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Optimizing ELISA Methods for Neutralizing Antibody (NAb) Detection

Challenges Of Developing an Optimized Drug Tolerant Neutralising Antibody Assay, with Cost, Complexity and Time Efficiency in Mind

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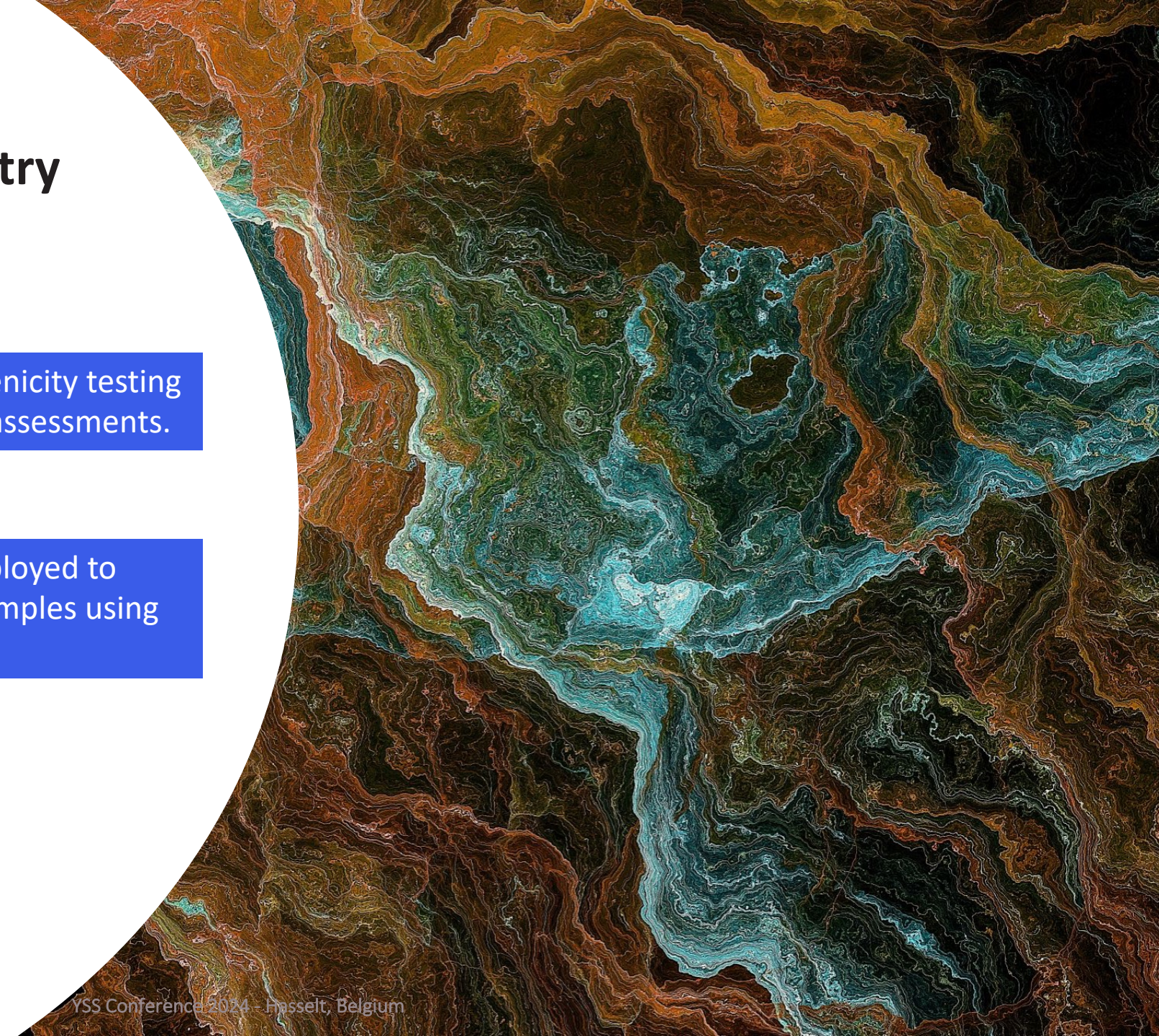
BioA and Immunochemistry

BioAnalytical methods crucial for immunogenicity testing of novel therapeutics for PK, PD and safety assessments.

The principles of immunochemistry are employed to analyze the immunogenicity of biological samples using BioAnalytical methods.



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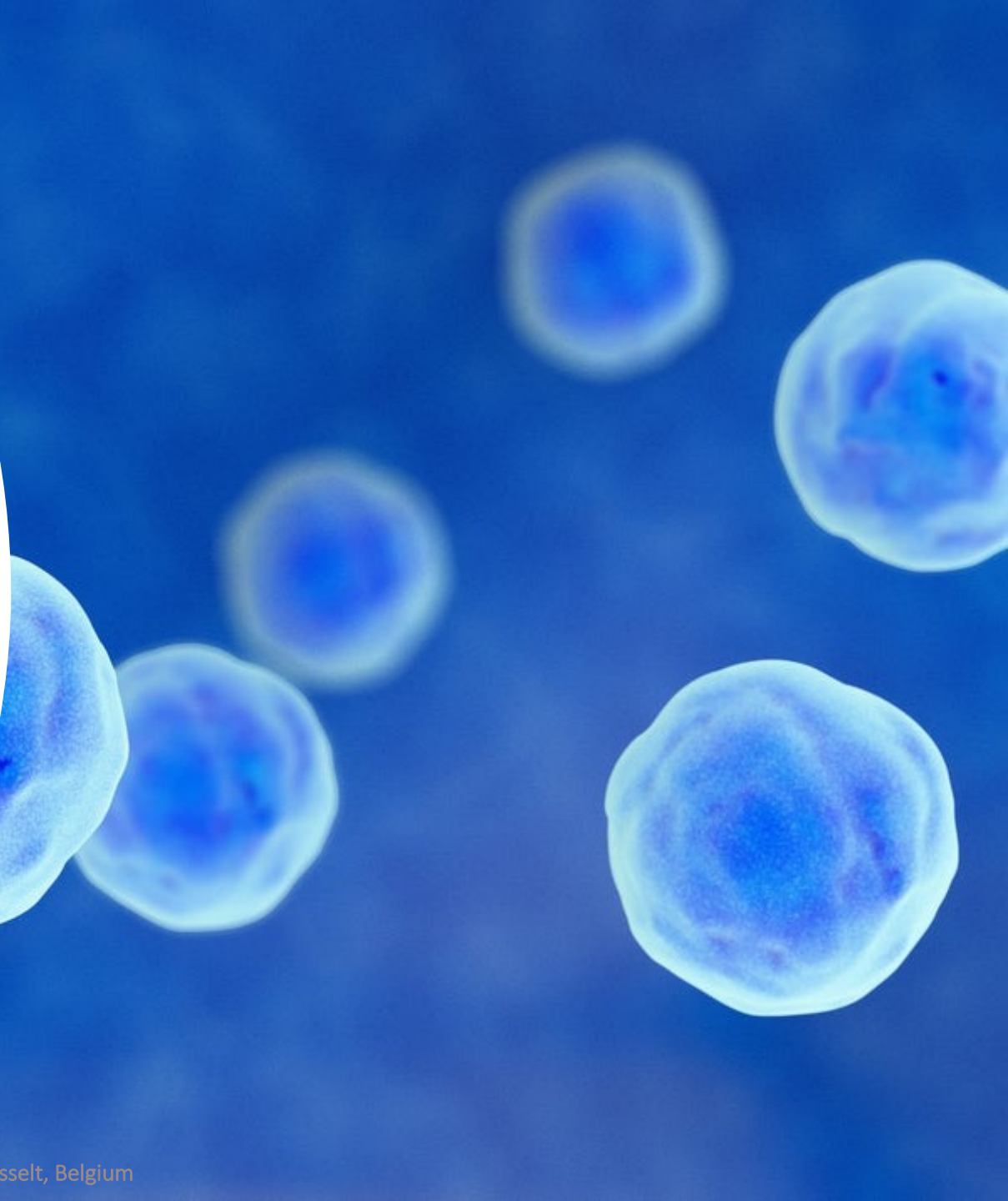
What is Immunogenicity?

Immunogenicity refers to the ability of exogenous or foreign proteins, such as a therapeutic drug or antigens, to induce a humoral and/or cell-mediated immune response.

Desired Immunogenicity:
NAbs raised against the SARS-CoV-2 virus.

Undesired Immunogenicity:
ADAs such as NAbs are secreted in response to therapeutic drugs.

