Design and Validation of a Non Cell-based Receptor Binding Assay for the Detection of Neutralizing Antibodies to a Biological Therapeutic

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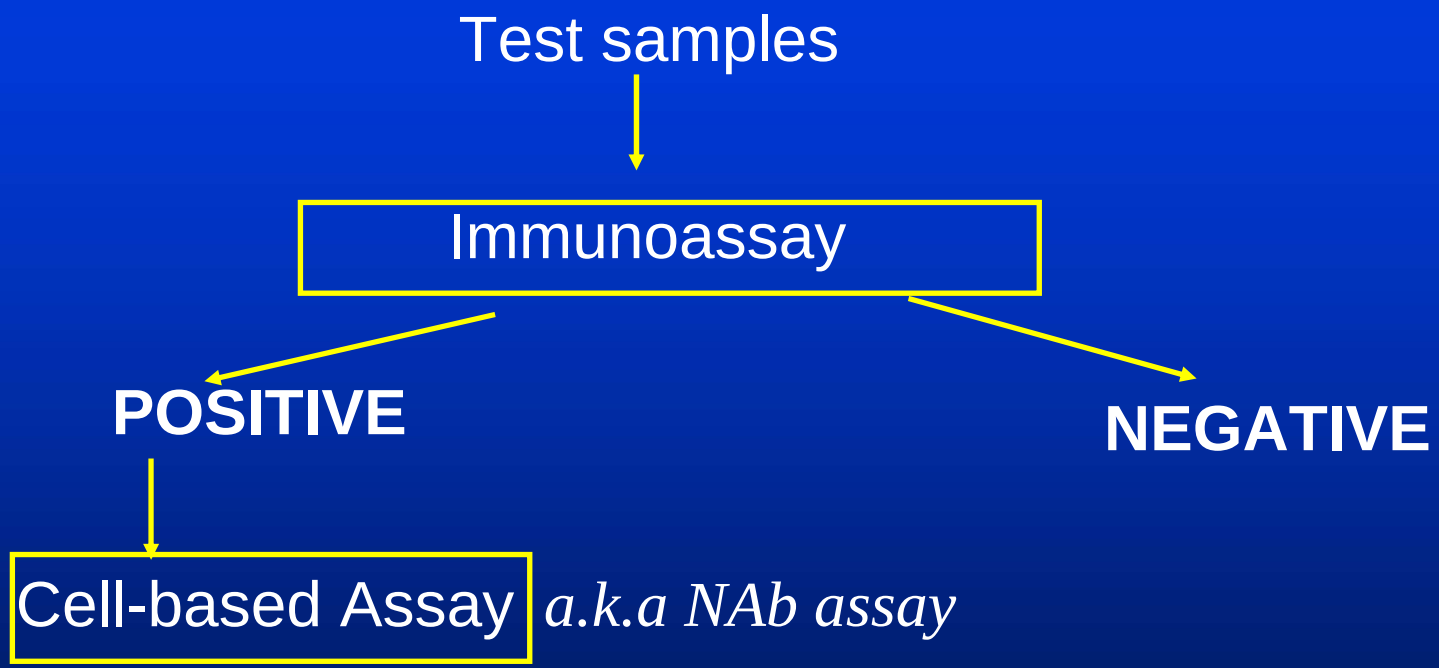
Immunogenicity Testing



Provide a functional biological system to assess if the Abs detected by the immunoassay have neutralizing capability

Cell-based NAb Assay Designs

- Utilize intracellular signaling events triggered within a cell line that responds to :
  - * the drug product (eg. cytokines)
  - * the ligand inhibited by the drug product (eg. MAbs)
- May also utilize study of extracellular binding events at the cell surface (eg. MAbs)

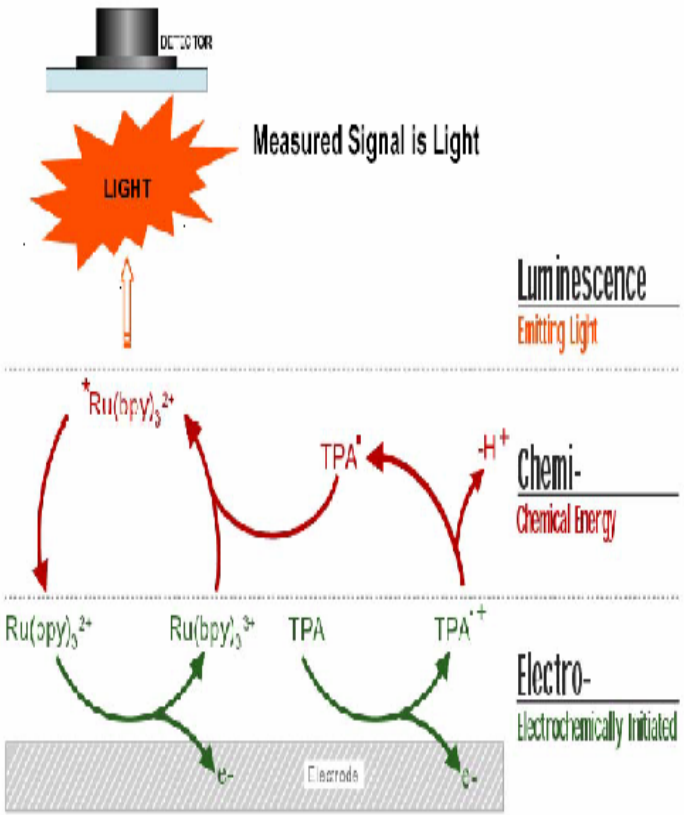
Requirements for Cell-based NAb Assays

- Stable cell line responsive to drug
- Measurable Readout (cpm, OD, conc of secreted protein)
- Robust signal to noise ratio in serum matrix
- Availability of a positive control antibody to the drug
- Medium to High Throughput
- Adaptable to Automation

Justification For a Non-cell Based NAb Assay

- Drug product : blocked ligand activity
- Limited choice of cell lines expressing the receptor for ligand that was target for drug product
- Only cell line available yielded at best 2-fold signal when treated with 100-200 ng/mL ligand
- Cell line had finite life span
- Difficult cells to culture
- Low throughput (24-well plates)
- Irreproducible bioassay results

Meso-Scale Discovery (MSD) Technology



Meso-Scale Discovery (MSD) technology employs microtitre plates fitted with a series of electrodes associated with the bottom of each well.

Using an MSD Sector PR™ plate reader, an electrical current is placed across the plate-associated electrodes in the presence of a Tripropylamine (TPA) containing buffer.

The result is a series of electrically induced oxidation-reduction reactions involving Ruthenium (from the captured complex) and TPA leading to a luminescent signal. The consequent electrochemiluminescent (ECL) signal is measured by photodiodes and is quantified as a relative unit (RU).

