Methods to Avoid Inactivation of Primary Amines

The most popular method of labeling olionucleotides is to use an amino-modifier phosphoramidite to produce the amino-modified oligonucleotide, which is then reacted with an appropriate N-Hydroxy-succinimide (NHS ester) to yield the oligonucleotide conjugate. The yield of the conjugation reaction is typically less than 80% and frequently below 50%. Why is the conjugation yield quite so poor? There are three predominate means by which an amino-modifier may be inactivated.

Disuccinimidyl Carbonate (DSC) contamination of the NHS labeling solution.

DSC is a starting material for the synthesis of NHS active esters. If the NHS-Label is poorly purified, DSC may be present and can react with the amino-modifier in lieu of the label. If DSC contamination is suspected, the NHS-Label can be reacted with Dansyl Cadaverine and analyzd by RP HPLC for the Dansyl-DSC adduct.

Transamidation

Customers have reported seeing different levels of labeling of an amino-modifier depending upon the sequence of the oligonucleotide. Such sequence dependence may be due to transamidation. The amine of the modifier could 'deprotect' a neighboring base such as dA or dG, and in doing so, be locked up in a non-hydrolyzable amide linkage.

Alkylation by acrylonitrile.

Acrylonitrile is produced in situ during deprotection of the ß-cyanoethyl protecting groups and is a potent electrophile. Typically, the acrylonitrile is eliminated through reaction by base, however, alkylamines, such as those in our family of amino-modifiers, are more reactive than ammonia and will preferentially react with the acrylonitrile during deprotection.

Our previous work had indicated the DSC contamination would lead to amine inactivation. To determine if either or both transamidation and reaction with acrylonitrile could also lead to amine-inactivation, the following experiment was done.

Two oligos were synthesized with the following sequences (1 μmole scale, DMT off, using amino-modifier

10-1906. An additional deblock of the MMT group was done manually for 85 seconds since MMT is slower to remove than DMT.

Amino-Modifier-AGT TTT TTT TTT
Amino-Modifier-TTT TTT TTT TTT

Each synthesis CPG was split in roughly equal portions. One portion of each synthesis was transferred to a Twist column and the other portion to a 4 mL vial. 10 mL of 10% diethylamine (DEA) in acetonitrile was pushed through each synthesis Twist column over a 30 second period into a waste vessel. This was repeated over a 60 second period using fresh 10% diethylamine. The columns were rinsed with ACN (10 mL or so), dried under an argon stream and the CPG transferred to 4 mL vials. Ammonium hydroxide (1 mL, 30% NH3) was added to each vial and the solutions were heated overnight at 55°C. The deprotected oligos were dried down and taken up in 500 μL of water. A 250 μL portion of each vial was labeled with 6 μL TAMRA NHS ester according to our Technical Bulletin and desalted. The labeled and unlabeled oligos were analyzed by RP HPLC.

Results:

The amino-modified-T12 oligo without DEA treatment showed a large peak that eluted just after the unlabeled oligo on RP HPLC. It comprised approximately 20% of the oligo and did not label with TAMRA. When treated with the DEA, this peak was reduced by approximately 90-95%. These data are consistent with the late eluting peak being the acrylonitrile adduct.

The amino-modified-AGT-T9 oligo without DEA treatment showed three peaks - all with longer elution times on RP HPLC. One of the peaks (approximately 8% in area) eluted a full minute after the unlabeled oligo whereas the other two - approximately 25% peak combined area - came out just after the unlabeled oligo where the acrylonitrile adduct was seen for the amino-modified-T12 probe. The fast eluting peaks were only partially resolved. When the support was treated with DEA, the large, fast eluting peak was substantially reduced, but a significant portion - approximately 10% or so peak area - remained. All of these peaks increased in percentage area when labeled with TAMRA indicating that they contain an inactive amine. These results are consistent with these peaks being the benzamide and isobutamide conjugates, the result of reaction with dA and dG respectively - with the isobutamide almost co-eluting with the acylonitrile adduct.

