CASSS Bioassay Workshop Introduction to Bioassay Development

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Disclosure

‘All studies shown were funded by AstraZeneca. All authors are employees of and may have stocks, or stock options in, AstraZeneca'.

Agenda

- CMC Control strategy: role of bioassay/potency
  - Potency
  - Characterization
- MOA surrogate
- Types of Assays

Agenda

- Method Lifecycle
- Method development
    - Basic steps
    - Dose response curve
  - Parameters and Criteria
    - Statistics (brief)
  - Training and Tech Transfer
  - Qualification/Validation
  - Bridging
  - Trending

Presenter Qualifications

- Scott Umlauf
  - Ph.D. in Cellular and Molecular Biology, University of Wisconsin-Madison
  - Post-doc and Cellular and Molecular Immunology, NIH
  - Positions at T Cell Sciences, Powderject Vaccines, VaxInnate, BMS
  - Currently Executive Director, Head of Bioassays, Biosafety & Impurities at AstraZeneca
- Patrick Hussmann
  - Ph.D. in Pharmacology, Georgetown University
  - Post-doc in Neuroscience at Johns Hopkins Medical Center
  - Positions at NIH-NINDS and MedImmune/AstraZeneca
  - Currently Associate Director, group lead in Bioassays, Biosafety & Impurities at AstraZeneca

Research Candidate

Warm Up

Developability Assessments

Candidate Drug Nomination

Start

Clinical Process, Analytical and Formulation Development

Clinical Manufacturing

Phase 1 Clinical Trials

P2 Clinical Manufacturing

Phase 2 Clinical Trials

Commercial Process, Analytical, Formulation and Device Development

Phase 3 Clinical Trials

Commercial Process Tech Transfer & Phase 3 Clinical Mfg

BLA Authoring & Submission

Responses To Regulatory Questions

PAI Inspection

Commercial Marketing Approval

Life Cycle Management

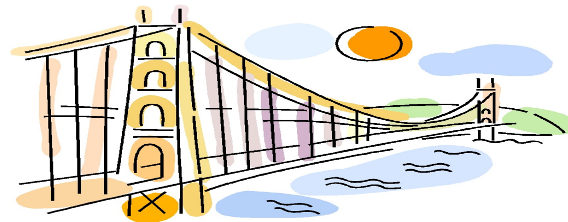
Bioassays in Drug Development

Clinical Material Supply

- Clinical supply
- Product testing
- Regulatory submissions

Delivery of Candidate Drugs (CDs)

- Candidate selection
- New molecular formats
- Research supplies



Product Support for Commercial Products

- Improvements
- Investigations

Development

- Cell lines
- Mfg process
- Formulation/delivery/device
- Assays
- Pre-clinical supplies

- Process scale up
- Fit to plant
- Tech transfer

Commercial and Beyond

Bioassay (Pharmacology): Screening Assays, HTS

Bioassay (Relative Potency)

Early Potency Assay Dev (Simple target binding Assay suitable)

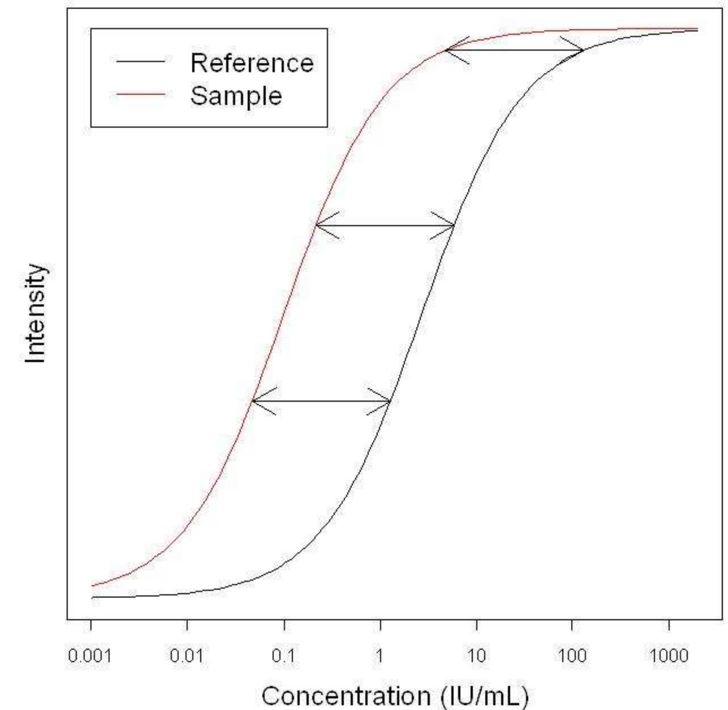
MoA Potency Release and Characterisation Assay dev

Method Qualification

Method Lifecycle

Bioassays Requirements for Drug Development

- Bioassays used in drug development ideally should be simple yet robust, easily transferable and fit for testing in CMO or CRO quality control settings.
- Should be suitable for measuring manufacturing consistency across batches/ lots of material produced in the course of the development process
- Method should be suitable for commercial release of the product



Potency assays as part of a typical biopharmaceutical manufacturing process

Cell culture

Bioreactor process

Clarification & purification

Formulation & packaging

Finding optimal purification & formulation

Enabling optimisation of manufacturing process

Enabling optimisation of manufacturing process

Analytical testing for release & characterisation

- Potency testing is a critical property of the drug and therefore is part of the formal control system within CMC (Chemistry, Manufacturing Process and Controls)

CMC Control strategy: role of bioassay/potency

Overall CMC Control Strategy

- Includes Process controls
  - Bioreactor conditions
  - Purification parameters
  - In process tests recorded in batch record
- Analytical Lot Release & Stability
  - Qualified assays
  - GxP regulations apply
- Characterization
  - Fit for purpose
- IND or BLA
  - Supports narrative of safety and efficacy throughout CMC sections

Major Differences Between Physicochemical Assays and Bioassays

- Physicochemical assays (e.g., HPLC) define and quantify the ‘protein’ content
  - Protein Concentration
  - Type and number of peaks that define ‘product’
  - Will determine clinical dosing
  - Values are in terms of weight/volume

- Bioassays/Potency Assays define and quantify ‘protein’ appropriateness
  - Quantification of ‘fitness for use’
  - Will not be used to determine dosing
  - Rather, is the content, used for dosing, ‘valid’
  - Values are in terms of activity/weight

ICH Q14 (Nov 2023)

Key Topics (not specific to Bioassays)

- Analytical Procedure Lifecycle
- Analytical Target Profile (ATP)
- Knowledge and Risk Management
- Robustness
- Control Strategy
- Definitions:
  - Accuracy
  - Precision
- Example of Potency assay for a Biologic (anti-TNF-α mAb)

CONTROL STRATEGY

A planned set of controls, derived from current product and process understanding, that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control. (*ICH Q10*)

RESPONSE

The response of an analytical procedure is its ability (within a given range) to obtain a signal which is effectively related to the concentration (amount) or activity of analyte in the sample by some known mathematical function. (*ICH Q2*)

Technical Requirements for Potency Assays for Biologics

Physicochemical Testing Considered Insufficient

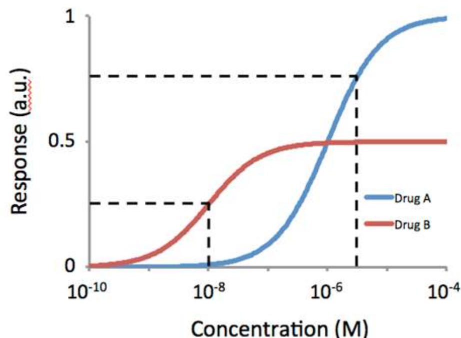
- Complex large molecules, produced in mammalian cells, depend on biological activity for their mode of action (MoA)

Have to reflect Drug MoA

- Lot-to-lot comparison at release, biochemical (competition, binding assay) or cell-based assays (black box, end to end), depending on phase and MoA

Simple to Perform and Robust

- For release and stability testing, Potency Assays are often transferred to and operated by CMOs or CROs, often with limited bioassay specific scope



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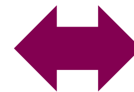
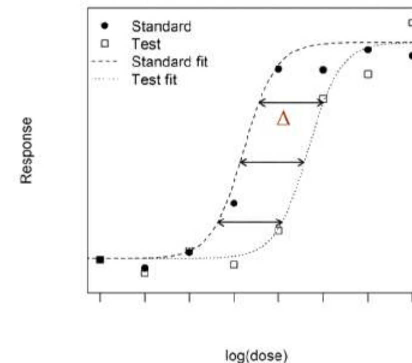
Screening/Selection Bioassays:
Resolve log scale differences between **different drugs**





https://www.quantics.co.uk/qubas-bioassay-software2/qubashelp/relative_potency_1.htm

CMC Potency Assays:
Resolve within 2 fold change between **different lots of the same drug**

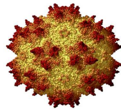
Bioassay and Quality

Bioassay Development

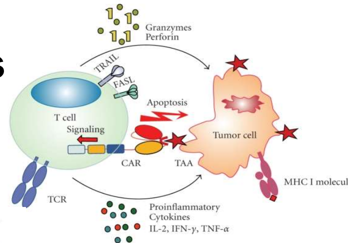
QC Lab (GMP testing)

DNA Plasmid

Virus

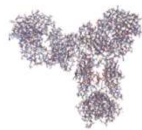


Cellular Therapies



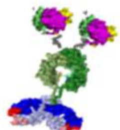
Cap Signal Sequence 3' UTR
5' ORF AAAA
5' UTR

mAb



Peptide Fusion

Bispecifics



Example of Division Between Method Development and QC

Understand Structure/Function Relationships

Develop and qualify MOA-reflective functional assays to assess critical attributes and enhance product understanding

- Characterize biological properties of product
- Understand the impact of post-translational modifications
- Perform analytical to enable manufacturing process, formulation and drug product development
- Examples include mAb, bispecific, Fc-based, peptide- and antibody-drug conjugates, vaccines, cellular and gene therapies

Transfer, GMP Support and Lifecycle Management

Technical transfer and support of analytical methods to clinical and commercial groups

- Provide GMP support for clinical programs, including release and stability testing, management of stability programs, generation of master specification and CoA, Quality audits and investigations
- Contribute to control system including specifications, comparability, and shelf-life strategies required by regulatory agencies
- Prepare regulatory filings and response to questions
- Support commercial lifecycle management

Analytical techniques for the analysis of biopharmaceuticals

- POTENCY:

  A measure of the ability of a material to elicit its function.

  - If that ability is to induce a biological response, then a potency assay should be a bioassay.
  - For some products (e.g. mAb therapeutics), binding assays may suffice as a potency assay, especially if ‘binding’ to its target is that molecule’s chief known mechanism of action.

- BIOASSAY:
  - WHO/NIBSC, *J. Immunol. Methods (1998), 216, 103-116. International consensus, Dev. Biol. Standard. (1999) vol 97*

  "A bioassay is defined as an analytical procedure measuring a biological activity of a test substance based on a specific, functional, biological response of a test system"

CMC lot-release potency assays

All biological therapeutics must meet prescribed requirements for safety, purity and potency

GMP potency testing is required for release of clinical and commercial lots, as well as:

- Stability testing
- Shelf-life determination
- Manufacturing process comparability

Potency testing is typically accomplished with cell- and/or non-cell based in vitro assays

- Results reported as % relative potency, as compared to a qualified reference standard
- Assays must meet established accuracy, precision, sensitivity, and specificity criteria, as demonstrated through phase appropriate method validation
- Assays must reflect the product's mechanism(s) of action

PHS Act section 351 and 21 CFR 210, 211, 600, 610

CMC requirements for controlling biological activity

All biotherapeutics must meet prescribed requirements for safety, purity and potency

GMP potency assays

- Cell or non-cell based in vitro assays
- Reflect primary MoA(s)
- Required for lot-release and stability testing
- Results reported as % relative potency (%RP)
- Assays must undergo phase-appropriate validation

Characterization bioassays

- Cell or non-cell based in vitro assays
- Reflect primary MoA via alternative endpoint
- Capture secondary MoA(s)
- Not required for lot-release, but other activities:
  - Process comparability
  - CQA evaluations
- Reportable results not confined to %RP
- Assays must demonstrate fit-for-purpose

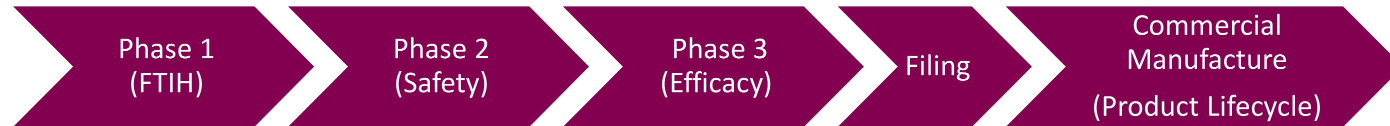
PHS Act section 351 and 21 CFR 210, 211, 600, 610

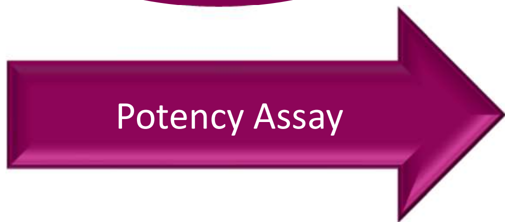
Potency vs Characterization Assays

- Typical potency assay requirements
  - Accuracy, precision, linearity, specificity
  - System suitability criteria (assay failure)
  - Frequently testing product vs a qualified Reference Standard (RS): Relative Potency (RP%) as reportable value
  - Ideally have Assay Control (AC) for trending
  - Product Specification (Out of Specification, OOS)
  - Phase-appropriate validation (qualification)
  - Robustness

- Typical characterization assay expectations
  - Accuracy, precision, linearity, specificity (may be less stringent than potency)
  - May have system suitability criteria
  - Sometimes testing product vs a qualified Reference Standard (RS): Relative Potency (RP%) as reportable value
  - No formal Product Specification
  - Fit for purpose

Phase Requirements



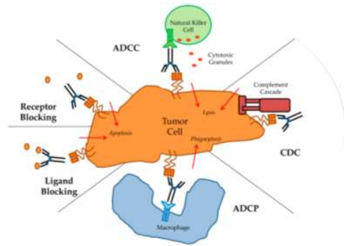


	Early Clinical	Pivotal Trial	Commercial
	• Demonstrate target engagement • Demonstrate Comparability of lots • Critical quality attribute assessment	• Measure relevant Mode of action • Ensure lot-to-lot consistency • Qualify manufacturing process and site(s)	• Maintain method to cGMP • Adjust to increased product understanding and additional indications
Potency Assay	• Binding assay usually sufficient • Qualified Assay	• Fully MoA reflective assay required (often cell based) • Validated Assay	• Periodic review and continuous improvement

Questions?

