

Chemical synthesis of oligonucleotides using acetone as a washing solvent

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Acetone is being proposed as the main washing solvent during automated synthesis of oligonucleotides to reduce acetonitrile usage in the assembly process and endure the impact of global acetonitrile shortage.

Since the publication of the phosphoramidite method for the chemical synthesis of oligonucleotides (1), acetonitrile has been used as the universal solvent to dissolve most of the monomers and modifiers reported in literature as well as most of the activating reagents. Sometimes a small amount of tetrahydrofuran (THF) or dichloromethane is added to dissolve poorly soluble compounds. However, most of the acetonitrile used in the assembly of oligonucleotides is not used to dissolve monomers or activating reagents. Instead it is used as a “washing solvent” to remove all reagents that remain impregnated to the synthesis support between the coupling, capping, oxidation and detritylation reactions that comprise a synthesis cycle.

Historically, acetonitrile has been used in oligonucleotide assembly for three main reasons: (i) as a polar organic aprotic solvent, it dissolves all reagents used in the process (monomer phosphoramidites, 1-H tetrazole or 5-ethylthio-1H-tetrazole, acetic anhydride, 1-methylimidazole, trichloroacetic acid and iodine); (ii) as a sub-product in the production of acrylonitrile, the price of acetonitrile was relatively low and it was always available; and (iii) the HPLC-grade reagent containing an average of 0.001% water is enough to perform reactions in practically anhydrous conditions as required for the coupling step.

Until the middle of 2008, acetonitrile supply was abundant and searching for other solvent options seemed unnecessary. However, at the end of that year, most of the big chemical companies announced a significant shortage, and its

price increased notably at the beginning of 2009. The acetonitrile shortage is a consequence of the reduction of acrylonitrile demand due to the global financial crisis.

The acetonitrile shortage has had not only a direct detrimental effect on many HPLC-based analytical techniques that require this solvent for the elution process, but also in molecular biology, where custom required oligonucleotides are an indispensable tool for staple laboratory techniques such as PCR and DNA sequencing. Therefore, the goal of this study was to look for an alternative economic solvent to reduce or eliminate acetonitrile usage for oligonucleotide assembly. Without this solution, oligonucleotide synthesis core facilities established in developing countries or poorly funded universities may need to stop their production.

One possible option was the well-known solvent acetone, which seems to never have been used in the automated assembly of oligonucleotides. Acetone is commonly used in laboratories of organic chemistry to perform final clean up of glassware used in experiments because it is an inexpensive and non-toxic solvent that dissolves several hydrophobic as well as hydrophilic materials. However, the water content of HPLC-grade acetone is 0.3% on average, which is very high for dissolution of the monomer-phosphoramidites and activating reagents, but suitable for the wash steps. To probe this idea, the acetonitrile bottle of a DNA synthesizer (model no. 394; Applied Biosystems, Foster City, CA) was replaced with acetone and the synthesis of three

oligonucleotides of different length and sequence was achieved. The stepwise average yield per coupling was 95% as determined by a colorimetric assay of the released 4,4'-dimethoxytrityl (DMTr) cation. The visual quality of the three oligonucleotides was acceptable as shown in lanes 2, 5, and 8 in the denaturing polyacrylamide gel shown in Figure 1A. In these three cases, a main band corresponding to the expected oligonucleotide size and minor bands corresponding to truncated chains were observed. In agreement with PAGE analysis, the ion exchange HPLC analysis displayed a main peak in each case and several minor peaks of shorter retention time (Figure 1B).

Therefore, short oligonucleotides such as those required for many PCR applications can be synthesized using acetone as unique washing solvent. Under our synthesis conditions using an old DNA synthesizer, this substitution saved 4.5 mL acetonitrile per coupling. Only 350 μ L acetonitrile distributed between the dissolved phosphoramidite and dissolved activating reagent were used per cycle of synthesis.

Unfortunately, this stepwise yield is not appropriate for the assembly of long oligonucleotides. To circumvent this limitation, a wash step with only 300 μ L acetonitrile, previous to the coupling step, was performed to remove water traces from the support and delivery lines. Since the original acetonitrile bottle was occupied with acetone, and bottle 10 for ammonium hydroxide is always empty in our synthesizer, acetonitrile was loaded into this bottle (Figure 2) and the synthesis program (see Supplementary Materials) was modified to perform the additional wash via a user function. The same oligonucleotide sequences were assembled and the average yield per coupling rose to 98.5%, only 0.4% less than when using acetonitrile for the entire process. PAGE analysis of these oligonucleotides loaded in lanes 3, 6, and 9—as well as ion exchange HPLC—revealed less deletion products than found in those oligonucleotides assembled only with acetone. The products were of similar quality to those synthesized with only acetonitrile (lanes 1, 4, and 7). Furthermore, a 65-mer assembled with the combination of acetone plus an acetonitrile rinse was obtained in high quality as shown in lane 10. Since this implementation in February 2009, more than 1000 oligonucleotides have

