Solid-Phase Synthesis of Branched Oligonucleotides

This unit describes the synthesis of nucleic acids containing vicinal 2′,5′- and 3′,5′-phosphodiester bonds. These molecules occur in the cell nucleus, and are formed during the splicing of precursor messenger RNA (pre-mRNA). As such they have many potential applications in nucleic acid biochemistry, particularly as tools for probing the substrate specificity of lariat debranching enzymes, and as tools for studying pre-mRNA splicing (e.g., Nam et al., 1994; Carriero et al., 2001). The assembly of these branched nucleic acids (bNAs) on a solid support can be achieved by following two strategies (Damha and Zabarylo, 1989; Braich and Damha, 1997). The first, referred to as the convergent strategy, is based on well-established automated phosphoramidite chemistry (UNITS 3.3 & 3.5). This method uses a ribonucleoside bisphosphoramidite as the branch-introduction synthon (see Basic Protocol 1). The branching reagent serves to couple together solid-support-bound chains, thus forming a branch juncture with the desired vicinal 2′,5′- and 3′,5′-phosphodiester bonds (see Basic Protocol 2). For efficient branching to occur, CPG supports with high nucleoside loadings are used (see Support Protocol 1). With this approach, Y- and V-shaped molecules having identical 2′ and 3′ chains are readily assembled.

The second method is a divergent approach that permits the regiospecific synthesis of bNAs using readily available phosphoramidite reagents (see Basic Protocol 3). An important feature of this method is the assembly of a linear DNA:RNA chimera containing a single 2′-O-silylribonucleoside residue in the middle of the chain. Subsequent removal of the 2-cyanoethyl and silyl protecting groups without detaching the nascent oligonucleotide from the solid support is another salient feature of this approach. This releases an internal 2′-OH group from which orthogonal synthesis of a branch can be carried out.

This unit also describes methods used in the authors’ laboratory for the deprotection (see Support Protocol 2), purification, and characterization of branched oligonucleotides. Preferred methods for purification of bNAs are anion-exchange HPLC (see Support Protocol 3) and polyacrylamide gel electrophoresis (see Support Protocol 4). The branched nature of the molecule is confirmed by enzymatic hydrolysis of the bNA to its constituent nucleosides using nuclease P1 (see Support Protocol 3). Further characterization may be conducted via nucleoside composition analysis using snake venom phosphodiesterase (UNIT 10.6) or MALDI-TOF-MS (UNIT 10.1).

SYNTHESIS AND CHARACTERIZATION OF THE ADENOSINE BRANCHING SYNTHON N⁶-BENZOYL-5′-O-(4,4′-DIMETHOXYTRITYL)-ADENOSINE-2′,3′-BIS-O-(2-CYANOETHYL-N,N-DIISOPROPYL) PHOSPHORAMIDITE

The authors’ group has been predominantly interested in the synthesis of branched RNA fragments related to the lariat intermediates formed during pre-mRNA splicing. Such intermediates contain almost exclusively adenosine at the branch point; therefore, the protocol given below describes the synthesis of the adenosine branching phosphoramidite synthon (BIS-A; S.3; Fig. 4.14.1) used for the synthesis of symmetrical, branched DNA and RNA oligonucleotides (Damha and Ogilvie, 1988). The same protocol may be adapted to the synthesis of the corresponding U, C, and G bisphosphoramidites.

The starting protected nucleoside, N⁶-benzoyl-5′-O-(4,4′-dimethoxytrityl)adenosine (S.1), is commercially available (ChemGenes). Alternatively, it may be synthesized from adenosine using the transient benzoylation procedure of Ti et al. (1982), followed by dimethoxytritylation of the 5′-hydroxyl group (Hakimelahi et al., 1982; Wu et al., 1989).
The synthesis of the branching synthon involves the phosphitylation of the 2' and 3' secondary hydroxyls of the ribose sugar (Fig. 4.14.1) using an excess of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (S.2). Reaction conditions, workup, chromatographic purification, and characterization of the product N6-benzoyl-5'-O-(4,4'-dimethoxytrityl)adenosine-2',3'-O-bis-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (S.3) are described.

**Materials**

- N6-Benzoyl-5'-O-(4,4'-dimethoxytrityl)adenosine (S.1; ChemGenes)
- 4-Dimethylaminopyridine (DMAP; 99%; Aldrich)
- Nitrogen or argon gas, dry
- Anhydrous THF (see recipe) in a septum-sealed distillation collection bulb
- Anhydrous DIPEA (see recipe)
- 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite (S.2; ChemGenes)
- 1:1 (v/v) dichloromethane/diethyl ether
- 20% (v/v) sulfuric acid (optional)
- Ethyl acetate prewashed with 5% (w/v) NaHCO3
- NaCl solution, saturated
- Sodium sulfate (Na2SO4), anhydrous
- 50:47:3 (v/v/v) CH2Cl2/hexanes/triethylamine
- Silica gel (230- to 400-mesh) in 50:47:3 CH2Cl2/hexanes/triethylamine
- 95% (v/v) ethanol
- Diethyl ether
- 50-mL oven- or flame-dried round-bottom flask with rubber septum
- Glass syringe and needle, oven dried
- 2 × 5 cm silica-coated thin-layer chromatography (TLC) plate with fluorescent indicator (e.g., Kieselgel 60 F254 aluminum sheets)
- 254-nm UV light source
- 500-mL separatory funnel
- Gravity filtration device and filter paper
250- and 500-mL round-bottom flasks
Rotary evaporator with a water aspirator
5 × 25–cm glass chromatography column with solvent reservoir bulb

Additional reagents and materials for thin-layer chromatography (TLC; APPENDIX 3D), column chromatography (APPENDIX 3E), 31P-NMR (UNIT 7.2), and mass spectrometry (UNITS 10.1 & 10.2)

**Phosphitylate 2′- and 3′-OH**

1. Place 1.3 g (2.0 mmol) N6-benzoyl-5′-O-(4,4′-dimethoxytrityl)adenosine (S.1) and 84 mg (0.7 mmol) DMAP into a 50-mL oven- or flame-dried round-bottom flask.

2. Cap the flask with a rubber septum and purge the flask with dry nitrogen or argon. *Care must be taken to avoid the presence of moisture throughout the entire reaction.*

3. Withdraw anhydrous THF from a septum-sealed distillation collection bulb using an oven-dried glass syringe and needle. Add 6.0 mL THF to the purged flask with stirring until the starting material is completely dissolved.

4. Add 3.6 mL (21 mmol) anhydrous DIPEA and stir the mixture.

5. Slowly add 1.8 mL (8.3 mmol) 2-cyanoethyl-\(\text{N},\text{N}\)-diisopropylchlorophosphoramidite (S.2).

   If the THF solution is sufficiently dry, a white precipitate should form after ~1 min. This is the diisopropylethylammonium hydrochloride salt that forms during the reaction.

6. Stir the mixture 1 hr at room temperature or until the reaction is complete. *Reaction of the secondary hydroxyls of the starting material with S.2 is fast. If TLC analysis (see below) reveals that the reaction is not complete after 1 hr, a further 0.5 mmol S.2 should be added dropwise and stirring continued until all of the starting material is consumed.*

7. Monitor reaction by TLC

   7. Spot the reaction mixture onto a precut 2 × 5–cm silica-coated TLC plate with fluorescent indicator and develop using 1:1 (v/v) dichloromethane/diethyl ether (APPENDIX 3D).

   Addition of 1% to 3% triethylamine may help prevent detritylation.

8. Visualize heterocyclic bases under a 254-nm UV light source. If desired, spray the plate with 20% sulfuric acid in order to visualize the dimethoxytrityl-bearing species. *CAUTION: Wear protective eyewear.*

   TLC analysis should indicate complete conversion to products, which exhibit larger \(R_f\) values than the starting protected nucleoside. Since the phosphitylation reaction gives rise to two new chiral centers (2′- and 3′-P), the product (S.3) consists of a mixture of four diastereomers. The products appear as two spots \((R_f = 0.51 \text{ and } 0.40)\) or as one dumbbell-shaped spot, because the solvent system partially resolves the four diastereomeric products.

   A minor side product forms, which migrates between the product (S.3) and starting material (S.1). This is likely the nucleoside-2′,3′-H-bis-phosphonate \((R_f = 0.25)\) that forms via hydrolysis of S.3.

**Work up reaction**

9. Transfer the reaction mixture to a 500-mL separatory funnel and add 100 mL prewashed ethyl acetate.

   The ethyl acetate is prewashed with 5% NaHCO₃ in order to prevent detritylation and/or activation of the phosphoramidite moiety.

10. Wash the ethyl acetate layer five times each with 100 mL saturated NaCl solution.

   The diisopropylammonium hydrochloride salt dissolves.
11. Dry the organic layer over anhydrous Na₂SO₄. Add more Na₂SO₄ if the salt crystals clump together upon swirling.

When the solution is dry, the nonhydrated crystals will float in solution upon swirling.

12. Gravity filter the resulting solution through filter paper into a 250-mL round-bottom flask. Rinse the Na₂SO₄ crystals with 10 to 20 mL ethyl acetate.

13. Remove the solvent under reduced pressure (i.e., in a rotary evaporator with a water aspirator) to yield the crude product as a yellow oil.

Isolate and characterize product

14. Prepare a 5 × 25–cm glass chromatography column by adding a slurry of 40 g silica gel in 50:47:3 CH₂Cl₂/hexanes/triethylamine. Precondition with the same solvent.

15. Dissolve the crude material in a minimum amount of 50:47:3 CH₂Cl₂/hexanes/triethylamine and load on column. Perform chromatography at a rate of ~1 in. solvent/min using a small amount of air pressure (APPENDIX 3E). Collect product in 10-ml fractions in small test tubes.

16. Combine product-containing fractions into a 500-mL round-bottom flask and concentrate to an oil on a rotary evaporator.

17. Remove residual triethylamine by co-evaporating the oil first with 50 mL of 95% ethanol followed by 50 mL diethyl ether, to provide the pure product as a pale yellow foam.

18. Store bisphosphoramidite at −20°C under an inert atmosphere protected from light.

Phosphoramidites are particularly sensitive to UV light; therefore, it is best to store the bisphosphoramidite in a dark bottle (or a bottle covered with aluminum foil) in a ~−20°C freezer. Under these conditions, the phosphoramidite may be stored for an indefinite period of time. Prior to use, its purity may be verified via TLC analysis. If partial decomposition has occurred, or the coupling reactions with S.3 are poor, the compound should be subjected to chromatography again as described above.

