on day 6 for 48 hours, compared to media with PBS, and LPS (100 ng/mL) and stained for CD80, CD83, and CD86 markers (black line), compared to isotype-matched control Ab (grey filled-in). These results have been reproduced using more than 3 donors.

[25] Figures 4A-4D show various cytokine/chemokine profiles of human DC. Cytokine/chemokine profiles of human DC induced by miR-17 (Figure 4A), miR-21 (Figure 4B), and at varying concentrations (0-15 μg/ml) of miRNA (Figure 4C). No activation of the specified TLRs by a mixture of miR-17, -21, and -191 at the concentrations shown, and particularly no activation by TLR4 indicated that activation of DC was not likely the result of contamination with known ligands such as LPS, an agonist of TLR4 (Figure 4D).

[26] Figures 5A and 5B show antibodies are present in mice immunized with miR-17. Three different Balb/c mice were immunized at about 8 weeks old and boosted two weeks later with miR-17 for detecting antibody to miR-17 (black bars) in serum collected two weeks after boost and diluted from 1:10, 1:50, and 1:250 in PBS-T using a 5-panel MagPlex-based multiplex bead assay (Figure 5A). RFI of IgG Ab against miR-17 over albumin in 3 mice immunized with miR-17. Average RFI from 3 naïve mice were used as control. A minimum cut-off of 50 MFI is required for signals regarded as above background MFI levels. For example, Figure 5 shows mouse serum #1 with detectable antibody to miR-17 (black bars) above the minimum 99% confidence level. No significant detection of antibodies to other miRNA such as miR-

21, miR-191, or miR-Control (white bars, randomized sequence) were observed both above the 99% confidence level, and above the 50 MFI cut-off. These two criteria using both the 99% confidence level and 50 MFI cut-off level provides the stringency needed for reducing false positives. In addition, a third criterion for a positive sample requires at least two out of three dilutions tested must satisfy the two criteria mentioned. Ab titers were measured using serum sample from a seropositive mouse after boosting (Figure 5B).

[27] Figures 6A-6C graphically show the characterization of the humoral immune response against miR-17 immunogen in miR-17-immunized and non-immunized mice. Binding kinetics to miR-17 ligand using BLItz interferometry (ForteBio) with (Figure 6A) mouse serum seropositive for miR-17antibody, (Figure 6B) naïve, non-immunized mouse serum, and (Figure 6C) comparison of seropositive (M1) and naïve mouse (N) serum-derived antibody affinity to miR-17 ligand at 41 nM autoAb concentration.

[28] DETAILED DESCRIPTION OF THE INVENTION

[29] The present invention generally relates to cancer-associated miRNAs and immune responses, e.g., autoantibodies (autoAbs), against the cancer-associated miRNAs. Prior to the present invention, it was unknown that cancer-associated miRNAs in subjects having cancers could induce autoAbs in the subjects.

[30] As disclosed herein, detectable humoral immune responses were found to be induced *in vivo* in mice immunized with a cancer-associated miRNA, miR-17, as compared to non-immunized, naïve mice detected using MagPlex-based multiplex immunoassay. These results were confirmed by BLItz interferometry binding kinetics which shows enhanced binding to miR-17 over naïve control and non-specific albumin protein in the nM-pM range binding affinity characteristic of Ab binding.

[31] Also as disclosed herein, cancer-associated miRNAs, such as miR-17, miR-21 and miR-191, were found to have a significant impact on the maturation of human DC. At the same time, autoAb responses against these cancer-associated miRNAs were found to be present in prostate and lung cancer patients, but not at statistically significant levels in healthy controls as evidenced by the diminished binding of healthy plasma to the miRNAs compared to lung cancer plasma demonstrated by xMAP-based assays and confirmed by BLItz interferometry.

[32] Thus, cancer-associated miRNAs endogenous to subjects having cancer are recognized as an immunogens by the subjects' own immune systems and result in

autoAbs specific against the cancer associated miRNAs. Therefore, in some embodiments, the present invention relates to cancer-associated miRNAs as immunogenic agents and in immunogenic compositions. In some embodiments, the present invention relates to cancer-associated miRNAs and autoAbs specific against the cancer-associated miRNAs as biomarkers for cancer. In some embodiments, the present invention relates to methods of detecting a cancer in a subject, diagnosing a subject as having a cancer, and/or characterizing the type of cancer in a subject which comprise using one or more cancer-associated miRNAs and/or one or more autoAbs specific against a cancer-associated miRNA as a biomarker to be detected and/or quantified in a sample from the subject.

[33] It should also be noted that prior to the present invention, miRNA array and quantitative real-time PCR (qRT-PCR) have been the two approaches used for quantifying miRNA (11). While these approaches are straightforward, they have given rise to conflicting data in different studies. These discrepancies might be due to the lack of an established endogenous miRNA control for normalization. In addition, because of the small size of the miRNA and their attachment to lipids and proteins, efficient and reproducible extraction methods remain elusive (Schwarzenbach, et al. (2011) Nat Rev Cancer 11:426-437). There has been no previously known art to quantify miRNA using antibodies or approaches to generating antibodies against target miRNA. However, as disclosed herein, cancer-associated miRNA are immunogenic (Figures 1A-1E2). Accordingly, in some embodiments, miRNA can be used as immunogens to induce antibodies (Figures 5A-5B) and/or for the quantification of miRNA using ELISA or xMAP-based approaches in the future.

[34] As used herein, an "epitope" is the part of a molecule that is recognized by a given antibody.

[35] As used herein, "autoantibody" refers to an antibody produced by a subject that is directed against one or more of the subject's own antigens (e.g., a tumor antigen). As used herein, "antibody" refers to an immunoglobulin molecule and immunologically active portions thereof (i.e., molecules that contain an antigen binding site that specifically bind the molecule to which antibody is directed against). As such, the term antibody encompasses not only whole antibody molecules, but also antibody multimers and antibody fragments as well as variants (including derivatives) of antibodies, antibody multimers and antibody fragments. Examples of molecules which are described by the term "antibody" herein include, but are not limited to:

single chain Fvs (scFvs), Fab fragments, Fab' fragments, $F(ab')_2$, disulfide linked Fvs (sdFvs), Fvs, and fragments comprising or alternatively consisting of, either a VL or a VH domain. The term "single chain Fv" or "scFv" as used herein refers to a polypeptide comprising a VH domain of antibody linked to a VL domain of an antibody. Antibodies of the invention may also include multimeric forms of antibodies. For example, antibodies of the invention may take the form of antibody dimers, trimers, or higher-order multimers of monomeric immunoglobulin molecules. The antibodies of the present invention can be natural or synthetic, polyclonal or monoclonal, or chimeric, and can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) or subclass of immunoglobulin molecule.

[36] As used herein, a molecule, e.g., an antibody, that "specifically binds" another molecule, means that the interaction is dependent upon the presence of a specific structure, e.g., an epitope, on the molecule being bound. For example, an antibody which specifically binds a protein is recognizing and binding a specific structure on the protein rather than indiscriminate binding that gives rise to non-specific binding and/or background binding. As used herein, "non-specific binding" and "background binding" refer to an interaction that is not dependent on the presence of a specific structure (e.g., a particular epitope).

[37] As used herein, a "biomarker" refers to a substance used as an indicator of a process, event, or condition. A biomarker can be a biomolecule such as a nucleic acid molecule (e.g., miRNA, genomic DNA, etc.), a protein, a polysaccharide, and the like. Biomarkers include tumor antigens and tumor markers.

[38] As used herein, "tumor antigens" refer to tumor-specific antigens (TSAs), which generally classified as antigens present only on tumor cells and tumor-associated antigens (TAAs), which are generally classified as antigens present on some tumor cells and also some normal cells.

[39] As used herein, a "tumor marker" is a substance that may be found in body tissues or bodily fluids that is produced by tumor cells or non-tumor cells in response to the presence of cancerous cells. Examples of tumor markers include AFP (in liver cancer), CA 125 (in ovarian cancer), CA 15-3 (in breast cancer), CEA (in ovarian, lung, breast, pancreas, and gastrointestinal tract cancers), and PSA (in prostate cancer). Tumor markers can be classified in two groups: cancer-specific markers and

tissue-specific markers. Tumor markers include tumor antigens. However, tumor markers might not induce an immune response.

[40] As used herein, a "subject suspected of having cancer" refers to a subject that presents one or more symptoms indicative of a cancer (e.g., a detectable lump or mass). A subject suspected of having cancer has generally not been tested for cancer. However, a subject suspected of having cancer may encompass one who has received an initial diagnosis (e.g., a CT scan or X-ray showing a mass) but for whom the type or stage of cancer is not known. A subject suspected of having cancer may also include one who once had cancer (e.g., individuals in remission). A subject suspected of having cancer may also be a subject at risk for cancer.

[41] As used herein, a "subject at risk for cancer" refers to a subject with one or more risk factors for developing a specific cancer. Risk factors include genetic predisposition, environmental exposure, preexisting non-cancer diseases, previous cancers, and lifestyle.

[42] As used herein, a "subject" is used interchangeably with "patient" and refers to a mammal such as a human.

[43] As used herein, the "stage of cancer" refers to the level of advancement of a given cancer as is recognized by those skilled in the art. Criteria used to determine the stage of a cancer include, but are not limited to, the size of the tumor, whether the tumor has spread to other parts of the body and where the cancer has spread (e.g., within the same organ or region of the body or to another organ).

[44] As used herein, "detecting the presence of cancer in a subject" refers to detecting the presence of a tumor antigen or autoantibody indicative of cancer.

[45] As used herein, a "subject diagnosed with a cancer" refers to a subject having cancerous cells. The cancer may be diagnosed using any suitable method, including but not limited to, the diagnostic methods of the present invention.

[46] The term "isolated" when used in relation to a nucleic acid molecule or a peptide or polypeptide refers to the given biomolecule that is separated from at least one component or contaminant with which it is ordinarily associated in nature.

[47] As used herein, a "purified" composition refers to the removal of components (e.g., contaminants) from the composition.

[48] As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from

animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like.

[49] As used herein, a "capture reagent" refers to a molecule which is used to specifically bind an analyte of interest. The capture reagent may be immobilized on a substrate. For example, if the analyte of interest is an antigen, the capture reagent may be an antibody which specifically binds the antigen and if the analyte of interest is an antibody, then the capture reagent may be an epitope which the antibody specifically binds.

[50] EXAMPLES

[51] *Methods*

[52] *miRNA and xMAP-based Ab Analysis Against the miRNA*

[53] All miRNA (Figure 1A) were modified with a 5' amino group to enhance the conjugation efficiency to xMAP Microspheres (Luminex Corporation, Austin, TX, USA) followed by a 6-carbon spacer to reduce steric hindrance by the conjugated bead and enhance accessibility for binding to the miRNA ligand. Furthermore, the RNA backbone was modified with 2'-O-methylation to minimize the susceptibility of RNA to hydrolysis by RNases, thereby enhancing the miRNA stability. These miRNA were synthesized by GenScript USA Inc. (Piscataway, NJ, USA) and duplicated by Bio-Synthesis Inc. (Lewisville, TX, USA) to ensure that the observed immune stimulatory functions are intrinsic properties of the miRNA not product-related contaminants. Biotinylated miRNA were products of Bio-Synthesis Inc. and GenScript USA Inc. Conjugation of miRNA to xMAP Microspheres was conducted according to the manufacturer's recommendations (Luminex Corporation). As a result, xMAP Microsphere region 050 was conjugated with human albumin (Central Laboratory Blood Transfusion Service Swiss Red Cross) or randomized miRNA, region 060 with miR-21, region 070 with miR-191, and region 080 with miR-17. As a positive control, xMAP region 012 was conjugated with a long NY-ESO-1 peptide epitope containing amino acid residues 1-75.

[54] Similarly, in the MagPlex-based multiplex assay MagPlex Microsphere region 018 was conjugated with human serum albumin (Sigma-Aldrich, St. Louis, MO, USA), region 025 with miR-17, region 035 with miR-21, region 045 with miR-191, region 055 with miR-Control of randomized miRNA. As a positive control, MagPlex region 015 was conjugated with mixed NY-ESO-1 peptide epitopes containing 7 overlapping regions from amino acid residues 1-180 for binding to a commercially-

available mouse anti-human NY-ESO-1 monoclonal Ab clone E978 (Upstate/EMD Millipore, Billerica, MA, USA).

[55] SeroMAP-based Ab assay was conducted using 96-well filter plates (Millipore, Billerica, MA, USA) as described previously (25). MagPlex-based Ab assay was conducted using 96-well clear, flat bottom plates (Bio-Rad Laboratories, Hercules, CA, USA). The SeroMAP plate was washed using a manual 96-well vacuum-filtration system and the MagPlex plate was washed using the automated Bio-Rad magnetic plate washer. The plate was read using a Bioplex-100 instrument (Bio-Rad Laboratories) to measure the median fluorescence intensity (MFI) for each xMAP region included in the multiplex assay. Human albumin or control miRNA conjugated to an xMAP bead was used in each multiplex assay to measure its non-specific MFI. Relative fluorescence intensity (RFI) for each xMAP region was calculated using the MFI of a miRNA xMAP region over non-specific fluorescence and graphically displayed as a dot plot or bar chart. As described previously (26, 27), positive serum and plasma for prostate and lung cancer detection of Ab reactive to miRNA was determined at a minimum 95% to 99% confidence level indicated by the red line in the dot plot. A positive detection with 99% confidence level was defined by RFI equal to or greater than three standard deviations above the average RFI of healthy donor serum and plasma in at least two of the three replicates. A 95% confidence level was two standard deviations above the average RFI of healthy donor samples in at least two of the three replicates.

[56] Many commercially available assay kits and materials are suitable for the assays described here. It is noted, however, that different assay materials, e.g., substrate platforms, may result in different sensitivities. For example, the MagPlex substrate was found to result increased sensitivity over xMAP and SeroMAP magnetic beads. Thus, in some embodiments, the assays according to the present invention employ MagPlex materials, e.g., MagPlex beads.

[57] *Patient Serum and Plasma*

[58] Historical prostate cancer patient serum, lung cancer plasma, healthy male donor serum, and healthy donor plasma were collected at our collaborating institutions as previously described (25) and David Geffen School of Medicine of UCLA under IRB approved protocols.

[59] *Binding Kinetics of Seropositive Plasma AutoAb to miR-191*

[60] BLItz interferometer instrument and software (ForteBio, Menlo Park, CA, USA) was used to perform the binding kinetics experiment. Streptavidin-coated sensor tips (ForteBio) were used to bind biotinylated miR-191 (20 μg/mL) for 200 seconds. A wash step for 30 or 250 seconds was used to remove unbound ligand from the sensor and establish the baseline prior to binding. Seropositive plasma from a lung cancer patient was serially diluted from 18.6 ng/μL to 1.55 pg/μL (proposed autoAb concentration based on the assumption that approximately 0.1% of human plasma is comprised of the specific IgG). Then the autoAb (analyte) was allowed to bind to the biotinylated miR-191 or biotinylated miR-17 immobilized by the streptavidin-coated sensor tip for 250 seconds to load the tip. After the association step, the PBS buffer used as a diluent was added to the sensor tip for the dissociation of the analyte from the biotinylated miR-191 or biotinylated miR-17 immobilized to the streptavidin sensor tip for 250 seconds. Disposable sensor tips were hydrated in PBS for a minimum of 10 minutes immediately prior to use per manufacturer recommendations. Each step was performed with a shaker speed of 2200 rpm.

[61] *Culture of DC and Analysis of DC Maturation*

[62] Human PBMC were obtained from donors of the UCLA virology core under an IRB-approved protocol. $CD14^+$ monocytes were positively selected from fresh PBMC using a magnetic beads-based approach (Miltenyi Biotech Inc., Germany), followed by tissue culture in the presence of human GM-CSF and IL-4 as previously described (28). On day 6, DC was subjected to treatment with the addition of miRNA (6 μg/ml or otherwise indicated), polyI:C (50-100 μg/ml unless otherwise indicated), or LPS (100 ng/ml).

