

Supplementary Information

Site-specific di-functionalization of structured RNAs yields probes for microRNA maturation

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1. General experimental details

Chemicals were purchased from Aldrich and TCI. Phosphoramidites were purchased from Thermo Fisher. The activator 5-benzylthiotetrazole was purchased from Biosolve. All oligonucleotides used in this work were synthesized with a MM12 synthesizer from Bio Automation Inc. on 1000 Å UnyLinker CPG from ChemGenes. The coupling time for phosphoramidites was 2 x 90 s. The oligonucleotides were purified on an Agilent 1200 series preparative HPLC on a Waters XBridge OST C-18 column, 10 x 50 mm, 2.5 µm at 65 °C. Buffer A: 0.1M aqueous triethylamine/acetic acid, pH 8.0; buffer B: 100 % MeOH; flow-rate: 5 mL/min. Gradient for the DMT-on and DMT-off purification: 5 % to 100 % buffer B over 10 min. Fractions containing the product were collected and dried in a miVac duo SpeedVac from Genevac. The oligonucleotides were analyzed by LC-MS (Agilent 1200/6130 system) on a Waters Acquity OST C-18 column, 2.1 x 50 mm, 1.7 µm, 65 °C. Buffer A: 0.4M HFIP, 15 mM triethylamine; buffer B: MeOH. Gradient: 5-80 % B in 10 min; flow-rate: 0.3 mL/min.

2. Oligonucleotide synthesis, incorporation of modified phosphoramidites and work-up of modified pre-miRNAs

10 solutions were prepared for these experiments directly prior use:

- Solution 1: PBS buffer/MeOH (1:1): 5 mL of PBS buffer, 5 mL of MeOH.
- Solution 2: TBTA [50 mM] in DMF: 0.27 mg of TBTA in 40 µL of DMF.
- Solution 3: Cy3 azide [50 mM] in DMF: 0.56 mg of Cy3 azide in 40 µL of DMF.
- Solution 4: CuSO₄·5H₂O [5 mM] in H₂O: 12.5mg of CuSO₄·5H₂O in 10 mL of water.
- Solution 5: Na-ascorbate [50 mM] in H₂O: 10mg of Na-ascorbate in 1 mL of water.
- Solution 6: NaN₃ [400 mM]: 0.65mg of NaN₃ in 110 µL of DMF.
- Solution 7: THPTA [50mM]: 1.1mg of THPTA in 50 µL of water.
- Solution 8: Cy5 alkyne [20mM]: 1.18mg of Cy5 alkyne in 100 µL of DMF.
- Solution 9: CuSO₄·5H₂O [20mM]: 5mg of CuSO₄·5H₂O in 1 mL of water.
- Solution 10: Na ascorbate [100mM]: 20mg of Na ascorbate in 1 mL of water.

Oligoribonucleotides were synthesized with regular 2'-O-TBDMS-phosphoramidites on a 50 nmol scale using 5 mg of CPG (1000Å). For 2'-O-propargyl cytidine phosphoramidite and 2'-O-methylenetriazolobutylbromide cytidine phosphoramidite, the coupling time was prolonged to 2 x 3 min. After synthesis, the CPG with the modified RNA was suspended in 300 µL of PBS buffer/MeOH (1:1) (solution 1) in an Eppendorf tube. Subsequently, freshly-prepared solutions of TBTA (solution 2, 40 µL), azide (solution 3, 40 µL), CuSO₄·5H₂O (solution 4, 10 µL) and Na-ascorbate (solution 5, 10 µL) were added to the suspension. The reaction mixture was shaken for 16 h at 45 °C in an Eppendorf shaker. CPG was filtered, washed three times with 0.5 mL of DMF, 0.1N aqueous EDTA, DMF, and acetonitrile.

CPG was transferred into an Eppendorf tube and suspended in a DMF solution of sodium azide (solution 6, 110 μ L). The reaction mixture was shaken vigorously for 6 h at 45°C in an Eppendorf shaker. The CPG was filtered and washed three times with successively 1 mL of DMF and acetonitrile. The CPG was transferred into an Eppendorf tube, dried under high vacuum for 2 h and treated with 200 μ L of ammonia (25% in H₂O) and 200 μ L of aqueous methylamine (40 % in H₂O) solutions for 6 h at room temperature. After filtration, the remaining RNA was washed from the solid support with 3 x 100 μ L H₂O/EtOH (1:1). To the solution was added 20 μ L of 1N Tris-base and it was evaporated to dryness in a SpeedVac. Desilylation was carried out by treatment with 130 μ L of a mixture of NMP (60 μ L), TEA (30 μ L) and TEA.3HF (40 μ L) at 70 °C for 90 min. The reaction was quenched with trimethylethoxysilane (160 μ L) for 20 min at room temperature on an Eppendorf shaker. Diethylether (1 mL) was added, the mixture was vortexed and centrifuged at 4 °C for 2 min. The supernatant was taken off and the precipitate was washed twice with 1 mL diethylether, vortexed and centrifuged. The oligonucleotide was dissolved in 200 μ L of water and purified DMT-on by RP-HPLC. The isolated product was dried in a SpeedVac and treated for 1 h with 40 % aq. acetic acid at room temperature. The Cy3/N₃-DMT_{off} pre-miRNA was dried in a SpeedVac and diluted in water.

To 2 nmol of modified RNA in 20 μ L of water was successively added PBS buffer (pH = 7.4, 28.5 μ L), THPTA (solution 7, 2.5 μ L), Cy5 alkyne (solution 8, 1.25 μ L), CuSO₄.5H₂O (solution 9, 1.25 μ L) and Na ascorbate (solution 10, 2.5 μ L). After incubation of the reaction mixture for 2 h at 65 °C, the solution was cooled down to room temperature. Water (150 μ L) was successively added and the solution extracted three times with 200 μ L of EtOAc. NaOAc (25 μ L of a 3M solution) and 800 μ L of EtOH:iPrOH (2:1) were added and RNA was precipitated for 30min at -80°C (alternatively, -20°C can be used for 12 h). After centrifugation (20min, 14000rpm), supernatant was removed, the RNA pellets dissolved in water (200 μ L) and purified by RP-HPLC to yield pure Cy3/Cy5 bis-labeled RNA.

3. Optimization of azidation reaction on pre-miR-21

3.1 Azidation of 3'-Br-pre-miR-21

Experimental procedure

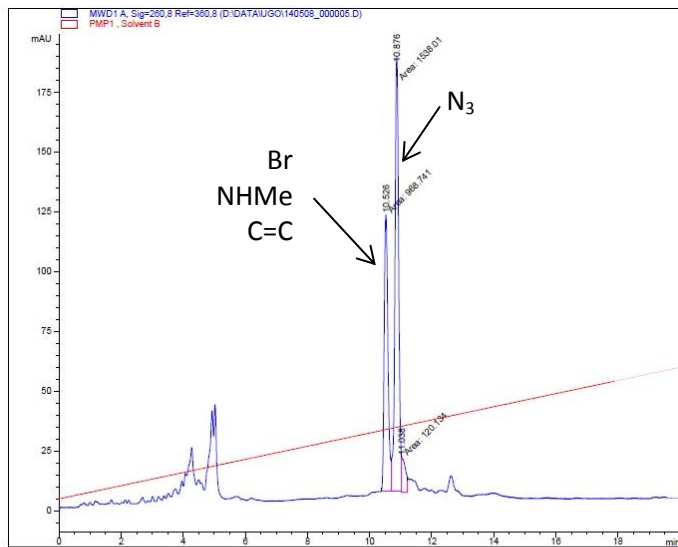
Two solutions were prepared for these experiments directly prior use:

- *Solution 1:* 1.3 mg of NaN_3 in 600 μL of DMF.
- *Solution 2:* 3 mg of NaI in 200 μL of DMF.

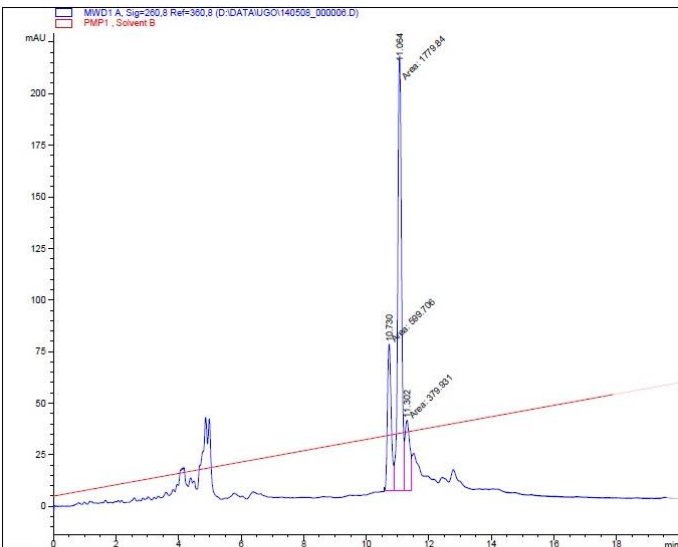
Two CPG columns (5 mg of 1000Å, 50 nmol syntheses) with the sequence of pre-miR-21 (5'-CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC_{Br}) (C_{Br}: 2'-*O*-methylenetriazolo-bromobutane) were prepared. CPGs were combined and mixed in an Eppendorf tube before being equally redistributed into 4 Eppendorf tubes. Sodium azide (150 μL of solution 1, final $[\text{NaN}_3] = 25\text{mM}$) and sodium iodide (50 μL of solution 2, final $[\text{NaI}] = 25\text{mM}$) were added. The reaction mixture was shaken vigorously for the corresponding time (respectively 1 h, 2 h, 4 h, 8 h) on an Eppendorf shaker. The CPGs were filtered and washed successively with 1 mL of DMF and acetonitrile. After cleavage from the solid support and RNA deprotection (aq. $\text{MeNH}_2/\text{NH}_4\text{OH}$, TEA.HF), the composition of the crude solution was evaluated by integration of peaks in the HPLC chromatograms (see following spectra).

HPLC-profile of crude 3'-N₃-pre-miR-21

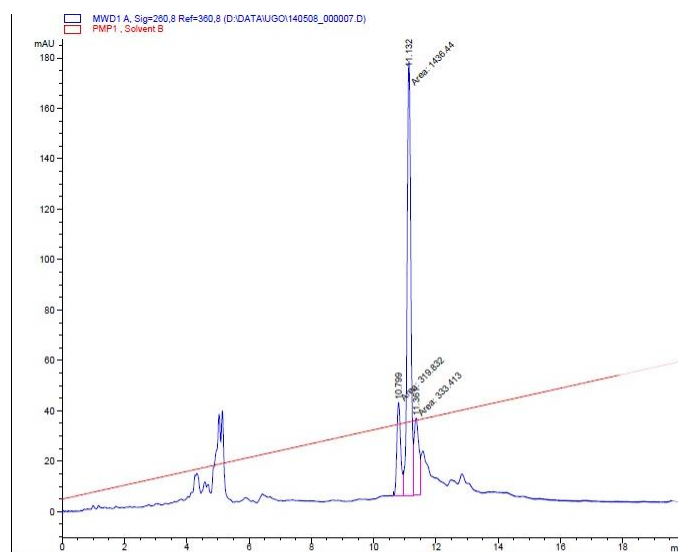
1 h, 45°C



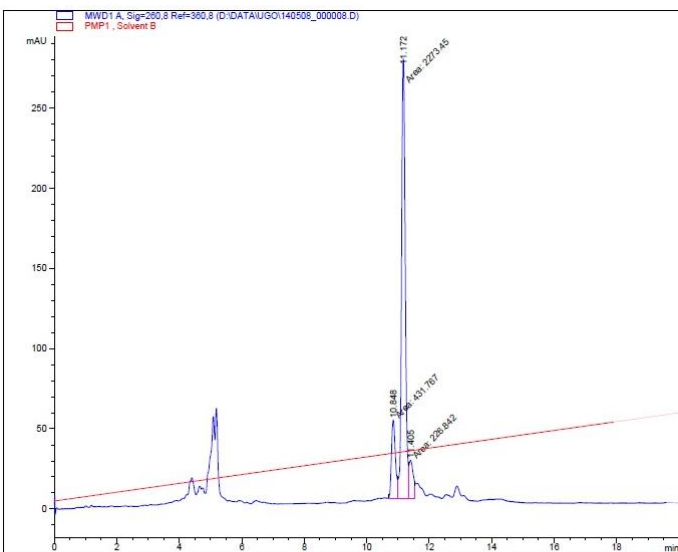
2 h, 45°C



4 h, 45°C



8 h, 45°C



3.2 Azidation of 3'-Br-pre-miR-21 in harsher conditions

Experimental procedure

8 solutions were prepared for these experiments directly prior use:

- *Solution 1*: 1.3 mg of NaN_3 in 400 μL of DMF.
- *Solution 2*: 3 mg of NaI in 400 μL of DMF.
- *Solution 3*: 0.65 mg of NaN_3 in 65 μL of DMF.
- *Solution 4*: 1.5 mg of NaI in 65 μL of DMF.
- *Solution 5*: Commercial solution LiN_3 20% wt in H_2O .
- *Solution 6*: 1.34 mg of LiI in 397.5 μL of DMF.
- *Solution 7*: 3.25 mg of NaN_3 in 200 μL of DMF.
- *Solution 8*: 7.5 mg of NaI in 200 μL of DMF.

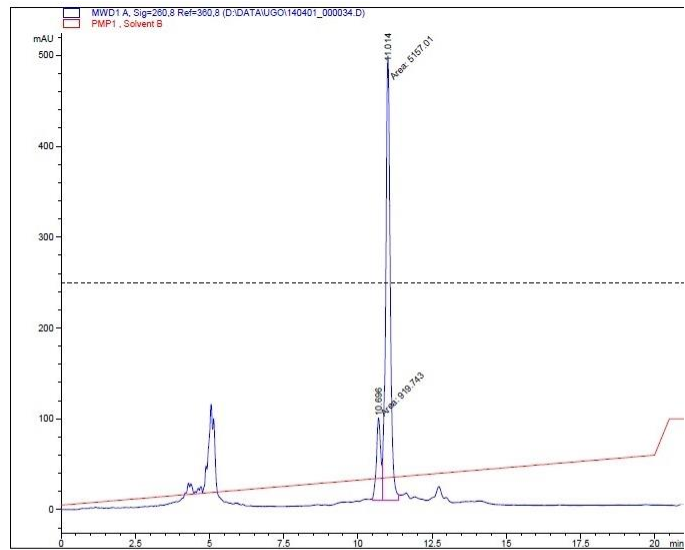
5 CPG columns (5 mg of 1000Å, 50 nmol syntheses) with the sequence of pre-miR-21 (5'-CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC_{Br}) (C_{Br}: 2'-*O*-methylenetriazolo-bromobutane) were prepared. CPGs were combined and mixed in an Eppendorf tube before being equally redistributed into 5 Eppendorf tubes. Respective azide and iodide solutions were added. The reaction mixtures were shaken vigorously for 6 h on an Eppendorf shaker at the corresponding temperature and the CPGs filtered and washed successively with 1 mL of DMF and acetonitrile. After cleavage from the solid support and RNA deprotection (aq. $\text{MeNH}_2/\text{NH}_4\text{OH}$, TEA.HF), the composition of the crude solution was evaluated by integration of peaks in the HPLC chromatograms (see following spectra).

Conditions:

- Conditions A (control experiment): $[\text{NaN}_3]=25\text{ mM}$, $[\text{NaI}]=25\text{ mM}$, 45°C. 200 μL of solution 1 and 200 μL of solution 2.
- Conditions B (increased concentration): $[\text{NaN}_3]=75\text{ mM}$, $[\text{NaI}]=75\text{ mM}$, 45°C. 65 μL of solution 3 and 65 μL of solution 4.
- Conditions C (lithium counter-ion): $[\text{LiN}_3]=25\text{ mM}$, $[\text{LiI}]=25\text{ mM}$, 45°C. 2.45 μL of solution 5 and 397.5 μL of solution 6.
- Conditions D (increased equivalence): $[\text{NaN}_3]=125\text{ mM}$, $[\text{NaI}]=125\text{ mM}$, 45°C. 200 μL of solution 7 and 200 μL of solution 8.
- Conditions E (increased temperature): $[\text{NaN}_3]=25\text{ mM}$, $[\text{NaI}]=25\text{ mM}$, 65°C. 200 μL of solution 1 and 200 μL of solution 2.

HPLC-profile of crude 3'-N₃-pre-miR-21

Conditions A (control experiment)

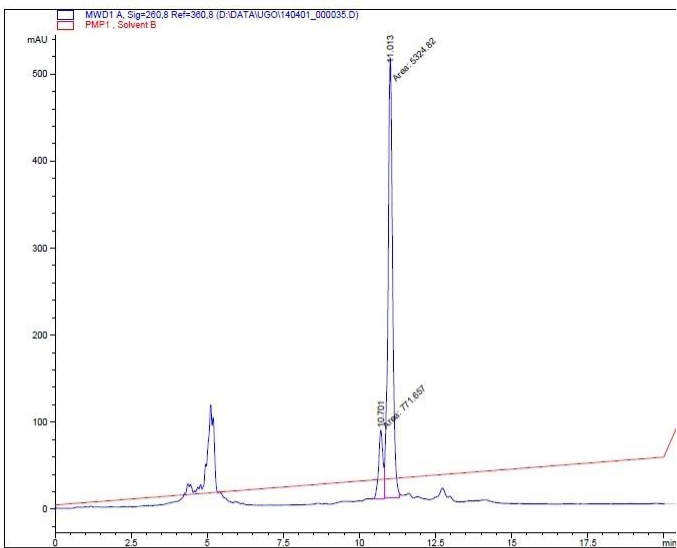


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.696	MM	0.1683	919.74261	91.07889	15.1354
2	11.014	MM	0.1759	5157.01074	488.51984	84.8646

Totals : 6076.75336 579.59872

Conditions B (increased concentration)

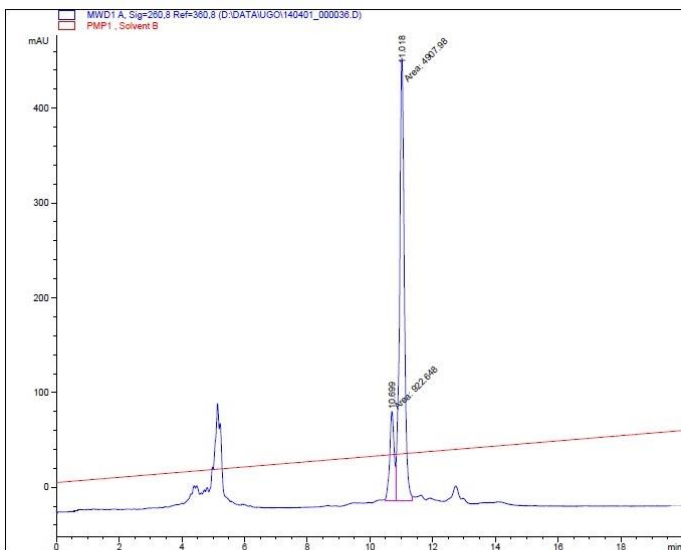


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.701	MM	0.1635	771.65680	78.68349	12.6574
2	11.013	MM	0.1754	5324.82178	505.97549	87.3426

Totals : 6096.47858 584.65899

Conditions C (lithium counter-ion)

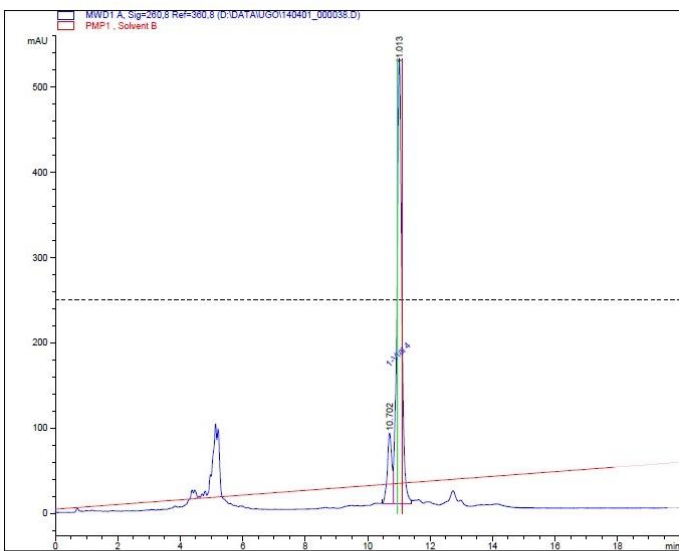


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.699	MM	0.1640	922.64752	93.76894	15.8241
2	11.018	MM	0.1753	4907.98242	466.67819	84.1759

Totals : 5830.62994 560.44714

Conditions D (increased equivalence)

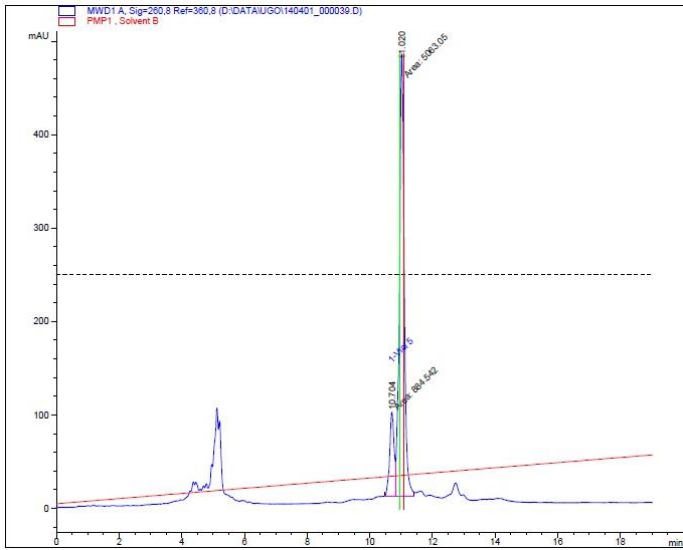


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.702	MM	0.1613	796.11285	82.26608	12.5523
2	11.013	MM	0.1772	5546.26758	521.61694	87.4477

Totals : 6342.38043 603.88302

Conditions E (increased temperature)



=====
Area Percent Report
=====

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=260.8 Ref=360.8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.702	MM	0.1613	796.11285	82.26608	12.5523
2	11.013	MM	0.1772	5546.26758	521.61694	87.4477

Totals : 6342.38043 603.88302

3.3 Azidation of 3'-Br-pre-miR-21 with and without NaI

Experimental procedure

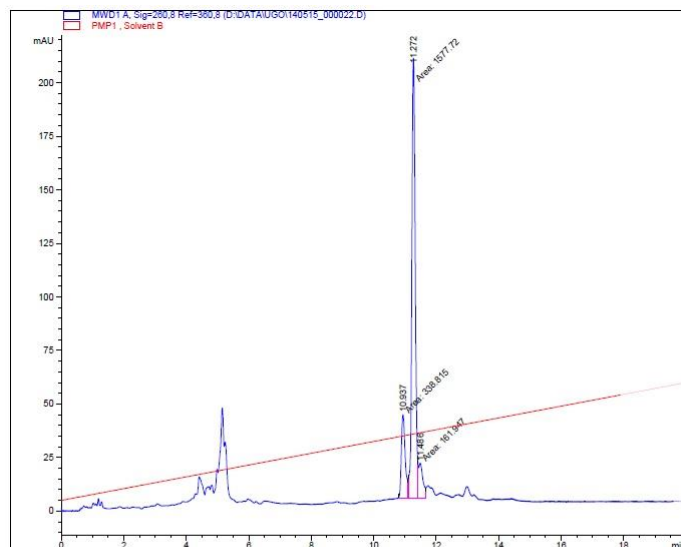
2 solutions were prepared for these experiments directly prior use:

- *Solution 1*: 2.6mg of NaN_3 in 440 μL of DMF.
- *Solution 2*: 6mg of NaI in 80 μL of DMF.

Two CPG columns (5 mg of 1000Å, 50 nmol syntheses) with the sequence of pre-miR-21 (5'-CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC_{Br}) (C_{Br}: 2'-*O*-methylenetriazolo-bromobutane) were prepared. CPGs were combined and mixed in an Eppendorf tube before being equally redistributed into 4 Eppendorf tubes. Sodium azide (110 μL of solution 1, final $[\text{NaN}_3] = 75\text{mM}$) was added to both samples while sodium iodide (20 μL of solution 2, final $[\text{NaI}] = 75\text{mM}$) was only added to one sample. The reaction mixture was shaken vigorously for 3 h at 45°C on an Eppendorf shaker. Solution were added a second time as described previously, the reaction mixtures shaken vigorously for 3 h at 45°C and the CPGs filtered and washed successively with 1 mL of DMF and acetonitrile. After cleavage from the solid support and RNA deprotection (aq. $\text{MeNH}_2/\text{NH}_4\text{OH}$, TEA.HF), the crude solution was evaluated by integration of peaks in the HPLC chromatograms (see following spectra).

