February 10, 2017

Jessica Walden
Materials Specialist, Standards Division
NOP, AMS
US Department of Agriculture

Dear Ms. Walden:

Attached, please find our response to your inquiry regarding our petition of October 12, 2016, which requests the addition of short DNA tracers to section 205.605 of the National Organic Program's (NOP) National List of Allowed and Prohibited Substances (National List). Specifically, you requested "supporting documentation that describes the source and manufacturing process of the primer oligonucleotides. Specifically, please confirm whether they are naturally or synthetically derived and/or produced."

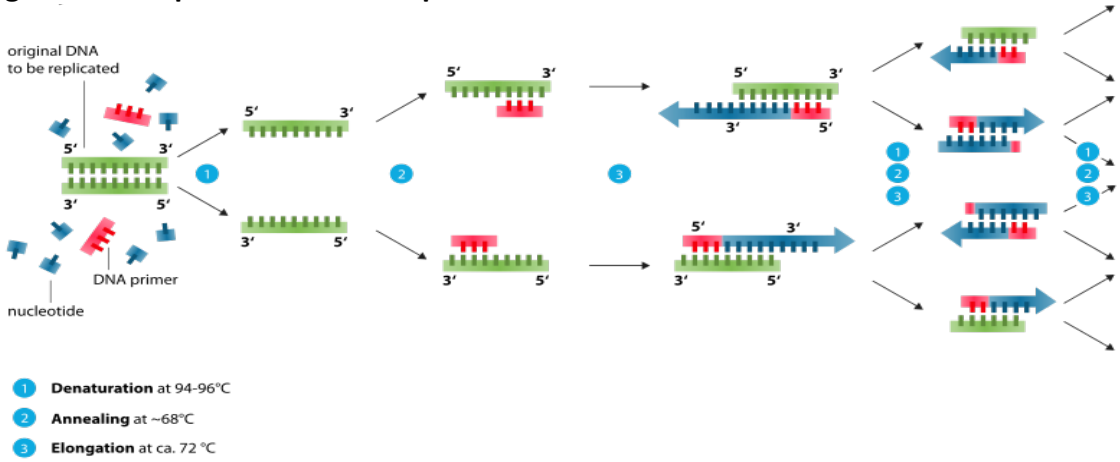
Explanation of Primers

Purpose

Short DNA tracers are short DNA sequences that are drawn from a natural source and are copied employing primer oligonucleotides. Primer oligonucleotides are short nucleic acid sequences that provide a starting point for the DNA production. Primer oligonucleotides are part of the natural sequence of the organism and they are used to bind to site-specific regions of the DNA that is being copied. Primer oligonucleotides are sequence specific and make copies only of the corresponding short DNA sequences. The use of a primer oligonucleotide is necessary, because the naturally-occurring enzymes that are used in the DNA production, called DNA polymerases, can only attach new DNA nucleotides to an existing strand of nucleotides. The primer oligonucleotide therefore serves to prime and lay a foundation for our DNA production.

Primer oligonucleotides are manufactured synthetically, as they are too short to reliably procure from the original natural source (from which the short DNA sequence originates). The primer oligonucleotides represent only about 25% of the final product.

Diagram of PCR process to include primers:



[Source: https://en.wikipedia.org/wiki/Polymerase_chain_reaction]

Manufacturing Process

The manufacturing process for the primers is called oligonucleotide synthesis. The technique is extremely useful in current laboratory practice because it provides a rapid and inexpensive access to custom-made oligonucleotides of the desired sequence. Whereas enzymes synthesize DNA and RNA only in a 5' to 3' direction, chemical oligonucleotide synthesis does not have this limitation, although it is, most often, carried out in the opposite, 3' to 5' direction. Currently, the process is implemented as solid-phase synthesis using phosphoramidite method and phosphoramidite building blocks derived from protected 2'-deoxynucleosides (dA, dC, dG, and T).

To obtain the desired primers, the building blocks are sequentially coupled to the growing oligonucleotide chain in the order required by the corresponding DNA sequence. The process has been fully automated since the late 1970s. Upon the completion of the chain assembly, the product is released from the solid phase to solution, deprotected, and collected. Products are often isolated by high-performance liquid chromatography (HPLC) to obtain the desired primer in high purity. Our primers are single-stranded DNA molecules around 15–25 bases in length.