19. Characterize by TLC (APPENDIX 3D), ³¹P-NMR (UNIT 7.2), and mass spectrometry (UNITS 10.1 & 10.2).

³¹P-NMR spectra (400 MHz) of S.3 were measured on a Varian XL-400 spectrometer using CD₃CN as the solvent (Fig. 4.14.2). Chemical shifts are reported in parts per million (ppm) and are downfield (positive value) from 85% H₃PO₄ (external standard). Fast atom bombardment mass spectrometry (FAB-MS) analysis was conducted on a Kratos MS25RFA high-resolution mass spectrometer using a p-nitrobenzyl alcohol (NBA) matrix.

N⁶-Benzoyl-5⁰-O-(4,4'‐dimethoxytrityl)adenosine-2',3'-bis-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (S.3); yield 52% (560 mg); Rₜ (1:1 CH₂Cl₂/diethyl ether): 0.51 and 0.40; ³¹P-NMR (400 MHz, CD₃CN): diastereomer 1, 152.3 and 150.7 ppm (J PP = 10.1 Hz); diastereomer 2, 151.9 and 150.5 ppm (J PP = 6.6 Hz); diastereomer 3, 151.8 and 151.0 ppm (J PP = 6.6 Hz); diastereomer 4, 151.3 and 151.2 ppm (J PP = 8.9 Hz); FAB-MS anal. calc’d: 1074.17; observed: 1074.57; [M]+.

CONVERGENT SYNTHESIS OF SYMMETRICAL BRANCHED NUCLEIC ACIDS

The procedure described below for the synthesis of bNAs is carried out on a 1-µmol scale using an ABI 381A DNA synthesizer (Damha and Zabarylo, 1989; Damha et al., 1992). The condensation of two adjacent linear oligonucleotides (prepared from standard RNA or DNA phosphoramidites; Fig. 4.14.3) with the adenosine bisphosphoramidite synthon (S.3; Fig. 4.14.1) produces bNAs that contain identical branches connected via vicinal 2',5'- and 3',5'-phosphodiester linkages (Fig. 4.14.4). The same protocol may be utilized for the synthesis of bNAs containing D-xylose or D-arabinose instead of D-ribose at the
branchpoint, using the appropriate bisphosphoramidite synthons (Damba and Ogilvie, 1988; Noronha, Carriero, Agha, and Damha, unpub. observ.).

Branched nucleic acid synthesis works very well on commercially available solid supports (i.e., LCAA-CPG) containing 20 to 40 µmol nucleoside per gram support; however, even better yields are attainable on CPG supports with higher loadings (e.g., 90 µmol/g; Fig. 4.14.5). Such supports can be prepared using HATU/DMAP as the coupling reagents (see Support Protocol 1), and are ideal for the synthesis of short-length bNAs (e.g., trimers) since, in this case, high loadings ensure proper distance between the neighboring CPG-bound nucleosides (Damha and Zabarylo, 1989).

**Materials**

- 5′-O-(4,4′-Dimethoxytrityl)-N-protected-2′-deoxyribonucleoside- or -ribonucleoside-derivatized succinyl-LCAA-CPG (ChemGenes; also see Support Protocol 1)
- Cap A and B capping reagents (see recipes)
- DNA and/or RNA 3′-phosphoramidites (S.4a-d and S.5a-d; Fig. 4.14.3)

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**Figure 4.14.2** 31P-NMR of N6-benzoyl-5′-O-DMTr-adenosine-2′,3′-bis-O-(2-cyanoethyl-N,N-di-isopropyl)phosphoramidite (S.3). Due to the two chiral phosphorus centers, the compound exists as a mixture of four diastereomers (2^2 = 4; Fig. 4.14.1). Each diastereomer displays two sets of phosphorus signals (4 isomers x 2 = 8 signals). The doubling or splitting of each signal (8 x 2 = 16) is due to long-range coupling between the two chiral 31P atoms (nuclear spin = 1/2) of the bisphosphoramidite. The spectrum was recorded on a Varian XL-400 spectrometer using CD3CN as the solvent. The chemical shift and coupling constants for the four diastereomers (shown as numbers near peaks) are as follows: diastereomer 1, 152.3 and 150.7 ppm (6J_P,P = 10.1 Hz); diastereomer 2, 151.9 and 150.5 ppm (6J_P,P = 6.6 Hz); diastereomer 3, 151.8 and 151.0 ppm (6J_P,P = 4.6 Hz); diastereomer 4, 151.3 and 151.2 ppm (6J_P,P = 8.9 Hz).
Figure 4.14.3  Chemical structures of DNA and RNA phosphoramidites used for bNA synthesis: 5′-O-DMTr-N-protected-2′-deoxyribonucleoside-3′-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidites (S.4a-d), 5′-O-DMTr-N-protected-2′-O-TBDMS-ribonucleoside-3′-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidites (S.5a-d), and the inverted phosphoramidites 3′-O-DMTr-N-protected-2′-deoxyribonucleoside-5′-O-(2-cyanoethyl-N,N-diisopropylamino)phosphoramidites (S.6a-d).

4-6a B = N6-benzoyladenin-9-yl
4-6b B = N2-isobutyrylguanin-9-yl
4-6c B = N4-benzoylcytosin-1-yl
4d, 6d B = thymin-1-yl
5d B = uracil

NOTE: 1H-Tetrazole is no longer commercially available in crystalline form. Solutions of 0.45 M 1H-tetrazole in anhydrous acetonitrile may be purchased from ChemGenes. For a list of supplementary phosphoramidite activating reagents, see UNIT 3.5.

CAUTION: All solutions for the DNA/RNA synthesizer should be prepared in a well-ventilated fume hood.
**Prepare columns for synthesis**

1. Transfer an accurately weighed amount of 5′-O-(4,4′-dimethoxytrityl)-N-protected-2′-deoxyribonucleoside- or -ribonucleoside-derivatized succinyl-LCAA-CPG for a 1-µmol synthesis to an assembled synthesis column.

The support-bound nucleoside represents the first nucleotide at the 3′-end of the oligonucleotide to be synthesized. See Support Protocol 1 for high-loading CPG supports.

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**Figure 4.14.4** Schematic representation demonstrating the convergent synthesis of V- and Y-shaped bDNA or bRNA oligonucleotides on a solid support. The method can also be used for the synthesis of short branched sequences—e.g., tetranucleoside triphosphates, NpA(2′pN)3′pN—particularly when the nucleoside loading on the solid support approaches 90 µmol/g. Abbreviations: A, bisphosphoramidite; Bz, benzoyl; DMTr, 4,4′-dimethoxytrityl; N, any nucleotide (RNA or DNA); TREAT-HF, triethylammonium trihydrofluoride; S.7, linear oligonucleotide; S.8 and S.10, full-length bNAs (V- and Y-shaped); S.9a-b and S.11a-b, unbranched linear failure sequences. P* indicates the presence of a 2′- or 3′-linked phosphate to the adenosine branch point, which is formed by the hydrolysis and oxidation of the residual phosphoramidite during solid-phase synthesis.
Figure 4.14.5  Effect of CPG loading on yield of the Y-shaped bDNA, $T_{10}A^{2',5'-T_{10}}_{3',5'-T_{10}}$ synthesized via the convergent strategy (see Basic Protocol 2). (A) PAGE analysis of the amount of Y-shaped product (i; S.10) and failure sequences (ii; S.11a-b) formed as a function of CPG loading on a 20% denaturing polyacrylamide gel. P* indicates the presence of a 2'- or 3'-linked phosphate. (B) Chart demonstrating the increase in the amount of S.10 (i) with increasing nucleoside-CPG loading. The chart also demonstrates the inverse relationship between the amount of extended isomeric linear failures (ii; S.11a-b) and nucleoside-CPG loading. The percentage oligonucleotide was determined by integration of the HPLC peak areas of the compounds in question (see Support Protocol 3) using gradient 3 (see Table 4.14.4).

Table 4.14.1  Automated Cycle on an ABI 381A DNA Synthesizer for Capping of Free CPG-Bound Amino and Hydroxyl Groups

| Synthesis step | Function                          | Time (sec) |
|----------------|-----------------------------------|------------|
| **Column washing steps:** |                                  |            |
| 1              | Acetonitrile to waste             | 5          |
| 2              | Acetonitrile to column            | 60         |
| 3              | Argon reverse flush               | 5          |
| 4              | Argon block flush                 | 5          |
| **Column capping steps:** |                                  |            |
| 5              | Cap A + cap B to column           | 15         |
| 6              | Wait                              | 300        |
| 7              | Repeat steps 5 and 6              |            |
| **Column washing steps:** |                                  |            |
| 8              | Argon reverse flush               | 5          |
| 9              | Acetonitrile to column            | 30         |
| 10             | Repeat steps 8 and 9              |            |
| 11             | Argon reverse flush               | 5          |
| 12             | Argon block flush                 | 5          |

*Alternatively, CPG may be capped manually using acetic anhydride (see Support Protocol 1).*
2. Acetylate any underivatized amino and hydroxyl groups on the solid support using an ABI 381A automated DNA synthesizer and the capping cycle given in Table 4.14.1 (APPENDIX 3C).

This step also removes traces of water from the solid support.

Alternatively, see Support Protocol 1 for the manual capping procedure on nucleoside-loaded CPG.

**Synthesize branched oligonucleotides**

3. Weigh out the appropriate amount of DNA (**S.4a-d**) and/or RNA (**S.5a-d**) 3′-phosphoramidites (Fig. 4.14.3) and dilute to the appropriate concentration with anhydrous acetonitrile as indicated in Table 4.14.2.

4. Transfer 100 mg BIS-A (**S.3**) to an oven-dried synthesizer bottle and dilute to 0.03 M with anhydrous acetonitrile.

Low concentrations of BIS-A should be employed in the branching reaction, as high concentrations minimize the yield of fully branched product (**S.8** and **S.10**) and favor the extended isomeric side products (**S.9a-b** and **S.11a-b**).

