Supporting Information For

Single Promoter System Co-expressing RNA Sensor with Fluorescent Protein for Quantitative mRNA Imaging in Living Tumor Cells

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Experimental Section:

Chemicals and materials

AmpliScribe T7 Flash Transcription Kit was obtained from Epicenter (WI, USA). SYBR Green II, precast 6% PAGE gel, Agarose gel, denaturing PAGE gel and RiboRuler Low Range RNA ladder were purchased from Life Technologies (MA, USA). YM155 were obtained from Sigma Aldrich (MO, USA). Docetaxel was obtained from J&K China Chemical Ltd. (Guangzhou, China). PureLink™ Quick Gel Extraction Kit was obtained from Promega (WI, USA). TaqDNA polymerase, 10×PCR Buffer (without Mg^{2+}, 100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8%(v/v) Nonidet), $MgCl_2$ (25mM), dNTP (10mM), Marker, 6×DNA Loading Dye, Rapid Competent Cell Preps Kit (one step) (SK9307), SanPrep Column Plasmid Mini-Preps Kit (SK8191), Gel Purification Kits (SK8131) and UNIQ-10 Column Trizol Total RNA Purification Kit (SK1321) were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Recombinant human β-actin protein was purchased from R&D Systems (Minneapolis, MN). MCF-7 (human breast adenocarcinoma cell line), HeLa cells (human cervical carcinoma cell line) and C166 cells (mouse endothelial cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). MDA-MB-435S (melanocyte cells, previously described as human breast carcinoma cell line) and MCF-10A cells (immortalized nontumorigenic human mammary epithelial cell line) was purchased from Cell Bank of the Committee on Type Culture Collection of Chinese Academy of Sciences (Beijing, China). RevertAid Premium Reverse Transcriptase Kit, Cell culture media and lipofectmine 3000 transfection reagent were purchased from Thermo Fisher Scientific (MA, USA). In-Fusion HD Cloning Kit. Sulforhodamine B-PEG_3-Dinitroaniline (SR−DN) conjugate was synthesized following a procedure previously reported.[S1,S2] Oligonucleotides used in this work were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). The synthesized oligonucleotide sequences are given in Supporting Information Table S1. All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All solutions and ultrapure water used were treated with DEPC and autoclaved to avoid RNA degradation.

Plasmid construction

The RNA sensors were constructed in a mammalian expression vector pcDNA3.1(+). Several cloning steps were performed to generate the DNA construct for the sensor. The DNA sequences for GFP from pEGFP-C (Clontech), tRNA-scaffolded RNA aptamer with or without two-domain spacer (a mRNA-stablizing triplex in the upstream domain and a dsRNA Dicer substrate in the downstream), tRNA-scaffolded RNA sensor with or without the two-domain spacer were obtained by PCR amplification from pUC-57 vector templates with the BamHI and HindIII restriction sites at the 5' and 3' ends, respectively. Then, the PCR products of GFP was cloned into the BamHI and HindIII sites of the CMV promoter in the pcDNA3.1(+) vector to form plasmid of pcDNA3.1(+)-GFP. After that, using In-Fusion HD Cloning Kit the PCR products of the tRNA-scaffolded RNA aptamer or tRNA-scaffolded RNA sensor were cloned into the pcDNA3.1(+)-GFP plasmids to form the interested plasmids, including pcDNA3.1(+)-GFP-Aptamer, pcDNA3.1(+)-GFP-Triplex-dsRNA-Aptamer, pcDNA3.1(+)-

GFP-Sensor, and pcDNA3.1(+)-GFP-Triplex-dsRNA-Sensor. After transfected in cells, these plasmids could generate the fusions of GFP mRNA and RNA aptamer or RNA sensor.

***In vitro* transcription of RNA sensor**

RNA sensor for *in vitro* assays was prepared using pUC57 vector with a linear DNA template encoding T7 promoter and the RNA sensor, and transcribed in vitro by T7 RNA polymerase. A 20 μL transcription reaction system was prepared containing 2 μL 0.5 μM DNA template, 2 μL 10× AmpliScribe T7 reaction buffer, 2 μL 10 mM DTT, 0.5 μL 20 U RNase inhibitor, 2 μL T7 RNA polymerase, 6 μL NTPs (10 mM for each NTP), with 5.5 μL RNase-free water. The transccription was performed for 2 h at 37 °C. Then the RNA transcripts were purified using Bio-Spin columns and quantified using absorbance values and the Quant-iT RiboGreen RNA Assay Kit. The purified RNA was stored at -80 °C until use.

