Electronic Supplementary Material (ESI) for RSC Advances.
This journal is © The Royal Society of Chemistry 2016

Electronic Supplementary Information

Azobenzene-modified antisense oligonucleotides for site-specific cleavage of RNA with photocontrollable property

Xingyu Wang,[†, §] and Xingguo Liang[†, *]

[†]School of Food Science and Engineering, Ocean University of China, Yushan Road No.5, Qingdao, Shandong, P.R.China

[§] College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi Changan Road No.620, Xi'an, Shaanxi, China

* To whom correspondence should be addressed. Tel/Fax: +86 532 82031086; Email: liangxg@ouc.edu.cn

Content

1. **Photoresponsive property of Azo-ASOs before and after UV irradiation.**

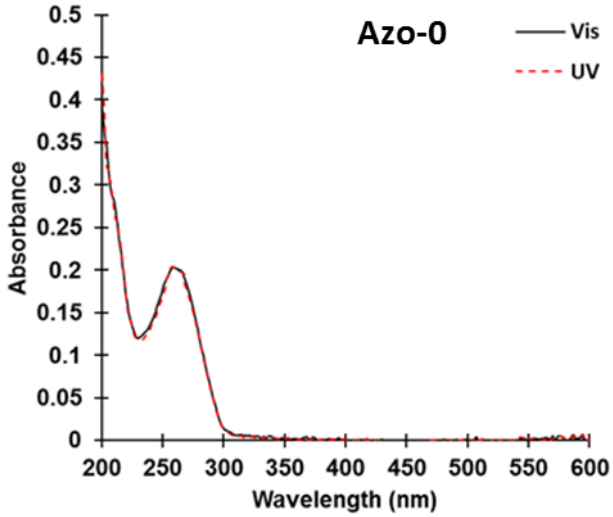
Materials: Azobenzene-modified oligonucleotides were synthesized and purified by Nihon Techno Service Co. LTD (Ibaraki, Japan). There sequences can be found in Table S1. DEPC treated water was purchased from Tiangen Biotechnology Co. LTD (Beijing, China). All other chemical reagents was provided by local commercial suppliers, and are of analytical grade.

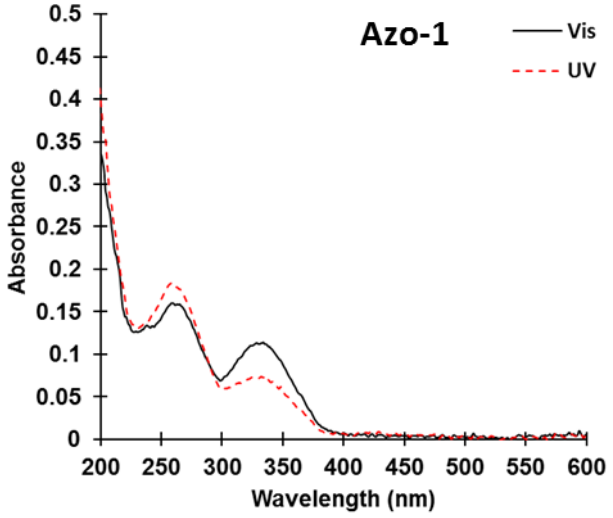
Table S1 Sequence of oligomers used in this study [a]

