Bioassay
APAC Managing your BIO Risk with USP

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Agenda

- Potency assay and related USP Standards
- Relative Potency assay development
- Statistical Models
- Robustness
- Bioassay Validation
- Additional USP Resources

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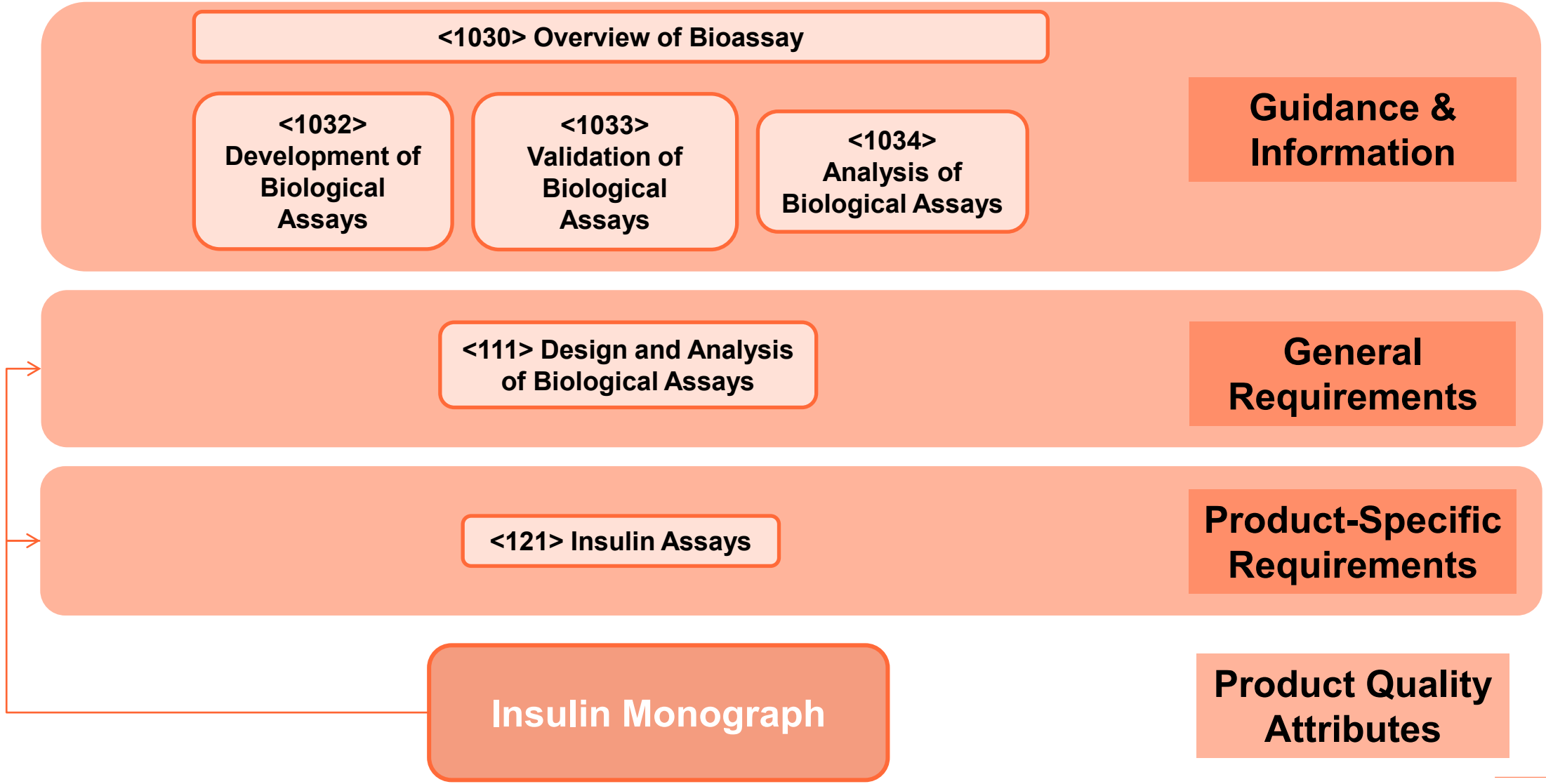
Potency assay and related USP Standards

How do General Chapters and Monographs Relate to One Another?



Product-Specific Potency Assays

- Bioassay General Chapters numbered between 1000 to 1999 are for informational purposes only. They contain no mandatory tests, assays, or other requirements applicable to any official article, regardless of citation in a general chapter numbered below 1000, a monograph, or in General Notices.
- USP product-specific potency assays can be found in a Monograph or a General Chapter
- Monograph requirements supersede Chapter requirements

Statistics Expert Committee_Bioassay SC: 2020-2025 Cycle

General Chapters / Stimuli		Update

<1033> Biological Assay Validation	• PF 45(4)
	• PF 48(6)
		• Major revision was completed based on the comments received from PF, SC members around validation requirements
		  • clarity to the distinction between a bioassay method versus procedure
		  • Move the Validation Example to an appendix
		• Target to republish at PF 50(1)

<1030> Introduction to Bioassays - Overview and Glossary	• Current official	• Revision was completed based on the comments received and discussion among the SC
		  • Definition of Bioassay
		  • Scope of the chapters
		  • Introduction of life cycle concept
		• Target to PF 50(1)

<1032> Design and Development of Biological Assays	• PF46(4)	• Comments received are under review by the SC • Target to republish in PF50(4)
<1034> Analysis of Biological Assays	• Current official	• Revision was completed based on the comments received and discussion among the SC • Target to PF 50(4)

