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Note d'application

# Synthetic mRNA Oligo-Mapping Using Ion-Pairing Liquid Chromatography and Mass Spectrometry

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## Abstract

Messenger RNA has quickly become an important modality for human medicine, as shown with its evaluation for cancer treatment and the FDA approval of mRNA vaccines for COVID-19. The rapid development of mRNA vaccines and other classes of mRNA therapeutics is supported by advances in analytical methodologies. One important aspect of such methodologies is confirming the identity, purity, and modification(s) of a therapeutic mRNA through mapping its sequence by liquid chromatography-mass spectrometry (LC-MS). Mass spectrometry-based sequencing of RNAs has an advantage over templated RNA sequencing by offering direct molecular detection of fragments, which can be used to localize nucleoside impurities and identify important structural attributes (5'-cap and poly-A tail). As such, we propose a workflow for comprehensive bottom-up LC-UV-MS characterization of mRNAs that yields mRNA component-annotated chromatograms derived from accurate-mass matching.

#### Benefits

 High chromatographic resolution and MS sensitivity with the use of ion-pairing reversed-phase chromatography in combination with the ACQUITY<sup>™</sup> Premier Oligonucleotide BEH<sup>™</sup> C<sub>18</sub> 300 Å Column Automated mRNA digest annotation based on accurate-mass matching as facilitated by *in silico* mRNA digestion calculations and application of waters\_connect/UNIFI™ Scientific Libraries

#### Introduction

The SARS-CoV-2 pandemic provided the impetus for the fast-paced development of nucleic-acid based medicine, especially synthetic mRNA.<sup>1</sup> Now, 40 years after its discovery in 1961 by Brenner et al.<sup>2</sup> [i], mRNA has evolved into being an important modality with massive potential, as shown with the start of an in-human clinical trial for cancer treatment<sup>1-3</sup> and the full approval of two COVID-19 mRNA vaccines by the US Food and Drug Administration in August 2021 and in January 2022, respectively. The rapid development of mRNA vaccines and other classes of mRNA therapeutics are supported by advances in analytical methodologies. One important aspect of such methodologies is confirming the identity, purity, and modification of a therapeutic mRNA through oligo-mapping and sequencing via liquid chromatography hyphenated to mass spectrometry (LC-MS). Nucleic acid sequencing technologies, like Sanger and next-generation sequencing (NGS), provide valuable information to drug developers. However, there is also a heightened level of analysis that can be achieved through the use of LC in combination with tandem MS (LC-MS/MS)<sup>4</sup> or MS<sup>E</sup> (alternating low and high collision energy)<sup>5</sup> based fragmentation. Similar to a proteomics bottom-up approach, LC-MS/MS or MS<sup>E</sup> based sequencing have the advantage of direct molecular detection of RNA fragments, including the detection and localization of nucleoside impurities and important structural attributes, like lipidated nucleobases,<sup>6</sup> endcapped residues, and polyA tail modifications.<sup>4,7</sup> Unlike bottom-up proteomics workflows, where a plethora of data processing solutions exist, options for RNA mapping are limited. We propose a workflow for oligo-mapping based on a bottom-up approach for the characterization of a given synthetic mRNA within a single platform comprising LC, UV detection and MS measurements. Digestion components were processed using an in-house developed, freely available in silico digestion library calculator, mRNAcalcondemand, in combination with waters\_connect™ to yield an annotated chromatogram. Here, we demonstrate this analytical approach for mRNA sequence mapping using RNase T1 digested luciferase mRNA.

### Experimental

### Sample Information

Approximately 90 µg of synthetic Cypridina luciferase mRNA (uncapped and not modified with a polyA tail); a gift from Bijoyita Roy (New England Biolabs, Ipswich, MA) was digested using 3'-guanosine specific ribonuclease RNaseT1 (Worthington Biochemical Corporation, Lakewood, NJ). Note that this workflow was repeated using 10 µg of TriLink Biotechnologies (CleanCap\* FLuc mRNA, San Diego, CA) firefly luciferase mRNA (untranslated sequences are proprietary) and comparable results were achieved. Luciferase mRNA was denatured prior to digestion using 20 µL urea (8 M) prepared in nuclease-free buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5 in water, Integrated DNA Technologies, Inc, Coralville, IA) at 80 °C for 5 minutes. Next, 24 µg (~10kU) of RNase T1 (Worthington Biochemical Corporation, Lakewood, NJ) resuspended in nuclease-free buffer was added to the denatured mRNA at room temperature and the mixture was then incubated at 37 °C for 30 minutes. Nucleasefree buffer (40 µL) was added at the end of the incubation period, bringing the total sample volume to 80 µL. The final aliquot was transferred to a polypropylene 300 µL autosampler vial (p/n: 186002639 < https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186002639-polypropylene-12x-32-mm-screw-neck-vial-with-cap-and-preslit-pt.html> ). The resulting digest was subjected to ion-pairing reversed-phase chromatography (IP-RPLC) without any further manipulation prior to MS detection in negative ion mode using the BioAccord<sup>™</sup> RDa<sup>™</sup> Detector.

#### LC Conditions

| LC system:          | ACQUITY UPLC <sup>™</sup> Premier BSM System (as part of the BioAccord System)          |
|---------------------|---|
| Detector:           | ACQUITY UPLC TUV Detector   |
| Wavelength:         | 260 nm  |
| Column:             | ACQUITY Premier Oligonucleotide BEH C18, 2.1 X<br>150 mm, 300 Å, 1.7 μm (p/n:186010541) |
| Column Temperature: | 70 °C   |
| Sample Temperature: | 4 °C  |

| Injection:     | 5 µL  |
|----------------|---|
| Flow Rate:     | 0.4 mL/min  |
| Mobile phase A | 0.1% N,N-diisopropylethylamine (DIPEA) as the IP<br>reagent and 1% 1,1,1,3,3,3-hexafluoroisopropanol<br>(HFIP) in deionized water |
| Mobile phase B | 0.0375% DIPEA and 0.075% HFIP in 65:35<br>acetonitrile/water  |

# **Gradient Table**

| Time (min) | mL/min | A (%) | B (%) | Curve |
|------------|--------|-------|-------|-------|
| Initial    | 0.4    | 97    | 3     | *     |
| 60         | 0.4    | 70    | 30    | 6     |
| 60.5       | 0.4    | 5     | 95    | 6     |
| 61         | 0.4    | 97    | 3     | 6     |
| 70         | 0.4    | 97    | 3     | 6     |

Run time = 70 minutes

# **MS** Conditions

MS system: Detector: Mode: BioAccord LC-MS System

ACQUITY RDa Detector

Full scan with fragmentation

| Polarity:                   | Negative                    |
|-----------------------------|-----------------------------|
| Cone voltage:               | 40 V                        |
| Fragmentation cone voltage: | 80–200 V                    |
| Mass range:                 | High (400-5000 <i>m/z</i> ) |
| Scan rate:                  | 2 Hz                        |
| Capillary voltage:          | 0.80 kV                     |
| Desolvation temperature:    | 400 °C                      |

### **Results and Discussion**

Ion-pairing reversed-phase chromatography (IP-RPLC) with a  $C_{18}$  stationary phase has become a tried-and-true approach for the analysis of oligonucleotides.<sup>4,7</sup> The mobile phase contains an ion pairing reagent, commonly an alkylamine, which adsorbs onto the  $C_{18}$  stationary phase<sup>8,9,10</sup> and thereby introduces a mixed mode like retention mechanism.<sup>8–10</sup> The N,N-diisopropylethylamine (DIPEA)/ 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) mobile phase system used in this application is compatible with both optical UV detection and negative ion mode mass spectrometry.<sup>4,7,-10</sup> HFIP is used to enhance electrospray ionization.<sup>8</sup>

RNase T1 digested luciferase mRNA was injected onto an ACQUITY Premier Oligonucleotide BEH C<sub>18</sub> (2.1 x 150 mm, 300 Å, 1.7 µm) Column and gradients were developed with an ACQUITY Premier Binary LC equipped with an ACQUITY UPLC TUV Detector. The ACQUITY Premier Oligonucleotide BEH C<sub>18</sub> Column used in this work is similar to ACQUITY Premier Oligonucleotide BEH C<sub>18</sub> 130 Å Column, but with a wider pore size, providing better resolution for longer oligonucleotide species. Data were acquired in triplicate using negative ion mode mass spectrometry with the ACQUITY RDa Detector of a BioAccord Benchtop LC-MS system. Moreover, the ACQUITY RDa Detector was programmed to acquire MS<sup>E</sup> data such that every other scan produced a high energy

fragment ion spectrum that could be later used to corroborate LC peak identifications. Figure 1 depicts total ion chromatograms (TIC) for an RNase T1 control sample (top trace), mRNA control sample (bottom trace) and the digested mRNA (middle trace). Oligonucleotide fragments resulting from the digestion of luciferase mRNA with RNase T1 were readily separated according to a separation with a 4-sigma peak capacity of 613. Overall, chromatographic peaks were sharp and symmetrical with ~0.01 minutes variation in retention time (RT) over triplicate injections. Digest components eluted between 2 and 23 minutes on a 60 minute gradient time method; some incompletely digested mRNA was seen to elute around 29 minutes and intact RNaseT1 eluted around 54 minutes. As can be seen on the top trace of Figure 1, an RNase T1 control sample showed signal only after a 50 minute retention time. This confirmed that RNase T1 would not introduce interference within the retention window of the mRNA digestion components. (Figure 1, middle trace). Likewise, the bottom trace of Figure 1 shows that intact luciferase mRNA elutes at approximately 38 minutes, confirming that the peak observed at 29 minutes in the digested sample (Figure 1, middle trace) corresponds to incompletely digested mRNA. We note here that in the case of synthetic mRNA comprising 5'-cap and poly-A tail structures, peaks at ~29 and 37 minutes are observed post digestion. The slight shift from ~38 minutes to ~37 minutes might be indicative of the undigested polyA structure (an investigation into this chromatographic behavior will be described in a future application note).



Figure 1: TIC for RNase T1 control sample (top trace), mRNA control sample (bottom trace) and the digest

(middle trace) obtained from ion-pairing reversed-phase chromatography (IP-RPLC) of luciferase mRNA digested with Rnase T1 and analyzed using the ACQUITY UPLC I-Class System (ACQUITY Premier Oligonucleotide BEH C<sub>18</sub> Column, 2.1 x 150 mm, 300 Å, 1.7 μm) and the BioAccord ACQUITY RDa Detector in negative ion mode.

The graphical user interface (GUI) of the *in-silico* digestion mRNA calculator, mRNAcalcondemand, is shown in Scheme 1. Next to the base sequence, a number of digestion parameters are specified, such as modification(s), enzyme, and missed cleavages. Based on this input, the calculator defaults to a number of MS specific settings, including charge state and m/z ranges, as well as the ability to conduct calculations based on monoisotopic or average mass. The generated output, in the form of a flat text csv file, can be utilized in UNIFI or waters\_connect software, or used for complementary downstream analysis.

| sequence and modifications  | MS settings   |  |
|---|---|--|
| AUGGAGGACGCCAAGAACAUCAAGAAGGGCCCCGCCCUUUUACCCCCUGGAGGAC<br>GGCACCGCCGGCGAGCAGCUGCACAAGGCCAUGAAGCGGUACGCCCUGGUGCCCGGC<br>ACCAUCGCCUUCACCGACGCCCACAUGAAGGUGGACAUCACCUACGCCGAGUACUUC<br>GAGAUGAGCGUGCGGCUGGCCGAGGCCAUGAAGCGGUACGGCCUGAACACCAACCA | Length Min Length 3 Length a-specific fragment Min Length 3 | Max Length<br>200<br>Max Length<br>200 |
| ACCUGCCCCCGGCUUCAACGAGUACGACUUCGUGCCCGAGAGCUUCGACCGGGACA Modifications/Fragments 0  | Charge<br>Min Charge  | - Max Charge                           |
| Modification  | Polarity negative   |  |
|   | m/z range   |  |
| Enzyme  | Min m/z range   | - Max m/z range                        |

#### Scheme 1: mRNA calculator GUI for in silico mRNA digest mass calculation.

digested\_sequence....csv

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Next, a library was created where the digest components are considered to be individual analytes, which was achieved by importing the output of the calculator as a spreadsheet into a UNIFI Scientific Library. The created libraries can be utilized in an HRMS Screening Analysis Method, targeting the digest compounds post-acquisition, using user-specified tolerances. The use of mass tolerance-based library matching is demonstrated below. As a result of the library search, an annotated chromatogram is automatically generated (Figure 2). A close-up, corresponding to a zoomed view of the 17 to 20 minute retention time window is represented in Figure

Reset Calculate



Figure 2: Annotated TIC of luciferase mRNA digest generated after matching, based on accurate mass, to a target component library. Luciferase mRNA was digested with Rnase T1 and analyzed using the ACQUITY Premier BSM LC (ACQUITY Premier Oligonucleotide BEH C<sub>18</sub> Column, 2.1 x 150 mm, 300 Å, 1.7 μm) and the BioAccord ACQUITY RDa Detector in negative mode. Target components were calculated using the mRNA MASS calculator.

