Method Development and Evaluation of the Techniques for Oligonucleotide Concentration Determination

Robert J. Duff, Chris Gutierrez, Michael Schneider and Jose G. Ramirez
Amgen Inc., 1 Amgen Center Drive, Thousand Oaks, CA 91320

ABSTRACT

Oligonucleotide-based therapeutics are emerging as promising modality against a variety of diseases. The determination of oligonucleotide concentration is a critical quality attribute as it directly relates to the patient's safety and efficacy. A typical dose may require a dosing concentration of > 150 mg/mL. Traditionally, concentration determinations have been assessed through a fixed path length instrument. Due to limitations of the spectrophotometer with these high absorbance products, sample concentrations in this range must involve a time-consuming dilution step. However, alternate technologies exist for high concentration samples which require no sample dilution, such as the NanoDrop™ and SoloVPE instruments using Slope Technology™.

A study was performed to 1) compare the range of oligonucleotide concentrations using the SoloVPE and NanoDrop™ instruments including the evaluation of multiple wavelengths and 2) evaluate the precision and accuracy of both instruments to a standard. The study encompassed an oligonucleotide concentration range up to ~200 mg/mL. Extinction coefficients of the target oligonucleotide at multiple wavelengths were determined. With the optimal wavelength determined, high concentration determinations were possible. Accuracy was assessed via a direct comparison between the instruments. Statistical analysis evaluated the equivalency of the two instruments

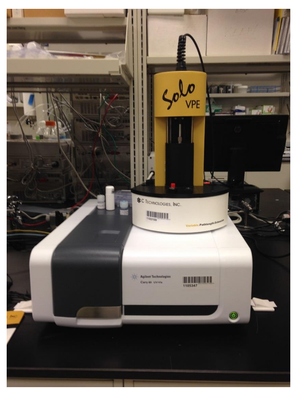
MATERIALS AND METHODS

Oligonucleotide was synthesized using standard techniques on an automated oligonucleotide synthesizer and purified to 97% by ion pairing reversed phase chromatography. Extinction coefficient was experimentally determined. Units were converted to $(mg/mL)cm^{-1}$ from $M^{-1}cm^{-1}$.

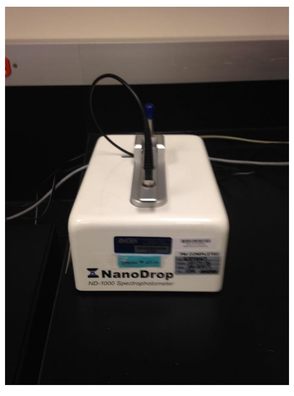
A Beckman DU800 was used to measure and establish the concentration of the stock solution of the oligonucleotide, measured at 2.944 mg/mL at 260 nm in phosphate-buffered saline at pH 7.4. This solution was used for all testing with the SoloVPE and NanoDrop instruments.

Statistical assessment was accomplished using SAS software.

SoloVPE with Agilent Cary 60 Spectrophotometer



NanoDrop



Method development centered on the use of the instruments with oligonucleotide concentrations ranging from 3 mg/mL to 200 mg/mL. Extinction coefficients are proportional through Beer's law to concentration and absorbance as given by Equation 1 [Ref 1].

Equation 1

$$A = \varepsilon bc$$

Where A is Absorbance;
ε is the extinction coefficient at single wavelength,
b is path length
c is concentration

Practical consideration of the optical range of the spectrophotometers was of a chief concern. For the spectrophotometers, the upper absorbances deviate from Beer's law at 1.5 – 2.0 AU as dependent on the instrument. If the absorbance is above this practical limit, the determination is affected by giving a lower than actual concentration.

RESULTS AND DISCUSSION

A study was performed to 1) compare the range of oligonucleotide concentrations using the SoloVPE and NanoDrop™ instruments including the evaluation of multiple wavelengths and 2) evaluate the precision and accuracy of both instruments to a standard. Alternately, an off-maxima wavelength was chosen with sufficient sensitivity and tested for reliability of measurement.

1) Comparison of Instruments over a Range of Oligonucleotide Concentrations

Using a representative solution of the oligonucleotide (concentration in the linear range of Beer's Law), Figure 1 shows the wavelengths of measured absorbances. Absorbances and calculated extinction coefficients for that sample are reported in Table 1. Dilution steps can be time-consuming and introduce additional error to the determination through pipetting.

Off-maxima extinction coefficients were calculated according to Equation 2 (subscripts denote terms of different wavelengths). The absorbances at off-maxima wavelengths as indicated in the figure were used for this calculation. Concentration and path length are constants as the absorbance is measured at two wavelengths with the same sample.

Figure 1. Ultraviolet Spectrum of the Oligonucleotide at 3 mg/mL.

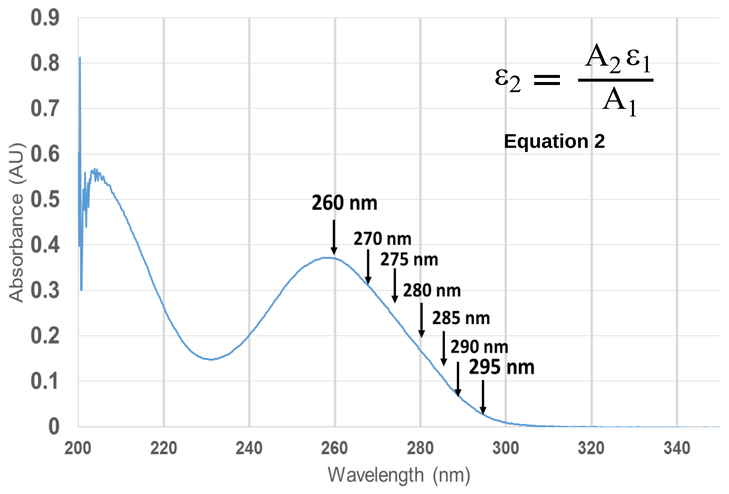


Table 1: Extinction Coefficients at Multiple Wavelengths

	Abs @ 260.0	Abs @ 270.0	Abs @ 275.0	Abs @ 280.0	Abs @ 285.0	Abs @ 290.0	Abs @ 295.0
Absorbance	0.3702	0.2865	0.2285	0.1677	0.1107	0.0583	0.0254
Extinction Coefficient (mg/mL) cm^{-1}	22.25	16.95	13.46	9.88	6.61	3.61	1.46

Off-maxima wavelength at 295 nm was deemed suitable for testing precision and accuracy of both instruments. Deviations of absorbance that could impact at this wavelength varied minimally as compared to 270 – 290 nm and expected to minimally affect Beer's Law. It is expected that deviations from Beer's Law at this wavelength will be mostly related to instrument capability. Extinction coefficient is reduced by a factor of 15 by using the off-maxima wavelength.

Suitability of the instruments for high concentrations was assessed over a range as based on maximum absorbance of the instrument (~2 AU) (Table 2).

Table 2: Instrument Readout at Multiple Wavelengths

	SoloVPE		NanoDrop	
Concentration	260 nm	295 nm	260 nm	295 nm
3 mg/mL	< 2 AU	< 2 AU	< 2 AU	< 2 AU
60 mg/mL	Above 2 AU	< 2 AU	Above 2 AU	Above 2 AU
200 mg/mL	Above 2 AU	~2 AU[1]	Above 2 AU	Above 2 AU

[1] Upper range of spectrophotometer, SoloVPE – linearity of absorbance versus path length passed manufacturer criteria for sample acceptance (R^2 >0.999), 10 data points were used [Ref 2]. Oligonucleotide concentration measured at 196.25 mg/mL.

- Both instruments were able to determine the oligonucleotide concentration without dilution for the 3 mg/mL sample at 260 nm and 295 nm.
- NanoDrop instrument required dilution to be under the 2 AU spectroscopic limit for 60 and 200 mg/mL samples. The NanoDrop was not able of reading higher concentrations (>3 mg/mL) at the 260 nm or 295 nm wavelengths.
- SoloVPE was capable of measuring the highest concentration by employing the smallest path lengths (5 μm to 50 μm). Cary 60 exceeded the 2 AU limit when testing the 200 mg/mL sample, but provided valid result (R^2 > 0.999) with 10 data points [Ref 2].

SoloVPE provides a robust technology for oligonucleotide concentration determination to 200 mg/mL

2) Evaluation of Precision and Accuracy for Oligonucleotide Concentration

The equivalency assessment focused on the use of 3mg/mL sample concentration and followed the strategy outlined in Figure 2. The data is listed in Table 2.

Figure 2. Testing Strategy

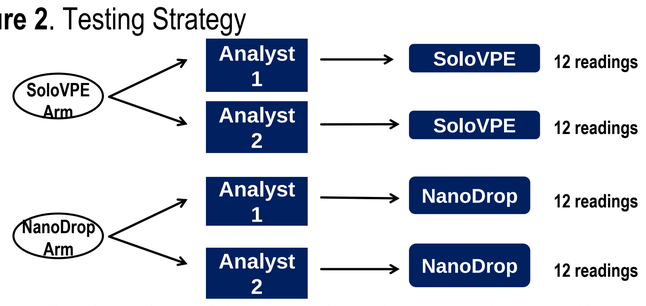


Table 2. Raw Data, Standard Deviation and Relative Standard Deviation for Oligonucleotide Concentration Determinations

	SoloVPE (Conc (mg/mL))				NanoDrop (Conc (mg/mL))			
	Analyst 1		Analyst 2		Analyst 1	Analyst 2	Analyst 1	Analyst 2
	260 nm	295 nm	260 nm	295 nm	260 nm	260 nm	295 nm	295 nm
1	2.870	3.078	2.883	3.121	N/A	N/A	3.075	3.151
2	2.882	3.120	2.845	3.140	N/A	N/A	3.116	3.137
3	2.896	3.114	2.860	3.116	N/A	N/A	3.130	3.144
4	2.856	3.135	2.849	3.137	N/A	N/A	3.082	3.151
5	2.869	3.132	2.900	3.122	N/A	N/A	3.103	3.144
6	2.859	3.204	2.874	3.138	N/A	N/A	3.089	3.096
7	2.887	3.097	2.937	3.141	N/A	N/A	3.164	3.171
8	2.873	3.110	2.846	3.126	N/A	N/A	3.116	3.130
9	2.869	3.075	2.896	3.125	N/A	N/A	3.199	3.130
10	2.845	3.139	2.856	3.121	N/A	N/A	3.123	3.123
11	2.862	3.127	2.922	3.137	N/A	N/A	3.110	3.130
12	2.873	3.118	2.879	3.132	N/A	N/A	3.075	3.144
Average	2.870	3.121	2.879	3.130	N/A	N/A	3.115	3.138
StDev	0.014	0.033	0.030	0.009	N/A	N/A	0.037	0.018
%RSD	0.485	1.071	1.048	0.281	N/A	N/A	1.178	0.585

Figure 3 is a box plot showing the means and the ranges of the analyses. Analyst 2 gives slightly higher average readings with less variation (shorter boxes) than Analyst 1, but both were within 2% RSD and 2% of Target (Figure 4). Lower concentration readings for the SoloVPE at 260 nm could possibly be due to instrument related Beer's Law deviations (i.e. spectrophotometer limitations).

Figure 3. Direct Comparison of Instrument Performance at 295 nm

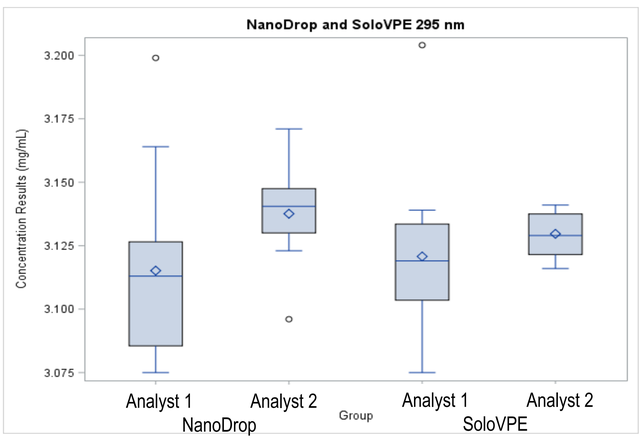
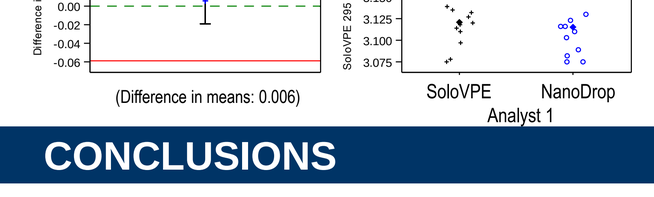


Figure 4 show the equivalence acceptance criteria (± 0.059 AU; red lines) within 2% of Target for both the SoloVPE and NanoDrop. Instruments are equivalent at 3 mg/mL.

Figure 4. Equivalence Plots



CONCLUSIONS

- **UV-based concentration determinations for highly concentrated oligonucleotide solutions using the SoloVPE and NanoDrop™ can be performed off-maxima with adequate precision and accuracy**
- **Using a sample concentration of 3mg/mL oligonucleotide, SoloVPE and NanoDrop™ results were equivalent at 295nm with <2%RSD and within 2% of target value.**
- **In contrast to NanoDrop™, the SoloVPE functioned at all concentrations of oligonucleotide, allowing for a streamlined analysis of highly concentrated samples.**

REFERENCES

1. Analytical Chemistry: An Introduction (Saunders Golden Sunburst Series) 7th Ed., by Douglas A. Skoog, Donald M. West, F. James Holler. **1999**.
2. Huffman, S., Soni, K., & Ferraiolo, J. (2014). UV-Vis Based Determination of Protein Concentration. *BioProcess International*, *12*, 8.

ACKNOWLEDGEMENTS

Han Ke
Sekhar Kanapuram
Tiffany Thiel
Shekar Ganesa