Thyrotropin and thyroglobulin testing

Stefan K. G. Grebe, MD, PhD
Professor of Laboratory Medicine and Pathology
Mayo Clinic, Rochester, MN
Conflicts of interest

• None pertaining to this talk
Off label use of FDA regulated in vitro diagnostic devices

• None
Content

• Strengths and limitations of immunoassays (IAs)
• Measurement of thyrotropin (TSH)
• Measurement of thyroglobulin (Tg)
Immunoassays - definition

IAs use antibodies to detect or quantify analytes
Immunoassays – antigen requirements

- Target must be (or be made) immunogenic
  - Not completely conserved across species
  - MW >6000: immunogenic
  - MW 2000-6000: ? carrier needed
  - MW <2000: carrier needed

- Target must contain specific features recognizable by antibodies

- Target must be (or be made) accessible
  - Location
  - Carrier proteins
  - Solubility
Immunoassays – antibody requirements

- ABs are sufficiently specific
- ABs have sufficient avidity (polyclonal) or affinity (monoclonal) to target
- ABs are compatible with the chosen detection methodology/system
## Immunoassays – basic assay configurations

<table>
<thead>
<tr>
<th>Immunometric IA</th>
<th>Competitive IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>• &gt;= 1 ABs (usually 2)</td>
<td>• 1 AB</td>
</tr>
<tr>
<td>• AB excess</td>
<td>• AB limited</td>
</tr>
<tr>
<td>• No exogenous AG added</td>
<td>• Exogenous AG added</td>
</tr>
<tr>
<td>• AB binds endogenous AG</td>
<td>• AB binds endogenous or exogenous AG</td>
</tr>
<tr>
<td>• Signal proportional to endogenous AG conc.</td>
<td>• Signal inversely proportional to endogenous AG conc.</td>
</tr>
<tr>
<td>• (Label on AB)</td>
<td>• (Label on exogenous AG)</td>
</tr>
</tbody>
</table>

**Immunoassays**

- **Competitive IA**
  - 1 AB
  - AB limited
  - Exogenous AG added
  - AB binds endogenous or exogenous AG
  - Signal inversely proportional to endogenous AG conc.
  - (Label on exogenous AG)
Immunoassays – basic assay configurations

Immunometric IA

- Immunometric Assay ('sandwich assay')
  - Detection AB
  - Capture AB
  - AG
  - Capture support

Competitive IA

- Antibody Assay
  - Anti-human AB labeled
  - Auto AB
  - AG
  - Capture support

- Competitive Binding Assay
  - AG labeled
  - Capture AB
  - Capture support
Immunoassays – basic assay configurations

![Immunometric Dose-response Graph]

- **Signal** vs. **Concentration**
- **NSB** indicates the non-specific binding level.
Immunoassays – basic assay configurations

Competitive Assay Dose Response Curve

% Bound
Total

CONCENTRATION

(B0)

NSB
Immunoassays – detection format

*: Label-free
&: Direct label
$: Indirect label

- Photometry, Nephelometry, Turbidimetry
- Direct & Fluorescence
- Direct & Chemiluminescence
- Fluorescence & Polarisation
- Time-resolved Fluorescence
- ¹²⁵I RIA or IRMA &
- Chemiluminescent or fluorescent EIA
- Direct Fluorescence
- Direct & Chemiluminescence

High Conc. $>10^0$ $10^{-3}$ $10^{-6}$ $10^{-9}$ $10^{-12}$ Low Conc.

Analyte concentration (g/L)
Immunoassays – problems & interferences

Assay format specific problems

- Competitive assays - limited dynamic range
- Immunometric assays - hook
Immunoassays – problems & interferences

Limited dynamic range of competitive assays – 1-2 logs

![Graph showing detector response (RLU) vs. free T4 (ng/dL)]
Immunoassays – problems & interferences

Hook effect

![Graph showing the Hook effect](image)
Immunoassays – interferences

- Non-specific
  - pH and salt extremes
  - physico-chemical interference with antigen-antibody binding
  - reporter signal enhancement or quenching

- Specific
  - cross reactivity
  - autoantibodies
  - heterophile antibodies
Specific Immunoassay Interferences - Cross Reactivity -

• Similar epitopes
  • Interfering analytes with high homology to the primary analyte
    Example: Steroid hormones

