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Establishing Reference Intervals in Autoimmune Assays

Dr Chris Bundell
PathWest Laboratory Medicine
QEII Medical Centre, Western Australia

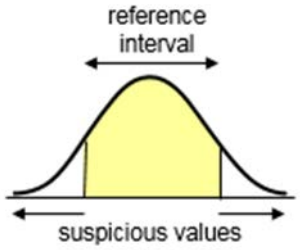
RCPA Pathology Update 2019

Outline

i. Background
ii. Review of the published guidelines
iii. Examples of setting reference intervals
iv. Summary

Reference intervals

- Range of measurements for a specific analyte from a population of representative healthy individuals
- specified interval of the distribution of values taken from a biological reference population (NATA AS Iso 15189 2013)



Decision level/limit

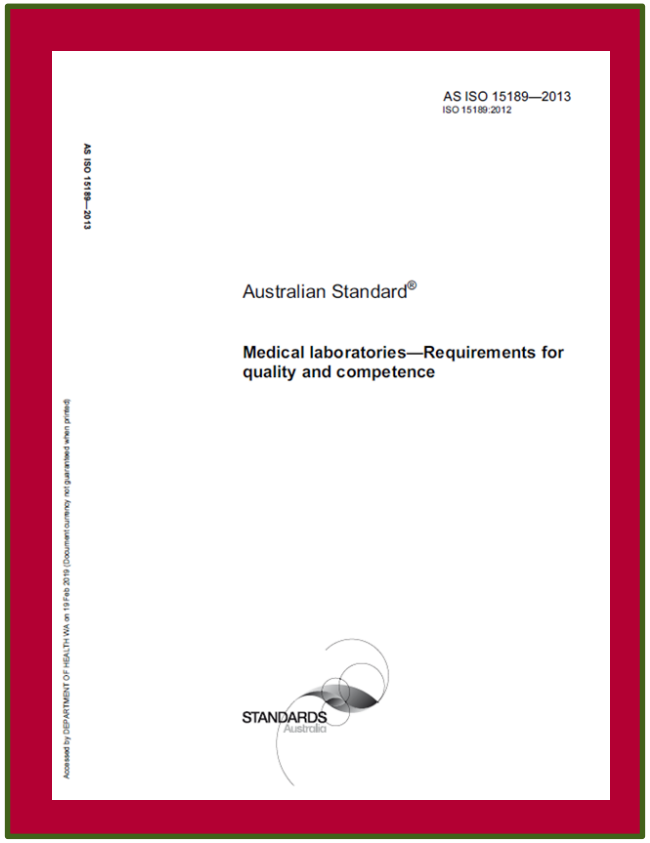
- Particular cut-off value for an analyte or test that enables individuals with a disorder or disease to be distinguished from those without the disorder or disease

- Certain tests have National Guidelines defining a “good” value, eg HbA1c for diabetic control

∴ In these cases there is no need to establish a reference interval for the analyte

NATA requirements for reference intervals

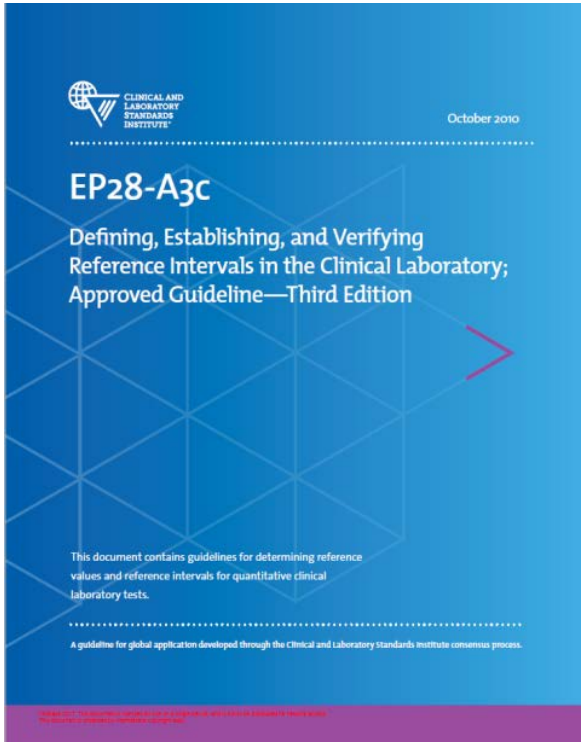
- ISO 15189 standard specifies that:
  - Reference intervals or limits must be included with the result report
  - The Laboratory must have a documented and monitored Quality System in place that covers information about the laboratory's reference intervals
  - Reference values should be established by the laboratory OR verified by the laboratory on the local patient population.



Guidelines for establishing reference ranges

- EP28-A3c Defining Establishing and verifying Reference Intervals in the Clinical Laboratory

- Approved Guidelines – 3rd Edition
  Published in 2010



CLSI. *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition.* CLSI document EP28-A3c. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.

When do we need to know how to define a reference interval?

- New assays
- New methods
- Diversifying population
- Need to be re-evaluated periodically

How do we establish a reference interval?

- Define the analyte
  - Clinical utility
  - Biological variability
  - Analytical interferences
- Select appropriate reference individuals
  - Determine number needed
  - Selection and exclusion criteria
  - Potential sub-categories / partitioning
  - Similar to population tested, eg in terms of age, gender etc
  - Sources include – blood bank, lab volunteers, students…

Sampling Methods

- Data collected from relatively healthy individuals
  - Blood donors
  - Individuals undergoing routine physical examination for periodic health screening
  - Individuals undergoing minor surgical procedures
  - Individuals undergoing genetic screening
- Get consent/Ethical Approval

What is healthy??

Sources of healthy individuals

- Blood donors
- Busselton Healthy Population Study
- Collected Patient Data

bpmri BUSSELTON POPULATION Medical Research Institute

Population Study Cohorts

Busselton Health Study collection

- A cross sectional whole population health survey which included the collection of sera and DNA samples.

