Oligonucleotide Mapping via LC-UV-MS/MS to Enable Comprehensive Primary Structure Characterization of an mRNA Vaccine Against SARS-CoV-2

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All authors and work were funded by Pfizer.
Outline

• Overview of the Comirnaty (BNT162b2) mRNA Vaccine Against SARS-CoV-2

• Oligonucleotide Mapping of BNT162b2 mRNA Primary Structure by LC-UV-MS/MS
  • Workflow
  • Core reportables providing direct primary structure understanding

• Oligonucleotide Mapping Method Development Highlights

• Additional Oligonucleotide Mapping Application Highlights
  • Batch Comparability
  • Variant Construct Comparisons
Overview of the Comirnaty mRNA Vaccine (BNT162b2) Against SARS-CoV-2
Basic Design of Pfizer/BioNTech mRNA Vaccine(s) against SARS-CoV-2

- Train patient’s immune system to recognize the virus, specifically the spike protein on the surface
- Give the “code” or “recipe” of the spike protein to your cells

**SARS-CoV-2 (3D Model)**

**Spike Protein**

mRNA for spike protein of coronavirus

mRNA is injected into muscle

The vaccine triggers production of the spike protein and antibodies specific to it

(Wrapp et al., 2020, Science)
Analytical Characterization of the Drug Substance (mRNA) is Critical for Development of a High Quality Manufacturing Process & Product

Drug Substance (mRNA)

Platform QC Assays

- Compendial methods
- **Purity** by Capillary Gel Electrophoresis
- **Purity** by Immunoblot
- **Concentration** by UV spectroscopy
- **Identity, Impurities** by PCR-based methods

Heightened Characterization

**Primary Structure**

- Oligonucleotide mapping (LC-UV-MS/MS)
- Nucleoside Analysis (LC-UV-MS)
- NextGen Sequencing (NGS)

**Higher Order Structure**

- Circular Dichroism (CD)

**Protein Expression**

- FACS
- Western Blot

The modulated 5' and 3' untranslated sequences optimize mRNA stability and translation efficiency. A 5' cap structure is added as a critical step that allows for large-scale production. All uridines are replaced by m1Ψ to further increase RNA stability and to reduce innate immune responses.

Figure adapted from Lewis LM, Badkar AV, Cirelli D, Comba R, Lerch TF. J Pharm Sci. 2023 Mar;112(3):640-647.
Oligonucleotide Mapping of mRNA Primary Structure by LC-UV-MS/MS is Applied in Three Ways to Support mRNA Vaccine Development

Direct Primary Structure Understanding
- 5’ terminus (capping)
- 3’ terminus (poly(A) – tail)
- Full-length mRNA

Batch Comparability Assessment
- Process changes
- Scale-up
- Scale-out

Orthogonal Identity
- BNT162b2 (Original)
- Variant constructs (Delta, Omicron)

Oligonucleotide Mapping of mRNA Primary Structure by LC-UV-MS/MS has Supported Regulatory Filings And Launches in 180+ Markets Globally

Figure adapted from Lewis LM, Badkar AV, Cirelli D, Combs R, Lerch TF. J Pharm Sci. 2023 Mar;112(3):640-647.
Oligonucleotide Mapping of BNT162b2 mRNA Primary Structure by LC-UV-MS/MS
**Simple, Robust, Semi-Automated Workflow**

- **Rapid One-Pot, One-Enzyme Sample Preparation**
  - Ribonuclease T1 Cleave 3’ to G

- **IP-RP-HPLC-UV** + **MS-MS/MS**

- **Semi-Automated Data Analysis**
  - Commercial Software + In-House VBA Tools

**Semi-Automated Data Analysis Workflow**

1. **Automated Search**
   - Mass table by retention time
   - Identifications (~70% Coverage)

2. **Automated LC-UV Annotation**
   - Match Peak IDs to Chromatogram
   - Reformatted Mass Table

3. **Supplement LC-UV Annotation**
   - Data mining & MS/MS Analysis Tools

4. **Supplement Missing Coverage**
   - Data mining & MS/MS Analysis Tools

5. **Add 5’ & 3’ Termini Characterization**
   - Match Peak IDs to Chromatogram
   - Reformatted Mass Table
   - Data mining & MS/MS Analysis Tools

