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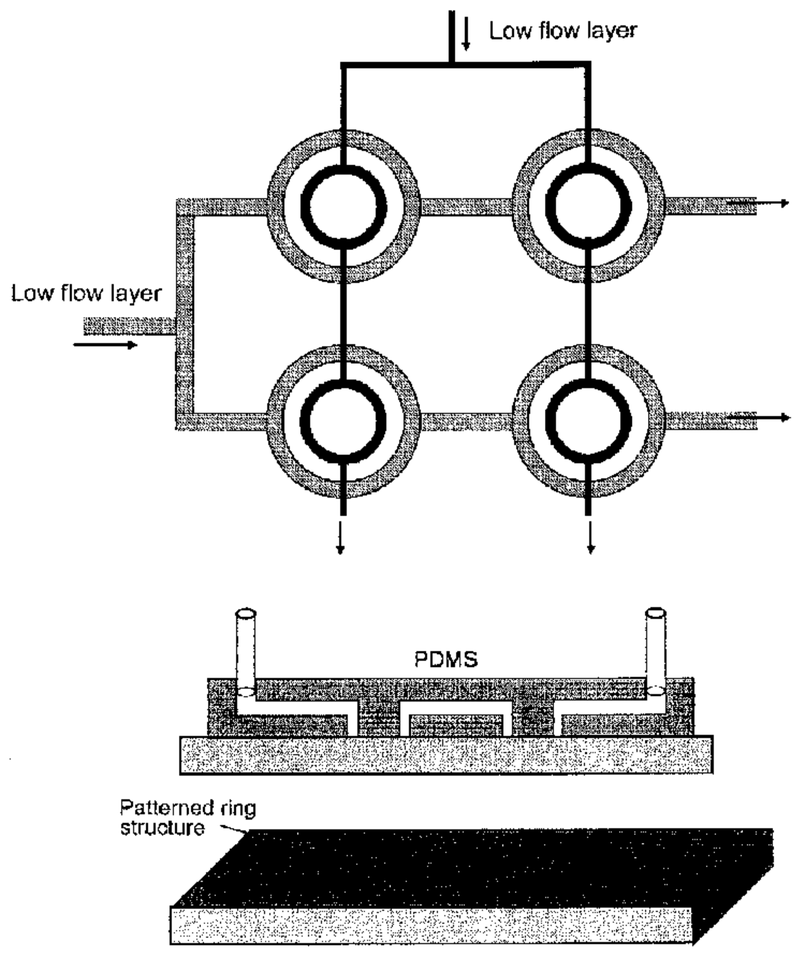

(57) Abrégé/Abstract:
The disclosure provides an array for the multiplexed analysis of a plurality of biomolecules, wherein each well provides a distinct signature of at least one cell or its cellular components. Exemplary arrays comprise a solid substrate comprising a surface and a plurality of primary antibodies attached thereto in a pattern. The plurality of primary antibodies bind different cellular components. Between 3 and 50 different primary antibodies are attached to the solid surface in the pattern. When a polydimethylsiloxane mold comprising a plurality of wells is releasably coupled with the solid substrate, the plurality of wells are oriented to intersect with the pattern of attached primary antibodies and each well is exposed to the pattern of attached primary antibodies. The selective binding of each primary antibody and its target biomolecule is labeled by a detectable secondary antibody. The resultant pattern of detectable secondary antibodies comprises the distinct signature of each well.

ABSTRACT

The disclosure provides an array for the multiplexed analysis of a plurality of biomolecules, wherein each well provides a distinct signature of at least one cell or its cellular components. Exemplary arrays comprise a solid substrate comprising a surface and a plurality of primary antibodies attached thereto in a pattern. The plurality of primary antibodies bind different cellular components. Between 3 and 50 different primary antibodies are attached to the solid surface in the pattern. When a polydimethylsiloxane mold comprising a plurality of wells is releasably coupled with the solid substrate, the plurality of wells are oriented to intersect with the pattern of attached primary antibodies and each well is exposed to the pattern of attached primary antibodies. The selective binding of each primary antibody and its target biomolecule is labeled by a detectable secondary antibody. The resultant pattern of detectable secondary antibodies comprises the distinct signature of each well.

Arrays Comprising a Plurality of Wells, Each Well Releasably Attached to a Patterned Plurality of Different Antibodies

By Rong Fan, Habib Ahmad, and James R. Heath

STATEMENT OF GOVERNMENT GRANT

[0002] The U.S. Government has certain rights in this disclosure pursuant to Grant No. CA119347 awarded by the National Institutes of Health.

TECHNICAL FIELD

[0003] The present disclosure relates to patterning of materials, performance of assays and in particular detection of target molecules in a sample. More specifically, it relates to arrays, devices, methods and systems for detecting a plurality of target molecules in a sample.

BACKGROUND

[0004] Detection of target molecules and in particular of biomarkers has been a challenge in the field of biological molecule analysis. In particular, qualitative and

quantitative detection of biomarkers is often a critical step in several applications ranging from diagnostics to fundamental biology studies.

[0005] In particular, qualitative and quantitative detection of multiple biomarkers has become increasingly important in several applications, such as clinical diagnostic wherein accurate detection of a plurality of biomarkers is desired. More particularly, in some of those applications detection of the multiple biomarkers is directed to identify a biological profile (e.g. proteome and/or metabolome) which can be associated to an indication of interest (e.g. a diagnostic indication).

[0006] Detection of multiple biomarkers is performed by several surface-bound assays known in the art. In those assays capture agents (e.g. primary antibodies) attached to a surface (e.g. a substrate surface) bind the targets of interest in capture agent binding complexes. The capture agent binding complexes are then detected, typically through further binding of the targets with labeling molecules (e.g. secondary antibodies coupled with fluorescent dyes).

[0007] A number of critical parameters is associated with successful execution of a surface-bound assay and include: a) sensitivity of the assay, or minimum concentration, of the biomolecule that can be detected, b) concentration range over which that biomolecule can be detected, c) numbers of different biomolecules that can simultaneously be detected, d) variability from measurement to measurement, e) numbers of different types of biomolecules (e.g. mRNAs, proteins, etc.) that can simultaneously be detected, f) minimum sample size required for the measurement, and g) speed at which the measurement can be performed.

[0008] A number of those assays are typically performed in a microfluidic environment. Microfluidics-based assays are particularly attractive for applications where minimum sample size and short time of execution are desired, because they require only small amounts of biological materials and small amounts of capture agents, materials and associated reagents.

SUMMARY

[0009] Provided herein, are devices, methods and systems for detection of a plurality of targets that allow a fast and sensitive detection of a large number of multiple targets in a sample and/or provide results in an easily readable fashion.

[0010] According to a first aspect, an array for detecting at least one target in a sample, and in particular a plurality of targets in a sample is disclosed. The array comprises, at least one capture agent or component thereof attached to a substrate, the at least one capture agent capable of specifically binding the at least one target to form a capture agent target binding complex. In the array, the at least one capture agent or component thereof arranged on the array so that capture agent target binding complexes are detectable along substantially parallel lines forming a barcoded pattern. The at least one target can be a plurality of targets, the capture agent can be a plurality of capture agents, with each capture agent of the plurality of capture agents bindingly distinguishable and positionally distinguishable from another and capable of specifically binding each target of the plurality of targets to form a capture agent target binding complex.

[0010.1] According to one embodiment of the first aspect, there is provided an array, comprising: a solid substrate comprising a surface and a plurality of primary antibodies attached to the surface in a pattern, wherein the plurality of primary antibodies attached in the pattern bind different cellular components, and wherein between 3 and 50 different primary antibodies of the plurality of primary antibodies are attached to the solid surface in the pattern; a polydimethylsiloxane (PDMS) mold comprising a plurality of wells releasably coupled with the solid substrate, wherein the plurality of wells are oriented to intersect with the pattern of attached primary antibodies, and wherein each well is exposed to the pattern of attached primary antibodies.

[0011] According to a second aspect, a microfluidic device is disclosed that comprises an array according to the present disclosure.

[0012] According to a third aspect, a system for the detection of a plurality of targets in a sample is disclosed. The system comprises an array disclosed herein and a device for detecting the barcoded pattern on the array.

[0013] According to a fourth aspect, a method for detecting a plurality of targets in a sample is disclosed. The method comprises: contacting said sample with an array

herein disclosed for a time and under conditions to allow binding of said plurality of targets with said plurality of capture agents to form capture agent target binding complexes; and detecting said capture agent target binding complexes.

[0014] According to a fifth aspect, a substrate is disclosed, the substrate for detecting a target, and in particular a plurality of targets, in a sample. The substrate is configured to allow attachment of the target on the substrate so that said target is detectable along substantially parallel lines forming a barcoded pattern.

[0015] According to a sixth aspect, a microfluidic device is disclosed that comprises a substrate according to the present disclosure.

[0016] According to a seventh aspect, a system for the detection of a target, and in particular a plurality of targets, in a sample is disclosed. The system comprises a substrate disclosed herein and a device for detecting the barcoded pattern on the substrate.

[0017] According to an eighth aspect, a method for detecting a target and, in particular, a plurality of targets, in a sample is disclosed. The method comprises: contacting said sample with a substrate herein disclosed for a time and under conditions to allow binding of said target with said substrate; and detecting said target attached to the substrate.

[0018] According to a ninth aspect, a method to attach a molecule on a microfluidic support along a predetermined microfluidic pattern is disclosed. The method comprises: providing a mold comprising microfluidic channels, the microfluidic channels having an inlet and an outlet, the outlets of the channels configured to form part of the predetermined pattern, providing the support, said support suitable to be coupled with the mold, coupling the mold with the support, providing the molecule in the microfluidic channels for a time and under conditions to allow attachment of the molecule on the support; and decoupling the mold from the support.

[0019] According to a tenth aspect a system to attach a molecule on a microfluidic support along a predetermined microfluidic pattern is disclosed. The system comprises: a mold comprising microfluidic channels, the microfluidic channels having an inlet and an outlet, the outlets of the channels configured to form part of the predetermined pattern, and a support suitable to be coupled with the mold.

[0019.1] According to an eleventh aspect, there is provided a composition comprising: a solid substrate comprising a surface and a plurality of primary antibodies attached to the surface in a pattern, wherein the plurality of primary antibodies attached in the pattern bind different cellular components, and wherein between 3 and 50 different primary antibodies of the plurality of primary antibodies are attached to the solid surface in the pattern; and a polydimethylsiloxane (PDMS) mold that releasably couples with the solid substrate, the mold having a plurality of channels into which a plurality of primary antibodies are introduced and exposed to the solid substrate surface whereby at least a portion of the plurality of primary antibodies are attached to the solid substrate in the pattern when the mold is uncoupled from the solid substrate.

[0020] The methods and systems for attaching a molecule on a support on a microfluidic support along a predetermined microfluidic pattern can be used to manufacture an array and/or a substrate according to the present disclosure, in embodiments wherein the pattern is composed of substantially parallel lines forming a barcoded pattern.

[0021] Arrays, substrates, devices, methods and systems herein disclosed provide information in a one-dimensional fashion which can be detected with a single line scan (line profile) perpendicular to the strip direction to complete reading all information. In this way, is possible to obtain all the necessary information without need of a precise move of a reader (e.g. a scan head) which is instead required in imaging 2D array of the art. This feature can allow, in certain embodiments, the reading of barcode DNA array as easy as scanning the product barcode in supermarket.

[0022] Arrays, substrates, devices, methods and systems herein disclosed can provide an increased concentration of capture agents suitable to bind the target and, therefore, increased detection sensitivity (e.g. up to 0.1 picomolar) when compared to prior art techniques.

[0023] Arrays, substrates, devices, methods and systems herein disclosed can allow an increased number of locations for a specific capture agent on a surface (herein also indicated as spots). Accordingly, the arrays, devices methods and systems herein disclosed also allow detection of an increased number of targets or target related parameters (e.g. 50 targets or more) in comparison with the ones detectable with prior art techniques.

[0024] Arrays, substrates, devices, methods and systems herein disclosed are also compatible with microfluidic fabrication techniques, since the spots can be placed in positions that can be defined not only with respect to each other, but also with respect to microfluidic channels and/or other structure on the surface.

[0025] Arrays, substrates, devices, methods and systems herein disclosed allow providing high density capture agents on a substrate, with a decreased level of impurities in comparison to prior art techniques.

[0026] Arrays, substrates, devices, methods and systems herein disclosed also allow detection of a larger number of biomarkers in a reduced time (e.g. about 9 minutes) with respect prior art techniques, in particular in embodiments wherein the array is integrated with microfluidics.

[0027] Arrays, substrates, devices, methods and systems herein disclosed allow detection from a sample reduced in size (e.g. 500 nano liter per barcode and/or protein sections from only one cell) in comparison to the samples analyzed with prior art techniques, in particular in embodiments wherein the array is integrated with microfluidics

[0028] Additionally, since the arrays, substrates, devices, systems and methods herein disclosed allow detection of multiple biomarkers within the same environment, and in particular the same microfluidics environment, using a single assay technique, the relative error associated with measurements of different biomarkers from the same sample is minimized.

[0029] The arrays, substrates, devices, methods and systems herein disclosed are applicable to performance of the detection of various types of target molecules that can bind to immobilized capture agents. Suitable target molecules include, but are not limited to, proteins, peptide, polypeptide, ligands, metabolites, nucleic acid, polynucleotide, carbohydrate, amino acid, hormone, steroid, vitamin, drug, drug candidate, virus, bacteria, cells, microorganisms, fragments, portions, components, products, epitopes of virus, bacteria, microorganisms and/or cells, polysaccharides, lipids, lipopolysaccharides, glycoproteins, cell surface markers, receptors, immunoglobulins, albumin, hemoglobin, coagulation factors, volatile gas molecules, particles, metal ions and the antibodies to any of the above substrates.

[0030] The arrays, substrates, devices, methods and systems herein disclosed are applicable to performance of assays including diagnostic assays, environmental monitoring assays, heath/drug response monitoring assays and assays performed for research purposes. Exemplary assays that can be performed include but are not limited to detection of cancer biomarkers (e.g. prostate cancer antigen (PSA), and human chorionic gonadotropin (hCG)), detection of liver toxicity biomarker C-reactive protein (CRP) and plasminogen, detection of immuno complement proteins like C3, detection of cytokines such as interferon gamma (IFN-gamma), tumor necrosis factor alpha (TNF-a), interleukin 1 alpha (IL-1 alpha), interleukin 1 beta (IL-1 beta), transforming growth factor beta (TGF beta), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), granulocyte macrophage colony stimulating factor (GM-CSF) etc, detection of chemokines: CCL2 (also called monocyte

chemoattractive protein -1, MCP-1), and demonstration of detection of complementary DNA molecules.

[0031] Additional applications of the arrays, substrates, devices, methods and systems herein disclosed include but are not limited to use the patterning technique to make a barcode array of gas selective polymers as gas sensors; patterning liquid crystal film for LCD, and assemble magnetic particle array using DNA-iron oxide nanoparticle conjugates (just like the antibody-DNA conjugates) for magnetic barcodes (product ID).

[0032] The details of one or more embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the present disclosure and, together with the detailed description, serve to explain the principles and implementations of the disclosure.

[0034] **Figure 1** shows a schematic representation of the method to manufacture a reversed or inversed phase barcoded array according to an embodiment herein disclosed. Panel A shows a barcode pattern including a number of stripes or bars corresponding to immobilized serum molecules from various patients. Panel B shows a barcode pattern wherein the bars are provided by microfluidic channels formed on top of the array of Panel A.

[0035] **Figure 2** shows a schematic representation of a method and equipment to detect a barcoded array according to an embodiment herein disclosed.

[0036] **Figure 3** shows a schematic representation of a comparative detection of a spot array and of a barcoded array according to an embodiment herein disclosed.

[0037] **Figure 4** shows a schematic representation of an exemplary passage in the pattering methods and systems for producing a barcoded array according to an embodiment herein disclosed.

[0038] **Figure 5** shows a schematic representation of the method to manufacture a patterned substrate, using a multi-layer fluidic channel device according to an embodiment herein disclosed.

[0039] **Figure 6** shows an exemplary array according to an embodiment herein disclosed.

[0040] **Figure 7** shows two images corresponding to an exemplary molecular detection using a 20μm barcoded array (panel A) and a 2μm barcoded array (panel B) according to an embodiment herein disclosed.

[0041] **Figure 8** shows a computer-aided design of the barcode array according to an embodiment herein disclosed and a related use. The panel on the bottom shows thirteen different capture agents (A-M) flowed into a set of parallel fluidic channels each channel having a width of 20μm. The top panel is the enlarged view of a selected area.

[0042] **Figure 9** shows the execution of multiple assays in twelve isolated wells using a barcoded array according to an embodiment herein disclosed. Panel A shows a barcoded array manufactured on a supporting glass slide. Panel B shows protein detection from the array of Panel A visualized by fluorescence imaging.

[0043] **Figure 10** shows a schematic representation of the method to detect target molecules using a group of distinct capture agents that are directly patterned into a barcoded array according to an embodiment herein disclosed.

[0044] **Figure 11** shows a schematic representation of the method to detect target molecules using a group of distinct capture agents that are immobilized onto the specific location of a pre-determined barcoded array via a set of linkers according to an embodiment herein disclosed. This is exemplified by the detection of target antigen using captured antibodies encoded by a set of complementary DNA molecules.

[0045] **Figure 12** shows a schematic representation of the method to vary the loading of capture agents and consequently the sensitivity and concentration range for the detection of targets using a barcoded array according to an embodiment herein disclosed.

[0046] **Figure 13** shows a schematic representation of a method to manufacture a device including a barcoded array according to an embodiment herein disclosed and a related use.

[0047] **Figure 14** shows an exemplary detection of protein targets according to an embodiment herein disclosed.

[0048] **Figure 15** shows an exemplary protein detection using a barcoded array according to an embodiment herein disclosed and comparison with the protein detection using a conventional pin-spotted array.

[0049] **Figure 16** shows an exemplary detection of target polynucleotides according to an embodiment herein disclosed.

[0050] **Figure 17** shows an exemplary multiplexed detection of multiple protein targets in a sample using a barcoded array according to an embodiment herein disclosed.

[0051] **Figure 18** shows an exemplary detection of a protein target according to an embodiment herein disclosed.

[0052] **Figure 19** shows an exemplary detection of multiple targets in a sample using a barcoded array according to an embodiment herein disclosed, and its comparison to the conventional array.

[0053] **Figure 20** shows a schematic representation of a method and system to detect targets according to an embodiment herein disclosed.

[0054] **Figure 21** shows an exemplary detection of a target in a series of samples according to an embodiment herein disclosed.

[0055] **Figure 22** an exemplary detection of a protein target in a series of samples over a large concentration range according to an embodiment herein disclosed.

[0056] **Figure 23** shows an exemplary detection of a biological profile according to an embodiment herein disclosed.

[0057] **Figure 24** shows an exemplary detection of a target at different concentration ranges according to an embodiment herein disclosed.

[0058] **Figure 25** shows data concerning the exemplary detection of a biological profile of Figure 20A.

[0059] **Figure 26** shows detection of a protein profiling in a time span according to an embodiment herein disclosed.

[0060] **Figure 27** shows an exemplary quantitative detection according to an embodiment herein disclosed.

[0061] **Figure 28** shows an exemplary elaboration of biological profiles detected according to the exemplary embodiment illustrated in Figure 21(A) embodiment herein disclosed.

[0062] **Figure 29** shows an exemplary detection of target proteins in a drop of fresh human blood.

[0063] **Figure 30** shows an exemplary detection of a human plasma proteome according to an embodiment herein disclosed.

[0064] **Figure 31** shows a schematic representation of the method to manufacture a patterned substrate according to an embodiment herein disclosed.

DETAILED DESCRIPTION

[0065] Arrays, substrates, devices, methods and systems for detecting a target, and in particular, a plurality of target molecules in a sample are herein disclosed.

[0066] The term "detect" or "detection" as used herein indicates the determination of the existence, presence or fact of a target or signal in a limited portion of space, including but not limited to a sample, a reaction mixture, a molecular complex and a substrate. A detection is "quantitative" when it refers, relates to, or involves the measurement of quantity or amount of the target or signal (also referred as quantitation), which includes but is not limited to any analysis designed to determine

the amounts or proportions of the target or signal. A detection is "qualitative" when it refers, relates to, or involves identification of a quality or kind of the target or signal in terms of relative abundance to another target or signal, which is not quantified.

[0067] The term "target" or "target molecule" as used herein indicates an analyte of interest. The term "analyte" refers to a substance, compound or component whose presence or absence in a sample has to be detected. Analytes include but are not limited to biomolecules and in particular biomarkers. The term "biomolecule" as used herein indicates a substance compound or component associated to a biological environment including but not limited to sugars, amino acids, peptides proteins, oligonucleotides, polynucleotides, polypeptides, organic molecules, haptens, epitopes, biological cells, parts of biological cells, vitamins, hormones and the like. The term "biomarker" indicates a biomolecule that is associated with a specific state of a biological environment including but not limited to a phase of cellular cycle, health and disease state. The presence, absence, reduction, upregulation of the biomarker is associated with and is indicative of a particular state. Exemplary biomarkers include breast cancer marker HER2, ovarian cancer marker CA125, and heart disease marker thrombin.

[0068] The term "sample" as used herein indicates a limited quantity of something that is indicative of a larger quantity of that something, including but not limited to fluids from a biological environment, specimen, cultures, tissues, commercial recombinant proteins, synthetic compounds or portions thereof.

[0069] In some embodiments, arrays, substrates, methods and systems are herein disclosed for the detection of multiple, distinct targets, such as biomolecules, or a panel of biomarkers. In the arrays, substrates, devices methods and systems herein disclosed each target is detected in a particular location on a surface, and the collection of detected biomolecules forms a pattern, or a barcode. In particular, the arrays, devices, methods and systems herein disclosed can apply to the detection of the biomarker panel within a micro fluidics environment.

[0070] In some embodiments of the arrays, substrates devices methods and systems herein disclosed a plurality of capture agents attached to a substrate.

[0071] The wording "capture agents" as used herein indicate a molecule capable of specific binding with a predetermined binding. Exemplary capture agents include but are not limited to polynucleotides and proteins, and in particular antibodies.

[0072] The term "polynucleotide" as used herein indicates an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof. The term "nucleotide" refers to any of several compounds that consist of a ribose or deoxyribose sugar, joined to a purine or pyrimidine base and to a phosphate group and that are the basic structural units of nucleic acids. The term "nucleoside" refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. The term "nucleotide analog" or "nucleoside analog" refers respectively to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or a with a different functional group. Accordingly, the term polynucleotide includes nucleic acids of any length DNA RNA analogs and fragments thereof. A polynucleotide of three or more nucleotides is also called nuclcotidic oligomcrs or oligonuclcotidc.

[0073] The term "polypeptide" as used herein indicates an organic polymer composed of two or more amino acid monomers and/or analogs thereof. The term "polypeptide" includes amino acid polymers of any length including full length proteins and peptides, as well as analogs and fragments thereof. A polypeptide of three or more amino acids is also called a protein oligomer or oligopeptide. As used herein the term "amino acid", "amino acidic monomer", or "amino acid residue" refers to any of the twenty naturally occurring amino acids including synthetic amino acids with unnatural side chains and including both D and L optical isomers. The term "amino acid analog" refers to an amino acid in which one or more individual atoms have been replaced, either with a different atom, isotope, or with a different functional group but is otherwise identical to its natural amino acid analog.

[0074] The term "protein" as used herein indicates a polypeptide with a particular secondary and tertiary structure that can participate in, but not limited to, interactions with other biomolecules including other proteins, DNA, RNA, lipids, metabolites, hormones, chemokines, and small molecules.

[0075] The term "antibody" as used herein refers to a protein that is produced by activated B cells after stimulation by an antigen and binds specifically to the antigen promoting an immune response in biological systems and that typically consists of four subunits including two heavy chains and two light chains. The term antibody includes natural and synthetic antibodies, including but not limited to monoclonal antibodies, polyclonal antibodies or fragments thereof. Exemplary antibodies include IgA, IgD, IgG1, IgG2, IgG3, IgM and the like. Exemplary fragments include Fab Fv, Fab' F(ab')2 and the like. A monoclonal antibody is an antibody that specifically binds to and is thereby defined as complementary to a single particular spatial and polar organization of another biomolecule which is termed an "epitope". A polyclonal antibody refers to a mixture of monoclonal antibodies with each monoclonal antibody binding to a different antigenic epitope. Antibodies can be prepared by techniques that are well known in the art, such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybridoma cell lines and collecting the secreted protein (monoclonal).

[0076] The wording "specific" "specifically" or specificity" as used herein with reference to the binding of a molecule to another refers to the recognition, contact and formation of a stable complex between the molecule and the another, together with substantially less to no recognition, contact and formation of a stable complex between each of the molecule and the another with other molecules.. Exemplary specific bindings are antibody-antigen interaction, cellular receptor-ligand interactions, polynucleotide hybridization, enzyme substrate interactions etc. The term "specific" as used herein with reference to a molecular component of a complex, refers to the unique association of that component to the specific complex which the component is part of. The term "specific" as used herein with reference to a sequence of a polynucleotide refers to the unique association of the sequence with a single polynucleotide which is the complementary sequence.

[0077] The term "attach" or "attached" as used herein, refers to connecting or uniting by a bond, link, force or tie in order to keep two or more components together, which encompasses either direct or indirect attachment such that for example where a first molecule is directly bound to a second molecule or material, and the

embodiments wherein one or more intermediate molecules are disposed between the first molecule and the second molecule or material.

[0078] The term "substrate" as used herein indicates an underlying support or substratum. Exemplary substrates include solid substrates, such as glass plates, microtiter well plates, magnetic beads, silicon wafers and additional substrates identifiable by a skilled person upon reading of the present disclosure.

[0079] In some embodiments, the capture agents used in the arrays, devices, methods and systems herein disclosed can be either directly deposited onto substrate to form an array or immobilized by linker molecules that are pre-deposited onto substrate and capable to specific binding to capture agent for form an array. Since they are functional to the attachment of capture agents to a substrate, linker molecules can be considered as capture agent components.

[0080] In the arrays, substrates, devices, methods and systems herein disclosed, wherein multiple capture agents are used, each capture agent can be bindingly distinguishable and/or positionally distinguishable from another.

[0081] The wording "bindingly distinguishable" as used herein with reference to molecules, indicates molecules that are distinguishable based on their ability to specifically bind to, and are thereby defined as complementary to a specific molecule. Accordingly, a first molecule is bindingly distinguishable from a second molecule if the first molecule specifically binds and is thereby defined as complementary to a third molecule and the second molecule specifically binds and is thereby defined as complementary to a fourth molecule, with the fourth molecule distinct from the third molecule.

[0082] The wording "positionally distinguishable" as used herein refers to with reference to molecules, indicates molecules that are distinguishable based on the point or area occupied by the molecules. Accordingly, positionally distinguishable capture agents are substrate polynucleotide that occupy different points or areas on the assaying channel and are thereby positionally distinguishable.

[0083] In arrays herein disclosed, each capture agent of the plurality of capture agents is capable of specifically binding each target of the plurality of targets to form

a capture agent target binding complex, and the plurality of capture agents arranged on the array so that capture agent target binding complexes are detectable along substantially parallel lines forming a barcoded pattern.

[0084] In other embodiments, substrates systems and methods are herein disclosed wherein the substrate is configured to allow attachment of targets (herein also reverse barcode or inversed-phase barcode), and in particular detectable targets, along substantially parallel lines forming a barcoded pattern. An exemplary illustration of reverse barcode is illustrated in **Figure 1,** wherein a barcoded pattern including a number of bars corresponding to immobilized serum molecules from various patients and microfluidic channels for providing various drugs to be contacted with the serum of the patients for a bio-assay, are shown.

[0085] In some embodiments, detection of the attached target and/or capture agent target complex is performed by providing a labeled molecule, which includes any molecule that can specifically bind a capture agent target complex to be detected (e.g. an antibody, aptamers, peptides etc) and a label that provides a labeling signal, the label compound attached to the molecule. The labeled molecule is contacted with the attached target and/or capture agent target complex and the labeling signal from the label compound bound to attached target and/or the capture agent-target complex on the substrate can then be detected, according to procedure identifiable by a skilled upon reading of the present disclosure and, in particular, of the Examples section.

[0086] In particular, the signal readout that is used in the arrays, devices, methods and systems herein disclosed can be realized using labels such as probes that transduce the capture event of target molecule into optical, electrical or magnetic signal. Exemplary probes include, but not limited to, fluorescent dyes, gold nanoparticles, silver nanoparticles, semiconductor nanoparticles (e.g. CdSe, ZnSe and/or their core-shell nanoparticles), and iron oxide nanoparticles.

[0087] The terms “label” and “labeled molecule” as used herein as a component of a complex or molecule refer to a molecule capable of detection, including but not limited to radioactive isotopes, fluorophores, chemoluminescent dyes, chromophores, enzymes, enzymes substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, nanoparticles, metal sols, ligands (such as biotin, avidin, streptavidin or haptens) and

the like. The term "fluorophore" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in a detectable image. As a consequence the wording and "labeling signal" as used herein indicates the signal emitted from the label that allows detection of the label, including but not limited to radioactivity, fluorescence, chemolumiescence, production of a compound in outcome of an enzymatic reaction and the likes.

[0088] In embodiments wherein one or more targets and/or a plurality of targets is detected described below in more details, the labeled molecule can be formed of a plurality of labeled molecules. Each labeled molecules comprises a molecule that specifically binds one target of the one or more targets/plurality of targets and a label compound attached to the molecule, the label compound providing a labeling signal, each labeled molecule detectably distinguishable from another.

[0089] The wording "detectably distinguishable" as used herein with reference to labeled molecule indicates molecules that are distinguishable on the basis of the labeling signal provided by the label compound attached to the molecule. Exemplary label compounds that can be use to provide detectably distinguishable labeled molecules, include but are not limited to radioactive isotopes, fluorophores, chemoluminescent dyes, chromophores, enzymes, enzymes substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, nanoparticles, metal sols, ligands (such as biotin, avidin, streptavidin or haptens) and additional compounds identifiable by a skilled person upon reading of the present disclosure.

[0090] In embodiments, wherein bindingly distinguishable capture agents are used different analytes can be detected by use of detectably distinguishable labeled molecules each specific to a separate analyte of interest.

[0091] In some embodiments, the detection method can be carried via fluorescent based readouts, in which the labeled antibody is labeled with fluorophore which includes but is not limited to small molecular dyes, protein chromophores and quantum dots. In other embodiments, on-chip detection can be performed with methods other than fluorescence based techniques. Exemplary suitable techniques include, colorimetric detection, enzyme-catalyzed production of different colored or fluorescent dyes (with different colors being associated with distinct analytes),

microparticle/nanoparticle based detection using electron microscopy, AFM, or dark-field microscopy, magnetic detection using magnetic micro/nanoparticles, electrical detection methods.

