
**Parallel Clamps and Polypurine Hairpins (PPRH) for Gene Silencing and Triplex-Affinity Capture: Design, Synthesis and Use.**

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**Significance Statement.**

Most of the applications involving oligonucleotide probes are based on hybridization with complementary targets to form a duplex. When the target sequence is a polypurine-polypyrimidine track it is possible to use special triplex forming oligonucleotides (TFO). Parallel clamps and polypurine reversed hairpins (PPRH) are special cases of TFOs in which the polypurine Watson-Crick strand is linked to either a polypyrimidine Hoogsteen strand or a polypurine reversed Hoogsteen strand. The high stability of the triplex formed upon binding of these TFOs with the polypyrimidine target allows targeting of single and double stranded nucleic acid sequences as these TFOs induce strand displacement. This targeting technology covers from gene silencing of genomic DNA and mRNA to detection of nucleic acids of biological interest.

**ABSTRACT**

Nucleic acid triplexes are formed when a DNA or RNA oligonucleotide binds to a polypurine-polypyrimidine rich sequence. Triplexes have wide therapeutic applications such as gene silencing or site-specific mutagenesis. In addition, protocols based on triplex-affinity capture have been used for detecting nucleic acids in biosensing platforms. In this unit we describe the design, synthesis and use of parallel clamps and polypurine reversed hairpins (PPRH) to bind to target polypyrimidine targets. The combination of the polypurine Watson-Crick strand with the triplex forming strand in a single molecule produces highly stable triplexes allowing targeting of single and double stranded nucleic acid sequences. On the other hand, PPRHs are easily prepared and work at nanomolar range, like siRNAs, and at a lower concentration than that needed for antisense ODNs or TFOs. However, the stability of PPRHs is higher than that of siRNAs. In addition, PPRHs circumvents off-target effects and are non-immunogenic.

**Keywords:** Triplex, polypurine hairpins, gene silencing, parallel clamps, triplex-affinity capture
INTRODUCTION. Triplex forming oligonucleotides are important tools in gene silencing and gene analysis. The most studied triplex is the so-called parallel triplex, where a polypuridine strand binds a polypurine-polypyrimidine duplex sequence by formation of C+: G.C and T:A.C triads. The stability of this triplex depends on the pH as cytosine needs to be protonated to form the C+: G.C triad. 8-Amino adenine and 8-aminoguanine stabilize the parallel triplex by a combined effect of the gain in one Hoogsteen purine-pyrimidine H-bond and the ability of the amino group to be integrated into the "spine of hydration" located in the minor-Major groove of the triplex structure (Añón, 2002). In order to use the beneficial triplex stabilization properties of 8-aminopurine, capture probes were designed as parallel clamps to bind to single-stranded polypyrimidine sequences. These clamps consisted in a purine strand carrying 8-aminopurines and the parallel stranded polypyrimidine sequence. Examples of the use of these clamps are the isolation of polypyrimidine RNA sequences of biological interest such as bacterial RNA (Carrascosa, 2012) and miRNA (Añón, 2016). The PolyPurine Reverse Hoogsteen (PPRH) technology is often used for targeting single and double stranded nucleic acid sequences (Villalobos, 2015). Several examples of PPRHs for the inhibition of gene expression have been described (Solé, 2017; Ciudad, 2017; Coma, 2005) as well as for capture of miRNA (Ribas 2018) and double-stranded DNA for the analysis of DNA methylation status (Huertas, 2018).

This unit describes the design, synthesis and use of parallel clamps and polypurine (PPRH) hairpins for gene silencing and triplex-affinity capture.

Basic protocol 1 describes the search of polypyrimidine-polypurine tracks and design of the appropriate clamps and hairpins (Figure 1). Basic protocol 2 describes the protocols for the synthesis of 8-amino-2′-deoxyadenine phosphoramidite. Basic protocol 3 describes the protocols for the synthesis of 8-amino-2′-deoxyguanosine phosphoramidite. Basic protocol 4 describes the synthesis of oligonucleotide clamps and polypurine hairpins. Basic protocol 5 describes the use of polypurine hairpins for gene silencing. Basic protocol 6 describes the functionalization of gold surfaces of SPR for detection.
Figure 1. Steps in the design of parallel clamps or the PPRH. First select the polypyrimidine track from sequence database. Then select if parallel clamps or the PPRH will be used and the linking strategy (tetra- or penta-thymidine loop or hexaethyleneglycol) and the ends that will be connected (A, B , C, or D). \( P_1, P_2, \ldots, P_n \) purine sequence; \( Y_1, Y_2, \ldots, Y_n \) pyrimidine sequence.

**BASIC PROTOCOL 1**

**DESIGN OF POLYPURINE REVERSE HOOGSTEEN HAIRPINS**

The protocol describes the design of PPRHs which are formed by two mirror-repeat sequences of polypurines linked by a pentathymidine bridge (Figure 1). This involves the search of polypurine stretches within a given gene and their localization in the chromosome and in its specific parts such as promoter, exonic or intronic sequences. Thus, the complementary sequence of each one of these
regions constitutes the polypyrimidine track which will be the target of the selected PPRHs. Then, the selection of sequences to build the PPRHs, among all the possible match found in the search, is made considering the length of the sequence, the number of pyrimidine interruptions and the specificity of that sequence within the genome of interest. Once a polypurine sequence is selected, the mirror repeat is determined by searching for its reverse sequence. Then, the final PPRH sequence is assembled by connecting these two sequences using a pentathymidine (T₅) spacer, which will produce a loop to allow the formation of the hairpin.

Materials

Computer processor
Internet connection
Triplex-Forming Oligonucleotide Target Sequence Search software: available at http://utw10685.utweb.utexas.edu/tfo/
Triplex target site Mapping and Integration software: available at http://ttsmi.bii.a-star.edu.sg

Design of PPRHs

1. Connect via web with http://utw10685.utweb.utexas.edu/tfo/ to look for Polypurine sequences using the TFO-searching Target Sequence Search.

2. Select the species and gene. By default, Species is set at Homo sapiens, but it can also be changed to Mus musculus. When searching for Polypurines in other species, the DNA sequence and the gene of interest has to be provided as a .txt file in FASTA format.

3. Enter the Gene name, either as a symbol or as Entrez Gene ID.

4. Set the parameters, which by default are i) a minimum of 15 nt length; ii) a minimum of %G: 50; iii) a maximum allowable of 3 Pyrimidine interruptions; and iv) taking 2000 bases for the putative promoter region length. These parameters can be changed according to your needs or results after a first iteration.

5. Select the gene in case you are offered with a variety of genes with similar names or with different transcripts and search for TFO target sequences.

6. A list of polypurine sequences will appear in decreasing order of nucleotide length. For each sequence, the following information is given: i) the length of the sequence including the number and position of polypyrimidine interruptions (in red) up to 3; ii) the % of G ; iii) the strand of the double strand DNA where the polypyrimidine track is found (either forward or
reverse) according to the chromosome orientation; iv) the position in the chromosome and in the gene; and the region of the gene where it is found (promoter, intron or exon).

7. To analyze the specificity of each sequence it is given the possibility to perform automatically a BLAST (Basic Local Alignment Sequence Tool) analysis from NCBI to determine the similarity of the selected sequence within the genome of interest.

8. BLAST analysis can also be performed manually using a chosen sequence from the TFO report or a selected fragment of a sequence. The query (input) sequence is compared with a database (e.g. nr: non-redundant) for the species of interest (e.g. human).

9. The BLAST report consists of list of genes in a decreasing order of similarity to the input sequence quantified by a score followed by a selection of the sequences producing significant alignments.

10. A Polypurine sequence is considered to be specific when the report of the BLAST finds out the gene of interest as the most similar among all the hits and with a significant distance in score with the next gene sequence. This distance in score in significance depends of the length of the sequence. For instance, for a sequence of 26 nt the difference can be around 15% less, corresponding to 4-5 mismatches or lack of identity.

11. To design a particular PPRH out of a selected TFO target, paste that sequence into the Reverse-Complement software and determine the Reverse sequence. This sequence will correspond to the mirror repeat of the original sequence.

12. Proceed to the design of the sequence of the PPRH in the following manner: Place the Polypurine sequence followed by 5 Ts which is then linked to the reverse sequence. The T₅ will act as a loop between the two mirror sequences that form the hairpin bound by intramolecular reverser Hoogsteen bonds.

13. If the chosen polypurine sequence is in the forward strand of the chromosome and the gene has forward orientation, then the designed PPRH is termed Template-PPRH because it would be directed against the polypyrimidine track in the template strand of the gene (Figure 2A).

14. If the chosen polypurine sequence is in the reverse strand of the chromosome and the gene has forward orientation, then the designed PPRH is termed Coding-PPRH because it would be directed against the polypyrimidine track in the coding strand of the gene, which will also correspond to the sequence and orientation of the transcribed RNA (Figure 2B).

15. If the chosen polypurine sequence is in the forward strand of the chromosome and the gene has reverse orientation, then the designed PPRH
is termed Coding-PPRH because it would be directed against the polypyrimidine track in the coding strand of the gene, which will also correspond to the sequence and orientation of the transcribed RNA (Figure 2C).

16. If the chosen polypurine sequence is in the reverse strand of the chromosome and the gene has reverse orientation, then the designed PPRH is termed Template-PPRH because it would be directed against the polypyrimidine track in the template strand of the gene (Figure 2D).

17. It is advisable to design several PPRHs directed against each gene, for instance in the promoter region, and in intron and exon sequences. Also, if possible, design template- and coding- versions of PPRHs if the target sequences exist. This will allow having a broader spectrum of PPRH sequences for screening of their potential gene silencing effect. Regularly, it is enough to test 3 PPRHs to get silencing effects.

