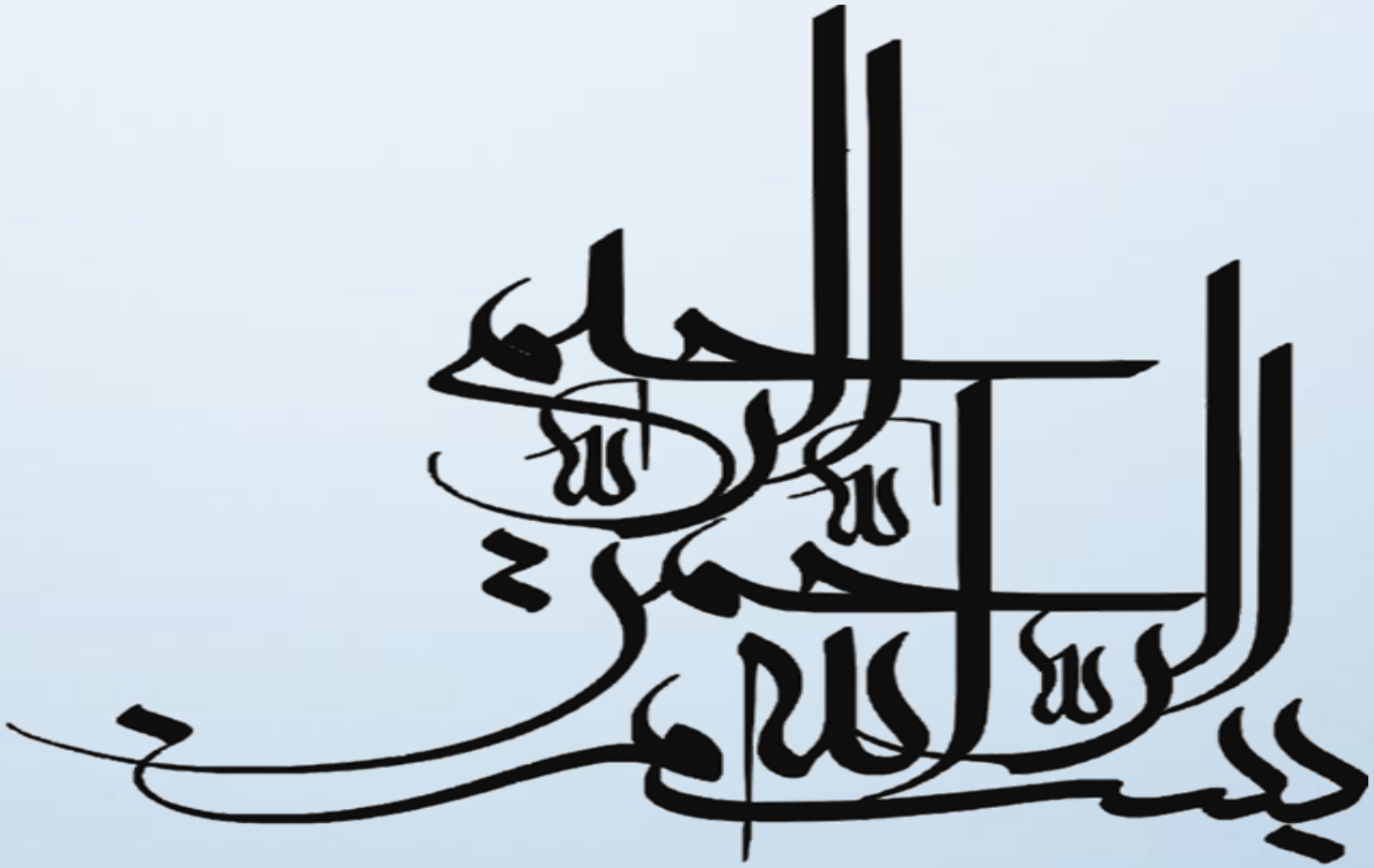


Thyroid function tests

Challenges in

Measurements & Interpretation

Maryam Tohidi
Associate professor of anatomical & clinical pathology
Research Institute for Endocrine Sciences
Shahid Beheshti University of Medical Sciences

February 2021
Tehran, Iran

Agenda

- *Pre- analytical considerations*
- *Factors affected TFT reference intervals*
- *Discordant thyroid function tests*
- *What technical pitfalls should the clinician be aware of ?*
- *T-uptake assay (TBI)*
- *Immunoassay methods at a glance*

Pre-analytical considerations (patient preparation)

- Does fasting is necessary for thyroid function testing?
- Does sampling time affect the TFT results?
- What about taking thyroid medication before sampling?

Pre-analytical considerations (patient preparation)

- Clinical guidelines for TFTs or laboratory guidelines for FT4 and TSH estimation do not give emphasis to the time of blood sampling or the fasting/non-fasting status of the patient.

A statement that be easily found here & there !

These tests may be measured any time of the day without fasting.

THYROID
Volume 25, Number 8, 2015

DOI: 10.1089/thy.2014.0589

Circadian and Circannual Rhythms in Thyroid Hormones: Determining the TSH and Free T4 Reference Intervals Based Upon Time of Day, Age, and Sex

Joel Ehrenkranz,[1] Phillip R. Bach,[2] Gregory L. Snow,[3] Alison Schneider,[4] Jo Lynn Lee,[5] Sarah Ilstrup,[2] Sterling T. Bennett,[2] and Salvatore Benvenga[6]

Biochemical and Biophysical Research Communications 506 (2018) 450–454

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

IF= 2.985

Effects of sex, age, sampling time, and season on thyroid-stimulating hormone concentrations: A retrospective study

Danchen Wang [a, 1], Dandan Li [a, 1], Xiuzhi Guo [a, 1], Songlin Yu [a], Ling Qiu [a, *], Xinqi Cheng [a], Tao Xu [b, **], Honglei Li [a], Hongchun Liu [c]

[a] Department of Clinical Laboratory, Peking Union Medical College Hospital, Peking Union Medical College & Chinese Academy of Medical Science, Beijing, 100730, China
[b] Department of Statistics, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100005, China
[c] Department of Medical Laboratory, First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan, 450052, China

Original Article

Does fasting or postprandial state affect thyroid function testing?

Rakesh Nair, Shriraam Mahadevan[1], R. S. Muralidharan, S. Madhavan

Department of General Medicine, Stanley Medical College, Chennai, [1]Department of Endocrinology, Sri Ramachandra Medical College and Research Institute, Chennai, Tamil Nadu, India

Indian Journal of Endocrinology and Metabolism / Sep-Oct 2014 / Vol 18 | Issue 5

- Aim: to evaluate whether TSH measured in fasting state or post-prandially would make a difference
- Fifty-seven adult ambulatory patients
- Exclusions: Patients with renal or liver dysfunction, steroid or thyroxine therapy
  - Group A (Normal freeT4 and TSH)
  - Group B (SCH with increased TSH and normal free T4)
  - Group C (overt hypothyroidism with low free T4 and high TSH).
- Sampling time & fasting state:
  - First simple: after an 8-12 hour overnight fast between 7:30-8:30
  - Second sample: 2 hours after breakfast between 10:30-11:00 am on the same day

❖ Results & conclusion:

- TSH levels showed a statistically significant decline post-prandially in comparison to fasting values.
- This may have clinical implications in the diagnosis and management of hypothyroidism, especially SCH.
- A fasting TSH sample may be preferred to random or postprandial estimations.

Table 1: Fasting and 2 hour post-prandial values (mean±standard deviation) of free T4 and TSH among the three groups

	Fasting	2 hour-postprandial	*P* value
Group A: (*n*=19)			
Free T4 (ng/ml)	1.06±0.11	1.05±0.11	0.07
TSH (mIU/L)	2.42±1.49	1.79±1.10	0.00*
Group B: (*n*=20)			
Free T4 (ng/ml)	0.89±0.20	0.88±0.21	0.35
TSH (mIU/L)	7.53±1.50	5.35±1.40	0.00*
Group C: (*n*=18)			
Free T4 (ng/ml)	0.57±0.18	0.56±0.17	0.75
TSH (mIU/L)	66.93±17.83	61.22±16.41	0.00*

Does Time of Sampling or Food Intake Alter Thyroid Function Test?

Shriraam Mahadevan[1,2], Dhalapathy Sadacharan[2,3], Subramanian Kannan[4], Anita Suryanarayanan[5]

[1]Department of Endocrinology, Diabetes and Metabolism, Sri Ramachandra Medical College and Research Institute, [2]Endocrine and Speciality Clinic, Madras Medical College, [3]Department of Endocrine Surgery, Madras Medical College, Chennai, Tamil Nadu, [4]Department of Endocrinology, Diabetology and Bariatric Medicine, Narayana Health City, Bengaluru, Karnataka, [5]Metropolis Health Care Limited, Chennai, Tamil Nadu, India

- Aim: to evaluate whether TSH measured in fasting state or post-meal would make a difference and whether this difference was related to the time of blood draw or the meal intake.
- 52 non-pregnant subjects without thyroid disorders
- Sampling time:

  First day: 8 am at fasting state & 10 am at with extended fasting

  Second day: 8 am at fasting state & 10 am post-prandial

Table 1: Thyroid-stimulating hormone values: Comparison between fasting and extended fasting (day 1) and fasting and postprandial (day 2)

	Day 1			Day 2		
	Fasting	2 h extended fasting	*P* (paired *t*-test)	Fasting	2 h postprandial	*P* (paired *t*-test)
TSH (mIU/L)	2.93±1.62	↓ 2.26±1.19	<0.001	2.46±1.32	↓ 1.89±1.01	<0.001

Values are mean±SD. TSH: Thyroid-stimulating hormone, SD: Standard deviation

- Results & conclusion:

- we noticed that there was a significant decline in TSH values when the sample was collected at around 10 am regardless of whether it was a fasting (extended fast) or post-meal sample.
- The timing of the sample regardless of meal intake affects TSH values and this should be factored in making decisions of subclinical hypothyroidism.

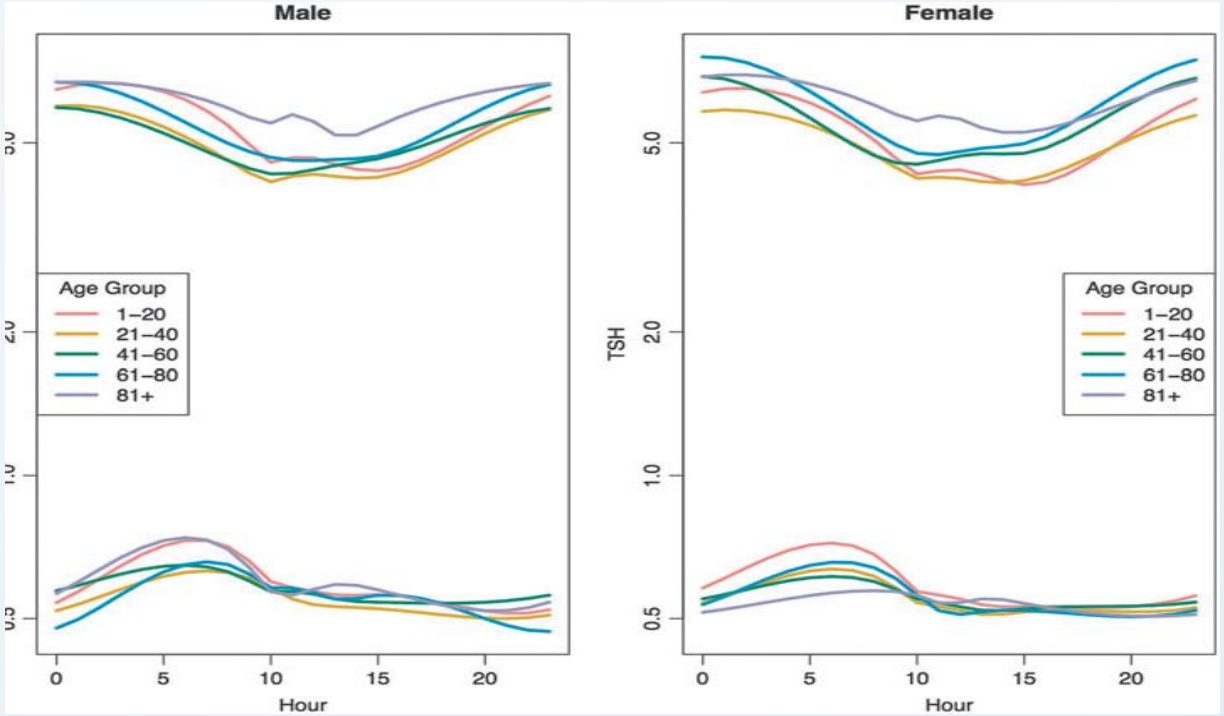
THYROID
Volume 25, Number 8, 2015

DOI: 10.1089/thy.2014.0589

Circadian and Circannual Rhythms in Thyroid Hormones: Determining the TSH and Free T4 Reference Intervals Based Upon Time of Day, Age, and Sex