HPLC-profile of crude 3'-N₃-pre-miR-21

With NaI

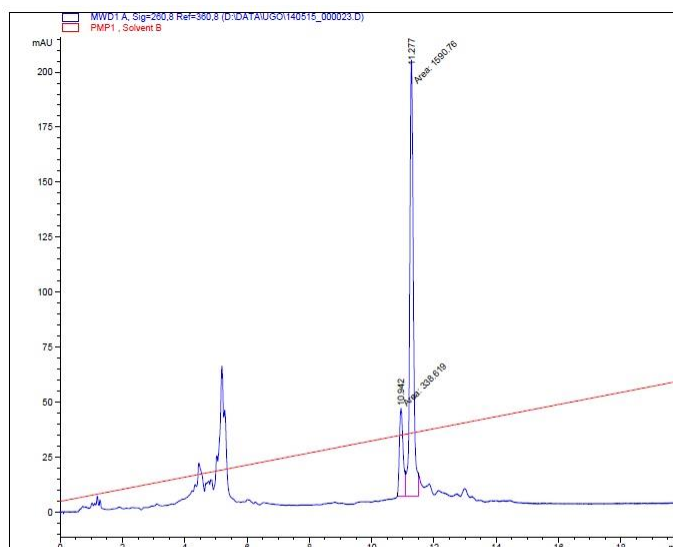


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.937	MM T	0.1437	338.81519	39.29510	16.3011
2	11.272	MM T	0.1279	1577.71741	205.63945	75.9073
3	11.486	MM T	0.1355	161.94681	16.28609	7.7916

Totals : 2078.47940 261.22064

Without NaI



Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.942	MM	0.1406	338.61874	40.13443	17.5507
2	11.277	MM	0.1337	1590.75879	198.33020	82.4493

Totals : 1929.37753 238.46463

3.4 Azidation of 3'-Br-pre-miR-21 in various solvents

Experimental procedure

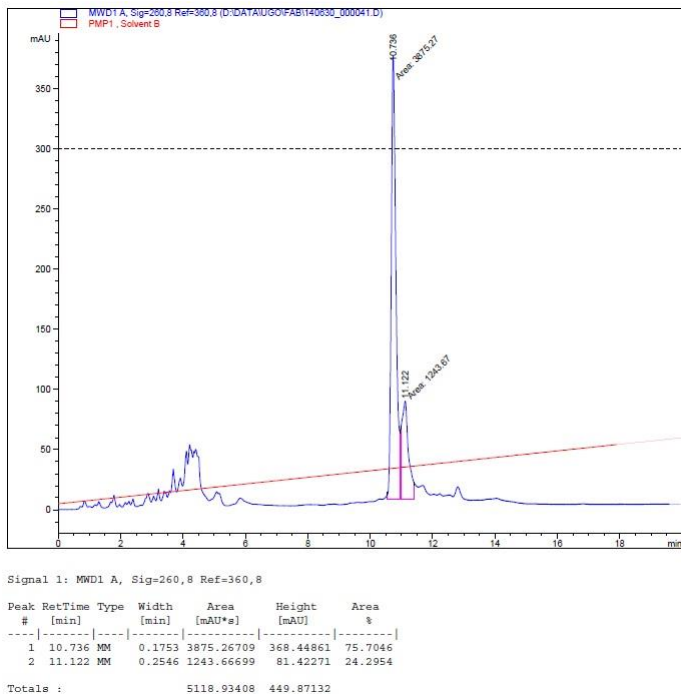
3 solutions were prepared for these experiments directly prior use:

- *Solution 1*: 0.65 mg of NaN₃ in 110 µL of THF.
- *Solution 2*: 0.65 mg of NaN₃ in 110 µL of DMSO.
- *Solution 3*: 0.65 mg of NaN₃ in 110 µL of EtOH.

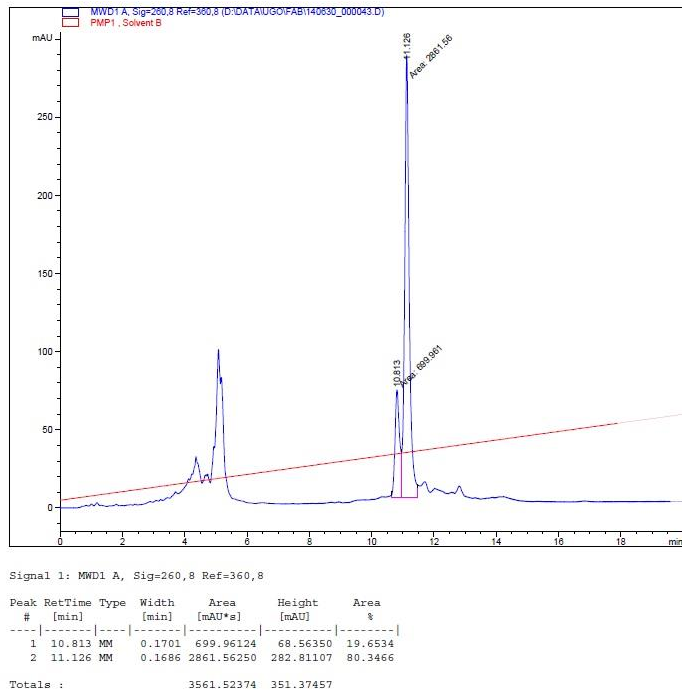
2 CPG columns (5 mg of 1000Å, 50 nmol syntheses) with the sequence of pre-miR-21 (5'-CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC_{Br}) (C_{Br}: 2'-*O*-methylenetriazolo-bromobutane) were prepared. CPGs were combined and mixed in an Eppendorf tube before being equally redistributed into 3 Eppendorf tubes. Sodium azide solutions (110 µL, end [NaN₃] = 75mM) were added. The reaction mixture was shaken vigorously for 6 h at 45°C on an Eppendorf shaker, filtered and washed successively with 1 mL of DMF and acetonitrile. After cleavage from the solid support and RNA deprotection (aq. MeNH₂/NH₄OH, TEA.HF), the composition of the crude solution was evaluated by integration of peaks in the HPLC chromatograms (see following spectra).

HPLC-profile of crude 3'-N₃-pre-miR-21

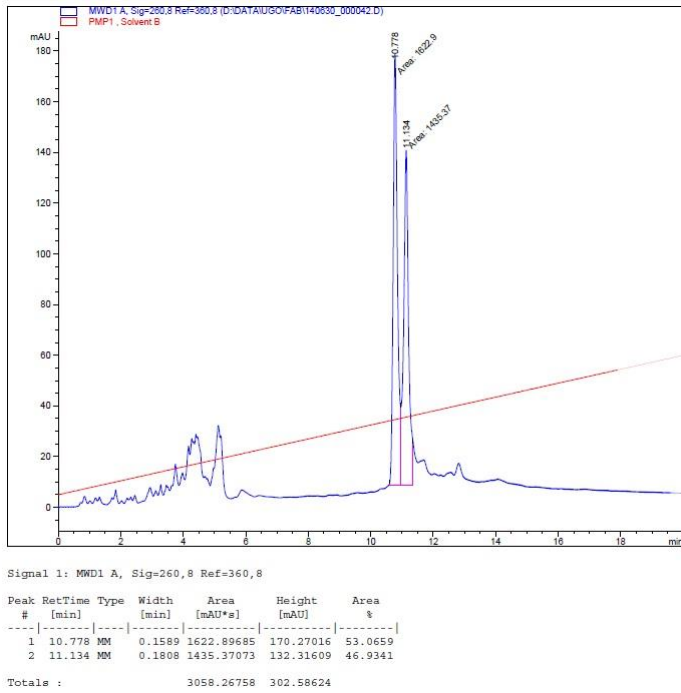
In THF



In DMSO



In EtOH



4. Optimization of reverse-click reaction in solution phase on pre-miR-21

Experimental procedure

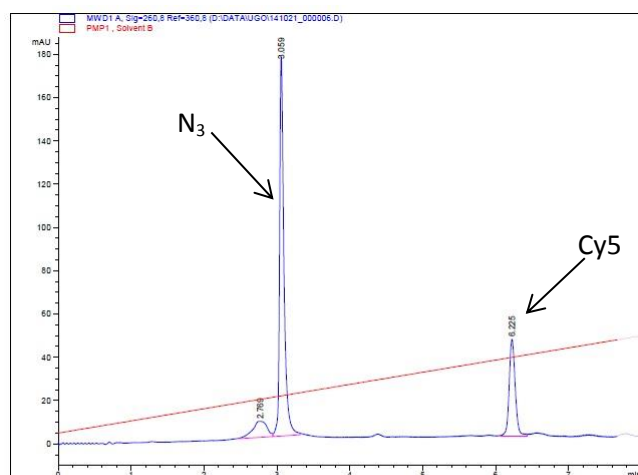
4 solutions were prepared for these experiments directly prior use:

- *Solution 1*: THPTA [50mM]: 1.1 mg of THPTA in 50 μ L of water.
- *Solution 2*: Cy5 alkyne [20mM]: 1.18 mg of Cy5 alkyne in 100 μ L of DMF.
- *Solution 3*: CuSO₄·5H₂O [20mM]: 5 mg of CuSO₄·5H₂O in 1 mL of water.
- *Solution 4*: Na ascorbate [100mM]: 20 mg of Na ascorbate in 1 mL of water.

To a water solution of 5'-N₃-pre-miR-21 (10 μ L, 1nmol) with the sequence (5'-C_{N3}CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGU) (C_{N3}: 2'-O-methylenetriazolo-azidobutane) was successively added water (15 μ L), PBS buffer (pH = 7.4, 28.5 μ L), THPTA (solution 1, 2.5 μ L, end [2.1mM]), Cy5 alkyne (solution 2, 1.25 μ L, end [0.8mM]), CuSO₄·5H₂O (solution 3, 1.25 μ L, end [0.8mM]) and Na ascorbate (solution 4, 2.5 μ L, end [4.2mM]). After incubation of the reaction mixture for the reported time and at the reported temperature, the solution was cooled down to room temperature. Water (150 μ L) was added and the solution extracted three times with 200 μ L of EtOAc. NaOAc (25 μ L of a 3M solution) and 800 μ L of a 2:1 EtOH/iPrOH mixture were added and RNA was precipitated for 30min at -80°C (alternatively, -20°C can be used for 12 h). After centrifugation (10min, 14000rpm), supernatant was removed and the RNA pellets dissolved in water (200 μ L) for HPLC purification (see following spectra).

HPLC profiles of crude 5'-Cy5-pre-miR-21

1 h at 25°C

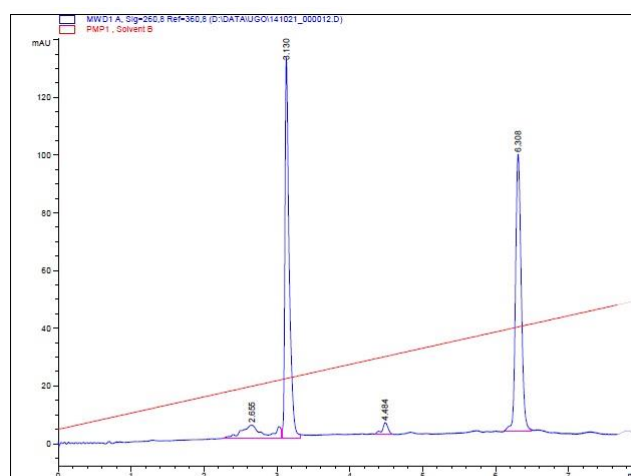


Signal 1: MWD1 A, Sig=260.8 Ref=360.8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.769	BV	0.1637	102.74428	7.43518	9.2772
2	3.059	VB	0.0620	727.90454	175.20293	65.7254
3	6.225	BB	0.0885	254.49306	44.66813	22.9792
4	8.694	VB	0.1116	5.60339	6.04891e-1	0.5060
5	9.599	BB	0.0742	16.74885	2.84406	1.5123

Totals : 1107.49412 230.75518

3 h at 25°C

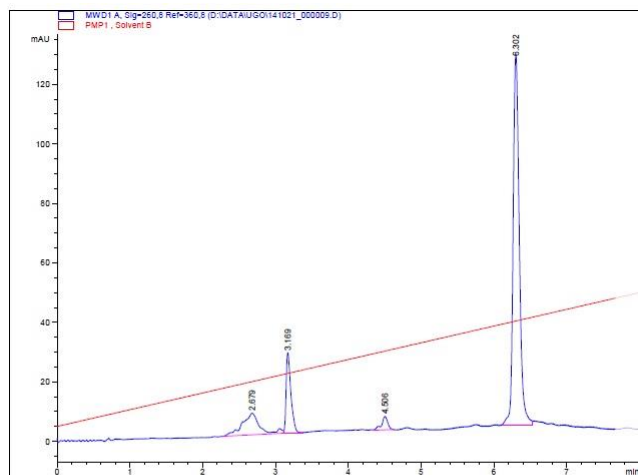


Signal 1: MWD1 A, Sig=260.8 Ref=360.8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.655	NM	0.3735	102.99064	4.59572	8.6947
2	3.130	NM	0.0675	533.90582	131.75653	45.0734
3	4.484	NM	0.0939	23.23036	4.12345	1.9612
4	6.308	NM	0.0909	524.39868	96.13461	44.2708

Totals : 1184.52550 236.61030

1 h at 45°C

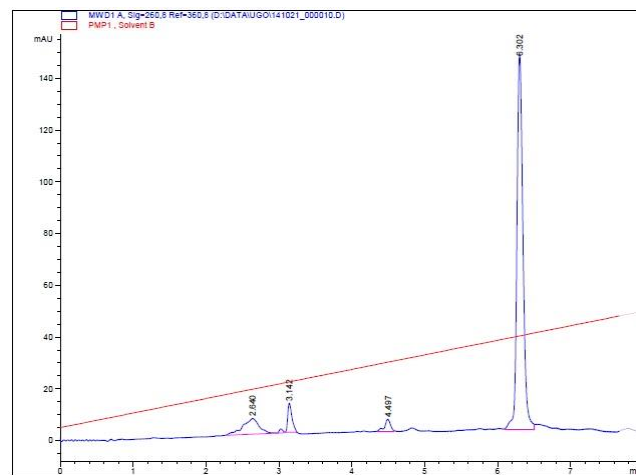


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.679	MM	0.2721	119.59730	7.32676	11.8747
2	3.169	MM	0.0764	124.02019	27.06338	12.3139
3	4.506	MM	0.0957	25.93965	4.51579	2.5755
4	6.302	MM	0.0983	737.60107	125.04221	73.2359

Totals : 1007.15821 163.94814

2 h at 45°C



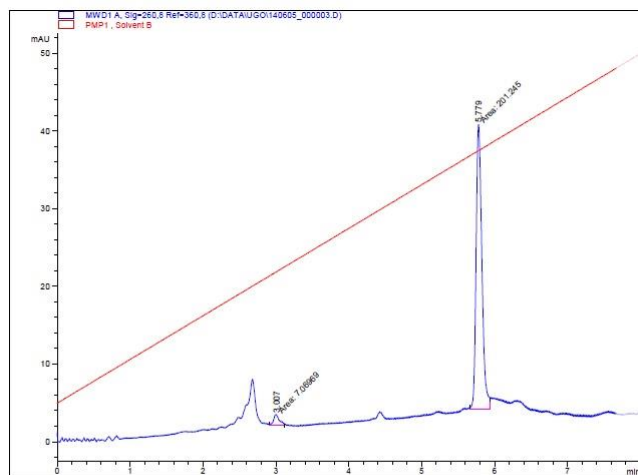
Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.640	MM	0.2763	100.04795	6.03406	10.0108
2	3.142	MM	0.0675	45.57722	11.24751	4.5605
3	4.497	MM	0.0947	27.30400	4.80786	2.7320
4	6.302	MM	0.0949	826.46924	145.08606	82.6967

Totals : 999.39842 167.17549

HPLC profiles of crude 3'-Cy5-pre-miR-21

2 h at 45°C



Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.007	MM	0.0881	7.06969	1.33799	3.3938
2	5.779	MM	0.0916	201.24489	36.61101	96.6062

Totals : 208.31458 37.94901

5. Evaluation of reverse-click reaction on solid support on pre-miR-21 in different conditions

Experimental procedure

7 solutions were prepared for these experiments directly prior use:

- *Solution 1:* CuSO₄·5H₂O [5 mM] in H₂O: 12.5 mg of CuSO₄·5H₂O in 10 mL of water.
- *Solution 2:* Na ascorbate [50 mM] in H₂O: 10 mg of Na ascorbate in 1 mL of water.
- *Solution 3:* TBTA [50 mM] in DMF: 2.7 mg of TBTA in 100 µL of DMF.
- *Solution 4:* THPTA [50 mM] in DMF: 2.2 mg of THPTA in 100 µL of DMF.
- *Solution 5:* H₂O/MeOH (1:1): 5 mL of H₂O, 5 mL of MeOH.
- *Solution 6:* PBS buffer/MeOH (1:1): 5 mL of PBS buffer, 5 mL of MeOH.
- *Solution 7:* Cy3 alkyne [50 mM] in DMF: 3.4 mg of Cy3 azide in 120 µL of DMF.
- *Solution 8:* Tris base [1M] in water: 121 mg of Tris in 1 mL of water
- *Solution 9:* NaN₃ [400 mM]: 4.55 mg of NaN₃ in 770 µL of DMF.

7 CPG columns (5 mg of 1000Å, 50 nmol syntheses) with the sequence of pre-miR-21 (5'-C_{Br}AGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC) were prepared. CPGs were transferred into 7 Eppendorf tubes and were suspended in a DMF solution of sodium azide (110 µL of solution 9). The reaction mixture was shaken vigorously for 6 h at 45°C on an Eppendorf shaker. The CPG was filtered and washed successively with 1 mL of DMF and acetonitrile. CPGs were combined and mixed in an Eppendorf tube before being equally redistributed into 7 Eppendorf tubes and successfully added solvent (H₂O/MeOH or PBS buffer/MeOH), DMF, ligand (TBTA or THPTA), Cy3 alkyne, Na ascorbate and copper sulfate. The reaction mixture was shaken vigorously for 16 h on an Eppendorf shaker and the CPGs filtered and washed successively with 1 mL of DMF, 0.1N aq. EDTA pH 8, DMF, and acetonitrile. After cleavage from the solid support and RNA deprotection (aq. MeNH₂/NH₄OH, TEA.HF), the composition of the crude solution was evaluated by integration of peaks in the HPLC chromatograms (see following spectra).

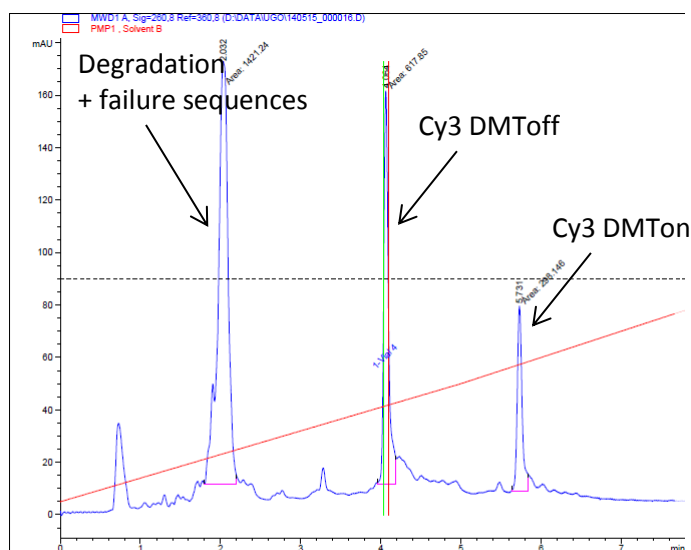
Conditions:

- Condition A (Control): 10 µL of solution 1, 10 µL of solution 2, 10 µL of solution 3, 300 µL of solution 5, 20 µL of solution 7, 50 µL of DMF.
- Condition B (Catalyst /5): 2 µL of solution 1, 2 µL of solution 2, 2 µL of solution 3, 300 µL of solution 5, 20 µL of solution 7, 50 µL of DMF.
- Condition C (TBTA x5): 10 µL of solution 1, 10 µL of solution 2, 50 µL of solution 3, 300 µL of solution 5, 20 µL of solution 7, 50 µL of DMF.

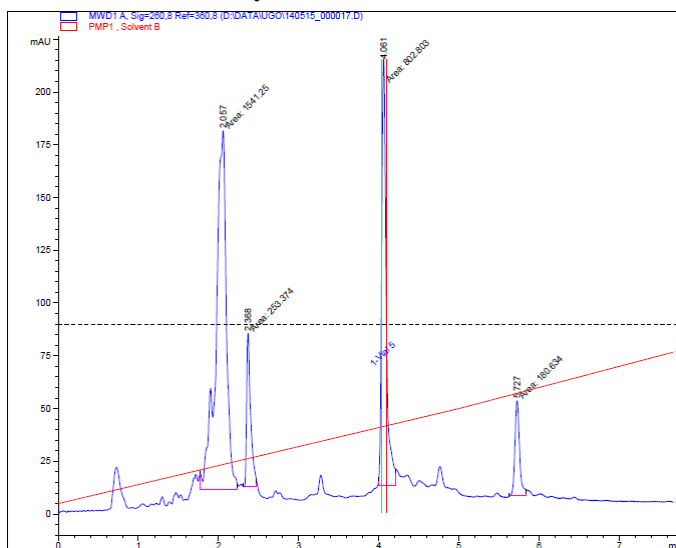
- Condition D (THPTA): 10 μ L of solution 1, 10 μ L of solution 2, 50 μ L of solution 4, 300 μ L of solution 5, 20 μ L of solution 7, 50uL of DMF.
- Condition E (PBS buffer): 10 μ L of solution 1, 10 μ L of solution 2, 10 μ L of solution 3, 300 μ L of solution 6, 20 μ L of solution 7, 50uL of DMF.
- Condition F (TRIS base): 10 μ L of solution 1, 10 μ L of solution 2, 10 μ L of solution 3, 300 μ L of solution 5, 20 μ L of solution 7, 10 μ L of solution 8, 50 μ L of DMF.

HPLC profiles of crude 5'-Cy3-pre-miR-21

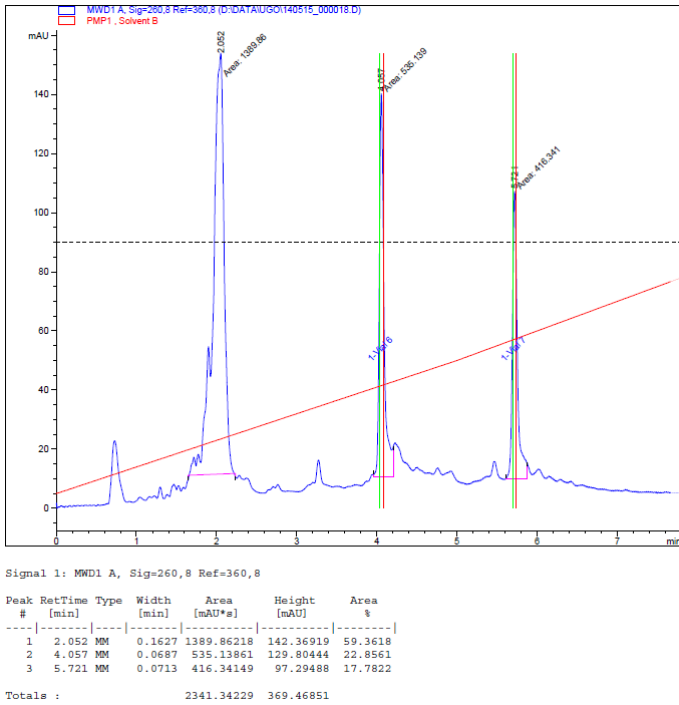
Condition A (control)



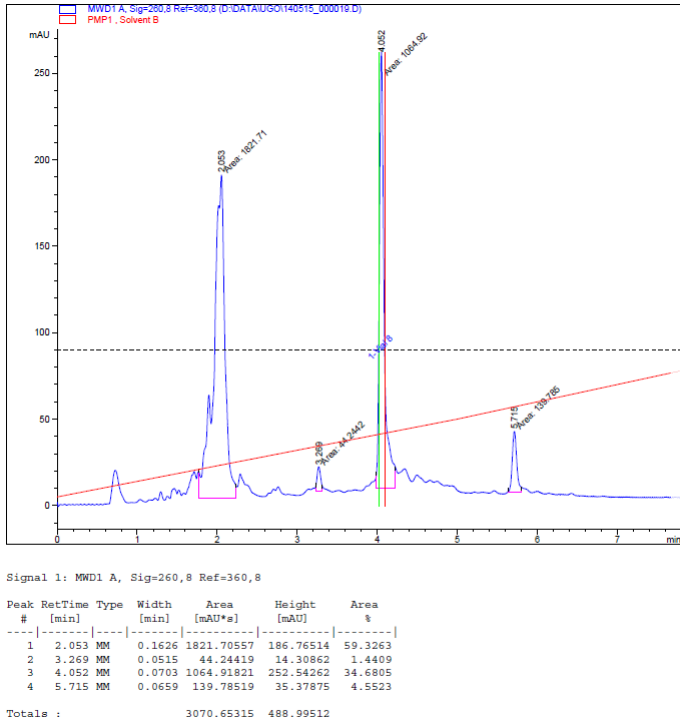
Condition B (Catalyst /5)



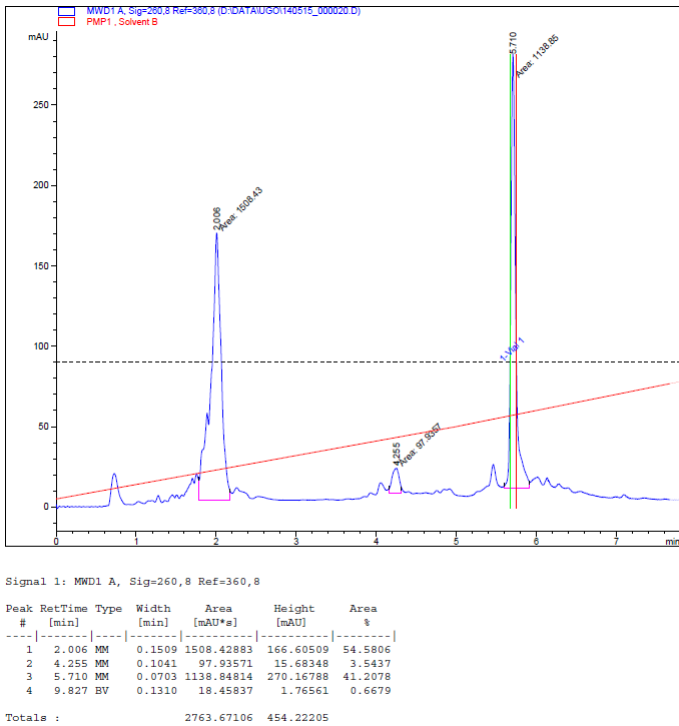
Condition C (TBTA x5)



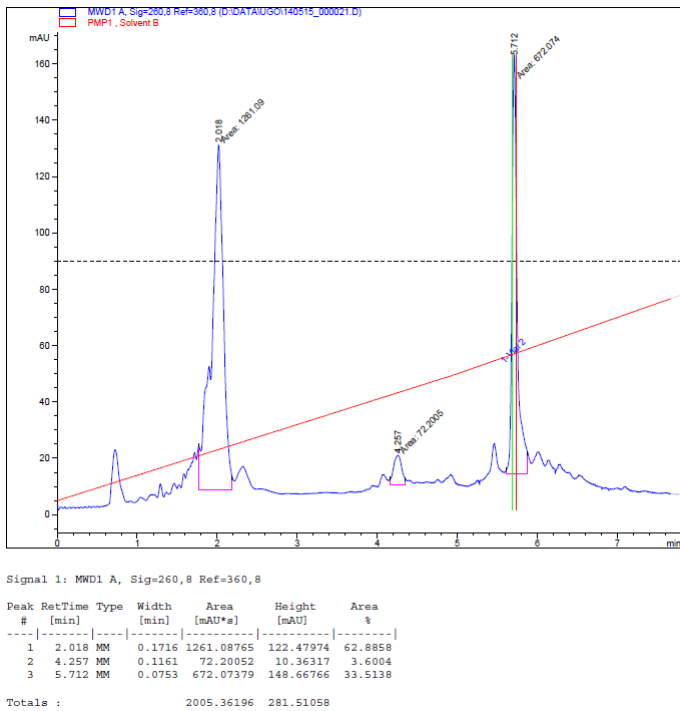
Condition D (THPTA)



Condition E (PBS buffer)



Condition F (TRIS base)



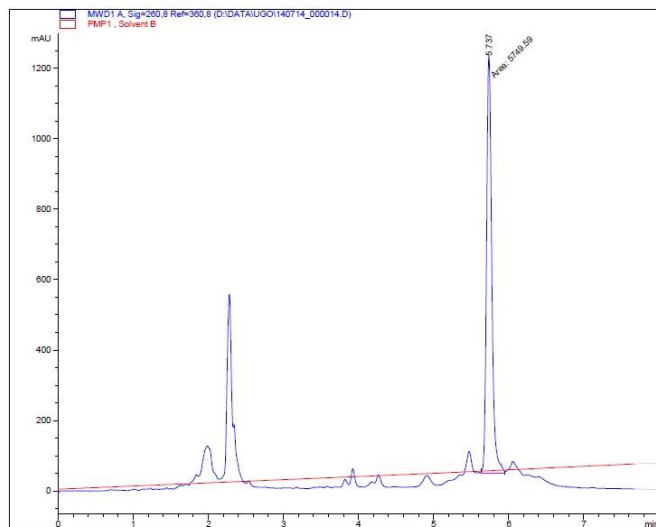
6. Evaluation of click/reverse-click protocol on pre-miR-21

Experimental procedure

Compounds were prepared following the reported procedure in section 2.

