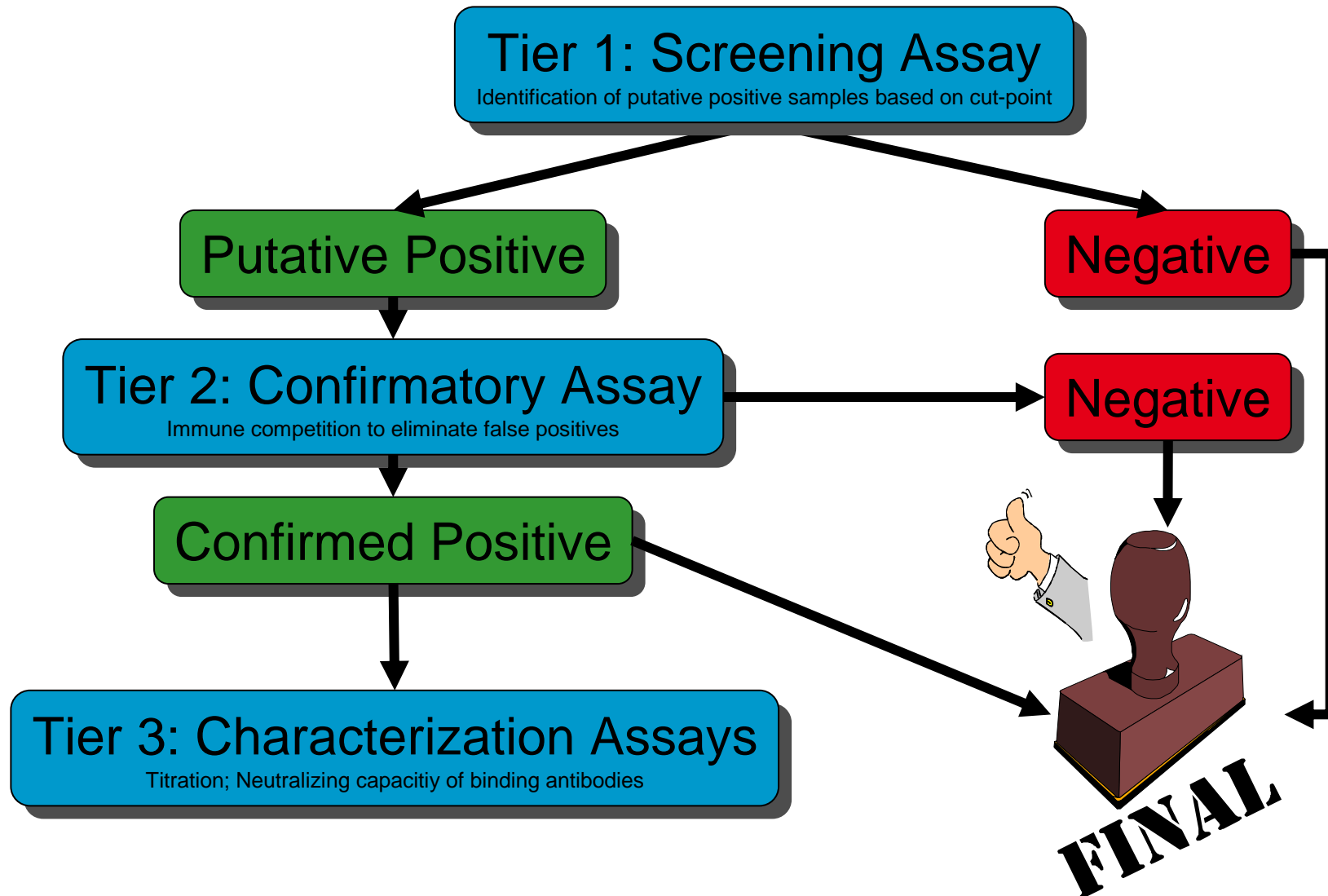


Binding Antibodies: Assay Methodologies, Screening Confirmation, Characterization of Anti-Drug-Antibodies

EIP Open Symposium Copenhagen 2012

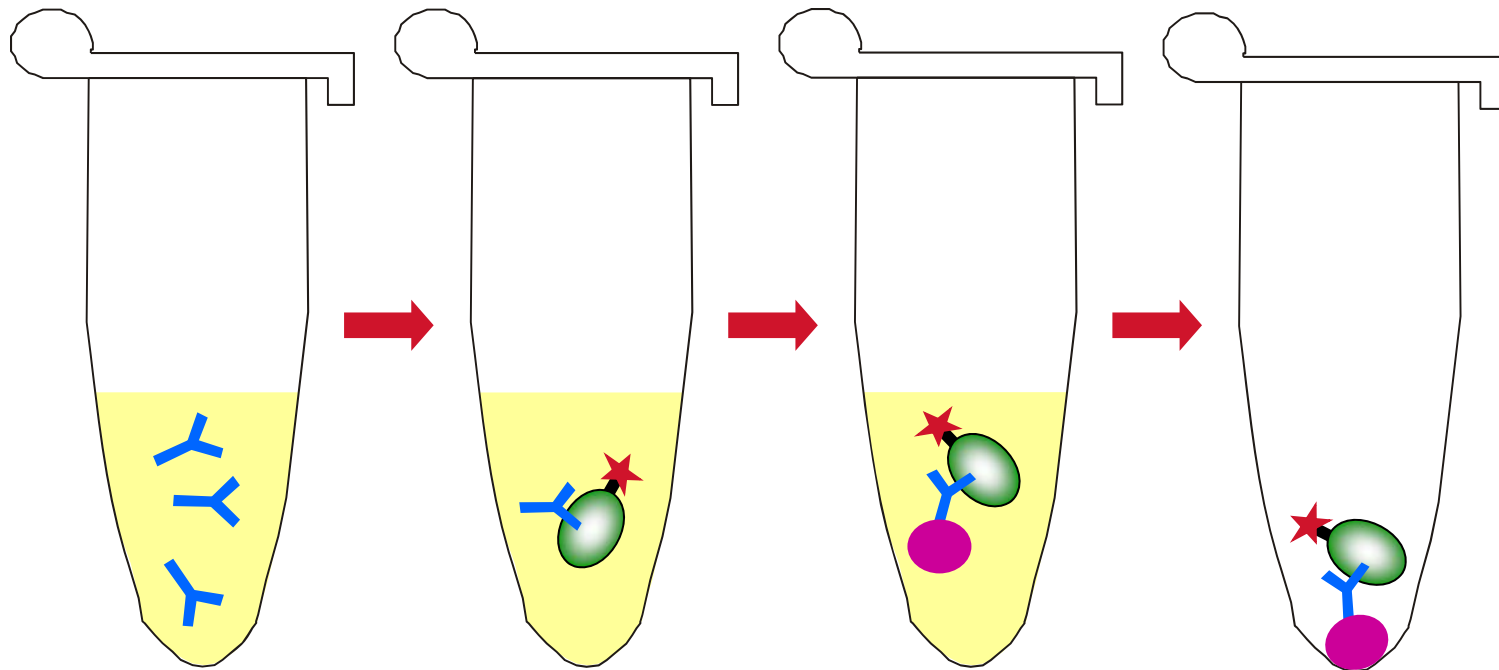
Daniel Kramer, Global DMPK, Merck Serono, Germany

Immunogenicity Testing – How...?



1st Tier: Screening Assay

Radio-Immuno-precipitation (RIP)



Add **iodine**-labeled **drug** to serum containing **anti-drug antibodies**

Add **protein A coated beads**

Centrifuge measure pellet

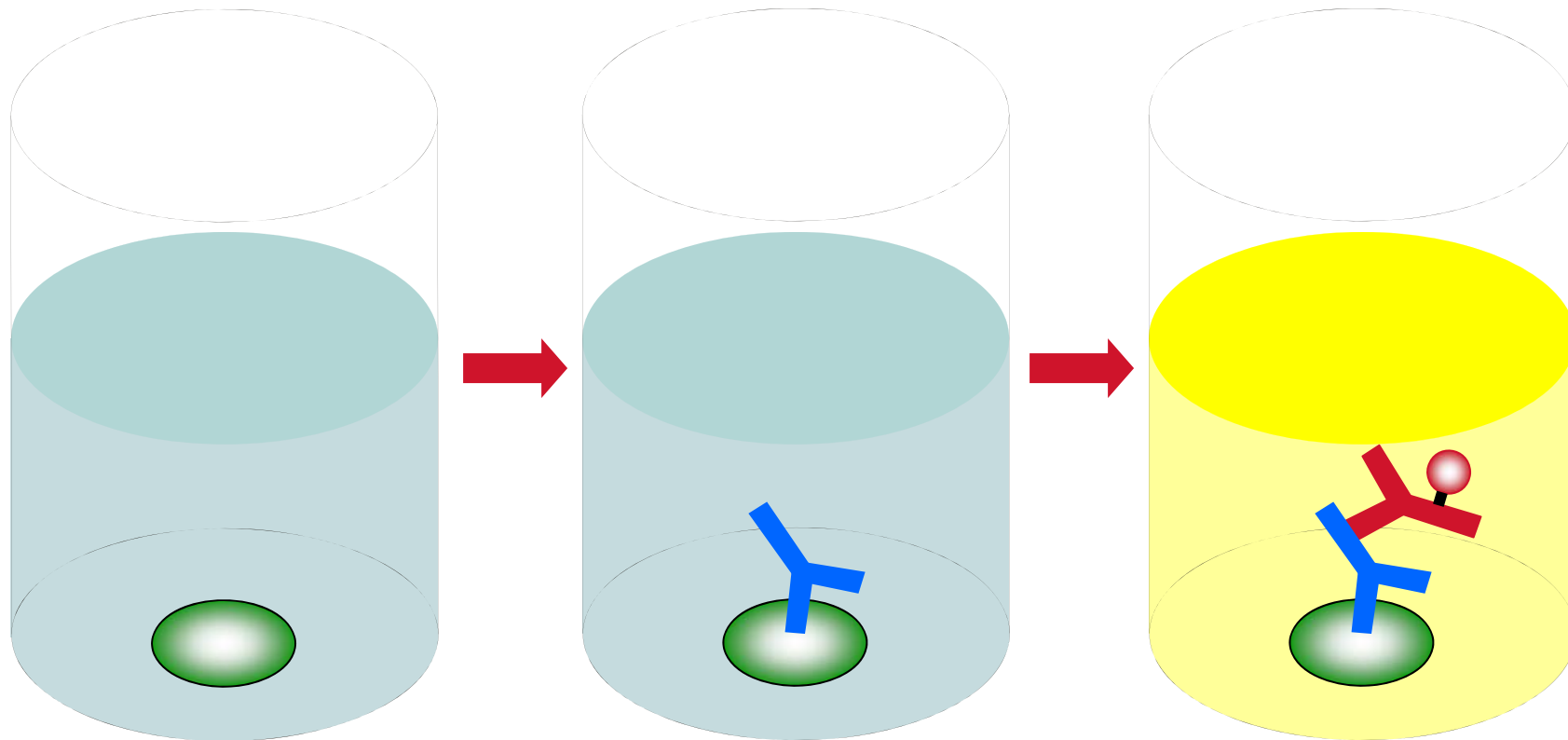
1st Tier: Screening Assay

Radio-Immunoprecipitation (RIP)

- Advantages
 - Sensitivity
 - Rather high drug tolerance
- Disadvantages:
 - Low throughput
 - Restricted availability of CROs
 - Specificity (prone to artefacts)
 - Radiolabelling process can mask/denature epitopes recognized by anti-drug antibodies
 - Protein A/G are known of having different affinities to different isotypes

