B. Free Thyroxine (FT4) and Free Triiodothyronine (FT3) Estimate Tests

Thyroxine in blood is more tightly bound to serum proteins than is T3, consequently the free T4 (FT4) bioavailable fraction is less than free T3 (0.02% versus 0.2%, FT4 versus FT3, respectively). Unfortunately, the physical techniques used for separating the minute free hormone fractions from the predominant protein-bound moieties are technically demanding, inconvenient to use and relatively expensive for routine clinical laboratory use. Those methods that employ physical separation of free from bound hormone (i.e. equilibrium dialysis, ultrafiltration and gel filtration) are typically only available in reference laboratories. Routine clinical laboratories typically use a variety of free hormone tests that estimate the free hormone concentration in the presence of protein-bound hormone. These free hormone estimate tests employ either a two-test strategy to calculate a free hormone "index" [see Section-3 B2] or a variety of ligand assay approaches *(14,145,147)*. In reality, despite manufacturers claims, most if not all FT4 and FT3 estimate tests are binding-protein dependent to some extent *(148,149)*. This binding protein dependence negatively impacts the diagnostic accuracy of the free hormone methods that are subject to a variety of interference's that can cause misinterpretation or inappropriate abnormal results (Table 1). Such interferences include sensitivity to abnormal binding proteins, in-vivo or in-vitro effects of various drugs [Section-3 B3(c)vi], high FFA levels and endogenous or exogenous inhibitors of hormone binding to proteins that are present in certain pathological conditions *(60)*.

1. Nomenclature of Free T4 (FT4) and Free T3 (FT3) Estimate Methods

Considerable confusion encompasses the nomenclature of free thyroid hormone tests. Controversy continues regarding the technical validity of the measurements themselves and their clinical utility in conditions associated with binding protein abnormalities *(145,147,148,150,151)*. Free hormone testing in clinical laboratories are made using either index methods that require two separate tests, test ligand assays or physical separation methods that isolate free from protein-bound hormone prior to direct measurement of hormone in the free fraction. Ligand assays are standardized either with solutions containing gravimetrically established concentrations of hormone, or use calibrators with values assigned by a physical separation method (i.e. equilibrium dialysis and/or ultrafiltration). Physical separation methods typically are manual, technically demanding and quite expensive for routine clinical use. Index and ligand assay tests are more commonly used in the clinical laboratory setting, where they are typically performed on automated immunoassay analyzer platforms *(17)*.

Guideline 11. Free Hormone Test Nomenclature

- The free hormone methods used by most clinical laboratories (indexes and immunoassays) do not employ physical separation of bound from free hormone and do not measure free hormone concentrations directly! These tests are typically binding protein dependent to some extent and should more appropriately be called **"Free Hormone Estimate"** tests, abbreviated FT4E and FT3E.
- In general, Free Hormone Estimate tests overestimate the FT4 level at high protein concentrations and underestimate FT4 at low protein concentrations.

Unfortunately, a confusing plethora of terms have been used to distinguish the different free hormone methods and the literature is filled with inconsistencies in the nomenclature of these tests. Currently, there is no clear methodologic distinction between terms such as "T7", "effective thyroxine ratio", "one-step", "analog", "two step", "backtitration", "sequential", "immunoextraction" or "immunosequestration", "ligand assay" because manufacturers have modified the original techniques or adapted them for automation *(147)*. Following the launch of the original one-step "analog" tests in the 1970s, the term "analog" became mired in confusion *(147)*. This first generation of hormone-analog tests were shown to be severely binding-protein dependent and have since been replaced by a new generation of labeled-antibody "analog" tests which are more resistant to the presence of abnormal binding proteins *(147,152)*. Unfortunately, manufacturers rarely disclose all assay constituents or the number of steps involved in an automated procedure so that it is not possible to use the method's nomenclature (two-step, analog etc) to predict its diagnostic accuracy for assessing patients with binding protein abnormalities *(152)*.

2. Index Methods: FT4I and FT3I

Index methods are free hormone estimate tests that require two separate measurements *(146)*. One test is a total hormone measurement (TT4 or TT3) the other is an assessment of thyroid binding protein concentration using either an immunoassay for TBG or a T4 or T3 "uptake" test called Thyroid Hormone Binding Ratio (THBR). Alternatively, indexes may be calculated from a TT4 measurement paired with an estimate of the free T4 fraction determined by isotopic dialysis. In this case, the quality and purity of the tracer employed critically impacts the accuracy of the index *(149,153,154)*.

(a) Indexes using TBG Measurement

Calculation of a FT4I using TBG only improves diagnostic accuracy compared with TT4 when the TT4 abnormality results from an abnormal concentration of TBG. In addition, the TT4/TBG index approach is not fully TBG independent, nor does it correct for non TBG-related binding protein abnormalities or for TBG molecules which have abnormal affinity *(141,155-158)*. Thus, despite the theoretical advantages of using a direct TBG measurement, TT4/TBG indexes are rarely used because TBG binding capacity can be altered independent of changes in the concentration of TBG protein, especially in patients with NTI *(99)*. In addition, TBG binding reflects 60 – 75% of the binding capacity thus relying on TBG alone excludes hormone binding to transthyretin and albumin.

