

Manual PNA Fmoc-synthesis

Preparation of Lysine Resin. This procedure adds a lysine residue to an MBHA resin for PNA synthesis. The lysine will become the C-terminus on all PNAs synthesized on this resin. The side chain amine of the lysine is protected with a Boc group that is not removed until the PNA is cleaved from the support.

1. Prepare the following solutions:

(A) 0.200 M Fmoc-Lys(Boc) in NMP:

(B) 0.202 M HATU in NMP: Weigh out 0.768 g HATU and dissolve in NMP to give 10 mL. This reagent is also used for PNA synthesis so some of it may already be available.

(C) 0.500 M DIEA in Pyridine: Mix 8.7 mL DIEA with NMP to give 100 mL total volume. This reagent is also used for PNA synthesis so some of it may already be available.

(D) 5% DIEA in DCM: Mix 5 mL DIEA with 95 mL DCM. This reagent is also used for PNA synthesis so some of it may already be available.

(E) Ac₂O/NMP/Pyridine: Mix 2 mL Ac₂O, 4 mL NMP and 4 mL Pyridine.

Swelling the resin. Soaking the Rink amide MBHA resin in DCM causes the polymer to swell, increasing the accessibility of reagents to the functional groups on the support.

2. Weigh out 1.0g of resin and place in a reactor

3. Add 10 mL DCM and agitate for 1 hour.

4. Drain the solvent, then wash the resin twice with DCM, once with 5% DIEA in DCM, and twice more with DCM. Add enough of each solvent/solution to disperse the resin, and mix the resin with a pipet prior to draining. After the last washing, open the reactor to vacuum only long enough to drain the solvent. (If the resin is completely dried, it will no longer be swelled).

Solutions for PNA synthesis

(1) **Monomer Solutions.** Monomers are dissolved in NMP at a concentration of 0.4M.

A (725 g/mol)

G (741.8 g/mol)

T (506 g/mol)

C (701 g/mol)

- *Prepare the solutions in microcentrifuge tubes or small glass vials. Calculate how much of each monomer you will need based on the PNA sequence and weigh out only what you will need. Some heating will be necessary to get the solids to completely dissolve.*

(2) **20 % piperidine in DMF.** This solution is used to remove Fmoc-protecting groups from the PNA N-terminus.

- *Mix 20 mL piperidine with 80 mL of DMF (dimethylformamide) and store in an amber bottle in the hood.*

(3) **0.8 M base solution.** This solution provides the base that is necessary for activation on the PNA monomer with HATU prior to coupling.

- *Use Base Solution purchased from Applied Biosystems.*

(4) **0.2M HATU in DMF.** This solution is used to generate the activated PNA monomer by esterifying the carboxyl group of the monomer with a hydroxybenzotriazolyl group. Displacement of this group by the N-terminus of the PNA is facile.

- *Dissolve 360 mg HATU in DMF (dimethylformamide) to give a total volume of 5 mL. Store in a glass vial wrapped in aluminum foil in the hood.*
- *Use HATU Solution purchased from Applied Biosystems*

(5) **Ac₂O/NMP/Pyridine (1:25:25).** This solution is used to cap any unreacted amino groups as acetamides, preventing elongation of failure sequences.

- *Mix 2 mL Ac₂O (acetic anhydride) with 50 mL each of NMP and pyridine. Store in an amber bottle in the hood.*

Coupling the lysine residue to the resin. The carboxylic acid group of the protected lysine must be activated prior to coupling to the resin. The amino acid is mixed with HATU to activate the carboxylic acid and then added to the resin.

5. Prepare the following solutions:

(F) 0.45 mL Lysine solution (A) + 0.46 mL DIEA solution (C) + 1.59 mL NMP

(G) 0.55 mL HATU solution (B) + 1.95 mL NMP

6. Mix solutions (F) and (G) for one min, then add to the resin.

7. Agitate the resin for one hour.

8. Wash the resin twice with DMF, four times with DCM, once with 5% DIEA in DCM (agitate for 30 seconds during this wash) and four more times with DCM.