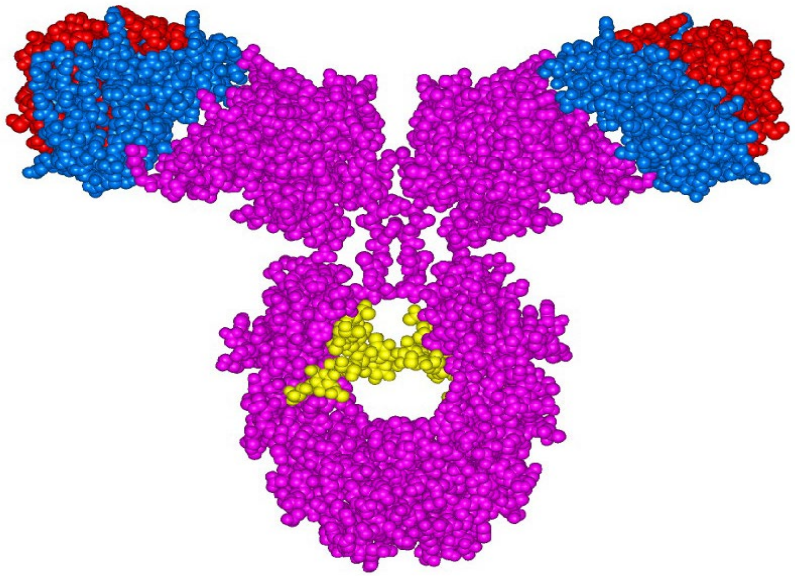
Biotherapeutics Characterization

Primary Structure

- Molecular weight
- Amino acid sequence
- Methionine oxidation
- Deamidation
- Non-glycosylation
- C- & N-terminal variants
- Disulfide linkage Mapping
- Free sulfhydryl group

Size/Charge heterogeneity

- High molecular weight
- Low molecular weight
- Acidic and basic variants



Fab-related Biological Activity

- Target neutralization activity
- Target binding activity
- Apoptosis activity

Higher order Structure

- Protein secondary
- tertiary structure
- Thermodynamic Stability

Fc-related Biological Activity

- Transmembrane TNF- α binding assay
- Fc Rn binding
- Fcγ RIIIa (V/V type) binding
- ADCC
- CDC
- C1q binding

Carbohydrate Structure and Composition

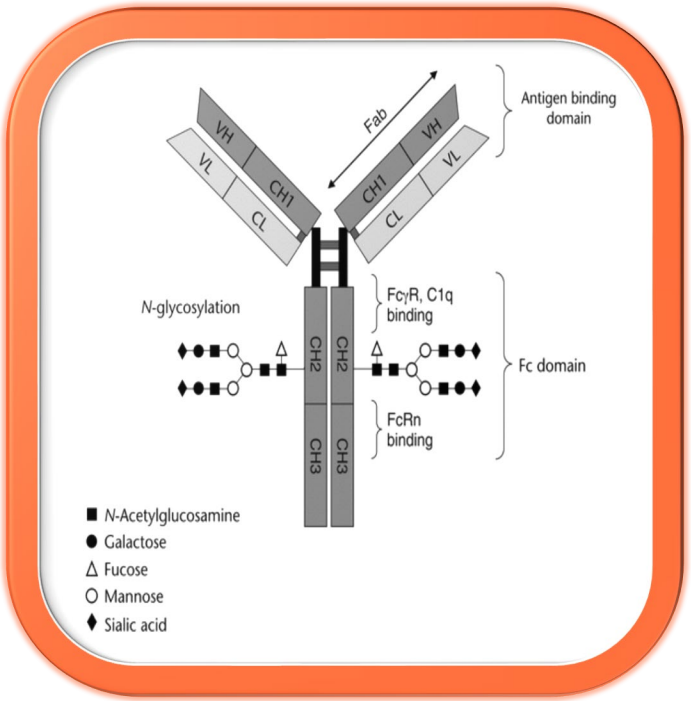
- N-linked glycosylation site determination
- N-glycan Identification
- N-glycan profile analysis

