2025.5.1-6 Pilot testing for C12 C15 reactivity in cells

Project: Project 1. Small molecule-binding RNA structural probing
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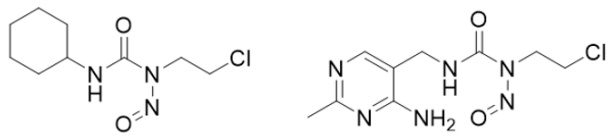
THURSDAY, 5/1/2025

Planned samples

	#	Covalent modifier	Covalent modifier (final)	Covalent rxn conditions	RT ion
1	1	DMSO (100%)	Ctrl	37°C for 15min on cells	Mg2+ (3mM)
2	2	DMSO (100%)	Ctrl	37°C for 15min on cells	Mn2+ (3mM)
3	3	C12 (100mM, stock)	C12 (5mM final)	37°C for 15min on cells	Mn2+ (3mM)
4	4	C15 (100mM, stock)	C15 (5mM final)	37°C for 15min on cells	Mn2+ (3mM)
5	5	C12 (10mM, 1:10 diluted)	C12 (0.5mM final)	37°C for 15min on cells	Mn2+ (3mM)
6	6	C15 (10mM, 1:10 diluted)	C15 (0.5mM final)	37°C for 15min on cells	Mn2+ (3mM)
7	7	DMSO (100%)	Ctrl	80°C for 15min on cells	Mg2+ (3mM)
8	8	DMSO (100%)	Ctrl	80°C for 15min on cells	Mn2+ (3mM)
9	9	C12 (100mM, stock)	C12 (5mM final)	80°C for 15min on cells	Mn2+ (3mM)
10	10	C15 (100mM, stock)	C15 (5mM final)	80°C for 15min on cells	Mn2+ (3mM)
11	11	C12 (10mM, 1:10 diluted)	C12 (0.5mM final)	80°C for 15min on cells	Mn2+ (3mM)
12	12	C15 (10mM, 1:10 diluted)	C15 (0.5mM final)	80°C for 15min on cells	Mn2+ (3mM)
13	13	DMSO	Ctrl	80°C for 5min on pure RNA	Mn2+ (3mM)
14	14	C12	C12 (0.1mM final)	80°C for 5min on pure RNA	Mn2+ (3mM)
15	15	C15	C15 (0.1mM final)	80°C for 5min on pure RNA	Mn2+ (3mM)

C12 Lomustine & C15 Nimustine



Cell treatment with RNA covalent modifiers:

- Treat "SARS-CoV2 5UTR"cells (SL5 HEK293) with TrypLE Express at room temp
- Collect and wash cells with 1x PBS
- Count cells: **2.9x10^6 cells/ml**
- Passage at 1:20, keep the extra on ice
- Prepare 2x10^5 cells/rxn, 12 rxns
- Resuspend cells for each rxn in 47.5ul 1x PBS
- Add 2.5ul DMSO or covalent modifier in DMSO (total volume=50ul)
- Incubate at designated temp for **15min**
- Chill on ice for 2min
- Add 300ul RLT Plus buffer (total volume=350ul)
- Vortex for 1 min to homogenize
- Store in -80

Note:

- When making 5mM solution with 1x PBS, C15 is transparent but C12 looks like emulsion at room temp, and both are transparent after 15min of 37°C or 80°C incubation.

FRIDAY, 5/2/2025

RNA extraction:

- Thaw cell lysate in RTL Plus buffer at RT
- Vortex for 1min to homogenize
- Transfer to DNA spin column, 8000xg 30s
- Add 350ul 70% EtOH to the flow-through
- Transfer to RNA spin column, 8000xg 15s, discard the flow-through
- Add 700ul RW1 buffer, 8000xg 15s, discard the flow-through
- Add 500ul 70% EtOH instead of RPE buffer, 8000xg 15s, discard the flow-through
- 500ul 80% EtOH, 8000xg 2min, discard the flow-through
- Full speed 5min
- Elute with 35ul water

- Nanodrop:

