INTEGRATED DNA TECHNOLOGIES

Oligonucleotide Yield, Resuspension, and Storage

Contents

1. Introduction

Scale refers to the amount of starting material which is composed solely of the most 3' nucleotide of a sequence attached to a solid support and housed within the column used to make the oligonucleotide. **Yield** refers to the amount of final product recovered after all of the synthesis and purification steps have been completed.

Other issues arising from custom oligonucleotide synthesis are **resuspension** of the lyophilized oligonucleotide once it reaches the hands of the researcher and **storage** of the resuspended material prior to use. In this report we address each of these issues and provide recommendations that we have found to be useful.

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2. Yields of Custom Synthesis Reactions

Custom oligonucleotide synthesis begins with specification of the desired sequence in an oligonucleotide synthesis platform. Specification is composed of three crucial elements: the actual sequence that is to be made, the identification of any desired modifications, and verification of the scale at which the synthesis is to be carried out. This third element determines the choice of a column in which the synthesis will be performed. Synthesis columns permit a one-way flow of reagents from the synthesis platform through a precisely defined physical space containing and confining the growing oligonucleotide. Columns are prepared with a fixed amount of the most 3' nucleotide attached to a solid support, the controlled pore

glass (CPG) bead. It is the amount of the most 3′ nucleotide present in the column, in nanomoles, that constitutes the synthesis scale.

Once the column is properly prepared with the correct 3′ nucleotide at the desired scale, it is placed in the synthesis platform and the rest of the oligonucleotide is synthesized by adding each base of the specified sequence one at a time in the 3′→5′ direction. Because of chemical and physical restraints, the coupling efficiency is less than 100% at each step in the synthesis. In addition, coupling efficiency varies for each base both by type and position in the growing oligonucleotide [1, 2]. Experience gained at Integrated DNA Technologies from the synthesis of millions of oligonucleotides shows that some sequences will result in better yields than others. For example, one 24mer may give twice the yield of another 24mer even if they are synthesized on the same machine, the same day, and with the same reagents. Another source of yield variation can be the specific machine on which the synthesis is carried out. For this reason, Integrated DNA Technologies maintains a rigorous maintenance and monitoring program designed to keep each synthesis platform functioning at peak efficiency at all times.

Even with the variables of custom oligonucleotide synthesis, it is possible to derive a theoretical yield for any given synthesis. Making the operational assumption that coupling efficiency for each nucleotide will be constant regardless of type and location, theoretical yield is given as,

$$Y = (\text{eff})^{n-1}$$

where (eff) is the average coupling efficiency and n is the number of bases in the oligonucleotide. Monitoring efforts at Integrated DNA Technologies confirm that our average coupling efficiency exceeds 99% for all oligonucleotides. Thus, theoretical yield for a 24mer will be 89.1% full-length product (FLP) at 99.5% average coupling efficiency and 79.4% FLP at 99.0% average coupling efficiency. A more complete picture of the relationship between coupling efficiency and %FLP for oligonucleotides of various lengths is shown in Figure 1. Two aspects of this relationship are readily apparent. First, the cumulative effect of even a 0.5% average coupling failure rate can be dramatic for longer oligonucleotides. Second, minor increases in average coupling failure rates will have a substantial net effect on even average length oligonucleotides. It is for this

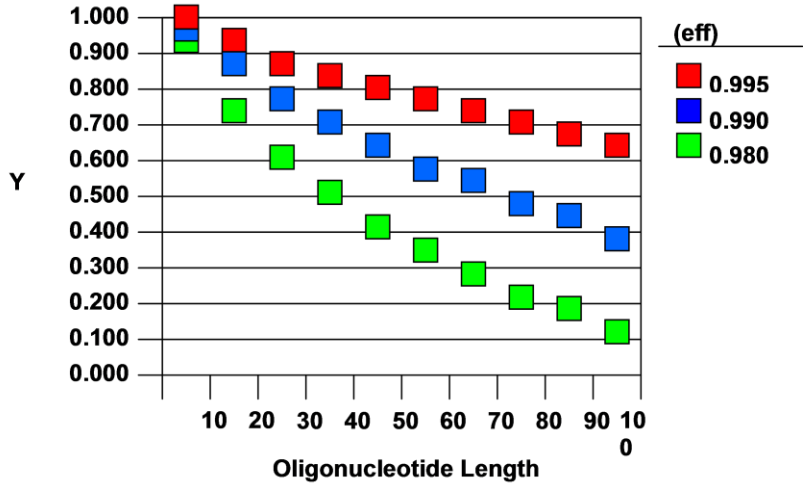

Figure 1. Relationship between average coupling efficiency and synthesis yield ($Y = (\text{eff})^{n-1}$) over a range of oligonucleotide lengths.

reason that Integrated DNA Technologies maintains constant, real-time monitoring of every custom synthesis reaction on every synthesis platform.

