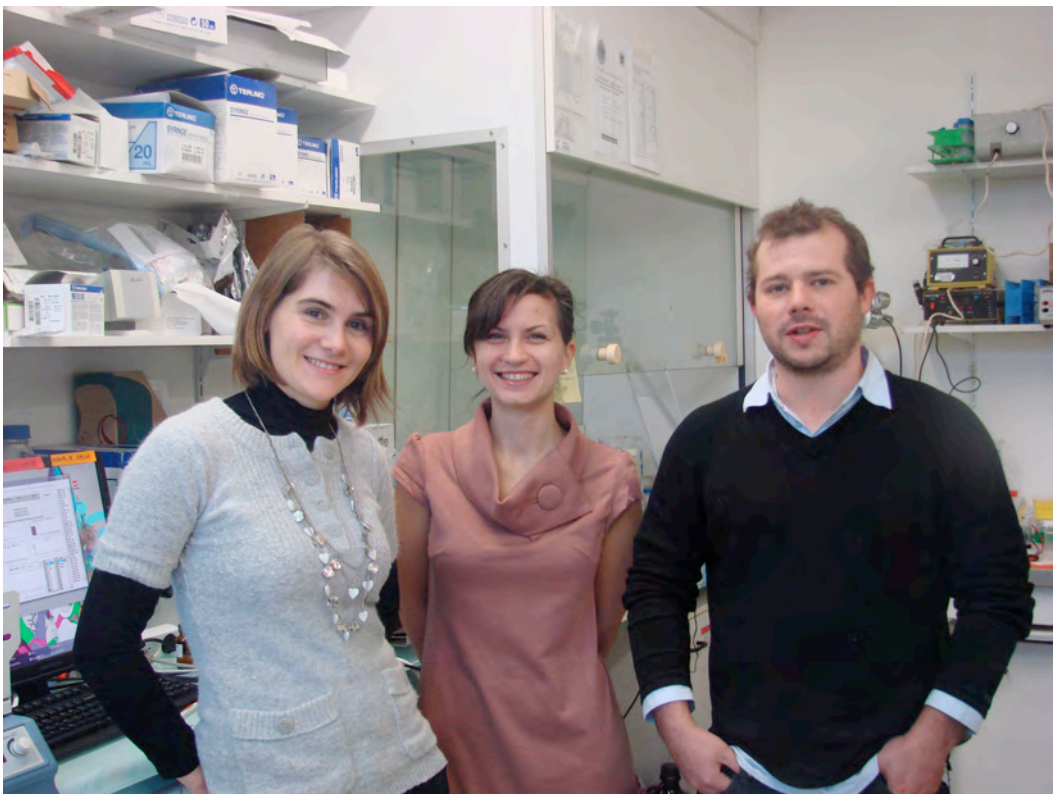
LNA containing probe synthesis (Prot53)



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Introduction

A successful PICh experiment requires the use of special probes which bind to the locus of interest with high stability and specificity. PICh probes are not commercially available, or may require custom synthesis by Exiqon, the inventor and owner of the LNA (Locked Nucleic acid) patent. Conventional DNA based FISH probes, such as the ones obtained by nick-translation, may not be suitable for PICh, as we think (and know) their binding to the target is not stable enough to maintain sufficient material bound during the course of the purification. PICh probes typically contain a mixture of DNA and LNA residues, a very long spacer and

desthiobiotin. The positions and content of LNA residues in the probe do not obey specific rules, however we are always designing our probes as follows:

-40-50% LNA content scattered along the length of the sequence (no 'blocks of LNA')

-25-35 nucleotides long with a Tm generally higher than 78°C.

Following these rules is not a guarantee PICh will work, since other critical parameters contribute to a successful purification. This design is however the best we found so far.

In the original paper, we were using a 108 atoms long spacer together with desthiobiotin. We are now using a different strategy, which works with the same efficiency: we are coupling 4 times a Spacer 18 and a Desthibiotin Tri-Ethylene-Glycol, making a ~100atoms long distance between the DSB and the oligo. These extremely long spacers are used to prevent steric hindrance issues that could potentially be encountered upon immobilization of chromatin on beads.

