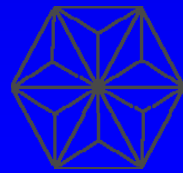


Best Practice Guidelines for the Validation of Immunoassays Assays for Pharmacokinetic Studies of Macromolecules

Russell S. Weiner, Ph.D.
Group Director
Biomarker and Bioanalytical Sciences



Bristol-Myers Squibb Company

Bioanalytical Method Validation History (Large Molecule)

2000: Macromolecule Workshop #1

- More differences than similarities

2000: AAPS Ligand Binding Assay Bioanalytical Focus Group

2001: FDA Guidance

- Small molecule focus with a touch of immunoassay
- Does this pertain to macromolecules?



Bioanalytical Method Validation History (Large Molecule)

2001: Macromolecule Workshop #1 Report

- Consensus or controversy?

2003: Macromolecule Workshop #2

- Presented AAPS BMV Subteam recommendations
- General Consensus

2003: AAPS BMV Subteam white paper published

- Recommendation for use of a Total Error approach. Data from the validation experiments used for setting in-study run acceptance criteria versus using a priori criteria.

DeSilva, B., Smith, W., Weiner, R., et al. Recommendations for the Bioanalytical Method Validation of Ligand-Binding Assays to Support PK Assessments of Macromolecules. *Pharm Res* 20:1185-1900 (2003)



Bioanalytical Method Validation History (Large Molecule)

2006: Bioanalytical (Crystal City) Workshop #3

- Focus broadened to include large and small molecules

2007: Bioanalytical Workshop #3 Report

Critical points include

- Recognized need to increase the in-study run acceptance criteria to something >15%
- Recognition of the Total Error approach
- Incurred Sample Reproducibility (ISR)



Can we use the small molecule guidance?

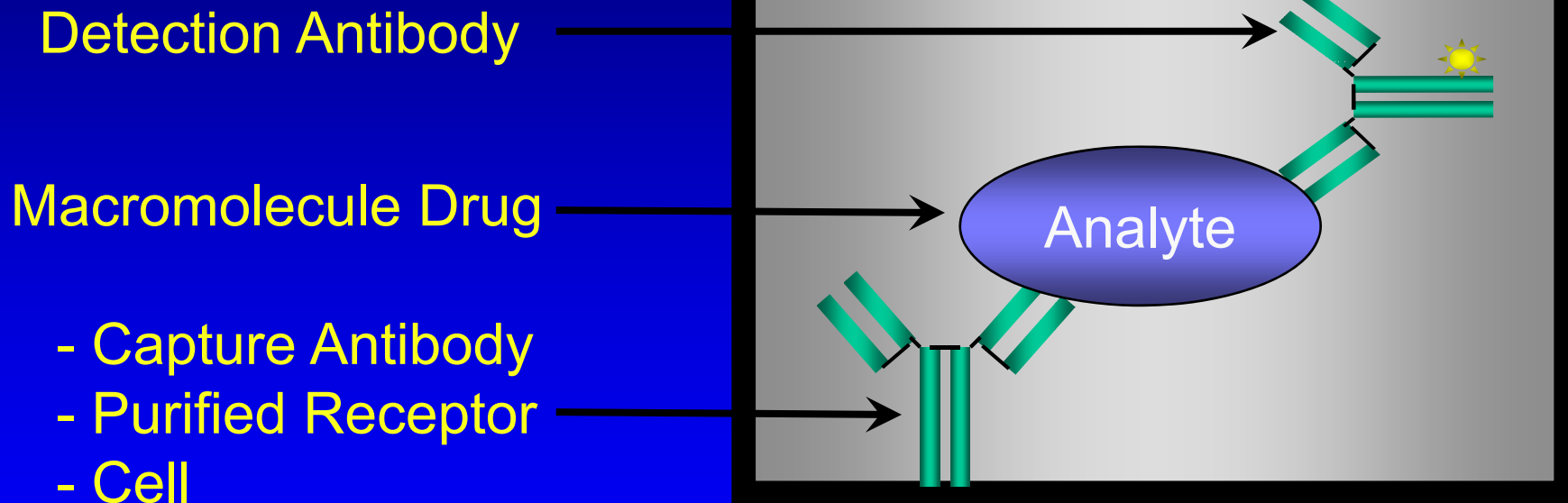
Of course you can with a few exceptions, but only under specific circumstances.