**Figure 2.** Different permutations and nomenclature (template or coding) of PPRHs depending on the orientation of the gene in the chromosome (forward or reverse) and in which strand the polypurine stretch is found. A) Forward gene and Polypurine (Ppu) sequence in the forward strand, thus the Polypyrimidine sequence (Ppy) is in the template strand and therefore the hairpin is termed template-PPRH; B) Forward gene and Ppu sequence in the reverse strand, thus the hairpin is binding to the coding strand and named coding-PPRH; C) Reverse gene and Ppu sequence in the forward strand, thus the PPRH binds to the coding strand; and D) Reverse gene and Ppu sequence in the reverse strand, thus the PPRH binds to the template strand. The arrows representing the PPRH are shown in the 5’ > 3’ orientation. TSS, transcription start site.

**ALTERNATE PROTOCOL 1.**
SEARCHING FOR POLYPURINE SEQUENCES USING THE TRIPLEX TARGET SITE MAPPING AND INTEGRATION SOFTWARE

This alternate protocol uses different software which is located in a different web location. The present description illustrates an alternate option to search of polypurine stretches. The main difference is that the present one gives only sequences with up to one pyrimidine interruption. Additionally, it can filter the maximum number of Gs in a row. Given that 3 interruptions are allowed for the binding among PPRHs and their target, the BP1 is preferentially used.

18. Connect via web with http://ttsmi.bii.a-star.edu.sg

19. Introduce the name of the gene using the gene symbol, synonym or RefSeq ID.

20. Establish the search criteria, which by default are: Percent guanine content, more than 40%; number of pyrimidine interruptions, 0; number of Gs in a row, less than 4. These parameters can be changed as desired, depending of the results after the first iteration, e.g. if there are no results, change from “0” to “1” pyrimidine interruption.

21. The filtering options can be accurately adjusted by selecting the percentage of Gs, the minimum length of the polypurine sequence, and the gene region annotation.

22. Submit the search to obtain the list of polypurine sequences including the information of the interruptions, length of the sequence, the %of Gs, and the gene regions where they are found.

23. Design the sequence of the PPRH: Place the Polypurine sequence and link it to its reverse sequence using a T₅ loop for connecting both sequences.

BASIC PROTOCOL 2

SYNTHESIS OF 8-AMINO-2’-DEOXYADENOSINE PHOSPHORAMIDITE

8-Amino-2’-deoxyadenosine stabilizes the parallel triplex by the gain in one Hoogsteen purine-pyrimidine H-bond and the ability of the amino group to be integrated into the "spine of hydration" located in the minor-Major groove of the triplex structure (Güimil-García, 1999). This protocol describes the preparation of the phosphoramidite of 8-amino-2’-deoxyadenosine (Güimil Garcia, 1999). The synthesis involves 2 basic steps: synthesis of 8-amino-2’-deoxyadenosine and synthesis of the N-protected phosphoramidite derivative (Figure 3). The preparation of 8-amino-2’-deoxyadenosine involves 3 reactions: 1) bromination of 2’-deoxyadenosine (dA), 2) displacement of the bromine by an azide group and 3) hydrogenation of the azide (Holmes, 1965; Long, 1967). Alternatively, it is possible to avoid hydrogenation by a simple treatment of 8-azido-2’-deoxyadenosine with 40% aqueous methylamine (Frieden, 2003). The synthesis of the N-protected phosphoramidite derivative involves more 3 reactions: 4) introduction of the
dimethylformamidino (dmf) group at N^6 and N^8 positions, 5) introduction of the dimethoxytrityl (DMT) group at the 5’-hydroxyl function and 6) preparation of the 3’-O-phosphoramidite derivative.

![Synthesis of the phosphoramidite of 8-amino-2’-deoxyadenosine](Figure 3)

**Figure 3.** Synthesis of the phosphoramidite of 8-amino-2’-deoxyadenosine

**Materials**

- 2’-Deoxyadenosine (dA)
- Bromine (Br₂)
- Sodium acetate
- Sodium bisulfite
- Methanol (MeOH)
- Ethanol (EtOH)
- Dichloromethane (DCM)
- Lithium azide (Kodak)
- N,N-Dimethylformamide (DMF)
- Pd (5%) on carbon (Aldrich)
- Celite™ (Fluka)
- Hydrogen (H₂)
- 40% methylamine aqueous solution
- Dimethylacetal-N,N-dimethylformamide
- Silica gel (230-400 mesh, Grade 60, Fluka)
- Anhydrous pyridine
- Dimethoxytrityl chloride
- Sodium bicarbonate (NaHCO₃)
- Sodium chloride (NaCl)
- Anhydrous sodium sulphate
- Triethylamine
- Anhydrous acetonitrile
Diisopropylethylamine
2-Cyanoethoxy-N,N-diisopropylamino chlorophosphine (Link Technologies)
Hexanes

100- and 250-mL round-bottom flasks
Magnetic stir bars
Magnetic stirrer /hotplate
Dropping funnel
Silicone bath
Dry ice/ acetone bath
Rubber septa
Syringe needles
Buchner funnels setup with vacuum source
Desiccator
Hydrogenation apparatus (if using the hydrogenation route)
Separation funnel
Equipment for column chromatography

**Synthesis of 8-Bromo-2'-deoxyadenosine (2).**

1. Prepare a buffered bromine solution as follows: 2 ml of bromine was mixed with 40 mL of a 1M sodium acetate solution in a 250-mL Erlenmeyer flask by magnetic stirring until complete dissolution.

2. Weight out 2.51 g (10 mmol) of 2'-deoxyadenosine (dA, 1, Figure 3) into a 250-mL round bottom flask and add 20 mL of a 1M sodium acetate solution with stirring. A suspension is formed. Add dropwise (over 30 min) the buffered bromine solution to the dA suspension using a dropping funnel.

3. After the addition of the bromine solution, stir the mixture for 2 hours at room temperature.

4. Stop the reaction by addition of around 10 mL of a 38% NaHSO₃ aqueous solution until the brown color of the solution changes to a pale yellow color.

5. Adjust the pH of the solution to pH 7

6. Cool the solution at -20 °C with a dry ice/acetone bath.

7. Filter the solution to obtain a precipitate and dry in a desiccator.

*The product (2) is a white-yellowish solid and it was obtained in 84% yield (2.77 g, 8.4 mmol). TLC (10% methanol (MeOH) / dichloromethane (DCM) Rf 0.42; (20% MeOH/ DCM) Rf 0.58. ¹H-NMR (250 MHz, DMSO-d₆) δ (ppm) 2.2 (m, 1H, H-2'), 3.2 (m, 1H, H-2'), 3.4 (m, 1H, H-5'), 3.6 (m, 1H, H-5'), 3.83 (m, 1H, H-4'), 4.42 (m, 1H, H-3'), 5.25 (wide s, 2H, 3'-OH and 5'-OH), 6.26 (t, 1H, H-1'), 7.52 (wide s, 2H, NH₂), 8.8 (s, 1H, H-2). ¹³C-NMR (62 MHz, DMSO-d₆) δ (ppm) 37.0 (C-2'), 62.1 (C-5'), 71.2 (C-3'), 86.4 (C-4'), 88.3 (C-
1), 119.7 (C-5), 126.6 (C-8), 149.9 (C-4), 152.4 (C-2), 155.1 (C-6). Mass spectra (electrospray +) expected for C₁₀H₁₂BrN₅O₃ 329.2 and 331.2 (2 isotopes of Br), found 330.02, 332.02 (M+H).

**Synthesis of 8-azido-2'-deoxyadenosine (3).**

8. Weight out 2.7 g (8.2 mmol) of 8-bromo-2'-deoxyadenosine (2) into a 250-mL round bottom flask and dissolve in 30 mL of N,N-dimethylformamide (DMF) with magnetic stirring. Add 1 g of lithium azide (20.4 mmol). Cap the flask with a septum.

9. Place the solution in a silicone bath placed on a hot plate and maintain the solution at 70ºC for 20 hr with magnetic stirring.

10. Cool the solution to room temperature and concentrate the solution to dryness under vacuum using a rotovap.

11. Dissolve the product in the minimal amount of hot water (around 40 mL) and cool the solution at 4 oC. Collect the resulting solid.

The product (3) was obtained in 77% yield (1.84 g, 6.3 mmol) as a white solid. TLC (10% EtOH / DCM) Rf 0.22; (20% EtOH/ DCM) Rf 0.60. ¹H-NMR (250 MHz, DMSO-d₆) δ (ppm) 2.1 (m, 1H, H-2'), 3.0 (m, 1H, H-2'), 3.42 (m, 1H, H-5'), 3.6 (m, 1H, H-5'), 3.82 (m, 1H, H-4'), 4.41 (m, 1H, H-3'), 5.2 (s, 2H, 3'-OH and 5'-OH), 6.1 (t, 1H, H-1'), 7.25 (s, 2H, NH₂), 8.05 (s, 1H, H-2).

¹³C-NMR (62 MHz, DMSO-d₆) δ (ppm) 37.1 (C-2'), 62.1 (C-5'), 71.0 (C-3'), 83.4 (C-4'), 88.1 (C-1'), 117.0 (C-5), 144.0 (C-8), 149.3 (C-4), 151.5 (C-2), 154.4 (C-6).

**Synthesis of 8-amino-2'-deoxyadenosine (4) by hydrogenation**

12. Weight out 3.5 g (12.2 mmol) of 8-azido-2'-deoxyadenosine (3) into a 250-mL round bottom flask and dissolve in 100 mL of a 1:1 mixture of EtOH/water with magnetic stirring.

