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Maternal Serum Screening; Approved Standard

This document addresses the steps required to provide reliable screening and reporting using examples of serum markers currently in common use (AFP, hCG, uE3, DIA). Outcome evaluation, information management, and calculation of risk are also emphasized in this standard.

A standard for global application developed through the NCCLS consensus process.

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Maternal Serum Screening; Approved Standard

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Abstract

NCCLS document I/LA25-A—*Maternal Serum Screening; Approved Standard* is written for clinical laboratorians who participate in prenatal screening for open neural tube defects and Down syndrome involving AFP, hCG, uE3, DIA, and/or PAPP-A measurements, as well as for clinicians and manufacturers who have a direct interest in the tests. The standard is intended to present necessary considerations: preanalytical, analytical, and postanalytical; and to ensure the reliability of the tests, including the risk calculation, the outcome evaluation, and the accuracy of the information management. If properly applied, the four biochemical determinations (or five, if performing integrated testing with PAPP-A from the first trimester), and the risk calculations can contribute constructively to the field of prenatal screening and to the welfare of pregnant women and the fetus.

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Foreword

The goal of this document is to update information on second-trimester maternal serum screening (MSS) for open neural tube defects (NTD), which occur at an incidence of 1 to 2 in 1000 live births, and Down syndrome (DS), which occurs at an incidence of 1.6 in 1000 live births. Even with the advent of first-trimester screening markers for DS, second-trimester testing is important due to the following:

- The need for second trimester AFP screening for NTD.

- A significant proportion of pregnant women do not seek prenatal care until the second trimester, thus the need for accurate determinations of biochemical markers of second-trimester MSS.

- First-trimester screening relies on, in addition to the biochemical markers choriogonadotropin free βhCG and pregnancy-associated plasma protein-A (PAPP-A), a nuchal translucency measurement which requires qualified ultrasonographers or physicians experienced in ultrasound which are not always available.

- Without a nuchal translucency measurement, screening using the two first-trimester biochemical markers alone has only about a 60% detection rate for a 5% false positive rate, and is, therefore, not recommended as a screening method. However, a screening protocol using the PAPP-A measurement from the first trimester together with the triple test markers (AFP, uE3, hCG) or the quadruple test markers (AFP, uE3, hCG, DIA) in the second trimester, offers an effective method of screening (the serum integrated test). This improves the performance of second-trimester screening.

This document updates, extends, and replaces NCCLS document I/LA17-A—*Assessing the Quality of Systems for Alpha-Fetoprotein (AFP) Assays Used in Prenatal Screening and Diagnosis of Neural Tube Defects; Approved Guideline.* This document offers guidance that may be used by manufacturers and clinical laboratories that provide prenatal diagnostic services. This document addresses the standards that should be maintained by manufacturers, laboratories, and clinicians when providing screening services used to evaluate pregnancies and risks of fetal disease. At this time, the principles of serum screening remain similar regardless of which assay(s) is/are used as part of the evaluative service. The standard addresses the steps required to provide reliable screening and reporting using examples of serum markers currently in common use (AFP, hCG, uE3, DIA). It is recognized that the list of assays and methods of pregnancy screening will continue to change. Outcome evaluation, information management, and calculation of risk are also emphasized in this standard. Screening for Down syndrome (T21) also includes the incidental detection of Edwards syndrome (T18).

Unlike diagnostic testing which is designed to make the diagnosis of a specific disorder, screening is intended to identify individuals with a sufficiently high risk of that disorder to benefit from further diagnostic testing. NCCLS document I/LA17-A—*Assessing the Quality of Systems for Alpha-Fetoprotein (AFP) Assays Used in Prenatal Screening and Diagnosis of Open Neural Tube Defects*; *Approved Guideline,* published in 1997, was the first NCCLS document to outline the use of specific testing during pregnancy to assess the welfare of the pregnant woman. The part of this document not related to maternal serum screening, but to AFAFP for detection of NTD, is given as an Appendix.

To maintain historical continuity in the field, the foreword from that document (with up-to-date corrections) is quoted below:

> "The aim of this document is to increase the reliability of alpha-fetoprotein (AFP) laboratory testing during the second trimester of pregnancy. Although this document primarily addresses the clinical laboratorians who perform AFP measurements for birth defect assessment, it should also be of value to clinicians and to manufacturers of reagents used in these tests. If properly applied, the measurement of AFP concentration in both maternal serum and amniotic fluid can contribute

constructively to the field of prenatal diagnosis and to the welfare of pregnant women and the fetus.

The value of AFP in prenatal diagnosis was first suggested in 1972 with a report by Brock and Sutcliffe, who documented that amniotic fluid alpha-fetoprotein (AFAFP) levels were increased in the presence of fetal open neural tube defects (NTD). The two major types of NTD are anencephaly and open spina bifida. Following confirmation of this discovery, measurement of AFAFP levels rapidly became part of second-trimester prenatal diagnosis. At that time, such testing was reserved for women who had already borne a child with a neural tube defect because these women are at increased risk for having another similarly affected child. AFP analyses were soon performed on amniotic fluid samples being processed primarily for other diagnostic purposes (e.g., chromosomal analysis).

Although AFAFP measurements proved to be diagnostically useful, occasional false-positive results led to the need for continuing reappraisal, including repeat amniocentesis in some cases. Fetal blood contamination of the amniotic fluid sample was found to be the most frequent explanation of such false-positive results. Other fetal lesions (e.g., open ventral wall defects and the Finnish type of congenital nephrosis) were also found to be associated with elevated AFAFP concentrations, thus diminishing the diagnostic specificity of the test but increasing the range of identifiable fetal problems. Requirements for sample collection and handling, as well as methodology, were such that existing prenatal diagnostic facilities in the United States were able to develop AFAFP testing capabilities with relative ease.

Leek, Ruoss, Kitau, et al, and Brock, Bolton, and Monaghan independently demonstrated increased maternal serum AFP (MSAFP) levels in the presence of fetal anencephaly. In 1974, Wald, Brock, and Bonnar, and Brock, Bolton, and Scrimgeour presented data showing that MSAFP levels were also increased when the fetus was affected by open spina bifida. Strengths and limitations of this screening procedure were addressed by numerous centers during the ensuing years, and it became clear that MSAFP measurement could be applied usefully as a routine prenatal screening test.

Two major United Kingdom collaborative studies, one in 1977 addressing MSAFP screening, and the other in 1979 involving AFAFP analysis, established the overall reliability of both analytical processes and formed a general basis for estimating detection rates and false-positive rates when applied to the United States pregnancy population. The application of AFP measurements to the antenatal diagnosis of NTD was comprehensively evaluated and reported at three international conferences (1977, 1978, and 1980) in Scarborough, Maine. As a prelude to the introduction of MSAFP screening in this country, Haddow and Macri, and Wald and Cuckle also reviewed the current state of knowledge concerning practical applications of MSAFP testing at that time. A National Conference on MSAFP was held in July of 1980 in Washington, D.C., to discuss scientific, medical, ethical, legal, and economic issues in the prenatal screening and diagnosis on NTD. Potentially, all pregnant women might be offered this blood test as a way of screening and diagnosing fetal NTD, with diagnostic ultrasound and AFAFP testing to be offered to women with positive screening results.

Although this disorder is one of the most common serious congenital malformations, people afflicted with open spina bifida may lead productive and satisfying lives. More than 2000 pregnancies are affected with this condition each year in the United States (another 2000 are affected with anencephaly). More than 95% of all open neural tube defects occur among pregnant women with no known risk factors (e.g., a neural tube defect in a close relative or previous pregnancy). All of the infants born with anencephaly will die at, or shortly after, birth. Each year, about 1400 of the infants born with open spina bifida will survive for at least five years, the majority with a significant handicap. Identifying open spina bifida prenatally allows the family to

> choose between terminating and continuing the pregnancy. When the latter choice is made, the family and physician can prepare for the birth of an affected child. This advance notice permits these women to have their babies in hospitals that can offer surgical, medical, and other care needed to minimize the infant's disability. In addition, elevated MSAFP levels may help to identify pregnancies at higher risk for perinatal complications and also about 50% of twin or higher multiple-birth pregnancies.
>
> When implementing an MSAFP screening program, it is necessary at the outset to educate physicians, nurses, laboratory staff, and the patient population of childbearing age. The differing prevalence of NTD for different populations and geographic regions is an important consideration, not only for the educational process, but also for decision making in clinical laboratories that are contemplating performing AFP analysis. AFP testing is no different from any other clinical laboratory procedure whose goal is to improve the quality of patient care. The modern laboratory needs not only to provide accurate assays, but also to aid in the appropriate interpretation of its results. By presenting guidelines aimed at assuring the quality of AFP laboratory testing, this document represents one step toward achieving that goal."

In 1984, it was reported that, on average, MSAFP levels are about 25% lower in DS-affected pregnancies than in unaffected pregnancies.[1] Subsequently, it was shown that fetal DS affects several other MSMs. In 1987, human choriogonadotropin (hCG) was found to be elevated in maternal serum from DS pregnancies.[2] In 1988, maternal serum unconjugated estriol (uE3) was shown to be significantly reduced in DS pregnancies.[3] In the same year, the triple test was described in which AFP, uE3, and hCG are used together with maternal age as a single screening test.[4] In 1995, the same markers were used to identify Trisomy 18 (T18).[5] In 1996, Wald[6] proposed Dimeric Inhibin-A (DIA) as a fourth screening marker and in 1999,[7] he proposed integrated screening using first- *and* second-trimester tests together to obtain a single screening result.

The capacity to measure substances derived from the embryo or fetus, through maternal blood collection, permitted the expansion of medical care to the prenatal, or antenatal, period of development. The use of MSS has expanded rapidly since the early 1970s when the first major works were published confirming that fetal alpha-fetoprotein could be measured in the maternal blood and that high levels were associated with NTD. Since then, additional markers have been identified, which permits the risk assessment of DS as well as the detection of other fetal abnormalities such as T18.

Prenatal screening services are best designed on a program basis that addresses all issues associated with prenatal care. These include a description of the population being screened, the conditions being screened, and regular audits of the screening program. In addition, attention must be given to the education of health professionals, the provision of appropriate information for women considering screening, and education of the public at large.

Key Words

Alpha-fetoprotein, amniocentesis, amniotic fluid, βhCG, chromosomal abnormalities, dimeric inhibin A, Down syndrome, human choriogonadotropin, inhibin-A, maternal serum screening, open neural tube defects, pregnancy-associated protein A, prenatal diagnosis, Trisomy 18, unconjugated estriol

Maternal Serum Screening; Approved Standard

1 Scope

This standard specifies requirements and recommendations for maternal serum screening to ensure that screening methods and quality control procedures are carried out to a high standard. It is the intent of this document to strike a balance between being sufficiently specific to be clear but not too prescriptive, allowing laboratory directors to use their professional judgment in setting policy.

The intended users of this standard are manufacturers, diagnostic laboratories, regulatory agencies, and public health authorities involved in providing or regulating prenatal screening services used to evaluate pregnancies and risks of fetal disease.

2 Introduction

Prenatal screening for serious fetal abnormalities has made significant advances over the last 25 years when maternal serum alpha-fetoprotein started to be used as a screening test for open neural tube defects. Additional maternal serum measurements have been shown to be useful, for example, in screening for Down syndrome. Laboratories have had to not only extend the range of measurements they perform, but also become involved in risk assessment using computer-assisted test interpretation so that clinicians can inform patients of their risk of having the disorders for which screening is being carried out.

The goal of this document is to update information on maternal serum screening (MSS) for neural tube defects (NTD) and Down syndrome (DS). NCCLS document I/LA17-A—*Assessing the Quality of Systems for Alpha-Fetoprotein (AFP) Assays Used in Prenatal Screening and Diagnosis of Open Neural Tube Defects* was the first NCCLS document to outline the use of specific testing during pregnancy to assess fetal well-being. Information related to amniotic fluid analyses taken from I/LA17-A are updated and addressed in the Appendix.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals.* Infection Control and Hospital Epidemiology. CDC. 1996;17(1):53-80 and *MMWR* 1988;37:377-388). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

4 Terminology

4.1 Definitions

Accuracy (of measurement) – Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93)[8]; **NOTE:** See the definition of **Measurand**, below.