Courtesy of Mesoscale Discovery, Gaithersburg, MD

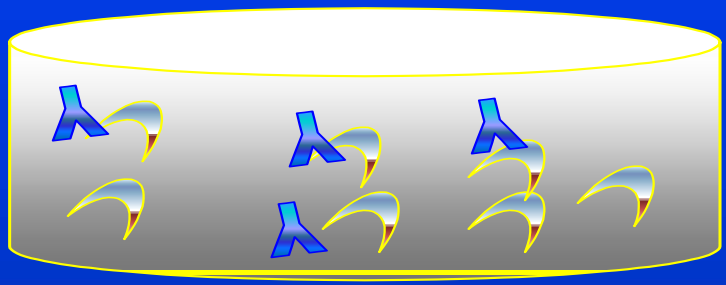
Assay Reagents

- Purified Soluble receptor (ruthenylated)
- Purified Ligand (biotinylated)
- Drug product (TP)
- Streptavidin-coated MSD Plates
- Cynomolgus monkey serum
- Affinity purified rabbit anti-TP antibody

Assay Methodology

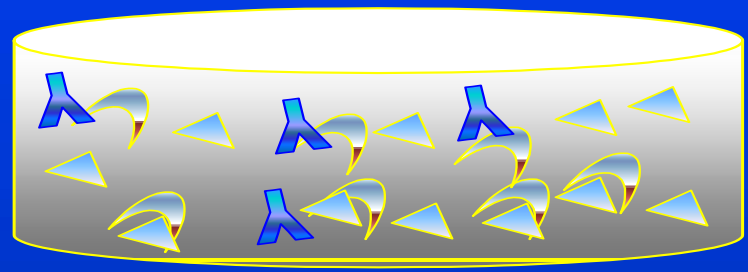
Assay Format

1.



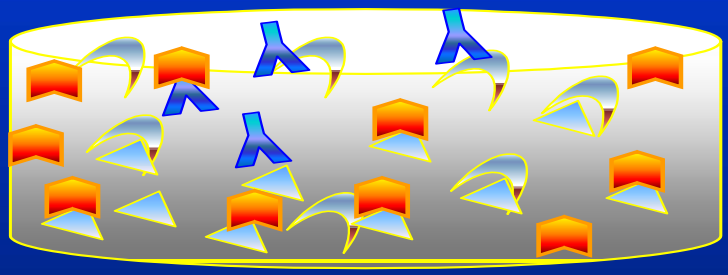
Incubate serum sample with TP – 15-30 Min

2.



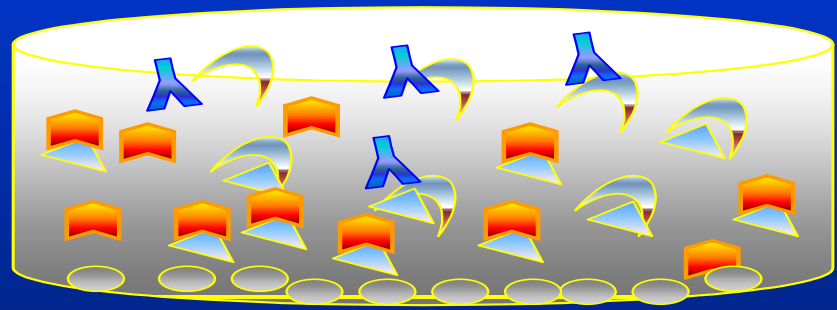
Add L-Biot – 15-30 Min

3.



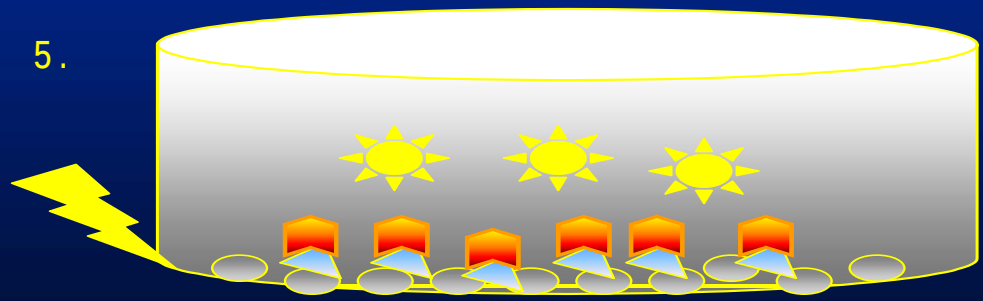
Add R-Ru – 15-30 Min

4.



Add Mixture to blocked SA-Coated Multiarray Plate – 30-60 Min

5.



Wash 2X and add TPA
Containing read buffer
Read on MSD reader.