The Western Australian Pregnancy Cohort (Raine) Study

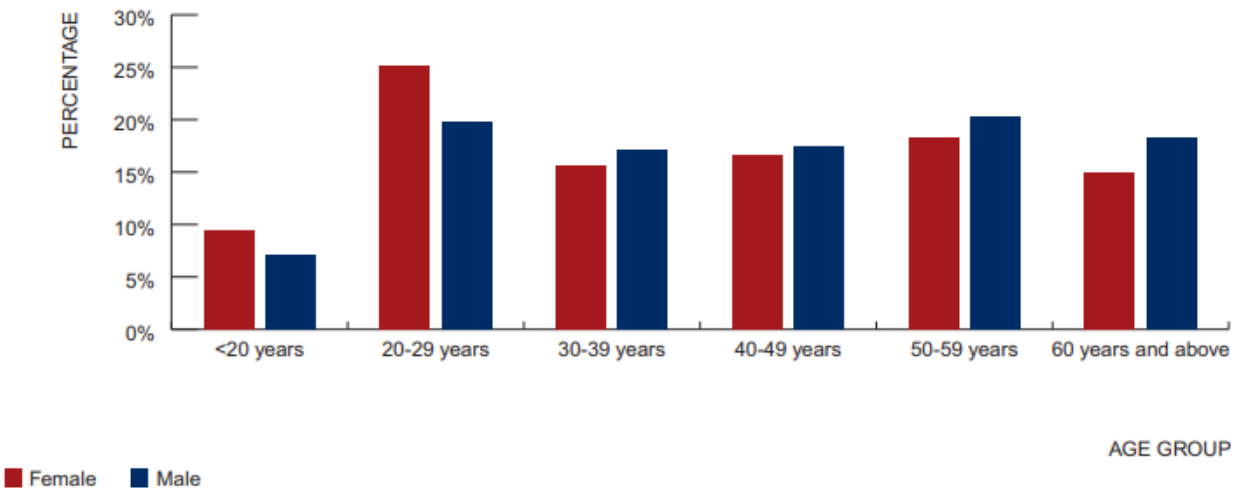
- Prospectively collected cohort of pregnancy, childhood, adolescence and now early adulthood to be carried out anywhere in the world. The cohort was established between 1989 and 1991

Demographics of a subset of the Busselton Reference Population Cohort

	Males	Females
Total number, *n* (%)	102 (51.5)	96 (48.5)
Mean age ± SD, years	51 ± 17	50 ± 17
Age group, years, *n* (%)		
<30	14 (7.1)	11 (5.5)
30–50	33 (16.7)	38 (19.2)
50–70	40 (20.2)	30 (15.2)
>70	15 (7.6)	17 (8.6)
Country of birth, *n* (%)		
Australia	79 (39.9)	83 (41.9)
Northwest Europe	17 (8.6)	10 (5.1)
Other	2 (1.0)	2 (1.0)
Not stated	4 (2.0)	1 (0.5)

Healthy Blood Donors

Figure 5 Distribution of blood donors in Australia by age group and sex, 2015



Among all blood donors who donated in 2015 there was:

- an equal proportion of males and females
  - a higher proportion of females among younger age groups (less than 20 years and 20-29 years),
  - a higher proportion of males in donors 30 years and above
- Overall, 35% of donors were from those aged 50 years and above;
  - the median age of male and female donors was 43 and 39 years, respectively

Source: Transfusion-transmissible infections in Australia: 2016 Surveillance Report. The Kirby Institute, UNSW Sydney, and Australian Red Cross Blood Service

Possible partitioning criteria

Partitioning criteria – characteristics of a reference population that can allow them to be divided into significant subclasses

- Age
- Sex
- Race

- Ethnic background
- Blood group

- Stage of pregnancy
- Stage of menstrual cycle

- Geographic location
- Circadian variation
- Diet
- Exercise
- Fasting or non-fasting

- Posture when sampled
- Tobacco use

CLSI EP28A-3c, 2010

Possible exclusion criteria

- Alcohol consumption
- Tobacco use
- Drug abuse
- Drugs, prescription or over the counter, oral contraception
- Vitamin abuse
- Hospitalisations, recent, current
- Illness, recent
- Surgery, recent
- Blood pressure, abnormal
- Obesity
- Pregnancy
- Lactation
- Environment
- Genetic factors
- Occupation
- Fasting or non-fasting (partitioning factor)
- Transfusion, recent
- (Blood donor)

CLSI EP28A-3c, 2010

Pre-analytical factors

- Subject presentation
- Prior diet
- Fasting or non-fasting
- Abstinence from pharmacological agents
- Drug regime
- Physical activity
- Sampling in relation to biological rhythms
- Rest period before collection
- Stress

Specimen collection

- Time
- Body posture
- Environmental conditions
- Specimen type
- Collection site
- Site preparation
- Blood flow
- Equipment

Specimen handling

- Transport
- Clotting
- Separation of serum / plasma
- Storage
- Preparation for analysis

CLSI EP28A-3c, 2010

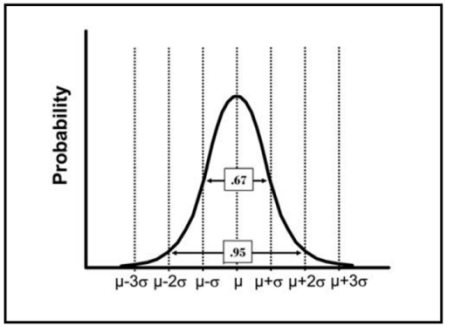
The next steps:

- Analyse reference data
- Identify possible data errors and outliers
- Document all of the above

Analysis of Data: Detection of outliers

- Assume that measured reference values represent a “homogeneous” collection of observations
- Some reference values arise from a different population of test results
  - Easily identifiable as outliers – lie well outside majority of reference values

Parametric Analysis



- For normally distributed data
  - Does the data have a Gaussian distribution?
    - Visual inspection
    - Evaluation of skewness/kurtosis
    - Chi-squared (goodness of fit) test
    - Kolmogorov-Smirnov test
- Mean $(\bar{x}) \pm 1.96$ x Std Deviation → 95% results
- Reference limits
  - 2.5th percentile = $\bar{x}$ – 1.96 SD
  - 97.5th percentile = $\bar{x}$ + 1.96 SD

