A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers

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INTRODUCTION

Microvolume spectrophotometers (MVS) are commonly used for the analysis of nucleic acid (NA) samples. They require a small sample volume (0.5–2.0 µl) and are economical, convenient and widely commercially available. Typically, they can measure NA concentrations as low as 1 ng/µl and they are compatible with various assays for all types of NA and proteins. Concentration and purity readings from MVS are often accepted as fact; however, a more detailed understanding of the readings can provide meaningful insight into the quality of the sample and its suitability for downstream applications.

This technical note provides detailed explanations and guidance on how to interpret and use MVS measurements, with a focus on samples purified using silica spin columns. Using simple experiments, we provide useful information on the following topics:

1. Optimal working range for concentration and ratio determination
2. Protein contamination in NA preps
3. Buffer contaminants in NA preps
4. Other contaminants in NA preps
5. RNA contamination in DNA samples
6. Measuring NA in water or buffer

Unless noted otherwise, MVS measurements were performed on a Nanodrop® One (Thermo Fisher Scientific®). Manufacturer's specifications for nucleic acid detection is a lower detection limit of 2 ng/µl and reproducibility of 2 ng/µl for the concentration range 2–100 ng/µl and 2% for values >100 ng/µl.

BACKGROUND

The standard technique for performing concentration and purity measurements is UV absorbance measurement with a spectrophotometer.

Nucleic acid concentrations are determined by measuring the absorbance of ultraviolet light. Derived from the Beer-Lambert law, the amount of light absorbed at 260 nm is proportional to the concentration of nucleic acid in solution. Extinction coefficients have been determined for dsDNA, RNA, and ssDNA using a 10 mm path length and allow the creation of conversion factors in the absence of a standard curve. These conversion factors are:

- 50 µg/ml for dsDNA
- 40 µg/ml for RNA
- 33 µg/ml for ssDNA

For example, a dsDNA sample with A_{260} = 1 will have a concentration of 50 ng/µl.

Additionally, as an indicator of sample purity, the ratios of the absorbance values of 260 nm vs 280 nm (A_{260}/A_{280}) and the 260 nm vs 230 nm (A_{260}/A_{230}) can be determined.

A_{260}/A_{280} Ratios

The A_{260}/A_{280} provides insight regarding the type of nucleic acid present (dsDNA or RNA) as well as providing a rough indication of purity. Typically, protein contamination can be detected by a reduction of this ratio; RNA contamination can be detected by an increase of this ratio. In buffered solutions, pure dsDNA has an A_{260}/A_{280} of 1.85–1.88 and pure RNA has a ratio of around 2.1.

A_{260}/A_{230} Ratios

The A_{260}/A_{230} is a sensitive indicator of contaminants that absorb at 230 nm. These contaminants are significantly more numerous than those absorbing at 280 nm, and include chaotropic salts such as guanidine thiocyanate (GTC) and guanidine hydrochloride (GuHCl), EDTA, non-ionic detergents like Triton™ X-100 and Tween® 20, proteins, and phenol. Substances like polysaccharides or free floating solid particles like silica fibers absorb at this wavelength, but will have a weaker effect.

In buffered solutions, pure dsDNA has slightly higher A_{260}/A_{230} ratios than RNA, with a value of 2.3–2.4 commonly reported for dsDNA and 2.1–2.3 for RNA. A_{260}/A_{230} ratios typically produce a higher standard deviation than A_{260}/A_{280} ratios and should be interpreted with care.

In common laboratory practice, DNA and RNA samples with A_{260}/A_{280} and A_{260}/A_{230} >1.8 are considered to be "clean", and suitable for use in most downstream applications.

1 OPTIMAL WORKING RANGE FOR CONCENTRATION AND RATIO DETERMINATION

Suppliers of MVS instruments define the detection limits between 0.5 to 2 ng/µl, but warn that below 20 ng/µl the reliability of the purity ratios is compromised. It is often discussed whether the use of fluorescence-based methods (e.g., "Qubit") may be a better alternative for lower concentrations. To address these concerns, a dilution series of 150 ng/µl down to 1 ng/µl in TE buffer was analyzed (Table 1 A–C, page 2). Measurements were carried out in triplicate and data were collected for concentration and purity ratios. For comparison, the average of triplicate Qubit measurements of the same DNA dilution series was included.

Concentration Measurements

The concentration data shown in Table 1A illustrate that for concentrations at or under 5 ng/µl (yellow), there is a large spread in the data, as illustrated by the relative standard deviations. However, if replicates are included, relatively accurate results can be obtained. Below 5 ng/µl, Qubit values do not show as large a deviation, but were slightly less accurate overall. Since fluorescence measurements are more time consuming and more costly, MVS is recommended as the preferred tool in the evaluation of concentration for samples ≥1 ng/µl that are sufficiently pure (e.g. after purification with a high-quality silica kit that successfully separates NA species).

Purity Ratio Measurements

A_{260}/A_{280} ratios are unusable at concentrations <20 ng/µl (**blue**), as indicated by the variability in triplicate measurements (Table 1B). Remarkably, the variability is still relatively high in the 20–50 ng/µl range (**yellow**), with averages tending to be too high. Only when working with higher concentrations (**green**), can consistent and reliable values be obtained.