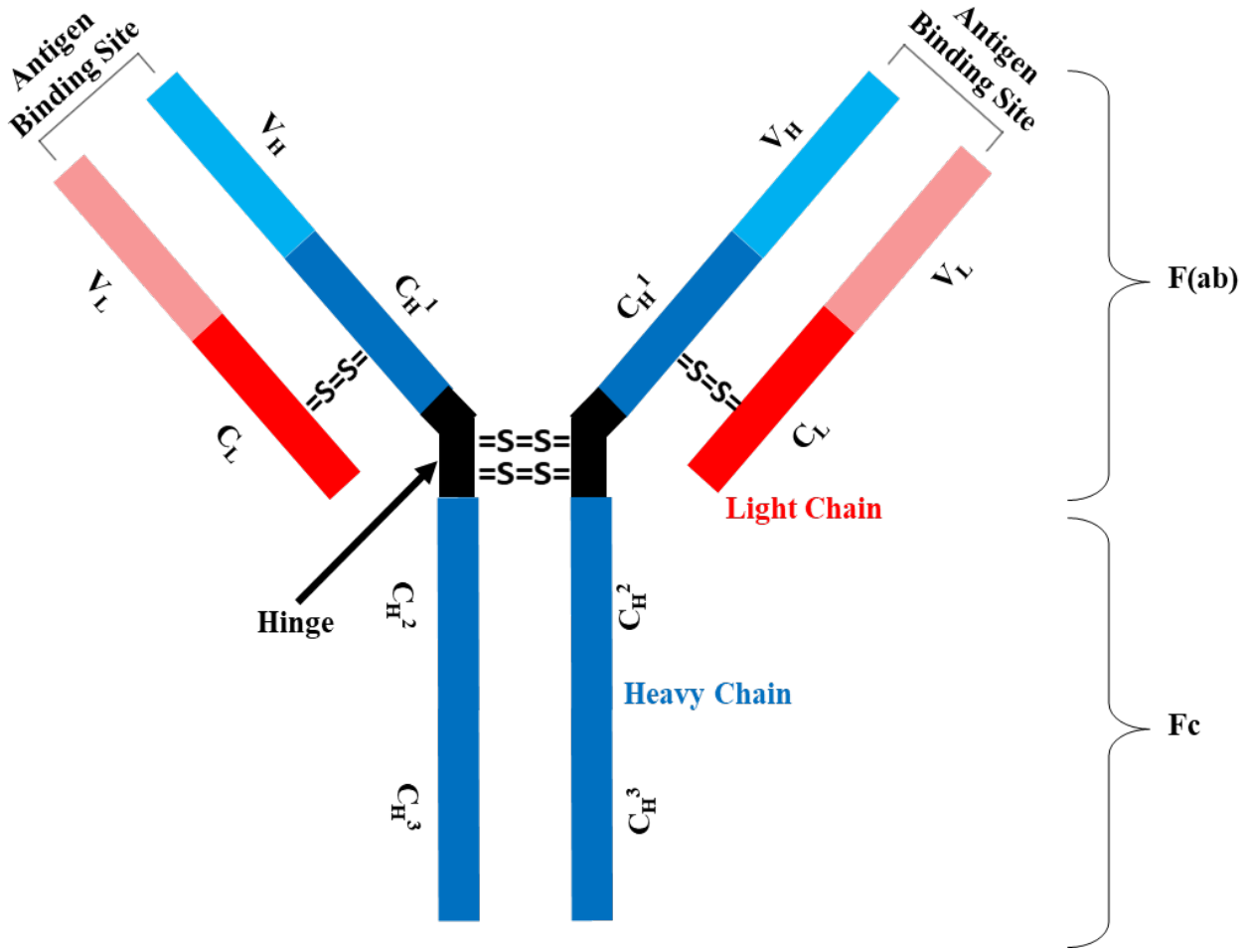


Introduction to Neutralising Antibodies (NAbs)

NAbs are a subset of ADAs produced by the immune system.

NAbs are studied to understand immune responses and to assess the efficacy of vaccines.

NAbs May Either:
a. Inactivate Therapeutic Antibodies
b. Antibody-dependent Enhancement of Infection
- Fc-mediated endocytosis rather than neutralisation



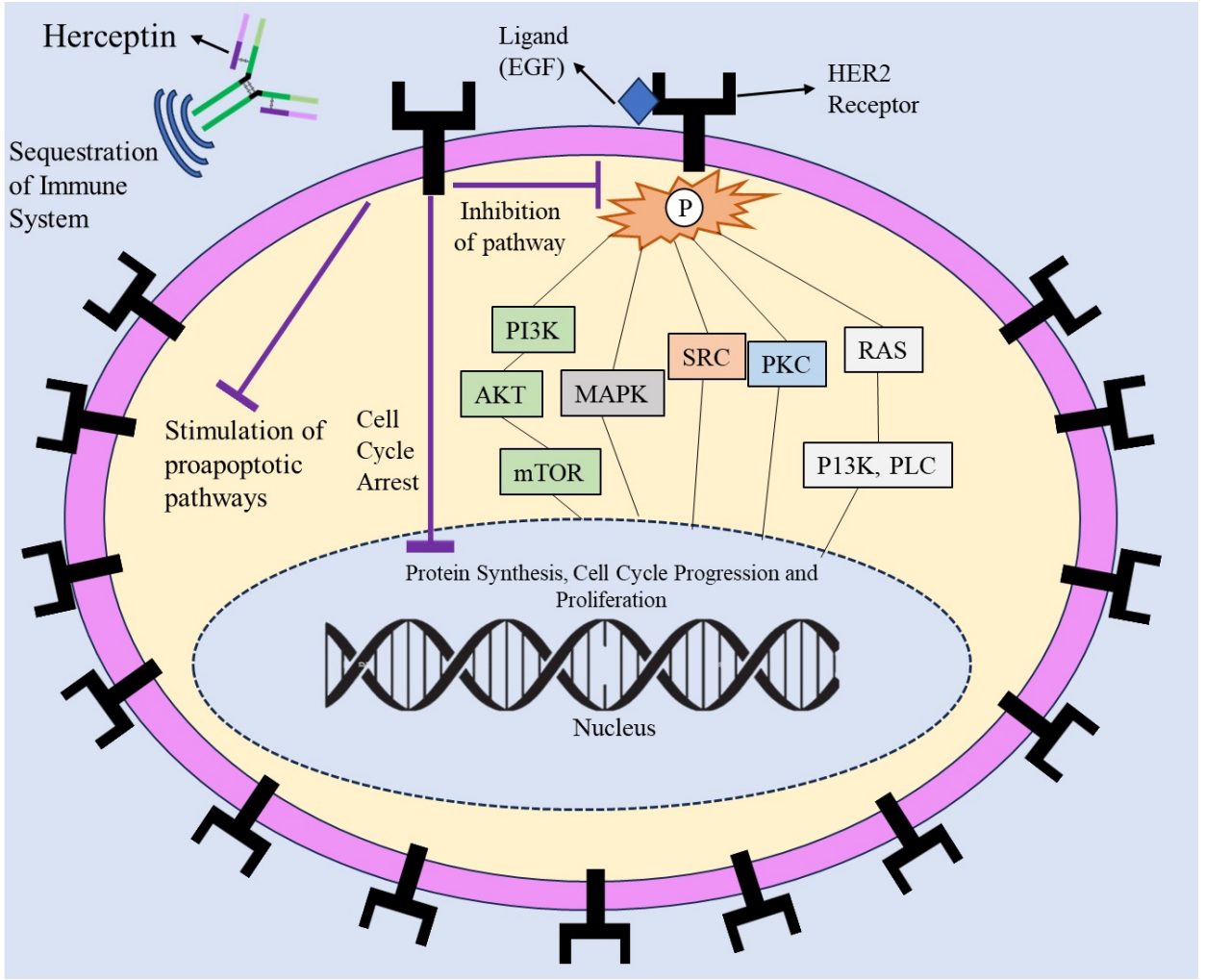
Background of Herceptin (Trastuzumab)

Herceptin (Trastuzumab)
Herceptin is a MAb which targets the extracellular domain of HER2 and is used in HER2-positive breast cancers.

Human Epidermal Growth Factor Receptor 2 (HER2)
Binding of EGF to HER2 results in cell growth and proliferation. Overexpression of ErbB2 gene increases HER2 receptors, as observed in 20-25% of breast cancers.

Neutralising Anti-Herceptin Antibodies (NAbs)
Anti-Herceptin antibodies are produced in response to Herceptin, they reduce drug efficacy by blocking its MOA.

