



US009804165B1

(12) United States Patent
Morre**(10) Patent No.: US 9,804,165 B1**
(45) Date of Patent: *Oct. 31, 2017**(54) METHODS AND COMPOSITIONS FOR TARGETED TWO-DIMENSIONAL WESTERN BLOT ANALYSIS FOR EARLY CANCER DETECTION AND CANCER DIAGNOSIS UP TO TEN YEARS IN ADVANCE OF CLINICAL SYMPTOMS OF MALIGNANT DISEASE****(71) Applicant: Mor-NuCo Enterprises, Inc.,** West Lafayette, IN (US)**(72) Inventor: D. James Morre,** West Lafayette, IN (US)**(73) Assignee: Mor-NuCo Enterprises, Inc.,** West Lafayette, IN (US)**(*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: 15/434,982**(22) Filed: Feb. 16, 2017****Related U.S. Application Data****(63)** Continuation of application No. 15/169,205, filed on May 31, 2016.**(51) Int. Cl.**
G01N 33/53 (2006.01)
G01N 33/574 (2006.01)
G01N 21/49 (2006.01)
C07K 16/40 (2006.01)**(52) U.S. Cl.**
CPC **G01N 33/57488** (2013.01); **C07K 16/40** (2013.01); **C12Y 106/00** (2013.01); **G01N 21/49** (2013.01); **C07K 2317/56** (2013.01); **C07K 2317/622** (2013.01); **G01N 2333/90209** (2013.01)**(58) Field of Classification Search**
None
See application file for complete search history.**(56) References Cited****U.S. PATENT DOCUMENTS**7,053,188 B2 5/2006 Morre et al.
9,283,257 B2 3/2016 Morre
9,459,256 B2 10/2016 Morre et al.
2003/0207340 A1 11/2003 Morre et al.
2009/0042209 A1 2/2009 Hostetler
2014/0212896 A1 7/2014 Morre et al.**FOREIGN PATENT DOCUMENTS**WO 2011109663 A1 9/2011
WO 2013052926 A2 4/2013**OTHER PUBLICATIONS**

Extended European Search Report of 16180364.8 dated Nov. 11, 2016, 12 pp.

Bird, et al., "Single-chain antigen-binding proteins." *Science*, Oct. 21, 1988, vol. 242, No. 4877, pp. 423-426.Braman, et al., "Site-Directed Mutagenesis Using Double-Stranded Plasmid DNA Templates." Chapter 3 of *Methods in Molecular Biology, In Vitro Mutagenesis Protocols*, 1996, vol. 57, pp. 31-44.
Bruno, et al., "Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes." *Biochemical Journal*, Jun. 15, 1992, vol. 284, No. 3, pp. 625-628.Cho, et al., "Monoclonal antibody to a cancer-specific and drug-responsive hydroquinone (NADH) oxidase from the sera of cancer patients." *Cancer Immunol. Immunother.*, May 2002, vol. 51, pp. 121-129.Chomczynski, et al., "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." *Anal. Biochem.*, 1987, vol. 162, pp. 156-159.Chueh, et al., "Molecular Cloning and Characterization of a Tumor-Associated, Growth-Related, and Time-Keeping Hydroquinone (NADH) Oxidase (tNOX) of the HeLa Cell Surface." *Biochemistry*, 2002, vol. 44, pp. 3732-374.Daugherty, et al., "Polymerase chain reaction facilitates the cloning, CDRgrafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins." *Nucleic Acids Research*, 1991, vol. 19, pp. 2471-2476.Davies, et al., "Spotlight on tNOX: A tumor-selective target for cancer therapies." *Drug News Perspect*, May 2006, vol. 19, No. 4, pp. 223-225.Davis, et al. "Single Chain Antibody (SCA) Encoding Genes: One-Step Construction and Expression in Eukaryotic Cells." *Bio/Technology*, Feb. 1991, vol. 9, pp. 165-169.Del Castillo-Olivares, et al., "A Drug-Responsive and Protease-Resistant Peripheral NADH Oxidase Complex from the Surface of HeLa S Cells." *Arch. Biochem. Biophys.*, Oct. 1, 1998, vol. 385, pp. 125-140.

GenBank Accession No. AF207881, 2002.

Glockshuber, et al., "A comparison of strategies to stabilize immunoglobulin Fv fragments." *Biochemistry*, 1990, vol. 29, pp. 1262-1367.Goncalves, et al., "Clinical Application of Proteomics in Breast Cancer: State of the Art and Perspectives." *Medical Principles and Practice*, 2011, vol. 20, pp. 4-18.Gough, NM "Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells." *Anal. Biochem.*, 1988, vol. 173, pp. 93-95.Hoopes, et al., "Staining Electrophoretic Gels for Laccase and Peroxidase Activity Using 1,8-Diaminonaphthalene." *Analyt. Biochem.*, May 1, 2001, vol. 293, No. 1, pp. 96-101.

(Continued)

Primary Examiner — Jacob Cheu**(74) Attorney, Agent, or Firm** — Edwin S. Flores; Chalker Flores, LLP**(57) ABSTRACT**

The present invention includes methods for detecting benign to malignant transformation of a cancer in a subject, comprising the steps of: collecting a sample from the subject prior to electrophoretic protein separation; activating electrophoretically separated ENOX2 transcript variants with an ENOX2 electron donor; and detecting the presence of the one or more activated ENOX2 transcript variants using a pan-ENOX2 detectable binding reagent, wherein the presence of one or more activated ENOX2 transcript variants in the sample is indicative of the malignant transformation of the cancer, whereby a 10 to 100 fold increase in detection sensitivity is obtained for the one or more activated ENOX2 transcript variants when compared to an equivalent non-activated ENOX2 transcript variant.

37 Claims, 11 Drawing Sheets

(56)

References Cited

OTHER PUBLICATIONS

- Hostettler, et al., "Cancer Site-Specific Isoforms of ENOX2 (tNOX), A Cancer-Specific Cell Surface Oxidase." Clin. Proteomics., 2009, vol. 5, pp. 46-51.
- Howlander, et al., Seer Cancer Statistics Review, 1975-2012, National Cancer Institute, Bethesda, MD, 2012.
- Huston, et al., "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in *Escherichia coli*." Proc. Natl. Acad. Sci. USA., 1988, vol. 85, pp. 5879-5883.
- Jiang, Z., et al., "Molecular Cloning and Characterization of a Candidate Human Growth-Related and Time-Keeping constitutive Cell Surface Hydroquinone (NADH) Oxidase." Biochemistry, Sep. 2008, vol. 47, No. 52, pp. 14028-14038.
- Jones, et al., "Rapid PCR-Cloning of Full-Length Mouse Immunoglobulin Variable Regions," Bio/Technology, Jan. 1991, vol. 9, pp. 88-89.
- Korean Intellectual Property Office, International Search Report and Written Opinion for PCT/US2012/059141 dated Mar. 25, 2013, 14 pp.
- Lee et al., "Effect of BRCA1/2 mutation on short-term and long-term breast cancer survival: a systematic review and meta-analysis." Breast Cancer Research and Treatment, Jul. 2010, vol. 122, No. 1, pp. 11-25.
- Morre, D.J., et al., "Inhibition of Plasma membrane redox activities and elongation of growth of soybean." Physiologia Plantarum., vol. 72, 1988, pp. 236-240.
- Morre, D.J., et al., "The antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenylurea) (LY181984) inhibits NADH oxidase activity of HeLa plasma membranes." Biochim. Biophys. Acta., 1995, vol. 1240, pp. 11-17.
- Morre, D.J., et al., "Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture." Proc. Natl. Acad. Sci., Mar. 1995, vol. 92, pp. 1831-1835.
- Morre, D.J., et al., "NADH Oxidase Activity from Sera Altered by Capsaicin Is Widely Distributed among Cancer Patients" Arch. Biochem. Biophys., Jun. 15, 1997, vol. 342, pp. 224-230.
- Morre, D.J., et al., "A circulating form of NADH oxidase activity responsive to the antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenylurea) (LY181984) specific to sera from cancer patients." J. Bioenerg. Biomemb., 1997, vol. 29, pp. 281-289.
- Morre, D.J. "NADH Oxidase: A Multifunctional Ecto-proteins of the Eukaryotic Cell Surface. In: Asard, H., Bérczi, A. and Caubergs, R., Eds., Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease." Kluwer Academic Publishers, Dordrecht, 1998, pp. 121-156.
- Morre, D.J., et al., "Use of dipyriddy-dithio substrates to measure directly the protein disulfide-thiol interchange activity of the auxin stimulated NADH: Protein disulfide reductase (NADH oxidase) of soybean plasma membranes." Molecular and Cellular Biochemistry, 1999, vol. 200, pp. 7-13.
- Morre, D.J., et al., "Glaucarubolone and Similikalactone D, Respectively, Preferentially Inhibit Auxin-Induced and Constitutive Components of Plant Cell Enlargement and the Plasma Membrane NADH Oxidase." Int. J. Plant Sci., 1999, vol. 160, No. 2, pp. 291-297.
- Morre, D.J., et al., "Surface NADH oxidase of HeLa cells lacks intrinsic membrane binding motifs." Arch. Biochem. Biophys., Aug. 15, 2001, vol. 392, No. 2, pp. 251-256.
- Morre, D.J., et al., "Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative disease." Free Radical Res., Aug. 2003, vol. 37, pp. 795-805.
- Morre, D.J., et al., "Cancer Type-Specific tNOX Isoform: A Putative Family of Redox Protein Splice Variants with Cancer Diagnostics and Prognostic Potential." Biofactors, 2009, vol. 34, No. 3, pp. 201-207.
- Morre, D.J., et al., "Early Detection: An Opportunity for Cancer Prevention Through Early Intervention." Cancer Prevention—from Mechanisms to Translational Benefits, Apr. 20, 2012, pp. 389-402.
- Morre, D.J., et al., "The Auxin-Stimulated ENOX and Auxin Stimulation of Plant Growth." Chapter 10 of Ecto-Nox Proteins: Growth, Cancer; Aging, Springer Science+Business Media New York, 2013, pp. 313-342.
- Morre, D.J., et al., "Cancer Diagnostic Applications of ENOX2 Proteins." Chapter 12 of Ecto-Nox Proteins: Growth, Cancer; Aging, Springer Science+Business Media New York, 2013, pp. 419-434.
- Morre, D.J., et al., "ENOX2-based early detection (ONCOblot) of asbestos-induced malignant mesothelioma 4-10 years in advance of clinical symptoms." Clin. Proteomics, Jan. 22, 2016, vol. 13, No. 2, 8 pp.
- Smith, PK, et al., "Measurement of protein using bicinchoninic acid." Anal. Biochem., May 15, 1987, vol. 163, No. 1, 279.
- Sock, J., et al., "Activity Staining of Blotted Enzymes by Reaction Coupling with Transfer Membrane-Immobilized Auxiliary Enzymes." Analyt. Biochem., 1988, vol. 171, No. 2, pp. 310-319.
- Tang, et al., "Alternative splicing as the basis for specific localization of tNOX, a unique hydroquinone (NADH) oxidase, to the cancer cell surface." Biochemistry, 2007, vol. 46, pp. 12337-12346.
- Tang, et al., "Molecular Cloning and characterization of human age-related NADH oxidase (arNOX) proteins as members of the TM9 suprefamily of transmembrane proteins." Advances in Biological Chemistry, Apr. 2013, vol. 3, pp. 187-197.
- Wang, et al., "NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms." Biochim. Biophys. Acta, 2001, vol. 1539, pp. 192-204.
- Weinberg, R., The Biology of Cancer, Chapter 11, "Multi-Step Tumorigenesis." 2007, pp. 399-462.
- Wu, et al., "Next-Generation Sequencing of MicroRNAs for Breast Cancer Detection." Journal of Biomedicine and Biotechnology, Mar. 24, 2011, vol. 2011, 7 pp.

