Immuno-qPCR analysis and immunogenicity assessment of gene therapeutics and their targeted delivery molecules

EBF YSS

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Who am I?

(Can someone please tell me?)

Background

- Nucleic acid-based biotherapeutic products are gaining interest, but bioanalytical methods and guidelines are yet to be defined.
  - Preclinical and clinical safety evaluation methods need to adapt to the new needs of the industry.

Background

- Anti-sense oligonucleotides (ASOs) are primarily administrated by parental injection.
  - After administration ASOs are tranferred into tissues predominantly by endocytotic uptake.
- It has been suggested that peptide conjugates can enhance cell penetration and improve the targeting of antisense agents.
  - Pharmacokinetic analysis
- Short oligonucleotides are not likely to elicit immune responses. However, polypeptide conjugates may have immunogenic properties.
  - Immunogenicity assessment

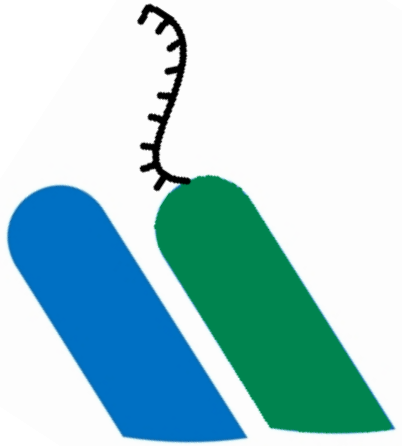
Aims

- Development of a pharmacokinetic analysis method for nucleic acid-based biopharmaceuticals and their carrier molecules.
  - Immuno-qPCR
- Anti-drug antibody (ADA) assay for the assessment of immunogenicity.
  - ADA Bridging assay

ADA bridging assay and quantitative immuno polymerase chain reaction (qIPCR)

