How to deal with the challenges of NAb assays: case studies evaluated

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INTRODUCTION

Immunogenicity assessment is a critical step in the safety and efficacy evaluation of biopharmaceuticals. Neutralizing antibodies (NAbs), a subset of anti-drug antibodies (ADAs), may block the biological activity of a therapeutic drug, resulting in altered pharmacokinetic and pharmacodynamic profiles. NAbs may also cross-react with the endogenous counterpart of the biotherapeutic, potentially leading to life-threatening consequences.

Establishing an optimal NAb assay using an appropriate format that reflects the drug's therapeutic mechanism of action (MOA) and fulfills all requirements, such as adequate sensitivity and free-drug tolerance, can be a challenging task. NAb assays are typically categorized in cell-based assays (CBA) and competitive ligand-binding assays (CLBA), both of which may require assay-specific optimization steps.

Here, we describe some of the most recent developments on NAb assays from our laboratory and share some challenges encountered during method development. Depending on the drug of interest, which ranged from endogenous cytokines and glycoproteins to therapeutic antibodies, we utilized either CBA or CLBA approaches. After troubleshooting steps, we improved assay sensitivity, reduce intra- and inter-assay variability and decreased inter-individual variability, reducing the rate of false-positive individuals.

CASE STUDY N°1: ENDOGENOUS GLYCOPROTEIN

ASSAY SETUP

We developed a CBA to detect NAbs directed against the drug, an endogenous glycoprotein in the first case study. We selected the iLite® cell-based system with a dual reporter gene readout for ease of use (i.e. cells can be directly used in the assay after thawing) and claimed robustness.

ILite® cells are genetically engineered to express Firefly luciferase enzyme under the control of a receptor-responsive promoter (Figure 1. A). The binding of the ligand to its receptor results in receptor activation, leading to a signaling cascade and subsequently expression of the Firefly luciferase reporter gene. When a substrate is added, the luciferase generates light (measured in RLU), proportional to the ligand activity and inversely proportional to the concentration of NAbs against the ligand present in the sample.

ILite® cells also contain a second reporter gene, Renilla Luciferase, which is controlled by a constitutive promoter. Renilla luciferase signal is used to normalize the Firefly luciferase signal. This helps compensate for cell number differences, differentiate between specific and non-specific cellular responses, and minimize individual sample variability.

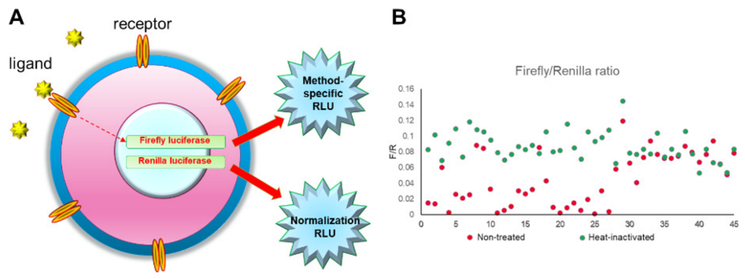

FIGURE 1. (A) Schematic representation of the iLite® reporter cell system. (B) Assay response from unspiked sera showing reduced variability upon heat-inactivation.

ASSAY CHALLENGES

During the early stages of assay development, we observed high variability between individuals when assessing the screening Cut Point (sCP) with unspiked individual sera. This variability can be strongly reduced by heat-inactivationg of the samples before analysis (Figure 2. B). Also, we could confirm that normalization of the Firefly luciferase signal to the Renilla luciferase signal further helped to minimize individual sample variability (data not shown).

When assessing assay performance, we observed a poor precision at the pLPC and HPC level, with 34 and 28 %CV, respectively (Table 1). We hypothesized that increasing the drug concentration, used to stimulate the cells from 1.25 ng/mL to 2.5 ng/mL, could improve day-to-day assay reproducibility by ensuring robust cell stimulation. Both tested drug concentrations were on the linear part of the drug-response curve, the first being slightly lower, the second being slightly higher than the calculated EC50 concentration. As expected, increasing the stimulatory drug concentration to 2.5 ng/mL improved assay precision.

However, when re-evaluating sCP, lower individual variability was observed, resulting in a smaller correction factor (rCP=0.86, compared to rCP=0.75 when using 1.25 ng/mL stimulatory drug), and a higher false-positive rate (FPR). To mitigate both issues, we tested an intermediate stimulatory drug concentration at 1.8 ng/mL and could indeed prove the best assay performance for precision and FPR for this condition.

	Drug: 1.25 ng/mL		Drug: 2.5 ng/mL		Drug: 1.8 ng/mL	
Sample	Average (S/NC)	%CV	Average (S/NC)	%CV	Average (S/NC)	%CV
NC	1.0	6.0	1.0	5.1	1.0	0.0
pLPC	0.6	34 !	0.6	2.3	0.6	11
MPC	0.4	16	0.5	0.8	0.4	4.5
HPC	0.3	28 !	0.2	7.6	0.2	8.8
rCP	0.75		0.86		0.77	
Note	Poor precision		High FPR !		Good precision No false positives	

Table 1: Comparing assay performance at different stimulatory drug concentrations close to the estimated EC50 from the dose-response curve.