Analyte – Component represented in the name of a measurable quantity (ISO 17511)[9]; **NOTES:** a) In the type of quantity "mass of protein in 24-hour urine," "protein" is the analyte. In "amount of substance of glucose in plasma," "glucose" is the analyte. In both cases, the long phrase represents the **Measurand** (ISO 17511)[9]; b) In the type of quantity "catalytic concentration of lactate dehydrogenase isoenzyme 1 in plasma," "lactate dehydrogenase isoenzyme 1" is the analyte **(**ISO 18153).[10]

Bias – The difference between the expectation of the test results and an accepted reference value (ISO 3534-1).[11]

Calibration – Set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring instrument or measuring system, or values represented by a material measure or a reference material, and the corresponding values realized by standards (VIM93)[8]; **NOTE:** According to the U.S. Code of Federal Regulations, calibration is the process of testing and adjustment of an instrument, kit, or test system, to provide a known relationship between the measurement response and the value of the substance being measured by the test procedure (42 CFR 493.1217).[12]

Imprecision – Dispersion of independent results of measurements obtained under specified conditions; **NOTE:** It is expressed numerically as standard deviation or coefficient of variation.

Measurand – Particular quantity subject to measurement (VIM93)[8]; **NOTE:** This term and definition encompass all quantities, while the commonly used term "analyte" refers to a tangible entity subject to measurement. For example, "substance" concentration is a quantity that may be related to a particular analyte.

Measurement error//(Error of measurement) – The result of a measurement minus a true value (or accepted reference value) of the measurand (VIM93)[8]; **NOTE:** Formerly, the term **Total error** was used.

Precision – Closeness of agreement between independent test results obtained under stipulated conditions (ISO 3534-1)[11]; **NOTE:** Precision is not typically represented as a numerical value but is expressed quantitatively in terms of imprecision—the standard deviation (SD) or the coefficient of variation (CV) of the results in a set of replicate measurements (ISO 3534-1).[11]

Primary standard – Standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity (VIM93).[8]

Reference material//Reference preparation (RM) – A material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Reproducibility (of results of measurements) – Closeness of the agreement between the results of measurements of the same measurand carried out under changed conditions of measurement (VIM93).[8]

Sample – One or more parts taken from a system, and intended to provide information on the system, often to serve as a basis for decision on the system or its production (ISO 15189)[13]; **NOTE:** For example, a volume of serum taken from a larger volume of serum (ISO 15189).

Screening – The systematic application of a test or inquiry, to identify individuals at sufficiently high risk of a specific disorder to benefit from further investigation or direct preventive action, among persons who have not sought medical attention on account of symptoms of that disorder.[14]

Screening test – A test to systematically identify individuals at sufficiently high risk of a specific disorder to benefit from further investigation or direct preventive action, among persons who have not sought medical attention on account of symptoms of that disorder.

Secondary standard – Standard whose value is assigned by comparison with a primary standard of the same quantity (VIM93).[8]

Sensitivity – Change in the response of a measuring system or instrument divided by the corresponding change in the stimulus (modified from VIM93)[8]; **NOTES:** a) The sensitivity may depend on the value of the stimulus (VIM93)[8]; b) The sensitivity depends on the imprecision of the measurements of the sample.

Specificity – The ability of a measurement procedure to measure solely the **Measurand**; **NOTE:** Specificity has no numerical value in this context; See also **Measurand,** above.

Specimen (patient) – The discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more quantities or characteristics to determine the character of the whole.

Trueness – Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1)[11]; **NOTE:** Trueness is usually expressed numerically by the statistical measure bias that is inversely related to trueness. See also **Accuracy, Bias,** above.

Validation – Confirmation through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled (ISO 9000)[15]; **NOTES:** a) WHO defines validation as the action {or process} of proving that a procedure, process, system, equipment, or method used works as expected and achieves the intended result (WHO-BS/95.1793)[16]; b) The components of validation are quality control, proficiency testing, validation of employee competency, instrument calibration, and correlation with clinical findings.

Verification – Confirmation through the provision of objective evidence that specified requirements have been fulfilled (ISO 9000)[15]; **NOTE:** A one-time process completed to determine or confirm test performance characteristics before the test system is used for patient testing.

4.2 Acronyms and Abbreviations

AChE	acetylcholinesterase
AFAFP	amniotic fluid alpha-fetoprotein
AFP	alpha-fetoprotein
βhCG	choriogonadotropin free beta-subunit
CG	choriogonadotropin
DIA	Dimeric Inhibin A
DS	Down syndrome
ELISA	Enzyme-Linked Immunosorbent Assay
hCG	human choriogonadotropin
IDDM	insulin-dependent diabetes mellitus
IU	International Units
LMP	last menstrual period
MoM	multiples of the median
MS	maternal serum
MSAFP	maternal serum alpha-fetoprotein
MSM	maternal serum markers
MSS	maternal serum screening
NTD	neural tube defects
PAPP-A	pregnancy-associated plasma protein-A

RIA	radioimmunoassay
T18	Trisomy 18
T21	Trisomy 21
uE3	unconjugated estriol
WHO	World Health Organization

5 Specimen Collection

It is usual to collect blood from an individual in the sitting or recumbent position. Specimens can be collected any time of the day. Specimens should not be collected after amniocentesis, since amniocentesis often produces procedure-related elevated MSAFP levels in pregnant women and may affect the analyte levels. As discussed later in this guideline, multiple fetuses, low birth weight, fetal death, and underestimation of gestational age are all associated with increased MSAFP levels.

6 Sample Handling and Preparation

6.1 Serum

Samples that are chylous or severely hemolyzed should be rejected.

Without prolonged application of a tourniquet, collect blood into an evacuated glass or plastic tube without anticoagulant. Allow the sample to stand at room temperature for 30 to 45 minutes or until the clot has retracted. Serum samples can be taken directly from above the retracted clot after brief centrifugation.

The biochemical markers routinely used for screening are sufficiently stable in maternal serum to allow shipment at ambient temperatures. However, serum should be separated from the clot promptly and stored refrigerated until assayed or shipped.

6.2 Plasma

Plasma is not a recommended patient sample, unless it is specifically referenced in the manufacturer's package insert.

6.3 Dried Blood Spots

Dried blood spot specimens are not recommended since they have not been shown to be suitable for uE3 measurement and no assay advantage has been shown with respect to the other biochemical markers.

6.4 Sample Storage

Serum samples may be shipped or stored at room temperature or 4 °C for up to six days, at -20 °C for up to six months,[17] or at -70 °C indefinitely, provided the tubes are securely stoppered. Whole blood samples should reach the laboratory within two days of collection. The serum analytes are reasonably stable with the exception that free βhCG tends to increase with storage at ambient temperatures due to its separation from total hCG.

7 Immunochemical Methods

7.1 Background

Maternal serum markers (MSMs) have traditionally been measured by some form of immunoassay. A few tests are still performed using radioimmunoassay (RIA) (e.g., uE3), but the majority of laboratories are using nonisotopic enzyme immunoassays. First-generation enzyme-linked immunosorbent assays (ELISAs) have been followed with advances in precision and sensitivity afforded by second- and third-generation assays. The methods of choice are one-step immunometric assays employing detection systems such as fluorescence (time resolved or not) or chemiluminescence that do not require the additional step for color development used in ELISAs, and confer the advantages of more rapid throughput and higher sensitivity.

7.2 Radioimmunoassay

The commonly employed competitive format of RIA combines high sensitivity, economy of reagents, and reproducibility. In this method, a fixed amount of radiolabeled antigen competes for a limited amount of specific antibody with the "cold" unlabeled antigen in the sample or calibrator. A standard curve is constructed using known amounts of unlabeled antigen from which unknown antigen concentrations can be computed.[18] The assay can be carried out in a liquid medium, incorporating a separation step, or on a solid phase.

7.3 Enzyme-Linked, Time-Resolved Fluorometric or Chemiluminescent Assays

Immunoassays can use either a "sandwich" style format or a competitive assay format. The most commonly used form is the two-site or sandwich assay that uses two antibodies that bind to different sites on the analyte. One of the antibodies is coated onto a solid support such as plastic beads or paramagnetic particles. The other antibody is attached to an enzyme-linked conjugate. During the reactions, the first binding site of the analyte will be immobilized on the solid phase. The antibody to the second binding site is usually attached to an enzyme conjugate. Unbound components are removed by repeated washing of the solid phase. The final phase of the reaction involves the addition of a colorimetric, fluorimetric, or chemiluminescent substrate, catalyzed by the enzyme bound to the solid phase analyte antibody complex. The resulting signal produced is directly proportional to the concentration of analyte present.

Competitive assays differ in that a single antibody is directed to a binding site(s) on the analyte. Sample is added to a reaction vessel along with analyte-enzyme conjugate and a solid phase coated with a limited quantity of immunoglobulin antianalyte complex. Analyte present in the sample competes with the analyte-enzyme conjugate for a limited number of binding sites on the solid phase. Again, unbound components are washed away, and the final signal produced is inversely proportional to the concentration of analyte present.

In time-resolved fluorometry, the analyte-antibody conjugate is chemically (rather than enzymatically) linked to Europium. Incubation of the washed bound analyte-antibody solid complex with an enhancement solution dissociates Europium ions from the labeled antibody into the solution where they form highly fluorescent chelates. The fluorescence is directly proportional to the analyte concentration.

7.4 Reference Materials to Calibrate MSM Assay Systems

7.4.1 Existing Calibrators

7.4.1.1 WHO Standard for AFP

The World Health Organization (WHO) Standard for Human AFP (72/225) is a lyophilized cord serum preparation that is available on a limited basis. In this preparation, International Units (IU) have been collaboratively assigned.[19,20]

7.4.1.2 British Standard

The First British Standard for Human Cord Serum (72/227) was produced from the same batch of cord serum as the WHO material, but it was lyophilized by a different technique. The British reference material is calibrated against WHO (72/225).

7.4.1.3 Pure AFP Standards

No pure standard of native AFP, calibrated in mass units, currently exists.

7.4.2 Composition of Proposed United States National Reference Materials

7.4.2.1 Matrix Conditions

Two body fluids are analyzed for AFP content during pregnancy: maternal serum and amniotic fluid. Stable calibrators traceable to the WHO (72/225) Standards that simulate the analytical conditions defined by the composition of these fluids are desirable.

7.4.3 hCG Standardization

Most CG Reference Standards are calibrated against the World Health Organization's (WHO) 1st IRP (75/537) and 3rd International Standard (IS) (75/537).[21]

From 1964 until early in 1982, the 2nd IS was used for reporting bioassay and immunoassay values. Biologically active hCG molecules comprise only approximately 20% of this standard. In 1974, the WHO Expert Committee on Biological Standardization established the 1st IRP (75/537) of highly purified, biologically active hCG.

Interpretation of results, especially when correlating kits from different manufacturers, should be made with the possible differences in standardization in mind. Commercial assays calibrated to the 1st IRP (75/537) and 3rd IS (75/537) may give results twice the value of assays calibrated using the 2nd IS.

The difference in interpretation of commercial reference standards is due to the variability of assay systems employed and the different hCG antibodies used. The lower values obtained with the 2nd IS are due to the impure nature of this standard, which contains large amounts of disassociated alpha and beta subunits in addition to intact hCG molecules.

For total βhCG assays, the appropriate reference is the WHO 3rd IS (75/537).

WHO (75/551) is the current standard used for the free βhCG subunit.

New WHO standards used for calibration are: 99/642, 650, 688, 692, 708, and 720.