[63] In assessment of DC maturation, DC were grown in a 8-well chamber slide (Lab-Tek/Nunc, Denmark) for 6 days, treated with miRNA, polyI:C, or LPS for 2 hours, followed by fixation and filamentous actin staining with phalloidin (Sigma-Aldrich, St. Louis, MO, USA). Routine flow cytometry procedures (28) were performed for staining with cell surface markers such as CD40, CD54, CD80, CD83, CD86, HLA-DR, B7-H1 (PD-L1), B7-H2, and B7-H4 using DC ($5x10^5$/well) that were treated with miRNA, polyI:C, or LPS for 48 hours. The culture supernatant were collected and analyzed for cytokine/chemokine secretion (EVE Technologies, Calgary, Canada; Biolegend, San Diego, CA, USA). Analysis of protein

phosphorylation was performed using lysate from $7x10^5$ DC that were treated with miRNA and polyI:C for about 90 minutes.

[64] *Immunization with miRNA*

[65] Female Balb/c mice of about 8 weeks old were used for immunization with a mixture of 4 μg of biotinylated miR-17 bound with streptavidin magnetic beads (Thermo Scientific Pierce, Rockford, IL, USA) in filter sterile PBS with 0.01% Tween-20 by intraperitoneal injection. Two weeks later, mice were boosted with the same amount of miRNA mixture. Two weeks following, mice were bled and serum-derived Ab against each miRNA and non-specific albumin protein binding for normalization was measured using MagPlex-based approach (Luminex Corp.) with PE-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as the detection Ab.

[66] RESULTS

[67] *miRNA Induces IgG Ab Responses in Animals*

[68] To determine whether miRNA has the potential to illicit immune responses *in vivo*, mice were immunized with an miRNA, i.e., miR-17, and their serum was measured for antibody reactive against the miRNA. In efforts to augment the presentation of small antigens such as miRNA to immune cells, increasing the proximity and number of accessible antigen or miRNA for binding to immune cells is vital for successful immunization. Therefore, biotinylated miR-17 (Bio-Synthesis or GenScript) bound to streptavidin-coated magnetic beads (Thermo Scientific Pierce) in PBS with 0.01% Tween-20 was administered to 3 Balb/c female mice in efforts to generate an immunoglobulin-specific response to miR-17. Tayapiwatana et al. have conducted immunization of mice using streptavidin magnetic beads for monoclonal antibody production previously (2006) (51).

[69] Mice serum were collected after boosting with miR-17 and stored at -20°C. Serum was diluted 1:10, 1:50, and 1:250 in PBS with 0.01% Tween-20 for measuring antibody reactive to the miRNA. Three mice (n=3) were used for immunization with miR-17 bound to streptavidin magnetic beads which resulted in one out of three (33%) immunized mice with detectable antibody reactive to miR-17 using the MagPlex-based assay (Figures 5A-5B). Mouse #1 serum diluted 1:10, 1:50, and 1:250 were found reactive to miR-17 (black bars) above the 99% confidence level. Naïve mice did not have such Ab responses (black bars); nor did the immunized

mouse recognize miR-Control (Figure 5A, white bars). In addition to the 99% confidence level, a positive result must include a mean fluorescence intensity (MFI) cut-off of 50 or above in the raw data to eliminate background binding and false positives. The data shown in Figures 5A-5B are RFIs or MFIs of antibody binding to the respective miRNA shown in the legend, normalized by albumin for non-specific MFI background. As a third criterion, two of three dilutions of serum must be positive for antibody to the miRNA. Here, antibody detected for binding to miR-17 was found in at least two of three dilutions (1:10, 1:50, Figure 5A). Therefore, the three stringent criteria used here to characterize serum-derived antibody binding to miR-17 suggest that mice immunized with miR-17 are capable of generating an immune response against miRNA *in vivo*. To confirm that this seropositive mouse serum against miR-17 is in fact an antibody, binding kinetics was used to confirm binding affinities characteristic of antibodies (nM-pM).

[70] Seropositive mouse serum from a miR-17-immunized Balb/c mouse (Figures 5A and 5B, mouse #1) was detected with relatively increased antibody binding to miR-17 ligand using BLItz interferometry (Figure 6A) compared to non-immunized, naïve Balb/c mouse serum (Figures 5A and 6B) at estimated antibody concentrations between 0.1 nM-10.3 nM. Approximately three times greater binding of antibody to miR-17 was detected in seropositive mouse serum (Figure 6A, about 1.5 nm shift) versus the naïve mouse serum (Figure 6B, about 0.5 nm binding shift). As mentioned previously, binding above 1 nm indicates a significant binding interaction.

[71] When mouse serum antibody is diluted to 41 nM (1:3 dilution), the highest estimated antibody concentration used here, the miR-17-immunized mouse serum (Figure 6C, M1) demonstrated a dramatically increased number of antibody binding to miR-17 (about 3 nm binding shift) compared to the naïve, non-immunized mouse serum (Figure 6C, N, about 0.5 nm binding shift). This observed difference in antibody binding to miR-17 represents about 6 times greater reactivity to miR-17 by the miR-17-immunized mouse serum compared to the non-immunized mouse serum.

[72] Surprisingly, the same observed difference in reactivity of mouse serum (six times the naïve serum) to miR-17 was found using BLItz Interferometry (Figure 6C) and MagPlex-based assay as shown in Figure 5A for 1:10 mouse serum dilution with 12.6 RFI detected for mouse #1 serum reactivity to miR-17 (black bar) compared to 1.5 RFI for naïve mouse serum (average of at least 3 naïve mouse serum, black bar). Interestingly, the estimated binding affinity of mouse antibody to miR-17 was

estimated at about 4 nM using BLItz software data analysis of Figure 6A and 6B, indicating that binding is characteristic of an Ab, and at lower levels when compared to control samples (Figure 6B). The nM range binding affinity detected with mouse serum to miR-17 confirms the immunoassay results (Figures 5A-5B) that detected an IgG-specific immune response using PE-labeled anti-mouse IgG.

[73] *Class Switched Ab Responses against Cancer-Associated miRNA in Prostate and Lung Cancer Patients*

[74] A Luminex xMAP-based Ab detection approach (Figure 1B) was used to measure Ab response in serum samples from 30 prostate cancer patients and the same number of healthy male controls, as well as plasma samples from 27 NSCLC patients and 15 sex- and age- matched healthy controls.

[75] Detection of autoantibody against miR-17 was found in 3 out of 30 (10%) prostate cancer patient sera, whereas no statistically significant autoantibody against miR-17 was detected in sera from 30 healthy donors (Figure 1C1). Each patient serum was diluted 1:10, 1:20, and 1:50 and tested for consistency of these results. The cutoff used for determination of seropositive prostate cancer patient sera was based on a minimum 99% confidence level (Figures 1C-1E, bars). The cutoff was calculated by summing the average relative fluorescence intensity (RFI) of the healthy donor sera to 3 times the standard deviation of the healthy donor sera. A seropositive result is determined with at least 2 of the 3 dilutions tested to equal the cutoff for a 99% confidence level or higher.

[76] Similarly, autoantibody against miR-21 was detected in 3 prostate cancer patient sera in all 3 dilutions as depicted in Figure 1D1. None of the 30 healthy donor sera were detected positive for autoantibody against miR-21 (Figure 1D1) using the criteria described. These 3 seropositive prostate cancer patient sera were determined with greater than 99% confidence level.

[77] Furthermore, autoantibody against miR-191 was detected in 2 prostate cancer patient sera in all 3 dilutions as depicted in Figure 1E1). Likewise, none of the 30 healthy donor sera was detected for autoantibody against miR-191 (Figure 1E1) at statistically relevant levels. These 2 seropositive prostate cancer patient sera were determined with greater than 99% confidence level.

[78] Seropositive patient sera with RFI equal to or greater than the 99% confidence level are represented with a colored symbol. Patient sera with RFI less than 99% confidence level are represented with a black dot.

[79] To explore whether this phenomenon of autoantibodies against miRNAs detected in prostate cancer patient sera is observed in other cancers, we repeated the assay using the same beads with non-small cell lung carcinoma (NSCLC) patient plasma and healthy donor plasma. Similarly, detection of autoantibodies against the 3 miRNAs, miR-17, miR-21, and miR-191, was discovered with lung cancer plasma and not healthy plasma with statistical levels of significance.

[80] Detection of autoantibody against miR-17 was found in 2 out of 27 (about 7%) NSCLC patient plasma, and 0 of 15 (0%) healthy donor plasma samples (Figure 1C2), calculated with 99% confidence level (bars) or higher. Similarly, detection of autoantibody against miR-21 was found in 2 out of 27 (about 7%) NSCLC patient plasma, and r 0 of 15 (0%) healthy donor plasma samples (Figure 1D2), calculated with 99% confidence level (bars). Additionally, autoAb against miR-191 was detected in 3 out of 27 (11%) NSCLC patient plasma, and 0 of 15 (0%) healthy donor plasma samples (Figure 1E2), calculated with 99% confidence level (bars) or higher.

[81] Expansion of the miRNA panel of potential biomarkers for serum-based detection of prostate, lung and other cancers is expected to greatly enhance our detection frequency of cancer when combining a minimum of 10 miRNA candidates in our assay. For each miRNA biomarker candidate used in this study when analyzed independent of one another, 2 to 3 out of 30 different prostate cancer patient serum (n=30) was found reactive (up to 10%). Interestingly, when the 3 miRNA biomarker candidates were combined for analysis, a total of 6 prostate cancer patients out of 30 (20%) were identified at 99% confidence level or higher. Therefore, the effect of increasing the number of miRNA biomarkers used in our preliminary study is perceived to drastically improve the overall number of seropositive prostate cancer patients detected. However, when combining the 3 miRNA targets for screening lung cancer patient plasma (n=27), the number of NSCLC patient plasma identified at 99% confidence level remained the same simply because one of the miRNA targets, miR-191, was reactive to all 3 NSCLC patient plasma (up to 11%). Still, it is our belief that more miRNA candidates are needed for screening to identify other novel miRNA biomarkers reactive to autoAb to improve the sensitivity, specificity, and accuracy of diagnosis for patients with prostate cancer, and other cancers.

[82] Therefore, several other miRNA candidates have been selected and are being screened against prostate and lung cancer patient sera to detect serum reactive autoantibodies. Furthermore, combining a panel of 10-35 miRNAs with the

previously established 6 TAAs of the A+PSA test is predicted to be a powerful and novel diagnostic and prognostic tool for high-throughput routine clinical applications. Moreover, it is important that this test includes both novel and well-studied biomarkers useful for distinguishing aggressive from non-aggressive forms of prostate, lung, and other cancers. Over 500 age-, race-, and sex-matched serum samples will be screened to conduct a comparison of cancer and healthy donor samples.

[83] *Characterization of the Observed Ab Responses Against miRNA in Cancer Patients*

[84] These observations were further verified using an alternate approach, such as BLItz interferometer. The interaction between miR-191 and a positive plasma sample was estimated to have an apparent affinity around 20 pM (Figure 2A), which is within the range of a typical antibody-antigen interaction. Several different dilutions of the positive lung cancer patient plasma sample were used to perform the binding kinetics assay. PBS was used as a diluent to perform a serial dilution of the plasma sample. The concentration of autoantibody present in plasma was based on current estimates of total IgG isotypes, 18.6 mg/mL, and with the assumption that 0.1% of total IgG proteins represent autoAb. Given the vast number of Ab repertoires circulating against a large number of TAAs, an even smaller fraction than 0.1% is reasonable to represent the TAA-specific autoAb population.

[85] Unfortunately, due to the complexity of serum and plasma samples, and low abundance of autoAb, the current technical and instrumental limitations deem these samples as difficult to work with. Depletion of these 'contaminating' high abundant immunoglobulin proteins with protein A, protein G, or protein L conjugated beads/columns to enrich for non-immunoglobulin proteins and other molecules of interest is the common method used to overcome samples of complex mixtures. However, we are in fact interested in the immunoglobulin proteins.

[86] To examine and confirm the interaction of autoAb with miRNA, we decided to measure the binding affinity. Binding kinetics is commonly measured using purified samples in large amounts using the widely accepted surface plasmon resonance (SPR)-based technique with instruments such as Biacore. However, unpurified, complex mixtures such as serum and plasma pose a challenge and further complicated by the low abundance of autoAb specific to a TAA. Therefore, the BLItz interferometer was an attractive alternative to Biacore to accomplish binding kinetics of an impure and low abundant molecule given its much greater sensitivity allowing

for an extraordinarily reduced amount of sample required. Moreover, the BLItz instrument has capabilities to measure complex samples, including cell lysate. The BLItz interferometer is a recently released singleplex instrument that measures one sample at a time, a smaller derivative of the more robust performing Octet instrument (ForteBio) for octoplexing samples. The principle of the Octet instrumentation and BioLayer Interferometry (BLI) is described recently (49). Therefore, the data presented (Figures 2A-2D and 6A-6C) using the newly released BLItz instrument (ForteBio) described here is the first report that we know of to date.

[87] A total of 6 different concentrations of autoAb were used for binding, ranging from 0.00103 nM (1.03 pM) up to 41.33 nM. At every successive increase in concentration used, an increase in binding was measured (y-axis) (Figure 2A). The y-axis represents the relative intensity in the wavelength shifted (nm) as a result of the increased number of analyte molecules (here, autoAb reactive to miR-191) bound to the biosensor tip's surface coated with ligand (here, streptavidin bound to biotinylated miR-191) as a function of time in seconds (Figures 2A-2D). The biosensor tip has an optic fiber that directs white light back to the opposite end of the tip where the detector is located. Two beams of light are detected, one from the tip as a reference (a constant), and the second from the ligand or analyte molecular layer bound to the tip surface (varies as a function of the number of molecules bound). The difference in the optical properties detected is a function of the thickness in the molecular layer formed and corresponds to the number of molecules binding on the sensor tip surface. This real-time measurement of binding was used to calculate the association and dissociation rates, which can be used to determine the dissociation constant K_D in Equation 1 (49):

$$K_D = k_d/k_a \qquad \text{(Equation 1)}$$

[88] The binding affinity between miR-191 and autoAb (NSCLC patient plasma) was measured at 6 different autoAb concentrations. The dissociation and association rate constants were calculated to determine the K_D in each of the samples measured, using global fitting and baseline correction at the start of association and dissociation steps. The highest concentration of autoAb measured (41.33 nM) appeared to be too concentrated and resulted in the greatest deviation from the 1:1 binding kinetics model used to fit the 6 different concentration curves and therefore concentrations lower than 41.33 nM were prepared and measured.

[89] To confirm the binding of miR-191 to autoAb present in seropositive NSCLC patient plasma is specific, purified BSA (New England BioLabs Inc., Ipswich, MA, USA) and PBS were used as a negative control (Figure 2B). The concentrations of BSA used to measure binding to miR-191 were similar to the two highest concentrations used to bind NSCLC autoAb, 1.493 nM and 14.93 nM. Using the highest concentrations of BSA protein to measure non-specific interactions is important when confirming this possible phenomenon of non-specific molecular interactions, normally seen at high concentrations.

[90] The most abundant protein found in human blood plasma is human serum albumin (HSA), a soluble, monomeric, 67 kDa protein. The range of albumin concentration in blood is 3.4-5.4 g/dL, equivalent to 34-54 g/L and 507-806 μM (undiluted). Here, the NSCLC patient plasma was diluted 1:3 (41.3 nM autoAb), then 1:4 (10.33 nM autoAb), 1:10 (1.03 nM), and so forth. Therefore, the highest concentration range of albumin expected in the NSCLC patient plasma when diluted to 1.03 nM autoAb (1:120 total serial dilution) used for binding miR-191 was 6.72 μM HSA. The concentration of albumin present in the NSCLC patient plasma when diluted to 1.03 pM (0.00103 nM, a total serial dilution of 1:111,120) would have been equivalent to the approximate range of 4.56-7.25 nM human serum albumin. Therefore, the binding (nm) measured for 1.03 pM autoAb (Figure 2A) is equivalent to contain between 1-10 nM human serum albumin. Therefore, the binding intensity (nm) of BSA between 1-10 nM (Figure 2B) should be lower than the binding intensity for 1.03 pM autoAb (Figure 2A). Otherwise, it is possible that the human serum albumin present in NSCLC plasma binds to miR-191 instead or in addition to autoAb present. However, nonspecific protein binding exhibits several fold orders in magnitude lower binding affinities than antibodies. It is doubtful that BSA would attribute a binding affinity greater than or similar to antibodies or autoAb in the μM to pM range.

