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Supplemental Information

Circular siRNAs for Reducing Off-Target Effects and Enhancing Long-Term Gene Silencing in Cells and Mice

Liangliang Zhang, Duanwei Liang, Changmai Chen, Yuan Wang, Gubu Amu, Jiali Yang, Lijia Yu, Ivan J. Dmochowski, and Xinjing Tang

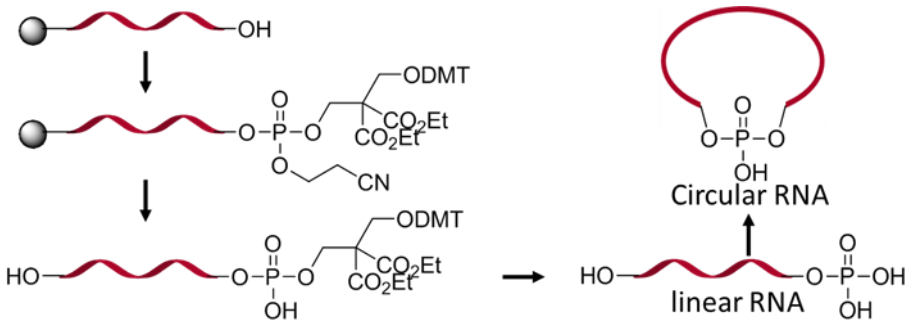


Figure S1. **Synthetic route of the circular single-stranded RNA**

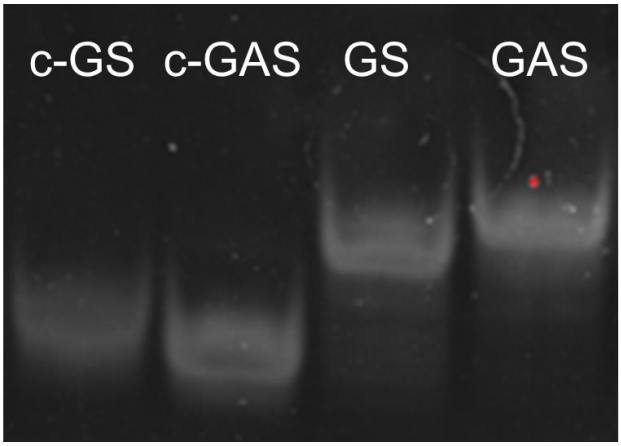
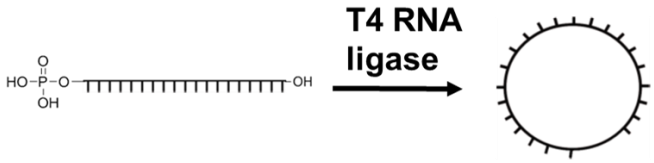


Figure S2. **Native page Gels analyze of single strand circular RNA(c-GS, c-GAS) and single strand linear RNA (GS, GAS). Result showed the circular RNA moved faster than linear RNA in the gel.**

Table S1. Synthesis of circular RNA and siRNAs used for GFP gene silencing in this study.



GS or GAS c-GS or c-GAS

Name	sequence
Sense circular/ Antisense(c-GS/GAS)	5'P –GAA CGG CAU CAA GGU GAA CUU UU CUU GCC GUA GUU CCA CUU G-P-5'
Antisense circular/ Sense(c-GAS/GS)	5'P –GAA CGG CAU CAA GGU GAA CUU UU CUU GCC GUA GUU CCA CUU G-P-5'
Positive Control(PC)	5'P –GAA CGG CAU CAA GGU GAA CUU UU CUU GCC GUA GUU CCA CUU G-P5'
Negative Control(NC)	5'P –UUC UCC GAA CGU GUC ACG UTT TT AAG AGG CUU GCA CAG UGC A -P5'

The single-stranded circular RNAs were dissolved in 1 ×PBS buffer and annealed with equal amount of the complementary RNA without chemical modification to form the circular siRNA (c-S/AS or c-AS/S) for further applications.

Table S2. The sequences of RNAs used in the study and their measured molecular weights (MW) using ESI-MS.

Name	sequence	Calculated MW	Measured MW
Sense(GS)	5'P –GAA CGG CAU CAA GGU GAA CUU	6839.2	6839.9
Antisense (GAS)	5'P–GUU CAC CUU GAU GCC GUU CUU	6643.9	6644.8
Sense circular(c-GS)	5'P –GAA CGG CAU CAA GGU GAA CUU	6821.2	6822.2
Antisense circular(c-GAS)	5'P–GUU CAC CUU GAU GCC GUU CUU	6625.9	6626.9
21LS	5'P-CCC UAU UCU CCU UCU UCG CUU	6500.7	6501.5
21LAS	5'P-GCG AAG AAG GAG AAU AGG GUU	6982.3	6983.0
24LS	5'P-AAC CCU AUU CUC CUU CUU CGC UUA	7488.4	7489.5
24LAS	5'P-UAA GCG AAG AAG GAG AAU AGG GUU	7946.9	7947.3
27LS	5'P-UUA ACC CUA UUC UCC UUC UUC GCU UAA	8429.9	8429.5
27LAS	5'P-UUA AGC GAA GAA GGA GAA UAG GGU UAA	8911.4	8912.3
c-21LS	5'P-CCC UAU UCU CCU UCU UCG CUU	6482.7	6484.3
c-21LAS	5'P-GCG AAG AAG GAG AAU AGG GUU	6964.3	6964.8

Single-stranded RNA (~ 0.2 nmol) were dissolved in water/acetonitrile (50:50, 20μL) containing 1% triethylamine to make a final concentration of 10 μM. The solutions were then analyzed with a Waters Xevo G2 Q-Tof spectrometer with electrospray ionization (ESI) in the negative ion mode.

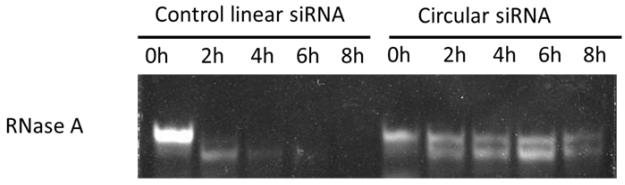


Figure S3. Native PAGE (10%) analyze of circular siRNA(c-GAS/GS) and linear siRNA(PC) were treated with RNase A. The circular structure of siRNA could increase the enzymatic stability of RNA.