Why?

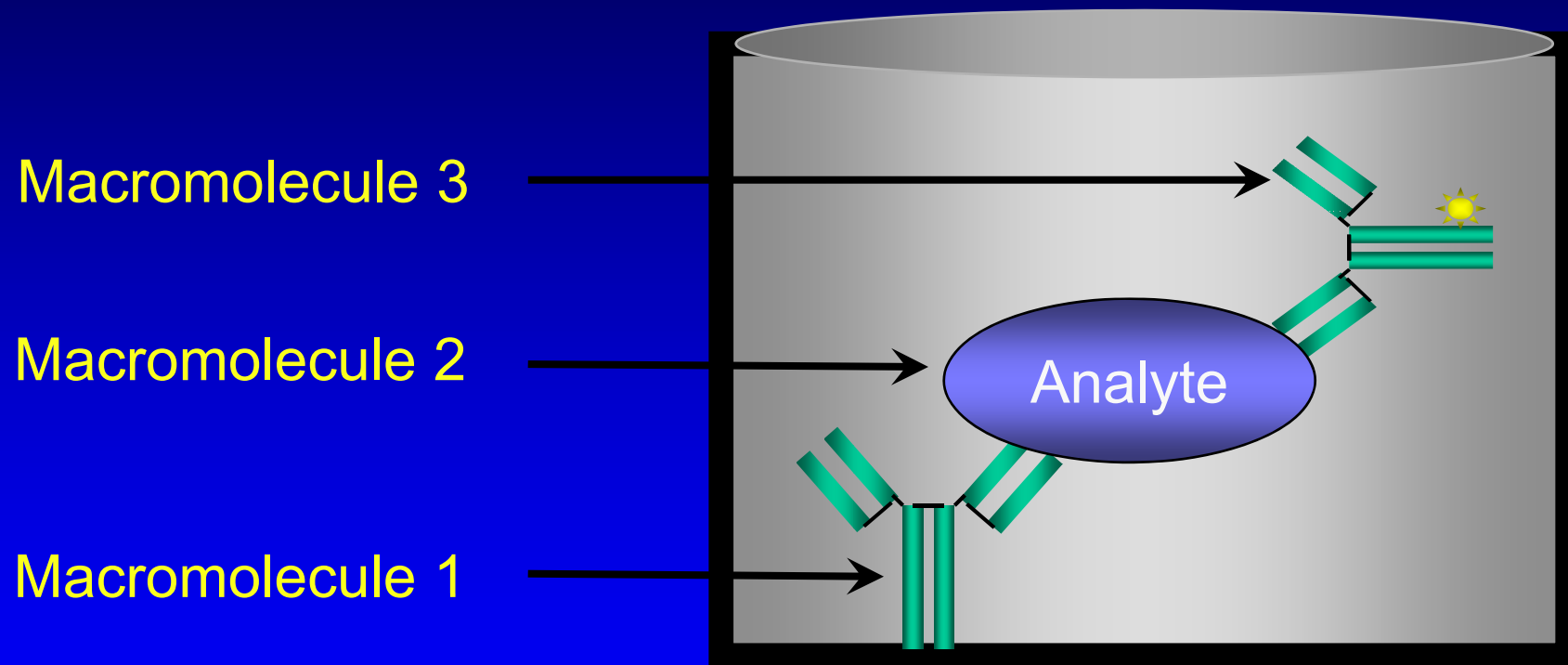
- Many of the concepts and practices are based on good scientific principles common to all assays



Immunoassay Parts



Immunoassay Parts



Specific Circumstances

Analyte

- Stable
- Manufacturing process is highly controlled
- Well characterized
- Not endogenous
- No binding proteins

Reagents

- Sourced internally and well characterized
- Sourced externally and well controlled with
- minimal lot-to-lot variability



But more importantly why not?