  (Rounded to 2 Standard deviations)

Upper and lower limit of immunoglobulins

IgG . . . . . 3.3-11.6 g/L
IgA . . . . . 0.14-1.10 g/L
IgM . . . . 0.41-1.62 g/L

One sided reference interval

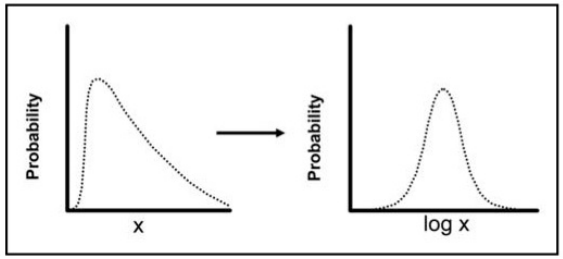
- If clinical interest is only in “low” or “high” results, one-sided intervals exclude only the 5% of the population in the “abnormal” tail of the distribution

Anti CCP	< 7 U/ml
Anti MPO	< 3.5U/ml
Anti PR3	< 2U/ml

Run on the Immunocap)

Data transformation

- Non-Gaussian data can be transformed into normally distributed data
  - Example – linear to log transformation
  - If data then looks Gaussian then treat as parametric



Non-parametric Analysis

- In data with a non-Gaussian distribution, the central 95% of the data can be determined by ordering the array from the lowest to the highest values and eliminating the lowest and highest 2.5% = rank order analysis

- *CLSI recommend*:
  - Sample size (minimum) = 120
  - Ranked according to magnitude and reference limits calculated as lower 2.5th percentile and upper 97.5th percentile
  - ie lowest and highest 3 values are eliminated

Outliers – retain or reject?

- Retain outliers unless there is known to be an aberrant observation, e.g. an analytical error
- Statistical techniques for identifying an outlier
  - Dixon's test
  - Block procedure
  - Tukey's 2-stage procedure (Gaussian data)

NB. If an outlier is rejected, remaining data needs to be re-tested for additional outliers

Validation study – small number of reference individuals

- Laboratory's test population, n = 20
- Need to:
  - Satisfy original exclusion and partitioning criteria
  - Statistically homogenous group (ie no outliers)

- Compare with original (larger) study:
  - ≥ 5 results outside 95% RR → re-assess analytical procedure, establish own RR
  - 3-4 outside 95% RR → choose another 20 reference specimens and repeat
  - ≤ 2 outside 95% RR → accept RR

Validation study – larger number of reference individuals

If an analyte ***reference interval*** is critically important for local assay interpretation

- Laboratory's test population, n = 60
- Need to:
  - Satisfy original exclusion and partitioning criteria
  - Statistically homogenous group (ie no outliers)
- Compare with larger study:
  - Examine 2 sets of values
  - Assess if statistically significant difference between mean and variance