It is important to prepare the BIS-A stock solution using ≥100 mg material to ensure that (unavoidable) traces of moisture do not consume significant amounts of BIS-A during the coupling (branching) step and reduce the overall yield of bNA synthesis (Fig. 4.14.6E). When the ABI 381A DNA synthesizer is used, this stock solution can be used for as many as 18 branching reactions (170 μL/addition). Once bNA synthesis is complete, the bottle containing the BIS-A reagent can be removed from the synthesizer, sealed, purged under an inert atmosphere, and left in a freezer (−20°C) for ~2 weeks. Alternatively, the stock solution can be evaporated under vacuum, and the solid bisamidite recovered for future use.

5. Place all synthesizer reagents (i.e., activator, capping, oxidant, and detritylation solutions, and acetonitrile) and diluted phosphoramidites (step 3) on the appropriate ports of the synthesizer.

6. Place the BIS-A phosphoramidite bottle on the spare phosphoramidite port (the “X” port on the 381A synthesizer).

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**Table 4.14.2**  Concentrations and Optimal Coupling Times of Phosphoramidites in Synthesis of bNAs on an ABI 381A DNA Synthesizer

| Phosphoramidite<sup>a</sup> | Mol. wt. (g/mol) | Concentration (g/mL) | Coupling time (sec) |
|-----------------------------|------------------|----------------------|---------------------|
| **DNA phosphoramidites (0.1 M in CH₃CN<sup>b</sup>):** | | | |
| S.4a, S.6a                  | 857.7            | 86                   | 90                  |
| S.4b, S.6b                  | 839.7            | 84                   | 120                 |
| S.4c, S.6c                  | 833.7            | 83                   | 90                  |
| S.4d, S.6d                  | 744.6            | 74                   | 90                  |
| **RNA phosphoramidites (0.15 M in CH₃CN):** | | | |
| S.5a                        | 988.2            | 148                  | 600                 |
| S.5b                        | 970.2            | 146                  | 900                 |
| S.5c                        | 964.2            | 145                  | 600                 |
| S.5d                        | 861.0            | 129                  | 600                 |
| **Bisphosphoramidite (0.03 M in CH₃CN):** | | | |
| S.3                         | 1074.2           | 32.2                 | 1800                |

<sup>a</sup>Phosphoramidite structures are shown in Figure 4.14.3.

<sup>b</sup>Final concentration of the first inverted DNA phosphoramidite coupled to the 2′-OH of the rA in divergent and regiospecific synthesis is 0.3 M (see 5′-pD<sup>′</sup>′ in Fig. 4.14.8).
| Synthesis step | Function                                      | Time (sec) |
|----------------|-----------------------------------------------|------------|
| **Detritylation of support-bound nucleoside:** |                                               |            |
| 1              | Acetonitrile to waste                         | 5          |
| 2              | Acetonitrile to column                        | 45         |
| 3              | Argon reverse flush                           | 5          |
| 4              | Argon block flush                             | 5          |
| 5              | Advance fraction collector                    | 1          |
| 6              | 3% TCA to waste                               | 10         |
| 7              | 3% TCA to column                              | 140        |
| 8              | Acetonitrile to column                        | 30         |
| 9              | 3% TCA to column                              | 80         |
| 10             | Argon block flush                             | 10         |
| **Column washing steps:** |                                               |            |
| 11             | Acetonitrile to waste                         | 5          |
| 12             | Acetonitrile to column                        | 120        |
| 13             | Argon reverse flush                           | 5          |
| 14             | Argon block flush                             | 5          |
| 15             | Acetonitrile to waste                         | 5          |
| 16             | Acetonitrile to column                        | 60         |
| 17             | Argon reverse flush                           | 5          |
| 18             | Argon block flush                             | 5          |
| **Phosphoramidite coupling steps:** |                                               |            |
| 19             | Phosphoramidite preparation                   | 3          |
| 20             | Activator to column                           | 5          |
| 21             | Phosphoramidite + activator to column         | 5          |
| 22             | Repeat steps 20 and 21 two times              |            |
| 23             | Activator to column                           | 3          |
| 24             | Wait<sup>a</sup>                              |            |
| 25             | Argon reverse flush                           | 5          |
| 26             | Argon block flush                             | 5          |
| **Column capping steps:** |                                               |            |
| 27             | Cap A + cap B to column                       | 17         |
| 28             | Wait                                          | 45         |
| 29             | Repeat steps 27 and 28                        |            |
| 30             | Acetonitrile to waste                         | 5          |
| 31             | Argon block flush                             | 5          |
| 32             | Acetonitrile to waste                         | 5          |
| 33             | Argon reverse flush                           | 5          |
| 34             | Argon block flush                             | 5          |
| **Oxidation steps:** |                                               |            |
| 35             | Oxidant to waste                              | 5          |
| 36             | Oxidant to column                             | 20         |
| 37             | Acetonitrile to waste                         | 5          |
| 38             | Argon block flush                             | 5          |
| 39             | Wait                                          | 20         |
| **Column washing steps:** |                                               |            |
| 40             | Acetonitrile to waste                         | 5          |
| 41             | Argon reverse flush                           | 10         |
| 42             | Argon block flush                             | 5          |
| 43             | Acetonitrile to waste                         | 5          |
| 44             | Acetonitrile to column                        | 18         |
| 45             | Argon reverse flush                           | 5          |
| 46             | Repeat steps 44 and 45 six times              |            |
| 47             | Argon block flush                             | 5          |

<sup>a</sup>See Table 4.14.2 for coupling times of various phosphoramidites.
7. Enter the sequence to be synthesized in the 5′-to-3′ direction, where the 3′-nucleotide corresponds to the nucleoside bound to the CPG.

For example, to synthesize the hypothetical V-shaped branched oligonucleotide (S.8) shown in Figure 4.14.4, enter the sequence 5′-T10A2-5′-GT9 by (A) anion-exchange HPLC (see Support Protocol 3) using gradient 3 (Table 4.14.4) and (B) 20% denaturing PAGE (see Support Protocol 4). (C-E) Analysis of a successful (C-D) and unsuccessful (E) synthesis of the mixed base Y-RNA 5′-CCCUACUAA2-5′-GUAUGCCC by (C) anion-exchange HPLC (see A for conditions) and (D-E) 20% denaturing PAGE. The regioisomeric extended failure sequences (ii) are resolved into two peaks by HPLC (A and C), but appear as one band by gel analysis (B, D, and E). In panel E, the major product is the unbranched 8-mer 5′-GUAUGCCC-3′, which accumulates due to the unsuccessful branching of the bisphosphoramidite. P* indicates the presence of a 2′- or 3′-linked phosphate.

8. Perform synthesis in the trityl-off mode according to the synthesis cycle outlined in Table 4.14.3 and utilizing the coupling times recommended in Table 4.14.2. Collect dimethoxytrityl solutions in 15-mL test tubes using an external fraction collector.

Turning the trityl mode off ensures that the last nucleotide at the 5′ end has a free hydroxyl group, which is desirable for purification using anion-exchange HPLC (see Support Protocol 3).
9. Upon completion of the synthesis, dry the CPG by manually conducting an argon reverse flush operation on the synthesizer for 10 min. Alternatively, dry the CPG under a stream of nitrogen or argon, or in a vacuum desiccator for 30 min.

10. Cleave the oligonucleotides from the support and deprotect the exocyclic amino, phosphate, and 2'-silyl (RNA only) protecting groups (see Support Protocol 2).

11. Purify the bNAs from failure sequences by anion-exchange HPLC (see Support Protocol 3) or denaturing PAGE (see Support Protocol 4).

*Typical HPLC and PAGE profiles for the synthesis of branched V-shaped and Y-shaped DNA and RNA molecules are demonstrated in Figures 4.14.6 and 4.14.7.*

**Measure branching efficiency by trityl color analysis**

12. Dilute the dimethoxytrityl solutions collected after each successive coupling (step 8) with 10 mL detritylation solution using a 50-mL buret.

13. Aliquot 100 µL into a quartz cuvette and dilute with 2 mL detritylation solution.

*Use only quartz cuvettes, as the 1,2-dichloroethane will dissolve disposable polystyrene cuvettes.*

14. Measure the absorbance of the solution on a UV-Vis spectrophotometer between 450 and 550 nm, and record the absorbance peak at ~505 nm for the dimethoxytrityl cation.

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**Figure 4.14.7** PAGE analysis of bDNA molecules synthesized via the convergent strategy (see Basic Protocol 2), demonstrating the mobilities of bNAs through a cross-linked 20% polyacrylamide gel. Lane 1, crude linear DNA 12-mer 5'-TACTAAGTATGT-3' (a); lane 2, crude V-DNA 13-mer 5'-A2',5',3',5'-GTATGT (c); lane 3, crude Y-DNA 18-mer TACTAA2',5',3',5'-GTATGT (d); lane 4, running dyes (xylene cyanol and bromphenol blue). The fastest-migrating sequence in lanes 2 and 3 is the 6-mer 5'-GTATGT-3' (b), which is the immediate precursor of the V-shaped molecule (c). Notice the large gap between the failure sequence (b) and product V-DNA (c) in lane 2, owing to more than doubling the molecular weight upon branching. In lane 3, extension of the failure sequence yields a separate band (a').
15. Determine the efficiency of each coupling step using the equation $\left(\frac{A_x}{A_{x-1}}\right) \times 100\%$, where $A_x$ is the absorbance of the trityl cation released at any given step and $A_{x-1}$ is the release in the previous step.

For efficient coupling, these values should be close to 100% for linear portions of the oligonucleotide (also see UNIT 10.3). For efficient branching, the value for the coupling of S.3 should be 50%, since one tritylated bisphosphoramidite is coupled to two nucleotide chains. For this step, trityl yields that are significantly greater than or less than 50% indicate little branch formation (e.g., 80% indicates the formation of mainly the extended isomeric compounds S.9a-d and S.11a-d).

### REGIOSPECIFIC SYNTHESIS OF BRANCHED NUCLEIC ACIDS

The protocol described below outlines the regiospecific and divergent synthesis of bNAs via phosphoramidite chemistry according to the method of Braich and Damha (1997; Fig. 4.14.8). This protocol allows for the synthesis of bNA molecules with different DNA sequences surrounding the branchpoint nucleotide. The methodology requires the use of standard DNA and RNA 3’-phosphoramidites (S.4a-d and S.5a-d) for the synthesis of a linear DNA strand incorporating a single ribonucleotide unit. Once this sequence is assembled, all of the 2-cyanoethyl phosphate-protecting groups are selectively removed by treatment with triethylamine. This step is necessary as phosphotriesters are susceptible to cleavage/modification by the ensuing fluoride treatment. The CPG-bound oligomer is then treated with fluoride ions to cleave the tert-butyldimethylsilyl group at the 2’ position of the ribose unit, from which another chain (2’-branch) can be synthesized. This is accomplished using “inverted” DNA phosphoramidites (deoxyribonucleoside 5’-phosphoramidites; S.6a-d), allowing branch synthesis to occur in the opposite (5’-to-3’) direction. With the exception of the decyanoethylation and desilylation steps, the entire process is conducted using an ABI 381A DNA synthesizer.