- Reference standard/reagent purity, stability and lot variability
 - We measure macromolecules with macromoleculesⁿ
- Matrix variability
- No internal standard to correct for assay process variables
- Aside from dilution, no sample pretreatment to remove matrix prior to analysis
- For antibody drugs, need to measure drug at **ng/pg** levels in the presence of **>10 mg/ml** of endogenous antibody



Analytical Challenges Unique to Biologics

Interference from:

- Pre-existing binding proteins
- Pre-existing anti-drug antibodies (e.g. rheumatoid factor)
- Pre-existing anti-reagent antibodies (e.g. anti-mouse Ig)
- Anti-drug antibodies (immunogenicity)
- If developing an endogenous therapeutic (e.g. cytokine), pre-existing endogenous levels are also recognized in the assay



Assay Development Considerations

- Reagent Identification
 - exp. date, COAs, lot#
- Reagent Stability
- Antibody Specificity
- Early Analyte Stability
- Diluents
- Plate Type (coating conditions)
- Detection System
- Matrix Qualification
 - type (serum, plasma, etc)
 - normal, disease
 - minimal dilution
- Sample Collection
 - Protease inhibitors?
- Sample Preparation
 - lipemic, hemolysis
- Standard Curve Range & Model
- Target Acceptance Criteria

Some of these experiments may be repeated during Pre-Study validation



Ask Yourself: Are these really needed?

Do plates need to be

- covered?
- shaken?
- kept in the dark?
- incubated at
 - room temperature?
 - 4°C?
 - 37°C?
 - Washed 3x, 5x or high

Don't just do it because that's the way it's always been done



Pre-Study Validation Considerations

Target acceptance criteria should be stated prior to beginning pre-study validation. The following parameters should be determined using a minimum of six assays

- **Required**

- Documentation
- Standard Curve
- Accuracy and Precision
- Range of Quantitation
- Selectivity
- Specificity
- Dilution Linearity
- Stability

- **Suggested Robustness and Ruggedness**

- Lot-to-lot variability of critical reagents
- Time, Temp, pH
- Inter-operator variability
- Inter-plate washer variability

