



OLIGO THERAPEUTIC EXTRACTION

User's Guide for Extracting Oligo Therapeutics from Biological Samples

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...breaking with tradition™



Extracting Oligo Therapeutics from Biological Samples

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Clarity OTX is patented by Phenomenex. U.S. Patent No. 7, 119,145. European Patent No. 1, 506, 239. Japanese Patent No. 4312605

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Extracting Oligo Therapeutics from Biological Samples

1.0 Clarity® OTX™ Overview

Clarity OTX was designed to extract and isolate a wide range of therapeutic oligonucleotides in a rapid four-step SPE method from matrix contaminants that interfere with LC/MS quantitation of a parent and its metabolites. This solution eliminates the need for LLE, can be automated for large sample sets, and results in recovery consistently greater than 80% with good sample-to-sample reproducibility.

2.0 Protocol

Components Required

- 100 mg / 3mL cartridges, or 100mg / 96-well plates*
- Lysis-Loading buffer** (Version 2.0, AL0-8579)
- Equilibration buffer
- Wash buffer
- Elution buffer
- Methanol (MeOH)
- Homogenization buffer or Proteinase K digest buffer if tissue samples are being analyzed

NOTE – The cartridges, 96-well plates, and Lysis-Loading buffer can be purchased from Phenomenex. The Equilibration, Wash, Elution, and Homogenization buffers should be prepared according to the recipes provided in Appendix 9.0.

** A larger scale version of Clarity OTX is now available in 500 mg/6 mL format.*

*** The exact components of the Lysis-Loading buffer are proprietary, but it does have the following properties.*

Lysis-Loading Buffer Properties	Yes	No
Cell Lysis	X	
Liposome disruption	X	
Protein denaturing	X	
Nuclease inhibition	X	
Protease inhibition	X	

Sample Preparation

Biological Fluids

1. Add at least equal volume of Lysis-Loading buffer to biological fluid matrix. Use additional volume of buffer if one needs to solubilize sample. Do not use water for dilution
2. Vortex briefly
3. Using a manifold (vacuum or positive pressure) or centrifuge, isolate and recover oligonucleotide via SPE extraction protocol

Tissue*

Option A – Homogenization

1. Finely mince tissue to be analyzed
2. Add Lysis-Loading buffer to tissue (approximately 3–5x tissue volume)
3. Homogenize tissue using a mechanical homogenizer
4. Using a manifold (vacuum or positive pressure), isolate and recover oligonucleotide via SPE** extraction protocol

Option B – Proteinase K Digest

1. Prepare stock solution of Tris/CaCl₂ digest buffer (directions for 100 mL volume are below)
 - Add 0.61 g of Tris to water and adjust to pH 8.0 with concentrated HCl
 - Add 0.55 g of CaCl₂ to the Tris buffer
2. Prior to digestion, add 0.86 mL of Tris/CaCl₂ digest buffer and 40 µL of Proteinase K (20 mg/mL concentration) to a 1.5 mL centrifuge tube,
3. Add ~100 mg of tissue per tube
4. Incubate @ 50 °C for 3 h
5. Centrifuge 10 minutes and collect supernatant
6. Add 0.9 mL of Lysis-Loading buffer to supernatant and vortex briefly
7. Using a manifold (vacuum or positive pressure), isolate and recover oligonucleotide via SPE extraction protocol

*These are just suggested procedures. Use of existing tissue-specific protocols are encouraged. Tissue homogenates using other methods can be combined at least 1:1 with LoadingLysis buffer prior to loading on OTX extraction cartridges. Call your local Phenomenex technical representative for additional information.

**Proteinase K Digest can be combined with mechanical homogenization for difficult samples. Addition of cheotropes can also improve Proteinase K digestion. See literature for additional homogenation ideas.

† See page 13 for additional information on Proteinase K Digest.

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Moving Liquid: Vacuum, Positive Pressure, or Centrifuge

Clarity OTX is manufactured in standard tube and 96-well plate formats that can be used with most types of SPE manifolds (vacuum or positive pressure) or centrifuges. For simplicity purposes, vacuum settings are suggested.

A slow and steady flow (1 drop/second or slower) to allow sample to interact with the sorbent greatly improves the performance of Clarity OTX products.