CASE STUDY N°2: FUSION ANTIBODY CONJUGATED TO A CYTOKINE

ASSAY SETUP

In the second case study, we developed a CBA using recombinant HEK-Blue™ reporter cells to detect NAbs directed against the drug, a fusion antibody conjugated to a cytokine. The HEK-Blue™ reporter cell system consists of HEK293 cells, which are genetically engineered to express the cytokine specific receptor together with the main signalling proteins to obtain a fully active signaling pathway (Figure 2. A). Activation of the receptor by the cytokine triggers downstream signaling and expression of the secreted embryonic alkaline phosphatase (SEAP) reporter gene. The amount of SEAP secreted in the cell supernatant can be measured with SEAP detection medium – a colorimetric enzyme assay - and is proportional to the cytokine activity and inversely proportional to the concentration of anti-cytokine NAbs

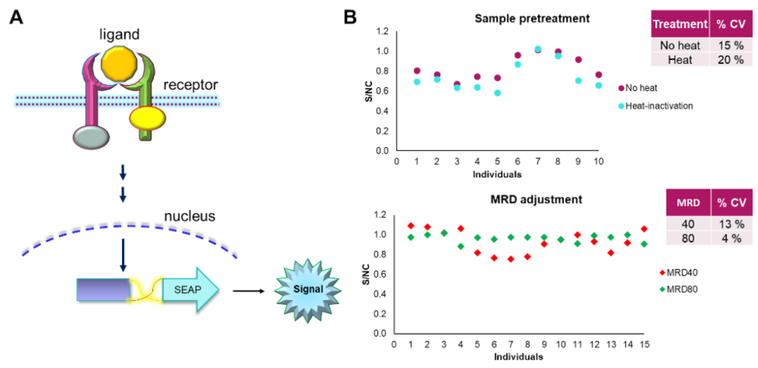

Figure 2. (A) Schematic representation of the HEK-Blue™ reporter cell system. (B) Assay response from unspiked sera showing reduced variability after increased sample dilution, but not after heat-inactivation.

ASSAY CHALLENGES

Similarly to the first case study, we observed individual-to-individual variability when evaluating unspiked sera (Figure 2. B). However, in this case, the samples' heat-inactivation did not help reduce the variability between individuals (20%CV with heat-inactivation vs 15% without heat-inactivation). Increasing the sample dilution from MRD40 to MRD80 provided a robust solution by reducing the variability from 13%CV (MRD40) to 4%CV (MRD80).

Next, we optimized the assay procedure to reduce the handling time. According to the original protocol, and to ensure the best assay performance, HEK-Blue™ reporter cells should be maintained in culture for several days before starting the assay. We tested whether cells can be used in the assay directly after thawing or with one-day recovery after thawing. We first compared cell viability by monitoring cell morphology and density 16-20 h after plating cells in the assay plate. While cultured cells and recovered cells were healthy and dense, cells thawed on assay day grew into clumps and were less dense, indicating a growth deficiency (Figure 3). Next, we assessed assay performance by comparing sensitivity curves at each condition. Similarly, we observed that the cell stimulation with the drug was less efficient with cells thawed on assay day (OD 1.4 at 0 ng/mL positive control) compared to both other conditions (OD 1.8 and OD 2.0 at 0 ng/mL positive control), thereby impacting assay sensitivity.

Altogether, these results indicate that cells thawed on the assay day are less viable and drug-responsive compared to other conditions. Therefore, either cultured cells or cells thawed one day before the assay should be used in the assay. The assay was successfully qualified and validated for both conditions.

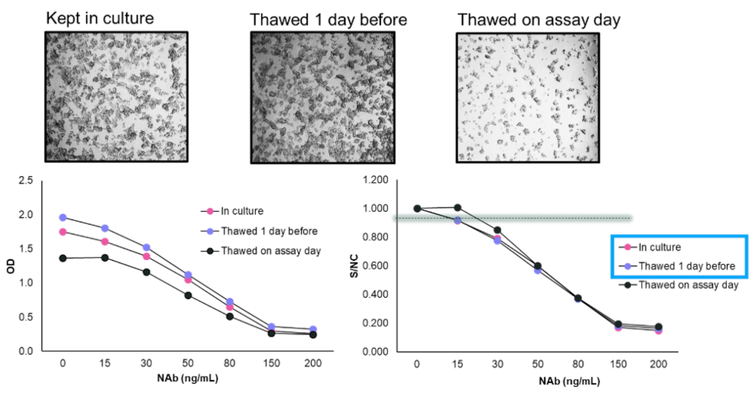

Figure 3: Comparing assay performance when using cells kept in culture, cells thawed 1 day before assay or cells thawed on assay day.