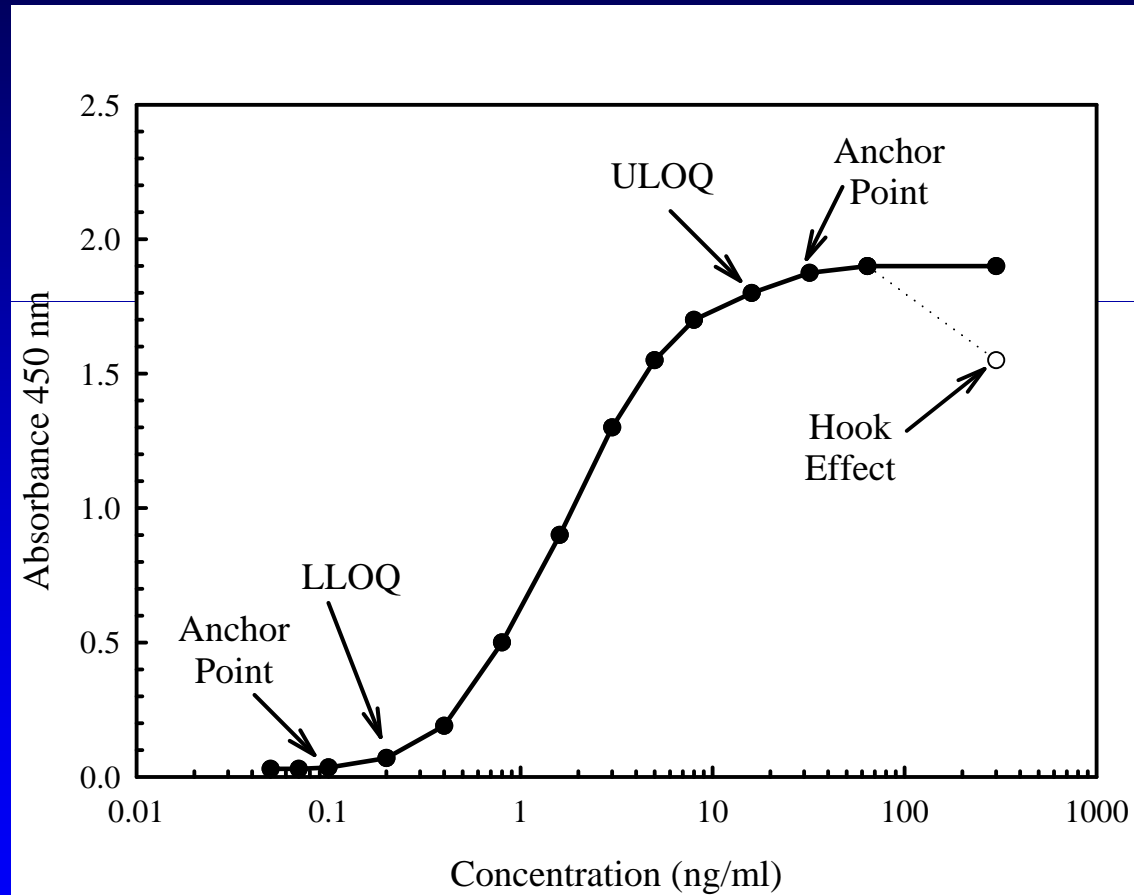


Standard Curve

- Should contain a minimum of 6 non-zero concentrations run in duplicate.
- Should be prepared in the same matrix as samples. If different then the absence of a matrix effect must be demonstrated.
- Anchor points may be included. However, concentrations can not be reported below the LLOQ or above the ULOQ.



Standard Curve Description



Dilution Linearity

At least 5 dilutions are recommended:

- 1 well above the ULOQ to rule out a prozone or “hook” effect.
- 3 that fall into the assay range
 - back-calculated concentration should be within 20% of nominal.
 - %CV between values should be within 20%.
- 1 below the LLOQ

