# CLARITY® QSP

Cartridges & 96 Well-Plates
**User's Manual** for Synthetic

# RNA purification

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# 1.0 Introduction



Advances in functional genomics have caused a dramatic surge for both synthetic ribo- and deoxyribonucleotides. The increase in nucleotide demand and their recent therapeutic applications have fostered a pressing need for more efficient and efficacious purification platforms. To better match the need of oligo manufacturers and their customers, Phenomenex introduces Clarity QSP; a newly developed cartridge-based format that offers a quick and simple alternative for obtaining highly purified and concentrated synthetic DNA and RNA. Utilizing trityl-on chemistry, this innovative approach provides in one-step complete discrimination of the full-length sequence from unwanted synthetic contaminants delivered in a physiological pH buffered solution. Clarity QSP is a next generation purification product that was designed specifically to complement contemporary synthetic processes.

## 1.1 RNA Synthesis

Present-day RNA synthesis utilizes solid-phase phosphoramidite chemistry to build oligoribonucleotides through a succession of phosphodiester linkages. While following a similar assembly process as DNA, ribonucleotide chemistry differs with the added protecting group at the 2' hydroxyl group on the pentose sugar of ribo-phosphoramidites. Accordingly, RNA construction requires an intricate synthetic design that secures 2' hydroxyl protection throughout the process while providing incremental removal of the 5' hydroxyl-protecting group necessary for sequence elongation. Generic RNA synthetic schemes rely primarily on the acid labile dimethyltrityl (DMT) for 5' hydroxyl protection and fluoride-labile silyl chemistry for 2' hydroxyl protection (TBDMS). In this strategy, the synthetic process begins with the initial protected ribonucleotide coupled to a solid porous support. Chain extension then proceeds with subsequent phosphoramidites assembled from 3' to 5' using sequential automated cycles of detritylation, coupling, capping, and oxidation. Upon sequence completion, the crude ribonucleotide is cleaved from the solid support in dilute ammonia then gently heated to remove base-labile protecting groups from the nucleobases and phosphate backbone. Depending on the purification method used, the final 5' DMT group can be retained (trityl-on) or removed (trityl-off) prior to cleavage and deprotection. The concluding step requires the release of 2' silyl protection, which necessitates the evaporation of the primary deprotecting components then incubation in a neutral pH balanced hydrofluoride solution.

Integrating modern synthetic chemistry with advanced fabrication technology has significantly improved assembly efficiencies and thus the quality of crude synthetic oligonucleotides. Yet, RNA synthesis still remains a challenge as instrument efficiency rates rarely exceed 99 % per coupling event. On most instruments, the typical yield for a 21 nt RNA sequence is only 80 %

of full-length product [(0.99)$^{20}$], whereas synthetic DNA operations can yield over 90 % of full-length sequences. Consequently, synthetic ribonucleotides contain considerably more contaminants than its DNA counterpart. These remnant impurities consist primarily of truncated sequences and depurinated fragments that require robust purification techniques to provide more acceptable purity and recovery yields in the final oligoribonucleotide product.

## 1.2 Purification Platforms

For years DNA has overshadowed its far less famous relative RNA. Not surprising, the majority of purification platforms used for synthetic oligonucleotides has focused primarily on DNA that can withstand rather rigorous cleaning regimens. However, the newly recognized biological importance of RNA has generated a tremendous demand for synthetic ribonucleotides, while also creating a quandary for more efficacious yet milder purification strategies. The instability of RNA and the high degree of tenacious impurities limits the purification methodologies available. Less stringent practices such as desalting and precipitation do not offer much improvement in the purity from that of the crude yield. Consequently, the most commonly practiced purification method for synthetic ribonucleotides has been limited to trityl-off HPLC using either reversed-phase or anion exchange chromatography.

Prior to the rise in popularity for RNA sequences, Wincott, et al. developed an improved 2' silyl deprotection method that allowed trityl-on purification to become a viable alternative. Since their publication, various substitute trityl-on purification formats have been introduced, yet many in the field maintain trityl-off serial HPLC as their preferred mode for RNA purification.

## 1.3 Reversed-Phase Trityl-On Cartridge Purification

Utilizing Wincott's approach, trityl-on RP cartridge purification was introduced as an efficient purification alternative for synthetic RNA. Designed to circumvent sequential HPLC purification, the cartridge-based format was to function as a cost effective process that was fast, efficacious, and tailored for parallel purification. However, hindered by reversed-phase perceptions, trityl-on RP cartridge systems have not delivered as advertised. The standard RP-cartridge design requires multiple solvents, sequential wash steps,

RNA introduces toxic and analytically problematic ion-pairing agents, but more importantly has failed to produce generally acceptable purity and recovery yields. Further diminishing the appeal, the vast majority of commercial RP cartridges lack the necessary format for serial high-throughput RNA purification. Consequently, those seeking alternative purification methods are rather disappointed with the performance of cartridge-based products that once promised unmatched ease and efficacy