(b) Indexes using a Thyroid Hormone Binding Ratio (THBR) or "Uptake" Test

"Uptake" tests have been used to estimate protein binding of thyroid hormones since the 1950s. Two different types of "uptake tests " have been used. "Classical" uptake tests add a trace amount of radiolabeled T3 or T4 to the specimen and allow the labeled hormone to distribute across the thyroxine binding proteins in exactly the same way as endogenous hormone *(146,154)*. Since only a trace amount of labeled T3 or T4 is used, the original equilibrium is barely disturbed. The distribution of the tracer is dependent upon the saturation of the binding proteins. Addition of a secondary binder or adsorbent (anion exchange resin, talc, polyurethane sponge, charcoal, or antibody-coated bead, etc.) results in a redistribution of the T3 or T4 tracer into a new equilibrium, that now includes the binder. The tracer counts sequestered by the adsorbent are dependent on the saturation of the binding proteins: the higher the saturation of the binding proteins, the greater the amount of tracer in the adsorbent. The uptake of added tracer into the absorbant results in an indirect measure of TBG. When the TBG concentration is low, TBG binding sites are highly saturated with T4 so that a smaller amount of added T3 tracer binding will bind to TBG and more will be being taken up by the adsorbent. Conversely, when the TBG concentration is high, TBG saturation with T4 is low, more tracer binds unoccupied TBG binding sites and less becomes bound to the adsorbent. Unfortunately the relationship between THBR and TBG concentration is non-linear, such that index testing usually does not correct TT4 abnormalities resulting from grossly abnormal TBG concentrations *(158)*.

It has been recommended that a normal serum sample standard be used to normalize the response of the assays and allow for the reporting of the result as a ratio to normal i.e. a "Thyroid Hormone Binding Ratio (THBR)" *(154)*. "Classic" uptake assays used T3 tracer because the lower T3-TBG binding affinity relative to T4-TBG resulted in a higher isotopic uptake by adsorbant and thus shorter counting times. However, since the validity of using a T3-uptake test to correct a TT4 value is questionable, some current non-isotopic assays use a "T4-uptake". Many manufacturers still use the "classical" approach to produce T3 uptake assays in which the mean normal percent uptake can vary from 25% to 40% (bound counts/total counts). Traditionally, the free thyroxine index, sometimes called a "T7" is derived from the product of a T3-uptake test and a TT4 measurement, often expressed as a % uptake (adsorbent bound counts divided by total counts).

Guideline 12. Thyroid Hormone Binding Ratio (THBR) or "Uptake" Tests

- "Uptake" tests should be called "Thyroid Hormone Binding Ratio" tests, abbreviated THBR and include an indication of which hormone is used, i.e. THBR (T4) or THBR (T3).
- A T4 signal is preferred over T3 for THBR measurements, to better reflect T4 binding protein abnormalities.
- THBR values should be reported as a ratio with normal serum, the latter having an assigned value of 1.00.
- THBR calculations should be based on the ratio between absorbent counts divided by the total minus absorbent counts, rather than the ratio between absorbent counts and total counts.
- The THBR result should be reported in addition to the total hormone and free hormone index value.
- THBR tests should not be used as an independent measurement of thyroid status, but should be interpreted in association with a TT4 and/or TT3 measurement and used to produce free hormone estimates (FT4 or FT3 indexes).

"Classic" T3-uptake or THBR tests are typically influenced by the endogenous T4 concentration of the specimen. This limitation can be circumvented by using a very large excess of a non-isotopically labeled T4 tracer with an affinity for thyroid binding proteins comparable to that of T4. Current THBR tests usually produce normal FT4I and FT3I values when TBG abnormalities are mild (i.e. pregnancy). However, some of these tests may produce inappropriately abnormal index values when patients have grossly abnormal binding proteins (congenital TBG high or low, familial dysalbuminemic hyperthyroxinemia (FDH), thyroid hormone autoantibodies and NTI) and in the presence of some medications that influence thyroid hormone protein binding [Section-3 B3(c)vi].

(c) Indexes using a Free Hormone Fraction Determination

The first free hormone tests developed in the 1960s were indexes, calculated from the product of the free hormone fraction from a dialysate multiplied by the TT4 measurement (made by PBI and later RIA) *(159,160)*. The free fraction index approach was later extended to measure the rate of transfer of isotopically-labeled hormone across a membrane separating two chambers containing the same undiluted specimen. The free hormone indexes calculated with isotopic free fractions are not completely independent of TBG concentration and furthermore are influenced by radiochemical purity, the buffer matrix and the dilution factor employed *(161,162)*.

3. Ligand Assays for FT4 and FT3 Estimation

These methods employ either a "two-step" or "one-step" approach. Specifically, two-step assays use a physical separation of free from protein-bound hormone before free hormone is measured by a sensitive immunoassay, or alternatively, an antibody is used to immunoextract a proportion of ligand out of the specimen before quantitation. In contrast, one-step ligand assays attempt to quantify free hormone in the presence of binding proteins. Two-step methods are less prone to non-specific artifacts. One-step methods may become invalid when the specimen and the standards differ in their affinity for the assay tracer *(60,145,150)*.

