T 01 Establishment of an assay to assess the immunogenicity of a therapeutic antisense oligonucleotide in clinical samples

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Abstract

The immunogenicity of biotherapeutics is an important aspect of the safety program in clinical trials and is first of all assessed by analyzing the occurrence of anti-drug antibodies (ADAs) in response to the treatment of patients. Although RNA therapeutics, such as antisense oligonucleotides (ASOs) are much smaller than recombinant proteins, they still are able to elicit ADAs in patients. E.g., in about 65 % of patients treated with mipomersen (approved by FDA as Kynamro[1]) the occurrence of ADA indicated an immunogenicity that can be even higher than that observed for most therapeutic antibodies. However, due to the small size of RNA therapeutics and their ability to bind serum proteins, it is often challenging to develop an assay to detect ADAs in human blood samples that meets regulatory acceptance criteria.

Here we present the technical hurdles when establishing a highly sensitive assay for detection of ADAs against an ASO.

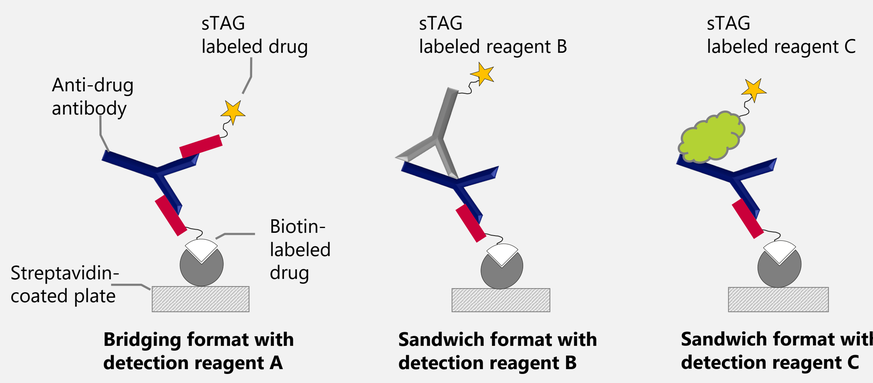
Method

A chemically modified antisense oligonucleotide (ASO) with 16 nucleotides was used to establish a method for detecting antibodies directed against RNA therapeutics (anti-drug antibodies, ADAs).

ADA were detected in human serum samples using the electrochemiluminescence (ECL) technology of Meso Scale Diagnostics (MSD) LLC (Rockville, Maryland, USA). ECL involves complexed Rhutenium ions (sTAG) that release photons upon electronic excitation when bound to the electrodes. The electrodes are placed on the bottom of 96 well plates.

Different ECL assay formats, set up as bridging or sandwich assay, were evaluated (Fig. 1). In brief, the ASO drug was biotinylated to be used as catching reagent and for detection sTAG was conjugated either to the drug or detection reagents A or B.

Fig. 1: ECL immunoassay formats



Bridging format with detection reagent A | **Sandwich format with detection reagent B** | **Sandwich format with detection reagent C**

In case of the bridging format, the binding reactions of catching and detection reagent with the positive control (PC) antibodies or serum samples were performed in solution. Thereafter, formed complexes of ADA (either from PC or sample) with catching and detection reagents were immobilized on the bottom of a streptavidin coated plate and ECL was measured after addition of READ buffer.

In case of the sandwich formats, first the catching reagent was immobilized on streptavidin coated plates, secondly ADA (either from PC or sample) added, and thirdly detected by incubation with the detection reagents B or C, addition of READ buffer. The emitted light increases proportionally with the bound ADA.

Positive Control

Four different positive controls (PCs) were generated by immunization of 4 rabbits (animals 30587 – 30590) with a special immunization scheme for small molecules such as oligonucleotides developed and performed by BioGenes GmbH (Berlin, Germany). The polyclonal antibodies were purified by affinity chromatography and provided for assay establishment in PBS. The four different PCs were tested in an ELISA with immobilized drug oligonucleotide and anti-rabbit antibodies for detection in absence of human serum.