• Shared epitopes
  • Analyte isoforms
    • Splice variants
    • Variant post-translational modifications
      Example: TSH
  • Analyte precursors
    Examples: procalcitonin/calcitonin
  • Analyte metabolites
    Example: PTH and its fragments
Specific Immunoassay Interferences
- Autoantibodies 1 - general mechanism -

Auto-ABs that do not bind to capture or detection epitopes:
No interference with detection, but antigen half-life prolonged
- falsely “elevated” results - ~50% of cases -
Specific Immunoassay interferences
- Autoantibodies 2 - general mechanism -

Auto-ABs that mask capture or detection epitopes:
  Detection fails
  - false negative results - ~50% of cases -
Specific Immunoassay Interferences - Heterophile Antibodies (HAB) -

• ‘heterophile’ = ‘friend of the other’: antibodies that can bind to animal antigens (including immunoglobulins)

• Three major categories:
  • High prevalence (up to 40% of the population) low specificity (‘polyspecific’), low to medium affinity antibodies to multiple human and animal antigens - e.g. ‘Paul Bunell-type ABs’
  • Antibodies to immunoglobulins (animal and human - mostly to Fc portion) - e.g. ‘rheumatoid factor’
  • Low prevalence (<1% of population) high specificity, medium to high affinity antibodies to very specific animal antigens from a single species (mostly to constant region of FAB) - e.g. human anti mouse antibodies (HAMA)
Immunometric assays - HAB interference -

- No interference by heterophile ABs
  - Detection AB
  - Capture AB

- Heterophile AB false positive (>80%)
  - Detection AB
  - Capture AB
  - Heterophile AB

- Heterophile AB false negative (<20%)
  - Detection AB
  - Capture AB
  - Heterophile AB
  - AG
  - Capture support
TSH measurement

- The best single marker to assess thyroid function status
The TSH response to changes in peripheral thyroid hormone levels is exponential. This makes it a very sensitive marker for changes in peripheral thyroid hormone levels.
TSH measurement

- The best single marker to assess thyroid function status
- A TSH within the ‘normal’ range should correlate with euthyroidism
No method to generate a reference range is perfect

TSH values in a cohort of subjects classified prior to TSH testing as: hyper-, eu-, or hypothyroid based on:
• clinical exam,
• TT4, FT4,
• TT3, FT3,
• rT3
• TRH-test

Values in the “gray-zones” are often observed in the absence of thyroid disease.
TSH secretion is pulsatile with diurnal variation

![Graph showing TSH (mIU/L) levels over time from 6:00 AM to 6:00 PM. The graph indicates a trend with peaks and troughs throughout the day.]
Individual reference ranges vs. population reference ranges

![Graph showing TSH (mIU/L) vs. Sample Number for different patients (Pt. 1 to Pt. 5).]
TSH assays differ from each other and also over time

- 10-20% average difference between different assays
  - Much larger difference may be observed
Differences between TSH assays

Before calibration adjustment

Thienpont et al.: Eur Thyroid J, 3:109, 2014
Differences between TSH assays

After calibration adjustment

Thienpont et al.: Eur Thyroid J, 3:109, 2014
TSH assays differ from each other and also over time

- 10-20% average difference between different assays
  - Much larger difference may be observed
- Lot-to-lot variability of +/- 5-10% is common, more might be observed occasionally
A 10% difference between reagent lots can double (or half) the number of positive (or negative) results.
Recommendations on interpreting TSH results and management - 1

• Re-measure borderline elevated or low TSH levels at least twice, regardless of reference range used

• A narrow TSH reference range is unsuitable in tertiary (and likely in secondary) care patient populations - about 20% of individuals would be classified as ‘biochemically hypothyroid’

• In treated hypothyroid patients, aim for a TSH between 0.3-3 mIU/L, if the patient is young/middle aged; higher levels in the elderly

• Avoid excessive micro-titration of TSH levels
Recommendations on interpreting TSH results and management - 2

• Overtreatment is likely with T4 doses of >150 mcg/day
Recommendations on interpreting TSH results and management - 2

Serum TSH levels at baseline and 30 min after thyrotropin releasing hormone
(5th_p, median, and 95th_p; * = 16th_p + 84th_p; ** = 30th_p + 70th_p)

TSH

(mIU/ml)