***In Vitro* assay with RNA sensor**

The reaction solution containing RNA sensor (5 μM) was incubated with RNA target of a given concentration in 50 μL Tris-HCl buffer (40 mM, pH 7.6) with 1 μM SR-DN conjugate, 100 mM KCl and 10 mM $MgCl_2$ at 37 °C for 30 min. The fluorescence spectra of the mixture were measured at room temperature using a quartz cuvette on an F-7000 fluorescence spectrophotometer. The excitation wavelength was 560 nm and the emission wavelength ranged from 565 nm to 650 nm with a slit width of 5 nm for both excitation and emission.

To evaluate selectivity, the RNA sensor was also used for detecting other co-existing cellular components including 50 μM let-7f, 50 μM miR-222, 50 μM miR-141, 10 μM β-actin protein and C166 cell lysate. The C166 cell lysate was prepared according to the following procedure: C166 Cells (1×10^6) were dispensed in an RNase-free 1.5 mL centrifuge tube, washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4), centrifuged at 2000 rpm for 2min, and then suspended in 100 μL RIPA lysis buffer containing 1% glycerol and 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). The resulting lysate (50 μL) was used immediately in the assay. Other components were directly used in the assay as received without purification.

Gel electrophoresis analysis

About 100 ng RNA sensor transcribed in vitro or a mixture of RNA sensor with *survivin* mRNA was loaded into a well of the precast 6% TBE PAGE gel, and electrophoresis was run at 100 V in 1× TBE buffer for 80 min.[S2] The RiboRuler Low Range RNA Ladder was used as the molecular weight standard. After electrophoresis, the gel was washed with water and stained for 30 min in 40 mM Tris-HCl buffer (pH 7.6) containing 1 μM SR-DN, 100 mM KCl, and 10 mM $MgCl_2$. The gel was visualized using a Tocan 240 gel imaging system (Tocan Biotechnol. Co., Shanghai, China). Next, the gel was stained again for 30 min with 10,000-fold diluted SYBR Green II in TBE buffer.

The RNA samples were analyzed using 1.5% agarose gel electrophoresis in TBE buffer at room temperature. The gel was stained using 0.5 μg/mL GoldView and 0.5 μg/mL ethidium bromide (EB). Electrophoresis was performed at a constant

voltage of 100 V for 90 min with a load of 10 μL of sample in each lane. After electrophoresis, the gel was visualized using a Tocan 240 gel imaging system (Tocan Biotechnol. Co., Shanghai, China).

Cell culture and transfection

The cells of HeLa, C166, MDA-MB-435S and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 g/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. MCF-10A cells were grown in Mammary Epithelial Cell Growth Medium (MEGM) containing BPE, rhEGF, insulin, hydrocortisone, GA-1000, and 100 ng mL^{-1} cholera toxin at 37 °C in humidified atmosphere containing 5% CO_2. The cells were plated on sterilized glass coverslips in 35 mm plates with 14 mm well and grown to 50–70% confluency in the corresponding medium at 37 °C in a humidified atmosphere containing 5% CO_2 followed by transfection with 500 ng of the plasmid, pcDNA3.1(+)-GFP-Aptamer, pcDNA3.1(+)-GFP-Triplex-dsRNA-Aptamer, pcDNA3.1(+)-GFP-Sensor, or pcDNA3.1(+)-GFP-Triplex-dsRNA-Sensor, by using lipofectamine 3000 transfection reagent. In order to alter the expression of *survivin* mRNA in the cells, docetaxel or YM155 of a given dose was co-incubated with the cells as needed. After transfection for 24 h or a clarified period, the cells were subjected to fluorescence imaging analysis.

Live cell imaging

The cells were washed three times with PBS, and incubated with a fresh medium containing 1 μM SR-DN conjugate, 100 mM HEPES, 10 mM $MgSO_4$ at 37 °C for 30 min. Then, the cells were washed and incubated with a fresh medium followed by imaging. All fluorescence images were acquired using a 100× objective on a confocal laser scanning fluorescence microscope with an Olympus FV1000 confocal scanning system. A 488 nm laser was used as the excitation source, and emission was collected using a 100 μm pinhole at two separate channels, green (500−550 nm) and red (575−625 nm).