### Materials

- 5’-O-(4,4’-Dimethoxytrityl)-N-protected-2’-deoxyribonucleoside-derivatized succinyl-LCAA-CPG (ChemGenes; also see Support Protocol 1)
- Cap A and B capping reagents (see recipes)
- DNA 3’-phosphoramidites (S.4a-d; ChemGenes; Fig. 4.14.3)
- Anhydrous acetonitrile (see recipe)
- RNA 3’-phosphoramidite (S.5a-d; ChemGenes; Fig. 4.14.3)
- Activator solution: 0.5 M 1H-tetrazole (sublimed) in anhydrous acetonitrile
- Oxidant solution (see recipe)
- Detritylation solution (see recipe)
- 4:6 (v/v) triethylamine/acetonitrile (see recipe)
- Anhydrous THF (see recipe)
- 1 M tetra-n-butylammonium fluoride (TBAF; Aldrich) in THF, fresh
- Inverted DNA 5’-phosphoramidites (S.6a-d; ChemGenes; Fig. 4.14.3)
- Argon or nitrogen gas (optional)
- Synthesis columns for 1 µmol scale synthesis, with seals and filters (PE Biosystems) and 13-mm aluminum seals (Chromatographic Specialties)
- ABI 381A automated DNA synthesizer (PE Biosystems)
- External fraction collector and 15-mL test tubes
- Empty DNA synthesizer bottles, oven-dried
- 10- and 1-mL disposable syringes
- 25-mL glass syringe
- Additional reagents and equipment for cleaving and deprotecting the oligonucleotide (see Support Protocol 2), anion-exchange HPLC (see Support
Figure 4.14.8  Schematic representation for the divergent and regiospecific synthesis of branched DNA. The branching synthon is a standard RNA phosphoramidite (S.5a-d; Fig. 4.14.3). Abbreviations: 3′-pD, DNA 3′-phosphoramidites (S.4a-d); 5′-pD′, inverted DNA 5′-phosphoramidites (S.6a-d); 5′-pD″, higher concentration (0.3M) of inverted DNA 5′-phosphoramidite as first nucleotide coupled to the ribose branch point; 3′-pR, RNA 3′-phosphoramidite (S.5a-d); DMTr, 4,4′-dimethoxytrityl; TBDMS, tert-butyldimethylsilyl.
Protocol 3) or denaturing PAGE (see Support Protocol 4), and measuring coupling efficiency by trityl color analysis (see Basic Protocol 2).

CAUTION: All solutions required for bNA solid-phase synthesis should be prepared in a well-ventilated fume hood.

**Synthesize linear DNA (S.12)**

1. Prepare synthesis column (1 μmol) with the appropriate 5′-O-dimethoxytrityl-2′-deoxyribonucleoside-derivatized succinyl-LCAA-CPG (see Basic Protocol 2, steps 1 and 2).

2. Weigh out the proper amount of DNA 3′-phosphoramidites (S.4a-d; Fig. 4.14.3) and dilute to 0.1 M with anhydrous acetonitrile (see Table 4.14.2).

   *For synthesis on the ABI 381A DNA synthesizer, the volume of each phosphoramidite addition to the column is 170 μL.*

3. Weigh out the appropriate amount of 3′-RNA phosphoramidite (S.5a-d; Fig. 4.14.3) and dilute to 0.15 M with anhydrous acetonitrile (see Table 4.14.2).

   The RNA 3′-phosphoramidite is the branching synthon. Any of the four standard RNA 3′-phosphoramidites (A, G, C, or U) may be used depending on the specific branch point to be introduced.

4. Install all reagents (i.e., activator, capping, oxidant, and detritylation solutions, and acetonitrile) and phosphoramidite solutions on the synthesizer, placing the RNA 3′-phosphoramidite on the spare port (the “X” port on the 381A synthesizer).

5. Enter the base sequence of the linear oligonucleotide to be synthesized in the 5′-to-3′ direction, where the last entry (3′ nucleotide) corresponds to the nucleoside bound to the CPG.

   *For example, to synthesize the hypothetical linear oligonucleotide (S.12) shown in Figure 4.14.8, enter the sequence 5′-NNNNNXNNNNNNN-3′, where N is any deoxyribonucleoside phosphoramidite and X represents the branch point of the RNA.*

6. Perform synthesis in the trityl off mode according to the synthesis cycle outlined in Table 4.14.3 and utilizing the coupling times shown in Table 4.14.2. Collect the dimethoxytrityl solutions in 15-mL test tubes in an external fraction collector.

   *Turning the trityl mode off ensures that the last nucleotide at the 5′ end has a free hydroxyl group, which is desirable for purification using anion-exchange HPLC (see Support Protocol 3).*

7. Acetylate the hydroxyl group at the 5′ terminus by running the automated capping cycle (Table 4.14.1).

   *Capping the free 5′-OH is necessary as it ensures that extension from this functional group will not occur during the synthesis of the “`orthogonal” 2′-branch.*

**Cleave 2-cyanoethyl protecting group**

8. Dry the CPG by manually conducting an argon reverse flush operation on the synthesizer for 10 min.

9. Remove the synthesis column from the synthesizer and connect it to a 10-mL disposable syringe filled with 4:6 (v/v) triethylamine/acetonitrile. Slowly push the deprotection solution through the column over a 90-min period.

   *Deprotection of the 2-cyanoethyl phosphate-protecting group converts the phosphotriester to the more stable phosphodiester, which withstands the conditions required for desilylation in the ensuing step. To ensure complete decyanoethylation, push the solution slowly through the column and then pull in on the syringe slightly in order to displace the CPG beads from the base of the column.*

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**Synthesis of Modified Oligonucleotides and Conjugates**

4.14.15
10. Wash the CPG beads extensively with 30 mL acetonitrile followed by 30 mL THF using a 25-ml glass syringe attached to the column via the syringe adapter.

**Cleave 2′-O-TBDMS group**

11. Push 1 mL of 1 M TBAF in THF through the column over a period of 10 min using a 1-mL disposable syringe.

   *It is essential to use fresh TBAF. In order to ensure complete desilylation, push the solution slowly through the column and then pull in on the syringe slightly in order to displace the CPG beads from the base of the column. Prolonged treatment with TBAF results in cleavage of the oligonucleotide chain from the solid support (Braich and Damha, 1997).*

   *Incomplete desilylation results in the accumulation of silylated linear DNA (S.12; Fig. 4.15.8), which does not allow branch extension from the 2′ position of the branch point (see Fig. 4.14.9B).*

12. Wash CPG beads with 50 mL THF followed by 50 mL acetonitrile.

13. Reinstall the column on the synthesizer.

**Synthesize 2′,5′-linked branch (S.13)**

14. Modify the DNA synthesis cycle such that synthesis step 15 becomes the first step in the cycle.

   *Steps 1 to 14 (TCA treatment) may be disregarded since the assembled chain lacks a dimethoxytrityl group.*

15. Weigh out the appropriate amounts of inverted DNA 5′-phosphoramidites (S.6a-d; Fig. 4.14.3) into the amidite bottles. Add acetonitrile to the first DNA phosphoramidite to prepare a 0.3 M solution. Add acetonitrile to make 0.1 M solutions of each of the remaining monomers.

   *The first inverted DNA 5′-phosphoramidite is added as a 0.3 M solution due to steric hindrance around the ribose 2′-hydroxyl group.*

16. Install all the inverted phosphoramidites on the synthesizer and place the first inverted phosphoramidite (0.3 M concentration) on the spare port (the “X” port on the 381A).

17. Enter the linear sequence to be synthesized in the 5′-to-3′ direction, where the last entry corresponds to the first phosphoramidite to be coupled to the 2′-hydroxyl group of the branch point.

   *For example, to synthesize the hypothetical branched DNA oligonucleotide (S.13) shown in Figure 4.14.8, enter the sequence 5′-NNNNN3′, where N is the first DNA 5′-phosphoramidite to be coupled to the 2′-hydroxyl group of ribose.*

18. Synthesize the 2′-branch in the trityl off mode using the modified synthesis cycle starting from step 15 of Table 4.14.3, and utilizing the coupling times shown in Table 4.14.2, except for the first phosphoramidite (0.3 M), which should have a coupling time of 30 min.

19. Upon completion of the synthesis, dry the CPG by reverse flushing the column with argon for 10 min. Alternatively, dry the CPG under a stream of nitrogen or argon, or in a vacuum desiccator for 30 min.

20. Cleave the oligonucleotides from the support and deprotect the amino- and phosphate-protecting groups (see Support Protocol 2).
21. Purify the bNAs from failure sequences by anion-exchange HPLC (see Support Protocol 3) or denaturing PAGE (see Support Protocol 4) and measure coupling efficiency by trityl color assay (see Basic Protocol 2, steps 12 to 15).

Typical PAGE profiles for the successful and unsuccessful regiospecific synthesis of a branched Y-shaped DNA molecule are demonstrated in Figure 4.14.9.

PREPARATION OF LCAA-CPG SUPPORTS WITH HIGH NUCLEOSIDE LOADINGS

The method described allows for the rapid derivatization of LCAA-CPG having nucleoside loadings up to \(\sim 90 \mu\text{mol/g}\), which is 3 to 4 times the loading found in commercially available solid supports. While commercial samples provide more than adequate yields of bNAs (Damha et al., 1992), those with higher loadings (50 to 90 \(\mu\text{mol/g}\)) provide the best results (Fig. 4.14.5). For example, the synthesis of small bNAs (i.e., trimers and tetramers) requires that the CPG be densely loaded so that efficient branching may occur. The protocol below, adapted from the work of Pon et al. (1999) and Damha et al. (1990), allows for the rapid esterification of 5\(^{\prime}\)-O-protected ribonucleosides and deoxyribonucleosides to succinyl-LCAA-CPG. The key condensing reagent is a mixture of HATU and 4-DMAP.