Joel Ehrenkranz,[1] Phillip R. Bach,[2] Gregory L. Snow,[3] Alison Schneider,[4] Jo Lynn Lee,[5] Sarah Ilstrup,[2] Sterling T. Bennett,[2] and Salvatore Benvenga[6]

- A retrospective analysis
- 465,593 TSH & 112,994 free T4 measurements
- Subjects ages 1–104 years with no thyroid disease
- Using a single TSH and free T4 immunoassay method

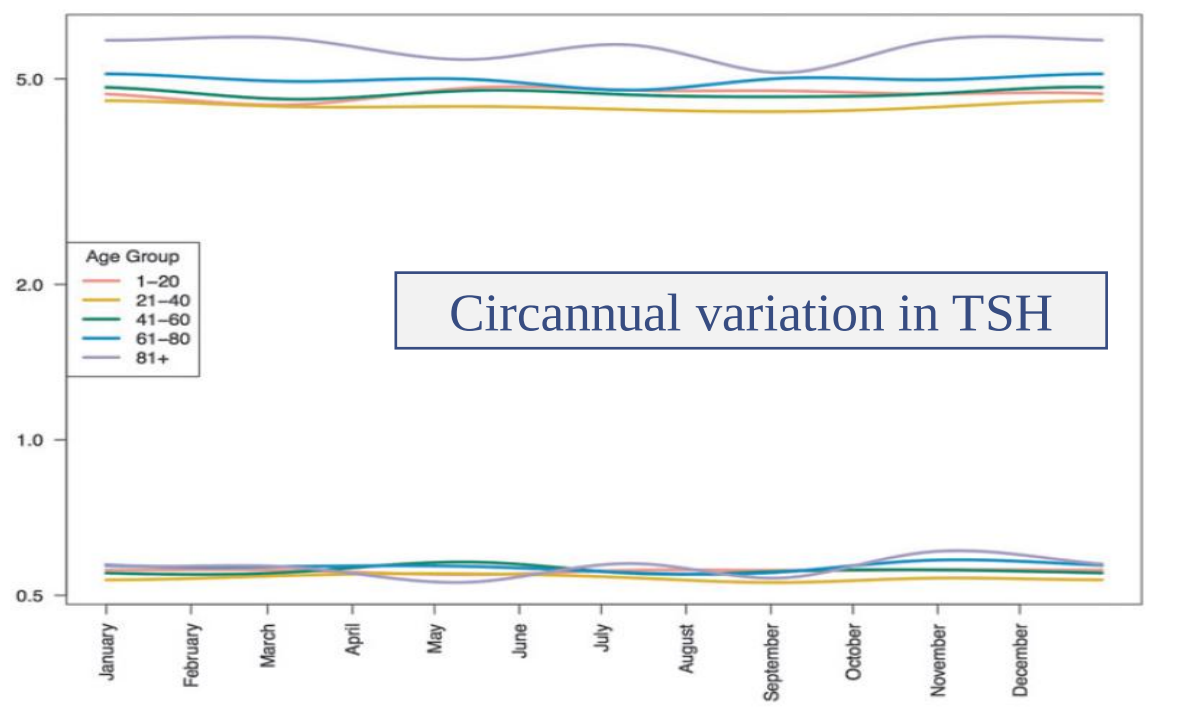


TSH circadian reference interval by sex, age, and time of day

- TSH exhibits a pronounced circadian rhythm in normal children and adults.
- Mean TSH levels increase with age.

TABLE 3. CIRCADIAN THYROTROPIN (MIU/L) REFERENCE INTERVALS BY AGE, SEX, AND TIME OF DAY

Age (years)	*Sex*	*Lower limit of reference interval (mIU/L)*	*Median lower limit (mIU/L)*	*Hour when nadir of reference interval lower limit occurs*	*Hour when peak of reference interval lower limit occurs*	*Upper limit of reference interval (mIU/L)*	*Median upper limit (mIU/L)*	*Hour when nadir of reference interval upper limit occurs*	*Hour when peak of reference interval upper limit occurs*
1–20	M	0.51–0.73	0.56	2100	0700	4.37–6.68	5.42	1500	0200
	F	0.53–0.72	0.56	1900	0600	4.08–6.51	5.13	1500	0200
21–40	M	0.50–0.63	0.53	2100	0700	4.14–5.99	4.92	1000	0100
	F	0.51–0.64	0.53	1300	0600	4.12–5.86	4.93	1400	0100
41–60	M	0.54–0.65	0.55	1800	0600	4.30–5.94	5.06	1000	0000
	F	0.52–0.61	0.54	1400	0600	4.51–6.90	5.34	1000	0000
61–80	M	0.47–0.66	0.56	2300	0700	4.59–6.71	5.42	1200	0000
	F	0.50–0.66	0.52	2000	0600	4.72–7.58	5.90	1100	0000
80+	M	0.52–0.74	0.58	2000	0600	5.19–6.72	6.20	1400	0100
	F	0.51–0.57	0.54	2100	0800	5.26–6.95	6.07	1400	0200

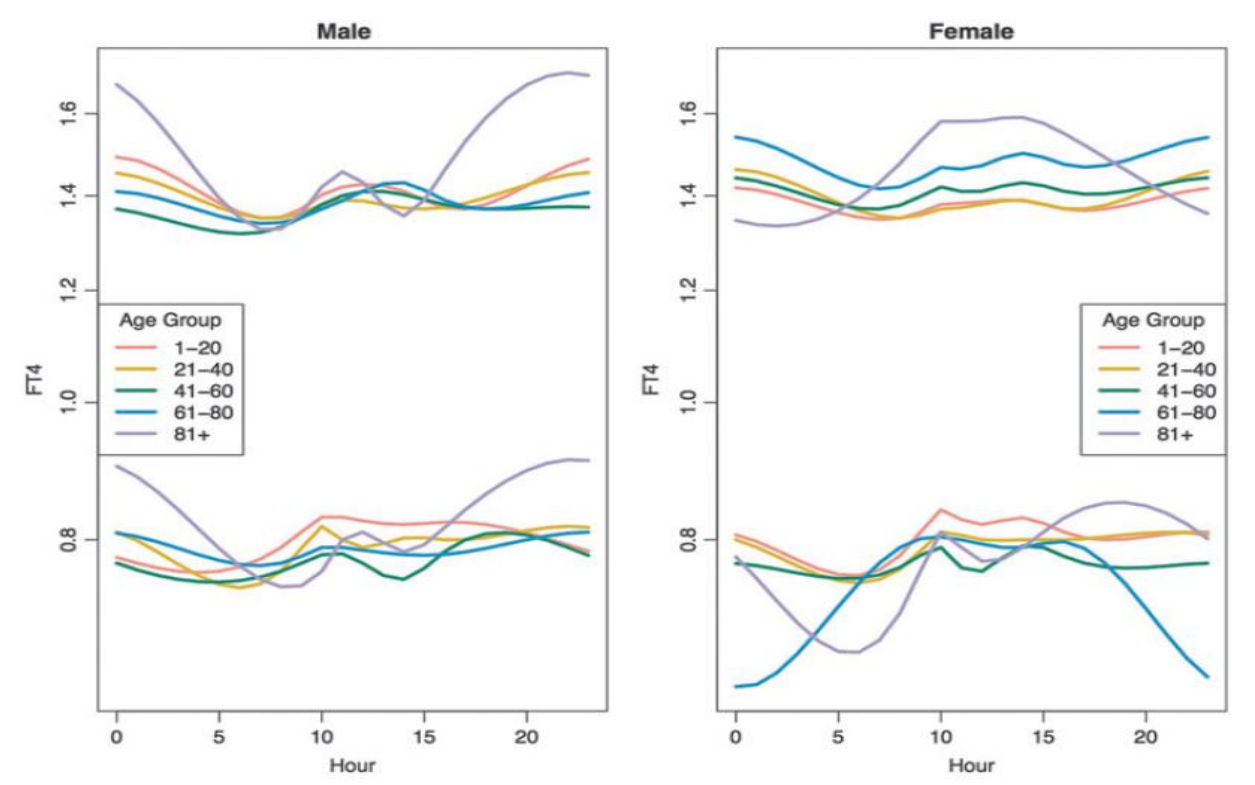


Circannual variation in TSH

- The upper limit of the 95% reference interval (both sexes and all ages) had a low value of 4.31 in August and peak value of 6.06 in December.
- The lower limit of the 95% reference interval for TSH was 0.5 throughout the year.
- Statistically significant differences in the upper limit of the monthly TSH reference interval were found ($p < 0.05$).
- No clinically significant differences in the circannual TSH reference range by sex or age.

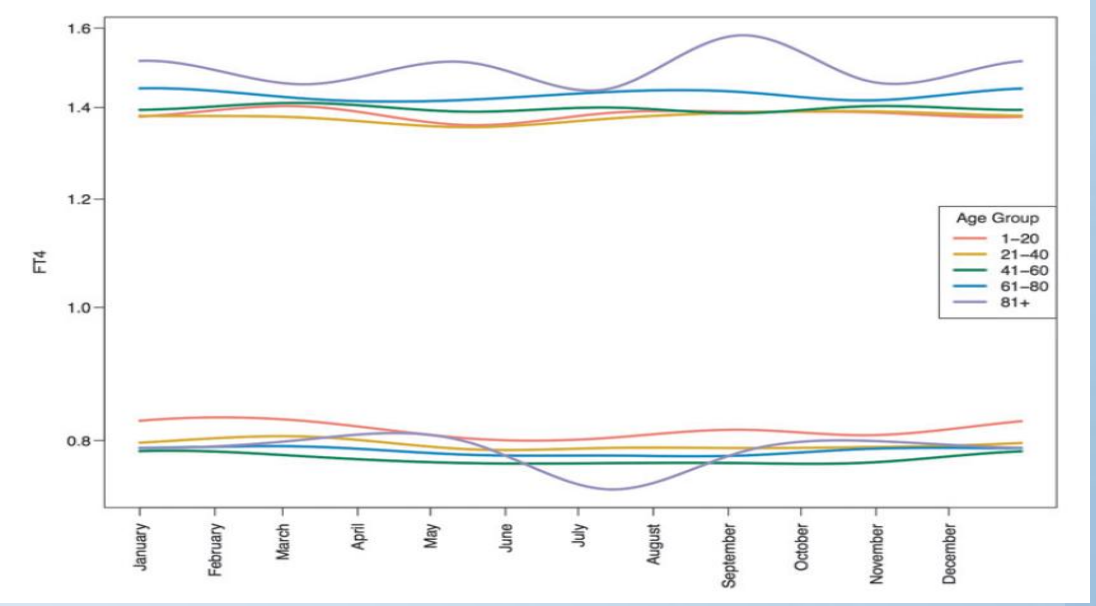
TABLE 4. CIRCANNUAL THYROTROPIN (MIU/L) REFERENCE INTERVALS BY AGE, SEX, AND MONTH

Age band (years)	*Sex*	*Jan.*	*Feb.*	*March*	*April*	*May*	*June*	*July*	*Aug.*	*Sept.*	*Oct.*	*Nov.*	*Dec.*
1–20	M	0.57–4.62	0.58–4.40	0.59–4.51	0.57–4.48	0.56–4.84	0.58–4.68	0.58–4.71	0.58–4.76	0.57–4.78	0.59–4.58	0.58–4.69	0.59–4.70
	F	0.54–4.70	0.55–4.48	0.56–4.59	0.54–4.56	0.53–4.92	0.55–4.76	0.55–4.79	0.55–4.84	0.54–4.86	0.56–4.66	0.55–4.77	0.56–4.78
21–40	M	0.55–4.46	0.57–4.47	0.56–4.36	0.57–4.27	0.58–4.43	0.57–4.33	0.55–4.29	0.56–4.23	0.55–4.33	0.56–4.41	0.56–4.33	0.55–4.42
	F	0.52–4.54	0.54–4.55	0.53–4.44	0.54–4.35	0.55–4.51	0.54–4.41	0.52–4.37	0.53–4.31	0.52–4.41	0.53–4.49	0.53–4.41	0.52–4.50
41–60	M	0.57–4.69	0.56–4.58	0.58–4.54	0.60–4.61	0.59–4.64	0.57–4.73	0.58–4.62	0.57–4.46	0.58–4.70	0.58–4.60	0.59–4.62	0.57–4.80
	F	0.54–4.77	0.53–4.66	0.55–4.62	0.57–4.69	0.56–4.72	0.54–4.81	0.55–4.70	0.54–4.54	0.55–4.78	0.55–4.68	0.56–4.70	0.54–4.88
61–80	M	0.57–5.02	0.58–4.93	0.58–4.91	0.60–4.92	0.59–5.06	0.58–4.64	0.57–4.88	0.58–4.85	0.57–4.92	0.59–4.94	0.60–4.94	0.62–5.15
	F	0.54–5.10	0.55–5.01	0.55–4.99	0.57–5.00	0.56–5.14	0.55–4.72	0.54–4.96	0.55–4.93	0.54–5.00	0.56–5.02	0.57–5.02	0.59–5.23
80+	M	0.61–6.20	0.59–5.72	0.60–5.81	0.56–5.62	0.57–5.37	0.55–5.82	0.59–5.71	0.57–5.20	0.55–5.12	0.64–5.85	0.58–5.58	0.62–5.98
	F	0.58–6.28	0.56–5.80	0.57–5.89	0.53–5.70	0.54–5.45	0.52–5.90	0.56–5.79	0.54–5.28	0.52–5.20	0.61–5.93	0.55–5.66	0.59–6.06



FT4 circadian reference interval by sex, age, and time of day.