**Final Reportables**

- Fully-Annotated Chromatographic Map
- Sequence Coverage Calculation & Map
- Curated Mass Table
- 5’ & 3’ terminus characterization
Result: 100% BNT162b2 Sequence Coverage Observed

- 232 oligonucleotides (48.8% Coverage)
- 70 oligonucleotides (51.2% Coverage)
- 46 oligonucleotides
- 14 oligonucleotides

Approximately half of consensus RNAseT1 cleavages map to one locus and half contain multiple loci

Missed-Cleavages & Non-Concensus Cleavages

V = N1-methyl pseudouridine
Oligonucleotide Mapping Enables Simultaneous Characterization of the 5’ Terminus Without Affinity Purification

Majority of 5’ terminus is capped
Oligonucleotide Mapping of mRNA Enables Simultaneous Characterization of the 3′ Terminus Without Affinity Purification

Poly(A) Tail “A30”

Original

A30 mis cleavage

Delta

Omicron

Poly(A) Tail “L70”

Poly(A) Tail Distribution Characterized

Moderate Shifts in Poly(A) Length Distribution Are Detectable
Detection of Multiple Loci
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th># of Loci</th>
<th>Monoisotopic Mass (Da)</th>
<th>Retention Time (min)</th>
<th>Ratio of XIC Areas Normalized to Isomer 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomer 1</td>
<td>CVAAG</td>
<td>1</td>
<td>1646.2453</td>
<td>28.9</td>
<td>1</td>
</tr>
<tr>
<td>Isomer 2</td>
<td>VCAAG</td>
<td>1</td>
<td></td>
<td>30.5</td>
<td>0.8</td>
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<td>Isomer 3</td>
<td>AVCAG</td>
<td>1</td>
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<td>33.8</td>
<td>1.1</td>
</tr>
<tr>
<td>Isomer 4</td>
<td>AVACG</td>
<td>1</td>
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<tr>
<td>Isomer 5</td>
<td>AACVG</td>
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<td>31.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Isomer 6</td>
<td>VACAG</td>
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<td>27.6</td>
<td>3.2</td>
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<tr>
<td>Isomer 7</td>
<td>CAAVG</td>
<td>3</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(V = N1\)-methyl pseudouridine

Measured XIC Areas of Non-Unique Sequence Isomers Correlate with their Number of Loci in the Full Length mRNA Sequence
Measured UV Areas Across Oligonucleotide Map Correlate with Theoretical UV Areas but only when Accounting for All Loci of Non-Unique Oligonucleotides

Theoretical peak areas were calculated using oligonucleotide compositions and NMR-derived extinction coefficients for pdG*, pdA*, pdC*, and N1-methylpseudouridine monophosphate\(^*\)


\(^*\)Empirically determined at Pfizer
Identification of Oligonucleotides by LC-MS/MS

The Challenge
MS/MS Fragmentation is Critical For Differentiating Oligonucleotide Sequence Isomers Generated Through Enzymatic Digestion by RNaseT1

Most Oligonucleotide Sequences are Unique

BNT162b2 Forward Sequence

26%

74% Shared

BNT162b2 Reverse Sequence

74% Unique

Most Oligonucleotide Masses are Not Unique

BNT162b2 Forward Sequence

BNT162b2 Reverse Sequence

99% Shared
Identification of Oligonucleotides by LC-MS/MS

HCD Fragmentation Study
Higher Energy Collision Dissociation (HCD) Parameters Optimized to Generate Fragmentation Appropriate for Oligonucleotide Mapping

Fragmentation of RNA Oligonucleotides is Complex

HCD Collision Energy Optimized at Stepped CE 17, 21, 25

Oligonucleotide Fragment Ion Coverage as a Function of HCD Energy & Length

Normalized BNT162b2 Sequence Coverage Across All Oligonucleotides as a Function of HCD Energy

Key
- 5' fragment ion
- 3' fragment ion
- Internal fragment ion

Charge densities are fixed at 0.4 charge / base

V = N1-methyl pseudouridine
Applying Optimized HCD to Differentiate 2 Sequence Isomers Differing by a Single Exchange in Base Positions