13. Transfer the solution in a hydrogenation apparatus and add 400 mg of Pd (5%) on carbon.

14. Maintain the suspension at 3 atmospheres of hydrogen during 72 hr with stirring.

15. Empty the hydrogen using vacuum and add a nitrogen atmosphere.

16. Open the hydrogenation apparatus and filter the mixture on Celite™. Wash the catalyst with 1:1 EtOH/ water solution.

17. Concentrate the resulting filtrates to dryness under vacuum using a rotovap.

18. Dissolve the product in the minimal amount of hot ethanol (around 40 mL) and cool the solution at 4 oC. Collect the resulting solid by filtration.
The product (4) was obtained in 92% yield (3 g, 11.3 mmol) as a white solid. TLC (20% EtOH / DCM) Rf 0; (50% EtOH/ DCM) Rf 0.45. $^1$H-NMR (250 MHz, DMSO-d$_6$) δ (ppm) 2.0 (m, 1H, H-2'), 2.7 (m, 1H, H-2'), 3.5 (m, 2H, H-5'), 3.9 (m, 1H, H-4'), 4.4 (m, 1H, H-3'), 6.25 (dd, 1H, H-1'), 6.6 (s, 2H, NH$_2$), 7.9 (s, 1H, H-2). $^{13}$C-NMR (62 MHz, DMSO-d$_6$) δ (ppm) 37.8 (C-2'), 61.8 (C-5'), 71.4 (C-3'), 83.3 (C-4'), 87.6 (C-1'), 117.1 (C-5), 148.5 (C-2), 149.0 (C-8), 151.5 (C-4), 152.4 (C-6).

Alternate synthesis of 8-amino-2'-deoxyadenosine (4) by treatment with a 40% methylamine aqueous solution

19. Weight out 5.1 g (17.4 mmol) of 8-azido-2'-deoxyadenosine (3) into a screw-cap tube and dissolve in 20 mL of 40% aqueous methylamine solution and 2 mL of dioxane.

20. Heat the solution overnight to 55 ºC

21. Cool the solution to room temperature and evaporated to dryness under vacuum using a rotovap.

22. Use the oily residue in the following step without further purification.

23. The product has the same spectroscopy properties than the product obtained above.

Synthesis of 8-amino-N$_6$N$_8$-bis(dimethylaminomethylidene)-2'-deoxyadenosine (5).

24. Dissolve the 8-amino-2'-deoxyadenosine obtained above (approx. 17.4 mmol) in 250 mL of DMF and add 12.1 mL of N,N-dimethylformamide dimethylacetal.

25. Stir the solution overnight at room temperature and then concentrate to dryness.

26. Dissolve the residue in DCM (20 mL) and purify the resulting compound using a silica gel column eluted with a 0-20% gradient of MeOH in DCM taking 20-mL fractions. The desired compound elutes at around 10% MeOH in DCM. Small amounts of 8-amino-2'-deoxyadenosine (4) may be observed at 20% MeOH in DCM. Combine the fractions containing the desired compound and concentrate to dryness under vacuum.

The product (5) was an oil and it was obtained in 88% yield (5.4 g, 15.3 mmol). TLC (10% EtOH / DCM) Rf 0.29. $^1$H-NMR (250 MHz, DMSO-d$_6$) δ (ppm) 2.05 (m, 1H), 3.05-3.2 (3s and m, 13H), 3.5-3.6 (m, 2H), 3.86 (m, 1H), 4.46 (m, 1H), 5.25 (m, 2H), 5.55 (m, 1H), 6.57 (t, 1H), 8.17 (s, 1H), 8.66 (s, 1H), 8.81 (s, 1H). $^{13}$C-NMR (62 MHz, DMSO-d$_6$) δ (ppm) 34.4, 34.5, 37.1, 40.4 (under solvent peak), 62.6, 71.7, 82.9, 87.8, 124.7, 148.4, 151.6, 155.6, 156.0, 157.1, 157.6. Elemental analysis, expected for C$_{16}$H$_{24}$N$_8$O$_3$. ½ H$_2$O C 49.86, H 6.54, N 29.07; Found C 49.52, H 6.57, N 28.88. UV (water) max 267 and 334 nm.
**Synthesis of 8-amino-N\(^6\),N\(^8\)-bis(dimethylaminomethylidene)-2′-deoxy-5′-(4,4′-dimethoxytrityl-adenosine (6).**

27. Dissolve 1.0 g (2.8 mmol) of compound 5 in 50 mL of anhydrous pyridine in a 100-mL round-bottom flask. The solution is stirred under nitrogen atmosphere on a magnetic stirrer and the flask capped with a rubber septum.

28. Add 1.08 g of 4,4′-dimethoxytrityl chloride (3.18 mmol) to the pyridine solution in several small portions.

29. After the last addition, stir for additional 2 hr at room temperature.

30. Stop the reaction by adding 1 ml of MeOH and concentrated the solution to dryness under vacuum using rotovap.

31. Dissolve the residue in DCM (40 mL) and wash the organic solution with 5% NaHCO\(_3\) aqueous solution (40 mL) and a saturated NaCl solution (40 mL) (brine) using a separation funnel.

32. Dry the organic solution with anhydrous Na\(_2\)SO\(_4\) (1 g) and concentrated to dryness.

33. Pack a silica gel column with 1% triethylamine in DCM. Dissolve the residue in DCM (10 mL) and purify the compound with a 0-4% gradient of MeOH in DCM collecting 10 mL fractions. The desired compound elutes at around 3-4% MeOH in DCM. Small amounts of DMT-containing products eluted at pure DCM. Combine the fractions containing the desired compound and concentrate to dryness under vacuum using rotovap.

The product (6) was obtained as white foam in 81% yield (1.5 g, 2.28 mmol). TLC (5% EtOH / DCM) Rf 0.6. \(^1\)H-NMR (250 MHz, Cl\(_3\)CD) \(\delta\) (ppm) 2.2 (m, 1H), 2.93, 3.02, 3.07, 3.16 (4s and m, 13H), 3.4 (m, 2H), 3.69 (s, 6H), 4.18 (m, 1H), 4.85 (m, 1H), 6.6-6.8 (m, 5H), 7.1-7.5 (m, 9H), 8.14 (s, 1H), 8.68 (s, 1H), 8.71 (s, 1H). \(^1^3\)C-NMR (62 MHz, Cl\(_3\)CD) \(\delta\) (ppm) 34.6, 34.8, 36.4, 40.6, 40.8, 55.0, 64.4, 73.2, 82.4, 85.3, 85.9, 112.8, 124.8, 126.4, 127.4, 128.0, 129.9, 136.0, 144.8, 149.2, 152.1, 153.3, 156.7, 156.8, 157.5, 158.1. Mass spectra (electrospray +) expected for C\(_{37}\)H\(_{42}\)N\(_8\)O\(_5\) 678.8, found 679.33 (M+H); 303.1 (DMT+).

**Synthesis of 8-amino-N\(^6\),N\(^8\)-bis(dimethylaminomethylidene)-2′-deoxy-5′-(4,4′-dimethoxytrityl-adenosine)-3′-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (7).**

34. Dissolve 1.16 g of compound 6 (1.7 mmol) in 20 mL of anhydrous acetonitrile and 0.9 mL (5.1 mmol) of disopropylethylamine in a 50-mL round-bottom flask. The solution is stirred under nitrogen atmosphere on a magnetic stirrer and the flask capped with a rubber septum.
35. Place the round-bottom flask in an ice bath and add 0.57 mL (2.55 mmol) of 2-cyanoethoxy-\(\text{N},\text{N}\)-diisopropylamino chlorophosphine with a syringe.

36. After the addition, stir the solution for 1 hr at room temperature.

37. Concentrate the solution to dryness under vacuum using rotovap.

38. Dissolve the residue in DCM (40 mL) and wash the organic solution with 5% NaHCO\(_3\) aqueous solution (40 mL) using a separation funnel.

39. Dry the organic solution with anhydrous Na\(_2\)SO\(_4\) (1 g) and concentrated to dryness.

40. Pack a silica gel column with 1% triethylamine in DCM/hexanes (9:1). Dissolve the residue in DCM (10 mL) and purify the compound eluting with DCM/hexanes (9:1) collecting 10 mL fractions. Combine the fractions containing the desired compound and concentrate to dryness under vacuum using rotovap.

The product (7) was obtained as white foam in 93% yield (1.4 g, 1.59 mmol). TLC (DCM /hexanes 9:1) Rf 0.6. \(^{31}\text{P-NMR}\) (101 MHz, \(\text{CDCl}_3\)) \(\delta\) (ppm) 144.96. Elemental analysis, expected for \(\text{C}_{46}\text{H}_{59}\text{N}_{10}\text{O}_{6}\text{P}\) C 62.86, H 6.77, N 15.93; Found C 62.58, H 6.80, N 15.64.