Abnormal HER2+ Breast Cancer Cell



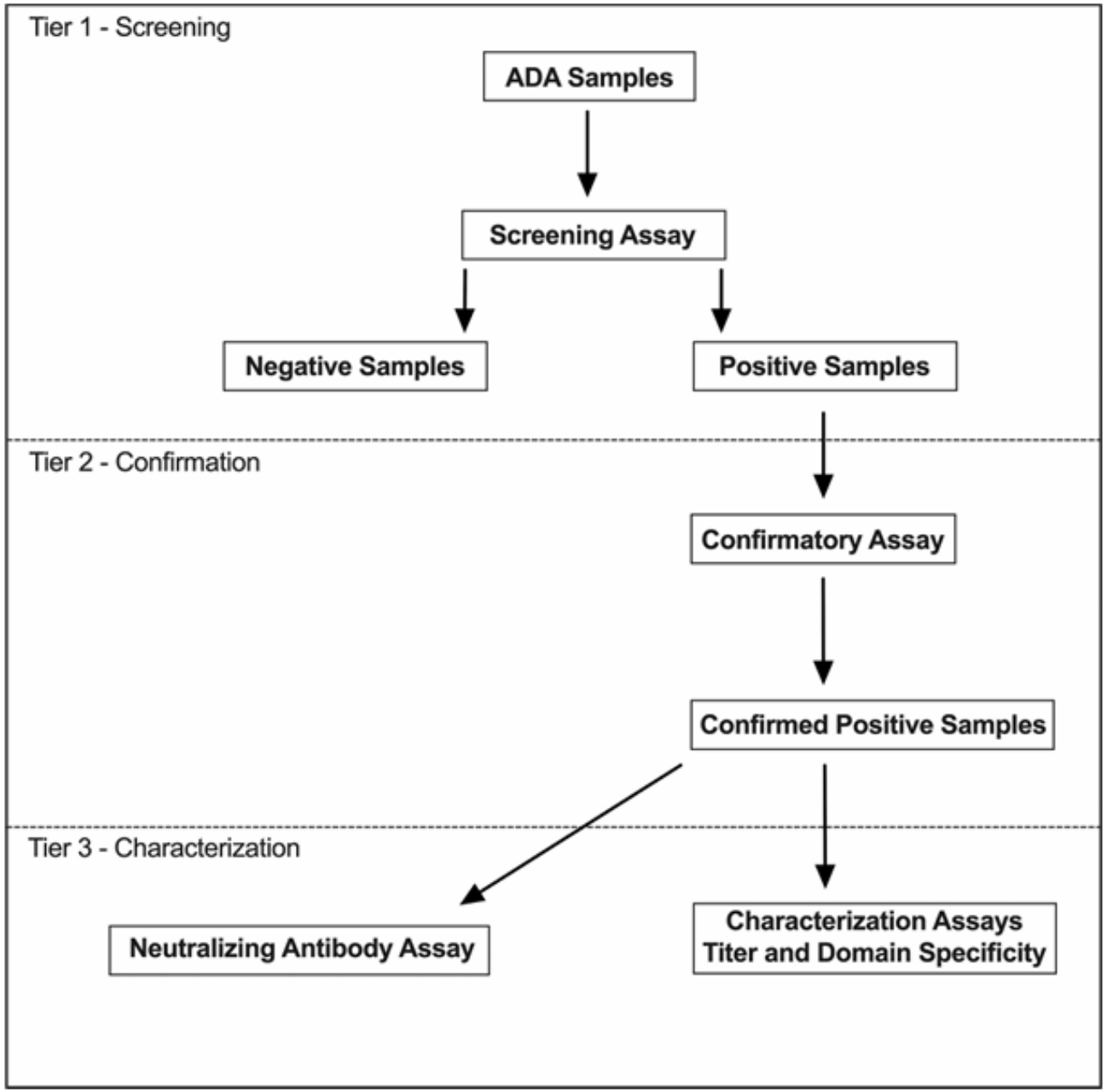
Excess HER2 receptors amplify signals for cell growth and proliferation

NAb Assays

NAb assays form the latter part of immunogenicity testing: Tier 3.

Traditional methods of NAb detection required cell-based assays.

Non-cell based Competitive Ligand Binding (CLB) Assays have a higher throughput and are more cost effective.



Importance of Achieving Drug Tolerance and Sensitivity

Drug tolerance = maximum concentration of free drug (Herceptin) which can be present in a sample without inhibiting the detection of NAbs.

Sensitivity = lowest concentration of NAb that yields a response at or below the assay cut-point level.

Drug tolerance

EB07

	Biotinylated Coating Concentration (0.2 µg/mL)											
	Glycine (0.5 M)											
	Sulfotagged Detection Concentration (0.05µg/mL)											
	MRD											
	1 in 2				1 in 4				1 in 10			
	1	2	3	4	5	6	7	8	9	10	11	12
A	0 ug/mL Drug, 0 ng/mL ADA		1.0 ug/mL Drug, 0 ng/mL ADA		0 ug/mL Drug, 0 ng/mL ADA		1.0 ug/mL Drug, 0 ng/mL ADA		0 ug/mL Drug, 0 ng/mL ADA		1.0 ug/mL Drug, 0 ng/mL ADA	
B	0 ug/mL Drug, 100 ng/mL ADA		1.0 ug/mL Drug, 100 ng/mL ADA		0 ug/mL Drug, 100 ng/mL ADA		1.0 ug/mL Drug, 100 ng/mL ADA		0 ug/mL Drug, 100 ng/mL ADA		1.0 ug/mL Drug, 100 ng/mL ADA	
C	0 ug/mL Drug, 250 ng/mL ADA		1.0 ug/mL Drug, 250 ng/mL ADA		0 ug/mL Drug, 250 ng/mL ADA		1.0 ug/mL Drug, 250 ng/mL ADA		0 ug/mL Drug, 250 ng/mL ADA		1.0 ug/mL Drug, 250 ng/mL ADA	
D	0 ug/mL Drug, 500 ng/mL ADA		1.0 ug/mL Drug, 500 ng/mL ADA		0 ug/mL Drug, 500 ng/mL ADA		1.0 ug/mL Drug, 500 ng/mL ADA		0 ug/mL Drug, 500 ng/mL ADA		1.0 ug/mL Drug, 500 ng/mL ADA	
E	0.5 ug/mL Drug, 0 ng/mL ADA		5.0 ug/mL Drug, 0 ng/mL ADA		0.5 ug/mL Drug, 0 ng/mL ADA		5.0 ug/mL Drug, 0 ng/mL ADA		0.5 ug/mL Drug, 0 ng/mL ADA		5.0 ug/mL Drug, 0 ng/mL ADA	
F	0.5 ug/mL Drug, 100 ng/mL ADA		5.0 ug/mL Drug, 100 ng/mL ADA		0.5 ug/mL Drug, 100 ng/mL ADA		5.0 ug/mL Drug, 100 ng/mL ADA		0.5 ug/mL Drug, 100 ng/mL ADA		5.0 ug/mL Drug, 100 ng/mL ADA	
G	0.5 ug/mL Drug, 250 ng/mL ADA		5.0 ug/mL Drug, 250 ng/mL ADA		0.5 ug/mL Drug, 250 ng/mL ADA		5.0 ug/mL Drug, 250 ng/mL ADA		0.5 ug/mL Drug, 250 ng/mL ADA		5.0 ug/mL Drug, 250 ng/mL ADA	
H	0.5 ug/mL Drug, 500 ng/mL ADA		5.0 ug/mL Drug, 500 ng/mL ADA		0.5 ug/mL Drug, 500 ng/mL ADA		5.0 ug/mL Drug, 500 ng/mL ADA		0.5 ug/mL Drug, 500 ng/mL ADA		5.0 ug/mL Drug, 500 ng/mL ADA	

Modifying assays formats to achieve drug tolerance is expected to induce variations in achievable drug tolerance.
Investigating various assay optimizations will contribute to an improved understanding of optimal conditions for precise detection.

Challenges in Achieving Drug Tolerance in NAb Assays

1

Diversity of NAbs:

NAbs can be highly diverse in terms of their specificity and affinity.

2

Low Concentrations:

NAbs are present in low titres, making it difficult to detect them using conventional assays.

3

Drug Interference:

Plasma proteins or the drug itself may interfere with NAb detection, leading to false positive, false negative results, or a reduced sensitivity.

4

Influence of Sample Storage and Handling:

Improper storage or handling of samples can impact NAb stability, leading to changes in their activity, potentially affecting assay results.

5

Difficulty in Standardization:

Variations in reagents, protocols, and sample types results in a lack of standardization, resulting in inconsistent results.

NAb Analysis Strategy

NAb assays have a NCPR of 0.9 from which assay sensitivity and drug tolerance are determined.

Raw data from NAb assay duplicates are averaged and processed into normalised ratios:

$$Normalised\ Ratio = \frac{\bar{x}\ \text{Sample Duplicates}}{ECL\ [NC]}$$

$\bar{x}$ = Average (Mean)

ECL [NC] = Electrochemiluminescence of Negative Control Sample

Bold = signal below cut point (drug tolerance).
Underlined = sensitivity has been achieved.
Highlighted = incidents of drug interference.