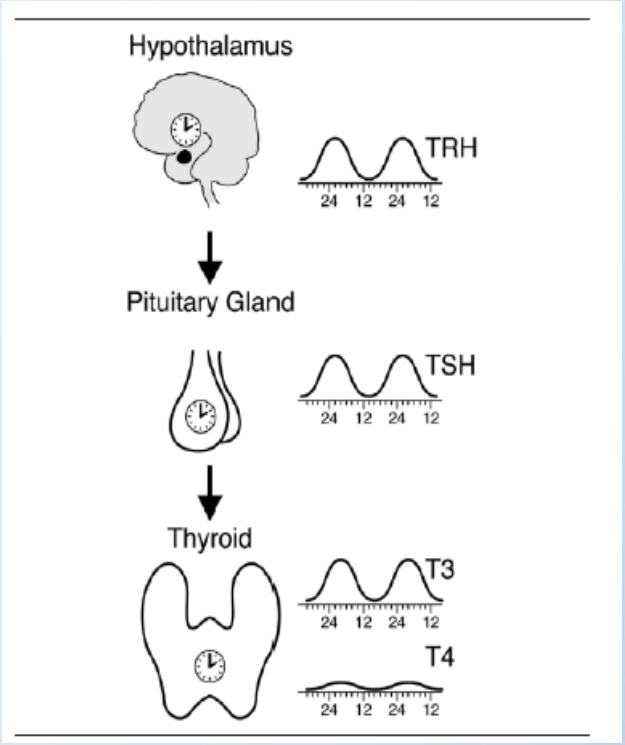
Free T4 circannual reference interval by age.



- Age, sex, hour of day and time of year do not affect the FT4 reference interval
- The circadian and circannual FT4 variations are modest in amplitude and the differences between age groups are not significant.

- To avoid incorrectly identifying TSH values as abnormal, the TSH reference range needs to take into account the subject's age and the time of day when the blood sample was drawn.
- For example, a TSH value of 7.5mIU/L would generally be considered elevated and indicative of subclinical hypothyroidism. However, if the sample was drawn from an 80 year old woman at midnight, a TSH of 7.5 mIU/L is within the 95% reference range and is in fact normal.
- Despite an age-related increase in median TSH concentration, the circadian rhythm and nocturnal surge in TSH secretion do not change with age.
- Clock time does not always correspond to biological time, particularly in individuals performing shift work or with jet lag or sleep disorders.

Diurnal variation in serum TSH



- TSH levels show a considerable diurnal variation.
- A decrease of up to 50% occurs from 8:00 to 9:30 AM.
- Thereafter the concentration remains relatively constant until evening with a smaller nadir in the late afternoon.
- Values tend to be lowest in the late afternoon and highest around the hour of sleep (midnight).
- ***Variations of serum TSH values within the normal range of up to 40%-50% do not necessarily reflect a change in thyroid status.***

*(*The vast majority of blood samples for thyroid hormone measurement are obtained between the hours of 7 AM and 8 PM)

- *Jensen E et al. Sampling Time Is Important but May Be Overlooked in Establishment and Use of Thyroid-Stimulating Hormone Reference Intervals. Clin Chem. 2007; 53(2):355-6.*
- *Sheehan MT. Biochemical Testing of the Thyroid: TSH is the Best and, Oftentimes, Only Test Needed - A Review for Primary Care. Clin Med Res. 2016;14(2):83-92.*
- *Philippe J, Dibner C. Thyroid Circadian Timing: Roles in Physiology and Thyroid Malignancies. J Biol Rhythms. 2015 ;30(2):76-83.*

Take Medication ?

- Serum T4 & FT4 concentrations peak 2-4 hours after an oral dose of LT4.

- They remain above normal for approximately 6 hours in patients receiving daily replacement therapy.

- TSH and T3 levels are not affected by LT4.

- ***So, it is recommended that blood sampling for T4 & FT4 measurements be performed before taking any thyroid medication.***

Take Medication ?

- It’s important to be aware that T3 has a half-life of around 8 hours.
- FT3 levels increases after taking T3-containing medication and reach a peak after 3-4 hours.
- Straight after taking a T3 containing medication, the TSH level begins to fall and then stays suppressed for as long as 5 hours.
- ***So, it is recommended to hold off taking T3-containing medication until after the blood sampling.***

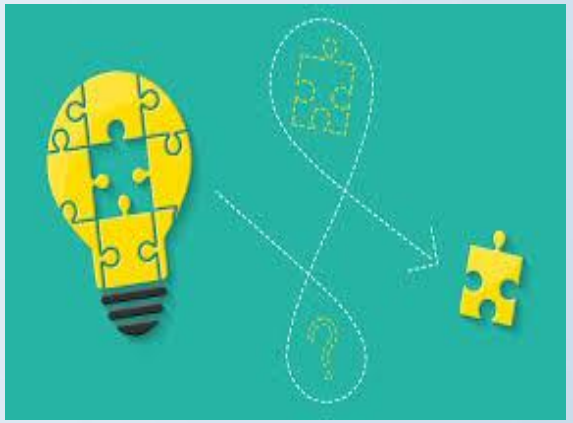
Suggestions

- At least 2-3 hours fasting before sampling
- Monitoring of TSH level in each patient at the same time, and under the same circumstances.
- For thyroid hormone measurements, blood sampling should be performed before taking thyroid medications.

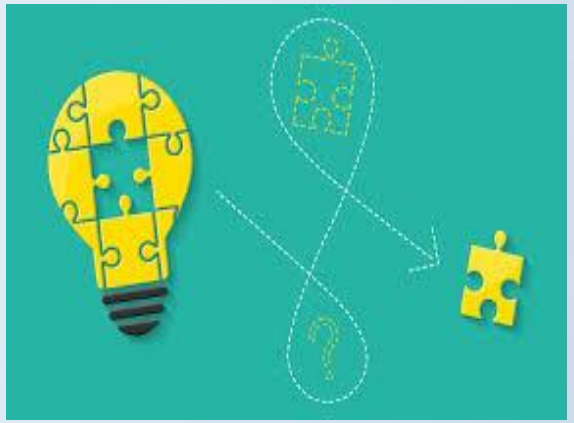
Discordant thyroid function tests

What should be suspected when thyroid function tests do not make sense?

❖ Clinical history



- ✓ *Age & proper reference values*
- ✓ *Physiology conditions affecting TFT (Pregnancy)*
- ✓ *Conditions associated with changes in TBG concentration*
- ✓ *Confounding medication*
  - *Altered TBG concentrations*
  - *Competition for hormone bonding sites on TBG*
- ✓ *Non-thyroidal illness*
- ✓ *Thyroid medication*
- ✓ *Subjects that are more likely to develop an interference (such as recent immunization, transfusion, autoimmune disease, monoclonal therapy, contact with pets)*



- **Biological variability**
- **Assay interference**
- **Rare genetic & acquired disorders of HPT axis**
  - ✓ *Thyroid hormone e resistance*
  - ✓ *TSHoma*

Biological variability

Biological variability

❖ Definition: The natural variability in a laboratory parameter due to physiologic differences among subjects and within the same subject over time.

I) Inter-individual variability:

Variability due to factors specific to individual subjects

(diet, genetics or immune status)

II) Intra-individual variability:

Variability due to biologic changes that cause analyte levels to fluctuate over time

❖ For some analytes such as TFTs, guidelines have been published as to what constitutes a clinically significant difference between two consecutive patient sample results.

Clinically Significant Difference between Two Consecutive Patient Results

Analyte	Change
Total T_4	2.2 µg/dL
Free T_4	0.5 ng/dL
Total T_3	35 ng/dL
Free T_3	0.1 ng/dL
TSH	0.75 mIU/L
Thyroglobulin	1.5 ng/mL

Data from Baloch Z, Carayon P, Conte-Devolx B, et al: Laboratory medicine practice guidelines: laboratory support for the diagnosis and monitoring of thyroid disease, *Thyroid* 13(1):3–126, 2003.

T_3, Triiodothyronine; *T_4,* thyroxine; *TSH,* thyroid-stimulating hormone.

Assay interference

When the suspicion of an interference raise up?

Discrepancy between:

- ✓ test values and previous results obtained with the same test.
- ✓ discrepancies with other biochemical parameters or clinical settings

Macro- TSH interference

- A large circulating form of TSH composed of monomeric TSH complexed with autoimmune anti-TSH antibodies.
- Macro-TSH is mostly composed of IgG-bound TSH.
- Prevalence: 0.6 to 1.6 %
- Macro-TSH, a large molecule of at least 150 kDa, accumulates in the circulation, compared to small bioactive molecule of TSH, 28 kDa
- Macro-TSH is currently considered to be inactive because auto-antibodies bound to TSH may prevent the activation of TSH receptors due to steric hindrance.
- Macro-TSH can lead to falsely high TSH results.
- Different assay platform show different sensitivity to presence of Macro-TSH.
- No ideal assay without cross reaction with macro-TSH is still developed.

A case:
Markedly elevated TSH [232 (0.45–5.0) mIU/liter)]
a low-normal FT4 [10 (10.0–23.0) pmol/liter]

- ✓subclinical hypothyroidism
- ✓malabsorption of L-thyroxine
- ✓use of certain drugs (e.g., amiodarone, lithium)
- ✓TSH resistance
- ✓biologically inactive TSH
- ✓non-thyroidal illness during the recovery phase
- ✓macro-TSH interference

Which cut off is suggested to suspect presence of macro-TSH ?

- ✓ A cutoff of 10 mUI/L for TSH concentration along with normal thyroid hormones could be proposed to suspect the presence of macro-TSH.
- ✓ This cutoff is not perfect, however; some macro-TSH cases have been reported with only a slight elevation of TSH.
- ✓ Hence, interference should be suspected in a patient with isolated TSH elevation (typically markedly elevated), with thyroid hormones in the upper half of the normal range, and without signs or symptoms of thyroid dysfunction.

Evaluation of presence of macro-TSH

1. Serial dilution

- ✓ Using diluent provided by the manufacturer or Phosphate-buffered saline (PBS), a buffer solution commonly used in biological research.
- ✓ Interpretation: An increased recovery of diluted samples showing non-linearity may be indicative of macro-TSH presence.
- ✓ Neither specific nor sensitive
- ✓ Lack of parallelism can be seen with other interfering antibodies (e.g., heterophilic antibodies, rheumatoid factor, anti-Ru antibodies)

Evaluation of presence of macro-TSH

2. Polyethylene glycol (PEG) precipitation method

- ✓ Interpretation: If TSH recovery is low, *the* ***presence of a high-molecular-weight interfering substance*** such as macro-TSH should be suspected.
- ✓ Although several authors have used a 40% cutoff for macro-PRL, others have proposed a lower cutoff of < 20% or < 25% for macro-TSH.
- ✓ However, concern has been raised in the literature regarding the use of recovery calculation.

