Rainbow™ Universal CPG: A Versatile Solid Support for Oligonucleotide Synthesis

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ABSTRACT

We have developed a universal solid support, termed Rainbow™ Universal CPG, for use in automated oligonucleotide synthesis. The universal solid support allows any oligodeoxyribonucleotide sequence to be synthesized from a single type of controlled pore glass (CPG) support. Deprotection of oligodeoxyribonucleotides was optimized using 0.5 M LiCl in concentrated ammonium hydroxide. PCR experiments using three different sets of primers proved that the 3′-hydroxyl function of oligodeoxyribonucleotides synthesized from Rainbow Universal CPG was retained. This universal solid support shows promise for replacing the standard nucleoside CPG supports.

INTRODUCTION

Solid supports used in automated oligonucleotide synthesis possess pre-attached nucleosides to provide chain initiation sites for oligonucleotide construction (4). Chain elongation occurs by sequential addition of monomeric phosphoramidite units on the 5′ hydroxyl. During the deprotection process of the oligodeoxyribonucleotide, the pre-attached nucleoside is cleaved from the solid support and retained on the oligodeoxyribonucleotide as the 3′-terminal base. However, since the 3′-terminal base can be any one of the four base variations, four separate solid supports are needed to accommodate all possible sequences. A universal solid support that could be used to synthesize any oligodeoxyribonucleotide regardless of the nature of the 3′-terminal base would have significant advantage (3).

In this paper, we introduce a novel and universal solid support, Rainbow™ Universal CPG, for use in automated oligonucleotide synthesis (Figure 1). The universal support is quite versatile, allowing any oligodeoxyribonucleotide sequence to be synthesized from a single type of controlled pore glass (CPG) support. It is stable under standard oligodeoxyribonucleotide synthesis conditions. Oligodeoxyribonucleotide cleavage from the support and deprotection is easily accomplished with a modified ammonium hydroxide procedure. Finally, incorporation of 3′-terminal mixed-base sites, labels or other modifications can be conveniently achieved through the use of phosphoramidite reagents.

MATERIALS AND METHODS

Biotin-VN Phosphoramidite, 3′-Biotin-VN CPG, Oligonucleotide Purification Elution Columns (OPEC™) and the Advantage™ Polymerase Chain Reaction (PCR) Kit were obtained from CLONTECH Laboratories (Palo Alto, CA, USA). All other chemicals were purchased from either Aldrich Chemical (Milwaukee, WI, USA) or Chem Impex International (Wood Dale, IL, USA). Ion-exchange HPLC analysis was performed on a Rabbit HPX System (Rainin Instruments, Emeryville, CA, USA) using a Mono Q® HR 5/5 Column (Pharmacia Biotech, Piscataway, NJ, USA). Oligonucleotide synthesis was performed on a Model 394 DNA/RNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA), and PCR amplification was accomplished on a Model 480 DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) .

Preparation of Rainbow Universal CPG

The succinate precursor was prepared from *cis*-3-O-(4,4′-dimethoxytrityl)tetrahydrofuran-4-ol using a modified procedure of Hardy et al. (5) and covalently attached to CPG (500 Å) through a novel approach using benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as coupling reagent (6). Thus, BOP (233 mg, 0.527 mmol), 1-hydroxybenzotriazole (HBT, 71 mg, 0.527 mmol), triethylamine (0.42 mL) and *cis*-3-O-(4,4′-dimethoxytrityl)tetrahydrofuran-4-yl succinate (304 mg, 0.6 mmol) were dissolved in 32 mL methylene chloride. Long chain alkyl amine (LCAA)-CPG (10 g) was added, and the mixture was agitated at room temperature for 16 h on an orbital shaker. The CPG resin was collected in a sintered glass funnel, washed (6× 100 mL methanol, 2× 100 mL ethyl ether) and dried under high vacuum for 30 min. Capping was accomplished by treating the resin with a pyridine/acetic anhydride/*N*-methylimidazole (50:10:6, vol/vol/vol) mixture for 2 h. The CPG resin was again collected in a sintered glass funnel and washed (3× 100 mL pyridine, 3× 100 mL dimethylformamide, 6× 100 mL methanol, 2× 100 mL ethyl ether) and then dried under high vacuum. The trityl loading was determined to be 32 µmol/g.

