Microsynth

## Troubleshooting Guide

# Synthesis of Nucleic Acids - Implications for Molecular Biology

### Background Information

Chemical synthesis of DNA features a few characteristics that are worth to know. In contrast to PCR reactions, synthesis of DNA oligonucleotides is performed from 3′ to 5′ end with the first nucleotide attached to a solid phase (controlled pore glass; see Box 1). Chain elongation occurs during the coupling cycle. This cycle encapsulates a series of chemical reactions, during which an incoming nucleotide is covalently connected to the growing strand. This sequential addition of nucleotides enables the synthesis of customized single-stranded DNA sequences.

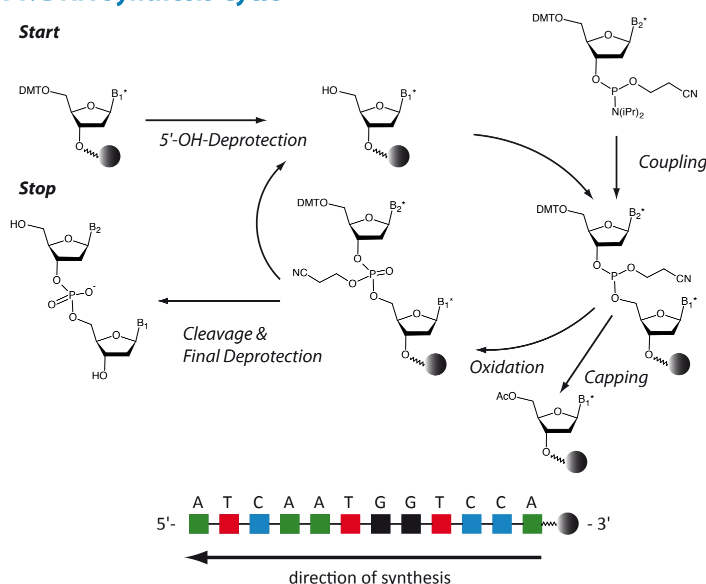
The coupling of an additional base to the elongating oligo is close too but never 100% as it is the fact for any chemical reaction. A small fraction of the oligo is not elongated in each cycle. To avoid the elongation in later coupling steps the oligo will be capped. Hence, any raw synthesis will include the full-length product as well as products truncated at the 5′ end (n-x products). The proportion of full-length product in the synthesis depends on the coupling efficiency and the oligo length (Box 2). For example, a 50 mer produced with a coupling efficiency of 99% will include 61% of the full-length product and the remaining 39% represent n-x products. In case that the n-x products are critical for your downstream analysis, an appropriate purification method is recommended.

As the automated synthesizer performs many liquid-handling steps, perfect function of the robot is crucial for the production of high quality oligonucleotides. For example, if a plug valve of the phosphoramidite reagent leaks, traces of an undesired nucleotide might be incorporated at a particular position within the DNA sequence. Please note that only a small fraction of the entire DNA supply would be affected by this mutation phenomenon. Therefore, proper inspection and maintenance of the synthesizers are essential to reach a constant high-level production standard.

For non-sensitive applications such as PCR, sequencing and/or hybridization, neither n-x products nor the small fraction of the oligo population resulting from malfunction of a synthesizer are of any relevance. However, n-x products may become visible if the oligos or PCR products are used for cloning experiments. Moreover, the down-stream process in your lab, i.e. PCR steps or cloning, are known to affect the final results you will see in the Sanger sequencing reads.

With this troubleshooting guide we want to help you to overcome potential problems that can occur during cloning experiments. The first part of this brochure gives you some additional background information about the quality control process of oligonucleotides as well as different purification techniques. In the second part we

**Box 1: DNA Synthesis Cycle**



focus on 5'-truncated (n-x) products and undesired effects observed in the PCR and cloning step.

