

Oligo Workflow Resource Guide

End-to-End Workflow Solutions for Oligonucleotide Analysis

From research discovery to production QA/QC



Synthetic oligonucleotides are widely used in research and genetic testing. This class of short nucleic acid polymers includes small interfering RNA, antisense oligonucleotides, aptamers, and CRISPR guides. The popularity of these macromolecules means there is a growing need for robust analytical methods and easy-to-use data analysis workflows to characterize them. Common attributes of interest include the mass, sequence, purity, and relative quantity of specific impurities.

Oligonucleotide purity analysis

Purification of synthetic oligonucleotides can be uniquely challenging. Impurities associated with synthesis of the full-length product often include molecules that display truncations, incomplete thiolation, and base loss. Such structures must be considered when developing a purification method.

Agilent has developed a systematic approach to optimize oligo separation (Figure 1). The method uses either strong anion exchange (SAX) or ion-pair reversed-phase (IP-RP) chromatography; the latter works well with mass spectrometry. In addition, Agilent has developed separate workflows for performing SAX fractionation of oligonucleotides and confirming sample purity using either LC/UV or LC/MS methods.

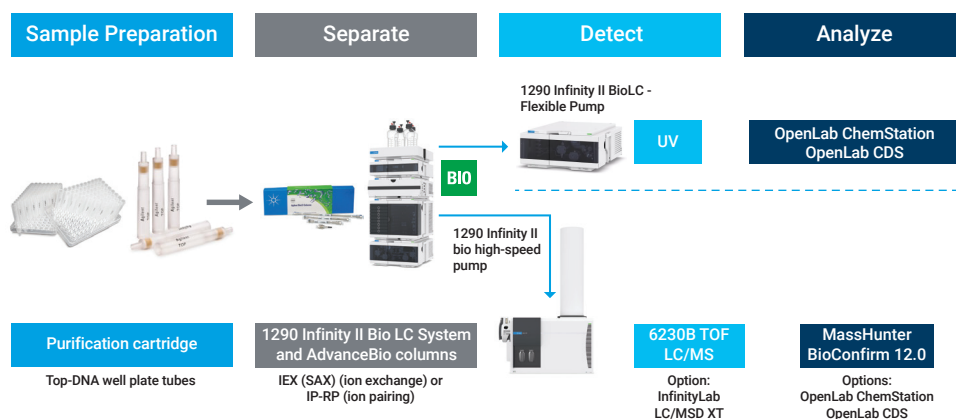


Figure 1. Workflow 1: Oligonucleotide purity analysis

Oligonucleotide Target Plus Impurities (TPI) analysis

The characterization of product-related impurities is an important task in the development of new biotherapeutics. Sources of common impurities include conversion of phosphorothioate to phosphodiester, truncations, extensions, and formation of abasic oligonucleotides.

Advanced analytical methods, such as LC/MS analysis, are indispensable for the characterization of target oligonucleotides and their impurities. Advanced techniques are needed because impurities are often numerous, present at very low abundances, and found in combination with one another. Because characterization of impurities can be challenging, software that supports and automates impurity profiling can be valuable. Figure 2 and Figure 3 show Agilent's workflow for analysis that includes impurities.

LC/MS – based approach for product-related impurities analysis

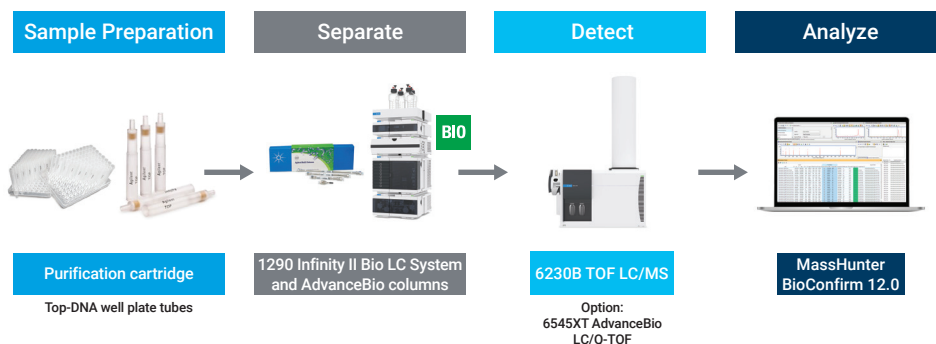


Figure 2. Workflow 2: Oligonucleotide Target Plus Impurities (TPI) analysis

Target Plus Impurities (TPI) data analysis workflow in Agilent MassHunter BioConfirm software 12.0