Oligodeoxyribonucleotide Synthesis

All synthetic oligodeoxyribonucleotides were prepared from Rainbow Universal CPG by the phosphoramidite method using standard conditions on a Model 394 DNA/RNA Synthesizer (PE Applied Biosystems). Oligodeoxythymidine 7-mers were synthesized for cleavage and deprotection studies. Control oligodeoxyribonucleotides were prepared from standard nucleoside CPG supports. All syntheses were performed on a 1.0-µmol scale and the TRITYL-ON mode was used to facilitate subsequent cartridge purification. 3′-Biotin modification was accomplished by using the universal CPG in conjunction with coupling Biotin-VN Phosphoramidite in the first cycle (7). A 3′-biotinylated control oligodeoxyribonucleotide possessing the identical structure was synthesized from the corresponding 3′-Biotin-VN CPG reagent. Coupling efficiencies were determined by measuring the dimethoxytrityl (DMT) cation concentration.

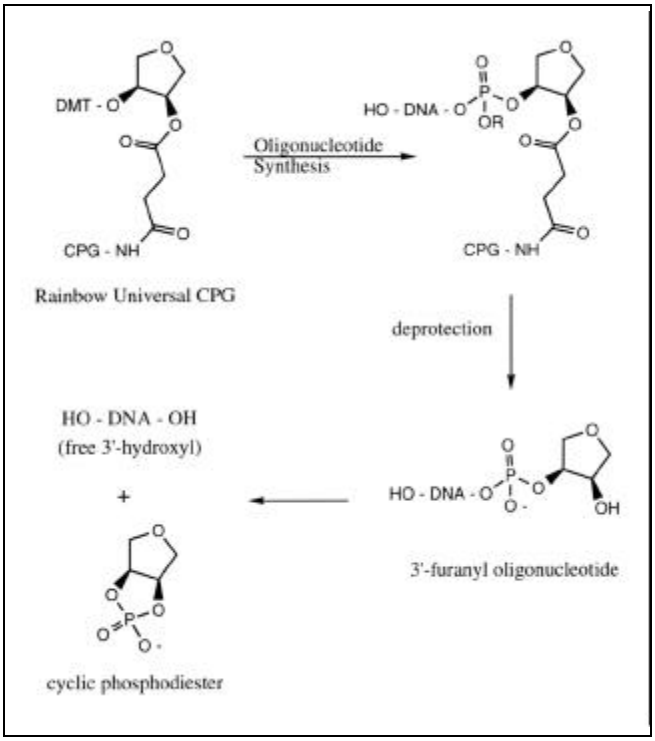

Figure 1. Illustration of the functional design of Rainbow Universal CPG in oligodeoxyribonucleotide synthesis. Scission of 3′-furanyl phosphodiester results in a free 3′-terminal hydroxyl.

Solid Support Cleavage and Deprotection

Oligodeoxyribonucleotides synthesized from Rainbow Universal CPG were cleaved from the solid support and deprotected by treatment with 0.5 M LiCl in concentrated ammonium hydroxide at 55°C for 16 h overnight. The addition of 0.23 M triethylamine (1:30 vol/vol) was added to effect a 1-h quick deprotection procedure. The 3′-furanyl cleavage efficiency was investigated by individually varying LiCl concentration, time and temperature parameters (Figure 2). Control oligodeoxyribonucleotides synthesized from standard nucleoside CPG were deprotected using standard ammonium hydroxide treatment.

Oligodeoxyribonucleotide Purification and Desalting

All oligodeoxyribonucleotides synthesized in the TRITYL-ON mode were purified by directly applying the deprotection solution to Oligodeoxyribonucleotide Purification Elution Columns and following the recommended procedures of the supplier. Alternatively, crude oligodeoxyribonucleotides (TRITYL-OFF mode) were conveniently worked up by evaporation of the deprotection solution, dissolution of the LiCl residue in 1 mL methanol, spinning down the suspended oligodeoxyribonucleotide to a pellet and decanting off the supernatant.

