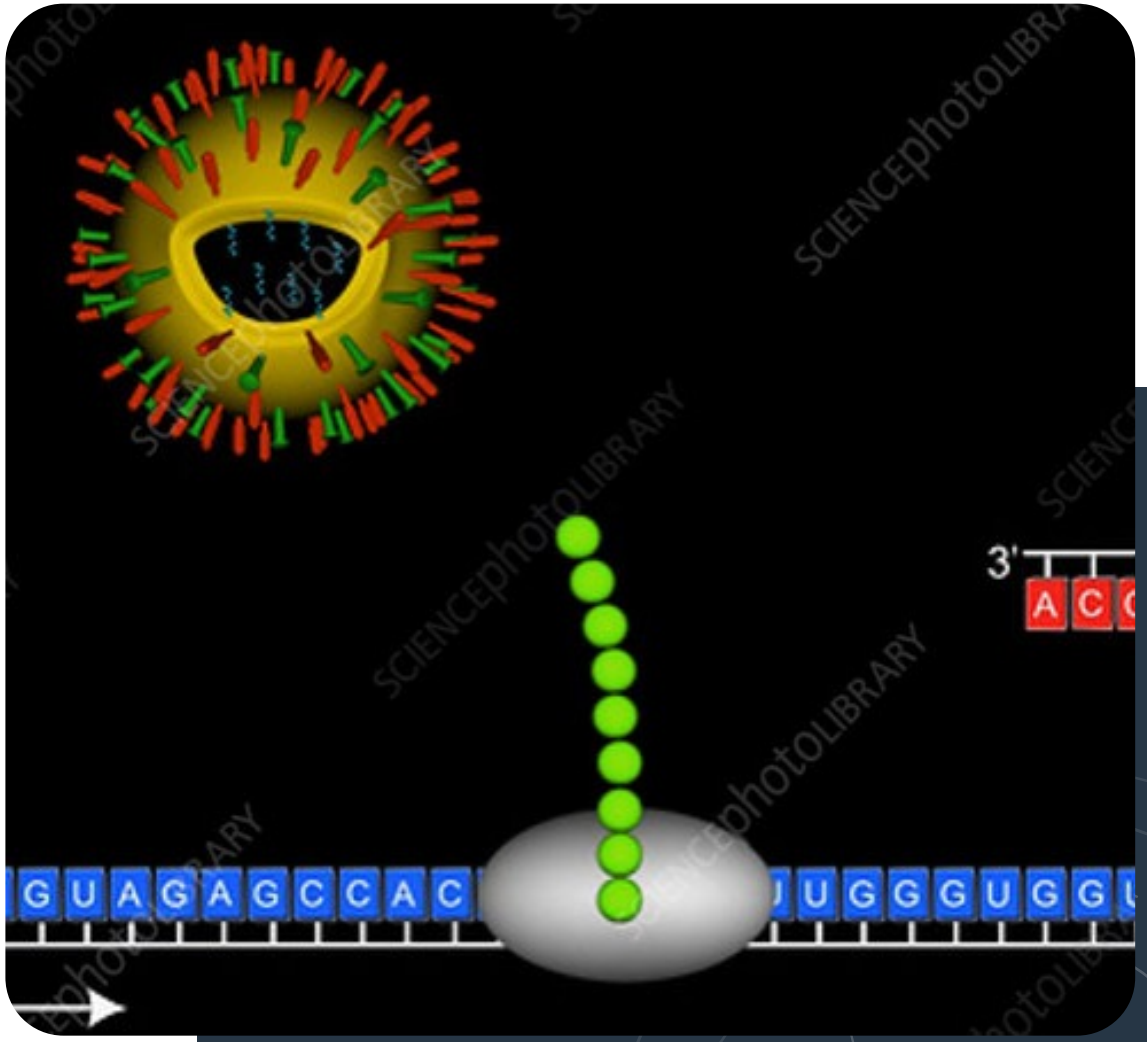
ARDENA

How to increase assay sensitivity of oligonucleotide Ligand Binding pharmacokinetic assay and how to develop strategies on dealing with ADA challenges of oligonucleotides - Ardena case study