[91] To ensure that the streptavidin-coated biosensor tips do not non-specifically bind to proteins, BSA was used at the highest protein concentration measured for autoAb (14.93 nM) (Figure 2B). The lack of BSA binding to the streptavidin-coated biosensor tip (Figure 2B) compared to autoAb (Figure 2A) confirms binding specificity of streptavidin and to biotinylated targets such as biotinylated miR-191.

[92] To confirm our results of decreased Ab responses to miRNA observed in healthy patient plasma measured by Luminex xMAP Microspheres, binding kinetics

using BLItz Interferometry was performed (Figure 2C). The same dilutions of healthy and cancer patient samples were prepared for consistent comparisons. The estimated autoAb concentrations used for binding to miR-191 ranged from 0.01 nM up to 10 nM as depicted in Figures 2A (NSCLC patient plasma) and 2C (healthy donor plasma). As shown in Figure 2C, healthy donor plasma diluted to an estimated 10 nM autoAb resulted in minimal binding (up to nearly 0.4 nm) compared to the same dilution of autoAb from NSCLC patient plasma (Figure 2A) which resulted in modest binding near 1.5 nm. A shift in binding of at least 1 nm is considered a significant binding interaction, per conversations with current BLItz instrument users studying Ab interactions. Interestingly, the similar binding affinity estimated by BLItz software as about 33 pM in healthy donor plasma (Figure 2C) and about 20 pM in NSCLC patient plasma (Figure 2A) is suggestive that autoAbs to miR-191 are present in the human plasma tested based on the pM affinity characteristic of Abs measured here. The enhanced binding properties of cancer patient plasma to miRNA compared to healthy plasma shown using BLItz interferometry (Figure 2A and 2C) confirms the initial studies detecting increased autoAb response to miRNA using the seroMAP-based assay (Figure 1C-E).

[93] In addition to BSA as a negative control for non-specific protein binding to miRNA, we also tested human serum albumin (HSA) protein. Not only is HSA a more relevant protein that is species-specific to this assay, but it is ubiquitously present in human serum and plasma samples. It is worth noting that BSA is still widely accepted as a non-specific binding protein throughout the scientific community and is used in numerous assays. Given the relatively high abundance of HSA protein expected in human plasma and serum (about507-806 μM), we wanted to eliminate the possibility of non-specific protein binding to miRNA.

[94] In Figure 2D we measured non-specific binding to miR-191 or miR-17 by HSA protein at various concentrations that reflect the expected levels of HSA estimated in the dilutions used for human blood measurements by BLItz interferometry for adequate comparison. HSA protein concentrations at 150 nM, 1.5 μM, and 7.5 μM were used to represent the equivalent HSA protein concentration in human plasma when diluted to roughly 1:3,000, 1:300, and 1:100 final dilutions, and roughly equivalent to 0.01 nM autoAb, 0.1 nM autoAb, 1.0 nM autoAb, respectively. For example, the estimated binding of 1.0 nM autoAb from NSCLC patient plasma to miRNA was nearly 1.5 nm in Figure 2A compared to nearly 0.4 nm by HSA in Figure

2D, suggesting that HSA non-specific binding to miRNA does not attribute to the level of that observed with increased binding to miRNA in NSCLC patient plasma. Moreover, the binding affinity was estimated by BLItz software for HSA binding (Figure 2D) as 5 mM, significantly lower binding affinity than the autoAb estimated pM affinities (Figure 2A) measured here by an estimated 9 orders in magnitude difference. Therefore, it is unlikely that non-specific proteins present in human plasma resulted in the observed binding to miRNA at the affinities measured, other than by an Ab.

[95] Therefore, binding kinetics using BLItz Interferometry was used here for the first time to confirm our results for observed Ab responses in cancer patient serum and plasma to miRNA measured by Luminex xMAP Microspheres. Reduced binding of healthy donor plasma to miRNA (Figure 2C) was conducted as further confirmation of enhanced miRNA binding to cancer patient plasma (Figure 2A).

[96] *miRNA Induces DC Maturation In vitro*

[97] To shed light on the mechanism of immunogenicity for miRNA involved in this study, we focused on testing a hypothesis that miRNA may simulate single- or double-stranded RNA from virus and activate the innate immune system, particularly DC. An *in vitro* co-culture experiment showed DC morphological changes induced by the presence of miRNA, similar to that induced by bacterial LPS, and by viral double-stranded RNA mimic, poly I:C (Figures 3A-G). Immature DC derived from human monocytes exhibited attachment to the culture dish and elongation of dendrites. Further experiments showed that this morphological change was accompanied with filamentous actin rearrangement as stained by phalloidin as early as 90 minutes after co-culturing (Figures 3H-L). Flow cytometry analysis (Figure 3M) was followed to investigate whether maturation and co-stimulatory molecules on the surface of immature DC were upregulated, typical to most maturation processes. No apparent upregulation was observed for cell-surface CCR7, CD40, CD86, B7-H1, B7-H2, B7-H3, B7-H4, and HLA-DR, compared to control-treated DC (PBS or miR-control). Interestingly, miR-17 and miR-21 induced upregulation of antigen presenting cell (APC) activation marker CD54 (data not shown), CD80 costimulatory molecule for T cell activation and survival, and DC-specific maturation marker CD83 (Figure 3M), molecules responsible for B cell maturation and CD4+ T cell longevity, and thus Ab responses.

[98] Additionally to the mass spectra data showing more than about 99% purity for the individual miRNA used in this study (data not shown), we obtained miRNA from two independent manufacturers to ensure that activation of immature DC were not caused by even a minute amount of contamination within the miRNA. Elimination of the possibility that the miRNA used contained contaminating substances that activate DC is demonstrated by the negative control used in this study. The control miRNA (miR-control) resulted in no activation of immature DC (Figures 3B and 3H). Furthermore, the absence for detectable activation of HEK-293/TLR4 cells by miR-17, -21, and -191 serves as an internal negative control to rule out LPS contamination (Figure 4D). Similar profiles of chemokine/cytokine were obtained using miRNA from two different sources. A panel of 41 cytokine/chemokine was screened from the supernatant of human DC co-cultured with miR-17, -21, -191, and -control. Significantly increased secretion of IL-6, IL-10, IP-10, MIP-1β, TNF-α, and RANTES for miR-17 as well as IL-6 for miR-21 (Figures 4B and D) suggested the activation of inflammatory pathways by extracellular miR-17 and miR-21. Activation of immature DC had typical concentration-dependence for miR-17 and less so for miR-21 (Figure 4C).

To investigate whether miR-17, -21, and -191 activated human DC through TLR, experiments were conducted using a mixture of miR-17, -21, and -191 co-cultured with HEK-293 cells encoding human TLR3, 4, 7, 8, and 9 individually with an NF-κB-driven SEAP reporter. No activation of the TLR-mediated SEAP was observed (Figure 4D) despite strong SEAP activities induced by known TLR ligands as positive controls. These results support the hypothesis that cancer-associated miR-17 and miR-21 function as extracellular danger signals to activate immature DC and may further induce adaptive immune responses in vivo.

[99] ASSAYS

[100] In some embodiments, the present invention is directed to methods for detecting the presence of and/or diagnosing a cancer in a subject which comprises directly or indirectly assaying one or more cancer-associated miRNAs in a sample from the subject. In some embodiments, the miRNAs are selected from the group consisting of miR-17, miR-21 and miR-191. In some embodiments, the subject is mammalian, preferably human. In some embodiments, the subject is suspected of having cancer. In some embodiments, the subject is at risk for cancer. In some embodiments, the cancer is selected from the group consisting of lung cancers (such

as NSCLC), glioblastomas, breast cancer, hepatocellular cancers, gastric cancers, prostate cancer, and colon cancers.

[101] In some embodiments, where the miRNAs are detected directly, one or more antibodies that specifically bind the miRNAs are used as the capture reagent. In some embodiments, the miRNAs are indirectly detected by detecting one or more autoAbs against the miRNAs. In some embodiments, the one or more autoAbs are detected using, as the capture reagent, one or more antibodies that specifically bind the autoAbs. In some embodiments, the one or more autoAbs are detected using, as the capture reagent, one or more nucleic acid molecules (e.g. synthetically made oligonucleotides having the same or substantially the same sequence(s) as the one or more miRNAs) to which the autoAbs specifically bind. In some embodiments, the capture reagents may be immobilized on a substrate, e.g. assay plate or well. In some embodiments, the capture reagents are provided in one or more assay panels which comprise a plurality of additional capture reagents for other biomarkers such as tumor antigens and tumor markers. See e.g., US 20110311998, which is herein incorporated by reference in its entirety. In some embodiments, the presence of a particular miRNA or a particular combination of miRNAs, alone or in combination with one or more other tumor antigens and/or tumor markers, is used to characterize the type of cancer and/or the stage of the cancer in the subject.

[102] MULTIPLEX ASSAYS

[103] In some embodiments, the methods and/or compositions of the present invention are combined with other assays to form multiplex assays. The methods and/or compositions of the present invention may be combined with that described in US 20110311998, which is herein incorporated by reference in its entirety.

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[157] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

[158] Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

We claim:

1. A method for detecting the presence of and/or diagnosing a cancer in a subject which comprises

directly or indirectly detecting and/or quantifying one or more cancer-associated miRNAs in a sample from the subject.

2. The method of claim 1, wherein the miRNAs are selected from the group consisting of miR-17, miR-21, and miR-191.

3. The method of claim 1, which comprises using one or more antibodies that specifically bind the one or more cancer-associated miRNAs as the capture reagent.

4. The method of claim 1, which comprises using one or more autoAbs to indirectly detect the one or more cancer-associated miRNAs.

5. The method of claim 4, wherein the one or more autoAbs specifically bind one or more nucleic acid molecules which have the same or substantially the same sequence as the one or more cancer-associated miRNAs.

6. The method according to any one of the preceding claims, wherein the capture reagents may be immobilized on a substrate.

7. The method according to any one of the preceding claims, and further comprising detecting and/or quantifying one or more additional biomarkers in the sample.

8. The method according to any one of the preceding claims, wherein the cancer is selected from the group consisting of lung cancers (such as NSCLC), glioblastomas, breast cancer, hepatocellular cancers, gastric cancers, prostate cancer, and colon cancers.

9. The method according to any one of the preceding claims, wherein the subject is mammalian, preferably human.

10. The method according to any one of the preceding claims, wherein the subject is suspected of having cancer or at risk for cancer.

miR-17: 5'-amino-6 carbon spacer-mCmAmAmAmGmUmGmCmUmUmAmCmAmGmUmGmCmAmGmGmUmAmG-3'

miR-21: 5'-amino-6 carbon spacer-mUmAmGmCmUmUmAmUmCmAmGmAmCmUmGmAmUmGmUmUmGmA-3'

miR-191: 5'-amino-6 carbon spacer-mCmAmAmCmGmGmAmAmUmCmCmCmAmAmAmAmGmCmAmGmCmUmG-3'

miR-Control: 5'-amino-6 carbon spacer-mUmAmGmUmCmAmUmGmAmAmGmUmUmGmGmAmCmCmAmAmGmU-3'

