

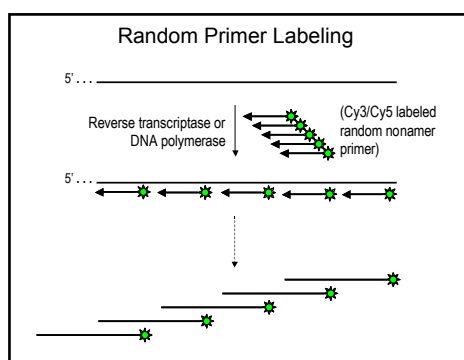
Randomer Oligonucleotides

By Richard Hogrefe, Ph.D., Terry Beck, Natasha Paul, Ph.D. and Alexandre Lebedev, Ph.D.; TriLink BioTechnologies

Introduction

There has been a recent resurgence in the interest in oligonucleotides containing sites with more than one base. These sites, known as either "random", "wobble" or "degenerate" sites, have long been a synthetic target for oligonucleotide chemists. The need originated from early probe studies using synthetic oligonucleotides to hunt for the gene of a particular protein. Each amino acid is encoded by a trinucleotide codon, the third and sometimes the second base of which can often consist of several different nucleosides and still correctly encode for the amino acid desired. This is called a degenerate site in the genetic code. If the specific sequence for the gene is not known, then it is necessary to probe for all potential codons that result in the correct amino acid sequence. Therefore the probe must also have degenerate sites, or mixtures of all possible bases, in order to identify a gene that has the code for the protein of interest.

Another application for oligonucleotides with degenerate sites is the random amplification of a genetic target to generate a pool of all the available sequences. This random amplification is done with oligonucleotides containing a number of degenerate sites containing all four nucleosides. A number of commercial diagnostic assays were developed using oligonucleotides containing degenerate sites, or "randomers" for short. One such assay was designed by Incyte. They developed an assay that could detect a single base mismatch polymorphism using a two dye nonamer randomer probe system coupled with their microarray detection system. The sample and a WT control were both amplified using the randomers, but with different dyes. The labeled amplicons were analyzed with a microarray, yielding a plot of data. The polymorphism was detected if the fluorescence intensity from the two plots deviated from each other (3,4,5,6).



Other companies have also employed dye labeled randomers in their microarray assay systems (1). These assays may, for example, use dye labeled random primers to map differences in chromosomes. Such differences in the chromosomes can be detected, the results of which have diagnostic implications.

In another application of randomers the sequence is only random in select sites, reducing the number of compounds needed to determine the sequence. A common requirement of all these applications is that the oligonucleotides are as random as possible, and are as reproducible as possible from lot to lot.

A Synthetic Challenge

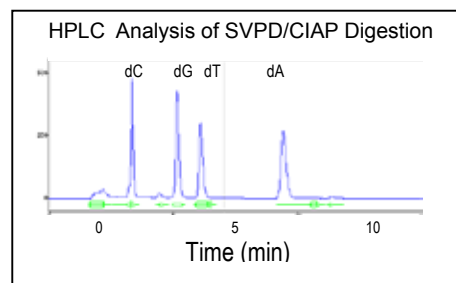
The synthetic problems stem mainly from the fact that there are B^n sequences in the mixture, where B = the number of bases (4 if totally degenerate) and n = the number of sites that

are random. Thus a nonamer randomer is actually 4^9 different sequences; 262,144 different individual oligonucleotides, nine bases in length. Many of the sequences will have similar properties during manufacturing. Some, however, like the homopolymers, will have vastly different characteristics.

The challenge lies in manufacturing a dye modified randomer oligonucleotide that is highly purified of shorter fragments, chemical modifications, and extra dye, while still maintaining a reproducible random mixture of sequences throughout the process which includes the step-wise chemical synthesis of the oligonucleotide, preliminary purification, followed by conjugation to the dye, repurification and then final processing. The randomness of the sequence is potentially changed at each step, requiring extreme care in order to manufacture a reproducible, high quality product.

Determining Base Composition

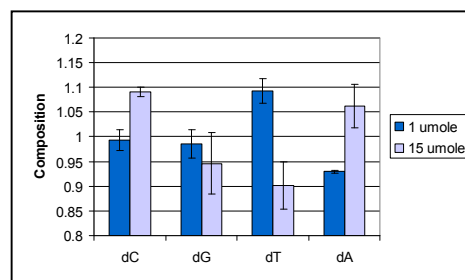
Determining base composition is accomplished by digesting the oligonucleotide using a combination of snake venom phosphodiesterase (SVPD) and alkaline phosphatase, yielding the free nucleosides which are quantified using RP-HPLC to yield the ratio of each base in the oligonucleotide (2).



