# HPLC purification of dye-labeled or Fmoc-protected peptide nucleic acids from solid-phase synthesis

Application Note

Marc Dauber, Jörg Hoheisel, Anette Jacob
*Deutsches Krebsforschungszentrum, Funktionelle Genomanalyse, Heidelberg, Germany*
Udo Huber, Edgar Nägele
*Agilent Technologies, Waldbronn, Germany*

**Abstract**

This Application Note demonstrates:

- Configuration of the Agilent 1200 Series Rapid Resolution LC (RRLC) analytical scale (AS) purification system.
- Use of the Agilent 1200 Series RRLC AS purification system with an optimized method for purification of peptide nucleic acids from solid-phase synthesis.


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## Introduction

Peptide nucleic acids (PNAs) are synthetic DNA mimics,[1] which are suitable tools for biological applications like transcription profiling, detection of single nucleotide polymorphisms (SNPs), fluorescence *in situ* hybridization (FISH), and so on. Due to the uncharged pseudopeptide backbone (figure 1) of the PNA, PNA/DNA or PNA/RNA double strands are more stable than DNA/DNA or DNA/RNA double strands. According to the literature, single mismatches in the PNA/DNA complex will decrease the melting temperature dramatically.[2] Therefore, for most applications, PNA probes can be much shorter than DNA probes. Because of the lack of interstrand repulsion, hybridization is also possible at lower salt concentrations.

PNA oligomers are synthesized according to standard peptide Fmoc chemistry (which uses the protecting group 9*H*-**f**luoren-9-yl**m**eth**o**xy**c**arbonyl) on a solid-phase.[3] The protected and activated monomer is coupled to a solid support, followed by an acetylation step to prevent mixed sequences. After selective removal of the N-terminal Fmoc protecting group, the next coupling step takes place.[4] After the last cycle, modifications like the introduction of fluorophors or special peptide sequences are possible. The crude product is cleaved from the solid support. This crude product contains the full-length PNA and some truncated sequences that are acetylated at the N-terminal side.

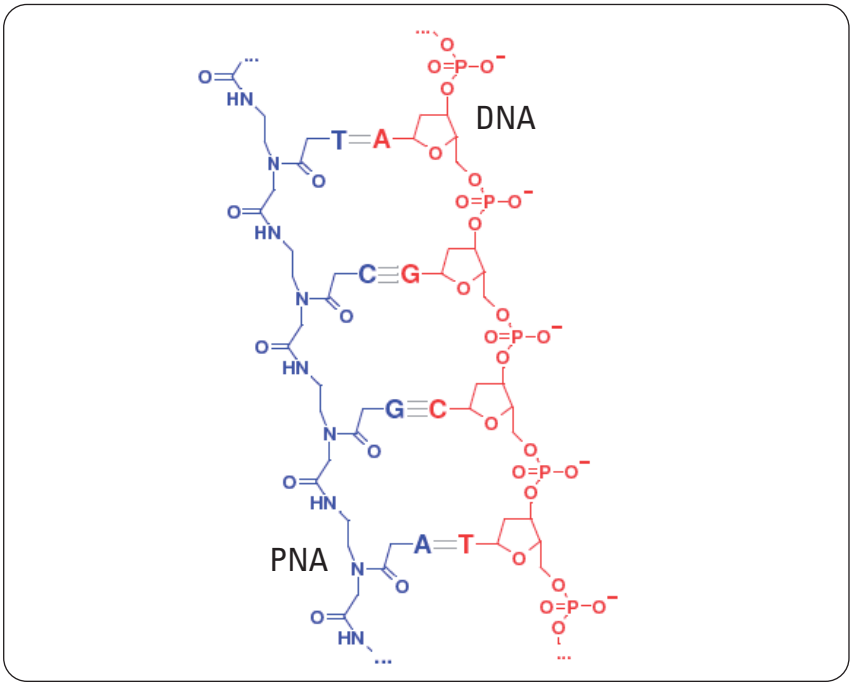


**Figure 1**
**Structure of PNA and DNA backbone.**

## Experimental

### Equipment

Agilent 1200 Series RRLC AS purification system consisting of:

- Agilent 1200 Series binary pump SL with degasser
- Agilent 1200 Series high-performance autosampler with cooler
- Agilent 1200 Series thermostatted column compartment
- Agilent 1200 Series diode array detector (DAD) SL, using a flow cell with 10-mm path length
- Agilent 1200 Series analytical scale fraction collector

### General method parameters

- Sample temperature: 4 °C
- Column temperature: 60 °C
- DAD: 260 ± 4 nm, Ref. 600 ± 4 nm
- Collection of fraction was triggered by UV signal. The system delay volume was determined by the procedure for delay volume calibration described in an Agilent Application Note.[5]