Examples of Establishing Reference Intervals

1. Existing Assay – establishing local reference values

Myositis Antibody Detection

PathWest Laboratory approach

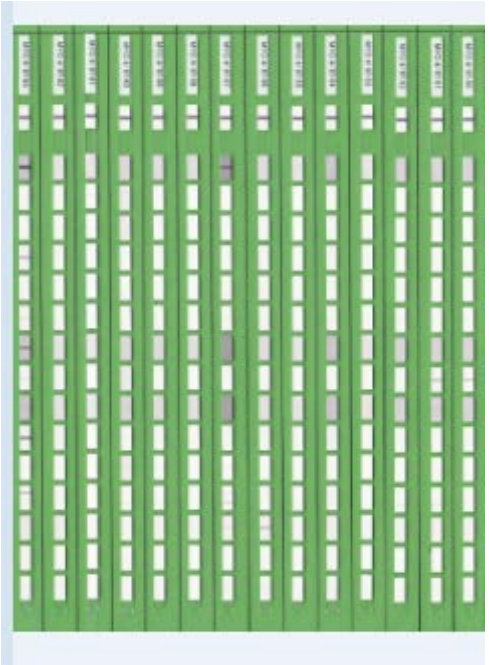
Method: Immunoblot

Detecting IgG antibodies

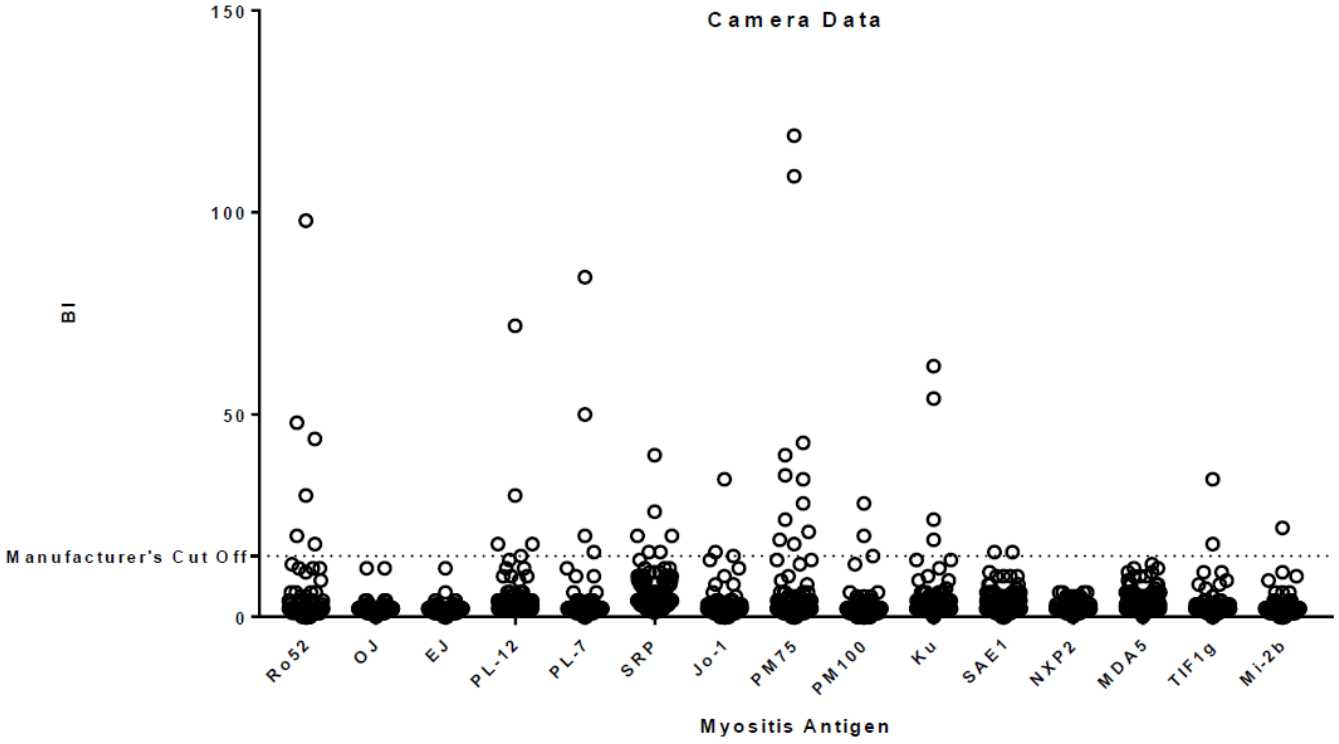
Readout: Band Intensity

- Preset cut off
- Control band to check assay performance

Ro52
OJ *
EJ *
PL-12
PL-7
SRP
Jo-1
PM-Scl 75
PM-Scl 100
Ku
SAE1
NXP2 *
MDA5 *
TIF1 gamma
Mi-2b*
Mi-2a



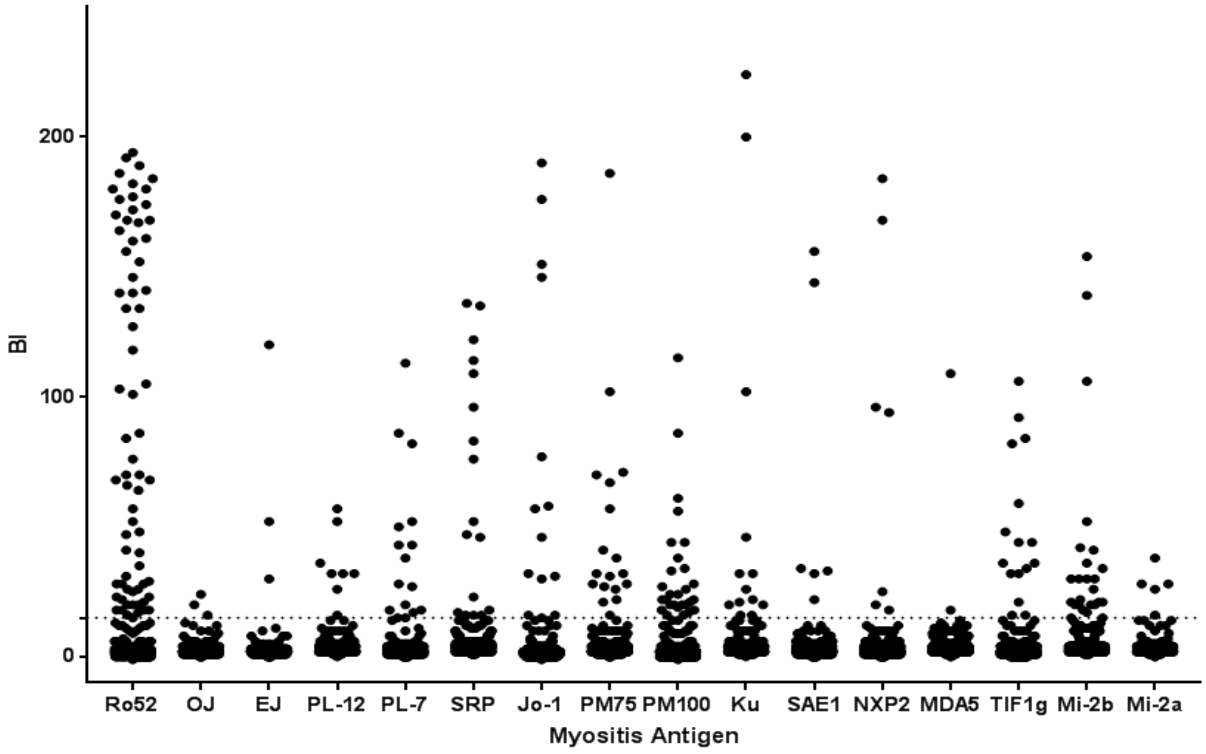
Population Reference Data
16 Ag Myositis Blot



Data for 191 individuals from the Busselton Health Study all run on the16 Ag Immunoblot

Myositis Immunoblot Clinical Data

Myositis Autoantibodies by Immunoblot



Myositis Blot Testing - Clinical samples
(n= 531: Nov 2017 – July 2018)

Antigen	I. Band Intensity
Ro52	52
OJ *	15
EJ *	15
PL-12	33
PL-7	53
SRP	27
Jo-1	17
PM-Scl 75	110
PM-Scl 100	21
Ku	55
SAE1	16
NXP2 *	15
MDA5 *	15
TIF1 gamma	19
Mi-2b*	15
Mi-2a	19

