Chk1 Sirna Containing Gemcitabine and Chemically Modified Nucleotides Shows Antitumoral Activity in Vitro and in Vivo

The following sequences were made using the synthesis and QC methods detailed below. The main manuscript describes the process in Materials and Methods.

Name	Sense Strand	Antisense strand
PGN6	5'-AAGAAAgAgau[GEM]UGUAU[GEM]AAU-3'	5'-AuUGAuACAGAUCuCuuucuu[GEM][GEM]dTdT-3'
PGN7	5'-AAGAAAgAgau[GEM]uGuAu[GEM]Aau-3'	5'-AuUGAuACAGAuCuCuUuCuU[GEM][GEM]dTdT-3'
PGN6 (no Gem)	5'-AAGAAAgAgaucUGUAUcAAU-3'	5'-AuUGAuACAGAUCuCuuucuudTdT-3'
PGN7 (no Gem)	5'-A*A*GAAAgAgaucuGuAucA*A*u-3'	5'-A*u*UGAuACAGAuCuCuUuC*u*UdTdT-3'
PGN7 S	5'-A*A*GAAAgAgau[GEM]uGuAu[GEM]A*a*u-3'	5'-Phos-A*u*UGAuACAGAuCuCuUuC*u*U[GEM][GEM]dTdT-3'
(Upper case letters are 2'OMe modified bases, small case letters are 2'-Fluoro modified bases; [GEM] – gemcitabine nucleoside; *=Phosphorothioate; Phos=phosphate).		

Table SI-OS-T 1: RNA Sequences

RNA Synthesis and Analysis: Instrument and Equipment

1. **Oligo Synthesizer**: Mermade 12, LGC Genomics LCC
2. **HPLC:** LC 1260 Infinity II System w/ Enh. Feat., Agilent Technologies
3. **HPLC Column:** DNA Pac PA200 Bio LC 4x250mm Analytical, Thermo Scientific
4. **LCMS**: 6230 LC/TOF, Agilent Technologies
5. **LC Column (for LCMS)**: AdvanceBio Oligonucleotide C18 column, 2.1 mm × 50 mm with a particle size of 2.7µm
6. **UV-Spectrophotometer:** Cary 3500 Multizone
7. **Temperature Probe:** Agilent in-cuvette temperature probe (part # G9889-60005)
8. **Cuvettes:** Quartz BEXCO, 3.5mL, spectral range: 190-2500 nm, 1cm
9. **pH Meter:** FiveEasy Plus FP20, Mettler Toledo
10. **pH Electrode:** LE410 with integrated temperature sensor, Mattler Toledo

Materials for RNA synthesis

1.	Oligo Synthesis Universal Support (CPG-1000A)	Glen Research 20-5141
2.	2'-OMe-A(Bz)-CE Phosphoramidite	Hongene PR1-001
3.	2'-F-A(Bz)-CE Phosphoramidite	Hongene PD1-001
4.	2'-OMe-Ac-C-CE Phosphoramidite	Hongene PR3-001
5.	2'-F-Ac-C-CE Phosphoramidite	Hongene PD3-001
6.	2'-OMe-U-CE Phosphoramidite	Hongene PR5-001
7.	2'-F-U-CE Phosphoramidite	Hongene PD5-005
8.	2'-OMe-G(dmf)-CE Phosphoramidite	Hongene PR2-001
9.	2'-F-G(dmf)-CE Phosphoramidite	Hongene PD2-001
10.	Gemcitabine Phosphoramidite	Hongene PD3-064

11. Solid Phosphorylation Reagent-II	Glen Research 10-1902-02
12. Diluent: Acetonitrile Anhydrous	Glen Research 40-4050
13. Acetonitrile Anhydrous (for DNA synthesis)	VWR BDH85501.400
14. 3% Dichloroacetic acid in dichloromethane	VWR BDH0609
15. ETT Activator	Millipore Sigma L030000
16. 0.05M DDTT/pyridine/Acetonitrile	Glen Research 40-4137-51
17. 0.02M Iodine/pyridine/THF/water	Millipore Sigma BI0420-P4000
18. Cap Mix A (80%THF/10%Acetic Acid/10% Pyridine)	Millipore Sigma 555339
19. Cap Mix B (THF/N-methylimidazole 84/16, v/v)	Millipore Sigma L050030
20. Argon	Roberts Oxygen R104D3
21. 5N ammonium hydroxide in methanol	Thermo Scientific 133710010
22. Ammonium Hydroxide (28-30%)	Millipore Sigma 221228
23. Methylamine (40% solution in water)	Sigma Aldrich 8220911000
24. Acetonitrile (for HPLC Grade)	VWR JT9012-3
25. 2.0M TEAA Solution	Glen Research 60-4110-62
26. GlenPack DNA Cartridges	Glen Research 60-5100-96
27. GlenPack RNA Cartridges	Glen Research 60-6100-96
28. Trifluoroacetic Acid (TFA)	Millipore Sigma T6508
29. Potassium Phosphate Diabasic	VWR BDH9266
30. Sodium Bromide	VWR JT3588-5
31. RNAase Free Water	ThermoFisher Scientific 4387936
32. 1.0N Sodium Hydroxide	VWR BDH7222-1
33. Glen Gel-Pak Desalting Columns	Glen Research 61-5025
34. 1,1,1,3,3,3-Hexafluoro-2-propanol (LCMS grade)	Millipore Sigma 18127-10ML
35. Acetonitrile (LCMS grade)	Millipore Sigma AX0156-6
36. Triethylamine (LCMS grade)	Fisher Scientific NC1417016
37. Phosphate buffered saline (PBS), 10X, pH 7.4	ThermoFisher Scientific AM9624

RNA Synthesis Cycle

(Detritylation → Coupling → Oxidation/Sulfurization →Capping

→Next Cycle)Detritylation Time:	90seconds x2
Coupling (2'OMe and regular amidites):	360seconds x 3
Coupling (2'F-amidites):	240seconds x 3
Coupling (Gemcitabine Amidite):	360seconds x 3
Coupling (Phosphorylation):	360seconds x 3
Oxidation:	60seconds x 1
Sulfurization:	240seconds x 2
Capping:	80seconds x1

Note: Acetonitrile washes were included between each step in the RNA synthesis cycle.

Glen DNA-Pak Cartridge Purification (Reference Glen Research)

1. Taken oligo as it was with cleavage and deprotection reagents and added 1mL of 100 mg/mL sodium chloride solutionfor a final volume ~2 -3mL.
2. Heated the sample at 55 °C for 15 minutes.
3. Conditioned Glen Pak-DNA cartridges using 1mL of acetonitrile followed by 2mL 2M TEAA.
4. Applied the hot deprotected oligo/salt mixture to the cartridge.
5. Washed the cartridge with 1mL of Salt Wash Solution (12% Acetonitrile in 100mg/mL salt solution).

