Five essentials for accurate oligonucleotide chemistry

The word *oligonucleotide* is derived from the Greek word *olígoi*, which means “few or small,” and the word nucleotide, which refers to the nucleic acid building blocks found in DNA and RNA. Synthetic oligonucleotides are DNA or RNA polymers that are connected in a repetitive, four-step synthesis reaction, where each nucleotide base (i.e., phosphoramidite, henceforth referred to as amidite) is added to the growing chain. The reaction can be visualized in this short animation. Although oligonucleotide synthesis is a straightforward, cyclic chemical process, many details can go wrong that lead to a low yield of your precious oligonucleotide or result in costly failed runs.

In this white paper, we share some of what we learned in our four decades of experience in flow-through synthesis of oligo manufacturing. Read on to explore five key areas in your oligonucleotide chemistry where improvements will have a huge influence on the quality outcome of your synthesis.

1. How to spot an issue: get to know your chromatogram

Process performance is monitored by continuously measuring UV, conductivity, pressure, and temperature in the flow path throughout the synthesis. These measurements collect multiple data points for each process step in each cycle, providing insights that need to be analyzed to understand the coupling efficiency specifically and how well the synthesis performed overall. Our UNICORN™ software sorts the data and presents them in a chromatogram to show you the integration of data in real-time (this software is available on all our oligo synthesis systems, and the newest systems have the most up-to-date version, UNICORN 7.10 software.) A preset value of percent coupling efficiency in the software pauses the system automatically if a specific threshold is not reached, which will save you time and reduce the consumption of expensive reagents and solvents.

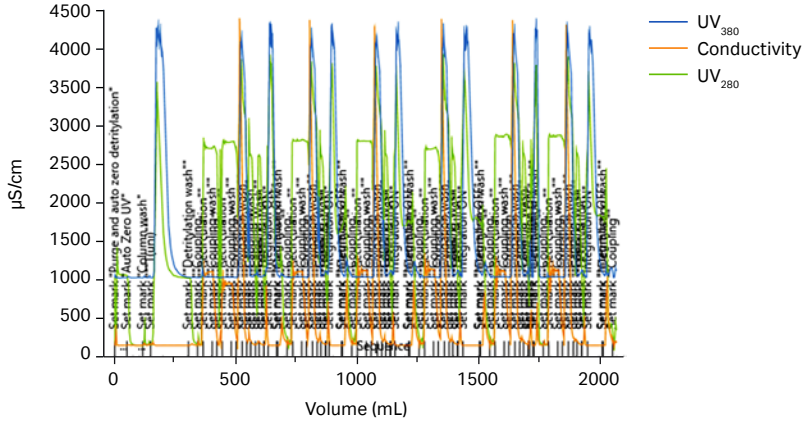

Fig 1. A detailed chromatogram of a full synthesis run. These data were generated using UNICORN 7.8 software.

The chromatogram shows the repetitive pattern of data from each cycle, which makes it easy to spot trends. The trends can provide good evidence that the synthesis is going according to plan or allow you to see when it is not. By zooming in on the chromatogram of a specific cycle, you can easily see data curves from each of the process steps.

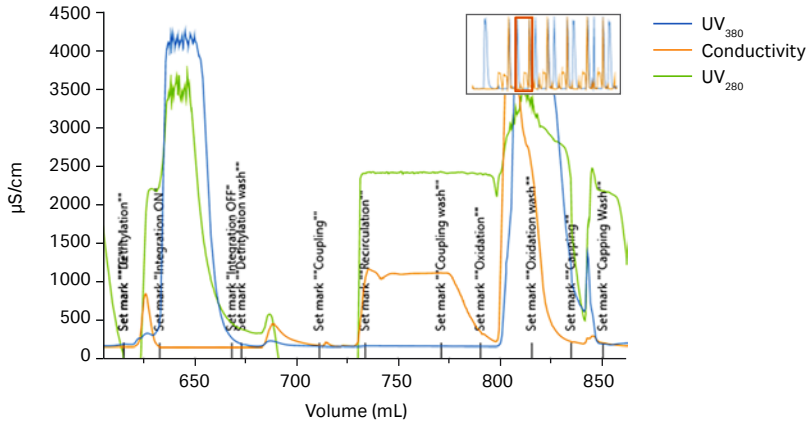

Fig 2. The same chromatogram as Figure 1, zoomed in on one cycle. These data were generated using UNICORN 7.8 software.