(a) Ligand Assays employing Physical Separation

FT4 methods that physically isolate free from protein-bound hormone before employing a sensitive immunoassay to measure the free hormone concentration are standardized using solutions containing gravimetrically prepared standard preparations of T4. The physical isolation of free from protein-bound hormone is accomplished with either a semi-permeable membrane using a dialysis chamber, an ultrafiltration technique, or a Sephadex LH-20 resin adsorption column *(161-165)*. An exceedingly sensitive T4 RIA method is needed to measure the picomole concentrations of FT4 in dialysates or free fraction isolates, as compared with total hormone measurements in the nanomole range. Although there are no officially acknowledged "gold standard" free hormone methods, it is generally considered that methods that employ physical separation are

least influenced by binding proteins, and by inference, provide free hormone values that best reflect the circulating free hormone level *(94,166)*. However, dialysis methods employing a dilution step may underestimate FT4 when binding inhibitors are present in the specimen and adsorption of T4 to membrane materials may be an issue *(94,166)*. In contrast, such methods may overestimate FT4 in sera from heparin-treated patients as a result of in-vitro generation of FFA [see Section-3 B3(c)vii] *(84,97,98,100,101,167-170)*. This in-vitro heparin effect is the primary cause of spuriously high FT4 values in NTI patients *(101)*. Physical separation methods are too labor intensive and expensive for routine use by clinical laboratories and are usually only available in reference laboratories. FT3 methods employing physical separation are only available in some specialized research laboratories *(102)*.

(b) Ligand Assays without Physical Separation

Most of the free hormone immunoassays in current use employ a specific, high affinity hormone antibody to sequester a small amount of total hormone from the specimen. The antibody unoccupied antibody-binding sites that are usually inversely proportional to the free hormone concentration and are quantified using the hormone labeled with radioactivity, fluorescence- or chemiluminescence. The signal output is then converted to a free hormone concentration using calibrators with free hormone values assigned by a method employing physical separation. The actual proportion of total thyroid hormone sequestered varies with the method design, but greatly exceeds the actual free hormone concentration and should be <1-2% in order to minimize perturbation of the free-bound equilibrium. The active sequestering of hormone by the anti-thyroid hormone antibody reagent in the assay results in a continuous stripping of hormone from binding proteins and perturbation of the bound to free equilibrium. The key to the validity of these methods is twofold. First, it is necessary to use conditions that maintain the free to protein-bound hormone equilibrium, and to minimize dilution effects that weaken the influence of any endogenous inhibitors present in the specimen. Secondly, it is important to use serum calibrators containing known free hormone concentrations that behave in the assay in an identical manner to the patient specimens. Three general approaches have been used to develop comparable FT4 and FT3 immunoassay methods: (i) two-step labeled-hormone; (ii) one-step labeled-analog; and (iii) labeled antibody.

Guideline 13. For Manufacturers Developing Free Hormone Estimate Tests

- Methods that do not physically separate bound from free hormone should extract no more than 1-2% of the total hormone concentration off the binding proteins, so that the thermodynamic equilibrium is maintained as much as possible.
- Minimize dilution effects that weaken the influence of any endogenous inhibitors present in the specimen.
- Use serum calibrators containing known free hormone concentrations that behave in the assay in an identical manner to the patient specimens.
- Perform the test procedure at 37°C.

(i) Two-Step, Labeled-Hormone/Back-Titration Methods

Two-step methods were first developed for research purposes in the late 1970s and were subsequently adapted to produce commercial FT4 and FT3 methods. During a first incubation step, these methods used a high affinity ($>1x10^{11}$ L/mol) anti-hormone antibody bound to a solid support (ultrafine Sephadex, antibody-coated tube or particles) to sequester a small proportion of total hormone from a diluted serum specimen. After a short incubation period, unbound assay constituents are washed away before the second step is performed in which sufficient labeled hormone is added to bind to all the unoccupied antibody-binding sites. After washing, the amount of labeled hormone bound to the solid-phase antibody is quantified relative to gravimetric standards or calibrators that have free hormone values assigned by a reference method. One-step labeled hormone-analog methods were introduced in the late 1970s. These new tests were less labor-intensive than two-step techniques. As a result, two-step methods lost popularity despite comparative studies showing that they were less affected by albumin concentration and binding protein abnormalities that negatively impact the diagnostic accuracy of the one-step analog tests *(147,171-173)*.

(ii) One-Step, Labeled Hormone-Analog Methods

The physicochemical validity of the one-step labeled hormone-analog tests were dependent upon the development of a hormone analog with a molecular structure that was totally non-reactive with serum proteins, but could react with unoccupied hormone antibody sites. When these conditions are met, the hormone-analog, which is chemically coupled to a signal molecule such as an isotope or enzyme, can compete with free hormone for a limited number of antibody-binding sites in a classical competitive immunoassay format. Though conceptually attractive, this approach is technically difficult to achieve in practice, despite early claims of success. The hormone-analog methods were principally engineered to give normal FT4 values in high TBG states (i.e. pregnancy). However, they were found to have poor diagnostic accuracy in the presence of abnormal albumin concentrations, FDH, NTI, high FFA levels or with thyroid hormone autoantibodies. Considerable efforts were made during the 1980s to correct these problems by the addition of proprietary chemicals to block analog binding to albumin or by empirically adjusting calibrator values to correct for protein-dependent biases. However, after a decade of criticism, most hormone-analog methods have been abandoned because these problems could not be resolved *(147)*.