6. Rinsed the cartridge with 2 x 1mL of 4% TFA to remove DMT group.
7. Washed the cartridge with deionized water 1mL x 5.
8. Eluted the purified oligo using 1mL 50% acetonitrile in water containing 0.5% ammonium hydroxide.
9. Concentrated/dried using speedvac.

Removal of phosphate tail and desalting (for 5' phosphate containing oligos)

1. Dissolved dried oligo sample completely in 1mL concentrated ammonium hydroxide solution.
2. Incubated it at 65 °C for 2 hours.
3. Cooled it to room temperature.
4. Evaporated the solvent till about 10% solution is left in the tube.
5. Dilute it to make volume about 500uL or about 1mL.
6. Desalted reconstituted sample using suitable desalting cartridge and the protocol provided by the desalting cartridgevendor.
7. Evaporate the solvent to get pure oligo.

HPLC Method and HPLC Profiles (after cartridge purification)

Mobile Phase A: 20 mM phosphate in 10% acetonitrile (90% water), pH 11.

Dissolved 5.4g of sodium phosphate dibasic heptahydrate in about 800mL deionized water. Adjusted the pH to 11 with 1N NaOHsolution in deionized water (needs about 3mL; add 250uL at a time and mix well each time). Added 100 mL acetonitrile and made up the volume to 1L using deionized water. Filtered through 0.22um filter before the use.

Mobile Phase B: 20 mM phosphate, 1M NaBr in 10% acetonitrile (90% water), pH 11.

Dissolved 5.4g of sodium phosphate dibasic heptahydrate and 102.9g of sodium bromide in about 800mL deionized water. Adjusted the pH to 11 with 1N NaOH solution deionized water (needs about 13mL; mix well at each addition). Added 100 mLacetonitrile and made up the volume to 1L using deionized water. Filter through 0.22um filter before use.

Flow Rate:	1mL/minute
Column Temperature:	Room Temperature
Autosampler Temperature:	Room Temperature
DAD wavelength:	260nm

Gradients: Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0	80	20
20	25	75
21	0	100
24	0	100
25	20	80
30	20	80

Note: Phosphorothioation of RNA sequences produced diastereomeric forms of RNAs, resulting in multiple peaks in HPLC analysis. LCMS analysis, therefore, was used for the confirmation of targeted RNA as a major product. In some cases, diastereomers co-elute to show a fewer or a single peak in HPLC analysis.

Sr. No.	RNA Name	Strand	Purity (%)	Reference Figure
1	PGN6	Sense Strand	95	Figure SI-OS-1
2	PGN6	Antisense Strand	91	Figure SI-OS-2
3	PGN7	Sense Strand	89	Figure SI-OS-3
4	PGN7	Antisense Strand	86	Figure SI-OS-4
5	PGN6 (no Gem)	Sense Strand	90	Figure SI-OS-5
6	PGN6 (no Gem)	Antisense Strand	87	Figure SI-OS-6
7	PGN7 (no Gem)	Sense Strand	88	Figure SI-OS-7
8	PGN7 (no Gem)	Antisense Strand	87	Figure SI-OS-8
9	PGN7S	Sense Strand	91	Figure SI-OS-9
10	PGN7S	Antisense Strand	88	Figure SI-OS-10

Table SI-OS-T 2: RNA IEX-HPLC purity by manual integration

IEX HPLC Profiles

Diastereomers are integrated as a single HPLC peak. High-Resolution Mass Analysis confirms thepresence of diastereomers over other impurities.