EBF Spring Workshop - 08 June 2023 - Malaga, Spain - Foka Venema

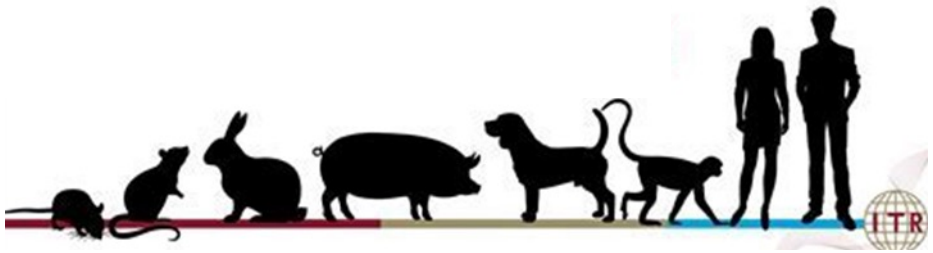
Introduction

- Antisense oligonucleotide (ASO)
- Binds target RNA sequence inside cells
- Gene silencing
- ASO in Phase 1/2a clinical trial
- ASO multiple disease models

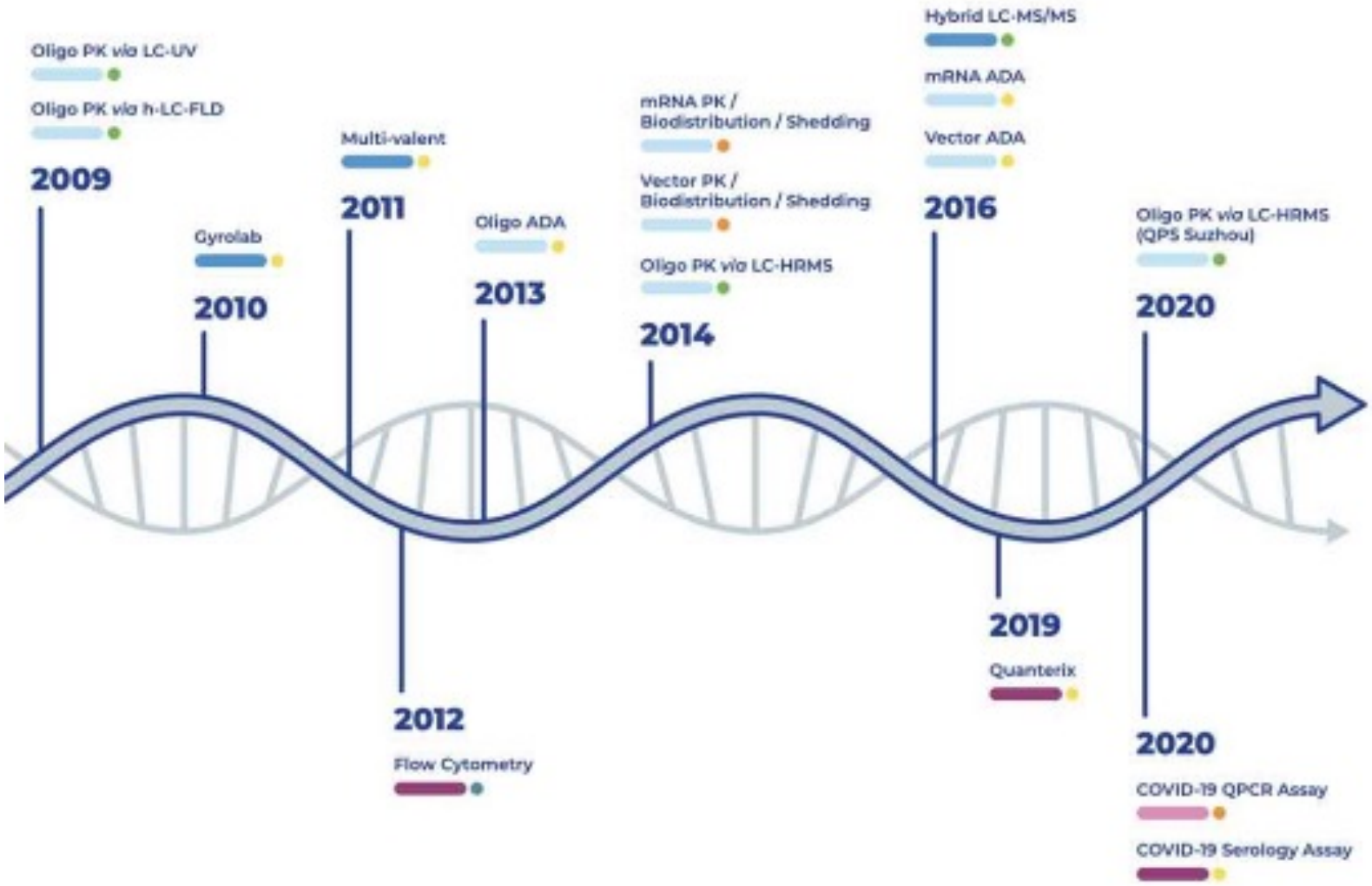


Antisense oligonucleotide PK assay

- Complex sample matrices plasma, CSF and preclinical brain, liver, kidney, spleen
- GLP compliant - oligonucleotide full length product (FLP) assay
  Required LLOQ < 1.00 ng/mL
  Human LLOQ < 0.100 ng/mL
- FLP assay does not fully distinguish between FLP, modified or truncated forms of ASO