A_{260}/A_{230} ratios show a similar trend (Table 1C). They are unusable at concentrations below 20 ng/µl (**blue**) and should be used with care between 20–50 ng/µl (**yellow**): the A_{260}/A_{230} ratio is too high and shows significant variability. Above 50 ng/µl the values are more reliable (**green**). Overall, A_{260}/A_{230} ratios have a higher standard deviation than A_{260}/A_{280} ratios. Similar trends were observed with RNA (Table 5B, page 7).

SUMMARY:

When working with mostly pure NA solutions:

- MVS can be used effectively in a concentration range down to 1 ng/µl
- Several replicates are required for accurate quantitation below 5 ng/µl
- In general, fluorescence measurements are not more accurate or reproducible, but for dilute samples, they offer more reproducible results
- A_{260}/A_{230} ratios are more variable than A_{260}/A_{280} ratios
- Both purity ratios are unusable below concentrations of 20 ng/µl, and are still relatively variable (with a tendency to be too high) at values up to 50 ng/µl. Replicate measurements are recommended.

2 PROTEIN CONTAMINATION

NA samples that have significant protein contamination will display a UV absorbance spectrum consistent with a protein/DNA mixture. Generally, there will be a strong effect on the A_{260}/A_{230} and a small effect on the A_{260}/A_{280}. The UV absorbance for protein is relatively low in comparison to NA absorbance, so if the A_{260}/A_{280} reflects signs of protein contamination, then relatively large amounts of protein are present. Additionally, this implies that at low NA concentrations, protein contamination has a large effect on purity ratios, but at high NA concentrations, it may be hardly detectable.

TABLE 1:
Assessment of DNA concentration and purity by MVS

A NIH3T3 gDNA stock in TE buffer was diluted with TE buffer to obtain the indicated DNA dilutions. Concentration values (OD_{260}) (A) and purity ratios 260/280 (B) and 260/230 (C) were determined in triplicate on a Nanodrop One. MVS concentration values were compared to triplicate fluorescence values obtained with the Qubit BR DNA kit using the same dilutions.

A

	MICROVOLUME SPECTROPHOTOMETER (MVS)					QUBIT	
SAMPLE CONCENTRATION (ng/µl)	MEASURED CONCONCENTRATION (ng/µl)			AVERAGE CONCENTRATION (ng/µl)	RELATIVE STANDARD DEVIATION (%)	AVERAGE QUBIT CONCENTRATION (ng/µl)	QUBIT REL. STANDARD DEVIATION (%)
	1	2	3				
1	0.3	1.2	1.6	1.0	66.9	0.7	3.8
2.5	1.9	2.9	2.8	2.6	21.3	2.0	4.2
5	4.9	5.2	5.2	5.1	3.5	4.6	4.1
10	9.1	9.9	9.9	9.6	4.5	9.1	1.7
15	13.8	15.2	14.2	14.4	5.0	13.7	1.9
20	19.7	19.5	19.6	19.6	0.5	19.0	2.1
25	25.0	24.9	25.0	25.0	0.3	23.8	0.3
30	29.0	29.5	29.2	29.2	0.9	28.4	1.9
40	38.4	39.8	39.8	39.3	2.1	37.3	1.2
50	48.1	48.4	49.3	48.6	1.3	47.5	1.0
75	71.8	72.3	74.5	72.9	2.0	72.7	3.2
100	93.0	98.4	97.8	96.4	3.1	99.4	1.5
150	147.4	149.2	147.9	148.2	0.6	151.2	0.7

B

SAMPLE CONCENTRATION (ng/µl)	260/280				RELATIVE STANDARD DEVIATION (%)
	1	2	3	AVERAGE	
1	5.91	1.11	2.83	3.28	74.2
2.5	3.80	1.89	1.60	2.43	49.1
5	1.93	1.85	1.70	1.82	6.5
10	1.95	1.85	1.78	1.86	4.7
15	1.80	1.86	2.04	1.90	6.6
20	1.86	1.77	1.93	1.85	4.4
25	1.86	1.86	1.92	1.88	1.7
30	1.85	1.87	1.92	1.88	1.9
40	1.91	1.85	1.88	1.88	1.5
50	1.86	1.84	1.90	1.87	1.6
75	1.86	1.84	1.86	1.86	0.8
100	1.86	1.86	1.86	1.86	0.3
150	1.87	1.86	1.86	1.86	0.2

C

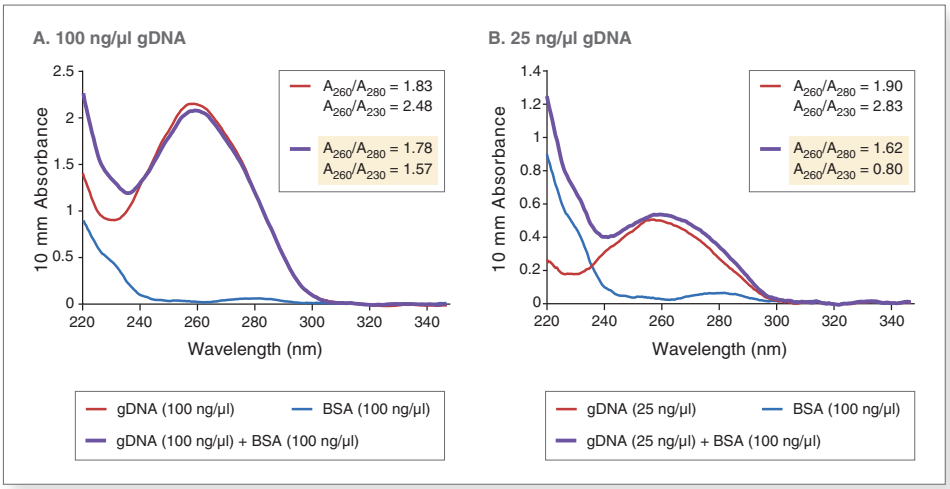
SAMPLE CONCENTRATION (ng/µl)	260/230				RELATIVE STANDARD DEV. (%)
	1	2	3	AVERAGE	
1	-0.52	137883.58	2.13	12628.4	173.2
2.5	-2.60	6.03	8.84	4.09	145.8
5	12.97	3.28	4.40	6.88	77.0
10	5.94	3.28	2.99	4.07	39.9
15	3.62	2.90	3.08	3.20	11.6
20	3.17	3.02	3.09	3.09	2.5
25	3.10	2.91	3.09	3.04	3.5
30	2.94	2.37	2.89	2.73	11.5
40	3.02	2.85	2.94	2.94	3.0
50	3.02	2.79	2.75	2.85	5.0
75	2.95	2.77	2.81	2.84	3.3
100	2.80	2.74	2.80	2.78	1.3
150	2.83	2.77	2.76	2.79	1.4