MATERIALS AND METHODS

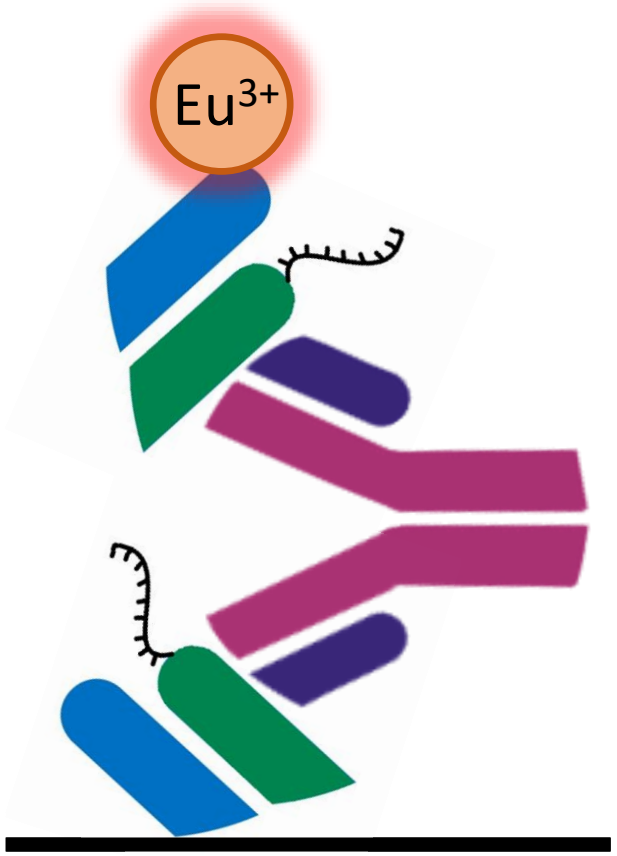
Test molecules



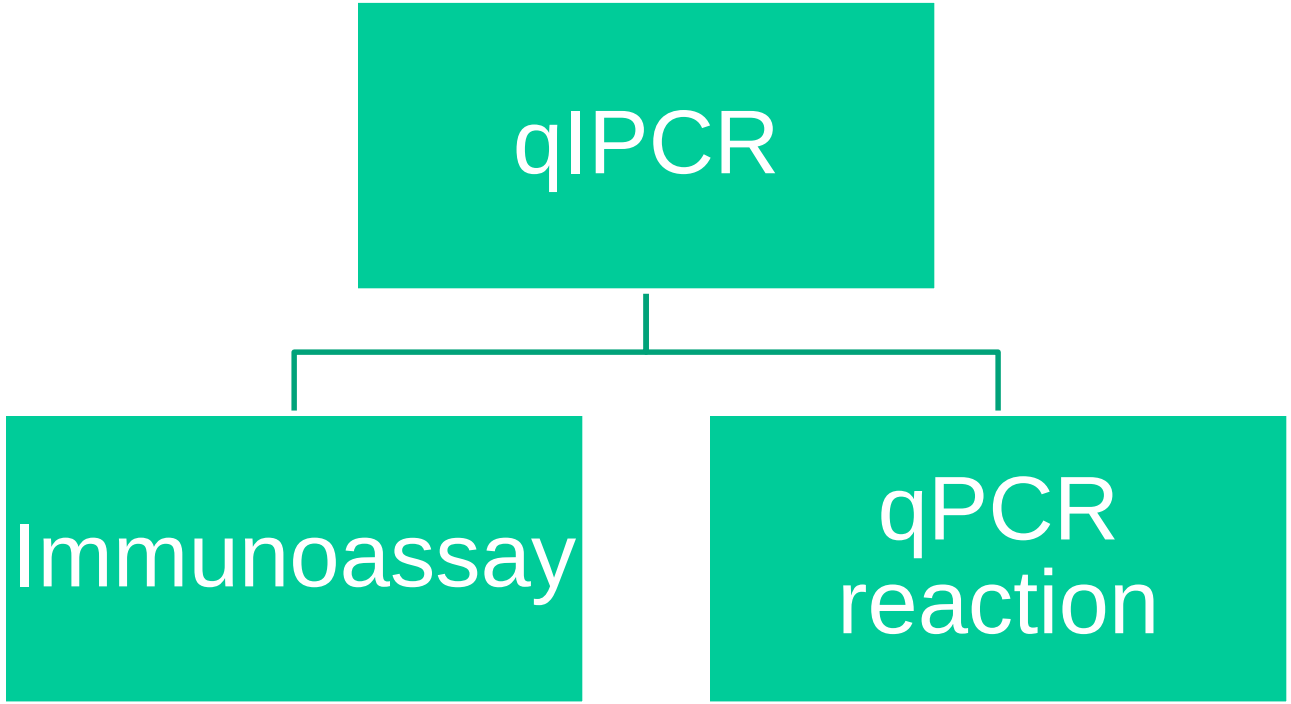
- Production of a fusion protein containing an antigen-binding fragment (Fab) and SpyCatcher in *E.Coli*.

