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Nucleotides, Oligonucleotides, and Polynucleotides

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NUCLEOTIDES

Nomenclature: De facto and Du jour

Lehninger (1975) provides a thorough discussion of proper nucleotide nomenclature and abbreviations. Unfortunately,

commercial catalogs and occasionally the research literature introduce different notations. Some consider "NTP" a general term for deoxynucleotides, but the absence of the letter "d" indicates a ribonucleotide to others. Commercial literature also describes ribonucleotides as "RTP's." If the letter "d" is present, the name describes a deoxynucleotide. If "d" is absent, check the literature piece closely to avoid a common purchasing error. Dideoxynucleotides are generically referred to as "ddNTP's."

What Makes a Nucleotide Pure?

Using dATP as an example, what categories of impurities could be present? One potential contaminant is a nucleotide other than dATP, such as dCTP. A second class of impurity could be the mono-, di-, or tetraphosphate form of the deoxyadenosine nucleotide. Since most if not all commercial nucleotides are chemically synthesized from highly analyzed precursors, contamination with a nucleotide not based on deoxyadenosine is very unlikely. A third class of impurities is the non-UV-absorbing organic and inorganic salts accumulated during the synthesis and purification procedures.

While essentially all commercial nucleotides are chemically synthesized, the final products are not necessarily identical. Manufacturing processes vary; raw materials and intermediates of the nucleotide synthesis reactions are subjected to different purification strategies and processes. It is these intermediate steps, and the scrutiny of the products' final specifications, that allow manufacturers to legitimately claim that nucleotides are extremely pure.

A formal definition of *extremely pure* does not exist, but commercial preparations of such products typically contain greater than 99% of the desired nucleotide in the triphosphate form. Contaminating nucleotides are rarely detected in commercial preparations, even using exceedingly stringent high-performance chromatography procedures, but some contaminants escape HPLC detection. Freedom from non-UV-absorbing materials is typically judged by comparison of a measured molar extinction (A_m) coefficient to published extinction coefficients (ε)values. Nuclear magnetic resonance (NMR) may also be used to monitor for contaminants such as pyrophosphate.

Are Solution Nucleotides Always More Pure Than Lyophilized Nucleotides?

Nucleotides were first made commercially available as solvent-precipitated powders. The lyophilized and extremely pure solution

forms appeared in the early 1980s. Some lyophilized preparations approach 98% purity or more but rarely match the >99% achieved by extremely pure solutions. Generally, solution nucleotides are purer than the lyophilized version, but unless supporting quality control data are provided, it should not be concluded that a solution nucleotide is extremely pure or even more pure than a lyophilized preparation.

Are Solution Nucleotides More Stable Than Lyophilized Nucleotides?

Peparations of deoxynucleoside triphosphates decompose into nucleoside di- and tetraphosphates via a disproportionation reaction. This reaction is concentration and temperature dependent. At temperatures above 4°C, lyophilized preparations of deoxynucleotides undergo disproportionation faster than nucleotides in solution. In contrast, the rate of degradation for both forms is less than 1% per year at –20°C and below (Table 10.1). Solutions of dideoxynucleotides and ribonucleotides are similarly stable for many months at temperatures of –20°C and below. Most, but not all, dideoxy- and ribonucleotides are stable for many months at 4°C.

Table 10.1 Storage Stability of Nucleotides

		% Triphosphate Form			
	Months	–70°C	–20°C	4°C	21°C
Powder					
dATP	54	99.44	99.14	97.47	93.93 (48 mo) 97.78 (3 mo)
dCTP	54	98.46	95.46	39.3 (33 mo)	39.45 (2.75)
dGTP	54	96.95	95.37	25.74 (27 mo)	34.4 (1.75)
dTTP	54	97.29	94.28	27.4 (30 mo)	39.45 (2.75 mo)
dUTP	N.A.	N.A.	N.A.	N.A.	N.A.
Solution (100 mM)					
dATP	54	99.2	98.75	95.3	91.8 (2 mo) 37.07 (39 mo)
dCTP	54	99.38	99.15	96.98	95.2 (2 mo) 21.25 (42 mo)

Table 10.1 (*Continued*)

	Months	% Triphosphate Form			
		–70°C	–20°C	4°C	21°C
Powder					
dGTP	54	99.63	98.83	95.47	90.5 (2 mo) 19.7 (42 mo)
dTTP	54	99.44	98.87	93.54	95.6 (2 mo) 0.07 (42)
dUTP	54	99.23	98.02	71.55	90.1 (1.2 mo) 40.13 (6 mo)
Solution (10 mM)					
dATP	15		99.68	99.59 (12 mo)	88.6 98.5 (2 mo)
dCTP	15		98.2	99.56 (12 mo)	86.11 98.85 (2 mo)
dGTP	15		98.6	99.51 (12 mo)	89.47 98.35 (2 mo)
dTTP	15		93.57	99.29 (12 mo)	81.05 98.86 (2 mo)
dUTP	15		93.8	99.45 (12 mo)	84.95 98.5 (2 mo)
Solution ddNTP (10 mM)					
ddATP	3		99.69	99.49	94.52
ddCTP	3		100	98.51	97.38
ddGTP	3		98.4	98.08	94.23
ddTTP	3		99.36	99.13	87.06
Solution ddNTP (5 mM)					
ddATP	3		99.77	98.12	68.56
	4		99.63	96.31	2
ddCTP	3		98.77	100	98.4
	4		99.27	99.46	93.72
ddGTP	3		95.61	98	96.67
	4		98.25	97.9	93.68
ddTTP	3		93.1	55.09	49.03
	4		94.25	63.23	3.6
RTP Solutions (100 mM)					
ATP	3		98.57	98.18	95.39
CTP	3		99.25	99.43	98.43
GTP	3		98.46	98.44	96.82
UTP	3		99.71	99.69	97.99

