Electronic Supplementary Material (ESI) for ChemComm.

Electronic supplementary information

Morpholino molecular beacon for specific RNA visualization *in vivo*

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Materials and Methods

DNA molecular beacon (DNA-MB), perfect matched RNA target (PM), and one-base mismatched RNA target (1MM) were synthesized and purified by Integrated DNA Technologies (Coralville, IA, USA). Ribonuclease H (RNase H) and single-stranded binding protein (SSB, T4 Gene 32 Protein) were purchased from NEB (New England Biolabs Inc. Ipswich, MA, USA). Deoxynuclease I (DNase I) were purchased from Promega (Madison, WI, USA). Texas Red was obtained from Sigma-Aldrich. 96-cell pure grade black microplates were purchased from BRAND (Wertheim, Germany). All other commercially available reagents were used as received.

UV-Visible absorption spectra and the concentration quantitation of MBs were recorded on BioTek CYTATION 3 Cell Imaging Multi-Mode Reader (Winooski, VT, USA) equipped with Take3 Trio Micro-Volume Plate. Steady-state fluorescent emission spectra were measured on a FluoroMax-4 spectrofluorometer (JobinYvon, Inc., Edison, NJ, USA). Fluorescent kinetics analysis was performed on BioTek CYTATION 3 with 96-cell pure grade black microplates. Thermal denaturation measurements was performed on Stepone™ and Steponeplus™ Real-time PCR Systems (Applied Bioystems, Inc. Waltham, MA, USA). Autoflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA, USA) was used to confirm the purified morpholino molecular beacon (MO-MB). The MO-MB in the embryonic cell lysate was determined with a Dionex UltiMate 3000 HPLC system (Thermo Scientific Dionex, Waltham, MA, USA) coupled to a Bruker micrOTOF-QII Mass Spectrometer system (Bruker Daltonics). The brightfield and fluorescence images were acquired under Fluorescence Stereo Microscope M205FA from Leica equipped with QImage CCD and QCapture Pro software from QImaging (Surrey, BC, Canada).

Design and Characterization of Morpholino Molecular Beacons

We chose a random sequence (25 bp) that does not have any exact match in the medaka

genome to design a shared-stem model MO-MB, in which one arm of the stem complements the target site. MO-MB was composed totally of morpholino. In order to compare the MO-MB with a conventional probe, we also prepared DNA-MB with the same sequence. The MBs were labelled with a FAM reporter dye at the 3'-end and a quencher DABCYL at the 5'-end. Sequences and RNA targets of the MBs used in this study are listed in table S1. Using Zuker's open access web server (http://mfold.rna.albany.edu/?q=mfold), we folded the molecular beacon sequence at fixed conditions (28 °C, [Na^+] =0.01M) [1-2]. The prediction output was shown in Figure S1. MO-MB was synthesized by Gene-tools (Philomath, OR, USA). Before use, MO-MB was further purified with MQ water through Amicon Ultra-15 Centrifugal Filter 3kDa NMWL by Merck Millipore (Billerica, MA, USA). The purified MO-MB was confirmed by MALDI-TOF mass spectroscope and UV-Vis absorption spectra. The major single peak at 9428 m/z (theoretical value: 9430 m/z) was observed in the mass spectrometry analysis (Figure S2). In the UV-Vis absorption spectra, MO-MB represented distinct absorption maxima at 265nm by morpholino and a broad peak around 490nm by FAM and DABCYL (Figure S3).

***In Vitro* Hybridization and Thermal Denaturation Profiles with RNA**

In vitro hybridization reactions for Fig. 1D were performed at 25 °C, in a 400 μL total volume containing 500 nM MO-MB and 5-fold excess RNA targets in hybridization buffer (10 mM phosphate buffer, pH=7.0, 100 mM NaCl, 3 mM $MgCl_2$). Fluorescence intensity was measured on a Fluoromax-4 photon-counting spectrofluorometer (Horiba-Jobin Yvon, Edsion, NJ, USA) from 510 nm to 650 nm with the excitation/emission wavelength of 490 nm/520 nm. Hybridization kinetics analysis for Fig. 1E were performed in a 200 μL total volume using CYTATION 3 Cell Imaging Multi-Mode Reader from BioTek (Winooski, VT, USA) with pure grade black microplates under the same buffer at both 25 °C (Fig. S4) and 28 °C (Fig. 1E). Hybridization kinetics were recorded with GFP filter (485/528) with sampling intervals of 15s.

Thermal denaturation Profiles were determined by StepOnePlus Real-Time PCR System from Applied Biosystems (Waltham, MA, USA). The fluorescence intensity of 20 μL the mixture of MBs (500 nM) and PM Targets (2.5 μM) in 10 mM phosphate buffer (pH=7.0) containing 0, 25, 100, 250 mM NaCl was monitored as a function of temperature. The temperature was brought to 30 °C and increase at 2 °C increments to 90 °C. For melt curve of MO-MB, the range of temperature was from 20 °C to 95 °C.

In Vitro Stability of Molecular Beacons

Nuclease Sensitivity

The nuclease sensitivity of MBs was examined in a 200 μL solution containing 500 nM DNA-MB or MO-MB, 40 mM phosphate buffer (pH=7.5), 10 mM $MgCl_2$ and 1 mM $CaCl_2$. Upon the addition of 2 Units DNase I, the fluorescence intensity of the solution was real-time monitored as a function of time at 37°C.

To test whether RNase H can digest the perfectly matched RNA target that MBs are bound, 500 nM DNA-MB or MO-MB was incubated with 2.5 μM the PM RNA target for 15 min 37 °C in a 200 μL buffer solution containing 50 mM phosphate buffer (pH=8.3), 70 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol. And then, 15 Units RNase H was added, the fluorescence signal was recorded as a function of time. For each enzyme, the outcome of fluorescence intensity was normalized on a scale 0 to 1.

Single-stranded DNA binding protein (SSB) disruption

To investigate the effect of SSB on MBs, 500 nM DNA-MB or MO-MB was incubated with 5-fold excess SSB (T4 Gene 32 Protein) for 2 h 37 °C in 200 uL 1× NEB buffer 4 (provided by manufacturer, 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1mM DTT, pH=7.9). Thereafter, the fluorescence changes were recorded.