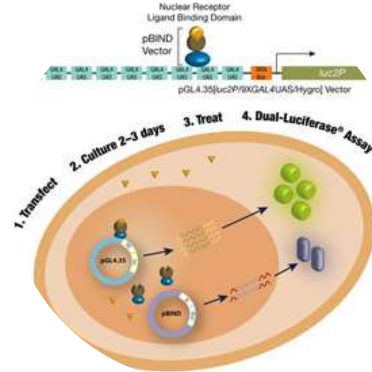
Method Development: MOA-reflective vs Surrogate Assays

Potency Assay Design

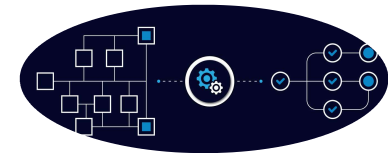


Define Drug MoA on molecular level:

Focus on Action vs Effect



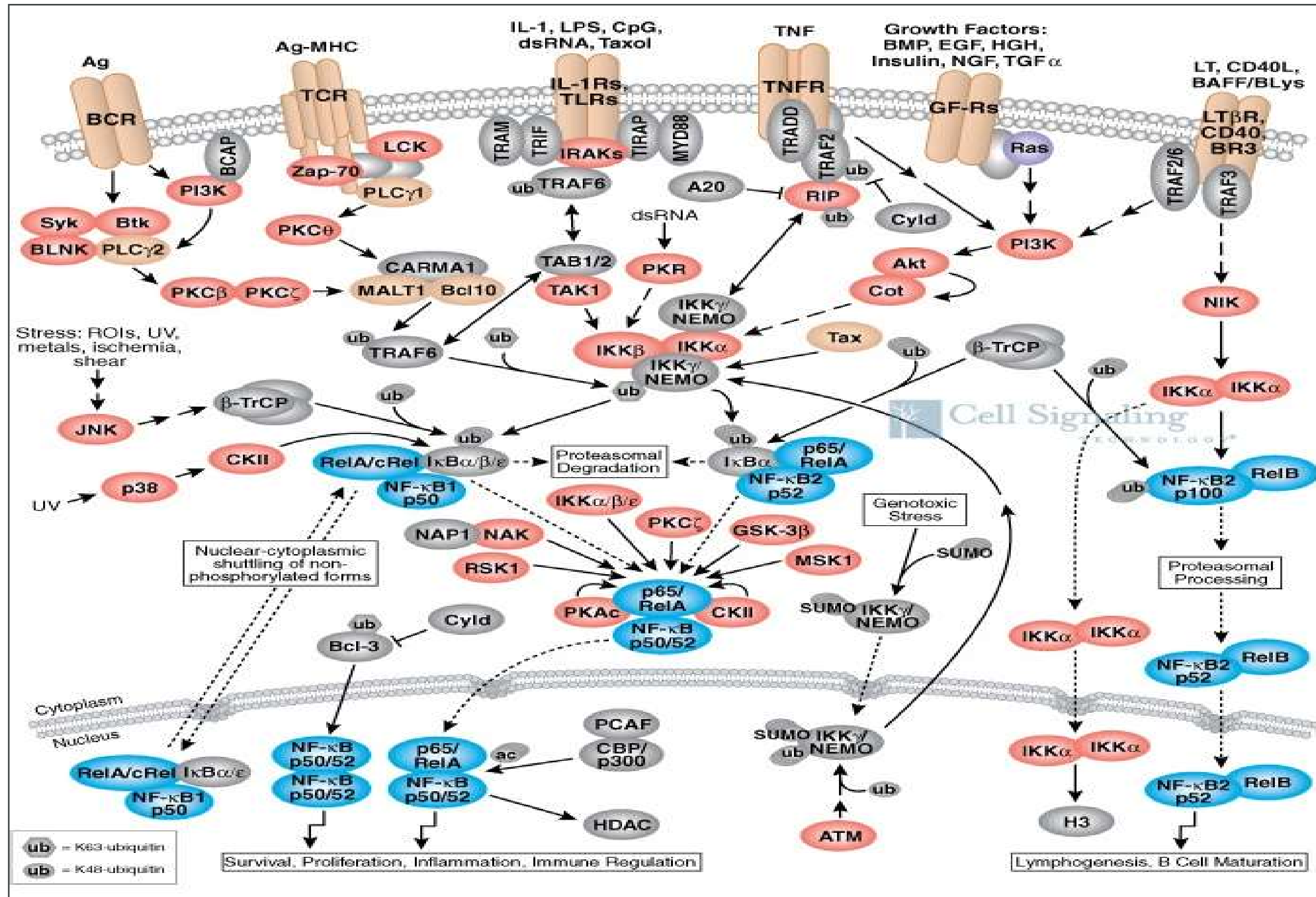
Devise a method to represent this MoA in response to drug and measure with high accuracy and precision



Reduce complexity and maximise control to ensure robust performance in release lab

Cell-based Reporter Assays Using Luciferase Biosensors

Output is light, excellent intrinsic signal/background, kits and plate readers widely available



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Describe principle of MOA

- Mechanism of Action not Clinical Mode of Action
  - Mechanism of Action: specific biochemical interaction through which a drug substance produces its pharmacological effect
  - Mode of Action: functional or anatomical changes, at the cellular level, resulting from the exposure of a living organism to a substance
- Doesn't need to completely recapitulate in vivo effect
- All Potency Assays Are Surrogates, in one way or another
- Example: T Cell Engagers (TCE) don't need to show direct killing in vitro
- Correlation studies may be needed to demonstrate relevance to function
  - Reporter assay as a stand-in for cytokine release

Many products have more than one MOA: primary and secondary. Ideally the primary MOA is represented in Potency Lot Release Assay, others as Characterization

Potency/activity of secondary MoAs may also require CMC control

Degree of testing and control depends on actual or perceived level of contribution to overall potency, efficacy, and/or safety

- Challenging to determine, especially during early development phases
- Usually requires in vivo and/or clinical data
- Health authorities may perceive significance of secondary MoA differently than AZ

Significance of secondary MoA to overall potency and efficacy	
Major	Minor

• Additional lot-release potency assay (or surrogate assay) likely required	• Additional lot-release potency assay (or surrogate assay) likely ***not*** required
	• Characterization bioassay required for:
	  • Manufacturing process comparability
	  • Reference Standard Qualifications
	  • Critical Quality Attribute Assessment

Types of Assays

List of Assay types

Binding

• Recombinant or synthetic targets	ELISA, DELFIA, SPR, BLI, HTRF, AlphaLISA
• Cell-based binding	Competition or indirect (use of 2° Ab) ELISA

Cell-based functional assays

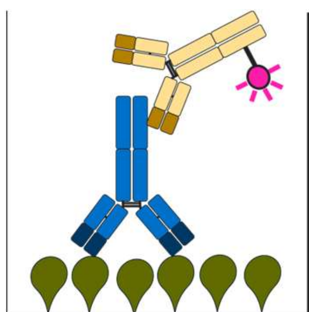
• Cytotoxicity	Fluorescent dyes, luciferase, LDH, MTT
• Cytokine release	ELISA or DELFIA
• Proliferation	MTT, luciferase
• Gene reporter	Luciferase, β-gal

Cell Properties

• Antigen staining	Flow cytometry

MOA/Surrogate Examples

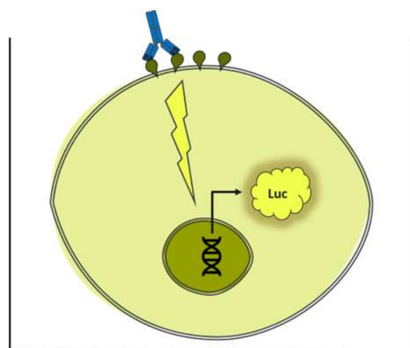
Binding



Generally sufficient for early clinical stages

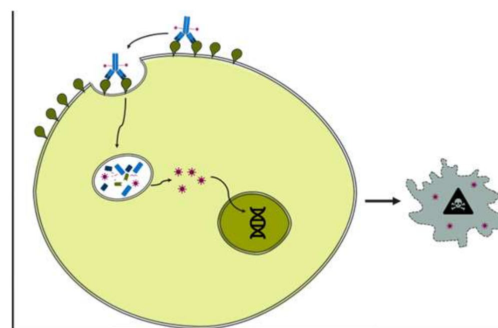
Expectation to have a cell-based assay by pivotal/commercial*

Cell-based Reporter



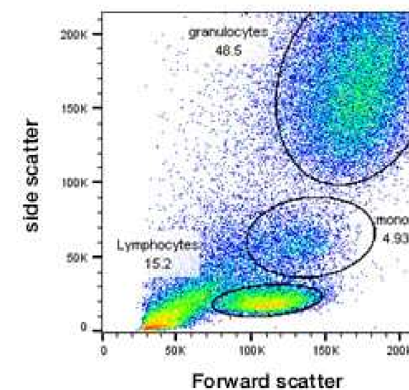
Surrogate for cell signaling assay. Widely accepted, very sensitive, kits available for many common targets

Cytotoxicity



Commonly used for ADC, CAR-T, TCE. Stable expression of antigen on target cells is critical

Flow Cytometry

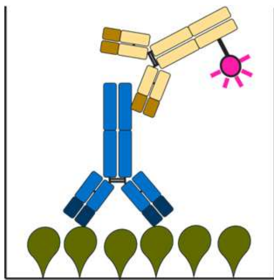


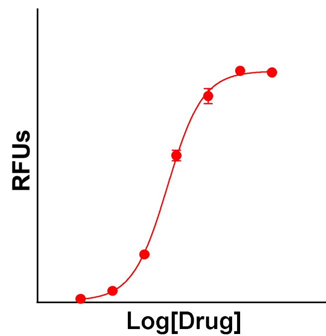
Go-to surrogate method for C>. Does not show function, but acceptable for early clinical stages

Binding Assay Types: Traditional

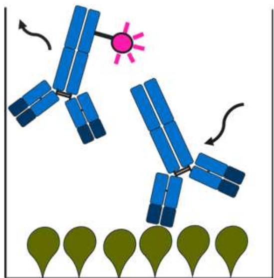
Formats

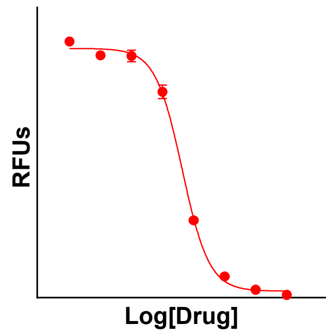
Indirect ELISA/Immunoassay





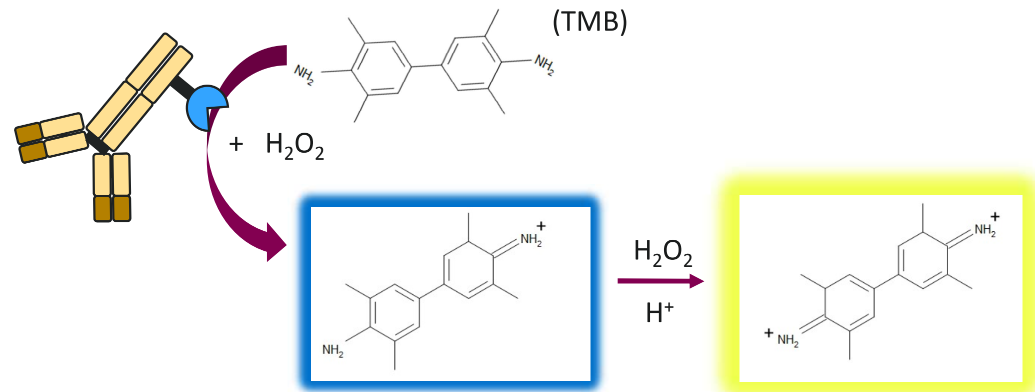
Competitive ELISA/Immunoassay



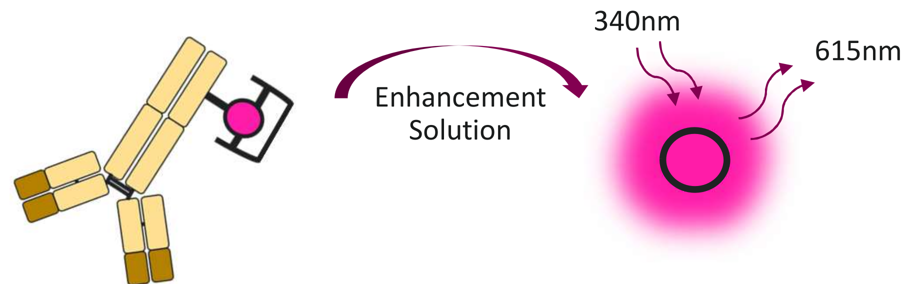


Readout Technologies

Absorbance (Horseradish Peroxidase)



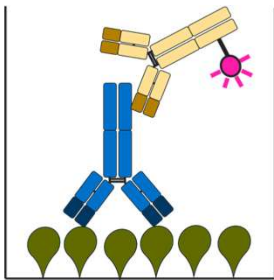
Time Resolved Fluorescence (DELIFIA® Europium Chelate)

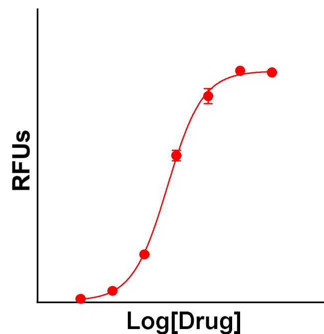


Binding Assay Types: Traditional

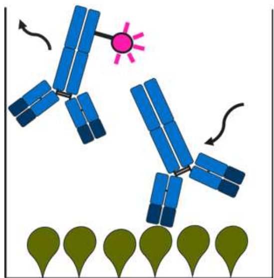
Formats

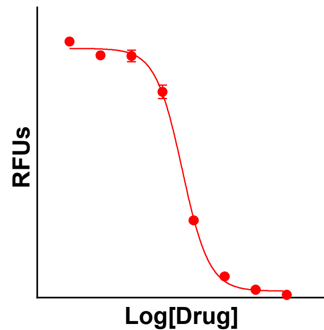
Indirect ELISA/Immunoassay





Competitive ELISA/Immunoassay





Readout Technologies

Absorbance (Horseradish Peroxidase)

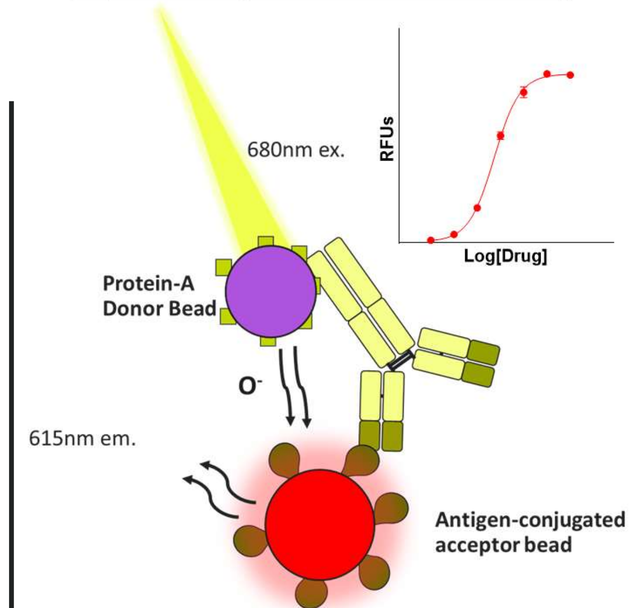
- Pro: Lots of experience across the industry
- Pro: Universally read on most microplate reader instruments
- Con: Lengthy and requires multiple washes and additions
- Con: Enzyme reaction can lead to assay variability

Time Resolved Fluorescence (DELIFIA® Europium Chelate)

- Pro: Robust and stable assay signal
- Pro: Not dependent on enzymatic reaction
- Pro: Quicker to develop and easier to automate
- Con: Not all microplate readers are optimized for TRF reading

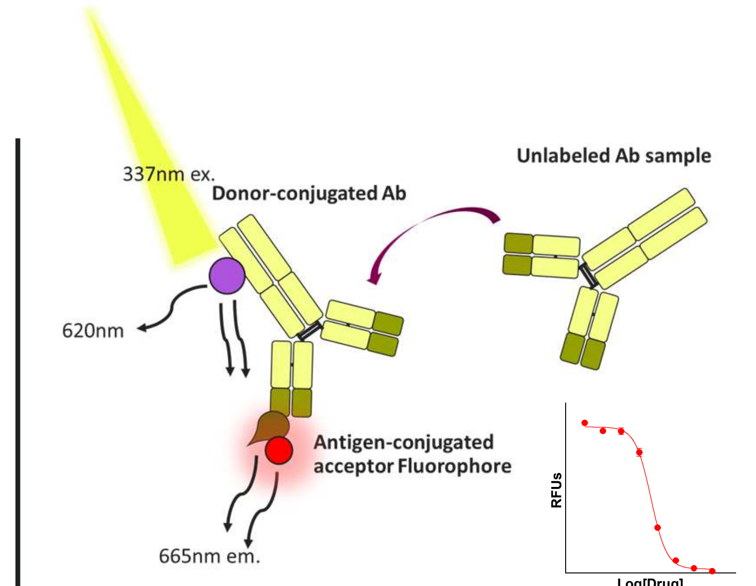
Binding Assay Types: Proximity-based Immunoassays

AlphaLISA (Chemiluminescence)



- Pro: No wash procedure
- Pro: Robust Signal
- Con: Notorious for hook-effect
- Con: Size of beads can induce steric interference
- Con: Beads are highly light-sensitive
- Con: Very limited microplate readers

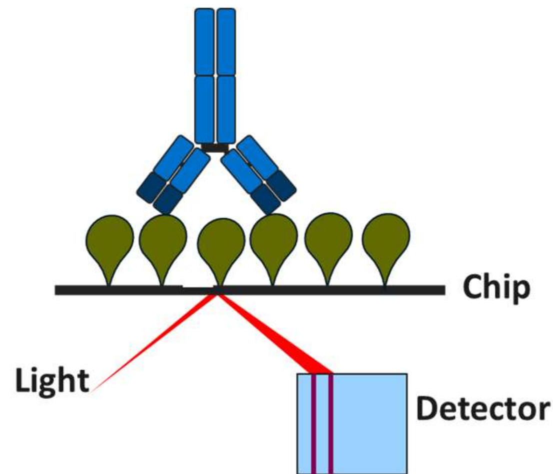
Homogeneous Time Resolved Fluorescence (HTRF / FRET)

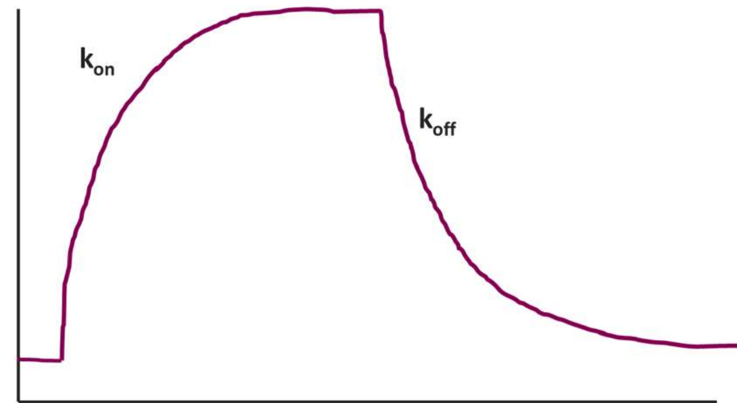


- Pro: No wash procedure
- Pro: Robust and stable signal
- Pro: Fluorophores are small and minimize impact to proteins
- Con: Small assay windows (background)
- Con: Limited microplate readers

Binding Assay Types: Surface Plasmon Resonance (SPR)

Surface Plasmon Resonsance (SPR)