- Bioconjugation of Fab-SpyCatcher with SpyTag-oligonucleotide

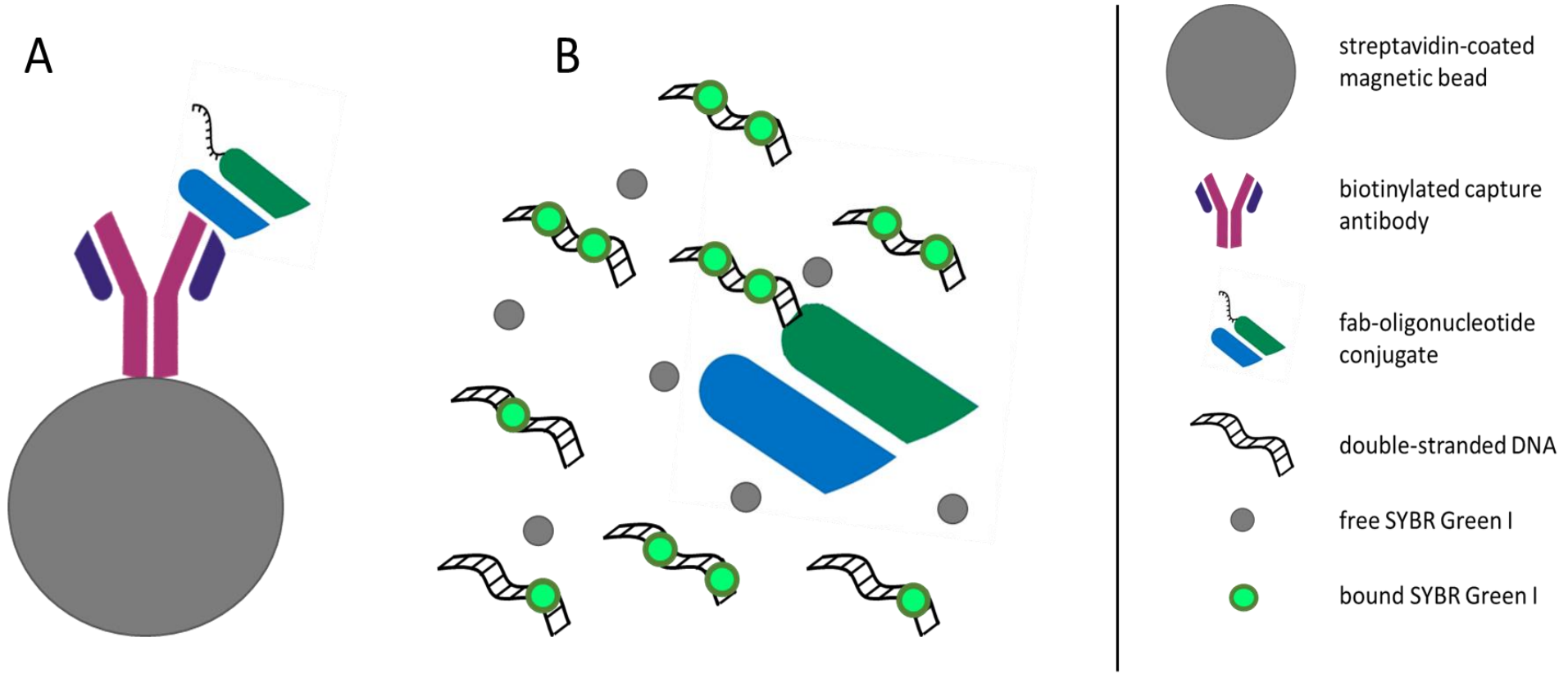
ADA bridging assay



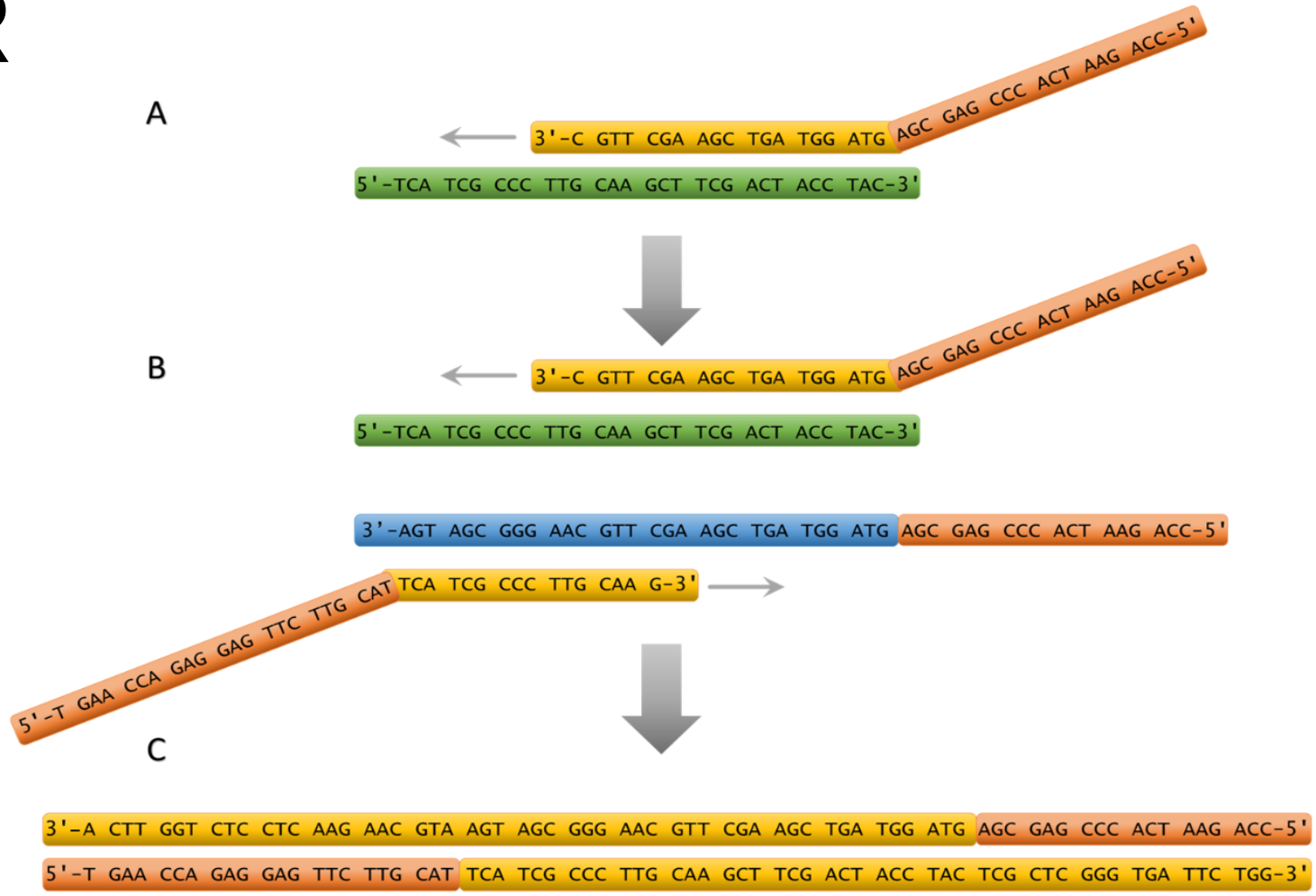
qIPCR



qIPCR


A
B
streptavidin-coated magnetic bead
biotinylated capture antibody
fab-oligonucleotide conjugate
double-stranded DNA
free SYBR Green I
bound SYBR Green I