Here we provide a synthesis protocol for making PICh probes in the lab using conventional nucleic acid synthesis methods.

Modern nucleic acid synthesizers allow using different types of chemistries, but phosphoramidite chemistry is the most popular and the easiest one to achieve efficient synthesis. Phosphoramidite monomers are stable and allow generating specific ribo- and deoxy-ribo oligonucleotides with almost any kind of modification of interest, provided they are available as amidites precursors (fluorescent label, small tags, spacers, etc…). The nucleotide chain grows from an initial protected nucleotide attached via its 3' end to a solid glass support (Controlled Pore Glass or CPG) in a column. Chemical synthesis of oligonucleotides always runs from the 3' toward the 5' end of the oligo.

Similar to a PCR reaction where each cycle is divided into Annealing/Polymerization/Denaturation steps, oligo synthesis is a step-wise chemical reaction where an amidite is added 5' of the previous nucleotide. In these reactions, spacers and biotin are considered similar to nucleotide since they are also used as phosphoramidites precursors. To obtain desired oligonucleotides, monomers are added following a stepwise chemical reaction called a cycle. A synthesis cycle consists of 5 steps (figure 1):

- Deblocking: The cycle begins with the removal of the acid-labile 4,4' DimethoxyTrityl (DMT) group from the 5' hydroxyl end.
- Coupling: The next protected phosphoramidite (with a 5' DMT) is delivered to the reaction column in the presence of tetrazole which activates the coupling reaction.
- Capping: Unreacted 5' hydroxyl ends, not engaged in a reaction with the incoming monomer must be permanently blocked. This step is necessary to minimize truncated products, to increase the synthesis yield and to facilitate the purification

process. Tetrahydrofuran (THF) with Methylimidazole and Pyridine are used to acetylate and block the unextended 5'hydroxyls.

- Oxidation: Phosphite triester are oxidized to yield the phosphor diester bond linking the two nucleosides.The cycle of monomer addition is then completed and other cycle begins with the removal of the 5'DMT from the previously added monomer.
- Capping: An extra capping step is necessary to ensure no hydroxyl group is left.

We are using The Expedite 8909 Nucleic Acid Synthesis System (Note1) operated by a PC computer running the "Expedite Workstation" software version 2.5, 1999 from Perseptive Biosystems. This system, although old and not sold anymore as a brand new instrument, can be obtained as refurbished equipment from several synthesizer companies. The 8909 has very low reagent consumption, making this a cost effective machine for PICh probe synthesis.

Two columns can operate independently allowing the simultaneous synthesis of two different sequences. PICh probes may also be made using other systems. Please refer to the manufacturer's instruction for adapting the protocol below to other machines.

Procedure

I. PICh probe synthesis

Starting up the Expedite 8909, preparation of the monomers

1. Before and after each run, the Expedite lines and valves have to be washed and dryed for optimal performance. We perform this step with anhydrous acetonitrile (ACN) in each line by using the "Prime" menu and "Prime all" function of the machine 3 times boths columns. ACN bottles are connected to each position on the synthesizer.

2. During the start-up, monomers can be resuspended slowly in appropriate buffers Under dry argon atmosphere. Use plastic syringes and needles to transfer ACN without opening the septum from bottles

DNA-A, T, C, G amidites, LNA-A, G, T amidites and spacers, desthiobiotin amidites are used as 0.07M solutions in anhydrous ACN.

For LNA-mC, the amidite is first dissolved in anhydrous THF. When a clear solution is obtained, anhydrous ACN is added to a final THF/ACN ratio of 25:75 (0.07M final). (Note 2)(Table 1).

3. When the machine is done pumping ACN into tubings, remove ACN bottles from reagents positions and place capping solutions bottles (CAP A and CAP B), oxidizer, activator and Wash bottles with anhydrous acetonitrile to the appropriate locations (Note 3)(figure 2).

4. Fill lines with reagents using “Prime” menu and “prime reagents” functions of the machine.

5. When monomers are completely dissolved, connect bottles to the machine at the appropriate position (see below).

6. Fill lines with each monomers using “Prime” menu and “prime monomers” functions of the machine.

Expedite 8909 is now ready for synthesis.

