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October 2004

# C45-A

## Measurement of Free Thyroid Hormones; Approved Guideline



This document addresses analytical and clinical validation of free (nonprotein-bound) thyroid hormone (FTH) measurement procedures.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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# Measurement of Free Thyroid Hormones; Approved Guideline

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## Abstract

Clinical and Laboratory Standards Institute document C45-A—*Measurement of Free Thyroid Hormones; Approved Guideline* is a guideline for free (nonprotein- bound) thyroid hormone (FTH) testing. The primary audience for this publication is personnel responsible for the development, manufacture, approval, and/or use of FTH measurement procedures. The guideline briefly discusses FTH measurement procedures with respect to design, factors confounding measurements, and specimen choice and collection, and addresses existing problems in definitions and specific nomenclature. However, it mainly emphasizes analytical and clinical validation of FTH measurement procedures. Assessment of analytical validity of an FTH measurement procedure not only implies demonstration of sufficient intrinsic quality or robustness against challenging factors, but also of metrological traceability to the Système International d'Unités (SI). In this respect, the guideline provides recommendations on the implementation of a reference measurement system in the development/validation process of *in vitro* diagnostic medical devices.

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## Contents

## Foreword

The measurement of free (nonprotein-bound) thyroid hormone (FTH) [free thyroxine (FT4) and free triiodothyronine (FT3)] concentrations in serum has been widely accepted to be clinically useful in differential diagnosis of thyroid disorders, monitoring therapeutic interventions, and follow-up of patients. However, along with the increasing use of FTH testing, confusion has arisen regarding definitions and specific nomenclature. Also, there is a continuing controversy regarding the validity of current routine measurement procedures. One major reason is that the design of some FTH routine measurement procedures insufficiently accounts for the Laws of Mass Action, and consequently lack a valid physicochemical basis. The lack of a generally accepted FTH reference measurement procedure for validation of routine measurement procedures additionally contributes to this controversy. Thus, there is an urgent need for clarifications and recommendations in these respects.

The validity of an FTH measurement procedure depends on two key requirements: (1) its capability to correctly determine serum FTH concentrations (i.e., with minimal analytical error and reflecting *in vivo* FTH); and (2) its capability to provide measurement results that are clinically useful. Therefore, designers/manufacturers urgently need minimum recommendations to assess/demonstrate both analytical and clinical validity of their measurement procedures. Furthermore, in an era of increasing efforts toward standardization of measurements in laboratory medicine, validity in the above terms extends to metrological traceability to a reference measurement system. Major drivers behind this concept have been the EC Directive on *In-Vitro* Diagnostic Medical Devices (98/79/EC) (IVD Directive) and supporting ISO/CEN standards. Consequently, the development of a reference measurement system for FTH measurement is of top priority. Following this, manufacturers of *in vitro* diagnostic medical devices shall be given guidance to implementing and demonstrating metrological traceability.

Therefore, the clarifications and recommendations given in this document are expected to benefit designers, manufacturers, regulators, and users of FTH measurement procedures.

### Key Words

Accuracy, analytical/clinical validation, free thyroid hormone, reference measurement system, thyroxine, traceability, triiodothyronine, trueness

# Measurement of Free Thyroid Hormones; Approved Guideline

## 1 Scope

This guideline, C45-A—*Measurement of Free Thyroid Hormones; Approved Guideline*, gives an overview of the pathophysiology of blood free thyroid hormones (FTH) and of the principles and limitations of current routine measurement procedures. Recommendations are included for specimen collection, stability, and storage. The main focus of the document is on the analytical and clinical validation of FTH measurement procedures and on their standardization by implementation of a reference measurement system. Specific recommendations for validating both analytical and clinical performance are provided. Direct equilibrium dialysis (ED) and direct ultrafiltration (UF) are proposed as candidate reference measurement procedures, and recommendations are provided for appropriate use and calibration of these measurement procedures. Lastly, the document provides a summary of the objectives and status of the European project to develop higher order reference measurement procedures for free thyroid hormones.