History of Measurement of ASO



Bioanalysis of ASO

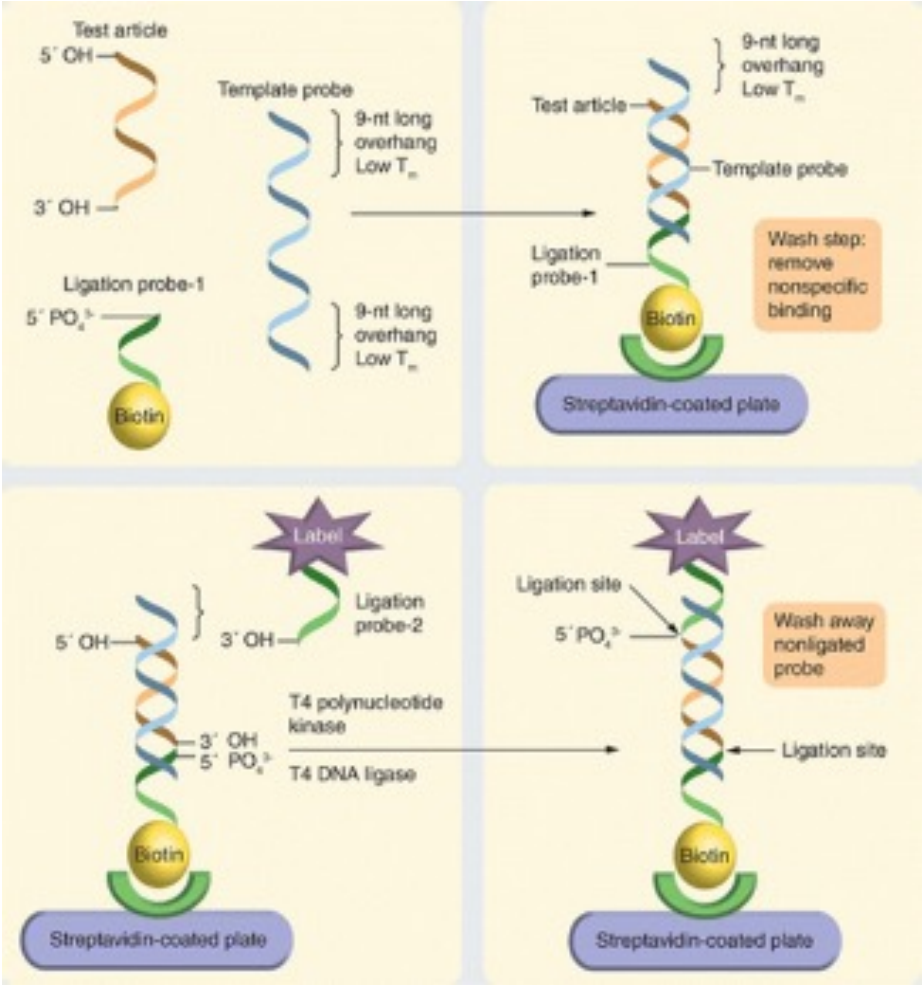
- Conventional approach is LBA
- Sensitivity - specificity

1. Spectrophotometer - exposure UV 260 nm - not validated
2. LC-MS/MS - Ardena - low ng/mL range - not validated since precision not within 20.0%
3. Ligation hybridization LBA - LLOQ of 200 ng/mL - fully validated EMA and FDA
4. Dual probe hybridization LBA - LLOQ of 3.00 ng/mL - fully validated EMA and FDA
5. Dual probe hybridization LBA with antibody - LLOQ of 0.0500 ng/mL - fully validated ICH M10