## QC and Purifications

### Quality Control (QC)

A stringent quality control system ensures the consistently high quality of all our oligonucleotides. Microsynth performs online trityl monitoring in order to check the coupling efficiency after each cycle. Molecular identity of oligonucleotides is verified either by MALDI-TOF MS (up to 50 bases) or by analytical PAGE (51 to 150 bases) following synthesis.

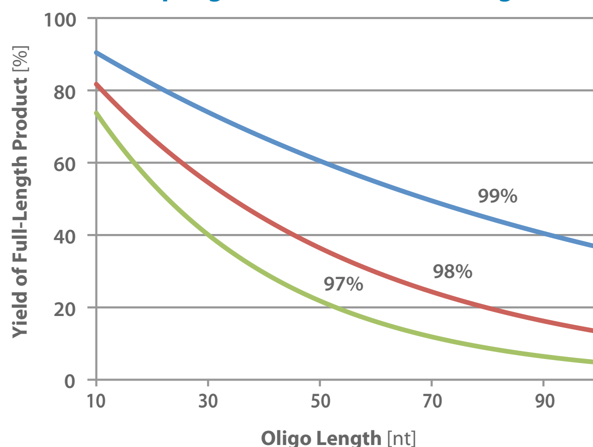
### Desalted

As minimum level of purification Microsynth offers desalted oligonucleotides. These oligonucleotides are free of low molecular weight compounds which form during synthesis. This level of purification is sufficient for oligonucleotides shorter than 30 and/or oligonucleotides used for non-critical applications such as PCR, sequencing, probing, mobility shift or hybridization. However, desalted oligos are not recommended for use in cloning projects.

### HPLC Purification

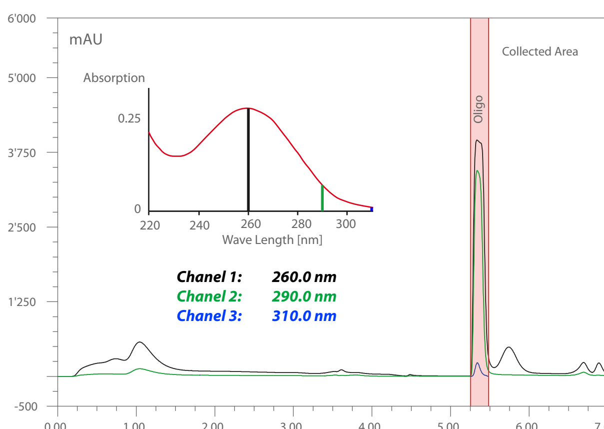
Oligos ≤50 bases in length can be well purified via Reverse-Phase HPLC. State of the art purification benefits from the hydrophobic DMT group which is (still) attached to the full-length DNA sequence. These oligonucleotides are much better retained on reverse phase material compared to 5'-truncated n-x compounds which lack the DMT group. This additional adhesion enables a straight forward separation of the desired oligonucleotide from side products and results in 90-95% purity. RP-HPLC is useful for more demanding down-stream applications such as cloning, DNA fingerprinting,

**Box 2: Effect of Coupling Efficiencies on Full-Length Product**

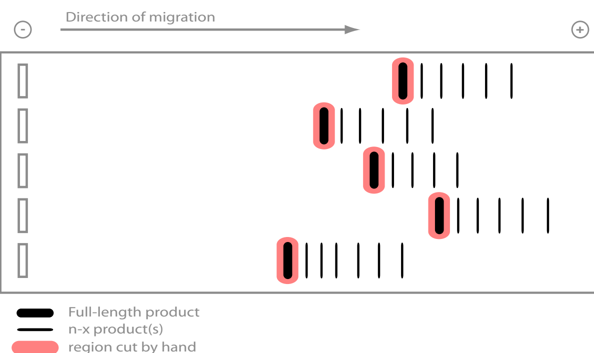



**Box 3: Different Purifications Applied to Oligonucleotides**

**HPLC Purification**




**PAGE Purification**

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real-time PCR, FISH etc.