**BASIC PROTOCOL 3**

**SYNTHESIS OF 8-AMINO-2’-DEOXYGUANOSINE PHOSPHORAMIDITE**

8-Amino-2’-deoxyguanosine has been shown to stabilize both parallel (Soliva, 2000) and antiparallel (Aviñó 2003) triplexes. This protocol describes the synthesis of the phosphoramidite of 8-amino-2’-deoxyguanosine needed for the preparation of oligonucleotides carrying 8-amino-2’-deoxyguanosine (Rao, 1994; Güimil García, 1998; Rieger, 1999, Soliva, 2000). As described above the synthesis involves 2 basic steps: synthesis of 8-amino-2’-deoxyguanosine and synthesis of the N-proTECTED phosphoramidite derivative (Figure 4). The preparation of 8-amino-2’-deoxyguanosine involves 3 reactions: 1) bromination of 2’-deoxyguanosine (dG), 2) displacement of the bromine by hydrazine and 3) reduction of hydrazine with Raney Nickel (Long, 1967). The synthesis of the N-protected phosphoramidite derivative involves more 3 reactions: 4) introduction of the dimethylformamidino (dmf) group at \(\text{N}^2\) and \(\text{N}^6\) positions, 5) introduction of the dimethoxytrityl (DMT) group at the 5’-\(\text{O}\)-hydroxyl function and 6) preparation of the 3’-\(\text{O}\)-phosphoramidite derivative (Rao, 1994; Güimil García, 1998; Rieger, 1999; Soliva, 2000).
Figure 4. Synthesis of the phosphoramidite of 8-amino-2'‐deoxyguanosine

Materials

- 2′-Deoxyguanosine (dG)
- Bromine (Br₂)
- Hydrazine hydrate (NH₂NH₂•H₂O)
- Methanol (MeOH)
- Ethanol (EtOH)
- Anhydrous ethyl ether
- Dichloromethane (DCM)
- Raney™-Nickel (Fluka)
- N,N-Dimethylformamide (DMF)
- Celite™ (Fluka)
- N,N-dimethylformamide dimethylacetal
- Silica gel (230-400 mesh, Grade 60, Fluka)
- Anhydrous pyridine
- Dimethoxytrityl chloride
- Sodium bicarbonate (NaHCO₃)
- Sodium chloride (NaCl)
- Anhydrous sodium sulphate (Na₂SO₄)
- Triethylamine
- Anhydrous acetonitrile
- Diisopropylethylamine
- 2-Cyanoethoxy-Ν,Ν-diisopropylamino chlorophosphine (Aldrich)

100- and 250-mL round-bottom flasks
Magnetic stir bars
Magnetic stirrer /hotplate
Dropping funnel
Desiccator
Rubber septa
Syringe needles
Buchner funnels setup with vacuum source
Equipment for column chromatography

**Synthesis of 8-Bromo-2’-deoxyguanosine (9).**

1. Prepare a bromine solution as follows: 1.4 g of bromine was mixed with 138.6 mL of water in a 500 mL-round-bottom flask by magnetic stirring until complete dissolution (15 min).

2. Weight out 5 g (18.8 mmol) of 2’-deoxyguanosine (dG, 8, Figure 4) into a 500 mL round bottom flask and add 30 mL of water with stirring. A suspension is formed. Add the bromine solution to the dG suspension in fractions of 15 mL each until the suspension becomes permanently yellow and a white precipitate is formed (around 30 min).

3. Filter immediately the suspension and collect the precipitate formed during the addition of the bromine solution. The precipitate is washed with cold water (50 mL) and cold acetone (20 mL). The solid is dried in a desiccator.

The product (9) is a white solid and it was obtained in 84% yield (5.46 g, 15.7 mmol). HPLC (see basic protocol 4, reversed phase, 5-50%B gradient in 20 min) single peak at 13.7 min. \(^1\)H-NMR (250 MHz, DMSO-d\(_6\)) \(\delta\) (ppm) 2.09 (m, 1H, H-2’), 3.15 (m, 1H, H-2’), 3.54 (m, 2H, H-5’), 3.78 (m, 1H, H-4’), 4.38 (m, 1H, H-3’), 5.24 (m, 2H, 3’-OH and 5’-OH), 6.14 (t, 1H, H-1’), 6.49 (s, 2H, NH\(_2\)), 10.8 (s, 1H, NH). \(^1^3\)C-NMR (62 MHz, DMSO-d\(_6\)) \(\delta\) (ppm) 36.5 (C-2’), 62.1 (C-5’), 71.0 (C-3’), 85.1 (C-1’), 87.9 (C-4’), 117.5 (C-5), 120.6 (C-8), 152.0 (C-4), 153.3 (C-2), 155.5 (C-6). Mass spectra (electrospray +) expected for C\(_{10}\)H\(_{12}\)BrN\(_5\)O\(_4\) 345.2 and 347.2 (2 isotopes of Br), found 346.01 and 348.01 (M+H), 368.00 and 370.00 (M+ Na), 409.02 and 411.02 (M+Na+K), 691.02, 693.02 and 695.01 (2M+H), 713.00, 715.00 and 717.00 (2M+ Na).

**Synthesis of 2’-deoxy-8-hydrazinoguanosine (10).**

4. Weight out 3.8 g (10.9 mmol) of 8-bromo-2’-deoxyguanosine (9) into a 250-mL round bottom flask. The solid is suspended in a mixture of 50 mL water and 100 mL of methanol with magnetic stirring. Add 21.2 mL of NH\(_2\)-NH\(_2\)•H\(_2\)O (438 mmol) and fit a reflux condenser.

5. Reflux the reaction mixture for 24 hr (approx. 90 °C), then allow to cool to room temperature.

6. Concentrate the reaction mixture to dryness under vacuum to obtaining a white solid.

7. The residual solid is dissolved with the minimum amount of boiling methanol (around 50 mL) and allow cooling to room temperature and then to 4°C, obtaining a white solid.
8. The precipitate is collected by filtration. The solid is washed with anhydrous ether (30 mL) and dried in a desiccator.

The product (10) as white solid was obtained in 56% yield (1.82 g, 6.1 mmol). HPLC (see basic protocol 4, reversed phase, 5-50%B gradient in 20 min) single peak at 5.4 min. UV (water) max 258 with shoulder at 280 nm. $^1$H-NMR (250 MHz, DMSO-$d_6$) $\delta$ (ppm) 2.11 (m, 2H, H-2''), 3.60 (m, 2H, H-5''), 3.77 (m, 1H, H-3''), 4.35 (m, 1H, H-3'), 6.14 (t, 1H, H-1''), 6.41 (s, 2H, NH$_2$), 6.61 (s, 2H, NH$_2$ hydrazino), 7.47 (s, 1H, NH hydrazine), 7.91 (s, 1H, NH).

$^{13}$C-NMR (62 MHz, DMSO-$d_6$) $\delta$ (ppm) 37.5 (C-2''), 61.2 (C-5''), 71.1 (C-3''), 82.4 (C-4''), 87.1 (C-1''), 112.1 (C-5), 150.7 (C-8), 151.5 (C-4), 152.5 (C-2), 155.9 (C-6). Mass spectra (electrospray +) expected for C$_{10}$H$_{15}$N$_7$O$_5$ 297, found 298 (M+H).

Synthesis of 8-amino-2'-deoxyguanosine (11)

9. Weight out 1.4 g (4.7 mmol) of 2'-deoxy-8-hydrazino-guanosine (10) into a 500-mL round bottom flask and dissolve in a mixture of 60 mL of water and 100 mL of MeOH.

10. Add 10 g of Raney$^\text{TM}$-Nickel and fit a reflux condenser.

11. Reflux the reaction mixture for 16 hr.

12. Filter the hot solution through a column filled with Celite$^\text{TM}$ to remove the catalyst. Washed the column with 100-200 mL of a hot solution of MeOH/water (1:1) collecting the filtrates.

13. Combine the filtrates and concentrate the mixture to dryness under vacuum.

14. The residual solid is dissolved with the minimum amount of boiling mixture of MeOH/water (1:1) (around 30 mL) and allow to cool to room temperature and then to 4ºC, obtaining a white solid.

15. The precipitate is collected by filtration and dried in a desiccator.

The product (11) as white solid was obtained in 81% yield (1.1 g, 3.8 mmol). TLC (20% MeOH in DCM) Rf 0.1. HPLC (see basic protocol 4, reversed phase, 5-50%B gradient in 20 min) single peak at 5.8 min. UV (water) max 259, 290 nm. $^1$H-NMR (250 MHz, DMSO-$d_6$) $\delta$ (ppm) 1.9 (m, 2H, H-2''), 3.4 (m, 2H, H-5''), 3.62 (m, 1H, H-4''), 4.33 (m, 1H, H-3''), 5.27 (d, 1H, 3'-OH), 5.44 (t, 1H, 5'-OH) 6.09 (t, 1H, H-1''), 6.16 (wide s, 2H, NH$_2$), 6.23 (wide s, 2H, NH$_2$), 10.7 (wide s, 1H, NH). $^{13}$C-NMR (62 MHz, DMSO-$d_6$) $\delta$ (ppm) not available lack of solubility. Mass spectra (electrospray +) expected for C$_{10}$H$_{14}$N$_6$O$_4$ 282.2, found 283.1 (M+H).

Synthesis of 8-amino-\(N^6,N^8\)-bis(dimethylaminomethylidene)-2'-deoxyguanosine (12).
16. Dissolve 1 g of 8-amino-2'-deoxyguanosine (11, 3.5 mmol) in 60 mL of DMF and add 2.3 mL of N,N-dimethylformamide dimethylacetal (17.5 mmol).

17. Stir the solution overnight at room temperature and then concentrate to dryness under vacuum.

18. Dissolve the residue in 10% EtOH in DCM (20 mL) and purify the resulting compound using a silica gel column eluted with a 10-15% gradient of EtOH in DCM taking 10 mL fractions. Combine the fractions containing the desired compound and concentrate to dryness under vacuum.

The product (12) was obtained as oil in 99% yield (1.4 g, 3.5 mmol). TLC (20% EtOH / DCM) Rf 0.41. $^1$H-NMR (250 MHz, DMSO-$d_6$) $\delta$ (ppm) 2.05 (m, 1H), 3.05-3.2 (3s and m, 13H), 3.5-3.6 (m, 2H), 3.86 (s, 1H), 4.46 (m, 1H), 5.25 (m, 2H), 5.55 (m, 1H), 6.57 (t, 1H), 8.17 (s, 1H), 8.66 (s, 1H), 8.81 (s, 1H). $^{13}$C-NMR (62 MHz, DMSO-$d_6$) $\delta$ (ppm) 34.2, 34.3 (2 CH$_3$ dmf group), 38.4 (C-2'), 40.3, 40.6 (CH$_3$ dmf group), 62.8 (C-5'), 72.3 (C-3'), 83.7 (C-4'), 87.4 (C-1'), 117.1 (C-5), 149.2 (C-8), 153.6 (C-4'), 155.1 (C-2'), 156.6 (CH, dmf), 157.2 (C-6), 157.4 (CH, dmf). Mass spectra (electrospray +) expected for C$_{16}$H$_{24}$N$_8$O$_4$ 392.4, found 393.2 (M+H), 785.3 (2M+ H).

