Centre for Biopharmaceutical Excellence

Biological Tests Validation and Controls

In-vivo Test

In-vitro Test

Sections of the Report

- Useful References for Biological Assays
- Differences between Biological and Analytical Assays
- Types of Assays and Tests
- Standards and Standardisation
- Validation of BioAssays
- Treatment of OOS and Outlier Data in Bioassays

Key Regulatory References

INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS
Q6B

Current *Step 4* version
dated 10 March 1999

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

WHO and Biological Standards

Useful Regulatory References for Test Methods and Assay Calculation Examples

- WHO – Manual Lab. Methods for Testing Vaccines – WHO/ VSQ/97.04
- USP <111> Design and Analysis of Biological Assays
- USP <1033> Biological Assay Validation
- USP <1034> Analysis of Biological Assays
- European Pharmacopoeia Section VIII.13 Statistical Analysis of Results of Biological Assays and Tests.
- British Pharmacopoeia (BP) 1993 Appendix XIV - Biological Assays and Tests
- Test Methods included in USP/BP/EP/WHO and US FDA 600 series
- EDQM – PLA Database for standardized calculation of parallel line assays

Analytical Method Validation USP<1225>

Analytical Performance Parameter	Category I Active	Category II Impurities and Degradation Products		Category III Performance	Category IV Identification
		Quantitative	Limit Tests	Dissolution etc..	
Precision	Yes	Yes		Yes	
Accuracy	Yes	Yes	*	*	
Detection Limit			Yes	*	
Quantitation Limit		Yes		*	
Specificity / Selectivity	Yes	Yes	Yes	Yes	Yes
Range	Yes	Yes	*	*	
Linearity	Yes	Yes		*	
Ruggedness	Yes	Yes	Yes	Yes	

* Maybe required depending on nature of specific test

Analytical Method Validation – WHO Draft

Type of analytical procedure	Identification	Testing for impurities	Testing for impurities	Assay — dissolution (measurement only) — content/potency
Characteristics		Quantitative tests	Limit tests	
Accuracy	–	+	–	+
Precision				
Repeatability	–	+	–	+
Intermediate precision[a]	–	+	–	+
Specificity	+	+	+	+
Detection limit	–	–[b]	+	–
Quantitation limit	–	+	–	–
Linearity	–	+	–	+
Range	–	+	–	+

– Characteristic is normally not evaluated;
+ Characteristic should normally be evaluated.
[a] In cases where a reproducibility study has been performed, intermediate precision is not needed.
[b] May be needed in some cases.

Why are biological assays different to chemical assays ?

- Bioassays are much more variable then chemistry
- Bioassays have multiple “factors” that impact the robustness of the method
- Generally the bioassay regression line is non-linear (lacks selectivity) compared to analytical regression
- Bioassays use multiple dilutions or “doses” to ensure the most sensitive range is used.
  - Analytical assays usually done at one dilution
- Bioassays compare the test sample to a standard reference sample with known activity.
  - assays are always comparative

Types of Biological Assays

Invivo - Animal Models — ↑ Variability ↑ Cost
- drugs and biologicals in living systems

Invitro - Biological Systems — → Variability → Cost
- microbiological or tissue response
- drug in biological (serum, plasma, urine etc.) matrix
- biological in biological matrix

Immunological (ELIZA) Assays — ↓ Variability → Cost

Enzymatic Assays

Microbiological Assays — ↓ Variability ↓ Cost
- Quantitative (enumeration)
- Qualitative (presence / absence)

Types of Bioassays

- **Quantal Assays**
  - Elicits an 'All or None' response in different animals e.g
    - Digitalis induced cardiac arrest in guinea pigs
    - Insulin Assay - hypoglycaemic convulsions in mice.
    - Antivenom assay - % dead vs. % survived
    - Calculation of LD_{50} in mice or rats

- **Graded Response Assays** [mostly on tissues/cells/plates or in animals]
  - **Parallel Line Assays** or **Slope Ratio Assays**
  - Graded responses to varying doses e.g
    - Antibiotic assay (relative zones of inhibition)
    - Heparin (relative clotting time)
    - Influenza Assay – Haemagglutination
    - % transmission or absorbance

Bio-Assay Variability – Lack of Ruggedness (Variation Sources)

- In-vivo tests subject to animal variability: sex, health, weight, season
- Lots under test often biologically produced and variable
- Preparations can become unstable on the bench
- Analyst technique plays a part in control of variation
- Tests use multiple reagents, different cell/ tissue lots, variable biological and "living" cultures/animal models etc.
- "Inter-Lot" variation is a major influence on assay Precision and sometimes accuracy.
  - Often forced to use different Lots of Reagents
  - Try to minimise Lot to Lot changes if possible
- Assay variation (lack of robustness) due to multiple factors plus the sample matrix (plasma, tissue etc.)
- The dose response is not linear in many cases