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3. Synthesis Scale, Purification, and Yield

3.1 Scale and Yield

Integrated DNA Technologies routinely offers yield guarantees based upon a combination of oligonucleotide length and synthesis scale. These guarantees are shown in Table 1. Synthesis of very long oligonucleotides (those greater than 60 bases) is problematic. Thus, Integrated DNA Technologies does not offer any yield guarantees for oligonucleotides greater than 60 bases at our highest synthesis scale and greater than 100 bases at any scale.

Table 1
Yield Guarantees (in Optical Density Units; ODs) by Synthesis Scale

Length	Unmodified, Desalted Scale			
	25 nmol	100 nmol	250 nmol	1 µmol
5–9			5	10
10–14		2	5	15
15–19	3	4	19	30
20–60	3	6	15	45
61–80		6	15	45
81–100			15	45
Modifications can affect yield; please contact Customer Care for yield on specific modified oligonucleotides (800-328-2661) or custcare@idtdna.com				

3.2 Purification and Yield

As a general rule, IDT recommends that any oligonucleotide longer than 40 bases should receive further purification. In addition, for demanding applications such as site-directed mutagenesis, cloning, and gel-shift protein-binding assays, additional purification is recommended even for oligonucleotides shorter than 40 bases. Our experience has shown that taking the time to purify an oligonucleotide used in the more demanding applications saves far more in terms of the precious commodities of time and research funds than it costs on the front end. Thus, additional purification should be considered for any oligonucleotide that is to be used for any application other than routine PCR or DNA sequencing.

IDT offers preparative-scale purification via denaturing polyacrylamide/urea gels (PAGE) and HPLC. While both of these methods will result in greatly increased oligonucleotide purity, one approach to purification may be superior to the other depending upon the intended use of the oligonucleotide and the presence or absence of modifications. A summary of general recommendations is presented in Table 2 below. Please note that additional purification will result in a decrease in final oligonucleotide yield. Yield guarantees for various combinations of synthesis scale and purification are presented in Table 3.

Table 2
General Oligonucleotide Purification Recommendations

	PAGE	**Purification Method RP-HPLC***	**IE-HPLC***
Basis of Purification:	Length	Hydrophobicity	Length
Mass Recovery (estimated):	20–50%	40–70%	30–60%
Pros	Best method to enrich the proportion of full-length product	Best method for oligonucleotides with a hydrophobic group	Good means of purifying large scale syntheses (≥10 µmole)
Cons	Only for syntheses ≤ 1 µmole	Does not remove (n-1)-mers very efficiently	Not good for oligonucleotides ≥ 80 bases
Recommendation	Use for any oligonucleotide ≥ 50 bases	Use for oligonucleotides ≤ 50 bases if intended for: - Site directed mutagenesis - Cloning, screening - Gel shift assays Use with any oligonucleotide modified with a hydrophobic group; i.e., Biotin, Digoxigenin, NHS-ester conjugates, Fluorescent dyes	Use for large scale syntheses and for *in vivo* applications

*RP-HPLC (reverse-phase), IE-HPLC (ion-exchange)

Table 3
Yield Guarantees (in Optical Density Units; ODs) by Synthesis Scale and Method of Purification. Note that these guarantees refer to unmodified oligonucleotides

Unmodified, HPLC-purified				
Length	**Scale**			
	25 nmol	100 nmol	250 nmol	1 µmol
5–9			2	2.5
10–14		1	3	5
15–19		1	4	10
20–60		1	4	20
61–65		1	4	20
66–70		1	3	15
71–75		1	3	15
76–80		1	2	10
81–100			2	10
Unmodified, PAGE-purified				
Length	**Scale**			
	25 nmol	100 nmol	250 nmol	1 µmol
5–9			0	1.3
10–14		0.5	1	2.5
15–19		0.5	1.5	5
20–50		1	2	10
51–60		0.5	2	10
61–65		0.5	2	10
66–70		0.5	1.5	7.5
71–75		0.5	1.5	7.5
76–80		0.5	1	5
81–100			1	5
Modifications can affect yield; please contact Customer Care for yield on specific modified oligonucleotides (800-328-2661) or custcare@idtdna.com				