R-Ru

Anti-TP Antibody

L-Biot

TP

Streptavidin

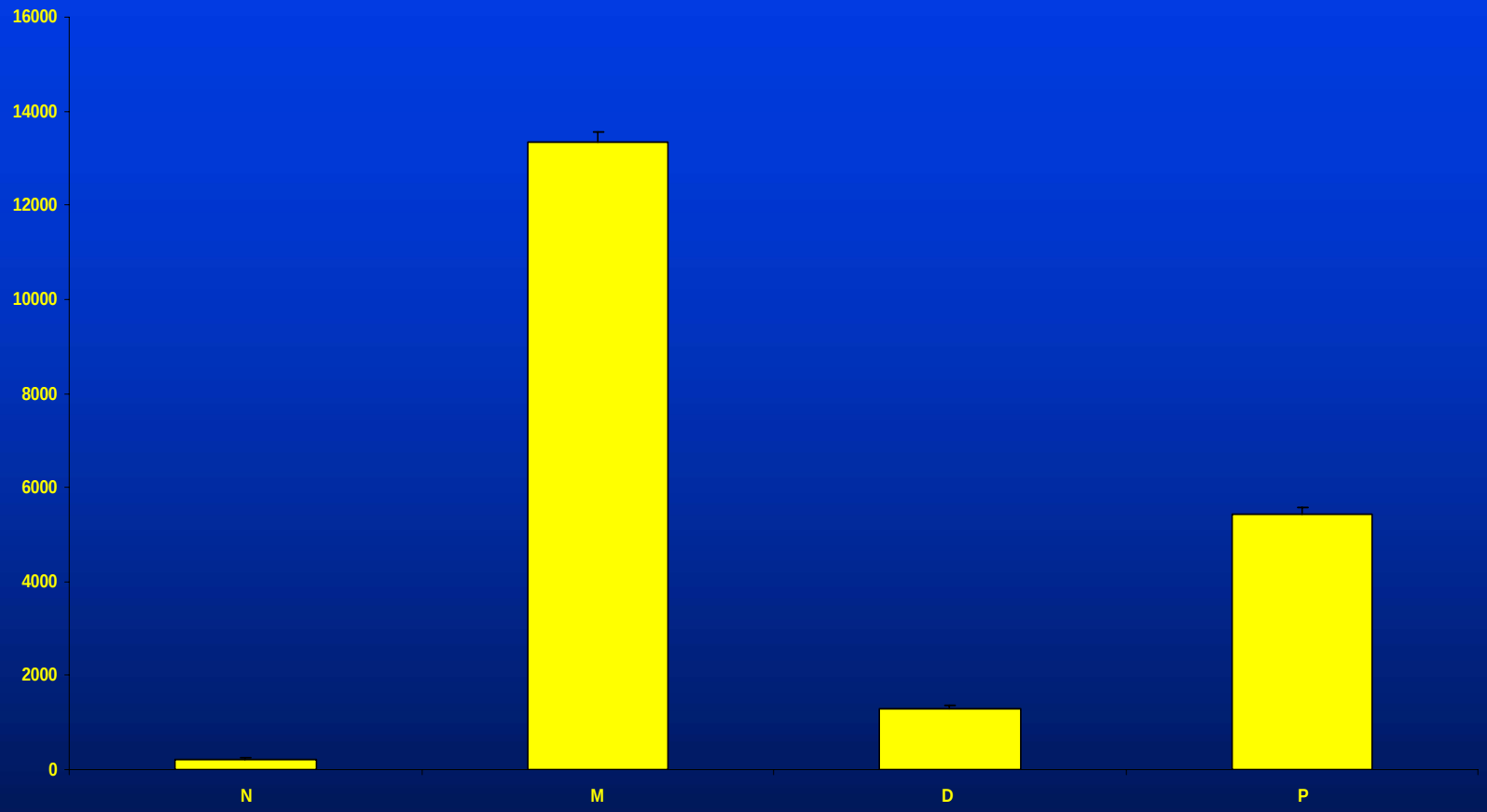
Assay Development

- Optimized ratio between Ru-R and Biot-L for optimal signal
- Used 50% cyno serum for initial development expts
- Due to donor (n=10) variability serum was reduced to 15%
- Optimized order of mixing of reagents (capture vs. homogenous)
- Improved precision and inter-day repeatability in homogenous format with assay controls

Optimized Assay Conditions

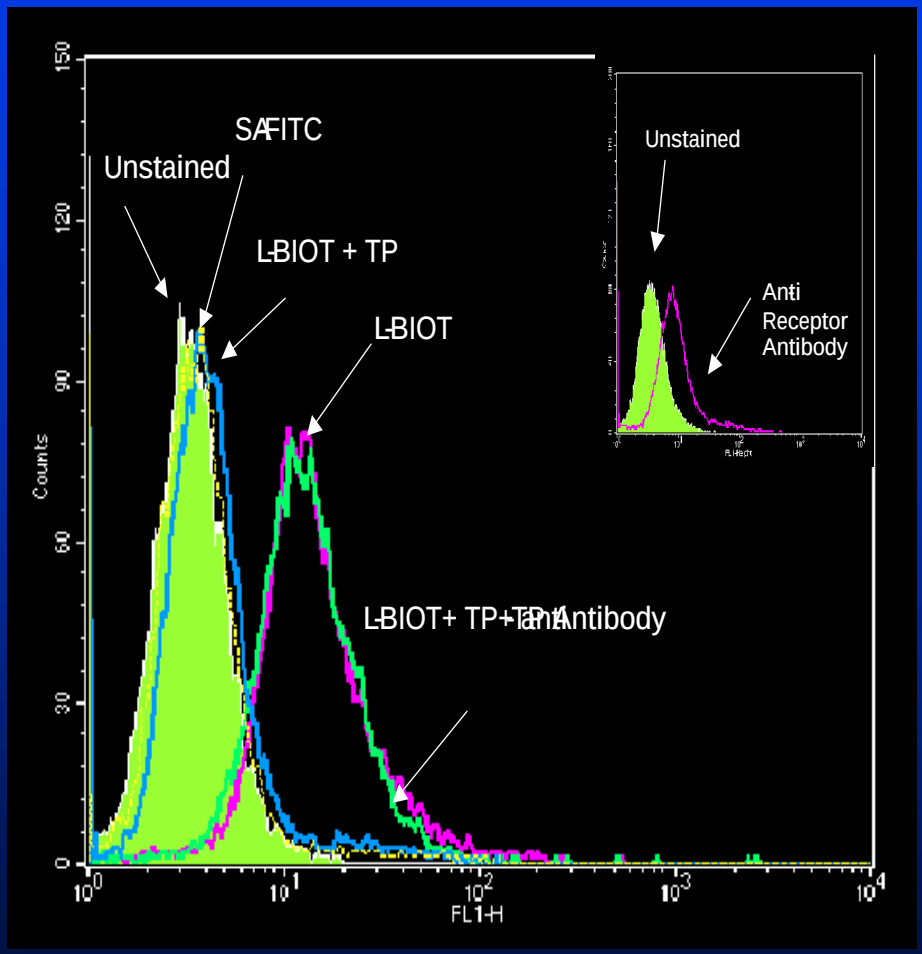
- 15% cynomolgus monkey serum
- 250 ng/mL Biot-Ligand
- 900 ng/mL Ru-R
- 80 ng/mL TP

Assay Controls



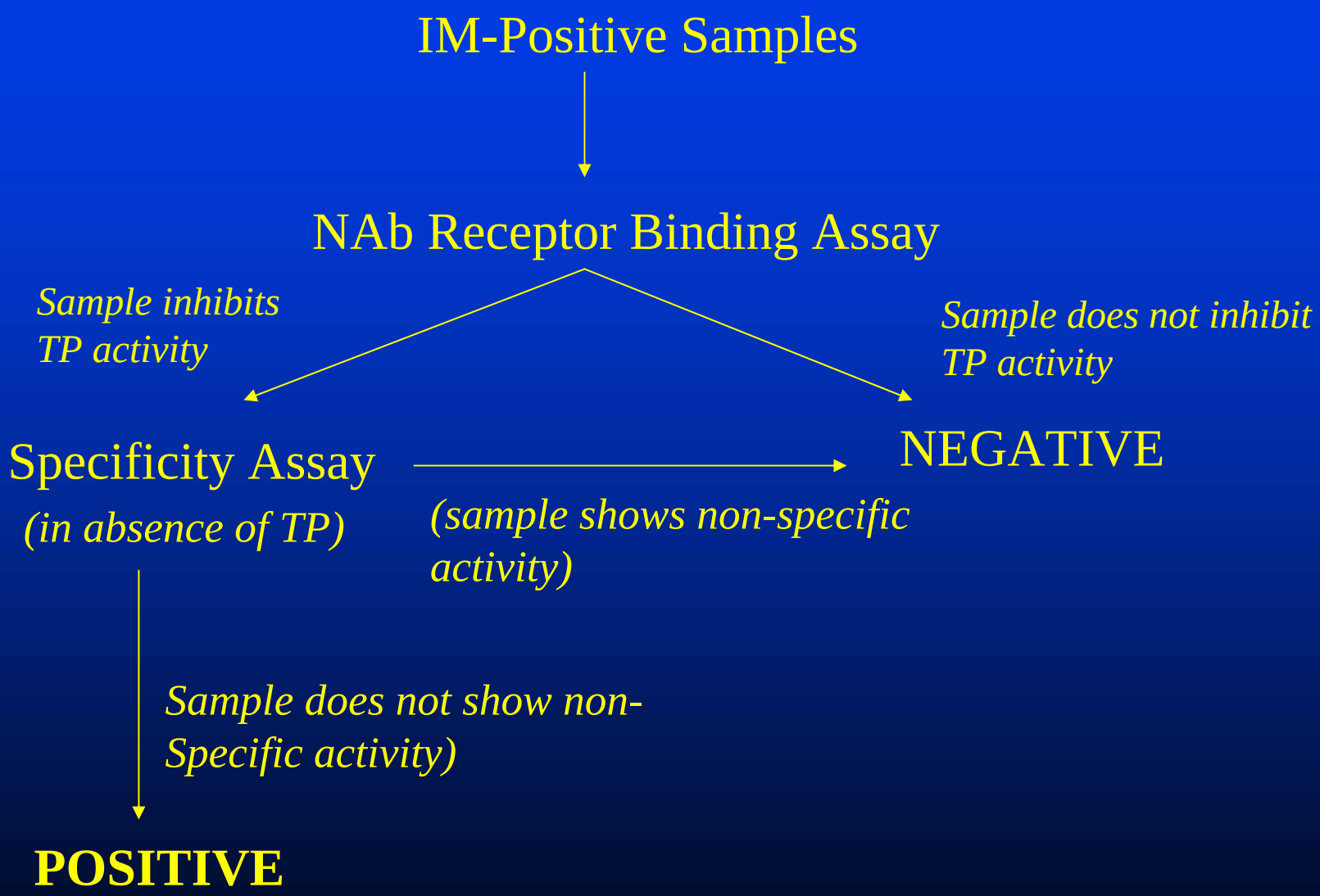
N = background (15% PCS with R-Ru)
M = maximum binding (15% PCS with R-Ru and L-biot)
D = drug control (15% PCS with TP, r-RU and L-biot)
P = positive control (D with anti-TP antibody)