A total of 436, 428, and 441 potential identifications (IDs) of digestion components were produced from each technical replicate upon screening data against an *in silico* library created with an allowance for up to 2 missed cleavages. Several criteria were considered for manual validation. 40 out of 441 identified components were rejected based on abundance and peak shape, *e.g.*, low abundant chromatographic peak shoulders. The majority of these rejected IDs (27 out of 40) were situated between 24 and 60 minutes. Overall, ~60% of the identified and validated components were within 10 ppm mass error (261 digest components). The RNase T1 control sample (Figure 1, top trace) was subjected to the same query and as expected did not yield any identifications. In addition to peak shape and abundance, we further validated the results based on high-confidence interpretation

of isotopic distributions to deduce charge assignments, which yielded 139 digest components (65% within 5 ppm mass error). Lastly, some assignments were not included in the analysis because they appeared to be redundant assignments triggered by repeated detection on broadened and shoulder-containing chromatographic peaks. The number of identifications was thereby reduced by another 16 components. In a couple instances, however, analyte masses showed up at multiple retention times to produce two unique IDs corresponding to two distinct, well-defined chromatographic peaks. Here, we also took note of the presence of isomeric IDs (species having the same chemical composition but different sequences) as well as isobaric IDs (species having different chemical compositions [different exact mass], yet similar nominal mass). Ultimately, 90 unique components could be identified based on accurate-mass matching, and these are reported in Table 1, including isomeric nucleotide sequences and isobaric ions (cells highlighted in grey in Table 1).