RNA yield

	#	RNA conc (ng/ul)	RNA 260/280	100ng RNA (ul)	Water to 8ul (ul)
1	1	35.9	2.03	2.8	5.2
2	2	42.9	2.08	2.3	5.7
3	3	45.9	2.06	2.2	5.8
4	4	41.8	2.05	2.4	5.6
5	5	40.6	2.04	2.5	5.5
6	6	41.3	2.07	2.4	5.6
7	7	29.4	2.03	3.4	4.6
8	8	26.2	2.07	3.8	4.2
9	9	36.5	2.09	2.7	5.3
10	10	15	2.09	6.7	1.3
11	11	41.9	2.10	2.4	5.6
12	12	20.6	2.18	4.9	3.1

Reverse transcription:

- Input: 100ng total RNA

Annealing

	Component	vol (ul)	13x (ul)
1	CMV-5UTR-SHAPE-Rv (10uM)	1	13
2	dNTP (10mM)	1	13
3	100ng RNA+water	8	8/EA
4	Total	10	10/EA

- 65°C 5min, immediately put on ice.

Extension

	Component	vol (ul)	13x (ul)
1	Annealing product	10	10/EA
2	375mM Tris/500mM KCl (5x buffer)	4	52
3	100mM DTT (10x)	2	26
4	Protoscript II	0.5	6.5
5	RNaseIN	0.2	2.6
6	Water	1.3	16.9
7	30mM MgCl2 or MnCl2	2	2/EA
8	Total	20	20/EA

- 42°C 1h, 65°C 20min, 4°C hold.
- store cDNA in -20.

MONDAY, 5/5/2025

PCR amplification of SL5:

System

	Component	Vol (ul)	13x (ul)	Note
1	5x Phusion HF buff	10	130	
2	10mM dNTP	1	13	
3	10uM F	2.5	32.5	COVID_6-28_F_56C
4	10uM R	2.5	32.5	CMV-5UTR_SHAPE_Rv
5	Water	23.5	305.5	
6	Phusion Pol	0.5	6.5	
7	cDNA	10	10/EA	Half RT product
8	Total	50	50/EA	

Program

	A	B	C
1	98°C	30s	
2	98°C	10s	32x
3	62°C	10s	
4	72°C	15s	
5	72°C	5min	
6	4°C	hold	

PCR purification:

- 250ul DNA Binding Buffer to 50ul PCR product (5:1)
- Transfer to Zymo-Spin Column, 10000xg 30s
- Add 200 ul DNA Wash Buffer, 10000xg 30s
- Repeat washing step
- Spin at full speed 2min
- Elute = 50ul water
- Nanodrop:

PCR purification

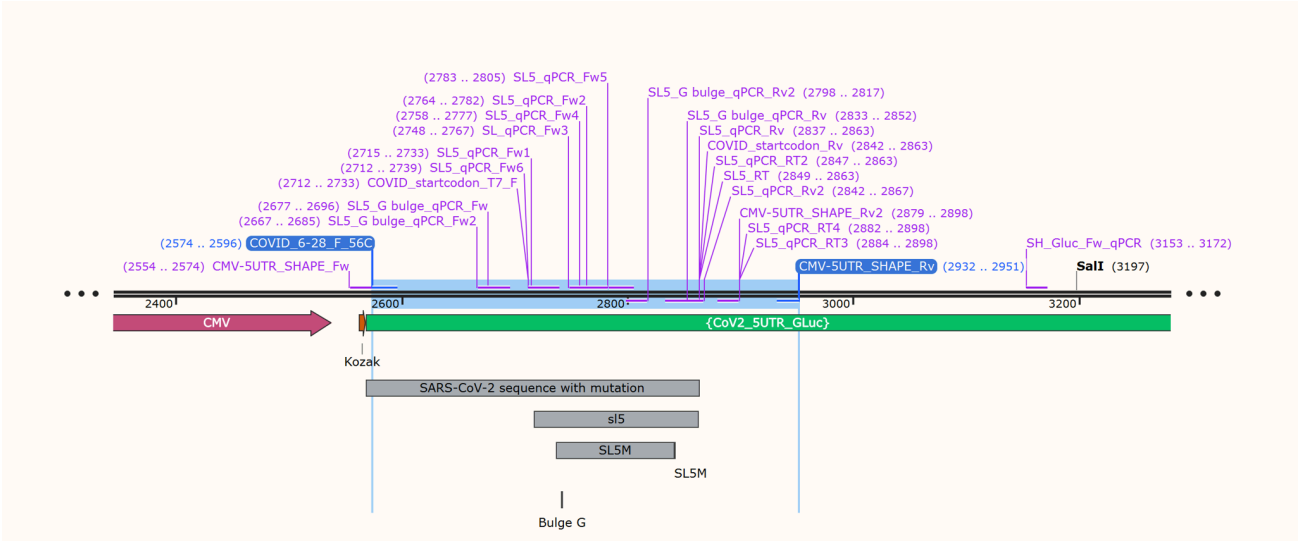
	#	DNA conc (ng/ul)	DNA 260/280	80ng= x ul	Waster to 20ul (ul)
1	1	21.1	2.06	3.8	16.2
2	2	12.5	2.02	6.4	13.6
3	3	12.0	2.14	6.7	13.3
4	4	11.1	2.06	7.2	12.8
5	5	15.2	1.85	5.3	14.7
6	6	11.7	2.01	6.8	13.2
7	7	24.8	1.90	3.2	16.8
8	8	12.0	1.87	6.7	13.3
9	9	12.2	2.02	6.6	13.4
10	10	10.5	2.13	7.6	12.4
11	11	12.6	2.07	6.3	13.7
12	12	13.1	2.08	6.1	13.9

Size verification:

- Gel: 1% agarose in 1x TBE, 1:20000 APExBIO Safe DNA Gel Stain
- Ladder: Zymo ZR 50bp DNA Marker

- Loading dye: Invitrogen 10x Blue Juice (9ul sample+1ul Blue Juice, or 7ul water+2ul 50bp Marker (1ug)+1ul Blue Juicer)
- Amplicon size=378bp
- Gel running: 100v 30min

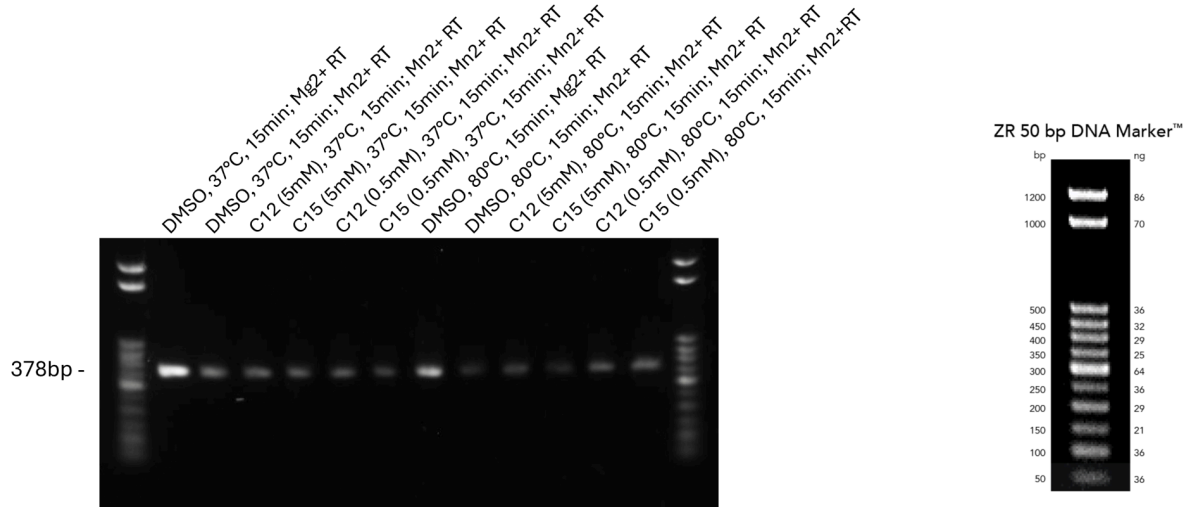
SL5 Amplicon



Gel result

2025.5.5 PCR Product

- Template: total RNA from SL5 HEK293 cells
- F primer: COVID_6-28_F_56C
- R primer: CMV-5UTR_SHAPE_Rv
- Expected size: 378bp