Established Local Cut Off values

Examples of Establishing Reference Intervals

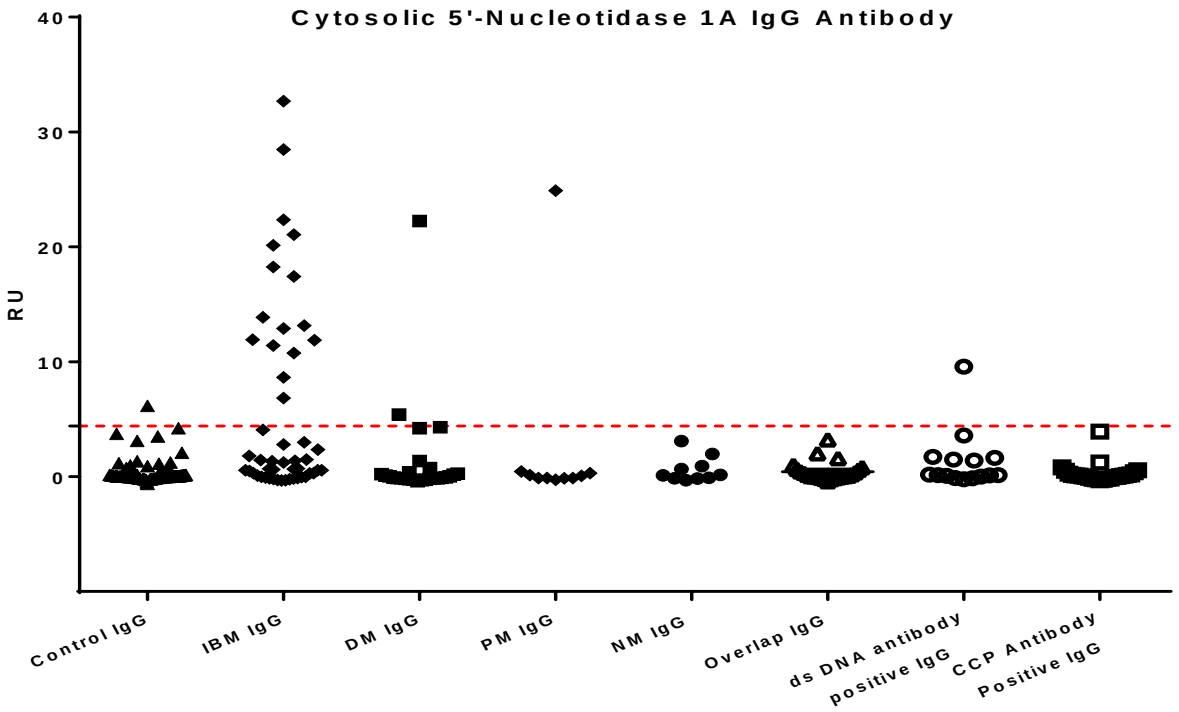
2. In house Assay –establishing a local reference value

IBM Antibody Assay

Method: In house ELISA

- Antigen: N terminal His-tagged cNIA/ NT5C1A protein
- Detection anti human IgG
- Readout OD of patient sample/OD of a reference serum (pool of 4 healthy individuals)

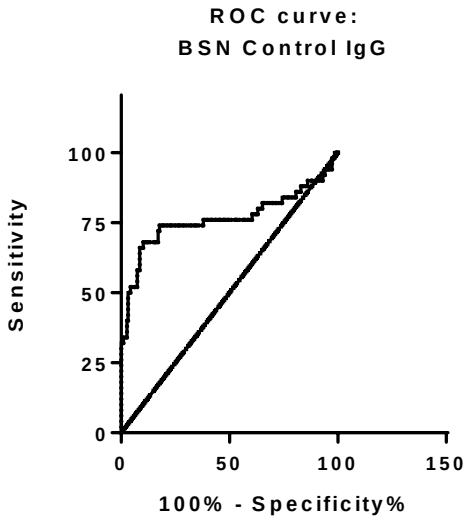
Population Control and Disease Control Data



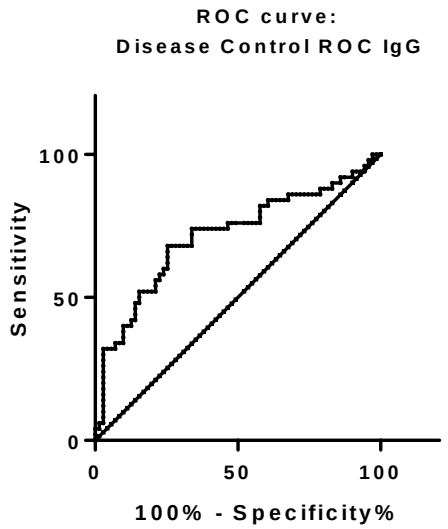
Control IgG

	Relative Units
	IgG
99% Percentile	4.4

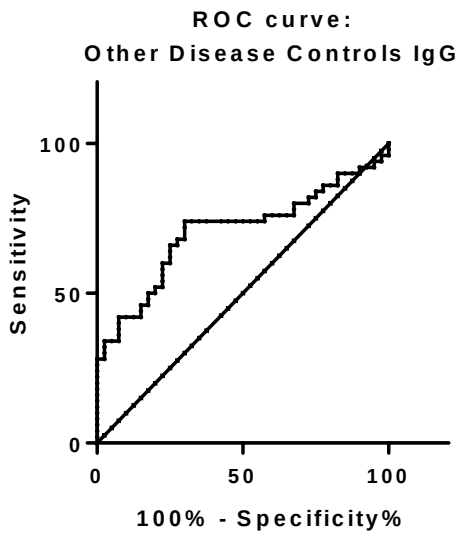
ROC Curve Analysis



Area under the ROC curve	
Area	0.76
Std. Error	0.05
95% confidence interval	0.67 to 0.86
P value	<0.0001
Data	
Controls (BSN Control IgG)	187
Patients (IBM IgG)	50



Area under the ROC curve	
Area	0.71
Std. Error	0.05
95% confidence interval	0.62 to 0.81
P value	<0.0001
Data	
Controls (IgG Control)	71
Patients (IBM IgG)	50



Area under the ROC curve	
Area	0.71
Std. Error	0.055
95% confidence interval	0.6 to 0.81
P value	0.0008
Data	
Controls (Other Disease IgG)	40
Patients (IBM IgG)	50

Specificity and and sensitivity of the assay using the ROC analysis

A. IgG Disease Cohort

Reference value (RU)	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 4.3	32	20% to 47%	94	86% to 98%	5.7
> 4.9	32	20% to 47%	96	88% to 99%	7.6
> 6.1	32	20% to 47%	97	90% to 100%	11
> 7.7	30	18% to 45%	97	90% to 100%	11
> 9.7	28	16% to 42%	97	90% to 100%	9.9
> 11	26	15% to 40%	97	90% to 100%	9.2
> 12	24	13% to 38%	97	90% to 100%	8.5