[0092] In some embodiments, detection can be performed by methods that use signal amplification such as gold nanoparticle based detection followed by gold or silver amplification. In particular, in some embodiments, in any of the methods and systems herein disclosed, detection can be carried out on gold nanoparticle-labeled secondary detection systems in which a common photographic development solution can amplify the gold nanoparticles as further described below. Also, if the readout comes from dark field scattering of gold particles, single molecule digital proteomics is enabled.

[0093] The detection can be performed with the aid of suitable equipments. In particular any equipment configured to read barcoded pattern can be used as long as the relevant sensitivity is applicable to the detection of choice.

[0094] For example, in some embodiments, reading the information of the arrays herein disclosed can be performed using a simple line-scan reader such as the laser line scanner schematically illustrated in **Figure 2**. The one-dimensional layout of the arrays renders a higher reliability as compared to the conventional circular spot arrays as schematically illustrated in **Figure 3.** In the illustration of **Figure 3**, is shown how a scan reading from a same line scanner (scan b) provides a higher reliability for a barcoded pattern (panel B) if compared with a spotted array (Panel A).

[0095] Additional equipment suitable to detect the array herein described can be identified by a skilled person upon reading of the present disclosure. For example. when fluorescent probes are used for signal readout, laser microarray scanner (such as. Axon Genepix 4000 series scanner, Affymetrix™ 300 scanner, etc), scanning laser confocal microscope (e.g. Nikon Eclipse C1si microscope) can be used to visualize the pattern. In particular, the parallel-stripe pattern allows a single scan of laser to read out full information with high fidelity and reliability as illustrated in **Figures 2 and 3**. This feature opens the possibility of implementing a simple laser line scanner similar as the barcode reader in supermarket for reading the barcode array described herein.

[0096] In other embodiments, wherein gold nanoparticles are used, light scattering microscope (such as Nikon® Eclipse LV100) can be used. In other embodiments, wherein electroless metal plating is used to enhance the nanoparticle signal, a flat bed scanner (such as Nanosphere Verigene® reader) can be used besides light scattering microscopes. In still other embodiments, wherein magnetic particles are used as probes, a magnetoresistive sensor similar to a scan head in a hard disk can be used to read out the barcode information.

[0097] Additional techniques are identifiable by a skilled person upon reading of the present disclosure and will not be further discussed in details.

[0098] Arrays and substrates herein disclosed can be manufactured using methods and systems to attach a material to a support along a predetermined pattern herein also disclosed (herein also indicated as patterning methods and systems). The methods and systems to attach material can be used to manufacture arrays and substrate according to any predetermined pattern. In embodiments, wherein the patterned material is configured along substantially parallel lines forming a barcoded pattern, the methods and systems herein disclosed can be used to manufacture barcoded arrays and substrates.

[0099] In some embodiments, the barcoded surface patterning can be performed as described below in the exemplary procedure illustrated with reference to microfluidics channels patterned from polydimethylsiloxane (PDMS) that are weakly or strongly bonded to glass substrates. A skilled person would understand that the patterning method is not limited the specific microfluidic features and materials used and that a different number of channels with different dimensions as well other materials, such as injection molded micro fluidics channels, semiconductor wafers, etc., all identifiable by a skilled person upon reading of the present disclosure, may all be utilized.

[00100] In some embodiments, a mold can be fabricated by molding a polymer such as a PDMS elastomer from a master template, to include microchannels each having an inlet and an outlet and each of the outlets is such that it forms a portion of the desired pattern (in particular a barcoded pattern).In some embodiments, the polymer is molded using photolithography to create a photoresist pattern on a silicon

wafer. Those embodiments, allow a particularly rapid prototyping. An exemplary illustration of a mold fabrication for the patterning methods and systems herein disclosed is illustrated in **Figure 4** wherein fabrication of a PDMS microchannel stamp for flow patterning of a barcode array is disclosed.

[00101] In another embodiment, the mold can be manufactured by providing a silicon "hard" master and by transferring the photolithographically-defined pattern into the underlying silicon wafer using a deep reactive ion etching (DRIE) process. Those embodiments allow a robust and reusable mold for higher throughput chip fabrication.

[00102] In some embodiments, the molded polymer can then be coupled and in particular bonded onto a support, such as a glass surface, which provides the floor for the channels of barcoded pattern. An exemplary illustration of a design two-layer PDMS fluidic channel device used for creating a multiple ring pattern (bull's eye) on a glass slide is shown in **Figure 5**.

[00103] In some of embodiments, the substrate can be pre-coated with a material of interest. For example in embodiments wherein a barcode is manufacture using the DEAL technology further illustrate below, a polyamine polymer or poly-L-lysine polymer (Sigma-Aldrich), can be pre-coated prior to bonding to increase DNA loading of the final barcoded pattern (see below and in particular Example 2)..

[00104] The number of microfluidic channels determines the size of the barcode array. In some exemplary embodiments the barcoded array comprises 13 to 20 parallel microchannels that wind back and forth to cover a large area (3cm x 2cm) of the support with the DNA barcode microarray.

[00105] In some embodiments, patterning can be performed by contacting the capture agent or molecule of choice on the support for a time and under conditions to allow attachment on the support. More particularly, in some embodiments patterning can be performed by providing solutions, each containing the molecule of choice (e.g. a different strand of primary DNA oligomers prepared in 1x PBS buffer in embodiments wherein the array is coupled with DEAL technology), can be flowed into each of the microfluidic channels. Then, the solvent of the solution can be allowed to evaporate, e.g. by placing the solution-filled chip in a dessicator to allow

solvent (e.g. water) to evaporate completely through the gas-permeable PDMS, leaving the molecules to be attached (e.g. DNA molecules) behind. In some embodiments, this process can take from several hours to overnight to complete.

[00106] Following patterning of the molecules, the mold is usually decoupled from the support. In some embodiments, once the mold is removed from the support the patterned molecule can be subjected to subsequent treatments (e.g. DNA molecule can be fixed to the glass surface by thermal treatment at 80C for 4 hours, or by UV crosslinking; removal of salts or other precipitates that might have formed during one or more of the previous operations which can be removed, for example, by rapidly dipping the slide in deionized water prior to bonding the blood-assay chip to the slide). An exemplary procedure of the patterning method herein disclosed is illustrated in Example 15.

[00107] In particular, in some specific embodiments, a series of microfluidics channels is patterned into PDMS, and those channels bonded onto a glass surface so that one out of the 4 channel walls is the glass surface itself. The numbers of micro fluidics channels determines the size of the barcoded array. In this way, a solution flowing through the micro fluidics channel will come into contact with the glass substrate. Typical dimensions of these micro fluidics channels for barcoded used for biological assays are 10 micrometers or larger. In particular, in embodiments where material is patterned to be subjected to a bio assay, the channel width defines the width of an individual bio-assay measurement area within the final bar code. In those embodiments, if the final measurement of the biomolecule is done using optical methods, then a 10 micrometer wide area constitutes a size that is readily imaged using low-cost optics. Larger and smaller bars are also possible.

[00108] A different material and in particular a different biological species (or a different concentration of the same biological species), such as DNA oligomers, can then be flowed in to each of the individual micro fluidics channels.

[00109] The biological species or other patterned material can then be attached to the glass surface areas within those microfluidics channels using electrostatic or other chemical interactions. The glass may be pre-coated with some molecular

component to increase the chemical interaction between the biological species and the glass surface (see above and below in particular Example 2).

[00110] The solvent from the solution containing the patterned material (e.g. the biological species) is then removed. If that solution is water and the fluidics (e.g. microfluidics) is fabricated from PDMS, then the water can be let naturally evaporate through the PDMS, leaving the patterned material attached to the substrate thus providing a the patterned array on the substrate. In some embodiments, it may be desirable to introduce additional channel (e.g. micro fluidics channels) at this point for handling and introducing the biological sample of interest.

[00111] The microfluidic bar-code patterning chip may be made by molding silicon elastomer from a master template. The master template may be fabricated from many materials. One method is to fabricate the master by using photolithography to expose an SU8 2015 photoresist. Regions of the photoresist are removed following lithographic exposure, and the remaining material constitutes the master. Alternatively, photolithographic patterning methods, coupled with deep reactive ion etching (DRIE), can be utilized to prepare a master from a silicon wafer. These various methods for preparing microfluidics molds and microfluidics channels from those molds are well known in the art. (Gael Thuillier and Chantal Khan Malek, *Microsys. Technol* 12, 180, 2005.)

[00112] The patterned material can comprise any substance of interest suitable to be attached to a support, including organic or inorganic substances, Exemplary inorganic material that can be patterned using the patterning methods and systems herein disclosed include but are not limited to gold nanoparticles that can attach to thiol functionalized substrate surface, iron oxide nanoparticles that can be deposited onto the substrate using magnetic field, and silica particles that can be immobilized by cationic polymer coated substrate, and so on.

[00113] Exemplary organic that can be patterned using the patterning methods and systems herein disclosed include but are not limited to living species and their mixtures such as cells, virus, bacteria and fungi, complex biospecimens and their mixtures such as tissue, tissue lysate, cell lysate, serum, saliva and joint fluid, monotypic molecule and their mixtures such as polynucleotides, proteins, antibodies,

glycoproteins, polysaccharides, lipopolysaccharides, ligands, peptides, polypeptides, lipids, drugs, drug candidates, antigens and the fragments, potions, and components or any of above. The organic materials can also include non-biological materials such as polymers, oligomers, dye molecules, conducting polymers, responsive polymer, gas sensing polymers, liquid crystals and metal organic frameworks (MOFs), carbon nanotube, fullerene, grapheme, and their nano/microstructures. In some embodiments, the patterned material comprises capture agents. In some embodiments, the patterned material comprises detectable targets. In other embodiments, the patterned material comprises a material, such as cells or other biological material to be assayed. In other embodiments, the patterned material can comprise other organic or inorganic substance for which the barcoded configuration is desired (e.g. liquid crystal for LCD manufacturing, or gas selective polymers to be used as gas sensors).

[00114] According to the patterning methods and systems herein disclosed, a pattern and in particular a barcoded pattern or array can be created on very small area and patterning of magnetic ID or other material can therefore be performed onto small-sizcd products.

[00115] In some embodiments, wherein the pattern is used for the detection through capture agents, the capture agent is formed by a polynucleotide and in particular a DNA polynucleotide, that bind about 10 to 20 consecutive bases of a target RNA via complementary hybridization. In some of those embodiments the arrays, substrates, methods and systems herein disclosed can be used to detect messenger RNA (mRNA) and in particular mRNA from a biospecimen (e.g. tissue lysate). In some of those embodiments, another labeled DNA stand (e.g. fluorescently labeled) is designed to bind to ~10-20 different bases of the captured mRNA for signal read out. In some embodiments, a multiplexed measurement of a panel of mRNA molecules can be performed on a barcode array patterned with stripes of their capture agent DNA.

[00116] In some embodiments, wherein the pattern is used for the detection, the target is a microRNA (miRNA) a type of short RNA molecules (22 bases) that regulate gene expression at the post-transcription level

[00117] In some embodiments, wherein the pattern is used for detection, the target can be a transcription factor, and the capture agent is a polynucleotide and in particular a DNA polynucleotide having the same sequence of the binding site of the transcription factor, or a portion thereof or an homologous sequence thereof. In some embodiments, fluorescence-labeled or biotin-labeled antibodies are then used for signal readout.

[00118] In some embodiments, the lines are formed by one or more channels configured to host the material to be patterned. In particular, in some embodiments the fluidic channel width can be made ranging from 0.5μm to 1cm. The height can be typically >1/10 of the channel width when a soft materials such as PDMS is employed, and can be less if a harder material (e.g. glass, silicon, polystyrene, PMMA, polycarbonate or epoxy) is used to make the fluidic channels.

[00119] In embodiments when a two-layer device is used for patterning arrays, the channel can be as short as 1mm and up to meters when the channel is shaped to cover the entire substrate (e.g. a glass slide 1"x 3") for example by turning back and forth on the substrate. In embodiments where a larger substrate is used, the channel length can be longer since the length is defined by the substrate and the application of interest.

[00120] The array can be in principle made into any custom-designed shapes such as stripes, rings, concentric rings (see for example the illustration of **Figures 5 and 6**), triangles, rectangles, polyhedrons, stars, cross-bars, letters, pictures on flat, convex, concaved or irregular substrates. In particular in **Figure 6** a multiple ring pattern suitable to application such as a bio-assay for detection of targets secreted by a sample such as a cell placed in the middle, is shown. In particular the images of **Figure 6** show the detection of proteins IL-2 and TNF-α visualized by Cy3 and Cy5 fluorescent probes.

[00121] In embodiments, wherein the channels are used to pattern polynucleotides (e.g. DNA) or proteins (e.g. antibodies), the channels width can be anywhere from 0.5μm to 1cm and the height can range from 1μm to 1cm, and the length can any that is allowed by the area of the given substrate. An exemplary 2-μm barcode array is shown in **Figure 7,** wherein a barcoded array of fluorescent DNA

molecules manufactured according to the teaching of the present disclosure, is illustrated. For optimum demonstrated performance of polynucleotide detection using a complementary DNA barcoded array, a channel width of 20μm and a height of 20μm are preferred when a 200-μM capture DNA solution is used and the developed array is visualized using fluorescence scanner. In embodiments, wherein a DNA barcoded array is used to immobilize DNA encoded antibodies and subsequent immuno-sandwich assay, the same channel width and height are preferred (see below description of DEAL technology).

[00122] In some embodiments, some or all of the substantially parallel lines are connected to one another through at least one of the ends. More particularly, in applications wherein the lines are formed by channels the substantially parallel lines can be connected to one another to form a single channels configured in a serpentine-like shape. Serpentine-like channels allow the fabrication of repeated barcode arrays over a large area, e.g. the entire glass slide (1"x 3"), in a single step of flowing capture agents. It represents a significant advantage in large-scale, low cost manufacture of barcoded arrays for detection applications. In addition, it allows an assay to be executed in multiple repeats at the same thus reduce the statistic errors. An exemplary illustration of a serpentine-like channel is shown in **Figure 8.** Additional connections between the substantially parallel lines of a pattern or multiple patterns (for example multiple barcoded patterns connected to form a pyramid to increase DNA loading in application wherein barcode is manufactured in connection with DEAL technology).

[00123] The material to be patterned can be disposed along the parallel lines according to a specific experimental design of choice. For example, in embodiments where a plurality of capture agents are patterned, the capture agents can be disposed with each capture agent disposed along one line, or with two or more capture agents located disposed along portions of a single channel. In other embodiments, the material to be tested (and in particular detected) can be patterned along one line or portion of a line of the barcode. Exemplary illustrations of those embodiments are shown in **Figures 1 and 7.**

[00124] In some embodiments, the patterned material can be used for target detection. In those embodiments, typically capture agents are patterned on the substrate, to form detectable capture agent target complexes. In other embodiments,

detectable targets are patterned directly on the material. For example, a number of serum samples from multiple patients can be patterned into a barcoded array. In such array, each stripe contains the biomolecules in the entire plasma proteome of that patient. This array can be exploited to screen for antibodies, ligands, drug candidates, and comparison of biological profiles among patients. Those embodiments are exemplified for the barcoded arrays, substrates, methods and systems of Examples 3-14 and illustrated in the related figures and further described below.

[00125] In some embodiments, assays are performed in a non-microfluidic environment. An exemplary illustration of those embodiments is shown in **Figure 9**, wherein execution of multiple assays in twelve isolated wells using a barcoded array is illustrated. In particular, the barcoded array illustrated in **Figure 9** is manufactured on a supporting glass slide including wells, wherein each well contains a different sample such as human serum. In the experiments illustrated in **Figure 9**, protein detection from the different samples is visualized by fluorescence imaging.

[00126] In some embodiment, assays are performed in microfluidics which allows handling particularly small amounts of biospecimens (such as a finger prick of blood, tissue from skinny needle biopsy, etc).

[00127] In some embodiments, the barcode array can be used to detect multiple proteins and/or genes from a single cell via on-chip single cell culture, lysis, mRNA and protein isolation/purification, in particular using an integrated microfluidic device such as the one described in the U.S. Application entitled "Microfluidic Devices, Methods and Systems for Detecting Target Molecules" Serial No. 12/174,598.

[00128] A further description of the arrays, substrates, devices methods and systems of the present disclosure is provided with reference to microfluidic applications wherein the sample is a material of biological origin (bio sample) and the targets are biomarkers. A person skilled in the art will appreciate the applicability of the features described in detail for microfluidics and biomarkers for non-microfluidic applications and/or for other biologic, organic and inorganic samples and targets.

[00129] In some embodiments, the arrays, devices methods and systems herein disclosed can be used to perform a surface bound bioassay based on detection a biomolecule of interest in some biomaterial, such as blood, serum, biological tissue, or as a component of a cell culture (herein also indicated as bio-barcode assay).

[00130] The biological material can be pretreated so as to release the biomolecules of interest, to remove biological material that can interfere with binding of the biomolecules in the surface bound bioassay. An exemplary pretreatment procedure includes separating blood cells from blood plasma (or serum), and then measuring the proteins from the plasma. In other procedures the separated cells could be further separated into white and red blood cells, which can be therefore subjected to further analysis. An exemplary surface bound bioassay can be carried out as follows: The biomolecule of interest is bound to a (primary or 1°) surface-bound capture agent molecule (e.g. an antibody or complementary single-stranded DNA oligomer) that specifically recognizes and binds to the biomolecule of interest. Typically, a secondary (or 2°) capture agent containing some label for detection, such as a fluorescent molecule, is introduced to bind to the surface-bound biomolecule.

[00131] The bio-barcode can be manufactured patterning the capture agents of choice on a substrate along substantially parallel lines. In certain microfluidic applications the substantially parallel lines can be formed by channels or channel portions. Exemplary illustration of different embodiments wherein capture agents are attached to a surface in a bio-barcode are shown in **Figures 10** (capture agents DNA molecules for detection of polynucleotide (e.g. mRNA and microRNA) to be configured in a barcoded array), **Figure 11** (DNA-encoding antibodies to enable immuno-sandwich assay on barcode array allowing detection of proteins, cell surface markers, glycoproteins, virus and bacteria in multiplex) and **Figure 12** (schematic illustration showing how increased DNA loading helps to enhance detection sensitivity in application wherein the bio-barcode is coupled with DEAL technology see below).

[00132] Patterning of capture agents, for example, antibody arrays for detecting proteins or complementary DNA arrays for detecting polynucleotides, results in an increased sensitivity of molecules such as polynucleotide, nucleic acid (mRNA, miRNA, DNA etc), An increased sensitivity could be in particular associated with two

factors: (1) the increased loading of capture DNA using poly-amine to coat substrate surface (for embodiments wherein the capture agent is a polynucleotide and in particular DNA) and (2) the reduced feature size with respect to conventional pin spotted arrays (e.g. 20μm in barcoded array vs. 200μm in conventional pin-spotted array) lowers the diffusion barrier and leads to high binding efficiency.

[00133] In some embodiments the capture agents include one ore more component. In particular, in some embodiments the capture agents can be formed by a substrate polynucleotide and a polynucleotide encoded-protein in application of the technology (herein also identified as DEAL) described in U.S. patent application Serial No. 11/888,502.

[00134] Accordingly, the wording "substrate polynucleotide" as used herein refers to a polynucleotide that is attached to a substrate so to maintain the ability to bind to its complementary polynucleotide. A substrate polynucleotide can be in particular comprised of a sequence that specifically binds and is thereby defined as complementary with an encoding-polynucleotide of a polynucleotide encoded protein.

[00135] The wording "polynucleotide-encoded protein" refers to a polynucleotide-protein complex comprising a protein component that specifically binds to, and is thereby defined as complementary to, a target and an encoding polynucleotide attached to the protein component. In some embodiments, the encoding polynucleotide attached to the protein is protein-specific. Those embodiments can be used to perform assays that exploit the protein-specific interaction to detect other proteins, cytokines, chemokines, small molecules, DNA, RNA, lipids, etc., whenever a target is known, and sensitive detection of that target is required. The term "polynucleotide-encoded antibody" as used herein refers to a polynucleotide-encoded protein wherein the protein component is an antibody.

[00136] In the polynucleotide-encoded proteins herein disclosed each protein specifically binds to, and is thereby defined as complementary to, a pre-determined target, and each encoding polynucleotide-specifically binds to, and is thereby defined as complementary to, a pre-determined substrate polynucleotide.

[00137] In embodiments wherein the protein is an antibody, the protein-target interaction is an antibody-antigen interaction. In embodiments wherein the protein is

other than an antibody, the interaction can be receptor-ligand, enzyme-substrate and additional protein-protein interactions identifiable by a skilled person upon reading of the present disclosure. For example, in embodiments where the protein is streptavidin, the protein-target interaction is a receptor-ligand interaction, where the receptor is streptavidin and the ligand is biotin, free or attached to any biomolecules. An exemplary schematic illustration is shown in **Figure 12**.

[00138] When coupled with the DEAL technique, the amount of polynucleotides that is deposited onto a given spatial location within the bio-barcode array can be controlled in view of the desired sensitivity and concentration range over which the biomolecule of interest can be detected. By using two or more stripes within the same bio-barcode array, each optimized to detect the same biomolecule but over different concentration ranges, the concentration range over which that protein can be detected, as compared to a conventional assay, can be dramatically increased.

[00139] The concentration range of DNA detectable with a Bio-Barcode array coupled with DEAL can be as low as 1pM to 100nM using 200μM loading of capture DNA on 20μm barcode stripes. Target molecules suitable for this technique include messenger RNAs, micro RNAs, the fragments of genomic DNAs, viral DNA, bacterial DNA, and synthesized polynucleotides.

[00140] Some embodiments wherein the Bio-Barcode is coupled with DEAL shows an increased sensitivity if compared with embodiments wherein protein capture agents are patterned directly on a substrate. In particular, in some embodiments wherein antibodies are patterned directly into barcoded array with fabrication methods that require application of high temperatures when the antibodies are attached to the substrate, all the target molecules that can be detected by DEAL are in principle detectable, but a lower sensitivity might be seen due to the poor stability of the antibody in a dry state.

[00141] When coupled with the DEAL technique, the bio-barcode array withstands the processing conditions associated with micro fluidics chip fabrication. As a consequence, the Bio Bar .Bar Code array can be advantageously manufactured as illustrated in the exemplary procedure outlined below with reference to an exemplary

array including 10 antibodies used as capture agents (10 CAs) labeled with single stranded DNA used as encoding polynucleotide.

[00142] The 10 antibodies against the biomarker of interest are chemically labeled with single-stranded DNA (ssDNA) oligomers. The complementary ssDNA' oligomers can be deposited onto regions of a surface. DNA hybridization assembles the 10 CAs onto those particular regions.

[00143] The 10 CAs are patterned using microfluidics channels. The channel widths and densities are limited by what can be patterned - smaller channels and higher densities than are practical using other methods are readily achieved. Typically channels of widths of at least 10 micrometers, spaced by distances of at least 50 micrometers, are most practical for typical bioassays, such as analyzing multiple proteins from serum. This allows for large numbers of measurements to be carried out in a relatively small microfluidics channel.

[00144] Spot sizes significantly smaller than 10 micrometers are also possible with this technique, as are significantly higher spot densities. These may be useful for more specialized applications, such as would be required for measuring a panel of protein biomarkers and other biomolecules from circulating tumor cells, cancer stem cells, and other extremely rare cell types.

[00145] The bio-barcode patterned microfluidics channels are readily aligned with other microfluidics channels, such as are used for the handling of the biological specimen from which the assays are performed. For example, alignment markers that are utilized to align the bio-barcode micro fluidics channels can also be utilized to assemble the microfluidics channels for handling the biological sample. This is standard fabrication practice.

[00146] The density of 1° CAs that can be deposited onto such a small spot can be significantly higher than what can be achieved using spotting methods. Repeated depositions of 10 CAs through the same microfluidics channels can achieve a very high surface loading of the 10 CAs. Conversely, the DEAL technique utilizes single-stranded DNA (ssDNA) oligomers as capture agents for the 10 CA antibodies that are, in turn, utilized to detect proteins. The DNA can be loaded at very high levels using

the bio-barcode Array because of the high solubility of DNA in water. This, in turn, can lead to very high coverage of the 1° antibody CAs.

[00147] Multiple numbers and classes of capture agents can be placed on specific, microscopic locations on a surface using microfluidic patterning of the 10 capture agents. In this way, the panel of biomolecules is detected by detecting labeling signals (for example, fluorescence) from the region of the surface where the pattern of 10 capture agents was placed.

[00148] In some embodiments, wherein the arrays, substrates methods and systems herein disclosed are performed in microfluidics, the capture agents can be attached on the location with a method to attach molecule along a predetermined pattern herein disclosed. In those embodiments, using a microchannel-guided flow-patterning approach, a barcode arrays can be manufactured that are at least an order of magnitude denser than conventional microarrays. In some embodiments, this result can be accomplished by creating a mold, e.g. a polydimethylsiloxane (PDMS) mold containing the desired number of microfluidic channels, e.g. 13-20 parallel microfluidic channels, with each channel conveying a different biomolecule capture agent. A skilled person will understand that the number of channels can readily be expanded to include 100 or more different capture agents; whereas in microcontact printing, the patterning difficulty increases exponentially as the number of proteins printed is increased, due to the challenges of aligning multiple stamps to print multiple proteins.

[00149] In some embodiments, the barcoded array is a DEAL barcoded array. In some of those embodiments poly-amine coated glass surfaces can be use to allow significantly higher DNA loading than do more traditional aminated surfaces. DNA "bars" of 2 micrometers in width could be successfully patterned. In some exemplary embodiments, described herein an about 20-micrometer (μm) channel width was chosen because the fluorescence microarray scanner has a resolution of 5μm.

[00150] In those embodiments a 10-fold increase in array density is achieved as compared to a typical pin-spotted DNA array (i.e. 150 μm spot diameters at 300 μm pitch), and greatly expands the numbers of proteins that can be measured within a microfluidic chip disclosed herein for a given sample size. In particular, in some

embodiments, simultaneous detection of 12 to 20, up to 50 or even more than 50 proteins. This feature can be used in applications where detection of multiple targets is desired, for example detection of a biological profiles but also a variety of waste gases (e.g. from car engine exhaustion) or pollutes in a sample.

[00151] The protein assay can be carried out on the 10 CAs array as described above. Use of DNA hybridization as an assembly strategy allows for multiple proteins to be detected within the same microenvironment, since the various 10 CA antibodies for the various proteins to be detected can be each labeled with a different ssDNA oligomer. Also use of DNA hybridization as an assembly strategy allows preparation of the substrate including ssDNA in early in the fabrication process so that a substrate including the ssDNA can be treated, dried out, heated, shipped and provided to the final user in a ready to use systems that also include complementary capture agents . Exemplary applications are described in Examples 1 to 7 and in the related figures describe the bar-code array patterning technique and DEAL bar-code chips for protein detection.

[00152] A person skilled in the art would understand that the array herein disclosed can include patterning a variety of biological materials, e.g. DNA, proteins, sera and tissue lysates, using micro fluidic channels. The Bio Bar-code Array method can be applied to the fabrication of bio-chips and integrated biosensing devices for high-density, multiplexed and sensitive detection of DNA and proteins in clinic diagnostics of human diseases like cancers, and for high-throughput drug screening. In some embodiments the patterning is based upon a new, yet simple and reliable approach – micro channel guided surface patterning of a large number of different biological species to fabricate a small-size, high-density array.

[00153] The systems herein disclosed can be provided in the form of arrays or kits of parts. An array sometimes referred to as a “microarray” includes any one, two or three dimensional arrangement of addressable regions bearing a particular molecule associated to that region. Usually the characteristic feature size for microarrays is micrometers.

[00154] In a kit of parts, various components can be comprised in the kit independently. In some embodiments, a patterned substrate can be provided together

with a label and/or other reagents suitable to perform detection. In some embodiments, a device suitable for detecting the pattern can also be included.

[00155] In embodiments, wherein the patterned substrate is integrated with deal technology a system can include polynucleotide-encoded proteins and a patterned substrate comprised in the kit independently. Molecules comprised in the kit (e.g. the polynucleotide-encoded protein) can in particular be included in one or more compositions, with each molecule in a composition together with a suitable vehicle carrier or auxiliary agent.

[00156] The substrate provided in the system can have substrate polynucleotides attached thereto or other molecule attached according to the desired pattern. In some embodiments, the substrate polynucleotides, or the material to be patterned can be further provided as an additional component of the kit. Additional components can include labeled polynucleotides, labeled antibodies, labels, microfluidic chip, reference standards, and additional components identifiable by a skilled person upon reading of the present disclosure. In particular, the components of the kit can be provided, with suitable instructions and other necessary reagents, in order to perform the methods here disclosed. The kit will normally contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, will usually be included in the kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials (i.e. wash buffers and the like).

[00157] Additional applications in which the patterned material is not limited to a biological sample will be identifiable by the person skilled in the art. In particular in some embodiments, the patterned material can be used for magnetic identity (ID) of small-sized products, which can include but are not limited to products carrying a biological material. For example, a magnetic ID bar has been widely used in tracking a product. But conventional magnetic ID pad is too large to be used for a small-sized subject such as a small camera CMOS chip, a fine jewel and a tiny artifact. Those embodiments are exemplified for the barcoded arrays, substrates, methods and systems in Example 15.

[00158] Further details concerning the identification of the suitable carrier agent or auxiliary agent of the compositions, and generally manufacturing and packaging of the kit, can be identified by the person skilled in the art upon reading of the present disclosure.

EXAMPLES

[00159] The methods and system herein disclosed are further illustrated in the following examples, which are provided by way of illustration and are not intended to be limiting the scope of the present disclosure.

Example 1: Fabrication and use of a Barcoded Chip with integrated DEAL technology

[00160] A Barcoded chip was fabricated according to the procedure schematically illustrated in **Figure 13 Panel A.**

[00161] A silicon elastomer (PDMS) stamp was molded from a lithographically patterned silicon master. Then it was thermally bonded onto a poly-amine coated glass slide on which different biomolecule solutions are flowed into the parallel microchannels. Once the solutions evaporate completely, the PDMS stamp is peeled off and the glass side will be baked to create a robust Bio-Bar-code array. The bar-code stripes can be made 2-20 μm in width and spacing, leading to increased array density compare to conventional microarrays. In principle, there is no limit for the number of primary molecules like DNA that can be patterned using this technique. It indeed enables the fabrication of a large-scale, high-density biomolecule array for systems biology and disease diagnostics.