qIPCR

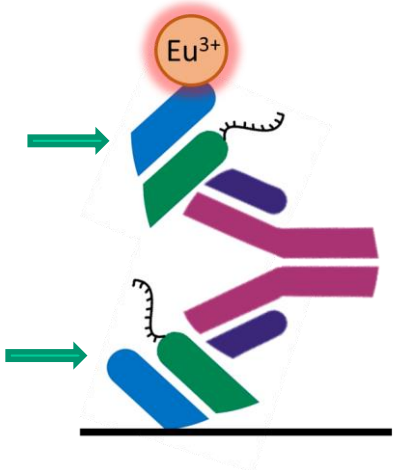

A
3'-C GTT CGA AGC TGA TGG ATG AGC GAG CCC ACT AAG ACC-5'
5'-TCA TCG CCC TTG CAA GCT TCG ACT ACC TAC-3'
B
3'-C GTT CGA AGC TGA TGG ATG AGC GAG CCC ACT AAG ACC-5'
5'-TCA TCG CCC TTG CAA GCT TCG ACT ACC TAC-3'
3'-AGT AGC GGG AAC GTT CGA AGC TGA TGG ATG AGC GAG CCC ACT AAG ACC-5'
5'-T GAA CCA GAG GAG TTC TTG CAT TCA TCG CCC TTG CAA G-3'
C
3'-A CTT GGT CTC CTC AAG AAC GTA AGT AGC GGG AAC GTT CGA AGC TGA TGG ATG AGC GAG CCC ACT AAG ACC-5'
5'-T GAA CCA GAG GAG TTC TTG CAT TCA TCG CCC TTG CAA GCT TCG ACT ACC TAC TCG CTC GGG TGA TTC TGG-3'

RESULTS

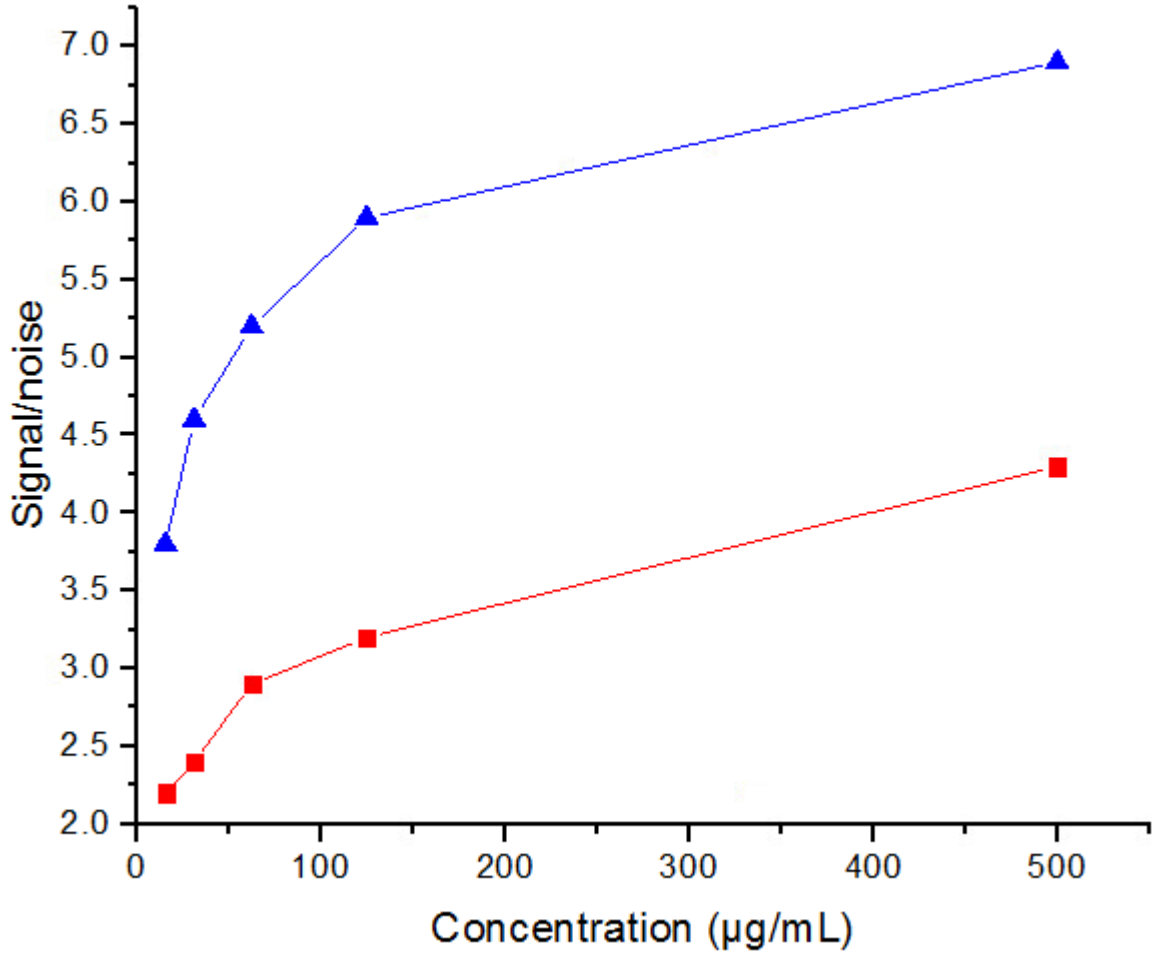
ADA bridging assay - Capture and tracer optimization

