New CDC standardization projects on PTH and thyroid function tests

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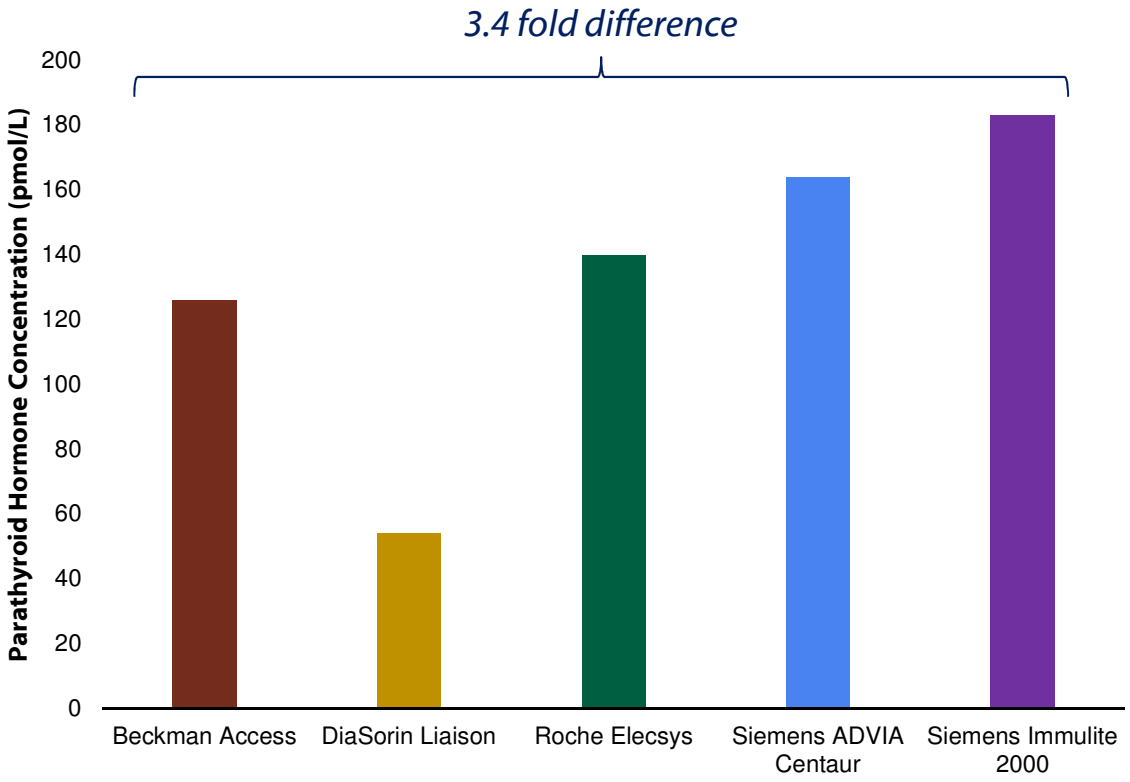
Overview

- Current Challenges in PTH Testing
- Developments to the CDC PTH Reference Method
- CDC's New Thyroid Reference Methods
- Future Directions

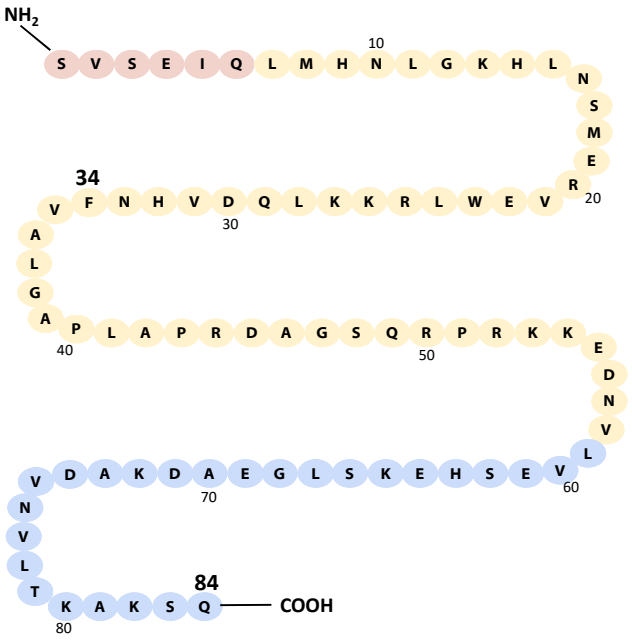
Studies suggest that current inaccuracy among PTH assays can lead to inconsistent diagnosis