HPLC profiles of crude DMTon Cy3/N₃-pre-miR-21

ORN3-Cy3/N₃ DMTon

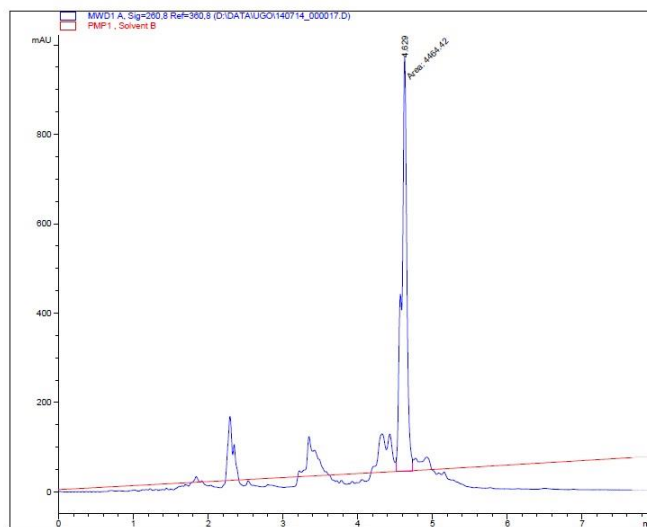


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.737	MM	0.0807	5749.58545	1187.02576	100.0000

Totals : 5749.58545 1187.02576

ORN4-Cy3/N₃ DMTon

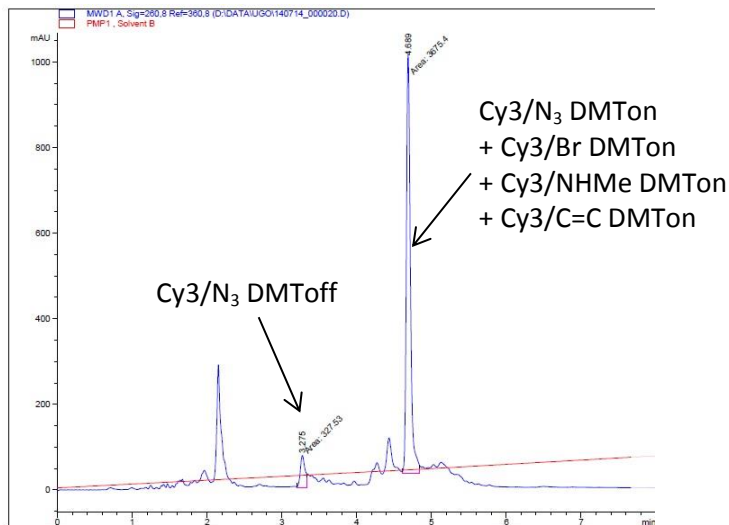


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.629	MM	0.0801	4464.41797	928.87146	100.0000

Totals : 4464.41797 928.87146

ORN5-Cy3/N₃ DMTon

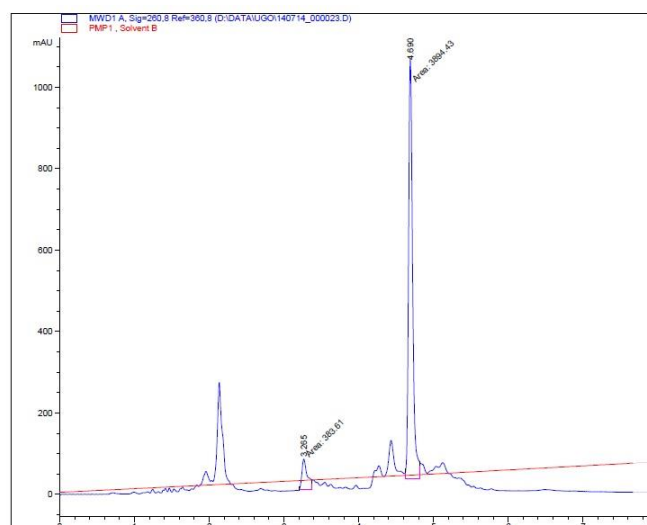


Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.275	MM	0.0730	327.52991	74.82005	8.1823
2	4.689	MM	0.0626	3675.40015	978.12073	91.8177

Totals : 4002.93005 1052.94077

ORN6-Cy3/N₃ DMTon



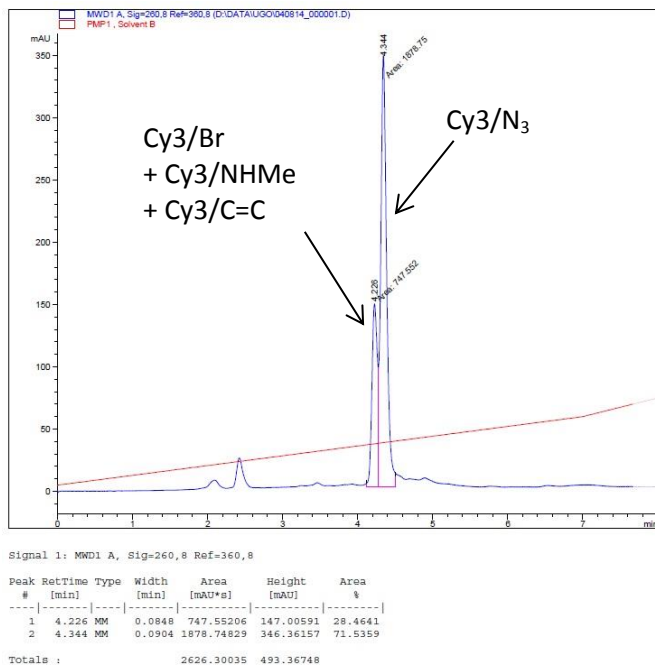
Signal 1: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.265	MM	0.0847	383.61002	75.50623	8.9669
2	4.690	MM	0.0630	3894.43408	1029.54114	91.0331

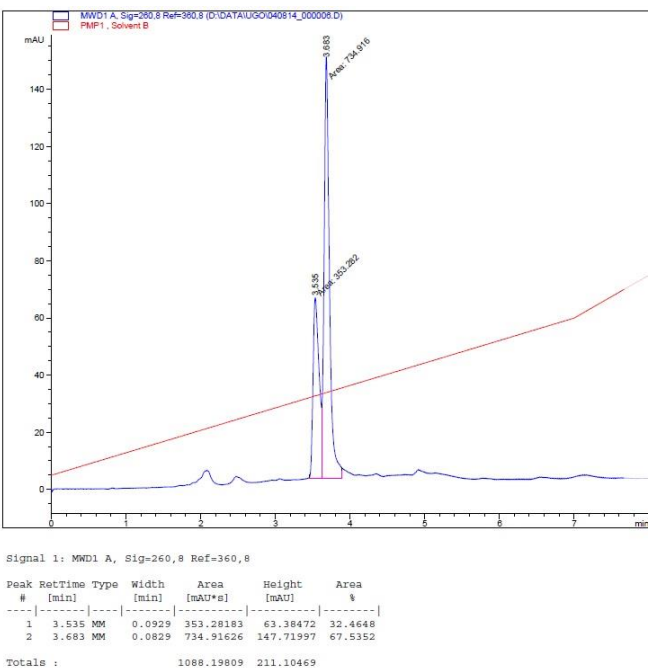
Totals : 4278.04410 1105.04737

HPLC profiles of crude DMToff Cy3/N₃-pre-miR-21

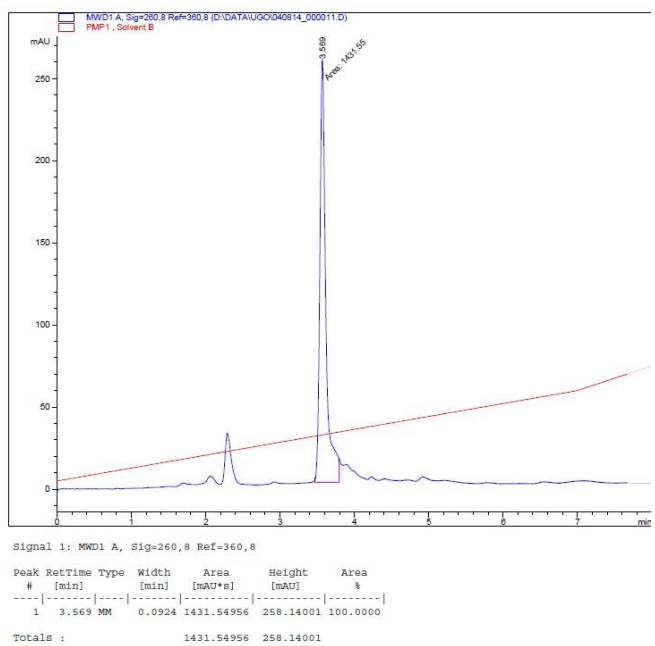
ORN3-Cy3/N₃



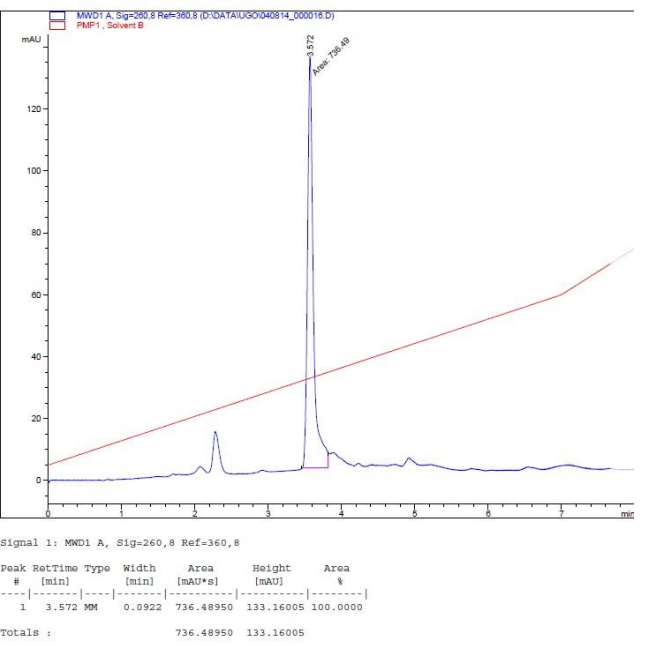
ORN4-Cy3/N₃



ORN5-Cy3/N₃

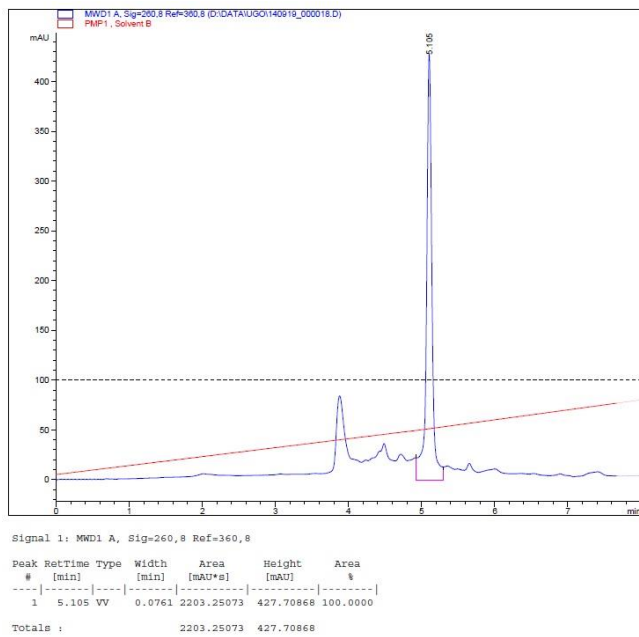


ORN6-Cy3/N₃

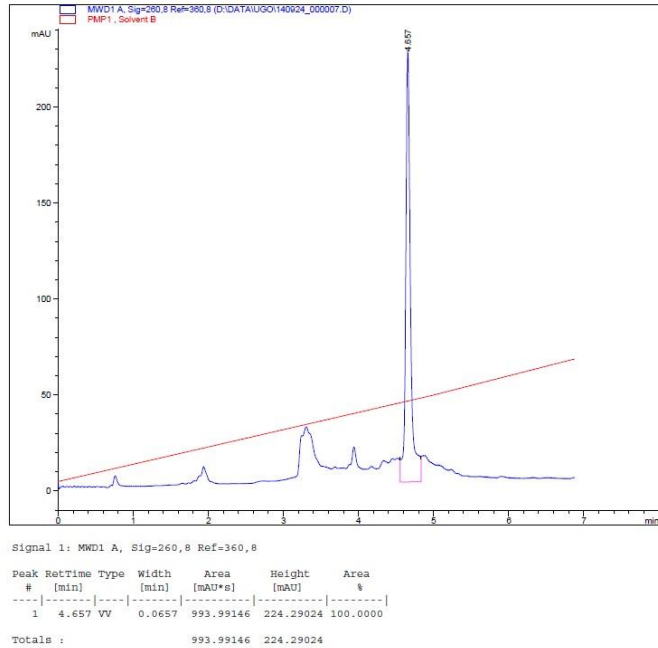


HPLC profiles of crude Cy3/Cy5-pre-miR-21

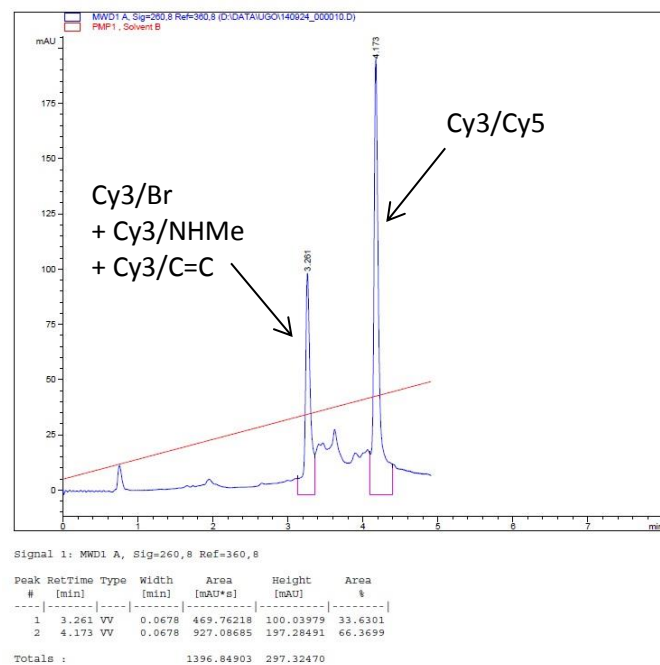
ORN3-Cy3/Cy5



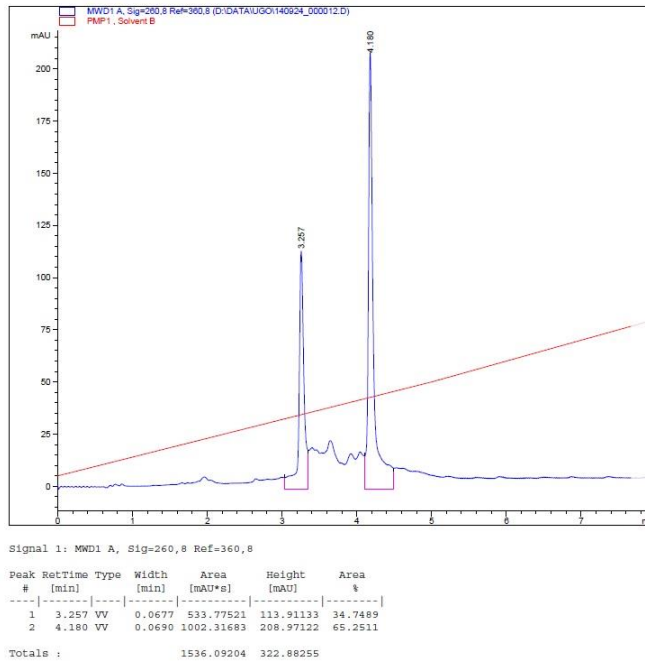
ORN4-Cy3/Cy5



ORN5-Cy3/Cy5

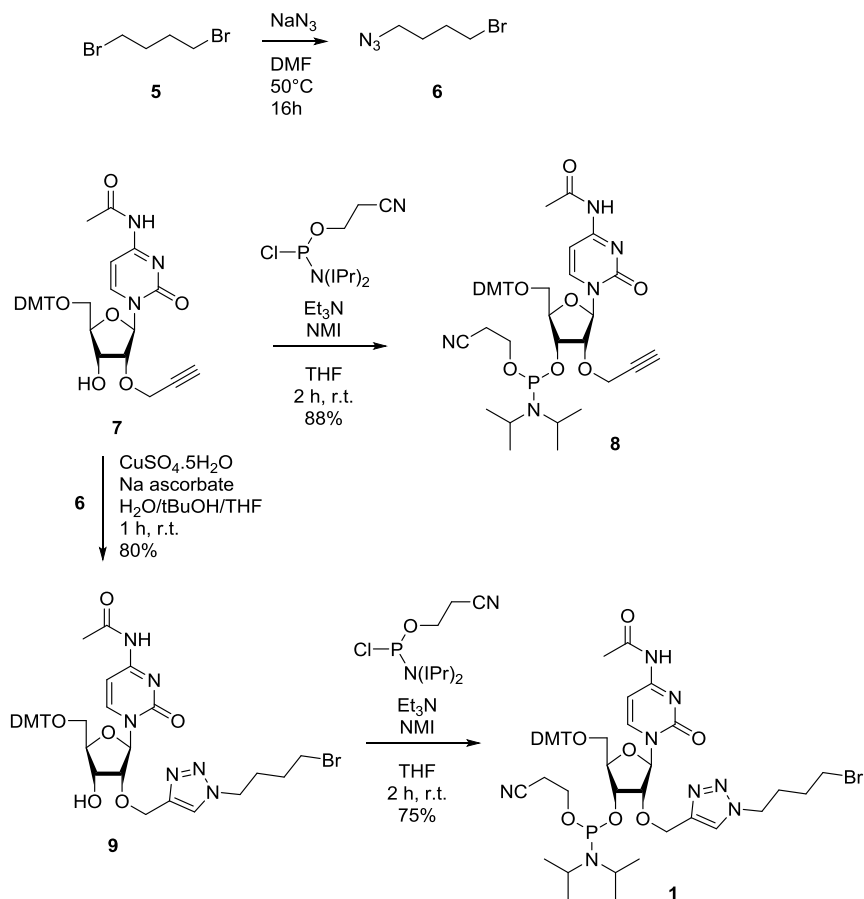


ORN6-Cy3/Cy5



7. Synthesis of reagents

7.1 2'-O-methylenetriazolobutylbromide cytidine phosphoramidite



Synthetic procedures

4-Azido-1-bromobutane **6** (CAS 116114-55-7)

4-Azido-1-bromobutane was prepared following the protocol of Agnew et al.¹

To a solution of 1,4-dibromobutane **5** (2 g, 9.26 mmol, 2 equiv.) in DMF (15mL) was added sodium azide (300 mg, 4.63 mmol). The reaction mixture was stirred at 50°C for 16 h and partitioned between water (100mL) and CH_2Cl_2 (200mL). The organic layer was washed three times with water, once with brine and dried over Na_2SO_4 . After filtration and evaporation of the volatiles, the crude residue was purified by silica gel column chromatography (up to 10% CH_2Cl_2 in hexanes, TLC revelation with KMnO_4) to give compound **6** (680 mg, 3.84 mmol, 83% yield) as a clear oil.

¹ Agnew, H.D.; Rohde, R.D.; Millward, S.W.; Nag, A.; Yeo, W.-S.; Hein, J.E.; Pitram, S.M.; Tariq, A.; Burns, V.M.; Krom, R.J.; Fokin, V.V.; Sharpless, K.B.; Heath, J.R. *Angew. Chem. Int. Ed.* **2009**, 48, 4944–4948

2'-O-propargyl-5'-dimethoxytrityl-N⁴-acetyl-cytidine 7

For a detailed synthesis in three steps starting from cytidine, see our previous report.²

2'-O-propargyl-5'-dimethoxytrityl-N⁴-acetyl-cytidine phosphoramidite 8

For a detailed synthesis in three steps starting from cytidine, see our previous report.²

2'-O-methylenetriazolobutylbormide-5'-dimethoxytrityl-N⁴-acetyl-cytidine 9

To a solution of compound **7** (1.5 g, 2.4 mmol) dissolved in a mixture of water (5 mL), tBuOH (5 mL), and THF (5 mL) at room temperature were successively added azidobutylbromide **6** (850 mg, 4.8 mmol, 2 equiv.), CuSO₄·5H₂O (60 mg, 0.24 mmol, 0.1 equiv.) and sodium ascorbate (95 mg, 0.48 mmol, 0.2 equiv.). After 1 h stirring at room temperature, the reaction mixture was partitioned between EtOAc (250 mL) and water (250 mL). The organic phase was washed twice with water, once with brine and dried over Na₂SO₄. After filtration and evaporation of the volatiles, the crude residue was purified by silica gel column chromatography (up to 5% MeOH in EtOAc to give compound **9** (1.544 g, 1.92 mmol, 80% yield) as a white amorphous solid. ¹H-NMR (400 MHz, CDCl₃) δ=9.57 (s, 1H), 8.45 (d, J = 7.3 Hz, 1H), 7.57 (s, 1H), 7.32 (d, J = 7.2 Hz, 2H), 7.34-7.14 (m, 8H), 7.04 (br, 1H), 6.76 (dd, J = 8.9, 1.6 Hz, 4H), 5.93 (s, 1H), 5.05 (d, J = 12.7 Hz, 1H), 4.97 (d, J = 12.7 Hz, 1H), 4.39 (br, 1H), 4.25 (t, J = 6.8 Hz, 2H), 4.07 (d, J = 9.1 Hz, 1H), 4.00 (d, J = 4.6 Hz, 1H), 3.72 (s, 3H), 3.71 (s, 3H), 3.48 (s, 2H), 3.30 (t, J = 6.4 Hz, 2H), 2.16 (s, 3H), 1.99-1.91 (m, 2H), 1.78-1.71 (m, 2H). ¹³C-NMR (100 MHz, CDCl₃) δ=170.30, 162.67, 158.59, 158.57, 155.14, 144.81, 144.26, 143.99, 135.46, 135.17, 130.03, 128.10, 127.94, 127.03, 123.25, 113.22, 96.57, 89.13, 86.88, 83.00, 81.52, 67.69, 63.25, 60.79, 55.16, 49.23, 32.44, 29.19, 28.56, 24.85. ESI-MS: negative mode 801.3 ([M-H]⁻). Calc.: 802.2.

