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(54) **Title**: ASSAY FOR DISEASE DETECTION

(57) **Abstract**: An assay for detecting a disease comprising obtaining exosomes from a biological sample; providing a support having an array of a plurality of antibodies or binding fragments of antibodies; capturing exosomes on the support by binding of exosomes to one or more of the plurality of antibodies or binding fragments of antibodies; treating the captured exosomes with an agent for a selection marker indicative of a cellular origin of an exosome; detecting a subset of exosomes having the selection marker bound to the support; and identifying an array binding profile of the subset of exosomes, wherein the array binding profile is indicative of the disease.

ASSAY FOR DISEASE DETECTION

Technical Field

The present invention relates to an assay for disease detection using exosomes from a mammalian subject, in particular a human subject.

Background Art

Proteomic biomarkers are a powerful and practical approach in systems medicine. Current proteomics techniques for measuring blood-based protein biomarkers include Western blot, ELISA assays, mass spectrometry, and a range of antibody and antigen microarrays.

Exosomes are tiny closed vesicles (30 – 100 nm) that are secreted into body fluids such as blood from a variety of normal tissues. Exosomes were first identified several decades ago (Pan BT, Blostein R, Johnstone RM. Loss of the transferrin receptor during the maturation of sheep reticulocytes in vitro. An immunological approach. Biochem J 1983;210(1):37-47) but it is only recently that their normal role in conveying communication between cells has been recognised (Record M, Subra C, Silvente-Poirot S, Poirot M. Exosomes as intercellular signalosomes and pharmacological effectors. Biochem Pharmacol 2011;81(10):1171-82). Exosomes contain proteins, both on the surface, and in soluble form inside, with other biological molecules such as microRNAs (miRNA) that may have roles in the regulation of expression of genes and messenger RNAs (mRNA). miRNA have been recently recognized as important regulators of cellular processes, providing an additional layer of control over aspects of cellular function (Thery C. Exosomes: secreted vesicles and intercellular communications. F1000 Biol Rep 2011;3:15). Exosomes are formed within cells from endosomes and gain their surface and internal proteins, and miRNA via an internal secretion pathway leading to their budding off the surface of cells of origin and passing into the intercellular fluid and then into blood, lymph or other body fluid. As exosomes pass through the outer plasma membrane of a cell, they pick up some of the surface molecules (CD antigens) of the cell and may therefore be coated with a repertoire of proteins that is similar to the cell from which they came.

Thus, exosomes may be considered as a normal part of an ever-increasing number of regulatory mechanisms that maintain cellular homeostasis in the human body. Cancer

cells also secrete exosomes, and in many cases far more exosomes are secreted per cell, perhaps because of the higher metabolic rate and proliferation of cancer cells relative to normal cells. It has been proposed that cancers may gain a selective advantage by secreting exosomes that reduce the activity of the immune system, reducing the level of 'immuno-surveillance' that normally provides the first line of defence against cancer. Thus, there may be selective pressure for cancers to produce high levels of exosomes to sustain a rapid rate of growth and metastasis.

Cluster of differentiation (CD) antibody microarrays (DotScan™, Medsaic Pty Ltd, Sydney, Australia) were developed to provide extensive surface profiles (immunophenotypes) of leukemia cells obtained from blood samples and bone marrow aspirates (WO 2000/039580, US 7560226, EP 1151300). Bound cells are viewed by an imaging device (WO 2004/077031) and sophisticated software allows differential diagnosis of disease by recognition of cell binding patterns. These profiles are disease signatures that enabled the unequivocal classification of ~30 common leukemias and lymphomas (Belov L, Mulligan SP, Barber N, Woolfson A, Scott M, Stoner K, et al. Analysis of human leukaemias and lymphomas using extensive immunophenotypes from an antibody microarray. Br J Haematol 2006;135(2):184-97). The diagnostic capability of DotScan™ was validated with a clinical trial involving 796 leukemia patients and normal subjects, with a >95% correspondence between the diagnoses made using DotScan™ alone, and the multiple diagnostic criteria routinely used by pathology laboratories. The DotScan™ assay is based upon the principle of capture of live cells by CD antibodies that are immobilized on a nitrocellulose-coated glass slide. Cells are only captured on an antibody dot when they express the corresponding CD antigen. Cell capture may take 60 minutes at room temperature, and then unbound cells are gently washed off. The resultant dot pattern is the surface profile for that population of cells. It is noteworthy that only live cells are captured, dead cells do not have mobility of CD antigens in their plasma membrane that is necessary for capping of the antigen on the side of the captured cell facing the immobilized antibodies. In addition, pseudopodia may inter-digitate the three-dimensional surface of the nitrocellulose, providing additional interactions for cell capture.

The DotScan™ procedure works well for high level leukemias where the white cell count in peripheral blood is $>10 \times 10^6$ cells/mL, and there is a predominant monoclonal population of leukemia cells. However, for low-level leukemias, or for suspensions of live cells made from solid tumours, a simple optical scan to obtain the dot pattern does not provide useful data. Such a profile would represent the average binding densities

due to capture of a variety of different cell types. In the case of cell suspensions derived from colorectal cancer (CRC), the captured cells include CRC, a variety of leukocytes, and stroma cells. These subsets of cells can be imaged separately by staining the captured cells on the microarray with a cocktail of fluorescently-labelled antibodies that distinguish between subsets of the cells. Using a suitable fluorescence scanner, individual dot patterns are obtained for each subset of cells. An alternative approach is to fractionate a subset of cells from the live cell suspension using an appropriate discriminatory antibody coupled to Miltenyi magnetic beads (50 nm, MACS). This subset of cells may subsequently be profiled on DotScan™ with the Miltenyi beads still attached.

Although the DotScan™ system provides a powerful tool for disease detection, it was considered that the system was limited to the capture of live cells. Previous attempts to capture cell membrane fragments using antibody arrays as a means for detecting disease were unsuccessful.

There is an ongoing need for assays for disease detection. The present inventors have found that various disease states may be identified by examining the differential expression of protein markers on the surface of isolated exosomes.

Disclosure of Invention

In a first aspect, the present invention provides an assay for detecting a disease comprising:

(a) capturing exosomes from a biological sample on a support having an array of a plurality of antibodies or binding fragments of antibodies;

(b) treating the captured exosomes with an agent for a selection marker indicative of a cellular origin of an exosome;

(c) detecting a subset of exosomes having the selection marker bound to the support; and

(d) identifying an array binding profile of the detected subset of exosomes, wherein the array binding profile is indicative of a disease.

In a second aspect, the present invention provides an assay for detecting a disease comprising:

(a) obtaining exosomes from a biological sample;

(b) providing a support having an array of a plurality of antibodies or binding fragments of antibodies;

(c) capturing exosomes on the support by binding of exosomes to one or more of the plurality of antibodies or binding fragments of antibodies;

(b) treating the captured exosomes with an agent for a selection marker indicative of a cellular origin of an exosome;

(e) detecting a subset of exosomes having the selection marker bound to the support; and

(f) identifying an array binding profile of the subset of exosomes, wherein the array binding profile is indicative of the disease.

Preferably, the assay detects a disease signature in a mammal. The mammal may be human, equine, bovine, murine, feline, porcine, canine, or ovine. Preferably, the mammal is a human.

Preferably, the disease signature is selected from cancer, cardiovascular disease, diabetes, and autoimmune disorders. Preferably, the cancer is selected from leukaemia, lymphoma, colorectal cancer, melanoma, lung cancer, pancreatic cancer, liver cancer, breast cancer, ovarian cancer, or prostate cancer.

Typically, exosomes include membrane particles or exosome-like vesicles or microvesicles. Preferably, the exosomes range in size from 30-100 nm, the membrane particles range in size from 50-80 nm, the exosome-like vesicles range in size from 20-50 nm and the microvesicles range in size from 100-1000 nm.

In preferred embodiments, the biological sample is selected from blood, urine, cerebrospinal fluid, mucous, bone marrow aspirate, purulent discharge, biopsy or surgical excision of solid tissue, tissue culture medium or ascities fluid. Preferably, the biological sample is blood, plasma, saliva or urine.

Preferably the exosomes are isolated from the biological sample prior to being captured on the support. Preferably, the exosomes are isolated by centrifugation and sedimentation equilibrium on gradients, or ultrafiltration and ultracentrifugation, or affinity capture, or affinity purification, or precipitation.

Exosomes may be isolated by centrifugation on an iodixanol Optiprep gradient. The centrifugation may include several centrifugation steps, for example 10 min at 300g, followed by 30 min at 1200g, 1 hour at 100,000g and 16 hours at 100,000g on an

iodixanol Optiprep gradient. The isolated exosomes may be finally collected by centrifugation for 3 hours at 100,000g.

Alternatively, exosomes may be isolated by sedimentation equilibrium centrifugation on linear sucrose gradients. Preferably partially purified by centrifugation for 10 min at 500g, 20 min at 16,500g, 70 min at 120,000g for sedimentation equilibrium on linear sucrose gradients (0.25-2.0 M) and then centrifugation for 15 hours at 100,000g.

A further preferred method for isolating exosomes is ultrafiltration using a 500 Dalton membrane and ultracentrifugation on 30% sucrose/deuterium oxide.

A further preferred method for isolating exosomes is isolation by affinity capture. Typically, antibodies or binding fragments of antibodies are used for such affinity capture. For example, A33 antibody may be used to capture exosomes. The affinity capture is preferably on magnetic beads.

A further preferred method for isolating exosomes is affinity purification. Typically, the affinity purification involves a selective filtration device containing affinity agents that tightly bind to high-mannose structures unique to the surface of exosomes often produced by cancer.

A further preferred method for isolating exosomes is precipitation using ExoQuick (System Biosciences, Mountain View, CA, USA).

In preferred embodiments, the support contains antibodies specific for cell surface markers. Preferably, the cell surface markers are cellular proteins or peptides being Cluster of Differentiation (CD) antigens, or other cell surface proteins. The CD antigens may be selected from Zola et al. (2007) (Zola H, Swart B, Nicholson I, Voss E (2007) Leukocyte and stromal cell molecules: the CD markers, John Wiley and sons Inc, New Jersey, USA).

Other cell surface proteins are preferably selected from the Human Protein Atlas (Fagerberg L, Jonasson K, von Heijne G, Uhlén M, Berglund L. Prediction of the human membrane proteome. Proteomics 2010;10: 1141-1149.).

Preferably the support has a microarray of a plurality of CD antibodies on a solid support. The solid support may be comprised of any suitable material such as glass, micro-porous silica, cellulose, ceramic material, nitrocellulose, polyacrylamide, nylon, polystyrene, polystyrene derivatives, polyvinylidene difluoride, methacrylate, methacrylate derivatives, polyvinyl chloride, or polypropylene. Preferably, the solid support comprises nitrocellulose. Typically, the antibodies are immobilized on the solid support.

The exosomes may be immobilised on a two-dimensional antibody microarray of anti-CD ligands (or other surface proteins) that bind to corresponding CD antigens (or other surface proteins) on the surface of exosomes. Typically, the ligands are placed on the microarray with a diameter of about 0.5 mm but may be smaller or larger in diameter.