Figure 1A

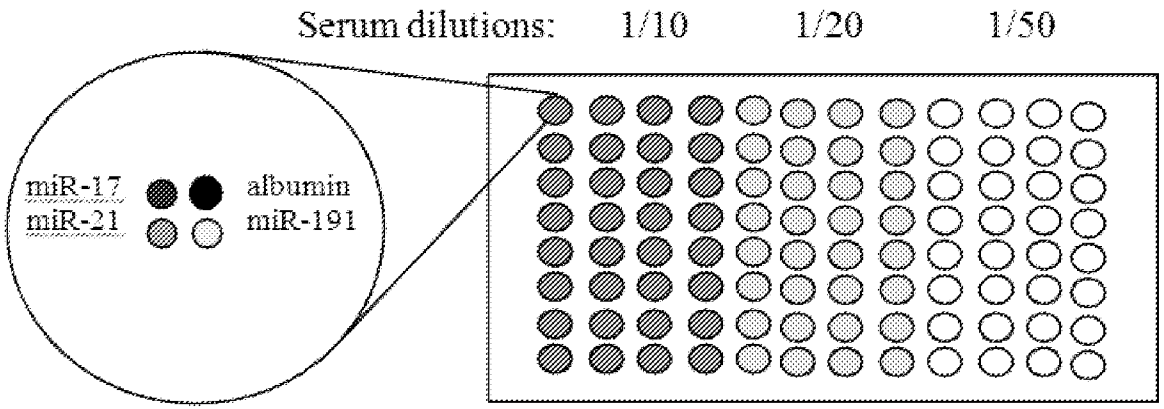


Figure 1B

WO 2014/100216

PCT/US2013/076193

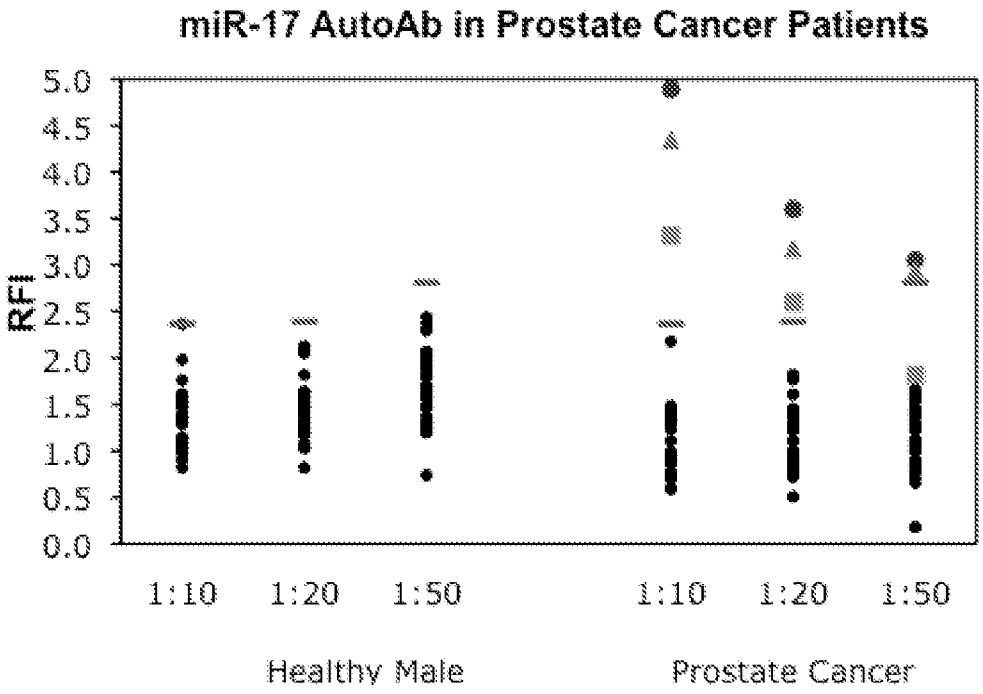


Figure 1C1

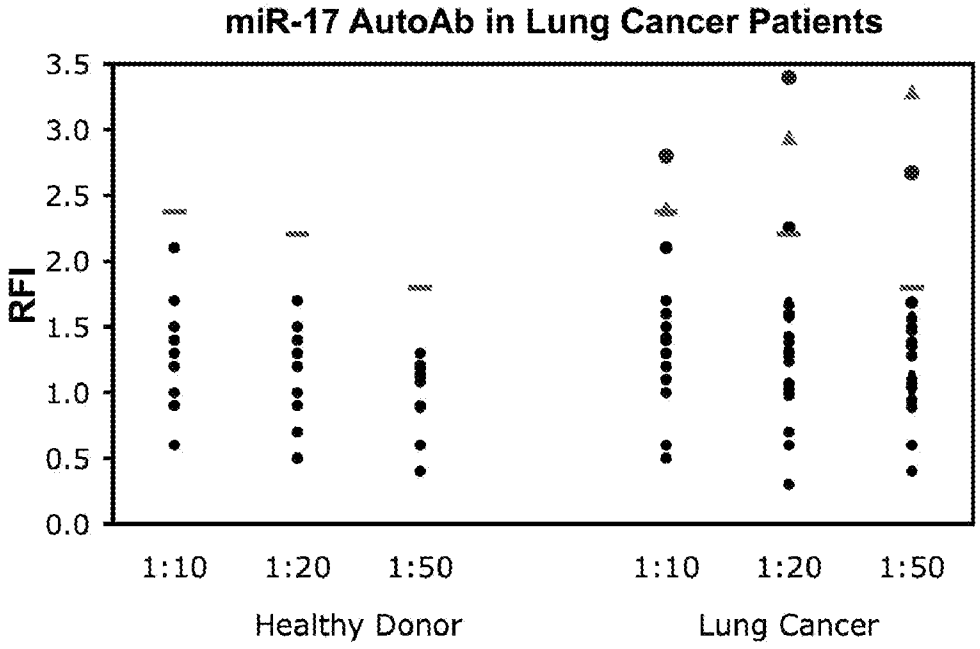


Figure 1C2

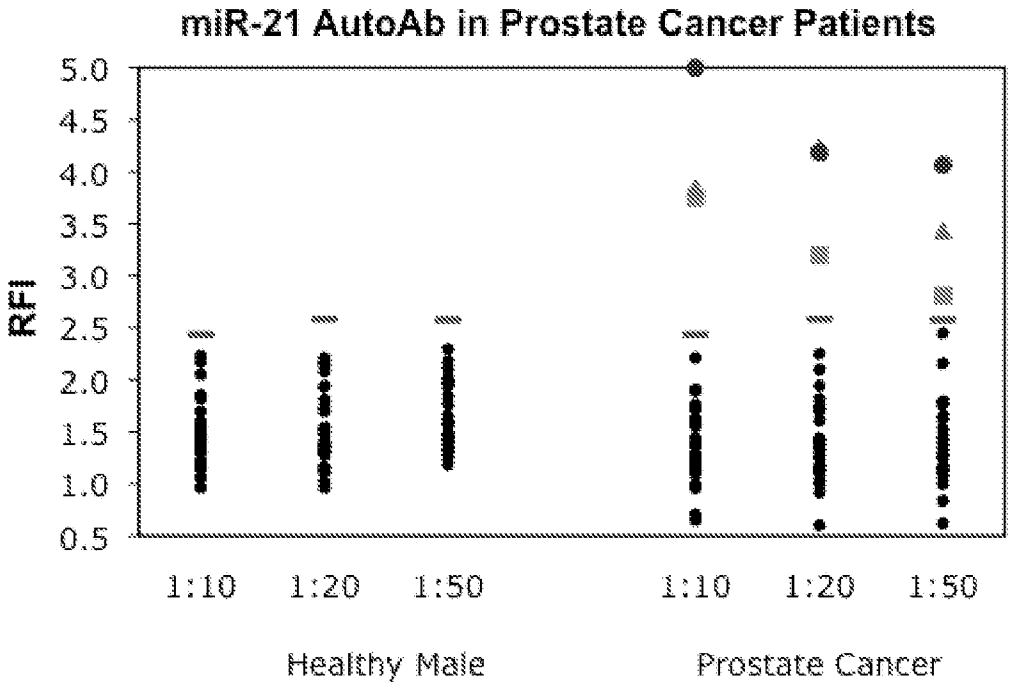


Figure 1D1

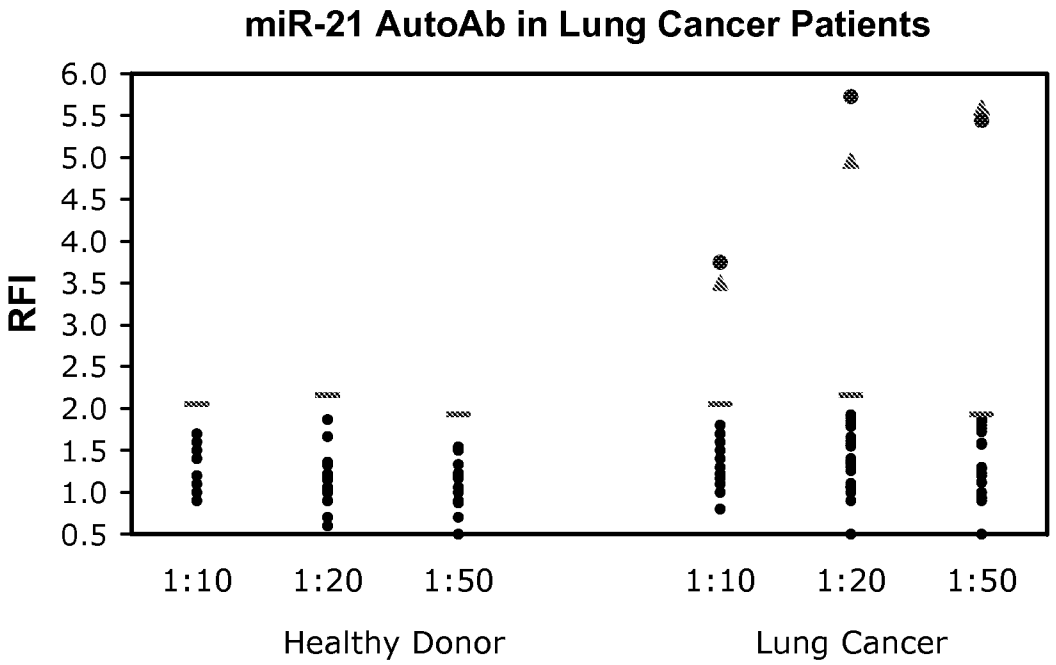


Figure 1D2

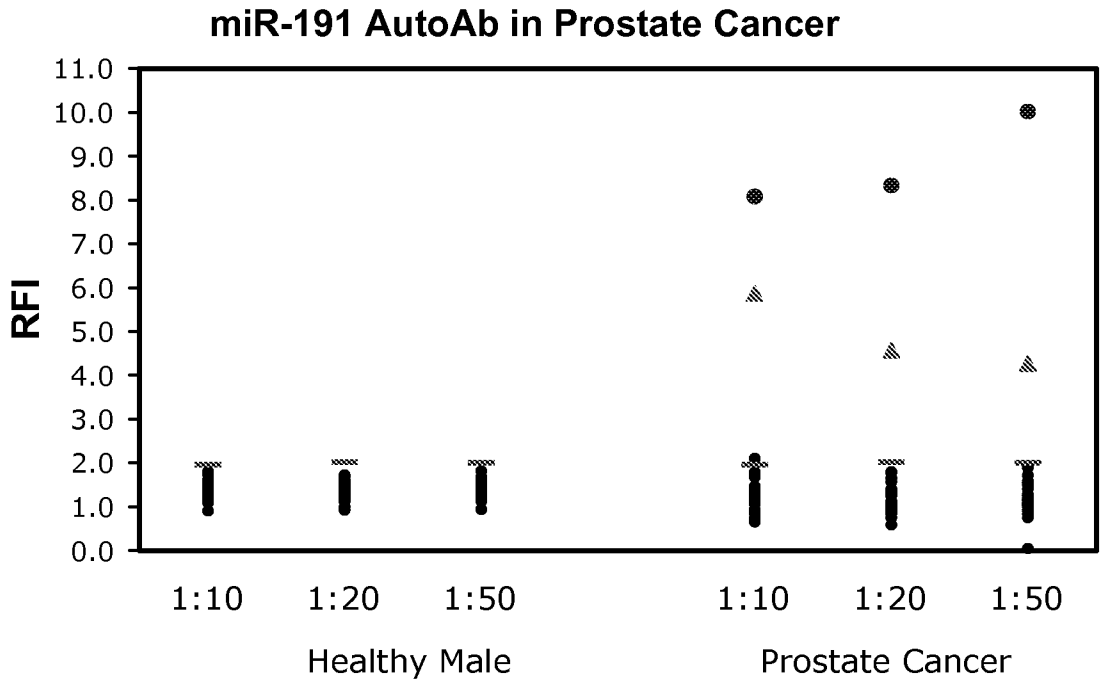


Figure 1E1

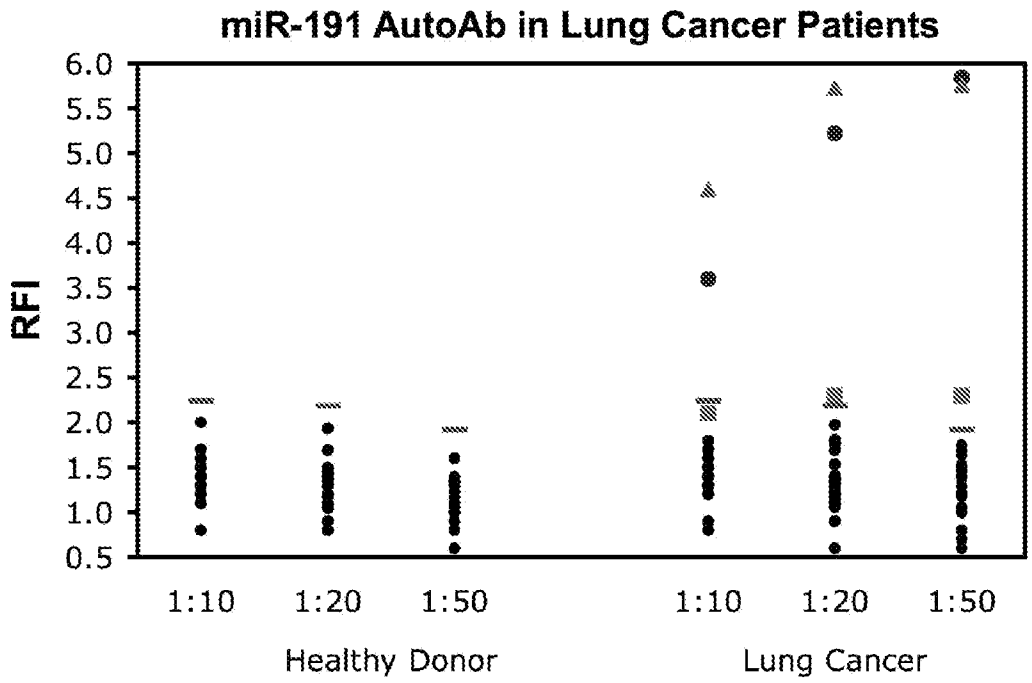


Figure 1E2

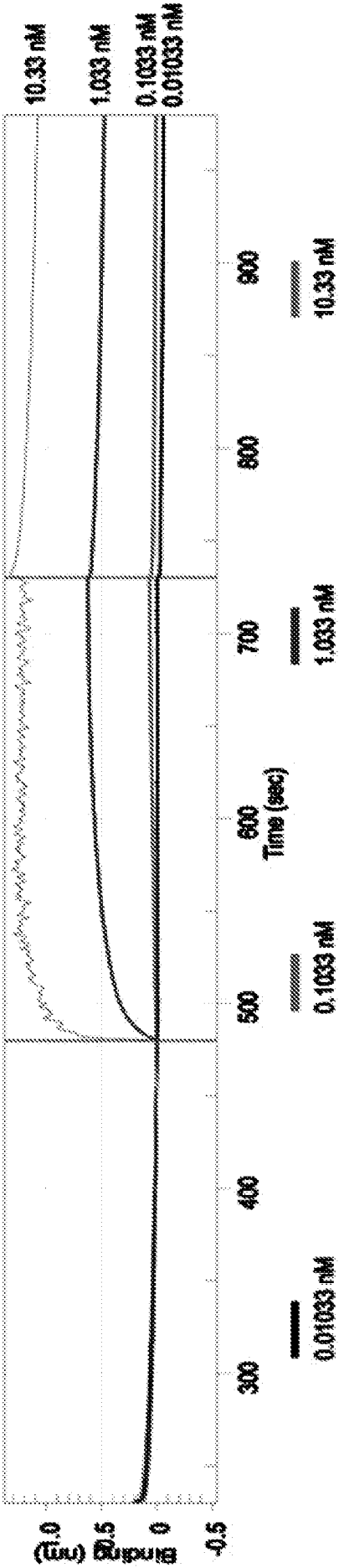