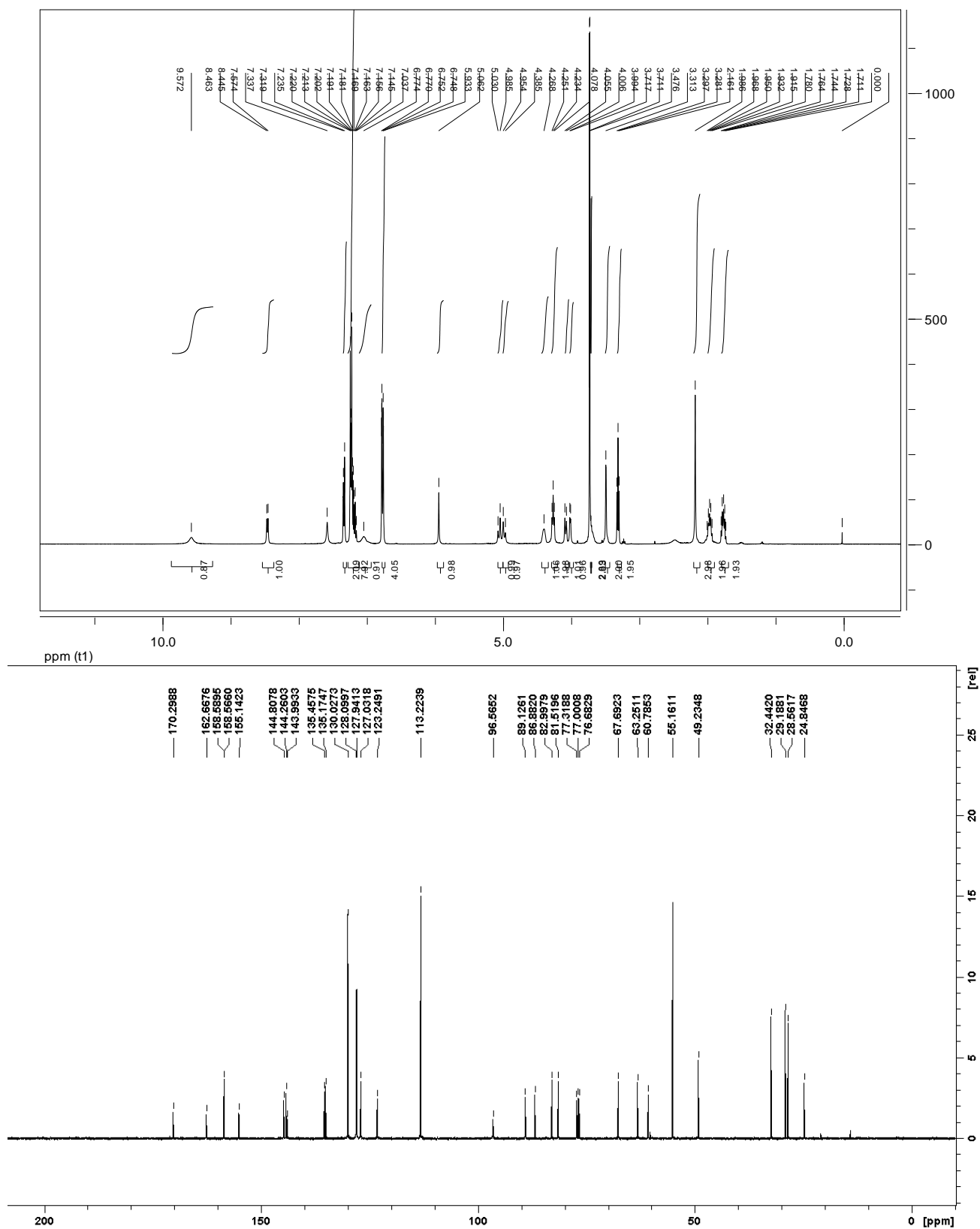
2'-O-methylenetriazolobutylbormide-5'-dimethoxytrityl-N⁴-acetyl-cytidine phosphoramidite 1

To a solution of 2'-O-methylenetriazolobutylbormide-5'-dimethoxytrityl-N⁴-acetyl-cytidine **9** (1.15g, 1.43 mmol, 1 equiv.) in THF (10 mL) at 0 °C were added Et₃N (0.96 mL, 7.16 mmol, 5 equiv.), 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (1.01 g, 4.29 mmol, 3 equiv.). After 2 h stirring at 0 °C, the reaction mixture was partitioned between a 5% NaHCO₃ aqueous solution (100 mL) and EtOAc (100 mL). The aqueous layer was extracted three times by EtOAc (3x 30 mL). The combined organic layer was washed twice with water (2x 20 mL) and once with brine (20 mL), dried over sodium sulfate, filtered and evaporated to dryness. The residue was purified by flash column chromatography with a gradient up to 2% MeOH in EtOAc (0.5% Et₃N) to afford phosphoramidite **1** (1.07 g, 1.07 mmol, 75% yield) as a white foam. ³¹P-NMR (66 MHz, CDCl₃) δ=150.39, 149.87. ESI-HRMS calculated for C₄₈H₆₀BrN₈NaO₉P positive mode ([M+Na]⁺) 1025.3291. Calc.: 1025.3296.

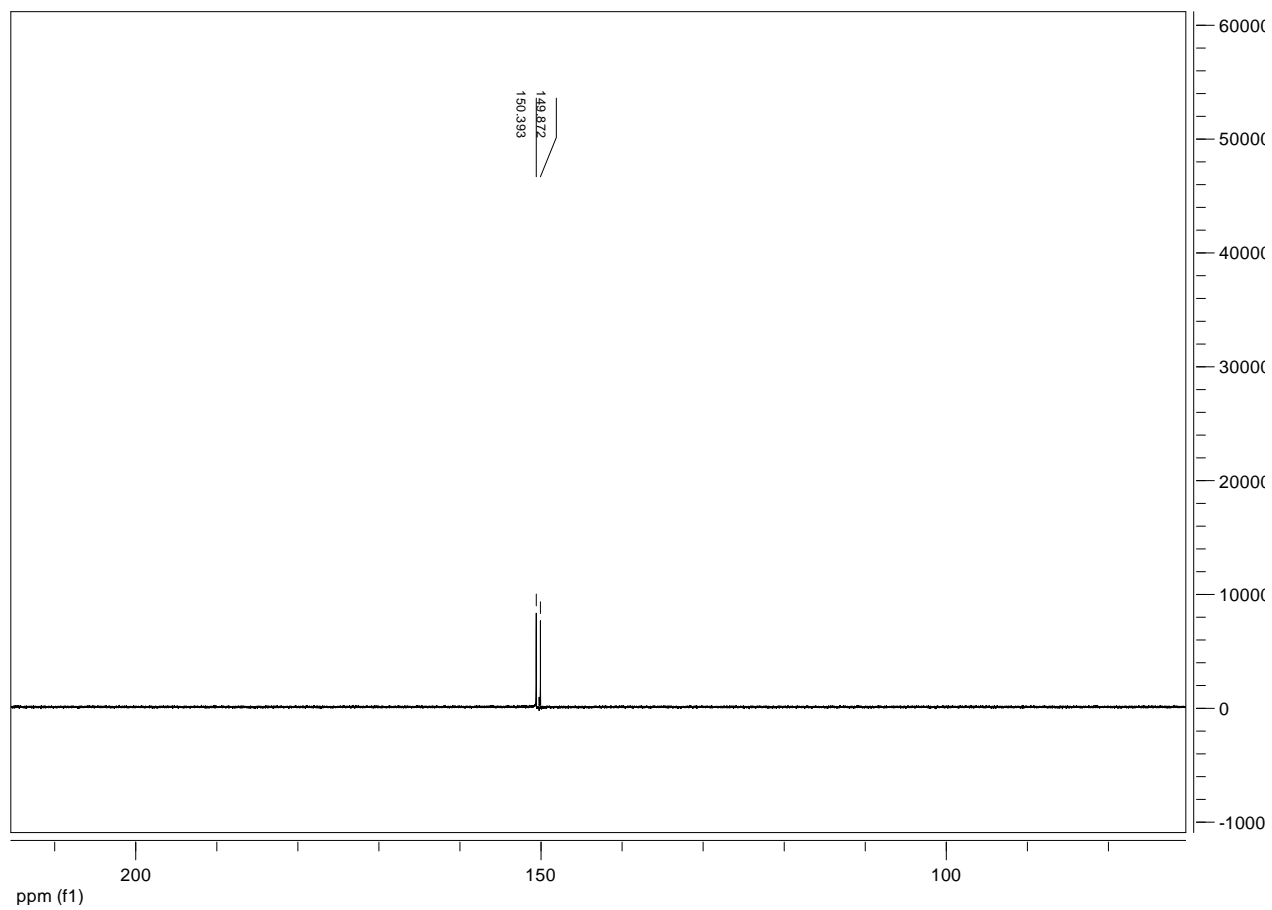
² Pradère, U.; Brunschweiler, A.; Gebert, L.F.R.; Lucic, M.; Roos, M.; Hall, J. *Angew. Chem. Int. Ed.* **2013**, 52, 12028–12032

NMR Spectras

2'-O-methylenetriazolobutylbormide-5'-dimethoxytrityl-*N*⁴-acetyl-cytidine 9

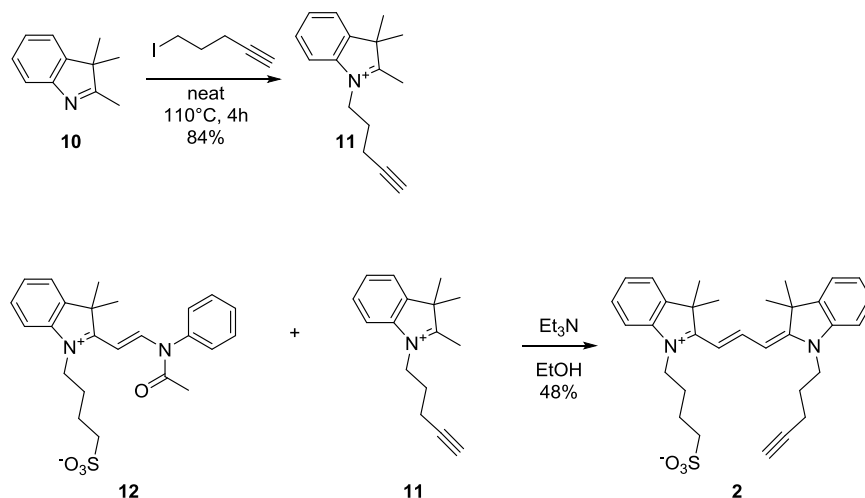


2'-O-methylenetriazolobutylbormide-5'-dimethoxytrityl-*N*⁴-acetyl-cytidine phosphoramidite 1



7.2 Cy3 alkyne

Synthetic procedures



5-iodopent-1-yne (CAS 2468-55-5)

5-iodopent-1-yne was prepared following a modified procedure of Barber et al.³

2,3,3-Trimethyl-1-(pent-4-yn)-3H-indolium **11** (CAS 1354932-44-7)⁴

To a 5 mL round bottom flask were added 2,3,3-trimethylindolenine **10** (372mg, 2.34 mmol, 1 equiv.) and 5-iodopent-1-yne (500 mg, 2.58 mmol, 1.1 equiv.). The reaction mixture was stirred at 110 °C for 4 h, cooled to room temperature and diluted with CH₂Cl₂ (3 mL) and MeOH (0.5 mL). Precipitation in Et₂O (75 mL), filtration and washing with Et₂O afforded compound **11** (440 mg, 1.76 mmol, 84%) as a purple solid.

2-[2-(Acetylphenylamino)ethenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium **12**

For a detailed synthesis of 2-[2-(Acetylphenylamino)ethenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium **12** in three steps starting from 2,3,3-trimethylindolenine **10**, see our previous report.²

2-[3-[1,3-Dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-1-propen-1-yl]-3,3-dimethyl-1-(4-azidobutyl)-3H-Indolium **2** (Cy3 alkyne)

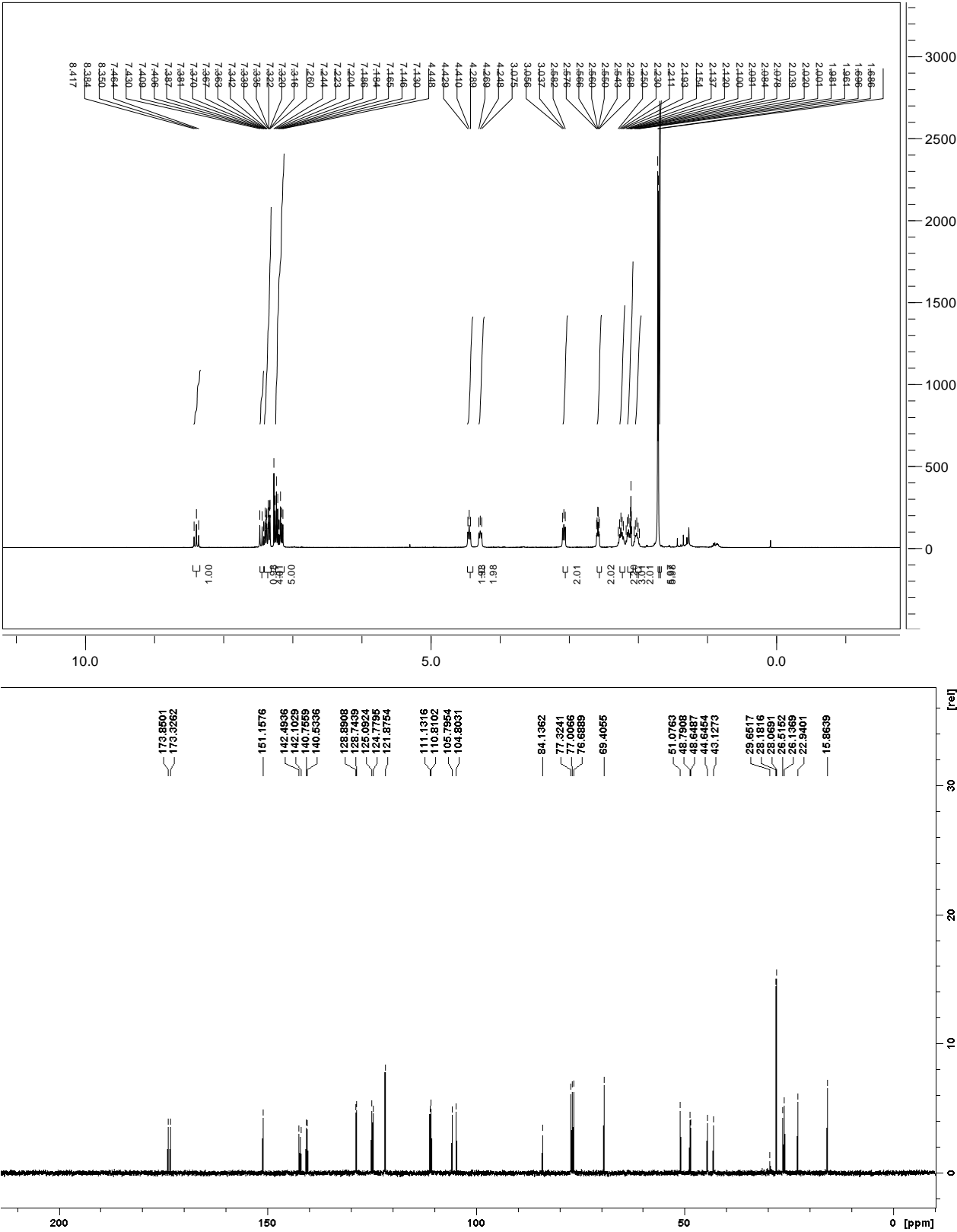
To solution of hemicyanine **12** (454 mg, 1.03 mmol, 1 equiv.) and compound **11** (225 mg, 1.03 mmol, 1 equiv.) in EtOH (20 mL) was added triethylamine (420 μL, 3.09 mmol, 3 equiv.). The reaction mixture was heated to 80 °C for 30 min, cooled to room temperature and evaporated to dryness. The residue was partitioned between water (50 mL)

³ Barber, D.M.; Sangane, H.J.; Dixon D.J. *Org. Lett.* **2012**, *14*, 5290–5293

⁴ Gerowska, M.; Hall, L.; Richardson, J.; Shelbourne, M.; Brown, T. *Tetrahedron* **2012**, *68*, 857–864

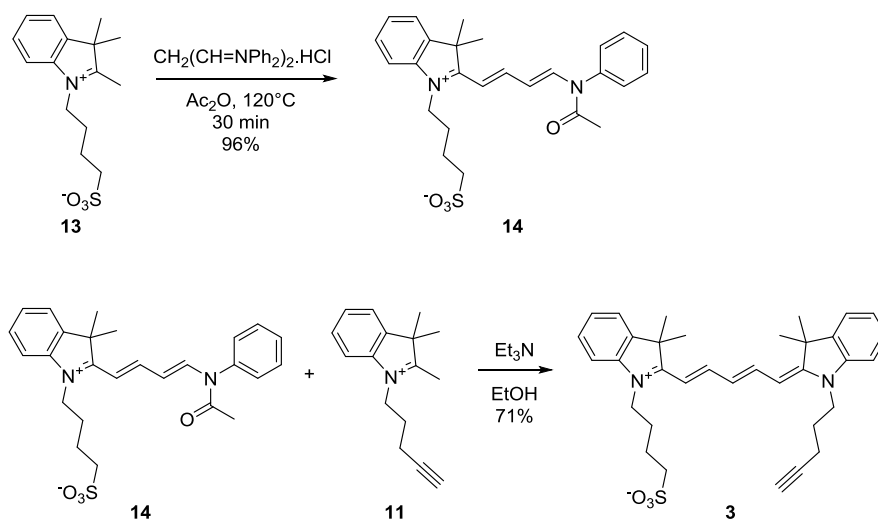
and CH₂Cl₂ (50 mL). The organic phase was washed twice with water (2x 25 mL), dried over Na₂SO₄ and evaporated to dryness. The crude residue was purified by silica gel column chromatography (gradient up to 5% MeOH in CH₂Cl₂) to afford Cy3 alkyne **2** (263 mg, 0.50 mmol, 48% yield) as a dark purple solid. ¹H-NMR (400 MHz, CDCl₃) δ=8.38 (t, J = 13.4 Hz, 1H), 7.45 (d, J = 13.5 Hz, 1H), 7.43-7.32 (m, 4H), 7.24-7.13 (m, 5H), 4.43 (t, J = 7.4 Hz, 2H), 4.27(t, J = 8.0 Hz, 2H), 3.06 (t, J = 7.7 Hz, 2H), 2.56 (td, J = 6.5, 2.5 Hz, 2H), 2.27-2.19 (m, 2H), 2.15-2.08 (m, 3H), 2.04-1.96 (m, 2H), 1.70 (s, 6H), 1.69 (s, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ=173.85, 173.33, 151.16, 142.49, 142.10, 140.76, 140.53, 128.89, 128.74, 125.09, 124.78, 121.88, 111.13, 110.81, 105.80, 104.80, 84.14, 69.41, 51.08, 48.79, 48.65, 44.65, 43.13, 29.65, 28.18, 28.07, 26.52, 26.14, 22.94, 15.86. ESI-HRMS calculated for C₃₂ H₃₉ N₂ O₃ S positive mode ([M]⁺) 531.2671. Calc.: 531.2676.

NMR Spectras of Cy3 alkyne 2



7.2 Cy5 alkyne

Synthetic procedures



2-[4-(acetylphenylamino)-1,3-butadien-1-yl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-Indolium **14** (CAS 120725-11-3)

Compound **13** was prepared following the procedure of Kvach et al.⁵

To a stirred solution of compound **13** (1.588 g, 5.38 mmol, 1 equiv.) in acetic anhydride (20 mL) was added malonaldehyde bis(phenylimine) hydrochloride (1.805 g, 7.0 mmol, 1.3 equiv.). After 30 min stirring at 120°C , the reaction mixture was evaporated to dryness and dissolved in dichloromethane (2 mL). Ether (50 mL) was added and the resulting gum was filtered. The gum was dissolved in dichloromethane (2 mL) and precipitated by addition of ether (100 mL) affording hemicyanine **14** (675 mg, 1.52 mmol, 86% yield) as a light brown solid.

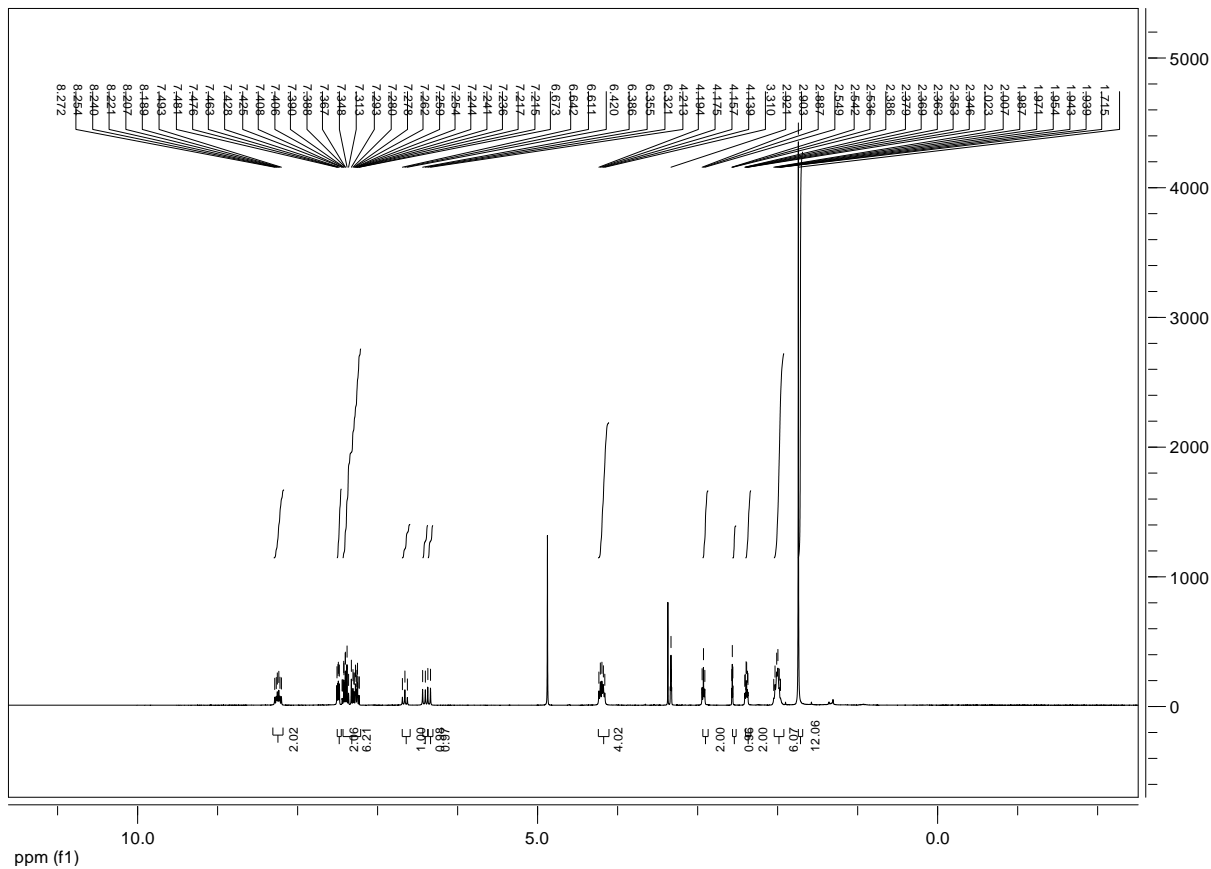
2-[5-[1,3-dihydro-1-(4-sulfobutyl)-3,3-dimethyl-2H-indol-2-ylidene]-1,3-pentadien-1-yl]-3,3-dimethyl-1-(4-pentenyl)-3H-Indolium **3** (Cy5 alkyne)

To solution of hemicyanine **14** (1 g, 2.14 mmol, 1 equiv.) and **11** (549 mg, 2.14 mmol, 1 equiv.) in EtOH (20 mL) was added triethylamine (864 μL , 6.42 mmol, 3 equiv.). The reaction mixture was heated to 80°C for 30 min, cooled to room temperature and evaporated to dryness. The residue was partitioned between water (200 mL) and CH_2Cl_2 (200 mL). The organic phase was washed twice with water (2x 50 mL), dried over Na_2SO_4 and evaporated to dryness. The crude residue was purified by silica gel column chromatography (gradient up to 8% MeOH in CH_2Cl_2) to afford Cy5 alkyne **3** (850 mg, 1.53 mmol, 71% yield) as a dark blue solid. $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ = 8.21 (dt, J =

⁵ Kvach, M.V.; Gontarev, S.V.; Prokhorenko, I.A.; Stepanova, I.A.; Shmanai, V.V.; Korshun, V.A. *Russ. Chem. Bull.* **2006**, 55, 159-163.

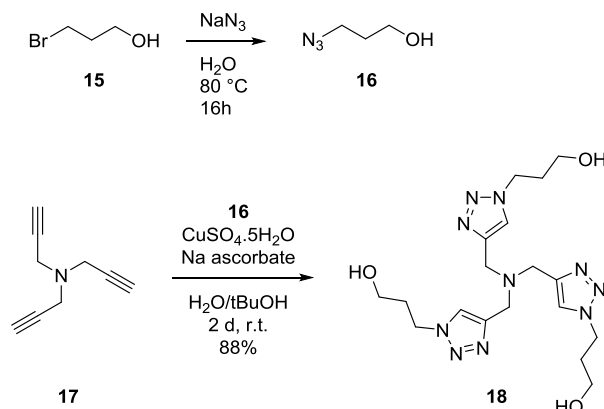
13.0, 7.5 Hz, 2H), 7.48 (dd, J = 7.0, 5.0 Hz, 2H), 7.43-7.22 (m, 6H), 6.64 (t, J = 12.4 Hz, 1H), 6.41 (d, J = 13.8 Hz, 1H), 6.34 (d, J = 13.6 Hz, 1H), 4.21-4.14 (m, 4H), 2.90 (t, J = 6.8 Hz, 2H), 2.54 (t, J = 2.6 Hz, 1H), 2.37 (dt, J = 6.6, 2.6 Hz, 2H), 1.98 (br, 6H), 1.71 (s, 12H). ¹³C-NMR (100 MHz, CDCl₃) δ=175.27, 174.16, 155.93, 155.16, 143.60, 143.42, 142.77, 142.48, 129.83, 129.68, 127.05, 126.44, 126.00, 123.39, 123.36, 112.34, 111.66, 105.10, 104.02, 83.79, 71.48, 51.68, 50.69, 50.38, 44.91, 43.60, 28.01, 27.89, 27.21, 23.50, 16.54. ESI-HRMS calculated for C₃₄H₄₁N₂O₃S positive mode ([M]⁺) 557.2836. Calc.: 557.2832.

NMR Spectras of Cy5 alkyne 3



7.4 THPTA

Synthetic procedures



3-azido-1-propanol **16** (CAS 72320-38-8)

3-azido-1-propanol was prepared following the procedure of Chen et al.⁶

THPTA **18** (CAS 760952-88-3)

THPTA was prepared using a modified synthetic procedure of Peacock et al.⁷

To solution of tripropargylamine **17** (393 mg, 3 mmol, 1 equiv.) and 3-azido-1-propanol **16** (1.33 g, 12 mmol, 4 equiv.) in a 1:1 $\text{H}_2\text{O}/\text{tBuOH}$ mixture (6 mL) was added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (75 mg, 0.3 mmol, 0.1 equiv.) and sodium ascorbate (119 mg, 0.6 mmol, 0.2 equiv.). The reaction mixture was stirred at room temperature for 2 days, evaporated to dryness and purified by silica gel column chromatography (gradient up to 30% MeOH in CH_2Cl_2 , detection at 210 nm) to afford THPTA **18** (1.15 g, 2.64 mmol, 88% yield) as a yellow solid.

