ARDENA

Positive controls in immunogenicity assays: case studies and best practices

EIP- 18 March 2026 - Esther Biemans, PhD

Overview

- Why positive controls (PCs) matter
- Types of PCs and regulatory expectations
- What good PC behavior looks like
- Modality specific PC strategies – case studies
- Key takeaways

Why positive controls matter

- PCs are used as critical reagents to characterize ADA assays:
  - Sensitivity
  - Selectivity and specificity
  - Drug tolerance
- PCs are essential for routine assay performance monitoring
  - Detect reagent drift
  - In validation and bioanalysis studies

If PCs fail, entire ADA assay is unreliable.

Types of PCs

Polyclonal (pAb)

Best mimic of clinical ADA

Due to heterogeneous epitopes and affinity profiles

Capture realistic variation in patient immune responses

Ideal for

Characterization of assay

Early-stage assay development

Limitations

Batch variability, limited supply and purification/characterization challenges

Monoclonal (mAb)

Highly reproducible

Consistent binding properties

Easier to characterize

Ideal for

Routine assay performance monitoring

Long-term clinical programs

Limitations

May not reflect clinical ADA heterogeneity

Regulatory expectations for PCs

FDA expectations

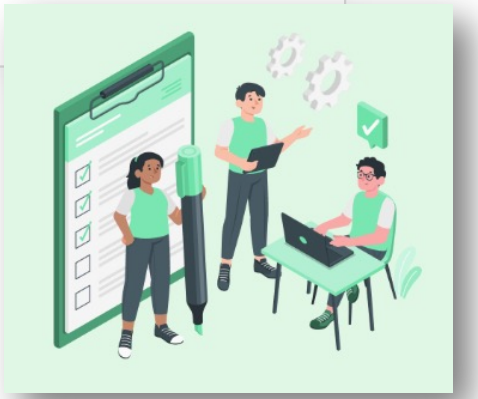
- PCs must be purified and have a known concentration
- Recommends affinity purified and therapeutic targeted pAbs where feasible
- Allows use of mAbs or panels of mAbs
- For therapeutic mAbs, PCs must bind the variable region (idiotype) and not Fc

EMA expectations

- Use of well characterized PCs for standardizing assays
- PCs are critical assay reagents, essential for assay validation
- PCs are needed for all assays (tiers)

Regulatory guidance is high level, but implementations are strict:

PCs must be scientifically justified, not arbitrary selected
Purity, concentration and epitope relevance must be documented
Realistic affinity is required, overly strong PCs are discouraged due to inflated sensitivity



What good PC behavior looks like

Stable and reproducible

PCs must fall within acceptance ranges

Variability in PC recovery indicates assay drift or reagent instability

Failed PC recovery results in failed runs

Realistic affinity and sensitivity

PCs must be purified and have a known concentration

High affinity PCs can artificially inflate sensitivity

PC affinity reflects true clinical ADA

Correct confirmatory inhibition and predictable titer curve

Poor PC inhibition by excess drug suggests issues with assay design or specificity

Titer curve should be smooth, monotonic and reproducible

Mimics expected ADA biology

PCs must represent heterogeneity of patient antibodies

Domain specific PCs for multi domain biologics

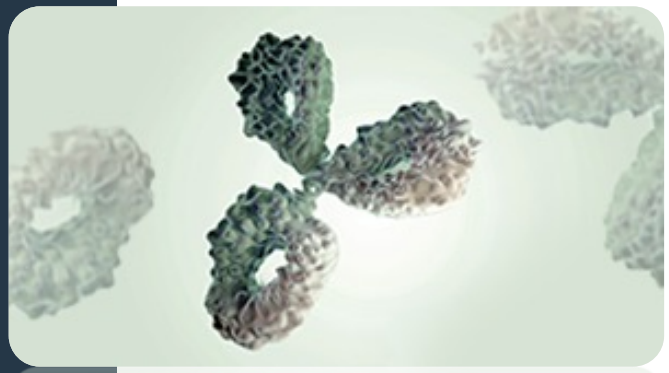
Modality specific PC strategies

Why? ADA responses vary across therapeutic modalities

- Single domain antibodies, multi domain antibodies, Fc-fusion proteins, bispecifics, oligonucleotides
- Different modalities expose different epitopes, use different MoA's and generate different ADA binding patterns

How? Where strategies start to diverge

- Epitope location and molecular structure
  - mAbs: CDR regions
  - BsAbs: separate arms
  - Fc fusions: different domains
- Clinical ADA expectations
  - strong anti-idiotypic ADA for mAbs
  - multi arm responses for BsAbs
  - chemistry driven responses for oligo’s



The more structurally complex the molecule, the more precise and multi component the PC strategy must be.

Case study 1 – therapeutic mAbs

PC Strategy

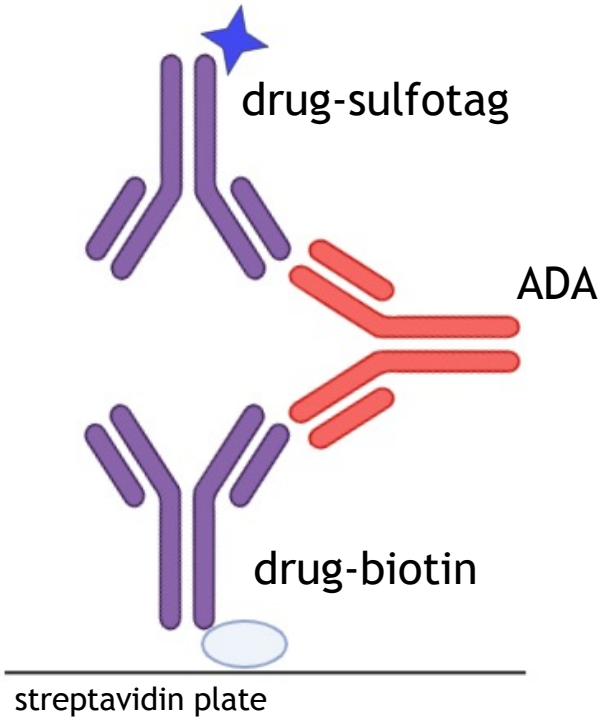
- *Use anti-idiotype antibodies (mAb or pAb) because most ADA are anti-idiotypic*
- *Must bind variable region, not Fc*