Sequences, protocols and synthesis

1. Prepacked columns contain a matrix called controlled pore glass (CPG). They are silica beads with a 3'-nucleoside attached. There are pores in these beads and the choice of poresize s will depend on the size of the oligo to synthesize. The CPG pore size we routinely use is 1000 Å to accommodate for the very long spacers. Remember synthesis proceeds from 3’ to 5’ and CPG columns have the DNA residue which will be the 3’ residue of your probe, Design PICh probe so that it ends in 3’ with a DNA. The scale of synthesis is usually 1 micromole. A successful synthesis typically yields 50-100 nmol of pure PICh probe. So if your probe finishes with a C, install a CPG-C, 1 micromol, 1000 Å column on the synthesizer. Probes terminating with a 3’ LNA can be made but require the use of universal CPGs (any amidite can be coupled to the support, including an LNA amidite), which are more expensive.

2. , Enter the sequence of the PICh probe in the Expedite Workstation software.To each monomer position on the machine corresponds a letter or a number code which the software uses. The Expedite 8909 has 9 amidite positions named “A”, “T”, “C”, “G”, “5”, “6”, “7”, “8”, “9”. Synthesizing a PICh probe containing 4 different DNA, 4 different LNA, spacers and DSB-TEG would require 10 different amidite positions (one for each species). We are therefore performing the synthesis in two stepsA, T, C and G DNA amidites bottles are installed on the A, T, C and G positions on the machine. We install A, T, C and G LNA monomers on positions 6, 7, 8 and 9 respectively. The spacer amidite is installed on position 5. The desthibiotin TEG is added during an independent, subsequant run where the DSB TEG bottle is also installed on position 5 in replacement of the spacer bottle.. (see figure 1).

For example:

A PICh probe with the following design: 5’-spacer-spacer-spacer-spacer-A-T-C-G-G-C-A-A-T-T-G-3’ (red letters are LNA monomers)

will be entered as follows in the Expedite workstation: 5’-5-5-5-5-6-T-7-G-8-C-6-A-9-T-G-3’

Synthesis is performed leaving the final 5’ DMT on the oligo (DMT-ON) to allow for coupling of the DSB TEG in a subsequent run. We use position 5 to add DSB-TEG. Here again, the final DMT is left on to maintain the yield of the probe during subsequent purification. (note 4)Protocols for synthesis

Each monomer amidite requires different timing for efficient coupling. We use the protocols below:

To couple DNA amidites, a standard coupling protocol is used:

Deblocking step: 150 pulses during 49 seconds
Coupling: 39 pulses during 116 seconds
Capping: 56 pulses during 15 seconds
Oxidizing: 30 pulses during 1 second
Capping: 54 pulses during 1 second
(1 pulse corresponds to approximately 16µL on the Expedite).

To couple LNA amidites:
Deblocking step: 152 pulses during 51.5 seconds
Coupling: 29 pulses during 174 seconds

Capping: 56 pulses during 15 seconds
Oxidizing: 50 pulses during 20 second
Capping: 54 pulses during 1 second

To couple spacers:
Deblocking step: 154 pulses during 52.5 seconds
Coupling: 39 pulses during 294 seconds
Capping: 56 pulses during 15 seconds
Oxidizing: 30 pulses during 1 second
Capping: 54 pulses during 1 second

To couple desthiobiotin:
Remove the spacer bottle from position '5' and replace with ACN. Wash the line using the 'prime monomer' tool on the machine. Install the DSB-TEG bottle and prime the monomer prior synthesis.
Deblocking step: 144 pulses during 51.5 seconds
Coupling: 25 pulses during 96 seconds
Capping: 48 pulses during 15 seconds
Oxidizing: 30 pulses during 1 second
Capping: 37 pulses during 1 second

Authors Notes:

Note1: Our synthesizer is working under dry Helium (5.0) pressure.