| DescriptionDescriptionDescriptionDescriptionDescriptionDevelopment13200420750.4.14.1302.43.4.1UCANCOCQPCelefinityConcord17200420750.4.14.1302.43.4.1Development1.3200420750.4.13.4.14.1303.4.13.4.1DevelopmentCelefinityConcord1.3.41.3.12.4.13.4.13.4.1DevelopmentCelefinityConcord2.3.41.3.1.31.4.14.1.14.1.1DevelopmentCelefinityConcord2.3.41.3.1.31.4.14.1.14.1.1DevelopmentCelefinityConcord2.3.41.3.1.31.4.14.1.14.1.1DevelopmentCelefinityConcord2.3.41.3.1.31.4.14.1.14.1.1DevelopmentCelefinityConcord2.3.11.3.1.11.4.11.4.14.1.1Development2.3.11.3.1.11.4.1.21.4.11.4.11.4.11.4.1Development2.3.11.3.1.11.4.1.21.4.11.4.11.4.11.4.1Development2.3.11.3.1.11.4.1.21.4.11.4.11.4.11.4.1Development2.3.11.3.1.11.4.1.11.4.1.11.4.11.4.11.4.1Development2.3.11.4.1.11.4.1.11.4.1.11.4.1.11.4.1.1Development2.3.1.11.4.1.11.4.1.11.4.1.11.4.1.11.4.1.1Development2.3.1.11.4  | Component name        | Formula            | Observed RT | Neutral mass | Observed   | Charge | Mass error | Additional ions         |
|---|-----------------------|--------------------|-------------|--------------|------------|--------|------------|-------------------------|
| Decomp.<  | 110110- 1             | Cont Landa Action  | (min)       | (Da)         | m/z        |        | (ppm)      | observed                |
| orrent of any and any   | ccuucecccer           | C030H48N14031P4    | 1.64        | 1320.15604   | 659.0689   | -2     | -4         | -2rl, -H                |
| constructure </td <td>UCUACUGCGn</td> <td>C84H107N30O66P0</td> <td>1.05</td> <td>2870 25772</td> <td>1434 1633</td> <td>-3</td> <td>-6.2</td> <td>-30</td>  | UCUACUGCGn            | C84H107N30O66P0    | 1.05        | 2870 25772   | 1434 1633  | -3     | -6.2       | -30                     |
| DAMPDecisionDisplayPart Part Part Part Part Part Part Part  | CCGGUCAAAGn           | C96H121N41O69P10   | 1.7         | 3261 45959   | 1086 1526  | -2     | -0.3       | -2H                     |
| Cale and controlDistant of the set of th                          | GAAGp                 | C40H50N20O27P4     | 2.1         | 1366.21047   | 682.0924   | -2     | -9.2       | -2H                     |
|   | CCGp_6                | C28H38N11O22P3     | 2.54        | 973.14057    | 485.567    | -2     | 6.6        | -2H, -H                 |
| CircleCircleSet ofSet   | GAGACACCCUAGp         | C115H145N49O82P12  | 2.54        | 3895.55339   | 1297.5089  | -3     | -3.2       | -3H                     |
| Cu0pCu0pCu0PartPa   | CUCGGp                | C47H61N18O37P5     | 2.55        | 1624.21331   | 1623.2076  | -1     | 1          | -H                      |
| abade <th< td=""><td>CUGp</td><td>C28H37N10O23P3</td><td>2.55</td><td>974.12459</td><td>973.1177</td><td>-1</td><td>0.4</td><td>-H, -2H</td></th<>  | CUGp                  | C28H37N10O23P3     | 2.55        | 974.12459    | 973.1177   | -1     | 0.4        | -H, -2H                 |
|   | GAGACAUCAUUGp         | C115H143N47O84P12  | 2.55        | 3897.52142   | 1298.1735  | -3     | 3.5        | -3H                     |
| ACDQ<br>CAPUSADD27.397.0097  | UAUUGAUAGCAGp         | C115H142N46O85P12  | 2.55        | 3898.50544   | 1948.2401  | -2     | -3.1       | -2H                     |
| CADBCapacity (Capacity (Capaci                          | ACGp                  | C29H38N13O21P3     | 2.73        | 997.1518     | 996.1407   | -1     | -3.8       | -H, -2H                 |
| AllogCapacity (Capacity (Capac                          | CAGp                  | C29H38N13O21P3     | 2.73        | 997.1518     | 996.1407   | -1     | -3.8       | -H, -2H                 |
| AABp -         Controlsmits Control         3.18         1000000         1000000  | AUGp                  | C29H37N12O22P3     | 2.92        | 998.13582    | 997.1285   | -1     | 0          | -H, -2H                 |
| ACCCCUMAADAG         CTININAMAGNIPP         3.4.4         JINEAR         JINEAR        JINEAR <thjinear< th="">        &lt;</thjinear<>  | AAGp_4                | C30H38N15O20P3     | 3.18        | 1021.16304   | 1020.153   | -1     | -2.7       | -H, -2H                 |
| Diade         Diade <thdiade< th="">         Diade         <thd< td=""><td>ACACCCUAGAAGp</td><td>C115H145N49O81P12</td><td>3.44</td><td>3879.55848</td><td>1292.17</td><td>-3</td><td>-8.8</td><td>-3H, -6H, -9H</td></thd<></thdiade<>   | ACACCCUAGAAGp         | C115H145N49O81P12  | 3.44        | 3879.55848   | 1292.17    | -3     | -8.8       | -3H, -6H, -9H           |
| CCCQp         CD191000102094         3.94         [27.11980         0.98         0.9 <td>UGAUCUUGp</td> <td>C75H94N26O60P8</td> <td>3.9</td> <td>2566.30045</td> <td>1282.1387</td> <td>-2</td> <td>-3.9</td> <td>-2H</td>  | UGAUCUUGp             | C75H94N26O60P8     | 3.9         | 2566.30045   | 1282.1387  | -2     | -3.9       | -2H                     |
| CLAUP         CLAUP <th< td=""><td>CCCGp</td><td>C37H50N14O29P4</td><td>3.91</td><td>1278.18186</td><td>638.0888</td><td>-2</td><td>6.9</td><td>-2H, -3H, -H</td></th<>   | CCCGp                 | C37H50N14O29P4     | 3.91        | 1278.18186   | 638.0888   | -2     | 6.9        | -2H, -3H, -H            |
| Database   | CCUGp                 | C3/H49N13O30P4     | 3.91        | 1279.16587   | 638.5822   | -2     | 9          | -2H, -3H, -H            |
| AMACCOUND         CONTRIPUTSOCIAPS         AUX         SEXTLAGEN         Line         Line <thline< th=""> <thline< th=""> <thline< th="">         Lin</thline<></thline<></thline<>  |                       | C75H95N27060P8     | 3.91        | 2581.31135   | 1289.6535  | -2     | 3,4        | -2H                     |
| Dam         Consistent/CODER*         P.20         BIZTURD #         DIZE         P.10         P.14         P.10         P.14         P.10         P.14         P.10         P.14         P.10         P.14         P.10         P.11         P.10         P.11  | Accecute              | C75H96N28O59P8     | 3.91        | 2080.32733   | 1289.1002  | -2     | 0.2        | -28                     |
| DAMAGE         Constraints Constraints         Dist Constraints <td>HAGCCGOGp</td> <td>C20H40N17029P4</td> <td>4.17</td> <td>122710024</td> <td>1312.075</td> <td>-2</td> <td>-1.1</td> <td>-20</td>   | HAGCCGOGp             | C20H40N17029P4     | 4.17        | 122710024    | 1312.075   | -2     | -1.1       | -20                     |
| CIUCUDP         CAMMENNOQUEPS         5.40         1945-1010         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1945-1100         1  | AAAGn                 | C40H50N20O26P4     | 4.75        | 1250 21556   | 1240 2040  | -1     | -2.5       | -H, -2H, -3H            |
| UDCOG         C4444891003795         59.43         1984.6776         79.1011         -22         4.1         -241414414           ACCCOp         C4740601703795         6.51         1900.2021         800.602         -2         -24141441           AUCCOp         C4740601703795         6.53         1600.2136         803.050         -2         6.11         -24141441           AUCCOp         C444167080395         6.52         4486.6975         119.210         -2         3         -2413144141           AUAAAA         C6497723010459         7.39         1980.23246         94.4118         -2         3         -2413144141           CCCCCAp         C569773010459         7.39         1980.23246         94.6114         -2         3         -2413144141           CCCCCAp         C569773010479         8.63         1901.2325         970.8375         -2         3         -2413144141           AUCCAp         C5697730230479         8.63         1901.2325         970.8375         -2         3         -2413144141           AUCCAp         C5697730230479         8.63         1901.2325         970.8375         -2         3         -2413144141           AUCCAAP         C5697730230  | CUCUGa                | C46H60N15O38P5     | 5.81        | 1585 19118   | 791 5962   | -2     | -2.5       | -2H -3H -H              |
| CACCOp         CHTME201003279         0.81         1802.234.04         0.82.214.0         -22         1.4         -24. <t< td=""><td>UCCCGp</td><td>C46H61N16O37P5</td><td>5.83</td><td>1584.20716</td><td>791.1011</td><td>-2</td><td>5.1</td><td>-2H, -3H, -H</td></t<>  | UCCCGp                | C46H61N16O37P5     | 5.83        | 1584.20716   | 791.1011   | -2     | 5.1        | -2H, -3H, -H            |
| AUCUGp         CH*ME01703279         6.51         M00.20241         09.20.97         -22         -24  | CACCGp                | C47H62N19O35P5     | 6.18        | 1607.23438   | 802.6146   | -2     | 4.8        | -2H, -3H, -H            |
| AUCCOp         C+FMENRIO309F         6.5.2         H80.2189         903.1076         -2.3         -3.1         -2.4         -3.4         -3.4         -3.4           CAAAAG         C494162/230.2389         6.79         1955.25844         928.025         -2.3         3.7         -241.341441.41            CCCUCOp         C59417241004498         7.38         1980.2246         941.422         -2.4         3.7         -241.341441.41           CUCCCCAp         C59417341004498         7.38         1980.22708         951.35         -2.4         3.6         -241.341441.41           AUCCAp         C5717312304278         8.88         1992.2770         956.84         -2         6.3         -241.341441.41           AUCCAp         C5947714004476         8.83         1992.2701         978.353         -2         3.6         -241.341441.41           AUCCAp         C5947712204278         8.80         1992.2701         978.353         -2         3.6         -241.341441.41           AUCCAp         C5947712204278         9.80         1992.2701         978.457         -2         3.7         -241.341441.41           AUCCAp         C5947712204278         9.80         1992.2701         978.457         -2         3   | AUCUGp                | C47H60N17O37P5     | 6.51        | 1609.20241   | 803.6021   | -2     | 9.2        | -2H, -3H, -H            |
| AAAACAUGUUUCCQ         CH44HT79NE0012919         6.82         4480.6779         199.201        2        2        2        2        2        2        2        2        2        2        2        2        2        2        2        2        2         -2        -2 <td>AUCCGp</td> <td>C47H61N18O36P5</td> <td>6.53</td> <td>1608.21839</td> <td>803.1076</td> <td>-2</td> <td>6.1</td> <td>-2H, -3H, -H</td>   | AUCCGp                | C47H61N18O36P5     | 6.53        | 1608.21839   | 803.1076   | -2     | 6.1        | -2H, -3H, -H            |
| CAAAGP         CodeWiteDates         0.79         1905.55444         89.025         -2.         3.7         -2.41.31.44.24.34           CUCCCGQP         CSMPT3MB004P8         7.91         189.2485         494.022         -2.8         3.7         -2.41.34.44.34.44           CUCCCGQP         CSMPT3MB004P8         8.83         193.27091         987.835         -2.8         3.74         -3.44.44.44           AUCCAGP         CSMPT3MB004P8         8.84         193.27091         987.835         -2.8         -3.44.34.44.44           AUCCAGP         CSMPT3MB004P8         8.84         191.52217         95.844         -2.8         -3.41.34.44.44           AUCCAGP         CSMPT3M2504P8         8.83         191.52218         97.8375         -2.8         3.2         -3.41.34.44.44           AUCCAGP         CSMPT3M2504P8         9.33         193.72701         967.834         -2.8         3.2         -3.41.34.44.44           AUCCAGP         CSMPT428039P6         9.33         193.72701         967.834         -2.8         3.2         -3.41.34.44.44           AUCCAGP         CSMPT428039P6         9.33         193.72701         967.834         -2.8         3.2         -3.41.34.44.44           AUCCAGP         CSMPT428039P7  | AAAAACAUGUUGCCGp      | C144H179N60O103P15 | 6.52        | 4860.66775   | 1619.2101  | -3     | -4.7       | -3H                     |
| CCUCUGp         CSHIT/XIN0-04P6         789         1980-2324         94.4145         4.2         5.1         -2434441           CCCCCGp         CSHIT/XIN0-04P6         8.29         1932.2766         95.5142         -2         3         -2434441           CCCCAGP         CSHIT/XIN0-04P6         8.28         1937.2761         97.61         -2         28         -2434441           CAULAGP         CSHIT/XIN0-04P6         8.28         1937.2751         97.61         -2         3         -2434441           AAUCAGP         CSHIT/XIN0-04P6         8.28         1937.2751         97.83.77         -2         3         -2434441           AAUCAGP         CSHIT/XIN0-04P6         8.28         1937.2791         97.83.77         -2         3         -2434441           AACCAGP         CSHIT/XIN0-04P6         9.26         1937.2791         97.847         -2         2         -2434441           ACCAAGP         CSHIT/XIN0-04P6         9.26         199.2226         193.159         -2         743434444           UCUUCUGP         CSHIT/XIN0-04P6         9.26         199.2226         193.159         -2         -243434444           UCUUCUGP         CSHIT/XIN0-04P6 <t< td=""><td>CAAAGp</td><td>C49H62N23O33P5</td><td>6.79</td><td>1655.25684</td><td>826.625</td><td>-2</td><td>3.7</td><td>-2H, -3H, -H</td></t<>   | CAAAGp                | C49H62N23O33P5     | 6.79        | 1655.25684   | 826.625    | -2     | 3.7        | -2H, -3H, -H            |
| CUCCCGp         CSHT7NN0-04P6         77.0         198-8448         943.272         2         37         2-44-44-4           AUCCAGP         CSHT7NN204P8         8.88         1937.2766         957.375         4         1         2         3         2         3         2         3<         2         3<         2         3<         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         3         2         2         3         2         3         2         3         2         3         2         3         2         3         3         3         3         3         3         3         3         3 <td>CCUCUGp</td> <td>C55H72N18O45P6</td> <td>7.89</td> <td>1890.23246</td> <td>944.1145</td> <td>-2</td> <td>5.1</td> <td>-2H, -3H, -4H, -H</td>  | CCUCUGp               | C55H72N18O45P6     | 7.89        | 1890.23246   | 944.1145   | -2     | 5.1        | -2H, -3H, -4H, -H       |
| CCCCApp         CSH/TANZ2042PP         8.29         1912/2766         95.732         2         3  | CUCCCGp               | C55H73N19O44P6     | 7.91        | 1889.24845   | 943.6212   | -2     | 3.7        | -2H, -3H, -4H, -H       |
| AUCCAGpC5/H73N2304988.8619372091987.0514.74.84.94  | CCCCAGp               | C56H74N22O42P6     | 8.29        | 1912.27566   | 955.1342   | -2     | 3          | -2H, -3H, -4H, -H       |
| CAUUAGp         CSMPT/N220498         8.8.0         1938.2440         1937.214         1         1.9         -H.4H.4H.           AAUCAGp         CSMPT/N250418         8.8.3         1951.2215         978.037         -2         3         -24H.4H.4H.           AAUCAGp         CSMPT/N2504186         8.8.8         1951.2215         978.037         -2         3         -24H.4H.4H.           AUCCAGp         CSMPT/N2504286         8.8.9         1937.2091         967.835         -2         2         -24H.4H.4H.           ACCCAGP         CSMPT/N2504286         9.8.0         1937.2091         978.44         -2         1.22         -24H.4H.4H.           ACCCAGP         CSMPT/N2504786         9.8.0         1977.218         109.159         -2         1.7         -24H.4H.4.           VUCUCUGP         CSMPT/N2504787         10.32         222.0280         109.159         -2         1.2         -24H.4H.4.           VUCUCUGP         CSMPT/N250477         10.33         2243.2861         110.64         -2         1.2         -24H.4H.4.           VUCUACUGP         CSMPT/N250487         11.22         2266.3234         112.05         -2         24H.4H.4.           VUCUACUGP         C  | AUCCAGp               | C57H73N23O42P6     | 8.58        | 1937.27091   | 967.6315   | -2     | 2.6        | -2H, -3H, -4H, -H       |
| AUVCUGp         C66477/H0040PP         8.88         1915.2271         956.514        2         6.9         -2413414414           AUCCAGp         C6947738250-04PP         8.88         1901.28215         979.375        2         3         -2413414414           AUCCAGp         C594773820-04PP         8.89         1901.28215         979.375        2         5.2         -2413414414           AACCMAGP         C594773820-04PP         8.89         1903.72001         967.433        2         5.2         -2413414444           ACCACAGP         C5947743420-04PP         9.82         1904.2008         991.1500         -2         2.7         -24134144           AUCUCUGp         C649483420505P7         10.33         2220.209         190.1509         -2         2.1         -24134144           AUUCCAGD         C6494420505P7         10.33         2243.20821         1120.441         -2         2.6         -24134144           AUUCCAGD         C6494420505P7         10.36         2243.20821         1120.441         -2         2.6         -24134144144           AUUCCAGD         C6494832049P7         11.25         2265.33421         1131.4168         -2         2.1         -24134144144   | CAUUAGp               | C57H72N22O43P6     | 8.58        | 1938.25493   | 1937.2514  | -1     | 1.9        | -H                      |