Source: Data based on chromatographic separation of nucleotide species via high performance chromatography on an Amersham Pharmacia Biotech FPLC® System.
Notes: Each sample, 0.2 μmoles (0.2 ml of a 1 mM solution) was injected onto a Mono Q® Ion Exchange column. Using the following buffers:

Buffer A, 5mM sodium phosphate, pH 7.0.
Buffer B, 5mM sodium phosphate, 1M NaCl, pH 7.0.

purification was achieved via a gradient of 5–35% NaCl over 15 minutes using a flow rate of 1 ml/min. Nucleotide peaks were detected at of 254 nm. (Data from Amersham Pharmacia Biotech, 1993a.)

Does Your Application Require Extremely Pure Nucleotides?

Only you can answer this question. Most applications have supporters and detractors for the use of extremely pure nucleotides.

How Can You Monitor Nucleotide Purity and Degradation?

Nucleotides produce very specific spectroscopic absorbance data. Absorbance ratios not within predicted ranges (Table 10.2) indicate a contaminated deoxy- or ribonucleotide, such as if dATP and dCTP were accidentally mixed together. This technique is adequate to quickly determine if a large contamination problem exists, but a high-performance liquid chromatography approach is required to detect minor levels of impurities.

The absorbance ratio will not indicate when the triphosphate form of a nucleotide breaks down into the di- and tetraphosphate forms. This form of degradation can be monitored most effectively

Table 10.2 Nucleotide Absorbtion Maxima

Nucleotide	Lambda Maximum (pH 7.0)	A_m (pH 7.0) molar extinction coefficient
2′-dATP	259 nm	15.2×10^{3d}
2′-dCTP	280 nm[a]	$13.1 \times 10^{3a,e}$
2′-dGTP	253 nm	13.7×10^{3f}
2′-dITP	249 nm	$12.2 \times 10^{3b,h}$
2′-dTTP	267 nm[b]	9.6×10^{3g}
2′-dUTP	262 nm	10.2×10^{3i}
c7-2′-ATP	270 nm	12.3×10^{3j}
c7-2′-dGTP	257 nm	10.5×10^{3c}
2′,3′-ddATP	259 nm	15.2×10^{3d}
2′,3′-ddCTP	280 nm[a]	$13.1 \times 10^{3a,e}$
2′,3′-ddGTP	253 nm	13.7×10^{3f}
2′,3′-ddTTP	267 nm	9.6×10^{3g}
ATP	259 nm	15.4×10^{3}
CTP	280 nm[a]	13.0×10^{3a}
GTP	252 nm	13.7×10^{3}
UTP	262 nm	10.2×10^{3}

Note: The spectral terms and definitions used are those recommended by the National Bureau of Standards Circular LCD 857, May 19, 1947.
[a] Spectral analysis done at pH 2.0.
[b] Spectral analysis done at pH 6.0.
[c] Value determined at Amersham Pharmacia Biotech.
[d] 2′-dAMP NRC reference spectral constants employed.
[e] 2′-dCMP NRC reference spectral constants employed.
[f] 2′-dGMP NRC reference spectral constants employed.
[g] 2′-dTMP NRC reference spectral constants employed.
[h] 2′-dIMP NRC reference spectral constants employed.
[i] 2′-dU NRC reference spectral constants employed.
[j] Leela and Kehne (1983).

by high-performance chromatography, but when such equipment is unavailable, thin layer chromatography can provide qualitative data (Table 10.3).

How Should You Prepare, Quantitate, and Adjust the pH of Small and Large Volumes of Nucleotides?

The following procedure can be used to prepare solutions of deoxynucleotides, ribonucleotides, and dideoxynucleotides provided that the different formula weights are taken into account.

A 100 mM solution of a solid nucleotide triphosphate is prepared by dissolving about 60 mg per ml in purified H_2O. The exact weight will depend on the formula weight, which will vary by nucleotide, supplier, and salt form. As solid nucleotide triphosphates are very unstable at room temperature, they should be stored frozen until immediately before preparing a solution.

Quantitation

Spectroscopy

The most accurate method of quantifying a solution is to measure the absorbance by UV spectrophotometry. A dilution should be made to obtain a sample within the linear range of the spectrophotometer. The sample should be analyzed at the specific λ_{max} for the nucleotide being used. The concentration can then be obtained by multiplying the UV absorbance reading by the dilution factor, and dividing by the characteristic A_m for that nucleotide. These data are provided in Table 10.2.