Note:

- After RT, the conc of total RNA should be 100ng/20ul=5ng/ul, given the LS5 RNA would be a small proportion, use high cDNA input for PCR.
- Mn2+ seemed to be less efficient than Mg2+ in terms of RT.

TUESDAY, 5/6/2025

Dr. Wang's feedback:

- Send all for Premium PCR sequencing, include positive controls from Zhichao
- Might be ideal to keep cDNA input no more than 5% for final PCR.
- Include C30 in the next next run.

Positive control samples (from Zhichao)

	#	Covalent modifier	Covalent modifier (final)	Covalent rxn temp	RT ion	cDNA conc (ul)	cDNA 260/280
1	13	DMSO ("D 1-1")	Ctrl	80°C (on naked RNA)	Mn2+ (3mM)	349.9	1.44
2	14	C12 ("12-2")	C12 (0.1mM)	80°C (on naked RNA)	Mn2+ (3mM)	385.2	1.50
3	15	C15 ("15-2")	C15 (0.1mM)	80°C (on naked RNA)	Mn2+ (3mM)	394.0	1.50

PCR amplification of positive controls:

System2

	Component	Vol (ul)	4x (ul)	Note
1	5x Phusion HF buff	10	40	
2	10mM dNTP	1	4	
3	2uM F+R	12.5	50	"148 PCR primer" (labeled on tube, from Zhichao); SMN2-GT-PCR-FW (GT2) + SMN1/2-GT-PCR-Rv (on map)
4	Water	23.5	94	
5	Phusion Pol	0.5	2	
6	cDNA	2.5	2.5/EA	5%
7	Total	50	50/EA	

Program2

	A	B	C
1	98°C	30s	
2	98°C	10s	35x
3	58°C	10s	
4	72°C	15s	
5	72°C	5min	
6	4°C	hold	

PCR purification:

- 250ul DNA Binding Buffer to 50ul PCR product (5:1)
- Transfer to Zymo-Spin Column, 10000xg 30s

- Add 200 ul DNA Wash Buffer, 10000xg 30s
- Repeat washing step
- Spin at full speed 2min
- Elute = 50ul water
- Nanodrop:

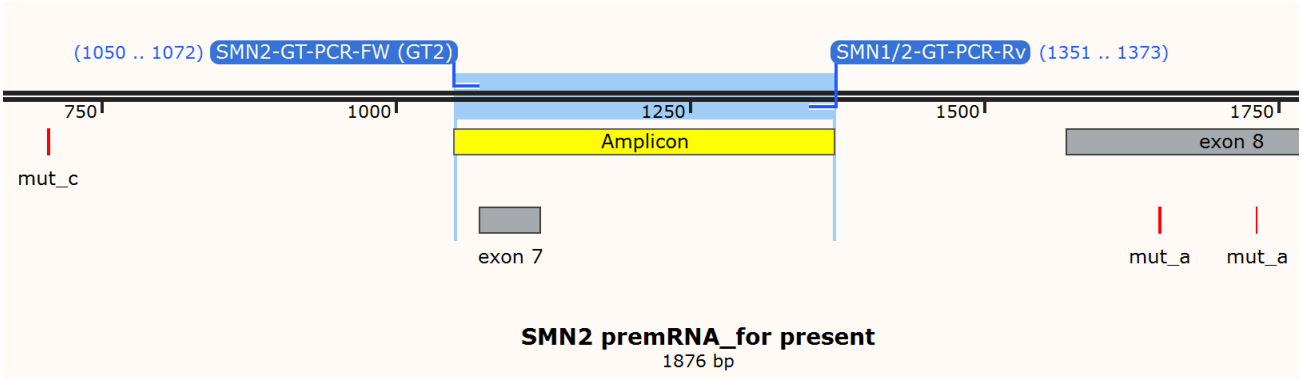
PCR purification2

	#	DNA conc (ng/ul)	DNA 260/280	80ng= x ul	Water to 20ul (ul)
1	13	31.5	1.85	2.5	17.5
2	14	35.3	1.85	2.3	17.7
3	15	38.5	1.87	2.1	17.9