Figure 1 shows UV-Vis spectra from samples containing either pure DNA (red), pure protein (blue), or mixtures at defined concentrations (purple). The typical UV-absorbance spectrum of proteins shows a strong peak in the 220–230 nm range and an approximately 10-fold lower shoulder at around 280 nm (Figure 1). The effect of protein contamination on purity ratios is significantly higher in the 25 ng/µl DNA sample than in the 200 ng/µl sample. Therefore, purity ratios should be taken as relative indicators of contamination and always be considered in relation to the NA concentration range under investigation.

Table 2 illustrates how the purity ratios of four different DNA dilutions are influenced by the addition of varying concentrations of BSA protein in TE buffer. At DNA concentrations of 100 ng/µl and 50 ng/µl, contaminating protein barely influences the A_{260}/A_{280} (**green**), but at a DNA concentration of 25 ng/µl, the same protein levels strongly affect the purity ratios and suggest significant contamination (**yellow**). Additionally, at low DNA concentrations, the presence of protein influences the measured DNA concentration (**blue**), whereas the relative influence for high DNA concentrations is much lower. Some systems, like the Nanodrop One, offer software tools that are able to correct for the influence of the protein on the A_{260} values.

FIGURE 1: **The effect of protein contamination on purity ratio is dependent on DNA concentration**

NIH3T3 gDNA stock in TE buffer was mixed with a Bovine Serum Albumin (BSA) protein solution (NEB #B9000) to reach the indicated concentrations, with EDTA concentration kept at 1 mM. Concentration values (A_{260}) and purity ratios A_{260}/A_{280} and A_{260}/A_{230} were determined in triplicate on a Nanodrop One. Individual spectra and ratios are shown from separate samples. Protein contamination is more detectable in the purity ratios when DNA concentration is lower.



NA samples derived from blood may contain traces of hemoglobin that will absorb around 410 nm. Additionally, the effects described above will be detected in the purity ratios. Elevated background absorbance values may also be observed.

SUMMARY:

- Protein contamination affects the A_{260}/A_{230} more strongly than the A_{260}/A_{280}, making the A_{260}/A_{230} more suitable for assessing protein contamination
- The effect of protein contamination on purity ratios is dependent on NA concentration:
  - Protein contamination of dilute NA solutions strongly influences concentration and purity determinations
  - With more concentrated DNA samples, the impact of protein contamination tends to be underestimated

TABLE 2: **The effect of protein contamination on purity ratio is DNA- concentration dependent**

NIH3T3 gDNA stock in TE buffer was mixed with BSA protein solution (NEB #B9000) to reach the indicated concentrations, with EDTA concentration kept at 1 mM. Concentration values (OD_{260}) and purity ratios 260/280 and 260/230 were determined in triplicate on a Nanodrop One.

INPUT gDNA CONCENTRATION (ng/µl)	BSA CONCENTRATION (mg/ml)	DETECTED NA CONCENTRATION (ng/µl)	260/A280	260/230
100	0	103.7	1.83	2.48
	200	105.3	1.73	1.13
	100	103.3	1.78	1.57
	50	100.7	1.82	2.04
	25	100.8	1.85	2.33
50	0	53.2	1.84	2.44
	200	55.3	1.60	0.76
	100	51.1	1.71	1.19
	50	50.8	1.78	1.66
	25	49.8	1.79	2.07
25	0	25.5	1.81	2.66
	200	29.4	1.45	0.48
	100	26.5	1.59	0.83
	50	25.0	1.67	1.21
	25	24.8	1.72	1.69
12.5	0	12.7	1.80	2.86
	200	16.3	1.21	0.29
	100	13.5	1.42	0.49
	50	12.2	1.55	0.77
	25	11.9	1.67	1.34

3 BUFFER CONTAMINANTS IN NA PREPS

Substances utilized in NA purification kit buffers may also influence the UV-absorbance spectra of the purified samples if not completely removed during the prep. Chaotropic salts, like guanidine thiocyanate (GTC) and guanidine hydrochloride (GuHCl), are frequently used in binding buffers. Non-ionic detergents, such as Triton X-100 and Tween 20, are often found in lysis buffers. Even EDTA, frequently used in DNA elution buffers, will affect absorbance spectra. Additionally, phenol, TRIzol® or similar reagents are often used in RNA purification kits and can influence UV-vis readings, the latter of which contains both phenol and GTC. To a

certain extent, ethanol, commonly used in wash buffers, may also influence absorbance readings and is therefore, included in this analysis.