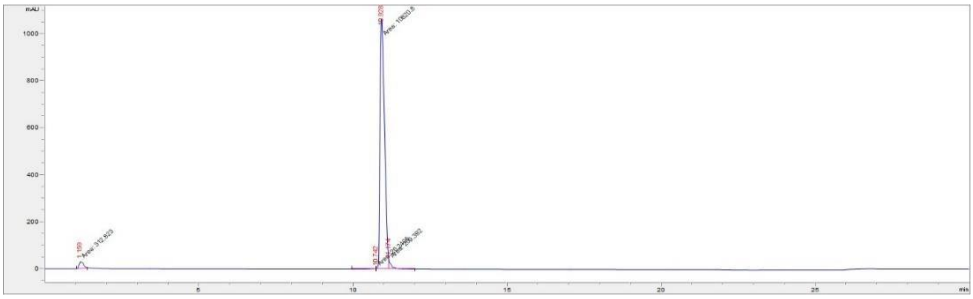

Figure SI-OS- 1: IEX -HPLC Purity Profile of PGN6 Sense Strand

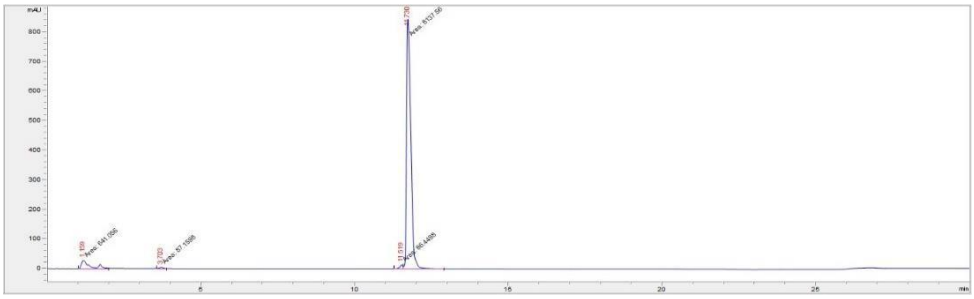

Figure SI-OS- 2: IEX -HPLC Purity Profile of PGN6 Antisense Strand

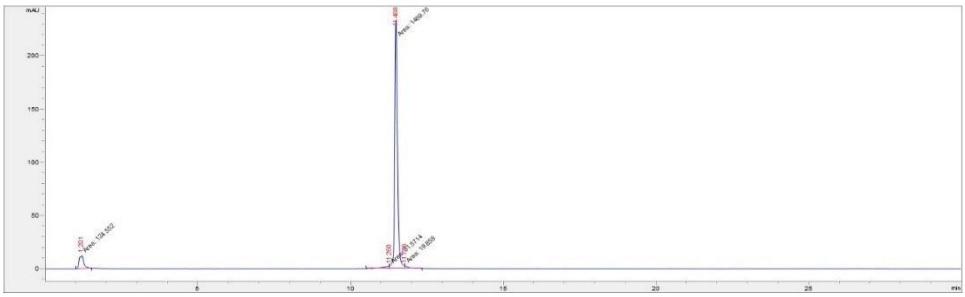

Figure SI-OS- 3: IEX -HPLC Purity Profile of PGN7 Sense Strand

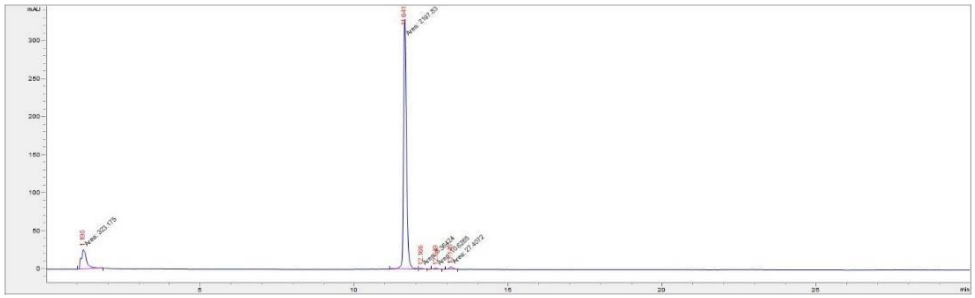

Figure SI-OS- 4: IEX -HPLC Purity Profile of PGN7 Antisense Strand

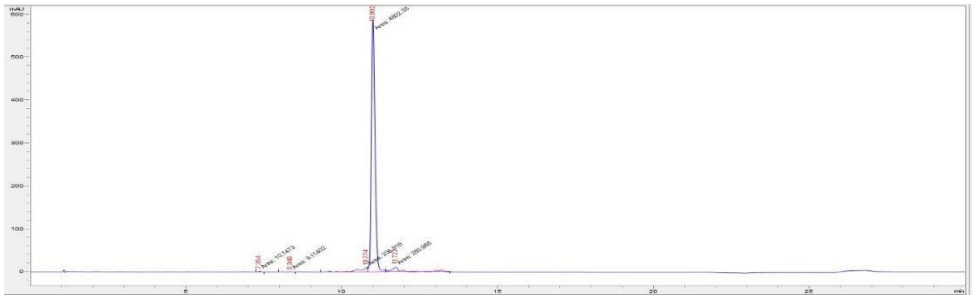

Figure SI-OS- 5: IEX -HPLC Purity Profile of PGN6 (no Gem) Sense Strand

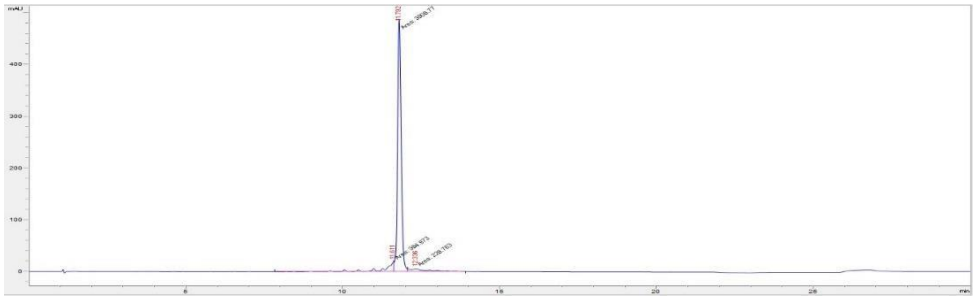

Figure SI-OS- 6: IEX -HPLC Purity Profile of PGN6 (no Gem) Antisense Strand

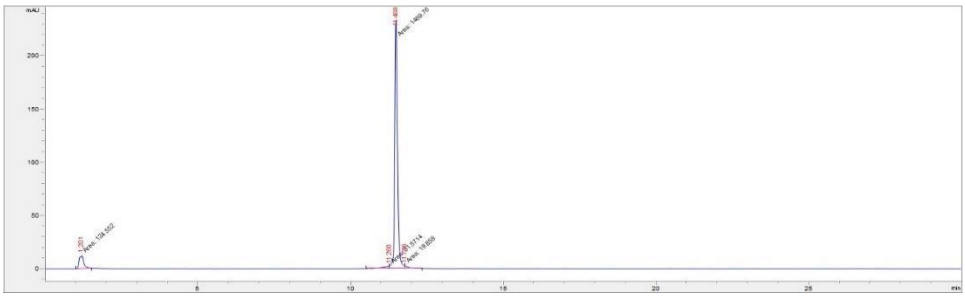

Figure SI-OS- 7: IEX -HPLC Purity Profile of PGN7 (no Gem) Sense Strand

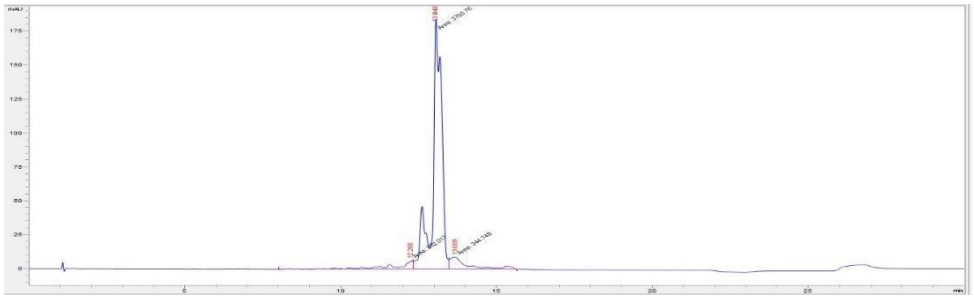

Figure SI-OS- 8: IEX -HPLC Purity Profile of PGN7 (no Gem) Antisense Strand

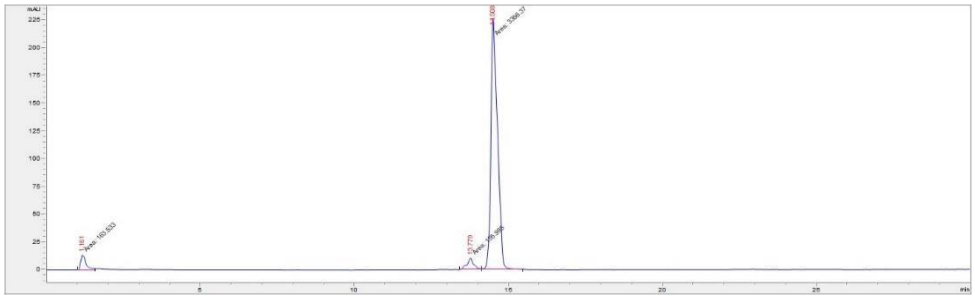

Figure SI-OS- 9: IEX -HPLC Purity Profile of PGN7S Sense Strand

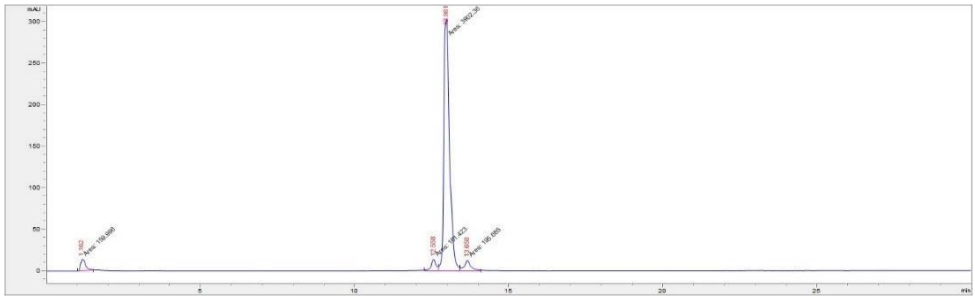

Figure SI-OS- 10: IEX -HPLC Purity Profile of PGN7S Antisense Strand

High-Resolution Mass Analysis

Mobile Phase A: 0.075% HFIP, 0.0375% TEA in water
0.469 mL (0.75 g) HFIP and 0.514 mL (0.0375 g) TEA were added to 1 L RNAase-free water.