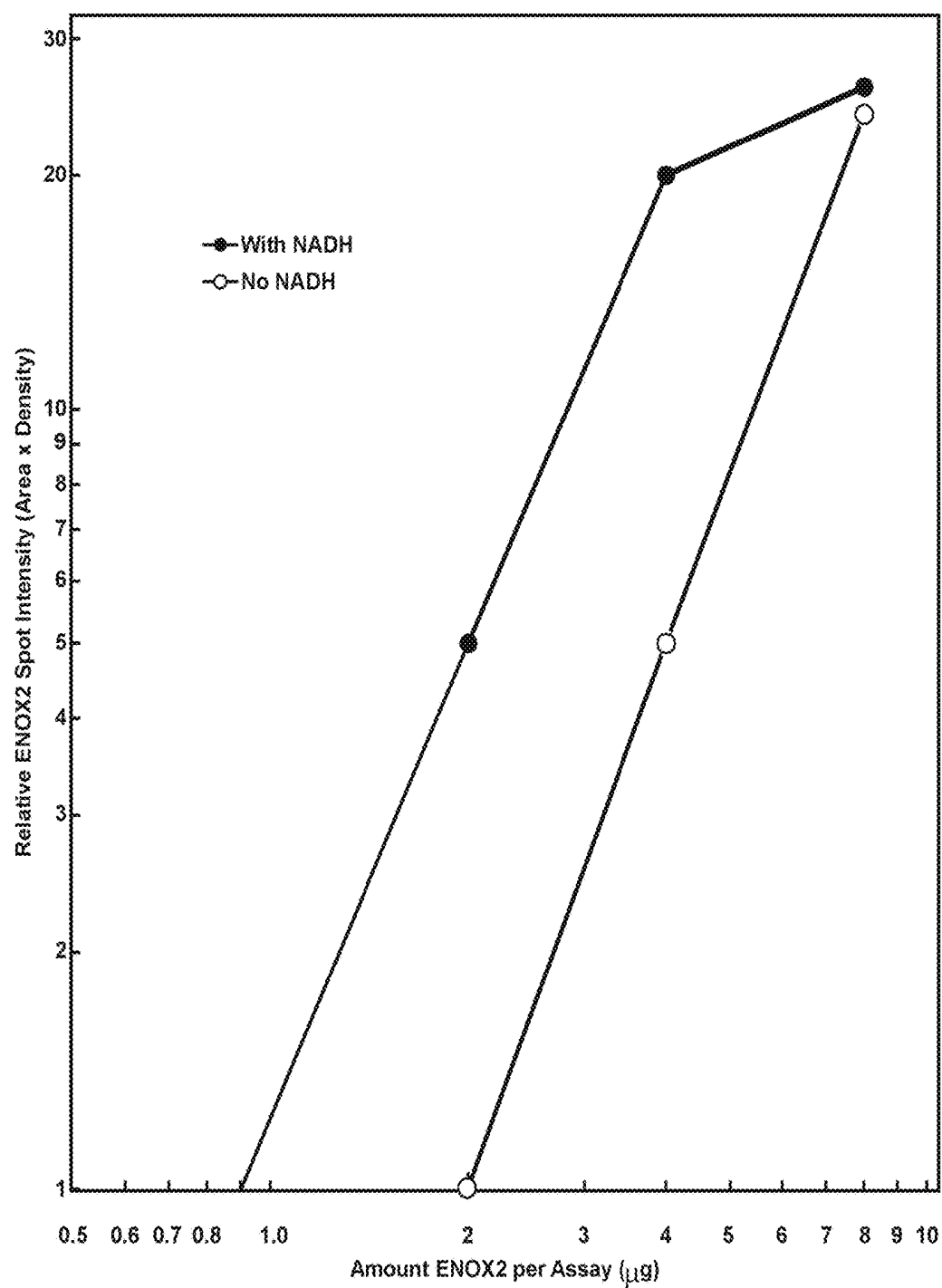


FIG. 1

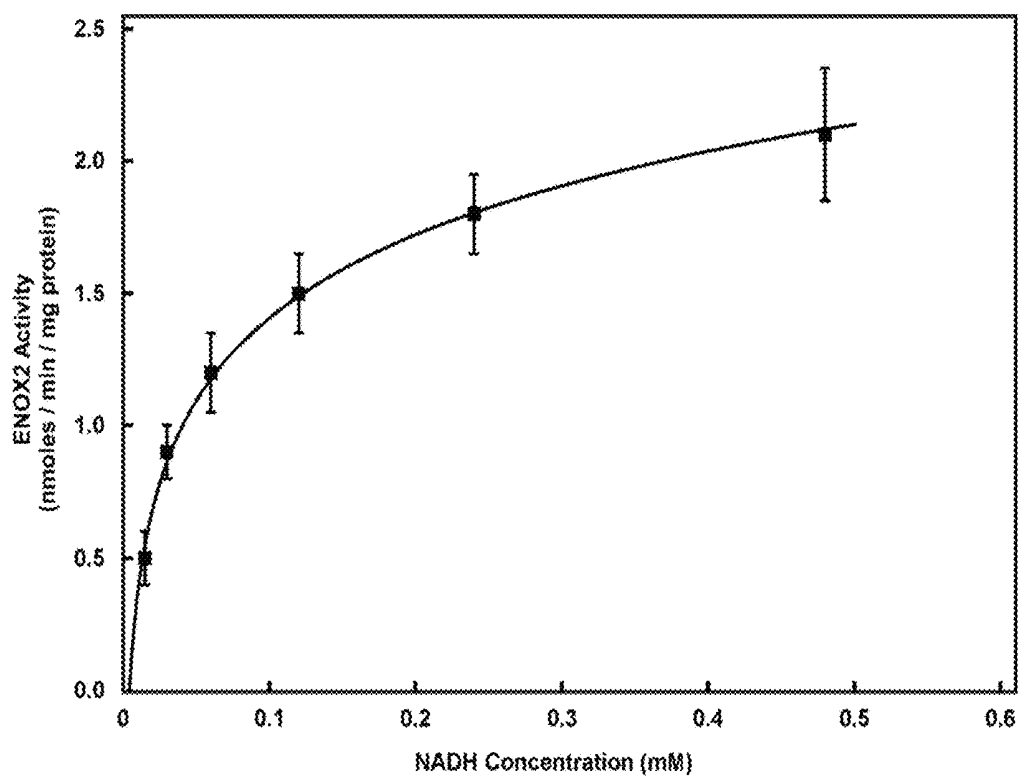


FIG. 2

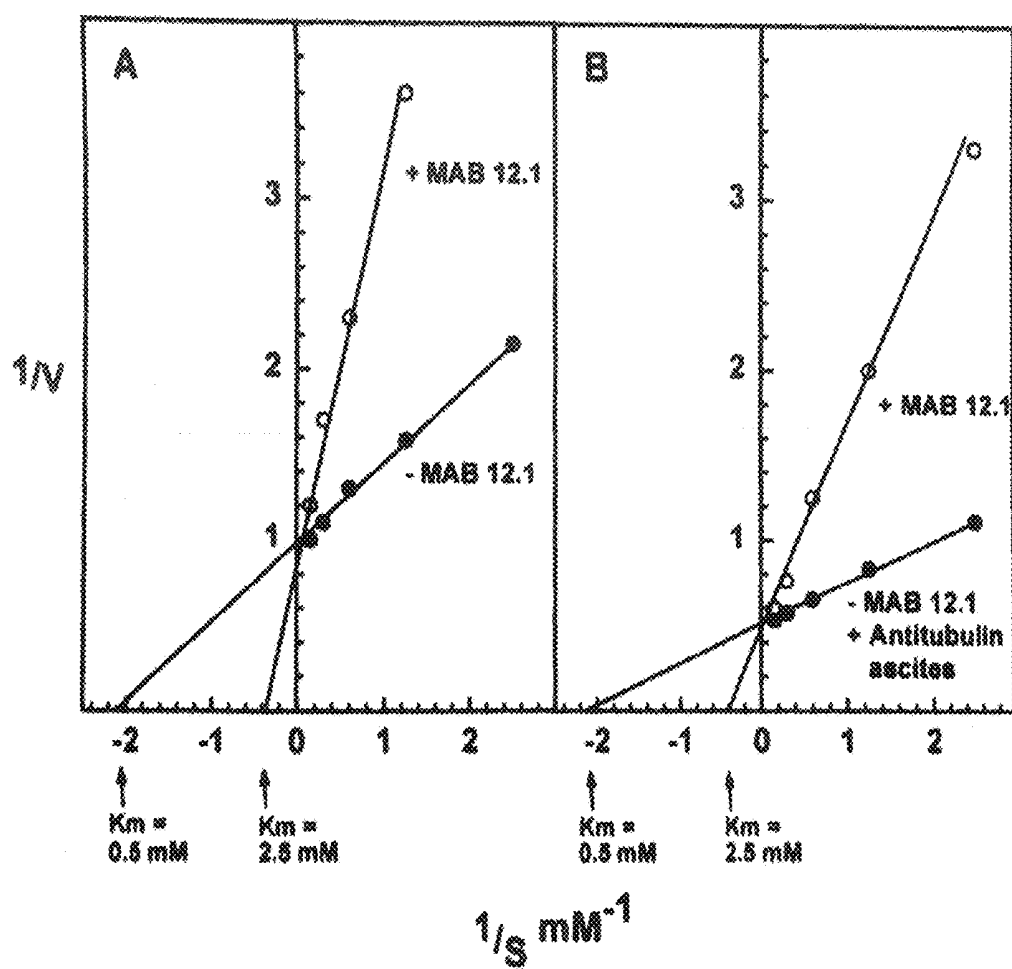


FIG. 3

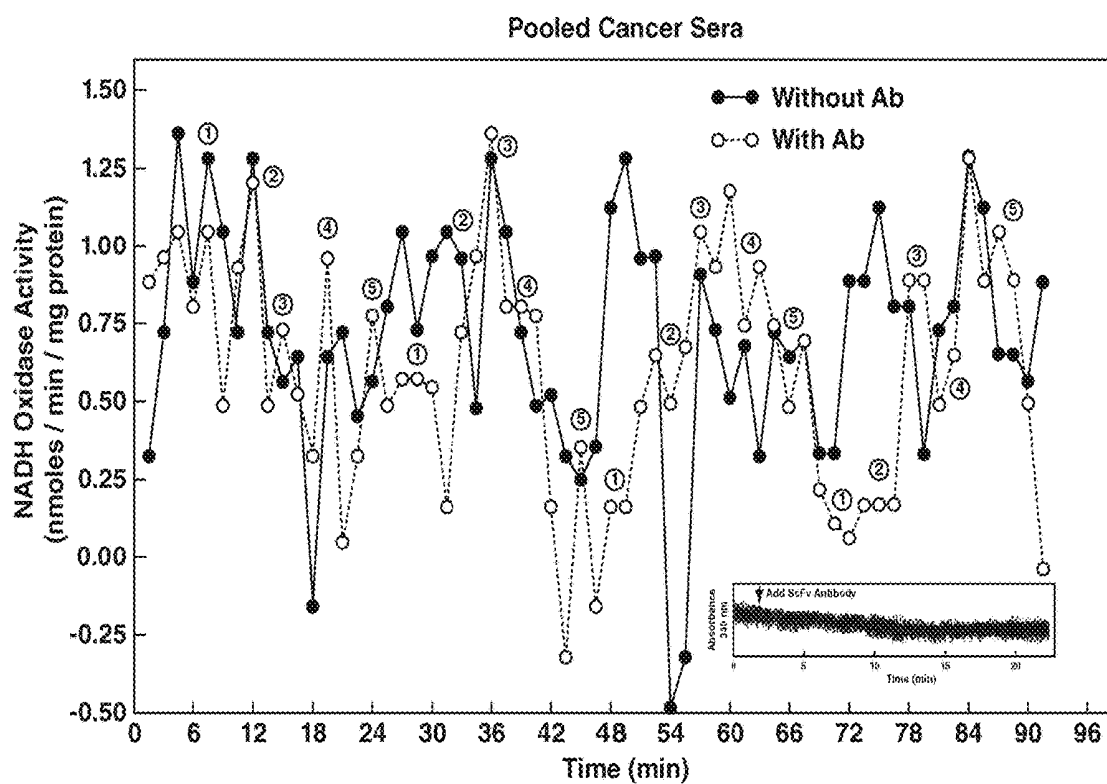


FIG. 4

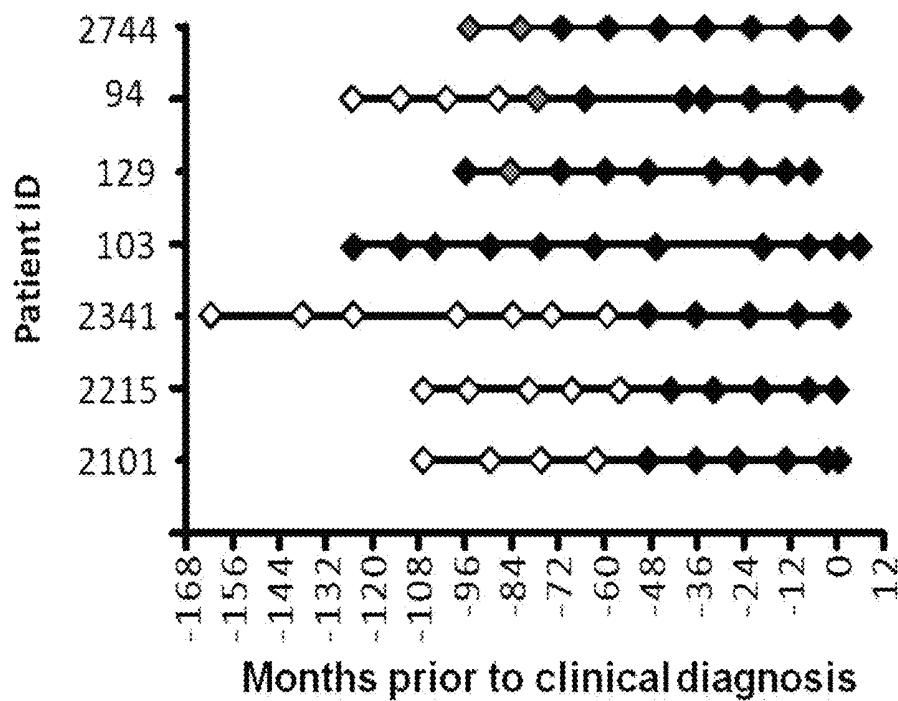


FIG. 5

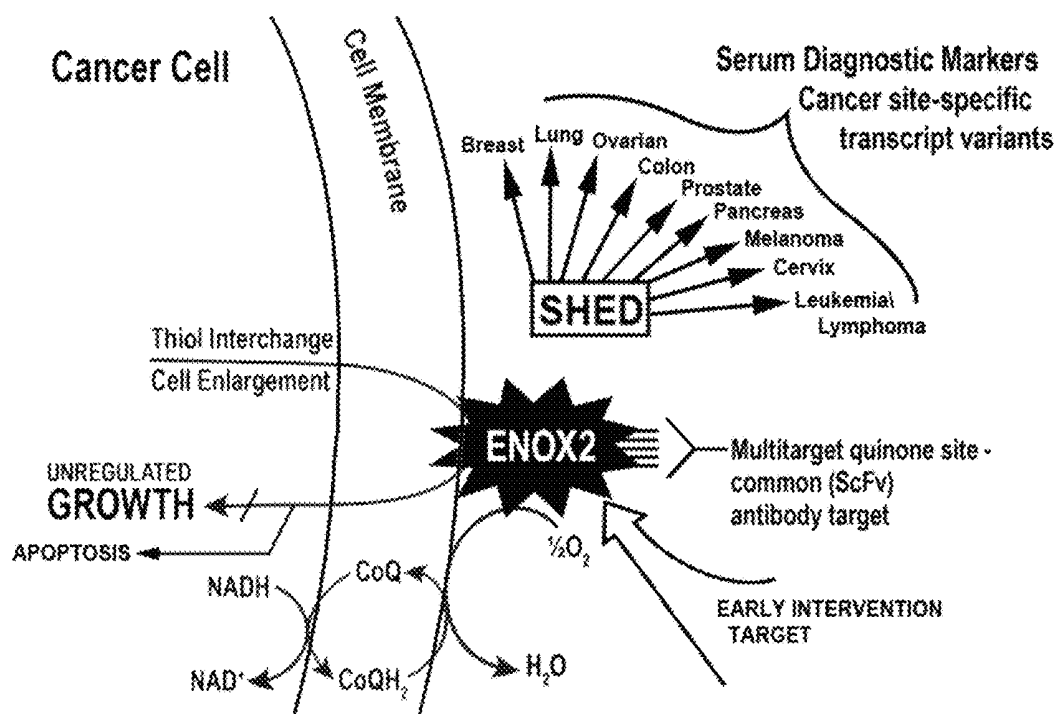


FIG. 6

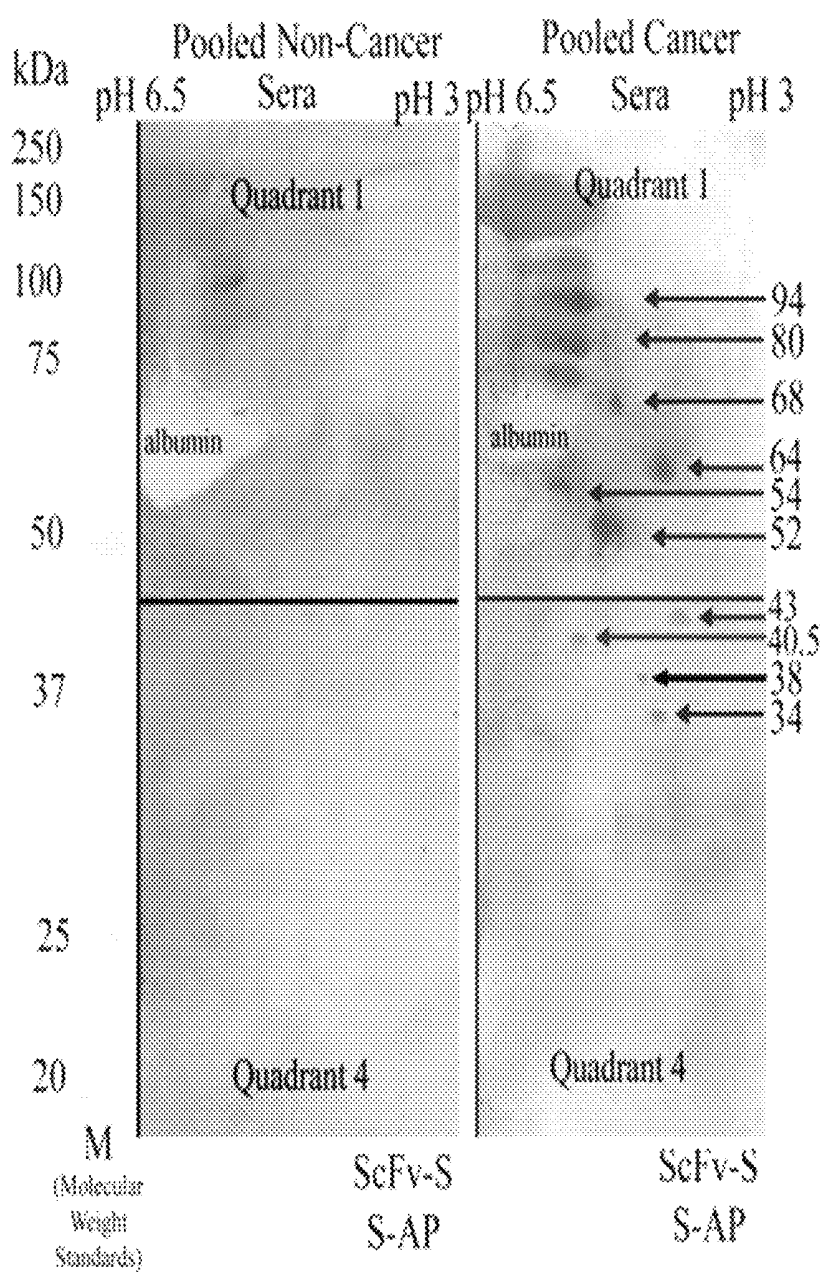
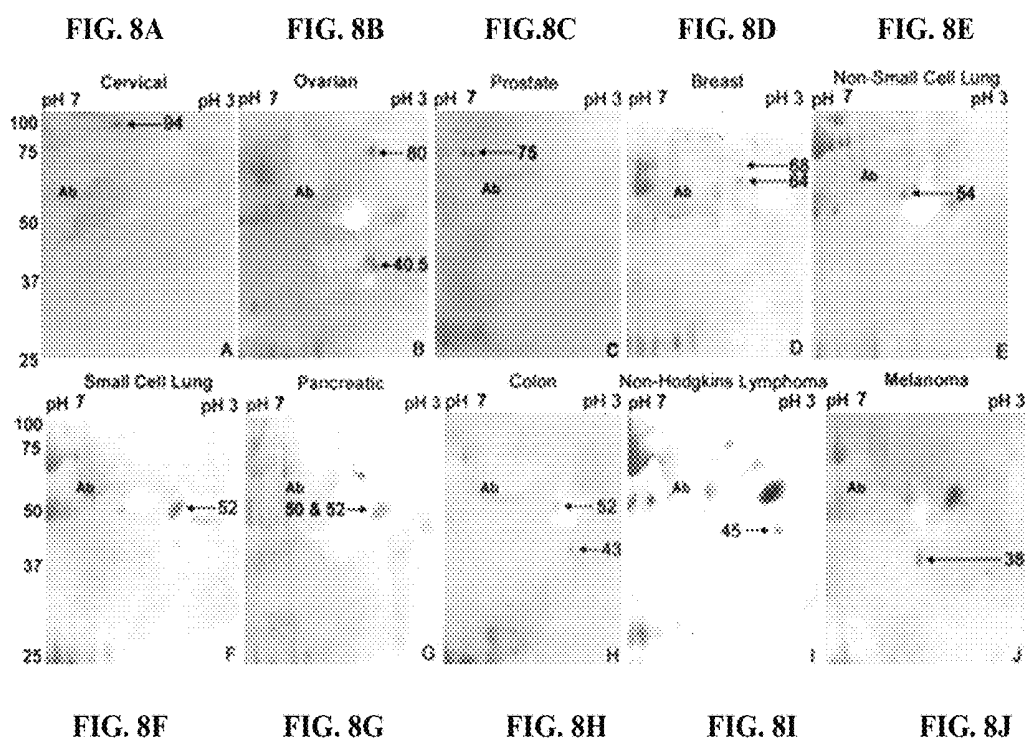


FIG. 7



FIGS. 8A – 8J

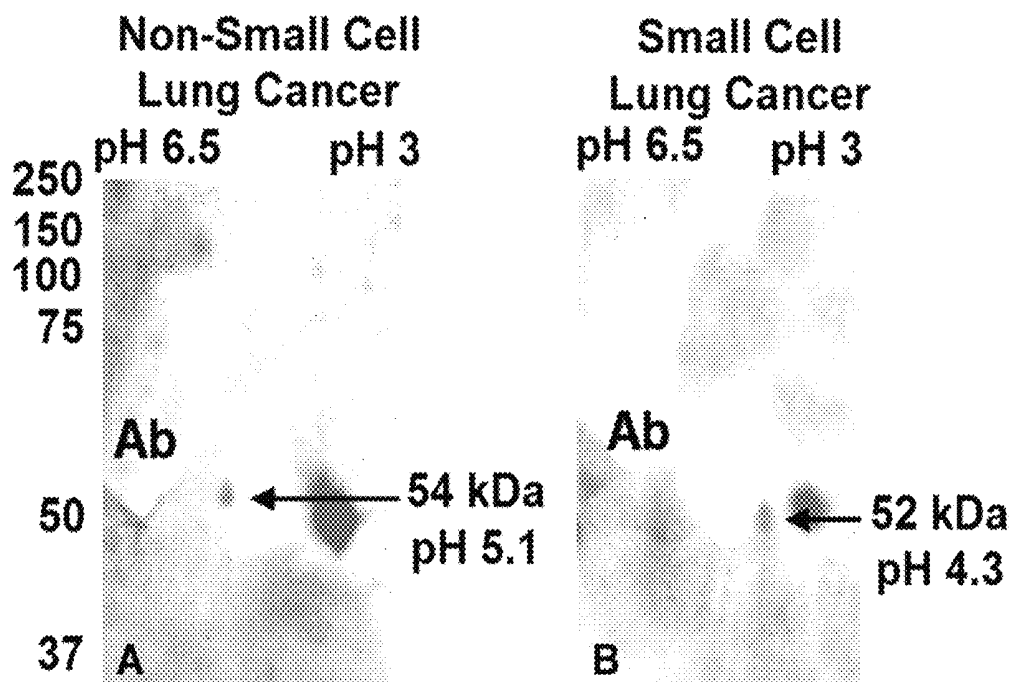


FIG. 9A

FIG. 9B

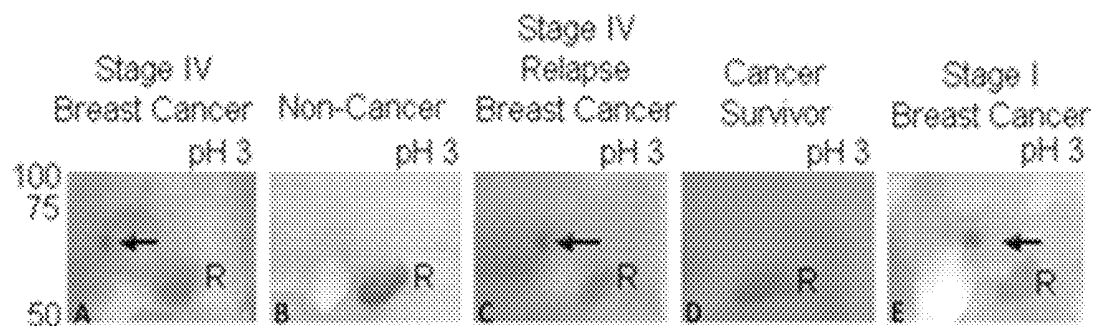


FIG. 10

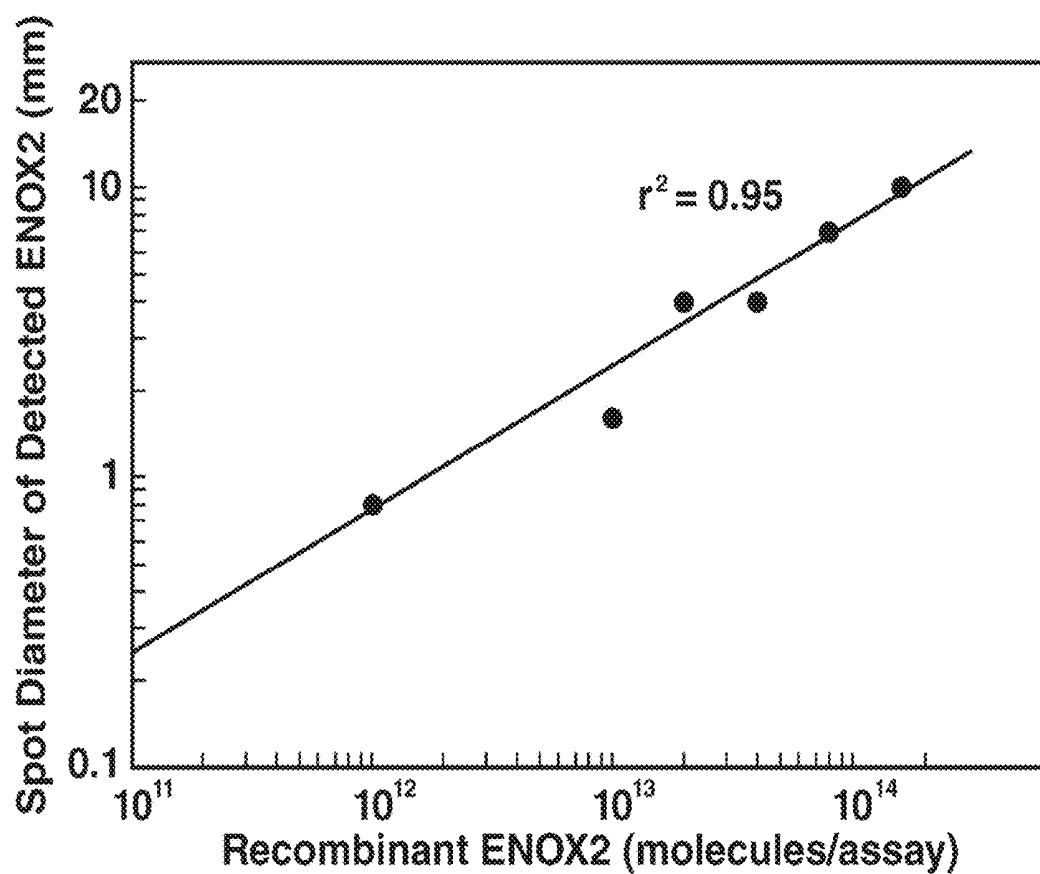
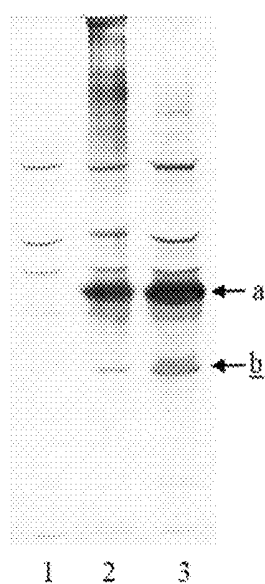


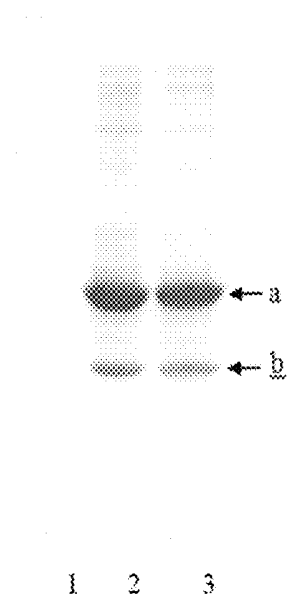
FIG. 11

A) Functional scFv (SC) Linker



Lane 1: Recombinant ENOX2
Lane 2: scFv(SC)
Lane 3: ENOX2 + scFv(SC)

B) Conventional scFv (S) Linker



Lane 1: Recombinant ENOX2(GST)
Lane 2: scFv(S)
Lane 3: ENOX2(GST) + scFv(S)

FIG. 12

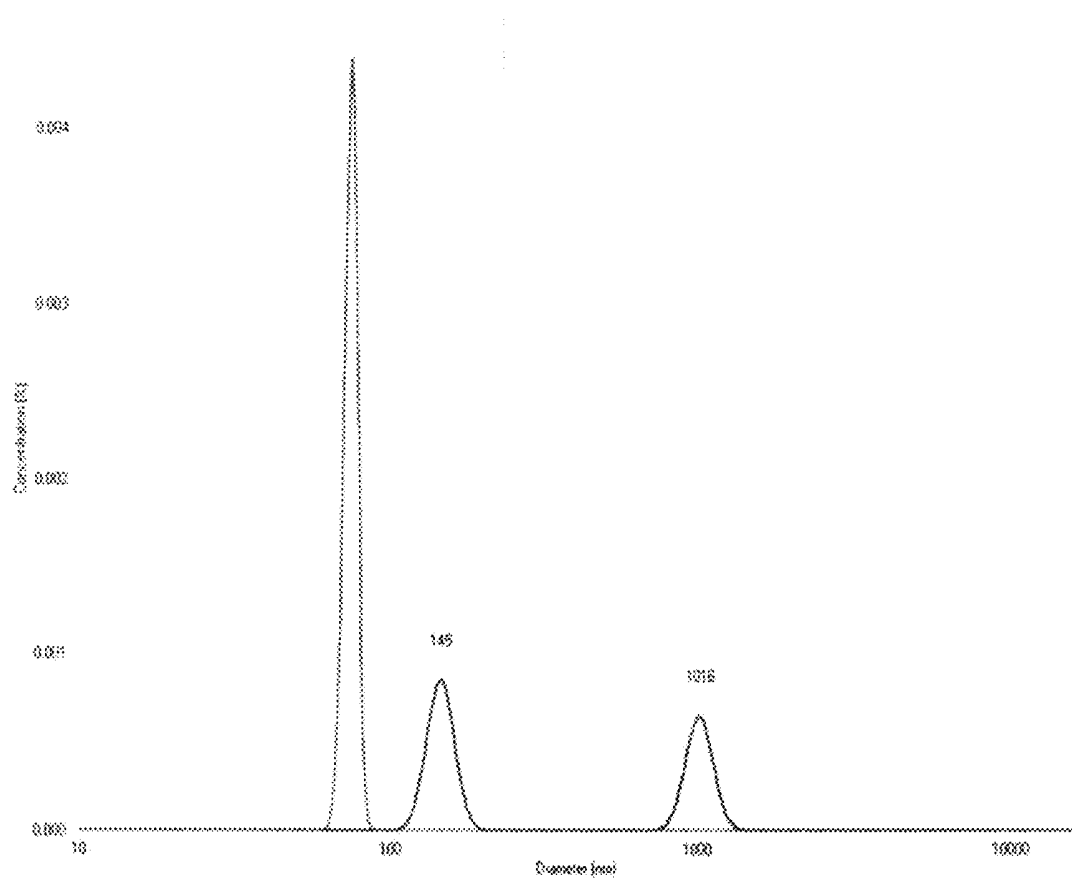


FIG. 13

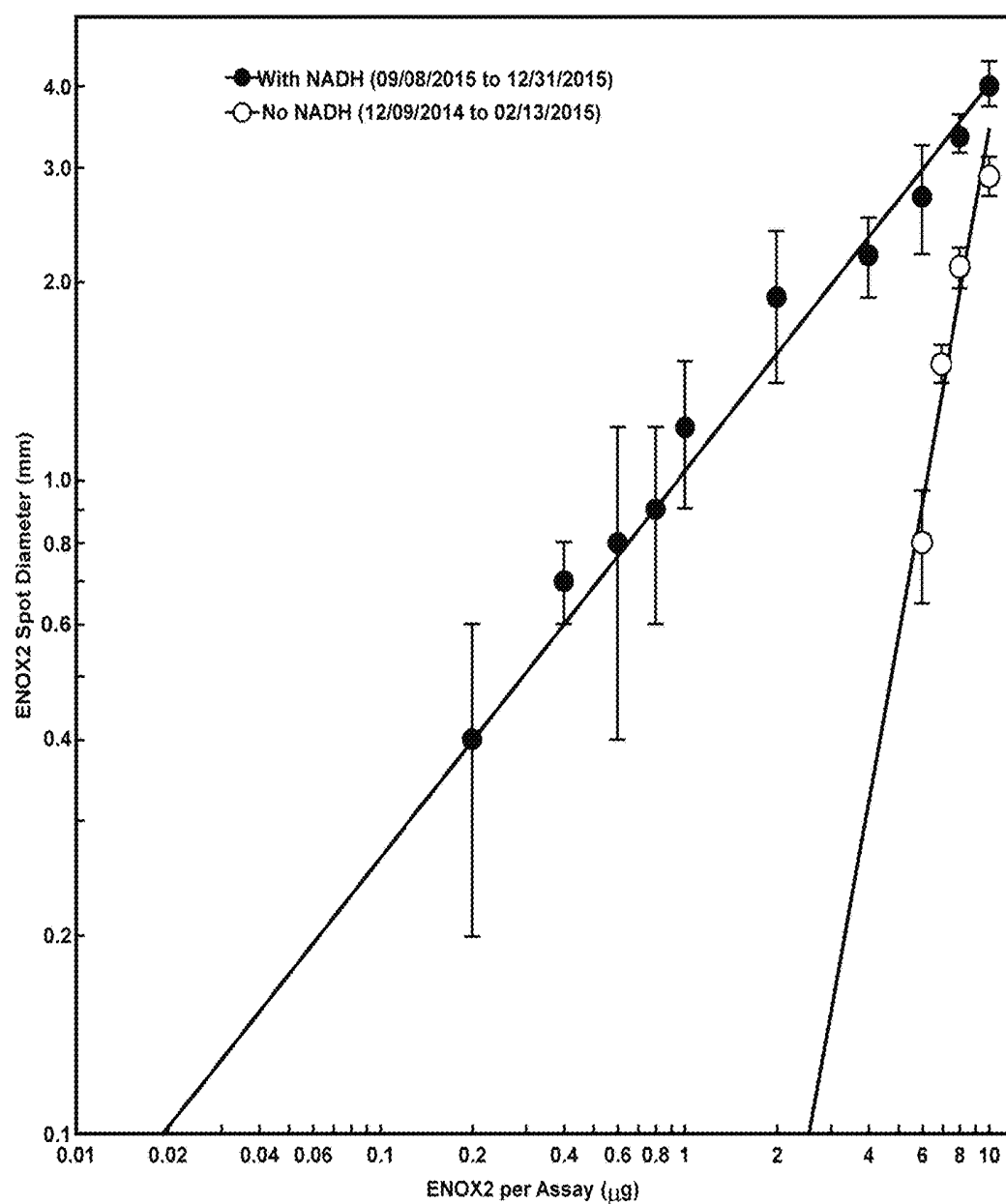


FIG. 14

1

**METHODS AND COMPOSITIONS FOR
TARGETED TWO-DIMENSIONAL WESTERN
BLOT ANALYSIS FOR EARLY CANCER
DETECTION AND CANCER DIAGNOSIS UP
TO TEN YEARS IN ADVANCE OF CLINICAL
SYMPTOMS OF MALIGNANT DISEASE**

**CROSS-REFERENCE TO RELATED
APPLICATIONS**

This patent application is a continuation of U.S. application Ser. No. 15/169,205 filed May 31, 2016, which is hereby incorporated by reference.

