Designing and Using Oligos

A guide to working with oligonucleotides

Content

Introduction

Oligonucleotides (or oligos for short) are synthetic strands of DNA or RNA that are commonly used in molecular biology techniques such as PCR, qPCR, Sanger sequencing, site-directed mutagenesis, gene construction or Next Generation Sequencing (NGS). Oligos are typically 20 to 100 nucleotides (nts) in length, although there are applications where longer oligos are needed, i.e. →ultramers or even →megamers.

Researchers can customize the oligonucleotide sequence, chemical modifications, and purification based on the intended application and experimental design. There are key factors to consider when designing oligos and universal best practices when handling them. Here, we describe the fundamentals of designing and using oligos and offer tips to avoid common issues.

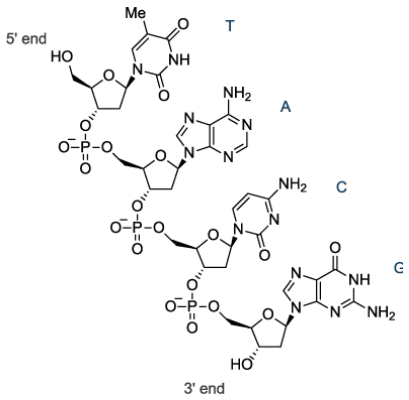

Figure 1: Chemical structure of the oligo dTACG.

Design Considerations

Application

Oligonucleotides are most commonly used as PCR primers for amplification of a specific DNA sequence. Other uses include oligos as sequencing primers, as probes for DNA microarrays and Fluorescence In Situ Hybridization (FISH), as guide or donor molecules for CRISPR, and as antisense therapy to inhibit protein synthesis. The intended use influences the optimal oligo design, which in turn dictates the oligo sequence, length, possible modifications and purification. The most important considerations for primer design are their specificity and melting temperature (Tm) value. Primers should also be free of strong secondary structures and self-complementarity.

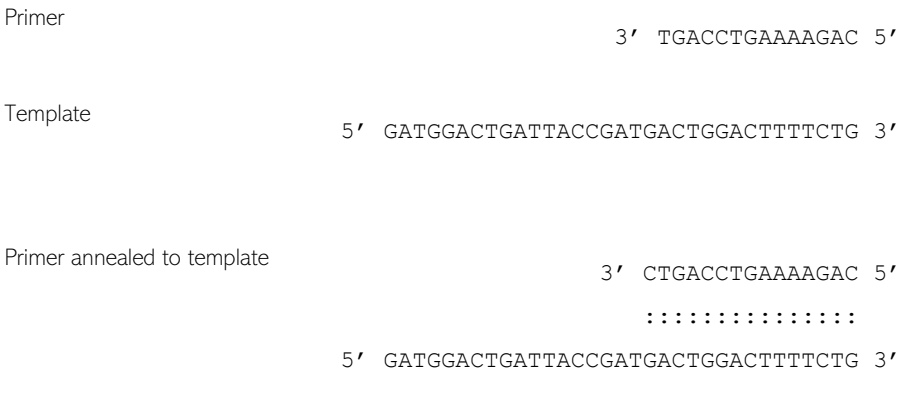

Figure 2: Annealing of a 14-mer oligo to a template sequence. In a PCR application, annealed oligos are extended in a 5' to 3' direction along the template by a DNA polymerase.

Specificity

Oligos should be unique to the target sequence to reduce off-target interactions and enhance probe efficiency.

Tips:

- ✓ Run a BLAST alignment to ensure your primers are unique to the target sequence - this can be done directly using IDT's → OligoAnalyzer Tool.
- ✓ When using oligos in PCR assays and panels for biologically related genes, avoid regions known to have a high rate of single nucleotide polymorphisms (SNPs) and span introns if possible.

Melting Temperature

Melting temperature (T_m) refers to the state where 50% of the oligonucleotides are annealed with their target sequence and 50% are in solution. Factors influencing the melting temperature include the oligo sequence, length, oligo concentration, and buffer composition. The ideal T_m depends on the application.

Tips:

- ✓ T_m increases with GC content (which is influenced by the full sequence and nearest neighbors, i.e. base stacking), with oligo concentration, and with salt concentration (Na^+ and Mg^{2+}).
- ✓ For more information on melting temperature visit: → Understanding melting temperature (Tm) or → PCR and qPCR Oligo Melting Temperature.

Complementarity and Secondary Structures

Avoid and evaluate secondary structures such as self-dimers, heterodimers, and hairpins, as they can reduce the efficiency of the oligo binding to the target sequence. The **Δ**G (free Gibbs energy) value of any self-dimers, hairpins, and heterodimers should be weaker (more positive) than –9.0 kcal/mole. Positive **Δ**G values indicate that the indicated secondary structure will not form at all.

