Automated Method Development of Oligonucleotide in Tissues Using Clarity® OTX™ 96-Well Plate and High Resolution Mass Spectrometry

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Introduction

As synthetic oligonucleotide (oligo) therapeutics move through the drug-development path, there has been an increasing need for TK and PK/PD studies in drug discovery and development. Because of the specificity, sensitivity, and shorter method development time which they afford, LC/MS/MS methods are preferred over ligand binding assays or ELISA. However, isolation of the oligonucleotide is required prior to analysis via LC/MS/MS; the clean and efficient extraction of synthetic oligonucleotides often proves challenging during method development (MD) due to low sample concentration, analyte recovery, and the possible co-extraction of matrix interferences. Previously reported methods use a combination of liquid-liquid extraction (LLE) and solid phase extraction (SPE) for the isolation of oligonucleotide therapeutics from biological matrices (1). However, this method is not amenable to automation, which is prohibitive for high-throughput analysis.

To accommodate faster drug discovery and developmental needs, we investigated an automated SPE method development for oligonucleotides in tissues using a Tecan Freedom EVO® liquid handling platform and a Phenomenex Clarity OTX 96-well plate. The simple, one step SPE process using Clarity OTX technology is amenable to automation platforms, and the experimental design presented provides short and efficient SPE method development for oligos from various tissues, including mini pig liver, kidney, and heart tissue. The optimization of method wash and elution conditions was completely automated, with the recoveries of oligos in tissue samples exceeding 70% in most of the case studies. This standardized MD maximized lab throughput and removed cell debris such as proteins, genomic DNA, and lipids, significantly improving the detection level of oligos at lower concentrations with various systems including FLD, LC/MS/MS, or LC/HR/AM MS.

Chemicals and Reagents

5-10-5 2'-MOE Gapmer and BNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). The sequences, including methylation and thioation modifications, are noted below. The specific oligos were selected because their modifications make extraction and characterization more challenging. These are also the types of oligos that are commonly used as therapeutics.

Sequence of 5-10-5 2'-MOE Gapmer (m.w. 6,689.4)
5´-mC*mG*mA*mC*mU*A*T*A*C*G*C*G*C*A*A*mU*mA*mU*mG*mG-3´

Sequence of BNA oligo (m.w. 6,039.1)
5´- /5BNA-T/AA/iBNA-meC/AC/iBNA-G/TC/iBNA-T/AT/iBNA-A/CG/iBNA-meC/CC/3BNA-A/ -3´

Experimental Conditions

Tissue homogenate samples were prepared as follows: Mini pig liver and kidney tissues were diluted 1:4 with Lysis Loading Buffer, pH 5.5 and homogenized.

The following two experiments were conducted on a 96-well Clarity OTX plate in parallel using the Tecan Freedom EVO liquid handling system. One oligo in the same tissue matrix per plate is required. Each set of experiments contains matrix blank, recovery sample and samples spiked with the oligo at a single concentration (compound dependent). The 96-well plate map of MD is shown below.

Table 1.
Clarity OTX Method Development Plate Map

	1	2	3	4	5	6	7	8
A	W1, E1	W2, E1	W3, E1	W4, E1	W1, E1	W2, E1	W3, E1	W4, E1
B	W1, E1	W2, E1	W3, E1	W4, E1	W1, E1	W2, E1	W3, E1	W4, E1
C	W1, E2	W2, E2	W3, E2	W4, E2	W1, E2	W2, E2	W3, E2	W4, E2
D	W1, E2	W2, E2	W3, E2	W4, E2	W1, E2	W2, E2	W3, E2	W4, E2
E	W1, E3	W2, E3	W3, E3	W4, E3	W1, E3	W2, E3	W3, E3	W4, E3
F	W1, E3	W2, E3	W3, E3	W4, E3	W1, E3	W2, E3	W3, E3	W4, E3
G	W1, E4	W2, E4	W3, E4	W4, E4	W1, E4	W2, E4	W3, E4	W4, E4
H	W1, E4	W2, E4	W3, E4	W4, E4	W1, E4	W2, E4	W3, E4	W4, E4