1st Tier: Screening Assay

Direct ELISA



Immobilize **drug**

Add serum containing
anti-drug antibodies

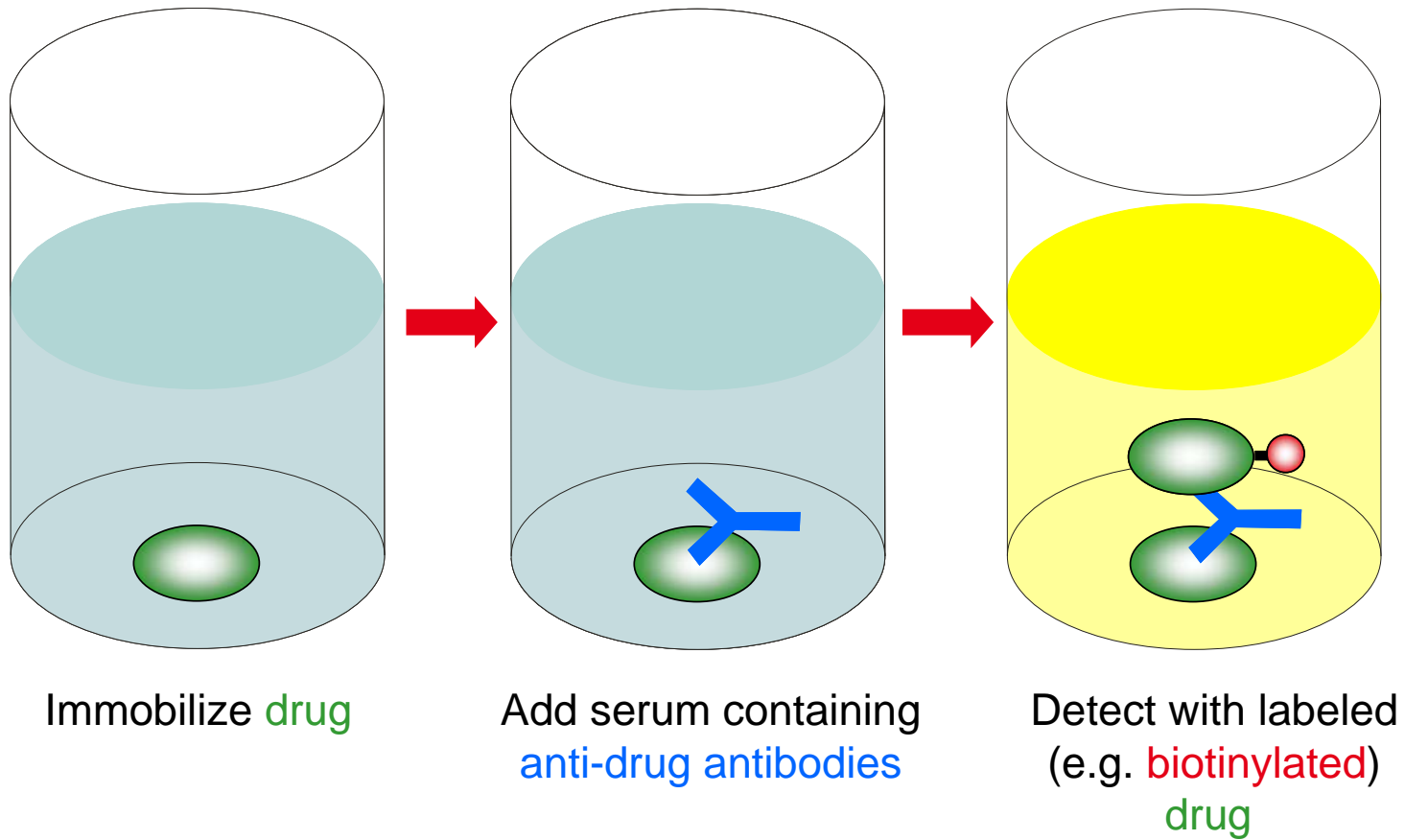
Detect with **enzyme labeled**
polyclonal secondary
antibody

1st Tier: Screening Assay

Direct ELISA

- Advantages:
 - Sensitivity
 - Commercial available secondary antibodies
- Disadvantages:
 - Source of the positive control has to be the same as that of the anti-drug antibodies
 - Specificity (unspecific binding to matrix components)
 - Restricted detection of low-affinity antibodies

1st Tier: Screening Assay Bridging ELISA



1st Tier: Screening Assay Bridging ELISA

■ Advantages

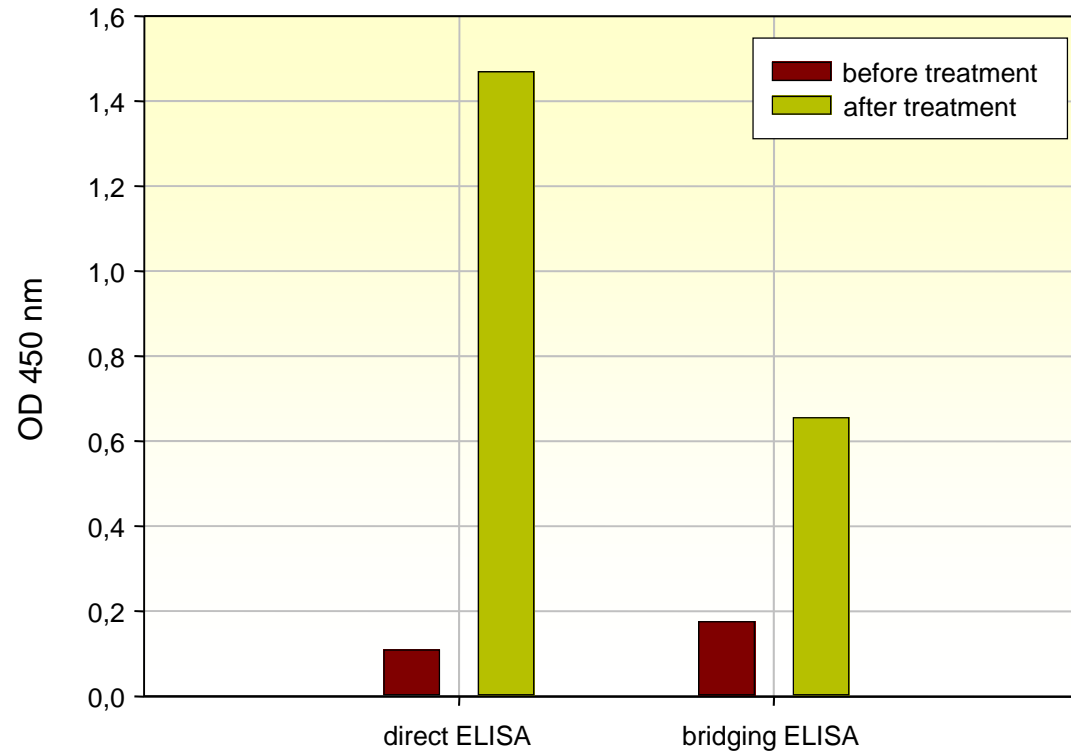
- High throughput
- Specificity (two-fold binding of drug required for signal)
- Possibility to use any positive control binding to the drug (independent of species)

■ Disadvantages

- Sensitivity (special orientation of immobilized drug required)
- Restricted detection of low-affinity antibodies
- Biotinylation might mask/denature epitopes recognized by anti-drug antibodies

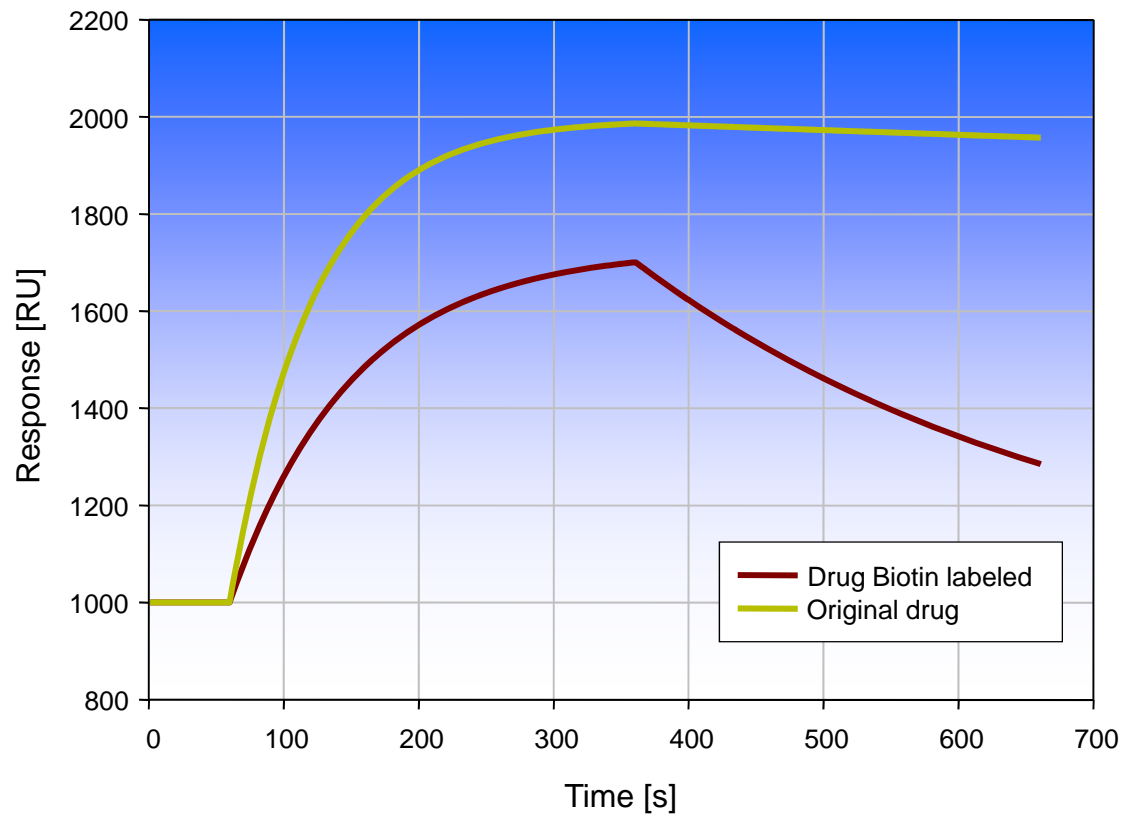
1st Tier: Screening Assay Bridging ELISA

