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Purification of Nucleic Acids by Hybridization to Affinity Tagged PNA Probes

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The use of affinity tagged PNA capture probes offers an efficient means for the purification of nucleic acids by hybridization. Two different approaches are described. A sequence specific method and a generic method. The sequence specific method requires sequence information on the target and synthesis of a dedicated PNA. It can be used to selectively purify the nucleic acid containing the target from non-related nucleic acids and other cellular components. The generic method uses a "universal" triplex forming PNA and requires no sequence information on the target. It can be used in the bulk purification of large nucleic acids.

Introduction

Biological material has been shown to contain many substances that can inhibit *in-vitro* enzymatic manipulation of nucleic acids (1-4). Methods that facilitate efficient purification of nucleic acids from complex starting material are therefore of considerable interest.

To provide maximum separation power the purification method should exploit a unique property of the nucleic acids. An obvious choice is the ability of single stranded nucleic acids to form Watson-Crick hydrogen bonded duplexes with complementary synthetic DNA probes. Using this property, homothymine DNA probes carrying affinity tags or coupled directly to solid supports have been used successfully to purify bulk mRNA by hybridization to the poly(A) tail (5,6). Although the "purification by hybridization" approach ought to be applicable to the purification of other classes of nucleic acids and specific target molecules, it does not appear from the literature to be widely used.

Part of the reason for this is that the use of DNA capture probes faces a number of practical problems. One of these are the need for high affinity capture probes that provide a probe/target stability that will survive the purification procedure (7-10). In practise this means that rather large DNA probes are required, the consequences of which are 1) a reduced ability of the probe to discriminate between closely related target sequences and 2) an increase in the complexity of preparing the probes. Another often encountered problem with DNA capture probes is poor accessibility of the target sequence which can be caused by undesired inter- or intra-molecular hybridization event involving the target. Capture of dsDNA, for instance, can be hampered by competition from the complement strand. Likewise, stable intramolecular structures, which occurs in several classes of nucleic acids like rRNAs, tRNAs and snRNAs - and which may also occur by chance in individual mRNAs - may limit target sequence accessibility.

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PNA is ideally suited to tackle these problem. Firstly, the high T_m of even moderately sized PNA probes (11) usually ensures adequate probe/target stability's whilst maintaining good specificity. The increased affinity further provides PNA with a competitive advantage over the corresponding nucleic acids in binding to its target sequence. When the PNA is able to form a triplex (*i.e.,* consisting of pyrimidine residues exclusively) and thus able to bind to dsDNA by "strand invasion" (12, 13) this competitive advantage is at its maximum.

Secondly, when PNA is used as capture probes, the hybridization conditions can be adjusted so as to unwind nucleic acid structures (thereby making them accessible for hybridization) whilst not affecting PNA binding (11, 14). This possibility is a consequence of the uncharged nature of the PNA oligomer, which allows hybridization in the absence of the counterions needed to stabilise nucleic acid duplexes. Taking advantage of these properties, we have developed two different PNA based methods for purifying nucleic acids by hybridization; a sequence specific method (14) and a generic method (15).

The sequence specific method uses duplex forming PNAs carrying His_6 affinity tags in combination with Ni-NTA affinity columns to purify the target nucleic acid. The method requires sequence information on the target nucleic acid and synthesis of a dedicated PNA. Using an oligonucleotide model system the method was shown to allow selective enrichment of a fully complementary target over a single base mismatched target oligo. Also, the use of a low ionic strength buffer was shown to facilitate efficient capture of a hairpin oligonucleotide which could not be captured under conditions normally used for nucleic acid hybridization. When *in vitro* transcribed RNAs, varying in sizes from 257 to 2224nt., were used as targets, an inverse relationship between the size of the target and the efficiency of capture was observed. Efficient capture of the large target, however, could be significantly improved by the simultaneous use of several different PNAs.

