Avoiding Depurination During Trityl-on Purification

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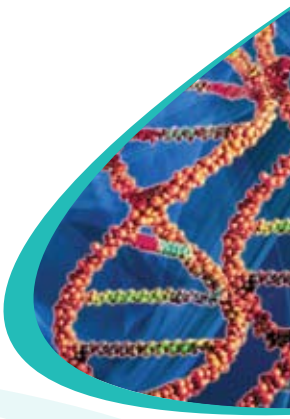


Introduction

Depurination in DNA and RNA sequences whether in nature or through fabrication is an alteration of the sugar-phosphate backbone in which hydrolysis of a purine base (adenine or guanine) occurs. In the typical mammalian cell, depurination is a rather common event as nearly one thousand purines are lost daily due to endogenous chemical reactions. [1] Systemic mechanisms, notably enzymes and amines, are in place that can effectively cleave voided fragments to prevent errant and potentially damaging transcriptions. [2] As in nature, synthetic oligonucleotides suffer from similar errors during the assembly process resulting in unwanted and harmful contaminants. Contemporary DNA and RNA synthesis utilizes solid-phase phosphoramidite chemistry to construct nucleotide sequences through a succession of phosphodiester linkages. By incorporating an acid labile 5' protecting group, dimethoxytrityl (DMT), nucleotide assembly is accomplished through sequential automated cycles of deprotection, coupling, capping, and oxidation. The greatest potential for damage to the sequence occurs during the deprotection stage (detritylation) where the oligonucleotide is exposed to dilute acid that may induce purine hydrolysis. Adding further complexity, the depurination rate of single stranded DNA (as synthetically produced) is four-fold greater than native double stranded DNA. [3] Unlike nature, the synthetic process lacks the necessary mechanism to selectively clear apurinic sites from the full-length sequence. Consequently, to deliver a pure and error-free product, purification techniques are required to remove depurinated fragments and other remnant impurities.

Crude synthetic oligonucleotides are purified by either trityl-on or trityl-off methodologies. In trityl-on practices, the final 5' DMT protecting group is retained on the nucleotide and removed during purification, whereas for trityl-off, the 5' protecting group is cleaved during the final cycle in the synthesis process. While both trityl-on and trityl-off techniques are compatible with serial liquid chromatography, the trityl-on modality does not require chromatographic instrumentation distinguishing it as a more accommodating method for high-throughput parallel processes. An advantageous feature of trityl-on separation is the lipophilic properties of the dimethoxytrityl group, which can serve as an idyllic handle to enable discrimination between the protected full-length sequence and unprotected impurities. The downside for trityl-on purification however is the potential for further depurination. During synthesis, the integrity of the ring structures of the purine bases are secured and shielded from acid hydrolysis with benzoyl and isobutyryl protecting groups. However, these groups are removed soon after support release, thereby increasing the likelihood of nucleobase cleavage when exposed to an acidic environment. Consequently, trityl-on techniques require a delicate balance of avoiding purine hydrolysis while ensuring complete detritylation. The heighten risk for nucleic acid damage is a principal concern and a limiting factor for broader acceptance of trityl-on techniques.