Table 10.3 TLC Conditions to Monitor dNTP Degradation

dNTP	R_f, Principal	R_f, Trace	Solvent System
dATP	0.25	0.35 (dADP)	A
dCTP	0.15	0.21 (dCDP)	A
dGTP	0.27	0.34 (dGDP)	B
dTTP	0.14	0.21 (dTDP)	A

Note: Solvent System A: Isobutyric acid/concentrated NH_4OH/water, 66/1/33; pH 3.7. Add 10 ml of concentrated NH_4OH to 329 ml of water and mix with 661 ml of isobutyric acid.
Solvent System B: Isobutyric acid/concentrated NH_4OH/water, 57/4/39; pH 4.3. Add 38 ml of concentrated NH_4OH to 385 ml of water and mix with 577 ml of isobutyric acid.
TLC Plates: Eastman chromagram sheets (#13181 silica gel and #13254 cellulose).

Weighing

One would think that the mass of an extremely pure nucleotide could be reliably determined on a laboratory balance. Not so, because during the manufacturing process, nucleotide preparations typically accumulate molecules of water (via hydration) and counter-ions (lithium or sodium, depending on the manufacturer), which signficantly contribute to the total molecular weight of the nucleotide preparation. Unless you consider the salt form and the presence of hydrates, you're adding less nucleotide to the solution than you think. The presence of salts and water also contribute to the molecular weights of oligo- and polynucleotides, which are also most reliably quantitated by spectroscopy.

pH Adjustment

The pH of a solution prepared by dissolving a nucleotide in water will vary, depending on the pH at which the nucleotide triphosphate was dried. An aqueous solution of nucleotide triphosphate prepared at Amersham Pharmacia Biotech will have a pH of approximately pH 4.5. The pH may be raised by addition of NaOH (0.1 N NaOH for small volumes, up to 5 N NaOH for larger volumes). Approximately 0.002 mmol NaOH per mg nucleotide triphosphate is required to raise the pH from 4.5 to neutral pH. If the pH needs to be lowered, addition of a H^+ cation exchanger to the nucleotide solution will lower the pH without adding a counter-ion. The amount of cation-exchanger resin per volume of 100 mM nucleotide solution varies greatly depending on the starting and ending pH. For very small volumes (<5 ml) of nucleotide solutions, a 50% slurry of SP Sephadex can be added dropwise. For larger volumes (>5 ml), solid cation exchanger can be added directly in approximately 0.2 cm^3 increments. The cation exchanger can be removed by filtration when the desired pH is obtained.

The triphosphate group gives the solution considerable buffering capacity. If an additional buffer is added, the pH should be checked to ensure that the buffer is adequate. The pH should be adjusted when the solution is at or near the final concentration. A significant change in the concentration will change the pH. An increase in concentration will lower the pH, and dilution will raise the pH, if no other buffer is present.

Similar results will be obtained for all of the nucleotide triphosphates. Monitor the pH of the solutions as a precaution; purines are particularly unstable under pH 4.5, and all will degrade at acid pH.

Example

To prepare a 10 mM solution from a 250 mg package of dGTP, the dGTP may be dissolved in about 40 ml of purified H_2O. The pH may then be adjusted from a pH of about 4.5 to the desired pH with 1 N NaOH, carefully added dropwise with stirring. About 0.5 ml of 1 N NaOH will be needed for this example. A dilution of 1:200 will give a reading in the linear range of most spectrophotometers. Spectroscopy should be performed at the nucleotide's absorbance maximum, which is 253 nm for dGTP. In this example an absorbance of about 0.700 is expected. The formula for determining the concentration is:

$$\frac{\text{Absorbance at } \lambda_{max} \times \text{ dilution factor}}{A_m} = \text{molar concentration}$$

Using the A_m for dGTP of 13,700, the concentration in this example is found to be

$$\frac{0.700 \times 200}{13,700} = 0.0102\,\text{M}, \quad \text{or} \quad 10.2\,\text{mM dGTP}$$

What Is the Effect of Thermocycling on Nucleotide Stability?

Properly stored, lyophilized and solution nucleotides are stable for years. The data in Table 10.4 (Amersham Pharmacia Biotech, 1993b) describe the destruction of nucleotides under common thermocycling conditions. Fortunately, due to the excess presence of nucleotides, thermal degradation does not typically impede a PCR reaction.

Is There a Difference between Absorbance, A_{260}, and Optical Density?

Readers are strongly urged to review Efiok (1993) for a thorough and clearly written discussion on the spectrophotometric quantitation of nucleotides and nucleic acids.