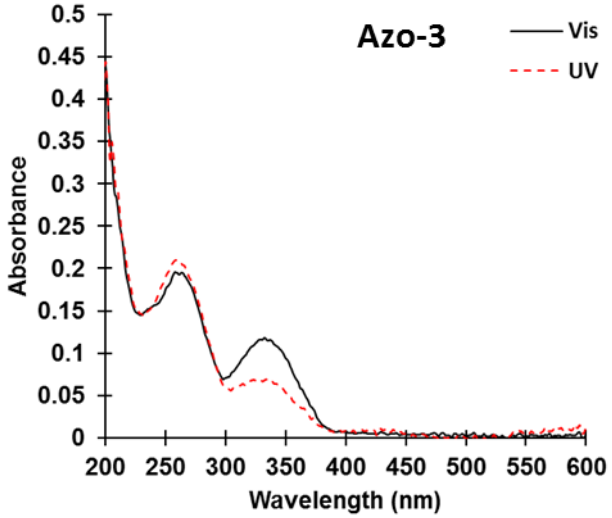
Name	Sequence (5' →3')	Length of oligonucleotides
RNA substrate	FITC-AAAAAGUAAAUAUUGGCGUAGCAGCACGU	29 nt
Azo-0	TGCTACGCCA	10 nt
Azo-1	TG**X**CT**X**AC**X**GC**X**CA	10 nt
Azo-2	T**X**GC**X**TA**X**CGCCA	10 nt
Azo-3	**X**TG**X**CT**X**ACGCCA	10 nt
Azo-4	T**X**G**X**CT**X**ACGCCA	10 nt
Azo-5	T**X**G**X**C**X**T**X**ACGCCA	10 nt
Azo-6	TG**X**CT**X**ACGCCA	10 nt
Azo-7	C**X**G**X**T**X**G**X**C**X**T**X**G**X**CT**X**ACGCCA	15 nt
Azo-8	TGCT**X**ACGCCA	10 nt
Azo-9	TGC**X**TACGCCA	10 nt
Azo-10	TG**X**CT**X**ACGCCA**X**AT**X**AT	14 nt
Azo-11	TG**X**CT**X**ACGCCAA**X**TA**X**T	14 nt

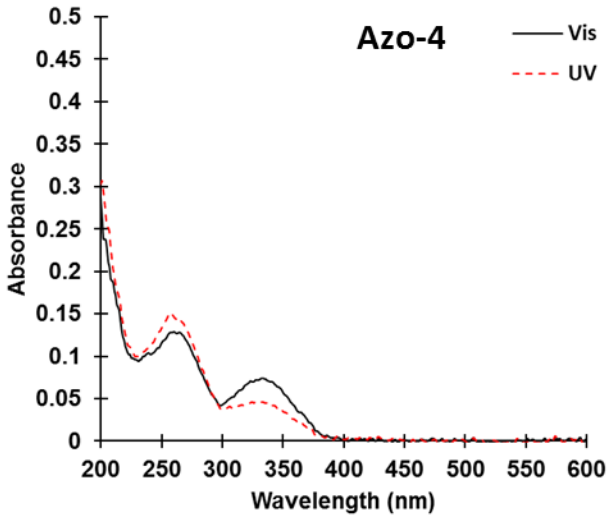
[a] "FITC" represents fluorescein isothiocyanate modification. The bold character "X" indicates azobenzene moiety inserted between bases.

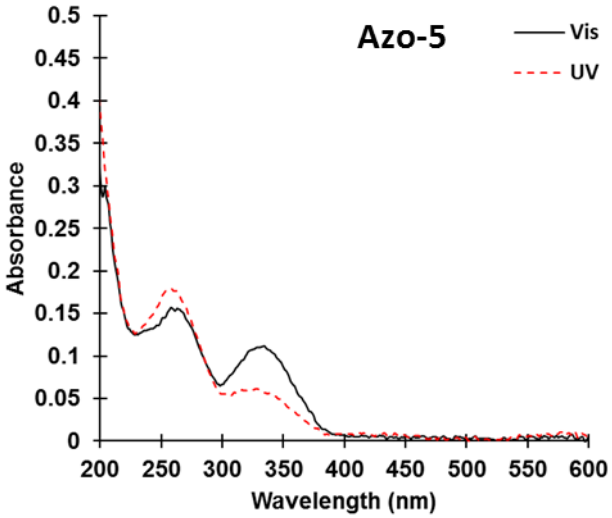
Method: Each Azo-ASO was dissolved in total volume of 500 μL buffer containing 50 mM Tris-HCl (pH=7.5), 2 mM EDTA, and the final concentration of Azo-ASO was 10 μM. The solution was pipetted into a quartz cuvette and loaded on the UV spectrophotometer. The absorbance spectra was obtained by scanning adsorption peaks from 200 nm to 600 nm with the accuracy of 1 nm. To measuring absorbance profile of Azo-ASO after UV irradiation, the solution was exposed to UV light at 60 ℃ for 10 min using a 8-W UV lamp (intensity of UV light was below 91.9 μW cm^{-2}), followed by wavelength scanning (200 nm ~ 600 nm) under the same conditions.