- pAbs for assay characterization:
  immunized rabbits with therapeutic mAb
    - pAb
    - idiotypic pAb (affinity purification against drug idiotype)

  mAbs for long-term use (lot consistency):
  produced in HEK293 cells, protein A purified
    - idiotypic mAb 1
    - idiotypic mAb 2

- Clinical method

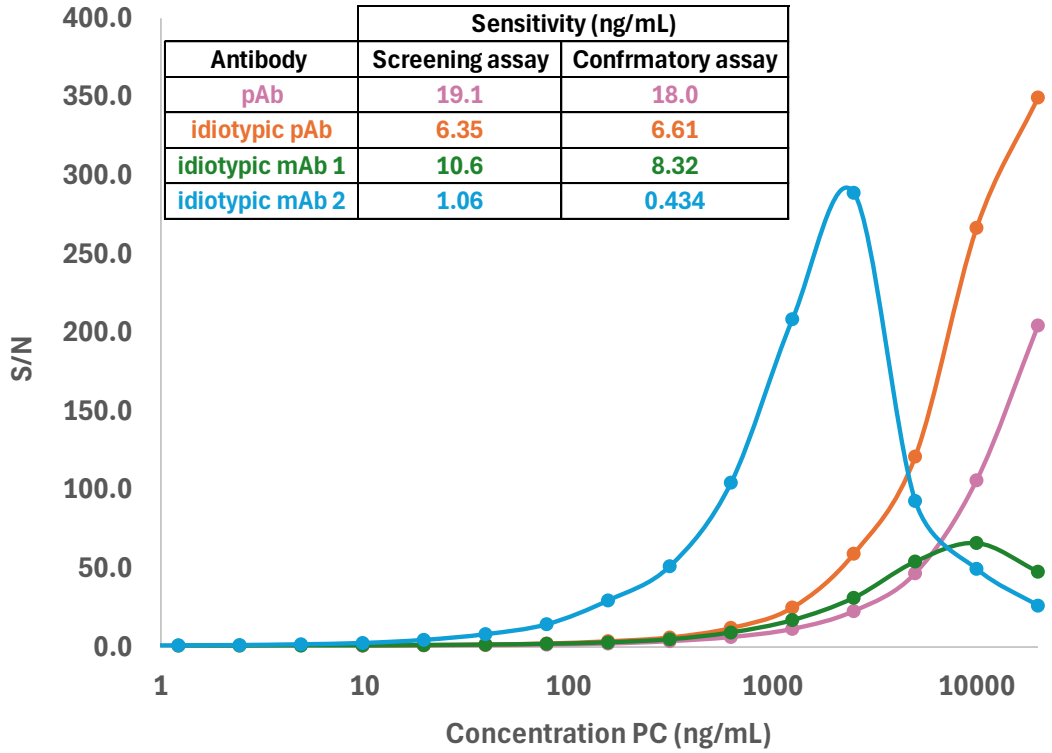
Assay format



Case study 1 – therapeutic mAbs

Sensitivity

Antibody	Sensitivity (ng/mL)	
	Screening assay	Confrmatory assay
pAb	19.1	18.0
idiotypic pAb	6.35	6.61
idiotypic mAb 1	10.6	8.32
idiotypic mAb 2	1.06	0.434



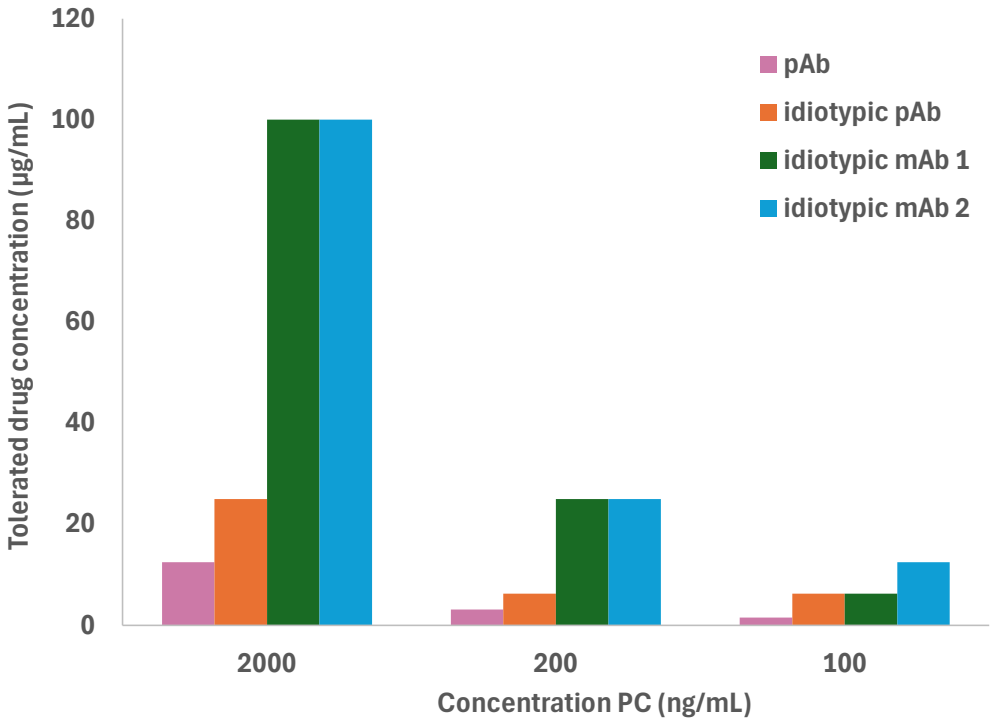
Conclusions

- Sensitivity is antibody dependent; best sensitivity for mAb 2
- mAb 1 shows more realistic sensitivity than mAb 2
- Idiotypic pAb shows better sensitivity than pAb
- Hook effect observed for mAbs

How are the different sensitivities related to drug tolerance?