TECHNICAL FIELD OF THE INVENTION

The present invention relates in general to the field of cancer detection and diagnosis, and more particularly, to a functionalized single chain variable region recombinant ENOX2 antibody based on NADH-targeted detection.

**STATEMENT OF FEDERALLY FUNDED
RESEARCH**

None.

**INCORPORATION-BY-REFERENCE OF
MATERIALS FILED ON COMPACT DISC**

The present application includes a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 30, 2016, is named MNCE1002.txt and is 18 Kilobytes in size.

BACKGROUND OF THE INVENTION

Without limiting the scope of the invention, its background is described in connection with cancer protein biochemistry.

There is a unique, growth-related family of cell surface hydroquinone or NADH oxidases with protein disulfide-thiol interchange activity referred to as ECTO-NOX proteins (for cell surface NADH oxidases) (Morre, 1998. *Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease* (Asard, H., Berczi, A. and Caubergs, R. J., Eds) pp. 121-156, Kluwer Academic Publishers, Dordrecht, Netherlands; Morre and Morre, 2003. *Free Radical Res.* 37: 795-805). One member of the ECTO-NOX family, designated ENOX2 (for tumor associated) is specific to the surfaces of cancer cells and the sera of cancer patients (Morre et al., 1995. *Proc. Natl. Acad. Sci.* 92: 1831-1835; Bruno et al., 1992. *Biochem. J.* 284: 625-628). The presence of the ENOX2 protein has been demonstrated for several human tumor tissues (mammary carcinoma, prostate cancer, neuroblastoma, colon carcinoma and melanoma) (Cho et al., 2002. *Cancer Immunol. Immunother.* 51: 121-129); and serum analysis suggest a much broader association with human cancer (Morre, et al. 1997. *Arch. Biochem. Biophys.* 342: 224-230; Morre and Reust, 1997. *J. Bioenerg. Biomemb.* 29: 281-289).

ENOX proteins are ectoproteins anchored in the outer leaflet or the plasma membrane (Morre, 1965. *Biochim. Biophys. Acta* 1240: 201-208; FIG. 1). As is characteristic of other examples of ectoproteins (sialyl and galactosyl transferase, dipeptidylamino peptidase IV, etc.), the ENOX proteins are shed. They appear in soluble form in conditioned

2

media of cultured cells (Cho et al., 2002. *Cancer Immunol. Immunother.* 51: 121-129) and in patient sera (Morre, et al., 1997. *Arch. Biochem. Biophys.* 342: 224-230; Morre and Reust, 1997. *J. Bioenerg. Biomemb.* 29: 281-289). The serum form of ENOX2 from cancer patients exhibits the same degree of drug responsiveness as does the membrane-associated form. Drug-responsive ENOX2 activities are seen in sera of a variety of human cancer patients, including patients with leukemia, lymphomas or solid tumors (prostate, breast, colon, lung, pancreas, ovarian, and liver) (Morre, et al., 1997. *Arch. Biochem. Biophys.* 342: 224-230; Morre and Reust, 1997. *J. Bioenerg. Biomemb.* 29: 281-289). The extreme stability and protease resistance of the ENOX2 protein (del Castillo-Olivares et al., 1998. *Arch. Biochem. Biophys.* 385: 125-140) may help explain its ability to accumulate in sera of cancer patients to readily detectable levels. In contrast, no drug-responsive NOX activities have been found in the sera of healthy volunteers or in the sera of patients with disorders other than neoplasia (Morre, et al., 1997. *Arch. Biochem. Biophys.* 342: 224-230; Morre and Reust, 1997. *J. Bioenerg. Biomemb.* 29: 281-289).

While the basis for the cancer specificity of cell surface ENOX2 was not previously determined, the concept was supported by several lines of evidence. Drug responsive ENOX2 activity has been rigorously determined to be absent from plasma membranes of non-transformed human and animal cells and tissues (Morre et al., 1995. *Proc. Natl. Acad. Sci.* 92: 1831-1835). ENOX2 proteins lack a trans-membrane binding domain (Morre, et al., 2001. *Arch. Biochem. Biophys.* 392: 251-256) and are released from the cell surface by brief treatment at low pH (del Castillo-Olivares et al., 1998. *Arch. Biochem. Biophys.* 358: 125-140). A drug-responsive ENOX2 activity has not been detected in sera from healthy volunteers or patients with diseases other than cancer (Morre, et al., 1997. *Arch. Biochem. Biophys.* 342: 224-230; Morre and Reust, 1997. *J. Bioenerg. Biomemb.* 29: 281-289). Several ENOX2 antisera have identified the immunoreactive band at 34 kDa (the processed molecular weight of one of the cell surface forms of ENOX2) by Western blot analysis or immunoprecipitation when using transformed cells and tissues or sera of cancer patients as antigen source (Cho et al., 2002. *Cancer Immunol. Immunother.* 51: 121-129; Morre, et al., 2001. *Arch. Biochem. Biophys.* 392: 251-256; Chueh et al., 2002. *Biochemistry* 44: 3732-3741). The immunoreactive band at 34 kDa is absent with Western blot analysis or immunoprecipitation when using transformed cells and tissues or sera from healthy volunteers or patients with disorders other than cancer (Cho et al., 2002. *Cancer Immunol. Immunother.* 51: 121-129; Morre, et al., 2001. *Arch. Biochem. Biophys.* 392: 251-256; Chueh et al., 2002. *Biochemistry* 44: 3732-374). These antisera include a monoclonal antibody (Cho et al., 2002. *Cancer Immunol. Immunother.* 51: 121-129), single-chain variable region fragment (scFv) which reacts with the cell surface NADH oxidase from normal and neoplastic cells, polyclonal antisera made in response to expressed ENOX2 (Chueh et al., 2002. *Biochemistry* 44: 3732-374) and polyclonal peptide antisera to the conserved adenine nucleotide binding region of ENOX2 (Chueh et al., 2002. *Biochemistry* 44:3732-374).

ENOX2 cDNA has been cloned (GenBank Accession No. AF207881: 11; U.S. Patent Publication 2003/0207340 A1). The derived molecular weight from the open reading frame is 70.1 kDa. Functional motifs include a quinone binding site, an adenine nucleotide binding site, and a CXXXXC cysteine pair as a potential protein disulfide-thiol inter-

change site based on site-directed mutagenesis (Chueh et al., 2002. *Biochemistry* 44: 3732-374). Based on available genomic information (Bird, 1999. Direct submission of human DNA sequence from clone 875H3 (part of APK1 antigen) to GenBank database at NCBI) the ENOX2 gene is located on chromosome X, and it is comprised of multiple exons (thirteen). It is known that there are a number of splice variant mRNAs and proteins expressed.

The hybridoma cell line that produces the tumor NADH oxidase-specific monoclonal antibody MAB 12.1 was deposited with the American Type Culture Collection., Manassas, Va., 20108 on Apr. 4, 2002, under the terms of the Budapest Treaty. This deposit is identified by Accession No. ATCC PTA-4206. The deposit will be maintained with restriction in the ATCC depository for a period of 30 years from the deposit date, or 5 years after the most recent request, or for the effective life of the patent, whichever is longer, and will be replaced if the deposit becomes non-viable during that period. This monoclonal antibody is described in U.S. Pat. No. 7,053,188, issued May 30, 2006, which is incorporated by reference herein.

Because cancer poses a significant threat to human health and because cancer results in significant economic costs, there is a long-felt need in the art for an effective, economical and technically simple system in which to assay for the presence and organ site of cancer.

SUMMARY OF THE INVENTION

The present invention includes methods for the analysis of a biological sample for the presence of particular transcript variants of the pan-cancer antigen known as ENOX2 (for tumor-specific NADU oxidase). The present method includes, e.g., a 2-dimensional gel electrophoresis and immunoblotting using an antibody specific for the pan-cancer ENOX2 antigen and the various isoforms, which characterize particular types of cancers. The methods of the present invention can also be applied to evaluate response to therapy, with decreasing amounts of ENOX2 isoform(s) reflecting successful treatment, as well as early detection of recurrent disease (reflected increased or reappearance of ENOX2-specific isoforms).

In one embodiment, the present invention includes a method for detecting benign to malignant transformation of a cancer in a subject, comprising the steps of: collecting sample from the subject prior to electrophoretic protein separation; activating electrophoretically separated ENOX2 transcript variants with an ENOX2 electron donor; and detecting the presence of the one or more activated ENOX2 transcript variants using a pan-ENOX2 detectable binding reagent, wherein the presence of one or more activated ENOX2 transcript variants in the sample is indicative of the

malignant transformation of the cancer, whereby a 10 to 100 fold increase in detection sensitivity is obtained for the one or more activated ENOX2 transcript variants when compared to equivalent non-activated ENOX2 transcript variants. In one aspect, the ENOX2 electron donor required to activate the ENOX2 transcript variants is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M. In another aspect, the method further comprises performing two dimensional (2D) electrophoretic protein separation and blotting onto a film or paper. In another aspect, the pan-ENOX2 detectable binding reagent comprises SEQ ID NO:1. In another aspect, the sample is a blood, serum, plasma, urine, cerebrospinal fluid or other body fluid. In another aspect, the pan-ENOX2 detectable binding reagent comprises an anti-ENOX2 scFv fusion protein that is: non-aggregating at between 4° C. and 40° C.; comprises a functional linker between heavy and light chain portions of the ScFv fusion protein comprised primarily of amino acids with small side chains (Gly/Ser) containing, in addition, two cysteine residues (C) spaced as either CXXXXC or CXXXXXC; and is capable of forming an interchain disulfide bond with the protein disulfide-thiol interchange functional motif of ENOX2. In another aspect, the pan-ENOX2 detectable binding reagent comprises an scFv of SEQ ID NO:11, wherein the stability, binding efficiency, and shelf life of the scFv of SEQ ID NO.1 is improved by storing in 50% at -70° C. glycerol to prevent aggregation. In another aspect, the step of concentrating the blood, serum, or plasma sample is defined further as concentrating the one or more ENOX2 transcript variants using a nickel-agarose substrate to bind and concentrate the one or more ENOX2 transcript variants. In another aspect, the method further comprises detecting the cancer and/or determining a tissue of origin of a human cancer at least one year in advance of clinical symptoms. In another aspect, the method further comprises detecting the cancer and/or determining a tissue of origin of a human cancer at least one year (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or 20 years) in advance of clinical symptoms. In another aspect, the method further comprises determining a tissue of origin of one or more cancers of unknown primary (CUP). In another aspect, the method further comprises screening for at least one of: early cancer in populations with family cancer histories, environmental exposures, or in a general population. In another aspect, the method further comprises determining a tissue of origin of the one or more activated ENOX2 transcript variants based on the presence of ENOX2 transcript variants as set forth in the following Table 1:

TABLE 1

Ranges for Molecular Weight (MW) and Isoelectric Point (Pi) Determined for Sera of Patients Diagnosed with 24 Different Cancers Representing 20 Different Tissues of Origin							
RANGES							
Cancers	N	Protein 1		Protein 2		Protein 3	
		MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)
Bladder	37	63-66	4.2-5.6 ¹	42-48	4.1-4.8 ¹		
*Blood Cell (Total)	(117)	34-47	3.5-4.6				

TABLE 1-continued

Ranges for Molecular Weight (MW) and Isoelectric Point (Pi) Determined for Sera of Patients Diagnosed with 24 Different Cancers Representing 20 Different Tissues of Origin							
RANGES							
Cancers	N	Protein 1		Protein 2		Protein 3	
		MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)
Breast	682	64-69	4.2-4.9				
Cervical	47	90-100	4.2-5.4				
Colorectal	125	80-96	4.4-5.4	50-65	4.2-5.3	33-46	3.8-5.2
Esophageal	26	42-47	4.6-5.2				
Gastric	26	120-188	4.7-5.5	50-62	4.5-5.6	45-53	2.4-3.6
Hepatocellular	31	58-70	4.5-5.0	34-40	4.1-5.2		
Leukemias	36	34-45	3.5-4.5				
**Lung, Non-Small Cell	55	53	4.7-5.3				
**Lung, Non-Small Cell	270	54-56	4.6-5.3				
Lung, Small Cell	27	52-53	4.1-4.6				
Lymphomas	56	43-45	3.5-4.5				
Melanoma	51	37-41	4.6-5.3				
Mesothelioma	27	60-68	3.8-4.1	38-44	3.8-4.6		
Myelomas	25	40-45	3.9-4.6				
Ovarian	115	72-90	3.7-5.0	37-47	3.7-5.0		
Pancreatic	75	48-51	3.9-5.4				
Prostate	361	71-88	5.1-6.5				
Renal Cell (Kidney)	31	69-73	4.7-5.4	54-61	4.1-5.2	38-43	3.7-4.3
Sarcoma	29	50-55	5.2-5.6	37-45	4.3-4.9		
Squamous Cell	51	57-68	5.0-5.4				
Testicular Germ Cell	25	61-62	5.0-5.4	40-45	4.4-4.7		
Thyroid Follicular	25	48-56	4.7-5.1	37-42	4.5-5.2		
Thyroid Papillary	27	56-67	4.5-5.0	37-44	3.2-3.6		
***Uterine (Endometrial)	26	63-66	4.2-4.9 ²	41-48	4.4-5.6 ²		
***Uterine (Endometrial)	57	67-71	4.2-5.1	41-48	3.7-5.4		
Total	2460						

*Blood cell cancers include lymphomas, leukemias and myelomas already represented in the totals.

**Non-Small Cell Lung cancers are in two subsets to avoid molecular weight overlap with small cell lung cancer.

***Uterine cancers are in two subsets based on molecular weight to avoid overlap with bladder cancer (see footnotes 1 and 2).

1. Isoelectric point pH of Protein 1 $\geq$ Protein 2.

2. Isoelectric point pH of Protein 1 < Protein 2.

In another aspect, the method further comprises resolving proteins at 53 and/or 79 to 85 kDa as total protein loading controls. In another aspect, the method further comprises distinguishing between distant metastases and multiple primary cancers based on the presence of the one or more activated ENOX2 transcript variants. In another aspect, the method further comprises detecting stage 0 and stage 1 cancers and determining the tissue of origin of the cancer. In another aspect, an incidence of false positives and confirmed false negatives of the predicted cancer is less than 1%. In another aspect, the sensitivity of the method for detecting the cancer is greater than 95%. In another aspect, the sensitivity of the method detects the presence of 2 million cancer cells or less. In another aspect, the method further comprises monitoring the reemergence of the cancer, wherein the presence of 2 million or less cancer cells is detectable based on the reemergence of the one or more activated ENOX2 transcript variants. In another aspect, the method further comprises determining between malignant and benign early neoplastic lesions, wherein if a benign lesion is detected a step of performing surgery, chemotherapy, or radiation therapy may be avoided. In another aspect, the method further comprises determining an early indication of prostate cancer. In another aspect, the method further comprises determining a ductile mammary carcinoma in situ and in need of curative early intervention to potentially avoid

surgery, the need for radiation or chemotherapy, prior to the development of ductal mammary carcinoma. In another aspect, the benign to malignant transformation is detected at least one year before development of clinical symptoms. In another aspect, the tissue of origin of the cancer detected is selected from at least one of bladder, blood cell, lymphomas, leukemias, multiple myelomas and myelomas breast, cervical, colorectal, esophageal, gastric, hepatocellular, lung, non-small cell, melanoma, mesothelioma, ovarian, pancreatic, prostate, renal cell (kidney), sarcoma, squamous cell, testicular germ cell, thyroid follicular, thyroid papillary, or uterine (endometrial).

Yet another embodiment of the present invention includes a calibrated method of quantitation of ENOX2 transcript variants on a western blot comprising: subjecting a sample from the subject concentrated sample to electrophoretic protein separation; western blotting the electrophoretically separated proteins onto a protein capture film or paper; activating one or more ENOX2 transcript variants on the protein capture film or paper with a reduced ENOX2 substrate; and detecting the presence of the one or more activated ENOX2 transcript variants using a pan-ENOX2 detectable binding reagent, wherein a presence of one or more activated ENOX2 transcript variants in the blood, serum, or plasma sample is indicative of the malignant transformation of the cancer, wherein a 10 to 100 fold

increase in detection sensitivity for ENOX2 is obtained when compared to the non-activated ENOX2, wherein the amount of the amount of one or more activated ENOX2 transcript variants is calibrated based on a spot diameter and an intensity of the ENOX2 transcript variant on the protein capture film or paper when compared to a known amount of the one or more ENOX2 transcript variants. In one aspect, the spot diameter is compared to a standard curve of recombinant ENOX2. In another aspect, the spot diameter and the log of the mass of ENOX2 are linearly correlated.

Yet another embodiment of the present invention includes a method for determining the tissue of origin of a cancer in a subject comprising: subjecting proteins in a sample from the subject to a two-dimensional (2D) molecular weight and isoelectric point electrophoretic protein separation; transferring the proteins to a protein capture film or paper; activating the electrophoretically separated ENOX2 transcript variants by incubation with an ENOX2 electron donor on the protein capture film or paper; detecting the activated ENOX2 transcript variants; and determining the tissue of origin of the cancer based on the presence of one or more activated ENOX2 transcript variants in the sample from the Table 1, wherein activation of the one or more ENOX2 transcript variants in situ increases the detection sensitivity by 10 to 100 fold when compared to non-activated ENOX2 transcript variants. In another aspect, the ENOX2 electron donor required to activate the ENOX2 transcript variants is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M. In another aspect, the method further comprises performing two dimensional (2D) electrophoretic protein separation and blotting onto a film or paper, wherein the ENOX2 electron donor is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M. In another aspect, the pan-ENOX2 detectable binding reagent comprises SEQ ID NO:11. In another aspect, the pan-ENOX2 detectable binding reagent comprises an anti-ENOX2 scFv fusion protein is: non-aggregating at between 4° C. and 40° C.; comprises a small side chain (Gly/Ser) amino acid residues containing two cysteines (C) spaced as either CXXXXC or CXXXXXC; and is capable of forming interchain disulfide bonds with the protein disulfide-thiol interchange functional motif of ENOX2. In another aspect, the pan-ENOX2 detectable binding reagent comprises an scFv of SEQ ID NO:11, wherein the stability, binding efficiency, and shelf life of the scFv of SEQ ID NO:11 is improved by storing in 50% glycerol at -70° C. to prevent aggregation. In another aspect, the step of concentrating the sample is defined further as concentrating the one or more ENOX2 transcript variants using a nickel-agarose substrate to bind and concentrate the one or more ENOX2 transcript variants. In another aspect, the method further comprises detecting the cancer and/or determining a tissue of origin of a human cancer at least one year in advance of clinical symptoms. In another aspect, the sample is a blood, serum, plasma, urine, cerebrospinal fluid or a body fluid.

Yet another embodiment of the present invention includes a blot comprising one or more electrophoretically separated proteins from a concentrated sample from a subject, wherein the blot is exposed to a pan-ENOX2 detecting reagent and

to a reducing substrate for ENOX2 that activates the ENOX2 transcript variants on the blot, wherein the blot is analyzed for the presence of one or more activated ENOX2 transcript variants from Table 1, wherein the presence of the one or more activated ENOX2 transcript variants is used to detect and determine a tissue of origin of a human cancer at least one year in advance of clinical symptoms, and the activation of the one or more ENOX2 transcript variants in situ increases the detection sensitivity by 10 to 100 fold when compared to non-activated ENOX2 transcript variants.

BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

FIG. 1 is a graph that demonstrates a two-fold increase in spot size and density of different amounts of ENOX2 in the assay as determined by 2-D gel electrophoresis/western blot analysis comparing incubation in the presence of 15 μ M NADH in the assay compared to no NADH.

FIG. 2 is a graph that depicts the relationship of ENOX2 activity and concentration of NADH. Maximum activity was achieved at about 500 μ M NADH with a K_M of approximately 0.8 μ M (see FIG. 3 for determination of K_M).

FIG. 3 is graph that shows a double reciprocal Lineweaver-Burke plot of ENOX2 activity (nanomoles/min/mg protein) V versus mM substrate concentration S yielding a K_M of approximately 0.8 mM. The ordinate intercept being the same or similar for with and without antibody demonstrate that the antibody competes with the NADH substrate for the active site providing evidence for the substrate and antibody combining site being in the same region of the ENOX2 sequence.

FIG. 4 is graph that shows a representation of ENOX2 activity as a function of time to illustrate five maxima of the ENOX2 activity cycle of pooled cancer sera. As quantitated in Table 1, inhibition occurs at maximum 4 within the 22 min activity cycle. Thus, antibody binding must occur exclusively during maximum 4 since maximum 5 is the first maximum to be inhibited. Antibody addition at maximum 5 had no effect nor did antibody addition before maximum 4.

FIG. 5 is graph that depicts early detection of malignant mesothelioma in asbestos workers up to 10 years in advance of clinical symptoms. Serial assays of seven male subjects, median age of diagnosis 67 years, beginning 168 to 96 months before diagnosis of asbestos-induced malignant mesothelioma are represented. Solid symbols—both protein 1 and protein 2 characteristic of malignant mesothelioma, evident. Open symbols—neither protein 1 nor protein 2 evident. Shaded symbols—only protein 1 evident.

FIG. 6 is a schematic representation of the utility of the ENOX2 family of cancer-specific, cell surface proteins for early diagnosis and early intervention of cancer. Cancer site-specific transcript variants of ENOX2 are shed into the serum to permit early detection and diagnosis. The ENOX2 proteins of origin at the cell surface act as terminal oxidases of plasma membrane electron transport functions essential to the unregulated growth of cancer. When the ENOX2 proteins are inhibited, as for example through EGCg/*Capsicum* synergies, the unregulated growth ceases and the cancer cells undergo programmed cell death (apoptosis).

FIG. 7 provides a 2-Dimensional gel/western blot of ENOX2 transcript variants comparing pooled non-cancer (left panels) and pooled cancer representing major carcinomas plus leukemias and lymphomas (right panels) patient

sera. The approximate location of unreactive (at background) albumin is labeled for comparison. ENOX2 reactive proteins are restricted to quadrants I and IV. Detection use recombinant ScFv-S (S-tag peptide: His-Glu-Ala-Ala-Lys-Phe-Gln-Arg-Glu-His) antibody linked with alkaline phosphatase. The approximately 10 ENOX2 transcript variants of the pooled cancer sera are absent from non-cancer (A) and are cancer site-specific.

FIGS. 8A to 8J show western blots of 2-D gel electrophoresis/western blots of sera from cancer patients analyzed individually. Cancer sites are presented in the order of decreasing molecular weight of the major transcript variant present. FIG. 8A. Cervical cancer. FIG. 8B. Ovarian cancer. FIG. 8C. Prostate cancer. FIG. 8D. Breast cancer. FIG. 8E. Non-small cell lung cancer. FIG. 8F. Small cell lung cancer. FIG. 8G. Pancreatic cancer. FIG. 8H. Colon cancer. FIG. 8I. Non-Hodgkin lymphoma. FIG. 8J. Melanoma. The approximate location of unreactive (at background) albumin (Ab) is labeled for comparison. Approximately 180 non-cancer patient sera were analyzed in parallel without evidence of proteins indicative of specific transcript variants for comparison as positive controls.

FIGS 9A and 9B show analytical gel electrophoresis and immunoblots of patient sera. FIG. 9A. Sera from a patient with non-small cell lung cancer contains a 54 kDa, isoelectric point pH 5.1 transcript variant (arrow). FIG. 9B. Sera from a patient with small cell lung cancer contains a 52 kDa, isoelectric point 4.3 transcript variant (arrow). The reference spots to the right, MW 52 kDa and isoelectric point pH 4.1 are α 1-antitrypsin inhibitor. Albumin and other serum proteins are unreactive.

FIG. 10 shows 2-D gel electrophoretic separations and detection of ENOX2 transcript variants specific for breast cancer by western blotting of patient sera. Arrow=66 to 68 kDa breast cancer specific transcript variant. R=52 kDa, isoelectric point pH 4.1 α -fetuin reference spot.