The generic method exploits the unique ability of short homopyrimidine PNAs to form extremely stable triplexes with homopurine DNA targets (12,13). The triplex between a PNA T_7 and a DNA A_7, for instance, exhibit a thermostability of 63.3°C. Since a 7 nt. sequence will on average occur by chance every 16.4 kb in nucleic acids (assuming a 50% GC content), small triplex forming PNAs ought to function as generic probes for the capture of large nucleic acids. This contention was verified using biotinylated bisPNA-T_7 (16,17) in combination with streptavidin-coated magnetic particles to efficiently purify human genomic DNA from whole blood. When lysed blood was added to the PCR reaction without prior purification as little as 5µl totally inhibited the amplification process. After PNA-T_7 purification, however, amplification could easily be conducted with an amount corresponding to 500µl of blood. In a prospective study of 12 individuals (6 males and 6 females) the method, in all cases, was found to provide DNA that could be PCR amplified using sets of primers directed against target genes located on chromosomes 1 and 7 and the X chromosome.

Protocols

Sequence Specific Method

1. The hybridization reaction (typically 200µl) contains 2M Urea, 20mM Na_2HPO_4 (pH 8.0), 0.5M NaCl, 0.1% Triton X-100, 1µM of His_6-PNA capture probe and lysed sample.
2. Heat to 95°C for 5 min. and hybridise for 10 min at the desired temperature. With the PNAs analysed so far, good specificity and fast hybridization kinetics have been observed at temperatures between 20 to 25°C below the measured T_m.
3. Load the hybridization reaction onto a Ni-NTA spin column (prepared in advance as described below) and centrifuge at 200*x g* for 30 sec (no incubation time on the Ni-NTA column is necessary).

4. Wash the column several times to remove non-specifically bound material by adding 200µl of hybridization buffer (2M Urea, 20mM Na_2HPO_4 (pH 8.0), 0.5M NaCl, 0.1% Triton X-100) followed by centrifugation at 200*x g* for 30 sec.
5. Elute the purified target nucleic acids by (i) adding 200µl of hybridization buffer to the column and (ii) incubating the column at 95°C for 5 min and (iii) centrifuging at 200*x g* for 30 sec.

The elution procedure is designed to maintain the His_6-PNAs on the column and only release the capture target nucleic acids. If desired, however, exchanging the hybridization buffer with 100mM of EDTA can elute the entire His_6-PNA/DNA complex.

Preparation of Spin Column

- Load 400µl of Ni-NTA resin (Qiagen GmbH, Hilden, Germany) onto an Eppendorf spin column (Durapore 0.45µm; Millipore Corp.) and centrifuge at 200*x g* for 30 sec to remove the Ni-NTA storage buffer.
- Wash the column several times by adding 200µl of water followed by centrifugation at 200*x g* for 30 sec.
- Add 200µl of hybridization buffer (2M Urea, 20mM Na_2HPO_4 (pH 8.0), 0.5M NaCl, 0.1% Triton X-100) to the column. Remove the hybridization buffer by centrifugation at 200*x g* for 30 sec immediately before use.

Generic Method

The generic method has been optimised for the (i) purification of human genomic DNA from whole blood and to allow (ii) direct PCR on the captured nucleic acids whilst bound to the streptavidin coated magnetic particles. Using a different lysis buffer and lysis procedure the method was found to facilitate efficient purification of the cryptic plasmid of *Chlamydia trachomatis* from urine samples (18).