Sensitivity

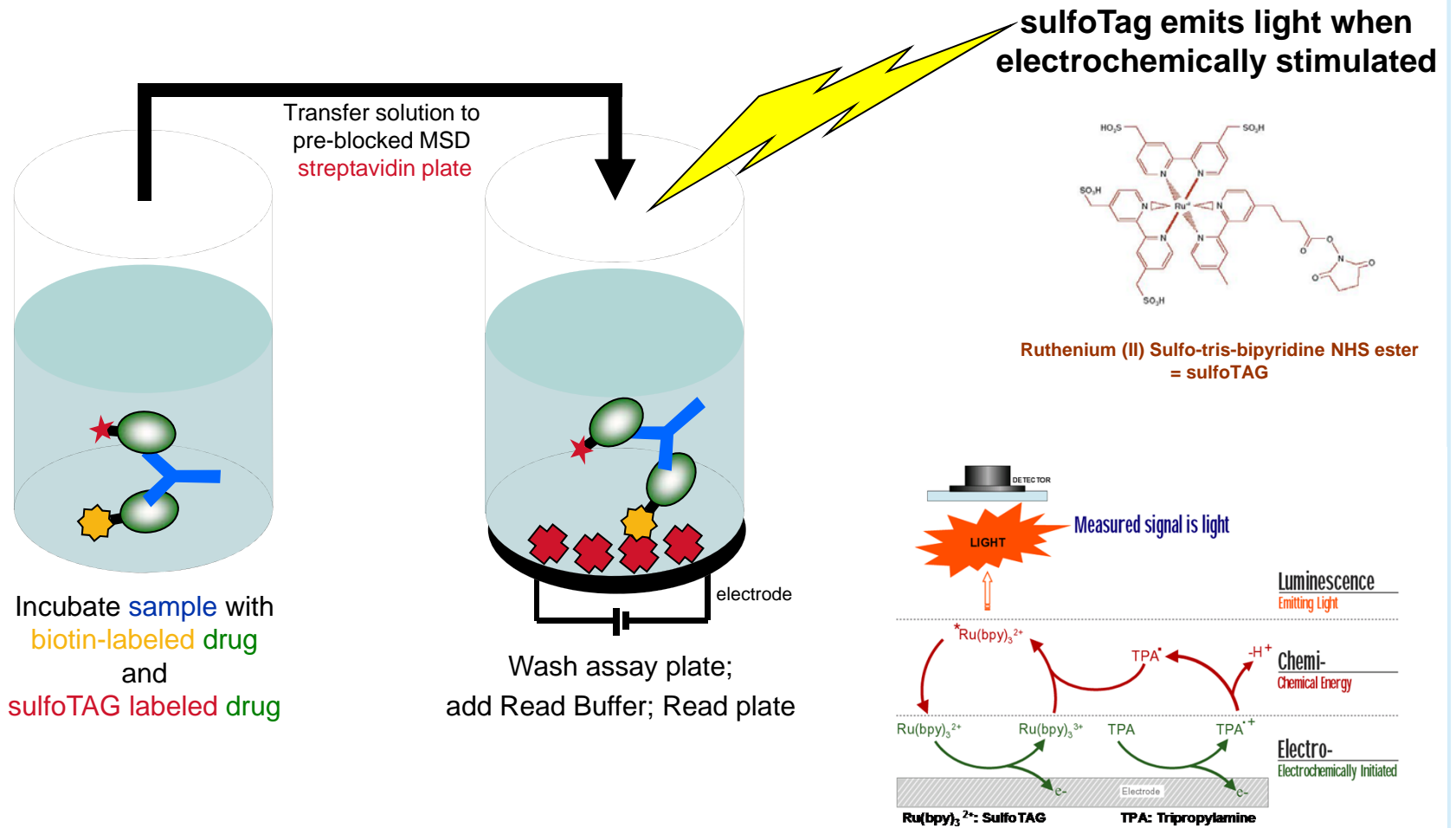


1st Tier: Screening Assay Bridging ELISA

Masking of binding epitopes by biotin



1st Tier: Screening Assay Electrochemiluminescence (ECL)



1st Tier: Screening Assay

Electrochemiluminescence (ECL)

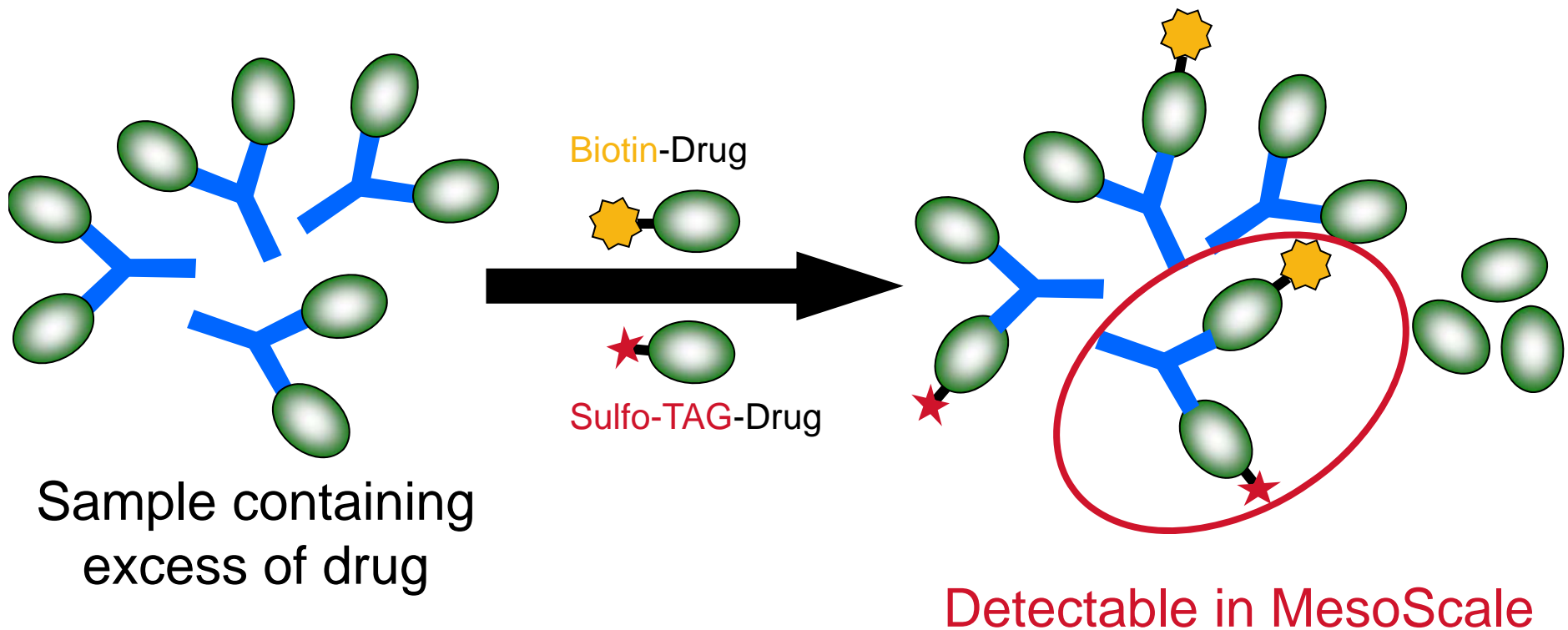
■ Advantages

- Electrochemiluminescence technology offers sensitivity and large dynamic range
- Less washing steps allow the detection of low affinity anti-drug antibodies
- Better tolerance for drug than ELISA
- Possibility for multiplexing (epitope mapping)

■ Disadvantages

- The use of two conjugated reagents increases the risk of masking of binding epitopes

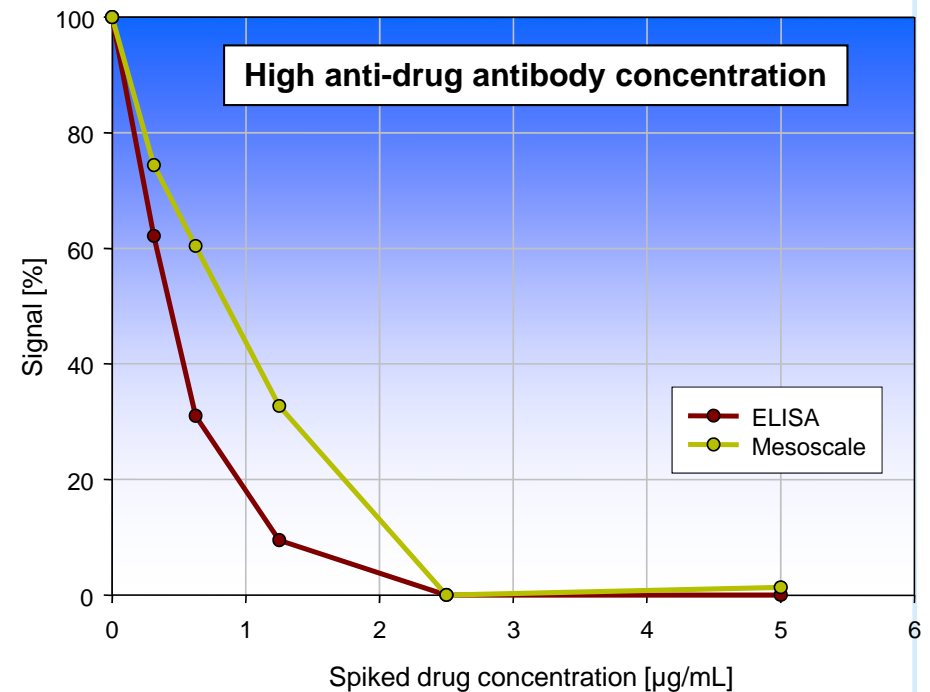
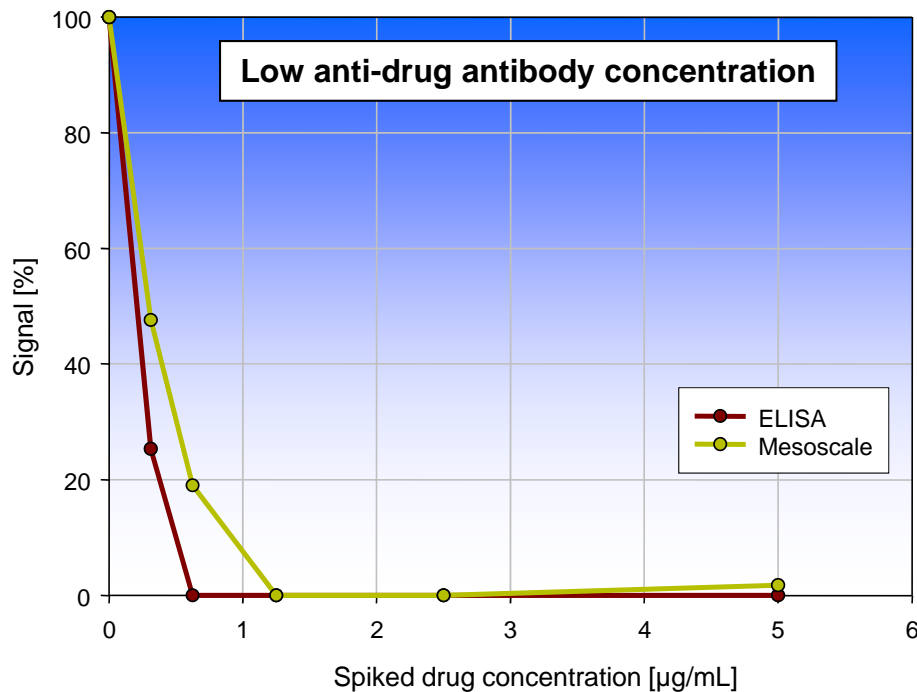
1st Tier: Screening Assay Electrochemiluminescence (ECL)