Case study 1 – therapeutic mAbs

Drug tolerance



Conclusions

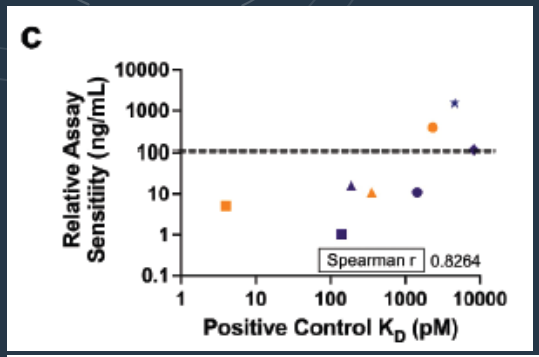
- For mAbs, drug tolerance is not related to sensitivity;
  mAb 2 is 10-fold more sensitive than mAb 1, but drug tolerance is comparable
- For pAbs, drug tolerance is more in line with sensitivity;
  idiotypic pAb is 3-fold more sensitive than pAb, drug tolerance is 2-fold better for idiotypic pAb

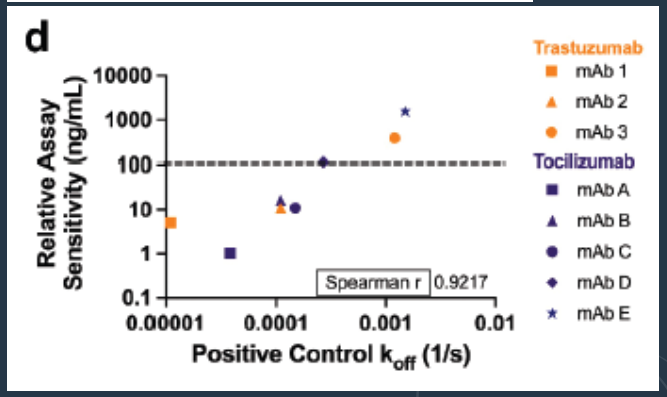
Proceed with method validation using mAb 1 and idiotpyic pAb

Observed mAb performance is in line with published literature

Case study 1 - PC affinity and sensitivity

A 2025 bioanalysis study demonstrated that PC binding kinetics (K_D and K_{off}) significantly influence ADA assay sensitivity

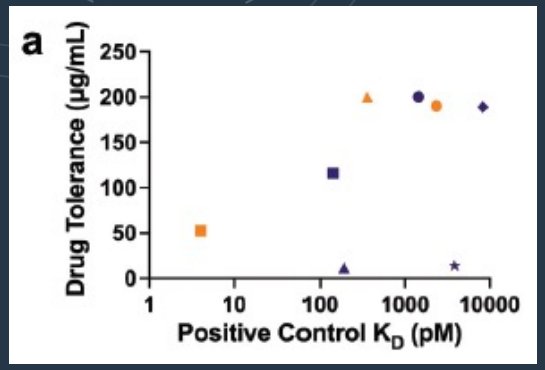


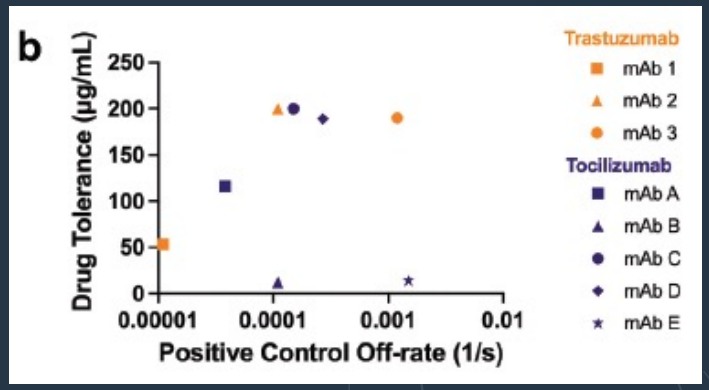


Correlation between higher affinity antibodies (low K_D and K_{off}) and increased assay sensitivity

Case study 1 - PC affinity and drug tolerance

A 2025 bioanalysis study demonstrated that PC binding kinetics (K_D and K_{off}) do not influence drug tolerance (at 100 ng/mL PC)





No clear correlation between higher affinity antibodies (low K_D and K_{off}) and increased drug tolerance

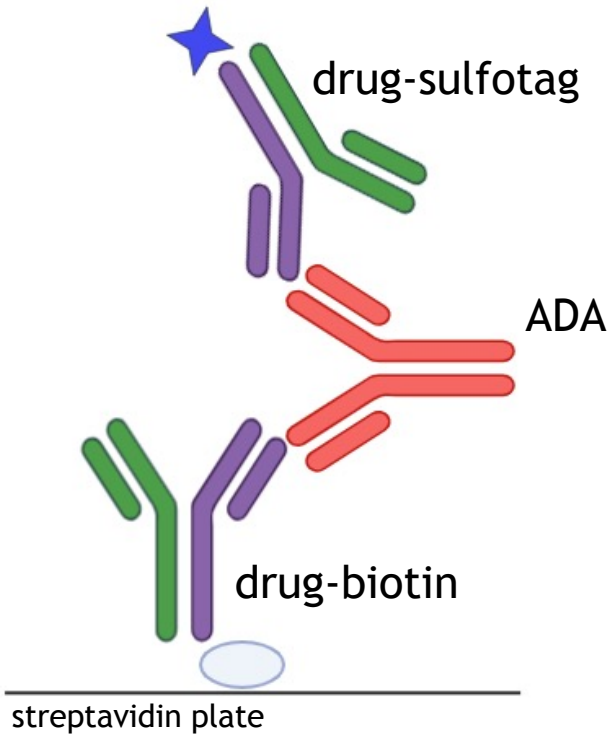
Other factors than binding affinity have an influence on drug tolerance

Case study 2 – bispecific antibodies

PC Strategy

- *PCs must enable domain specific ADA classification, use PCs against different domains (arm A and arm B)*
- *PC affinity must be carefully chosen to avoid altered bridging behaviour*
- Recombinant idiotypic mAbs for assay characterization and assay performance monitoring
- Mix mAbs against arm A and B to generate domain specific PCs (1:1 molar ratio)
- Clinical method