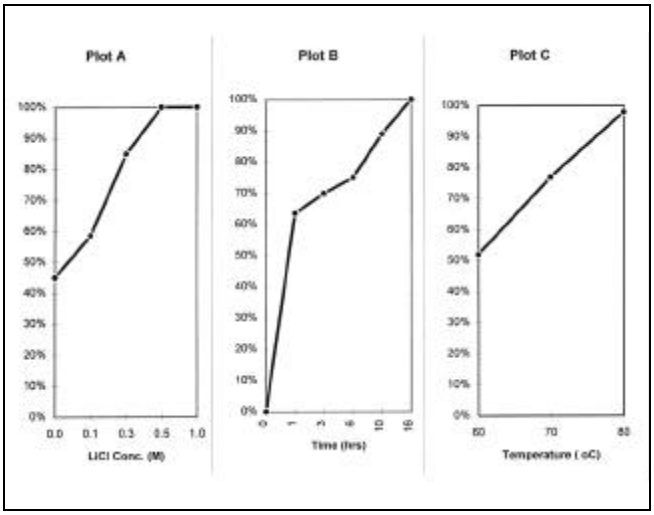

Figure 2. Percent cleavage of the 3′-furanyl phosphodiester vs. LiCl concentration, time and temperature parameters. Poly(dT)$_7$-mers synthesized from Rainbow Universal CPG and deprotected in concentrated ammonium hydroxide using the following conditions. Plot A: Varying concentration of LiCl conducted at 55°C over a 16-h period. Plot B: Varying time conducted at 55°C with 0.5 M LiCl. Plot C: Varying temperature conducted in 0.5 M LiCl and 0.23 M triethylamine over a 1-h period.

Ion-Exchange HPLC Analysis

Poly(dT)$_7$ oligodeoxyribonucleotides synthesized using Rainbow Universal CPG were analyzed by ion-exchange HPLC on a Mono Q HR 5/5 Column (5 mm × 5 cm). The mobile phases used were A = 50 mM Tris-HCl (pH 7.5) and B = 800 mM NaCl, 50 mM Tris-HCl (pH 7.5). A gradient of 5%–45% B over 40 min at a flow rate of 1.0 mL/min was used. Peaks corresponding to the free 3′-OH oligodeoxyribonucleotide vs. the 3′-furanyl-derivatized oligodeoxyribonucleotide were fully resolved. The efficiency of cleaving the 3′-terminal phosphodiester (furanyl group) was determined by area integration. A (dT)$_7$ oligodeoxyribonucleotide synthesized from standard deoxythymidine CPG was used as a control.

Enzymatic Digestion of Oligodeoxyribonucleotides

Oligodeoxyribonucleotides 1–6 were digested with snake venom phosphodiesterase/alkaline phosphatase and analyzed by reversed-phase HPLC according to the procedures of Beaucage (2).

PCR Amplification

The PCR amplifications were carried out in 50-μL reactions using Advantage™ KlenTaq Mix/cDNA PCR Kit and protocol (CLONTECH). The final PCR mixture included KlenTaq Polymerase Mix (1×), 40 mM tricine-KOH (pH 9.2), 15 mM KOAc, 3.5 mM Mg(OAc)$_2$, 75 μL/mL bovine serum albumin (BSA), 200 μM of each dNTP, 0.01 mg/mL human placental cDNA template and 1 μM of each primer. After overlaying with 50 μL of mineral oil, the reactions were subjected to PCR under the following conditions: 1 min at 94°C, then 28 cycles of 94°C for 30 s and 68°C for 5 min. After the reaction was completed, 5 μL of loading dye were added to each reaction. Ten microliters of each reaction were then loaded on a 1.2% agarose gel and electrophoresed in 1× TBE buffer (pH 8.0).

RESULTS AND DISCUSSION

Design and Preparation of the Universal Solid Support

We designed Rainbow Universal CPG by combining a previous approach from deBear et al. (3) and the cyclic *cis* diol phosphodiester cleaving mechanism of Hardy et al. (5). Thus, we replaced the pre-attached nucleoside of standard CPG solid supports with a non-nucleosidic anhydroerythritol (furanyl) adapter. The adapter eliminates the sequence restrictions due to the four possible base variations and is easily cleaved from the resin with ammonium hydroxide treatment. After cleavage from the solid support, the adapter is retained on the 3′ terminus of the oligodeoxyribonucleotide. This provides a scission mechanism to cleave the 3′-terminal phosphodiester, yielding a free 3′-hydroxyl oligodeoxyribonucleotide and a cyclic phosphodiester by-product. This is critical to the design because many applications using synthetic oligodeoxyribonucleotides such as PCR and sequencing require a free 3′-hydroxyl for enzymatic synthesis. The entire deprotection process is illustrated in Figure 1. Furthermore, incorporation of a DMT protection group at the chain elongation site allows use of standard deblocking conditions and direct coupling efficiency measurements of the first base. Lastly, the universal solid support is compatible with standard procedures and chemical reagents used in oligodeoxyribonucleotide synthesis.

Oligodeoxyribonucleotide Synthesis Using the Universal Solid Support

The Rainbow Universal CPG was successfully used in automated solid-phase synthesis of oligodeoxyribonucleotides. Standard procedures using a commercial synthesizer gave average phosphoramidite couplings at greater than 99%. Since the universal CPG possesses a DMT-protected hydroxyl, we were able to directly measure the first base phosphoramidite couplings at greater than 98% efficiency. Overall we found the performance of Rainbow Universal CPG to be very convenient. High coupling efficiencies and recoveries of pure oligodeoxyribonucleotides were observed.

