



IEX Purification of RNA Base(s) Containing DMT-on Oligonucleotide Single Strand Using a One Step On-Column Detritylation Technique

Citation

Shaikh, Sarfraz. 2019. IEX Purification of RNA Base(s) Containing DMT-on Oligonucleotide Single Strand Using a One Step On-Column Detritylation Technique. Master's thesis, Harvard Extension School.

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:42004244>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)

IEX Purification of RNA Base(s) containing DMT-ON Oligonucleotide Single
Strand Using a one step on-column Detritylation Technique

Sarfraz Basir Shaikh

A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2019

Abstract

The purity of a drug product is an important aspect for successful drug development to maximize drug efficacy and reduce potential toxicity arising from impurities. In the case of oligonucleotide synthesis, shorter oligomers may be produced along with final full-length oligonucleotides upon completion of the synthesis process. This is potentially due to incomplete reactions and reagents used in the synthesis process. It is important to design strategies to optimize drug product quality through implementation of appropriate synthesis and purification steps. Automated solid phase oligonucleotide synthesis is a commonly used approach to generate small and large-scale single strand material and has been established as a robust method for synthesizing oligonucleotide at various scales. In this process, to ensure correct chain growth, acid labile Di-methoxy-trityl (DMT) group is utilized to protect the 5' Hydroxyl (OH) group of the ribose sugar in the growing oligonucleotide chain. The 5' hydroxyl group is kept on the incoming phosphoramidite and removed prior from the nucleoside present on the solid support to grow the sequence in the 3'-5' direction. Oligonucleotide sequences can be synthesized either with the 5' Di-methoxy-trityl (DMT) group remaining "on" or "off" at the 5' end position for the final base. This is done by avoiding the final acid treatment (deprotection) to remove the DMT group of the last base added at the end of synthesis cycle. Presence of the DMT group on the last base of oligonucleotides, increases the

hydrophobicity significantly and provides a great chromatographic handle to aid hydrophobicity-based chromatography. Here, we propose to evaluate a purification procedure for oligonucleotides by incorporating the DMT at the 5' end, such that it can act as an anchor and provide strong binding to the chromatographic stationary phase used in IEX chromatography. In general, Purification of DMT-on oligonucleotides requires a multistep, time consuming process to remove undesirable impurities. We anticipate that DMT-on purification on IEX stationary phases will allow for a direct on column detritylation step as part of the purification process, hence eliminating the need of additional post purification steps, which are difficult to scale and with the potential to improve the product purity without compromising yield.

Dedication

I dedicate this Thesis to the motivation behind my vision and hard work, my family. To my parents, your guidance, encouragement and supports have enabled me to obtain graduate degree. My brothers Iliyas and Arif for supporting me every path of the way and pushed me to work harder to achieve my goals through hard work. My wife Anisha, for offering me a never-ending support throughout the most stressful time of my life. You Always encouraged me to work harder and you took care of our children when I was heavily involved in study, your patience has always provided me the additional motivation to pursue the graduate degree at Harvard University.

Acknowledgements

I would like to sincerely thank Dr. Klaus Charissé for his support, assistance and mentorship for this thesis project. Without his guidance, I would not have been able to complete this project. Dr. Charissé, you pushed me to read more, improve my writing and most importantly been very patient while I was learning to synthesize proposal and thesis. I would like also to thank Dr. Satya Kuchimanchi for sharing his knowledge on technical part of this project. Your mentorship and guidance always have been very valuable to me. I would like to thank Dr. Steven Denkin for his insight and feedback on the proposal writing. I want to acknowledge all the professors whom I have taken classes from for sharing their knowledge and providing meaning full feedback on my learning process. Lastly, I want to acknowledge all my colleague at small scale synthesis, medium scale synthesis and analytical department at Alnylam pharmaceuticals for their support.

Table of Contents

Abstract	3
Dedication	v
Acknowledgements	vi
Table of Contents	vii
List of Figures	xi
List of Tables	x
I. Introduction.....	1
Anion Exchange IEX Chromatography- A Purification Technique.....	2
Automated Synthesis of Oligonucleotides.....	3
DMT-on Synthesis and Its Advantages for Purification.....	5
Traditional DMT on Purification Approach- A Cumbersome Process	7
II. Materials and Methods	15
Crude and Reference materials	15
Calculations of Column Volume	16
Stationary phases	16
Analytical Methods.....	19

Key Steps	24
Step 1: Loading of crude Oligo followed by 100% Buffer A Wash	24
Step 2: DMT-on Wash.....	25
Step 3: 100% A Wash.....	26
Step 4: Detritylation (Acid treatment)	26
Step 5: DI H ₂ O Wash.....	27
Step 6: 100% A Wash.....	27
Step 7: Separation Gradient/Fractionation.....	28
Step 8: 100% B Wash	28
Step 9: Final column conditioning and Column storage	28
Demonstrate on Column Retention of DMT-on Single Strand.	29
Explore “DMT-on” Wash- a Wash to remove Maximum Amount of Impurities Without Affecting the Binding of the DMT-on Full Length Sequence.....	29
Investigate Optimal Column Volume (CV) of 80% Aqueous Acetic Acid to Demonstrate Complete on Column Trityl (DMT) Removal	30
Explore Different Gradient Conditions to Achieve Best Purity and Highest Isolated Yields.....	32
III. Results and Discussion	33

	Retention of Crude DMT-On Single Strand Oligonucleotide on	
	Column	33
	Optimization of DMT-on wash step for Maximizing DMT-Off	
	Impurities Removal	39
	Gradient Conditions for Achieving Best Purity and Highest Isolated	
	Yields	44
	Evaluate Q Sepharose® FF and TSK gel® for the Purification of	
	DMT- on Single Strands	50
IV.	Conclusion	57
V.	References.....	59

List of Tables

Table 1. Physio-chemical Characteristics of Stationary Phase	19
Table 2: AX- HPLC parameters	20
Table 3: Ion Pairing Liquid chromatography mass spectrometry (IPRP-LCMC) method	20
Table 4: Purification mobile phases	46

List of Figures

Figure 1. General Synthesis Scheme of Oligonucleotides.....	10
Figure 2. Schematic of Oligonucleotide Purification	11
Figure 3. Oligonucleotide Chromatography Steps.....	12
Figure 4. Structures of RNA bases	13
Figure 5. Structure of Protecting Groups.....	13
Figure 6. Non-Thiolated (phosphodiester) Vs Thiolated Phosphate Group Nucleotide.....	13
Figure 7. 3' to 2' bond migration on the ribose sugar of RNA under extended exposure at lower pH	14
Figure 8. Illustration of Oligonucleotide sequence with GalNAc at 3' end and the DMT group at 5' end	16
Figure 9. Process overview of a one step on column purification and DMT group removal of a DMT-on containing Single strand (Legend: Blue line = absorbance at UV _{280nm} , brown line = conductivity, Red line = fractions)	24
Figure 10: AX-HPLC Chromatogram of DMT-on Crude Single Strand.....	35
Figure 11: AX- HPLC Chromatogram of DMT-off Crude Single Strand	35

Figure 12. Overlay of AX- HPLC Chromatograms of DMT-on Crude Single Strand and DMT-off Crude Single strand	36
Figure 13. Mass Identification using LC-MS of DMT-on Crude Single Strand	37
Figure 14. AX HPLC Analysis of flow through from Loading Step and additional wash Step (3CV of mobile phase A)	38
Figure 15. LC-MS Analysis of flow through from Loading and 3CV of mobile phase A	39
Figure 16. Elution Profile of Different the DMT-on Washes and their AX-HPLC Analysis	41
Figure 17: AX-HPLC Analysis of 25% mobile phase B Wash (3CV)	42
Figure 18. LC-MS Analysis of Eluent of 25% mobile phase B Wash (3CV). Investigation of Optimal CV of 80% Aqueous Acetic Acid to Demonstrate Complete on Column the DMT group Removal.....	43
Figure 19: AKTA Purification Profile Using Q Sepharose FF ® stationary phase Legend: Blue line = absorbance at UV280nm, brown line = conductivity, Red line = fractions)	46
Figure 20. Distribution of Mass Balance during the Salt Gradient using Q Sepharose FF ® stationary phase	47
Figure 21. AX-HPLC Analysis of the fractions from the gradient using using Q Sepharose FF ® stationary phase	48

Figure 22. Overlay of AX-HPLC Profiles of main peak Fractions indicating Removal of Front and Late Impurities for the Purification using Q Sephacrose FF ® stationary phase	49
Figure 23. Comparison of Crude (DMT-on) vs Purified (DMT-off) single strand using Q Sepharose FF ® stationary phase	49
Figure 24. AKTA Purification Profile using TSK gel ® stationary phase (Legend:	52
Figure 25. Distribution of Mass Balance during the Salt Gradient using TSK gel ® stationary phase	53
Figure 26. Chromatographic Separation of FLP and other Moieties during the Gradient and their AX HPLC Analysis for Purification using TSK gel ® stationary phase.....	54
Figure 27. Comparison of Crude (DMT-on) vs Purified (DMT-off) single strand using TSK gel ® Stationary phase.....	55
Figure 28: LC-MS Analysis of Purified single strand (DMT-off) from TSK gel ® Experiments to confirm Identity. (calc. M.W of DMT-off Single Strand = 8541 D)	56

Chapter I

I. Introduction

The success of any drug development not only depends on satisfactory clinical trials, but also on the quality and proper characterization of the drug substance (Cramer, 2013). According to the International Conference on Harmonization (ICH) guideline, any actual impurity present in drug substance at or above an apparent level of 0.1% needs to be characterized and controlled (Cramer, 2013). Understanding the origin of impurities in a given drug substance is not only critical for crafting efficient strategies of their removal, but also plays a crucial role in the mandatory impurity and process control (Cramer, 2013).

Automated oligonucleotide synthesis on solid support utilizes a repetitive synthesis cycle for the addition of one base at a time to generate a single strand of desired length. Even though automated solid phase synthesis schemes and protocols have been optimized over time to provide single strands with the highest quality possible, not every incorporation does complete 100% successful (Matteucci, 1981) (Carlton H. Paul, 1996) (Salon, 2011) . Due to this incomplete coupling efficiency, a small percentage of failed

sequences (shorter fragments) are always obtained in the final crude product (Carlton H. Paul, 1996) (Salon, 2011). It is therefore essential to have scalable, easy and cost-efficient post-synthesis purification procedures for effective isolation of highly pure single strands.

Anion Exchange IEX Chromatography- A Purification Technique

Anion exchange (IEX) chromatography is a widely used technique for the purification of crude oligonucleotides, especially for oligo-ribo-nucleotides. IEX chromatography separates oligonucleotides based on negative charge differences resulting from variability of the number of phosphate groups present between shorter sequences and the desired full-length product (Cramer, 2013). Separation of a crude mixture through IEX chromatography is achieved by slowly increasing the ionic strength of the mobile phase to weaken the interactions between the oligonucleotide (poly-anion) and the cationic stationary phase (Cramer, 2013). When increasing the ionic strength in a linear gradient fashion, longer oligonucleotides having a higher charge elute later in the gradient compared to shorter oligonucleotides, which elutes earlier.

IEX chromatography provides greater advantages over other chromatographic techniques such as reverse phase chromatography (RPC), which separates oligonucleotide based on hydrophobic interactions. RPC can also be used effectively to purify oligonucleotides, is widely known for its reproducibility and broad applicability, but has various disadvantages compared to IEX chromatography (Matteucci, 1981). For oligonucleotides

of 15-25base lengths, IEX chromatography provides greater separation strength between full-length oligonucleotide and oligonucleotides with single base deletions, compared to RPC. IEX chromatography can also differentiate between sequences with differing chemical backbones such as thiolated vs non-thiolated phosphate groups (Figure 3) so called phosphorothioates whereas RPC does not perform very well due to the minor changes in hydrophobicity between thiolated and non-thiolated compounds. When scaling for larger scale purification, IEX provides additional advantages compared to RPC, such as higher loading capacity of oligonucleotides per mL of stationary phase (chromatographic media), more cost-effective chromatographic media, and, IEX chromatography requires lesser to no amount of organic solvent in the mobile phase compared to RPC which mainly requires organic (polar) mobile phases. This provides another cost, safety and environmental advantage for IEX chromatography. Hence, IEX chromatography is a preferred method of choice for purification for large-scale crude oligonucleotides.

Automated Synthesis of Oligonucleotides

Automated oligonucleotide synthesis is a commonly used approach to generate small and large-scale single strand material and has been established as a robust method of synthesizing oligonucleotide at various scales. Automated programming using a synthesizer can be implemented to instruct an instrument to robotically deliver reagents

and building blocks to synthesize oligonucleotides with desired length. For over 35 years the established chemistry applied to synthesize RNA or DNA oligonucleotides is the phosphoramidite chemistry introduced by Matteucci and Caruthers in 1981, and later modified by Koesters in 1984. The phosphoramidite building blocks utilize an orthogonal protection scheme, to allow the one directional growth of the oligonucleotide chain from the 3' to 5' end and at the same time protecting the exocyclic and phosphate reactive groups present in the nucleotide (Matteucci, 1981) (Sinha, 1984). A typical synthesis cycle and its steps are shown below in Figure 1. A critical protecting group for the correct chain growth is the acid labile Di-methoxy-trityl (DMT) group protecting the 5' Hydroxyl group of the ribose sugar ring. The 5' hydroxyl group is kept on the incoming phosphoramidite and removed prior from nucleotide present on support to grow the chain (Cramer, 2013) (Sinha, 1984). As a result, oligonucleotides can be synthesized with the 5' Di-methoxy-trityl (DMT) group remaining "on" or "off" at the 5' end position for the final base by programming the instrument with appropriate instructions. To be specific, "DMT on" single strands have the 5' OH protecting DMT group remaining on the 5' end position of the last base of the sequence. This is achieved by avoiding the final acid treatment to remove DMT group of the last base added at the end of synthesis cycle. In any case acid treatment to remove DMT group can also be done as a separate post synthesis step after purification for example. This is a commonly used technique for the purification of DNA sequences where RPC purification is most often applied. The additional strong hydrophobicity introduced through the terminal DMT group is a great chromatographic handle to aid in the reverse phase separation. In addition to this, ideally, only the material of the desired full length carries the final DMT group. There are two

main ways of generating shorter sequences carrying 5' DMT sequences impurities during the oligonucleotide synthesis. Firstly, due to incomplete detritylation and secondly shorter sequences which have been created by imperfect capping and / or incomplete coupling of the free 5' hydroxyl groups (Micura, 2002) (Carlton H. Paul, 1996). For an RNA containing sequence, it is necessary to remove the additional 2' protecting group from the hydroxyl of the 2' position in ribose. In order to achieve "DMT on" oligonucleotides when RNA bases are present a slight modification to the deprotection method is needed. In RNA containing oligos the DMT group can easily be lost during the 2' deprotection cleavage step using triethylammonium x 3HF, the most commonly used reagent to remove the *tert*-butyl-di-methyl-silyloxy (TBDMS) group . But if appropriate steps are taken the DMT group can remain stable on the final 5' base and utilized in a similar fashion as a chromatographic additional handle in IEX than in RPC chromatography.