Given the primary problem associated with trityl-on purification, the focus of this discussion is to examine the root causes of depurination. While a variety of trityl-on formats are available, such as reversed-phase and ion exchange HPLC, our investigation is directed at cartridge-based and high throughput purification (HTP) platforms. The modality among the mentioned techniques differs, but shared with all trityl-on procedures is the addition of a dilute protic acid to release the trityl group. In cartridge-based and HTP techniques, for complete detritylation, the consensus has been to add 2 % - 5 % of TFA and incubate at room temperature for five minutes. The rationale for the rather high acid concentration is effective detritylation requires one molecule of acid per monomer unit of oligonucleotide. [4] Moreover, thymine retains the trityl group more readily than the other nucleobases; accordingly sequences with abundant thymidine can often require longer exposure to acidic conditions to successfully cleave the trityl group. Therein lies the trityl-on paradox, provide complete trityl release without depurination. The unlikely scenario has led many oligo manufactures and end-users to become leery of trityl-on cartridge and HTP purification. While dismissing the technique for fear of possible sequence damage is of course understandable, such reasoning foregoes a very efficient and efficacious purification platform. Reversing this trend will require a departure from conventional wisdom and a re-examination of alternative conditions that may in fact accomplish the unthinkable, depurination-free trityl-on purification. A recently introduced high-throughput and cartridge-base trityl-on purification product, Clarity® QSP™, recommends detritylation and eluting conditions that substantially minimize depurination. Despite the common use of trityl-on techniques, comparative data regarding depurination is not readily available. Therefore, to better gauge the success or failure of a new detritylation procedure, a thorough examination using an appropriate array of analytical techniques was warranted. Borrowing from nature, polyamine hydrolysis was incorporated with anion exchange chromatography and MS analysis to qualify and quantitative the level of depurination of various DNA sequences following Clarity QSP purification.

Materials and Methods

Materials

USC Norris Cancer Center DNA Core Lab, Los Angeles, CA and Integrated DNA Technologies, Coralville, IA provided 200 nmole synthesis scale of crude trityl-on ssDNA. All DNA sequences were retained in concentrated NH_4OH following support cleavage and primary deprotection. Solvents, methanol and acetonitrile were from Burdick and Jackson. NH_4HCO_3 was from Fluka, Na_2CO_3, Na_2HCO_3, Hepes, and Tris were received from Sigma. Spermine was purchased from Alfa Aesar. Purification was performed using Clarity QSP 1 mL/50 mg cartridges and 96-well plates with DNA loading buffer, Phenomenex. For SPE apparatus both a 12 slot and 96-well plate vacuum manifolds were used, Phenomenex. Vacuum was provided by a KNF brand filtration pump, and UV quantitation was performed using a Beckman Coulter 700 series Spectrophotometer. MS verification was performed on a LTQ™ (Thermo Scientific).

HPLC instrumentation:	Agilent® HP® 1100 quaternary system
Column:	IEX column, DNAPac® PA200 250 x 4.0 mm
Mobile Phase:	A: Water / C: 0.25 M Tris-HCL pH 8 / D: 0.375 $NaClO_4$
Gradient:	A: 80 %, C: 10 %, D: 10 %-65 % in 20 minutes
Flow Rate:	1.2 mL/min
Detection:	UV @ 260 nm

Purification Method

Sample preparation for Clarity QSP purification required the addition of an equal volume of DNA loading buffer to the NH_4OH solution containing the tested oligonucleotide. The volume of NH_4OH used for support cleavage and primary deprotection was based on 150 µL per 100 nmole synthesis scale. Total working volume including loading buffer was 600 µL. DCA (dichloroacetic acid) was used for detritylation with concentrations of 1 % and 3 %. Acid exposure time was limited to 1 minute during cartridge purification and 10 minutes for the automated 96-well plate procedure. Solutions used to recover the detritylated final product were composed of either A) water / 40 % acetonitrile, B) a buffered 40 % acetonitrile solution containing Na_2CO_3 or C) a buffered 20 % acetonitrile solution containing 20 mM NH_4HCO_3.

Performed on a 12-slot manifold with vacuum source, **QSP cartridge purification** involved the following:

- **Sorbent Conditioning: 1 mL MeOH (0.5 mL x 2)**
- **Sorbent Equilibration: 1 mL Water (0.5 mL x 2)**
- **Loading vol of Oligo (600 µL)**
- **Detritylation: 3 % aqueous DCA (1 mL)**
- **Rinse: 1 mL Water (0.5 mL x 2)**
- **Dry sorbent @ 10" Hg (~1 min)**
- **Elution: 1 mL A, B, or C formulations**

Materials and Methods *(cont'd)*

Performed using an automated 96-well plate format with vacuum source, **QSP 96-well plate** purification consisted of:

- **Sorbent Conditioning: 1 mL MeOH (0.5 mL x 2)**
- **Sorbent Equilibration: 2 mL Water (1 mL x 2)**
- **Loading vol of Oligo (600 µL)**
- **Detritylation: 1 % aqueous DCA (1 mL)**
- **Rinse: 1 mL Water (0.5 mL x 2)**
- **Dry sorbent @ 10" Hg (~1 min)**
- **Elution: (C formula only) 1 mL 20 mM NH_4HCO_3 / 20 % Acetonitrile**

Analysis

Polyamine Hydrolysis

Depurination was precisely identified by hydrolysis of apurinic sites and detected with chromatographic and MS techniques. Following purification and detritylation, 200 µL aliquots of the 1 mL final elution volumes were dried using an N_2 purge. Each pellet was reconstituted in 200 µL of 50 mM Hepes / 2 mM Spermine / 1 mM EDTA (pH 8.1). The solution was incubated at 37 °C for 1 hour. 100 µL of the hydrolysis mixture was diluted with 900 µL with DI water (1:10) and 50 µL was injected on column and analyzed via IEX chromatography.

IEX Chromatography

Crude sample analysis: Prior to purification 50 µL aliquot of DNA trityl-on was diluted (1:10) with DI water. 100 µL was injected on column and analyzed using IEX chromatography.

Detritylated purified DNA: The final 1 mL volume was collected and stored at room temperature. 50 µL aliquot of purified detritylated DNA elution was diluted (1:10) with DI water. 100 µL was injected on column and analyzed with IEX chromatography.

ESI-MS

The final 1 mL volume of purified and detritylated DNA from the automated format was collected and dried then reconstituted in water and analyzed via MS using the Novatia™ Oligo HTCS system (which uses a Thermo Scientific LTQ™ for analysis).

DNA Sequences

Four DNA 20 mer sequences were synthesized with a lone internal adenosine or guanosine to monitor site-specific purine hydrolysis following the Clarity QSP procedure. Pyrimidine compositions were also varied among the sequences to detect possible neighboring influences. Our investigation also compared the differences of depurination rates in sequences containing internal and terminal purine locations. Two DNA 21 mer sequences containing a 5' positioned purine and two internal purines were analyzed post QSP purification. 96 DNA 30 mer sequences containing at least 40 % purine nucleobases were analyzed via ESI-MS following the automated well plate format.

Results and Discussion

Crude and purified DNA sequences were analyzed using anion exchange chromatography; however, the resulting chromatograms failed to distinguish depurinated sequences from other impurities. Therefore, our study utilized polyamine hydrolysis prior to chromatography as we found the technique to be far superior for accurately monitoring apurinic site cleavage. Presented in ***Table 1*** is a summary of the results of the four sequences with lone internal purines after QSP cartridge purification and subsequent chain cleavage and analysis. The data revealed a consistent pattern throughout in which minimal site-specific hydrolysis had occurred during the detritylation step or within the first hour following elution, regardless of the eluting conditions. While guanosine is reported to release at a slightly faster rate than adenosine [5], we observed no difference, as the level of depurination was equivalent among the four sequences. Adjacent pyrimidine groups were also found to have no influence on depurination rates among the sequences.

Table 1

SEQUENCE	DETRITYLATION	ELUANT	TIME	DEPURINATION (% AREA)
CTTCTCTCC**G**CTCTCTCTCT	3 % DCA	Water / Acetonitrile	1hr 24 hr 48 hr 72 hr	1.3 % 17.5 % 29.6 % 41.5 %
CTTCTCTCC**G**CTCTCTCTCT	3 % DCA	Na_2CO_3 / Acetonitrile	1hr 24 hr 48 hr 72 hr	0.5 % 0.3 % 0.8 % 0.4 %
CTTCTCTCT**A**TTCTCTCTCT	3 % DCA	Water / Acetonitrile	1hr 24 hr 48 hr 72 hr	0.3 % 13.7 % 28.4 % 39.1 %
CTTCTCTCT**G**TTCTCTCTCT	3 % DCA	Na_2CO_3 / Acetonitrile	1hr 24 hr 48 hr 72 hr	0.0 0.0 0.0 0.0