[00162] More particularly, a polydimethylsiloxane (PDMS) mold containing 13-20 parallel microfluidic channels, with each channel conveying a different DNA oligomer as DEAL code, was fabricated by soft lithography. The PDMS mold was bonded to a polylysine-coated glass slide via thermal treatment at 80°C for 2 hours. The polyamine surfaces permit significantly higher DNA loading than do more traditional aminated surfaces. DNA "bars" of 2 micrometers in width have been successfully patterned using this technique. In the present study, a 20-micrometer (μm) channel width was chosen because the fluorescence microarray scanner used by

applications has a resolution of 5μm. Nevertheless, the current design already resulted in a DNA barcode array an order of magnitude denser than conventional microarrays fabricated by pin-spotting. The coding DNA solutions (A-M for the cancer serum test and AA-HH for the finger-prick blood test) prepared in 1xPBS were flowed into individual channels, and then allowed to evaporate completely. Finally, the PDMS was peeled off and the substrate with DNA barcode arrays was baked at 80°C for 2-4 hours. The DNA solution concentration was ~100μM in all experiments except in the hCG test, leading to a high loading of ~$6x10^{13}$molecules/cm^2 (assuming 50% was collected onto substrate).

[00163] The array so created was used in a bio assay as illustrated in **Figure 13 Panel B**. An integrated microfluidic device was placed onto the bio-bar-code chip microfluidic channels. There was no need of fine alignment to integrate the bio-bar-code pattern with the microfluidic systems. Different samples such as patient sera, tissue lysates can be assayed in each microfluidic channels, respectively. The array depicted in **Figure 13 panel B** enables high-through biodetection with minimum sample consumption.

[00164] The experiments described above can be modified to modulate sensitivity and detectable range of targets according to the experimental design of choice. A possible modification is illustrated in **Figure 8** which shows a schematic illustration of a mask design of a 13-channel patterning chip, wherein the letter A-M indicate the channels for flowing different DNA molecules. Additional modifications include subjecting the array to poly-amine surface modification, e.g. with the procedure exemplified in Example 2 below, to allow increased DNA loading. This modification leads to higher sensitivity and broader dynamic range as illustrate in the exemplary procedure of Example 3 below.

Example 2: Fabrication of a DEAL Barcoded Chip with an increased DNA Loading

[00165] During microchannel-guided flow-patterning of the DEAL barcode arrays, the glass surface was modified by treatment with poly-L-lysine (a poly-amine), yielding a three-dimensional matrix for DNA adsorption and markedly increasing the amount of DNA loading

[00166] The results are illustrated in **Figure 14**, which shows the effects of poly-lysine coating on an assay performed with DEAL technology. More particularly, **Figure 14** shows detection of protein targets using the barcoded array manufactured with low and high loading of primary DNA molecules and the resulting difference in the protein detection. As shown in the schematic illustration of panel (a) polylysine coating of the PDMS support results in an increased loading of DNA oligomer codes.

[00167] In particular, the DNA-loading density is estimated to be $6x10^{13}$molecules/cm^2 in our experiments, an order of magnitude higher than typical loading densities on amino-silane coated glass slides. As a result, the protein detection sensitivity was improved by an order of magnitude, and the dynamic range was increased to 4 orders of magnitude, as compared with 2-3 orders of magnitude for the small-molecule amine (i.e. amino-propyl-triethoxyl silane, APTES) functionalized glass surface. Exemplary results of this comparative analysis is illustrated in **Figure 14** Panel (b) detection of three human cytokines (IFN-γ, TNF-α, and IL-2) using substrates coated with amino-silane and polylysine, respectively is shown.

Example 3: Barcoded Chip with ELISA-like Sensitivity

[00168] A series of experiments performed by the applicants showed that a barcode chip integrated with DEAL technology renders a high density array for multiplexed protein measurements. Moreover, the DEAL barcoded chip also demonstrates a marked improvement in sensitivity as compared to conventional pin-spotted microarrays.

[00169] In particular, a side-by-side comparison study was performed by running DEAL assays on three cytokines under identical conditions. Using the microchannel-guided flow patterning method, a glass slide was patterned with DNA oligomers **A, B, C** and a blank control **O**. Each bar was 20μm in width. The DNA solutions were all 50-100μM. The pin-spotted array was printed at the Institute for Systems Biology at 100μM concentration. The typical spot size was 150-200 μm. Six sets of spots were printed corresponding to oligomers **A, B, C, D, E,** and **F**. Poly-l-lysine coated slides were used for both types of arrays.

[00170] Before the DEAL assay, the capture antibodies were conjugated to DNA oligomer codes as follows: **A'** to IFN-γ, **B'** to TNF-α and **C'** to IL-2. Protein standards were diluted in 1% BSA/PBS solution at concentrations ranging from 1fM to 1nM. The incubation time for each step (blocking, conjugate hybridization, sample binding, detection-antibody binding, and fluorescent-molecule binding) was 30min. The bar width was 20μm.

[00171] The results are illustrated in **Figure 15** wherein immunoassays run on DEAL barcode arrays is shown. In particular, as illustrated in Panel (a) detection of three human cytokines (A: IFN-γ, B: TNF-α, C: IL-2, O: negative control) was proven to be concentration dependent. In the illustration of Panel (a) the bar-code array has a sequence of ABCOABCOABCOA (herein, "0" denotes that no 1° DNA was flowed in such microchannel). This data show proteins can be detected at concentration as low as 1pM. Concentration dependence is indicated by the diagram of Panel (b) where quantitation of fluorescence intensity is plotted versus TNF-α concentration. The line profile for the results obtained with 1-pM protein sample as indicated in Panel (a), is shown in the diagrams of Panel (c).

[00172] As a further comparison, the sensitivity obtained in ELISA assays (using antibody pairs and protein standards from eBioscience) is projected to be ~10pg/mL (0.8pM) for TNF-α. Therefore, those experiments show that the DEAL barcode array combines ELISA-like sensitivity with a high degree of multiplexing for protein measurements.

[00173] In addition, the TNF-α detection sensitivity of the DEAL barcode arrays was higher and the projected sensitivity limit was better than 1pM, as compared to 10-100pM for conventional microarrays as illustrated in the comparative assay performed under the same condition using a conventional pin-spotting method of Panel (d) further illustrated in the comparative Example 6 below. These results confirmed that the barcoded chip has much higher sensitivity and increased linear range for protein measurements, as compared with a conventional assay.

Example 4: Use of a barcoded Array for DNA detection

[00174] A barcoded array was used in a bio assay for detection of DNA. In particular, a polynucleotide (DNA) was patterned on a substrate and used to detect a complementary polynucleotide in a sample. The results illustrated in **Figure 16** show that the patterned DNA oligomers exhibit a high affinity for binding their complementary strands.

[00175] In particular, in **Figure 16 panel A**, fluorescence images are reported taken before and after hybridization of an A' strand to its Alexa 532 labeled complementary stand. Three different strands of DNA oligomers, nonfluorescent A, Alexa 532 labeled B(red) and Alexa 635 labeled (dark green) were flow-patterned on a polyL- lysine slide to form this bar-code chip. "0" denotes a non-patterned channel for bland control. After applying the Alexa-532 labeled A' molecule s (its concentration is 1 nanomolar, these DNA molecules are complementary to the surface bound A stands), a clear and strong green fluorescence band emerges, indicating highly effective and specific sensing of A' DNA molecules.

[00176] The line profile of fluorescence intensity across the whole set of bar-code array is shown in **Figure 16 Panel B.** In the illustration of **Figure 16**, A' is the target polynucleotide that was added into sample b and detected by fluorescence change in the location indicated by an asterisk.

Example 5: Use of Barcoded Array for protein detection

[00177] A barcoded array assembled as disclosed herein was used for protein detection according to an experimental approach developed by the applicants.

[00178] In particular, applicants developed a multiplexed assay of 12 plasma proteins using DEAL barcode arrays. In a first test, the level of cross-reactivity of each antigen with DEAL stripes that are not specific to that antigen was assessed. DNA-encoding capture antibodies and biotinylated detection antibodies for all 12 antigens were used as usual, but a distinct antigen (10nM) was added to each assay lane. Cy5-Streptavidin (red-fluorescence tag) was run as usual to visualize the extent of analyte capture.

[00179] The reference marks (DNA strand M) were visualized in all lanes with fluorescent green Cy3-M' DNA molecules. The 12 proteins showed a negligible

extent of cross-talk. In a second test, assays were performed on serial dilutions of all 12 proteins on the DEAL barcode chip in view of the limitation imposed by the particular devices used, each allowing a maximum of 12 parallel assays to be executed. In the specific experimental approach of choice for this setting 6 lanes were used for cross-talk validation and 6 lanes were used for dynamic range studies.

[00180] The results are illustrated in **Figure 17** which shows cross-reactivity check and dilution curves for all 12 proteins. In particular, the DEAL barcode images and line profiles from a single device of panel (a) show minimal cross-talk and a series of standard antigens ranging from 1nM to 1pM for all 12 proteins. In the experiments shown in panel (a), 2 proteins were combined in each assay lane (**Figure 17 panel (a)**).

[00181] All proteins were assayed on the same chip over the concentration range of 1nM down to 1pM (except PSA and TGF-b: 5nM to 5pM), and quantified the fluorescence signal vs. concentration for all 12 antigens as illustrated in **Figure 17 panel (b)**, where dilution curves for all 12 proteins are shown.

[00182] In this experiment, all the concentrations were imaged using the Genepix scanner at the same laser power (55 for 635nm, 15 for 532nm), optical gain (500 for 635nm and 400 for 532nm), and brightness/contrast (92/90) in order for quantitative comparison. Apparently, the estimated sensitivity varies a lot from ~0.3pM (e.g. IL-1β and IL-12) to 30pM (TGF-β) largely depending on the antibodies being used. For example, the TGF-b antibody pair has a relatively lower binding affinity and a poorer detection limit in ELISA (according to the spec sheet, it is ~70pg/mL compared to 5-10pg/mL for most other cytokines). Predictably, this gave rise to a poorer performance in the DEAL assay. Although these curves clearly show a dynamic response of DEAL signals with respect to antigen concentrations, the variation remains pretty large as compared to bulk-scale immuno-assay such as ELISA.

[00183] Detection probes are not limited to fluorescent dyes, but can be any others that are capable to transduce signal from captured targets to optical, magnetic or electrical read out.

[00184] In particular, an alternative method of detection is provided by use of gold nanoparticles as probes. An exemplary illustration of detection performed using gold

nanoparticles is shown in **Figure 18**, wherein detection of target protein IL-1β using gold nanoparticles as the probe is shown.

[00185] In particular, in the example of **Figure 18**, 40-nm gold nanoparticles were used to visualize the captured protein (e.g. IL-1β) of interest from human serum).

[00186] Additional examples of labels and method of detections are illustrated the U.S. Application entitled "Methods and Systems for Detecting and/or Sorting Targets" Serial No. 11/888,502 filed on August 1, 2007.

Example 6: Comparative example related to use of a barcoded array and a conventional microarray for protein detection

[00187] Comparative experiments were performed on the barcode array of example 3 and a conventional microarray printed using pin-spotting technique. The results illustrated in **Figure 15 panel d,** show how apparently, the conventional microarray only achieved sensitivity 1-2 orders of magnitude worse than the DEAL barcoded chips.

[00188] A side-by-side comparison study was performed by running DEAL assays on three cytokines under identical conditions on a barcoded and a pin spotted microarrays under the experimental conditions illustrated in Example 3. The pin-spotted array was printed at the Institute for Systems Biology at 100μM concentration. The typical spot size was 150-200μm. Six sets of spots were printed corresponding to oligomers A, B, C, D, E, and F. Poly-l-lysine coated slides were used for both types of arrays. Further details are illustrated in Example 3.

[00189] The results illustrated in **Figure 15 panel e** show that barcoded array exhibits greater performance with higher sensitivity than does the conventional array.

[00190] . In particular, these results demonstrate that the detection sensitivity of the DEAL barcode arrays was higher and the projected sensitivity limit was better than 1pM, as compared to 10-100pM for conventional microarrays (**Figure 15 panel e**).

[00191] The only difference between the barcoded and conventional pin-spotted platforms used in the experiment shown in **Figure 15** is the feature size. The barcode array has a line-width of 20μm, whereas the spot size in conventional arrays is more than 150μm. The mechanism for improved sensitivity in the DEAL barcode assay is not completely understood. A possible explanation which is not intended to be limited is that the improved sensitivity could be attributed to a reduced kinetic barrier and decreased diffusion time. These results are consistent with a recent report which demonstrated that DNA microarrays with smaller spot sizes could detect DNA with increased sensitivity.

Example 7: Use of a barcoded Array for detection of multiple different targets

[00192] A barcoded array integrated with DEAL technology was used to detect multiple proteins as illustrated in **Figure 19**. In particular **Figure 19** shows the use of DEAL bar-code immunoassay for the detection of five different proteins. The proteins are detected within an area that is less than would be required for the detection of a single protein using a conventional spotted microarray.

[00193] The results illustrated in **Figure 19** show in particular multiple proteins simultaneously detected using a DEAL bio-barcode. Panel A shows a schematic illustration of DEAL bar-code array for co-detection of a variety of proteins at the same time, including cytokines, chemokines, growth factors, intracellular signaling molecules and cancer markers. Panel B shows a multiparameter DEAL Bar-code immunoassays of 5 proteins at the same time, detected from human reference serum that was spiked with the five proteins: hCG, TNF-α., IL-2, IL-a, and IL-l β. In principle, bar-code array can provide high density assay of a much greater number of protein s simply by increasing the number of microchannel s used in flow patterning.

[00194] The detection of multiple targets was performed according to the schematic representation of **Figure 20** that shows the microfluidic device used in patient serum measurement In particular, **Figure 20 panel A** shows. the schematic of the operation of a microfluidic device that is bonded onto a barcode array glass slide.

[00195] **Figure 20 Panel B** shows a schematic illustrating the method to introduce fluid into microfluidic devices for molecular detection and in particular

interfacing the outside sample loading/injection systems to the microfluidic device using plastic tubing and metal pins.)

Example 8: Use of a barcoded array to detect proteins over a broad dynamic concentration range

[00196] A bio-barcode integrated with DEAL technology was used to detect biomarkers as illustrated in **Figure 21**. In particular **Figure 21** illustrates the increased dynamic range of a barcoded array when it is utilized with DEAL technology. The data show measurements of hCG, a pregnancy test marker, in human serum using the DEAL bar-code immunoassay that can cover the huge dynamic range >4 orders of magnitude.

[00197] In particular, the results illustrated in **Figure 21**, show that an expanded range of concentrations that can be detected from a single DEAL-based bio-barcode, demonstrated here for the detection of hCG. hCG is a pregnancy test marker, as well as a serum cancer marker. By varying the primary DNA oligomer concentration that binds the 1° antibody capture agent during the initial flow patterning step, a single set of bar-code can distinguish the hCG concentration spanning from 25000mIU/mL to O.25mIU/mL(not shown) in a single step.

Example 9: Barcoded array for detecting a biological profile: detection of human chorionic gonadotropin(hCG) over a period of time

[00198] Applicants performed a test on a series of standard human chorionic gonadotropin (hCG) spiked human serum samples provided by the National Cancer Institute (NCI). hCG is widely used for pregnancy testing, and also serves as a biomarker for gestational trophoblastic tumors and germ cell cancers of the ovaries and testes.

[00199] The results from these hCG assays are shown in **Figure 22**, which illustrate measurement of human chorionic gonadotropin(hCG) spiked in sera using a microfluidic DEAL barcode chip on an integrated platform including a barcoded array manufactured as described in U.S. Application entitled "Microfluidic Devices, Methods and Systems for Detecting Target Molecules" Serial No. 12/174,598.

[00200] In Panel a of **Figure 22**, fluorescence images of DEAL barcodes used in measuring standard hCG samples and two unknowns, are shown. The bars used to measure hCG were patterned with DNA strand A at different concentrations. TNF-α encoded by strand B was employed as a negative control. The lane indicated with REF represents the reference marker, while the other lanes indicate hCG test results in which the DNA was patterned from solutions at concentrations that varied from 2μM - 200μM. A negative control using TNF-α was also included.

[00201] ELISA-like sensitivity (~1mIU/mL), but with a broader detectable concentration range (~10^5), was demonstrated by quantitating fluorescence intensity. Moreover, even without photon integration, the analyte concentrations over a large range can be readily estimated by eye through pattern-recognition of the full barcode (See also indication in Example 5).

[00202] Quantitation of fluorescence signals obtained at different DNA loading was also performed as indicated in panel (b) of **Figure 22**. In such a barcoded array, the bar with high DNA-loading rendered great sensitivity at low analyte concentrations, whereas the bar with low DNA-loading was used to readily discriminate samples with high analyte concentrations. The two unknowns were also assayed and the results are in good agreement with ELISA tests run at NCI Laboratories.

[00203] Applicants noted that the hCG level is tracked during pregnancy, with concentrations in the blood increasing from ~5mIU/mL in the first week of pregnancy to ~$2x10^5$ mIU/mL in ten weeks. The microfluidic barcoded arrays used in the experiments herein described can accurately cover such a broad physiological hCG range.

Example 10: Barcoded array for detecting a biological profile: protein profiling in cancer patients

[00204] A barcoded array was used to detect a biological profile as illustrated in **Figure 23**. In particular, **Figure 23** shows the use of an integrated microfluidic DEAL barcoded device for human serum protein profiling. The serum samples from 12 cancer patients were measured in such prototype clinic test platform.

[00205] The protein profile obtained from this experiment exhibits unique patterns for individual patients, suggesting the efficacy of DEAL bar-code assay for serum-based cancer diagnosis and personalized medicine. This result displays a great indication for using a barcoded device and in particular an integrated DEAL barcode device for diagnostics and in particular human disease diagnostics.

[00206] In particular, the results of **Figure 23** show that the integrated DEAL Bio bar-code device can be used for rapid, sensitive and high-throughput protein measurements out of cancer patient sera. Panel A illustrates the design of the integrated microfluidic device that can conduct a dozen of serum assays at the same time in a highly automated fashion. Blue denotes the microfluidic channels for delivery of all reagents and samples. Magenta shows the control channel for pressure-actuated valves where they intersect the microfluidic channels. Overlay is a representative image of DEAL bar-code chip visualized by Cy5 fluorescence probes.

[00207] Measurement of 12 proteins out of 11 cancer patient serum samples and reference serum is illustrated in Panel B. The number denotes each individual lanes used for protein detection out of a patient sample.

[00208] Statistics of 12 protein level present in the serum samples from 12 different patients(S I-S 12), among which S1-5 are breast cancer patients while S6-S 11 are prostate cancer patients, is shown in Panel C. Each patient displays a unique pattern of serum proteins that are thought to be associated with their unique molecular origin of cancer.

[00209] A chart listing the specification and medical history of cancer patients is shown in panel D. Several unique signatures can be seen by comparing the medical record and the serum protein profile measured from DEAL bar-code assay.

Example 11: Barcoded array for detecting a biological profile: additional protein profiling in cancer patients

[00210] To further assess the utility and reproducibility of barcoded array for clinical blood samples, applicants measured a panel of 12 proteins from small amounts of serum from 24 cancer patients in a DEAL barcoded microfluidic device. The proteins in this panel included prostate specific antigen (PSA), as well as eleven

proteins secreted by various white blood cells. Each barcode was measured many times for each serum sample.

[00211] The stored serum samples from 11 breast cancer patients(all female) and 11 prostate cancer patients(all male) were acquired from Asterand. Two unknowns were acquired from Sigma-Aldrich. Nineteen out of 22 patients were Caucasian and the remaining three were Asian, Hispanic and African-American. The medical history is summarized in the supplementary materials.

[00212] Finger pricks were performed using BD Microtainer Contact-Activated Lancets. Blood was collected with SAFE-T-FILL capillary blood collection tubes (RAM Scientific), which we pre-filled with 80 μL of 25 mM EDTA solution. A 10 μL volume of fresh human blood from a healthy volunteer was collected in this EDTA-coated capillary, dispensed into the tube, and rapidly mixed by inverting a few times. The spiked blood sample was prepared in a similar means except that 40 μL of 25 mM EDTA solution and 40 μL of recombinant solution were mixed and pre-added in the collection tube. Then 2 μL of 0.5 M EDTA was added to bring the total EDTA concentration up to 25mM.

[00213] Execution of blood separation and plasma protein measurement was performed using an integrated platform extensively described in U.S. entitled "Microfluidic Devices, Methods and Systems for Detecting Target Molecules" Serial No. 12/174,598.

[00214] The integrated platforms were first blocked with the buffer solution for 30-60 minutes. The buffer solution prepared was 1% w/v Bovine Serum Albumin Fraction V (Sigma) in 150 mM 1x PBS without calcium/magnesium salts (Irvine Scientific). Then DNA-antibody conjugates (~50-100nM) were flowed through the plasma assay channels for ~30-45min. This step transformed the DNA arrays into capture-antibody arrays. Unbound conjugates were washed off by flowing buffer solution through the channels. At this step, the integrated platform was ready for the blood test. Two blood samples prepared as mentioned above were flowed into the integrated platforms within 1 minute of collection. The integrated platform quickly separated plasma from whole blood, and the plasma proteins of interest were captured in the assay zone where DEAL barcode arrays were placed. This whole process from

finger-prick to plasma protein capture took <10 minutes. In the cancer-patient serum experiment, the as-received serum samples were flowed into the integrated platforms without any pre-treatment (i.e. no purification or dilution). Afterwards, a mixture of biotin-labeled detection antibodies (~50-100nM) for the entire protein panel and the fluorescence Cy5-straptavidin conjugates (~100nM) were flowed sequentially into the integrated platforms to complete the DEAL immunoassay. The unbound fluorescence probes were rinsed off by flowing the buffer solution for 10 minutes. At last, the PDMS chip was removed from the glass slide. The slide was immediately rinsed in ½ x PBS solution and deionized water, and then dried with a nitrogen gun. Finally, the DEAL barcode slide was scanned by an Axon Instruments Genepix Scanner.

[00215] The serum samples from 24 cancer patients were assayed using two chips, each containing 12 separate assay units that were operated in parallel. In every assay unit, 50 sets of DEAL barcodes were placed in the detection channel for statistical sampling of the serum. In all experiments, 25μL of patient serum, or 500 nanoliters per barcode, was used for each assay. The white-blood cell secreted proteins included inflammatory molecules and cytokines. These proteins are employed by immune cells for intracellular communication, and have significant implications in tumor microenvironment formation, and in tumor progression and metastasis. Thus, this panel provides information on both cancer and the immune system.

[00216] Experiments were repeated at least 2-3 times. In every integrated platform, multiple sets of barcode arrays were patterned in a single assay channel to allow simultaneous parallel measurements. For example, 50 sets of barcode were used in assaying a cancer patient serum sample, with each barcode detecting the full panel of proteins. Quantitation of fluorescence signal was performed using either the Genepix software or imageJ (NIH). In processing the cancer-patient data, the background intensity for each channel was individually identified, and then re-assigned to a common background level of 20 arbitrary units. The intensities of all "bars" in a given channel are normalized to that channel's background. Therefore, the data in Figure 10 corresponds to the bar's fluorescence intensity differences relative to its own channel's background, plus the common background level of 20. This treatment minimizes interference from non-specific background signal, but could make it indistinguishable between the positive results with high background (e.g. B10) and the true negative results (e.g. B9 and B11).

[00217] The results are illustrated in **Figures 24 and 25**, which show the related profile of cancer patients (**Figure 24**) together with their medical history (**Figure 25**).

[00218] In particular, fluorescence images each showing four sets of representative barcodes obtained from the 24 patient samples are shown in **Figure 24**. The proteins measured include cancer marker PSA and eleven cytokines also indicated in details in **Figure 25**. In the barcode image panel, the left two columns were performed on the same chip while the right two were from the other. The samples were randomly picked in the assay to minimize arbitrary bias. B01-B11 denote 11 samples from breast cancer patients, P01-P11 denote those from prostate cancer patients, whereas the S01 and S02 are unknown samples from a different supplier.

[00219] The medical records for all patients are summarized in **Figure 24** which shows a brief summary of cancer patient medical records. The two unknowns are not included in the table.

[00220] A more detailed medical history of the patients is included in the following table 1.

Table 1 Medical Record of cancer patients.

PATIENT	CANCER	GENDER/AGE	RACE	UICC STAGE	GLEASONS SCORE	OTHERS
B01	Breast	Female/62	Caucasian	T2N0M0		wine 200mL/day
B02	Breast	Female/79	Caucasian	T4N2M0		
B03	Breast	Female/71	Caucasian	T1cNXM0		1-2 drinks/day
B04	Breast	Female/72	Caucasian	T2NXM0		hypertension
B05	Breast	Female/89	Caucasian	T3N0MX		arthritis
B06	Breast	Female/56	Asian	T1NXM0		
B07	Breast	Female/54	Caucasian	T2N2M0		hypertension, obesity
B08	Breast	Female/55	Caucasian	T2NxM0		1-5 cigs/day, wine 200mL/day
B09	Breast	Female/83	Caucasian	T4N0M0		Coronary artery disease, cerebral atherosclerosis
B10	Breast	Female/63	Hispanic	T3N2MX		6-10cigs/day, hyperthyroid, hypertension, osteoarthritis
B11	Breast	Female/63	Caucasian	T1NXM0		arterial hypertension
P01	Prostate	Male / 51	Caucasian	T2cNXM0	4+3=7	
P02	Prostate	Male / 64	Caucasian	T3bN0MX	3+4=7	
P03	Prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	hypertension
P04	Prostate	Male / 55	Caucasian	T2bN0M0	3+3=6	11-20 cigs/day
P05	Prostate	Male / 73	Caucasian	T3aNXMX	4+4=8	hypertension,11-20 cigs/day
P06	Prostate	Male/64	Caucasian	T3N0M0		chronic bronchitis, 11-20cigs/day
P07	Prostate	Male/60	Caucasian	T3aN0M0	3+4=7	gastroesophageal reflux
P08	Prostate	Male/72	African Am.	T2aNXMX	3+3=6	1-5cigs/day
P09	Prostate	Male/78	Caucasian	T3aN1MX	4+3=7	hypertension, atrial fibrillation
P10	Prostate	Male/66	Caucasian	T2aN0MX	3+3=6	hypertension, 11-20 cigs/day
P11	Prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	hypertension
S01	Unknown					
S02	Unknown					

[00221] Many proteins were successfully detected with high signal-to-noise, and the barcode signatures are distinctive among patients. Most assays show a relatively low fluorescence background. However, the assays on P05, P04, P10 and B10 were characterized by a high, interfering background. These high background assays all correlate with patients that were heavy smokers (~11-20cigs/day); only one serum sample from a heavy smoker did not exhibit a high background (P06). The reason for this high background fluorescence remains unclear. A possible cause is the elevated blood content of the fluorescent carboxyhemoglobin formed in lung. While this identification of smokers constitutes unexpected information from the IBBCs, it also means that, for these patients, some pre-purification of the plasma or serum will be required in order to assay serum protein levels.

[00222] The protein panels used in the cancer-patient serum experiment (panel 1) and finger-prick blood test (panel 2), the corresponding DNA codes, and their sequences are summarized in Tables 2 and 3. These DNA oligomers were synthesized by Integrated DNA Technologies (IDT), and purified by high pressure liquid chromatography (HPLC). The quality was confirmed by mass spectrometry.

Table 2. List of protein panels and corresponding DNA codes.

DNA-code	Human Plasma Protein	Abbreviation
Panel (1)		
A/A'	Interferon-gamma	IFN-γ
B/B'	Tumor necrosis factor-alpha	TNF-α
C/C'	Interleukin-2	IL-2
D/D'	Interleukin-1 alpha	IL-1α
E/E'	Interleukin-1beta	IL-1β
F/F'	Transforming growth factor beta	TGF-β
G/G'	Prostate specific antigen (total)	PSA
H/H'	Interleukin-6	IL-6
I/I'	Interleukin-10	IL-10
J/J'	Interleukin-12	IL-12
K/K'	Granulocyte-macrophage colony stimulating factor	GM-CSF
L/L'	Monocyte chemoattractant protein -1	MCP-1
M/M'	Blank control/reference	
Panel (2)		
AA/AA'	Interleukin-1beta	IL-1β
BB/BB'	Interleukin-6	IL-6
CC/CC'	Interleukin-10	IL-10

DD/DD'	Tumor necrosis factor-alpha	TNF-α
EE/EE'	Complement Component 3	C3
FF/FF'	C-reactive protein	CRP
GG/GG'	Plasminogen	Plasminogen
HH/HH'	Prostate specific antigen (total)	PSA

Table 3. List of DNA sequences used for spatial encoding of antibodies

Sequence Name	Sequence)	SEQ ID NO	Tm (50mM NaCl) °C
A	5'- AAA AAA AAA AAA AAT CCT GGA GCT AAG TCC GTA-3'	1	57.9
A'	5' NH3- AAA AAA AAA ATA CGG ACT TAG CTC CAG GAT-3'	2	57.2
B	5'-AAA AAA AAA AAA AGC CTC ATT GAA TCA TGC CTA -3'	3	57.4
B'	5' NH3AAA AAA AAA ATA GGC ATG ATT CAA TGA GGC -3'	4	55.9
C	5'- AAA AAA AAA AAA AGC ACT CGT CTA CTA TCG CTA -3'	5	57.6
C'	5' NH3-AAA AAA AAA ATA GCG ATA GTA GAC GAG TGC -3'	6	56.2
D	5'-AAA AAA AAA AAA AAT GGT CGA GAT GTC AGA GTA -3'	7	56.5
D'	5' NH3-AAA AAA AAA ATA CTC TGA CAT CTC GAC CAT -3'	8	55.7
E	5'-AAA AAA AAA AAA AAT GTG AAG TGG CAG TAT CTA -3'	9	55.7
E'	5' NH3-AAA AAA AAA ATA GAT ACT GCC ACT TCA CAT -3'	10	54.7
F	5'-AAA AAA AAA AAA AAT CAG GTA AGG TTC ACG GTA -3'	11	56.9
F'	5' NH3-AAA AAA AAA ATA CCG TGA ACC TTA CCT GAT -3'	12	56.1
G	5'-AAA AAA AAA AGA GTA GCC TTC CCG AGC ATT-3'	13	59.3
G'	5' NH3-AAA AAA AAA AAA TGC TCG GGA AGG CTA CTC-3'	14	58.6
H	5'-AAA AAA AAA AAT TGA CCA AAC TGC GGT GCG-3'	15	59.9
H'	5' NH3-AAA AAA AAA ACG CAC CGC AGT TTG GTC AAT-3'	16	60.8
I	5'-AAA AAA AAA ATG CCC TAT TGT TGC GTC GGA-3'	17	60.1
I'	5' NH3-AAA AAA AAA ATC CGA CGC AAC AAT AGG GCA-3'	18	60.1
J	5'-AAA AAA AAA ATC TTC TAG TTG TCG AGC AGG-3'	19	56.5
J'	5' NH3-AAA AAA AAA ACC TGC TCG ACA ACT AGA AGA-3'	20	57.5
K	5'-AAA AAA AAA ATA ATC TAA TTC TGG TCG CGG-3'	21	55.4
K'	5' NH3-AAA AAA AAA ACC GCG ACC AGA ATT AGA TTA-3'	22	56.3
L	5'-AAA AAA AAA AGT GAT TAA GTC TGC TTC GGC-3'	23	57.2
L'	5' NH3-AAA AAA AAA AGC CGA AGC AGA CTT AAT CAC-3'	24	57.2
M	5'-Cy3-AAA AAA AAA AGT CGA GGA TTC TGA ACC TGT-3'	25	57.6
M'	5' NH3-AAA AAA AAA AAC AGG TTC AGA ATC CTC GAC-3'	26	56.9
AA'	5' NH3-AAAAAAAAAAGTCACAGACTAGCCACGAAG-3'	27	58
BB	5'-AAAAAAAAAAGCGTGTGTGGACTCTCTCTA-3'	28	58.7
BB'	5' NH3-AAAAAAAAAATAGAGAGAGTCCACACACGC-3'	29	57.9
CC	5'-AAAAAAAAAATCTTCTAGTTGTCGAGCAGG-3'	30	56.5
CC'	5' NH3-AAAAAAAAAACCTGCTCGACAACTAGAAGA-3'	31	57.5
DD	5'-AAAAAAAAAAGATCGTATGGTCCGCTCTCA-3'	32	58.8

Table 3. List of DNA sequences used for spatial encoding of antibodies

Sequence Name	Sequence)	SEQ ID NO	Tm (50mM NaCl) °C
DD'	5' NH3-AAAAAAAAAATGAGAGCGGACCATACGATC-3'	33	58
EE	5'-AAAAAAAAAAGCACTAACTGGTCTGGGTCA-3'	34	59.2
EE'	5' NH3-AAAAAAAAAATGACCCAGACCAGTTAGTGC-3'	35	58.4
FF	5'-AAAAAAAAAATGCCCTATTGTTGCGTCGGA-3'	36	60.1
FF'	5' NH3-AAAAAAAAAATCCGACGCAACAATAGGGCA-3'	37	60.1
GG	5'-AAAAAAAAAACTCTGTGAACTGTCATCGGT-3'	38	57.8
GG'	5' NH3-AAAAAAAAAAACCGATGACAGTTCACAGAG-3'	39	57
HH	5'-AAAAAAAAAAGAGTAGCCTTCCCGAGCATT-3'	40	59.3
HH'	5' NH3-AAAAAAAAAAAATGCTCGGGAAGGCTACTC-3'	41	58.6

* All amine-terminated strands were linked to antibodies to form DNA-antibody conjugates using SFB/SANH coupling chemistry as described by R. Bailey et al.[1] Codes AA-HH were used in the experiment which examined fresh whole blood from a heathy volunteer. Codes A-M were used for the molecular analyses of cancer patient serum samples.

