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BIOPROCESSING

Advancements in Oligonucleotide Synthesis

Tutorial: A Comparative Study of Commercially Available Universal Supports

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Standard oligonucleotide synthesis uses a solid support that contains the first nucleoside covalently bound to the support by a linker that is hydrolyzed during the cleavage step following solid-phase synthesis. This support-bound nucleoside becomes the 3'-terminal residue of the final oligonucleotide after the cleavage and deprotection steps.

This approach requires the use of at least four solid supports for general DNA synthesis along with an additional four supports for RNA synthesis. Various solid supports containing unusual nucleosides

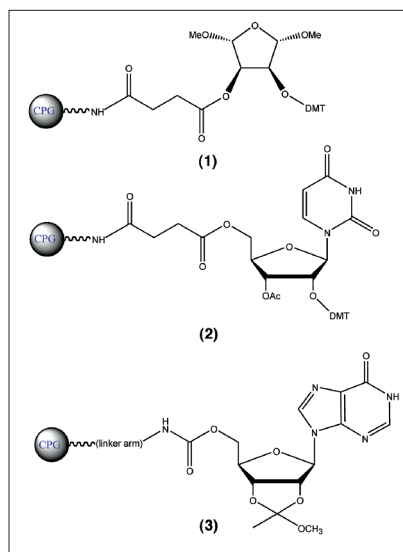


Figure 1a. Universal supports—dephosphorylation after cleavage.

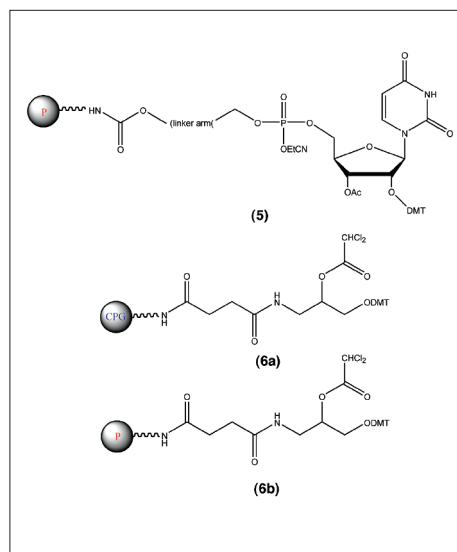


Figure 1b. Universal supports—dephosphorylation during cleavage.

for specific applications are also required.

A universal support does not have the intended 3'-nucleoside attached. Rather, the 3'-nucleoside or residue is added in the first cycle, generating an undesired phosphate linkage between this nucleoside and the universal support.

This approach requires that this phosphate linkage be removed during the

cleavage and/or deprotection steps. However, the universal support strategy offers clear advantages: it eliminates the possibility of errors in parallel synthesis applications where up to 384 wells may contain different supports; it eliminates the need for at least four supports for DNA synthesis and four supports for RNA synthesis; and it simplifies the preparation

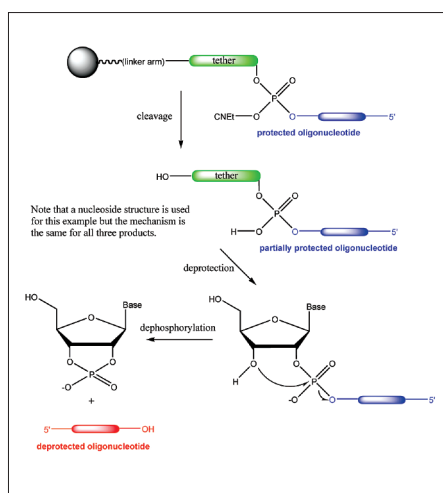


Figure 2a. Cleavage/dephosphorylation of oligonucleotides synthesized on universal supports 1-3.

of oligonucleotides with modified or unusual nucleosides at the 3'-terminus.

Universal Supports

Several universal supports have been described in the literature¹⁻⁵ and are commercially available (Figures 1 a,b). These supports fall into two categories, depending on the timing of the dephosphorylation step that generates the 3'-hydroxyl of the target oligonucleotide.

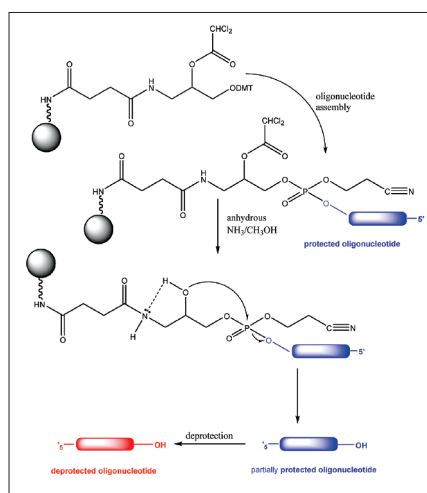


Figure 2b. Cleavage/dephosphorylation of oligonucleotides synthesized on universal supports 6a and 6b.

CLEAVAGE THEN DEPROTECTION AND DEPHOSPHORYLATION In the first category, the regular cleavage step of oligonucleotide synthesis predominantly leaves the tether attached to the 3'-nucleoside through a phosphodiester linkage. The dephosphorylation step along with elimination of the unwanted tether takes place during the deprotection step and usually requires stronger conditions than normal deprotection.