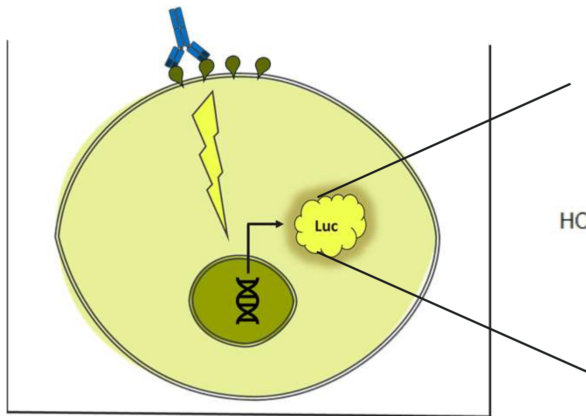


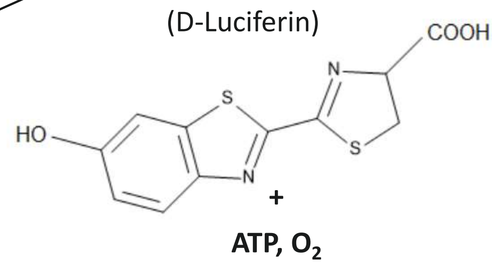


- Pro: Most direct measurement of binding
- Pro: Highly sensitive
- Con: Expense
- Con: Requires specialized training/SME
- Con: Difficulty in separating specific vs. non-specific interactions

Functional Assays Types: Reporter Gene Assays

Luciferase (Luminescence)

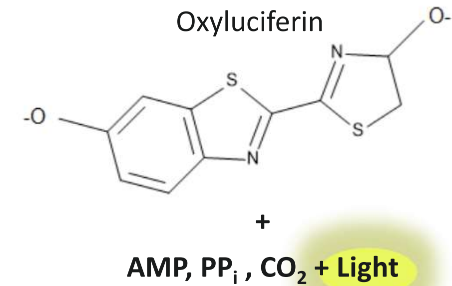




+

ATP, O_2

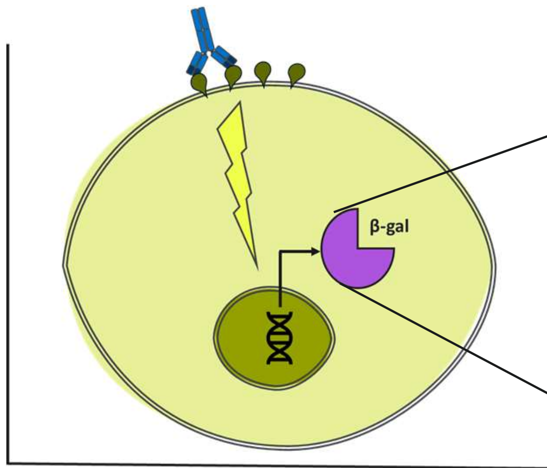
Luciferase

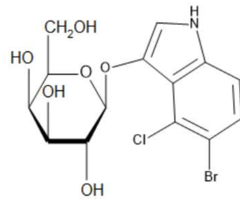


+

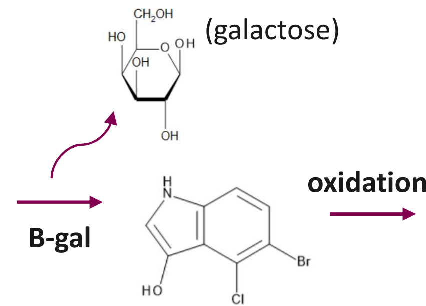
AMP, PP_i, CO_2 + Light

B-galactosidase (Absorbance)



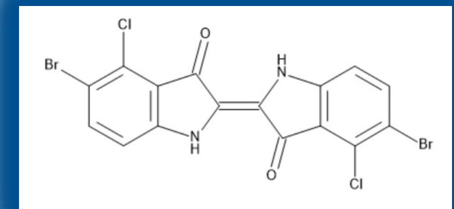


(Xgal)



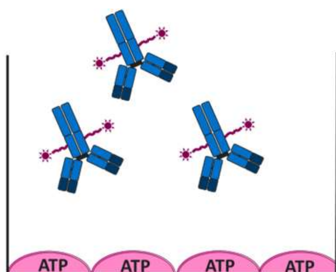
(5-bromo-4-chloro-3-hydroxyindole)

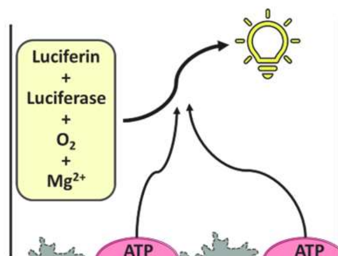
(5,5'-dibromo-4,4'-dichloro-indigo)



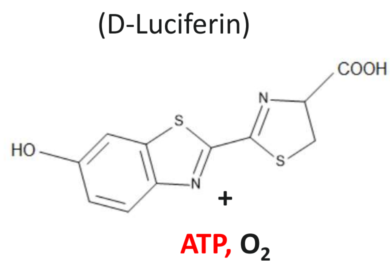
Functional Assays Types: Cytotoxicity/Proliferation

CellTiter Glo (Luminescence)





(D-Luciferin)



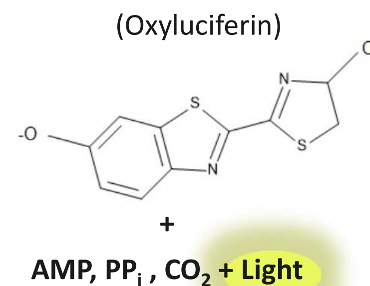
+

ATP, O_2

Mg^{2+}

Luciferase

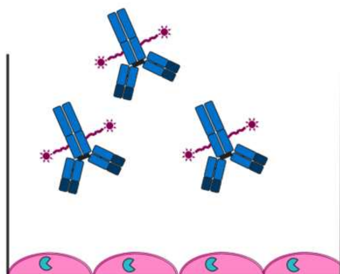
(Oxyluciferin)

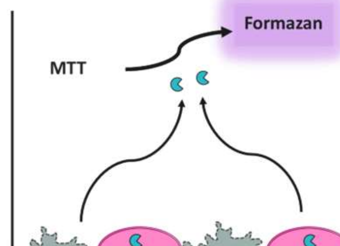


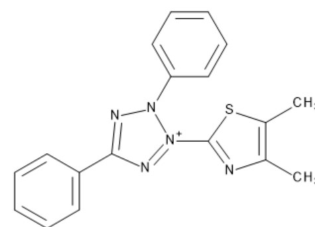
+

AMP, PP_i , CO_2 + Light

MTT (absorbance)



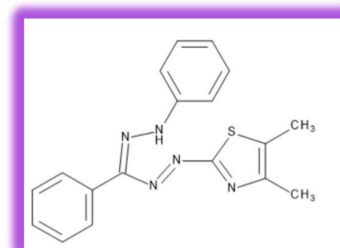




(MTT)

NADH NAD+

Mitochondrial Reductase

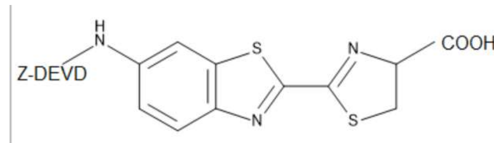


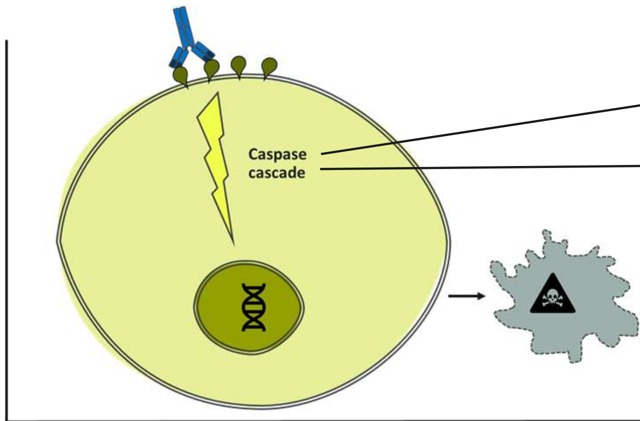
(Formazan)

Functional Assays Types: Cytotoxicity/Proliferation

Caspase 3/7 Glo

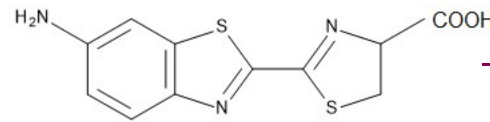
(D-Luciferin)





Caspase 3/7

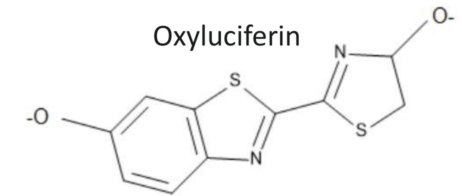
Z-DEVD



(amino-Luciferin)

Luciferase

Oxyluciferin



+

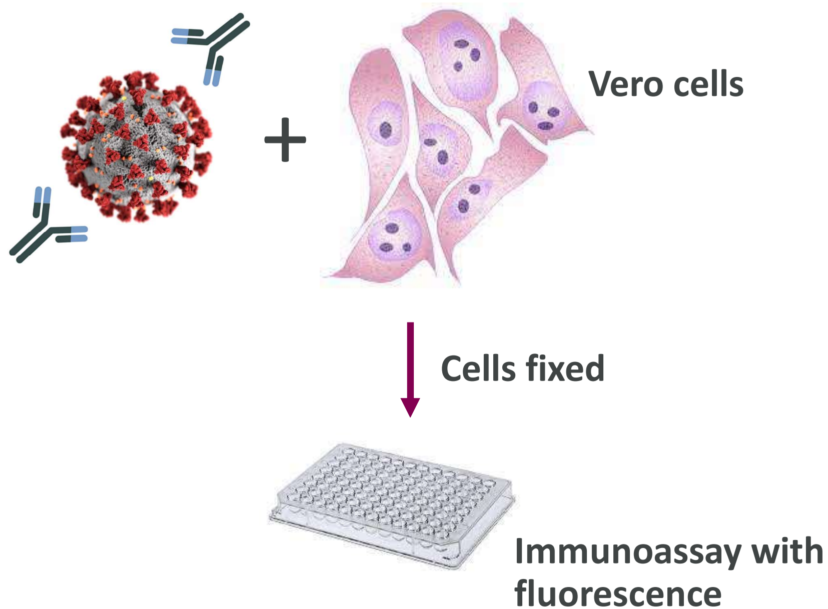
AMP, PP_i , CO_2 + Light

(Formazan)

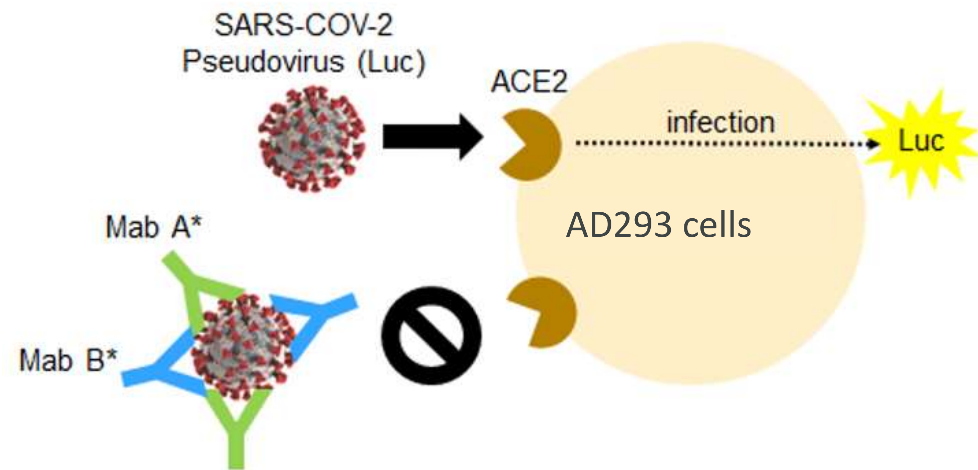
Functional assays specific to infectious disease

Virus Neutralization Assays

Live Virus Killing Assay – BSL3

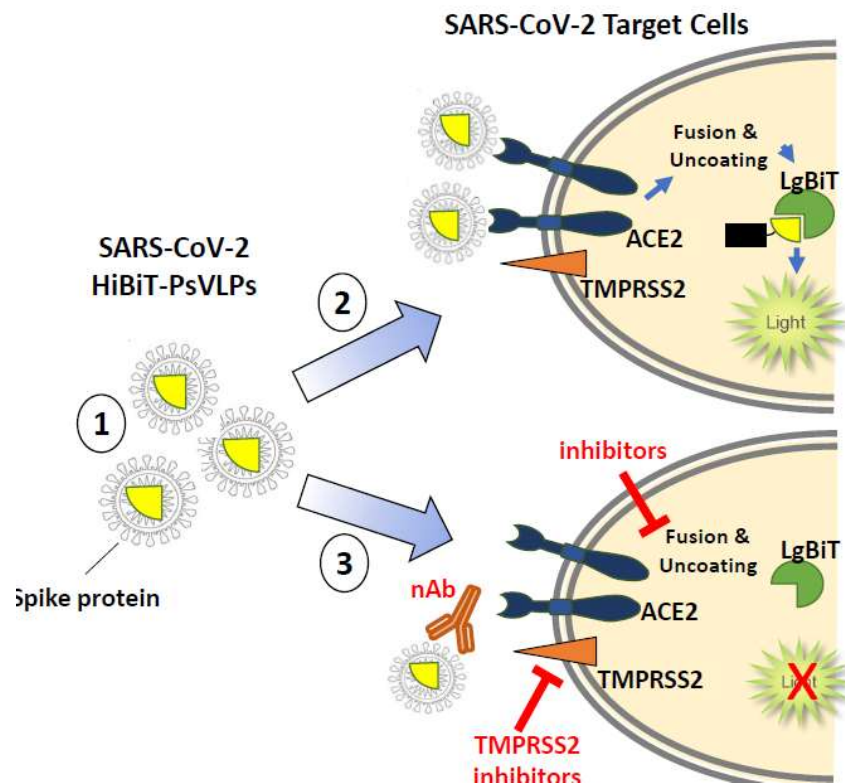


Pseudovirus Neutralization



Functional assays specific to infectious disease

VLP Assays



Assay Design:

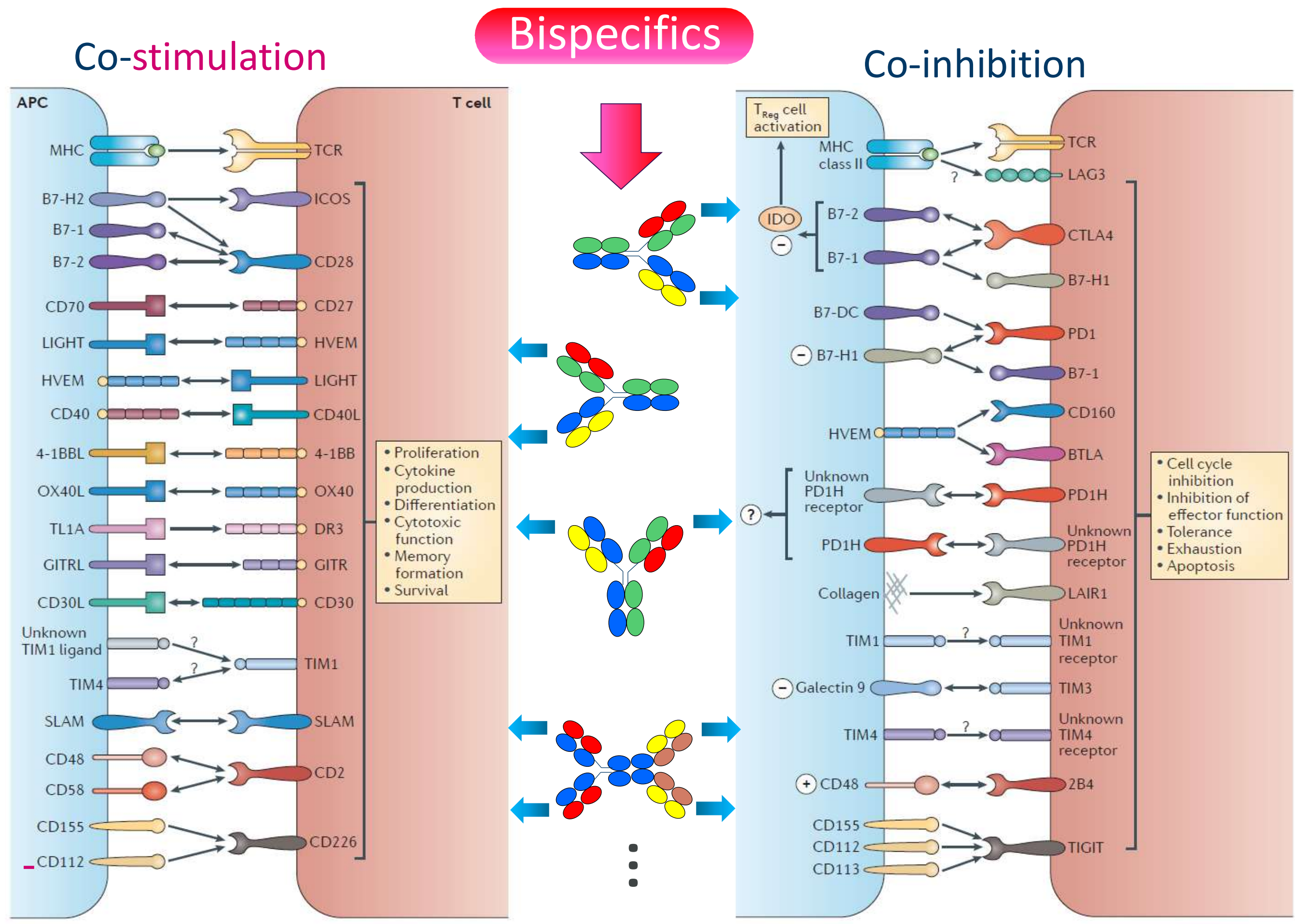
1. HiBiT-tagged VLPs pseudotyped with SARS-CoV-2 Spike protein are added to SARS-CoV-2 Target Cells
   - HiBiT is packaged inside the PsVLPs
2. In the absence of inhibitors or neutralizing antibodies (nAbs), SARS-CoV-2 HiBiT-PsVLPs bind to target cells via Spike/ACE2 interaction and undergo membrane fusion mediated by cellular proteases. HiBiT is released into target cells and binds to LgBiT to generate a luminescent signal in the presence of substrate.
3. In the presence of inhibitors or nAbs of SARS-CoV-2 entry, the entry/fusion processes of PsVLPs are blocked, thereby preventing HiBiT release. No luminescent signal is produced.

https://www.promega.com/applications/infectious-diseases/viral-research/sars-cov2-therapeutic-vaccine-discovery

How do assay types or strategies change with products composed of more complex structures?