Recent advances in purification technology have led to the development by an IFCC working group of the 1st WHO reference reagents that selectively target hCG and its specific subunits.[22] The reference reagent for hCG (99/688) includes no nicked hCG (hCGn) and negligible free subunits. These new standards have the following codes: hCG 99/688, βhCG 99/650, hCGα 99/720, hCGn 99/642, hCGβn 99/692, hCG core fragment (hCGcf) 99/708. At the time of publication of this standard, few manufacturers of hCG assays had converted from using the 3rd IS in their primary standard preparations to using the new WHO 1st reference reagents.

7.4.4 Estriol Standards uE3

There is no "gold standard" for estriol assays. Many assays are standardized to exhibit optimal correlation of a select patient panel to a Gas Chromatography-Mass Spectroscopy (GCMS) method.[21]

Additionally, matrix issues may exist with many immunoassay methods for measuring unconjugated estriol. Most unconjugated estriol assays are designed to measure naturally occurring unconjugated estriol in maternal serum. Artificial samples, including calibrators from other kits, certain proficiency samples, and certain controls, may under-recover by as much as two to three times lower than stated. Proficiency surveys must be graded by peer-group, rather than across all methods.

To convert results from ng/mL to nmol/L, multiply by 3.467.

8 Clinically Significant Conditions Associated with Elevated or Suppressed Values of Maternal Serum Markers

8.1 MSAFP

8.1.1 Open Neural Tube Defects (NTD)

The original and continuing purpose for measuring AFP in maternal serum has been to identify NTD, particularly open spina bifida in the fetus. The median value for sera from NTD pregnancies is approximately 4 times the normal single-birth median, and from anencephalic pregnancies it is 8 times the normal median.

The first United Kingdom Collaborative Study[23] (MSAFP screening) has determined detection rates for open spina bifida as follows: 70% using 3 times the median as the cutoff level, 79% using 2½ times the median, and 91% using 2 times the median. Detection rates are higher for anencephaly. No single cutoff level is used today by all testing centers. False-positive rates have been the major consideration for differing policies on cutoff levels. Approximately 1% of MSAFP measurements from unaffected pregnancies will be above the cutoff at 3 times the median, about 2% at 2½ times the median, and 2% at 2 times the median.

Subsequent testing (e.g., ultrasonographic identification of multiple pregnancies or reinterpretation of the MSAFP measurement due to corrected gestational age) will cause a proportion of unaffected pregnancies that were initially screen-positive to be reclassified as screen-negative. It will also reclassify true positives as false negatives. This loss in detection among women already alerted to the risk of having an affected pregnancy should be avoided if at all possible. To this end, laboratories only alter the gestational age estimate among women with screen-positive results if the discrepancy between LMP and ultrasound is greater than a specified period ranging from 7 to 17 days in different laboratories. It is best to avoid the problem in the first place by obtaining an ultrasound estimate of gestational age before the screening test is performed.

8.1.2 Prevalence of NTD

The birth prevalence of neural tube defects has been declining in many countries even in the absence of screening and selective abortion. This has arisen at least in part due to increases in folate and folic acid consumption. A general estimate of the birth prevalence in the absence of antenatal screening and selective abortion would be about 1 to 2 in 1000 births. Recent data put the NTD birth prevalence at 1.1%.[24] The Food and Drug Administration authorized the addition of synthetic folic acid to grain products in March 1996 with mandatory compliance by January 1998 resulting in an approximate 20% reduction in risk of an NTD pregnancy.

8.1.3 Multiple Pregnancies

The median MSM values for twin pregnancies are approximately 2 times the single-birth median.[25] MSAFP levels are about 3 times higher in triplet pregnancy[26] but there are insufficient data for the other markers in multiple gestational pregnancies other than twins. Once a twin pregnancy has been identified, a separate interpretation needs to be made, based on reference ranges for such pregnancies.

8.1.4 Fetal Demise, Pregnancies at Risk for Spontaneous Abortion, and Low Birth-Weight/ Premature Outcomes

These conditions are also associated with elevated MSAFP levels.[27,28] Only 19% of pregnancies in one study with MSAFP levels above three times the median resulted in normal single-birth term deliveries of infants weighing ≥ 2.5 kg. The clinical implications of these results are unclear. Extremely high and low values of the MSMs have been associated with fetal demise.[29]

8.1.5 Other Major Fetal Malformations Identified in Association with MSAFP Elevations

Open ventral wall defects are the next most common major fetal malformation identified via MSAFP screening. Congenital nephrosis of the Finnish type[30] is a rare but lethal problem identified as part of MSAFP screening.

8.2 Other Maternal Serum Markers

Interest in maternal serum markers of fetal DS began shortly after maternal serum alpha-fetoprotein (MSAFP) screening for neural tube defects came into use; a chance observation in one patient led to the finding that, on average, MSAFP levels are about 25% lower in DS-affected pregnancies than in unaffected pregnancies.[1] Subsequently, it was shown that several other maternal serum markers are affected by fetal DS, with hCG being among the most strongly affected.[2,31] In 1987, human choriogonadotropin (hCG) was found to be elevated in maternal serum from DS pregnancies; levels are, on average, about twice as high in DS-affected pregnancies.[2] In 1988, maternal serum unconjugated estriol (uE3) was shown to be significantly reduced in DS pregnancies; levels of uE3 are about 25% lower in DS pregnancies, making this marker separation equivalent to MSAFP but the distribution of uE3 is tighter than for MSAFP and therefore, the discrimination between affected and unaffected pregnancies is greater.[3] In the same year, the triple test was described in which AFP, uE3, and hCG are used together with maternal age as a single screening test.[4] Later, in 1995, the same markers were used to identify Trisomy 18.[5] In 1996, Wald[6] proposed Dimeric Inhibin-A (DIA) as a fourth screening marker, and in 1999,[7] he proposed integrated screening using first- *and* second-trimester tests together to obtain a single screening result.

8.2.1 Maternal Serum hCG in Down Syndrome

hCG is almost undetectable in nonpregnancy. Initially, hCG is secreted by the trophoblast, and later by the chorion and placenta. The rapid rise following implantation is the basis of the clinical laboratory test

for pregnancy. The function of hGC is to maintain the corpus luteum, which is necessary for the production of both progesterone and estrogen.

Maternal serum hCG levels in nonpregnant females are typically less than 10 IU/L, and most pregnancy tests are optimized for the concentration range of 10 to 500 IU/L. During pregnancy, hCG levels rise dramatically to peak at 10 weeks of gestation at levels of 100 000 to 200 000 IU/L. In the early mid-trimester, the levels fall to a plateau of approximately 20 000 IU/L (or 20 IU/mL) at 18 weeks gestation, which is then maintained throughout the pregnancy. For convenience, hCG concentrations in the mid-trimester are usually expressed in the units of International Units per milliliter (IU/mL).

CG dimer, βtCG, and βhCG assays are all suitable for DS screening application. A βhCG assay has the advantage that dilution of specimens is generally not required, whereas the other assay types are generally optimized for the detection limit, and require about 100-fold dilution to run second-trimester specimens. Some data suggest that βhCG offers slightly better DS discrimination,[32] but there are insufficient data at this time to establish a particular type of CG assay as being the best.[33-35] Multiple marker testing, including measurement of CG dimer or subunits, could be effective for trisomy screening in the first trimester, but there is less experience with this practice.[36-42] One 1994 study reported exceptionally good discrimination of DS achieved by measurement of the free βhCG subunit in urine.[43]

In the second trimester, free βhCG can be used instead of total hCG. In the first trimester βhCG is a somewhat better screening marker than total hCG. Free βhCG is widely used outside the U.S. in antenatal screening for Down syndrome.

Many different CG assay kits are suitable for DS screening; but most are not yet FDA-approved for this purpose at the time of this writing, which places additional responsibility on individual laboratories for ensuring the validity of the test. It is recommended that laboratories follow, as closely as possible, protocols for which the efficacy has been verified in large prospective studies.

8.2.2 Maternal Serum uE3 in Down Syndrome

uE3 is produced by a metabolic pathway that involves fetal adrenal tissue, fetal liver, and the placenta. The fetal adrenal precursor for estriol production is dehydroepiandrosterone sulfate (DHEAS), which is hydroxylated in the fetal liver to 16α-OH DHEAS. The hydroxylated DHEAS is then metabolized to estriol by the placenta, and a fraction of the estriol diffuses across the placenta into the maternal circulation. Essentially, all of the maternal serum uE3 is of fetal origin.

The reliance on fetal liver and the placenta for the production of estriol has enabled this compound to be used as a marker of fetal placental function in later pregnancy. Most assays for uE3 were originally optimized for the third-trimester levels that are approximately 5 to 10 times higher than the midtrimester levels. They have now been enhanced in order to attain the necessary performance at the lower levels required for DS screening in the second trimester.

In 1988, Canick, et al demonstrated that the levels of maternal serum uE3 are lower in DS pregnancies than those observed in unaffected pregnancies.[3] In their retrospective analysis of frozen maternal serum from 22 DS pregnancies, the median uE3 concentration was 0.79 times the unaffected control median. In a separate study published in the same year, these same collaborators extended their study to 77 affected cases, and found a median of 0.73 multiples of the unaffected median.[44] In addition, this study showed that the uE3 concentrations in both populations fitted Gaussian distributions. A number of studies have subsequently confirmed the lower levels of uE3 in DS pregnancies.[45-47] The cause of the lower concentrations has been speculated to be a relative immaturity of the tissues involved in its production.[3]

8.2.3 Maternal Serum DIA in Down Syndrome

Inhibins are dimeric glycoprotein hormones consisting of disulphide-linked subunits α and βA (termed dimeric inhibin A or DIA) or α and βB (termed dimeric inhibin B). Both forms suppress follicle-stimulating hormone (FSH) production by the anterior pituitary gland and have local modulating effects on gonadal steroidogenesis. Testicular and ovarian cells are the main source of inhibin. In primates, the corpus luteum is also an important source during the normal cycle and early pregnancy.

During pregnancy, the placenta secretes significant amounts of inhibin, especially in later pregnancy when most of the maternal serum inhibin is placental in origin. The function of inhibin is still unknown, but there is speculation that there are some local autocrine/paracrine actions at the level of the placenta and the corpus luteum. Since inhibin is a placental product, and placental products are increased in pregnancies associated with DS, it has proved to be an effective screening marker in combination with existing serum markers and maternal age. Assays that selectively measure inhibin A are more discriminating for DS in the second trimester than nonspecific assays.

In 1996, Wald[48] demonstrated that the median DIA concentration in DS pregnancies was 1.79 MoM, significantly higher than that in unaffected pregnancies. In a subsequent study[49,50] refining the methods used to derive their data, Wald found that adding DIA to AFP, uE3, and either hCG or free βhCG, and maternal age, with marker values adjusted for maternal weight and gestational age determined by ultrasound, gave a detection rate of 75% with a false-positive rate of 5%.

8.2.4 Prevalence of Down Syndrome

The birth prevalence of Down syndrome has been increasing in many countries as women have tended to have their babies at an older age. As a result, the overall birth prevalence (in the absence of screening and selective abortion) is now higher than it was. A reasonable estimate would be about 1.6 in 1000 births.

8.2.5 Pregnancy-Associated Plasma Protein (PAPP-A)

Recently, second-trimester maternal screening has been shown to be enhanced by the inclusion of a PAPP-A measurement at about 10 to 11 weeks of pregnancy where PAPP-A measurement is then used together with the second-trimester measurements in a single integrated test.

PAPP-A is a large molecular weight placenta-derived glycoprotein. During pregnancy, it is produced in high concentrations by the trophoblast and released into maternal circulation. PAPP-A levels in maternal serum rise steadily with gestational age, most noticeably during the last part of pregnancy. The functional significance of PAPP-A is unclear. However, studies suggest that reduced concentrations of PAPP-A in pregnancy are associated with chromosomal abnormalities in the fetus, especially Down syndrome. The correlation was found to be significant at weeks 10 to 14 of pregnancy, and thus was used together with serum free βhCG subunit, in the first trimester, or together with the second-trimester markers in an integrated screening.[48]

9 Factors That Influence the Biochemical Marker Measurements During Pregnancy

9.1 Maternal Serum Measurements

In the early second trimester, gestational age is the main factor that affects changes in the concentration of serum markers. Two of the serum markers increase with gestational age (AFP about 15% and uE3 about 25% per week), one (hCG) decreases exponentially, and the fourth (DIA) changes little.