(iii) Labeled Antibody Methods

Labeled antibody methods also measure free hormone as a function of the fractional occupancy of hormone-antibody binding sites. This competitive approach uses specific immunoabsorbents to assess the unoccupied antibody binding sites in the reaction mixture. A related approach has been the use of solid-phase unlabeled hormone/protein complexes (sometimes referred to as "analogs") that do not react significantly with serum proteins, to quantify unoccupied binding sites on the anti-hormone antibody in the liquid-phase. The physiochemical basis of these labeled-antibody methods suggests that they may be as susceptible to the same errors as the older labeled-hormone analog methods. However, physicochemical differences arising from the binding of analog to the solid support confer kinetic differences that results in decreased analog affinity for endogenous binding proteins and a more reliable free hormone measurement. The labeled antibody approach is currently the favored free hormone testing approach on most automated platforms.

(c) Performance of FT4 and FT3 Tests in Different Clinical Situations

The only reason to select a free thyroid hormone method (FT4 or FT3) in preference to a total thyroid hormone test (TT4 or TT3) is to improve the diagnostic accuracy for detecting hypo- and hyperthyroidism in patients with thyroid hormone binding abnormalities that compromise the diagnostic accuracy of total hormone measurements *(60)*. Unfortunately, the diagnostic accuracy of current free hormone methods cannot be predicted from either their method classification (one-step, two-step, labeled antibody etc) or by in-vitro tests of their technical validity, such as a specimen dilution test. The index tests (FT4I and FT3I) as well as current ligand assay methods, are all protein dependent to some extent, and may give unreliable values when binding proteins are significantly abnormal *(148)*. Free hormone tests should be performed at 37°C since tests performed at ambient temperature falsely increase values when specimens have a very low TBG concentration *(174,175)*.

The impetus for developing free hormone tests has been the high frequency of binding-protein abnormalities that cause discordance between total and free thyroid hormone concentrations. Unfortunately, no current FT4 method is universally valid in all clinical conditions. When the concentration of TBG is abnormal, most FT4 methods give results that are more diagnostically useful than TT4 measurement. However, pre-analytical or analytical assay artifacts arise in many situations associated with binding protein abnormalities: when the binding of the assay tracer to albumin is abnormal; in the presence of medications that displace T4 from TBG; during critical phases of NTI; and in pregnancy (see Table 1). The frequency of these FT4 assay artifacts suggests that TSH or the TSH/FT4 relationship is a more reliable thyroid parameter to use than an estimate of FT4 alone.

When it is suspected that a FT4 result is discrepant, FT4 should be checked using a different manufacturer's method (usually measured in a different laboratory). Additionally, or alternatively the FT4/ TT4 relationship can be checked for discordance since interference seldom affects both measurements to the same degree and in the same direction.

Guideline 14. Clinical Utility of Serum Free T3 Estimate Tests

Serum T3 measurement has little specificity or sensitivity for diagnosing hypothyroidism, since enhanced T4 to T3 conversion maintains normal T3 concentrations until hypothyroidism becomes severe. Patients with NTI or caloric deprivation typically have low total and free T3 values. Serum T3 measurements, interpreted together with FT4, and are useful to diagnose complex or unusual presentations of hyperthyroidism and certain rare conditions:

- A high serum T3 is often an early sign of recurrence of Graves' hyperthyroidism.
- The TT3/TT4 ratio can be used to investigate Graves' versus non-Graves' hyperthyroidism. Specifically, a high TT3/TT4 ratio (>20 ng/µg metric or >0.024 molar) suggests thyroidal stimulation characteristic of Graves' disease.
- Serum T3 measurement can be used to monitor the acute response to treatment for Graves' thyrotoxicosis.
- A high or paradoxically normal serum T3 may indicate hyperthyroidism in an NTI patient with suppressed TSH (< 0.01 mIU/L).
- A high or paradoxically normal serum T3 may indicate amiodarone-induced hyperthyroidism.
- Patients with goiter living in areas of iodide deficiency should have FT3 measured in addition to TSH to detect T3 thyrotoxicosis caused by focal or multifocal autonomy.
- A high serum T3 is frequently found with congenital goiter, due to defective organification of iodide (TPO defect) or defective synthesis of thyroglobulin.
- A high serum T3 usually precedes iodide-induced thyrotoxicosis when patients have multinodular long-standing goiter.
- A high serum T3 is often seen with TSH-secreting pituitary tumors.
- A high serum T3 is often seen in thyroid hormone resistance syndromes that usually present without clinical hyperthyroidism.
- Serum T3 measurement is useful for monitoring compliance with L-T3 suppression therapy prior to ^{131}I scan for DTC.
- Serum T3 measurement is useful for distinguishing mild (subclinical) hyperthyroidism (low TSH/ normal FT4) from T3-toxicosis, sometimes caused by T3-containing health-foods.
- Serum T3 measurement is useful for investigating iodide deficiency (characterized by low T4/high T3).
- Serum T3 measurement can be useful during antithyroid drug therapy to identify persistent T3 excess, despite normal or low serum T4.
- Serum T3 measurement can be used to detect early recurrence of thyrotoxicosis after cessation of antithyroid drug therapy
- Serum T3 measurement can be used to establish the extent of T3 excess during suppressive L-T4 therapy or after an intentional T4 overdose.