## 2 Introduction[1-13]

When considering the concentration of free T4 (FT4) in serum or plasma, a clear distinction must be made between the *in vivo* situation and the *in vitro* condition that holds once a blood sample has been taken. In both, a dynamic equilibrium exists between free and bound T4 that depends on the concentration of the transport proteins as well as the affinity of their binding sites (which depends on the presence of competing ligands, pH, temperature, and other factors).

The *in vivo* relationship between FT4 and TT4 must be considered in terms of the free hormone hypothesis, which in its original and simplest form states that the biological effect (including disposal) of (thyroid) hormone is governed by its free, nonprotein-bound moiety, as can be measured *in vitro*. Especially this last statement is now considered a gross oversimplification, since it implies that everywhere in the body the same conditions prevail, as in the bulk of the circulation with unlimited availability of T4 and instantaneous equilibration. More sophisticated models postulate a complex interplay between flow, diffusion, and dissociation as means by which the hormone is made available to the target tissues.[13] The pivotal point here is the recognition that relative depletion of hormone may arise at the interface of cell structures within the microcirculation, when the hormone uptake rate by these cells is high. The magnitude of this depression inversely relates to the concentration of reversibly bound hormone. Nevertheless, these models should be considered as refinements or improvements of the free hormone hypothesis as long as hormone uptake by the cells is supposed to take place via the free form of the hormone, in contrast with models in which the hormone-transport protein complex enters the cell in its entirety or where hormone is transferred directly from the transport protein to a membrane transporter molecule.

The *in vivo* situation constitutes an open system into which the thyroid releases T4, while on the other hand, T4 is removed from the system by degradative processes. These, analogous to the biological effects, depend on the uptake rate of free hormone at the interface of the microcirculation and the tissues that harbor these processes. Notwithstanding the fact that local FT4 levels may differ from those in the bulk of the circulation, changes in serum T4 binding capacity initially lead to modified FT4 concentration(s) and therefore, modified uptake and ensuing T4 elimination. With T4 production remaining constant, the size of the circulating TT4 pool as well as FT4 levels change until a new steady state is attained in which the original serum FT4 (and thus T4 elimination rate) are restored. In this new steady state, TT4 has been adjusted corresponding to the change in serum T4 binding capacity.

In contrast, once a blood sample has been collected, it constitutes a closed system. Therefore, the TT4 concentration cannot change. Thus, while *in vivo*, the TT4 depends on the FT4 concentration, *in vitro*, the

opposite is true. Given a certain level of TT4, FT4 *in vitro* varies with the addition of binding sites and the presence of either inhibitors or promoters of binding.

The very first commercially available direct FT4 assay was marketed by Lepetit in 1977 and was based on sequestration to equilibrium of T4 from serum to porous polymerized dextran particles, followed by immunoassay of the T4 eluted. This procedure worked by virtue of the fact that the internal space of the dextran matrix was accessible to unbound T4 only. The necessary assay sensitivity could be attained, because the matrix concentrates T4 by a constant factor of about 40 in a dose-independent manner. In most if not all respects, this assay was theoretically equivalent to ED followed by T4 immunoassay in the dialysate. During the 1980s, there was great interest in immunoassay of FT4 based on assessment of antibody occupancy. If the T4-binding antibody constitutes a single order of noninteracting binding sites, the ambient FT4 concentration is directly proportional to the antibody occupancy ratio (i.e., the fraction of the total binding capacity that is actually occupied by the ligand) divided by the unoccupied binding capacity. This follows from the Law of Mass Action:

$$K_{diss} = [\text{Free ligand}] \cdot [\text{Free binding sites}]/[\text{Ligand-protein complex}]$$

Or:
$$[\text{Free ligand}] = K_{diss} \cdot [\text{Ligand-protein complex}]/[\text{Free binding sites}] =$$

$$= K_{diss} \cdot \{\text{Occupancy ratio}\}$$

In this relationship, the free ligand (FT4) concentration is the ambient concentration (i.e., the concentration in the equilibrium state that results from the effects of all factors involved). In real life assays, this may differ appreciably from the original concentration in the sample, as will be discussed.