Note2: It is important to dissolve amidites correctly, with fresh synthesis grade anhydrous acetonitrile and THF. Make sure they are completely dissolved, in particular the spacer (no 'oil' remaining in the bottle). Also add dry Argon in these bottles to prevent oxidation. Keep them at room temperature on an orbital shaker for 5-10min before using.

Note 3: Appropriate action must be taken to exclude water from any part in contact with reagents (bottles, lines, etc…). The presence of minute amounts of water will lead to synthesis failure. We are adding molecular sieves in most bottles of reagents. We previously dry them at 120°C for one day. Bottles used for amidites are also dried in the oven.

Note 4: Don't forget to prime DSB-TEG in the tubing after having cleaned the line with ACN. For this hook up ACN of position '5', prime the position '5' three times and install the DSB-TEG on this position. Prime once on position '5' to fill the line with DSB-TEG).

Material and reagents

Table1: Dissolution of amidites and products

	Molecular weight	Company	Product number	Dissolve in	To obtain a 0.07M solution
LNA-A (250mg)	885.9	Exiqon	EQ-0063-250	Anhydrous ACN	4.2mL
LNA-C (250mg)	875.9	Exiqon	EQ-00666-250	THF/CAN 25/75 (v/v)	4.3mL
LNA-G (250mg)	852.9	Exiqon	EQ-0082-250	Anhydrous ACN	4.4mL
LNA-T (250mg)	772.8	Exiqon	EQ0064-250	Anhydrous ACN	4.8mL
DNA-A (250mg)	857.9	Glen research	10-1000-250	Anhydrous ACN	5mL
DNA-C (250mg)	833.9	Glen research	10-1010-250	Anhydrous ACN	5mL
DNA-G (250mg)	839.9	Glen research	10-1020-250	Anhydrous ACN	5mL
DNA-T (250mg)	744.8	Glen research	10-1030-250	Anhydrous ACN	5mL
Spacer Phosphoramidite 18 (100µmole)	784.93	Glen research	10-1918-90	Anhydrous ACN	1,5mL
DesthiobiotinTEG phosphoramidite	980,19	Glen research	10-1952-90	Anhydrous ACN	1,5mL
CAP A		Glen research	40-4012-57		
CAP B		Glen research	40-4122-57		

oxidizer		Glen research	40-4032-57		
activator		Glen research	30-3140-45		
wash		Glen research	40-4050-57		
deblocking solution		Glen research	40-4040-62		
ACN		Glen research	40-4050-50 40-4050-57		
THF		Sigma	401757		

Figures

Figure 1: Synthesis cycle for preparation of oligonucleotides by phosphoramidite method.