Adapted from Almond, A. et al. Annals of Clinical Biochemistry **2012**, *49, 63-67.*

Blood contains intact PTH and a range of different PTH fragments/modifications that can affect measurement accuracy

PTH variants, found in circulation, can be reactive to the PTH receptor and henceforth be of biological relevance



Adapted from Shrader, S. P. et al. Annals of Pharmacotherapy **2005**, *39, 1511-1516.*

Some ratio of PTH fragments to PTH(1-84) may have potential for use in the diagnosis of:

- Hyperparathyroid-Associated Bone Loss
- Non-Dynamic Bone Disease
- Severe or End-stage Renal Disease

PTH Fragment Type	Amino Acid Range	
Intact	1-84	
N-terminal	1-34 1-44	
Mid-range	7-34 34-77 35-64	37-77 38-77 44-68
C-terminal	4-84 7-84 28-84 34-84 35-84	37-84 38-84 45-84 48-84 53-84

Phosphorylation and oxidation can occur at serine and methionine

The candidate reference measurement procedure needs to meet specific criteria that are specific to PTH testing

Method Criteria	Laboratory Approach
Specific for intact PTH without interference Able to detect PTH fragments	UHPLC/MS/MS with stable-isotope dilution quantitation
Measure PTH in native blood samples	Isolation of the analyte from sample matrix
Meet ISO and other relevant standards	Use certified reference materials for traceability

Desirable Specification		
Specimen	*Imprecision*	*Inaccuracy*
Serum	6-7%	4-5%
Plasma	6-7%	6-7%

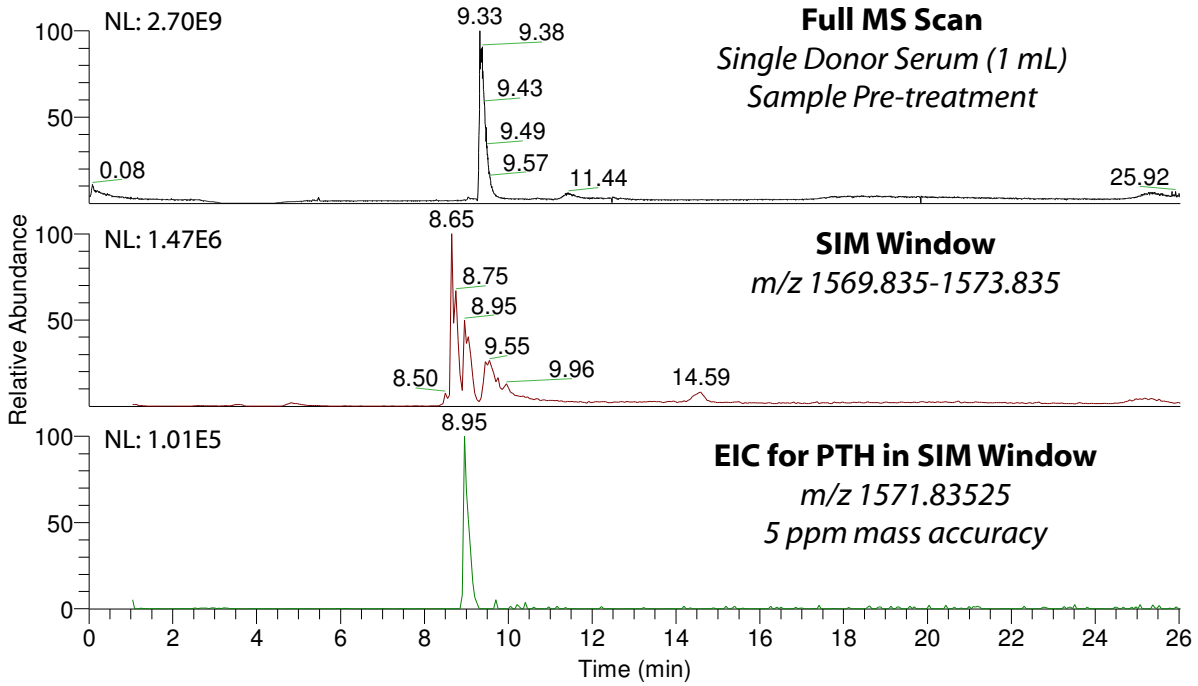
Requirements are based on biological variability from the references listed