The curves are directly linked to each of the reactions taking place in the four-step, cyclic process:

1. Detritylation, or deprotection, of the last base of the growing oligonucleotide makes the base available for coupling with the next base. UV absorbance at 380 nm (A_{380}) measures the 5′ dimethoxytrityl (DMTr) protection group coming off, shown by the dark blue curve in the chromatogram. This curve is automatically integrated to measure coupling efficiency in real time. Detritylation is followed by a thorough wash with acetonitrile to ensure no deprotection liquid is present before the next step starts.
2. In the coupling step, the activator is monitored by its conductivity, shown by the light orange curve. The curve is extended during recirculation when the activator is partly consumed and partly diluted in the recirculation path. Note that the amidite itself is not tracked with conductivity, but it is possible to track with UV.
3. During the oxidation step, which happens after the activator and amidite are washed away by acetonitrile (peak of pressure at the end of coupling reaction), the oxidizer is monitored by UV absorbance (in the current chromatogram the oxidizer is monitored by UV absorbance at 280 nm [A_{280}] and 380 nm [A_{380}]), and the sample conductivity is also measured. Oxidation is a quick reaction, 1 min for DNA and 2 min for RNA. Be sure to keep the timing short and to wash the flow path thoroughly before the next reaction is initiated.
4. The final capping step eliminates any trityl deprotected, yet unreacted, 5′ends of the oligonucleotides. Capping reagents are monitored at A_{280}. Again, wash the flow path before the next cycle starts.

UNICORN software is designed to assist in process development and optimization. It has tools to speed up the evaluation of data either comparing synthesis cycles or different runs of the same sequence.

2. Amidites and reagents: quality and flow path washes

One of the root causes for low yield of a crude oligonucleotide or failed runs is the quality of the reagents and solvents used. The quality of each raw material must meet set specifications to optimize each reaction and reduce the risk of side reactions. We can provide the specifications for reagents and solvents, and we have strong relationships with several suppliers of high-quality reagents. Please be sure to reach out to your local Cytiva representative if you need support.

You also need to properly prepare and store raw materials to eliminate any other risks that could impact the quality of your oligonucleotide. Acetonitrile is the high-volume solvent used in flow-through synthesis, and it needs to be in its driest form. The presence of water interferes with your coupling reaction and ultimately reduces the coupling efficiency. The maximum limit of water is 10 ppm (1), and we recommend molecular sieves in acetonitrile, amidite, and the activator bottles to keep the water content low over time.
You use acetonitrile to wash the flow path thoroughly after each reaction. This wash prevents cross-contamination of different reagents. It also provides you with a clear separation of the different synthesis steps and reduces the risk of unplanned reactions caused by undesired conditions.

The acetonitrile wash also reduces precipitates, which can build up in the system. For example, thiolation reagents often precipitate within a couple of days. A simple way to prevent precipitation is to prepare fresh reagents when they are needed, and you should always rinse the inlet with acetonitrile after adding the reagents. In our experience, filters on the inlet minimize the risk of precipitates entering the flow path.

Amidites should also be made fresh for each run and to the right concentration. Use the optimized equivalents developed for your process and align the volume with the column volume for the scale you intend to synthesize. Low coupling efficiency cannot be overcome by extending the recirculation time. Instead, increase the equivalents and/or concentration to increase efficiency. However, keep the total volume of amidite and activator to 1 to 1.5 column volumes. Once the prepared amidite is connected to the oligonucleotide system, expect a shelf life of 1 to 2 weeks if the amidite is stored correctly in dry conditions. Over time, amidites can clog the inlet line or other parts of the flow path. Clogging can impact delivery of the individual amidites and other reagents depending on where the clog is in the synthesizer.