Evaluation of presence of macro-TSH

2. PEG precipitation method

- ✓ Recovery calculation can lead to mismanagement of thyroid conditions in certain patients, and normalization of hormone concentration after PEG precipitation should also be taken into account.
- ✓ A fraction of ~25% of free analyte is co-precipitated upon PEG precipitation.
- ✓ So, the reference range provided by manufacturers cannot be used & adjusted post-PEG reference ranges must be established for each immunoassay, because the susceptibility to macro-complexes varies between platforms.

Evaluation of presence of macro-TSH

2. PEG precipitation procedure

Some important issues:

- ✓ Patientswho have both macro-TSH and primary hypothyroidism
- ✓ An increase in globulin concentration can enhance the fraction of precipitated TSH, thus resulted in misclassification.
- ✓ The preferred method for identifying macro-TSH remains GFC, and low recovery after PEG treatment should always be confirmed by GFC.
- ✓ GFC is expensive, not widely available, and can confound macro-TSH with human anti-mouse antibodies (HAMAs).
- ✓ *Hattori N et al. Macro TSH in patients with subclinical hypothyroidism. Clin Endocrinol (Oxf). 2015;83(6):923–930*

Of 117 patients with low PEG recovery of TSH > 25%, only seven had evidence of high-molecular-mass TSH > 100 kDa on GFC.

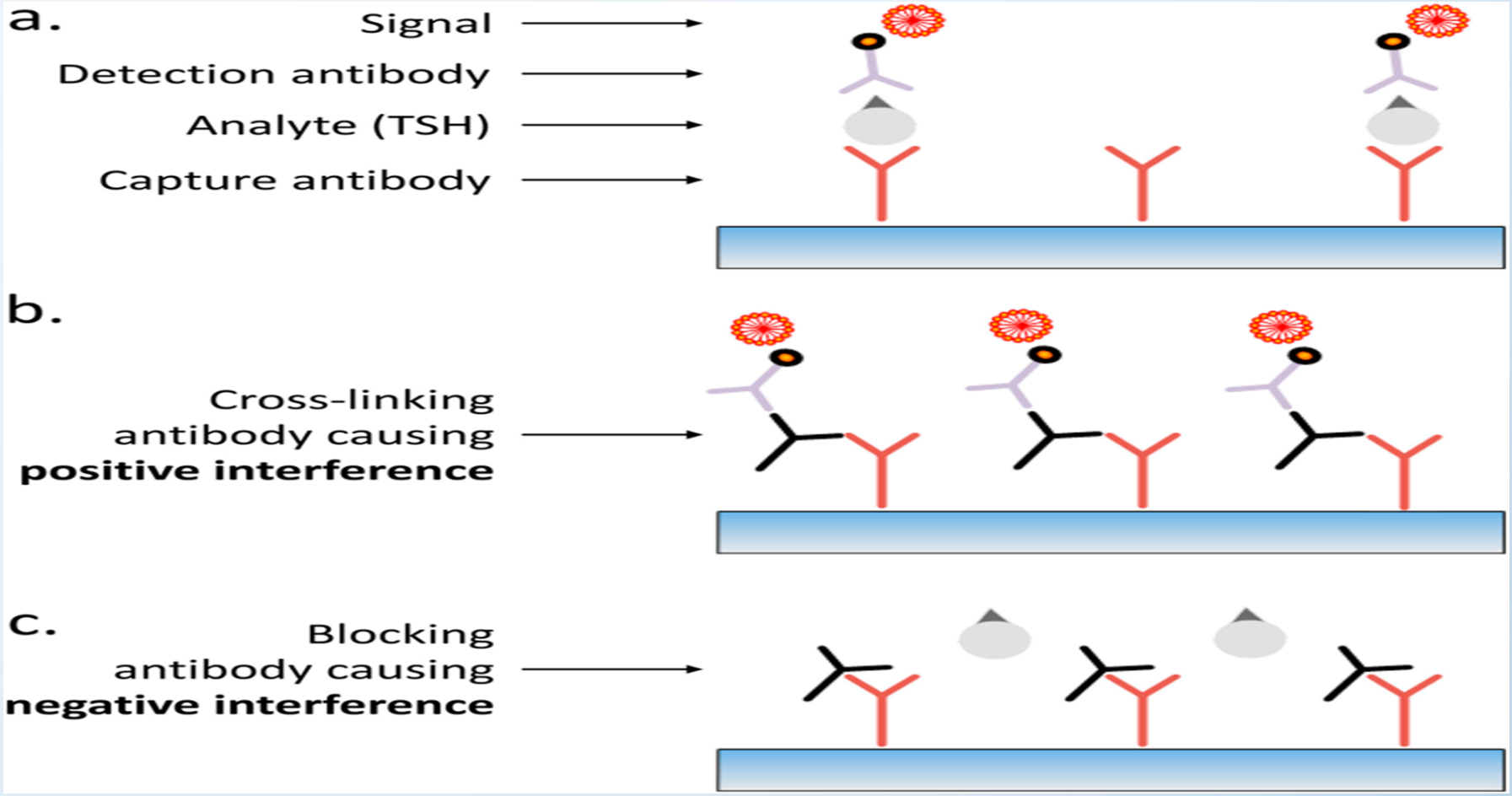
Evaluation of presence of macro-TSH

2. PEG precipitation method

- ✓ Incubating serum supposed to contain interference for 4 hours with a serum from a patient known to exhibit hypothyroidism (i.e., high TSH levels) in a 1:1 ratio enables differentiation between macro-TSH and heterophilic antibody interference.
- ✓ Interpretation: Decreased recovery after incubation suggests excess TSH binding capacity (i.e., macro-TSH), which is usually not found with heterophilic antibody interference

Interference in TSH measurement

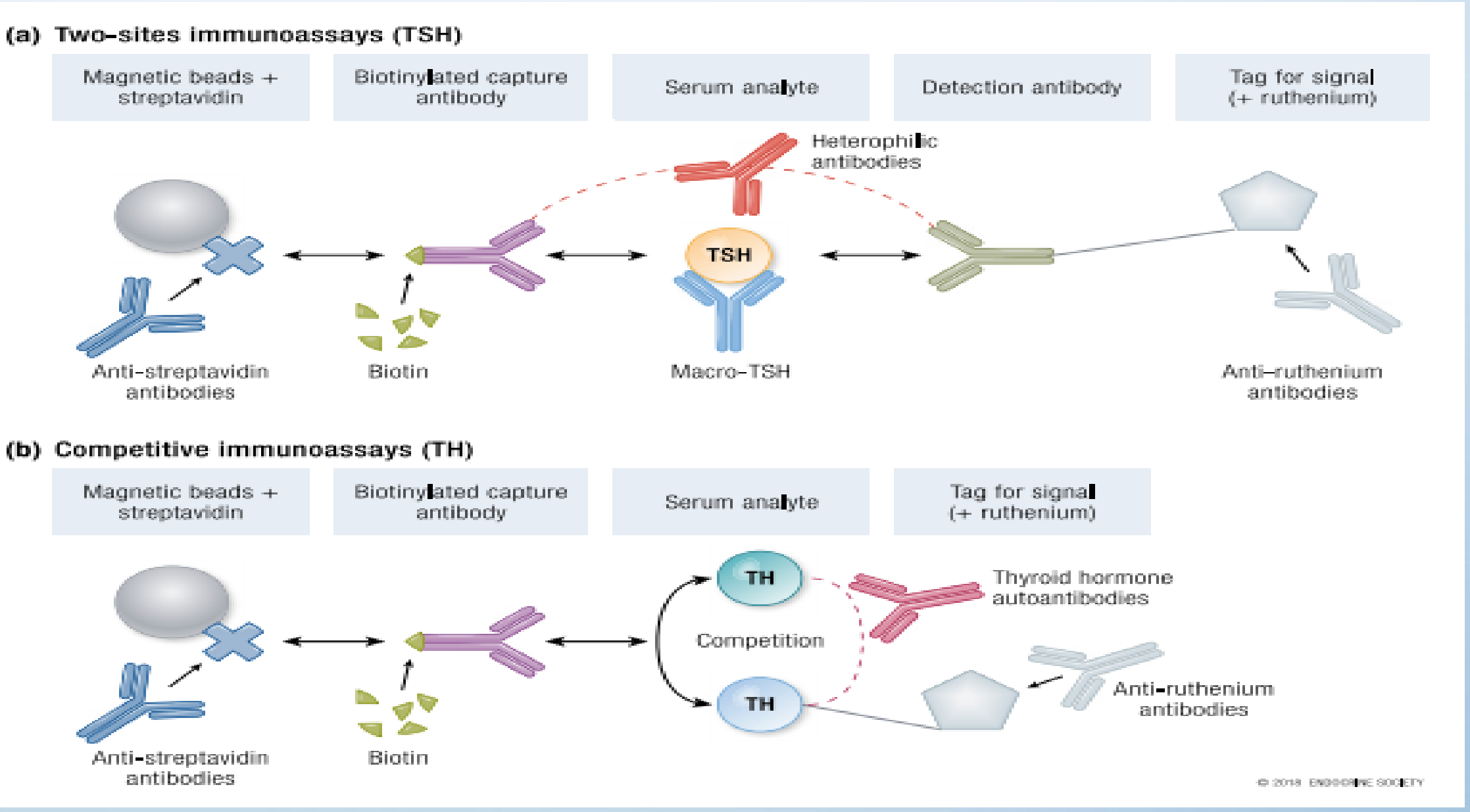
Human anti-animal antibodies (HAAs) in a patient's serum if directed against the same species as the assay antibodies



Best Practice & Research Clinical Endocrinology & Metabolism 2013 27, 745-762

Biotin interference

- Biotin (or vitamin H, B7,or B8) is a small (244.3-Da), soluble, essential decarboxylase enzyme cofactor synthesized by bacteria in the gut and directly bio-available from food intake.
- Adequate intake: 30-35mg/day in adults and 20-25/day in children
- Treatment with high biotin doses (100-300 mg/day) in different conditions:
  - Multiple sclerosis
  - Rare metabolic disorders (i.e.,bio-tinidase deficiencies and propionic acidemia)
  - Dietary supplement for alopecia or to improve nail and skin texture
- The high affinity of the non-covalent biotin-streptavidin interaction has been widely used in two-site and competitive in-vitro immunoassays as an immobilizing system.
- The most popular immunoassays are biotin based.
- The prevalence of biotin interference is currently not known but considering the high frequency of thyroid function testing for thyroid dysfunction, the scale of the problem seems enormous.


(a) Two-sites immunoassays (TSH)
Magnetic beads + streptavidin
Biotinylated capture antibody
Serum analyte
Detection antibody
Tag for signal (+ ruthenium)
Heterophilic antibodies
TSH
Anti-streptavidin antibodies
Biotin
Macro-TSH
Anti-ruthenium antibodies
(b) Competitive immunoassays (TH)
Magnetic beads + streptavidin
Biotinylated capture antibody
Serum analyte
Tag for signal (+ ruthenium)
TH
TH
Competition
Thyroid hormone autoantibodies
Anti-ruthenium antibodies
Anti-streptavidin antibodies
Biotin
© 2019 ENDOCRINE SOCIETY

Biotin interference

- Interestingly, biotin has been reported to act as an interfering factor in certain immunoassay platforms.
- In TSH sandwich assays, excess biotin displaced biotinylated antibody-antigen complexes from streptavidin-coated microparticles, resulting in falsely low TSH levels (as the assay signal is directly related to TSH concentration).
- In contrast, in competitive assays of FT3 and FT4, excess biotin caused overestimation of both hormones (as the signal is inversely proportional to hormone concentrations).
- It is essential to note that the impact of biotin is directly related to the type of platform used:
  - All TSH, FT3, and FT4 affected by excess biotin
  - Either TSH or free hormones affected by excess biotin
  - Platform uses a preformed streptavidin-biotin complex not sensitive to the presence of biotin.
  - Immunoassays that are not affected by biotin, because the biotin-streptavidin immobilization system is not used for TSH, FT3 and FT4 measurements.