Detection limits were determined for each of the aforementioned contaminants, and to enable comparison of spectra, the concentrations needed to reach an OD_{230}=1 were also determined (Table 3). The latter values enable comparison of the contaminant spectra amongst each other (Figure 2). As little as 10–50 µM of GTC or EDTA can be detected. Other substances, like GuHCl, must be present in amounts >50 mM to influence the purity ratios of a purified NA. Since it is unlikely to find GuHCl at concentrations in the 50 mM to 1 M range in silica prep eluates, contaminations with GuHCl are usually not detected by UV-vis spectrometry. In the investigated concentration range, most contaminants only affect the A_{260}/A_{230}, while Triton X-100 has an effect on both purity ratios.

Figure 2 shows the spectra of each substance alongside a spectrum of a 100 ng/µl gDNA solution containing the same substance. All combined spectra are consistent with contributions from both the gDNA and the contaminant. The only exception is ethanol, where the spectrum shows a uniform increase in signal.

Guanidine Salts/Detergents

In contrast to previously published data (1), GTC has an absorbance maximum in the 220-230 nm range and not at 240–260 nm. We believe the assignment of GTC absorbance maxima between 240–260 nm is an artifact of the Nanodrop system software that cuts off part of the signal in the 220–230 nm range (2).

Although substances like GTC or non-ionic detergents may influence the UV absorbance spectrum heavily, their impact on downstream applications is often negligible. Von Alflen and Schlumpberger demonstrated that carryover of significant amounts of chaotropic salt into qPCR assays showed no effect on qPCR reaction efficiency (2).

Phenol

Phenol, used frequently in RNA purification protocols, absorbs heavily in the UV-Vis range. When the aqueous phase over phenol is diluted approximately 10,000-fold, the spectrum is still visible. The spectrum of TRIzol/Tri-reagent is very similar to the phenol signal, but shows additional absorbance in the 220–230 nm range, typical for GTC. In Figure 3, an RNA sample mixed with known amounts of phenol or TRIzol is shown as a typical example of contaminated RNA. If phenol is present at higher concentrations, a clear shift of the 260 nm peak in the spectrum towards 270 nm can be observed, with a slight reduction of both purity ratios. TRIzol has similar effects on the 260 nm peak, but the presence of GTC will lead to strong absorbance in the 220–230 nm range and result in a strong reduction of the A_{260}/A_{230}.

EDTA

When working with DNA, EDTA is commonly used to reduce nuclease activity. EDTA chelates divalent cations such as Mg^{2+} or Ca^{2+}. However, once EDTA is complexed with Mg^{2+} or Ca^{2+}, the UV absorbance of these EDTA-cation complexes is lower than the absorbance of free EDTA. Therefore, if the DNA solution contains Mg^{2+} and Ca^{2+} ions, which act as counterions of the NA of interest, it will show a lower 230 nm

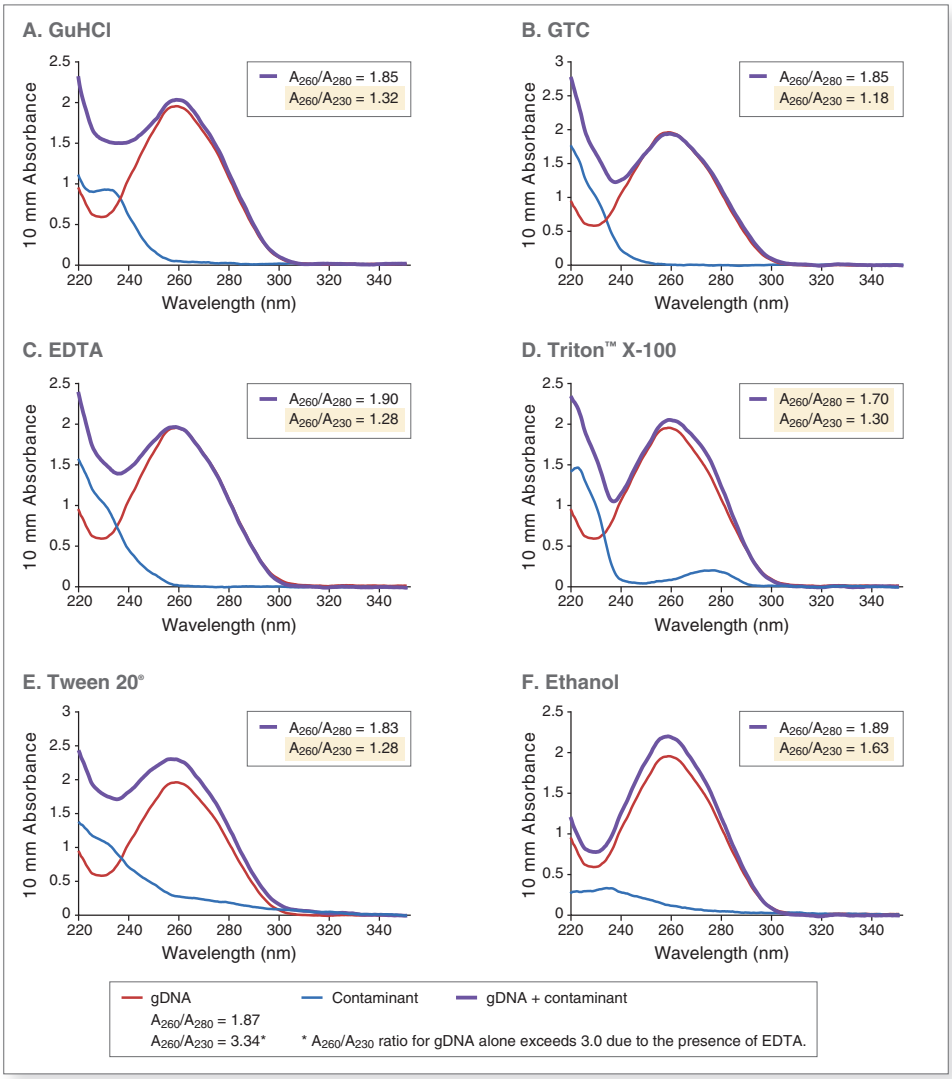
TABLE 3: **Detection limit of common contaminants**