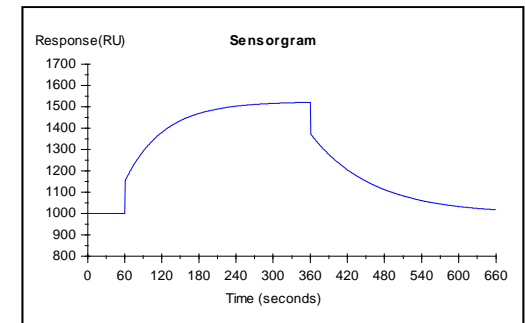
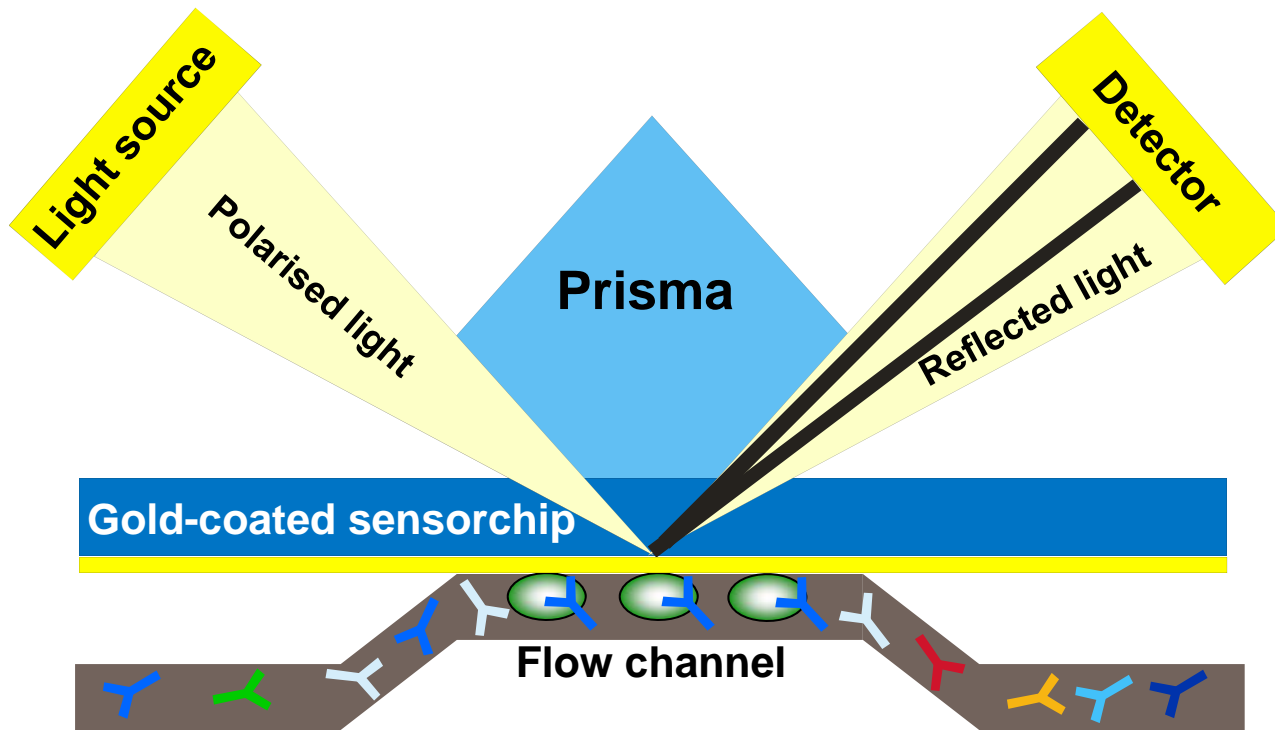
1st Tier: Screening Assay Electrochemiluminescence (ECL)

Drug Interference

Positive samples were spiked with increasing amounts of drug and analyzed in Mesoscale and (bridging) ELISA