Preferably, the array of antibodies includes ten or more antibodies selected from TCRα/β, TCRγ/δ, CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD15s, CD16, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD28, CD29, CD31, CD32, CD33, CD34, CD36, CD37, CD38, CD40, CD41, CD42a, CD43, CD44, CD44v6, CD45, CD45RA, CD45RO, CD47, CD49b, CD49c, CD49d, CD49e, CD49f, CD49f, CD51, CD55, CD59, CD58, CD63, CD66a, CD66b, CD69, CD52, CD54, CD55, CD56, CD57, CD60, CD61, CD62L, CD62E, CD62P, CD63, CD64, CD65, CD66c, CD66e, CD71, CD77, CD79a, CD79b, CD80, CD82, CD86, CD87, CD88, CD95, CD98, CD102, CD103, CD104, CD117, CD120a, CD122, CD126, CD128, CD130, CD134, CD135, CD138, CD151, CD154, CD166, CD175s, CD177, CD184, CD200, CD227, CD235a, CD244, CD255, CD261, CD262, CD324, CD326, CD340, Annexin II, beta-catenin, CA 19.9, CA-125, Claudin-4, DCC, EGFR, ErbB3, FAP, Galectin-3, Galectin-4, Galectin-8, HLA-ABC, HLA-DR, HLA-G, FMC7, kappa and lambda light chains, Mabthera, MAGE-1, MICA, MMP-14, PIGR, surface immunoglobulin, TSP-1 and isotype control antibodies.

It will be appreciated as new cell surface antigens are detected, antibodies to these new cell surface antigens may be used for the present invention.

In preferred embodiments, the array may contain about 10, or 15 or 20, or 30, or 40, or 50, or 60, or 70, or 80, or 90, or 100 or more different antibodies at known locations on the microarray.

In various other preferred embodiments, the array may comprise from about 20 to up to about 1000 different antibodies, each being specific for a single cell surface marker at known locations on the array. For example, the array may comprise from 20 up to about 500, or from 20 up to about 200, or from 20 to up to about 100, or from 20 to up to about 50 different antibodies each being specific for a single cell surface marker.

In particular preferred embodiments, the array comprises from about 100 up to about 180, for example about 160, different protein binding antibodies.

A ligand specific agent for a cellular protein or peptide found on exosomes can be used to detect bound exosomes. Typically, the cellular protein or peptide is selected from a tumour marker, or other surface marker found on cells. In a preferred form, the marker

is part of a particular disease signature, for example, for cardiovascular disease or diabetes. For example for colorectal cancer (CRC), the tumour marker is selected from EpCAM (epithelial cell adhesion molecule), CEA (carcino-embryonic antigen), or A33.

Preferably the agent is an antibody for a cell surface marker indicative of a cancer cell type.

Table 1 shows examples of CD markers that can be used for the detection of exosomes derived from different cancers.

Table 1

Cancer	**Marker**
colorectal	EpCAM
	A33
	CEA
prostate	TAG-72
	PSMA
	PSCA
breast	CA15-3
	CA27.29
	HER-2/neu
melanoma	MAGE
	tyrosinase
	NG-2
ovarian	CA-125
	Ca-MOv18
	Bcl-2

Preferably, a subset of captured exosomes is labelled with a detectable marker. Preferably, the label is a fluorescent marker. Typically the fluorescent marker is selected from Alexa dyes, Cy dyes, Quantum Dots, FITC, or phycoerythrin. These fluorophores may be attached to appropriate soluble antibodies to label the exosomes captured on the microarray.

In preferred embodiments, two or more fluorophores may be used.

The exosomes captured on the support may be detected by use of a secondary antibody coupled to horse radish peroxidase (HRP) with detection of luminescence using high sensitivity X-ray film. Alternatively, a Biotin-conjugated secondary antibody can be used together with Streptavidin-poly HRP.

The exosomes captured on the support may also be detected by rolling circle amplification from a secondary antibody coupled to a segment of DNA of defined sequence which is amplified with DNA polymerase, with detection involving hybridisationof complementary oligonucleotides coupled to a fluorophore or horse radish peroxidase (Schweitzer B, Roberts S, Grimwade B, Shao W, Wang M, Fu Q, Shu Q, Laroche I, Zhou Z, Tchernev VT, Christiansen J, Velleca M, Kingsmore SF. Multiplexed protein profiling on microarrays by rolling-circle amplification. Nat Biotechnol. 2002 Apr; 20(4): 359-65).

In a preferred embodiment, the sample is pre-treated to remove abundant exosomes derived from platelets.

The array binding profile of a given exosome population is used to generate a specific disease signature.

An array binding profile of a selected subset of exosomes is characteristic of a given disease signature. As a subset of exosomes is derived from a given cell type and forms a characteristic binding pattern on an array of ligands, the detection of a given array binding profile can be used for disease diagnosis or detection.

In a preferred embodiment, the array is analysed using a commercially-available fluorescence scanner, microscopically by staining, histochemically, biochemically or immunologically. Preferably, the array is analysed with a scanner. The array analysis may be either manual or automated. Further analysis may be achieved with confocal microscopy of fluorescently-labelled CD antibodies or by scanning electron microscopy (SEM) of exosomes fixed on the array.

In a third aspect, the present invention provides an assay for detecting a change in a health status of a subject comprising:

carrying out the assay according to the first or second aspects of the present invention on a biological sample of a subject to obtain a first disease signature of the subject;

carrying out the assay according to the first aspect of the present invention on a subsequent biological sample of the subject to obtain a second disease signature of the subject; and

comparing the first surface profile and second profile obtained, wherein a change in a disease signature is indicative of a change in the health status of the subject.

For example, change in the health status of the subject may be remission or relapse. Similarly, the change in status may be from progress of clinical treatment of the patient.

This time-dependent aspect of the present invention is useful in monitoring progression of disease or treatment of a disease in a single subject.

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

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this specification.

In order that the present invention may be more clearly understood, preferred embodiments will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 shows addresses of antibodies in the DotScan™ colorectal cancer antibody microarray. Numbers in the key refer to CD antigens. Other abbreviations are TCR, T-cell receptor; κ, λ, immunoglobulin light chains; sIg, surface immunoglobulin; DCC, deleted in colorectal cancer protein; EGFR, epidermal growth factor receptor; FAP, fibroblast activation protein; HLA-A,B,C, HLA-DR, human leukocyte antigens DR and A,B,C respectively; MICA, MHC class I chain-related protein A; MMP-14, matrix metallopeptidase 14; PIGR, polymeric immunoglobulin receptor; TSP-1, thrombospondin-1; Mabthera, humanised anti-CD20. Alignment dots around the microarrays consist of a mixture of CD44 and CD29 antibodies.

Figure 2 shows dot patterns of exosomes from human LIM1863 colorectal cancer cells, and live intact colorectal cancer cells from a surgical sample captured on a CD antibody microarray (DotScan™, Medsaic Pty Ltd, Sydney, NSW 2006, Australia). Captured exosomes and live cells were stained with an EpCAM antibody labelled with Alexa 647. DotScan microarrays were scanned with a Typhoon 8600 Variable Mode Imager using a 633 nm laser. Black dots indicate saturation of antibodies with exosomes or cells, grey dots are partially saturated. (a) Exosomes (8 µg protein) purified from the culture medium of the CRC cell line LIM1863 and captured on a DotScan microarray. (b) Live intact CRC cells ($4X10^6$) prepared from a surgical sample of CRC (ACP stage A3, moderately differentiated) captured on DotScan™.

Figure 3 shows surface profiles for LIM1863 exosomes and live patient CRC cells. Levels of expression for the indicated CD antigens are shown for exosomes (red/grey) and CRC cells (black). Note that CD47 and HLA-ABC were negative on exosomes, and CD13 and CD26 were negative on CRC cells. Values for intensity (y-axis) were obtained by analysis of the scanned image files in Figure 2 using DotScan software (Medsaic Pty Ltd, Sydney, Australia).

Figure 4 shows surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of LIM1215 CRC visualised with CD9-FITC plus CD63-FITC antibodies.

Figure 5 shows surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of DU145 prostate cancer visualised with EpCAM-Alexa647 antibody.

Figure 6 shows surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of MCF-7 breast cancer visualised with EpCAM-Alexa647 antibody.

Figure 7 shows surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of Me4405 melanoma visualised with CD9-FITC plus CD63-FITC antibodies.

Figure 8 shows surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of ES-2 ovarian cancer visualised with CEACAM5-phycoerythrin antibody.

Figure 9 shows surface profiles of platelet-derived exosomes from the plasma of a normal subject. Exosomes were prepared by the three-step differential centrifugation procedure and captured on the DotScan™ antibody microarray by standard procedures

(Figure 9a). Some of these exosomes were also bound to CD61 antibody Miltenyi beads and purified in a magnetic field prior to capture of the exosomes bound to beads on DotScan™ microarray (Figure 9b). The exosomes on the CD antibody microarrays were visualised with CD9-FITC plus CD63-FITC or CD61-PE antibody.

Figure 10 shows visualisation of CRC exosomes using an antibody conjugated to Biotin and detected with Streptavidin-poly HRP (Thermo Scientific) by chemiluminescence. Images were acquired on a GS-800 Calibrated Densitometer (Biorad).

Mode(s) for Carrying Out the Invention

DEFINITIONS

'Capture' as used herein relates to exosomes or cells involving multiple antigen-antibody interactions where the antibody is immobilized as part of an antibody microarray.

'CD' - cluster of differentiation.

'CD antigens' - surface molecules found on human cells.

'CRC' - colorectal cancer.

'DotScan™ microarray' - a CD antibody microarray that captures exosomes and live cells via antibodies immobilised on a support.

'Profile' as used herein relates to a set of intensities of binding for exosomes or cells on a microarray.

'Signature' as used herein relates to a profile that is characteristic of a particular disease state.

MATERIALS AND METHODS

Methodology for exosome isolation and purification is described in detail in a published paper Mathivanan *et al* (2010), and fluorescence multiplexing in Zhou *et al* (2010).

Mathivanan S, Lim JW, Tauro BJ, Ji H, Moritz RL, Simpson RJ. Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature. Mol Cell Proteomics 2010;9: 197-208.

Zhou J, Belov L, Huang PY, Shin J-S, Solomon MJ, Chapuis PH, Bokey L, Chan C, Clarke C, Clarke SJ, Christopherson RI. Surface antigen profiling of colorectal cancer

using antibody microarrays with fluorescence multiplexing. J Immunol Methods 2010;355: 40-51.

Clinical colorectal cancer sample

A fresh CRC sample was collected from a patient with informed consent. The CRC sample was excised from the macroscopic lesion and the remaining tissue was processed for histological diagnosis. Normal mucosal strips at least 10 cm from the site of the tumor were taken as controls. Pathological examination of the resected specimen followed the Australian Clinico-Pathological (ACP) Staging protocol for CRC. The final diagnosis for the surgical CRC sample was ACP stage A3, moderately differentiated.

Production of a viable CRC cell suspension from solid tissue

A patient tissue sample was collected and stored in Hanks' balanced salt solution (HBSS; Sigma-Aldrich) at 4°C for no longer than 3 hours to maintain cell viability. The sample was cut into 2 mm strips and incubated with occasional gentle mixing for 60 min at 37°C with an equal volume of RPMI 1640 medium containing 2% (v/v) collagenase type 4 (Worthington, Lakewood, NJ, USA) and 0.2% (w/v) deoxyribonuclease I from bovine pancreas (DNAse I; Sigma-Aldrich). The semi-digested tissue was then forced gently through a fine wire mesh using the plunger from a 20 mL syringe; cells were washed through with HBSS. The resulting cell suspension was sequentially passed through 200 µm and 50 µm Filcon filters (BD Biosciences) to remove cell aggregates. Cells were stored frozen at −80°C in heat-inactivated FCS with 10% dimethyl sulphoxide (DMSO). Thawed cell suspensions were treated with 0.1% (w/v) DNAse I. Cell viability was determined using trypan blue exclusion (Sigma-Aldrich, Castle Hill, NSW, Australia).

