Clarity™ Biosolutions

User Guide for Extracting Oligo Therapeutics from Biological Samples

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Extracting Oligo Therapeutics from Biological Samples

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 compound and its metabolites. This solution eliminates the need for liquid-liquid extraction (LLE), can be automated for large sample sets, and facilitates recoveries that are consistently greater than 80 % with excellent sample-to-sample reproducibility.

Components Required

- Cartridge Formats: 100 mg / 3 mL, 500 mg / 6 mL
- 96-Well Plate Formats: 2 mg / well, 25 mg / well, 100 mg / well
- Lysis-Loading buffer*
- Equilibration buffer
- Wash buffer
- Elution buffer
- Methanol (MeOH)
- Homogenization buffer (Lysis-Loading buffer, pH 5.5)
- Recommended for cartridges: 1.5 mL Eppendorf DNA LoBind Collection Tube

The cartridges, 96-well plates and Lysis-Loading buffers can be purchased from Phenomenex.
**Lysis-Loading buffer properties are listed below (individual components are proprietary).*

Lysis-Loading Buffer Properties

Lysis-Loading Buffer Property	Yes	No
Cell lysis	X	
Liposome disruption	X	
Protein denaturing	X	
Nuclease inhibition	X	
Protease inhibition	X	
Reduction of protein / peptide disulfide bonds		X

Extracting Oligo Therapeutics from Biological Samples

Sample Pre-Treatment

While the Lysis-Loading buffer inhibits the majority of nuclease activity in a sample, some scientists working with RNAi-based therapeutics may require complete nuclease activity inhibition. To do so it is strongly recommended to add TCEP-HCl (tris (2-carboxyethyl) phosphine hydrochloride) to the Lysis-Loading buffer (2.87 g/L).

For scientists working with phosphorothioates, it is recommended to add cysteine to the Lysis-Loading buffer to prevent desulfurization during sample processing (1 g/L).

Sample Preparation

Biological Fluids:

1. Add an equal volume of Lysis-Loading buffer to the biological fluid matrix
   Note: If using the reducing agent TCEP-HCl, allow 5-15 minutes at room temperature for complete reduction before loading to plate / cartridge
2. Vortex briefly.
3. Using a vacuum or positive pressure manifold, isolate and recover oligonucleotide via extraction protocol (see p. 5).

Tissue Samples:

Option A: Proteinase K

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 at 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 vacuum manifold or positive pressure manifold, isolate and recover oligonucleotide via extraction protocol (see p. 5).

Option B: Homogenization

1. Homogenize tissue in 0.9 mL of Lysis-Loading buffer. Many tools are available for the homogenization of samples; bead homogenizers will work best for tissue samples.
2. Dilute your sample 1:1 or 1:2 (v/v), with Lysis-Loading buffer and vortex briefly.
3. Using a vacuum manifold or positive pressure manifold, isolate and recover oligonucleotide via extraction protocol (see p. 5).

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Extraction Protocol for Biological Fluids

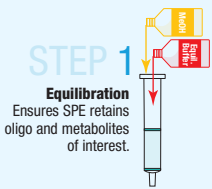
Sample Pre-Treatment	Apply appropriate sample pre-treatment (see p. 4) and dilute with Lysis-Loading buffer (1:1 or 1:2)		
Step	**Solvent**	**100 mg 96-Well Plates**	**3 mL Cartridges**
Condition	Methanol	1 mL	1 mL
Equilibrate	50 mM Ammonium Acetate (pH 5.5)	1 mL	1 mL
Load	Pre-treated sample	Up to 0.5 mL	0.4 - 3 mL
Wash	50 mM Ammonium Acetate (pH 5.5) with 50 % Acetonitrile	3x 1 mL (total volume 3 mL)	2x 3 mL (total volume 6 mL)
Elute	100 mM Ammonium Bicarbonate, (pH 9.5) with 40 % Acetonitrile and 10 % Tetrahydrofuran	1 mL	1 mL

