FLARE: A label-free FLuorescence Assisted method for RNA Engineering of three-way junctions

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Materials and methods

All the DNA sequences were either ordered as single stranded DNA from Sigma or synthesised by K&A H6 DNA/RNA synthesizer. Oligonucleotides were dissolved at 100 µM stock concentration in autoclaved Milli-Q water. The oligonucleotide stocks were stored at -80° C for long-term storage. dNTPs (DNTP100), Taq DNA Polymerase (D4545) and DFHBI (SML1627) were purchased from Sigma. MinElute PCR Purification Kit was purchased from QIAGEN (28006). In vitro transcription was carried out using Ampliscribe T7 High Yield Transcription Kit (AS3107, Lucigen) and for 2`F modified RNA transcription was carried out using DuraScribe transcription kit (DS010925). For cleavage assay Cas9 was purchased from Sigma (CAS9PROT), target eGFP dsDNA was PCR amplified from plasmid PX552 purchased from addgene (#60958). For cell viability doxorubicin was purchased from Hi-Media (TC420) and for confocal imagine cell mask was purchased from Thermo fisher scientific (C37608), DAPI was purchased from sigma (D9542). All RNAs were dissolved or resuspended in DEPC (D5758) treated water.

In vitro transcription of RNA

PCR amplified dsDNA containing T7 Promoter sequence were used as template for in-vitro transcription to synthesize RNA using Ampliscribe T7 high yield transcription kit following kit protocol. To assess the size and purity, transcription products were resolved on a denaturing polyacrylamide gel electrophoresis on an 8% gel containing 8M urea. Desired bands were excised using UV-shadowing for visualization, and purified by crush and soak method or using RNA purification columns. The concentration was determined using Nanodrop spectrophotometer.

Fluorescence Measurements

The samples for fluorescence measurements were prepared in 1 X FLARE buffer composed of 10 mM Tris, 100 mM KCl, 10 mM $MgCl_2$, pH 7.2. Individual reactions were assembled using 0.25 µM of respective RNAs and 2.5 µM of DFHBI in 1 X FLARE buffer. The reactions were incubated at RT for 30 minutes followed by fluorescence measurements using a multi-mode microplate reader (BioTek Synergy H1). Fluorescence excitation was carried out at 450 nm, and fluorescence emission was recorded at 505 nm. All measurements were performed in triplicate to ensure reproducibility of the data.

Assembly and UV-Melting analysis of three-way junction (3WJ) RNA Constructs

Three-way junction (3WJ) RNA constructs were assembled by annealing equimolar concentrations (5 µM) of each of the three oligonucleotide strands. The samples were subjected to initial denaturation at 95 °C for 5 minutes, followed by a gradual cooling phase at a rate of 1 °C per minute until ambient temperature (25 °C) was reached, for optimal hybridization and formation of the 3WJ structure. The 3WJ assembly was checked using polyacrylamide gel electrophoresis on an 12% gel. Thermal stability of the annealed 3WJ constructs (5 µM) were assessed using UV-thermal melting analysis using Agilent Cary 3500 UV-Vis Multicell Peltier spectrophotometer. Absorbance at 260 nm was monitored as a function of temperature using a controlled heating ramp from 20 °C to 90 °C at a uniform rate of 1 °C per minute. The resulting melting profiles were plotted using origin software, with temperature (°C) represented on the y-axis and absorbance at 260 nm on the x-axis.

CD-Melting analysis of three-way junction (3WJ) RNA Constructs

Circular dichroism (CD) spectra and thermal melting studies were done on a JASCO J-1500 CD spectrometer equipped with a Peltier temperature controller. The 3WJ RNA constructs (5 μM) were used for CD spectra and CD-melting studies. CD spectra were measured from 200nm to 350 nm at a scan speed of 100nm/min with a 1 nm bandwidth, 1s response time, and 1 nm data interval. Thermal melting studies were monitored at 270 nm (ellipticity) with a temperature ramp rate of at 1 °C/min over a range of 20 °C to 90 °C with continuous data collection every 1 °C. The resulting melting profiles were plotted using origin software.

In Vitro eGFP DNA Cleavage Assay using CRISPR-Cas9 system

In vitro cleavage assay was performed in a total reaction volume of 10 μL. The reaction mixture consisted of 1 X cleavage buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 6.5), 100 nM Cas9 protein, 100 nM of either wild type sgRNA or engineered 5S sgRNA, and incubated at 25 °C for 10 minutes to allow Cas9-sgRNA complex formation. Subsequently, 20 nM of target eGFP DNA was added to the reaction mixture, and the mixture was incubated at 37 °C for 2 h. The reaction was terminated by the addition of 1 μL proteinase K, followed by incubation at 56 °C for 10 minutes. Cleavage products were resolved via agarose gel electrophoresis, and DNA band intensities were quantified using the Amersham Typhoon ImageQuant imaging system software.

$$Cleavage\ efficiency\ (\%) = \frac{Intensities\ of\ cleaved\ products}{Intensities\ of\ cleaved\ products + uncleaved\ product}$$

Construction of engineered 5S triangle

Engineered 5S triangle was assembled by annealing equimolar concentration (1 μM) of each of 2` F modified RNA strands. For hybridization and formation of the engineered 5S triangle the 2` F modified RNA strands were subjected to initial denaturation at 95 °C for 5 minutes, followed by a gradual cooling phase at a rate of 1 °C per minute until 12 °C was reached. The formation of triangle was checked using agarose gel electrophoresis on 1.5% gel.

Cell Viability assay

A549 and HeLa cancer cell lines were seeded into 96-well plates. Cells were incubated at 37 °C with 5% CO_2 until 80% confluency was reached. Cells were gently washed with phosphate-buffered saline (PBS) to remove residual media and unattached cells. Actively proliferating cells were then treated with either free doxorubicin or doxorubicin-loaded engineered 5S triangle that was assembled using 2 μM Dox and 100 nM of engineered 5S triangle and incubating for 15 mins. Untreated cells and cells treated with only the engineered 5S triangle were used as controls.