DMT-on Synthesis and Its Advantages for Purification

Usually in IEX chromatography "DMT off" crude oligonucleotides are used, and separation is achieved only using the charge differences between shorter and longer oligos and varying the concentration of salt containing mobile phase for elution (Cramer, 2013). Oligonucleotides synthesized as "DMT on" have the terminal 5' DMT group still remaining. In this case, IEX can be used in a similar fashion, however, the purified

product must undergo an additional step for the removal of the DMT functionality, before final IEX gradient purification see Figure 2. This removal usually requires a short and mild acid treatment step. Additionally, commercially used IEX stationary phases, especially the phases used at large scale, are mainly based on hydrophobic polystyrene or similar polymer cores. The resulting hydrophobic properties can be explored, like RPC, as an additional chromatographic handle in combination with the very hydrophobic DMT group to aid in separation. As shown in Figure 3, the ideal scenario would be to load the crude material on an IEX column, apply a salt gradient strong enough to wash away non-DMT carrying shorter sequences, but retain the DMT full length product on the column. After removal of the DMT group in place on the column, a second gradient is applied to separate any remaining shorter impurities and collect the desired final full-length material. The success of this “DMT on IEX chromatography” will highly depend on the critical chromatographic steps, such as:

- Determining the amount of “DMT on” crude, (loading capacity) that can be loaded to stationary phase without overloading the column (so called breakthrough) or effecting separation efficiency
- Effectiveness of DMT-on Wash which is used for the elution of short sequence and impurities without affecting binding of the DMT-on full length sequence and therefore final product purity achievable compared to regular IEX
- Ease, effectiveness and safety of procedure used for on column detritylation without affecting yields and product quality
- Overall purity and yield achievable compared to regular IEX procedures.

Even though on column detritylation is used as a technique in the IEX purification for DNA base(s) containing oligonucleotide sequence, and at the smaller scale RPC for oligonucleotide sequence, applying this technique directly as a one step process to RNA base(s) containing oligonucleotide has not been demonstrated in the available literature.

Traditional DMT on Purification Approach- A Cumbersome Process

Traditionally DMT on purification of RNA containing single strand oligonucleotides employs a two-step process. First, reverse phase chromatography (RPC), is used for the purification of the crude RNA sequence with the DMT group remaining on at the 5' end terminal of the full-length oligo sequence. The crude DMT-on RNA sequence is loaded onto the RP- silica gel column for the separation of DMT-on and DMT-off shortmer sequences. This step is done very effectively at small scale as the hydrophobic DMT group provides a chromatographic handle for strong binding to the chromatographic media compared to the majority of shorter failure strand which do not carry the DMT group (Micura, 2002) (Thazha P. Prakash, 2008). Once the purification using the DMT-on product is complete, fractions are analyzed for the purity and pooled according to the desired purity. Pooled fractions then are dried to completion in preparation of the detritylation step for the removal of the DMT group. Usually, the Detritylation step requires 20-80% acetic acid solution in water for 20min to 2hours,

depending on acetic acid concentration to remove the DMT group completely from the RNA containing single strand (Micura, 2002) (Reiko Iwase, 2006) (Thazha P. Prakash, 2008). The reaction can be easily monitored for completion by either HPLC or LC-MS methods. Once detritylation is completed- the single strand reaction mixture needs to be neutralized. In order to avoid the use of amines or ammonia ions, which must be removed via an additional cation exchange step, due to their toxic nature *in-vivo*, sodium bicarbonate is the method of choice. This is a very lengthy and cumbersome process, requiring careful titration of diluted sodium bicarbonate solution (15-20ml of solution for 1mL of acetic acid used). Once neutralization is complete, the compounds need to undergo regular desalting using size exclusion chromatography to remove excess salt. In general, this two-step process requires much longer time due to the additional neutralization and chromatographic steps which cannot be scaled easily. Additionally, very small particle size range (2.5-10µm) of silica gel columns are not suitable for large scale purification due to the high-pressure generation when utilizing high flow rates and the high cost of the media. Furthermore, the silica gel media is not suitable for on column detritylation, as silica is not stable against the low pH of strong acid. Also, the use of RPC for purification has some disadvantages such as RPC does not perform very well for the separation of thiolated and non-thiolated compounds due to their minor difference in hydrophobicity (Sinha, 1984). When scaling for larger scale purifications, IEX provides additional advantages, such as higher loading capacity of oligonucleotides per mL of stationary phase (chromatographic media) and more cost-effective chromatographic media. Additionally, IEX chromatography requires lesser or no amount of organic solvent in the Mobile phase compared to RPC which utilized organic (polar) Mobile

phases (Matteucci, 1981) . At larger scale, the neutralization step becomes time limiting and poses a potential for 3' to 2' phosphate bond migration on the ribose sugar which can occur on RNA under extended exposure at lower pH (Figure 7). Impurity generated due to this bond migration have isobar which make it impossible to detect by LC-MS technique. Additionally, this impurity is associated with potential toxicity which make it critical to avoid its generation at first step (Gabor Butora, 2011) (Walter R. Stapps, 2008). Furthermore, there is a potential risk of over-titrating into high pH which is also equally destructive for the RNA, as RNA is base labile and easily will hydrolyze under those conditions. Therefore, the titration step needs to be closely monitored.

We believe that the DMT-on purification combined with on-column detritylation can be done efficiently and without the need of two step purification. It substantially reduces hands on time and simplifies the challenging neutralization step which could affect stability of RNA structure integrity. With the proposed one step IEX purification using an on column detritylation technique, we see the potential to improve product purity without compromising yield. Therefore, this method can be used as a practical applicable, cost effective and scalable additional purification techniques to get high purity RNA containing single strands.

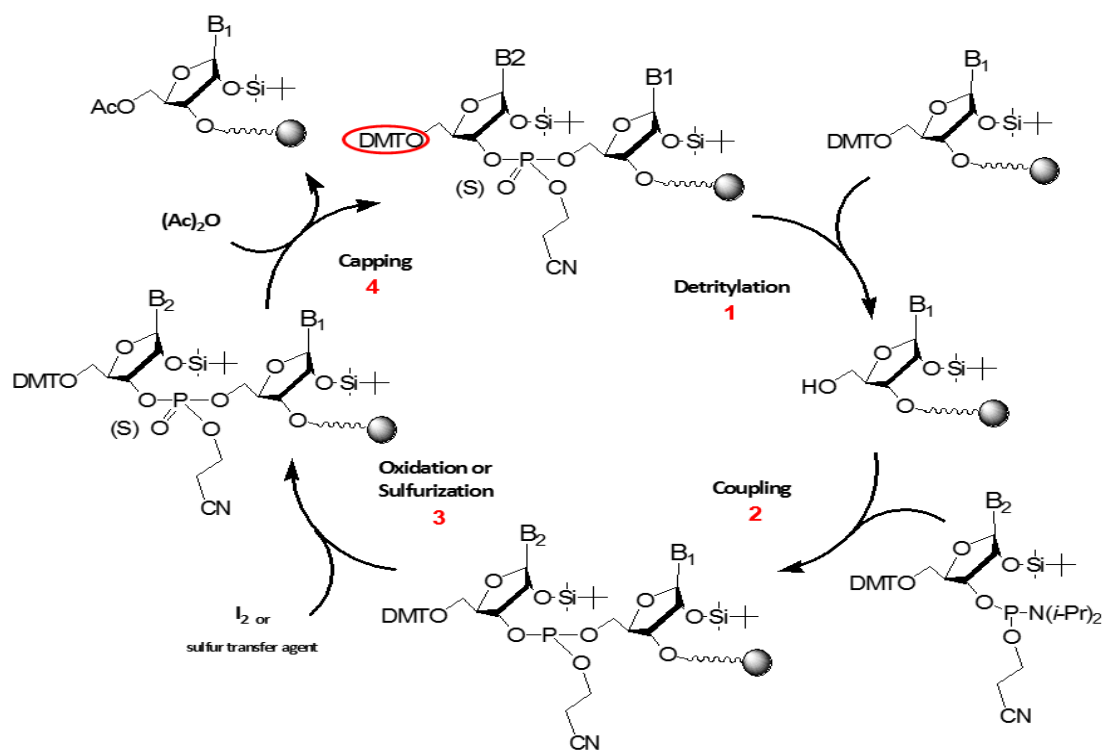


Figure 1. General Synthesis Scheme of Oligonucleotides

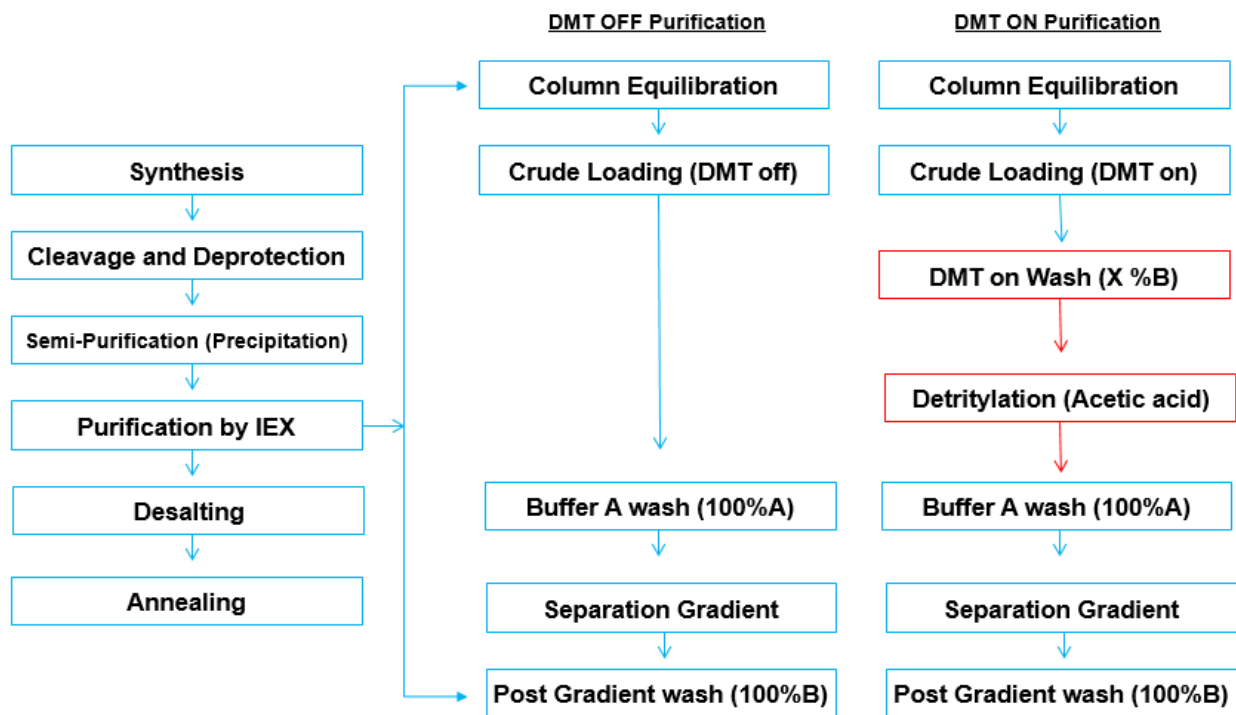


Figure 2. Schematic of Oligonucleotide Purification

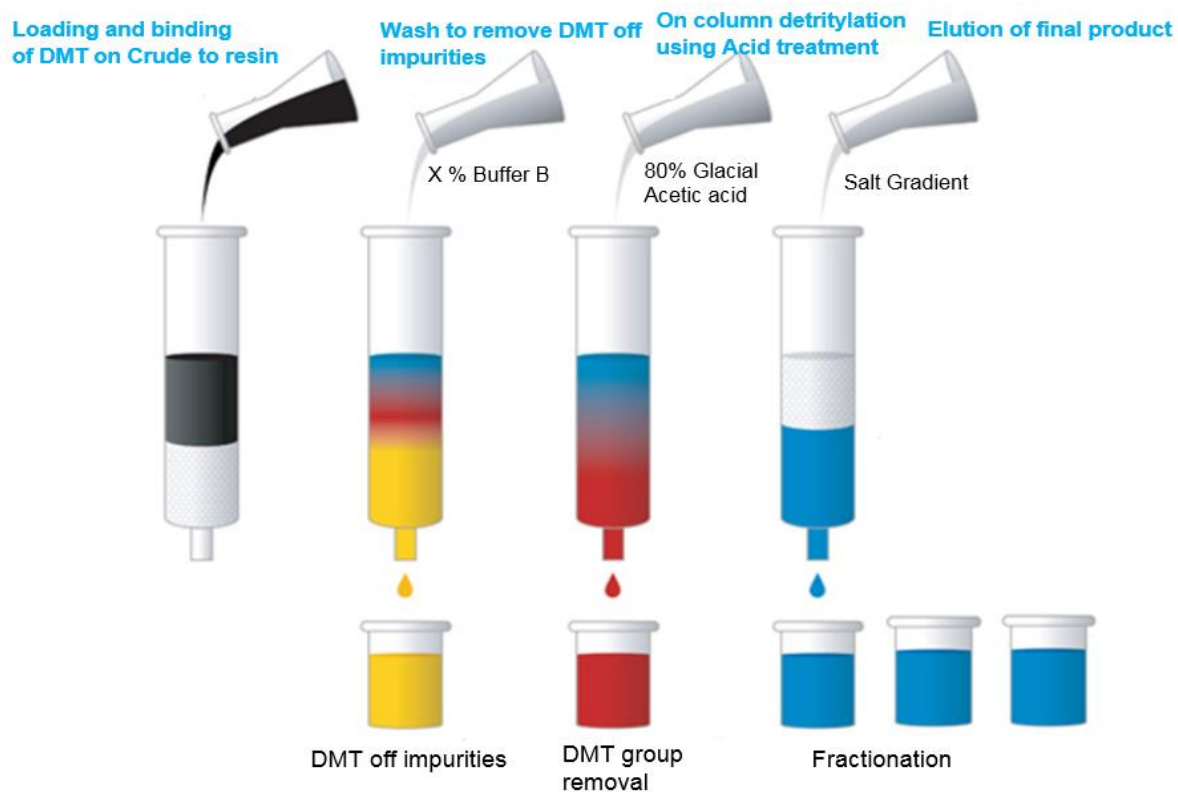


Figure 3. Oligonucleotide Chromatography Steps

Ribo Adenosine (rA)	Ribo Guanosine (rG)	Ribo Cytidine (rC)	Uridine (rU)
<chem>Nc1ncnc2n(cnc12)[C@@H]3O[C@H](CO)[C@@H](O)[C@H]3O</chem>	<chem>O=c1nc(=O)[C@@H]2[C@H](CO)[C@@H](O)[C@H]2n1</chem>	<chem>Nc1cc[nH]c1=O[C@@H]2[C@H](CO)[C@@H](O)[C@H]2O</chem>	<chem>Nc1nc2[nH]cnc2c(=O)[nH]1[C@@H]2[C@H](CO)[C@@H](O)[C@H]2O</chem>

Figure 4. Structures of RNA bases



Figure 5. Structure of Protecting Groups

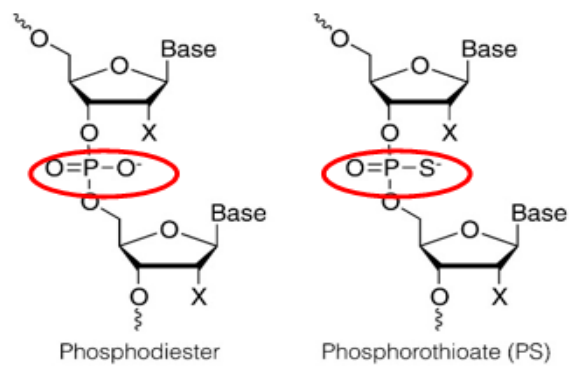


Figure 6. Non-Thiolated (phosphodiester) Vs Thiolated Phosphate Group Nucleotide.