Drug Tolerance & Sensitivity Achieved < 0.9
Drug Tolerance & Sensitivity Not Achieved > 0.9

EB05b
Reference Material Curve: Observed Response
EB05b : 0.2 µg/mL Biotinylated Herceptin / 0.05 µg/mL sTag HER2

Herceptin Concentration (µg/mL)	Observed Response (ECL)				Approximate Cut-Point (ECL) [X1]
	Blank	NAb (ng/mL)			
		100.00	250.00	500.00	
0.00	41656	39768	36991	36820	37,490
0.50	40201	41521	41310	41720	
1.00	40685	40880	40803	39902	
5.00	39636	40961	40891	42139	

EB05b
Reference Material Curve: Observed Response
EB05b : 0.2 µg/mL Biotinylated Herceptin / 0.05 µg/mL sTag HER2

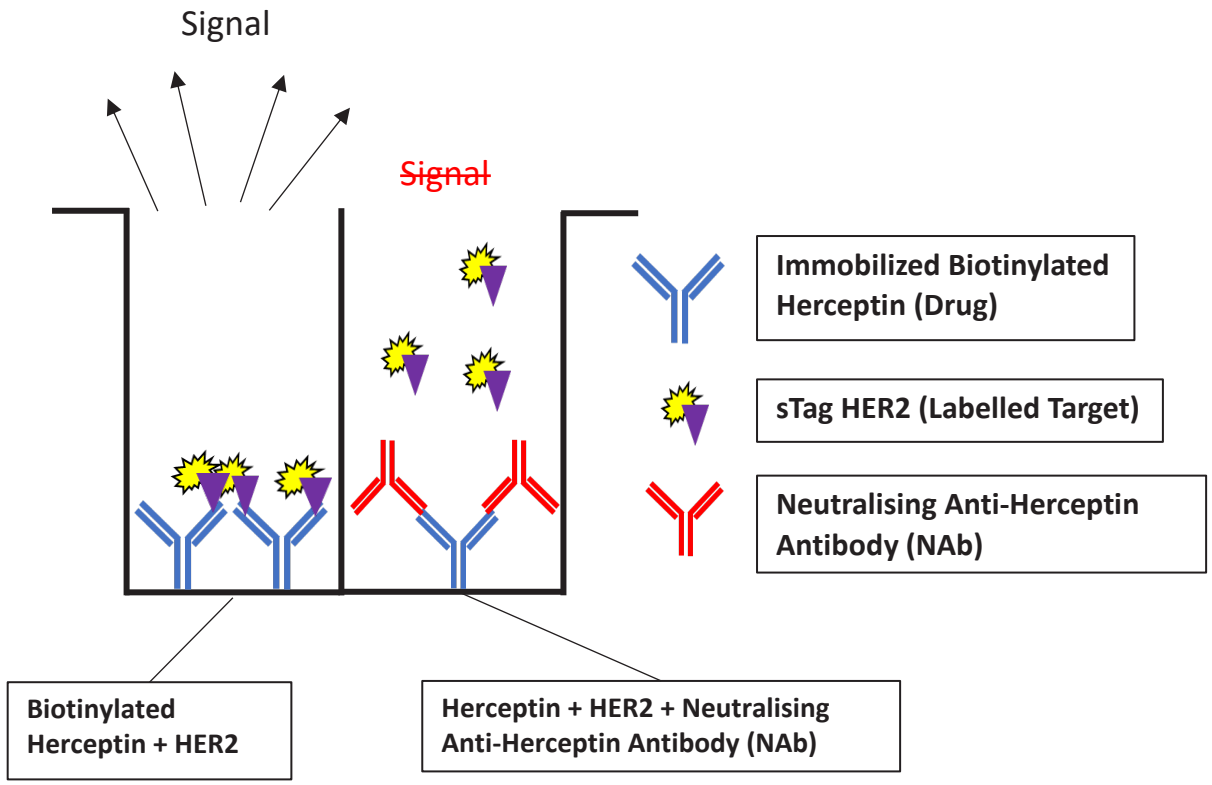
Herceptin Concentration (µg/mL)	Observed Response (ECL)				Approximate Cut-Point (ECL) [X1]
	Blank	NAb (ng/mL)			
		100.00	250.00	500.00	
0.00	-	0.95	0.89	0.88	0.90
0.50	0.97	1.00	0.99	1.00	
1.00	0.98	0.98	0.98	0.96	
5.00	0.95	0.98	0.98	1.01	

ELISA Detection of NAbs (No Acid Dissociation & Acid Dissociation)

Direct Non-Cell Based CLB Assay (ELISA Format):

Principle: Detects NAbs in patient samples using immobilized Biotinylated Herceptin as capture antibody. sTag HER2 is added as detection reagent.

Detection: Binding of sTag HER2 is quantified in Level of Electrochemiluminescence (ECL). A decreased in ECL is indicative of NAb presence.

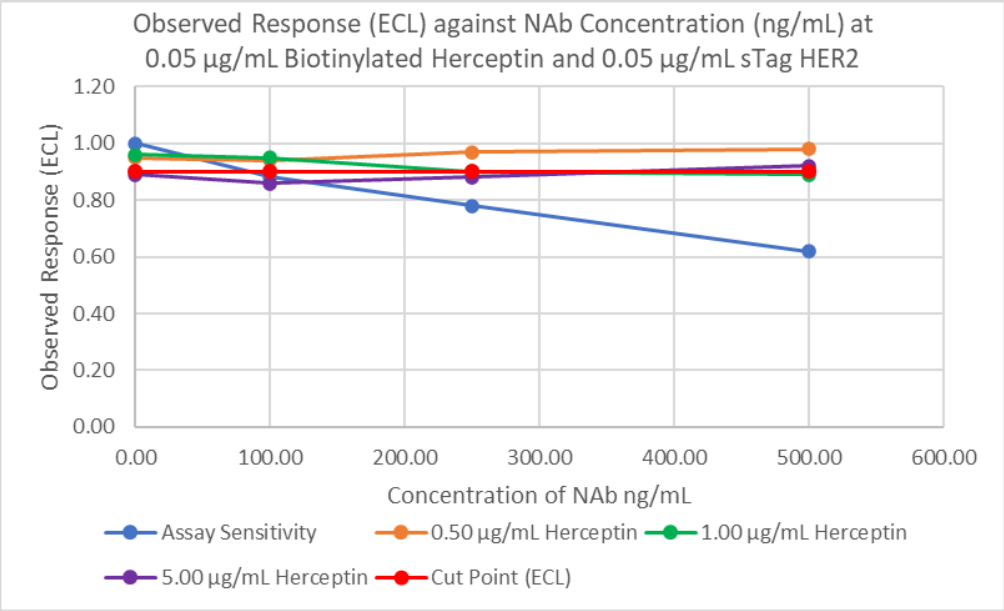


Optimization of Reagent Concentrations: No Acid Dissociation

Reagents Tested Up To 500.00 µg/mL Herceptin

EB01a : 0.05 µg/mL Biotinylated Herceptin / 0.05 µg/mL sTag HER2

Herceptin Concentration (µg/mL)	Observed Response (ECL)				Approximate Cut-Point (ECL) [X1]
	Blank	NAb Concentration (ng/mL)			
		100.00	250.00	500.00	
0.00	-	0.88	0.78	0.62	0.90
0.50	0.95	0.94	0.97	0.98	
1.00	0.96	0.93	0.90	**0.89**	
5.00	**0.89**	**0.86**	**0.88**	0.92	



EB04a : 0.05 µg/mL Biotinylated Herceptin / 0.05 µg/mL sTag HER2

Herceptin Concentration (µg/mL)	Observed Response (ECL)				Approximate Cut-Point (ECL) [X1]
	Blank	NAb Concentration (ng/mL)			
		100.00	250.00	500.00	
0.00	-	0.93	0.87	0.72	0.90
0.25	1.01	1.03	1.01	1.01	
0.50	1.00	1.02	0.99	1.01	
1.00	1.00	1.01	1.01	1.01	
3.00	0.95	0.99	0.99	0.99	
5.00	0.98	1.00	0.99	1.01	
10.00	0.91	0.91	0.91	0.95	
25.00	**0.88**	0.92	0.90	0.92	
50.00	**0.8**	**0.83**	**0.80**	**0.80**	
100.00	**0.72**	**0.75**	**0.76**	**0.74**	
500.00	**0.29**	**0.37**	**0.30**	**0.34**	

Significant Drug Interference Was Detected Upwards from 25.00 µg/mL Herceptin

Optimization of MRD (1 in 10): No Acid Dissociation Assay

Dilution increased to 1:10 to reduce drug interference

EB03 : 0.05 µg/mL Biotinylated Herceptin / 0.05 µg/mL sTag HER2 - 1 in 10 MRD

Herceptin Concentration µg/mL	Observed Response (ECL)				Approximate Cut-Point (ECL) [X1]
	Blank	NAb Concentration (ng/mL)			
		100.00	250.00	500.00	
0.00	-	0.97	0.91	0.87	0.90
0.25	0.96	0.97	0.96	0.99	
0.50	0.98	0.99	0.98	0.94	
1.00	0.95	0.94	0.96	0.99	
3.00	0.96	0.97	0.98	0.98	
5.00	0.96	0.96	0.96	0.95	