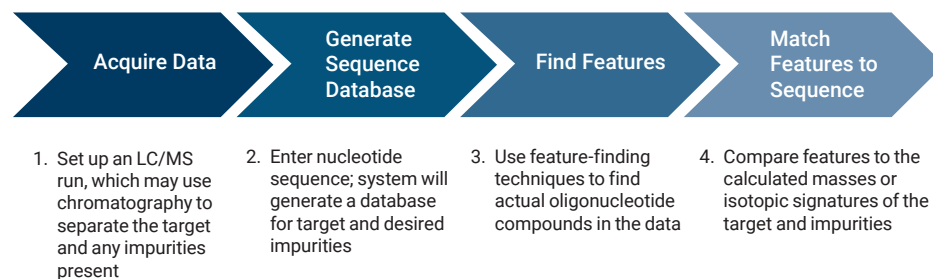


Figure 3. Workflow 2: Target Plus Impurities (TPI) data analysis workflow in Agilent MassHunter BioConfirm software 12.0

Oligonucleotide sequence confirmation

This sequence confirmation workflow uses an Agilent 1290 Infinity II bio LC system coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF (Figure 4 and Figure 5). The workflow uses fragment confirmation at the MS2 level by matching isotope patterns against expectations that are calculated from the oligo sequence. This matching capability is a new feature of the BioConfirm software. It demonstrates the power of coupling a high-resolution accurate mass system with targeted MS/MS data. In this approach, oligonucleotides are structurally characterized by confirming heavily modified sequences and determining the positions of specific chemical groups.

LC/MS/MS-based approach for analysis of full-length products and their impurities

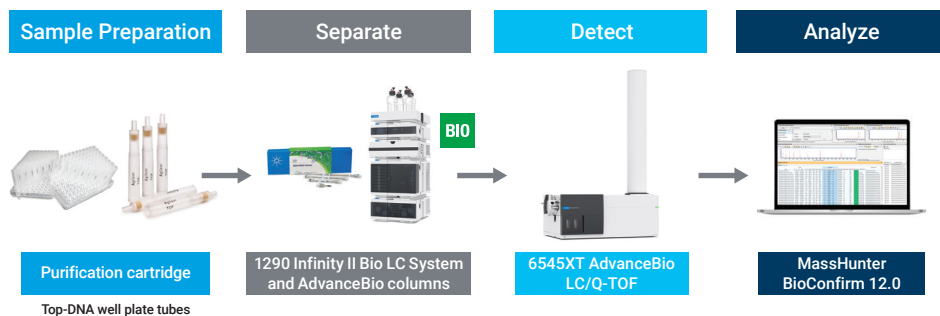


Figure 4. Workflow 3: Oligonucleotide sequence confirmation

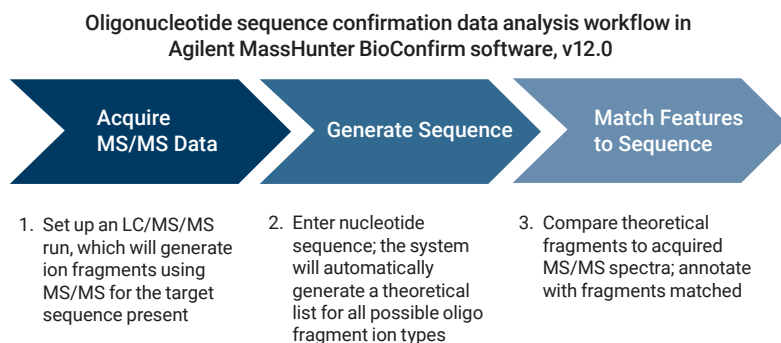


Figure 5. Workflow 3: Oligonucleotide sequence confirmation data analysis workflow in Agilent MassHunter BioConfirm software 12.0

Oligonucleotide purification solutions

Agilent offers the most comprehensive portfolio of flexible and reliable solutions for sample purification by liquid chromatography. No matter what scale of LC purification you are working at, Agilent has high-performance instrumentation, columns, software, and services that ensure highest purity and maximum recovery. Figure 6 presents Agilent's workflow for oligonucleotide purification, and Figure 7 compares analyte quantities and flow rates for Agilent systems operating at different scales.