Bispecifics

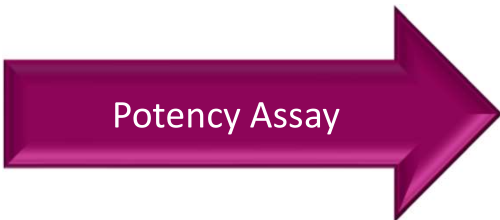
Bispecific Antibodies and Regulation of T Cell Function



Adapted from Nat Rev Immunol. 13(4):227-42 (2013)

APC = Antigen presenting cell

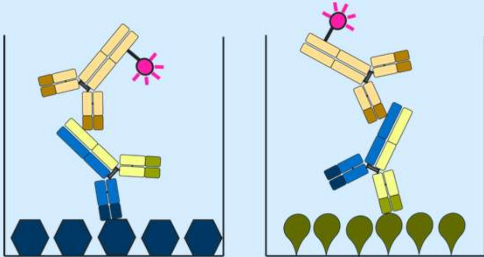
Mode of Action Categories of Multi-Specific Drugs



Synergy	Additive	Complex
Targeting moiety with active moiety	Two parallel activities resulting in novel approach	Active moiety with inhibiting moiety
Co-expression targeting	Combining two drugs reducing cost of goods	Delivery of several active components
Measures dependency on all specificities	Independent tests appropriate	Reflects complexity

Potential binding assay strategies for bispecifics

Separate Antigen Binding Assays



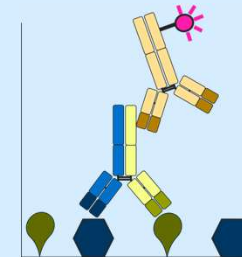
Pros:

- Leverage traditional methods
- Measure individual affinities

Cons:

- Juggle multiple GMP assays and specifications (x2 for mAb int. *and* ADC)
- Does not capture avidity
    - Overly sensitive to changes?

Combined Binding Assay



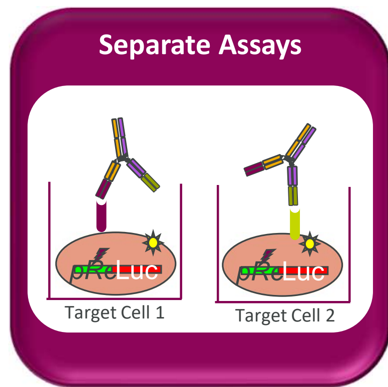
Pros:

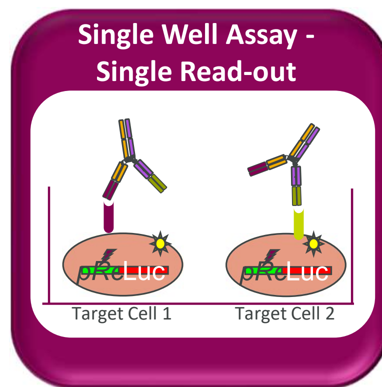
- Single GMP assay and binding specification
- Captures avidity

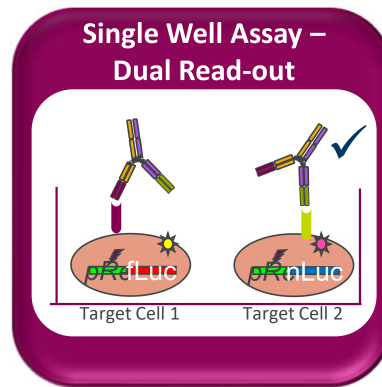
Cons:

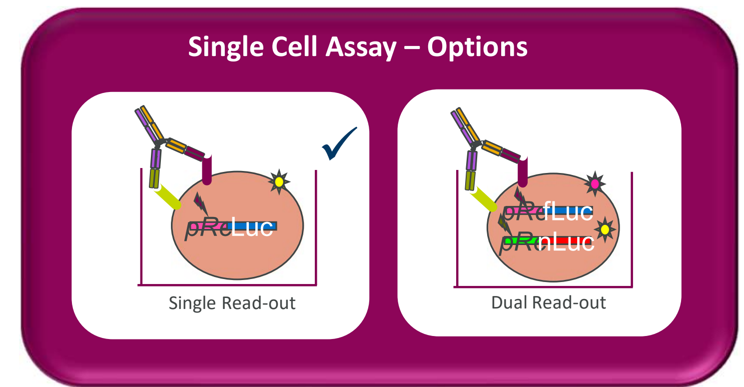
- Complex method development
- Cannot separate individual binding affinities
- May require separate assays for characterization

Potential strategies for bispecific reporter gene assay









Pro:
- Easy to develop
- Sensitive to individual changes

Con:
- Two assays to develop
- Two assays to administer
- Often not MoA reflective (e.g. synergy?)

Pro:
- Relatively easy to develop
- Only one assay (QC✓)

Con:
- Less sensitive to individual changes
- Which specificity is impacted?
- Often not MoA reflective (e.g. synergy?)

Pro:
- Relatively easy to develop
- Sensitive to single change
- Only one assay (QC✓)
- Same assay for characterisation

Con:
- More cloning required
- Often not MoA reflective (e.g. synergy?)

Pro:
- Fast and easy to run
- Only one cell line (QC ✓)
- Likely MoA reflective

Con:
- Hard to develop and clone
- Less sensitive to individual changes
- Which specificity is impacted?
- Characterisation assays

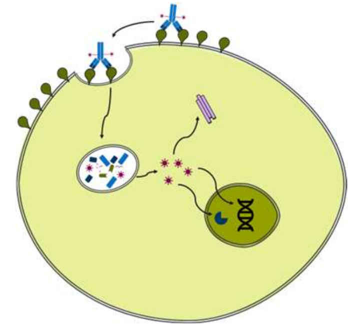
Pro:
- Sensitive to individual changes
- Only one assay and cell line (QC ✓)
- Same characterisation assay
- Likely MoA reflective

Con:
- Hard to develop
- Only different pathways
- Lots of cloning for cell line generation

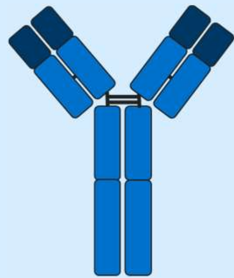
How do assay types or strategies change with products composed of intermediates?

Antibody Drug Conjugates

Standard GMP potency assays for ADCs



mAb intermediate

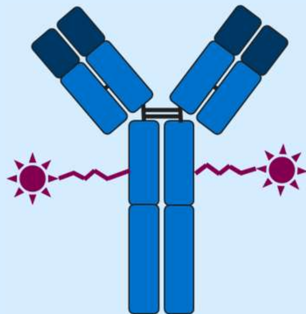


1) Target-antigen binding

- Cell or non-cell based
- Ensures potency before conjugation
- Common methods: ELISA
- Common readouts: Fluorescence or colorimetric

ADC

(DS and DP)



2) Target-antigen binding

- Identical assay as mAb intermediate
- Ensures conjugation does not impact target binding
- Goal to remove from commercial specification

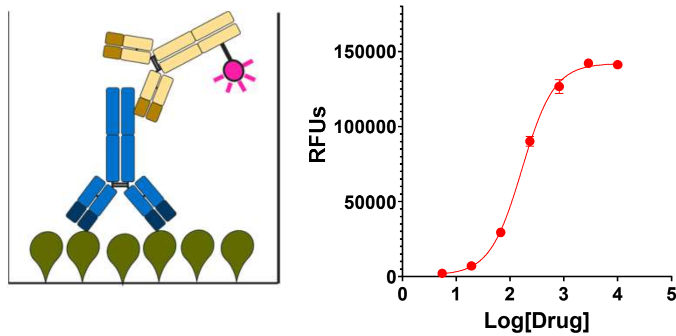
3) Cytotoxicity assay

- Cell-based. Does not need to be from same tissue as indication
- Common endpoints: ATP production, membrane integrity, caspase activity
- Common readout: Luminescence or colorimetric

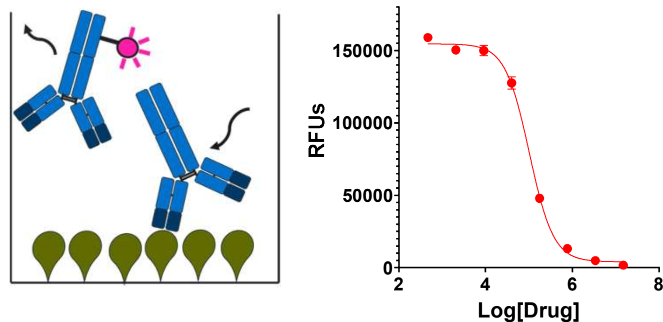
Traditional methods for ADC lot-release potency testing

Binding

Indirect ELISA/Immunoassay

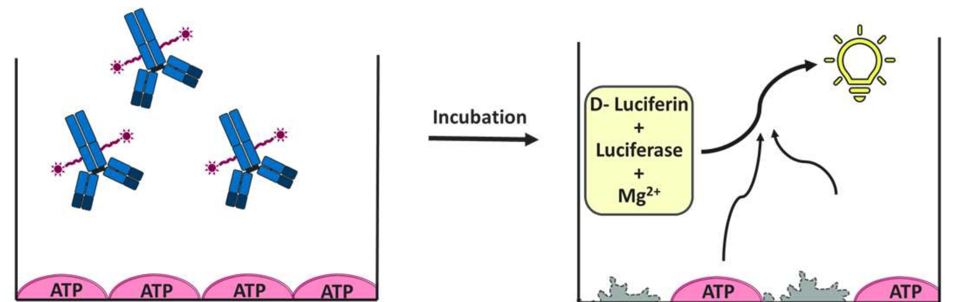


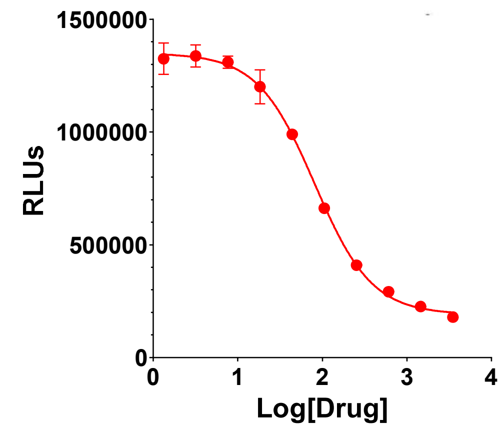
Competitive ELISA/Immunoassay



Cytotoxicity

CellTiter Glo





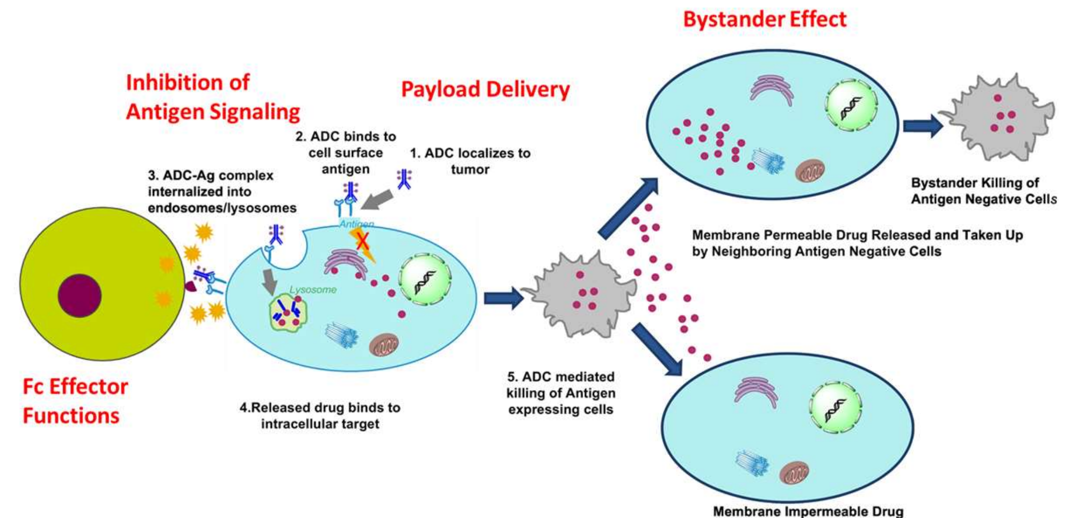
Potential secondary MoAs of ADCs

Bystander effect
- CMC characterization and control not expected by Health Authorities

Inhibition of Antigen Signaling
- CMC characterization may be expected by Health Authorities
- Control strategy, if needed:
  - Demonstrate that lot-release binding and cytotoxicity assays inherently control for this activity

Fc effector functions (e.g. ADCC, CDC, ADCP)
- CMC characterization and control expected by health authorities
- Strategies for control, if needed:
  - Implement characterization bioassays
  - Identify bioassays or surrogate assays that could be used for lot-release, if requested by health authorities

Potential secondary MoAs of ADCs



Early characterization is critical to make decisions whether an ADC has the desired quality attributes

CAR-T cell manufacturing process (example)

Apheresis
Wash/fill
CRYO
T isolation Activation
Lentivirus
Recovery Harvest
Form/Fill
CRYO

Cryo CAR-T

Drug Product

QC test

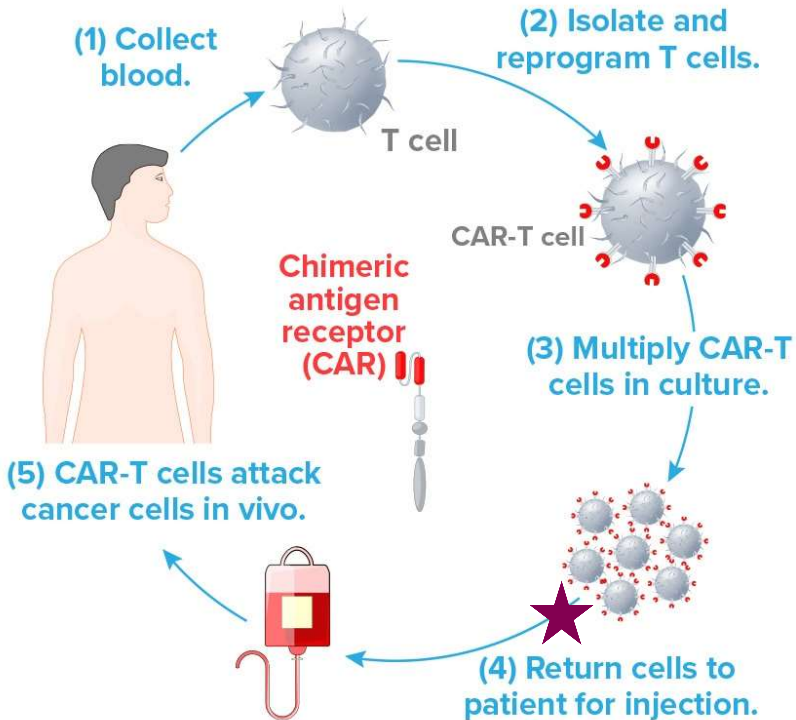
FLOW CYTOMETRY

Current Vein to Vein Time:
3- 4 weeks

Infusion

QA release

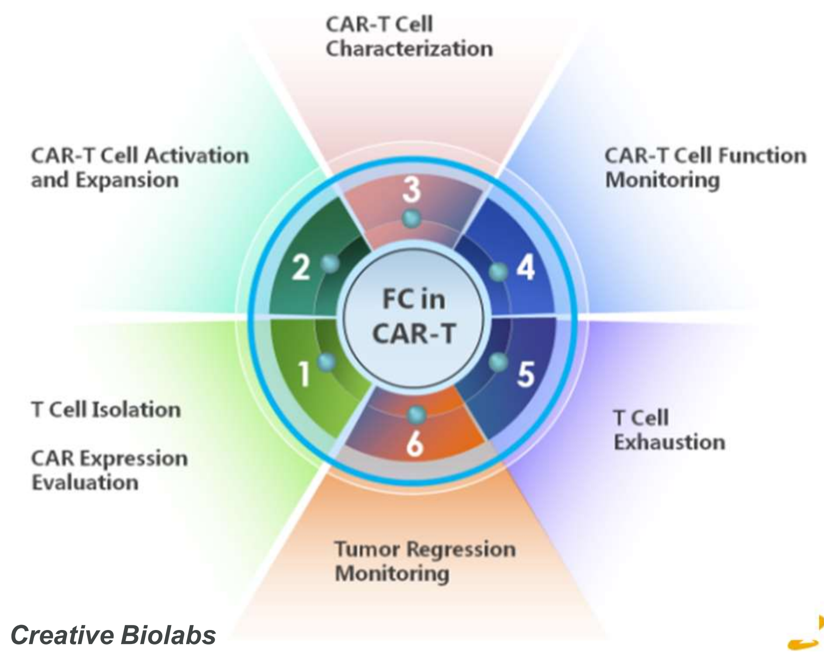
Flow cytometry is an essential technique for lot release and characterization of cell therapy products