Figure 2A

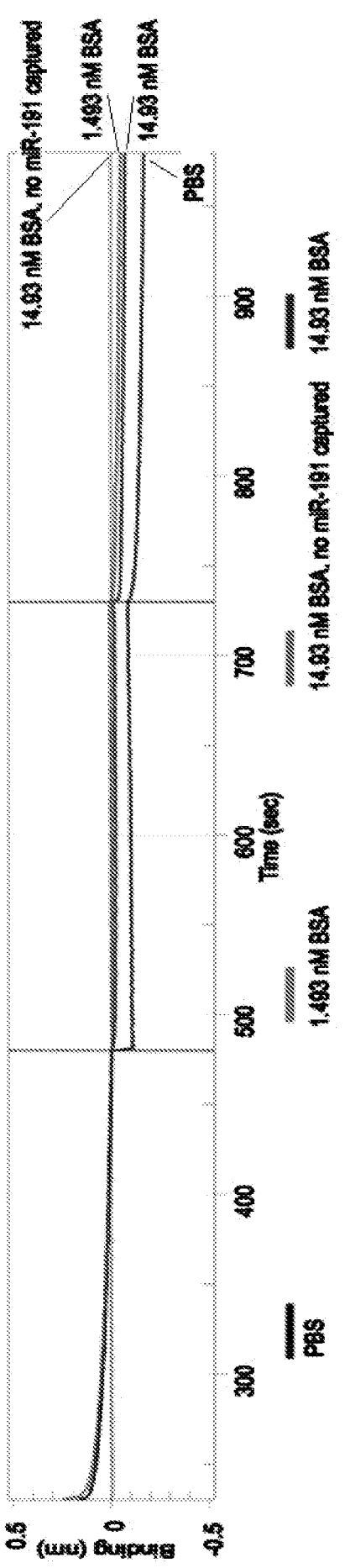


Figure 2B

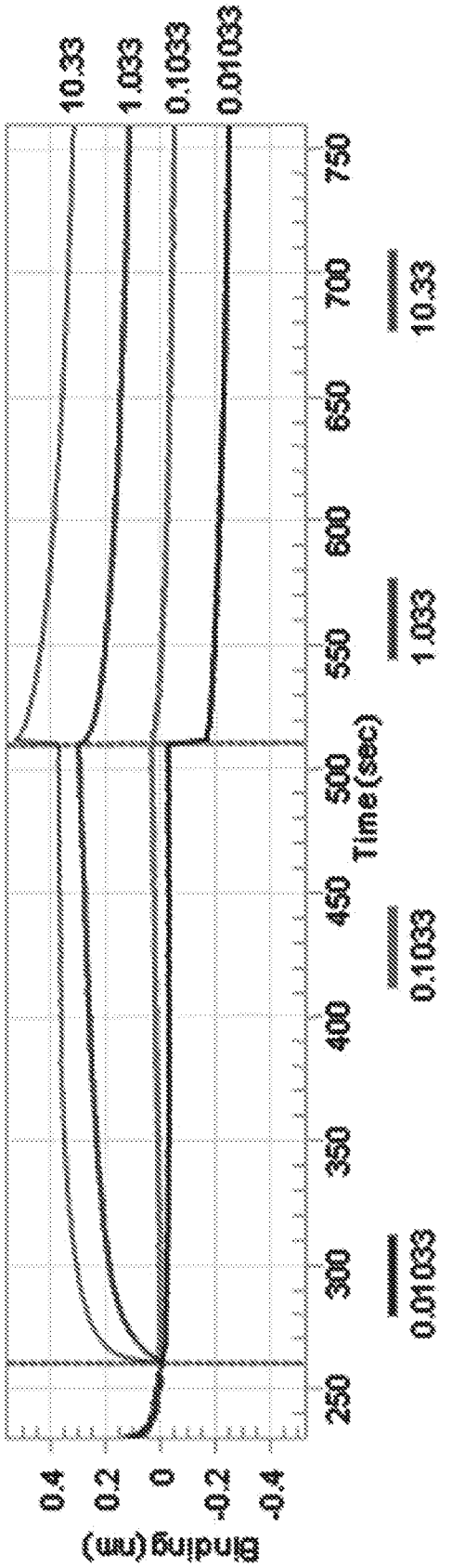


Figure 2C

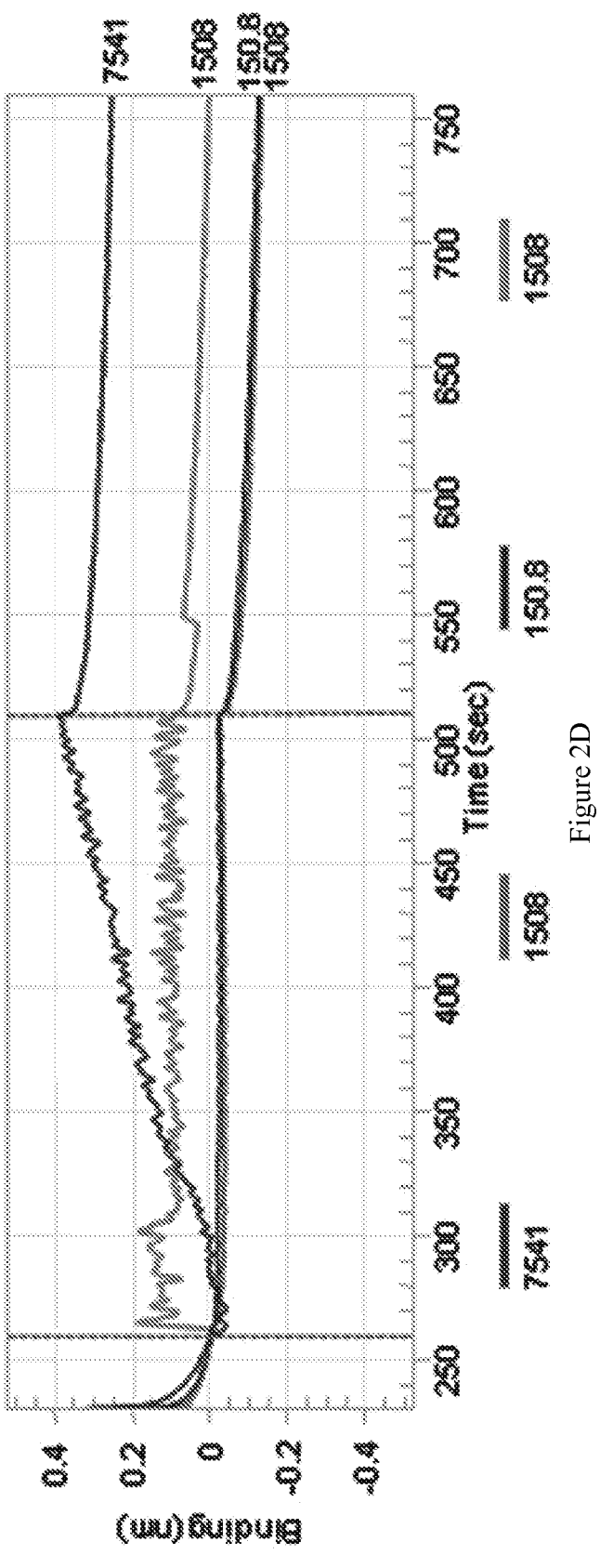
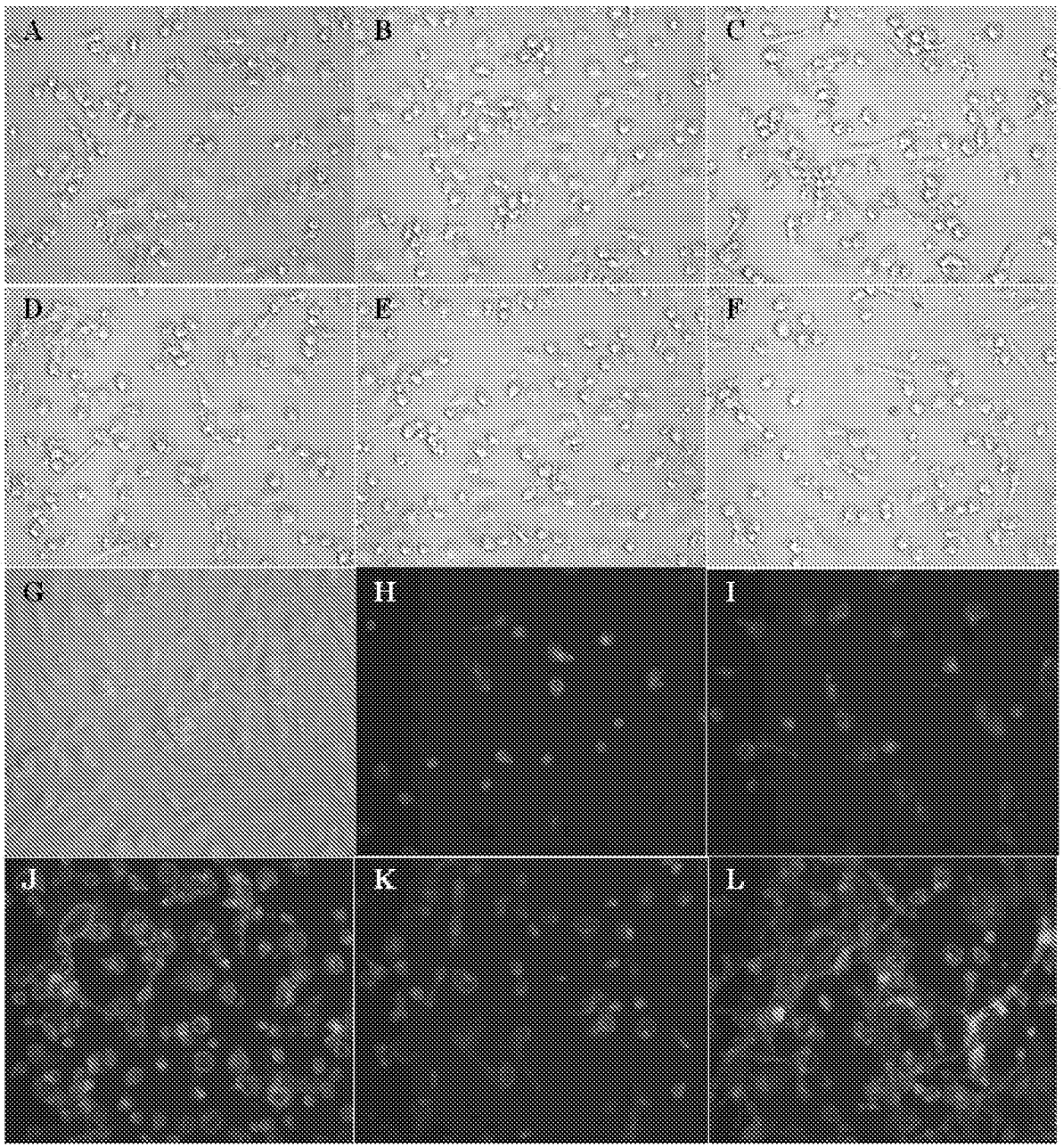


Figure 2D



Figures 3A-3L

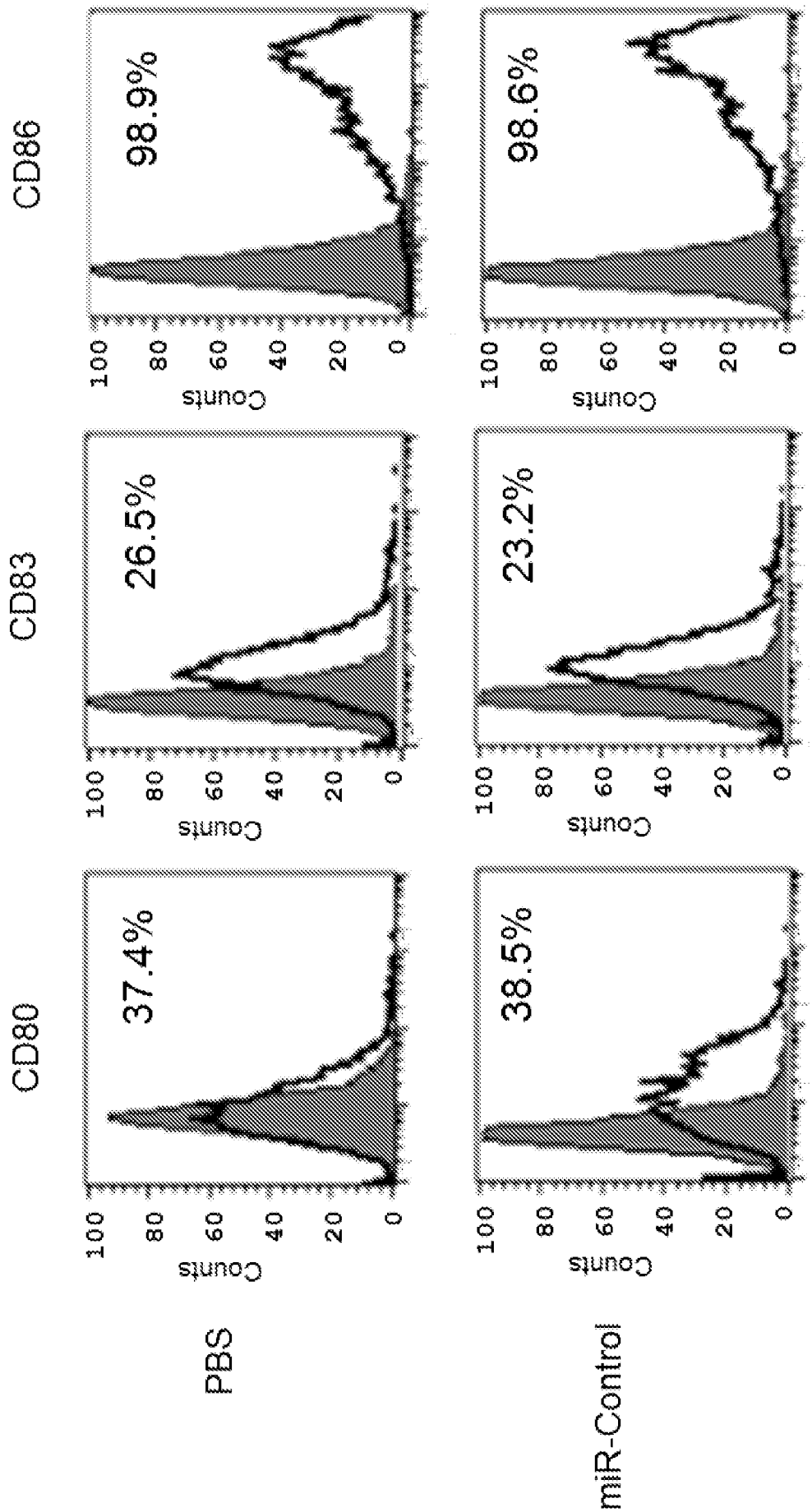

CD80
CD83
CD86
PBS
miR-Control
37.4%
26.5%
98.9%
38.5%
23.2%
98.6%
Counts
100
80
60
40
20
0

Figure 3M

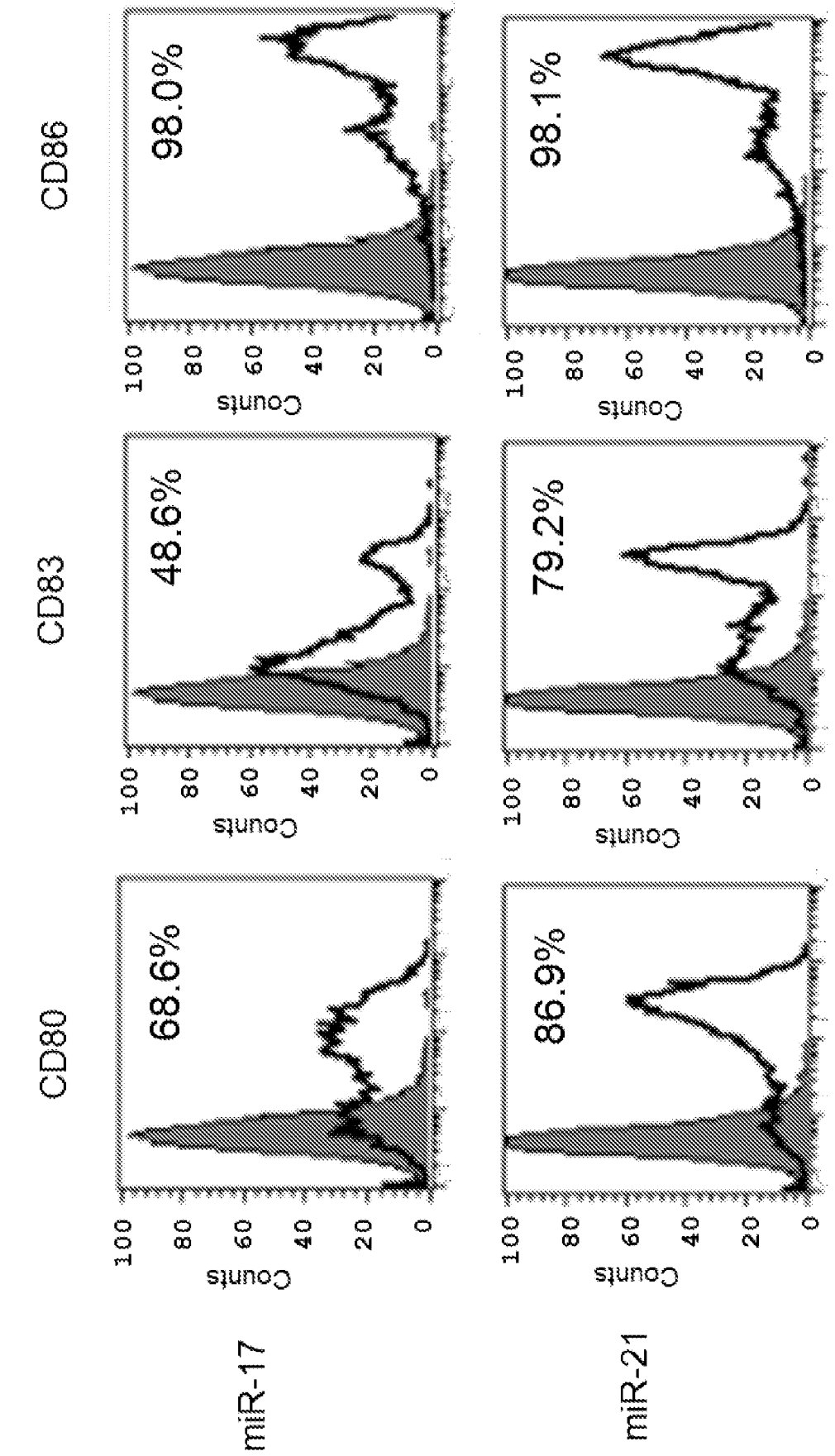


Figure 3M cont.

