ONCOblot®
Early Cancer Detection

The ONCOblot® Test

A single blood test that detects over 26 forms of cancer and determines tissue of origin.

The ONCOblot® Tissue of Origin Cancer Test (ONCOblot®) is based on the discovery of the cancer-specific universal cancer marker ENOX2:

- Coincides with the onset of malignancy (uncontrolled invasive growth).
- Located at the cancer cell surface and shed into circulation.
- Present in all forms of cancer thus far examined as a family of transcript variants where each variant is specific to a particular tissue of origin.
- More than 26 cancer types identified.
- Cancer-specific intervention target. Cancer cells cannot enlarge when ENOX2 is inhibited and the resultant small cells following division are unable to divide again and, subsequently undergo apoptosis.

The ONCOblot® Test is:

- Sensitive. Detects as few as 2 million cancer cells (0.8 mm cancer).
- Direct molecular visualization. Based on two dimensional gel electrophoresis with detection using an ENOX2-specific recombinant antibody to minimize false positives.

UNIVERSAL CANCER MARKER - DETECTS 26+ FORMS OF CANCER IN A SINGLE SCREEN - SERUM TEST - NON-INVASIVE - CANCER SPECIFIC - COINCIDES WITH ONSET OF MALIGNANCY - INDICATES BOTH CANCER PRESENCE AND ORGAN SITE - NOVEL INTERVENTION TARGET

- **Available since January 2013**
- **CLIA Certified and CAP Accredited**
- **Meets FDA guidelines as a Laboratory-Developed Test**
- **Investigational Device Exemption determined by FDA**
- **510(K) submission to the FDA in progress**
- **Currently seeking coverage from Medicare and insurance companies**

Please visit our website for more information:
www.oncoblotlabs.com

ONCOblot®
Early Cancer Detection
www.oncoblotlabs.com

Section One: Why ENOX2 is a cancer marker

The Short Story

The ENOX2 gene
The human ENOX2 gene is located on the reverse strand of chromosome X. It is expressed only in malignant cells and tissues. The transcription products (protein) of the ENOX2 gene consist of a family of transcript variants. The variants are specific to the tissue of origin of the cancer.

The ENOX2 protein
ENOX2 proteins are located on the surface of cancer cells and being ECTO or surface proteins, are shed into the circulation. As such the pattern of ENOX2 proteins in a patient's sera not only serves as a molecular indicator of cancer presence but precisely identifies the tissue of origin of the cancer.

The Long Story

ENOX—A family of enzymes
Ecto-**N**icotinamide Dinucleotide **Ox**idase Disulfide-Thiol Exchanger (**ENOX**) is a family of enzymes having two distinct activities (Morré and Morré, 2013). They alternate between protein disulfide-thiol exchange and plasma membrane electron transport (PMET) activity (See figure 1) both of which affect cell growth and cell division.

Activity One
The Protein Disulfide-thiol Exchange Cycle demonstrated by ENOX enzymes effectively interchange electrons with other cell surface proteins that play a role in cell enlargement and ultimately cell growth. If this activity is unregulated, the result is the unregulated cell growth that defines all forms of cancer.

Activity Two
During PMET activity, ENOX enzymes function as terminal oxidases whereby electrons coming from cytosolic reduced pyridine nucleotide (NADH) are transferred to membrane-located coenzyme Q (CoA) with eventual transfer of electrons and protons to oxygen to form water. The released energy is utilized to drive the enlargement phase of cell growth. This activity is an absolute requirement for cells to increase in size. When inhibited, cells cannot divide and subsequently undergo apoptosis.

ENOX1—Cells under control
All non-cancer cells express a cell-surface ENOX protein designated as ENOX1. ENOX1 is extremely resistant to inhibition (only one specific ENOX1 inhibitor is known).

ENOX1 form is responsive to growth factors and hormones such as epidermal growth factor (EGF) and insulin (Bruno et al., 1992) while ENOX2 is growth factor and hormone unresponsive and appears constitutively active. ENOX1 is the healthy form needed for homeostasis and normal cell function while ENOX2 is seen only in cancer cells (See Figure 2).