Recommended Specifications (ICH Q6B plus and specific monographs)

Bulk Substance

- *Identity*
- *Appearance and description*
- *Purity and impurities*
  - *Process related*
  - *Product related*
- *Potency*
- *Sterility (or bulk bioburden)*
- *Endotoxin*
- *Toxicity*
- *Protein Content Mass*

Product

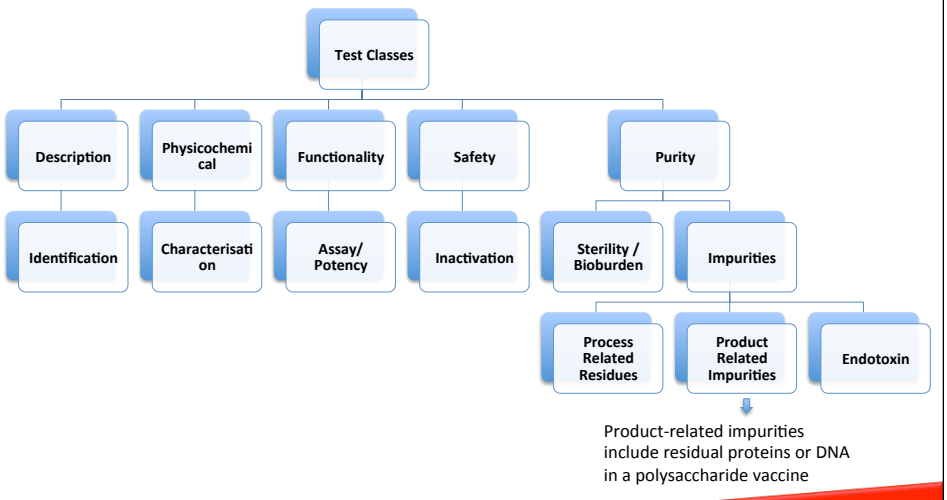
- *Identity*
- *Appearance and description*
- *Purity and impurities*
  - *Process related*
  - *Product related*
- *Potency*
- *Quality (% protein in product)*
- *Sterility*
- *Endotoxin*
- *Container / closure integrity***
- *Preservative content*
- *100% physical inspection container*
- *Content(weight/ volume)*
- *General tests.*
  - *pH, osmolarity, specific gravity*

** in development only

Testing of Vaccine Bulk vs Finished Dose (FDA Guidance – 1997)

- With CBER concurrence, testing of the final formulated bulk vaccine may be substituted for testing in the final container when additional processing has been shown to have no effect on the potency of the final product.
- However, in some cases such as a lyophilized product, demonstration of the potency of the product in the final container is necessary.
- Tests for potency should detect any component interactions that may have a potentiating or interfering effect on any other component.

USPNF <1045> Biotechnology Products (Suggested Classes of Test)



Flash Quiz

	Biological Assays	Your Selection
1	Which **one** of these statements is true (a) Biological assays (bioassays) are less robust than equivalent chromatographic methods. (b) In-vivo assays are higher cost, but more reliable than in-vitro tests (c) ELIZA tests are high cost and high variability (d) In-vivo assays should be repeated 3 times	
2	Which of these can contribute to bioassay variability (there may be more than one) (a) Analyst to Analyst (b) Change of reagent lot numbers (c) Change of animal (change of sex or weight range) (d) Change of suppliers test kits	
3	We are expected to validate bioassays before use	TRUE/FALSE
4	We are not required to validate a bioassay if we use a control sample in the test	TRUE/FALSE

Biological International Standards

- Various sources of reference standards (primary & working) for biological activity: eg. WHO, USP, EP, NIBSC
- Use of a particular standard may be specified in regulatory requirements, eg pharmacopeial monograph
- Various standards may be different materials

- **International Standards (IS)**: assigned in international units (IU)
- IS are used to calibrate local (working) standards – NOT used as working standards themselves
  - Supplies are limited, and replacement of IS risks causing discontinuity

Refer to:

www.who.int/biologicals/en/reference materials

www.nibsc.ac.uk products biological reference materials

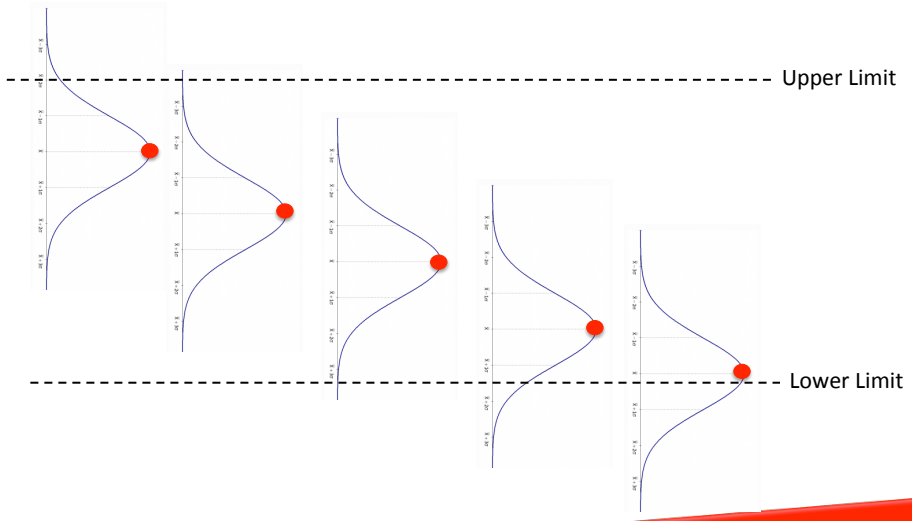
https://www.edqm.eu/en/Biological-Standardisation-Programme-mission-60.html

Biological Standards Management

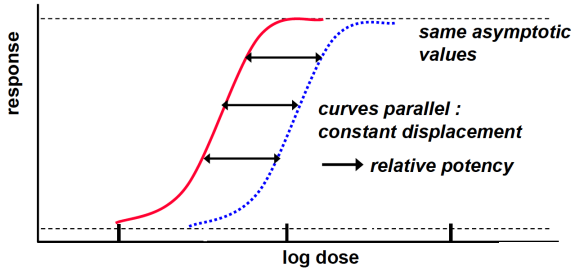
- International/WHO 1º standards available
- Utilise 2º working (in-house) standards
  - Cross standardisation program – must have an SOP
  - Re-standardise against 1º standard annually
- Define storage conditions (Fluid, Frozen, Freeze Dried)
- Standards should have similar functional characteristics and matrix as the sample under test.
- Verify long term stability of the standard/sample/control
  - Use freeze thaw cycles up to 5 cycles - sample between cycles
  - Verify bench stability once reconstituted

Problem of Standard "Creep"

(Sequential calibrations of successive standards)

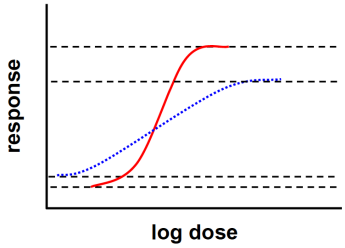


Comparing Functional Similarity of Standard and Sample Matrix



Functional similarity of the reference standard and sample in a bioassay is a fundamental condition for determination of a valid relative potency assay

- Functional Similarity is important
- Look for parallel lines (similar dose response curves
- Look for same asymptotic values – top and bottom
- Look for same transformation of response



Use of Controls

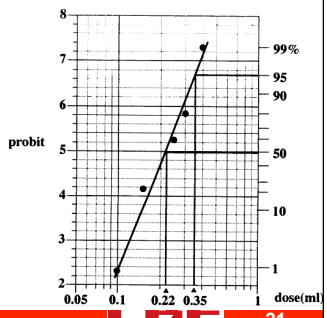
- Many bioassay designs use controls (of known potency) as well as reference standards.
- Control is usually a batch of the same matrix as the product
- The purpose of a control is to verify that the assay is performing with expected parameters
- Control limits are often set at +/-2sd and +/-3sd from the mean
- Must also look for trends or drifts of controls up or down
- Can apply control chart rules (SPC) to manage trending and responses

Binomial/Quantal (Pass/Fail) Tests

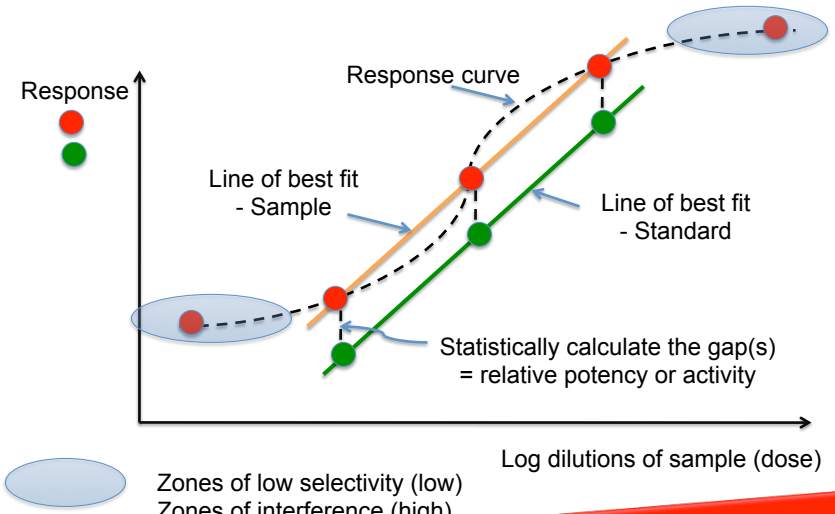
- Binomial tests are common in biological systems e.g determine the LD_{50} of a substance or use in Quantal assays
- Convert % responders to a 'Probit" value from Tables
- This normalises the data so straight lines can be drawn
- Can directly compare test and standard response curves
- Can compute the relative potency (M) and Confidence Interval
- Example use is in Insulin assays – response is % mice that are hypoglycemic or convulse in 90 minutes when dosed with drug – use a 3 dose interval.