## 1.4 Clarity QSP Trityl-On Purification

Developed to be a versatile, gentle, and more effective trityl-on purification process, Clarity QSP delivers near impurity-free, concentrated full-length RNA sequences in a stable media suitable for *in-vivo* applications and downstream analysis conducive for MS, NMR, CE, and HPLC. Simple in practice and in theory, the product offers speed and efficacy in formats that can be readily automated for high-throughput parallel purification and is suitable for both combinatorial-scale and large-scale purifications. Clarity QSP consists of two components, a loading buffer and a polymeric sorbent. Housed in three cartridge formats and a 96-Well plate, the QSP resin is pH-stable and purifies RNA sequences of lengths ranging from 10 nt to 100 nt. In addition, the QSP media has enhanced flow characteristics to ensure consistent flow rates for increased analyte contact time resulting in unfailing performance. The accompanying loading buffer is composed of biological compatible agents and is free of toxic and meddlesome ion-pairing agents. Together, the sorbent and buffer create a simple three-step process that in minutes delivers highly purified synthetic RNA with exceptional recovery yields.

## 1.5 The QSP Process

QSP purification of trityl-on RNA begins after an equal volume of loading buffer is mixed with a quenched 2' deprotection solution. After brief conditioning of the resin with methanol and water, the solublized crude oligo is passed through the sorbent. The compatibility of the buffer with silyl deprotecting solutions provides selective retention of the full-length trityl-on RNA sequence, while eliminating tenaciously bound unlabeled truncated sequences and damaged fragments. Depending on the quality of the crude product, the improved cleaning proficiency of the buffer can alleviate subsequent wash steps leaving only detritylation and elution to follow. The result is a final product of synthetic RNA sequences with purities ranging from 89 % to 93 %[1] and reliable recovery values of 75 % or higher[2].

1. Ion-exchange chromatography and capillary electrophoresis.

2. $OD_{260}$ used for quantitation.

## 2.1 Clarity QSP Components

Clarity QSP RNA Loading Buffer (ion-pairing free)
- 100 mL
- 1 L

Clarity QSP Cartridges
- 50 mg/ 1 mL
- 150 mg/ 3 mL
- 5 g/ 60 mL

Clarity QSP 96-Well Plate
- 50 mg/ well

## 2.2 Equipment and Materials Required

Vacuum manifold
- 12- or 24- position
- 96-Well Plate

Vacuum pump

Reagents
- Methanol
- Water (Nuclease free)
- DCA (Dichloroacetic acid)
- Acetonitrile

Recommended eluting buffer solutions and their intended applications.

| Buffer | pH | Primary Application | Dry Down | Recon-stituting Solvent |
|---|---|---|---|---|
| 15 mM $Na_2CO_3$ / 50 % Acetonitrile | 7.5 - 8 | In-vivo & tissue based investigations | YES | Water |
| 20 mM $Na_2HCO_3$ / 50 % Acetonitrile (24 hr shelf-life) | 7.5 | In-vivo & tissue based investigations | YES | Water |
| 10 mM Tris pH 8 / 50 % Acetonitrile | 8 | In-vivo & tissue based investigations | YES | Water |
| 20 mM $NH_4HCO_3$ / 50 % Acetonitrile (24 hr shelf-life) | 7.5 | MS, LC/ MS- ESI, MALDI / NMR | Customer Preference | Water |
| 15 mM $NH_4HCO_3$ / 50 % Acetonitrile | 7.5 - 8 | MS, LC/ MS- ESI, MALDI / NMR | Customer Preference | Water |
| 20 mM $NH_4CH_3CO_2$ pH 8 / 50 % Acetonitrile | 7 | MS, LC/ MS- ESI, MALDI / NMR | Customer Preference | Water |

RNA

## For Synthesis Scales: ≤ 0.2 µmole

**IMPORTANT:** For trityl-on RNA purification it is imperative that the final 5' DMT group is retained following synthesis completion.

### 1. Support cleavage and primary deprotection:

- Add an appropriate volume of EtOH:$NH_4OH$ (1:3) to CPG column according to synthesis scale. Typically, the volume used is 150 µL per 100 nmole.
- Allow 17 hours for room temperature incubation or 2 hours at 55 °C.

### 2. Secondary 2' deprotection (Wincott, et al. Nucleic Acids Research, 1995)

#### Reagents

n-Methylpyrrolidone
Triethylamine
Triethylamine-trihydrofluoride (TEA.3HF)
Ammonium bicarbonate

- After primary deprotection (solid support, and amino protecting group cleavage), evaporate EtOH/$NH_4OH$, drying RNA oligo to pellet.
- Prepare fresh RNA deprotecting cocktail in volumes according to the table below.
- Always add TEA.3HF last to prevent coagulation of the mixture.

| 2' Deprotection Reagents | Number of RNA Oligonucleotides | | | |
|---|---|---|---|---|
| | 4 | 8 | 16 | 32 |
| NMP | 188 µL | 375 µL | 750 µL | 1500 µL |
| TEA | 94 µL | 187.5 µL | 375 µL | 750 µL |
| TEA.3HF | 125 µL | 250 µL | 500 µL | 1000 µL |

- Add 100 µL of RNA deprotection solution to RNA pellet.
- Vortex / mix briefly
- Heat @ 65 °C for 1.5 hours (agitation optional)
- Cool to room temperature
- Slowly add 400 µL of 1.5 M ammonium bicarbonate to quench.
- Add an equal volume of Clarity RNA loading buffer (0.5 mL) to quenched deprotection solution. [Final volume 1 mL]
- Proceed to trityl-on cartridge or Well plate purification (pp. 9-12).