0.01

0.10

1.00

10.0

30.0

25 50 75 100 125 150 175 200 250 300 400 (μg T4/d)

 euthyroid

n =

B A

203 136 30 18 126 79 114 78 226 167 27 21 44 32 21 18 52 32 19 11 11 8 9 6

B = basic TSH; A = 30 min after i.v. TRH-application
Recommendations on interpreting TSH results and management - 2

• Overtreatment is likely with T4 doses of >150 mcg/day

• In most thyroid cancer patients do not aim for complete TSH suppression
  • Suppression to <0.1 mIU/L is only indicated for high risk patients and those with persistent disease
Measurement of thyroglobulin (Tg)
The role of Tg measurements

- Thyroid tumor marker
- Detection of tumor recurrence
Tg - Biology

• Principal protein made in thyrocytes
Tg - Biology

Follicular lumen

TPO

Tg-I

Tg-I-T4/T3

Tg

Tg - proteolysis

I-

Pendrin

Na-I symporter

Basement membrane

T4

T3

rT3
Tg - Biology

- Principal protein made in thyrocytes
- Thyroid specific – no significant expression in any other tissue
Tg - Biology

- Principal protein made in thyrocytes
- Thyroid specific – no significant expression in any other tissue
- NOT tumor specific
  - Circulating levels correlate with thyroid size
  - Elevated in conditions of disordered thyroid growth or glandular destruction
    - Goiter
    - Graves’ disease, thyroiditis
Tg – Clinical role

• Follow-up of treated thyroid cancer patients
Tg – Clinical role

- Follow-up of treated thyroid cancer patients
  - Should be undetectable in athyrotic thyroid cancer patients (total thyroidectomy +/- RRA)
  - Unstimulated or stimulated (thyroid hormone withdrawal or rhTSH) Tg >1 ng/mL suspicious of recurrence, but positive predictive value only 5-10%
  - Unstimulated or stimulated Tg >10 ng/mL highly predictive of persistent/recurrent disease
Tg measurements - limitations

• Residual benign thyroid tissue
  • Each 1 g of residual tissue contributes to serum Tg levels:
    • ~0.5 ng/mL if serum TSH <0.1 mIU/L
    • ~1 ng/mL if serum TSH >=0.1 mIU/L
Tg measurements - limitations

• Residual benign thyroid tissue

• Autoantibody interferences – pot. false low Tg
  • ~25% of thyroid cancer patients have detectable anti thyroglobulin auto-antibodies (TgAb)

• Always measure both Tg & TgAb
  • Use LOQ of TgAb assay as cut-off for TgAb positivity, rather than manufacturer recommended cut-offs

• “True” Tg conc. might be impossible to determine in TgAb+ patients
Tg measurements - limitations

• Residual benign thyroid tissue
• Autoantibody interferences – pot. false low Tg
• Other limitations/interferences
  • As discussed in the first section:
    • Non-specific interferences
    • Hook
    • Heterophile antibody interferences
Tg measurements - limitations

• Residual benign thyroid tissue
• Autoantibody interferences – pot. false low Tg
• Other limitations/interferences
• Poor low-end sensitivity necessitates stimulated Tg measurements for some assays
Assay precision profiles for two different Tg assays

CV (%) vs Tg (ng/mL)

Functional Sensitivity

Least significant change between 2 measurements at functional sensitivity:
~39% (LSC=1.96*\sqrt{2*CV})
Tg measurements - limitations

- Residual benign thyroid tissue
- Autoantibody interferences – pot. false low Tg
- Other limitations/interferences
- Poor low-end sensitivity necessitates stimulated Tg measurements in some assays
- Calibration differences between different Tg assays, or reagent lot-to-lot variability can lead to false positive and false negative test results
Tg measurements – current ATA Guidelines (2015) and other sources

- Use assay calibrated against CRM457
  - All current assays fulfil this criterion
Tg measurements – current ATA Guidelines (2015) and other sources

- Use assay calibrated against CRM457
- Always measure TgAb alongside Tg
  - This ensures that potentially falsely low biased Tg measurements are identified
Tg measurements – current ATA Guidelines (2015) and other sources

• Use assay calibrated against CRM457

• Always measure TgAb alongside Tg

• Tg assay with a LOQ of ≤0.2 ng/mL preferred
  • Assays with higher LOQ often will force use of stimulated Tg measurements
Tg measurements – current ATA Guidelines (2015) and other sources

