Technical standards and guidelines: Prenatal screening for open neural tube defects

This new section on "Prenatal Screening for Open Neural Tube Defects," together with the new section on "Prenatal Screening for Down Syndrome," replaces the previous Section H of the American College of Medical Genetics Standards and Guidelines for Clinical Genetics Laboratories*

Linda A. Bradley, PhD, Chair[1], Glenn E. Palomaki, BS[2], and Geraldine A. McDowell, PhD[3], ONTD Working Group, ACMG Laboratory Quality Assurance Committee

Key Words: *open neural tube defects, maternal serum screening, prenatal diagnosis, genetic screening*

Disclaimer: *These standards and guidelines are designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to these standards and guidelines does not necessarily ensure a successful medical outcome. These standards and guidelines should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical molecular geneticist should apply his or her own professional judgment to the specific clinical circumstances presented by the individual patient or specimen. It may be prudent, however, to document in the laboratory record the rationale for any significant deviation from these standards and guidelines.*

This specific technical standards and guidelines statement is intended to augment the current general American College of Medical Genetics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories and to address validation guidelines specific to second trimester maternal serum screening. Individual laboratories are responsible for meeting the CLIA/CAP quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency, and quality control measures.

ONTD2 BACKGROUND ON NEURAL TUBE DEFECTS

ONTD2.1 OMIM NUMBERS. 182940, Spina bifida; 206500 Anencephaly

ONTD2.2 BRIEF CLINICAL DESCRIPTION. Neural tube defects (NTDs) result when the embryonic neural tube fails to close properly by approximately 4 (four) weeks' gestation. The clinical consequences of NTDs are dependent on the site and severity of the defect. Failed closure of the anterior neural tube (anencephaly) is lethal and usually results in miscarriage, stillbirth, or early death. Defects lower along the neural tube may be open or have a thin covering membrane (about 80% of cases) or may be closed. Clinical effects of open spina bifida (OSB) cover a wide spectrum that can include paralysis, hydrocephalus, and incontinence. A second trimester maternal serum screening program can aid in the identification of open neural tube defects (ONTD): OSB and anencephaly.[1]

ONTD2.3 MODE OF INHERITANCE. Most cases of NTDs are sporadic and inheritance most likely is multifactorial. Kindreds have been described that suggest autosomal or X-linked recessive inheritance. NTDs also can be associated with specific single gene disorders and chromosome disorders. Environmental factors that increase the risk that a fetus will be affected with an NTD include exposure to anticonvulsant medications (e.g., valproate) and inadequate maternal folate intake. Preconceptional folic acid supplementation/fortification can reduce the incidence of NTDs up to 80%, dependent on dose.[2]

ONTD2.4 LABORATORY DIRECTOR. Although the prenatal screening laboratory utilizes clinical chemistry methods such as enzyme immunoassays, the role of the laboratory extends beyond the performance of the tests because the results require a unique kind of interpretation. This interpretation puts the results of the test into the appropriate context of a priori risks as determined by race, gestational age, and family history. The laboratory director is often called upon to provide consultation regarding these risks and options for further action. To address these unique requirements, the laboratory director should meet the standards set out in Section B3 of the ACMG Guidelines. When prenatal screening for ONTDs is performed in a clinical chemistry laboratory in which the director does not meet these standards, the laboratory should have a demonstrated relationship with an individual who does meet the standards set out in Section B3 and who is available in a timely

From [1]Clinigene Laboratories, Hauppauge, New York, [2]Foundation for Blood Research, Scarborough, Maine; and [3]LabCorp of America, Research Triangle Park, North Carolina.

G.E. Palomaki's present address is Women and Infants Hospital, Providence, Rhode Island.

American College of Medical Genetics, 9650 Rockville Pike, Bethesda, MD 20914-3998.

**Find ACMG standards and guidelines for clinical genetics at http://www.acmg.net.*

Go to www.geneticsinmedicine.org for a printable copy of this document

Approved by the Board of Directors October 26, 2004

fashion to aid in interpretation and provide consultation when requested.

ONTD2.5 SCREENING VERSUS DIAGNOSTIC TESTING. Prenatal testing for ONTDs by measurement of alpha-fetoprotein (AFP) is considered a screening test when performed in maternal serum and a diagnostic test when performed in amniotic fluid. The distinction between a screening and a diagnostic test is important because the goals and expectations for sensitivity, specificity, costs, and acceptable level of invasiveness differ. Maternal serum AFP screening results are not diagnostic of any condition. Rather, the screening process identifies pregnancies that are at sufficient risk for ONTDs to warrant genetic counseling and the offer of additional testing such as ultrasound and amniocentesis. The detection and false-positive rates of this screening test will be a function of several factors, including assay precision, the gestational age when the sample is obtained, the method of gestational age assignment, and the AFP cut-off level used to determine "screen positive" results. The measurements of AFP and acetylcholinesterase (AChE) in amniotic fluid, in combination with ultrasound, are diagnostic for fetal ONTDs.[3]

ONTD3 SECOND TRIMESTER MATERNAL SERUM SCREENING FOR OPEN NEURAL TUBE DEFECTS

Prenatal screening for ONTDs is best implemented in the context of a comprehensive program that coordinates preanalytic, analytic, and postanalytic components of the process.

ONTD3.1 PATIENT AND PROVIDER INFORMATION.

ONTD3.1.1 Patient information. Laboratories should either provide educational materials (e.g., brochures, videotape) for use by patients in consultation with their providers or, at a minimum, provide information about where such materials can be obtained. Many laboratories and professional organizations (e.g., ACOG, National Society of Genetic Counselors, regional genetics groups) have produced, and in some cases formally evaluated, materials that are in effective formats, at appropriate reading levels, and available in multiple languages. These materials provide general information about the disorder, test performance, patient rights, eligibility, test interpretation, treatment options, costs, risks and benefits of testing, and what to expect if the screening test is positive.

ONTD3.1.2 Informational materials for health care providers. Laboratories should supply providers with informational materials that include the following:

ONTD3.1.2 (**a**) Detailed information about the sampling process and how samples should be labeled and transported to the laboratory;

ONTD3.1.2 (**b**) Samples of test requisitions that must accompany samples to provide information needed for identification and accurate test interpretation;

ONTD3.1.2 (**c**) General information on testing, such as laboratory turn-around time and whether results will be phoned/faxed or mailed;

ONTD3.1.2 (**d**) Information about expectations for test performance (sensitivity, specificity, and failure rate) and reporting formats.

ONTD3.1.3 Informed consent. Patients should be informed about the benefits and limitations of prenatal screening before testing. It is the duty of the health care professional, not the laboratory, to inform and obtain consent for testing, but the laboratory may be required to document such consent (e.g., in New York state). It is the laboratory's responsibility to provide sufficient information about prenatal screening to the health care provider to ensure that an appropriate specimen is obtained and to facilitate educating the patient and obtaining informed consent.

ONTD3.1.4 Requisition forms and intake information. For the most reliable interpretation, laboratories should have a mechanism to collect pretest clinical information that includes:

ONTD3.1.4 (**a**) Basic required demographic information (see Sections C2.4 and C3);

ONTD3.1.4 (**b**) Gestational age (see also Section ONTD3.5.3.3);

ONTD3.1.4 (**c**) Maternal weight;

ONTD3.1.4 (**d**) Maternal race (at least Caucasian and African American);

ONTD 3.1.4 (**e**) Presence of maternal insulin dependent diabetes mellitus prior to pregnancy;

ONTD3.1.4 (**f**) Number of fetuses;

ONTD3.1.4 (**g**) Previous screening in the current pregnancy (e.g., initial or repeat serum sample);

ONTD3.1.4 (**h**) Family history of NTDs.

The laboratory may choose to contact health care providers if critical patient information does not accompany the specimen. If the laboratory does not obtain this information, the written report should indicate that the information is missing and what information, if any, was used in the interpretation. In some cases, including information on the report about the potential impact of the missing information may be warranted (e.g., maternal weight, race). In other cases, full interpretation may not be possible (e.g., no gestational age).

ONTD3.2 SPECIMEN COLLECTION AND TRANSPORTATION.

ONTD3.2.1 Specimen collection. Blood samples should be collected using standard phlebotomy techniques. The labora-

tory should specify what samples are acceptable (e.g., whole blood, serum separator tube, spun serum separator tube). Specimen containers should be labeled with the patient's name and draw date.

ONTD3.2.2 Specimen transportation. Acceptable specimen handling from collection site to the laboratory should be specified, including packaging, mode of transportation (e.g., courier, United States mail, overnight transport), and temperature range (AFP is very stable and samples can be shipped at ambient temperature).

ONTD3.3 SPECIMEN PROCESSING AND STORAGE.