Ankrah-Tetteh T, et al. *Ann Clin Biochem* **2008**;45:167-169.

Viljoen A, et al. *Clin Chem Lab Med* **2008**;46:1438-42.

Low endogenous concentrations of PTH in serum require highly sensitive and specific methodologies

High-Resolution Mass Spectrometry allows for excellent intact PTH specificity



An immunocapture strategy is being evaluated as part of the sample preparation workflow for the PTH Reference Method

Sample Collection

Single-donor serum

1 mL

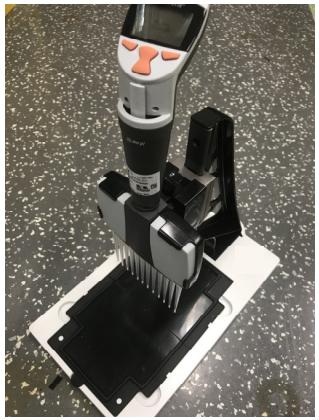
Internal Standard Addition

Spike in the following:

1. [^{15}N] Stable-Isotope Labeled Internal Standards
2. PBS/BSA Buffer

750 µL

PTH Isolation from Matrix



Mass-Spectrometry Immunoassay (MSIA)-Tips
Embedded with monoclonal antibodies for PTH

80 µL

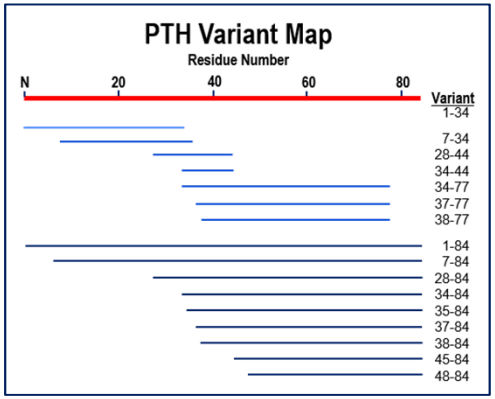
PTH Elution

Perform Elution of PTH from MSIA-Tips into 96 well plates

80 µL

Mass Spectral Analysis of Intact PTH

UHPLC-HRMS analysis of Intact PTH without enzymatic digestion



Adapted from Lopez, M. F., et al. Clinical Chemistry **2010**, *56, 281-290*

Initial assessments demonstrate excellent linearity, reproducibility, and sufficient sensitivity of the UHPLC-HRMS system

Normal Biological Levels of PTH

	Concentration
iPTH	10 – 65 pg/mL
N-terminal	8 – 24 pg/mL
C-terminal	50 – 330 pg/mL