The concentration of buffer components that are still detectable in water varies significantly. In some cases, (e.g., GuHCl) the amount required for detection is well above the amount normally expected in eluates of silica preps.

CONTAMINANT	DETECTION LIMIT	OD_{230}=1
GuHCl	50 mM	2.5 M
GTC	10 µM	0.6 mM
EDTA	20 µM	1.25 mM
Triton X-100	0.0002%	0.012%
Tween 20	0.02%	1.50%
Ethanol	2%	NA (20% used)

FIGURE 2: **UV-absorbance spectra of common contaminants alone and mixed with DNA**

Contaminants were diluted in TE buffer to be at A_{230} = 1 and subsequently measured alone and mixed with 100 ng/µl of gDNA from NIH3T3 cells. All mixed spectra (except that of ethanol) reflect the absorbance of both the DNA and the contaminant. Contaminants mainly affect the A_{260}/A_{230} while leaving the A_{260}/A_{280} mostly unchanged.



absorbance than the blank. Hence, although the A_{260}/A_{230} of clean DNA usually does not exceed 2.4 without EDTA, it may exceed 3.0 when in solution with EDTA and chelated Mg^{2+} or other divalent cations. In routine analyses, we often find that pure gDNA in TE buffer may have a A_{260}/A_{230} ratio of 2.6–3.0, whereas if measured in 10 mM Tris pH 8.5, the ratios are 2.3–2.4. With higher DNA concentrations, the effect of EDTA on the A_{260}/A_{230} will decrease.

Ethanol

Ethanol diluted in water shows UV absorbance with peak values of 230-240 nm. However, when present in DNA solutions, the effect on the UV absorbance spectra is reduced, and other than decreased A_{260}/A_{230}, cannot be visually differentiated. A similar effect can be observed with isopropanol (not shown).

SUMMARY:

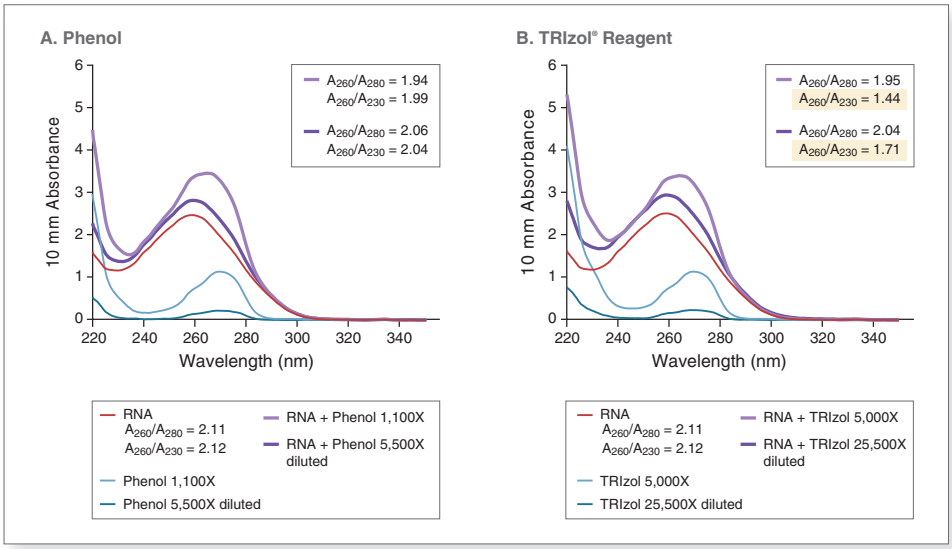
- **GTC, EDTA, phenol and Triton X-100 significantly affect UV-absorbance spectra and particularly the A_{260}/A_{230}**
- **Other contaminants like GuHCl or Tween 20 can only be detected at high concentrations – their detection in silica preps is unlikely**
- **In contrast to protein contamination, the impact of contamination from silica prep buffer components on downstream applications is often overestimated**
- **Minute differences in free EDTA concentration in dilute DNA eluates vs blanks will influence the A_{260}/A_{230} strongly; ratios >3.0 are not unusual in TE buffer**
- **Ethanol cannot be distinguished clearly in NA absorbance spectra, but is detectable with MVS when measured alone or diluted in water.**

4 OTHER CONTAMINANTS

Polysaccharides (e.g., plants materials, agarose, etc.) and lipopolysaccharides (e.g., bacteria, yeast, etc.) may be present in NA preps and will result in background absorbance. Some MVS devices (e.g., Trinean Dropsense™ 16, Unchained Labs Lunatic) are able to filter out this background signal and display it separately (Figure 4). However, not all

FIGURE 3: **UV-absorbance spectra of phenol and TRIzol mixed with RNA**

Phenol was mixed with water, the aqueous upper phase was taken and used for further dilutions. TRIzol and the phenol aqueous phase were diluted until A_{270} ~1 was reached. A 5X higher dilution was also included. Both dilutions were mixed with 100 ng/µl of RNA from rat liver. Phenol affects both purity ratios moderately. TRIzol more strongly affects the A_{260}/A_{230} because of the presence of GTC.