Mobile Phase B: 0.075% HFIP, 0.0375% TEA in acetonitrile (65%) and water
0.469 mL (0.75 g) HFIP and 0.514 mL (0.0375 g) TEA were added to 0.65 L acetonitrile and 0.35 L RNAase-free water.

Flow Rate: 0.3 mL/minute
Column Temperature: Room temperature
Autosampler Temperature: 4°C
Mobile Phase Gradient: Isocratic (Mobile Phase A 20% and Mobile Phase B 80%)
LC Column Usage: For Desalting of Samples

Sr. No.	RNA Name	Strand	Calculated Molecular Weight (Da)	Observed Molecular Weight (Da)	Reference Figure
1	PGN6	Sense Strand	7010.3	7010.1	Figure SI-OS-11
2	PGN6	Antisense Strand	8001.7	8001.1	Figure SI-OS-12
3	PGN7	Sense Strand	6950.2	6950.0	Figure SI-OS-13
4	PGN7	Antisense Strand	8025.7	8025.1	Figure SI-OS-14
5	PGN6 (no Gem)	Sense Strand	6974.3	6974.2	Figure SI-OS-15
6	PGN6 (no Gem)	Antisense Strand	7351.4	7351.1	Figure SI-OS-16
7	PGN7 (no Gem)	Sense Strand	6990.1	6990.0	Figure SI-OS-17
8	PGN7 (no Gem)	Antisense Strand	7519.3	7519.0	Figure SI-OS-18
9	PGN7S	Sense Strand	7014.1	7014.0	Figure SI-OS-19
10	PGN7S	Antisense Strand	8169.6	8170.0	Figure SI-OS-20

