

## Endocrine Lab Testing 2017: Tips, Tricks, and Traps

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Assoc. Clin. Prof. (Voluntary) of Pediatrics & Endocrinology, UCSD

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### DISCLOSURE STATEMENT Speaker: Jon Nakamoto, M.D., Ph.D.

Dr. Nakamoto has disclosed the following relevant financial relationships. Any real or apparent conflicts of interest related to the content of this presentation have been resolved. There will be no discussion of unapproved or off-label, experimental, or investigational use.

Affiliation/ Financial Interest	Organization
Employee & stockholder	Quest Diagnostics Nichols Institute

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### Case 1: The Horrendous hCG Hunt

- 22 y/o ♀ w/ abdominal pain, vaginal bleeding
- Elevated serum hCG ( = 250 mIU/mL)
  - U/S, laparoscopy, D&C – nothing found
- 5 months later: hCG = 300 mIU/mL
- Referral to trophoblastic disease expert
  - Empiric methotrexate chemoRx x 4 months
- hCG still elevated; levels confirmed by two other labs (\*\*remember this fact\*\*)

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### Horrendous hCG Hunt (continued)

- Hysterectomy
  - hCG falls to 137 mIU/mL, then rises again to 250
- New chemoRx (5 agents) – no decrease in hCG
- CT scan chest/abdomen: 2 nodules seen
- Thoracotomy & partial lung resection – nodules benign
- Lab director becomes suspicious
  - hCG falls more rapidly than predicted when sample is diluted
- Split sample is sent to yet another laboratory
  - hCG *undetectable* (on a different assay platform)
  - Urine hCG also negative
  - Culprit is an **interfering antibody**

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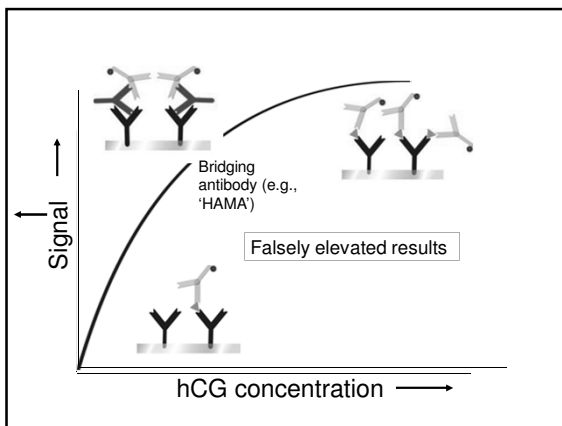
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### A sobering case: the outcome

- 16.2 million dollar award (upheld on appeal)
- 8.1 million against the manufacturer for “failure to warn”
  - **Not** for a “faulty assay”
- 8.1 million against the physicians/hospital

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## Antibody interference: practical aspects

When should one consider the possibility of interference?

- When the results aren't clinically concordant
- *When you're going to make a critical clinical decision on the basis of a single immunoassay*

What can one do?

- Fastest: ask your lab to have the sample re-tested using a different manufacturer's platform (e.g., Beckman vs Siemens)
- If available, have the lab re-test using a "HAMA" (heterophilic antibody) blocking agent prior to assay
- I do not see great value in assays that measure HAMA
- Some labs may be willing to assay serial dilutions (if validated)
- As mass spectrometry becomes more available for some proteins, consider comparison using this different method – less prone to interference from HAMA and autoantibody

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## Morals of the Story

- Always consider in your differential diagnosis the possibility of interference from antibodies or other factors
- Know when it's worth checking for possible interference, and how to do this most efficiently
- Find a sympathetic ally in your lab who can help you figure out what best to do

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## Case 2: Alexa's Autoantibody Anxiety

- Alexa had a total thyroidectomy for thyroid cancer
- A few months later, her surgeon orders a thyroglobulin assay – it's undetectable (thyroglobulin antibody is negative)
- Around the same time, Alexa goes to see her endocrinologist, who also orders a thyroglobulin – again, undetectable, but this time her thyroglobulin antibodies are *positive*
- Was the first thyroglobulin antibody assay "bad"?