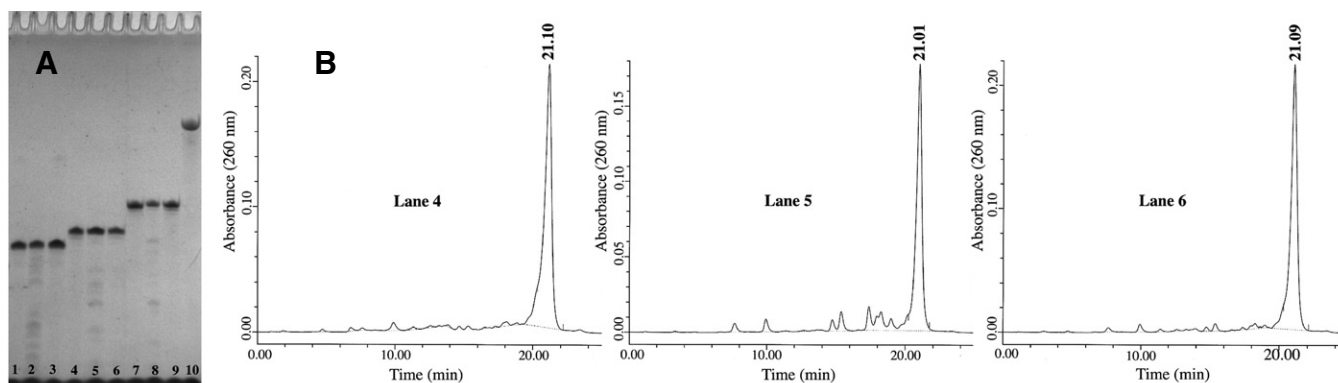


Figure 1. Oligonucleotide analysis. (A) Denaturing 15% polyacrylamide gel visualized by UV shadowing. Lanes 1, 4, and 7 show oligonucleotides synthesized using acetonitrile as the washing solvent. Lanes 2, 5, and 8 show oligonucleotides synthesized with acetone as the washing solvent. Lanes 3, 6, 9, and 10 show oligonucleotides synthesized with the combination of acetone washing with an acetonitrile rinse as described in the text. The spots seen in the bottom of the gel are bromophenol blue from the tracking dye. Oligonucleotide sequences are as follows: Lanes 1–3, 23-mer, AGAAGCTTGGAGACCAATGCAA; Lanes 4–6, 25-mer, CATCCGCGAGTTGACTTCGGTCGTC; Lanes 7–9, 30-mer, GATCCTGCAGCGGTGATTGATTGAGCAAGC; Lane 10, 65-mer, CTCTGCACAGCATATGCACCATCACCATCACCATGGTGCTAGCAAAGGAGAAGAACTCTTCAC. (B) Ion exchange HPLC of the samples shown in lanes 4–6.

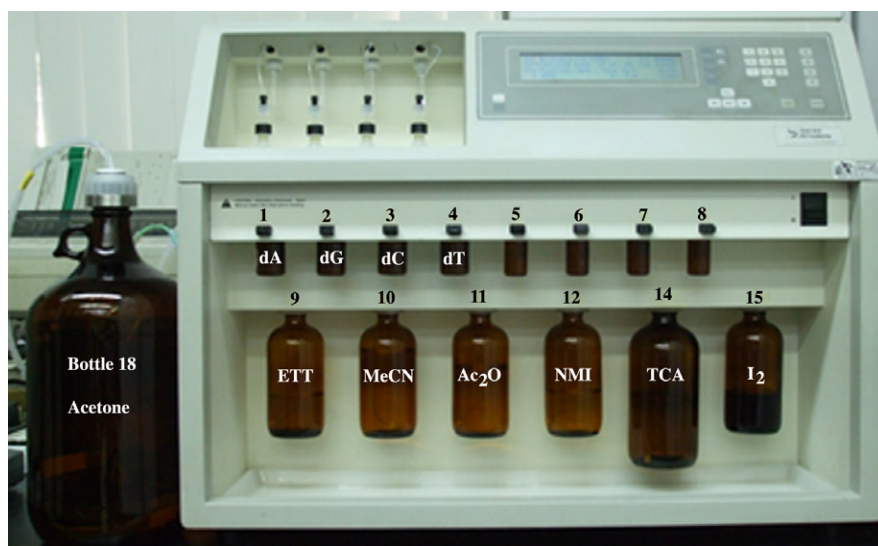


Figure 2. DNA synthesizer implemented for the use of acetone as the main “washing solvent.” Vials 1, 2, 3, and 4 were loaded with standard 80 mM DMTr-protected monomer phosphoramidites of dA, dG, dC and dT respectively, dissolved in acetonitrile. (DMTr stands for the 4,4'-dimethoxytri-tyl protecting group.) Bottle 9, 0.25 M 5-ethylthio-1H-tetrazole (ETT) in acetonitrile; bottle 10, acetonitrile (MeCN); bottle 11, 10% acetic anhydride (Ac_2O) in 8:1 tetrahydrofurane:pyridine; bottle 12, 16% 1-methylimidazole (NMI) in tetrahydrofurane; bottle 14, 2% trichloroacetic acid (TCA) in dichloromethane; bottle 15, 20 mM iodine (I_2) in 8:1:1 tetrahydrofurane:pyridine:water; bottle 18, acetone.

been assembled in our synthesis core facility. These have been used successfully in site-directed mutagenesis, PCR amplifications, and DNA sequencing with no differences when compared with oligonucleotides assembled only with acetonitrile.

In conclusion, HPLC-grade acetone was successfully used to replace most of the acetonitrile wasted in the washing steps during oligonucleotide synthesis. It is possible that an anhydrous form [such as the extra-dry acetone available from Fisher Scientific (Waltham, MA, USA)] will be able to replace acetonitrile for the whole process, even for the dissolution of the key phosphoramidites and activating reagents.

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