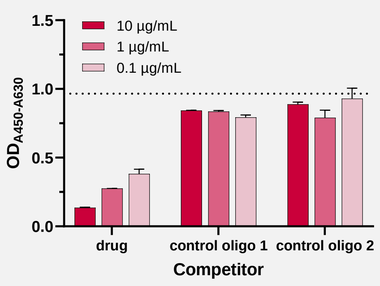
Based on purified antibodies from animal 30588 high and low PC samples were generated by spiking 6000 ng/mL (high PC) and 25 ng/mL (Low PC-1), 50 ng/mL (Low PC-2), and 75 ng/mL (low PC-3) of ADA into pooled human serum. NC represents the pooled human serum.

Animal 30588 produced the highest titer of ADA yielding 23.6 mg of purified, polyclonal antibodies against the ASO. Because no difference between the ADA preparations from the four animals was observed (Fig.2), the antibody preparation of animal 30588 was used as PC for assay establishment

Tab. 1: ADA yield

Animal	Amount of purified polyclonal ADA
30587	6.1 mg
30588	23.6 mg
30589	6.4 mg
30590	8.1 mg

Fig. 2: PC binding characteristics in ELISA (animal 30588)



Results

Optimal Assay Design

Fig. 1 gives an overview of the ECL immunoassay formats tested. Instead of the bridging format that is commonly used in ADA assays, the sandwich assay format with detection reagent C revealed the best results (Fig. 3).

Fig. 3A: Sandwich assay with detection reagent C
Serial dilutions between 10,000 and 2.4 ng/mL ADA (PC) twice independently in presence of 10% serum

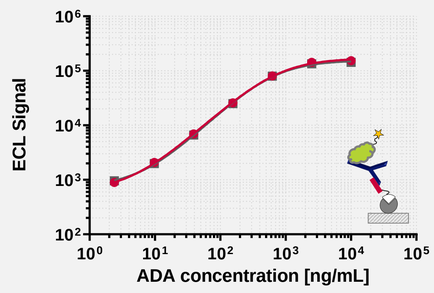
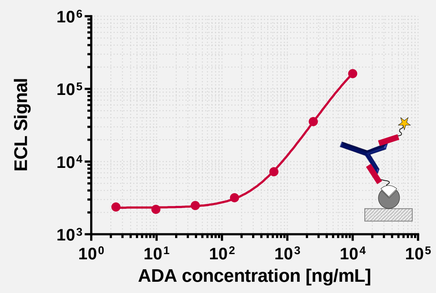
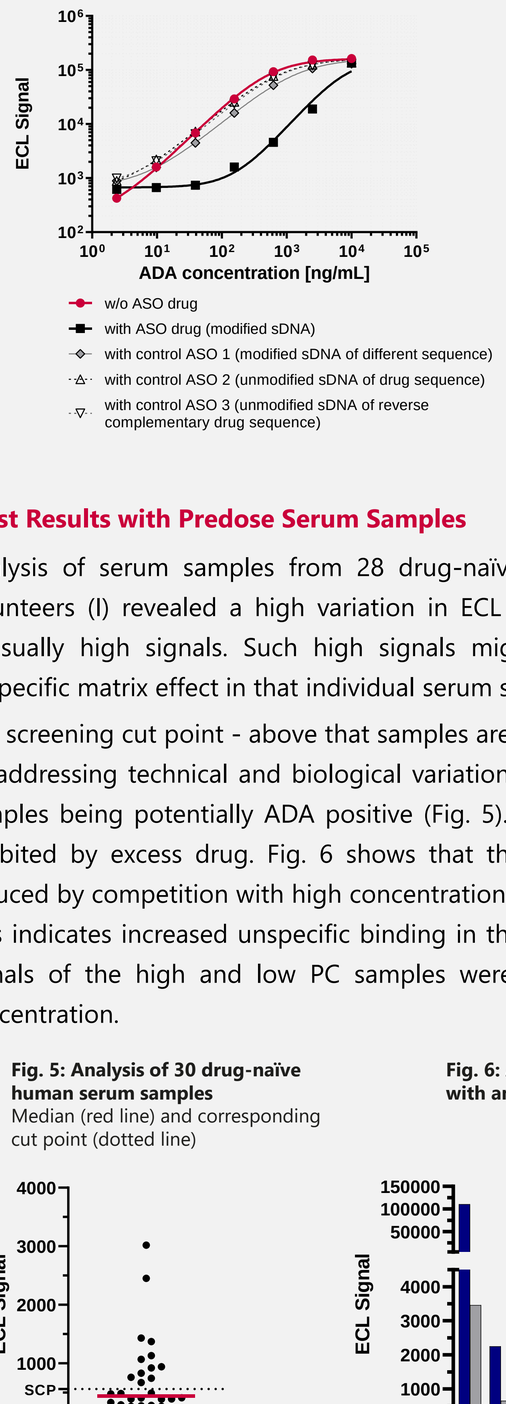