*Absorbance (*A*)*

Absorbance (A), also referred to as optical density (OD), is a unitless measure of the amount of light a solution traps, as measured on a spectrophotometer. The Beer-Lambert equation (Efiok, 1993) defines absorbance in terms of the concentration of the solution in moles per liter (C), the path length the light travels through the solution in centimeters (l), and the extinction coefficient in liter per moles times centimeters (E):

Table 10.4 Breakdown of Nucleotides under Thermocycling Conditions

		% Purity of Triphosphate	
	Nucleotides	0 PCR Cycles	25 PCR Cycles
Experiment 1	dATP	99.31	92.41
	dCTP	99.47	93.64
	dGTP	99.14	92.43
	dTTP	99.06	93.38
Experiment 2	dATP	99.56	94.17
	dCTP	99.80	95.36
	dGTP	99.78	94.02
	dTTP	99.60	94.17
Experiment 3	dATP	99.40	92.02
	dCTP	99.66	93.84
	dGTP	99.39	92.68
	dTTP	99.15	93.69
Experiment 4	dATP	99.44	92.77
	dCTP	99.59	93.89
	dGTP	99.43	92.88
	dTTP	99.19	93.65

Source: Data from Amerhsam Pharmacia Biotech (1993b).
Note: Each nucleotide was mixed with 10× PCR buffer from the GeneAmp® PCR Reagent Kit (Perking Elmer catalogue number N801-0055)to give a final nucleotide concentration of 0.2 mM in 1× PCR buffer. Noncycled control samples (0 cycles) were immediately assayed. Test samples were cycled for 25 rounds in a Perkin Elmer GeneAmp® PC System 9600 using the cycling program of 94°C for 10 seconds, 55°C for 10 seconds, and 72°C for 10 seconds. After cycling, the samples were stored on ice until assayed.

For analysis, samples were diluted to give a nucleotide concentration of 0.133 mM. The diluted samples were then assayed on FPLC® System using a MonoQ® column. The assay time for a sample was 10 minutes using a sodium chloride gradient (50–400 mM) in 20 mM Tris-HCl at pH 9.0. Nucleotide peaks were detect using a wavelength of 254 nm.

$$A = ClE$$

Since the units of C, l, and E all cancel, A is unitless.

Absorbance Unit

Also referred to as an optical density (OD) unit, an absorbance unit (AU) is the concentration of a material that gives an absorbance of one and therefore is also a unitless measure. Typically, when working with nucleic acids, we express the extinction coefficient in ml per mg times cm:

$$E = \frac{\text{ml}}{\text{mg} \times \text{cm}}$$

Using an extinction coefficient expressed in these terms, one A_{260} unit of double-stranded DNA has a concentration of DNA of 50 μg/ml.

For practical reasons, suppliers typically define the total volume of material to be one milliliter when selling their nucleic acids.

Note that from a supplier's perspective, an A_{260} unit specifies an amount of material and not a concentration. It is the amount of material in one milliliter that gives an absorbance of one. The A_{260} unit value provided by a supplier cannot be substituted into the Beer-Lambert equation to calculate concentration. If this substitution is done, the concentration will be off by a factor of 1000.

*Extinction Coefficient (*E*)*

Also known as absorption coefficient, absorptivity, and absorbency index, the proportionality constant E is a constant value inherent to a pure compound. E will not vary between different lots of a chemical. The units of E are typically ml/mg-cm or L/g-cm. It is experimentally measured by utilizing a method that is not affected by the presence of a contaminant. For example, the extinction coefficient of a nucleotide can be determined by measuring the amount of phosphorous present.

As in the Beer-Lambert equation, the concentration (C) of a solution in mg/ml or g/L = A/El.

Molar Extinction Coefficient (ε) versus A_m

The molar extinction coefficient (also referred to as molar absorbtivity) describes the absorbance of 1 ml of a 1 molar solution measured in a cuvette with a 1 cm path length. For practical reasons a manufacturer may measure a molar coefficient by weighing an amount of the solid material, mixing into a solution and measuring the absorbance of that solution. This way, a molar coefficient is calculated that is not a true molar extinction coefficient because it is affected by the presence of contaminants. To set this measured coefficient apart from a true molar extinction coefficient, companies use the symbol A_m. The A_m for a given chemical will vary from preparation to preparation depending on the presence of contaminants. Using nucleotides as an example, the number of sodium and water molecules present in the finished product can vary from lot to lot, causing the A_m values to also vary slightly between lots. The units of A_m are L/mol-cm.

*Suppose that you have 100 μl of a 5 mM solution of a nucleotide with a molar extinction coefficient of 10.4×10^3, how many A_{260} units do you have? Using the Beer-Lambert equation, the undi-

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luted 5 mM solution of this nucleotide will have an absorbance of 52. $A = 10.4 \times 10^3\,\text{L/(mol} \times \text{cm)} \times 0.005\,\text{M} \times 1\,\text{cm} = 52$. This measure of absorbance is a unitless measure of the opacity of the solution and is independent of the volume of the solution.

To calculate the A_{260} units present as a supplier would define an A_{260} unit, the volume of the solution must be taken into account. This is simply done by multiplying the volume of the solution in milliliters by the absorbance measurement. For the 100 μl of a solution with an absorbance of 52, the number of A_{260} units present is 5.2 units (i.e., 52×0.1 ml = 5.2 units).

Why Do A_{260} Unit Values for Single-Stranded DNA and Oligonucleotides Vary in the Research Literature?

The A_{260} unit values are generated by rearranging the Beer-Lambert equation as per Efiok (1993):

$$OD = ECL$$

$$\frac{C}{OD} = \frac{1}{E} = \frac{1}{\text{AU}}$$

Substituting the value of $E_{1\text{cm}}^{1\text{mg/ml}}$ in Table 10.5 generates the conversion factors to A_{260} data into mg/ml of nucleic acid.