Following is a video excerpt from GeneArt™ that describes the oligonucleotide manufacturing process in simple terms. https://vimeo.com/203522121

The Primers undergo a rigorous quality control process to ensure that they meet specification, such as the process described here:
https://www.thermofisher.com/us/en/home/products-and-services/product-types/primers-oligos-nucleotides/invitrogen-custom-dna-oligos/technical-resources-for-oligonucleotides/oligo-quality-control.html

The process includes 100% in-process trityl monitoring with up to 12 measurements taken for every base addition and additional CE, MS, and OD to verify yield. Consistent oligonucleotide quality is guaranteed and a comprehensive Certificate of Analysis accompanies each order, indicating quality, molecular weight, extinction coefficient, sequence, and melting temperature.

Additional Features of our Process

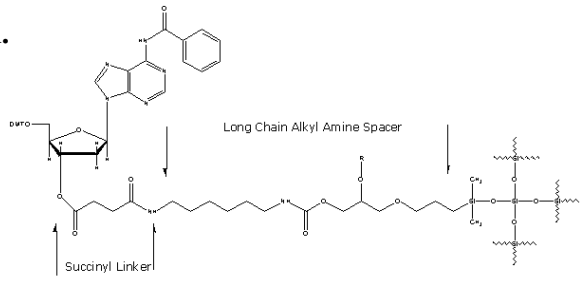
- Final testing of statistically selected oligos by capillary electrophoresis or by mass spectroscopy to further ensure the quality.
- Certificate of Analysis includes oligo name; scale of synthesis; sequence and length; melting temperature (TM); amount in ODs, µg, and nmole; and molecular weight and extinction coefficient calculated from your exact sequence, including modification.
- Label information on each vial is durably printed on clear transparent stock to allow full view of contents.
- Fluorescent oligos shipped in amber tubes to protect light sensitive oligos"

For the technically inclined audience, a detailed description of the manufacturing process follows: The manufacturing process for primers is based on the use of phosphoramidite monomers as building blocks and the use of tetrazole catalysis. (https://www.idtdna.com/pages/docs/technical-reports/chemical-synthesis-of-oligonucleotides.pdf?sfvrsn=4)

A phorphoramidite is a normal nucleotide but with protection groups, such as a trityl group, added to its reactive amine, hydroxyl, and phosphate groups. These protection groups prevent unwanted side reactions and force the formation of the desired product during synthesis. The protection groups are removed after the completion of the synthesis process. The link to the solid support is made through the 3' carbon and synthesis proceeds 3' to 5' rather than the 5' to 3' synthesis used previously (Figure 1). The solid support is a 5 micron controlled pore glass bead (CPG) with holes and channels where the protected nucleotide is attached (Figure 1B). The advances in oligonucleotide synthesis chemistries have resulted in substantial increases in quality and yield with the added advantage of decreasing cost. This is particularly important since each oligonucleotide has to be custom made dependent on the needs of the individual researcher.

Phophoramidite synthesis begins with the 3'-most nucleotide and proceeds through a series of cycles composed of fours steps that are repeated until the 5'-most nucleotide is attached. These steps are deprotection, coupling, **capping**, and **stabilization**.

A.



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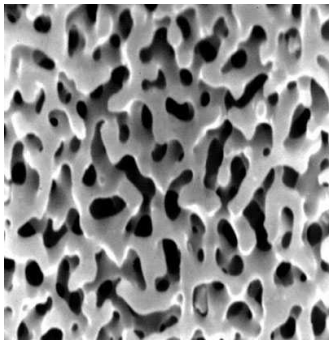


Figure 1A. A protected nucleoside attached to a CPG. 1B. An electron photomicrograph of the surface of a CPG bead. The scale of this picture is 10 millionths of an inch square.

Deprotection

In the classic deprotection step, the trityl group, which is attached to the 5' carbon of the pentose sugar of the recipient nucleotide, is removed by trichloroacetic acid (TCA) leaving a reactive hydroxyl group to which the next base is added.

Coupling

It is here that the advent of tetrazole activation replaces the use of condensing agents like DCC. Berner et al. showed that tetrazole, a weak acid, attacks the coupling phosphoramidite nucleoside forming a tetrazolyl phosphoramidite intermediate [3]. This structure then reacts with the hydroxyl group of the nucleoside attached to the CPG bead and the 5' to 3' linkage is formed (Figure 2). The unbound base and by-products are washed out, the tetrazole is reconstituted, and the process continues. The use of tetrazole increased coupling efficiency to greater than 99% which allowed longer and longer oligonucleotides to be synthesized.