Host Cell Protein Analysis

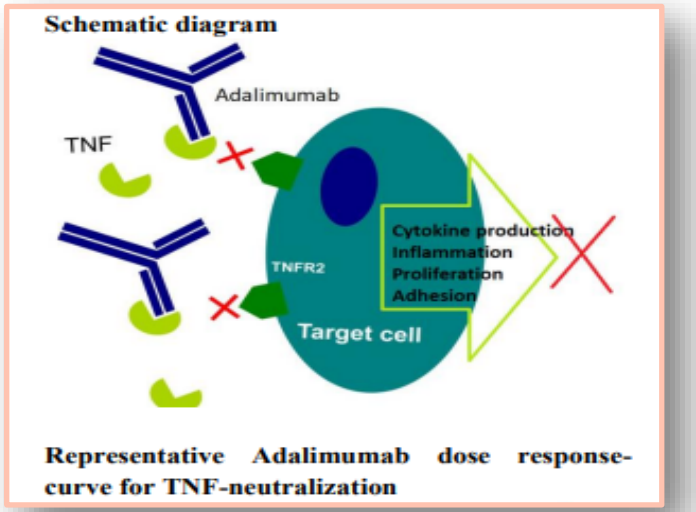
Residual DNA measurement

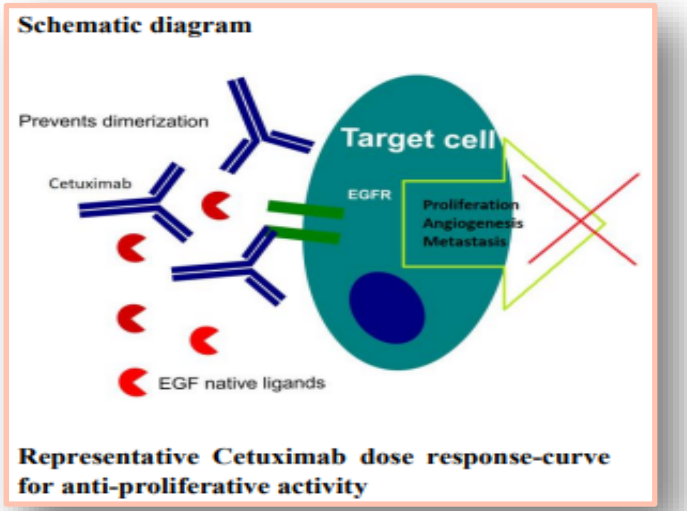
Monoclonal Antibody – Potency assays

Basic Structure of an
IgG1 Monoclonal Antibody

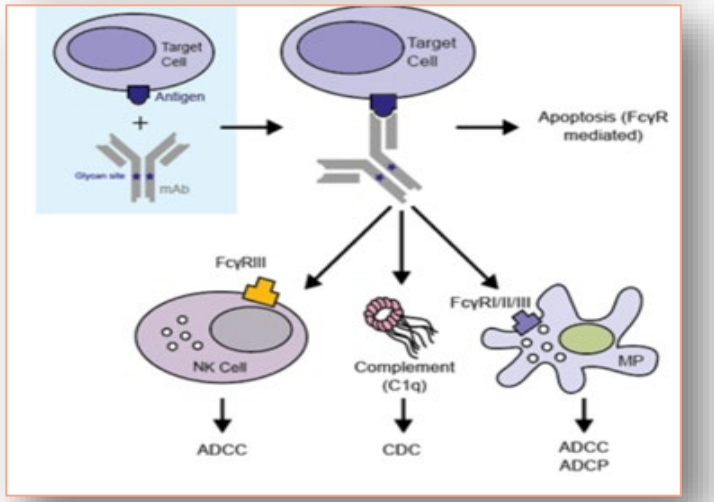


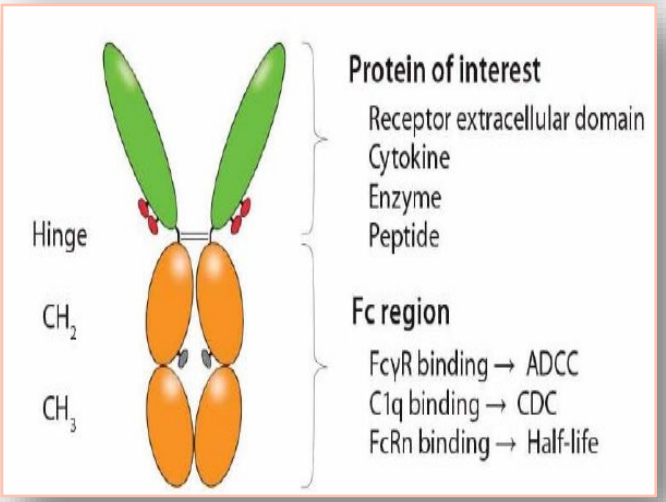
Fab-mediated Potency Assays*





Fc-mediated Potency Assays# (Immune effector functions)





*Agata Burzawa et. al - Role of cell-based potency assay in functional characterization of therapeutic monoclonal antibodies (mAbs), Bioreliance, Merck

USP GC <1108>Assays to evaluate fragment crystallizable (fc)-mediated effector function.

Immunoglobulin Fc-Fusion Proteins Part 2: Therapeutic Uses and Clinical Development: https://bioprocessintl.com/manufacturing/cell-therapies/manufacture-and-regulation-of-cell-gene-and-tissue-therapies-part-1-chemistry-manufacturing-and-control-challenges-for-atmps/

Connecting MOA and Potency assay - Example

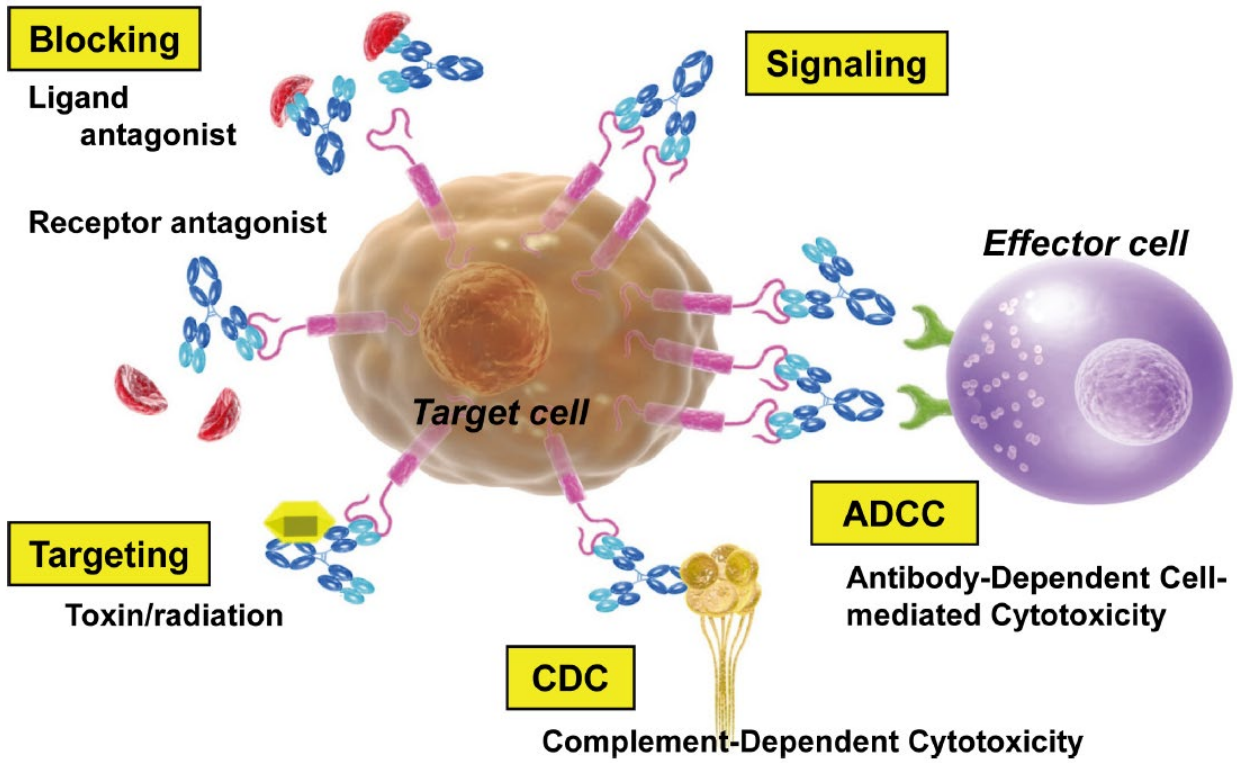


TABLE 4 Functional characterization of a proposed biosimilar to Remicade (Jung et al., 2014)

Category	Quality attribute	Techniques
Target and receptor binding	TNF binding	ELISA and cell-based binding assay
	FcRN	SPR
	C1q	ELISA
Bioactivity	TNF neutralization	Cell-based TNF neutralization assay
	Apoptosis	Cell-based apoptosis assay
	CDC	Cell-based CDC assay

CDC, complement dependent cytotoxicity; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TNF, tumor necrosis factor.

Masami Suzuki, Chie Kato, and Atsuhiko Kato. J Toxicol Pathol 2015; 28: 133–139 Concise Review.
Kirchhoff, C. F., Wang, X. M., Conlon, H. D., Anderson, S., Ryan, A. M., & Bose, A. (2017). Biosimilars: Key regulatory considerations and similarity assessment tools. *Biotechnology and Bioengineering*, *114*(12), 2696–2705.