**Synthesis of 8-amino-N$^2$N$^8$-bis(dimethylaminomethylene)-2'-deoxy-5'-(4,4'-dimethoxytrityl)-guanosine (13).**

19. Dissolve 1.4 g of compound 12 (3.56 mmol) in 50 mL of anhydrous pyridine in a 100-mL round-bottom flask. The solution is stirred under nitrogen atmosphere on a magnetic stirrer and the flask capped with a rubber septum.

20. Add 1.44 g of 4,4'-dimethoxytrityl chloride (4.3 mmol) to the pyridine solution in several small portions.

21. After the last addition, stir for additional 2 hr at room temperature.

22. Stop the reaction by adding 1 ml of MeOH and concentrated the solution to dryness under vacuum.

23. Dissolve the residue in DCM (40 mL) and wash the organic solution with 5% NaHCO$_3$ aqueous solution (40 mL) and a saturated NaCl solution (40 mL) using a separation funnel.

24. Dry the organic solution with anhydrous Na$_2$SO$_4$ (1 g) and concentrated to dryness under vacuum.

25. Pack a silica gel column with 1% triethylamine in DCM. Dissolve the residue in DCM (10 mL) and purify the compound with a 0-10% gradient of MeOH in DCM collecting 10 mL fractions. The desired compound elutes at around 5% MeOH in DCM. Small amounts of DMT-containing products eluted at pure DCM. Combine the fractions containing the desired compound and concentrate to dryness under vacuum.
The product (13) was obtained as white foam in 54% yield (1.35 g, 1.94 mmol). TLC (10% EtOH / DCM) Rf 0.24, (20% EtOH / DCM) Rf 0.64. $^1$H-NMR (250 MHz, CD$_3$CD) $\delta$ (ppm) 2.1 (m, 2H, H-2'), 2.86 (s, 3H, CH$_3$ dmf), 3.06 (s, 3H, CH$_3$ dmf), 3.2 (m, 2H, H-5'), 3.71 and 3.72 (2s, 6H, methoxy DMT), 3.87 (m, 1H, H-4'), 4.50 (m, 1H, H-3'), 5.23 (m, 1H, 3'-OH), 6.47 (t, 1H, H-1'), 6.7-7.3 (m, 13H, DMT), 8.29 (s, 1H, CH dmf), 8.38 (s, 1H, CH dmf), 11.1 (s, 1H, NH). $^{13}$C-NMR (62 MHz, CD$_3$CD) $\delta$ (ppm) 34.2 and 34.5 (2 CH$_3$ dmf), 37.3 (C-2'), 40.2 and 40.7 (2 CH$_3$ dmf), 54.6 (methoxy DMT), 64.8 (C-5'), 72.4 (C-3'), 81.7 (C-4'), 85.0 (C-1'), 85.6 (Cq, DMT), 112.6 (DMT), 116.9 (C-5), 126.3 (DMT), 127.3 (DMT), 127.7 (DMT), 129.7 (DMT), 135.8 (DMT), 144.6 (DMT), 149.5 (C-8), 153.4 (C-4), 154.2 (C-2), 156.1 (CH, dmf), 157.1 (CH dmf and C-6), 158.0 (DMT). Mass spectra (electrospray +) expected for C$_{37}$H$_{42}$N$_8$O$_6$ 694.8, found 695.3 (M+H); 1389.6 (2M+H).

Synthesis of 8-amino-N$_2$N$_8$-bis(dimethylaminomethylidene)-2'-deoxy-5'-(4,4'-dimethoxytrityl)-guanosine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite (14).

1. Dissolve 1.27 g of compound 13 (1.8 mmol) in 20 mL of anhydrous acetonitrile and 1 mL (5.5 mmol) of diisopropylethylamine in a 50-mL round-bottom flask. The solution is stirred under nitrogen atmosphere on a magnetic stirrer and the flask capped with a rubber septum.

2. Place the round-bottom flask in an ice bath and add 0.6 mL (2.73 mmol) of 2-cyanoethoxy-N,N-diisopropylamino chlorophosphine with a syringe.

3. After the addition, stir the solution for 1 hr at room temperature.

4. Concentrate the solution to dryness under vacuum.

5. Dissolve the residue in DCM (40 mL) and wash the organic solution with 5% NaHCO$_3$ aqueous solution (40 mL) using a separation funnel.

6. Dry the organic solution with anhydrous Na$_2$SO$_4$ (1 g) and concentrated to dryness under vacuum.

7. Pack a silica gel column with 1% triethylamine in DCM. Dissolve the residue in DCM (10 mL) and purify the compound eluting with 0-1% MeOH gradient in DCM taking 10 mL fractions. Combine the fractions containing the desired compound and concentrate to dryness under vacuum.

The product (14) was obtained as white foam in 61% yield (1.0 g, 1.11 mmol). TLC (5% MeOH / DCM) Rf 0.17, (10% MeOH / DCM) Rf 0.75. $^1$H-NMR (250 MHz, CD$_3$CD) $\delta$ (ppm) 1.1-1.2 (several d, 12H), 2.2-2.4 (m, 2H, H-2'), 2.6 (m, 2H, -CH$_2$CN), 2.99 (several s, 6H, CH$_3$ dmf), 3.06 (several s, 6H, CH$_3$ dmf), 3.1-3.3 (m, 2H, H-5'), 3.57 (m, 2H, OCH$_2$), 3.73 (several s, 6H, methoxy DMT), 4.1-4.2 (m, 1H, H-4'), 4.9 and 5.0 (2 diastereoisomers, 2m, 1H, H-3'), 6.60 and 6.77 (2 diastereoisomers, 2m, 1H, H-1'), 6.7-7.3 (m, 13H, DMT), 8.31 and 8.34 (2 diastereoisomers, 2s, 1H, CH dmf), 8.56 and
$8.58 \ (2 \ diastereoisomers, \ 2s, \ 1H, \ CH \ dmf)$. $^{31}$P-NMR (101 MHz, $\text{CD}_3$CD) $\delta$ (ppm) 145.7 and 145.5 (two diastereoisomers). Mass spectra (electrospray +) expected for $C_{46}H_{59}N_{10}O_7P$ 895.0, found 895.4 (M+H), 1790.9 (2M+ H).

**BASIC PROTOCOL 4**

**SYNTHESIS, PURIFICATION AND CHARACTERIZATION OF POLYPURINE HAIRPINS**

This protocol describes the synthesis of polypurine hairpins on solid-supports (Figure 4). As described in Figure 1 PPRH have two moieties: the complementary strand and the reversed Hoogsteen strand that can be linked by a polynucleotide (such as tetrathymidine or pentathymidine) loop or an organic linker such as hexaethyleneglycol (HEG) or similar. The connection can be done by any of the ends (configurations A or B from Figure 1). In any case the starting nucleotide is the one that has the 3’-end free and the synthesis on solid-phase is performed by regular protocols (Figure 5). Most of the times PPRHs do not contain 8-aminopurine modifications and they can be easily obtained from commercial sources. On the other hand, it has been described that 8-aminoguanine residues in the Watson-Crick purine strand have a positive effect in the triplex affinity capture of double stranded DNA for detection of DNA methylation (Huertas 2018). This positive effect due to the antiparallel-triplex stabilization of 8-aminoguanine may be useful in future applications of PPRHs and for this reason the synthesis of PPRHs carrying 8-aminoguanines is also included.
Figure 5. Scheme of the steps needed for the assembly of PPRHs A and B (Figure 1) and parallel clamps C and D (Figure 1). $P_1$, $P_2$, ..., $P_n$ purine sequence; $Y_1$, $Y_2$, ..., $Y_n$ pyrimidine sequence. CPG: controlled pore glass supports. DMT: 4,4'-dimethoxytrityl group.

Materials

8-Amino-$N^2$, $N^6$-bis(dimethylaminomethylene)-2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-guanosine-3'-O-(2-cyanoethyl)-$N$, $N$-disopropylphosphoramidite (14 or Berry Associates) (if needed)
5'-O-(4,4'-Dimethoxytrityl)-3'-O-(2-cyanoethyl)-$N$, $N$-disopropyl phosphoramidites (Link Technologies):
$N^2$-Isobutyryl-2'-deoxyguanosine (ibu-dG-CE phosphoramidite)
$N^6$-Benzoyl-2'-deoxyadenosine (Bz-dA-CE phosphoramidite)
Thymidine (dT-CE-phosphoramidite) (if needed for the loop)
(4,4'-Dimethoxytrityl)-hexaethyleneglycol-2-cyanoethyl)-$N$, $N$-disopropyl phosphoramidite (C18 spacer, Link Technologies) (if needed for the loop)
DNA controlled-pore glass (CPG) supports (Link Technologies)
ibu-dG-CPG (Link Technologies)
Bz-dA-CPG (Link Technologies)
Thymidine (dT 3'-CE-phosphoramidite)
Reagents for DNA synthesis (Applied Biosystems, see also unit 3.1 and other units of Chapter 3):
Acetic anhydride in THF/pyridine
16% 1-Methylimidazole in THF
0.02 M Iodine in THF/pyridine/water
1H-Tetrazole in anhydrous acetonitrile
3% Trichloroacetic acid in CH$_2$Cl$_2$
Anhydrous CH$_2$Cl$_2$
Anhydrous acetonitrile
Concentrated ammonia
Illustra™ NAP™-10 column
1M Triethylammonium acetate (TEAA) pH 7.0 (stock solution, unit A.2A.13)

DNA synthesizer (e.g., Applied Biosystems Model 3400)
Argon source
HPLC column Nucleosil 120 C18 (250x8mm)
Heating block
SpeedVac concentrator
Hamilton gas-tight syringe
3-mL glass vials

**Solid-phase Synthesis of PPRH**

1. Dissolve 100 µmol of 14 (if needed) in 1 mL of anhydrous acetonitrile in a 10-mL vial used in the synthesizer capped with a rubber septum using a gas-tight syringe. Load the vial in the synthesized using the change bottle protocol.

