Neutralizing Antibody Assays: To Be Cell Based or Ligand Binding, That's the Real Question!

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Introduction

Neutralizing antibodies (nAbs) against a therapeutic drug might have detrimental effects by abrogating the drug`s functionality, or by posing a safety risk for patients if the therapeutic drug is similar to a non-redundant endogenous protein. In consequence, the reliable detection of nAbs is necessary for highly immunogenic therapeutics.

Regulatory bodies (FDA/EMA) are particularly keen on nAb assays respecting the drug`s mode of action (MOA). Many drugs exert their function by binding to cell membrane components or specific receptors. A preferred method to assess nAbs on these cytoactive drugs is by functional cell-based assays (CBA). CBA methods, however, suffer from major limitations such as low assay robustness, low sensitivity, and low free drug tolerance. In such cases using a competitive ligand binding assay (CLB) instead might be a valuable alternative to a CBA.

In the present study we developed and compared two different assay formats for the detection of nAbs against a humanized therapeutic antibody with an antibody-dependent cell-mediated cytotoxicity (ADCC) MOA in serum samples:

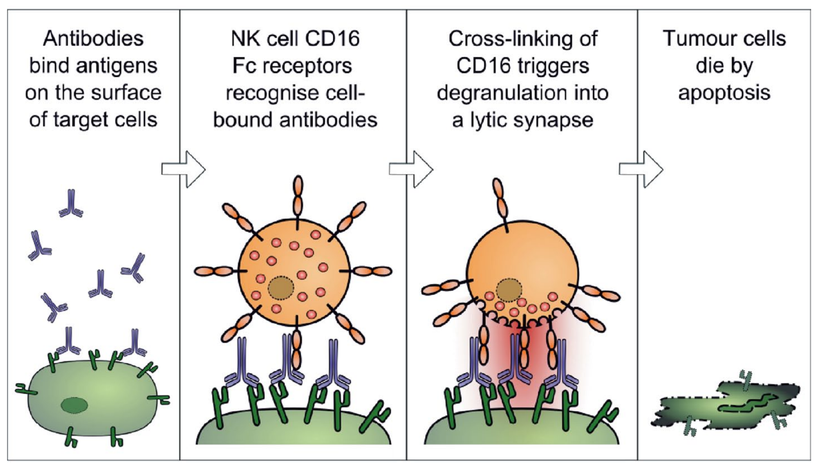
- A CBA using a commercial ADCC kit, with inhibition of ADCC effector cells` downstream luciferase gene activation as the nAbs readout
- An electrochemiluminescent immunoassay (ECLIA) CLB, with inhibition of antigen-drug binding as the nAbs readout

Here we present the challenges, solutions and special considerations regarding the development of CBA versus CLB nAb assays, and we compare the two assays side-by-side for the key parameters robustness and sensitivity.

Analytical Methods

The drug under investigation, a humanized therapeutic antibody, has an ADCC MOA, where it binds to cell surface antigens on target cells, followed by binding of immune effector cells (typically NK cells), which induce target cell apoptosis (Figure 1). ADCC is a desirable mechanism for killing cancer cells using antibody-based therapy. However, nAbs would prevent the antibody drug from binding to its antigens on target cells, and therefore prevent target cells apoptosis, leading to a reduced drug efficacy.