Oligodeoxyribonucleotide Deprotection and Scission of the 3′-Furanyl Phosphodiester

It was anticipated that normal ammonium hydroxide deprotection conditions would not effect complete scission of the 3′-furanyl phosphodiester to give a free 3′-hydroxyl (Figure 1). When we used literature procedures that used methylamine as an additive to ammonium hydroxide during the deprotection step, only 82% scission was effected (5). This was unacceptable since the efficiency of the scission mechanism

must be essentially quantitative for the universal CPG to have any practical value. Therefore, we investigated the use of lithium chloride to facilitate the scission of the 3′-furanyl phosphodiester. The use of metal ions to facilitate cleavage of phosphodiester linkages has been demonstrated previously (3,8).

Several different concentrations of lithium chloride in ammonium hydroxide were tested in the deprotection experiments, and the results are shown in Figure 2 (Plot A). The degree of 3′-phosphodiester scission was quantitated by analytical ion-exchange HPLC using $(dT)_7$ oligodeoxyribonucleotides synthesized from Rainbow Universal CPG. Both 3′-furanyl phosphodiester and free 3′-hydroxyl $(dT)_7$ peaks were fully resolvable, and quantitation was accomplished by area integration. Complete scission of the 3′-furanyl phosphodiester to give a free 3′ hydroxyl was observed to readily occur at 0.5 M LiCl. The lithium cation indeed facilitates the formation of the cyclic phosphodiester yielding a free 3′-terminal hydroxyl (Figure 1).

Time and temperature parameters of the 0.5 M LiCl/ammonium hydroxide deprotection procedure were also investigated, and the results are plotted in Figure 2. Plot B demonstrates that 16 h (overnight) at 55°C are needed to achieve complete cleavage of the 3-furanyl phosphate group. We were also able to successfully effect a 1-h quick deprotection scheme that used triethylamine as a second additive to facilitate deprotection (1). Plot C demonstrates 98% scission of the 3′-furanyl phosphodiester using 0.23 M triethylamine and 0.5 M LiCl at 80°C.

Oligodeoxyribonucleotide Purification

TRITYL-ON oligodeoxyribonucleotides could be purified on a cartridge by directly applying the LiCl/ammonium hydroxide deprotection mixture and using commercially recommended elution procedures. Alternatively, for crude oligodeoxyribonucleotide (TRITYL-OFF) workup, the LiCl could be removed easily after evaporation of ammonium hydroxide by dissolution in methanol and centrifuging the precipitated oligodeoxyribonucleotide.

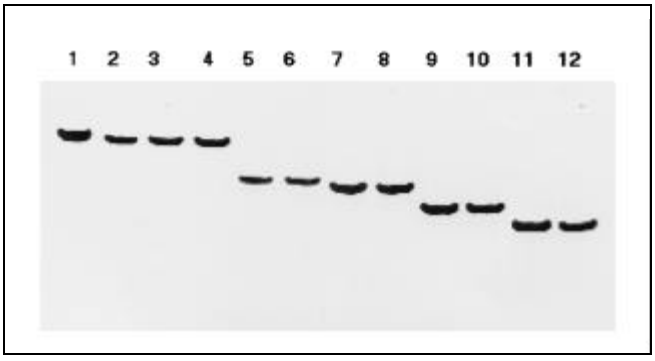

Figure 3. Polyacrylamide gel electrophoresis analysis of oligodeoxyribonucleotides 1–6 prepared from Rainbow Universal CPG. Sequences ATCTGGCACCACACCTTCTACAATGAGCTGCG, CGTCATACTCCTGCTTGCTGATCCACATCTGC, TTCTCATGGAAGCTATGGGTATCACAT, CCACCATCTCGGTCATCAGGATTGCCT, GCTTACATGTCTCGATCCCACTTAA and CTCGCGCTACTCTCTCTTTCTGG correspond to oligodeoxyribonucleotides 1, 2, 3, 4, 5 and 6, respectively. Oligodeoxyribonucleotides 1, 2, 3, 4, 5 and 6 correspond to even-numbered lanes 2, 4, 6, 8, 10 and 12, respectively. Odd-numbered lanes are control oligodeoxyribonucleotides having the same sequence as its immediate neighbor on the right, but synthesized using standard nucleoside CPG.