Illustrated in ***Figure 1***, depurination was drastically amplified in the water / acetonitrile eluted sequences following ambient overnight incubations, while in the Na_2CO_3 buffered elution, under similar conditions, we observed no evidence of further depurination, ***Figure 2***. As represented in the table, the trend continued throughout the 72-hour incubation period and remained consistent among the sequences and conditions. The rationale is rather straightforward as the water / acetonitrile solution does not elevate the pH to an appropriate physiological level to circumvent further acid cleavage. Following detritylation in 3 % DCA, eluants composed of an organic solvent and water reach a pH of approximately 5.5 and while no immediate depurination occurred, continuous exposure did induce significant acid cleavage. Such findings are consistent with other published accounts that observe depurination and chain cleavage in a pH 5 solution at elevated temperatures [6]. In sharp contrast, eluting and maintaining in a physiological accommodating solution of pH 7.5 prevented additional depurination throughout the 3-day cycle.

Figure 1: CTTCTCTCC**G**CTCTCTCTCT

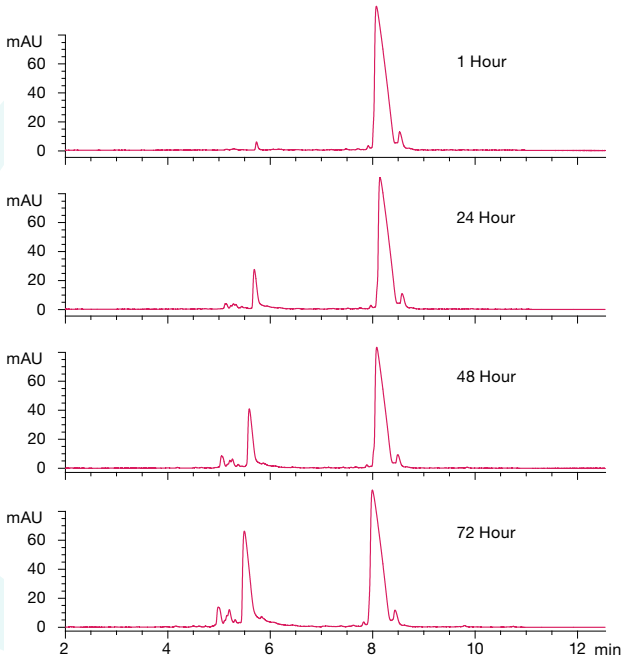
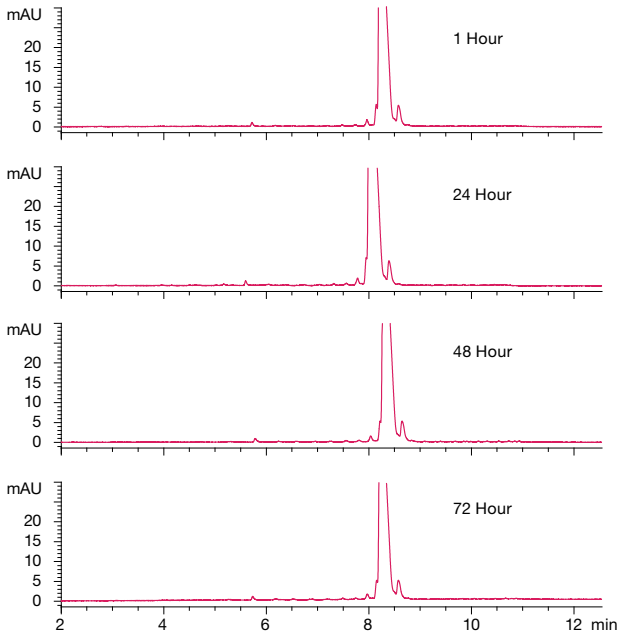


Figure 2: CTTCTCTCC**G**CTCTCTCTCT



Results and Discussion *(cont'd)*

Table 2 outlines the tabulated results following QSP cartridge purification and subsequent analysis of the two sequences composed of internal purine groups and a 5' terminal purine.