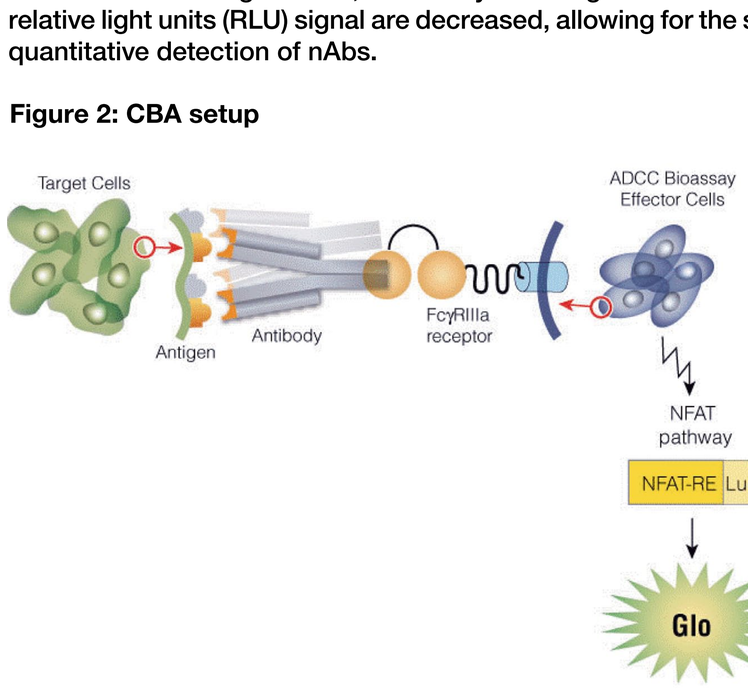
Figure 1: ADCC mechanism



CBA setup

The CBA is based on a commercially available ADCC kit (Promega ADCC Reporter Bioasay). Antigen-expressing target cells are incubated with a fixed concentration of the antibody drug and with genetically engineered ADCC effector cells, which express luciferase upon antibody binding and downstream NFAT pathway activation (Figure 2, Promega©). In the presence of nAbs-containing serum, antibody binding and luciferase relative light units (RLU) signal are decreased, allowing for the semi-quantitative detection of nAbs.

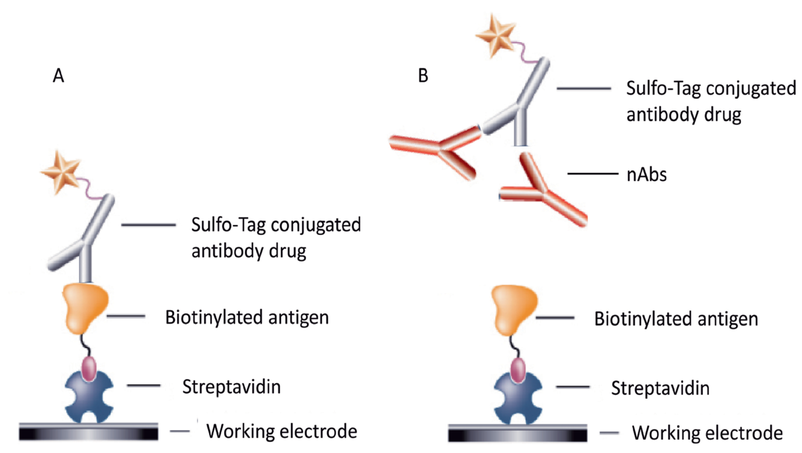
Figure 2: CBA setup



CLB setup

The CLB assay is based on ECLIA detection of antigen-antibody drug binding using a streptavidin-coated Meso Scale Discovery plates, biotinylated antigen, and a fixed concentration of Sulfo-Tag-labeled antibody drug (Figure 3A). In the presence of nAbs-containing serum, antibody binding and ECLIA RLU signal are decreased, allowing for the semi-quantitative detection of nAbs (Figure 3B). The CLB setup uses the antigen – antibody drug binding readout, which is the critical step in ADCC MOA.

Figure 3: CLB setup in the (A) absence and (B) presence of nAbs



Analytical Challenges and Solutions

1. Free drug tolerance

Problem: Very low free drug tolerance and false positives were expected in both CBA and CLB setups because of sample free drug concentrations expected in the range of 3.5 - 10 µg/mL.

Solution: Remove free drug from samples using a depletion procedure prior to nAb detection. The optimized free drug depletion procedure is presented in Figure 4.

Result: The effects of the procedure on the CBA and the CLB setups are presented in Figures 5 and 6, respectively.