⁶ Chen, P.; Li, C.; Liu, D.; Li, Z. *Macromolecules* **2012**, *45*, 9579–9584

⁷ Peacock, H.; Maydanovych, O.; Beal, P.A. *Org. Lett.* **2010**, *12*, 1044–1047

8. Sequences of ORN1 to ORN14

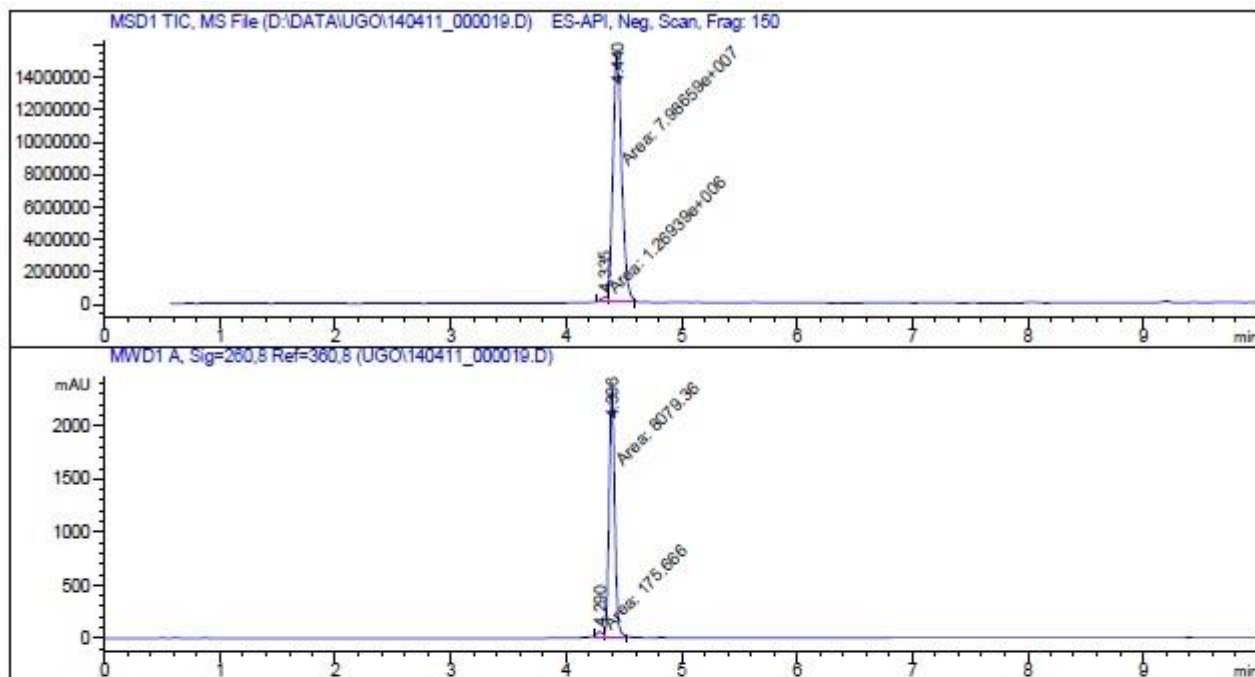
ORN1, pre-miR-21	UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAG UCGAUGGGGCUGU <u>C</u>
ORN2, pre-miR-21	<u>C</u> AGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAG UCGAUGGGGCUGUC
ORN3, pre-miR-21	<u>C</u> AGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAG UCGAUGGGGCUGU <u>C</u>
ORN4, pre-miR-21	UAGCUUAUCAGACUGAUGUUGACUGUUGAAU <u>C</u> UCAUGGCAACACCAG UCGAUGGGGCUGU <u>C</u>
ORN5, pre-miR-21	UAGCUUAUCAGA <u>C</u> UGAUGUUGACUGUUGAAUCUCAUGGCAACAC <u>C</u> AG UCGAUGGGGCUGUC
ORN6, pre-miR-21	UAGCUUAUCAGA <u>C</u> UGAUGUUGACUGUUGAAUCUCAUGGCAACACCAG U <u>C</u> GAUGGGGCUGUC
ORN7, pre-miR-106a	AAAAGUG <u>C</u> UUACAGUGCAGGUAGCUUUUUGAGAUCUACUGCAAUGUA AGCA <u>C</u> UUCUUAC
ORN8, pre-miR-106a	AAAAGUG <u>C</u> UUACAGUGCAGGUAGCUUUUUGAGAU <u>C</u> UACUGCAAUGUA AGCACUUCUUAC
ORN9, pre-miR-124	CGUGUU <u>C</u> ACAGCGGACCUUGAUUUAAAUGUCCAUAACAAUUAAGGCAC G <u>C</u> GGUGAAUGCC
ORN10, pre-miR-124	CGUGUUCACAGCGGACCUUGAUUUAAAUGUC <u>C</u> AUACAAUUAAGGCAC G <u>C</u> GGUGAAUGCC
ORN11, pre-miR-20b	CAAAGUG <u>C</u> UCAUAGUGCAGGUAGUUUUGGCAUGACUCUACUGUAGUA UGGGCA <u>C</u> UUCCAG
ORN12, pre-miR-20b	CAAAGUG <u>C</u> UCAUAGUGCAGGUAGUUUUGG <u>C</u> AUGACUCUACUGUAGUA UGGGCACUUCCAG
ORN13, pre-miR-122	UGGAGUGUGA <u>C</u> AAUGGUGUUUGUGUCUAAACUAUCAACGCCAUUAU CA <u>C</u> ACUAAAUA
ORN14, pre-miR-122	UGGAGUGUGA <u>C</u> AAUGGUGUUUGUGUCUAAA <u>C</u> AUCAACGCCAUUAU CACACUAAAUA

9. LCMS traces of ORN1 to ORN14

ORN1-N₃, pre-miR-21

UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

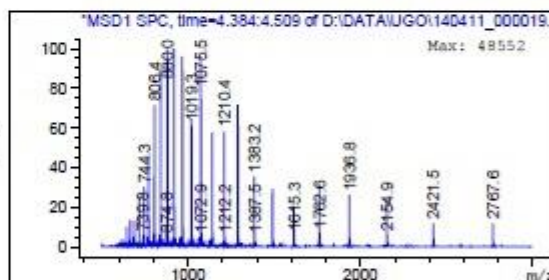
Mass calculated 19381.72, found 19379.63



Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	4.335	MM	0.0750	1.26939e6	2.82258e5	1.5645
2	4.440	MM	0.0839	7.98659e7	1.58586e7	98.4355

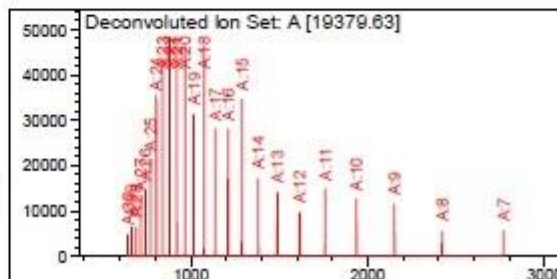
Totals : 8.11353e7 1.61408e7



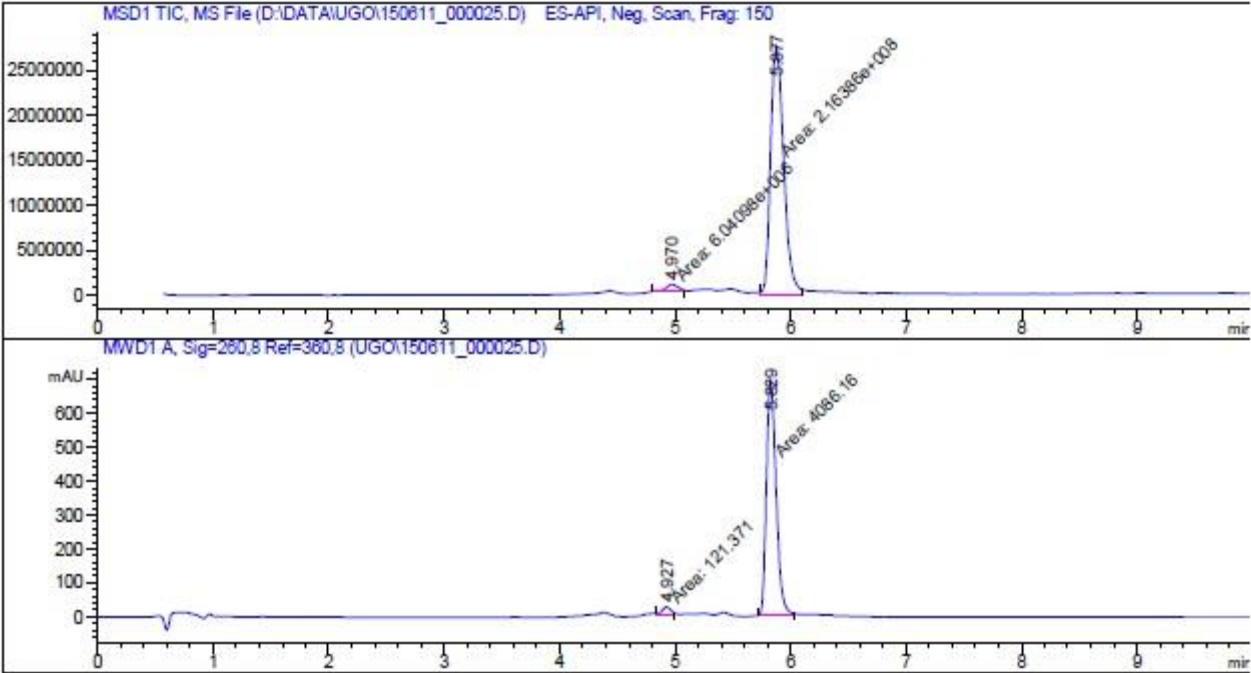
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.290	MM	0.0614	175.66582	47.64628	2.1280
2	4.396	MM	0.0572	8079.36035	2355.74341	97.8720

Totals : 8255.02617 2403.38969

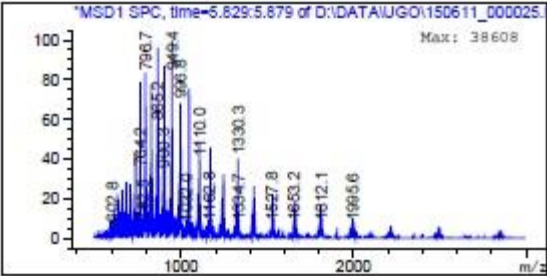


ORN1-Cy5, pre-miR-21
 UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC
 Mass calculated 19938.48, found 19939.31



Signal 1: MSD1 TIC, MS File

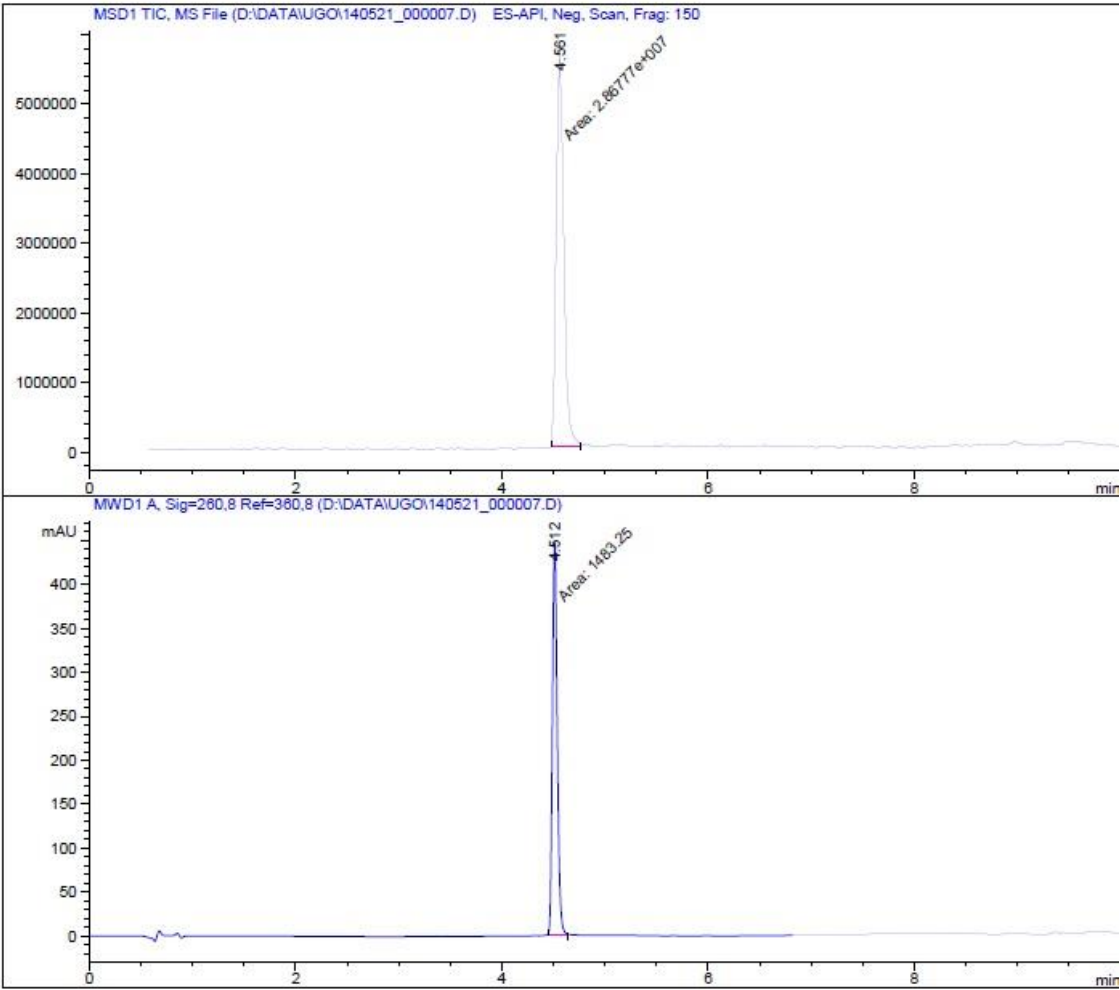
Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	4.970	MM	0.1257	6.04098e6	8.01086e5	2.7159
2	5.877	MM	0.1296	2.16386e8	2.78220e7	97.2841
Totals :				2.22427e8	2.86231e7	



ORN2-N3, pre-miR-21

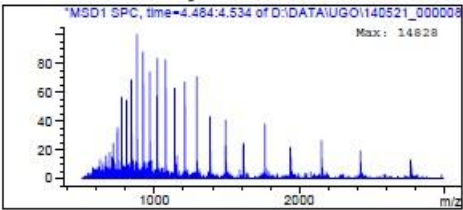
CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

Mass calculated 19380.72, found 19379.85



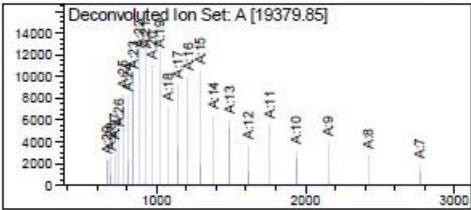
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	4.561	MM	0.0820	2.86777e7	5.83159e6	100.0000
Totals :				2.86777e7	5.83159e6	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.512	MM	0.0547	1483.24731	451.76257	100.0000
Totals :				1483.24731	451.76257	

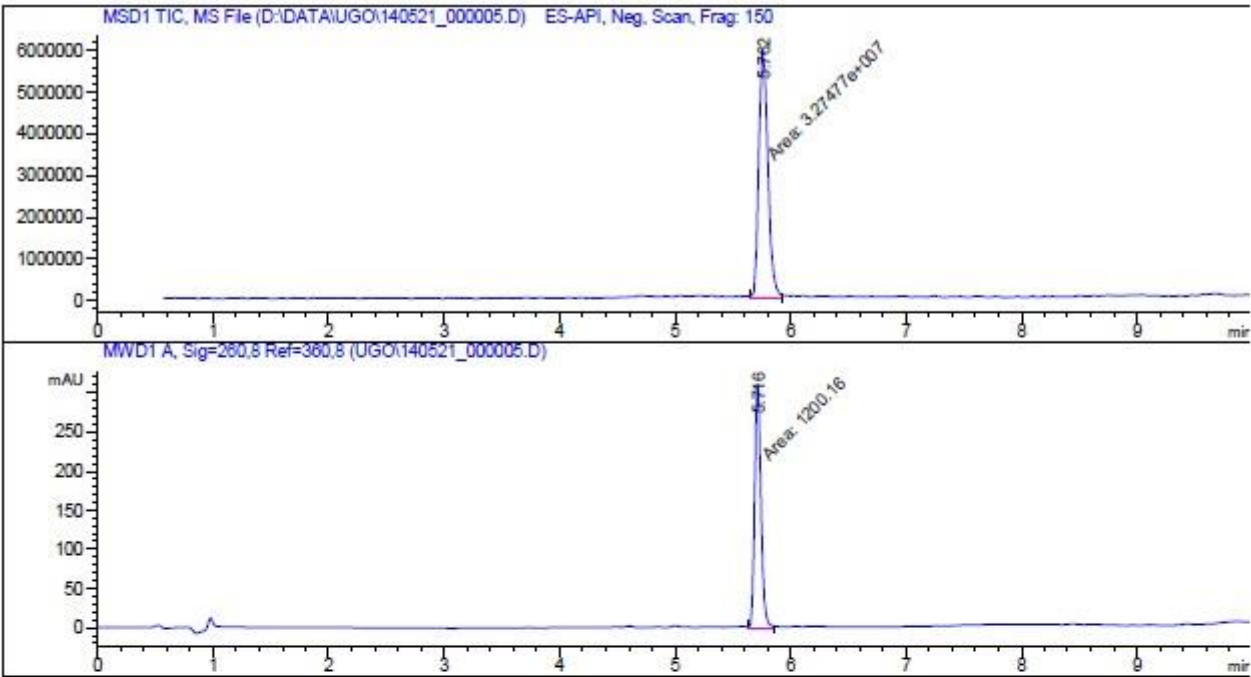


Component	Molecular Weight
A	19379.85

ORN2-Cy3, pre-miR-21

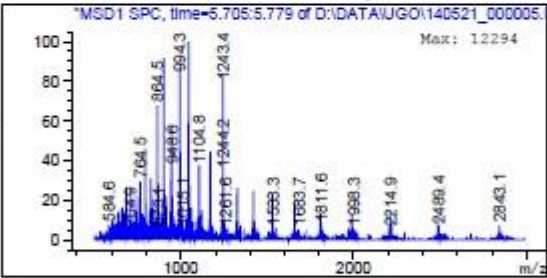
CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

Mass calculated 19911.44, found 19910.22



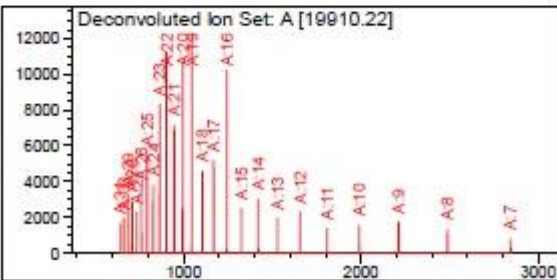
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.762	MM	0.0878	3.27477e7	6.21792e6	100.0000
Totals :				3.27477e7	6.21793e6	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.716	MM	0.0637	1200.15576	314.08551	100.0000
Totals :				1200.15576	314.08551	

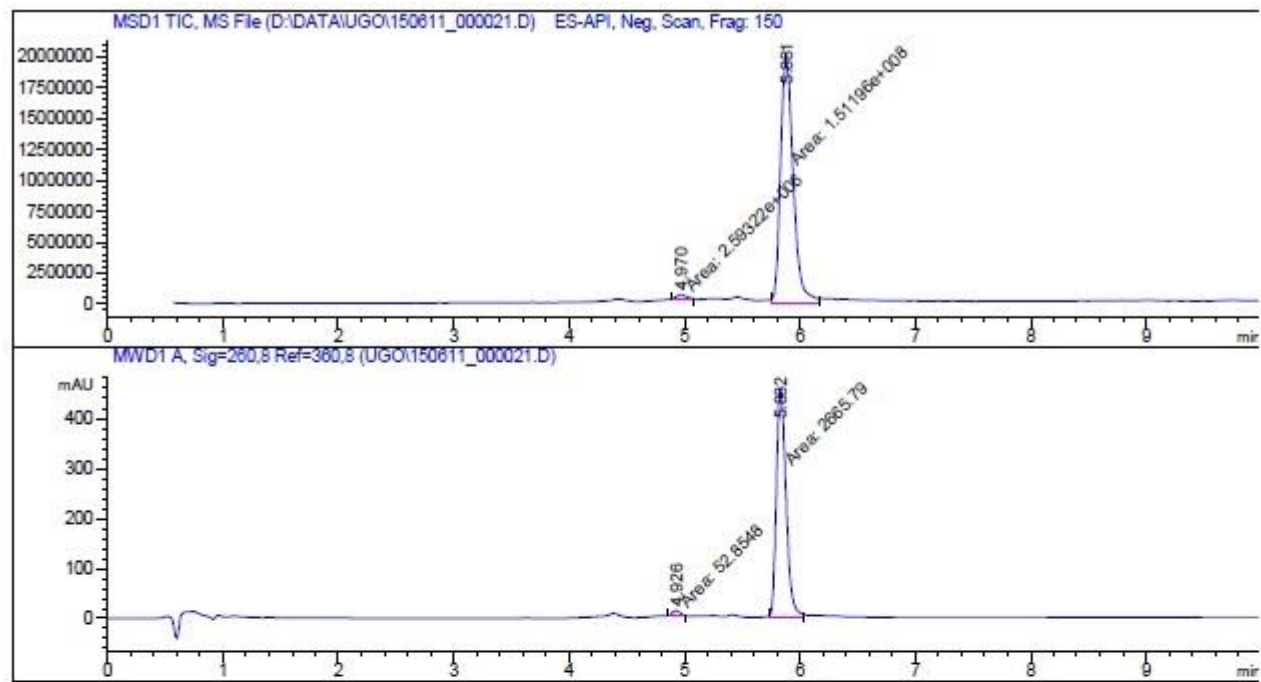


Component	Molecular Weight
A	19910.22

ORN2-Cy5, pre-miR-21

CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

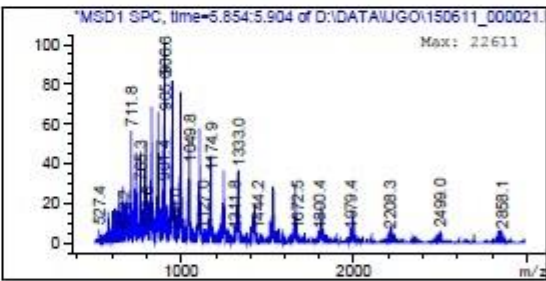
Mass calculated 19937.48, found 19936.63



Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	4.970	MM	0.1088	2.59322e6	3.97135e5	1.6862
2	5.881	MM	0.1240	1.51196e8	2.03205e7	98.3138

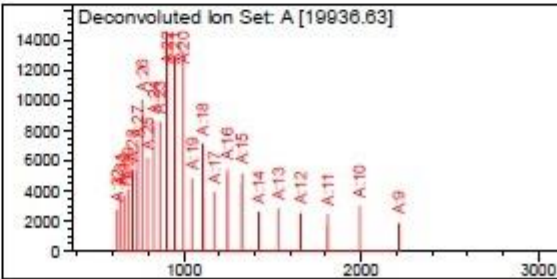
Totals : 1.53789e8 2.07176e7



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.926	MM	0.0832	52.85483	10.59320	1.9442
2	5.832	MM	0.0970	2665.78882	457.91339	98.0558

Totals : 2718.64365 468.50659

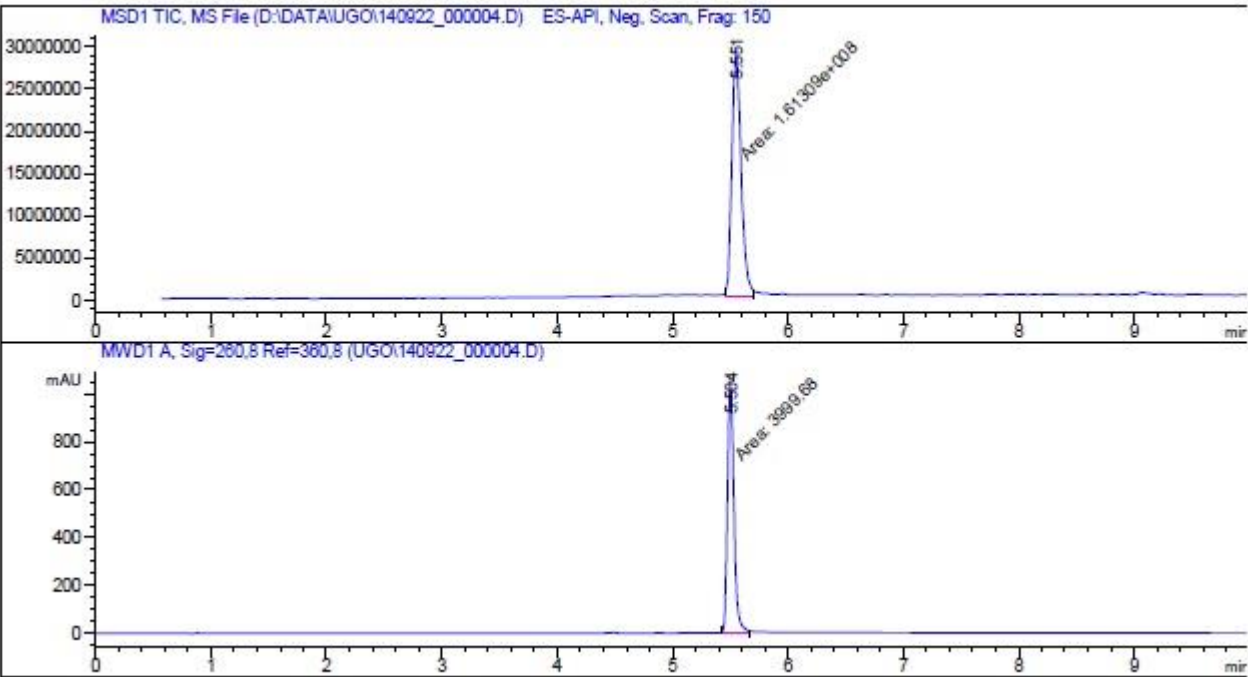


Component Molecular Weight
A 19936.63

ORN3-Cy3/N₃, pre-miR-21

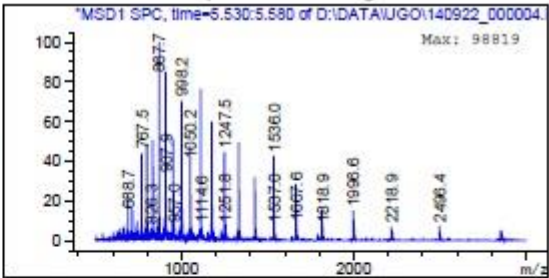
CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