ONTD3.3.1 Criteria for sample rejection. Variables that can affect the acceptability of a sample for ONTD screening or a specific AFP assay protocol should be established by the laboratory and may include both clinical (e.g., gestational age out of range) and sample-related characteristics (e.g., inappropriate sample type, insufficient quantity, gross hemolysis). See also Sections C2.4 through C2.6.

ONTD3.3.2 Specimen processing. Protocols should be designed to avoid contamination, tampering, or substitution. Handling samples must be in accordance with OSHA guidelines, with the express understanding that any human fluids may harbor infectious agents.

ONTD3.3.3 Sample stability. AFP can be reliably determined in sera stored at 4° to 8°C for several days and at −20°C for years. See also Sections C2.5 through C2.6.

ONTD3.3.4 Establishment of laboratory policies regarding specimen retention. See Sections C2.7 through C2.8 for more information.

ONTD3.4 ASSAY METHODOLOGIES.

ONTD3.4.1 Detailed analytic procedures. Guidance on developing assay protocols is available. See C5, C6, and C8.3 (Validation).

ONTD3.4.2 Methodology and reagents. In the United States, the FDA licenses AFP kits as an aid in the diagnosis of ONTDs. As Class III devices, these kits are approved to reliably measure AFP in second trimester maternal serum samples and amniotic fluid. Available kits include immunometric and radioimmunoassay methods, all capable of measuring AFP reliably in the range of values important for ONTD screening (25 to 150 IU/mL).

ONTD3.4.3 Standards and calibration procedures. AFP standards can be calibrated in either mass units (ng/mL) or International Units (IU/mL). Each AFP kit manufacturer provides a factor for converting mass units into International Units. Conversion factors should be considered manufacturer-specific. Commercially available AFP kits provide calibrators and specific calibration protocols. Laboratories utilizing assays developed in their own laboratory ("home brew") or modifying AFP kit assay protocols, are responsible for determining calibration protocols and validating performance.

ONTD3.4.4 Preparation, characterization, and use of controls.

ONTD3.4.4.1 Assay controls. In-house pooled controls, commercially available controls, or controls received in kits serve as checks on reagents and technical performance. Advantages of in-house pooled controls include a sample matrix that more closely resembles patient samples, AFP levels set for ONTD clinical action points, and control lots prepared with long expiration dating to aid in assessment of kit master reagent lot changes and long-term assay drift. An alternative for long-term monitoring is commercial controls bought in sufficient quantity to last a year or more.

ONTD3.4.4.2 Repeat assay controls. Repeat assay controls (RACs) are also helpful for monitoring performance variability. To assess short-term performance, unfrozen patient samples are chosen at random from recent assays and reassayed to monitor intra- and interassay precision. Because serum AFP levels are stable when frozen and thawed, reassaying stored patient samples from the time period when the current median values were established can help to identify long-term drift and determine if reference data need to be updated.

ONTD3.4.4.3 Concentration of AFP. Each batch assay should contain at least two quality control samples that fall at clinical action points (three controls may be required to comply with some licensure requirements). For example, low controls could be targeted at serum AFP values falling at 0.5 multiples of the median (MoM) for 16 weeks of gestation. Normal or midrange controls could be targeted near the 16-week median (1.0 MoM), and high controls at a value near commonly used ONTD cut-off levels (2.0 to 2.5 MoM).

ONTD3.4.4.4 Characterization of control materials. After preparation and aliquoting, performance ranges for in-house pooled controls can be set using standard clinical laboratory quality control approaches. Controls received with AFP kits have an acceptable target range specified by the manufacturers but laboratories may wish to establish an in-house range. This information is used to accept or reject individual control results or a whole assay so care should be taken to set appropriate ranges and avoid unnecessary result rejection.

ONTD3.4.5 Quality control.

ONTD3.4.5.1 Type and frequency of quality control assessments. Standard approaches used in the clinical laboratory are appropriate for internal quality control (QC) of AFP assays, including the type and frequency of assessments.

ONTD3.4.5.2 Measures of repeatability both within and between batch assays. As part of the initial method validation, the laboratory should demonstrate that intra- and interassay variation reported by the manufacturer can be reproduced.

ONTD3.4.5.3 Routine equipment calibration and preventive maintenance. Standard approaches to routine equipment calibration and preventive maintenance used in the clinical laboratory are appropriate. In many cases, calibration and maintenance protocols are set by the product/equipment manufacturer (see Section ONTD3.4.3).

ONTD3.5 ASSAY RESULTS.

ONTD3.5.1 Converting assay results to multiples of the median. In order for AFP measurements to be interpreted, each result in mass or International Units must first be converted to a MoM for a given gestational age. The resulting MoM levels can then be adjusted for other factors, such as maternal weight.

ONTD3.5.2 Normative data. It has been established that values obtained from different lots from the same manufacturer or from different manufacturers may demonstrate systematic bias. Therefore, it is essential that each laboratory establish its own normative data or, at a minimum, demonstrate that data obtained from another source are appropriate for its screened population.

ONTD3.5.2.1 Source of medians. Maternal serum AFP levels increase by a constant percentage per week (approximately 10% to 15%) in the second trimester.

ONTD3.5.2.2 Sample size. Package insert (commercial) medians should not be used, even for a short time. Several methods exist that can be utilized to establish reliable medians.

ONTD3.5.2.3 Computing medians. Ideally, 100 samples for each gestational week from 15 through 20 would be used to calculate median values. Because AFP is stable, it is possible to use stored frozen specimens collected over several years. It is not necessary that all samples used be from unaffected singleton pregnancies. Using regression analysis (see ONTD3.5.2.4) allows use of fewer samples (e.g., 300 over the 15- to 20-week period) to establish reasonable medians.[4]

ONTD3.5.2.4 Expected change in medians by gestation. "Smoothing" the observed median values by weighted log-linear regression analysis (logarithms of medians regressed vs. gestational age in days or completed weeks, weighted by the square root of the number of observations in each category) provides reliable and accurate medians. This method also allows median values to be extrapolated for weeks in which little data are available. Using median values that are specific to each day of gestation will further improve screening performance.

ONTD3.5.3 Variables that have significant impact on calculation of the MoM level.

ONTD3.5.3.1 Time of testing. The optimal time for ONTD screening is 16 to 18 weeks but screening is acceptable between 15.0 and 20.9 weeks.[1] Screening performance is significantly decreased in the 14th week of gestation. Under special circumstances, laboratories may accept samples later than 20 weeks gestation with the understanding that clinical options may be limited.

ONTD3.5.3.2 Gestational age. Gestational age may be expressed in completed weeks (15 weeks and 5 days is 15 completed weeks). Expressing results in rounded weeks (15 weeks and 5 days is 16 weeks) is not recommended. Screening performance is improved by expressing gestational age as weeks and days or decimal weeks (15 weeks and 5 days is 15.7 weeks).

ONTD3.5.3.3 Dating method. The most common method for determining gestational age is dating by the first day of the last menstrual period (LMP). Although LMP dating is sufficiently accurate for ONTD screening, gestational age estimation based on ultrasound measurements is more accurate and its use improves both the sensitivity and specificity of screening. Ultrasound measurement of crown-rump length (CRL) in early pregnancy provides an accurate estimate of gestational age. In the second trimester, ultrasound dating based on multiple measurements (composite) is accurate to within 10 days. Composite ultrasound dating is preferable to LMP dating. For ONTD screening, ultrasound dating based on biparietal diameter (BPD) measurement alone is recommended for pregnancies at 14 weeks gestation or later, or when the LMP is uncertain or discrepant with physical examination. BPD dating rules out anencephaly and because OSB cases have, on average, BPD measurements equal to a 2-week younger fetus, dates based on BPD can significantly improve detection of open spina bifida.[5,6]

ONTD3.5.3.4 Incorporating dating method. The method of determining gestational age can be taken into account when providing interpretations in two ways. First, separate medians can be calculated for those pregnancies dated by LMP and those dated by ultrasound measurements. Secondly, separate Gaussian population parameters can be utilized in determining risk (see Sections ONTD3.5.5.2 through ONTD3.5.5.3).

ONTD3.5.4 Factors that may be used to adjust the MoM levels. The following are interpretive refinements based on patient demographics and other pregnancy-related information that are less critical than taking gestational age into account, but will improve screening performance by optimizing the interpretation. Currently, most laboratories take the following factors into account.

ONTD3.5.4.1 Maternal weight. AFP levels are, on average, higher in lighter weight women and lower in heavier weight women. Adjusting AFP values for maternal weight improves ONTD screening performance and is recommended.[7–9] Laboratories should only utilize published weight adjustment formulas for a short time until in-house data are collected and new laboratory-specific formulas derived.