Figure 4: Free drug depletion procedure prior to nAbs detection

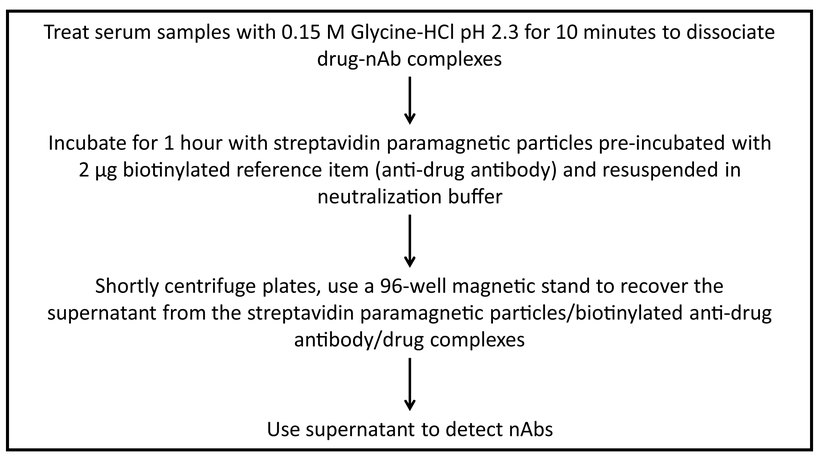


Figure 5: Effect of drug depletion procedure on 3.5 µg/mL drug samples on the CBA drug dilution curve. (A) RLU; (B) signal-to-noise (S/N) ratio

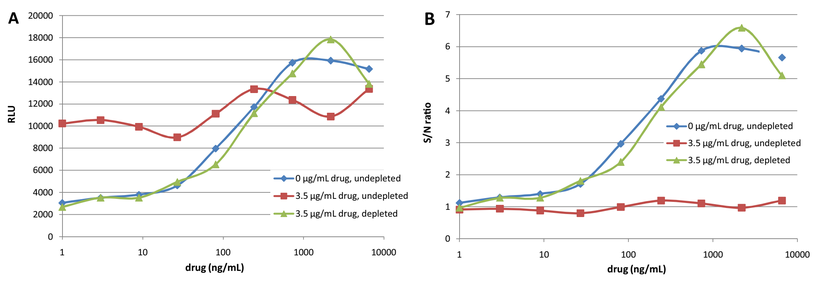
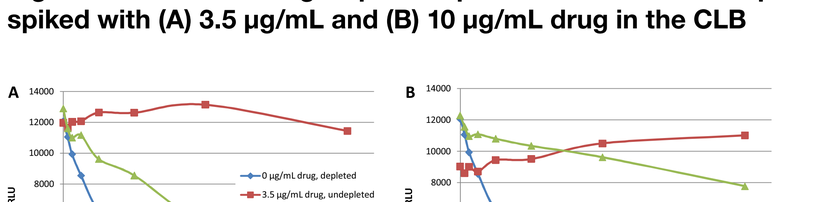


Figure 6: Effect of drug depletion procedure on nAb samples spiked with (A) 3.5 µg/mL and (B) 10 µg/mL drug in the CLB



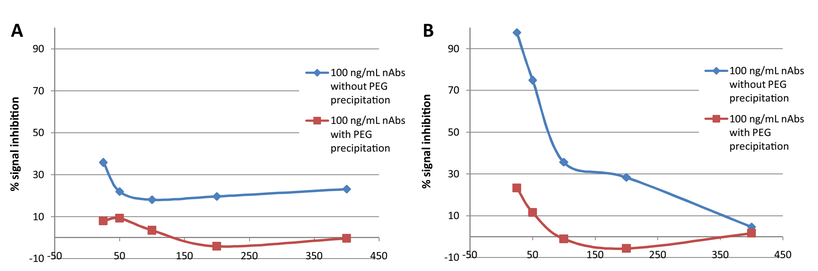
2. Matrix interference

Problem: Severe matrix/buffer interference of processed samples was observed in the CBA setup. No matrix interference was observed in the CLB.

Solution: Precipitate sample supernatant after the free drug depletion procedure with 16% PEG and resuspend sample pellets in CBA assay buffer.