| AAUCAGp         C58H73/82504HP6         8.88         1961.2285         978.4378         -2         3         -2413H441H           AUCCAGp         C57H73/82204PP         8.89         1937.2201         967.435         -2         6.5         -2413H441H           AUCCAGp         C57H73/82204PP         9.84         1996.22813         973.445         -2         2.2         -2413H441H           ACCAAGp         C58H74/82204PP         9.84         1996.22813         973.445         -2         2.2         -2413H441H           ACCAAGp         C54H74/82204PP         9.84         1997.1287         1997.182         -2         3.5         -2413H44144           UCUACUGp         C64H82/10506PT         10.33         2220.269         1903.150         -2         2         -2413H441           UCUACUGp         C64H84/25050PT         10.33         2242.32811         1120.441         -2         2.2         -2413H441           UUCACU         C64H84/25050PT         10.36         2243.29821         1120.441         -2         2.2         -2413H441           UUCACU         C64H84/25050PT         10.35         2243.29821         1120.441         -2         2.2         -2413H441 <t< td=""><td>AUUCUGp</td><td>C56H71N19O45P6</td><td>8.63</td><td>1915.22771</td><td>956.614</td><td>-2</td><td>6.9</td><td>-2H, -3H, -4H, -H</td></t<>   | AUUCUGp               | C56H71N19O45P6     | 8.63        | 1915.22771   | 956.614    | -2     | 6.9        | -2H, -3H, -4H, -H       |
| UACAAGp         C58H73N25041PF         8.88         1991.2825         979.6375         -2         3         2H3H, -4H, -H           AACCUGp         C57H73N22042PF         9.03         1937.2091         967.635         -2         6.5         2H3H, -4H, -H           AACCUGp         C59H741420204PF         9.26         1996.2981         979.457         -2         2.2         2H3H, -4H, -H           ACCAAGp         C59H741420203PF         9.26         1996.2981         979.182         -2         2.7         3.5         2H, -3H, -4H, -H           AUCUCUGp         C68H83N22052P7         10.33         2220.299         109.1590         -2         2.1         2H, -3H, -4H           UCUACUGp         C68H83N22052P7         10.63         2243.29821         1120.641         -2         2.2         2H, -3H, -4H           AUUCCAGp         C68H83N2050P7         10.53         2243.29821         1120.641         -2         2.2         2H, -3H, -4H, -4H           AUUCCAGp         C68H83N2060P7         11.2         2266.3234         1121.403         -2         4.5         2H, -3H, -4H, -4H           AUUCCAGp         C67H88N2040P7         11.2         2266.3234         1121.403         -2         4.5         2H, -3H, -4H, -4H   | AAUCAGp               | C58H73N25O41P6     | 8.88        | 1961.28215   | 979.6375   | -2     | 3          | -2H, -3H, -4H, -H       |
| AUCCAGp         Control Mac2042PP         B.B         1937/2019         997/AB3         -2         B.B.         -24, -34, -44, -44, -44, -44, -44, -44, -4  | UACAAGp               | C58H73N25O41P6     | 8.88        | 1961.28215   | 979.6375   | -2     | 3          | -2H, -3H, -4H, -H       |
| AACCUOp         CSF/TAR23042PP         1003         1003/CU01         1007/CU01   | AUCCAGp               | C57H73N23O42P6     | 8.89        | 1937.27091   | 967.6353   | -2     | 6.5        | -2H, -3H, -H            |
| NCRCARAGE         Communication         Base         Implementary        2         -21         -2   | AACCUGp               | C57H73N23O42P6     | 9.03        | 1937.27091   | 967.634    | -2     | 5.2        | -2H, -3H, -4H, -H       |
| NCARAGID         Communication         Communication         Operation  | ACACAGP               | C58H74N26O40P6     | 9.26        | 1960.29813   | 9/9.145/   | -2     | 3.2        | -2H, -3H, -4H, -H       |
| Current Current         Constraint         Constraint   | нениенся              | C64U92N10O54D7     | 9.52        | 2107 24179   | 1007.6192  | -2     | 2.1        | -2H, -3H, -4H, -H       |
| Decompting         Constraints         Decompting         Constraints         Decompting         Constraints         Decompting         Constraints         Decompting         Dec   | AUCUCUGp              | C65U93N22O52P7     | 9,09        | 2107.24170   | 11097.0102 | -2     | 71         | -2H, -3H, -4H           |
| COULDUCUP         CoSH83N2202597         10.81         2202.059         109.1302         1-2         3         3         4           AUUCAGQ         C66H64M22050677         10.96         2243 29021         112.0441         -2         6.6         -2         2         -2H         -3H         -4H         -4H           AAACUGAG         C67H68N2040877         11.2         2266 32342         113.10655         -2         2         -2H         -3H         -4H         -2         1.5         -2H         -3H         -4H         -4H<   | UCUACUGo              | C65H83N22O52P7     | 10.23       | 2220.269     | 1109.1359  | -2     | 71         | -2H -3H -4H             |
| AUUCCAGp         C66H84N25050P7         10.53         2243.29021         1120.643         -2         1.2         -2H, -3H, -4H           CAUACUGp         C66H84N25050P7         10.63         2243.29021         1120.643         -2         1.2         -2H, -3H, -4H           AUUCCAGP         C66H83N23051P7         10.95         2244.28023         1121.141         -2         6.6         -2H, -3H, -4H           AUUCCAGP         C67H85N23048P7         11.22         2266.32343         1132.1003         -2         4.5         -2H, -3H, -4H, -4H           ACUACAGP         C67H85N23048P7         11.22         2266.32431         113.1003         -2         4.5         -2H, -3H, -4H, -4H           ACUACAGP         C7H85N23048P7         11.84         213.31881         155.672         -2         1.2         -2H, -3H, -4H           AAACAGP         C7H98N20056P8         11.89         2525.31021         125.1683         -2         0.3         -2H, -3H, -4H           AAUCCUGP         C7H98N20056P8         12.83         2572.34673         125.1683         -2         0.3         -2H, -3H, -4H           ACUACUGP         C7H98N20056P8         12.86         2572.34673         125.1683         -2         1.2         -2H, -3H, -4H, -5H   | UCUACUGn              | C65H83N22O52P7     | 10.61       | 2220.269     | 1109.1302  | -2     | 2          | -2H, -3H, -4H           |
| CAUALUGp         C06H644X50500P7         10.53         2243 29821         110.04.41         -2         12         -2H         3H         -4H           CAUAUUGp         C06H84X50500P7         10.96         2243 29821         1120.6441         -2         6.6         -3H         -3H, -4H           CAUAUGp         C0FH85X2064P7         11.22         2266 32244         1132.1603         -2         4.5         -2H         -3H, -4H           CAUACAGp         C0FH85X2064P7         11.22         2266 32244         1132.1603         -2         4.5         -2H         -3H, -4H           CCCACAAGp         C0FH85X2064P7         11.25         2265 33942         1250.1665         -2         1.1         -2H         -3H, -4H           CUCUUCUGC         C7H498X2059P8         11.99         2252.51076         -2         1.2         -2H, -3H, -4H           CAUACUGp         C7H998X2059P8         12.9         2427.3549         122.6162         -2         3.1         -2H, -3H, -4H           CAUACUGp         C7H998X2059P8         12.85         272.34873         125.1683         -2         0.3         -2H, -3H, -4H           CAUACUGp         C7H998X2059P8         12.85         272.4477         125.1676         -2         <  | AUUCCAGp              | C66H84N25O50P7     | 10.53       | 2243.29621   | 1120.643   | -2     | 1.2        | -2H, -3H, -4H           |
| CAUACUGa         Co64984/N2S050P7         10.96         2244 29021         110.0441         -2         6.2         2.4         -4H           AUUUCAGp         Co64983N2405IP7         11.22         2265.22441         1132.1603         -2         4.5         -2H         -3H         -4H         -4H           ACULACAGp         Co7H65N20048P7         11.22         2265.322441         1132.1603         -2         4.5         -2H         -3H         -4H         -4H           ACULACAGp         C07H65N2004P8         11.82         2265.33244         1132.1603         -2         1.5         -2H         -3H         -4H         -4H           UCUUCUCGp         C7H95N2005P8         11.89         2255.31028         122.8747         -2         1.5         -2H         -3H         -4H         -4H           AAAACAGp         C7H95N20056P8         12.63         2272.34873         1285.168         -2         0.3         -2H         -3H         -4H         -4H           ACUAUCUGp         C7H96N30056P8         12.65         2572.34873         1285.168         -2         0.3         -2H         -3H         -4H           ACUAUCUGp         C7H96N30056P8         12.65         2522.34873         1285.1683   | CAUACUGp              | C66H84N25O50P7     | 10,53       | 2243.29621   | 1120.643   | -2     | 1.2        | -2H, -3H, -4H           |
| AUULCAGp         C66H33N2001P7         10.95         22422023         11.141         -2         6.8         -2H, -3H, -4H, -H           AACCUGp         C67H85N2004P7         11.22         2266.32343         1132.1603         -2         4.5         -2H, -3H, -4H, -H           ACUACAGP         C67H85N2004P7         11.82         2265.3342         113.1665         -2         1.1         -2H, -3H, -4H, -5H           AAAACAGP         C69H80N3045P7         11.84         2313.36188         155.672         -2         1.2         -2H, -3H, -4H, -5H           AAAACAGP         C69H80N3054P7         11.84         2313.36188         155.672         -2         1.2         -2H, -3H, -4H, -5H           CCACCAUGP         C75H97N20056P8         11.92         2252.31627         128.5183         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGP         C76H98N30056P8         12.63         2572.34873         128.5183         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGP         C76H98N30056P8         12.64         192.25450         54.0663         -3         6.0         -3H         -2C         -22         -2H, -3H, -4H, -5H           ACUACUGP         C64H738/1044P6         12.64         192.25450         54.2   | CAUACUGp              | C66H84N25O50P7     | 10.96       | 2243.29621   | 1120.6441  | -2     | 2.2        | -2H, -3H, -4H           |
| AAACCUGp         C67H85R2048P7         11.22         2268.3234         112.1003         -2         4.5         -2H.3H4HH           CCACAAGp         C67H85R2047P7         11.26         2268.3342         113.1665         -2         4.5         -2H.3H4HH           CCACAAGp         C73H98R2047P7         11.26         2268.3342         113.1655         -2         1.5         -2H.3H4H           UCUCUUCGp         C73H98R2059P8         11.84         213.3688         1155.6762         -2         1.5         -2H3H4H           UCUCUACGp         C7H98R2059P8         11.99         2252.31028         122.81501         -2         3.1         -2H3H4H           UCUCUACGp         C7H98R3056P8         12.63         2572.34873         128.5183         -2         0.3         -2H3H4H5H           ACAUACUGp         C7H98R3056P8         12.85         2572.34873         128.5176         -2         2.1         -2H3H4H5H           ACAUACUGp         C6H73821044P6         12.84         129.24589         42.0633         -2         2.1         -3H3H4H5H           ACAUACUGp         C6H73821044P6         13.87         285.3787         1425.6931         -2         7.1         -2H3H4H5H   | AUUUCAGp              | C66H83N24O51P7     | 10.95       | 2244.28023   | 1121.141   | -2     | 6.6        | -2H, -3H, -4H, -H       |
| ACUACAGp         CP1498N2904P7         11.22         2268.3243         11.21003         -2.2         4.5         -2.414141           CACAAAGp         CP1498N2904P8         11.86         2265.23497         125.0385         -2         2         -2.4131445H           AAAACAGp         C694980N3045P7         11.84         2250.2302         126.1670         -2         1.2         -243H445H           CACUCUUCCp         C74499N2005P8         11.92         2257.3302         126.1670         -2         1.2         -243H445H           CACUACUGP         C74499N2005P8         12.83         2572.34973         1285.1683         -2         0.3         -243H445H           ACAUACUGP         C74499N30056P8         12.85         2572.34973         1285.1683         -2         0.2         -2         2         -2   | AAACCUGp              | C67H85N28O48P7     | 11.22       | 2266.32343   | 1132.1603  | -2     | 4.5        | -2H, -3H, -4H, -H       |
| CCACAAGp         C07H08N20047P7         11.26         228.339.24         11.31665         -2         2         -2H, 3H, -4H           CUCUUCUCUCUCUC         C7H49N2200FP         11.64         2502.230.7         1250.1365         -2         1.1         -2H, 3H, -4H, -5H           AAAACAAp         C9H98N32036P7         11.84         2313.3088         156.376         -2         1.2         -2H, 3H, -4H, -5H           CCACCAUGP         C7H97N29056P8         11.23         2547.35349         122.5472         2.2         3.1         -2H, -3H, -4H, -5H           ACAUACUGP         C7H96N30056P8         12.63         2572.3477         128.5163         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGP         C7H96N30056P8         12.65         2572.3477         128.5167         -2         2.1         -2H, -3H, -4H, -5H           ACAUACUGP         C6H73N104056P8         13.64         192.25459         142.5681         -2         2.1         -2H, -3H, -4H, -5H           ACUAUCUGP         C6H73N1064P9         13.87         138.591841         137.1869         -2         7.1         -2H, 3H, -4H, -5H           UCACCAUCGP         C6H10N12064P9         13.87         285.37879         1425.693         -2         7.1         -2H, 3H, -  | ACUACAGp              | C67H85N28O48P7     | 11.22       | 2266.32343   | 1132.1603  | -2     | 4.5        | -2H, -3H, -4H, -H       |
| UCUCUUCQC         C73H94H22001P8         11.89         250228307         125.818         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2         1.5         -2   | CCACAAGp              | C67H86N29O47P7     | 11.25       | 2265.33942   | 1131.6655  | -2     | 2          | -2H, -3H, -4H           |
| AAAACAsp         C69H86N30245F7         11.84         213.36188         115.5722         -2         1.5         -2H, -3H, -4H           UCCUULCQU         C74H97N2005F8         11.99         225.3102         126.15.01         -2         1.2         -24         -3H, -4H, -4H           CAACCAUGP         C75H97N2005F78         12.32         2257.34873         125.1683         -2         0.3         -2H, -3H, -4H, -5H           ACAUCCUGP         C76H98N0056F78         12.85         2272.34873         1255.1683         -2         2.2         -2H, -3H, -4H, -5H           ACAUACUGP         C76H98N0056F78         12.86         2272.34873         1255.1987         -2         2.1         -2H, -3H, -4H           ACAUACUGP         C76H98N0056F78         12.86         2272.34873         1255.1981         -2         2.1         -2H, -3H, -4H           CGCC6p         C66H73N2C04F78         13.37         1355.1947         125.5981         -2         7.1         -2H, -3H, -4H, -5H           UCACCAUCGP         C64H100N1064F9         13.87         2853.37879         142.5693         -2         7.1         -2H, -3H, -4H, -5H           UCACCAUCGP         C64H100N1064F9         14.19         2853.37879         142.5693         -2         4.5  | UCUCUUCGp             | C73H94N22O61P8     | 11.69       | 2502.28307   | 1250.1365  | -2     | 1.1        | -2H, -3H, -4H, -5H      |