MVS systems can perform this deconvolution. Therefore, any sample with polysaccharide contamination may lead to reduced A_{260}/A_{230} ratios, slightly increased A_{260}/A_{280} ratios, and greater variability in the measurements.

Plant Samples

NA extractions derived from plant samples that are not sufficiently pure may show either strong phenol-like signals because of residual polyphenols, or weak signals caused by the presence of polysaccharides. This mainly affects that A_{260}/A_{230} and causes background scattering. Polyphenols and polysaccharides can be removed by using specific lysis chemistries during extraction. (Figure 4).

Silica Fibers

Similar background signals can be detected if NA samples contain silica fibers. Such fibers may be released from the silica membrane during the elution step, and if present in large quantities, can be observed in the eluate as a white cloud. Silica fibers can be removed from the solution by centrifugation. The effect of silica fibers on the UV absorbance spectra is similar to that of polysaccharides. Figure 5B shows a gDNA eluate isolated with Qiagen® DNeasy® Blood and Tissue Kit that contains silica fibers. The Monarch® Genomic DNA Purification Kit (NEB #T3010) columns contain a specific membrane layer that removes these fibers, resulting in cleaner gDNA eluates (Figure 5A).

FIGURE 4: **UV-Vis spectra from partially purified gDNA samples from tomato leaves obtained with a Trinean Dropsense 16**

Gray lines represent the background signal caused by the polysaccharides. Some of that signal attributes to increased absorbance values over the range 230–280 nm and leads to the unusual shape of the absorbance curve.

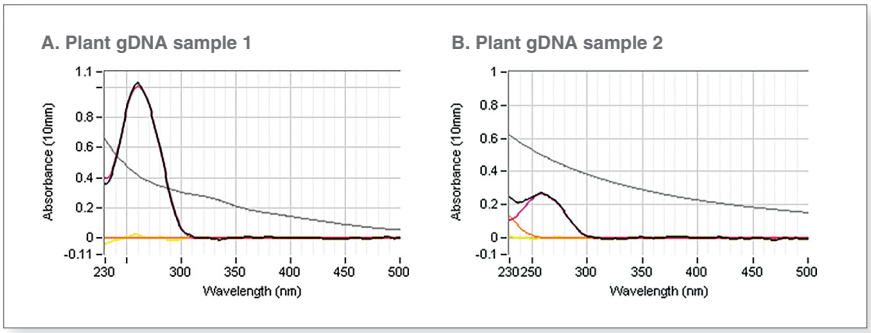
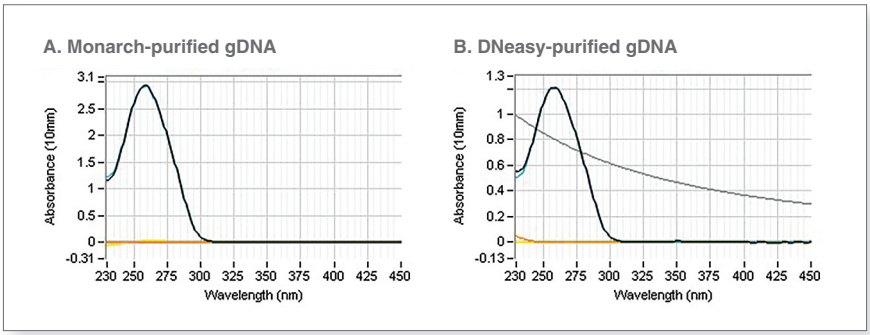


FIGURE 5: **gDNA samples purified with certain silica kits sometimes contain silica fibers**

Comparison of Monarch-purified genomic DNA (A) and Qiagen DNeasy-purified genomic DNA (B). The DNeasy purified DNA contains silica fibers represented by the gray line. The fibers lead to a slightly increased A_{230} (black vs blue line) After spinning the samples for 1 minute at 15,000 x g the DNeasy sample contained a small white pellet. Data were collected with a Trinean Dropsense 16.



Substances without absorbance

The last wash buffer that is used in many silica kits for NA purification usually contains ethanol and, in many cases, Tris. Occasionally, NaCl may also be included in these buffers. Even though the silica membrane is spun dry after the last wash step, trace amounts of each of the wash buffer components can be found in the eluates. This is illustrated by the fact that RNA eluted from silica columns, even though eluted in water, still shows A_{260}/A_{280} ratios ~2.1, values typical for buffered solutions (Table 5B).

In addition to Tris and NaCl, other substances such as different buffers and buffer components, common salts, various detergents (e.g., SDS) and antioxidants (e.g., beta-mercaptoethanol) may be used in NA preps during various steps. Their presence or absence cannot be detected by the spectrophotometer.