FACS Analysis of a Cell Line Naturally Expressing the Target Receptor



- **A cell line naturally expressing the receptor was used to demonstrate:**
  - **L-Biot binding, inhibition of binding by TP**
  - **Restoration of binding by the anti-TP NAb.**
- **Binding of L-Biot to the receptor was detected using a SA-FITC conjugate.**
- **Receptor expression was confirmed using a biotinylated goat polyclonal anti-receptor Ab and detected with SA-FITC.**
- **Binding of L-Biot to the target receptor was inhibited by the TP and restored by the anti-TP NAb.**

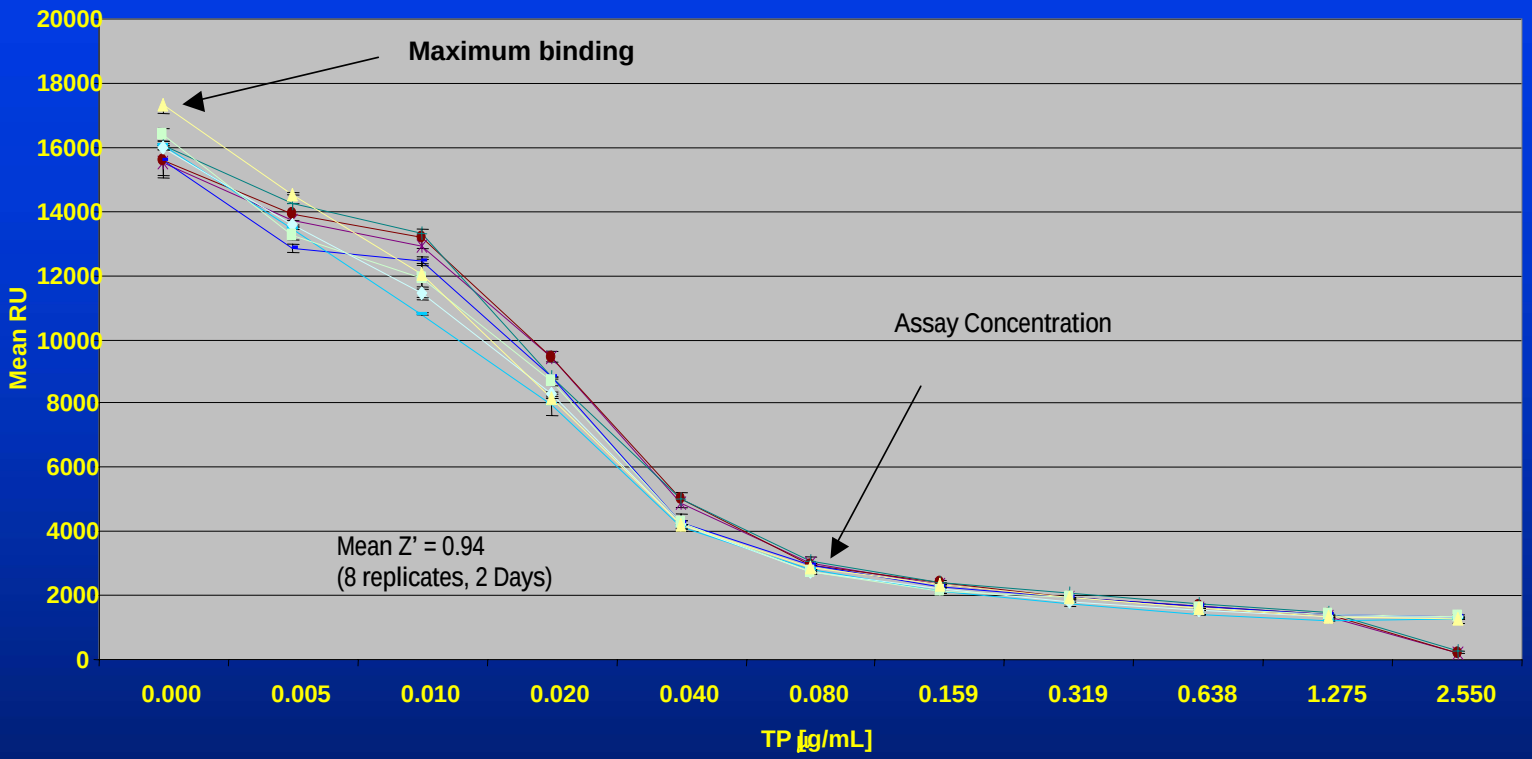
NAb Testing Strategy



Validation Experiments

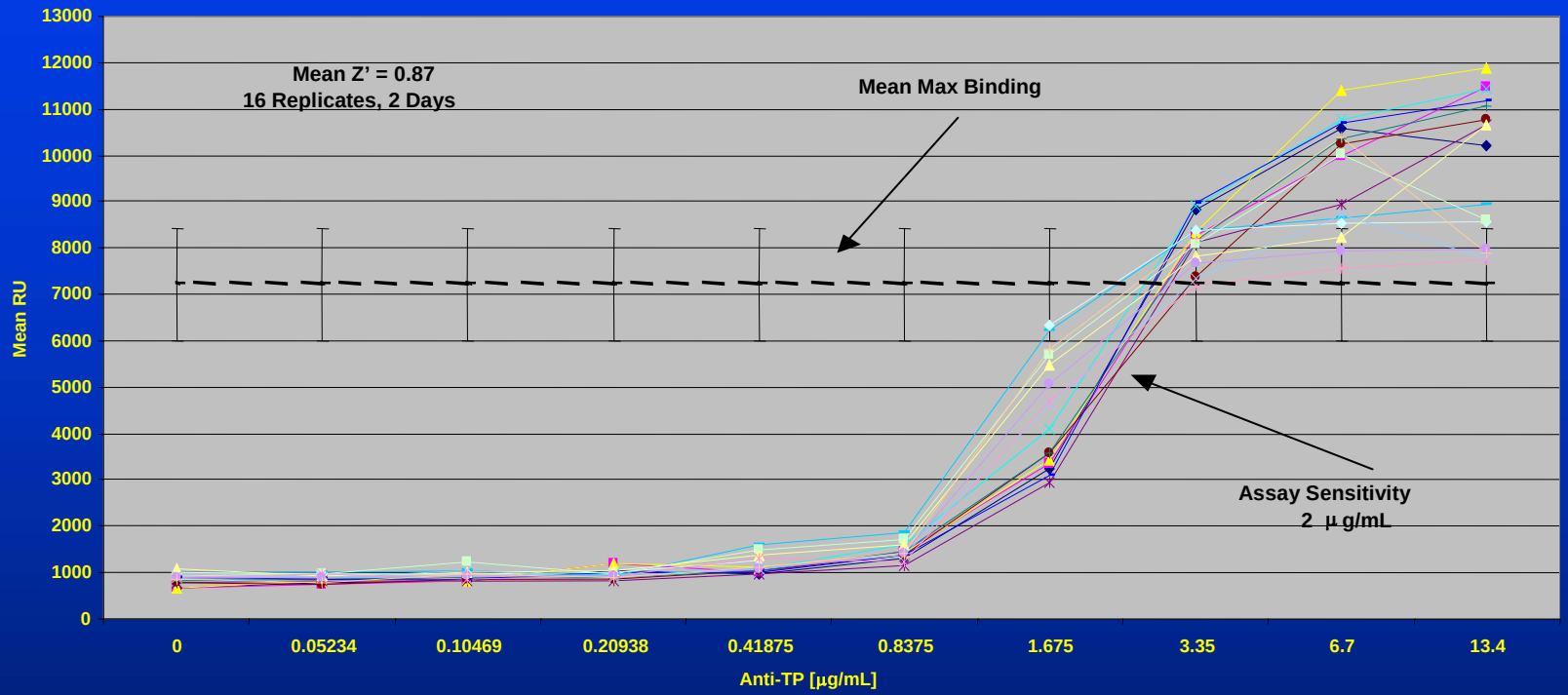
- TP dose response curves
- Positive Control Ab curves
- NAb assay cutoff
- Specificity Assay Cutoff
- Interference by drug product
- Freeze-thaw stability