1st Tier: Screening Assay Biacore

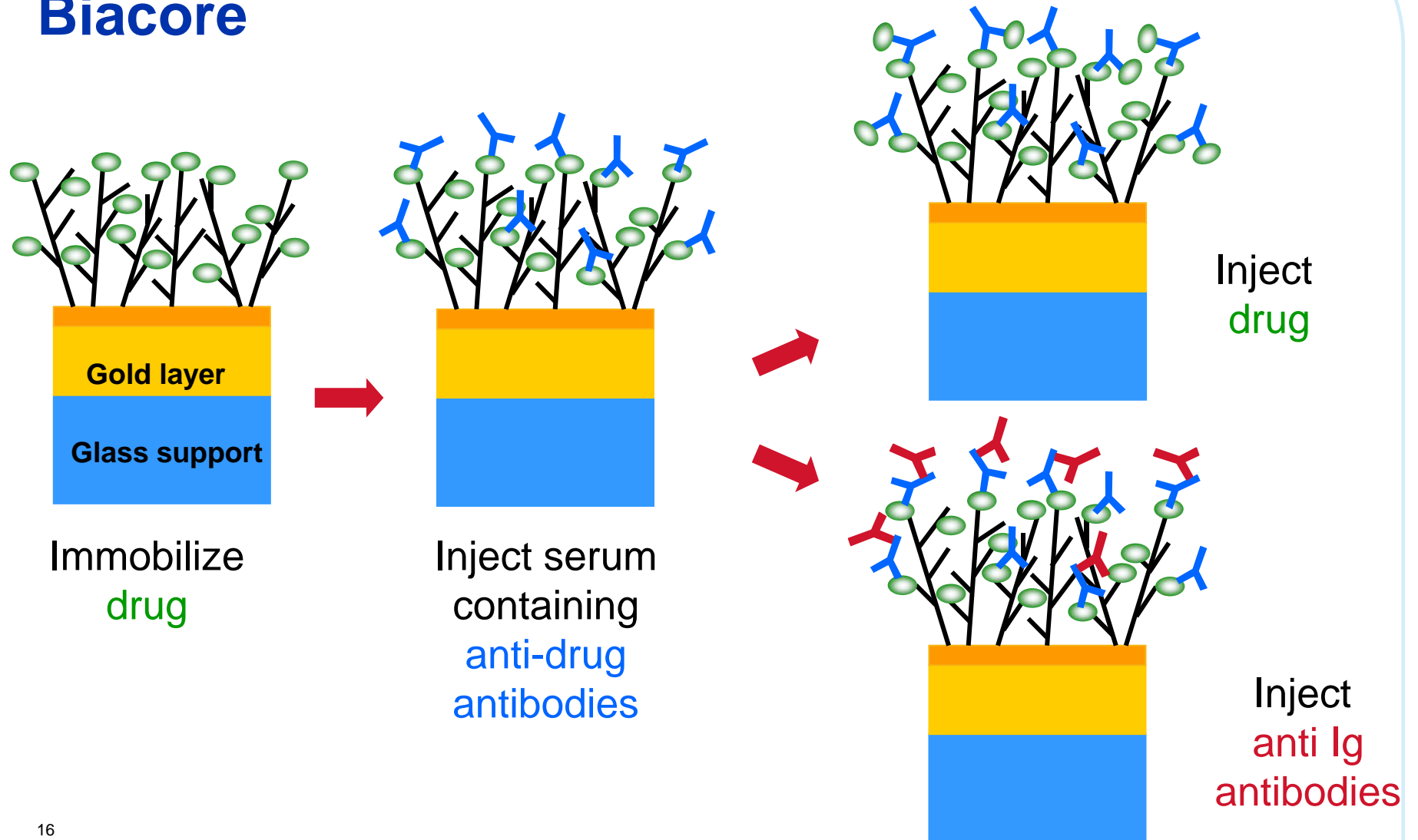


Drug immobilized on sensorchip

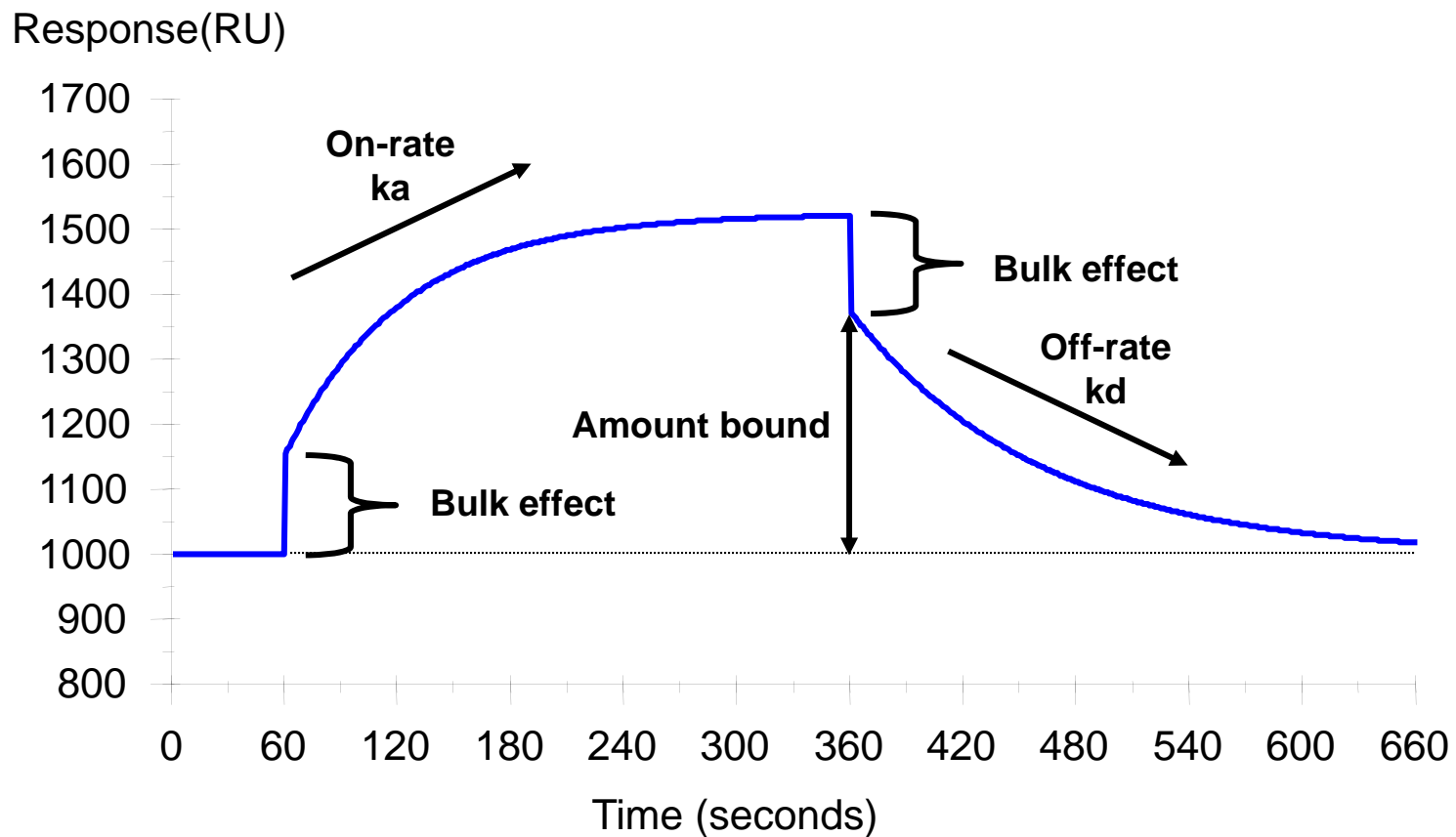


Injection of serum containing anti-drug antibodies

1st Tier: Screening Assay Biacore



1st Tier: Screening Assay Biacore



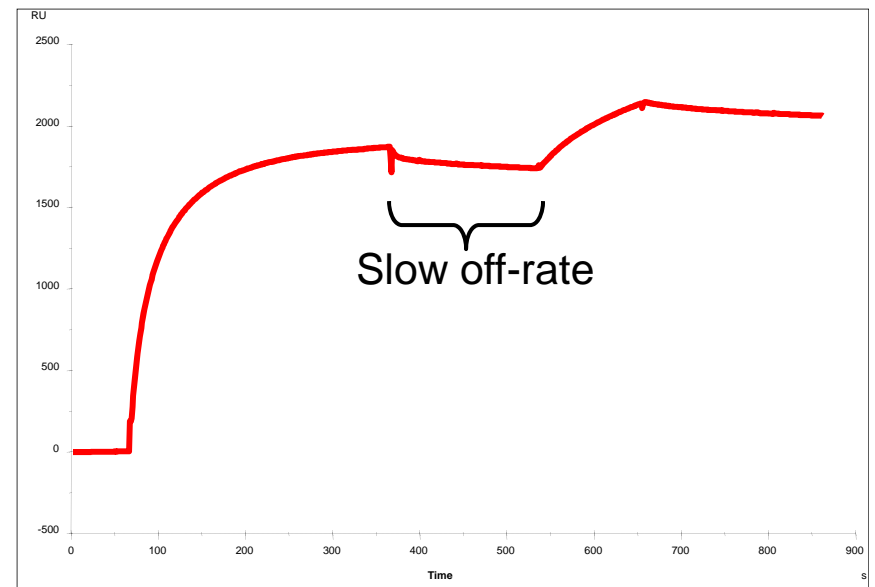
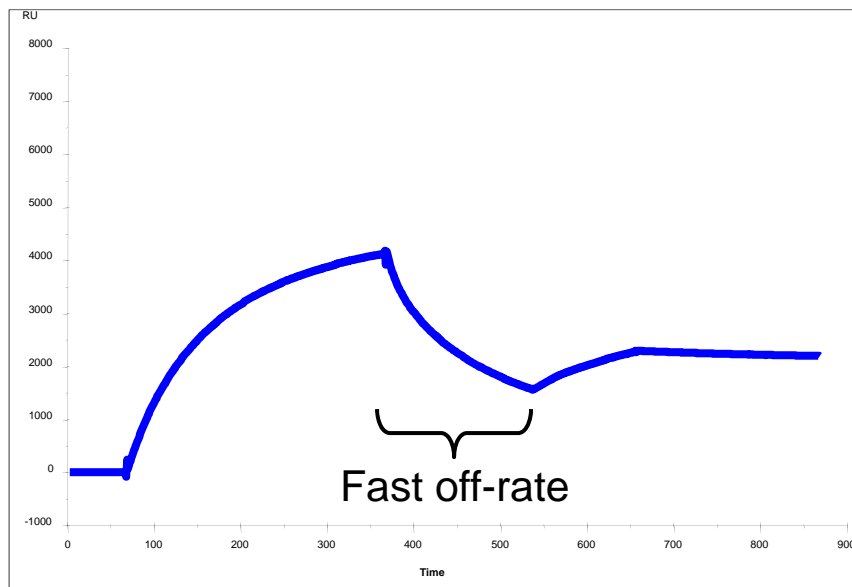
1st Tier: Screening Assay

Biacore

- Advantages:
 - Large dynamic range
 - No secondary reagents required
 - Detection of low affinity antibodies
 - Sensograms include information about affinity of anti-drug antibodies
 - Easy procedure for isotyping
 - Easy procedure for epitope mapping
- Disadvantages:
 - Structure of drug might be influenced by chemical coupling
 - Less sensitive than ELISA
 - Time consuming
 - Costs

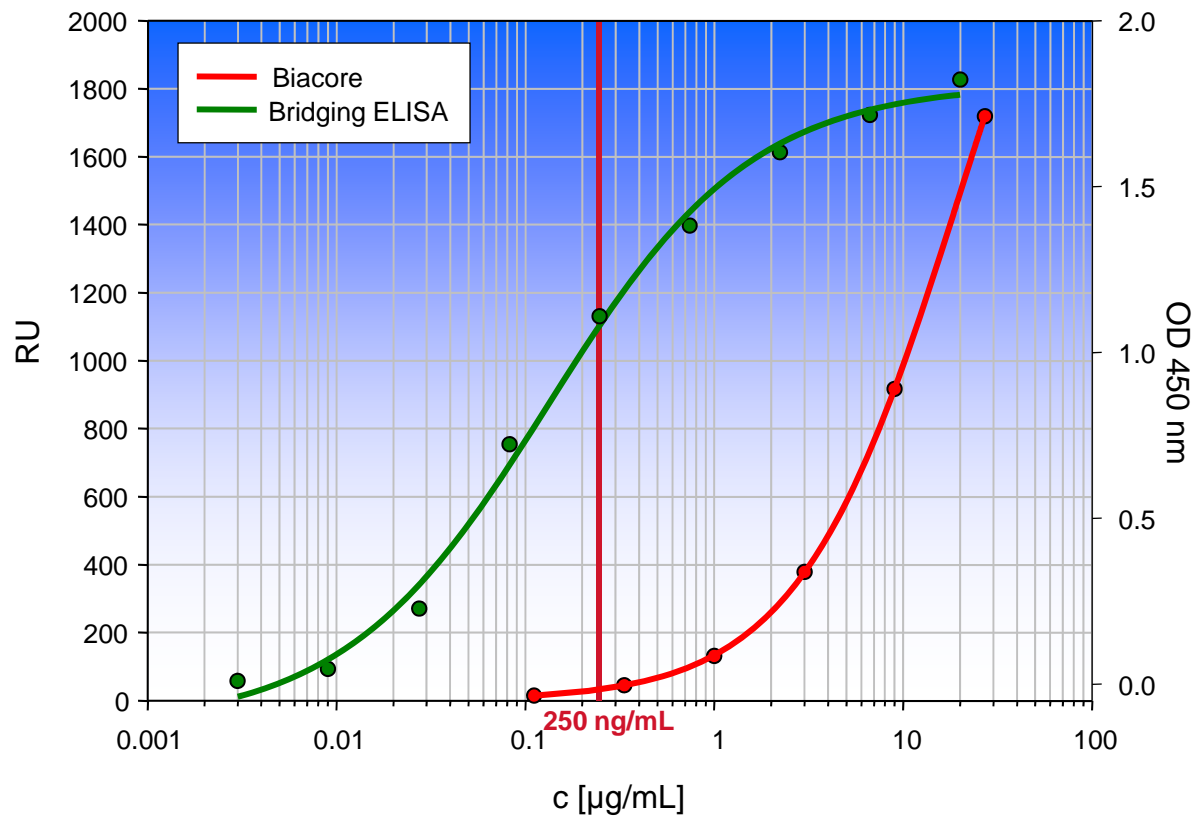
1st Tier: Screening Assay Biacore

- Sensograms contain information about affinity of anti-drug antibodies



1st Tier: Screening Assay Biacore

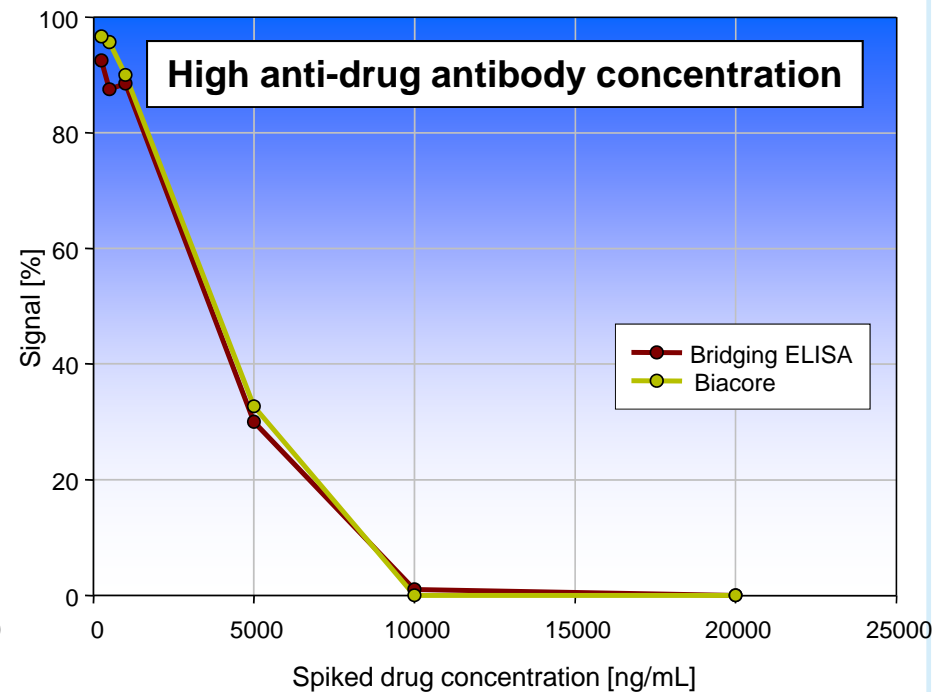
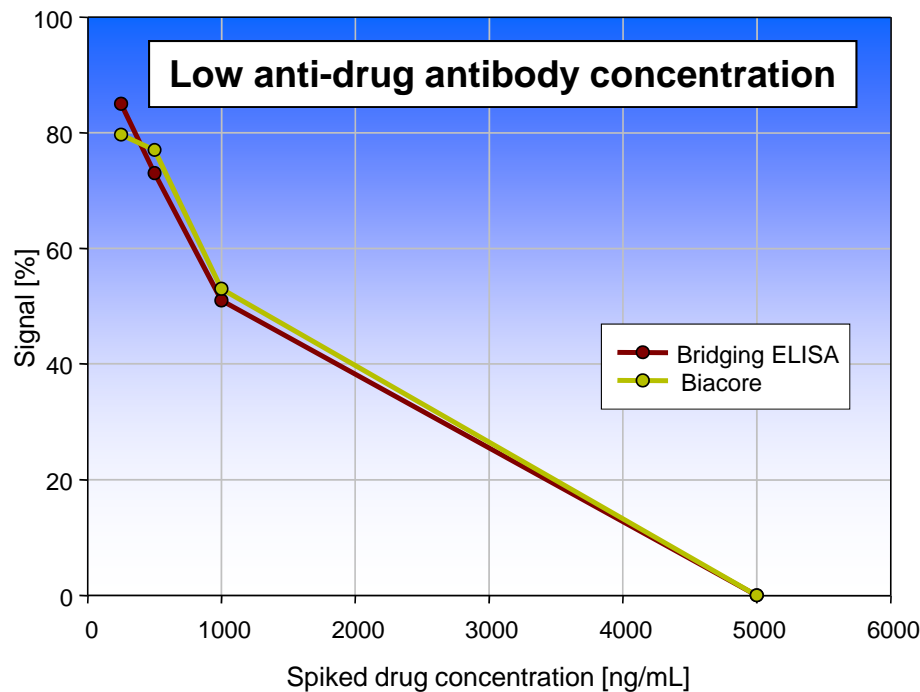
Sensitivity / Dynamic range



1st Tier: Screening Assay Biacore

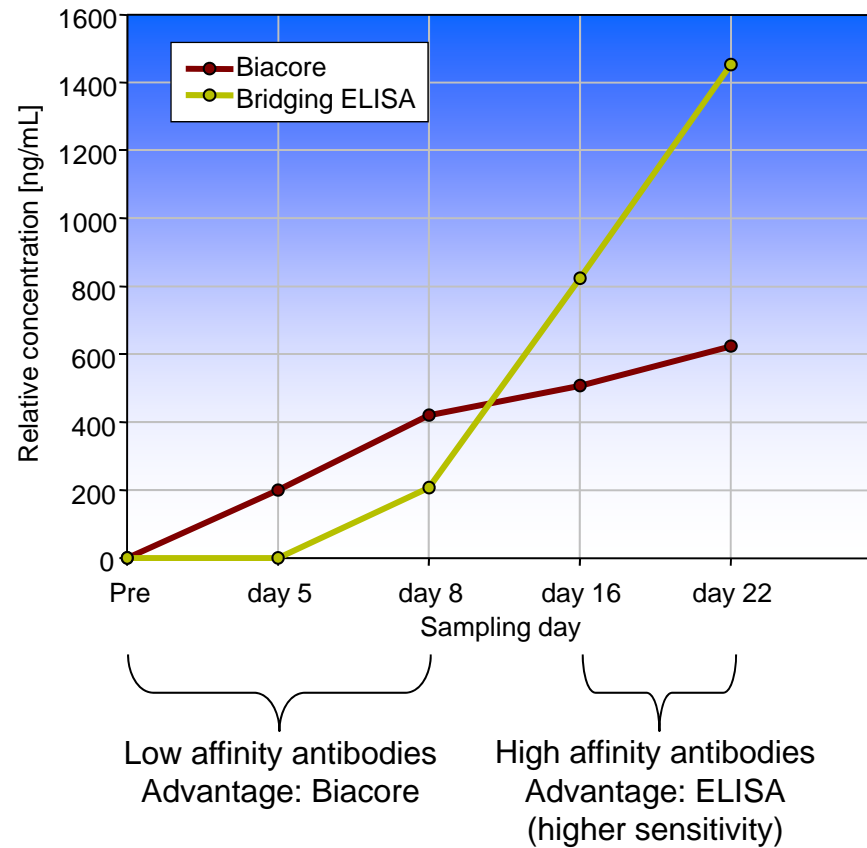
Drug Interference

- Positive samples were spiked with increasing amounts of drug and analyzed in Biacore and (bridging) ELISA