**Materials**

- 5\(^{\prime}\)-O-(4,4\(^{\prime}\)-Dimethoxytrityl)-N-protected-ribonucleoside or -2\(^{\prime}\)-deoxyribonucleoside (ChemGenes)
- O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; PE Biosystems)
- 4-Dimethylaminopyridine (DMAP; 99%, Aldrich)

![Figure 4.14.9](image_url) 20% denaturing PAGE analysis of a successful (A) and a less successful (B) synthesis of bNAs. (A) Lane 1, pure bNA (i) prepared by the divergent synthesis method (see Basic Protocol 3); lane 2, pure bNA (i) prepared by the convergent synthesis method (see Basic Protocol 2); lane 3, crude bNA (i) prepared by the divergent synthesis method; lane 4, running dyes (xylene cyanol and bromphenol blue). (B) Preparative PAGE purification of bNA (iii) prepared via the divergent (regiospecific) approach. The diminutive amount of fully branched product (iii) is due to the incomplete desilylation of the 2\(^{\prime}\)-O-TBDMS group (Si) on the RNA branching synthon, as seen by the significant amount of (iv) that remains.
Succinylated long-chain-aminoalkyl controlled-pore glass (succinyl-LCAA-CPG; *UNIT 3.2*)

Anhydrous acetonitrile (see recipe)
Dichloromethane, reagent grade (Fisher)
Methanol, reagent grade (Fisher)
Cap A and B capping reagents (see recipes)
15-mL glass bottle with septum
1-mL syringe and needle
Wrist-action shaker
Sintered glass funnel or Buchner funnel with filter paper

Additional reagents and equipment for quantitation of released trityl groups (*UNIT 3.2*) and acetylation of support (*UNIT 3.2*; optional)

1. In a 15-mL glass bottle, combine the following:

   - 0.1 mmol 5′-O-(4,4′-dimethoxytrityl)-N-protected-ribonucleoside or -2′-deoxyribonucleoside
   - 0.1 mmol HATU
   - 12 mg DMAP
   - 250 mg succinyl-LCAA-CPG.

2. Cap the bottle with a septum and add 1 mL anhydrous acetonitrile via a 1-mL syringe and needle.

3. Shake 2 to 4 hr on a wrist-action shaker at room temperature.

   *Do not stir the slurry with a magnetic stir bar as this will break up the glass beads into fine particles that may clog the frit on the DNA synthesizer.*

4. Vacuum filter succinyl-LCAA-CPG into a side-arm filter flask through a sintered glass funnel or Buchner funnel with filter paper.

5. Wash the CPG sequentially with 25 mL reagent-grade dichloromethane, 25 mL methanol, and 25 mL dichloromethane.

   *It has been reported that the free carboxylic groups are inconsequential and do not react during phosphoramidite synthesis (Lyttle et al., 1997).*

6. Transfer the CPG to a glass vial and dry under vacuum using in a desiccator attached to a vacuum pump.

   *The derivatized CPG may be stored indefinitely at room temperature, preferably in a vacuum desiccator.*

7. Determine nucleoside loading through the quantitation of the released trityl groups from the support-bound nucleoside (*UNIT 3.2*).

8. Acetylate the solid support with cap A and B capping reagents on a DNA synthesizer using the capping cycle outlined in Table 4.14.1. Alternatively, perform capping as described in *UNIT 3.2*. 

*Solid-Phase Synthesis of Branched Oligonucleotides*

4.14.18

Supplement 9 Current Protocols in Nucleic Acid Chemistry
COMPLETE DEPROTECTION OF BRANCHED OLIGONUCLEOTIDES
(DNA AND RNA)

This protocol describes the steps necessary for cleaving the bNA from the solid support and removing the protecting groups from the heterocyclic bases and sugar-phosphate backbone. The first step is treatment of the solid support with concentrated aqueous ammonia to concomitantly cleave both the bNA from the support and the N-acyl and 2-cyanoethyl phosphate-protecting groups. A subsequent deprotection step with triethyl-ammonium trihydrofluoride (TREAT-HF) cleaves the 2′-O-tert-butyldimethylsilyl (TBDMS) protecting groups from branched oligoribonucleotides (Gasparutto et al., 1992). The desilylated material is then precipitated directly using 1-butanol (Sproat et al., 1995). A procedure for the quantitation of oligonucleotides is also described.

Materials

- Branched oligonucleotide attached to CPG (bNA-CPG; see Basic Protocols 2 and 3)
- 29% ammonium hydroxide, 4°C (store up to 1 month at 4°C)
- 70% and 100% (v/v) ethanol, former at 4°C
- DEPC-treated water (optional; APPENDIX 2A)
- Autoclaved water
- Triethylammonium trihydrofluoride (TREAT-HF; 98%; Aldrich)
- 3 M sodium acetate, pH 5.5 (APPENDIX 2A)
- 1-Butanol, analytical grade, 4°C
- 1.5-mL screw-cap microcentrifuge tubes with O-ring seals (preferred)
- Wrist-action shaker
- Speedvac evaporator (Savant)
- Double-beam UV spectrophotometer, calibrated

Cleave and deprotect oligonucleotide

1. Transfer bNA-CPG to a 1.5-mL screw-cap microcentrifuge tube, preferably with an O-ring seal.
2. Add 750 µL cold 29% ammonium hydroxide and 250 µL of 100% ethanol, screw the cap on tightly, and incubate 24 to 48 hr at room temperature on a wrist-action shaker.
   
   *If the branched oligonucleotide sequence contains isobutyryl-protected guanosine nucleotides, then deprotection must proceed for \( \geq 48 \text{ hr} \), room temperature.

   *The ammonium hydroxide should be relatively fresh (<1 month old). Seal the cap on the ammonium hydroxide solution tightly and store at 4°C.

3. Microcentrifuge 1 min at maximum speed, room temperature, and cool 30 min on dry ice or 1 to 2 hr at −20°C.

   *The contents must be cooled before the screw cap is released to prevent volatile ammonium hydroxide from boiling over, which could result in loss of product.

4. Remove supernatant and transfer to a fresh 1.5-mL microcentrifuge tube.
5. Wash CPG pellet with 500 µL of 100% ethanol, microcentrifuge to settle the CPG, and transfer the supernatant wash to a second 1.5-mL tube. Repeat two more times (total four tubes).
6. Cool all four tubes for 1 hr on dry ice, then dry in a Speedvac evaporator.

   *Introducing the vacuum too quickly will lead to bumping of the ammonia solution and sample loss.
7. Pool in a total of 1 mL water. For branched RNAs, use DEPC-treated water that has been autoclaved to remove residual DEPC.

The product is a crude deprotected branched DNA or partially deprotected branched RNA. For RNA, DEPC-treated water should be used in all subsequent steps.

**Quantitate oligonucleotide**

8. Dilute crude bNA 10 or 100 fold with autoclaved water and measure the absorbance at 260 nm ($\text{A}_{260}$) on a calibrated, double-beam, UV spectrophotometer using autoclaved water as a reference.

If using a single-beam spectrophotometer, a baseline should be run with a sample containing autoclaved water (blank).

9. Determine the concentration of the stock solution using the dilution factor and Beer’s Law ($\text{A}_{260} = \varepsilon bc$), where $\varepsilon$ is the molar extinction coefficient, $b$ is the UV cell pathlength (typically 1 cm), and $c$ is the concentration of oligonucleotide present.

The molar extinction coefficients (liter/mol cm) and molecular weights of the oligonucleotides can be obtained from http://paris.chem.yale.edu/extinct.html and http://medstat.med.utah.edu/masspec/oligoii.htm.

**Cleave 2’-O-TBDMS group**

10. Evaporate bRNA samples (step 7) using a Speedvac evaporator and resuspend in 5 µL TREAT-HF per $\text{A}_{260}$ unit (step 8).

11. Cover the microcentrifuge tubes with aluminum foil and incubate 24 to 48 hr at room temperature on a wrist-action shaker.

Alternatively, the 2’-O-TBDMS group may be removed using a mixture of TREAT-HF and N-methylpyrrolidinone according to the method of Wincott et al. (1995).

For large bRNAs ($\geq$10-mers):

12a. Precipitate the oligonucleotides directly from the desilylation reaction by adding 25 µL of 3 M sodium acetate, pH 5.5, followed by 1 mL cold analytical-grade 1-butanol. Vortex 1 min and cool 3 to 6 hr at $-20^\circ$C.

A white precipitate should be clearly visible after addition of 1-butanol.

13a. Microcentrifuge 10 min at maximum speed, 4°C, carefully remove the supernatant, and wash the white pellet twice with 500 µL cold 70% ethanol each.

Disruption of the pellet during washing steps could result in loss of sample.

14a. Dry the pellet in a Speedvac evaporator, resuspend in 1 mL autoclaved water, and requantitate the amount of crude oligonucleotide (steps 8 and 9).

Once the 2’-O-TBDMS group has been removed, the fully deprotected RNA is sensitive to nucleases and base hydrolysis, which will cause strand cleavage. In order to prevent this, special considerations for working with RNA (APPENDIX 2A) must be observed. Water used for RNA dissolution and buffer preparation should be of the highest quality available (Milli-Q) and should be treated with DEPC.

For small bNAs (<10-mers):

12b. Quench the desilylation reaction with an equal volume of autoclaved water and evaporate in a Speedvac evaporator. Resuspend in 1 mL autoclaved water and requantitate the amount of crude oligonucleotide (steps 8 and 9).

Shorter sequences (i.e., trimers and tetramers) do not precipitate efficiently.
ANALYSIS AND PURIFICATION OF BRANCHED OLIGONUCLEOTIDES USING ANION-EXCHANGE HPLC

Branched oligonucleotides may be easily and efficiently analyzed and purified from the crude mixture by anion-exchange HPLC as described below (Fig. 4.14.6). bNAs of high purity are attainable (>95%; Fig. 4.14.10). The bNA of interest can be readily isolated from the reaction mixture failure sequences. The resultant product is precipitated directly by the addition of 1-propanol (Sproat et al., 1995). This direct precipitation method works very well for the direct isolation of large bNAs (>10 nt). Smaller bNAs do not precipitate out efficiently and must be further purified by size-exclusion chromatography (or reversed-phase Sep-Pak cartridges) subsequent to HPLC separation. As an alternative to HPLC purification, the bNAs may be purified by denaturing PAGE (see Support Protocol 4).