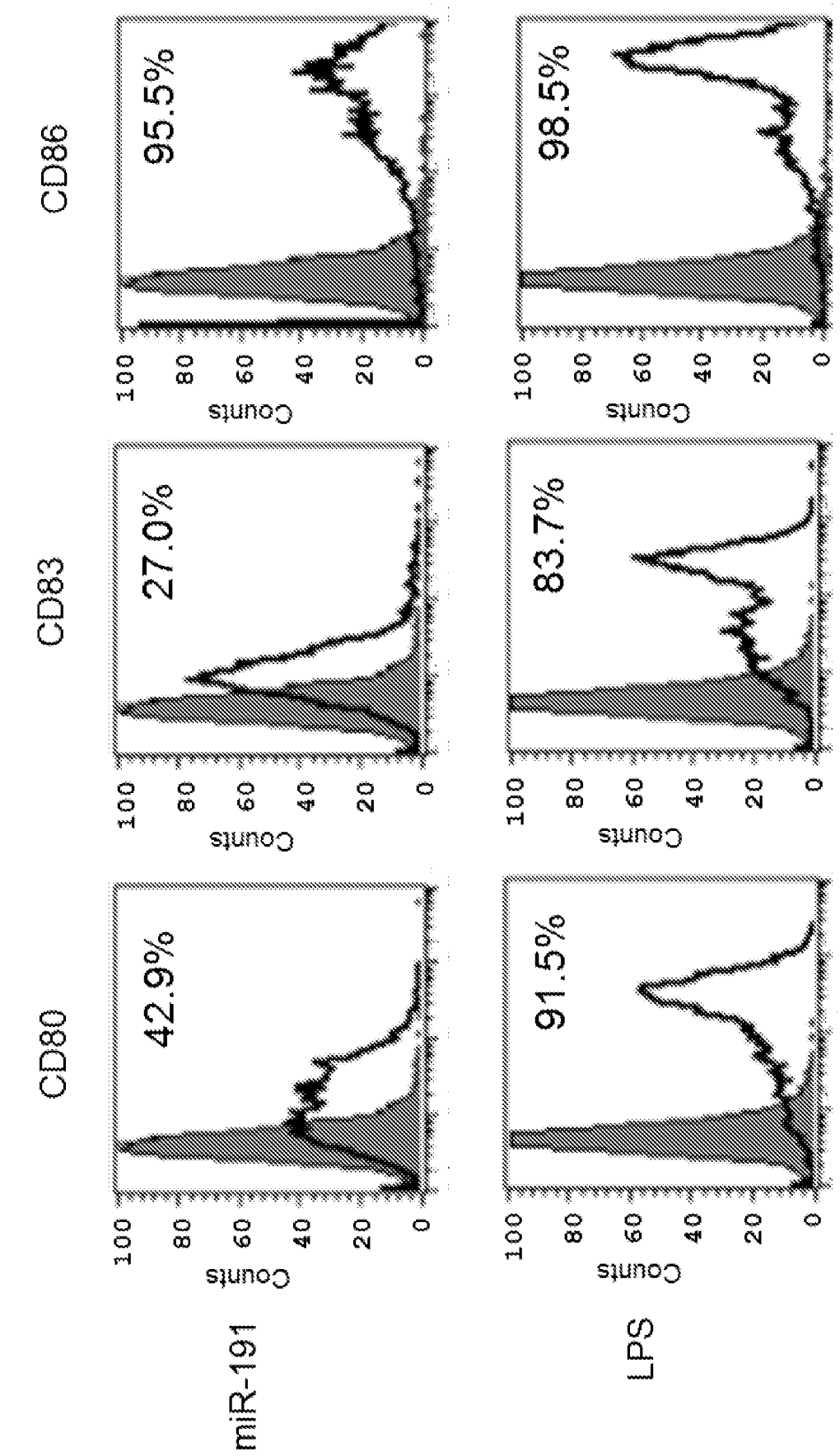


Figure 3M cont.

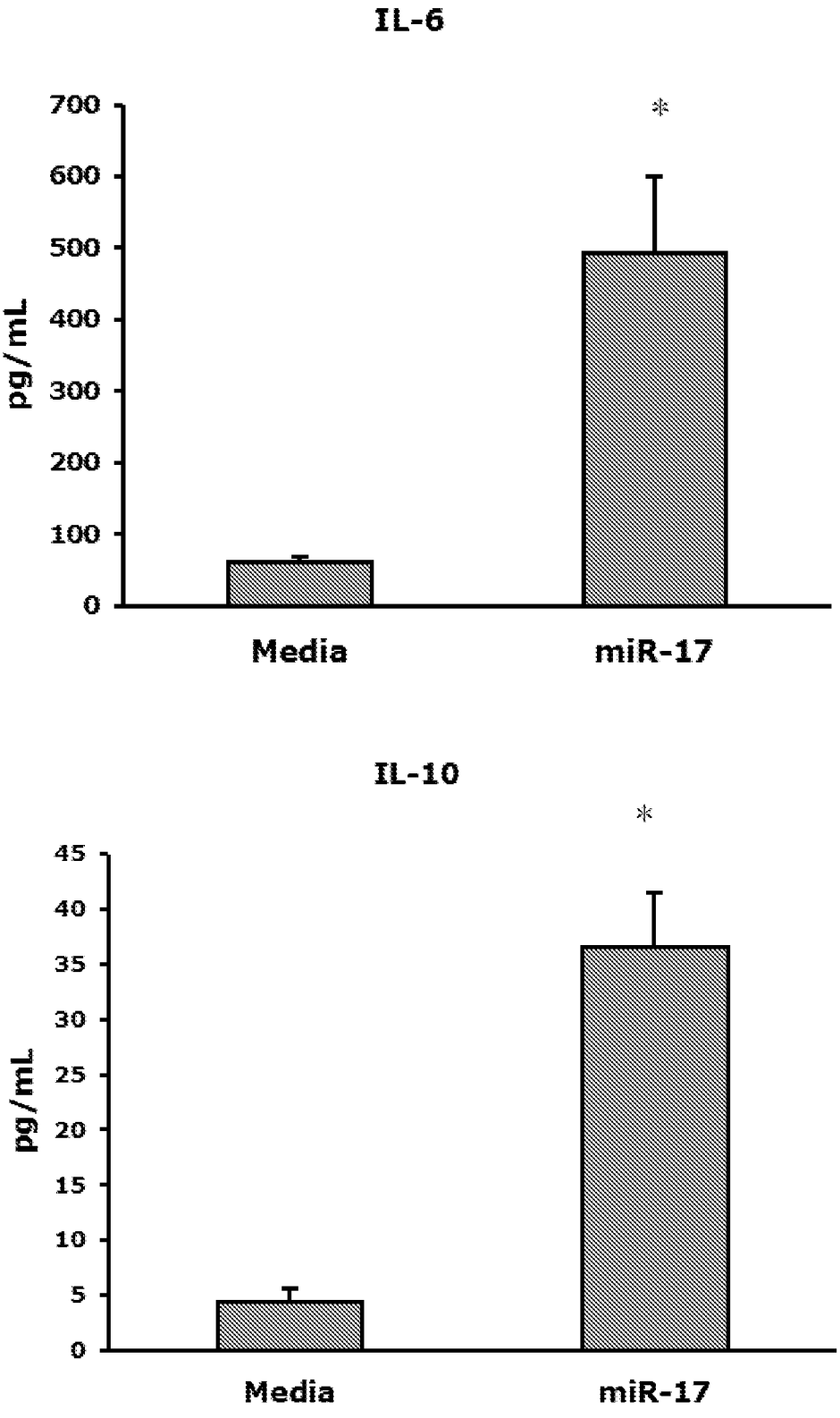

IL-6
700
600
500
400
300
200
100
0
pg/mL
*
Media
miR-17
IL-10
45
40
35
30
25
20
15
10
5
0
pg/mL
*
Media
miR-17

Figure 4A

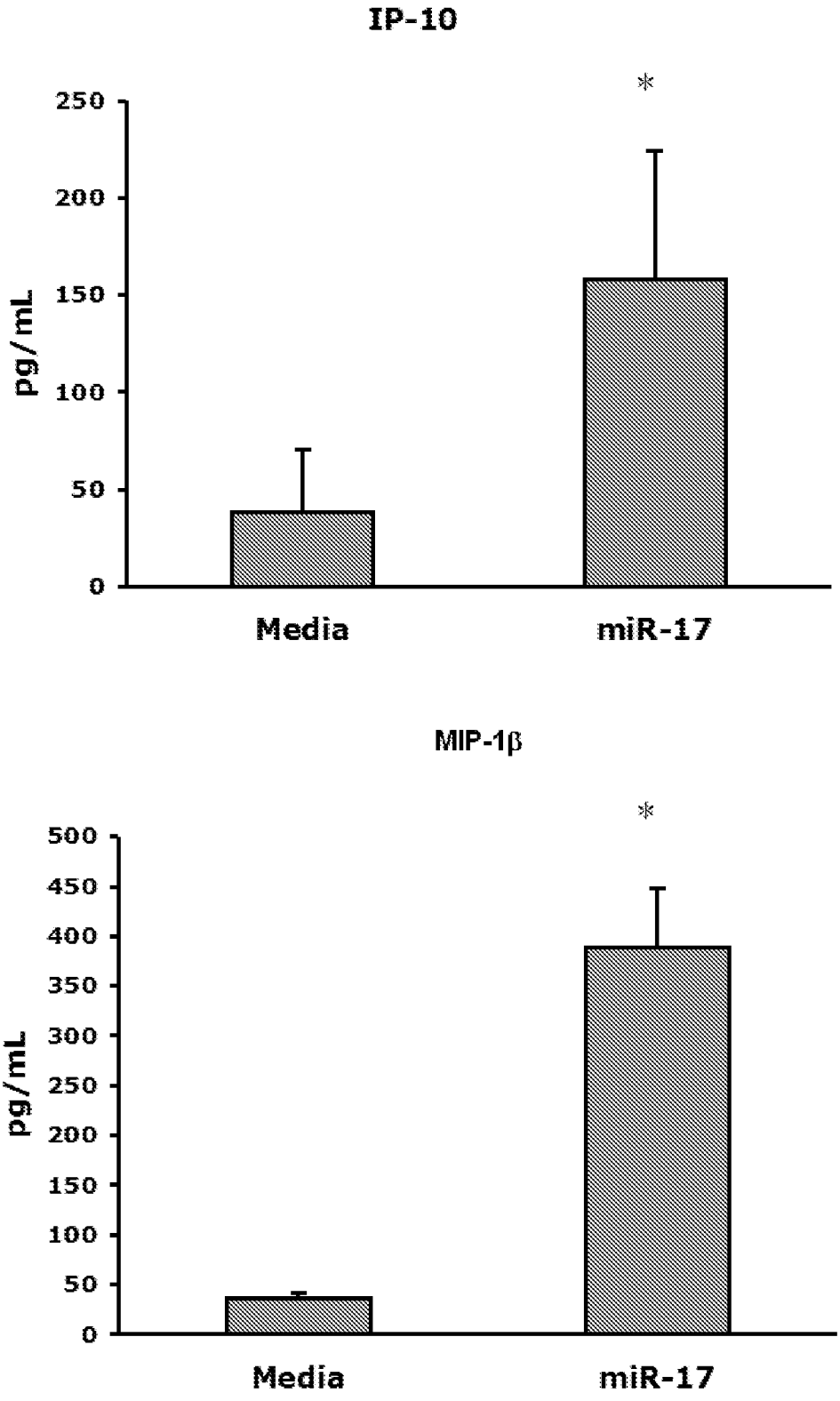
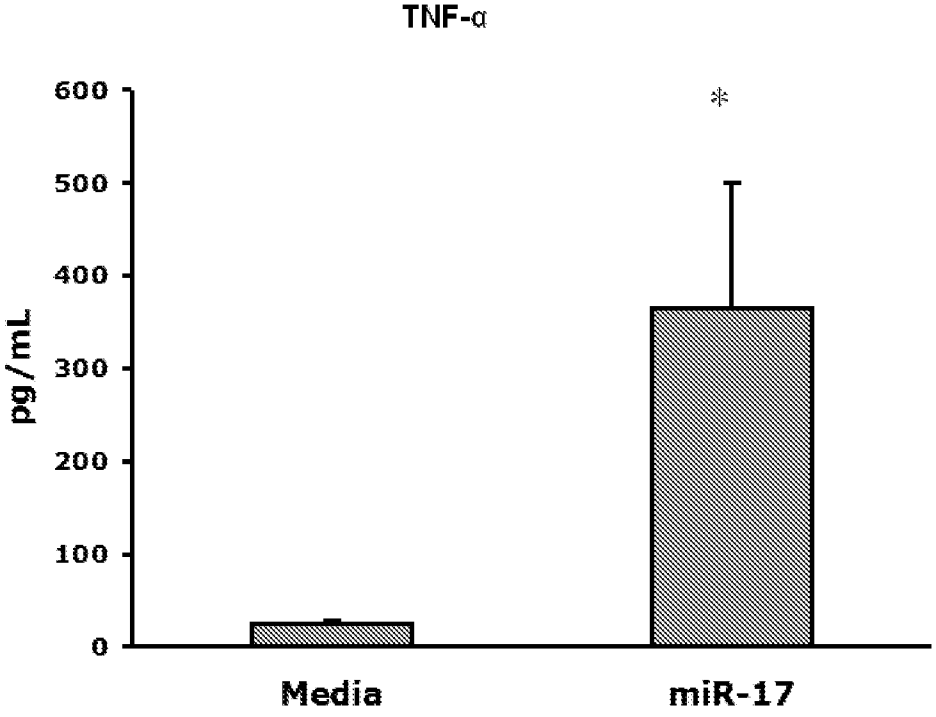


Figure 4A cont.



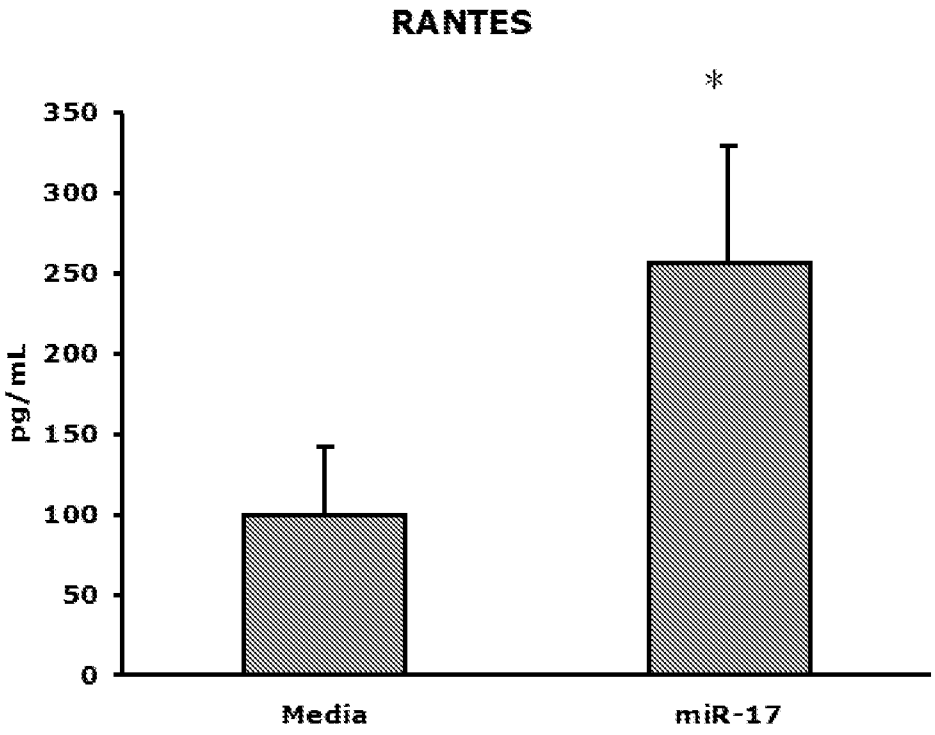


Figure 4A cont.