(i) Pregnancy

The increase in serum TBG and the low albumin concentrations associated with pregnancy results in wide method-dependent variations in FT4 measurements [see Section-2 A3] *(47,59)*. Albumin-dependent methods can produce low FT4 values in up to 50 percent of patients and are unsuitable for assessing thyroid status during pregnancy because of the marked negative bias attributable to the progressive decline in the serum albumin concentration by the third trimester *(59)*. Conversely, methods such as tracer dialysis tend to show a positive bias in relation to standards, possibly due to tracer impurities *(60)*. The use of method- and trimester-specific reference ranges might improve the diagnostic accuracy of free hormone testing in pregnancy. However, few if any manufacturers have developed such information for their methods.

(ii) Premature Infants

A low thyroxine level without an elevated TSH is commonly encountered in premature infants of less than 28 weeks gestation *(39,176)*. There is some clinical evidence to suggest that L-T4 treatment may improve neurological outcome *(176)*. However, as described above, method differences in FT4 methods are likely to compromise the reliability of detecting hypothyroxinemia of prematurity.

Guideline 15. Abnormal Thyroid Hormone Binding Proteins Effects on FT4 Tests

Binding protein abnormalities cause pre-analytical or analytical FT4 assay artifacts. Thyroid function should be assessed from the TSH-TT4 relationship when:

- The binding of assay tracer to albumin is abnormal (i.e. FDH).
- The patient is taking medications that displace T4 from TBG, i.e. Phenytoin, Carbamazepine or Furosemide (Frusemide).
- The patient has a critical or severe non-thyroidal Illness.

(iii) Genetic Abnormalities in Binding Proteins

Heredity and acquired variations in albumin, and TBG with altered affinity for either T4 or T3 can cause abnormal total hormone concentrations in euthyroid subjects with normal free hormone concentrations *(141)*. The albumin variant responsible for familial dysalbuminemic hyperthyroxinemia (FDH) has a markedly increased affinity for T4 and numerous T4-analog tracers, resulting in spuriously high serum free T4 estimates with these tracers *(145,177)*. In FDH, serum TT4 and FT4I values, as well as some FT4 ligand assays, give supra-normal values, whereas serum TT3, FT3, TSH and FT4 measured by other methods, including equilibrium dialysis, are normal *(177)*. Failure to recognize the presence of the FDH albumin variant that can occur with a prevalence of up to 1:1000 in some Latin-American populations can result in inappropriate thyroid test result interpretation leading to thyroid gland ablation *(178)*.

(iv) Autoantibodies

Some patient sera contain autoantibodies to thyroid hormone that result in methodologic artifacts in total or free hormone measurements *(143,145)*. Such antibody interferences are method-dependent. Tracer T4 or T3 bound to the endogenous antibody is falsely classified as bound by adsorption methods, or free by double antibody methods, leading to falsely low or falsely high serum TT4 or TT3 values, respectively *(144,145)*. The T4 tracer analogs used in some FT4 tests may bind to these autoantibodies, leading to spuriously high serum FT4 results. There have even been reports of anti-solid phase antibodies interfering in labeled-antibody assays for free thyroid hormones *(179)*.

(v) Thyrotoxicosis and Hypothyroidism

The relationship between free and total T4 and T3 in thyrotoxicosis is non-linear. In severe thyrotoxicosis, the elevations in TT4 and FT4 are disproportionate. This non-linearity reflects both a decrease in TBG levels and an overwhelming of the TBG binding capacity despite increased binding to TTR and albumin *(180)*. Similarly, FT3 concentrations may be underestimated as a result of high T4-TBG binding. The converse situation exists in severe hypothyroidism, in which there is reduced occupancy of all binding proteins *(180)*. In this situation, an excess of unoccupied binding sites may blunt the FT4 response to treatment. This suggests that an initial L-T4 loading dose is the most rapid approach for restoring a therapeutic FT4 level in a hypothyroid patient.

(vi) Drugs that Compete for Thyroid Hormone Binding

Some therapeutic and diagnostic agents such as Phenytoin, Carbamazepine or Furosemide/Frusemide may

competitively inhibit thyroid hormone binding to serum proteins in the specimen. The reduced binding-protein availability results in an acute increase in FT4 and in some instances increased hormone action as evidenced by a reduction in TSH *(181)*. The increased FT4 measurements are influenced by the serum dilution used by the method and are also seen with dialysis methods *(182,183)*. During the chronic administration of such competitor drugs, there is enhanced clearance of hormone. However, eventually the system re-establishes a "normal" equilibrium and FT4 levels normalize at the expense of a low TT4 concentration. The withdrawal of drug at this point would cause an initial fall in FT4 as more carrier protein becomes available, with re-normalization of FT4 as the equilibrium is re-established through an increased release of hormone from the thyroid gland. The time-scale and magnitude of these competitor effects differ with the half-life of the competitor agent.

A number of medications and factors compete with the binding of T4 and T3 to TBG causing an acute increase in the availability of FT4 or FT3. Many of these competing agents of thyroid hormone binding are frequently prescribed therapeutic agents that differ in their affinity for TBG relative to T4 *(96,184)*. Furosemide, for example binds to TBG but with an affinity that is about three-fold less than T4 whereas aspirin binds seven-fold less than T4 *(170,185)*. The competition in vivo observed with such agents relates to their affinity for TBG rather than their therapeutic levels, the free fraction or their affinity for non-TBG proteins, especially albumin *(170,186)*.