Preparation of exosomes from human plasma

Blood (10 ml) was collected into EDTA tubes and centrifuged at 400 g for 10 min to remove cells. Plasma was further centrifuged at 1,200 g for 20 min and then 10,000 g to remove platelets and large microparticles, respectively. In some cases this last step (10,000 g centrifugation) was omitted. Plasma was then diluted 1 in 4 in 0.1 mM EDTA/PBS and centrifuged at 100,000 g for 6-16 h at 4°C to pellet exosomes. Pellets were washed once in PBS to remove residual plasma. In some cases affinity purification of CRC-derived exosomes was carried out on CRC patient plasma, using EpCAM-coated MACS beads (Miltenyi). Platelet-derived exosomes were purified from normal control plasma using CD61-coated MACS beads.

DotScan™ CRC antibody microarrays

Microarrays were made by Medsaic Pty Ltd (Eveleigh, NSW, Australia), using FAST slides from Whatman (Sanford, ME, USA). The first section of each microarray consisted of the original 82 antibodies of the DotScan™ Leukaemia microarray (Belov *et al.*, 2003). An additional 40 antibodies, selected on the basis of published prognostic potential for the corresponding surface proteins, were added as a CRC 'satellite' microarray (Figure 1). A third section contained several dilutions of isotype control antibodies. All antibody solutions contained 0.1% bovine serum albumin (BSA) and 0.1% sodium azide.

Cell capture and fluorescence multiplexing on microarrays

Suspensions of CRC cells were applied to moistened DotScan™ CRC microarrays and incubated for 1 h at room temperature. Disaggregated CRC and control intestinal mucosa cell suspensions ($4X10^6$ viable cells in 300 µL of RPMI 1640 containing 10% FCS) were incubated with microarrays for 1 h at 37 °C. Unbound cells were gently washed off in PBS (10–20 s) and bound cells were fixed with 3.7% (w/v) formaldehyde in PBS for 20–30 min. Fixative was thoroughly removed with at least 3 gentle washes in PBS (total 5 min). While still moist, microarrays were blocked for 20 min at room temperature with 200 µL blocking buffer (2% w/v BSA and 2% heat inactivated AB serum in PBS for Alexa 647 and then incubated at room temperature for 30 min with 150 µL of blocking buffer containing Alexa conjugated antibody at an optimal dilution (generally 1/7.5–1/15). Microarrays were gently washed with 3–5 changes of PBS (1–5 min total) and allowed to dry in the dark.

Imaging of binding patterns

Alexa 647-anti-EpCAM 'stained' microarrays were scanned on a Typhoon 8600 Variable Mode Imager (Amersham-Pharmacia, Castle Hill, NSW, Australia) using a 633 nm laser and a 670 BP30 emission filter. The images were scanned using DotScan™ software and the dot fluorescence intensities for each microarray were normalized against the brightest dot set as 100% intensity.

Growth and harvesting of cell lines

Human cancer cells lines were either obtained from ATCC (ES-2, 5637) or were gifts from Prof Rob Sutherland (Garvan Institute of Medical Research, Darlinghurst, Australia; MCF7), Prof Geoff Pietersz (Burnet Institute, Centre for Immunology, Melbourne,

Australia; SK-BR-3), Dr Briony Forbes (University of Adelaide, Adelaide, Australia; LIM1215), Dr Stephen Assinder (University of Sydney, Sydney, Australia; DU145), Dr John Allen (Centenary Institute, University of Sydney, Sydney, Australia; Mel-RM and Me4405), and Dr Helen Rizos (Westmead Millennium Institute, Westmead, Australia; WMM1215L). The cells were cultured in medium recommended by ATCC or the lab of origin.

For the isolation of exosomes, the cells were grown to about 70% confluency, washed twice in PBS and then cultured for 2-3 days in fresh medium containing depleted FBS (FBS subjected to centrifugation at 100,000 g overnight at 4°C to remove bovine exosomes; Invitrogen, Mulgrave, VIC, Australia). The medium was then harvested and the exosomes prepared as described below.

Preparation of exosomes by four-stage centrifugation

Growth medium containing the secreted exosomes was centrifuged at 300 x g for 10 min to remove cells, then at 1500 x g for 10 min to pellet any cell debris and finally at 10,000 x g for 30 min to remove any residual small fragments. The supernatant was filtered through a 0.22 µm filter and concentrated using a stirred cell with a 100K membrane (Merck-Millipore, North Ryde, NSW, Australia) before the exosomes were pelleted in an ultracentrifuge overnight at 100,000 x g and 4°C.

Capture and imaging of exosomes on DotScan™ antibody microarrays

Pelleted exosomes were resuspended in cell culture medium and incubated on a rehydrated DotScan™ array for one hour at 37°C. The exosomes were then fixed onto the membrane by washing the array three times in fixative (3.7% formaldehyde / PBS) followed by a 20 min incubation at room temperature (RT). After washing three times in PBS and blocking for 20 min at RT, the detection antibodies (FITC anti-human CD9, FITC anti-human CD63, phycoerythrin anti-human CEACAM5 and phycoerythrin anti-human NG2; all BioLegend, San Diego, CA, USA) were diluted in blocking solution (1% BSA, 0.1% azide / PBS) and incubated with the array for 20 min at RT and 10 min at 37°C in the dark. Washed and dried arrays were scanned using a Typhoon FLA 9000 Biomolecular Imager (GE HealthCare Bio-Sciences AB, Sweden) to visualize bound exosomes.

EXAMPLES

Example 1

Using the methodology described above taken from Mathivanan et al (2010) and Zhou et al (2010), surface profiles were obtained for a sample of exosomes from the human CRC cell line LIM 1863 and a surgical CRC sample from a patient (ACP stage A3, moderately differentiated). The addresses of the antibodies in the DotScan CRC microarray are shown in Figure 1. The dot pattern obtained for LIM1863 exosomes captured on the DotScan microarray is shown in Figure 2a. The captured exosomes were stained with an EpCAM antibody labelled with the fluorophore Alexa 647. The dot pattern obtained is clear and provides a 'signature' for these exosomes derived from the culture medium of a CRC cell line.

Using the same procedure, live intact CRC cells from a surgical sample from a patient were profiled on a separate DotScan™ antibody microarray. The captured cells were stained with EpCAM antibody-Alexa 647 and the resultant fluorescent scan for captured cells expressing EpCAM is shown in Figure 2b. Again, the dot pattern obtained is clear and the surface profile provides a 'signature' for these cells.

Using DotScan™ software (Medsaic Pty Ltd), the intensities of the dots in Figure 2a and Figure 2b were transformed to bar charts that are compared in Figure 3. The similarity between these two profiles is striking with exosomes and CRC cells expressing in common CD9, CD15, CD29, CD44, CD71, CD49f, CD55, CD63, CD66e, CD104, CD151, CD326, and CD98.

This common signature of 13 CD antigens (surface molecules) shared by exosomes from a CRC cell line, and CRC cells (EpCAM-positive) from a surgical sample from a patient, indicates that exosome signatures from blood or other body fluids, could be used to identify (diagnose) CRC, perhaps at an early stage before metastasis. The quite extensive (13) CD signature in common between exosomes and a clinical CRC sample was unexpected, but provides the basis for the use of (blood) exosomes to diagnose at an early stage a variety of human diseases based solely upon exosome surface profiles.

Example 2

Profiling of exosomes derived from the growth medium of the human LIM1863 CRC cell line (obtained from Prof Richard Simpson, Ludwig Institute, Melbourne) on DotScan™ microarrays, and the similarity with the surface profile of CRC cells from a surgical sample. Results are shown in Figure 3.

Example 3

The following human cancer cell lines were grown in culture, and exosomes were purified from the growth medium:

(a) LIM1215 CRC visualised with CD9-FITC plus CD63-FITC antibodies;

(b) LIM1215 CRC visualised with EpCAM-Alexa647 antibody;

(c) ES-2 ovarian cancer visualised with CD9-FITC plus CD63-FITC antibodies;

(d) ES-2 ovarian cancer visualised with CEACAM5-phycoerythrin antibody;

(e) MCF-7 breast cancer visualised with EpCAM-Alexa647 antibody;

(f) SK-BR-3 breast cancer visualised with CD9-FITC plus CD63-FITC antibodies;

(g) DU145 prostate cancer visualised with CD9-FITC plus CD63-FITC antibodies;

(h) DU145 prostate cancer visualised with EpCAM-Alexa647 antibody;

(i) 5637 bladder cancer visualised with CD9-FITC plus CD63-FITC antibodies;

(j) Me4405 melanoma visualised with CD9-FITC plus CD63-FITC antibodies;

(k) Mel-RM melanoma visualised with CD9-FITC plus CD63-FITC antibodies;

(l) Mel-RM melanoma visualised with NG2-phycoerythrin antibody;

(m) WMM1215L melanoma visualised with CD9-FITC plus CD63-FITC antibodies; and

(n) WMM1215L melanoma visualised with NG2-phycoerythrin antibody.

The findings are exemplified in Figure 4 showing surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of LIM1215 CRC visualised with CD9-FITC plus CD63-FITC antibodies, Figure 5 showing surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of DU145 prostate cancer visualised with EpCAM-Alexa647 antibody, Figure 6 showing surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of MCF-7 breast cancer visualised with EpCAM-Alexa647 antibody, Figure 7 showing surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of Me4405 melanoma visualised with CD9-FITC plus CD63-FITC antibodies, and Figure 8 showing surface profiles of human cancer cell lines grown in culture, and exosomes purified from the growth medium of ES-2 ovarian cancer visualised with CEACAM5-phycoerythrin antibody.

A similarity between the cancer cell line profile and the corresponding exosome profile was found to be quite striking; in general the exosome profile was a subset of the cell (of origin) profile (Figure 4, Figure 5, Figure 6, Figure 7 and Figure 8). These results have allowed the present inventors to recognise disease signatures from the characteristic binding patterns of exosomes from difference cancer cell origin.

Example 4

Surface profiles of platelet-derived exosomes from the plasma of a normal subject. Exosomes were prepared by the four-step differential centrifugation procedure and captured on the DotScan™ antibody microarray by standard procedures (Figure 9a). Some of these exosomes were also bound to CD61 antibody Miltenyi beads and purified in a magnetic field prior to capture of the exosomes bound to beads on DotScan™ microarray (Figure 9b). The exosomes on the CD antibody microarrays were visualised with CD9-FITC/CD63-FITC or CD61-PE antibody. Results are shown in Figure 9.

Example 5

Visualisation of CRC exosomes using a Biotin-conjugated secondary antibody and detected with Streptavidin-poly HRP chemiluminescence..

Exosomes derived from the LIM1215 CRC cell line were incubated on the DotScan CRC microarray. Bound exosomes were detected with a Biotin-conjugated EpCAM antibody followed by Streptavidin-poly HRP. Chemiluminescence images were acquired on a GS-800 Calibrated Densitometer (Biorad). Results are shown in Figure 10.

Example 6

Visualisation of CRC exosomes using a secondary antibody conjugated to DNA for rolling circle amplification.

Briefly, a primer oligonucleotide is conjugated to a specific antibody, which binds to exosomes captured on the microarray. Using a circular DNA template, the primer can then be extended by continuous DNA synthesis, creating a long single-stranded DNA fragment with sequence repeats. Complementary 5' and 3' HRP-modified detection oligonucleotides hybridize to the repeats and multiple oligonucleotides enhance the chemiluminescence reaction.