1. The lysis reaction contain up to 100µl of whole blood and 100µl of lysis buffer (100mM Tris.HCl (pH 8.5), 50mM KCl, 6mM $MgCl_2$, 0.02% Triton X-100 and 1mg/ml Proteinase K (Boehringer Mannheim; added immediately before use), 100pmol of biotinylated bisPNA T_7, and water to a final volume of 200µl in an Eppendorf tube.
2. Incubate the reaction at 55°C for 15 min., then at 100°C for 10min to simultaneously denature the genomic DNA and inactivate the proteinase K (active proteinase K may digest the streptavidin on the magnetic particles added in step 6).
3. Centrifuge the sample at 12000*x g* to pellet cellular debris (when less than 25µl of blood is used this step can be omitted).
4. Transfer the supernatant to a new Eppendorf tube and incubate for 10 min. at room temperature to allow the PNA to hybridise.
5. Wash 20µg of streptavidin-coated magnetic beads (Dynabeads ^{R} M-280; Dynal A/S, Oslo, Norway) in 200µl of wash buffer (20mM Tris-HCl, (pH 8.3), 100mM KCl, 3mM $MgCl_2$, 0.1% zwittergent) and resuspend in 50µl wash buffer.
6. Add the 50µl of beads to the hybridization reaction and shake at room temperature for 10 min at 1200 rpm to allow the Bio-PNA/DNA complex to bind to the beads.
7. Collect the beads with a magnetic beads concentrator (Dynal A/S) and wash twice with 200µl of wash buffer.
8. Resuspend the beads in 20µl of water and transfer directly to the PCR reaction.

Examples

One of the potentially most useful features of PNA in sample preparation is its ability to hybridise to the target nucleic acid in the absence of counterions. Thus, by simply lowering the salt concentration in the hybridization buffer one can

selectively unwind nucleic acid structures that may otherwise limit the access of the PNA to its target sequence.

To demonstrate the practicability of the "low salt" approach we attempted to capture a "hairpin" oligonucleotide in which the entire 15 nt. PNA target sequence was part of the stem structure (Figure 1). The oligonucleotide was radioactively labelled with ^{32}P and incubated for 10 min at room temperature in the presence of a complementary His_6-PNA in either high salt (20mM Na_2HPO_4 (pH 8.0), 500mM NaCl, 0.1% Triton X-100 and 2M urea) or low salt (1mM Na_2HPO_4 (pH 8.0), 0.1% Triton X-100 and 2M urea). After hybridization the sample was loaded onto a Ni-NTA column and taken through the procedure outlined above. Finally, the radioactivity in the run-through, washes and eluents were counted in a scintillation counter. As shown in Figure 2 essentially all the radioactive material is found in the run-through and first wash when hybridization is conducted in the high salt buffer were the hairpin structure should be stable. In contrast, more than 50% of the radioactivity is recovered in the eluent when hybridization is conducted in the low salt buffer where the hairpin structure should be significantly destabilised.

Application and Limitations

Purification by hybridization is an appealing approach whereby nucleic acids can be efficiently separated from non-nucleic acid components in a biological sample. With PNA there are two options; sequence specific or generic purification. Which method will be most suitable depends on the objective. If small nucleic acids are to be purified then the generic approach might fail because of lack of the target sequence. In this case, and in cases where the objective is to purify a specific target nucleic acid from a background of unrelated nucleic acids, the sequence specific method must be used. The efficiency of this method, however, decreases with increasing size of the target molecule. Hence, when large nucleic acids are to be purified, or the objective is to obtain bulk nucleic acids then the generic method is most suitable.

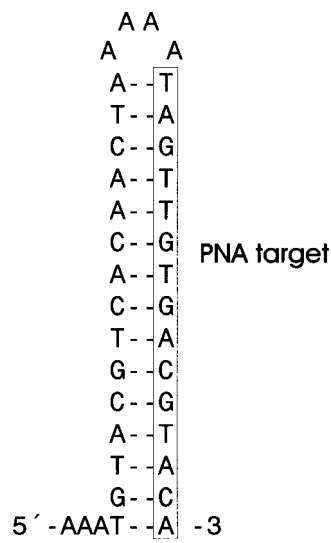


Figure 1. Sequence and predicted secondary structure of the 37 mer synthetic target oligonucleotide.