BioProcess International

- Identity
- Purity
- Potency
- T cell characterization (in-process & final DP)

Miltenyi MACSQuant



Creative Biolabs

Flow Cytometry

Percent of positive population (e.g. CAR-T+) are quantified and reported directly, or calculated as %RP against an RS

A

Stained Sample

Sheath Fluid

Hydrodynamic Focusing

Laser

To waste

Mirrors Filters

Detectors

Data Aquisition and Analysis

Electronics System

B

Detector

Side Scatter (SSC)

Laser

Forward Scatter (FSC)

Detector

C

Granulocytes

Monocytes

Lymphocytes

SSC-A

FSC-A

From: Leukemia

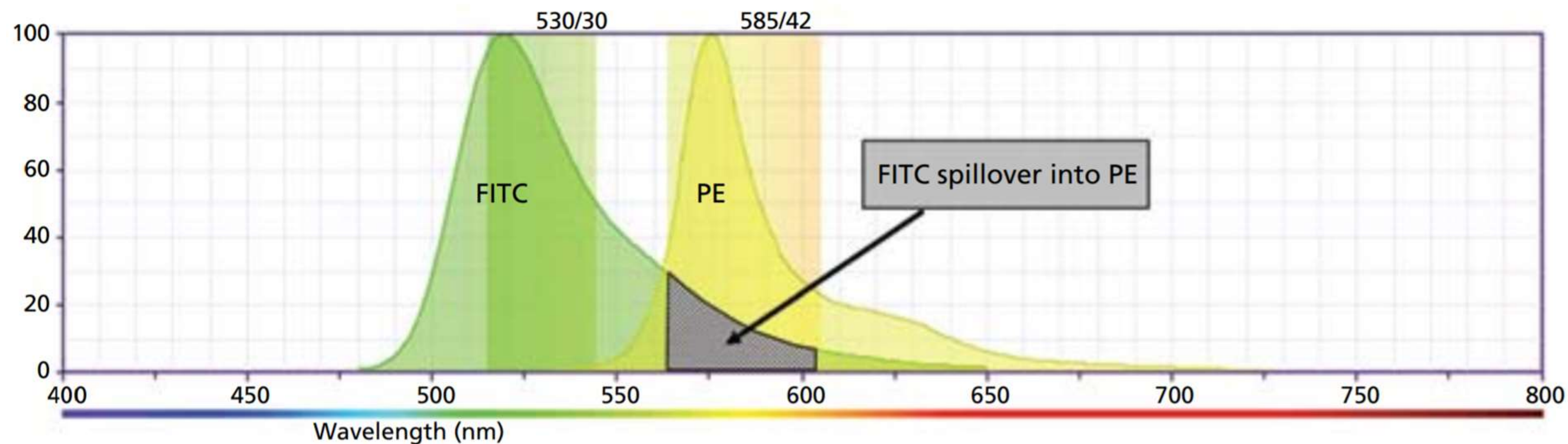
Li W, editor, **Brisbane, 2022**

Chapter 4 Flow Cytometry in the Diagnosis of Leukemias

Weijie Li

In multicolor flow cytometry, when >1 marker is expressed on a single cell Spectral Spillover may occur

- Spillover is when the fluorescence emission of one fluorochrome is detected in a detector (channel) being used to measure signal from another fluorochrome



- Different fluorescent reagents can contribute significant optical background in proportion to their brightness – brighter fluorochromes may cause more spillover

Questions?

Break

Method Lifecycle

Method Lifecycle

- Initial CMC Method development
- Method "lock" followed by pre-qualification, tech transfer to clinical QC lab (GMP)
- Qualification or co-qualification
- Set Ph I specifications
- Release and stability testing, trending
- Tech support for QC lab, address any robustness issues
- Method robustness study
- Verify lack of impact for DS and/or DP process changes, formulation etc
  - e.g. high concentration formulation
- Tech transfer to commercial GMP lab
- Validation
- On market support

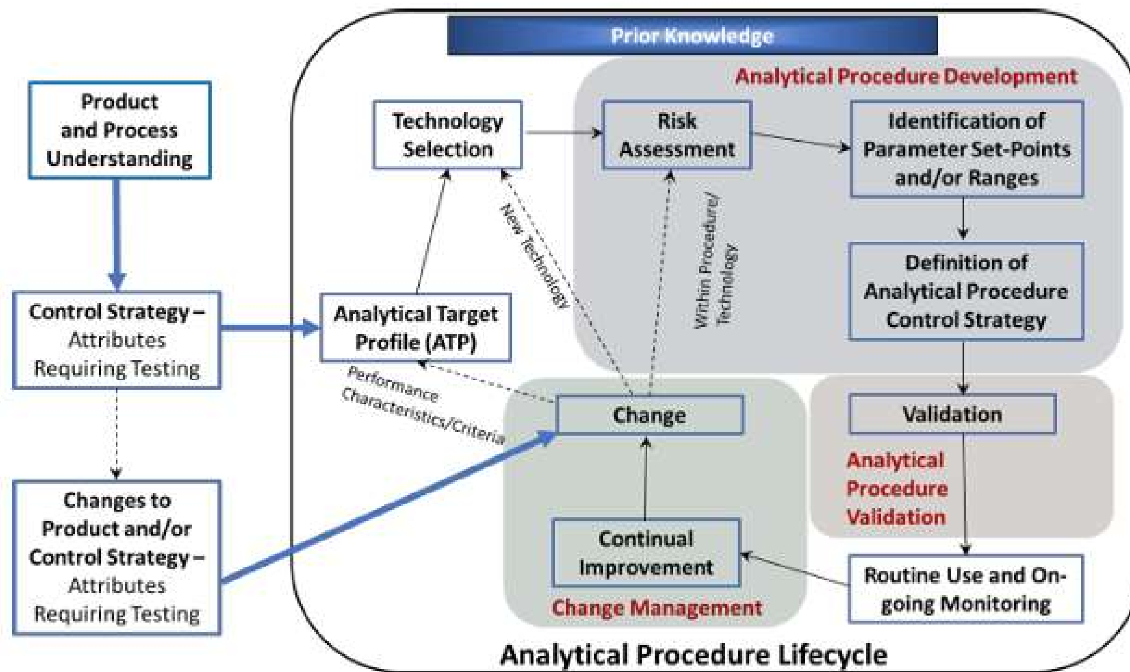
Method Lifecycle—common issues

- “Potency-like” assays may be developed and deployed in Discovery phase
  - May be good starting point for potency or characterization assays
  - Different purposes, less stringent control in Discovery
  - Greater use of primary cells
- Method for Ph I filing needs to have some relationship to MOA but can be simplified
  - Target binding is frequently sufficient
  - Main purpose is to show lot-to-lot consistency and stability of drug
-

Method Development

Analytical Methods Require Updates thought the drug development lifecycle

Figure 1: The analytical procedure lifecycle



Also see:

USP <1032>Design and Development of Biological Assays

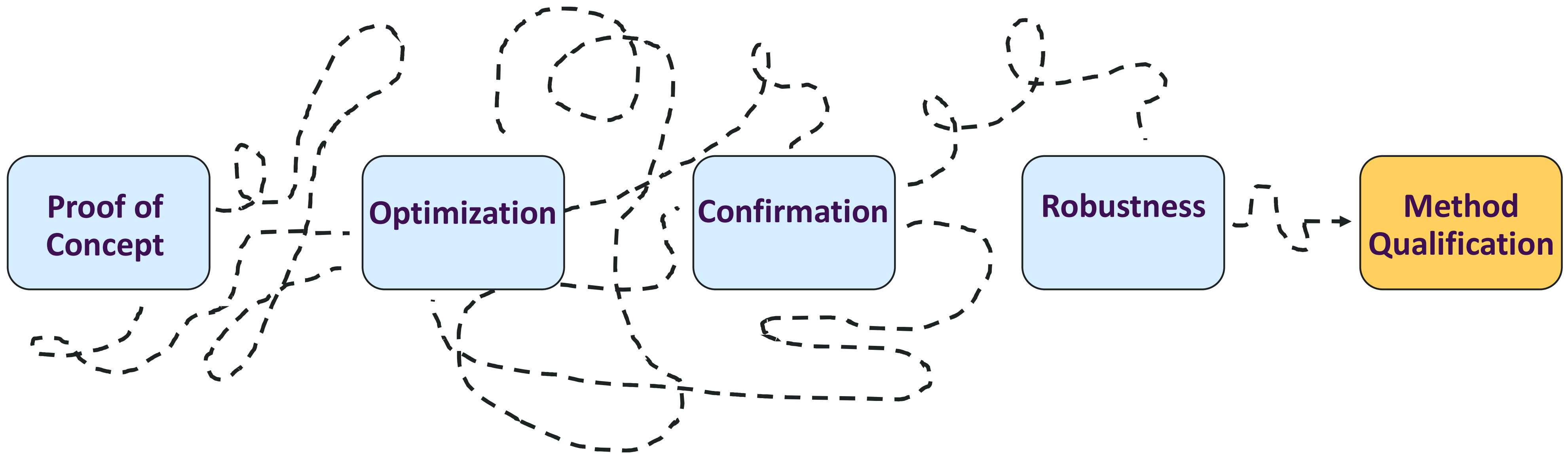
and

USP<1034> Analysis of Biological Assays

ICH Q14, 01 Nov 2023

Ideal road to developing a lot-release potency method

Reality to developing a lot-release potency method



Common challenges to bioassay development

Reagents and consumables
- Lot-lot consistency
- Availability
- Comparable vendors
- Navigating IP

Cells
- Biological variability
- Consistency across passages
- Optimal culturing procedures
- Navigating IP

Assay variables
- Temperature
- Time
- pH
- Light

Analyst and Lab variability
- Instruments
- Technique
- Experience

Basic Tools

- Analyst Training
- Documentation
- Equipment
- Critical Reagents
- Reference Standards
- Assay Formats
- Cells

All are sources of variability

Method Development "Basics"

- Screening initial conditions does not require full dose response curves: typically high, medium, low (negative) drug concentrations are sufficient to establish optimal signal to noise (dynamic range)
- Ideal 4 PL dose response curve has well-anchored upper and lower asymptotes, 2 points on each
- 4 PL curve is intrinsically symmetric, clustering of points near the center of the dynamic range is not necessary
- Uneven dilution steps are often one of the most powerful tools!
  - Wide dilution steps at top and bottom anchor asymptotes, smaller dilution steps near the center provide points in the dynamic range
- Slope (B) is typically the most difficult parameter to adjust, it is largely an intrinsic property of the biochemical mechanism

Selection of Cells for CBA

- Cell line stability is one of the most important considerations. The method needs to be robust, consistent between labs (including GMP) and over time (product stability studies run for ≥ 2years)
- Generally transformed or immortalized cells are selected, primary tissues should be avoided whenever possible. Carefully document cell line engineering for transfer in Commercial operations
- Safety considerations
  - Commonly used human and animal cell lines (HEK293, HeLa, CHO) are "grandfathered", known not to constitutively generate infectious agents. BSL-2
- Banking
  - Labs generally take a tiered approach
  - Master Cell Bank (MCB) → Working Cell Bank (WCB) → Assay Ready Cell Bank (ARCB, if appropriate)
- Licenses may not be needed for clinical stage but typically required for Commercial—check with vendor!

Potency assay development

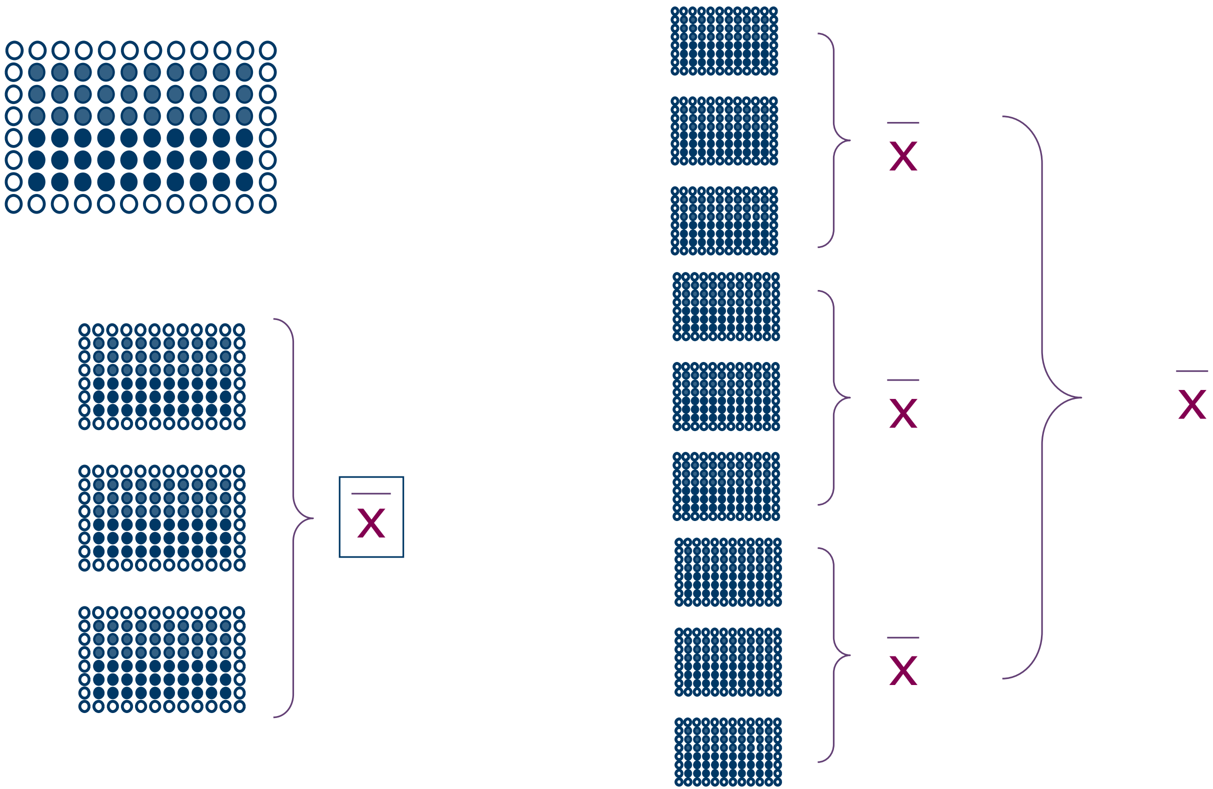
Can be performed with JMP

Experiments	Data analysis
Screening reagents and assay conditions	Screening DoE
Establish PoC dose-response curve	Curve fit analysis
Establish plate layout	Plate uniformity and bias
Lock plate layout and experimental conditions	Robustness DoE
Assay validation	<USP 1033>

- Accuracy
- Linearity
- Precision
- Range
- Specificity
- Stability - indicating potential

Assay Characteristics or Parameters – Plate Replicates

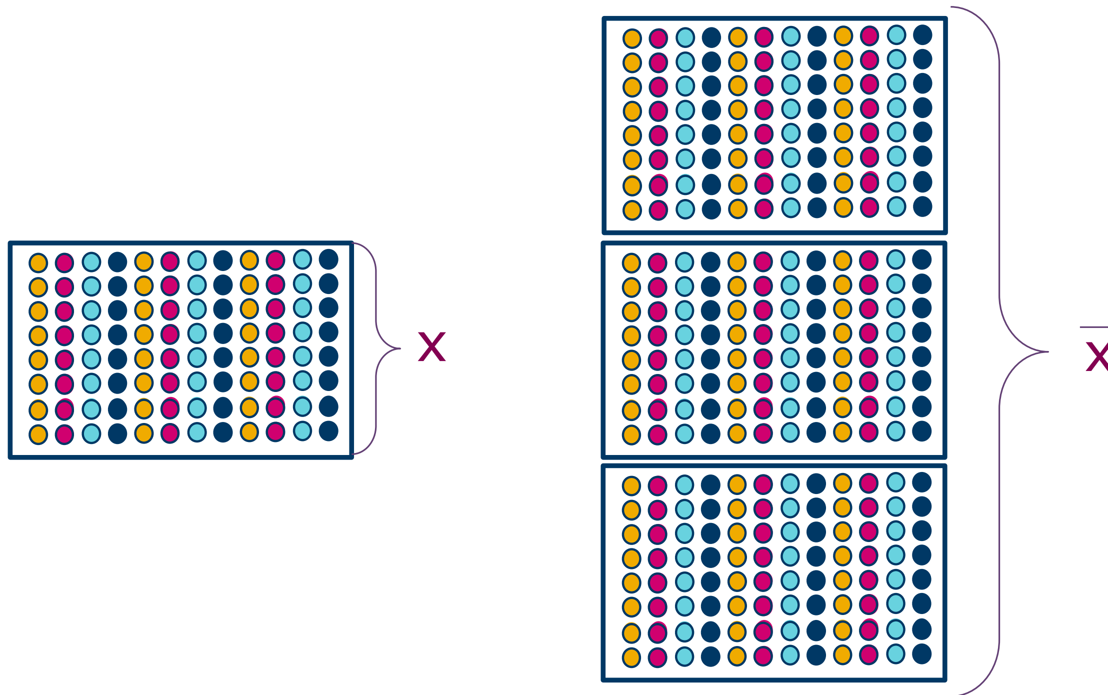
One Plate, Three Plates or Nine Plates?