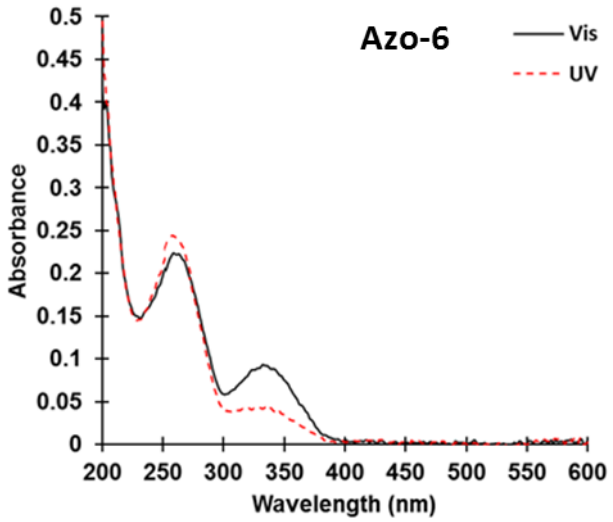

Azo-0
Vis
UV
Absorbance
Wavelength (nm)


Azo-1
Vis
UV
Absorbance
Wavelength (nm)


Azo-3
Vis
UV
Absorbance
Wavelength (nm)


Azo-4
Vis
UV
Absorbance
Wavelength (nm)


Azo-5
Vis
UV
Absorbance
Wavelength (nm)


Azo-6
Vis
UV
Absorbance
Wavelength (nm)