Quantitative RT-PCR (qRT-PCR) analysis of RNA sensor

The expressions of the RNA sensor in HeLa cells after transfection for 24 h or a clarified period (24 h, 48 h, 60 h, 72 h) was investigated by RT-PCR analysis. HeLa cells grown on a 10 cm dish were transfected with the plasmid and then were harvested. Total RNA from the cells was isolated using the UNIQ-10 column Trizol Total RNA Purification Kit. The cDNAs were reverse-transcribed from the purified RNA with an oligo-dT primer for mRNA and an anti-aptamer primer for the sensor using the PrimeScript™ II 1st strand cDNA Synthesis Kit according to the manufacturer's protocol. Briefly, a total volume of 11 μL solution containing 5 μL of the total RNA, 1 μL primer (0.2 μM), and 5 μL nuclease-free water was incubated at 10 °C for 5 min followed by ice bath for 10 s. Then, a total volume of 20 μL reaction mixture was obtained by adding 1 μL RNase inhibitor (20 U/μL), 2 μL NTPs (10 mM for each NTPs), 4 μL 5× AMV Reverse Transcriptase buffer and 2 μL AMV Reverse Transcriptase (10 U/μL). The reaction mixture was incubated at 37 °C for 5 min and 42 °C for 60 min followed by heat inactivation of reverse transcriptase for 10 min at 70 °C.

qRT-PCR analysis was performed with SybrGreen PCR Master Mix according to the manufacturer's instructions on an ABI StepOnePlus qPCR instrument (CA, USA). The 20 μL reaction mixture was composed of 2 μL cDNA, 10 μL SybrGreen qPCR Master Mix, 0.4 μL upstream primer (10 μM), 0.4 μL downstream primer (10 μM) and 7.2 μL nuclease-free water. Primers were given in Supporting Information Table S2. The qPCR conditions were as follows: staying at 95 °C for 3 min, then followed by 40 cycles of 95 °C for 15 s, 57 °C for 20 s and 72 °C for 30 s.

qRT-PCR of *survivin* mRNA

The copies of *survivin* mRNA in cells were determined according to a standard curve established based on qRT-PCR results and using β-actin gene as an endogenous reference to validate *survivin* concentrations. Before establishing the standard curve of *survivin* mRNA, standard products for *survivin* mRNA and *β*-actin gene were prepared as follows: Total RNA was extracted from cell samples using UNIQ-10 column Trizol Total RNA Purification Kit following the manufacturer's instructions. The 1st strand cDNAs were reverse-transcribed from the purified RNA using the RevertAid Premium Reverse Transcriptase Synthesis Kit, according to its indicated protocol. Briefly, a total volume of 14.5 μL solution containing 2 μL the total RNA, 1 μL Random Primer (100 pmol), 1 μL dNTP Mix (0.5 mM final concentration) and 10.5 μL nuclease-free water was incubated at 65 °C for 5 min followed by ice bath for 2 min, centrifuged for 3~5 s, and added to 4.0 μL 5X RT Buffer, 0.5 μL Thermo Scientific RiboLock RNase Inhibitor (20 U), 1.0 μL RevertAid Premium Reverse Transcriptase (200 U) in a total volume of 20 μL reaction mixture. The qPCR conditions were as follows: staying at 25 °C for 10 min followed by cDNA synthesis, 50 °C for 30 min, and 85 °C for 5 min. Standard products for *survivin* mRNA and β-actin gene were then obtained by amplifying a fragment from the cDNA template, using the common PCR primers given in Supporting Information Table S2. The 25 μL reaction mixture was composed of 0.5 μL cDNA, 0.5 μL upstream primer (10 μM), 0.5 μL downstream primer (10 μM), 0.5 μL dNTP (10 mM), 2.5 μL Taq Buffer (10×), 2 μL $MgCl_2$ (25 mM), 0.2 μL Taq polymerase (5 U/μL) and 18.3 μL nuclease-free water. The PCR amplification conditions were as follows: staying at 95 °C for 3 min, then followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, 72 °C for 8 min repair extend. The cDNA amplification products were recovered and purified using Gel Purification Kits and cloned into pMD® 18-T vector according to the manufacturer's guidelines, and then further transformed into the TOP10 competent Escherichia coli (Invitrogen) and plated onto LB plates. Transformants were screened by selecting white colonies and performing colony PCR. Corresponding colonies were cultured overnight in liquidLB medium supplemented with 50 μg/mL ampicillin. Plasmid isolation was performed using the PureLink Quick Plasmid DNA MiniprepKit (Invitrogen) and DNA was quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific).