Mass calculated 19980.52, found 19980.11



Signal 1: MSD1 TIC, MS File

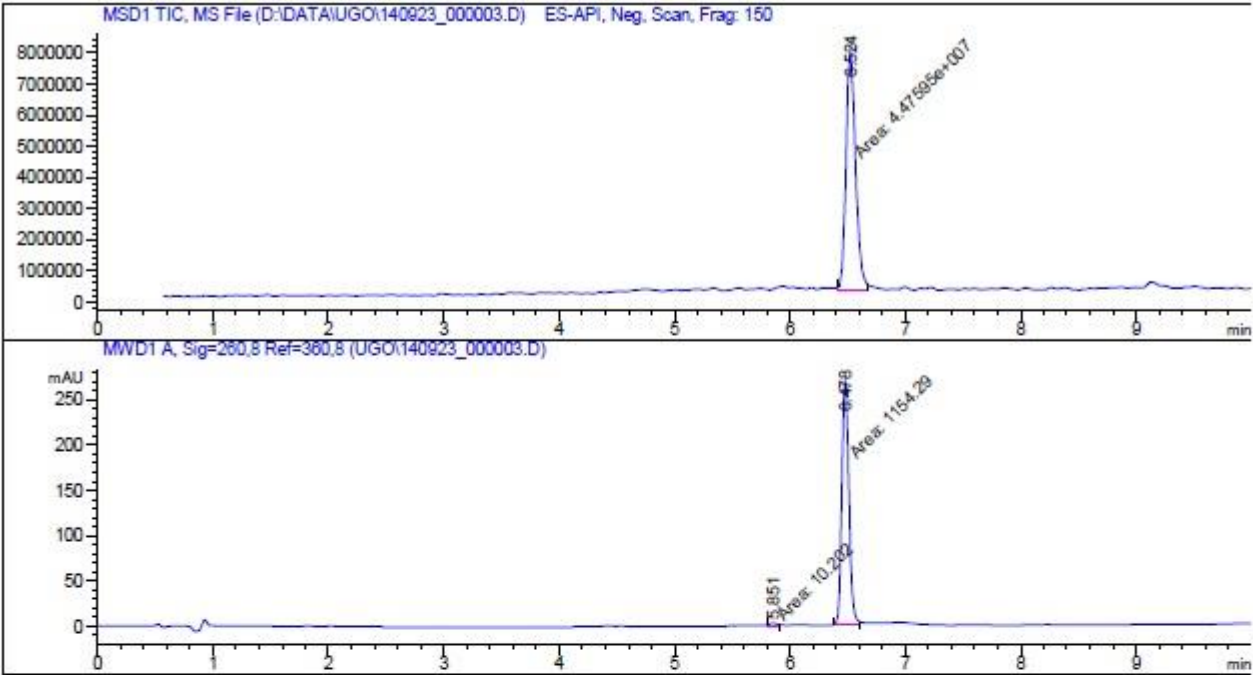
Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.551	MM	0.0900	1.61309e8	2.98775e7	100.0000
Totals :				1.61309e8	2.98775e7	



ORN3-Cy3/Cy5, pre-miR-21

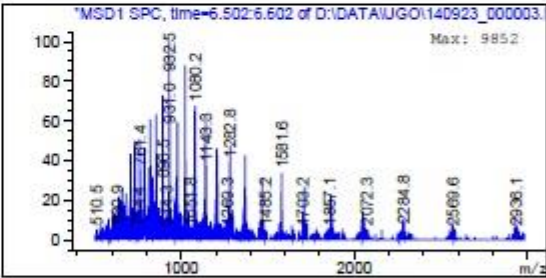
CAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

Mass calculated 20537.28, found 20537.65



Signal 1: MSD1 TIC, MS File

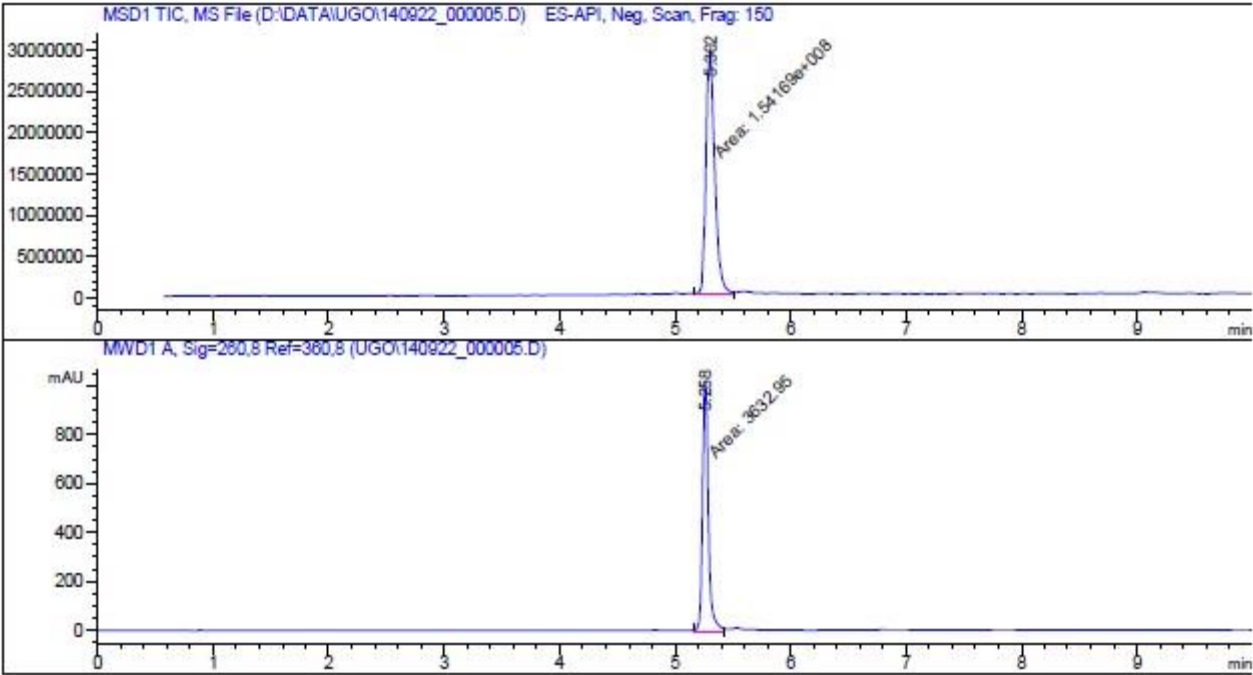
Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	6.524	MM	0.0938	4.47595e7	7.95428e6	100.0000
Totals :				4.47595e7	7.95428e6	



ORN4-Cy3/N₃, pre-miR-21

UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

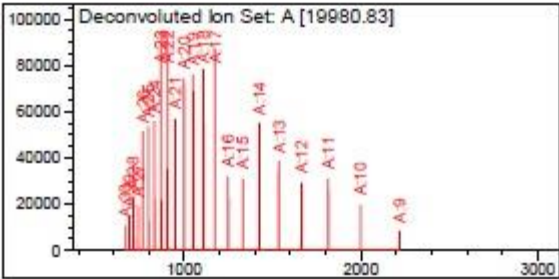
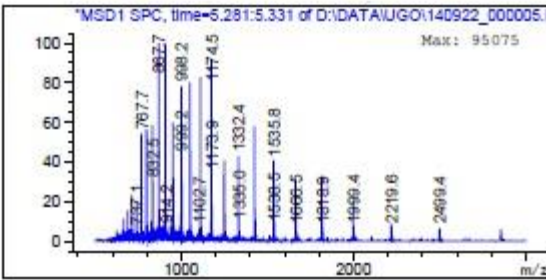
Mass calculated 19981.52, found 19980.83



Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.302	MM	0.0838	1.54169e8	3.06742e7	100.0000
Totals :				1.54169e8	3.06742e7	

Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.258	MM	0.0589	3632.95215	1027.27783	100.0000
Totals :				3632.95215	1027.27783	

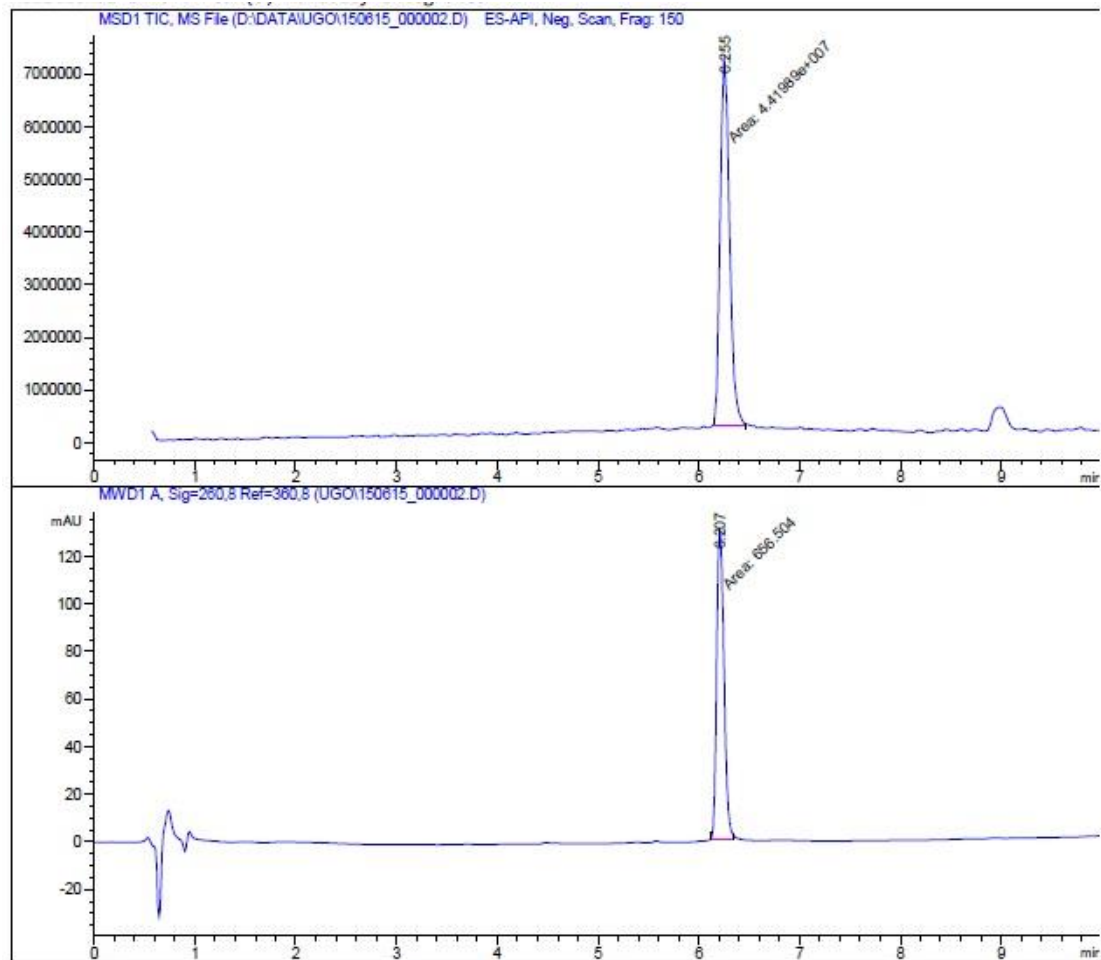


Component	Molecular Weight
A	19980.83

ORN4-Cy3/Cy5, pre-miR-21

UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

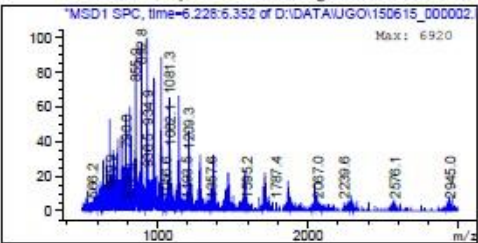
Mass calculated 20538.28, found 20538.48



Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	6.255	MM	0.1034	4.41989e7	7.12255e6	100.0000

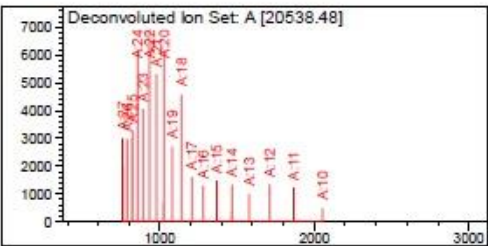
Totals : 4.41989e7 7.12255e6



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.207	MM	0.0842	656.50354	130.00429	100.0000

Totals : 656.50354 130.00429

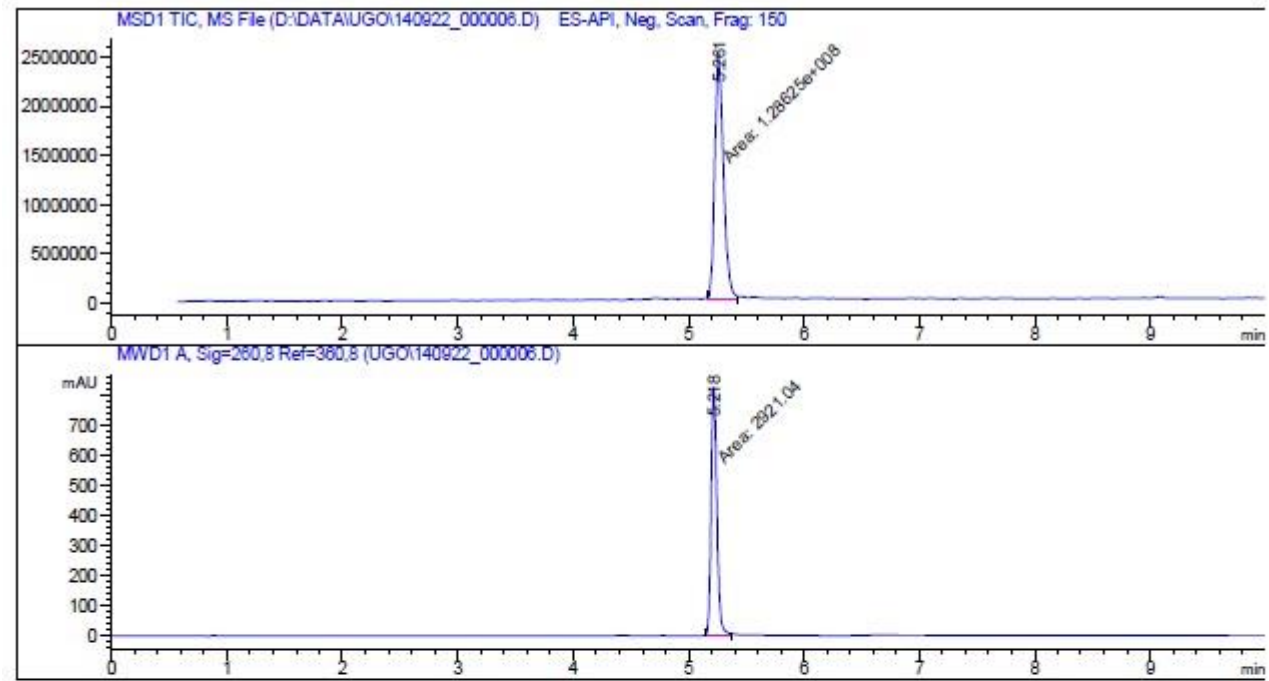


Component	Molecular Weight
A	20538.48

ORN5-Cy3/N3, pre-miR-21

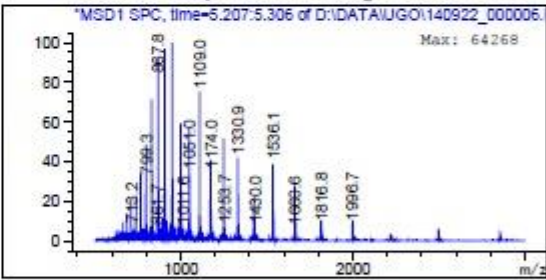
UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC

Mass calculated 19981.52, found 19980.29



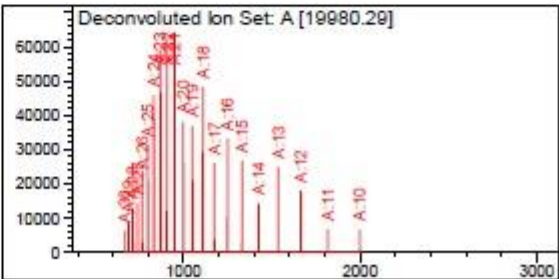
Signal 1: MS1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.261	MM	0.0829	1.28625e8	2.58583e7	100.0000
Totals :				1.28625e8	2.58583e7	



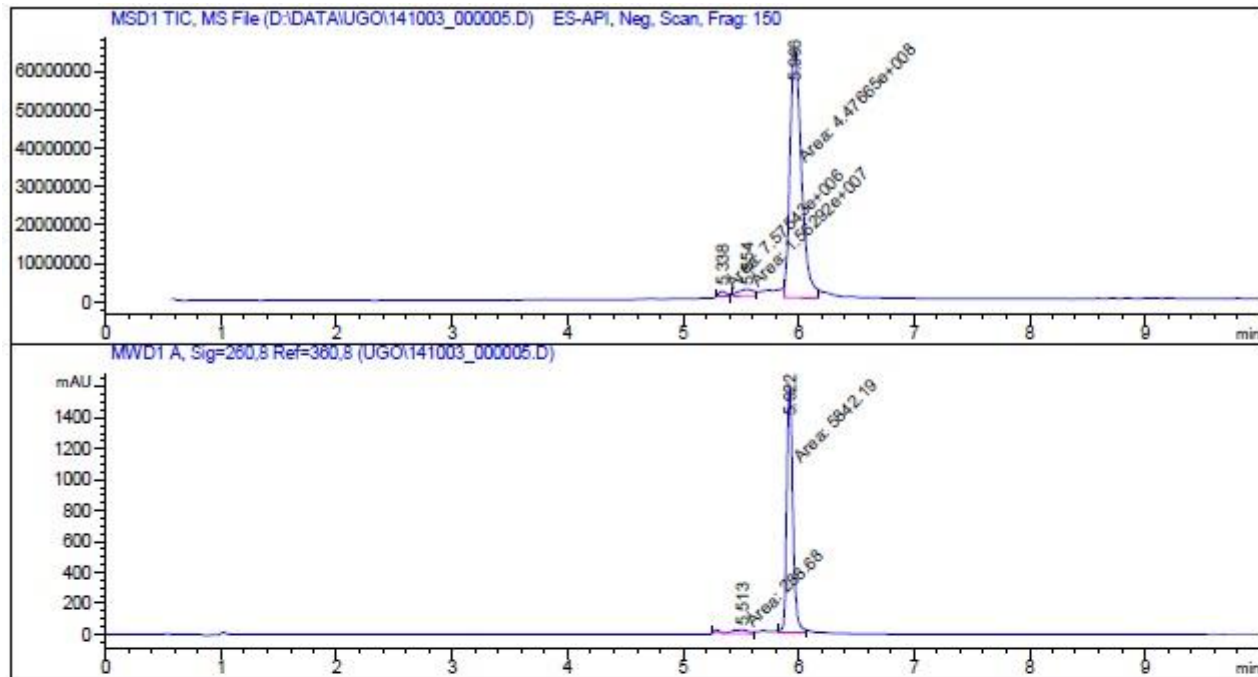
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.218	MM	0.0584	2921.03760	833.48517	100.0000
Totals :				2921.03760	833.48517	

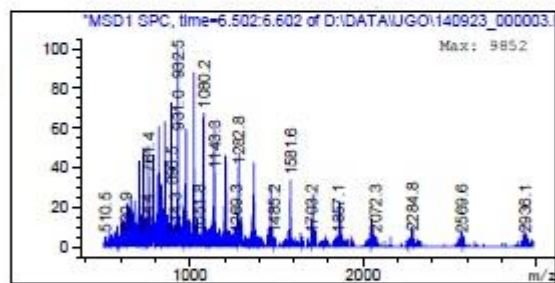


Component	Molecular Weight
A	19980.29

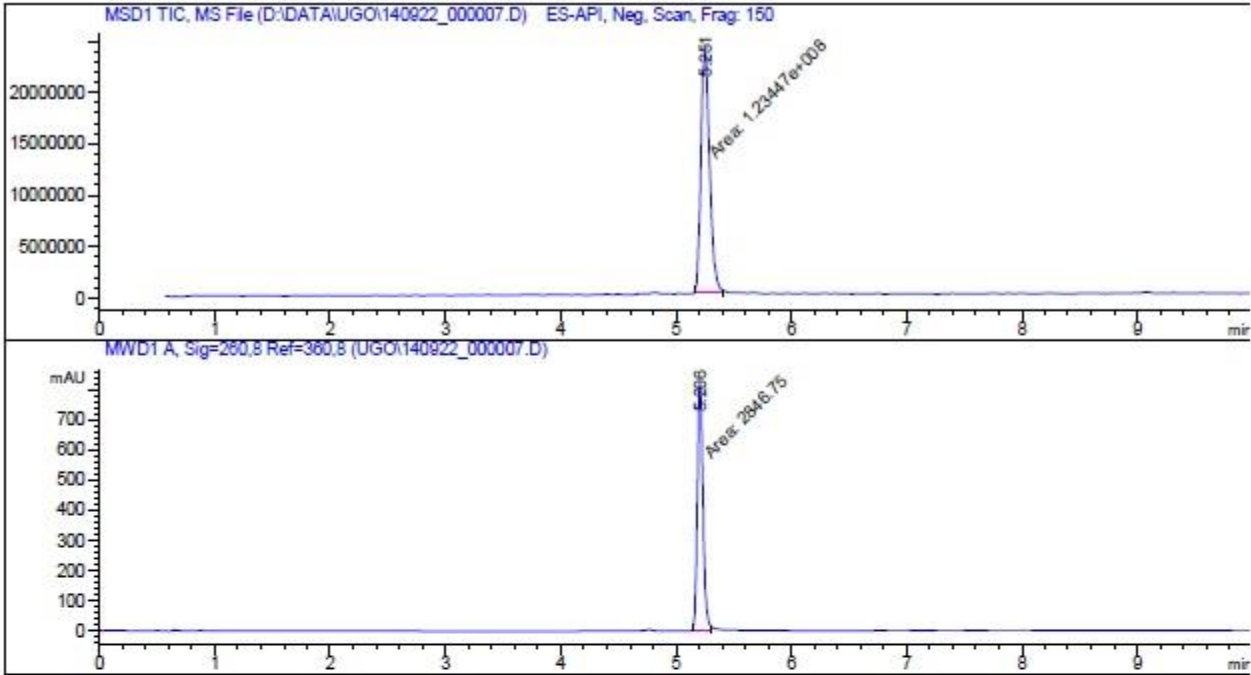
ORN5-Cy3/Cy5, pre-miR-21
UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC
Mass calculated 20538.28, found 20537.65



Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.338	MM	0.0801	7.57543e6	1.57605e6	1.6088
2	5.554	MM	0.1526	1.56292e7	1.70723e6	3.3192
3	5.966	MM	0.1095	4.47665e8	6.81623e7	95.0720
Totals :				4.70869e8	7.14456e7	

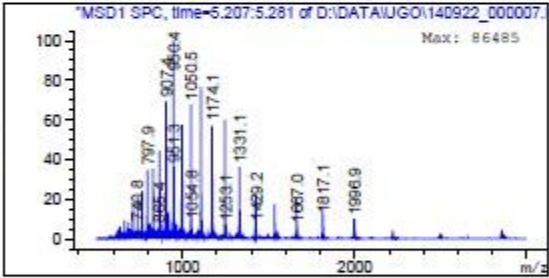


ORN6-Cy3/N3, pre-miR-21
 UAGCUUAUCAGAGCUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC
 Mass calculated 19981.52, found 19979.70



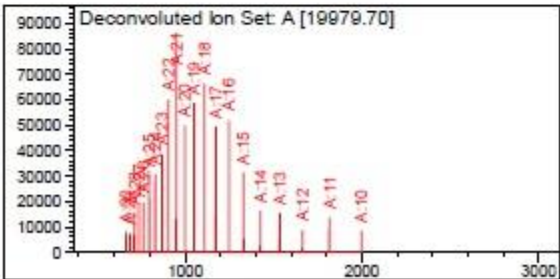
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.251	MM	0.0833	1.23447e8	2.47119e7	100.0000
Totals :				1.23447e8	2.47119e7	



