Research Article

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

High-sensitivity quantification of antisense oligonucleotides for pharmacokinetic characterization

Shilpi Mahajan*[‡,1], Hui Zhao[‡,1], Kristina Kovacina[1], Eric Lachacz[1], Sany Hoxha[1], Josolyn Chan[1] & Meina Liang**[,1]

[1]Integrated Bioanalysis, Clinical Pharmacology & Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, South San Francisco, CA 94080, USA

*Author for correspondence: Tel.: +1 650 379 3089; shilpi.mahajan@astrazeneca.com

**Author for correspondence: Tel.: +1 925 389 0605; meina.liang@AmadorBio.com

‡Authors contributed equally

Aim: Antisense oligonucleotides (ASOs) are a fast-growing drug modality. Pharmacokinetic characterization and accurate quantification of ASOs is critical for drug development. LC–MS and hybridization immunoassays are common methods to quantify ASOs but may lack sensitivity. In this study we aimed to develop an ASO quantification method with improved sensitivity. **Methods:** We developed a branched DNA approach for ASO quantification and compared it with hybridization immunoassays. **Results:** The branched DNA assay showed significantly improved sensitivity, with LLOQ 31.25 pg/ml in plasma, 6.4-and 16-fold higher than dual-probe hybridization electrochemiluminescence and single-probe hybridization ELISA, respectively, with adequate precision, accuracy, selectivity and specificity and acceptable matrix interference. **Conclusion:** Branched DNA for ASO quantification has significantly higher sensitivity and lower hemolysis interference.

Plain language summary: Disease can be caused by genetic mutations that lead to overproduction or underproduction of an aberrant protein. Antisense oligonucleotides (ASOs) are a relatively new class of drugs. While most current drugs act at the protein level, ASOs work at the RNA level and minimize synthesis of the aberrant protein. ASOs are small synthetic nucleotides that specifically bind and modify the target RNA. Quantification of ASOs is important in drug development to understand how much of the drug is in circulation or in the body after a certain period of time. While there are methods available to quantify ASOs, they lack sensitivity. We developed a method called 'branched DNA' to quantify ASOs, and compared it with known ASO quantification methods. We found that the branched DNA method showed improved sensitivity compared with other existing methods and is a reliable method to quantify ASOs. This method may be used in clinical trials when improved sensitivity quantification is needed and thus facilitate the ASO drug development field.

Tweetable abstract: Branched DNA (bDNA) assay for ASO quantification was developed and compared with hybridization immunoassays. bDNA has significantly higher sensitivity and lower hemolysis interference and may be used in drug development for pharmacokinetic analysis.

First draft submitted: 17 February 2022; Accepted for publication: 8 April 2022; Published online: 17 May 2022

Keywords: antisense oligonucleotides (ASOs) • bioanalysis • branched DNA (bDNA) • dual-probe hybridization immunoassay (H-ECL) • high-sensitivity quantification • single-probe hybridization nuclease protection assay (H-ELISA)

Oligonucleotides can be used to modulate gene expression and have potential therapeutic applications for myriad indications [1]. Antisense oligonucleotides (ASOs) are synthetic short, single-stranded DNA, RNA or hybrid DNA–RNA molecules with chemical modifications that are designed to be complementary to the mRNA of interest and can prevent protein translation. After the first ASO drug, fomivirsen (Vitravene), was approved by the US FDA in

newlands press

1998, five more ASOs were approved for different indications between 2012 and 2019 [2], and many more clinical trials have evaluated ASOs in a broad variety of diseases [3,4]. With advancements in ASO chemistry and improved stability, ASOs as drugs are becoming more potent, requiring reduced doses at lower concentrations. Although significant technical advancements have been achieved to measure drug levels for pharmacokinetics (PK) in clinical trials [5], quantifying ASOs with methods that have higher sensitivity remains a challenge due to the use of reduced drug concentrations and non-intravenous routes of delivery.

Current approaches to ASO quantification can be divided into two major categories: hybridization-based immunoassays and chromatography-based methods [6–9]. These two commonly used approaches have different advantages and challenges. Chromatography-based quantification assays use high-performance or ultra-high-performance LC for separation, and UV detection and MS or MS/MS for analysis. Chromatography-based assays have several advantages for the quantification of ASOs as they are easier to develop, can specifically quantify and differentiate between intact ASOs and their metabolites within a wide dynamic range, and are useful for assessment of tissue distribution [9,10]. However, this type of quantification is not very sensitive, having a typical sensitivity in the low ng/ml range [8,9].

Hybridization-based assays are generally more sensitive than chromatography-based approaches, especially if electrochemiluminescence (ECL) is used for detection [6]. Two traditional hybridization techniques to quantify ASOs are dual-probe ECL and single-probe nuclease protection-based methods. In the dual-probe method, one probe hybridizes with and captures ASOs, while a second detection probe is conjugated with a tag that generates a chemiluminescent signal (see schematic in Supplementary Figure 1). This method results in increased sensitivity compared with LC–MS. However, dual-probe methods can be challenging when shorter ASO sequences are used, because the hybridization stability of the probe is limited by the length of the ASO. Moreover, this method may not be able to differentiate between a full-length ASO and its metabolites.

Nuclease-protected, single-probe assay is another hybridization-based method to quantify ASOs. This method consists of digoxigenin-labeled probes that can specifically bind the target ASO sequence and S1 nuclease, which specifically cleaves single-stranded, unhybridized nucleic acids (see schematic in Supplementary Figure 2). The remaining double strand can be recognized by an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody and quantified using an AP-generated signal [6,11]. This method can potentially work better with shorter ASO sequences and can measure only full-length, unmetabolized ASOs. Although both methods offer greater sensitivity than LC–MS, there is still a need for more sensitive methods for PK characterization of ASOs.