Then, standard curves for *survivin* mRNA and β-actin gene were established using the prepared plasmids. The sequences of the constructed plasmids of standard *survivin* mRNA and β-actin gene were confirmed by sequencing. The concentrations of plasmids were determined according to OD260 values and conversed into copy numbers (copies/μL).[S3] After that, ten-

fold dilutions of the plasmids, ranging from 10^3 to 10^8 copies, were used as DNA standard sample templates of RT-PCR. The conditions of qPCR were the same as above. Let Ct value as the ordinate, lgC referring to the logarithm of the concentration as the abscissa, 6 points were selected to establish the standard curves of *survivin* mRNA and β-actin gene, respectively.

To determine the copies of *survivin* mRNA in different cells according to the standard curve, total RNA was extracted from the cells of MDA-MB-435S, MCF-7, MCF-10A, and HeLa (HeLa cells were treated with different concentrations of YM155 or docetaxel) using UNIQ-10 column Trizol Total RNA Purification Kit following the manufacturer's instructions. A total volume of 14.5 μL solution containing the total RNA (800 ng), 1 μL Random Primer (100 pmol), 1μL dNTP Mix (0.5 mM of final concentration) and nuclease-free water was incubated at 65 °C for 5 min followed by ice bath for 2 min, centrifuged 3~5 s, and added into 20 μL reaction mixture containing 4.0 μL 5X RT Buffer, 0.5 μL Thermo Scientific RiboLock RNase Inhibitor (20 U), 1.0 μL RevertAid Premium Reverse Transcriptase (200 U). A total volume of 20 μL cDNA products was obtained and its ten-fold dilutions were used as template for qPCR. Primers used for RT-PCR were given in Supporting Information Table S2. The 20 μL reaction mixture was composed of 2 μL cDNA, 10 μL SybrGreen qPCR Master Mix, 0.4 μL upstream primer (10 μM), 0.4 μL downstream primer (10 μM) and 7.2 μL nuclease-free water. The qPCR conditions were as follows: staying at 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s, 57 °C for 20 s and 72 °C for 30 s. After that, the copy numbers of *survivin* mRNA and *β*-actin were determined according to their standard curves.

References:

S1. A. Arora, M. Sunbul, A. Jaschke, *Nucleic acids Res.* 2015, **43**, e144.
S2. Z. M. Ying, Z. Wu, B. Tu, W. H. Tan and J. H. Jiang, *J. Am. Chem. Soc.*, 2017, **139**, 9779–9782.
S3. T. D. Schmittgen, K. J. Livak, *Nature protocols*., 2008, **3**, 1101–1108.