Fish Strains and Maintenance

Work with fish followed the guidelines on the Care and Use of Animals for Scientific Purposes of the National Advisory Committee for Laboratory Animal Research in Singapore

and was approved by this committee (permit number 27/09). Briefly, for embryo collection, medaka adults (strains *af*, *HdrR* and *HdrR II*) were maintained in ~20 L tanks of static water (fresh dechlorinated tap water with hardness under 100 mg/L $CaCO_3$ equivalent) under an artificial photoperiod of 14-h/10-h light/darkness at 26～28 °C. 15～20 females with 4～5 males were kept in each tank. Brine shrimp was fed three times daily. Freshwater snails were kept in each tank for better water quality. One-third of water in each tank was changed each week during the removal of debris from the bottom of the tank. The pH of water was monitored weekly and was maintained to be 6.5～7.0. For detail information about medaka maintenance, a monograph[3] can be referred to.

Microinjection and Imaging

Microinjection of medaka embryos was performed as described [4]. Briefly, to prepare the medaka embryos for microinjection, male and female fish were separated one night before the microinjection and were mixed to allow courtship and embryos spawning right before the experiment. The embryos were collected soon after spawned. They were then separated and arranged in agarose grooves (made with 1.5% agarose using customized moulds of 0.8 × 1mm ridges) submerging in 1x Yamamoto Ringer's solution (NaCl 7.5 g, KCl 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, and $NaHCO_3$ 0.02 g in 1000 mLH_2O, pH=7.3). These embryos were rotated with their cytoplasm facing upwards. Glass capillary needles were pulled with a Micropipette Puller P-87 (Sutter Instrument, USA). Solution to be injected into the embryos was loaded to the glass capillary needles by first heating the needles to expand the air inside and then cooling it so that air contraction could load the solution in the needles. After loading the solution, the needles were assembled to the Mechanical Manipulators by Leica Microsystems (Wetzlar, Germany) with a self-made micro-injector using mineral oil as pressure transmitter. Then, the tips of the needles were opened through touching slightly on hard surfaces and microinjection could begin. Usually, 0.4 ～1 nL of the solution would be injected into the cytoplasm of 1-cell

or 2-cell stage embryos. After injection, the embryos were incubated at 28 ºC with embryo rearing medium (NaCl 1.00 g, KCl 0.03 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.16 g, and optionally, Methylene blue 0.0001 g in 1000 mL H_2O, pH=7.3).

Embryos were immobilized on agarose grooves or with 3% methylcellulose during photography. Images were acquired and analyzed by using QImage CCD and QCapture Pro software from QImaging (Surrey, BC, Canada) under Fluorescence Stereo Microscope M205FA from Leica.

***In Vivo* Toxicity Assays**

Based on the OECD Fish Embryo Acute Toxicity Test [5] for zebrafish embryos, we designed and performed *in vivo* toxicity experiments of molecular beacons on medaka fish as followed. 100 μM or 250 μM probes were mixed with 5 mg/mL Texas Red and were injected to 1-cell stage *HdrRII* embryos. Texas Red of 5 mg/mL was also injected as control group to evaluate microinjection caused damage. Right after injection, the injected embryos were screen under fluorescent microscope to remove unsuccessful injection samples. To evaluate the toxicity of probes, abnormality and death rate of each injection groups at 1 dpf, 5 dpf and 3 dph were recorded. In this evaluation, embryos with abnormal somite and head development, or from 5 dpf onwards with disordered blood vessel formation, lack of pigment, and irregular heartbeat would be recorded as abnormal, while embryos without body axis on 1 dpf and without heartbeats from 5 dpf onwards would be recorded as dead.

***In Vivo* Stability Assays**

To compare the stability of DNA-MB and MO-MB *in vivo*, 100 μM MO-MB or DNA-MB were mixed with 5 mg/mL Texas Red, respectively, and were microinjected to 1-cell stage *HdrRII* embryos. Images of the green and red channels of the injected embryos were taken every 15 min to monitor the degradation rate of each probe. All images were taken under the same exposure condition: 36 bit, 700 ms exposure time for green channel and 200 ms for red channel. The original image files were cropped into 100×100 pixels, remaining the center parts

of the cell with uniform fluorescence intensity. The cropped images were subjected to brightness analysis using a Python script to get a green/red ratio by averaging brightness value of all pixels.

To further investigate the stability of MO-MB in developing embryos after 120 min post injection, LC-MS was used to determine the existence of MO-MB. 250 μM MO-MB was injected to 1-cell stage HdrRII embryos. Around 40 embryos were dechorionated by forceps at 6 h and 24 h after injection with the cell mass separated from the yolk and subjected to overnight lysate by Proteinase K in 0.05% SDS. The embryonic cell lysate was centrifuged at 14000 g for 10 min and 100 μL supernatant was taken for LC-MS analysis. The conditions of LC-MS:

HPLC column: Phenomenex Aeris Widepore 3.6u XB-C8

Aqueous running buffer: A: H_2O+0.1% formic acid; B: CH_3CN+0.1% formic acid

Flow rate: 0.2 mL/min

Effective gradient program:

0～5 min 15% buffer B

6～10 min 60% buffer B

11～25 min 100% buffer B

Standard MO-MB of 50 μM was used as reference and uninjected 6 hpf embryos were used as negative control under the same experimental conditions.

***In Vivo* Target Specificity Assays**

For *in vivo* target specificity assays of MO-MB, 200 μM MO-MB was injected to 1-cell stage *HdrRII* embryos. Injected embryos were checked under fluorescence microscope to confirm successful injection. The embryos were then incubated at 28 °C for three hours to reach 16-cell stage. One single cell of the 16-cell embryos was injected with 10 μM perfect matched RNA target or 2-base mismatched RNA target mixed with 5 mg/mL Texas Red as

indicator. The images of embryos were acquired 10 min after the injection of the targets at 28 °C.

References

1. Markham, N. R. & Zuker, M. (2005) DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res.*, 33, W577-W581.

2. Markham, N. R. & Zuker, M. (2008) UNAFold: software for nucleic acid folding and hybridization. In Keith, J. M., editor, *Bioinformatics, Volume II. Structure, Function and Applications*, number 453 in *Methods in Molecular Biology*, chapter 1, pages 3–31. Humana Press, Totowa, NJ. ISBN 978-1-60327-428-9.