FIG. 11 illustrates the log-log relationship between spot diameter and amount of ENOX2 protein loaded on the 2-D gel. Varying amounts of recombinant ENOX2 were analyzed by immunoblot. The log of the diameter of the detected ENOX2 spot was then plotted as a function of the log of the amount of ENOX2 loaded.

FIG. 12 shows Western blots of mixing experiments to demonstrate efficacy of ScFV with -CXXC-XX-C linker (FIG. 12A) compared to the conventional XXXXXX linker (FIG. 12B). Under non-reducing conditions for both (to preserve S—S bonds), the ScFV recombinant antibody with the functional cysteine linker when incubated with recombinant ENOX2 showed reduced levels of 53 kDa ScFV (a) and a 46 kDa truncated ScFV from a downstream initiation site (b) predominantly by disulfide bond formation between the full length ScFV and the 72 kDa full length ENOX2 in a ratio of one ENOX2 molecule plus two ScFV molecules (one to each of the two protein disulfide isomerase binding sites of ENOX2) for a combined molecular weight of about 176 kDa. In FIG. 12B, with the conventional linker lacking cysteines, no evidence of cross-linking under identical reaction conditions was observed.

FIG. 13 is particle distribution as concentration (%) of antibody based on size (nm) determined by light scattering (Agilent 7080 Particle Size Spectrophotometer) showing predominately the major contribution of unaggregated antibody when stored in 50% glycerol at -70°C .

FIG. 14 is a comparison of the NADH-targeted two-dimensional western blot analysis described herein compared with a conventional two-dimensional western blot analysis as described by Hostetler et al. 2009 (*Clin. Pro-*

teomics 5: 46-51) validating a 10-fold increase in sensitivity of ENOX2 transcript variant detection.

DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

The field of this invention is a serum-based assay for early pan-cancer diagnosis of cancer that permits detection of cancer up to ten years in advance of clinical symptoms of malignant disease based on a specific functionalized single chain variable region recombinant antibody targeted to a cancer-specific family of cell surface ENOX2 proteins whereby molecular weights and isoelectric points of tissue of origin-specific transcript variants determined by using two-dimensional gel electrophoresis with an ENOX2-specific recombinant scFv antibody combined with NADH-targeted technology providing ten- to 100-fold enhanced sensitivity over the previously developed protocols.

Ecto-Nicotinamide Adenine Dinucleotide Oxidase Disulfide Thiol Exchanger 2 (ENOX2) (GenBank accession no. AF207881; Chueh et al., 2002) also known as Tumor Associated Nicotinamide Adenine Dinucleotide Oxidase (ENOX2) is ideally suited as a target for early diagnosis as well as for early preventive intervention (FIG. 6). The proteins are expressed on the cell surface of malignancies and detectable in the serum of patients with cancer (Cho et al., 2002). ENOX2 proteins are terminal hydroquinone oxidases of plasma membrane electron transport. From the standpoint of early intervention, they are important in the growth and enlargement of tumor cells (Morre and Morre, 2003; Tang et al., 2007. *Biochemistry* 46:12337-12346; 2008. *Oncol. Res.* 16:557-567). Our approach using ENOX2, as a target for both early detection and for early interventions, is based on these properties (Cho et al., 2002; Morre and Morre, 2003; reviewed by Davies and Bozzo, 2005. *Drug News Perspect* 19: 223-225). While ENOX2 presence provides a non-invasive approach to cancer detection, without methodology to identify cancer site-specific ENOX2 forms, it did not offer an indication as to cancer type or location.

The present invention provides a method for the analysis of a biological sample for the presence of particular isoforms of the pan-cancer antigen known as ENOX2 (for tumor-specific NADH oxidase). The present method entails 2-dimensional gel electrophoresis and immunoblotting using an antibody specific for the pan-cancer ENOX2 antigen and the various isoforms which characterize particular types of cancers. As specifically exemplified, about 30 μL of sera are loaded for analysis.

ENOX2 transcript variants are identified on the basis of their molecular weights and isoelectric point with detection using a ENOX2-specific monoclonal antibody (MAb) (U.S. Pat. No. 7,053,188), using single chain variable region (scFv) fragment which recognizes all cell surface NOX proteins (both age-related, normal cell and neoplasia specific NADH oxidase) or using polyclonal sera raised against ENOX2. The ECTO-NOX proteins are first enriched and concentrated from a biological sample, desirably a serum sample, by binding to nickel-agarose and then eluting. After release of the proteins from the nickel agarose by vortexing, the proteins are separated in the first dimension by isoelectric focusing and in the second dimension by polyacrylamide gel electrophoresis. As specifically exemplified herein, the isoelectric focusing step is over a pH range from 3 to 10, and size separation is over a 10% polyacrylamide gel. Most of the cancer-specific ENOX2 isoforms exhibit isoelectric points in a very narrow range between pH 3.9 and 6.3 but differ in molecular weight from 34 to 136 kDa. In the 2D gel system specifically exemplified, the cancer-specific isoforms are located in Quadrants I (relatively high molecular weight material) and IV (lower molecular weight material, notably the range of 30 to 50 kDa. IgG heavy chains (Quadrant II and IgG light chains (Quadrant III) cross react with the scFv antibody and along with reference proteins at 53 and 79 to 85 kDa serve as loading controls. The absence of all ENOX2 isoforms indicates the absence of cancer or a cumulative cancer size below the limit of detection in the assay. The presence of an ENOX2 isoform indicates the presence of cancer. The particular molecular weight present in a serum sample or a particular combination of isoforms provides an indication of the cell type or tissue of origin of the cancer. The method not only determines cancer presence, but also the method of the present invention provides diagnostic information concerning the tissue of origin. At present there are no other pan cancer (all forms of human cancer) tests with these particular capabilities. ENOX2 transcript variants with apparent molecular weights in the range of 64-69 kDa, pH in the range of 4.2-4.9 are associated with breast cancer. ENOX2 transcript variants of 52-53 kDa, pH 4.1-4.6 is associated with small cell lung cancer. An ENOX2 transcript variant of 54-56 kDa, pH 4.6-5.3 is associated with non-small cell lung cancer. Two ENOX2 transcript variants of about 72-90 kDa and 37-47 kDa, isoelectric point pH 3.7-5.0 for both, characterize ovarian cancer. ENOX2 transcript variants of 71-88 kDa, pH 5.1-6.5 are associated with prostate cancer. An ENOX2 transcript variant of about 90-100 kDa, pH 4.2-5.4 signals cervical cancer. Three ENOX2 transcript variants of about 80-96 kDa, pH 4.4-5.4; 50-65 kDa, pH 4.2-5.3; and 33-46 kDa, pH 3.8-5.2 are characteristic of colon cancer. Where a patient is suspected of having cancer, the 2D gel electrophoresis/immunological analysis of a biological sample, advantageously a serum sample will reveal both cancer presence and organ site with the present invention.

Method 1

Ten- to 100-fold Increase in Sensitivity and Specificity of the Western Blot Detection Protocol

Using the present invention a ten- to 100-fold increase in sensitivity and specificity of the western blot detection protocol was derived from an observation that there was a delay time of the inhibition of NADH oxidation following exposure of serum ENOX2 to an antibody combining site inhibitory ligand. The inhibition was observed to occur at

various time between 0 and 22 minutes (22 minutes is the length of the ENOX2 activity cycle; (Wang et al., 2001. *Biochim. Biophys. Acta* 1539:192-204) during a 30 minute incubation. Thus, the time during each 22 min activity cycle where the EEMTE antibody combining site is available for combination with antibody is relatively brief, and in the order of less than 5 minutes. Also, the ENOX2 protein must be active in order for the antibody to gain access to the binding site during this relatively short window of opportunity.

Table 2 shows the activity of ENOX2 at the five activity maxima during 5 successive 22 minutes activity cycles following antibody addition.

	nanomoles/min/mg protein				
	Maximum 1	Maximum 2	Maximum 3	Maximum 4	Maximum 5
Cycle 1					1.00
Cycle 2	1.00	1.25	0.75	0.90	0.40
Cycle 3	0.50	0.25	0.10	0.25	0.25
Cycle 4	0.10	0.50	0.25	0.25	0.05
Cycle 5	0.10	0.05	0.30	0.00	0.30

Inhibition occurs at maximum 4 within the 22 min activity cycle. Thus, antibody binding must occur exclusively during maximum 4 since maximum 5 is the first maximum to be inhibited. Antibody addition at maximum 5 had no effect nor did antibody addition before maximum 4. A corollary of the above result is that antibody will not bind or will bind only poorly to inactive ENOX2 transcript variants. Therefore, steps were initiated to render ENOX2 on western blots active during incubation with antibody.

In order to maintain an active ENOX2 during western blotting with antibody, following blocking for 10 minutes with 1% milk and two rinses with TBST to remove excess milk, reduced pyridine nucleotides [NAD(P)H] were added at a concentration of 15 μ M during incubation with the primary scFv antibody. NADH is preferred at a final concentration in the range of 10 μ M. Incubation of the blot with antibody with or without shaking at any temperature between 4° C. and 40° C. for times of 1 to 16 hours depending on the concentration of NADH, the temperature and the antibody titer, preferably with incubation at 4° C. overnight (approximately 16 hours) with shaking.

The cancer diagnostic system of the present invention utilizes two-dimensional polyacrylamide gel electrophoretic techniques for the separation of proteins in human sera to generate cancer-specific isoform patterns and compositions indicative of cancer presence, tumor type, disease severity and therapeutic response. The protocol is designed for the detection of at least 26 cancer-specific patterns of ENOX2 transcript variants each denoting a different tumor type representing 20 different tissue of cancer origin, which are resolved to indicate cancer presence and origin of the primary cancer. This specification illustrates the process of the transcript—resolving two-dimensional gel electrophoresis protocol and subsequent immunoanalysis to detect ENOX2 transcript variants that reflect particular cancers.

Two-dimensional gel electrophoresis separates by displacement in two dimensions oriented at right angles to one another and immunoblotting identifies the ENOX2 isoforms. In the first dimension variants are separated by isoelectric point (pI) according to charge by isoelectric focusing (IEF). The variants are then separated according to molecular weight in kilodaltons (kDa) by SDS-PAGE in a second

dimension. The isoforms are then blotted onto a nitrocellulose membrane for further analysis using the pan-cancer specific antibody preparation containing a functional linker that stabilizes antibody binding to the transcript variants.

The opportunity to simultaneously determine both cancer presence and cancer tissue of origin emerged as a result of 2-dimensional gel electrophoretic separations where western blots with a pan ENOX2 recombinant single chain variable region (scFv) antibody carrying an S tag (FIG. 7) was employed for detection (Hostetler et al., 2009. *Clin. Proteomics* 5: 46-51). The antibody cross reacted with all known ENOX2 transcript variants from hematological and solid tumors of human origin but, of itself, did not differentiate among different kinds of cancers. Analyses using this antibody, when combined with two-dimensional gel electrophoretic separation, revealed specific species of ENOX2 proteins subsequently identified as transcript variants, each with a characteristic molecular weight and isoelectric point indicative of a particular form of cancer (Table 1).

Method 2

ENOX Transcript Variants of Specific Molecular Weights and Isoelectric Points are Produced Uniquely by Patients with Cancer

The transcript variants are shed into the circulation and have the potential to serve as definitive, non-invasive and sensitive serum markers for early detection of both primary and recurrent cancer in at risk populations with a low incidence of both false positives, and false negatives as they are molecular signature molecules produced specifically by cancer cells and absent from non-cancer cells.

As the 2-D-western blot protocol detects cancer early, well in advance of clinical symptoms, the opportunity to combine early detection with early intervention as a potentially curative prevention strategy for cancer is provided whereby elimination of the disease in its very earliest stages is uniquely possible.

FIG. 2 is a graph that depicts the relationship of ENOX2 activity and concentration of NADH. Maximum activity was achieved at about 500 μ M NADH with a K_M of approximately 0.8 μ M (see FIG. 3 for determination of K_M).

FIG. 3 is graph that shows a double reciprocal Lineweaver-Burke plot of ENOX2 activity (nanomoles/min/mg protein) V versus mM substrate concentration S yielding a K_M of approximately 0.8 mM. The ordinate intercept being the same or similar for with and without antibody demonstrate that the antibody competes with the NADH substrate for the active site providing evidence for the substrate and antibody combining site being in the same region of the ENOX2 sequence.

FIG. 4 is graph that shows a representation of ENOX2 activity as a function of time to illustrate five maxima of the ENOX2 activity cycle of pooled cancer sera. As quantitated in Table 1, inhibition occurs at maximum 4 within the 22 minutes activity cycle. Thus, antibody binding must occur exclusively during maximum 4 since maximum 5 is the first maximum to be inhibited. Antibody addition at maximum 5 had no effect nor did antibody addition before maximum 4.

FIG. 5 is graph that depicts early detection of malignant mesothelioma in asbestos workers up to 10 years in advance of clinical symptoms. Serial assays of seven male subjects, median age of diagnosis 67 years, beginning 168 to 96 months before diagnosis of asbestos-induced malignant mesothelioma are represented. Solid symbols—both protein 1 and protein 2 characteristic of malignant mesothelioma,

evident. Open symbols—neither protein 1 nor protein 2 evident. Shaded symbols—only protein 1 evident.

Analytical 2-D gel electrophoresis and immunoblotting of ENOX proteins from a mixed population of cancer patients (bladder, blood cell (leukemias, lymphomas, and myelomas), breast, cervical, colorectal, esophageal, gastric, hepatocellular, lung, melanoma, ovarian, pancreatic, prostate, renal cell (kidney), sarcoma, squamous cell, testicular germ cell, thyroid, and uterine (endometrial) revealed multiple species of acidic proteins of molecular weight between 34 and 100 kDa in quadrants I and IV (FIG. 7, right panels), none of which were present in sera of non-cancer patients (FIG. 7, left panels). Separation in the first dimension was by isoelectric focusing over the pH range of 3 to 10 and separation in the second dimension was by 10 percent SDS-PAGE. Isoelectric points of the ENOX2 transcript variants were in the range of 3.2 to 6.3. The principal reactive proteins other than the ENOX2 forms were a 53 kDa isoelectric point pH 4.1, mostly phosphorylated alpha-fetuin (Labeled "R" in FIG. 8) which served as a convenient loading control and isoelectric point reference and a 79-85 kDa, isoelectric point pH 6.8 serotransferrin which served as a second point of reference for loading and as an isoelectric point reference. The two cross reactive reference proteins are present in a majority of sera and plasma of both cancer and non-cancer subjects. Albumin and other serum proteins normally do not react when present in low amounts. On some blots, the recombinant scFv was weakly cross-reactive with heavy (ca. 52 kDa) and light (ca. 25 kDa) immunoglobulin chain proteins.

FIGS. 8A to 8J show western blots of 2-D gel electrophoresis-western blots of sera from cancer patients analyzed individually. Cancer sites are presented in the order of decreasing molecular weight of the major transcript variant present. FIG. 8A. Cervical cancer. FIG. 8B. Ovarian cancer. FIG. 8C Prostate cancer. FIG. 8D. Breast cancer. FIG. 8E. Non-small cell lung cancer. FIG. 8F. Small cell lung cancer. FIG. 8G. Pancreatic cancer. FIG. 8H. Colon cancer. FIG. 8I. Non-Hodgkin lymphoma. FIG. 8J. Melanoma. The approximate location of unreactive (at background) albumin (Ab) is labeled for comparison. Approximately 180 non-cancer patient sera were analyzed in parallel without evidence of proteins indicative of specific transcript variants for comparison as positive controls.

FIGS. 9A and 9B show analytical gel electrophoresis and immunoblots of patient sera. FIG. 9A. Sera from a patient with non-small cell lung cancer contains a 54 kDa, isoelectric point pH 5.1 transcript variant (arrow). FIG. 9B. Sera from a patient with small cell lung cancer contains a 52 kDa, isoelectric point 4.3 transcript variant (arrow). The reference spots to the right, MW 52 kDa and isoelectric point pH 4.1 are α 1-antitrypsin inhibitor. Albumin and other serum proteins are unreactive.

FIG. 10 shows 2-D gel electrophoretic separations and detection of ENOX2 transcript variants specific for breast cancer by western blotting of patient sera. Arrow=66 to 68 kDa breast cancer specific transcript variant. R=52 kDa, isoelectric point pH 4.1 α -fetuin reference spot.

Sera from individual patients with various forms of cancer were analyzed by 2-D gel electrophoresis and immunoblotting to assign each of the ENOX2 isoforms of FIG. 7 to a cancer of a particular tissue of origin (see Table 3). ENOX2 transcript variants with apparent molecular weights in the range of 64-69 kDa, pH in the range of 4.2-4.9 are associated with breast cancer. ENOX2 transcript variants of 52-53 kDa, pH 4.1-4.6 is associated with small cell lung cancer. An ENOX2 transcript variant of 54-56 kDa, pH 4.6-5.3 is

15

associated with non-small cell lung cancer. Two ENOX2 transcript variants of about 72-90 kDa and 37-47 kDa, isoelectric point pH 3.7-5.0 for both, characterize ovarian cancer. ENOX2 transcript variants of 71-88 kDa, pH 5.1-6.5 are associated with prostate cancer. An ENOX2 transcript variant of about 90-100 kDa, pH 4.2-5.4 signals cervical cancer. Three ENOX2 transcript variants of about 80-96 kDa, pH 4.4-5.4; 50-65 kDa, pH 4.2-5.3; and 33-46 kDa, pH 3.8-5.2 are characteristic of colon cancer. Where a patient is suspected of having cancer, the 2D gel electrophoresis/immunological analysis of a biological sample, advantageously a serum sample, will reveal both cancer presence and organ site with the present invention.

The protein sequence of ENOX2 exhibits similarity with the two reference proteins alpha-fetuin and serrotransferrin reactive with the pan ENOX2 scFv recombinant antibody. Regions of similarity are restricted to a 7 amino acid sequence (underlined) adjacent in ENOX2 to the E394EMTE398 quinone inhibitor-binding site, which serves as the antigen sequence to which the specific scFv antibodies bind.

(SEQ ID NO: 1)
ENOX2 EEMTETKETEESALVS underlined AA 399-406

(SEQ ID NO: 2)
Alpha-fetuin GTDCVAKEATEAAKCN underlined AA 210-216

(SEQ ID NO: 3)
Serrotransferrin CLDGTGTRKPVVEEYANCH underlined AA 588-595

A. Sample Preparation

1. Prepare/thaw re-hydration solution (−20, 1.5 mL tube labeled RB).

a. Add 1% Dithiothreitol (DTT) to solution before use (0.01 g/1.0 mL).

2. Add 120 μL of Rehydration Buffer to a 1.7 ml tube.

3. Add 30 μL of sera to tube.

4. Vortex solution until fully mixed.

5. Remove Immobililine DryStrips from freezer (−20° C., pH 3-10) and allow strips to equilibrate to RT for 5 minutes.

a. Do not leave strips at RT for more than 10 minutes.

6. Record ID# from strip.

7. Load 130 μL of sample to tray per 7 cm DryStrip. Ensure tray is level.

8. Place DryStrips gel-side down over sample.

9. Ensure sample is evenly spread throughout strip by carefully lifting strip in and out of sample a few times if needed.

10. If samples are concentrated in one region of the strip, redistribute sample by pipetting.

11. Remove air bubbles by gently pressing down on DryStrip with pipette tip.

12. Place lid on tray and place tray in plastic bag with ddi-H₂O soaked paper towels.

13. Seal bag.

14. Allow sample to re-hydrate overnight at RT on a level surface allowing strips to absorb sample for 12-24 hours.

B. Isoelectric Focusing (First Dimension)

1. Turn on IPGphor (ensure proper startup of machine).

2. Place strips on Manifold focusing tray as follows:

a. Gel side up.

b. positive (acidic) end towards back.

c. Strips are aligned.

d. Between metal strips (so electrodes fit and touch metal strip)

16

3. Obtain 2 Paper Wicks per strip.

4. Wet wicks with 150 μL di-H₂O per wick.

5. Place wicks over anodic and cathodic ends of gel (approx. 0.3 cm).

6. Place electrodes on wicks, but away from gel (be sure prong is on metal plate), and lock in place.

7. Cover strips with DryStrip Cover fluid.

a. Fill strips entire lane with oil.

b. Ensure strips are fully covered.

8. Close lid.

9. IEF run with IPGphor II.

a. Maximum amperage: 50 μAmps.

b. Temperature: 20° C.

c. Ensure correct assembly by checking initial voltage.

d. As needed, pause run and replace wicks, continue run until dye front disappears.

Run Parameters:

	Step	Voltage	Time/Vhrs
7 cm Strip pH 3-10 (Run #1)	1-Stp.	250 V	250 Vhrs.
	2-Stp.	500 V	500 Vhrs.
	3-Stp.	1000 V	1000 Vhrs.
	4-Grd.	4000 V	3 hrs.
	5-Stp.	4000 V	25,000 Vhrs.
	6-Stp.	500 V	Hold

C. Prepare SDS-Page Gels (for Second Dimension)

1. Prior to use, wash and scrub plates very well in soap and hot water.

2. Rinse in di-H₂O.

3. Leave the plates to air dry or wipe with ethanol-soaked Kimwipes.

4. Order plates in Protean-plus Multi-Gel casting Chamber (Bio-Rad) as per manual (with a spacer between each plate and block).

5. Ensure screws are fully tightened.

6. Add gel solution.

7. Stop pouring when gel is about 1-1.5 cm from top of glass plates.

8. Gently overlay gels with ethanol.

9. Cover with Saran Wrap.

10. Allow gels to polymerize for at least one hour (best if overnight).

D. Equilibration (First Dimension)

1. Remove strips from tray and place on Kimwipe to remove excess oil.

1. Place strips gel side up on Kimwipe.

2. Overlay strips with a second Kimwipe and gently blot to remove oil.

2. Place strips in equilibration plate gel side up; freeze or equilibrate.

1. Freeze: Wrap plate in plastic wrap, store at −80° C.

a. Thaw strips prior to equilibration (clear when thawed).

2. Equilibrate: continue to next step.

3. Cover strips with equilibration buffer, about 1.5 mL per strip.

4. Heat up Agarose until it is liquefied.

5. Shake 20 min at RT.

E. SDS-PAGE (Second Dimension)

1. Prepare left (pH 10 side) markers by adding 8 μL of standards on Whatman 3MM chromatography paper cut to about 3 cm×0.75 cm. Standards should be added to bottom of paper, about 1 cm high.

2. Prepare right (pH 3 side) markers by adding 8 μL of standards on Whatman 3MM chromatography paper cut to

17

approximately 3 cm×0.075. Standards should be added to bottom of paper, about 1 cm high.

3. Pour off Equilibration Buffer.

4. Cover strips in SDS Running buffer to rinse away excess Equilibration Buffer

5. Remove SDS Running buffer from strips.

6. Repeat SDS Running buffer rinse.

7. Carefully place strips gel side out on back plate of SDS-PAGE gel.

8. Overlay strips with 1% low melting agarose once it has cooled enough to touch skin

1. Ensure no air bubbles have formed under the gel.

2. Use ruler to tap gel and remove air bubbles.

9. Insert marker's next to appropriate end of IEF strip, ensuring marker is flush to the gel on the strip.

10. Allow polymerization of agarose.

11. Continue for each strip to be loaded in 2nd dimension.

12. Place gels in Dodeca tank, HINGED SIDE DOWN

13. After all gels have been put in tank, ensure gels are covered in entirety by SDS running buffer.

14. 2nd dimension run is done at 13° C.

1. 250 V.

2. 1-1.5 hour (allow gel to run until gel front approaches tubing in lid of tank).

F. Protein Transfer (for Western Blot)

1. Remove gel from Dodeca tank.

2. Cut gel to desired size.

3. Fill tray (large enough to fit gel) with transfer buffer.

4. Place sponges in transblot cell—2 sponges per gel.

5. Fill tank with transfer buffer to allow sponges to saturate with transfer buffer.

6. Soak pre-cut transfer membrane.

7. Assemble transfer cassette as follows.

1. Black side down.

2. Sponge soaked in transfer buffer.

3. Filter paper.

4. Gel.

5. Nitrocellulose membrane—once placed on gel do not move membrane.

6. Filter Paper.

7. Sponge.

8. Ensure all air bubbles have been removed between gel and membrane.

9. Place tray in transblot tank, black side (gel side) of tray to black tank side.

10. Transfer at 4° C. and following conditions (transfer can be done in an ice bath if needed).

1. 90 V for 50 minutes.