Maternal weight influences the concentration of the serum markers due to a dilutional effect related to increased blood volume with increasing weight. There are also ethnic differences independent of weight or other known variables (e.g., women of African descent have higher MSAFP levels than are found in Caucasian women). Insulin-dependent diabetes mellitus (IDDM) in a pregnant woman is associated with lower MSAFP levels (by about 20%). Each of these factors should be taken into account when interpreting serum marker levels in screening.

During each week of gestation, there is overlap in the distributions of AFP concentrations between unaffected pregnancies and pregnancies affected by NTD. This is particularly true for maternal sera and, to a much lesser extent, for amniotic fluid. Consequently, the selected AFP concentration cutoff level carries a definable false-positive rate and detection rate.

9.2 The Effect of Maternal Weight on uE3, hCG, and DIA

Since AFP, uE3, hCG, and DIA are fetal/placental in origin, the same rationale for the effect of maternal weight applies to all; namely, that the circulating maternal plasma volume will affect their concentration. A number of studies have been published on the effect of maternal weight.[51-53] All studies showed the expected inverse relationship between maternal weight and serum marker levels. The relationship between hCG and DIA, and maternal weight was similar in magnitude to that seen with AFP, whereas the effect was less with uE3. It has been postulated that the shorter half-life of uE3 might be the cause of its lesser dependence on maternal weight.

The overall effect of maternal weight correction on multiple marker screening performance has been shown to be slight.[52] This can be explained best by example: in a larger woman, weight correction will raise the MoM values of all markers. This will reduce the risk calculated on the basis of AFP and uE3, but it will increase the risk calculated on the basis of hCG and DIA. (Women who are smaller than average will have the opposite effect on marker concentrations.) The combined offsetting effects on the risk estimate are essentially equal. However, since most NTD screening protocols require weight correction, weight correction of the other markers seems advisable if the information has been provided. Weight correction is more important if only two serum markers are in use; this is particularly true if only AFP and uE3 are used. In addition, weight adjustment is important for Trisomy 18.

9.3 Expressing the Concentration of Markers as Multiples of the Median (MoM)

The concept of the MoM (multiples of the median) was first described in 1976[54] and was used in the United Kingdom Collaborative Studies.[23,55] These studies contain a detailed description of the manner in which MoM values are derived. Although reference ranges for MSAFP concentrations are different for each week of gestation during the early second trimester, individual AFP values could be expressed as a multiple of the median derived from women of the same gestational age tested in the same laboratory.

In this way, MoM provides a common basis for transforming all marker levels across different gestational ages and different laboratories. The MoM has the convenience of being unitless (since it is the ratio of two concentrations) and directly providing information on whether a given value is high or low, and by how much. The MoM value can also be readily adjusted to take into account the other variables that affect the interpretation of biochemical markers (e.g., maternal weight, IDDM, ethnicity).

The MoM value is also used to calculate individual patient-specific risks. The College of American Pathologists Laboratory Accreditation Program for Prenatal Screening with AFP requires the following with regard to the MoM: "documentation that the laboratory has established its own normal median AFP values or verified the manufacturer's package insert or other source for the population being screened and that these medians are updated at least annually."

Other methods of normalizing test results, such as means and standard deviations or percentiles, are less robust and are not recommended. The biochemical markers under consideration contain skewed distributions that require special attention to defining percentiles of normal versus the total population. It is usually best to calculate medians separately for gestational ages based on "dates" (time from last menstrual period) or using ultrasound measures such as crown-rump or biparietal diameter.

The same considerations apply to expressing the concentrations of uE3, DIA, and hCG as discussed for AFP. uE3 levels rise during the midtrimester at approximately 20% per week; therefore, median levels are required and the patient concentrations are expressed as the multiple of the unaffected median (MoM). In contrast, hCG levels fall during the early weeks of the midtrimester, until they reach a plateau concentration at 18 to 20 weeks of screening. Serum levels of DIA increase during the first trimester until week 10, then decline to stabilize from week 15 to week 25, and rise again peaking at term. Although DIA concentration is relatively stable in the second trimester, there is still enough change in the weekly medians to require transformation of DIA measurements to MoM.

About 100 data points are required for each week of gestation in order to estimate the median concentration of each marker. When each laboratory introduces screening or adopts a new set of reagents, it is reasonable to use about 100 data points between 16 and 18 weeks and then reestimate the weekly medians as larger numbers accumulate. As discussed for AFP, the median levels change in a predictable manner from week to week, and the change can be modeled through the use of mathematical equations. This modeling enables the extrapolation of median values out to gestational ages that have fewer observations. The mathematical expressions also enable the calculation of daily medians by interpolation within weeks. The estimation of gestational age is more critical with multiple markers than with the single marker MSAFP; if three markers are used, the gestational age is factored into the final risk estimate three times. For this reason, the use of medians by gestational days (as opposed to completed weeks) is recommended for multiple marker screening.

Different software programs for median calculation allow either the use of stored medians by completed weeks or the calculation of the medians by days based on the slope and intercept of the regression line. Even if medians by completed weeks are used, it is still advisable to regress the observed data prior to storing weekly median values.[56]

9.4 Time of Gestation When Test is Performed

9.4.1 Methods of Dating

Two conventions are presently in use for defining which gestational week to assign a given serum sample sent for biochemical marker measurements. In the first method, completed weeks determine assignment of gestational age (e.g., 17 weeks and 4 days equals 17 weeks). In the second, gestational age is rounded to the nearest week (e.g., 17 weeks and 4 days equals 18 weeks). Results from proficiency testing programs in the United States indicate that completed weeks of gestation are used by almost all laboratories and this approach should be generally adopted.

Gestational age is estimated from the first day of the last menstrual period (LMP). Because three of the four biochemical markers continuously change in concentration in the second trimester of pregnancy, errors in estimating gestational age will tend to produce falsely high and low values when expressed in MoM. These errors will tend to reduce screening performance. Ultrasound estimation improves screening performance by reducing these errors. Therefore, it is recommended that ultrasound estimation of gestational age be obtained before the biochemical results are obtained. A biparietal diameter is the best ultrasound measure of gestational age in the second trimester of pregnancy; in the first trimester, it is the crown-rump length. The ultrasound dating scan prior to screening will also identify multiple pregnancies and allow women to decide whether they still wish to be screened.

Wald, et al[57] reported on an added benefit of using a biparietal diameter in screening for spina bifida. On average, fetuses with spina bifida have biparietal diameters that are smaller than would be predicted based on LMP (two weeks younger, on average). Using biparietal diameter measurements for routine pregnancy dating, therefore, increases serum AFP levels in spina bifida pregnancy by about 40% when expressed in MoM. This increases the spina bifida detection rate by about 15%.

9.4.2 Gestational Age for Screening

In MSAFP screening for NTDs, the best time for AFP measurement is at 16 to 18 weeks. It is unreliable at less than 15 weeks and is not recommended beyond 21 weeks because of a lack of normative data and difficulties in completing subsequent diagnostic testing within a reasonable time. There are no valid biochemical markers for NTD in the first trimester.

In multiple-marker screening for DS using the triple or quadruple test, the best time is 14 to 18 weeks. These two sets of tests are not recommended before 14 weeks but can be used up to 21 weeks, although with the same concerns noted above. There are useful biochemical markers for DS in the first trimester, particularly PAPP-A at 10 to 11 weeks of pregnancy. If samples are to be interpreted for both NTD and DS, then 15 weeks is the earliest that samples should be interpreted.

9.5 Second Trimester Protocol

Screening for both NTD and DS can be satisfactorily accomplished with a collection of a single blood specimen early in the second trimester. For NTD, an AFP cutoff (usually between 2.0 and 2.5 MoM) is used to define screen-positive results. For DS, a cutoff based on risk is used which combines information obtained from multiple markers and risk for DS based on the woman's age at delivery.[30] Repeat screening measurements are not recommended because they have little impact on the performance of screening and may lead to the situation where women who truly have an affected pregnancy and who initially received positive screening results are then told their screening results are negative. Therefore, if the screening result is positive for either disorder, a diagnostic amniocentesis is indicated. If the AFP is elevated, a detailed ultrasound examination is also indicated for the diagnosis of spina bifida.

A protocol commonly used for elevated MSAFP values in some screening centers uses two consecutive MSAFP measurements, sampled at least one week apart, before further diagnostic steps are suggested. A better protocol is to proceed directly to diagnosis.

9.5.1 Integrated Testing Protocol

The efficacy and safety of screening using the second-trimester quadruple test (or triple test) can be significantly improved by including a PAPP-A measurement obtained at 10 to 13 weeks (preferably at 10 to 11 weeks) to the second-trimester measurements when calculating risk. All the measurements from both trimesters are integrated into a single test result which is interpreted together with maternal age. This test (the serum integrated test[7]) has a higher detection screening rate and a lower false-positive rate (85% detection rate for a 2.7% false-positive rate[29]) than tests currently available in either the first or second trimester alone. If a satisfactory nuchal translucency measurement at 10 to 13 weeks is available, it can be added to the serum-integrated test to further improve screening performance, reducing the false-positive rate to 1.2% for the same 85% detection rate. Adding a nuchal translucency measurement in this way, when it is available, is similar to improving second-trimester screening performance by using an ultrasound estimate of gestational age when it is available.

The integrated test requires that a woman have an NT measurement and a blood draw for PAPP-A measurement between 10 and 13 gestational weeks, and then return for a second blood draw ideally at 15 or 16 weeks. However, the second blood can be drawn as late as 21 gestational weeks. The results of NT and PAPP-A measurements are not reported until they can be integrated with the quad marker results

from the second trimester blood sample into a single estimate of risk. Alternatively, if the serum version of the integrated test is done, the test simply involves two separate blood draws.

With data from the U.K., SURUSS trial,[58] and the U.S., FASTER trial,[59] it now seems that markedly safer screening is possible. Rather than subjecting 5% or more of pregnant women to the risks of chorionic villus sampling (CVS) or amniocentesis, the rate of diagnostic intervention can be reduced by as much as 75 to 80%, to only 1% or 2%. To achieve such improvement in performance, the test must be done in two parts, with two to six weeks intervening before the result is reported. Amniocentesis, rather than CVS, is offered to screen positive women so that diagnosis of Down syndrome, if present, is made at the same time in pregnancy as is usual following second trimester screening. In addition, screening for open neural tube defects with AFP is part of the protocol.

9.6 Patterns of Second Trimester Marker Concentrations

Certain patterns of maternal serum marker concentrations may indicate certain kinds of fetal developmental defects as shown in the following table.

Serum Markers			**Possible Indication**
AFP	**uE3**	**hCG**	
low	low	high	Trisomy 21
low	low	high	Overestimated GA
low	very low	very low	Trisomy 18
very low	very low	negligible	Nonpregnancy
initially high then very low	very low	very low	Fetal demise
slightly reduced	very low	high	Turner (with hydrops)
slightly reduced	very low	low	Turner (no hydrops)
high	-	-	NTD
-	low	very low	Nonmolar Triploidy
-	low	high	Partial Molar plus Triploidy
normal	undetectable	normal	Placental sulfatase deficiency, Smith-Lemli-Opitz Syndrome, Contiguous gene deletion syndromes, or other mutations
very high	very low	low	Anencephaly

9.7 Calculation of Down Syndrome Risk

In their 1988 report of multiple-marker screening for DS, Wald, et al[4] demonstrated that the relative frequency distributions of the MoM for AFP, uE3, and hCG (expressed in logs) for the unaffected and DS populations fitted Gaussian curves. The parameters (means, standard deviations) that describe these curves can be used in the formula for a Gaussian distribution curve to obtain likelihood ratios for each marker and with the correlation coefficients to obtain the multivariant likelihood ratios for the markers combined. The MoM truncation limits for calculating likelihood ratios for DS are presented in the table in Section 9.8.