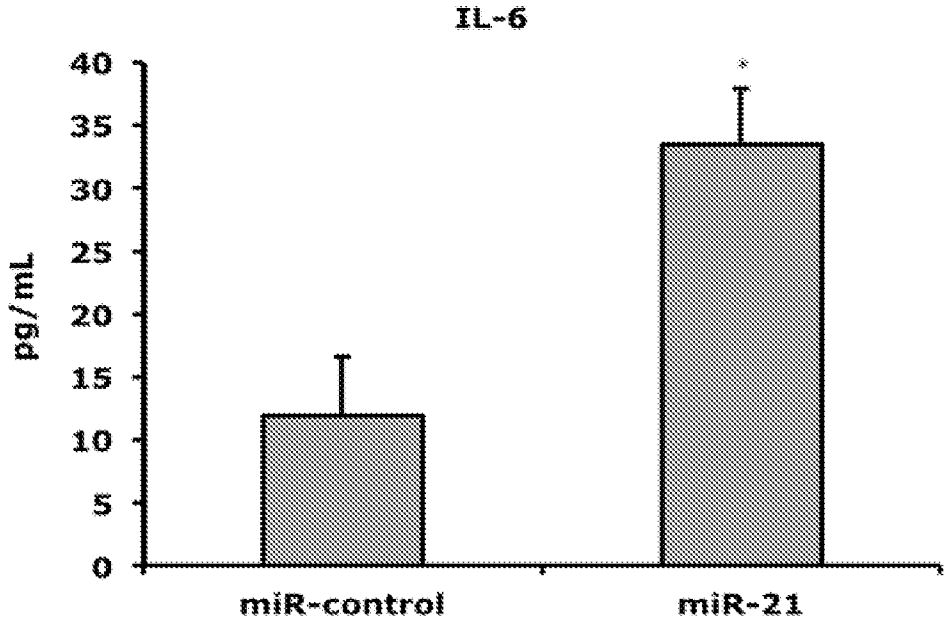


Figure 4B

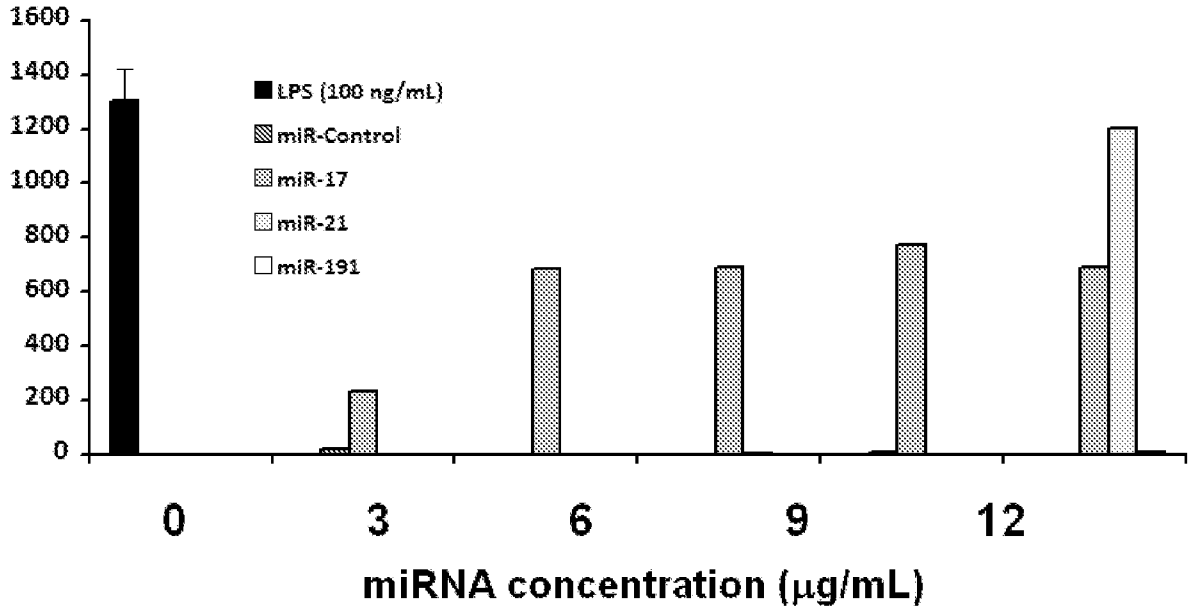
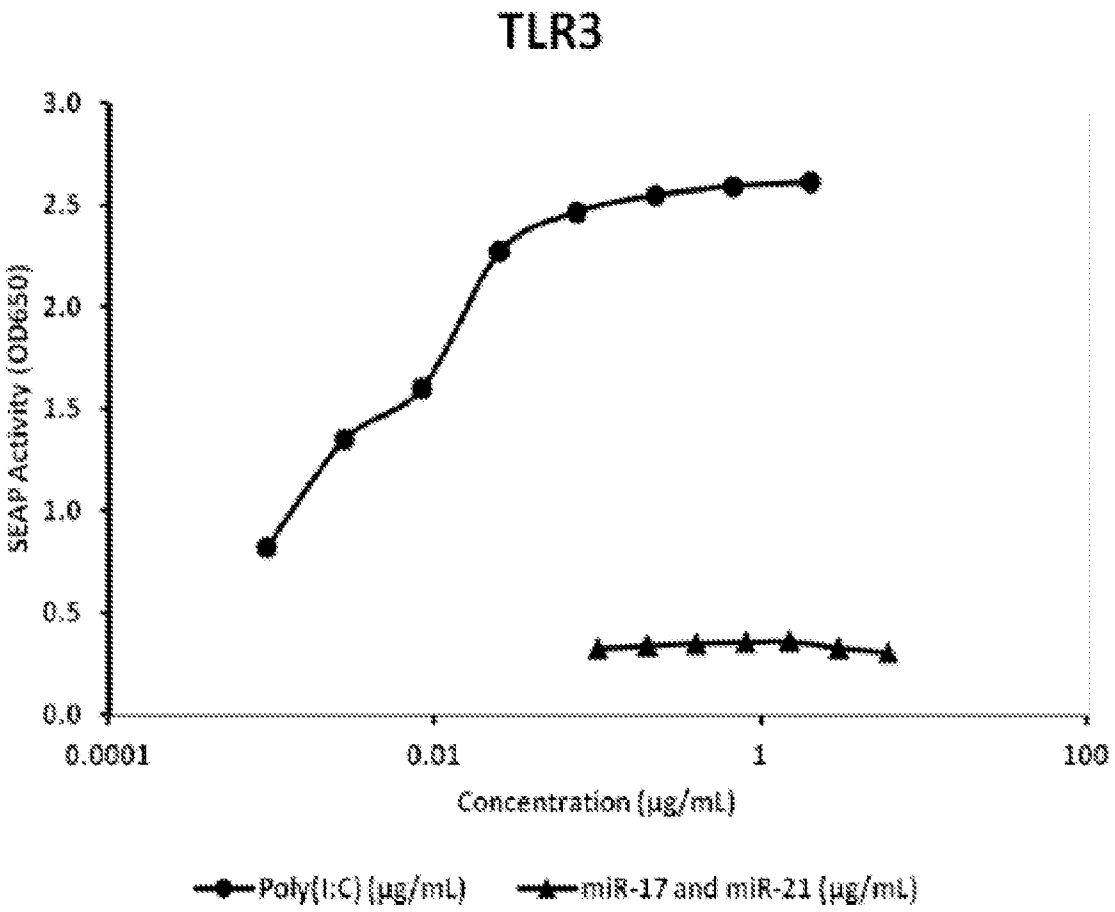


Figure 4C


TLR3
SEAP Activity (OD650)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
Concentration (µg/mL)
Poly(I:C) (µg/mL)
miR-17 and miR-21 (µg/mL)

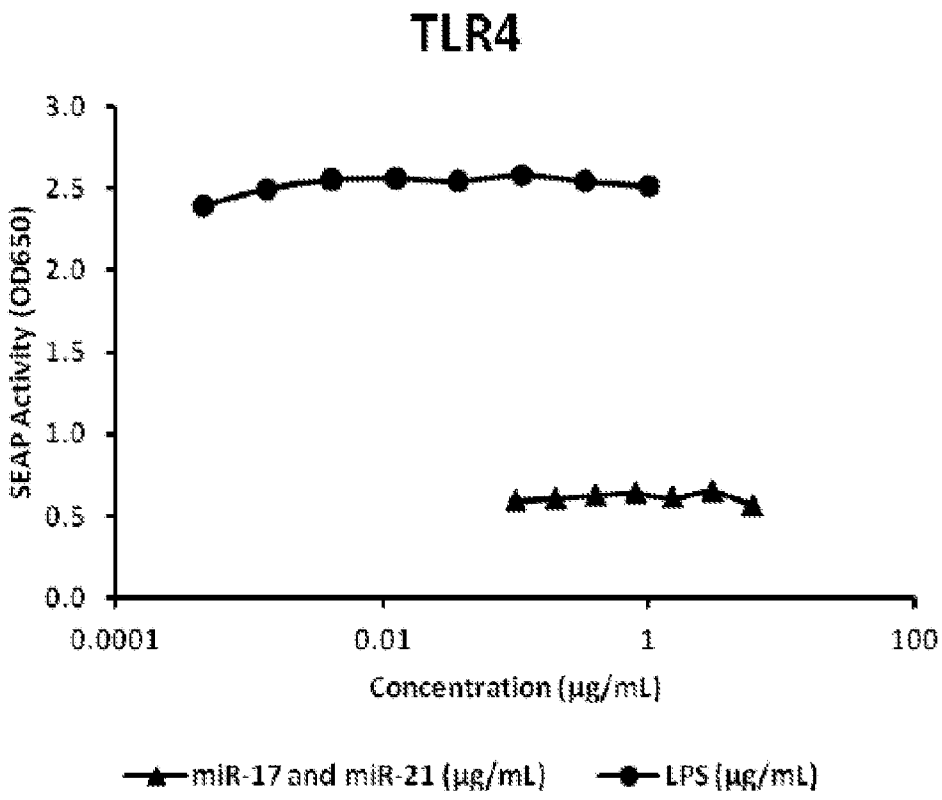
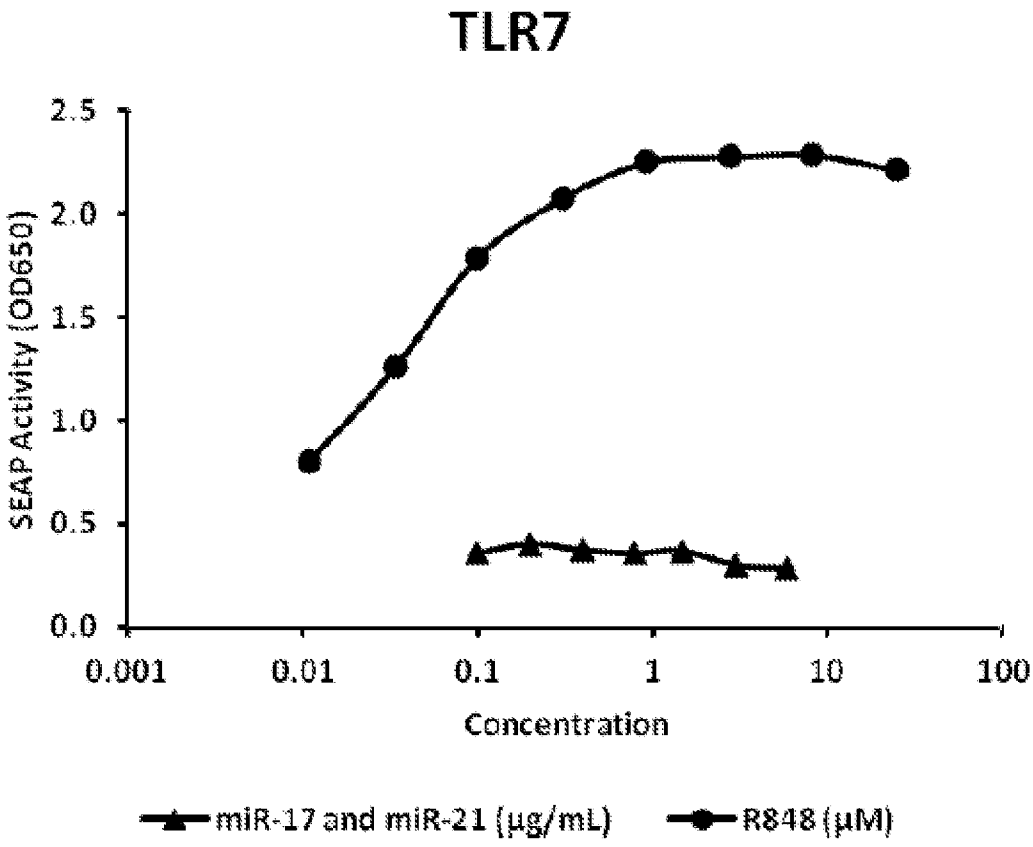

TLR4
SEAP Activity (OD650)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
Concentration (µg/mL)
miR-17 and miR-21 (µg/mL)
LPS (µg/mL)

Figure 4D



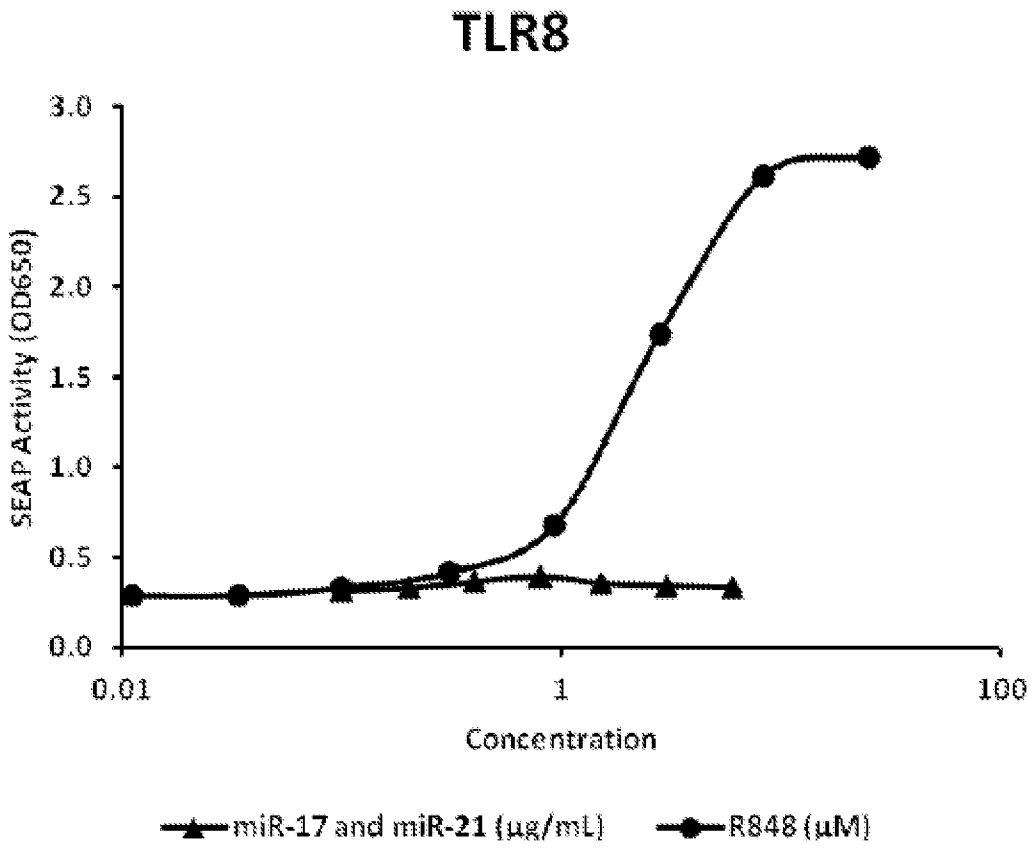


Figure 4D cont.