Example 12: Barcoded array for detecting a biological profile: quantitative protein profiling in cancer patients

[00223] The blood barcodes measured throughout the experiments illustrated in Example 10 were unique for each patient.

[00224] **Figures 26 to 28** show quantitation and clustering of cancer patient barcode data obtained using a barcode array designed as exemplified in Example 8. In particular, **Figure 26** shows layout of the barcode array used in this study. Strand M denotes the reference (control). **Figure 27** illustrates a plot showing quantitation of fluorescence signals of all proteins (left axis) detected as shown in Figure 21A for all cancer patients (from left: B01-B11, P01-P11, S01 and S02). S01 and S02 are two unknown serum samples. **Figure 28** shows an exemplary manual clustering of cancer patients derived on the basis of protein patterns. First, all prostate cancer patients are clearly identified by PSA. Second, both breast and prostate cancer patients exhibit possible subpopulations with distinct cytokine profiles.

[00225] The fluorescence signals intensity for all the patient samples are plotted in **Figure 26**. The cancer marker, PSA, clearly distinguishes between the breast and prostate cancer patients, and allowed for the unknown samples, S01 and S02, to be assigned to prostate cancer patients. Applicants then performed a manual

clustering of patients on the basis of protein signals and generated the map schematically illustrated in **Figure 27** to assess the potential of this technology for patient stratification. This approach is only going to be as good as the biomarker panel itself, and the number of serum samples profiled is small. Nevertheless, the results are encouraging. For example, the measured profiles of breast cancer patients can be classified into three subsets – non-inflammatory, IL-1β positive, and TNF-α positive. The prostate cancer patient data exhibits a generally higher level of inflammation, and those inflammation-positive samples can also be classified as shown in **Figure 27**. An interesting observation is the lack of IL-10 signal for most patients. IL-10 is a cytokine production suppressor that functions as an anti-inflammatory mediator, and its absence may reflect deviation from normal immune homeostasis in local tumor sites. Applicants have initiated studies involving a larger number of proteins and a much larger number of blood samples. Researches have been focused on developing technologies for multiplexed measurement of cytokines, and serum cytokine profiling has shown relevance in cancer diagnostics and prognostics. The results described above have clearly demonstrated that integrated platforms can be applied to the multiparameter analysis of human health-relevant proteins.

[00226] The principal goal behind developing the integrated platform was to be able to measure the levels of a large number of proteins in human blood within a few minutes of sampling that blood, so as to avoid protein degradation that can occur when plasma is stored. In a typical 96 well plate immunoassay, the biological sample of interest is added, and the protein diffuses to the surface-bound antibody. Under sufficient flow conditions, diffusion is no longer important, and the only parameter that limits the speed of the assay is the protein/antibody binding kinetics (the Langmuir isotherm), thus allowing the immunoassay to be completed in just a few minutes.

Example 13: Barcoded array for detecting a biological profile: human plasma proteome

[00227] Use of a barcoded array was tested to verify improved sensitivity for plasma protein assays.

[00228] The human plasma proteome is comprised of three major classes of proteins – classical plasma proteins, tissue leakage proteins, and cell-cell signaling

molecules (cytokines and chemokines). Cell-cell signaling molecules are biologically informative in a variety of physiological and pathological processes, i.e. tumor host immunity and inflammation.

[00229] The results of a first series of experiments performed by the Applicants are illustrated in **Figure 29,** wherein a detection of target protein other than cytokines TNF-α, and Interleukins such as IL-6, IL-10 is shown. In particular, **Figure 29**, shows detection of molecules such as CRP, C3 and plasminogen associated with biological profile such inflammation response (CRP), complement system (C3) and liver toxicity response (CRP and plasminogen).

[00230] The results of a second series of experiments performed by the Applicants is summarized in the diagram of **Figure 30**, showing a schematic of human plasma proteome (refer to N.L. Anderson and N.G. Anderson, Molecular & Cellular Proteomics 11, 845, 2001).

[00231] As shown in **Figure 30**, the concentration range of plasma proteins spans 12 orders of magnitude and the lowest end is approximately at the detection limit of mass spectrometry – a high-throughput protein profiling technique. The state-of-the-art for clinical protein measurements is still the ELISA assay. Yet ELISA is a low-throughput process, requiring a large amount of sample and long duration to complete a multiparameter plasma protein measurement. The high performance of the DEAL barcode chip, especially its increased sensitivity, is a key to realizing highly multiplexed measurements of a panel of proteins, including the low abundance cytokines, from small quantities of clinical blood samples.

[00232] Applicants therefore concluded that the DEAL barcode assay has a markedly high sensitivity, comparable to ELISA, leading to the feasibility of multiplexed detection of plasma proteins including low-abundance cell-cell signaling molecules, e.g. cytokines and chemokines, from a small quantity of sample.

Example 14: Assay performed in a barcoded array

[00233] For the assays shown in the Examples 3-13 illustrated in the related figures, a DEAL immunoassay was used. To detect each protein, a pair of antibodies was chosen. One is conjugated to the secondary DNA strands that are complementary to the primary DNA strands flow-patterned on glass slides. This antibody also serves to capture proteins being detected, and then the biotin-labeled detection came in to

bind to the same protein creating immunosandwich structure. Finally, Cy-3 or Cy5labled fluorescent streptavidin was used to visualize the results of bar-code through streptavidin-biotin binding.

[00234] Detection of human cytokine proteins prepared at different concentrations was first tested (**Figure 15**). The results show the detection is highly specific, and exhibits increased sensitivity comparable to ELISA. Then, a multiparameter (up to 5 proteins) detection was demonstrated as in **Figure 16**. TNF-a exhibits the best signal intensity due to the high affinity of the 10 anti-TNF-a AB. Having the high loading of primary DNA oligomers and by varying DNA concentrations in flow-pattering step, it is shown the a single bar-code can detect protein like hCG across a huge dynamic range, several orders of magnitube better than any conventional protein detection methods (**Figure 21**). Finally, an integrated microfluidic device was fabricated, which comprises of a two-layer PDMS microfluidic chip bonded on to a bar-DEAL barcode glass chip, that allows rapid, sensitive detection of 13 different proteins at the same time out of 12 different human serum samples. The DEAL bar-code devices for the first time provide a highly multiplexed (as in protein microarray and mass spectrometry) method for protein detection at an ultra-high sensitivity as good as the state-of-art ELISA assay.

[00235] Barcoded array patterning is a generic technique that can be exploited to pattern DNA, protein, or even sera and tissue lysates. The inverse-phase bar-code array (serum or lysate array) can be used for high throughput drug screening and biomarker discovering.

Example 15: Manufacturing a barcoded array for magnetic ID

[00236] A schematic representation of a method to manufacture a magnetic ID barcode on a small object such as a ring is shown in **Figure 31.**

[00237] A PDMS microfluidic channels with a small exposed contact area can be manufactured using two-layer lithography (it means there are two layers of fluidic channels. The bottom layer can be contacted with the substrate e.g. the small-sized product and the fluid can be introduced from the upper layer that contains embedded fluidic channels to join the bottom layer channels at the small contact area to the large inlets at the sides of the PDMS device.

[00238] Once this PDMS device is attached onto the small subject, a number of distinct different molecules were flowed to the contact arca to create a DNA barcoded array. Next, a library of complementary DNA-magnetic nanoparticle conjugates can be synthesized.

[00239] Therefore, the fabrication of magnetic barcode can be realized by simply immersing the small-sized subject patterned with DNA barcodes into a solution that contains several complementary DNA-magnetic nanoparticle conjugates. The different combination of complementary DNA-magnetic nanoparticle conjugates gives rise to a distinct magnetic ID barcode that can be readily read with a magnetoresistive scan head.

[00240] The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the devices, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure.

[00241] In summary, in some embodiments, arrays and substrates comprising a material are disclosed and related methods and systems. In the arrays and substrates the material can be formed in particular by capture agents and/or detectable targets and can be attached to the substrates along substantially parallel lines forming a barcoded pattern.

[00243] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the disclosure pertains.

[00245] It is to be understood that the disclosures are not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. The term "plurality" includes two or more referents unless the content clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the specific examples of appropriate materials and methods are described herein.

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An array, comprising:

a solid substrate comprising a surface and a plurality of primary antibodies attached to the surface in a pattern, wherein the plurality of primary antibodies attached in the pattern bind different cellular components, and wherein between 3 and 50 different primary antibodies of the plurality of primary antibodies are attached to the solid surface in the pattern;

a polydimethylsiloxane (PDMS) mold comprising a plurality of wells releasably coupled with the solid substrate,

wherein the plurality of wells are oriented to intersect with the pattern of attached primary antibodies, and

wherein each well is exposed to the pattern of attached primary antibodies.

2. The array of claim 1, further comprising an average of one cell per well of the plurality of wells, wherein the cell in each well is exposed to the pattern of attached primary antibodies.

3. The array of claim 2, wherein the cell in each well is exposed to the pattern of attached antibodies under reaction conditions that result in specific interactions between said cell and said attached primary antibodies.

4. The array of claim 2 or 3, further comprising a composition comprising a labeled secondary antibody specific for each primary antibody of the plurality of primary antibodies, wherein each labeled secondary antibody detects a specific interaction between a primary antibody and the cell or a component thereof and wherein a discrete cellular signature comprises a plurality of specific interactions.

5. The array of any one of claims 1 to 4, wherein the plurality of wells are oriented to intersect with the pattern of attached primary antibodies in a repeating manner such that a discrete cellular signature is formed at each well.

6. The array of any one of claims 1 to 5, wherein the primary antibodies specifically recognize selected biomolecules present in at least a portion of the plurality of wells.

7. The array of claim 6, wherein the biomolecules comprise proteins, peptides, nucleic acids, lipids, chemokines, cytokines, or growth factors, or any combination thereof.

8. The array of any one of claims 2 to 7, wherein each well comprises secreted cellular components.

9. The array of any one of claims 1 to 7, wherein each well comprises biomolecules prepared in buffer solution.

10. The array of any one of claims 1 to 8, wherein each well comprises biomolecules in a fluid, and wherein the fluid comprises blood.

11. The array of any one of claims 1 to 8, wherein each well comprises biomolecules in a fluid and wherein the fluid comprises plasma.

12. The array of any one of claims 1 to 8, wherein each well comprises biomolecules in a fluid and wherein the fluid comprises serum.

13. The array of any one of claims 1 to 8, wherein each well comprises biomolecules in a fluid and wherein the fluid comprises blood, plasma and serum.

14. The array of any one of claims 2 to 7, wherein each well comprises a single cell and associated cellular components.

15. The array of any one of claims 4 to 14, wherein the labeled secondary antibodies comprise fluorescent, gold, or silver labels.

16. A composition comprising:

a solid substrate comprising a surface and a plurality of primary antibodies attached to the surface in a pattern, wherein the plurality of primary antibodies attached in the pattern bind different cellular components, and wherein between 3 and 50 different primary antibodies of the plurality of primary antibodies are attached to the solid surface in the pattern; and

a polydimethylsiloxane (PDMS) mold that releasably couples with the solid substrate, the mold having a plurality of channels into which a plurality of primary antibodies are introduced and exposed to the solid substrate surface whereby at least a portion of the plurality of primary antibodies are attached to the solid substrate in the pattern when the mold is uncoupled from the solid substrate.

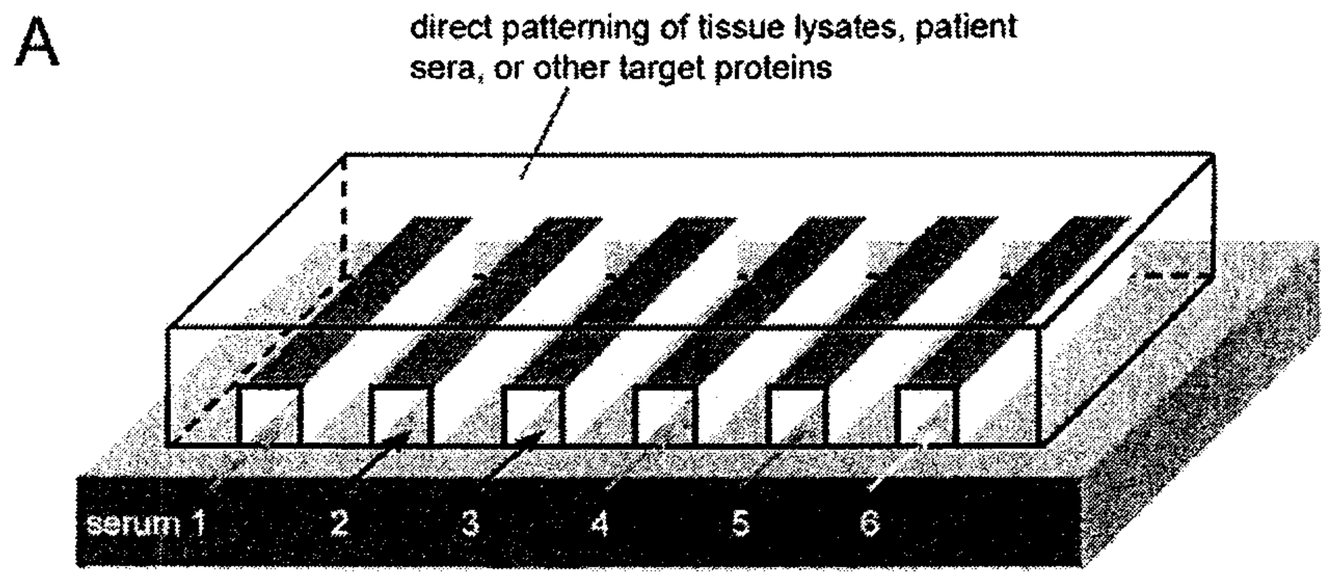

A
direct patterning of tissue lysates, patient sera, or other target proteins
serum 1
2
3
4
5
6
B
Drug A
Drug B
Drug C
Drug D
Drug E
Drug F
Drug G
serum array
microfluidic channel

FIG. 1

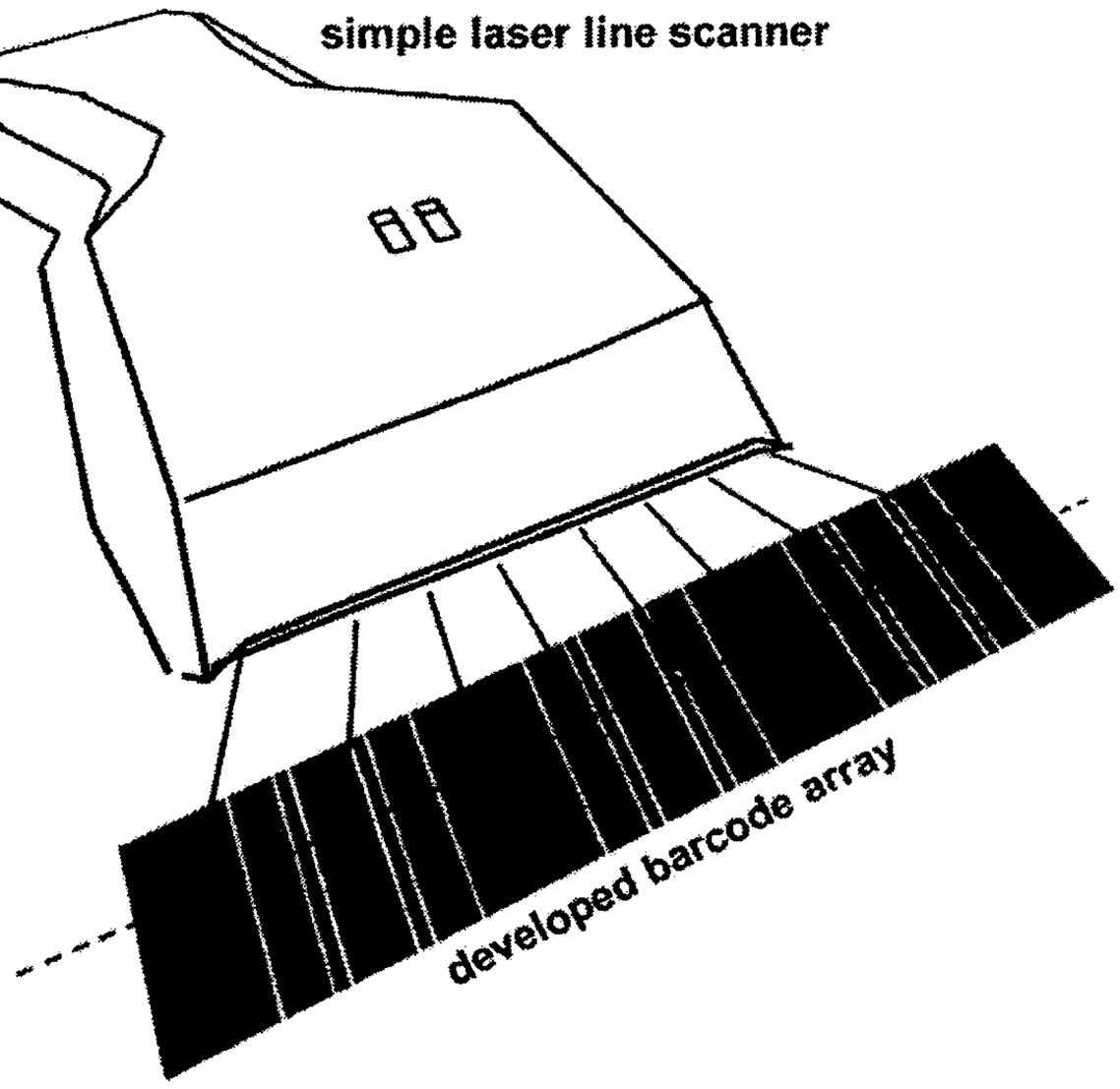


FIG. 2

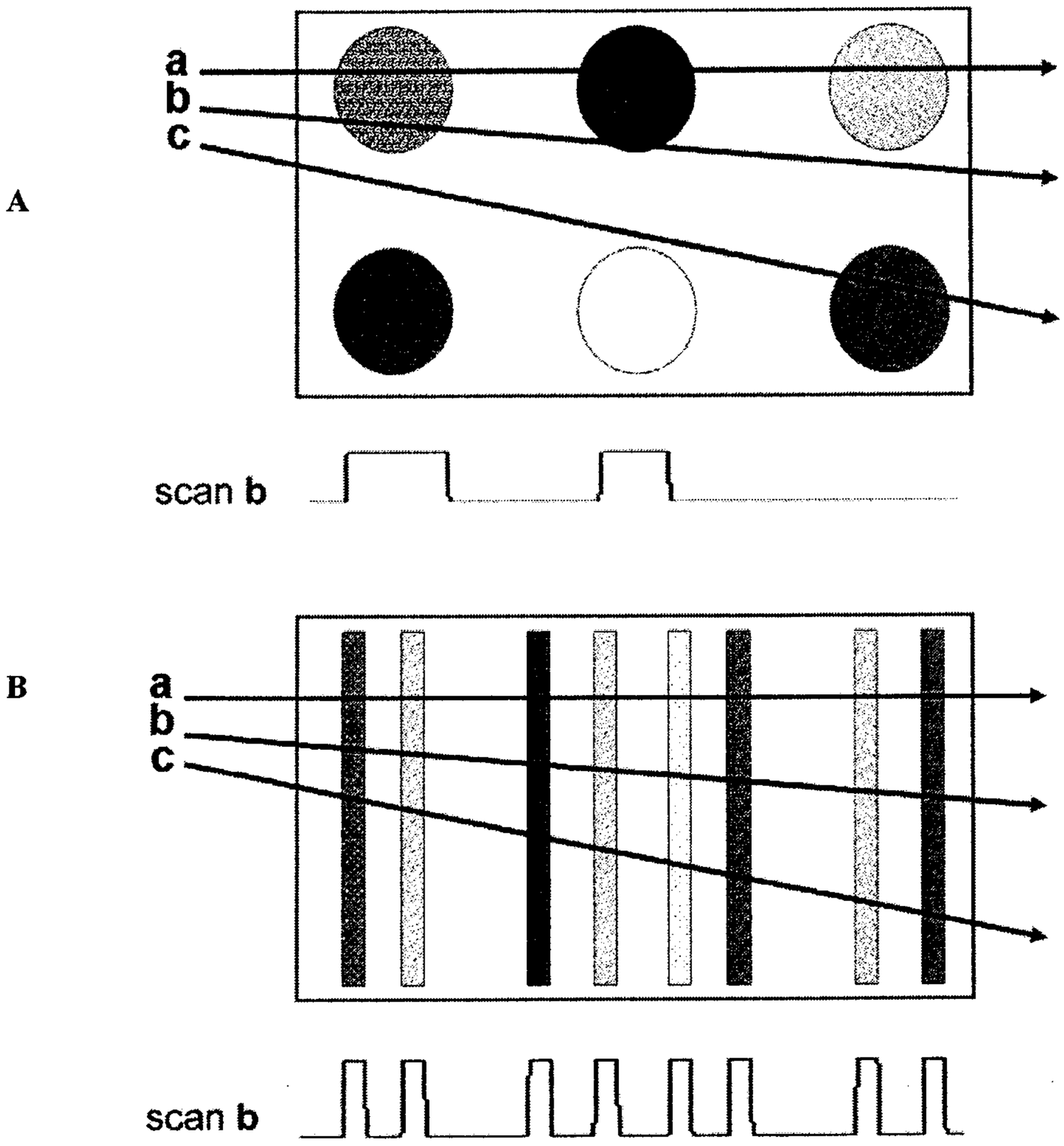


FIG. 3

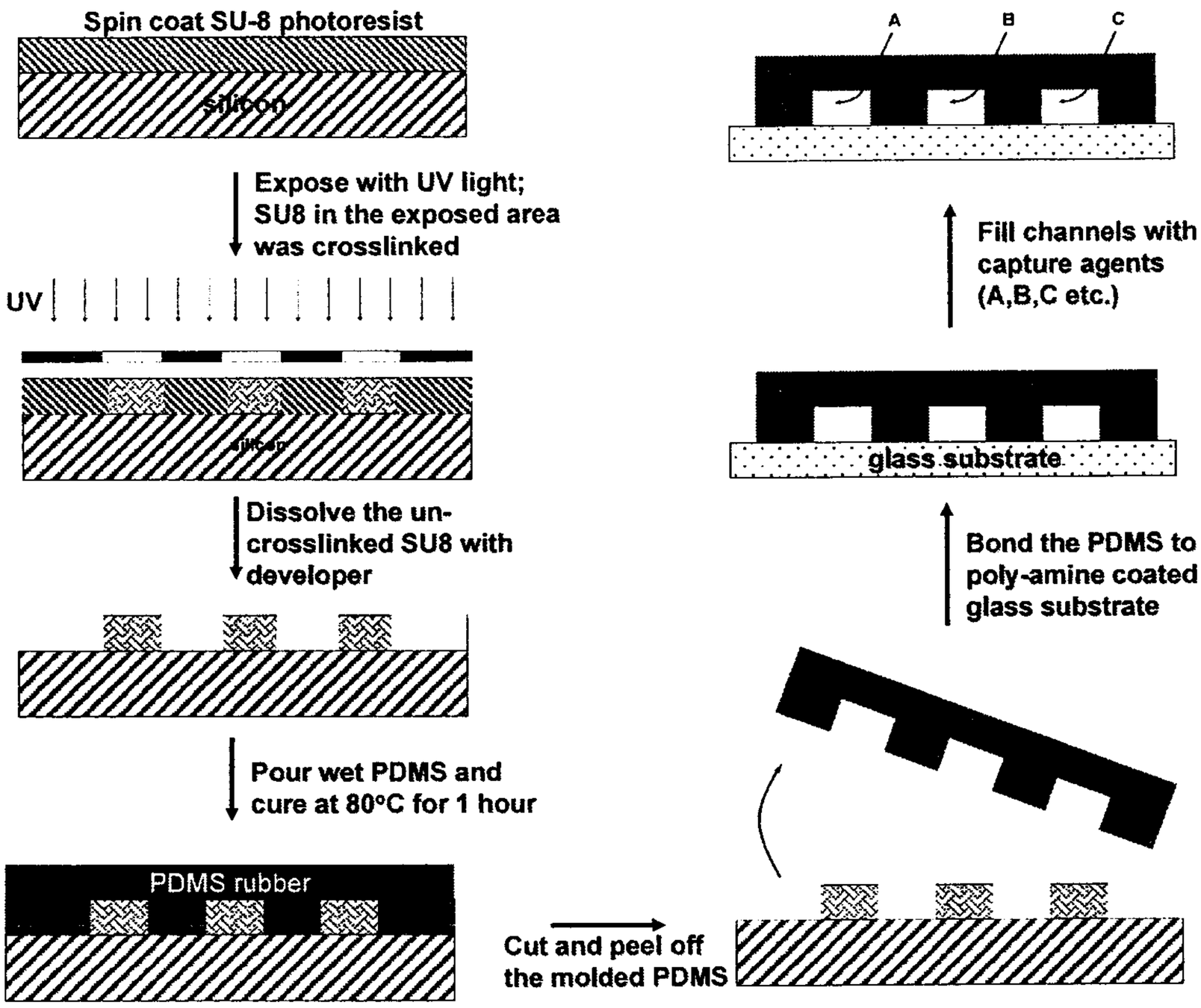

Spin coat SU-8 photoresist
silicon
Expose with UV light;
SU8 in the exposed area
was crosslinked
UV
Dissolve the un-
crosslinked SU8 with
developer
Pour wet PDMS and
cure at 80°C for 1 hour
PDMS rubber
Cut and peel off
the molded PDMS
A
B
C
Fill channels with
capture agents
(A,B,C etc.)
glass substrate
Bond the PDMS to a
poly-amine coated
glass substrate

