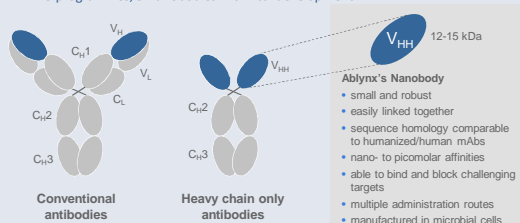# An innovative approach for detecting neutralizing antibodies directed to antibody-derived therapeutics based on the conventional bridging anti-drug antibody (ADA) assay format

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Background: Nanobodies

- The isolated variable domains (VHH) of camelid heavy-chain only antibodies are stable and fully functional
- Nanobodies represent the next generation of antibody-derived biologics
- > 45 programmes, 8 Nanobodies in clinical development

Ablynx's Nanobody
- small and robust
- easily linked together
- sequence homology comparable to humanized/human mAbs
- nano- to picomolar affinities
- able to bind and block challenging targets
- multiple administration routes
- manufactured in microbial cells

Conventional antibodies | Heavy chain only antibodies | VHH 12-15 kDa

Background: Immunogenicity testing

- Neutralizing antibodies (NAb) are defined as antibodies (Ab) able to affect the function of the Nanobodies by blocking its ability to bind to target, including Ab binding to complementary determining region (CDR)
- Development of a NAb assay based on drug-target interaction is challenging and often requires extensive pre-treatment steps in order to obtain the required drug and target tolerance for proper detection of NAb during clinical trials
- Such an assay set-up often introduces a sensitivity difference between the ADA assay and the NAb assay
- The sensitivity gap complicates ADA data interpretation as a discrepancy between ADA and NAb results can either reflect presence of non-neutralizing antibodies only or reflect neutralizing antibodies that are left undetected in the NAb assay
- An alternative NAb assay format was developed based on the conventional bridging ADA assay format which allows unambiguous comparison of the levels of total ADA and NAb

Figure 1: Bridging ADA assay

The alternative NAb assay

The proposed alternative NAb assay is based on the conventional bridging ADA assay format

- An excess amount of the null variant of the Nanobody, i.e. a variant of the Nanobody which is non-functional for target binding and which is identical to the Nanobody with the exception of altered CDR's of the target binding Nanobody domain, is added to the reagent master mix
- Non-neutralizing antibodies are complexed with the null variant of the Nanobody and are left undetected (similar to a drug-displacement set-up as confirmatory assay of the conventional ADA assay)
- Positive assay signals reflect antibodies with neutralizing potential only

Figure2: Null variant of Nanobody (2 different angles)

Figure 3: Alternative NAb assay

The conventional NAb assay

Assay was developed to be fit-for-purpose according to following requirements
- Drug tolerance: predicted maximum drug levels of 30 µg/mL
- Target tolerance: predicted maximum target levels of 1500 ng/mL
- Sensitivity: 250 – 500 ng/mL positive control antibody in absence of drug

Assay format
- Competitive ligand binding assay (CLBA) with a complex pre-treatment step to achieve drug and target tolerance

Flow scheme

Sensitivity gap between ADA assay and conventional NAb assay
- Both assays have been fully validated according to FDA guidelines

[drug] (ng/mL)	NAb assay sensitivity (ng/mL)	ADA assay sensitivity (ng/mL)	Sensitivity difference ADA versus NAb assay
60 µg/mL	1890	<36	At least 50-fold
30 µg/mL	1374	<36	At least 40-fold
15 µg/mL	779	<36	At least 20-fold
600 ng/mL	417	<36	At least 10-fold
30 ng/mL	429	<36	At least 10-fold
0	521	<36	At least 10-fold

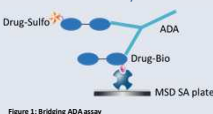## Alternative NAb assay – fit for purpose using mAbs

Fit for purpose of assay format demonstrated using a panel of established neutralizing and non-neutralizing Ab

- Neutralizing potential of the monoclonal antibodies (mAb) is based on the ability to block target interaction as determined in the CLBA (in buffer without pre-treatment)
- NAb are detected at the same sensitivity as compared to the ADA assay
- Non-neutralizing Ab are left undetected; some residual binding can be detected at very high Ab concentrations, however these levels are not expected to be clinically relevant

Neutralizing Ab	Assay	Assay response (ECL) at mAb concentration (ng/mL)									Sensitivity	Sensitivity in conventional NAb assay
		20000	4000	1000	500	250	125	63	0		
mAb 1	ADA	19714	4122	1140	599	375	242	178	109	< 63 ng/mL	
	NAb	23151	4633	1266	640	383	246	173	92	< 63 ng/mL	556 ng/ml
mAb 2	ADA	55587	10405	2786	1405	754	448	273	106	< 63 ng/mL	
	NAb	62575	12217	3097	1596	854	466	278	89	< 63 ng/mL	556 ng/ml
mAb 3	ADA	646	214	139	122	118	117	110	116	400 ng/mL	
	NAb	671	203	127	105	103	99	94	94	543 ng/mL	> 5 µg/mL
mAb 4	ADA	757	250	147	136	125	119	116	107	165 ng/mL	
	NAb	807	251	140	127	117	108	103	95	111 ng/mL	> 5 µg/mL