Inhibition of L-Biot Binding to R-Ru By TP in 15% Pooled Cynomolgus Monkey Serum



- **TP was incubated at increasing concentrations with 250 ng/mL L-Biot followed by incubation with 900 ng/mL R-Ru in 15% PCS.**
- **TP showed a dose dependent inhibition of L-Biot/R-Ru binding.**
- **A concentration of 80 ng/mL TP was chosen to be used for the Screening Assay.**

Neutralization of TP and Restoration of Ligand Binding by Anti-TP Neutralizing Antibody in Pooled Cynomolgus Monkey Serum



- **Rabbit Polyclonal anti-TP Antibody (NAb) was added to neat PCS.**

- **The Ab containing PCS was diluted to 15% and preincubated with 80 ng/mL TP followed by sequential incubations with 250 ng/mL L-Biot and 900 ng/mL R-Ru.**

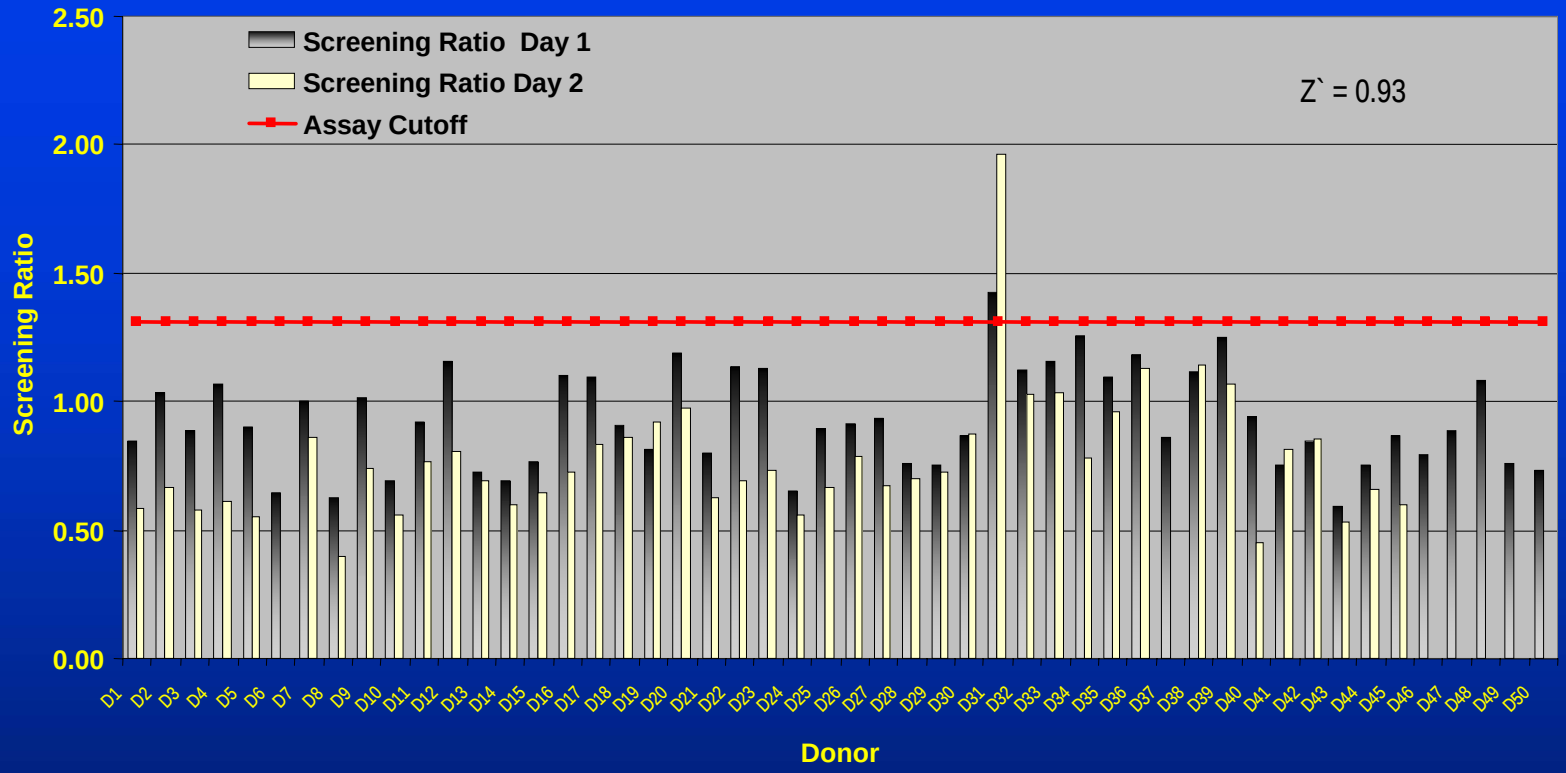
- **Anti-TP antibody showed a dose dependent restoration of L-Biot/R-Ru binding to maximal binding signal (above curves represent 2 days testing)**