Characterization of the bNAs is conveniently done via MALDI-TOF-MS as described in UNIT 10.1. The matrix and co-matrix typically used are 6-aza-2-thiothymine (ATT) and dibasic ammonium citrate, respectively (Lecchi et al., 1995).

The branched nature of the molecules may also be confirmed via the yeast debranching enzyme (yDBR), a phosphodiesterase specific to hydrolysis of the 2′,5′-phosphodiester bond of oligonucleotides that contain vicinal 2′,5′- and 3′,5′-phosphodiester linkages (Nam et al., 1994; Ooi et al., 2001). Nucleoside composition analysis of bNA is carried out using snake venom phosphodiesterase (SVPD) according to the method of Eadie et al. (1987; UNIT 10.6). This enzyme cleaves bDNA or bRNA from the 3′ termini yielding 5′-monophosphates, which can then be converted to their constituent nucleosides by in situ treatment with alkaline phosphatase (AP). The resulting nucleoside mixture is analyzed by reversed-phase HPLC as described in UNIT 10.5. Alternatively, bNA can be digested with nuclease P1 from Penicillium citrinum, an endonuclease that cleaves bNA to produce the constituent nucleoside 5′-monophosphates and its branch core trinucleoside diphosphate—i.e., A(2′p5′N)3 p5′N (Damha et al., 1992). The released branched trinucleoside diphosphate structure can be readily synthesized (see Basic Protocol 2) and used as a standard during HPLC analysis of the enzyme digest (UNIT 10.6).

NOTE: For branced RNAs, use DEPC-treated water throughout (APPENDIX 2A).

NOTE: For branced RNAs, use DEPC-treated water throughout (APPENDIX 2A).

Figure 4.14.10  Analysis of the purity of a bNA synthesized via the convergent strategy. Purification was conducted using anion-exchange HPLC (see Support Protocol 3) with linear gradient 2 (Table 4.14.4). The chromatogram was obtained using the same conditions.
### Materials

- Deprotected branched oligonucleotide (see Support Protocol 2)
- Autoclaved water
- 1 M LiClO₄ (see recipe)
- Reagent-grade 1-propanol, 4°C (>5-mers; Fisher)
- Sephadex G-25 columns (Amersham Pharmacia Biotech) or Sep-Pak cartridges (Waters Chromatography) for <5-mers
- Nuclease P1 buffer (see recipe)
- 0.3 U/µL *Penicillium citrinum* nuclease P1 (NP1)
- 9 U/µL alkaline phosphatase (AP)
- 0.1 M triethylammonium acetate (TEAA), pH 7.0
- Acetonitrile, HPLC grade
- 50°C water bath or heating block
- High-performance liquid chromatograph (HPLC) with:
  - Anion-exchange column (7.5 × 75–mm Waters Protein-Pak DEAE-5PW)
  - UV detector with adjustable range or dual-wavelength detection
  - Sample loop
  - Column heater
  - Reversed-phase C18 column (e.g., Whatman Partisil ODS-2, 10-µm, 4.6 × 250–mm; Chromatographic Specialties)
  - Syringe
  - Speedvac evaporator (Savant)
- Additional reagents and equipment for reversed-phase chromatography (UNIT 10.1 & 10.6) and quantitation of bNAs by UV spectrophotometry (see Support Protocol 2)

### Analyze bNAs by anion-exchange HPLC

1. Aliquot 0.3 to 0.6 $A_{260}$ units of deprotected branched oligonucleotide (25 to 100 µL volume) into a sterile 1.5-mL microcentrifuge tube.

2. Heat the sample 1 min in a 50°C water bath or heating block.

   *Heating the sample disrupts any intramolecular secondary structures.*

3. To settle any particulates, microcentrifuge 2 min at maximum speed, room temperature.

   *It is important to avoid loading any small particulates into the injector of the HPLC as they may clog the injection loop.*

4. Preequilibrate HPLC anion-exchange column with initial buffer conditions (Table 4.14.4) and set the UV detector wavelength to 260 nm.
5. Load the sample into the sample loop using an appropriate syringe and inject. Elute bNA at 50°C with a flow rate of 1 mL/min and the gradient and run time specified in Table 4.14.4. Record the chromatogram.

The branched DNA or RNA product should be very well resolved from the extended linear failure sequences and other failure sequences present in the crude mixture. Full-length bNAs elute last (i.e., highest retention time). Typical chromatograms for a 31-mer bDNA and 25-mer bRNA are demonstrated in Figure 4.14.6A and C. Note the excellent separation between the bNA of interest and the failure sequences.

**Purify bNAs by anion-exchange HPLC**

6. Dissolve 40 to 60 A$_{260}$ units crude bNA mixture in 1 mL autoclaved water. Heat and microcentrifuge as in steps 2 to 3.

Loading >60 A$_{260}$ units may overload the column and compromise the separation of the bNA from the linear failure sequences.

7. Run the sample as described (steps 4 and 5), but set the detector to 290 nm and collect 1-ml fractions from the peaks of interest in sterile 1.5-mL microcentrifuge tubes.

The detector wavelength is set to 290 nm in order to avoid saturation of the detector signal. If the HPLC is equipped with a detector capable of monitoring dual wavelengths, monitor both the 260- and 290-nm profiles.

The anticipated retention time should be very similar to that obtained during routine HPLC analysis.

8. Pool peak fractions and dry them in a Speedvac evaporator. Add 250 µL autoclaved water.

**For large bRNAs (≥5-mers):**

9a. Precipitate from perchlorate salts (in sample) by adding 4 vol (1 mL) reagent-grade cold 1-propanol and cooling 4 to 6 hr at −20°C.

Lithium perchlorate (LiClO$_4$) is much more soluble in organic solvents than other perchlorate salts, making precipitation easy and efficient, and thus preventing a final desalting step. The DNA or RNA isolated is in its lithium salt form.

10a. Microcentrifuge 10 min at maximum speed, room temperature, carefully remove the supernatant, and wash the white pellet twice with 500 µL cold 1-propanol.

Disrupting the pellet during washing steps can result in loss of sample.

11a. Dry in a Speedvac evaporator, resuspend in 1 mL autoclaved water, and proceed to step 12.

**For small bNAs (<5-mers):**

9b. Desalt by size-exclusion chromatography on Sephadex G-25 columns according to manufacturer’s instructions or by reversed-phase chromatography on Sep-Pak cartridges (UNIT 10.1). Proceed to step 12.

Small bNAs do not precipitate out of solution efficiently.

**Characterize bNAs**

12. Quantitate the bNA sample by UV spectrophotometry (see Support Protocol 2, steps 8 and 9).

13. Dissolve 0.5 A$_{260}$ units bNA in 20 mL of nuclease P1 buffer.

14. Add 3 µL (0.9 U) NP1 and 1 µL (9 U) AP. Mix well and incubate 24 hr at 37°C.

The alkaline phosphatase may be contaminated with adenosine deaminase, which converts adenosine into inosine. The retention time of an appropriate inosine nucleoside control should be obtained prior to HPLC analysis of the digestion mixture (UNIT 10.6).
15. Dry the sample in a Speedvac evaporator and dissolve residue in 15 µL autoclaved water.

16. Analyze the mixture by reversed-phase HPLC on a C18 column using the mobile phases 0.1 M TEAA, pH 7.0, and acetonitrile with the gradient described in UNIT 10.6.

17. Calculate the relative ratios of nucleoside to branched trinucleotide diphosphate by dividing the area of each peak by the extinction coefficients specified in UNIT 10.6.

The extinction coefficient for the branched trinucleotide diphosphate may be calculated using the oligonucleotide extinction coefficient calculator at http://paris.chem.yale.edu/extinct.html. The extinction coefficients for bDNA and bRNA are assumed to be the same as their linear counterparts.

ANALYSIS AND PURIFICATION OF BRANCHED OLIGONUCLEOTIDES BY DENATURED PAGE

A method for the analysis and purification of bNAs by denaturing polyacrylamide gel electrophoresis (PAGE) is described (also see UNIT 10.4 and APPENDIX 3B). PAGE is a very convenient way to assess efficiency of bNA synthesis since the molecular weight of oligonucleotides bound on the solid support more than doubles after a convergent branching reaction—e.g., reaction of neighboring decathymidylic acid chains with bisphosphoramidite synthon S.3 to give a 21-unit-long bNA molecule (Fig. 4.14.4 and product ii in Fig. 4.14.6B). This generates a gap between the desired bNA product and its precursor molecules, greatly facilitating the separation process. Any type of standard laboratory electrophoresis equipment may be utilized. Most bNAs of >10 nucleotides in length are very well resolved on a 20% denaturing polyacrylamide gel. If shorter sequences must be purified, better resolution is achieved with a 24% denaturing polyacrylamide gel. Setup and polymerization of the gel along with electrophoretic separation conditions are described. The resultant bands may be visualized by UV shadowing and photographed. A technique for the rapid extraction of oligonucleotides from the gel matrix is also described (Chen and Ruffner, 1996).

Materials

- 20% (w/v) denaturing acrylamide gel solution (see recipe)
- Deprotected branched oligonucleotide sample, dry (see Support Protocol 2)
- Formamide loading buffer (see recipe)
- Gel extraction buffer (see recipe)
- Running dye (see recipe)
- Gel electrophoresis equipment (APPENDIX 3B) with:
  - 16 × 18–cm glass plates
  - Spacers: 0.75 mm (analysis) or 1.5 mm (purification)
  - Gel combs: 0.75 mm thick with 12 to 20 wells (analysis) or 1.5 mm thick with one to three large wells (purification)
- Sonicator or ∼50°C water bath (optional)
- 20 × 20–cm silica-coated thin-layer chromatography (TLC) plate with fluorescent indicator (e.g., Kieselgel 60 F_{254} aluminum sheets)
- Handheld UV lamp (254 nm)
- Camera equipped with UV filter (optional)
- 90°C water bath or heating block (optional)
- UV shadow box
- Wrist-action shaker (optional)
- Speedvac evaporator (Savant)
- Sephadex G-25 (Amersham Pharmacia Biotech) or reversed-phase cartridges (e.g., Sep-Pak cartridges; Waters Chromatography)
Additional reagents and equipment for denaturing PAGE (UNIT 10.4 and APPENDIX 3B), reversed-phase chromatography using Sep-Pak cartridges (UNIT 10.1), UV spectrophotometry (see Support Protocol 2), MALDI-TOF-MS (UNIT 10.1), and enzymatic digestion (see Support Protocol 3)

CAUTION: Acrylamide and N,N′-methylenebisacrylamide are neurotoxins. Prepare all solutions containing these two reagents in a fume hood. Minimize exposure and contact to both crystalline forms and solutions by conducting all handling (including weighing) in a well-ventilated area and wearing disposable gloves at all times.