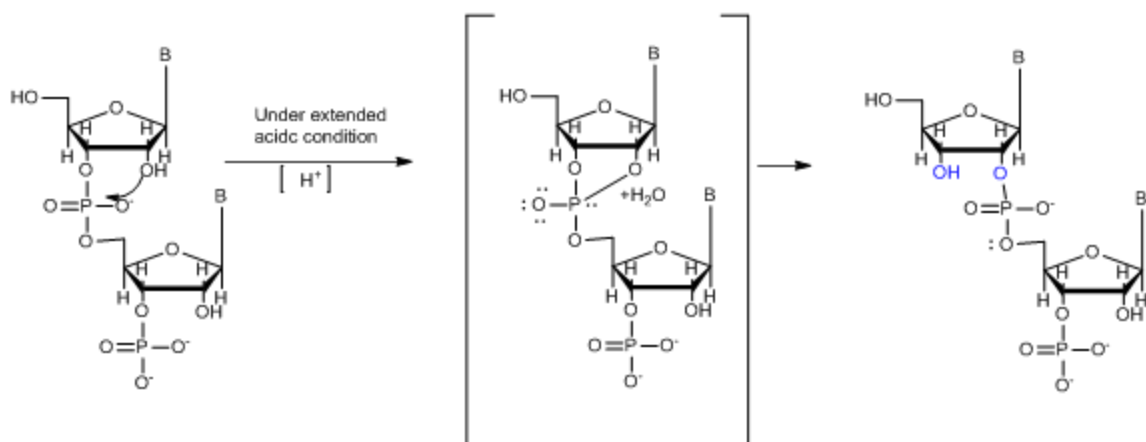


Figure 7. 3' to 2' bond migration on the ribose sugar of RNA under extended exposure at lower pH

Chapter II

II. Materials and Methods

It is ideal to obtain the enough materials (oligonucleotide single strands) to execute planned experiments in one batch so the preliminary tests could be done to assess the quality of the starting materials. Based on the design of the experiments, starting materials could be divided into smaller lots to provide starting materials for each experiment.

Crude and Reference materials

Approximately 1250 milligrams (30,000OD) of Crude DMT-on oligonucleotide single strand containing 21 bases (Figure 8) was obtained from large-scale synthesis group here at Alnylam Pharmaceuticals Inc. The material was split into smaller batches to provide the same starting point for the planned experiments in support of this project. Identical oligonucleotide single strand, without the DMT group at 5' end terminal (DMT-off) was also obtained from the medium scale synthesis group and used as reference

material. Single strand sequence mass identification, purity, and concentration analysis was carried out using techniques such as Liquid chromatography-Mass spectrometry (LC-MS), High-Performance Liquid Chromatography (HPLC) and UV spectrometry.



Figure 8. Illustration of Oligonucleotide sequence with GalNAc at 3' end and the DMT group at 5' end

Calculations of Column Volume

$$\text{Column Volume (CV)} = \pi r^2 L$$

$$\text{CV} = 3.14 * (0.5\text{cm})^2 * 17\text{cm} = 13.3\text{mL}.$$

$$\pi = 3.14, r = \text{column radius}, L = \text{Length of column (or packed bed height)}$$

Stationary phases

Two identical columns of one-centimeter (cm) diameter and 17cm bed height and total column volume (CV) of 13mL were used for these experiments. We have used two very different stationary phases for these experiments which have very different

physio-chemical characteristics (Table 1). As a result, chromatographic behavior of both stationary phases is very different, however both stationary phases find application depending on the desired outcome of the purification and are widely used in the oligonucleotide field.

One column was packed with Q Sepharose ® FF (GE life science) stationary phase which is a strong anion exchange stationary phase and contains a highly cross-linked agarose base matrix. The matrix contains a non-swellable particle with the average size of 90µm and functionalized with quaternary amines. Having a larger particle size results in a smaller number of theoretical plates for a given bed height, therefore provides less selectivity, but allows for a higher flow rate without generating high back pressure for the pumps. In general, Q Sepharose ® FF stationary phase provides a great alternative and good separation profiles when the quality of the starting crude materials is relatively high, and no critical impurities close to the full-length material is to be purified. Due to its fast process time and less mobile phase consumption, it's a cost-effective stationary phase for the large-scale applications.

The second column was packed with TSK gel® (Tosoh Bioscience) stationary phase which is also a strong anion exchange stationary phase and composed of non-swellable, highly cross-linked polymethacrylate beads that have also been functionalized with quaternary amines. An average particle size of TSK gel ® stationary phase is 25µm which allows for a high number of theoretical plates therefore provides higher separation capability. However, operating at higher flow rate becomes difficult and can add additional time to the chromatography as operational flow rates must be lower. TSK gel®

provides great advantages when critical impurities (hard to separate) need to be purified or the target purity of single strands needs to be high.

With the comparison of Q Sepharose ® FF and TSK gel ® stationary phase, we cover the two most commonly used stationary phases in the industry. Q Sepharose ® FF with its large particle size and lower price does represents a cost-effective solution for large-scale purification. In contrast TSK gel® (Tosoh Biosciences) due to its smaller particle size, is known to provide a better separation of oligonucleotide single strands, however, it is a much more expensive and therefore less cost-effective option at a larger scale. Depending on the desired outcome, both stationary phases do find wide application in the oligonucleotide industry.

Both columns were tested on GE AKTA explorer purification system (GE Healthcare life science), to provide the chromatographic setup for all experiments. Only one column was tested (used) at a time. Column packing was checked visually prior to the start of the experiments to confirm that both columns are packed properly and do perform equally well.

Table 1. Physio-chemical Characteristics of Stationary Phase

	TSK Gel ® Super Q 5pw- Tosoh Bioscience	Q Sepharose ® Fast Flow- GE life Science
Vendor	Sigma-Aldrich	Sigma-Aldrich
Matrix	highly crosslinked polymethacrylate beads	cross-linked agarose
Ave. Particle size	25µm (20-30µm), Hydrophilic	90µm (45-165µm)
Functional Group	-R-N ⁺ -(CH ₃) ₃ , R=Proprietary	-CH ₂ -N ⁺ -(CH ₃) ₃
Cost	\$ 5500/liter	\$ 2400/liter

Analytical Methods

AX- HPLC Mobile phase	mobile phase A {20 mM Sodium Phosphate, 10% ACN, pH 11} mobile phase B {20 mM Sodium Phosphate, 1M NaBr, 10% ACN, pH 11}
-----------------------	---

Column	Thermo Scientific, DNAPac-PA200 analytical column, 8 μ m, 4 $\times$ 250 mm Product No. 063000,
Column volume	3.14 mL
Column Temperature	30 °C
Flow Rate	1.2 mL/min.
Detection, Signal	260 nm, BW = 4 nm
Reference, Signal	400 nm, BW = 50 nm
Injection Volume	25 μ L
Run Time	12 min

Table 2: AX- HPLC parameters

AX-HPLC Gradient table:

Time (min)	Flow (ml/min)	% mobile phase A	% mobile phase B
initial	1.200	77	23
0.50	1.200	77	23
2.00	1.200	60	40
9.00	1.200	45	55
9.10	1.200	0	100
9.20	1.200	80	20
12.00	1.200	80	20

Table 3: Ion Pairing Liquid chromatography mass spectrometry (IPRP-LCMC) method

An optimized denaturing IP RP-UPLC-MS method was applied on the Agilent 1290 UPLC that was coupled with an Agilent 6230 TOF mass spectrometer for mass identification. The experimental conditions are described below.

UPLC parameters:

mobile phase	A: {550 mM 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 13 mM triethylamine in 90:10 H ₂ O: methanol} B: {100% methanol}		
Column	Waters Acquity UPLC BEH C18 1.7 µm, 2.1×50 mm (Part No. 186002350)		
Column Temperature	80 °C		
Flow Rate	0.40 mL/min		
Detection, Signal	260 nm, BW = 4 nm		
Reference, Signal	360 nm, BW = 100 nm		
Injection Volume	20 µL		
Run Time	13 min		
Time (min)	%A	% B	Curve
0.0	98	2	NA
1.0	98	2	Linear
9.5	86	14	Linear
9.9	40	60	Linear
10.4	40	60	Linear
10.8	98	2	Linear
13.0	98	2	Linear

MS ion source parameters:

Dual AJS ESI (Seg)	Gas Temp: 350 °C Drying Gas: 12 L/min Nebulizer: 35 psig Sheath Gas Temp: 300 °C Sheath Gas Flow: 11 L/min
Dual AJS ESI (Expt)	VCap: 3750 V Nozzle Voltage (Expt): 1200 V
MS TOF (Expt)	Fragmentor: 250 V Skimmer: 65 V OCT 1 RF Vpp: 750 V

The mass spectrometer acquisition was set to negative ion mode with the MS scan range of 900 - 3200 *m/z*. Using Agilent MassHunter Qualitative analysis software, the obtained spectrum of the main impurity peak was deconvoluted by the function of “Resolved Isotope”.

UV Spectrometry:

Instrument: Beckman Coulter DU800 Spectrophotometer or equivalent

Wavelength: 260nm

Cuvette path length: 1cm

If sample absorbance at 260nm is exceeding values higher than 1.2AU the sample should be diluted for most accurate measurements, Abs at 260nm between the range of 0.2 to 1.2 should be targeted and the dilution factor needs to be included in the calculations of final sample concentration. The total OD is calculated by multiplying the OD/ml times total volume tested.

For example: dilute sample 1 μ L in 100 μ L so the dilution factor is 100.

Sample concentration in OD/mL = Abs at 260nm * dilution factor

Total ODs = Abs at 260nm * dilution factor * total volume

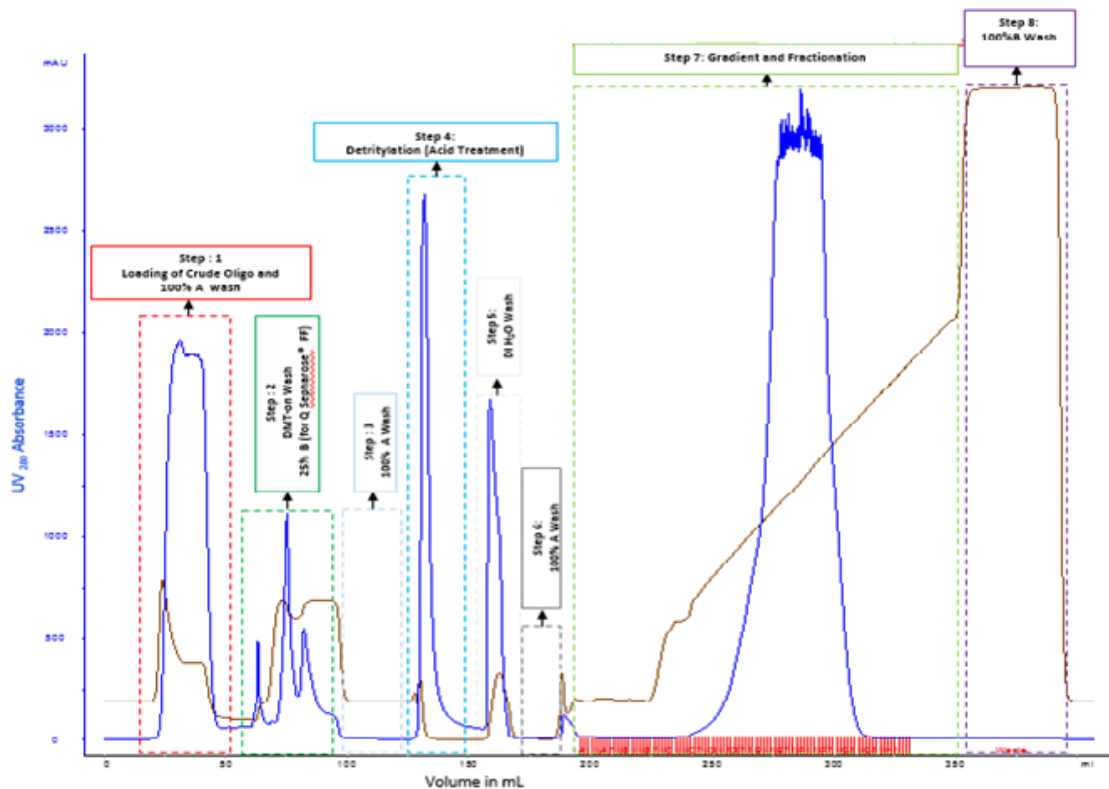


Figure 9. Process overview of a one step on column purification and DMT group removal of a DMT-on containing Single strand (Legend: Blue line = absorbance at UV_{280nm}, brown line = conductivity, Red line = fractions)

Key Steps

The key steps of the purification using an on-column detritylation techniques are: 1.) loading and retaining of crude oligo sequence on the column packed with stationary phase, 2.) The DMT-on wash to remove as much DMT-off impurities as possible, as this simplifies greatly the actual purification step and 3.) the on-column detritylation to remove the DMT group from the loaded oligo sequences for final purification gradient and isolation of high purity DMT-off oligo sequences.

Step 1: Loading of crude Oligo followed by 100% Buffer A Wash

Rational, general steps and parameters: Ensure complete binding of the DMT-on single strand material on column, without breakthrough of any Full-length material when washing with 100% Buffer A necessary to successful remove o any other synthesis impurities which will not bind to the column.

Loading of Crude Oligo (Crude concentration = 4.6mg/mL, 111OD/mL, total volume = 11.7mL) was achieved using the loading line of Akta explorer at a flow rate of 1mL/min. Flow-through from the column was continually collected for further analysis.

In General, crude oligo will completely and strongly bind to the stationary phase. Extremely short failure sequences along with the cleavage products from the protecting groups of the RNA bases do not bind to the column and can be identified in the flow-through using AX-HPLC analysis. A flow rate of 1mL/min was utilized, which is lower than the flow rate of the following chromatographic steps, as the crude material is viscous and will otherwise generate high back pressure at higher flow rates. Once loading is completed, 3 CV of Buffer A wash utilize to wash off any nonbinding materials from the column.

Step 2: DMT-on Wash

Rational, general steps and parameters: Removal of all non-DMT containing shorter strands. The buffer salt content needs to be optimized in strength to remove the maximum amount of impurities without losing any of the full length DMT-on strand material.