All oligonucleotides were purchased from IDT, Coralville, IA, USA. The primer was coupled to a human EpCAM antibody (R&D Systems Inc., Minneapolis, MN, USA) using a kit (Antibody-Oligonucleotide All-in-One Conjugation Kit, Solulink, San Diego, USA). CircLigase II ss DNA Ligase (Epicentre, Madison, WI, USA) can be utilized to circularize the template, followed by digestion of remaining linear fragments, according to the manufacturers' instructions. The antibody-primer conjugate can be used in the same way as a detection antibody as described in "Capture and imaging of exosomes on DotScan antibody microarrays" above. The primer is annealed with the circular template for 30 min at 37°C in T7 DNA polymerase buffer, and then the array will be washed twice in PBS, before adding the reaction mix for the DNA synthesis containing T7 DNA polymerase (NEB, Ipswich, MA, USA) and dNTPs in T7 DNA polymerase buffer. After 45 min at 37°C, the array is washed twice in PBS and then incubated for another 30 min at 37°C to allow binding of the detection antibody before two final washes in PBS. The chemiluminescence reaction is carried out as described above.

SUMMARY

The present invention involves the use of DotScan™ to obtain surface profiles of exosomes that carry a disease signature, for example, a specific type of cancer. Initial experiments involved the fractionation of exosomes from the culture medium of human cancer cell lines growing in culture. The purified exosomes were incubated over DotScan™ antibody microarrays and were found to be captured as are live human cells as described above. This observation is surprising, as exosomes are not alive and would not be expected to be captured as it has been found that dead intact cells are not captured. The difference is that exosomes (30 – 100 nm) are much smaller than human cells (~40 µm), and the shearing forces would be greatly reduced on the much smaller exosomes, enabling their capture with many fewer antibody-(CD antigen) interactions. Exosomes captured on particular antibody dots of a microarray are not visible by optical scanning, and can be visualised by post-staining with one or more fluorescently-labelled antibodies, for example, as can be achieved with mixed populations of live cells.

The second important observation was that the extensive surface profile so determined for exosomes corresponded closely with the surface profile of the human cancer cell line from which the exosomes were secreted. It therefore follows that a primary cancer in the human body could secrete exosomes into the blood, and with sensitive detection, a surface profile or disease signature for a particular cancer could be identified before metastasis. Surface profiles obtained for CRC from surgical samples using

fluorescence multiplexing also showed quite close correspondence to the profiles of CRC cell lines and the exosomes purified from the culture medium of these cells.

The detection of surface profiles from the plasma of CRC patients with metastatic disease will typically employ detection methods with much greater sensitivity. Examples include use of a secondary antibody coupled to horse radish peroxidase (HRP) with detection of luminescence using high sensitivity X-ray film. Alternatively, rolling circle amplification (RCA) from a secondary antibody coupled to a segment of DNA of defined sequence, with detection involving hybridisation of complementary oligonucleotides coupled to a fluorophore or HRP could be used.

Exosomes derived from platelets have been profiled (Figure 9, Figure 10). Such exosomes were detected using a fluorescent antibody against CD61. These exosomes have also been purified by binding to CD61 antibody coupled to Miltenyi magnetic beads. As for live cells, exosomes on Miltenyi beads may be captured on DotScan™ and profiled without removal of the beads. Platelet-derived exosomes are the most abundant exosomes found in plasma (George JN, Thoi LL, McManus LM, Reimann TA. Isolation of human platelet membrane microparticles from plasma and serum. Blood 1982;60(4): 834-40), and they probably arise during the differentiation (fragmentation) of megakaryocytes into platelets in the bone marrow. The sensitivity for detection of CRC-derived exosomes in plasma from a patient could be enhanced by removal of highly abundant exosomes prior to capture on DotScan™ microarrays. The removal of platelet-derived exosomes on CD61 antibody-Miltenyi beads would enhance the sensitivity for detection of less abundant subsets of exosomes.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. An assay for detecting a disease comprising:

obtaining exosomes from a biological sample;

providing a support having an array of a plurality of antibodies or binding fragments of antibodies;

capturing exosomes on the support by binding of exosomes to one or more of the plurality of antibodies or binding fragments of antibodies;

treating the captured exosomes with an agent for a selection marker indicative of a cellular origin of an exosome;

detecting a subset of exosomes having the selection marker bound to the support; and

identifying an array binding profile of the subset of exosomes, wherein the array binding profile is indicative of the disease.

2. The assay according to claim 1 wherein the exosomes are obtained from the biological sample by isolation using centrifugation, sedimentation equilibrium on gradients, ultrafiltration, ultracentrifugation, affinity capture, affinity purification, or precipitation.

3. The assay according to claim 3 wherein the exosomes are obtained by centrifugation, filtration and concentration.

4. The assay according to any one of claims 1 to 3 wherein the biological sample is selected from blood, plasma, serum, urine, cerebrospinal fluid, mucous, bone marrow aspirate, purulent discharge, biopsy or surgical excision of solid tissue, tissue culture medium or ascities fluid.

5. The assay according to claim 4 wherein the biological sample is plasma.

6. The assay according to any one of claims 1 to 5 wherein the disease is cancer, cardiovascular disease, diabetes, or an autoimmune disorder.

7. The assay according to claim 6 wherein the cancer is selected from leukaemia, lymphoma, colorectal cancer, melanoma, lung cancer, pancreatic cancer, liver cancer, breast cancer, ovarian cancer, or prostate cancer.

8. The assay according to any one of claims 1 to 7 wherein the antibodies or antibody fragments are specific for cell surface markers being Cluster of Differentiation (CD) antigens.

9. The assay according to claim 8 wherein the array of antibodies includes TCRa/b, TCRg/d, CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD15s, CD16, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD28, CD29, CD31, CD32, CD33, CD34, CD36, CD37, CD38, CD40, CD41, CD42a, CD43, CD44, CD44v6, CD45, CD45RA, CD45RO, CD47, CD49b, CD49c, CD49d, CD49e, CD49f, CD49f, CD51, CD55, CD59, CD58, CD63, CD66a, CD66b, CD69, CD52, CD54, CD55, CD56, CD57, CD60, CD61, CD62L, CD62E, CD62P, CD63, CD64, CD65, CD66c, CD66e, CD71, CD77, CD79a, CD79b, CD80, CD82, CD86, CD87, CD88, CD95, CD98, CD102, CD103, CD104, CD117, CD120a, CD122, CD126, CD128, CD130, CD134, CD135, CD138, CD151, CD154, CD166, CD175s, CD177, CD184, CD200, CD227, CD235a, CD244, CD255, CD261, CD262, CD324, CD326, CD340, Annexin II, beta-catenin, CA 19.9, CA-125, Claudin-4, DCC, EGFR, ErbB3, FAP, Galectin-3, Galectin-4, Galectin-8, HLA-ABC, HLA-DR, HLA-G, FMC7, kappa and lambda light chains, Mabthera, MAGE-1, MICA, MMP-14, PIGR, surface immunoglobulin, TSP-1 and isotype control antibodies.

10. The assay according to any one of claims 1 to 9 wherein the support contains an microarray of 10, or 20, or 30, or 40, or 50, or 60, or 70, or 80, or 90, or 100 or more different antibodies at known locations on the support.

11. The assay according to any one of claims 1 to 10 wherein the agent is an antibody for a cell surface marker indicative of a cancer cell type.

12. The assay according to claim 11 wherein the cell surface marker for colorectal cancer is EpCAM, A33, or CEA; the cell surface marker for prostate cancer is TAG-72, PSMA, or PSCA; the cell surface marker for breast cancer is CA15-3, CA27.29, or HER-2/neu; the cell surface marker for melanoma is MAGE, tyrosinase, or NG-2; and the cell surface marker for ovarian cancer is CA-125, Ca-MOv18 or Bcl-2.

13. The assay according to claim 12 wherein the agent is labelled with a detectable marker.

14. The assay according to claim 13 wherein the label is a fluorescent marker.

15. The assay according to claim 14 wherein the fluorescent marker is selected from Alexa dyes, Cy dyes, Quantum Dots, FITC, or phycoerythrin.

16. The assay according to any one of claims 1 to 15 wherein the array having bound exosomes is analysed by fluorescence scanning, microscopically, histochemically, biochemically or immunologically.

17. The assay according to claim 16 wherein the array is analysed by fluorescence scanning .

18. The assay according to any one of claims 1 to 15 wherein the exosomes captured on the support are detected by use of a secondary antibody coupled to horse radish peroxidase (HRP) with detection of luminescence using high sensitivity X-ray film.

19. The assay according to any one of claims 1 to 12 wherein the exosomes captured on the support are detected by rolling circle amplification from a secondary antibody coupled to a segment of DNA of defined sequence, with detection involving hybridisation of complementary oligonucleotides coupled to a fluorophore or horse radish peroxidase.

20. The assay according to any one of claims 1 to 19 wherein the biological sample is pre-treated to remove abundant exosomes derived from platelets.

21. An assay for detecting a change in a health status of a subject comprising:

carrying out the assay according to any one of claims 1 to 20 on a biological sample from the same subject to obtain a first disease signature of the subject;

carrying out the assay according to the first aspect of the present invention on a subsequent biological sample of the subject to obtain a second disease signature of the subject; and

comparing the first surface profile and second profile obtained, wherein a change in a disease signature is indicative of a change in the health status of the subject.

22. The assay according to claim 21 wherein the change in the health status of the subject is remission, relapse or progress of clinical treatment.

Figure 1

TCR α/β	TCRγ/δ	1a	2	3	4	5
7	8	9	10	11a	11b	11c
13	14	15	16	19	20	21
22	23	24	25	28	29	31
32	33	34	36	37	38	40
41	42a	43	44	45	45RA	45RO
49d	49e	52	54	56	57	60
61	62L	62E	62P	64	65	66c
71	77	79a	79b	80	86	88
95	102	103	117	120a	122	126
128	130	134	135	138	154	235a
HLA-DR	FMC7	κ	λ	sIg		

15s	26	44-v6	47	49f	55	59
63	69	66e (CEA)	82	87	104	151
166	175s	227	244	261	262	324
326 (EpCAM)	340	98	β catenin	Annexin II	CA-125	Claudin-4
DCC	EGFR	FAP	Galectin-3	Galectin-4	Galectin- 8	HLA-A,B,C
MICA	MMP-14	PGR	TSP-1	CA19-9		

mIgG1 500	mIgG1 200	mIgG1 50	mIgG2a 500	mIgG2a 200	mIgG2a 50
mIgG2b 200	mIgG3 500	mIgM500	mIgM 200	mIgM 50	MabThera

