Application note

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Measuring modified mRNA concentration for therapeutics

NanoDrop Spectrophotometers

Introduction

Advances in infectious disease vaccines and cancer therapeutics are shifting focus to incorporating mRNA because it is non-integrating and does not require nuclear localization.[1-2] By nature, mRNA is functionally difficult for many applications because RNA is less stable than DNA due to the presence of the hydroxyl group on the 2' carbon of the ribose sugar (Figure 1).

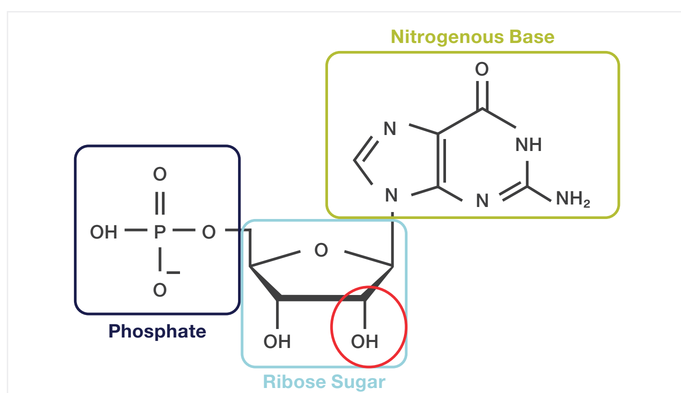

Figure 1. RNA chemical structure. The presence of the 2' hydroxyl group (circled in red) makes RNA less stable than DNA. Image created with BioRender.com.

For use in therapeutics, mRNA must be translated with high efficiency to produce enough protein to elicit the desired response.[3,4] To improve the stability and translation efficiency of mRNA for use in therapeutics, the 5' cap, 3' poly A tail, untranslated region (UTR), or the translated region (coding sequence) can be manipulated and modified (Figure 2).[1,3-5]

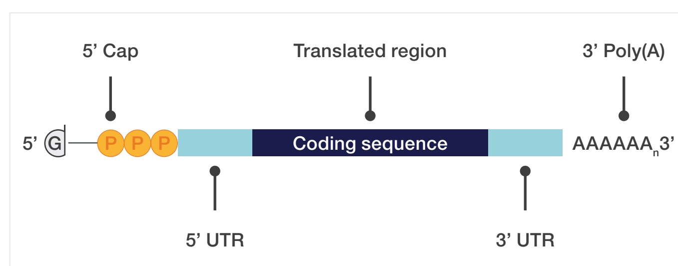

Figure 2. Schematic of mRNA highlighting modifiable components. Image created with BioRender.com.

Modifications to the 5' cap include utilizing anti-reverse cap analogs (ARCAs) and replacing oxygen in the phosphate bridge with sulfur to confer resistance to decapping enzymes and improve binding to translation initiation factors, respectively.[1,4] Poly(A) tails that contain modified adenosines such as 8-azaadenosine reduces the risk of deadenylation and degradation.[1] Human α- and β-globin mRNAs contain regions in the 3' UTR sequences that can be incorporated into the modified mRNA to increase stability.[4] For the translated region, nucleoside chemical modifications, notably pseudouridine for uridine and 5-methyl-cytosine for cytosine, change the mRNA secondary structure to evade the innate immune response and increase translation efficiency.[5,6] Finally, methylation of the 2' hydroxyl group on the ribose sugar (2'-OMe) is one of the most common modifications to improve stability of the RNA molecule.[1]

After the mRNA has been sufficiently modified, the mRNA can be quantified for application in downstream therapies.

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Sequence	Modification	Extinction Coefficient ($L \cdot mole^{-1} \cdot cm^{-1}$)	Molecular Weight (g/mol)	Concentration Factor (µg/mL)
rGrUrC rCrCrC rCrUrU rGrCrC rGrUrC rCrCrA rArGrC rA	None	194900	6888.2	35.34
rGmUrC mCrCmC rCmUrU mGrCmC rGmUrC mCrCmA rAmGrC mA	Alternating methylation	194900	7042.5	36.13
rGrU/iMe-dC/ /iMe-dC//iMe-dC//iMe-dC/ / iMe-dC/rUrU rG/iMe-dC//iMe-dC/ rGrU/iMe-dC/ /iMe-dC//iMe-dC/rA rArG/iMe-dC/ rA	5-methyl-cytosine	175800	6866.5	39.06
rG/ipsU/rC rCrCrC rC/ipsU//ipsU/ rGrCrC rG/ipsU/rC rCrCrA rArGrC rA	Pseudouridine	194900	6888.2	35.34

Table 1. Extinction coefficients, molecular weights, and concentration factors of RNA oligos with or without modifications. Calculated using Integrated DNA Technologies' OligoAnalyzer.

Beer-Lambert Law and extinction coefficients

Quantifying mRNA can be quickly performed with UV-Vis spectrophotometry. The Beer-Lambert Law (Beer's Law) describes the following relationship between absorbance and concentration:

$$A = \varepsilon b c \quad \text{or} \quad c = \frac{A}{\varepsilon b}$$

Equation 1.

Through rearranging Beer's Law, concentration (c) is determined by dividing the absorbance (A) by the pathlength (b) and the sample-specific extinction coefficient (ε). The extinction coefficient is a measure of how strongly a sample absorbs light; for RNA, the mass extinction coefficient is 0.025 $(ng/\mu L)^{-1}$ cm^{-1} at 260 nm. The pathlength is the length the light travels through the sample; traditional spectrophotometers typically use a 1.0 cm pathlength cuvette but microvolume spectrophotometers implement a range of pathlengths to accommodate higher sample concentrations. Based on Beer's Law, one absorbance unit at 260 nm is equal to an RNA concentration of 40 ng/µL when measured in a 1.0 cm cuvette.

Nucleic acid absorbance is measured at 260 nm due to the intrinsic aromaticity of the purine and pyrimidine nitrogenous bases (see Figure 1). Most changes to the base chemistry will affect the extinction coefficient and molecular weight of RNA, rendering the 0.025 $(ng/\mu L)^{-1}$ cm^{-1} mass extinction coefficient incorrect for the sample type. For short oligonucleotides (< 80 nucleotides), the extinction coefficient will be highly sequence dependent. To determine the correct extinction coefficient for a base-modified RNA oligonucleotide, Integrated DNA Technologies' OligoAnalyzer tool calculates the molar extinction coefficient in $L \cdot mole^{-1} \cdot cm^{-1}$ and determines the molecular weight of an oligo that contains modifications. Table 1 outlines the calculated extinction coefficient, molecular weight, and concentration factor specific to the mRNA sequence and modifications. The concentration factor, which is used for application to UV-Vis spectrophotometry, is calculated as shown:

$$\text{Concentration Factor } \mu g/mL = \frac{\text{Molecular Weight} \left(\frac{g}{mol}\right) * 1000}{\text{Extinction coefficient } (M^{-1}\ cm^{-1})}$$

Equation 2.

The factor (f) can then be applied to Beer's Law in the following formula:

$$c = \frac{Af}{b}$$

Equation 3.

The extinction coefficient for alternating methylation remains the same as the control because the modification is on the 2' carbon of the ribose sugar, not the nitrogenous base. However, the molecular weight does change because the 2'-OH group was replaced with a 2'-O-CH_3 group (Figure 3A), thus the concentration factor increased. For 5-methyl-cytosine, the nitrogenous base is modified with an additional methyl (CH_3) group (Figure 3B), which changes the molar extinction coefficient. The pseudouridine modification does not affect the extinction coefficient or molecular weight because the nitrogen joining the nitrogenous base with the 1' carbon on the ribose sugar exchanges locations with the carbon originally located at the 5' position in uridine, yielding a C-C bond with the sugar and nitrogenous base (Figure 3C).[7]

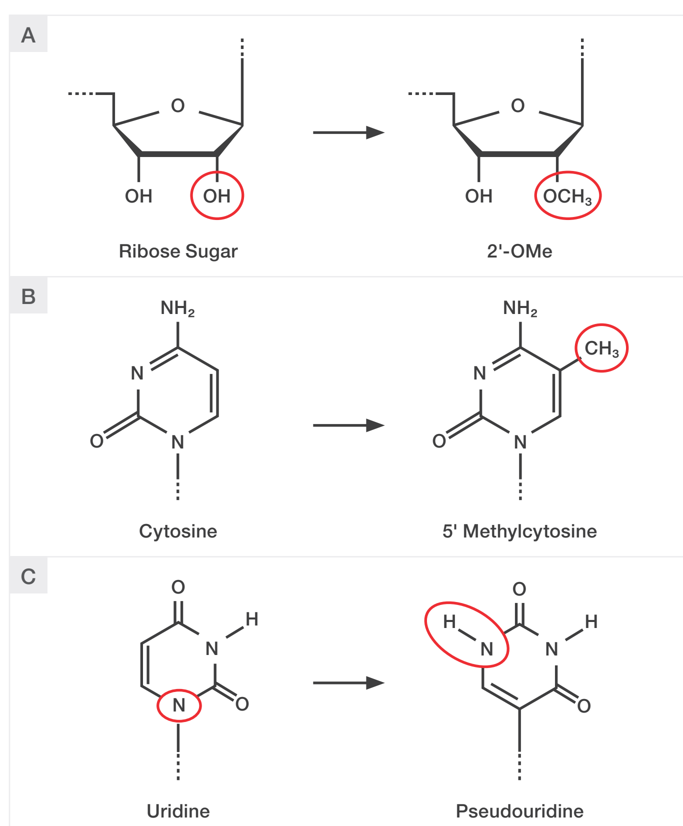

Figure 3. Chemical structure modifications for A) 2'-OMe, B) 5-methyl-cytosine, and C) pseudouridine. Image created with BioRender.com.

Measuring modified RNA with the NanoDrop Spectrophotometers

The Thermo Scientific™ NanoDrop™ One/One^C and NanoDrop Eight Microvolume UV-Vis Spectrophotometers allow for quick nucleic acid measurements using only 1-2 µL sample volumes. The NanoDrop software is fitted with a Custom Factor application, making the process of measuring the concentration of modified mRNA only a few steps. In Figure 4 from the NanoDrop One/One^C software, the concentration factor that is specific to the modified oligonucleotide calculated in Table 1 can be entered to obtain an absorbance and concentration result.

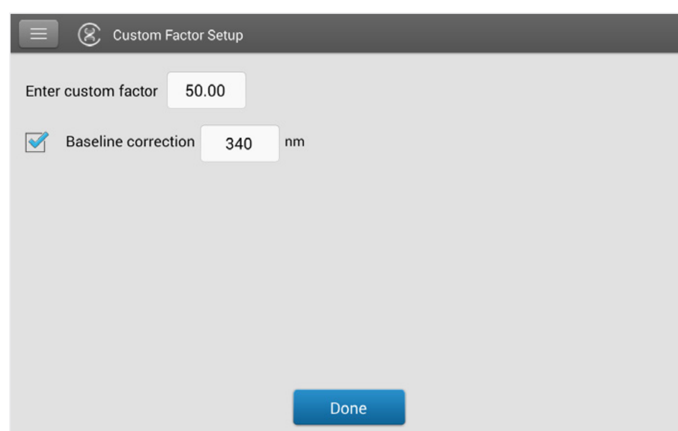

Figure 4. Custom Factor setup screen on the NanoDrop One/One^C instrument (local control) software.

Unmodified oligonucleotides can be measured using the Oligo DNA or Oligo RNA applications. The sequence can be typed into the software and the sequence-specific extinction coefficient and concentration factor will be automatically calculated. This eliminates the need for an online calculator to determine the proper extinction coefficient.

When measuring nucleic acid samples with UV-Vis spectrophotometry, the purity ratios, A260/A280 and A260/A230, provide general guidelines for a purity assessment. However, purity ratios are not applicable to oligonucleotides because the sequence varies the shape of the UV spectrum.[8] As an alternative, oligonucleotide purity is often checked via auxiliary techniques such as mass spectrometry or capillary electrophoresis.[9-10]

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Conclusions

The rising popularity of mRNA-based therapeutic research has led to improvements in the molecular stability of RNA through a wide array of modifications. Since some modifications to the aromatic rings of RNA change the specific extinction coefficient or molecular weight, the concentration factor "40" most commonly used for RNA is not always applicable. It is crucial to utilize the correct extinction coefficient for measuring concentration with UV-Vis spectrophotometry to ensure accurate and reproducible data. Online extinction coefficient and molecular weight calculators, such as that provided by Integrated DNA Technologies, are helpful tools for calculating the correct concentration factor. The Custom Factor application built into the NanoDrop One/One^{C} and NanoDrop Eight software performs quick and sensitive measurements of modified mRNA when the specific extinction coefficient is known.

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