## For Synthesis Scales: 1 µmole

**IMPORTANT:** For trityl-on RNA purification it is imperative that the final 5' DMT group is retained following synthesis completion.

## 1. Support cleavage and primary deprotection:

- Add an appropriate volume of EtOH:$NH_4OH$ (1:3) to CPG column according to synthesis scale. Typically, the volume used is 150 µL per 100 nmole.
- Allow 17 hours for room temperature incubation or 2 hours at 55 °C.

## 2. Secondary 2' deprotection (Wincott, et al. Nucleic Acid Research, 1995)

### Reagents

n-Methylpyrrolidone
Triethylamine
Triethylamine-trihydrofluoride (TEA.3HF)
Ammonium bicarbonate

- After primary deprotection (solid support, and amino protecting group cleavage), evaporate EtOH/$NH_4OH$, drying RNA oligo to pellet.
- Prepare fresh RNA deprotecting cocktail in volumes according to the table below.
- Always add TEA.3HF last to prevent coagulation of the mixture.

| 2' Deprotection Reagents | Number of RNA Oligonucleotides | | | |
|---|---|---|---|---|
| | 3 | 6 | 9 | 12 |
| NMP | 375 µL | 750 µL | 1125 µL | 1500 µL |
| TEA | 187.5 µL | 375 µL | 562.5 µL | 750 µL |
| TEA.3HF | 250 µL | 500 µL | 750 µL | 1000 µL |

- Add 250 µL of RNA deprotection solution to RNA pellet.
- Vortex / mix briefly
- Heat @ 65 °C for 1.5 hours (agitation optional)
- Cool to room temperature
- Slowly add 1 mL of 1.5 M ammonium bicarbonate to quench.
- Add an equal volume of Clarity RNA loading buffer (1.25 mL) to quenched deprotection solution. [Final volume 2.5 mL]
- Proceed to trityl-on cartridge purification (pp. 13-14)

## For Synthesis Scales: 10-50 µmole

**IMPORTANT:** For trityl-on RNA purification it is imperative that the final 5' DMT group is retained following synthesis completion.

### 1. Support cleavage and primary deprotection:

- Add an appropriate volume of EtOH:$NH_4OH$ (1:3) to CPG column according to synthesis scale. Typically, the volume used is 150 µL per 100 nmole.
- Allow 17 hours for room temperature incubation and 2 hours at 55 °C.

### 2. Secondary 2' deprotection (Wincott, et al. Nucleic Acids Research, 1995)

#### Reagents

n-Methylpyrrolidone
Triethylamine
Triethylamine-trihydrofluoride (TEA.3HF)
Ammonium bicarbonate

- After primary deprotection (solid support, and amino protecting group cleavage), evaporate EtOH/$NH_4OH$, drying RNA oligo to pellet.
- Prepare fresh RNA deprotecting cocktail in volumes according to the table below.
- Always add TEA.3HF last to prevent coagulation of the mixture.

| 2' Deprotection Reagents | Synthesis Scale (µmole) | | | |
|---|---|---|---|---|
| | 10 | 20 | 30 | 50 |
| NMP | 1.5 mL | 3 mL | 4.5 mL | 7.5 mL |
| TEA | 0.75 mL | 1.5 mL | 2.25 mL | 3.75 mL |
| TEA.3HF | 1 mL | 2 mL | 3 mL | 5 mL |

- Add 2.50 mL of RNA deprotection solution per 10 µmole scale to RNA pellet.
- Vortex / mix briefly
- Heat @ 65 °C for 1.5 hour (agitation optional)
- Cool to room temperature
- Add 7.5 mL of 1.5 M ammonium bicarbonate per 10 µmole scale to quench.
- Add an equal volume of Clarity RNA loading buffer to quenched deprotection solution.
- Proceed to trityl-on cartridge purification (pp. 15-16)

## 4.1 Clarity QSP for RNA Purification

**Format:** 96-Well Plate (50 mg/ well)
**Synthesis Scale:** ≤ 0.2 µmole

The 96-Well plate format can be performed using automated liquid handling systems or may also be used with manual 96-Well plate manifolds. The protocol can perform using either vacuum or positive pressure systems. The optimal vacuum or pressure settings differ between the various available automated systems; therefore the following serve only as suggestions and modifications may be required for each system.