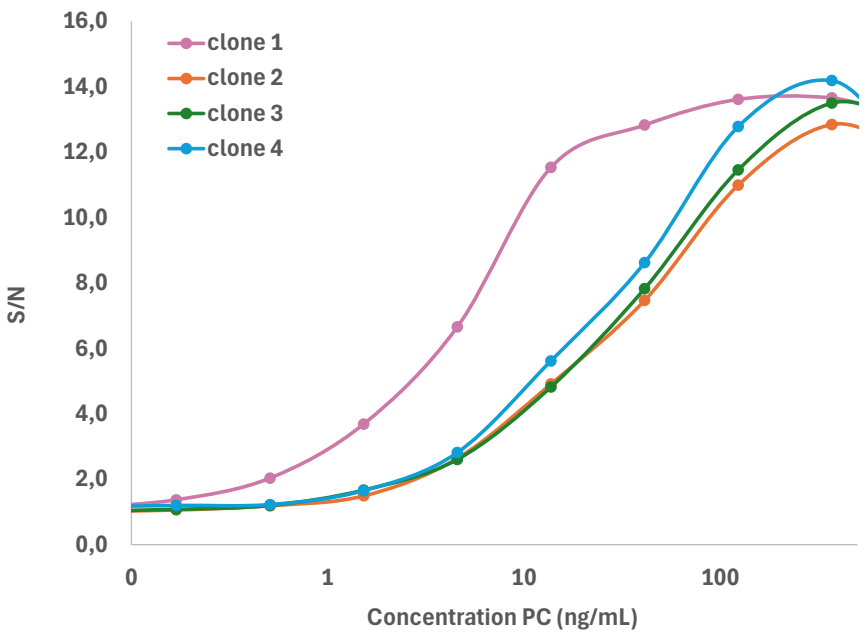
Assay format



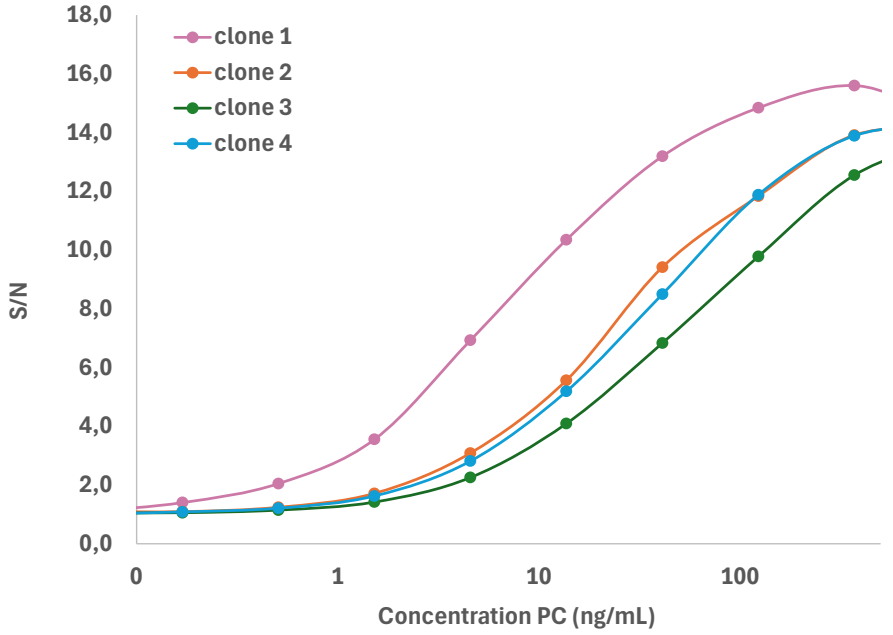
Case study 2 – bispecific antibodies

Screening of clones

Anti-arm A



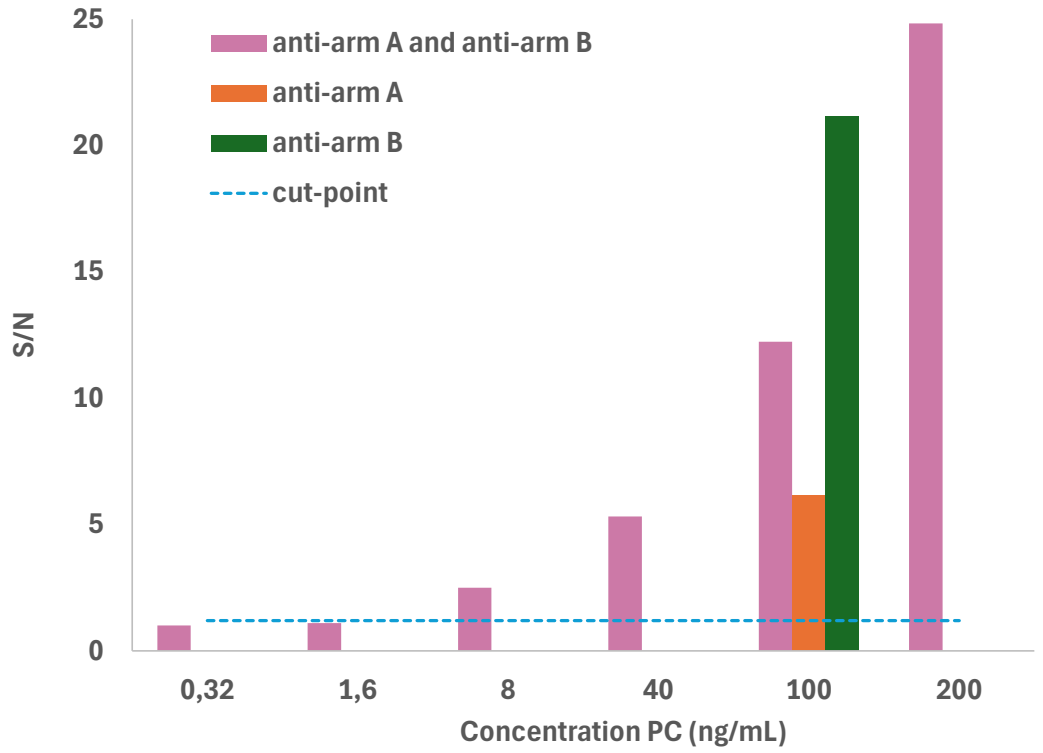
Anti-arm B



Clone 4 was selected for both arm A and arm B;
not highest affinity, resulting in realistic sensitivity

Case study 2 – bispecific antibodies

Sensitivity



Conclusions

- Sensitivity of mixed PC is estimated around 3 ng/mL
- Mixed PC response is approximately average response of individual mAbs at PC 100 ng/mL
- No altered bridging behaviour is observed using the mixed PC;
  Assay sensitivity can differ depending on which arm is used for capture vs. detection if mAbs have different affinities.