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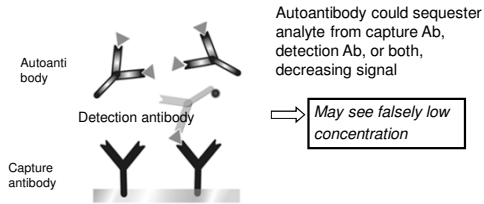
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### Thyroglobulin ICMA & Tg antibody: what happens?




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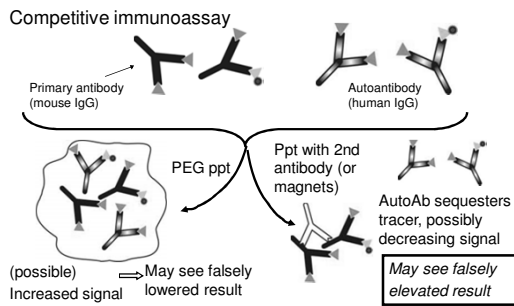
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### Thyroglobulin RIA & Tg antibody: what happens?




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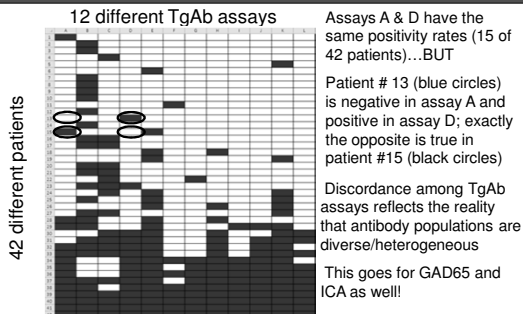
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### TgAb assays do not always agree

(see Spencer *et al* JCEM 2005; 90: 5566)




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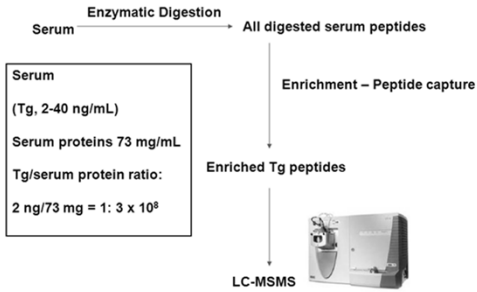
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Evolving solution: use non-immunoassay approaches that don't "see" antibody interferences




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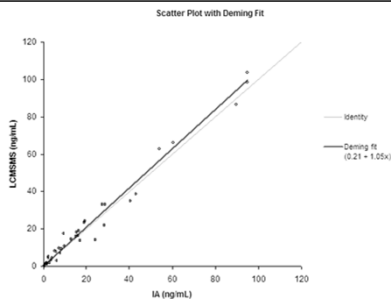
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Tg by immunoassay and Tg-LCMSMS correlate well when there are no Tg antibodies




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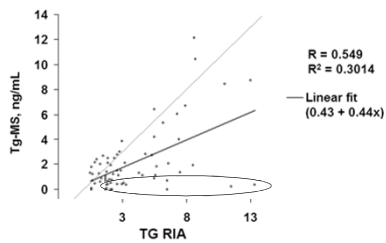
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Tg by RIA and Tg-LCMSMS do not always correlate when Tg antibodies are present




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## Moral of the Story

The detection of autoantibodies (TgAb, diabetes-related autoantibodies, adrenal antibodies, etc) is not a cut-and-dried process – but some help is on the way

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## Case 3: Tiana's Terrible Total Testosterones

- 7 year old Tiana has a bit of pubic hair
- A total testosterone is sent and the result is around 60 ng/dL, with the upper limit for age cited as  $\leq 20$  ng/dL
  - Even adult females are generally below 45 ng/dL
- By accident, some of the same sample is sent to a different lab and the testosterone is 20 ng/dL
- What's the difference?