Result: The additional PEG precipitation step in the CBA lead to nAb signal loss/decreased sensitivity as evaluated in both the CBA and the LBA setups (Figure 7).

Figure 7: Effect of PEG precipitation on 100 ng/mL nAbs signal in the (A) CBA and (B) CLB setup



Results

Screening cutpoint with 1% false positives (Table 1) and sensitivity of a polyclonal anti-drug antibody used as the reference item (Table 2) were evaluated in both CBA and CLB setups.

Table 1: Screening cutpoint evaluation in CBA vs. CLB

	distribution	mean	SD	%CV	median	MADn
CBA	normal	40909.03	8698	21.261	39681.75	7043.09
CLB	not normal	14809.39	1569	10.5932	N/AP	N/AP

	cutpoint	cutpoint calculation	NC mean	CF
CBA	23297.0695	median (Ind) - 2.326 x MADn	38365.38	0.60724
CLB	10788.985	CP = percentile (Ind, 0.01)	14503.75	0.74388

Table 2: Sensitivity evaluation in CBA vs. CLB

reference item (ng/mL)	CBA	CLB
3200	16150.5	54.5
1600	26750	282
800	33114.5	2020
400	39059.5	6011
200	36477	9292.5
100	30875	12428.5
negative control (NC)	37909	13472.5
	28663.5	15200
correction factor (CF)	0.607242065	0.743875549
run-specific cutpoint (rCP)	20212.81118	10664.38585
concentration at rCP (ng/mL)	**2586.79203**	**156.2536401**

Based on the lower sensitivity, the higher variability and the more complex CBA setup (additional PEG precipitation leading to loss of nAb signal), the CLB setup was chosen for further development and qualification. Qualification results are presented in Table 3.

Table 3: CLB nAb qualification results

screening cut point				10788.9850
correction factor				0.7439 by division
inter-assay precision	at NC	0	ng/mL	14749 ± 9.2
mean RLU ± %cv	at LPC	200	ng/mL	9208 ± 6.6
	at MPC	400	ng/mL	4812 ± 12.2
	at HPC	800	ng/mL	1603 ± 12.2
		LPC/rCP		0.8140 ± 8.7
intra-assay precision	at NC	0	ng/mL	11.1
%cv	at LPC	200	ng/mL	7.4
	at MPC	400	ng/mL	13.3
	at HPC	800	ng/mL	6.2
sensitivity (two runs)		conc at rCP		126.6 ng/mL
		estimated sensitivity		269 ng/mL
selectivity	at LPC	200	ng/mL	10 of 10 and pool
	at MPC	400	ng/mL	10 of 10 and pool
	at HPC	800	ng/mL	10 of 10 and pool
recovery	at LPC	200	ng/mL	107
	at MPC	400	ng/mL	117
	at HPC	800	ng/mL	107
free drug tolerance		of LPC		0.46875 µg/mL
		of HPC		3.75 µg/mL

Discussion and Conclusions

The following issues were observed during method development of the CBA and CLB setups:

- Poor free drug tolerance and high probability of false positives in both assays, which were significantly improved by addition of a sample drug depletion procedure prior to nAb detection
- Severe matrix/buffer interference in the CBA, which was only partially improved by an additional sample precipitation step prior to nAb detection
- Due to the precipitation step and to higher assay variability of the CBA (cutpoint run %CV was 21.26% compared to 10.59% in the CLB), the sensitivity in the CBA was considerably worse than in the CLB (2.587 µg/mL vs. 0.156 µg/mL)

We demonstrated superiority of the CLB over the CBA nAb assay against a therapeutic antibody with ADCC MOA for the key parameters robustness and sensitivity. Based on this comparison, the CLB method was chosen for further development and was successfully qualified.

Considering the MOA of the drug and the limitations encountered with the functional CBA, our comparison study showed that a robust and sensitive CLB assay can be the format of choice when investigating nAbs.

References

Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products. Guidance for Industry. Draft Guidance. US FDA 2016

Poster presentation at EBF 2018