Table 3.2 Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09



What do a Parallel Line Bioassay look like ?



Types of Graded Response Designs

- Standard Calibration Curve + single test point
- 2 standard dose + single test point = 2+1 design
- 2 standard + 2 test (2_{std}+$2_{test\ (T)}$+ $2_{test\ (U)}$ ….) = 2+2+2+...
- 3 standard + 3 test ((3_{std}+$3_{test\ (T)}$+ $3_{test\ (U)}$ ….) = 3+3+3+...

Selection is a balance between cost and quality of information. General rules:

- Standard curve and single point – not reliably quantitative
- 2+1 designs are semi quantitative only – best for estimates
- 2+2 designs are suitable for very reliable assays with little variation e.g heparin assays
- 3+3 designs are used when the assay has variability or full quantiation is required e.g potency assays.

Biological Assay Validation and Control

- Can be difficult to “validate” a biological assay
- Must show:
  - **significance of slope (regression)**
  - **estimates of precision**
  - **equivalent selectivity (standard and sample)**
  - **linearity of responses over the dose range**
- difficult to demonstrate ruggedness of bioassays

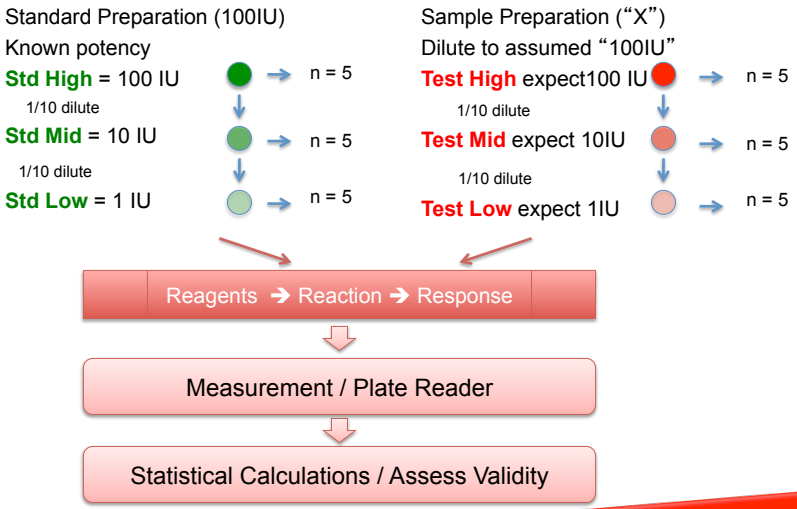
- alternative is assessment of each assay for “validity” on a case by case basis, using controls

Biological Assay Design
(some basic considerations)

1. Always include a standard or control - comparative assays
2. There should be more than one dose level of standard and test preparations ie:
   - 3 levels standard + 3 levels of unknown (3 X 3)
   - 2 levels standard + 2 levels of unknown (2 X 2)

3. Equal number replicates per dose & equal numbers of doses
4. Dose "interval" of Standard & Test (sample) should be the same
5. Always conduct at least duplicate assays to average variation
6. Evaluate potential for response bias in the design of the assay
   - Order of the "run"
   - Make up of the doses (time / temperature/ bench stability)
   - "Location" or position in the assay
   - Method of reading results / human readers

Example Bio-assay Set Up



Example Bioassay Plate Design

	1	2	3	4	5	6	7	8	9	10	11	12
A		Chigh	Chigh	Chigh	Cmid	Cmid	Cmid	Clow	Clow	Clow	-	
B	-	Shigh	Thigh	Uhigh	Smid	Tmid	Umid	Slow	Tlow	Ulow	P	-
C	-	Shigh	Thigh	Uhigh	Smid	Tmid	Umid	Slow	Tlow	Ulow	P	-
D	+	Shigh	Thigh	Uhigh	Smid	Tmid	Umid	Slow	Tlow	Ulow	P	+
E	+	P	Ulow	Tlow	Slow	Umid	Tmid	Smid	Uhigh	Thigh	Shigh	+
F	-	P	Ulow	Tlow	Slow	Umid	Tmid	Smid	Uhigh	Thigh	Shigh	-
G	-	P	Ulow	Tlow	Slow	Umid	Tmid	Smid	Uhigh	Thigh	Shigh	-
H		Clow	Clow	Clow	Cmid	Cmid	Cmid	Chigh	Chigh	Chigh	-	