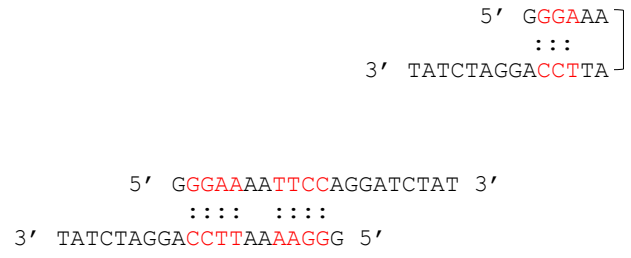

Figure 3: An oligo with hairpin structure (3 bp, ΔG = -0.1 kcal/mol and an oligo with a tendency to form dimers (4 bp, ΔG = -6.6 kcal/mol)

Tips:

- ✓ To reduce secondary structures, avoid oligo-end complementarity (e.g. an oligo with 5' end: CCG and 3' end: GGC).
- ✓ Aim to have 40%-60% GC content within your oligo, and promote binding to the target sequence by ending the 3' end of the oligo with a G or C.

Length

Oligo length is designated by the suffix -mer (e.g. an oligo with 25 nucleotides is a 25-mer). Oligos typically range from 6-60 bases, but the ideal length depends on the application. For example, most PCR primers are 18- to 30-mers, FISH probes are generally 20- to 30-mers, and donor DNA for CRISPR applications are frequently >100-mers. While length is an important consideration, specificity and melting temperature are the most important factors when designing oligonucleotides, which conversely, can be enhanced based on length. For more challenging applications, IDT offers extra-long oligos: →ultramers (up to 200 nucleotides)and →megamers (up to 2000 nucleotides).

Modifications

A variety of modifications can be incorporated into an oligo during synthesis, others must be attached after synthesis, which requires HPLC purification and often results in lower yields. Common modifications include linkers, fluorophores, modified bases, phosphorylation, spacers, click-chemistry modifications, and phosphorothioate bonds. Melting temperature and sequence specificity can change with the addition of modifications. Read more about → Oligo modifications.

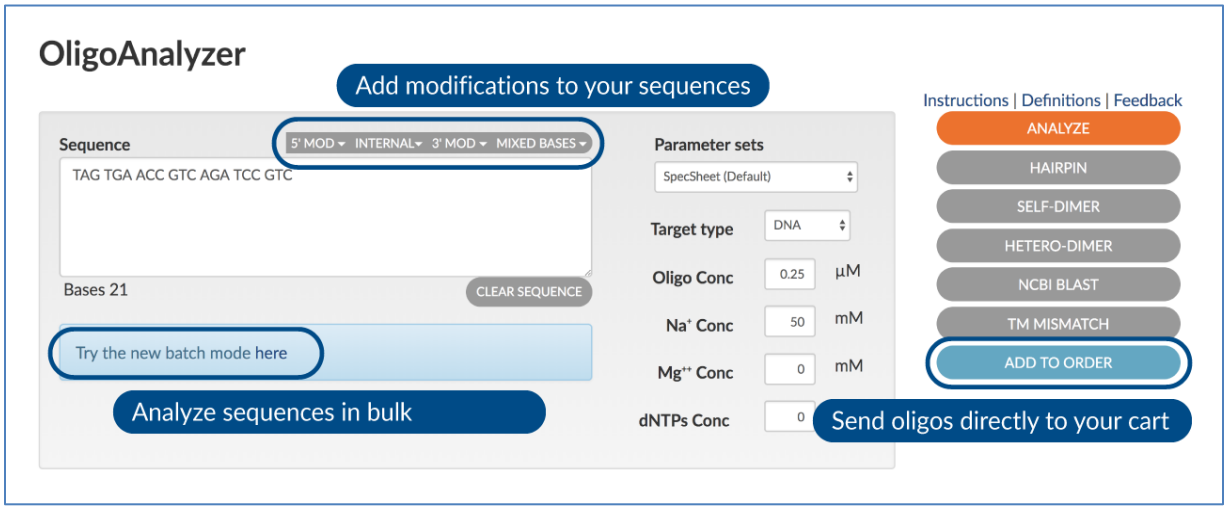

Figure 4: The OligoAnalyzer Tool from IDT is a valuable tool to design, test and optimize your oligo sequences.