"A rose is a rose is a rose" – but a testosterone is not necessarily a *testosterone* is not necessarily a testosterone

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## Testosterone immunoassays

- "Direct" testosterone immunoassay
  - Simple, often 'walkaway' automated procedure
  - Equipment is generally more affordable
  - Fast!
  - ...but limited accuracy below 100 ng/dL (typically overestimates levels)
- Testosterone RIA after extraction/chromatography
  - More complex and labor-intensive
  - Higher cost
  - Longer procedure
  - ...but high accuracy even at low levels, high specificity, generally high sensitivity

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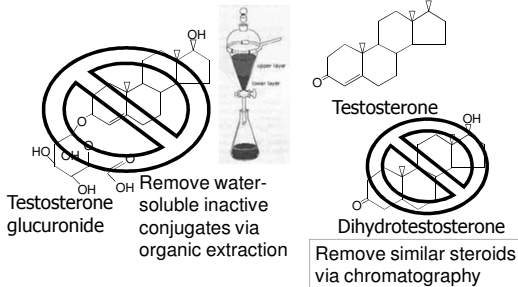
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When it comes to steroids,  
antibodies need help to be selective and specific




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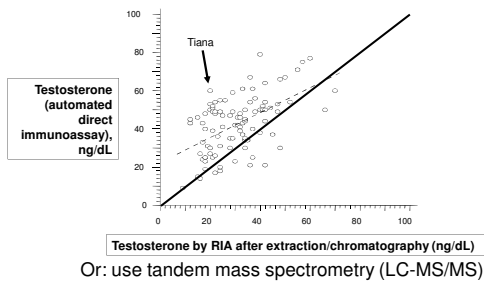
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“Direct” immunoassays tend to overestimate at lower steroid levels




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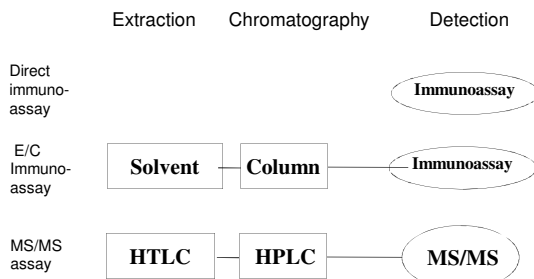
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How do we get specificity & accuracy?




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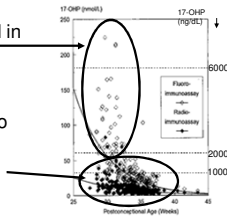
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## What causes false positives on CAH NB screening?

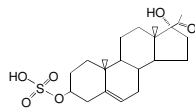
Non-extraction fluoroimmunoassay (FIA) used in blood spot screening (or often even on venous draw) overestimates 17-OHP in premature infants (compared to RIA after extraction/chromatography or LCMSMS)



(Al Saedi *et al.*, Pediatrics 1996; 97:100-2)

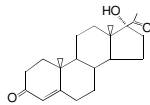
Why?

## Here's one culprit



17- $\alpha$ -hydroxypregnenolone sulfate

Produced in large amounts in the fetal zone of the immature adrenal cortex



17- $\alpha$ -hydroxyprogesterone

But most 17-OHP assays have low cross-reactivity to other steroids. Why, therefore, is there interference observed?