Blank Well

S = Standard
T = Test Sample #1
U = Test Sample #2
C = Control (Known)
+ = positive control
-= blank
P = placebo or matrix

Std High Dose

Treatment of Data in Bioassays

- Pharmacopeias allow limited use of the outliers test to remove "*aberrant*" data points:
  - USP <111> Design and Analysis of Biological Assays
  - USP <1033> Biological Assay Validation
  - USP <1034> Analysis of Biological Assays
- This acknowledges their inherent variability;
- Thee must be specific rules about removal of points – must be in an SOP or the test method;
- Removal is strictly limited with an explanation, approved by laboratory management;
- The primary use of the outlier test is to identify a discordant result from a homogeneous sample. If an outlier is tested and the outcome is statistical outlier it would be scientifically in error to average it in since by definition it was not part of the population.

Treatment of Data in Bioassays
The USP States:

- *"For biological assays having a high variability, an outlier test may be an appropriate statistical analysis to identify those results that are statistically extreme observations."*
- *The USP describes outlier tests in the general chapter on Design and Analysis of Biological Assays <111>.*
- *The USP also states that "arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias… the rejection of observations solely on the basis of their relative magnitudes is a procedure to be used sparingly" (USP <111>)."*
- *"Occasionally, an outlier test may be of some value in estimating the probability that the OOS result is discordant from a data set, and this information can be used in an auxiliary fashion, along with all other data from the investigation, to evaluate the significance of the result."*

Combining Replicate Bioassays
USP <1034>

- *"In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value.*
- *That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria."*
- This statement acknowledges to possibility that, on infrequent occasions, a replicate bioassay result may fall outside a specification limit, based on inherent variability in the method, rather than a laboratory error or manufacturing error. Under these circumstances it is scientifically valid to combine the replicate with other estimates to determine a reportable result.

How do I average (combine) Independent Biological Assays ?

1. Unweighted Geometric Mean

Potency	Log Potency
1. 80 units	1.90308
2. 120 units	2.07918

Av. log potency (M)
= 3.98226/2
= 1.99113

antilog (M) = 97.97 units

2. Weighted Geometric Mean

Potency	Log Potency (R)	Weight (W)
1. 80 units	1.90308	100
2. 120 units	2.07918	500

$$\text{Log Potency (M)} = \frac{\Sigma (R * W)}{\Sigma (W)} = \frac{190.3 + 1039.59}{600} = 2.0498$$

Potency = antilog (M) = 112.2 units

What does assay "Weighting" mean ?

- Biological assays, because of their design, can be statistically analysed for validity and "quality".
  - Not all assays have the same "quality"
- The quality of an assay is somewhat dependent on:
  - The significance of the slope – how responsive is the assay system to the dose level. (Higher is better)
  - The level of precision within replicates and between replicates (Lower is better)
- The ratio of slope / precision gives us "weight" factor
- The weight is used to:
  - grade assays
  - Calculate fiducial limits (confidence intervals)
  - combine 2 or more assays (weighted geometric)

Flash Quiz

	Biological Assays	Your Selection
1	Which of these **one** statements is true: a) Bioassays are best conducted by one expert technician b) The robustness of bioassays should always be independent of the analyst technique c) Bioassays should only be "read" or assessed by one technician to reduce bias d) The reliability of bioassays generally rely upon a high level of technician training	
2	Which **one** of the following is generally considered the least reliable biological assay a) Animal (in vivo) model b) Laboratory (in vitro) model c) ELIZA (enzymatic) laboratory test d) Identity test	
3	Bioassay monographs allow the application of the outliers test to remove data points	TRUE/FALSE
4	The monographs for bioassays specifically preclude the use of outliers tests as they introduce bias into the method.	TRUE/FALSE

Features of a Valid Biological Assay

(Demonstrate these features in development and validation)

1. Significant and consistent slope (dose response curve)
2. All assays pass all validity criteria
   - parallelism of test and standard
   - significant regression
   - lack of curvature
3. Sufficient precision between replicates - Consistent "weight" and fiducial limits
4. Same transformations for response
4. Replicate assays pass X^2 test – homogeneous replicates
5. Sufficient "weight" to satisfy limits for replicate assays

Validation of Bioassay Methods

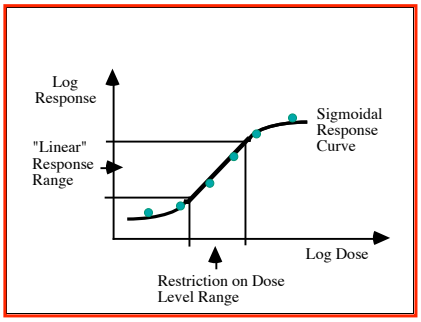
(some basic rules)