**Remember:** Follow RNA sample preparation protocol in section 3.0 to ensure highest purity and recovery.

**IMPORTANT:** For each of the following steps, allow the entire volume to pass through the sorbent.

**1. Condition Cartridge:** 1 mL Methanol (0.5 mL x 2)
Initiate vacuum to 2-3" Hg. Flow at 2 drops / second

**2. Equilibrate Cartridge:** 2 mL Water (1.0 mL x 2)
Flow at 2 drops / second, vacuum setting 3-4" Hg

**3. Load RNA:** 1 mL
Monitor vacuum to ensure flow rate of 1 drop / 2-3 second (typically 3" Hg).
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

**Wash (optional):** 1.0 mL (0.5 mL x 2) dilute RNA loading buffer (60 % water/ 40 % RNA buffer) flow at 1 drop / second, vacuum setting 3" Hg
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *For crude purity yields of less than 70 % it is recommended to wash following the load step*

RNA

**4. Detritylate:** 1 mL Aqueous 0.5 % or 1.0 % DCA
Flow at 2 drops / second, vacuum setting 4-5" Hg.
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *A faint orange band will appear at the top half of the sorbent indicating DMT retention.*

***Note:*** *Automated parallel systems will experience a longer exposure to acid. Consequently it is suggested to lower the concentration of DCA to the lowest level possible that still allows complete DMT removal.*

**5. Rinse:** 1 mL Water (0.5 mL x 2)
Flow at 2 drops / second, vacuum setting 4-5" Hg

**6. Dry sorbent:**
Increase vacuum to 10" Hg for 1 minute

**7. Elution:** 1 mL 20 mM $NH_4HCO_3$ / 50 % Acetonitrile (see section 2.2)
Flow at 1 drop / second, vacuum setting 3-4" Hg.
***Note:*** *The initial eluting volumes may appear cloudy, but as the pH increases the solution will become water clear.*

***Caution:*** *During and after detritylation the unprotected RNA analyte is exposed to low pH, which over time can cause depurination and other modifications (please see section 5.0 for more information). It is therefore strongly recommended to elute in an appropriate buffer to ensure a final pH between 7 & 8. The various buffer salts mentioned in section 2.0 can be used at the discretion of the end user.*

**TIP:** It is not recommended to load more than 70 $ODs_{260}$ of crude synthetic RNA on 50 mg of resin.

**TIP:** Please contact Phenomenex for any additional assistance in optimizing your vacuum or pressure settings.

## 4.2 Clarity QSP for RNA Purification

**Format:** 50 mg/ 1 mL Cartridge
**Synthesis Scale:** ≤ 0.2 µmole

**Remember:** Follow RNA sample preparation protocol in section 3.0 to ensure highest purity and recovery.

**IMPORTANT:** For each of the following steps, allow the entire volume to pass through the sorbent.

**1. Condition Cartridge:** 1 mL Methanol (0.5 mL x 2)
Initiate vacuum to 2-3" Hg. Flow at 2 drops / second

**2. Equilibrate Cartridge:** 1 mL Water (0.5 mL x 2)
Flow at 2 drops / second, vacuum setting 3-4" Hg

**3. Load RNA:** 1 mL
Monitor vacuum to ensure flow rate of 1 drop / 2-3 second (typically 3" Hg).
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

**Wash (optional):** 1.0 mL dilute RNA loading buffer (60 % water / 40 % RNA Buffer) flow at 1 drop / second, vacuum setting 3" Hg
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *For crude purity yields of less than 70 % it is recommended to wash following the load step.*

RNA

**4. Detritylate:** 1 mL Aqueous 1 % or 2 % DCA
Flow at 2 drops / second, vacuum setting 4-5" Hg.
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *A faint orange band will appear at the top half of the sorbent indicating DMT retention.*

**5. Rinse:** 1 mL Water (0.5 mL x 2)
Flow at 2 drops / second, vacuum setting 4-5" Hg

**6. Dry sorbent:**
Increase vacuum to 10" Hg for 1 minute

**7. Elution:** 1 mL 20 mM $NH_4HCO_3$ / 50 % Acetonitrile (see section 2.2)
Flow at 1 drop / second, vacuum setting 3-4" Hg.
***Note:*** *The initial eluting volumes may appear cloudy, but as the pH increases the solution will become water clear.*

***Caution:*** *During and after detritylation the unprotected RNA analyte is exposed to low pH, which over time can cause depurination and other modifications (please see section 5.0 for more information). It is therefore strongly recommended to elute in an appropriate buffer to ensure a final pH between 7 & 8. The various buffer salts mentioned in section 2.0 can be used at the discretion of the end user.*

**TIP:** It is not recommended to load more than 70 $ODs_{260}$ of crude synthetic RNA on 50 mg of resin.

## 4.3 Clarity QSP for RNA Purification

**Format:** 150 mg/ 3 mL Cartridge
**Synthesis Scale:** ≤ 1.0 µmole

**Remember:** Follow RNA sample preparation protocol in section 3.0 to ensure highest purity and recovery.

**IMPORTANT:** For each of the following steps, allow the entire volume to pass through the sorbent.

**1. Condition Cartridge:** 3 mL Methanol (1.5 mL x 2)
Flow at 2 drops / second, vacuum setting 2-3" Hg

**2. Equilibrate Cartridge:** 3 mL Water (1.5 mL x 2)
Flow at 2 drops / second, vacuum setting 3-4" Hg

**3. Load RNA:** ~2.5 mL
Monitor vacuum to ensure flow rate of 1 drop / 2-3 second (typically 3-4" Hg).
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