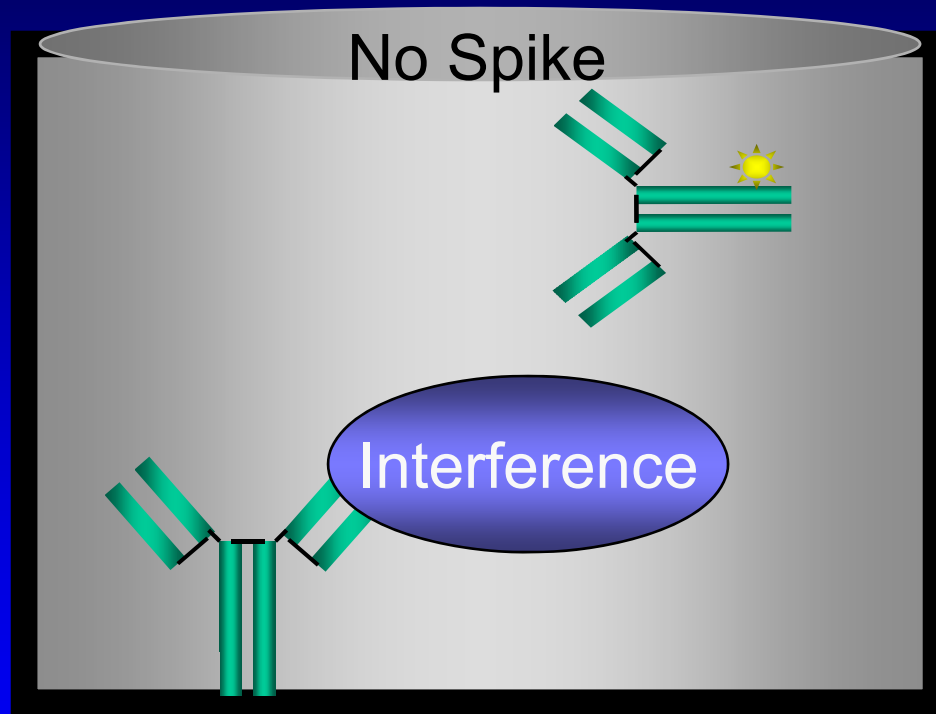


Selectivity/Specificity

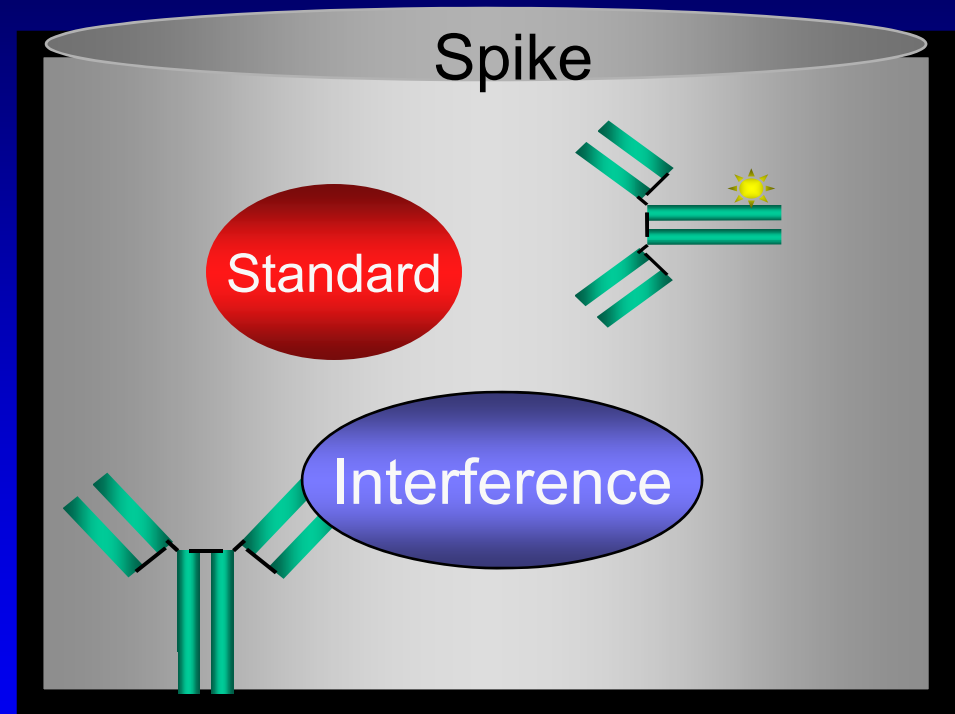
- At least 6 individual sources of the sample matrix should be evaluated with and without being spiked (at or near the LLOQ)
- Samples should be spiked with structurally similar compounds that are expected to be present in study samples.
- Strong recommendation to evaluate sample matrix from disease state patients.
- Competition assays are generally more susceptible to interference.



Selectivity/Specificity - continued



Results: <LLOQ



<Nominal

Stability

- Must be done in neat sample matrix
- Stability experiments should be modified to reflect the actual sample collection, shipment and storage conditions.
- Although preliminary stability analysis is done during the development stage, the final evaluation should be done using a validated method.
 - **Short Term:**
 - 4°C and ambient for 24 hours
 - 3 Freeze-thaw cycles
 - Sample processing simulation (whole blood)
 - **Long Term:** -30°C and/or -70°C >2 weeks



Validations Differences

Full

- Change in species within matrix (rat to monkey plasma)
- Change in matrix within a species (rat plasma to rat urine)

Partial

- Change in anticoagulant
- Change in lots of reagents (may need to reoptimize)