Branched DNA (bDNA) technology offers a highly sensitive detection method due to signal amplification (Figure 1). This technology has been approved by the FDA for sensitive and specific detection of HIV and hepatitis C virus load [12]. bDNA has also been used to detect mRNA levels of the target gene after gene silencing, from animal tissue biopsies [13]. The latest bDNA technology uses target-specific oligonucleotides (i.e., capture extender and label extender) to hybridize with and capture the target sequence. The signal is amplified via multiple specific hybridizations and is then detected by chemiluminescence from an AP reaction. The specificity and sensitivity of the bDNA assay is achieved through multiple sequence-specific hybridization steps without amplification of the target sequence as in PCR. One of the advantages of bDNA technology is that nucleic acid can be directly measured in the matrix (e.g., whole blood, plasma) without sample purification. Despite these advantages, quantification of ASOs using bDNA has never been tested.

In this work, we used a 16-bp ASO, AZD2373, as an example for developing a high-sensitivity quantification method that can be readily applied to other ASOs when higher sensitivity is desirable. AZD2373 is an ASO designed to treat a genetically associated form of kidney disease and is under investigation in a first-in-human study in healthy male subjects of African ancestry [14]. We evaluated the feasibility of bDNA technology and compared it with single- and dual-probe immunoassays.

Materials & methods

Reagents

Human dipotassium-EDTA plasma samples (ten individual and one pooled) and hemolyzed samples were purchased from BioIVT (NY, USA). Nuclease-free water was purchased from Ambion (cat. no. AM9932; Life Technologies, CA, USA). Trizma base (cat. no. 22643, US Biochemical Ultrapure), Dulbecco's phosphate-buffered saline (cat. no. 14190250; Gibco, MD, USA), Blocker A (cat. no. R93BA-1), antibody diluent (cat. no. R50AA-3) and streptavidin-coated 96-well plates (cat. no. L15SA-1) were obtained from Meso Scale Discovery (MSD; MD, USA). Anti-digoxigenin–AP Fab fragment (cat. no. 11093274910) was obtained from Millipore Sigma (MO,