B. IgG BSN Cohort

RU	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 2.6	38	25% to 53%	97	94% to 99%	14
> 2.9	36	23% to 51%	97	94% to 99%	13
> 3	34	21% to 49%	97	94% to 99%	13
> 3.3	34	21% to 49%	98	95% to 99%	16
> 3.6	34	21% to 49%	98	95% to 100%	21
> 3.9	34	21% to 49%	99	96% to 100%	32
> 4.1	32	20% to 47%	99	96% to 100%	30
> 5.2	32	20% to 47%	99	97% to 100%	60

C. IgG Unrelated Disease Cohort

RU	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 3.8	34	21% to 49%	95	83% to 99%	6.8
> 4	34	21% to 49%	98	87% to 100%	14
> 5.5	32	20% to 47%	98	87% to 100%	13
> 7.7	30	18% to 45%	98	87% to 100%	12
> 9.1	28	16% to 42%	98	87% to 100%	11

Examples of Establishing Reference Intervals

3. Existing Assay: A review of Reference Values for Assays run in the Autoimmunity Lab

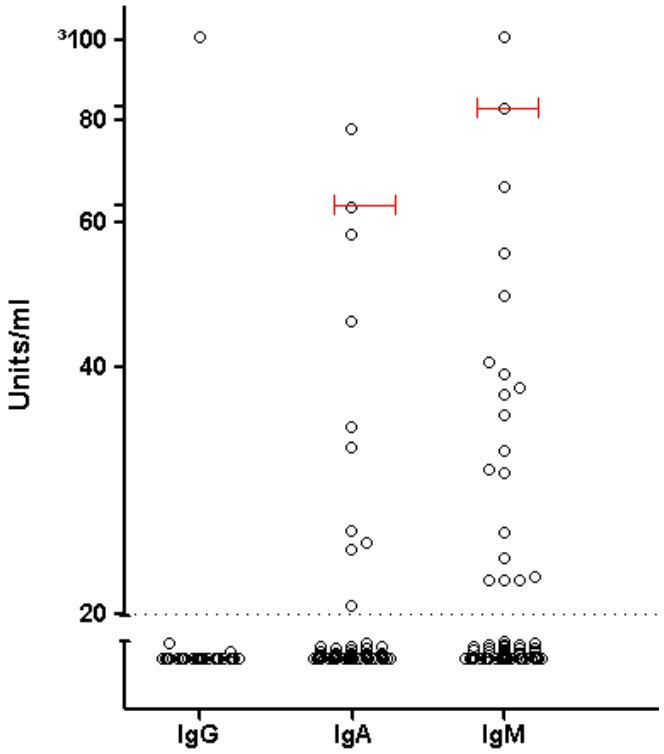
Method

- ELISA - REAADS®, Corgenix,
- IgG, IgM and IgA
- Kit Ref value <20 units

Kit Details: Cut Off Determined from 120 healthy blood donors

B2GPI Antibody Reference Interval

	SCGH Cohort	General Population Cohort
Total number n (%)	676	198
Male:Female Ratio	I : 1.2	1:0.95
Mean age ± SD (years)	53.65 ± 17.1	50.4 ± 17

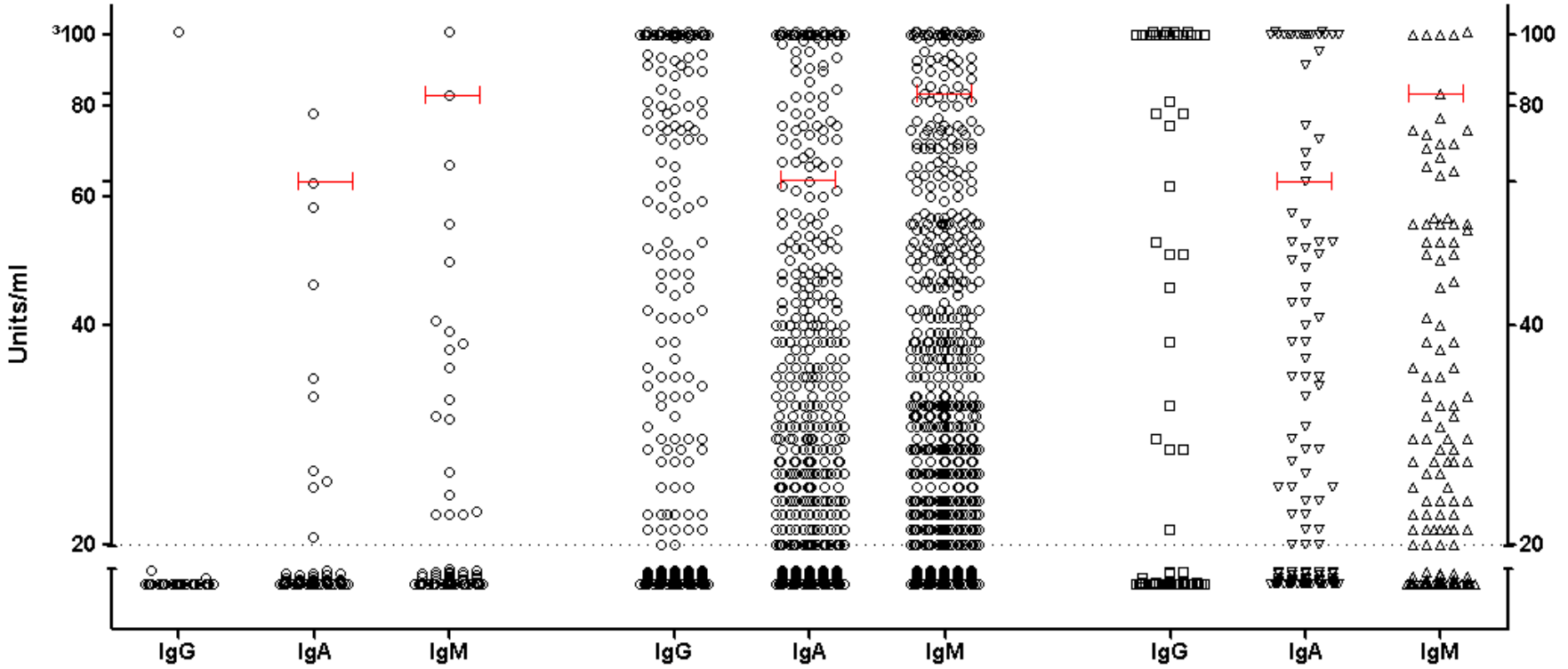


	General Population		
	IgG	IgA	IgM
<20	197	188	179
≥20	1	10	19
%	0.5	5	9.6

Reference Population

- β2GPI ab level > 99th percentile
  - A >63 units
  - G >20 units
  - M >83 units

Brusch A, Bundell C, Hollingsworth P. Immunoglobulin G is the only anti-beta-2-glycoprotein I isotype that associates with unprovoked thrombotic events among hospital patients. Pathology. 2014;46(3):234-9.