### LC method for PNA purification

- Column: Agilent ZORBAX Eclipse SB-C18, 2.1 x 100 mm, 1.8 µm particle size
- Injection volume: 10 µL (~6 % of a 0.4-µmol synthesis scale)
- Solvent A: 0.1 % aqueous trifluoroacetic acid ($TFA_{aq}$) (v/v). Solvent B: 0.085 % TFA/acetonitrile (v/v)
- Gradient:
  0 min, 7 %B
  20 min, 10 %B
  24 min, 30 %B
  25 min, 100 %B
  28 min, 100 %B
  29 min, 7 %B
  32 min, 7 %B
- Stop time: 32.0 min
- Post time: 3 min
- Flow: 0.5 mL/min

### LC method for Fmoc-PNA and dye-PNA purification

- Column: Agilent ZORBAX Eclipse XDB-C18, 4.6 x 50 mm, 1.8 µm particle size
- Injection volume: 5 µL (~2.5 % of a 0.4-µmol synthesis scale)
- Solvent A: 0.1 % $TFA_{aq}$ (v/v). Solvent B: 0.085 % TFA/acetonitrile (v/v)
- Gradient:
  0 min, 5 %B
  0.2 min, 15 %B
  1.5 min, 30 %B
  1.7 min, 100 %B
  2.5 min, 100 %B
  3.0 min, 5 %B
- Stop time: 3.0 min
- Post time: 1 min
- Flow: 2 mL/min

## Results and discussion

Fluorescence-labeled and unlabeled PNAs were synthesized in multi-well filter plates according to standard peptide Fmoc-chemistry.[4] After cleavage from the resin, the compounds were transferred into another multi-well plate and purified by HPLC. For smaller PNA probes, HPLC purification works reasonably well, but with increasing length, purification becomes more and more difficult. Figure 2 demonstrates HPLC purification of a 20-mer, which takes 35 minutes from injection to injection. MALDI-TOF MS analyses of the crude and the purified product are shown on the right side in figure 2.

For other sequences or even longer PNAs (> 20-mers), purification was in some cases not possible under these conditions. Here, an Fmoc-on HPLC purification method,[6] analogous to a dimethoxytrityl (DMT)-on oligodeoxynucleotide purification, leads to an easy and faster separation of the full-length product. The following figures show purifications of a crude Fmoc-protected PNA and two dye-labeled PNAs. Due to the increased inter-

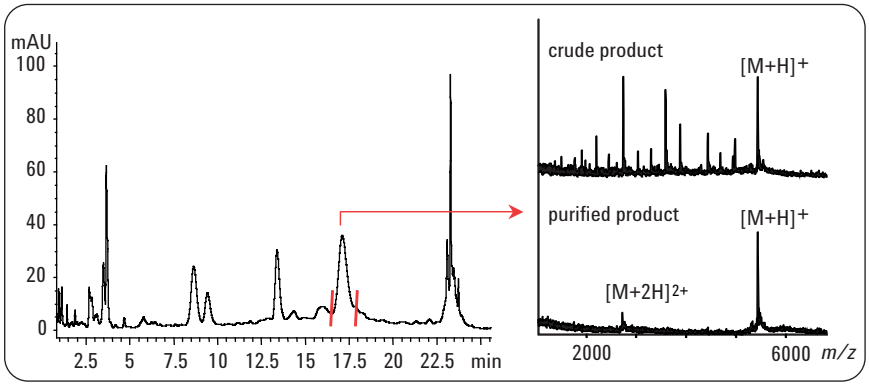


**Figure 2**
**Chromatogram of the HPLC purification (left) of a crude PNA 20-mer (TACCTGGGTGGCGTTCTATC) and MALDI-TOF MS analyses (right) of the crude and the purified product.**

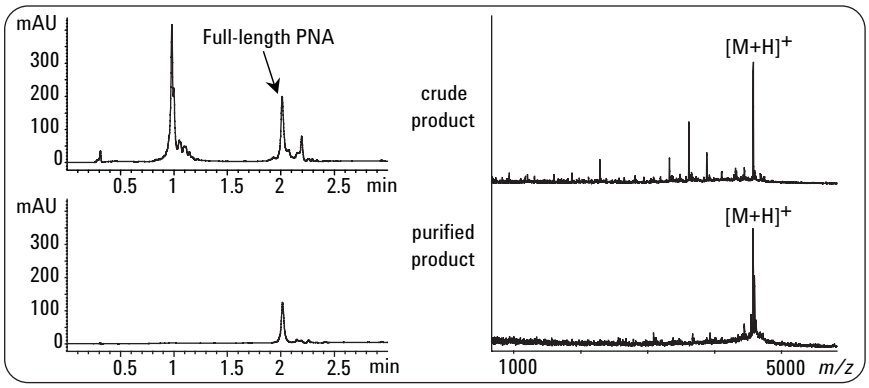


**Figure 3**
**HPLC purification of a crude Fmoc-protected 17-mer (Fmoc-CysXTTGGTCTTGCGACAG, where X=AEEOH-linker) and reanalysis of the purified product by HPLC (left) with MALDI-TOF MS analyses for both samples (right).**