Continue with method development and check altered bridging behaviour for drug tolerance and target interference

Case study 3 – Fc fusion proteins

PC Strategy

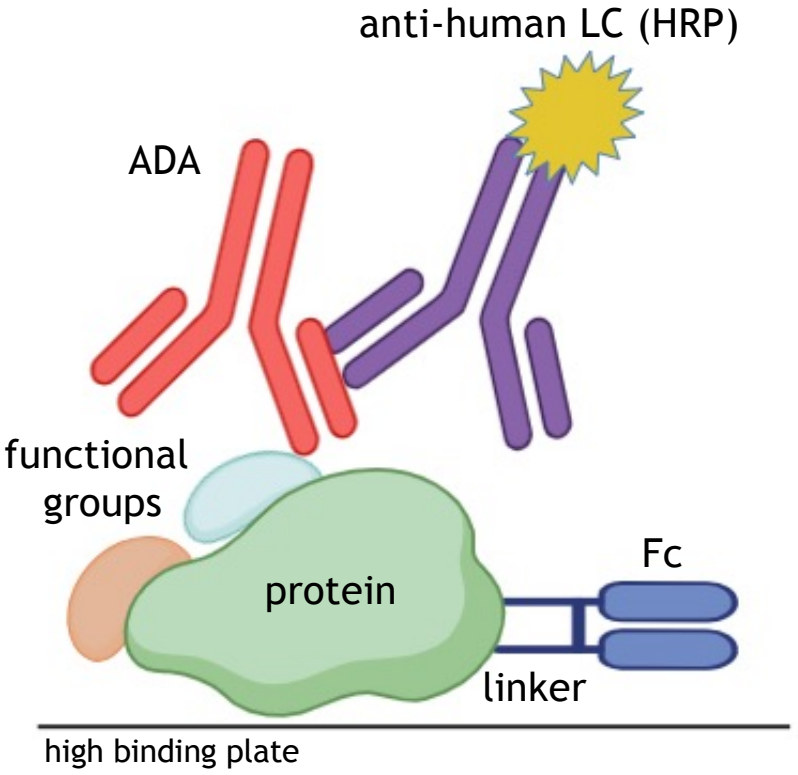
- *ADA may target different domains*
- *Need PCs that target the biologically relevant part (usually non-Fc domain)*
- *Multiple PCs may be required*

- Idiotypic mAbs (human IgG backbone) against each part (domain) of the Fc fusion protein

- Mix mAbs to generate domain specific PCs (1:1 molar ratio)

- Preclincial methods (NHP and rat)

Assay format



Case study 3 - concern is antibody interference in mixed PC

Epitope competition

Antibodies binding same/overlapping epitope

High affinity antibodies dominate, loss of diversity

Steric hindrance

Antibodies on nearby epitopes physically block each other

Common on small antigens/surfaces, reduced binding

Allosteric effects

Binding of one antibody alters antigen confirmation

Can expose or hide other epitopes

Affinity dominance

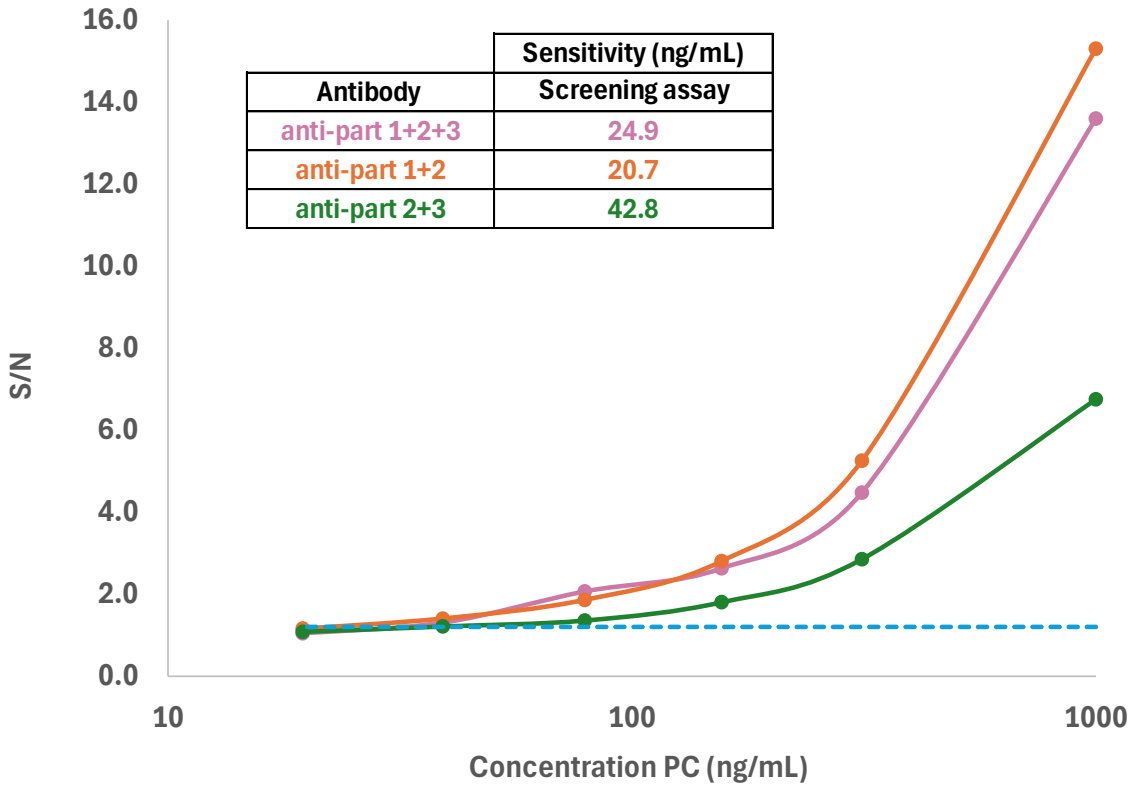
High affinity antibodies monopolize binding

