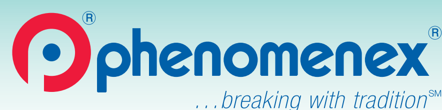
Oligo Therapeutic Extraction

User's Guide for Extracting Oligo Therapeutics from Biological Samples



Version 2.0

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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 / 3 mL cartridges or 100 mg / 96-well plates
- Lysis-Loading buffer* (version 2.0)
- Equilibration buffer
- Wash buffer
- Elution buffer
- Methanol
- Homogenization buffer or Proteinase K digest buffer (for Tissues)

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

**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	
Reduction of protein / peptide disulfide bonds		X

NOTE – Version 1.0 of the Lysis-Loading buffer required the addition of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and cysteine (CYS) to reduce protease and oxidative modifications. Version 1.0 has been replaced by Version 2.0, which comes preformulated with both TCEP and CYS.

Sample Preparation

Biological Fluids

1. Add an equal volume of Lysis-Loading buffer to biological fluid matrix
2. Vortex briefly
3. Using a vacuum manifold, isolate and recover oligonucleotide via extraction protocol

Tissue

Option A – Homogenization

1. Homogenize tissue in 0.9 mL of 0.1 M Tris buffer pH 8.0
2. Add an equal volume of Lysis-Loading buffer and vortex briefly
3. Using a vacuum manifold, isolate and recover oligonucleotide via extraction protocol

Option B – Proteinase K Digest

1. Prepare stock solution of Tris/$CaCl_2$ digest buffer (directions for 100 mL volume are below)
   - Add 1.21 g of Tris to water and adjust to pH 8.0 with concentrated HCl
   - Add 0.55 g of $CaCl_2$ to the Tris buffer
2. Prior to digestion, add 0.86 mL of Tris/$CaCl_2$ 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 vacuum manifold, isolate and recover oligonucleotide via extraction protocol

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 µL – 1.8 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: 6 mL Wash buffer (2 mL x 3) (Vacuum 3-4" Hg)
   96-well plates: 6 mL Wash buffer (1 mL x 6) (Vacuum 3-4" Hg)
6. Vacuum: 10-15" Hg for ~1 minute
7. Elution: 1 mL Elution buffer: (Vacuum ~5" Hg)
8. Dry down to near dryness or lyophilize and reconstitute in 100 µL water or aqueous buffer. For enhancing MS signal it is recommended to add 100 µM of EDTA to your reconstitution buffer

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

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

Tissue

For improved cleaning efficacy, it is recommended to modify the Equilibration buffer with the addition of 0.5 % 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 µL of 0.5 % Triton X-100 and 20 mg of 0.1 % Cysteine to it.

1. Condition: 1 mL MeOH (Vacuum ~2” Hg)
2. Equilibrate: 1 mL of *Modified* Equilibration buffer
3. Load sample:
   Cartridges: 0.4 mL - 3 mL volume (Vacuum ~3” Hg)
   96-well plates: 200 µL – 1.8 mL volume (Vacuum ~3” Hg) for
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
6. Wash: 6 mL Wash buffer (3 mL x 2)
7. Rinse: 4 mL Equilibration buffer (2 mL x 2)
8. Vacuum: ~15” 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 µL water or aqueous buffer. For enhancing MS signal it is recommended to add 100 µM of EDTA to your reconstitution buffer

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

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.	Retain 3-5 µL of solution in the sample and then reconstitute with 100 µL water or appropriate buffer.
Low (< 75 %) or No recovery	Sample not completely dissolved after reconstitution.	Ensure sample is completely dissolved after reconstitution.
(typical recovery is 80-90 %)	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 was dried down to completeness and has bonded to the polypropylene collection tube.	Retain 5-10 µL of elution buffer in tube.
Chromatograms indicate that biological matrix contaminants are present	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 can be added to the protocol to help remove unwanted contaminants without adversely affecting recovery.

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. The Lysis-Loading and Equilibration buffers are tested for endo- and exo-nuclease contamination before packaging. 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 Calibration curves are linear over the concentration range of 5-2000 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. However, contact your local Phenomenex representative for method optimization tips.

Q Is a 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 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 manifold, vortex, centrifuge, N_2 dry down station and/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 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.

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. Based on in-house data and customer feedback the extraction protocol can deliver sensitivity down to 50 ng/mL. However, with a very sensitive mass spec there should be no issue with achieving below 10 ng/mL. A linearity range of 5-2000 ng/mL for plasma and 10 - 100 µg/ g of liver have been investigated, though we are confident wider ranges can be achieved with more sensitive MS instrumentation.

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 Both methods work well and don't influence the recovery. It is at the discretion of the scientist. If drying down the sample, never dry to completeness, always retain 5-10 µL 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 µL of Proteinase K to 0.86 mL of 0.1 M Tris/ 5 mm CaCl buffer. Digest sample for 3 hours at 50 °C.

8.0 Ordering Information

Part No.	Description		Unit
KS0-8494	Clarity® OTX™ Starter Kit	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
8E-S103-EGA	Clarity OTX	100 mg/ 96-Well Plate	1/Box
8E-S103-EBJ	Clarity OTX	100 mg/ 3 mL Tubes	50/Box
AL0-8579	Clarity OTX Lysis-Loading Buffer Version 2.0	1 L	Ea

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

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

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 Manifold	25/pk
AH0-7195	96-Well Pierceable Sealing Mat	Square Well	50/pk

9.0 Buffer Recipes

Bufffers	MW	Molarity	Volume (L)	Grams	Water	ACN	THF	50 % NaOH Solution	28 % NH_4OH Aqueous Solution
Equilibration									
50 mm NaH_2PO_4 / 2 mm NaN3 (pH 5.5)	—	—	—	—	100 %			4-5 drops	
NaH_2PO_4	138	0.05	1	6.90	—			—	
NaN_3	65.01	0.002	1	0.13	—			—	
Wash									
50 mm NaH_2PO_4 / Acetonitrile (pH 5.5)	—	—	—	—	50 %	50 %		1-2 drops	
NaH_2PO_4	138	0.05	1	6.90	—	—		—	
Elution									
100 mm NH_4HCO_3 / 10 % THF / 40 % Acetonitrile (pH 8.8)	—	—	—	—	50 %	40 %	10 %		4-5 drops
NH_4HCO_3	79.06	0.1	1	7.91	—	—	—		—

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