Table 2

SEQUENCE	DETRITYLATION	ELUANT	TIME	DEPURINATION (% AREA)
A**GG**CTTCCTCCTCCTCTT	3 % DCA	Water / Acetonitrile	1hr 24 hr 48 hr 72 hr	15.4 % 50.0 % 76.6 % 88.0 %
A**GG**CTTCCTCCTCCTCTT	3 % DCA	Na_2CO_3 / Acetonitrile	1hr 24 hr 48 hr 72 hr	0.0 0.0 0.0 0.0
G**AA**CTTCCTCCTCCTCTT	3 % DCA	Water / Acetonitrile	1hr 24 hr 48 hr 72 hr	1.6 % 41.7 % 70.4 % 84.3 %
G**AA**CTTCCTCCTCCTCTT	3 % DCA	Na_2CO_3 / Acetonitrile	1hr 24 hr 48 hr 72 hr	0.0 0.0 0.0 0.0

Figure 3 represents the water / acetonitrile eluant that clearly depicts depurination and sequence cleavage occurring within one hour. Throughout the 72-hour period, purine hydrolysis had commenced at a considerable faster rate in comparison with the previous lone internal purine positioned sequences. Our observation has been seen previously and is proposed to be the result of the 5 'phosphate group at the a purinic site acting as an electron-withdrawing group to greatly accelerate chain cleavage. [7] Our data clearly supports such a mechanism, as the sequences evaluated with pyrimidine groups at the 5' position did not produce similar levels of depurination and site cleavage. When the same sequence was eluted and maintained in a Na_2CO_3 buffered acetonitrile solution, depurination and chain cleavage was absent throughout the period, ***Figure 4***. Moreover, as evident in ***Table 2***, a reversal of the purine groups in the sequence did not adversely affect depurination occurrence. From our investigations we can affirm that while sequence composition and in particular purine location does influence depurination rates, the primary source for depurination however is the surrounding environment of the oligonucleotide.

Figure 3: ACTC**GG**CTTCCTCCTCCTCTT

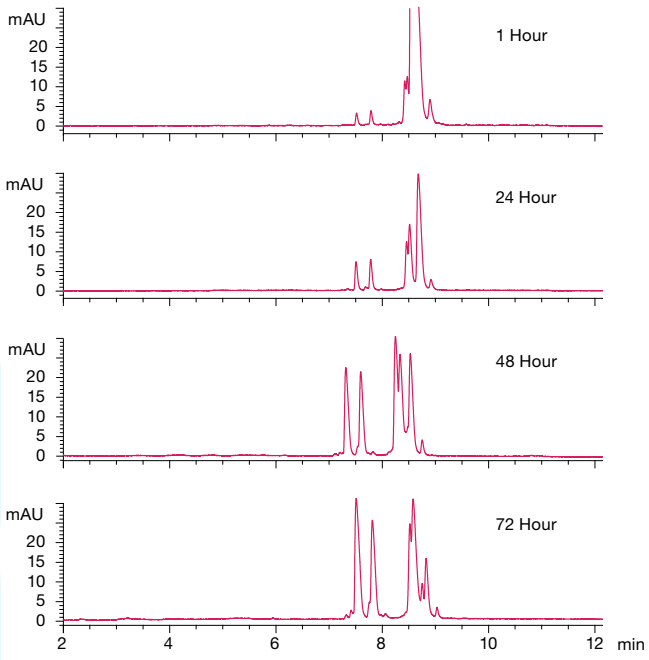
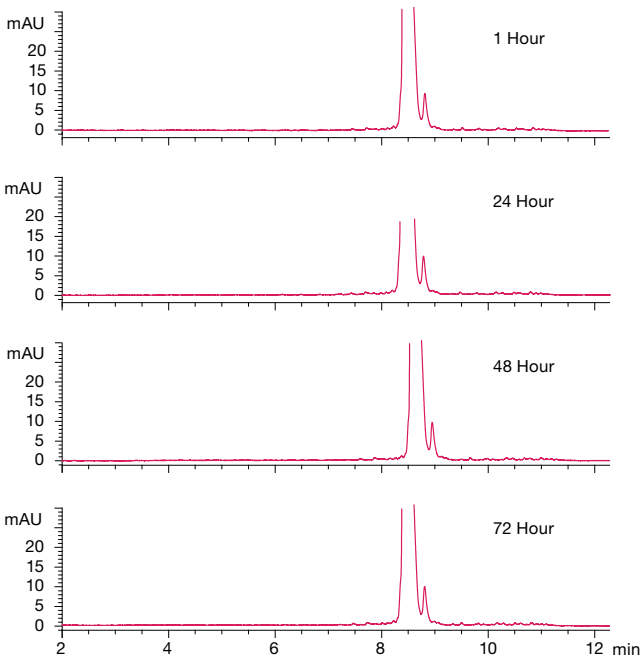