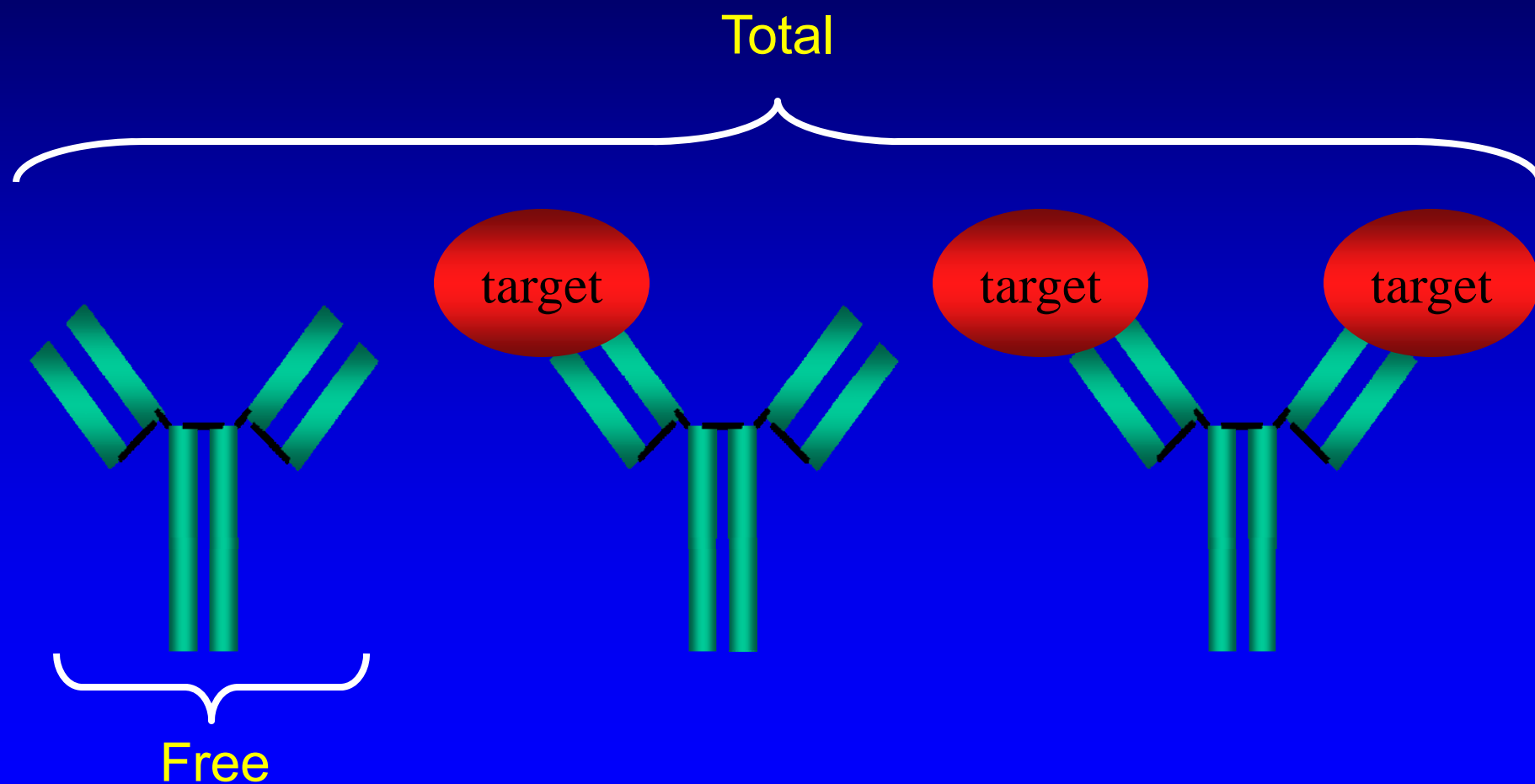


Do you or the Pharmacokineticist know what to measure?

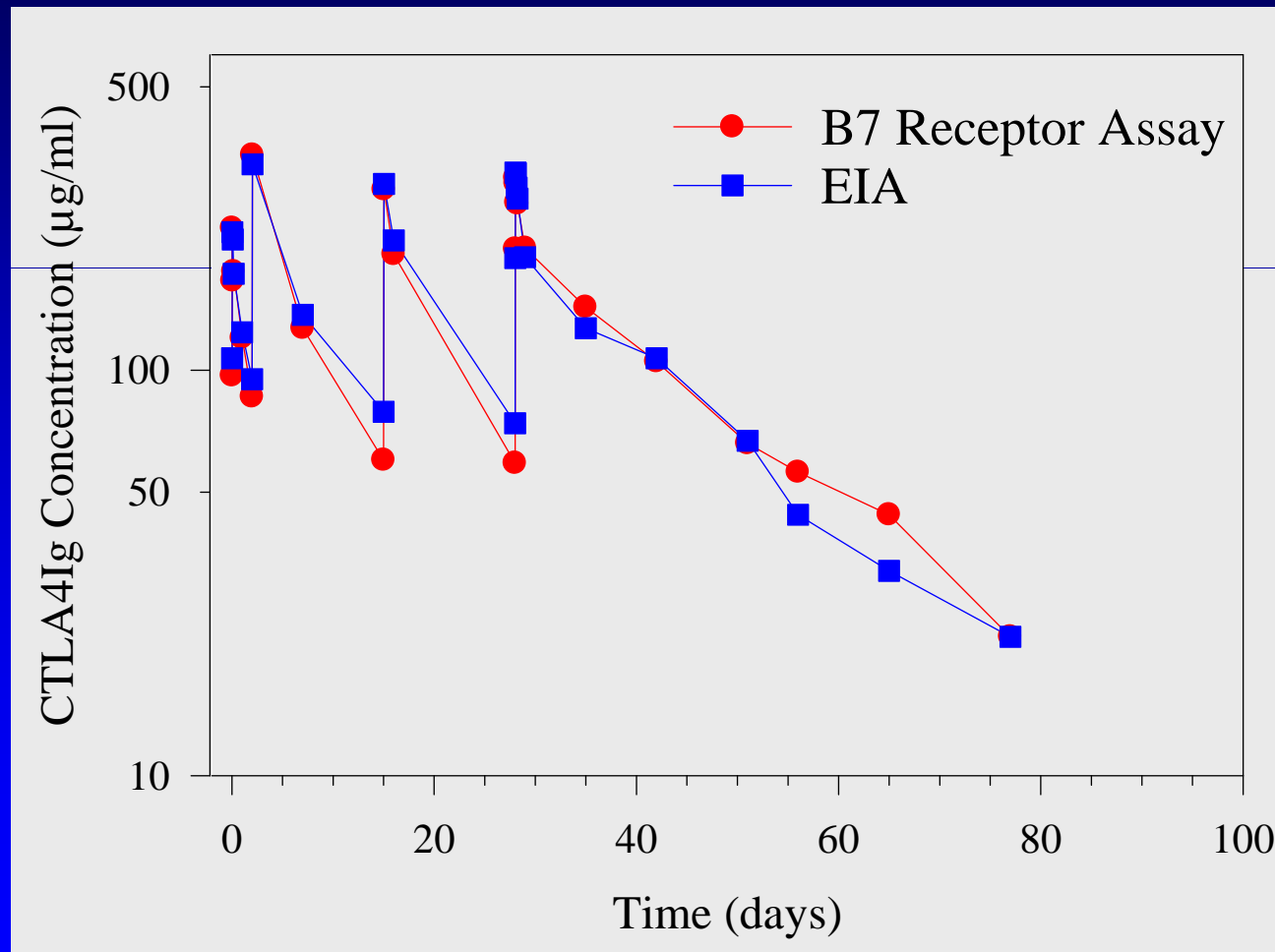
Know what you need to measure and
Know what you are measuring