Biotin interference

- The extent of biotin interference depends on several factors:
  - ✓ sample volume (the lower the volume, the lower the biotin concentration)
  - ✓ Sandwich or competitive assays (excess antibody reagent in two site immunoassays)
  - ✓ one-step or two-step format
  - ✓ wash or no-wash procedure
- Manufacturers often provide the biotin cutoff point above which interference may be observed.
  - It remains difficult, however, to evaluate which daily doses these cutoffs correspond to.
  - These cutoffs have been determined in vitro and may thus not translate to in vivo conditions.
- Biotin interference in immunoassays is not expected with normal dietary intake of biotin.

Evaluation of Biotin interference

I. Question of whether the patient is taking biotin.

- ✓ The problem is that biotin is not always considered a medication or not necessarily documented on dietary supplements designed to improve the quality of hair, skin, or nails.

II. Dilution test with the manufacturer's diluent

III. A comparison with another method not using the biotin-streptavidin interaction can be used.

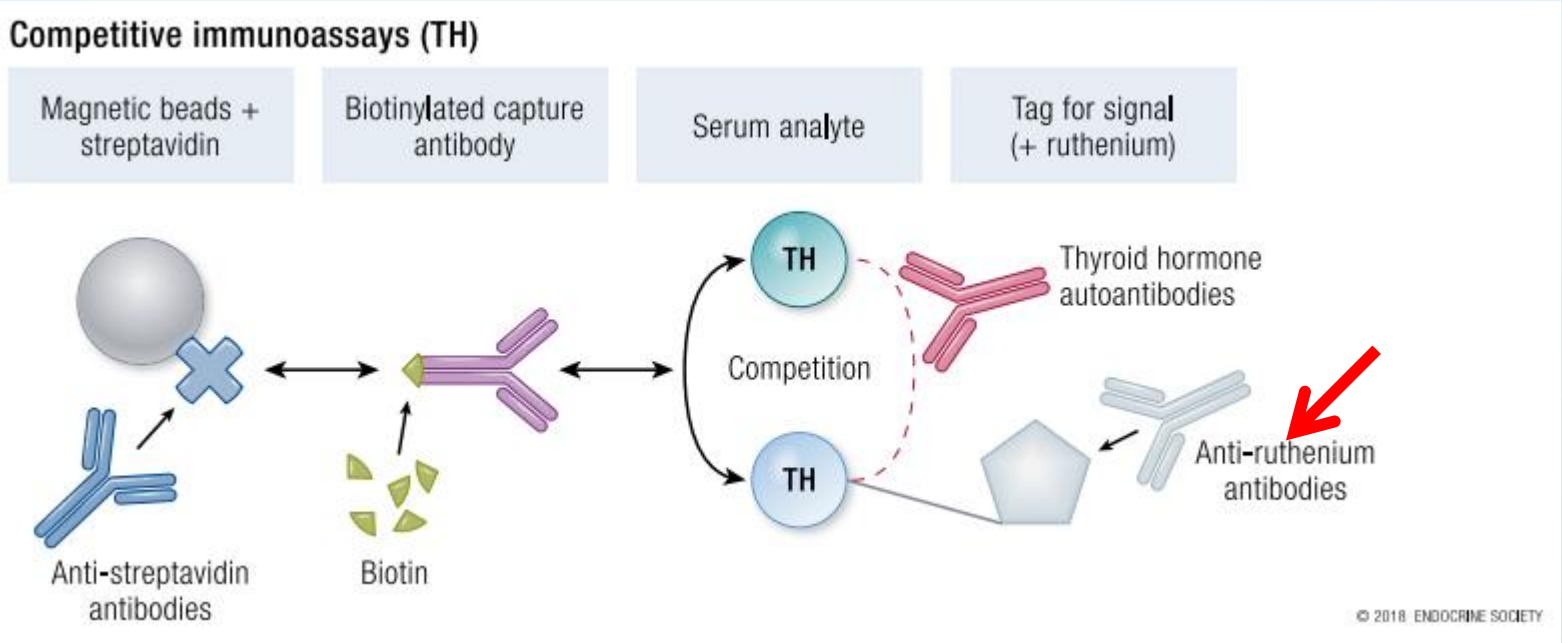
IV. Washout period may be advisable to be free of this interference.

- ✓ Suggested different washout periods of 8 hours, 16 hours, 25 hours, 2 days, 3days, or even more rendering the implementation of washout guidelines problematic.

V. Incubating the sample potentially containing biotin with streptavidin beads (recycled from the manufacturer's kits) and then re-assayed on the same platform. If biotin is present, a substantial change from baseline is expected.

Thyroid hormone autoantibody (THAAb) Interference

- anti-T3 and anti-T4
- THAAbs are IgG iso-types
- More prevalent in patients with autoimmune disorders.
- The prevalence of THAAbs in the general population is low (1.8 %) vs. up to 40 % in autoimmune thyroid diseases.
- Tg-Ab or TPO-Ab have been found in THAAb- positive samples in up to 80-100% of cases.
- Presence of THAAbs should suspected in patients with autoimmune disorders if any interference is suspected .



- In the absence of THAAbs, the labeled tracer and free hormones in the sample compete for binding sites on the capture antibody.

- In the presence of anti-T3 and anti-T4, autoantibodies may bind to both the measured analyte and labeled tracer, thereby skewing the exact concentration of thyroid hormones.

THAAb Interference

- The one-step immunoassays, the patient's serum and labeled hormone analog are added to the reaction chamber at the same time and compete for the solid phase antibody. The unbound material is then washed away, with only the bound analog measured.

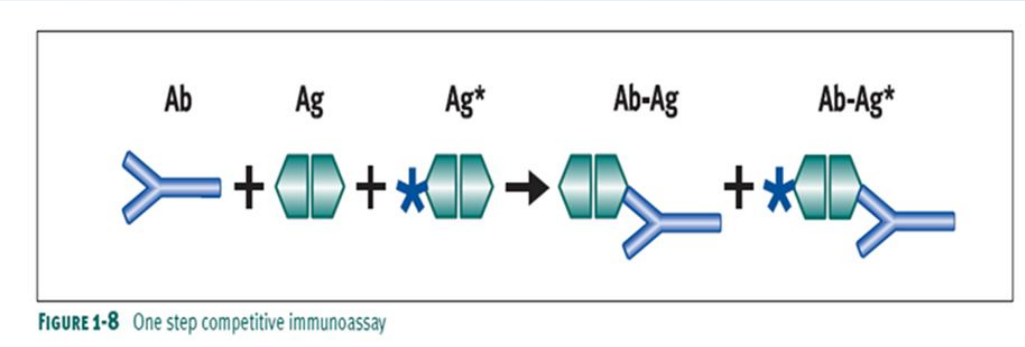


FIGURE 1-8 One step competitive immunoassay

- THAAbs bind to analogs because they are less available for competition. The signal, therefore, is reduced, yielding a falsely elevated hormone value (free and total hormone concentration), given that there is an inverse relationship between signal and analyte concentration .

THAAb Interference

- Assays in which there is no contact between the patient's serum and analog tracer (i.e., two-step assays) are considered insensitive toward these autoantibodies.

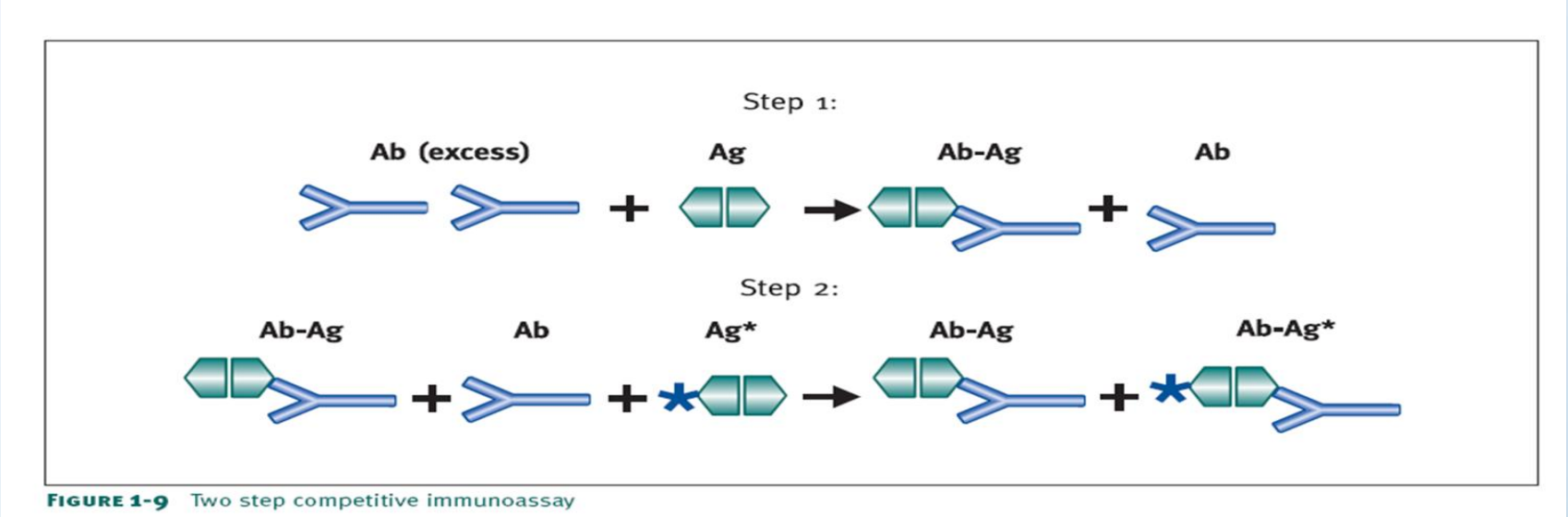


FIGURE 1-9 Two step competitive immunoassay

- Therefore, in theory, only one-step immunoassays are likely affected by THAAb interference.

How to evaluate the THAAb interference?

If specious results are suspected:

- ✓ Is the used immunoassay a ‘one-step’ competitive method?

- ✓ If yes, using a ‘two-step’ (‘back titration’) assay method is a logical first step.

In two- step method, a wash step prior to tracer addition, may reduce but not completely eliminate interference.

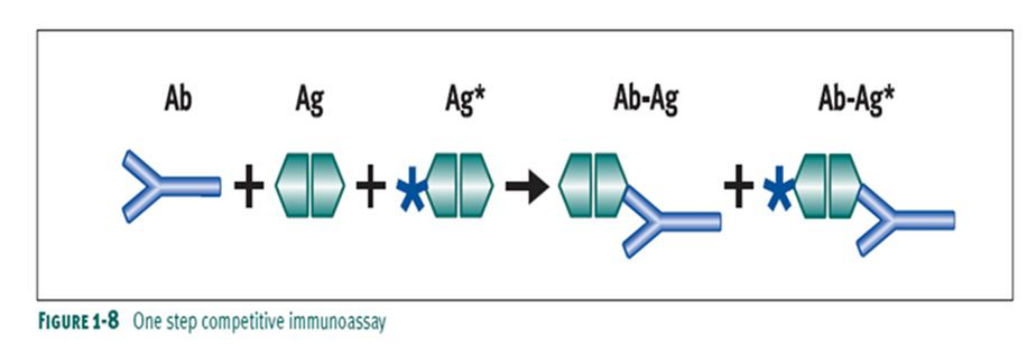


FIGURE 1-8 One step competitive immunoassay

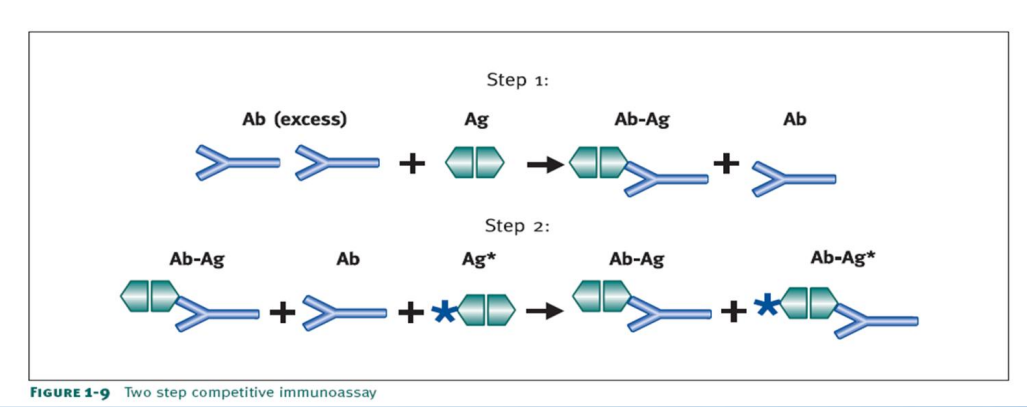


FIGURE 1-9 Two step competitive immunoassay

How to evaluate the THAAb interference?