2. Membrane can be left in tank overnight at 4° C. after transfer.

G. Immunological Analysis for Western Blot Using scFv with S-Tag Linked to Alkaline Phosphatase or Alkaline Phosphatase Linked Anti S-Tag so as to Amplify Signal

1. Remove membrane from transfer.

2. Rinse membrane in 1% milk (enough to cover membrane) and block, 10 minutes, RT.

3. Prepare antibody solution (According to Titer instructions on Ab).

4. Remove blocking solution (save at 4° C.).

5. Place membrane into container with antibody solution.

6. Incubate at 4° C. overnight (usually 8-12 hours).

H. Development of Western Blot and Scanning

1. Remove 1° antibody.

2. Wash membrane 4 times.

1. Cover membrane with TBST.

2. Gently shake at RT for 5 minutes.

3. Cover membrane with Western Blue.

18

4. Allow to develop until reference spots reach maximum intensity.

5. Stop develop by rinsing with di-H₂O.

6. Dry membrane.

7. Scan membrane.

Solutions Used for First Dimension

I. Rehydration Buffer pH 7 (25 mL):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Urea	10.51 g	60.06	7M
Thiourea	3.81 g	76.12	2M
CHAPS	0.5 g	614.88	2%
asb-14	0.125 g	434.68	0.5%
40% Ampholytes	330 µL	N/A	0.5%
IPG Buffer	125 µL	N/A	0.5%
ddi-H ₂ O	To 25 mL	18.02	N/A
Bromophenol Blue	3 mg	669.96	0.012%

Dissolve Urea in minimal ddi-H₂O (do not heat over 30° C.)

Dissolve Thiourea in Urea/ddi-H₂O solution, and then add remaining chemicals.

Q.S. with ddi-H₂O to 25 mL and aliquot to 1 mL tubes and store at -80° C.

Add 1% DTT - 10 mg (0.01 g) before use.

Solutions Used for Second Dimension

Tris Buffer (1.5 M, pH 8.8) (1 L):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Trizma	181.65 g	121.14	1.5M
HCL	pH to 8.8	36.46	N/A
ddi-H ₂ O	To 1 L	18.02	N/A

Dissolve Trizma in 750 mL ddi-H₂O and adjust pH to 8.8 with HCL.

Q.S. to final volume of 1 L with ddi-H₂O and store at 4° C.

Equilibration Buffer (400 mL):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Tris-Buffer {1.5M, pH 8.8}	134 mL	N/A	0.5M
Urea	144.14 g	60.06	6M
Glycerol	120 mL (150 g)	92.09	30%
SDS	10 g	288.38	2.5%
ddi-H ₂ O	To 400 ml	18.02	N/A
Bromophenol Blue	Trace amount	669.96	N/A

Q.S. to final volume of 4 L with ddi-H₂O.

Add Bromophenol Blue (add with pipette tip to give trace of blue).

Aliquot to 15 mL tubes and store at -20° C.

Acrylamide Gel (20 cm×20 cm; 1-mm Thick)

Gel %	# gels	Tris Buffer 1.5M, pH 8.8 (mL)	30% Acrylamide (mL)	ddi-H ₂ O (mL)	10% APS (mL)	TEMED (mL)
10	6	100	133.33	166.67	4	0.4
	8	125	166.67	208.33	5	0.5
	10	150	200	250	6	0.6
	12	175	233.33	291.67	7	0.7

Formulations for Protean II gels.

19

10% Alkaline Phosphatase Substrate (APS)

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
APS	2 g	228.18	10%
ddi-H ₂ O	To 20 mL	18.02	N/A

Q.S. to final volume of 20 mL with ddi-H₂O.

Agarose Solution (1%):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Agarose	1.5 g	N/A	1%
SDS-Running Buffer	150 mL	N/A	N/A
Bromophenol Blue	Trace Amount	669.96	N/A

Combine agarose and SDS-Run Buffer.

Microwave to heat and dissolve.

Add Bromophenol Blue (add with pipette tip to give trace of blue).

10× SDS Running Buffer (4 L):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Trizma	121.2 g	121.14	0.25M
Glycine	576 g	75.07	1.92M
SDS	40 g	288.38	1%
ddi-H ₂ O	To 4 L	18.02	N/A

Q.S. to final volume of 4 L with ddi-H₂O.

Solutions for Western Blot

Western Transfer Buffer (4 L):

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Trizma	121.2 g	121.14	0.025M
Glycine	576 g	75.07	0.192M
Methanol	480 mL	32.04	0.12%
SDS	3 g	288.38	.075%
di-H ₂ O	To 4 L	18.02	N/A

Q.S. to final volume of 4 L with di-H₂O.

Blocking Buffer (5% BSA)

Chemical	Mass	Molar Mass (g/mol)	Final Value
BSA	5 g	66500	5%
N ₃ Na	0.2 g	65.01	0.2%
TBST	To 100 mL	18.02	N/A

Q.S. to final volume of 100 mL with TBST.

10× Tris Buffered Saline with Tween-20 (TBST) (4 L)

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
Trizma	48.4 g	121.14	100 mM
NaCl	350.6 g	58.44	1.5M
Tween 20	20.6 g	1227.54	0.5%

20

-continued

Chemical	Amount Added	Molar Mass (g/mol)	Final Value
HCl	pH to 7.5	36.46	N/A
ddi-H ₂ O	To 4 L	18.02	N/A

Add Trizma and NaCl to 3.5 L ddi-H₂O.

pH to 7.5 by HCl

Add Tween 20.

10 Q.S. to 4 L ddi-H₂O.

Method 3

2-D Gel Electrophoresis Western Blot Early
Detection Protocol

Serum was prepared from 5 ml of blood collected by venipuncture (with tourniquet) in standard B & D 13×100 (7 ml) vacutainer clot tubes (or equivalent) with or without hemoguard closure. After approximately 30 minutes at room temperature to allow for clotting, the clot was pelleted by centrifugation for 5 to 10 minutes at 2,500 to 3,000 rpm. Clot-free serum was decanted into a clean tube, labeled and analyzed fresh or stored frozen.

For western blot analysis, 30 μL of sera was added to 120 μL of Rehydration Buffer (7M urea, 2 M thiourea, 2% (w/v) CHAPS [(3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate), a nondenaturing zwitterionic detergent], 0.5% (w/v) ASB-14 (amidolulfobetaine-14, a zwitterionic detergent), 0.5% (v/v) ampholytes pH 3-10 (Bio-Rad), 0.5% (v/v) immobilized pH gradient (IPG) buffer pH 3-10 (Amersham-Pharmacia Biotech) and 65 mM dithiothreitol). The samples were quickly vortexed to mix sera with Rehydration Buffer. Four to six mg of protein were loaded for analysis. The samples were electrophoresed in the first dimension by using a commercial flatbed electrophoresis system (Ettan IPGphor 3, Amersham-Pharmacia Biotech) with IPG dry strips (Amersham). A linear pH range of 3 to 10 on 7 cm IPG strips was used. The IPG strips were rehydrated with the samples overnight at room temperature. The strips were then focused at 50 mA per strip and at increasing voltage of 250 V for 250 Vhrs, 500 V for 500 Vhrs, 1,000 V for 1,000 Vhrs and 4,000 V for 3 hours. The samples were then focused at a constant 4,000 V for 28,000 Volt-hours. After isoelectric focusing, the IPG strips were re-equilibrated for 20 minutes in 2.5% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, 100 mM Tris-HCl (pH 8.8). The strips were placed onto linear SDS-PAGE gels (10% (w/v) polyacrylamide) and electrophoresed at a constant 250 V for 75 minutes. The samples were then transferred to nitrocellulose membranes by electroblotting using the Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The membranes were blocked using milk protein (1% low fat dry milk) at room temperature for 10 minutes. Detection was with recombinant anti-ENOX2 single chain variable region of antibody (scFv) that was alkaline phosphatase-linked overnight at 4° C. After washing, detection was performed with Western Blue nitroretazolium (NBT) substrate (Promega, Madison, Wis.; Cat. No. 53841) at room temperature. Images were scanned and processed using Adobe Photoshop. Quantitation utilized an algorithm developed for this purpose. Reactive proteins appeared reddish blue. For interpretative purposes, the blots were divided into quadrants I-IV with unreactive serum albumin at the center (FIG. 7).

21

EXAMPLES

Example 1

Two-fold increase in spot size and density of different amounts of ENOX2 in the assay as determined by 2-D gel electrophoresis/western blot analysis comparing incubation in the presence of 15 μ M NADH in the assay compared to no NADH (FIG. 1).

Example 2

Analyses of sera from 1,626 cancer patients with confirmed diagnoses of cancer (Table 1, repeated from the Summary of the Invention) showing non-lapping patterns of number of transcript variants (protein 1 to protein 3), molecular weights and isoelectric points that distinguish 26 different cancers and 20 different tissues of origin.

TABLE 1

repeated from the Summary of the Invention: Ranges for Molecular Weight (MW) and Isoelectric Point (Pi) Determined for Sera of Patients Diagnosed with 24 Different Cancers Representing 20 Different Tissues of Origin							
RANGES							
		Protein 1		Protein 2		Protein 3	
Cancers	N	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)
Bladder	37	63-66	4.2-5.6 ¹	42-48	4.1-4.8 ¹		
*Blood	(117)	34-47	3.5-4.6				
Cell (Total)							
Breast	682	64-69	4.2-4.9				
Cervical	47	90-100	4.2-5.4				
Colorectal	125	80-96	4.4-5.4	50-65	4.2-5.3	33-46	3.8-5.2
Esophageal	26	42-47	4.6-5.2				
Gastric	26	120-188	4.7-5.5	50-62	4.5-5.6	45-53	2.4-3.6
Hepatocellular	31	58-70	4.5-5.0	34-40	4.1-5.2		
Leukemias	36	34-45	3.5-4.5				
*Lung, Non-Small Cell	55	53	4.7-5.3				
**Lung, Non-Small Cell	270	54-56	4.6-5.3				
Lung, Small Cell	27	52-53	4.1-4.6				
Lymphomas	56	43-45	3.5-4.5				
Melanoma	51	37-41	4.6-5.3				
Mesothelioma	27	60-68	3.8-4.1	38-44	3.8-4.6		
Myelomas	25	40-45	3.9-4.6				
Ovarian	115	72-90	3.7-5.0	37-47	3.7-5.0		
Pancreatic	75	48-51	3.9-5.4				
Prostate	361	71-88	5.1-6.5				
Renal Cell (Kidney)	31	69-73	4.7-5.4	54-61	4.1-5.2	38-43	3.7-4.3
Sarcoma	29	50-55	5.2-5.6	37-45	4.3-4.9		
Squamous Cell	51	57-68	5.0-5.4				
Testicular Germ Cell	25	61-62	5.0-5.4	40-45	4.4-4.7		
Thyroid Follicular	25	48-56	4.7-5.1	37-42	4.5-5.2		
Thyroid Papillary	27	56-67	4.5-5.0	37-44	3.2-3.6		
***Uterine (Endometrial)	26	63-66	4.2-4.9 ²	41-48	4.4-5.6 ²		

22

TABLE 1-continued

repeated from the Summary of the Invention: Ranges for Molecular Weight (MW) and Isoelectric Point (Pi) Determined for Sera of Patients Diagnosed with 24 Different Cancers Representing 20 Different Tissues of Origin

RANGES							
		Protein 1		Protein 2		Protein 3	
Cancers	N	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)	MW (kDa)	Pi (pH)
**Uterine (Endometrial)	57	67-71	4.2-5.1	41-48	3.7-5.4		
Total	2460						

*Blood cell cancers include lymphomas, leukemias and myelomas already represented in the totals.

**Non-Small Cell Lung cancers are in two subsets to avoid molecular weight overlap with small cell lung cancer.

***Uterine cancers are in two subsets based on molecular weight to avoid overlap with bladder cancer (see footnotes 1 and 2).

1. Isoelectric point pH of Protein 1 $\geq$ Protein 2.

2. Isoelectric point pH of Protein 1 < Protein 2.

Example 3

The ONCOblot test correctly identified cancers as to tissue of origin with both state 0 and Stage I cancers (Table 3). Often referred to as cancer in situ, with stage 0 and stage I cancers, the disease has not spread beyond the tissue of origin. Thus, the ONCOblot Cancer Test signals cancer earlier than Circulating Tumor Cell tests that detect only cancer cells in the blood.

TABLE 3

The tissue of origin stage 0 and stage I cancers analyzed			
Stage 0 Cancers	n	Stage I Cancers	n
Bladder	2	Bladder	1
Blood Cell	3	Blood Cell	1
Breast	6	Breast	16
Cervix	3	Colorectal	5
Hepatocellular (Ampullary)	1	Lung	1
Lung	1		
Melanoma	1		
Renal Cell	2		
Squamous Cell (Vulvar)	2		
Uterine	1		

Example 4

A 64 year old male was followed longitudinally for 16 years by using archived serum samples. The starting PSA was 0.8 ng/mL. No clinical symptoms were recorded until year 16 when prostate cancer was confirmed by biopsy. ENOX2 was first detected in year 8 with a PSA of 4 ng/ml 8 years in advance of clinical symptoms.

Example 5

Malignant mesothelioma is an aggressive, almost uniformly fatal cancer caused primarily by exposure to asbestos. Sequential serum samples collected from asbestos-exposed individuals prior to the development of frank mesothelioma were assayed for ENOX2 presence by 2-D gel-immunoblot analysis to determine how long in advance of clinical symptoms mesothelioma-specific transcript variants could be detected. Two mesothelioma-specific tran-

script variants were detected in the sera of asbestos-exposed individuals 4 to 10 years prior to clinical diagnosis of malignant mesothelioma (average 6.2 years). Either one or both ENOX2 protein transcript variants indicative of malignant mesothelioma were absent in 14 of 15 subjects diagnosed with benign pleural plaques either with or without accompanying asbestosis.

Example 6

Analysis of patient sera with cancer of unknown primary (CUP). The 2-D gel from a patient with cancer where the primary tumor was unknown revealed the presence of 80 and 42.5 kDa, both with transcript variants of isoelectric points of pH 4.2 and 4.1 to indicate that the primary cancer was ovarian cancer, which was confirmed later by biopsy.

Example 7

Successful validation and quantitation of ENOX2 presence in 1,587 ONCOblot analyses comparing 20 different tissues of origin (Table 1).

Example 8

Early detection of cancer in a healthy population of young adults in advance of clinical symptoms. Seta from 100 subjects (50 male and 50 female) between the ages of 20 and 39 years were analyzed for ENOX2 presence by the technology described herein. In the age group 20-29 years, none of the 25 females and only one (colorectal) of the 25 males exhibited ENOX2 proteins indicative of cancer presence. Similarly, in the age group 30-39 years, one (blood cell) of the 25 females and one (prostate) of the 25 males exhibited ENOX2 proteins. Therefore, the overall incidence of ENOX2 presence within all 100 serum samples was 3%. The overall incidence of newly diagnoses cancers within a population of men and women between 20 and 29 years old is approximately 2% (Howlander et al. SEER Cancer Statistics Review, 1975-2012, Nation Cancer Institute, Bethesda, Md.).

Method 4

Generation of Functionalized Recombinant Antibody with Protein Disulfide-Thiol Interchange Functional Motif Represented in Linker

To further enhance the affinity and specificity of the binding of the recombinant antibody to ENOX2 on western blots a linker was designed to specifically mimic the protein disulfide-thiol interchange functional motif of the ENOX2 family of proteins.

Monoclonal antibody generated against ENOX2 NADH oxidase tumor cell specific was produced in sp-2 myeloma cells; however, the monoclonal antibody slowed the growth of sp-2 myeloma cells that were used for fusion with spleen cells after 72 hours. This phenomenon made it difficult to produce antibody in quantity. To overcome this problem, the coding sequences of the antigen-binding variable region of the heavy chain and the light chain (Fv region) of the antibody cDNA were cloned and linked into one chimeric gene, upstream of the S-tag coding sequence. The Fv portion of an antibody, consisting of variable heavy (VH) and variable light (VL) domains, can maintain the binding specificity and affinity of the original antibody (Glockshuber et al. 1900. *Biochemistry* 29:1262-1367).

For a recombinant antibody, cDNAs encoding the variable regions of immunoglobulin heavy chain (VH) and light chain (VI), were cloned using degenerative primers. Mammalian immunoglobulins of light and heavy chain contain conserved regions adjacent to the hypervariable complementary defining regions (CDRs). Degenerate oligoprimers allow these regions to be amplified using PCR (Jones et al. 1991. *Bio/Technology* 9:88-89; Daugherty et al. 1991. *Nucleic Acids Research* 19:2471-2476). Recombinant DNA techniques have facilitated the stabilization of variable fragments by covalently linking the two fragments by a polypeptide linker (Huston et al. 1988. *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Either VL or VH can provide the NH2-terminal domain of the single chain variable fragment (scFv). The linker should be designed to resist proteolysis and to minimize protein aggregation. Linker length and sequences contribute and control flexibility and interaction with scFv and antigen. The preferred linker was uniquely comprised as a functional linker between heavy and light chain portions of the ScFv fusion protein comprised primarily of amino acids with small side chains (Gly/Ser) containing, in addition, two cysteine residues (C) spaced as either CXXXXC or CXXXXXC; that forms interchain disulfide bonds with the protein disulfide-thiol interchange functional motif of ENOX2.

Total RNA was isolated from the hybridoma cells producing ENOX2-specific monoclonal antibodies by the following procedure modified from Chomczynski et al. (1987) *Anal. Biochem.* 162:156-159 and Gough (1988) *Anal. Biochem.* 176:93-95. Cells were harvested from medium and pelleted by centrifugation at 450× g for 10 minutes. Pellets were gently resuspended with 10 volumes of ice cold PBS and centrifuged again. The supernatant was discarded and cells were resuspended with an equal volume of PBS. Denaturing solution (0.36 ml of 2-mercaptoethanol/50 ml of guanidinium stock solution-4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl) 10 ml per 1 g of cell pellet was added prior to use and mixed gently. Sodium acetate (pH 4.0, 1 ml of 2M), 10 ml of phenol saturated water and 2 ml of chloroform:isoamyl alcohol (24:1) mixtures were sequentially added after each addition. The solution was mixed thoroughly by inversion. The solution was vigorously shaken for 10 seconds, chilled on ice for 15 minutes and then centrifuged 12,000× g for 30 minutes. The supernatant was transferred and an equal volume of 2-propanol was added and placed at -20° C. overnight to precipitate the RNA. The RNA was pelleted for 15 minutes at 12,000× g, and the pellet was resuspended with 2-3 ml of denaturing solution and 2 volumes of ethanol. The solution was placed at -20° C. for 2 hours, and then centrifuged at 12,000× g for 15 minutes. The RNA pellet was washed with 70% ethanol and then 100% ethanol. The pellet was resuspended with RNase-free water (DEPC-treated water) after centrifugation at 12,000× g for 5 minutes. The amount of isolated RNA was measured spectrophotometrically and calculated from the absorbance at 280 nm and 260 nm.

A poly(A)mRNA isolation kit was purchased from Stratagene. Total RNA was applied to an oligo(dT) cellulose column after heating the total RNA at 65° C. for 5 minutes. Before applying, the RNA samples were mixed with 500 µL of 10× sample buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 M NaCl). The RNA samples were pushed through the column at a rate of 1 drop every 2 seconds. The eluates were pooled and reapplied to the column and purified again. Preheated elution buffer (65° C.) was applied, and mRNA was eluted and collected in 1.5 ml of centrifuge tubes on ice.

The amount of mRNA was determined at OD₂₆₀ (1 OD unit=40 µg of RNA). The amounts of total RNA and mRNA obtained from 4×10⁸ cells were 1328 µg and 28 µg, respectively.

Next, mRNA (1-2 µg) dissolved in DEPC-treated water was used for cDNA synthesis. mRNA isolated on three different dates was pooled for first-strand cDNA synthesis. The cDNA synthesis kit was purchased from Pharmacia Biotech. The mRNA (1.5 µg/5 µL of DEPC-treated water) was heated at 65° C. for 10 minutes and cooled immediately on ice. The primed first strand mix containing Murine Leukemia Virus (MuLV) reverse transcriptase (11 µL) and appropriate buffers for the reaction were mixed with the mRNA sample. DTT solution (1 µL of 0.1 M) and RNase-free water (16 µL) also were added to the solution. The mixture was incubated for 1 hour at 37° C.

Degenerate primers for light chain and heavy chain (Novagen, Madison, Wis.) were used for PCR. PCR synthesis was carried out in 100 µL reaction volumes in 0.5 ml microcentrifuge tubes by using Robocycler (Stratagene, La Jolla, Calif.). All PCR syntheses included 2 µL of sense and anti-sense primers (20 picomoles/µL), 1 µL of first-strand cDNA as a template, 2 µL of 10 mM of dNTPs, 1 µL of Vent polymerase (2 units/µL), 10 µL of 10× PCR buffer (100 mM Tris-HCl, pH 8.8 at 25° C., 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 82 µL of H₂O. Triton X-100 is t-octylphenoxy polyethoxyethanol. All PCR profiles consisted of 1 min of denaturation at 94° C., 1 minute of annealing at 55° C., and 1 minute of extension at 72° C. This sequence was repeated 30 times with a 6-minute extension at 72° C. in the final cycle. PCR products were purified with QIAEX II gel extraction kit from Qiagen, Valencia, Calif. PCR amplification products for heavy and light chain coding sequences were analyzed by agarose gel electrophoresis and were about 340 base pair (bp) long and 325 bp long, respectively.

Total RNA or DNA was analyzed by agarose gel electrophoresis (1% agarose gels). Agarose (0.5 g in 50 ml of Tris-acetate-EDTA (TAE) buffer, 40 mM Tris-acetate, 1 mM EDTA) was heated for 2 minutes in a microwave to melt and evenly disperse the agarose. The solution was cooled at room temperature, and ethidium bromide (0.5 µg/ml) was added and poured into the apparatus. Each sample was mixed with 6× gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water). TAE buffer was used as the running buffer. Voltage (10 v/cm) was applied for 60-90 minutes.

According to the proper size for heavy and light chain cDNAs, the bands were excised from the gels under UV illumination, and excised gels were placed in 1 ml syringes fitted with 18-gauge needles. Gels were crushed to a 1.5 ml Eppendorf tube. The barrel of each syringe was washed with 200 µL buffer-saturated phenol (pH 7.9±0.2). The mixture was thoroughly centrifuged and frozen at -70° C. for 10 minutes. The mixture was centrifuged for 5 minutes, and the top aqueous phase was transferred to a new tube. The aqueous phase was extracted again with phenol/chloroform (1:1). After centrifuging for 5 minutes, the top aqueous phase was transferred to a clean tube, and chloroform extraction was performed. Sodium acetate (10 volumes of 3 M) and 2.5 volumes of ice-cold ethanol were added to the top aqueous phase to precipitate DNA at -20° C. overnight.

Purified heavy and light chain cDNAs were ligated into plasmid pSTBlue-1 vector and transfected into NovaBlue competent cells (Stratagene). Colonies containing heavy and light chain DNAs were screened by blue and white colony selection and confirmed by PCR analysis. Heavy and light chain DNAs were isolated and sequenced using standard techniques.

PCR amplification and the assembly of single scFv gene was according to Davis et al. (1991) *Bio/Technology* 9:165-169. Plasmid pSTBlue-1 carrying VH and VL genes were combined with all four oligonucleotide primers in a single PCR synthesis. Following first PCR synthesis, one tenth of the first PCR product was removed and added to a second PCR reaction mixture containing only the primer a (VH sense primer) and primer d (VL Antisense primer). The product of the second PCR synthesis yielded single scFv gene. The single scFv gene was ligated to plasmid pT-Adv (Clontech, Palo Alto, Calif.). pT-Adv carrying scFv gene was used for DNA sequencing.

The complete functionalized scFv gene was assembled from the VH, VL and linker sequences to yield an scFv cDNA with a high degree of efficacy and sensitivity.

Total RNA or DNA was analyzed by agarose gel electrophoresis (1% agarose gels). Agarose (0.5 g in 50 ml of TAE buffer, 40 mM Tris-acetate, 1 mM EDTA) was heated for 2 minutes in a microwave to melt and evenly disperse the agarose. The solution was cooled at room temperature, and ethidium bromide (0.5 µg/ml) was added and poured into the apparatus. Each sample was mixed with 6× gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water). TAE buffer was used as the running buffer. Voltage (10 v/cm) was applied for 60-90 minutes.