Assessment of antibody occupancy *per se* is not required. There is even no need for actual attainment of equilibrium—though this may be desirable in view of assay stability—nor is it necessary to have a measurement signal that follows a simple relationship with antibody occupancy, as long as it is an unambiguous measure of occupancy; that is, samples with equal FT4 should give the same signal irrespective of their TT4 concentration. The unknown sample's signal is compared with the signal produced using a standard series of samples with known FT4. Therefore, these measurement procedures must be considered as "comparative."

A full account of all known assay principles can be found in Ekins' standard text.[4] There are three principal ways to obtain a measure of antibody occupancy:

1. *Two-step, back-titration:* In the first step, the serum sample is incubated with anti-T4 antibody, usually coupled to a solid support. The antibody will be occupied by a certain amount of T4, depending on the ambient FT4 concentration. The sample is washed away, and the solid-phase antibody (fractionally occupied by T4) is incubated with labeled T4. In this second step, binding of the label to the antibody exclusively depends on how much T4 has become bound during the first incubation and thereby exclusively depends on (ambient) FT4. Labeled T4 as a tracer may be replaced by a suitable analog of T4 that has a lower affinity for the antibody in order to attain better assay precision. Since the sample has been completely removed before addition of tracer, there are no special requirements of inertness toward serum components, such as a labeled analog has to meet in a one-step format.

2. *One-step, labeled analog:* Similar to the above principle, the occupancy of the antibody is reflected in the amount of label bound to the antibody. However, this principle differs from the previous one in that the sample is not separated from the antibody before addition of the label. Since binding of the label to the antibody must depend exclusively on the antibody-bound T4, the label must not interact with any sample constituent. Labeled analogs of T4 have been prepared for this purpose, but it has

## 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 HS1—*A Quality 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. 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 |

C45-A addresses the 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 following page.

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment | Process Improvement | Service & Satisfaction | Facilities & Safety |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | X<br>EP7 | | | | | | M29 |

Adapted from NCCLS document HS1—*A Quality 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, GP26-A2 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.

C45-A addresses the clinical laboratory path of workflow steps 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 following page.

| **Preanalytic** | | | | | **Analytic** | | **Postanalytic** | |
|---|---|---|---|---|---|---|---|---|
| Patient Assessment | Test Request | Specimen Collection | Specimen Transport | Specimen Receipt | Testing Review | Laboratory Interpretation | Results Report | Post-test Specimen Management |
| | H3 | H1<br>H3<br>H4 | H18 | H18 | | | | |

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

## Related NCCLS Publications*

**EP6-A** **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline (2003).** This document provides guidance for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

**EP7-A** **Interference Testing in Clinical Chemistry; Proposed Guideline (2002).** Provides background information, guidance, and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.

**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.

**H1-A5** **Tubes and Additives for Venous Blood Specimen Collection; Approved Standard—Fifth Edition (2003).** This standard contains requirements for blood collection tubes and additives including heparin, EDTA, and sodium citrate.

**H3-A5** **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. It also includes recommendations on order of draw.

**H4-A5** **Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens; Approved Standard—Fifth Edition; (2004).** This document provides a technique for the collection of diagnostic capillary blood specimens, including recommendations for collection sites and specimen handling and identification. Specifications for disposable devices used to collect, process and transfer diagnostic capillary blood specimens are also included.