Relative Potency assay development

Bioassay and Relative Potency

- **Biological activity is a critical quality attribute** for biopharmaceuticals and is often determined by a **biological assay called bioassay or potency assay**.
- Specifically, potency is the biological activity or capacity of a product to affect a given result, typically reflecting the mechanism of action.
- Because of the inherent variability in biological test systems an absolute measure of potency is more variable than a measure of activity relative to a standard.
- This has led to the adoption of the relative potency methodology. Assuming that the standard and test materials are biologically similar, statistical similarity should be present, and the test sample can be expected to behave like a concentration or dilution of the standard.
- The reference standard can be internal developed by the manufacturer, a pharmacopeial standard (e.g., USP), or a higher order standard prepared by WHO when available.

Decisions about Fitness for Use

- Based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay
- For lot release, a linear-model bioassay may allow sufficient assessment of similarity, but this should be demonstrated
- For stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes – assess similarity using entire concentration-response curve including the asymptotes (if present)

Important Tools & Prerequisites

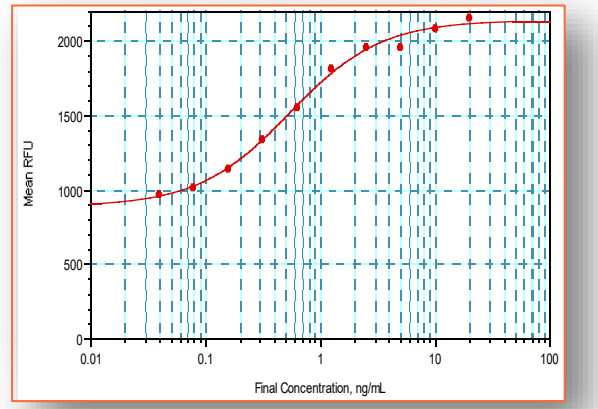
- Reference standards and other critical reagents - qualification of new lots/multiple storage
- Equipment – maintenance and periodic calibration
- Reagents – qualification of serum & other critical supplements
- Software – validation and periodic qualification of templates
- Qualified analysts – training records
- Labeling format – cell banks/plates/flasks, etc.

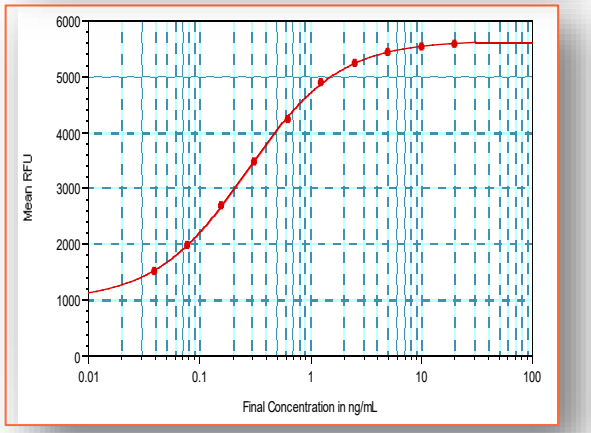
Cell Line
Understanding and defining cell line requirements

- Selection/Establishing a cell line:
  - Screen several cell lines based on mechanism of action and prior knowledge and select the one that shows a good dose-dependent response to the drug
  - Sub-clone, if necessary for better stability and/or signal: noise ratio (S/N; “working window”)
- If necessary, engineer cell lines by transfecting with appropriate receptors and/or signaling proteins
  - Suitability and stability testing
  - Clone stability over a period/passages
- Adaptation
- Sub-culturing optimization: a detailed history of the cell line is required so if needed it can be reproduced in the future

Improvement in the signal post-adaptation

Pre-Adaptation



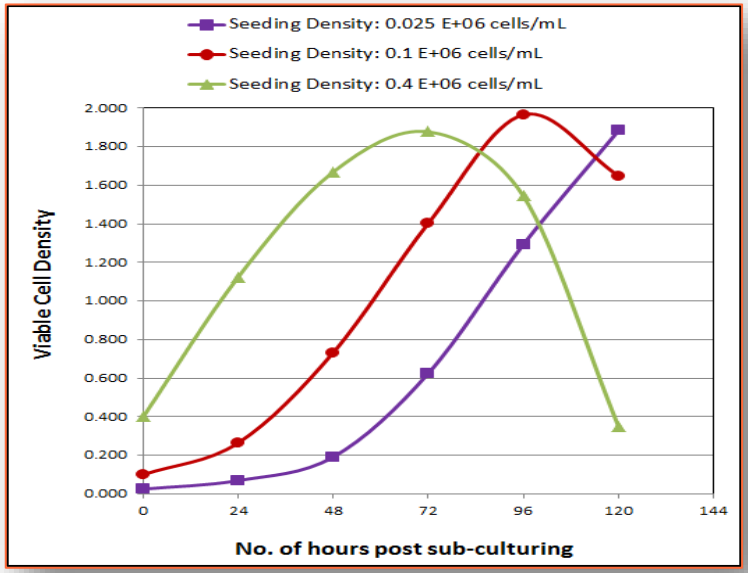


Post Adaptation (~5 weeks)

Cell Line
Cell banking & Characterization

- Tiered system
  - 2 [Master (MCB) and Working Cell Bank (WCB)] or 3 (starting with seed stock)
- Growth curve analysis
  - Try at least 3 Seeding densities, morphology & viability – photographs/cell counts
- Morphology & Viability
- Sterility (bacteria & fungi) & Mycoplasma testing
- Effect of cell passage

Growth Curve Analysis & Sub-culturing optimization example



No. of days in culture	Sub-culturing	
	For Assay	For Maintenance
1	$0.4 - 0.5 \times 10^6$ cells/mL	-
2	$0.2 - 0.4 \times 10^6$ cells/mL	0.1×10^6 cells/mL
3	0.1×10^6 cells/mL	$0.025 - 0.1 \times 10^6$ cells/mL
4	0.1×10^6 cells/mL	0.025×10^6 cells/mL

Screening & Optimization

- **Dose Response Investigation**
  - **Assay parameters:**
    - Cell density
    - Drug concentration
    - Media components (e.g., FBS concentration, etc.)
    - Fold dilution of drug
    - Incubation time in presence of drug
    - Quantification Reagent – Choice, Concentration, Volume, Incubation time, etc.
- **Other critical reagents, if any**

Design of Experiment

- Screen factors that influence assay
  - Find out a little about many factors
  - Which factors have largest effect on response?
- Optimize conditions
  - Combine factors and levels
  - Optimal/Stable operating conditions
  - What is the relationship between factor and response?