Following treatment, cells were incubated for 24 hours at 37 °C with 5% CO_2. After incubation cells were treated by adding MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at a final concentration of 0.5 mg/mL to each well. Cells were incubated for an additional 4 hours at 37 °C to allow the formation of insoluble purple formazan crystals. After incubation, the medium was carefully aspirated without disturbing the formazan crystals. Dimethyl sulfoxide (DMSO) was then added to each well to solubilize the crystals. Absorbance was measured at 570 nm using a microplate reader. All experiments were performed in triplicate to ensure reproducibility and statistical significance.

Confocal Microscopy Imaging

Confocal images were taken using Leica TCS SP8 laser scanning confocal microscope equipped with a 40 X oil immersion objective. A549 cancer cells were seeded in 12-well plates with cover slip and cultured until 80% confluency was reached. The cells were then treated with doxorubicin-loaded engineered 5S triangle and incubated for 6 h. Cells were gently washed with phosphate-buffered saline (PBS), and were stained with CellMask™ membrane dye, and incubated further for 90 minutes. The cells were gently washed with phosphate-buffered saline (PBS), and cells were fixed using 4% paraformaldehyde and counterstained with DAPI to visualize nuclei. Images were recorded by excitation at 408 nm for DAPI, 488 for doxorubicin and 633 for CellMask. The respective emission wavelength ranges were 430-480 for DAPI, 580-620 for doxorubicin and 650-680 for CellMask. Acquired images were processed and analyzed using ImageJ software.

Sequences of different RNA used:

FLARE constructs used for optimization	
Main strands of FLARE:	
FLARE 1	CCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGUAGUUCGCUACUGUUGGUA GAGUGUGAGGUCCAUCAAUCAUGGCAA
FLARE 2	CCCACAUACUUUGUUGAUCAAGGACGGGUCCAGUAGUUCGCUACUGCCUAGUAGA GUGUGAGGUUCAAUCAUGGCAA
FLARE 3	CCCACAUACUUUGUUGAAAGGACGGGUCAGUAGUUCGCUACUGUUGAGUAGAGUG UGAGUCAAUCAUGGCAA
FLARE 4	UCAGCCCACAUACUUUGUUGAAAGGACGGGUCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
FLARE 5	GCAGUCAGCCCACAUACUUUGUUGAAAGGACGGGUCAGUAGUUCGCUACUGUUGA GUAGAGUGUGAGUCAAUCAUGGCAAGUGCUUGG
FLARE 6	Strand 1: CCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGU
	Strand 2: ACUACUGUUGGUAGAGUGUGAGGUCCAUCAAUCAUGGCAA
FLARE 7	Strand 1: GCAGUCAGCCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGU
	Strand 2: ACUACUGUUGGUAGAGUGUGAGGUCCAUCAAUCAUGGCAA
FLARE 8	Strand 1: GGACCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGUAG
	Strand 2: CUACUGUUGGUAGAGUGUGAGGUCCAUCAAUCAUGGCAACAC
FLARE 9	Strand 1: UCAGCCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGU
	Strand 2: ACUACUGUUGGUAGAGUGUGAGGUCCAUCAAUCAUGGCAAGUGC
FLARE 10	Strand 1: GCAGUCAGCCCACAUACUUUGUUGAUGGUCAAGGACGGGUCCAGU
	Strand 2: ACUACUGUUGGUAGAGUGUGAGGUCCAUCAAUCAUGGCAAGUGCUUGG
Upper strands of FLARE:	
FLARE 1, 2, 3, & 6	UUGCCAUGUGUAUGUGGG
FLARE 4 & 9	GCACUUGCCAUGUGUAUGUGGGCUGA
FLARE 5 & 10	CCAAGCACUUGCCAUGUGUAUGUGGGCUGACUGC
FLARE 7	UUGCCAUGUGUAUGUGGGCUGACUGC
FLARE 8	GUGUUGCCAUGUGUAUGUGGGUCC

Mismatches and modifications used in upper strand of FLARE 4	
Upper strand FLARE 4	GCACUUGCCAUGUGUAUGUGGGCUGA
Mismatches used:	
MM1	GCACUUGCCUUGUGUAUGUGGGCUGA
MM2	GCACUUGCCACGUGUAUGUGGGCUGA
MM3	GCACUUGCCAUAUGUAUGUGGGCUGA
MM4	GCACUUGCCAUGUAUAUGUGGGCUGA
MM5	GCACUUGCCAUGUGCAUGUGGGCUGA
MM6	GCACUUGCCAUGUGUUUGUGGGCUGA
Nucleotide Modifications used:	
Bulge U Deleted	GCACUUGCCAUG_GUAUGUGGGCUGA
Bulge A	GCACUUGCCAUGAGUAUGUGGGCUGA
Bulge G	GCACUUGCCAUGGGUAUGUGGGCUGA
Bulge C	GCACUUGCCAUGCGUAUGUGGGCUGA

Modification used in FLARE 4 keeping upper strand of FLARE 4 unchanged	
Mod 1	UCAGCCCACAUACUUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGA GUGUGAGUCAAUCAUGGCAAGUGC
Mod 2	UCAGCCCACAUACAUUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 3	UCAGCCCACAUACGUUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 4	UCAGCCCACAUACCUUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 5	UCAGCCCACAUACUCUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 6	UCAGCCCACAUACUUAGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 7	UCAGCCCACAUACUUGGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 8	UCAGCCCACAUACUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGAG UGUGAGUCAAUCAUGGCAAGUGC
Mod 9	UCAGCCCACAUACUUUAUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAAUCAUGGCAAGUGC
Mod 10	UCAGCCCACAUACUUUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAG AGUGUGAGUCAACCAUGGCAAGUGC
Mod 1.1	UCAGCCCACAUACAUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGA GUGUGAGUCAAUCAUGGCAAGUGC
Mod 1.2	UCAGCCCACAUACGUGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGA GUGUGAGUCAAUCAUGGCAAGUGC
Mod 1.3	UCAGCCCACAUACUAGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGA GUGUGAGUCAAUCAUGGCAAGUGC
Mod 1.4	UCAGCCCACAUACUGGUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUGAGUAGA GUGUGAGUCAAUCAUGGCAAGUGC