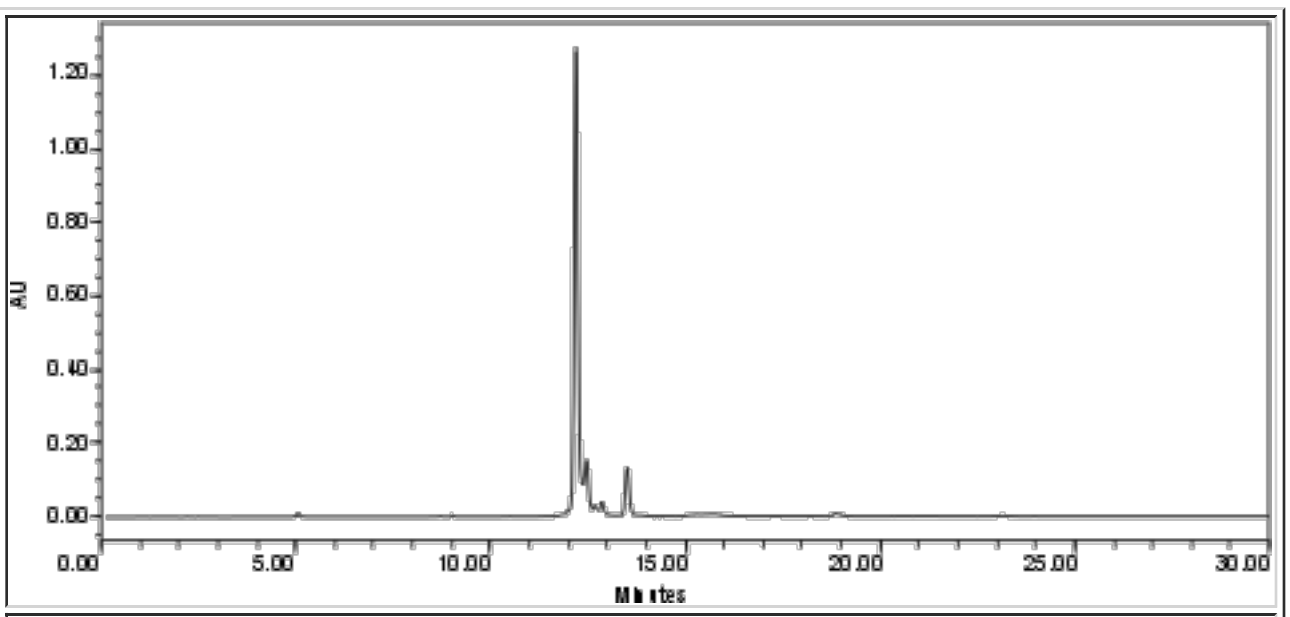

FIGURE 1: DEA-treated amino-modified-AGT-T9 oligo

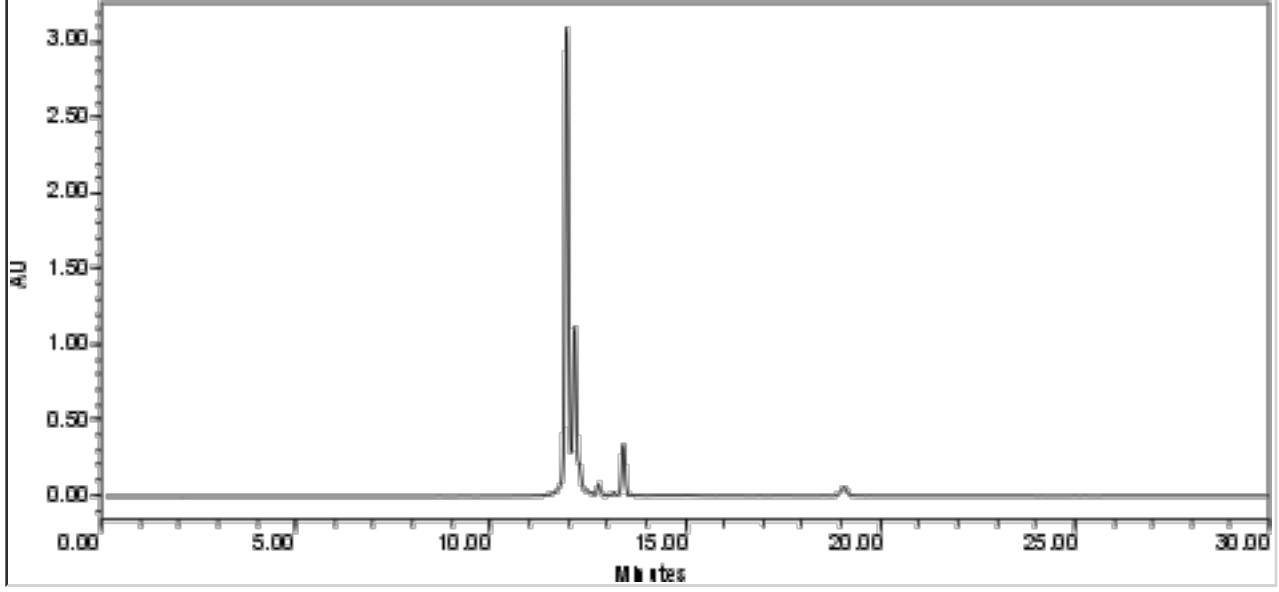

FIGURE 2: amino-modified-AGT-T9 oligo without DEA treatment

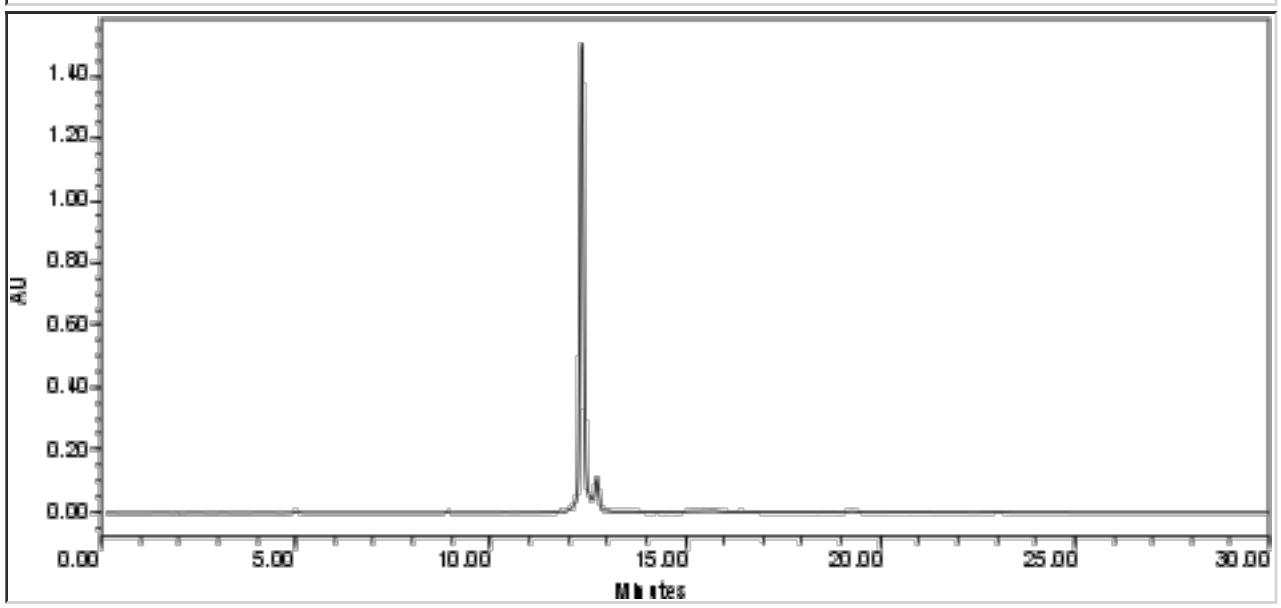

FIGURE 3: DEA-treated amino-modified-T12 oligo

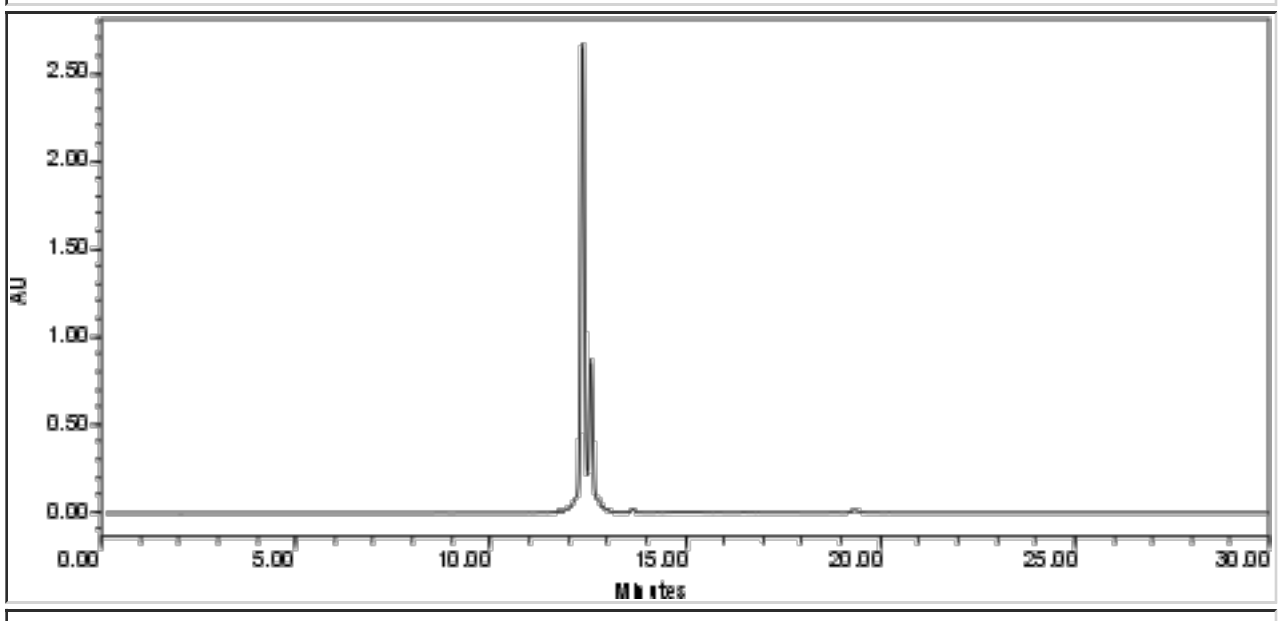

FIGURE 4: amino-modified-T12 oligo without DEA treatment

Conclusion:

In summary, our data suggest that inactivation of amino-modifiers can occur by transamidation and reaction with acrylonitrile. The best solution to avoid inactivation is to keep the MMT group on the amine during

deprotection, reducing the temperature to 40 °C and extending the deprotection to 17 hours. In situations where that is not possible - e.g., a TFA-protected amine - the greatest labeling efficiency will be obtained by first treating the newly synthesized oligo with 10% DEA in ACN while still on the support. We have found that a simple 5 minute treatment with 1 mL of 10% DEA in acetonitrile, followed by a rinse with ACN will remove all acrylonitrile. The oligo is then cleaved and deprotected using ammonium hydroxide/methylamine in UltraFast conditions which minimizes transamidation. And finally, where possible, choose sequences where A's and G's are not adjacent to the amino-modifiers.