**PAGE Purification**
Polyacrylamide gel electrophoresis (PAGE) purification is generally required for long oligonucleotides (≥50 nt) and for all primers with critical sequences at their 5'-end (restriction endonuclease sites, RNA promoters). It is the best method to separate full-length products from aborted sequences (n-x oligos) since the migration behavior of oligonucleotides is mainly based on charge and molecular weight. Its excellent resolution yields products with an average purity of 95-99%. This type of purification is highly recommended for sequence-sensitive experiments such as cloning, mutagenesis, DNA fingerprinting, in situ hybridization, etc.

## Troubleshooting Guide

| Observation | Possible Cause(s) | Recommendation(s) |
|---|---|---|
| **5' truncated oligonucleotide sequence after cloning** | **PCR artefact:**<br>Preferred priming of 5' truncated oligonucleotides during PCR.<br><br>**n-x products:**<br>May be the result of 5' exonuclease activity of polymerases or not appropriately purified oligos. | **1) Pick additional clones**<br>If the first two clones did not show the expected clone, pick another couple of clones and sequence them. It's easier to sequence another 3-10 clones before repeating the time consuming process of oligonucleotide re-synthesis and cloning. The chance is high to recover the correct clone.<br><br>**2) Do not perform your PCR overnight**<br>Even if you keep the finished PCR reaction at 4°C, the 5' exonuclease activity of your polymerase may lead to degradation of your PCR amplicon.<br><br>**3) Use HPLC or PAGE purified oligonucleotides**<br>HPLC (<50mers) or PAGE purifications (≥50mers) effectively remove n-x products. |
| **Restriction enzyme does not cut the PCR product** | **PCR artefact:**<br>Preferred priming of 5' truncated oligonucleotides during PCR.<br><br>**n-x products:**<br>May be the result of 5' exonuclease activity of polymerases or not appropriately purified oligos.<br><br>**Missing overhang:**<br>Restriction site located at the 5' end of the oligo | **1) See recommendations above**<br><br>**2) Restriction enzymes needs overhang**<br>Please be aware that some restriction enzymes need overhangs for efficient cutting. In such cases, we suggest that you extend your primer sequence by 3 to 6 bases in front of the restriction site. |

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## Troubleshooting Guide (continued)

| Observation | Possible Cause(s) | Recommendation(s) |
|---|---|---|
| **3' truncated oligo sequence** | **PCR artifact:**<br>Some DNA polymerases (e.g. Taq DNA polyerase) exhibit a relatively high error rate. | **1) Use proof reading polymerase**<br>As the DNA synthesis proceeds 3' to 5' and starts with the first nucleotide bound on a glass bead it is highly unlikely that 3' truncation originates from chemical synthesis. In contrast, a PCR artifact may be responsible and the usage of Pfu or blended DNA polymerase are recommended. |
| **Mutations at non-specific sites within the oligonucleotide sequences after cloning** | **PCR artifact:**<br>Some DNA polymerases (e.g. Taq DNA polyerase) exhibiting a relatively high error rate | **1) Use proof reading polymerase**<br>Pfu or blended DNA polymerase are recommended because they exhibit very low error rates. |
| **Mutations at specific sites within oligo sequences after cloning and sequencing** | **PCR artifact:**<br>Some DNA polymerases (e.g. Taq DNA polyerase) exhibit a relatively high error rate.<br><br>**Secondary structure(s):**<br>Hairpin structrue(s) in the oligonucleotide increases the likelihood to introduce mutations in your construct during PCR and cloning. | **1) Pick additional clones**<br>Pick another couple of clones and sequence them. It's easier to sequence another 3-10 clones before repeating the time consuming process of oligonucleotide re-synthesis and cloning. The chance is high to recover the correct clone.<br><br>**2) Disintegrate hairpin**<br>Hairpins may be (partially) disintegrated by introducing silent mutations (make use of the redundancy of the genetic code). |
| **Multiple-base deletions within oligo sequences after cloning and sequencing** | **Selection for mutants:**<br>Recombinant protein produced within *E. coli* is harmful or even lethal to its host and therefore favors the growth of clones carrying mutants. | **1) Pick additional clones**<br>Pick another couple of clones and sequence them. It's easier to sequence another 3-10 clones before repeating the time consuming process of oligonucleotide re-synthesis and cloning.<br><br>**3) Pick small cultures**<br>If you see a dimorphim in *E. coli* growth on your plate try to pick some of the small colonies |