FIG. 4

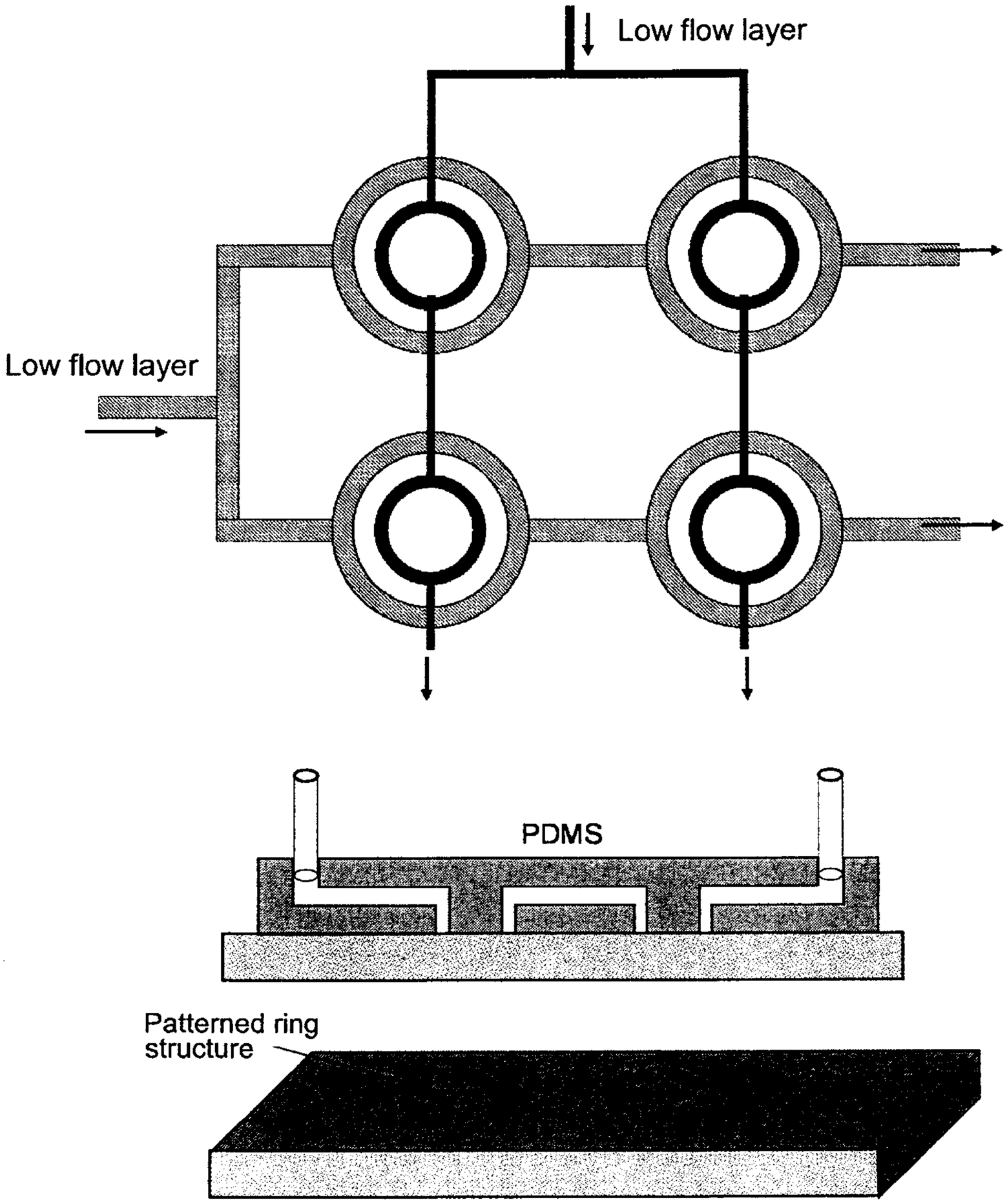

Low flow layer
Low flow layer
PDMS
Patterned ring structure

FIG. 5

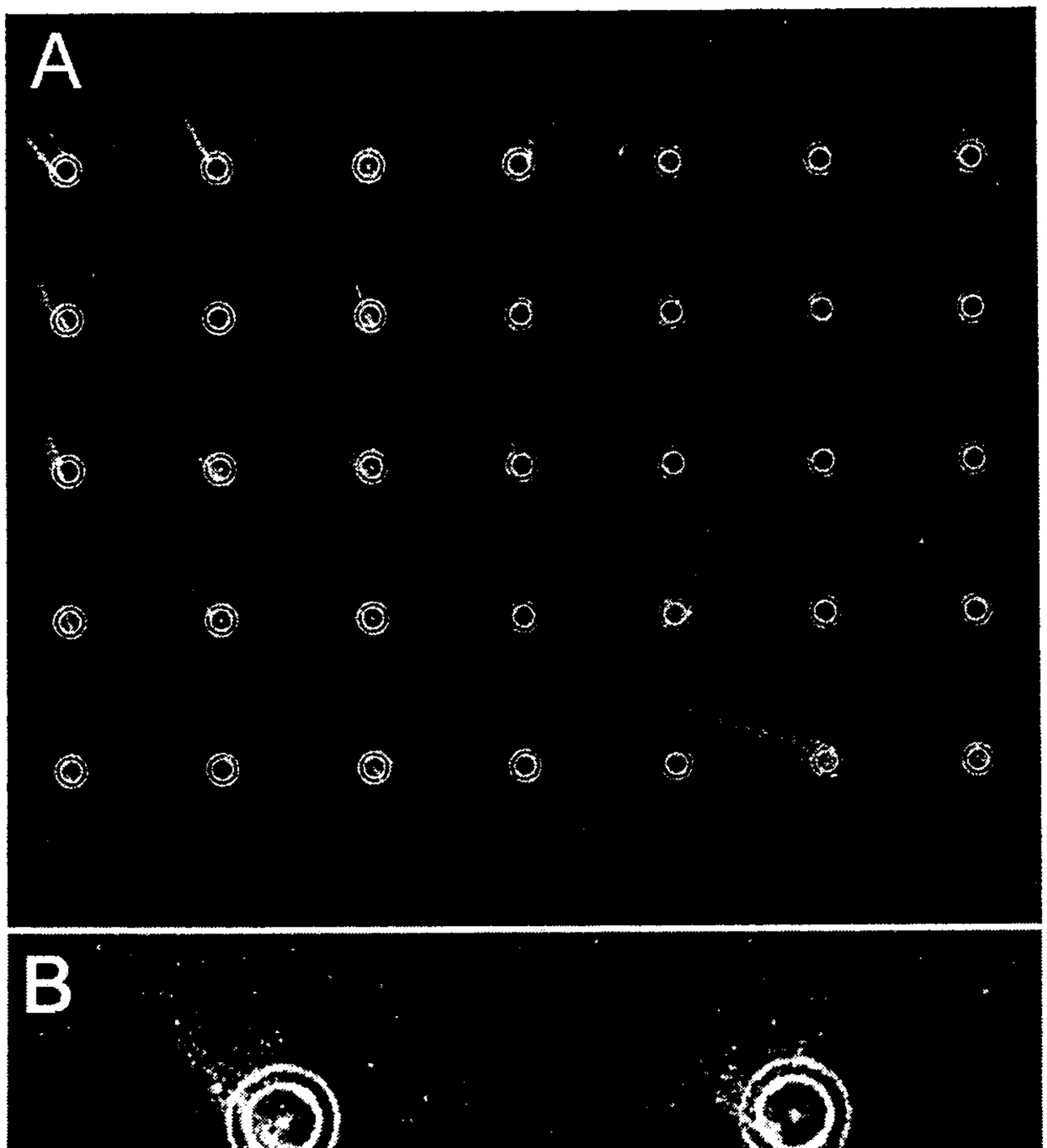


FIG. 6

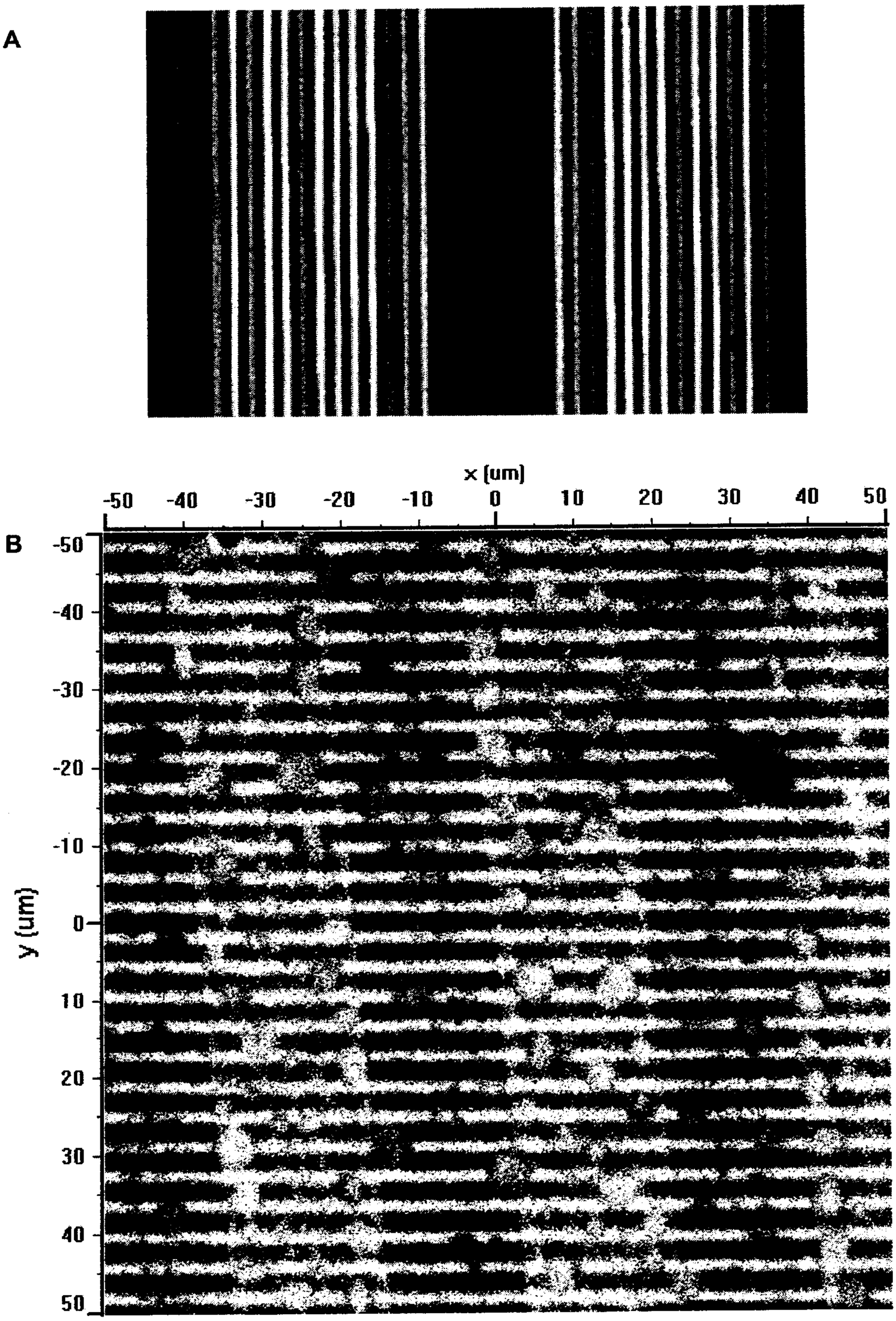


FIG. 7

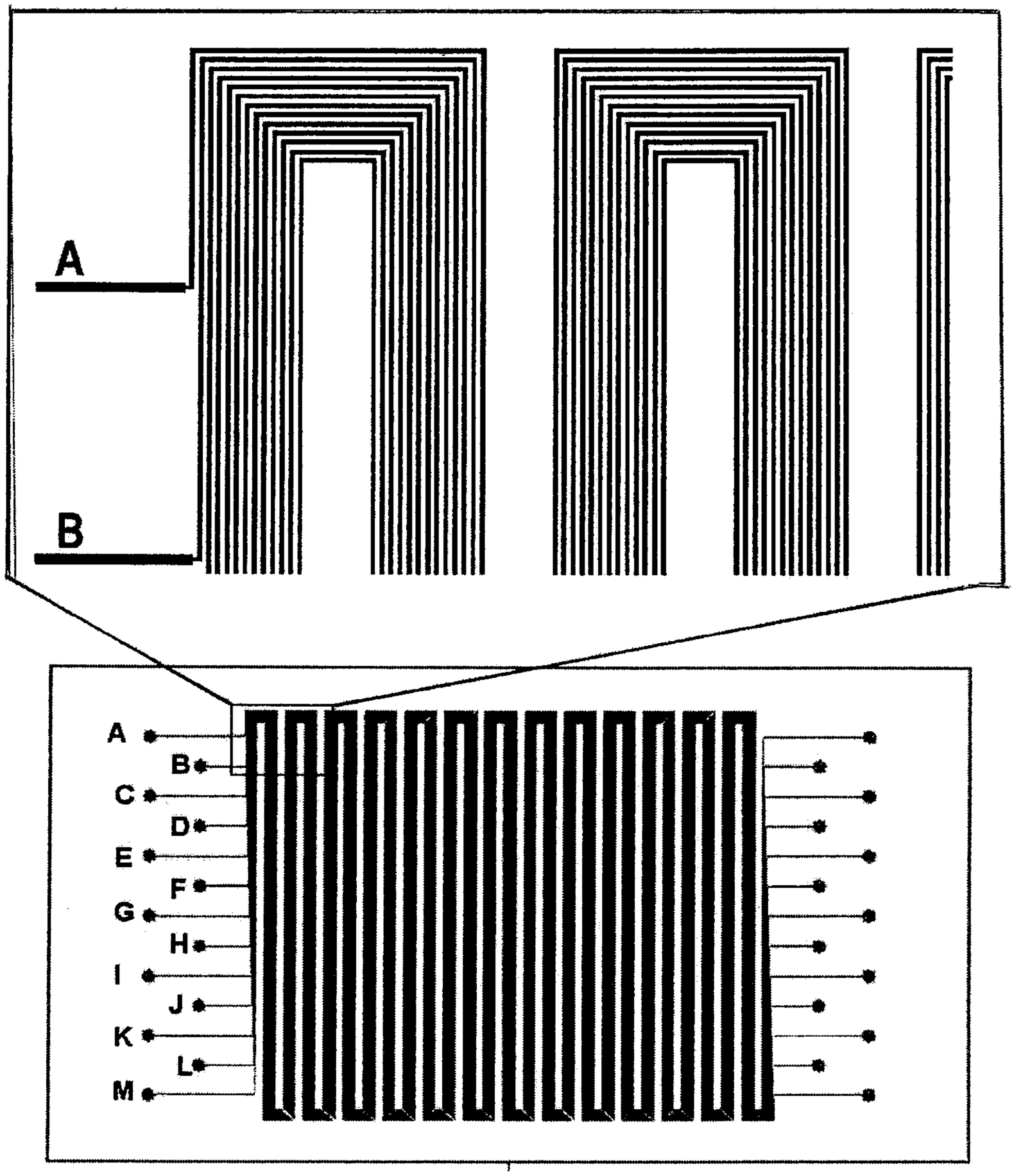


FIG. 8

A

Each well is used to test a different sample

B

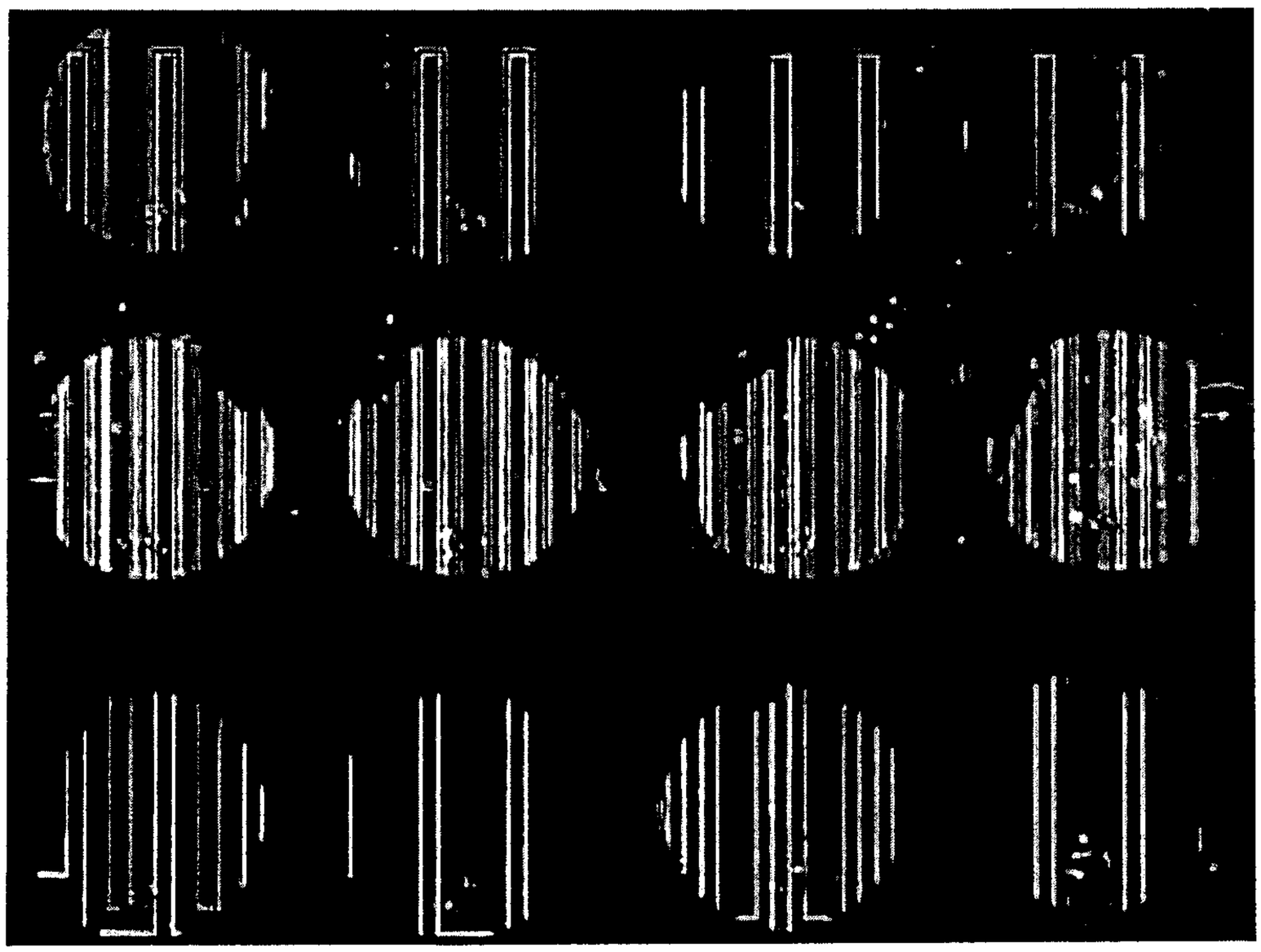


Fig. 9

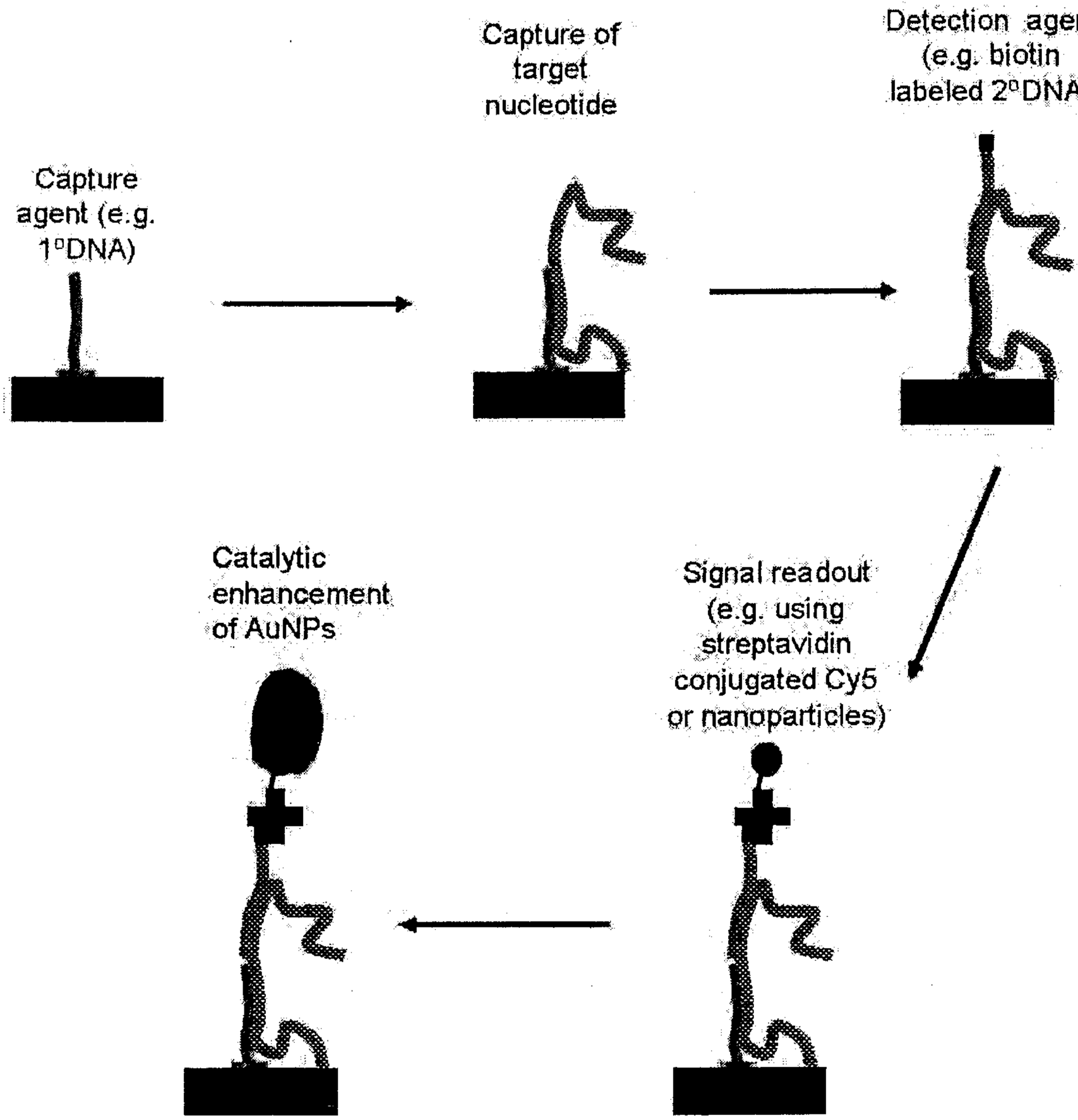

Capture of target nucleotide
Detection agent (e.g. biotin labeled 2°DNA)
Capture agent (e.g. 1°DNA)
Catalytic enhancement of AuNPs
Signal readout (e.g. using streptavidin conjugated Cy5 or nanoparticles)

FIG. 10

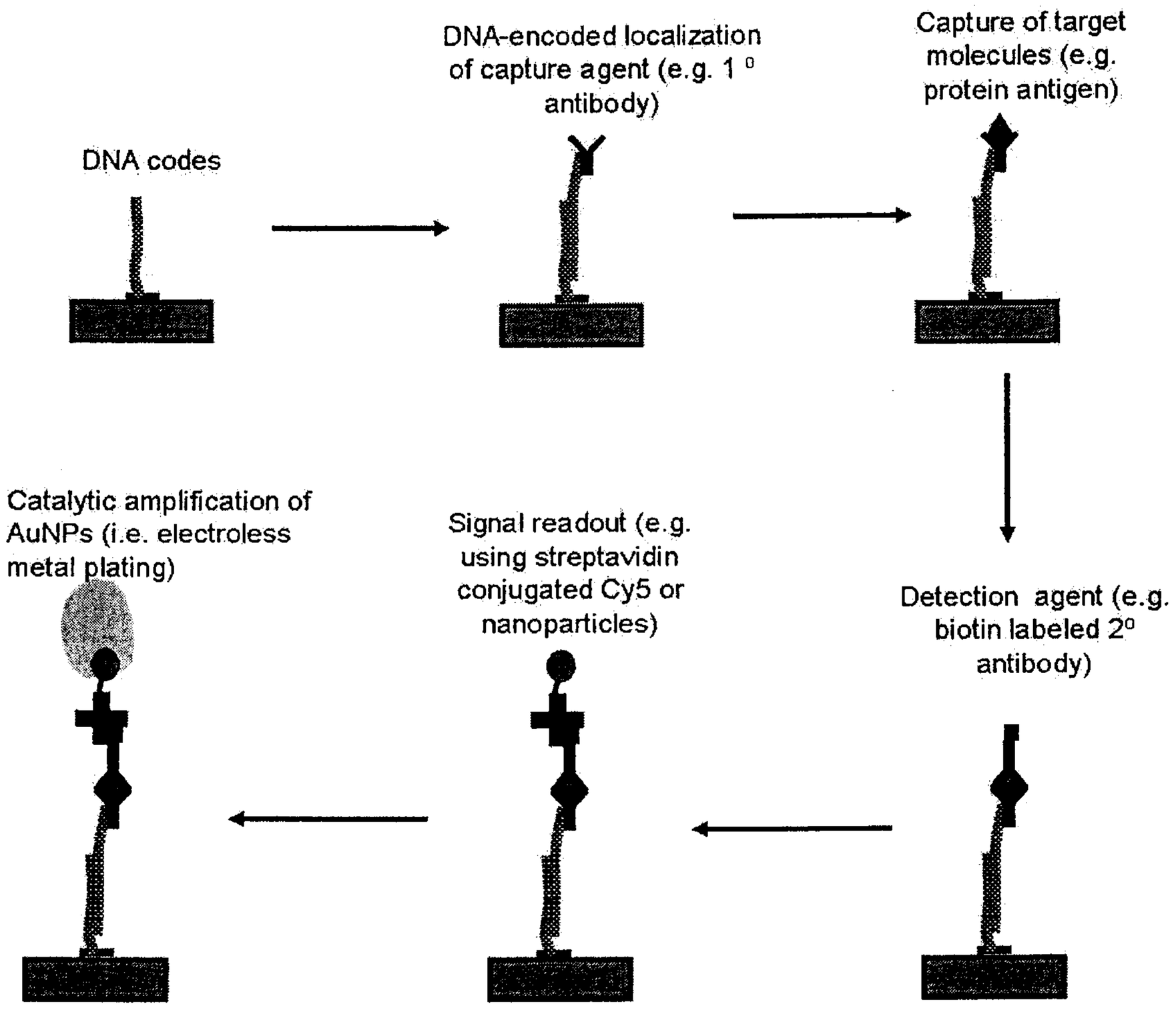

DNA codes
DNA-encoded localization of capture agent (e.g. 1° antibody)
Capture of target molecules (e.g. protein antigen)
Detection agent (e.g. biotin labeled 2° antibody)
Signal readout (e.g. using streptavidin conjugated Cy5 or nanoparticles)
Catalytic amplification of AuNPs (i.e. electroless metal plating)

FIG. 11

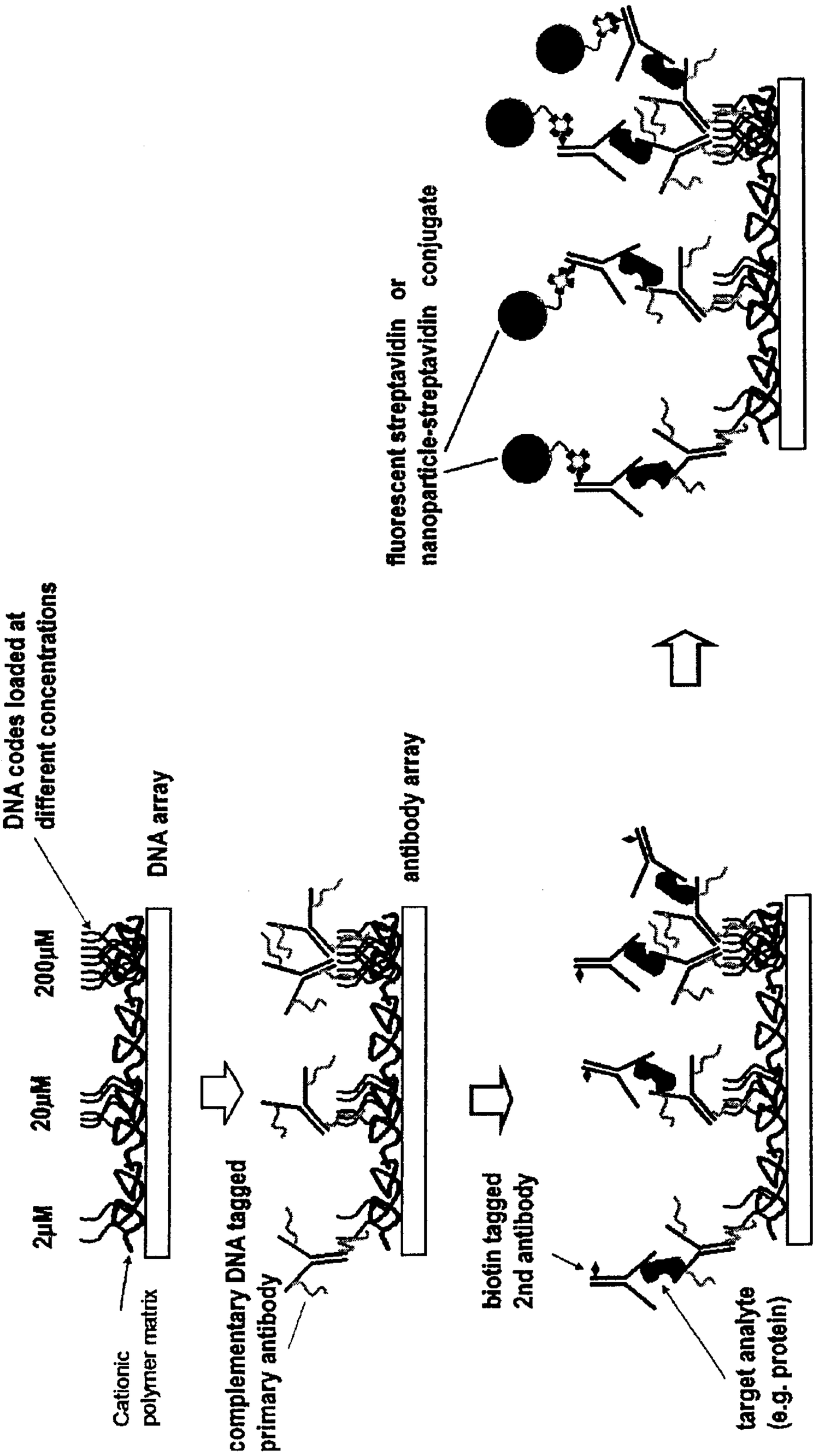

fluorescent streptavidin or
nanoparticle-streptavidin conjugate
DNA codes loc
different conc
20µM
200µM
2µM
DNA array
Cationic
polymer matrix
antibody array
complementary DNA tagged
primary antibody
biotin tagged
2nd antibody
target analyte
(e.g. protein)