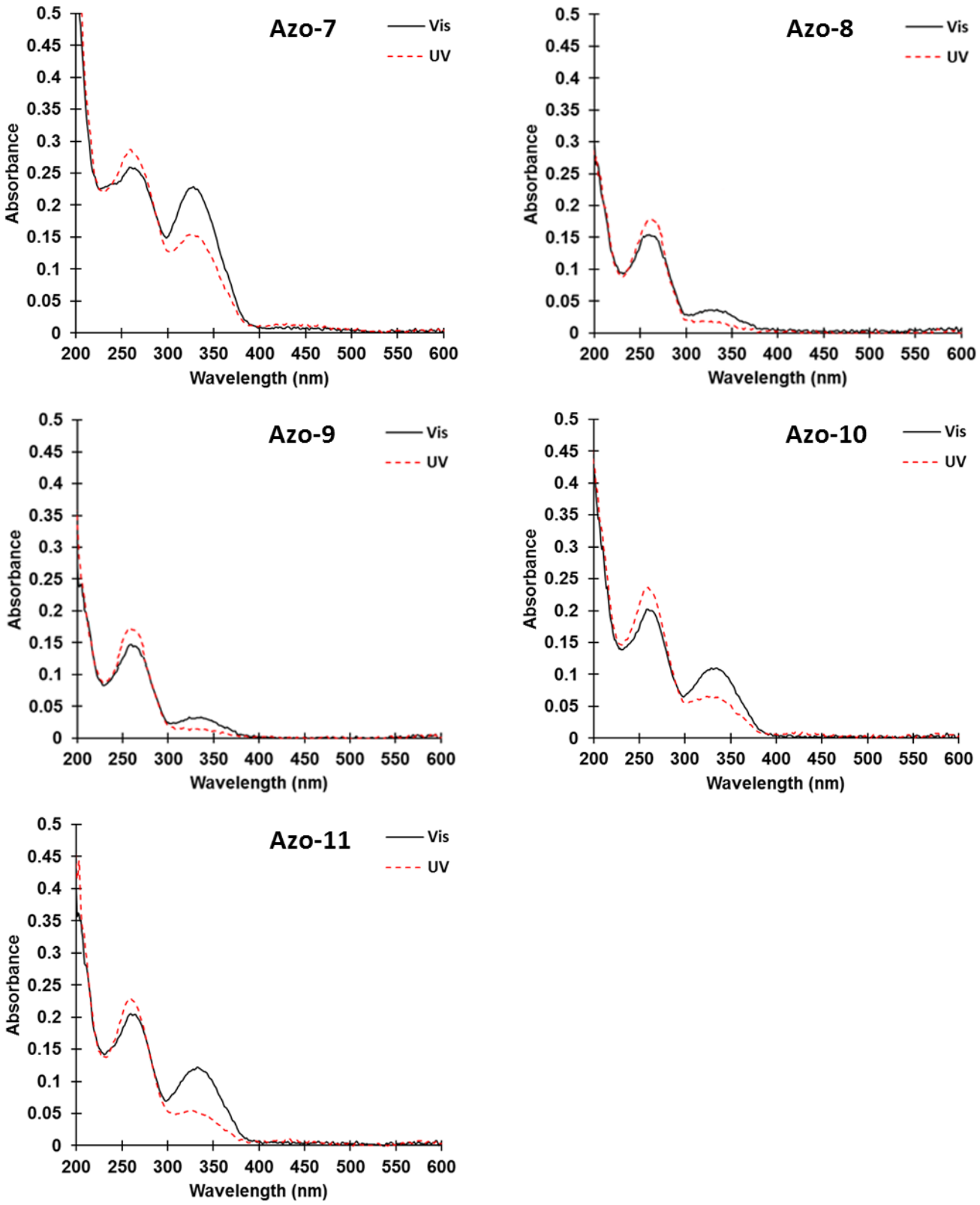


Figure S1. Absorbance spectra of Azo-ASOs under visible or UV light irradiation. The solid black lines represent adsorption profile when azobenzene unit adopts *trans* from, the red dotted lines indicate absorbance spectra when some azobenzene moieties are in *cis* form induced by UV irradiation.

2. **Detailed procedure of RNase H assay mediated by azobenzene-modified ASO**

Materials: Azobenzene-modified oligonucleotides were synthesized and purified by Nihon Techno Service Co. LTD (Ibaraki, Japan). FITC-labelled RNA oligomers used as sense sequence were obtained from Genescript Biotechnology Co. LTD (Nanjing, China). Common oligomers were provided by Sangon Biological Engineering Technology Co. LTD (Shanghai, China). Sequence of all the oligomers used in this study were listed in Table S1. Ribolock^{TM} RNase inhibitor, RNase H and RNase free water were purchased from Thermo Scientific Co. LTD (Beijing, China). All solutions were prepared using Milli-Q purified water (resistance of 18.2 MΩ cm^{-1}).

Protocol of RNA cleavage by Azo-ASO and RNase H: Azo-ASO of 0.05 μM, RNA substrate of 1.0 μM and 2.0 U RNase H were mixed in 10 μL of 1 × RNase H buffer (20 mM Tris-HCl pH=7.8, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) containing 10 U RNase inhibitor (not inhibit RNase H), the resulting mixture was incubated at 37 ℃ for 40 min, followed by inactivation of RNase H at 65 ℃ for 10 min. The cleavage products were placed on ice before subjected to electrophoresis on 20% denaturing PAGE gel containing 8 M urea. After electrophoresis, the gel was photographed under ultraviolet illumination at 494 nm with a Bio-rad molecular imager.