## Low cross-reactivity but very high concentration $\rightarrow$ interference

Steroid monosulfates in term neonatal plasma (Wong *et al.*, Clin Chem 1992; 1830-7)

Steroid	Concn, $\mu$ mol/L	nmol/L	17-OHP in a 3 day old term infant $\approx$ 13 nmol/L (429 ng/dL) – this compound is swamped by other closely related steroid conjugates
Pregn-5-ene-3 $\beta$ ,20 $\alpha$ -diol	8.36	8360	
16 $\alpha$ -OH pregnenolone	7.83	7830	
16 $\alpha$ -OH dehydroplandrosterone	7.41	7410	
Pregn-5-ene-3 $\beta$ ,20 $\alpha$ ,21-triol	5.57	5570	
16 $\beta$ -OH dehydroplandrosterone	4.72	4720	
Androst-5-ene-3 $\beta$ ,16 $\alpha$ ,17 $\beta$ -triol	4.04	4040	
Pregn-5-ene-3 $\beta$ ,20 $\beta$ ,21-triol	3.48	3480	
Dehydroplandrosterone	3.46	3200	
Pregnenolone	3.20	2480	
Pregnanetriol	2.48	2110	
17 $\alpha$ -OH pregnenolone	2.11	820	
Pregn-5-ene-3 $\beta$ ,16 $\alpha$ ,20 $\alpha$ -triol	0.82	750	
Pregn-5-ene-3 $\beta$ ,15 $\beta$ ,17 $\alpha$ -triol-20-one	0.75	360	
Androst-5-ene-3 $\beta$ ,17 $\beta$ -diol	0.36		



## Evolving solution: a formal push for accuracy

- 1980's-1990's: Fussy (pediatric) endocrinologists
- Early 2000's: Clinical chemists

See editorial, Immunoassays for Testosterone in Women: Better than a Guess?, Clin Chem 2003; 49: 1251

"Taieb et al. are the first to show that for every commercially available testosterone assay studied, the values are in error – by a factor of 2 on average, and in some cases by a factor of almost 5. Are assays that miss target values by 200-500% meaningful? Guessing would be more accurate and additionally could provide cheaper and faster testosterone results for females – without even having to draw the patient's blood."

- 2007: Endocrine Society Position Statement
- 2010: CDC/Endo Soc Consensus Conf. & JCEM article
- 2011+: Partnership for Accurate Testing of Hormones
- 2013: JCEM request for use of more accurate assays

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JCEM Oct 2013

SPECIAL FEATURE  
Editorial

### Requirement for Mass Spectrometry Sex Steroid Assays in the *Journal of Clinical Endocrinology and Metabolism*

D. J. Handelsman and L. Wartofsky

ANZAC Research Institute (D.J.H.), Concord Hospital, University of Sydney, Sydney, NSW 2139, Australia, and Department of Medicine, Washington Hospital Center (L.W.), Washington, DC 20010

- sex steroid measurements. Effective January 1, 2015,
- manuscripts reporting sex steroid assays as important end-
- points must use MS-based assays including reporting or
- citing their methods with sufficient detail to allow them to
- be reproduced together with standard quality control,
- specificity, and reproducibility metrics. Some limited ed-
- itorial discretion may be exercised for rare circumstances,
- such as where a study reports hormone measurements
- made long ago and where samples no longer exist; how-
- ever, that would not extend to the sort of high-quality,
- well-designed clinical studies that the JCEM expects to
- publish where it is expected that extra serum samples
- would be stored for key endpoints. It is anticipated that
- this requirement for MS-based assays will extend to ad-
- renal steroids and vitamin D in the near future.

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## But the world was not yet ready...

### Important Notice on JCEM Mass Spectrometry Sex Steroids Assays Requirement

In the October 2013 issue of the *Journal of Clinical Endocrinology and Metabolism (JCEM)*, a policy requiring mass spectrometry sex steroid assays was published. This new JCEM policy raises important and valuable scientific issues that require broader consideration. In order to properly address the complexity of the issues, the requirement for using mass spectrometry sex steroid assays, which was scheduled to go into effect on January 1, 2015, is suspended pending further scientific review.

A task force of experts is being convened to review scientific policies regarding the reporting of sex hormone measurements in the Society's journals. Once this review is completed, any new policies and/or clarifications will be announced at that time. In instituting a time line for any policy changes, we will be sensitive to the needs of clinical investigators who are conducting and planning studies. We anticipate that the task force will complete their scientific review by June 30, 2014.