Optimization

- Number of doses
- Replication and dilution strategy
- Randomization
- Uniformity
- Blocking
- Plate layout
- Assay controls
- Outliers - technical & statistical

➢ **Number of doses** –
- Number of doses should support desired modeling and dynamic range:
  - » 4 or more doses for linear modeling
  - » 8 or more doses to support wider dynamic range 4PL or 5PL
- Spacing of doses (fold-dilution) should be examined with each proposed model and the expected range of potencies

pseudo replicates vs independent replicates:

The potential bias due to location effects can be moderated through blocking and randomization:

A higher risk plate layout

Reference (R) and test samples (A & B) grouped together on the plate; plate effect is likely to impact both series so can only be used for very well characterized assays

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	
R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	
R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	

Strip plot design

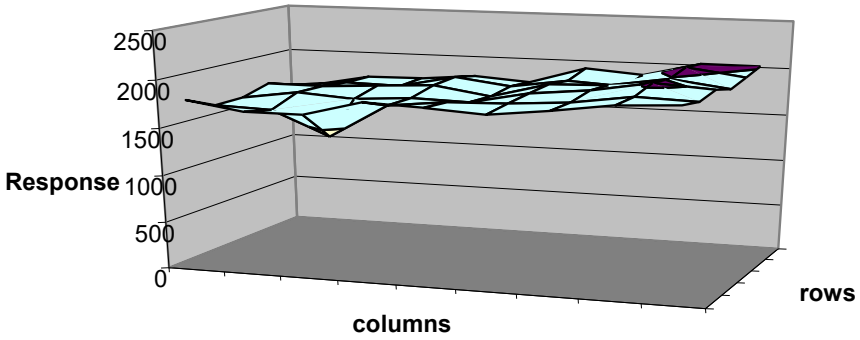
Randomize samples to rows to decrease potential plate effects. Reverse dilutions in the bottom half of the plate

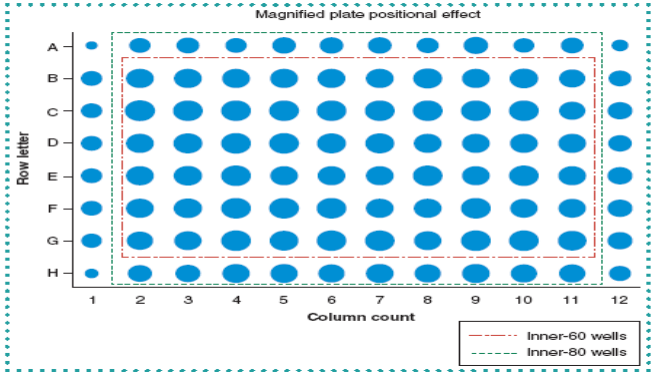
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	
R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	
R10	R9	R8	R7	R6	R5	R4	R3	R2	R1	
A10	A9	A8	A7	A6	A5	A4	A3	A2	A1	
B10	B9	B8	B7	B6	B5	B4	B3	B2	B1	

Optimization

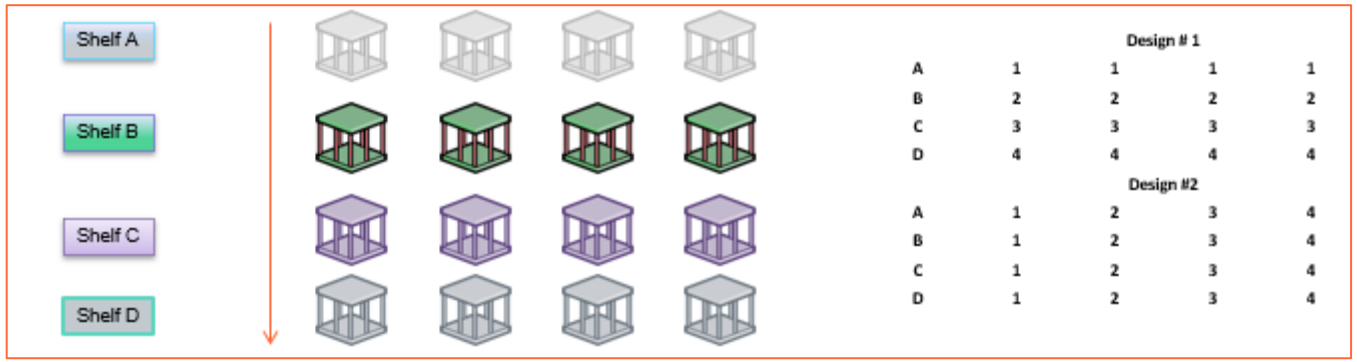
- Number of doses
- Replication and dilution strategy
- Randomization
- Uniformity
- Plate layout
- Blocking
- Assay controls

Uniformity and Edge Effect





- **Blocking - the grouping of related experimental units in experimental designs**
  - Blocking is often used to reduce the contribution of variability associated with a factor not of primary interest
  - The goal is to isolate by statistical design and analysis, a systemic effect, such as SHELF, so that it does not obscure the comparisons of interest



Assay/System Suitability Criteria

System suitability is different from sample suitability:

- System suitability is for standard
- Sample suitability is for the unknown (test sample)
- Should be based on several assays and dose response curves
- Broad ranges until qualification

- For example:
  - Upper/Lower (background) signals
  - Assay Controls & Control samples
  - Asymptotes, Slope, EC50 / IC50
  - GoF (Residual plot)

Reference :
USP General Chapter <1032>,

Data Analysis during Assay Development

Step 1	• Choose an appropriate statistical model.
Step 2	• Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance. Transform the data as necessary.
Step 3	• Screen for outliers and remove as appropriate.
Step 4	• Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.
Step 5	• If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear
Step 6	• Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

Statistical Models

Relative potency model

Relative Potency [RP]