Figure 2

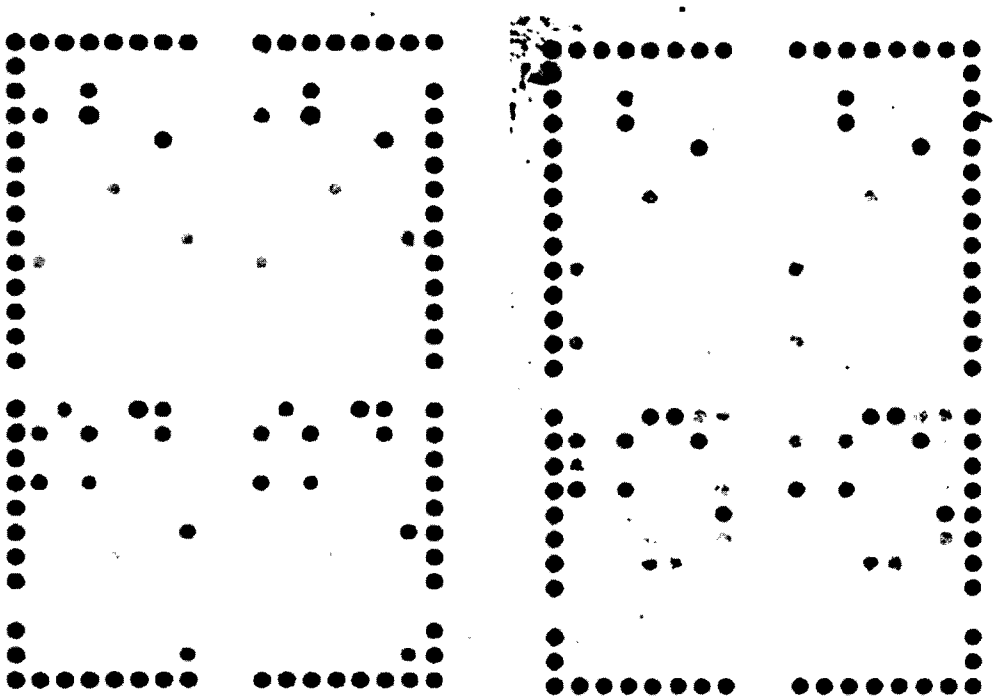

8µg LIM1863 exosome
ACP stage A3
Moderately differentiated

Figure 3

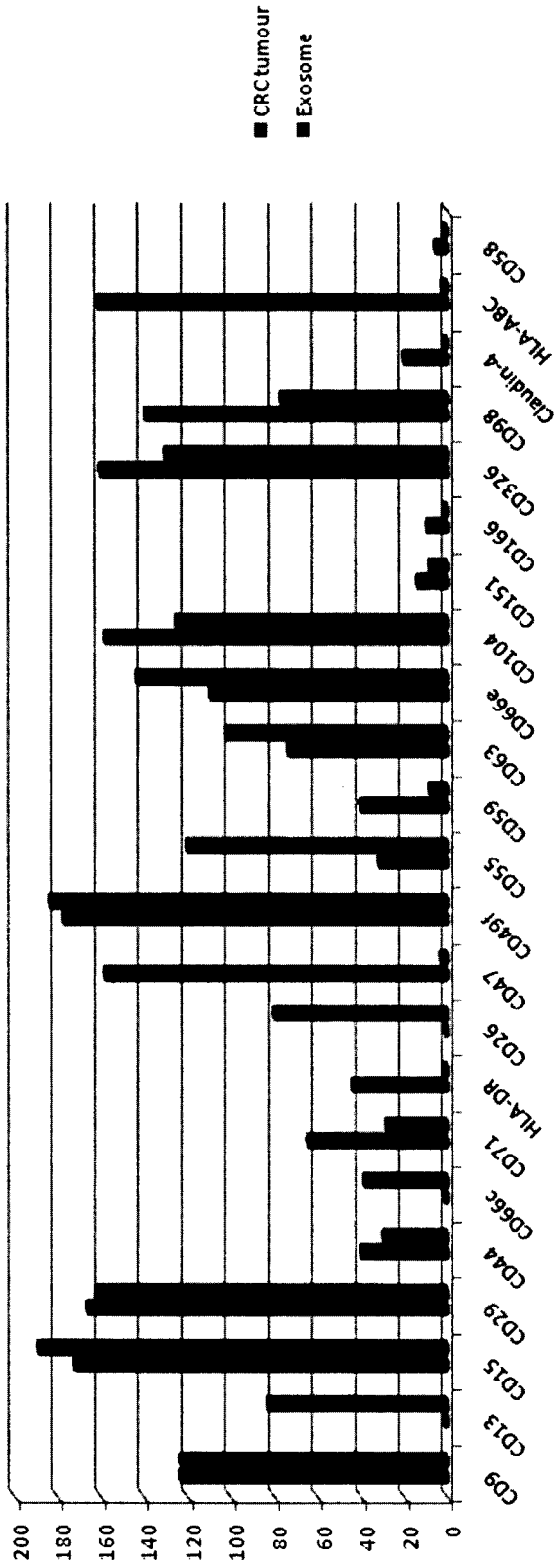

CRC tumour
Exosome
CD58
HLA-ABC
Claudin-4
CD98
CD326
CD166
CD151
CD104
CD66e
CD63
CD59
CD55
CD49f
CD47
CD26
HLA-DR
CD71
CD66c
CD44
CD29
CD15
CD13
CD9
200
180
160
140
120
100
80
60
40
20
0