Table S1. Sequences of synthesized DNA templates and RNA

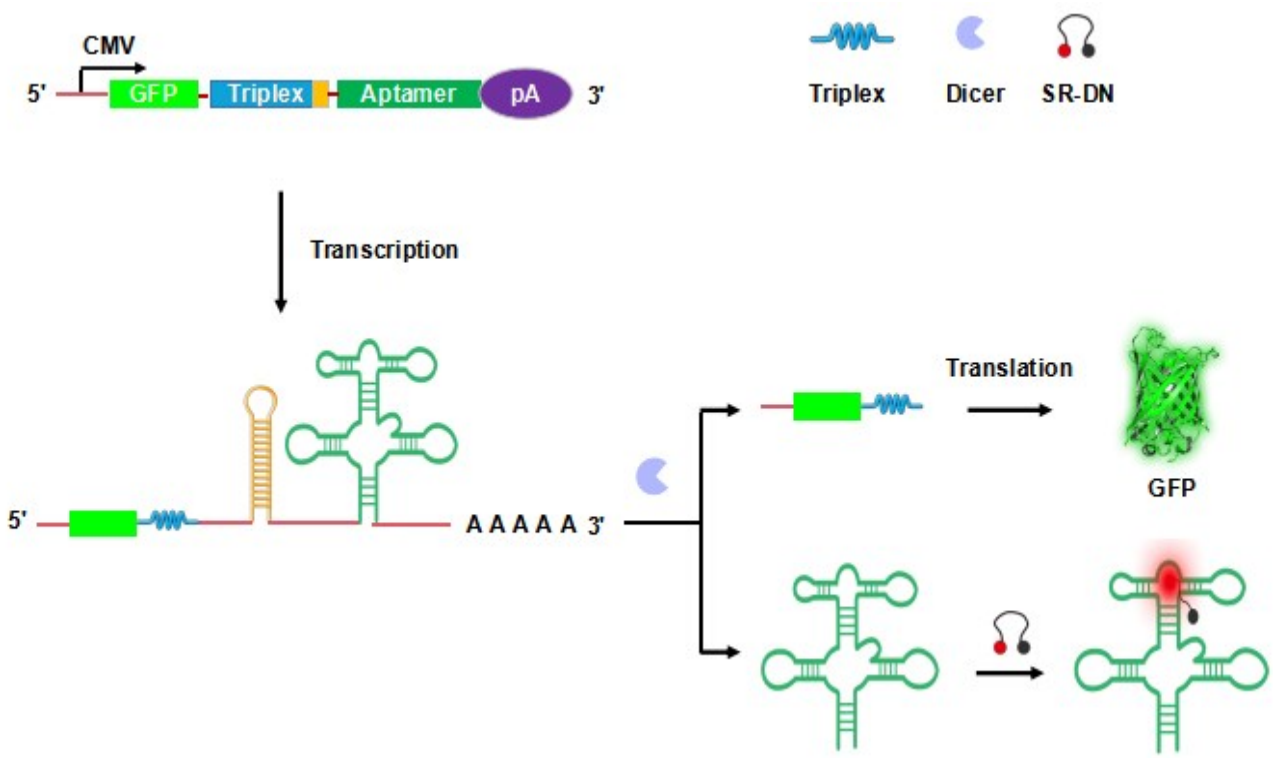
Name	Sequences (5'-3')
mRNA-stablizing triplex template	ACC GGT GAT TCG TCA GTA GGG TTG TAA AGG TTT TTC TTT TCC TGA GAA AAC AAC CTT TTG TTT TCT CAG GTT TTG CTT TTT GGC CTT TCC CTA GCT TTA AAA AAA AAA AAG CAA AA
dsRNA Dicer substrate template	CTC ACC GAG GCA GTT CCA TAC AAG ATC CTG GTA TAT TCT CCG AAC GTG TCA CGT TAG CAT TAG CTG CTA ACG TGA CAC GTT CGG AGA ATG CTA GCG TTT TAG AGC TAG AAT TTT TTT C
tRNA-scaffolded RNA aptamer template	GCC CGG ATA GCT CAG TCG GTA GAG CAG CGG CCG CAC CTC GCT TCG GCG ATG ATG GAG AGG CGC AAG GTT AAC CGC CTC AGG TGC GGC CGC GGG TCC AGG GTT CAA GTC CCT GTT CGG GCG CCA
tRNA-scaffolded RNA sensor template	GCC CGG ATA GCT CAG TCG GTA GAG CAG CGG CCG AAA AAG AGG TCA TGT AGA GAT GCG GTG GTC GAC CTC GCT TCG GCG ATG ATG GAG AG G CGC AAG GTT AAC CGC CTC AGG TCA AAA ACG GCC GCG GGT CCA GGG TTC AAG TCC CTG TTC GGG CGC CA
T7 promoter sequence	TAA TAC GAC TCA CTA TAG GG
Survivin mRNA	GAC CAC CGC AUC UCU ACA UUC A
miR-141	UAA CAC UGU CUG GUA AAG AUG G
miR-222	CUC AGU AGC CAG UGU AGA UCC U
let-7f	UGA GGU AGU AGU AGA UUG UAU AGU U

The underlined bases indicate the triplex sequence. The red bases indicate the cDNA template of double-stranded RNA sequence for Dicer enzyme digestion. Green bases indicate the SR aptamer sequence. The brown bases indicate $tRNA^{Lys}$ scaffold. The blue bases indicate *survivin* mRNA response region.