Figure 4: ACTC**GG**CTTCCTCCTCCTCTT



50 µL Injected: IEX column, DNAPac® PA200 250 x 4.0 mm
Mobile Phase: A: Water / C: 0.25 M Tris-HCL pH 8/ D: 0.375 $NaClO_4$
Gradient: A: 80 %, C: 10 %, D: 10 %-65 % in 20 minutes
Flow Rate: 1.2 mL/min
Detection: UV @ 260 nm

Results and Discussion *(cont'd)*

Today, the majority of synthetic oligonucleotides are produced in combinatorial-style multiplex formats enabling oligo manufactures to produce tens of thousands of sequences per day. To meet their purification demands, manufacturers are relying on parallel platforms to work in concert with automated liquid handling systems. The Clarity QSP product offers a 96-well plate format (HTP) designed specifically for robotic systems. While virtually similar to the cartridge protocol, the HTP methodology does have variations. For this discussion, the more relevant difference is the alternative conditions required during detritylation. In automated systems, time exposure among samples may vary substantially; consequently, some sequences may be exposed to acidic conditions much longer than others. Accounting for such variations, the QSP procedure recommends a lower acid strength from 3 % DCA to 1 % DCA. To verify the performance of these conditions, a 96-well plate composed of 30 mer DNA sequences containing 40 % purine groups and a 5' terminal guanosine were processed using the Perkin-Elmer MultiPROBE robotic liquid handling system. During the detritylation process, acid exposure times of samples in the first two rows (A & B) were observed to be the nearly three times longer than samples in the remaining wells. Accordingly, samples from these rows were analyzed and compared against later rows. After eluting in 1 mL of 20 mM NH_4HCO_3 / 20 % acetonitrile, samples were dried using an N_2 purge. Select sequences were analyzed following our in-house polyamine-HPLC protocol while others were outsourced for ESI-MS. ***Figures 5 & 6*** represent MS data of sequences from well positions A2 and H4 respectively. As shown, only minimal depurination was found in well A2, while there was no depurination detected in well H4. As expected, the longer exposure to the acid accounted for the slight variation between depurination levels. We observed consistent results as previously discussed that when eluting in physiological pH conditions sequence composition and purine location depurination have virtually no affect on elevating the rate of depurination. Moreover, the spectra also illustrates the lower recommended acid strength did not adversely affect trityl release.