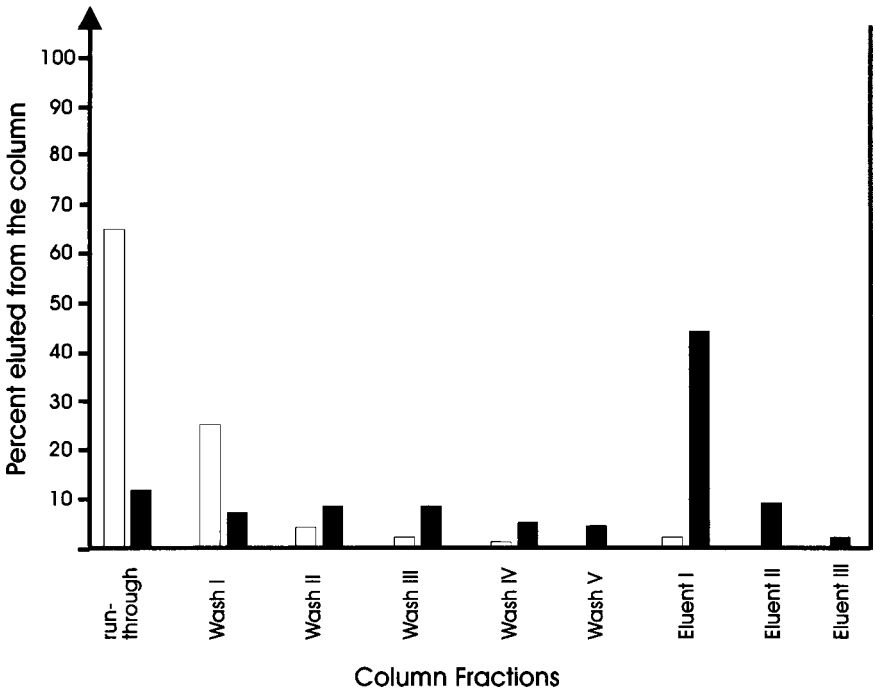


Figure 2. Capture efficiency of the hairpin oligonucleotide depicted in Figure 1 as a function of the ionic strength of the hybridization buffer. The radioactivity in each of the fractions is represented as the percentage of total radioactivity in all fractions. White bars: High salt, Black bars: Low salt.

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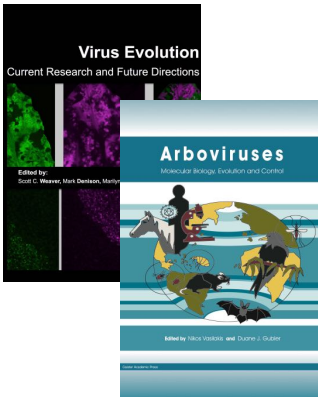
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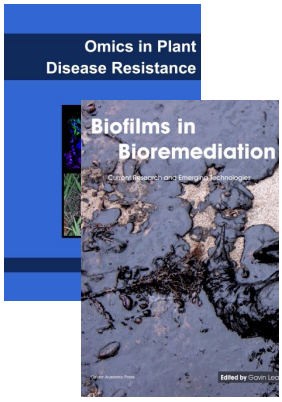
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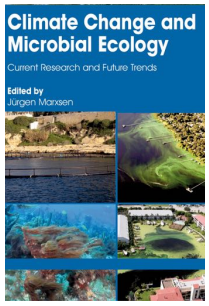
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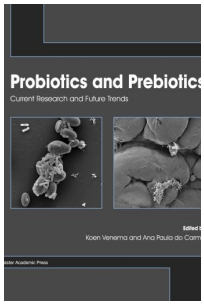
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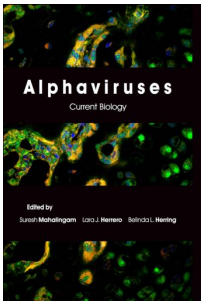
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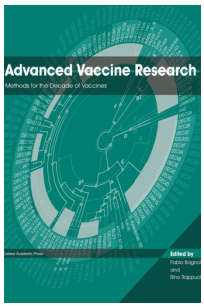


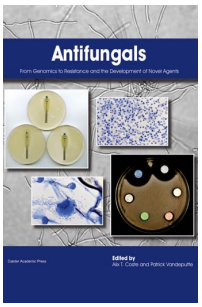












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