According to the proper size for heavy and light chain cDNAs, the bands were excised from the gels under UV illumination, and excised gels were placed in 1 ml syringes fitted with 18-gauge needles. Gels were crushed to a 1.5 ml Eppendorf tube. The barrel of each syringe was washed with 200 µL of buffer-saturated phenol (pH 7.9±0.2). The mixture was thoroughly centrifuged and frozen at -70° C. for 10 minutes. The mixture was centrifuged for 5 minutes, and the top aqueous phase was transferred to a new tube. The aqueous phase was extracted again with phenol/chloroform (1:1). After centrifuging for 5 minutes, the top aqueous phase was transferred to a clean tube, and chloroform extraction was performed. Sodium acetate (10 volumes of 3 M) and 2.5 volumes of ice-cold ethanol were added to the top aqueous phase to precipitate DNA at -20° C. overnight.

Purified heavy and light chain cDNAs were ligated into plasmid pSTBlue-1 vector and transfected into NovaBlue competent cells (Stratagene). Colonies containing heavy and light chain DNAs were screened by blue and white colony selection and confirmed by PCR analysis. Heavy and light chain DNAs were isolated and sequenced using standard techniques.

PCR amplification and the assembly of single scFv gene was according to Davis et al. (1991) *Bio/Technology* 9:165-169. Plasmid pSTBlue-1 carrying VH and VL genes were combined with all four oligonucleotide primers in a single PCR synthesis. Following first PCR synthesis, one tenth of the first PCR product was removed and added to a second PCR reaction mixture containing only the primer a (VH sense primer) and primer d (VL Antisense primer). The

product of the second PCR synthesis yielded single scFv gene. The single scFv gene was ligated to plasmid pT-Adv (Clontech, Palo Alto, Calif.). pT-Adv carrying scFv gene was used for DNA sequencing.

The complete functionalized scFv gene was assembled from the VH, VL and linker sequences to yield a scFv cDNA by PCR as follows:

DNA and Amino Acid Sequence of scFv(S)(GST) with Gly.Ser.Cys Linker [scFv(SC)]

Linker Sequence

DNA:

5' GGCGGGGGTG GTAGCTGCGG CGGTGGATCG TGTGGCGGTG SEQ ID NO: 4

GCAGT 3'.

Amino acid:

GlyGlyGlyGlySerCytGlyGlyGlySerCytGlyGlyGlySer. SEQ ID NO: 5

DNA and Amino Acid Sequence of scFv(GST)

GST-tag

SEQ ID NO: 6

1 ATGTCCCCTA TACTAGGTTA TTGGAATAATT AAGGGCCTTG TGCAACCCAC

51 TCGACTTCTT TTGGAATATC TTGAAGAAAA ATATGAAGAG CATTGTGTATG

101 AGCGCGATGA AGGTGATAAA TGGCGAAACA AAAAGTTTGA ATTGGGTTTG

151 GAGTTTCCCA ATCTTCCTTA TTATATTGAT GGTGATGTTA AATTAACACA

201 GTCTATGGCC ATCATACTGT ATATAGCTGA CAAGCACAAAC ATGTTGGGTG

251 GTTGTCCAAA AGAGCGTGCA GAGATTTCAA TGCTTGAAGG AGCGGTTTTG

301 GATATTAGAT ACGGTGTTTC GAGAATTGCA TATAGTAAAG ACTTTGAAAC

351 TCTCAAAGTT GATTTTCTTA GCAAGCTACC TGAAATGCTG AAAATGTTCTG

401 AAGATCGTTT ATGTCATAAA ACATATTTAA ATGGTGATCA TGTAACCCAT

451 CCTGACTTCA TGTTGTATGA CGCTCTTGAT GTTGTTTTAT ACATGGACCC

501 AATGTGCCTG GATGCGTTCC CAAAATTAGT TTGTTTTTAA AACGTATTG

551 AAGCTATCCC ACAAATTGAT AAGTACTTGA AATCCAGCAA GTATATAGCA

601 TGGCCTTTGC AGGGCTGGCA AGCCACGTTT GGTGGTGGCG ACCATCCTCC

651 AAAATCGGAT CTGGTTCCGC GTGGATCCCC AGGAATTCCC

scFv heavy chain (VH)

SEQ ID NO: 7

691 GAGGTCAAGC TGCAGGAGTC AGGAACGTAA GTGGTAAAGC CTGGGGCTTC

741 AGTGAAGTTG TCCTGCAAGG CTTCTGGCTA CATCTTCACA AGTTATGATA

791 TAGACTGGGT GAGGCAGACG CCTGAACAGG GACTTGAGTG GATTGGATGG

841 ATTTTTCCTG GAGAGGGGAG TACTGAATAC AATGAGAAGT TCAAGGGCAG

891 GGCCACACTG AGTGTAGACA AGTCCTCCAG CACAGCCTAT ATGAGACTCA

941 CTAGGCTGAC ATCTGAGGAC TCTGCTGTCT ATTTCTGTGC TAGAGGGGAC

991 TACTATAGGC GCTACTTTGA CTTGTGGGGC CAAGGGACCA CGGTACCCGT

1041 CTCCTCA

-continued

Linker Sequence
DNA:

GCAGT 3'.

5' GGCGGGGGTG GTAGCTGCGG CGGTGGATCG TGTGGCGGTG

SEQ ID NO: 4

Amino acid:

SEQ ID NO: 5

5 GlyGlyGlyGlySerCytGlyGlyGlySerCytGlyGlyGlySer.

scFv light chain (VL)

SEQ ID NO: 8

1093 GAAAAATGTGC TCACCCAGTC TCCAGCAATC ATGTCTGCAT CTCACGGGGA
1143 GAGGGTCACC ATGACCTGCA GTGCCAGCTC AAGTATACGT TACATATATT
1193 GGTACCAACA GAAGCCTGGA TCCTCCCCCA GACTCCTGAT TTATGACACA
1243 TCCAACGTGG CTCCTGGAGT CCCTTTTCGC TTCAGTGCCA GTGGGTCTGG
1293 GACCTCTTAT TCTCTCACA TCAACCGAAT GGAGGCTGAG GATGCTGCCA
1343 CTTATTACTG CCAGGAGTGG AGTGGTTATC CGTACACGTT CGGAGGGGGG
1393 ACCAAGCTGG AGCTGAAAGC G

DNA sequence of scFv(SC) with GST tag

SEQ ID NO: 9

1 ATGTCCCCTA TACTAGGTTA TTGGAAAATT AAGGGCCTTG TGCAACCCAC
51 TCGACTTCTT TTGAATATC TTGAAGAAAA ATATGAAGAG CATTGTATG
101 AGCGCGATGA AGGTGATAAA TGGCGAAACA AAAAGTTTGA ATTGGGTTTG
151 GAGTTTCCCA ATCTTCCTTA TTATATTGAT GGTGATGTTA AATTAACACA
201 GTCTATGGCC ATCATACGTT ATATAGCTGA CAAGCACAAAC ATGTTGGGTG
251 GTTGTCCTAA AGAGCGTGCA GAGATTTCAG TGCTTGAAGG AGCGGTTTGG
301 GATATTAGAT ACGGTGTTTC GAGAATTGCA TATAGTAAAG ACTTTGAAAC
351 TCTCAAAGTT GATTTTCTTA GCAAGCTACC TGAAATGCTG AAAATGTTCTG
401 AAGATCGTTT ATGTCATAAA ACATATTTAA ATGGTGATCA TGTAACCCAT
451 CCTGACTTCA TGTGTATGA CGCTCTTGAT GTTGTTTTAT ACATGGACCC
501 AATGTGCCTG GATGCGTTC CAAAATTAGT TTGTTTTTAA AACGTATTG
551 AAGCTATCCC ACAAATTGAT AAGTACTTGA AATCCAGCAA GTATATAGCA
601 TGGCCTTTGC AGGGCTGGCA AGCCACGTTT GGTGGTGGCG ACCATCCTCC
651 AAAATCGGAT CTGGTTCCGC GTGGATCCCC AGGAATTCCC GAGGTCAAGC
701 TGCAGGAGTC AGGAACTGAA GTGGTAAAGC CTGGGGCTTC AGTGAAGTTG
751 TCCTGCAAGG CTTCTGGCTA CATCTTCACA AGTTATGATA TAGACTGGGT
801 GAGGCAGACG CCTGAACAGG GACTTGAGTG GATTGGATGG ATTTTTCCTG
851 GAGAGGGGAG TACTGAATAC AATGAGAAGT TCAAGGGCAG GGCCACACTG
901 AGTGTAAGCA AGTCCTCCAG CACAGCCTAT ATGGAGCTCA CTAGGCTGAC
951 ATCTGAGGAC TCTGCTGTCT ATTTCTGTGC TAGAGGGGAC TACTATAGGC
1001 GCTACTTTGA CTTGTGGGGC CAAGGGACCA CGGTACCGT CTCCTCAGGC
1051 GGGGGTGGTA GCTGCGGCGG TGATCGTGT GCGGTGGCA GTGAAAATGT
1101 GCTCACCAG TCTCCAGCAA TCATGTCTGC ATCTCCAGGG GAGAGGGTCA
1151 CCATGACCTG CAGTGCCAGC TCAAGTATAC GTTACATATA TTGGTACCAA
1201 CAGAAGCCTG GATCCTCCCC CAGACTCCTG ATTTATGACA CATCCAACGT
1251 GGCTCCTGGA GTCCTTTTTC GCTTCAGTGG CAGTGGGTCT GGACCTCTT

-continued

1301 ATTCTCTCAC AATCAACCGA ATGGAGGCTG AGGATGCTGC CACTTATTAC
1351 TGCCAGGAGT GGAGTGGTTA TCCGTACACG TTCGGAGGGG GGACCAAGCT
1401 GGAGCTGAAA GCG

Translation of scFv(S)(GST) with Gly.Ser.Cys Linker
DNA Sequence SEQ ID NO:10 and Amino Acid Sequence
SEQ ID NO:11:

1 atgtccctatactaggttattggaaaattaagggccttgtgcaaccactcgacttctt
1 M S P I L G Y W K I K G L V Q P T R L L
61 ttggaatatcttgaagaaaaatatgaagagcatttgtatgagcgcgatgaagtgataaa
21 L E Y L E E K Y E E H L Y E R D E G D K
121 tggcgaacaaaaagtttgaattgggtttggagtttcccaatcttccttattatattgat
41 W R N K K F E L G L E F P N L P Y Y I D
181 ggtgatgttaaattaacacagtcctatggccatcatagcttatatagctgacaagcacaac
61 G D V K L T Q S M A I I R Y I A D K H N
241 atgttgggtggttgcacaaagagcgtgcagagatttcaatgcttgaaggagcggttttg
81 M L G G C P K E R A E I S M L E G A V L
301 gatattagatacgggttttcgagaattgcatatagtaaagactttgaaactctcaaagtt
101 D I R Y G V S R I A Y S K D F E T L K V
361 gattttcttagcaagctacctgaaatgctgaaaatgttcgaagatcgtttatgtcataaa
121 D F L S K L P E M L K M F E D R L C H K
421 acatattttaaattggtgatcatgtaaccatcctgacttcatgttgatgacgctcttgat
141 T Y L N G D H V T H P D F M L Y D A L D
481 gttgttttatacatggacccaatgtgcctggatgcgttcccaaaattagtttgttttaa
161 V V L Y M D P M C L D A F P K L V C F K
541 aaacgtattgaagctatcccacaaattgataagtaacttgaatccagcaagtataragca
181 K R I E A I P Q I D K Y L K S S K Y I A
601 tggccttttcaggggtgcaagccacgttttggtgggtggcgaccatcctccaaaatcgat
201 W P L Q G W Q A T F G G G D H P P K S D
661 ctggttccgcgtggatccccaggaattcccaggtcaagctgcaggagtcaggaactgaa
221 L V P R G S P G I P E V K L Q E S G T E
721 gtggtaaaacctgggggttcagtgaagttgtcctgcaaggcttctggtacatcttcaca
241 V V K P G A S V K L S C K A S G Y I F T
781 agttatgatatagactgggtgaggcagacgcctgaacagggaacttgagtgattggatgg
261 S Y D I D W V R Q T P E Q G L E W I G W
841 attttctcggagaggggagtactgaatacaatgagaagttcaagggcaggccacactg
281 I F P G E G S T E Y N E K F K G R A T L
901 agttagacaagtcctccagcacagcctatatggagctcactaggtgacatctgaggac
301 S V D K S S S T A Y M E L T R L T S E D
961 tctgctgtctatttctgtgctagaggggactactataggcgtactttgacttggtgggc
321 S A V Y F C A R G D Y Y R R Y F D L W G
1021 caagggaccacggtcacctctcctcaggcgggggtggtagctgcggcggtggatcgtgt
341 Q G T T V T V S S G G G G S C G G G S C
1081 ggcggtggcagtgaaaatgtgctcaccagtcctccagcaatcatgtctgcatctccagg
361 G G G S E N V L T Q S P A I M S A S P G
1141 gagagggtccatcatgactgcagtgccagctcaagtatacgttacatatattggtaccaa
381 E R V T M T C S A S S S I R Y I Y W Y Q
1201 cagaagcctggatcctccccagactcctgatttatgacacatccaacgtggctcctgga
401 Q K P G S S P R L L I Y D T S N V A P G
1261 gtcccttttcgcttcagtggtggcagtggtctgggaaccttattctctcacaatcaaccga
421 V P F R F S G S G S G T S Y S L T I N R

- continued

1321 atggaggctgaggatgctgccacttattactgccaggagtggagtgggtatccgtacacg
 441 M E A E D A A T Y Y C Q E W S G Y P Y T

1381 ttccggagggggaccaagctggagctgaaagcg
 461 F G G G T K L E L K A

Translated Protein Sequence:

SEQ ID NO: 11
 1 MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL EFPNLPYYID
 61 GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL DIRYGVSRIA YSKDFETLKV
 121 DFLSKLPEML KMFEDRLCHK TYLNGDHVTH PDFMLYDALD VVLYMDPMCL DAFPKLVCFK
 181 KRIEAIPQID KYLKSSKYIA WPLQGWQATF GGGDHPKSD LVPRGSPGIP EVKLQESGTE
 241 VVKPGASVKL SCKASGYFT SYDIDWVRQT PEQGLEWIGW IFPGEGSTEY NEKFKGRATL
 301 SVDKSSSTAY MELTRLTSED SAVYFCARGD YYRRYFDLWG QGTTTVVSSG GGGSCGGGSC
 linker
 361 GGGSENVLQ SPAIMSASPG ERVTMTCSAS SSIRYIYWYQ QKPGSSPRLL IYDTSNVAPG
 421 VPFRRSGSGS GTSYSLTINR MEAEDAATYY CQEWSGYPYT FGGGKLELK A

25

Analysis of Protein Sequence:

Length in amino acids: 477
 Molecular weight in kD: 53.52
 Iso-electric point (IP): 5.87
 Molar absorption coefficient (in L/mol/cm); all cysteine residues as disulfide bonds: 1.02E+05
 Molar absorption coefficient (in L/mol/cm); 50 percent of cysteine residues as disulfide bonds: 1.02E+05
 Molar absorption coefficient (in L/mol/cm); no disulfide bonds: 1.01E+05

1. Primer design
 Linker with Gly.Ser.Cys
 Amino acid sequence: SEQ ID NO: 5
 GlyGlyGlyGlySerCytGlyGlyGlySerCytGlyGlyGlySer
 DNA sequence: SEQ ID NO: 4
 5'-GGCGGGGGTG GTAGCTGCGG CGGTGGATCG TGTGGCGGTG
 GCAGT-3'
 Design for Primers

1. Forward primer for scFv(S) heavy chain for PCR amplification
 scFv(S) heavy chain N-terminus (primer 15)

SEQ ID NO: 12

5' GGA TCC CCA GGA ATT CCC GAG GTC AAG CTG CAG GAG TCA GGA 3'
 EcoRI

2. Reverse primer for scFv(S) heavy chain for PCR amplification
 scFv(S) heavy chain C-terminus plus linker 1

Amino acid seq: GGTTTVSS SEQ ID NO: 13
 heavy chain C-terminus

GGGSCGGGSCGGGS SEQ ID NO: 14
 linker

1 GGGACCACGG TCACCGTCTC CTCAGGCGGG GGTGGTAGCT GCGGCGGTGG
 51 ATCGTGTGGC GGTGGCAGT SEQ ID NO: 15

1 GGGACCACGGTCACCGTCTCCTCAGGCGGGGGTGGTAGCTGCGGCGGTGGATCGTGTGGC
 1 G T T V T V S S G G G G S C G G G S C G

61 GGTGGCAGT
 21 G G S SEQ ID NO: 16

35

Reverse primer for scFv(S) heavy chain for PCR amplification (primer 18):

SEQ ID NO: 17
5' ACC GCC ACA CGA TCC ACC GCC GCA GCT ACC ACC CCC
GCC TGA GGA GAC GGT GAC CGT GGT CCC 3'

3. Forward primer for scFv(S) light chain PCR amplification
Linker plus scFv(S) light N-terminus SEQ ID NO: 18
Amino acid seq: G GSC GGG SCG GGS ENV LTQ SP
Linker light chain N-terminus

1
GGTGGTAGCTGCGGCGGTGGATCGTGTGGCGGTGGCAGTGAAAAATGTGCTCACCCAGTCT
1 G G S C G G G S C G G G S E N V L T Q S
61 CCA SEQ ID NO: 19
21 P SEQ ID NO: 20

36

-continued

1) PCR of heavy chain	2) PCR of light chain
5 pGEX4T-scFv(SE)	0.2 µl pGEX4T-scFv(SE)
pfu DNA polymerase	0.25 µl pfu DNA polymerase
	0.25 µl

Forward primer for scFv(S) light chain for PCR amplification (primer 19):
SEQ ID NO: 21
5' GGT GGT AGC TGC GGC GGT GGA TCG TGT GGC GGT GGC
AGT GAA AAT GTG CTC ACC CAG TCT CCA 3'

4. Reverse primer for scFv(S) light chain for PCR amplification (primer 17)
Sense DNA seq:
SEQ ID NO: 22
5' GAACGCCAGCACATGGACAGCTGACTCGAGCGGCCGGTG 3'
Antisense DNA seq:
SEQ ID NO: 23
5' CACCGGCCGCTCGAGTCAGCTGTCCATGTGCTGGCGTTC 3'
XhoI

Primers were ordered from Integrated DNA Technologies (Coralville, Iowa). PCR amplification of heavy chain (V_H) and light chain (V_L) of scFv(SC). Heavy chain and light chain of scFv(SC) were amplified by polymerase chain reaction (PCR).

Conditions for PCR were:

1) PCR of heavy chain	2) PCR of light chain
10 x pfu DNA polymerase	5 µl
buffer	5 µl
dNTP mix	1 µl
Forward primer	1 µl
(Primer 15)	(Primer 19)
Reverse primer	1 µl
(Primer 18)	(Primer 17)

-continued

1) PCR of heavy chain	2) PCR of light chain
Taq DNA polymerase (10x diluted)	0.2 µl Taq DNA polymerase (10x diluted)
Water	41.35 µl Total
Total	50 µl

PCR cycle		
94° C.	3 minutes	1 cycle
94° C.	30 seconds	30 cycles
64° C.	30 seconds	
72° C.	2 minutes	
72° C.	10 minutes	1 cycle

Heavy and light chain DNAs of scFv(SC) was analyzed by agarose gel electrophoresis (0.9%). DNA bands were excised, frozen, thawed and centrifuged. scFv(SC) DNA was amplified by extending heavy chain and light chain DNAs by PCR.

Conditions for PCR were:

10 x pfu DNA polymerase	10 µl		
buffer			
dNTP mix	2 µl		
Forward primer (Primer 15)	2 µl	94° C.	3 minutes
Reverse primer (Primer 17)	2 µl		
Heavy chain DNA	5 µl	94° C.	30 seconds
Light chain DNA	5 µl	64° C.	30 seconds
pfu DNA polymerase	0.5 µl	72° C.	2 minutes
Taq DNA polymerase (10x diluted)	0.1 µl	72° C.	10 minutes
Water	74.4 µl		
Total	100 µl		

The amplified scFv(SC) DNA was analyzed by agarose gel electrophoresis (0.9%). DNA bands were excised, purified by QIAquick Gel Extraction Kit (QIAGEN) and solubilized in 30 µl of TE buffer.

1) Digestion with EcoRI	Digestion was 2 hr at 37° C.
10 x NE4 buffer	5 µl Enzyme was heat inactivated at 65° C.
scFv(SC)	25 µl 15 minutes.
EcoRI HF (20 Units)	1 µl DBNA was precipitated by ethanol
Water	19 µl precipitation
	50 µl
2) Digestion with XhoI	Digestion was 1 hr 24 min at 37° C.
10 x React 2 buffer	5 µl Solution was heated at 65° C. 20 min.
scFv(SC)	44 µl DBNA was precipitated by ethanol
XhoI (10 Units)	1 µl precipitation.
	50 µl DNA was purified by agarose gel
	electrophoresis (0.9%) and purified.
	Concentration was 26.4 ng/µl in 30 µl
	of TE buffer.

Ligation of scFv(SC) to pGEX-4T2 [pGEX4T-scFv(SC)]. scFv(SC) was ligated to pGEX-4T2. The ligation reaction was as follows:

Digestion with EcoRI	2 µl
10 x T4 DNA ligase buffer	2 µl
pGEX-4T2 digested with EcoRI and XhoI (200 ng)	1.13 µl
scFv(SC) digested with EcoRI and XhoI (100 ng)	25 µl
T4 DNA ligase	1.5 µl
Water	11.5 µl
	20 µl

Ligation was 16 hours 50 minutes at 15° C. After ligation, *E. coli* BL21 (DE3) cells were transformed with pGEX4T-scFv(SC) and plated on LB/ampicillin plate. Cells were grown 37° C. for 20 hours. Screening of colonies for carrying pGEX4T-scFv(SC). Seven colonies were screened for carrying plasmid pGEX4T-scFv(SC) by PCR.

5 x Green GoTaq DNA	30 µl
polymerase buffer	
25 mM MgCl ₂	15 µl
dNTP mix	3 µl 94° C. 2 min. 1 cycle
Forward primer (Primer 15)	3 µl
Reverse primer (Primer 18)	3 µl 94° C. 30 sec
Heavy chain DNA	5 µl 64° C. 30 sec
Colony DNA	35 µl 72° C. 45 sec
Taq DNA polymerase	1.5 µl 72° C. 10 min 1 cycle
Water	59.5 µl
Total	150 µl

Reaction volume was 20 µl per colony.

Cells were picked from colony 1, grown in 100 ml of LB/ampicillin media and plasmid was purified using plasmid purification kit (Plasmid Midi Kit, Qiagen). scFv(SC) DNA sequence was performed by Purdue University. The sequenced DNA was analyzed by Alignment algorithm (NCBI) and showed no mutation.