Upper strand modifications for Mod 1	
Upper strand for Mod 1	GCACUUGCCAUGUGUAUGUGGGCUGA
Mod 1.5	GCACUUGCCAUGAGUAUGUGGGCUGA
Mod 1.6	GCACUUGCCAUGGGUAUGUGGGCUGA
Mod 1.7	GCACUUGCCAUGCGUAUGUGGGCUGA

Modification used in 5S FLARE main strand.	
5S FLARE	UCAGCCCACAAGCGUUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUG UUGAGUAGAGUGUGAGUCAAGUUCUGGCAAGUGC
5S-Mod 2	UCAGCCCACAAGCGUUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUG UUGAGUAGAGUGUGAGUCAAGCUCUGGCAAGUGC
5S-Mod 3	UCAGCCCACAAGCUUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGU UGAGUAGAGUGUGAGUCAAGUCUGGCAAGUGC
5S-Mod 4	UCAGCCCACAAGCGUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGU UGAGUAGAGUGUGAGUCAAGUUCUGGCAAGUGC
5S-Mod 5	UCAGCCCACAAGCGCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUU GAGUAGAGUGUGAGUCAAGUUCUGGCAAGUGC
5S-Mod 6	UCAGCCCACAAGCCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUUG AGUAGAGUGUGAGUCAAGUCUGGCAAGUGC
5S-Mod 11	UCAGCCCACAAGCGUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGU UGAGUAGAGUGUGAGUCAAGCUCUGGCAAGUGC
5S-Mod 12	UCAGCCCACAAGCGCUUGAAAGGACGGGUCCAGUAGUUCGCUACUGUU GAGUAGAGUGUGAGUCAAGCUCUGGCAAGUGC
5S-Mod 13	UCAGCCCACAAGCGUUCUUGAAAGGACGGGUCCAGUAGUUCGCUACUG UUGAGUAGAGUGUGAGUCAAGUCUCUGGCAAGUGC
Upper strand modifications used in 5S-FLARE	
5S-FLARE	GCACUUGCCACAUAGCUGUGGGCUGA
5S-Mod 1	GCACUUGCCAGAUAGCUUGUGGGCUGA
Upper strand modifications used for 5S-Mod 2	
5S-Mod 7	GCACUUGCCAGAUUGCUUGUGGGCUGA
5S-Mod 8	GCACUUGCCAGAUGCUUGUGGGCUGA
5S-Mod 9	GCACUUGCCAGAAGCUUGUGGGCUGA
5S-Mod 10	GCACUUGCCAGAGCUUGUGGGCUGA

RNA used for 3WJ construction for UV-T_m and thermal stability on 8M, 10 M UREA-PAGE gel electrophoresis	
Phi29 3WJ	
Phi29 3WJ-S1	CCCACAUACUUUGUUGAUCC
Phi29 3WJ-S2	GGAUCAAUCAUGGCAA
Phi29 3WJ-S3	UUGCCAUGUGUAUGUGGG
5S rRNA 3WJ	
5S rRNA 3WJ-S1	CCCACAAGCGUUCUUGAUCC
5S rRNA 3WJ-S2	GGAUCAAGUUCUGGCAA
5S rRNA 3WJ-S3	UUGCCACAUAGCUGUGGG
5S Mod 2 3WJ	
5S Mod 2 3WJ-S1	CCCACAAGCGUUCUUGAUCC
5S Mod 2 3WJ-S2	GGAUCAAGCUCUGGCAA
5S Mod 2 3WJ-S3	UUGCCAGAUAGCUUGUGGG

sgRNA sequence	
WT sgRNA	GGAGCGCACCAUCUUCUUCAGUUUUAGAGCUAGAAAUAGCAAGUUAA AAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGU GCUUUU
5S sgRNA	GGAGCGCACCAUCUUCUUCAGUUUUAGAGCUAGAAAUAGCAAGUUAA AAUAAGGCUAGUCCGUUAUCAACUUGGCCCACAAGCGUUCUUGAUCC UUUGGAUCAAGCUCUGGCAAUUUUUGCCAGAUAGCUUGUGGGCCAAG UGGCACCGAGUCGGUGCUUUU

eGFP target dsDNA sequence obtained from plasmid PX552 showing target site in bold
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACG GCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGG CAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTC GTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA**GGAGCGCACCATCTTCTTCA**A GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAAC CGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGG AGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAA GGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTAC CAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCA CCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTT CGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG

Strands used for nano-triangle construct (2' F modified)	
5S-Mod 2 Tr-A	GGAUCAAGCUCCGGGAAGAGCCUAUGCCCAUCCAGCGUUCUUGAUCC
5S-Mod 2 Tr-B	GGAUCAAGCUCCGUAUCACCAUGGGCAGUUGAGAGCGUUCUUGAUCC
5S-Mod 2 Tr-C	GGAUCAAGCUCCGGUAUUGGACGGCCUCGCAUGAGCGUUCUUGAUCC
5S-Mod 2 Tr-D	GCCGUCCAAUACCGGAUAGCUGGAUGGGCAUAGGCUCUUCCCGGAUA GCUCUCAACUGCCCAUGGUGAUACGGAUAGCUCAUGCGAG

RNAs used for 3WJ construction for UV-T_m (to compare with FLARE Fluorescence)	
Mod 1 3WJ	
Mod 1 3WJ-S1	CCCACAUACUUGUUGAUCC
Mod 1 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 1 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 2 3WJ	
Mod 2 3WJ-S1	CCCACAUACAUUGUUGAUCC
Mod 2 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 2 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 3 3WJ	
Mod 3 3WJ-S1	CCCACAUACGUUGUUGAUCC
Mod 3 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 3 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 4 3WJ	
Mod 4 3WJ-S1	CCCACAUACCUUGUUGAUCC
Mod 4 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 4 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 5 3WJ	
Mod 5 3WJ-S1	CCCACAUACUCUGUUGAUCC
Mod 5 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 5 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 6 3WJ	
Mod 6 3WJ-S1	CCCACAUACUUAGUUGAUCC
Mod 6 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 6 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 7 3WJ	
Mod 7 3WJ-S1	CCCACAUACUUGGUUGAUCC
Mod 7 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 7 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 8 3WJ	
Mod 8 3WJ-S1	CCCACAUACUGUUGAUCC
Mod 8 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 8 3WJ-S3	UUGCCAUGUGUAUGUGGG