Assay Characteristics or Parameters – Plate Replicates

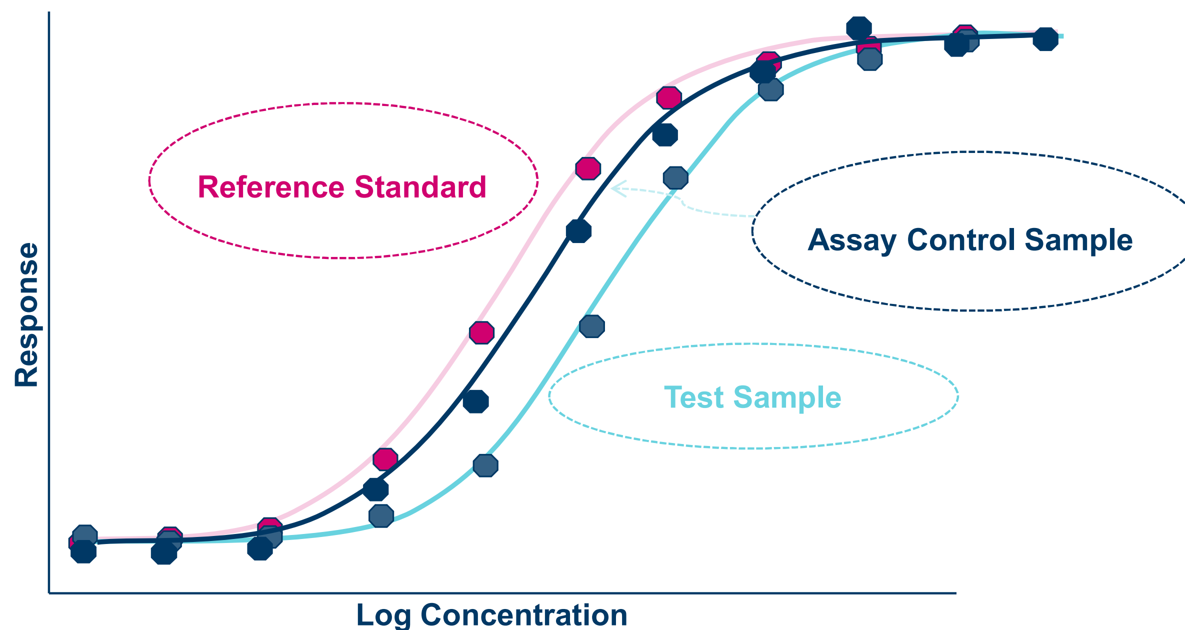
Or, if you've optimized the assay for all 96 wells,

One Plate or Three Plates?



Criterion of similarity of dose-response curves

Note: "Linearity" of the method does not refer to the primary analysis of the raw data, which is frequently non-linear by nature, Instead, it refers to the analysis of an adjusted value, commonly



Potency Value is Typically

"Percent Relative Potency" (RP%) Calculated as the ratio of EC50s of a 4 PL fit:

Sample/RS X 100

Parallelism of assay control sample & reference standard
+ ACS potency value → essential AAC

Parallelism of test sample & reference standard → essential SAC

Slide borrowed and adapted from Jane Robinson

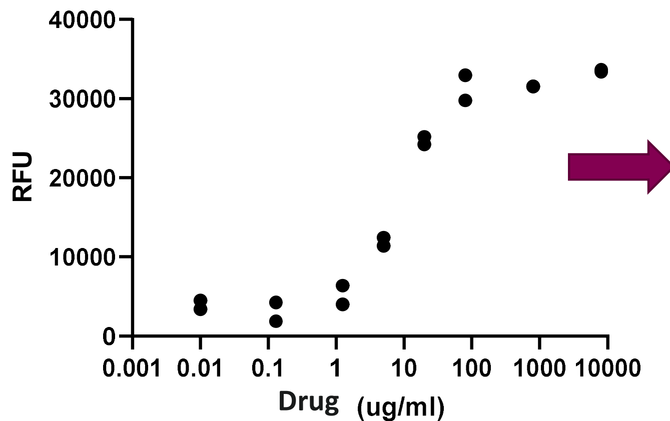
66

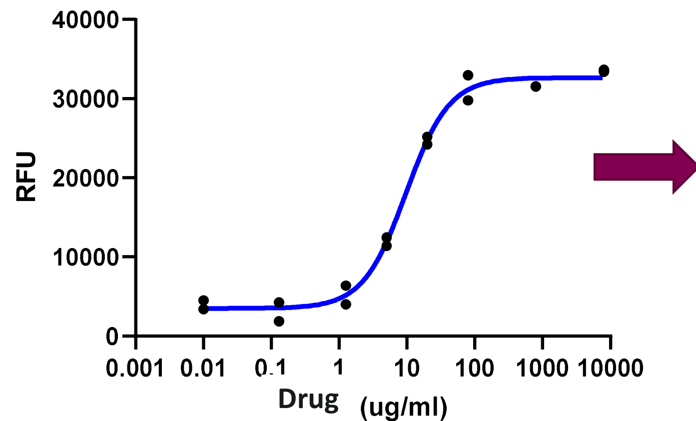
Relative comparisons (biological activity assays)

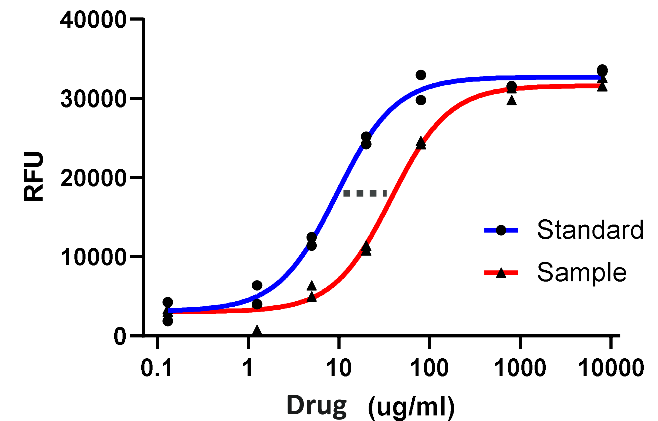
Measuring response

Fitting dose-response

Comparing standard and tested sample fits







- Assessing quality of the unconstrained standard and sample fits
- Dose response curves must be similar to each other to allow meaningful comparison (parallelism)
- Readout: horizontal shift along the log(dose) axis of constrained curves

Non-liner Curve Selection

4 Parameter Logistic Equation

y = ((A - D) / (1+ ((x/C)^B))) + D

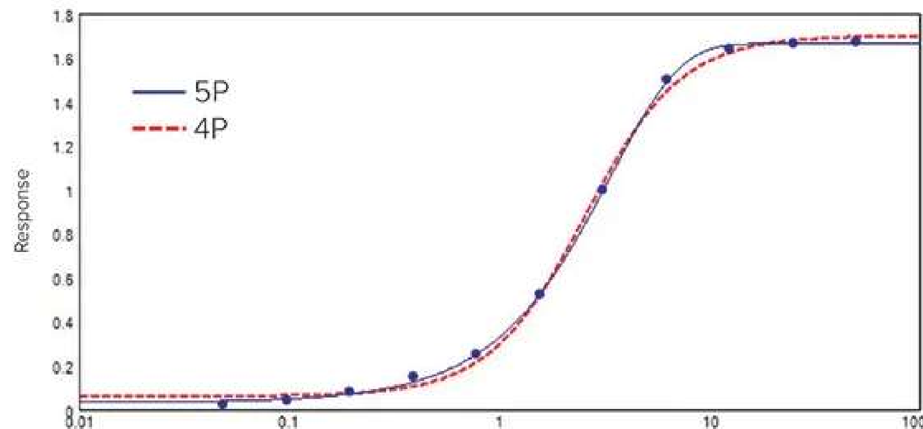


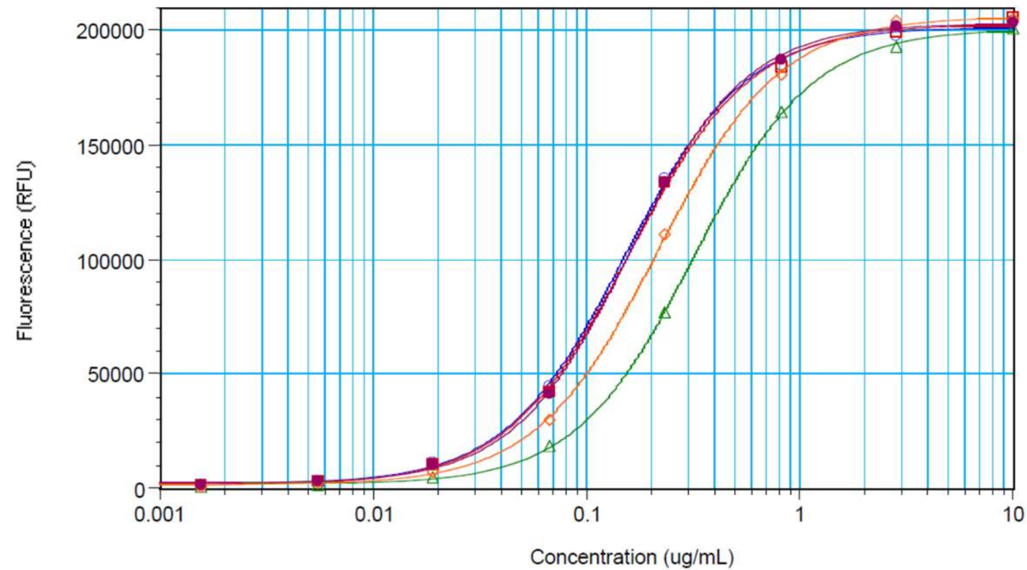
Figure 2. Concentration-response curve fitted with the 4P and the 5P curve fit models for comparison. Although the 4P model gives a smooth symmetrical curve, data are clearly asymmetrical. Therefore, the 5P model gives a better fit.

Molecular Devices: https://www.moleculardevices.com/en/assets/app-note/br/selecting-best-curve-fit-in-softmax-pro-7-software

5 Parameter Logistic Equation addresses *consistent* asymmetry, but care should taken before selecting this option, as parallelism becomes much more challenging to demonstrate

Example of Curve Development
8-point curve, gives 2 or 3 points in dynamic range

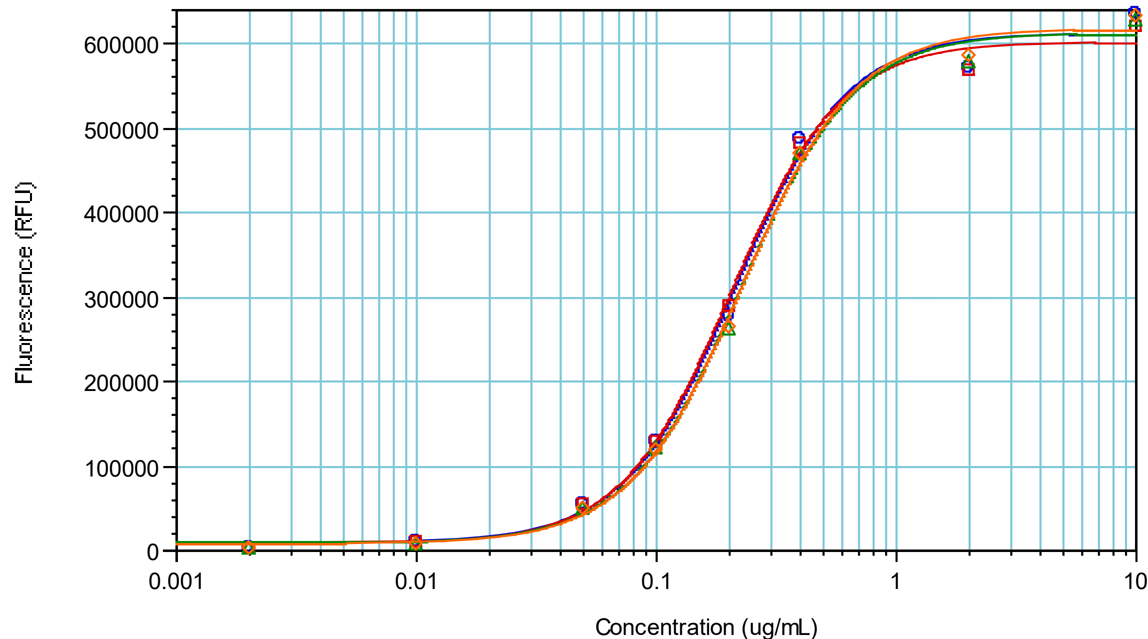
- Would more points help?



<u>All 8 points</u> contribute to final potency value via 4 PL fit, the 4 PL equation does <u>not</u> weight points in the dynamic range

Adjusted Dilution Series

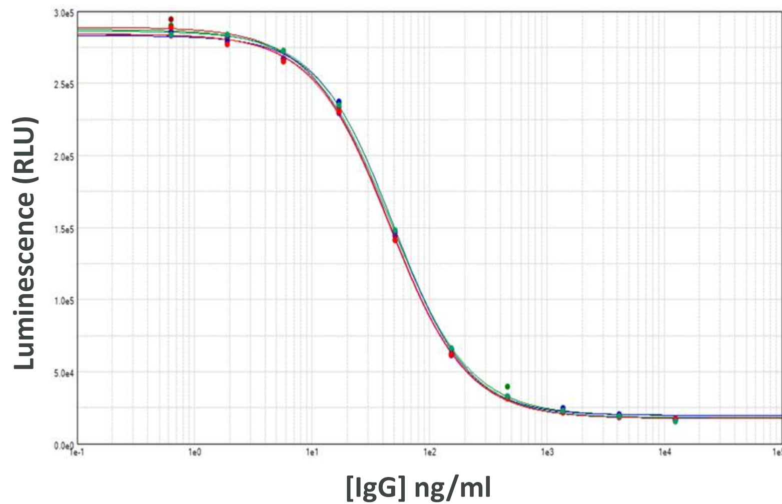
Once maximum and minimum concentrations are established, distribution of points between can be modeled. Even (uniform) dilution intervals are appealing from a pipetting standpoint, but may be less robust with regard to linearity.



- Example of Non-uniform dilution series
- (2x 1:5, 3x 1:2, 2x 1:5)

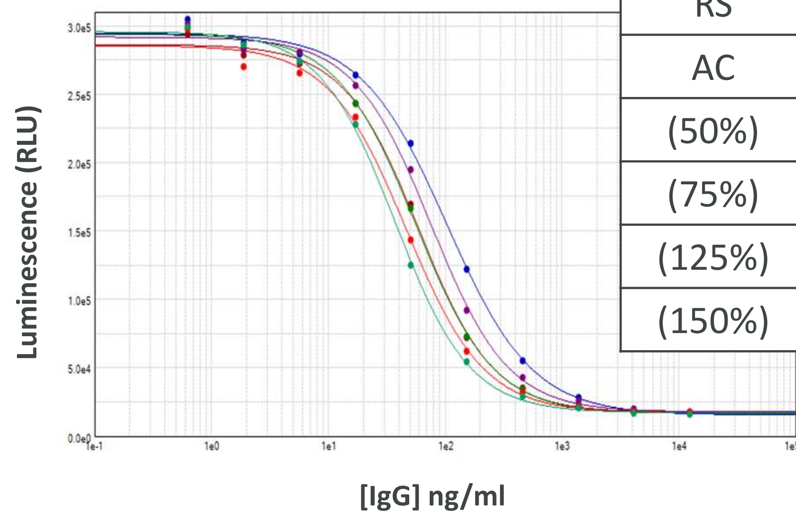
Example of locked method: Repeatability and Linearity

Repeatability



Average accuracy = 95%

Linearity

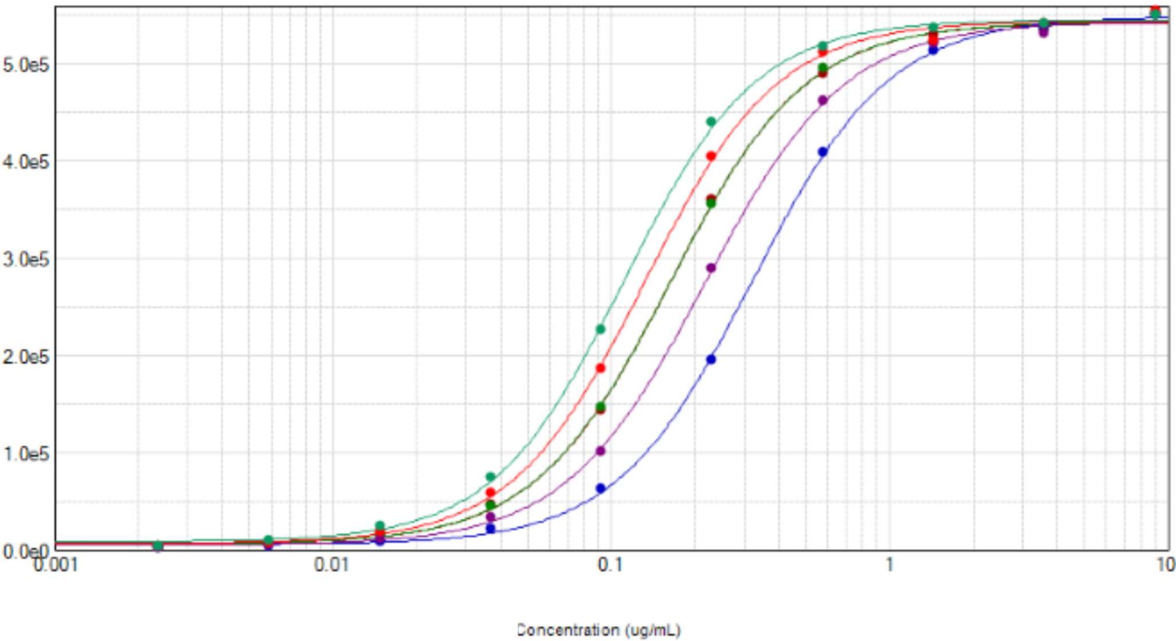


sample	% RP	% accuracy
RS	NA	NA
AC	101	101
(50%)	54	108
(75%)	74	99
(125%)	128	102
(150%)	147	98

Average accuracy = 102%

Assay system produces very accurate results with little optimization required.