FIG. 12

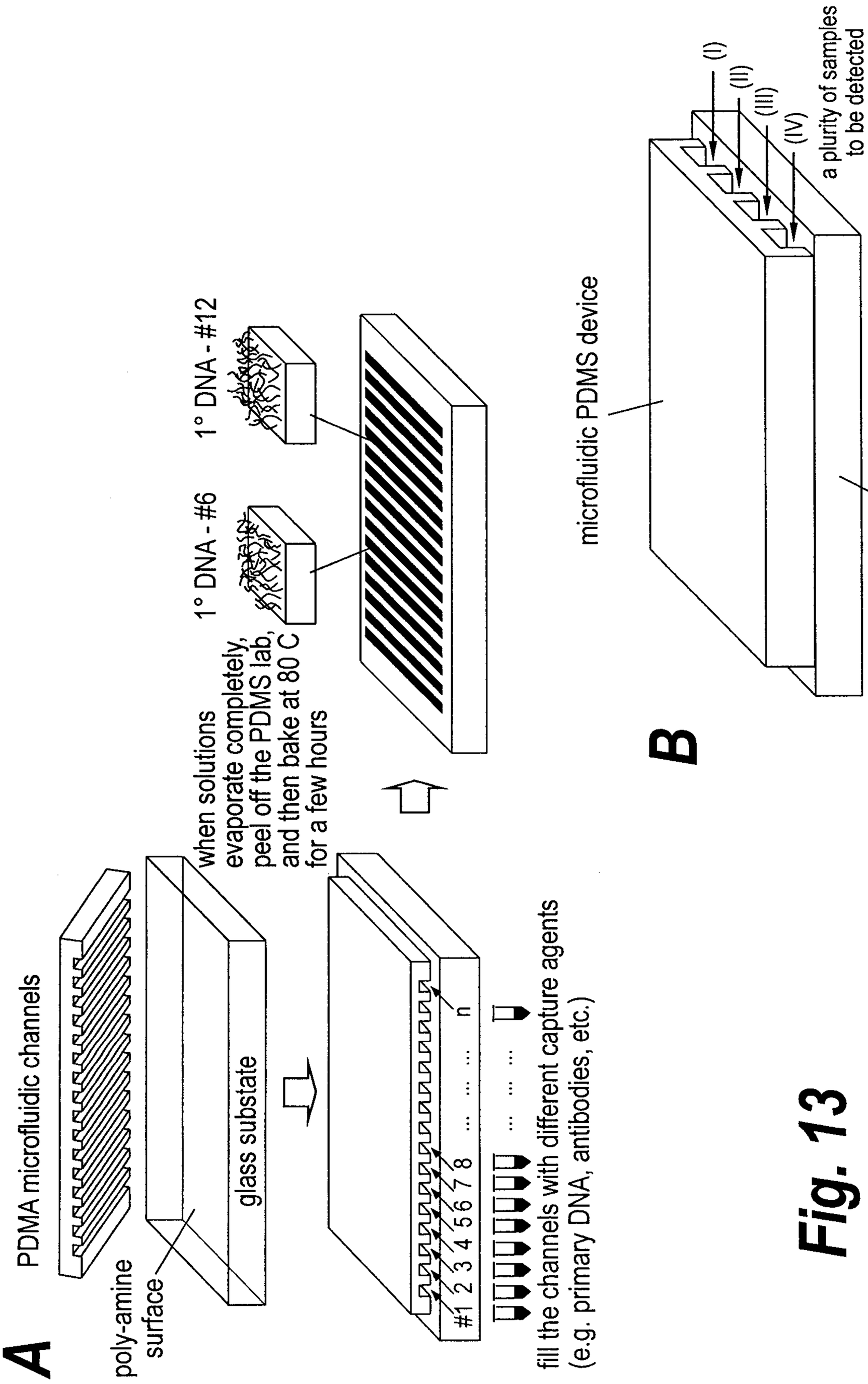

A
PDMA microfluidic channels
poly-amine surface
glass substate
when solutions evaporate completely, peel off the PDMS lab, and then bake at 80 C for a few hours
1° DNA - #6
1° DNA - #12
fill the channels with different capture agents (e.g. primary DNA, antibodies, etc.)
#1 2 3 4 5 6 7 8
n
B
microfluidic PDMS device
High density barcode chip
a plurity of samples to be detected
(I)
(II)
(III)
(IV)

Fig. 13

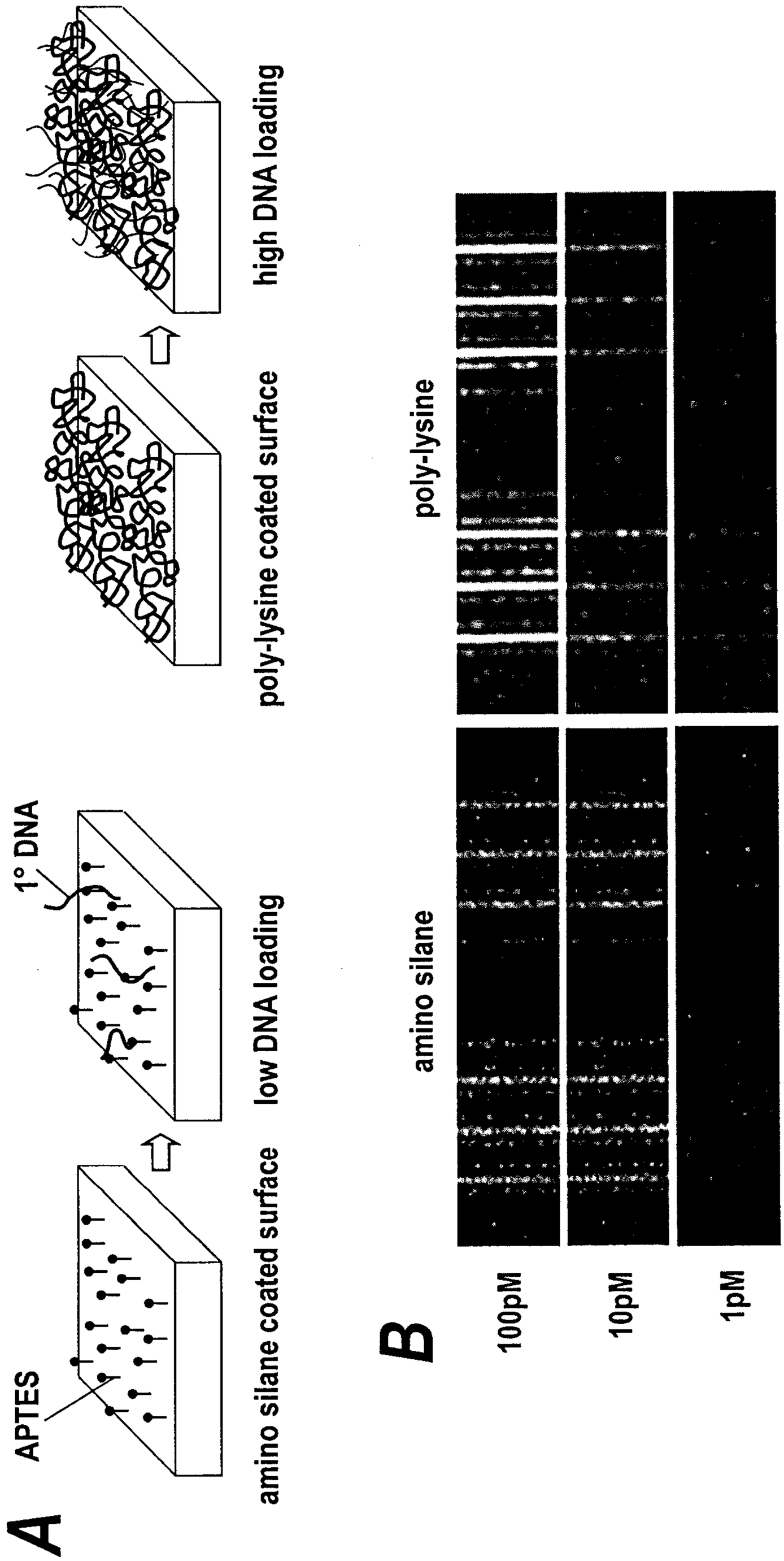

A
APTES
1° DNA
amino silane coated surface
low DNA loading
B
amino silane
100pM
10pM
1pM
poly-lysine coated surface
high DNA loading
poly-lysine

Fig. 14

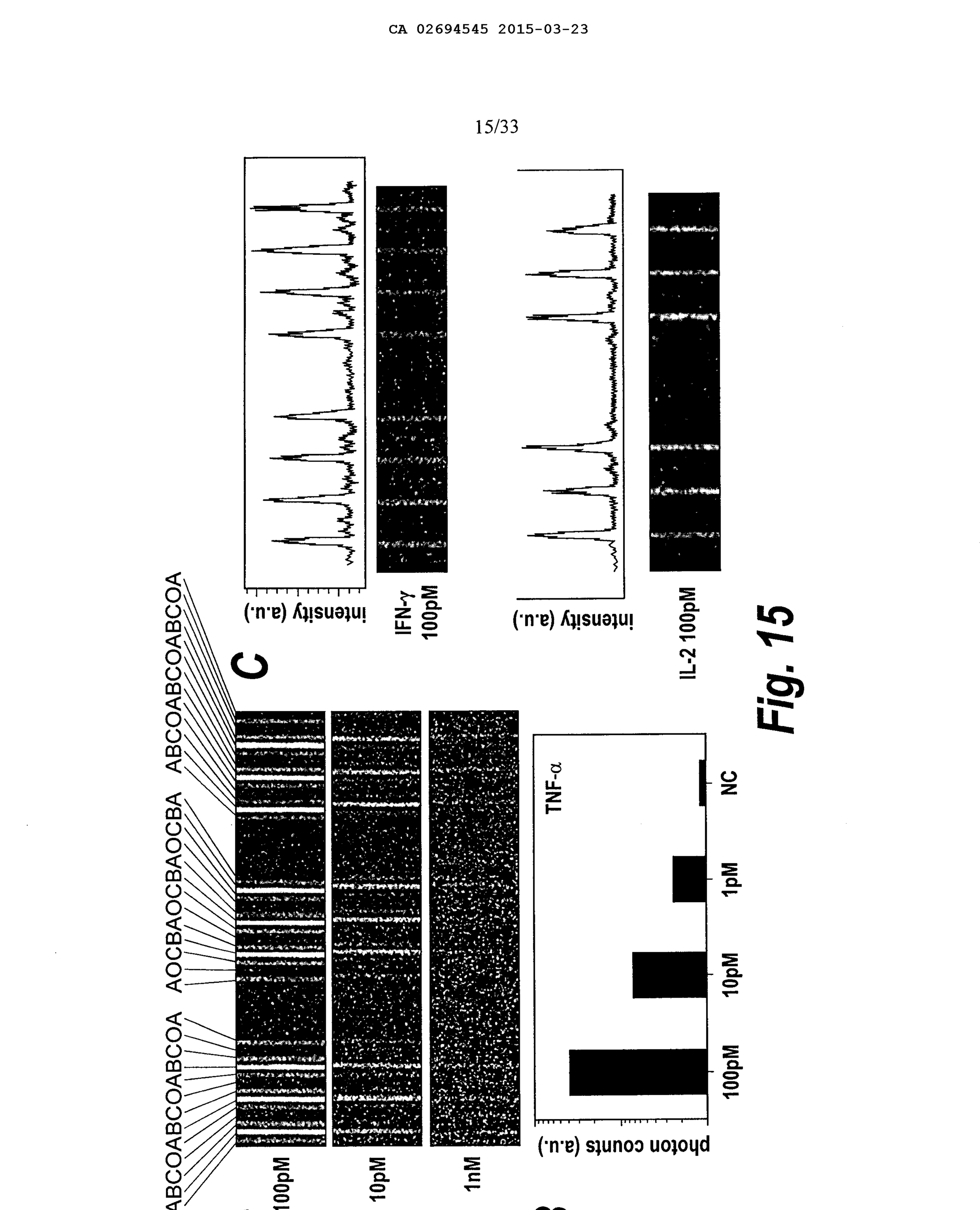

C
ABCOABCOABCOA AOCBAOCBAOCBA ABCOABCOABCOA
100pM
10pM
1nM
A
ABCOABCOABCOA
100pM
10pM
1nM
B
photon counts (a.u.)
TNF-α
NC
1pM
10pM
100pM
IL-2 100pM
intensity (a.u.)
IFN-γ
100pM
intensity (a.u.)

Fig. 15

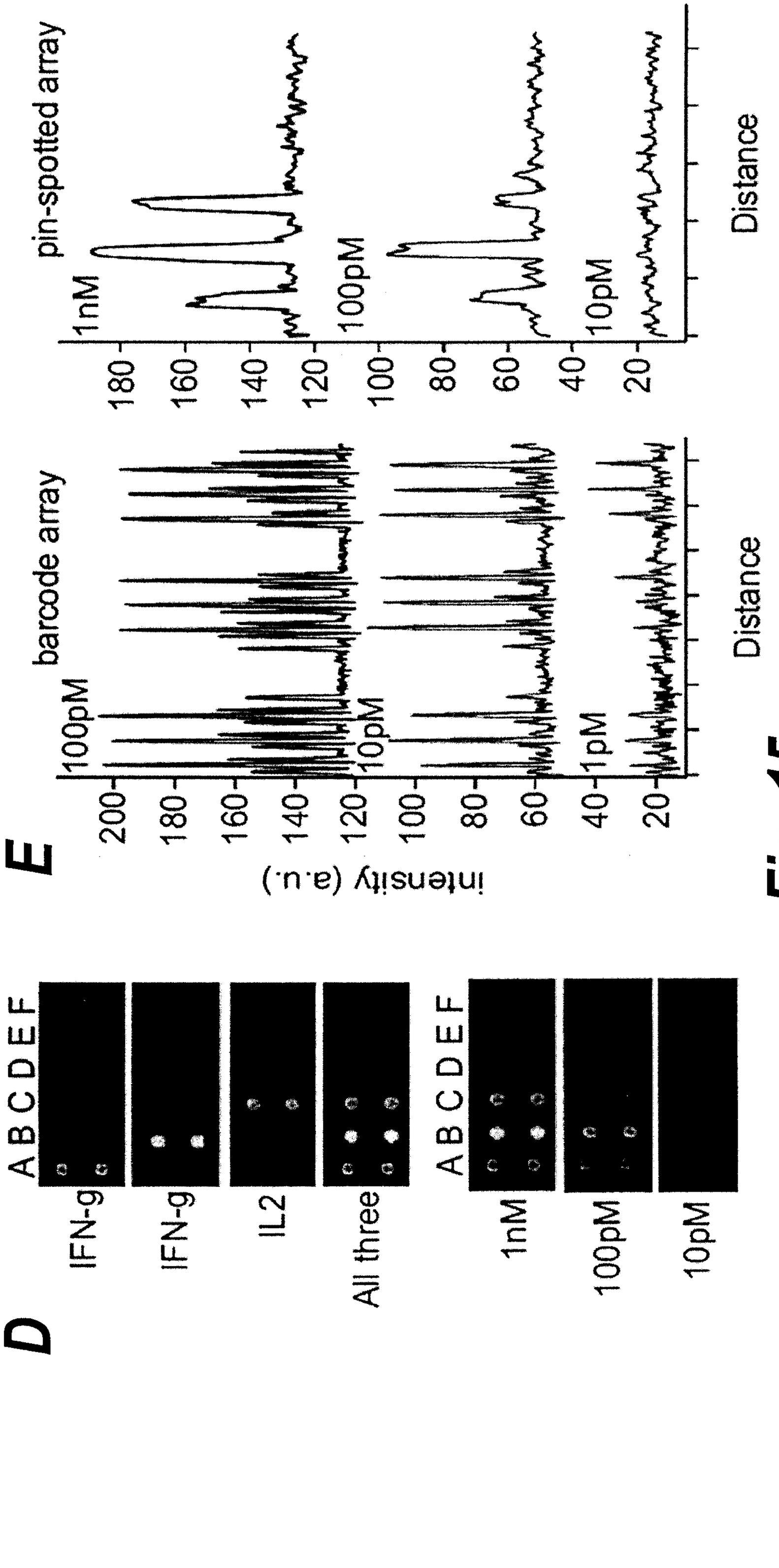
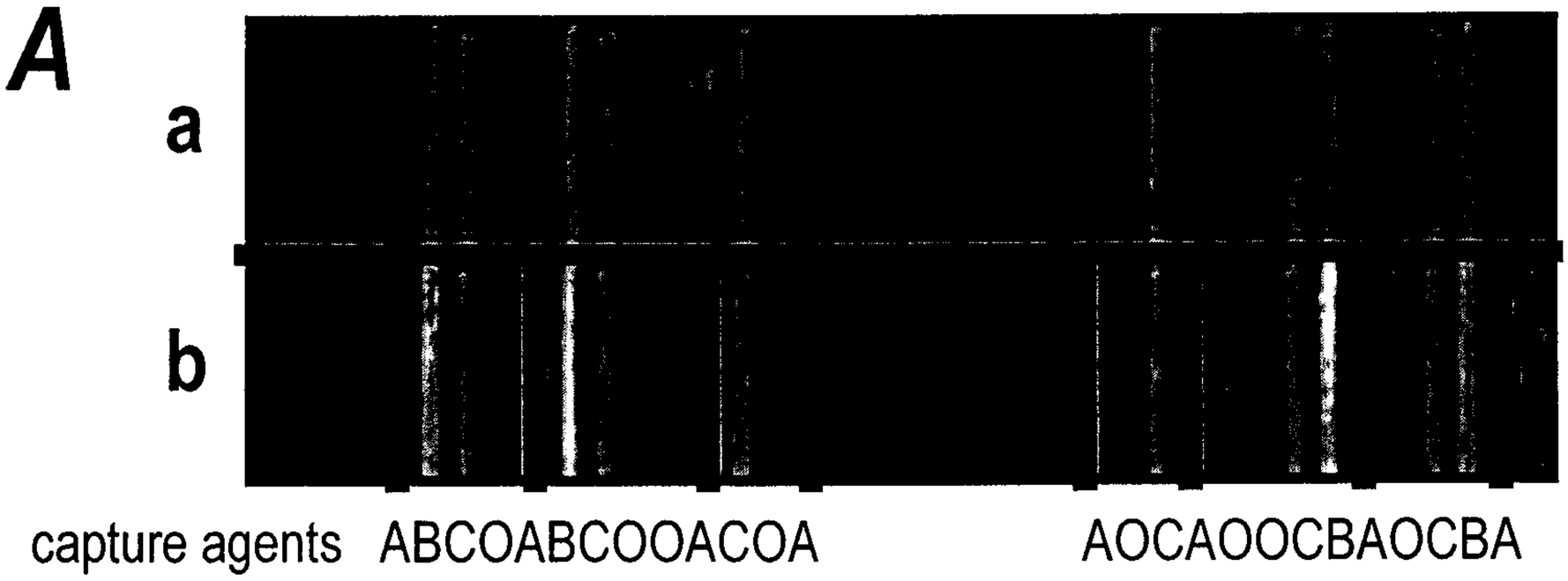
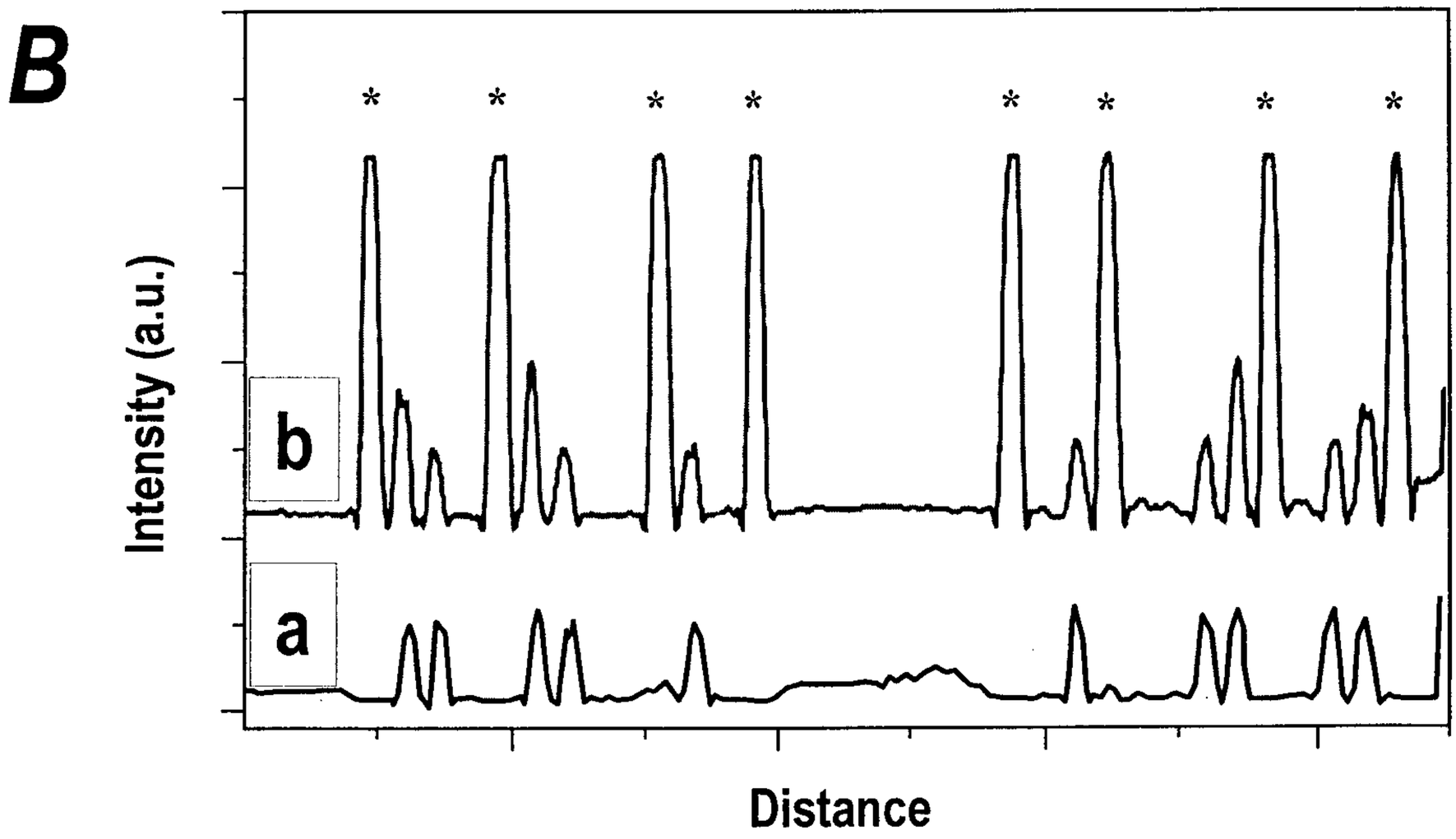

D
IFN-g
IFN-g
IL2
All three
A B C D E F
1nM
100pM
10pM
E
intensity (a.u.)
pin-spotted array
barcode array
1nM
100pM
10pM
100pM
10pM
1pM
Distance
Distance

Fig. 15





a: add blank sample with no target DNA; b: add sample with target DNA (A')
* denotes the peak corresponding to target DNA
The bars B and C are employed as reference.

Fig. 16

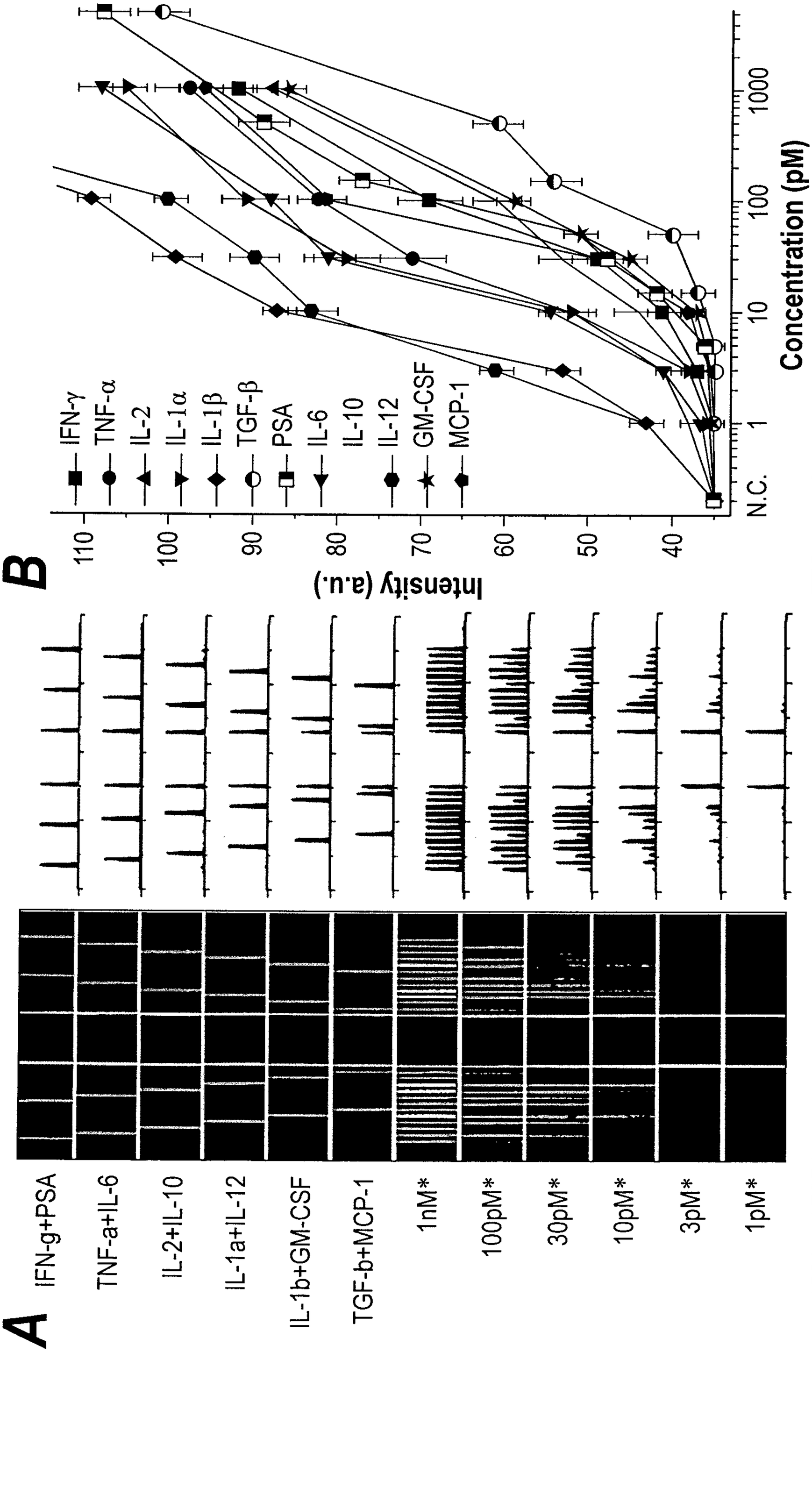
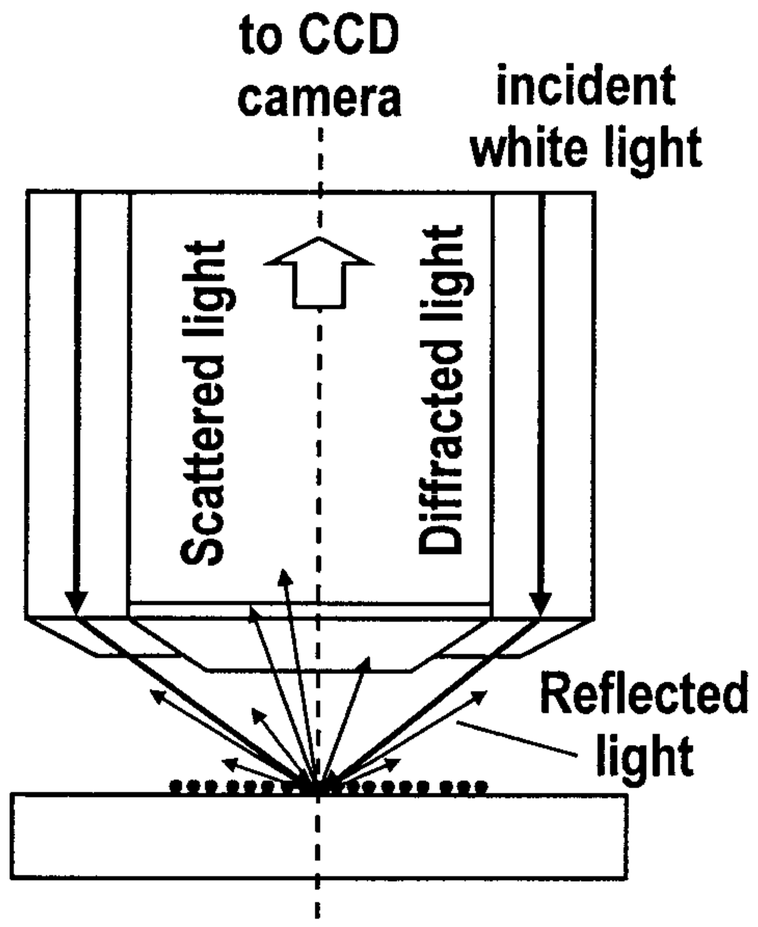
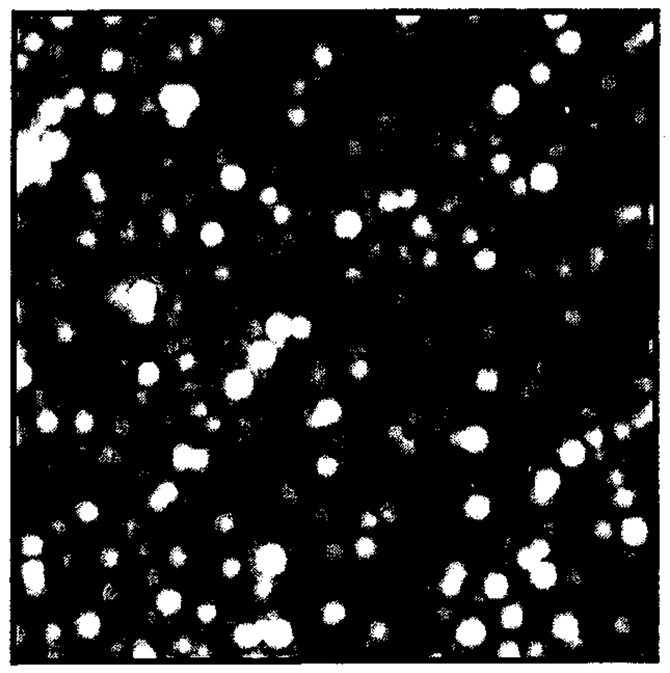
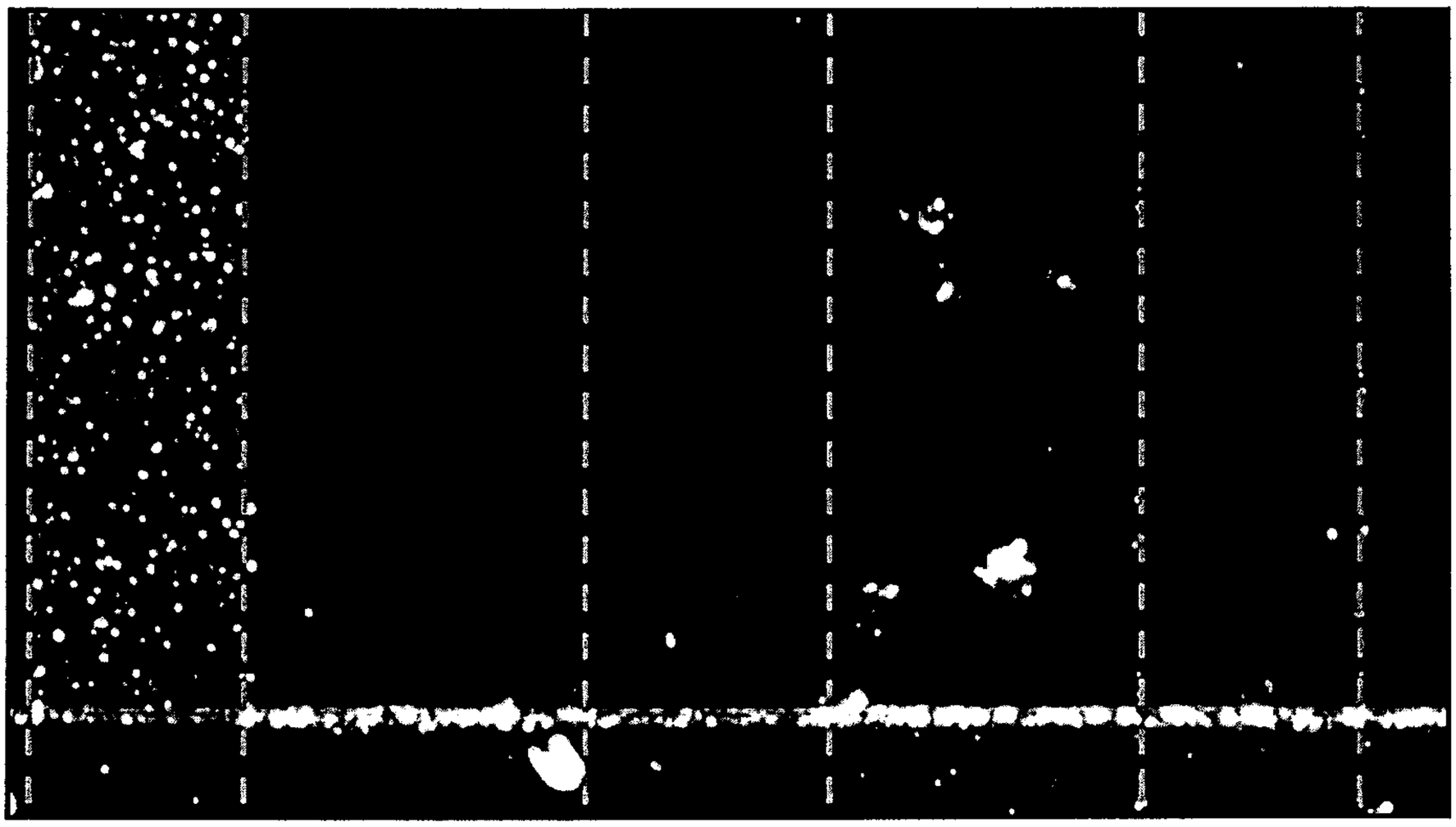