ONTD3.5.4.2 Maternal race. Correction for maternal race is recommended, because AFP levels in Black/African Ameri-

can women are about 10% to 15% higher than in Caucasian women.[7,10] If sufficient data are available, the preferred adjustment method is to calculate a separate set of medians for each of the groups. If too few observations are available in one of the groups, a correction factor can be applied to the MoM when screening those pregnancies.

ONTD3.5.4.3 Maternal insulin-dependent diabetes mellitus. Correction for maternal insulin-dependent diabetes mellitus (IDDM) is recommended. AFP levels have been reported to be 10% to 20% lower in women with IDDM before and during pregnancy.[10] Most programs take this into account by using an adjustment factor for AFP MoM levels in IDDM women. There is no consensus on whether this correction should be applied to gestational diabetic women.

ONTD3.5.4.4 Use of multiple correction factors to calculate the MoM. Calculating MoM results for a 200-pound, Black/African American woman with IDDM would require at least three adjustments to the AFP MoM.[7,10] Although data are sparse, programs can make the assumption that the effects are independent. Although most data are derived from studies of mainly Caucasian women, the assumption is usually made that a similar effect will be seen in Black/African American women.

ONTD3.5.5 Prenatal screening software for computing and reporting patient-specific ONTD risk. Laboratories should be able to compute risks for ONTD and OSB even though they may not be routinely reported.[11] Some licensing agencies require routine reporting of the risk, and it is important to be able to provide this information to health care professionals for clinical counseling and pregnancy management. The use of specialized software applications is generally considered a necessity for ONTD screening, due to the complex nature of calculating and interpreting the results, the need for patient-specific interpretive reports, and because of the large number of samples processed.

ONTD3.5.5.1 ONTD risks. Patient-specific risks are generated by complex mathematical algorithms that are integral to prenatal screening. Software to perform these calculations can be obtained commercially or developed in-house. Software must be verified before routine clinical use.

ONTD3.5.5.2 Risk algorithm. The commonly used algorithm to assign a patient-specific risk utilizes the MoM results (adjusted for variables such as weight and race as discussed above) to calculate a likelihood ratio based on the overlapping Gaussian distributions defined by the affected and unaffected distribution parameters. The population or prior risk for OSB or anencephaly is then multiplied by the corresponding likelihood ratio to generate the patient-specific risks.[11]

ONTD3.5.5.3 Population parameters. Risk algorithms utilize published or in-house population parameters for AFP, expressed as log means and log standard deviations of AFP distributions in unaffected pregnancies and in pregnancies affected with OSB or anencephaly. Population parameters for each of these disorders can vary based on the gestational age at the time of testing and gestational dating method (see ONTD3.5.7.1 and ONTD3.5.7.7).

ONTD3.5.5.4 Combinations of factors. There is no formal consensus on which adjustments to the result or prior risk to include, specifically how to include them or how inclusion influences screening performance. These decisions are left to the laboratory director.

ONTD3.5.6 Selection of screening cut-off levels. Definition of screen Positive and negative results: for ONTD screening, determination of "screen positive" results most commonly relies on AFP MoM cut-off levels. Few laboratories choose the ONTD risk estimate as the screening variable.

ONTD3.5.6.1 AFP cut-off levels. Reasonable AFP cut-off levels for ONTD screening range between 2.0 and 2.5 MoM. Screen positive results are defined as those with an AFP MoM greater than or equal to the cut-off level.

ONTD3.5.6.2 Prior risk. The prevalence, or background risks may be higher or lower in certain populations. In such cases, the screening cut-off level may be modified in order to keep the risk of an ONTD at the cut-off roughly equal (isorisk screening). Either approach is acceptable, but the laboratory should understand the trade-offs associated with the different approaches. For example, Black/African American pregnancies are at about half the risk of ONTDs. Screening programs that use a 2.0 MoM cut-off level in Caucasian pregnancies may use 2.5 MoM for Black/African American pregnancies because the risk is similar for the two groups at these specified levels. Another approach is to keep the cut-off level the same in order to maintain the detection rate for ONTDs at the cut-off equivalent (isodetection screening). In the previous example, modifying the cut-off level in Black/African American pregnancies would reduce both detection and false-positive rates. Keeping the cut-off level at 2.0 MoM would maintain both rates.

ONTD3.5.7 Modifying factors are important to understand. These may be genetic or environmental. Several of these have been discussed already.

ONTD3.5.7.1 Time of testing. The optimal time for ONTD screening is 16 to 18 weeks.[1] Before that time, the unaffected and OSB populations have more overlap. This has the effect of decreasing screening performance, particularly in the 14th week of gestation. After 18 weeks of gestation, performance is relatively unchanged but fewer clinical options may be available.

ONTD3.5.7.2 Maternal weight. Recent large studies have shown that obese women have a 2- to 3-fold increase risk of ONTDs.[12,13]

ONTD3.5.7.3 Maternal race. The birth prevalence of ONTDs in the Black/African American population is approximately half of the prevalence in Caucasians.[14]

ONTD3.5.7.4 Maternal IDDM. The birth prevalence of ONTDs is increased several-fold in women with IDDM.[15,16]

ONTD3.5.7.5 Multiple gestation. Twin pregnancies are known to have AFP levels approximately two times the levels in singleton pregnancies. Distribution parameters for serum AFP measurements have been defined for unaffected twin pregnancies and for twin pregnancies in which one or both of the fetuses are affected with OSB or anencephaly.[17] The birth prevalence of ONTDs is also higher in twin pregnancies, with one report estimating that an ONTD is 2.28 times more likely than in a singleton pregnancy. Maternal serum AFP cut-off levels for recommendation of amniocentesis should be determined separately for twin pregnancies (generally, cut-off levels fall between 4.0–5.0 MoM). Other factors, such as the acceptability of performing amniocentesis on twin gestations and the difficult options should one affected fetus be identified, should also be considered.

ONTD3.5.7.6 Repeat testing. Obtaining a second specimen for repeat testing may be beneficial when the initial specimen has a slightly elevated AFP (relative to the screening cut-off level) and the gestational age is early enough to allow time for appropriate follow-up. Most laboratories do not combine results for the two tests but rather use a simple set of rules for interpreting results of repeat testing. Methods for combining the results of the two tests have been published,[11] and the laboratory should develop a policy that indicates the method to be used. If the pregnancy was originally misdated and the new gestational age is too early for interpretation (e.g., 14 weeks or earlier), the subsequent sample can be considered to be the first usable sample.

ONTD3.5.7.7 Family history. Family history of ONTDs may increase the prior risk, depending on the number of affected relatives and the degree of relatedness. Family history can be incorporated into the ONTD risk estimate using published algorithms. The laboratory may include a recommendation for genetic counseling if a positive family history is identified.

ONTD3.5.7.8 Method of assigning gestational age. Assigning gestational age based on ultrasound measurements has the effect of "tightening up" the distribution of AFP measurements in both unaffected and affected pregnancies. For this reason, separate sets of distribution parameters can be used for LMP and ultrasound dated pregnancies. Ultrasound dating based on biparietal diameter (BPD) measurement reduces the false-positive rate and significantly increases the detection rate for open spina bifida. BPD dating rules out anencephaly and (because spina bifida cases have, on average, BPD measurements equal to a 2-week younger fetus) gestational age estimates based on BPD improve detection on OSB at any screening cut-off level. Other ultrasound measurements (e.g., crown rump length or multiple second trimester measurements) can reliably date the pregnancy but do not have this unique advantage of BPD dating.

ONTD3.5.7.9 Geographic location. In the United States, the overall incidence of ONTD is between 0.5 and 1.5 per 1000 births. The frequency is lowest in the Western United States and increases to the South and East, with the highest rates occurring in the Southern Appalachian states.[14]

ONTD3.5.8 Technical limitations of the methodology for the intended use. Because of the impact of as little as 10% systematic change in assay results on detection and screen positive rates, laboratories need to select AFP kits for maternal serum screening to meet performance requirements that are more stringent than for other intended uses. Kits need to be both precise and relatively accurate (different kits need not give identical values on the same sample provided in-house reference data are established using the same kit). Because AFP MoM values are calculated using reference data collected in the past, it is also important that kits/reagents are stable over a long period of time, and that lot-to-lot variability is minimized.

ONTD3.5.9 Long-term assessment of variability and performance.

ONTD3.5.9.1 Assay controls. In-house pooled controls (or commercial products obtained in sufficient quantity to last a year or more) and repeat assay controls (RACs) are valuable for monitoring long-term assay drift and lot-to-lot variability (see ONTD3.4.4.1 through ONTD3.4.4.4).