| UGCULACSOp         CP4H98H20059P8         11.99         225231028         121.6501         -2         12.         -2H, -3H, -4H, -5H           AAAUCCUQp         C75H98N20056P8         12.23         2547.3594         122.65183         -2         3.1         -2H, -3H, -4H, -5H           ACAUACUGp         C76H96N30056P8         12.83         2572.34873         1285.1683         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGp         C76H96N30056P8         12.96         2572.34873         1285.1676         -2         -0.2         -2H, -3H, -4H, -5H           ACAUACUGp         C76H96N30056P8         12.96         2572.34873         1285.1676         -2         -0.2         -2H, -3H, -4H, -5H           ACAUACUGp         C76H96N30056P8         12.96         2572.34873         1285.1877         -2         5         -3H         -3H           GGCGp         C39H50N1602P9         13.37         1358.19415         157.1878         -2         5         -2H, -3H, -4H, -5H           UCACCAUCGp         C64H100N1064P9         13.87         2853.37879         1425.6893         -2         4,5         -2H, -3H, -4H, -5H           UUCACCAUCGp         C64H100N1064P9         14.1         2853.37879         1425.6893         -2         4,5<  | AAAACAGp              | C69H86N33O45P7     | 11.84       | 2313.36188   | 1155.6762  | -2     | 1.5        | -2H, -3H, -4H           |
| CCACCAUGp         CFH97M20969F8         12.32         284735340         172.6742         -2         3.3         -2H, -3H, -4H           AAUUCCUGp         CFH99N30056F8         12.63         2572.34673         1285.1683         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGp         CFH99N30056F8         12.63         2572.34673         1285.1683         -2         0.3         -2H, -3H, -4H, -5H           ACAUACUGp         CFH99N30056F8         12.86         2572.34673         1285.1676         -2         2.1         -2H, -3H, -4H           ACAUACUGp         C5H73N21044P6         12.86         2572.34673         1285.1678         -2         7.1         -2H, -3H, -4H           ACAUACUGp         C5H100N30264P9         13.37         1356.1815         157.1868         -2         7.7         -2H, -3H, -4H, -5H           CUCACAUCGp         C6H100N3064P9         13.87         2853.37879         1425.693         -2         7.1         -2H, -3H, -4H, -5H           UCACCAUCGp         C6H100N3064P9         14.19         2853.3787         1425.693         -2         5.6         -2H, -3H, -4H, -5H           UCACCAUCGp         C6H10N3064P9         14.19         2853.3787         1425.693         -2         5.6         -2H, -3H, -  | UCCUUACGp             | C74H95N25O59P8     | 11.99       | 2525.31028   | 1261.6501  | -2     | 1.2        | -2H, -3H, -4H, -5H      |
| AAUCCUG0p         CPH198430058P8         12.63         252.3473         128.1683         -2         0.3         -24, -34, -44, -54           ACUUACUG0p         CPH198430058P8         12.85         2572.3473         128.51675         -2         0.3         -24, -34, -44, -54           ACUUACUG0p         CPH198430058P8         12.86         2572.3473         128.51676         -2         2.1         -24, -34, -44           CUGCCC0p         C9H1984030058P8         12.86         2572.3473         128.51676         -2         2.1         -24, -34, -44           CUGCC0p         C9H1984030058P8         13.84         128.25         0.42         7.1         -5.8         -7.4         -3.4         -44         -54           CUGCCAUCGp         C94H100813064P9         13.87         2853.3779         1425.6931         -2         7.1         -24, -34, -44, -54           UCACCAUCGp         C94H100813064P9         14.1         2853.3779         1425.6931         -2         6.4         -24, -34, -44, -54           UCACCAUCGp         C94H100813064P9         14.1         2853.3779         1425.6931         -2         6.4         -24, -34, -44, -54           UCACCAUCGp         C94H100812064P9         14.19         2853.3056         -2 <td< td=""><td>CCACCAUGp</td><td>C75H97N29O56P8</td><td>12.32</td><td>2547.35349</td><td>1272.6742</td><td>-2</td><td>3.1</td><td>-2H, -3H, -4H</td></td<>  | CCACCAUGp             | C75H97N29O56P8     | 12.32       | 2547.35349   | 1272.6742  | -2     | 3.1        | -2H, -3H, -4H           |
| ACAUACUGp         CFM4984000569F8         12.63         252:24873         1285.1683         -2         0.3         -24r.3H.4H6H.           ACAUACUGp         CFM4984000569F8         12.96         2572:24873         1285.1765         -2         -0.2         -24r.3H.4H4H.           ACAUACUGP         CFM4984000569F8         12.96         2572:34873         1285.1765         -2         0.2         -2H.3H4H.           ACAUACUGP         CGM4908100569F8         12.96         1929:2526         642:068         -3         8.8         -3H           GGCGp         CGM4100813064F9         13.87         2853.37879         1425.6931         -2         7.1         -2H.3H4H5H           UCACCAUCGp         CG4H100813064F9         13.87         2853.37879         1425.6931         -2         7.1         -2H.3H4H5H           UCACCAUCGP         CG4H100813064F9         13.87         2853.37879         1425.6931         -2         4.5         -2H.3H4H5H           UCACCAUCGP         CG4H100813064F9         13.87         2858.33081         1427.179         -2         4.5         -2H.3H4H5H           UCACCAUCGP         CG4H100813064F9         13.93         283.30362         147.179         -2         4.5         -2H.3H4H5H   | AAAUCCUGp             | C76H96N30O56P8     | 12.63       | 2572.34873   | 1285.1683  | -2     | 0.3        | -2H, -3H, -4H, -5H      |
| Number         Christmann (Christmann (Christmann)         Carbon (Christmann) <td>ACAUACUGp</td> <td>C76H95N30056P8</td> <td>12.63</td> <td>2572.34873</td> <td>1285.1683</td> <td>-2</td> <td>0.3</td> <td>-2H, -3H, -4H, -5H</td>  | ACAUACUGp             | C76H95N30056P8     | 12.63       | 2572.34873   | 1285.1683  | -2     | 0.3        | -2H, -3H, -4H, -5H      |
| ACAUACUGUp Criveres Criveres Constraints of the constraint of the | ACAUACUGp             | C76H96N30O56P8     | 12.85       | 2572.34873   | 1285.1676  | -2     | -0.2       | -2H, -3H, -4H           |
| UNAULUUP         COMMUNICIPATION         IL.044         1929/25459         642,0063         C-3         B.9         C-3H           GGCGCp         C3H50N180229H         13.37         1356,1941         1557,189         -1         1.5         -H           CCULCACCQD         C64H109X12063P9         13.84         2852,3947         1425,593         -2         7.1         -2H, -3H, -4H, -5H           UCACCAUCGD         C64H109X12064P9         13.87         2853,3779         1425,693         -2         7.1         -2H, -3H, -4H, -5H           UCACCAUCGD         C64H109X12064P9         14.19         2853,3787         1425,693         -2         4.5         -2H, -3H, -4H, -5H           AULCCUUUGD         C64H109X12064P9         14.19         2876,3764         1438,1666         -2         4.5         -2H, -3H, -4H, -5H           AULCCUUUGD         C64H109X12064P9         14.19         2876,3764         1432,1266         -2         6.8         -2H, -3H, -4H, -5H           AUCCAUUGD         C64H109X1206P1         14.19         2864,3963         142,1719         -2         6.8         -2H, -3H, -4H, -5H           AUCCAUUGD         C64H109X13061P9         14.40         2877,4566         1432,17248         -2         5.8         -2H, -3H, -4H,   | ACAUACUGp             | C76H96N30056P8     | 12.96       | 25/2.34873   | 1285.1705  | -2     | 2.1        | -2H, -3H, -4H           |
| CCCUCACCC         Construction         Construction <td>GGCGn</td> <td>C39H50N18O20D4</td> <td>12.04</td> <td>1358 10.415</td> <td>042.0803</td> <td>-3</td> <td>9.8</td> <td>-50<br/>_H</td>  | GGCGn                 | C39H50N18O20D4     | 12.04       | 1358 10.415  | 042.0803   | -3     | 9.8        | -50<br>_H               |
| Dest-norm         Dest-norm         Expose         Expose         Fragment         <   | CCUCACACGo            | C84H109N32O63P9    | 13.87       | 2852.30477   | 1425 1981  | -1     | 5          | -2H -3H -6H -5H         |
| Decomposition         Decompos  | ACAUCCUCGp            | C84H108N31064P9    | 13.87       | 2853.37979   | 1425 6931  | -2     | 71         | -2H, -3H, -4H -5H       |
| UCACCAUCGp         C64H108N310684P9         14.1         2853.37927         H25.689         -2         6.2         -2H         -3H         -2H         -4H         -5H           AUUCUUUGp         C64H106N32068P9         13.93         2283.3082         1415.653         -2         6.4         -2H         -3H         -4H         -5H           AUUCUUUGp         C64H106N32068P9         14.19         2876.37404         1438.1666         -2         -9.7         -2H         -4H           AUUCUUUGp         C64H106N32067P9         14.19         2876.37404         1438.1666         -2         -3         -6.6         -3H           AUUCUUUGp         C64H106N32067P9         14.43         2879.35005         1438.8666         -2         -8.8         -2H         -3H         -4H         -5H           AUACCUUAGp         C65H100N3060F9         14.43         2879.35005         1438.8666         -2         5         -2H         -3H         -4H         -5H           AAACAUCGp         C68H100N3060F9         14.92         290.4724         1449.2135         -2         5         -2H         -3H         -4H         -5H           AAACAUCGp         C68H10N300699         14.96         284.42847         144.2135 <td>UCACCAUCGp</td> <td>C84H108N31064P9</td> <td>13.87</td> <td>2853.37879</td> <td>1425.6931</td> <td>-2</td> <td>7.1</td> <td>-2H, -3H, -4H, -5H</td>  | UCACCAUCGp            | C84H108N31064P9    | 13.87       | 2853.37879   | 1425.6931  | -2     | 7.1        | -2H, -3H, -4H, -5H      |
| AULCUUUGp         C93H104X5068P9         13.93         2833.30362         H16.653         -2         5.4         -2H, -3H, -4H, -5H           ACAUCAUUUGp         C68H107N32064P9         H.19         2853.30362         H16.653         -2         -2H, -3H, -4H, -5H           ACAUCAUUUGp         C68H107N32064P9         H.19         2856.3083         H27.719         -2         -2H, -3H, -4H, -5H           UGAUGAUUCUUUGp         C58H108N3065P9         H.419         2865.3083         H37.719         -2         -8.6         -3H           UGAUGAUUCUUUGp         C58H108N3062P9         H.44         2879.3060         H37.27048         -2         5.8         -2H, -3H, -4H, -5H           ACACCCUAGp         C68H109N3001P9         H.44         2879.406         H37.27048         -2         5.8         -2H, -3H, -4H, -5H           ACACCUAGp         C69H109N30050P9         H.46         2924.428247         H412135         -2         5.8         -2H, -3H, -4H, -5H           ACAACAUCGp         C69H109N30050P9         15.25         4749.81122         H38.21         5.9         -28         5.8         -2H, -3H, -4H, -5H           UUCCAUCAGp         C64H1079N510107P15         15.25         4749.8152         1590.7068         -2         4.7         -21, -3H, -4H   | UCACCAUCGp            | C84H108N31064P9    | 14.1        | 2853.37879   | 1425.6893  | -2     | 4.5        | -2H, -3H, -4H, -5H      |
| ACAUCAUUGp         C65H107X2064P9         14.19         2878.37404         1438.866         -2         -9.7         -2H           AUUCUUAUGp         C64H105X2064P9         14.19         2856.33083         1427.1719         -2         -9.7         -2H           AUUCUUAUGp         C63H105X2064P9         14.19         2856.33083         1427.1719         -2         -3         -6.6         -3H           AUUCUUAUGp         C63H105X1065P9         14.43         2875.3505         1438.6666         -2         5.8         -2H3H,-4H           AUACAUUGp         C65H100X1065P9         14.43         2875.3505         1438.6666         -2         5.8         -2H3H,-4H,-5H           AACAAUCGp         C66H100X3065P9         14.42         2804.4287         1461.215         -2         5.8         -2H3H,-4H,-5H           AACAAUCGp         C68H100X1058P9         14.96         2947.4559         1472.7265         -2         3.5         -2H3H,-4H,-5H           AAACAAUCGp         C68H100X1058P9         14.96         2947.4559         1472.7265         -2         3.5         -2H3H,-4H,-5H           AAAAAUGp         C68H1070X1058P17         15.55         3183.41532         1500.706         -2         1.2         -2, H3H,-4H,-5H  | AUUCUUUUGp            | C83H104N25O69P9    | 13.93       | 2833.30362   | 1415.653   | -2     | 5.4        | -2H, -3H, -4H, -5H      |
| ALUICUUUUADp         Ca4H106x8007P9         H.19         2658.33053         H27719         -2         0.1         -2H3H4H           UGAUGAUUCUUUUQp         C13H182N440108PH         H.19         4464.50161         H477128         -3         -6.6         -3H           MACAUUUGP         C03H108N106SP9         H.4.43         2275.36605         H338.6866         -2         9.8         -2H3H6H           ACACACUCARp         C08H100N3062P9         H.4.44         2270.406         H37.2048         -2         5         -2H3H4H5H           ACACACUCAP         C08H100N3060P9         H.96         2242.42847         H412.335         -2         5         -2H3H4H5H           AAACAAUCGP         C08H100N3050P9         H.96         2242.42847         H412.335         -2         5.8         -2H3H4H5H           AAACAAUCGP         C08H100N3050P9         H.96         2244.42847         H412.335         -2         5.8         -2H3H4H5H           AAAACAAUGP         C08H100N3050P9         H.92         244.568         H72.7265         -2         4.2         -2H3H4H5H           AAACAAUGP         C08H100N3050P1         H.5.8         388.4632         H50.7086         -2         4.2         -3H4H5H <t< td=""><td>ACAUCAUUGp</td><td>C85H107N32O64P9</td><td>14.19</td><td>2878.37404</td><td>1438.1666</td><td>-2</td><td>-9.7</td><td>-2H</td></t<>  | ACAUCAUUGp            | C85H107N32O64P9    | 14.19       | 2878.37404   | 1438.1666  | -2     | -9.7       | -2H                     |
| UGAUGAUUCUUUUG         C131H152N440105P14         14.19         444.6.50161         1471.220         -3         -6.6         -3H           AUACAUUUGU         C68H106N31065P9         14.43         2879.35605         1438.6666         -2         5.8         -2H3H,-5H           ACACCCUAGP         C68H106N31065P9         14.43         2879.35605         1437.2048         1-2         5.8         -2H3H,-4H,-5H           AACACUCGp         C68H109N3060FP9         14.72         290.047724         1449.2039         -2         5.8         -2H3H,-4H,-5H           AACAAUCGp         C68H109N3050FP9         14.96         2924.24294         1481.2033         -2         5.8         -2H3H,-4H,-5H           AACAAUCGp         C68H110N1058P9         14.96         2924.4297         1473.225         -2         5.8         -2H3H,-4H,-5H           CAAACAAUGP         C68H110N1058P9         15.25         2446.4917         1473.226         -2         6.2         -2H3H,-4H,-5H           CUGACCCUAUCAUCAGP         C140H178N510107P15         15.55         3183.4152         190.7086         -2         1.4         -2H3H,-4H,-5H           AUACAUUGACAAAGP         C64H119N35071010         15.64         3204.4571         160.3200         -2         1.2  | AUUCUUAUGp            | C84H105N28O67P9    | 14.19       | 2856.33083   | 1427.1719  | -2     | 9.1        | -2H, -3H, -4H           |
| AUACAUUUGp         C65H100R10056P9         14.43         2879 35805         143.8686         -2         8.8         -243H9H9H           ACACCCUAG         C65H100R10056P9         14.43         2876 3505         1432.8046         -2         5.8         -243H9H         -5H           AACACCUCGp         C66H100R1060P9         14.72         2800.41724         1449.2093         -2         5         -2H3H4H5H           AAACAAUCGp         C6H100R130506P9         14.96         2924.42847         144.1213         -2         3.0         -2H3H4H5H           AAACAAUGA         C6H100R130506P9         15.85         2944.4397         147.2225         -2         5.8         -2H3H4H5H           AAAACAAUGp         C68H100R10059P9         15.25         2448.4397         147.3225         -2         -3         -5         -3H           UUUCACAUCAUCG         C69H107RN50107P15         15.55         318.4152         1500.706         -2         1.2         -2H3H4H5H           AUACAUUUGACAAGp         C64H110R3507P10         15.54         320.41577         160.2107         -2         1.2         -2H3H4H5H           AUACAUUUGACAAAGp         C64H110R3507P10         15.64         320.45377         164.2191  | UGAUGAUUCUUUUGp       | C131H162N44O105P14 | 14.19       | 4464.50161   | 1487.1526  | -3     | -6.6       | -3H                     |