Bioanalysis of ASO

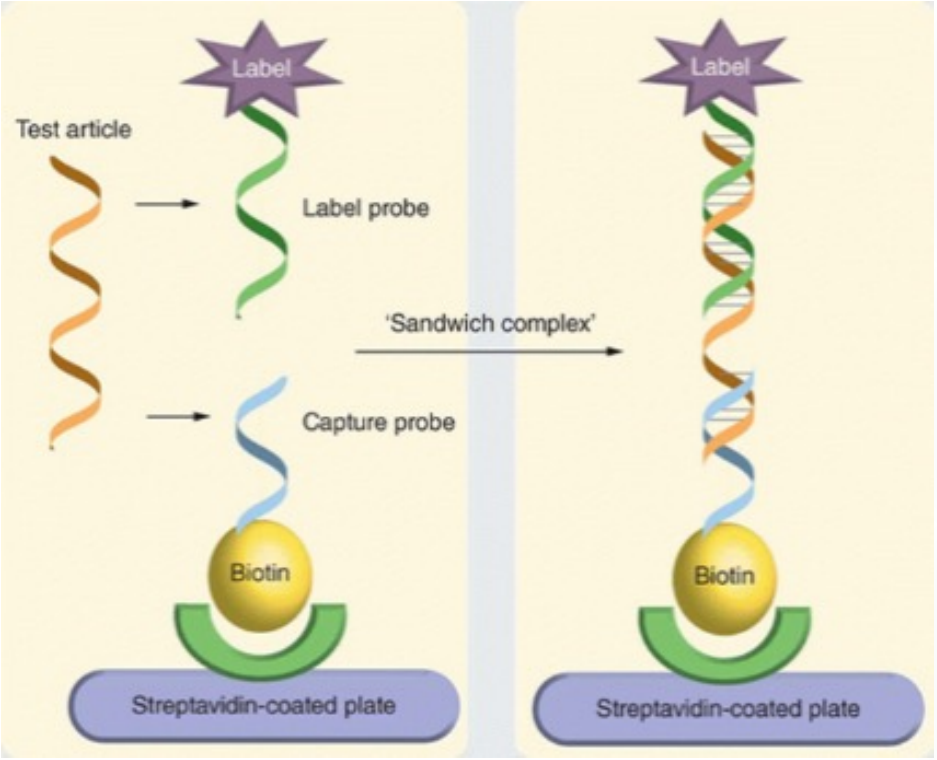
- Hybridization method for low LLOQ pharmacokinetic assay
- Design of experimental procedure
- Selection and quality of critical reagents
- Absorption measurement
- Electrochemiluminescence (MSD-ECL)
- Optimized matrix interference and background signal (S/N)
- Specificity 3’ metabolites

Ligation hybridization



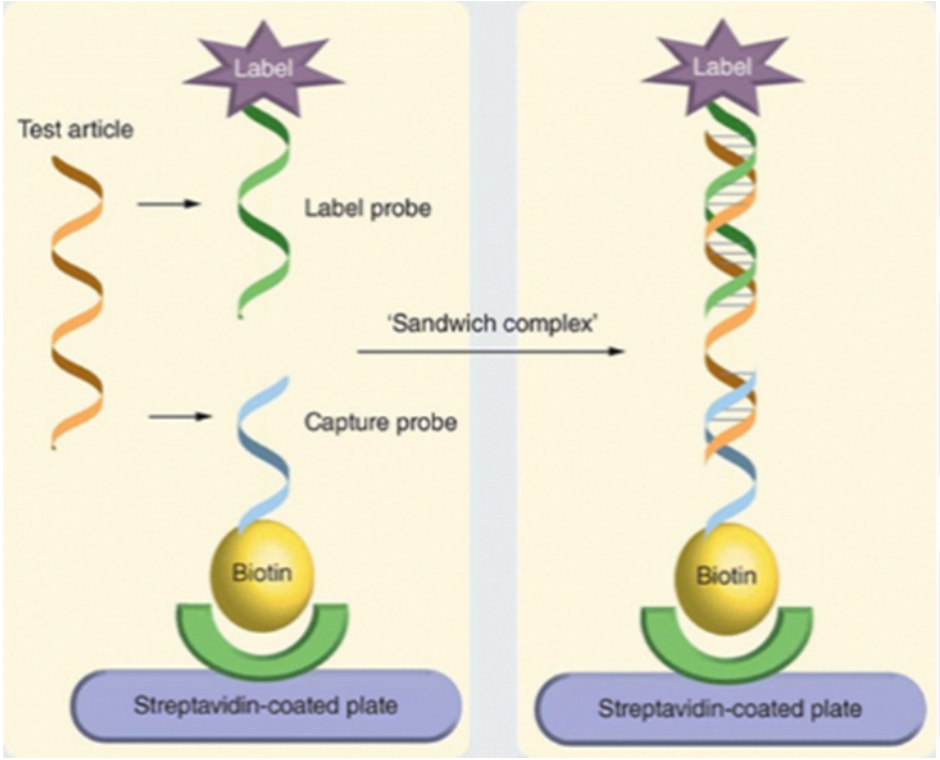
- Intact 3’ end oligonucleotide
- Ligation probe
- Single template probe binds oligonucleotide
- Complementary to 5’ overhang template probe
- Overhang 5’ end
- Hybridized oligonucleotide with T4 DNA Ligase

Dual probe hybridization



- Biotin-labelled capture probe
- Probe = single-stranded RNA with known sequence
- ASO
- Stability of hybrid
- Labeled detection probe
- Improved assay sensitivity