Mod 9 3WJ	
Mod 9 3WJ-S1	CCCACAUACUUUAUUGAUCC
Mod 9 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 9 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 10 3WJ	
Mod 10 3WJ-S1	CCCACAUACUUUGUUGAUCC
Mod 10 3WJ-S2	GGAUCAACCAUGGCAA
Mod 10 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 1.1 3WJ	
Mod 1.1 3WJ-S1	CCCACAUACAUGUUGAUCC
Mod 1.1 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 1.1 3WJ-S3	UUGCCAUGUGUAUGUGGG
Mod 1.3 3WJ	
Mod 1.3 3WJ-S1	CCCACAUACGUGUUGAUCC
Mod 1.3 3WJ-S2	GGAUCAAUCAUGGCAA
Mod 1.3 3WJ-S3	UUGCCAUGUGUAUGUGGG
5S Mod 1 3WJ	
5S Mod 1 3WJ-S1	CCCACAAGCGUUCUUGAUCC
5S Mod 1 3WJ-S2	GGAUCAAGUUCUGGCAA
5S Mod 1 3WJ-S3	UUGCCAGAUAGCUUGUGGG
5S Mod 4 3WJ	
5S Mod 4 3WJ-S1	CCCACAAGCGUCUUGAUCC
5S Mod 4 3WJ-S2	GGAUCAAGUUCUGGCAA
5S Mod 4 3WJ-S3	UUGCCAGAUAGCUUGUGGG
5S Mod 5 3WJ	
5S Mod 5 3WJ-S1	CCCACAAGCGCUUGAUCC
5S Mod 5 3WJ-S2	GGAUCAAGUUCUGGCAA
5S Mod 5 3WJ-S3	UUGCCAGAUAGCUUGUGGG
5S Mod 11 3WJ	
5S Mod 11 3WJ-S1	CCCACAAGCGUCUUGAUCC
5S Mod 11 3WJ-S2	GGAUCAAGCUCUGGCAA
5S Mod 11 3WJ-S3	UUGCCAGAUAGCUUGUGGG
5S Mod 12 3WJ	
5S Mod 12 3WJ-S1	CCCACAAGCGCUUGAUCC
5S Mod 12 3WJ-S2	GGAUCAAGCUCUGGCAA
5S Mod 12 3WJ-S3	UUGCCAGAUAGCUUGUGGG

Figure S1: Assessing FLARE functionality for upper strand U-bulge deleted (U_{del}) 3WJ.

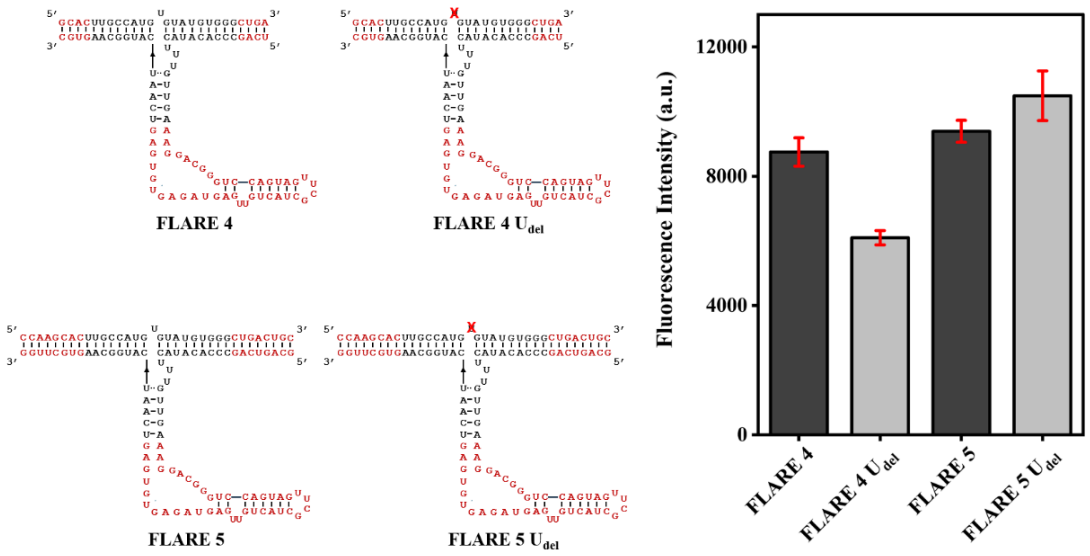

Figure S1: Fluorescence response of FLARE 4 and FLARE 5 using U-bulge deleted (U_{del}) 3WJ. FLARE 4 exhibited a significant decrease in fluorescence intensity correlating with the literature findings.

Deletion of the U bulge in upper strand of the Phi29 3WJ destabilize the 3WJ.[1-2] Thus, FLARE 4 and FLARE 5 were compared to assess their functionality utilizing upper strand U-bulge deleted (U_{del}) 3WJs.