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Using the correct activator type and concentration is important as they can impact the amidite quality during recirculation. If your amidite quality it too low, impurities can form. Using a higher acidity strength or molar concentration of activator and longer recirculation time can lead to detritylation of amidite, which will also couple and cause formation of N+1 peaks (oligonucleotides with more than the intended number of nucleotides).

3. Reduce risk: stop water from entering the flow path

As stated in the previous section, water can negatively affect your coupling reaction. Therefore, in addition to limiting water content in the solvent and reagents, you should also exclude air from the reagent bottles when setting up the synthesizer.

All our bottles of oligonucleotide consumables are connected to a dry, inert gas (argon or nitrogen) applied with a slight overpressure to act as a blanket between reagents and the outside atmosphere. You can check for the presence of an overpressure in a specific bottle by opening it under properly ventilated conditions and listening for an exhaust of air. Conversely, if the gas line is blocked or clogged, a vacuum is created inside the bottle when the liquid is pumped into the system. This vacuum leads to the delivery of incorrect volumes, which causes significant quality issues on the crude oligonucleotide.

In addition, make sure there are no loose connectors or bottle caps that could cause evaporation of organic solvent from the bottles. Evaporation will impact concentration and volumes (which were previously optimized), and hence affect the reaction in your column.

4. Integrated data: how is my synthesis performing?

Retention and duration of integrated data in the chromatogram should be stable between cycles throughout the synthesis. If this is not the case, then there might be an issue with reaction in the background of the integration.

If the quality of your crude oligonucleotide is poor, start troubleshooting by looking at the chromatogram to observe the in-process analytics. Check the different curves in the chromatogram of the synthesis run to see if they follow a repeating pattern. If this repetitive pattern is disrupted, you can determine which cycle is problematic and which specific process step (or steps) within the cycle is the issue. Using the chromatogram to understand the oligo chemistry will give you good insight into the root cause of any issues.

Incomplete couplings are the main source of impurities and loss of yield. As mentioned, the detritylation peak is integrated in real-time, and the integration gives you a signal whether or not the previous coupling was completed as expected.

Figure 3 shows an example of a declining trend in the detritylation peak after the fifth coupling, which indicates poor couplings. Specific data like these show you where to begin method optimization or troubleshooting. You should initially focus on the two most influential reactions, coupling and detritylation, to understand the underlying issue.

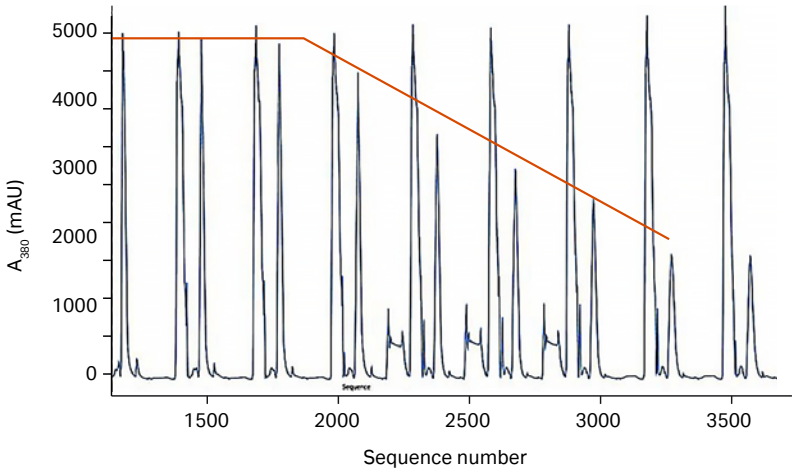

Fig 3. The orange line in this example highlights a negative trend in the detritylation peak area. These data were generated using UNICORN 7.8 software.