3.Kinoshita, M., Murata, K., Naruse, K. & Tanaka, M. *Medaka* 31–66 (John Wiley & Sons, Ltd., 2009).

4. Porazinski, S. R., Wang, H. & Furutani-Seiki, M. Microinjection of Medaka Embryos for use as a Model Genetic Organism. *J. Vis. Exp.* (2010). doi:10.3791/1937

5. OECD. *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. (Organisation for Economic Co-operation and Development, 2013).

Table S1. Molecular beacon sequences and their RNA targets

Name	Sequence (5'-3')
MO-MB	DABCYL-G^{m}C^{m}G^{m}C^{m}G^{m}C^{m}T^{m}A^{m}T^{m}A^{m}C^{m}A^{m}C^{m}G^{m}A^{m}C^{m}A^{m}C^{m}A^{m}G^{m}C^{m}G^{m}C^{m}G^{m}C^{m}-FAM
DNA-MB	DABCYL-GCGCGCTATACACGACACAGCGCGC-FAM
PM	rUrGrUrGrUrCrGrUrGrUrArUrArGrCrGrCrGrC
1MM	rUrGrUrGrUrCrGrU***r*C**rUrArUrArGrCrGrCrGrC

m: morpholino; r: RNA; PM: perfect matched target; 1MM: single-base mismatched target; Underlined letters are bases for stem; Red letters represent target sequences; italic nucleotides represent mismatched bases.

Table S2. Raw data of *in vivo* toxicity of MO-MB.

Injection	Time	Dead (%)	Defect (%)	Normal (%)	Total
None	1 dpf	6 (3.8)	1 (0.7)	147 (95.5)	154
	5 dpf	6 (3.9)	3 (1.9)	145 (94.2)	
	3 dph	11 (7.2)	5 (3.2)	138 (89.6)	
Texas Red	1 dpf	5 (3.5)	4 (2.7)	137 (93.8)	146
	5 dpf	5 (3.5)	4 (2.7)	137 (93.8)	
	3 dph	28 (19.1)	2 (1.4)	116 (79.5)	
100 μM MO-MB	1 dpf	0 (0)	11 (7)	146 (93)	157
	5 dpf	2 (1.2)	10 (6.4)	145 (92.4)	
	3 dph	20 (12.8)	4 (2.5)	133 (84.7)	
250 μM MO-MB	1 dpf	5 (3.2)	7 (4.3)	149 (92.5)	161
	5 dpf	7 (4.3)	7 (4.3)	147 (91.4)	
	3 dph	20 (12.3)	5 (3.2)	136 (84.5)	

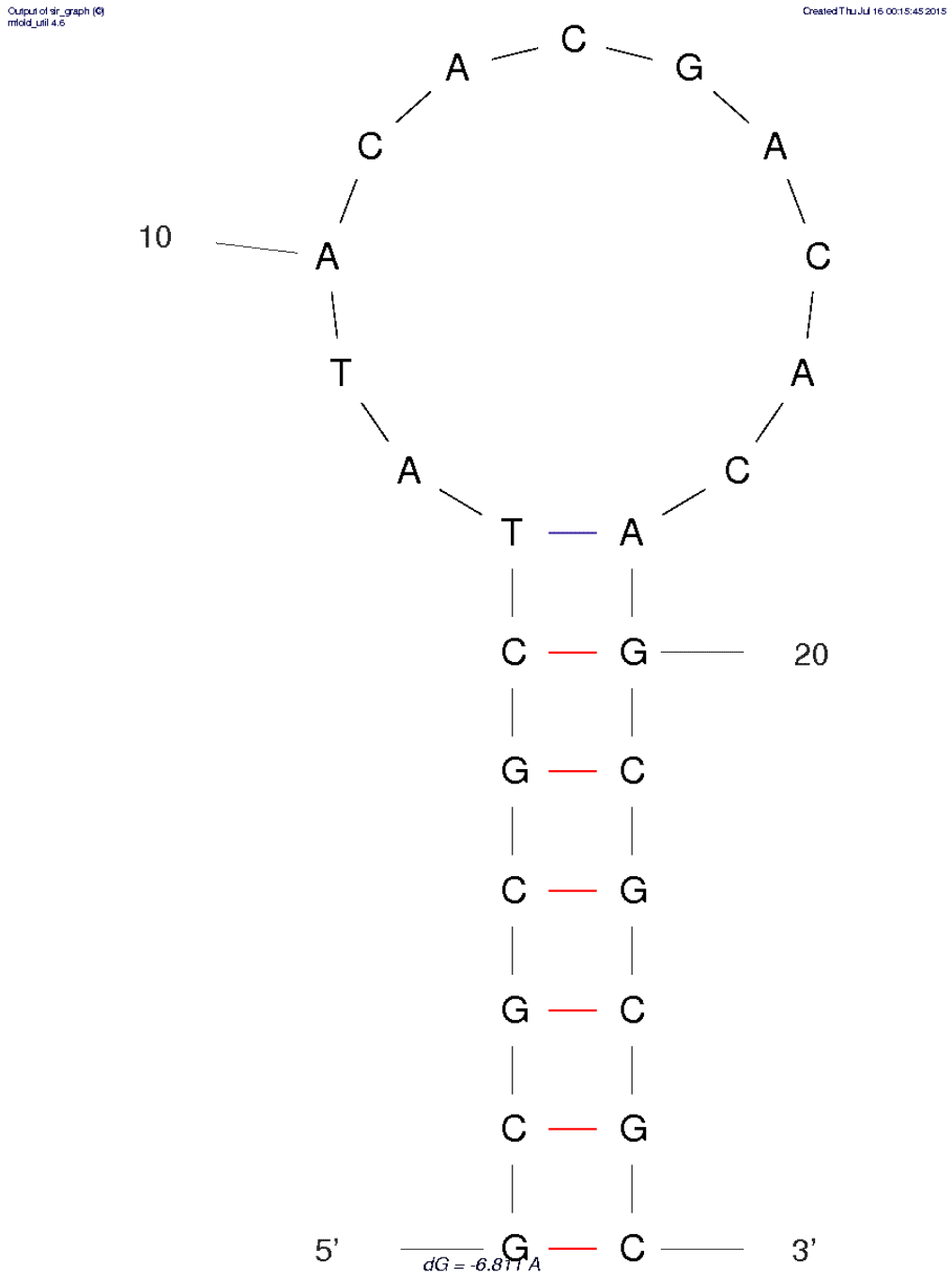

Figure S1. The predicted structure of molecular beacon by *mflod* system.