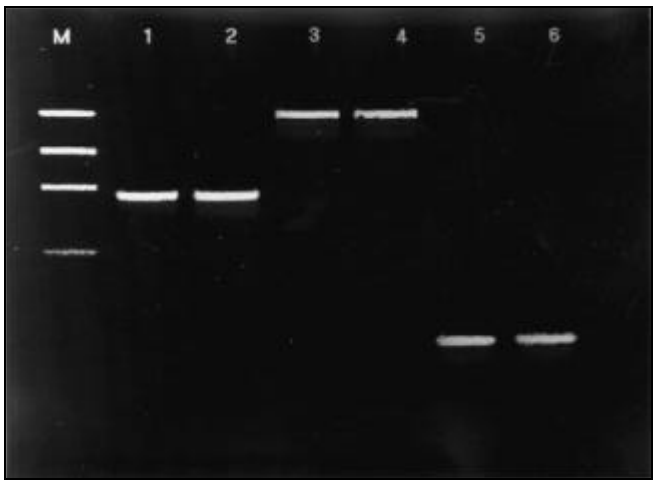

Figure 4. PCR amplification of human placental cDNA using primers prepared from Rainbow Universal CPG. Odd-numbered lanes correspond to primers synthesized using the universal CPG. Even-numbered lanes correspond to control primers of identical sequence prepared from standard nucleoside CPG. Lanes 1 and 2: primers 1 and 2 (β-Actin). Lanes 3 and 4: primers 3 and 4 (transferrin receptor). Lanes 5 and 6: primers 5 and 6 (β_2-microglobulin). Lane M: ϕX174 marker (*Hae*III digest, from top 1.35, 1.08, 0.872, 0.603 and 0.310 kb).

PCR Amplification

The functional integrity of the 3′-hydroxyl was examined by PCR amplification. Three sets of PCR primers (1–6) were synthesized using Rainbow Universal CPG, and the sequences are listed in the legend of Figure 3. The syntheses were performed in the TRITYL-ON mode for convenient purification with polymeric columns. Each primer prepared from the Universal CPG was deprotected by treatment with 0.5 M LiCl/ammonium hydroxide at 55°C for 16 h. Control primers were synthesized using standard nucleoside CPG and standard deprotection conditions. Polyacrylamide gel electrophoresis analysis revealed that primers prepared using the Universal CPG appeared identical to control primers prepared from standard nucleoside CPG methods (Figure 3). The integrity of oligodeoxyribonucleotides 1–6 was confirmed by digesting each with snake venom phosphodiesterase/alkaline phosphatase followed by HPLC analysis (2).

We used each of the three different primer sets in PCR amplification to test the functional integrity of the 3′-terminal hydroxyl groups (Figure 4). The three primer sets corresponded to the human β-actin, transferrin receptor and β_2-microglobulin genes. In all cases, the primer sets prepared from the Rainbow Universal CPG amplified identically to the primers synthesized with standard CPG, indicating no change in the function of the 3′-terminal hydroxyl groups.

3′-Terminal Mixed-Base and Biotinylation Modifications

Additional utility was demonstrated by conveniently preparing oligodeoxyribonucleotides having 3′-terminal mixed-base sites using Rainbow Universal CPG and programming a mixed-base position for the first amidite coupling. This improves the current method, which requires manual mixing of all four nucleoside CPGs to accomplish the synthesis of a 3′ mixed base. Also, biotinylation at the 3′ end [$(dT)_7$-mer] was achieved by coupling Biotin-VN Phosphoramidite in the first cycle (7). Structural identity of the 3′-biotinylated oligodeoxyribonucleotide was verified by synthesizing the same oligodeoxyribonucleotide from the corresponding 3′-Biotin-VN CPG reagent and comparing them by ion-exchange HPLC and polyacrylamide gel electrophoresis (results not shown).

CONCLUSION

In summary, Rainbow Universal CPG has been successfully used in solid-phase oligodeoxyribonucleotide synthesis under standard conditions. The universal solid support is versatile, allowing any oligodeoxyribonucleotide sequence to be synthesized from a single type of CPG support regardless of the nature of the 3′-terminal base. A simple cleavage and deprotection procedure using 0.5 M LiCl/ammonium hydroxide results in quantitative deprotection to give free 3′-hydroxyl oligodeoxyribonucleotides. The function of the 3′ terminus is retained and is identical to oligodeoxyribonucleotides synthesized using standard nucleoside CPG. Furthermore, 3′ mixed-base positions and other 3′ modifications can be achieved easily through phosphoramidite additions, eliminating the need for special 3′ CPG labeling reagents. Rainbow Universal CPG represents a significant improvement in the simplicity and economy of oligodeoxyribonucleotide synthesis, and we are currently expanding its scope of applicability to RNA synthesis.

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