| ACACCCUAGp         C65H1001X300EP9         14.44         287.640E         147.2248         -2         5.8         -241.3H, -4415H           ACACACUCGQ         C69H1001X300EP9         14.72         2900.4724         1442.039         -2         5.8         -241.3H, -4415H           AAACAAUCGp         C69H1001X300EP9         14.96         2924.42847         1461.2135         -2         5.8         -2H.3H, -4H5H           AAACAAUCGp         C68H1001X40059P9         14.96         2924.42847         1472.225         -2         6.2         -2H.3H, -4H, -5H           AAAACAUGp         C68H1001X40059P9         15.25         244.4397         1473.2225         -2         6.2         -2H.3H, -4H, -5H           UACAUUGACACAGp         C64H1101X5071P10         15.55         3183.41521         1590.7086         -2         4.7         -2H.3H, -4H, -5H           UACAUUGACACAAGP         C69H1101X5071P10         15.84         3208.41521         150.2007         -2         4.7         -2H.3H, -4H, -5H           UACAUUGACACAAGP         C69H1201X60068P10         16.02         323.45377         161.42191         -2         3.1         -3H, -6H           UCACAUUAGP         C69H1201X60350P17         16.42         3645.42339         171.7058         -2         <   | AUACAUUUGp            | C85H106N31065P9    | 14.43       | 2879.35805   | 1438.6866  | -2     | 9.8        | -2H, -3H, -5H           |
| AACAAUCGp         C66H109N3060FP         14.72         290.41724         144.2028         -2         5         -2H.3H4H5H           AACAAUCGp         C71100N3060FP         14.96         2924.4287         1461.213         -2         3.9         -2H.3H4H5H           AACAAUCGP         C88H10N41058PP         14.96         2924.4287         1472.725         -2         3.9         -2H.3H4H5H           AAAAAUGP         C88H10N41058PP         15.25         2944.4597         1472.225         -2         0.2         -2H.3H4H5H           AAAAAUGP         C84H10N30507P15         15.25         244.4597         1582.19         -3         -5.8         -3H           UUUCACAUCADCP         C94H119N35071P16         15.55         318.34532         1590.708         -2         1.2         -2H3H4H5H           AUACAUUUGACAAAGP         C94H119N35071P16         15.54         3204.4577         160.20         -2         1.2         -2H3H4H5H           AUACAUUUGACAAAGP         C94H120N40068P1         11.02         4484.56868         164.2194         -2         1.8         -2H3H4H5H           GGUGGP         C48H61120307P5         16.02         1664.21946         1663.208         -1         -2.5         -H.3H4H5H  | ACACCCUAGp            | C85H109N34O62P9    | 14.44       | 2876.406     | 1437.2048  | -2     | 5.8        | -2H, -3H, -4H, -5H      |
| AAACAAUCap         C07H109N38000P9         14.96         2924.42847         146.1235         -2         3.9         -2H.3H4H5H           AAACAAUCap         C8H110N40059P         14.96         2947.4566         1472.726         -2         3.5         -2H.3H4H5H           AAAACAAUGp         C8H110N40059P         15.25         2448.4397         1472.225         -2         4.7         -2H.3H4H5H           AAAAACAUGp         C9H119N35071P10         15.25         2448.4397         1472.225         -2         4.7         -2H.3H4H5H           UUUCACACAGp         C9H119N35071P10         15.55         3183.41532         1590.7066         -2         4.7         -2H3H4H5H           UACAUUCACAACAGP         C9H119N35071P10         15.84         3260.41057         160.2         33         -31         -31         -31         -3H6H           UACAUUCAAAAQ         C9H112N1500508P10         16.02         320.45377         161.4219         -2         -2         -2H3H4H5H           UCCACUUAAGP         C9H120N40058P10         110.2         323.4530         171.705         -2         1.8         -2H3H4H5H           UCCACUUAGQ         C04H120N303079P1         172.4         333.4509         1.77.724         -2   | AACAACUCGp            | C86H109N36O61P9    | 14.72       | 2900.41724   | 1449.2093  | -2     | 5          | -2H, -3H, -4H, -5H      |
| CAAACAAGp         CBH110N1036PP         19.96         29.4745660         147.27265         -2         -24         -34         -44, -5H           AAAACAUQC         CBH110N1036PP         15.25         2946.439         1473.222         -2         6.2         -24         34, -44, -5H           AUAAACAUQC         CBH110N10307P15         15.25         2946.439         1473.222         -2         6.2         -24, -3H, -4H, -5H           UUUCACACAGp         C94H119N35071P10         15.25         3183.41532         1590.7086         -2         1.2         -2H, -3H, -4H, -5H           UUUCACACAGp         C94H119N35071P10         15.84         3283.41552         1503.2007         -2         1.2         -2H, -3H, -4H, -5H           UUACACALAAGp         C94H12NN500107P15         15.02         4845.5585         1614.2191         -3         3.3         -3H, -5H           UUACALAAAGp         C94H12NN500107P15         15.02         4845.5585         1614.2191         -2         -0.8         -2H, -3H, -4H, -5H           UACAUUALAAGP         C34H12NN50012P15         16.02         123.04577         1614.2191         -2         -0.8         -2H, -3H, -4H, -5H           UACAUAUAUGA         C104H130N35037P11         17.4         3513.4506         175.724   | AAACAAUCGp            | C87H109N38O60P9    | 14.96       | 2924.42847   | 1461.2135  | -2     | 3.9        | -2H, -3H, -4H, -5H      |
| AAAAACUGp         CBH109N80059P9         15.25         2948.4397         147.3225         2-2         6.2         -2H.3H4H5H           CUGACCCUALCQC         C104H17NN51017915         15.25         4749.6192         158.21         -3         -3         -3H           LUCACACAAp         C34H119NS5071P10         15.55         318.4152         1590.708         -2         1.2         -2H.3H4H5H           LUCACAULUAAA         C34H119NS5071P10         15.54         338.4152         1590.708         -2         1.2         -2H3H4H5H           ALACAUUUGACAAAGP         C144H178N590103P15         16.02         320.45577         16H.2191         -2         3.1         -3H6H           CUACUUAAACp         C9H1201480068P10         16.02         320.45577         16H.2191         -2         -2.0         -2H3H4H5H           GGCUdp         C48H18003579P1         16.02         323.45387         171.705         -2         1.2         -2H3H4H5H           UCCACUUAUGA         C104H190.1305080P11         116.49         3654.2338         173.7105         -2         1.1         -2.5         -2H3H4H5H6H           AUCUUAAAUGP         C105H130N1077P11         17.45         353.4508         197.7274         -2         0.8 <td>CAAACAAAGp</td> <td>C88H110N41058P9</td> <td>14.96</td> <td>2947.45569</td> <td>1472.7265</td> <td>-2</td> <td>3.5</td> <td>-2H, -3H, -4H, -5H</td>   | CAAACAAAGp            | C88H110N41058P9    | 14.96       | 2947.45569   | 1472.7265  | -2     | 3.5        | -2H, -3H, -4H, -5H      |
| CUUQACUCADUCAUCUQUE         CH0HIT/RNIS10107P15         15.25         4749.01192         158.219         -3         -38         -384           UUUCACACADQ         C94H110M35071P10         15.55         3133.4152         1590.7086         -2         4.7         -241, -341, -44, -5H           UUCACACADQ         C94H110M35071P10         15.84         3303.41557         1614.2191         -2         4.7         -241, -341, -44, -5H           UUCACALCAADQ         C94H11078050103P15         16.02         12320.45377         1614.2191         -2         -0.8         -241, -341, -441, -5H           GGCUGQ         C94H101X030078P5         16.02         12320.45377         1614.2191         -2         -0.8         -241, -341, -441, -5H           GGCUGQ         C94H101X030078P1         16.02         1334.5186         1757.216         -2         0.8         -24, -341, -44, -5H, -6H           ALCALUUCALUGG         C104H130X130078P11         17.4         3537.46309         1767.7284         -2         0.8         -241, -3H, -4H, -5H, -6H           AUCUJAAALUGAD         C104H130X13078P11         17.45         3534.5808         1910.7484         -2         0.8         -2H, -3H, -4H, -5H, -6H           CUGUGGp         C7H712422045P6         18.45         1910.7284  | AAAAACAUGp            | C88H109N40O59P9    | 15.25       | 2948.4397    | 1473.2225  | -2     | 6.2        | -2H, -3H, -4H, -5H      |
| UJUUCALAUAUp         C94H119N3507/H10         15.55         318.34/532         15007086         -2         -24         -34         -24         -34         -44         -54           LACAUAUUGACAAACp         C94H119N3507/H10         15.54         3208.41652         1603.2007         -2         1.2         -24         -34         -34         -64           AUACAUUUGACAAACp         C144H178N500103P15         16.02         4845.65685         1614.2191         -3         -31         -34, -64         -34         -64           CUCACULAAACp         C44H178N500103P15         16.02         1664.21946         1663.208         -1         -2.5         -44, -34, -44, -54           GGCUGp         C49H61N20037P5         16.02         1664.21946         1663.208         -1         -2.5         -44, -34, -44, -54           ACAUUCUAUGC         C104H130N30508P11         17.48         2351.45168         175.724         -2         1.1         -24, -34, -44, -54, -54           AUCUUAAAUGC         C164H130N30307P11         17.45         3537.4509         1907.754         -2         0.68         -24, -34, -44, -54           AUCUUAADUGC         C164H143N4503891271         18.45         1970.2447         190.2478         -2         0.68         -24, -34, -44, -54 </td <td>CUGACCCUAUCAUCGp</td> <td>C140H178N510107P15</td> <td>15.25</td> <td>4749.61192</td> <td>1582.19</td> <td>-3</td> <td>-5.8</td> <td>-3H</td>  | CUGACCCUAUCAUCGp      | C140H178N510107P15 | 15.25       | 4749.61192   | 1582.19    | -3     | -5.8       | -3H                     |
| Own.cov.cov.ov.print         CVSHITERISKOV (TPT0         15:.84         32268.41057         160.32.007         -2         -24         -34         -44         -54           AUACAUUCACALAAGP         C1441178NS90102915         16:.02         242.03.4537         1614.2191         -3         3.1         -31         -64           CUACAULAAGP         C1441178NS90102915         16:.02         323.04537         1614.2191         -2         3.1         -31         -64           CUACAULAAGP         C04H120NA9008P10         16:.02         126.320.45377         1614.2191         -2         -4         -4         -4         -54         -4           UCCACUCUAUGP         C102H130N3500P11         116.49         3645.4239         173.10705         -2         1.0         -24         -4 <t< td=""><td>UUUCACACAGp</td><td>C94H119N35071P10</td><td>15.55</td><td>3183.41532</td><td>1590.7086</td><td>-2</td><td>4.7</td><td>-2H, -3H, -4H, -5H</td></t<>   | UUUCACACAGp           | C94H119N35071P10   | 15.55       | 3183.41532   | 1590.7086  | -2     | 4.7        | -2H, -3H, -4H, -5H      |
| Anachovolation         Control  |                       | C95H118N36071P10   | 15.84       | 3208.41057   | 1603.2007  | -2     | 1.2        | -2H, -3H, -4H, -5H      |
| Cumunanapi<br>GGCUap         Caming                              | MUMCAUUUUGACAAAGp     | C14401/00090103P15 | 16.02       | 4040.65685   | 1014.2191  | -3     | 3.1        | -30,-00                 |
| Concord         Concord <t< td=""><td>COACHURAABUP</td><td>C49U61N20027P5</td><td>16.02</td><td>3230.453/7</td><td>1662 200</td><td>-2</td><td>-0.8</td><td>-2n, -3h, -4h, -5h</td></t<>  | COACHURAABUP          | C49U61N20027P5     | 16.02       | 3230.453/7   | 1662 200   | -2     | -0.8       | -2n, -3h, -4h, -5h      |
| AccAUUCUAQ0p         Clock1130N390781         III         Clock1130N390781         IIII         Clock1130N390781         IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII  | UCCACUCUAUGe          | C102H130N35080P11  | 16.02       | 3465.42030   | 1731 7005  | -1     | -2.5       | -2H -3H -6H -6H -6H     |
| AutouudaAadap         Closhiisonationin         Intel         District         Intel         District         Intel         District   | AACAUUCUAUGo          | C104H130N39O78P1   | 17.24       | 3513 45186   | 1755 7214  | -2     | 11         | -2H -3H -4H -5H -6H     |
| CAUACUAUCAGp         C1141143Na5038712         10.45         3841,520,417         10.76,44         -2         0.6         -2         0.471,341,-491,-591,-681           CUGUGGp         C5171/212/2045P6         18.45         1970,2447         1990,2473         -2         0.6         -2         0.4         -4         -4         -6         -4         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         0.6         -2         -2         -4         -  | AUCUUAAAAUGn          | C105H130N41077P11  | 17.45       | 3537,46309   | 1767.7284  | -2     | 19         | -2H, -3H, -4H -5H       |
| Cuguidgp         C57H72N22045P6         18.45         1970.2476         1969.2473         -1         5         -H           SUGGp         C3H494W7030P4         19.55         1359.17017         1388.1705         -1         5         -H           GGGp         C3H494W7030P4         19.55         1359.17017         1388.1705         -1         -0.3         -H           GGGp         C4H60x20028P4         19.39         1382.2053         690.0948         -2         -2         -2         -2         -2         -2H         -H           CAGGp_1         C4H60x20028P4         19.39         1382.2053         690.0948         -2         -2         -2H         -1         -         -H           UAUUGABUUCUCUAAACGG         C1894234M80144P2         19.39         1382.2053         690.0948         -2         -2         -2H         -H           UAUUGABUUCUCUCUCAACGG         C24H150N1089P13         19.43         496.95897         138.8648         -3         3.8         -3H         -4H         -H         -H           UACULACAUUCUCUCAACACGA         C24H150N1089P13         19.43         4196.95897         138.8648         -3         -3H         -H         -H         -H         -H         -H  | ACAUACUAUCAGo         | C114H143N45O83P12  | 18.45       | 3841,52036   | 1919.7549  | -2     | 0.6        | -2H, -3H, -4H, -5H, -6H |
| GUGGp         C39H49NI7030P4         18.55         1354/1701         1358.1705         -1         -0.3         H           AGG6p         C40H50N20028F4         19.39         1382.20539         690.0948         -2         -2         -2H, -H           AGG6p.1         C40H50N20028F4         19.39         1382.20539         690.0948         -2         -2         -2H, -H           UCAUUGAGUUCUCAAACUGp         C188H234N680144P20         19.39         6366.78303         2121.2642         -3         3.8         -3H           JACUACAACCAGp         C18H15010802B13         19.43         469.58887         1388.8648         -3         -3H, -2H, -5H, -6H           JAGCUACAACCAGp         C18H15010802B13         119.43         4169.58887         1388.8648         -3         -3H, -2H, -4H, -5H, -6H  | CUGUGGp               | C57H72N22O45P6     | 18.45       | 1970.24476   | 1969.2473  | -1     | 5          | -H                      |
| AGGGp         C40H50N20028P4         19.39         1382.20539         690.0948         -2         -2         -2H         -H           CAGGp_1         C40H50N20028P4         19.39         1382.20539         690.0948         -2         -2         -2H, -H           CAGGp_1         C40H50N20028P4         19.39         1382.20539         690.0948         -2         -2         -2H, -H           UCJUUGACUUCUCAAACGG         CB81234H69104P20         19.39         5856.7530         2112.2424         -3         3.8         -3H           UAACUACACCAGp         C124H156N1080P13         19.43         4469.58887         138.8648         -3         -3H, -4H, -5H, -6H           CAGGp         C34H50N18028P13         1342.3942         13411366         -1         -4.4         +1, -2H   | GUGGp                 | C39H49N17O30P4     | 18.55       | 1359.17817   | 1358.1705  | -1     | -0.3       | -н                      |
| GAGGp_1         C40H50N20028P4         19.39         1382.2053         690.094         -2   | AGGGp                 | C40H50N20O28P4     | 19.39       | 1382,20539   | 690.0948   | -2     | -2         | -2H, -H                 |
| UCAUUGAGUUCUUCAAACUGp C188H234N680144P20 19.39 536578303 2121.2642 -3 3.8 -3H<br>UAACUACAACCAGp C124H150N1088P13 19.43 4169.58887 1388.8648 -3 4.8 -3H, -2H, -4H, -5H, -6H<br>CAGGp C39H50N18028P1 42.3.8 134219924 13411865 -1 -4.1 ++7H   | GAGGp_1               | C40H50N20O28P4     | 19.39       | 1382.20539   | 690.0948   | -2     | -2         | -2H, -H                 |
| UAACUACAACCAGp C124H156N51088P13 19.43 4169.58887 1388.8648 -3 4.8 -3H, -2H, -4H, -5H, -6H<br>CAGGp C39H50N18028P4 22.38 134219924 13411865 -1 -4.1 -H2H  | UCAUUGAGUUCUUCAAACUGb | C188H234N68O144P20 | 19.39       | 6366.78303   | 2121.2642  | -3     | 3.8        | -3H                     |
| CAGGp C39H50N18028P4 22.38 134219924 1341.1865 -1 -4.1 -H2H   | UAACUACAACCAGp        | C124H156N51O88P13  | 19.43       | 4169.58887   | 1388.8648  | -3     | 4.8        | -3H, -2H, -4H, -5H, -6H |
|   | CAGGp                 | C39H50N18O28P4     | 22.38       | 1342.19924   | 1341.1865  | -1     | -4.1       | -H, -2H                 |