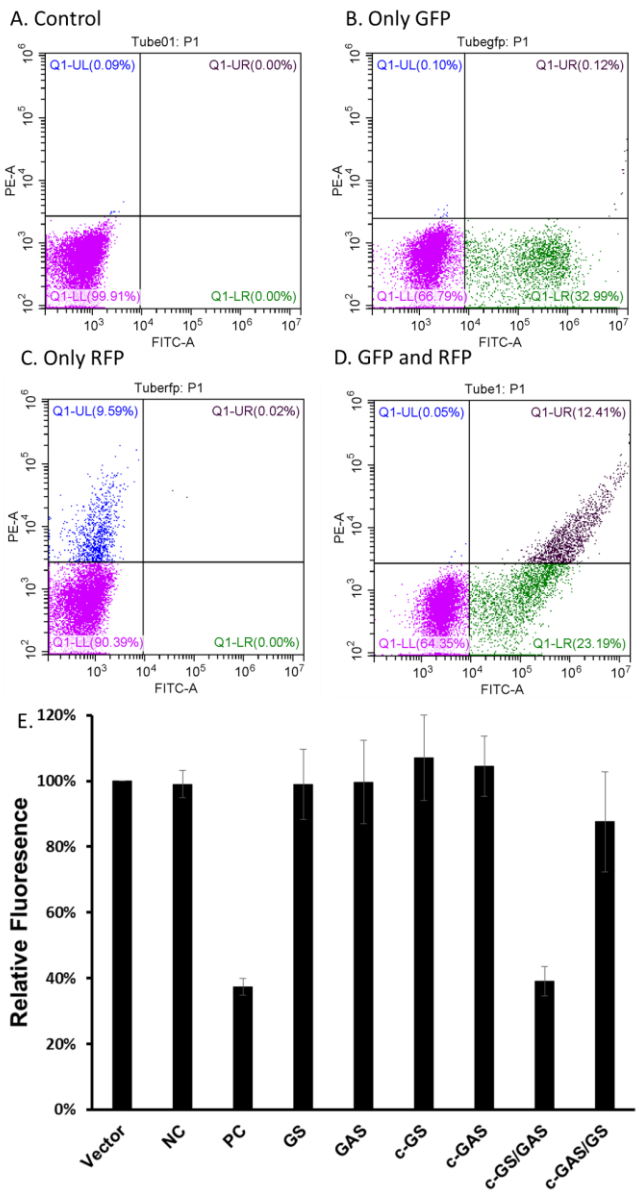


Figure S4. Selection of the HEK 293A cells subpopulations by the expression values of both the GFP and RFP genes. A) Transfected with no vector. B) Transfected with only pEGFP-N1 vector. C) Transfected with only pDsRed-N2 vector. D) Transfected with both pEGFP-N1 and pDsRed-N2 vector. The average GFP expression values of the selected cells in Q1-UR area. E) Quantified results of average GFP expression value were taken by flow cytometry. Each result is the average of three individual experiences. The concentrations of siRNAs were 2.5 nM. Error bar is indicated as standard derivation.

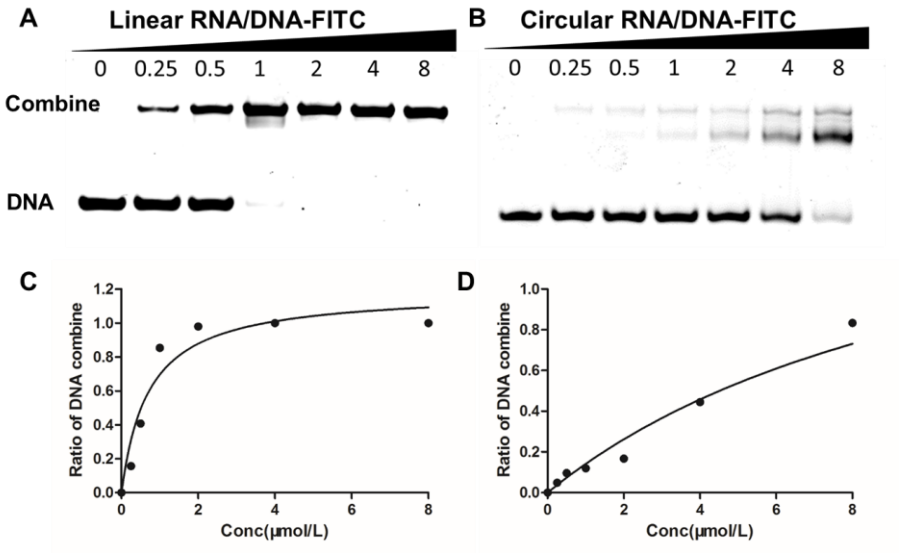


Figure S5. 20% PAGE Gel analyze the ability of the linear strand leaving its circular or linear RNA partner. A) The combine ability of linear DNA-FITC to different ratio of linear RNA(0.25, 0.5, 1, 2, 4, 8). B) The combine ability of linear DNA-FITC to different ratio of circular RNA(0.25, 0.5, 1, 2, 4, 8). C)Ratio changes of DNA-FITC combine following the increased concentration of linear RNA . D) Ratio changes of DNA-FITC combine following the increasing concentration of circular RNA. RNA/DNA duplex was annealed in PBS. The data of C or D were fit to a hyperbola function by non-linear curve fitting method of GraphPad PRISM.