There are tools available in UNICORN software for setting percent lower limits for efficiency. If the integration is below these limits, your system will pause to save solvent (to reduce waste and cost) and time (also a cost to your business or research). Depending on where in the cycle the system paused, you might be able to resume the cycle after you've reviewed the issue.

If making longer oligos or specific oligos with higher purine oligonucleotide content, then using higher wavelengths for trityl integration or using volume-based trityl integration may be helpful. If using watch parameters (start and end UV parameters) for trityl integration, the detection wavelength can be adjusted based on sequence to have optimum detritylation reaction time. Standard watch settings are 300 mAU start and 100 mAU end, but changing (e.g., end to 300 mAU) can reduce the detritylation reaction time. Increasing wavelength from 380 nm to 500 nm will also have different exposure to detritylation reaction time. One can increase the flow rate for detritylation solution (standard is 300 to 400 cm/h) to reduce the detritylation solution exposure time or use a fixed column volume (CV) for detritylation if the sequence and methods are optimized (2).

5. Analytics: is my oligo the right quality?

The quality standards for oligonucleotides as active pharmaceutical ingredients (APIs) demand precision and accuracy from the analytical technologies and methods used in the analysis to ensure their safe and effective use as well as manufacturing quality.

Your synthesis could be a huge success, but then the analysis might show that the purity is not correct. If this happens, try these troubleshooting steps:

- To increase purity, start by using anion exchange chromatography (AIEX) or ion-paring chromatography, and a gradient of 20 to 30 min for shorter oligos. For longer oligos, you'll need more time and a higher temperature for improved resolution. Use a chromatography resin composed of small particles to get an optimized resolution. To maximize it further, make the product peak around 100 mAU.
- The purification resin to choose also depends on whether the oligonucleotide has the DMTr protection group on or off. In simplest terms, DMTr on and the 5'end provides hydrophobicity to the oligonucleotide and hydrophobic interaction chromatography (HIC) can be used. We use the HIC resin Capto™ Phenyl ImpRes for this. DMTr off and the negative charge on the phosphate group is used for an AIEX. The AIEX resin we recommend is Capto Q ImpRes.
- For checking the yield (at 260 nm absorbance or with optical density [OD] measurements), it makes sense to use a standard spectrophotometer and a 1 cm cuvette instead of a microvolume cuvette as small volumes may impart higher error in determining yield. The cleavage and deprotection solutions contain large amounts of ammonium hydroxide. So, when diluting them, avoid using small volumes due to difficulties pipetting when ammonia is present. A more accurate result will come from diluting 500 to 1000 µL volumes.
- Sometimes during a run, trityl data look normal and there are no other issues, but the analysis still shows a low coupling efficiency. In this case, you probably have a problem related to oxidation/thiolation. Therefore, you need to check the conductivity profile and fine-tune it. There can also be a pressure build up because of this, and to correct it, you need to change the contact time.

What is contact time and why does it matter?

Our synthesizers are based on a flow-through principle: the reagent is pushed into the column and then leaves the column. Contact time is the time the reagent was inside the column. Contact time has a large impact on several steps during synthesis.

- If purity or yields are low, it might be because of incomplete cleavage and deprotection, which will look like impurities in high-performance liquid chromatography (HPLC). If you observe this, use fresh cleavage and deprotection solutions.

Summary

In summary, the promise of oligonucleotide therapeutics to transform human health is locked into their specificity. To unlock this potential, the oligonucleotides produced using the cyclic synthesis process must have precise chemistry and sequences. Cytiva is a strong oligo technology provider, and we can support you in meeting your oligo chemistry goals thanks to our decades of application experience. We are ready to assist and can offer basic training as well as more advanced assistance throughout your synthesis journey. The numerous recent and high-profile regulatory approvals have demonstrated that oligonucleotide therapeutics can be used clinically for diverse indications.

References

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