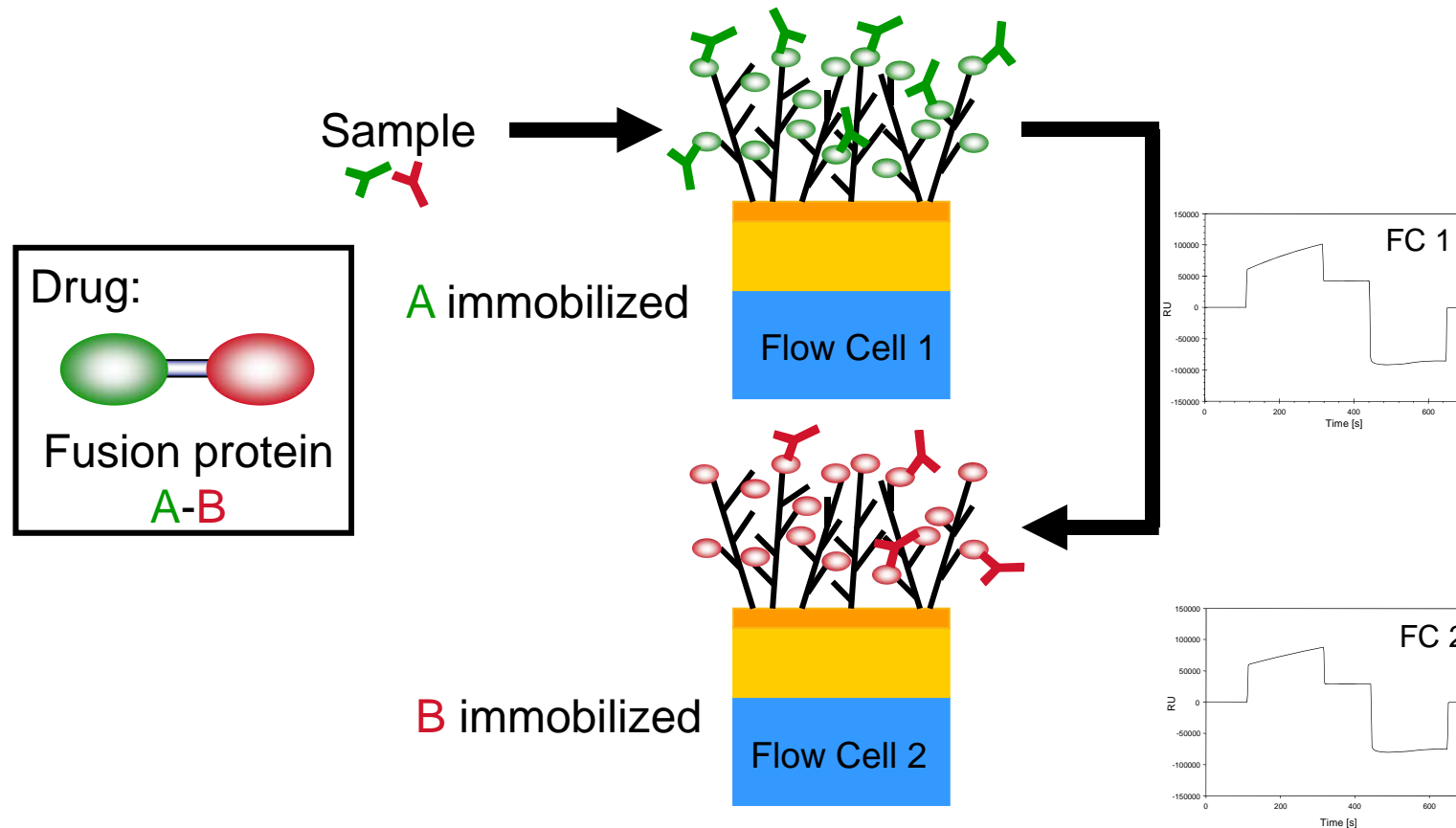
1st Tier: Screening Assay Biacore

Low Affinity Antibodies

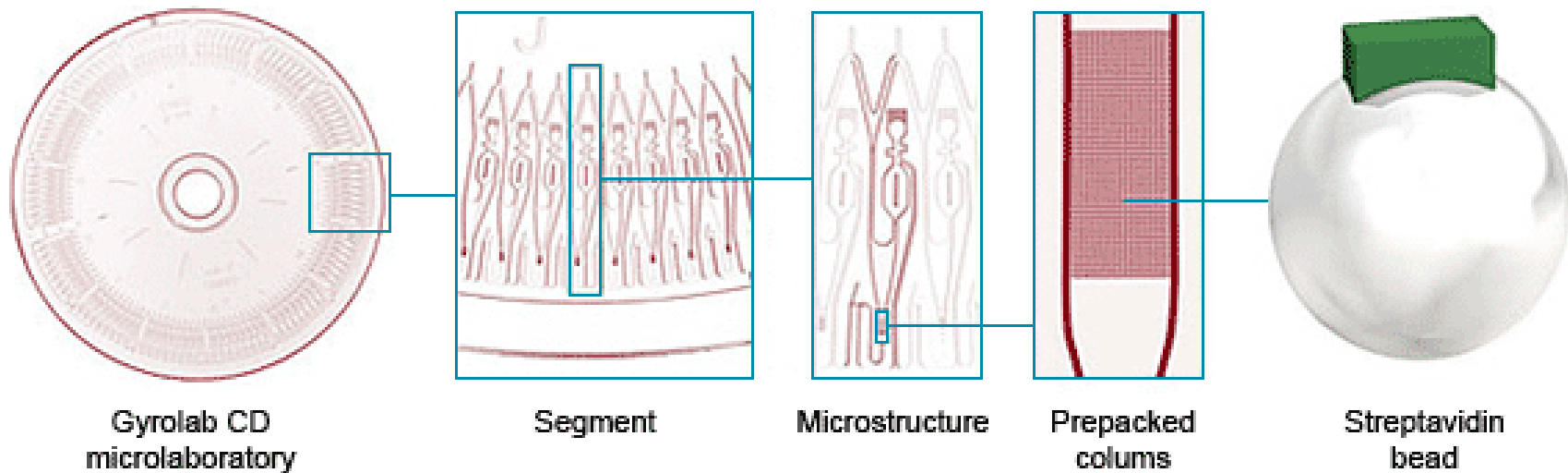


1st Tier: Screening Assay Biacore

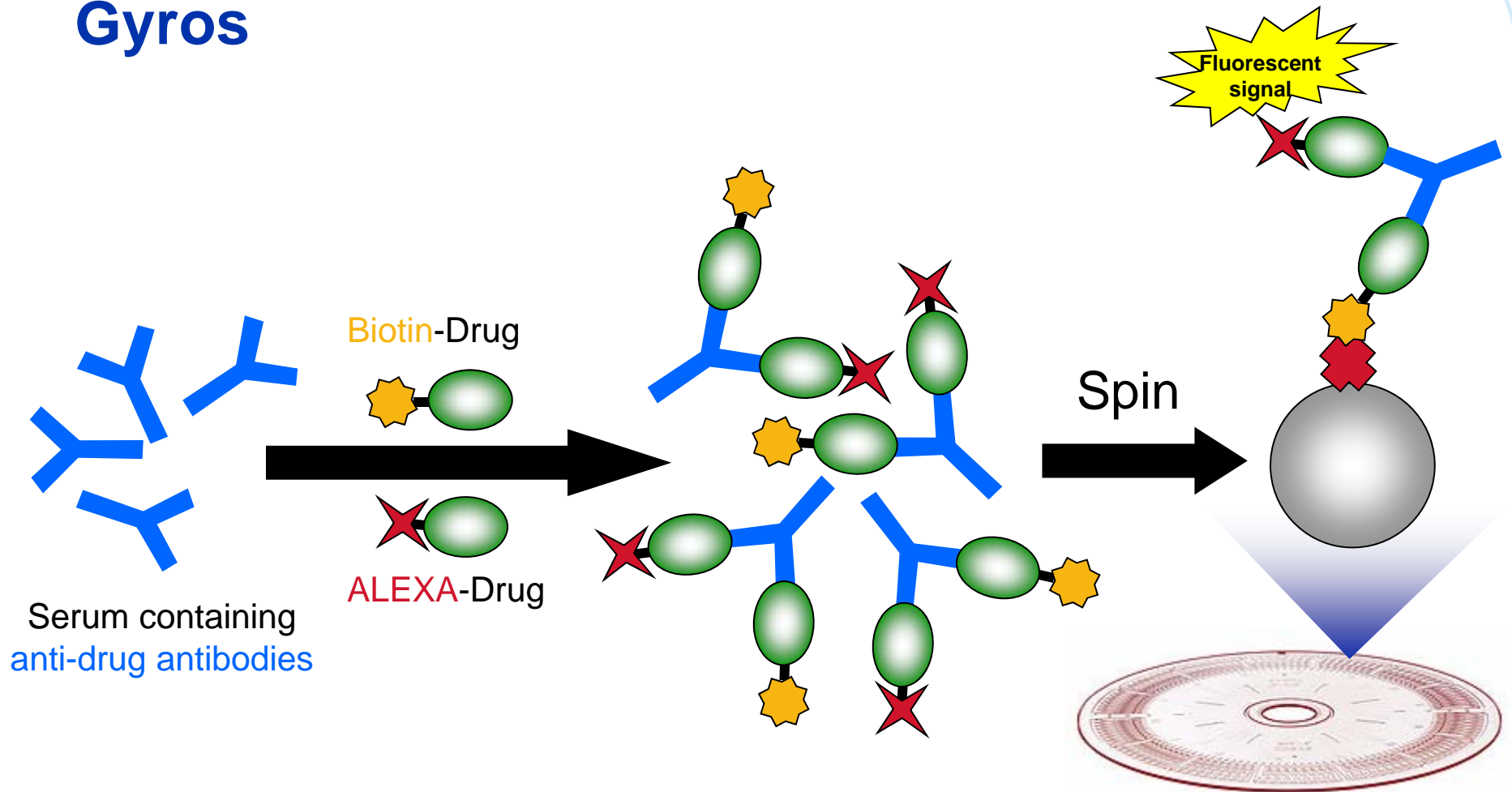
- Epitope Mapping



1st Tier: Screening Assay Gyros



1st Tier: Screening Assay Gyros



1st Tier: Screening Assay

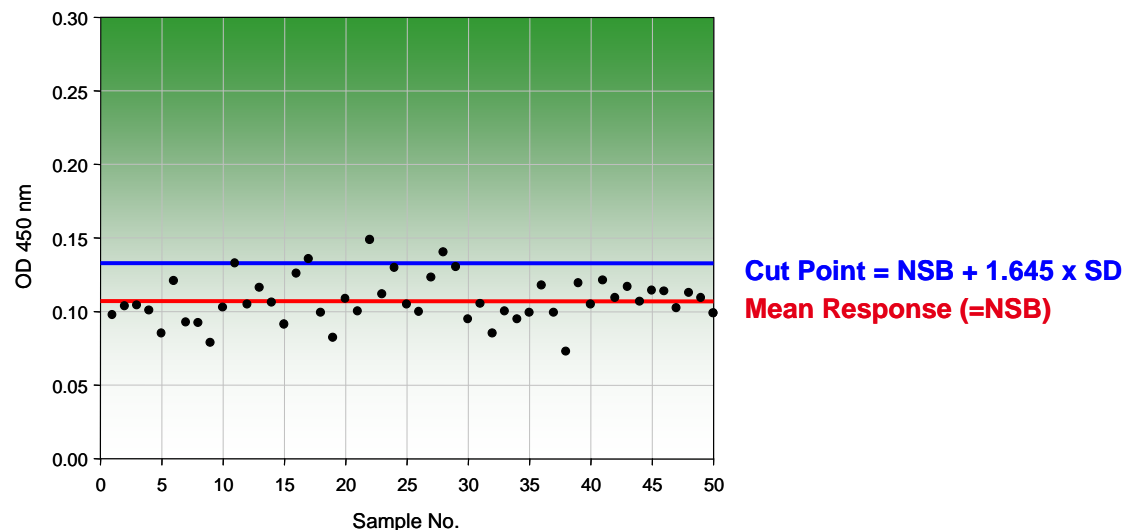
Gyros

- Advantages:
 - The Gyros technology offers sensitivity and large dynamic range
 - Detection of low affinity antibodies (homogenous format)
 - Rather high drug tolerance (homogenous format)
 - Requires only small sample volumes
 - Epitope mapping possible
 - High throughput
 - Automatization reduces variability (less manual pipetting steps)
- Disadvantages:
 - The use of two conjugated reagents increases the risk of masking of binding epitopes
 - Costs
 - Carry over

1st Tier: Screening Assay Cut-Point

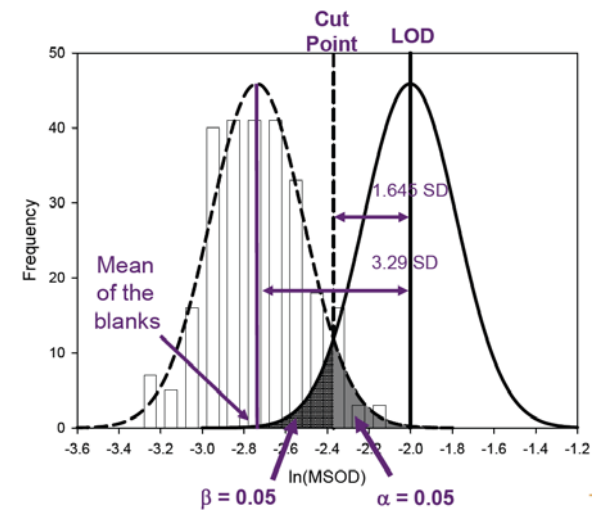
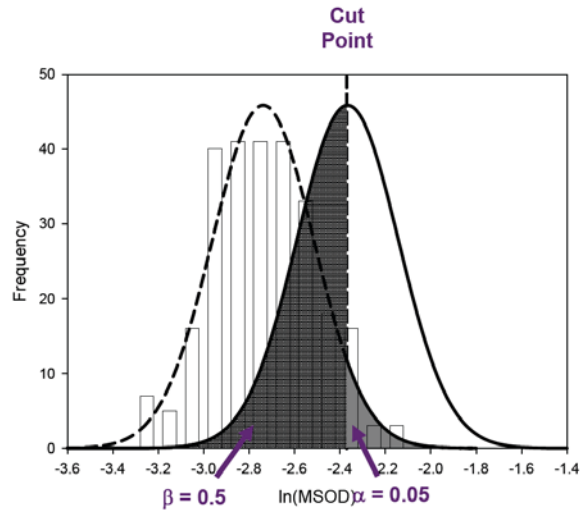
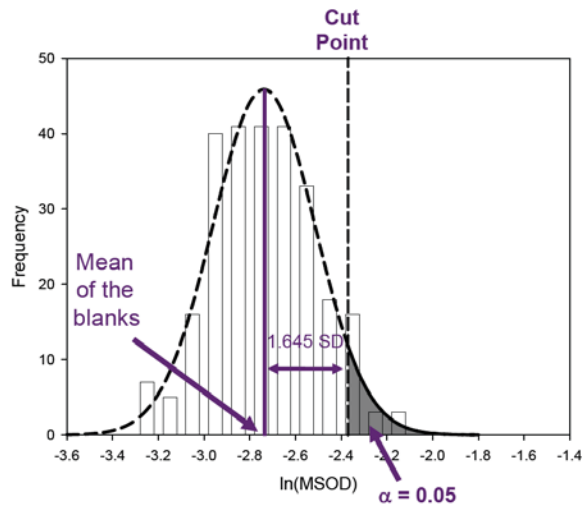
Problem: Immunogenicity is a relative thing => criteria for positive samples needed

- Determination of the „non specific background“ (NSB) by testing of 50 serum samples of untreated animals or patients on three different days
- Cut-Point: $NSB + 1.645 \times \text{Standard Deviation}$ (=> 5 % false positives)
- Positive: Response \geq Cut-Point



1st Tier: Screening Assay

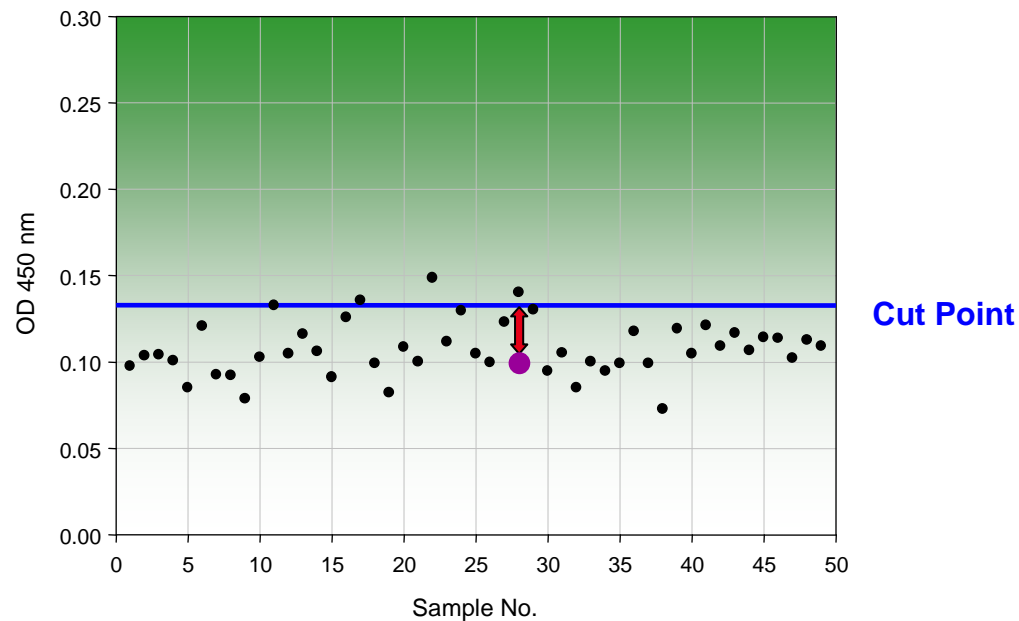
Some Statistics



1st Tier: Screening Assay

Normalization of Cut-Point