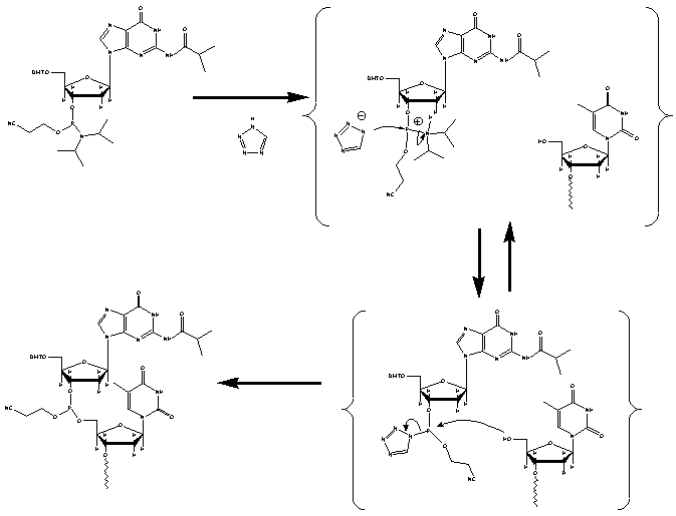


Figure 2. The pathway of tetrazole phosphoramidite-intermediate coupling. The phosphoramidite is introduced in the presence of tetrazole which protonates diisopropylamine leading to the formation of the tetrazole phosphoramidite intermediate. Coupling of the intermediate to the growing oligonucleotide is the final step which returns the tetrazole to its original state.

Capping

While the increased efficiency afforded by the advent of tetrazole phosphoramidite-intermediate coupling was a major advance in oligonucleotide synthesis, it was still a chemical process and so had a finite failure rate. A coupling failure results when an oligonucleotide retains a reactive hydroxyl group on its 5'-most end. If this were to remain freely reactive, it would be able to couple in the next round and would result in a missing base in the synthesis. Thus, coupling failures must be removed from further participation in the synthesis. This is accomplished by adding an acetylating reagent composed of acetic anhydride and N-methyl imidazole. This reagent reacts only with free hydroxyl groups to irreversibly cap the oligonucleotides in which coupling failed.

Stabilization

Once the capping step is accomplished, the last step in the cycle is oxidation which stabilizes the phosphate linkage between the growing oligonucleotide chain and the most recently added base. The phosphate linkage between the first and second base must be stabilized by making the phosphate group pentavalent. This is achieved by adding iodine and water which leads to the oxidation of the phosphite into phosphate leaving the phospho-triester bond stabilized.

Cleavage, Detritylation, Deprotection

This cycle is repeated for each nucleotide in the sequence. At the end of the synthesis the oligonucleotide exists as, for example, a 25-mer with the 3' end still attached to the CPG and the 5' end protected with a trityl group. In addition, protecting groups remain on three of the four bases to maintain the integrity of the ring structures of the bases. The protecting groups are benzoyl on A and C and N-2-isobutyryl on G (Figure 3). Thymidine needs no protecting group. The completed synthesis is detritylated and then cleaved off the CPG leaving a hydroxyl on both the 3' and 5' ends. At this point the oligo (base and phosphate) is deprotected by base hydrolysis using ammonium hydroxide at high temperature. The final product is a functional single-stranded DNA molecule.

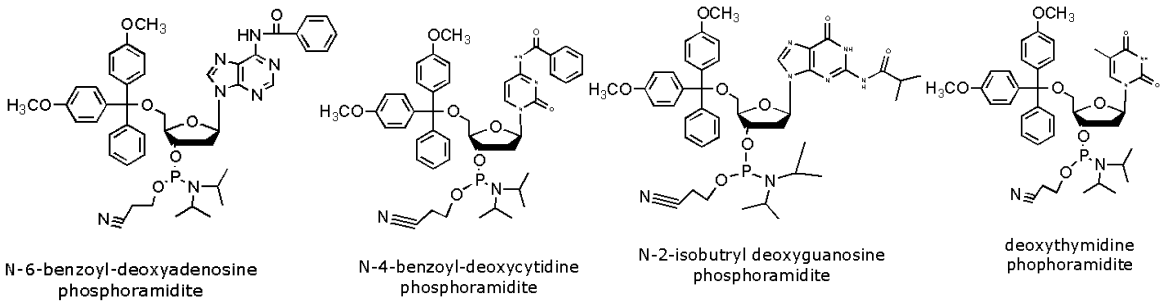


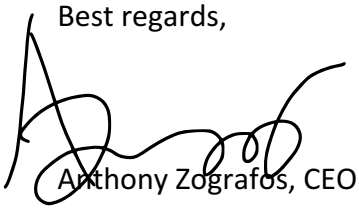
Figure 3. Structures of the four nucleoside phosphoramidite monomers. The benzoyl and isobutyryl protecting groups on the A, C, and G monomers are shown.

Desalting

Although deprotection removes the protecting groups, they remain with the oligonucleotide as organic salts. The process of removing these contaminants is called **desalting**.

I hope that this description answers your questions. Please let me know if you have additional questions.

Best regards,



Anthony Zografos, CEO