9.8 MoM Truncation Limits to Be Used

	AFP		hCG		uE3		βhCG		DIA	
	Lower Limit	Upper Limit	Lower Limit	Upper Limit	Lower Limit	Upper Limit	Lower Limit	Upper Limit	Lower Limit	Upper Limit
Down syndrome[6]	0.3	3.3	0.2	5.0	0.5	2.0	0.33	3.0	0.4	3.5
Down syndrome[60]	0.4	2.0	0.5	4.0	0.4	2.0	0.5	4.0	0.7	2.5
Trisomy 18	0.33	2.0	0.2	2.5	0.5	1.5				

When calculating the risk of DS with AFP as the sole marker, the likelihood ratio for the AFP concentration is multiplied by the patient's prior risk to yield the final risk estimate. This simple process is possible because the maternal age risk and the AFP risk are independent assessments. With multiple serum markers, the situation is more complex. Not surprisingly, the serum markers are not totally independent assessments of risk; instead, they are weakly correlated. This means that the likelihood ratios for the markers, cannot be simply chained together without the allowance for their correlation. Correlation coefficients must be calculated, one for each pair of markers, and these are used to modify the final risk calculation. Two sets of such parameters have been published and are recommended for quadruple markers: Wald, 1996,[49,61] and Haddow, Palomaki, 1998.[60,62]

9.8.1 Screening for Down Syndrome in Twin Pregnancies

A "pseudo-risk" can be calculated for DS in twin pregnancies but it is not as accurate or reliable as for singletons. Since DS is rare in twin pregnancies, it is difficult to collect data from affected pregnancies. The detection rate appears to be higher in monozygotic affected pregnancies and lower for dizygotic singletons. The calculation is done by dividing each of the analyte MoM by the average level for that analyte in twin pregnancies. The resulting "pseudo-MoM" are combined with the age-associated risk, and reported on the sample as if it were for a singleton pregnancy. Studies have shown that MSAFP levels in twin pregnancies are about twice (2.17 times) the average singletons: uE3 levels are 1.64 times as high, hCG levels are 1.94 times as high,[63] βhCG are 2.165 times as high, and DIA levels are 1.99 times as high.

10 Quality Assurance

Knight[18] and a report by the U.S. National Institute of Child Health and Human Development[64] give comprehensive general instructions for quality control in clinical laboratories.

10.1 Reference Values

Reference values in MSM screening consist of a set of median values calculated for each week of gestation and each day of a gestational week using the laboratory's own MSM assay values, measured

preferably on the population to be screened. Use of incorrect reference data is a common cause of incorrect interpretation of MSM screening measurements by laboratorians. It is, therefore, essential that the screening laboratory establish its own reference data and/or demonstrate that reference data obtained from another source are valid for the population being screened. Individual MSM test results are then expressed as multiples of the unaffected population median (MoM), which is obtained by dividing each individual MSM value by the median value for the relevant gestational week. Strategies are outlined below for establishing median values for both maternal serum (see Section 10.1.1) and amniotic fluid (see Appendix).

10.1.1 Establishing Median Values in Maternal Serum

Proficiency testing programs find that MSM measurements vary by as much as 15% between laboratories when results are expressed directly in the units of measurement (e.g., ng/mL). The major contributing factor to explain these differences is a bias among manufactured test kits. The use of median values obtained from published sources as reference data is, therefore, contraindicated. Laboratories may be tempted to use the median values provided in package inserts as a source of reference data. Such median values have been documented to be widely in error for some kits, resulting in inappropriately large numbers of false-positive (or false-negative) screening test results. Data provided in manufacturers' package inserts are not appropriate for establishing median values because of factors such as the variability in reliability of gestational dating. It has, therefore, been recommended that each laboratory establish its own reference data using the kit chosen for screening and samples obtained from the population to be screened.

WARNING: Care should be taken in assuming that the results of proficiency testing on artificial samples are equivalent to those found in patient samples.

In obtaining median values, it is not necessary to ensure that all samples come from unaffected, single-birth pregnancies. The prevalence of conditions that result in outlying MSM values, such as twins, fetal death, open fetal defects, and IDDM, is only a few percent, collectively. If the measurements are on an unselected population, this will have minimal impact on the median value. In practice, screening program medians can be established by obtaining about 100 samples from the local population for each gestational week, from 16 to 18 weeks (if available, data at earlier and later weeks should be included). These data should be used in a weighted log-linear regression (for AFP and uE3) to estimate normal medians as follows: median values are first calculated using available MSM values. Then an appropriate regression analysis is carried out, in which each median is weighted according to the number of data points at each gestational week, and then extrapolation is performed to obtain the equation. This mathematical procedure produces "smoothed" median values for each day of the gestational stage (it is very important to store the slope and intercept of the calculated regression line). This log-linear relationship holds over the gestational age range from 14 to 25 weeks, allowing median values to be extrapolated for weeks for which data are sparse. This method has been published.[18] For hCG, it should be an exponential regression, and for DIA, it should be a quadratic regression.

Until sufficient samples are available to accomplish this on the local population, laboratories can obtain serum samples from another screening laboratory, assay them, and establish median values. This approach has the weakness that patient samples may differ in different populations. Alternatively, laboratories may make arrangements with an established laboratory that uses the same kit to share its median values. About 50 to 100 samples with assigned MSM values should be obtained and reassayed. If the MSM assay values agree within ±5%, the median values can be adopted to begin screening. When sufficient data are available, laboratories should calculate their own median values in the standard way.

Occasionally, two laboratories using the same assay will find a systematic but consistent bias in MSM values for reasons that may not be readily apparent. The linear regression analysis method is applicable in these cases as well, provided the two laboratories are certain that the assay differences are real and

consistently reproducible. For an in-depth discussion of method comparison, refer to the most current version of NCCLS document EP9—*Method Comparison and Bias Estimation Using Patient Samples.*

10.2 Quality Control

Quality control sera can be obtained from a variety of sources. Control sera supplied with MSM test kits serve as a check on reagents and technical performance. However, the control sera are of limited value for monitoring long-term shifts because the range of acceptance values given by the manufacturer for each lot of controls is generally established using the kit lot accompanying the controls.

Consequently, quality control sera that are independent from those provided by the manufacturer are strongly recommended. These can be commercial controls bought in sufficient quantity to last for one year or more, or aliquoted samples made from pools of maternal serum.

The laboratory can also use individual patient sera to monitor long-term assay drift by reassaying aliquots of stored patient samples from the more distant past. The individual patient sera should be selected from the period of time when the data used to calculate the medians currently in use were obtained. Shifts in assay values of more than 5 to 8% and, by extension, corresponding shifts in median values and screening cutoff levels, should be investigated to determine if recalculation or adjustment of reference data is necessary.

If the shift in assay values is consistent and associated with a change in median values of more than 5%, medians should be recalculated using only data collected after the time that the shift occurred. If the sample numbers measured after the shift are insufficient to calculate a reliable set of medians, applying an adjustment factor corresponding to the percentage change observed in the median values to the old medians is a reasonable substitute.

Shifts in assay values can also occur during the period of time in which the reagent is being pipetted because of time-dependent changes in assay reagents and/or technician performance (short-term drift). Such short-term drift is more likely to be a problem when relatively large numbers of tubes are being assayed (e.g., >100 tubes). To monitor such shifts, it is recommended that controls be included at the beginning and end of the run, and optionally for high volume workloads, at intervals throughout the run.

10.2.1 Internal Assay Quality Control[65]

Standard internal laboratory quality control procedures should be adopted. These should include ensuring satisfactory standard curves and monitoring within-batch and across-batch assay coefficient of variation (CV) using control sera that span the operating ranges of the analytes, less than 0.5 MoM and more than 2.0 MoM.

10.2.2 Quality Control of Risk Estimation

Risk estimation depends upon the statistical model used, which in turn depends on the distribution parameters (means, standard deviation, and correlation coefficients) of the MSMs in affected and unaffected pregnancies. Laboratories should be aware of which parameters they are using and ensure that the ones they are using are the most appropriate. For example, the standard deviation (SD) of AFP has declined in recent years with improvements in assay performance; thus, most recently reported parameters should be the best ones. Also, depending on whether gestational age was determined using LMP or ultrasound, the appropriate parameter set for each can be used. This will ensure that laboratories are achieving the best screening performance available (i.e., the highest detection rate for a given false-positive rate achievable with the information available).

10.2.3 External Quality Control

Laboratories performing screening and diagnostic MSM assays should, as part of good laboratory practice, participate in one of the presently available maternal MSM external quality control (proficiency testing) programs. Laboratories should be aware of the applicable regulations in the countries or states in which they provide laboratory services as to specific proficiency testing programs in which they must successfully participate. Formal proficiency testing survey programs are designed to monitor a laboratory's performance by periodically comparing its quantitative results statistically with those obtained by all participating laboratories and by a panel of recognized reference laboratories.

10.3 Screening Workload

Laboratories considering performing MSM measurements should screen at least 25 women per week so that they can perform the necessary epidemiological monitoring (see below) and to ensure sufficient experience and the ability to audit the screening program.

When attempting to define the minimum number of specimens required to perform reliable MSM screening, it is necessary to separate the assay component from the interpretive component. MSM assays are among the more reliable immunoassays available, with between-batch coefficients of variation of less than 10% routinely found in proficiency testing programs.

Therefore, even laboratories testing relatively few samples should be able to obtain reliable MSM test values. It is more difficult for laboratories that test small numbers of specimens to interpret the MSM value properly. Serum MSM values vary with gestational age, maternal weight, race, the presence of insulin-dependent diabetes, and multiple gestation. Each of these variables must be taken into account for proper interpretation of serum MSM test results.

10.4 Laboratory Technical Requirements

In addition to the standard laboratory expertise, screening laboratories need to include the following:

- use locally derived reference data;
- take account of the variables that affect MSM values;
- produce patient-specific risks for DS;
- provide epidemiological monitoring; and
- audit the screening program.

While it is mathematically possible to estimate patients' risk of NTD, the accuracy of the estimates may be uncertain because of changes and variations in prevalence of NTDs, and because an ultrasound examination prior to screening may identify NTD pregnancies and selectively remove them from the screened population.

10.5 Clinical Performance

It is impractical for most laboratories to monitor all the outcomes of pregnancies of all the women screened. Since in screening, the prevalence of the disorders being screened for (DS and NTD) is low (about 1 in 1000), nearly all positives are false positives; the positive rate and the false-positive rate are, therefore, almost the same. For example, if the false-positive rate were 3%, there would be 30 false

positives in 1000 women screened and one true positive (if this were detected in screening), so the screen-positive rate would be 3.1% (31 in 1000 instead of 30 in 1000).

Laboratories can therefore use their screen-positive rate to monitor their performance knowing that it accurately reflects the false-positive rate. If, for example, this were found to be 7% when an expectation is that it should be 3%, the laboratory should investigate the problem; it may be due to a shift in the normal median value.

The rationale for this epidemiological monitoring is that the screen-positive rate should fall within limits obtained by large, experienced screening programs. For example, a laboratory whose MSAFP screening cutoff level is at 2.0 MoM should have a rate of about 2%. If the cutoff level is at 2.5 MoM, the rate should be 1 to 3%.

The screen-positive rate is sensitive to changes in precision and accuracy of the MSM assay, long-term assay drift, and inappropriate reference data (median values). Often, the first indication of a decline in assay quality is a change in the screen-positive rate. All laboratories should therefore routinely monitor the screen-positive rate. Rates should be monitored monthly if the number of samples screened is sufficient to establish a statistically reliable screen-positive rate (300 to 500 specimens). Monitoring a moving window of screen-positive rates is recommended in addition to monthly monitoring.