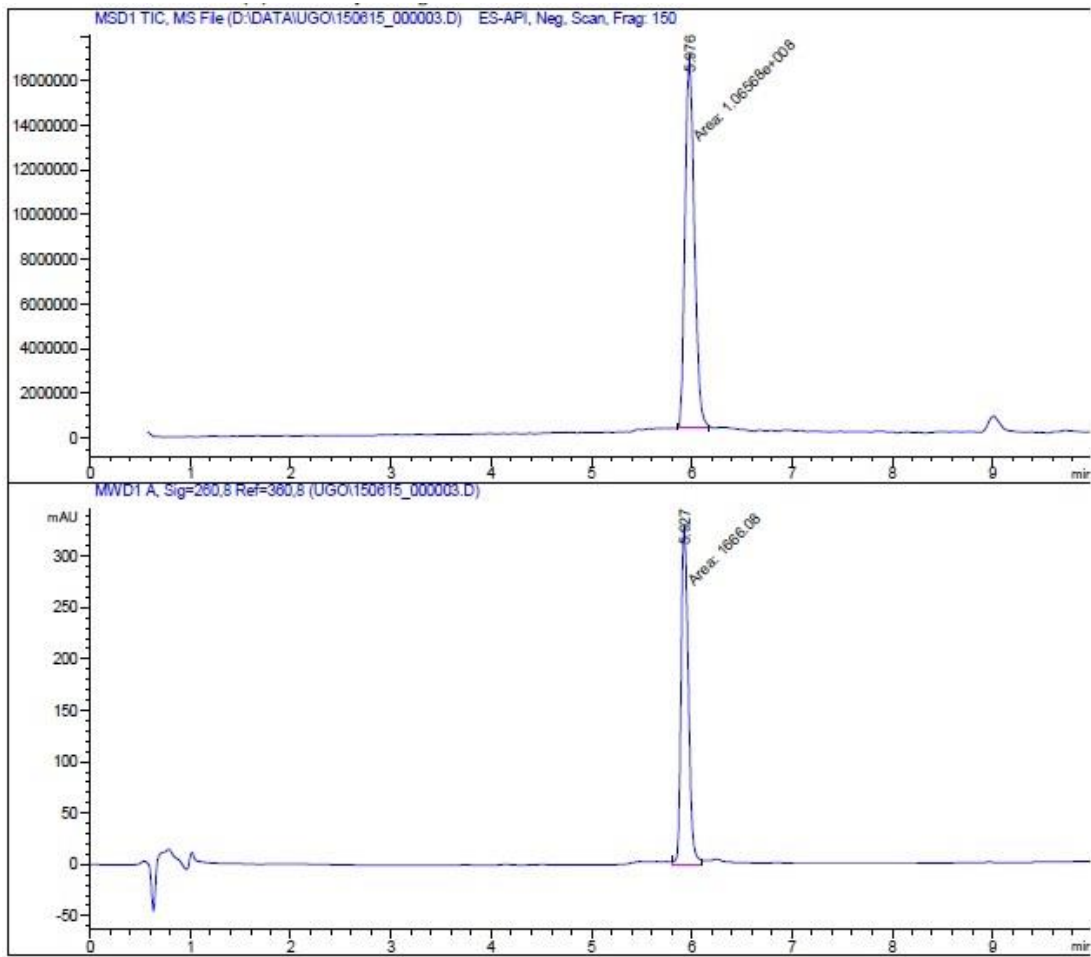
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.206	MM	0.0570	2846.75171	832.68756	100.0000
Totals :				2846.75171	832.68756	



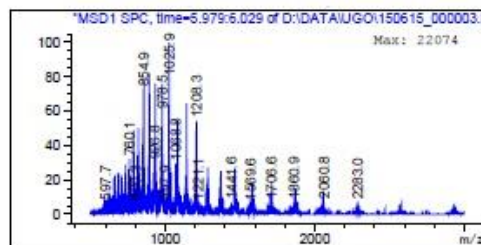
Component	Molecular Weight
A	19979.70

ORN6-Cy3/Cy5, pre-miR-21
UAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUC
Mass calculated 20538.28, found 20536.52



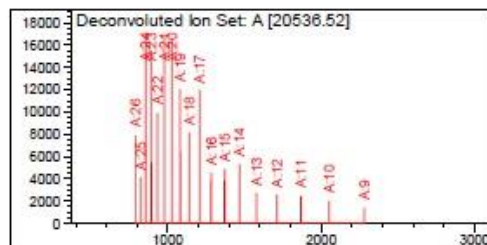
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.976	MM	0.1045	1.06568e8	1.69985e7	100.0000
Totals :				1.06568e8	1.69985e7	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.927	MM	0.0842	1666.08313	329.68893	100.0000
Totals :				1666.08313	329.68893	

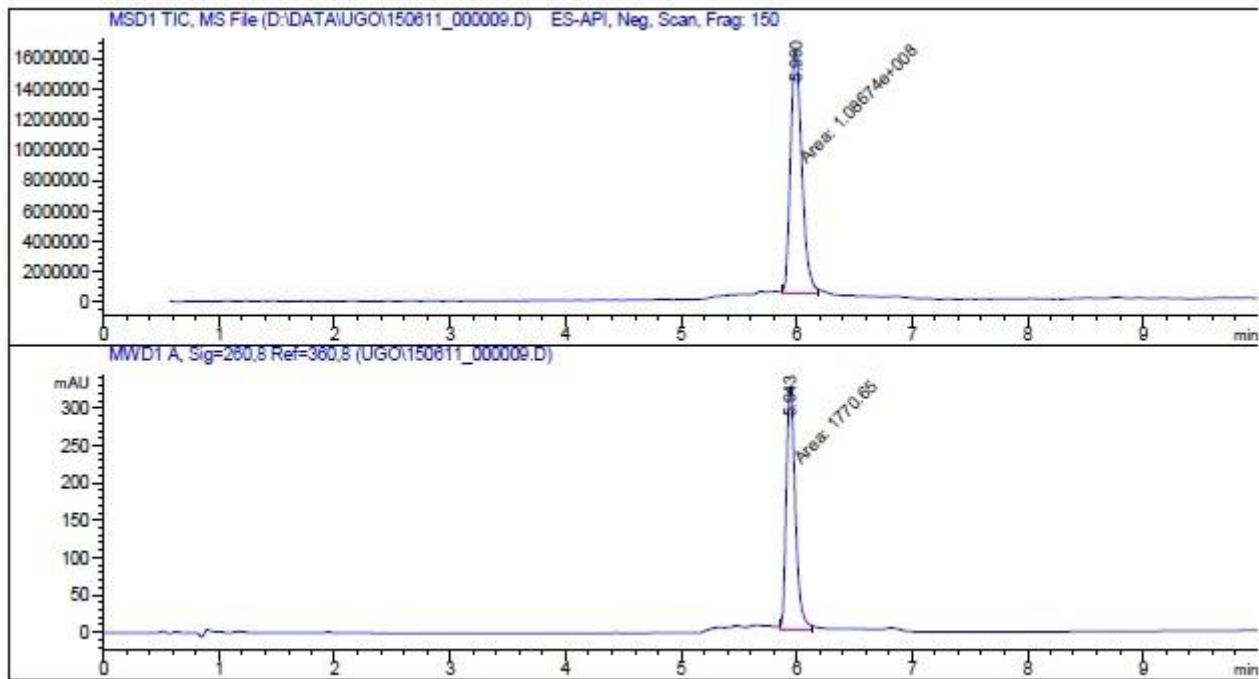


Component	Molecular Weight
A	20536.52

ORN7-Cy3/Cy5, pre-miR-106a

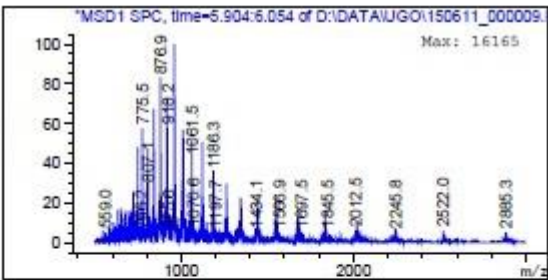
AAAAGUGCUUACAGUGCAGGUAGCUUUUUGAGAUCUACUGCAAUGUAAGCACUUCUUAC

Mass calculated 20186.08, found 20186.35



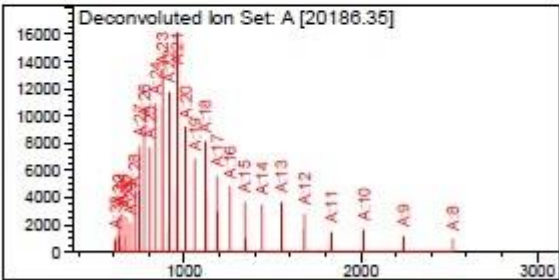
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.990	MM	0.1094	1.08674e8	1.65627e7	100.0000
Totals :				1.08674e8	1.65627e7	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.943	MM	0.0901	1770.64746	327.39304	100.0000
Totals :				1770.64746	327.39304	

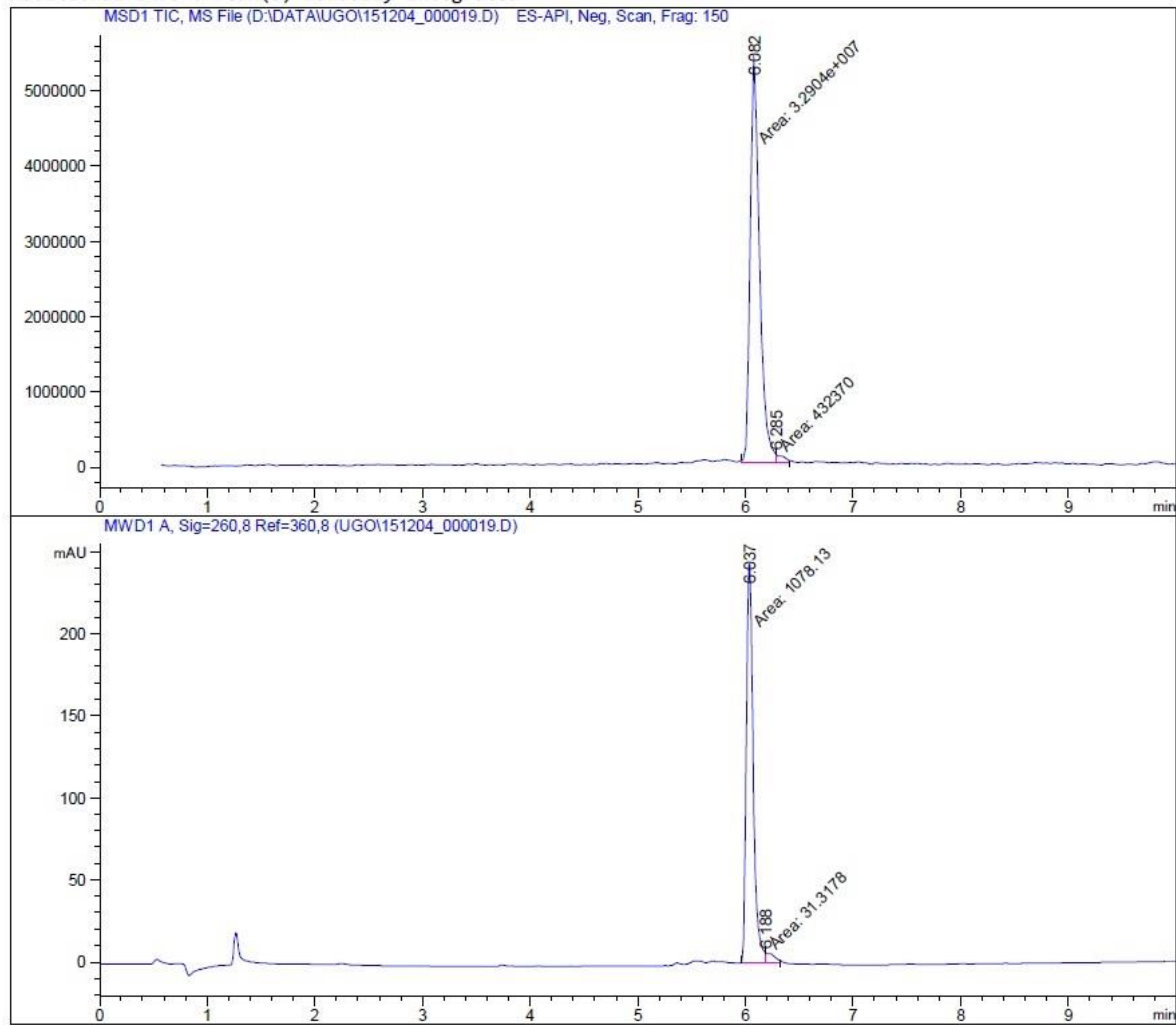


Component	Molecular Weight
A	20186.35

ORN8-Cy3/Cy5, pre-miR-106a

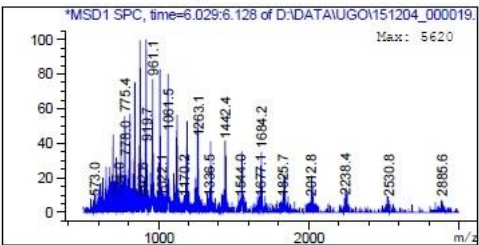
AAAAGUGCUUACAGUGCAGGUAGCUUUUUGAGAUCUACUGCAAUGUAAGCACUUCUUAC

Mass calculated 20186.08, found 20208.43 (+ Na⁺), 20184.60



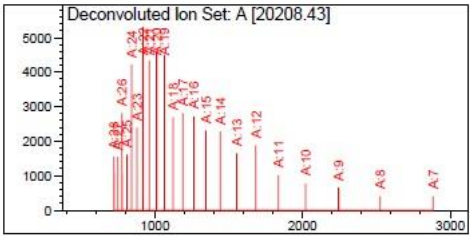
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	6.082	MM	0.0993	3.29040e7	5.52002e6	98.7030
2	6.285	MM	0.0720	4.32370e5	1.02299e5	1.2970
Totals :				3.33364e7	5.62232e6	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.037	MM	0.0734	1078.13025	244.89534	97.1772
2	6.188	MM	0.0678	31.31784	5.97308	2.8228
Totals :				1109.44809	250.86842	

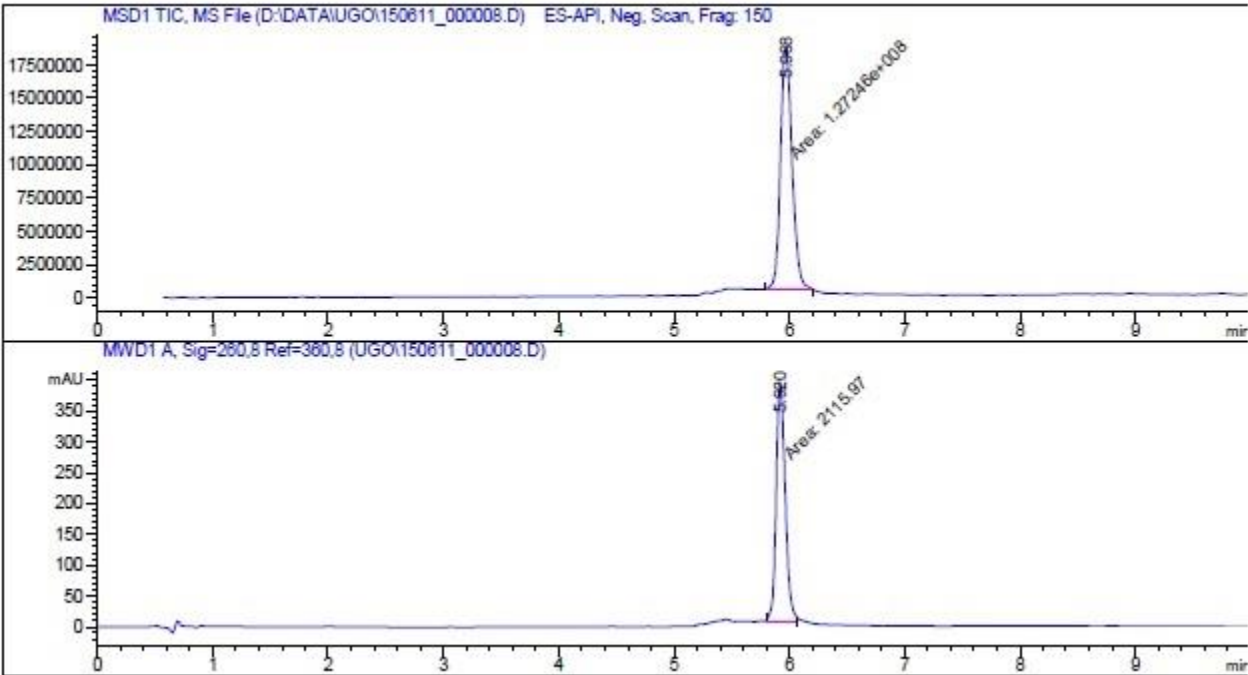


Component	Molecular Weight
A	20208.43
B	20184.60

ORN9-Cy3/Cy5, pre-miR-124

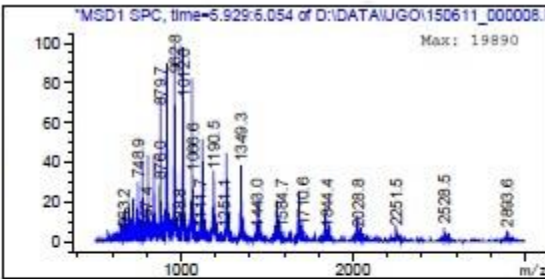
CGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCC

Mass calculated 20238.18, found 20238.71



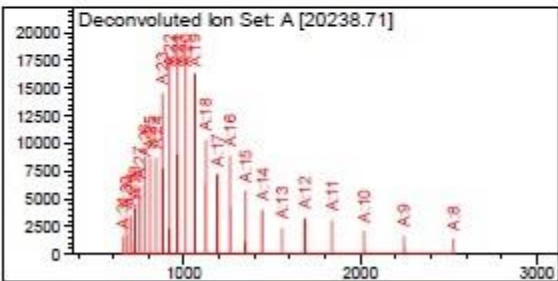
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.968	MM	0.1132	1.27246e8	1.87402e7	100.0000
Totals :				1.27246e8	1.87402e7	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.920	MM	0.0910	2115.97119	387.58792	100.0000
Totals :				2115.97119	387.58792	

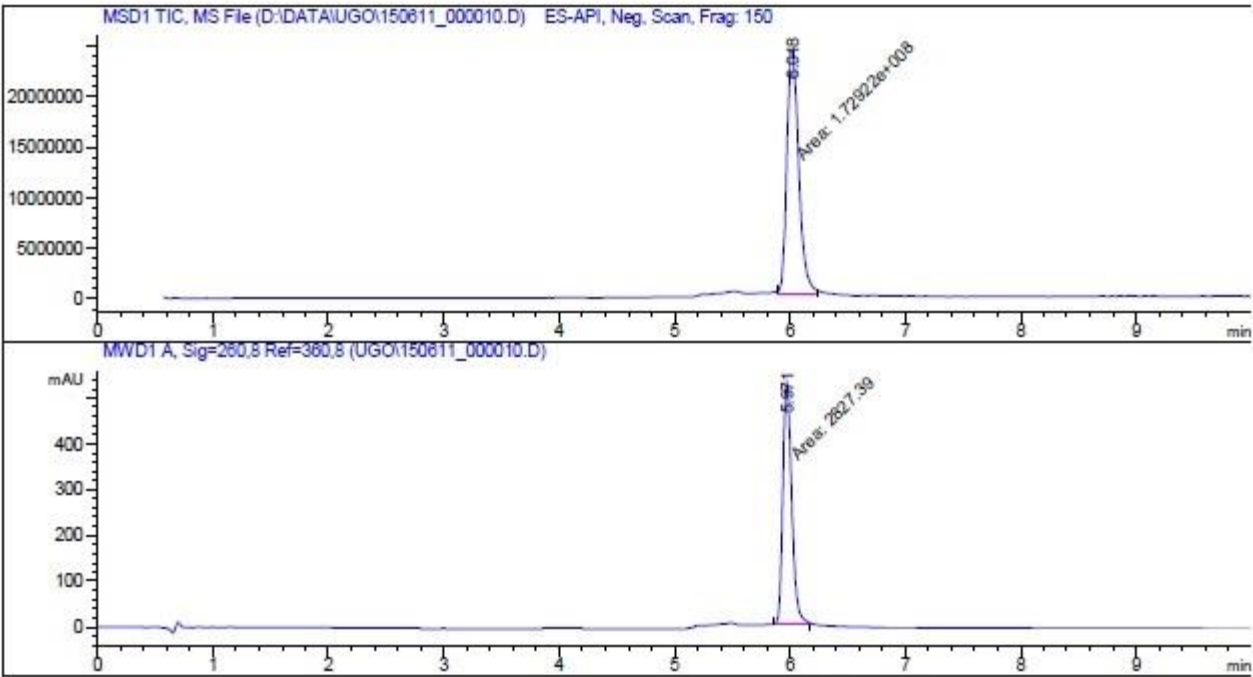


Component	Molecular Weight
A	20238.71

ORN10-Cy3/Cy5, pre-miR-124

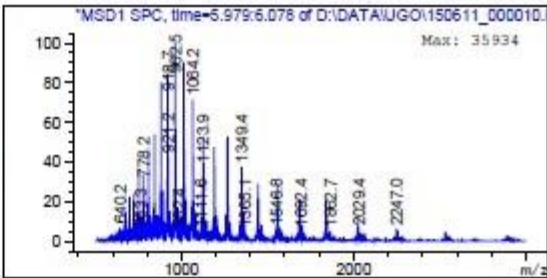
CGUGUUCACAGCGGACCUUGAUUUAAAUGUCCAUACAAUUAAGGCACGCGGUGAAUGCC

Mass calculated 20238.18, found 20237.22



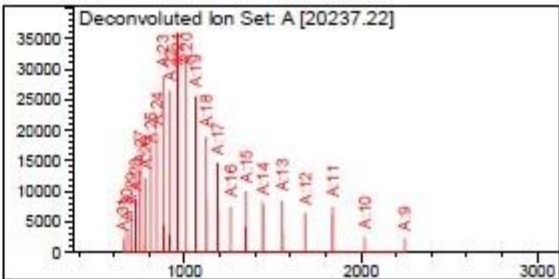
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	6.018	MM	0.1157	1.72922e8	2.49136e7	100.0000
Totals :				1.72922e8	2.49136e7	



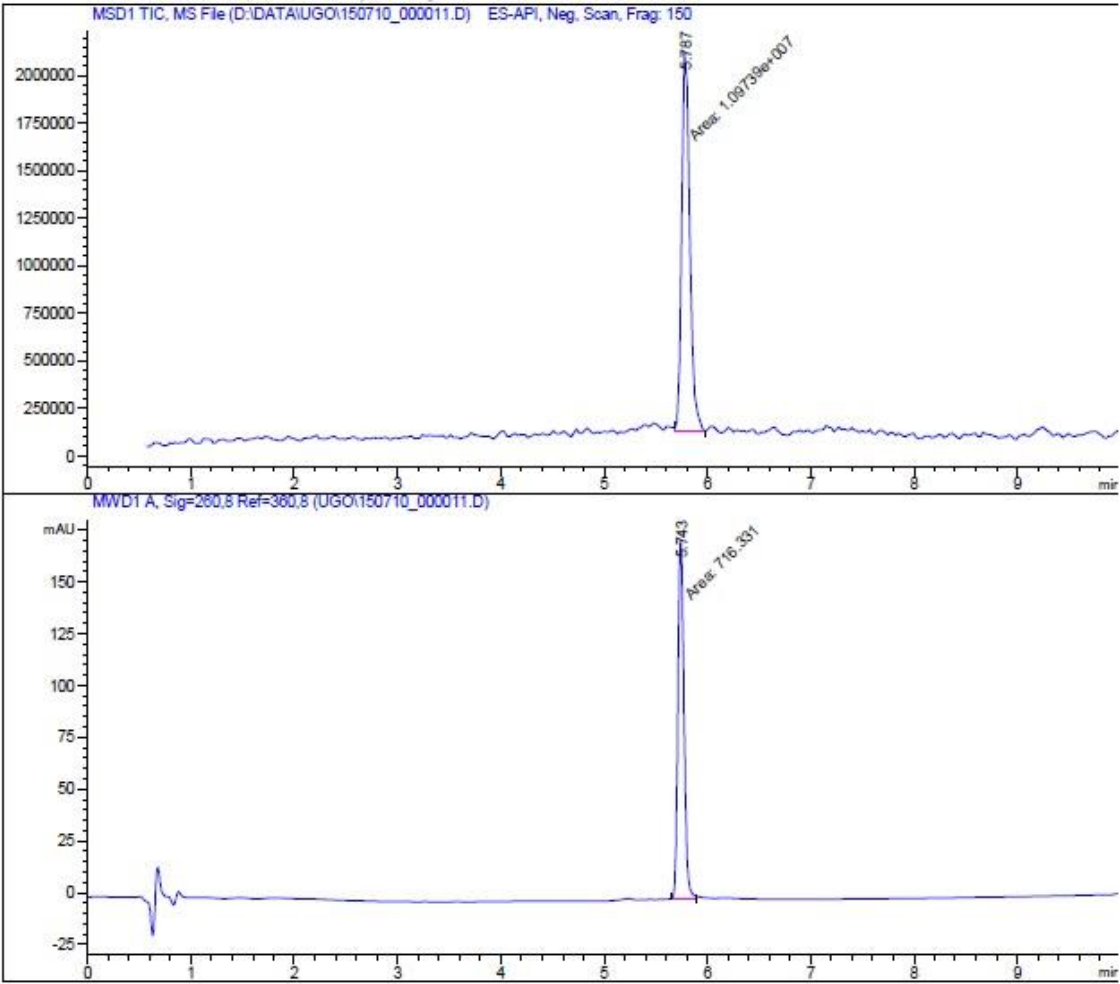
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.971	MM	0.0893	2827.38794	527.46393	100.0000
Totals :				2827.38794	527.46393	



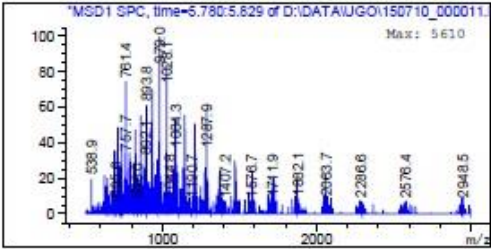
Component	Molecular Weight
A	20237.22

ORN11-Cy3/Cy5, pre-miR-20b
 CAAAGUGCUCAUAGUGCAGGUAGUUUUGGCAUGACUCUACUGUAGUAUGGGCACUCCAG
 Mass calculated 20578.38, found 20576.07