In specific instances, IDT requires additional purification of an oligonucleotide synthesis. One of these is any oligonucleotide of any length that is modified with phosphorothioate intended for use as an anti-sense agent. The impurities resulting from phosphorothioate syntheses are toxic in tissue culture as well as in *in vivo* applications. Other circumstances in which IDT requires additional purification of an oligonucleotide synthesis are presented in Table 4 below. Also presented in Table 4 are circumstances for which we strongly recommend additional purification

Table 4
IDT Purification Requirements and Recommendations

	5′ Modifications	Internal Modifications	3′ Modifications
Purification Is Required	Biotin dT Biotin-TEG Dual Biotin Fluorescein dT I-Linker 5-Br dU *Bodipy® 630/650-X *Bodipy® 650/665 Cy3™ Cy5™ Cy5.5™ 2,6,-Diaminopurine Digoxigenin NHS JOE NHS *Oregon Green™ 488-X *Oregon Green™ 514 *Rhodamine Green-X *Rhodamine Red-X ROX NHS TAMRA NHS Texas Red-X NHS	Biotin dT Fluorescein dT 5-Br dU *Bodipy® 630/650-X *Bodipy® 650/665 2,6,-Diaminopurine JOE NHS *Oregon Green™ 488-X *Oregon Green™ 514 *Rhodamine Green-X *Rhodamine Red-X ROX NHS TAMRA NHS Texas Red-X NHS	Biotin-TEG Dabcyl *Bodipy® 630/650-X *Bodipy® 650/665 Cy3™ CPG Cy5™ CPG Cy5.5™ CPG Digoxigenin NHS JOE NHS *Oregon Green™ 488-X *Oregon Green™ 514 *Rhodamine Green-X *Rhodamine Red-X ROX NHS TAMRA CPG/NHS Texas Red-X NHS
Purification Is Optional	Acrydite™ Amino C6 Amino C12 Amino dT 2-Aminopurine Biotin C3 Spacer dSpacer 6-FAM HEX Inosine 5-Me-dC 5-Nitroindole Phosphate Spacer 18 TET Thiol C6 Uridine Uni-Link Amino	Amino dT 2-Aminopurine C3 Spacer dSpacer Inosine 5-Me-dC 5-Nitroindole Spacer 18 Uridine	Amino C7 Biotin Dideoxycytidine 6-FAM Inosine Inverted dT Phosphate 3' Ribo Bases Thiol C3 Uridine
*** NHS ester dye from Molecular Probes, Inc.**			

4. Resuspension, Quantification, and Storage

4.1 Resuspension

Oligonucleotides are shipped in dry (lyophilized) form unless otherwise requested. Dried DNA is usually very easy to resuspend in an aqueous solution. However, not all oligonucleotides dry identically and some can take a bit more time to completely go into solution than others. In addition, if the oligonucleotide solution freezes during the dry-down process in the Speed-Vac it will appear as a white powder similar in appearance to a piece of tissue or a kimwipe. In such cases it is possible for the dried oligonucleotide to become dislodged from the tube during shipping. Thus, it is very important to spin every oligonucleotide prior to opening the tube for resuspension.

We recommend using TE buffer (10 mM Tris pH 8.0; 0.1 mM EDTA; pH 8.0) because the buffer will maintain a constant pH. The oligo should not be exposed to conditions that are either too acidic or too basic. Alternative, sterile water can be used for resuspending dry oligonucleotides. If using water for resuspension, be sure to use only nuclease-free water, pH 7.0 (HPLC-grade is preferable). DEPC water will harm oligonucleotides and water from deionizing systems can be acidic with pH as low as 5.0.

Oligonucleotides should be aliquoted into portions for immediate use and those for longer term storage in order to avoid contamination. Stock concentrations can be made using the yield information contained on the "spec sheet." There you will find the actual yield of the oligonucleotide synthesis in three forms: optical density (OD) units; mass in milligrams (mg); and copy number in nanomoles (nm). We routinely resuspend dry oligonucleotides to a storage stock of 100 µM and then dilute working stocks accordingly. Adding TE or water at ten times the number of nanomoles will give a 100 µM final concentration. This concentration provides 100 pmoles of oligonucleotide per µL. Most PCR reactions will use 10 to 50 pmoles of each primer per reaction.

To make a 100 µM concentration: Take the number of nmoles of oligo in the tube and multiply that by 10. This number will be the number of µL of buffer to add to get a 100 µM solution. For example, if you have 9 nmoles of oligo, add 90 µL of buffer to make a 100 µM solution.