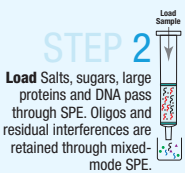
** Please refer to application notes AN48200722_W and AN48210722_W respectively for 2 and 25 mg 96-well plate protocols.*

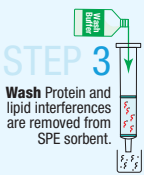
Extraction Protocol for Tissue Samples

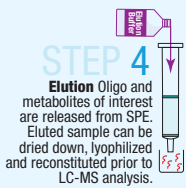
Sample Pre-Treatment	Apply appropriate sample pre-treatment (see p. 4) and dilute with Lysis-Loading buffer (1:1 or 1:2)		
Step	**Solvent**	**100 mg 96-Well Plates**	**3 mL Cartridges**
Condition	Methanol	1 mL	1 mL
Equilibrate	50 mM Ammonium Acetate (pH 5.5) with 0.5 % Triton® X-100*	1 mL	1 mL
Load	Pre-treated sample	Up to 0.5 mL	0.4 - 3 mL
Wash 1	50 mM Ammonium Acetate (pH 5.5)	3x 1 mL (total volume 3 mL)	2x 3 mL (total volume 6 mL)
Wash 2	50 mM Ammonium Acetate (pH 5.5) with 50 % Acetonitrile	3x 1 mL (total volume 3 mL)	2x 3 mL (total volume 6 mL)
Elute	100 mM Ammonium Bicarbonate, (pH 9.5) with 40 % Acetonitrile and 10 % Tetrahydrofuran	1 mL	1 mL

** It is advised to modify the equilibration buffer with 0.5 % Triton X-100 when working with tissue samples. To prepare, add 100 µL 0.5 % Triton X-100 and 20 mg of 0.1 % Cysteine to 20 mL of Equilibration Buffer.*









Extracting Oligo Therapeutics from Biological Samples

Method Optimization

Publication	Matrix	Oligo	Sample Pre-treatment	Modifications
Boos JA, Kirk DW, Piccolotto ML, et al.	Various Organs	miRNA/siRNA	Lysing matrix A tubes (MP Biomedicals) 1:10 Lysis-Loading buffer w/ TCEP FastPrep-24 (MP Biomedicals) Spin down @ 12,000 rpm	N/A
R. Wheller et al. / International Journal of Mass Spectrometry 345–347 (2013) 45–53	Plasma	siRNA (2'-OMe/F)	N/A	Equilibration: 50 mM Ammonium Acetate, 2 mM Sodium Azide, pH 5.5 Wash: 50 mM Ammonium Acetate / Acetonitrile (50:50) Elution: 100 mM Ammonium Bicarbonate / Acetonitrile / Tetrahydrofuran (50:40:10, v/v/v)
Christensen J, Litherland K, Faller T, et al.w	Plasma	siRNA-LNP	0.1 % Triton® X-100 was added to the Lysis-Loading buffer	N/A
Jiao K, Rashid A, Basu SK, et al.	Liver Tissue	DsiRNA	TissueLyser II homogenization homogenized in 100 mL of TEKnova denaturing buffer (TE buffer), pH 7.2	N/A
Perepelyuk M, Thangavel C, Liu Y, et al.	Tissue	Encapsulated siRNA	Homogenization in 0.1 M Tris buffer, pH 8.0 1:1 Lysis-Loading buffer	N/A

For additional support, chat with a live technical expert 24/7 www.phenomenex.com/chat