Tips:

- ✓ Always Calculate T_m. The T_m values provided on product spec sheets are calculated for specific conditions that likely differ from your reaction conditions. Use a design tool, such as the IDT → OligoAnalyzer Tool, to make accurate calculations based on your own experimental conditions.
- ✓ Tm is affected by the concentration of ions and compounds in solution. Take these into account when calculating T_m.
- ✓ When possible, design your primer and probe sequences around SNPs. When this is not possible, position SNP locations towards the 5' end of the primer or probe sequence where they will have less effect on amplification.
- ✓ Certain modifications, such as locked nucleic acids, provide good mismatch discrimination.
- ✓ You can design probes to the sense or antisense strand, but you must still evaluate their T_m, which may differ.

Purifications

Post-production purification is important for applications that may be sensitive to the presence of truncated oligos and for oligos that are longer than 40 bases or contain modifications such as non-standard bases, fluorescent dyes, and linkers. Common purification methods include high percentage polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC). Purification will result in decreased product yield, but this is offset by the quality of the product.

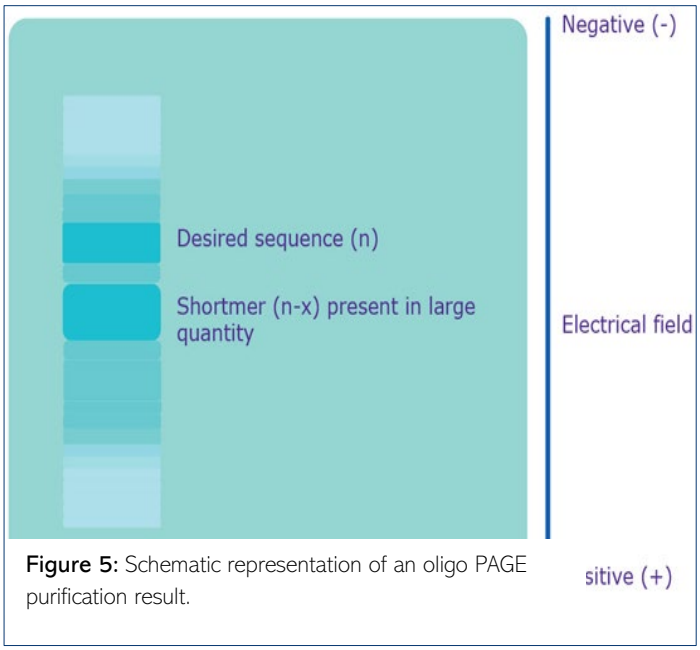

Figure 5: Schematic representation of an oligo PAGE purification result.

PAGE: Recommended for unmodified oligos >60 bases in length or if they will be used for cloning. Routinely achieves >90% purity.

HPLC: Recommended for modified oligos and generally results in a higher yield compared with PAGE. The ideal type of HPLC (reverse-phase vs. ion-exchange), retention time, and phase composition vary depending on the oligo characteristics.

Tips:

- ✓ Standard desalting (performed for every oligo ordered from IDT) is sufficient for most PCR and sequencing applications and for cloning applications with oligos < 60 bp. For cloning applications with oligos > 60 bp or complex applications such as mutagenesis or gel shift assays, PAGE purification is recommended. Oligos containing modifications should always be subjected to HPLC purification.
- ✓ For additional information, visit → Which type of oligo purification should I choose?

Using Oligos

Resuspending Oligos

Oligos are typically shipped lyophilized and should be spun down before opening the tube to avoid dislodging the oligo. Resuspension solutions should not be too acidic or too basic. TE buffer (10 mM Tris pH 8.0; 0.1 mM EDTA; pH 8.0) is the ideal resuspension solution because it will maintain a constant pH, alternatively, nuclease-free water, pH 7.0 can be used. A common best practice is to resuspend the oligo in enough buffer to generate a stock solution of 100 µM (this results in 100 pmoles of oligo per µL) and generate a diluted working stock for the intended application (PCR reactions typically require 10 to 50 pmoles of each primer).

Tips:

- ✓ To make a 100 µM stock concentration, multiply the number of nmoles of oligo by 10. This is the number of µL of buffer to add to the dry oligo. If ordering from IDT, the number of nmoles can be found on the oligo spec sheet.
- ✓ Equation for 100 µM stock: nmoles of oligo * 10 = µL buffer.
- ✓ Avoid making working stocks <1 µM, as plastic tubes can absorb oligos, which will alter the concentration of the stock.
- ✓ Try heating oligos at 55°C for 1-5 minutes and vortexing if oligos are not easily resuspended.
- ✓ If you plan to form a duplex of two oligos, use IDT's duplex buffer for dissolving the oligos rather than standard TE buffer.

Quantifying Oligos

The most accurate way to assess the concentration of an oligo solution is by measuring the optical density at 260 nm (OD_{260}) and using the proper molar extinction coefficient (ε_{260}) in your calculations. For approximating oligo concentration, duplex DNA is about 50 µg/OD_{260}, single-stranded RNA is approximately 40 µg/OD_{260}, and single-stranded DNA is approximately 33 µg/OD_{260}. To measure the concentration, resuspend the oligo in 1.0 mL buffer/nuclease free water and measure the absorbance using a quartz cuvette (1 cm path-length) in a spectrophotometer at 260 nm. Alternatively, use a NanoDrop™ or Qubit® Fluorometer to measure the concentration.

