Robust and cost-efficient oligonucleotide synthesis

An application of Process Analytical Technology (PAT)

Introduction

Oligonucleotides are short strands of DNA or RNA that play a major role in drug discovery and molecular diagnostic chip technology. They are used from initial research and screening through to target validation and drug production. Developers of oligonucleotide-based drugs and molecular diagnostic kits have a clear need for regulatory-compliant and cost-effective production methods. The United States Food and Drug Administration (FDA) has formed a working group to develop guidelines for encouraging or requiring the adoption of Process Analytical Technology (PAT) by the biotechnology and pharmaceutical production industries.

PAT has been extensively utilized in the petroleum and chemical industries for more than ten years. Simply stated, PAT calls for continuous rather than end-stage testing and monitoring of a process – potentially from a remote location. Ideally, that monitoring is cybernetic, i.e. the monitoring process is tied to a self-correcting mechanism to solve problems as they are encountered (or to discontinue the process if it is flawed or contaminated). A cybernetic, continuous remote monitoring system produces significant cost savings by minimizing quality-related product discards and lost production time.

PAT will be an important tool to meet the requirements of regulatory bodies like the FDA. This Application Note shows how common monitors like UV and pressure monitors, together with a flexible control software, can be used for PAT in oligonucleotide synthesis.

Table 1. Synthesis step vs. critical process factors monitored and controlled with PAT.

	Critical process factors									
	Yield/Purity					Time		Integrity		
	1. Coupling efficiency	2. Wash efficiency	3. Flow distribution	4. Reagent quantity control	5. Solid support capacity	6. Flow rate	7. Reagent feed, time minimization	8. Solid support	9. Reactants	10. UV-VIS monitor
Synthesis step										
0 Initialization								X		X
1 Detritylation	X			X	X		X			X
2 Wash		X				X				X
3 Coupling			X	X			X		X	X
4 Wash		X				X				X
5 Oxidation/Thiolation				X			X			X
6 Wash		X				X				X
7 Capping				X			X			X
8 Wash		X				X				X

Principles of oligonucleotide solid-phase synthesis

Solid-phase oligonucleotide synthesis is performed in a flow-through column reactor using a pump-driven system. Prior to synthesis, the column reactor is filled with a solid support resin with the first nucleoside already attached. This is the anchor for the growing oligonucleotide. Synthesis is then carried out in a number of steps as shown in the left-hand column of Table 1. These steps are described below.

1. Detritylation

The first reaction, detritylation, removes a 5'-dimethoxytrityl protecting group from the support-bound nucleoside. The release of the protecting group generates a bright red-orange color and its relative quantities can be measured on-line by UV-VIS. See the first peak in Figure 1 labeled Detritylation.

2. Wash

After detritylation, the support is washed with acetonitrile to remove the detritylation reagent.

3. Coupling

The second reaction involves coupling the appropriate phosphoramidite monomer (A, C, G, or T) mixed with an activator. In this reaction, a phosphite triester internucleotide bond is formed in very high yields, usually over 99 %. For DNA, coupling is most often performed in a single pass of reagents through the column. With slower coupling kinetics, e.g. RNA, a longer reaction time is required. This is achieved by re-circulating coupling reagents over the column. See Coupling in Figure 1.

4. Wash

After coupling, the support is washed with acetonitrile.

5. Oxidation/thiolation

The newly-formed phosphite triester internucleotide bond is then converted to the corresponding phosphorothioate or phosphodiester with a thiolating or oxidation reagent respectively. See the peak labeled 'Ox' in Figure 1.

6. Wash

After oxidation/thiolation, the support is washed with acetonitrile.

7. Capping

The final step in the synthesis cycle is capping the unreacted 5'-hydroxyl groups. This can be seen as the peak labeled 'Cap' in Figure 1.

8. Wash

After capping, the support is thoroughly washed with acetonitrile before beginning the next cycle.

Synthesis steps 1–8 can be repeated until the full-length oligonucleotide has been synthesized.

PAT applied to the process

The complete process can be visualized by applying multi-wavelength/channel UV monitoring during the synthesis cycle. Visualization is based on UV-VIS monitoring, an example of which is shown in Figure 1.