SUMMARY:

- Samples containing polysaccharides will show background absorbance influencing the A_{260}/A_{230} and will lead to higher signal variability
- Polysaccharides and lipopolysaccharides usually remain undetected because they only minimally affect OD readings
- Other soluble or insoluble substances like silica fibers may influence absorbance measurements in a similar way

5 RNA CONTAMINATION IN DNA SAMPLES

Effect of RNA contamination on the purity ratios of gDNA solutions

To monitor how increasing amounts of RNA added to a gDNA solution influence quantitation and purity ratios, a series of gDNA and RNA mixtures were generated. RNA ranged from 0–50% of the total NA content, and the gDNA concentration was held constant at 100 ng/µl. EDTA concentration was 1 mM (Table 4).

TABLE 4: **The addition of increasing amounts of RNA to DNA solutions leads to a minimal increase of the A_{260}/A_{280} ratio**

A 100 ng/µl gDNA of NIH3T3 DNA in TE buffer was mixed with increasing amounts of RNA as indicated in the table. Amounts of RNA are indicated as % of total NA. Triplicate measurements of DNA concentration (A_{260}) and purity ratios were carried out and averages are displayed. To reach an A_{260}/A_{280} of >1.90, an RNA content >15% of total NA needs to be present.

%RNA OF TOTAL NA	AVERAGE CONCENTRATION (ng/µl)	AVERAGE 260/280	AVERAGE 260/230
0	101.1	1.86	3.03
1%	99.7	1.87	3.02
2.5%	101.9	1.87	3.02
5%	104.7	1.87	3.00
7.5%	108.1	1.88	2.98
10%	110.9	1.88	2.90
15%	117.5	1.89	2.83
20%	126.4	1.91	2.78
25%	135.3	1.92	2.69
30%	147.7	1.93	2.62
40%	175.6	1.95	2.48
50%	215.6	1.99	2.40

The results in Table 4 illustrate that the total NA concentration measured is higher than expected. This is because the MVS software uses the conversion factor 50 for dsDNA, while increasing amounts of RNA with a conversion factor of 40 are added. The A_{260}/A_{280}, which is normally used to analyze which NAs are present, starts at 1.86 when only gDNA is present. Although this ratio increases slightly when low amounts of RNA are added, only when RNA reaches 20% of the total NA, the A_{260}/A_{280} ratios exceed 1.90 (yellow). MVS is therefore not suitable to detect small amounts of contaminating RNA. If the A_{260}/A_{280} rises to over 1.90 because of RNA contamination, this means that at least 15–20% of the total nucleic acid may be RNA.

While the A_{260}/A_{280} rises with increasing RNA amounts, the A_{260}/A_{230} falls from 3.0 to 2.4 in TE buffer, which is consistent with the observation that pure RNA has a lower A_{260}/A_{230} than pure gDNA in buffered solutions (Table 5B).

SUMMARY:

- RNA contamination of <15% does not raise the A_{260}/A_{280} ratio to over 1.90, and is difficult to identify in routine NA analysis
- RNA has a lower A_{260}/A_{230} ratio than DNA: Increasing RNA amounts in gDNA solutions lead to a reduction of the A_{260}/A_{230} ratio

6 MEASURING IN WATER OR IN BUFFERED SOLUTIONS

Purity ratios are impacted by pH (3). When spectrophotometric measurements of NAs are carried out in water or under acidic solutions, A_{260}/A_{280} ratios tend to be approximately 0.3–0.4 units lower than in buffered and mildly alkaline solutions (4). Elution in water is a common practice when working with RNA, ostensibly to avoid alkaline hydrolysis of RNA. To document the effect of dilution of RNA in water on the concentration values and purity ratios measured, a total RNA sample isolated from rat liver using the Monarch Total RNA Miniprep Kit (NEB #T2010) was used to create an RNA dilution series in water. Subsequent measurements of sample concentration, shown in Table 5A, page 7 demonstrate that measured values in water are inaccurate and show a high degree of variation (yellow). In fact, concentration values

under 20 ng/µl progress toward an underestimation, while values over 20 ng/µl trend toward overestimation. However, when the same dilution series is created with 10 mM Tris pH 8.0, concentration values are significantly more accurate and reproducible, thereby eliminating the need to carry out fluorescence measurements as an alternative.

A_{260}/A_{280} ratios were 1.75–1.80 and A_{260}/A_{230} ratios were 1.95–2.0, both with high variation (**yellow**). When using Tris buffer, both purity ratios are around 2.1 with sufficiently concentrated samples (**green**) (Table 5B).

The most concentrated RNA samples in water showed higher A_{260}/A_{280} ratios. The RNA sample that was used for the dilution series had a A_{260}/A_{280} of 2.1. This observation is consistent with RNA eluted from the column in water being buffered by residual components from the wash buffer. Once the eluate is diluted 10-fold in water, the remaining buffering components lose their influence. A similar effect is observed with DNA.

SUMMARY:

- RNA samples that are dissolved in water have A_{260}/A_{280} ratios that are 0.3–0.4 unit lower than buffered and mildly alkaline samples
- RNA and DNA measurements in water are inaccurate and highly variable
- Using Tris buffer for spectrophotometric analysis is a good alternative to water and eliminates the need for fluorescence measurements
- Water eluates from silica columns are still slightly buffered by residual buffer components from previous wash steps

TABLE 5: **Solution of RNA in water leads to low A_{260}/A_{280} ratios and inaccurate and highly variable concentration values**

Total RNA isolated from rat liver using the Monarch Total RNA Miniprep Kit was eluted in water and diluted with water or 10 mM Tris-Cl pH 8.0 to the RNA concentrations indicated. Concentrations (A) and purity ratios (B) were measured in triplicate on the Nanodrop One MVS and averages were listed in the respective tables. Concentration values were compared to triplicate concentration measurements carried out with the Qubit BR RNA Kit.