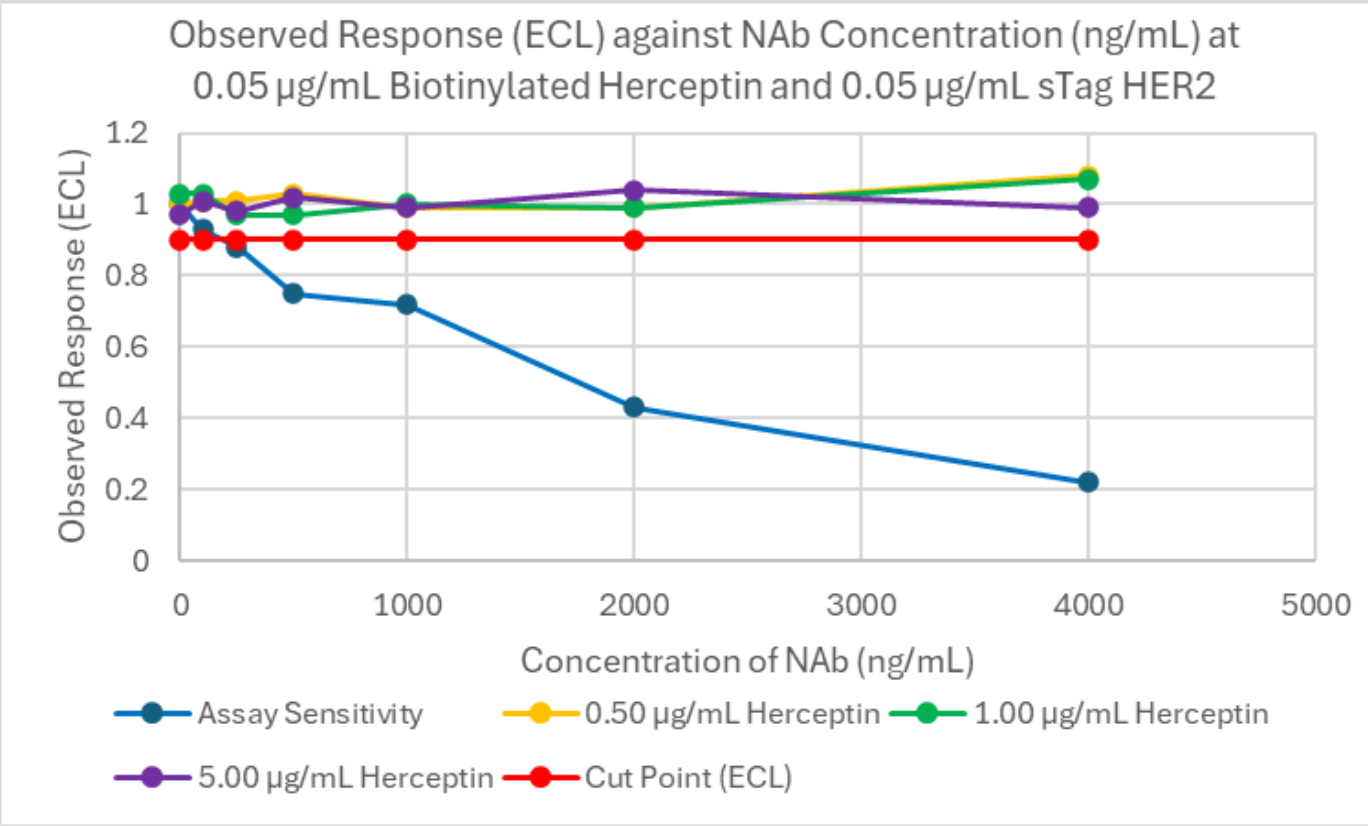
Successful reduction of Drug Interference

Sensitivity Achieved at 500.00 ng/mL NAb

Zero Drug Tolerance Achieved

Higher Concentration of PC's: No Acid Dissociation

Concentration of NAb was increased to 4000.00 ng/mL

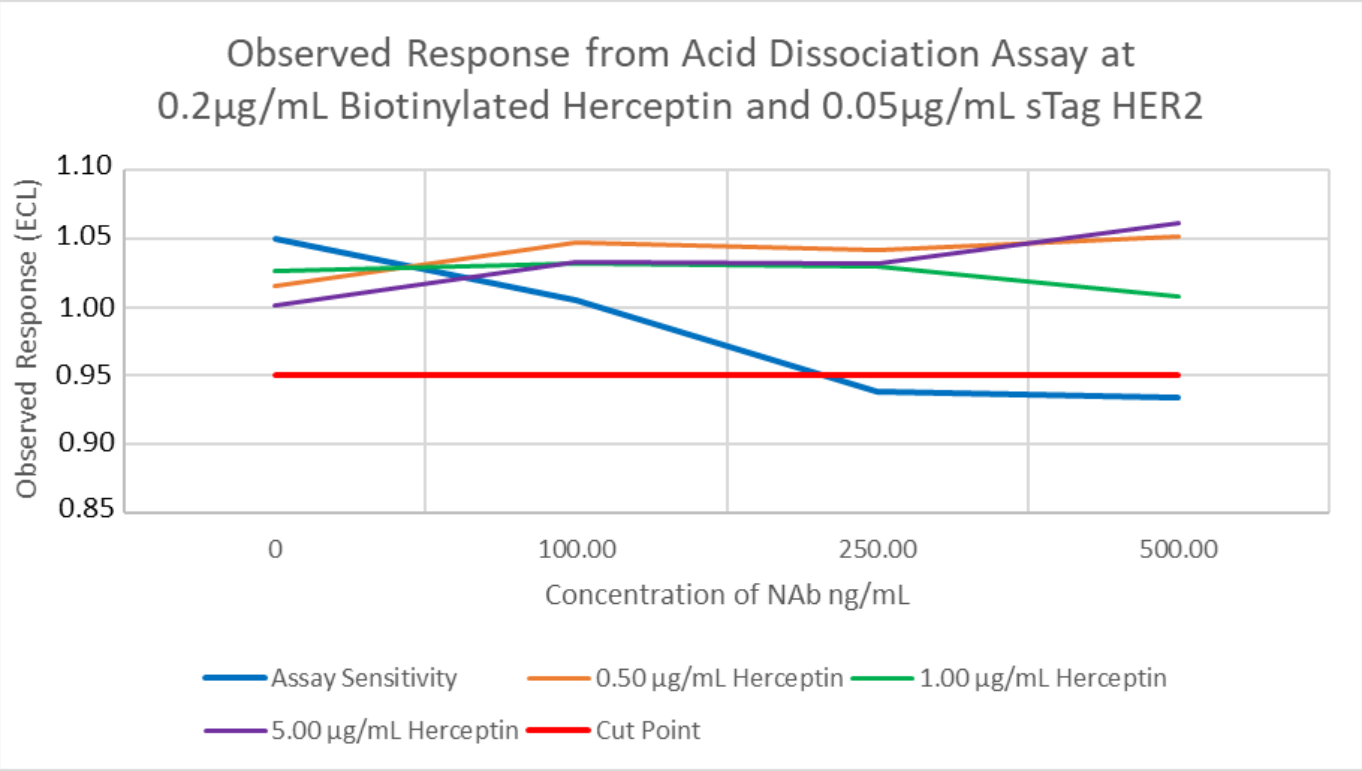


Sensitivity Achieved at 250.00 ng/mL of NAb (Improved)