Manufacturer technical bulletins (Amersham Pharmacia Biotech, 2000) and protocol books (Ausubel et al., 1995; Sambrook, Fritsch, and Maniatis, 1989) frequently cite different values for single-stranded DNA and oligonucleotides. Since nucleotide sequence and length alter the value of an extinction coefficient, the variability amongst A_{260} conversion factors is likely caused by the use of different nucleic acid samples to calculate the extinction coefficient. In practice, this means that it probably does not matter which value you use for your work as long as you consistently use the same value for the same type of nucleic acid. However, consider the existence and impact of different conversion factors when attempting to reproduce the work of another researcher.

Table 10.5 Nucleic Acid

	$E_{1\text{cm}}^{1\text{mg/ml}}$ 1	A_{260} (μg/ml)
Double-stranded DNA	20	50
Single-stranded DNA or RNA (>100 nucleotides)	25	40
Single-stranded oligos (60–100 nucleotides)	30	33
Single-stranded oligos (<40 nucleotides)	40	25

Source: From Effiok (1993).

OLIGONUCLEOTIDES

How Pure an Oligonucleotide Is Required for Your Application?

During standard solid phase oligonucleotide (oligo) synthesis, nucleotides are coupled one at a time to a growing chain attached at its 3′ end to a solid support (unlike enzymatic DNA synthesis, chemical DNA synthesis occurs in the 3′ to 5′ direction). To prepare an oligonucleotide where the majority of the product is full length, a coupling efficiency of ≥98% at each nucleotide addition is required. At lower coupling efficiencies, the synthesis will yield a significant amount of oligos that are not full length (failure sequence).

Oligonucleotide impurities may consist of various forms of the desired sequence as well as impurities from the reagents used in synthesis. The ammonium hydroxide that detaches the oligonucleotide from the solid support of a DNA synthesizer and buffer salts carried over from a purificaton process can also be troublesome. Ammonium ions are inhibitory to T4 Polynucleotide kinase, so if the the oligo isn't properly de-salted, subsequent end-labeling reactions will fail.

Your application dictates the level of acceptable purity. The ammonium ions carried over from detaching the oligo from the solid support can completely inhibit end labeling but not other reactions. An oligo preparation that contains less than 50% full-length product will produce miserable sequencing results, but might function as a PCR primer. If your oligo functions reproducibly and verifiably generates data, it's sufficiently pure.

What Are the Options for Quantitating Oligonucleotides?

The concentration of oligonucleotides is most commonly approximated by applying the Beer-Lambert law and a conversion factor ranging from from 25 to 37 μg per A_{260} unit. This approach is inexact, but it is reliable for common molecular biology techniques as long as its limitations are considered. Computer software that predicts an extinction coefficient based on nucleotide sequence and nearest-neighbor analysis is also available. Such predictive software should be employed with caution, since it does not take into account a number of factors, such as the degree of base stacking and the presence of alternate structures commonly found among nucleic acids, that significantly influence the magnitude of the extinction coefficient.

If an exact extinction coefficient is required, a method that directly calculates the quantity of the nucleic acid is required. The

phosphate analysis method of Griswold et al. (1951) is described below.

The method of Griswold et al. (1951) is based on a colorimetric assay (A_{820}) employing ANS (aminonaphtosulfonic acid) dissolved in a sulfite/bisulfite solution. The reaction requires the presence of molybdate prepared in 10 N sulfuric acid. A carefully prepared phosphate solution is utilized to obtain a standard curve by serial dilution (10–100 μM phosphate). DNA test solutions of known absorbance at 260 nm are digested with nuclease P1 and alkaline phosphatase. The phosphate released from the digestion is quantified by monitoring the blue color development at 820 nm following reaction with ANS solution in the presence of molybdate in acidic solution and incubation at 95°C for 10 minutes. The extinction coefficient is determined in accordance with the following equation:

$$E_{260} = \frac{A_{260\,\mathrm{nm}}}{\text{phosphate}\,(\mu\mathrm{M}) \times (n-1)}$$

where A_{260nm} is the original absorbance of the DNA solution, phosphate (μM) represents the value obtained in triplicate of the digested DNA solution extrapolated from the standard phosphate curve, and n is the number of bases comprising the oligonucleotide.

As with nucleotides, determining the amount of an oligo is best done by measuring the absorbance. If you prefer to measure the mass on a very accurate analytical balance, take into account the presence of contaminating salts and water.

What Is the Storage Stability of Oligonucleotides?

The fundamentals of safe DNA storage are discussed in Chapter 7, "DNA Purification," and RNA storage is discussed in Chapter 8, "RNA Purification." Lyophilized oligonucleotides are stable for months or years stored at –20°C and colder in frost-free or non-frost-free freezers. Solutions of DNA oligonucleotides are best stored at –20°C and below at neutral pH. Non-frost-free freezers are preferred to eliminate potential nicking due to freeze–thawing.