For those researchers who prefer to work in mass units, the amount of oligonucleotide present in each tube in OD units and weight can be used. A 20mer oligonucleotide primer with random base composition will have a molecular weight of ~6100 and a molar extinction coefficient of 196900 L/mole-cm. 1 OD_{260} unit of this oligonucleotide will therefore correspond to 31 µg, or 5 nmoles, of DNA. Dissolving 500 µg of DNA (16 OD units) in 500 µL of TE will yield a stock primer concentration of 1 µg/µL, or about a 160 µM solution. This converts to 160 pmoles of oligonucleotide per µL. Most PCR reactions set up using mass units will use about 60 ng of each primer. Working stocks should be set up accordingly. A dilution calculator is also available in SciTools on the IDT website.

An oligo can be stored at any concentration. However, concentrations lower than 1 µM may change over time as some of the oligo may be absorbed into the plastic of the tube. In addition, a 5–10 mM solution is generally the highest concentration at which an oligo will go into solution.

For hard to suspend oligos, heat the oligo at 55^{o} C for 1–5 minutes, then thoroughly vortex. If this does not work, the tube might have Trityl (flakey appearance) or CPG (a pellet at bottom of tube). Neither of these should affect the performance of the oligo, and both can be removed with a Sephadex G50 column, or by removing the supernatant.

4.2 Verifying the Spec Sheet

The heterocyclic ring structures in DNA and RNA absorb light with a maximum absorbance near 260 nanometers (nm). The most accurate means of assessing the amount of oligonucleotide present following synthesis is to measure the optical density of the final product at 260 nm. This value is provided on the spec sheet and it is determined only after purification since unincorporated nucleotides and protecting groups can lead to inaccurate estimates of oligonucleotide mass.

While an estimate of mass will suffice for many applications in which oligonucleotides are used, it may be desirable for the researcher to verify mass after receiving the synthesis. One OD_{260} unit is defined as the amount of oligonucleotide which, when resuspended in a volume of 1.0 mL, results in an absorbance of 1.0 when measured at 260 nm in a 1 cm path-length quartz cuvette. Once an oligonucleotide is resuspended according to the data provided on the spec sheet, replicate samples can be measured in a spectrophotometer at 260 nm and the mass amount can be calculated using the molar extinction coefficient, ε.

The relationship between measured OD_{260}, molar extinction coefficient (ε_{260}), and oligonucleotide concentration is given as,

$$OD_{260} = \varepsilon_{260} * \text{Concentration}$$

Molar extinction coefficient is a unique physical property of every oligonucleotide determined by the sequence. Purine nucleotides will absorb more strongly than pyrimidine nucleotides regardless of whether they occur in DNA or RNA. ε_{260} values for both DNA and RNA nucleotides are,

dA = 15,400, dC = 7,400, dG = 11,500, dT = 8,700
rA = 15,400, rC = 7,200, rG = 11,500, rU = 9,900

in L/mole-cm [3, 4].

However, interactions between neighboring bases alter absorbance in the same manner as such neighbor effect will alter the melting temperature (T_m) of DNA:DNA and RNA:DNA bases pairing. Thus, extinction coefficient is ultimately determined both by base composition and

base order. Taking base stacking interactions into account, nearest-neighbor values for ε_{260} among dinucleotides are;

5′→3′	dA	dC	dG	dT
dA	27,400	21,200	25,000	22,800
dC	21,200	14,600	18,000	15,200
dG	25,200	17,600	21,600	20,000
dT	23,400	16,200	19,000	16,800

5′→3′	rA	rC	rG	rU
rA	27,400	21,000	25,000	24,000
rC	21,000	14,200	17,800	16,200
rG	25,200	17,400	21,600	21,200
rU	24,600	17,200	20,000	19,600

again, in L/mole-cm [5]. In general, then, calculation of the extinction coefficient of an oligonucleotide of length n can be given by the expression,

$$\varepsilon_{260} = \sum_{n-1}^{1} (\varepsilon_{\text{nearest neighbor}}) - \sum_{n-1}^{2} (\varepsilon_{\text{individual}}) \qquad (1)$$

where the second term is a necessary correction for internal bases being counted more than once when nearest-neighbor doublets are summed. A numerical example of this calculation is presented in Appendix I.