Table 1: Tentatively identified and validated luciferase mRNA digest components within a 10 ppm mass error

based on accurate-mass matching. Cells corresponding to isomeric sequences or isobaric ions are highlighted in grey.

Figure 3 shows example data that produced an identification of digestion component UCCACUCUAUGp. This component eluted at 16.49 minutes as seen on the top left chromatogram and was identified from the *in silico* digestion library based on 5 ions carrying 2 to 6 negative charges (Figure 3, left bottom trace) at *m/z* 576.5732 ([M-6H]<sup>6-</sup>), 692.0876 ([M-5H]<sup>5-</sup>), 865.3603 ([M-4H]<sup>4-</sup>), 1154.1438 ([M-3H]<sup>3-</sup>) and 1731.7095 ([M-2H]<sup>2-</sup>). Isotopic distributions for [M-5H]<sup>5-</sup> and [M-2H]<sup>2-</sup> ions are illustrated to show data in support of charge state assignments (Figure 3, right).



Figure 3: Identification of digest component UCCAUCACCCUGp eluting at 16.49 minutes (left top trace). The component was identified from the generated in silico digest based on 5 ions carrying 2 to 6 negative charges (left bottom trace) at m/z 576.5732 ([M-6H]<sup>6-</sup>), 692.0876 ([M-5H]<sup>5-</sup>), 865.3603 ([M-4H]<sup>4-</sup>), 1154.1438 ([M-3H]<sup>3-</sup>) and 1731.7095 ([M-2H]<sup>2-</sup>). The experimentally observed isotopic distributions for [M-5H]<sup>5-</sup> and [M-2H]<sup>2-</sup> ions are depicted on the right top and bottom traces respectively.

Ambiguities resulting from the presence of isomeric or isobaric ions were resolved using the waters\_connect CONFIRM Sequence<sup>™</sup> application for the interpretation of MS<sup>E</sup> spectra as reported in Table 2. Isomeric sequences at position 623–631 (ACAUCCUCGp) and 551–559 (UCACCAUCGp) are predicted from the RNAse T1 *in silico* digestion and both are assigned to the same retention time of 13.87 minutes. The correct assignment cannot be made using the intact mass analysis. MS<sup>E</sup> data from the same injection were used to elucidate the

correct sequence for this assignment using the waters\_connect<sup>™</sup> CONFIRM Sequence application, wherein high energy fragment ions are predicted for each sequence and matched to isotope clusters of the integrated raw data via a bespoke algorithm. Confirmed fragment ions are represented on a Dot-Map allowing a rapid assessment of the sequence coverage (Figure 4, B): full sequence coverage was obtained for UCACCAUCGp such that it could be readily validated as the correct assignment (Table 2, index #63).

Neutral precursor masses obtained for manually validated digestion components (Table 1) spanned from 973.1406 Da (CCGp, RT 2.54 minutes) to a 20-mer nucleotide at 6366.7830 Da (UCAUUGAGUUCUUCAAACUGp, RT 19.39 minutes). The earliest eluting component was UGUGp, and it was identified with an observed neutral mass of 1320.1560 and a retention time of 1.64 minutes. The last luciferase mRNA digest component to elute within the manually validated set of IDs (Table 1) was CAGGp (1342.1992Da), and it was observed to elute at 22.38 minutes. Elution of CAGGp at such a late retention time was unexpected since the closely related sequence UAAGp eluted at ~4 minutes. In order to address the issue of false positives, we used the waters\_connect CONFIRM Sequence application to further characterize components IDs. Validated based on MS data with MS<sup>E</sup> data. As it can been seen in Table 2, 34/90 components, including CAGGp, did not generate an adequate number of MS<sup>E</sup> fragments to further validate the sequence assignment, although accurate-mass matching supported the assignment. Another interesting example is the observation within the same dataset of assigned sequences AAAAACAUGUUGCCGp (4860.6678 Da, 15-mer, 9 purines) and AUACAUUUGACAAAGp (4845.6568 Da, 15-mer, 9 purines) with identical purine content but eluting 10 minutes apart. Using MS<sup>E</sup> data, we were able to rule out AAAAACAUGUUGCCGp as a false positive and confirm the identification of AUACAUUUGACAAAGp. This demonstrates the importance of strategically using both accurate-mass matching and fragmentation spectra to aid the unambiguous identification of components resulting from digested mRNA sequences. Additionally, our observations have led us to recognize that the retention of digested mRNA components may not be as predictable as first thought and that there is an urgent need for the interactions between oligonucleotides and chromatographic stationary phases to be further studied and modeled.