Figure 4

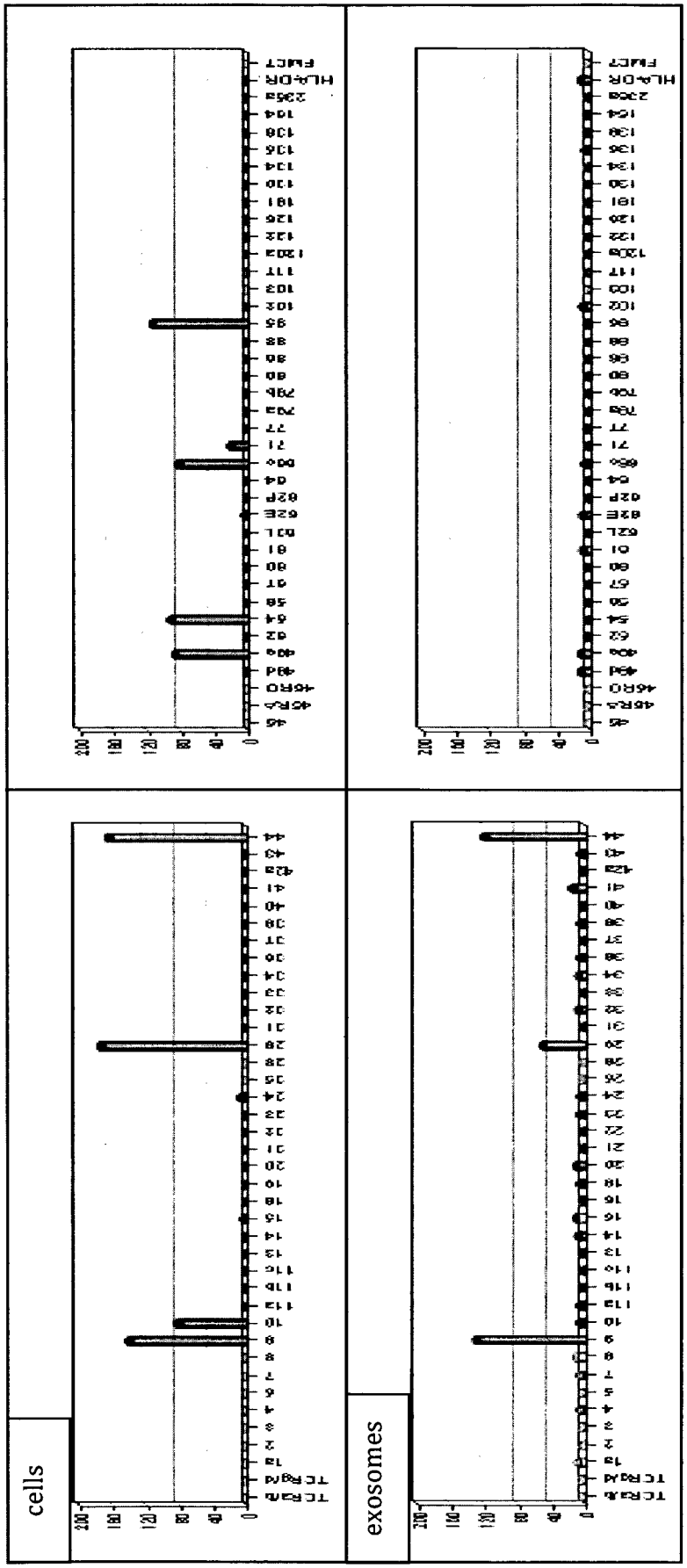


Figure 4 cont'd

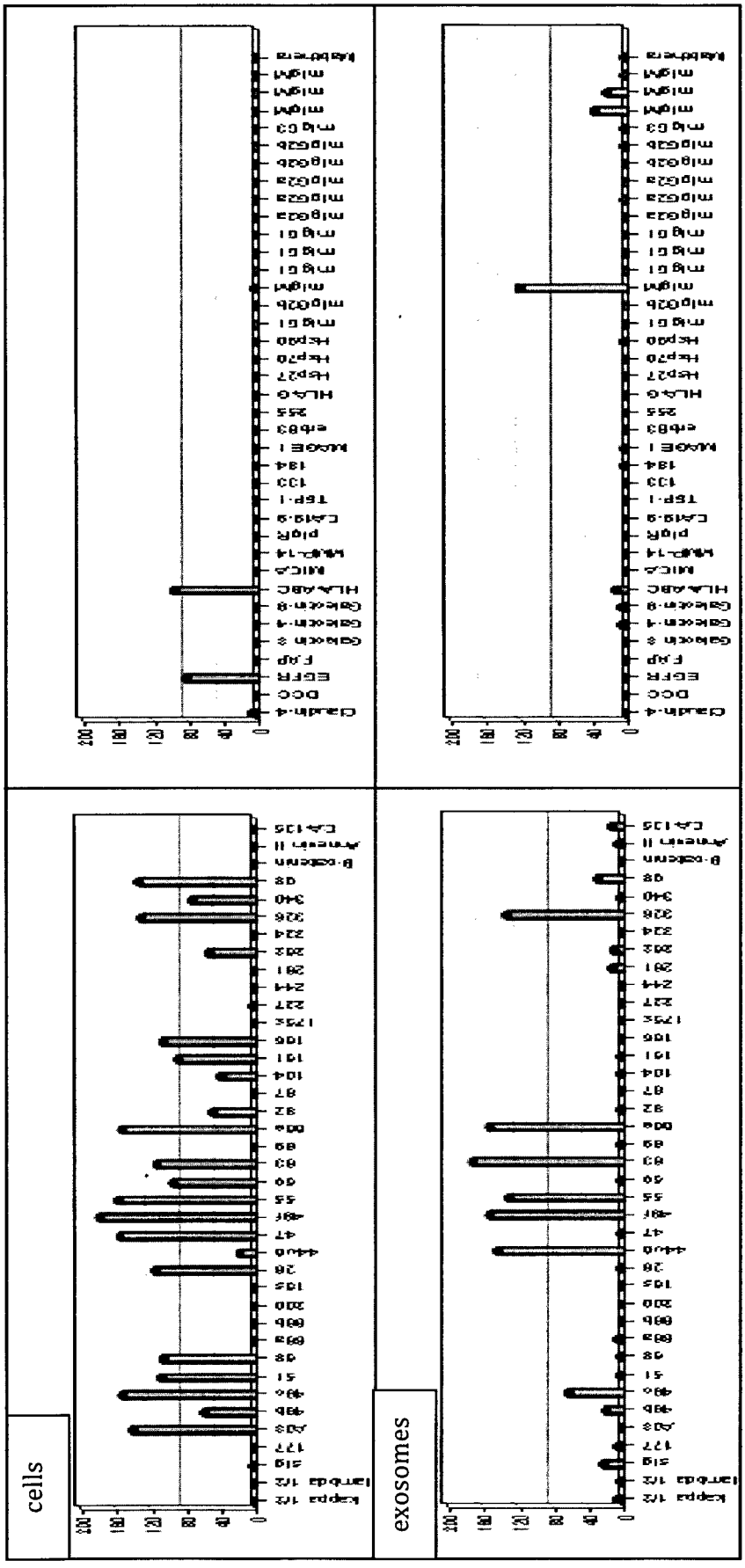


Figure 5

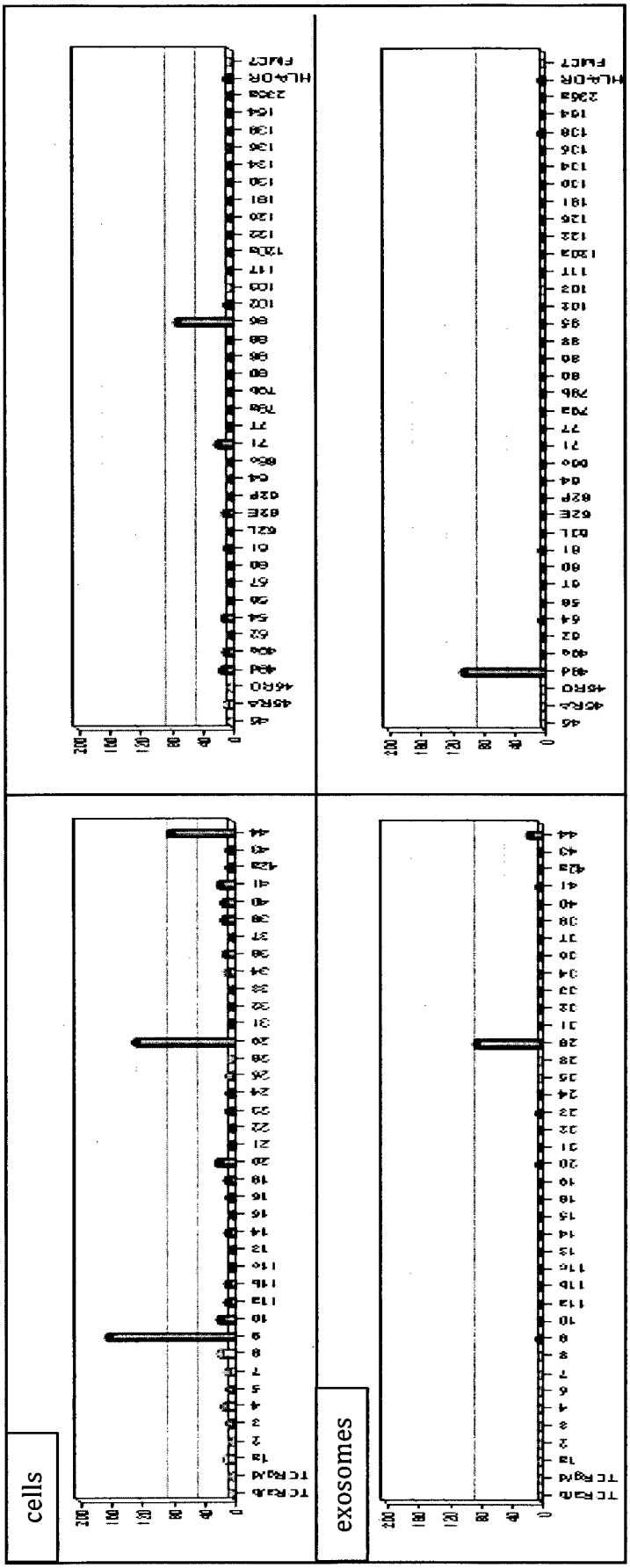


Figure 5 cont'd

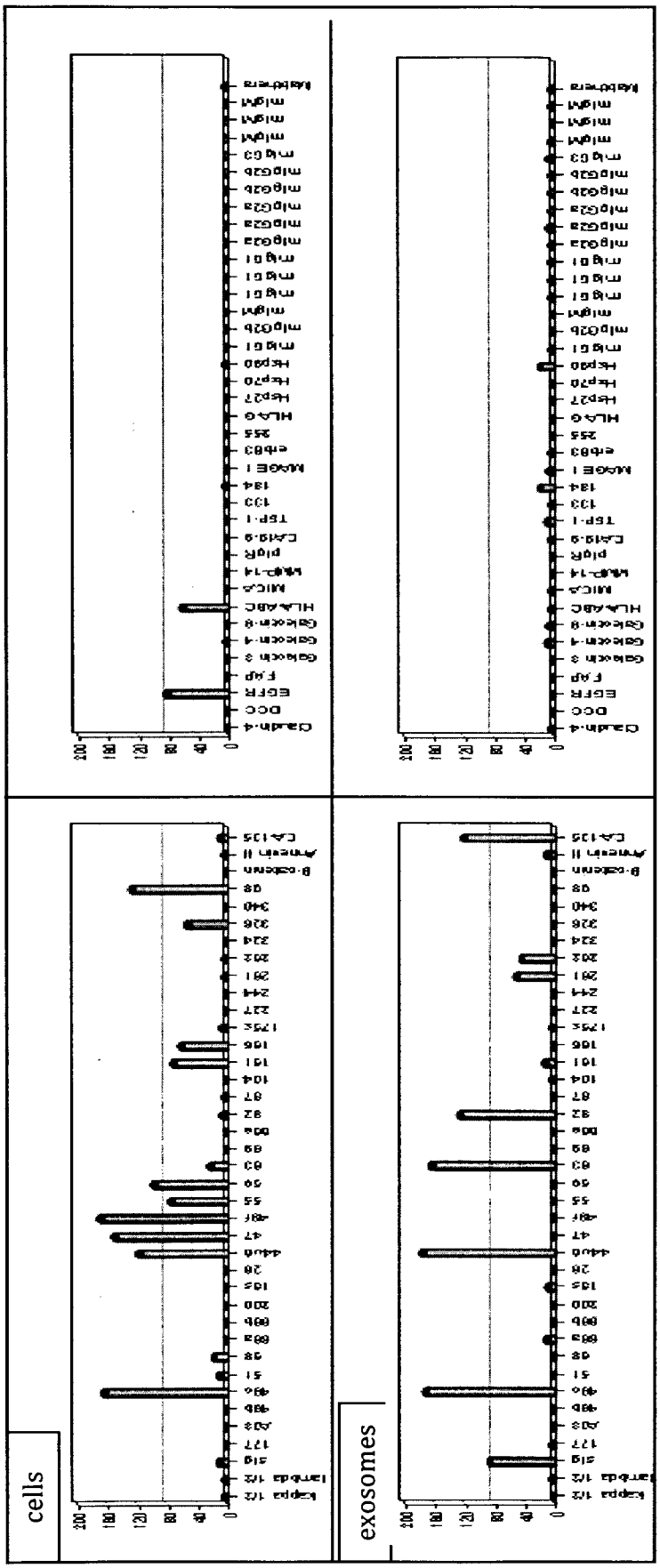


Figure 6

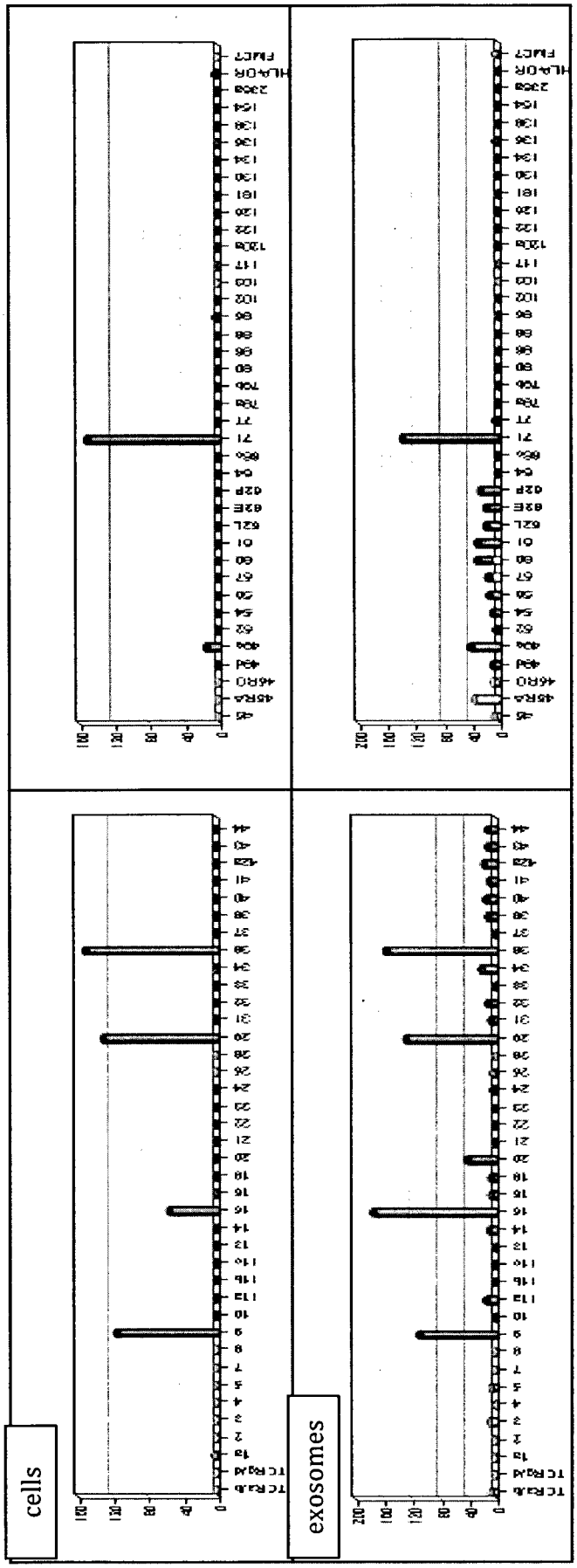
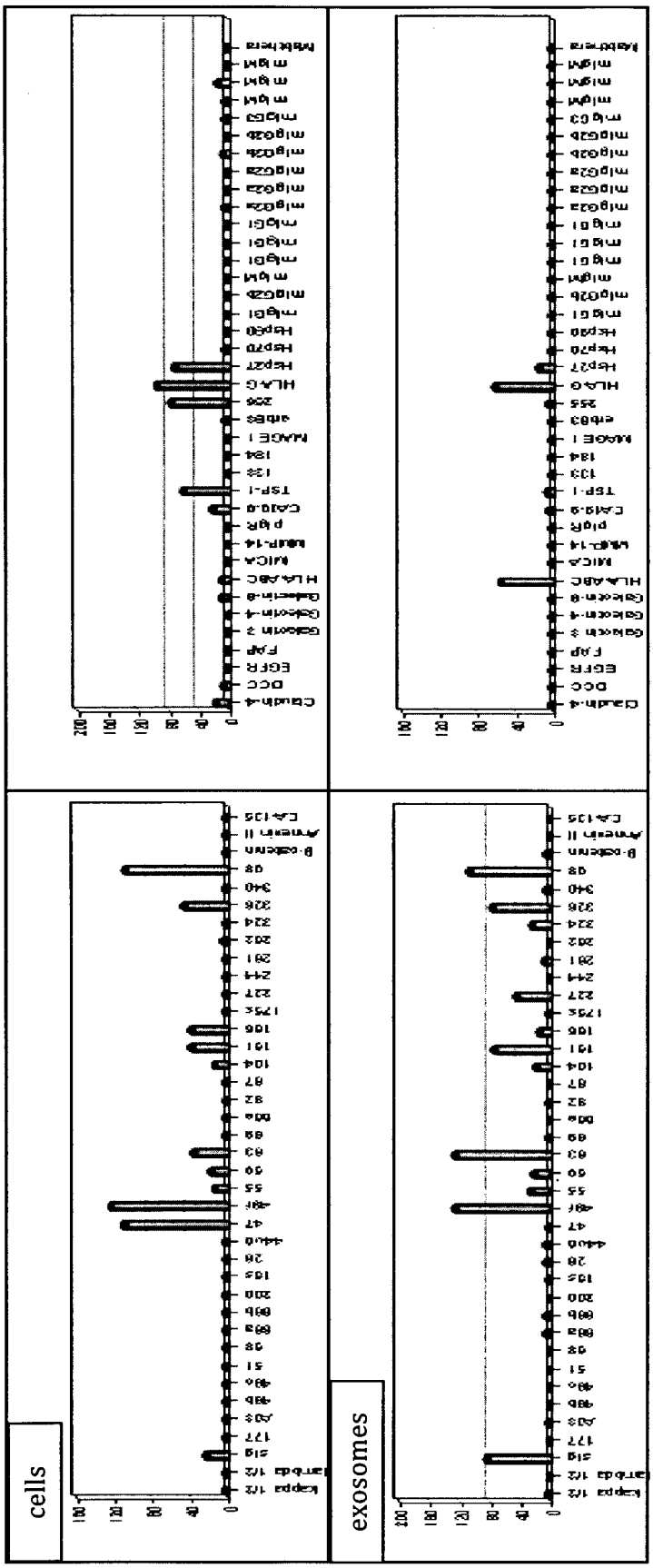