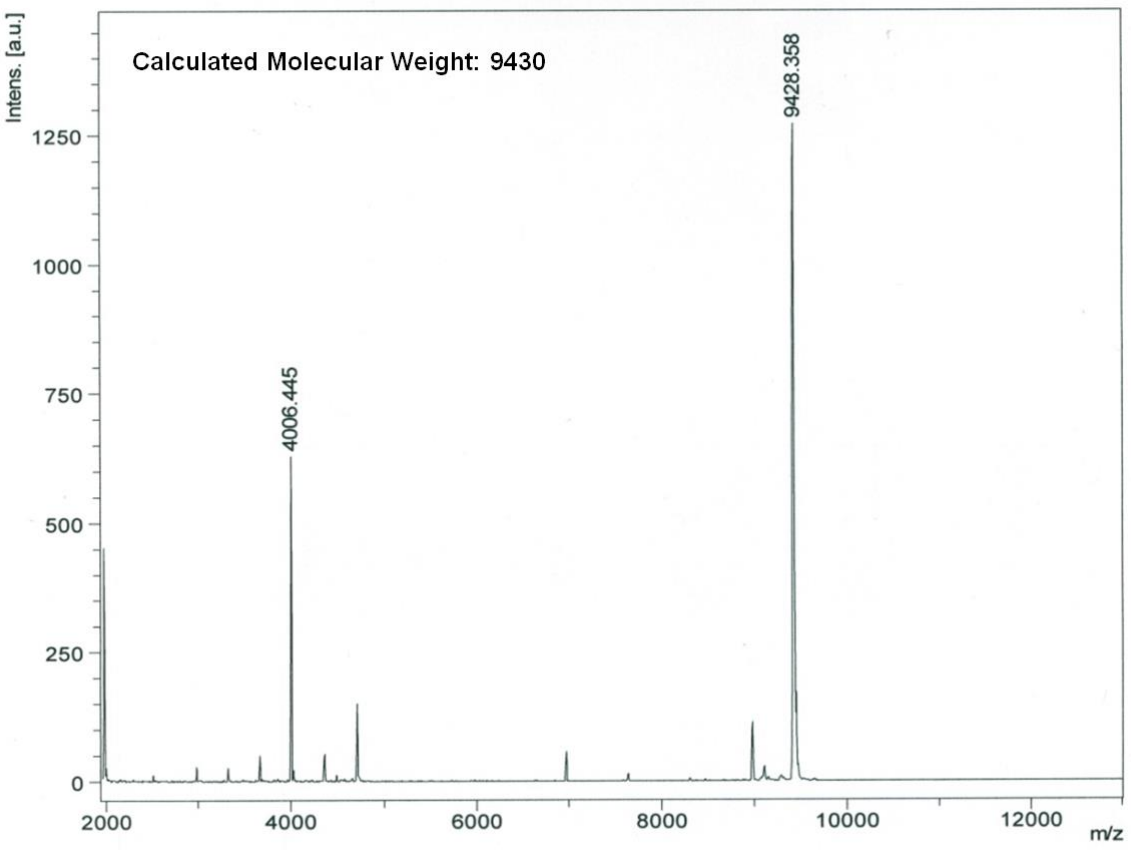

Figure S2. MALDI-TOF MS of MO-MB.

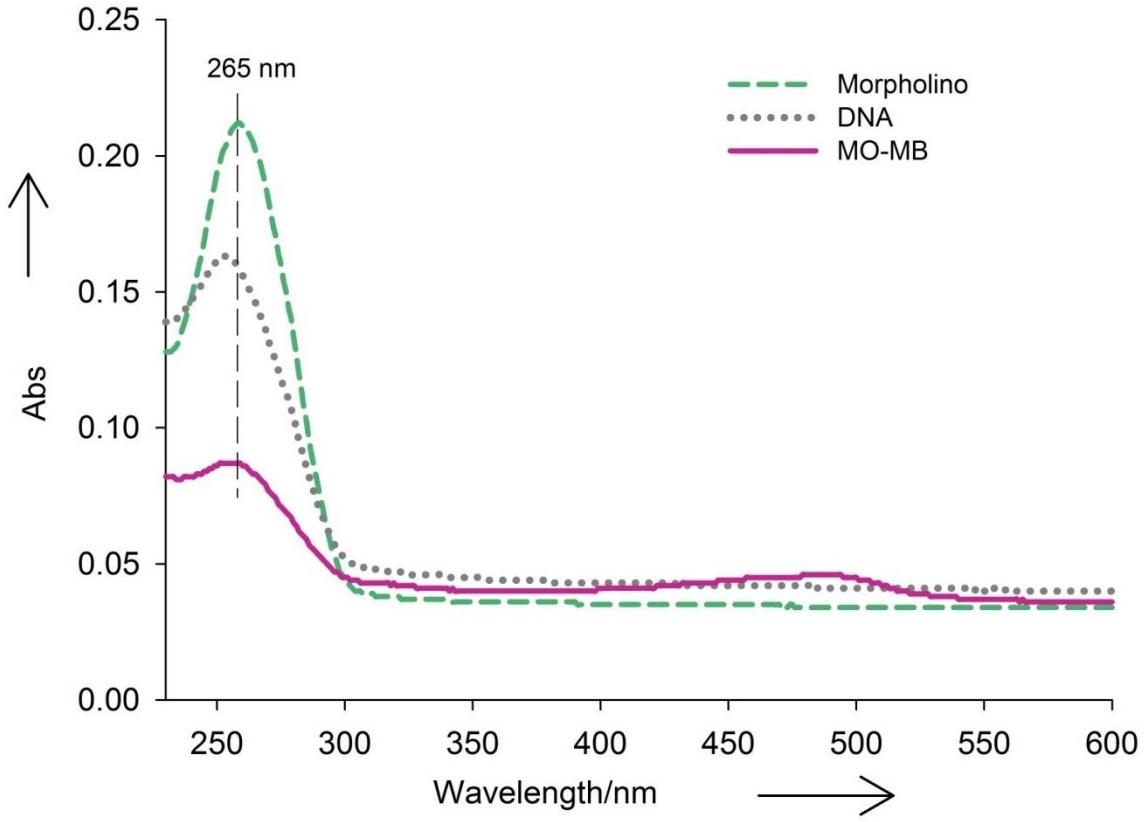

Figure S3. UV-Visible spectra of 10 μM morpholino (dotted line), DNA (dash line), and MO-MB (solid line).