Analyze bNAs by denaturing PAGE
1. Assemble a gel sandwich using 16 × 18-cm glass plates separated by 0.75-mm spacers.

   See APPENDIX 3B and UNIT 10.4 for a thorough description of denaturing PAGE.

2. Transfer 30 mL of 20% denaturing acrylamide gel solution to an Erlenmeyer flask and add 200 µL fresh 10% (w/v) APS immediately followed by 20 µL TEMED. Swirl and degas the solution by attaching the Erlenmeyer flask to a house vacuum line. Maintain rapid stirring to avoid bumping.

   The gel solution may also be degassed prior to APS and TEMED addition by placing the 20% acrylamide gel solution on a sonicator for 5 to 10 min. If using larger glass plates, adjust the volumes of acrylamide, APS, and TEMED accordingly.

3. Pour the solution between the plates, insert a 0.75-mm-thick comb, and allow gel to polymerize (30 to 45 min).

4. Place gel in electrophoresis apparatus, removing the comb and bottom spacer, rinse the wells, and prerun the gel 30 min at 500 V, room temperature.

5. Dissolve 0.6 A260 units deprotected bNA sample in 10 µL formamide loading buffer. If the dissolution process is not immediate, place the sample in a sonicator bath 1 to 2 min or heat the samples briefly at ~50°C.

6. Load the samples into the wells. Load an equal amount of running dye in the first and last well as an external reference marker. Run the gel at 500 V until the bromphenol blue dye is 3/4 of the way down the gel.

7. Disassemble the glass plates and wrap the gel in plastic wrap. Place the wrapped gel over a 20 × 20–cm silica-coated TLC plate with fluorescent indicator and visualize the bands by shining a handheld UV lamp over the gel. Take a picture of the gel using a camera equipped with a UV filter.

   CAUTION: Wear safety glasses to avoid eye burn.

A typical crude bNA reaction mixture will consist of at least three bands as shown in Figures 4.14.6 and 4.14.7. The fastest-migrating band is the linear precursor sequence (S.7: Fig. 4.14.4), followed by the extended isomeric failure sequences (S.9a-b for the synthesis of V-shaped bNAs and S.11a-b for the synthesis of Y-shaped bNAs), and finally the products (S.8 and S.10). If the coupling efficiencies between successive nucleotides is less than optimal, a ladder of failure sequences will be evident below the extended branch failure and linear sequences.

Note that the extended isomeric failure sequences are a mixture of regioisomers (S.9a-b and S.11a-b) that are sometimes resolved into two close-moving bands (this is also evident by HPLC analyses). The slowest of the predominant bands is the bNA of interest. If linear markers are run alongside the purified bNA, one observes that bNA has retarded mobility relative to a linear oligonucleotide of identical composition (length and sequence composition). This is due to the increased frictional effects of the bNA relative to the linear sequences as they move through the highly cross-linked gel environment.
**Purify bNAs by denaturing PAGE**

8. Assemble and run a preparative gel as for the analytical gel (steps 1 to 6) with the following modifications:
   
a. Increase gel thickness (spacers and comb) to 1.5 cm and scale up volume of gel to 50 mL gel solution, 350 µL APS, and 35 µL TEMED.
   
b. Use up to 100 A\textsubscript{260} units crude bNA dissolved in 100 µL formamide loading buffer.
   
c. Use a comb with one large well to purify 60 to 100 A\textsubscript{260} units, one well of a two-well comb for 30 to 60 units, and one well of a three-well comb for <30 units.

9. Disassemble the glass plates, remove the gel, and wrap it in plastic wrap. Place the wrapped gel over a silica-coated TLC plate and visualize the bands by shining UV light over the gel. If desired, photograph the gel using a camera equipped with a UV filter.

   CAUTION: Wear safety glasses to avoid eye burn.

10. Excise the slowest moving band and place the gel piece into a sterile 15-mL tube. Crush the gel piece using a sterile spatula and soak in 3 mL gel extraction buffer.

11. Heat 5 min in a 90°C water bath or heating block and rapidly freeze 5 min in dry ice. Thaw contents rapidly at 90°C and centrifuge 10 min at maximum speed \( \times g \) in a tabletop centrifuge to settle the crushed gel pieces. Alternatively, after extracting in 3 mL gel extraction buffer (step 10) or water, shake the slurry 12 to 16 hr at room temperature on a wrist-action shaker.

   The rapid “crush and soak” method described has been outlined in a paper by Chen and Ruffner (1996).

12. Extract the supernatant and dry in a Speedvac evaporator. Desalt on a Sephadex G-25 column according to manufacturer’s instructions, or via reversed-phase chromatography using Sep-Pak cartridges according to UNIT 10.1.

13. Quantitate the amount of bNA recovered via UV spectrophotometry (see Support Protocol 2, steps 8 and 9).

14. Characterize bNAs by MALDI-TOF-MS (UNIT 10.1) and/or enzymatic digestion of the constituent nucleotides with SVPD/AP and NP1/AP (see Support Protocol 3).

**REAGENTS AND SOLUTIONS**

*Use deionized, double-distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Acetonitrile, anhydrous**

Predry by refluxing over reagent-grade phosphorus pentoxide (Fisher) followed by refluxing and distillation over calcium hydride (Aldrich) under inert atmosphere. Prepare fresh before each use.

Alternatively, low-water acetonitrile suitable for use on a DNA synthesizer can be purchased (VWR Canlabs or BDH) and used as such, or refluxed and distilled from calcium hydride under inert atmosphere.

**Cap A capping reagent**

Prepare anhydrous 2,4,6-collidine (Aldrich) by refluxing and distillation over calcium hydride. Store up to 6 months at room temperature under inert atmosphere over activated 4A molecular sieves. Before use mix 1 part dry 2,4,6-collidine with 1 part acetic anhydride and 8 parts anhydrous THF (see recipe).
Cap B capping reagent
Prepare anhydrous N-methylimidazole (Aldrich) by stirring over calcium hydride for 24 hr, followed by vacuum distillation. Dilute to 16% (v/v) in anhydrous THF (see recipe). Store up to 6 months at room temperature under inert atmosphere over activated 4A molecular sieves.

Denaturing acrylamide gel solution, 20% (w/v)
20 g acrylamide
1 g N,N'-methylenebisacrylamide
21.02 g electrophoresis-grade urea (final 7 M)
5 mL 10× TBE electrophoresis buffer (APPENDIX 2A)
H₂O to 50 mL
Store up to 1 to 2 months at 4°C
CAUTION: Acrylamide and N,N'-methylenebisacrylamide are neurotoxins. Prepare all solutions in a well-ventilated fume hood, and take precautions to minimize exposure. Wear gloves at all times.
To aid in dissolution, the acrylamide solution can be heated in a warm (<50°C) water bath with constant stirring. Do not overheat as this will cause the acrylamide to polymerize.

Detritylation solution
3% (w/v) trichloroacetic acid (TCA, 99%, Aldrich)
1,2-Dichloroethane (analytical grade, Fisher)
Store up to 6 months at room temperature

Diisopropylethylamine (DIPEA), anhydrous
Dry by mild heating (50°C to 60°C) and stirring 16 hr over calcium hydride followed by distillation under vacuum. Store up to 2 to 4 months at 4°C over 4A activated molecular sieves in septum-sealed bottles.
Alternatively, 99.5% redistilled DIPEA can be purchased (Aldrich) and kept anhydrous by storage over activated 4A molecular sieves up to 1 year at 4°C.

Formamide loading buffer
Deionize 10 mL formamide by adding 1 g mixed bed resin (Bio-Rad) and stirring 30 min. Filter through Whatman filter paper. Mix 4 vol deionized formamide with 1 vol of 10× TBE electrophoresis buffer (APPENDIX 2A). Store up to 1 year at −20°C.

Gel extraction buffer
363 mg Tris base (final 30 mM)
1.75 g NaCl (final 300 mM)
112 mg disodium EDTA (final 3 mM)
H₂O to 100 mL
Store up to 6 months at 4°C
pH to 7.5

Lithium perchlorate (LiClO₄), 1 M
Dissolve 106.4 g LiClO₄ (99%, Aldrich) in 1 liter water. Store up to 1 month at room temperature. Filter through a 0.45-μm filter before use.

Nuclease P1 buffer
1.21 g Tris base (final 0.1 M Tris-Cl)
13.6 mg ZnCl₂ (final 1 mM)
H₂O to 100 mL
Adjust pH to 7.2 with HCl
Store up to 6 months at −20°C
Oxidant solution
1.23 g iodine crystals (0.1 M)
25:20:2 (v/v/v) tetrahydrofuran/pyridine/water
Store up to 6 months at room temperature

Running dye
9.8 mL deionized formamide
0.2 mL 10× TBE electrophoresis buffer (APPENDIX 2A)
10 mg xylene cyanol (final 0.1%)
10 mg bromphenol blue (final 0.1%)
Store up to 1 year at −20°C

Tetrahydrofuran (THF), anhydrous
Dry by continuous reflux and distillation over sodium metal and benzophenone under an inert atmosphere until a purple color persists. Prepare fresh before each use.

Triethylamine/acetonitrile, 4:6 (v/v)
Dry triethylamine by refluxing and distillation over calcium hydride. Store up to 1 year at 4°C over activated 4A molecular sieves. Mix 8 mL anhydrous triethylamine and 12 mL anhydrous acetonitrile (see recipe) in a septum bottle and seal. Prepare fresh. To use, withdraw via syringe through the septum.