ENOX2—Cells with uncontrolled growth
In contrast to ENOX1, ENOX2 is unregulated. Any cell/tissue expressing the ENOX2 protein will experience uncontrolled, malignant (cancerous) cell growth. ENOX2 activities are inhibited by most cancer chemotherapeutic agents including cisplatin and doxorubicin, while the normal ENOX1 protein is drug resistant (Jiang et al., 2008).

ENOX1 and ENOX2 are genetically "normal"
ENOX1 gene is located on chromosome 13 while ENOX2 is located on the reverse strand of chromosome X. These are discreet genes on different chromosomes that code for similar proteins with similar activities. Therefore, the presence of ENOX2 is not the result of a gene mutation or any event at the genetic level. Rather, ENOX2 gene is universally present in the human genome, which is why genomic cancer screens do not reveal ENOX2 gene as a cancer marker. ENOX2 is a normal gene coding for an oncofetal protein that appears in very early embryogenesis and then disappears only to be re-expressed in cancer.

ENOX2—Splicing makes all the difference
It is alternative splicing of ENOX2 during transcription of the gene into mRNA that accounts for the cancer-specific expression of the ENOX2 protein. The ENOX2 protein is expressed only in malignant cells and tissues.

Alternative splicing events also explain the presence of various forms of ENOX2 proteins. These transcript variants are specific to the type of tissue and reveal the origin of the cancer. Each variant is unique in the number of variants produced (one to several), molecular weight and isoelectric point.

All transcript variants include an exon 4 deletion splicing event that allows for down-stream initiation and expression. Without the exon 4 deletion, ENOX2 mRNA is not translated into protein (Tang et al., 2007; 2008). Consequently, the exon 4 deletion is the basis for cancer specificity.

ENOX2 proteins—Released into the bloodstream

ENOX2 proteins are not permanently bonded to their location on the outer surface of the cell membrane and they are released into the circulation. This makes the proteins an attractive marker for diagnostic testing. Therefore, a simple blood draw and subsequent testing can reveal their presence.

ENOX2 proteins—Reveal origin of cancer

Furthermore, circulating ENOX2 proteins have been detected in sera of patients representing all major forms of cancer including blood cancers. All ENOX2 proteins share a common antigenic determinant recognized by an engineered recombinant antibody expressed in bacteria and derived from an ENOX2-specific monoclonal antibody-producing hybridoma cell line (Hostetler et al, 2009).

Figure 1

ENOX enzymes have two distinct activities which effect cell growth and cell division.

ENOX2 proteins are shed into the bloodstream.