Zero Drug Tolerance Achieved

Optimization of Reagent Concentrations: Acid Dissociation

Dissociation of NAb-Herceptin complexes using acid for improved NAb detection

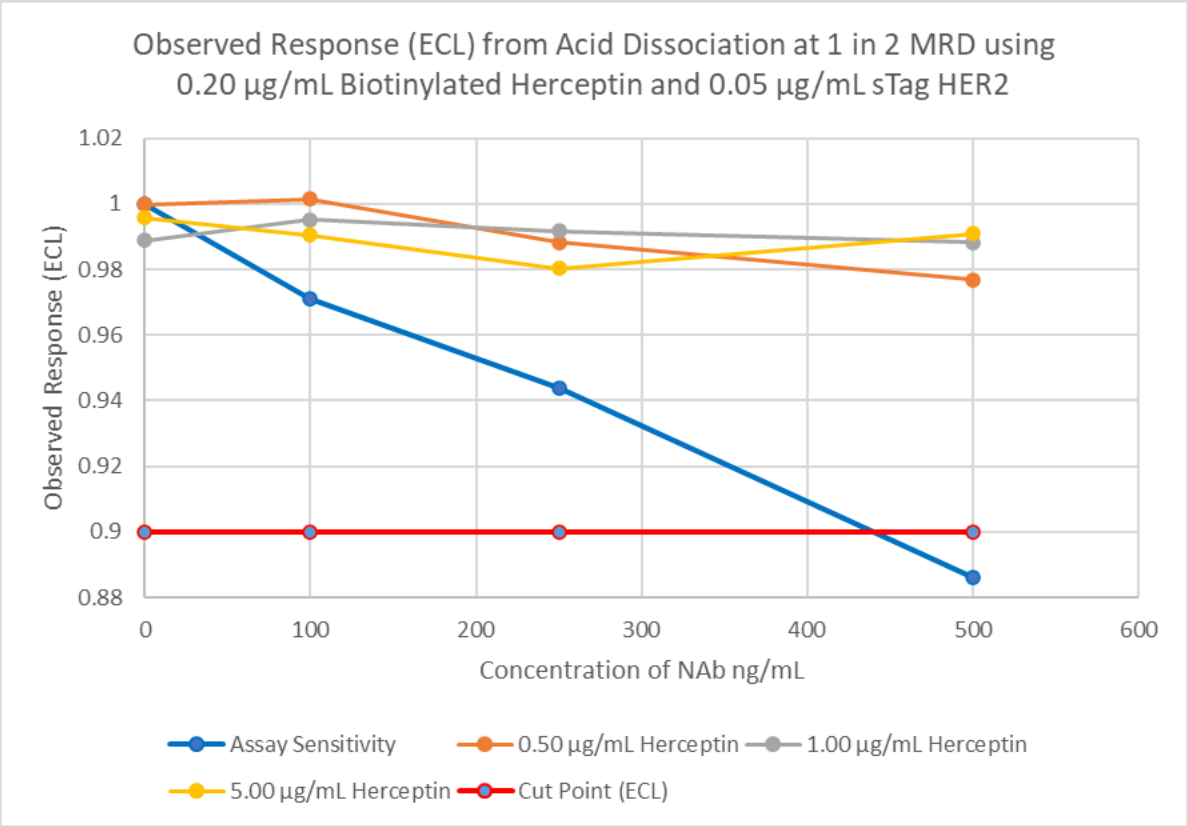


Sensitivity Achieved at 250.00 ng/mL of NAb

Zero Drug Tolerance Achieved

Acid Dissociation: MRD Optimization

Dilution decreased to 1:2 to increase NAb concentration for analysis using 0.5M Glycine HCl (pH 2.2) Acid

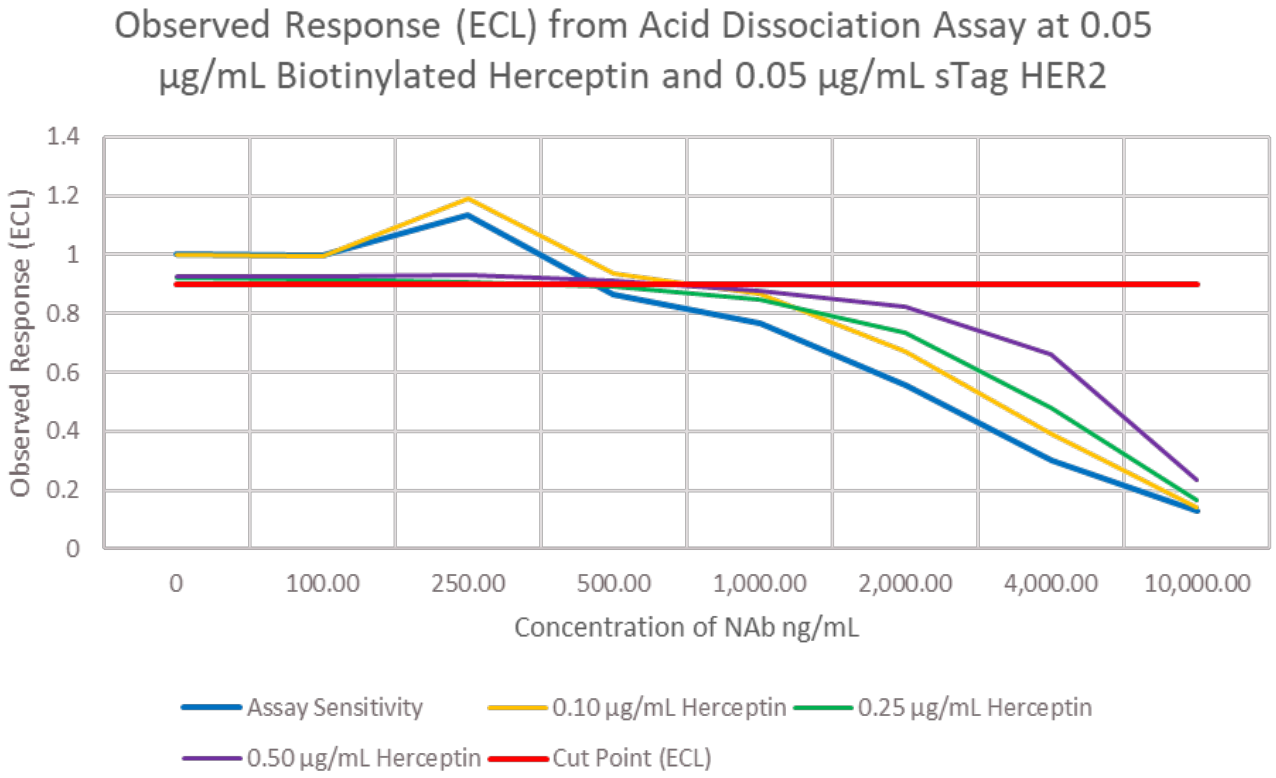


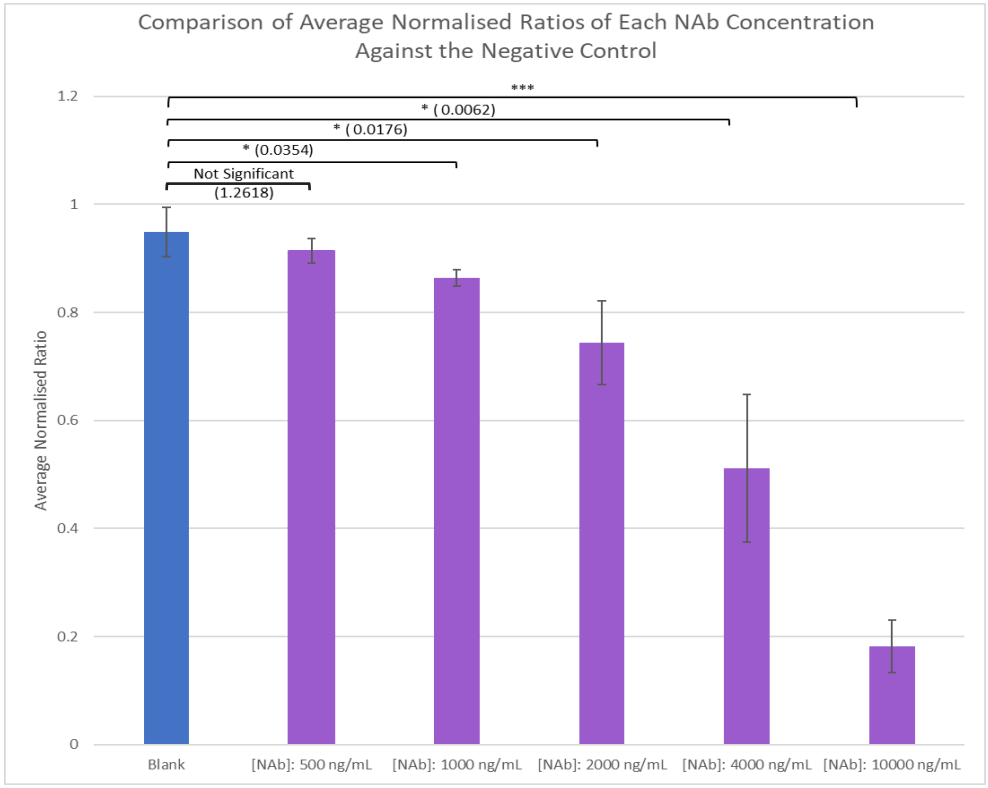
Sensitivity Achieved at 250.00 ng/mL of NAb

Zero Drug Tolerance Achieved

Acid Optimization at Higher Concentration PC's

Assay performed at NAb concentrations up to 10000.00 ng/mL





Sensitivity Achieved at 500.00 ng/mL of NAb

Drug Tolerance Achieved at 1000.00 ng/mL of NAb and above

Affinity Capture Elution with Acid Dissociation (ACE)

