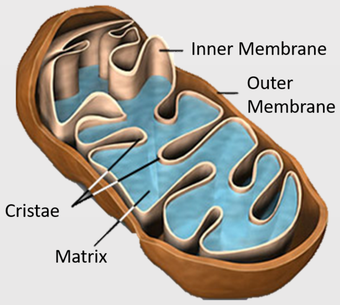

Separation of Nucleic Acids by Ion Pair Reversed Phase High Performance Liquid Chromatography (IP RP HPLC)

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Introduction

There are currently many time-consuming methods for the separation and quantification of oligonucleotides available. Separation of oligonucleotides is usually performed by agarose gel electrophoresis followed by ethidium bromide visualization (1). Ethidium bromide is a toxic chemical that requires many safety precautions prior and during use. Quantification of oligonucleotides is usually performed either through measuring the absorbance at 260 nm or through phosphorimager analysis after electrophoresis (2). Phosphorimager analysis requires radioactive labelling of the oligonucleotides in order to visualize them (3). The analysis of oligonucleotides by IP RP HPLC has been shown to reliably separate DNA oligos with single base pair resolution much quicker and safer than traditional methods (4, 5). Figure 1 shows a representative figure for oligonucleotide analysis by IP RP HPLC. The mobile phase contains a positively charged ion-pairing reagent that adsorbs to the stationary phase forming a pseudo ion-exchange layer. This layer interacts with the polar bases of the oligonucleotides allowing for separation to occur. **Our goal is to separate and quantify RNA molecules generated via in-vitro transcription reactions.** Here we show a method to separate and quantify DNA samples of 54 and 58 nt.

Methods and Materials

Samples were dissolved in diethyl pyrocarbonate (DEPC)-treated water, heated at 65 °C for 5 minutes, then immediately put on ice for at least 1 minute.

Ion-pair reversed-phase high performance liquid chromatography was performed on a Shimadzu LC-2010HT Liquid Chromatography system with an Xbridge Oligonucleotide BEH C18 (130 A, 2.5µ, 4.6 x 50 mm; Waters) column. Quantification of peaks was performed through integration of the peak areas. IP RP HPLC was performed using a two-buffer eluent system:

- Buffer A: 0.1 M triethylammonium acetate (TEAA) in DEPC-treated water (pH 7.0)
- Buffer B: 0.1 M TEAA with 25 % (v/v) acetonitrile (ACN) in DEPC-treated water (pH 7.0)

The gradient conditions used were: 38-39% B from 0 to 45 minutes, to 40% B from 45 to 55 minutes, to 100% B from 55 to 60 minutes, followed by a 10-minute re-equilibration to initial conditions. Flow rate was 0.5 mL/min. Column temperature was 50 °C. Oligonucleotides were kept at 4 °C prior to analysis.

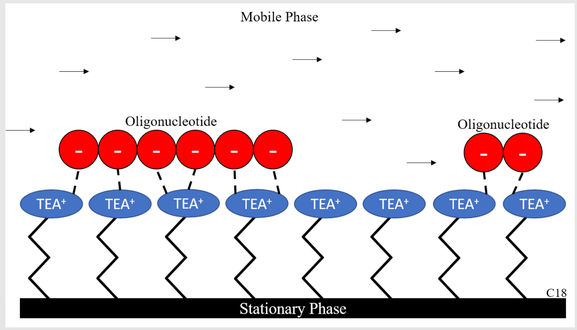

Figure 1. Representative figure for analysis of oligonucleotides by IP RP HPLC. The mobile phase, TEAA, contains the ion-pairing reagent TEA^+ (blue circles) which adsorbs to the non-polar stationary phase forming a positively charged pseudo ion-exchange layer. This layer interacts with the polar bases of the oligonucleotides (red circles) allowing for separation to occur

Results

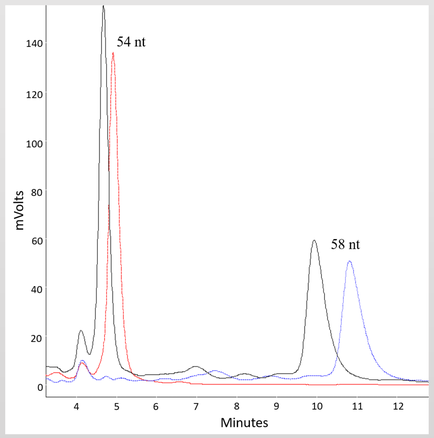

Figure 2. IP RP HPLC analysis of ssDNA samples. Injections of 1000 ng of 54 nt DNA sample (red), 1000 ng of 58 nt DNA sample(blue), and an injection consisting of 1000 ng each 54 nt and 58 nt (black)

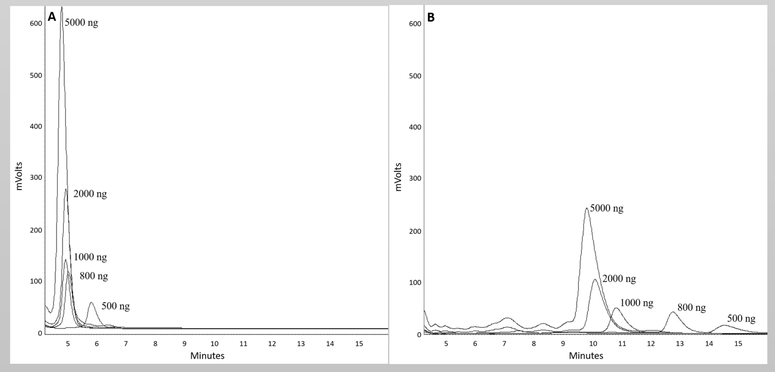

Figure 3. Overlay of IP RP HPLC chromatograms. (**A**) IP RP HPLC chromatograms of 54 nt DNA sample with varying injection sizes from 500 ng to 5000 ng. (**B**) IP RP HPLC chromatograms of 58 nt DNA sample with varying injection sizes from 500 ng to 5000 ng. Integrated peak areas for each DNA sample yielded an linear (R^2 > 0.999) response with respect to injected mass of DNA.

Table 1. Quantification results from varying injections of 54 nt DNA samples after IP RP HPLC analysis. Quantification results determined through integration of peak areas. Correlation coefficient (R^2) for the data is 0.9999.

DNA injected (ng)	Integrated Peak Area (mVs)	Calculated DNA amount (ng)
500	1338083	489.9
800	2214286	803.1
1000	2741980	991.6
2000	5625227	2022.0
5000	13940253	4993.5

Table 2. Quantification results from varying injections of 58 nt DNA samples after IP RP HPLC analysis. Quantification results determined through integration of peak areas. Correlation coefficient (R^2) for the data is 0.9999.

DNA injected (ng)	Integrated Peak Area (mVs)	Calculated DNA amount (ng)
500	960860	513.0
800	1688220	827.5
1000	2037595	978.6
2000	4332044	1970.7
5000	11361591	5010.4

Future Directions

Going forward we hope to optimize the current method and apply it to separate RNA samples of similar sizes to the DNA samples tested. **The ultimate goal is to utilize this method and IP RP HPLC to separate and quantify RNA transcripts of unknown sizes generated via in-vitro transcription reactions.**

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