Tips:

- ✓ Sequence, length and modifications can alter the absorbance; use IDT's OligoAnalyzer program to calculate the extinction coefficient and the sequence-specific OD conversion rate (µg/OD260).
- ✓ Confirm the concentration by measuring your stock solutions and working stock solutions following resuspension/dilution and prior to experiments.
- ✓ The most accurate quantification results can be achieved by measuring diluted oligonucleotides with an absorbance <12.5. Use the Beer-Lambert equation to calculate the absorbance:
- ✓ $A = \varepsilon b c$; where A = absorbance, ε = Molar attenuation coefficient (L/(mole·cm), b = path length (cm), c = concentration (mole/L).
- ✓ For more information visit → Oligo quantification - getting it right and if using a NanoDrop™, visit → Tips for accurate oligonucleotide quantification

Storing Oligos

Oligo stability mainly depends on storage buffer and temperature. Oligos resuspended in TE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA), are more stable than dry oligos at room temperature or 4°C; storing oligos in non-DEPC water is the least stable. The optimal storage temperature is -20°C, but oligos are generally stable at 4°C for 60 weeks.

Tips:

- ✓ Avoid contamination and degradation by dividing resuspended samples into smaller aliquots and storing samples at -20°C.
- ✓ Avoid exposure to ambient and UV light by storing oligos in the dark.
- ✓ Store oligonucleotides in polypropylene tubes rather than polystyrene tubes to avoid DNA being absorbed into tube walls and losing material.

Annealing Oligos

Making double-stranded DNA from two complementary oligos is a common protocol. To anneal oligos, centrifuge dried oligos and resuspend in nuclease-free duplex buffer (100 mM Potassium Acetate, 30 mM HEPES pH 7.5) to the desired concentration (10-100 µM). Mix the two oligo strands together in equal molar amounts, heat the oligos to 94°C for 2 minutes in a heating block or water bath and vortex; gradually cool at room temperature. You can verify if your oligos successfully annealed by running them on a 2% non-denaturing PAGE gel with appropriate molecular weight markers, side by side with single-stranded oligo, or using a stain to visualize the bands.

Tips:

- ✓ To avoid secondary structures, slowly cool samples by keeping the oligo mix in the water bath or heat block and turning off the machine.
- ✓ The same method can be used to generate siRNA duplexes.
- ✓ For more information visit: → Annealing oligonucleotides

Troubleshooting

Try these recommendations if you run into trouble with your oligos. Additional troubleshooting information can be found online on →IDT's FAQ page.

- ✓ Check the concentration: Use a spectrophotometer (OD=260nm) and verify the extinction coefficient to get an accurate reading of the concentration prior to starting your next experiment.
- ✓ Quality control: Check the purity of the oligo using mass spectrometry (MALDI-TOF or ESI) or capillary electrophoresis.
- ✓ Buffer choice: Generate a new working stock solution using the appropriate buffer that maintains a proper pH.
- ✓ Adjust the Annealing Temperature (T_a): Use an annealing temperature about 5°C below the T_m of your primers, where T_m of primer is the melting temperature of the less stable primer-template pair. Even better, use a thermocycler with a gradient block to run a set of identical reactions with different Annealing Temperatures. A good starting point is to use 1-2 °C steps – up from T_m if the reaction is unspecific, down from T_m if no PCR product is visible.

Summary

Oligos are used in a vast array of molecular biology applications and are customized to be experiment-specific. Scientists face several considerations when designing oligos and should abide by best practices when using them. IDT offers an abundance of helpful tools and resources for designing, using and troubleshooting oligos. For more information visit: → SciTools Web Tools and → OligoAnalyzer Tool.

Resources

Tools

- → SciTools Web Tools
- → OligoAnalyzer Tool
- → Sequence Alignment - BLAST
- → Resuspension and Dilution Tool

Guides and Useful Information

- → Understanding melting temperature (Tm)
- → PCR and qPCR Oligo Melting Temperature
- → Oligo Modifications
- → PAGE and HPLC Purification
- → Choosing the Purification Method
- → Resuspension and Dilution of Oligos
- → Oligo quantification—getting it right
- → Tips for accurate oligonucleotide quantification
- → Storing Oligos
- → Annealing oligonucleotides
- → Designing Probes for PCR and qPCR
- → IDT Guides and Protocols
- → IDT Oligo FAQs
- → Considering SNPs in Oligo Design