**Wash (optional):** 1.5 mL dilute RNA loading buffer (60 % water/ 40 % RNA buffer) flow at 1 drop / second, vacuum setting 3" Hg
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *For crude purity yields of less than 70 % it is recommended to wash following the load step.*

RNA

**4. Detritylate:** 1.5 mL Aqueous 2 % or 3 % DCA
Flow at 2 drops / second, vacuum setting 4-5" Hg.
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *A faint orange band will appear at the top half of the sorbent indicating DMT retention.*

**5. Rinse:** 2 mL Water (1.0 mL x 2)
Flow at 2 drops / second, vacuum setting 4-5" Hg

**6. Dry sorbent:**
Increase vacuum to 10" Hg for 2 minutes

**7. Elution:** 2 mL 20 mM $NH_4HCO_3$ / 50 % Acetonitrile (see section 2.2)
Flow at 1 drop / second, vacuum setting 3-4" Hg.
***Note:*** *The initial eluting volumes may appear cloudy, but as the pH increases the solution will become water clear.*

***Caution:*** *During and after detritylation the unprotected RNA analyte is exposed to low pH, which over time can cause depurination and other modifications (please see section 5.0 for more information). It is therefore strongly recommended to elute in an appropriate buffer to ensure a final pH between 7 & 8. The various buffer salts mentioned in section 2.0 can be used at the discretion of the end user.*

**TIP:** It is not recommended to load more than 200 $ODs_{260}$ of crude synthetic RNA on 150 mg of resin.

## 4.4 Clarity QSP for RNA Purification

**Format:** 5 g/ 60 mL Cartridge
**Synthesis Scale:** 10-50 µmole

**Remember:** Follow RNA sample preparation protocol in section 3.0 to ensure highest purity and recovery.

**IMPORTANT:** For each of the following steps, allow the entire volume to pass through the sorbent.

**1. Condition Cartridge:** 30 mL Methanol (15 mL x 2)
Initiate vacuum to 2-3" Hg. Flow at 2 drops / second.

**2. Equilibrate Cartridge:** 30 mL Water (15 mL x 2)
Flow at 2 drops / second, vacuum setting 3-4" Hg

**3. Load RNA:** (Volume dependant upon scale)
Monitor vacuum to ensure flow rate of 1 drop / 2-3 second (typically 3-4" Hg).
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

**Wash (optional):** 15 mL dilute RNA loading buffer (60 % water / 40 % RNA Buffer) flow at 1 drop / second, vacuum setting 3" Hg.
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *For crude purity yields of less than 70 % it is recommended to wash following the load step*

RNA

**4. Detritylate:** 15 mL Aqueous 2 % DCA
Flow at 2 drops / second, vacuum setting 4-5" Hg.
***Important:*** *After the volume has passed through the sorbent, increase vacuum to ~5" Hg for approximately 30 seconds to expel remaining liquid.*

***Note:*** *A faint orange band will appear at the top half of the sorbent indicating DMT retention.*

**5. Rinse:** 20 mL Water (10 mL x 2)
Flow at 2 drops / second, vacuum setting 4-5" Hg

**6. Dry sorbent:**
Increase vacuum to 10" Hg for 2 minutes

**7. Elution:** 10 mL 20 mM $NH_4HCO_3$ / 50 % Acetonitrile (see section 2.2)
Flow at 1 drop / second, vacuum setting 3-4" Hg.
***Note:*** *The initial eluting volumes may appear cloudy, but as the pH increases the solution will become water clear.*

***Caution:*** *During and after detritylation the unprotected RNA analyte is exposed to low pH, which over time can cause depurination and other modifications (please see section 5.0 for more information). It is therefore strongly recommended to elute in an appropriate buffer to ensure a final pH between 7 & 8. The various buffer salts mentioned in section 2.0 can be used at the discretion of the end user.*

## 5.0 Depurination

While depurination is less of a concern for RNA versus DNA, acid and base induced modifications are a legitimate concern for anyone using Trityl-On purification. Other commercial Trityl-On purification products use long incubations with TFA to remove the Trityl group and thus see high level of unwanted modifications generated during purification. In the DNA case, depurination is often the concern; in the case of RNA, 2' to 3' isomerization is the major concern, with modifications

occurring during extended exposure to acid or base. The extensive amount of modifications in other Trityl-On products has caused many oligo producers and end users to spurn trityl-on purification particularly, cartridge-based formats.