Figure 5:
Well A2 - GTGGATCTGCGCACTTCAGGCTCCTGGGCC

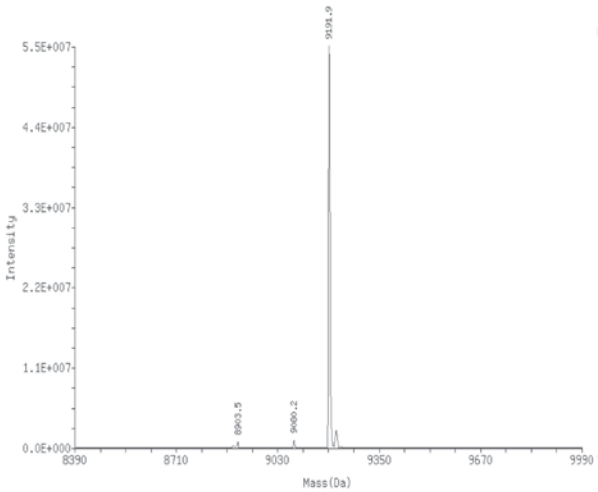
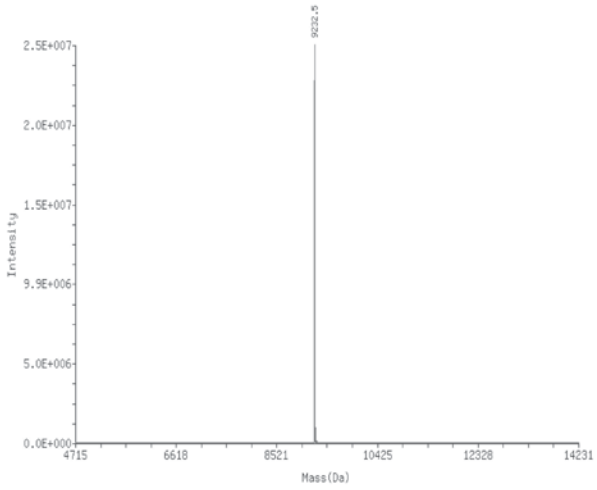


Figure 6:
Well H4 - GTGGATCTGCGCACTTCAGGCTCCTGGGCG



	Well A2				Well H4	
Mass Da	9191.9	9214.4	9080.2	8903.5	9232.5	9254.0
Std. Dev	0.2	0.3	0.4	0.5	0.2	0.8
Intensity	5.53E+07	2.38E+06	9.42E+05	7.92E+05	2.48E+07	9.29E+05
Identity	Target Mass	Na adduct	C depyrimidination	Depurination	Target Mass	Na adduct
Delta Mass	0	22.5	-111.7	-288.4	0	21.5
% Relative	100	4.3	1.7	1.4	100	3.7
% Total	93.1	4	1.6	1.3	96.4	3.6

Results and Discussion *(cont'd)*

Further validation of minimal depurination after purification is evident in ***Figures 7 & 8*** following polyamine hydrolysis and anion exchange chromatography. Comparative analysis was performed using a second row located sequences against later well samples. Corroborating the MS data, only trace depurination is observed in well B12 and completely absent in well G9. Also observed was complete trityl release among the sequences.