Horizontal Plate layout



- 2-3 Plates/assay
- Well-anchored asymptotes and 4+ points in dynamic range
- High accuracy

<table>
<tr><th>LATE 1</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
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<tr><td></td><td></td><td colspan="10">RS Start at 9 ug/ml, 1:2.5 dilution series (160 into 240)</td><td></td></tr>
<tr><td></td><td></td><td colspan="10">CNTRL</td><td></td></tr>
<tr><td></td><td></td><td colspan="10">S1</td><td></td></tr>
<tr><td></td><td></td><td colspan="10">S2</td><td></td></tr>
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<table>
<tr><th>LATE 2</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td></td><td colspan="10">S2</td><td></td></tr>
<tr><td>C</td><td></td><td colspan="10">S3</td><td></td></tr>
<tr><td>D</td><td></td><td colspan="10">S4</td><td></td></tr>
<tr><td>E</td><td></td><td colspan="10">RS Start at 9 ug/ml, 1:2.5 dilution series (160 into 240)</td><td></td></tr>
<tr><td>F</td><td></td><td colspan="10">CNTRL</td><td></td></tr>
<tr><td>G</td><td></td><td colspan="10">S1</td><td></td></tr>
<tr><td>H</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Good for longer assays (≥ 48 hours) where outer wells can give lower signal

Example Vertical Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D	RS	AC	S#1	S#2	S#3	S#4	RS	AC	S#1	S#2	S#3	S#4
E												
F												
G												
H												

- 1 Plate/assay
- Well-anchored asymptotes and 2+ points in dynamic range
- High accuracy

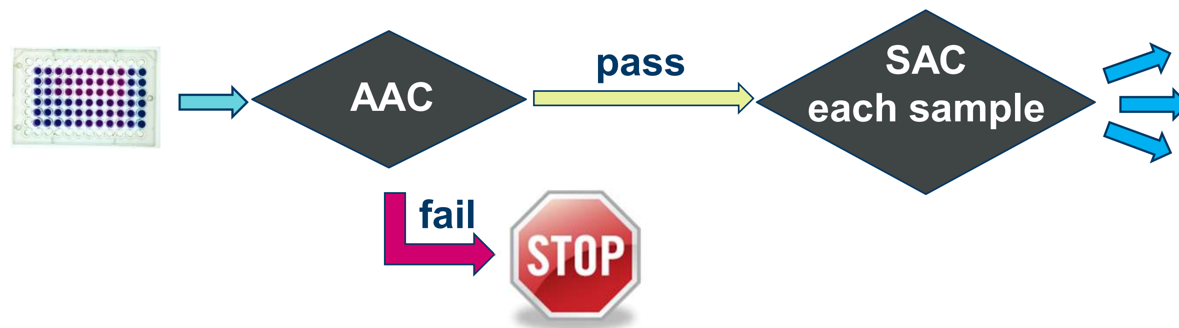
Good for shorter assays (< 48 hours) where no edge effects observed

Questions?
Break

Parameters and Criteria

Assay acceptance & sample acceptance
→ Flow Diagram

- Assay Acceptance Criteria (AAC) based on responses of control samples and reference standard
- Sample Acceptance Criteria (SAC) based on responses of each separate sample



- If the plate fails AAC, then there is no processing of test sample data
- If one test sample fails the SAC, then that particular test sample potency quantification fails. Other test samples on the plate are assessed separately

Slide borrowed and adapted from Jane Robinson

System Suitability

- Test assumption of parallelism between RS and sample curves (and independent Assay Control, if available)
- Must be performed on unconstrained curves
- Typical parameters include
  - r^2 of each unconstrained curve separately (RS, AC, sample)
  - Upper and Lower Asymptotes (A and D, often adjusted)
  - Slope of tangent line through mid-point (B)*
- Template criteria can be used for a “platform” method, but each assay should be assessed at the pre-qualification stage

* Only applies to 4 PL fit. 5 PL fit requires additional calculations to determine an approximate slope

Example Criteria (by category)

Parallelism

Equivalence Tests

- Slope Ratio
- Upper Asymptote Ratio
- Effective Asymptote Ratio

Adequacy of Model

Variability of Data

- %CV of replicates (rarely used)

Goodness of Fit

- R^2 Reference
- R^2 Control

Run Quality

Control Relative Potency

Other

Signal to Noise (actually signal to background)

%(G)CV of individual potency for combining to reportable

Application of System Suitability Criteria and Ranges

Method Development

- Assessing Assay quality during development, i.e. is my method fit-for-purpose?
- Consistent passing of System suitability signals transfer readiness
- Provides control of data quality during process development and CQA testing

Clinical Release and Stability

- Control of data quality
- Assay failure
- False OOS
- Evaluate Alert Limits (Historical Limits)
- Set Development Target Limits for platform knowledge

Late Phase, PPQ and Commercial

- False OOS during late and commercial phase
- Implement Alert Limits (Historical Limits)

Example System Suitability

	Criteria Name	Formula	Limits
Unconstrained	Signal-to-Noise of Reference Curve	$\frac{\boldsymbol{D_{ref}}}{\boldsymbol{A_{ref}}}$	>2
Unconstrained	R^2 for Reference Curve	-	>0.97
Unconstrained	Upper Asymptote Ratio	$\frac{\boldsymbol{D_{sam}}}{\boldsymbol{D_{ref}}}$	0.85 - 1.15
Unconstrained	Effective Asymptote Ratio	$\frac{\boldsymbol{D_{sam} - A_{sam}}}{\boldsymbol{D_{ref} - A_{ref}}}$	0.85 - 1.15
Unconstrained	Slope Ratio	$\frac{\boldsymbol{D_{sam} - A_{sam}}}{\boldsymbol{D_{ref} - A_{ref}}} \times \frac{\boldsymbol{B_{sam}}}{\boldsymbol{B_{ref}}}$	0.70 - 1.30
Unconstrained	R^2 for Control Curve	-	>0.97
Constrained	% Relative Potency of Control	$\frac{\boldsymbol{C_{sam}}}{\boldsymbol{C_{ref}}} \times \mathbf{100\%}$	70% - 130%

Qualification (Phase-appropriate Validation)

ICH Q2 (March 2022)

Key Topics (not specific to Bioassays)

- Analytical Procedure Validation
  - Range
  - Specificity
  - Accuracy and Precision (option to be combined)
  - Robustness

  Consider “Phase-appropriate validation”, sometimes called “Qualification”

  - Same critical properties assessed but expectations can be lower than for “full validation”
    - Example, fewer analysts and runs, less stringent criteria for accuracy and precision

Table 1: Typical performance characteristics and related validation tests for measured product attributes

Type of measured product attribute / Analytical Procedure Performance Characteristics to be demonstrated (2)	IDENTITY	IMPURITY (PURITY) Other quantitative measurements (1)		ASSAY content/potency Other quantitative measurements (1)
		Quantitative	Limit	
Specificity (3) Specificity Test	+	+	+	+
Working Range Suitability of Calibration model	-	+	-	+
Lower Range Limit verification	-	QL (DL)	DL	-
Accuracy (4) Accuracy Test	-	+	-	+
Precision (4) Repeatability Test	-	+	-	+
Intermediate Precision Test	-	+ (5)	-	+ (5)

Footnotes :

- signifies not normally evaluated

+ signifies is normally evaluated

() signifies normally not evaluated, complex cases recommended

(1) Impurity scheme, can be applied to other measurements

(2) Performance characteristics can be substituted for physchem assays with justification

(3) A combined approach can be use for accuracy and precision assessment

(4) Lack of specificity in one method can be compensated with one or more other procedures

(5) Reproducibility and intermediate precision can be performed together

Guidance for Qualification of Potency Methods

- This document provides guidance for the qualification of methods used to determine the potency of drug substance and drug product samples in support of clinical lot release and stability testing.

- The qualification of a potency method evaluates the method's suitability for use in **clinical lot release** and **stability testing** of **drug substance** and **drug product.**

- This evaluation includes parameters such as
  - **Specificity**
  - **Linearity**
  - **Accuracy**
  - **Intermediate precision**
  - **Repeatability**
  - **Assay range**

- All potency methods must be qualified prior to use for GMP testing.

Guidance for Qualification of Potency Methods

This guidance document is based on recommendations for method qualification described in:

- USP<1033> "Biological Assay Validation"
- ICH Q2(R1), "Validation of Analytical Procedures Methodology"

Example of assays to be performed in Assay qualification

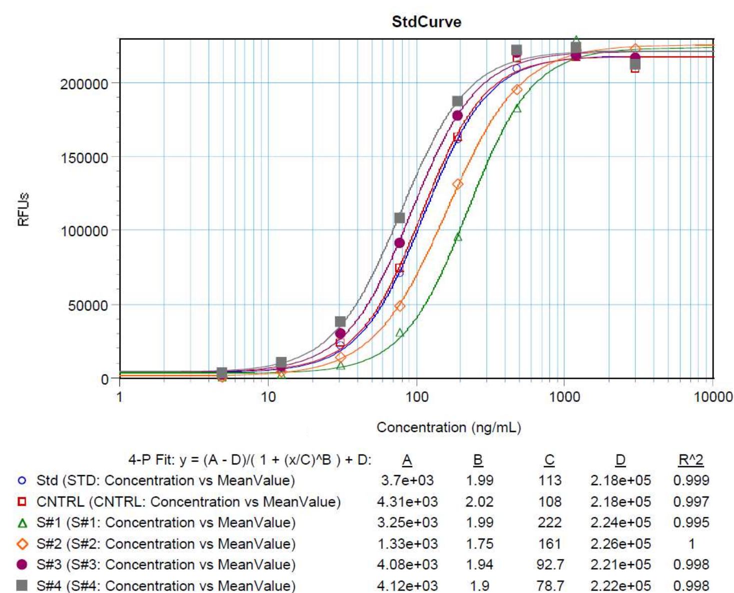
A minimum of two analysts should each test the simulated potency samples
- Analyst(s) should use different cell preparations
- The 100%RP sample test will be needed in addition to the assay control

Table 4 Example of Assays to be Performed in Assay Qualification

Description / Sample ID	Assays 1-2	Assays 3-4	Assays 5-6	Assays 7-8	Assays 9-10	Assays 11-12
Purpose of Assay	Linearity, Precision, & Accuracy				Stability Indicating properties / Specificity	Repeatability
Location Assay to be Performed	1 Assay each at Transferring and Receiving Lab				Transferring Lab	Receiving* or Transferring Lab
Reference	RS	RS	RS	RS	RS	RS
Assay control	RS	RS	RS	RS	RS	RS
Samples	• 50% • 75% • 100% • 125%	• 150% • 50% • 75% • 100%	• 125% • 150% • 50% • 75%	• 100% • 125% • 150% • TBD	• T_0 • Deg • FB (~10x) • AZDXXX	• 100%(R) • 100%(R) • 100%(R) • TBD

Deg = stressed sample or accelerated stability sample together with a T_0 timepoint when applicable; FB = formulation buffer; AZDXXX= not-target specific but structurally similar product; 100%(R) = 100%RP repeatability sample; TBD = can be used for re-testing invalid sample(s) from the previous runs.

*Receiving lab is preferred



Specificity and Stability runs

Specificity

Non-specific test samples (structurally related, functionally distinct) and formulation buffer alone should not generate dose-dependent responses in the assay that are similar to the relevant test article response with regard to Sample Acceptance Criteria.

Figure 1 **Example of specificity data**

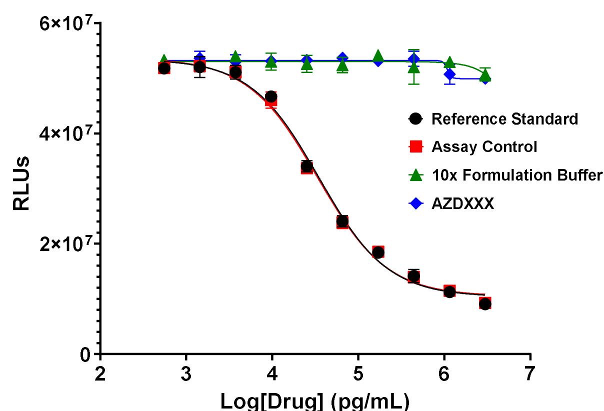


Table 1 **Example of Specificity Results Table**

Test Article	Antibody Subtype	Antibody Target	Lot/Batch	Concentration	Response
Formulation buffer (10X)	NA	NA	XXX	NA	None detected
AZD XXX	IgG1κ	XXX	XXX	10.2 mg/mL	None detected

Stability

The ability of the assay to detect changes in potency should be assessed by analysis of stressed samples and/or accelerated stability samples (if available).

When possible, samples possessing degradation consistent with known degradation pathways of the test article such as deamidation, oxidation should be used.

Figure 2 **Example of Dose Response Curves from Stability Indicating Study**

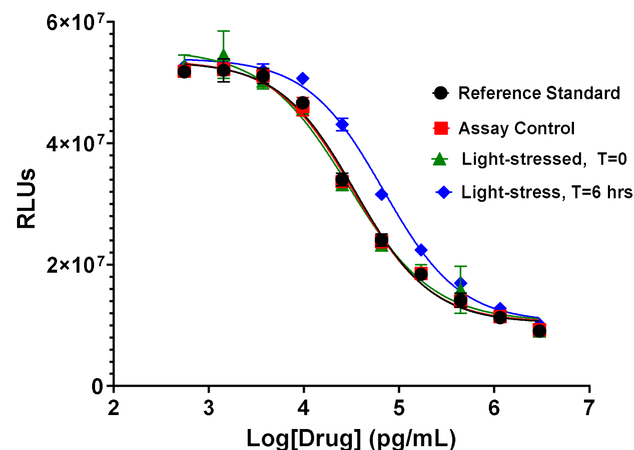


Table 2 **Example of Results from Stressed Samples**

Test Article	Stress Condition	Sample Acceptance Criteria (Pass/Fail, Assay1/2)	RP
AZD XXX (Lot [illegible]0)	Light stressed, T=0hrs	Pass, Pass	109%
AZD XXX (Lot 0)	Light Stressed, T=6hrs	Pass, Pass	52%

In case of a Non-Reportable RP value, add explanation here such as potency value is outside of the assay range. In the case of a sample that failed sample acceptance criteria add description of what failed e.g., parallelism.

Qualification summary (example)

Table 3 Qualification Summary

Qualification Parameter	Qualification Target	Qualification Result
Linearity	**Fit quality:** $R^2 \geq 0.970$ for overall linear regression plot of mean observed vs. expected relative potency values.	The R^2 value of the overall linear regression plot is > 0.999.
Repeatability and Intermediate Precision	**Repeatability:** The %CV should be ≤15%. **Intermediate Precision (IP):** The upper bound of 95% CI of the total %CV (run-to-run and intra-run) should be ≤25%.	**Repeatability:** The %CV is 3.9%. **IP:** The upper bound of 95% CI of the total %CV is 9.3%.
Accuracy	**Accuracy:** The 90% CI of mean accuracy should fall between 80 – 120% for overall and 75 – 125% at each potency level.	The 90% CI of mean accuracy: Overall: 50%RP level: 75%RP level: 100%RP level: 125%RP level: 150%RP level:
Range	Assay must meet targets for acceptable linearity, accuracy and precision within the 50% - 150% potency range.	Assay demonstrates acceptable linearity, accuracy, and precision within the 50% - 150% potency range.
Specificity	No meaningful response from formulation buffer and/or functionally irrelevant antibody	No concentration-response to formulation buffer was observed. No concentration-response to functionally irrelevant antibodies was observed.
Stability Indicating Potential	Assay detects a change in potency of stressed samples.	The assay is able to detect changes in potency for samples subjected to stress (such as UV or thermal).