CASE STUDY N°3: BISPECIFIC ANTIBODY

ASSAY SETUP = ASSAY CHALLENGE

We developed a CLBA using electrochemiluminescence (ECL) to detect NAbs directed against a bispecific antibody in the third case study. First, the assay was set up in the absence of positive control using the target of each arm for capture or detection. In the first assay format, Target 1 is coated on a high-bind ECL plate to capture one arm of the bispecific antibody, while the second arm is detected using Target 2 labeled with SulfoTag (Figure 4). Surprisingly, an atypical dose-response curve was obtained using this assay format: no assay saturation could be observed, even at high drug concentrations.

The inversed assay format was then tested. In brief, target 2 was coated on the plate to capture the drug, and biotinylated target 1 was used for detection. Incubation with streptavidin-SulfoTag (SA-STAG) enabled detection of the immune-complex (Figure 5). Interestingly, using this assay format, assay saturation could be reached at high drug level. A drug concentration on the linear part of the drug-response curve was selected as the baseline assay level. The NAb assay was then successfully qualified and validated using this second assay format.

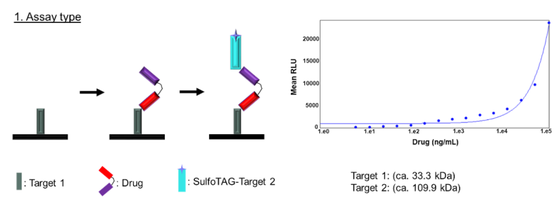

Figure 4: Drug—response curves evaluated with the first assay format.

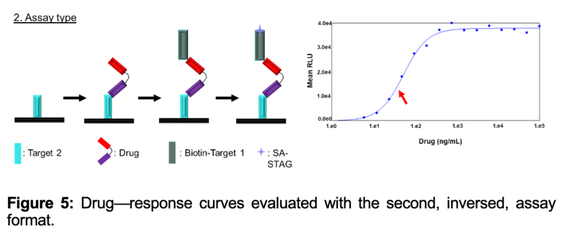

Figure 5: Drug—response curves evaluated with the second, inversed, assay format.

CASE STUDY N°4: COVID-19

ASSAY SETUP = ASSAY CHALLENGE

Similarly to the third study case, the challenge faced during developing a NAb assay against SARS-CoV-2 was about the choice of the assay format. We tested two ELISA formats monitoring the binding of the Spike protein S1 (catalyzing virus-to-receptor attachment) to the cellular receptor ACE2 (Figure 6. A and B). Both assays used different recombinant Spike protein S1: his-tagged full-length S1 subunit (nCoV Spike S1-His TAG) or His-tagged receptor-binding domain (RBD) of the S1 subunit (nCoV Spike RBD-His TAG). The best dose-response curve could be obtained with nCoV Spike RBD-His TAG (Figure 6. B), which was selected for further method development and qualification.

The results of the qualification are summarized in Figure 7. Notably, the assay could reliably detect NAb against SARS-CoV-2 in 9 out of 10 COVID-19 patients.

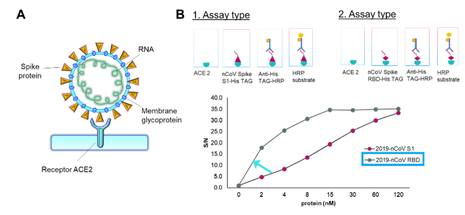

Figure 6: (A) Schematic representation of SARS-CoV-2 binding to its cellular receptor. (B) Drug-response curve evaluated for two different assay formats.

sCP	Sensitivity	Selectivity		Free-drug tolerance
%CV = 10.10	87 ng/mL	Blank	10/10 accepted	10 ng/mL free-drug* tolerated at LPC
sCP = 0.84		200 ng/mL	10/10 accepted	80 ng/mL free-drug* tolerated at HPC
		800 ng/mL	10/10 accepted	* 2019-nCOV RBD

Precision	0 ng/mL NC	200 ng/mL LPC	400 ng/mL MPC	800 ng/mL HPC
n	4	3	3	3
mean	1	0.7	0.4	0.2
%cv	1.8	7.4	14	17
intra-assay precision	2.7	1.9	4.6	2.3

Individual	S/NC	<rCP?
1	0.10	yes
2	0.04	yes
3	0.16	yes
4	0.94	no
5	0.22	yes
6	0.10	yes
7	0.02	yes
8	0.03	yes
9	0.04	yes
10	0.03	yes

COVID-19 individuals

Figure 7: Method qualification results.

DISCUSSION & CONCLUSIONS

Developing robust and reliable assays to detect NAbs is a crucial part of the immunogenicity testing strategy of biotherapeutics. By optimizing specific parameters, we established optimal NAb assays for a variety of different therapeutical drugs. In addition, one of our assays can be to characterize the antibody response against SARS-CoV-2 in the human population, without the need for high biosafety requirements.

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Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-drug Antibody Detection. Guidance for Industry. US FDA 2019