Non-neutralizing Ab												
mAb 5	ADA	14731	5182	1811	530	301	209	102	< 63 ng/mL		
	NAb	128	101	94	88	88	89	92	96	6898 ng/mL	N/A
mAb 6	ADA	1624	435	206	165	139	131	120	< 63 ng/mL		
	NAb	96	92	95	93	96	97	99	97	> 20 µg/mL	N/A
mAb 7	ADA	1197	344	170	141	128	116	120	113	211 ng/mL	
	NAb	96	89	90	93	94	93	94	95	> 20 µg/mL	N/A
mAb 8	ADA	106020	20679	5109	2625	1314	733	446	110	< 63 ng/mL	
	NAb	117	104	102	96	98	89	87	89	11022 ng/mL	N/A

Alternative NAb assay – fit for purpose using polyclonals

Neutralizing antibody fraction sensitively detected in polyclonal antibody samples
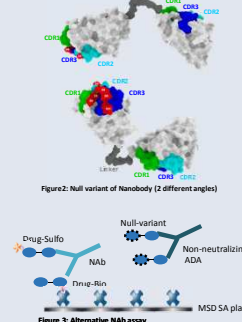
- The polyclonal rabbit Ab was specifically generated by immunization to be used as NAb positive control antibody
- The pre-clinical study samples (rhesus monkey) originating from a disease model shown to be prone to development of ADA, were shown to contain neutralizing activity via PD and efficacy markers
- The neutralizing fraction within the ADA positive samples (Rabbit pAb and pre-clinical study samples) can be determined by titer and/or sensitivity determination

	Assay	Assay response (ECL) at pAb concentration (ng/mL)								Sensitivity	Sensitivity in conventional NAb assay
		4000	1000	500	125	31	13	5	0		
Rabbit pAb	ADA	4694	1192	659	230	135	117	114	105	<5.0 ng/mL	
	NAb	1520	416	267	129	98	88	95	92	31 ng/mL	5 µg/mL

Rhesus Monkey study samples	Assay	Assay response (ECL) at pAb dilution								Log10 (titer)
		100	400	1600	6400	25600	102400	409600		
Sample 1	ADA	253499	25133	4526	812	236	121	105		5.1
	NAb	95876	15281	3032	581	176	106	92		5.0
Sample 2	ADA	714810	61990	9795	1711	389	155	110		5.5
	NAb	268224	41932	7437	1363	309	123	99		5.4
Sample 3	ADA	61076	8754	1496	333	144	112	98		4.9
	NAb	35866	6523	1197	274	125	96	87		4.8

ADA and alternative NAb assay qualification data

Assay qualification reveals similar precision, sensitivity and drug tolerance characteristics as compared to the ADA assay

- Similar sensitivity and drug tolerance, compliant to current regulatory guidelines: < 100 ng/mL positive control in presence of highest anticipated drug levels

Conc. mAb 2 (ng/mL)	ADA – Mean responses (ECL) Concentration drug					Alternative NAb – Mean responses (ECL) Concentration drug				
	30.0 µg/mL	15.0 µg/mL	0.6 µg/mL	0.03 µg/mL	No drug	30.0 µg/mL	15.0 µg/mL	0.6 µg/mL	0.03 µg/mL	No drug
20000.0	27386	25746	41832	38100	34203	32166	38126	64889	67705	66243
5000.0	7264	9463	10162	10956	12297	7329	8712	13118	16188	16898
500.0	701	881	858	1210	1470	858	966	1164	1649	1747
250.0	441	525	512	545	745	456	482	619	817	1044
72.0	185	220	228	207	316	197	205	250	268	346
36.0	139	148	145	146	180	137	147	160	182	212
0.0	94	99	94	98	103	94	86	96	92	88
Sensitivity	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL	<36.0 ng/mL

- Target tolerance characteristics similar as the ADA assay. In case of a monomeric target, this NAb format is target tolerant
- Intra-run and inter-assay precision: ≤ 20%

Conc. mAb 2 (ng/mL)	ADA			Alternative NAb		
	Overall mean (ECL)	Overall intra-run precision (%)	Inter-batch precision (%)	Overall mean (ECL)	Overall intra-run precision (%)	Inter-batch precision (%)
HiQC	20000.0	46942	4.8	15.8	64390	8.9	19.2
LoQC2	72.0	245	3.4	9.5	306	5.6	14.9
LoQC1	36.0	168	3.0	6.8	196	4.9	10.6
BLK	0.0	97	3.8	N/A	85	4.2	N/A

Conclusion

- The alternative NAb assay format is based on the conventional bridging ADA assay format. The assay is performed in presence of excess of a null variant of therapeutic drug added to the reagent master mix
- The null variant of the Nanobody is non-functional for target binding and is identical to the Nanobody with the exception of altered CDR's of the target binding Nanobody domain
- Fit for purpose of the assay format was demonstrated using an established panel of neutralizing and non-neutralizing Ab for which the (non)neutralizing potential is based on their (in)ability to block target interaction via a CLBA
- Signals generated in this assay reflect antibodies that bind to the CDR region and have a potential for neutralization.
- Non-neutralizing are left undetected as complexed with the null variant, in a way comparable to the conventional drug displacement set-up (confirmatory assay)
- Allows unambiguous immunogenicity data interpretation as potential NAb are detected with same assay characteristics as ADA assay
- While these assays represent a significant advance in methodology, the clinical relevance of the results must be viewed in relation to effects on drug PK, PD, efficacy and safety