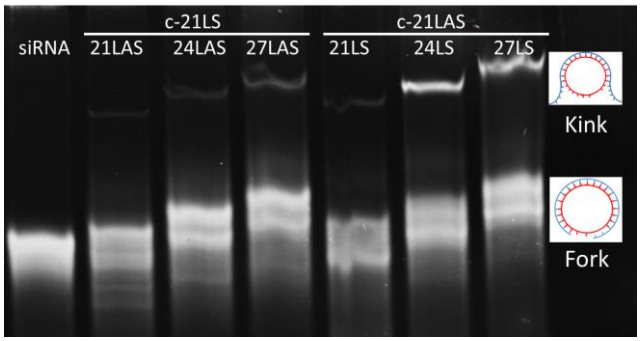
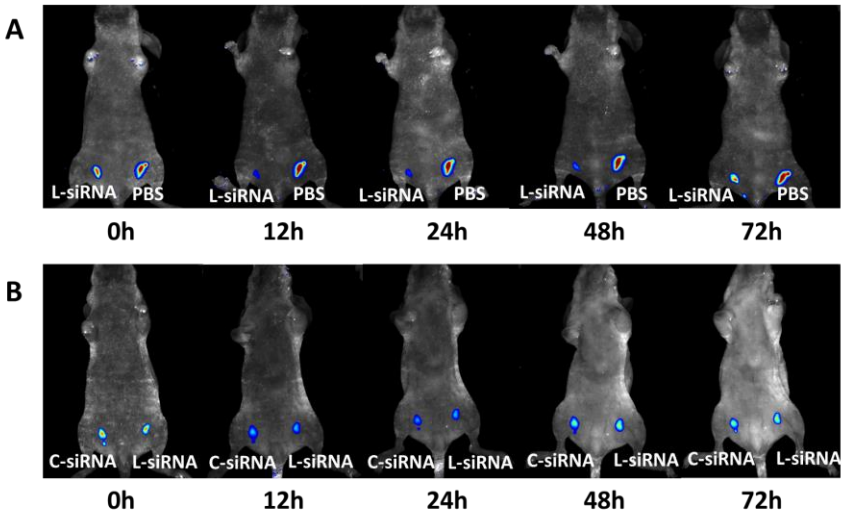


Figure S6. 20% PAGE Gel analysis of the binding ability of circular RNA (c-21LS, c-21LAS) with the different length of the linear single strand RNA (21 mer, 24 mer, 27 mer) or the linear siRNA control. The top band was the kink conformer, the bottom band was the fork conformer which moved faster due to the fully folded structure.



C

mice 1		0h	12h	24h	48h	72h
tumor A	linear siRNA	9.48E+06	8.15E+06	7.42E+06	1.29E+07	2.16E+07
tumor B	0.1×PBS	4.49E+06	7.05E+04	3.95E+04	2.25E+06	5.79E+06
mice 2		0h	12h	24h	48h	72h
tumor A	linear siRNA	2.90E+06	1.45E+06	1.82E+06	4.36E+06	9.75E+06
tumor B	0.1×PBS	7.02E+06	5.99E+06	4.62E+06	7.11E+06	1.48E+07
mice 3		0h	12h	24h	48h	72h
tumor A	linear siRNA	9.21E+06	1.25E+06	1.40E+06	1.88E+06	4.88E+06
tumor B	0.1×PBS	1.73E+07	2.23E+07	2.27E+07	2.35E+07	2.35E+07
mice 4		0h	12h	24h	48h	72h
tumor A	Liner siRNA	9.33E+06	6.03E+06	6.37E+06	1.08E+07	9.98E+06
tumor B	Circular siRNA	1.34E+07	7.93E+06	6.27E+06	1.03E+07	9.82E+06
mice 5		0h	12h	24h	48h	72h
tumor A	Linear siRNAs	8.76E+06	2.56E+06	4.90E+06	4.88E+06	1.42E+07
tumor B	Circular siRNAs	1.47E+07	7.66E+06	7.50E+06	7.35E+06	6.64E+06
mice 6		0h	12h	24h	48h	72h
tumor A	Linear siRNAs	6.31E+06	748900.48	755539	1.88E+06	3.65E+06
tumor B	Circular siRNAs	9.22E+06	5.67E+06	5.09E+06	4.20E+06	7.33E+06

Figure S7. Typical in vivo real-time fluorescent imaging at the indicated time point after intratumor injection. (A) PBS on the right and linear siRNAs (L-siRNA) on the left separately (B) linear siRNAs (L-siRNA) on the right and circular siRNAs (C-siRNA) on the left separately. （C）Fluorescence intensity of tumors for each experiments.

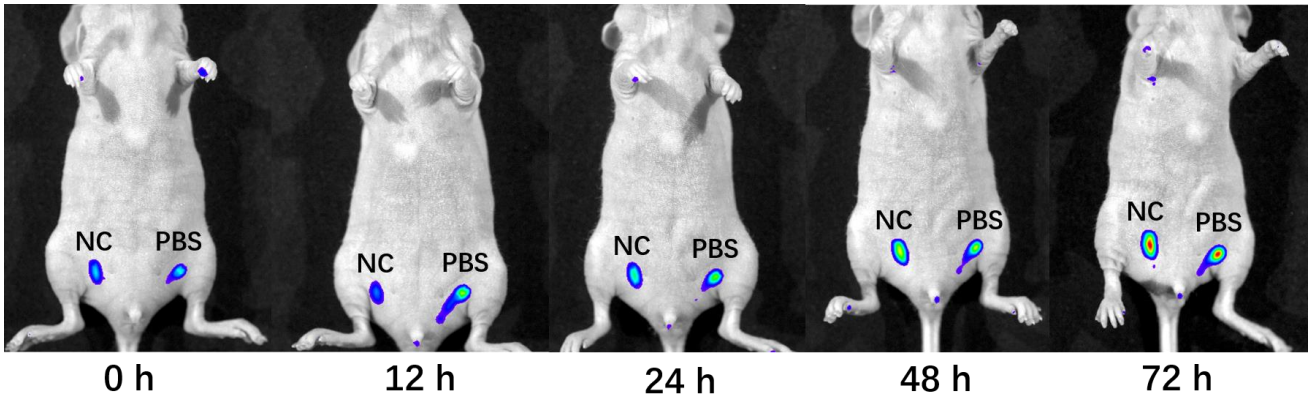


Figure S8 in vivo real-time fluorescent imaging at the indicated time point after intratumor injection of negative control siRNA (NC, left, 3 nmol) and 0.1xPBS (right) according to the similar injection protocol of circular and linear siRNAs. After injection, the mice were imaged at different time points (12h/ 24h/ 48h/ 72h) using the Maestro Automated In-Vivo Imaging system