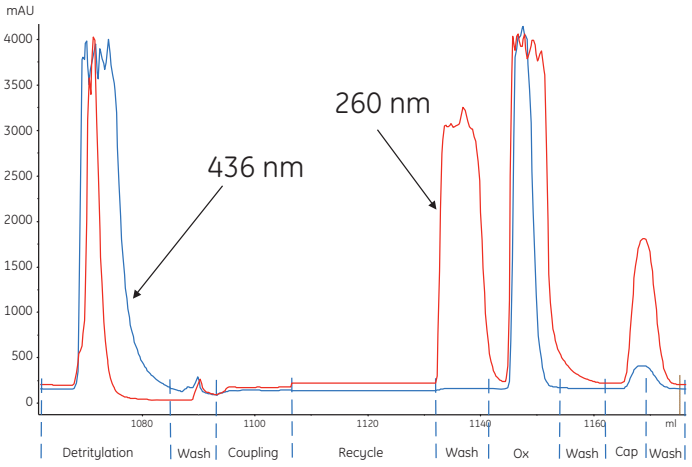

Fig 1. Spectra from the synthesis of oligonucleotides.

A cybernetic control solution ensures that maximum yield and purity, as well as cost-efficient production, is obtained. This solution takes advantage of the continuous monitoring and conditional programming of the control software.

Table 1 lists the critical factors for each of the synthesis steps. These factors are discussed below.

Yield/purity optimization

Process yield is a result of high yield in repeated synthesis steps in relation to the number of bases in the nucleotide synthesized. There are five critical factors for yield/purity:

1. Coupling efficiency
2. Wash efficiency
3. Flow distribution in column reactor
4. Reagent quantity control
5. Solid support capacity

1. Coupling efficiency

Release of the 5'-dimethoxytrityl protecting group is measured by UV and the signal is integrated on-line by the control software. The amount of dimethoxytrityl eluted is a direct measure of the oligonucleotide sequences still active in the column reactor and available for further base coupling. This can be used as a quantitative measurement of the ongoing synthesis. The information can also be used to terminate the ongoing synthesis should the integrated value drop below a pre-set limit, an important function to avoid costly prolongation of an oligonucleotide that would not meet final quality control criteria. The detritylation peak can be clearly seen in Figure 1. To ensure that complete detritylation has occurred, conditional programming can be used in such a way that the detritylation reagent

will continue to be pumped through the column until the absorbance at 436 nm falls below a pre-set level.

2. Wash efficiency

Incomplete wash-out of reagents between synthesis steps could generate unwanted side reactions and modifications to the oligonucleotide. Required wash volumes are dependent on known physical parameters, e.g. column reactor diameter and/or bed height (column volume), and on harder-to-predict changed diffusion characteristics during the synthesis. It is possible to monitor the wash performance on-line and control execution of the next step conditionally based on a fully-complete wash by using the UV signal and control software with conditional method programming. See the wash steps depicted in Figure 1.

3. Flow distribution control

For maximum utilization of the reactor solid support, flow distribution in the column is controlled with a function for dynamic back pressure. By using a flow restriction valve after the column, the back pressure for maximum column performance can be adjusted continuously.

4. Reagent quantity control

To ensure optimal process yield for each synthesis scale, conditions have to be adapted to the amount of solid support (column volume) and available sites for coupling (synthesis scale). Current methods give an average yield in each cycle of at least 98–99%, depending on the type of solid-phase synthesis chemistry.

The same column volume may represent different synthesis scales since, in addition to variations in physical parameters such as diameter and bed height, there are also different nucleoside loadings required for synthesizing different length oligonucleotides. As the system hold-up volume is static, the ratio of system volume to column volume varies. Reagent volumes vary depending on synthesis scale and flow rates vary to achieve the correct linear flow rate for different column diameters.

The front and end of the reagent zone can be monitored by pre-column UV monitoring. As can be seen in Figure 1, different synthesis reagents require different wavelengths.

For syntheses requiring recirculation of reagents, e.g. monomers, the UV monitor can be used to ensure that monomers are kept in the loop, i.e. that they enter the loop before it is closed, or are not pushed too far to waste.