	General Population		
	IgG	IgA	IgM
<20	197	188	179
≥20	1	10	19
%	0.5	5	9.6

Clinical Cohort		
IgG	IgA	IgM
5,410	5,209	5,041
202	403	571
3.6	7.2	10.2

SCGH Clinical Cohort		
IgG	IgA	IgM
646	613	593
30	63	83
4.4	9.3	12.3

Summary

- Reference intervals are important for the differentiation of healthy individuals from individuals having a disease associated with the analyte investigated
- Reference cohorts may not be readily available
- Partition of intervals may be required
- Sample size is important to ensure the estimated Reference Interval reflects the distribution of results for the reference population
- Transference and Verification:
  - Quoted reference intervals need to be validated/verified for the local population
  - Involves smaller number of samples.
- Statistical methods need to take into account the characteristics of the data and any outliers.

Acknowledgements

Prof Michaela Lucas
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References

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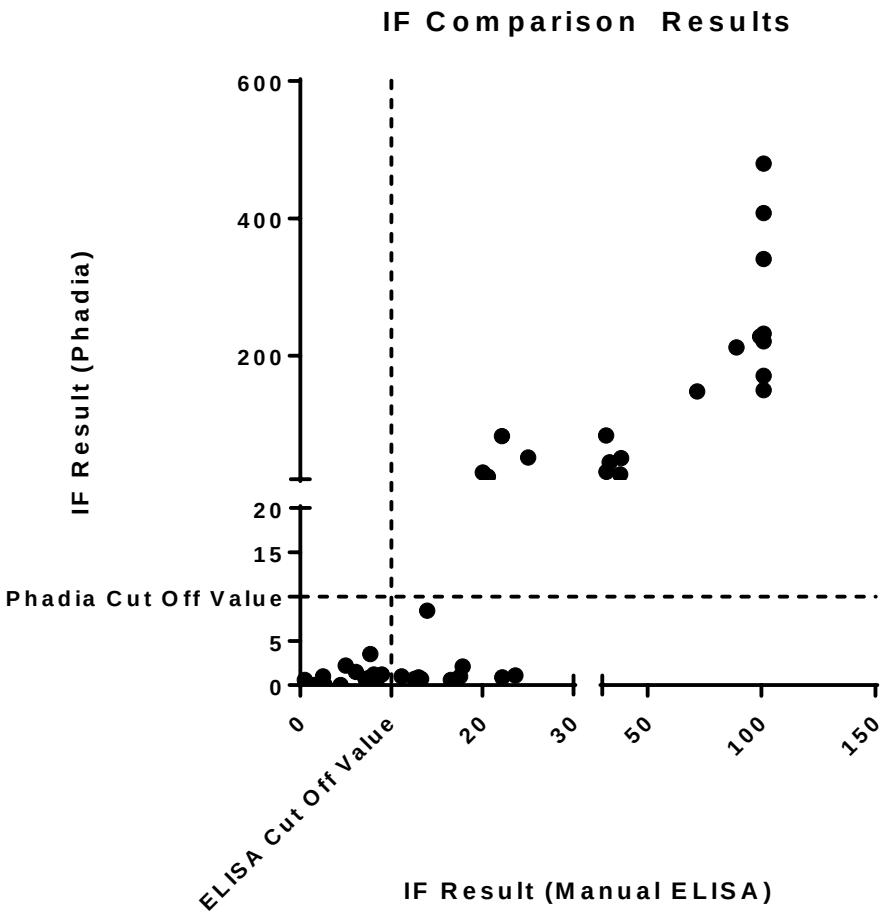
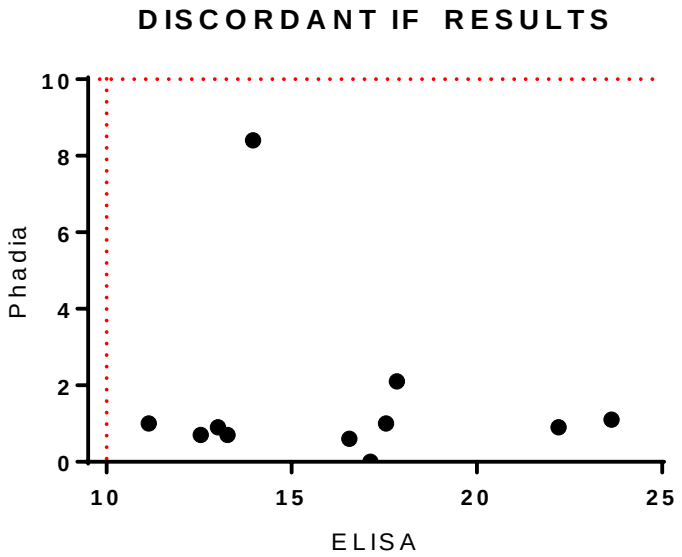
New Reference Intervals

- Need to periodically review reference intervals in all laboratories
  - Method changes
  - New analytes
- Who needs to know if a reference intervals changes?
- How is this advised?
  - Test directory
  - GP information
  - Document notice
  - Clearly stated on result reports
- QAP program
- Notification is required for NATA accreditation





Comparison of results from the manual Intrinsic Factor method (Genesis) compared to the Phadia automated IF results.