BLK Matrix (n=1)
Sample at 6 µg of oligo per vial (n=2)
100% relative recovery sample (Red Font n=1)

Wash Solvent Optimization

To optimize washing conditions, loading and elution parameters remained constant. Maintaining pH 5.5 during the wash is critical for the interaction of the oligo to the Clarity® OTX™ mixed mode anion-exchange sorbent. As such, the parameter adjusted and varied was the organic solvent, using stronger elution strength to disrupt any hydrophobic interactions that may potentially retain and elute during the elution step.

The following wash solvents were used:

Wash Solvent 1 (W1): 50 mM Ammonium acetate pH 5.5: Acetonitrile (50:50 v/v)
Wash Solvent 2 (W2): 50 mM Ammonium acetate pH 5.5: Acetonitrile (30:70 v/v)
Wash Solvent 3 (W3): 50 mM Ammonium acetate pH 5.5: Acetonitrile: MeOH (50:40:10 v/v/v)
Wash Solvent 4 (W4): 50 mM Ammonium acetate pH 5.5: Acetonitrile: EtOAC (50:40:10 v/v/v)

Elution Solvent Optimization

To optimize the elution conditions, loading and washing conditions remained constant. Elution solvents investigated varied in both organic solvent (i.e., differing amounts of THF) as well as an increase in pH since Bartlett et al suggest a higher pH than the recommended pH 8.8 yields optimal recovery.

The elution solvents used were as follows:

Elution Solvent 1 (E1): 100 mM NH_4HCO_3 (pH 8.8): Acetonitrile: THF (50:40:10 v/v/v)
Elution Solvent 2 (E2): 100 mM NH_4HCO_3 (pH 8.8): Acetonitrile: THF (50:25:25 v/v/v)
Elution Solvent 3 (E3): 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:40:10 v/v/v)
Elution Solvent 4 (E4): 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:25:25 v/v/v)

Based on the Clarity OTX sorbent design, the above selected wash and elution solvents cover most quantities of oligos needed.

Automation and LC/MS/MS Conditions

Automated Solid Phase Extraction (SPE) Conditions using Tecan Freedom EVO®

96-Well Plate/Cartridge: Clarity OTX 96-Well Plate

Part No.: 8E-S103-EGA

Condition: *950 µL MeOH

Equilibrate: 950 µL 50 mM Ammonium acetate pH 5.5/w 0.5 % Triton™ X-100

Load: 950 µL Lysis loading buffer, then 400 µL tissue sample homogenate, mix with Tecan tips in the Clarity OTX 96-well plate

Wash 1: 950 µL 50 mM Ammonium acetate pH 5.5/w 0.5 % Triton X-100

Wash 2: 4 X 950 µL Wash Solvent 1-4 (W1-W4) according to the MD plate map (Table 1.)

Wash 3: 4 X 950 µL 50 mM Ammonium acetate pH 5.5

Dry: 3 minutes

Elute: 950 µL Elution Solvent 1-4 (E1-E4) according to the MD plate map (Table 1.)

Frozen:** Cap the sample plate, put in < -20 °C freezer over night

Dry Down:** Lyophilize the plate at – 80 °C

Reconstitute: 500 µL 1.0 % HFIP & 0.1 % DIEA with 10 µM EDTA in water

* 950 µL is the limitation of Tecan aliquot volume using 1 mL filter tip
**Dry down also can be performed on speed vacuum under N_2 if lyophilize is not available

LC/MS/MS method development was performed using a Clarity Oligo-XT column using a Shimadzu® Nexera® X2 UHPLC system (Shimdazu Corporation, Columbia, MD, USA) with an upper pressure limit of 1000 bar. MS analysis was performed using a Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA)

LC/MS/MS Conditions (cont'd)