SPE Extraction Protocol

Biological Fluids

1. Condition: 1 mL MeOH (Vacuum ~2" Hg)
2. Equilibrate: 1 mL Equilibration buffer (Vacuum ~3" Hg)
3. Load sample:
Cartridges: 0.4 mL - 3 mL volume (Vacuum ~3" Hg) 96-well plates: 200 μ L - 1.5 mL volume (Vacuum ~3" Hg)
4. Vacuum: ~10" Hg for ~10 seconds to completely evacuate solution through cartridge or 96-well plate
5. Wash:
Cartridges: 4 mL Wash buffer (2 mL x 2) (Vacuum 3-4" Hg) 96-well plates: 3 mL Wash buffer (1.5 mL x 2) (Vacuum 3-4" Hg)
- 5a. Optional additional washes of equilibration and/or wash buffer can be implemented to reduce background
6. Vacuum: 10-15" Hg for 1 minute
7. Elution: 1 mL Elution buffer: (~3" Hg)
8. Dry down to near dryness or lyophilize and reconstitute in 100 μ L water or aqueous buffer. Reconstitution in urea/ EDTA can improve recovery.*

NOTE – If drying down the sample, never dry to completeness. Always retain 5-10 μ L of elution buffer.

NOTE – The extraction protocol for both biological fluids and tissues can be optimized based on oligonucleotide chemistry and/or biological matrix. Contact your local Phenomenex representative for optimization recommendations.

NOTE - A large volume Clarity OTX cartridge (500 mg/6 mL) is now available (P/N 8B-S103-HCH). A modified protocol using 4X volumes of the existing method can be used.

** See page 8 for additional information.*

SPE Extraction Protocol (cont'd)

Tissue

For improved cleaning efficacy, it is suggested to modify the Equilibration buffer with the addition of some Triton[®] X-100. Prepare this Modified Equilibration buffer by allocating 20 mL of Equilibration buffer (provided with starter kit or via provided formulation) and adding 100 μ L of 0.5 % Triton X-100 (Modified Equilibration buffer final composition: 50 mM NaHPO₄, pH 5.5, 0.0025 % Triton)

1. Condition: 1 mL MeOH (Vacuum ~2" Hg)
2. Equilibrate: 1 mL of Modified Equilibration buffer (see above)
3. Load sample:
Cartridges: 0.4 mL - 3 mL volume (Vacuum ~3" Hg) 96-well plates: 200 μ L - 1.5 mL volume (Vacuum ~3" Hg)
4. Vacuum: ~10" Hg for 10-15 seconds to completely evacuate solution through cartridge or 96-well plate
5. Rinse: 1 mL Modified Equilibration buffer (see above)
6. Wash: 4 mL Wash buffer (2 mL x 2 for cartridges or 1.5 mL x 2 for 96-well plates)
7. Rinse: 4 mL Equilibration buffer (2 mL x 2 for cartridges or 1.5 mL x 2 for 96-well plates)
8. Vacuum: ~10" Hg for 1 minute
9. Elution: 1 mL Elution Buffer (Vacuum ~3" Hg)
10. Dry down to near dryness or lyophilize and reconstitute in 100 μ L water or aqueous buffer. Reconstitution in urea/EDTA solution can help improve recovery

NOTE - If drying down the sample, never dry to completeness. Always retain 5-10 μ L of elution buffer.

NOTE - A large volume Clarity OTX cartridge (500 mg/6 mL) is now available (P/N 8B-S103-HCH). A modified protocol using 4X volumes of the existing method can be used.

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3.0 Troubleshooting

Problem	Cause	Solution
Following N ₂ dry-down, the white pellet did not completely dissolve	The pellet was evaporated to complete dryness.	DO NOT DRY TO COMPLETENESS. Reconstitute in 10-20 μ L of 8M urea/0.1 NH ₄ CO ₄ /0.5 mM EDTA. Then dilute 4X before LC/MS injection
Low (< 75%) or No recovery (typical recovery is 80-90%)	Sample not completely dissolved after reconstitution. Therapeutic sequence has a unique modification. The oligo did not load properly onto the sorbent. The oligo was dried down to completeness and has bonded to the polypropylene collection tube.	Ensure sample is completely dissolved after reconstitution. <u>Contact Phenomenex to discuss possible alternative elution formulations.</u> The pH of the sample loaded is too high or too low. The pH of the sample to be loaded on the sorbent, after mixing the sample with the Lysis-Loading buffer, should be ~5.5. Reconstitute as described above.
Chromatograms indicate that biological matrix contaminants are present	Loading buffer was not completely evacuated from SPE media diminishing the efficacy of subsequent steps. Appropriate buffer volumes were not administered. Biological sample is extremely complex and dirty.	Increase vacuum to 10-15" Hg immediately after loading sample on SPE media. Ensure the appropriate buffer volumes outlined in the protocol were used. Additional wash volumes of both wash and equilibration buffer can be added to the protocol to help remove unwanted contaminants. An additional high pH wash with minimal organic can be added to reduce background.