Dixon's test or "Reed rule"

- Calculation of the D/R ratio
  - D = absolute difference between the extreme observation and the next observation
  - R = range of all observations, including extremes

- Interpretation
  - D/R ≥ 1/3 → reject result
  - D/R < 1/3 → retain result

- Example: D = 20 – 17 = 3; R = 20 – 5 = 15
  D/R = 3 / 15 = 0.2 → retain

Block procedure

- If 2 or 3 outliers exist on one side of the distribution
- Apply the D/R rule to the least extreme outlier
  - If this value is rejected → all rejected
  - If this value is retained → all retained or apply a test that considers all outliers together

Tukey's procedure

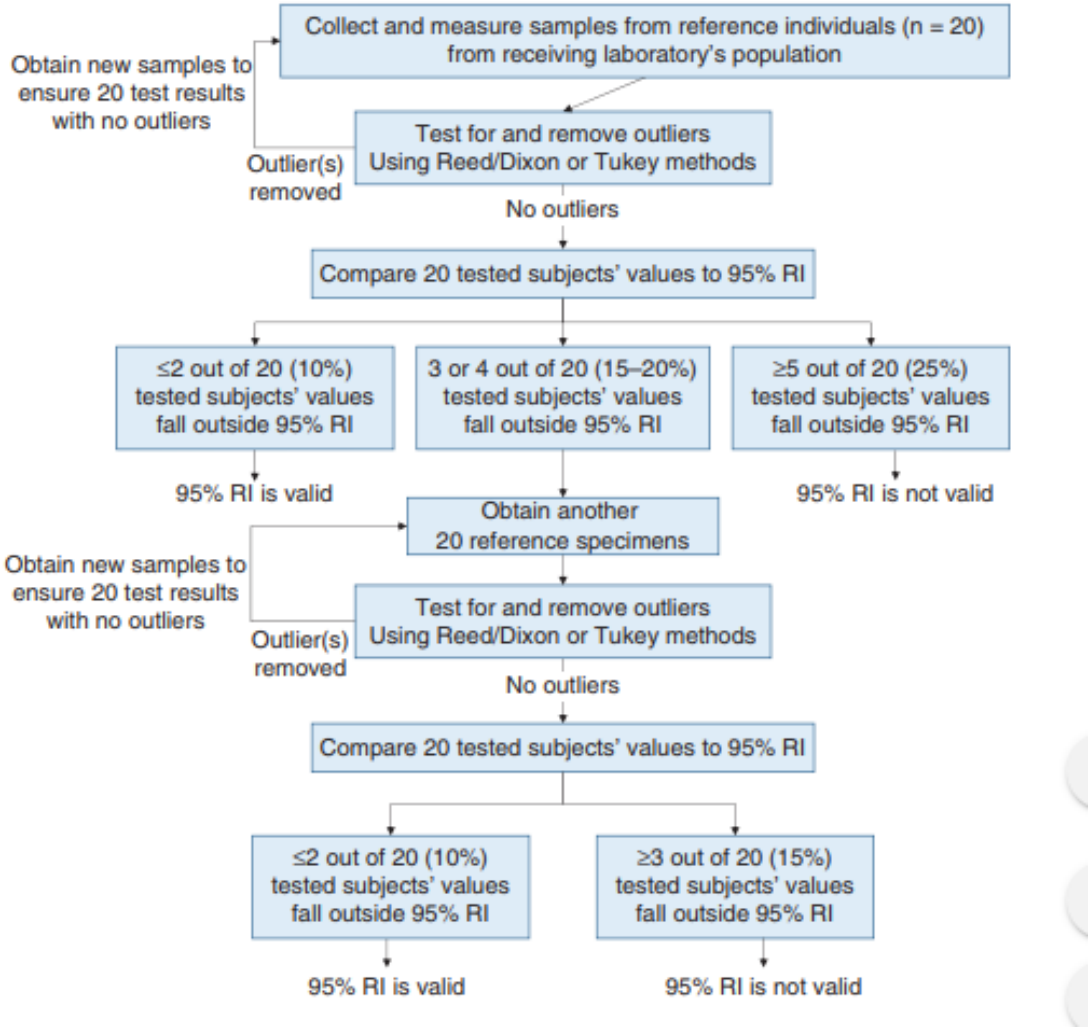
- Considers all outliers together
- Reduces / eliminates the potential masking effect of multiple outliers on one side of the distribution
- Appropriate only if the data has Gaussian distribution (nb. can transform non-Gaussian data)

- Uses middle 50% of sample
  - Calculate Q1 (25th centile) and Q3 (75th centile) of the data set
  - IQR (interquartile range) = Q3 – Q1
- Lower boundary = Q1 – 1.5 x IQR
- Upper boundary = Q3 + 1.5 x IQR
- Only those values between the lower and upper boundaries are considered

Rank order calculation

- Example:
  - Smallest value $r = 1$
  - Largest value $r = n$
  - Lower 2.5th percentile $r_1 = 0.025(n + 1)$
  - Upper 97.5th percentile $r_2 = 0.975(n + 1)$

- Therefore, if n = 120:
  - $r_1 = 0.025(120 + 1) = 3$
  - $r_2 = 0.975(120 + 1) = 118$


Collect and measure samples from reference individuals (n = 20) from receiving laboratory's population
Obtain new samples to ensure 20 test results with no outliers
Test for and remove outliers Using Reed/Dixon or Tukey methods
Outlier(s) removed
No outliers
Compare 20 tested subjects' values to 95% RI
≤2 out of 20 (10%) tested subjects' values fall outside 95% RI
3 or 4 out of 20 (15–20%) tested subjects' values fall outside 95% RI
≥5 out of 20 (25%) tested subjects' values fall outside 95% RI
95% RI is valid
95% RI is not valid
Obtain another 20 reference specimens
Obtain new samples to ensure 20 test results with no outliers
Test for and remove outliers Using Reed/Dixon or Tukey methods
Outlier(s) removed
No outliers
Compare 20 tested subjects' values to 95% RI
≤2 out of 20 (10%) tested subjects' values fall outside 95% RI
≥3 out of 20 (15%) tested subjects' values fall outside 95% RI
95% RI is valid
95% RI is not valid