Capping Unreacted Sites. It is critical to cap any unreacted sites on the resin, otherwise, PNAs will be synthesized that lack the C-terminal lysine residue. The capping reaction is done by adding acetic anhydride to the resin, resulting in conversion of all unreacted sites to the acetamides.

9. Add enough of the capping solution (E) to disperse the resin, then agitate for 1.5 hours

10. Wash the resin twice with DCM.

11. Remove a small portion of the resin and add to a microcentrifuge tube.

12. Add two drops each of Kaiser solutions (A) and (B).

13. Heat to 100 °C for two minutes

14. If the test is negative (yellow), wash the resin twice more with DCM and then allow to dry by opening to vacuum for 30-60 min.

15. If the test is positive (blue), repeat steps 9-11 and perform another Kaiser test.

16. After the resin is dry, place into a vial, cover with parafilm and store in a dessicator.

Kaiser Test. The “Kaiser Test” is a colorimetric test for the presence of amino groups; we use it to make sure that each coupling step in PNA synthesis goes to completion. It is based on the reaction of ninhydrin with amino groups to form a blue adduct. Therefore, an incomplete coupling cycle will lead to a positive Kaiser test, demonstrated by the development of a blue color, while coupling to completion will yield a negative (yellow) test.

Prepare the following solutions:

1. Dissolve 8 g phenol in 2 mL absolute ethanol. Warming of the solution will be required to completely dissolve the phenol. (Note: It is easiest to weigh out phenol when it is still cold. Still, the large crystals make it difficult to weigh out exactly 8 g. Try to get close and adjust the volume of ethanol accordingly.)
2. Dissolve 13 mg KCN in 20 mL water. (Transfer the KCN into a tared vial in the hood, then carry to the balance with the cap on!) Check in the hood before preparing this solution as there might already be some available.
3. Dilute 20 μL of aqueous KCN solution with 980 μL pyridine, then add to 100 μL of the phenol/ethanol solution. This is the Kaiser (A) solution.
4. Dissolve 1.0 g ninhydrin in 20 mL absolute ethanol. This is the Kaiser (B) solution
5. Store Kaiser (A) and (B) solutions in amber dripper bottles.

Once the solutions are prepared, perform the following procedure:

6. Set a heating block to 100 $^{\circ}\text{C}$
7. Remove a small amount of resin from the reactor and place in a microcentrifuge tube.
8. Add two drops each of Kaiser (A) and (B) solutions.
9. Mix by tapping the tube.
10. Place in the heat block for two minutes.
11. Remove and observe the color: Blue = positive (*i.e.* amino groups present); Yellow = negative (*i.e.* no amino groups present).

Cleavage of PNA oligomers from MBHA Resins.

This procedure uses a combination of trifluoroacetic acid (TFA) and m-cresol to cleave the PNA oligomers from its solid support. The procedure also removed the Boc protecting group from the side chains.

1. Prepare a solution of TFA:m-cresol (4:1) (*800 μ L TFA with 200 μ L m-cresol*)
2. Add the solution to the resin and let stand for 2 hours.
3. Elute liquid into conical glass centrifuge tube. Use pipet bulb to push liquid through frit.
4. Repeat steps 2-3, combining the eluted fractions.
5. Add at least a 5-fold excess of dry ether.
6. Mix and place on dry ice for 10 min.
7. Centrifuge in hood for 5 min.
8. Carefully pour off supernatant.
9. Add dry ether to 10 mL and mix to resuspend pellet.
10. Place on dry ice for 5 min.
11. Centrifuge in hood for 5 min.
12. Carefully pour off supernatant.
13. Repeat steps 9-12 four times with the following dry ice incubation times: 5 min, 2 min, 0 min, 0min.
14. Dry PNA with gentle stream of N₂.
15. Store dried PNA in refrigerator until ready for HPLC purification.