For the DMT-on wash, a 25% of mobile phase B for Q Sepharose ® FF and 27% of mobile phase B for TSK gel ® for 3 CV was performed. In general, this step and volume should be kept short, so that there is no unnecessary peak broadening occurring due to diffusion over the column. Therefore, an amount of 3 CV was chosen as fixed volume to achieve the maximal removal of those impurities. This step was performed at the flow rate of 2mL/min. Collected fractions were analyzed using AX-HPLC method and UV 260nm to investigate impurity removal and quantity.

Step 3: 100% A Wash

100% A Wash, post-DMT-on Wash is a 3CV mobile phase A wash needed to remove any salt from previous DMT-on Wash, and to establish the column in a no-Salt condition in preparation of the detritylation procedure.

Step 4: Detritylation (Acid treatment)

Rational, general steps and parameters:

Detritylation which can also be referred to as acid treatment was done using 80% glacial acetic acid. Stronger acids such as e.g. Dichloro- or trichloro- acidic acids, which have a faster cleavage kinetic, could be used as well and are also compatible with the column material, but do not find application at larger scale. Reason being primarily cost, but also the higher risk of damaging the RNA strand due to the stronger acidic condition and the prolonged exposure at higher scales. In order to avoid these strong alternative acids, we decide to utilize the weak acetic acid (80% glacial acetic acid) with a maximum pH of around 3.5 to remove the DMT group from 5'-hydroxyl groups of oligo base. This greatly minimizes the risk of 2'-3' migration and / or backbone cleavage (Salon, 2011). Due to the presence of multiple chemistries in our oligonucleotides, identifying the optimum deprotection of the DMT group from the hydroxyl group without affecting the quality is challenging (Salon, 2011). The 80% acetic acid provides a good balanced acid with reasonable cleavage kinetics, and low overall acidity and potential to negatively affect the quality of oligonucleotides. Additionally, its low toxicity, good

water solubility, low cost and ease of neutralization are additional plus points and further warrant its use.

First one CV of detritylation solution was provided at the 2mL/min flow rate followed by 2CV with a slower 0.5mL/min rate. Idea behind using fast flow rate first, is to quickly expose the DMT-on oligo to an acidic condition for the DMT group removal. Once the DMT group is starting to cleave a slower flow rate provides active force to help wash the cleaved the DMT group off the column. This is needed as the relatively un-polar DMT cation formed is still somewhat interacting with the column matrix and is moving only slow over the column Once the first CV of Detritylation is done, visible pale orange color could be seen from eluent coming off the column. Collected fractions were checked by UV to confirm the removal of the DMT group.

Step 5: DI H₂O Wash

2 CV of Deionized Water (DI H₂O) was passed through the column, post detritylation to remove all acid from the column. This step was done using the flow rate of 2mL/min and the column was washed until neutral pH was reached

Step 6: 100% A Wash

100% of mobile phase A wash for 3CV was done using the flow rate of 2mL/min. The rationale behind this wash was to establish the column back to buffered low salt conditions and prepare the column for separation gradient/fractionation step.

Step 7: Separation Gradient/Fractionation

The purpose behind the gradient step is to isolate the highest purity of DMT-off full length single strands. The gradient was established by varying the starting and ending gradient condition of mobile phase B over the desired column volume. For example from 5% to 60%B over 65 minutes ($10\text{cv} * 13\text{mL} = 130\text{mL}$, 130mL (total volume of gradient length), $130 / 2\text{mL/min} = 65 \text{ min}$). The Flow rate for this step is 2mL/min . Fractions collection was done using Auto-fractionation features of Unicorn software ® and fraction size was kept at 1.5mL . Fractions were collected in a 96 well deep well plate with well capacity of 2mL . Each collected fraction was analyzed by UV260nm for total OD and by analytical AX-HPLC method for their composition.

Step 8: 100% B Wash

100% B is post- gradient wash step which was done using 2mL/min for 3CV. This wash ensured elution off any impurities/un-eluted single strands that might still be bound on the column post gradient step

Step 9: Final column conditioning and Column storage

100% mobile phase A to re-condition the column for the next run for no less than 3CV or for overnight storage followed by 20% ethanol in Water for long term storage.

Demonstrate on Column Retention of DMT-on Single Strand.

Once column packing was confirmed, an amount of approximately 55mg (1300 OD) of DMT-on single strand, that is 4.2mg (100 OD) per one ml of stationary phase, was loaded to the column. The successful retention of DMT-on oligonucleotide single strand was confirmed by collecting and analyzing any UV active fractions (at 260nm) for possible single strand material eluting off the column. AX HPLC and/or LC-MS analysis was used as an analytical tool. Once confirmed the binding of DMT-on oligonucleotide single strand to the resin of the column, the next steps of the chromatography were investigated.

Explore “DMT-on” Wash- a Wash to remove Maximum Amount of Impurities Without Affecting the Binding of the DMT-on Full Length Sequence

One of the critical steps was to identify the ideal percentage of high salt mobile phase B to remove the maximum amount of impurities (Sequences without the DMT group on (DMT-off sequence). The ideal salt concentration and gradient should not elute any full-length DMT-on single strand, but most of the above-mentioned impurities. As mentioned earlier, Full-length single strand, which does contain the DMT group, has tighter binding to the stationary phase than sequences without the DMT group. A series of experiments were performed on both columns (one packed with Q Sepharose® FF

and another column packed with TSK gel®) to investigate the ideal concentration of mobile phase B, at the same time keeping the volume fixed to 3CV. Different percentage of mobile phase B, starting from 15%, 20%, 25%, and 30% were applied to decrease ionic interaction of single-strand oligo to the stationary phase using a simple step gradient. Collected fractions after the step gradient were tested using AX-HPLC and UV spectrometry to quantitate and investigate the composition of the eluted materials. Based on UV and HPLC analysis of the eluted fractions from step gradient, 25% mobile phase B was chosen as the ideal concentration to perform the “DMT-on” wash for the column packed with Q Sepharose ® FF. Based on another set of experiments, 27% mobile phase B was found to be the most effective for the column packed with TSK gel® stationary phase. For the column packed with Q Sepharose ® FF, there were no traces of full-length DMT-on single strand in collected fractions using 25% mobile phase B Wash, however, 30% mobile phase B wash did elute the DMT-on single strands which were confirmed by AX-HPLC analysis of the collected fraction.

Investigate Optimal Column Volume (CV) of 80% Aqueous Acetic Acid to Demonstrate Complete on Column Trityl (DMT) Removal

Once the “DMT on wash” was successfully established to remove the maximum amount of impurities, the next step was to remove the DMT group from the full-length single strand. It is very well known from established literature that on column detritylation for DNA containing oligonucleotide is fast and effectively achieved using

80% aqueous acetic acid (Salon, 2011). This concentration provides a mild enough condition to not harm the oligonucleotide or the chromatographic media but has enough strength to quickly and effectively remove the DMT group. For this reason and others stated before, 80% of aqueous Acetic Acid was used in all experiments to test the effective removal of the DMT group. Removal of the DMT group using acid treatment can be easily monitored by UV at the 260nm wavelength (due to many benzyl rings present in the DMT). It also has a characteristic visible faint orange color, resulting from the stable DMT cation formed, which can be additionally utilized to visually monitor the removal of the DMT group. Based on the UV monitoring and UV analysis of the Fractions collected, 3 column volumes of 80% aqueous acetic acid was established as the most effective volume to remove all the DMT groups. The use of a higher 2ml/min flow rate for 1 CV followed by a slower 0.5ml/min flow rate for another 2CV turned out to be the most effective way to ensure 100% DMT group removal. This was confirmed by the absence of no UV260nm signals at the completion of 3CV of 80% aqueous acetic acid treatment. Therefore, we considered that the DMT group has been completely removed. Once the acid treatment is completed, the column was washed using 2CV of water, to completely remove acetic acid from the stationary phase of the column. upon completion, the column was equilibrated using 3CV of mobile phase A to prepare for the gradient step.

Explore Different Gradient Conditions to Achieve Best Purity and Highest Isolated Yields

Once the removal of the DMT group had been confirmed, linear gradient conditions with high salt buffer B were tested to identify the ideal gradient conditions (chromatographic separation) providing the highest purity and maximum yields. Gradients with various starting and ending buffer B concentrations (5% buffer B to 70% buffer B) have been tested to achieve the highest purity and yield of full-length single strands. Collected fractions were analyzed for their purity, mass identity and quantity using HPLC and LC-MS and UV Spectrometry techniques respectively. Only fractions with equal or greater than 75 % of purity of full-length single strand were polled. The resulting overall yield and purity was determined from the combined fractions. The resulting data was compared for different gradient conditions to identify the optimal conditions.

Chapter III

III. Results and Discussion

Results and observations from planned experiments to investigate experimental aims are discussed here. AX-HPLC and LCMS analysis has been key techniques to investigate the each experimental procedures.

Retention of Crude DMT-On Single Strand Oligonucleotide on Column

The retention time and purity and impurity profile analysis using the AX-HPLC method was performed for the DMT-on and DMT-off crude single strand. Furthermore, mass identification using LC-MS was performed to confirm the DMT-on single stand molecular weight. As shown in Figure 10 and Figure 11, (AX-HPLC chromatograms) the retention time of the DMT-on crude strand was 4.7 minutes and the retention time of DMT-off crude was, as expected, shorter with 3.2 minutes respectively.

Figure 12 shows the overlay of DMT-on crude and DMT-off crude sequence in overlay (AX HPLC analysis). Calculated mass of the DMT-on single strand is 8844 Da and for the DMT-off single strand is 8541 Da. LC-MS analysis of the DMT-on single strand (Figure 13) shows the observed mass of 8842.4Da, $\pm$ 2.0 Dalton of the calculated mass, therefor confirms the identity of the DMT-on single strand.

Once the DMT-on single strand was loaded onto the column, 3 CV of mobile phase A was passed through the column so any unbound materials would wash out of the column. All flow-through mobile phase from loading step and additional wash step (3CV mobile phase A) was collected and analyzed by AX HPLC and LC-MS to identify any possible eluents. As shown in Figure 14 and Figure 15, no UV260nm active materials was observed which matches the retention time of the DMT-on single strand. This observation confirms the complete retention of the DMT-on single strand on the column. The LC-MS analysis further supported the retention of the DMT-on single strand to the column, as no corresponding mass of the full-length DMT-on strand was found.