Dual probe hybridization + anti-digoxigenin antibody



- Biotin-labelled capture probe
- Streptavidin plate
- Digoxigenin-labelled detection probe
- SULFO-TAG labelled anti-digoxigenin antibody
- Readbuffer, captured labels to emit light
- At least two SULFO-TAG for signal amplification

Pharmacokinetic considerations

- Instability ASO linkage by nucleases
- Increased binding affinity for complementary nucleic acids
- Specificity
- Quality of critical reagents
- Difference between matrices
- Compliance FDA Guidance for Industry and EMA
- Human matrices assay compliance ICH M10
- LBA not fully distinguish between full-length oligonucleotide and metabolites
- ASO can be bound to plasma proteins
- No sample extraction
- Tissue homogenization sample volume 10.0 µL

Method development PK

- Response ASO compared to blank matrix (S/N)
- Optimalisation of wash buffers, incubation temperature, incubation time
- Probe design, technological development; ≥ 2 SulfoTAG coupled to antibody
- Sensitivity required < 0.100 ng/mL in CSF - impact is lower ULOQ
- ULOQ increased by dilutional linearity
- Optimize blockers and diluents for optimal S/N
- Different plate blockers and sample diluents tested
  (ChonBlock, casein in PBS, Low Cross buffer, Monster Block, Starting Block, Super Block, Neptune Block, SynBlock, BSA/HSA, PBS, TBS, PBS)

Method development PK

- Decrease matrix interference by increasing dilution factor
- Identify biological outliers
- **Reduce** wash step for optimal sensitivity
- Reduce assay time
- Optimize assay temperature (RT, 37°C - 45°C)
- **Add** wash step to wash away unbound
- Use deep well plates
- Use anti static device

Results ASO PK

→ Life-cycle PK assay, PK assay validation results Plasma, CSF and tissue

Validation Results	2013-2020	2021	2023
Species	Cynomolgus, rat, mouse	Cynomolgus	Human
Platform	Absorption	MSD-ECL	MSD-ECL
Capture	Ligation probe	Capture probe	Capture probe
Detection	Hybridized + T4 DNA Ligase	Detection probe	Detection probe + Ab
Calibration range Plasma	40.0 - 800 ng/mL	0.500 - 230 ng/mL	0.0500 - 150 ng/mL
Calibration range CSF	200 - 4000 ng/mL	3.00 - 230 ng/mL	0.0500 - 150 ng/mL
A & P (n=6)	< 20.0% (<25.0%)	< 20.0% (<25.0%)	< 20.0% (<25.0%)
Stability (BT, FT, LTS)	72 h, 6 FT, 525 days ≤-70°C	25 h; 6 FT, 323 days ≤-70°C	BT, FT, LTS
MRD	20	1	1