Protocol for photoregulated RNA cleavage: The RNase H assays were carried out as following: A mixture containing 0.05 μM Azo-ASO, 10 U RNase inhibitor in 1 × RNase H buffer (20 mM Tris-HCl pH=7.8, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) was subjected to UV light irradiation at 37 ℃ for 20 min by using a 8-W UV lamp (intensity of UV light was below 91.9 μW cm^{-2}). Then certain amount of FITC-labeled RNA substrate and 2.0 U RNase H were added to the mixture. The whole procedure was conducted in a dark cubicle and the total volume of the reaction mixture was 10 μL, with the final concentrations of the RNA substrate and Azo-ASO were 1.0 μM and 0.05 μM, respectively. [1] The resulting mixture was incubated at 37 ℃ for 40 min, followed by inactivation of RNase H at 65 ℃ for 10 min. The parallel assay with no UV irradiation was carried out according to the same procedure, except that the mixture containing Azo-ASO was exposed to visible light at 37 ℃ for 20 min before adding the RNA substrate and RNase H. Azo-0 represented RNase assays using the same ASO sequence but without azobenzene modification. Same amount of RNA substrate used in RNase H assay was loaded on the gel serving as a reference (Ref.) for relative quantification. Relative quantification of RNA digestion was carried out by measuring the intensity of electrophoresis bands, and then comparing them with that of Ref. All the assays were repeated three times and the error bars indicating the standard deviations in Figure 4B.

Protocol for reversible photomodulating of RNA cleavage: Azo-5 that directed site-specific RNA cleavage was used for photoswitching assay. A mixture of 80 μL volume containing 0.5 μM Azo-5, 1.0 μM RNA substrate, 0.2 U/μL RNase H, 1.0 U/μL RNase inhibitor and 1 × RNase H buffer (20 mM Tris-HCl pH=7.8, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) was prepared. Then the mixture was incubated at 37 ℃ in the dark, with alternate visible (2 min) and UV (10 min) light irradiation at designated time point. Every 10 min, aliquot of 10 μL solution was pipetted out, and subjected to RNase H inactivation at 65 ℃ for 10 min for the following denaturing PAGE analysis.

3. **Preparation of RNA ladder using 2'-O-methylated oligonucleotides to cleave RNA substrate.**

Materials: FITC-labelled RNA oligomers used as sense sequence was obtained from Genescript Biotechnology Co. LTD (Nanjing, China). 2'-O-methylated antisense oligonucleotides (OM-ASO) were synthesized and purified by Sangon Biological Engineering Technology Co. LTD (Shanghai, China). Ribolock^{TM} RNase inhibitor, RNase H and RNase free water were purchased from Thermo Scientific Co. LTD (Beijing, China). All solutions were prepared using Milli-Q purified water (resistance of 18.2 MΩ cm^{-1}).

Table S2 Sequence of RNA and 2'-O-methylated oligomers used in preparation of RNA ladder [a]

Name	Sequence (5' →3')	Length
RNA substrate	FITC-AAAAAGUAAAUAUUGGCGUAGCAGCACGU	29 nt
Om-ASO-24	**CUACGUG**CTGCT	12 nt
Om-ASO-22	**ACGUGCU**GCTAC	12 nt
Om-ASO-18	**GCUGCUA**CGCCA	12 nt
Om-ASO-16	**UGCUACG**CCAAT	12 nt
Om-ASO-15	**GCUACGC**CAATA	12 nt

[a] "FITC" represents fluorescein isothiocyanate modification. The bold-underlined characters indicate nucleotides 2' -O-methyl modification. The 2'-O-methylated oligonucleotides are designated by "Om-ASO-n", where "n" represents the length of the cleavage product.