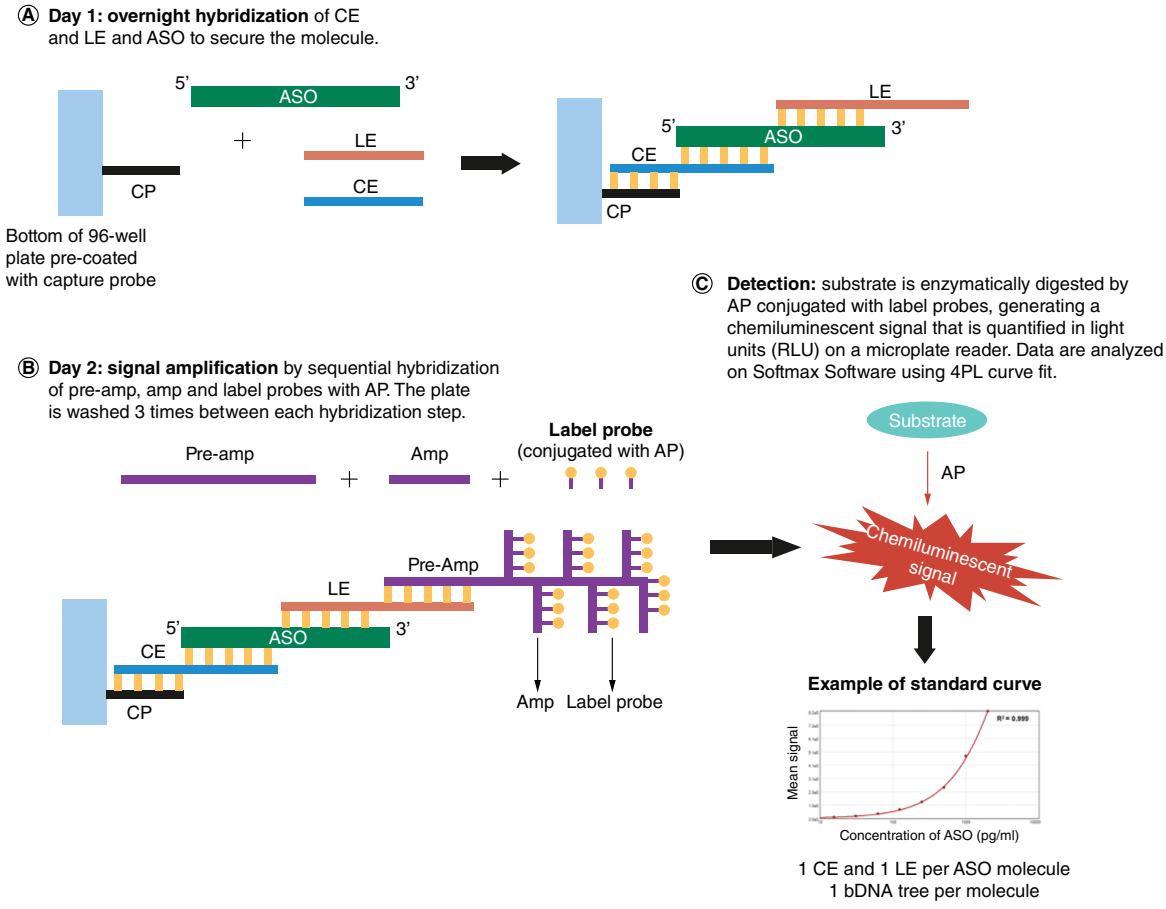

Figure 1. Schematic of the branched DNA assay. The technology that drives the branched DNA (bDNA) assay is essentially signal amplification via a series of highly specific hybridizations. Capture probe is pre-adsorbed to the bottom of the 96-well plate. Within the probe mix, capture extender oligonucleotides, which are generated against the sequence of interest (in this case, an antisense oligonucleotide [ASO]), anneal to the capture probe. These two molecules capture the target ASO sequence via co-operative hybridization. Once the target is captured, a bDNA 'tree' is built onto the target sequence by a series of sequential hybridizations of highly specific oligonucleotides. First, label extender probes bind to the ASO sequence. A pre-amplifier (the 'trunk' of the bDNA tree) is then hybridized to the label extenders. Subsequently, several amplifiers (tree branches) are hybridized onto the pre-amplifier, and several label probes conjugated to AP are hybridized onto the amplifier. Each bDNA tree contains hybridization sites for 400 AP-conjugated label probe molecules. Before the plate is read, substrate that is enzymatically digested by AP and generates a chemiluminescent signal is added to the well. This signal is quantified in light units on a microplate reader.
AP: Alkaline phosphatase; Amp: Amplifier; ASO: Antisense oligonucleotide; bDNA: Branched DNA; CE: Capture extender; CP: Capture probe; LE: Label extender; 4PL: Four-parametric logistical fit; Pre-amp: Pre-amplifier.

USA). The AttoPhos® AP fluorescent system (cat. no. S1000) was obtained from Promega (WI, USA). Pierce (IL, USA) black 96-well neutravidin-coated plates (cat. no. 15117) and SuperBlock™ Blocking Buffer in Tris Buffered Saline with Tween-20 (cat. no. 37536) were obtained from Thermo Fisher Scientific (MA, USA). The QuantiGene™ Singleplex assay kit (cat. no. QS0009) was obtained from Thermo Fisher Scientific (Life Technologies). Sodium phosphate, dibasic, sodium chloride, Tween 20, SSPE 20 × concentrate and S1 Nuclease kit were purchased from Sigma (MO, USA).

Quantification of ASO with bDNA

Standards, quality control (QC) and test plasma samples were diluted tenfold in 1 × LSK buffer (1 × lysis buffer containing proteinase K at a concentration of 0.25 mg/ml). These samples were incubated at 60°C for 1 h. Capture plates were removed from storage at 4°C and equilibrated to room temperature by placing them on a bench top before plating. The samples that had been incubated at 60°C were also equilibrated to room temperature

before plating on the capture plate. QuantiGene capture extender and label extender probes (from Thermo Fisher Scientific) designed specifically against AZD2373 were added to the capture plate, along with a lysis mixture and a blocking reagent to prevent nonspecific hybridizations. Standards and samples were then added to the plate and incubated for overnight hybridization (18 ± 2 h) at 46°C. Next day, the unbound probes were removed by washing the plate three times with wash buffer. For bDNA assay, all the plate wash steps were performed three times. After washing, 2.0 pre-amplifier was added to the plate and incubated for 1 h at 46°C. The plate was washed, 2.0 amplifier was added, and the plate was incubated at 46°C for 1 h. After incubation the plate was washed, label probe was added and the plate was incubated for 1 h at 46°C. After incubation the plate was washed and substrate was added to the plate, which was incubated for 6 min at room temperature. The plate was read on a luminometer (2103 EnVision® Multilabel Plate Reader; Perkin Elmer, MA, USA) within 15 min of substrate addition at 0.2 s. ASO concentrations were interpolated from the standard curve by using SoftMax® PRO GxP Software (Molecular Devices, CA, USA). The standard curves included eight non-zero concentrations: 2000 (anchor), 1000 (ULOQ), 500, 250, 125, 62.5, 31.25 (LLOQ) and 15.63 pg/ml (anchor). The five levels of QC concentrations in plasma were 1000 (ULOQ), 750 (high QC), 250 (middle QC), 95 (low QC) and 31.25 pg/ml (LLOQ).

Quantification of ASOs by dual–probe hybridization ECL

Dual-probe hybridization ECL is based on two-step hybridization of the analyte, first with the 5′-end biotinylated 8-mer capture probe (Integrated DNA Technologies, IA, USA) complementary to the 3′-end of the ASO. The hybridized complex was immobilized on a streptavidin-coated MSD plate and subsequently a second hybridization was performed with the 8-mer detection probe (Integrated DNA Technologies) labeled with MSD tag (ruthenium) on the 3′ end and complementary to the 5′ end of the ASO. First, 10 nM capture probe was incubated with dipotassium-EDTA plasma samples spiked with various concentrations of ASO at 65°C for 30 min, followed by incubation at 21°C for an additional 30 min. The hybridized complex was immobilized on streptavidin-coated MSD plates for 30 min at room temperature. After the plate wells were washed, prewarmed detection probe (5 nM) was added and the plates were incubated for 60 min at room temperature to allow hybridization between the detection probe and the unhybridized portion of the analyte that was captured on the plate. After three washes, MSD Read Buffer (MSD) was added to each well and the plate was exposed to electrical stimulation that resulted in light emission by the detection tag reporter. Electrical stimulation and signal capture were performed with MSD Sector Imager. Standard curves had eight non-zero concentrations: 200 (ULOQ), 100, 50, 10, 5, 1, 0.2 (LLOQ) and 0.1 ng/ml (anchor). The five QC samples were 200 (ULOQ QC), 140 (high QC), 40 (middle QC1), 3 (middle QC 2), 1 (low QC 2), 0.5 (low QC 1) and 0.2 ng/ml (LLOQ QC).