In one instance, which was not further investigated, approximately 10% of the phosphate groups were lost from the 5′ ends of phosphorylated oligo dT (approximately 15 nucleotides in length) after 12 months of storage at –20°C (Amersham Pharmacia Biotech, unpublished observations).

Your Vial of Oligonucleotide Is Empty, or Is It?

Lyophilization does not always produce a neat pellet at the bottom of the vial. The material might be dispersed throughout the inner walls of the vial in a very thin layer that is difficult to see. The best method to confirm the absence of the material is to dissolve the vial's contents by thoroughly pipetting the solvent on the vial's inner walls and measuring the absorbance at 260 nm.

SYNTHETIC POLYNUCLEOTIDES

Is a Polynucleotide Identical to an Oligonucleotide?

Manufacturers typically define polynucleotides as single- or double-stranded nucleic acid polymers whose length exceeds 100 nucleotides. Double-stranded polymers can be comprised solely of DNA or RNA, or DNA : RNA hybrids. As illustrated in Figure 10.1, a single preparation of a synthetic polynucleotide contains a highly disperse population of sizes. In comparison, oligonucleotides are almost always single-stranded molecules (RNA or DNA) shorter than 100 nucleotides and typically comprised of a nearly homogeneous population in length and sequence.

Polymer nomenclature is not universally accepted, but the major suppliers apply the following strategy:

- Poly dA—single-stranded DNA homopolymer containing deoxyadenosine monophosphate.
- Poly A—single-stranded RNA homopolymer comprise of adenosine monophosphate.
- Poly A · oligo $dT_{12\text{-}18}$—Double-stranded molecule, with one strand comprised of an RNA homopolymer of adenosine

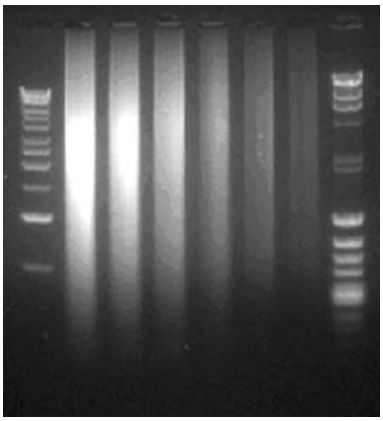

Figure 10.1 Lane 1–1 kb ladder; lane 2–7 poly (dI-dC) · (dI-dC); lane 2–2.0 μg; lane 3–1.5 μg; lane 4–1.0 μg; lane 5–0.5 μg; lane 6–0.25 μg; lane 7–0.125 μg; lane 8–Lambda HindIII/phi X174 Hinc II marker.

monophosphate; a mixture of DNA oligonucleotides 12 to 18 deoxythymidine monophosphates in length and randomly bound throughout the poly A strand.

- Poly dA-dT single-stranded DNA polymer comprised of alternating deoxyadenosine and deoxythymidine monophosphates.
- Poly dA·dT double-stranded DNA polymer containing deoxyadenosine monophosphate in one strand, and deoxythymidine monophosphate in the complementary strand.
- Poly (dA-dT)·(dA-dT) double-stranded DNA polymer comprised of alternating deoxyadenosine and deoxythymidine monophosphates in each strand.

Do double-stranded polynucleotides possess blunt or sticky ends? Yes to both, as explained below.

How Are Polynucleotides Manufactured and How Might This Affect Your Research?

The length of commercially produced polynucleotides varies from lot to lot. Polynucleotides are synthesized by polymerase replication of templates or by the addition of nucleotides to the 3′ ends of oligonucleotide primers by terminal transferase or poly A polymerase. These enzymatic reactions are difficult to regulate, so polymer size significantly varies between manufacturing runs. A second factor that affects the size of double-stranded polynucleotides is that these polymers are affected by annealing conditions. Double-stranded polymers may be produced by synthesizing each strand indpendently and then annealing the two independent strands. In reality, the annealing reaction consists of annealing two populations of strands, each with its own distribution of sizes. Depending on the actual composition of these two populations and the exact annealing conditions, the resulting population of the annealed double-stranded polymer may vary widely (see the discussion about structural uncertainty below for a related case).

Manufacturers apply analytical ultracentrifugation, gel electrophoresis, or chromatography to analyze polymer length. Commercial suppliers provide an average size of the polymer population, but they usually don't indicate the proportion of the different size polymers within a preparation. For example, two lots might have an average size of 500 bp; lot 1 might have a larger proportion of 800 bp polymers and lot 2 a larger proportion of polymers 300 bp in length. Will this affect your experiments? This question can be answered conclusively only at the lab bench, so it

is a good idea to consider performing control experiments when using a new lot of polymer for the first time.

Structural Uncertainty

What is the basic structure of a double-stranded polymer? Is it blunt ended? Will it have overhangs? How long are the overhangs? There is no single answer to these questions due to the heterogeneous nature of the product and the impact of the exact conditions used for dissolving the polymer. The buffer composition, temperature of dissolution, and volume of buffer used will all affect the final structure of the dissolved polymer.