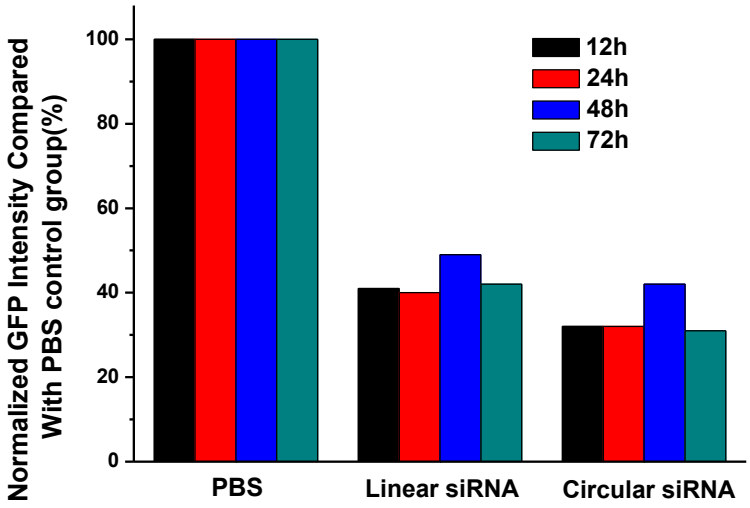
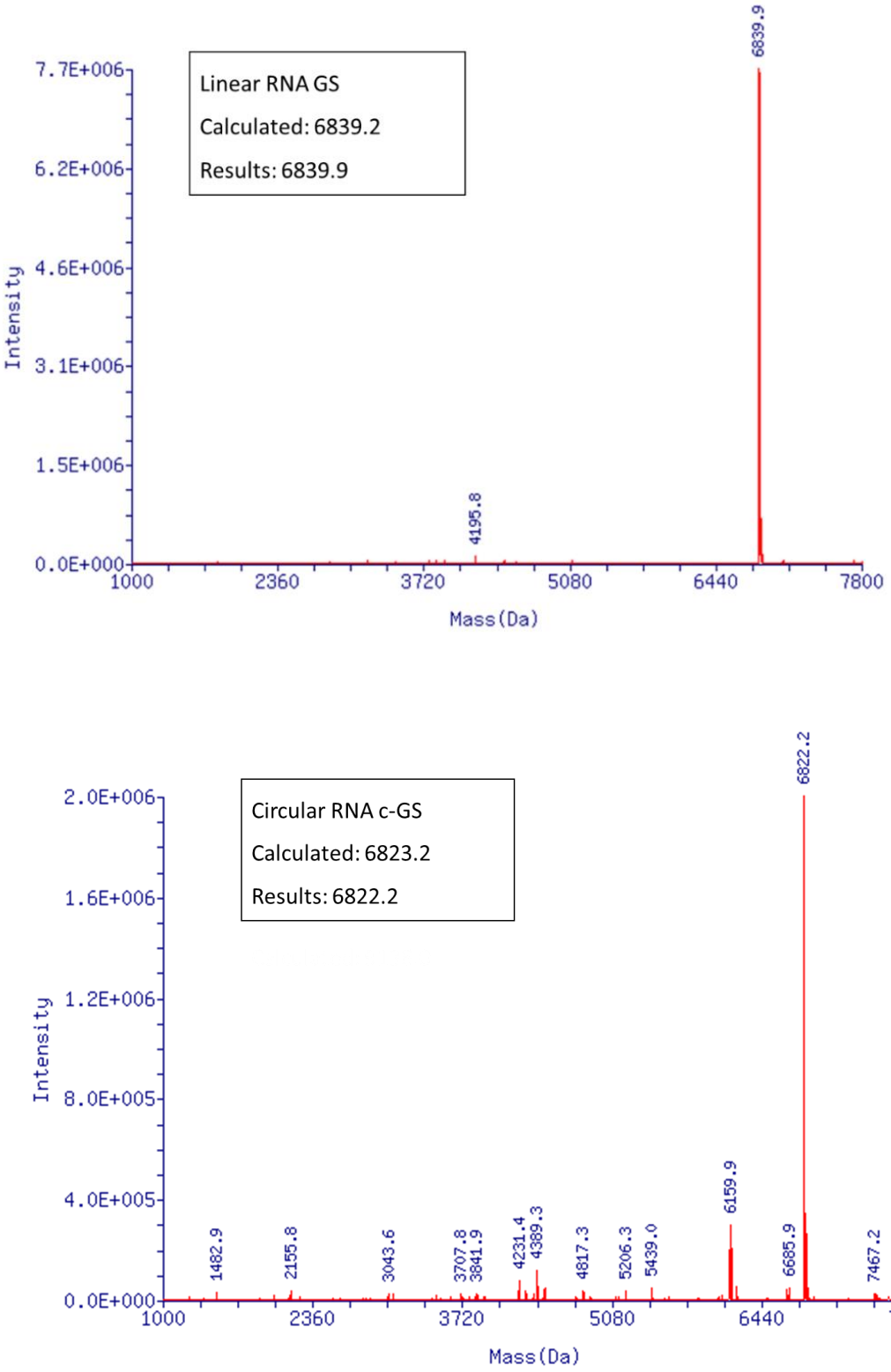
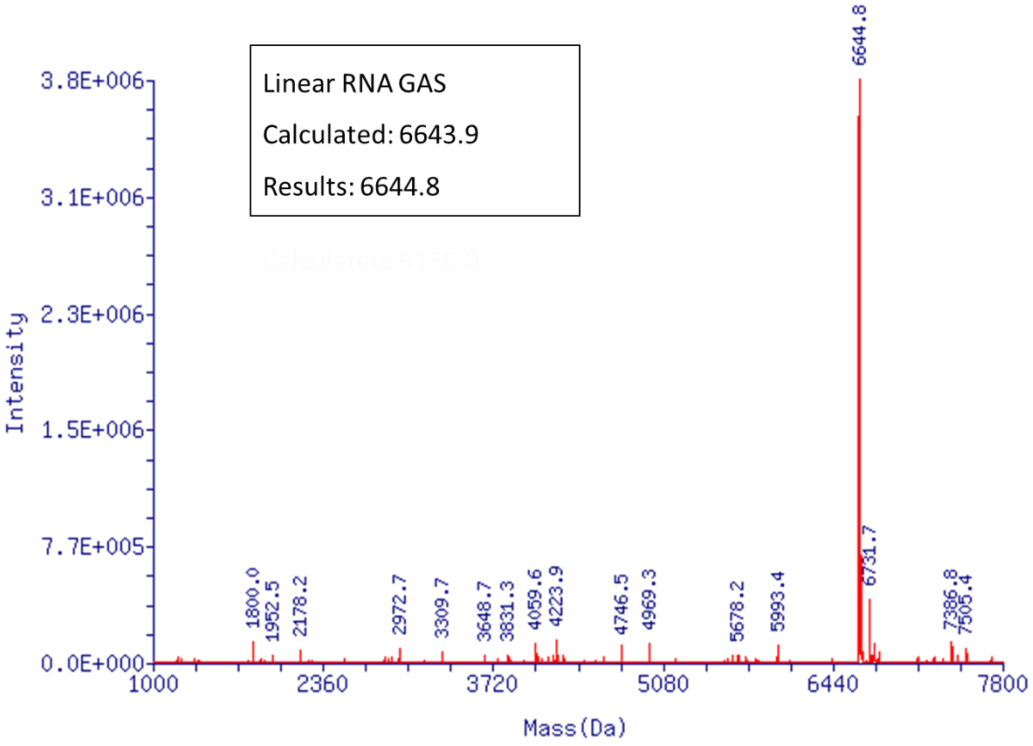


Figure S9. GFP fluorescence intensity of tumors (U87-GFP) after linear siRNA and circular siRNA after normalized to the GFP level of PBS control group at different time.