Figure S2: Thermal melting curves of wild-type and engineered phi29 3WJ

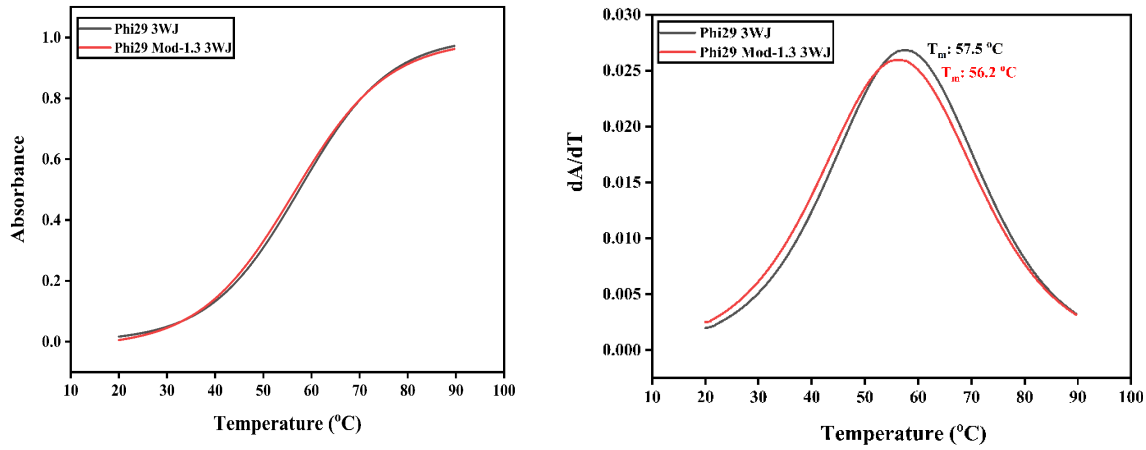

Figure S2: Sigmoidal and derivative curve showing UV melting temperatures of Phi29 3WJ and Phi29 Mod 1.3 3WJ

Figure S3: Step-wise assembly of wild-type and engineered 3WJ's

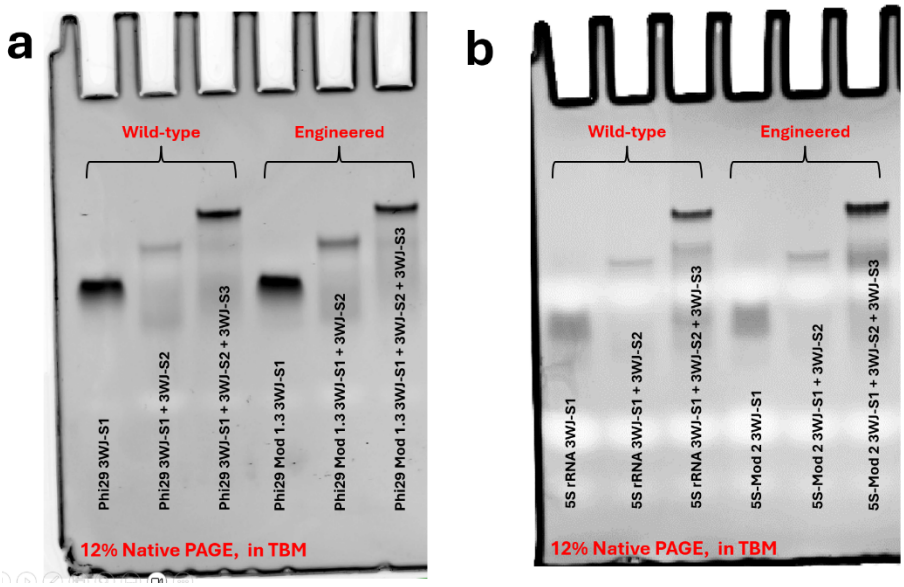

Figure S3: Step-wise assembly of Phi29 3WJ, Phi29 Mod 1.3 3WJ, 5S rRNA 3WJ and 5S-Mod 2 3WJ. Gels were run in TBM buffer on 12% polyacrylamide gel.

Figure S4: Temperature dependent CD spectra of Phi29 3WJ, 5S rRNA 3WJ and engineered 5S Mod 2 3WJ