B
Intensity (a.u.)
110
100
90
80
70
60
50
40
IFN-γ
TNF-α
IL-2
IL-1α
IL-1β
TGF-β
PSA
IL-6
IL-10
IL-12
GM-CSF
MCP-1
N.C.
1
10
100
1000
Concentration (pM)
A
IFN-g+PSA
TNF-a+IL-6
IL-2+IL-10
IL-1a+IL-12
IL-1b+GM-CSF
TGF-b+MCP-1
1nM*
100pM*
30pM*
10pM*
3pM*
1pM*
*PSA and TGF-b concentrations are 5x higher
Distance

Fig. 17





light scattering image of
40nm gold nanoparticles



Capture agent: anti-human IL-1β / E' conjugate
Sample: contain recombinant of human IL-1β

Fig. 18

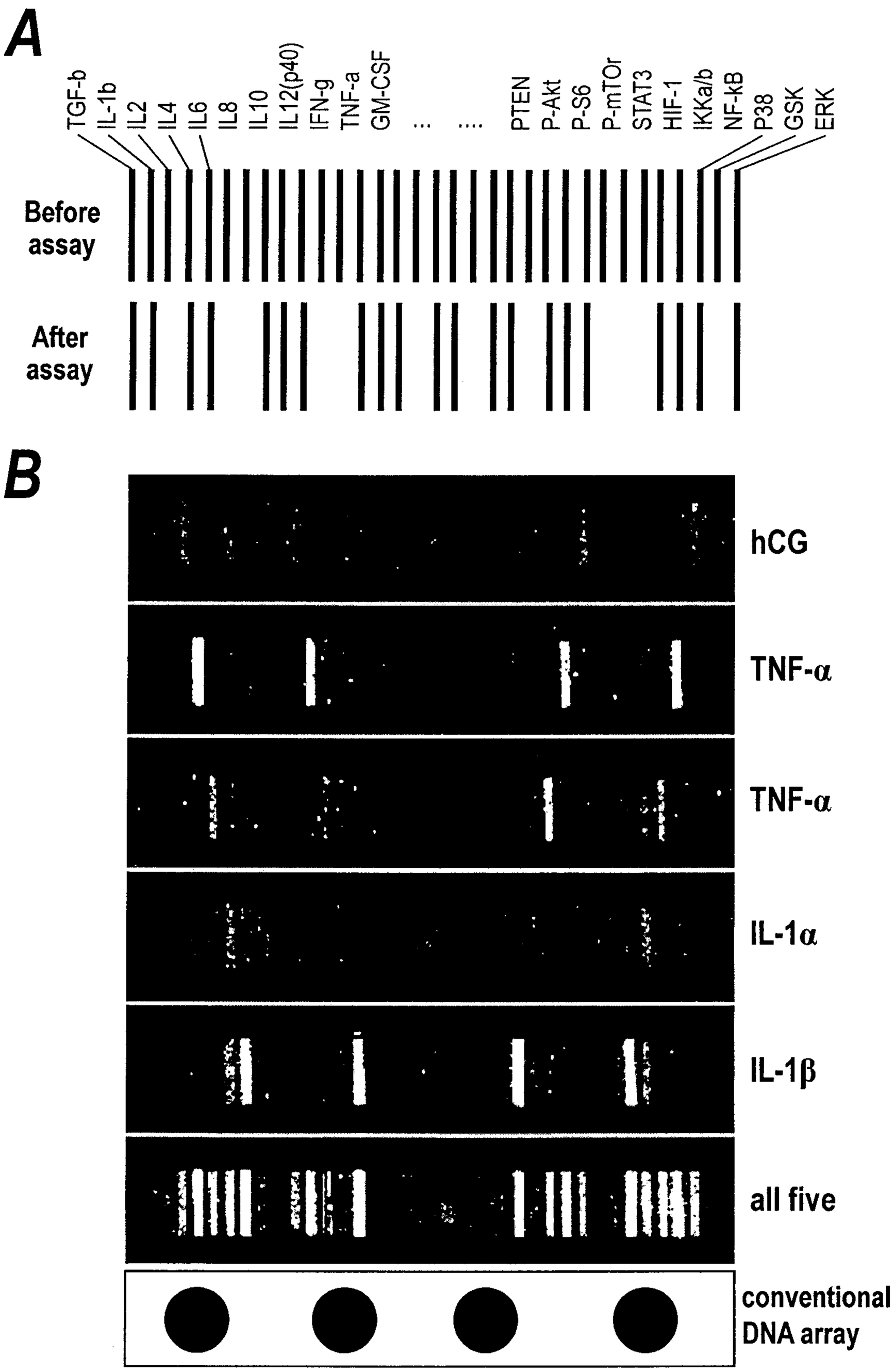
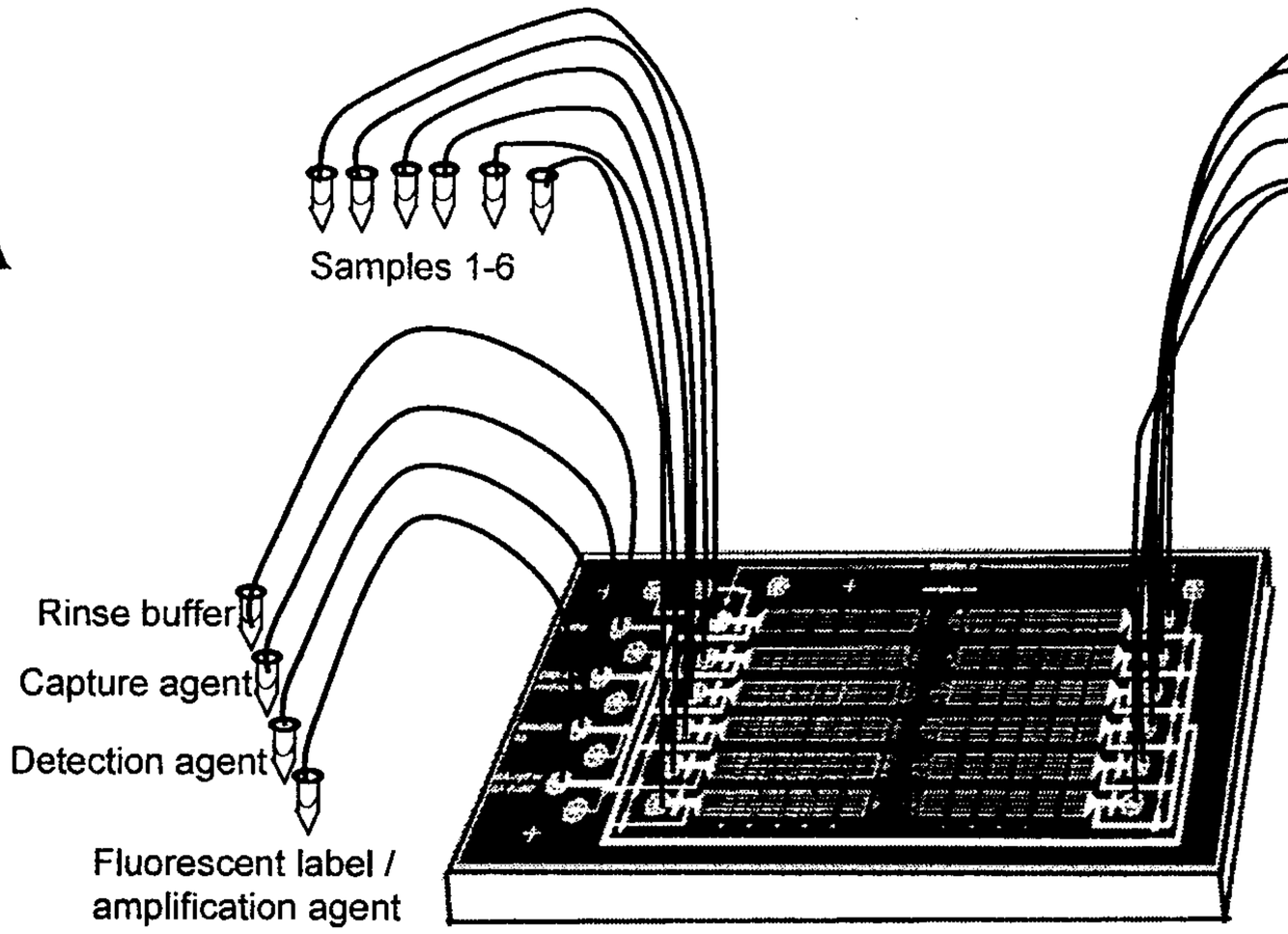

A
TGF-b
IL-1b
IL2
IL4
IL6
IL8
IL10
IL12(p40)
IFN-g
TNF-a
GM-CSF
PTEN
P-Akt
P-S6
P-mTOr
STAT3
HIF-1
IKKa/b
NF-kB
P38
GSK
ERK
Before assay
After assay
B
hCG
TNF-α
TNF-α
IL-1α
IL-1β
all five
conventional DNA array

Fig. 19

A


Samples 1-6
Samples 7-12
Rinse buffer
Capture agent
Detection agent
Fluorescent label / amplification agent

B

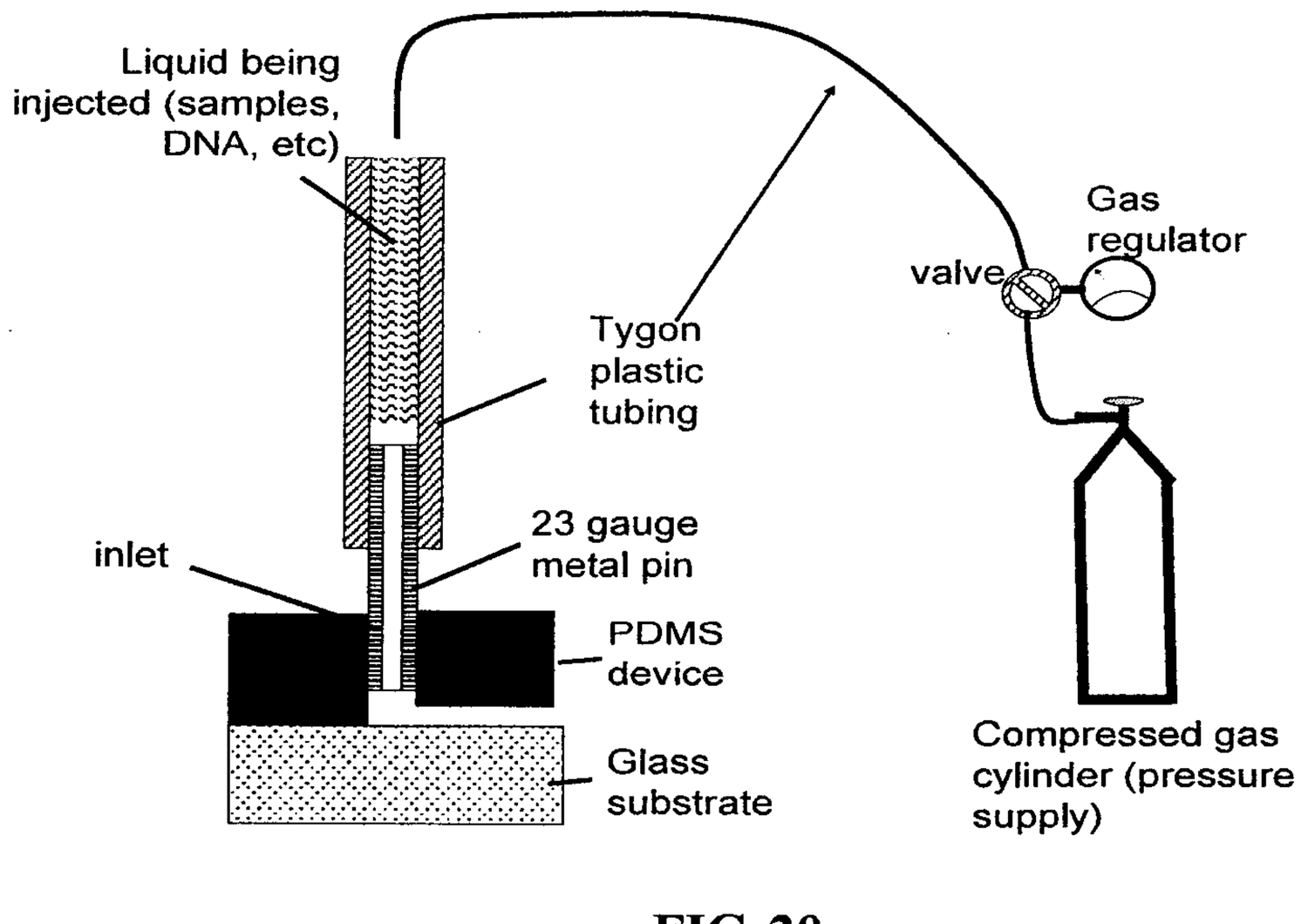

Liquid being injected (samples, DNA, etc)
Tygon plastic tubing
valve
Gas regulator
inlet
23 gauge metal pin
PDMS device
Glass substrate
Compressed gas cylinder (pressure supply)

FIG. 20

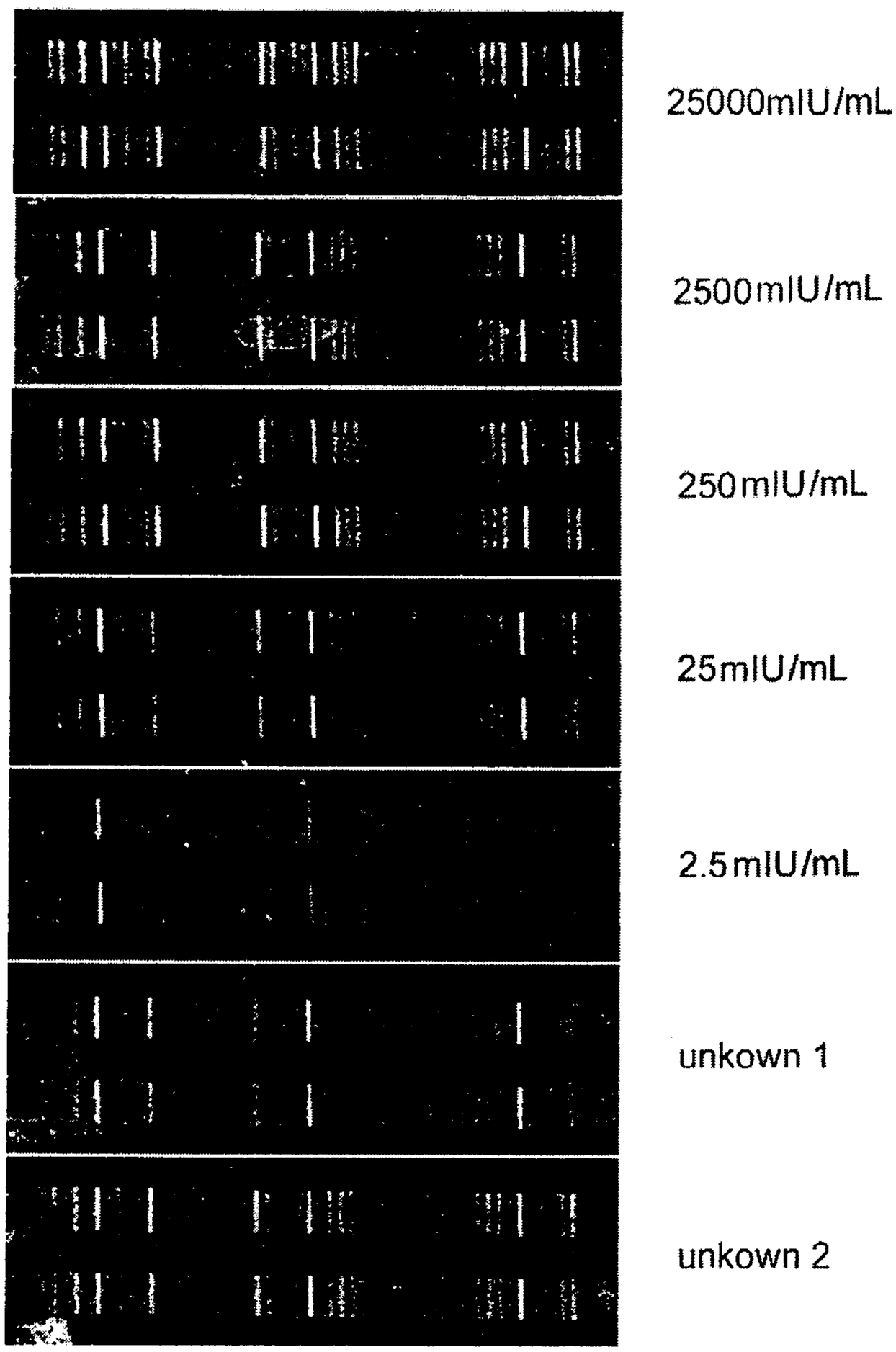

detection of human hCG from sera
25000mIU/mL
2500mIU/mL
250mIU/mL
25mIU/mL
2.5mIU/mL
unkown 1
unkown 2

FIG. 21

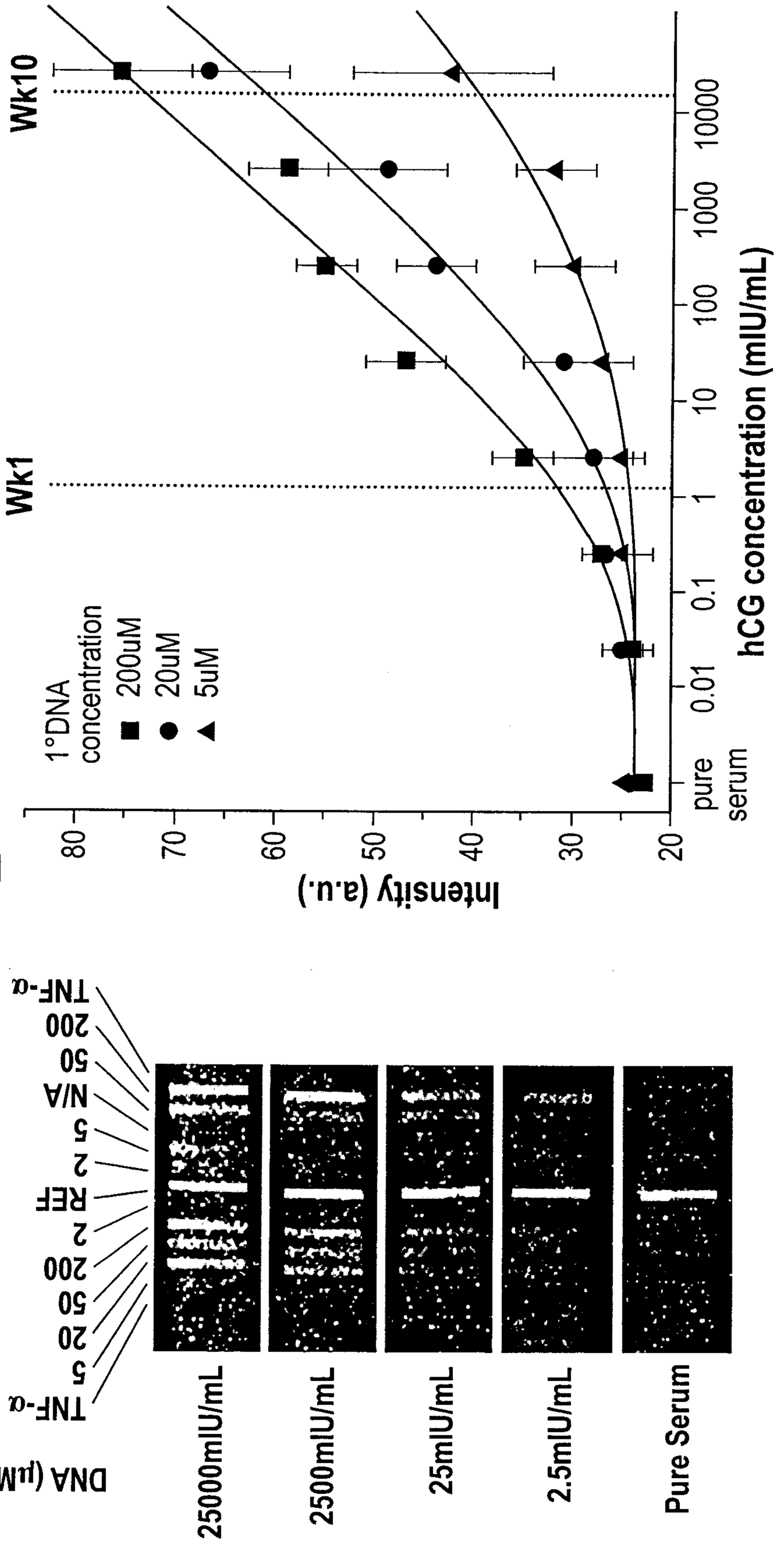

Wk10
Wk1
1°DNA concentration
200uM
20uM
5uM
pure serum
0.01
0.1
1
10
100
1000
10000
hCG concentration (mIU/mL)
Intensity (a.u.)
80
70
60
50
40
30
20
B
TNF-α
200
50
N/A
5
2
REF
2
200
50
20
5
TNF-α
DNA (μM)
25000mIU/mL
2500mIU/mL
25mIU/mL
2.5mIU/mL
Pure Serum
A

Fig. 2

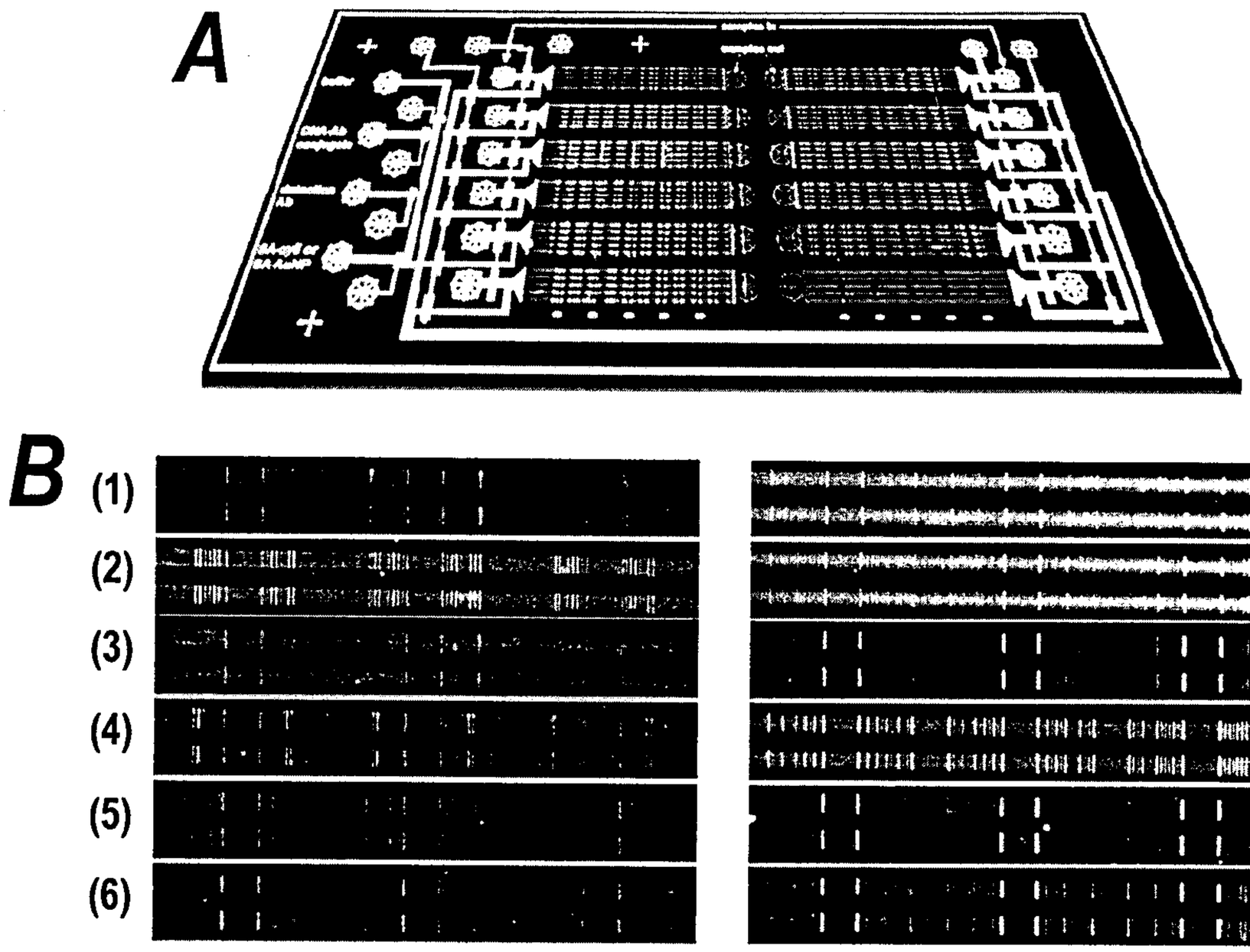

A
B
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
C
Intensity
(a.u.)
400
200
0
proteins
IFN-g
TNF-a
IL-2
IL-1a
IL-b
TGF-b
PSA
IL-6
IL-10
IL-12
G-CSF
MP-1
M'-ref
patient
S12
S11
S10
S9
S8
S7
S6
S5
S4
S3
S2
S1

Fig. 23

D

Patient	Lane	type	gender / age	race	UICC stage	Gleasons Score	others
S1	(3)	breast	Female / 62	Caucasian	T2N0M0		wine 200mL/day
S2	(5)	breast	Female / 79	Caucasian	T4N2M0		
S3	(6)	breast	Female / 71	Caucasian	T1cNXM0		1-2 drinks/day
S4	(11)	breast	Female / 72	Caucasian	T2NXMO		hypertension
S5	(1)	breast	Female / 89+	Caucasian	T3N0MX		arthritis
S6	(9)	prostate	Male / 51	Caucasian	T2cNXM0	4+3=7	
S7	(4)	prostate	Male / 64	Caucasian	T3bN0M0	3+4=7	Hypertension
S8	(2)	prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	Hypertension
S9	(8)	prostate	Male / 55	Caucasian	T2bN0M0	3+3=6	11-20 cigs/day
S10	(7)	prostate	Male / 73	Caucasian	T3aNXMX	4+4=8	hypertension, 11-20 cigs/day
S11	(10)	prostate	Male / 47	Caucasian	T2cN0M0	3+3=6	hypertension,
S12	(12)	Sigma Ref					

Fig. 23

CA 02694545 2015-03-23

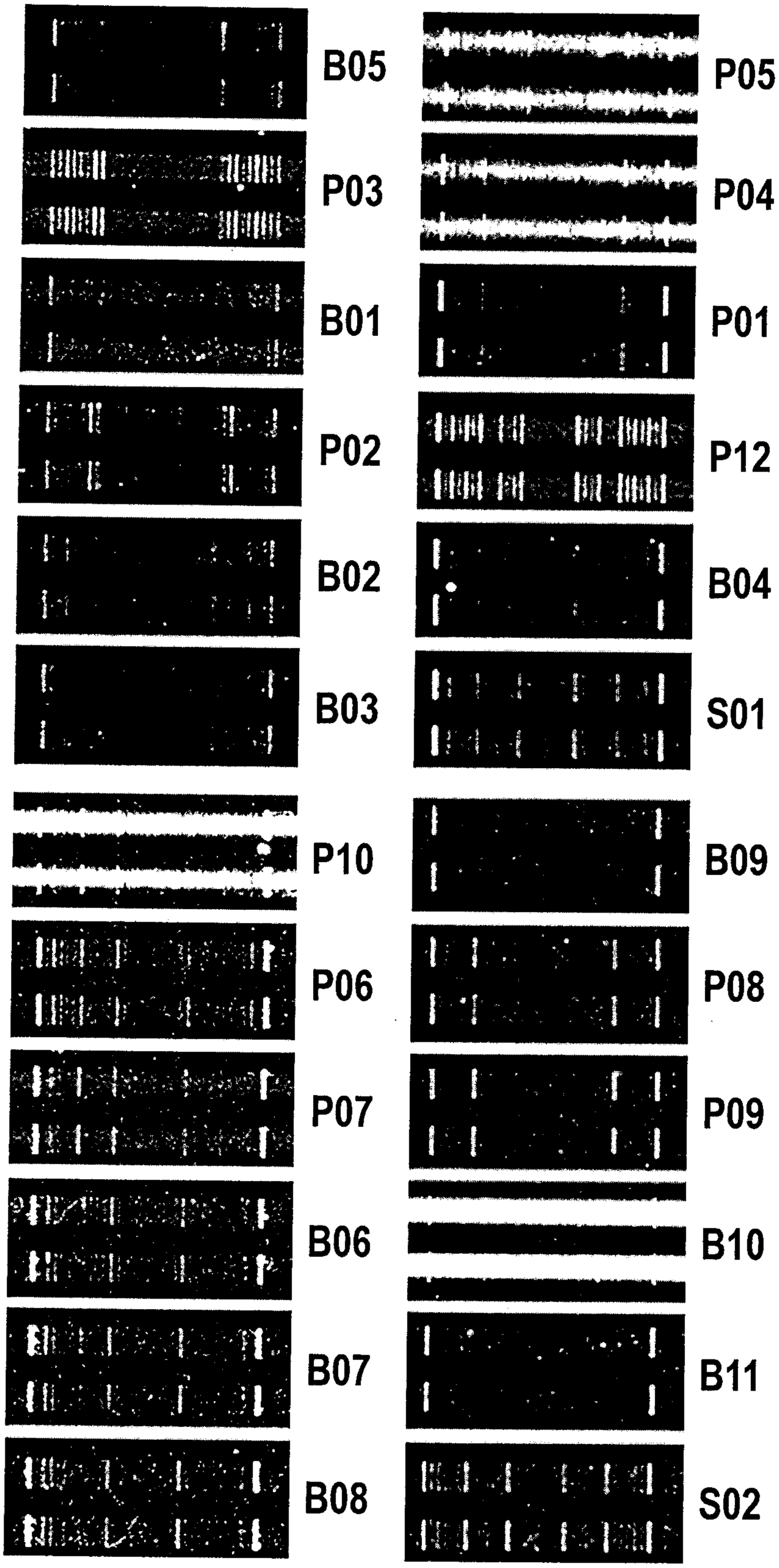

B05
P05
P03
P04
B01
P01
P02
P12
B02
B04
B03
S01
P10
B09
P06
P08
P07
P09
B06
B10
B07
B11
B08
S02

Fig. 24

PATIENT	UICC STAGE	MEDICAL HISTORY
B01	T2N0M0	wine 299m/day
B02	T4N2M0	
B03	T1cNXM0	1-2 drinks/day
B04	T2NXM0	hypertension
B05	T3N0M0	arthritis
B06	T1NXM0	
B07	T2N2M0	hypertension, obesity
B08	T2NxM0	1-5 cigs/day, wire 200mL/day
B09	T4N0M0	Coronary artery disease, cerebral atherosclerosis
B10	T3N2MX	6-10cigs/day, hyperthyroid, hypertension, osteoarthritis
B11	T1NXM0	arterial hypertension