Fig. 3B: Bridging assay
Serial dilutions between 10,000 and 2.4 ng/mL ADA (PC) in presence of 10% serum



Specificity

Fig. 4: Serial dilution of PC with 100 ng/mL control ASOs



Assay Parameters

Tab. 2: Characteristics of sandwich assay with detection reagent C

Sensitivity:	ca. 10 ng/mL
MRD:	10
Precision:	**inter-run:** 7.2% CV at 25 ng/mL and 11.9% CV at 6000 ng/mL
	Intra-run: 9.5% CV at 25 ng/mL and 5.2% CV at 6000 ng/mL
Drug tolerance:	at least 100 ng/mL drug are tolerated to detect 25 ng/mL ADA

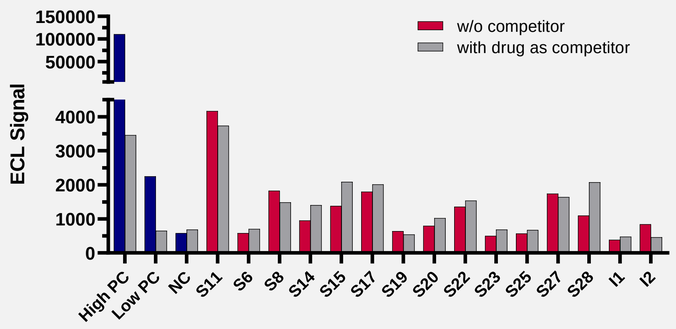
First Results with Predose Serum Samples

Analysis of serum samples from 28 drug-naïve patients (S, taken predose) and 2 healthy volunteers (I) revealed a high variation in ECL signals and a large number of samples with unusually high signals. Such high signals might be caused by pre-existing ADAs or any unspecific matrix effect in that individual serum sample.

The screening cut point - above that samples are considered ADA positive - is calculated as 561 by addressing technical and biological variation. Applying this cut point, resulted in 12 of 30 samples being potentially ADA positive (Fig. 5). True positivity can be proven, if the signal is inhibited by excess drug. Fig. 6 shows that the high signals observed in fig. 5, cannot be reduced by competition with high concentrations of the drug or the control oligos (not shown). This indicates increased unspecific binding in these individual serum samples. For control, the signals of the high and low PC samples were very efficiently inhibited at the same drug concentration.

Fig. 5: Analysis of 30 drug-naïve human serum samples
Median (red line) and corresponding cut point (dotted line)

Fig. 6: Analysis of the high signal serum samples of fig. 5 with and without 1000 ng/mL drug for competition



Conclusion

- Four high titer polyclonal positive controls could be generated without differences in antigen binding detectable by ELISA and high specificity for the ASO.
- To detect ADAs in human patient serum samples, the sandwich format with detection reagent C turned out to be superior compared with use of detection reagent B or performance of the bridging format.
- Using the optimal assay format a preliminary sensitivity of ca. 10 ng/mL, a high intra- and inter-run precision also at very low concentration, and a high drug tolerance of at least 100 ng/mL of the ASO was observed.
- Analysis of the drug-naïve serum samples from 30 individual patients / volunteers revealed 12 individuals with increased background signal due to unspecific matrix effects.

1 European Medicine Agency, Committee for Medicinal Products for Human Use (CHMP), Assessment report about Kynamro, EMA/305826/2013