Method: Aim of using the specially-made RNA ladder is to provide an accurate reference of electrophoretic band mobility to analyze the cleavage sites by Azo-ASO**.**[2] Certain amount of Om-ASO and RNA substrate were added into 1× RNase H buffer (20 mM Tris-HCl pH=7.8, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) to guarantee the final concentration of Om-ASO and RNA substrate were 0.1 μM and 2.0 μM respectively. The mixture was also added with 2.0 U RNase H and 10 U RNase inhibitor (not inhibit RNase H) to the final volume of 10 μL. The resulting mixture was incubated at 37 ℃ for 40 min, followed by inactivation of RNase H at 65 ℃ for 10 min. Aliquots of cleavage products (5 μL) of different Om-ASO were mixed, and the resulting solution was subject to lyophilization. The lyophilized powder was then dissolved by RNase free water to final volume of 10 μL containing 10 U RNase inhibitor.

4. **RNase H assay using natural ASOs with the same length as the Azo-ASOs.**

Materials: FITC-labelled RNA oligomers used as sense sequence was obtained from Genescript Biotechnology Co. LTD (Nanjing, China). All the natural antisense oligonucleotides (NA) were synthesized and purified by Sangon Biological Engineering Technology Co. LTD (Shanghai, China). Ribolock™ RNase inhibitor, RNase H and RNase free water were purchased from Thermo Scientific Co. LTD (Beijing, China).

Table S3 Sequence of RNA substrate and natural antisense oligonucleotides used in RNase H assay[a]

Name	Sequence (5' →3')	Length
RNA substrate	FITC-AAAAAGUAAAUAUUGGCGUAGCAGCACGU	29 nt
NA-1	TGCTACGCCAATAT	14 nt
NA-2	CGTGCTGCTACGCCA	15 nt

[a] "FITC" represents fluorescein isothiocyanate modification.

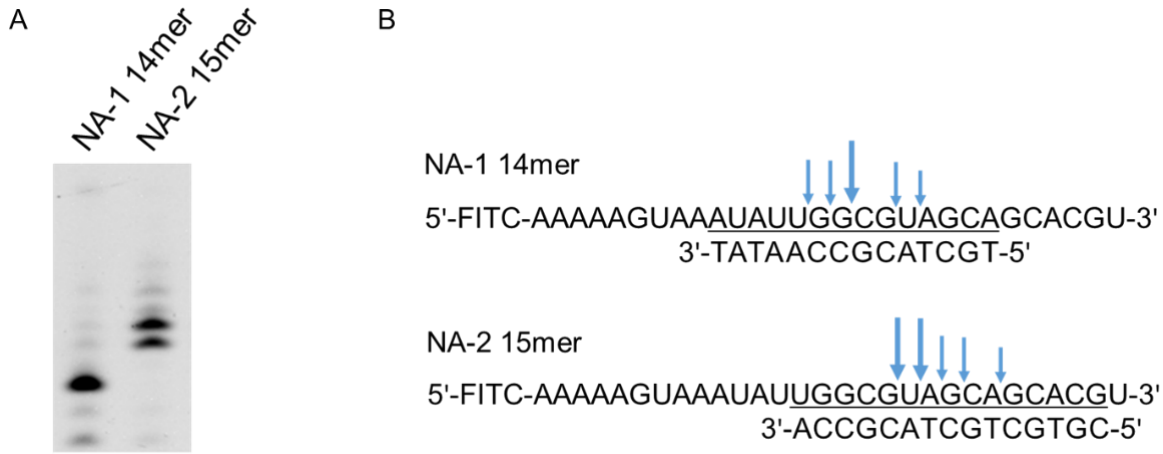


Figure S2. RNA cleavage with RNase H mediated by natural antisense oligonucleotides (NA). (A) Cleavage products analyzed on 20% denaturing PAGE with 8 M urea. Condition: 0.05 μM of NA, 1.0 μM of RNA substrate and 2.0 U of RNase H, incubated at 37 °C for 40 min. (B) Summary of the cleavage sites. The blue arrows indicate the positions of RNA cleavage, and their sizes are in direct proportion to cleavage extent. The underlined nucleotides indicate the part of RNA sequence where an NA hybridizes on to form a heteroduplex.