Heterogeneous Nature

If you add equimolar amounts of a disperse mixture of poly dA and a disperse mixture of poly dT, what are the odds that two strands bind perfectly complementary to form a blunt-ended molecule? What's the likelihood of generating the same overhang within the entire population of double-stranded molecules? Does one strand of poly dA always bind to one strand of poly dT, or do multiple strands interact to form concatamers? See Figure 10.2 for examples. Considering the heterogeneous population of the starting material, one should assume that a highly heterogeneous population of double-stranded polymers forms.

Buffer Composition

Double-stranded polynucleotides are usually supplied as lyophilized powders that may or may not contain buffer salts. The pH, salt concentration, and temperature of the final suspension affect the structure of the dissolved polymer. For example, at any specific temperature, the strands of poly dA·dT resuspended in water dissociate much more frequently than the same polymer dissolved in 100 mM sodium chloride. Heating a polymer solution to 85°C for 10 minutes followed by quick chilling on ice produces a different population of polymers compared to poly dA·dT dissolved in the same buffer at room temperature.

Consider these solution variations when attempting to reproduce your experiments and those cited in the literature.

Figure 10.2 Variable products when annealing synthetic polynucleotides.

Would the World Be a Better Place If Polymer Length Never Varied?

Poly (dI-dC)·(dI-dC) is commonly applied to reduce nonspecific binding of proteins to DNA in band shift (gel retardation) experiments. The polymer's average size varies from hundreds of base pairs to several kilobase pairs. Two researchers from one laboratory used the same lot of poly (dI-dC)·(dI-dC) in experiments with different protein extracts. This one lot of poly (dI-dC)·(dI-dC) produced wonderful band shift results for the first scientist's protein extract, and miserable results for the second researcher's extract. Is this Nature's mystique or a lack of optimized band shift conditions?

Oligonucleotides Don't Suffer from Batch to Batch Size Variation. Why Not?

Oligonucleotides are almost always chemically synthesized on computer-controlled instruments, minimizing variation between batches. Different batches of the same oligonucleotide are identical in sequence and length provided that they are purified to homogeneity.

How Many Micrograms of Polynucleotide Are in Your Vial?

At least one manufacturer of polymers reports the absorbance units/mg specification for each lot of polymer. The data from three lots of poly (dI-dC)·(dI-dC) are listed below:

	Absorbance units/mg	μg/absorbance unit
Lot *A*	9.0	111
Lot *B*	13.7	73
Lot *C*	10.4	96

Why is there so much μg/unit variation among the three lots? How should you calculate the mass of material in different lots of this polymer? Should you use 50 μg/unit as you would for double-stranded DNA, or the μg/unit calculated above?

In the tradition of answering one question with another, ponder this. Why do manufacturers quantitate most of their polymer products in terms of absorbance units rather than micrograms? What are the possible explanations?

- It's easier to quantitate polymers on a spectrophotometer than to weigh them on a scale.

- DNA isn't the only material present in the polymer preparation.
- 100 units sounds more generous than 5 mg.

Despite multiple purification procedures that include extensive dialysis, other materials such as water and salts can accumulate in polynucleotide preparations. Since polynucleotides absorb light at 260 nm and the common contaminants do not, manufacturers package polymers based on absorbance units to guarantee that researchers get a consistent amount of nucleic acid.

So, if you choose to define experimental conditions using mass of polymer, use spectrophotometry and a conversion factor. Common conversion factors are 50 μg/absorbance unit (260 nm) for double-stranded DNA polynucleotides, 37 or 33 μg/absorbance unit for single-stranded DNA, and 40 μg/absorbance unit for single-stranded RNA. A conversion factor for synthetic RNA:DNA hybrids has not been defined. Some researchers apply 45 μg/absorbance unit, a compromise between the RNA (40 μg) and DNA (50 μg) values.

Be careful about weighing out an amount of polymer for use in an experiment, or quantitating polymers based on the absorbance units/mg reported within the package insert of a commercial product. Both approaches assume that the polymer is 100% pure and are likely to give higher variation in experimental conditions when changing lots of polymer from the same manufacturer or switching between manufacturers of a polymer.

Is It Possible to Determine the Molecular Weight of a Polynucleotide?

Once the average length of the polymer is known, a theoretical average molecular weight can be calculated based on the molecular weight of each strand or the molecular weight of nucleotide base pairs. Just remember that these calculations are based on the average lengths of disperse populations of polymers.

What Are the Strategies for Preparing Polymer Solutions of Known Concentration?

Suppose that your task was to prepare a 10 μM solution of poly dT. Theoretically you could prepare a solution that was 10 μM relative to the poly dT polymer (molarity calculations would be based on the average molecular weight reported on the manufacturer's certificate of analysis), or 10 μM relative to the deoxythymidine monophosphate (dT) nucleotide that comprises the polymer.

The preferred approach for preparing a polymer solution of a particular molar concentration is to express all concentrations in a concentration of bases or base pairs. The reason for this is that the best way to determine the amount of polymer present is by measuring absorbance. In addition, since the population of polymer molecules is so disperse, approximating the concentration of polymer based on strands of polymer may be misleading. Finally, this approach will maximize the reproducibility of your experiments between different lots of polymer and for those who try to reproduce your work.