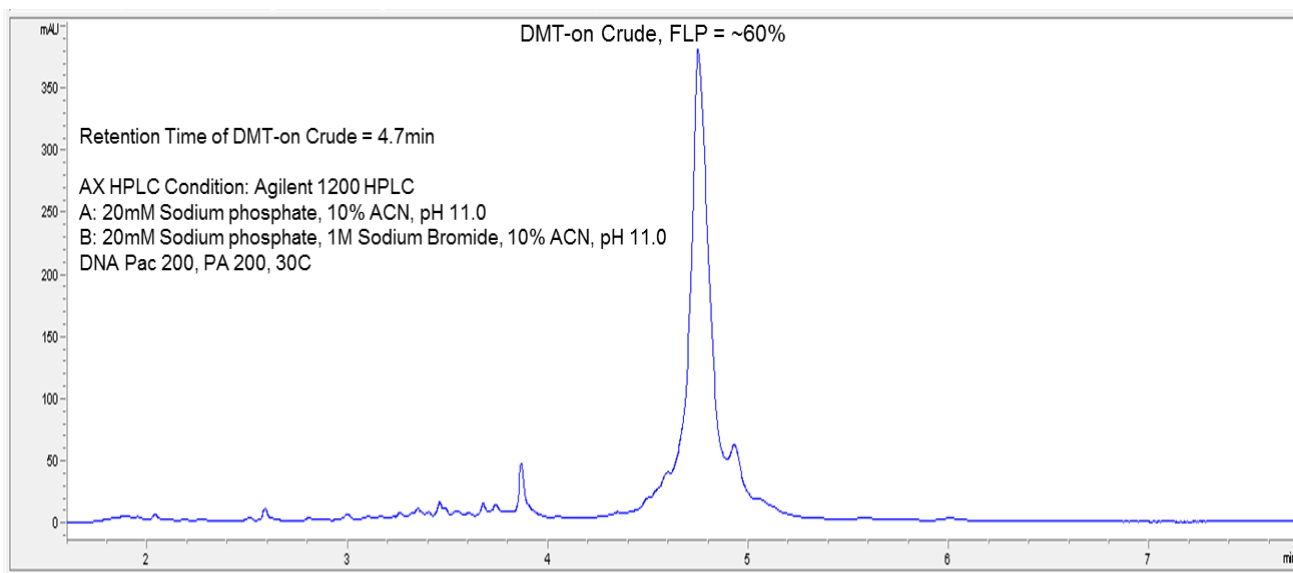


Figure 10: AX-HPLC Chromatogram of DMT-on Crude Single Strand

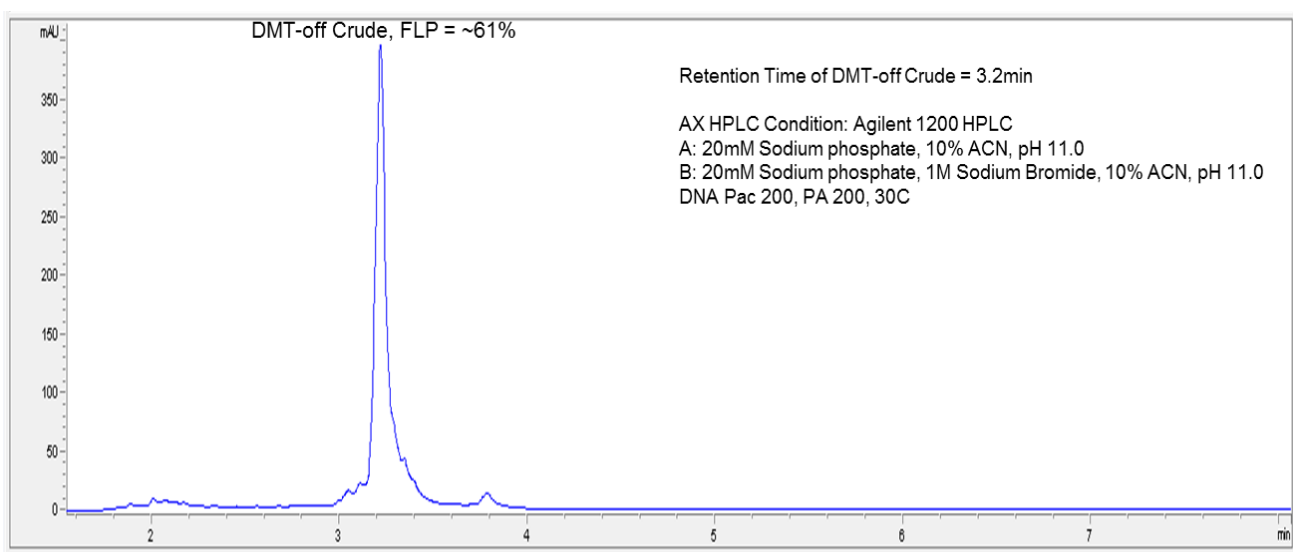


Figure 11: AX- HPLC Chromatogram of DMT-off Crude Single Strand

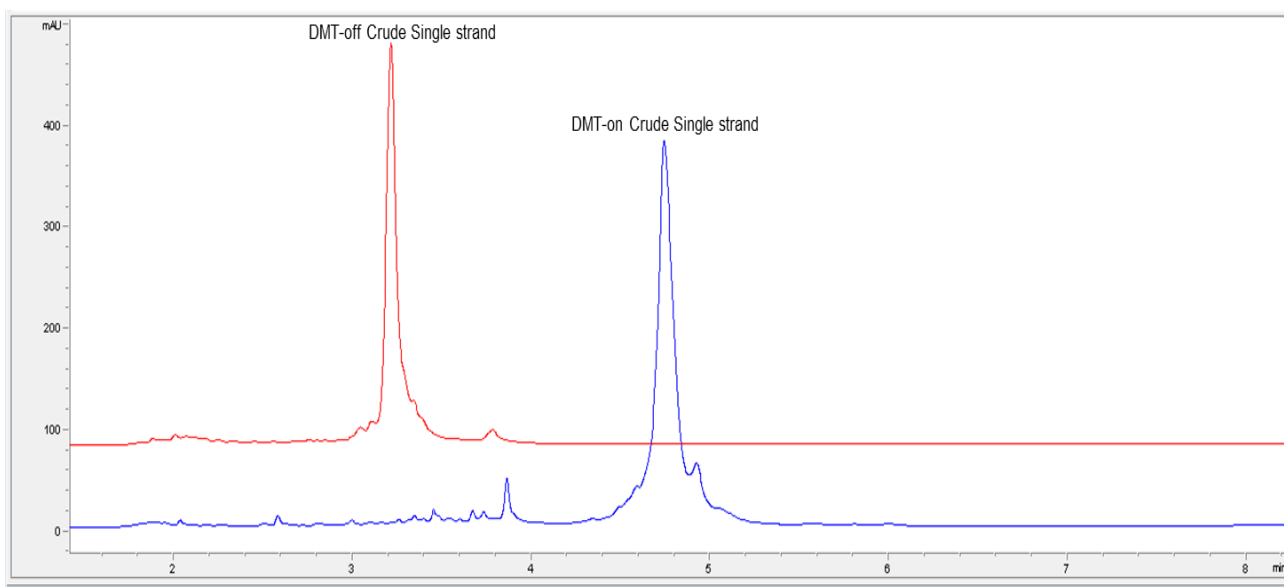


Figure 12. Overlay of AX- HPLC Chromatograms of DMT-on Crude Single Strand and DMT-off Crude Single strand

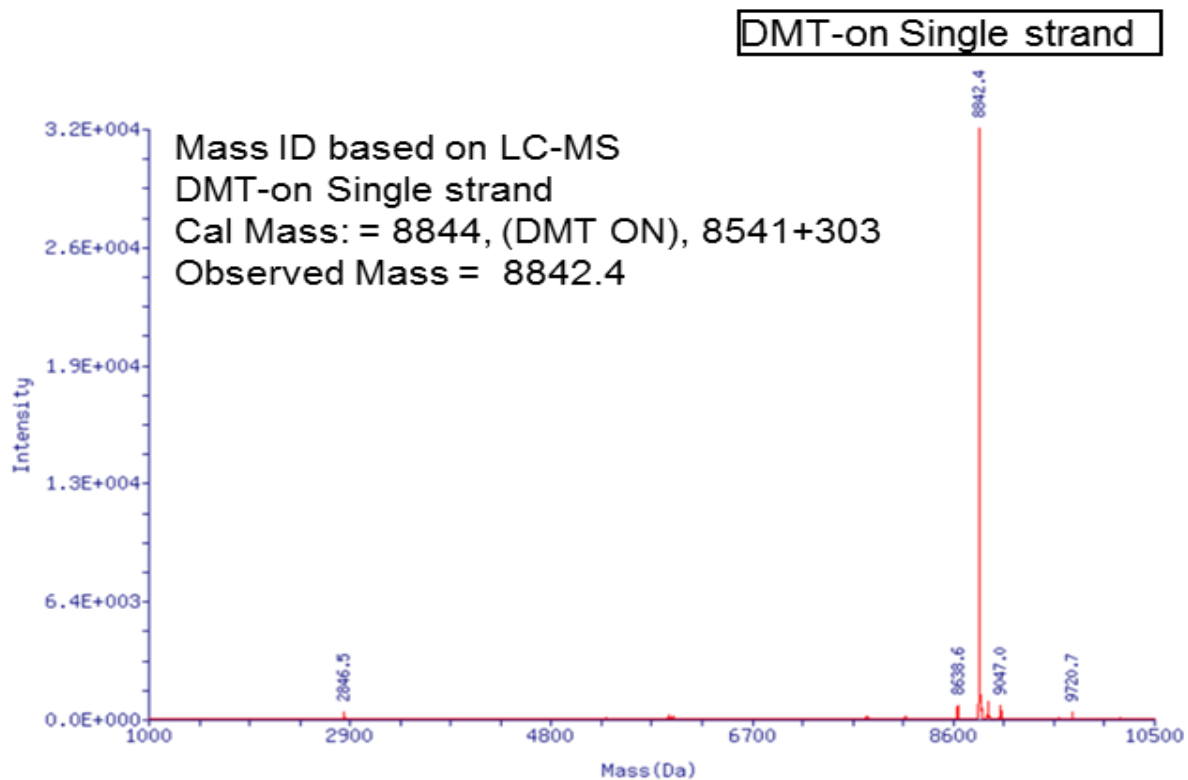


Figure 13. Mass Identification using LC-MS of DMT-on Crude Single Strand

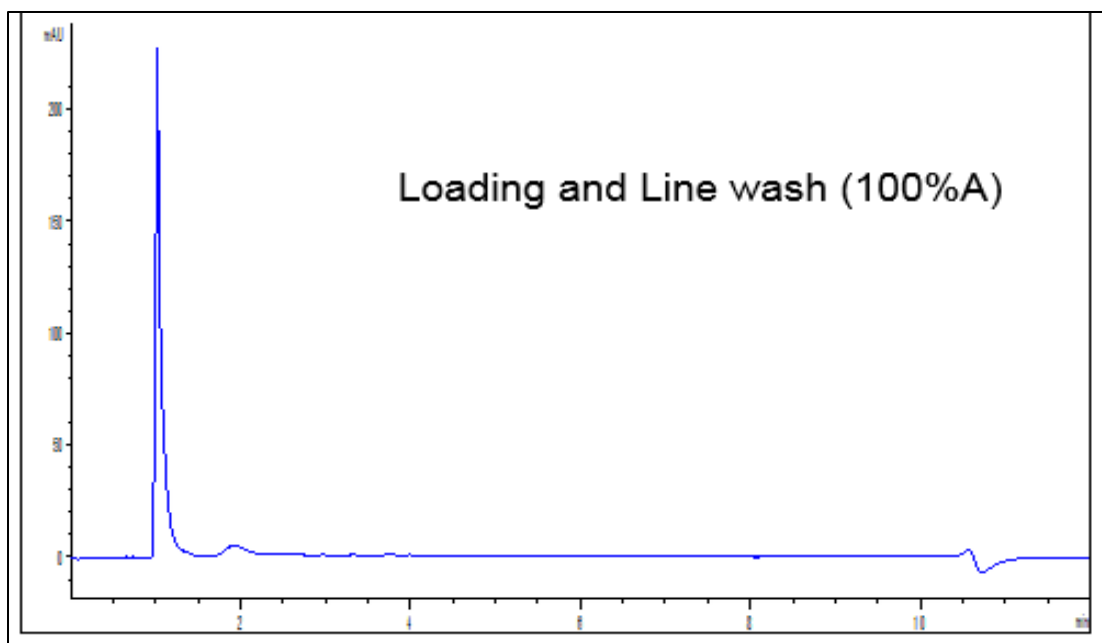


Figure 14. AX HPLC Analysis of flow through from Loading Step and additional wash Step (3CV of mobile phase A)

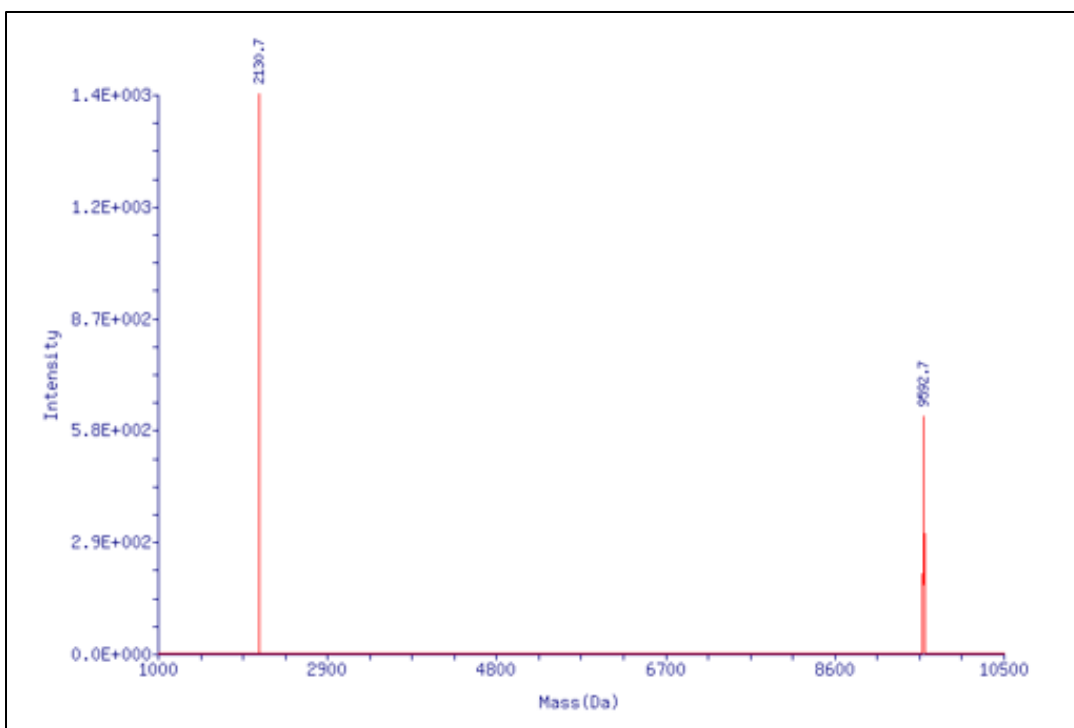


Figure 15. LC-MS Analysis of flow through from Loading and 3CV of mobile phase A

Optimization of DMT-on wash step for Maximizing DMT-Off Impurities Removal

Once retention of the DMT-on single strand onto the column was confirmed, different ratios of mobile phase A and mobile phase B were applied to the column to identify the optimum percentage of mobile phase B for the DMT-on wash step. This was done as a short simple step gradient. The ideal ratio of mobile phase A and mobile phase B would effectively remove maximum amount of the impurities without affecting the binding of the full-length DMT-on sequence. From DMT off containing material we do know that 15% Buffer B will not elute any full-length material. Therefore it was considered safe to set the starting conditions to 15% of mobile phase B (ratio of 85:15 ::

A: B). Sets of step-gradient experiments were performed by increasing the amount of mobile phase B by increments of 5% at a time. Collected fractions from these step gradient experiments were analyzed using AX HPLC and UV spectrometry. AX HPLC chromatogram of DMT-on crude was compared to the AX HPLC chromatograms of fractions collected from each step gradient experiment. As shown in Figure 16, 15% mobile phase B, 20% mobile phase B, 25% mobile phase B and 30% of mobile phase B were applied to the column as a post-loading DMT-on wash. As shown in Figure 17, AX HPLC profile of collected fractions from step gradient indicates that $\leq 25\%$ mobile phase B does not affect the binding of the DMT on single strand. There were no chromatographic peaks observed corresponding to retention time of full-length material at 4.7 minutes, indicates that no full-length material had eluted (See AX HPLC analysis part Figure 16). However, AX HPLC analysis of fraction collected from 30% mobile phase B showed the presence of a significant amount of DMT-on single strand (Retention time 4.7minutes). Furthermore, Quantitative analysis of all fractions by UV spectrometry confirms the elution of almost 90% of loaded DMT-on single strand. Based on these data we concluded, that the 30% mobile phase B cannot be used as a DMT-on wash. The AX HPLC profile of fraction collected from step gradient with 25% mobile phase B (Figure 17) did not have any eluents with the retention time of the DMT-on single strand. Eluted materials mainly show the retention time of shorter sequences which furthers shows the effectiveness of this composition. LC-MS analysis shown in Figure 18 further confirms these observations as all the masses observed corresponds to the shorter sequences and no masses of full-length single strand were identified. Based on this finding it was decided

that the 25% mobile phase B was ideally suited for the maximal removal of impurities without affecting the binding of the DMT-on single strand.

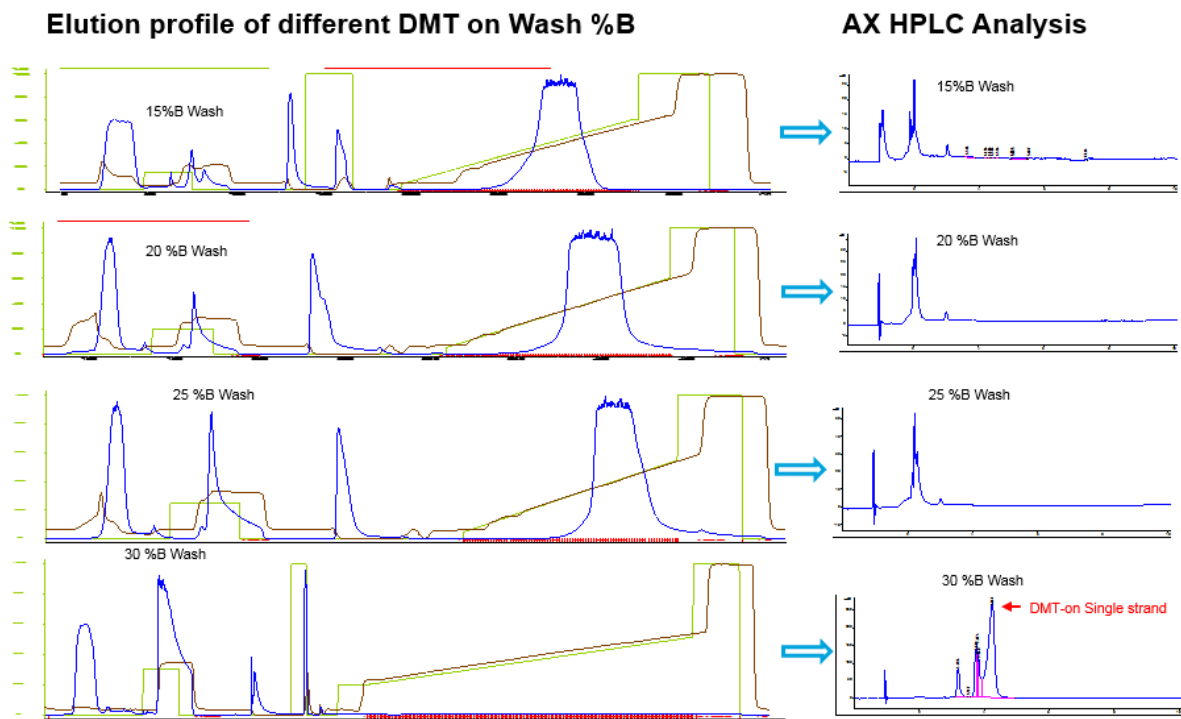


Figure 16. Elution Profile of Different the DMT-on Washes and their AX-HPLC Analysis