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### Moral of the Story

- Know when you need to be highly selective about the methods used for the assays you order
- Conversely, be aware that you don't always need the "gold standard" for every assay you order

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### Case 4: Isaac's Inconsistent Insulins

- Isaac, 17 y/o ♂ w/ recurrent hypoglycemia
  - Assay Platform E**
  - No ketones, but insulin is undetectable
  - C-peptide (and proinsulin) is undetectable
  - Metabolic workup is unrevealing
- A sample is accidentally sent to a different lab for insulin assay – it's *high!* **Assay Platform C**
- A split of the sample is sent to yet another lab – it's detectable, but not particularly high... **Assay Platform I**

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### Insulin analogs: typical % cross-reactivity on different insulin assay platforms...

	Insulin Assay Platform		
	E170 (Roche)	Centaur (Siemens)	Immulite (Siemens)
Insulin aspart	< 0.7 %	125 %	15 %
Insulin glargine	< 0.7	140	11
Insulin lispro	< 0.7	89	15

Clin Chem 2004; 50: 257-259

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## Insulin Immunoassay Discordances

Insulin Assay	Serum sample: low	Serum sample: high
Abbott AxSYM	13.9	1583.5
Siemens ADVIA Centaur	14.9	1559.7
Biosource EASIA	38.0	1387.0
Dakocytomation	11.5	1088.3
Siemens Immulite 1000	12.0	1188.0
Linco ELISA	12.0	878.4
Linco RIA	31.2	1565.2
Mercodia Iso-insulin	6.2	1343.8
Molecular Light Technol	12.1	1301.0
Roche E170	6.1	1394.0
Tosoh ST AIA (UK)	6.0	1076.4
Tosoh ST AIA (US)	1.7	1128.3

Manley SE, *et al.* Clin Chem 2007; 53: 922-932

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## What are the consequences of using a “non-standardized” insulin assay?

- Clinical thresholds and diagnostic strategies may vary depending upon the assay used
- Research studies will need to stress that findings may apply only to the specific assays used in the study

→ Can we ever really achieve a true “insulin resistance index” without standardization?

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## Standardization efforts ongoing...but slow

Reviews/Commentaries/ADA Statements

COMMENTARY

Diabetes Care January 2010 vol. 33  
no. 1 203-206

### Insulin Assay Standardization

Leading to measures of insulin sensitivity and secretion for practical clinical care

MARLENE A. STERN, MD<sup>1</sup>  
MICHAEL P. STERN, MD<sup>2</sup>  
W. GAIL MULLER, PhD<sup>3</sup>  
MICHAEL W. STEFFES, MD, PhD<sup>4</sup>

SCOTT E. CAMPBELL, PhD<sup>5</sup>  
FOR THE INSULIN STANDARDIZATION  
WORKGROUP

An effort is underway by the American Diabetes Association to standardize insulin assays so that this barrier to advancing research is removed. Once the insulin assays are standardized, research

accuracy, and cross-reactivities (1). Reasons for discrepancies in the results among commercially available assay methods were likely multifactorial and thus not explained by a single analytical performance characteristic. Improvement in standardization of insulin assay results will require an ongoing effort to achieve traceability to the isotope dilution mass spectrometry high-level reference measurement procedure calibrated with pure recombinant insulin and for manufacturers to address immunoassay specificity and response characteristics over the measuring interval when necessary (1).