Sequenced scFv(SC) DNA with primers 15 and 17
 >L_132420_Primer15_025.ab1 1464 21 860 ABI
 SEQ ID NO: 24
 AAAAAACAATGCAAAGTGGTAAAGCCTGGGGCTTCAGTGAAGTTGTCTT
 GCAAGGCTTCTGGCTACATCTTCACAAGTTATGATATAGACTGGGTGAGG
 CAGACGCCTGAACAGGACTTGAGTGGATTGGATGGATTTTCCTGGAGA
 GGGGAGTACTGAATACAATGAGAAGTTCAAGGGCAGGGCCACTGAGTG

-continued

TAGACAAGTCCCTCCAGCACAGCCTATATGGAGCTCACTAGGCTGACATCT
 GAGGACTCTGCTGTCTATTTCTGTCTAGAGGGGACTACTACAGGCGCTA
 5 CTTTGACTTGTGGGGCCAAGGACCACGGTCACCGTCTCCTCAGGCGGGG
 GTGGTAGCTGCGGCGGTGGATCGTGTGGCGGTGGCAGTGAAAATGTGCTC
 ACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAGGGTCACCAT
 10 GACCTGCAGTGCCAGCTCAAGTATACGTTACATATATTGGTACCAACAGA
 AGCCTGGATCCTCCCCAGACTCCTGATTTATGACACATCCAACGTGGCT
 CCTGGAGTCCCTTTTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTATTC
 15 TCTCACAATCAACCGAATGGAGGCTGAGGATGCTGCCACTTATTACTGCC
 AGGAGTGGAGTGGTTATCCGTACACGTTTCGGAGGGGGGACCAAGCTGGAG
 CTGAAAGCGAAAGAACTGCTGCTGCTAAATTGAAACGCCAGCACATGGA
 20 CAGCTGACTCGAGCGGCCGCATCGTGACTGACTGACGATCTGCCTCGCGC
 GTTTCGGTGATGACGGTGAAACCTCTGACACATGCAGCTCCCGGAGACG
 GTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGG
 25 CGCGTCAGCGGGTGTGGCGGGGTCTCGGGCCCCGCCATGACCCAGTCAC
 GTAGCGATAGCGGAGTGATAATTCTTGAAGACGAAAGGGCCTCGTGAAT
 CGCCTATTTTATAGGGTAATGTCATGATAATAATGGTTTCTTAAACGTC
 30 AAGGGGCACTTTTTCGGGAAATGTGCGCCGAAACCCCTAATTTGTTTTA
 TTTTCAAAATCCATTTCAATTAAGTTTCCCCCTCATGGAACAATAACC
 CCTGGAAAAAAGGCTTTTATTAAATATTGAAAAAAGGAAAAAAGTTAAA
 35 AATTATTTCAAAATTTTCCCTGTGTTCCCCCTTTATTTTCCCTTTTTTT
 TTGCGGGCCATTTTTCGCCCTTTTCCCTGGTTTTTTTTTGGCCCCCCCCC
 CAAAAAACCCCTGGGGTGAAAAAATAAAAAAATATGCTTTT
 40 AAAAAAATTTTCCAATTTTGTGGGGGGGGGCCCCCAAGAGTTG
 GGGGGGTTTTTAAACCACTTCTCCCAAAACAACATTGGGTGGAGATATT
 TCTTCTTCAAAAA
 45 >L_132420_Primer17_026.ab1 1492 22 854 ABI
 SEQ ID NO: 25
 GGTGGGTAAATTTAGCAGCAGCAGTTTCTTTTCGCTTTCAGCTCCAGCTTG
 50 GTCCCCCTCCGAACGTGTCCGGATAACCACTCCACTCTGGCAGTAATA
 AGTGGCAGCATCCTCAGCCTCCATTCGGTTGATTGTGAGAGAATAAGAGG
 TCCCAGACCACTGCCACTGAAGCGAAAAGGGACTCCAGGAGCCACGTTG
 55 GATGTGTCATAAATCAGGAGTCTGGGGGAGGATCCAGGCTTCTGTGTGTA
 CCAATATATGTAACGTATACTTGAGCTGGCACTGCAGGTGATGGTGAACC
 TCTCCCTGGAGATGCAGACATGATTGCTGGAGACTGGGTGAGCACATTT
 TCACTGCCACCGCCACACGATCCACCGCCGAGCTACCACCCCGCCTGA
 60 GGAGACGGTGACCGTGGTCCCTTGGCCCCACAAGTCAAAGTAGCGCCTGT
 AGTAGTCCCTCTAGCACAGAAATAGACAGCAGAGTCTCAGATCTCAGC
 CTAGTGAGCTCCATATAGGCTGTGCTGGAGGACTTGCTACACTCAGTGT
 65 GGCCCTGCCCTTGAACCTCTCATTGTATTTCAGTACTCCCTCTCCAGGAA

-continued

AAATCCATCCAATCCACTCAAGTCCCTGTTAGGCGTCTGCCTCAGCCAG
TCTATATCATAACTTGTGAAGATGTAGCCAGAAGCCTTGACAGACAATT
CACTGAAGCCCCAGGCTTTACCACTTCAGTTCTGACTCCTGCAGCTTGA
CCTCGGAATTCCAGAAAGGCGCTGCTTGAACCTTCCTTTGCTTCTTCC
ATATCTTTCTCATGGGCGGATCCACGCGGAACAGATCCGATTTTGGAGG
ATGGTCGCCACCACCAACGTGGCTTGCCAGCCCTGGCAAGGCCATGCTA
TATACTTGCTGGATTCAAGTACTTATCAATTTGTGGGAATAGCTTCTTT
ACGTTTTTTTTAAACAACTAATTTTGGGAACGATCCAGGCCATTGGG
TCCATGTATTAACACCATCAAAACCTCTACAACATGAAATTCGGGA
AGGGGTTTACATAATCCCCATTTTAAATATTGTTTTATGACATAAAAC
CAATCTTTCCAACTTTTTTTCACATTTTCCAGGTAACCTTGGTAAAAA
AAAACCACTTTTGAAGTTTTCAAAATCTTTTATTAAATGCAAAT
TTTCCGAAAAAACCCCTTATTCTAAAAATCCCAAAACCCGCTCC
CTTTCCAGCCCTTTGAAAAATTTCTTGCCCCCCCCCTCTTTTGGGG
AACAAAACCCCCCCCCACAACTTGGGTTGGGGTGGTTTTTGTGTCCAC
GCCCAATATAAAAAACAGTATAAATTAAGATGGGGCCCCACATATAA
AAAACGGGGGGGTTTTTTTATATAATTTTTTAAACACAACATCCCC
CCCCCCCCCATCCACACAAATAAATATATAATAAA

Alignment of scFv(SC) with Sequenced DNA Expression of scFv(SC)

BL21(DB3) cells carrying plasmid pGEX4T-scFv(SC) was inoculated in 10 ml of LB/carbenicillin (100 µg/ml). Cells were grown for 6 hr and centrifugal at 6,000 rpm (3,700×g) for 6 minutes. Supernatant was discarded and pellet was resuspended in fresh 2 ml of LB/ampicillin (100 µg/ml) and transferred to two 100 ml LB/amp media in 250 ml-Erlenmyer flask. Cells were grown 3 hours at 37° C. at 250 rpm. Temperature was lowered to 25° C. and IPTG was added (0.15 mM final concentration). Cells were grown additionally 15 hours, harvested and resuspended in 20 ml of 20 mM Tris-HCl, pH 8.0 buffer. Proteins were extracted by French Pressure (3 passages at 20,000 psi). Expression of scFv(SC) was analyzed by SDS-PAGE followed by Ponceau S staining and western blot.

scFv(SC) was expressed both as soluble protein and as insoluble inclusion bodies.

1D Western blot analysis of scFvSC. Reactivity of scFv(SC) toward recombinant ENOX2 was analyzed by 1 dimensional western blot. Recombinant ENOX2 was separated by SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with scFv(SC) for 17 hours and alkaline phosphatase linked S-protein for 2

hours. Detection was with Western Blue (Promega). scFv(SC) reacted toward recombinant ENOX2. For comparison, scFv(S) was included in the western blot analysis.

FIG. 12 shows Western blots of mixing studies to demonstrate efficacy of ScFv with -CXXC-XX-C linker (FIG. 12A) compared to the conventional XXXXXXX linker (FIG. 12B). Under non-reducing conditions for both (to preserve S—S bonds), the ScFv recombinant antibody with the functional cysteine linker when incubated with recombinant ENOX2 showed reduced levels of 53 kDa ScFv (a) and a 46 kDa truncated ScFv from a downstream initiation site (b) predominantly by disulfide bond formation between the full length ScFv and the 72 kDa full length ENOX2 in a ratio of one ENOX2 molecule plus two ScFv molecules (one to each of the two protein disulfide isomerase binding sites of ENOX2) for a combined molecular weight of about 176 kDa. In FIG. 12B, with the conventional linker lacking cysteines, no evidence of cross-linking under identical reaction conditions was observed.

2D Western blot analysis of scFvSC. Reactivity of scFv(SC) toward recombinant ENOX2 and transcript variant of serum of prostate cancer patient was analyzed by 2 dimensional western blot. Normal serum spiked with 6 µg of recombinant and serum from prostate cancer patient were separated first by isoelectric focusing and then SDS-PAGE and then transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with scFv(SC) for 17 hours and alkaline phosphatase linked S-protein for 2 hours. Detection was with Western Blue (Promega).

Method 5

Enhancement of the Physical Stability of Concentrated Antibody Solutions

An important embodiment of the present invention is to enhance the physical stability of concentrated solutions, while maintaining chemical stability and biological potency. The invention provides scFv antibodies which are at least 99% identical to SEQ ID NO:11 and have a kDa for ENOX2 protein of 7×10^{-7} M and a purity of at least 95%.

A major factor, however, in determining the efficacy of the scFv is its propensity to aggregate. After only ten days of storage at either -70° C. or 4° C., the binding of the purified scFv to recombinant ENOX2 protein was reduced by more than 90%. After much study, this loss of efficacy was traced solely to protein aggregation. Aggregated antibodies do not combine specifically with ENOX2 proteins during western blotting. Aggregation of the functionalized scFv antibody as determined from light scattering measurements is achieved by storage in 50% glycerol and stored at -70° C., essential ingredients in the overall protocol to achieve the desired level of sensitivity and specificity. Activity is lost rapidly by storage in the absence of glycerol.

TABLE 4

Detection of ENOX2 transcript variants and reference proteins comparing functionalized scFv recombinant antibodies stored in the presence or absence of 50% glycerol.								
Cancer	scFv stored in the absence of 50% glycerol				scFv stored in the presence of 50% glycerol			
	Ref. pH 4.1	Ref pH 6.8	Ref 3	Spot	Ref. pH 4.1	Ref pH 6.8	Ref 3	Spot
Prostate	+	+	-	+/-	+	+	+	+/-
Colorectal	+	+	-	+/-	++	++	+	++
Breast, Bladder	+	+	-	+	++	++	+	+

TABLE 4-continued

Detection of ENOX2 transcript variants and reference proteins comparing functionalized scFv recombinant antibodies stored in the presence or absence of 50% glycerol.								
scFv stored in the absence of 50% glycerol				scFv stored in the presence of 50% glycerol				
Cancer	Ref. pH 4.1	Ref pH 6.8	Ref 3	Spot	Ref. pH 4.1	Ref pH 6.8	Ref 3	Spot
NSC Lung	+	+	-	+	++	++	+	+
Testicular	+	+	-	+/-	++	++	+	+
Blood Cell	+	+	-	+/-	++	++	+	+
Overall	+	+	-	+/-	++	++	+	+

With storage in the presence of 50% glycerol (right), all three reference proteins were approximately two times stronger than in the absence of glycerol (left). Transcript variants (spots) were clearer with storage in 50% glycerol (right) than in its absence (left)

FIG. 13 is particle distribution as concentration (%) of antibody based on size (nm) determined by light scattering (Agilent 7080 Particle Size Spectrophotometer) showing predominately the major contribution of unaggregated antibody when stored in 50% glycerol at -70°C.

These three elements, addition of NADH during antibody binding, modification of the scFv linker peptide to resemble the ENOX2 protein disulfide-thiol interchange functional motif and the use of 50% glycerol to prevent scFv aggregation combine to provide an unprecedented sensitivity of the ONCOblot test to permit detection of cancers more than 10 years in advance of clinical symptoms.

Method 6

Determination of the Sensitivity and Limit of Detection of the Functionalized Antibody in the Presence of NADH

The incidence of confirmed false positives and confirmed false negatives is low, less than 1% each (D. J. Morre and D. S. Gilmartin. 2015 *ONCOblot Reports* 1 (1):1-2). For several cancers, two or more ENOX2 transcript variants must be present within the ONCOblot Tissue of Origin Cancer Test to permit the correct identification of the tissue of origin. These include bladder, colorectal gastric, mesothelioma, ovarian, renal cell and uterine cancer. If one or more ENOX2 transcript variants are absent or below the limit of detection, the tissue of origin of the cancer may be misidentified. Of the most recent ONCOblots analyzed under the current protocol, the rate of misidentification is 2.8%. Thus, the overall accuracy of the test with clinically diagnosed cancers is at least 95%.

When proteins are separated by two-dimensional (2-D) gel electrophoresis and detected by immunoblot, visualized proteins appear as small spherical or oval immunoreactive regions termed "spots." The average diameter of the spot produced by an ENOX2 protein is proportional to the amount of ENOX2 protein present. To determine the limit of ENOX2 detection by ONCOblot, a standard curve of spot diameter was generated. To this end, a functional 46 kDa form of human ENOX2 was first produced in *E. coli* and purified to near homogeneity. Various amounts of this recombinant ENOX2 protein were then assayed by ONCOblot. The log of the resulting spot diameter was then plotted against the log of the amount of ENOX2 protein assayed (FIG. 11) and a strong linear correlation ($r^2=0.95$) was found among the values. The practical lower limit of detection of

an ENOX2 protein assayed by ONCOblot is a spot on the order of 0.25 mm, which by comparison to FIG. 11 correlated to the detection of 120 femtomoles (1.2×10^{11} molecules) of ENOX2 per assay. This value is approximately equivalent to a lower limit of 100 femtomoles of ENOX2 per assay.

FIG. 11 illustrates the log-log relationship between spot diameter and amount of ENOX2 protein loaded on the 2-D gel. Varying amounts of recombinant ENOX2 were analyzed by immunoblot. The log of the diameter of the detected ENOX2 spot was then plotted as a function of the log of the amount of ENOX2 loaded.

FIG. 12 shows Western blots of mixing experiments to demonstrate efficacy of ScFv with -CXXC-XX-C linker (FIG. 12A) compared to the conventional XXXXXXX linker (FIG. 12B). Under non-reducing conditions for both (to preserve S—S bonds), the ScFv recombinant antibody with the functional cysteine linker when incubated with recombinant ENOX2 showed reduced levels of 53 kDa ScFv (a) and a 46 kDa truncated ScFv from a downstream initiation site (b) predominantly by disulfide bond formation between the full length ScFv and the 72 kDa full length ENOX2 in a ratio of one ENOX2 molecule plus two ScFv molecules (one to each of the two protein disulfide isomerase binding sites of ENOX2) for a combined molecular weight of about 176 kDa. In FIG. 12B, with the conventional linker lacking cysteines, no evidence of cross-linking under identical reaction conditions was observed.

FIG. 13 is particle distribution as concentration (%) of antibody based on size (nm) determined by light scattering (Agilent 7080 Particle Size Spectrophotometer) showing predominately the major contribution of unaggregated antibody when stored in 50% glycerol at -70°C.

ENOX2 proteins within sera of 25 Stage 0 and Stage 1 cancer patients were detected by ONCOblot (Morre, D. J and Taggart, D. J. 2015. *ONCOblot Reports* 1(4):1-2). The average concentration of ENOX2 within these sera samples was approximately 990 femtomoles (6×10^{11} molecules) per 150 μ L assay by comparison to the standard curve of FIG. 11. By definition, Stage 0 and Stage 1 cancers are typically less than 20 mm in diameter (Cancer Facts and Figures. 2014. *Amer Can Soc* 2-3). However, as the average size of Stage 0 or Stage 1 cancer is more likely to be 5 to 10 mm in diameter, the lower limit of detection of ENOX2 would be predicted to be produced by a 0.8 mm to 1.6 mm diameter tumor (1.2 mm average). A comparison of the lower limit of detection of recombinant ENOX2 between the previous version of the ONCOblot test (Hosteller et al., 2009. *Clinical Proteomics* 5: 46-51) and the current version of the test with NADH and functionalized scFv antibody is provided in FIG. 14. With NADH and the functionalized scFv antibody the lower limit of detection of recombinant ENOX2 in the protocol is about 5 μ g. With the functionalized test using

NADH and the modified linker the limit of detection is approximately 10-fold lower (curve on the right).

If the tumor cells are treated as spheres, then the number of cells in a solid tumor can be calculated by using equation 1, where V_{tumor} is the volume of the tumor, V_{cell} is the average volume of a cancer cell, D is the tumor diameter and d is the average diameter of a cancer cell estimated to be 10 μ m, then a 1.2 mm diameter cancer is calculated to contain approximately 2 million cells.

$$\# \text{ tumor cells} = \frac{V_{tumor}}{V_{cell}} = \frac{\frac{4}{3}\pi\left(\frac{D}{2}\right)^3}{\frac{4}{3}\pi\left(\frac{d}{2}\right)^3} = \left(\frac{D}{d}\right)^3 = \left(\frac{\frac{D}{2}}{\frac{d}{2}}\right)^3 = \left(\frac{D}{d}\right)^3 \quad \text{Equation 1}$$

Method 7

How Early Might the Functionalized Test with NADH and Modified Linker Detect Cancer in Advance of Clinical Symptoms

Evidence summarized herein has determined that the capability of the test in its present configuration is to detect cancer between 10 and 20 years in advance of clinical symptoms consistent with the 10-fold increased sensitivity provided.

In order to estimate how far in advance if clinical symptoms it would be possible to detect cancer presence by the current functionalized version of the test, a study was designed to determine, by comparison to NCI Seer Cancer Statistics Review (Howlander, N. et al. SEER Cancer Statistics Review, 1875-2912, National Cancer Institute, Bethesda, Md.) estimation of how closely the current test results correlated with overall risk of being diagnosed with cancer comparing six age groups consisting of equal numbers of male and female participants. Actual diagnoses were compared to those predicted by the Seer Cancer Statistics Review of predicted risk of being diagnosed with cancer with increasing age.

A total of 300 healthy volunteers without clinical evidence of cancer, 25 males and 25 females in each of six age groups, were analyzed for cancer presence by the current 2-D gel-western blot test with NADH and functionalized linker. Sera were collected by venipuncture and analyzed using IRB approved protocols and informed consent. Findings summarized in Table 5 reveal a relatively high incidence of early cancer detection with the normal healthy population consistent with cancer detection 10 to 20 years in advance of clinical symptoms (see also FIG. 5).

TABLE 5

Incidence of early cancer detected by the current 2-D gel-Western blot test with NADH and functionalized linker in a normal population of healthy male and female volunteers.			
Tests positive for cancer (%)			
Age at time of test (y)	Males	Females	Total
21-29	12	8	10
30-29	4	16	10
40-49	40	28	34
50-59	20	24	22

TABLE 5-continued

Incidence of early cancer detected by the current 2-D gel-Western blot test with NADH and functionalized linker in a normal population of healthy male and female volunteers.			
Tests positive for cancer (%)			
Age at time of test (y)	Males	Females	Total
60-69	20	28	24
70-79	12	32	22

Includes both solid tissue and blood cell cancers.

The overall incidence of positive test was 10% in the 21 to 29 year age group and stabilized at about 22% between 50 and 79 years. The marked increase in positive ONCOblots in the 40 to 49 year old males was due to a disproportionate number of early prostate indications within this population, a number of which would likely never develop in overt disease. Otherwise, for all age groups, the observed incidence of cancer matched closely the incidences predicted from the National Cancer Institute Seer Cancer Statistics Review (Table 6) for between 10 and 20 years rather than the previous estimates of 7 to 10 years to be provided by the 2-D gel-western blot protocol lacking the NADH/functionalized linker modified version of the present invention consistent with the 2- to 3-fold increase in sensitivity of the test over that of the original test.

TABLE 6

Risk of being diagnosed with cancer for different age groups from the National Cancer Institute Seer Cancer Statistics Review (Howlander, N. et al. SEER Cancer Statistics Review, 1875-2912, National Cancer Institute, Bethesda, MD).				
Current Age	+10 years	+20 years	+30 years	Ever
20	0.46	1.52	4.13	40.73
30	1.08	3.72	9.64	40.77
40	2.70	8.75	19.44	40.55
60	13.47	26.28	34.95	37.10
70	17.16	27.91	—	30.61
80	16.15	—	—	20.40

The findings show that the 2-D gel-western blot test as described in the present invention has the potential to detect cancer earlier than any test previously reported. Certainly, not all of the early detected cancers would be expected to develop into clinically diagnosed disease but, based on the comparisons provided, in the order of 50% would have a high probability of so doing. At least with prostate cancer, positive ONCOblot results have been recorded for subjects with PSA values as low as 0.5 ng/ml in the age group of 40 to 49 year old males as one explanation for the exceptionally high incidence of cancer indicated by the functionalized ONCOblot test.

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the

specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. In embodiments of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. As used herein, the phrase “consisting essentially of” requires the specified integer(s) or steps as well as those that do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited integer (e.g., a feature, an element, a characteristic, a property, a method/process step or a limitation) or

group of integers (e.g., feature(s), element(s), characteristic(s), property(s), method/process steps or limitation(s)) only.

The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

As used herein, words of approximation such as, without limitation, “about”, “substantial” or “substantially” refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skill in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least ± 1 , 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 25

<210> SEQ ID NO 1

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

Glu	Glu	Met	Thr	Glu	Thr	Lys	Glu	Thr	Glu	Glu	Ser	Ala	Leu	Val	Ser
1			5				10						15		

<210> SEQ ID NO 2

<211> LENGTH: 16

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Gly	Thr	Asp	Cys	Val	Ala	Lys	Glu	Ala	Thr	Glu	Ala	Ala	Lys	Cys	Asn
1			5				10						15		

<210> SEQ ID NO 3

-continued

<211> LENGTH: 16
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Cys Leu Asp Gly Thr Arg Lys Pro Val Glu Glu Tyr Ala Asn Cys His
 1 5 10 15

<210> SEQ ID NO 4
 <211> LENGTH: 45
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

ggcggggggtg gtagctgcgg cggtaggatcg tgtggcggtg gcagt 45

<210> SEQ ID NO 5
 <211> LENGTH: 15
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Gly Gly Gly Gly Ser Cys Gly Gly Gly Ser Cys Gly Gly Gly Ser
 1 5 10 15

<210> SEQ ID NO 6
 <211> LENGTH: 690
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

atgtccccta tactaggtta ttgaaaaatt aagggccttg tgcaaccac tcgacttctt 60
 ttggaatatc ttgaagaaaa atatgaagag catttgtatg agcgcgatga aggtgataaa 120
 tggcgaaaca aaaagtttg attgggtttg gagtttccca atcttcctta ttatattgat 180
 ggtgatgtta aattaacaca gtctatggcc atcatacgtt atatagctga caagcacaac 240
 atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcgggtttg 300
 gatattagat acggtgtttc gagaattgca tatagtaaag accttgaaac tctcaaagtt 360
 gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa 420
 acatatatta atgggtgatca tgtaacccat cctgacttca tgttgatga cgctcttgat 480
 gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa 540
 aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca 600
 tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat 660
 ctggttcgcg gtggatcccc aggaattccc 690

<210> SEQ ID NO 7
 <211> LENGTH: 357
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

gaggtcaagc tgcaggagtc aggaactgaa gtggtaaagc ctggggcttc agtgaagttg 60
 tcctgcaagg cttctggcta catcttcaca agttatgata tagactgggt gaggcagacg 120
 cctgaacagg gacttgagtg gattggatgg attttctctg gagaggggag tactgaatac 180
 aatgagaagt tcaagggcag ggccacactg agttagtaca agtcctccag cacagcctat 240
 atggagctca ctaggtgac atctgaggac tctgctgtct atttctgtgc tagaggggac 300

-continued

tactataggc gctactttga cttgtggggc caagggacca cggtcaccgt ctctca 357

<210> SEQ ID NO 8
 <211> LENGTH: 321
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

gaaaatgtgc tcacccagtc tccagcaatc atgtctgcat ctccaggga gagggtcacc 60
 atgacctgca gtgccagctc aagtatacgt tacatatatt ggtaccaaca gaagcctgga 120
 tcctccccc gactcctgat ttatgacaca tccaacgtgg ctctggagt cctttttcgc 180
 ttcagtggca gtgggtctgg gacctcttat tctctcaca tcaaccgaat ggaggctgag 240
 gatgctgcc cttattactg ccaggagtgg agtggttatc cgtacacgtt cggagggggg 300
 accaagctgg agctgaaagc g 321

<210> SEQ ID NO 9
 <211> LENGTH: 1413
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

atgtccccta tactaggtta ttgaaaaatt aagggccttg tgcaaccac tcgacttctt 60
 ttggaatata tgaagaaaa atatgaagag cttttgtatg agcgcgatga aggtgataaa 120
 tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat 180
 ggtgatgtta aattaacaca gtctatggcc atcatacgtt atatagctga caagcacaaac 240
 atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcggttttg 300
 gatattagat acggtgtttc gagaattgca tatagtaaag actttgaaac tctcaaagtt 360
 gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa 420
 acatatatta atgggtgatca tgtaacccat cctgacttca tgtgtgatga cgctcttgat 480
 gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa 540
 aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca 600
 tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat 660
 ctggttccgc gtggatcccc aggaattccc gaggtcaagc tgcaggagtc aggaactgaa 720
 gtggtaaagc ctggggcttc agtgaagttg tctgcaagg cttctggcta catcttcaca 780
 agttatgata tagactgggt gaggcagacg cctgaacagg gacttgagtg gattggatgg 840
 atttttctg gagaggggag tactgaatac aatgagaagt tcaagggcag ggccacactg 900
 agtgtagaca agtcctccag cacagcctat atggagctca ctaggctgac atctgaggac 960
 tctgctgtct atttctgtgc tagaggggac tactataggc gctactttga cttgtggggc 1020
 caagggacca cggtcaccgt ctctcaggc gggggtggtg gctgcggcgg tggatcgtgt 1080
 ggcggtggca gtgaaaatgt gtcacccag tctccagcaa tcatgtctgc atctccaggg 1140
 gagaggggtc ccatgacctg cagtgcacg tcaagtatac gttacatata ttggtaccaa 1200
 cagaagcctg gatcctcccc cagactcctg atttatgaca catccaacgt ggctcctgga 1260
 gtcccttttc gcttcagtgg cagtgggtct gggacctctt attctctcac aatcaaccga 1320
 atggagctg aggatgctgc cacttattac tgccaggagt ggagtgttga tccgtacacg 1380
 ttcggagggg ggaccaagct ggagctgaaa gcg 1413