1. .

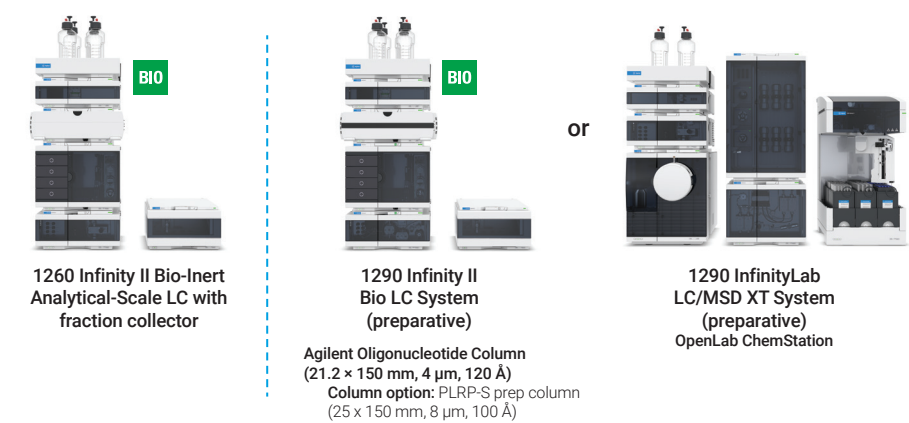


Figure 6. Workflow 4: Oligonucleotide purification

	Analytical		Semi-preparative		Preparative
Productivity Range	Micrograms (µg)	Milligrams (mg)	Grams (g)		
Agilent 1290 Infinity II Preparative LC System	1 – 50 mL/min		4 – 200 mL/min		
Agilent 1260 Infinity II Preparative LC System	1 – 50 mL/min				
Agilent 1220/1260/1290 Infinity II Analytical Scale LC Purification System	0.01 – 10 mL/min				
Column Inner Diameter (mm)	4.6	10	20 – 25	30	50
Typical Flow Rate (mL/min)	1	4.7	20 – 25	42	118

Figure 7. Comparison of analyte quantities and flow rates for Agilent systems operating at different scales

References

Oligonucleotide purity analysis

1. Hsiao, J.; Apffel, A.; Turner, M. Optimizing Separation of Oligonucleotides with Anion-Exchange Chromatography. Agilent Technologies application note, publication number [5994-4753EN](#), 2022.
2. Schneider, S. Comparability Studies for the Analysis of Nucleotides on Four Different LC Systems. Agilent Technologies application note, publication number [5994-4392EN](#), 2021.
3. Pulliere, F.; Christopher Welsby, C. Identification of Commercially Available Oligonucleotide Starting Materials Directly Through Containers. Agilent Technologies application note, publication number [5994-4239EN](#), 2021.
4. Best Practice for Nucleic Acid Thermal Stability Measurements Using the Cary 3500 UV-Vis Spectrophotometer. Agilent Technologies white paper, publication number [5994-4028EN](#), 2022.
5. Boden, J.; Meixner, J. Analysis of Oligonucleotides by Capillary Gel Electrophoresis with the Agilent 7100 Capillary Electrophoresis System. Agilent Technologies application note, publication number [5994-3864EN](#), 2021.
6. Feith, A. Analyzing Raw Material for Oligonucleotide Synthesis. Agilent Technologies application note, publication number [5994-3498EN](#), 2021.
3. Rye, P. High-throughput, Ion-Pairing-Free, HILIC Analysis of Oligonucleotides Using Agilent RapidFire Coupled to Quadrupole Time-of-Flight Mass Spectrometry. Agilent Technologies application note, publication number [5994-4945EN](#), 2022.
4. Wong, D. L.; Rye, P. An Integrated Workflow for the Analysis of Oligonucleotides and Their Impurities by Agilent High-Resolution LC/(Q-)TOF Mass Spectrometry. Agilent Technologies application note, publication number [5994-4817EN](#), 2022.
5. Hsiao, J.; Apffel, A.; Turner, M. Optimizing Separation of Oligonucleotides with Anion-Exchange Chromatography. Agilent Technologies application note, publication number [5994-4753EN](#), 2022.
6. Schneider, S. Comparability Studies for the Analysis of Nucleotides on Four Different LC Systems. Agilent Technologies application note, publication number [5994-4392EN](#), 2021.
7. Liao, B. Rapid Analysis of mRNA 5' Capping with High Resolution LC/MS. Agilent Technologies application note, publication number [5994-3984EN](#), 2021.
8. Rye, P.; Yang, Y. High-throughput Mass Spectrometry Analysis of Synthetic Oligonucleotides. Agilent Technologies application note, publication number [5994-3753EN](#), 2022.
9. Vanhoenacker, G.; Lecluyse, C.; Debyser, G.; Sandra, P.; Sandra, K.; Schipperges, S.; Schneider, S.; Huber, U. Evaluation of Different Ion-Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides. Agilent Technologies application note, publication number [5994-2957EN](#), 2021.