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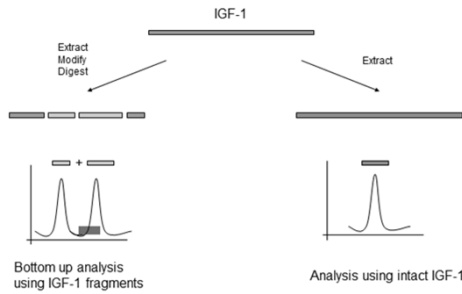
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## More than one way to measure small to medium-sized proteins by mass spectrometry



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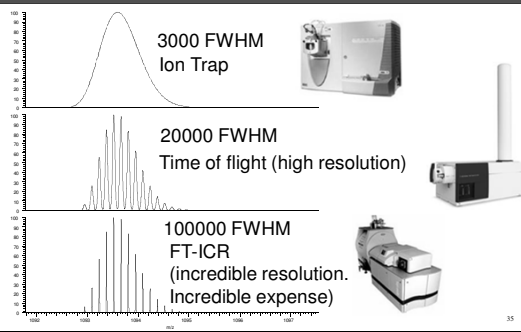
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## Mass spec: Improved resolution → more specificity



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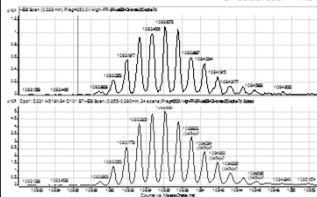
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## Isotope pattern matching to confirm identity

Isotope	Mass [Da]	% Abundance	Isotope	Mass [Da]	% Abundance
<sup>1</sup> H	1.007825	99.985	<sup>16</sup> O	15.994915	99.76
<sup>2</sup> H	2.014102	0.015	<sup>17</sup> O	16.999131	0.038
<sup>12</sup> C	12. (exact)	98.90	<sup>18</sup> O	17.999159	0.20
<sup>13</sup> C	13.003355	1.10	<sup>31</sup> P	30.973763	100.
<sup>14</sup> N	14.003074	99.63	<sup>32</sup> S	31.972072	95.02
<sup>15</sup> N	15.000109	0.37	<sup>33</sup> S	32.971459	0.75
			<sup>34</sup> S	33.967866	4.21



Actual

Predicted

By chance alone, some stable isotopes will be present in some of the molecules, changing the mass → multiple peaks that we can predict

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## Read this mini-review!

Available for free at  
<http://genomemedicine.com/content/5/6/53>

Savayri et al. *Genome Medicine* 2013, 5:53  
<http://genomemedicine.com/content/5/6/53>



### OPINION

## The emergence of top-down proteomics in clinical research

John P Savayri<sup>1</sup>, Adam D Catherman<sup>1</sup>, Paul M Thomas<sup>1</sup>, Michael M Abecassis<sup>2</sup> and Neil L Kelleher<sup>1\*</sup>

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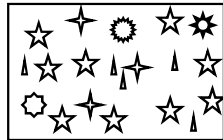
## Older examples of specificity questions: Why couldn't we ever standardize GH assays?

There is no single form of GH in the circulation

☆ GH 22 kDa form

✦ GH 20 kDa form

▲ GH fragments,  
dimers, higher-  
order oligomers,  
acetylated forms,  
deamidated  
forms, etc...



Assay 1 detects only 22 kDa GH  
(finds nine stars)

Assay 2 detects 20 & 22 kDa GH  
(finds twelve stars)

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## Bonus Case: Connie's Confusing Cortisols

- Connie is weaning off prednisone, and you want to measure her A.M. cortisol level
- You see that your hospital's cortisol immunoassay only has a 1% cross-reactivity with prednisone, and you feel pretty good about that
- But: hepatic metabolism transforms prednisone to prednisolone – and the cortisol immunoassay in question has a 33% cross-reactivity with prednisolone!

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### Morals of the story

- (1) Proteins are complicated – is it any wonder that antibody-based assays don't agree?
- (2) Mass-based assays may help – but we need to define exactly what isoforms and fragments we want to measure – so someone has to define exactly what things we want to measure
- (3) When you're trying to measure something you're giving your patient, don't forget about "biotransformation"

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### "Molecular" testing for CAH diagnosis

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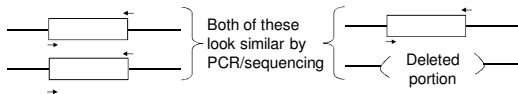
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### Molecular Misses...