ONTD3.5.9.2 Normative data review. Median values should be reviewed at regular intervals by the laboratory and recalculated at least annually. Medians should be recalculated if there is a shift in AFP values $>$ 10%, or a shift between 5% and 10% that is consistent over time (whether due to observed assay drift or reagent lot change). Shifts in AFP values can be monitored by computing the overall median MoM level (see ONTD3.5.9.4). Observations from the past should be used to calculate medians only if epidemiological monitoring shows the median MoM has been stable for the time period over which the median values are calculated. Alternative methods of revising medians may be necessary if a significant shift has been observed (see ONTD3.5.9.4).

ONTD3.5.9.3 Evaluating medians with new AFP reagent lots. Between 25 and 50 patient samples and current controls can be assayed on the old and new kit/reagent lot and the relationship between the two examined using techniques of regression analysis and method comparison. This relationship can then be applied to the existing medians to derive new medians that can be used until sufficient data are available for the proper optimum analysis (see ONTD3.5.2.2).

ONTD3.5.9.4 Epidemiological monitoring. In order to monitor assay and program performance and to identify possible areas of concern, screening programs must perform epidemiological monitoring.[4] Such monitoring, at a minimum, should include the following:

ONTD3.5.9.4 (a) Periodic computation (monthly or weekly, depending on numbers of samples processed) of the median AFP MoM, determination of the statistical significance of any deviation from 1.00, and documentation of any necessary corrective action;

ONTD3.5.9.4 (b) Periodic computation of the rate of initial positive results and comparison of that rate to expected published rates, after taking into account variables such as the screening cut-off level used and the proportion of pregnancies dated by ultrasound.

ONTD3.5.10 Long-term monitoring. In recent years, stricter privacy and confidentiality policies and in some cases laws have made it much more difficult to collect pregnancy outcome information and even information regarding follow-up of medical procedures (such as ultrasound and amniocentesis) performed subsequent to positive screens. If possible, laboratories should collect pregnancy outcome information on the women with initial screen positive results. This information might include the proportion of pregnancies reclassified as screen negative, the diagnostic testing uptake rate, and the number of affected pregnancies identified either in the second trimester or at term. For those laboratories that have sufficient resources, complete pregnancy follow-up is recommended and will allow the determination of the ONTD detection rates.

An alternative approach acknowledged by some regulatory agencies is to utilize epidemiological monitoring data as performance measures.[18] This can be accomplished by comparing published rates with in-house statistics for such measurements as the median MoM for each analyte, population parameters (log means and log standard deviations) and the initial and revised positive rates (see DS3.5.7.4).

ONTD3.5.11 Failure rates for different sample types. Few published data exist from screening programs but AFP kit manufacturers do provide information about acceptable sample types (e.g., serum vs. plasma), minimum sample volumes required, and conditions that can affect assay performance (e.g., hemolysis). Because laboratories should have specific sample processing protocols, many identifiable problem samples will be rejected before testing. Other testing "failures," such as results falling below the lower limit of sensitivity of the assay due to a sampling error, are likely to be uncommon and can be resolved by repeat testing. In rare cases, a second sample may be requested.

ONTD3.5.12 External proficiency testing. Each laboratory must participate in one or more of the external proficiency testing programs that evaluate assay performance for serum AFP in the second trimester. If this is not possible, the laboratory must utilize other recommended external proficiency testing methods, such as scheduled interlaboratory comparisons or split sample analysis with another laboratory (see Section C4).

ONTD3.6 ANALYTIC VALIDITY. The analytic validity of a genetic test defines its ability to accurately and reliably measure a specific analyte that is to be used clinically. Each laboratory is responsible for in-house validation of a test methodology but information in the package insert of an FDA-approved kit or from the literature can be used as supporting evidence.

ONTD3.6.1 Analytic sensitivity. Analytic sensitivity is commonly defined in the laboratory as an assay's lower limit of detection. However, in the context of maternal serum screening, we are defining analytic sensitivity as the proportion of samples with elevated AFP levels that are correctly classified as being high. Analytic sensitivity can be determined using samples with high consensus AFP levels (e.g., selected proficiency testing samples).

ONTD3.6.2 Analytic specificity. Analytic specificity is commonly defined in the laboratory as the extent to which a method measures an analyte exclusively and does not cross-react with other related compounds. However, in the context of maternal serum screening, we are defining analytic specificity as the proportion of samples with low or normal AFP levels that are correctly classified as being low or normal.

ONTD3.6.3 Confirmatory testing. Samples with results less than the lower limit of sensitivity of the assay must be repeated to rule out a technical error (e.g., sampling probe error) and to confirm the value. Results above the highest standard on the calibration curve must be repeated at dilution. Samples with a high coefficient of variation between replicate values (generally $> 10\%$) are routinely repeated by some laboratories to confirm the value. Many laboratories also repeat samples with AFP MoM levels greater than the specified ONTD cut-off level. Confirmatory testing is a consideration for the majority of laboratories utilizing methodologies that test in singlicate, in order to minimize analytic errors.

ONTD3.6.4 Assay robustness. Assay robustness measures how resistant testing is to small changes in preanalytic and analytic variables. In an attempt to define performance requirements and minimize possible impact on assay performance (e.g., analytic validity, reproducibility, failure rates), laboratories should consider the effects of common variables, such as sample type, sample handling (e.g., transit time or conditions), sample quality, reagent lots, or minor changes in assay conditions (e.g., timing or temperature).

ONTD3.7 CLINICAL VALIDITY. The clinical validity of a test defines its ability to accurately and reliably identify the clinical phenotype of interest. In this instance, it is the ability of maternal

serum AFP measurements to identify pregnancies in which the fetus is affected with an ONTD.

ONTD3.7.1 Clinical sensitivity. Clinical sensitivity is the proportion of pregnancies with an ONTD that have a positive test result.

ONTD3.7.2 Clinical specificity. Clinical specificity is the proportion of unaffected pregnancies identified by the test as being negative.

ONTD3.7.3 Screening performance. Clinical sensitivity (detection rate) and clinical specificity (1 − false-positive rate) will depend on many factors, including the MoM cut-off level chosen, the method of estimating gestational age, and the gestational age at screening.

ONTD3.7.3.1. Using either common ONTD screening cut-off level (2.0 or 2.5 MoM), the detection rate for anencephaly is expected to be 95% or greater. The detection rate for OSB is expected to be between 75% and 90% using a 2.0 MoM screening cut-off level, and between 65% and 80% using a 2.5 MoM cut-off level. False-positive rates are expected to be between 2% and 5% and between 1% and 3%, respectively.[4] These rates are influenced by many factors (e.g., gestational age at screening, dating method) that have been discussed earlier.

ONTD3.7.3.2. As the proportion of ultrasound dating increases (especially the use of biparietal diameter), the detection rate moves toward the upper end of the above ranges and the false positive rate moves toward the lower end of these ranges.

ONTD3.7.4 Positive predictive value and negative predictive values. The positive predictive value and negative predictive value of AFP testing in the target population measure the ability of the test to give accurate clinical information.

ONTD3.7.4.1 Positive predictive value. The positive predictive value (PPV) is the proportion of positive test results that correctly identify a pregnancy with an ONTD [true positives / (true positives + false positives)]. The PPV can also be expressed as an odds ratio and is referred to as the odds of being affected given a positive result (OAPR). The PPV and OAPR are computed using the prevalence of OSB and anencephaly and the respective clinical sensitivity and specificity. In general, PPVs are between 0.5% and 1% (OAPR of 1:200 to 1:100). As an example, consider a screening cut-off level that is found to have a clinical sensitivity for OSB of 80% with a corresponding clinical specificity of 96%. The prevalence of OSB is 1:2000 pregnancies. The PPV is approximately equal to [clinical sensitivity / (1 − clinical specificity) × prevalence]. For this example, that would be 80/4:2000 or 20:2000 or 1:100 or about 1%. Standard epidemiology texts provide a more complete discussion.

ONTD3.7.4.2 Negative predictive value. The negative predictive value (NPV) is the proportion of negative tests that correctly identify an unaffected pregnancy [true negatives / (true negatives + false negatives)]. Because the prevalence of the conditions being screening for (OSB and anencephaly) is low, the NPV is generally not computed.

ONTD3.7.5 Factors that impact testing. It is important to understand any genetic, environmental or other modifying factors that impact testing.

ONTD3.7.5.1 Race and family history. Race and family history can impact testing by changing the prior risk for ONTDs. See Sections ONTD3.5.4.2, ONTD3.5.6.2, ONTD3.5.7.2, and ONTD3.5.7.6.

ONTD3.7.5.2 Twin pregnancies. Twin pregnancies are at increased prior risk for ONTD. See ONTD3.5.6.2 and ONTD3.5.7.5.

ONTD3.7.5.3 Folate deficiency contributes to the ONTD risk . Preconceptional supplementation/fortification with folic acid reduces the incidence of ONTDs by up to 80%, dependent on dose and NTD prevalence.[2] Based on preliminary findings, most geographic locations in North America can expect a reduction of approximately 20% to 30% from fortification.[19] Incorporating data regarding folate fortification or supplementation into patient specific risk calculations is left to the discretion of the laboratory director.