Quantification of ASO by single-probe hybridization ELISA

For single-probe hybridization ELISA, plasma samples were mixed with 6 × 0.24% saline sodium phosphate–EDTA and 3.75 nM capture/detection probe (Integrated DNA Technologies) for 1 h at room temperature and then added to the black Reacti-Bind neutravidin plate (Pierce) and incubated for 1 h at room temperature. The plate was washed, S1 nuclease solution (300 U/ml; 1 × nuclease buffer, 0.6 M NaCl) was added to each well, and the plate was incubated for 2 h at room temperature. The plate was washed again, antidigoxigenin-AP solution (0.75 U/ml, 1.5 M NaCl, SuperBlock Blocking Buffer with Tween-20) was added to each well, and the plate was incubated for a further 30 min at room temperature. After washing, AttoPhos reagent was added to each well and the plate was incubated for 20 min at room temperature with shaking, after which 100 µl of stop solution (25% w/w dibasic sodium phosphate in water) was added and the plate was incubated for 1 min at room temperature with shaking. The plate was then read in the Envision plate reader at excitation 430 nm, emission 579 nm and gain 150 at high-concentration mode. Standard curves had eight non-zero concentrations: 80 (ULOQ), 70, 30, 10, 3, 1 and 0.5 ng/ml (LLOQ). The five QC samples were 80 (ULOQ QC), 60 (high QC), 5 (middle QC), 1.5 (low QC) and 0.5 ng/ml (LLOQ).

Method performance evaluation

Accuracy & precision

For each method, accuracy and precision were assessed by measuring at least five QC levels (ULOQ QC, high QC, middle QC, low QC, LLOQ QC) from a minimum of three independent assays conducted over multiple days. Each accuracy and precision run included freshly prepared standards and at least two sets of each of the five QC

Table 1. Summary of branched DNA assay performance.

Assay parameter		bDNA
Working range	LLOQ (ng/ml)	0.03
	ULOQ (ng/ml)	1
Selectivity	10/10 samples spiked at LLOQ recovered ±25%	100%
Specificity	10/10 unspiked matrix recovered below LLOQ	100%
Precision (CV)	Inter-assay	5.7–12.0
	Intra-assay	5.2–14.3
Accuracy (% recovered)	Inter-assay	95.0–103.4
	Intra-assay	90.4–99.5
Hemolysis interference (%)	≤5%	No
	≥10%	No

bDNA: Branched DNA.

levels in a plate. Accuracy was measured by percentage bias (back-calculated concentration), whereas precision was measured by the percentage CV. QC samples were prepared in the same biological matrix as the samples.

Intra-assay accuracy & precision

Intra-assay accuracy was evaluated by the percentage bias of each level within a run. Intra-assay precision is defined by the percentage CV of each level within a run. Intra-assay accuracy and precision were determined from three or more sets of each of the five QC levels in duplicate wells within one or more assays.

Specificity & selectivity

Specificity was evaluated by measuring drug concentrations in unspiked plasma samples from ten individuals. Selectivity is defined as the ability of the assay to measure the analyte of interest in the presence of unrelated compounds in the matrix. A minimum of ten individual human plasma samples were spiked with AZD2373 at LLOQ concentrations.

Data analysis

Raw data generated by the instruments were analyzed with SoftMax software, using four-parametric logistical curve fitting with a weighting factor of $1/y^2$.

Results

Quantification of ASOs with bDNA assay

We evaluated the feasibility of the bDNA method for AZD2373 quantification and assay sensitivity. The bDNA method requires two probes to simultaneously hybridize with the short ASO sequence: one to capture the target (capture extender) and a second probe (label extender) to bind to the ASOs for signal amplification (Figure 1). AZD2373 is a short ASO with only 16 nucleotides. To test the bDNA method for AZD2373 quantification, probes were designed specifically to capture AZD2373, and assay performance was evaluated first in the assay buffer at a tenfold dilution of the drug and the scrambled control. The results showed a robust assay window with a signal-to-background ratio of more than 200 and an adequate dynamic range for AZD2373 quantification. In contrast, scrambled ASOs produced signal only at background levels, suggesting that the assay is specific (Figure 2A). Next, we evaluated the assay's performance for detection of AZD2373 in human plasma. The standard curve and five QC levels demonstrated recovery within the acceptable criteria of ±20% at high, middle and low QC levels and within ±25% at the ULOQ and LLOQ (Table 1). The working range of the assay was from 1 (ULOQ) to 0.03 ng/ml (LLOQ).

Ten individual plasma samples were then used to evaluate assay selectivity and specificity (Table 1). All ten (100%) of the unspiked individual samples showed values below the LLOQ of 0.03 ng/ml, demonstrating the specificity of the assay. The same ten samples were spiked with AZD2373 at the LLOQ, 0.03 ng/ml. All of the spiked samples (100%) showed recovery within ±25% (75–125%) of the nominal concentration, demonstrating selectivity for this ASO in human plasma (Table 1). Multiple tests also showed good intra- and inter-assay precision and accuracy (Table 1). A separate test by a second analyst confirmed the performance of the bDNA method, suggesting that this assay is robust. The bDNA assay was very sensitive, having a quantification range of 0.03–

