The ONCOblot® Test
A single blood test that detects 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 serum marker ENOX2:

- Coincides with the onset of malignancy (uncontrolled invasive growth).
- Located on the cancer cell surface and shed into circulation.
- Present in all forms of cancer thus far examined as a family of protein transcript variants where each variant is specific to a particular tissue of origin.
- More than 26 cancer types identified.

The ONCOblot® Test is:

- Sensitive. Estimated to detect as few as 2 million cancer cells (approximately 2 mm solid tumor).
- Direct molecular visualization. Based on two dimensional gel electrophoresis with detection using an ENOX2-specific recombinant antibody to identify ENOX2 proteins.

DETECTS 26 FORMS OF CANCER IN A SINGLE SERUM TEST
NON-INVASIVE - CANCER SPECIFIC - INDICATES CANCER PRESENCE AND TISSUE OF ORIGIN

CLIA Certified and CAP Accredited
Meets FDA guidelines as a Laboratory Developed Test

Please visit our website for more information:
www.oncoblotlabs.com
Section One: Why ENOX2 is a cancer marker

Summary

The ENOX2 gene
The human ENOX2 gene is located on the X chromosome. It is expressed only in malignant cells and tissues. The transcription products (mRNA) of the ENOX2 gene are differentially spliced within various tissues giving rise to multiple tissue-specific ENOX2 transcript variants.

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 tissue-specific ENOX2 proteins in a patient's sera not only serve as molecular indicators of cancer presence but also serve to indicate the tissue of origin of the cancer.

The ENOX Proteins

ENOX—A family of enzymes
Ecto-Nicotinamide Dinucleotide Oxidase Disulfide-Thiol Exchanger (ENOX) proteins are a family of enzymes that possess two distinct activities (Morré and Morré, 2013). They alternate between protein disulfide-thiol exchange and a 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 exchange 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 Plasma Membrane Electron Transport (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 cells express a cell-surface ENOX protein designated as ENOX1. ENOX1 is extremely resistant to inhibition (only one specific ENOX1 inhibitor is known).

ENOX1 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 needed for homeostasis and normal cell function while ENOX2 is detected 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 possess the potential for uncontrolled, malignant (cancerous) cell growth. ENOX2 activities are inhibited by most cancer chemotherapeutic agents, 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 X chromosome. 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. 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 by cancer cells.

ENOX2—Splicing makes all the difference
Alternative splicing during transcription of the ENOX2 gene into mRNA 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 primary origin of the cancer. Each tissue-specific ENOX2 protein expression pattern 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 translational initiation and protein 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-specific expression of ENOX2 proteins.

ENOX2 proteins—Released into the bloodstream
ENOX2 proteins are not permanently bonded to 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 epitope recognized by a recombinant antibody derived from an ENOX2-specific monoclonal antibody-producing hybridoma cell line (Hostetler et al, 2009).

**Cancers in the database as of October 2015 (26):**
Bladder, Breast, Cervical, Colorectal, Endometrial, Esophageal, Gastric, Hepatocellular, Kidney, Leukemia, Non-Small cell, Lung Small cell, Lymphoma, Melanoma, Mesothelioma, Multiple Myeloma, Myeloma, Ovarian, Pancreatic, Prostate, Sarcoma, Squamous Cell, Follicular, Uterine, Papillary, Testicular Germ Cell
ENOX enzymes have two distinct activities which affects cell growth and cell division.

ENOX2 proteins are shed into the bloodstream.
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 not regulated by growth factors and hormones so it remains constitutively active. This leads to uncontrolled cell growth characteristic of cancer cells.
Section Two: How ONCOblot® Reveals Tissue of Origin

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

Part One
Two dimensional gel electrophoresis to separate blood serum proteins by molecular weight and isoelectric point

Part Two
A proprietary ENOX2-specific recombinant antibody 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 identifies the tissue of origin of the cancer. If cancer is present, the western blot will reveal one or more ENOX2 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 the presence of an ENOX2 protein produced by lung cancer.
How large is the current database?
The current database (as of October 2015) contains more than 1500 entries from clinically confirmed cancer patients representing more than 26 different cancers and 20 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 (Morré and Morré, 2013). The reference proteins must be present within a correct range of molecular weights and isoelectric points for a valid ONCOblot®.

Are there controls to avoid false positives?
Yes. For each 2-D gel-western blot analysis, a non-cancer serum sample is included.

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

How much serum is required for the test?
The test requires 150 ul (0.15 cc) of sera but one tiger top tube (approximately 2 to 4 ml) 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 and a large number of controls are required to minimize false positives and false negatives.

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.

Does the test distinguish distant metastasis from localized disease?
No. The metastatic cells continue to produce the ENOX2 transcript variants typical of the primary tissue of origin.
Is the test useful in staging cancer?
No. Detection of ENOX2 by the ONCOblot test is quite sensitive (see Figure 3). However, the concentration of ENOX2 in blood serum is not necessarily indicative of cancer stage.

What about ENOX2 proteins not in the database?
ENOX2 proteins not in the database thus far, 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 blood cells share a common progenitor (hematopoietic stem cells), the ONCOblot® Tissue of Origin Cancer Test does not distinguish among different blood cancers.

Is the test covered by Medicare or insurance providers?
No. ONCOblot Labs 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.
These data establish the lower limits of detection of ENOX2 proteins on ONCOblots® to be approximately 100 femtomoles of ENOX2 equivalent to 120 billion ENOX2 molecules. We estimate that 2 million cancer cells in the body, equivalent to a 2 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


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.

Average Molecular Weight (MW) and Isoelectric Point (pl) of ENOX2 Proteins Produced by Select Cancers

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Molecular weight, MW</th>
<th>Isoelectric point, pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prot. 1</td>
<td>Prot. 2</td>
</tr>
<tr>
<td>Cervical</td>
<td>94</td>
<td>40.5</td>
</tr>
<tr>
<td>Ovarian</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Prostate</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Non-small cell lung</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Small cell lung</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Blood cell</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

ENOX2: Reference the following publication regarding ENOX2.

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

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