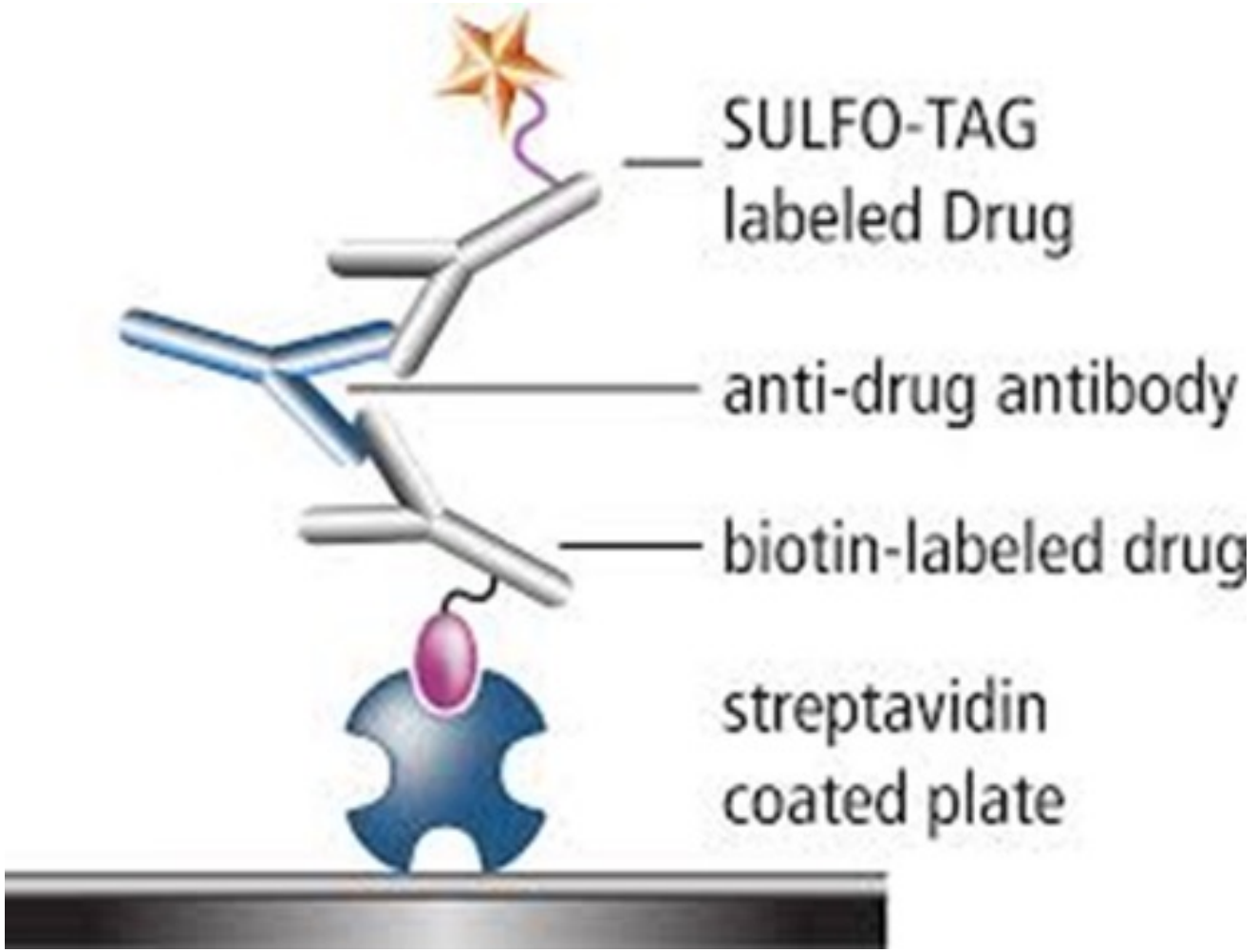
Results ASO PK

→ Life-cycle PK assay, PK assay validation and PK bioanalytical results

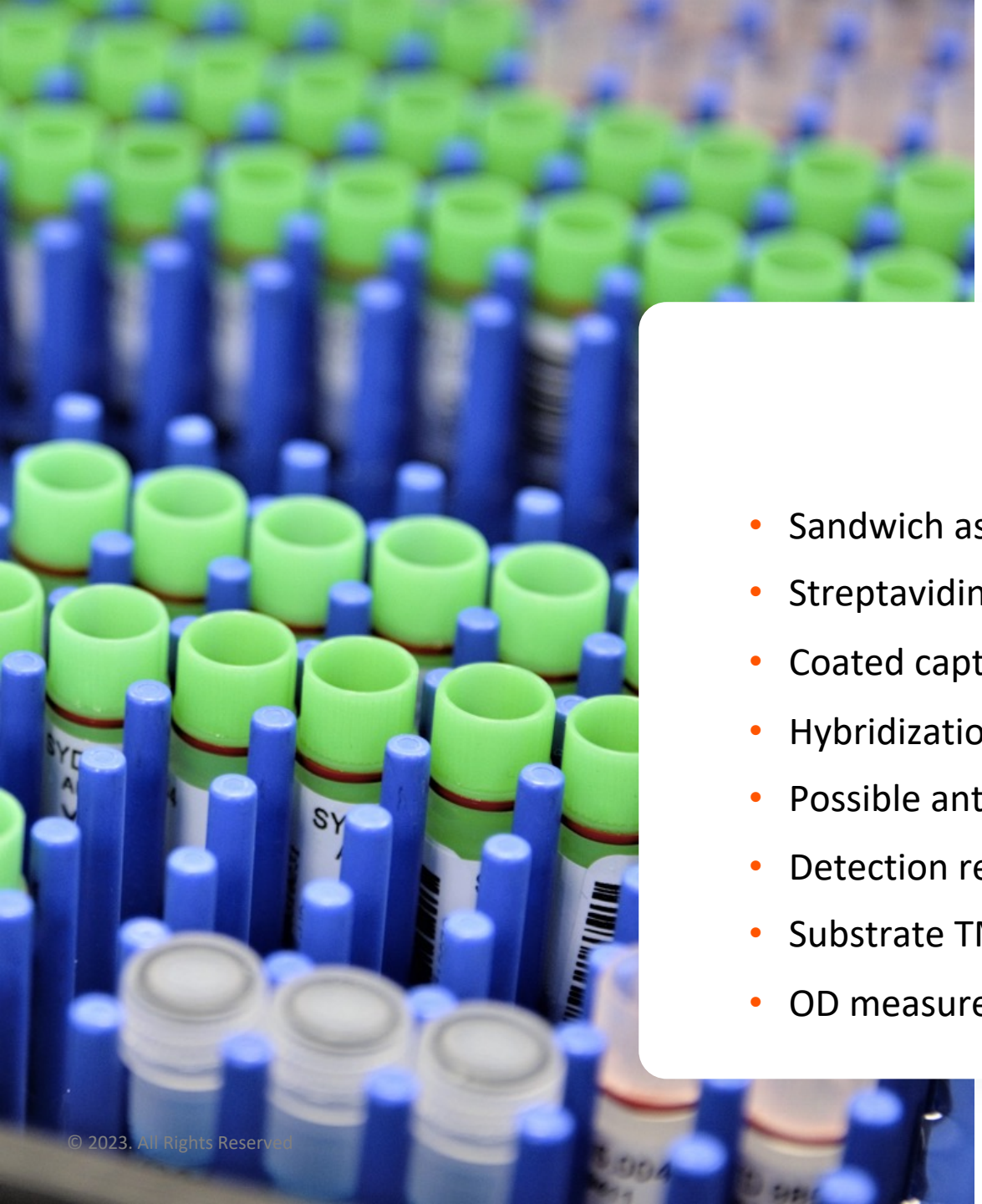
Validation Results	2013-2020	2021	2023
Selectivity	10 of 10 lots	6 of 6 lots, no effect of hemolysis	10 lots
Specificity (n-1)	N/A	3'n-1 metabolite of ASO -29.0% bias for 0.500 ng/mL and 8.3% bias for 230 ng/mL	May 2023
Bioanalysis	Plasma, CSF and tissue	Plasma, CSF and tissue	Plasma and CSF
ISR	Yes	Yes	Yes
C_{max}	C_{max} CSF 1000-fold higher than plasma C_{max}		Q3 2023
% samples < LLOQ plasma	82.0% < 40.0 ng/mL	28.8% < 0.500 ng/mL	Q3 2023
% samples < LLOQ CSF	96.6% < 200 ng/mL	48.6% < 3.00 ng/mL	Q3 2023