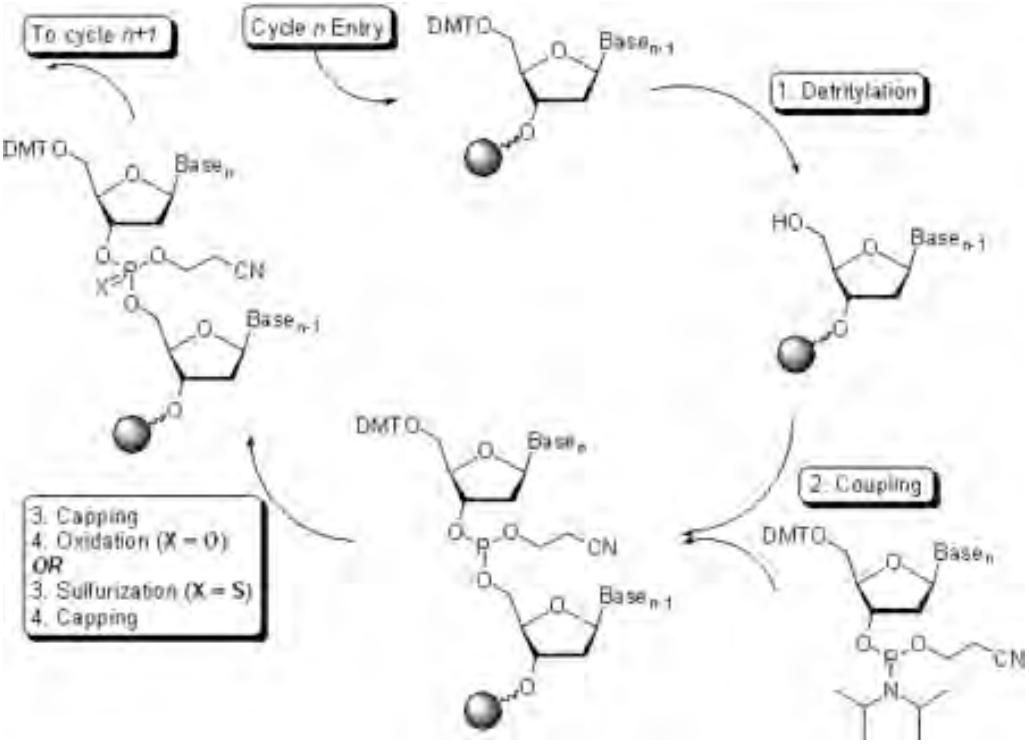
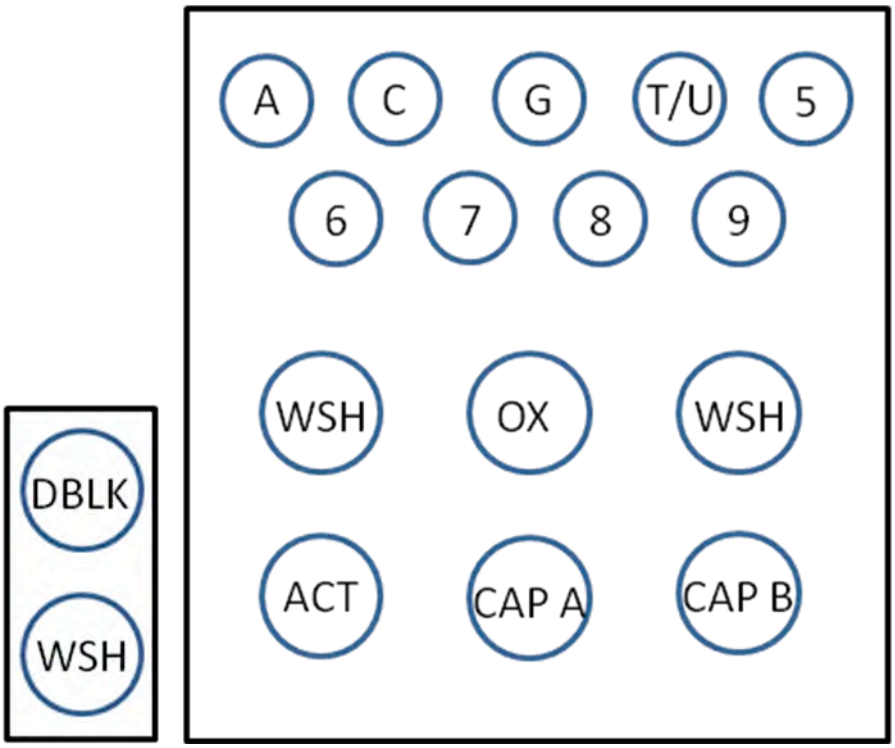


Figure 2: Reagents Reservoir Positions



DBLK: Deblocking solution,

WSH: Wash solution, anhydrous acetonitrile

OX: Oxidizer

ACT: Activator

CAP A and B: Capping solution

II. Purification of oligonucleotides

Deprotected nucleic acids may be purified and isolated by a variety of methods (precipitation, HPLC, gel purification…).The method of choice will depend on the purity required.
In the lab, we are purifying our probes by gel purification because it's the method of choice to obtain the purest full length probes.