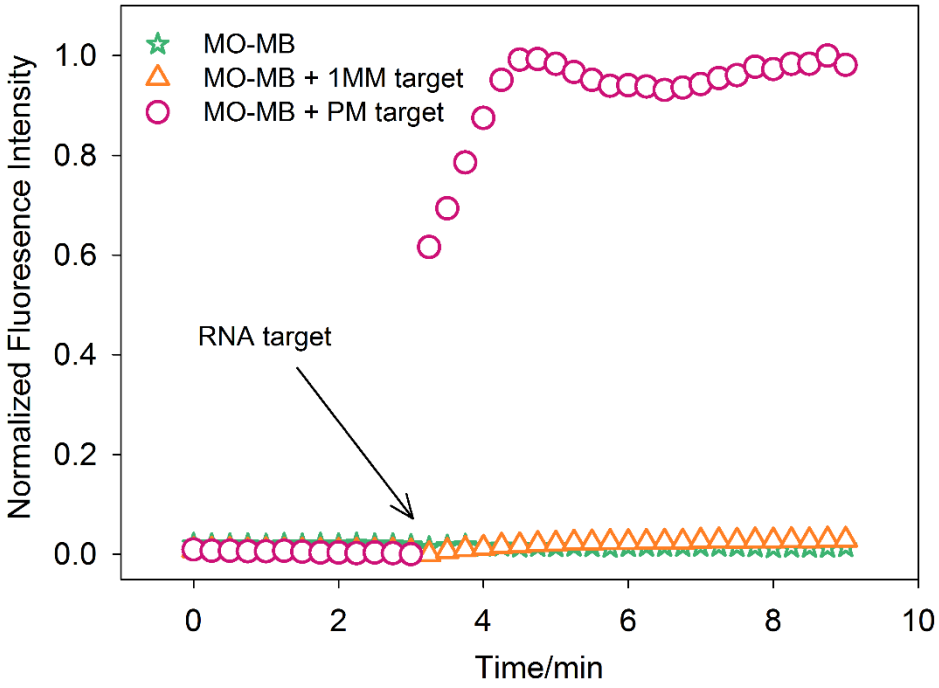

Figure S4. Normalized hybridization kinetics of MO-MB with perfect matched (PM,○) and single-base mismatched (1MM, △) RNA target at 25 ℃.

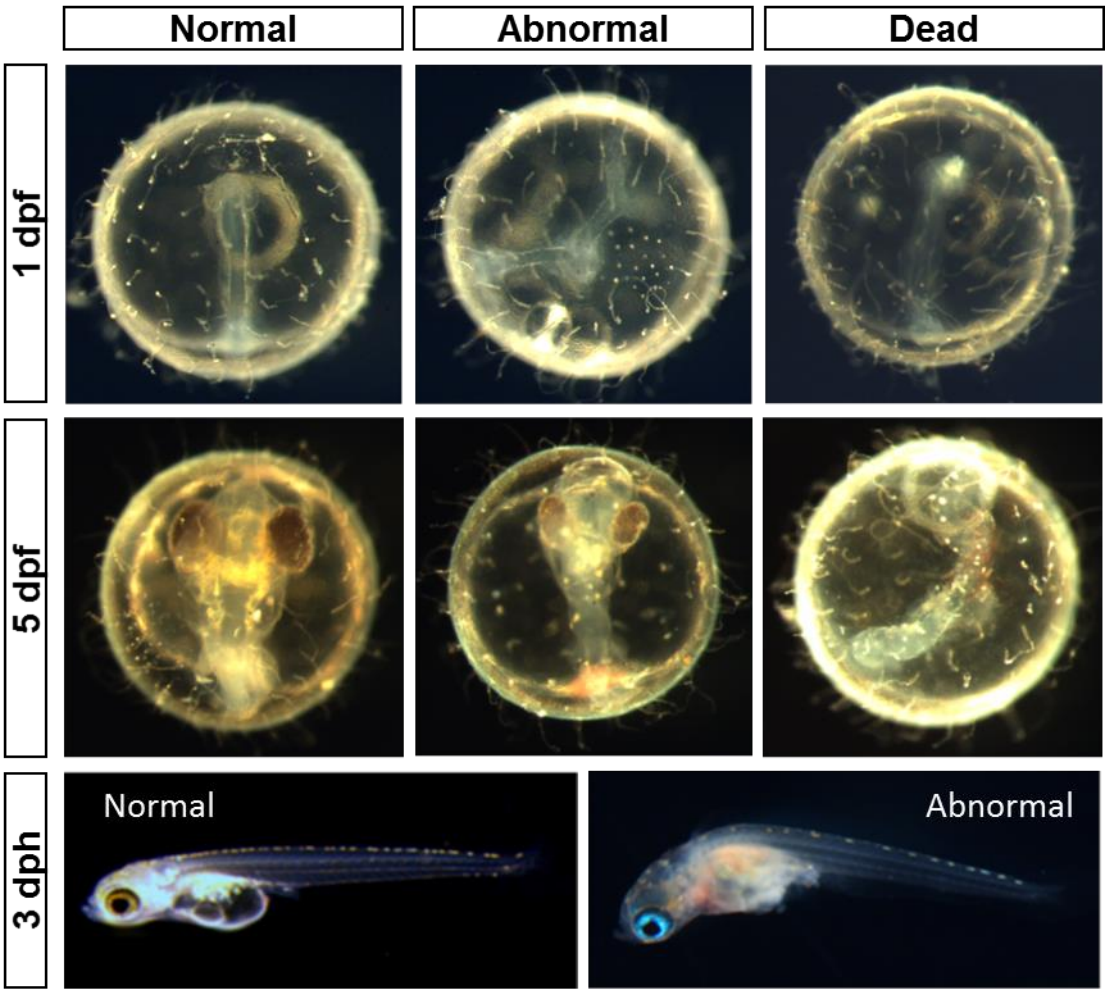

Figure S5. Typical examples of normal, abnormal and dead embryos and fry.