Troubleshooting

Problem	Cause	Solution
After homogenization of tissue sample, a white precipitate is observed, resulting in clogging of cartridge or plate.	Homogenate has not been completely solubilized, or incomplete homogenization.	Increase the volume of Lysis-Loading buffer; it is not uncommon to treat 1:4 (sample:LL buffer) prior to SPE. Consider the use of Proteinase K in addition to bead homogoenization, especially for any samples with connective tissue which may require more harsh homogenization and sample pretreatment.
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 µL of 8 M Urea/0.1 M Ammonium Bicarbonate/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.	Ensure sample is completely dissolved after reconstitution.
	Therapeutic sequence has a unique modification.	Contact Phenomenex to discuss possible alternative elution formulations.
	The oligo did not load properly onto the sorbent.	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.
	The oligo did not elute from the sorbent.	Increase the pH and/or change the organic solvent concentration of the elution buffer. Please contact Phenomenex for any specific recommendations depending on oligo type.
	The oligo was dried down to completeness and has bonded to the polypropylene collection tube.	Reconstitute as described above.
Chromatograms indicate that biological matrix contaminants are present	Lysis-Loading buffer was not completely evacuated from SPE media diminishing the efficacy of subsequent steps.	Increase vacuum to 10-15" Hg immediately after loading sample on SPE media.
	Appropriate buffer volumes were not administrated.	Ensure the appropriate buffer volumes outlined in the protocol were used.
	Biological sample is extremely complex and dirty.	Additional wash volumes of both wash and equilibration buffer can be added to the protocol to help remove unwanted contaminants. Depending on oligo type, modification of wash buffer can help to minimize matrix contamination.

Storage and Stability

Cartridges & 96-Well Plates

Store at room temperature (~25 °C).

Buffers

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

- Lysis-Loading buffer
- Equilibration buffer

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

- Wash buffer
- Elution buffer

Safety and Handing

Cartridges & 96-Well Plates

The SPE media housed in the cartridges and 96-well plates requires no special handling nor do they 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 SDS sheets available by contacting Phenomenex or by visiting www.phenomenex.com/documents/library

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. Certificate of Analysis documents can be found at www.phenomenex.com/QD using the batch number on the Clarity box.

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 clean-up 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 4 nt to 35 nt.

Q. Can double stranded oligonucleotides be extracted using Clarity OTX?

A. Yes, but only those sequences with less than 40 total base pairs are viable with Clarity OTX.

Q. What is the concentration range that can be detected?

A. Although linear dynamic ranges will vary depending on LC and MS instrumentation, calibration curves can be linear well over the 1 - 1000 ng/mL.

Q. Do the included buffers provide nuclease inhibition?

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

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

A. No. The Clarity OTX SPE media and buffers were developed to work in unison. Alternative solutions will not provide effective isolation or extraction of oligonucleotides.

Q. Is a vacuum source required?

A. Yes. The Clarity OTX media particle size is not suitable for gravity flow. A vacuum source that can provide at least 10" Hg is required. Alternatively, a positive pressure manifold may be used if no vacuum source is available.

Frequently Asked Questions

Q. What other equipment is necessary?

A. Vacuum manifold (or positive pressure manifold), vortex, centrifuge, N_2 dry down station and / or SpeedVac or Lyophilizer.

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.

Q. What type of mechanism is used to isolate the oligo therapeutics from the biological matrix?

A. The Clarity OTX polymeric sorbent is a mixed-mode, anion exchanger. It works by selectively retaining the oligo based on its inherent chemical properties.

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.

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

A. Preferably lyophilize. While evaporation techniques are practiced, drying to complete dryness can adversely affect the oligonucleotide.

Ordering Information

Clarity OTX™ Products

Part No.	Description		Unit
KS0-8494	Clarity OTX Starter Kit -Tubes	Includes: 100 mg / 3 mL cartridges (x50) Lysis-Loading buffer (100 mL) Equilibration buffer (250 mL) Wash buffer (350 mL) Elution buffer (100 mL)	ea
KS0-9253	Clarity OTX Starter Kit -96-Well Plate	100 mg/ 96-well plate (x1) Lysis-Loading buffer (100 mL) Equilibration buffer (250 mL) Wash buffer (350 mL) Elution buffer (100 mL)	ea
8E-S103-EGA	Clarity OTX	100 mg/well 96-well plate	1/ Box
8E-S103-CGA	Clarity OTX	25 mg/well 96-well plate	1/ Box
8M-S103-4GA	Clarity OTX	2 mg/well Microelution 96-well plate	1/ Box
8B-S103-EBJ	Clarity OTX	100 mg / 3 mL	50/ Box
8B-S103-HCH	Clarity OTX	500 mg / 6 mL	30/ Box
AL0-8579	Clarity OTX Lysis-Loading buffer, v2.0	1 L	ea

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

Note - The individual Clarity OTX 96-well plates & Lysis-Loading buffer are recommended for large sample volumes (> 100 µL) and for amenability to liquid handling robots.