2. Load the rest of the phosphoramidites, the rest of the DNA synthesis reagents and a DNA synthesis column with 1 µmol of DNA controlled-pore glass (CPG) support corresponding to the first nucleoside at the 3’-end of the DNA sequence onto the synthesis port of the synthesizer.

3. Perform the automated solid-phase assembly of the oligonucleotides from 3’ to 5’ using either DMT-on or DMT-off protocols.

4. Remove the column from the synthesizer and dry the column with a stream of argon or nitrogen.

5. If the PPRH does not contain 8-aminoguanine, transfer the CPG support to a 3-mL vial, add 1 mL of concentrated aqueous ammonia and incubate the vial at 55 °C overnight. If the PPRH contains 8-aminoguanine residues, use 0.1M DTT (dithiothreitol) or 2-mercaptoethanol solution in concentrated aqueous ammonia (Rieger 1999) and incubate for 24 hrs at 55°C.

**Purification of PPRH**

6. Purify the sample by reversed-phase high performance liquid chromatography (RP-HPLC, Unit 10.5) using Nucleosil 120 C18 column eluted at a flow rate of 3mL/min with a 20 min linear gradient from 0-50% B and 5 min isocratic at 50% B. Solutions were as follows. Solvent A: 5% CH$_3$CN
(Merck) in 100 mM triethylammonium acetate (TEAA) pH 7.0 and solvent B: 70% CH₃CN in 100 mM TEAA pH 7.0.

7. Pool the fractions containing the desired oligonucleotide, concentrate the solution using a SpeedVac concentrator, and desalt by passing through an Illustra™ NAP™-10 column.

8. Analyze the molecular weight by mass spectrometry (MALDI-ToF)

**ALTERNATE PROTOCOL 2**

**SYNTHESIS, PURIFICATION AND CHARACTERIZATION OF PARALLEL OLIGONUCLEOTIDE CLAMPS**

This protocol describes the synthesis of oligonucleotide parallel clamps on solid-supports (Figure 4). As described in Figure 1 parallel clamps have two moieties: the purine complementary strand and the pyrimidine Hoogsteen strand that can be linked by a polynucleotide (such as tetrathymidine or pentathymidine) loop or an organic linker such as hexaethyleneglycol (HEG) or similar. The connection can be done by any of the ends giving rise to two configurations (configuration C with 5′-5′ links or D with 3′-3′ links, Figure 1). Sometimes parallel clamps may contain 8-aminoguanine and/ or 8-aminoadenine residues in the Watson-Crick purine strand for stabilization purposes. The preparation of 5′-5′ hairpins (C configuration) is performed in three parts (Figure 5): First, the preparation of the purine part carrying the modified 8-aminopurines using standard phosphoramidites for the natural bases and the 8-aminopurine phosphoramidites. The phosphoramidites of 8-aminoadenine or 8-aminoguanine are prepared as described above or obtained from commercial sources. After the assembly of the purine part, a hexaethyleneglycol linker or a tetra- or penta thymidine sequence is added using the appropriate phosphoramidite. Finally, the pyrimidine part is assembled using reversed C and T phosphoramidites (Figure 5). For the preparation of 3′-3′ hairpins (D configuration) a similar approach is used (Figure 5). In this case, the pyrimidine part is assembled on solid supports functionalized with reversed C or reversed T (linked to the support through the 5′ end) and reversed phosphoramidites (Figure 6). This is followed by the addition of the hexaethyleneglycol or the addition of the tetra- or pentathymidine sequence. The purine part is the last part to be assembled using standard phosphoramidites for the natural bases and the 8-aminopurine phosphoramidites (Figures 5).


**Figure 6.** Scheme of a 5'-O-reversed phosphoramidite and controlled-pore glass (CPG) solid support functionalized with nucleoside 5'-O-succinate. DMT: 4,4'-dimethoxytrityl group.

**Materials**

8-Amino-N²,N⁶-bis(dimethylaminomethylidene)-2'-deoxy-5'-((4,4'-dimethoxytrityl)-guanosine)-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite (14 or Berry Ass. / Glen Research) (if needed)

8-Amino-N⁶,N⁸-bis(dimethylaminomethylidene)-2'-deoxy-5'-(4,4'-dimethoxytrityl)-adenosine)-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite (7, or Berry Ass./Glen Research) (if needed)

5'-O-(4,4'-Dimethoxytrityl)-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidites (Link Technologies):

- N²-Isobutyl-2'deoxyguanosine (ibu-dG-CE phosphoramidite)
- N⁶-Benzoyl-2'-deoxyadenosine (Bz-dA-CE phosphoramidite)
- Thymidine (dT-CE phosphoramidite) (if needed for the loop)

(4,4'-Dimethoxytrityl)-hexaethyleneglycol-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite (C18 spacer, Link Technologies) (if needed for the loop)

3'-O-(4,4'-Dimethoxytrityl)-5'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidites (Link Technologies):

- N⁴-Benzoyl-2'-deoxycytidine (Bz-dC-CE 5'- phosphoramidite)
- Thymidine (dT 5'-CE phosphoramidite)

DNA controlled-pore glass (CPG) supports (Link Technologies):

- ibu-dG-CPG (Link Technologies)
- Bz-dA-CPG (Link Technologies)
- 5'-Bz-dC-CPG (Link Technologies)
- 5'-dT-CPG (Link Technologies)

Reagents for DNA synthesis (Applied Biosystems, see also unit 3.1 and other units of Chapter 3):

- Acetic anhydride in THF/pyridine
- 16% 1-Methylimidizole in THF
- 0.02 M Iodine in THF/pyridine/water
- 1H-Tetrazole in anhydrous acetonitrile
- 3% Trichloroacetic acid in CH₂Cl₂
- Anhydrous CH₂Cl₂
- Anhydrous acetonitrile
- Concentrated ammonia
- Illustra™ NAP™-10 column
- 1M Triethylammonium acetate (TEAA) pH 7.0 (stock solution, unit A.2A.13)

DNA synthesizer (e.g., Applied Biosystems Model 3400)

- Argon source
- HPLC column Nucleosil 120 C18 (250x8mm)
- Heating block
- SpeedVac concentrator
- Hamilton gas-tight syringe
- 3-mL glass vials
Solid-phase Synthesis of 5’-5’ and 3’-3’ Parallel Clamps

1. Dissolve 100 μmol of 7 and/or 14 (if needed) in 1 mL of anhydrous acetonitrile in a 10-mL vial used in the synthesizer capped with a rubber septum using a gas-tight syringe. Load the vial in the synthesized using the change bottle protocol.

2. Load the rest of the phosphoramidites, the rest of the DNA synthesis reagents and a DNA synthesis column with 1 μmol of DNA controlled-pore glass (CPG) support corresponding to the first nucleoside at the 3’-end of the DNA sequence onto the synthesis port of the synthesizer.

3. Perform the automated solid-phase assembly of the oligonucleotides from 3’ to 5’ using either DMT-on or DMT-off protocols.

4. Remove the column from the synthesizer and dry the column with a stream of argon or nitrogen.

5. If the PPRH does not contain 8-aminoguanine, transfer the CPG support to a 3-mL vial, add 1 mL of concentrated aqueous ammonia and incubate the vial at 55 ºC overnight. If the PPRH contains 8-aminoguanine residues, use 0.1M DTT or mercaptoethanol solution in concentrated aqueous ammonia (Rieger 1999) and incubate for 24 hrs at 55ºC.

Purification of Parallel Clamps

6. Purify the sample by reversed-phase high performance liquid chromatography (RP-HPLC, Unit 10.5) using Nucleosil 120 C18 column eluted at a flow rate of 3mL/min with a 20 min linear gradient from 0-50% B and 5 min isocratic at 50% B. Solutions were as follows. Solvent A: 5% CH₃CN (Merck) in 100 mM triethylammonium acetate (TEAA) pH 7.0 and solvent B: 70% CH₃CN in 100 mM TEAA pH 7.0.

7. Pool the fractions containing the desired oligonucleotide, concentrate the solution using a SpeedVac concentrator, and desalt by passing through an Illustra™ NAP™-10 column.

8. Analyze the molecular weight by mass spectrometry (MALDI-ToF)

BASIC PROTOCOL 5,

USE OF CLAMPS OR POLYPURINE HAIRPINS IN GENE SILENCING EXPERIMENTS

This protocol describes the preparation of PPRHs for gene silencing purposes. The design of the different types of PPRHs is already described in section 1. Here, we detail the steps associated to the dissolution of the synthesized PPRH in an aqueous buffer and the formation of a complex with a cationic liposome to transfect the
PPRHs into the cells to achieve the silencing of the selected gene. The PPRH DNA
which is negatively charged interacts with the positive charges carried by the
cationic liposome, here illustrated by DOTAP, and after a brief incubation the mix is
added to the cells. An important factor is the ratio between the concentrations of
PPRH DNA and that of the liposome, which for PPRHs is around 1/100
(DNA:liposome). The intracellular delivery can be easily followed visually with
Fluorescent PPRHs (F-PPRH), which are obtained commercially (e.g. Sigma/Merck)
using either a fluorescence microscope or a flow cytometer at different times after
the beginning of the incubation. Depending of the parameter that needs to be
determined, different periods of times are chosen for stopping the incubation or
taking samples. The quantification of the remaining mRNA or protein compared to
the control cells are carried out by optimized techniques of RT-qPCR or Western blot
analysis which has been described thoroughly elsewhere (Rodriguez, 2013).