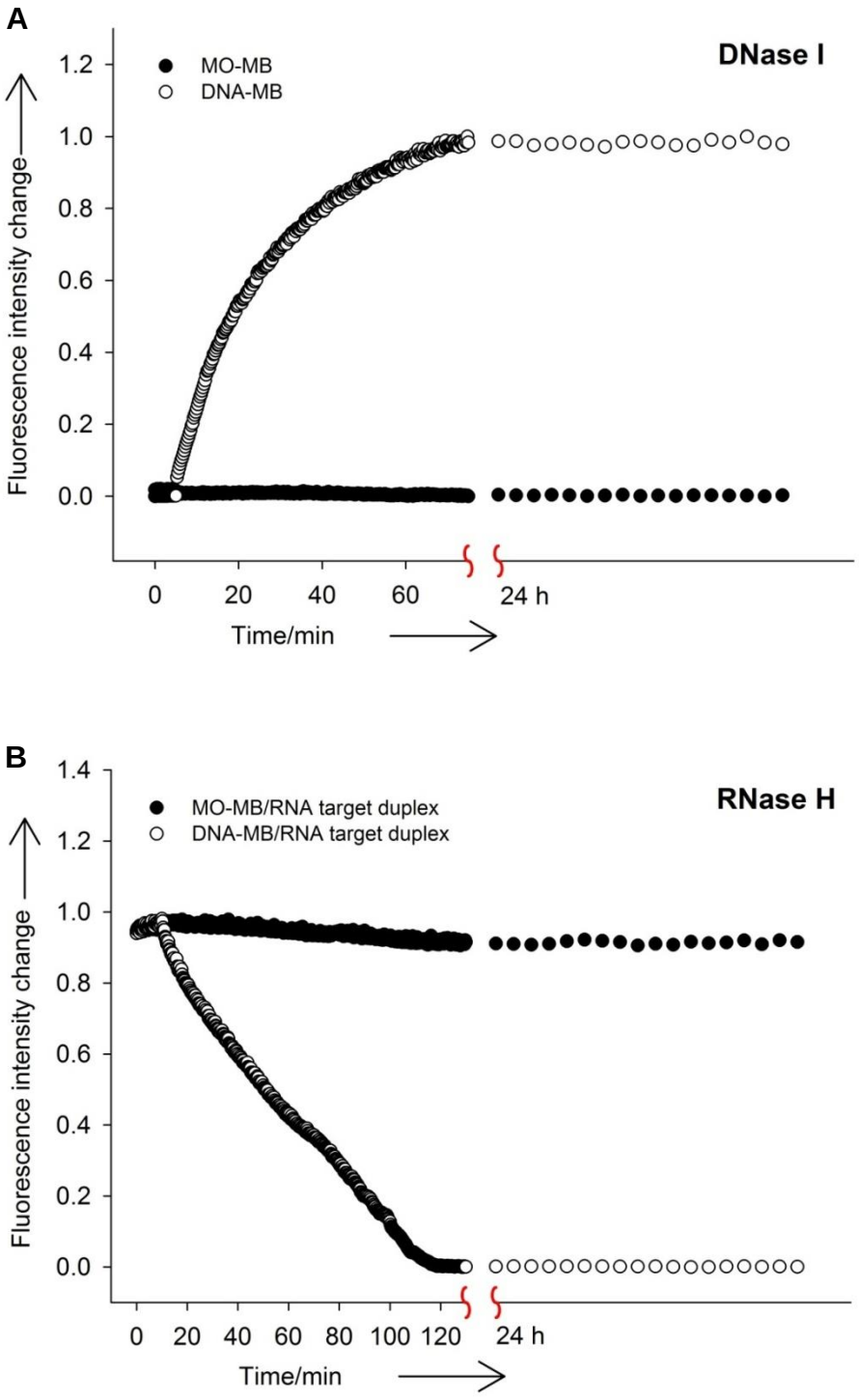

Figure S6. (A) Normalized fluorescence change of MO-MB (●) and DNA-MB (○) in the presence of 2 units DNase I. (B) Normalized fluorescence change of MO-MB/RNA (●) and DNA-MB/RNA (○) duplex targets on the addition of 15 units RNase H.