Validation design should include the following:

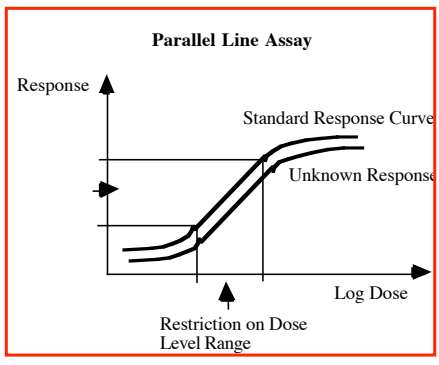
- Establish a standard “calibration” curve across multiple dilutions – define expected slope (does response)
- the assay layout is “randomised” - see plate design
- the assay includes <u>at least</u> three different dilutions of the standard preparation and three dilutions of the sample preparation – to demonstrate linearity
- Include replicates at each dose level eg. triplicate - precision
- if the test sample is presented in serum or formulated with other components, the standard should be likewise prepared
- the test includes blanks
- Include a known control in the assay if possible
- the assay is performed at least in triplicate
- Perform over multiple days by at least 2 analysts
- Where possible introduce different lots of reagents to confirm robustness

Dose Response Curves - Regression

Sigmoidal Calibration Curve



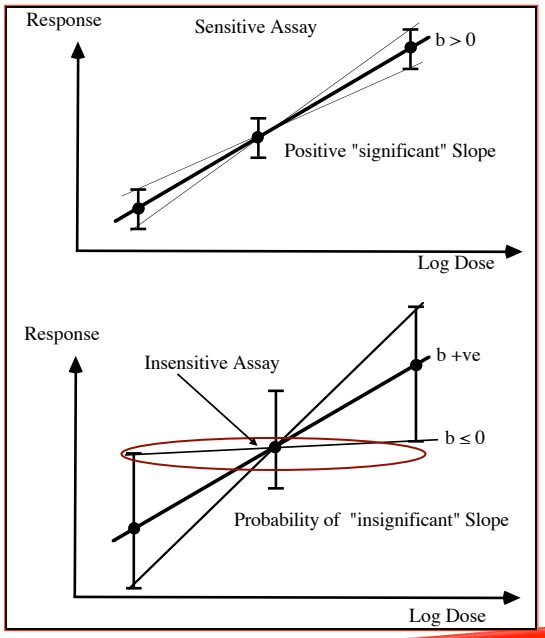
Parallel Line Comparative Assay



Biological Assay Sensitivity and Selectivity

The slope or dose response line is an important feature of a biological assay. **The larger the slope the better the assay.**

The "within dose" repeatability or precision has a direct effect on the confidence internal for the assay.
If the replication is too variable then the "significance" of the slope is poor and therefore the reliability of the test is subsequently poor.



Tests for Biological Assay Validity

For a biological test to be **"valid"** it must:

- exhibit significant regression (slope) - **sensitivity and precision**
- the slopes of the standard/unknown must be "parallel" - **selective**
- the responses must be linear with respect to dose ie not curved - **linearity, accuracy and range**
- the random error (residual) must be more significant than the error associated with dose level or treatment - **precision**

These statistical tests should be assessed during validation of the method and applied to each assay performed to confirm validity - this approach is part of a combined strategy of **validation** and **verification** or control.

Analysis of Variance

(Assessment of Validity - reference BP/EP Monograph

Example (3 x 3 x 3) assay.

Randomised Block Design

Analysis of Validity of the Assay**

- significant regression ($p < 0.01$)
- no significant departure from parallelism ($p > 0.05$)
- no significant departure - non opposed curvature ($p>0.05$)
- no significant departure - opposed curvature ($p>0.05$)

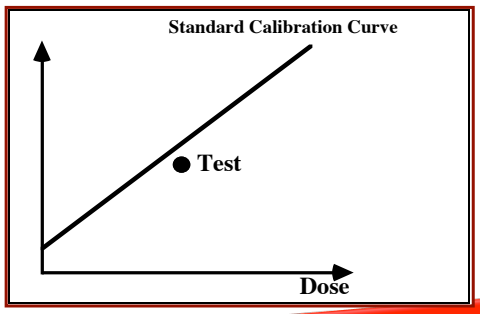
Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F	P
Preparations	2	176.26	**88.13**		
Regression	1	30976.0	**30976**	**25770**	**<0.01**
Parallelism	2	7.17	**3.59**	**2.99**	**>0.05**
Quadratic	1	2.37	**2.37**	**1.97**	**>0.05**
Difference of Quadratic	2	1.91	**0.96**	**0.8**	**>0.05**
Treatments	8	31163.5	**3895.5**		
Blocks	5	127.4	**25.48**	**21.2**	**<0.01**
Errors	40	48.1	**1.202**		
Total	53	31339.2			