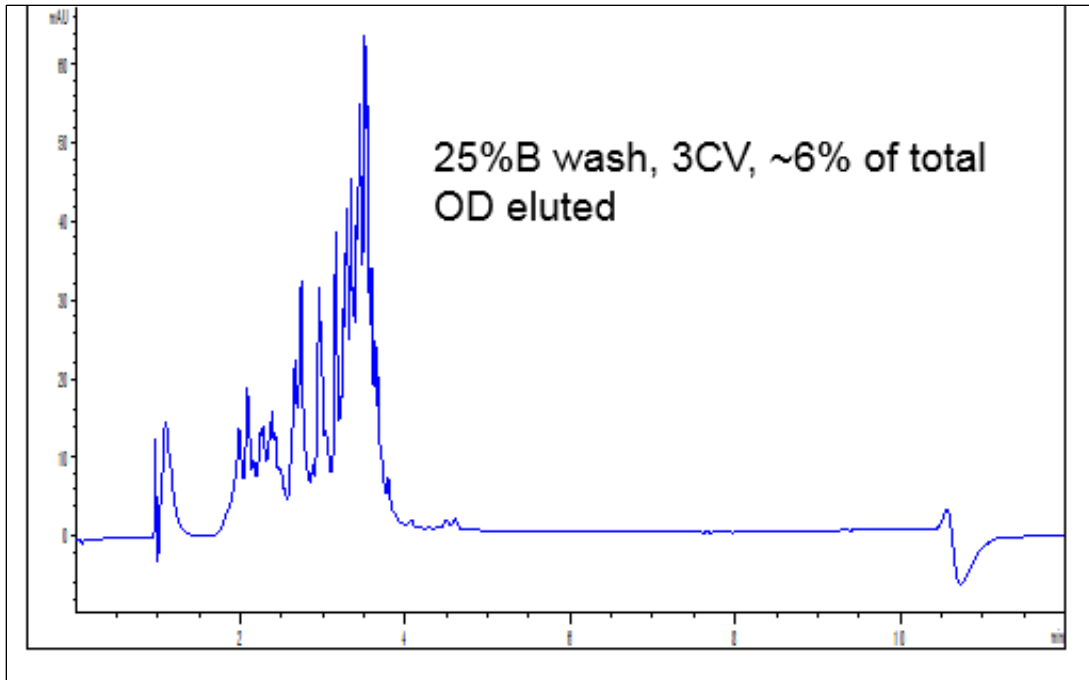
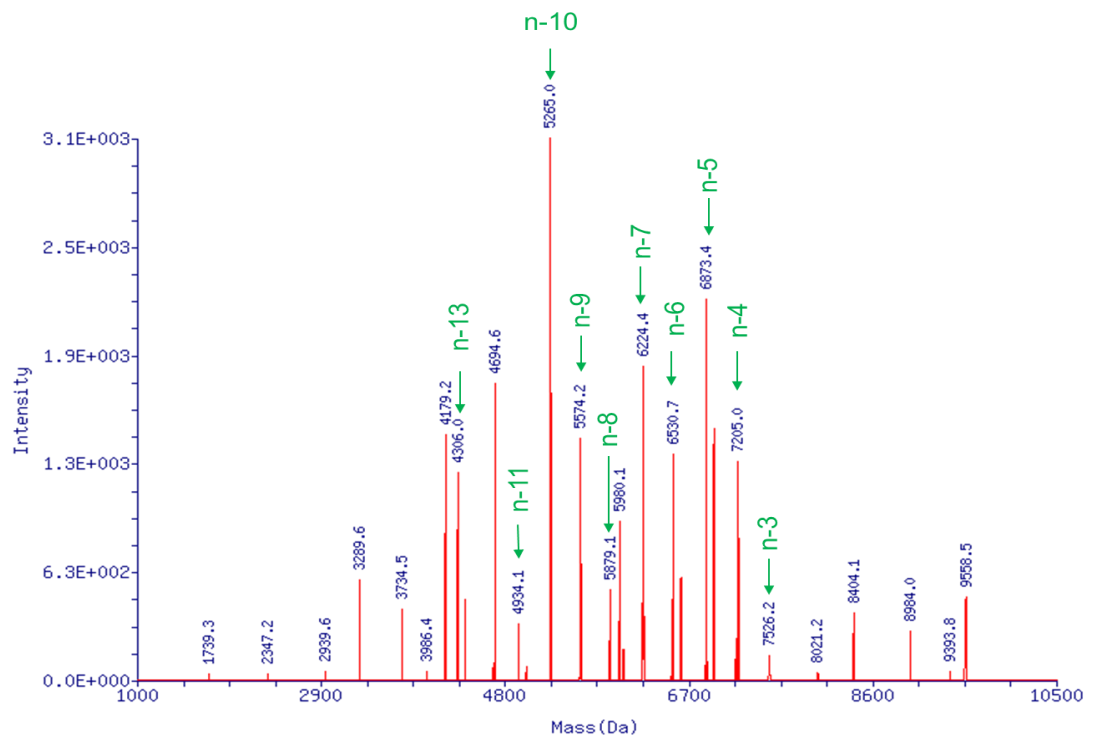


Figure 17: AX-HPLC Analysis of 25% mobile phase B Wash (3CV)



Note: n = full length sequence

Note: n-13 = 13 bases short sequences from 5' end

Figure 18. LC-MS Analysis of Eluent of 25% mobile phase B Wash (3CV). Investigation of Optimal CV of 80% Aqueous Acetic Acid to Demonstrate Complete on Column the DMT group Removal

Once, the DMT-on wash was optimized and fixed at 25% Mobile phase B, the next step was to investigate the amount of 80% aqueous acetic acid needed to quickly and effectively remove the DMT group from the DMT-on single strand. As mentioned in the material and method section, the removal of the DMT group using acid treatment can be easily monitored by UV at 260nm wavelength. It also has a characteristic visible faint orange color, which can be utilized to visually monitor the removal of the DMT group. The 80% aqueous acetic acid was applied in steps of one column volume at a time and all flow through was collected to check the UV absorbance at 260nm and monitor the removal of the DMT group. Based on UV absorbance reading at 260nm, it was determined that 3CV of acid treatment was enough to effectively and completely remove the DMT group completely from the loaded single strand oligo. Increasing the 80% aqueous acetic acid treatment beyond 3CV did not result in fractions with any 260nm UV value. Therefore, it was concluded that all the DMT groups had been completely removed from the full-length oligo sequence.

Gradient Conditions for Achieving Best Purity and Highest Isolated Yields

The final step is the purification of the now DMT-off single strands using a linear salt gradient. Aim is to find the ideal gradient conditions with highest purity and yield.

In general, two general buffer types, neutral pH (pH = 7.4) or high pH (pH=11), are used for the chromatography the single strand oligonucleotides. Compositions are shown in Table 3. Both types of buffer systems were evaluated for their performance, the neutral pH / higher organic containing mobile phase and the high pH/lower organic containing mobile phase. Using the neutral pH mobile phase, the higher organic content was needed for effective denaturing of the single strands and therefore sufficiently enhance the binding of the single strand oligo to the stationary phase of the column. In the high pH buffer, the higher pH is enough to denature the oligo already, allowing for a, lower organic content. All previously established experimental conditions such as column size, loading of crude oligo, DMT-on wash and amount of 80% aqueous acetic acid wash volume were kept constant.

First set of experiments were performed using a column packed with Q Sepharose FF ® stationary phase and neutral pH (pH=7.4) mobile phase. Based on a series of experiments, best purity and isolated yield were achieved by a gradient of 5%B to 60% B over the length of 10CV. Fractions were collected in 96 deep well plate with 2ml well size. Size of the fraction was kept 1.5ml to avoid any overflow of the well. Total of 89 fractions were collected per run. The UV absorbance of each fraction at 260nm was measured for mass balance purpose (total load versus recovery). Figure 19 shows the step

by step profile of the purification process; the blue line represents the absorbance at 260nm on the y-axis. The x-axis shows the fraction and respectively column volume (mL). UV absorbance at 260nm was also performed for every step of the purification. Figure 20 shows the resulting mass balance distribution of the complete purification run. The OD distribution chart indicated that mid-section of the gradient eluted the higher concentration fractions. Approximately 97% of total loaded materials was eluted and recovered, between the two impurity washing steps (the DMT-on wash (3CV) and separation gradient) which suggested that there was very little loss of full-length material during other steps of the purification process. Figure 21 shows chromatographic separation of FLP and other moieties during the gradient and their AX HPLC analysis. As anticipated, fractions from the middle portion of the gradient provided the highest purity. Figure 22 shows the overlay of AX HPLC profile of pure fractions, fractions 5 and 6 indicates the removal of front impurities (shown as a red dotted arrow and box) and reduced the impurities which eluted as a back shoulder (shown as blue dotted arrow) compare to other less pure fractions which again showed the effectiveness of the gradient. Figure 23 shows AX HPLC profile for the comparison of crude DMT-on single strand vs purified DMT-off single strand (highest purity fractions pooled together) using Q Sepharose FF ® stationary phase. Based on the data shown here, it has been demonstrated that the optimized purification conditions purified most of the early eluting impurities and reduced the back-shoulder impurities and provides good quality DMT-off single strands using on column detritylation technique.

mobile phase pH	mobile phase composition
pH: 7.4	Buffer A: 20 mM Tris, 1mM EDTA, 10mM NaClO ₄ , 20% ACN Buffer B: 20 mM Tris, 1mM EDTA, 500mM NaClO ₄ , 20% ACN
pH: 11	Buffer A: 20mM Sodium phosphate, 10%ACN, Buffer B: 20mM Sodium phosphate, 1M Sodium Bromide,10%ACN

Table 4: Purification mobile phases

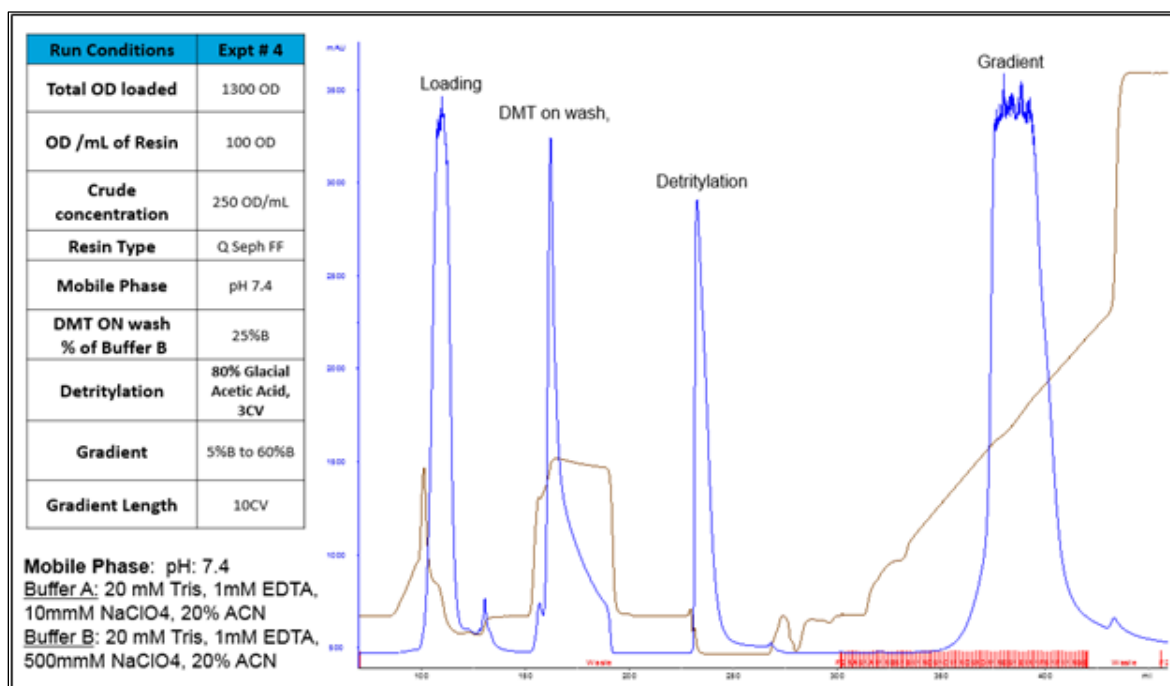


Figure 19: AKTA Purification Profile Using Q Sepharose FF ® stationary phase Legend: Blue line = absorbance at UV280nm, brown line = conductivity, Red line = fractions)

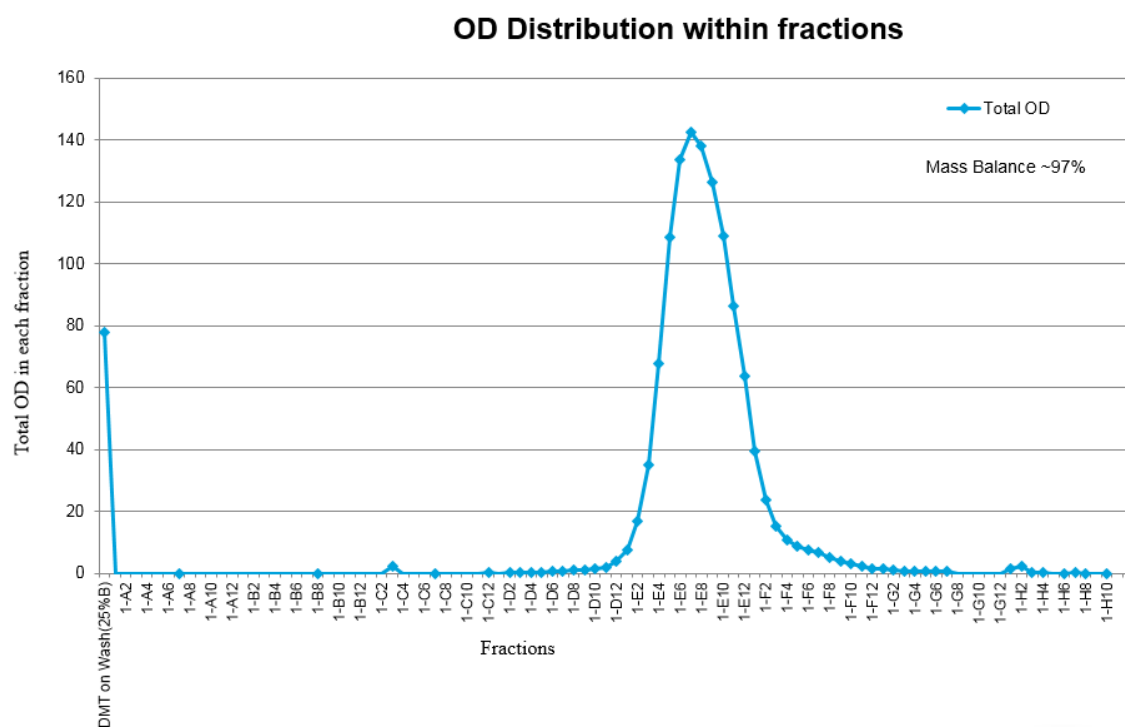


Figure 20. Distribution of Mass Balance during the Salt Gradient using Q Sepharose FF[®] stationary phase