Current FT4 assays that employ a dilution factor may fail to detect an elevation in FT4 secondary to the presence of binding-protein competitors. For example, a specimen containing both T4 (free fraction 1:4000) and a competitive inhibitor (free fraction 1:100) subjected to stepwise dilution will sustain the FT4 concentration up to a 1:100 dilution, secondary to progressive dissociation of T4 from binding proteins. In contrast, the free drug concentration would decrease markedly only after a 1:10 dilution. Thus the hormone-displacing effect of drugs competing for T4 binding will be underestimated in FT4 assays employing high specimen dilution. The use of symmetric equilibrium dialysis and ultrafiltration of undiluted serum can minimize this artifact *(94,165,187,188)*.

(vii) Heparin Treatment Artifacts

It is well known that in the presence of a normal albumin concentration, non-esterified fatty acid (FFA) concentrations > 3mmol/L will increase FT4 by displacing the hormone from TBG *(84,97,98,100,101,167-170)*. Serum from patients treated with heparin, including low-molecular weight heparin preparations, may exhibit spuriously high FT4 values secondary to in vitro heparin-induced lipase activity that increases FFA. This problem is seen even with heparin doses as low as 10 units and is exacerbated with storage of the specimen. Increased serum triglyceride levels, low serum albumin concentrations or prolonged assay incubation at 37°C can accentuate this problem.

(viii) Critical Nonthyroidal Illness

There is a large body of evidence collected over more than two decades, that report on the specificity of various FT4 methods in hospitalized patients with NTI [Section-2 B2]. This literature can be confusing, and is complicated by the heterogeneity of the patient populations studied and the method-dependence of the results. Manufacturers have progressively modified their methods over time, in an attempt to improve their specificity in this setting and other situations when binding proteins are abnormal. However, the exact composition of current methods remains proprietary and it is difficult for manufacturers to obtain pedigreed specimens from such patients to rigorously test their methods. In one recent FT4 method comparison study, a marked method-dependent difference was seen on the seventh day following bone marrow transplantation in euthyroid subjects receiving multiple drug therapies that included heparin and glucocorticoids *(101)*. In this study, the TT4 concentrations were normal in most of the subjects (95%) and the serum TSH was < 0.1 mIU/L in approximately half of the subjects. sThis was consistent with the glucocorticoid therapy the patients were receiving. In contrast, both elevated and subnormal values were reported by different FT4 methods. It appeared that the supranormal FT4 estimates reported by some methods in 20 to 40% of patients, probably reflected the

I.V. heparin effect discussed above [Section-3 B3(c)vii]. In contrast, analog tracer methods that are subject to the influence of tracer binding to albumin, gave subnormal FT4 estimates in 20-30% of patients *(101)*. Such FT4 measurement artifacts, giving rise to a discordance between FT4 and TSH results, increase the risk of an erroneous diagnosis of either thyrotoxicosis or secondary hypothyroidism and suggest that TT4 measurements may be more reliable in the setting of a critical illness.

(d) FT4 Method Validation

Unfortunately, most free hormone estimate methods receive inadequate evaluation prior to their introduction for clinical use. Manufacturers rarely extend the validation of their methods beyond the study of ambulatory hypo- and hyperthyroid patients, pregnant patients and a catchall category of "NTI/hospitalized patients". However, there is currently no consensus as to the best criteria to use for evaluating these free T4 estimate methods. It is insufficient to merely demonstrate that a new method can distinguish between hypothyroid, normal and hyperthyroid values, and to show comparability with existing methods - any free hormone estimate method will satisfy these criteria without necessarily giving information about the true physiologic free hormone concentration.

Guideline 16. For Manufacturers: Assessment of FT4 Estimate Test Diagnostic Accuracy

- The diagnostic accuracy of the method should be tested using pedigreed specimens from ambulatory patients with the following binding protein disturbances:
  - TBG abnormalities (high estrogen & congenital TBG excess and deficiency)
  - Familial Dysalbuminemic Hyperthyroxinemia (FDH)
  - Increased Transthyretin (TTR) affinity
  - T4 and T3 Autoantibodies
  - Rheumatoid Factor
- Test the method for interference with normal serum specimens spiked with relevant concentrations of common inhibitors at concentrations that cause displacement of hormone from binding proteins in undiluted serum, effects which are lost after dilution i.e.:
  - Furosemide (Frusemide) 30 µM
  - Disalicylic acid 300 µM
  - Phenytoin 75 µM
  - Carbamazepine 8 µM
- List all known interferences with the magnitude and direction of resulting errors
- Document in-vitro heparin effects on NEFA generation during the assay incubation