Materials
Polypurine reverse Hoogsteen Hairpins (PPRHs)
TE (Tris/EDTA) buffer, pH 8.0 (see appendices unit A.2A.13)
DOTAP, \(N-[1-(2,3\text{-dioneolloyxy})propyl]-N,N,N\text{-trimethylammonium methylsulphate}
(1 mg/ml) (from Biontex ) (C_{43}H_{83}NO_{8}S, MW: 774.21)
Culture medium for eukaryotic cells e.g. DMEM, RPMI, F12
Fetal Calf Serum
Automatic pipettes to measure between 1µl and 1000µl
Glass pipettes to measure from 1 ml to 10 ml
Culture dishes of 100 mm diameter for cell maintenance
Culture dishes of 35 mm diameter for cell incubation with the PPRHs

Preparation of PPRHs for gene silencing purposes

1. After designing the different PPRHs, either template- or coding-, against a
determined gene, proceed to synthesize them as non-modified
oligonucleotide which can be also obtained from an oligonucleotide synthesis
service (e.g. Sigma/Merck) and can be ordered at a scale of 0.05 micromol
using desalting as purification method. 8-aminoquinine modification could
offer an extra binding capability although they are only commercially
available by specialized companies (e.g. Bio-Synthesis, Inc. Lewisville, Texas,
USA) or they have to be synthesized using BP3 and BP4. In general, the
range of length of PPRHs lie between 20 and 30 nucleotides, aiming if at all
possible, for a length of 25 nt that confers high specificity.

2. Resuspend the powder containing each PPRH to a final concentration in the
tube of 100 µM (stock solution) using sterilized TE (Tris-EDTA, 10/1mM, pH
8.0) buffer as solvent.

3. Prepare a working solution of 10µM of PPRH, by making a dilution 1/10 from
the 100 µM stock solution.
4. Calculate the number of µL of the working solution to attain a final concentration in the cell medium of 100 nM; e.g. 10 µL for 1 ml of incubation medium, or 20 µL for 2 ml of incubation medium.

5. Mix 10 µL of 10 µM PPRH with 7.75 µL of cationic liposome DOTAP and incubation medium without Fetal Calf Serum (FCS) up to 200 µL and incubate in an Eppendorf for 20 min at room temperature.

6. Add the 200 µL mixture containing the PPRH/DOTAP to 800 µL of fresh medium with 10% FCS recently added to the incubation dish including the desired number of cells (1,000, 3,000, 10,000, 30,000, or 60,000) depending on the parameter to be determined, in a 35 mm diameter plate. This procedure will deliver a final concentration of 100 nM of the oligonucleotide with 10 µM DOTAP (ratio 1/100 DNA: liposome) when incubating the cells in 1 ml of medium. Scale the volume as needed. A wide range of cells lines have been transfected with PPRHs, such as human SKBR3, MCF7 and MDA-MB-468 from Breast cancer, PC3 prostate cancer, MiaPaCa from pancreas, HT29 and HCT116 colon cancer, monocytes THP-1, HEK293 and 786-O from kidney, K562 and Jurkat hematopoietic cells, HepG2, and HeLa cells, HUVEC primary cells, and also CHO K1 from Hamster.

7. Incubate the PPRHs with the cells for different periods of time, depending on the parameter to be determined, e.g. 24 h for cellular uptake; 24-48h for mRNA; 48-72h for protein; 3-5 days for cell viability.

**BASIC PROTOCOL 6**

**GOLD SURFACE FUNCTIONALIZATION WITH OLGONUCLEOTIDE CLAMPS FOR SURFACE PLASMON RESONANCE.**

Surface plasmon resonance (SPR) is one of the most commonly used biosensors for nucleic acid detection. They detect biological interaction by measuring the variations of the optical light and consist of an optical system for excitation, the plasmonic transducer that is a thin film of gold that incorporates the biomolecular recognition element on its surface and finally a fluidic system for sample confinement at the sensing surface and flow delivery system for sample injection and delivery. For SPR for measurements of biological interest refer to Unit 20.4, Current protocols in Molecular Biology and for overview of Biacore systems and their applications and to Unit 19.13, Current Protocols in Protein Science. This protocol describes the biofunctionalization of oligonucleotide clamps on gold surfaces of a SPR biosensor and their hybridization with the target sequence. The SPR biosensor employed (Carrascosa, 2012; Aviñó, 2016; Huertas, 2018) is an integrated platform based on the Kretschmann configuration with a real-time monitoring of the intensity of the reflected light at a fixed angle of incidence. The synthesis of the probes includes the clamp sequence followed by a poly-thymidine spacer and a thiol group on the 5’-end. The spacer moves the clamp away from the surface and facilitates the accessibility of the clamp to the target sequence. Immobilization of thiolated probes can be generated ex-situ or in-situ the sensor
apparatus. For the optimization of the clamp coverage and surface properties a horizontal spacer of 6-mercaptohexanol (MCH) or thiolated oligoethyleneglycol are commonly used. After immobilization of the probe, the addition of the target capture produces an increase in the refractive index of signal that is directly related to its concentration in the analyzed sample. Subsequent experiments consisted in dehybridization of the target with a regeneration solution, liberating the biosensor surface for further analysis with the same sensor.

**Materials**

Sensor chips made of a glass surface (10 × 10 × 0.3 mm) coated with 2 nm of chromium and 45 nm of gold (SSens, Enschede –The Netherlands)

Oligonucleotides clamps with either 3'- or 5'-thiol group (see UNIT S 4.2 and 4.5)

Acetone

Ethanol

Formamide

Buffer solutions were prepared by using milliQ water incubated overnight with 2% diethylpyrocarbonate (DEPC) and autoclaved at 121ºC for 1 hour

5x Sodium chloride citrate (5XSCC) buffer: 750 mM NaCl, 75mM sodium citrate, pH 7

Phosphate buffer saline (PBS) 50 mM: 50 mM phosphate buffer, 0.75 mM NaCl, pH 7

Thiolated methyl-PEG (SH-PEG-CH₃, MW: 2000 g/mol)

6-Mercapto-1-hexanol (MCH)

Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich)

UV/O₃ Generator (Bioforce Nanosciences, USA)

SPR instrument (Huertas, 2016)

**Gold Surface cleaning**

1. Clean the gold surface by sonication cycles (1 min) with solvents of increasing polarity (i.e. acetone, ethanol and MilliQ H₂O) previously heated up to their boiling point.

2. Dry under nitrogen flux and place the sensing surface in an UV/O₃ generator (Bioforce Nanosciences, USA) for 20 min. Rinse the sensor chip with ethanol and water and dry.

**Clamp immobilization on gold chips and target hybridization**

3. Incubate ex-situ 100 µl of thiolated DNA probe with thiolated methyl-PEG or MCH (1:1) at total concentrations of 1µM in 50 mM PBS buffer (pH 7) in a humid chamber overnight at room temperature. Rinse the gold sensing surface with water and dry the chip prior setting up in the SPR instrument.

4. Inject the sample containing the target sequence dissolved in 5XSSC buffer to hybridize (1-100 µM) to hybridize with the immobilized clamp probe.

5. Measure the binding curves.
Surface regeneration

   6. After the measure the surface is regenerated by washing with 20% formamide in 5X SSC buffer. Alternatively, a solution of 20 mM NaOH may be used to disrupt target-probe interaction.

REAGENTS AND SOLUTIONS

TEAA buffer (triethylammonium acetate), 1M, pH 7.0 (stock solution) see Appendices unit A.2A.13

SSC (Sodium chloride/sodium citrate), 20x (stock solution) see Appendices unit A.2A.12

PBS (phosphate buffered saline), see Appendices unit A.2A.8

TE (Tris/EDTA) buffer, pH 8.0, see Appendices unit A.2A.13

COMMENTARY

Background Information

Polypurine-polypyrimidine sequences are able to form a non-canonical structure constituted by three strands (Triplex). The third strand can be either a polypyrimidine (Hoogsteen strand) or a polypurine (reversed Hoogsteen strand) sequence forming parallel or antiparallel triplexes (see Figure 7). In both cases specific hydrogen bonds between the Watson-Crick purine strand and the Hoogsteen or reversed-Hoogsteen strand are formed. The triplex structure anticipates the potential use of a triplex-forming oligonucleotide (TFO) to bind to double stranded genomic DNA for therapeutic (antigene strategy) or for diagnostic (triplex affinity capture) purposes (Duca, 2008). The design of novel oligonucleotide derivatives for antigen inhibition of gene expression resulted in the discovery of peptide nucleic acids (PNA) (Hyrup, 1996) that have a strong affinity to DNA to form duplex PNA-DNA molecules but also very stable PNA-DNA-PNA triplexes (for RNA binding see Unit 4.60). The strong stability of PNA-DNA-PNA triplexes lead to the discovery of bis-PNA clamps (Nielsen, 2010) constituted by the Watson-Crick polypyrimidine sequences linked to the polypyrimidine Hoogsteen strand (Figure 6). These PNA clamps bind the target polypurine sequences in both single-stranded mRNA and double-stranded genomic DNA by strand displacement (Nielsen, 2010). The same strategy was described for the binding of polypyrimidine sequences but in this case the purine Watson-Crick strand is linked with the Hoogsteen or reversed Hoogsteen strand to form the so-called parallel clamps (Kandimalla, 1996; Aviño, 2002) or polypurine reversed hairpin (PPRH) (Coma, 2005) (Figure 7). The binding properties of the parallel clamps depend on the pH being more stable at slightly acidic pH as Hoogsteen cytosines are protonated in the parallel triplex (Robles, 2002). The triplex stabilization properties of 8-aminopurine derivatives modulate this pH dependence observing parallel triplex at neutral pH (Aviño, 2002; Güimil-
The stabilization of the triplex at neutral pH by a combined effect of the gain in one Hoogsteen purine-pyrimidine H-bond and the ability of the amino group to be integrated into the "spine of hydration" located in the minor-Major groove of the triplex structure (Aviñó, 2002). Moreover 8-aminoguanine also stabilizes antiparallel triplex (Aviñó, 2003). In order to use the beneficial triplex stabilization properties of 8-aminopurine, capture probes were designed as parallel clamps to bind to single-stranded polypurimidine sequences. Examples of the use of these clamps are the isolation of polypurimidine RNA sequences of biological interest such as bacterial RNA (Carrascosa, 2012) and miRNA (Aviñó, 2016). The same polypurimidine sequences can be targeted by antiparallel hairpins. In this case the hairpins are formed by two antiparallel purine strands in which the stability of triplex does not depend on pH. The Polypurine Reverse Hoogsteen (PPRH) technology is used for targeting single and double stranded nucleic acid sequences as PPRHs induce strand displacement (Coma, 2005). PPRHs work at nanomolar range, like siRNAs, and at a lower concentration than that needed for antisense ODNs or TFOs. However, the stability of PPRHs is higher than that of siRNAs, as evidenced by their longer half-life. In addition, PPRHs are target-specific and non-immunogenic (Villalobos, 2014). They represent a tool to validate genes in proliferation and cancer (Villalobos, 2015) and can be used to target genes related to resistance (de Almagro, 2009), as chemosensitizers (de Almagro, 2011), and in immunotherapy (Bener, 2016; Medina, 2018). Several examples of PPRHs for the inhibition of gene expression have been described (Rodríguez, 2013; Rodríguez, 2015; Solé, 2017; Ciudad, 2017; Felix, 2018) as well as for the capture of miRNA (Ribas, 2018) and double-stranded DNA for the analysis of DNA methylation status (Huertas, 2018).