For example, a laboratory screening 300 specimens per month could calculate a moving six-month window by averaging the screen-positive rates obtained for the current month and the screen-positive rates from the previous five months. When the rate differs from expectation, the laboratory needs to identify the source of the discrepancy. Epidemiological monitoring is a powerful addition to traditional quality control procedures and should be an integral part of all DS and NTD screening programs.

The positive rate will be determined by the screening policy and the cutoff selected, which must always be judged with the knowledge of the detection rate expected for a given false-positive rate.

11 Management of Women With Screen-Positive Results

Women can be screen-positive for either NTD or DS, and rarely for both.

11.1 Women Screen-Positive for NTD

11.1.1 Those Dated by Ultrasound

(1) Detailed ultrasound to identify the cranial markers of spina bifida and to determine whether spina bifida is present or absent (anencephaly will already have been identified from the dating ultrasound if a biparietal diameter measure is sought). The main non-NTD abnormality detected through a high serum AFP is ventral wall defect which can be identified on ultrasound. This examination should distinguish gastroschisis from omphalocele.

(2) Amniocentesis for AFAFP measurement and, if the AFAFP is ≥ 2.0 MoM, AChE determination.

11.1.2 Those Dated by LMP

(1) Ultrasound scan to identify anencephaly, and if absent to estimate gestational age from a biparietal diameter.

(2) Alter the screening results only if the difference between ultrasound estimate of gestational age and LMP estimate is greater than some predetermined number of days (laboratories differ in their

practice, ranging from 7 to 17 days). Reinterpret the test based on the revised gestational age and MoM only if the preset difference in gestational age is exceeded.

(3) Follow the steps outlined in Section 11.1.1.

11.2 Women Screen-Positive for DS

11.2.1 Those Dated by Ultrasound

Amniocentesis should be performed for a rapid diagnosis of DS using PCR or FISH followed by a karyotype.

11.2.2 Those Dated by LMP

(1) Ultrasound scan to estimate gestational age from a biparietal diameter.

(2) If the difference between ultrasound estimate of gestational age and LMP estimate is less than seven days, do not alter the screening results. If the discrepancy is greater than 7 to 17 days (laboratories differ in their practice), revise gestational age and recalculate risk. Use this value to reinterpret the test.

(3) Follow the steps outlined in Section 11.2.1.

12 Incidental Detection of Edwards Syndrome (T18)

The MSMs used to screen DS can also be used, using a different algorithm, to identify pregnancy with high risk for T18. Those women with risk estimates exceeding a specified value can have a diagnostic amniocentesis.

12.1 Identification of Trisomy 18

T18 (Edwards syndrome) is an essentially lethal genetic condition. There is some controversy surrounding screening for it; however, if screening for DS is being performed, it can be justified both medically and economically. It is associated with spina bifida or omphalocele in 25% of affected fetuses. Many are growth-retarded and, with the high fetal loss in the third trimester, can pose a danger to the mother due to increased Caesarian section rate in undiagnosed cases. Early diagnosis allows the family time to make informed choices on terminating the pregnancy.

12.1.1 Trisomy 18 Prevalence

Population prevalence of T18 in the second trimester is 1:2400 and at term is 1:8000, the discrepancy due to a fetal loss in the third trimester of 70%. Over 50% of live-born infants with T18 die within 10 days, and over 90% within 100 days.

12.1.2 Risk-Based Screening Method

Risk-based screening methods, based on triple marker and maternal age, identify 60% of affected pregnancies at a risk cutoff level of 1:100, and a false-positive rate of 0.2%. The strongest marker is uE3, followed by hCG, AFP, and maternal age in that order. In cases without associated open defects, second-trimester maternal serum levels of AFP, uE3, and hCG are on an average lower, 0.65, 0.43, and 0.36 MoM respectively, as opposed to the pattern seen in DS where hCG levels are on an average higher. In affected pregnancies with open defects, the AFP level can be elevated to as much as 2.5 MoM, although the AFP levels are not significantly higher in affected pregnancies with omphalocele. This pattern of high,

low, low is not detected in cutoff screening methods as T18 positive. Risk-based methods detect nearly twice (65% vs 39%) as many affected pregnancies as cutoff-based screening methods at the same false-positive rate of 0.2%.[5]

12.1.3 Calculation of Trisomy 18 Risk

Palomaki, et al reported that the values of each analyte for affected pregnancies fit a log-Gaussian distribution. The risk is calculated in a manner very similar to that of DS. The age-specific risk at term for T18 is one-tenth that of DS, or 1:384 for a 35-year-old. Since approximately 70% of affected fetuses spontaneously abort, the risk of a 35-year-old woman in the second trimester is 1:[3,840 x] (1.0 - 0.7) or 1:1152. The MoM truncation limits for calculating likelihood ratios for T18 are presented in the table in Section 9.8.

13 Outcome Evaluation

The evaluation of the screening services is ultimately based on the clinical writing of results reported. Ideally, outcome information on all results classified as at high risk of a defect should be collected to determine the false-positive rate. This information can be collected anonymously by the use of a simple postcard, but such data collection is obviously not a mandatory part of the proposed standard. Laboratories should be familiar with or conduct special studies that attempt to follow all participants and report false positives, false negatives, and detection rates at different risk cutoffs for each of the disorders included in the reporting scheme used by the laboratory.

The Initial Positive Rate (IPR) for each disorder for which a risk is given in the report is an indirect way to evaluate outcomes. The acceptable IPR must be derived from the experience reported in the medical literature for each disorder. Tables are frequently given which correlate MoM cutoff selected with IPR for NTD, and which correlate false-positive rates with detection rates for chromosomal disorders.[66]

Tables for background risk by maternal age and gestational age (beginning with 10 weeks to 40 weeks and increasing day by day) are available for both DS and T18.[67] Once the background (prior) risk is known, the adjusted risk by prenatal biochemical screening, a previous baby with DS, and/or an ultrasound finding may be calculated using the following approach:

Adjusted Risk = Background Risk + Other Prior Risks

Example:

A woman has a DS risk of 1/500 (0.2 %) and a previous baby with DS (1%). Her adjusted risk is:

1/500 + 1/100 = 1/500 + 5/500 = 6/500 = 1/83.

To adjust the background risk to include a targeted biochemical marker risk, the following formula is used:

Test-specific risk (as odds) = background risk x likelihood ratio (LR), where LR is the risk of the outcome associated with the biochemical marker finding.

Example:

A woman has a DS risk of 1/500 and a second-trimester marker which increases her risk by twofold (LR=2). Her test-specific risk is:

2 x 1/499= 2/499 approx. = 4/1000

With additional markers, the approach is the same but allowance needs to be made for correlations between markers in pregnancies with Down syndrome and in unaffected pregnancies.

13.1 Information Management

The efficiency of the screening process is critically dependent on the accurate collection of all the necessary variables for reliable calculation of risks and interpretation of results. The use of multiple marker screening requires a basic information technology system to enter the data and make adjustments for variables, such as weight, race, and gestational age as determined by menstrual dates or ultrasound measurement, and calculate risks. In addition to producing reports, the system should be capable of storing all data and generating evaluative data as needed. The evaluation should include the breakdowns of results by race, gestational age, and maternal age. The system should provide assessment and reassessment periodically of the gestational medians and MoM calculations for each analyte. There are commercial software products that can be used for this purpose. The data produced can be used to validate the methodology used as required by CLIA and for monitoring quality of testing over time.

13.2 *In Vitro* Fertilization

Increasing maternal age is associated with a greater risk for Down syndrome because women are more likely to release eggs with this (and other) genetic anomaly as they age. For this reason, the birth date (or age) of the egg donor rather than that of the woman carrying the child should be entered in the Patient Data Entry.

13.3 Reporting

The content of reports is decided by each screening laboratory. Reports should clearly indicate whether the risk given is a *term* risk (the risk of giving birth to a baby with DS) or the second-trimester risk (the risk that the fetus has DS); these differ because of increased spontaneous abortion of fetuses with DS. Reports should be simple and clear, presenting the marker levels in units of assay measurement and in MoM values and a categorization into screen-positive or screen-negative according to preassigned criteria. Risk assessment should be given for Down syndrome screen-positives and optionally for screen-negatives. Because of the difficulties in estimating risk in women with raised serum AFP levels (because of variation due to use of folic acid and prior ultrasound screening), this may not be appropriate. Laboratories are advised to consult with physicians, genetic counselors, and ultrasonographers to formulate a report that takes into account the needs of each group involved in the screening process. Particular care should be placed on the wording of recommendations made on the report, since this is usually a matter for individual discussion between patient and health professional.

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Appendix. Amniotic Fluid Alpha-Fetoprotein (AFAFP) for Detection of Open Neural Tube Defects (NTD)

A1 Sample Handling and Preparation

Collect the fluid by a standard amniocentesis technique. Record the presence of blood in the fluid, if it is visible to the eye.

In most situations, amniotic fluid can be handled similarly to serum. However, AFAFP is less stable than in serum, and problems can arise with sample degradation if fluids are left at ambient or elevated temperatures. Fluids should be promptly placed and stored in the refrigerator after collection until assayed. Fluids sent to other laboratories for analysis should be shipped on ice if elevated temperatures or long delays are anticipated.

Fetal blood contamination is a common cause of false-positive AFAFP results. Such samples should be centrifuged as soon as possible to remove red cells prior to assay or shipping. However, the presence of blood should be indicated on the laboratory slip, and the red cell pellet should be forwarded to the laboratory for possible testing for fetal hemoglobin if a positive amniotic fluid result is obtained (i.e., elevated AFP or NTD band in the AChE gel).

Attention needs to be paid to the possibility of contaminating amniotic fluid with fetal calf serum due to transfer from pipettes that may themselves be contaminated with amniotic fluid culture media, which contain fetal calf serum. Fetal calf serum can produce false-positive gel acetylcholinesterase results, and it is usual practice to check for this among samples that yield such results by testing for the presence of bovine albumin.

A2 Gestational Timing

A2.1 Methods of Dating

The spina bifida fetus has, on average, a smaller biparietal diameter than the unaffected fetus. Gestational dates calculated using this parameter tend to reduce detection of open spina bifida slightly, when they are used for interpreting AFAFP levels.[1-3]

As mentioned previously for maternal serum (see Section 9.4.1), two conventions are presently in use for defining which gestational week to assign a given sample and either can be used, as long as it is used consistently.

A2.2 Recommended Time for Testing

The recommended time for detecting NTD via amniotic fluid analysis is 16 to 22 weeks gestation.

A2.3 Clinically Significant Conditions Associated with Elevated Values of AFAFP

A2.3.1 Open Neural Tube Defects (NTD)

The most common major fetal malformations associated with AFAFP elevations are NTD. The Second United Kingdom Collaborative Study[4] established that a detection rate of 98% for both anencephaly and open spina bifida could be achieved with a sliding scale of amniotic fluid cutoff levels. However, false-positive results can also occur because other causes for elevated amniotic fluid results exist.

At 17 weeks gestation, the concentration of AFP in fetal blood is about 150 times that of amniotic fluid. At this time, contamination with fetal blood can cause significant elevations in the concentration of AFP in amniotic fluid. A high percentage of false-positive results is attributable to contamination with fetal blood. As a result, many can be properly classified by direct analysis of red blood cells using the Kleihauer-Betke test or by immunoassay for hemoglobin F in the fluid.

However, because about 40% of anencephalic and 20% of open spina bifida pregnancies are associated with samples contaminated with fetally derived blood, the presence of fetal blood does not itself rule out the presence of an NTD.

The most reliable and specific approach for ruling out false-positive AFAFP results is measurement of the neural enzyme acetylcholinesterase (AChE). Cerebrospinal fluid contains high concentrations of AChE. In cases of fetal neural tube defect, cerebrospinal fluid leaks from the lesion, resulting in the presence of AChE in amniotic fluid. This AChE can be visualized using polyacrylamide gel electrophoresis of amniotic fluid.[5]

The Second Report of the Collaborative Acetylcholinesterase Study established that use of a diagnostic AFAFP cutoff level of 2.0 MoM, followed by AChE confirmatory testing on the positive results, was capable of detecting 96% of open spina bifida cases with a false-positive rate of only 0.06% in nonblood-stained samples.[6] AChE analysis is, therefore, an essential confirmatory test for all amniotic fluid samples with positive AFP results. AChE analysis is generally required for less than 5% of all fluids for a typical screening program, and small laboratories may find it difficult to maintain proficiency for this type of testing. Small laboratories should, therefore, consider sending out fluids to large experienced laboratories for AChE analysis.