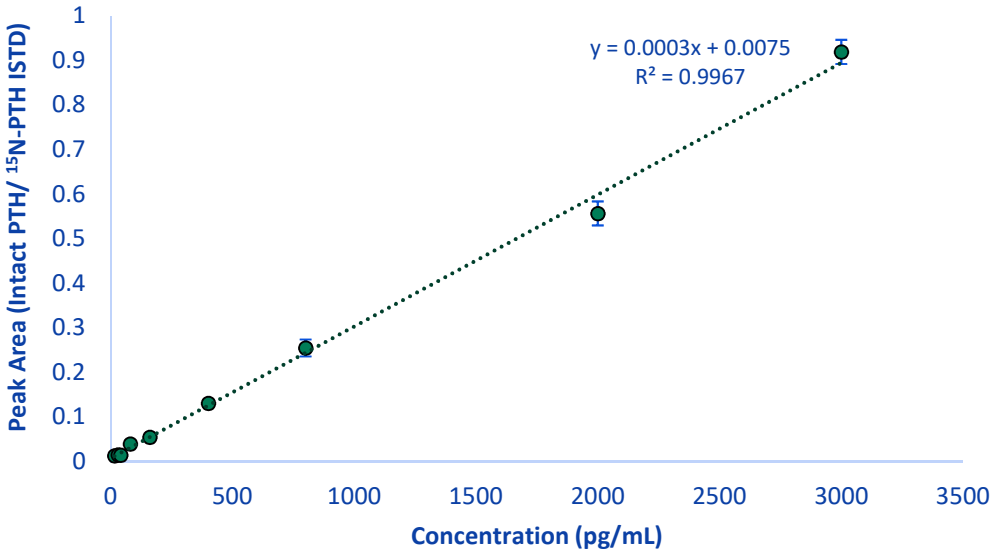
Pagana, K. D. P., T. J.; Pagana, T. N. (2015). Parathyroid Hormone. In Mosby's Diagnostic and Laboratory Test Reference (12 ed., pp. 689). St. Louis, Mo.: Mosby.

Target Levels of Intact PTH in KDOQI Guidelines for CKD-MBD

CKD Stage	Concentration
Stage 3	35 – 70 pg/mL
Stage 4	70 – 110 pg/mL
Stage 5	150 – 300 pg/mL

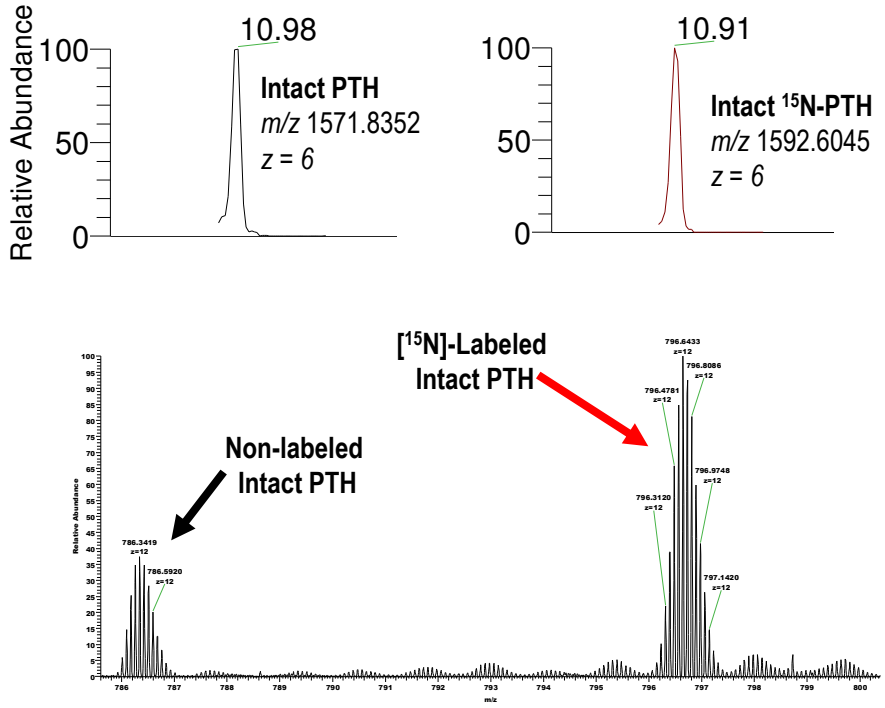
KDOQI Guidelines. NKF Am J Kidney Dis 2003, 42 (suppl 3), S1-201.