Oligonucleotide Target Plus Impurities (TPI) analysis

1. Grossman, J.; Herck, N. V.; Thibaut, F.; Verluyten, W.; Deckers, A.; Boon, J. P. and Hellings, M. Advanced Utilization of the Custom Calculator Tool for OpenLab CDS: An Oligonucleotide Example. Agilent Technologies application note, publication number [5994-5654EN](#), 2023.
2. Rye, P.; Schwarzer, C. MS1 Oligonucleotide Characterization Using LC/Q-TOF with HILIC Chromatography. Agilent Technologies application note, publication number [5994-5631EN](#), 2023.
10. Wong, D. L. Mass Spectrometric Characterization of Antibody-siRNA Conjugates using the Agilent 6545XT AdvanceBio LC/Q-TOF. Agilent Technologies application note, publication number [5994-2155EN](#), 2020.
11. Duong, P.; Bidlingmeyer, B. A.; Zhu, A.; Luke, S. Fast and High-Resolution Reversed-Phase Separation of Synthetic Oligonucleotides. Agilent Technologies application note, publication number [5991-6006EN](#), 2017.

Oligonucleotide sequence confirmation analysis















1. Li, G.; Rye, P. MS/MS Oligonucleotide Sequencing Using LC/Q-TOF with HILIC Chromatography. Agilent Technologies application note, publication number [5994-5632EN](#), 2023.
2. Wong, D. L.; Rye, P. Comprehensive and Integrated Workflow for Oligonucleotide Sequence Confirmation by Agilent High-Resolution LC/Q-TOF. Agilent Technologies application note, publication number [5994-5071EN](#), 2022.
3. Liao, B. Analysis of mRNA Poly-A Sequence Variants by High-Resolution LC/MS. Agilent Technologies application note, publication number [5994-3005EN](#), 2021.

Oligonucleotide purification









1. Rieck, F. Fast and Selective Purification of Oligonucleotides Using Preparative HPLC/MS and Software Support. Agilent Technologies application note, publication number [5994-4877EN](#), 2022. Coffey, A. Dynamic Binding Capacity of Oligonucleotides on PLRP-S Columns and Stationary Phases. Agilent Technologies technical overview, publication number [5994-4526EN](#), 2022.
2. Purify Your Way: Agilent Lock & Load Columns. Agilent Technologies brochure, publication number [5994-3907EN](#), 2021.
3. Rieck, F. Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography. Agilent Technologies application note, publication number [5994-3514EN](#), 2021.
4. Krieger, S.; Dickhut, C. Direct Analysis of In-Process Oligonucleotides Without Manual Purification. Agilent Technologies application note, publication number [5991-9490EN](#), 2018

Optimized workflows: Configuration and ordering information





Workflow 1: Oligonucleotide purity analysis

Bundle	Workflow 1A – LC/UV		Workflow 1B – LC/MSD XT		Workflow 1C – LC/TOF	
Sample Prep	TOP-DNA well plate tubes for 1 µmol scale, 150 mg, 96/pk (7572915C)					
Column	 AdvanceBio Oligonucleotide (659750-702) or Agilent PL-SAX (PL1951-1502)	 AdvanceBio Oligonucleotide (659750-702) or Agilent PLRP-S (PL1912-1502)	 AdvanceBio Oligonucleotide (659750-702) or Agilent PLRP-S (PL1912-1502)			
LC	 1290 Infinity II BioLC Required: G7131A or G7132A, G7137A, and G7116B Either: G7114B (VWD) or G7117B (DAD) 	 1290 Infinity II BioLC Required: G7131A or G7132A, G7137A, and G7116B Optional: G7114B (VWD) or G7117B (DAD) 	 1290 Infinity II BioLC Required: G7131A or G7132A, G7137A, and G7116B Optional: G7114B (VWD) or G7117B (DAD) 			
MS		 LC/MSD XT: G6135BA (with OpenLab ChemStation) or G6135CA (with OpenLab CDS)	 6230B LC/TOF: G6230BA			
SW	 In case customer has 3rd party CDS, if UV only, below Software is optional. OpenLab CDS: M8414AA or OpenLab ChemStation: M8380AA, M8510AA	 OpenLab ChemStation (G6135BA) or OpenLab CDS (G6135CA) is included. Add Deconvolution (M8363AA)	 BioConfirm 12 (M6025AA) Single workstation: M6026AA or M6027AA For network workstation: M6035AA or M6036AA or M6037AA			