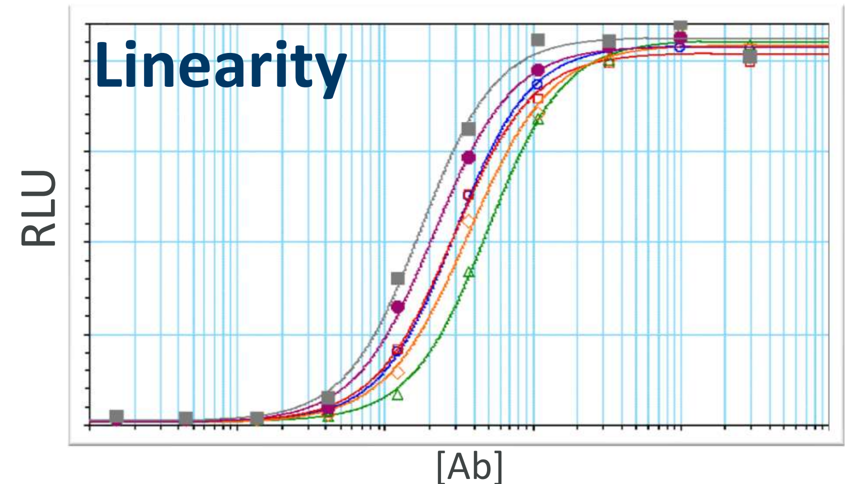
Consult biostatistician to define the number of valid independent runs (N) required for generating a reportable %RP value based on:

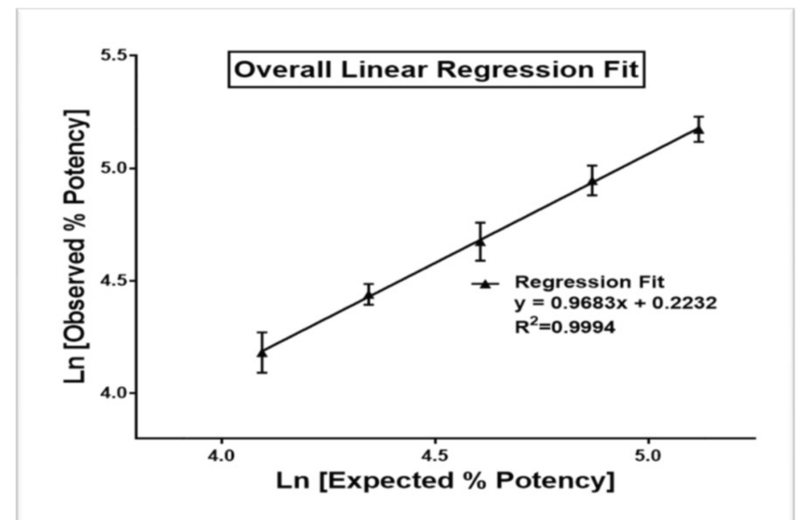
- Analytical method qualification data analysis
- Expected product variability
- Proposed potency specification

Qualification of Reporter Assay

Reporter assay successfully qualified:

- Linearity
- Assay range
- Accuracy
- Precision
  - Repeatability
  - Intermediate Precision
- Specificity
- Stability Indicating





With Reference Standard and Assay Control Sample in Mind

- Need to arrange, as soon as possible, for Reference Standard and Assay Control lots
  - Early on, it will likely be PD development lots, of some sort.
  - Ideally, lot size(s) will be large enough to get through assay development, optimization and
    pre-PAV, as well as PD support
  - Will need to generate sufficient supporting data to derive equivalence gates (ideally by pre-PAV)
    - Don't want to be switching lots midstream
  - Smaller lots will require extra time and work to bridge activity (potency) between lots
  - Once work/projects move on toward clinical phases and CTM release, assay will move towards PAV
    - Will need a more representative reference standard and assay control
    - Likely stronger documentation

Reference Standard, things to watch for…

Reference Standard (RS)

- Make sure there is enough (originating lab retains)
- May or may not be well characterized, can be non-GMP
- May evolve along with product development
- May not be the same formulation as the drug product
- Transfer from originating lab to recipient lab in batches
- Monitor stability – freeze/thaw → bioassay + 2nd method?
- Document stability as part of a protocol
- Monitor results with Statistical Process Control (SPC) chart
- Qualify new/next RS lot with a protocol
  - Characterize with all methods (analytical)
  - How many bioassay runs constitute an assignable value?

Statistics (only what you need for Potency!)

How to Determine Proper N for Reportable Mean?

- In order to reduce the risk of "false OOS", the effective variation of the method can be used
- Intermediate precision from the initial qualification is the best estimate of method variability when the method is first implemented in a GMP lab
- Method trending (ideally with an independent AC) will provide a "real world" update on true variability
- Using the method variability, the impact of different N for reportable mean can be modeled for N=1 to 5, for example, with the goal of reducing the false OOS rate while maintaining a reasonable work flow
- Typical potency methods will use N = 2-4

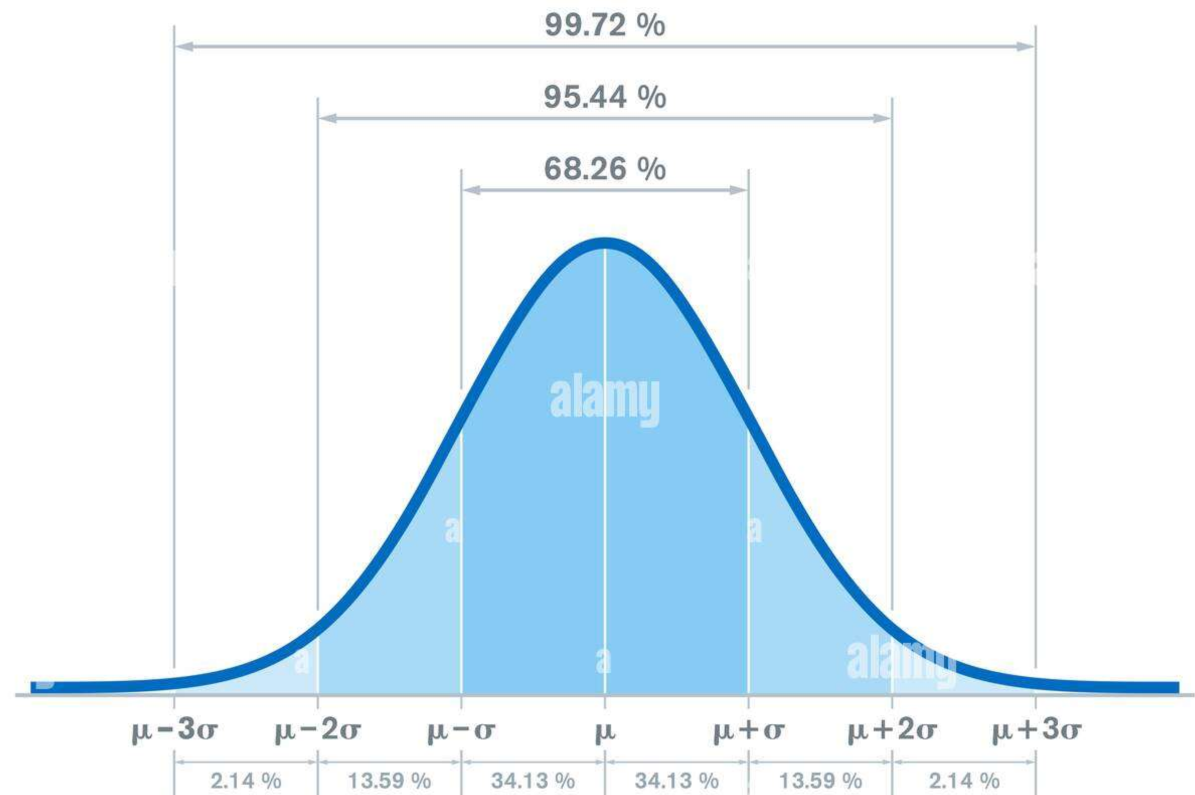
Setting Specifications

- Two major elements go into setting specifications
  - Manufacturing variability
  - Assay variability
- For protein or nucleic acid-based biologics, manufacturing variability is generally small, with a well-controlled process. Only testing of multiple lots and processes will determine
- For these modalities, bioassay variability will often be higher than manufacturing variability
- For C>, manufacturing (and starting material) variability can be quite high
- Qualification data allows estimate of method variability (intermediate precision), but N is small
- For initial IND, setting a wide specification, e.g. 60-140% RP is often acceptable with the expectation they will tightened using method and product trending
- Confidence or Tolerance intervals can be used with sufficient data (e.g. ≥7 lots)

Reportable Mean is based on reducing “false OOS” rate

Ultimate goal is a very low “false OOS”* rate in GMP testing for lot release

For example ≤ 1%



* “False OOS” is a sample that has a true potency value within the specifications, but the experimental reportable mean is outside of specs, due to statistical variation

Commonly Used Statistical Tests and Assumptions

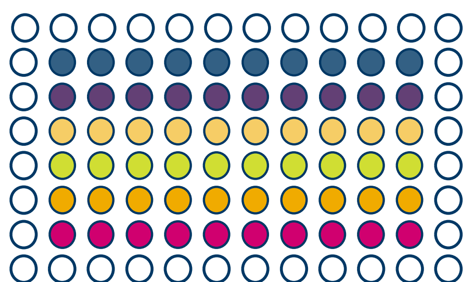
- T test
  - Assumes normal distribution of data, appropriate sample size and homogeneity of variance
  - Raw data in potency methods is often log-normally distributed, transformation can be appropriate
  - Intended for pair-wise comparisons, correction factors for muti-group
- ANOVA (analysis of variance)
  - Also assume normal distribution of data and homogeneity of variance
  - Better for multi-group analysis
- Confidence Interval--a method of estimating the probability that a given interval contains the true value being measured, at a given level (e.g. 95%)
  - Commonly used for setting criteria for qualification, bridging, tech transfer, etc.
- Tolerance interval---statistical interval within which a specific sample proportion falls, with some confidence (e.g. 99% confidence that sample is within 95% of the sample distribution)
  - Commonly used for setting specifications with $N \geq 7$ manufacturing lots

Assay Characteristics or Parameters

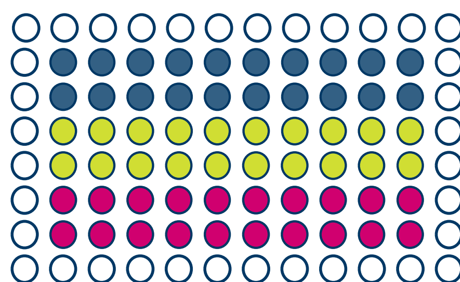
Reportable Value

- The value reported on a product’s Certificate of Testing/Certificate of Analysis
- Defined in SOP
- Any OOS is based on Reportable Value

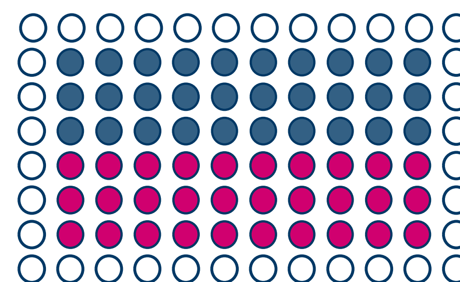
Is your data generated as



Singlicates



Duplicates



Triplicates

If replicates are used, are they true/independent replicates, or pseudo/technical replicates

Is your Reportable Value based on the result from one plate, or…

Questions?

Training and Tech Transfer

Analyst Training

- Minimum of two analysts trained
  - Bioassay SOP
  - Data analysis understanding
    - Review good, bad and ugly data examples

- Cross-training at originating vs. recipient lab
  - Stable platform at originating lab
    - Critical reagents, equipment and data analysis computer systems
  - Comfort level for new analystes at recipient lab

Analyst Training

- Training SOP documents number of bioassay runs and results expectations.
  - Start with simple assays using the Reference Standard or Training Samples.
  - Training Samples can be made from previously tested lots.
    - Label as Trainging Samples with newly coded identification numbers.
  - Train up to a standard/routine bioassay run.
  - What is the expectation regarding the size of a standard/routine bioassay run?
    - Three plates with Reference Standard, Controls and six samples?
    - Train as you will perform with fully burdened bioassay runs that include the Reference Standard and previously tested “controls“.
  - Does the training data fall within the range described in the Training SOP?

Analyst Training

- Required interactions
  - Face-to-face cross-training – combination of scientists with analyst-to-analyst interactions
  - Follow-up visits & audits
  - Monitoring of method performance
- Additional considerations for cell-based bioassays
  - Specific cell culture SOP training
  - Culture examples/ pictures, know/ understand pitfalls
  - What do good and bad cultures look like?
- Master the cell culture methods prior to performing the bioassay
- Practice seeding cells in plates with multichannel pipets and/ or conduct training on liquid handling machines

Equipment

- Installation/Operational/Performance Qualifications (IOPQ)
  - Plate readers/spectrophotometers, cell counters
  - Flow cytometer, surface plasmon resonance system, aggregometer
  - Data analysis software
- Temperature mapped equipment
  - Incubators; including the room temperature ones
  - Freezers
  - Refrigerators
  - Vapor phase liquid nitrogen storage

Critical Reagents

Definition:

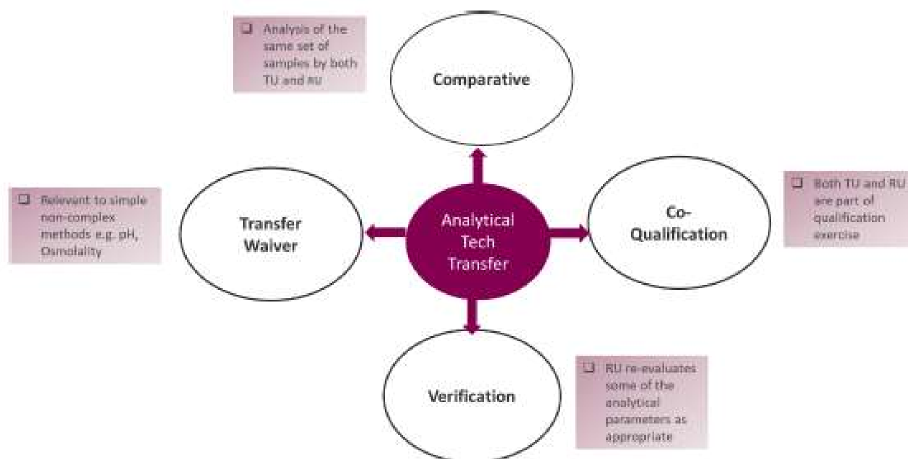
- Physical, (column), chemical (cytokine, media, serum) or biological system (cells) interacting with the active substance that could impact system suitability.

Or

- Any reference or calibration standard (pH, IS) from which a result for the active substance is interpolated.

Technology Transfer (Tech Transfer)

- Formal transfer of analytical procedure from transferring unit (TU) to a receiving unit (RU)
- Common stages
  - Development lab to QC testing lab, e.g. prior to qualication
  - Internal QC lab to CRO
  - Clinical QC lab to commercial unit
    - Can be direct to commercial QC lab or to a technical support operations group



Key considerations

- Sample type, typically RS and/or AC. 100% and at least one lower level
- Equipment and software equivalence
- Compliance level, protocol required?
- Statistical test
  - Criteria tight enough to ensure suitable performance at RU, but consider relatively small N

Bridging

Reason for Needing a Bridging Study

- Demonstrate statistically comparable performance between two methods
- Comparison can be between %RP, not underlying values
- Common example:
  - Binding assay used for early clinical stage
  - Cell-based assay expected for pivotal clinical stage or marketing application

Key Elements of Bridging Study

- Demonstrate comparable linearity/range: accuracy
  - Example: paired T-test of percent recovery at each level
- Demonstrate comparable lot release for historical and/or new DS and DP lots
  - Example: paired T-test of official lot release (qualified method) vs. tested with new method
- Demonstrate similar stability-indicating properties
  - Do not recommend using statistical comparison, compare trends of relevant accelerated stability or forced degradation samples

Suggestions

- Use qualification linearity samples and data from new method as part of bridging study
  - Clearly articulate in Bridging Protocol intention to use qualification data in bridge
  - Test aliquots of linearity samples using established method
- Retains from historical lots of DS and DP will be tested with the new method after qualification
- Ideally stability samples will be the same for both methods
  - Frozen retains from the old method can be reused for the new method, or fresh samples can be generated for both
- Try to minimize period of GMP testing with two methods: risk of false OOS increased, discrepant results
  - Coordination with Regulatory team to coincide bridging with scheduled IND amendments or annual updates.
  - Allow ≥30 days for agency to respond before removing established method

Method Trending

Trending Provide the most accurate picture of real-world assay performance

- Independent Assay Control (AC) is preferred, vs independent dilution of RS
- Potency of AC can show changes in variability and drift in assay performance
- Separate from trending Stability of DS or DP
- Additional parameters to trend
  - System suitability criteria: ratio of asymptotes, slope ratio,

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