The Synthesis Step

Although there is a subroutine on most commercial DNA synthesizers that will allow for the synthesis of degenerate sites consisting of mixes of 2, 3 or all 4 bases, you would be ill-advised to use it. Most machines apply pulses of each of the required nucleoside phosphoramidites to the column sequentially. Any deviation in flow rate between reagent bottles caused by restrictions can cause major changes in the resulting ratio of bases. It is much better to pre-mix the bases in the ratio you desire in a single bottle. Be sure to take into account the different molecular weights of each base.

Scale plays a bigger role in determining the ratio in instruments that use a single, gas driven flow through the column, like the Expedite 8909. In the case shown below, the same mix of reagents in the same machine gave considerably different ratios of each base at 1 and 15 μ mole scales. (The ratios of the bases were not optimized to synthesize a 1:1:1:1 mixture in this experiment.)



Another consideration is the starting ratio of bases on the solid support. It is important that the mixture is as close to 1:1:1:1 as possible, and that the support itself is well mixed to ensure uniformity throughout the batch. The proper mixture of supports needed to prepare a perfect 1:1:1:1 ratio of bases is best determined using empirical data.

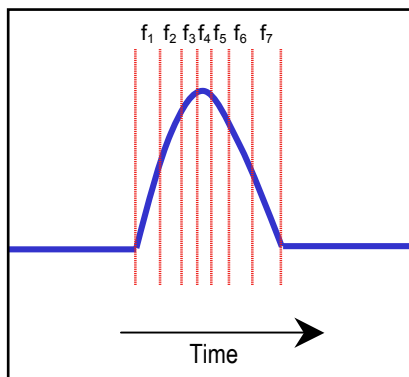
The Purification Step

A much larger potential impact on lot to lot variability can come from the purification step. Each oligonucleotide that is purified undergoes selection in order to remove impurities to the degree required to achieve the desired quality. The most common contaminants include shorter fragments from incomplete synthesis, oligonucleotides with some protecting groups still intact, and inadvertently modified oligonucleotides. These contaminants often elute close to the desired product, necessitating tight cuts in order to obtain high quality material. Unfortunately, in the case of randomers, which contain many different sequences, the elution profile of the desired product is much broader than a discrete oligonucleotide product. In fact, often the elution profile of the product overlaps that of the n-1 side-product elution profile making absolute removal of that contaminant impossible if complete integrity of the randomness of the desired product is to be retained.

To determine if the randomness of the mixture changes across an HPLC peak a nonamer randomer that originally yielded an overall base mixture close to 1:1:1:1 was fractionated by RP-HPLC as shown in the figure below.

The results show that in the beginning of the peak the guanosine nucleoside was enriched, while the back of the peak showed a lack of the sequences bearing guanosine. The adenosine, cytosine and thymidine ratios were essentially even throughout the fractions, equally sharing the change in the guanosine ratio through the elution profile.

It is very important to include the entire peak when purifying by HPLC. To do so, the synthesis must be very good to minimize side products and allow maximal recovery of product.



The Conjugation Step

The next step in which the ratio of bases can be inadvertently changed is during the conjugation step where a succinimidyl activated dye is attached to an amino labeled nonamer. We were concerned that the conjugation would skew the ratio of bases, especially if one or more bases are more reactive than the others.

We tested that concept by removing aliquots of oligonucleotide at various points of the conjugation reaction to see if the base composition of the resulting dye labeled product changed. We found very little difference in the base composition of the oligonucleotide as the efficiency of the reaction went from 20% to 100% completion, suggesting that there is no preference. However, the step immediately after conjugation is purification to remove the excess dye, which is another fractional purification as described above.

Summary

In order to prepare a successful randomer oligonucleotide, it is necessary to take the following steps:

1. Bring an assay in-house such as the described enzymatic digestion assay to test ratios.
2. Prepare your support by mixing it very well, and test random aliquots to assess both mixing and the base ratio. Adjust accordingly.
3. Prepare your amidite mixtures in one bottle and test the coupling of the prepared mixtures to ensure less than 10% overall variation in base ratio.
4. Ensure your DNA synthesizer is running very well to ensure high coupling efficiency.
5. Purify all of the full length material without cutting out the side fractions, hence the requirement for a near perfect synthesis.
6. Ensure a clean conjugation, purification, desalting process to remove excess dye and maximize yield.

A more simple solution would be to buy your randomer oligonucleotides from TriLink, of course. That way you can work on your next experiment, instead of your next oligonucleotide synthesis.

References

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