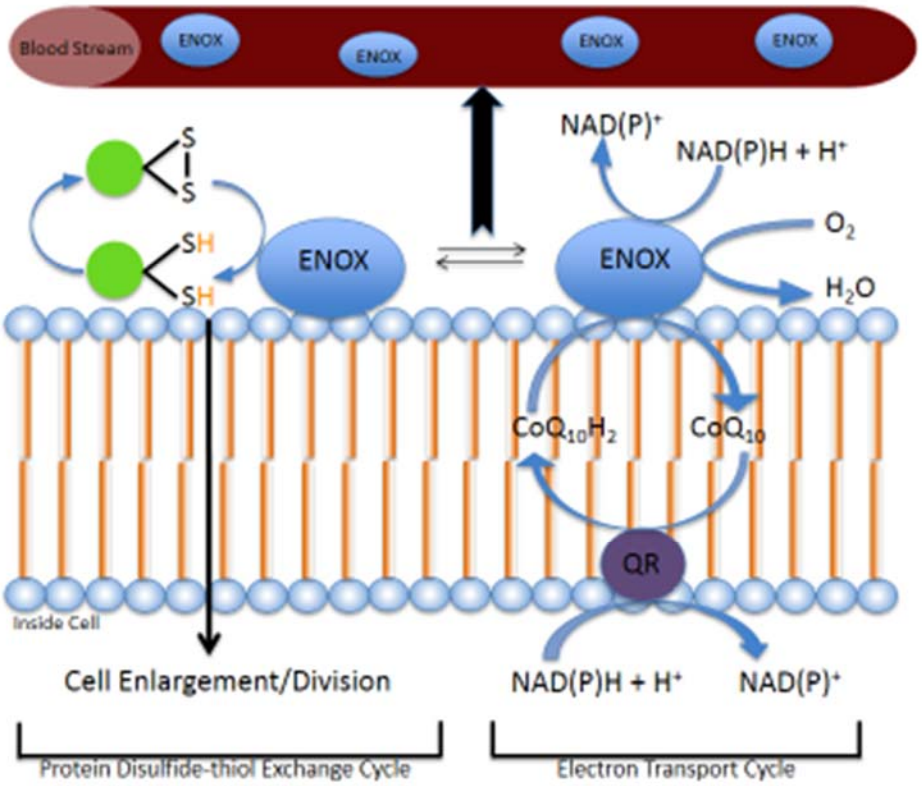
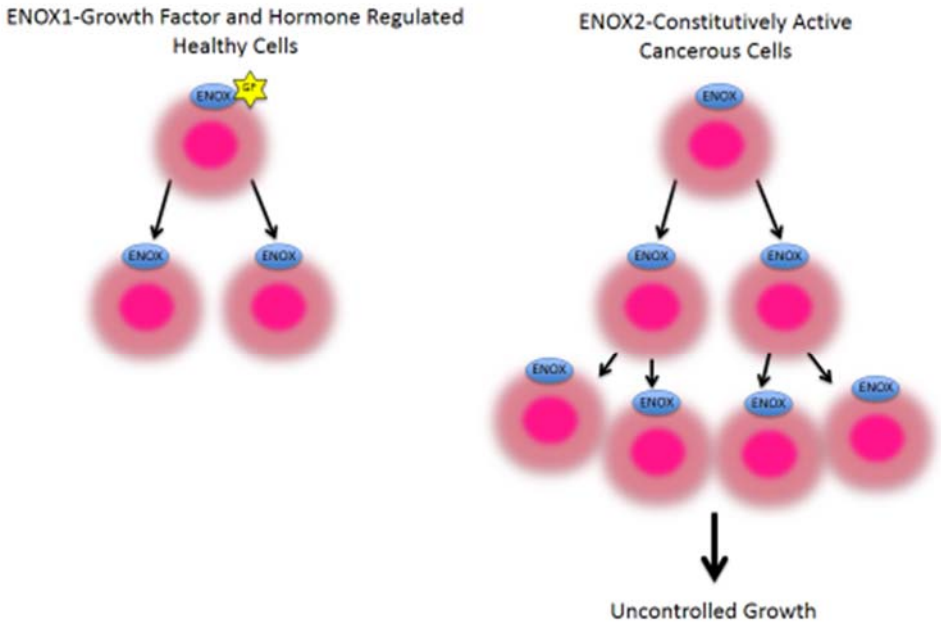


Figure 2

ENOX1 is responsive to growth factors and hormones and functions as an enzyme associated with normal cell enlargement and division in healthy cells. ENOX2, on the other hand, is a variant form of ENOX1 that is not regulated by growth factors and hormones so it remains constitutively active. This leads to uncontrolled cell growth characteristic of cancer cells.



ONCOblot®
Early Cancer Detection
www.oncoblotlabs.com

Section Two: How ONCOblot® Reveals Tissue of Origin

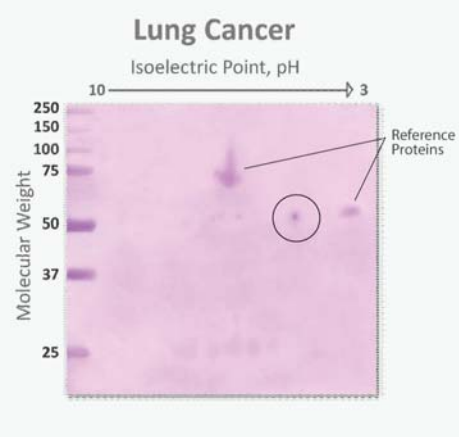
The test that reveals ENOX2 is the ONCOblot® Tissue of Origin Cancer Test. The ONCOblot® Test has two major working parts:

Part One
Two dimensional gel electrophoresis to determine molecular weight and isoelectric point

Part Two
A proprietary ENOX2-specific recombinant antibody engineered and reproducibly expressed in bacteria is used to probe western blots after transfer of proteins from gels to a nitrocellulose membrane. Presence of any ENOX2 proteins are recognized as dark spots on a light background.

These two parts are known collectively as 2-D gel electrophoresis with western blot detection.

How does the test reveal ENOX2?
The test results reveal a pattern of ENOX2 proteins in a patient's sera. This serves not only as a molecular indicator of cancer presence but precisely identifies the tissue of origin of the cancer in this innovative diagnostic test. If cancer is present, the western blot will reveal one or more spots.



The gel separation yields two dimensions in the x-y plane: (X) the isoelectric point with separation based on protein charge as a result of isoelectric focusing and (Y) the molecular weight with separation based on size of protein as a result of SDS-PAGE electrophoresis. The gel with separated proteins is then taken through a transfer process that moves the proteins from the gel onto a nitrocellulose membrane. This effectively places the separate proteins on a support matrix that can be probed with the ENOX2 specific antibody. In the figure, the circled spot indicates lung cancer.

How large is the current database?
The current database contains more than 1000 entries from clinically confirmed cancer patients representing more than 26 different cancers and 23 different tissues of origin.

How are individual western blots validated to minimize errors?
Human sera contain two proteins, alpha-fetuin and serotransferrin with a six amino acid sequence immunologically cross-reactive with a similar sequence in all ENOX2 transcript variants located adjacent (Morré and Morré, 2013). The reference proteins must be present within a correct range of molecular weight and isoelectric point for a valid ONCOblot®.

Are there controls to avoid false positives?
Yes. For each 2-D gel-western blot analysis of each 6 gel set, a non-cancer serum is included. There must be no unaccounted for extraneous spots and the reference proteins must be within range and of the correct intensity.

Are there controls to avoid false negatives?
Yes. For each of the non-cancer serum gels that accompany an amount of recombinant ENOX2 near the limit of detection of the assay are added. Detection of the recombinant ENOX2 ensures a level of sensitivity sufficient to avoid false negatives.

How much sera are required for the test?
The test requires 20 ul (0.02 cc) of sera but one tiger top tube is routinely collected for convenience and to provide sufficient sera for repetitions if necessary.

How much time is required to carry out the test?
Normally 15 business days is allowed from receipt of sample to delivery of the report.

What is the cost of the test?
The cost of the test is $850.

Why is the test that expensive?
The test is very labor intensive with volume restricted to a relatively small number of tests generated by each skilled laboratory director working full time, the large number of controls required to minimize false positives and false negatives, and the complicated and labor intensive interpretative skills required for blot analysis.

Why not simply perform an ELISA?
There is evidence that most, if not all, cancer patients generate IgM autoantibodies to ENOX2 early in cancer progression (Figure 12.7 from Morré and Morré, 2013). The autoantibodies do not block the growth of the cancer but render antibody recognition in an ELISA impossible. For the ENOX2 antibody to react with ENOX2 in patient sera, the ENOX2 protein must be separated from the bound autoantibody by isoelectric focusing. Hence, the antibody works very

effectively on western blots but not at all in an ELISA format. A similar limitation for ENOX2-based immunotherapy is imposed by the naturally-occurring autoantibodies.

Is it possible to detect cancer without determining the organ site?
Yes. In about 4% of the ONCOblot® analyses, particularly in early stage cancer and in late stage cancer with successful therapy, a common, fully processed form of ENOX2 is found in the sera to the exclusion of organ site-specific transcript variants as found in later stages of cancer progression. Generic ENOX2 indicates cancer even in the absence of clinical symptoms. Most often the cancer is in its earliest stages most susceptible to early intervention.