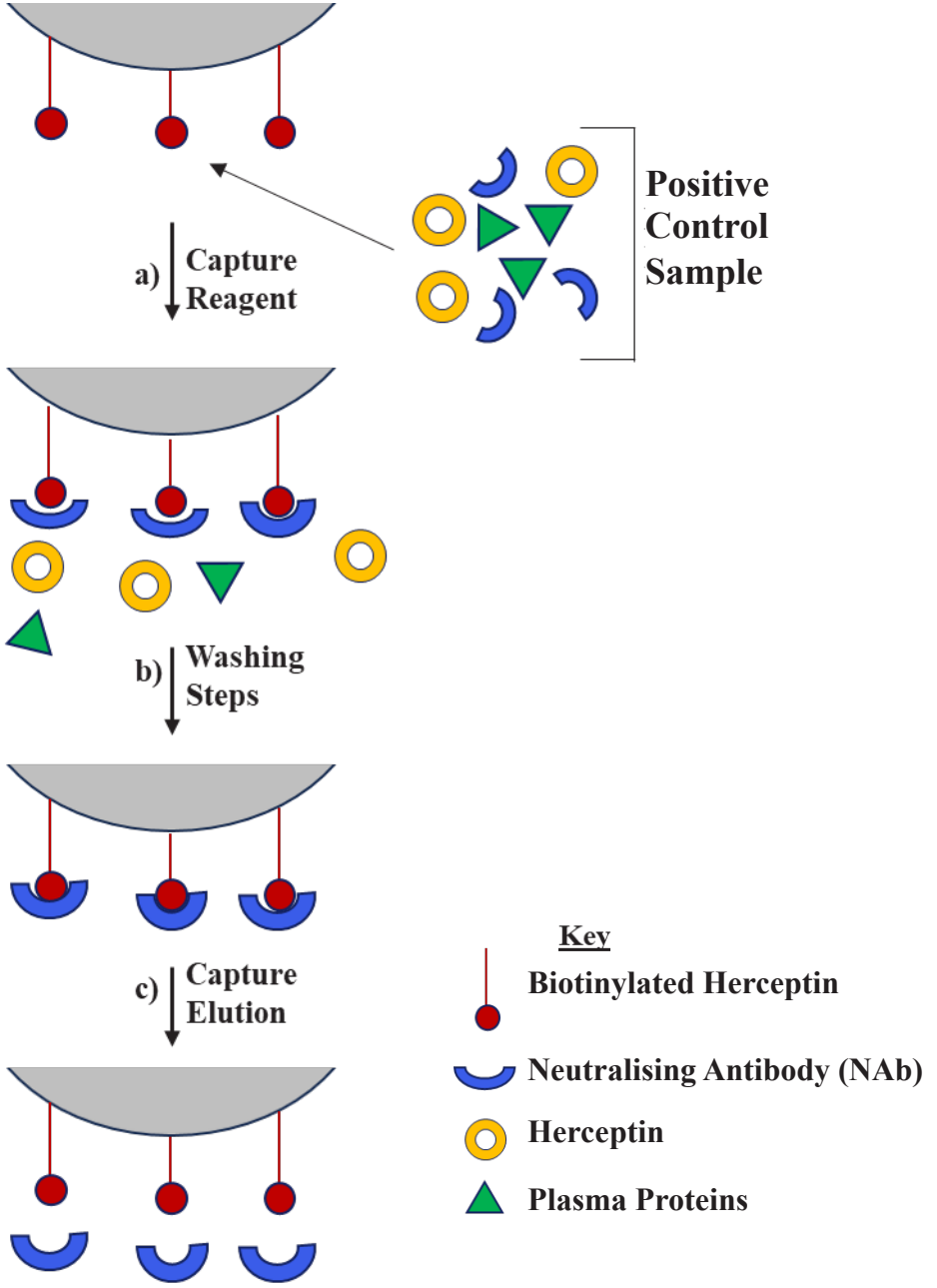
ACE assays capture specific molecules of interest with high affinity and subsequently release them for further analysis

a. **Capture:** Overnight incubation of PC samples with Biotinylated-Herceptin capture solution
b. **Wash:** Unbound serum proteins are washed off the plate
c. **Elution:** NAbs are eluted onto an MSD plate coated with Biotinylated Herceptin
d. **Detection:** Addition of sTag HER2 solution for detection and analysis of NAbs

No Sensitivity Achieved for all Optimizations

Zero Drug Tolerance Achieved For All Optimizations

No Incidents Of Drug Interference



BEAD Extraction with Acid Dissociation

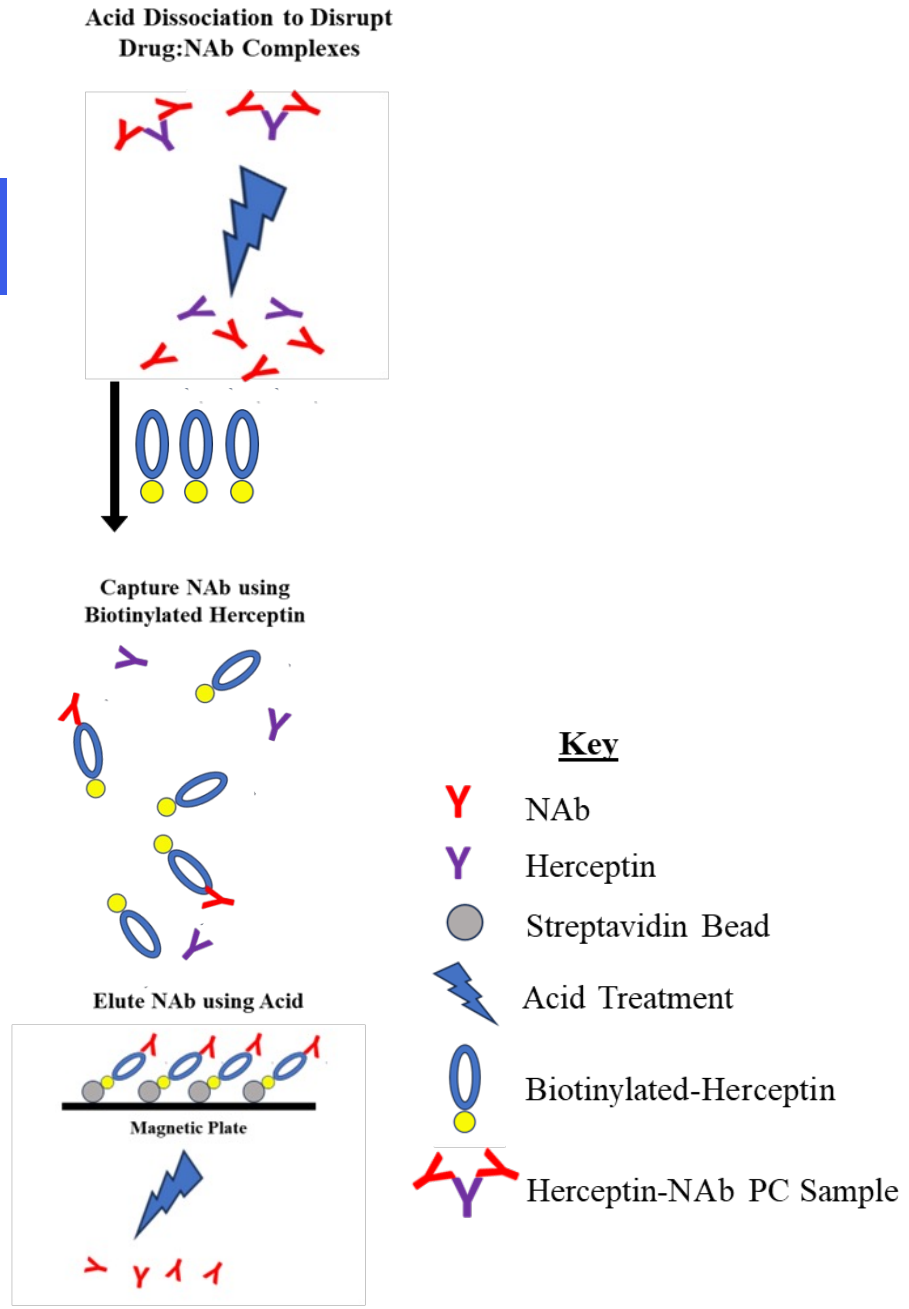
BEADs assays capture specific molecules of interest using a highly immobilised surface with high affinity and subsequently release them for further analysis

a. **Acid Dissociation:** Disruption the NAb-Drug Complexes
b. **Capture Incubation:** Biotinylated Herceptin coated Beads Solution is added to capture the NAb
c. **Elution:** NAb is eluted from beads using acid, neutralized, then eluted from the Biotinylated Herceptin Capture Solution, followed by neutralization.
d. **Detection:** Addition of sTag HER2 solution for detection and analysis of NAbs