Weak affinity antibodies appear underrepresented

Case study 3 – Fc fusion proteins

Sensitivity

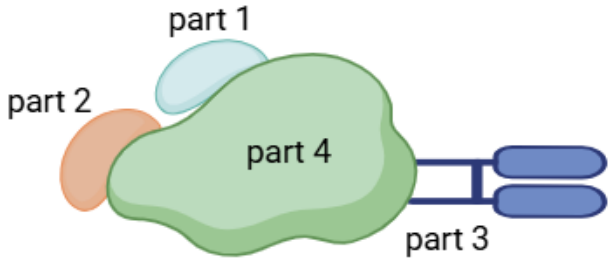
Antibody	Sensitivity (ng/mL) Screening assay
anti-part 1+2+3	24.9
anti-part 1+2	20.7
anti-part 2+3	42.8



Conclusions

- NHP method, focus on screening
- Sensitivity varies depending on PC target region
- Anti-part 3 has lowest contribution to sensitivity

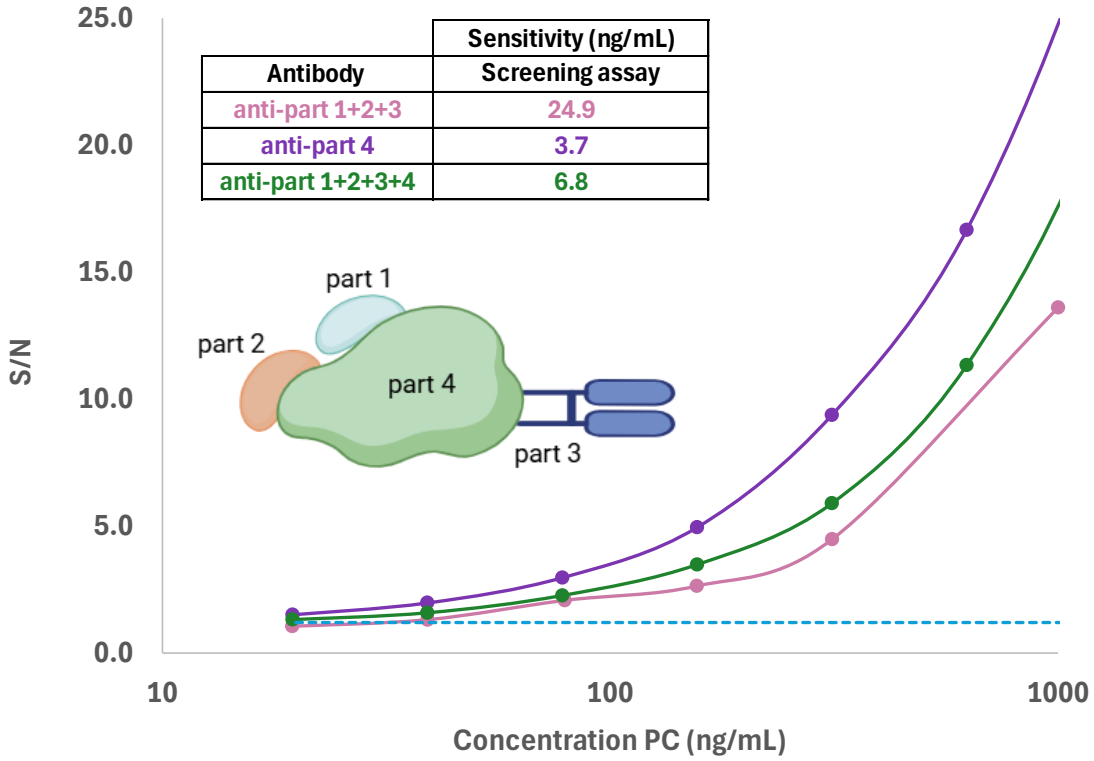
What will be the effect when antibody against part 4 is added to the mix?



Case study 3 – Fc fusion proteins

Sensitivity

Antibody	Sensitivity (ng/mL) Screening assay
anti-part 1+2+3	24.9
anti-part 4	3.7
anti-part 1+2+3+4	6.8