Table SI-OS-T 3: HRMS Results Summary

High-Resolution Mass Spectra

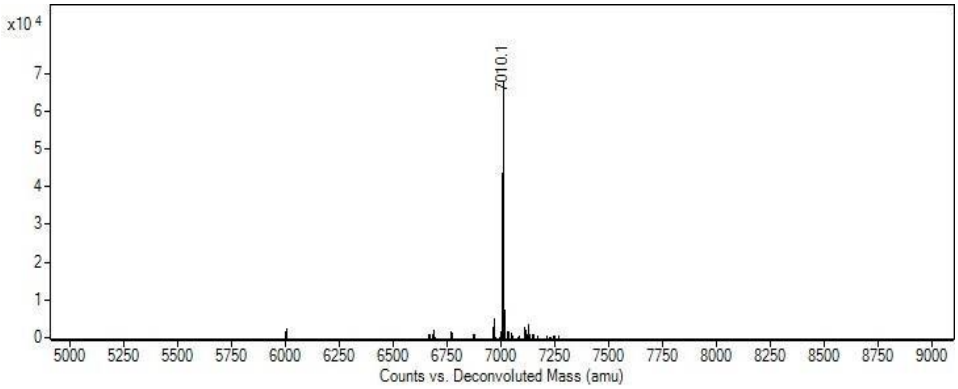

Figure SI-OS- 11: HRMS Spectrum of PGN6 Sense Strand

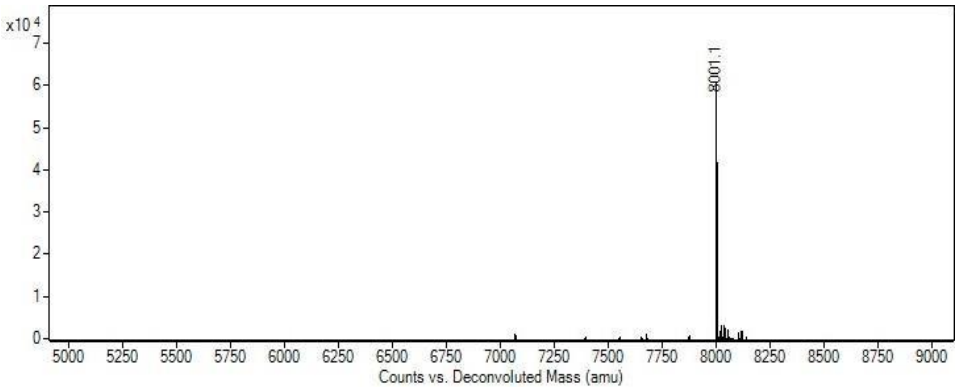

Figure SI-OS- 12: HRMS Spectrum of PGN6 antisense Strand

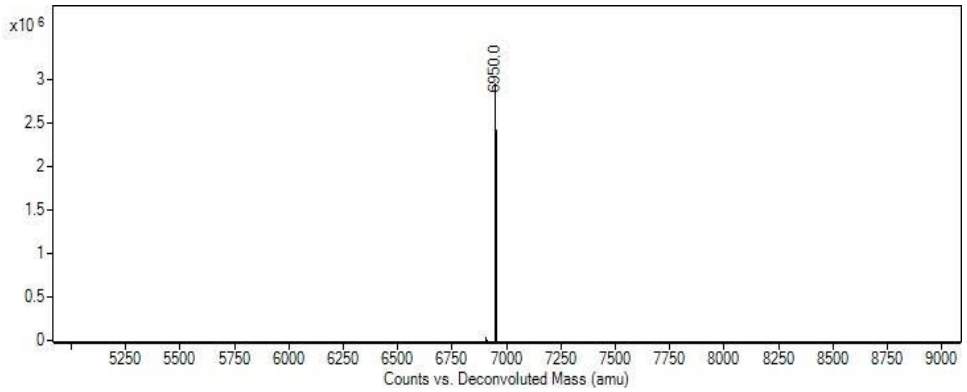

Figure SI-OS- 13: HRMS Spectrum of PGN7 Sense Strand

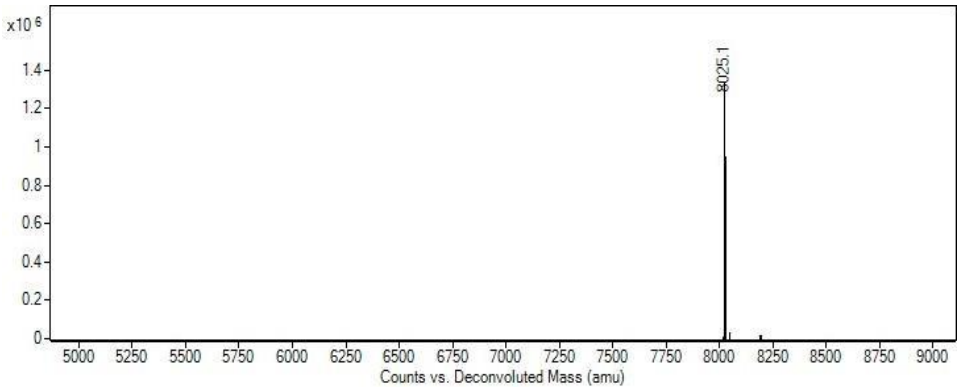

Figure SI-OS- 14: HRMS Spectrum of PGN7 Antisense Strand

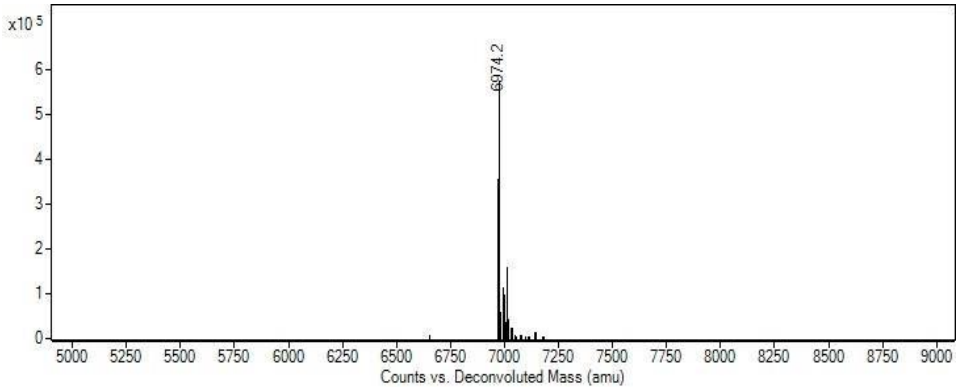

Figure SI-OS- 15: HRMS Spectrum of PGN6 (no Gem) Sense Strand

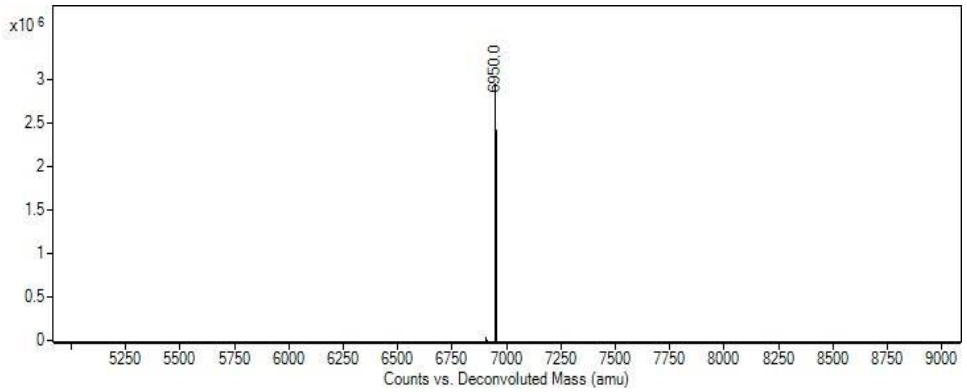

Figure SI-OS- 16: HRMS Spectrum of PGN6 (no Gem) Antisense Strand

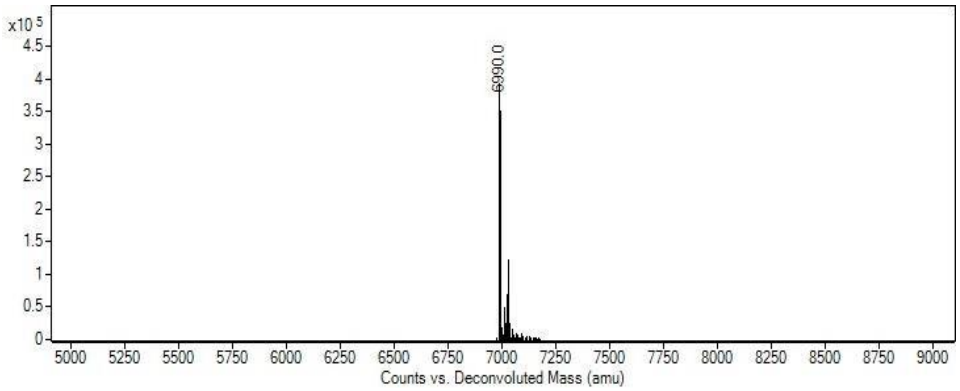

Figure SI-OS- 17: HRMS Spectrum of PGN7 (no Gem) Sense Strand

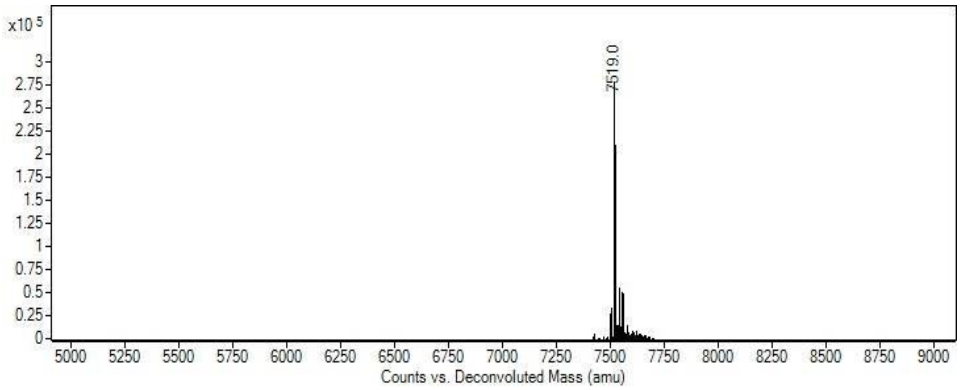

Figure SI-OS- 18: HRMS Spectrum of PGN7 (no Gem) Antisense Strand

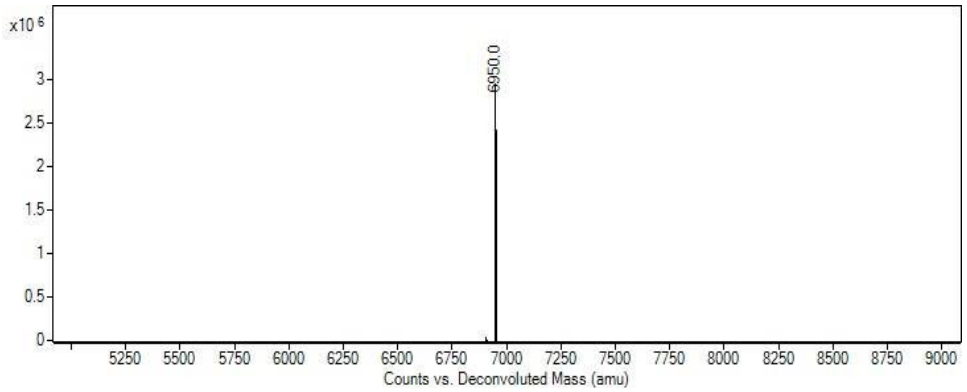