New methods should either be tested with pedigreed clinical samples, especially those that may challenge the assay validity, or alternatively, by manipulating the constituents of a normal serum sample to test a particular criterion *(148)*. Whichever approach is adopted, the key questions relate to the similarity between samples and standards, because all assays are generally comparable. Other approaches include testing the quantitative recovery of added L-T4, or determining the effects of serum dilution, since a 100-fold dilution of a "normal" serum theoretically causes an insignificant reduction (less than 2%) in the FT4 concentration *(94,152) (58,189)*. These approaches however, just test the "protein dependence" of the method, i.e. the degree to which free T4 is dependent on the dissociation of free from bound hormone *(148)*. These approaches will predictably give an unfavorable assessment of methods that involve a high degree of sample dilution compared to those methods that minimize sample dilution. There is no evidence however, to document whether these approaches truly reflect diagnostic accuracy of the method when used to evaluate difficult clinical specimens. Ultimately, as with any diagnostic method, the specificity of a free T4 method will only become evident after testing a full spectrum of specimens from individuals with and without thyroid dysfunction associated with binding protein abnormalities or medications known to affect thyroid hormone binding to plasma proteins. An unexpected interference may only be noted after methods have been in use for some time, as in the effects of rheumatoid factor that can produce spuriously high serum free T4 estimates *(112)*. Non-specific fluorescence due to the presence in the blood of substances such as organic acids in patients with uremia can be another cause of non-specific interference *(190)*.

The preferred approach is to pay particular attention to specimens that are likely to cause non-specific interference in the assay result *(98)*. Ideally, in the ambulatory patient setting these would include samples that have: a) TBG abnormalities (pregnancy, oral contraceptive therapy, and congenital TBG excess and deficiency); b) Familial Dysalbuminemic Hyperthyroxinemia (FDH); c) T4 and T3 autoantibodies; d) interfering substances such as rheumatoid factor and and e) a wide spectrum of drug therapies. In the hospital setting, three classes of patients should be tested: a) patients without thyroid dysfunction but with low or high TT4 due to NTI; b) patients with documented hypothyroidism associated with severe NTI and, c) patients with documented hyperthyroidism associated with NTI. However, it is prohibitively difficult for manufacturers to obtain pedigreed specimens from such patients. Since no manufacturers have tested their methods adequately in critically sick patients, it is difficult for physicians to have confidence that abnormal FT4 results in such patients reflects true thyroid dysfunction rather than NTI. Thus in hospitalized patients with suspected thyroid dysfunction, a combination of serum TSH and TT4 measurements may provide more information than only a FT4 test, provided that the TT4 value is interpreted relative to the degree of severity of the illness. Specifically, the low TT4 state of NTI is usually restricted to severely sick patients in an intensive care setting. A low TT4 value in a patient not critically ill should prompt a consideration of pituitary dysfunction. In ambulatory patients, serum FT4 measurements are often more diagnostically accurate than a TT4 measurement. However, when an abnormal FT4 result does not fit the clinical picture, or there is an unexplained discordance in the TSH to FT4 relationship, it may be necessary to order a TT4 test as confirmation. Alternatively, the laboratory could either send the specimen to a different laboratory that uses a different manufacturer's FT4 method, or to a reference laboratory that can perform a FT4 measurement using a physical separation method, such as equilibrium dialysis or ultrafiltration.

(e) Interferences with Thyroid Tests

Ideally, a thyroid hormone test should display zero interference with any compound, drug or endogenous substance (i.e. bilirubin) in any specimen, at any concentration. Studies available from manufacturers vary widely in the number of compounds studied and in the concentrations tested. Usually the laboratory can only proactively detect interference from a "sanity check" of the relationship between the FT4 and TSH result. If only one test is measured, interference is usually first suspected by the physician who observes an inconsistency between the reported value and the clinical status of the patient. Classic laboratory checks of verifying the specimen identity and performing dilution, may not always detect interference. Interferences with either TT4 or FT4 measurements typically elicit inappropriately abnormal values in the face of a normal serum TSH level (Table 1). Interferences with competitive or non-competitive immunoassays fall into three classes: (i) cross-reactivity problems, (ii) endogenous analyte antibodies and (iii) drug interactions *(191)*.

(i) Cross-reactivity

Cross-reactivity problems result from the inability of the antibody reagent to discriminate selectively between analyte and a structurally related molecule *(192)*. Thyroid hormone assays are less susceptible to this type of interference than TSH, because iodothyronine antibody reagents are selected for specificity by screening with purified preparations. The availability of monoclonal and affinity-purified polyclonal antibodies has reduced the cross-reactivity of current T4 and T3 tests to less than 0.1% for all studied iodinated precursors and metabolites of L-T4. However, there have been reports of 3-3',5-triiodothyroacetic acid (TRIAC) interfering in FT3 assays and D-T4 interference in FT4 assays *(14,135)*.

(ii) Endogenous Autoantibodies

Endogenous autoantibodies to both T4 and T3 have been frequently found in the serum of patients with autoimmune thyroid as well as non-thyroidal disorders. Despite their high prevalence, interference caused by such autoantibodies is relatively rare. Such interferences are characterized by either falsely low or falsely high values, depending on the type and composition of the assay used *(193)*.

(iii) Drug Interferences

Drug Interferences can result from the in-vitro presence of therapeutic or diagnostic agents in the serum specimen in sufficient quantities to interfere with thyroid tests *(67,68)*. Thyroid test methods employing fluorescent signals may be sensitive to the presence of fluorophor-related therapeutic or diagnostic agents in the specimen *(190)*. In the case of I.V. heparin administration, the in vitro activation of lipoprotein lipases results in the generation of FFA in vitro that may falsely elevate FT4 values [see Section-3 B3(c)vii] *(84,97,98,100,101,167-170)*.