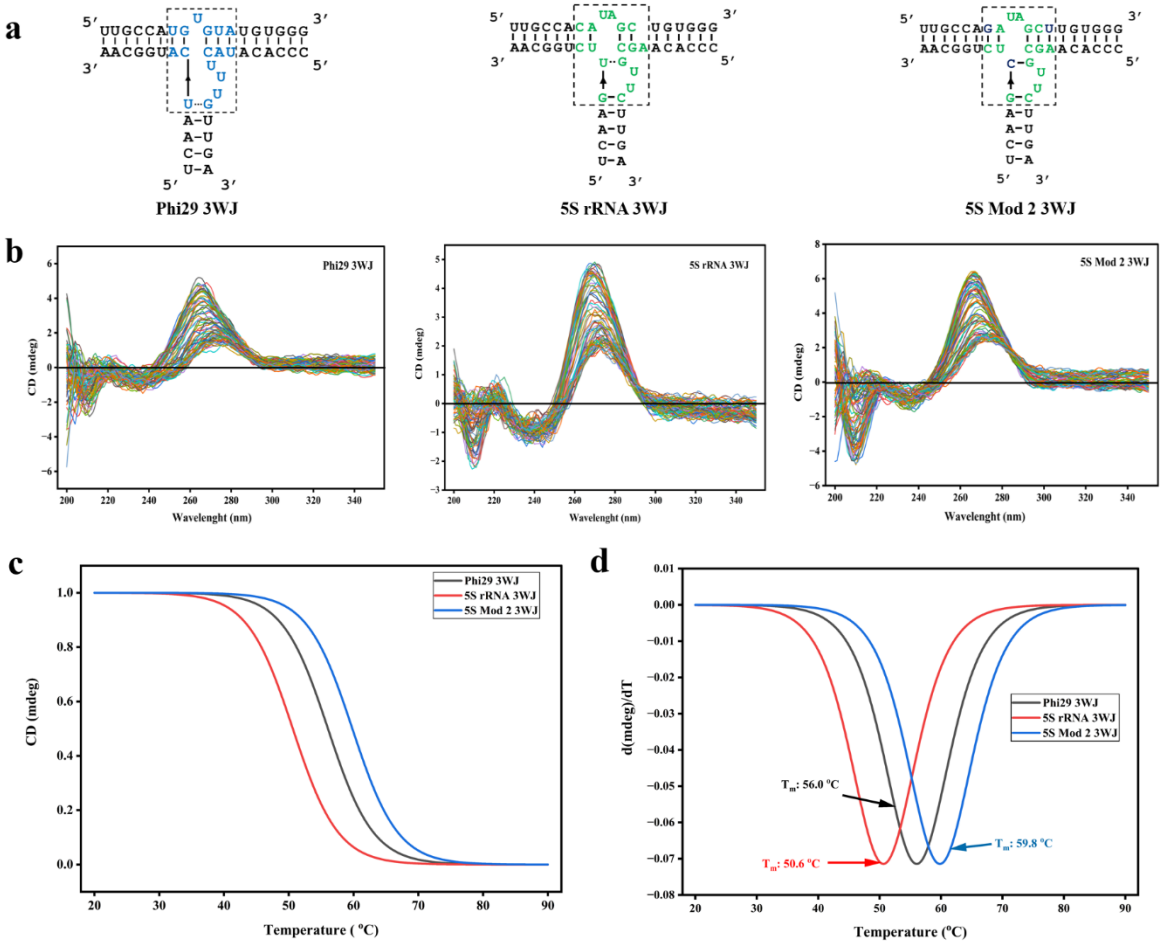

Figure S4: a) Sequence of Phi29 3WJ, 5S rRNA 3WJ and engineered 5S Mod 2 3WJ and b) their corresponding overlaid CD spectra obtained at different temperatures ranging from 20°C to 90°C showing a positive intense band around 265 nm and two negative bands around 240 nm and 210 nm indicative of an A-type helical structure c) CD thermal melting curves of Phi29 3WJ, 5S rRNA 3WJ and engineered 5S Mod 2 3WJ d) Derivative plots of CD melting curves Phi29 3WJ, 5S rRNA 3WJ and engineered 5S Mod 2 3WJ

Figure S5: Thermal melting curves of engineered Phi29 3WJ and engineered 5S rRNA 3WJ

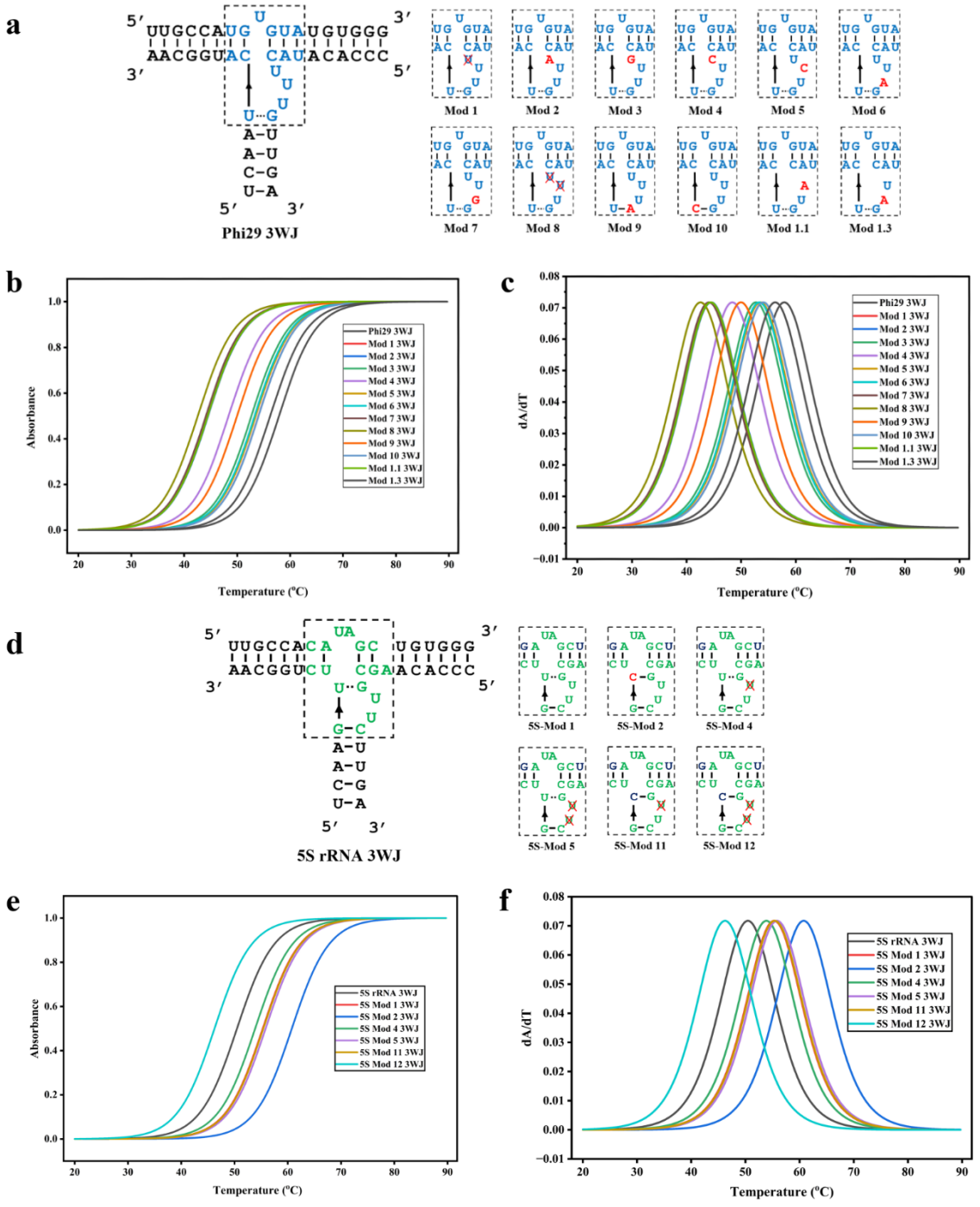

Figure S5: a) Sequences of isolated Phi29 3WJ and its modified constructs. b) & c) UV melting profiles showing sigmoidal and corresponding derivative curves, respectively of all the Phi29 3WJ constructs. d) Sequences of isolated 5S rRNA 3WJ and its modified constructs. e) & f) UV melting profiles showing sigmoidal and corresponding derivative curves, respectively of all the 5S rRNA 3WJ constructs.