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# *Examples*

## Example 1: 5' Truncated Oligonucleotide Sequences after Cloning and Sequencing

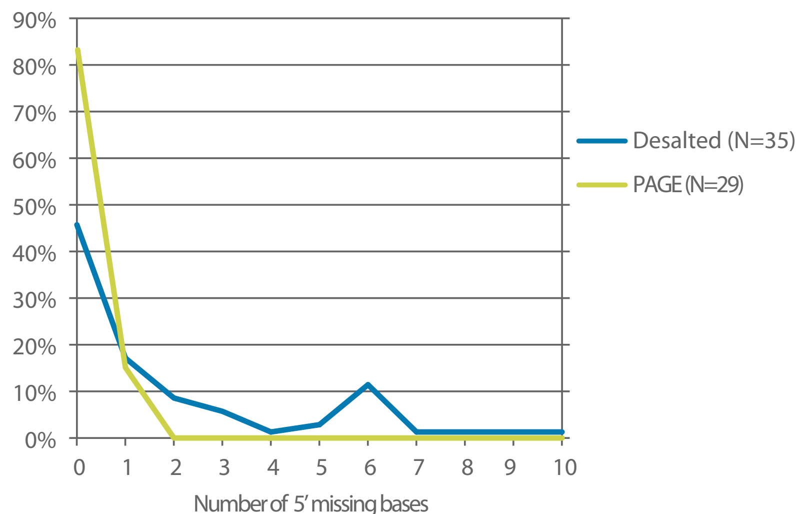
**Material and Methods**

Two 30 mer oligos - either desalted or PAGE purified - were used as PCR primers on an artifical template using high-fidelity polymerase. Blunt-end PCR products were cloned and sequenced and 5' missing bases were recorded.

**Results and Discussion**

As expected the success rate with PAGE purified oligos is improved in favor of the non-truncated clone (46% vs 93%; see Box 4). Therefore, the number of clones that need to be sequenced in order to recover a correct clone may be reduced in general when thoroughly purified oligos are used. We recommend HPLC purification for oligomers ≤50 and PAGE for >50 mer, respectively. In addition, for oligos <30 bases also a desalted oligo can be used if at least 2-3 clones are picked as the fraction of n-x products is small.

### Box 4: Distribution of n-x sequences after PCR and cloning




## Example 2: Effect of Hairpin Structure in Oligonucleotide Sequences

**Material and Methods**

A long oligo (79 bases) including a strong hairpin structure (see sequence 1 in Box 4) was synthesized at Microsynth and an external supplier. The oligo sequence was originally used for the shRNA techniques. The same oligo was re-synthesized at Microsynth and the hairpin was broken by exchanging three bases in the sequence (sequence 2). All oligos were PAGE purified at Microsynth and PCR amplified using a high-fidelity polymerase. After cloning, 53 (sequence 1, Microsynth), 63 (sequence 1, external supplier) or 81 clones (sequence 2) were sequenced, respectively.

**Results and Discussion**

The results clearly show that the hairpin structure has a profound effect on the number of mutations observed in the sequenced clones. The vast majority of clones are not identical for sequence 1 indenpendent of the company running the synthesis and the majority of mutations accumulate in the hairpin. In contrast, the oligo with the disrupted hairpin shows 85% correct clones. As the chemical synthesis is performed under non-native conditions (organic solvents) and the Watson-Crick sites of the nucleobases are masked with protecting groups, secondary structures do not form during the assembly and hence are not interfering with solid phase synthesis.