Vacuum Manifolds, Collection Plates, and Sealing Mats

Part No.	Description		Unit
AH0-8950	96-Well Plate Manifold	Acrylic	ea
VM24	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

Presston™ 1000 Positive Pressure Manifold

Part No.	Description
AH1-7033	Presston 1000 Positive Pressure Manifold, 96-Well Plate

Phenomenex warrants the Presston 1000 Positive Pressure Manifold against defects in materials and workmanship under normal installation, use, and maintenance for a period of 12 months following delivery.

*Please visit **www.phenomenex.com/prestonwarranty** for complete warranty information.*

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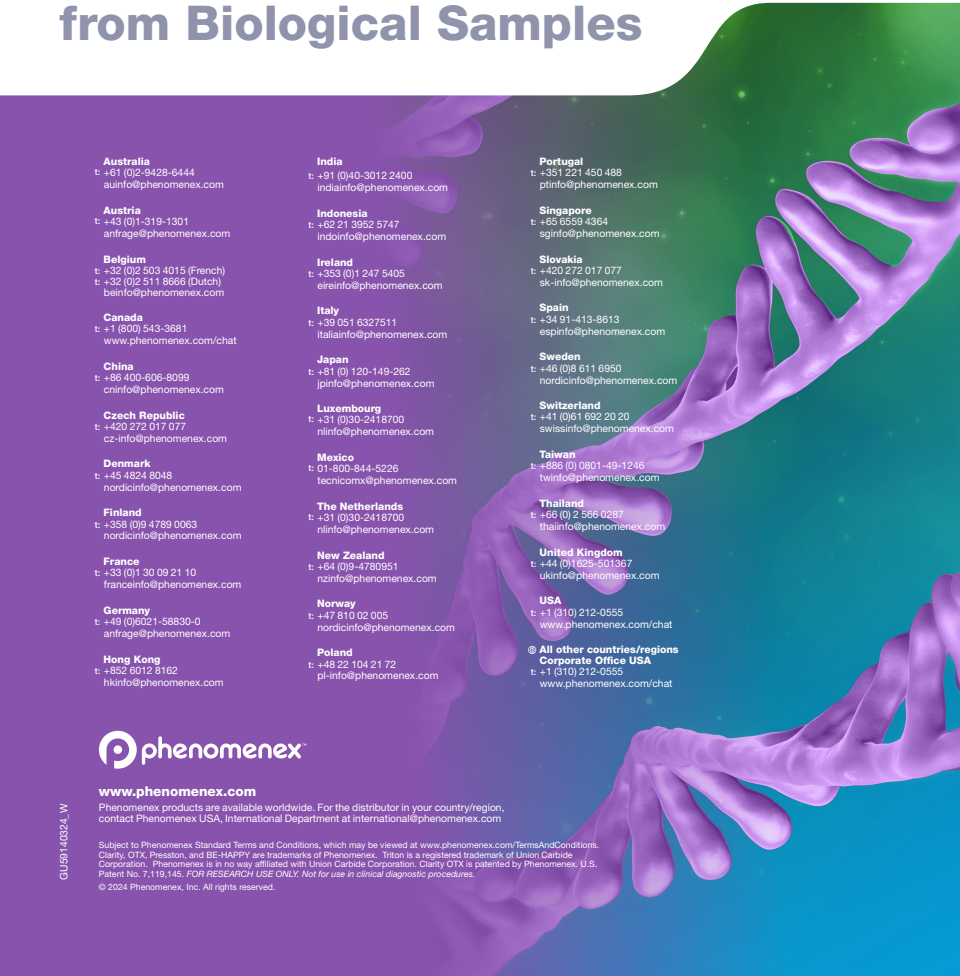

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