Figure SI-OS- 19: HRMS Spectrum of PGN7S Sense Strand

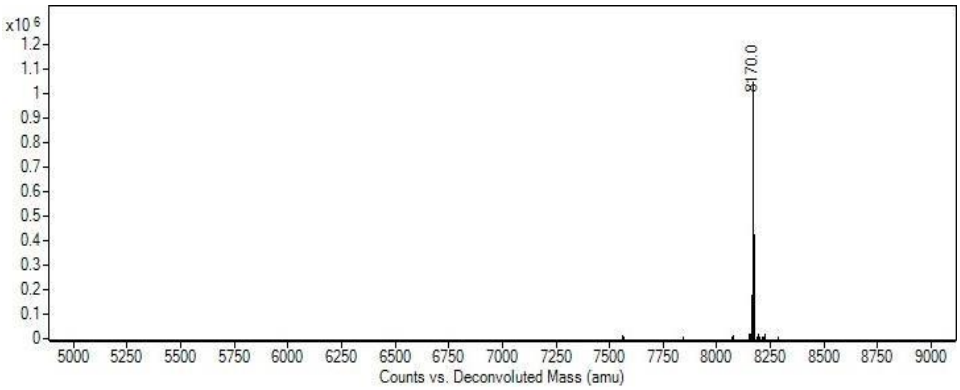

Figure SI-OS- 20: HRMS Spectrum of PGN7S Antisense Strand

Melting Temperature (Tm) Measurement

Solvent Media:	Phosphate buffered saline diluted to 1X (~ 0.01M), pH 7.4
Wavelength:	260 nm
Start Temperature:	25 °C
End Temperature:	100 °C
Return Temperature:	25 °C
Temperature Control:	In-cuvette temperature probe
Temperature Ramp Rate:	5 °C/min
Data Interval:	1 °C
Signal Averaging Time:	0.1 s
Smoothing Filter:	11
Smoothing Data Interval:	0.5 °C
Derivative Filter:	11
Derivative Data Interval:	0.5 °C

Results Tm Measurement

Name	Tm	Reference Figures
PGN6	74.6 °C	Figure SI-OS- 21 and 22
PGN7	74.0 °C	Figure SI-OS- 23 and 24
PGN6 (no Gem)	81.6 °C	Figure SI-OS- 25 and 26
PGN7 (no Gem)	80.1 °C	Figure SI-OS- 27 and 28
PGN7S	73.6 °C	Figure SI-OS-29 and 30
STD1	71.5 °C	Figure SI-OS- 31 and 32
CHK1	65.0 °C	Figure SI-OS- 33 and 34