ONTD3.8 RESULT REPORTING.

ONTD3.8.1 Recommended report formats. See Section C8.5.7 (Validation). Final reports of test results must be clear to a nongeneticist professional and must include the following:

ONTD3.8.1 (a) Patient's name, date of birth, and other unique identifiers;

ONTD3.8.1 (b) Name of referring physician/health center to receive the report;

ONTD3.8.1 (c) The test that is ordered;

ONTD3.8.1 (d) Type of specimen;

ONTD3.8.1 (e) Date when sample was obtained;

ONTD3.8.1 (f) Laboratory accession number(s);

ONTD3.8.1 (g) Demographic and pregnancy-related information used in the interpretation (e.g., gestational age, method of dating, maternal race, maternal weight);

ONTD3.8.1 (h) Analytic results in both mass units (e.g., ng/mL) and interpretive units (i.e., MoM) upon which all adjustments/corrections have been performed;

ONTD3.8.1 (i) Clinical interpretation, including whether the result is screen positive or screen negative, the AFP MoM level, and the MoM cut-off level. Reporting a patient-specific risk is optional, but laboratories should be able to perform this computation when requested

ONTD3.8.2 Reporting screen negative results. Written reports of screen negative results can be transmitted to the referring physician by US mail, courier, electronic transmission, or overnight carrier.

ONTD3.8.3 Reporting screen positive results. Screen positive results should be promptly transmitted to the referring health care provider, usually by phone and/or fax, within one working day after completion of the test. Appropriate recommendations for follow-up of screen positive results may include the following:

ONTD3.8.3 (**a**) If not already done, a dating ultrasound to confirm gestational age and fetal viability and to rule out twins, anencephaly, and other fetal defects;

ONTD3.8.3 (**b**) Repeat sampling when appropriate;

ONTD3.8.3 (**c**) Genetic counseling;

ONTD3.8.3 (**d**) Referral for targeted ultrasound examination;

ONTD3.8.3 (**e**) Amniocentesis with amniotic fluid AFP and acetylcholinesterase testing.

ONTD3.8.4 Reclassification of positive results. Laboratories should be aware of the potential problems associated with reclassifying screen positive women as screen negative. There is a chance of reclassifying a true positive as negative. Reclassification usually occurs when an LMP-dated pregnancy is subsequently dated by ultrasound, and the difference between the LMP and ultrasound dating exceeds a set standard. As guidance to laboratories, reclassification should not be considered unless the revised estimate of gestational age is different by at least a week. Many laboratories use 10 days (e.g., 1.5 weeks) as the standard. One way to help avoid reclassification and improve overall screening performance is to encourage physicians to base their initial gestational age estimates on ultrasound measurements.

ONTD3.8.5 Conditions other than ONTDs associated with positive screening results.

ONTD3.8.5 (**a**) Pregnancy unaffected with ONTD; however, women with unexplained elevated AFP levels have an increased incidence of poor pregnancy outcome (e.g., low birth weight, preterm labor).

ONTD3.8.5 (**b**) Underestimated gestational dating.

ONTD3.8.5 (**c**) Multiple gestation.

ONTD3.8.5 (**d**) Recent fetal demise.

ONTD3.8.5 (**e**) Other abnormalities (e.g., omphalocele, gastroschisis).[20]

ONTD3.9 CLINICAL UTILITY. Clinical utility addresses the risks and benefits associated with testing in routine clinical practice. This information may be requested by those ordering or paying for testing and the laboratory should be able to provide a reasonably accurate summary of the published literature. When clear gaps in knowledge exist, the laboratory may want to collect data in such a way as to answer these questions in the future. The following is a list of selected clinical utility topics that often are applicable:

ONTD3.9 (a) Knowing whether pilot trials have been undertaken and, if so, what the results were;

ONTD3.9 (b) Establishing or adopting quality assurance processes that monitor the effectiveness of the laboratory's ongoing testing activities;

ONTD3.9 (c) Understanding possible adverse health or psychosocial consequences of testing;

ONTD3.9 (d) What follow-up testing or interventions in persons with positive and negative test results might be reasonable;

ONTD3.9 (e) Understanding what is known about the financial costs and economic benefits of testing.

ONTD3.10 ETHICAL, LEGAL, AND SOCIAL IMPLICATIONS. The laboratory should be familiar with the ethical, legal, and social issues regarding genetic testing in general and those specifically applicable to maternal serum screening for ONTD. These may include informed consent, insurability, discrimination, labeling, confidentiality, obligations to disclose, and complex counseling issues. Legal issues such as patents, licensing, sample ownership and storage, proprietary testing, and reporting requirements should be carefully examined.

ONTD4 PRENATAL DIAGNOSTIC TESTING FOR OPEN NEURAL TUBE DEFECTS IN AMNIOTIC FLUID

ONTD4.1 PATIENT AND PROVIDER INFORMATION.

ONTD4.1.1 Patient information. Patient information concerning prenatal diagnostic testing is commonly provided as part of counseling before amniocentesis. Many prenatal diagnostic centers and professional organizations (e.g., ACOG, regional genetics groups) have produced and, in some cases, formally evaluated educational materials (e.g., brochures, videotapes) on amniocentesis and follow-up of positive screening test results that are in effective formats, at appropriate reading levels, and available in multiple languages.

ONTD4.1.2 Informational materials for health care providers. Laboratories should supply informational materials for providers that include the following:

ONTD4.1.2 (a) Detailed information about how samples should be collected, packaged, and transported to the laboratory;

ONTD4.1.2 (b) Samples of test requisitions that must accompany samples to provide information needed for accurate test interpretation;

ONTD4.1.2 (c) General information on testing, such as when reflex acetylcholinesterase (AChE) and fetal hemoglobin testing will be performed, turn-around times, and when results will be phoned/faxed;

ONTD4.1.2 (d) Information about reporting formats and expectations for amniotic fluid AFP and AChE test performance.

ONTD4.1.3 Informed consent. Information about diagnostic testing for ONTDs is included as part of general informed consent for amniocentesis. Collecting informed consent is not the responsibility of the laboratory but laboratories may want or need to document that the process has occurred.

ONTD4.1.4 Requisition forms and intake information. For the most accurate result interpretation, laboratories should have a mechanism to collect information that includes the following:

ONTD4.1.4 (a) Name and other unique identifying information;

ONTD4.1.4 (b) Sample collection date;

ONTD4.1.4 (c) Information to date the pregnancy, preferably by ultrasound measurement(s) (e.g., the date of the ultrasound and assigned gestational age on that day);

ONTD4.1.4 (d) Indication for testing (e.g., elevated serum AFP, abnormal ultrasound findings, family history of NTD, maternal anticonvulsant therapy);

ONTD4.1.4 (e) Name, address, and telephone/fax number of referring health care provider or prenatal diagnostic center;

ONTD4.1.4 (f) Sample type and condition (e.g., spun or unspun amniotic fluid, visibly bloodstained).

ONTD4.2 SPECIMEN COLLECTION AND TRANSPORTATION.

ONTD4.2.1 Specimen collection. Unspun amniotic fluid aliquots are usually received from the cytogenetics laboratory. Tubes should be labeled with the patient's name and draw date. Amniotic fluid color and presence of any visible blood should be noted; reddish or brown color may indicate the presence of maternal or fetal blood contamination.

ONTD4.2.2 Specimen transportation. Acceptable specimen handling from the cytogenetics laboratory should be specified, including acceptable aliquot tubes, mode of transportation (e.g., courier, US mail, overnight transport), and temperature. AFP in amniotic fluid is not as stable as in maternal serum. Consequently, amniotic fluid samples should not be held at room temperature any longer than necessary. Samples should be shipped on cool packs or kept frozen. Avoid exposure to high temperatures if possible. Next day delivery is advisable. AChE and pseudocholinesterase (PChE) is reasonably stable.

ONTD4.3 SPECIMEN PROCESSING AND STORAGE.

ONTD4.3.1 Criteria for sample rejection. Variables that can affect the acceptability of a sample for a specific amniotic fluid AFP testing protocol, or for AChE testing, should be established by the laboratory (e.g., too long in transit).

ONTD4.3.2 Specimen processing. Protocols should be designed to avoid contamination, tampering, or substitution. Handling samples must be in accordance with OSHA guidelines, with the express understanding that any human fluids may harbor infectious agents.

ONTD4.3.3 Sample stability. AFP and AChE can be reliably assayed in AF stored at 4° to 8°C for days and at −20°C for years.

ONTD4.3.4 Establishment of laboratory policies regarding specimen retention. See Sections C2.7 and C2.8 for more information.

ONTD4.4 ASSAY METHODOLOGIES.