4.0 Storage and Stability

Cartridges and 96-Well plates

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

Buffers

Store the following buffers tightly closed in the refrigerator (~4 °C) for up to 24 months.

- Lysis-Loading buffer (light sensitive)
- Equilibration buffer

Store the following buffers tightly closed at room temperature (~25 °C) for up to 24 months.

- Wash buffer
- Elution buffer

5.0 Safety and Handling

Cartridges and 96-Well plates

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

Buffers

Lysis-Loading buffer: Avoid contact and inhalation. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.

Equilibration buffer: Avoid contact and inhalation. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.

Wash buffer: Keep away from heat, sparks, and open flame. Avoid contact and inhalation. Do not get in eyes, on skin, or on clothing. Avoid prolonged or repeated exposure. Do not use if skin is cut or scratched. Wash thoroughly after handling.

Elution buffer: Keep away from heat, sparks, and open flame. Avoid contact and inhalation. Do not get in eyes, on skin, or on clothing. Avoid prolonged or repeated exposure. Do not use if skin is cut or scratched. Wash thoroughly after handling.

NOTE – for more information, refer to the MSDS sheets available by contacting Phenomenex.

6.0 Quality Assurance

The QA/QC of the SPE media includes determination of the physical characteristics and a % recovery evaluation. All buffers are tested for conductivity and pH to ensure they are within specification.

NOTE: Request Certificate of Analysis for SPE cartridges / 96-well plates and the buffers for more detailed information.

7.0 Frequently Asked Questions

Q What types of therapeutic oligonucleotides can be extracted from biological fluids with Clarity® OTX™?

A DNA, RNA, miRNA, siRNA, phosphorothioates, LNA, single stranded, duplexed, and encapsulated oligonucleotides. As long as there is a phosphodiester or phosphorothioate backbone, the extraction protocol should provide excellent cleanup and recovery. If you have a question about a specific oligo type, please contact Phenomenex to discuss further.

Q What sequence-lengths can be used with Clarity OTX?

A Clarity OTX is designed for isolating and extracting therapeutic sequences ranging from 4nt to 50nt.

Q Can double stranded oligonucleotides be extracted using Clarity OTX?

A Yes, but only those sequences with less than 50 total base pairs are viable with Clarity OTX.

Q What is the concentration range that can be detected?

A For some oligonucleotides, a linear calibration curve from 5-2000 ng/mL have been observed. As every oligonucleotide is different and matrix challenges of each fluid/tissue are unique, different calibration ranges may be realized.

7.0 Frequently Asked Questions (*cont'd*)

Q Do the included buffers provide nuclease inhibition?

A Yes. The Lysis-Loading buffer is now formulated to provide cell lysis and remove all protease activity in biological fluids.

Q Can alternative lysis and/or load buffers be used?

A Yes. However, the Clarity[®] OTX[™] SPE media and buffers were developed to work in unison. Contact your local Phenomenex representative for method optimization tips.

Q Is a positive pressure or vacuum source required?

A Yes, in most cases. The Clarity OTX media particle size is not suitable for gravity flow. A vacuum source that can provide at least 10" Hg is required. Centrifugation or positive pressure can be used if vacuum is not available. Centrifuge speed should be optimized so that liquid evacuation does not exceed 2 minutes per step.

Q What other equipment is necessary?

A Vacuum or positive pressure manifold, vortex, centrifuge, speed vac evaporator or lyophilization.

Q Can the Clarity OTX cartridges and 96-well plates be re-used?

A No. Unlike current extraction procedures, Clarity OTX provides an on-sorbent isolation and extraction of oligonucleotides from biological fluids. Matrix contaminants are retained on the media while the targeted oligo sequence is extracted. Consequently, those contaminants cannot be effectively removed even with stringent and continued washing. Thus, re-using would pollute subsequent samples.

7.0 Frequently Asked Questions (*cont'd*)

- Q Can the Wash buffer be reformulated not to include NaH_2PO_4 in order to be more mass spec friendly?**
- A Yes. Ammonium acetate can be used as an alternative; however it is crucial that the final pH of the wash buffer be at 5.5.
- Q What type of mechanism is used to isolate the oligo therapeutics from the biological matrix?**
- A The Clarity® OTX™ sorbent is a mixed-mode, anion exchanger. It works by selectively retaining the oligo based on its inherent chemical properties.
- Q What is the white powder I'm seeing after drying down or lyophilizing the sample?**
- A Predominately salts, which should resolubilize after dry down or freeze drying. If particulates remain, brief centrifuging is recommended. However, for best recovery, never speed-vac to complete dryness.
- Q What linearity range and sensitivity can be achieved using the Clarity OTX extraction protocol?**
- A Both the linearity range and the sensitivity that can be achieved are highly dependent on the mass spectrometer being used. A linearity range of 5-2000 ng/mL for plasma and 1-100 µg/g of liver have been reported, though we are confident wider ranges can be achieved with more sensitive MS instrumentation dependant on the oligonucleotide and tissue being analyzed.