The present unit describes the design, synthesis and properties of the parallel clamps and PPRHs. In addition to the hairpin geometry described, other oligonucleotide configurations have been described (Figure 7). These include cyclic oligonucleotides (Grimau, 2005), wedged (Rodriguez, 2015) and tail-clamps (Nadal, 2005; Nadal, 2006). In all these cases a higher affinity polypyrindine target and an increase in specificity has been observed. The methodology described in this unit is based on the sequential addition of the appropriate phosphoramidites. In the case of the synthesis of parallel clamps the synthetic protocol needs to assemble one of the strands in the reversed (5'-3') direction. The strategy for the synthesis of these clamps implies the preparation of the polypurimidine Hoogsteen strand using 5'-O-phosphoramidites. Alternatively, it has been described the use of asymmetric branching units. These are diol derivatives protected with fluorenyloxycarbonyl (Fmoc) and DMT groups. These derivatives allow the synthesis of one of the strands elongating the DMT-protected part, and the assembly of the second strand in the normal 3'-5' direction avoiding the need of reversed 5'-phosphoramidites (Aviñó, 2004). Another alternative method for the preparation of parallel clamps avoiding the use of 5'-phosphoramidite is the preparation of both polypurine and polypurimidine strands in a separate support and link them using Cu(I)-catalyzed cycloaddition reaction (Click reaction) (Alvira, 2007).
An important issue in the design of clamps and PPRHs is the presence of interruptions in the target polypurine sequence. In parallel clamps it has been suggested the use of the complementary base in the Watson–Crick strand and the use of C in the Hoogsteen strand for G interruptions and the use of G in A interruptions (Aviñó, 2002). In PPRH it has been suggested to add an A in the Watson–Crick position and another A in the Hoogsteen position (de Almagro, 2009). However, in later studies it was demonstrated that maintaining the pyrimidine interruption within the PPRH sequence conferred higher binding affinity and silencing effectiveness to the hairpin (Rodríguez, 2015). In Figure 8 it is shown the sequence and the mirror repeat structure of a PPRH designed against a polypurine track present in the promoter of the human \textit{survivin} gene (BIRC5). This polypurine sequence contains 3 pyrimidine interruptions in each strand.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target gene</th>
<th>Sequence (5‘-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HpsPr-C-WT</td>
<td>Survivin (BIRC5)</td>
<td>AGGGGAGGGATGGAGTGCAAGTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGGGGAGGGATGGAGTGCAAGTT</td>
</tr>
</tbody>
</table>

\textbf{Figure 7.} Triplex forming oligonucleotides (TFO), clamps, PPRH, tail-clamps, cyclic and wedged oligonucleotides.

\textbf{Figure 8.} Example of the sequence and the mirror repeat structure of a PPRH designed against a polypurine track present in the promoter of the human
survivin gene (BIRC5). This polypurine sequence contains 3 pyrimidine interruptions in each strand.

PPRHs are usually G-rich sequences. A concern on G-rich sequences is the possibility to have competing G-quadruplex structures that may lower the efficacy of triplex formation. This subject was studied in detail by Solé et al. 2017 demonstrating that PPRHs can form triplexes with their target sequences even under conditions where they fold into G-quadruplexes.

**Critical Parameters and Troubleshooting**

Careful attention to the details of synthetic procedures is advised. Familiarity with routine organic synthesis protocols and solid phase oligonucleotide synthesis is recommended as they involve the use of flammable solvents and toxic chemicals. Adherence to good laboratory safety practices is required. All reactions should be conducted in ventilated fume hoods. The use of personal protective equipment including gloves, safety goggles, and a laboratory coat is required. Special attention is needed for catalytic hydrogenation and Raney™-Nickel reduction reactions as well as the manipulation of azides, hydrazine and bromine. Anhydrous solvents are required in most organic reactions. It is recommended to use good quality and anhydrous solvents in Sure/Seal bottles and to acquire the reagents for oligonucleotide synthesis in specialized DNA synthesis distributors.

When using the dmf-protected 8-aminoguanine phosphoramidite, it is important to add 0.1 M 2-mercaptoethanol or DTT in the concentrated ammonia solution used in the final deprotection and to extent the deprotection time to 24 hrs to achieve completed removal of the dmf group (Rieger, 1999).

**Understanding Results**

The protocols described in this unit provide details on the preparation of the modified and unmodified parallel clamps and PPRHs. The assembly of the oligonucleotides proceeds in a similar manner than the assembly of standard oligonucleotides of similar sizes (20-50 bases long). There is no need to change the protocols for the purification of the oligonucleotides as they behave as standard oligonucleotides. In this unit we described the purification by reverse-phase HPLC, but ion exchange chromatography and gel electrophoresis as well as simple desalting over Sephadex™ columns can also be used as alternative purification methods. Yields are similar to the one obtained for standard oligonucleotides (around 30-60%) after HPLC purification. Sometimes parallel clamps may be obtained is slightly lower yields as reversed phosphoramidites may have lower coupling yields. Characterization of oligonucleotides is done by analysis of the homogeneity (single peak in HPLC or single band on gel electrophoresis) and determination of the molecular mass by MALDI mass spectrometry. The binding properties of the clamps and PPRHs can be measured by hybridization with the target polypyrimidine strand and performing denaturation studies followed by UV spectrophotometry or circular dichroism (CD). Examples of these measures can be found in the bibliography (Aviñó, 2002, see also Unit 7.12). Usually two transitions can be observed on triplexes formed by a single oligonucleotide bound to a double-
stranded DNA. The first transition is the dissociation of the triplex-forming strand and the second transition is the denaturation of the duplex to random coil. When the triplex-forming strand is linked to the purine Watson-Crick strand (parallel clamps and PPRHs) the denaturation profiles shown only a single transition that corresponds to the triplex denaturation to random coil (Aviñó, 2002; Coma, 2005). In order to demonstrate triplex formation a control clamp with scrambled Hoogsteen or reversed Hoogsteen strand can be made and confirm that the melting temperature of the control sequence is lower than the melting temperature of the triplex (Aviñó, 2002; Coma, 2005). In addition, the formation of the parallel triplex can be demonstrated by the appearance of a band at 210 nm in the CD spectra (Figure 9) and a change in the UV spectra at 295 nm (Nadal, 2006).

Figure 9. CD spectra of A) a parallel clamp (B22G, 3′-AGAGGAGGAAG-5′-(EG)_6-5′-CTTCCTCCTCT-3′) and B) a control clamp (B22Acontrol, 3′-AGAGGAGGAAG-5′-(EG)_6-5′-TTTTTCCCCC-3′) alone and bound to their target polypyrimidine sequence (S11WC, 3′-CTTCCTCCTCT-5′). Upon binding to their target, the parallel clamp generates a negative band around 210 nm) while the control sequence do not generate this band (Aviñó 2002). Buffer conditions 50 mM NaCl, 10 mM MgCl2, 0.1 M sodium phosphate pH 6. Adapted with permission from Aviñó, 2012. Copyright 2012, Oxford University Press.

Time Considerations

With prior experience in basic organic techniques each 8-aminopurine phosphoramidite can be prepared in 1 month. Oligonucleotides can be prepared in one working day. HPLC purification will need one day more. Parallel clamps cannot be easily obtained from commercially sources and there is a need for reversed 5′-O-phosphoramidites and 5′-linked supports. Unmodified PPRH are easier to prepare and can be obtained from any oligonucleotide synthesis service company. As the 8-aminopurine phosphoramidites can be obtained from commercial sources (Berry
associates), PPRH carrying 8-aminoguanine can be obtained from specialized oligonucleotide synthesis services.

ACKNOWLEDGEMENTS

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LITERATURE CITED


Kandimalla, E.R. and Agrawal, S. 1996. Hoogsteen DNA duplexes of 3’-3’ and 5’-5’-linked oligonucleotides and triplex formation with RNA and DNA pyrimidin...


KEY REFERENCES


INTERNET RESOURCES

Triplex-Forming Oligonucleotide Target Sequence Search software: available at http://utw10685.utweb.utexas.edu/tfo/

Triplex target site Mapping and Integration software: available at http://ttsmi.bii.a-star.edu.sg