- **The lowest concentration of Anti-TP Nab to restore binding by twofold was 1 µg/mL**

Precision and Analytical Recovery of the PAb

Ab (ug/mL)	0.055	0.11	0.22	0.44	0.88	1.75	3.5	7	14
AR	356%	228%	117%	138%	98%	99%	98%	87%	68%
%CV									
Inter-day	19%	29%	17%	8%	11%	2%	4%	25%	77%
Inter-assay	35%	40%	44%	16%	8%	3%	7%	0.00	49%
Intra-assay	14%	20%	14%	13%	10%	2%	9%	26%	104%

Screening Assay Cutoff



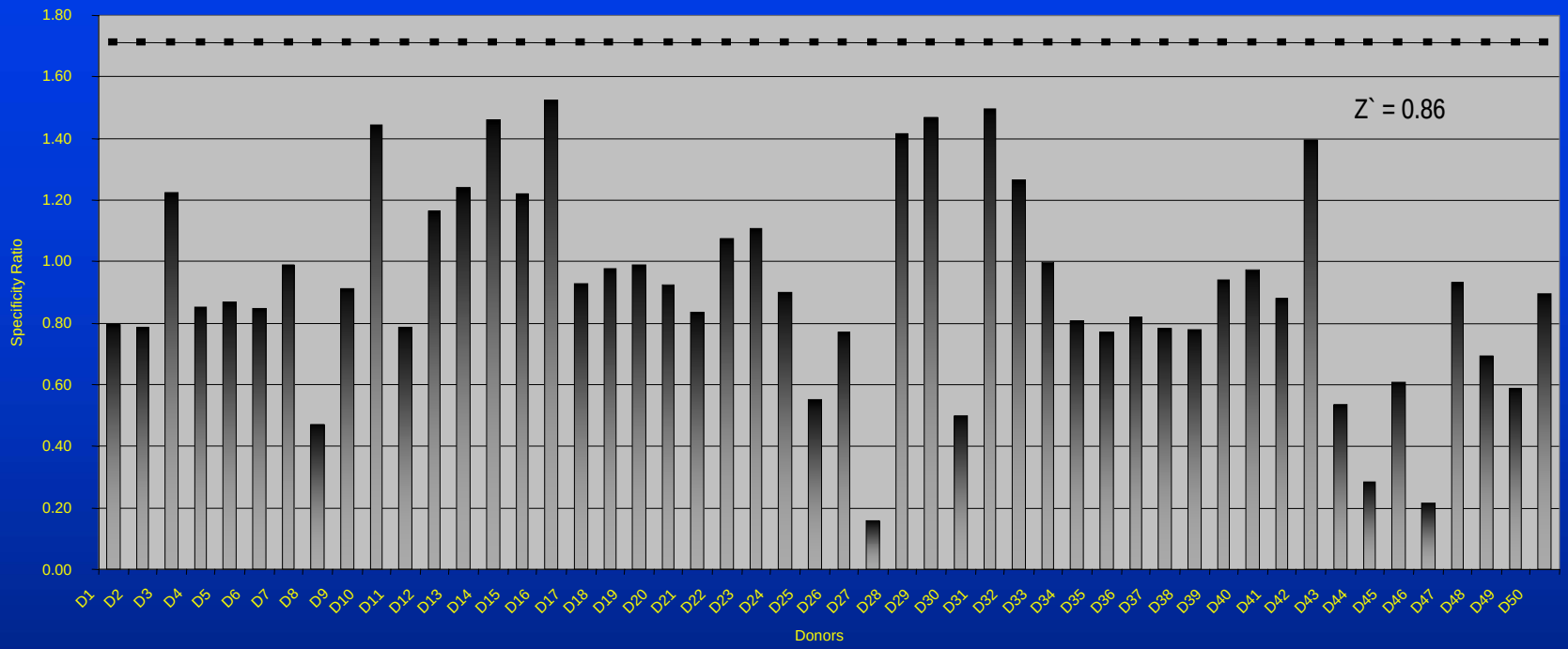
- Serum from 50 individual cynomolgus monkeys was tested at a final dilution of 15% with 80 ng/mL TP, 250 ng/mL L-Biot and 900 ng/mL R-Ru.

- Samples results were compared with control results in PCS to by dividing Sample Results/Control Results (Screening Ratio).

- Samples were tested on two separate days and the Ratio 1 values were combined to establish an assay cutoff using the upper bound of the 95% prediction limit.

- The assay cutoff (Screening Ratio) was determined to be 1.26.

Specificity Assay Cutoff



- **Serum from 50 individual cynomolgus monkeys was tested at a final dilution of 15%, 250 ng/mL L-Biot and 900 ng/mL R-Ru in the absence of the TP to assess for nonspecific enhancement of ligand binding.**

- **Samples results were compared with control results in PCS to by dividing Sample Results/Control Results (Ratio 3).**

- **The Ratio 3 values were used to establish an assay cutoff using the upper bound of the 95% prediction limit.**