Size verification:

- Gel: 1% agarose in 1x TBE, 1:20000 APExBIO Safe DNA Gel Stain
- Ladder: Zymo ZR 50bp DNA Marker
- Loading dye: Invitrogen 10x Blue Juice (200ng sample or 1ug 50bp Marker)
- Amplicon size=324bp
- Gel running: 100v 30min

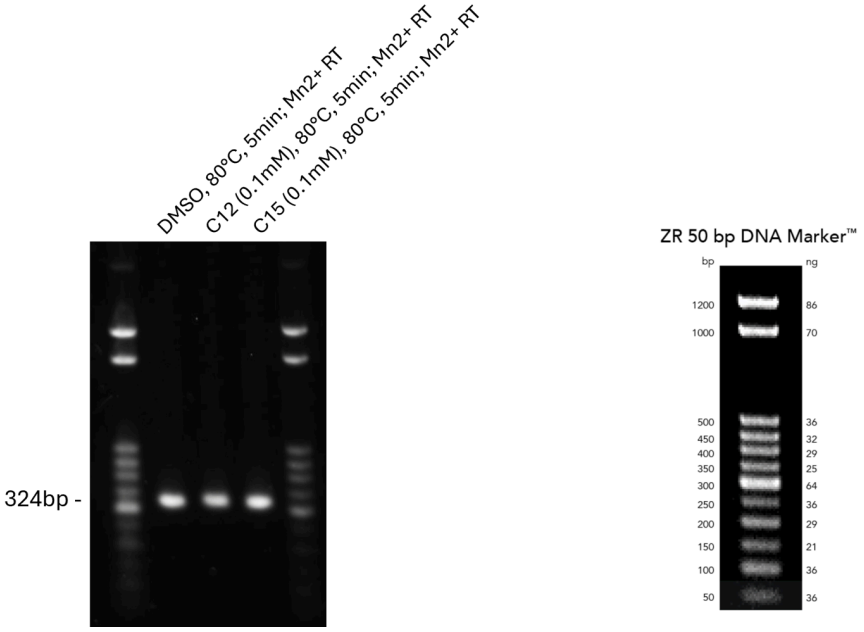
SMN2 exon7 amplicon



Gel result2

2025.5.6 PCR Product

- Template: cDNA from Zhichao (covalent modification on naked RNA)
- F/R primer: “148 PCR primer” from Zhichao
- Expected size: 324bp



Send for Premium PCR sequencing:

Order Premium_PCR_HC_20250506

	Samples
1	01_SL5_DMSO_37C_15min_Mg
2	02_SL5_DMSO_37C_15min_Mn
3	03_SL5_C12_5mM_37C_15min_Mn
4	04_SL5_C15_5mM_37C_15min_Mn
5	05_SL5_C12_0.5mM_37C_15min_Mn
6	06_SL5_C15_0.5mM_37C_15min_Mn
7	07_SL5_DMSO_80C_15min_Mg
8	08_SL5_DMSO_80C_15min_Mn
9	09_SL5_C12_5mM_80C_15min_Mn
10	10_SL5_C15_5mM_80C_15min_Mn
11	11_SL5_C12_0.5mM_80C_15min_Mn
12	12_SL5_C15_0.5mM_80C_15min_Mn
13	13_Ctrl_DMSO_80C_5min_Mn
14	14_Ctrl_C12_0.1mM_80C_5min_Mn
15	15_Ctrl_C15_0.1mM_80C_5min_Mn