Does the test distinguish distant metastasis from localized disease?
No. The metastatic cells continue to produce the ENOX2 transcript variants typical of the tissue of origin.

Is the test useful in staging cancer?
No. At very low levels of ENOX2 in the serum, the spot size is proportional to the logarithm of spot diameter (see Figure 3). However, with clinically diagnosed disease, the spot size reaches a maximum independent of cancer stage.

What about ENOX2 proteins not in the database?
ENOX2 proteins not in the database thus far, like generic ENOX2, indicate cancer presence but of undetermined tissue origin. They might represent rare cancers not in the database or transcript variants from yet undefined atypical regions in a tissue of origin already represented in the database.

Does ONCOblot® detect blood cancers?
Yes. ONCOblot® detects all forms of blood cancers thus far investigated including various forms of leukemia, various forms of lymphoma, and myelomas. However, as these cancers share a common tissue of origin, blood, the ONCOblot® Tissue of Origin Cancer Test does not distinguish among the different blood cancers.

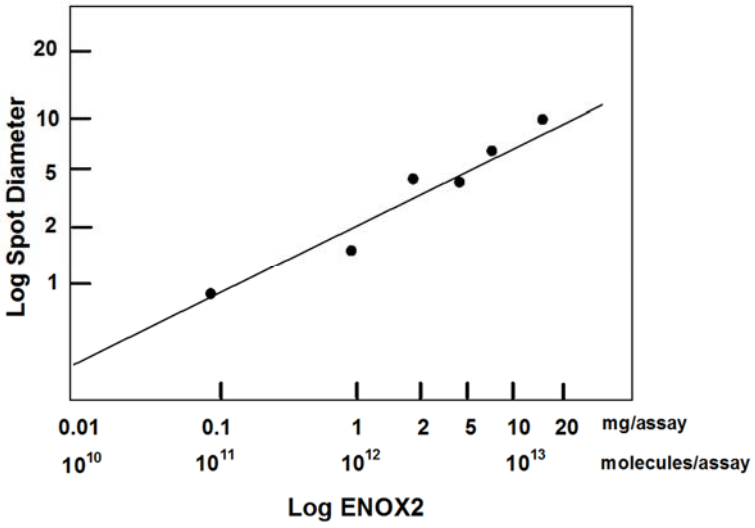
Is the test paid by Medicare or insurance providers?
No. Mor-NuCo representatives are currently working with Medicare to develop appropriate Medicare Codes.

Is the test approved by the FDA?
The test is CLIA certified and CAP accredited. It meets FDA guidelines as a Laboratory-Developed Test and a 510(k) submission to the FDA is in progress.

Figure 3

These data establish the lower limits of detection of ENOX2 proteins on ONCOblots® to be less than 100 femptomoles of ENOX2 equivalent to 60 billion ENOX2 molecules in 20 microliters of serum. We estimate that 2 million cancer cells in the body, equivalent to a 0.8 mm diameter cancer, would be reproducibly detected by the method (Hanau et al., 2014).

On logarithmic scales, ENOX2 spot diameter is proportional to the number of ENOX2 molecules present in the serum up to 10 trillion molecules per blot, after which, the spot size reaches a plateau that limits the usefulness of the test to stage advanced cancers once clinical symptoms appear.



References

1. Morré DJ, Morré DM. ECTO-NOX Proteins. New York, Springer; 2013:507.

2. Bruno M. Brightman AO, Lawrence J, Werderitsh D, Morré DM, Morré DJ. Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. Biochem J 1992, 284:625-628.

3. Jiang Z, Gorenstein NM, Morré DM, Morré DJ. Molecular cloning and characterization of a candidate human growth-related and time-keeping constitutive hydroquinone (NADH) oxidase. Biochemistry 2008, 47:14028-14038.