Lastly, we manually estimated sequence coverage by comparing the matched, manually validated digest components to the mRNA sequence. A preliminary estimate of sequence coverage for the 401 initial matches produced a coverage value of approximately 76%. When the rigorously validated matches were checked against the mRNA sequence, a coverage value of ~22% was obtained. Many of the observed digestion components mapped to more than one location in the luciferase mRNA sequence (Table 2) and the redundancy was expected because there are only four unique residues in a modified or fully modified nucleic acid sequence.

|                      | Validated luciferase mRNA digest                            |   |                                 | Validation using MSe data  |                    |                 |             |        |
|----------------------|---|---|---------------------------------|--|--------------------|-----------------|-------------|--------|
|                      | components using accurate mass                              | Observed RT<br>(min)  | on mRNA                         | Additional position(s) on mRNA sequence  |                    |                 | coverage (% | 6)     |
|                      | matching (10 ppm mass error)                                |   | sequence                        |  | Yes / No           | Peak 1          | Peak 2      | Peak 3 |
| 1                    | UGUGp_1   | 1.64  | 217-229                         | 1553-1556  | yes                | 50              |             |        |
| 2                    | CCUUCGCCCGp   | 1.65  | 896-905                         | NA   | no                 | 0               |             |        |
| 3                    | CCGGUCAAAGn   | 1.7   | 1520-1529                       | NA   | no                 | 10              |             |        |
| 5                    | GAAGp   | 2.1   | 378-381                         | 445-448,635-638,712-715  | ves                | 25              |             |        |
| 0                    | CCCp 8  | 2.54  | 50.61                           | 246-248, 284-286, 838-840, 855-857, 1073-1075, 1076-1078, 1223-1225,   |                    | 0               |             |        |
| 0                    | CCGp_6  | 2.54  | 59-01                           | 1520-1522  | no                 | 0               |             |        |
| 7                    | GAGACACCCUAGp   | 2.54  | 1667-1678                       | NA   | no                 | 0               |             |        |
| 8                    | COCGGp  | 2.55  | 25-29                           | NA 402 402 402 402 402 404 512 514 500 502 002 004 000 040 040   | no                 | 0               |             |        |
| 9                    | CUGp  | 2.55  | 177-179                         | 422-424, 427-429, 452-454, 512-514, 560-562, 852-654, 906-908, 940-942, 965-967, 1430-1432, 1493-1495  | yes                | 50              |             |        |
| 10                   | GAGACAUCAUUGp   | 2.55  | 455-466                         | NA   | no                 | 16.67           |             |        |
| 11                   | UAUUGAUAGCAGp   | 2.55  | 165-176                         | NA   | no                 | 0               |             |        |
| 12                   | ACGp  | 2.73  | 779-781                         | 809-811, 920-922, 958-960  | no                 | 0               |             |        |
| 13                   | CAGp  | 2.73  | 174-176                         | 273-275, 287-289   | yes                | 50              |             |        |
| 14                   | AUGp  | 2.92  | 209-211                         | 291-293, 407-409, 571-573, 709-711, 806-808, 1100-1102, 1193-1195, 1271-1273, 1367-1369, 1415-1417, 1427-1429, 1467-1469, 1581-1583, 1688-1690 | yes                | 100             |             |        |
| 15                   | AAGp_4  | 3.18  | 43-45                           | 146-148, 155-157, 379-381, 446-448, 636-638, 713-715, 1105-1107, 1139-1141, 1220-1222, 1304-1306, 1433-1435, 1475-1477, 1490-1492, 1679-1681   | yes                | 100             |             |        |
| 16                   | ACACCCUAGAAGp   | 3.44  | 1670-1681                       | NA   | no                 | 16.67           |             |        |
| 17                   | UGAUCUUGp   | 3.9   | 1530-1537                       | NA   | yes                | 50              |             |        |
| 18                   | CCUGp   | 3.91  | 902-905                         | NA<br>1420 1442 1564 1567 1500 1602  | no                 | 0               |             |        |
| 20                   | CUCUGGUGo   | 3.91  | 1307-1314                       | NA   | no                 | 12.5            |             |        |
| 21                   | GGUCCCUGp   | 3.91  | 1048-1055                       | NA   | no                 | 12.5            |             |        |
| 22                   | AAGCCGUGp   | 4.17  | 1220-1227                       | NA   | no                 | 12.5            |             |        |
| 23                   | UAAGp_1   | 4.79  | 644-647                         | 1371-1374  | yes                | 50              |             |        |
| 24                   | AAAGp   | 5.06  | 312-315                         | 1147-1150, 1645-1648   | yes                | 100             |             |        |
| 25                   | CUCUGp  | 5.81  | 1307-1311                       | NA   | yes                | 60              |             |        |
| 26                   | UCCCGp  | 5.83  | 1232-1236                       | NA   | no                 | 0               |             |        |
| 27                   | AUCUGn  | 6.18  | 620 640                         | 1445-1449  | no                 | 0               |             |        |
| 28                   | AUCCGp  | 6.53  | 30-34                           | NA   | yes                | 20              |             |        |
| 30                   | AAAAACAUGUUGCCGn  | 6,52  | 234-248                         | NA   | no                 | 0               |             |        |
| 31                   | CAAAGp  | 6.79  | 822-826                         | NA   | yes                | 40              |             |        |
| 32                   | CCUCUGp   | 7.89  | 687-692                         | NA   | yes                | 50              |             |        |
| 33                   | CUCCCGp   | 7.91  | 968-973                         | NA   | yes                | 33.33           |             |        |
| 34                   | CCCCAGp   | 8.29  | 653-658                         | NA   | no                 | 16.67           |             |        |
| 35                   | AUCCAGp   | 8.58  | 734-739                         | NA   | yes<br>(coelution) | 33.33           | 33.33       |        |
| 36                   | CAUUAGp   | 8.58  | 65-70                           | NA   | yes                | 100             |             |        |
| 37                   | AUUCUGp   | 8.63  | 1204-1209                       | NA   | yes                | 66.67           |             |        |
| 38                   | AAUCAGp   | 8.88  | 1155-1160                       | NA   | yes                | 66.67           |             |        |
| 39                   | UACAAGp   | 8.88  | 841-846                         | NA   | yes                | 100             |             |        |
| 40                   | AUCCAGp   | 8.89  | 734-739                         | NA   | yes                | 33.33           | 33.33       |        |
| 41                   | ACACAGe   | 9.03  | 659-664                         | 332-337, 400-405, 984-989<br>NA  | yes                | 16.67           |             |        |
| 43                   | ACAAAGp   | 9.52  | 1025-1030                       | NA   | ves                | 33.33           |             |        |
| 44                   | UCUUCUGp  | 9.89  | 828-834                         | NA   | yes                | 71.43           |             |        |
| 45                   | AUCUCUGp  | 10.23   | 679-685                         | NA   | по                 | 14.29           |             |        |
| 46                   | UCUACUGp  | 10.23   | 71-77                           | NA   | yes                | 57.14           | 28.57       |        |
| 47                   | AUUCCAGp  | 10.53   | 318-324                         | NA   | no                 | 14.29           |             |        |
| 48                   | CAUACUGp  | 10.53   | 815-821                         | NA   | yes                | 57.14           |             |        |
| 49                   | AUUUCAGp  | 10.95   | 1406-1412                       | NA   | yes                | 71.43           |             |        |
| 50                   | ACUACAGe  | 11.22   | 047 052                         | NA<br>1295 1201  | yes                | 71.43           |             |        |
| 52                   | CCACAAGo  | 11.25   | 1557-1563                       | NA   | ves                | 14.29           |             |        |
| 53                   | UCUCUUCGp   | 11.69   | 1512-1519                       | NA   | ves                | 37.5            |             |        |
| 54                   | AAAACAGp  | 11.84   | 1197-1203                       | NA   | yes                | 71.43           |             |        |
| 55                   | UCCUUACGp   | 11.99   | 102-109                         | NA   | yes                | 37.5            |             |        |
| 56                   | CCACCAUGp   | 12.32   | 35-42                           | NA   | yes                | 25              |             |        |
| 57                   | AAAUCCUGp   | 12.63   | 798-805                         | NA   | no                 | 12.5            |             |        |
| 58                   | ACAUACUGp   | 12.63   | 1277-1284                       | NA   | yes                | 14.29           | 62.5        | 40     |
| 59                   | COGCCGP   | 12.64   | 852-857                         | NA NA  | no                 | 0               |             |        |
| 61                   | CCUCACACGo  | 13.84   | 929-937                         | NA   | VPS                | 22.22           |             |        |
| 62                   | ACAUCCUCGp  | 13.87   | 623-631                         | NA   | yes                | 33.33           |             |        |
| 63                   | UCACCAUCGp  | 13.87   | 551-559                         | NA   | yes                | 100             | 33.3        |        |
| 64                   | AUUCUUUUGp  | 13.93   | 1418-1426                       | NA   | yes                | 55.56           |             |        |
| 65                   | ACAUCAUUGp  | 14.19   | 458-466                         | NA   | yes                | 22.22           |             |        |
| 66                   | AUUCUUAUGp  | 14.19   | 1063-1071                       | NA   | yes                | 44.44           |             |        |
| 67                   | UGAUGAUUCUUUUGp   | 14.19   | 1413-1426                       | NA   | no                 | 7.14            |             |        |
| 68                   | ACACCCUAGe  | 14.43   | 1016-1024                       | NA   | yes                | 55.56           |             |        |
| 70                   | AACAACUCGp  | 14.44   | 740-749                         | NA   | 011<br>VPS         | 22.22           |             |        |
| 71                   | AAACAAUCGp  | 14.96   | 1162-1170                       | NA   | yes                | 55.56           |             |        |
| 72                   | CAAACAAAGp  | 14.96   | 665-673                         | NA   | yes                | 77.78           |             |        |
| 73                   | AAAAACAUGp  | 15.25   | 234-242                         | NA   | yes                | 77.78           |             |        |
| 74                   | CUGACCCUAUCAUCGp  | 15.25   | 512-526                         | NA   | no                 | 0               |             |        |
| 75                   | UUUCACACAGp   | 15.55   | 1109-1118                       | NA   | yes                | 80              |             |        |
| 76                   | UACAUAUUAGp   | 15.84   | 1346-1355                       | NA   | yes                | 80              |             |        |
| 77                   | CUACAUOUGACAAAGp  | 16.02   | 1018-1030                       | NA NA  | yes                | 20              |             |        |
| 78                   | GGCUGn  | 16.02   | 420-424                         | NA   | yés                | 00              |             |        |
| 80                   | UCCACUCUAUGD  | 16.49   | 786-796                         | NA   | yes                | 72.73           |             |        |
| 81                   | AACAUUCUAUGp  | 17.24   | 300-310                         | NA   | yes                | 63.64           |             |        |
| 82                   | AUCUUAAAAUGp  | 17.45   | 698-708                         | NA   | yes                | 63.64           |             |        |
| 83                   | ACAUACUAUCAGp   | 18.45   | 197-208                         | NA   | yes                | 50              |             |        |
| 84                   | CUGUGGp   | 18.45   | 177-182                         | NA   | no                 | 0               |             |        |
| 85                   | GUGGp   | 18.55   | 506-509                         | 914-917, 1312-1315   | no                 | 0               |             |        |
|                      |   | the second se | 1602 1606                       | NA   | no                 | 0               |             |        |
| 86                   | AGGGp   | 19.39   | 1003=1000                       | 1014 1017  |                    |                 |             |        |
| 86<br>87             | AGGGp<br>GAGGp_1  | 19.39<br>19.39  | 632-635                         | 1214-1217<br>NA  | no                 | 0               |             |        |
| 86<br>87<br>88<br>89 | AGGGp<br>GAGGp_1<br>UCAUUGAGUUCUUCAAACUGp<br>UAACUACAACCAGp | 19.39<br>19.39<br>19.39<br>19.43  | 632-635<br>593-612<br>1392-1404 | 1214-1217<br>NA<br>NA  | no<br>no<br>yes    | 0<br>0<br>69.23 |             |        |

Table 2: Identification and validation of luciferase mRNA digest components based on accurate-mass matching

and further validation using the waters\_connect CONFIRM Sequence application and collected MS<sup>E</sup> spectra.



Figure 4: (A) Digested fragment components at position 623–631 (ACAUCCUCGp) and 551–559 (UCACCAUCGp) are predicted from RNAse T1 digest and are assigned to the same RT peak in the TIC. It is not possible to determine the correct assignment using intact mass information. (B) MS<sup>E</sup> data from the same injection can be used to elucidate the correct sequence for this assignment. Using the waters\_connect CONFIRM Sequence application, high energy fragment ions are predicted using McLucky annotation<sup>11</sup> for each sequence and matched to isotope clusters of the integrated raw data via a bespoke algorithm. The software presents confirmed fragment ions on a Dot-Map to quickly assess the sequence coverage.

# Conclusion

In the present work, we established a robust analytical workflow for oligo-mapping of synthetic mRNA using IP-RPLC and MS.

· Synthetic mRNAs were reproducibly digested using RNase T1, starting with as little as 10 µg of material, and

injected without additional sample clean-up onto an ACQUITY Premier Oligonucleotide BEH  $C_{18}$  (2.1 x 150 mm, 300 Å, 1.7 µm) Column

- High chromatographic resolution was achieved using ion-pairing reversed-phase chromatography on an ACQUITY Premier LC such that digest components could be readily separated from incompletely digested mRNA and residual enzyme and efficiently detected with a BioAccord ACQUITY RDa Detector
- Annotated mRNA digest chromatograms were generated based on accurate-mass matching as facilitated by in silico mRNA digestion calculations and the application of waters\_connect/UNIFI Scientific Libraries
- Assigned sequences for digested components were further validated based on MS<sup>E</sup> spectra using the waters\_connect CONFIRM Sequence application. In addition, Dot-Map visualization was used to quickly check the fragment ion coverage of potential assignments

With this work, it was our intent to establish the chromatographic, detection, and data interpretation approaches that would be needed to facilitate the bottom-up characterization of an mRNA molecule. RNase T1 digestion was applied only as a first example and a proof of concept on establishing a workflow for data collection and analysis. That said, there is ample opportunity to more comprehensively probe a given mRNA structure by (1) using multiple, different nucleases to generate orthogonal and additive sequence mapping information and (2) adopting a multiplexing approach for data acquisition. These aspects, aimed at achieving comprehensive sequence coverage, will be explored in future work.

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