Table SI-OS-T 4: *Tm Results*

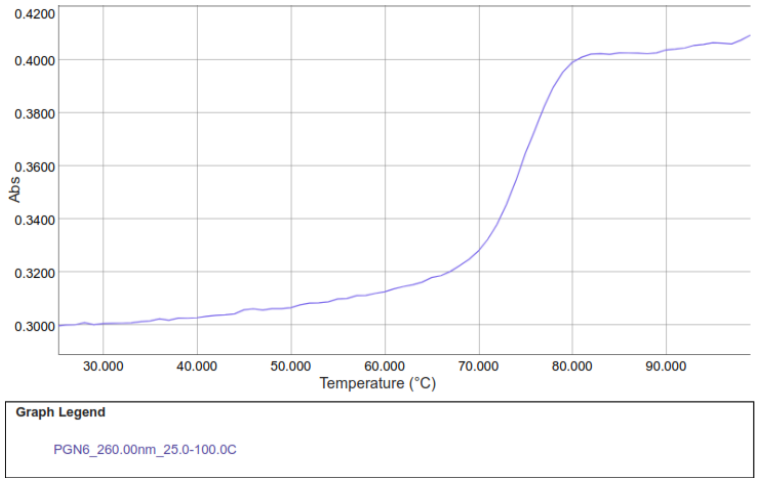

Figure SI-OS- 21: PGN6 Absorbance vs Temperature

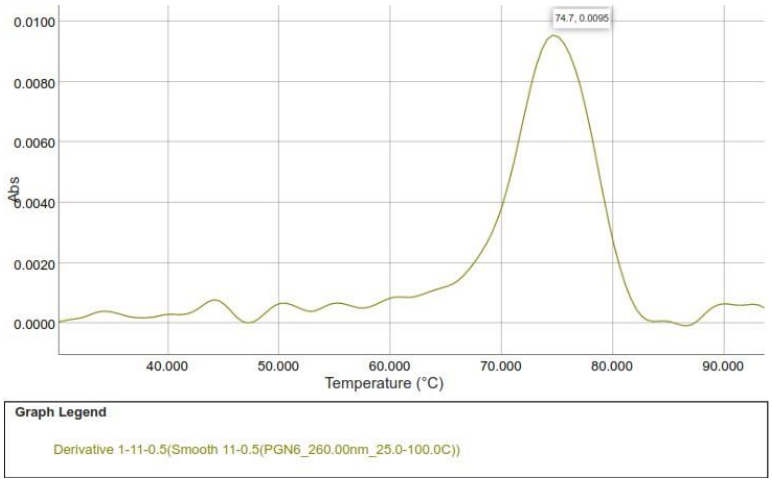

Figure SI-OS-22: PGN6 first derivative as a function of temperature ramp rate

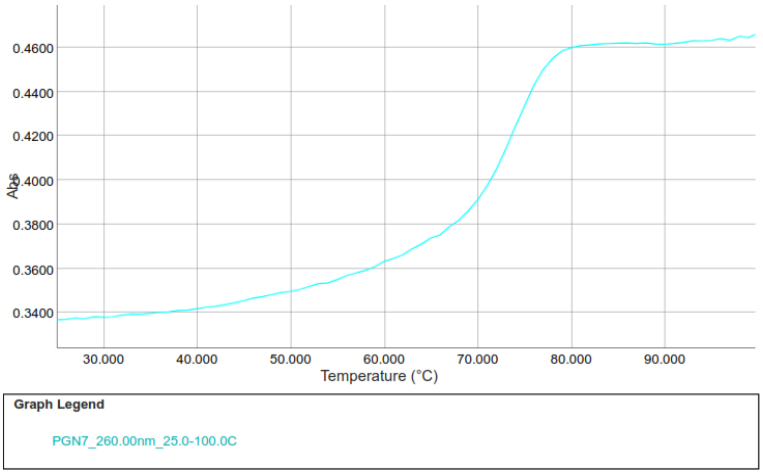

Figure SI-OS- 23: PGN7 Absorbance vs Temperature

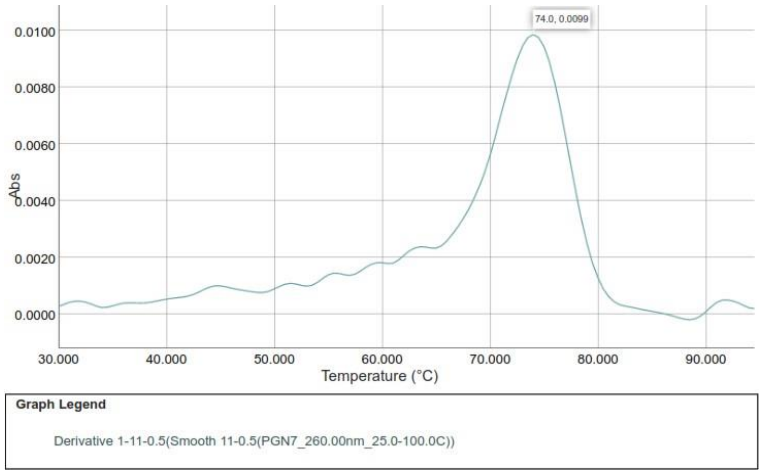

Figure SI-OS- 24: PGN7 first derivative as a function of temperature ramp rate

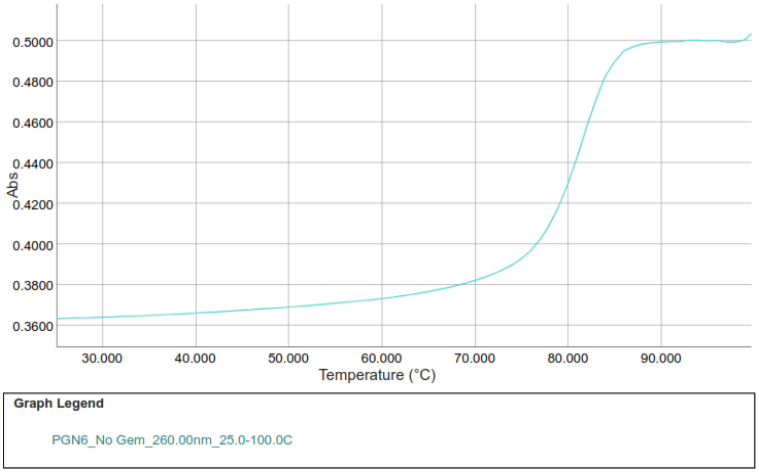

Figure SI-OS- 25: PGN6_No Gem Absorbance vs Temperature

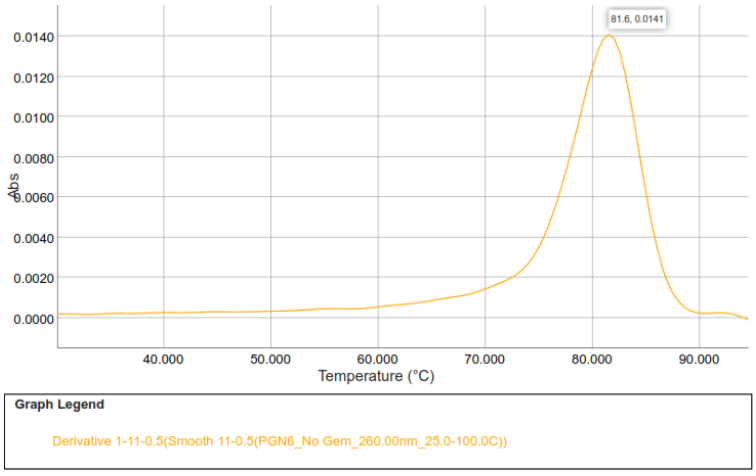

Figure SI-OS- 26: PGN6_No Gem first derivative as a function of temperature ramp rate

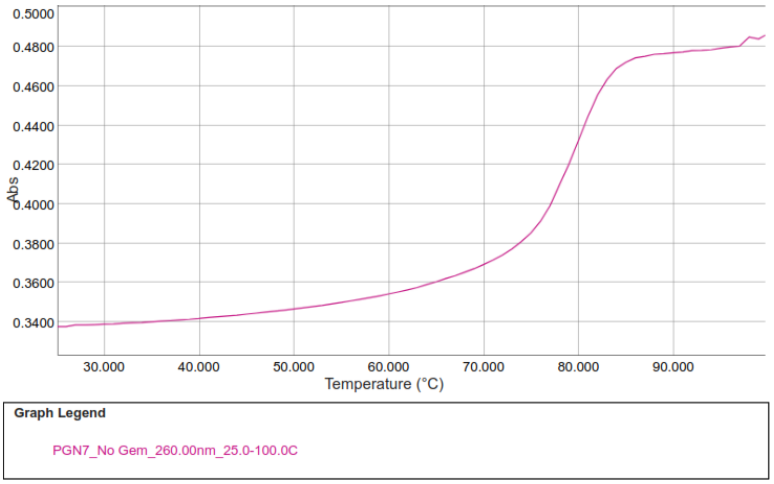

Figure SI-OS- 27: PGN7_No Gem Absorbance vs Temperature

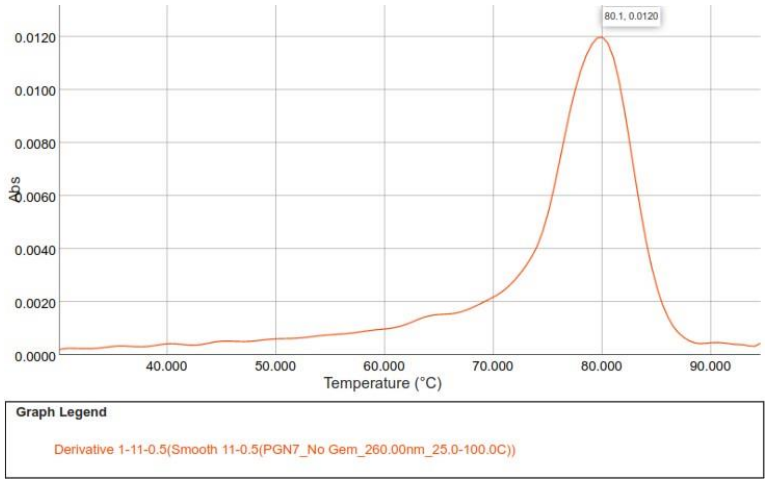

Figure SI-OS- 28: PGN7_No Gem first derivative as a function of temperature ramp rate