In developing Clarity QSP, significant effort was made to monitor the causes and minimize the degree of damage to the oligonucleotide during detritylation. By focusing on reducing depurination in the DNA protocol, isomerization of RNA is also reduced.While various factors influence modification such as sequence composition, repeated studies using the **Clarity QSP protocol revealed that lower acid concentrations and limited exposure times significantly minimized depurination and other modifications, yet still provided complete trityl release.** Using ESI-MS, IEC, and polyamine hydrolysis for analysis, effective DCA strengths were varied from 1 % to 3 % with a 1-minute (on cartridge) oligo exposure time. While maintaining complete detritylation, we observed less than 2 % depurination using 1 % DCA whereas using 3 % DCA concentration, depurination had increased to 5 %. When using 96-Well plates with automated liquid handling systems, oligo exposure time to the acid treatment will vary among the loaded samples with some seeing a five-fold increase in duration to nearly five minutes. Accordingly, an investigation was performed using 0.5 % DCA and incubated for 5 and 15 minutes at room temperature. Using the same analytical techniques described above, less than 1 % depurination was present after either 5 or 15 minutes with complete trityl detachment occurring at both time intervals. For cartridge formats, we do not recommend using this low of acid concentration, as it may not be sufficient for complete detritylation.

**Another important feature of the Clarity QSP protocol is the introduction of pH buffered solutions in the final elution.** During and after the detritylation step, the oligonucleotide is exposed to a low pH environment. Subsequent water washes do not effectively elevate the retained oligo to a more appropriate physiological pH. It was observed again using MS and polyamine hydrolysis, that eluants in pH of at least 7 contained no additional depurination even after 72 hours at room temperature. In sharp contrast, when eluting in a water and acetonitrile solution and left at room temperature, within hours substantial depurination had occurred.

Unavoidably, base cleavage of oligonucleotides is an inherent occurrence in not only trityl-on purification but during synthesis as well. The recommended QSP protocol will not prevent depurination from occurring however; **our investigation does present a methodology that will improve detritylation efficiencies, while also drastically reducing the level of unwanted modifications in the final purified product.** From our outcomes we hope to attract former users of cartridge-based products to once again consider the efficacy and efficiency advantages of trityl-on purification.

RNA

| Problem | Cause | Solution |
|---|---|---|
| Orange band observed throughout entire sorbent | Possible overloading of sample on the resin. | Do not exceed recommended oligo concentration as described for the given synthesis scales in section 4.0. |
| Breakthrough of DMT full-length sequence observed in load elution | Flow rate too fast. | Ensure flow rate of 1 drop / 2 seconds. Some patience is required. |
| Incomplete removal of DMT observed in final product | Detritylation step: Acid strength too weak | Gradually increase DCA concentration. Do not exceed 3 % aqueous DCA as higher acid strength will increase depurination. |
| Final product is not as clean as advertised | Impurities were not eluted in the load eluant.<br><br>Wash was not used | During each step, allow solutions to pass completely through the sorbent.<br><br>Prepare wash solution by diluting RNA buffer (60% water / 40 % RNA buffer). Add volume according to synthesis scale. One wash should suffice. However, multiple washes can be performed without loss of the trityl-on full-length sequence. |
| Following detritylation, cloudy precipitate observed in the water wash | Exceeded recommended volume of water in rinse step. | Use the appropriate volumes as outlined for each scale. |
| Orange color was not present after acid addition. No recovery of full-length sequence in final elution. | No DMT group. | Ensure DMT was retained following synthesis. Do not adjust the pH in the deprotection cocktail. A pH below 5 will cleave the DMT group. Do NOT heat above 85 °C for extended period of time, or loss of trityl group may occur. |
| Flow rate too slow following conditioning and equilibration | Air is trapped in sorbent | Slowly increase vacuum to remove air pocket. A constant flow should then resume. There is no need to re-condition or re-equilibrate. |

## 7.1 Clarity QSP Sorbent

Store at room temperature (~25 °C) indefinitely.

## 7.2 Clarity RNA Loading Buffer

Store at room temperature (~25 °C) indefinitely.

# 8.0 Quality Assurance

The QA/QC of the sorbent include determination of the physical characteristics and a % recovery evaluation. The RNA buffer solution is tested for endo- and exo-nuclease contamination before packaging.

***NOTE:*** *See Certificate of Analysis included with cartridges and the loading buffer for more information*

# 9.0 Safety and Handling

The resin housed in the cartridges and plates requires no special handling nor does it impose any chemical or biological hazards.

The buffer is composed of biological compatible agents and is nonflammable. Do not however store at elevated temperatures (above 40 °C) for extended periods of time. This can cause pressure to build causing a sudden and possible violent release of vapors upon opening.

***NOTE:*** *For more information, refer to the MSDS sheets*

RNA

**Q Why use trityl-on cartridge purification?**

**A.** When properly designed, trityl-on cartridge formats offer the most efficient purification method available for synthetic oligonucleotides. Unlike HPLC or PAGE, the versatility of the cartridge format allows the end-user to tailor their purification requirements whether off-line or continuous on-line purification. Moreover, when automated, cartridge platforms can effectively purify thousands of samples per day, thereby substantially increasing the productivity for high-throughput manufacturers.

**Q. Can Clarity QSP Cartridges and 96-Well Plates be re-used?**

**A.** No. Strongly bound lipophilic and hydrophobic contaminants will remain on the sorbent thus causing cross contamination if re-used.

