

# Traceability and Standardisation of Immunoassays: A major challenge

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# Comparability of immunoassays:

- The RCPA-AACB Australian External Quality Assurance Program represents some 238 laboratories from ~44 countries.
- Assays from all major manufacturers are represented.
- The total percentage variation about target values for the various analytes by routine assays are listed below:

<u>Analyte</u>	<u>Target 1</u>	<u>(% Variation)</u>	<u>Target 2</u>	<u>(% Variation)</u>
Cortisol (nmol/L)	190	(78 to 122)	1084	(95 to 107)
Testosterone (nmol/L)	1.5	(50 to 287)	42	(73 to 111)
FT4 (nmol/L)	11.1	(35 to 233)	52	(80 to 119)
FT3 (pmol/L)	1.9	(63 to 253)	12.2	(83 to 123)
TSH (IU/L)	0.12	(0 to 733)	18	(89 to 138)
FSH (IU/L)	3.3	(55 to 142)	76	(89 to 126)
LH (IU/L)	3	(17 to 400)	75	(75 to 133)
hCG (IU/L)	5	(40 to 400)	370	(92 to 120)
PSA ( $\mu$ g/L)	0.7	(57 to 371)	19.9	(63 to 115)
HbA <sub>1c</sub> (%)	5.3	(85 to 118)	12.7	(93 to 111)

## Clinical role of immunoassays:

- Analytes of clinical interest in biological fluids are present at a wide range of concentrations from at least  $10^{-1}$  M to  $10^{-12}$  M
- Immunoassay technology was the first to measure analytes at levels  $<10^{-6}$  M
- Automation in immunoassay technology has increased its use, such that some 20% of assays in a routine clinical automated laboratory utilise this technology

# Principles of immunoassay (1):



Competitive assays require the binding agent to be limiting

Metric assays require the binding agent to be in excess

Law of mass action determines the rate at which equilibrium is achieved

## Principles of immunoassay (2):

Any factors that modify the rate of achieving equilibrium are likely to disrupt measurement by immunoassay

Biological fluids contain such factors

e.g. Cholesterol is present in serum at  $10^{-3}$  M and shares structural similarity with steroid hormones present at  $10^{-7}$  M or lower

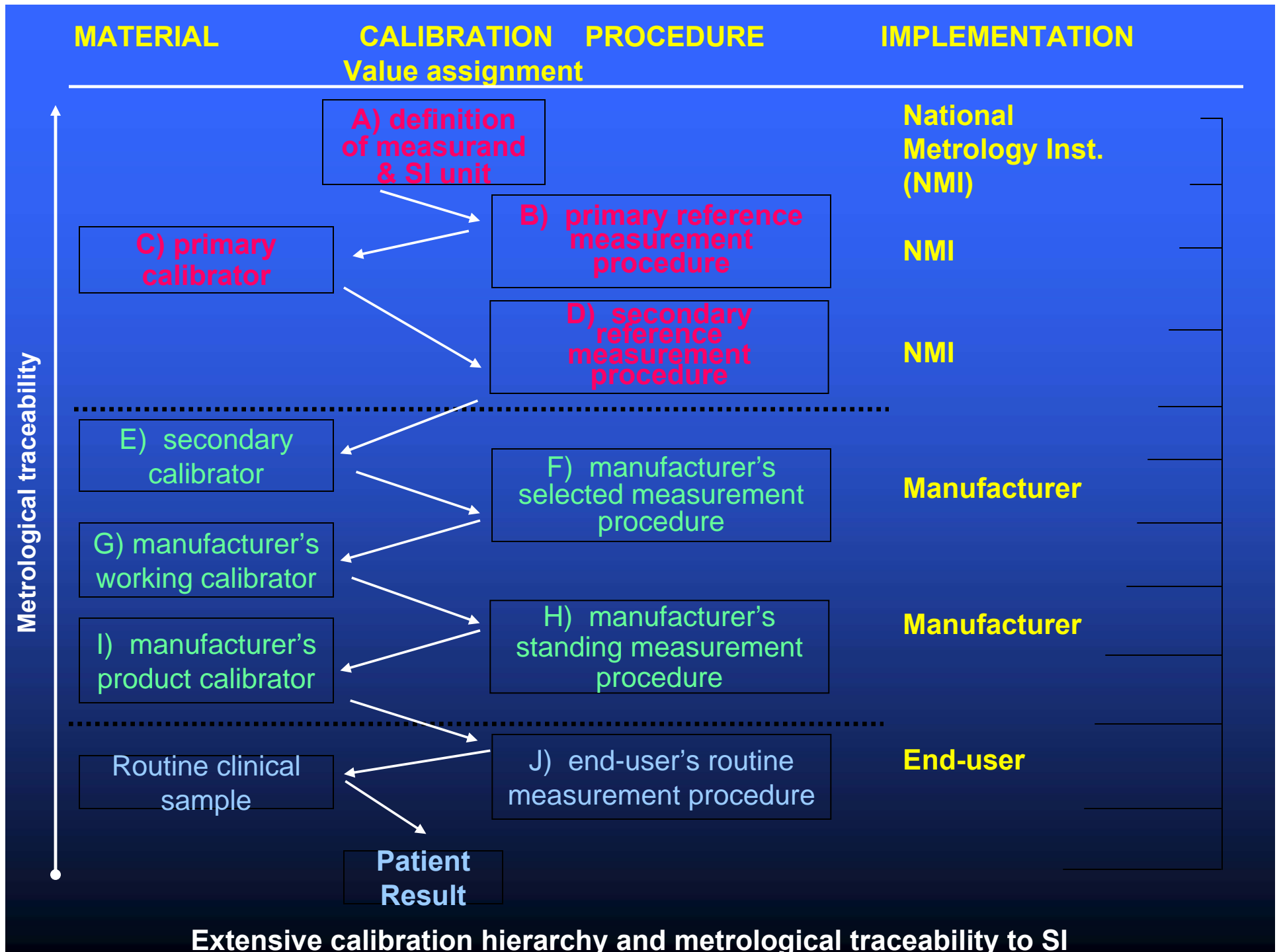
These disruptions are known as matrix effects