ONTD4.4.1 Detailed analytic procedures. Guidance on developing assay protocols is available. See Sections C5, C6, and C8.3 (Validation).

ONTD4.4.2 Methodology and reagents.

ONTD4.4.2.1 Amniotic fluid AFP. See Section ONTD3.4.1 and ONTD3.4.2. Because amniotic fluid AFP levels are approximately 250 times higher than in maternal serum at 16 weeks, samples must be routinely diluted 1:50 to 1:200, depending on the methodology used.

ONTD4.4.2.2 Acetylcholinesterase. The polyacrylamide slab or disc gel electrophoresis method for identifying acetylcholinesterase (AChE) is a second diagnostic test used along with amniotic fluid AFP to detect ONTDs. AChE testing is indicated when the amniotic fluid AFP levels exceed a specific cut-off level, usually 2.0 MoM. Occasionally, AChE analysis also is indicated when there is an increased prior risk of an ONTD (e.g., elevated MSAFP, abnormal ultrasound, or family history), despite a normal amniotic fluid AFP level.

ONTD4.4.2.2 (a) AChE and pseudocholinesterase (PChE) are part of a group of isoenzymes, called cholinesterases, which can be separated on polyacrylamide gel electrophoresis and then incubated with a specific substrate, acetylthiocholine. The resulting white bands can be directly visualized and photographed. Alternatively, the gels can be stained and photographed or preserved.

ONTD4.4.2.2 (b) PChE is normally found in the fetal and maternal circulation, and is routinely visualized in all amniotic fluid samples as a single band near the application site on polyacrylamide gel electrophoresis. In the absence of an open defect, amniotic fluid contains only the nonspecific pseudocholinesterases.

ONTD4.4.2.2 (c) In pregnancies affected with an ONTD, AChE leaks into the amniotic fluid from the open neural tube or other open fetal defect (e.g., open ventral wall defect). The AChE appears on polyacrylamide gel electrophoresis as a distinctive band below the PChE band.

ONTD4.4.2.2 (d) Occasionally, a nonspecific cholinesterase band can appear in the AChE location on the gel (e.g., from maternal blood), and if not correctly identified, can lead to a false-positive test result. For this reason, identified AChE bands are routinely confirmed using a specific AChE inhibitor (i.e., BW282C51).

ONTD4.4.2.3 Fetal Hemoglobin/Kleihauer-Betke. A major cause of borderline amniotic fluid AFP elevations and/or false-positive AChE results is contamination of the sample with fetal blood. Fetal blood AFP levels are more than 100 times higher than amniotic fluid levels. False-positive AChE results occur in about 2% of visibly bloodstained samples compared to only about 0.2% of samples that are not visibly bloodstained. Testing for the presence of fetal hemoglobin is indicated for samples with elevated amniotic fluid AFP levels, visible red cell contamination, and some other indications (e.g., unexplained elevated maternal serum AFP, reddish colored amniotic fluid). Two approaches can be used to identify fetal blood staining:

ONTD4.4.2.3 (a) If sufficient red cells are recovered from an amniotic fluid specimen, the Kleihauer-Betke cytochemical staining method can be used to determine whether the red cells are maternal or fetal.

ONTD4.4.2.3 (b) In many cases, however, the amniotic fluid sample will have been centrifuged before referral to the AFP laboratory, and blood staining will not be evident. In such cases, methods such as radial immunodiffusion using an antibody to fetal hemoglobin can be utilized to detect soluble hemoglobin in the supernatant.[21]

ONTD4.4.2.3 (c) Kits are commercially available for both Kleihauer-Betke (e.g., Sigma Diagnostics Fetal Hemoglobin Kit) and soluble fetal hemoglobin (e.g., Helena Laboratories HbF Quiplate Kit) testing.

ONTD4.4.3 Standards and calibration procedures.

ONTD4.4.3.1 Amniotic fluid AFP. See Section ONTD3.4.3.

ONTD4.4.3.2 AChE. Not applicable; AChE is a nonquantitative method.

ONTD4.4.3.3 Fetal hemoglobin. Fetal hemoglobin standards (0.5%, 5% and 10%) for calculating the reference curve are commercially available.

ONTD4.4.4 Preparation, characterization, and use of controls

ONTD4.4.4.1 Amniotic fluid AFP. An in-house pooled amniotic fluid control should be prepared, aliquoted, and stored frozen at −20°C. This material should be run with each batch of AF samples as a control of the dilution process, whether the assay is performed manually or on-board an automated system. See also repeat assay controls, ONTD3.4.4.2.

ONTD4.4.4.2 AChE. Positive controls are AChE-positive or weak-positive samples that have been coded to protect patient confidentiality, aliquoted, and stored frozen at −20°C. Negative samples from recent assays can be used as negative controls.

ONTD4.4.4.3 Fetal Hemoglobin/Kleihauer-Betke. Positive and negative soluble fetal hemoglobin controls can be obtained from positive patient samples that have been coded to protect patient confidentiality, aliquoted, and stored frozen at −20°C, or by spiking amniotic fluid with fetal blood or fetal hemoglobin calibrator material. Adult and fetal control red cell smears for Kleihauer-Betke are prepared from adult whole blood and fetal cord blood. The slides are dried, fixed, and stored frozen.

ONTD4.4.5 Quality control.

ONTD4.4.5.1. Standard approaches used in the clinical laboratory are appropriate for internal QC/QA of amniotic fluid AFP, AChE, and fetal blood staining assays.

ONTD4.4.5.2. As part of the initial method validation, the laboratory should demonstrate that intra- and interassay variation reported by the AFP kit manufacturer can be reproduced.

ONTD4.4.5.3. Standard approaches to routine equipment calibration and preventive maintenance used in the clinical laboratory are appropriate. In many cases, calibration and maintenance protocols are set by the product/equipment manufacturer (see ONTD3.4.3).

ONTD4.5 ASSAY RESULTS.

ONTD4.5.1 Normative amniotic fluid AFP data. In order for amniotic fluid AFP measurements to be interpreted, they are first converted to MoM for a given gestational week. It has been established that amniotic fluid AFP values obtained by different kit manufacturers or different lots from the same manufacturer may demonstrate systematic bias. Therefore, it is essential that each laboratory establishes its own normative data or at least demonstrates that data obtained from another source are appropriate for its screened population.

ONTD4.5.1.1. Amniotic fluid AFP concentrations decrease 13% to 15% per week from 15 to 22 weeks of gestation. Package insert (commercial) medians should not be used, even for a short time.

ONTD4.5.1.2. Ideally, 100 samples for each gestational week from 15 through 20 would be used to calculate median values. Because AFP is stable, it is possible to use stored frozen specimens collected over several years. However, collecting the above number of amniotic fluid samples is difficult, particularly at the later gestational weeks, and fewer samples are likely to be available. Regression analysis allows use of fewer samples (e.g., 300 over the 15- to 20-week period) to establish reasonable medians.

ONTD4.5.1.3. "Smoothing" the observed median values by weighted log-linear regression analysis (logs of medians regressed vs. gestational age in completed weeks, weighted by the square root of the number of observations in each category) provides reliable and accurate medians, and allows median values to be extrapolated for later gestational weeks in which little data are available and for which the log-linear relationship continues to be valid.

ONTD4.5.1.4. The median amniotic fluid AFP values from 12 to 14 weeks do not follow this model. Formulae have been published for extrapolating medians for these earlier weeks based on the smoothed medians obtained for 15 to 20 weeks.[22]

ONTD4.5.2 Variables that impact results.

ONTD4.5.2.1 Time of testing. Amniotic fluid AFP results can be reported in MoM levels from 11 to 25 weeks of gestation. The optimal time for detection of ONTDs is 13 to 22 weeks of gestation. The accuracy of testing before 13 weeks is not well established and results should be reported with a warning that diagnostic performance is poorly defined.[23–25]

ONTD4.5.2.2 Gestational age. For calculation of amniotic fluid AFP MoM levels, gestational age is most commonly expressed in completed weeks (15 weeks and 5 days is 15 completed weeks).

ONTD4.5.2.3 Blood contamination. Because some elevations of amniotic fluid AFP and false-positive AChE results can be explained by fetal blood contamination, health care providers and cytogenetic laboratories should be encouraged to submit unspun amniotic fluid samples for AFP analysis in order to allow for the detection of fetal blood to be directly detected. Alternatively, reflex testing to rule out fetal blood contamination using an assay for fetal hemoglobin should be performed on samples with AFP elevations and/or positive AChE results, particularly if there is a weak-positive AChE or inconsistency of the pattern of results (e.g., elevated amniotic fluid AFP with negative AChE).