A

	NANODROP WATER			NANODROP TRIS			QUBIT		
RNA INPUT CONCENTRATION (ng/µl)	NANODROP CONCENTRATION (ng/µl)	DEVIATION (%)	RELATIVE STANDARD DEVIATION (%)	NANODROP CONCENTRATION (ng/µl)	DEVIATION (%)	RELATIVE STANDARD DEVIATION (%)	NANODROP CONCENTRATION (ng/µl)	DEVIATION (%)	RELATIVE STANDARD DEVIATION (%)
1	0.54	-46.12	63.5	1.08	8.32	11.6	0.88	-11.53	7.1
2.5	1.53	-39.00	25.3	2.27	-9.31	1.5	2.17	-13.04	11.1
5	3.71	-25.77	15.3	4.37	-12.60	3.8	4.60	-8.04	6.1
7.5	6.11	-18.55	11.6	6.72	-10.42	2.1	7.14	-4.78	4.7
10	8.53	-14.72	6.0	9.16	-8.42	2.9	9.72	-2.82	5.0
15	14.35	-4.34	9.0	13.65	-8.99	2.9	15.07	0.46	4.5
20	20.32	1.60	6.8	19.34	-3.28	1.1	20.14	0.71	3.3
25	25.22	0.86	3.0	23.71	-5.17	1.2	24.26	-2.96	4.0
30	30.78	2.61	2.3	28.64	-4.52	0.8	29.49	-1.71	3.7
40	45.27	13.17	1.2	39.32	-1.70	0.4	42.48	6.20	1.1
50	53.92	7.83	0.4	51.26	2.51	1.2	49.97	-0.05	0.4
100	108.96	8.96	1.4	105.08	5.08	0.3	105.45	5.45	2.6
200	215.39	7.69	0.1	203.29	1.65	0.1	198.07	-0.96	1.1

B

	NANODROP WATER		NANODROP TRIS			NANODROP WATER		NANODROP TRIS	
RNA CONCENTRATION (ng/µl)	260/280	RELATIVE STANDARD DEVIATION (%)	260/280	RELATIVE STANDARD DEVIATION (%)	RNA CONCENTRATION (ng/µl)	260/230	RELATIVE STANDARD DEVIATION (%)	260/230	RELATIVE STANDARD DEVIATION (%)
1	1.40	91.9	4.70	83.4	1	-0.98	-210.6	6.51	85.3
2.5	1.75	25.2	2.45	16.0	2.5	2.83	201.6	2.18	69.9
5	1.86	8.7	2.18	7.2	5	1.85	58.6	2.50	45.0
7.5	1.87	3.9	2.27	5.7	7.5	2.27	38.7	2.23	10.4
10	1.75	10.1	2.09	8.4	10	2.05	19.5	2.14	4.0
15	1.77	10.5	2.16	6.7	15	1.86	24.9	2.17	5.6
20	1.76	13.4	2.12	2.9	20	1.93	22.1	2.07	3.3
25	1.71	8.8	2.12	3.2	25	1.97	17.6	2.00	0.5
30	1.73	3.0	2.11	2.2	30	1.95	12.4	2.05	3.1
40	1.75	3.4	2.09	2.5	40	2.03	9.8	2.04	1.5
50	1.80	4.2	2.09	1.5	50	2.02	7.1	1.94	6.1
100	1.89	1.2	2.09	0.4	100	1.99	3.5	2.06	0.8
200	1.96	0.8	2.10	0.2	200	2.04	1.4	2.08	0.5

CONCLUSION

Microvolume spectrophotometers are an excellent and convenient way to quantitate NA samples and analyze their purity. However, as described above, for both quantitation and purity analysis, there are limitations that should be considered.

- Microvolume spectrophotometers can be used for concentration of samples down to 1 ng/µl, but dilute samples should be repeated for accuracy
- Purity ratios of samples below 20 ng/µl are not reliable and remain quite variable for samples between 20–50 ng/µl
- The impact of protein contamination on purity ratios is relative to DNA or RNA concentration; dilute NA samples will look highly contaminated, concentrated NA samples may look almost clean
- In contrast to the widely held belief about the role of the A_{260}/A_{280} when evaluating protein contamination, the A_{260}/A_{230} is a more sensitive indicator for protein contamination
- The UV absorbance spectrum can be used to assess the presence of buffer components like GTC, Triton X-100, EDTA or phenol, but often, the detected components do not negatively affect downstream applications
- Proteins and polysaccharides can be present in substantial amounts but may only have limited impact on the purity ratios
- Slight variations in free EDTA concentration in DNA samples may result in unusually high A_{260}/A_{230} ratios (3.0 or higher)
- The A_{260}/A_{280} can be used as an indicator for RNA contamination in DNA solutions. Significant changes in this ratio, however, are only seen with RNA contaminations >15%.
- When NAs are eluted from the silica membrane with water, they may still be slightly buffered from previous wash steps
- Measuring NAs in water leads to highly inaccurate and variable values and significantly lower A_{260}/A_{280} ratios. Measuring them in Tris buffer solves these issues.

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