** Consider EDQM PLA Software

Single Test Point Assays

(some precautions)

- Best used as "Limit of Detection" tests only
- Assume linearity (parallelism) of control and test - major assumption
  - same selectivity profile
  - same matrix
- Assume there is no non-opposed curvature
- Cannot obtain measures of precision



Flash Quiz

	Biological Assays	Your Selection
1	Regarding the validation of biological test methods which **ONE** statement is most TRUE a) Companies should strictly follow USP <1225> requirements b) Companies should strictly follow ICH Q2 requirements c) Bioassays do not need to be validated as they have in-built controls d) Validation of biological assays requires assessment of the consistency of the dose response curve	
2	**Choose the one True statement from the following:** a) If a biological assay fails by junior analyst (A) due to not meeting acceptance criteria but passes by senior analyst (B) the reason must be analyst error or training b) When conducting repeat testing overwhelm the OOS result i.e. conduct 5 repeats and average all results including the original OOS c) If a biological assay fails, but also fails the test acceptance criteria i.e. %CV <20% for Endotoxin test fails, then it is not an OOS but an invalid test d) Using the Dixons outlier test is the 1st step in investigating a biological OOS	
3	Generally at least 2 replicate bioassays are conducted	TRUE/FALSE

Vaccine Potency - Human Use

Conventional test method	Alternative Test Method: Name & Description	Validation Status	Regulatory Status	Effect or Potential Effect on Animal Use	Last Reviewed
Lethal/paralytic challenge test in the batch potency testing of tetanus toxoid vaccines for human use (Methods A and B in PhEur 2.7.8)	ELISA procedure for Batch Potency Testing of Tetanus Vaccines Description and references	**EU:** Endorsed as valid by ECVAM (2000)	**EU:** Accepted into PhEur (Method C, 2.7.8) (2003)	**Reduction** (single-dilution test instead of multi-dilution) **Refinement** (Does not rely on death as an endpoint)	May 2012
	Toxin Binding Inhibition (ToBI) Test for Batch Potency Testing of Tetanus Vaccines Description and references	**EU:** Endorsed as valid by ECVAM (2000)	**EU:** Accepted into PhEur (Method C, 2.7.8) (2003) **US:** 21 CFR 610.10	**Reduction** (single-dilution test instead of multi-dilution) **Refinement** (Does not rely on death as an endpoint)	May 2012
Lethal/Intradermal Challenge Test in the Batch Potency Testing of Diphtheria Vaccine (Methods A and B in PhEur 2.7.6)	ELISA Procedure for Potency testing of Diphtheria Vaccines Description and references	No information	**EU:** Accepted into PhEur	**Reduction** **Refinement**	May 2012
	Vero Cell Assay for Potency testing of Diphtheria Vaccines Description and references	**EU:** Norwegian Medicines Agency presently conducting validation	**EU:** Accepted into PhEur	**Reduction** **Refinement**	May 2012
Combination Tetanus and Diphtheria vaccines – separate serology tests for each vaccine	Tetanus and Diphtheria Serology test – single test for combination vaccine Description and references	No information	**EU:** Accepted into PhEur	**Reduction**	May 2012
Potency Test of Hepatitis B Vaccine (Mouse)	Serological Antigen Quantification Description and references	No information	**EU:** Accepted into PhEur (2.7.15)	**Reduction** **Refinement**	May 2012

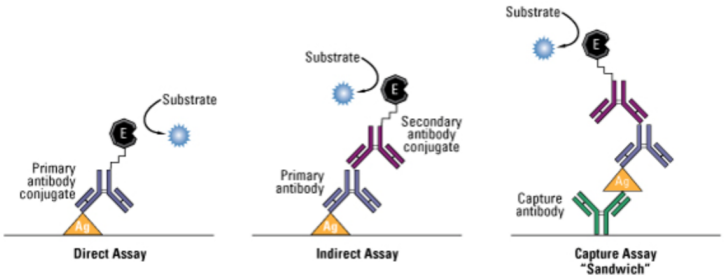
http://3rs.ccac.ca/en/testing-and-production/alternative-test-methods/vaccines/vaccine-potency-human-use/

Switch from In-Vivo to Alternative Test Methods

- Alternative Tests must be validated
- Cell based or ELIZA tests
- Some referenced in EP and US CFR610 Series
- Must refer to National Regulatory Authority (NRA)

ELIZA Tests

Diagram of common ELISA formats (direct vs. sandwich assays)



Common ELISA formats. In the assay, the antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or a matched set of unlabeled primary and conjugated secondary antibodies (indirect detection).

https://www.thermofisher.com/au/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

Advantages of Biological Assays

Advantages

- Can grade (weight) assay quality - relatively
- Can assess "validity" assay by assay - control