5. Solid support capacity quantification

A critical parameter for reaction quality is the capacity of the solid support in the reactor. This is measured continuously in the process (once every cycle). The critical parameter is the amount of available sites for coupling on the solid support. This is a dynamic value that changes (decreases) during the synthesis. If the value falls below a set threshold, an alarm can be set to stop the process.

Process time minimization

Reducing process time is related to flow rate and reaction time. Table 1 shows which synthesis steps are affected by each of the time-critical factors. Critical factors for process time are:

6. Flow rate
7. Reagent feed time minimization

6. Flow rate

Back pressure over the column reactor changes during the synthesis process, which results in a set volumetric flow rate generating an increasing reactor pressure. This problem can be reduced by utilizing pressure flow control where a pressure limit (rather than a flow set point) is set to reduce the overall process time. This means that pumps controlled by the software will ramp up and generate the set maximum pressure. The effect of this is that maximum flow rate is obtained throughout the process without exceeding system pressure limits. In practice, this means that the actual flow rate during the process reduces over time.

7. Reagent feed time minimization

An initial high reagent flow rate can be used to reduce process time. As the reagent front is detected by a pre-column UV monitor, the flow rate can be reduced to the required linear flow rate for the reagent to pass through the column.

Online integrity check

The integrity of the process is built on the integrity of a number of units and a set of critical parameters. Control is achieved with the WATCH command in the UNICORN method concept, with specific algorithms in the UNICORN configuration strategy, and with equipment providing hardware integrity data. Table 1 shows which synthesis steps are affected by integrity-critical factors:

8. Solid support
9. Reactants
10. UV-VIS monitor

8. Integrity of the solid support

At the very beginning of a new synthesis run (initialization), the first detritylation provides an integrity test of the solid support packed in the column reactor. The expected dimethoxytrityl measurement by UV is a theoretical 100 % sites available for further processing. If this is not the case, it can be assumed that the integrity of the solid support is in some way compromised.

9. Tracking reactants

It is possible to distinguish between A, C, G and T monomers by making a quotient from two different UV wavelengths. Figure 2 shows various quotient peak heights according to which monomer is present. By using control software conditional programming applied to the observed signal, rules can be set for on-line identification of the monomer added.

10. Integrity of the UV monitor

The UV monitor is the most important monitor in the system and its integrity is managed with three built-in functions for lamp surveillance. Lamp signal is continuously monitored and a 'Low lamp' warning is issued if light intensity falls below a specific limit. Exceeding the lamp life-time issues an '8000 h Exceeded' warning. If the lamp signal is below 0.1 % of max value, a 'No Lamp' warning is issued.

Conclusions

The examples described in this Application Note show how PAT can be applied to the process of oligonucleotide synthesis. These techniques have been implemented in UNICORN™ software used to control oligonucleotide synthesis systems from GE Healthcare, e.g. ÄKTA™ oligopilot™, Oligopilot 400 and OligoProcess™.

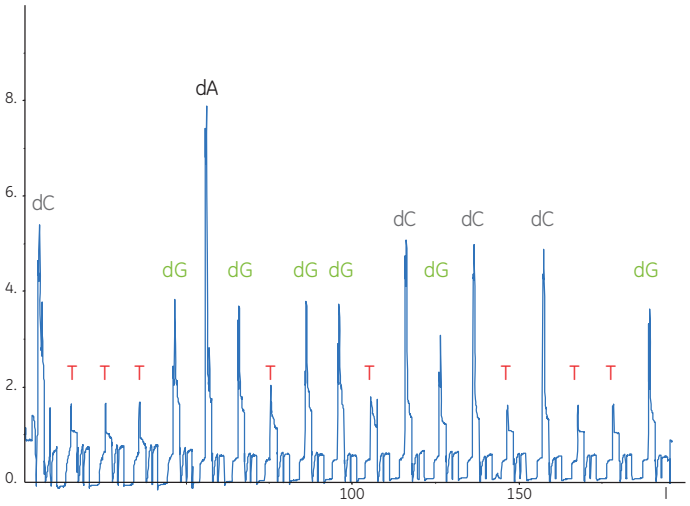

Fig 2. UV quotient diagram showing reagent identity.

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