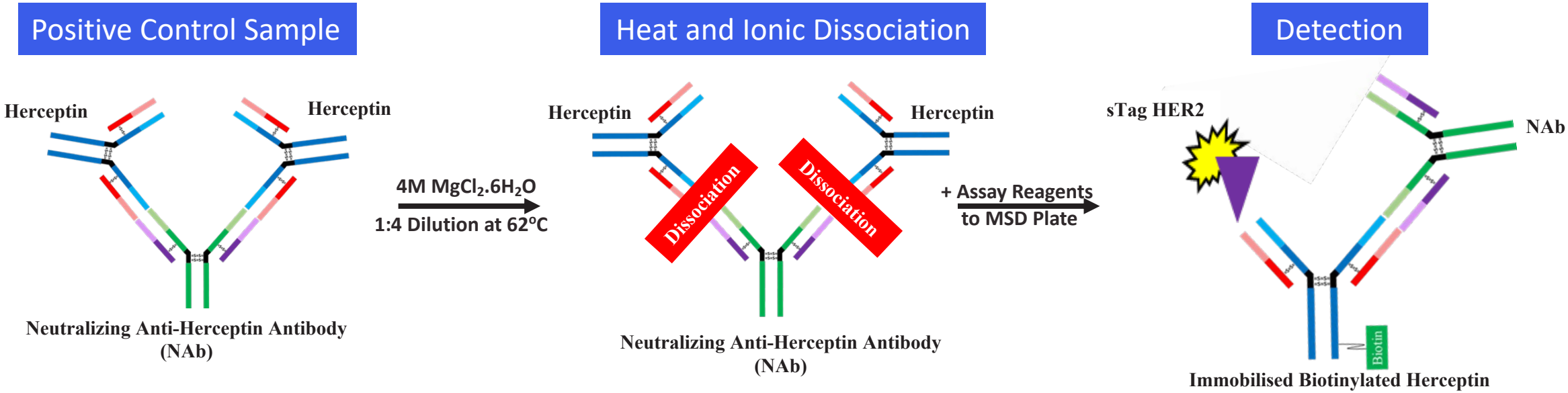
No Sensitivity Achieved for all Optimizations

Zero Drug Tolerance Achieved For All Optimizations

No Incidents Of Drug Interference



High Ionic Strength Dissociation Assay (HISDA)



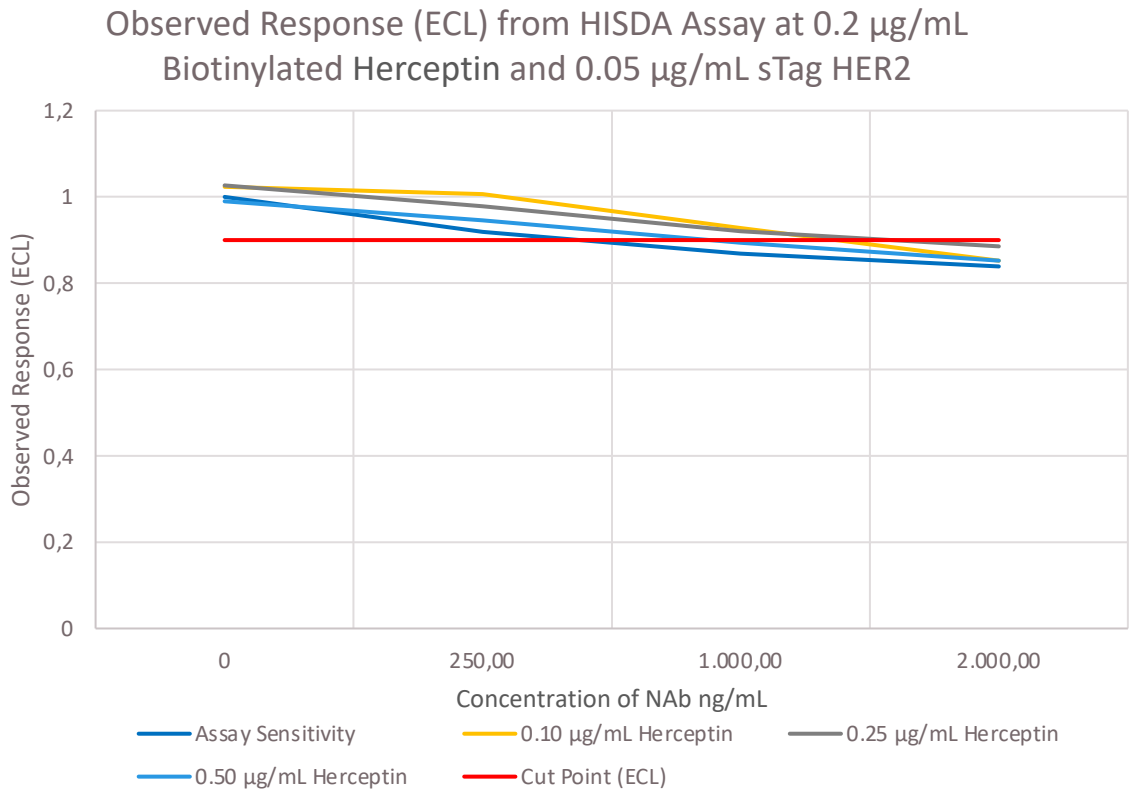
1. **Ionic and Heat Dissociation:** Samples are diluted in Magnesium Chloride Hexahydrate ($MgCl_2.6H_2O$) and incubate at 62°C
2. **Elution of NAbs:** Dissociated NAbs are eluted from the PCR plate and added to an MSD plate coated in Biotinylated Herceptin
3. **Detection of NAbs:** sTag HER2 detection solution is added, and the plate is read on an MSD reader

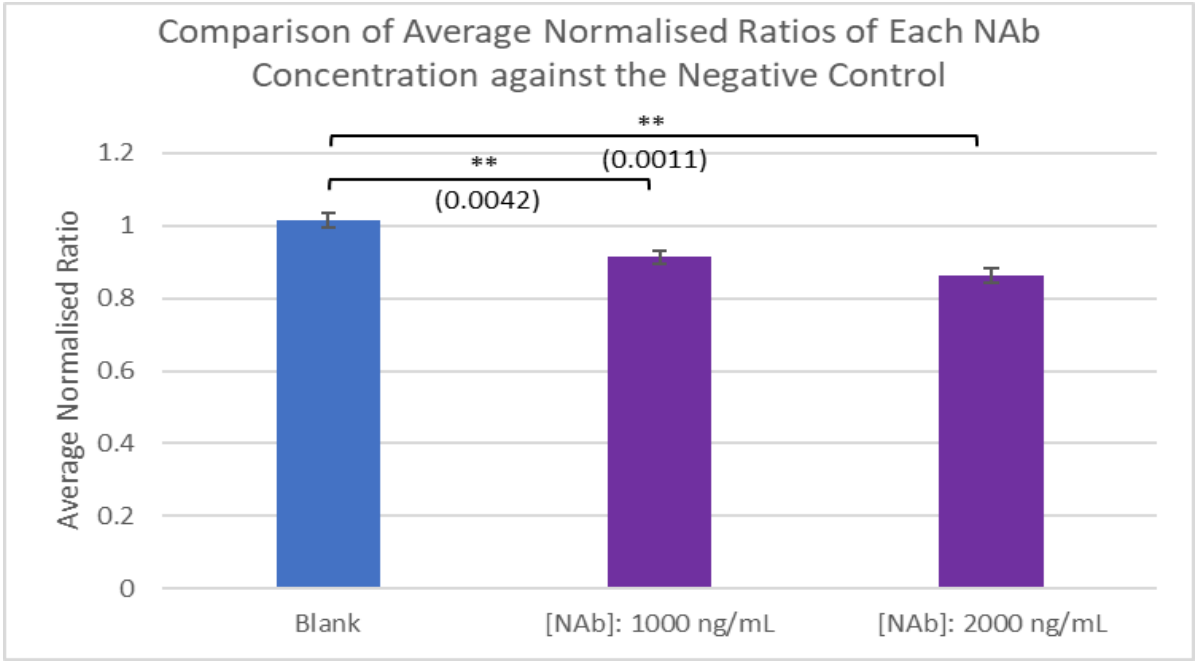
No Sensitivity Achieved

Zero Drug Tolerance Achieved

Acid Optimization at Higher Concentration PC's

Assay performed at NAb concentrations up to 2000.00 ng/mL





Sensitivity Achieved at 1000.00 ng/mL of NAb

Drug Tolerance Achieved at 1000.00 ng/mL and 2000.00 ng/mL of NAb

Final Comparison of Formats

Most Promising Assays for NAb Detection:
Acid Dissociation and HISDA

↓

Acid Dissociation Assay is More Cost Effective
Due to less cost of reagents (i.e. $MgCl_2$) and
Equipment (i.e. Eppendorf Thermomixer R)

↓

HISDA Has a Higher Throughput Due to Shorter
Incubation Times

↓

Both Assays Exhibit High Procedural Simplicity

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Thank You!

Special thanks to Sarah Malpas of Labcorp Drug Development, without whom this project would not have been possible