Column: Clarity® 5 µm Oligo-XT
Dimensions: 50 x 2.1 mm
Part No.: 00B-4745-AN
HPLC system: Shimadzu® Nexera® X2 UHPLC
Mobile Phase: A: 1.0 % HFIP & 0.1 % DIEA with 10 µM EDTA in Methanol
B: 1.0 % HFIP & 0.1 % DIEA with 10 µM EDTA in Methanol Water (50:50 v/v)

Gradient:

Time (min)	% B
0.5	30
2.5	60
3.0	100
3.5	100
4.0	30
5.0	30

Flow Rate: 500 µL/min
Inj. Volume: 10 µL
Temperature: 40 °C
Detection: Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, HESI, negative polarity

MS/MS Conditions:

Parameter	Setting
Spray Voltage (-):	3500.0
Capillary Temperature (-):	400
Sheath Gas (-):	60.00
Aux Gas (-):	20.00
Spare Gas (+ or +-):	30.00
Spare Gas (-):	1.00
Max Spray Current (-):	100.00
Probe Heater Temp. (-):	450.00
S-Lens RF Level:	55.00
Ion Source:	HESI

Result and Discussion

Figure 1 shows oligo extraction recoveries of BNA from mini pig liver and kidney homogenate using 4 different wash solvents and 4 different elution solvents. The wash solvent and elution solvent yielding the highest recovery for BNA in liver homogenate were wash solvent 1 - 50 mM Ammonium acetate pH 5.5: acetonitrile (50:50 v/v) and elution solvent 4 - 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:25:25 v/v/v); The wash solvent and elution solvent yielding the highest recovery for BNA in kidney homogenate were wash solvent 1 and elution solvent 3 - 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:40:10 v/v/v)

Figure 2 shows extracted oligo recoveries of 2'-MOE Gapmer from mini pig liver and kidney homogenate using 4 different wash solvents and 4 different elution solvents. The solvent and elution solvent yielding the highest recovery for 2'-MOE Gapmer in liver homogenate were wash solvent 1 - 50 mM Ammonium acetate pH 5.5: acetonitrile (50:50 v/v) and elution solvent 3 - 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:40:10 v/v/v); The wash solvent and elution solvent yielding the highest recovery for 2'-MOE Gapmer in kidney homogenate were wash solvent 1 and elution solvent 4 - 100 mM NH_4HCO_3 (pH 10): Acetonitrile: THF (50:25:25 v/v/v).

Figure 3 shows representative of chromatogram of STD1 at 1 ng/mL of BNA in mini pig liver homogenate sample. The first integrated peak was BNA, second peak was analog internal standard. **Figure 4** shows the represented curve (n=2) of BNA in mini pig liver homogenate with quadratic regression at 1 – 1000 ng/mL.