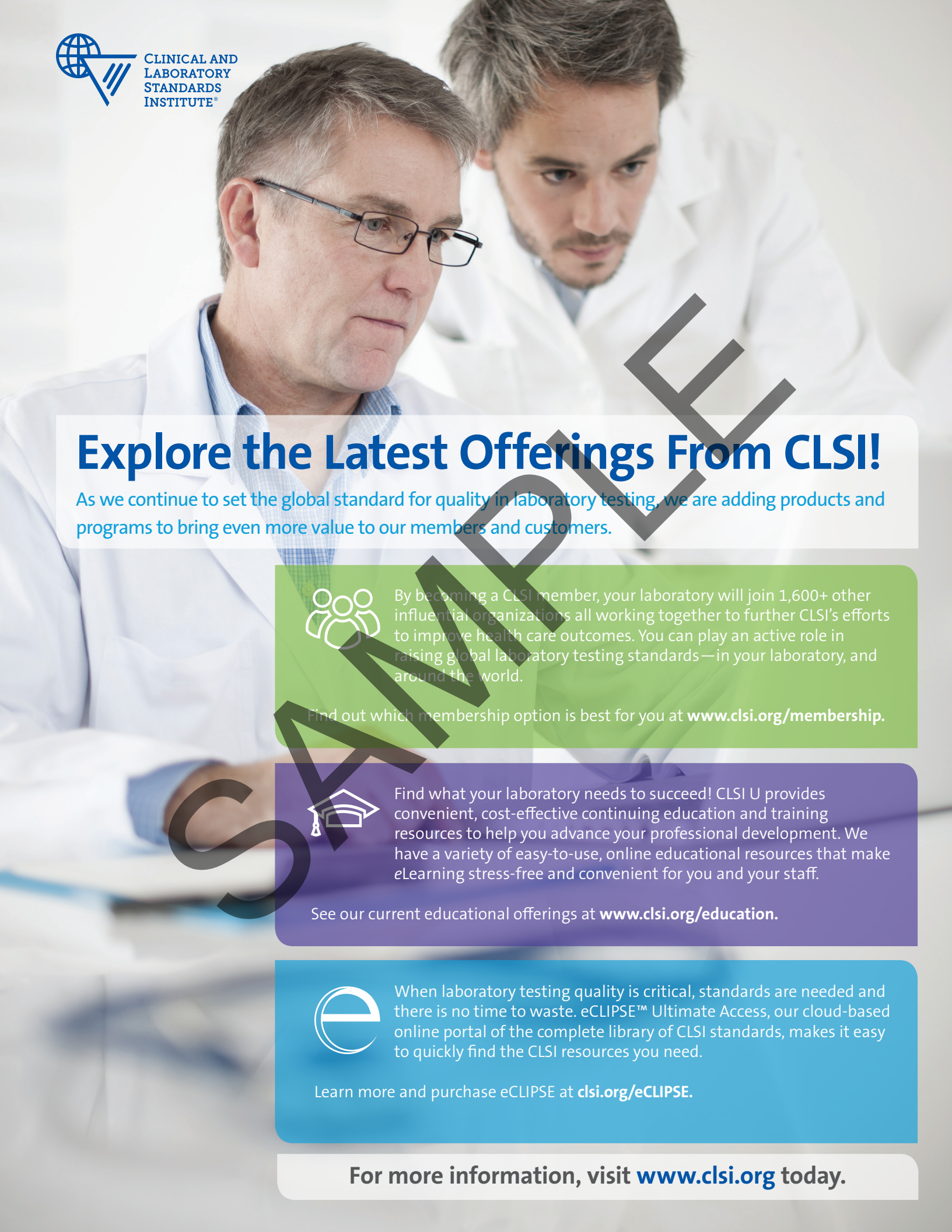
**H18-A2** **Procedures for the Handling and Processing of Blood Specimens; Approved Guideline—Second Edition (1999).** This guideline addresses multiple factors associated with handling and processing specimens, as well as factors that can introduce imprecision or systematic bias into results.

**M29-A2** **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (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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