Figure S10. The MS of the RNAs target GFP gene with ESI-MS for single-stranded linear and circualr RNA




Linear RNA GAS
Calculated: 6643.9
Results: 6644.8
Intensity
3.8E+006
3.1E+006
2.3E+006
1.5E+006
7.7E+005
0.0E+000
1000
2360
3720
5080
6440
7800
Mass(Da)
6644.8
6731.7
1800.0
1952.5
2178.2
2972.7
3309.7
3648.7
3831.3
4059.6
4223.9
4746.5
4969.3
5678.2
5993.4
7386.8
7505.4

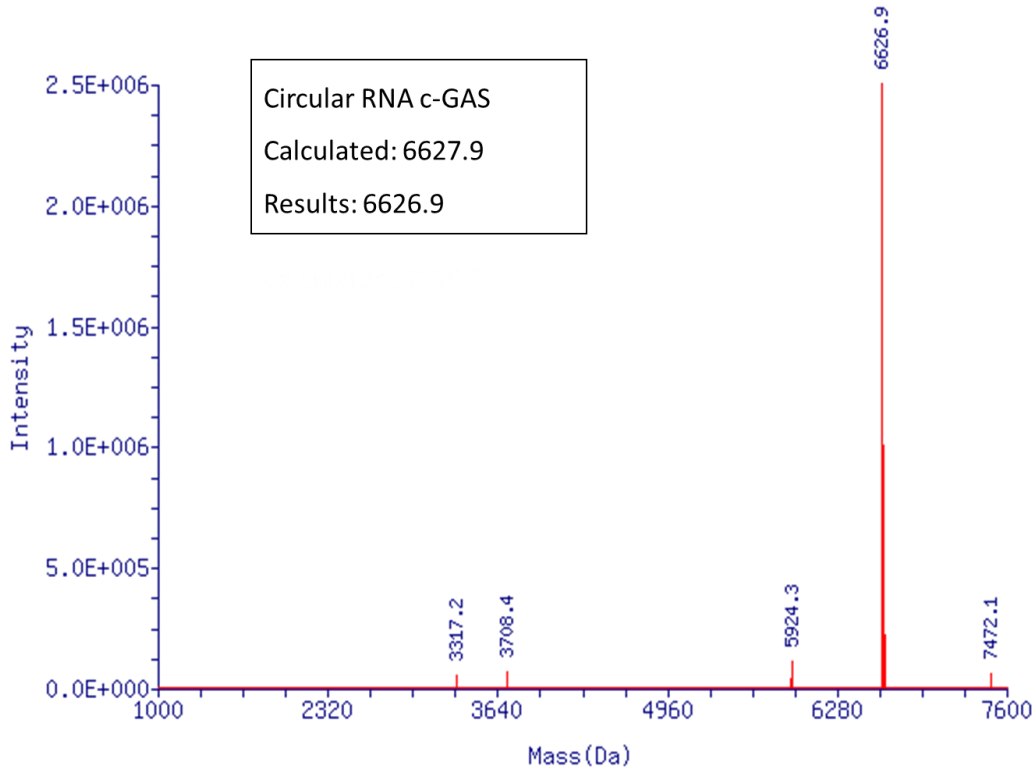
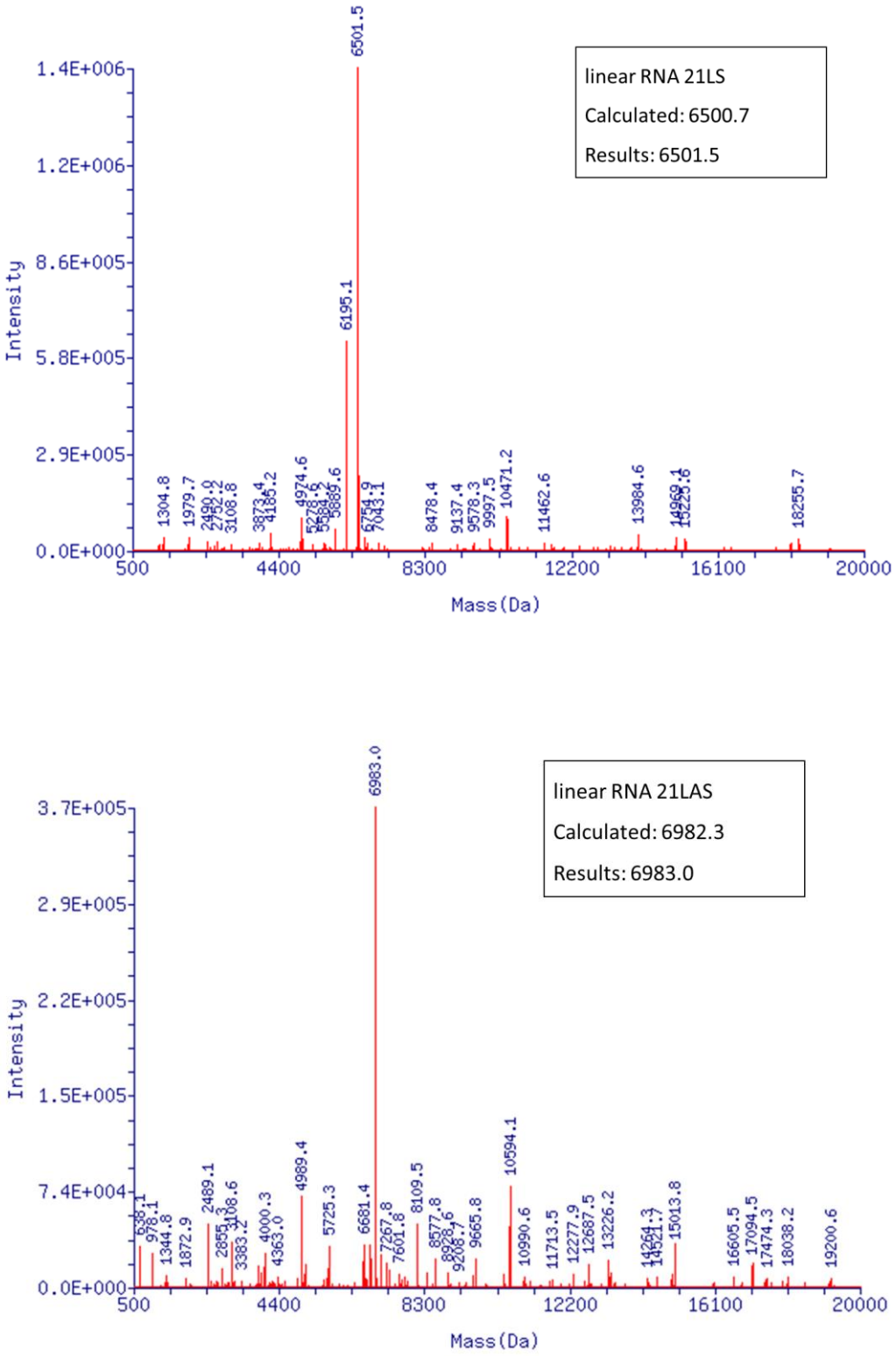
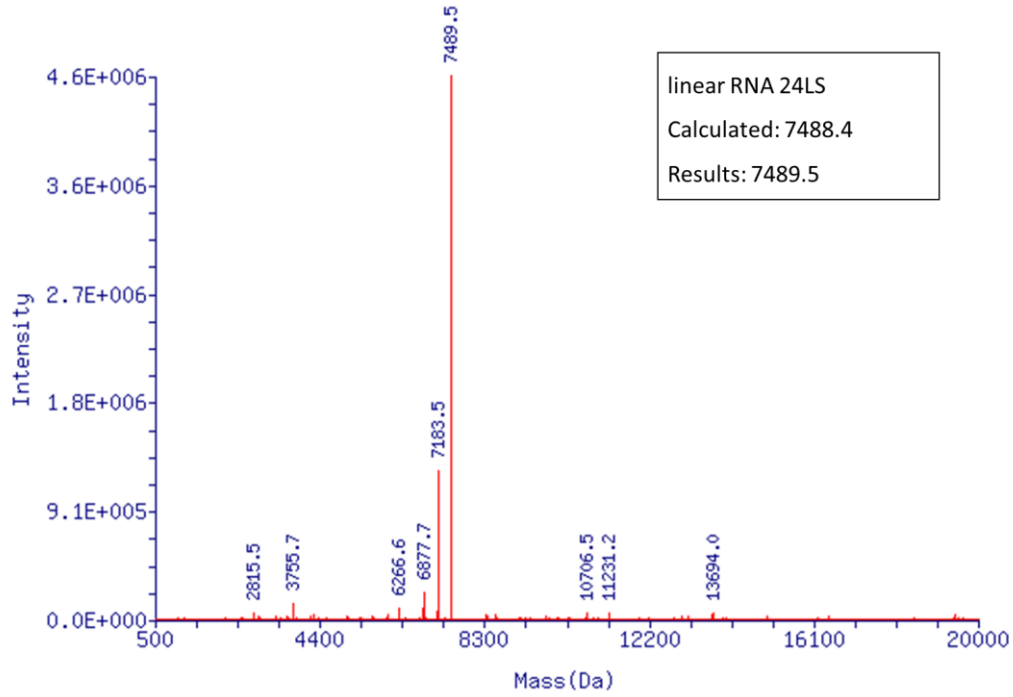
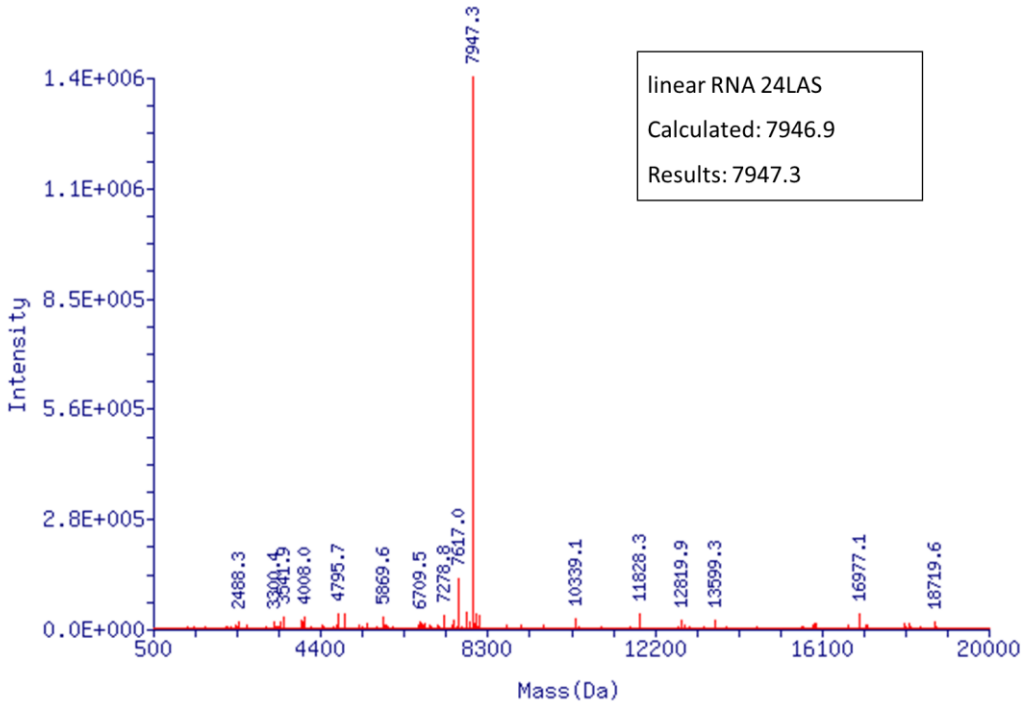
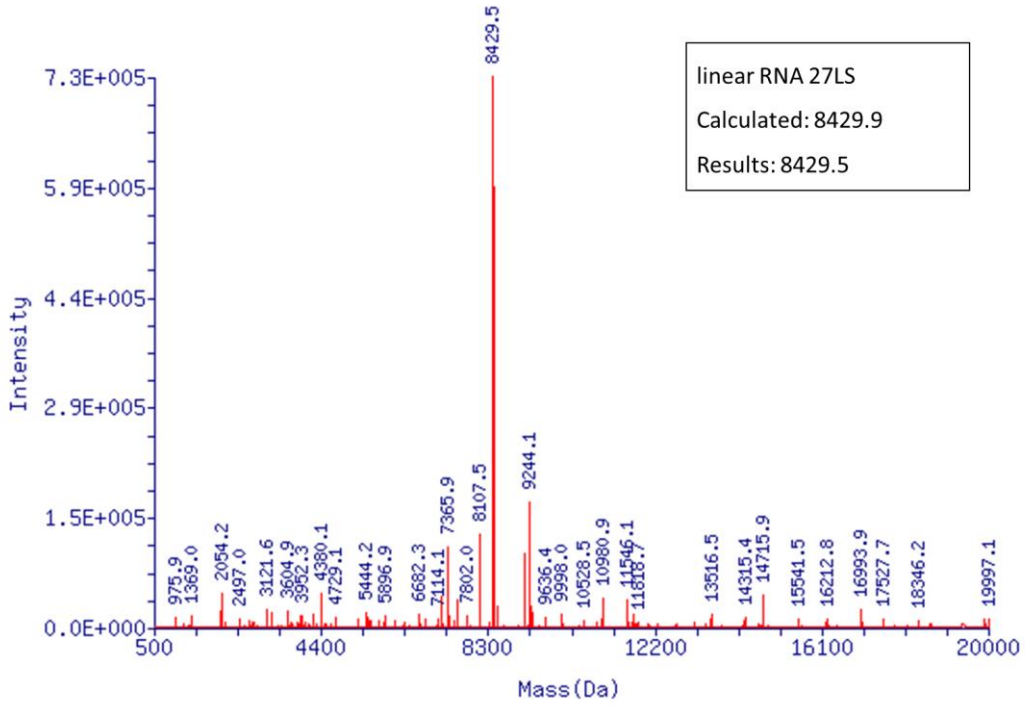