- Use assay calibrated against CRM457
- Always measure TgAb alongside Tg
- Tg assay with a LOQ of ≤0.2 ng/mL preferred
- Perform postoperative Tg measurement
  - ≥3-4 weeks post-op
  - On T4 or stimulated
  - Unstimulated Tg <0.2 ng/mL or stimulated Tg <1 ng/mL denote persistent disease risk <4%
  - Higher values do not distinguish between remnant and persistent disease
Tg measurements – current ATA Guidelines (2015) and other sources

- Use assay calibrated against CRM457
- Always measure TgAb alongside Tg
- Tg assay with a LOQ of ≤0.2 ng/mL preferred
- Perform postoperative Tg measurement
- Regular follow-up Tg measurements
  - Use the same assay in a given individual
  - Re-baseline if assay is changed
  - Usually unstimulated measurements
- Low/intermediate risk patients 6-12 monthly, later 12-24 monthly
  - Incomplete thyroidectomy: use patients previous values and doubling time, rather than fixed cut-offs
- High risk patients at least 6-12 monthly
(Partially) overcoming TgAb interference: Tg measurement by mass spectrometry (MS)

- MS can overcome the TgAb and heterophile AB (HAB) interferences that plague Tg IAs
(Partially) overcoming TgAb interference: Tg measurement by mass spectrometry (MS)

- MS can overcome the TgAb and heterophile AB (HAB) interferences that plague Tg immunoassays
  - Trypsin digestion of sample before MS cleaves Tg, TgAb and HAB (along with all other serum proteins)
  - MS detects Tg-proteotypic peptides after digest with high specificity
Pre-analytical phase 1:
- Selective large protein precipitation ("salting out")
- OR size selection chromatography
- Remove and discard supernatant and re-dissolve precipitate
- OR Buffer-change/ dissolve desired fraction
- Add trypsin and digest for several hours
- Stop reaction and add non-radioactive isotopic internal standards of the Tg proteotypic tryptic peptides that are to be measured

Pre-analytical phase 2:
- Dissolve dissociated peptides in suitable buffer for HPLC-MS/MS
- Dissociate peptides from beads/columns with organic solvent or acid
- Wash beads/columns
- Immuneaffinity capture of one or more proteotypic tryptic Tg peptides with AB-coated beads or immune-affinity column(s)

HPLC-MS/MS:
- Multiple reaction monitoring for selective and specific detection of target peptide(s) and internal standard

Triple Quadrupole Mass Spectrometer
- Q1
- Q2
- Q3

Tg at a concentration of 1.1 ng/mL
Fold difference from non-TgAb sample

Tg Assay
- LC-MS/MS
- Beckman Access
- Roche Elecsys
- Siemens Immulite
- Thermo Kryptor
- UK-RIA
- USC-RIA

Tg 1 ng/mL

TgAb (IU/mL)

Tg 100 ng/mL

TgAb (IU/mL)

Tg 25 ng/mL

TgAb (IU/mL)
Example MS vs IA – samples with Tg > LOQ: Tg-MS & Roche Tg II

Roche Tg II – Tg LC-MS/MS)/Tg LC-MS/MS

Folded Probability

TgAB-neg by all TgAb IAs (N = 113)

TgAB-pos by all TgAb IAs (N = 42)
Example MS vs IA – samples with Tg > LOQ: Tg-MS & Roche Tg II

Roche Tg II – Tg LC-MS/MS)/Tg LC-MS/MS

Folded Probability

TgAB-pos vs. TgAB-neg
~60% false-low bias

TgAB-neg
by all TgAb IAs
(N = 113)

TgAB-pos
by all TgAb IAs
(N = 42)
MS does not solve all Tg measurement problems

- LOQ of current Tg MS assays is suboptimal – 0.5 ng/mL
- In patients with known persistent disease who are Tg negative by IA, Tg MS fails to find Tg in ~40% of cases
Most patients with undetectable Tg by IA have low Tg values by Tg MS

N=105, 20 with detectable Tg by LC-MS/MS

Individual specimens, all with Tg <0.1 ng/mL by Beckman IA
Recommendation for use of Tg MS assays

- Test Tg and TgAb by IA with an LOQ $\leq 0.2$ ng/mL
- Use Tg MS primarily in patients who are TgAb positive and have Tg immunoassay values of $\leq 1$ ng/mL
- A negative Tg test by MS does not exclude active disease in patients who have clinical or imaging evidence that suggests cancer persistence or recurrence
Thank You

Questions?