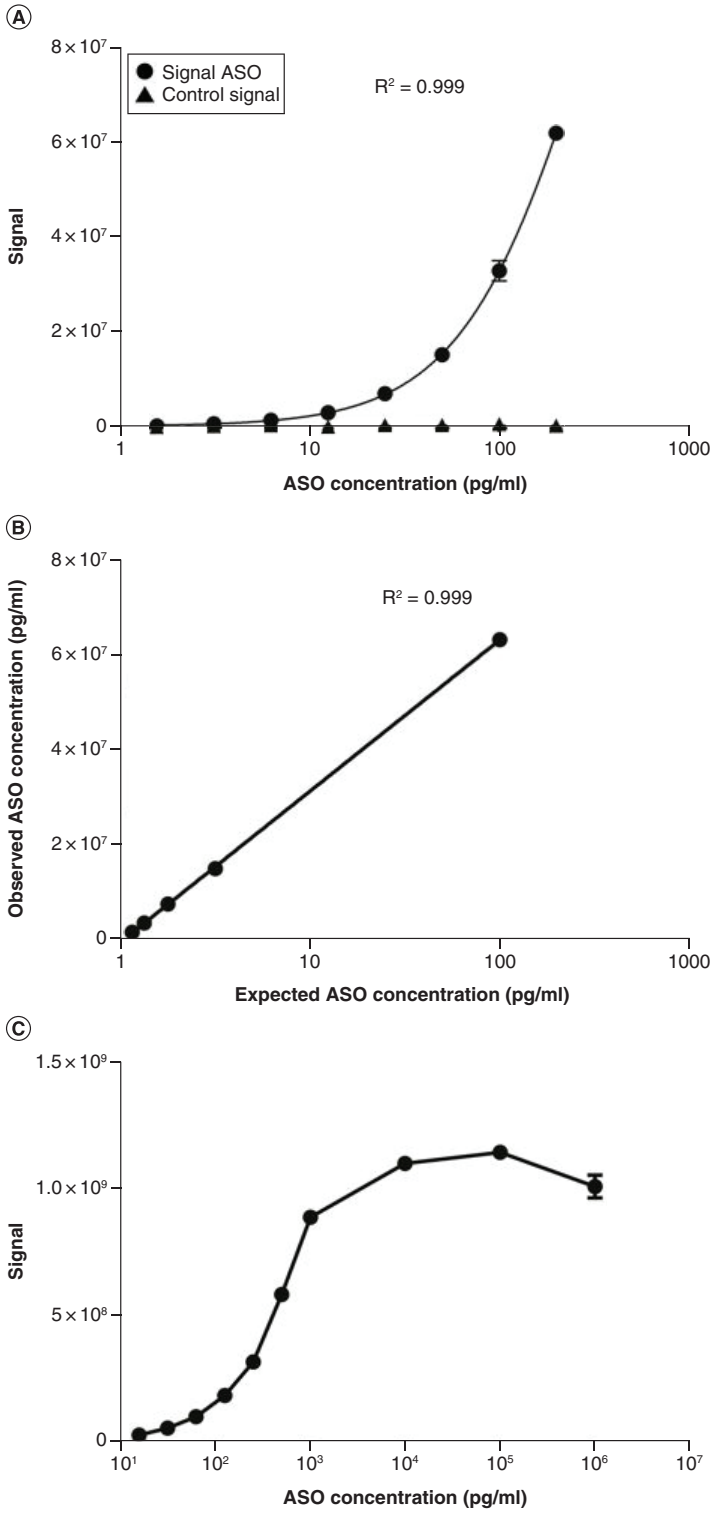

Figure 2. The branched DNA assay was specific and linear in the assay quantitative range for antisense oligonucleotide quantification in buffer and human plasma. (A) Signal generated from ASO was specific compared with a scrambled ASO control in the assay buffer. **(B)** Dilutional linearity of the branched DNA assay. AZD2373 was spiked into pooled normal human plasma at a final concentration of 1 μg/ml and then diluted with the indicated dilution factors. The graph shows the expected and observed concentrations of AZD2373 after linear dilutions of the drug starting at 1 μg/ml in plasma. Values are in the assay quantitative range. **(C)** Hook effect was also evaluated. The signal plateaus at concentrations >1 ng/ml.
ASO: Antisense oligonucleotide.

Table 2. Linearity of dilution via branched DNA assay.†

Dilution factor	AZD2373 concentration (μg/ml)		% recovery	% CV
	Expected	Observed		
1000	1.00	1.19	119.20	1.55
4000	1.00	1.15	114.60	5.34
8000	1.00	1.18	117.93	6.03
16,000	1.00	1.15	114.53	4.97
32,000	1.00	1.16	115.61	5.27

†AZD2373 was spiked in pooled normal human plasma at a final concentration of 1 μg/ml and then diluted by the indicated dilution factor.
bDNA: Branched DNA.

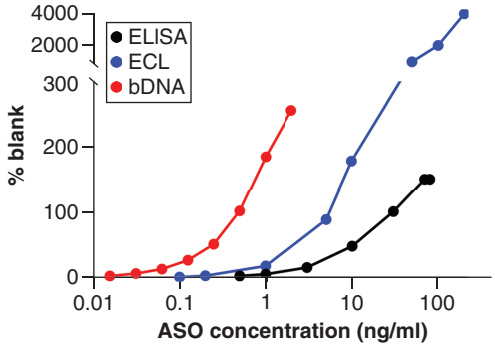

Figure 3. The branched DNA method versus the hybridization ELISA and electrochemiluminescence methods. The signals at the indicated ASO concentrations were normalized against the blank values to obtain the signal-to-background values for each method.
ASO: Antisense oligonucleotide; bDNA: Branched DNA; ECL: Electrochemiluminescence.

Table 3. Comparison of antisense oligonucleotide quantification methods.

Assay parameter	bDNA	ECL	ELISA
Sensitivity (ng/ml)	0.03	0.2	0.5
Assay range (ng/ml)	0.03–1	0.2–200	0.5–80
Assay window	30	1000	160
Specificity†	Met guideline requirement	Met guideline requirement	Met guideline requirement
Selectivity at LLOQ‡	Met guideline requirement	Met guideline requirement	Met guideline requirement
Hemolysis interference (10%)	No	Yes	No
Lipemia interference	No	No	No
ADA interference	Not likely	Minimum	Likely
Metabolite interference	Likely	Likely	Likely
ASO length challenge	More challenge for shorter	More challenge for shorter	Less

†Naive unspiked samples recovered below LLOQ, undetectable in at least 80% of samples.
‡Plasma samples spiked at LLOQ recovered within ±25% in at least 80% of samples.
ADA: Antidrug antibody; ASO: Antisense oligonucleotide; bDNA: Branched DNA; ECL: Electrochemiluminescence.

1 ng/ml. To quantify samples above the ULOQ of the assay, the linearity of dilution for the assay was assessed. AZD2373 was spiked into pooled human plasma to a final concentration of 1 μg/ml (1000-fold higher than the ULOQ) and was then serially diluted with the plasma. The drug was recovered well within the assay quantitative range of 0.03–1 ng/ml, suggesting that the drug levels can be accurately measured after dilution with untreated plasma (Figure 2B & Table 2). Furthermore, a plateau was observed at concentrations of >1 ng/ml, and no hook effect was observed (Figure 2C).