- In practice, several one-step immunoassays are not sensitive toward THAAbs, whereas some two-step assays might be affected by their presence.
- Contributing factors:
  - ✓ The nature & heterogeneity of the tracer
  - ✓ method of detection
  - ✓ affinity of the antibodies
- Other methodologies used to detect interference in literature review:
  - ✓ dilution test
  - ✓ acid-charcoal treatment
  - ✓ protein A chromatography, heterophilic blocking tube, THAAb measurement, IgG purification and THAAb measurement

Interference in free hormone measurements

- Free hormone assays are designed such that the equilibrium between T4 and its binding proteins is preserved during measurement, so that the amount of tracer displaced reflects the 'free' rather than 'total' hormone concentration.

- The presence of factors in serum which affect this equilibrium will confound hormone measurement, such as:

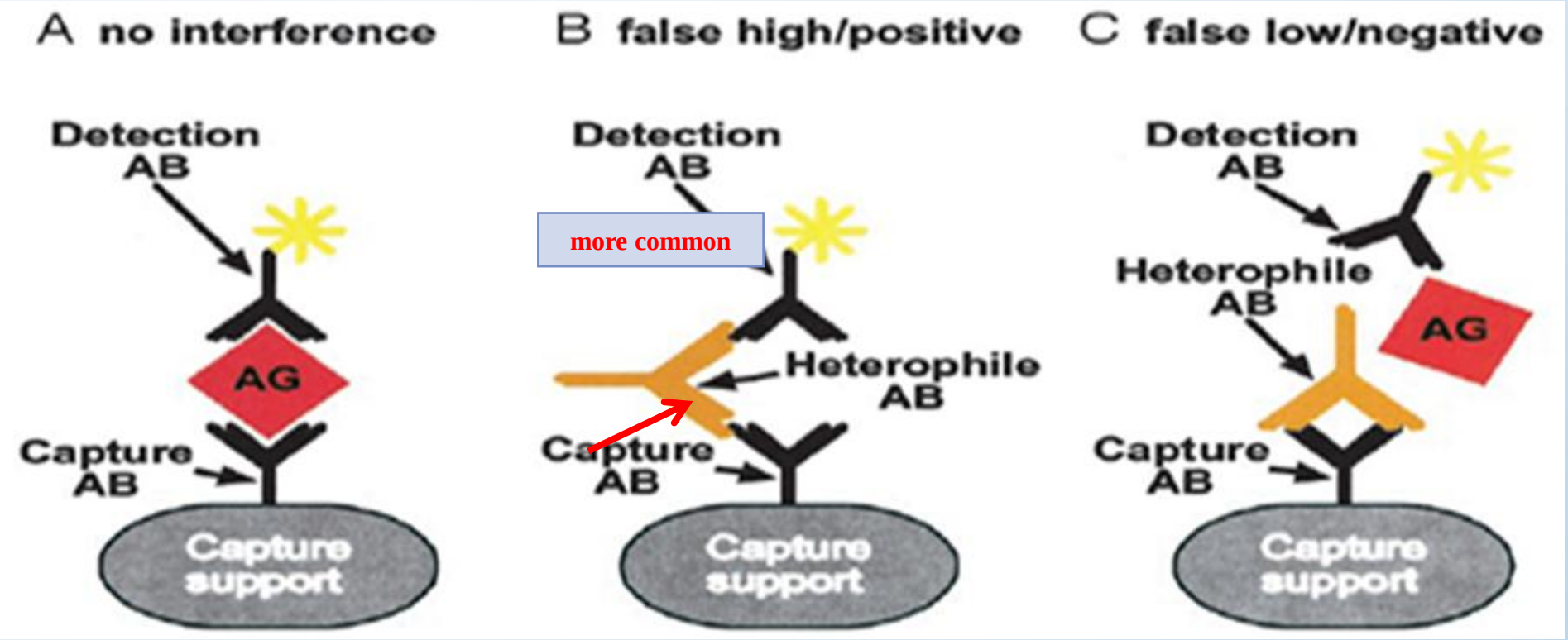
  - ✓ *Heparin and other displacing agents*
  - ✓ *HAAs or heterophile antibodies*
  - ✓ *Variant thyroid hormone binding proteins with altered affinity for T4 (e.g. albumin in familial dysalbuminaemic hyperthyroxinaemia)*
  - ✓ Anti-iodothyronine antibodies, which can bind the tracer

- Hormone measurement following equilibrium dialysis remains the gold-standard for eliminating free hormone assay interference.

Heterophilic Antibody Interference

- The definition of heterophilic, human anti-mauce antibodies (HAMAs), and human anti-animal antibodies (HAAAs) is vaguely used in the literature.
- HAAAs are monospecific, high-affinity antibodies directed against animal epitopes from goats, rabbits, sheep, horses, or, more frequently, mice.
- Heterophilic antibodies are weak polyspecific antibodies (usually of low titer) formed early in the immune response prior to affinity maturation. They typically react with immunoglobulins derived from at least two species.
- In daily laboratory practice, the term heterophilic antibody is typically used whenever one suspects a patient's sample contains antibodies that cause false results by binding to the assay antibodies.

Interference due to heterophilic antibodies may lead to falsely low or high analyte levels in one or more assay systems, depending on the interference site within the reaction.



Best Practice & Research Clinical Endocrinology & Metabolism 2013 27, 745-762

Which thyroid test are more affected by heterophil Ab interference?

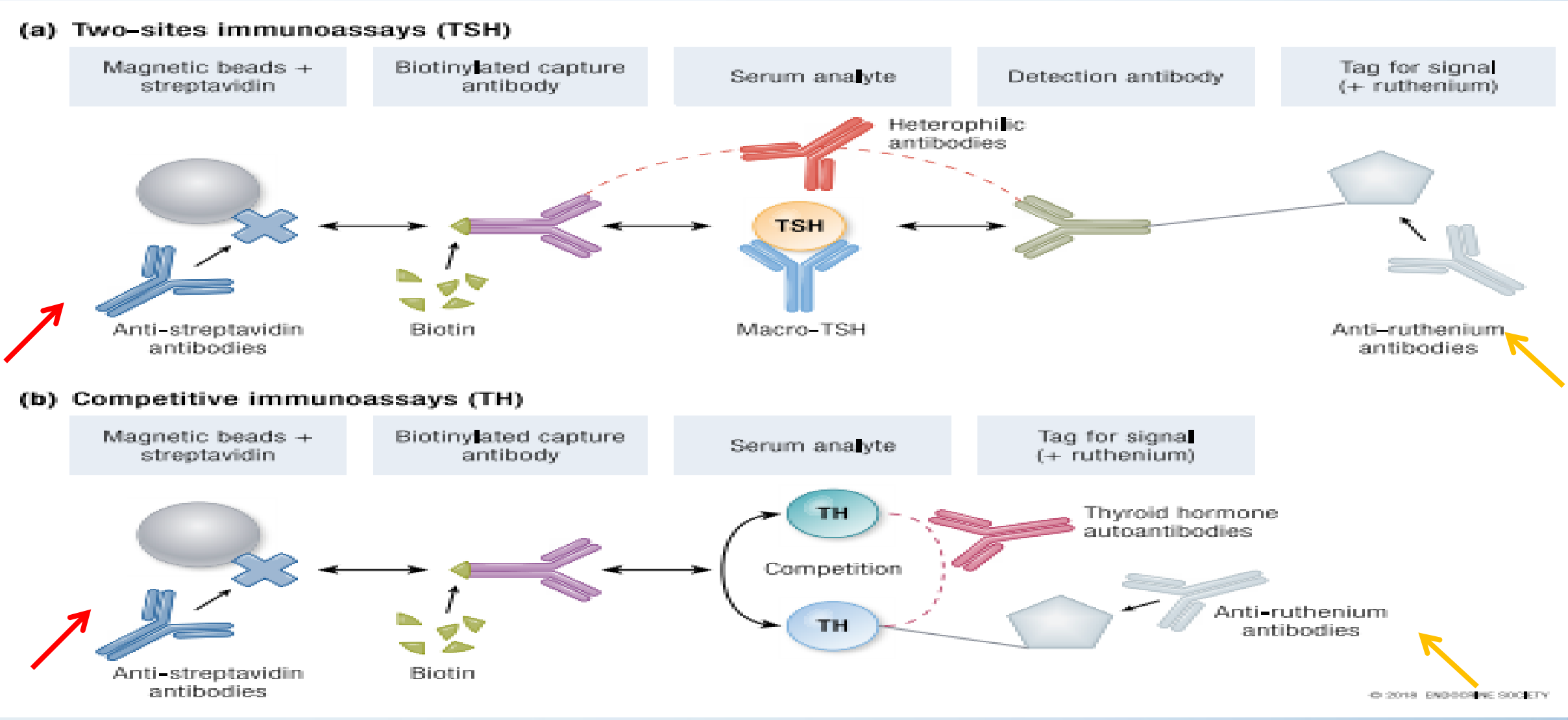
- Two-site immunoassays (typically TSH assays) are more sensitive toward heterophilic antibodies.
- FT3 and FT4 assays are less prone to being affected by these interfering agents.

How to evaluate the Heterophilic Antibody interference?

I. Comparison against an assay using other antibody species
II. Dilution test
III. Using the heterophilic blocking tube (HBT) test

- ✓ The presence of heterophilic antibodies may persist for a prolonged time (e.g., 4-12 months).
- ✓ Heterophilic antibodies may pass the placenta, interfering with thyroid function tests in newborns.
- ✓ Different strategies to remove these interferences in assay kits such as adding nonspecific animal immunoglobulins; heat aggregated, nonspecific, murine monoclonal antibodies, …. .
- ✓ Samples contain very high amounts of interfering antibodies that may still show interference in the assay.

Other interferences



Anti-Ru Interference

- ✓ Ruthenium is a chemical element and rare transition metal have its place in the platinum group.
- ✓ It is largely used as a chemical catalyst in electrical contacts, thick-film chip resistors, and platinum alloys.
- ✓ Ru may also be found in the food chain and clothing residues.

How to evaluate the Heterophilic Antibody interference

I. Using an alternative non-Ru method
II. PEG precipitation

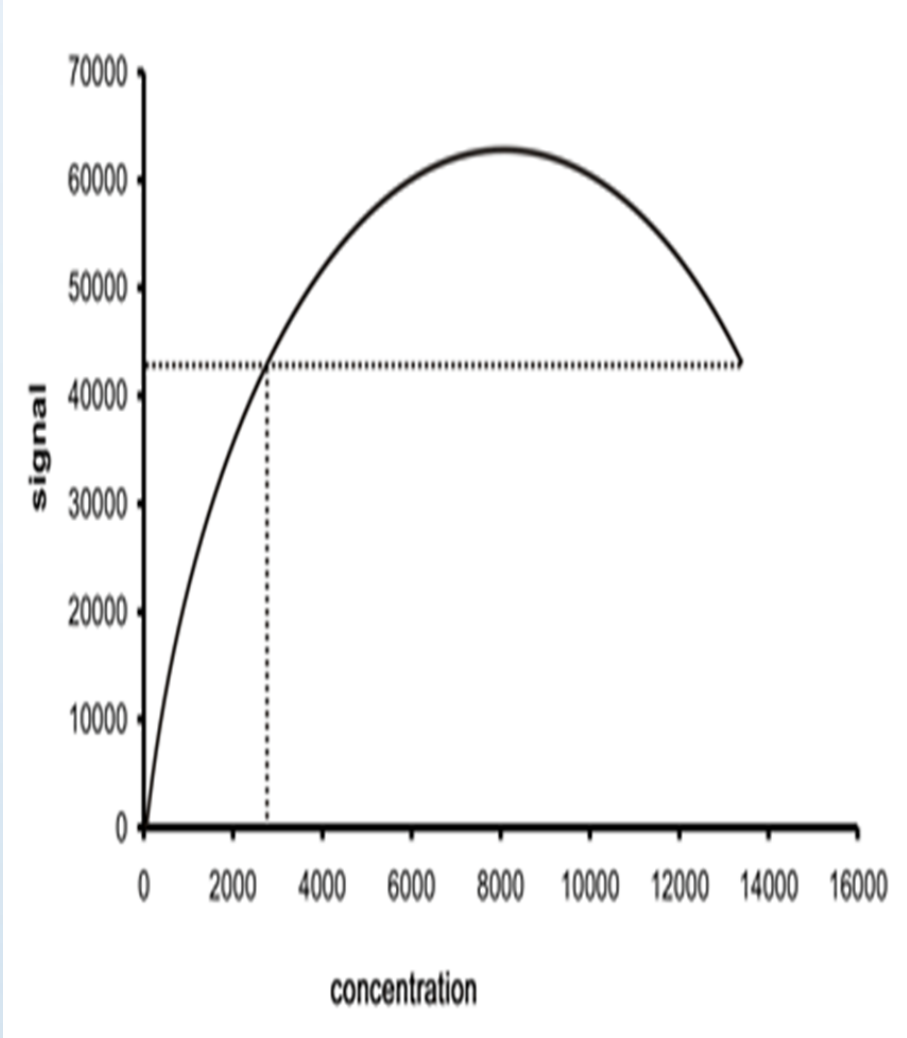
Hook effect

- Roche (ECLIA):

✓ No high-dose hook effect at TSH concentrations up to 1000 µIU/mL.