A2.3.2 Fetal Death

Fetal death is associated with elevated AFAFP levels. Fetal death produces a characteristic smeared pattern on AChE gel analysis.

A2.3.3 Aspiration of Maternal Urine

Unmeasurably low AFAFP concentrations are found most often when maternal urine has been aspirated by mistake. Maternal urine may be identified because of a low pH (if fresh aspirate) or by failure to give a positive “fern” test. To perform a fern test, a drop of specimen is placed on a microscope slide, allowed to dry, and then examined under the microscope. Amniotic fluid has a characteristic crystalline structure that suggests the frond of a fern, while urine does not.

A2.3.4 Fetal Malformations

Other major fetal malformations are also associated with elevated amniotic fluid results. Open ventral wall defects are the next most common fetal malformation identified. Turner’s syndrome with cystic hygromas results in AFP elevations when the hygroma ruptures or when fluid from the hygroma is inadvertently aspirated.

A2.4 Establishing Median Values in Amniotic Fluid

Obtaining sufficient numbers of amniotic fluid samples to calculate median values can be much more difficult than for maternal serum. For example, a laboratory that provides MSAFP screening to 3000 women per year will identify only 2 to 4% of those women as candidates for amniocentesis. Consequently, it is not possible for the typical screening laboratory to obtain enough samples to calculate in-house median values in a reasonable time period. However, amniotic fluid samples sent for AFP

analysis by cytogenetic laboratories can be used to supplement those obtained via the MSAFP screening program because nearly all such specimens will be from unaffected pregnancies.

In the event that the laboratory cannot obtain sufficient samples using its own resources, it should consider sending their samples to a reference laboratory with validated reference ranges.

AFAFP values are expressed as multiples of the median in the same way as described for maternal serum. The relationship of the median values and gestational age is also log-linear for gestational weeks 15 to 22, but AFAFP values decline rather than increase with gestational age.

A2.4.1 Preparation of United States Reference Materials and Assignment of AFP Values

A2.4.1.1 Working Curve Preparations

WHO (72/225), or the operationally equivalent U.S. National Reference Material, should be the working reference material intended for daily use in diagnostic laboratories.

Results from proficiency testing surveys in Europe indicate that reporting AFP test results in international units (IU) using conversion factors supplied in package inserts reduces between-kit variance. Most commonly used kits show reasonable agreement in mass units. However, some kits deviate significantly from the consensus mass unit values, and for this reason, users of these kits have failed proficiency testing exercises. On the other side of the argument are the data provided by the proficiency testing survey FBR/CAP FP-A 2004 which shows 185 laboratories reporting in ng/mL have a CV of 8.6%, while the 25 laboratories reporting in IU/mL have a CV of 8.9%. Thus, it is unlikely that most laboratories in the U.S. will change from mass units to IU at this time.

A2.4.1.2 Diluent Preparation

AFP content in amniotic fluid samples is generally measured using high-sensitivity assays designed to measure the lower AFP levels found in maternal serum. This requires an initial dilution step (1/100 to 1/200) for amniotic fluid samples. Most manufacturers supply diluent with their kits, or they sell diluent separately. *If a diluent other than that recommended or supplied by the manufacturer is used, the laboratory must show by direct comparison that the alternative diluent is suitable*. Laboratories should also include a dilution control to monitor the accuracy of this step in the analytical process.

A2.5 Normative Data and Assay Optimization

A2.5.1 Normative Data

On average, the normal median AFAFP concentration decreases by about 12% per week during the second trimester. Nearly all amniotic fluid samples from normal pregnancies contain between 5 and 40 kIU/mL during the recommended time of testing; the normal median at 17 weeks completed gestation is about 10 kIU/mL.

NOTE: 1 kIU = 1000 IU.

A2.5.2 Statistical Methods for Expressing Amniotic Fluid Alpha-Fetoprotein Results

The recommended statistical method for AFAFP is the same as for MSAFP (see Section 8.1.1). The Second United Kingdom Collaborative Study[3] has chosen multiples of the unaffected population median (MoM) to express individual AFAFP results. As with MSAFP, this method allows laboratories to compare results despite calibration differences (from different reference materials and/or other sources of bias).

References for Appendix

1) Wald NJ, Cuckle H, Boreham J, Stirrat GM. Small biparietal diameter of fetuses with spina bifida: Implications for antenatal screening. *Br J Obstet Gynecol*. 1980;87:20-21.

2) Wald NJ, Cuckle HS, Haddow JE. Should ultrasound be used to estimate gestational age in the screening and antenatal diagnosis of open neural tube defects? *Lancet.* 1980;ii:690.

3) Knight GJ, Palomaki GE. Maternal serum alpha-fetoprotein screening for fetal Downs Syndrome. *J Clin Immunol.* 1990;13:23-29.

4) United Kingdom Collaborative Study on Alpha-Fetoprotein in Relation to Neural Tube Defects. Amniotic fluid alpha-fetoprotein measurement in antenatal diagnosis of anencephaly and open spina bifida in early pregnancy—Second report. *Lancet.* 1979;ii:651-662.

5) Smith AD, Wald NJ, Cuckle HS, Stirrat GM, Bobrow M, Lagercrantz H. Amniotic fluid acetylcholinesterase as a possible diagnostic test for neural tube effects in early pregnancy. *Lancet.* 1979;i:684-688.

6) Collaborative Acetylcholinesterase Study. 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.

NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Consensus/Delegate Comments and Committee Responses

I/LA25-P: *Maternal Serum Screening; Proposed Standard*

General

1. The document fulfills a need in the laboratory community in that existing standards for maternal serum screening are out of date. However, note that the American College of Medical Genetics has updated standards currently undergoing their approval process.

- **It was the subcommittee's goal to provide recommendations that manufacturers, diagnostic laboratories, regulatory agencies, and public health authorities should consider to ensure that maternal serum screening is carried out to a high standard.**

2. Content wise, the document does fulfill its stated purpose, it is informative and useful. However, in my opinion, the flow of the document could be improved. Specifically, I would prefer that the topic of AFP be dealt with in it's entirety before moving onto uE3, hCG, etc.

- **The subcommittee considers it important to deal with all topics when possible in the way presented in order to be efficient and to be consistent with other NCCLS documents.**

3. Some data should be provided on the stability of the serum analytes during transportation, after arrival and long-term.

- **The following statement has been added to Section 6.4: "The serum analytes are reasonably stable with the exception that free βhCG tends to increase with storage at ambient temperatures due to its separation from total hCG."**

4. Need to address computing medians separately for LMP and US dated pregnancies in addition to recommending that separate parameters be used to estimate risk.

- **This has been addressed in Section 9.3 with the addition of the following two sentences:**

  "It is usually best to calculate medians separately for gestational ages based on "dates" (time from last menstrual period) or using ultrasound measures such as crown-rump or biparietal diameter."

  "When each laboratory introduces screening or adopts a new set of reagents, it is reasonable to use about 100 data points between 16 and 18 weeks and then reestimate the weekly medians as larger numbers accumulate."

5. The problems associated with contamination with fetal calf serum need to be addressed and methods to identify the problem described.

- **This has been addressed in Section A1 of the Appendix with the addition of the following paragraph:**

  "Attention needs to be paid to the possibility of contaminating amniotic fluid with fetal calf serum due to transfer from pipettes that may themselves be contaminated with amniotic fluid culture media, which contain fetal calf serum. Fetal calf serum can produce false-positive gel acetylcholinesterase results, and it is usual practice to check for this among samples that yield such results by testing for the presence of bovine albumin."

6. There should be more information on free HCG used now in Europe for maternal screening.

- **The following sentences have been added to the third paragraph of Section 8.2.1: "In the second trimester, free βhCG can be used instead of total hCG. In the first trimester, βhCG is a somewhat better screening marker than total hCG. Free βhCG is widely used outside the U.S. in antenatal screening for Down syndrome."**

7. The new WHO standards for HCG should be mentioned, i.e., WHO: 99/642, 650, 688, 692, 708, and 720 used for calibration.

- **Reference to these standards has been added to Section 7.4.3.**

8. Throughout the document, the rate of neural tube defects is reported to be 1 to 2 per 1000. This rate should be for open neural tube defects, and recent comprehensive reports put the rate lower at 1.1 per 1000 (Williams, et al. *Terat.* 2002;66:33-9 after restricting to states with prenatal ascertainment). This is especially important because of the routine fortification of grain products in the United States.

- **The following sentence has been added to Section 8.1.2: "Recent data put the NTD birth prevalence at 1.1%."**

9. Throughout the document, maternal serum AFP is sometimes abbreviated as MSAFP while uE3 is never abbreviated as MSuE3. This might be because AFP is also measured in amniotic fluid, but even in the Appendix where that is addressed, it is referred to as AFAFP, AFP in amniotic fluid, and just AFP (Section A2.3.4, paragraph 1, line 3). I suggest that just three letters be used for all analytes and if there might be confusion, use the phrases *amniotic fluid* or *maternal serum* as needed.

- **Revisions have been made to consistently use MSAFP or AFAFP which are necessary for efficiency.**

10. The use of MoMs is often incorrect. For example, "the cutoff level is at 2.0 MoMs." This would translate into 'multiples of the medians.' These should be changed to just "at 2.0 MoM."

- **The subcommittee agrees with the recommendation to change "MoMs" to "MoM" and this revision has been made throughout the document.**

Foreword

11. Page vii, bullet 1: The phrase "neural tube defect" is used throughout the document. In most instances, the correct phrase is "open neural tube defect." An example is, "The need for second trimester AFP screening for NTD."

- **As specified in the foreword, "open neural tube defect" is abbreviated as NTD.**

Section 2, Introduction

12. Line 4: The phrase "use of specific testing during pregnancy to assess the welfare of the pregnant woman" is not correct. It could be rewritten as "use of specific testing during pregnancy to assess fetal well-being."

- **The sentence has been revised as suggested**

Section 4.2, Acronyms and Abbreviations

13. Some of the abbreviations are nonstandard and, given the length of the document, seem unnecessary. Especially troublesome are DS, MSS, and MSM.

- **Abbreviations for DS, MSS, and MSM are necessary and accepted in the medical and scientific community.**

Section 8.2, Other Maternal Serum Markers

14. Line 9: It is stated that "this marker [uE3] is roughly equivalent to MSAFP in discriminatory power." Although the separation is similar, the distribution of results for uE3 are much tighter than for AFP and therefore, the discrimination is substantially better.

- **The sentence has been revised to read: "In 1988, maternal serum unconjugated estriol (uE3) was shown to be significantly reduced in DS pregnancies; levels of uE3 are about 25% lower in DS pregnancies, making this marker separation equivalent to MSAFP but the distribution of uE3 is tighter than for MSAFP and therefore, the discrimination between affected and unaffected pregnancies is greater."**

Section 8.2.5, Pregnancy-Associated Plasma Protein (PAPP-A)

15. The section describing the assay methodology and results for PAPP-A measurements in the first trimester is out of place in a second trimester document. This is confirmed by the lack of any references to PAPP-A in any of the other sections describing reference ranges, adjustment, etc.

- **The importance of including PAPP-A in this document is described in an additional sentence which reads: "Recently, second-trimester maternal screening has been shown to be enhanced by the inclusion of a PAPP-A measurement at about 10 to 11 weeks of pregnancy where PAPP-A measurement is then used together with the second trimester measurements in a single integrated test."**

Section 9.2, The Effect of Maternal Weight on uE3, hCG, and DIA

16. Paragraph 2: When referring to the need for weight adjustment, the document should include the need for weight adjustment for Trisomy 18 as a reason to adjust all markers. Also, all of the variances are reduced somewhat, making screening more efficient, even if the adjustment has 'offsetting effects.'