- A measure obtained from the comparison of a test sample to a standard based of the capacity to produce the expected biological activity
- RP is unit-less and is given definition for any test material solely in relation to the reference material and the assay:
  - The master standard is usually assigned a potency equal to 1.0 or 100%
  - May occasionally be assigned based on another property (e.g., protein content)
- A standard should be incorporated early into the bioassay:
  - Helps to reduce the variability produced by inter-assay factors which impact response
  - Standardizes potency throughout the lifecycle of use (including between laboratories)

Statistical Models

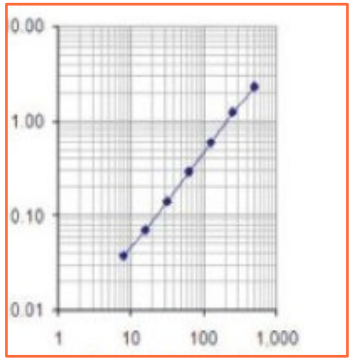
- Relative Potency: "Statistical Method in Biological Assay" by D.J. Finney (Second Edition-1971), page 61
- $F_T(z)=F_S(pz)$ where
  - Z = dose
  - F_T = dose response regression function for the test
  - F_S = dose response regression function for the standard
  - p = potency of the test relative to the standard
- This is a statement of the condition of "similarity":
  - Test function is a translation of the standard

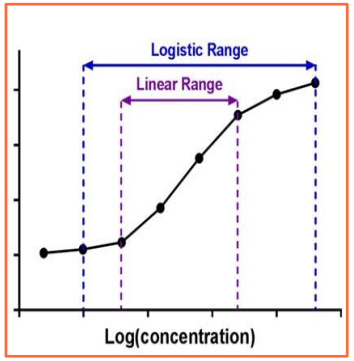
Statistical Models - Curve fit

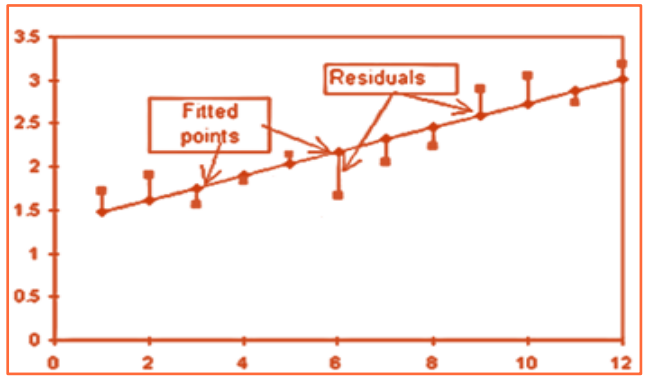
- Statistical models for dose response functions:
  - Mathematical “explanations” of relationships
- Dose response models explain variability
  - Proportion of the variability in “y” data attributed to “dose”
  - The leftover variability in “y” is called residual error (unexplained)
- R2 = The percent of the variance in the “y” data that can be explained by fitting the chosen model

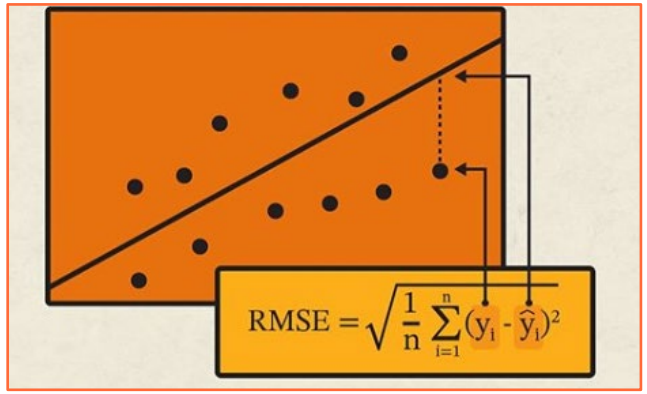
$$\frac{\text{1- Unexplained}}{\text{total}} = \text{Proportion of total explained by the model}$$

- A well fit model will have very little residual error:
  - The data points fall on or near the curve fit line
  - Root mean square error (RMSE) is low









Relative Potency Models

When To Use Each Model

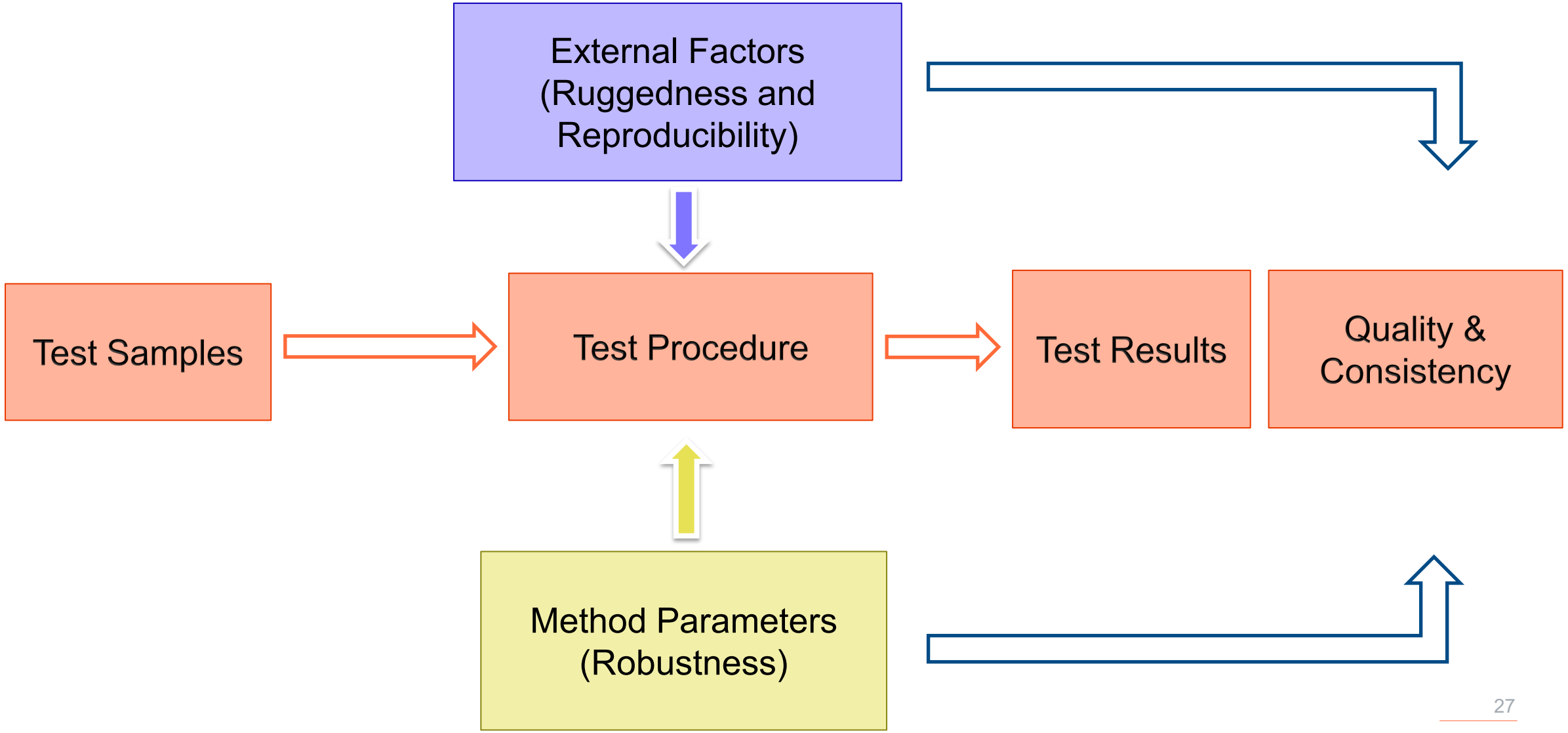
- Parallel Line Assay
  - Linear response (or linear after transformation). Can also be used for the linear portion of a non-linear response
- Parallel Curve (4-PL)
  - Non-linear response. Typically used for assays with asymptotes at low and high concentrations
- Slope Ratio
  - The expected response is linear in the dose, not its logarithm. The response will be straight lines of different slopes and common intercept
- Quantal
  - The assay involves 'all or none' response (ex. life or death)