Disadvantages

- Expensive to perform
- Subject to Bias (Conscious/Unconscious)
- Lack accuracy and precision (relatively)
- Difficult to Control
- Lack Ruggedness (day to day, lab to lab)

Example 3x3x3x3 Parallel Line Assay (Design and Raw Data)

Example Assay Calculation and Constants for Parallel Line - 3X3X3X3 Assay (loge dose vs Linear Response transform)

	Shigh	Smid	Slow	Chigh	Cmid	Clow	Thigh	Tmid	Tlow	Uhigh	Umid	Ulow	Total
# Replicates/dose = n	3	3	3	3	3	3	3	3	3	3	3	3	36
# Prep' s = h	4												
# Treatments =	12												
# Doses/prep'n = d	3												
Dose Interval =	2.0												
Assigned Potency =	C=	1.00		T=	2.00		U=	2.00					
t (60 @0.05)=	2.000												

= Allowed entry cells
= Automatic Entry cells (locked)
= Hidden Calculation Cells (locked)
= Locked Calculation Cells

Log10 transform Data	Shigh	Smid	Slow	Chigh	Cmid	Clow	Thigh	Tmid	Tlow	Uhigh	Umid	Ulow	
	1.2385	0.8365	0.4835	1.2625	0.7775	0.4415	1.7585	1.3205	0.8345	1.4575	1.1085	0.7265	
	1.2895	0.8745	0.4755	1.2505	0.7835	0.4555	1.7945	1.3575	0.8325	1.5025	1.0935	0.7485	
	1.3125	0.8375	0.4605	1.2825	0.7955	0.4725	1.8425	1.3375	0.8235	1.6035	1.1175	0.7265	
Sum													1.03655556
Average	1.2802	0.8495	0.4732	1.2652	0.7855	0.4565	1.7985	1.3385	0.8302	1.5212	1.1065	0.7338	12.44
Average square													14.803
													sum squares

Response Totals and Contrasts

			Std		Control		Test T		Test U	Totals			
	Low	Slow =	0.4732	Tlow=	0.4565	Ulow=	0.8302	Clow =	0.7338				
	Mid	Smid =	0.8495	Tmid =	0.7855	Umid =	1.3385	Cmid =	1.1065				
	High	Shigh =	1.2802	Thigh =	1.2652	Uhigh =	1.7985	Chigh =	1.5212				squares
Preparations	(P)	Ps=	2.6028	Pt=	2.5072	Pu=	3.9672	Pc=	3.3615	12.439	= Sum P		
Linear Contrast	(L)	Ls=	0.8070	Lt =	0.8087	Lu =	0.9683	Lc =	0.7873	3.371	= Sum L		2.86275

Example 3x3x3x3 Parallel Line Assay (ANOVA)

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F F ratio	P Validity Test	Hp = 1.0 / HL = 1.5		
Preparations	3	1.41861	0.4729		should be:	Limits	p = 0.05	P = 0.01
Regression	1	4.262	4.2622	4789.89	<0.01	4.24		**
Non - Parallelism	3	0.0319	0.0106	11.96	>0.05	2.99	**	
Non- Linearity	4	0.0149	0.0037	4.18	>0.05	2.75	**	
Treatments	11	5.7276						
Residual Errors	24	0.0214	0.0009					
Total	35	5.7490						

** convert Fratio to p values @0.05 or 0.01 level

go to the following web site:

http://www.graphpad.com/quickcalcs/index.cfm

Example 3x3x3x3 Parallel Line Assay (Potency Estimates and CIs)

Calculation of Potency Ratio and Fiducual Limits

Potency Calculations		Control		Batch T		Batch U
Common slope b =	0.6080					
+/- log FL		0.04580		0.05261		0.04990
Log Ratio (M) =	Mt =	-0.0525	Mu =	0.7480	Mc =	0.4160
Potency Ratio =	Potency	0.949	Potency	2.11	Potency	1.52
Assigned Potency (units) =		0.95		4.23		3.03
% of Assigned Potency =		94.9%		211.3%		151.6%
Log Fiducial Limits =	Upper	-0.0067	Upper	0.8013	Upper	0.4662
	Lower	-0.0983	Lower	0.6960	Lower	0.3664
Potency Fiducial Limits =	Upper	0.99333	Upper	2.228326	Upper	1.59392
	Lower	0.90638	Lower	2.005792	Lower	1.44254
Potency % of Assigned Potency =	Upper	99%	Upper	222.8%	Upper	159.4%
	Lower	90.6%	Lower	200.6%	Lower	144.3%
Potency % of Found Potency =	Upper	104.7%	Upper	105.4%	Upper	105.1%
	Lower	95.5%	Lower	94.9%	Lower	95.1%

0.69315	I = ln dose interval
1.0008	C =
2.56242	2V =