COMMENTARY
Background Information

Discovery and applications of bNAs
The body of work devoted to branched nucleic acids (bNAs) indicates that they are of current interest in chemistry and biochemistry. Branched ribonucleic acids (bRNAs) were first detected in nuclear RNA in 1983 (Wallace and Edmonds, 1983). It was later discovered that an RNA “lariat” is the first biochemical intermediate in the splicing of precursor messenger RNA (Ruskin et al., 1984). Several other bRNA structures such as Y-shaped trans-splicing intermediates and multicopy single-stranded DNAs (msDNA) were subsequently discovered in eukaryotes and prokaryotes, respectively (Yee et al., 1984; Inouye et al. 1987). These branched molecules all contain a common structural feature, namely vicinal 2′,5′- and 3′,5′-phosphodiester bonds at a branch point nucleotide. A novel polypeptide termed the RNA lariat debranching enzyme selectively hydrolyzes the 2′,5′-phosphodiester bond at the branch point of excised intron lariats, thus converting them into linear molecules (Ruskin and Green, 1985; Chapman and Boeke, 1991; Nam et al., 1994).

The novelty of bRNAs has raised interest as to their possible role in regulating RNA processing. Recently, there has been increasing interest in synthetic bDNA and bRNA for use in diagnostic applications (Urdea et al., 1991) and biosensor development (Uddin et al., 1997), as tools for studying mRNA splicing (Carriero et al., 2001) and debranching (Nam et al., 1994; Ooi et al., 2001), and as “molecular anchors” for inducing the formation of novel triplex (e.g., Hudson et al., 1995) and tetraplex structures (Robidoux et al., 1997).

Chemical synthesis of bRNA
The stepwise assembly of the vicinal 2′,5′- and 3′,5′-internucleotide linkages present in bRNA via traditional phosphotriester methods can be difficult, as cyclic phosphate may be formed (see Damha et al., 1992, and references therein). This problem was circumvented by using phosphodiester intermediates, which are more stable to nucleophilic attack by vicinal 2′-OH groups relative to phosphotriesters.

Short bRNA molecules were first prepared by solution-phase methods. Ogilvie and co-workers originally prepared branched tri- and tetranucleotides having identical nucleotides at the branch point 2′ and 3′ positions (symmetric bRNA; Damha et al., 1985; Damha and Ogilvie, 1988). Their method was also adapted to the synthesis of bRNA isomers having different nucleotide units at the 2′ and 3′ positions (Damha and Ogilvie, 1988). Regiospecific synthesis of a tetranucleotide of natural branch point sequence was performed by Kierzek et al.
Compound Characterization

Analysis of crude and purified bNAs is conveniently carried out via anion-exchange HPLC (see Support Protocol 3). The buffer concentration required for the elution of bNA is dependent upon the molecular weight and charge of the molecule being purified (Table 4.14.4). Separation of bNA from failure sequences is greatly facilitated by the relatively large molecular weight of the bNA species. Gel electrophoresis is another convenient way to isolate/purify bNA fragments (see Support Protocol 4). In this case, the bNA of interest exhibits significant retarded mobility relative to the failure sequences as a result of frictional effects as the bNA molecules migrate through the cross-linked gel environment. Further characterization is conducted by MALDI-TOF-MS (UNIT 10.1) using 6-aza-2-thiothymine (ATT) and dibasic ammonium citrate as matrices (Leccch et al., 1995). Nucleoside composition analysis is conducted via enzymatic hydrolysis with the exonuclease snake venom phosphodiesterase (Eadie et al., 1987; UNIT 10.6) and the endonuclease nuclease P1 (see Support Protocol 3). The yeast debranching enzyme (yDBR), a phosphodiesterase specific to the 2′,5′-phosphodiester bond of oligonucleotides containing vicinal 2′,5′- and 3′,5′-phosphodiester linkages, may also be used to confirm the branched nature of the molecules according to the method of Ooi et al. (2001).

Critical Parameters and Troubleshooting

Basic Protocol 1

The synthesis of the branch synthon (S.3) from 5′-O-DMTr-N6-benzoyl-riboadenosine is extremely moisture sensitive. Thus, it is imperative that all solvents, reagents, and apparatuses (e.g., syringes, needles, reaction vessel) be anhydrous. The phosphitylating reagent (stored at −20°C) should be warmed to room temperature in a desiccator prior to use. If the isolated yield of S.3 is significantly lower than expected, verify that the phosphitylating chlorophosphoramidite is a clear viscous liquid, and that no crystalline material is present (this residue is indicative of hydrolysis).

Basic Protocol 2

There are two important considerations when synthesizing bNA by the convergent approach. Firstly, the yield of bNA is dependent upon the molar concentration of the branching chlorophosphoramidite synthon S.3. Dilute solutions (0.03 M) of S.3 must be employed to maximize coupling with adjacent CPG-bound oligonucleotide chains (branching). If higher
concentrations are used, yields of branched products are reduced, and mainly extended isomeric side products are formed. The efficiency of the branching reaction can be assessed during synthesis by quantitation of the trityl cations released immediately before and after the branching reaction. Theoretically, 100% branch formation should give an apparent coupling yield of 50% since the trityl absorption following branching should be half of the previous absorption (during branching two nucleotide chains become joined to a single bisphosphoramidite synthon). Thus, trityl yields significantly greater or less than 50% indicate little branch formation (e.g., 80% apparent trityl yield indicates the formation of mainly the extended isomeric compounds). It is important to prepare the bisphosphoramidite stock solution (0.03 M) using at least 100 mg S.3. This ensures that (unavoidable) traces of moisture will not use up significant amounts of bisphosphoramidite during the branching step and reduce the overall yield of synthesis. The purity of the bisphosphoramidite can be assessed prior to use via TLC analysis or 31P-NMR. If partial decomposition has occurred, or if the branching reactions during synthesis are poor, the compound should be rechromatographed as described (see Basic Protocol 1).

Secondly, solid supports having a high degree of derivatization (>50 µmol/g) provide the best results for the synthesis of short bNA fragments (<7 nt). This is because highly substituted supports ensure the appropriate distance between the reactive 5′-OH end groups of the immobilized oligonucleotide chains. Supports with a nucleoside loading of 30 to 50 µmol/g give very good results for the synthesis of medium-size branched oligonucleotides.

**Basic Protocol 3**

The divergent approach requires synthesis of a linear oligonucleotide chain followed by backward synthesis from the 2′-hydroxyl of an internal ribonucleotide residue. This is carried out with commercially available inverted nucleoside 5′-phosphoramidites. Key to the success of this synthesis is the ability to cleave the 2′-O-silyl protecting group of the internal ribonucleotide residue and the use of an excess of the first inverted 5′-phosphoramidite reagent to force the branching reaction to a maximal extent. This is in sharp contrast to the convergent strategy, where branching requires a delicate control of reaction conditions.

Incomplete removal of the 2′-O-TBDMS group from the RNA branch point (Fig. 4.14.8) results in the accumulation of the linear DNA-RNA-DNA precursor (S.12), which is easily detected by HPLC and PAGE. To ensure complete 2′-O-desilylation of the CPG-bound oligonucleotide, it is essential to use fresh TBAF reagent and to introduce the TBAF solution slowly to the synthesis column (via syringe), pushing and pulling the piston slightly to displace the CPG beads stuck at the base of the column. This allows all of the CPG-bound oligonucleotide to come in contact with the TBAF reagent. It is important not to expose the CPG-bound bNA to the TBAF reagent for >10 min, as prolonged treatment results in significant cleavage of the oligonucleotide from the solid support. After washing steps, the first inverted phosphoramidite is introduced as a 0.3 M acetonitrile solution (as opposed to 0.1 M for all other DNA 3′- and 5′-phosphoramidite couplings) and allowed to react with the CPG-bound oligomer for 30 min (as opposed to 90 and 120 sec for all other DNA phosphoramidites).

**Anticipated Results**

The anticipated results of a convergent bNA synthesis are provided in Figures 4.14.6 and 4.14.7. Syntheses carried out on a 1-µmol scale will characteristically yield 50 to 80 A260 units crude material, while the isolated yields (bNA) generally fall in the range of 5 to 20 A260 units. bNAs that have been purified by anion-exchange HPLC are of high purity, usually >95% (Fig. 4.14.10). In the case of divergent bNA synthesis, typical crude yields are in the range of 50 to 100 A260 units. A standard gel analysis of bNA prepared by the divergent method is shown in Fig. 4.14.9. After HPLC or gel purification, 5 to 20 A260 units of bNA are typically recovered from divergent syntheses (slightly higher yields are obtained with HPLC). As for convergent synthesis, purity is >95%.

**Time Considerations**

Provided that all reagents and materials required for each step are available, most of the procedures are simple and rapid. The synthesis of S.3 from 5′-DMTr-N6-benzoyl-riboadenosine requires 2 to 4 hr to complete, including the workup. The reaction should not be allowed to proceed overnight, as decomposition may occur. Column chromatography requires ~1 to 2 hr including setup. Ideally, column purification should be conducted immediately following the phosphorylation reaction.

When the branching synthon S.3 and all the other phosphoramidite derivatives are ready to
use, the time required to prepare, purify, and isolate a Y-shaped RNA or DNA via the divergent approach is 3 days and 4 to 6 days, respectively. The preparation of bNA via the divergent approach requires 3 days or more.

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Nam et al., 1994. See above.

Reports on the substrate and sequence specificity of the yeast lariat debranching enzyme (γDBR), a unique 2′,5′-phosphodiesterase. The enzyme accepts a variety of substrates including group II intron lariats, msDNA, and synthetic bNAs.

Padgett, R.A., Konarska, M.M., Grabowski, P.J., Hardy, S.F., and Sharp, P.A. 1984. Lariat RNA’s as intermediates and products in the splicing of messenger precursors. Science 225:898-903.

This report provides evidence that the branched lariat structure is an intermediate of splicing of an adenovirus ML2 RNA transcript. Specifically demonstrated is that the excised intron contains an unusual nuclease-resistant core consisting of a branched trinucleotide structure with vicinal 2′,5′- and 3′,5′-phosphodiester linkages.

Sharp, P.A. 1994. Split genes and RNA splicing. Cell 77:805-815.

A Nobel lecture. A paramount review describing the splicing of introns from nascent RNA, the evolutionary significance of introns, and the plethora of factors involved in post-transcriptional processing.

Wallace and Edmonds, 1983. See above.

A first account demonstrating the occurrence of a branched nuclear polyadenylated RNA containing vicinal 2′,5′- and 3′,5′-phosphodiester bonds. Such molecules were absent from cytoplasmic polyadenylated RNA, implicating these structures as intermediates during mRNA processing.

Internet Resources
http://paris.chem.yale.edu/extinct.html
A useful site for the calculation of molecular weights of oligonucleotides and peptides as well as the determination of extinction coefficients (ε).

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