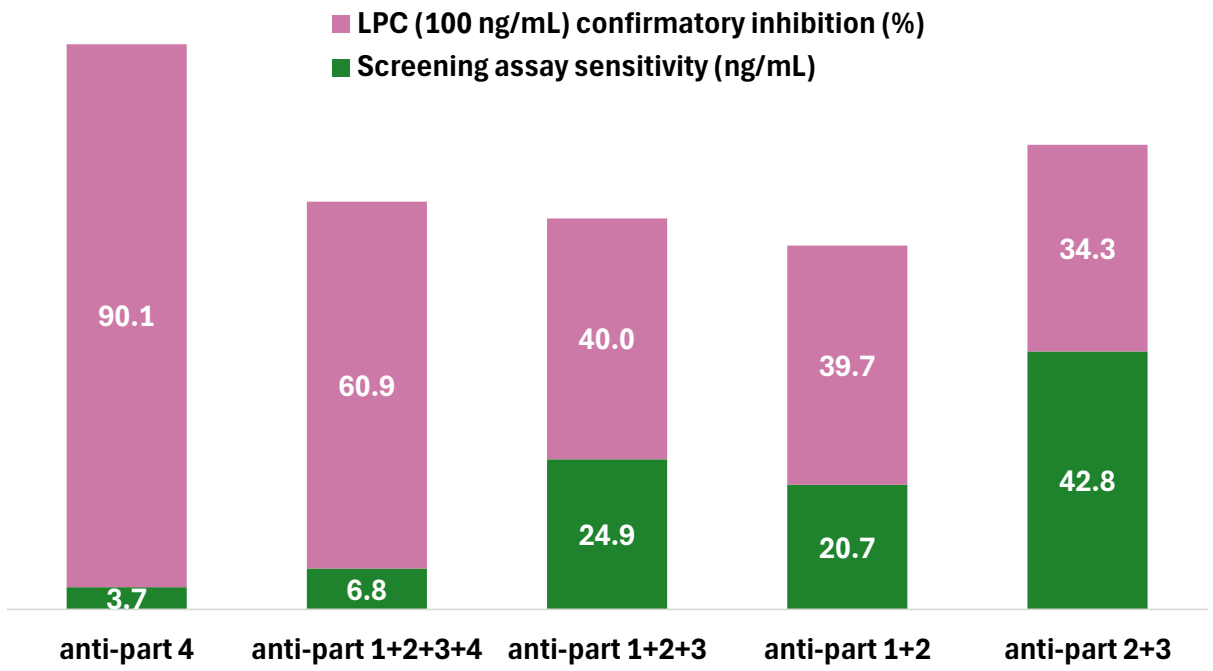
Conclusions

- Anti-part 4 shows greatest contribution to overall sensitivity
- Sensitivity of 4 antibody mixture is in line with average performance of each individual antibody:
  - ✓ sensitivity is primarily driven by affinity
  - ✓ no interference of antibodies in mixed PC is observed in screening assay

What about interference in the confirmatory assay?

Case study 3 – Fc fusion proteins

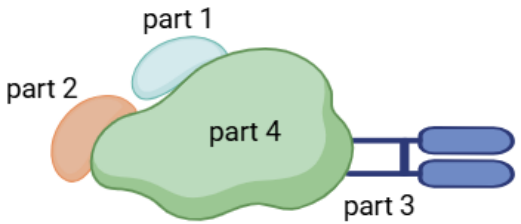
Inhibition



Conclusions

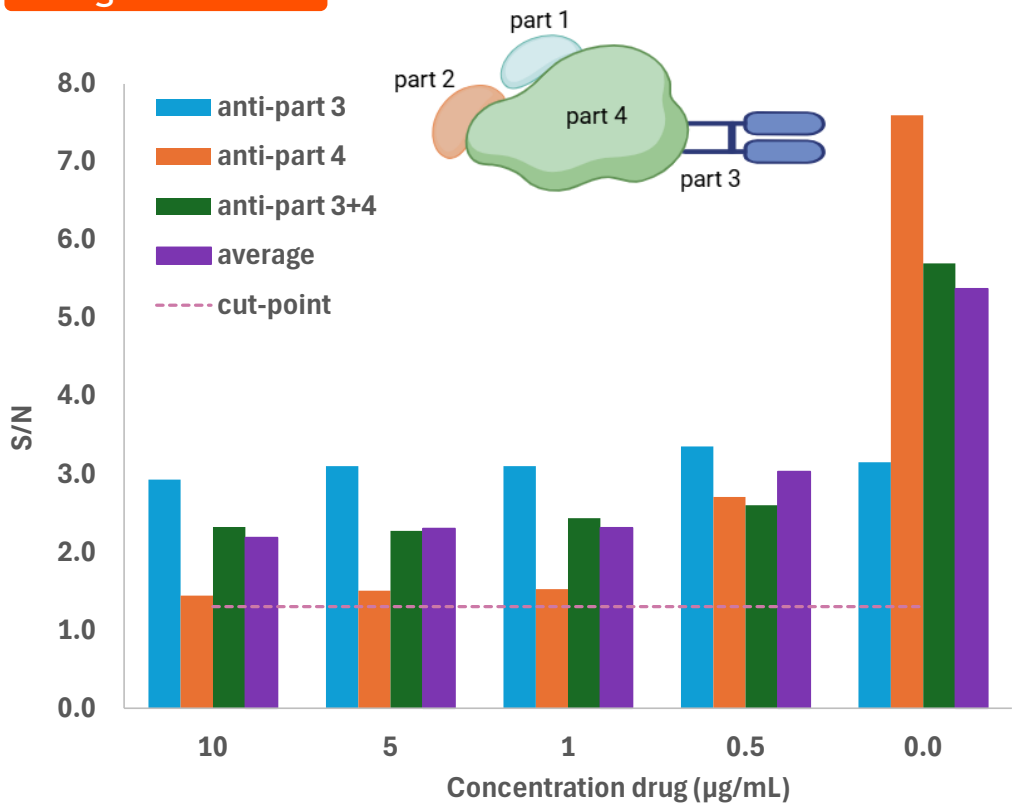
- Strength of ADA-drug binding (specificity) is proportional to sensitivity
- No interference of antibodies in mixed PC is observed in confirmatory assay

What about interference in drug tolerance?



Case study 3 – Fc fusion proteins

Drug tolerance



Conclusions

- Rat method, anti-part 1 and 2 excluded
- Anti-part 3 antibody is very drug tolerant (lowest sensitivity, lowest affinity)
- Anti-part 4 antibody is less drug tolerant (highest sensitivity, highest affinity)
- Drug tolerance is averaged out in mixed PC, no interference of antibodies