1. After synthesis, the oligonucleotide has to be cleaved from the column and the protecting groups have to be removed by a treatment with ammonia. CPG is collected and is treated with 1mL of 30% ammonium hydroxide at 55°C overnight in a screw cap eppendorf tube.
2. NEVER open a tube containing 30% ammonia while hot, as this could vaporize. Cool down the samples by keeping the tubes at -20°C for 15 minutes before opening.
3. Load samples (Ammonia and CPG) on a centrifugal filter units (0.22 µ m GV, Millipore) and centrifuge 1 minute at maximum speed to separate the beads from the flow-through (which contains the oligo).
4. Transfer the flow-through to a clean tube.
5. Wash beads with 200µL of water
6. Centrifuge 1 minute at maximum speed. Pool with the flow-through.
7. Evaporate water and ammonium hydroxide in a speed vac: to speed up the process, deep freeze your samples on dry ice and place it in the vacuum while frozen.
8. Resuspend the solid residue in 200µL of denaturing sample loading buffer (without xylene cyanol but with bromophenol blue) and denature at 85°c for 5 minutes. Be careful when pipeting as the sample can be very gewy and clog the pipet tip.
9. Prepare a 15 % denaturing polyacrylamide/ urea 20cmX20cm gel (any homemade recipe is fine, we are using premixed SequaGel UreaGel System from National diagnostics). Pre-run the gel for 15-20 minutes at 30 W.
10. Load your sample and run the gel at 30W (plates should be about 50°C). Load an empty lane with sample buffer containing bromophenol blue and xylene cyanol.
11. Migration can be stopped when bromophenol blue is about to exit the gel. Remove the gel from the plates, and wrap it in Saran film. Place wrapped gel on top of a fluorescent Thin layer chromatography plate and illuminate at 254 nm in a dark room. UV shadowing is used to detect oligos on the gel. Full-length oligos such as the ones we prepare for PICh are usually running at the same size as xylene cyanol. A successful synthesis results in the appearance of a unique band. Truncated products (usually a consequence of an inefficient coupling of spacers or DSB-TEG) will appear

as a ladder of n-1, n-2, n-3, etc… products. In this case and if synthesis cannot be performed again, only cut the top band.

12. Cut the appropriate band from the gel.
13. Place gel pieces into a PVDF 0.22µm column (Millipore) and grind the pieces until a fine powder of acrylamide is obtained. Leaving bigger pieces of gel will lower the elution yield. Add 900µL Tris-EDTA and 100µL NaCl 5M in each column (block the column exit using a small piece of parafilm). Leave them overnight at room temperature.
14. Centrifuge columns during 15 minutes at 3000g.
15. 15. Precipitate oligos by adding 1:10 (v/v) of 3M NaOAc, 20 mg of Glycogen and 3 volumes of 100% Ethanol. Vortex before leaving samples 30 minutes on dry ice.
16. Centrifuge 15 minutes at maximum speed at 4°C.
17. Remove supernatant and wash pellet with 80% Ethanol.
18. Centrifuge and resuspend pellet in 200µL of 80% acetic acid. Leave sample 1h at room temperature.
19. After incubation, add NaCl until 0.5M, glycogen and 1mL of 100% ethanol. Vortex.
20. Incubate 30 minutes on dry ice and centrifuge 20 minutes at maximum speed.
21. Remove supernatant and wash with 80% Ethanol.
22. Centrifuge 10 minutes at maximum speed.
23. Discard supernatant, air dry and resuspend pellet in 0.1X TE to the desired concentration.
24. Oligo probe may be further characterized using MALDI spectrometry.

Buffers:

Loading buffer:
95% deionised formamide
0.025% Bromophenol Blue
0.5mM EDTA

Tris EDTA

Sequagel buffer systems

Glycogen

Reviewer comments:

Review by: Jose Antao

Department of Molecular Biology, Simches Research Center, Boston

The most important aspect of the synthesis is that the whole system and reagents be kept free from any humidity. Some of the reagents used in the synthesis are very labile, and care should be taken not to use old reagents. If a bottle has been opened 1-2 months previously, use a new bottle instead. This is particularly critical for the Activator solution.

When using the Expedite 8909 machine, one way to do the synthesis in one step is to design the oligos with only 3 LNA bases and use the 4th bottle for the DSB TEG. I usually synthesize oligos without the C-LNA residue, as I have seen that oligos containing C-LNA and no G-LNA don't work as well as oligos containing G-LNA and no C-LNA.