- **The assay cutoff (Specificity Ratio) was determined to be 1.71.**

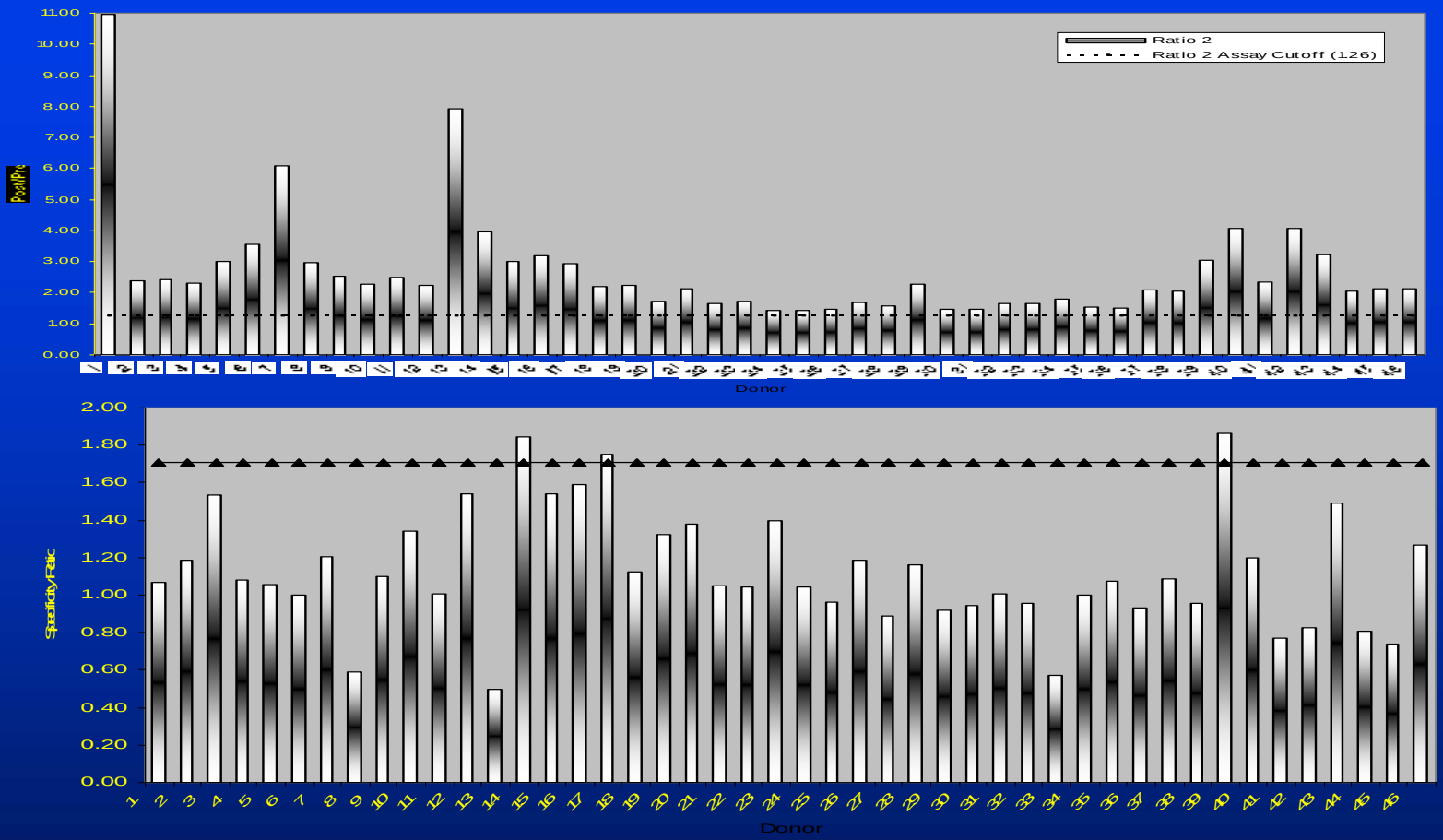
AP Criteria for Positive/Negative

- **Assay Acceptance Criteria**
  - **M/N $\geq$ 10**
  - **M/D $\geq$ 2.0**
  - **P/D $\geq$ 1.26**

Predose: Ratio 1 $\geq$ 1.26
Ratio 3 < 1.71
Postdose: Ratio 2 $\geq$ 1.26
Ratio 3 < 1.71

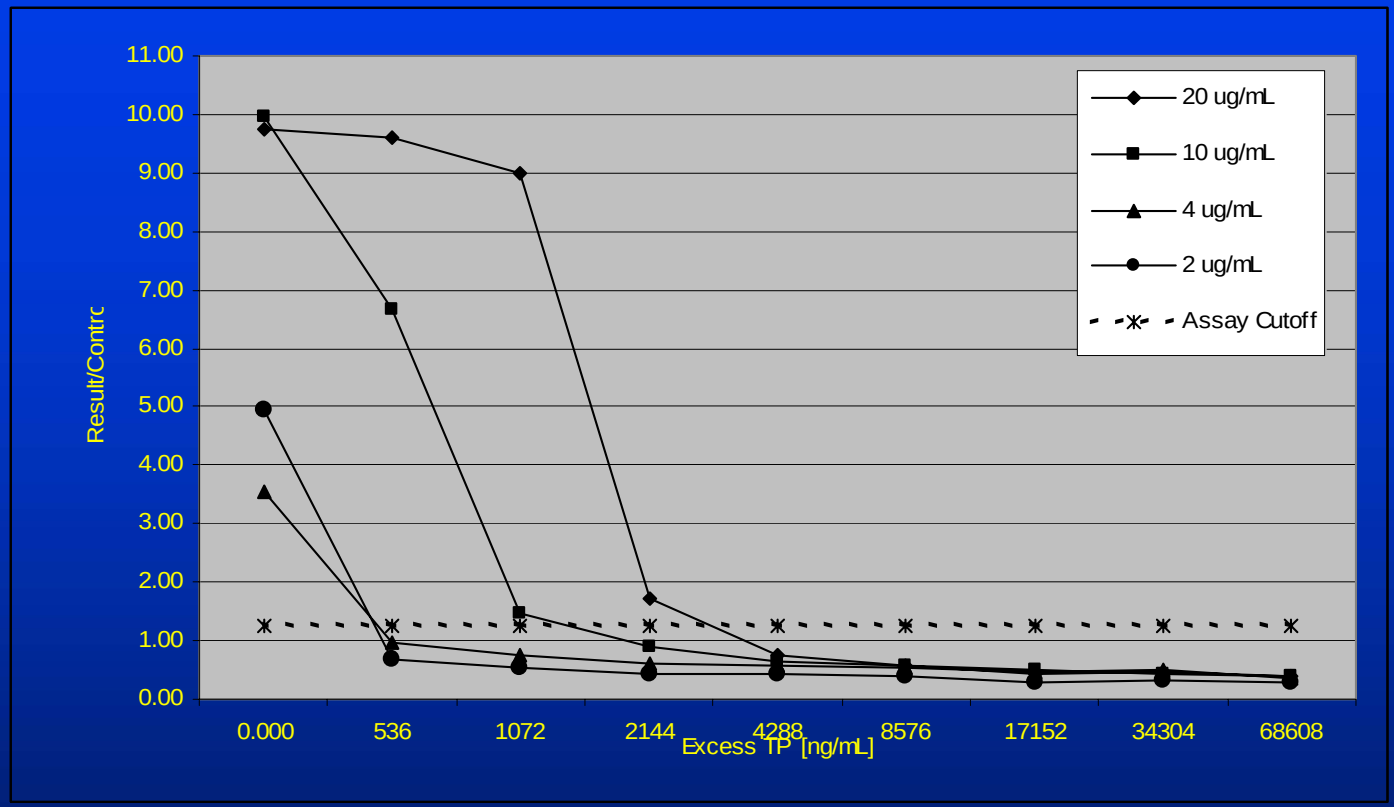
Post/Pre used because some individual animals yielded extremely low Ratio 1 values

Antibody Detection in Individual Monkeys



- Serum from 50 individual cynomolgus monkeys was tested treated and untreated with 2 μg/mL anti-TP NAb at a final dilution of 15% in the Screening or Specifcity Assay.
- A Ratio of Treated/Untreated samples (Post/Pre) was calculated and the Screening Ratio cutoff value was applied (1.26).
- Samples results were compared with Post/Pre and Specificity Ratio cutoff values.
- All samples treated with anti-TP NAb were above the Post/Pre Ratio cutoff.
- All samples treated with anti-TP NAb were below the Specificity Ratio cutoff except for three. These represent false negative samples.
- All untreated samples were below the Screening and Specificity Ratio cutoff (data not shown).