## Definition of unit for immunoassays:

1. Metrological principles are based on the definition of the SI unit or the 'SI-analyte' which requires a substance of known chemical structure and high purity ie 'Analytical Grade'
2. Many analytes measured by immunoassay lack an unequivocally recognized entity
  - WHO developed the International Unit (IU)
  - Function is defined by a bioassay and 1 IU was assigned to 0.1  $\mu\text{g}$  of the 1<sup>st</sup> preparation
  - The IU is not a constant unit: as new preparations with improvements in purification became available, 1 IU was now equivalent to a lower mass of the new preparation

## Traceability of hapten immunoassays:

- Haptens are small molecular weight substances, that are non-immunogenic until linked to a large MW carrier-substance.
- Molecular structure and SI unitage are/ can be defined
- Examples include cortisol, thyroid hormones and many therapeutic drugs etc.
- List of primary reference materials by JCTLM, continuously being upgraded
- Primary reference measurement procedure is gravimetry
- Secondary reference measurement procedure may be ID-GC / HPLC with MS detection
- Therefore current technology is capable of meeting all the needs for a traceable assay



# Current issues with traceability for hapten assays (1):

Matrix effects:

It is ideal for reference materials to be prepared in a matrix-free format, with commutability of the reference material with the native sample.

This is not achieved in immunoassays - matrix effects.

One solution is the 'split-sample measurement design' where native samples (serum base) are assigned values by a matrix-independent reference method, itself calibrated with the primary standard.

These native samples are used as manufacturer's working calibrators.

A reliable calibration must be demonstrated including uniformity of values across groups and low imprecision

## Current issues with traceability for hapten assays (2):

Most hapten analytes exist as 'free' and 'bound' fractions in serum with the 'free' fraction being biologically active and of greatest clinical interest.

Some clinical assays require reporting of the 'free' fraction, for example free T4.

Total T4 = bound T4 + free T4

Separation of the free fraction can be achieved by equilibrium dialysis or ultrafiltration. Current proposal to adopt a standard method for separating free from bound analyte

Can separation be achieved without disturbing the equilibrium and can this separation technique be standardised?

## Traceability of protein hormone assays (1):

Proteins are most often large molecular weight measurands.

This property:

- Precludes analyses of the total molecule by higher order analytical techniques
- Difficult to define structure and SI unit
- Many exist as a family of isotypes, with no current knowledge of the biologically active peptide, for example hCG

## Traceability of protein hormone assays (2):

Two approaches have been used to introduce harmonization or traceability to these assays

1. Identification of the biologically active or clinically relevant element. This approach can result in the development of a reference measurement system and traceability
2. Accept a strategy of harmonization through preparation of purified individual proteins for standardisation of routine assays.