Table 1: Comparison of Universal Solid Supports for Oligonucleotide Synthesis Using Conditions Constrained to a Maximum of 5 h at 55°C

Universal Support	Conditions of Cleavage/Deprotection (c/d)	Percentage of Full length oligo with 3'-OH after c/d	Percentage of full length oligo with 3' tether after c/d	Relative Yield of all UV ₂₆₀ absorbing material after c/d [#]
1	1 mL conc. NH ₃ /H ₂ O containing 15 mg of LiCl for 30 min at r.t., then 5h at 55°C	45%	31%	82%
2	1 mL conc. NH ₃ /H ₂ O containing 15 mg of LiCl for 30 min at r.t., then 5h at 55°C	64%	15%	95%
3	1 mL conc. NH ₃ /H ₂ O containing 15 mg of LiCl for 30 min at r.t., then 5h at 55°C	42%	35%	70%
4*	1 mL conc. NH ₃ /H ₂ O for 30 min at r.t., then 5h at 55°C	58%	22%	82%
5	1 mL conc. NH ₃ /H ₂ O for 30 min at r.t., then 5h at 55°C	71%	0.5%	46%
6a	100 μL of 2M NH ₃ /MeOH for 30 min at r.t., then 1 mL conc. NH ₃ /H ₂ O for 5h at 55°C	83%	0%	98%
6b	100 μL of 2M NH ₃ /MeOH for 30 min at r.t., then 1 mL conc. NH ₃ /H ₂ O for 5h at 55°C	87%	0%	100%

The oligonucleotide prepared was 5'-TTTTTACCGCCCGTACACCTTTT-3'.

[#] The yield of the target oligonucleotide, generated from the polystyrene Universal Support 6b, was taken as 100%. Contents of oligonucleotides in the crude mixtures were determined by ion-exchange HPLC.

* The structure of Universal Support 4 was not disclosed by the manufacturer.

These universal supports¹ are either non-nucleosidic but incorporating a five-membered ring similar to the ribose ring found in nucleosides or nucleoside-based supports, or protected nucleosides configured for base-mediated elimination (Figure 1a).

CLEAVAGE BY DEPHOSPHORYLATION THEN DEPROTECTION In the second group, the dephosphorylation step is the cleavage step, and the only oligonucleotides released into solution already have a 3'-hydroxyl group. Further conventional deprotection leads to the fully deprotected oligonucleotide. The first example⁴ of this type of support uses a nucleotide attached to the support by a non-cleavable linker. The second example⁵ is a novel non-nucleosidic support (Figure 1b).

Discussion

All of these solid supports function similarly: regular detritylation, the addition of the first nucleoside monomer and then the remaining oligonucleotide preparation steps proceed without any changes from standard procedures.

CLEAVAGE THEN DEPROTECTION AND DEPHOSPHORYLATION In the case of the supports in Figure 1a, the elimination of the terminal phosphodiester group utilizes the same reagents, as needed for routine deprotection of oligonucleotides, as shown in Figure 2a. However, much more aggressive and lengthy conditions are typically required.

Upon the completion of oligonucleotide assembly, the 3'-terminal phosphotriester group is first converted into the phosphodiester function by β-elimination of the cyanoethyl protection group. Only upon the release of the 3'-hydroxyl of the tether nucleoside and hydrolysis of the linker to the CPG does

the intramolecular nucleophilic attack on the phosphorous atom of the phosphodiester group take place to effect dephosphorylation.

This dephosphorylation reaction is a relatively slow process, requiring lengthy, aggressive treatment with ammonium hydroxide if the presence of 3'-tethered product along with the target oligonucleotide in the final mixture is to be avoided.

Universal support 4 was also tested in this study but its structure was not revealed by the manufacturer. However, its behavior during testing indicated that it fell into this first category. Using the same tests outlined in *Table 1*, this support also generated mixtures of the target oligomer and 3'-tethered oligomer in ratios similar to those found for universal supports 1–3.

The product profile can be improved by adding metal ions to the mix. Li^+ , Na^+ and Zn^{2+} have all been used to speed up the elimination reaction, presumably by stabilizing the five-membered transition state. However, the speed and simplicity of evaporation of the deprotection solution to give the crude oligonucleotide with no need for desalting is not possible with these ionic additives. All of these facts make these solid matrices unattractive for high throughput oligonucleotide manufacturing.