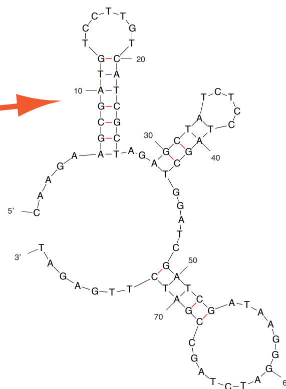
### Box 5: Effect of Secondary Structures

Sequence 1: b ' ' f ' AGCGATGs b b s s f s CATCGCT' f ' f b s ' s b s b b s ' f b s f f ' s b f ' s b f ' s ' ' f L
Sequence 2: b ' ' f ' AGCGATGs b b s s f s CAGTGTT' f ' f b s ' s b s b b s ' f b s f f ' s b f ' s b f ' s ' ' f L

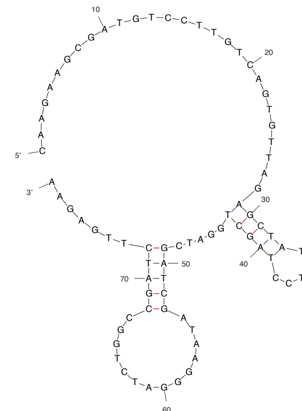
### Box 5: Effect of Secondary Structures (continued)

52% or 68% of all mutations were located in the hairpin structure (positions 20-26) for the oligo synthesized at Microsynth or an external supplier

Sequence 1:
~15% correct clones (N=53/63)



Sequence 2:
85% correct clones (N=81)

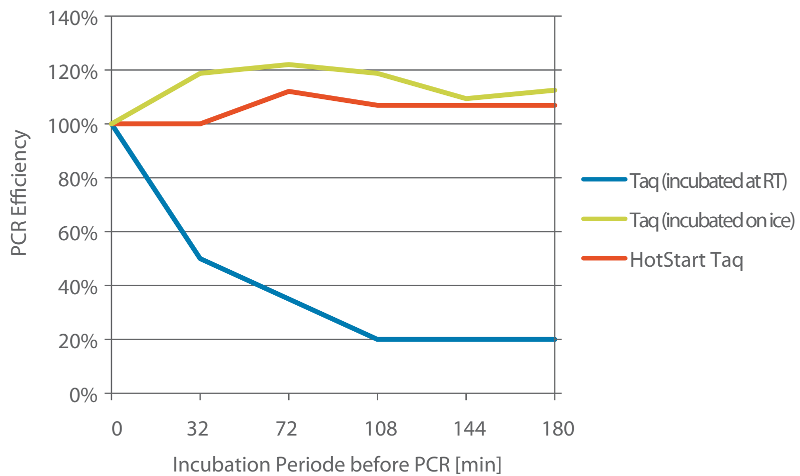


## Example 3: Exonuclease Activity of Polymerases

**Material and Methods**
The influence of the intrinsic exonuclease activity of the Taq polymerase on PCR efficiency was analyzed. PCR reaction mixtures were incubated for different periods at room temperature as well as at 4° C before starting the PCR cycles. The PCR efficiency was determined from the concentration of the PCR product and compared with the results obtained from a HotStart polymerase. As template bacterial genomic DNA was used.

**Results and Discussion**
The results clearly show a reduction of the amplification efficiency (100% = efficiency at 0 min incubation time) when the standard polymerase is exposed to the PCR mixture at room temperature. In contrast, the efficiency remains stable for the HotStart polymerase and the standard polymerase incubated on ice. These observations agree with the hypothesis that the PCR primers are digested via the exonuclease activity of the standard polymerase.

### Box 6: Exonuclease Activity of Polymerases




**Need More Information?**
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