4. Tang X, Tian Z, Chueh PJ, Chen S, Morré DM, Morré DJ. Alternative splicing as the basis for specific localization of tNOX (ENOX2), a unique hydroquinone (NADH) oxidase to the cancer cell surface. Biochemistry 2007, 46:12,337-12,346.

5. Tang X, Morré DJ, Morré DM. Antisense experiments demonstrate an exon 4 minus splice variant mRNA as the basis for expression of tNOX (ENOX2), a cancer-specific cell surface protein. Oncol Res 2008, 26:557-567.

6. Hostetler B, Weston N, Kim C, Morré DM, Morré DJ. Cancer site-specific isoforms of ENOX2 (tNOX), a cancer-specific cell surface oxidase. Clin Proteomics 2009, 5:46-51.

7. Hanau C, Morré DJ, Morré DM. Cancer prevention trial of a synergistic mixture of green tea concentrate plus *Capsicum* (Capsol-T) in a random population of subjects ages 40-84. Clin Proteomics 2014, in press

8. Morré DJ, Morré DM. Early detection: an opportunity for cancer prevention through early intervention, In Cancer Prevention, Edited by Georgakilas AG, Rijeka: In Tech: 2012:389-402.

9. ENOX2 (ecto-NOX disulfide-thiol exchanger2). Peer Reviewed and Published in Atlas of Genetics and Cytogenetics in Oncology and Haematology.
http://atlasgeneticsoncology.org/Genes/ENOX2ID40134chXq26.html

Section Three: Data

800 ONCOblots®

Background: To test for accuracy, The ONCOblot® Test was performed on serum samples from over 800 different patients all with clinically diagnosed cancers. The type of cancer was blinded. Samples were obtained as follows: 1) under contract to Greater Baltimore Medical Center. 2) through the Early Cancer Detection Network of the National Cancer Institute 3) from the Goshen Cancer Center, Goshen, IN 4) from Mishiwaka Cancer Clinic, Mishiwaka, IN 5) from Saint Elizabeth Hospital, Lafayette, IN, and assorted participating clinics and physicians and local volunteers.

Summary: The ONCOblot® Test revealed the presence of ENOX2 in 99.3% of the samples from patients with confirmed cancers. Of those testing positive for ENOX2, the organ site of the cancer was determined correctly in 96% of the cases.

Data Collected:

Table 1. Molecular weight and isoelectric point ranges (99 percentile) from ONCOblot database of ENOX2 transcript variants for tissues of origin encountered in study. From Hanau et al., 2014 updated as of July 1, 2013. See references 6, 7 and 8 for additional information and examples of ONCOblots® representing cancers of different tissues of origin.

Cancer	n	Molecular weight	Isoelectric point, pH
Bladder	9	63-66 and 42-48 kDa	4.2-5.8 and 4.1-4.8
Blood Cell	80	38-48 kDa	3.6-4.5
Breast	355	64-69 kDa	4.2-4.9
Cervical	18	90-100 kDa	4.2-5.4
Colorectal*	88	80-96, 50-60 and 33-46 kDa	4.5-5.3, 4.2-5.1 and 3.8-5.2
Melanoma	27	37-41 kDa	4.6-5.3
Mesothelioma	10	59-62 and 38-41 kDa	3.8-4.1 and 4.4-4.6
Non-small Cell Lung	75	53-56 kDa	4.7-5.3
Ovarian	78	72-90 and 37-47 kDa	3.7-5.0 and 3.7-5.0
Papillary Thyroid	5	56-66 and 37-44 kDa	4.5-4.9 and 3.2-3.7
Prostate	79	71-88 kDa	5.1-6.5
Squamous Cell	10	54-68 kDa	5.0-5.4
Uterine	9	64-69 and 36-48 kDa	4.2-4.9 and 4.5-5.6

*All three transcript variants or, frequently, only one or the other of the two higher molecular weight species, may be present.

****

ENOX2

ENOX2 gene included in Atlas of Genetics and Cytogenetics in Oncology and Haematology.

http://atlasgeneticsoncology.org/Genes/ENOX2ID40134chXq26.html