Figure 1.
SPE Wash and Elution Solvent Optimization – BNA in tissue sample

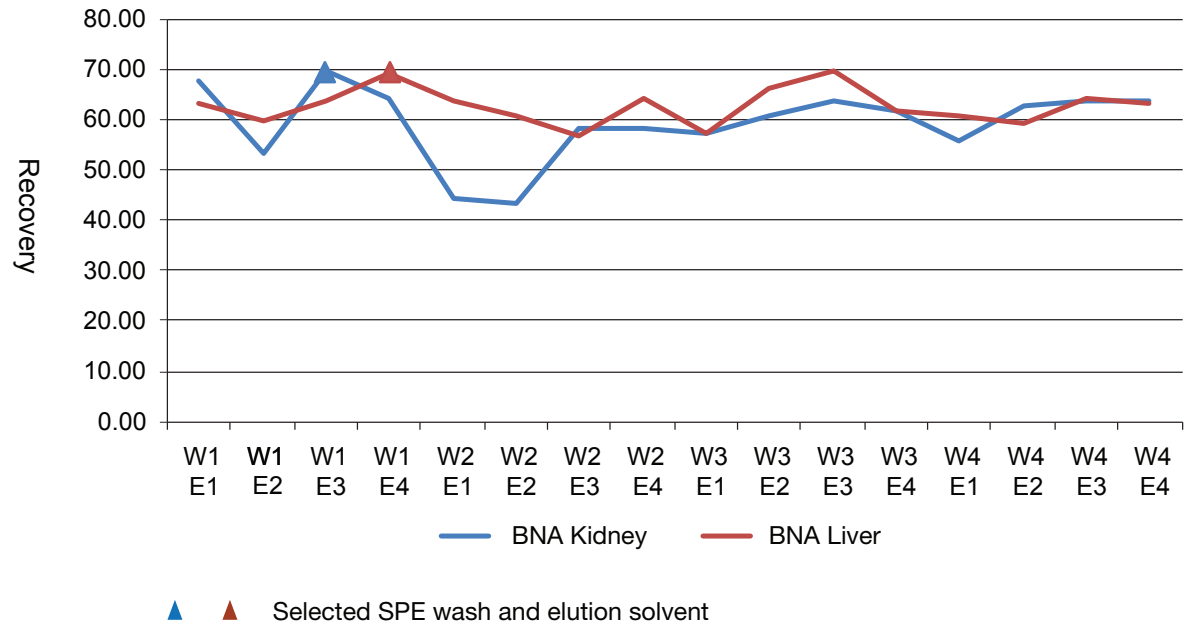


Figure 2.
SPE Wash and Elution Solvent Optimization - 2'-MOE Gapmer in tissue sample

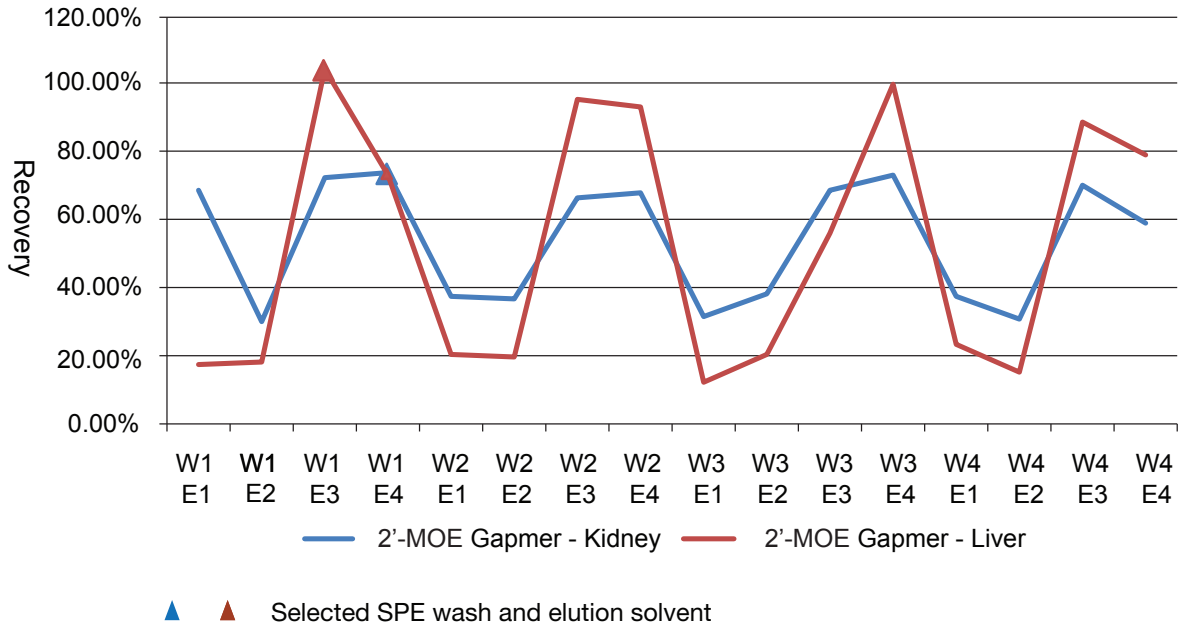


Figure 3.
Representative Chromatogram of BNA STD 1 (1 ng/mL) in mini pig liver homogenate