-continued

<210> SEQ ID NO 10
 <211> LENGTH: 1413
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

```

atgtccccta tactaggtta ttggaaaatt aagggccttg tgcaaccac tcgacttctt    60
ttggaatata ttgaagaaaa atatgaagag catttgatg agcgcgatga aggtgataaa    120
tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat    180
ggatgatgta aattaacaca gtctatggcc atcatagctt atatagctga caagcacaa    240
atgttggttg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcgggtttg    300
gatattagat acgggtgttc gagaattgca tatagtaaag accttgaaac tctcaaagtt    360
gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa    420
acatatatta atggtgatca tgtaacccat cctgacttca tgttgatga cgctcttgat    480
gttggtttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgtttttaa    540
aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca    600
tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat    660
ctggttcctg ctggatcccc aggaattccc gaggtcaagc tgcaggagtc aggaactgaa    720
gtggtaaagc ctggggcttc agtgaagttg tctgcaagg cttctggcta catcttcaca    780
agttagata tagactgggt gaggcagacg cctgaacagg gacttgagtg gattggatgg    840
atctttcctg gagaggggag tactgaatac aatgagaagt tcaagggcag ggccacactg    900
agttagaca agtcctccag cacagcctat atggagctca ctaggctgac atctgaggac    960
tctgctgtct atttctgtgc tagaggggac tactataggc gctacttga cttgtggggc   1020
caagggacca cggtcacagt ctctcaggc ggggggtgta gctgcggcgg tggatcgtgt   1080
ggcgggtggc gtgaaaatgt gctcaccag tctccagcaa tcatgtctgc atctccaggg   1140
gagaggggtc ccatgacctg cagtgccagc tcaagtatac gttacatata ttggtaccaa   1200
cagaagcctg gatcctcccc cagactcctg atttatgaca catccaactg ggctcctgga   1260
gtcccttttc gcttcagtgg cagtgggtct gggacctctt attctctcac aatcaaccga   1320
atggaggctg aggatgctgc cacttattac tgccaggagt ggagtggta tccgtacacg   1380
ttcggagggg ggaccaagct ggagctgaaa gcg                                1413

```

<210> SEQ ID NO 11
 <211> LENGTH: 471
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

```

Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
1           5           10          15

Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
          20          25          30

Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
          35          40          45

Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
          50          55          60

Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65          70          75          80

```

-continued

Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
 85 90 95
 Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
 100 105 110
 Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
 115 120 125
 Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
 130 135 140
 Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
 145 150 155 160
 Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
 165 170 175
 Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
 180 185 190
 Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
 195 200 205
 Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Val Pro Arg
 210 215 220
 Gly Ser Pro Gly Ile Pro Glu Val Lys Leu Gln Glu Ser Gly Thr Glu
 225 230 235 240
 Val Val Lys Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly
 245 250 255
 Tyr Ile Phe Thr Ser Tyr Asp Ile Asp Trp Val Arg Gln Thr Pro Glu
 260 265 270
 Gln Gly Leu Glu Trp Ile Gly Trp Ile Phe Pro Gly Glu Gly Ser Thr
 275 280 285
 Glu Tyr Asn Glu Lys Phe Lys Gly Arg Ala Thr Leu Ser Val Asp Lys
 290 295 300
 Ser Ser Ser Thr Ala Tyr Met Glu Leu Thr Arg Leu Thr Ser Glu Asp
 305 310 315 320
 Ser Ala Val Tyr Phe Cys Ala Arg Gly Asp Tyr Tyr Arg Arg Tyr Phe
 325 330 335
 Asp Leu Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly
 340 345 350
 Gly Ser Cys Gly Gly Gly Ser Cys Gly Gly Gly Ser Glu Asn Val Leu
 355 360 365
 Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly Glu Arg Val Thr
 370 375 380
 Met Thr Cys Ser Ala Ser Ser Ser Ile Arg Tyr Ile Tyr Trp Tyr Gln
 385 390 395 400
 Gln Lys Pro Gly Ser Ser Pro Arg Leu Leu Ile Tyr Asp Thr Ser Asn
 405 410 415
 Val Ala Pro Gly Val Pro Phe Arg Phe Ser Gly Ser Gly Ser Gly Thr
 420 425 430
 Ser Tyr Ser Leu Thr Ile Asn Arg Met Glu Ala Glu Asp Ala Ala Thr
 435 440 445
 Tyr Tyr Cys Gln Glu Trp Ser Gly Tyr Pro Tyr Thr Phe Gly Gly Gly
 450 455 460
 Thr Lys Leu Glu Leu Lys Ala
 465 470

<210> SEQ ID NO 12

<211> LENGTH: 42

<212> TYPE: DNA

-continued

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

ggatccccag gaattcccga ggtcaagctg caggagtcag ga

42

<210> SEQ ID NO 13

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Gly Thr Thr Val Thr Val Ser Ser
1 5

<210> SEQ ID NO 14

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Gly Gly Gly Gly Ser Cys Gly Gly Gly Ser Cys Gly Gly Gly Ser
1 5 10 15

<210> SEQ ID NO 15

<211> LENGTH: 69

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

gggaccacgg tcaccgtctc ctcaggcggg ggtggtagct gcggcgggtg atcgtgtggc 60

ggtggcagt 69

<210> SEQ ID NO 16

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Cys Gly Gly
1 5 10 15Gly Ser Cys Gly Gly Gly Ser
20

<210> SEQ ID NO 17

<211> LENGTH: 63

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

accgccacac gatccaccgc cgcagctacc acccccgctt gaggagacgg tgaccgtggt 60

ccc 63

<210> SEQ ID NO 18

<211> LENGTH: 21

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

Gly Gly Ser Cys Gly Gly Gly Ser Cys Gly Gly Gly Ser Glu Asn Val
1 5 10 15Leu Thr Gln Ser Pro
20

-continued

<210> SEQ ID NO 19
 <211> LENGTH: 63
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

ggtggtagct gcggcggtgg atcgtgtggc ggtggcagtg aaaatgtgct caccagctct 60
 cca 63

<210> SEQ ID NO 20
 <211> LENGTH: 21
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 20

Gly Gly Ser Cys Gly Gly Gly Ser Cys Gly Gly Gly Ser Glu Asn Val
 1 5 10 15
 Leu Thr Gln Ser Pro
 20

<210> SEQ ID NO 21
 <211> LENGTH: 63
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 21

ggtggtagct gcggcggtgg atcgtgtggc ggtggcagtg aaaatgtgct caccagctct 60
 cca 63

<210> SEQ ID NO 22
 <211> LENGTH: 39
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 22

gaacgccagc acatggacag ctgactcgag cgcccggtg 39

<210> SEQ ID NO 23
 <211> LENGTH: 39
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

caccggccgc tcgagtcagc tgtccatgtg ctggcgcttc 39

<210> SEQ ID NO 24
 <211> LENGTH: 1464
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

aaaaaacaat gcaaagtgg aaagcctgg ggcttcagtg aagttgtcct gcaaggettc 60
 tggctacatc ttcacaagtt atgatataga ctgggtgagg cagacgcctg aacagggact 120
 tgagtggatt ggatggattt ttcctggaga ggggagtact gaatacaatg agaagttcaa 180
 gggcagggcc aactgagtg tagacaagtc ctccagcaca goctatatgg agctcactag 240
 gctgacatct gaggactctg ctgtctatct ctgtgctaga ggggactact acaggcgcta 300
 ctttgacttg tggggccaag ggaccacggt caccgtctcc tcaggcgggg gtggtagctg 360

-continued

cggcgggtgga tcgtgtggcg gtggcagtgga aaatgtgctc acccagtcctc cagcaatcat	420
gtctgcacat ccaggggaga gggtcaccat gacctgcagt gccagctcaa gtatacgtta	480
catatattgg taccaacaga agcctggatc ctccccaga ctctgattt atgacacatc	540
caacgtggct cctggagtcc cttttcgctt cagtggcagt gggctctggga cctcttatc	600
tctcacaatc aaccgaatgg aggctgagga tgctgccact tattactgcc aggagtggag	660
tggttatccg tacacgttcg gaggggggac caagctggag ctgaaagcga aagaaactgc	720
tgctgctaaa ttogaacgcc agcacatgga cagctgactc gageggccgc atcgtgactg	780
actgacgac tgccctgcgc gtttcggtga tgacggtgaa aacctctgac acatgcagct	840
cccgagacg gtcacagctt gtctgtaagc ggatgccggg agcagacaag cccgtcaggg	900
cgcgtcagcg ggtgttggcg ggtgtccggg ccccgccatg acccagtcac gtagcgatag	960
cggagtgtat aattcttgaa gacgaaaggg cctcgtgaat cgcctatctt tatagggtaa	1020
tgtcatgata ataatgtttt cttaaacgtc aagggggcac tttttcggga aatgtgcgcc	1080
gaaaccccta atttgtttta tttttcaaaa tccatttcaa ttaagtttcc cctcatgga	1140
aacaataacc cctggaaaaa aggccttcat taaatattga aaaaaaggaa aaaagttaa	1200
aattatttca aaattttccc tgtgttcccc cctttatctt cccctttttt ttgcgggcca	1260
ttttttgccc ttttcctggg ttttttttgc ccccccccc caaaaaacc cccctggggt	1320
gaaaaaaaaa taaaaaaaaa aaatgtcttt aaaaaaaaaa ttccaatttt tgtggggggg	1380
gggccccccc aaaagagttg ggggggtttt ttaaaccaact tctccaaaaa caacattggg	1440
tggagatatt tcttcttcac aaaa	1464

<210> SEQ ID NO 25

<211> LENGTH: 1492

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

gggtgggtaaa tttagcagca gcagtttctt tcgctttcag ctccagcttg gtccccctc	60
cgaacgtgtc cggataacca ctccactcct ggcagtaata agtggcagca tctcagcct	120
ccattcggtt gattgtgaga gaataagagg tcccagacct actgccactg aagcgaaaag	180
ggactccagg agccacgttg gatgtgcat aaatcaggag tctgggggag gatccaggct	240
tctgttggtta ccaatatatg taacgtatac ttgagctggc actgcaggtc atggtgacct	300
tctcccttg agatgcagac atgattgctg gagactgggt gagcacattt tcaactgccac	360
cgccacacga tccaccgcg cagctaccac ccccgctga ggagacggtg accgtggtcc	420
cttgccccca caagtcaaag tagcgctgt agtagtcccc tctagcacag aaatagacag	480
cagagtctc agatgtcagc ctagtgagct ccatatagc tgtgctggag gacttgtcta	540
cactcagtg ggccctgccc ttgaacttct cattgtatc agtactcccc tctccaggaa	600
aaatccatcc aatccactca agtccctgtt caggcgtctg cctcaccag tctatatcat	660
aacttgtgaa gatgtagcca gaagccttg aggacaaact cactgaagcc ccaggcttta	720
ccacttcagt tctgactcc tgcagcttga cctcggaat tccagaaagg gctgcttga	780
acttctcctt tgcttcttcc atatctttct catggcgga tccacgcgga accagatccg	840
attttgagg atggtcgcca ccacaaaacg tggcttgcca gccctggcaa ggccatgcta	900
tatacttgct ggatttcaag tacttatcaa tttgtggaa tagcttctt acgttttttt	960
aaaacaaact aattttggga acgcatccag gccatttgg tccatgtatt aaaacaccat	1020

-continued

caaaaacctc ctacaacatg aaattccgga aggggtttac ataatcccc attttaata	1080
ttgtttttat gacataaaac caatctttcc aaactttttt tcacattttc caggtaacct	1140
tggtaaaaaaaa aaaccactt ttgaaaagtt ttcaaaatt ctttttatta aaatgcaa	1200
tttcccgaaa aaacccctt atttctaaaa attcccaaaa acccgctccc ctttccaagc	1260
cctttgaaaa atttctttgc cccccccctc ttttttgggg aacaaaaccc cccccacaa	1320
acttgggttg ggggtggttt ttgtgtccac gcccaaatat aaaaaacag tataaattaa	1380
gatggggccc ccacataata aaaactgggg ggggtttttt tatataattt tttaaacac	1440
aacatcccc ccccccccc acatcccaca caaaataaaa tatataataa aa	1492

What is claimed is:

1. A method for detecting benign to malignant transformation of a cancer in a subject, comprising the steps of:

collecting a sample from the subject prior to electrophoretic protein separation;

activating electrophoretically separated ENOX2 transcript variants with an ENOX2 electron donor; and

detecting the presence of the one or more activated ENOX2 transcript variants using a pan-ENOX2 detectable binding reagent, wherein the presence of one or more activated ENOX2 transcript variants in the sample is indicative of the malignant transformation of the cancer, whereby a 10 to 100 fold increase in detection sensitivity is obtained for the one or more activated ENOX2 transcript variants when compared to an equivalent non-activated ENOX2 transcript variant.

2. The method of claim 1, wherein the ENOX2 electron donor required to activate the ENOX2 transcript variants is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M.

3. The method of claim 1, further comprises performing two dimensional (2D) electrophoretic protein separation and blotting onto a film or paper.

4. The method of claim 1, wherein the pan-ENOX2 detectable binding reagent comprises SEQ ID NO:11.

5. The method of claim 1, wherein the sample is a blood, serum, plasma, urine, cerebrospinal fluid or a body fluid.

6. The method of claim 1, wherein the pan-ENOX2 detectable binding reagent comprises an anti-ENOX2 scFv fusion protein that is:

non-aggregating at between 4° C. and 40° C.;

comprises a small side chain (Gly/Ser) of amino acid residues containing two cysteines (C) spaced as either CXXXXC or CXXXXXX; and

is capable of forming an interchain disulfide bond with the protein disulfide-thiol interchange functional motif of ENOX2.

7. The method of claim 4, wherein the pan-ENOX2 detectable binding reagent comprises an scFv of SEQ ID NO:1, wherein the stability, binding efficiency, and shelf life of the scFv of SEQ ID NO:11 is improved by storing in 50% glycerol to prevent aggregation.

8. The method of claim 1, wherein the step of concentrating the blood, serum, or plasma sample is defined further as concentrating the one or more ENOX2 transcript variants

using a nickel-agarose substrate to bind and concentrate the one or more ENOX2 transcript variants.

9. The method of claim 1, further comprises detecting the cancer and/or determining a tissue of origin of a human cancer at least one year in advance of clinical symptoms.

10. The method of claim 1, further comprises determining a tissue of origin of one or more cancers of unknown primary (CUP).

11. The method of claim 1, further comprises screening for at least one of: early cancer in populations with family cancer histories, environmental exposures, or in a general population.

12. The method of claim 1, further comprises determining a tissue of origin of the one or more activated ENOX2 transcript variants based on the presence of ENOX2 transcript variants as set forth in Table 1.

13. The method of claim 1, further comprises resolving proteins at 53 and/or 79 to 85 kDa as total protein loading controls.

14. The method of claim 1, further comprises distinguishing between distant metastases and multiple primary cancers based on the presence of the one or more activated ENOX2 transcript variants.

15. The method of claim 1, further comprises detecting stage 0 and stage 1 cancers and determining the tissue of origin of the cancer.

16. The method of claim 1, wherein an incidence of false positives and confirmed false negatives of the predicted cancer is less than 1%.

17. The method of claim 1, wherein the sensitivity of the method for detecting the cancer is greater than 97%.

18. The method of claim 1, further comprises monitoring the reemergence of the cancer, therein the presence of 2 million or less cancer cells is detectable based on the reemergence of the one or more activated ENOX2 transcript variants.

19. The method of claim 1, wherein the tissue of origin of the cancer detected is selected from at least one of bladder, blood cell, lymphomas, leukemias, multiple myelomas and myelomas breast, cervical, colorectal, esophageal, gastric, hepatocellular, lung, non-small cell, melanoma, mesothelioma, ovarian, pancreatic, prostate, renal cell (kidney), sarcoma, squamous cell, testicular germ cell, thyroid follicular, thyroid papillary, or uterine.

20. A calibrated method of quantitation of ENOX2 transcript variants on a western blot comprising:

subjecting a sample from the subject concentrated sample to electrophoretic protein separation;

western blotting the electrophoretically separated proteins onto a protein capture film or paper;
activating one or more ENOX2 transcript variants on the protein capture film or paper with a reduced ENOX2 substrate; and

detecting the presence of the one or more activated ENOX2 transcript variants using a pan-ENOX2 detectable binding reagent, wherein a 10 to 100 fold increase in detection sensitivity for ENOX2 is obtained when compared to the non-activated ENOX2, wherein the amount of one or more activated ENOX2 transcript variants is calibrated based on a spot diameter and an intensity of the ENOX2 transcript variant on the protein capture film or paper when compared to a known amount of the one or more ENOX2 transcript variants.

21. The method of claim 20, wherein the spot diameter is compared to a standard curve of recombinant ENOX2.

22. The method of claim 20, wherein the spot diameter and the log of the mass of ENOX2 are linearly correlated.

23. The method of claim 20, wherein the quantitation is achieved by a combination of spot diameter and ENOX2 activity.

24. The method of claim 20, wherein the sample is a blood, serum, plasma, urine, cerebrospinal fluid or a body fluid.

25. The method of claim 20, wherein the quantitation is achieved by cutting out ENOX2 antibody reactive spots and quantitating the amount of ENOX2 using elution and reaction with silver tetroxide to form silver nanoparticles and quantitating the nanoparticles by light scattering using an UV-vis spectrophotometer or a light scattering spectrophotometer by comparison to a corresponding spot that is free of ENOX2.

26. The method of claim 25, wherein a spot percentage above background greater than 7% confirms the presence of an ENOX2 transcript variant in the spot.

27. The method of claim 20, further comprises detecting and determining a tissue of origin in the spot of a human cancer at least one year in advance of clinical symptoms.

28. A method for determining the tissue of origin of a cancer in a subject comprising:

subjecting proteins in a sample from the subject to a two-dimensional (2D) molecular weight and isoelectric point electrophoretic protein separation;

transferring the proteins to a protein capture film or paper; activating the electrophoretically separated ENOX2 transcript variants with an ENOX2 electron donor on the protein capture film or paper;

detecting the activated ENOX2 transcript variants; and determining the tissue of origin of the cancer based on the presence of one or more activated ENOX2 transcript variants selected from Bladder, Blood Cell, Breast, Cervical, Colorectal, Esophageal, Gastric, Hepatocellular, Leukemias, Lung Non-Small Cell, Lung Small Cell, Lymphomas, Melanoma, Mesothelioma, Myeloma, Ovarian, Pancreatic, Prostate, Renal Cell (Kidney), Sarcoma, Squamous Cell, Testicular Germ Cell, Thyroid Follicular, or Thyroid Papillary, wherein activation of the one or more ENOX2 transcript variants in situ increases the detection sensitivity by 10 to 100 fold when compared to non-activated ENOX2 transcript variants.

29. The method of claim 28, wherein the ENOX2 electron donor required to activate the ENOX2 transcript variants is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M.

30. The method of claim 28, further comprising the steps of performing two dimensional (2D) electrophoretic protein separation and blotting onto a film or paper, wherein the ENOX2 electron donor is selected from at least one of a reduced pyridine nucleotide, NADH, NAD(P)H, or a synthetic substrate that is an ENOX2 electron donor, and detecting the one or more activated ENOX2 transcript variants on the 2D blot, wherein the final concentration of NADH or NAD(P)H is in the range 10 μ M to 50 μ M.

31. The method of claim 28, wherein the pan-ENOX2 detectable binding reagent comprises SEQ ID NO:11.

32. The method of claim 28, wherein the sample is a blood, serum, plasma, urine, cerebrospinal fluid or a body fluid.

33. The method of claim 28, wherein the pan-ENOX2 detectable binding reagent comprises an anti-ENOX2 scFv fusion protein is:

non-aggregating at between 4° C. and 40° C.;

comprises a small side chain (Gly/Ser) amino acid residues containing two cysteines (C) spaced as either CXXXXC or CXXXXXC; and

is capable of forming an interchain disulfide bond with the protein disulfide-thiol interchange functional motif of ENOX2.

34. The method of claim 28, wherein the pan-ENOX2 detectable binding reagent comprises an scFv of SEQ ID NO:11, wherein the stability, binding efficiency, and shelf life of the scFv of SEQ ID NO:1 is improved by storing in 50% glycerol to prevent aggregation.

35. The method of claim 28, wherein the step of concentrating the blood, serum, or plasma sample is defined further as concentrating the one or more ENOX2 transcript variants using a nickel-agarose substrate to bind and concentrate the one or more ENOX2 transcript variants.

36. The method of claim 28, further comprises detecting the cancer and/or determining a tissue of origin of a human cancer at least one year in advance of clinical symptoms.

37. A blot comprising one or more electrophoretically separated proteins from a concentrated sample from a subject, wherein the blot is exposed to a pan-ENOX2 detecting reagent and to a reducing substrate for ENOX2 that activates the ENOX2 transcript variants on the blot, wherein the blot is analyzed for the present of one or more activated ENOX2 transcript variants from the following Table 1, wherein the presence of the one or more activated ENOX2 transcript variants is used to detect and determine a tissue of origin of a human cancer at least one year in advance of clinical symptoms, and the activation of the one or more ENOX2 transcript variants in situ increases the detection sensitivity by 10 to 100 fold when compared to non-activated ENOX2 transcript variants.

* * * * *

专利名称(译)	用于早期癌症检测和癌症诊断的靶向二维蛋白质印迹分析的方法和组合物，其在恶性疾病的临床症状之前提前十年		
公开(公告)号	US9804165	公开(公告)日	2017-10-31
申请号	US15/434982	申请日	2017-02-16
[标]申请(专利权)人(译)	铁道部NUCO企业		
申请(专利权)人(译)	MOR-NUCO ENTERPRISES , INC.		
当前申请(专利权)人(译)	MOR-NUCO ENTERPRISES , INC.		
[标]发明人	MORRE D JAMES		
发明人	MORRE, D. JAMES		
IPC分类号	G01N33/53 G01N21/49 C07K16/40 G01N33/574		
CPC分类号	G01N33/57488 G01N21/49 C07K16/40 C12Y106/00 G01N2333/90209 C07K2317/622 C07K2317/56 G01N33/574 G01N33/6893 C12Q1/26 G01N33/561 G01N33/57492 G01N2333/47		
代理人(译)	弗洛雷斯，EDWIN S.		
外部链接	Espacenet USPTO		

摘要(译)

本发明包括用于检测受试者中癌症的良性至恶性转化的方法，包括步骤：在电泳蛋白质分离之前从受试者收集样品；用ENOX2电子供体激活电泳分离的ENOX2转录物变体；使用pan-ENOX2可检测的结合试剂检测一种或多种活化的ENOX2转录物变体的存在，其中样品中一种或多种活化的ENOX2转录物变体的存在指示癌症的恶性转化，其中10至10当与等等的未活化的ENOX2转录物变体相比时，对于一种或多种活化的ENOX2转录物变体获得100倍的检测灵敏度增加。

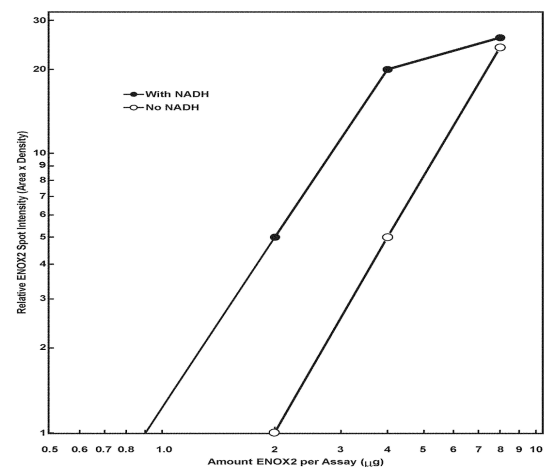


FIG. 1