We tend to treat DNA sequencing as a straightforward procedure giving us a black and white answer...but it ain't necessarily so...



...so you could miss a heterozygous deletion, unless the assay was designed to capture a specific deletion. Or you could do fluorescent in-situ hybridization (FISH) for larger deletions, or a Southern blot...

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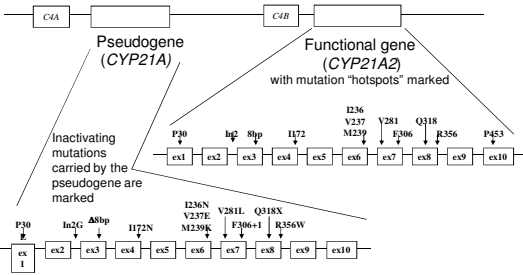
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## Why genetic testing for 21-OHase deficiency is efficient




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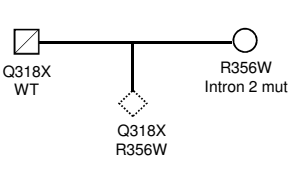
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## But where can genetic testing lead us astray?



- Mother affected with salt-losing CAH
- Father a "carrier"
- Infant was treated after birth; 17-OHP not elevated (but this was drawn after steroid Rx)
- 17OHP and PRA were "too easy" to suppress with very low-dose Rx

We re-tested the father and the infant, but this time using an assay that didn't ask "is there a mutation in CYP21A2?" (which there is) but rather, asked "is there an intact CYP21A2 present?" (which there is)

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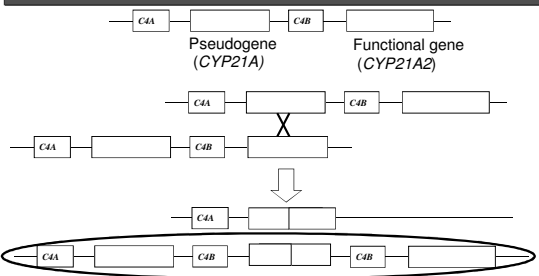
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## How CYP21A2 duplications arise



This isn't vanishingly rare, either! If the CAH mutation analysis isn't designed to pick this up, you could miss it

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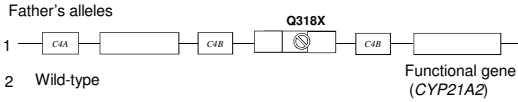
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Duplications of CYP21A2 are more common than we ever knew!



In a German study (Kleinle et al JCEM 2009; 94: 3954), 32 out of 38 unrelated individuals carrying Q318X mutations had a duplicated CYP21A2

PLoS ONE 2008; 3(5): e2136  
Published online May 14, 2008. doi: 10.1371/journal.pone.0022136

PMCID: PMC256463

High Frequency of Copy Number Variations and Sequence Variants at CYP21A2 Locus: Implication for the Genetic Diagnosis of 21-Hydroxylase Deficiency

Silvia Parales,<sup>1</sup> Cecilia Quintela,<sup>1</sup> Fernando Dominguez,<sup>1,2</sup> and Lourdes Loidi<sup>1\*</sup>

In a study of 144 randomly selected Spanish subjects, 7% had a chromosome with a duplicated CYP21A2

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## Moral of the story

Nothing is as simple as it first seems

We're all waiting to see how many of the issues are solved by using next-generation sequencing – and what new issues this methodology will bring

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## Take-home Messages

- \*\*Know your lab and your "go-to" lab person
- Keep antibody interference in your DDx
- Autoantibody detection is not straightforward
- The inherent specificity of mass spectrometry helps accuracy and assay harmonization
- The inherent specificity of mass spectrometry for proteins requires that we define what to measure
- Molecular genetic testing is not always cut-and-dried – recognize how you can be fooled

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Questions?



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