Preliminary Standard Curve for Intact PTH(1-84)



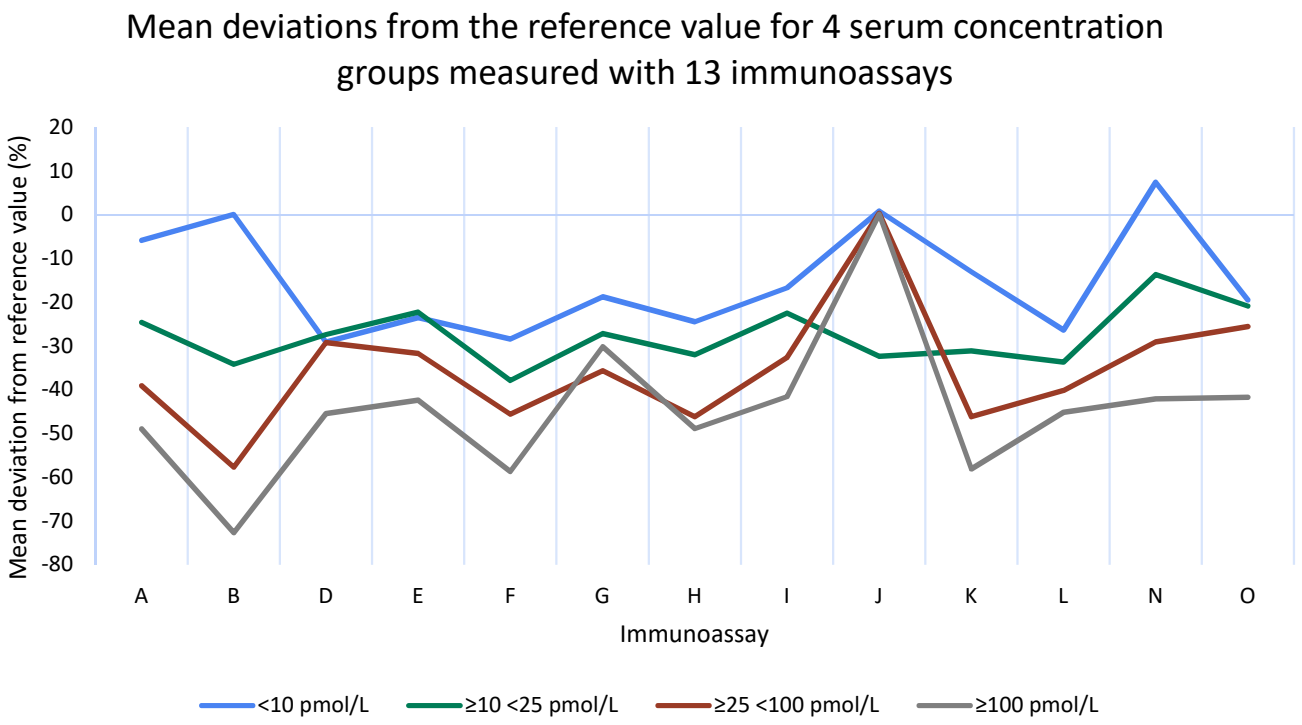
UHPLC-HRMS System Linear Range: 15 – 3000 pg/mL

Immunoaffinity purification procedures allows for analysis of intact PTH and PTH-derived peptides in the same analytical run



Fragment Name		Neutral *m/z*	RT (min)
N-terminal	PTH(1-34)	4115.1308	9.01
Mid-Range	PTH(7-34)	3471.8131	8.77
	PTH(28-44)	1816.9948	8.82
	PTH(34-44)	1110.6549	8.66
	PTH(34-77)	4712.4633	8.62
	PTH(37-77)	4395.2894	8.37
	PTH(38-77)	4282.2053	8.12
C-terminal	PTH(7-84)	8775.6468	8.87
	PTH(28-84)	6175.2525	8.67
	PTH(34-84)	5468.9127	8.60
	PTH(35-84)	5321.8443	8.50
	PTH(37-84)	5151.7387	8.45
	PTH(38-84)	5038.6547	9.66
	PTH(45-84)	4376.2683	9.35
	PTH(48-84)	4133.1828	8.92

Inaccurate free T4 measurements may lead to incorrect diagnosis of patients



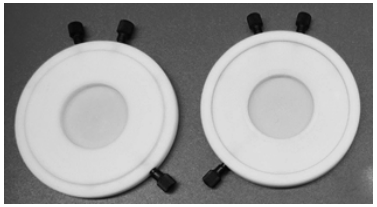
Adapted from De Grande, L. et al. Clinical Chemistry **2017**, *63:10, 1642-1652.*