CLEAVAGE BY DEPHOSPHORYLATION THEN DEPROTECTION When rationalizing the drawbacks of the first class of supports described above, two intrinsic problems must be emphasized. First, the universal support should be designed in such a way that the process of cleavage/3'-dephosphorylation should release only the desired product. Secondly, the 3'-dephosphorylation reaction should proceed quickly. In other words, the processes

Table 2: Comparison of Universal Solid Supports for Oligonucleotide Synthesis Following the Manufacturers' Recommended Procedure

Universal Support	Conditions of Cleavage/Deprotection (c/d)&	Percentage of Full length oligo with 3'-OH after c/d	Percentage of full length oligo with 3' tether after c/d	Relative Yield of all UV ₂₆₀ absorbing material after c/d [#]
1	1 mL of AMA 17h at 55°C	66%	0%	85%
2	1 mL of conc. $\text{NH}_3/\text{H}_2\text{O}$ containing 15 mg of LiCl for 6h at 65°C	79%	0.3%	71%
3	1 mL of conc. $\text{NH}_3/\text{H}_2\text{O}$ containing 17 mg of LiCl for 6h at 75°C	77%	0.5%	93%
4*	1 mL of conc. $\text{NH}_3/\text{H}_2\text{O}$ for 8h at 60°C	81%	1.3%	73%
5	1 mL of conc. $\text{NH}_3/\text{H}_2\text{O}$ for 8h at 60°C	74%	0.5%	74%
6a	1 mL of 3M NH_3/MeOH for 8h at 60°C	87%	0%	91%
6b	1 mL of 3M NH_3/MeOH for 8h at 60°C	87%	0%	100%

The oligonucleotide prepared was 5'-TTTTTTCACCGCCCGGTACACCTTTT-3'.

& Cleavage/deprotection conditions given in Table 2 were recommended by the manufacturers.

The yield of target oligonucleotide, generated from the polystyrene Universal Support 6b, was taken as 100%.

Contents of oligonucleotides in the crude mixtures were determined by ion-exchange HPLC.

* The structure of Universal Support 4 was not disclosed by the manufacturer.

of cleavage and 3'-dephosphorylation have to be the same fast process.

A nucleotide-based universal support (Support 5 in *Figure 1b*) with a non-cleavable attachment to a polystyrene support offers a significant improvement over the supports described above. In this case, the universal linker is attached to the polymer via a phosphotriester group.

Upon aqueous ammonium hydroxide treatment, this phosphotriester group, along with the oligonucleotide 3'-terminal phosphotriester group, are first converted into the phosphodiester functions by β -elimination of the cyanoethyl protection groups. Subsequent deprotection reactions are standard.

The other phosphodiester group linking the universal tether to the support is stable under conditions of cleavage/dephosphorylation. As a result, even after heating at 60 °C for 8 h, only about 0.5% of 3'-tethered oligonucleotide is present in the mixture, along with 74% of 3'-dephosphorylated target oligomer (*Table 2*).

This universal support affords reason-

able quantities of a target oligomer, free from the 3'-tethered product, in a reasonable time and looks more attractive for high throughput applications than the first set.

The most recently described universal support (Supports 6a and 6b in *Figure 1b*) may be the most likely to meet all of the criteria. The cleavage and 3'-dephosphorylation appear to be the same fast process (20–30 min), facilitated by a solution of anhydrous ammonia in methanol.

The labile dichloroacetyl group is cleaved prior to the β -elimination of the cyanoethyl protection group of the phosphate moiety (closest to the spacer, linked to the solid matrix). This is followed by the rapid intramolecular nucleophilic attack on the phosphotriester function by the hydroxyl group. This reaction is additionally assisted by the neighboring amide function.

All of these factors result in the fast cleavage/dephosphorylation of the oligonucleotide, which is still predominantly nucleic base-protected (*Figure 2b*).

After removing the solid support, further deprotection procedures employ either standard protocols, e.g., addition of aqueous ammonium hydroxide to the solution of oligonucleotide in methanolic ammonia and heating the mixture at 55°C for 5h, or simply continued deprotection with methanolic ammonia (60°C for 8 h).

It is noteworthy that a number of additional benefits were found using complete deprotection in anhydrous ammonia in methanol. First, final evaporation of ammonia in methanol takes much less time than the evaporation of aqueous ammonia (4–5 times faster).

Second, longer oligonucleotides (>50mer) precipitate from the methanolic ammonia nearly quantitatively in the course of deprotection of nucleic bases. This allows separation of the product oligomers by centrifugation in less than 5 min.

Conclusion

The main impediment to the adoption of a universal support has been the aggressively basic conditions required to

complete the elimination reaction to release the terminal hydroxyl group. The standard reagents used in oligonucleotide deprotection are ammonium hydroxide and aqueous methylamine, which are popular since they are completely volatile.

Using these reagents to carry out the elimination reaction requires either high temperature, with attendant high pressure, or extended reaction times. In addition, lithium chloride has been used to speed up the elimination reaction. However, the addition of salts to the deprotection solution requires an additional desalting step for the crude oligonucleotides and may be damaging to siRNA oligos.

The group of universal supports tested performed very well when used according to the manufacturers' guidelines. However, the results outlined in *Tables 1 and 2* show that only universal supports 5 and 6a,b are candidates to be truly universal. Universal supports 6a,b performed the best of the group, generating the best yields of oligonucleotide under

the mildest conditions.

This support type would be appropriate for the production of DNA oligos, long and short, as well as those requiring mild deprotection. It is also compatible with the synthesis of RNA and siRNA. The reagent used for the cleavage/dephosphorylation step is commercially available and the procedures described are fully compatible with high throughput synthesis. Universal Supports 1 and 6a,b are available from **Glen Research** (Sterling, VA). **GEN**

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