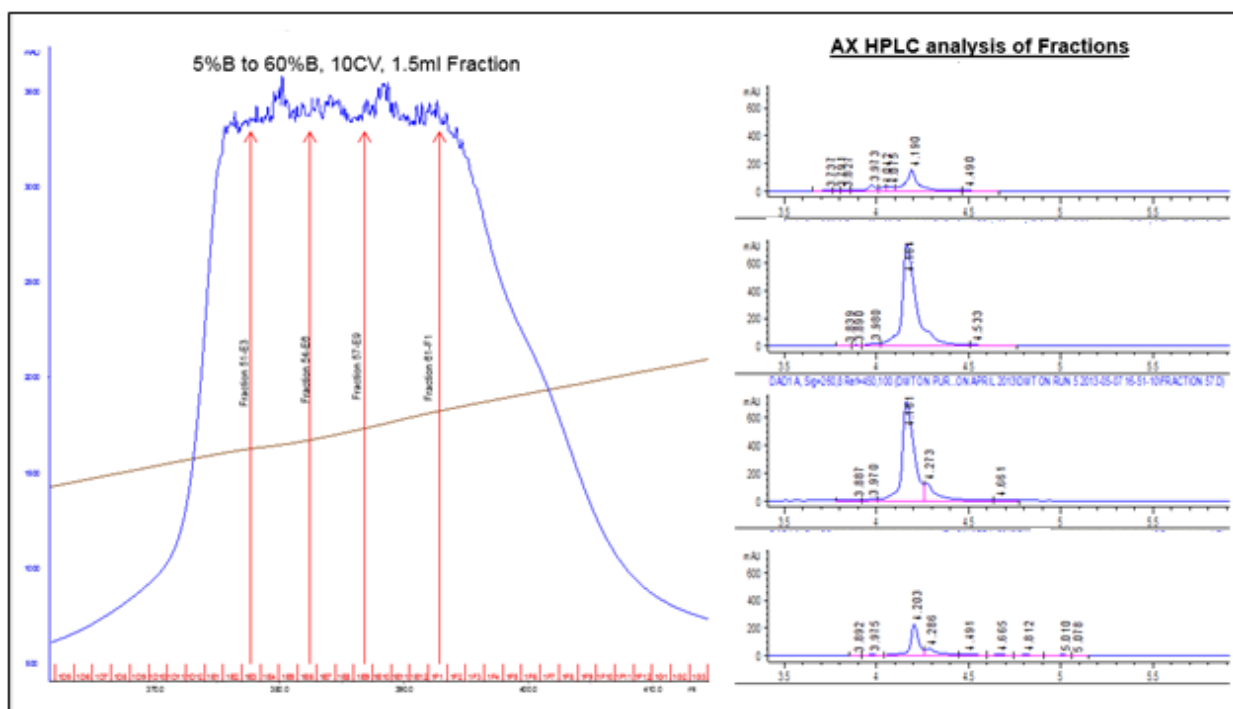


Figure 21. AX-HPLC Analysis of the fractions from the gradient using using Q Sepharose FF ® stationary phase

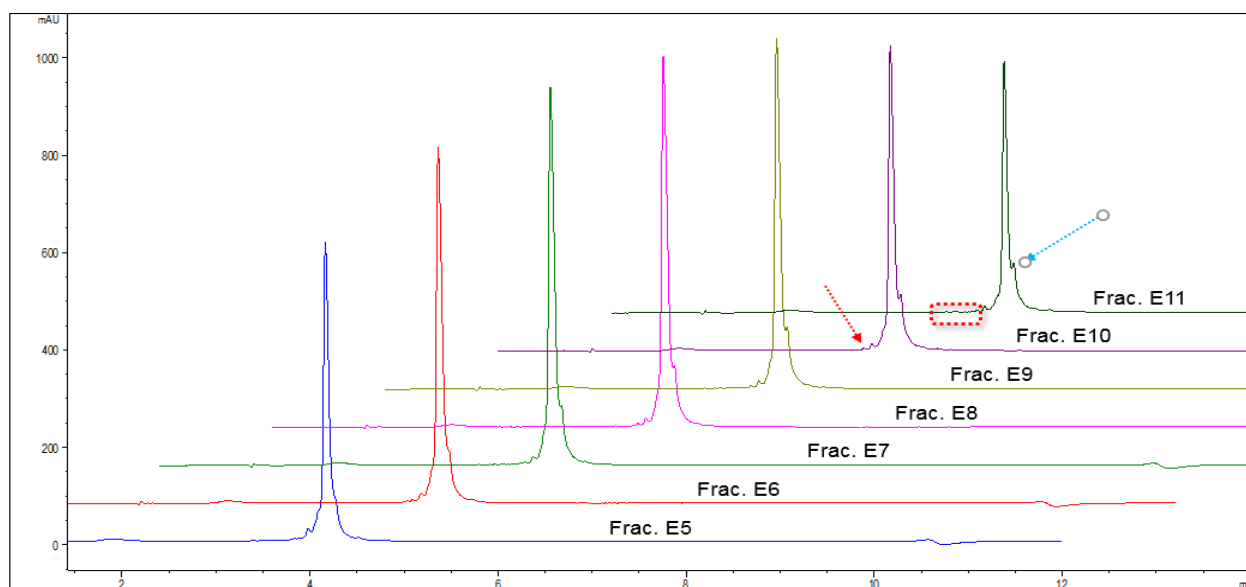
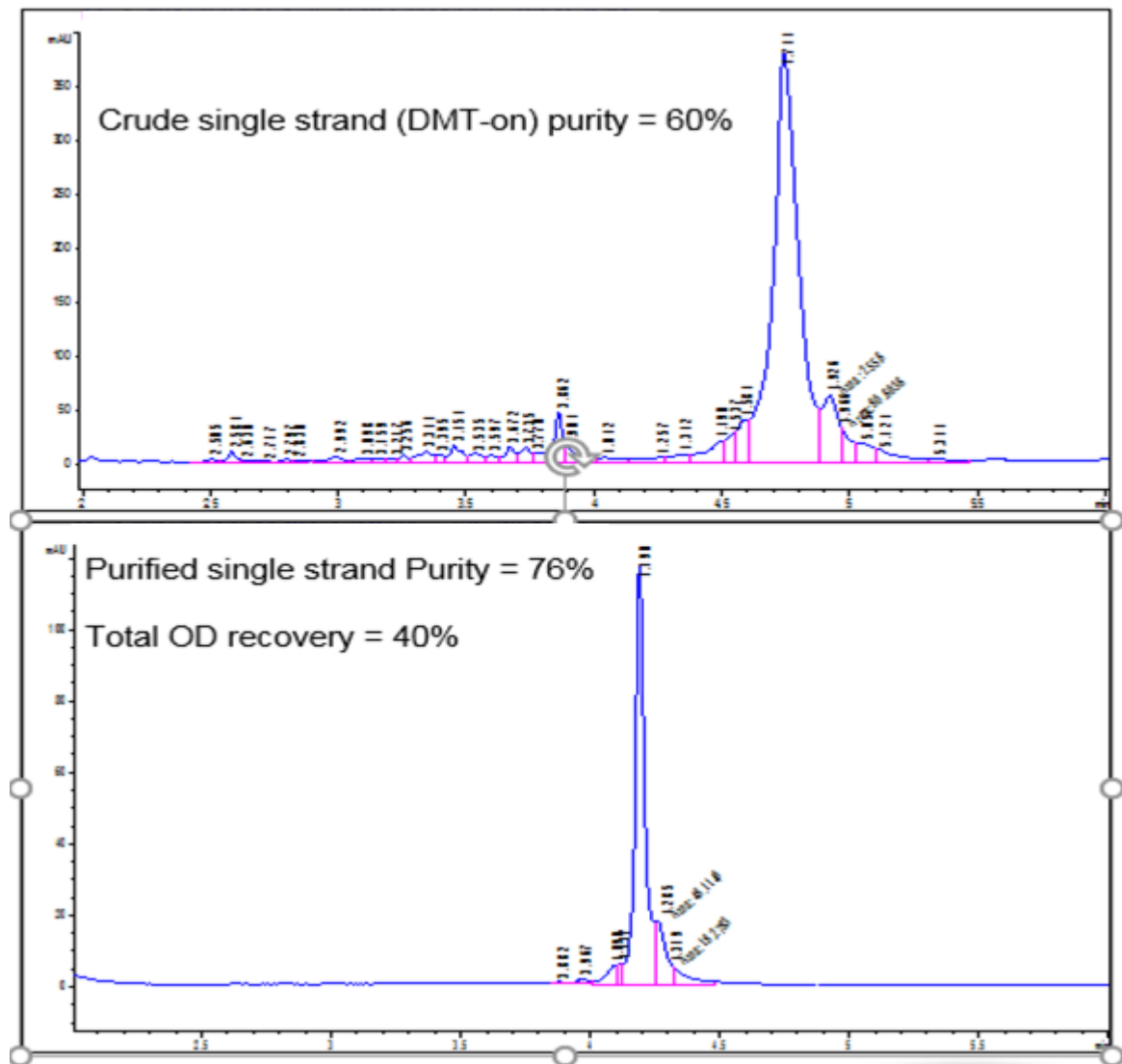


Figure 22. Overlay of AX-HPLC Profiles of main peak Fractions indicating Removal of Front and Late Impurities for the Purification using Q Sepharose FF® stationary phase



Note: Purified single strand are all fractions with FLP purity >75% pooled together.

Figure 23. Comparison of Crude (DMT-on) vs Purified (DMT-off) single strand using Q Sepharose FF® stationary phase

Evaluate Q Sepharose® FF and TSK gel® for the Purification of DMT- on Single Strands

For this evaluation, another 13.3mL column (identical in size) packed with TSK gel ® stationary phase was used. Majority of Chromatographic steps except DMT-on wash and gradient were kept constant. Initial experiments suggested that higher pH (pH = 11.0) mobile phase performed better therefor higher pH mobile phase were chosen for this set of experiments. As mentioned earlier, the physical and chemical characteristic of both stationary phases is different thus we anticipate to observed different chromatographic behavior from both stationary phases.

Firstly, the set of experiments were performed to investigate the optimum percentage of mobile phase B for the DMT-on wash. Based on the observations for these experiments, 27% mobile phase B was chosen to be effective for the impurity removal. TSK gel ® contains very fine particle therefore theoretical plates numbers are higher which provides better separation. Also due to a high number of theoretical plates, longer gradients are required for achieving the separation. Another set of experiments were performed to identify separation gradient conditions which obtain the highest purity of DMT-off single strands with the highest possible yield. Based on this experiments, gradient condition of 20% mobile phase B to 45% mobile phase B with the gradient length of 20CV showed the best result (20%B to 45%B over 130min). Step by step

profile of the purification process using TSK gel ® stationary phase and high pH mobile phase is illustrated in Figure 24. The blue line represents the UV absorbance at 260nm on the Y-axis. The X-axis shows the column volume in mL. Absorbance at 260nm was checked after loading, DMT-on wash and detritylation to confirm the retention of DMT-on single strand on the column. Figure 25 shows the mass balance distribution of complete purification process. OD distribution chart indicated that higher OD elution occurred during the middle portion of gradient. Approximately 98% of total loaded materials was recovered during the DMT-on wash and separation gradient step. Figure 26 shows the chromatographic separation of FLP and other moieties during the high salt gradient and their AX HPLC analysis from the collected fractions. As anticipated, fractions from middle portions of gradient provided the highest purity fractions which is also very similar to observations from Q sepharose FF ® experiments. Finally, Figure 27, shows the comparison of crude DMT-on single strand vs purification DMT-off single strand using on column detritylation technique with the TSK gel ® stationary phase. The identity of the purified single strand was also confirmed using LC-MS analysis (Figure 28), observed mass matches the molecular weight of the DMT-off single strand. With optimized gradient conditions, and the better chromatographic media, we were able to achieve much higher overall purity containing fractions, however overall yield was lower compared to the Sepharose experiment. Comparing the chromatographic performances of both stationary phases, TSK gel ® stationary provides higher purity by removing the close eluting impurities to full length single strand which was not removed using Q Sepharose FF ® stationary phase, however for larger scale chromatography Q sepharose

FF ® stationary phase still provides a good option when target purity is not of utmost requirements and overall yield is more critical.

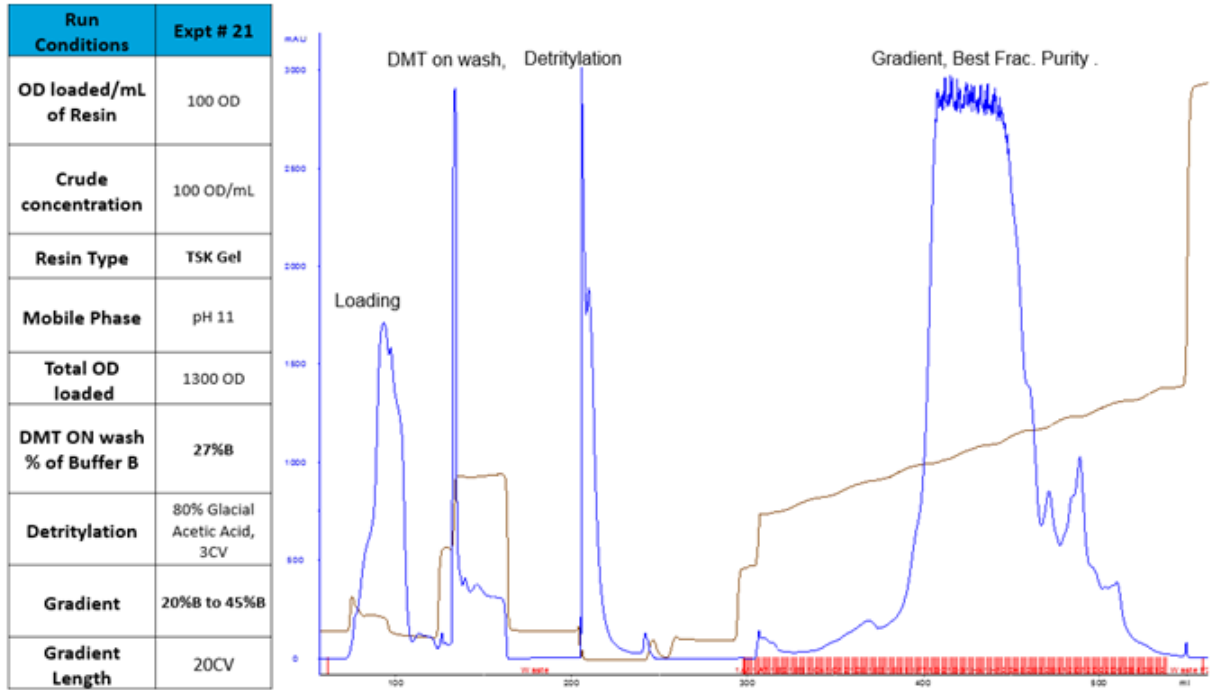


Figure 24. AKTA Purification Profile using TSK gel ® stationary phase (Legend: Blue line = absorbance at UV280nm, brown line = conductivity, Red line = fractions)