**Q Can other 5' chemistries be used with Clarity QSP?**

**A.** Yes. The QSP protocol can be used with any 5' protecting group providing a lipophilic handle for hydrophobic discrimination between contaminants and full-length sequences.

**Q Can the QSP sorbent be used for DNA?**

**A.** Yes. The sorbent is suitable for both RNA and DNA and can be used for sequences ranging from 10 nt to 100 nt.

**Q Can the RNA buffer be used for DNA?**

**A.** No. The RNA buffer is formulated specifically to work in 2' deprotecting cocktails and will not provide the same efficacy if used in alkaline DNA cleavage and deprotecting solutions.

**Q Does Clarity QSP remove trityl-on contaminants?**

**A.** No. The QSP process discriminates only trityl-off contaminants and cannot discern trityl-on impurities that may co-elute with the full-length sequence. Please do note that the peak often seen eluting immediately to the right of full-length sequences is in fact a deprotection modification of acrylonitrile reacting with the nucleobases. Widely mistaken as an n+1 trityl-on contaminant, this by-product actually results from extended exposure to alkaline conditions at high temperatures. (Bhan, et al. US Patent 6,887,990 B1, 5/3/05)

**Q Will sequence composition effect purity or recovery?**

**A.** No. During the development of QSP we evaluated thousands of sequences with varied combinations of the nucleobases. Purine or pyrimidine concentrations as well as varied sequence orders showed no difference in obtaining consistently high purity and recovery yields.

**Q What other acids can be used for detritylation?**

**A.** While we highly recommend using dilute aqueous DCA for our protocols, it is by no means a mandate. Dilute concentrations of TCA and TFA are effective for detritylation; however, in our research we observed higher rates of depurination among these acids than with similar concentrations of DCA. Acetic acid has been mentioned with other commercial products, but we strongly discourage its usage. Acetic acid requires extreme concentrations of at least 50 % for complete trityl

release. At this concentration, unwanted early elution of the full-length sequence occurs in the detritylation step resulting in substantial sample loss.

**Q Will the Clarity QSP protocol for RNA also work for purifying 2' ACE synthesized oligonucleotides?**

**A.** While the current protocol does not address 2' ACE chemistry, future protocols are being investigated for this chemistry.

## 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/ 1mL Cartridge | 50/Box |
| 8B-S102-SBJ | Clarity QSP | 150 mg/ 3mL 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 |

### *Accessories:*

| Part No. | Description | | Unit |
|---|---|---|---|
| AH0-7284 | 96-Well Plate Manifold | Acrylic | Ea |
| AH0-6024 | 24-Position Vacuum Manifold | Complete Set | Ea |
| AH0-7194 | 96 Square Well Collection Plate | 2 mL/Well (Polypropylene) | 50/pk |
| AH0-7408 | Solvent Waste Reservoir Tray | For Well Plate Manifolds | 25/pk |
| AH0-7195 | 96-Well Pierceable Sealing Mat | Square Well | 50/pk |

RNA

## 11.1 Conversions:

RNA 1 $OD_{260}$= 33 µg, 0.038 mg
Nanomoles = [milligrams / molecular weight] x $10^6$

## Molecular weights

Purines
A: 251
G: 267
Pyrimidines
U: 262
C: 230

## Molecular weight calculation of an oligonucleotide:

(nA x 251) + (nU x 262) + (nG x 267) + (nC x 230) + (61 x (n-1)) + (54 x n) + (17 x (n-1)) + 2.

Where: nA, nU, nG, and nC equal number of given base in the sequence.
n = total number of bases
(61 x (n-1)): molecular weight of phosphate group
(54 x n): Water molecules per nucleotide
(17 x (n-1)): $NH_4$ cations associated with phosphate groups

## Cumulative Population Failure Equation:

Purity Yield = (Coupling Efficiency Rate) $^{Couplings}$

## 11.2 RNA Applications & Chromatograms

## Quantitative Analysis: $OD_{260}$

## Qualitative (Purity)

IEC Chromatography
DNAPac® 200
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
VWD @ 260 nm

## Enclosed Data:

IEC Chromatograms
OD quantitation

## Clarity QSP 150 mg/ 3 mL cartridge with RNA loading buffer

RNA-21nt GGAGAACCUGCCAGCCGCTT MW: 6089 [1 µmole]

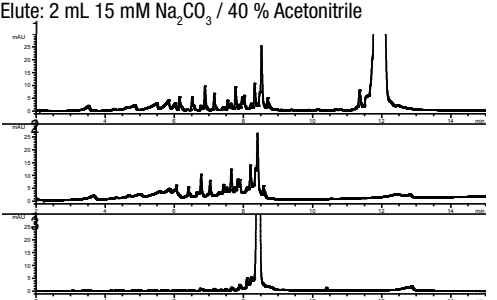
## Trityl-on RNA Sample Preparation

**2' Deprotection:**

- Added 250 µL of RNA deprotection (TEA.3HF) solution to dried RNA pellet
- Vortex / mix briefly
- Heated @ 65 °C for 1.5 hr
- Cooled to room temp
- Added 1 mL of 1.5 M ammonium bicarbonate to quench
- Added an equal volume of Clarity RNA loading buffer (1.25 mL) to quenched deprotection solution. [Final volume 2.5 mL]
- Proceeded to trityl-on cartridge purification