10 μM of the dT Nucleotide

As described above, polymer solutions are best quantitated via a spectrophotometer. Before you go to the lab, grab some paper and perform a couple of quick calculations. First, using the molar extinction coefficient, calculate the absorbance of a 10 μM solution. The molar absorbtivity of poly dT is 8.5×10^3 L/mol-cm-base at 264 nm and pH 7.0. This means one mole of dT monomers in one liter will give an absorbance of 8500. Therefore a 10 μM solution (i.e., 0.000010 M) will have an absorbance of 0.085 (i.e., 8500×0.000010).

Next calculate the dilution required of 50 absorbance units to give the absorbance of a 10 μM solution (i.e., 0.085). If you have a vial with 50 absorbance units of polymer and you dissolved the entire 50 absorbance units in 1 ml of buffer, the spectrophotometer would hypothetically measure an absorbance close to 50. To obtain an absorbance of 0.085, the total dilution of the 50 absorbance units would be 588-fold (i.e., 50/0.085 = 588).

In the lab you would never dissolve the entire 50 absorbance units in 588 ml. First, this would limit you to using the polymer at concentrations of 10 μM or less. Second, the dilution may not work as you theoretically calculated. And finally, if the dilution did work as you expected, the solution would have an absorbance of less than 0.100 and therefore not be reliably measured by a spectrophotometer. In practice, you would prepare a stock solution of approximately 10 times the final desired concentration and then dilute to a range that can be measured by a spectrophotometer.

Your Cuvette Has a 10 mm Path Length. What Absorbance Values Would Be Observed for the Same Solution If Your Cuvette Had a 5 mm Path Length?

Half the path length, half the absorbance.

Why Not Weigh out a Portion of the Polymer Instead of Dissolving the Entire Contents of the Vial?

As discussed earlier, would you be weighing out DNA polymer or DNA polymer and salt? Also DNA polymers are very stable in solution when stored at –20°C or colder. (If you have a choice, store unopened vials of polymer at –20°C or colder; see below.) Aliquot your polymer stocks to avoid freeze–thaw nicking and contamination problems.

Is a Phosphate Group Present at the 5′ End of a Synthetic Nucleic Acid Polymer?

Synthetic DNA and RNA polymers are produced by adding nucleotides to the 3′ end of an oligonucleotide primer or by replicating a template by a nucleic acid polymerase. If the primer is phosphorylated, and if the mechanism of the DNA polymerase produces 5′ phosphorylated product, one could conclude that the polymer contains a 5′ phosphate. If your purpose is to end-label a polymer via T4 polynucleotide kinase, it's safest to assume that a phosphate is present, and either dephosphorylate the polymer or perform the kinase exchange reaction (Ausubel et al., 1995).

What Are the Options for Preparing and Storing Solutions of Nucleic Acid Polymers?

Synthetic polymers comprised of RNA and DNA are most stable (years) when stored as lyophilized powders at –20°C or –70°C. Polymer solutions are stable for several months or longer when prepared and stored as described below.

Double-Stranded Polymers

Concentrated Stock Solutions

To maintain principally the double-stranded form of synthetic DNA and DNA–RNA hybrids requires a minimum of 0.1 M NaCl, or lower concentrations of bivalent salts present in the solution (Amersham Pharmacia Biotech, unpublished observations). In the absence of salt, the two strands within a polymer can separate (breathe) throughout the length of the molecule. While its presence won't harm polymers during storage, salt could hypothetically interfere with future experiments. If this is a concern, polymers destined for use in double-stranded form can also be safely stored for months or years in neutral aqueous buffers (i.e., 50 mM Tris, 1 mM EDTA) at –20°C or –70°C, even though they will likely be in principally single-stranded form when heated to room temperature and above.

Preparing Solutions for Immediate Use

DNA alternating co-polymers such as poly (dI-dC)·poly (dI-dC) can be prepared in the salt buffers described above, heated to 60°–65°C, and slowly cooled (no ice) to room temperature to reanneal the strands. Duplexes of poly (dA)·poly (dT) require the salt buffers above, and should be heated to 40°C for 5 minutes, and slowly cooled to room temperature. Duplexes of poly (dI)·poly (dC) and RNA·DNA hybrids require salt buffers and heating to 50°C for 5 minutes, followed by slow cooling. Poly (dG)·poly (dC) can be difficult to dissolve. Even after heating to 100°C and intermittent vortexing, some polymer would not go into solution (A. Letai and J. Fresco, Princeton University, 1986, personal communication).

Single-Stranded Polymers

Single-stranded DNA and RNA polymers are stable in neutral aqueous buffers. Depurination will occur if DNA or RNA polymers are exposed to solutions at pH 4 or lower. In addition, for RNA polymers, pH of 8.5 or greater may cause cleavage of the polymer. Carefully choose your buffer strategy for RNA work, since the pH of some buffers (i.e., Tris) will increase with decreasing temperature.

If a single-stranded DNA polymer is difficult to dissolve in water or salt, heat the solution to 50°C. If heating interferes with your application, make the polymer solution alkaline, and after the polymer dissolves, carefully neutralize the solution (Amersham Pharmacia Biotech, unpublished observations).

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