No relevant interference of antibodies in mixed PC observed in all 3 tiers

Case study 4 – Oligonucleotides

PC Strategy

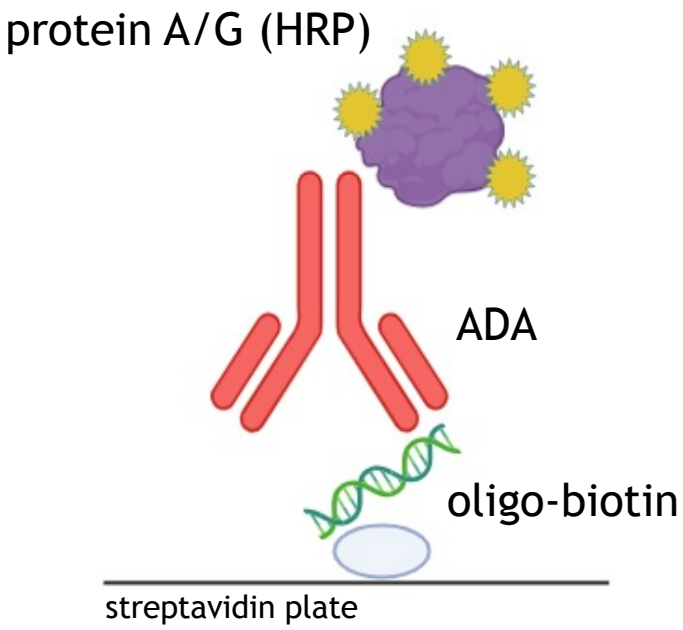
- *ADA can target chemical groups, linker modifications, or unexpected conjugate impurities*
- *PC must recognize either the backbone chemistry and chemical modification, or the sequence if sequence dependent immune recognition is expected*

- Idiotypic mAbs for assay characterization and assay performance monitoring

- Recombinant monoclonal antibodies generated against complete oligonucleotide and produced in mammalian cells

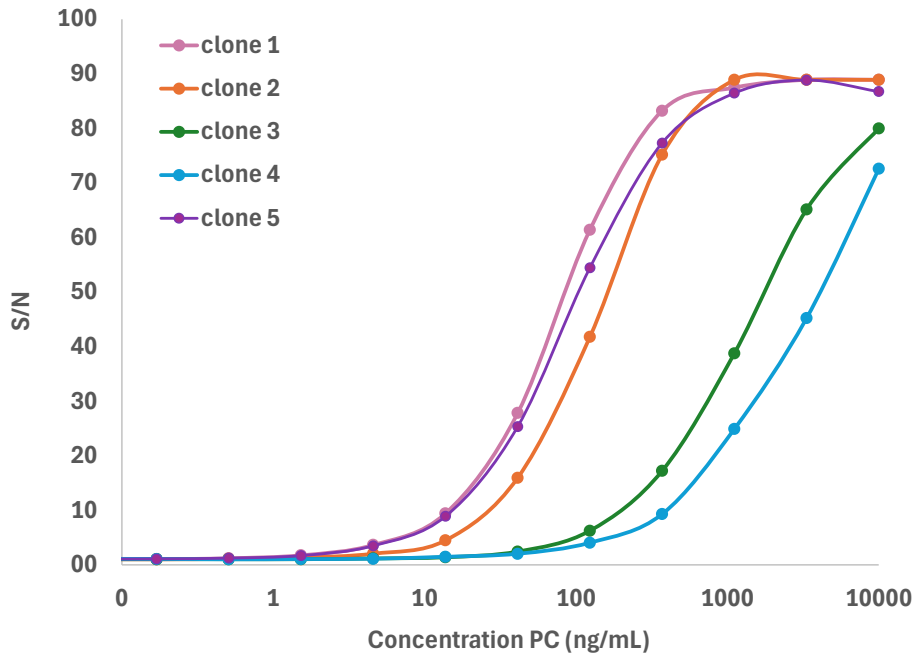
- Clinical method

Assay format



Case study 4 – Oligonucleotides

Screening of clones



Conclusion

- Clone 5 was selected;
  Higher affinity clone needed to achieve sensitivity of 100 ng/mL; high expected variation in the assay due to assay format

Case study 4 – Oligonucleotides

Validation results

Validation parameters	Screening	Confirmation	Titration
Cut-point	1.49	34.4%	2.41
Sensitivity	77.9 ng/mL	50.9 ng/mL	290 ng/ml
LPC level determination	350 ng/mL	350 ng/mL	800 ng/mL
Selectivity	Unspiked(5/5) Spiked at LPC(5/5) and LPCt (5/5) Hemolyzed (1/1 for both unspiked, spiked at LPC and LPCt) Lipemic (1/1 for both unspiked, spiked at LPC and LPCt)		
Precision - within run CV%	NC: 10.9 LPC: 1.9 MPC: 3.5 HPC: 1.5	NC:19.2 LPC: 3.1 MPC: 2.0 HPC: 0.9	LPCt: 6.1
Precision - between run CV%	NC: 5.3 LPC: 15.2 MPC: 16.1 HPC: 11.2	NC: 23.2 LPC: 14.3 MPC: 2.3 HPC: 1.5	LPCt: 6.3
Drug tolerance	350 ng/mL PC: 125 µg/mL drug		
Prozone	Not observed		
Stability	Bench-top stability - 19 hours at roomtemperature Freeze/thaw stability - 9 freeze/thaw cycles		

Immunogenicity method is successfully used for clinical phase 1 and 2 studies

PC strategy – risk and structure driven

Targeting variable regions

Anti-idiotypic PCs aligned to CDR/variable regions

pAbs for characterization of assay

mAbs for long-term assay performance

Arm-specific coverage

PCs required for each arm

Optional whole molecule PC to assess bridging behavior

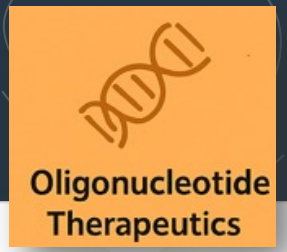
Enables domain-specific ADA classification

Domain and linker coverage

ADA may target functional domain, Fc region and linker

PCs aligned to clinical risk regions

Requires multiple PCs for full characterization



Chemistry driven immunogenicity

ADA often recognize backbone chemistries

PCs aligned to exact chemical modifications

Sequence specific PCs only when biologically justified

Key takeaways

- **PCs are essential for assay credibility**
  - They define sensitivity, specificity, and drug tolerance
  - All ADA tiers depend on correct PC behavior
- **PC selection must be fit for purpose**
  - Match to molecule biology and assay format
  - Balance realism (pAb) and reproducibility (mAb)
- **Affinity realism matters**
  - Overly strong PCs inflate sensitivity
- **Complex modalities require domain aligned PCs**
  - Bispecifics, Fc fusions, and oligos need domain- or chemistry specific controls
- **Think lifecycle, not only a quick selection**
  - Stability, trending and lot bridging ensures long-term performance

ARDENA

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