## Protocol followed

- Condition: 3 mL Methanol (1.5 mL x 2)
- Equilibrate: 3 mL Water (1.5 mL x 2)
- Load Vol of Oligo (2.5 mL)
- Detritylate: 1.5 mL 3 % DCA
- Rinse: 2 mL Water (1 mL x 2)
- Dried sorbent @ 10" Hg (~1 min)
- Elute: 2 mL 15 mM $Na_2CO_3$ / 40 % Acetonitrile



1.Crude Trityl-on
2. Load fraction
3. Detritylated final elution

$OD_{260}$

| Crude Trityl-on | Load Fraction | Detritylated final elution | Recovery | Purity (Peak area) |
|---|---|---|---|---|
| 62.8 | 9.91 | 43 | 81 % | 89 % |

RNA

## Clarity QSP 50 mg/ 1 mL cartridge with RNA loading buffer

RNA-21nt GGCUCCCCUCAACAACUUCTT MW: 6525 [200 nmole]

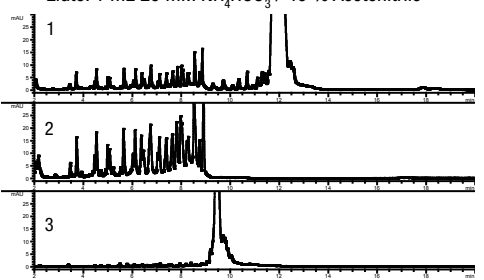
## Trityl-on RNA Sample Preparation

**2' Deprotection:**

- Added 100 µL of RNA deprotection (TEA.3HF) solution to dried RNA pellet
- Vortex / mix briefly
- Heated @ 65 °C for 1.5 hr
- Cooled to room temp
- Added 400 µL of 1.5 M ammonium bicarbonate to quench
- Added an equal volume of Clarity RNA loading buffer (500 µL) to quenched deprotection solution. [Final volume 1 mL]
- Proceeded to trityl-on cartridge purification

## Protocol followed

- Condition: 1 mL Methanol (0.5 mL x 2)
- Equilibrate: 1 mL Water (0.5 mL x 2)
- Load Vol of Oligo (1 mL)
- Detritylate: 1 mL 2 % DCA
- Rinse: 1 mL Water (0.5 mL x 2)
- Dried sorbent @ 10" Hg (~1 min)
- Elute: 1 mL 20 mM $NH_4HCO_3$ / 40 % Acetonitrile




1.Crude Trityl-on
2. Load fraction
3. Detritylated final elution

$OD_{260}$

| Crude Trityl-on | Load Fraction | Detritylated final elution | Recovery | Purity (Peak area) |
|---|---|---|---|---|
| 42.8 | 8.65 | 28.6 | 83.7% | 85 % |

## Clarity QSP 96-Well Plate with RNA loading buffer

RNA-28nt GACUCACAUCAACUACGAUCGAGCACTT MW: 8861 [200 nmole]

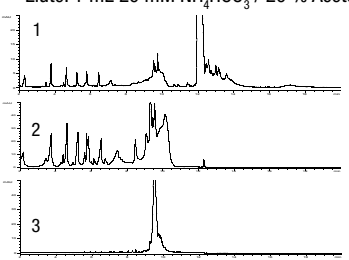
## Trityl-on RNA Sample Preparation

**2' Deprotection:**

- Added 100 µL of RNA deprotection (TEA.3HF) solution to dried RNA pellet
- Vortex / mix briefly
- Heated @ 65 °C for 1.5 hr
- Cooled to room temp
- Added 400 µL of 1.5 M ammonium bicarbonate to quench
- Added an equal volume of Clarity RNA loading buffer (500 µL) to quenched deprotection solution. [Final volume 1 mL]
- Proceeded to trityl-on cartridge purification

## 96-Well plate Protocol

- Condition: 1 mL Methanol (0.5 mL x 2)
- Equilibrate: 2 mL Water (1 mL x 2)
- Load Vol of Oligo (1 mL)
- Wash: 0.5 mL 40/60 [RNA buffer/Water] (0.5 mL x 1)
- Detritylate: 1 mL 1 % DCA
- Rinse: 1 mL Water (0.5 mL x 2)
- Dried sorbent @ 10" Hg (~1 min)
- Elute: 1 mL 20 mM $NH_4HCO_3$ / 20 % Acetonitrile




1.Crude Trityl-on
2. Load fraction
3. Detritylated final elution

$OD_{260}$

| Crude Trityl-on | Load Fraction | Detritylated final elution | Recovery | Purity (Peak area) |
|---|---|---|---|---|
| 61 | 24 | 26.7 | 72.8% | 81 % |

RNA

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