5. **Cleavage of RNA at elevated temperature by RNase H and azobenzene-modified ASO.**

Materials: Azobenzene-modified oligonucleotides were synthesized and purified by Nihon Techno Service Co. LTD (Ibaraki, Japan). FITC-labelled RNA oligomers used as sense sequence were obtained from Genescript Biotechnology Co. LTD (Nanjing, China). Common oligomers were provided by Sangon Biological Engineering Technology Co. LTD (Shanghai, China). Ribolock^{TM} RNase inhibitor, RNase H and RNase free water were purchased from Thermo Scientific Co. LTD (Beijing, China). All solutions were prepared using Milli-Q purified water (resistance of 18.2 MΩ cm^{-1}).

Method: Azo-ASO of 0.05 μM, RNA substrate of 1.0 μM and 2.0 U RNase H were mixed in 10 μL of 1 × RNase H buffer (20 mM Tris-HCl pH=7.8, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) containing 10 U RNase inhibitor (not inhibit RNase H), the resulting mixture was incubated at the designated temperatures for 40 min, followed by inactivation of RNase H at 65 ℃ for 10 min. The cleavage products were placed on ice before subjected to electrophoresis on 20% denaturing PAGE gel containing 8 M urea. After electrophoresis, the gel was photographed under ultraviolet illumination at 494 nm with Bio-rad molecular imager.

Results:

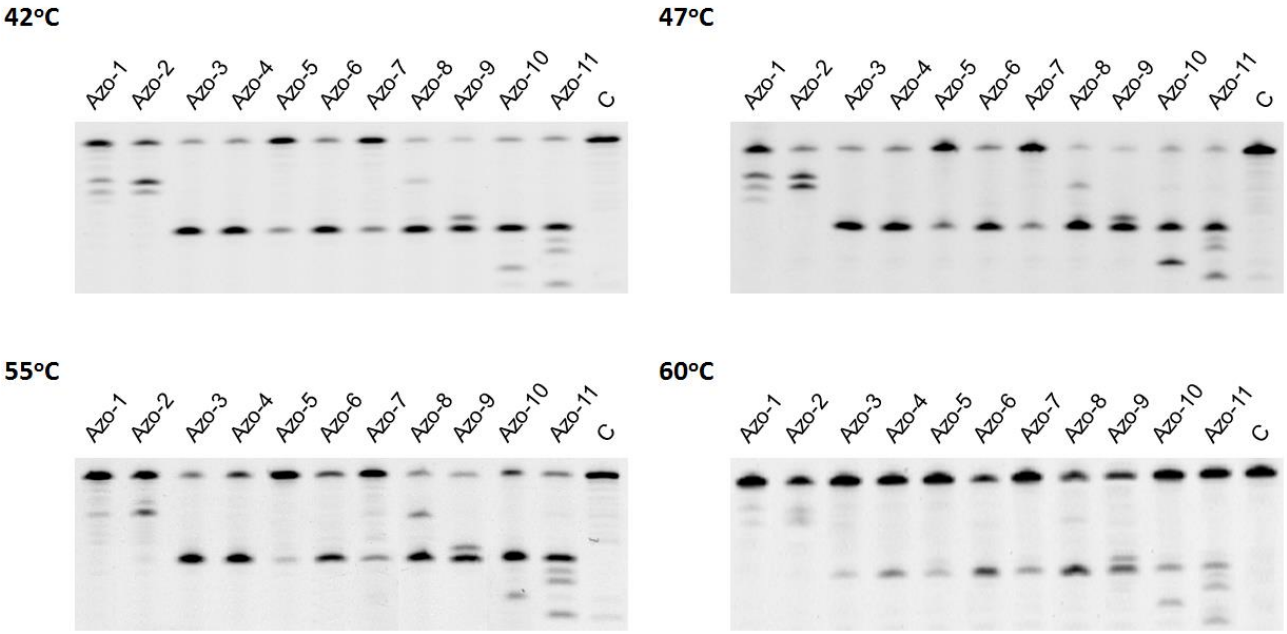


Figure S3. Cleavage of RNA with RNase H guided by Azo-ASOs at elevated temperatures. "C" indicated the control RNase H assay carrying out without adding ASO. The cleavage products were analyzed on 20% denaturing PAGE gel containing 8 M urea and photographed on a Bio-rad molecular imager

6. **Measuring the melting temperature of the azobenzene-modified ASOs.**

Materials: Azobenzene-modified oligonucleotides were synthesized and purified by Nihon Techno Service Co. LTD (Ibaraki, Japan), and their complementary RNA oligomers were provided by Integrated DNA Technologies Co. LTD (Caralville, USA). DEPC treated water was purchased from Tiangen Biotechnology Co. LTD (Beijing, China). All other chemical reagents was provided by local commercial suppliers, and are of analytical grade. All solutions were prepared using Milli-Q purified water (resistance of 18.2 MΩ cm^{-1}), and retreated with DEPC protocol to deactivate ribonucleases. T_m measuring was carried out on a Shimadzu UV spectrophotometer (Kyoto, Japan), equipped with a cooling water circulation system provided by Scientz Biotechnology Co. LTD (Ningbo, China).

Method: Equal mole of Azo-ASOs and complementary RNA were added to the same buffer solution as that for RNase H assay (except for the absence of DDT) in total volume of 500 μL containing 20 mM Tris-HCl, 40 mM KCl, 8 mM $MgCl_2$ (pH=7.8). The final concentration of Azo-ASO and complementary RNA were 1.0 μM. Adsorption profile at 260 nm was recorded from 0 ℃ to 90 ℃ with the thermal ramping at a rate of 1 ℃/min. The data were processed with T_m analysis of Lab solution software package authorized by Shimadzu Corporation. For measuring the melting temperature of *cis* form (T_ms) of Azo-ASOs, the solution containing Azo-ASO and its complementary RNA was subjected to UV irradiation at 37 ℃ for 20 min, followed by being pipetted into a quartz cuvette and then loaded on the UV spectrophotometer for T_m analysis.

Table S4 Melting temperature of Azo-ASOs used in this study

Name	Melting temperature (℃)		ΔT_m[a] (℃)
	Visible light (T_m)	UV light (T_ms)	
Azo-0[b]	55.4		
Azo-1	40.6	13.7	26.9
Azo-2	42.9	19.4	23.5
Azo-3	49.3	24.8	24.5
Azo-4	47.4	19.4	28.0
Azo-5	39.4	7.8	31.6
Azo-6	50.7	29.7	21.0
Azo-7	38.6	Not Detected [c]	--
Azo-8	51.2	40.4	10.8
Azo-9	52.3	43.6	8.7
Azo-10	49.3	14.5	34.8
Azo-11	47.0	11.7	35.3

[a] $\Delta T_m = T_m$(*trans*)- T_ms(*cis*); [b] Azo-0 is a natural ASO sequence with no azobenzene modification. [c] For the multiple azobenzene tethered in Azo-7, when they adopt *cis* form, the heteroduplex structure containing Azo-7 and RNA hardly formed in the conditions of T_m measuring.

REFFERENCE

1. Zhou M, Liang X, Mochizuki T, et al. A Light-Driven DNA Nanomachine for the Efficient Photoswitching of RNA Digestion [J]. *Angew. Chem. Int. Edit.*, **2010**, 122(12): 2213-2216.

2. Shibahara S, Mukai S, Nishihara T, et al. Site-directed cleavage of RNA [J]. *Nucleic Acids Res*., **1987**, 15(11): 4403-4415.