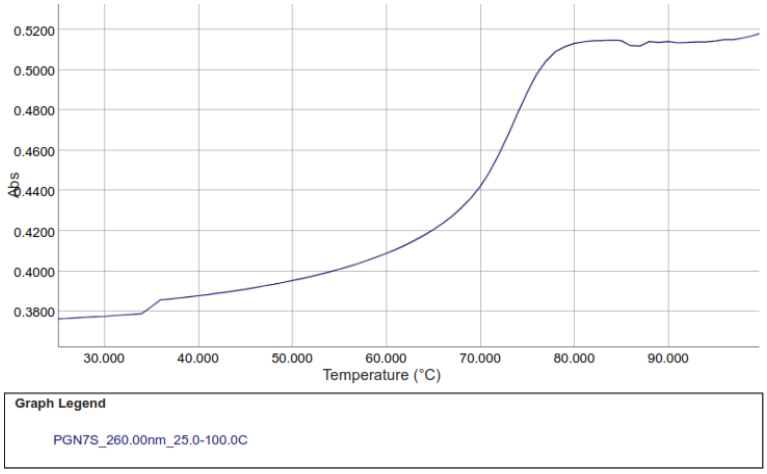

Figure SI-OS-29: PGN7S Absorbance vs Temperature

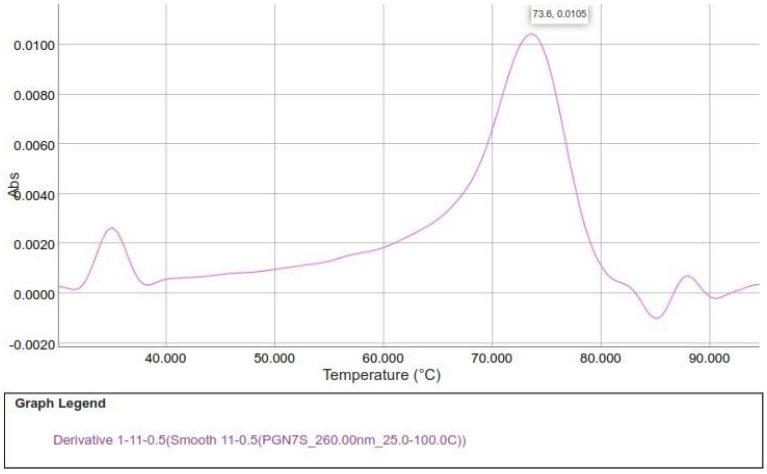

Figure SI-OS- 30: PGN7S first derivative as a function of temperature ramp rate

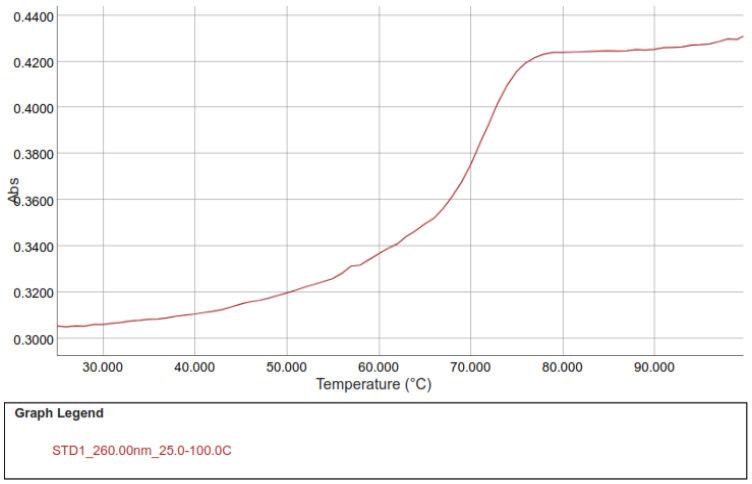

Figure SI-OS- 31: STD1 Absorbance vs Temperature

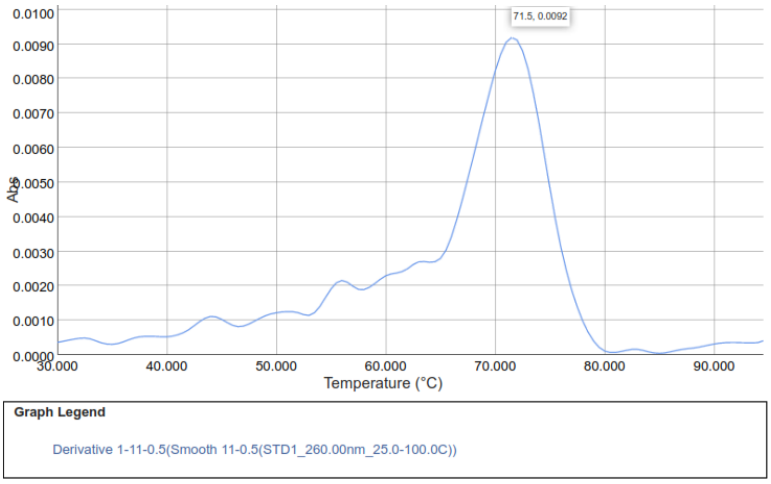

Figure SI-OS- 32: STD1 first derivative as a function of temperature ramp rate

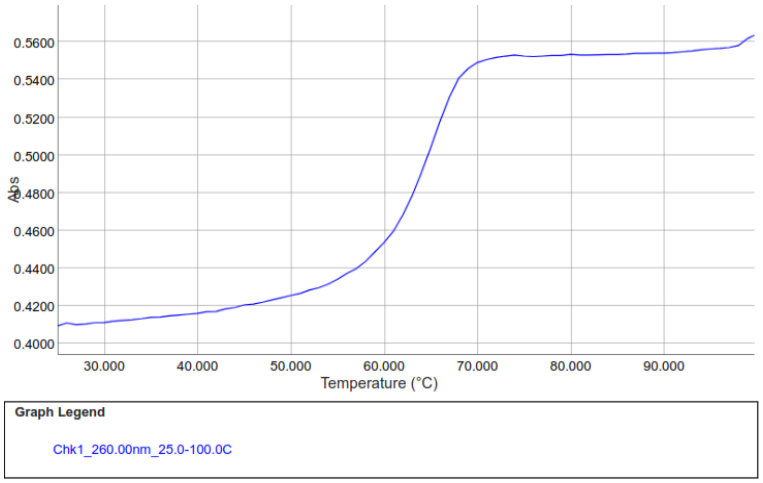

Figure SI-OS- 33: CHK1 Absorbance vs Temperature

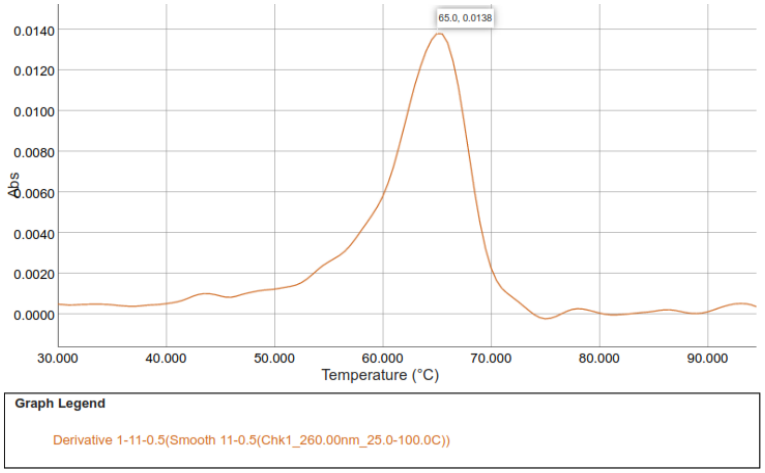

Figure SI-OS- 34: CHK1 first derivative as a function of temperature ramp rate