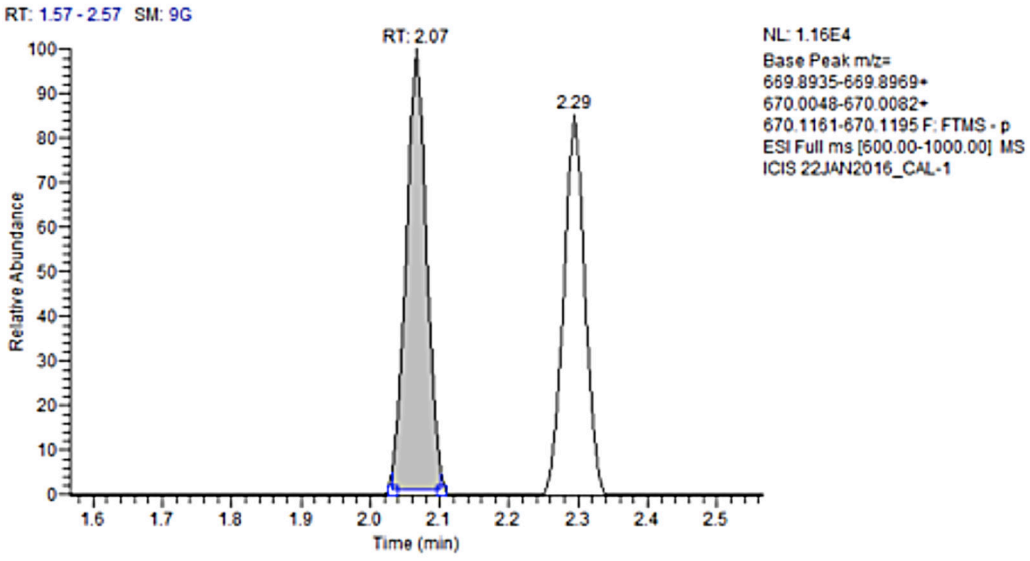
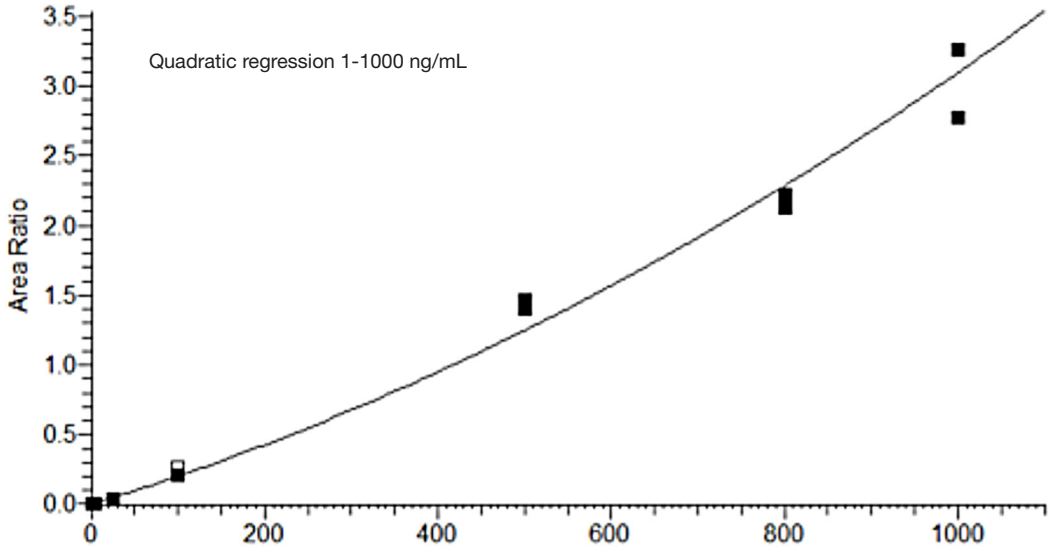