- **A sentence has been added which reads, "In addition, weight adjustment is important for Trisomy 18."**

Section 9.3, Expressing the Concentration of Markers as Multiples of the Median (MoM)

17. Computing reference ranges by gestational age is addressed inconsistently in different sections. "A minimum of 100 data points is required for each week" (Section 9.3, paragraph 6, line 1). Later, this is reduced to "about 100 samples…from 16 to 18 weeks (if available, data at earlier and later weeks should be included" (Section 10.1.1, paragraph 2, line 5). No mention is made of the more common and reasonable methodology of taking a consecutive series of 300 or so samples and deriving medians (ref).

- **Text has been revised in Section 9.3 to make it consistent with Section 10.1.1. Monitoring a moving window of screen-positive rates using 300 to 500 specimens is addressed in Section 10.5.**

18. Paragraph 6: Earlier, the guidelines recommend using day-specific reference ranges but later they seem to suggest usage of medians by completed week (Section 10.1, paragraph 1, line 1).

- **The first sentence in Section 10.1 has been revised to read: "Reference values in MSM screening consist of a set of median values calculated for each week of gestation and each day of a gestational week using the laboratory's own MSM assay values, measured preferably on the population to be screened."**

Section 9.4.1, Methods of Dating

19. Paragraph 1, Line 3: Gestational age by rounding is strongly discouraged and its use is not considered acceptable in the FBR/CAP proficiency testing. I suggest being consistent with this standard. In addition, the majority of laboratories (FP-A, 2004, 179 labs report in decimal weeks while 21 report in completed weeks) participating in FBR/CAP FP survey use day-specific dating (not completed week of gestation). This should be changed.

- **The last sentence in the first paragraph in Section 9.4.1 has been revised to read: "Results from proficiency testing programs in the United States indicate that completed weeks of gestation are used by almost all laboratories and this approach should be generally adopted."**

Section 9.4.2, Gestational Age for Screening

20. Paragraph 1, Line 2: One of the reasons that laboratories do not screen for open NTD beyond 21 weeks is not correct ("because of a lack of normative data"). It is too late to screen at that time in pregnancy (as stated), and therefore, few samples are received. If it were acceptable and useful, normative data would be available.

- **The subcommittee does not think any amendment is needed here. The text says that there is a lack of normative data, which is true, and it is also late in pregnancy to intervene, which is also true.**

21. Paragraph 2: This paragraph should include a statement that if samples are to be interpreted for both open NTD and Down syndrome, then 15 weeks is the earliest that samples should be interpreted.

- **The suggested statement has been added.**

22. Paragraph 2: Lack of normative data should not preclude NTD screening beyond 21 weeks. The log-linear relationship between MSAFP and gestational age holds from 14 to 25 weeks. Therefore, a weighted regression analysis can be used to extrapolate medians to later gestational ages. The difficulties obtaining subsequent diagnostic testing are really the only reasons here to limit the gestational weeks for testing.

- **This is essentially the same point as mentioned in Comment 20 above. There are too few data in the literature showing the separation of AFP values in affected and unaffected pregnancies after about 21 weeks.**

Section 9.5.1, Integrated Testing Protocol

23. The last paragraph seems to be out of place. Indicating whether risk is term or second trimester applies to all DS screening, not just the integrated test. Should this be in Section 13.3?

- **The subcommittee agrees with this recommendation and the last paragraph in Section 9.5.1 has been moved to Section 13.3.**

Section 9.5.2, Timeliness of Reporting

24. This section contains no real guidance. What is an unnecessary delay? How should the results be transmitted?

- **The subcommittee agrees with the commenter and the section has been deleted.**

Section 9.6, Patterns of Second-Trimester Marker Concentrations

25. Possible indication for undetectable uE3: The most common cause of undetectable uE3 in a viable pregnancy is Steroid Sulfatase Deficiency. This can be part of a contiguous gene syndrome, but can be an isolated condition caused by mutations other than deletions and therefore should be specifically mentioned rather than lumped with CGDs.

- **The subcommittee agrees with the commenter and the phrase "or other mutations" has been added.**

26. The table showing patterns in selected outcomes should be removed. It essentially suggests that it might be warranted to look for partial molar pregnancies with triploidy when following up low uE3 and high hCG results. Perhaps replace it with a reference to a published source that contains summaries of this information.

- **The subcommittee believes this table is very important. It was included in this document as requested by representatives of the laboratory community.**

Section 9.8, MoM Truncation Limits to Be Used

27. I thought that the lower recommended lower truncation limit for DIA was 0.5 rather than 0.4? I am surprised that the document is so specific as to list acceptable truncation limits when it is not that specific for other important factors such as corrections for race and IDDM status.

- **See the response to Comment 26 above. The laboratory community requested that the document include truncation limits. Corrections for race and IDDM status are also discussed in detail in the document.**

28. The table providing truncation limits should be removed. Truncation limits are already included in the parameter sets recommended for use later in the paragraph (those references should be Haddow 1998 and Knight 1998). Those for Trisomy 18 are included in the reference in that section as well.

- **See responses to Comments 26 and 27 above.**

Section 10.1.1, Establishing Median Values in Maternal Serum

29. Line 1: Care should be taken in assuming that the results of proficiency testing on artificial samples are equivalent to those found in patient samples. A warning is needed.

- **The subcommittee agrees with the recommendation and the proposed warning statement has been added.**

Section 10.2.2, Quality Control of Risk Estimation

30. This section needs to be more specific to be helpful to laboratories. Just how does a laboratory ensure that it is using the most appropriate parameters? The comment about the SD of AFP declining in recent years suggests that more recently reported parameters will be the best ones. Should that be explicitly stated? Any other considerations? Also, it might be too strong to say that labs "should" use different parameters depending on the method for determining gestational age. Are laboratories that don't do this doing a bad job of screening? Perhaps the standard should say that laboratories "can" do this to achieve the best screening performance.

- **The subcommittee agrees with the recommendations and the text has been revised to read: "For example, the standard deviation (SD) of AFP has declined in recent years with improvements in assay performance; thus, most recently reported parameters should be the best ones. Also, depending on whether gestational age was determined using LMP or ultrasound, the appropriate parameter set for each can be used."**

Section 10.3, Screening Workload

31. Paragraph 2: It is uncommon to have CVs of 10% or less for any sample in a given distribution of the CAP/FBR FP survey, either in mass units or in MoM. Actual reports should be examined prior to revising this section.

- **The second paragraph in Section 10.3 should refer to "between-batch coefficients of variation" rather than between-laboratory coefficients of variation. This revision makes the 10% figure correct. Also, the text addressing CAP/FBR FP surveys has been revised accordingly.**

Section 10.5, Clinical Performance

32. Paragraph 3: The positive rates quoted for open neural tube defect screening are too high 'at 2.0 MoM should have a rate of 3 to 5%' and inconsistent '5% at two times the median' (Section 8.1.1, paragraph 2, line 7). This result is especially important to get right, given the statement in the guidelines that "the standard deviation of AFP has declined in recent years" (Section 10.2.2, line 4).

- **Approximately 0.5% of MSAFP measurements from unaffected pregnancies will be at or above a cutoff of 2.5 MoM and about 2% of unaffected pregnancies will be at or above 2.0 MoM. The text has been revised for consistency in Section 10.5 and Section 8.1.1.**

Section 11.1.1, Those Dated by Ultrasound

33. Paragraph 1: The statement "anencephaly will already have been identified from the dating ultrasound" will often not be true if the dating is by CRL—a reasonable option.

- **The statement has been revised to read: "Detailed ultrasound to identify the cranial markers of spina bifida and to determine whether spina bifida is present or absent (anencephaly will already have been identified from the dating ultrasound if a biparietal diameter measure is sought)."**

Section 11.2.1, Those Dated by Ultrasound

34. The recommendation that all amniocenteses should be followed by PCR or FISH should be removed, and reference made to recommendations by professional organizations.

- **The subcommittee does not agree with this comment. Many professional organizations recommend that all amniocenteses should be followed by PCR or FISH.**

35. Is it within the scope of this document to specifically recommend PCR or FISH testing followed by a karyotype? That seems like a topic for a document on prenatal diagnosis of chromosome disorders.

- **See the response to Comment 34 above.**

Section 12.1.2, Risk-Based Screening Method

36. The statement "Maternal age does not appear to be significant, although median levels may be lower in older women, especially for hCG" can be misinterpreted as meaning that the risk of Trisomy 18 is not associated with age. There is no reference for the second part of the sentence; it should be provided or the phrase removed.

- **This sentence has been removed.**

Section 13, Outcome Evaluation

37. There are far better methods available to take into account a previous baby with Down syndrome 'previous baby with T21 (1%).' T21 is not an acceptable abbreviation for Down syndrome.

- **Examples of calculations are provided and "T21" has been replaced by "DS."**

References

38. The references need to be carefully checked and corrected. For example, the first name in the first reference is incorrect. "Merkotz" should be "Merkatz" and "Mocri" should be "Macri." In the fourth reference, the journal is "BMJ" not "GMJ."

- **Reference citations have been corrected.**

Appendix, Amniotic Fluid Alpha-Fetoprotein (AFAFP) for Detection of Open Neural Tube Defects (NTD)

Section A2.2, Recommended Time for Testing

39. The recommended time for testing AFP in amniotic fluid is now known to extend earlier than "16 to 22 weeks gestation." Also, add the difficulties in interpretation encountered with late first trimester samples.

- **Testing AFP in amniotic fluid should not be done earlier than 13 or 14 weeks gestation.**

Section A2.4, Establishing Median Values in Amniotic Fluid

40. Paragraph 1, Line 3: Should amniotic fluids from screen positive women be used for reference ranges?

- **The subcommittee does not understand the point of this comment.**

41. Paragraph 2, Line 1: The text refers to 'options B and C' that do not seem to exist in the document. It should be removed.

- **The subcommittee agrees with the recommendation and the text referring to "options B and C" has been removed.**

42. Paragraph 2: Amniotic fluid medians are only log-linear from 15 weeks on, not "log-linear for gestational weeks 14 to 22.

- **The text has been corrected to read: "…log-linear for gestational weeks 15 to 22…"**

Section A2.4.1.1, Working Curve Preparations

43. Paragraph 2: There are important reasons why reporting in IU is likely to increase variability rather than reduce it. "It is recommended that all proficiency testing results be reported in IU." Also, proficiency testing results do not show that IU reduces variance for AFP (FBR/CAP FP-A survey 2004 shows 185 labs reporting in ng/mL have a CV of 8.6%, while the 25 labs reporting in IU/mL have a CV of 8.9%).

- **Section A2.4.1.1 has been revised to include both results from Europe and the United States by deleting the last sentence in the first and second paragraphs and revising the second paragraph to describe the preferred unit usage in Europe and in the U.S.**

Section A2.5.3, Assay Optimization

44. The last two paragraphs seem out of place as they refer to serum testing and the Appendix deals with amniotic fluid. They should be moved or removed.

- **The subcommittee agrees with the comment and Section A2.5.3 has been removed.**

NOTES

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any healthcare service's path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

I/LA25-A addresses the following quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the next page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X EP9	M29	M29	X	X M29		M29

Adapted from NCCLS document HS1—*A Quality Management System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytic, analytic, and postanalytic. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

I/LA25-A addresses the following steps within the clinical laboratory path of workflow:

Preanalytic					**Analytic**		**Postanalytic**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Posttest Specimen Management
X	X	X	X	X	X	X	X	X

Adapted from NCCLS document HS1—*A Quality Management System Model for Health Care*.

Related NCCLS Publications*

EP9-A2 **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Second Edition (2002).** This document addresses procedures for determining the bias between two clinical methods or devices, and for the design of a method comparison experiment using split patient samples and data analysis.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections—Second Edition; Approved Guideline (2001).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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