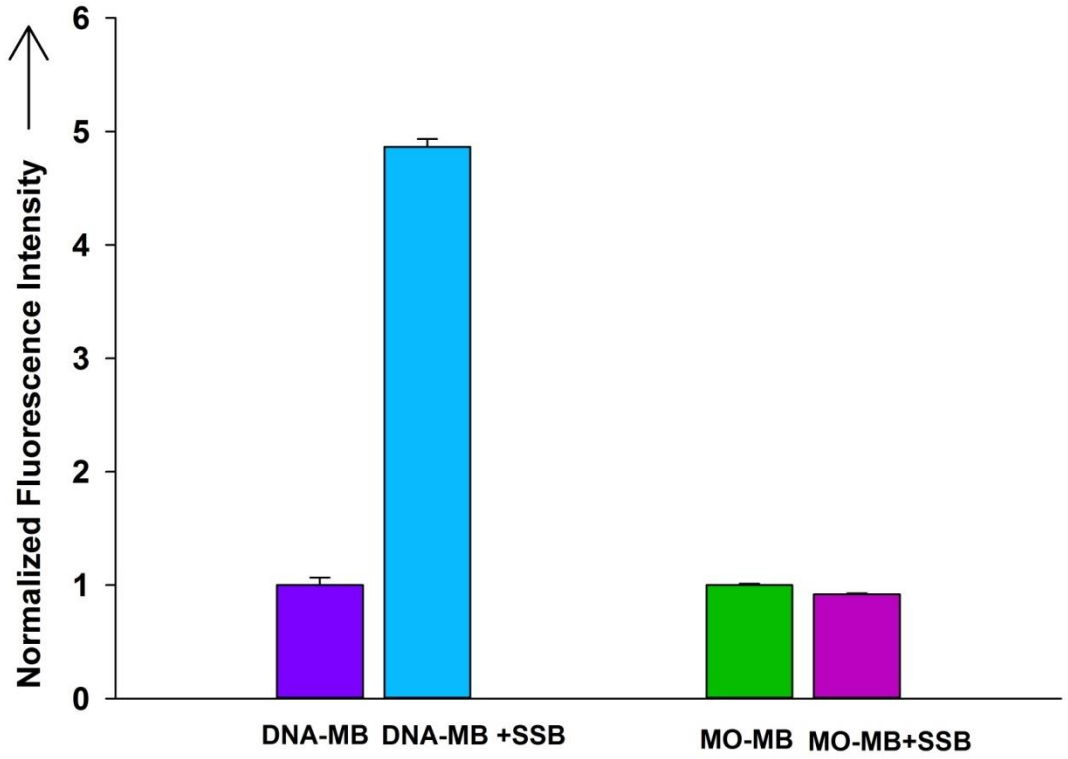

Figure S7. Fluorescent response of MO-MB and DNA-MB to the single-stranded binding protein (SSB).

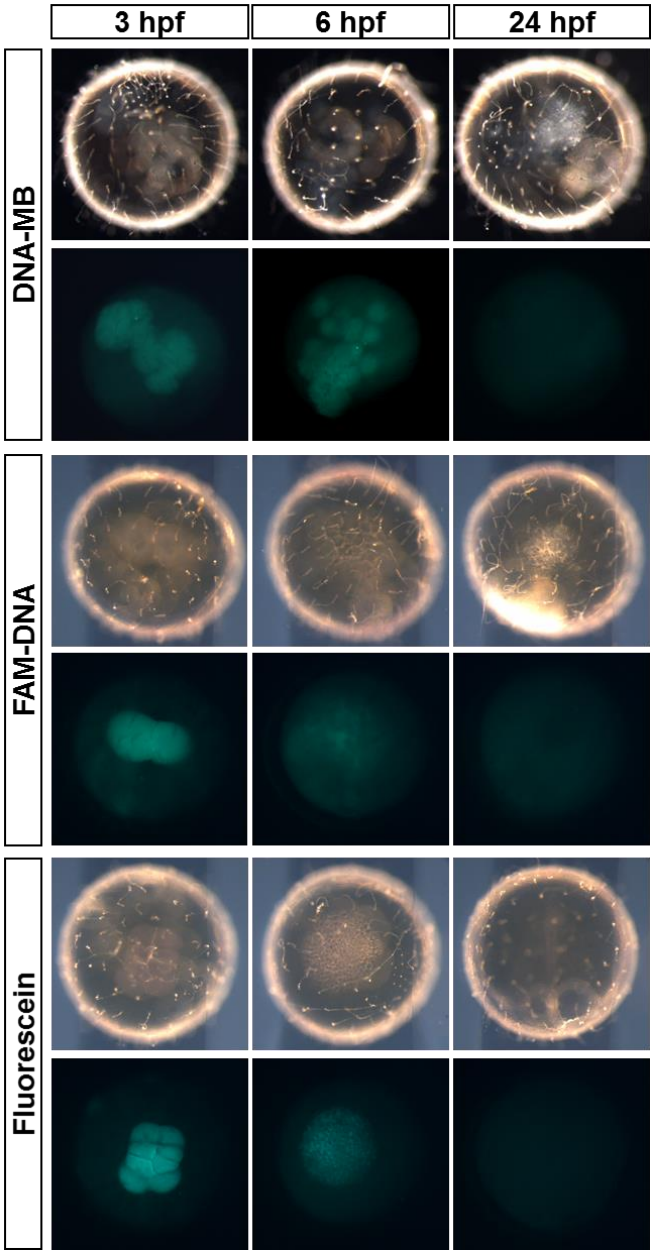

Figure S8. Decrease of the fluorescent signal over time in medaka embryos with injection of DNA-MB, FAM-tagged DNA oligonucleotide, or fluorescein. This could be due to the instability of fluorescein under *in vivo* environment. In addition, abnormal early development can be seen in embryos injected with DNA-MB or FAM-DNA, which were dead with nearly no cell structure seen after 24 h, indicating the toxicity fo DNA oligonucleotides to live embryos.