Similarity Criteria

- USP does not provide acceptance criteria or acceptance value for assessing similarity.
- Some common criteria used include:
  - Ratio of slopes from the unconstrained model.
  - Ratio of Lower or Upper asymptotes from the unconstrained model.
  - The difference between the Upper and Lower Asymptote from constrained model
- Regardless of the similarity criteria, equivalence bounds should be set based on data such as development runs.
- Any criteria chosen should be sensitive to non-similarity. In other words, should fail when data is shown to not be similar.

Robustness

Robustness

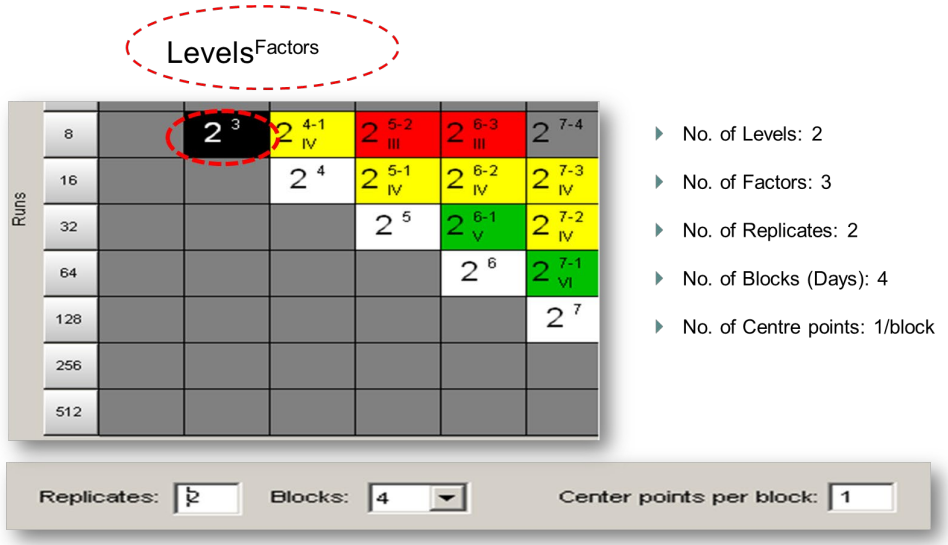


Full factorial - Robustness

Name	Units	Type	Low	High
Cell Density #	10(6) cells/mL	Numeric	0.09	0.11
Cell Passage		Numeric	2	20
Incubation time with protein	hrs	Numeric	40	44

*As cell passage was checked very early during screening, hence it was included in the robustness study

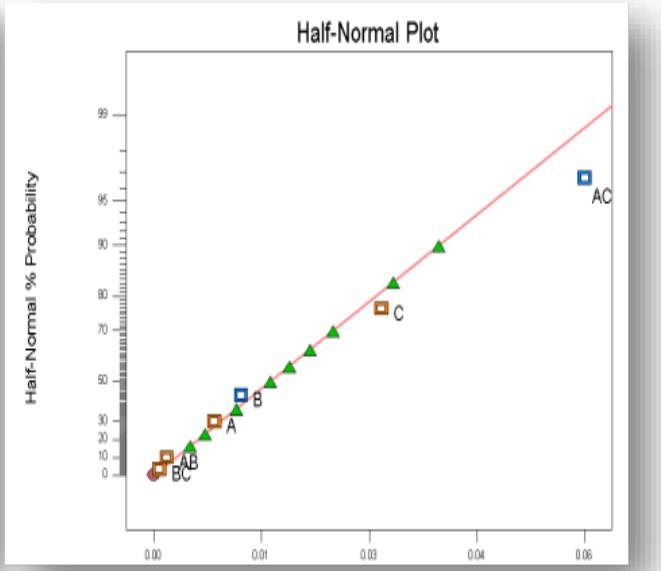
Plating cell density 3800 cell/well



- No. of Levels: 2
- No. of Factors: 3
- No. of Replicates: 2
- No. of Blocks (Days): 4
- No. of Centre points: 1/block