✓ No data regarding hook effect on T4, T3 or free hormone assays.

- There is no high-dose hook effect at Tg concentrations up to 120000 ng/mL.



Drug interference

- ***Increased TBG:***
  - Tamoxifen
  - Raloxifene
  - Estrogen
  - Fluorouracil
  - Clofibrate
  - Heroin/methadone
  - Mitotane

- D***ecreases TBG:***
  - Nicotinic acid
  - Asparaginase
  - Chronic glucocorticoid therapy
  - Androgens/anabolic steroids

- ***Displacing agents*** that affect the equilibrium between T3 or T4 and their binding proteins, thus resulting in altered free TH concentrations.
  - Aspirin,
  - Furosemide
  - Carbamazepine
  - Phenobarbital, Phenytoin
  - NSAID
  - Phenylbutazone
  - Heparin (fractionated or unfractionated)

Discordant TFTs

Step 1: Re-evaluate clinical history

Age

Consider:
- neonatal period
- elderly

Pregnancy changes

Consider:
- ↓TSH (1st trimester; 2° to ↑hCG)
- ↑TT4 & ↑TT3 (from 1st trimester; 2° to ↑TBG)
- changes in FT4 & FT3
- pregnancy RR

Thyroxine therapy

Consider:
- confounding dietary factors or medications
- malabsorption syndromes
- altered TH metabolism
- non-compliance
- other factors (see Table 2)

Confounding medications

Consider:
- amiodarone
- furosemide
- heparin
- corticosteroids
- dopamine
- others (see Table 3)

Non-thyroidal illness (NTI)

Step 2: Re-assess thyroid status

? hypothyroid

? euthyroid

? hyperthyroid

Step 3: Decide which TFT is most likely to be discordant

Step 4: Exclude TH &/or TSH assay interference (consider specialist laboratory input)

Step 5: Investigate for rare genetic/acquired disorders of HPT function (consider referral to specialist centre)

Best Practice & Research Clinical Endocrinology & Metabolism 2013 27, 745-762DOI: (10.1016/j.beem.2013.10.003)

Interferences With Thyroid Function Immunoassays: Clinical Implications and Detection Algorithm.
Julien Favresse et al. (Endocrine Reviews 39: 830 – 850, 2018)

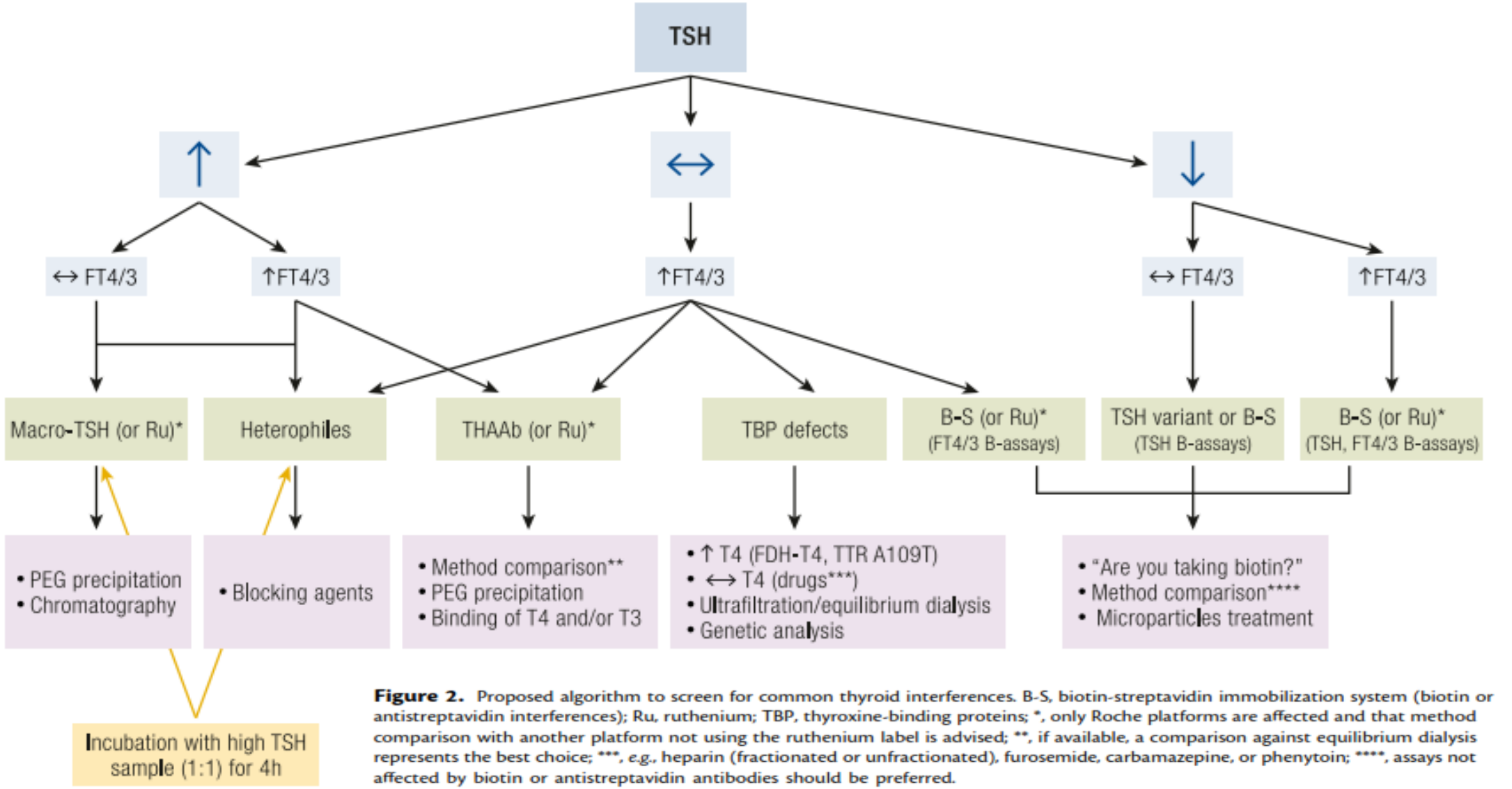


Figure 2. Proposed algorithm to screen for common thyroid interferences. B-S, biotin-streptavidin immobilization system (biotin or antistreptavidin interferences); Ru, ruthenium; TBP, thyroxine-binding proteins; *, only Roche platforms are affected and that method comparison with another platform not using the ruthenium label is advised; **, if available, a comparison against equilibrium dialysis represents the best choice; ***, *e.g.*, heparin (fractionated or unfractionated), furosemide, carbamazepine, or phenytoin; ****, assays not affected by biotin or antistreptavidin antibodies should be preferred.

T-uptake test
&
Thyroxine binding capacity (TBC)

T-uptake assay

- The T-Uptake assay is an immunological method for measuring available thyroxine-binding sites. (Thyroxine binding capacity, TBC).
- In which exogenous T4 is added to saturate the transport protein TBG.

T- uptake (%)

$$FT4I = \text{Total T4 patient} \times \frac{\text{T3RU patient}}{\text{T3RU mean control}}$$

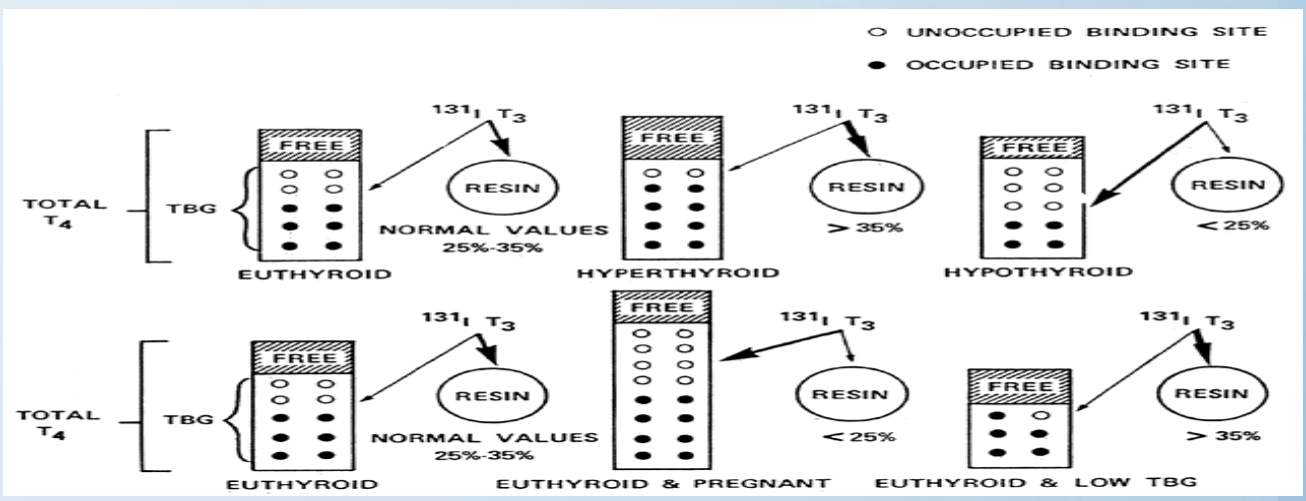
Mid-normal control T3RU for the laboratory on that day

Free T4 Index = Total T4 x T3 uptake (%)

T4 = 13.0, T-uptake= 29%

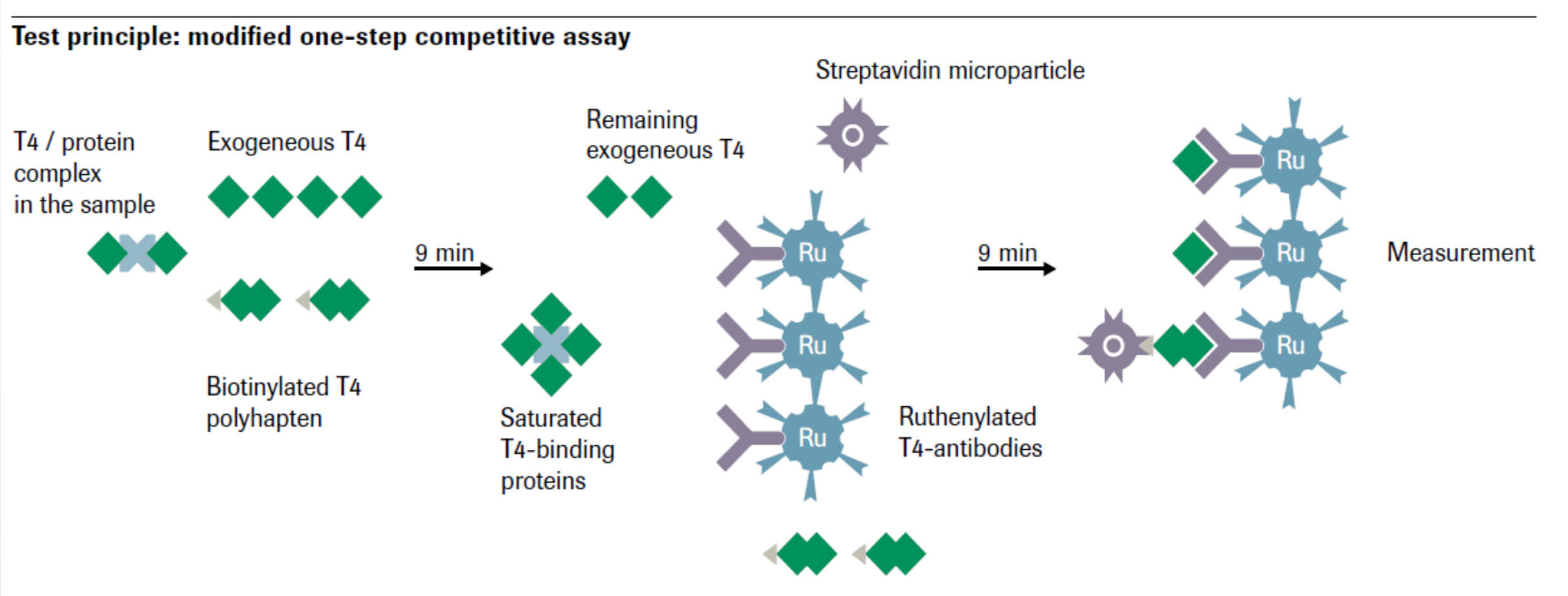
FT4I: 13.0 X 29%= 3.77

(1.33-4.68)



TBC (units are in Thyroxine Binding Index: TBI)

Immunoassay for the in vitro quantitative determination of thyroxine-binding capacity in human serum or plasma.