Extracted Ion Chromatogram

3 sequence isomers

MS/MS Spectra

Highly similar spectra

Narrow MS/MS isolation window avoids confounding fragment ions

Full Scan Mass Spectra of Precursor Ions

Other oligonucleotides

\( \vee = N1\text{-methyl pseudouridine} \)
Optimal Fragmentation Enables Differentiation of Highly Similar Sequence Isomers

Observed 5' MS/MS fragments

Key 5' fragment ion 3' fragment ion internal fragment ion

<table>
<thead>
<tr>
<th>1</th>
<th>5'VVCAAAGG3' [M-4H]4-</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>(319.0)</td>
<td>(337.0)</td>
</tr>
<tr>
<td>(559.1)</td>
<td>(577.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2</th>
<th>5'VVVAAACG3' [M-4H]4-</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>(319.0)</td>
<td>(337.0)</td>
</tr>
<tr>
<td>(559.1)</td>
<td>(577.1)</td>
</tr>
</tbody>
</table>

\( \vee \) = N1-methyl pseudouridine

**Observed 5' MS/MS fragments**

- **Position 2 Same**
- **Relative Intensity (%)**
  - 0
  - 10
  - 20
  - 30
  - 40
  - 50
  - 60
  - 70
  - 80
  - 90
  - 100

**Relative Intensity (%)**

- 0
- 10
- 20
- 30
- 40
- 50
- 60
- 70
- 80
- 90
- 100
Fidelity of Automated Oligonucleotide Identifications Comprehensively Verified by Decoy Searching

Decoy search **excluding** BNT162b2 mRNA construct

- Common Oligonucleotide Region
- Unique Oligonucleotide Region

Decoy 1
Decoy 2
Decoy 3

No Preferential Match

Decoy search **including** BNT162b2 mRNA construct

- Common Oligonucleotide Region
- Unique Oligonucleotide Region

Decoy 1
Decoy 2
Decoy 3
BNT162b2

Preferential Match to BNT162b2
Oligonucleotide Mapping of BNT162b2 mRNA Primary Structure by LC-UV-MS/MS

Batch Comparability Assessment
Oligonucleotide Mapping Enables Assessment of mRNA Batch Comparability

Oligonucleotide Maps Demonstrate Comparability of Multiple BNT162b2 mRNA Drug Substance Batches

- Side-by-side analyses are highly robust
- Chromatographic peaks overlay well
Oligonucleotide Mapping of BNT162b2 and Variant Construct Primary Structure by LC-UV-MS/MS

Orthogonal Identity Assessment
Oligonucleotide Mapping Enables Comparison of mRNA for Variant Constructs

**Key**
- Present in Original, Delta, & Omicron
- Present in Original & Delta
- Present in Original & Omicron
- Present in Delta
- Present in Omicron

**Oligonucleotide mapping detects:**
- Oligonucleotides unique to one or more constructs
- Differences in number of copies of multi-loci (non-unique) oligonucleotides
Conclusion

• Oligonucleotide mapping via LC-UV-MS/MS directly interrogates the primary structure of RNA, enabling enhanced structural understanding for mRNA vaccines, genetic therapies, and other RNA molecules.

• Semi-automated workflow produces a reproducible and fully-annotated oligonucleotide map:
  • Fully-annotated LC/UV chromatogram
  • Sequence coverage map (up to 100% sequence coverage - e.g. BNT162b2)
  • Microheterogeneity assessment of 5’ terminus capping and 3’ terminus poly(A) tail length

• MS/MS fragmentation was optimized and fidelity of identifications verified by decoy sequence searching.

• Oligonucleotide mapping assisted the development and commercialization of the Comirnaty® vaccine against SARS-CoV-2:
  • Elucidation of Structure (3.2.S.3.1)
  • Comparability (3.2.S.2.6)
  • Data supported regulatory filings to health authorities in 180+ markets

• Gau, B.C., Dawdy, A.W., et al. Sci Rep 13, 9038 (2023)
  • Step-by-step protocol and VBA-enabled data analysis tools are publicly available
Special Thanks

Brian Gau (Pfizer)
Lead oligonucleotide map co-developer & mass spectrometry lead on Comirnaty mRNA vaccine against SARS-CoV-2

BioNTech

ThermoFisher Scientific
Protein Metrics
Agilent
Waters