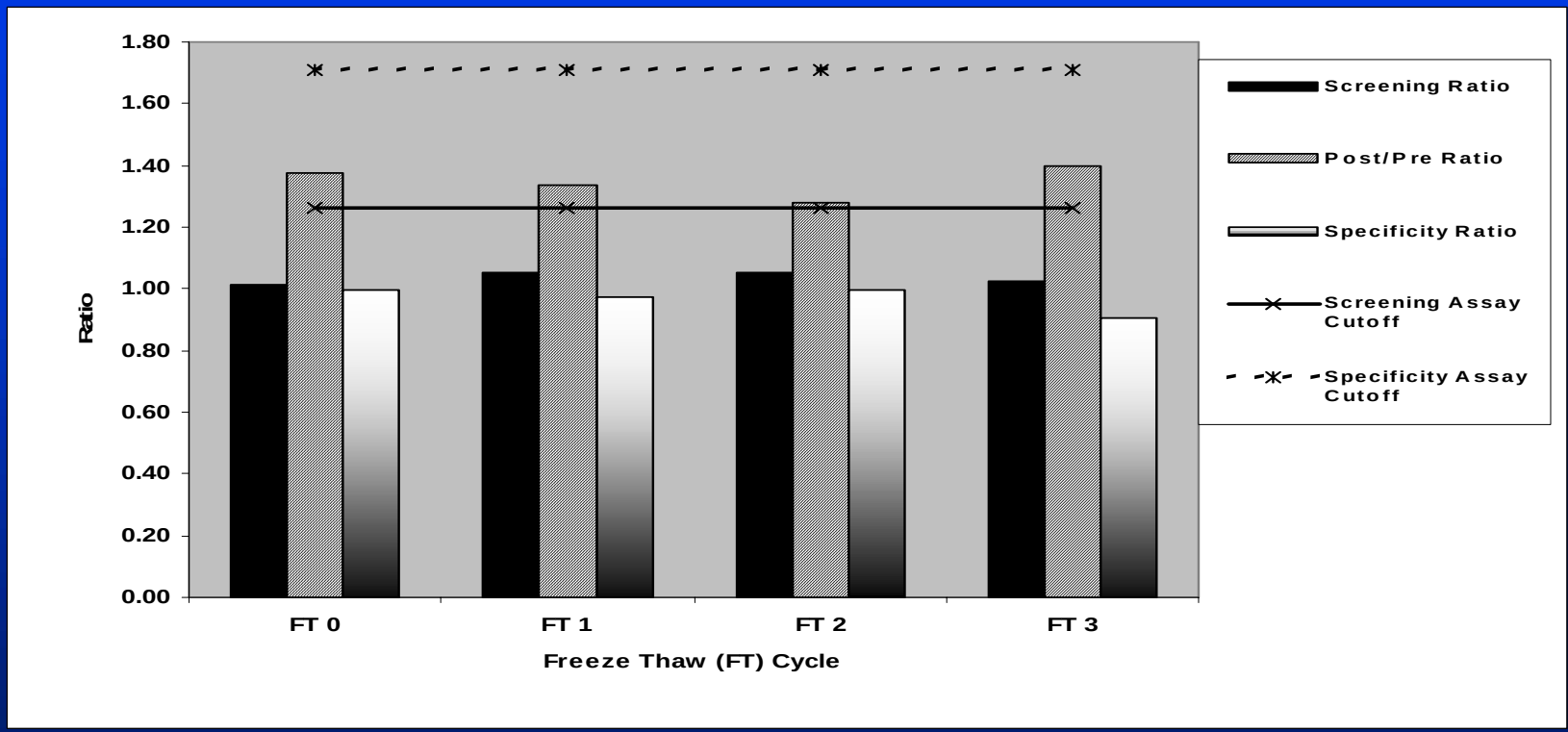
Excess Drug Interference



•Rabbit polyclonal anti-TP antibody was prepared in neat PCS at 20, 10, 4 and 2 μg/mL. At each concentration of antibody, the TP was titred from 68.6, 34.3, 17.15, 8.576, 4.288, 2.144, 1.072, 0.536 and 0 μg/mL μg/mL in neat PCS .

•The ability of the assay to detect 2 and 4 μg/mL anti-TP antibody was inhibited by 80 ng/mL excess TP (536 ng/mL in neat serum). The assay is able to detect 10 μg/mL anti-TP antibody in the presence of up to 160 ng/mL (1.07 μg/mL in neat serum) excess TP and 20 μg/mL in the presence of up to 320 ng/mL (2.14 μg/mL in neat serum) excess TP.

Effects of Freezing and Thawing on Antibody Detection



- Positive control anti-TP antibody was added to PCS at the LOD of the assay (2 μg/mL).
- All samples containing antibody were positive up to 3 freeze thaw cycles while all untreated samples remained negative

Study Results

Animal	Dose Group	Immunoassay Result	Neutralizing Antibody Result
1	Low	Negative	Not Analyzed
2	Low	Negative	Not Analyzed
3	Low	Negative	Not Analyzed
4	Mid	Negative	Not Analyzed
5	Mid	Negative	Not Analyzed
6	Mid	Negative	Negative
7	High	**Positive**	**Positive**
8	High	Negative	Not Analyzed
9	High	Negative	Not Analyzed

- **Serum Samples from Cynomolgus Monkeys dose with TP that were found to be Reactive in a Screening Immunoassay were tested in the Receptor-Binding Assay**

- **Of the samples tested one sample was found to be positive for neutralizing antibodies which correlated with the Immunoassay result.**

Conclusions

A robust and sensitive receptor binding assay was develop for the detection of neutralizing antibodies to a TP in cynomolgus monkey serum

The assay was able to detect 2 μg/mL rabbit polyclonal anti-TP antibody in monkey serum in the presence of 80 ng/mL TP.

The successful implementation of the assay detected antibodies in 1 animal from a preclinical pharmacokinetic study (11% incidence) .

Concluding Comments

- Dependent upon quality of reagents
- Biological activity of reagents should be evaluated
- Sequence of assay steps extremely important
- Serum still an important factor
- Quick readout
- Assay development requires careful consideration
- Ratio of ligand/therapeutic important
- Not a slam dunk!

Acknowledgements

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