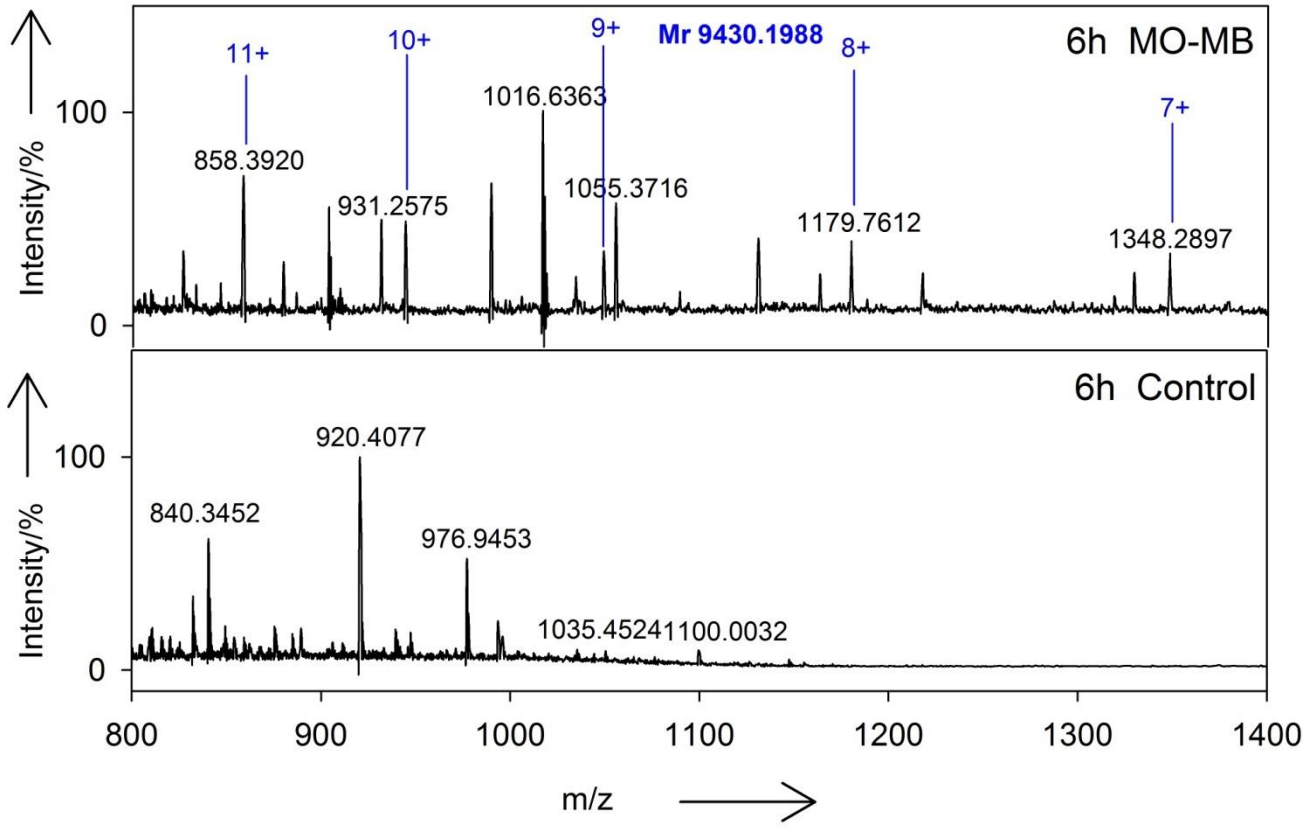

Figure S9. The mass spectrometry spectra of 6 h uninjected control sample and the embryonic cell lysate at 6 h after injection.

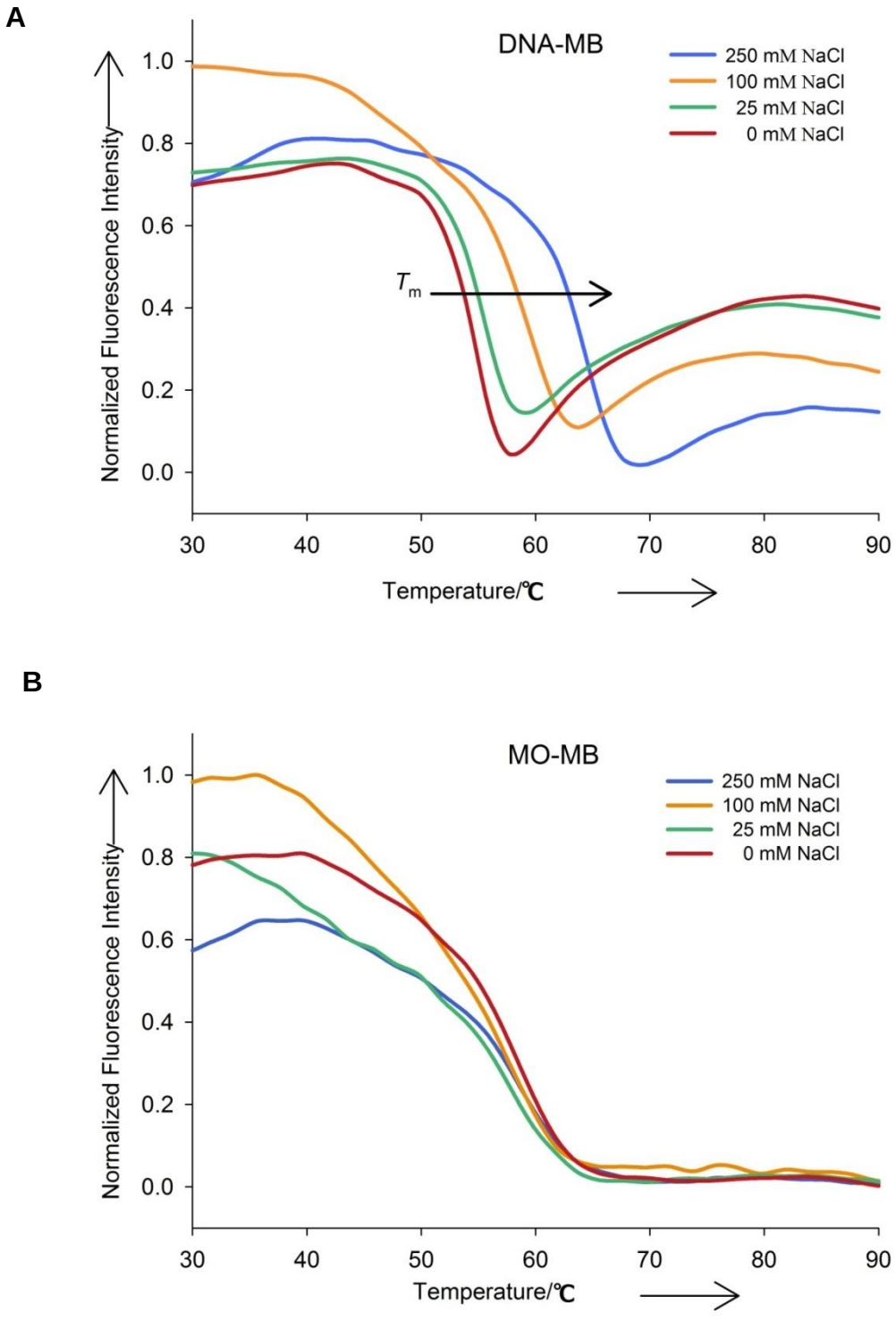

Figure S10. Effects of ionic strength on the melting temperature (T_m) of DNA-MB/RNA duplex (A) and MO-MB/RNA duplex (B). Conditions: 0 ~ 250 mM NaCl in 10 mM phosphate buffer (pH=7.0).