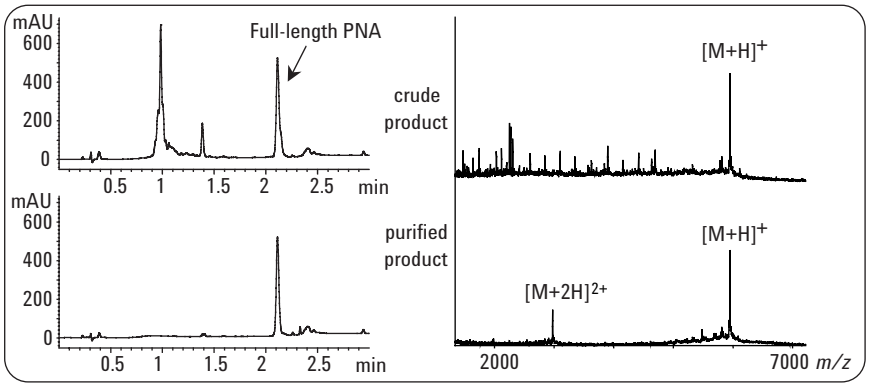


**Figure 4**
**HPLC purification of a crude dye-labeled 20-mer (dye647-XXAATCAACCCGAGTGCAAT, where X=AEEOH-linker) and reanalysis of the purified product by HPLC (left) with MALDI-TOF MS analyses for both samples (right).**

action of the lipophilic Fmoc or dye moieties with the C18-material of the column, a steeper gradient can be applied and shorter purification times are possible (4 minutes from injection to injection). Furthermore, retention times of the full-length "Fmoc-on" and dye-labeled PNAs are reproducibly greater than retention times of all truncated sequences and byproducts, thereby allowing for an easy automation of high-throughput purification of PNAs by automated peak detection.

As shown in figures 3, 4 and 5 the full-length Fmoc-protected or dye-labeled product was clearly separated from all truncated sequences within 2.5 minutes. Fractions of the products were collected and the purity was confirmed by HPLC and MALDI-TOF MS analysis.

## Conclusion

This Application Note demonstrates the use of the Agilent 1200 Series Rapid Resolution LC analytical scale purification system for the purification of PNAs from solid-phase synthesis. For longer PNAs (~20-mer), an easier and faster purification is achieved by purifying the "Fmoc-on" product. The same HPLC method can also be used for dye-labeled PNAs. The desired products can be isolated from the crude mixture in high purity by an automated purification process that is triggered by a UV signal. The purity of the isolated PNA can be confirmed by reanalysis of the fraction.

The Agilent 1200 Series RRLC allows for fully automated purification of Fmoc-protected and fluorescence-labeled PNAs within 4 minutes, including washing and equilibration of the column. With an additional alternating column regeneration,[7] purification of a PNA will only take 2.5 minutes per sample. As the spectra indicate, even shorter purification times can be established by using steeper gradients, thereby diminishing the overall purification time for high-throughput applications.

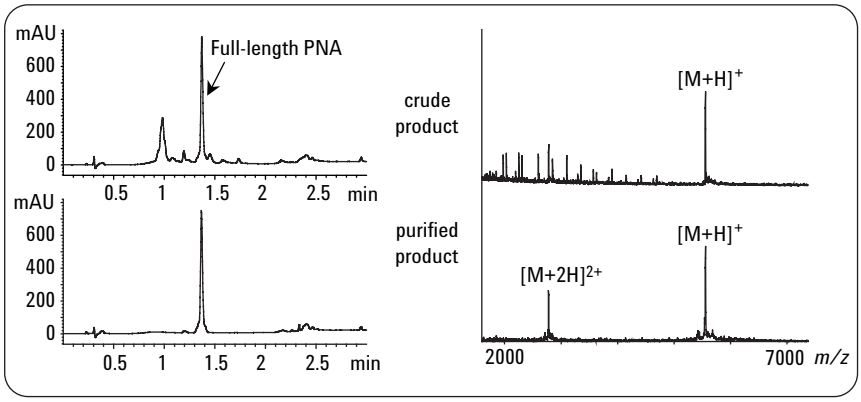


**Figure 5**
**HPLC purification of a crude dye-labeled 20-mer (oregon-green-XXAATCAACCCGAGTGCAAT, where X=AEEOH-linker) and reanalysis of the purified product by HPLC (left) with MALDI-TOF MS analyses for both samples (right).**

## References


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*Marc Dauber, Jörg Hoheisel, Anette Jacob are Scientists at Deutsches Krebsforschungszentrum, Funktionelle Genomanalyse, Heidelberg, Germany; Udo Huber and Edgar Nägele are Application Scientists at Agilent Technologies, Waldbronn, Germany.*


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