Estimation of Lower Limit of Detection of ENOX2 in Serum

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MorNuCo, Inc. continues its monthly report for participating physicians and health professionals in order to answer common questions relating to the ONCOblot® Tissue of Origin (Cancer) Test.

Based upon detection of purified, recombinant protein, the lower limit of detection of ENOX2 for the ONCOblot® test is <200 femtomoles per assay or a concentration of 1.3 nM of an ENOX2 protein within human serum. This steady-state concentration of ENOX2 within an average adult is estimated to be produced by approximately 2 million cancer cells, which is equivalent to a solid tumor 1.2 mm in diameter. By comparison, while estimates vary, 7 mm diameter breast cancers were detected approximately 50% of the time by mammography whereas tumors larger than 32 mm were detected 100% of the time (1). For comparison, tumors that are 7 mm and 32 mm in diameter are calculated to contain 350 million and 33 billion cells, respectively.

**Determination of the lower limit of ENOX2 protein detection**

When proteins are separated by two-dimensional (2-D) gel electrophoresis and detected by immunoblot, visualized proteins appear as small circles or ovals 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. The complete amino acid sequence of a full-length 72 kDa form of ENOX2 is available from GenBank under Accession No. AF207881. Various amounts of this recombinant ENOX2 protein were then assayed by ONCOblot®. The log of the resulting ENOX2 spot diameter was then plotted against the log of the amount of ENOX2 protein assayed (Fig. 1) and a strong linear correlation \( r^2 = 0.95 \) was found among these 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 Figure 1, correlates to the detection of 170 femtomoles \( (1.0 \times 10^{11} \text{ molecules}) \) of ENOX2 per assay. This value is in agreement with the previously reported lower limit of ENOX2 protein detection by ONCOblot® of approximately 100 femtomoles of ENOX2 per assay (2). By comparison, the largest ENOX2 spots produced by the analysis of human sera from late-stage cancer patients are approximately 3 mm in diameter, equivalent to \( 3.0 \times 10^{13} \text{ molecules per assay} \) or 300 times more ENOX2 protein than the lower limit of detection.
Estimation of the size of a solid tumor at the lower limit of detection

Previously, ENOX2 proteins within sera from 25 Stage 0 and 25 Stage I cancer patients were detected by ONCOblot® (3). The average concentration of ENOX2 within these sera samples was determined to be approximately 990 femtomoles (6.0 X 10^{11} molecules) per 150 μL assay, by comparison to the standard curve (Fig. 1). Although ENOX2 concentration within blood serum is not a predictive measure of tumor size, this finding can be used to estimate the smallest tumor that can be detected by the ONCOblot test by assuming that ENOX2 production is proportional to tumor size during early stages of disease and tumors possess a uniform blood supply. By definition, Stage 0 and Stage I solid tumors are typically less than 20 mm in diameter (4). If each of these Stage 0 and Stage I tumors were the maximum size of 20 mm in diameter, the lower limit of detection of 170 femtomoles of ENOX2 per assay would then be predicted to be produced by a 3.3 mm cancer. However, if the average Stage 0 or State I tumor was a more common 5 mm 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). These findings are consistent with a previous estimate of the minimum solid tumor size detected by ONCOblot® of 0.8 mm in diameter (2).

If 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 tumor cell. Although the average size of mammalian cells varies according to cell type, if the average diameter of a cancer cell is estimated to be 10 μm, then a 1.2 mm diameter cancer is calculated to contain approximately 2 million (2 X 10^6) cells.

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\text{\# tumor cells} = \frac{V_{tumor}}{V_{cell}} = \frac{\frac{4}{3} \pi D^3}{\frac{4}{3} \pi d^3} = \left(\frac{D}{d}\right)^3 = \left(\frac{D}{d}\right)^3\text{ Eq. 1}
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Summary

Based on the lower limits of detection of recombinant ENOX2, the lower limit of detection of the ONCOblot® test is approximately 170 femtomoles of ENOX2 protein (1.0 X 10^{11} molecules) in 150 μL of serum. This concentration of ENOX2 is predicted to be produced by solid tumors approximately 1.2 mm in diameter.

References