Figure 4.
Representative calibration curve of BNA in mini pig liver homogenate



Conclusion

The recent increase of large molecule quantitation and characterization often use HRMS. To achieve expected lower quantitation levels, focus should remain on concentrating and cleaning up oligos from biological samples. While the method development of oligo extraction can be challenging and time consuming, utilizing Clarity OTX 96-well plates and Tecan Freedom EVO offers a simplified experimental design. The Clarity OTX 96-well plate with developed methods offers more than 70% recovery of challenging oligonucleotides in tissue samples of complex matrices like mini pig liver and kidney homogenate.

References:

1. Zhang, G. et al. “Strategies for Bioanalysis of an Oligonucleotide Class Macromolecule from Rat Plasma Using Liquid Chromatography–Tandem Mass Spectrometry.” Anal. Chem., 2007, 79 (9): 3416–3424. DOI: 10.1021/ac0618674. Web: March 30, 2007

2. Chen, Buyun, and Michael Bartlett. “A One-Step Solid Phase Extraction Method for Bioanalysis of a Phosphorothioate Oligonucleotide and Its 3′ N-1 Metabolite from Rat Plasma by uHPLC–MS/MS.” The AAPS Journal 14.4 (2012): 772–780. PMC. Web. 13 May 2016.

Ordering Information

Clarity Oligo-OTX

Part No.	Description		Unit
KS0-8494	Clarity® OTX™ Starter Kit–Cartriges	**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
KS0-9253	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 Cartridge	50/Box
8B-S103-HCH	Clarity OTX Cartridge	500 mg/ 6 mL Cartridge	30/Box
AL0-8579	Clarity OTX Lysis-Loading Buffer Version 2.0	1 L	Ea

Accessories

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.

Ordering Information

Clarity® Oligo-XT

1.7 µm Minibore Columns (mm)			SecurityGuard™ ULTRA Cartridges‡
Phase	50 x 2.1	100 x 2.1	3/pk
Oligo-XT	00B-4747-AN	00D-4747-AN	AJ0-9515
			For 2.1 mm ID

2.6 µm Minibore and Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡	
Phase	50 x 2.1	100 x 2.1	50 x 4.6	100 x 4.6	3/pk	3/pk
Oligo-XT	00B-4746-AN	00D-4746-AN	00B-4746-E0	00D-4746-E0	AJ0-9515	AJ0-9514
					For 2.1 mm ID	For 4.6 mm ID

5 µm Minibore and Analytical Columns (mm)			SecurityGuard ULTRA Cartridges‡	
Phase	50 x 2.1	150 x 4.6	3/pk	3/pk
Oligo-XT	00B-4745-AN	00F-4745-E0	AJ0-9515	AJ0-9514
			For 2.1 mm ID	For 4.6 mm ID

5 µm Semi- Preparative Columns (mm)				SecurityGuard SemiPrep Cartridges*
Phase	50 x 10	100 x 10	150 x 10	3/pk
Oligo-XT	00B-4745-N0	00D-4745-N0	00F-4745-N0	AJ0-9516
				For 10 mm ID

5 µm Axia™ Packed Preparative Columns (mm)					SecurityGuard PREP Cartridges**	SecurityGuard PREP Cartridges***
Phase	100 x 21.2	150 x 21.2	250 x 21.2	150 x 30	/ea	/ea
Oligo-XT	00D-4745-P0-AX	00F-4745-P0-AX	00G-4745-P0-AX	00F-4745-U0-AX	AJ0-9517	AJ0-9518
					For 21.2 mm ID	For 30 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

* SemiPrep SecurityGuard Cartridges require holder, Part No.: AJ0-9281

** PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8223

*** PREP SecurityGuard Cartridges require holder, Part No.: AJ0-8277

guarantee

If analytical Clarity LC products do not provide at least an equivalent separation as compared to a competing product of the same particle size, similar phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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