7.0 Frequently Asked Questions (*cont'd*)

Q What types of biological matrices can Clarity[®] OTX[™] extract oligos from?

A The extraction protocol has worked effectively with tissue and most biological fluids, notably plasma, serum, urine, and sputum. However, specific tissues and oligos WILL need optimization to maximize recovery.

Q Is it better to dry down or lyophilize the sample after the elution step?

A Both methods work well and don't influence the recovery. It is at the discretion of the scientist. If drying down the sample by speed vac evaporation, never dry to completeness, always retain 5-10 μ L of elution buffer.

Q How much Proteinase K is needed for digestion? What buffer do I use?

A Proteinase K from Qiagen (P/N 19133) is recommended. For each digested sample, add 40 μ L of Proteinase K to 0.86 mL of 0.05 mM Tris/5 mM CaCl buffer. Digest sample for 3 hours at 50 °C.

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8.0 Ordering Information

Part No.	Description		Unit
KSO-8494	Clarity [®] OTX [™] Starter Kit–Cartridges	Includes: 100 mg/ 3 mL cartridges (x50) Lysis-Loading buffer (60 mL) Equilibration buffer (250 mL) Wash buffer (350 mL) Elution buffer (60 mL)	Ea
KSO-9253	Clarity OTX Starter Kit–96-Well Plate	Includes: 100 mg/ 96-well plate (x1) Lysis-Loading buffer (60 mL) Equilibration buffer (250 mL) Wash buffer (350 mL) Elution buffer (60 mL)	Ea
8E-S103-EGA	Clarity OTX 96-Well Plate	100 mg/Well	1/Box
8E-S103-EBJ	Clarity OTX Cartridge	100 mg/ 3 mL	50/Box
8B-S103-HCH	Clarity OTX Cartridge	500 mg/ 6 mL	30/Box
AL0-8579	Clarity OTX Lysis-Loading Buffer Version 2.0	1 L	Ea

Part No.	Description		Unit
AH0-8950	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
AH0-8637	Solvent Waste Reservoir Tray	For Well Plate Manifold	25/pk
AH0-7195	96-Well Pierceable Sealing Mat	Square Well	50/pk

NOTE – The Clarity OTX Starter Kit is recommended for validating proof of concept or for extracting small numbers of samples (< 100)

NOTE – The individual Clarity OTX 96-well plates and Lysis-Loading buffer are recommended for large numbers of samples (> 100) and for amenability to liquid handling robots.

9.0 Buffer Recipes

Buffers	MW	Molarity	Volume (L)	Grams	Water	ACN	THF	50% NaOH Solution	28% NH ₄ OH Aqueous Solution
Equilibration*									
50 mm Na ₂ HPO ₄ / 2 mm NaH ₂ PO ₄ (pH 5.5)	—	—	—	—	100%			4-5 drops	
Na ₂ HPO ₄	138	0.05	1	6.90	—			—	
NaH ₂ PO ₄	65.01	0.002	1	0.13	—			—	
Wash*									
50 mm Na ₂ HPO ₄ / Acetonitrile (pH 5.5)	—	—	—	—	50%	50%		1-2 drops	
Na ₂ HPO ₄	138	0.05	1	6.90	—	—		—	
Elution									
100 mm NH ₄ HCO ₃ / 10% THF / 40% Acetonitrile (pH 8.8)	—	—	—	—	50%	40%	10%		4-5 drops
NH ₄ HCO ₃	79.06	0.1	1	7.91	—	—	—		—

Buffers	MW	Molarity	Volume (L)	Water	Conc. HCl
Proteinase K Homogenization Buffer					
50 mM Tris / 5 mM CaCl ₂ (pH 8.0)	—	—	1	100%	~2 mL
Tris	121.14	0.05	—	6.05	—
CaCl ₂	110.98	0.005	—	0.55	—

* NOTE – Ammonium acetate can be substituted for phosphate buffer to reduce Na⁺ adduct formation on MS. However, monitor pH closely to verify pH is maintained at pH 5.5.

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