## Traceability by identifying the biologically active peptide - the example of HbA<sub>1c</sub> (1)

- The first approach to standardizing HbA<sub>1c</sub> assays was to adopt a harmonization strategy
- The NGSP adopted a particular chromatographic method to which all routine assays were harmonized.
- The technology of this method quickly became outdated and transferability of the reference method was limited

## Traceability by identifying the biologically active peptide - the example of HbA<sub>1c</sub> (2)

- IFCC adopted metrological principles
- HbA<sub>1c</sub> and HbA<sub>0</sub> were defined as the globin  $\beta$ -chain amino-terminal hexapeptide with HbA<sub>1c</sub> containing an extra 1-deoxyfructose
- Gravimetry is a suitable primary reference method for the preparation of primary reference materials for each peptide
- Secondary reference measurement procedures utilising tryptic digestion of haemoglobin and quantification by HPLC/ MS and CE/MS

## Traceability by identifying the biologically active peptide - the example of HbA<sub>1c</sub> (3)

- A full reference measurement system is in place including a network of reference laboratories
- Agreement has been reached with clinical organisations to adopt the reference procedure internationally
- Agreement has been reached with the IVD industry to fully implement introduction of clinical assays traceable to this system by 1 Jan 2011

# Harmonization of immunoassays:

- WHO has been active since 1920's supporting the calibration of preparations of biologically active peptides in terms of International Units (IU) using bioassays
- Subsequent preparations have been calibrated in IU's although the mass of peptides present differs which is acceptable for bioassays
- However immunoassays do not measure biological activity but mass
- Harmonization of immunoassays with these preparations has been problematic due to the variation in content of the preparations and variation of specificity of antibodies used in the assays

## Harmonization of immunoassays for heterogeneous antigens - the example of hCG

- hCG is a complex heterogeneous family of polypeptides with a high degree of carbohydrate, samples include intact hCG plus dissociated and degraded hCG-related peptides
- The cross-reactivity of the different polypeptides vary with different antibodies used in the many commercial assays available

## Harmonization of immunoassays for heterogeneous antigens - hCG (2)

- Identification of the biologically active component of hCG has not been completed
- It has been argued that it is impossible to standardize assays for heterogeneous antigens (Ekins 1991)

## Harmonization of immunoassays for heterogeneous antigens - hCG (3)

Aims of the IFCC WG for Standardization of hCG included:

- To investigate the possibility of standardization of such assays using hCG as an example
- Introduce a uniform nomenclature for hCG
- Prepare new standards
- Assign values to these standards in mol/L

## Harmonization of immunoassays for heterogeneous antigens - hCG (4)

- New standard preparations for purified intact hCG, hCG $\alpha$ , hCG $\beta$ , the hCG core fragment of hCG $\beta$ , and 2 'nicked' forms are available as WHO 1<sup>st</sup> Research Reagents (1<sup>st</sup> RR)
- Use of 1<sup>st</sup> RR to harmonize 9 commercial hCG assays reduced variation from 1.4 to 1.6-fold to 1.27-fold
- Concentrations of 1<sup>st</sup> RRs have been defined in mol/L necessary for the levels of the various forms of hCG to be compared

## Harmonization of immunoassays for heterogeneous antigens - hCG (5)

Factors contributing to variation of hCG assays

- Use of impure assay calibrators
- Differences in antibody specificities used in various assays – requirement for manufacturers to describe the epitopes recognized by their antibodies

A proposal to evaluate the feasibility of a reference immunoassay method and calibrated serum panels to improve harmonization has been made

## What are the responsibilities of the clinical laboratory scientist with regard to traceability of results?

- In order to provide the best service to our patients, it is our responsibility to identify the traceability of assays in our laboratory
- That means looking at the test information literature and asking the industry representatives the reference method and the reference laboratory supporting that method
- It also means ensuring that the reference laboratory is performing satisfactorily and therefore participating in external quality assurance programs – you should be able to review their performance

# Traceability in Immunoassay:

## Conclusions (1):

- It is technically feasible to establish traceable total hapten immunoassays – the issue is availability of resources
- Research into ‘free’ hapten immunoassays is required to determine the feasibility of standardized techniques for separating ‘free’ and ‘bound’ fractions that are clinically relevant

# Traceability in Immunoassay:

## Conclusions (2):

Immunoassays for heterogeneous analytes require considerable work and research:

- Purification and characterization of family members
- Characterization of epitopes of antibodies
- Investigation of standard immunoassays

# Traceability in Immunoassay:

## Conclusions (3):

- The IFCC has an enviable record in this area with WG's either in the past or currently investigating the following analytes:

Cortisol

PSA

hCG

Thyroxine

CDT

cTnI

Cystatin-C

Albumin in Urine

PAPP-A

GH

Insulin