Anti-drug antibody assay

- Immunogenicity assay
  Clinical relevance
- Immunogenicity assay
  Interpretation of PK data
- MSD-ECL bridging assay could not be developed
  Bivalent anti-ASO antibody

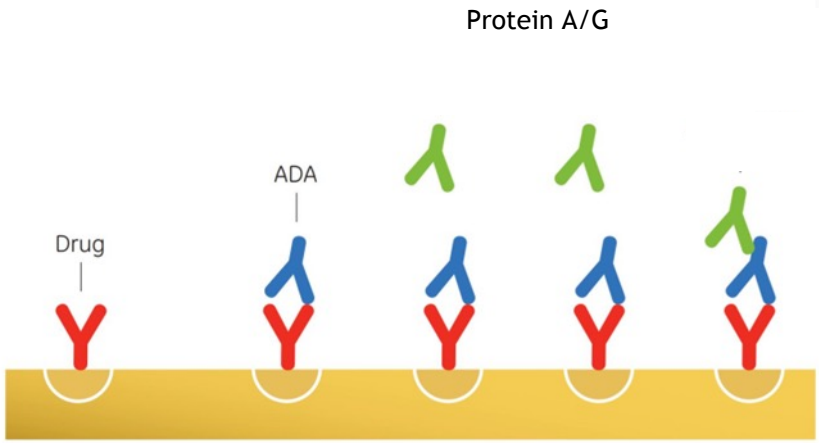


MSD-ECL bridging assay



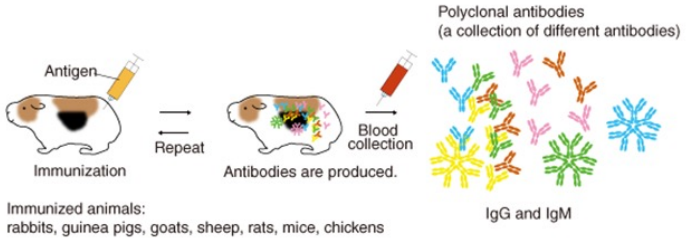
Method development ADA

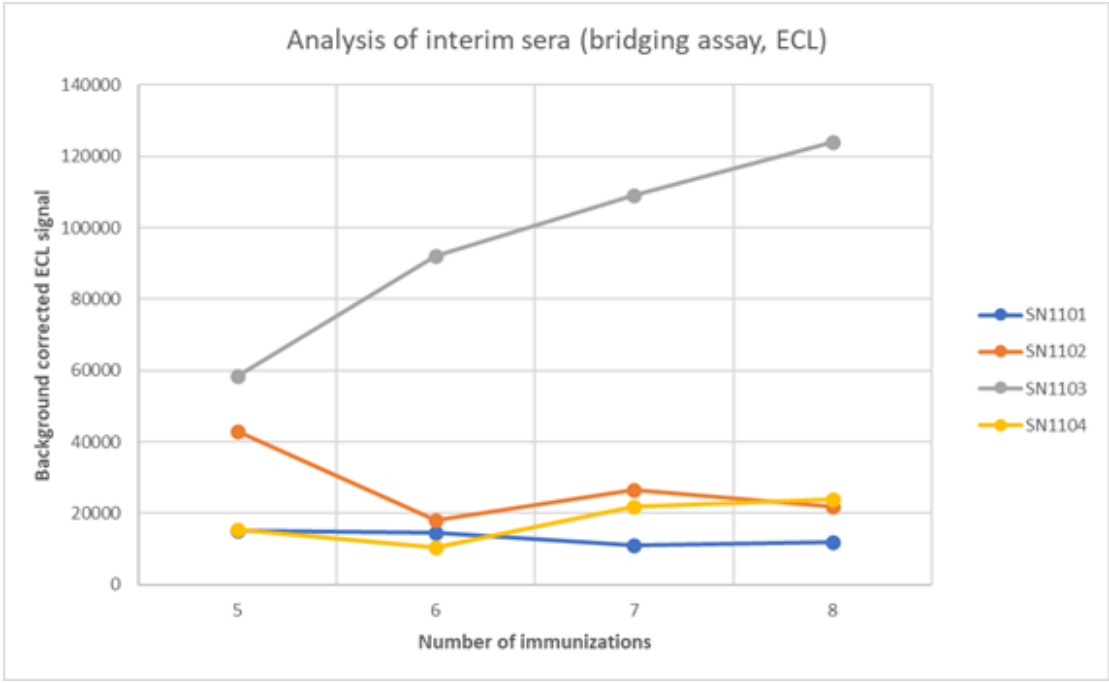
- Sandwich assay developed
- Streptavidin plate
- Coated capture probe-biotin
- Hybridization ASO
- Possible anti-ASO antibodies bound
- Detection recombinant Protein A/G, Peroxidase Conjugated
- Substrate TMB
- OD measured λ 450 nm



Positive Control generation ADA assay

- 4 rabbits immunized
  Antigen ASO with carrier KLH
- 1 out of 4 animals
  showed response
- Polyclonal Antibody
  as Positive Control





Results ASO Immunogenicity

→ Validation anti-ASO antibody results cynomolgus plasma

Validation Results	Absorption	Absorption	Absorption
Immunogenicity assay	Screening	Confirmation	Titer
Type cut point	Floating	Fixed	Floating
Cut point	SCPF 1.48	CCP 24.1%	TCP 8
Sensitivity	688 ng/mL	522 ng/mL	688 ng/mL
Selectivity	6 lots	6 lots	6 lots
Precision PC (NC)	≤ 25.0% (30.0%)	≤ 25.0% (30.0%)	≤ 25.0% (30.0%)
Drug tolerance	0.0100 - 0.500 µg/mL	N/A	0.0100 - 0.500 µg/mL
Stability	18 h, 6 FT ≤-70°C	18 h, 6 FT ≤-70°C	18 h, 6 FT ≤-70°C

Results ASO Immunogenicity

→ Bioanalytical anti-ASO antibody results cynomolgus plasma

Bioanalytical Results	Absorption	Absorption	Absorption	
Classification	Screening assay	Confirmation assay	Titer assay	
Positive	Potential 30.7%	Confirmed 14.2%	Titer < 1 n=1	Titer 32 n=8
Negative	69.3%	85.8%	Titer 2 n=2	Titer 64 n=3
Inconclusive	0%	0%	Titer 4 n=2	Titer 128 n=3
			Titer 8 n=6	Titer 256 n=1
			Titer 16 n=6	Titer 64 n=3

Conclusion

- Antisense oligonucleotide (ASO)
- Validation and bioanalytical results discussed
- Lessons learned preclinical applied to human PK and ADA assays
- Sensitive human PK assay plasma and CSF (LLOQ 0.0500 ng/mL)
- Anti-ASO antibodies in cynomolgus plasma detected

Contact Details

Foka Venema
Project Manager

foka.venema@ardena.com
T +31 (0) 592 344 211 – M +31 649 335 033

Melloney Dröge
Managing Director

melloney.droge@ardena.com
T +31 (0) 592 344 211 – M +31 649 334 578

Ardena Assen
W.A. Scholtenstraat 7
9403 AJ Assen, Netherlands
www.ardena.com