Figure 7:
Well B12 - GTGGATCTGCGCACTTCAGGCTCCTGGGCA

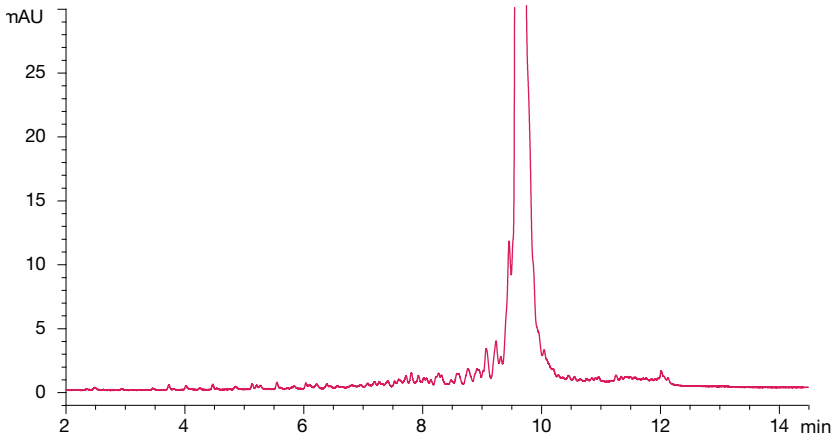
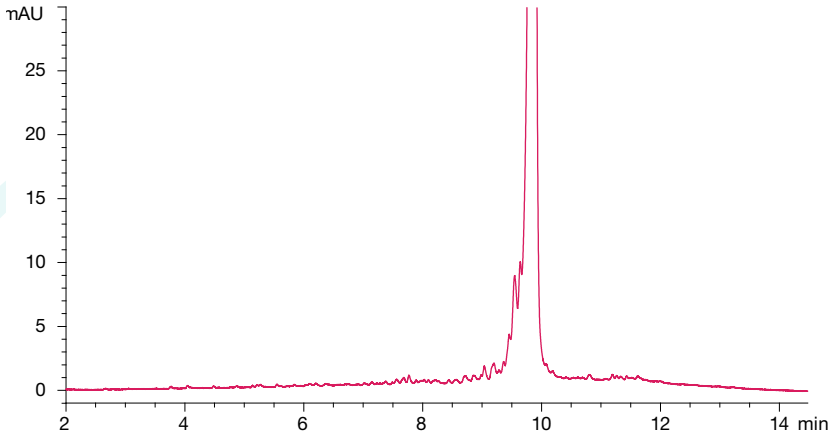


Figure 8:
Well G9 - GTGGATCTGCGCACTTCAGGCTCCTGGGCT



50 µL Injected:	IEX column, DNAPac® PA200 250 x 4.0 mm
Mobile Phase:	A: Water / C: 0.25 M Tris-HCL pH 8/ D: 0.375 $NaClO_4$
Gradient:	A: 80 %, C: 10 %, D: 10 %-65 % in 20 minutes
Flow Rate:	1.2 mL/min
Detection:	UV @ 260 nm

Conclusion

Since their commercial introduction, cartridge-based and HTP trityl-on products have received differing views regarding depurination from end-users. Some have mentioned finding little or no depurination while others have observed considerable apurinic sequences following trityl-on purification. Our investigation served to address the causes behind the conflicting opinions about trityl-on techniques and depurination. By reducing acid concentration, limiting exposure times, and utilizing physiologically accommodating elution buffers, we were able to substantially minimize if not eliminate depurination from occurring during purification. In addition and to our surprise, sequence composition considered by many as a primary cause of purine hydrolysis was discovered to be of little influence under appropriate conditions. Trityl-on purification has its fair share of opponents and proponents and our study is not aimed to sway beliefs but to merely evaluate the true concerns regarding trityl-on purification and its assumed proliferation of depurination. Unavoidably, base cleavage of oligonucleotides whether native or synthetic is an inherited phenomenon. The recommended QSP protocol will not guarantee depurination prevention however; it is a methodology that demonstrates trityl-on purification can be a safe and effective purification methodology for synthetic oligonucleotides.

Ordering Information

Formats

Part No.	Description		Unit
8E-S102-DGB	Clarity QSP	50 mg/ 96-Well Plate	1/Box
8B-S102-DAK	Clarity QSP	50 mg/ 1 mL Cartridge	50/Box
8B-S102-SBJ	Clarity QSP	150 mg/ 3 mL Cartridge	50/Box
8B-S042-LFF	Clarity QSP	5 g/60 mL Cartridge	16/Box

Buffer

Part No.	Description		Unit
AL0-8279	Clarity QSP DNA Loading Buffer	100 mL	Ea
AL0-8280	Clarity QSP DNA Loading Buffer	1 L	Ea
AL0-8281	Clarity QSP RNA Loading Buffer	100 mL	Ea
AL0-8282	Clarity QSP RNA Loading Buffer	1 L	Ea
AH0-7858	Clarity Nuclease Free Water	1 L	Ea

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