Finally, oligonucleotide modifications such as fluorescent dyes increase OD_{260} absorbance values. When calculating extinction coefficients for modified oligonucleotides, it is important to use values for OD_{260} and <u>not</u> the maximum absorbance of the modification. Correct molar extinction coefficients for any modified or unmodified oligonucleotide can be obtained using the on-line OligoAnalyzer 3.0 tool available in SciTools on the IDT website.

4.3 Other Quantification Methods

As noted, the most reliable means of determining oligonucleotide mass and concentration is to use OD_{260}. However, such estimates can also be obtained by running oligonucleotides against known mass standards on polyacrylamide gels. The best results will be achieved using denaturing urea-based gels (7 M urea, 1X TBE) as these gels will, for the most part, eliminate the problem of secondary structure formation. DNA bands can be visualized using UV backshadowing against a TLC plate where the DNA will appear as dark bands against a light background. At Integrated DNA Technologies we examine every oligonucleotide synthesis at the 250 nmole scale or larger using this method. While it is a reasonably reliable method, UV visualization on polyacrylamide gels does require a substantial amount of the synthesis (5–6 mg) which is non-recoverable.

Agarose gels electrophoresis with ethidium bromide visualization is not a reliable method for quantifying oligonucleotides because ethidium bromide is an intercalating agent requiring double-stranded structures. This means that only oligonucleotides having secondary structures can be visualized while those that do not form secondary structures are unable to provide a target for ethidium bromide intercalation.

Finally, there are other fluorescent dyes that will bind to DNA but most of them, like ethidium bromide are useful only against double-stranded substrates. Some dyes, like OliGreen and SYBR Green II will preferentially bind single-stranded species but the fluorescence they produce is not directly quantitative. In order to produce even reasonable quantification using these reagents, they must be run against known masses so that a set of standard curves can be produced for comparison.

4.4 Storage

Store resuspended oligonucleotides in several small aliquots at –20°C.

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5. References

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2. Hecker KH and Rill RL. (1998) Error analysis of chemically synthesized polynucleotides. Biotechniques, 24(2): 256–260.
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6. Appendix I

A Numerical Example of the Molar Extinction Coefficient
As an example of the method IDT uses to calculate the molar extinction coefficient, consider the M13 forward sequencing primer (–20). The sequence is:

5'–GTA AAA CGA CGG CCA GTG–3'

Applying equation 1 with the empirically derived ε_{260} values shown in the text, we see that,

$$\varepsilon_{260} = (\varepsilon_{GT} + \varepsilon_{TA} + \varepsilon_{AA} + \varepsilon_{AA} + \varepsilon_{AA} + \varepsilon_{AC} + \varepsilon_{CG} + \varepsilon_{GA} + \varepsilon_{AC} + \varepsilon_{CG} + \varepsilon_{GG} + \varepsilon_{GC} + \varepsilon_{CC} + \varepsilon_{CA} + \varepsilon_{AG} + \varepsilon_{GT} + \varepsilon_{TG}) - (\varepsilon_{T} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{A} + \varepsilon_{C} + \varepsilon_{G} + \varepsilon_{A} + \varepsilon_{C} + \varepsilon_{G} + \varepsilon_{G} + \varepsilon_{C} + \varepsilon_{C} + \varepsilon_{A} + \varepsilon_{G} + \varepsilon_{T})$$

ε_{260} = (20,000 + 23,400 + 27,400 + 27,400 +27,400 + 21,200 + 18,000
+ 25,200 + 21,200 + 18,000 + 21,600 + 17,600 + 14,600 + 21,200
+ 25,000 + 20,000 + 19,000) – (8,700 + 15,400 + 15,400 + 15,400
+ 15,400 + 7,400 + 11,500 + 15,400 + 7,400 + 11,500 + 11,500
+ 7,400 + 7,400 + 15,400 + 11,500 + 8,700)

= (368,000) – (185,400) = 182,600

Knowing this value and the total number of OD_{260} units from the synthesis, the total number of molecules present (in millimoles) can be derived. If, for example, there are 16.1 OD_{260} units from a synthesis of the M13 (–20), then,

16.1 / 182,600 = 0.00008817 mmoles = **88.17 nmoles**.

Finally, knowing the number of molecules and the molecular weight of the oligonucleotide will provide the total weight, in milligrams, of the synthesis. The M13 (–20) weighs 5557.7 g/mole. Thus,

(0.00008817) (5557.7) = 0.49002722 mg = **490.03 μg**

These values, accounting for minor rounding errors, are what are provided by OligoAnalyzer 3.0 available on-line in IDT SciTools if the M13 (–20) sequence is entered.