CDC is establishing a new reference methods for free and total T4 using equilibrium dialysis combined with UHPLC-MS/MS

Free T4 Method

Equilibrium Dialysis

- 5 kDa regenerated cellulose membranes
- PTFE 1 mL cells
- HEPES dialysis buffer (pH 7.5 ± 0.03 at RT)
- Dialysis at 37°C/4 hr



Total T4 Method

IS Addition

- $^{13}C_6$ T4
- Matched to approximate T4 concentration

Cleanup & Analysis

- SPE (C18)
- LLE (ethyl acetate)
- LC-MS/MS

CDC Method is aligned with the reference method established at the University of Ghent

[Clin. Chem. Lab. Med., 49, 1275–1281 (2011)]

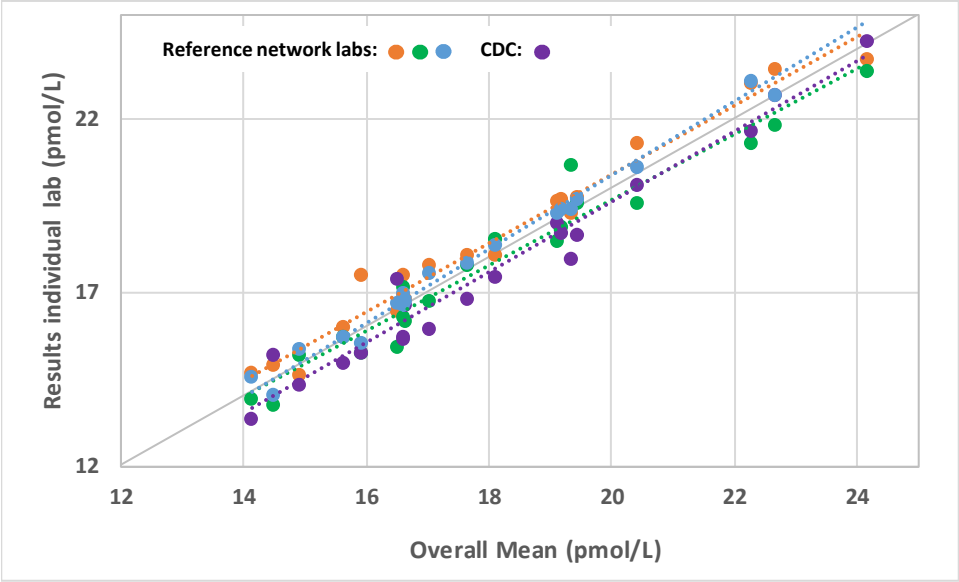
CDC's new reference methods meet or exceed performance requirements for reference methods

- Highly specific by use of isotope-dilution LC-MS/MS
- Traceable to SI: IRMM-468- certified, pure-compound reference materials as calibrators

Parameter	Total T4	Free T4
Accuracy	-0.5 %	-0.2%
Precision	0.8 % (inter-day) 0.9 % (intra-day)	1.1% (inter-day) 2.7% (intra-day)
Analytical Measurement Range	1-20 mg/dL	0.3-8.0 ng/dL

CDC's new reference method for FT4 is in good agreement with other reference labs

- Blinded euthyroid samples (20) measured by 4 reference labs show good correlation



- The CDC mean bias to the overall laboratory mean is within ±2.5% bias, and the bias is consistent across the 14–24 pmol/L concentration range

Next Steps

PTH

- Assess alternative sample preparation approaches to improve method sensitivity
- Measure additional PTH-related variants

Free T4

- Complete 2nd comparison with reference network using hypo/hyper/euthyroid samples
- Create reference materials for a thyroid hormone certification program to be launched in 2019

TSH

- Develop a harmonization program

Acknowledgements

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- Scientific Division Working Group for PTH
- Committee for Bone Metabolism
- Committee for the Standardization of Thyroid Function Tests

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THANK YOU

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