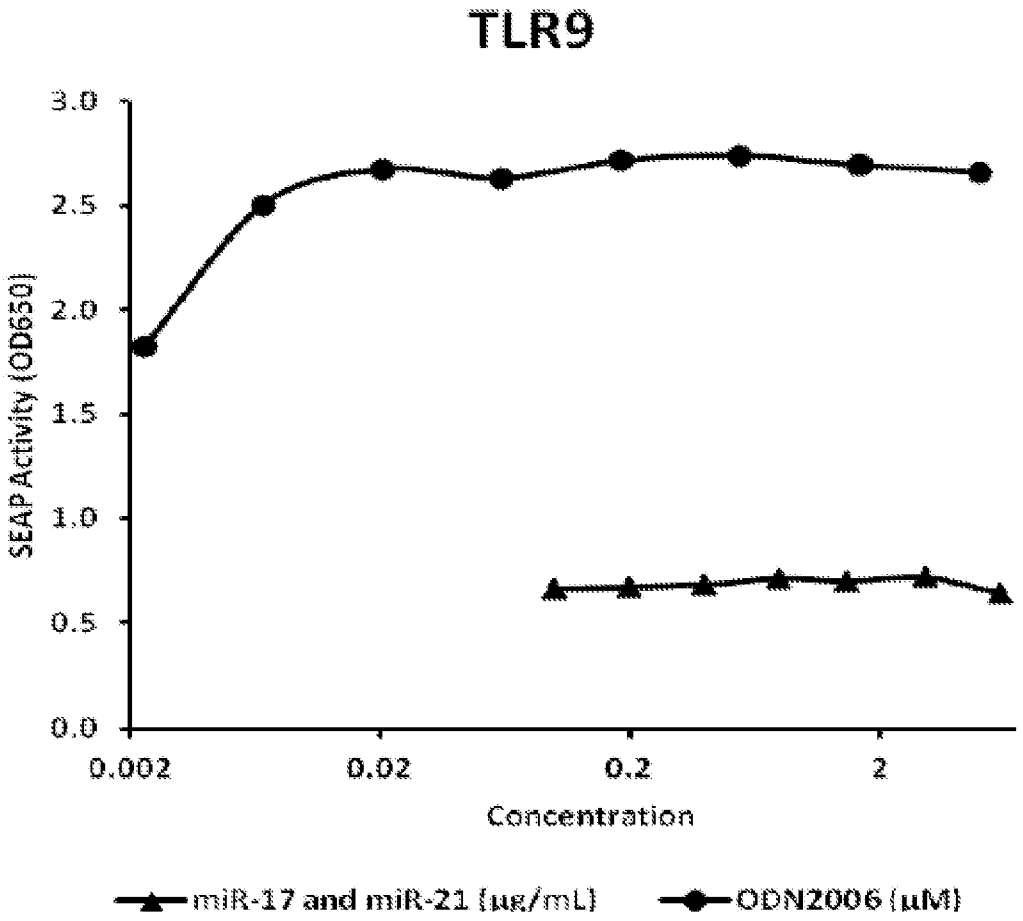


Figure 4D cont.

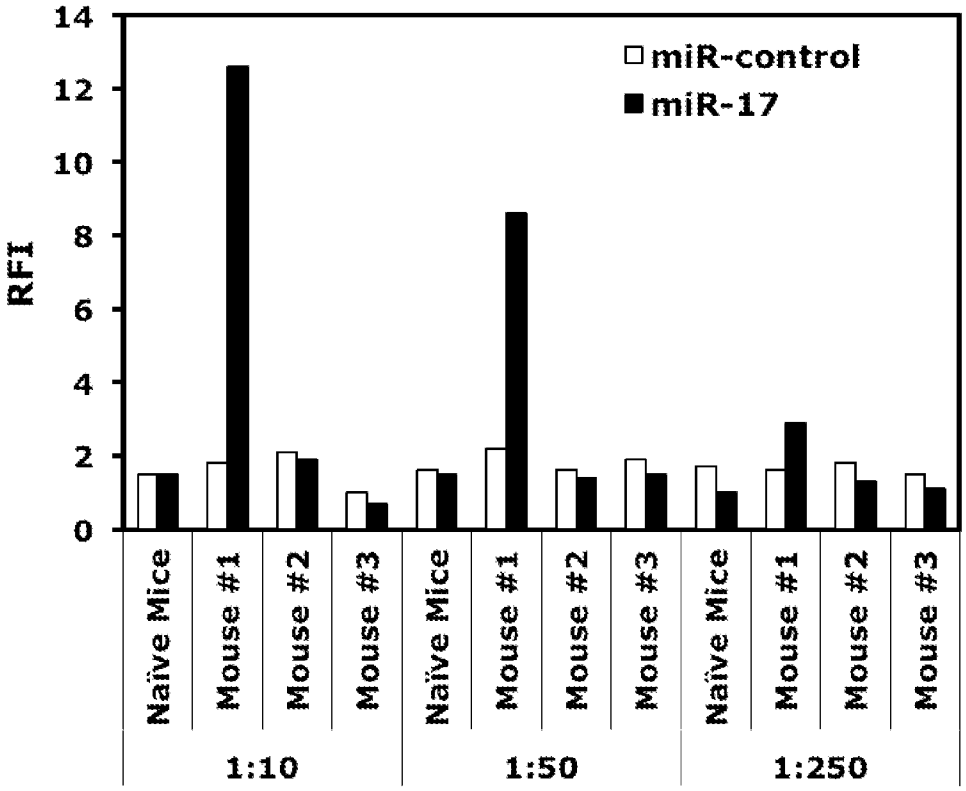


Figure 5A

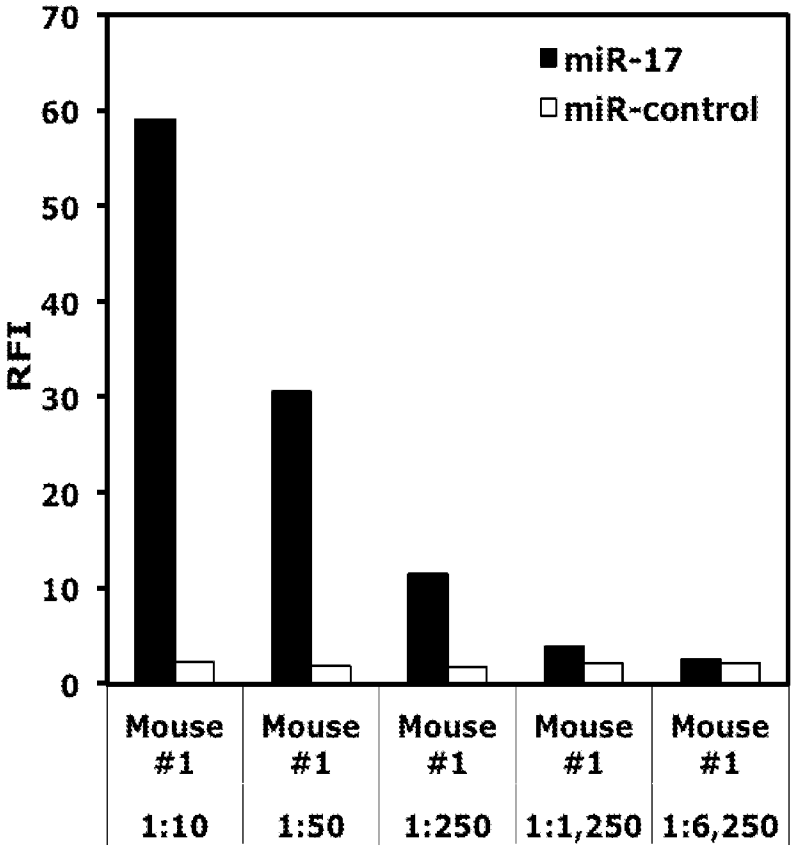


Figure 5B

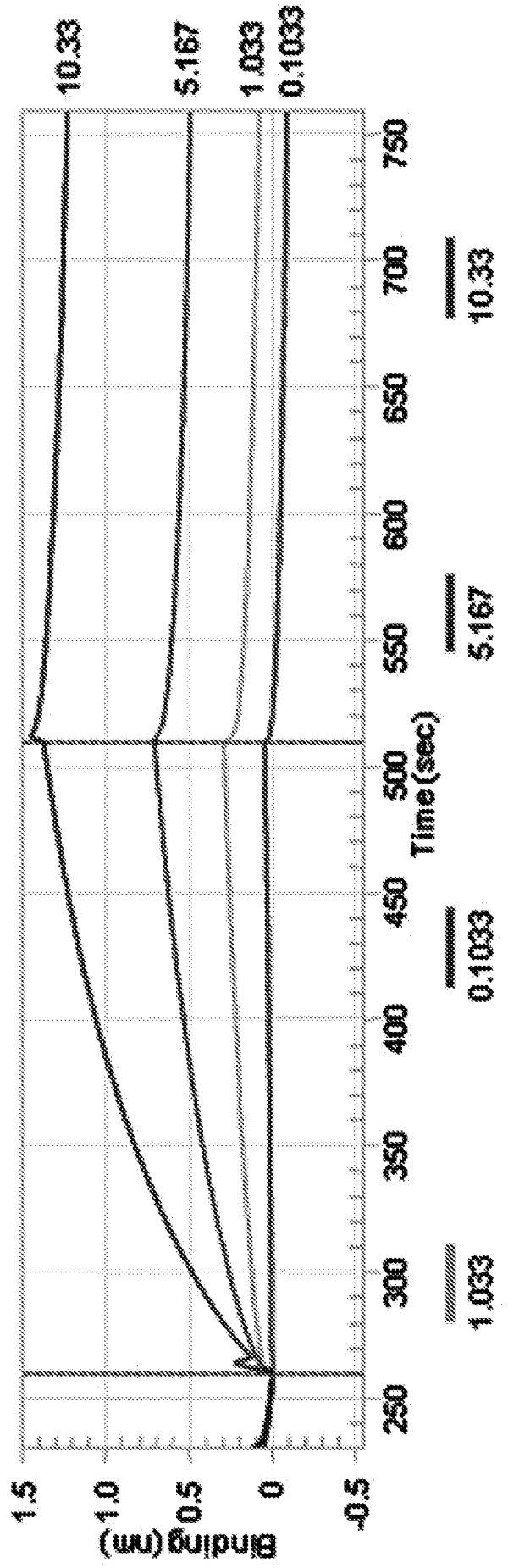


Figure 6A

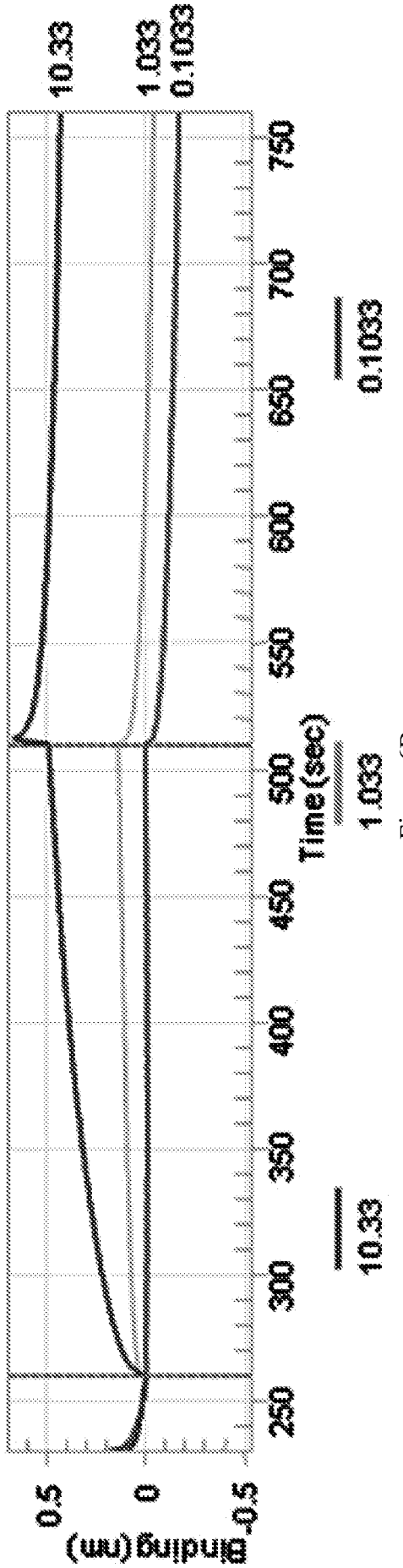


Figure 6B

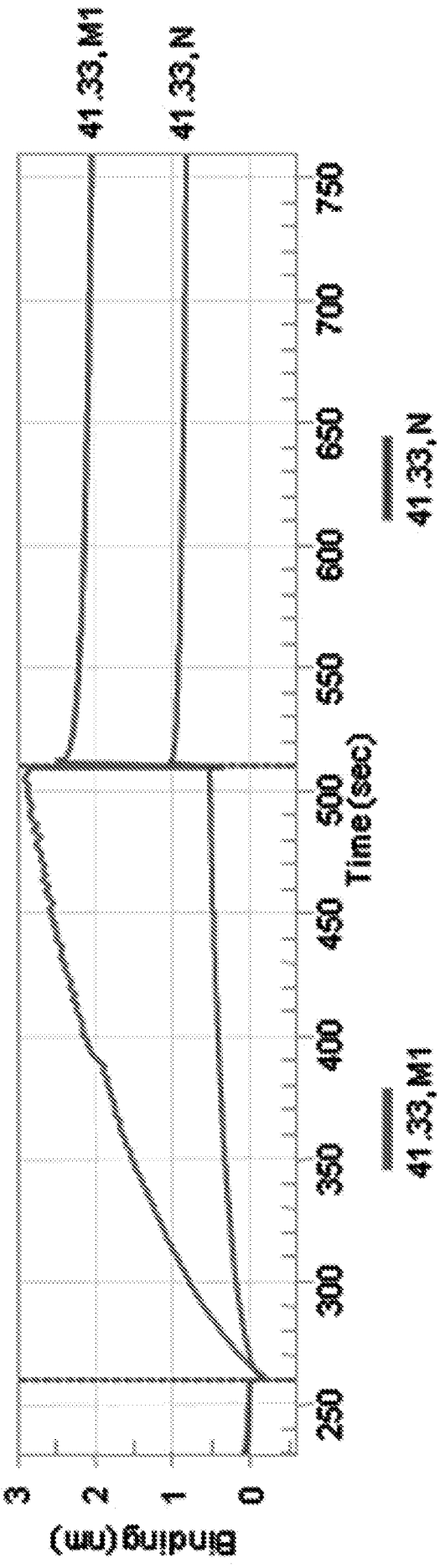

41.33,M1
41.33,N
Binding (nm)
3
2
1
0
250
300
350
400
450
500
550
600
650
700
750
Time (sec)
41.33,N
41.33,M1

Figure 6C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/076193

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i, G01N 33/574(2006.01)i, C12N 15/11(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68; A61K 38/16; G01N 33/574; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: cancer, miRNA, miR-17, miR-21, miR-191, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010-0285471 A1 (CROCE et al.) 11 November 2010 See abstract; paragraphs [0040], [0156]; claims 1-5.	1-3,6
Y		4,5
Y	WO 2011-073901 A1 (KONINKLIJKE PHILIPS ELECTRONICS N.V.) 23 June 2011 See abstract; claims 1-12, 24, 25.	4,5
A	RYU et al., 'Elevated microRNA miR-21 levels in pancreatic cyst fluid are predictive of mucinous precursor lesions of ductal adenocarcinoma' Pancreatology, Vol.11, pp.343-350 (12 July 2011) See abstract.	1-6
A	XI et al., 'Prognostic values of microRNAs in colorectal cancer' Biomarker Insights, Vol.1, pp.113-121 (2006) See abstract.	1-6
A	US 2010-0257618 A1 (CROCE et al.) 7 October 2010 See abstract; claims 1-9.	1-6

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 2014 (27.03.2014)	**01 April 2014 (01.04.2014)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
KIM, Seung Beom
Telephone No. +82-42-481-3371

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/076193

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-10
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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