ONTD4.5.2.4 Fetal calf serum contamination. In rare cases, falsely positive AChE result may result from contamination of amniotic fluid with fetal calf serum (FCS).[26,27] FCS is a component of the culture medium used by cytogenetic laboratories for culturing fetal cells and has a high concentration of AChE. The usual source of FCS contamination of the amniotic fluid aliquot is a tube or pipette in the cytogenetics laboratory that has a residue of culture medium. Confirming FCS contamination is performed by counter-immunoelectrophoresis or electroimmunodiffusion utilizing an antibody to bovine serum albumin (a protein found in FCS).

ONTD4.5.3 Definition of positive and negative test results.

ONTD4.5.3.1 Amniotic fluid AFP. Abnormal amniotic fluid AFP results usually are defined as elevations of 2.0 MoM or greater. Amniotic fluid AFP results can be reliably interpreted between 13 to 25 weeks of gestation, provided the laboratory has sufficient data to establish medians for the later gestational ages. At a minimum, laboratories should be able to interpret results up to 22 weeks of gestation.

ONTD4.5.3.2 AChE. A negative test result is defined by the presence of a PChE band and no AChE band. A positive test is defined by the presence of a PChE band and a weak or strong positive AChE band. The test is considered uninterpretable if the PChE band is absent or very weak. This pattern may be observed in amniotic fluid samples that are diluted with fetal urine, obtained very early in pregnancy, or mishandled (e.g., exposed to heat in transit).

ONTD4.5.3.2 (a) AChE results can be reliably interpreted between 13 and 22 weeks of gestation. If the laboratory is able to interpret amniotic fluid AFP up to 25 weeks of gestation, ACHE results may be interpreted to 25 weeks as well.[23–25]

ONTD4.5.3.2 (b) While testing at 11 to 12 weeks has some predictive power, sensitivity for predicting open defects is not well defined, and the false-positive rate for AChE may be significantly higher at this time.[23–25] A limited interpretation, with appropriate cautions, can be provided for amniotic fluid samples at 23 weeks and later.

ONTD4.5.3.3 AChE densitometry. The relative densities of the AChE and PChE bands, expressed as the AChE/PChE ratio, are useful in distinguishing between ONTDs and open ventral wall defects (OVWDs).[28–30] Nearly all ONTDs have PChE and

AChE bands and AChE/PChE ratios > 0.13. Nearly all OVWDs have unusually heavy PChE bands and weak-positive AChE bands, and ratios of 0.13 or lower. Ratios do not help to identify false-positive AChE bands, found more commonly in early amniocentesis (11 to14 weeks) and in samples contaminated with fetal blood.

ONTD4.5.4 Technical limitations of the methodology for the intended use. Because amniotic fluid AFP MoM values are calculated using reference data collected in the past, it is important that the kits/reagents selected are stable over a long period of time and that lot-to-lot variability is minimized. It is also important to determine that automated systems can perform the necessary dilutions of amniotic fluid samples and achieve the precision and relative accuracy needed. This is especially important if samples are being run in singlicate.

ONTD4.5.5 Long-term assessment of variability and performance.

ONTD4.5.5.1 Amniotic fluid AFP assay controls. In-house amniotic fluid pooled dilution control and RACs are valuable for monitoring long-term assay drift and lot-to-lot variability (see ONTD3.4.4.1 through ONTD3.4.4.4).

ONTD4.5.5.2 Normative amniotic fluid AFP data review. Median values should be reviewed at regular intervals by the laboratory director, and recalculated at least annually. Medians should be recalculated if there is a significant shift in amniotic fluid AFP values (whether due to observed assay drift or reagent lot change). Observations from the past should be used only if epidemiological monitoring shows the median MoM has been stable (see ONTD3.5.9.4). Alternative methods of revising medians may be necessary if a significant shift has been observed (see ONTD3.5.9.3).

ONTD4.5.5.3 Evaluating medians with new AFP reagent lots. Comparison data for amniotic fluid AFP can be used applying the same procedure as for serum AFP (see ONTD3.5.9.3).

ONTD4.5.6 Epidemiological monitoring. As part of epidemiological monitoring, each laboratory should periodically (weekly or monthly, depending on the number of samples tested) compute the median amniotic fluid AFP MoM, determine the statistical significance of any deviation from 1.00, and document any necessary corrective action.

ONTD4.5.7 Failure rates. Failure rates for amniotic fluid AFP and AChE testing are low, probably 1 in 1000 samples or less. Testing failures are usually related to inadequate specimens, due to sample mishandling (e.g., heating), gross contamination with fetal blood, or a specimen that is not amniotic fluid (e.g., urine).

ONTD4.5.8 External proficiency testing. Each laboratory must participate in an external proficiency testing program(s) that evaluates assay performance for amniotic fluid AFP, AChE, and fetal hemoglobin, or must utilize other recommended external proficiency testing methods, (e.g., evaluation of adult and fetal control red cell smears prepared from adult whole blood and fetal cord blood on slides that are dried, fixed, and stored frozen; scheduled interlaboratory comparisons; or split sample analysis with another laboratory). See Section C4.

ONTD4.6 ANALYTIC VALIDITY. See ONTD3.6.

ONTD4.6.1 Analytic sensitivity. See ONTD3.6.1.

ONTD4.6.2 Analytic specificity. See ONTD3.6.2.

ONTD4.6.3 Confirmatory testing. See ONTD3.6.3.

ONTD4.6.4 Assay robustness. See ONTD3.6.4.

ONTD4.7 CLINICAL VALIDITY. See ONTD3.7.

ONTD4.7.1 Clinical sensitivity and specificity. See ONTD3.7.1.

ONTD4.7.1.1 Screening performance for amniotic fluid AFP alone. Using a cut-off level of 2.0 MoM, detection rates for OSB are expected to be 90% at 13 to 15 weeks, 96% at 16 to 18 weeks, and 99% at 19 to 21 weeks. Associated false-positive rates are expected to be about 1%, 2%, and 5%, respectively. The detection rate for anencephaly at this cut-off level is even higher.

ONTD4.7.1.2 Screening performance for amniotic fluid AFP and AChE. When AChE testing is performed on all amniotic fluid specimens that have an AFP level of 2.0 MoM or greater and are free of fetal blood contamination, the detection rate for OSB is 97% with a false-positive rate of < 0.1%. In the presence of visible blood contamination, the detection and false-positive rates for OSB are about 91% and 1.2%, respectively.[3] The detection rate for anencephaly using this algorithm is even higher.

ONTD4.8 RESULT REPORTING.

ONTD4.8.1 Recommended report formats. See Section C8.5.7 (Validation). Final reports of test results must be clear to a nongeneticist professional and must include the following:

ONTD4.8.1 (a) Patient's name, date of birth, and other identifying information;

ONTD4.8.1 (b) Name of referring health care provider/health center to receive the report;

ONTD4.8.1 (c) Type of specimen;

ONTD4.8.1 (d) Date when sample was obtained;

ONTD4.8.1 (e) Laboratory accession number(s) to uniquely identify the sample;

ONTD4.8.1 (f) Pregnancy-related information used in the interpretation (e.g., gestational age);

ONTD4.8.1 (g) Indication for testing/procedure;

ONTD4.8.1 (**h**) Analytic results in both mass units (e.g., KIU/mL or uG/mL) and interpretive units (i.e., MoM);

ONTD4.8.1 (**i**) Clinical interpretation including recommendations for further studies.

ONTD4.8.2 Negative test results. Negative results are usually not associated with reflexive testing, although there are situations (e.g., family history of NTD) in which AChE and/or fetal hemoglobin are routinely run even if amniotic fluid AFP results are not elevated. Written reports of negative results can be transmitted to the referring health care provider by US mail, courier, electronic transmission, or overnight carrier.

ONTD4.8.3 Positive test results. All positive test results (i.e., elevated amniotic fluid AFP and/or positive AChE) should be promptly transmitted to the referring health care provider, usually by phone and/or fax within one working day after completion of the test. Optimally, elevated amniotic fluid AFP results are not reported until all reflexive testing is completed. If preliminary amniotic fluid AFP results are reported, there should be a clear indication on the report that full interpretation is not possible until the results of reflexive testing are complete.

ONTD4.8.4 Other conditions associated with elevated amniotic fluid AFP and/or positive AChE.

ONTD4.8.4 (**a**) Normal variation.

ONTD4.8.4 (**b**) Open ventral wall defects. Gastroschisis is associated with very elevated amniotic fluid AFP; AChE is positive in over 95% of cases with A/P ratio < 0.13. Omphalocele can have normal to elevated amniotic fluid AFP; AChE is negative in over 95% of cases but A/P ratio is < 0.13 when present.[31,3]

ONTD4.8.4 (**c**) Congenital nephrosis. This is a rare condition associated with very elevated amniotic fluid AFP and negative AChE.[31]

ONTD4.8.4 (**d**) Fetal demise. AChE may be present and inhibits but there is usually also a smeared and streaked pattern with some prominent anodal bands.