(f) Serum FT4 and FT3 Normal Reference Intervals

Physical separation methods are used to assign values to the calibrators employed for most FT4 estimate tests. There is closer agreement between the reference intervals of the various ligand assays used by clinical laboratories than there is between the various methods that employ physical separation. Reference intervals for FT4 immunoassay methods approximate 9-23 pmol/L (0.7 –1.8 ng/dL). In contrast, the upper FT4 limit for methods such as equilibrium dialysis that employ physical separation extends above 30 pmol/L (2.5 ng/dL). Reference intervals for FT3 immunoassay methods approximate 3.5-7.7 pmol/L (0.2 – 0.5 ng/dL). FT3 methods that employ physical separation are currently only available as research assays *(102)*.

(g) Standardization or Calibration

There are no internationally developed standard materials or methods for free hormone measurements. Although candidate reference methods have been suggested for TT4 measurements, it will be difficult to adapt such methods for free hormones *(139)*. Each method and manufacturer approaches the problem of standardization from its own unique perspective.

FT4 estimate methods that require two independent assays (tracer equilibrium dialysis and ultrafiltration as well as index methods) use a total hormone measurement and a measurement of the free fraction of the hormone. Total hormone assays are standardized with gravimetrically prepared calibrators from high purity hormone materials, which are commercially available. The free fraction is determined as radioactive counts in a dialysate or ultrafiltrate. Alternatively, in the case of the index methods, the saturation or binding capacity of the binding protein(s) is measured using a thyroid hormone binding ratio (THBR) test, sometimes referred to as an "uptake" test. THBR tests are standardized against sera with normal binding proteins and assigned a value of 1.00 [Section-3 B2(b)].

The more complicated situation occurs with the ligand free hormone estimate assays. In general these tests are provided with standards that have known or assigned free hormone values determined by a reference method (usually equilibrium dialysis with RIA of the FT4 concentration of the dialysate). This is typically performed by the manufacturer for the purpose of establishing free hormone values for the human serum based calibrators containing the hormone and its binding protein(s) for inclusion in the kit. Alternatively, in the case of highly bound hormones, such as thyroxine, the Law of Mass Action can be used to calculate the free hormone concentration *(194)*. The total hormone concentration, a measurement of the total binding capacity for the hormone in that serum sample, and the equilibrium constant provide the necessary information to calculate the free hormone concentration. This approach is valid for calibrators and controls manufactured in human serum that contains a normal TBG binding capacity. This allows the manufacturer to make calibrators and controls at fixed levels.

The use of calibrators, prepared as described above, also compensates for the over-extraction of hormone from their binding proteins. Specifically, in the case of thyroxine and triiodothyroninine, the antibody in the kit may bind the free hormone and extract a significant amount (~1-2%) of the bound hormone. If assayed directly, the concentration of free hormone would be elevated due to the over extraction. However, the use of calibrators with known free hormone levels and in human serum permits the assignment of the specific signal levels from the assay readout system (whether, isotopic, enzymatic, fluorescence, or chemiluminescence) to specific known concentrations of free hormone in a proportional relationship. However, this will only be valid if the percent of hormone extracted from the calibrator is identical to that from the patient specimen. This is often not the case for specimens containing binding protein abnormalities (i.e. congenital high and low TBG, FDH, NTI etc).

4. Free Hormone Measurement – the Future

The era of immunoassay methods for the quantification of thyroid and steroid hormones in biological fluids began in the 1970's. That era is now drawing to a close. The era of advanced mass spectrometry for quantifying hormones in biological fluids is now emerging *(138)*. There is no reason to doubt that mass spectrometry will provide better quantification because of greater analytical specificity and less analytical interference than immunoassays. So far such techniques have only being applied to TT4 determination *(139)*. However, for total hormone assays, the requirement for complete hormone release from protein-hormone complexes will remain. For free hormone assays, the requirement for a physical separation of free hormone and protein bound hormone, prior to quantification will also remain. In order to accomplish the later, new separation technology will be needed, before any method can be regarded as a gold standard. The implicit dilution of small molecules is a limitation of equilibrium dialysis that needs to be overcome. Ultrafiltration shows promise, but current methods are either too leaky or too impractical for this task. Mass spectrometry measurements of hormones that form complexes with serum proteins will only be as good as the specimen preparation steps associated with the quantification. However, the ideal free hormone reference method would be a technique that employs ultrafiltration at 37°C, to avoid dilution effects and the direct measurement of free hormone in the ultrafiltrate by mass spectrometry.

Guideline 17. For Laboratories Performing FT4 and FT3 testing

- Physicians should have ready access to information on the effects of drugs and the diagnostic accuracy of the test used for assessing the thyroid status of patients with various binding protein abnormalities and severe illnesses.
- When requested by the physician, the laboratory should be prepared to confirm a questionable result by performing a total hormone measurement or by re-measuring FT4 by a reference method that physically separates free from bound hormone, such as direct equilibrium dialysis or ultrafiltration.
- Questionable results on specimens should be checked for interference by re-measurement made with a different manufacturer's method. (Send out to a different laboratory if necessary.)