Figure 6 cont'd



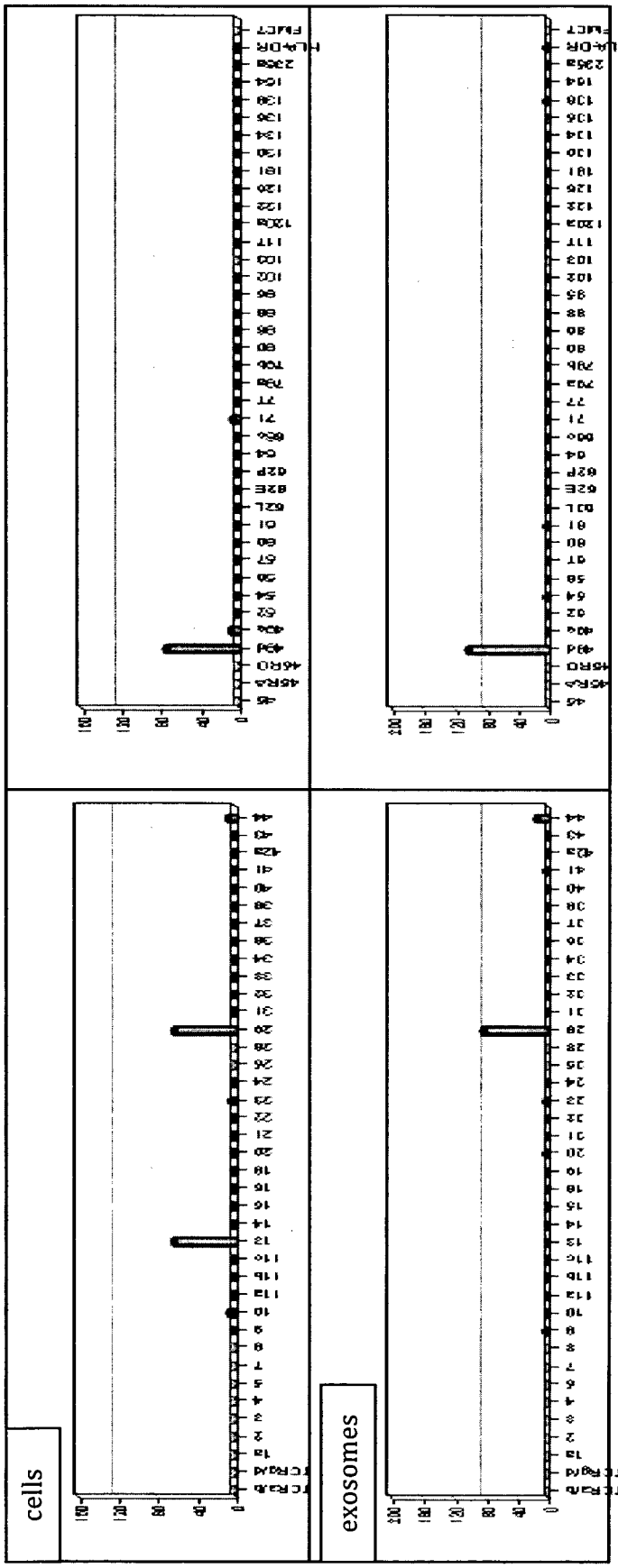


Figure 7

Figure 7 cont'd

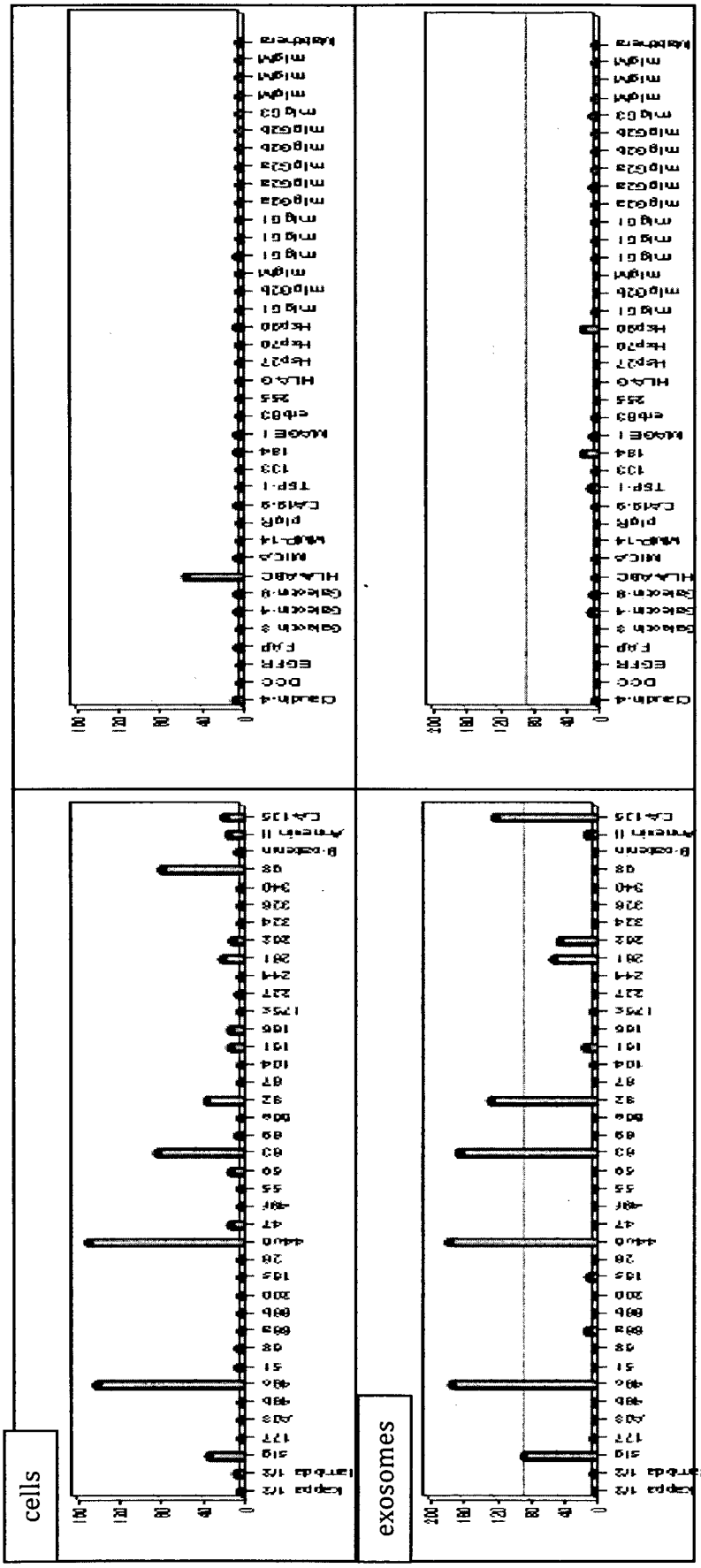


Figure 8

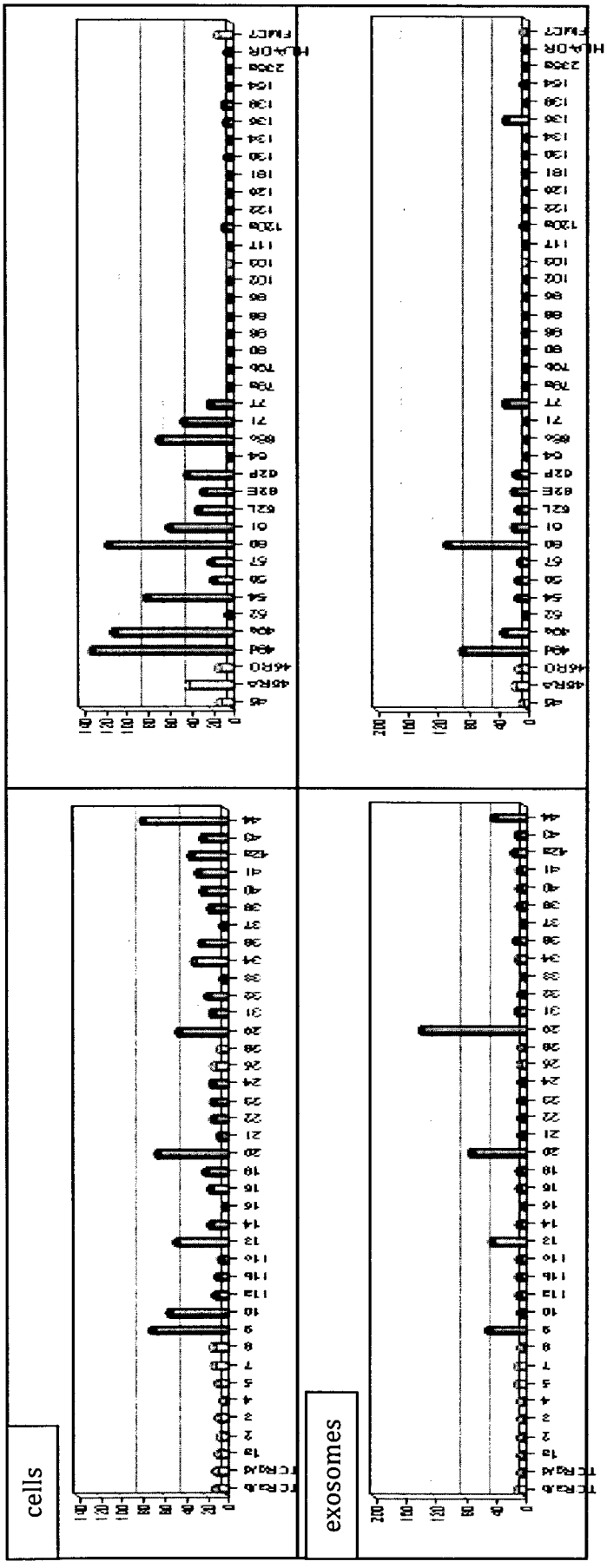


Figure 8 cont'd

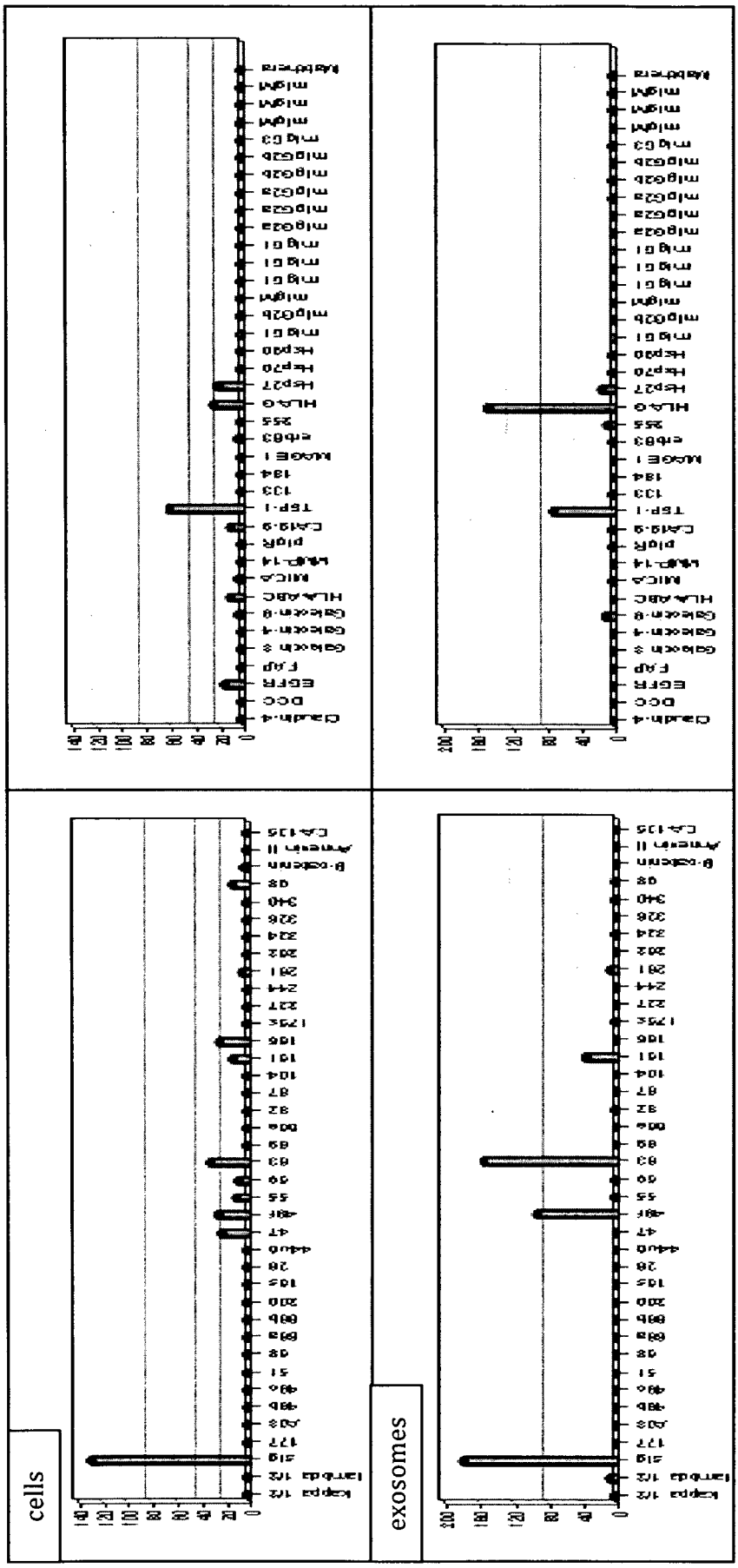


Figure 9

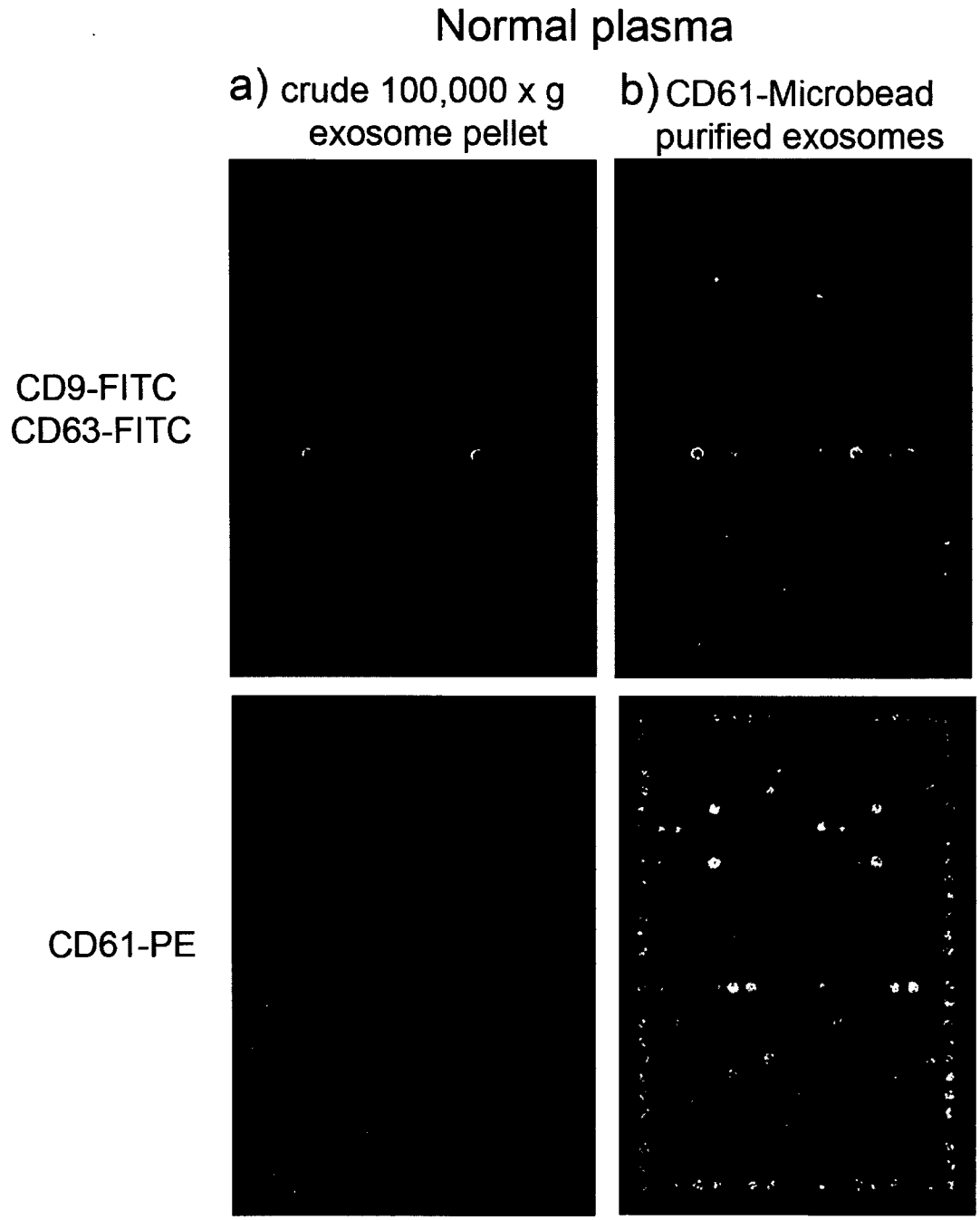
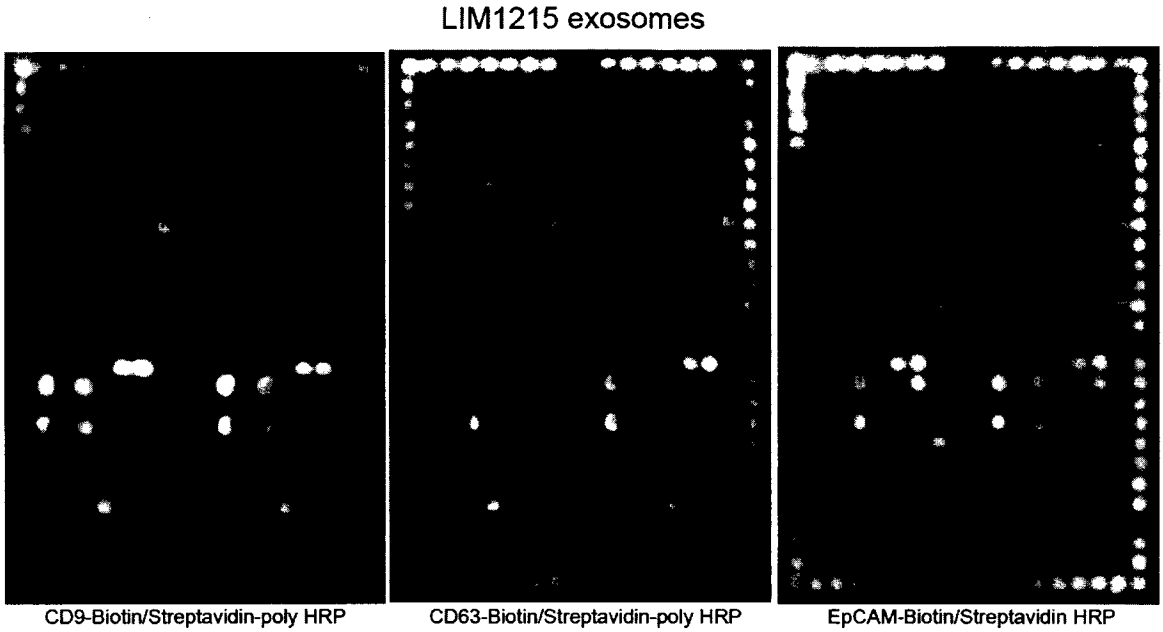

Normal plasma
a) crude 100,000 x g exosome pellet
b) CD61-Microbead purified exosomes
CD9-FITC
CD63-FITC
CD61-PE

Figure 10


LIM1215 exosomes
CD9-Biotin/Streptavidin-poly HRP
CD63-Biotin/Streptavidin-poly HRP
EpCAM-Biotin/Streptavidin HRP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/001300

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/552 (2006.01) ***C40B 30/04*** (2006.01)

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)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC, WPI, MEDLINE, HCAPLUS, BIOSIS, EMBASE, BIOTECHABS: exosome, array, disease and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/056337 A2 (CARIS MPI, INC.) 20 May 2010 See the whole document, particularly the abstract, [0005]-[0012], [0093]-[00100], [00105], [00115]-[00116], [00125]-[00126], [00129]-[00131], [00135], [00144]-[00146], [00174]-[00177], [00180]-[00183], [00259], [00285]-[00288], [00338], [00683]-[00689], [00694], [00704], [00767], [00778], [00788], [00790] table 1, figures 1a-1c, 3 and 6c, example 1 and the claims	1-22
A	MATHIVANAN, S. et al., 'Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature', Molecular & Cellular Proteomics, February 2010, Vol. 9, pages 197-208 See the whole document, particularly the abstract, page 204 second paragraph to page 206 first paragraph and figure 4	