ONTD4.8.4 (**e**) Cystic hygroma fluid. Inadvertent aspiration of hygroma fluid will result in very elevated amniotic fluid AFP level and positive AChE (may appear like anencephaly).

ONTD4.8.4 (**f**) Fetal blood contamination (see ONTD4.5.2.3).

ONTD4.8.4 (**g**) Maternal blood contamination; can occasionally result in a cholinesterase band in the AChE region, but this band will not be inhibited by BW284C51.

ONTD4.8.4 (**h**) Fetal calf serum contamination (see ONTD4.5.2.4).

ONTD4.8.5 Conditions associated with decreased or absent amniotic fluid AFP.

ONTD4.8.5 (**a**) Maternal urine. Performance of fern test will help to identify the presence of urine.

ONTD4.8.5 (**b**) Congenital absence of AFP.

ONTD4.9 CLINICAL UTILITY. See ONTD3.9.

ONTD4.10 ETHICAL, LEGAL, AND SOCIAL IMPLICATIONS. See ONTD3.10.

ONTD5 EXISTING GUIDELINES

American Academy of Pediatrics, Committee on Genetics: Alpha-Fetoprotein Screening. *Pediatrics* 1987;80:444–445.

American College of Obstetricians and Gynecologists: Alpha-Fetoprotein. ACOG Technical Bulletin 154. Washington, DC: ACOG, April 1991.

American Public Health Association: Maternal serum alpha-fetoprotein screening, 1990 Available at: http://www.apha.org/legislative/policy/policysearch/index.cfm?fuseaction=view&id=1213.

American Society of Human Genetics: Policy statement for maternal serum alpha-fetoprotein screening programs and quality control for laboratories performing maternal serum and amniotic fluid alpha-fetoprotein assays. *Am J Hum Genet*. 1987;40:75–82.

New York State Department of Health, Wadsworth Center, Laboratory Standards, Rev 2000 Available at: http://www.wadsworth.org/labcert/clep/Survey/standardseff12605.pdf Accessed April 30, 2005.

References

1. Wald NJ, Cuckle HS, Brock JH, Peto R, Polani PE, Woodford FP. Maternal serum alpha-fetoprotein measurement in antenatal screening for anencephaly and spina bifida in early pregnancy: Report of the U.K. Collaborative Study on Alpha-fetoprotein in relation to neural tube defects. *Lancet* 1977;1:1323–1332.
2. Wald NJ, Law MR, Morris JK, Wald DS. Quantifying the effect of folic acid. Lancet 2001;358:2069–2073. MRC Vitamin Study Research Group Prevention of neural tube defects: results of the Medical Research Council vitamin study. *Lancet* 1991; 338:131–137.
3. Wald N, Cuckle H, Nanchahal K. Amniotic fluid acetylcholinesterase measurement in the prenatal diagnosis of open neural tube defects: Second report of the collaborative acetylcholinesterase study. *Prenat Diagn* 1989;9:813–829.
4. Knight, Palomaki GE. Epidemiologic monitoring of prenatal screening for neural tube defects and Down syndrome. *Clin Lab Med* 2003;23:531–551.
5. Wald N, Cuckle H, Boreham J. Small biparietal diameter of fetuses with spina bifida: implications for antenatal screening. *Br J Obstet Gynaecol* 1980;87:219–221.
6. Wald NJ, Cuckle HS, Boreham J. Alpha-fetoprotein screening for open spina bifida: effect of routine biparietal diameter measurement to estimate gestational age. *Rev Epidem et Sante Publ* 1984;32:62–69.
7. Crandall BF, Lehbhez RB, Schroth PC, Matsumoto M. Alpha-fetoprotein concentrations in maternal serum: relation to race and body weight. *Clin Chem* 1983;29: 531–533.
8. Neveux LM, Palomaki GE, Larrivee DA, Knight GJ, Haddow JE. Refinements in managing maternal weight adjustment for interpreting prenatal screening results. *Prenat Diagn* 1996;16:1115–1119.
9. Johnson AM, Palomaki GE, Haddow JE. The effect of adjusting maternal serum alpha-fetoprotien levels for maternal weight in pregnancies with fetal open spina bifida: A United States collaborative study. *Am J Obstet Gynecol* 1990;163:9–11.
10. Wald NJ, Kennard A, Hackshaw A, McGuire A. Antenatal screening for Down's syndrome. *J Med Screen* 1997;4:181–246.

11. Estimating an individual's risk of having a fetus with open spina bifida and the value of repeat AFP testing. Report of the Fourth UK Collaborative Study on AFP in relation to neural tube defects. *J Epidem Comm Health* 1982;36:87–95.
12. Waller DK, Mills JL, Simpson JL Cunningham GC, Conley MR, Lassman MR et al. Are obese women at higher risk for producing malformed offspring? *Am J Obstet Gynecol* 1994;170:541–548.
13. Werler MM, Louik C, Shapiro S, Mitchell AA. Prepregnant weight in relation to risk of neural tube defects. *JAMA* 1996;275:1089–1092.
14. Greenberg F, James LM, Oakley GP Jr. Estimates of birth prevalence rates of spina bifida in the United States from computer-generated maps. *Am J Obstet Gynecol* 1983;145:570–573.
15. Kucera J. Rate and type of congenital anomalies among offspring of diabetic women. *J Reprod Med* 1971;7:73–82.
16. Mills J, Baker L, Goldman A. Malformations in infants of diabetic mothers occur before the seventh gestational week: Implications for treatment. *Diabetes* 1979;28:292–293.
17. Cuckle H, Wald N, Stevenson JD, May HM, Ferguson-Smith MA, Ward AM et al. Maternal serum alpha-fetoprotein screening for open neural tube defects in twin pregnancies. *Prenat Diagn* 1990;10:71–77.
18. NY State Standards and Guidelines. Available at: http://www.wadsworth.org/labcert/dep/survey/standards eff12605.pdf. Accessed April 30, 2005.
19. Williams LJ, Mai CT, Edmonds LD, Shaw GM, Kirby RS, Hobbs CA et al. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology* 2002;66:33–39.
20. Palomaki GE, Hill LE, Knight GJ, Haddow JE, Carpenter M. Second-trimester maternal serum alpha-fetoprotein levels in pregnancies associated with gastroschisis and omphalocele. *Obstet Gynecol* 1998:71:906–909.
21. Kellner LH, Weiss RR, Neuer M, Canick JA, Panizza DS, Pulkkinen A et al. Determination of fetal blood contamination of second trimester amniotic fluid by radial immunodiffusion using Helena Laboratories HbF Quiplate. *Clin Chem* 1994;40:1066.
22. Palomaki GE, Knight GJ, Haddow JE. Calculating amniotic fluid alpha-fetoprotein median values in the first trimester. *Prenat Diagn* 1993;13:887–889.
23. Burton BK, Nelson LH, Pettenati MJ. False-positive acetylcholinesterase with early amniocentesis. *Obstet Gynecol* 1989;74:607–610.
24. Crandall BF, Chua C. Detecting neural tube defects by amniocentesis between 11 and 15 weeks gestation. *Prenat Diagn* 1995;15:339–343.
25. Drugan A, Syner FN, Greb A, Evans MI. Amniotic fluid alpha-fetoprotein and acetylcholinesterase in early genetic amniocentesis. *Obstet Gynecol* 1988;72:35–38.
26. Barlow RD, Cuckle HS, Wald NJ. The identification of false-positive amniotic fluid acetylcholinesterase results due to fetal calf serum contamination. *Br J Obstet Gynaecol* 1984;91:986–988.
27. Brock DJH, Barlow RD, Wald NJ, Smith AD, Haddow JE, Goldfine C et al. Fetal calf serum as cause of false positive amniotic fluid acetylcholinesterase gel tests. *Lancet* 1982;2:1044.
28. Goldfine C, Miller WA, Haddow JE. Amniotic fluid gel cholinesterase density ratios in fetal open defects of the neural tube and ventral wall. *Br J Obstet Gynaecol* 1983;90:238–240.
29. Goldfine C, Haddow JE, Hudson GA, Miller WA. Densitometry as an aid in amniotic fluid gel acetylcholinesterase analysis. *Am J Obstet Gynecol* 1983;145:317–318.
30. Wald NJ, Barlow RD, Cuckle HS, Turnbull AC, Goldfine C, Haddow JE. Ratio of amniotic fluid acetylcholinesterase to pseudocholinesterase as an antenatal diagnostic test for exomphalos and gastroschisis. *Br J Obstet Gynaecol* 1984;91:882–884.
31. Crandall BF, Chua C. Risks for fetal abnormalities after very and moderately elevated AF-AFPs. *Prenat Diagn* 1997;17:837–841.