Circular RNA c-GAS
Calculated: 6627.9
Results: 6626.9
Intensity
2.5E+006
2.0E+006
1.5E+006
1.0E+006
5.0E+005
0.0E+000
1000
2320
3640
4960
6280
7600
Mass(Da)
6626.9
3317.2
3708.4
5924.3
7472.1

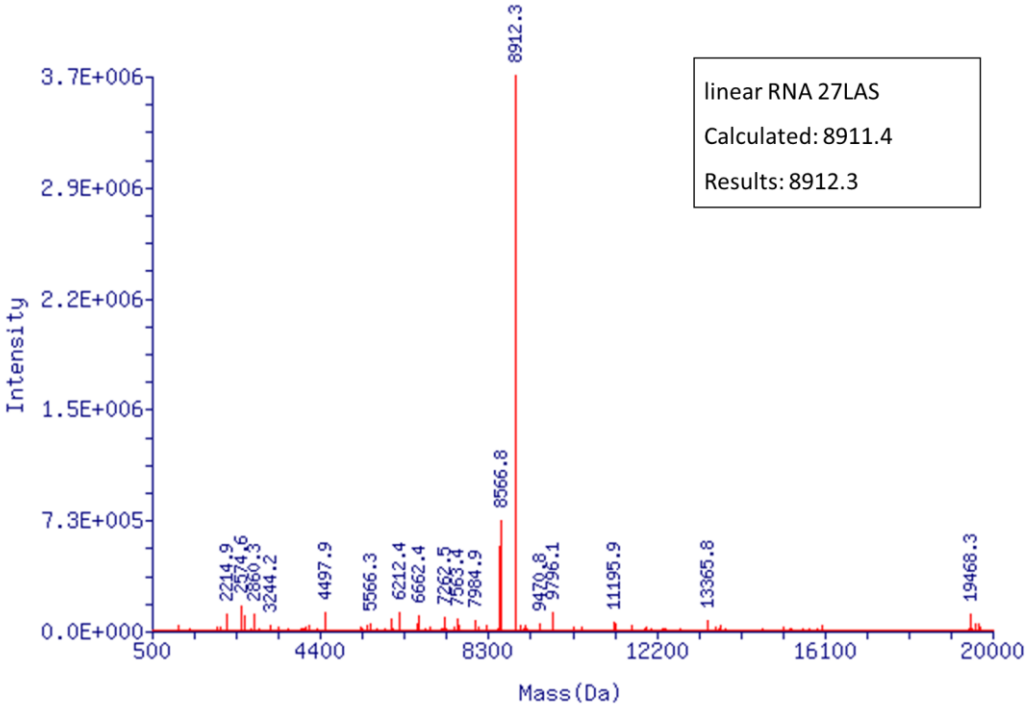
Figure S11. The MS of the RNAs target firefly luciferase with ESI-MS for single-stranded linear or circular RNA