Comparison of bDNA & immunoassay methods

Nuclease-protected, single-probe hybridization ELISA and dual-probe hybridization ECL are two commonly used immunoassays to measure ASO levels in circulation. To compare the bDNA method with these two methods, we also evaluated the performance of ELISA and ECL in the detection of AZD2373 levels in human plasma (Figure 3 & Table 3). The ECL method with two probes for capture and detection showed the best assay range, 0.2–200 ng/ml, which is consistent with the range of many ECL-based assays. This method also showed the best signal-to-background ratio (Figure 3). For the ELISA method, a single probe was used to hybridize with the ASOs,

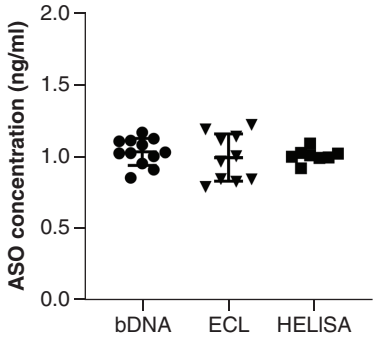

Figure 4. Correlation of branched DNA, hybridization ELISA and electrochemiluminescence methods. AZD2373 was spiked into pooled normal human plasma at 1 ng/ml. The measured concentration by each method is shown. The difference among the methods was evaluated by *t* test. ASO: Antisense oligonucleotide; bDNA: Branched DNA; ECL: Electrochemiluminescence; HELISA: Hybridization enzyme-linked immunosorbent assay.

and unhybridized probes were cleaved by S1 nucleases. The ELISA method had an assay range of 0.5–80 ng/ml but also had the lowest signal-to-background ratio (Figure 3 & Table 3). Both methods showed good accuracy, precision and robustness, which was further confirmed by the second analyst (data not shown).

Ten individual plasma samples were used to evaluate specificity and selectivity (Table 3). All of the unspiked plasma samples (100%) showed detection levels below the LLOQ for each method (0.5 ng/ml for hybridization ELISA and 0.2 ng/ml for ECL), demonstrating good assay specificity. AZD2373 was spiked into these individual plasma samples at the LLOQ of each respective method. ASOs could be recovered in at least 80% of plasma samples within 25% of the spiked concentration (Table 3), suggesting good assay selectivity.

Investigating the matrix effects of hemolysis and lipemia in plasma is essential to ensure accurate quantification. Hemolysis is the release of hemoglobin due to the breakdown of red blood cells in plasma or serum. It can occur during sample processing or due to an inherent medical condition. Hemolysis can interfere with the accuracy and precision of LC–MS-based PK measurements due to its effect on ion suppression or enhancement and should be evaluated during assay development [15]. Hemolysis can also interfere with immunoassay results due to the release of large amounts of hemoglobin, causing nonspecific coloration. To investigate the impact of hemolysis on AZD2373 detection in these assays, hemolyzed plasma was mixed with normal plasma at 2, 5 and 10% (Tables 1 & 3) to mimic different hemolysis conditions, and AZD2373 was spiked into the hemolyzed plasma samples at their LLOQs. Hemolyzed samples with up to 10% hemolysis did not affect the detection of AZD2373 at 0.03 ng/ml by the bDNA method or at 0.5 ng/ml by hybridization ELISA (Table 3). ECL had the disadvantage of hemolysis interference, which was detectable at 10% hemolyzed matrix (Table 3). It was found that ≤5% hemolysis did not have a significant effect on assay sensitivity.

Lipemia leads to turbid samples due to the accumulation of lipoproteins, and can interfere with the analytical method by physically interfering with probes or the spectrophotometric measurement [16]. We also evaluated the effect of lipemia on ASO quantification (Table 3). AZD2373 at each LLOQ concentration was spiked into either human lipemic plasma or in a 1:2 or 1:4 mixture with normal human plasma. All three methods recovered ASO within 25% of the nominal concentration, suggesting that lipemia has little effect on any of these methods.

All three methods – single-probe hybridization ELISA, dual-probe ECL and bDNA – showed good performance as quantification assays for AZD2373. To determine whether the results from these three assays are consistent in sample testing, AZD2373 was spiked into pooled human plasma to a final concentration of 1 ng/ml, and the concentrations derived from the three methods were compared (Figure 4). The results from the ELISA, ECL and bDNA methods were almost identical, suggesting that the three methods are comparable.

Discussion

ASOs are a promising new class of therapeutics. Development of a method that provides an adequate quantification of ASOs is crucial for accurate assessment of their PK parameters, which are needed for the development of ASO therapeutics. Advancements in the technology of ASO generation, along with improved stability, potency and target tissue delivery, enable lower doses and new routes of drug administration, such as oral dosing [17]. However, these drug administration approaches also raise bioanalytical challenges due to low circulating concentrations resulting from low dose or poor bioavailability. Methods with higher sensitivity may be needed to quantify ASOs. Current methods for ASO quantification, including LC–MS and immunoassays, may not be sufficient to address sensitivity challenges. Although LC–MS allows for the precise detection of both full-length ASOs and their metabolites in plasma and tissue within the early post-absorption/distribution phase, it may not provide sufficient sensitivity to

measure ASO levels during the elimination phase. In addition, a highly sensitive method is often required for ASO quantification for low-dose cohorts during the dose-escalation phase of clinical studies. Hybridization-based methods can improve quantification sensitivity by about tenfold, which may not be adequate in some cases. Hence there is a need for the development of higher sensitivity platforms for ASO quantification.

The bDNA assay that we developed to quantify ASO levels in human plasma showed the highest sensitivity of all the evaluated methods. The bDNA assay allows for high sensitivity and has been used to measure mRNA and viral load [12,13]. Each bDNA signal amplification unit is built through sequential hybridization of different types of oligonucleotides (capture extenders, label extenders, pre-amplifier and amplifier), followed by hybridization of the label probes conjugated with AP, which mediates the degradation of the chemiluminescent substrate. We therefore hypothesized that the generated signal could accurately measure a small number of ASO molecules.

Our results showed that all three methods – hybridization ELISA, ECL and bDNA – performed well for ASO quantification and fit FDA guidelines for PK assays in terms of calibration curves, QC and selectivity and specificity, and the results from these three methods were highly consistent with each other. Each method has unique advantages and may be preferred in different settings. LC–MS offers a wide dynamic quantification range and is able to detect ASO metabolites and short ASOs. For these reasons, it may be preferred for preclinical studies, where sensitivity is not critical due to high doses, as well as for analyzing tissue biodistribution of the ASO and its metabolites. ASOs are extensively taken and accumulate in tissues such as liver and kidney, where the concentrations of ASOs are higher than in plasma and well within the detection limits of LC–MS [18].

Immunoassay-based methods are generally preferred when higher sensitivity is required. Nuclease-protected hybridization ELISA may be preferred for shorter ASOs due to the fact that only one probe is used. In our study, the ECL method tended to have a wide assay range of about 3 logs, whereas the bDNA assay had the best sensitivity.

Matrix interference is a common challenge for assay development [15]. Hemoglobin is a common contaminant in plasma and serum due to hemolysis, and the evaluation of its interference for a PK assay is a regulatory recommendation [19]. Hemolysis can also occur in people with metabolic conditions such as chronic kidney diseases, which makes it even more important to evaluate its effect for therapeutics that are intended for those indications. Different assay methods have different levels of tolerance for hemolysis. We also observed some differences in the effect of hemolysis on the performance of other assays (Table 3). Both the hybridization ELISA and bDNA methods showed more resistance to hemolysis than the ECL method.

Lipemia causes the accumulation of lipids in the blood and is common in some metabolic diseases such as hyperlipidemia and diabetes. Our results suggest that lipemia did not interfere with the performance of any of the three assays.

Many factors should be considered when selecting an appropriate assay platform for ASO quantification in clinical trials (Table 3). Sample hemolysis may not happen very often, but it did have some negative effect on the ECL method in our study, whereas the bDNA and hybridization ELISA methods suffered the least interference from hemolysis. Another consideration is potential interference from antidrug antibodies on the assay. For the bDNA method, samples were treated with lysis buffer containing proteinase K and were incubated at 60°C before being added to the plates for hybridization, which should remove all anti-ASO antibodies present. These steps should also eliminate the interference of antidrug antibodies with ASO quantification. The hybridization ECL method also includes a 65°C incubation step [20] that should denature IgG molecules to reduce the effect of anti-ASO antibodies. In contrast, the hybridization ELISA method does not include proteinase K treatment or a heat inactivation step, which makes it vulnerable to interference by antidrug antibodies. It might be of interest to investigate the effect of proteinase K treatment on this assay's performance in the future. ASOs have different lengths, which could affect the consideration if one method is more preferred. Our results showed that a 16-mer ASO was sufficient for bDNA and dual-probe ECL methods; however, ASOs with shorter lengths may present difficulties for optimal binding. It might be of interest to determine the minimal length required for optimal bDNA and ECL assays. In addition, it is not clear how ASO metabolites affect bDNA assay performance. Hybridization-based immunoassays presents difficulty in distinguishing between full-length ASOs and their N-1 or N-2 metabolites [11]. Whether the bDNA assay has similar issues certainly warrants further evaluation.

In conclusion, our results suggest that we have more bioanalytical methods to choose from for measuring the wide PK profile range of ASOs. LC–MS is a preferred method for nonclinical studies due to its simplicity of development, wide dynamic range, amenability to any ASO length and ability to evaluate ASO tissue distribution and metabolites. Although hybridization ELISA is often used for clinical samples because its sensitivity is better than that of LC–MS, ECL readout offers higher sensitivity and wider dynamic range. The bDNA method is a

novel approach for ASO detection, with superior sensitivity compared with existing LC–MS and immunoassay methods. Although both the ECL and bDNA methods offer high sensitivity, they may encounter challenges for quantification of short ASOs.

Overall, both hybridization ELISA and ECL are commonly used for ASO quantification in clinical studies. If sensitivity is not a concern, LC–MS is the method of choice due to its simplicity and robustness. When ultra-sensitive measurement is needed for ASOs administered at low doses and/or via a non-intravenous route, bDNA may be considered.

Future perspective

As new drug modalities become more prevalent in the biopharmaceutical industry, they also bring new challenges for bioanalysis. New advances in ASO technologies and novel dosing routes make it necessary to have a highly sensitive method to quantify ASO levels in different matrices. Current available options include chromatography-based approaches and hybridization immunoassays, with each one having its advantages and disadvantages. ASO quantification platforms can be selected based on the development stage of the ASO molecule, studies, matrices and sensitivity requirements. Key characteristics of the bDNA method for ASO measurement include high sensitivity and resistance to some common matrix effects. However, the bDNA method also has a small dynamic range and a possible limitation in measuring short ASOs. Thus further efforts are warranted to find a method with high sensitivity similar to that of bDNA but with a wider assay range and which is more amenable for the quantification of shorter ASOs. Hybridization-based immunoassays and the bDNA method are affected by the length of ASOs (i.e., shorter ASOs tend to present challenges for quantification), whereas chromatography-based methods such as LC–MS are not affected but have lower sensitivity. Developing a method with good assay sensitivity and dynamic range regardless of ASO length will be crucial for the bioanalysis of novel ASOs with novel delivery routes.

Summary points

- Antisense oligonucleotide (ASO) quantification methods with improved sensitivity are needed to assess pharmacokinetic profiles, especially when low-dose and non-intravenous ASOs are administered.
- The branched DNA assay showed significantly improved sensitivity, with LLOQ 31.25 pg/ml in plasma, significantly higher than that of single-probe ELISA and dual-probe electrochemiluminescence hybridization assays, with adequate precision, accuracy, selectivity, specificity and acceptable matrix interference.
- Although both the electrochemiluminescence and branched DNA methods offer high sensitivity, they may encounter challenges for quantification of short ASOs.
- When sensitivity is not a concern, LC–MS may be the method of choice due to its wide dynamic range, amenability to any ASO length and ability to evaluate ASO tissue distribution and metabolites (e.g., for nonclinical studies).
- Developing a method with good assay sensitivity and dynamic range regardless of ASO length will be crucial for the bioanalysis of new ASOs with new delivery routes.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2022-0035

Acknowledgments

The authors wish to thank I Vainshtein and K Maloney for discussions on ASO bioanalysis, and S Chaukulkar for being the second analyst for the bDNA assay. Technical support for the synthesis of probes for the bDNA assay was provided by Thermo Fisher Scientific. Editorial support was provided by D Shuman of AstraZeneca. AZD2373 is being developed by AstraZeneca in collaboration with Ionis Pharmaceuticals.

Financial & competing interests disclosure

All authors are or were employees of AstraZeneca at the time of this study and may hold stock ownership or stock options in the company. D Shuman is an AstraZeneca employee and hence source of funding is AstraZeneca. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as: •• of considerable interest

1. Roberts TC, Langer R, Wood MJA. Advances in oligonucleotide drug delivery. *Nat. Rev. Drug Discov.* 19(10), 673–694 (2020).
2. Yin W, Rogge M. Targeting RNA: a transformative therapeutic strategy. *Clin. Transl. Sci.* 12(2), 98–112 (2019).
•• **Extensive overview of advancements in oligonucleotide therapeutics and the antisense oligonucleotide (ASO) drug class.**
3. Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* 50, 259–293 (2010).
4. Watts JK, Corey DR. Silencing disease genes in the laboratory and the clinic. *J. Pathol.* 226(2), 365–379 (2012).
5. Wang L, Ji C. Advances in quantitative bioanalysis of oligonucleotide biomarkers and therapeutics. *Bioanalysis* 8(2), 143–155 (2016).
6. Norris DA, Post N, Yu RZ, Greenlee S, Wang Y. Bioanalysis considerations on the pharmacokinetic evaluation of antisense therapeutics. *Bioanalysis* 11(21), 1909–1912 (2019).
7. Tremblay GA, Oldfield PR. Bioanalysis of siRNA and oligonucleotide therapeutics in biological fluids and tissues. *Bioanalysis* 1(3), 595–609 (2009).
8. Wang L, Meng M, Reuschel S. Regulated bioanalysis of oligonucleotide therapeutics and biomarkers: qPCR versus chromatographic assays. *Bioanalysis* 5(22), 2747–2751 (2013).
9. Welink J, Xu Y, Yang E *et al.* 2018 White paper on recent issues in bioanalysis: a global bioanalytical community perspective on last decade of incurred samples reanalysis. Part 1: small molecule regulated bioanalysis, small molecule biomarkers, peptides & oligonucleotide bioanalysis. *Bioanalysis* 10(22), 1781–1801 (2018).
•• **Industry white paper which contains a section on bioanalysis strategy and technology considerations for ASO drugs and provides a wider context for the work described here.**
10. Ewles M, Goodwin L, Schneider A, Rothhammer-Hampl T. Quantification of oligonucleotides by LC–MS/MS: the challenges of quantifying a phosphorothioate oligonucleotide and multiple metabolites. *Bioanalysis* 6(4), 447–464 (2014).
11. Wei X, Dai G, Marcucci G *et al.* A specific picomolar hybridization-based ELISA assay for the determination of phosphorothioate oligonucleotides in plasma and cellular matrices. *Pharm. Res.* 23(6), 1251–1264 (2006).
•• **Describes the use of a nuclease-protected hybridization ELISA assay for quantifying ASOs in biological samples. It provided the framework for the evaluation of this method for our research work.**
12. Tsongalis GJ. Branched DNA technology in molecular diagnostics. *Am. J. Clin. Pathol.* 126(3), 448–453 (2006).
•• **Describes the history of bDNA technology and provides additional context for the use of the bDNA assay for our study.**
13. Coles AH, Osborn MF, Alterman JF *et al.* A high-throughput method for direct detection of therapeutic oligonucleotide-induced gene silencing *in vivo*. *Nucleic Acid Ther.* 26(2), 86–92 (2016).
•• **Describes a bDNA assay for quantifying mRNA expression from tissue biopsies. The adaptation of the bDNA platform for ASO quantification is a highlight of our current work.**
14. ClinicalTrials.gov Identifier: NCT04269031. A First-in-human Study to Assess the Safety, Tolerability, Pharmacokinetics and Pharmacodynamics of AZD2373 After Single Dose Administration in Healthy Male Subjects of African Ancestry. https://clinicaltrials.gov/ct2/show/NCT04269031
15. Hughes NC, Bajaj N, Fan J, Wong EY. Assessing the matrix effects of hemolyzed samples in bioanalysis. *Bioanalysis* 1(6), 1057–1066 (2009).
16. Nikolac N. Lipemia: causes interference mechanisms, detection and management. *Biochem. Med. (Zagreb)* 24(1), 57–67 (2014).
17. Tillman LG, Geary RS, Hardee GE. Oral delivery of antisense oligonucleotides in man. *J. Pharm. Sci.* 97(1), 225–236 (2008).
18. Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv Drug Deliv Rev.* 87, 46–51 (2015).
19. US FDA.Bioanalytical Method Validation Guidance for Industry (2018). https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry (
20. Akazawa-Ogawa Y, Nagai H, Hagihara Y. Heat denaturation of the antibody, a multi-domain protein. *Biophys. Rev.* 10(2), 255–258 (2018).