- Free vs. Total
- Active vs. Inactive vs. Total
- Integrity: Is it the correct MW

Free -vs.- Total: Antibody Drug Against a Soluble Target



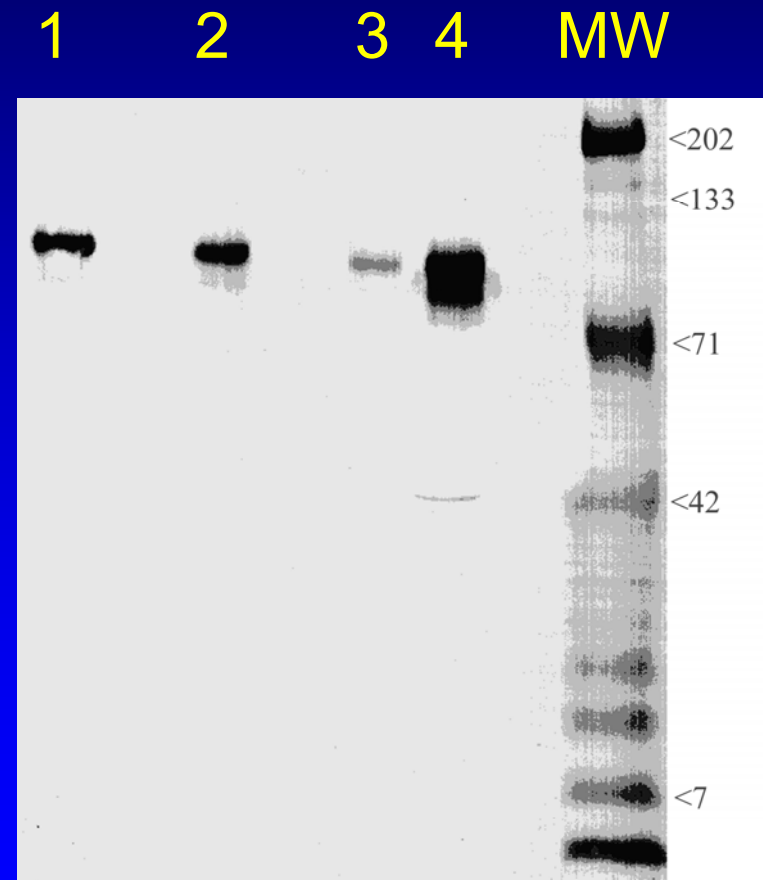
Activity via Receptor Binding Assay



Integrity via Western Blot Analysis

Lane

- 1. Patient A
- 2. Patient B
- 3-4. Drug Reference Standard



Recommendations

- You're measuring macromolecules with macromolecules start early!
- Not all sera are equal - look at disease state serum
- Prepare as many specific reagents as practical
- Start stability as soon as you have drug
- Know what you need to measure
- Know what you are measuring