Signal-to-noise ratios with different Bio-Fab-ON/Eu-Fab-ON amounts.

	Bio-Fab-ON (ng/reaction)					
Eu-Fab-ON (ng/reaction)	**70**	**100**	**125**	**150**	**175**	**200**
50	4.3	5.2	5.8	6.6	7.2	7.1
100	4.1	4.6	5.3	5.9	6.4	6.5
150	3.8	4.3	4.9	5.3	5.9	6.1
200	3.4	4	4.4	4.8	5.4	4.9



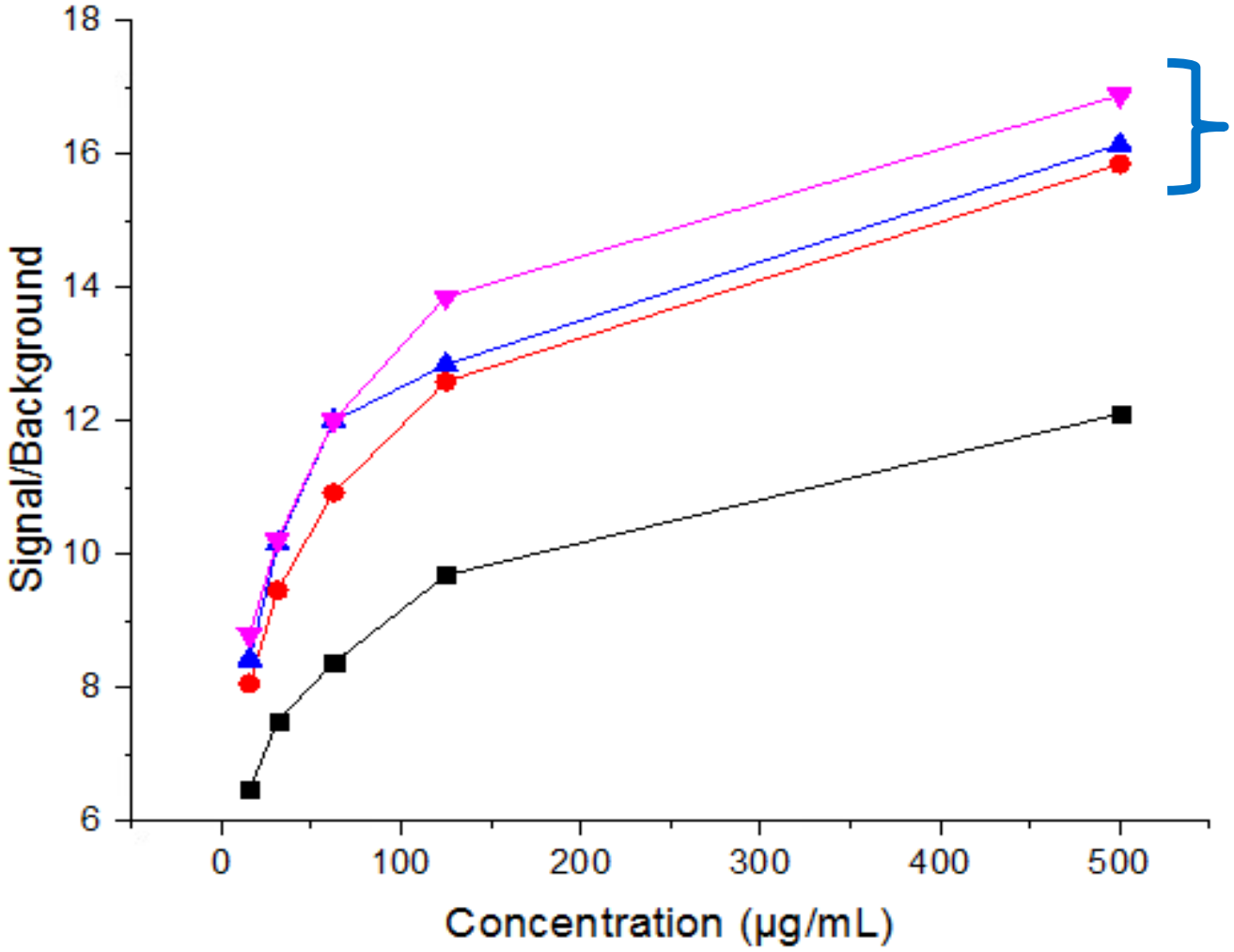
ADA bridging assay - Sample incubation



2 hours

1 hour

ADA bridging assay - Tracer incubation



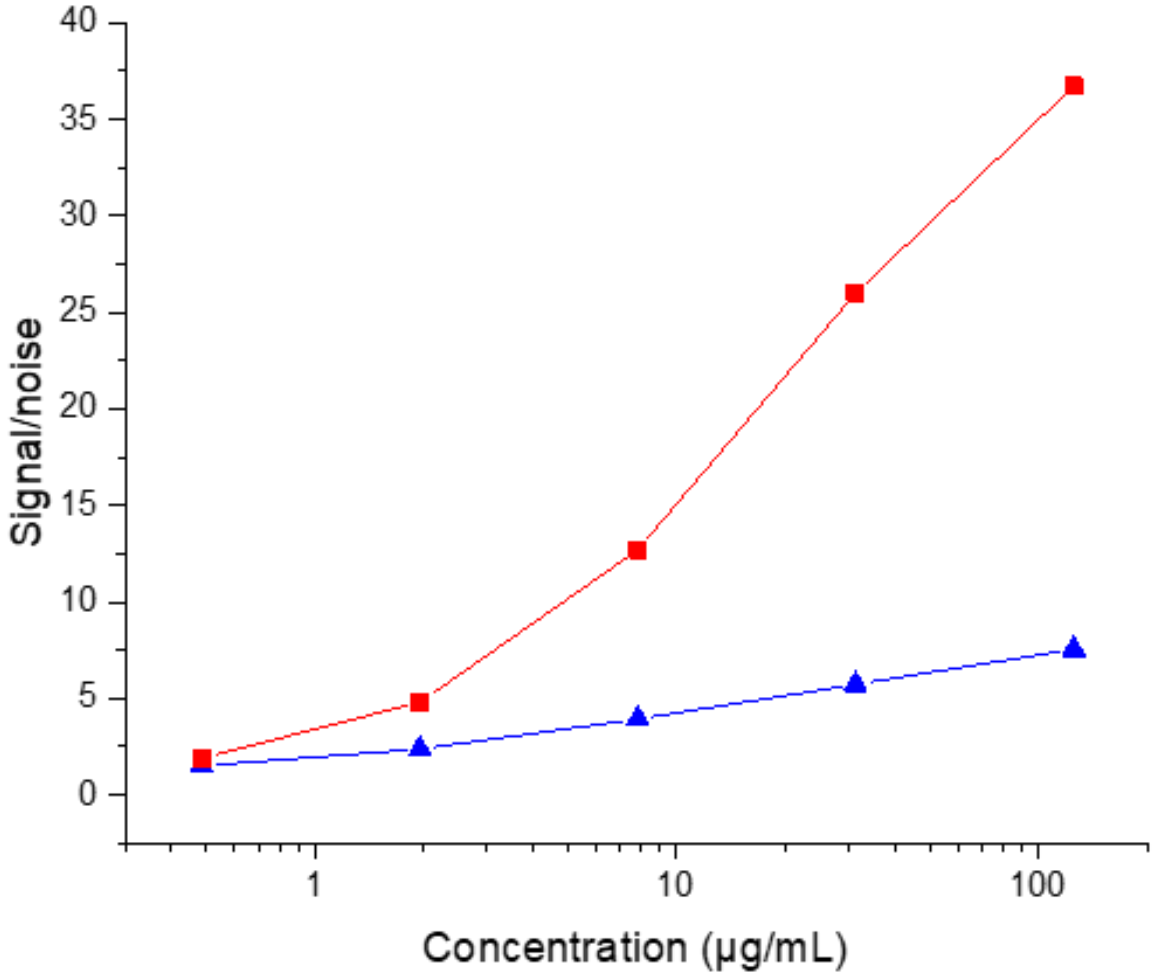
120 min

90 min

60 min

30 min

ADA bridging assay



Polyclonal antibody was tested as positive control due to low signals with monoclonal antibody

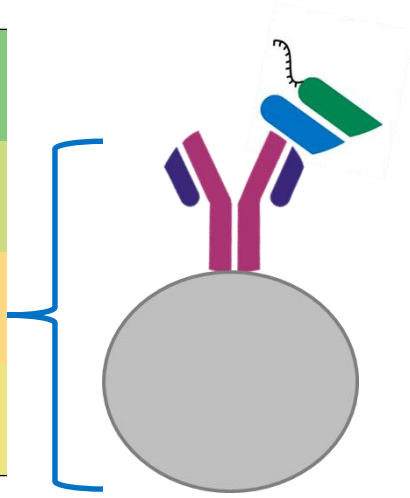
Cut point analysis

- Validated Excel spreadsheet based on Devanarayan V, Smith WC, Brunelle RL, Seger ME, Krug K, Bowsher RR. Recommendations for Systematic Statistical Computation of Immunogenicity Cut Points. AAPS J. 2017 Sep;19(5):14871498
  - 50 individual rat serum samples and 10 samples from a rat serum pool were measured.
  - Data was normally distributed.
  - Cut point 1.16 (mean + 1.645 x SD) at 5 % false positive rate.
  - Sensitivity 241 ng/mL (Cut point + 3 x SD).