Workflow 2: Oligonucleotide Target Plus Impurities (TPI) analysis

Bundle	Workflow 2A – LC/TOF		Workflow 2B – LC/Q-TOF	
Sample Prep	TOP-DNA well plate tubes for 1 μmol scale, 150 mg, 96/pk (7572915C)			
Column	 AdvanceBio Oligonucleotide (659750-702) or Agilent PLRP-S (PL1912-1502)	 AdvanceBio Oligonucleotide (659750-702) or Agilent PLRP-S (PL1912-1502)		
LC	 BIO 1290 Infinity II BioLC Required: G7131A or G7132A, G7137A, and G7116B Optional: G7114B (VWD) or G7117B (DAD)	 BIO 1290 Infinity II BioLC Required: G7131A or G7132A, G7137A, and G7116B Optional: G7114B (VWD) or G7117B (DAD)		
MS	 6230B LC/TOF: G6230BA	 6545XT LC/Q-TOF: G6549AA		
SW	 BioConfirm 12 (M6025AA) Single workstation: M6026AA or M6027AA For network workstation: M6035AA or M6036AA or M6037AA	 BioConfirm 12 (M6025AA) Single workstation: M6026AA or M6027AA For network workstation: M6035AA or M6036AA or M6037AA		

Workflow 3: Oligonucleotide sequence confirmation

Bundle	Workflow 3 – LC/Q-TOF	
Sample Prep	TOP-DNA well plate tubes for 1 µmol scale, 150 mg, 96/pk (7572915C)	
Column		AdvanceBio Oligonucleotide (659750-702) or Agilent PLRP-S (PL1912-1502)
LC		1290 Infinity II BioLC Required: G7132A, G7137A, and G7116B Optional: G7114B (VWD) or G7117B (DAD)
MS		6545XT LC/Q-TOF: G6549AA
SW		BioConfirm 12 (M6025AA) Single workstation: M6026AA or M6027AA For network workstation: M6035AA or M6036AA or M6037AA

Workflow 4: Oligonucleotide analytical & prep scale purification

Bundle	Workflow 4A – Analytical LC/UV		Workflow 4B – Prep LC/UV		Workflow 4C – Prep LC/MSD XT	
Column		AdvanceBio Oligonucleotide (659750-702)		AdvanceBio Oligonucleotide (671150-702) or PLRP-S Prep Column (PL1212-3800)		AdvanceBio Oligonucleotide (671150-702) or PLRP-S Prep Column (PL1212-3800)
LC		1260 Infinity II Bio-Inert Analytical-Scale LC Purification System with fraction collector G5654 & G5664B		1290 Infinity II Preparative LC G7161B, G7158B, G7114A, G7163B, G7166A (optional)		1290 Infinity II Preparative LC G7161B, G7111B, G7158B, G7114A, G7115A, G7170B, G9324A, G1170A, G4738A, G7163B, G7166A (optional)
MS						LC/MSD XT: G6135BA (with OpenLab ChemStation)
SW		OpenLab ChemStation M8380AA & M8510AA		Automated Purification Software for OpenLab ChemStation M8368AA		Automated Purification Software for OpenLab ChemStation M8368AA

Learn more:

www.agilent.com/oligonucleotides

Buy online:

www.agilent.com/chem/store

Get answers to your technical questions and
access resources in the Agilent Community:

community.agilent.com

U.S. and Canada

1-800-227-9770

agilent_inquiries@agilent.com

Europe

info_agilent@agilent.com

Asia Pacific

inquiry_lsca@agilent.com

www.agilent.com

DE73547804

This information is subject to change without notice.

© Agilent Technologies, Inc. 2023
Printed in the USA, December 1, 2023
5994-5790EN