Backup

White Papers/Conference Reports

Describing Immunoassay Best Practices

Validation of Immunoassays for Bioanalysis: A Pharmaceutical Industry Perspective.

J. Findlay, W. Smith, J. Lee, G. Nordblom, I Das, B. Desilva, M. Khan, R. Bowsher Journal of Pharmaceutical and Biomedical Analysis 21:1249-1273 (2000)

Workshop on Bioanalytical Method Validation for Macromolecules for Macromolecules: Summary Report.

K. Miller, R. Bowsher, A. Celniker, J. Gibbons , S. Gupta, J. Lee, S. Swanson, W. Smith, and R. Weiner. Pharmaceutical Research 18:1373-1900-1383 (2001)

Recommendations for the Bioanalytical Method Validation of Ligand-Binding Assays to Support Pharmacokinetic Assessments Of Macromolecules.

B. DeSilva, W. Smith, R. Weiner, M. Kelley, J. Smolec, B. Lee, M. Khan, D. Tacey, H. Hill, A. Celniker. Pharmaceutical Research 20:1885-1900 (2003)

Workshop/Conference Report- Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays.

C. Viswanathan, S. Bansal, B. Booth, A. Destefano, M. Rose, J. Sailsted, V. Shah, J. Skelly, P. Swann, and R. Weiner. The AAPS Journal 9: E30-E42 (2007)



Differences Between Small and Large Molecule Assays

	Immunoassay	LC/MS
Sample Preparation	None aside from dilution No extraction or IS	Samples extracted IS (stable label available)
Reference Standard	CoA issued Can have lot-to-lot variability.	Well characterized Limited lot-to-lot variability
Regression Model	Sigmoidal (4 or 5-Parameter logistic)	Linear or quadratic
Assay range	EIAs <2 orders of magnitude New platforms >2 orders of magnitude	2-3 orders of magnitude
Dilution Issues	Hook effects (at high conc.)	No issues
QC Criteria	Within 20 – 25% of nominal (majority) (LLOQ within 25-30%)	Within <<15% of nominal (LLOQ within 20%)



Where We Are Today

Performance Characteristic	LBABFG Consensus Report	Crystal City III Conference Report
Validation QCs %RE	± 20% ± 25% (LLOQ)	± 20% ± 25% (LLOQ & ULOQ)
Validation QCs %CV	≤ 20% ≤ 25% (LLOQ)	≤ 20% ≤ 25% (LLOQ & ULOQ)
Validation QCs %TE	± 30% ± 40% (LLOQ)	± 30% ± 40% (LLOQ & ULOQ)
In-Study Stds %RE	≥ 75% of std points are: ± 20% ± 25% (LLOQ);	≥ 75% of std points are: ± 20% ± 25% (LLOQ & ULOQ)
In-Study QCs	4-6-30 rule; At least 50% of QCs are valid at each level	4-6-20 rule; At least 50% of QCs are valid at each level

Courtesy of R. Bowsher & B. Nowatzke



In-Study Validation Considerations

- Standard Curve Acceptance Criteria
- QC Samples
 - Must be in the same matrix (neat) as the unknowns
 - Surrogate matrices must be scientifically justified.
 - Stored under sample storage conditions.
- QC Acceptance Criteria
- Incurred sample reproducibility (parallelism)



Incurring Sample Reproducibility (ISR)

- ISR (parallelism) differs from dilution linearity in that it can only be assessed with incurred “authentic” samples.
- A tolerance limit for acceptable non-parallelism should be established prior to the assessment.
- The degree of non-parallelism that is acceptable depends on the application.
- Methods for determining parallelism could include:
 - percent recovery at each dilution
 - slope comparisons
- In addition to performing as part of validation, there is general agreement to perform when changing matrix, species and disease.