Problem: Usually assay signal will vary between runs => Cut-Point normalization necessary

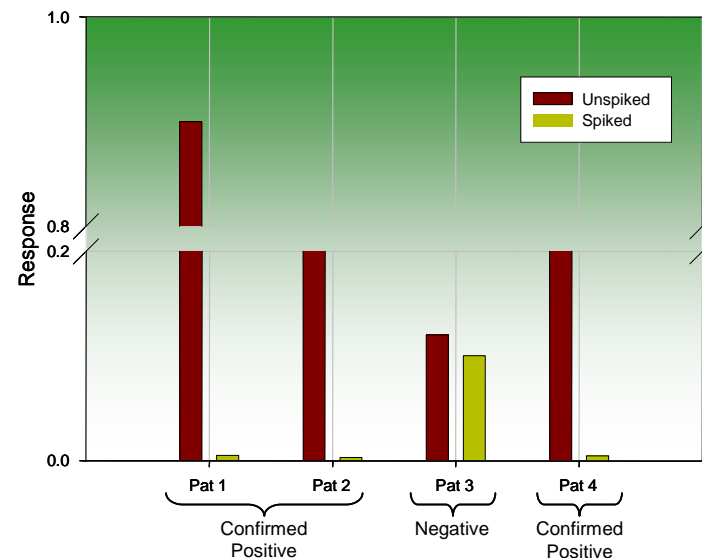


Normalization factor = relative response Cut-Point – relative response negative control

For each batch the normalization factor is added to the relative response of the negative control to set the cutpoint

2nd Tier: Confirmatory Assay

- Due to the 5% false-positive rate built into the screening cut point, samples showing a response at or above the assay cut-point can just be considered “putative positive” for the presence of BAbs.
- The confirmation of true positives among the putative positive samples requires the demonstration of specific binding to the drug:
 - A putative positive sample is re-tested in the presence and absence of an excess of drug.
 - The specificity cut point is defined as the percent inhibition at or above which a sample is considered as “confirmed positive”.

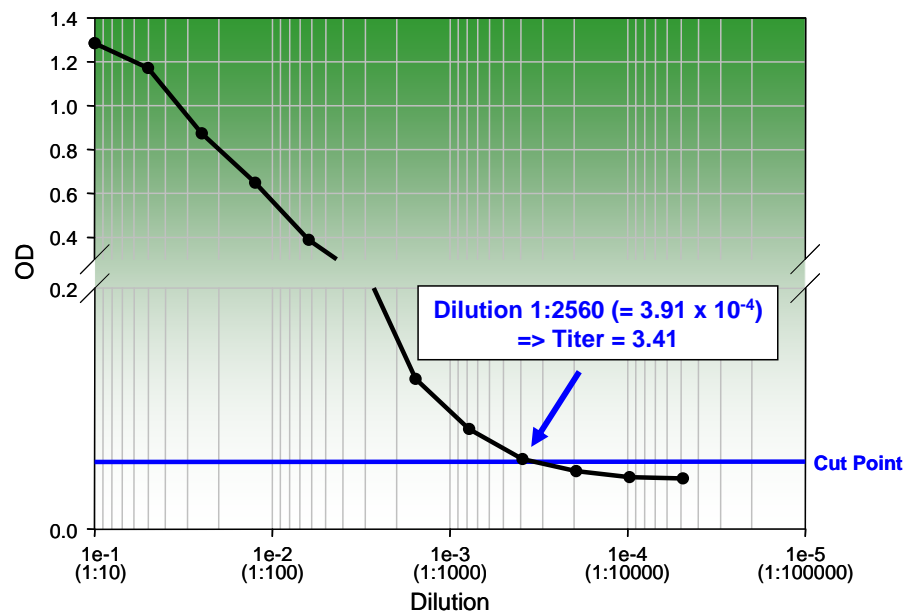


2nd Tier: Confirmatory Assay Specificity Cut-Point

- Spike all individual samples from the cut-point determination (preferable in the same experiment) with an excess amount of drug and calculate the percent inhibition per sample: $100 \times [1 - (\text{spiked}/\text{unspiked})]$
- Calculate the specificity cut-point from the percent inhibition of all samples:
Upper bound of a one-sided 99.9 % prediction interval (parametric: mean + 3.09 x SD or non-parametric: 99.9th percentile)
- A real sample in study showing a higher % inhibition after spiking of drug than the specificity cut-point is defined as „confirmed positive“