PATIENT	UICC STAGE	MEDICAL HISTORY
P01	T2cNXM0	
P02	T3bN0M0	
P03	T2cN0M0	hypertension
P04	T2bN0M0	11-20 cigs/day
P05	T3aNXMX	hypertension, 11-20 cigs/day
P06	T3N0M0	chronic bronchitis, 11-20cigs/day
P07	T3aN0M0	gastroesophageal reflux
P08	T2aNXMX	1-5cigs/day
P09	T3aN1MX	hypertension, atrial fibrillation
P10	T2aN0MX	hypertension, 11-20 cigs/day
P11	T2cN0M0	hypertension

Fig. 25

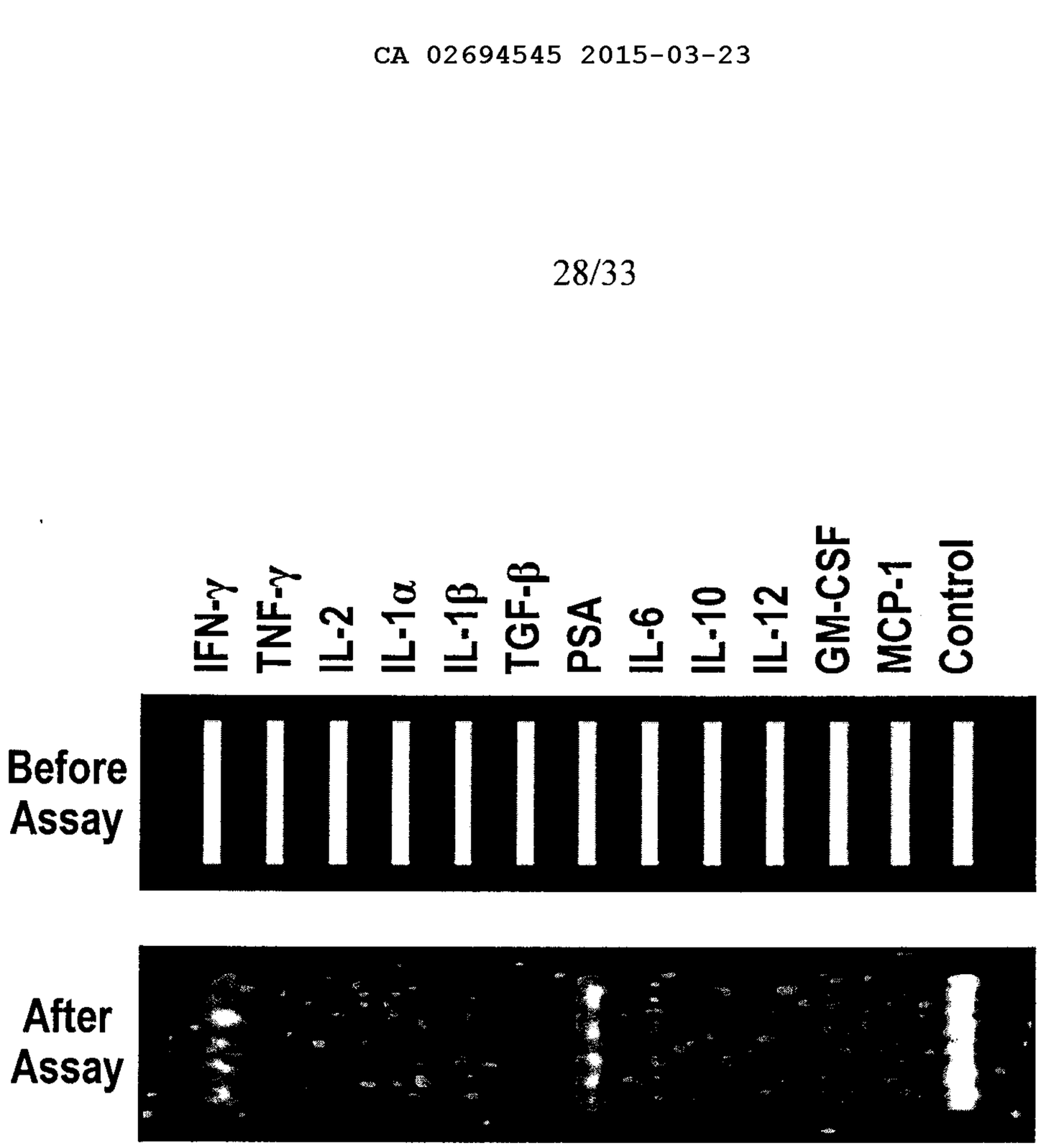

IFN-γ
TNF-γ
IL-2
IL-1α
IL-1β
TGF-β
PSA
IL-6
IL-10
IL-12
GM-CSF
MCP-1
Control
Before Assay
After Assay

Fig. 26

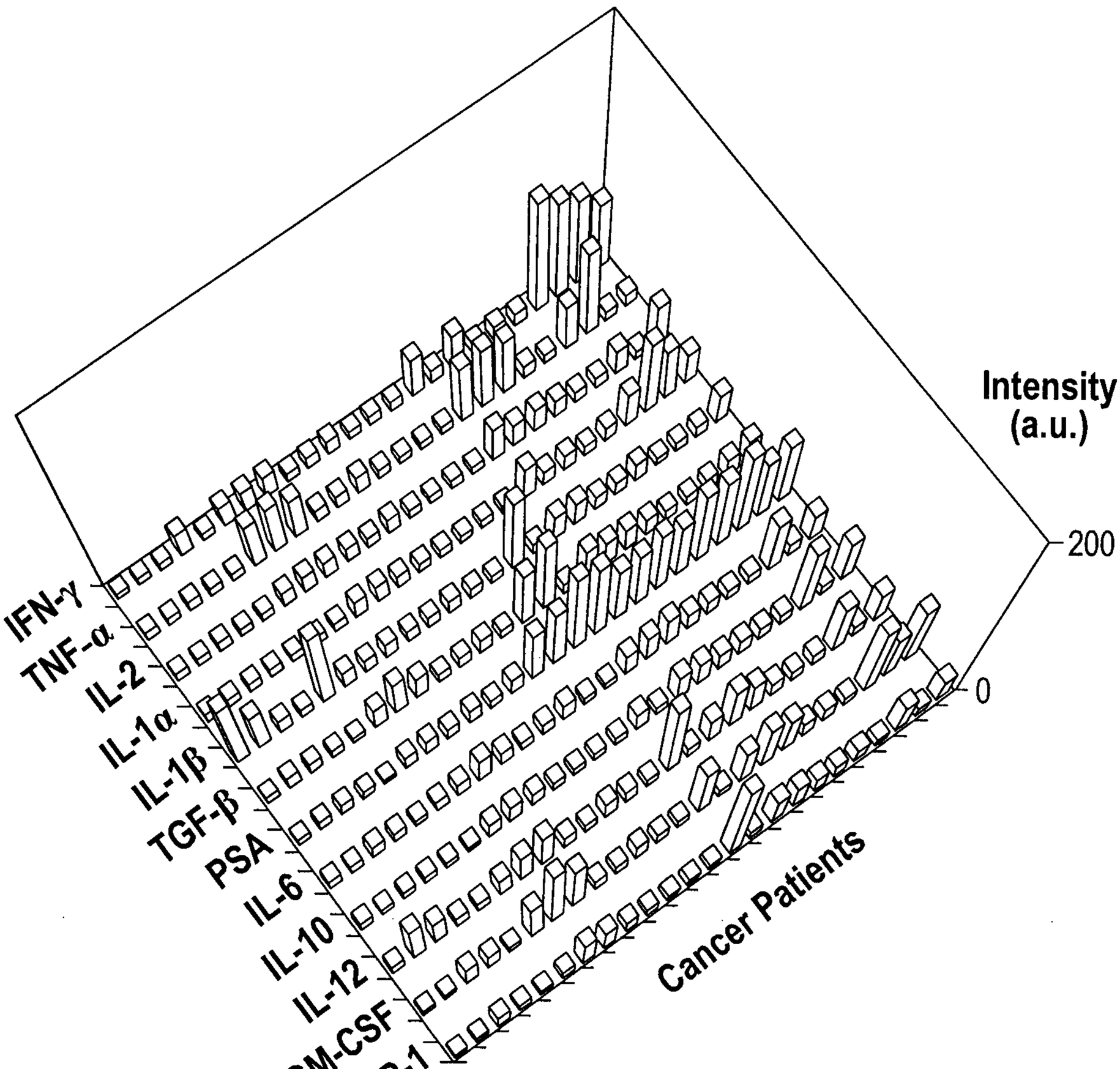

IFN-γ
TNF-α
IL-2
IL-1α
IL-1β
TGF-β
PSA
IL-6
IL-10
IL-12
GM-CSF
MCP-1
Intensity (a.u.)
200
0
Cancer Patients

Fig. 27

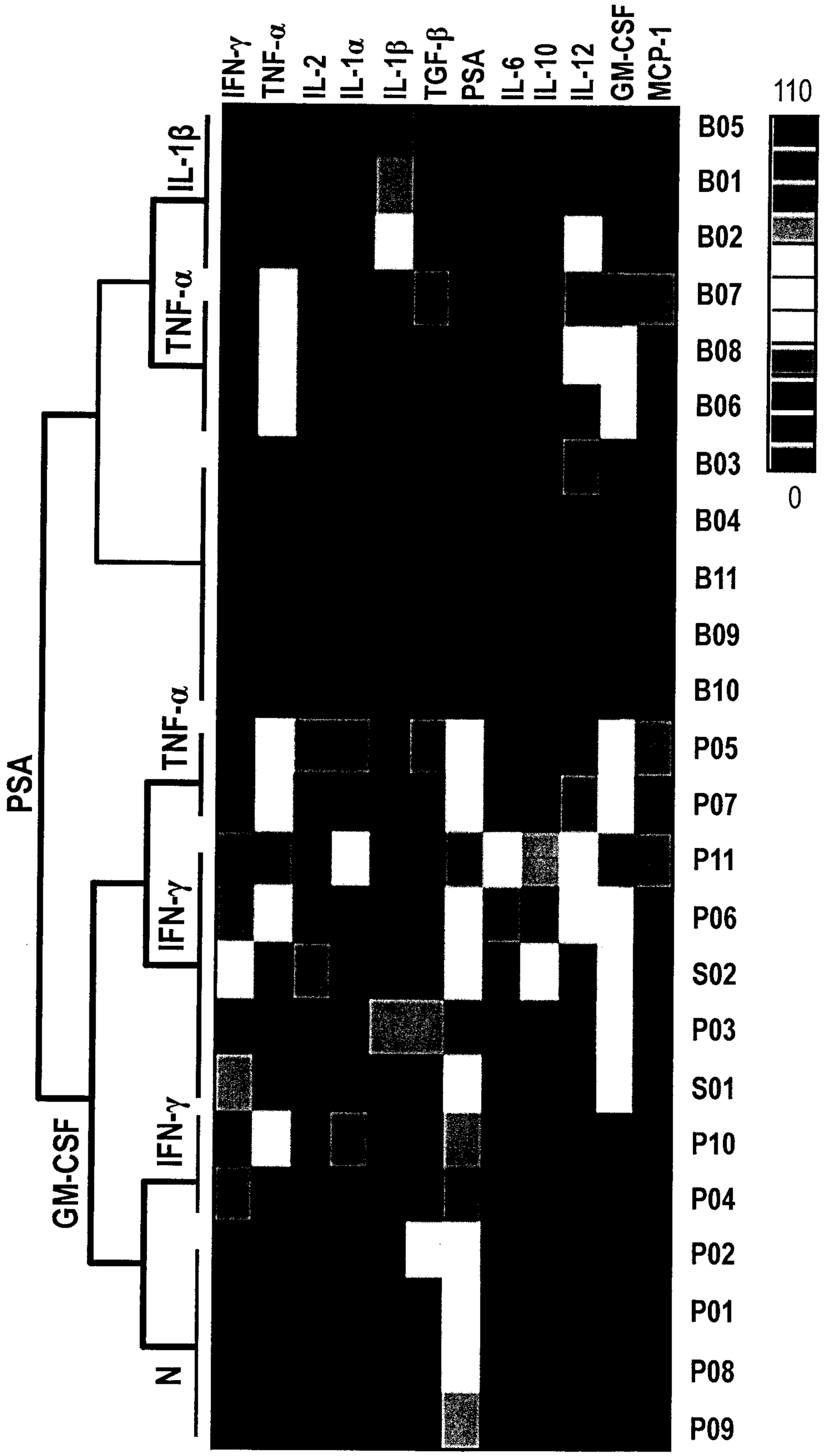

IFN-γ
TNF-α
IL-2
IL-1α
IL-1β
TGF-β
PSA
IL-6
IL-10
IL-12
GM-CSF
MCP-1
110
0
B05
B01
B02
B07
B08
B06
B03
B04
B11
B09
B10
P05
P07
P11
P06
S02
P03
S01
P10
P04
P02
P01
P08
P09
IL-1β
TNF-α
PSA
TNF-α
IFN-γ
GM-CSF
IFN-γ
N

Fig. 28

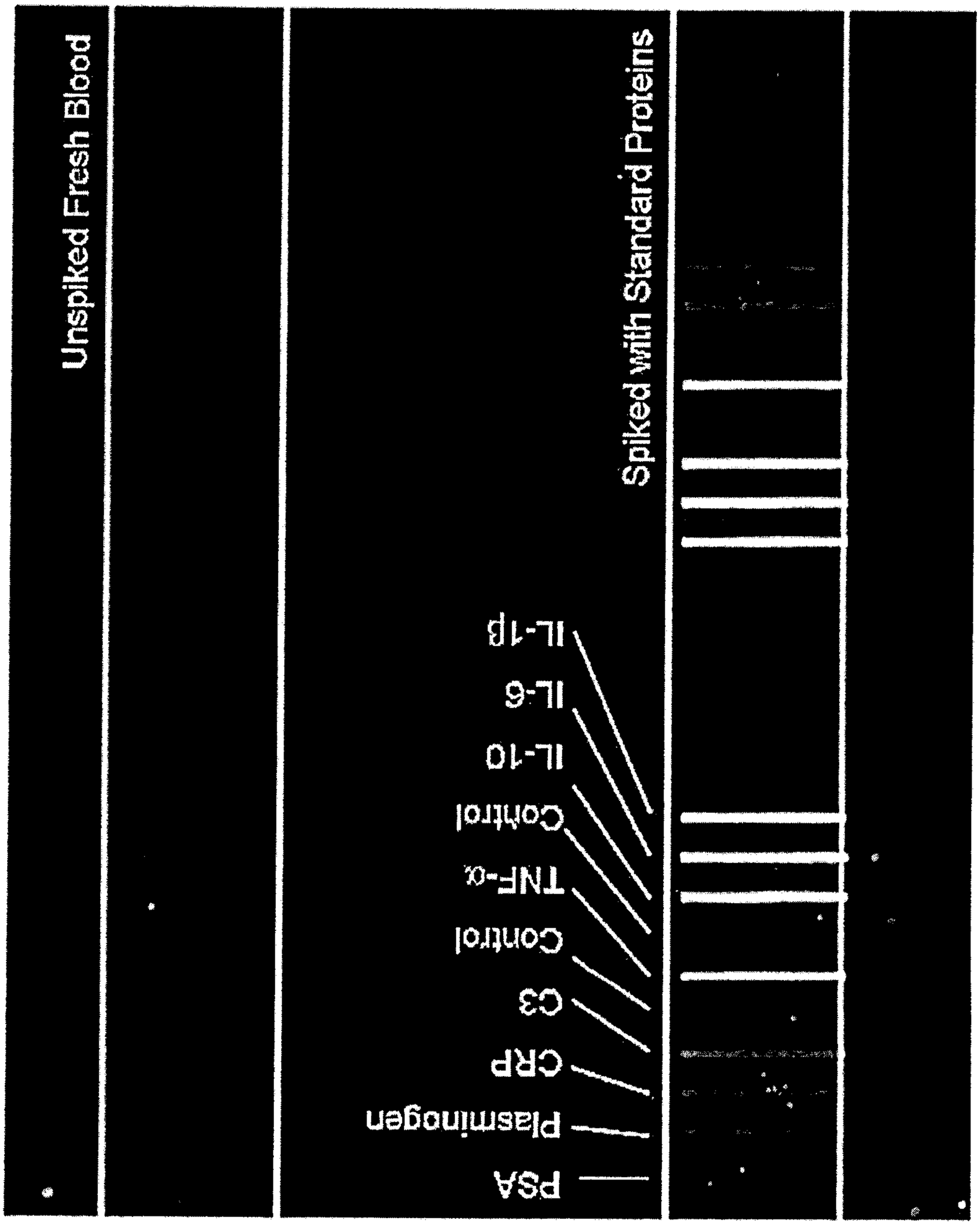

Unspiked Fresh Blood
Spiked with Standard Proteins
PSA
Plasminogen
CRP
C3
Control
TNF-α
Control
IL-10
IL-6
IL-1β

Fig. 29

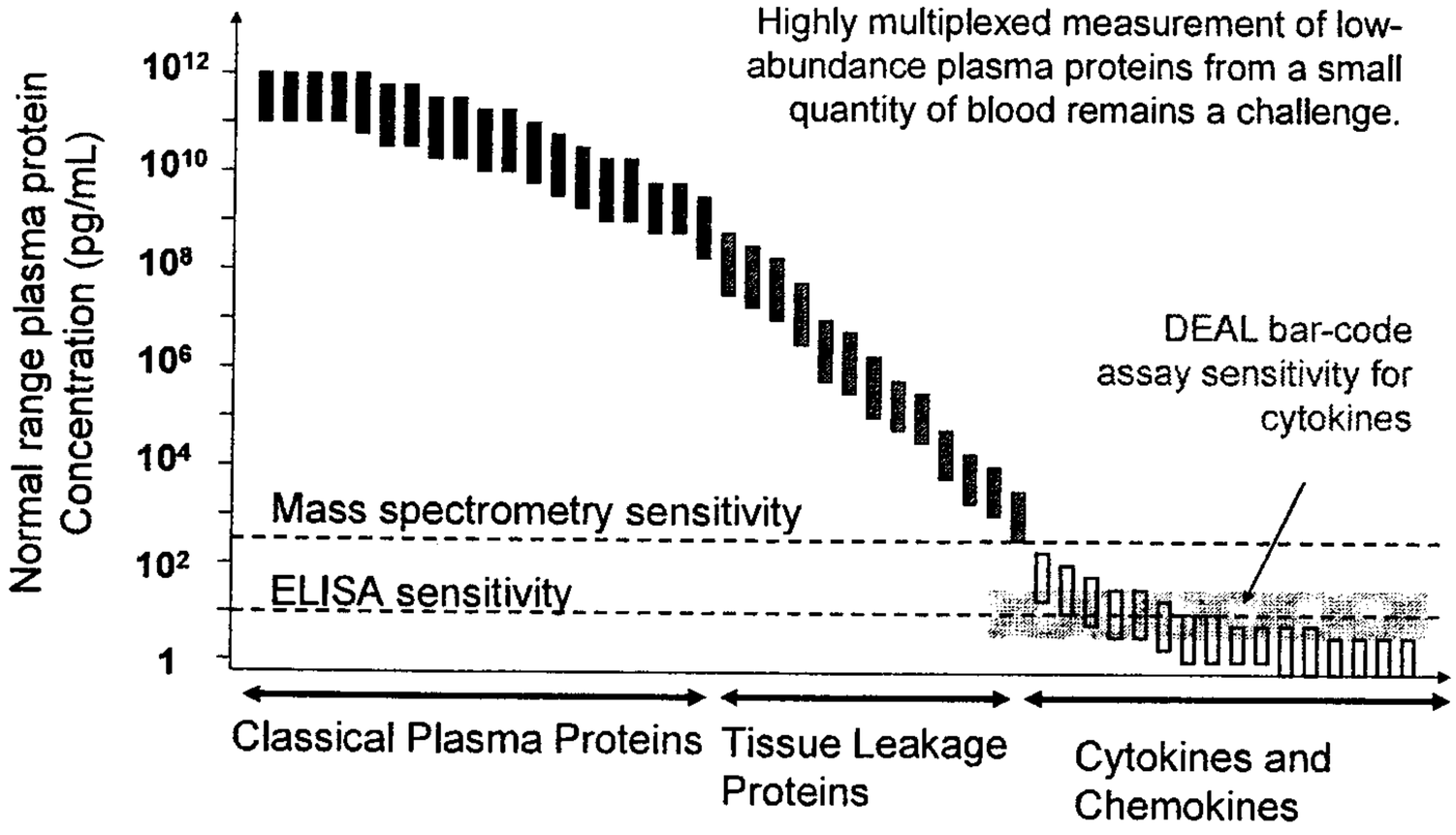

Highly multiplexed measurement of low-abundance plasma proteins from a small quantity of blood remains a challenge.
Normal range plasma protein Concentration (pg/mL)
10^12
10^10
10^8
10^6
10^4
10^2
1
DEAL bar-code assay sensitivity for cytokines
Mass spectrometry sensitivity
ELISA sensitivity
Classical Plasma Proteins
Tissue Leakage Proteins
Cytokines and Chemokines

FIG. 30

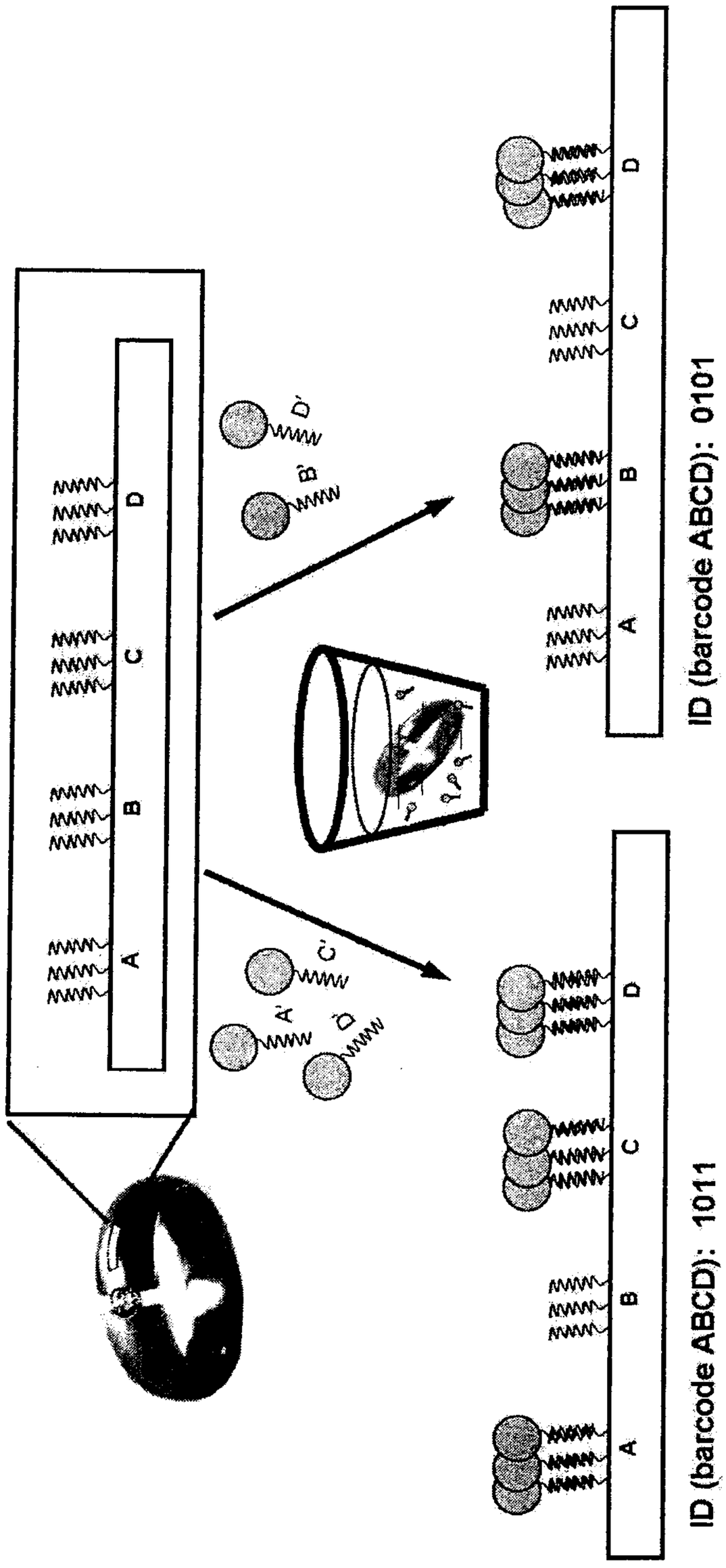
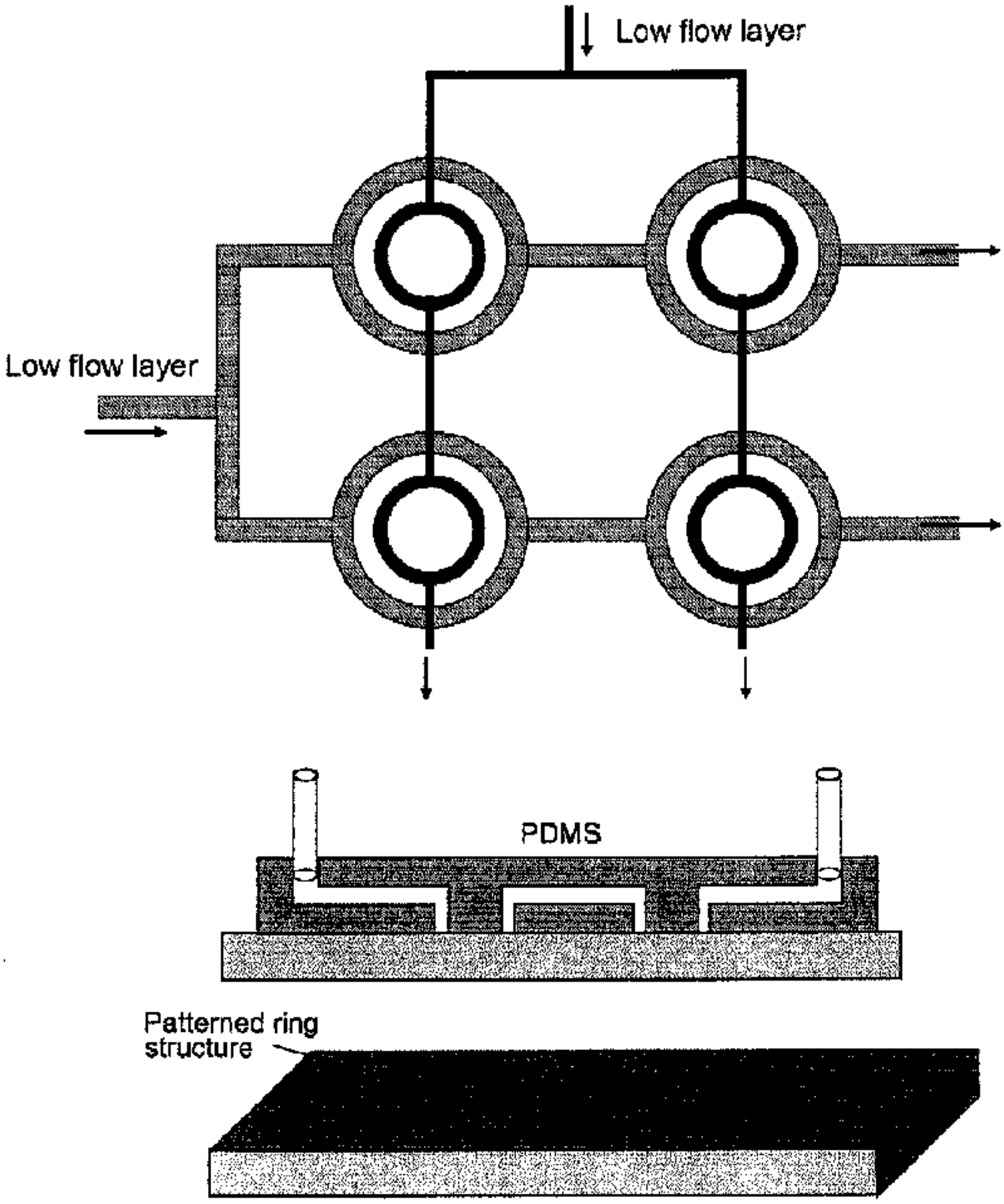


FIG. 31


Low flow layer
Low flow layer
PDMS
Patterned ring structure