Table 1: Head-to-head comparison of Fluorescence fold vs UV-T_m of selected 3WJs

S/N	Modification	FLARE Fluorescence fold (descending order)	UV-T_m (^oC) of isolated 3WJs
Phi29			
1	Phi29	9.6	57.5 ± 0.7
2	Mod 1.3	8.9	56.2 ± 0.7
3	Mod 1	8.8	54 ± 0.6
4	Mod 5	8.4	53.5 ± 0.7
5	Mod 3	7.8	52.6 ± 0.7
6	Mod 10	7.7	54 ± 0.5
7	Mod 6	6.3	53.2 ± 0.7
8	Mod 4	3.2	48.3 ± 1.1
9	Mod 9	3.2	49.9 ± 0.5
10	Mod 7	1.8	44 ± 1.2
11	Mod 2	1.5	44 ± 0.6
12	Mod 8	1.3	42.5 ± 1.2
13	Mod 1.1	1.1	44.4 ± 0.7
5S rRNA			
14	5S Mod 2	10.1	60.8 ± 0.6
15	5S Mod 11	7.5	55.4 ± 0.7
16	5S Mod 5	6.1	55.8 ± 0.6
17	5S Mod 4	4.8	53.8 ± 0.8
18	5S Mod 12	2.4	46 ± 0.6
19	5S Mod 1	2.2	55.2 ± 0.7
20	5S rRNA	1.8	50.4 ± 07

Figure S6: 8M and 10M urea stability of wild-type and engineered 3WJ's

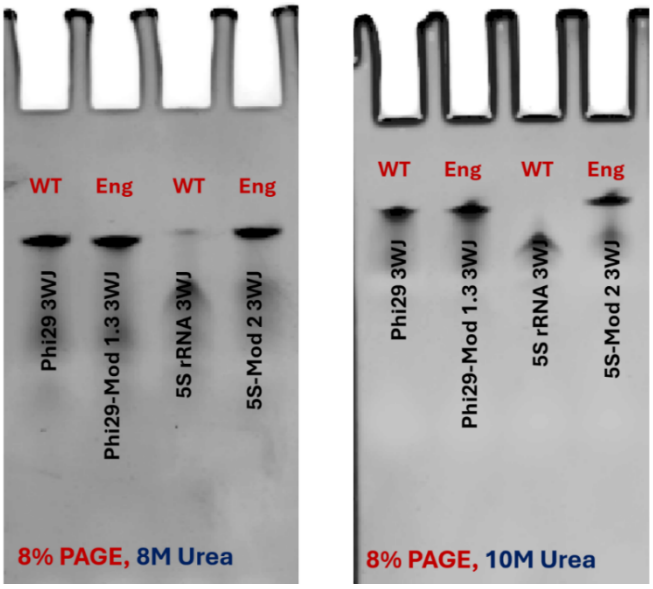

Figure S6: 8M and 10M Urea denaturation gel of Phi29 3WJ, Phi29 Mod 1.3 3WJ, 5S rRNA 3WJ and 5S-Mod 2 3WJ

Figure S7: Secondary structure prediction and thermodynamic parameters of WT sgRNA and 5S sgRNA

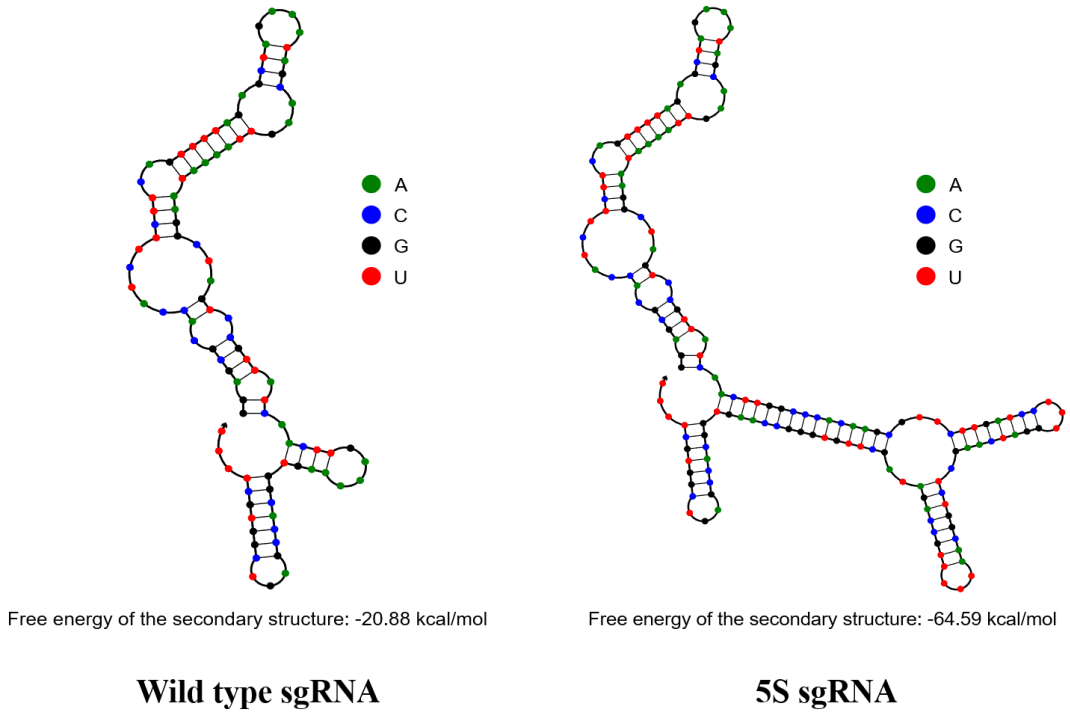

Figure S7: Secondary structure prediction and minimum free energy of WT sgRNA and 5S sgRNA using NUPACK webtool for predicting RNA folding.

Figure S8: To determine hydrodynamic size of the engineered 5S triangle.

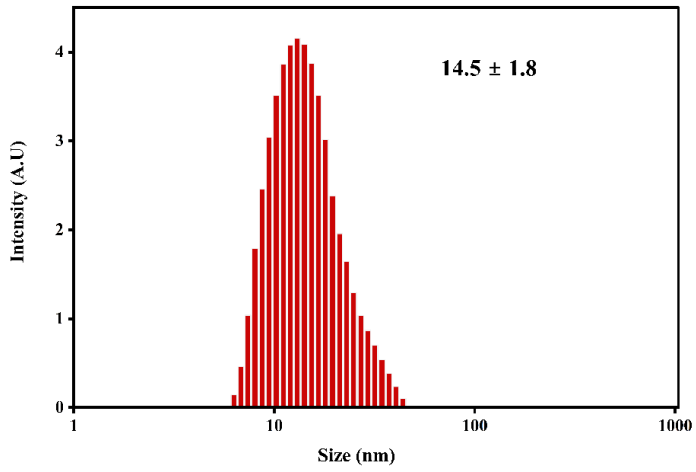

Figure S8: DLS graph showing the size distribution of engineered 5S triangle.