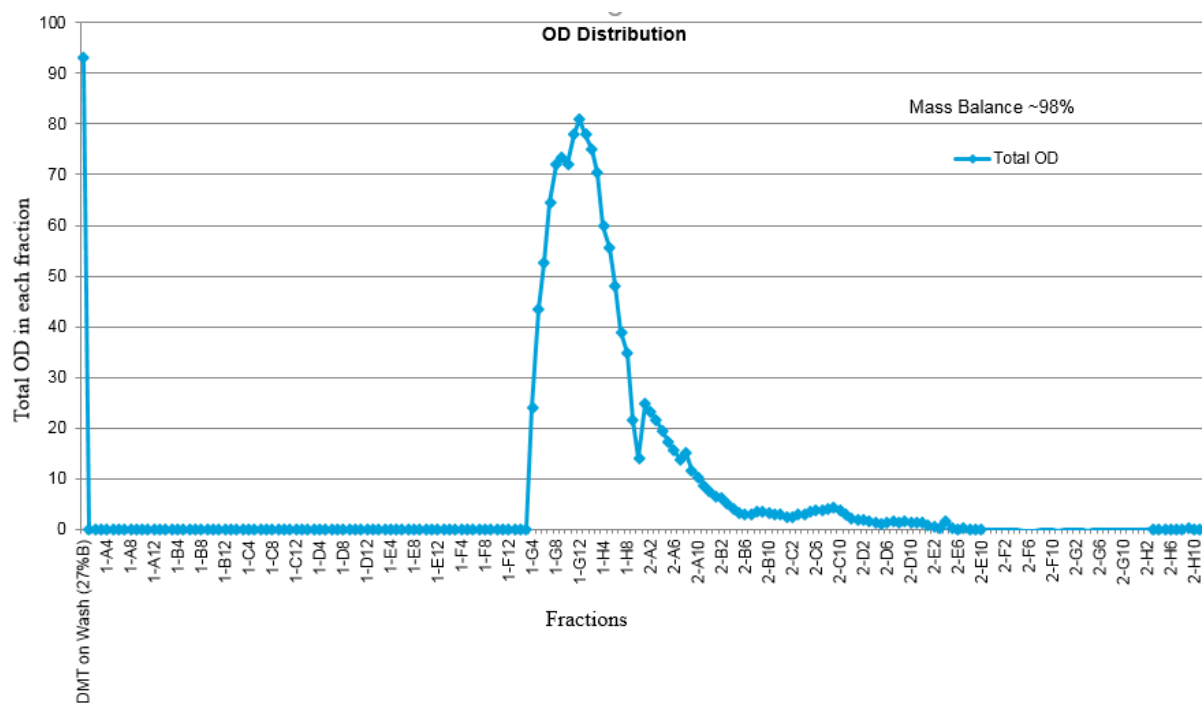


Figure 25. Distribution of Mass Balance during the Salt Gradient using TSK gel ® stationary phase

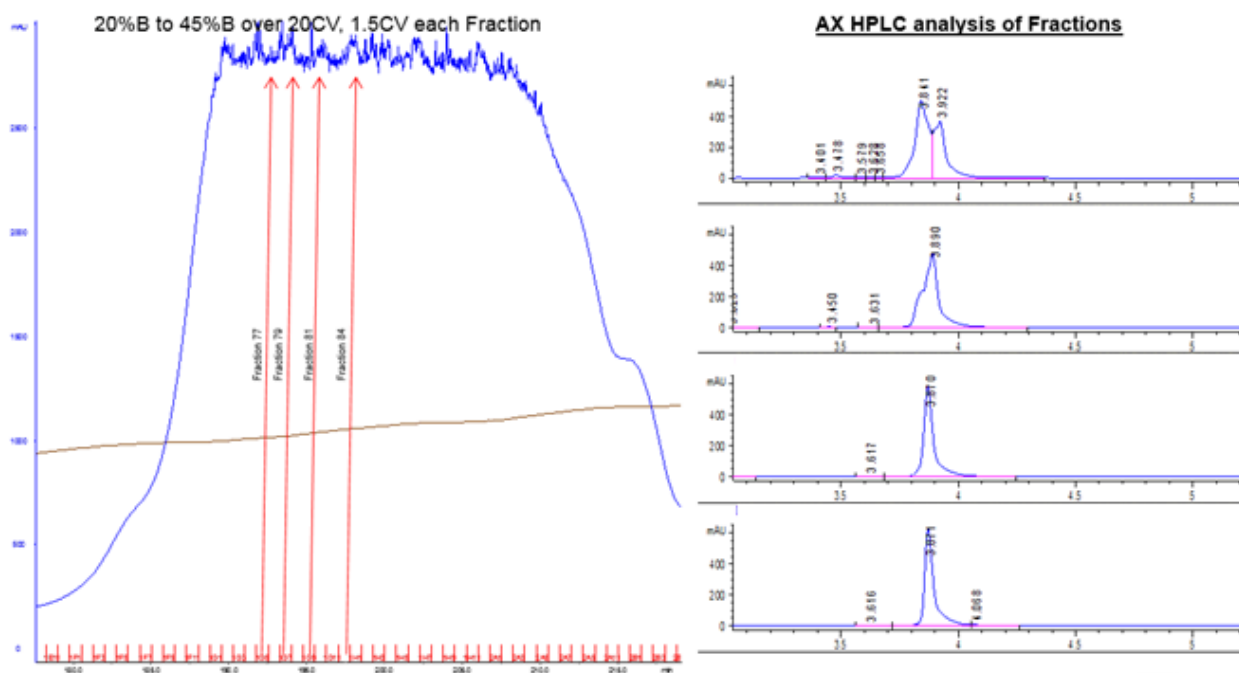


Figure 26. Chromatographic Separation of FLP and other Moieties during the Gradient and their AX HPLC Analysis for Purification using TSK gel® stationary phase

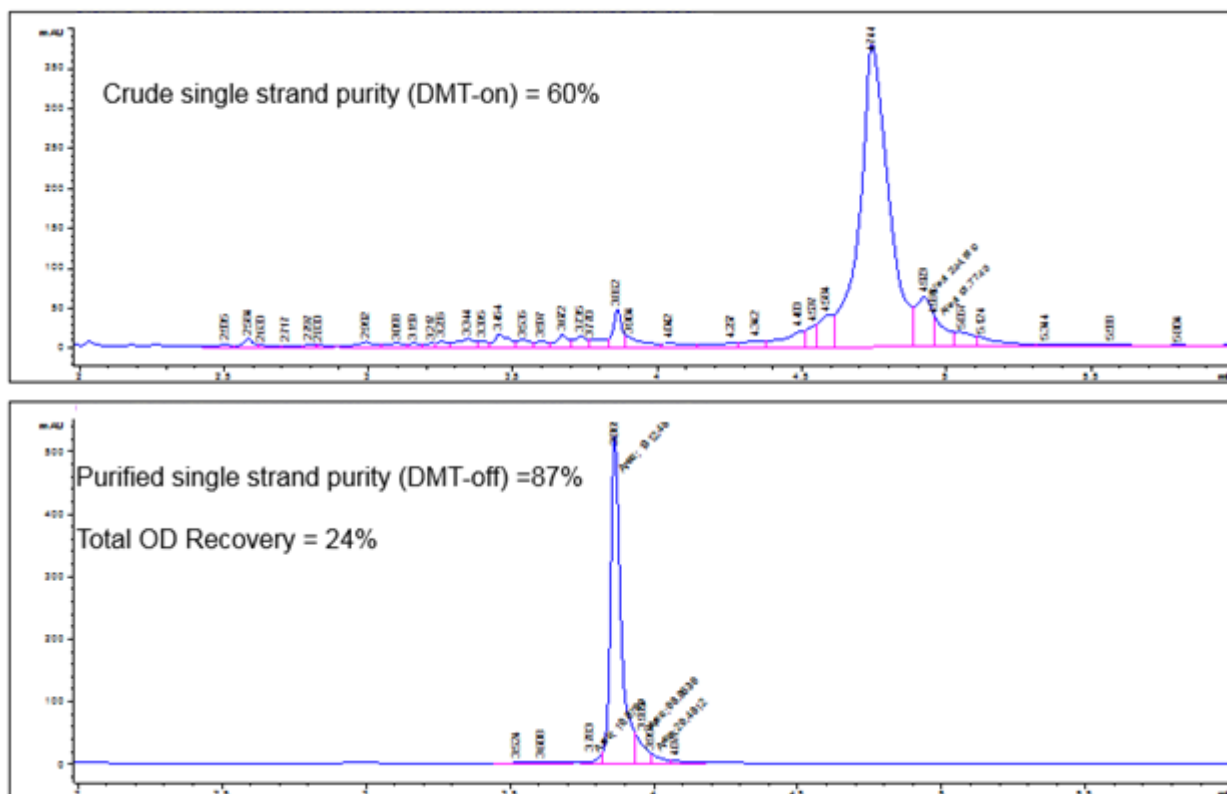


Figure 27. Comparison of Crude (DMT-on) vs Purified (DMT-off) single strand using TSK gel ® Stationary phase

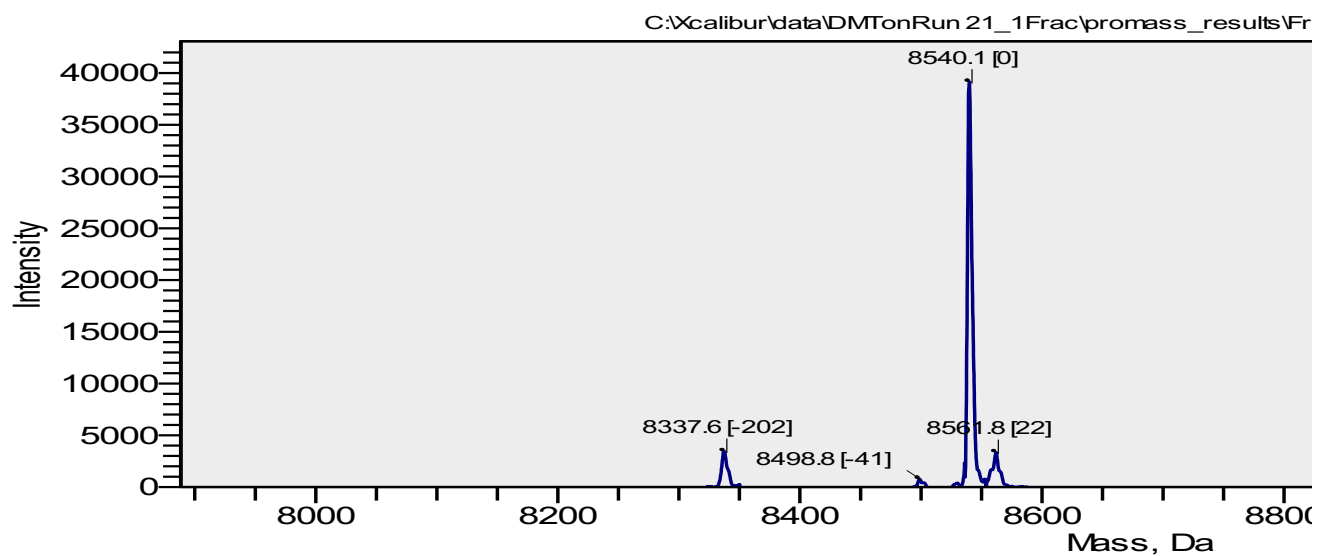


Figure 28: LC-MS Analysis of Purified single strand (DMT-off) from TSK gel[®] Experiments to confirm Identity. (calc. M.W of DMT-off Single Strand = 8541 D)

Chapter IV

IV. Conclusion

In conclusion, we developed a continuous and simple purification process utilizing the DMT group as an additional chromatographic anchor to aid in the purification process.

The DMT group is naturally present at the 5' end of the full-length product from the oligo-synthesis. Having the DMT group present on the 5' end of the sequence increases the hydrophobicity and provides an additional handle in chromatography. DMT-on single strands binds to the chromatographic stationary phase more strongly and allows simple and fast removal of most of the shorter failure sequences and synthesis impurities. Removal of shorter sequences and impurities prior to the gradient purification allows for an easier and faster separation of the desired FLP from impurities without the loss of overall yield. Using on-column detritylation technique, the DMT group can easily be removed from the single strand. The use of on-column detritylation technique eliminates the need for a two-step purification process. Therefore, this method offers a time and cost-effective alternative to the two-step purification process, as it eliminates the

additional purification, collection and concentration steps necessary typical done in the two-step process. It also minimizes the risk of a less robust neutralization step, which especially at larger scale could affect the stability of RNA structure integrity due to prolonged exposure to low pH.

IEX purification of RNA base(s) containing DMT-on oligonucleotide single strand using on column detritylation technique provides an easily implementable, cost-effective and scalable technique to obtain RNA containing single strands with high purity and yield.

Chapter V

V. References

- Carlton H. Paul, A. T. (1996). Acid Binding and Detritylation During Oligonucleotide Synthesis. *Nucleic Acids Research*, 3048-3052.
- Cramer, H. K. (2013). Oligonucleotide impurities and their origin. *Analysis of Oligonucleotides and their Related Substances*, 21-100.
- Gabor Butora, D. M. (2011). Nucleoside optimization for RNAi: A High-Throughput Platform. *J.Am.Chem.Soc*, 10.1021.
- Matteucci, M. a. (1981). Synthesis of deoxyoligonucleotides on a polymer support. *Journal of the American Chemical Society*, 3185-3191.
- Micura, R. (2002). Small interfering RNAs and their chemical synthesis. *Angewandte Chemie International Edition*, 2265-2269.
- Reiko Iwase, H. M. (2006). Synthesis and properties of modified siRNA having amide linked oligoribonucleosides at their 3' overhang regions. *Nucleic Acids Symposium Series N*, 175-176.
- Salon, J. Z. (2011). Mild detritylation of nucleic acid hydroxyl groups by warming up. *Nucleosides, nucleotides & nucleic acids*, 271-279.

- Sinha, N. (1984). Polymer support oligonucleotide synthesis XVIII: use of beta-cyanoethyl-N,N-dialkylamino-/N-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acids Research*, 4539-57.
- Thazha P. Prakash, A. M. (2008). Comparing in vitro and in vivo activity of 2'-O-[2-(Methylamino)-2-oxoethyl]-and 2'-O-Methoxyethyl-Modified antisense oligonucleotides. *J.Med.Chem.*, 2766-2776.
- Walter R. Stapps, V. P. (2008). The siRNA sequence and guide strand overhangs are determinants of in vivo duration of silencing. *Nucleic Acids Research*, 4788-4797.