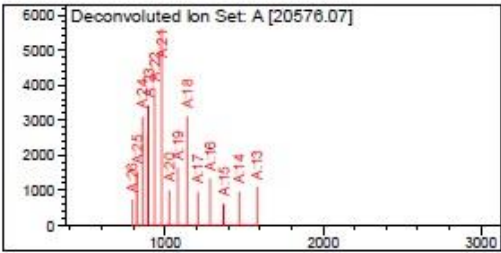
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.787	MM	0.0881	1.09739e7	2.07550e6	100.0000
Totals :				1.09739e7	2.07550e6	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.743	MM	0.0685	716.33148	174.23294	100.0000
Totals :				716.33148	174.23294	

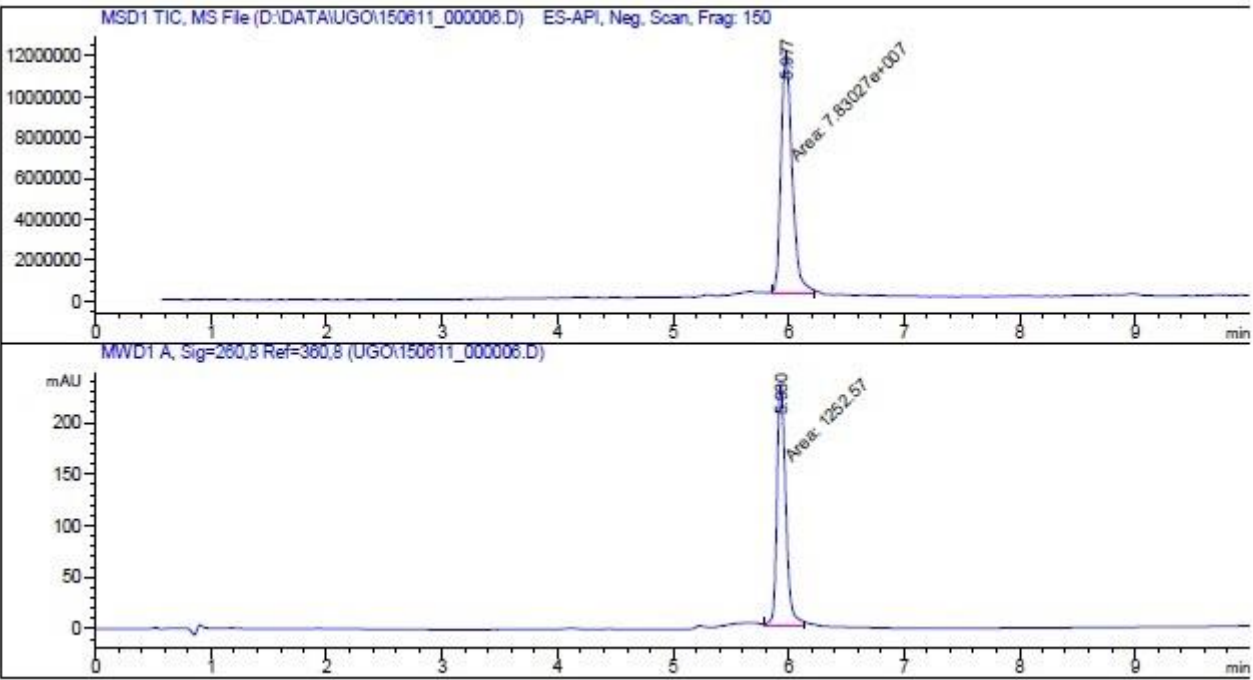


Component	Molecular Weight
A	20576.07

ORN12-Cy3/Cy5, pre-miR-20b

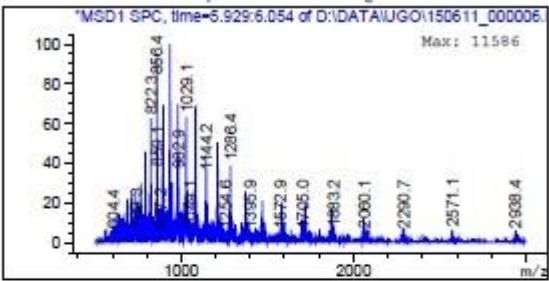
CAAAGUGCUCAUAGUGCAGGUAGUUUUGGCAUGACUCUACUGUAGUAUGGGGCACUCCAG

Mass calculated 20578.38, found 20575.76



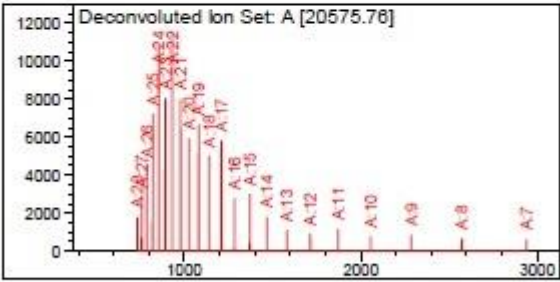
Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.977	MM	0.1082	7.83027e7	1.20657e7	100.0000
Totals :				7.83027e7	1.20657e7	



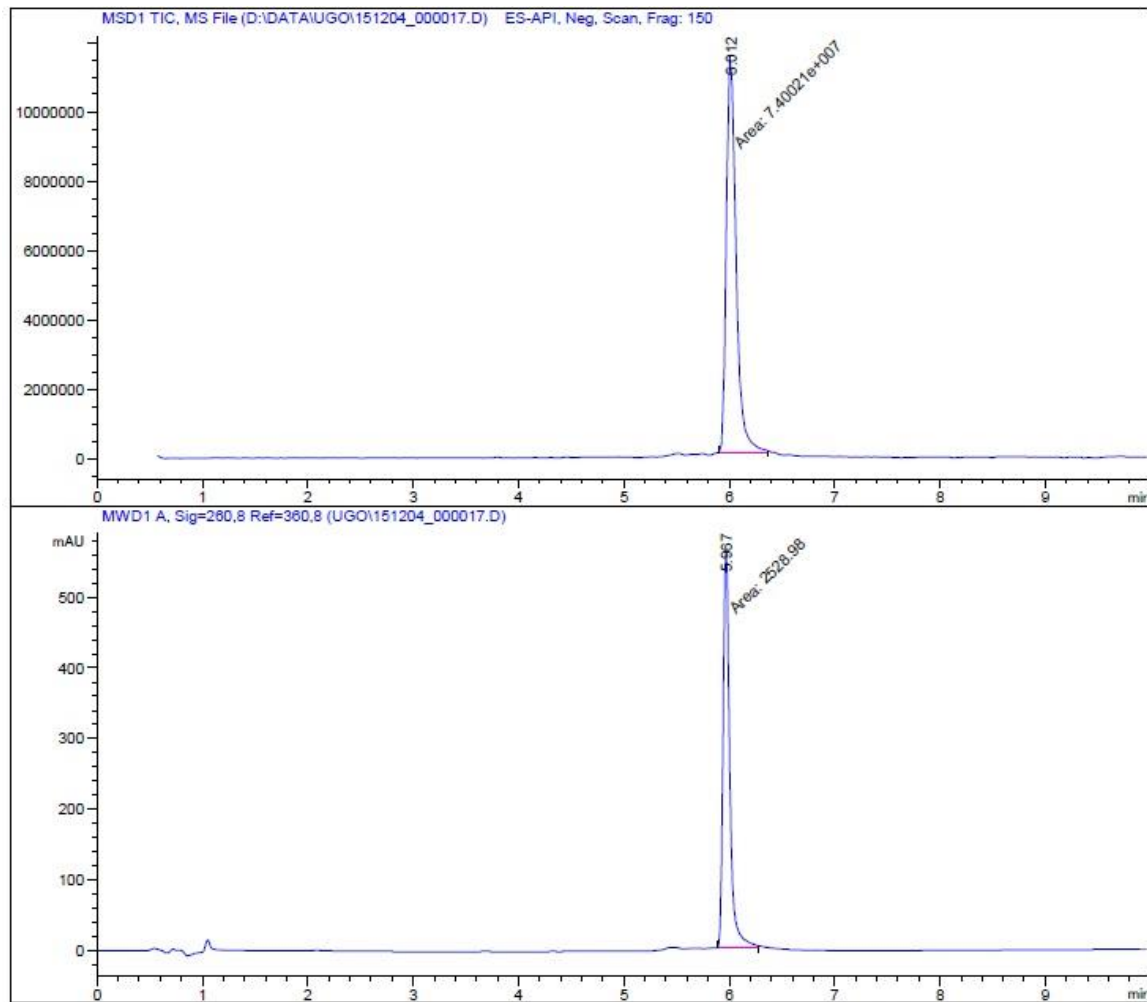
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.930	MM	0.0890	1252.56982	234.68275	100.0000
Totals :				1252.56982	234.68275	



Component	Molecular Weight
A	20575.76

ORN13-Cy3/Cy5, pre-miR-122
 UGGAGUGUGACAAUGGUGUUUGUGUCUAAACUAUCAAAACGCCAUUAUCACACUAAAUA
 Mass calculated 19887.20, found 19887.25

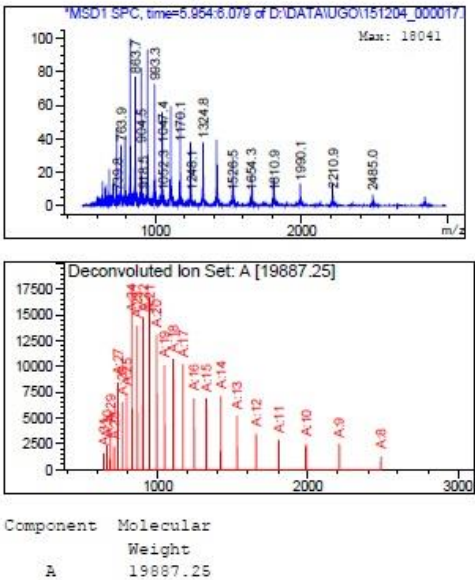


Signal 1: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	6.012	MM	0.1039	7.40021e7	1.18678e7	100.0000
Totals :				7.40021e7	1.18678e7	

Signal 2: MWD1 A, Sig=260,8 Ref=360,8

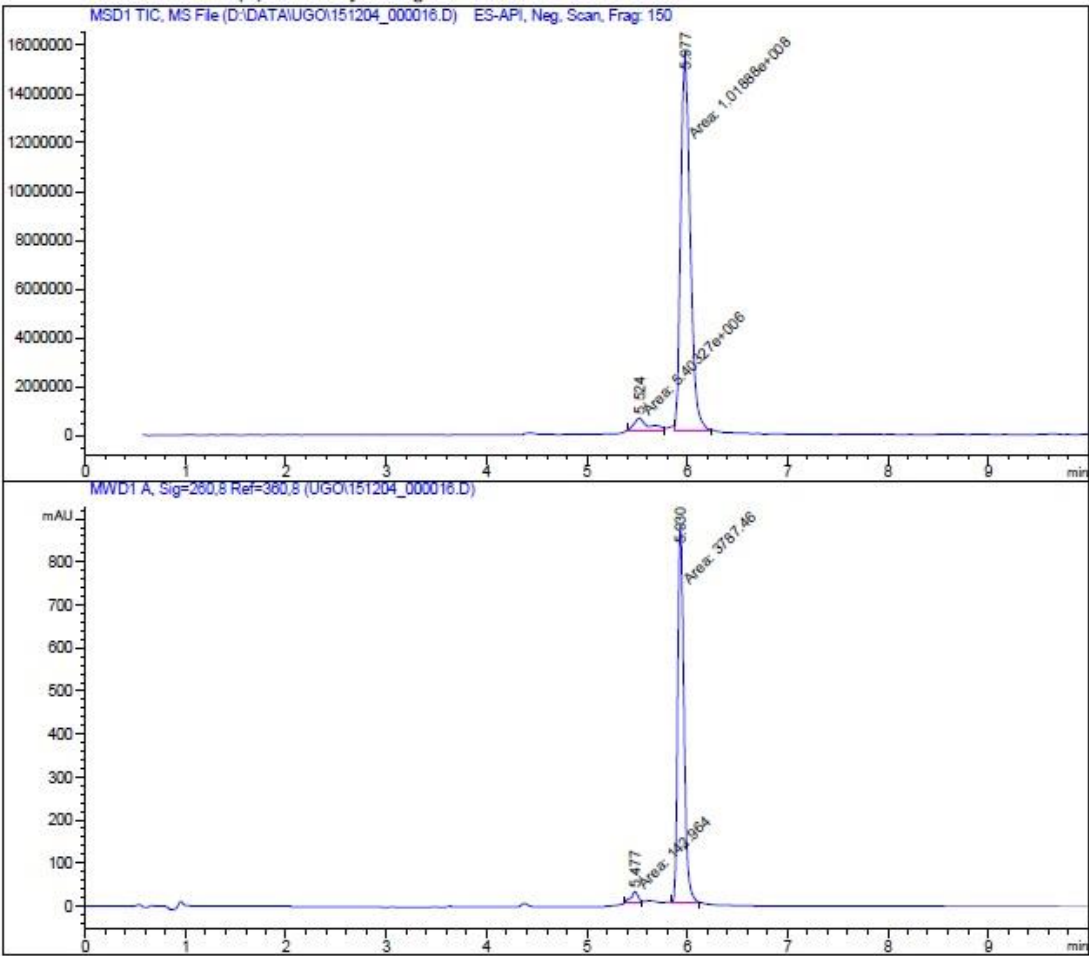
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.967	MM	0.0748	2528.97681	563.85748	100.0000
Totals :				2528.97681	563.85748	



ORN14-Cy3/Cy5, pre-miR-122

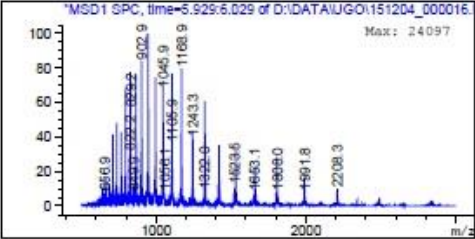
UGGAGUGUGACAAUGGUGUUUGUGUCUAAACUAUCAACGCCAUUAUCACACUAAAUA

Mass calculated 19887.20, found 19886.65



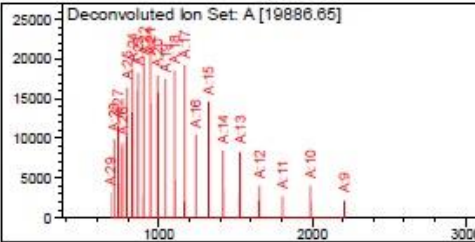
Signal 1: MS/MS TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	5.524	MM	0.1704	5.40327e6	5.28455e5	5.0361
2	5.977	MM	0.1075	1.01888e8	1.57965e7	94.9639
Totals :				1.07292e8	1.63249e7	



Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.477	MM	0.0899	142.96437	26.51037	3.6374
2	5.930	MM	0.0718	3787.45581	879.68030	96.3626
Totals :				3930.42018	906.19067	



Component	Molecular Weight
A	19886.65

10. *RNase digestion assay*

Experimental procedure

1 μL of a 20 μM solution of RNA was diluted in 15 μL of water. Fluorescence was measured every minute for 5 min on a Tecan Plate-reader Infinite M1000 at 675 nm (5 nm bandwidth) after excitation at 548 nm (Cy3 excitation, FRET) and 640 nm (direct Cy5 excitation). 1 μL of Fermentas RNase A enzyme was added to the solution, incubated at room temperature for 1 minute and the fluorescence was measured every minute for 5 min as described previously.

11. *Dicer assays with ORN13-Cy3/Cy5 and ORN14-Cy3/Cy5*

Experimental procedure

Dicer processing experiments were conducted with a Gelantis Recombinant Turbo Dicer Enzyme Kit (Cat # T520002) following manufacturer recommendations with minor modifications.

1.25 µg of ORN-Cy3/Cy5 was deposited into an Eppendorf and dried on a SpeedVac. Successively 3 µL of RNA free water, 1 µL of 10mM ATP solution, 1 µL of 5X BSA, 2 µL of 50mM MgCl₂ and 1 µL of Turbo Dicer Reaction Buffer were added. After mixing, 1.6 µL of the solution was removed from the solution and stored separately as starting material reference for fluorescence measurements (sample 1). The remaining solution was added 2 µL of Turbo Dicer Enzyme (1 unit) and incubated for 2 h at 37 °C under gentle shaking. The enzymatic reaction was stopped by the addition of 2 µL of a Turbo Dicer Stop Solution. 2 µL of the solution was removed from the solution and stored separately for fluorescence measurements (sample 2).

The remaining solution was analyzed by LC-MS with a gradient of 5 to 55% buffer B in buffer A over 10 min then 55% to 100% over 2.5 min (buffer A: 0.4M HFIP, 15 mM triethylamine; buffer B: MeOH).

Samples 1 and 2 were dissolved in water to reach a final volume of 15 µL and analyzed on a Tecan Plate-reader Infinite M1000 at 675 nm (5 nm bandwidth) after excitation at 548 nm (Cy3 excitation, FRET) and 640 nm (direct Cy5 excitation). To sample 2 was added 1 µL of a 200 µM solution of a 2'-MeO antisense oligonucleotide (fully complementary 5p antisense, sequence: 5'-UAUUUAGUGUGAUA AUGGCGUU-3', end concentration 12.5 µM). The solution was incubated for 15 min and fluorescence measurements were acquired as described previously. 4 µL of the antisense oligonucleotide solution were added to the solution (end concentration 50 µM) and fluorescence was measured once more as described previously. Fluorescence was normalized by dividing the fluorescence signal at 675 nm after excitation at 548 nm by the fluorescence signal at 675 nm after excitation at 640 nm.

LCMS traces

ORN13-Cy3/Cy5 ^P = phosphate group

UGGAGUGUGAC_{Cy3}AAUGGUGUUUGUGUCUAAACUAUCAAACGCCAUUAUCAC_{Cy5}ACUAAAUA

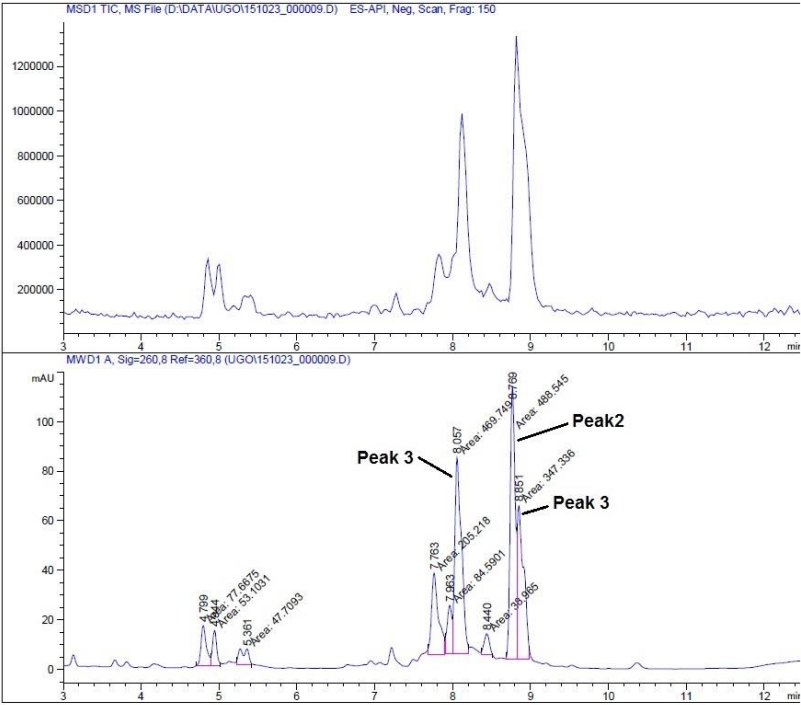
m/z 19887.2

UGGAGUGUGAC_{Cy3}AAUGGUGUUUG ^PUGUCUAAACUAUCA ^PAACGCCAUUAUCAC_{Cy5}ACUAAAUA

m/z 7715.3

m/z 4375.7

m/z 7752.3



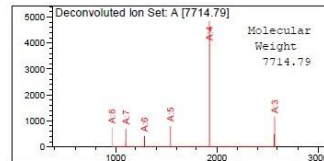
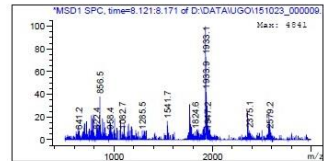
Signal 1: MSD1 TIC, MS File

Signal 2: MWD1 A, Sig=260,8 Ref=360,8

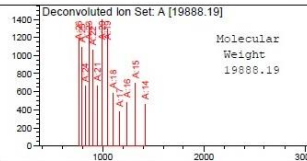
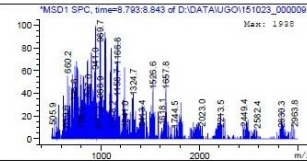
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	4.799	MM	0.0799	77.66751	16.20817	4.2842
2	4.944	MM	0.0621	53.10308	14.25176	2.9292
3	5.361	MM	0.1273	47.70932	6.24467	2.6317
4	7.763	MM	0.1038	205.21797	32.95470	11.3200
5	7.963	MM	0.0715	84.59008	19.71201	4.6661
6	8.057	MM	0.0982	469.74890	79.74553	25.9117
7	8.440	MM	0.0781	38.96497	8.31680	2.1493
8	8.769	MM	0.0738	488.54495	110.39530	26.9485
9	8.851	MM	0.0933	347.33624	62.07744	19.1593

Totals : 1812.88303 349.90639

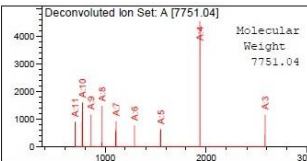
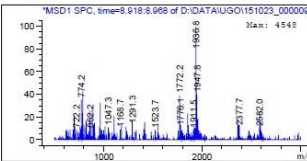
Peak 1



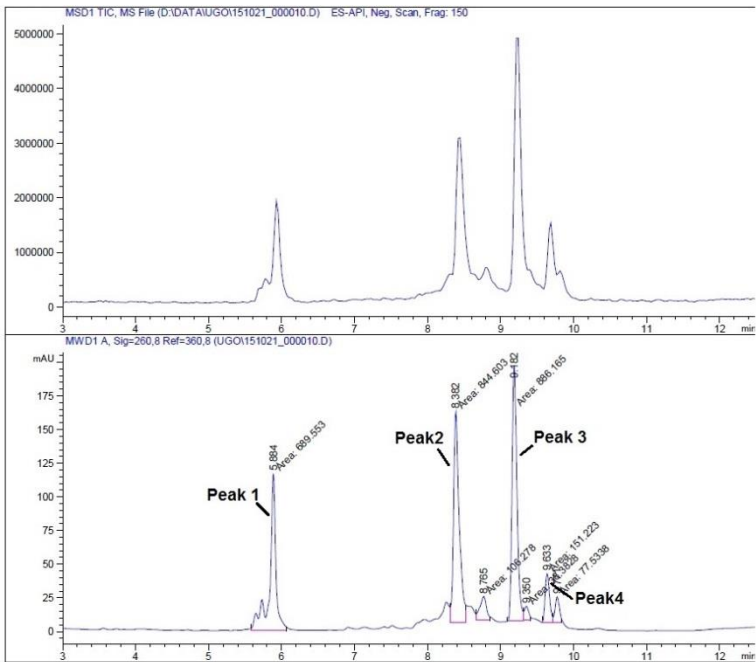
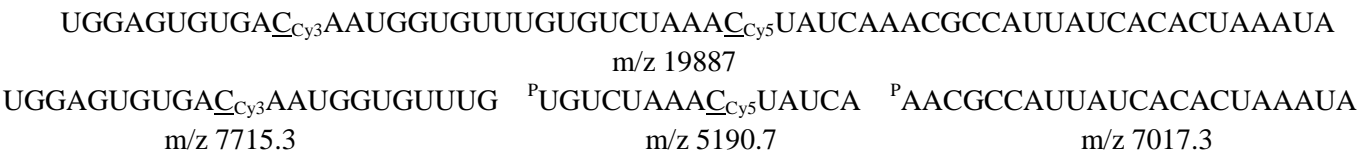
Peak 2



Peak 3

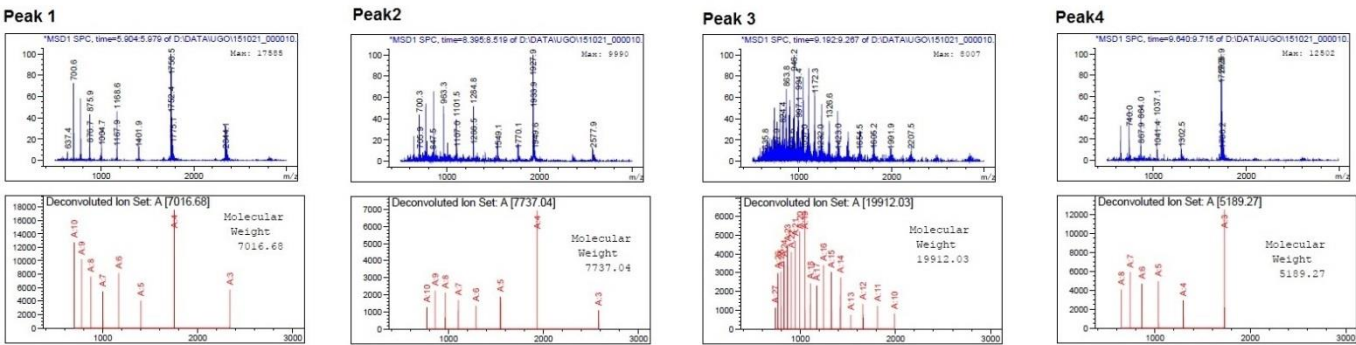


ORN14-Cy3/Cy5



Signal 1: MSD1 TIC, MS File
Signal 2: MWD1 A, Sig=260,8 Ref=360,8

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.884	MM	0.0983	689.55316	116.87001	24.6821
2	8.382	MM	0.0896	844.60260	157.09349	30.2320
3	8.765	MM	0.1020	106.27811	17.36560	3.8042
4	9.182	MM	0.0777	886.16492	190.17201	31.7197
5	9.350	MM	0.0635	38.38276	10.06780	1.3739
6	9.633	MM	0.0691	151.22289	36.49559	5.4129
7	9.771	MM	0.0678	77.53381	19.07162	2.7753
Totals :				2793.73825	547.13612	



Fluorescence measurments