3rd Tier: Titration

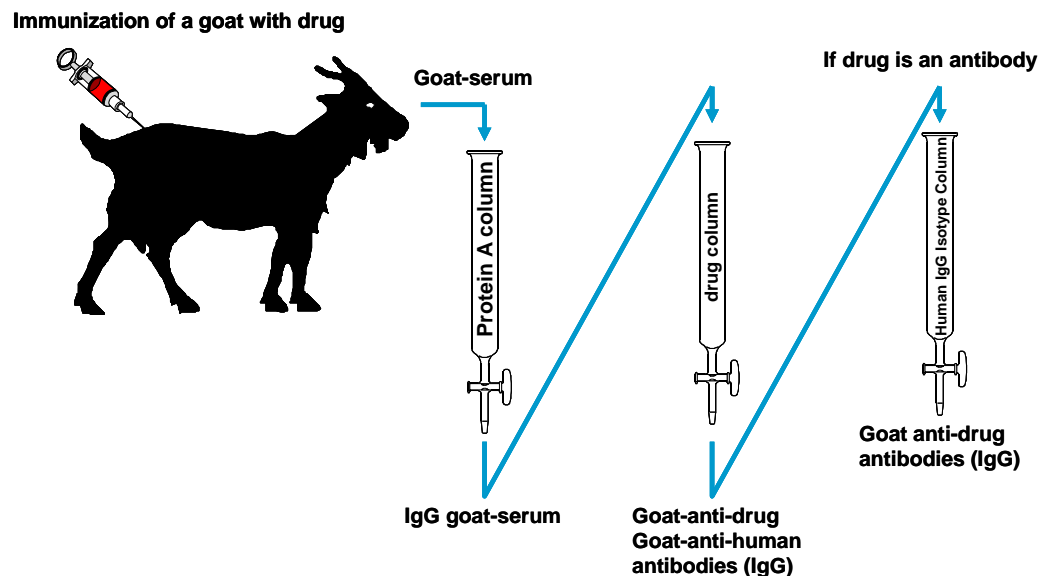
- Aim:
 - Retrieve quasi-quantitative information for confirmed positive samples
- Procedure:
 - Serial dilution of confirmed positive samples
 - Titer = $-\log$ dilution factor of the last dilution that tests positive



Challenges In Immunogenicity Testing

Positive Control

- In contrast to PK assays the analyte is not available in purified form
- Serum from animals (e.g. goats) hyperimmunized with the drug is used as control instead
- This surrogate control substantially differs from the measured human anti-drug antibodies in respect to affinity and avidity
- Consequently no exact numbers (e.g. for sensitivity) can be reported for Immunogenicity assays (but numbers relative to the positive control)



Challenges in Immunogenicity Testing

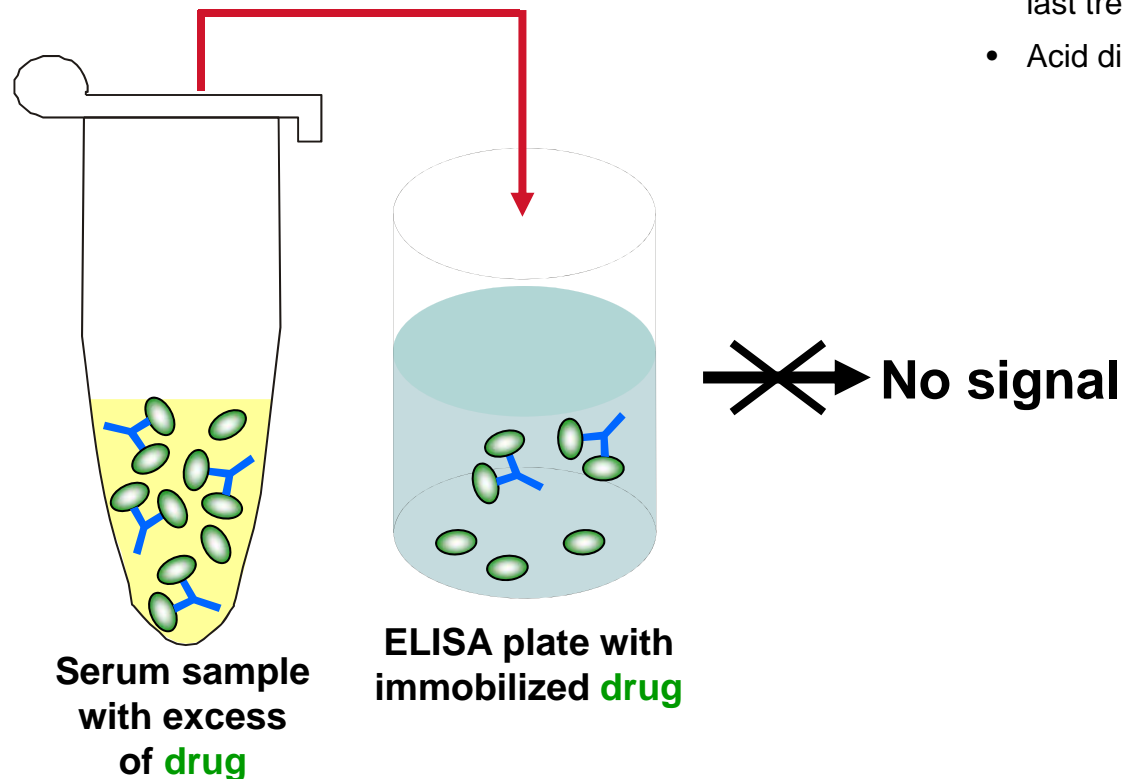
Drug Interference

- The presence of major amounts of drug interferes with the detection of anti-drug antibodies and leads to “false negatives”

- Solutions:

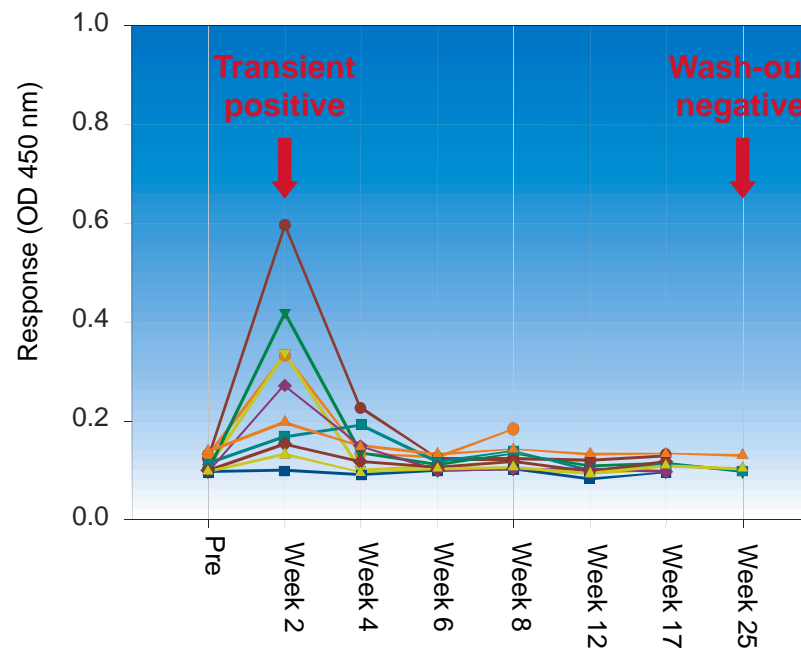
- Wash-Out Samples

- Draw blood samples for the detection of anti-drug antibodies several days/weeks after the last treatment (5-6 x $t_{1/2}$)
- Acid dissociation of the immunocomplexes

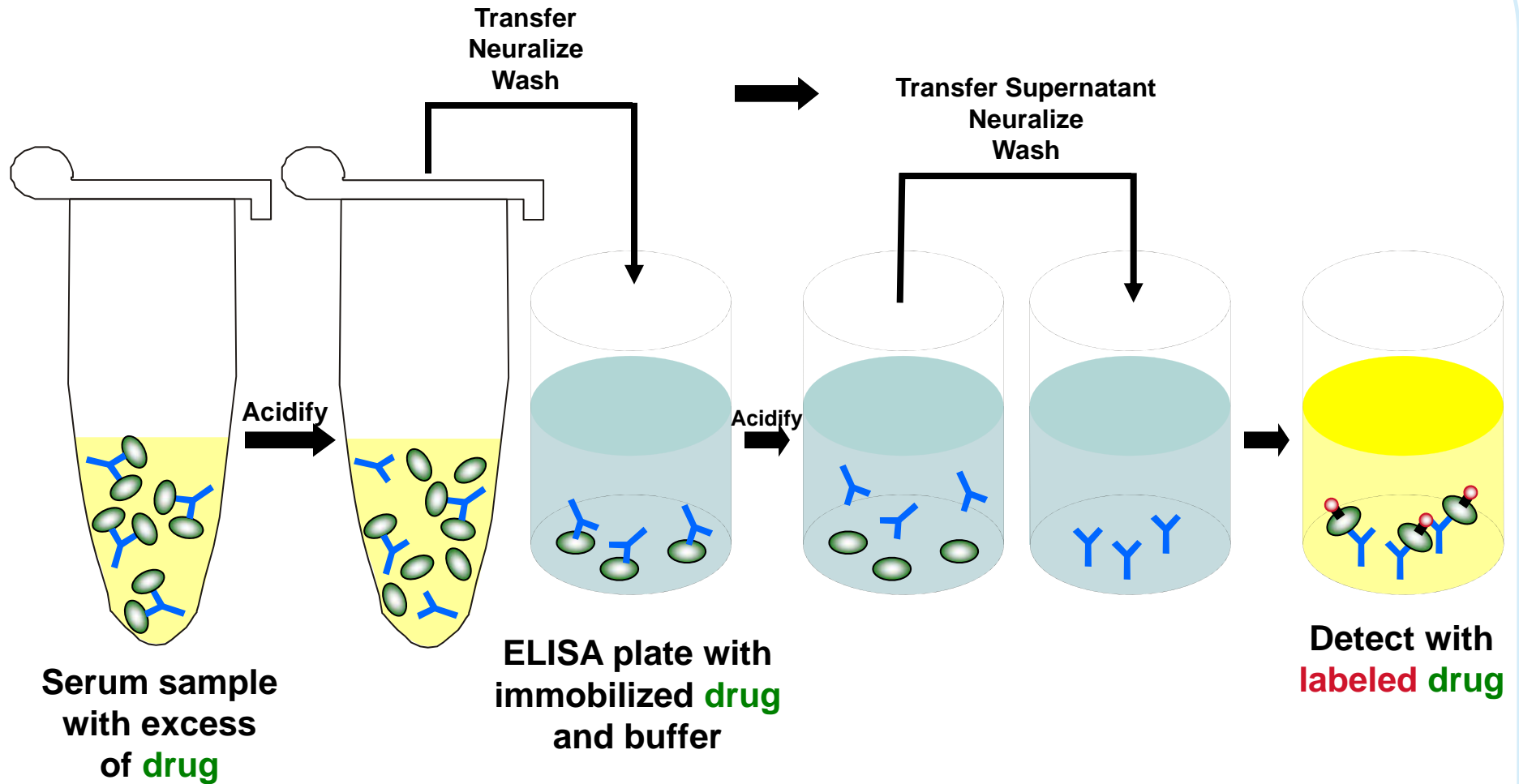


Wash-Out Samples

- Draw blood samples for the detection of anti-drug antibodies several days/weeks after the last treatment (5-6 x $t_{1/2}$)
- Problem: A transient immune response might not be detected in wash-out samples => acid dissociation assays might be needed



ACE Acid Dissociation Assay



THANK YOU !