- Total number of combinations = (2^3) * 2 replicates + 4 center points = 20

Run	Block	Factor 1 A:Cell Density 10(6) cells/mL	Factor 2 B:Cell Passage	Factor 3 C:Incubation ... hrs
1	Day 1	0.09	2.00	40.00
2	Day 1	0.10	11.00	42.00
3	Day 1	0.11	20.00	40.00
4	Day 1	0.09	20.00	44.00
5	Day 1	0.11	2.00	44.00
6	Day 2	0.11	2.00	40.00
7	Day 2	0.09	2.00	44.00
8	Day 2	0.09	20.00	40.00
9	Day 2	0.11	20.00	44.00
10	Day 2	0.10	11.00	42.00
11	Day 3	0.11	20.00	40.00
12	Day 3	0.09	20.00	44.00
13	Day 3	0.10	11.00	42.00
14	Day 3	0.11	2.00	44.00
15	Day 3	0.09	2.00	40.00
16	Day 4	0.09	20.00	40.00
17	Day 4	0.10	11.00	42.00
18	Day 4	0.09	2.00	44.00
19	Day 4	0.11	20.00	44.00
20	Day 4	0.11	2.00	40.00



Examples of Acceptance Criteria:

- The potency (CQA) of any combination should be within the acceptable limit (± 10 %) set from the center point condition (Calculate % Recovery)
- Model should be significant for Negative effects (p<0.05) at 95% CI.
- Curvature should be not significant for the selected model (p>0.05) at 95% CI

5 Validation

Preparations for validation

Pre-validation Assessment

- Equipment- Suitable for expected accuracy?
- Reference Materials- Suitably characterized?
- Analytical method- Is procedure finalized?
- Validation Protocol- Management / QA approved?

Validation: Parameters

- Specificity/Selectivity:
  - With formulation buffer, process intermediates, related and unrelated molecules
  - Stability investigation – minimum two attributes, if possible (should address functional attributes, if applicable)
- Precision:
  - Repeatability (with-in run variation)
  - Intermediate Precision (Overall – within run & inter-run in the same lab)
  - Reproducibility (inter-lab precision; not required if only one lab will always run samples later)
- Accuracy (or sample dilution linearity)
- Range

Note: Precision, accuracy, & range can be efficiently addressed through a common experimental setup

Validation: Experimental Design & Acceptance Criteria

Parameter	Experimental Design	Acceptance criteria
<u>Precision:</u> Repeatability & Intermediate Precision	▶ Prepare different potent samples (minimum three and optimum five) and assay using 100% as standard ▶ Note 1: No. of runs should be determined based on expected IP and %recovery (idea about %IP and %recovery to be obtained from development data/Qualification data) ▶ Note 2: Run combination to be decided based on no. of analysts and critical factors ▶ Calculate Geometric Mean, Intra run %GCV (repeatability), overall %GCV (intra-run and inter-run) and Upper 95% confidence limit for %GCV using variance component estimates obtained by standard one way ANOVA	Intra-run/overall %GCV should be within the expected limit
Accuracy or Dilutional Linearity	▶ Experimental design same as Precision ▶ Calculate Average Potency, %Recovery (relative bias), and 90% confidence interval for relative bias for each potency level ▶ Plot 90% confidence intervals for relative bias versus the acceptance criteria ▶ Plot a graph of Estimated Potency vs. Expected Potency and perform linear regression analysis to determine correlation coefficient, slope and y-intercept ▶ Plot a graph of Residuals vs. Estimated potency to observe the distribution of residuals	▶ Relative bias at each potency level (% recovery) should be within the specified interval and a trend should not appear in relative bias across potency levels ▶ A linear relationship should be observed between Estimated vs. Expected Potency with acceptable slope and Correlation coefficient ▶ Intercept should not be significantly different from zero ▶ The plot of Residuals vs. Estimated Potency should show a random distribution about zero
Range	▶ No additional experiments are needed	The range of the method is demonstrated when the precision, accuracy, and dilutional linearity of the method meet the given acceptance criteria at each potency level

Additional USP Resources

Additional USP General Chapters

- ⟨111⟩ Design and Analysis of Biological Assays
- ⟨121⟩ Insulin Assays
- ⟨123⟩ Glucagon Bioidentity Tests
- ⟨124⟩ Erythropoietin Bioassays
- ⟨126⟩ Somatropin Bioidentity Tests
- ⟨1010⟩ Analytical Data—Interpretation and Treatment
- ⟨1044⟩ Cryopreservation of Cells
- ⟨1210⟩ Statistical Tools for Procedure Validation
- ⟨1103⟩ Immunological test methods—enzyme-linked immunosorbent assay (ELISA)
- ⟨1105⟩ Immunological Test Methods—Surface Plasmon Resonance

Bioassay Design, Development and Validation (On-Demand)
BIO-1030-03

Duration: 7 hours, 20 minutes
Cost: $500

Course Description:
This bioassay course will focus on factors to be considered in the design, development and validation of bioassays. The course introduces related USP general chapters, terminology, bioassay life cycle, important statistical tools and best practices, followed by a detailed discussion on the topics of design and development, robustness, validation and post-validation, with examples of USP pharmacopeial bioassays. The course reflects statistical tools in USP General Chapters <111>, <1030>, <1032>, <1033> and <1034>.

Who should participate:
This course is designed for professionals who perform, supervise, manage, audit, or oversee the development and validation of bioanalytical assays.
This course is designed for attendees with a minimum of three (3) years of bioassay experience; five (5) years is recommended.

https://uspharmacopeia.csod.com/LMS/catalog/Welcome.aspx?tab_page_id=-67&tab_id=20000495

By taking this course, you will be able to:

- ✓ Understand the structure of USP bioassay general chapters
- ✓ Recognize applicable terminology
- ✓ Explain concepts of relative potency
- ✓ Understand the bioassay life cycle
- ✓ Identify important tools and prerequisites
- ✓ Recognize bioassay best practices
- ✓ Explain bioassay design and development:
  - ✓ Fitness for use/potential challenges
  - ✓ Screening and optimization
  - ✓ Number and spacing of standards for the curve
  - ✓ Replication, uniformity, outlier detection, optimization, experimental design concepts and randomization
  - ✓ Data and assumptions, variance heterogeneity
  - ✓ Goodness for fit and measurement of uncertainty
  - ✓ Normality, transformation and weighting
  - ✓ Validity/assay/system/sample suitability criteria
  - ✓ Acceptance criteria
- ✓ Understand robustness (experimental design concepts)
- ✓ Explain bioassay validation and post-validation:
  - ✓ Identifying and measuring significant sources of error
  - ✓ Experimental design and acceptance criteria
  - ✓ Statistical considerations involved
- ✓ Provide examples of USP pharmacopeial bioassays

Thank You

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