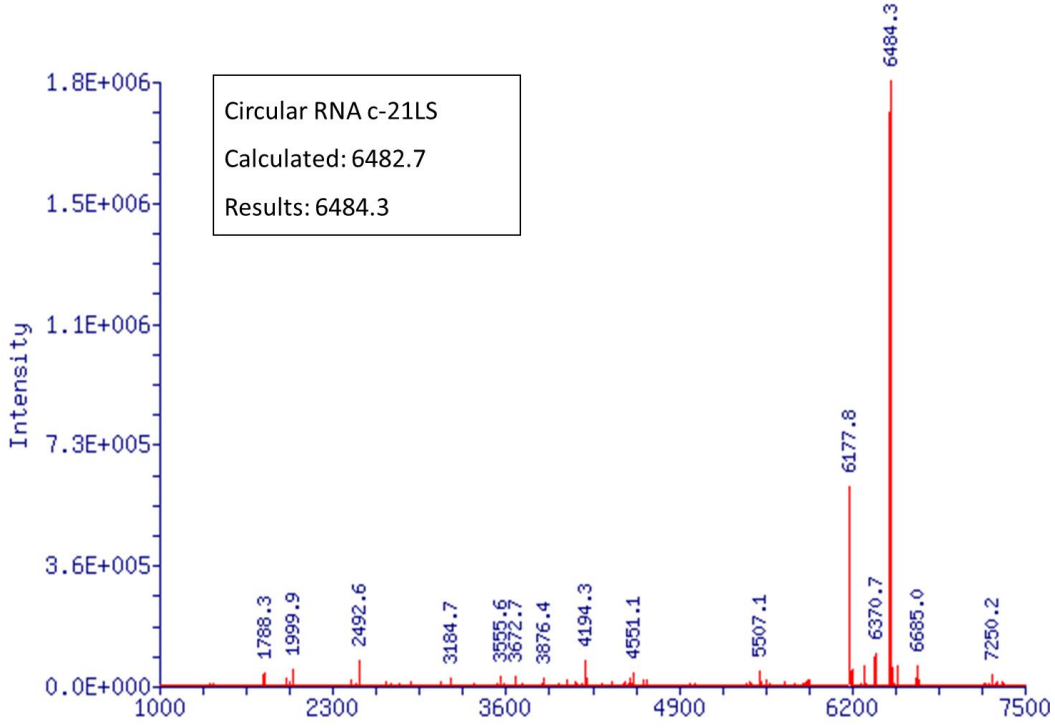



linear RNA 24LS
Calculated: 7488.4
Results: 7489.5
Intensity
Mass(Da)
4.6E+006
3.6E+006
2.7E+006
1.8E+006
9.1E+005
0.0E+000
500
4400
8300
12200
16100
20000
7489.5
7183.5
2815.5
3755.7
6266.6
6877.7
10706.5
11231.2
13694.0


linear RNA 24LAS
Calculated: 7946.9
Results: 7947.3
Intensity
Mass(Da)
1.4E+006
1.1E+006
8.5E+005
5.6E+005
2.8E+005
0.0E+000
500
4400
8300
12200
16100
20000
7947.3
7617.0
2488.3
3300.4
3541.9
4008.0
4795.7
5869.6
6709.5
7278.8
10339.1
11828.3
12819.9
13599.3
16977.1
18719.6


linear RNA 27LS
Calculated: 8429.9
Results: 8429.5
Intensity
Mass (Da)
7.3E+005
5.9E+005
4.4E+005
2.9E+005
1.5E+005
0.0E+000
500
4400
8300
12200
16100
20000
8429.5
9244.1
8107.5
7365.9
2054.2
4380.1
10980.9
13516.5
14715.9


linear RNA 27LAS
Calculated: 8911.4
Results: 8912.3
Intensity
Mass (Da)
3.7E+006
2.9E+006
2.2E+006
1.5E+006
7.3E+005
0.0E+000
500
4400
8300
12200
16100
20000
8912.3
8566.8
11195.9
13365.8
19468.3


Circular RNA c-21LS
Calculated: 6482.7
Results: 6484.3
Intensity
1.8E+006
1.5E+006
1.1E+006
7.3E+005
3.6E+005
0.0E+000
1000
2300
3600
4900
6200
7500
6484.3
6177.8
1788.3
1999.9
2492.6
3184.7
3555.6
3672.7
3876.4
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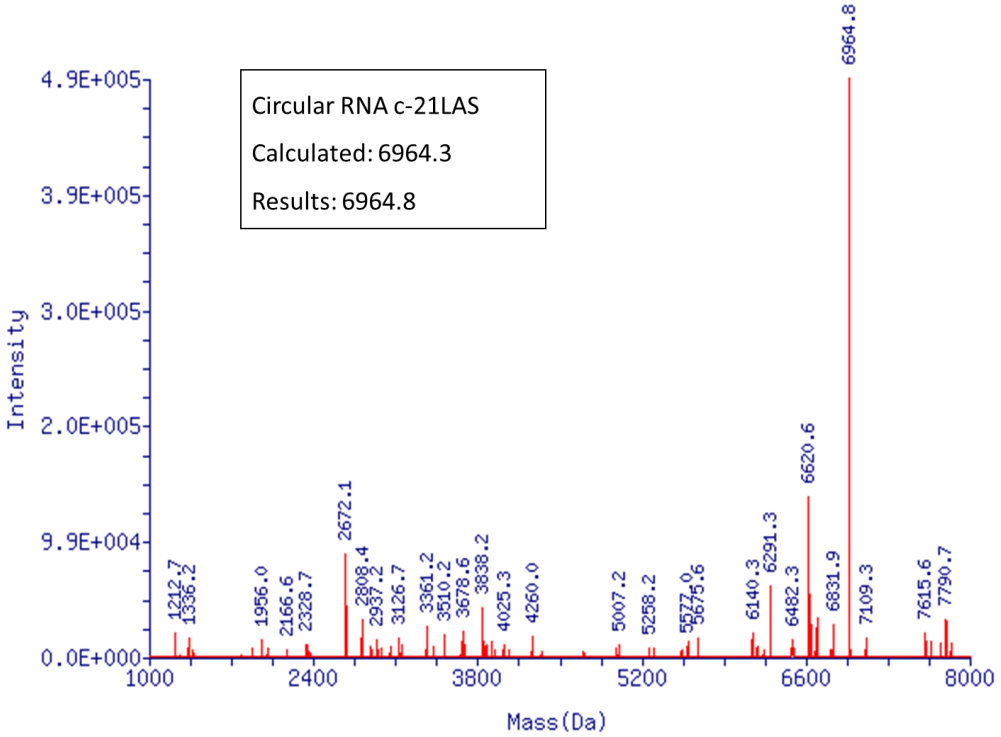
4551.1
5507.1
6370.7
6685.0
7250.2


Circular RNA c-21LAS
Calculated: 6964.3
Results: 6964.8
Intensity
4.9E+005
3.9E+005
3.0E+005
2.0E+005
9.9E+004
0.0E+000
1000
2400
3800
5200
6600
8000
Mass(Da)
6964.8
6620.6
6291.3
2672.1
1212.7
1336.2
1956.0
2166.6
2328.7
2808.4
2937.2
3126.7
3361.2
3510.2
3678.6
3838.2
4025.3
4260.0
5007.2
5258.2
5577.0
5675.6
6140.3
6482.3
6831.9
7109.3
7615.6
7790.7