A	WO 2009/092386 A2 (HANSABIOMED OÜ) 30 July 2009 See the whole document, particularly the abstract, claim 1 and examples 4-5	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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"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
5 December 2011	13 DECEMBER 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **JONATHAN KONRAD** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2601

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/001300

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2000/039580 A1 (UNIVERSITY OF SYDNEY) 6 July 2000 See the whole document, particularly page 5 lines 15-22, page 7 lines 3-10, the examples and figures 3a-4 and 7a-8g	
A	WO 2004/077031 A1 (MEDSAIC PTY LTD) 10 September 2004 See the whole document, particularly page 21 lines 12-20 and figures 3, 3A, 7-8 and 10A-10C	
A	BELOV, L. et al., 'Analysis of human leukaemias and lymphomas using extensive immunophenotypes from an antibody microarray', British Journal of Haematology, 2006, Vol. 135, pages 184-197 See the whole document, particularly the abstract and figure 1	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2011/001300

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2010056337	CA	2742324	CA	2743211	EP	2350320
		EP	2358912	GB	2465088	GB	2463401
		US	2010113290	US	7888035	US	2010184046
		US	7897356	US	2010203529	US	2011151460
		US	2011159506	US	2011237450	WO	2010062706
WO	2009092386	AU	2009207927	CA	2713196	EP	2245458
		JP	2011510309	MX	2010008164	US	2009220944
WO	0039580	AU	22703/00	BR	9916449	CA	2356286
		CN	1334922	EP	1151300	HK	1041922
		JP	2002533723	NZ	512472	NZ	525421
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		CN	1748138	EP	1604188	JP	2006519365
		KR	20050105176	NZ	540405	US	2006238846
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX