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# Accurate Quantification of Nucleic Acids Using Hypochromicity Measurements in Conjunction with UV Spectrophotometry

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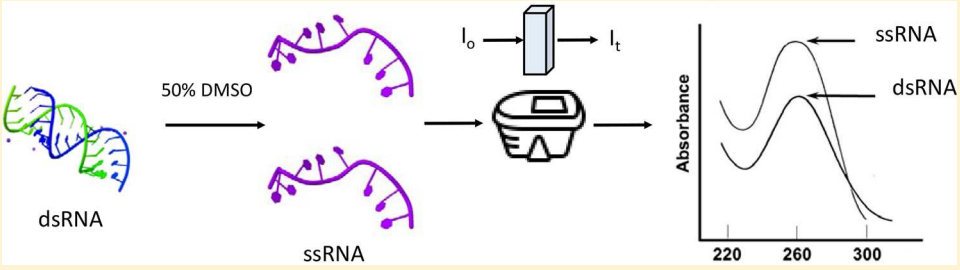
Supporting Information




**ABSTRACT:** UV absorbance spectrophotometry is widely used for the quantification of nucleic acids. For accurate quantification, it is important to determine the hypochromicity of the oligonucleotide or complex nucleic acid structure. The use of thermal denaturation studies in conjunction with UV spectrophotometry to determine hypochromicity requires prolonged, elevated temperatures, which may cause partial hydrolysis of RNA. In addition, dsRNA is difficult to denature even at elevated temperature, and the extinction coefficients of nucleic acids are also affected by temperature, which makes it difficult to accurately determine the nucleic acid concentration. To overcome these caveats, we have utilized the chemical denaturant dimethyl sulfoxide which, in conjunction with a short thermal denaturation, prevents renaturation of the duplex nucleic acids (dsDNA/RNA). Using this approach, we have measured the absorbance of both the unstructured and structured nucleic acids to accurately measure their hypochromicity and determine their extinction coefficients. For a range of different dsRNA, we have for the first time determined values of 46.18−47.29 $\mu$g/mL/$A_{260}$ for the quantification of dsRNA using UV spectrophotometry. Moreover, this approach enables the accurate determination of the relative proportion of duplex nucleic acids in mixed ds/ss nucleic acid solutions, demonstrating significant advantages over current methods.

UV absorbance spectrophotometry remains one of the most popular methods for the rapid quantification of nucleic acids.[1−3] The absorbance of DNA and RNA at 260 nm is measured and the concentration determined using the Beer−Lambert law ($A = \varepsilon \cdot C \cdot l$) in conjunction with the molar extinction coefficient ($\varepsilon$) of constituent nucleotides. However, inaccuracies in the extinction coefficients can reduce the accuracy of results obtained.[4,5] Three common methods are currently used to calculate nucleic acid mass concentration/$A_{260\ nm}$. The first method ignores base composition and assumes that the average molar mass and extinction coefficient of nucleotides is 330 g/mol and 10 $mmol^{-1}$ $cm^{-1}$, respectively.[6,7] For an absorbance ($A_{260\ nm}$) of 1, a concentration of 33 $\mu$g/mL is obtained for single-stranded (ss) oligonucleotide using the Beer−Lambert equation.[4,6] The second method assumes that $\varepsilon$ is the sum of nucleotide extinction coefficients weighted by the number of times each base appears in the sequence.[6] Both methods do not account for potential hypochromicity in the oligonucleotide or complex nucleic acid structures. For accurate quantification, it is important to determine the hypochromicity of the oligonucleotide or complex nucleic acid structure. The extinction coefficient of double-stranded nucleic acids is less than the sum of the extinction coefficients of the corresponding two single-stranded nucleic acids.[8] This hypochromic effect of nucleotides is attributed to dipole induced dipole interactions resulting from base stacking.[9] In addition to using the sum of the nucleotide extinction coefficients for a given sequence a factor of 0.9 is often applied to take into account base-stacking interactions in single-stranded nucleic acids.[5] The third method described as the near-neighbor calculation attempts to account for hypochromicity.[5] It has been reported to yield extinction coefficients within 20% of the experimentally measured extinction coefficients.[4,6]

Hypochromicity measurements can be made by comparing the absorbance of the nondenatured and denatured nucleic acid and determining the melting profile using UV spectrophotometry.[10,11] However, at high temperatures, partial hydrolysis of RNA may occur, whereas at moderate temperatures, complete denaturation is not guaranteed. Furthermore, very high temperatures are required to denature large dsRNA, and

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the extinction coefficients of nucleic acids are affected by temperature, which makes it difficult to accurately determine the RNA concentration.[12] Alternatively, the hypochromicity and concentration of the RNA or DNA can be measured on the basis of the absorbance of the intact molecule with their corresponding nucleosides generated from hydrolysis or enzymatic reactions. Treatment with 0.3 M NaOH at 37 °C or nucleases can be used to achieve nucleic acid hydrolysis.[6,13] The limitation in the NaOH hydrolysis method is that deamination of C to U can potentially occur.[4] Hydrolyses of nucleic acids at neutral pH have been reported; however, it requires long incubation at 95 °C.[14] Moreover, potential incomplete hydrolysis of complex and modified nucleic acid structures may result in inaccurate quantification.[6]

A previous study using NMR to accurately determine extinction coefficient yielded more accurate coefficients for nucleotide monophosphates.[4] The same study looked at the deviations between predicted and measured extinction coefficient and recommended mass concentration/$A_{260\ nm}$ coefficients of 37 and 38 $\mu$g/mL/$A_{260\ nm}$ for calculation of ssDNA and ssRNA concentrations, respectively. To our knowledge, there is no consensus accurate value for mass concentration/$A_{260}$ unit ($\mu$g/mL) of long dsRNA. However, values of 40 $\mu$g/mL/$A_{260}$ and 50 $\mu$g/mL/$A_{260}$, are commonly used for RNA and dsDNA, respectively.[2] Moreover, there is no current method that accurately determines and validates the extinction coefficients/hypochromicity of dsDNA, dsRNA and mixed ds/ss-nucleic acids.[4]

In this study, we have developed a high-throughput method for the accurate quantification of nucleic acids using UV spectrophotometry in conjunction with hypochromicity measurements. In addition, our method enables the accurate quantification of duplex nucleic acids in mixed ds/ss nucleic acid solutions demonstrating significant advantages over current methods.

## EXPERIMENTAL SECTION

**Chemicals and Materials.** Genes were synthesized by GeneArtGene Synthesis (Invitrogen Life Technologies). Ampicillin sodium salt, tetracycline hydrochloride, isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) ≥ 99%, sodium dodecyl sulfate (SDS) sodium chloride (NaCl), dimethyl sulfoxide (DMSO), RNA from baker's yeast (*S. cerevisiae*) and phenylalanine specific tRNA from brewer's yeast were all obtained from (Sigma-Aldrich, Poole, U.K.). TRIzol Max, isopropanol, and ethanol (ThermoFisher Scientific, NJ, U.S.A.), were used for nucleic acid purifications. Oligonucleotides were purchased from Eurofins Genomics, Ebersberg, Germany, and both tRNAs were from Sigma-Aldrich, Poole, U.K. Nucleic acids used in this study are shown in Table 1.

**Expression of dsRNA Using *E. coli* HT115 (DE3).** The *E. coli* strain, HT115 (DE3)[15] was obtained from Cold Spring Harbor Laboratory, NY, U.S.A. Plasmids pCOIV and pDome11 that contain in-house designed 686 bp and 481 bp sequences, respectively, flanked on both sides with T7 promoters were ordered from Gene Art Gene synthesis (Invitrogen). The *E. coli* HT115 (DE3) cells were transformed with either pCOIV or pDome11. The pCOIV and pDome11 transformed cells were grown in culture and induced with IPTG to express dsRNAs as previously described.[16]

**Analysis of Purified dsRNA and Total RNA.** RNA quantification was determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific). RNA concentrations were determined by absorbance at 260 nm. Absorbance data using a NanoDrop 2000c spectrophotometer were normalized to a 1.0 cm (10.0 mm) path. The $A_{260/280}$ and $A_{260/230}$ ratios were obtained using the NanoDrop instrument. Additional analysis of the RNA was performed using ion-pair reverse-phase chromatography using a 10 $\mu$L injection from 100 $\mu$L of eluted/resuspended RNA.

**Table 1. Nucleic Acids Used in This Study**

| nucleic acid | sequence/composition |
|---|---|
| NTPs | equimolar ATP/CTP/GTP/UTP |
| dNTPs | equimolar dATP/dCTP/dGTP/dTTP |
| 15mer RNA oligo | CAAAAGUCCGUGAGA (A:6; C:3; G: 4; U: 2) |
| 13mer DNA oligo | AGCTAGCTAGCTA (dA:4; dC:3; dG: 4; T: 2) |
| ssRNA (521 ntr) | (A:131; C:144; G102; U:144) |
| dsRNA (521 bp) | (A:275; C:246; G:246; U:275) |
| dsDNA (518 bp) | (dA:275; dC:243; dG:243; T:275) |
| dsRNA (686 bp) | (A:261; C:425; G:425; U:261) |
| dsRNA (481 bp) | (A:190; C:291; G:291; U:190) |
| ssRNA (600 ntr)* | (A:150; C;150; G150; U:150) |
| ssRNA (550 ntr)* | (A:150; C:100; G150; U:150) |
| ssDNA (600 ntr)* | (dA:150; dC;150; dG150; T:150) |
| ssDNA (550)* | (dA:150; dC;100; dG150; T:150) |

*theoretical sequences

**Ion-Pair Reverse-Phase High-Performance Liquid Chromatography (IP-RP HPLC).** Samples were analyzed by IP-RP-HPLC on an Agilent 1100 series HPLC using a Proswift RP-1S Monolith column (50 mm × 4.6 mm I.D. ThermoFisher). Chromatograms were generated using UV detection at a wavelength of 260 nm. The chromatographic analysis was performed using the following conditions: Buffer A 0.1 M triethylammonium acetate (TEAA) pH 7.0 (Fluka, UK); Buffer B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile (ThermoFisher). RNA was analyzed using the following gradients. Gradient (1) starting at 22% buffer B to 27% in 2 min, followed by a linear extension to 62% buffer B over 15 min, then extended to 73% buffer B over 2.5 min at a flow rate of 1.0 mL/min at 50 °C. Gradient (2) starting at 22% buffer B to 27% in 2 min, followed by a linear extension to 72% buffer B over 15 min, then extended to 73% buffer B over 2.5 min, at a flow rate of 1.0 mL/min at 50 °C.

**Determination of Nucleic Acid Hypochromicity.** Nucleic acids synthesized in vitro and in bacteria were purified using RNASwift as previously described[17] in order to remove contaminating proteins and salts. The nucleic acid samples ($A_{260\ nm}$ = 5−6) were mixed with DMSO (to 50% DMSO final concentration). Samples were heated at 95 °C for 1 min. Identical samples mixed with DMSO were also prepared but without heating. Identical control samples mixed with an equal volume of nuclease-free water were also prepared (no heating). Triplicate biological replicates were performed for all samples analyzed. Absorbance measurements were as follows.

$A_0$ = absorbance $A_{260}$ of nucleic acid in $H_2O$

$A_1$ = absorbance $A_{260}$ of nucleic acid in DMSO

$A_2$ = absorbance $A_{260}$of nucleic acid in DMSO + heat

$$\text{hypochromicity factor } (H) = A_2/A_0 \quad (1)$$

$$A_2/A_1 = (A_{260}\text{DMSO + heat})/(A_{260}\text{DMSO}) \quad (2)$$



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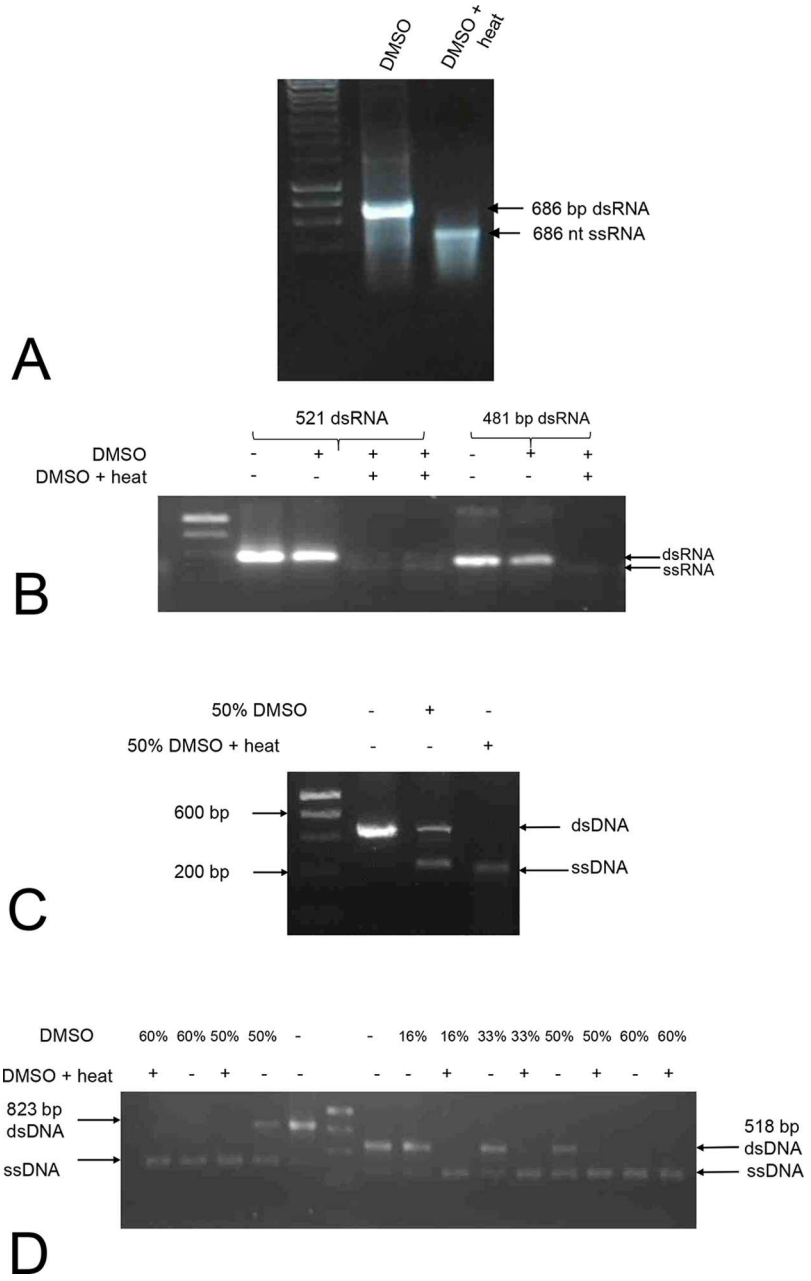


**Figure 1.** Agarose gel electrophoresis analysis of the effects of DMSO on dsRNA (A) 50% DMSO was added to purified dsRNA (686 bp). Thermal denaturation was performed at 95 °C for 1 min. (B) 50% DMSO was added to purified dsRNA (481 bp and 518 bp) dsRNA with and without additional thermal denaturation at 95 °C for 1 min. (C) Agarose gel electrophoresis analysis of the effects of 50% DMSO on dsDNA (518 bp). (D) A range of DMSO concentrations were added to dsDNA (518 bp and 823 bp) with and without additional thermal denaturation at 95 °C for 1 min.

$$A_1/A_0 = (A_{260}\mathrm{DMSO})/(A_{260}\mathrm{H_2O}) \quad (3)$$

The nucleic acid extinction coefficient ($\varepsilon$) was determined from the sum of the individual nucleotide extinction coefficients as determined by.[4]

$$\varepsilon_{\mathrm{sum}} = n_A\varepsilon_A + n_G\varepsilon_G + n_C\varepsilon_C + n_U\varepsilon_U \text{ or } n_T\varepsilon_T \quad (4)$$

where $n_N$= number of nucleotides, N, in the nucleic acid and then corrected for the hypochromicity using

$$\varepsilon_{\mathrm{nucleic\ acid}} = (1/H)^*\varepsilon_{\mathrm{sum}} \quad (5)$$

The concentration was determined using the Beer−Lambert equation using the predicted and corrected extinction coefficient in conjunction with the sum of the mononucleoside phosphates masses to determine the molecular mass of the nucleic acid.

**Propagation of Error.** Propagation of error in all experimental ratios was determined by first calculating the covariation of error and then the standard deviations. Standard deviations of the ratios shown were determined by propagation of the standard deviations of the absorbance values recorded in multiple measurements of aliquots of the same sample using UV spectrophotometry.[18]

$$\mathrm{SD}\ (a/b) = a/b\sqrt{((\mathrm{SD}_a/a)^2 + (\mathrm{SD}_b/b)^2)} \quad (6)$$

**Agarose Gel Electrophoresis.** Agarose gels (1%) were used for gel electrophoresis. RNA loading dye 2× (NEB) was added to RNA samples and loaded on the gel. TAE buffer (1×,



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40 mM Tris (pH 7.6), 20 mM acetic acid and 1 mM EDTA) was used to perform electrophoresis at 100 V for 45 min. The agarose gels were prestained with ethidium bromide, and images were obtained using a UV imaging system fitted with charge coupled device (CCD) camera (Biospectrum Multi-spectral Imaging System).

**Preparation of Model/Standard Curves.** An $A_2/A_1$ value of 1.105 would be obtained for an RNA sample containing 50% $A_{260\ nm}$ of dsRNA and 50% $A_{260\ nm}$ of ssRNA where 1.0 and 1.21 are the $A_2/A_1$ values for pure ssRNA and dsRNA, respectively (50%*1.21 + 50%*1.0)

A model equation is therefore proposed:

$$A_2/A_1 = 1.21(\%\mathrm{Abs}_{260}\ \text{of dsRNA}/100) + 1.0(1 - \%\mathrm{Abs}_{260}\ \text{of dsRNA}/100) \quad (7)$$

$$\text{or \%dsRNA Abs260} = (A_2/A_1 - 1)/0.0021 \quad (8)$$

By substituting a range of hypothetical $\%\mathrm{Abs}_{260}$ of dsRNA values in the equation, a range of hypothetical $A_2/A_1$ values are obtained. Using these hypothetical data and plotting $A_2/A_1$ values against $\%\mathrm{Abs}_{260}$ of dsRNA values, we generate a model linear calibration curve. Similarly, a standard curve is obtained by plotting $\%\mathrm{Abs}_{260}$ of dsRNA against experimentally derived $A_2/A_1$ values.

Concentration of dsRNA was determined using the %dsRNA $Abs_{260}$ as determined above in conjunction with the total $A_{260}$ to calculate the corresponding $A_{260}$ for dsRNA. Overall concentration was subsequently calculated using the value of 46.52 $\mu$g/mL/$A_{260}$:

$$\text{mass concentration} = \%\text{dsRNA Abs}_{260}/100 \times \text{total RNA } A_{260} \times 46.52\mu\text{g/mL}/A_{260} \quad (9)$$

## RESULTS AND DISCUSSION

**Validation of a Method Using DMSO and Heat To Measure the Hypochromicity of dsRNA.** High DMSO concentrations (>75%) have previously been shown to disrupt the structure and stability of RNA[10,19] and DNA,[20,21] consistent with disruption of base-stacking interactions and increased flexibility. In order to perform hypochromicity measurements using DMSO in conjunction with thermal denaturation, a range of dsRNAs synthesized, in either *E. coli* or by in vitro transcription, were purified using a combination of RNase T1 and solid-phase extraction to remove contaminating ssRNA and NTPs (see Figure S1). Following purification, the dsRNA (686 bp) was heated at 95 °C for 1 min in 50% DMSO and analyzed using agarose gel electrophoresis (see Figure 1A). The results show the effective denaturation of the duplex dsRNA into the corresponding ssRNA preventing reannealing of the dsRNA. In addition, decreased ethidium bromide fluorescence was observed in the corresponding ssRNA owing to reduced intercalation.

To further study the effects of DMSO, a range of dsRNA (521 bp and 481 bp) were incubated in the presence and absence of DMSO with and without heating (see Figure 1B). The results show a small relative reduction in the ethidium bromide fluorescence for dsRNA in the presence of DMSO without heating but no dissociation of strands (see Figure 1B). These results demonstrate that in the presence of 50% DMSO, it is not sufficient to disrupt the duplex structures. However, it destabilizes base-stacking interactions, resulting in reduced intercalation of the ethidium bromide and subsequent fluorescence.

In addition to denaturation of dsRNA, the same approach was used to denature dsDNA (518 bp). dsDNA was generated by PCR, purified using solid-phase extraction to remove ssDNA and dNTP impurities and analyzed using IP RP HPLC (see Figure S2). The dsDNA was added to 50% DMSO including a thermal denaturing step to denature the dsDNA to its corresponding ssDNAs prior to analysis using gel electrophoresis (see Figure 1C). The results reveal that, unlike dsRNA, a proportion of dsDNA dissociates into ssDNA upon addition of 50% DMSO in the absence of heat. However, in the presence of 50% DMSO and thermal denaturation, the dsDNA duplex completely dissociates into the corresponding ssDNA. These results highlight the difference in the stability of the dsDNA vs dsRNA duplex attributed to increased base-stacking interaction energy in the A-form conformation of dsRNA. We therefore used a range of DMSO concentrations to determine

**Table 2. Hypochromicity Measurements of ssRNA and dsRNA[a]**

| nucleic acid $A_{260}$ | $A_1/A_0$ | $A_2/A_0$ | $A_2/A_1$ |
|---|---|---|---|
| dsRNA (521 bps) | 1.24 ± 0.005 | 1.50 ± 0.007 | 1.21 ± 0.005 |
| dsRNA (481 bps) | 1.24 ± 0.004 | 1.48 ± 0.006 | 1.20 ± 0.005 |
| dsRNA (686 bps) | 1.21 ± 0.003 | 1.45 ± 0.004 | 1.21 ± 0.004 |
| | $A_1/A_0$ | $A_2/A_0$ | $A_2/A_1$ |
| ssRNA A (521 nt) | 1.22 ± 0.005 | 1.22 ± 0.006 | 1.00 ± 0.005 |
| ssRNA B (521 nt) | 1.22 ± 0.006 | 1.23 ± 0.006 | 1.00 ± 0.005 |
| ssRNA C (521 nt) | 1.22 ± 0.007 | 1.21 ± 0.008 | 1.01 ± 0.008 |
| average | 1.22 ± 0.010 | 1.22 ± 0.012 | 1.00 ± 0.011 |
| (dsRNA $A_2/A_0$)/(ssRNA $A_2/A_0$) | | 1.48/1.22 = 1.21 | |

[a] $A_{260}$ values were obtained in the absence of DMSO ($A_0$), the presence of DMSO ($A_1$), and DMSO + heat ($A_2$) to denature the dsRNA to their corresponding ssRNAs. The hypochromicity factor ($H$) was determined by calculating the ratio $A_2/A_0$. Absorbance values are shown as means ($n$ = 3) ± propagated SD.

**Table 3. Summary of the Ratios of the $A_{260}$ Measurements[a]**

| sample | $A_1/A_0$ | $A_2/A_0$ | $A_2/A_1$ |
|---|---|---|---|
| NTPs | 1.00 ± 0.002 | 1.00 ± 0.002 | 1.00 ± 0.002 |
| dNTPs | 1.00 ± 0.004 | 1.00 ± 0.005 | 1.00 ± 0.004 |
| ssRNA oligoribonucleotide (15 mer) | 1.21 ± 0.003 | 1.21 ± 0.007 | 0.99 ± 0.006 |
| ssDNA oligonucleotide (13 mer) | 1.21 ± 0.007 | 1.21 ± 0.005 | 1.00 ± 0.007 |
| ssRNA (521 nt) | 1.22 ± 0.010 | 1.22 ± 0.012 | 1.00 ± 0.011 |
| dsRNA (521 bp) | 1.24 ± 0.005 | 1.50 ± 0.007 | 1.21 ± 0.005 |
| dsRNA (481bp) | 1.24 ± 0.004 | 1.48 ± 0.006 | 1.20 ± 0.005 |
| dsRNA (686 bp) | 1.21 ± 0.003 | 1.45 ± 0.004 | 1.20 ± 0.004 |
| dsDNA (518 bp) | 1.40 ± 0.004 | 1.56 ± 0.002 | 1.11 ± 0.003 |
| phenylalanine tRNA (*S. cerevisae*) | 1.22 ± 0.003 | 1.22 ± 0.003 | 1.00 ± 0.003 |
| total tRNA (*S. cerevisae*) | 1.20 ± 0.003 | 1.21 ± 0.003 | 1.00 ± 0.001 |

[a] $A_{260}$ values were obtained in absence of DMSO ($A_0$) the presence of DMSO ($A_1$) and DMSO + heat ($A_2$) to denature the dsRNA to their corresponding ssRNAs. This enables an accurate $A_{260\ nm}$ measurement of both the unstructured denatured nucleic acids and the intact non-denatured dsRNA (see Table 2). The hypochromicity factor ($H$) was determined by calculating the ratio of $A_2/A_0$. Absorbance values are shown as means ($n$ = 3) ± propagated SD.



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Table 4. Determination of the Extinction Coefficient ($\varepsilon$) and Concentration of Nucleic Acids Using Hypochromicity Factors ($H$) in Conjunction with $A_{260}$ Measurements[a]

| nucleic acid | sequence | ($H$) | $\varepsilon$ ($mM^{-1}$ $cm^{-1}$) | $\varepsilon$* ($mM^{-1}$ $cm^{-1}$) | conc ($\mu g/mL/A_{260}$) | conc* ($\mu g/mL/A_{260}$) |
|---|---|---|---|---|---|---|
| ssRNA (600 nt) | (A:150;C:150; G:150;U:150) | 1.22 | 6574.50 | 5388.93 | 30.98 | 37.80 |
| ssRNA (550 nt) | (A:150;C:100; G:150;U:150) | 1.22 | 6221.00 | 5099.18 | 30.14 | 36.77 |
| ssDNA (600 nt) | (dA:150;dC;150; dG:150;T:150) | 1.21 | 6435.00 | 5318.18 | 29.504 | 35.70 |
| ssDNA (550 nt) | (dA:150;dC;150; dG:150;T:150) | 1.21 | 6080.00 | 5024.79 | 28.702 | 34.73 |
| ssRNA (15 nt) | CAAAAGUCCGUGAGA | 1.21 | 178.90 | 147.91 | 28.80 | 34.85 |
| ssDNA (13 nt) | AGCTAGCTAGCTA | 1.21 | 147.40 | 121.80 | 28.53 | 34.53 |
| ssRNA (521 nt) | (A:131; C:144; G102; U:144) | 1.22 | 5608.90 | 4597.46 | 31.36 | 38.27 |
| dsRNA (521 bp) | (A:275; C:246; G:246; U:275) | 1.50 | 11497.90 | 7665.27 | 30.78 | 46.18 |
| dsRNA (481 bp) | (A:190; C:291; G:291; U:190) | 1.48 | 10261.85 | 6933.68 | 31.95 | 47.29 |
| dsRNA (686 bp) | (A:261; C:425; G:425; U:261) | 1.45 | 14580.23 | 10055.33 | 32.08 | 46.52 |
| dsDNA (518 bp) | (dA:275; dC:243; dG:243; T:275) | 1.56 | 11180.54 | 7167.01 | 29.65 | 46.25 |

[a]The nucleic acid extinction coefficient ($\varepsilon$) was determined from the sum of the individual nucleotide extinction coefficients as determined in ref 4. The concentration was determined using the overall predicted extinction coefficient in conjunction with the sum of the mononucleoside phosphate masses to determine the molecular mass of the nucleic acid. *The calculated nucleic acid extinction coefficient and concentration determined using the hypochromicity factors ($H$).

the appropriate concentration to disrupt the base-stacking interactions without dissociating the duplex strands (see Figure 1D). The results show that using a lower % of DMSO (16%) reduced the amount of the dsDNA that was denatured but still caused strand separation upon heating.

**Quantitative Analysis of the Hypochromicity of dsRNA.** Following the successful demonstration that the addition of 50% DMSO with a short thermal denaturation step prevents reannealing of the dsRNA, we used this method to determine the dsRNA hypochromicity in conjunction with UV spectrophotometry. $A_{260}$ values were obtained in the absence of DMSO ($A_0$), the presence of DMSO ($A_1$), and DMSO + heat ($A_2$) to denature the dsRNA to their corresponding ssRNAs. This enables an accurate $A_{260}$ measurement of both the unstructured denatured nucleic acids and the intact nondenatured dsRNA (see Table 2). The hypochromicity factor ($H$) was determined by calculating the ratio of $A_2/A_0$. Values between 1.45 and 1.50 were obtained for a range of different dsRNA and 1.21 for ssRNA. The $A_1/A_0$ ratios observed for both ds and ssRNA were typically 1.22, demonstrating the reduction in single-stranded base-stacking interactions in ssRNA and a reduction in base-stacking interactions in the duplex dsRNA with the addition of DMSO. Furthermore, heating of the ssRNA in the presence of DMSO does not change the $A_{260}$ ratios, demonstrating that no further change in hypochromicity is observed. However, heating of the dsRNA in the presence of DMSO causes a further increase in absorbance as the duplex strands are denatured to their corresponding single strands.

**Quantitative Analysis of the Hypochromicity of Structured Yeast tRNAs.** A high degree of secondary structure in specific RNA molecules can lead to a significant hypochromicity that is not accounted for in the standard methods to calculate extinction coefficients using nearest-neighbor effects, which results in a systematic underestimation of RNA concentrations. In addition, previous data have demonstrated that the thermal denaturation of riboswitch RNAs is not a convenient or reliable method for determining the UV absorbance without the hypochromic effect from base-pairing interactions.[12,14] For RNAs with a high degree of secondary structure, thermal hydrolysis to the component mononucleoside phosphates has been used for quantification.[14] The ability of the developed method in this study to rapidly determine the hypochromicity of structured RNAs was analyzed using phenylalanine tRNA and total tRNA from *S. cerevisae* (see Table 3). The results show hypochromicity factors of 1.22 and 1.21 for phenyl tRNA and total tRNA, similar to values obtained for larger ssRNA molecules. In addition, similar to ssRNA, further heating of the tRNAs in the presence of DMSO does not significantly change the $A_{260}$ ratios, demonstrating that no further change in hypochromicity is observed. These results demonstrate that for structured RNAs such as tRNAs, the addition of 50% DMSO is sufficient to destabilize their base-stacking interactions and intramolecular folding.

**Quantitative Analysis of the Hypochromicity of dsDNA, Oligonucleotides, and Oligoribonucleotides.** The hypochromicity factor ($A_2/A_0$) for dsDNA was determined to be 1.56 and is higher than that obtained for dsRNA (see Table 3). It is noteworthy that the $A_1/A_0$ ratio is higher than that obtained for dsRNA owing to the significant ssDNA generated upon addition of 50% DMSO. In addition to the analysis of large ss/ds nucleic acids typically >400 nt/bp, hypochromicity measurements of short oligonucleotides (<20 nts) were also obtained (see Table 3). The results show that similar hypochromicity values of 1.21 were obtained for oligonucleotides/oligoribonucleotides when compared to larger ssRNA and ssDNA molecules. This suggests that intramolecular interactions/ss base-stacking interactions in short oligonucleotides are similar to those in large ssDNA and ssRNA. Furthermore, hypochromicity factors for NTPs and dNTPs were also determined (see Table 3). The results show no change in $A_{260\ nm}$ were observed, demonstrating that there is no alteration in the extinction coefficients in the presence of DMSO and as expected they do not exhibit hypochromicity upon heating in DMSO.

**Determination of Nucleic Acid Extinction Coefficients and Concentration Using Hypochromicity Measurements.** In this study, we have used the measurements of hypochromicity outlined previously to accurately determine the overall extinction coefficient and mass concentration/$A_{260}$ for a range of nucleic acids. In each of the nucleic acids analyzed, the base sequence and composition is known; therefore, we have used the sum of the monomer extinction coefficients as determined in[4] to compare the calculated and measured extinction coefficients based on the hypochromicity measure-



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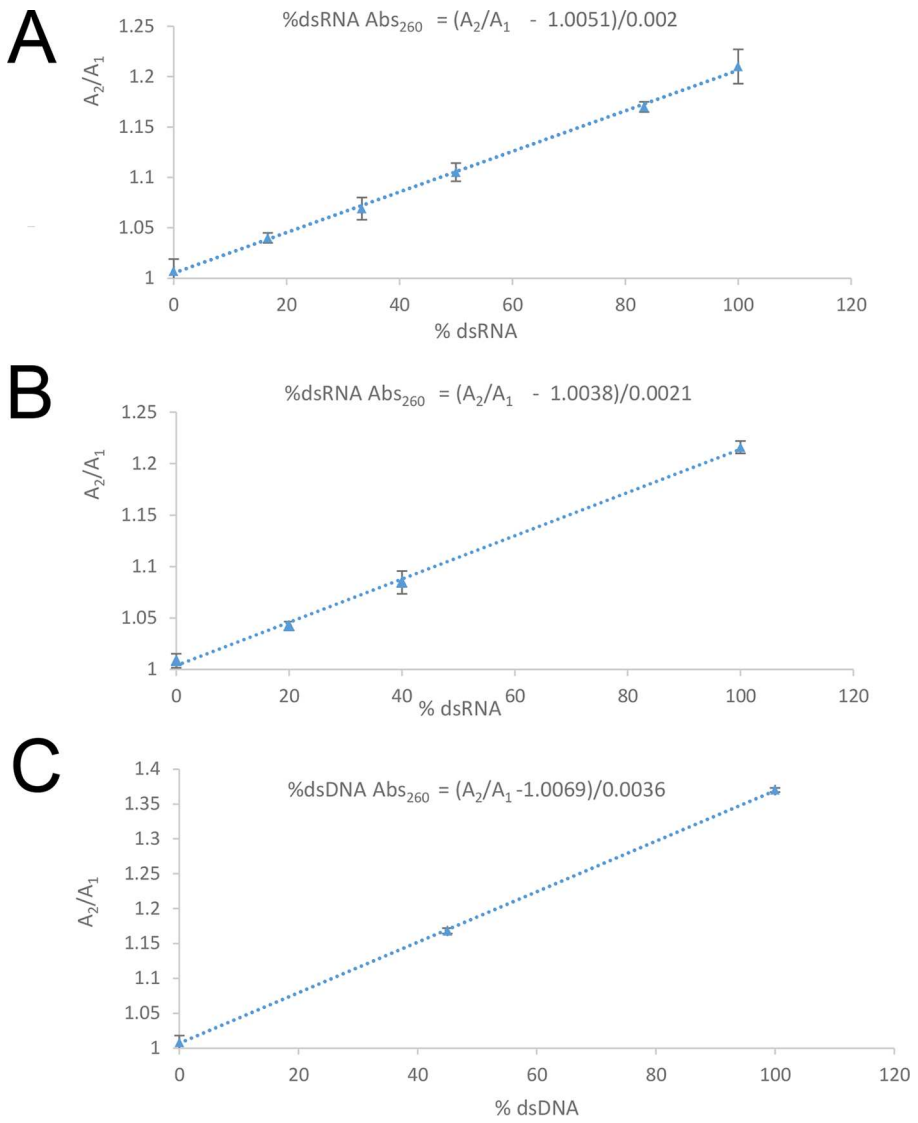


**Figure 2.** Quantification of ds RNA/DNA in nucleic acid mixtures using spectrophotometry. (A) Standard curve generated using mixtures of dsRNA (521 bp) and ssRNA (521 nt) standards. Following the experimental determination of the $A_2$ and $A_1$ values, the ratio $A_2/A_1$ of the corresponding mixtures were plotted against % dsRNA and the equation obtained is given by %dsRNA $Abs_{260} = (A_2/A_1 - 1.0051)/0.002$). (B) Standard curve generated using mixtures of 521 bp dsRNA and ssRNA (*E. coli* total RNA). $A_2/A_1$ ratios of the corresponding mixtures were plotted against % dsRNA and the equation is given by %dsRNA $Abs_{260} = (A_2/A_1 - 1.0038)/0.0021$). (C) Standard curve generated using mixtures of dsDNA and ssDNA (oligonucleotides). Following the experimental determination of the $A_2$ and $A_1$ values for dsDNA, the ratio $A_2/A_1$ were plotted against percentage dsDNA and the equation is given by %dsDNA $Abs_{260} = (A_2/A_1 - 1.0069)/0.0036$).

ments (see Table 4). In addition, using the sum of the mononucleoside phosphate masses, we have determined the mass concentration/$A_{260}$ and the hypochromicity corrected extinction coefficients (see Table 4).

The results obtained in Table 4 demonstrate that for a number of different dsRNA sequences, typical values of 46.18−47.29 (median = 46.52) $\mu$g/mL/$A_{260}$/ were obtained. To our knowledge, we believe this is the first time an accurate value for the calculation of the concentration of dsRNA directly from $A_{260}$ measurements has been experimentally obtained. For larger ssRNAs, a value of 37.80 $\mu$g/mL/$A_{260}$ (equimolar mononucleotide) was obtained which is lower than the typically reported value often used for RNA 40 $\mu$g/mL/$A_{260}$. For ssRNA oligoribonucleotides (13 mer), a value of 34.85 $\mu$g/mL/$A_{260}$ was obtained. For dsDNA (dA:275; dC:243; dG:243; T:275), a value of 46.25 $\mu$g/mL/$A_{260}$ was determined compared to previously reported values typically 50 $\mu$g/mL/$A_{260}$.[2]

The results show that for ssDNA (13 mer) a typical value of 34.53 $\mu$g/mL/$A_{260}$ and ssDNA (550 nt) 34.73 $\mu$g/mL/$A_{260}$ was obtained. For ssRNA and ssDNA oligonucleotides containing equimolar base composition, a typical value of 37.80 and 35.70 $\mu$g/mL/$A_{260}$ was predicted using this approach. For structured tRNAs, values of 37.80 and 37.18 $\mu$g/mL/$A_{260}$ were obtained taking into account the hypochromicity measurement.

**Quantification of ds and ss Nucleic Acids in Complex Mixtures.** Previous results showed that in the presence of 50% DMSO further heating of ssRNA resulted in no hyperchromic effect ($A_2/A_1$ = 1.0) while heating of dsRNA in 50% DMSO resulted in a hyperchromic effect ($A_2/A_1$ = 1.2). Therefore, it is proposed that a sample containing a mixture of dsRNA and ssRNA upon heating in DMSO will have a hyperchromic effect between 1.0 to 1.2 depending on the relative proportion of



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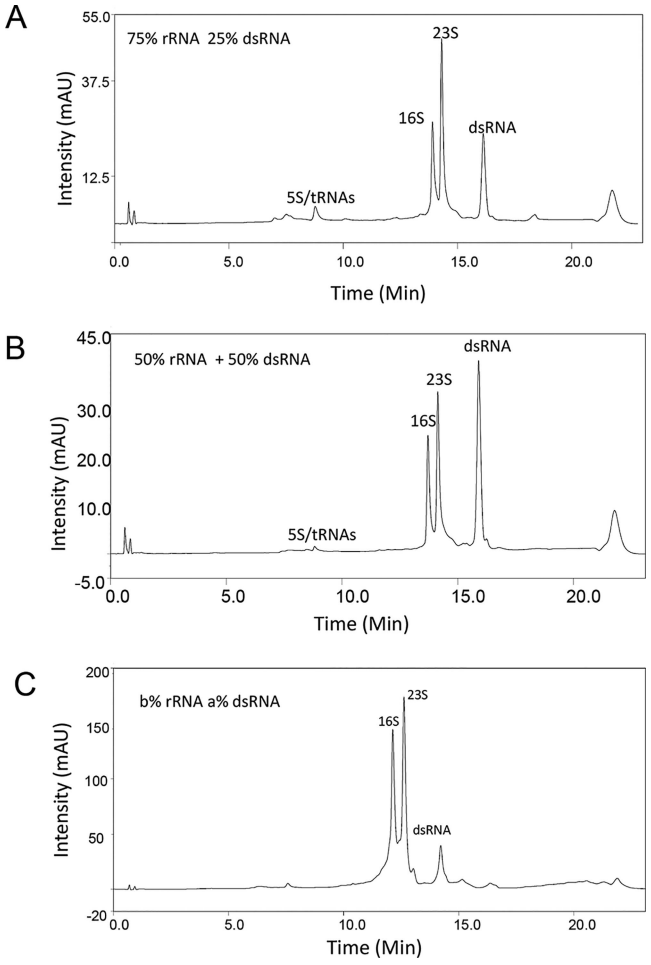


**Figure 3.** IP RP HPLC analysis of nucleic acid mixtures containing varying proportions of dsRNA (521 bp) and ssRNA (*E. coli* total RNA). (A) IP RP HPLC analysis (using gradient 1) of a mixture containing 25% dsRNA (521 bp) (based on $A_{260}$). The $A_2/A_1$ ratio for this nucleic acid mixture predicted 24.38% dsRNA, corresponding to a dsRNA concentration of 281.08 ng/$\mu$L (B) IP RP HPLC analysis (using gradient 1) of a mixture containing 50% dsRNA (521 bp) (based on $A_{260}$). The $A_2/A_1$ ratio for this complex mixture predicted 49.05% dsRNA corresponding to a dsRNA concentration of 565.52 ng/$\mu$L. (C) IP RP HPLC analysis (using gradient 2) of a complex mixture containing an unknown amount of dsRNA (481 bp). The $A_2/A_1$ ratio predicted 15.24% dsRNA corresponding to a concentration of 175.71 ng/$\mu$L.

dsRNA in the sample. We can therefore generate a model linear curve of ($A_2/A_1$) against % dsRNA values (see Figure S3). To experimentally validate the model, standards containing a range of known proportions of dsRNA relative to ssRNA were prepared (see Figure S4), and $A_2/A_1$ ratios were determined as previously described and plotted against the relative % of dsRNA (see Figure 2A). The results show the expected linear relationship between the relative % of dsRNA and $A_2/A_1$ ratio.

We used this approach to determine an unknown percentage and concentration of dsRNA in a complex mixture (see Figure 2B, Figure 3). To demonstrate the accuracy of method, the relative proportion and concentration of total dsRNA and dsRNA were determined as 24.4% (281.1 ng/$\mu$L) and 49.1% (565.5 ng/$\mu$L) for known RNA standards containing 25% and 50% dsRNA respectively. Samples containing an unknown quantity of dsRNA were determined to be 15.2% (175.7 ng/$\mu$L) dsRNA.

Similar to the method described above, the same approach can be used to measure the amount of dsDNA present in complex ds/ss DNA mixtures using the linear relationship between the $A_2/A_1$ ratio and % dsDNA absorbance. For a known DNA standard containing 45% dsDNA, the relative proportion of total dsDNA was determined as 44.75% (see Figure 2C).

It should be noted that although a small concentration of the dsDNA is denatured at 16% DMSO, this does not affect the determination of the relative proportion dsDNA; the hypochromicity due to the denatured DNA is reflected in the $A_1/A_0$ ratio (1.37) and accounted for in the $A_2/A_1$ = 0.0036 (dsDNA $Abs_{260}$) + 1.0069. This provides a rapid, high-throughput quantitative and qualitative approach to analyzing the amount of dsRNA/DNA present in all mixed DNA or RNA nucleic acid samples. No separation or purification of the dsRNA/DNA is required prior to accurate quantification. To our knowledge, this is the first time UV spectrophotometry has been used for accurate quantification of duplex structures in mixed ds/ss nucleic acid solutions.

## CONCLUSIONS

UV spectrophotometry has been used to rapidly determine the hypochromicity of a wide range of nucleic acids including ssDNA/RNA, oligonucleotides, structured RNA, and dsRNA. We have utilized the chemical denaturant dimethyl sulfoxide in conjunction with short thermal denaturation which prevents renaturation of the duplex nucleic acids (ds DNA/RNA) to measure the absorbance of both the unstructured and structured nucleic acids. This approach does not require prolonged, elevated temperatures, which may cause partial hydrolysis of RNA or incomplete denaturation of the dsRNA. In addition, this method is not affected by changes in the extinction coefficients of nucleic acids with temperature, as all $A_{260}$ measurements are performed at room temperature. Using this approach, we have used the measurements of hypochromicity outlined previously to accurately determine the overall extinction coefficient and mass concentration/$A_{260}$ for a range of nucleic acids. We have for the first time determined a median value of 46.52 $\mu$g/mL/$A_{260}$ for the quantification of dsRNA using UV spectrophotometry, enabling the accurate determination of the relative proportion of duplex nucleic acids in mixed ds/ss nucleic acid solutions and demonstrating significant advantages over current methods. This provides a rapid, high-throughput quantitative and qualitative approach to analyzing the amount of dsRNA present in all RNA samples. No separation or purification of the dsRNA is required prior to accurate quantification. To our knowledge this is the first time UV spectrophotometry has been used for accurate quantification of duplex structures in mixed ds/ss nucleic acid solutions.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04000.

IP RP HPLC analysis of experimental RNA and DNA samples, model linear curves for the quantification of ds and ssRNA/DNA in complex nucleic acid mixtures, IP RP HPLC analysis of nucleic acid mixtures containing varying proportions of dsRNA and *E. coli* total RNA (PDF)



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### Notes

The authors declare no competing financial interest.

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