1st incubation

- ✓ Sample, excess exogeneous T4, and biotinylated T4-polyhapten are incubated. While the polyhapten does not bind to the carrier proteins, T4 occupies the free binding sites in the serum sample.

2nd incubation

- ✓ A ruthenylated T4-specific antibody is added and a competition between the exogeneous T4 and the polyhapten takes place.
- ✓ The more exogeneous T4 remains from the previous step, the less biotinylated polyhapten will react to form a complex.
- ✓ The immunological complex becomes bound to the added streptavidin-coated microparticles via interaction of biotin and streptavidin.

TBC assay (by TBI unit)

Result of Thyroxine binding capacity (TBI)

(Thyroxine binding index = result of the T- uptake determination)

- 0-19 years: 0.8-1.2 TBI
- > or =20 years: 0.8-1.3 TBI

FT4 index: T4/ (T-uptake) TBI (4.6-12.7 µg/dL)

Differ in different races

Immunoassay methods at a glance

Immunoassay, an analytical technology

- Using the binding between an molecule of interest as antigen and its homologous antibody.
- ***Labeled materials:***

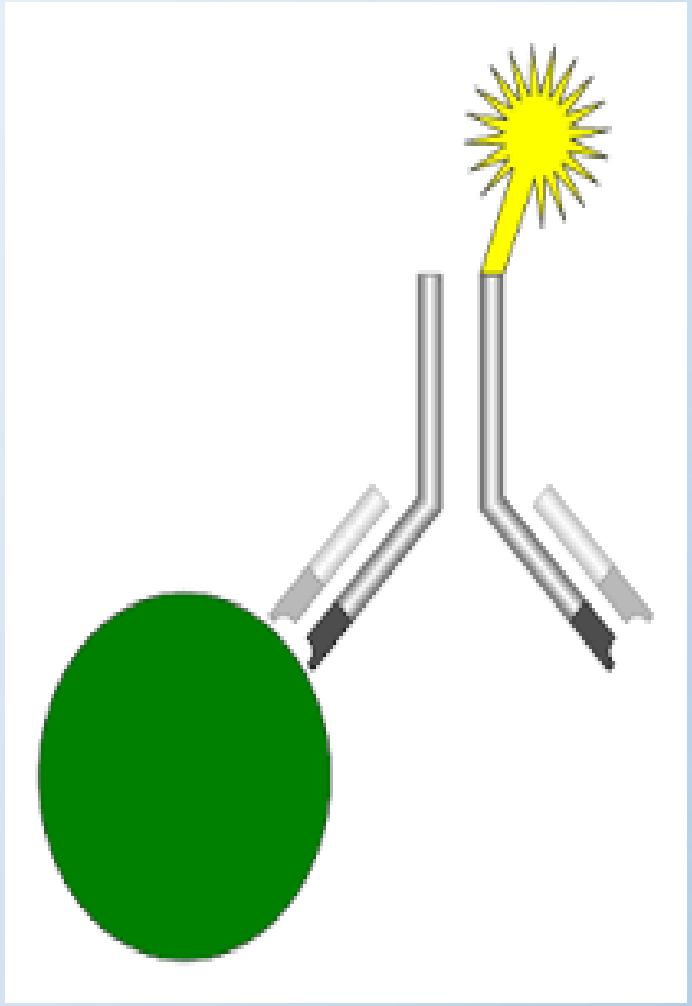
✓ Radioisotope e.g. I-125, I-131 (Radioimmunoassy)

✓ Enzyme e.g. Alkaline phosphatase (Enzyme- linked immunosorbent assay- ELISA)

✓ Fluorophore e.g. fluorescein (Fluorescence immunoassay)

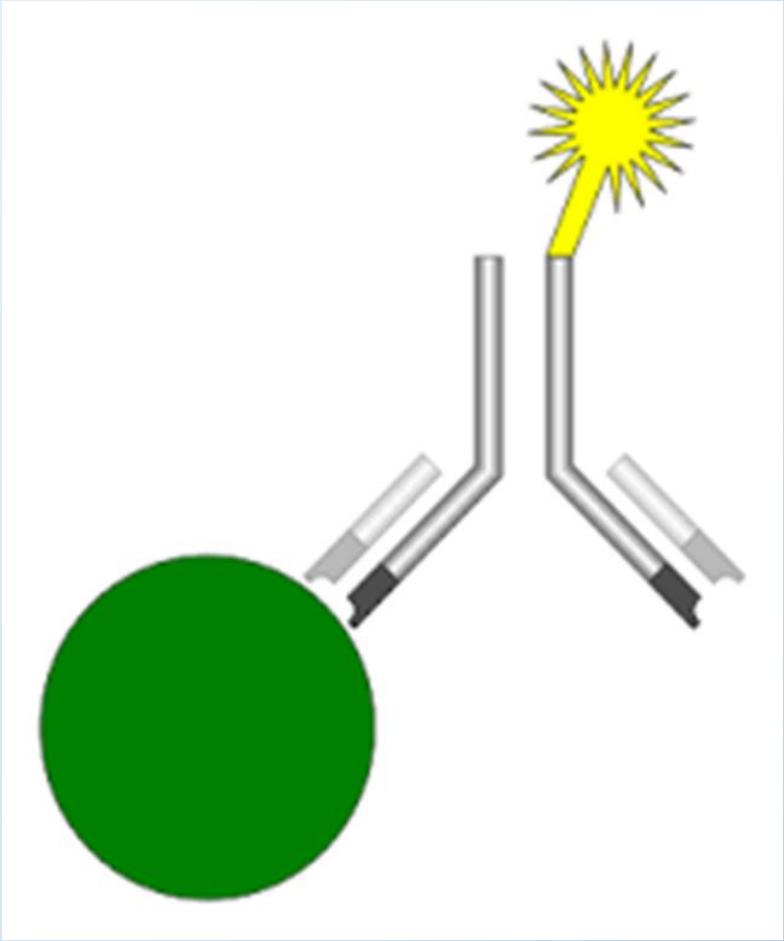
✓ Luminescent species e.g. Luminol (Chemiluminescent immunoassay- CLIA)

✓ Electrochemiluminescence [electrogenerated chemiluminescence (ECLIA or ECL)]: a kind of luminescence produced during electrochemical reactions in solutions



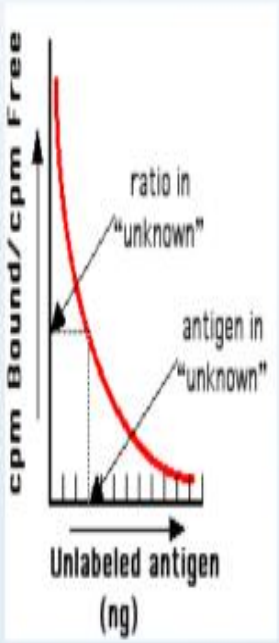
Label materials (types of immunoassays)

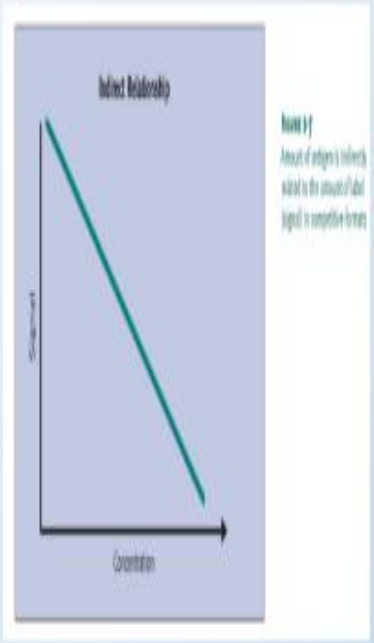
- ✓ Radioisotope e.g. I-125, I-131 (Radioimmunoassy)
- ✓ Enzyme e.g. Alkaline phosphatase (Enzyme- linked immunosorbent assay- ELISA)
- ✓ Fluorophore e.g. fluorescein (Fluorescence immunoassay)
- ✓ Luminescent species e.g. Luminol (Chemiluminescent immunoassay- CLIA)
- ✓ Electrochemiluminescence [electrogenerated chemiluminescence (ECLIA or ECL)]: a kind of luminescence produced during electrochemical reactions in solutions



Competitive immunoassays

The amount of antigen in the test sample is inversely related to the amount of label measured in the sample.





Non- competitive immunoassays

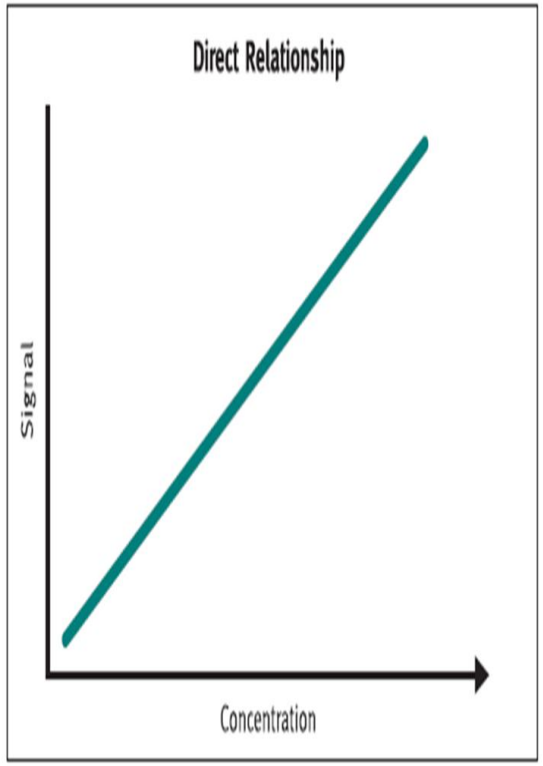


FIGURE 1-11 Amount of antigen is directly related to the amount of label (signal) in competitive formats

In noncompetitive assays, the measurement of labeled analyte, usually antibody, is directly proportional to the amount of antigen present in the sample. This can be represented by a dose response curve (Figure 1-11). The X-axis plots concentration of an antigen. The Y-axis plots response, which in this case is signal. Thus, the more antigen that is present, the more labeled antibody that will bind. This direct proportionality is in contrast with the indirect proportionality of competitive immunoassays discussed earlier.

Factors that affect the performance characteristics of an immunoassay

- ✓ Type of assay principle (competitive versus metric assay)
- ✓ One step versus two steps format
- ✓ Type of Ab (monoclonal versus polyclonal)
- ✓ Monoclonal Ab for single epitope versus multiple epitopes
- ✓ Specificity & affinity of the Ab
- ✓ Automated versus manual

Other important characteristics of an assay method

- ✓Traceability to reference standard material
- ✓Detection limit
- ✓Functional sensitivity
- ✓Specificity (cross reaction)
- ✓Measuring range
- ✓Sample volume
- ✓Test time
- ✓Expected values (age & sex- specific)

Take home messages

- For laboratory results that are discordant to the clinical pictures or appear incongruent with other results, revisiting the clinical history, confounding factors (physiologic, concurrent diseases, medication), and laboratory interference is essential.

- The Communication between clinicians and laboratory is essential to provide quality care to their patients.


Thank You
For Your Atten