qIPCR - bead and capture antibody optimization

Cq cycles of 31.3 µg/mL samples with different Dynabead/pAb ratios

	Bio-Anti-Human-IgG pAb					
Dynabeads	75 ng	100 ng	125 ng	150 ng	175 ng	200 ng
2.5 µg	12.2	12.9	11.95	11.45	13.95	10.5
5 µg	11.8	11.7	12.5	11.6	11.5	11.1
7.5 µg	12.1	11.25	10.8	10.2	11.9	11.9
10 µg	11.0	11.6	12.0	11.4	11.0	11.4



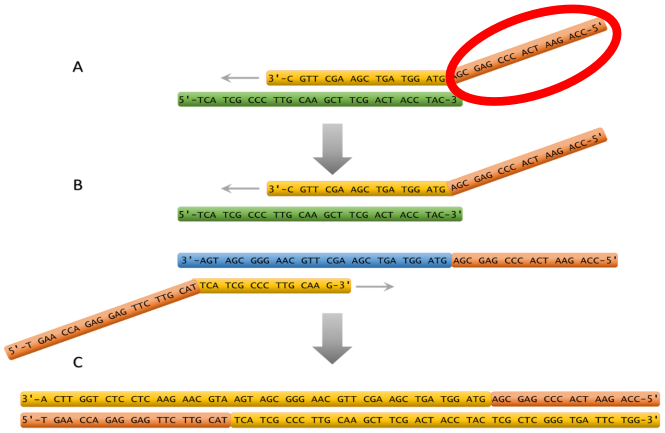
qIPCR – bead dissolving volume

%CV's of samples with the same amount of beads dissolved in 20 µL vs. 50 µL per reaction

	Concentration (ng/mL)			
Bead dissolving volume (µL)	1467	146.7	14.67	1.467
50	**37.3**	1.3	**95.8**	**22.1**
20	10.7	**27.7**	2.0	16.9