Figure S9: To calculate loading efficiency of doxorubicin on the engineered 5S triangle

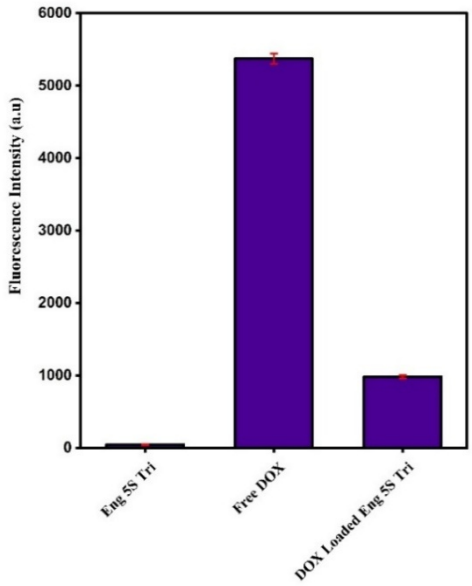

Figure S9: Bar graph showing fluorescence intensities of engineered 5S triangle, Free doxorubicin and doxorubicin loaded engineered 5S triangle.

$$\text{Loading efficiency} = \frac{\text{FI of free dox} - \text{FI of dox loaded Eng 5S tri}}{\text{FI of free dox}} * 100$$

Figure S10: Schematic representation 5S sgRNA

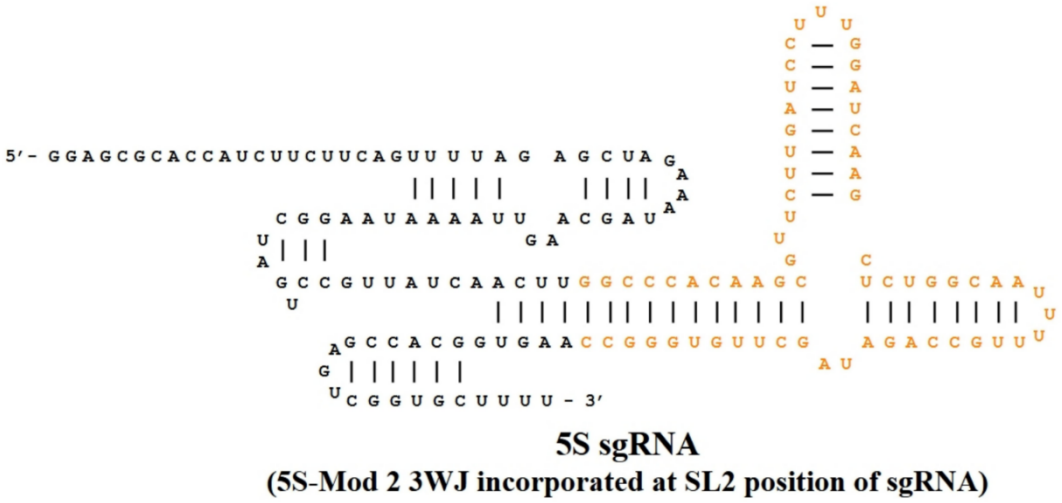

Figure S10: 5S sgRNA nucleotide sequence, orange colour indicates closed 5S-Mod 2 3WJ region at SL2 position of sgRNA

Figure S11: Schematic representation of engineered 5S triangle

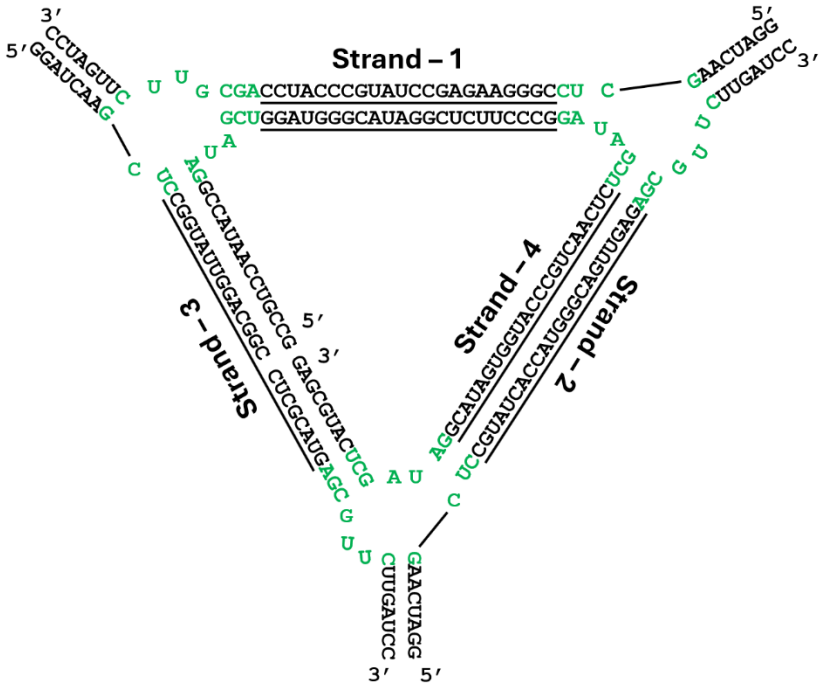

Figure S11: Engineered 5S triangle illustration showing all the sequence detail, highlighting the engineered 5S-core nucleotide sequence in green.

1. Hill, A. C.; Schroeder, S. J., Thermodynamic stabilities of three-way junction nanomotifs in prohead RNA. *RNA* **2017,** *23* (4), 521-529.
2. Shu, D.; Shu, Y.; Haque, F.; Abdelmawla, S.; Guo, P., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics. *Nature nanotechnology* **2011,** *6* (10), 658-667.