Underlined: >20 %

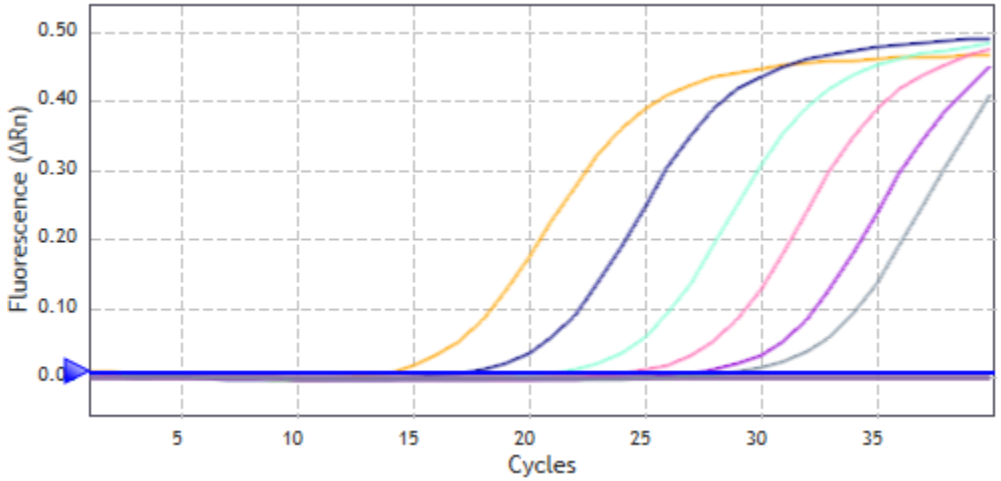
qIPCR – primer length

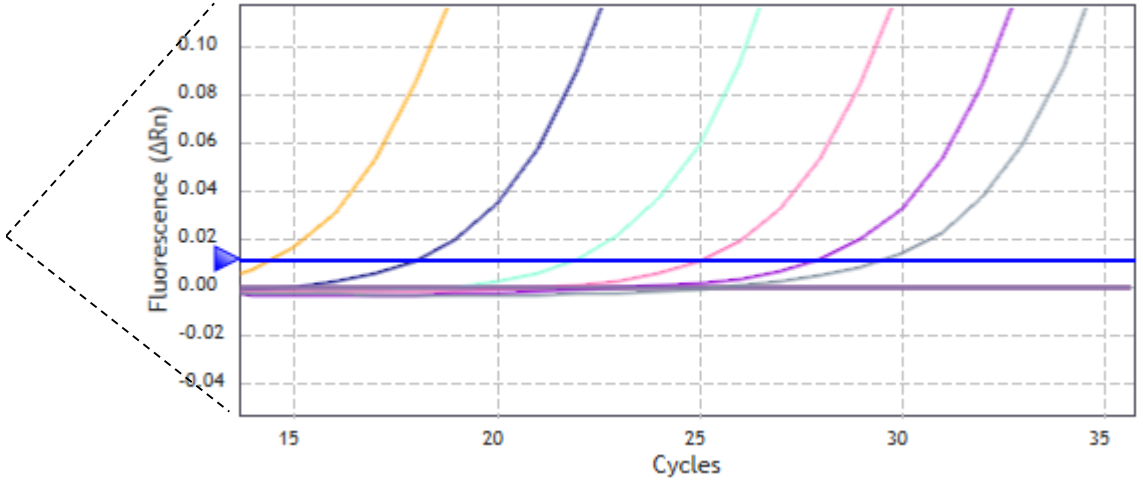


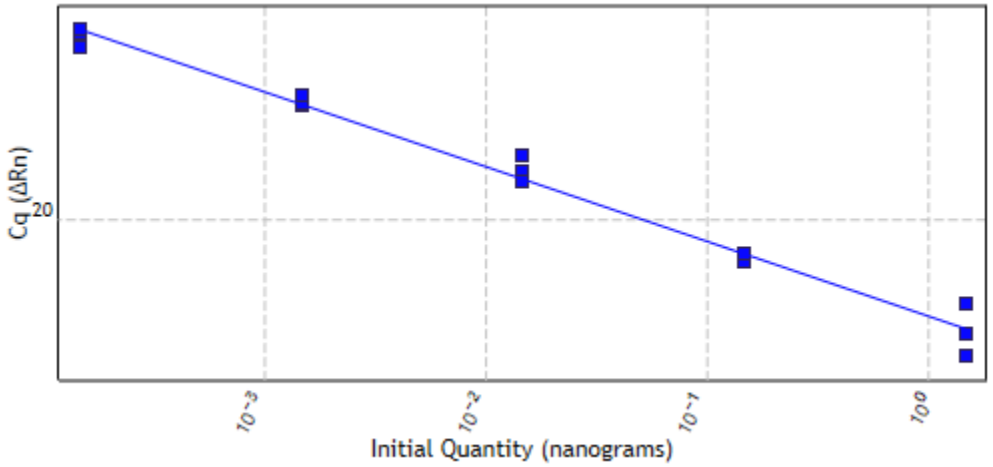
Cycles of quantification with different primer sets:
Overhanging primers and Overlapping primers

	Standard concentration (ng/mL)					
	147 000	14 700	1 470	147	14.7	0
Overhanging primers	10.6	13.8	16.2	20.9	24.6	33.1
Overlapping primers	14.8	17.0	19.2	24.9	30.3	31.0

qIPCR – final results







	Concentration (µg/mL)				
	1.47	0.147	0.0147	0.00147	0.000147
Cq	15.0	18.3	22.3	25.4	28.3
Calculated concentration	1.95	0.166	0.0115	0.00134	0.000184
%CV	**58.8**	9.2	**31.7**	12.9	24.1
Recovery	**132.8**	113.2	**78.7**	91.0	**125.3**

CONCLUSIONS

ADA bridging assay

- SpyCatcher fusion protein might block mAb from binding the Fab-Oligonucleotide molecule.
- Quasi-quantitative assay.
  - In practice anti-drug antibodies vary between individuals.
  - Results above cut point are considered positive.
- Assay meets the regulations for preclinical testing.
  - Sensitivity 241 ng/mL at 5 % false positive rate.

qIPCR

- Implementation of qIPCR assay was successful.
- qIPCR offers a super sensitive method for oligonucleotide-protein conjugate detection.
  - Less than 2 ng/mL of test molecule was detectable
- Problems with deviation.
  - Immunoassay phase-derived.
  - Beads may increase deviation.
- Changing the magnetic beads to a different solid phase might solve the deviation problem.

Future aspects

- Autumn: oligonucleotide extraction method development.
- Future: bioanalysis of oligonucleotide-based biotherapeutic products.

Acknowledgements

- Supervisor Nina Sirkka, Syrinx Bioanalytics
- Scientific Director Timo Piironen, Syrinx Bioanalytics
- Pasi Virta and Antti Äärelä, Department of Chemistry, University of Turku
- Tuomas Huovinen and Anastasiia Kushnarova-Vakal, Biotech. unit, Department of Biochemistry, University of Turku

For futher questions, don’t hesitate to contact me (e.g. via LinkedIn)!

THANK YOU!