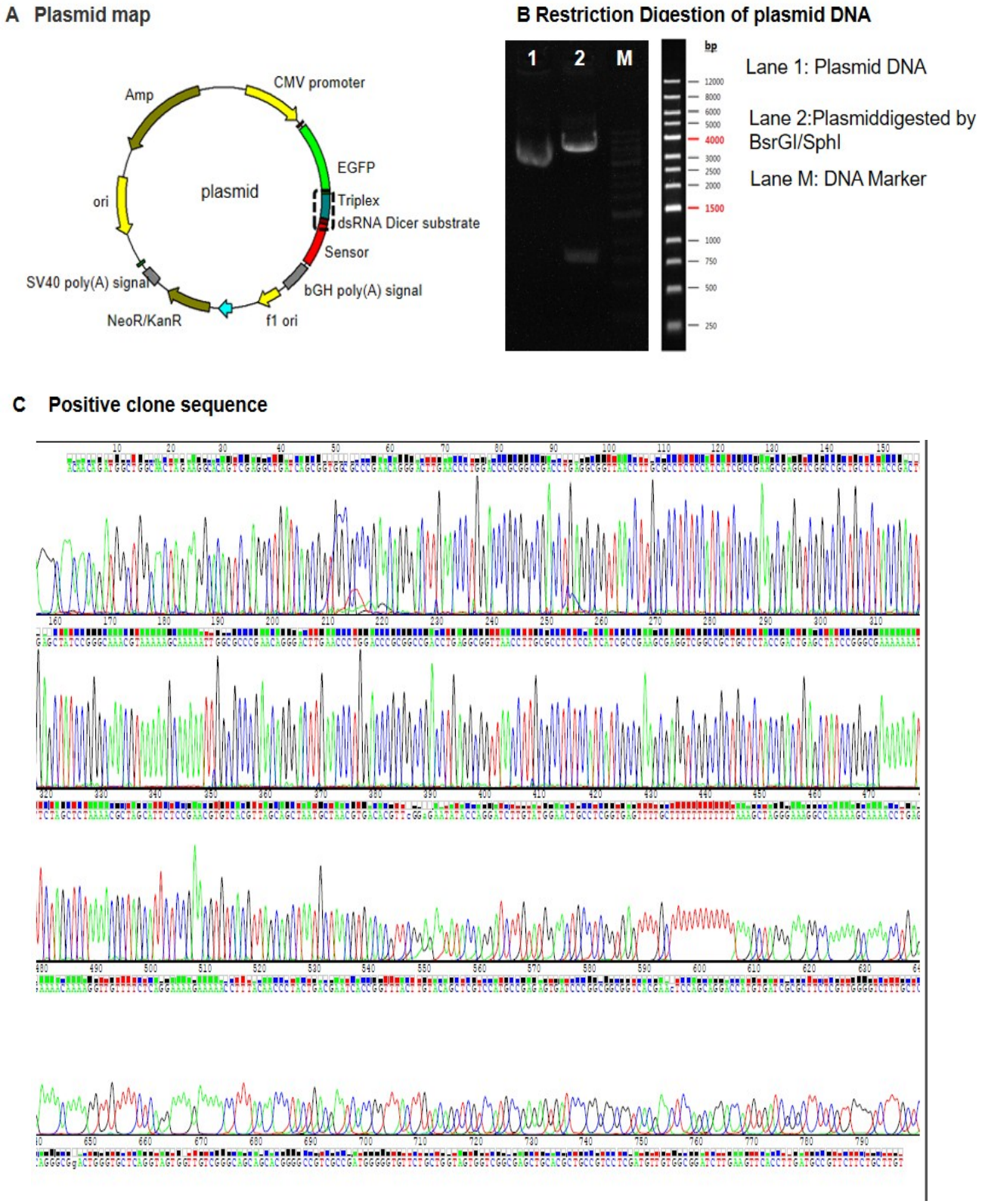
Table S2. Synthesized DNA primers for RT-PCR analysis

Name	Sequences (5'-3')
RNA aptamer F primer	TAA CCG CCT CAG GTC AAA AA
RNA aptamer R primer	ACC GCA TCT CTA CAT GAC CTC T
GFP mRNA F primer	AGA AGA ACG GCA TCA AGG TG
GFP mRNA R primer	TCT CGT TGG GGT CTT TGC T
dsRNA Dicer substrate F primer	CTC ACC GAG GCA GTT CCA TA
dsRNA Dicer substrate R primer	TCT AGC TCT AAA ACG CTA GCA TTC
Fusion of GFP mRNA and RNA aptamer F primer	AGA AGA ACG GCA TCA AGG TG
Fusion of GFP mRNA and RNA aptamer R primer	ACC GCA TCT CTA CAT GAC CTC T
Survivin mRNA F primer	TTT CTC AAG GAC CAC CGC A
Survivin mRNA R primer	TTC TCA GTG GGG CAG TGG A
β-actin F primer	TAG TTG CGT TAC ACC CTT TCT TG
β-actin R primer	TCA CCT TCA CCG TTC CAG TTT

Scheme S1. Illustration of single-promoter system for co-expression of RNA aptamer and GFP mRNA.



Scheme S2. Single-promoter plasmid with a Dicer substrate site for expressing splittable fusion of GFP mRNA and RNA sensor. (A) Plasmid structure. (B) Gel electrophoresis image after restriction endonuclease cleavage of the plasmid, Lane 1: Plasmid DNA, Lane 2: Plasmid digested by BsrGI/SphI, Lane M: DNA Marker. (C) Positive clone sequence data of the plasmid.



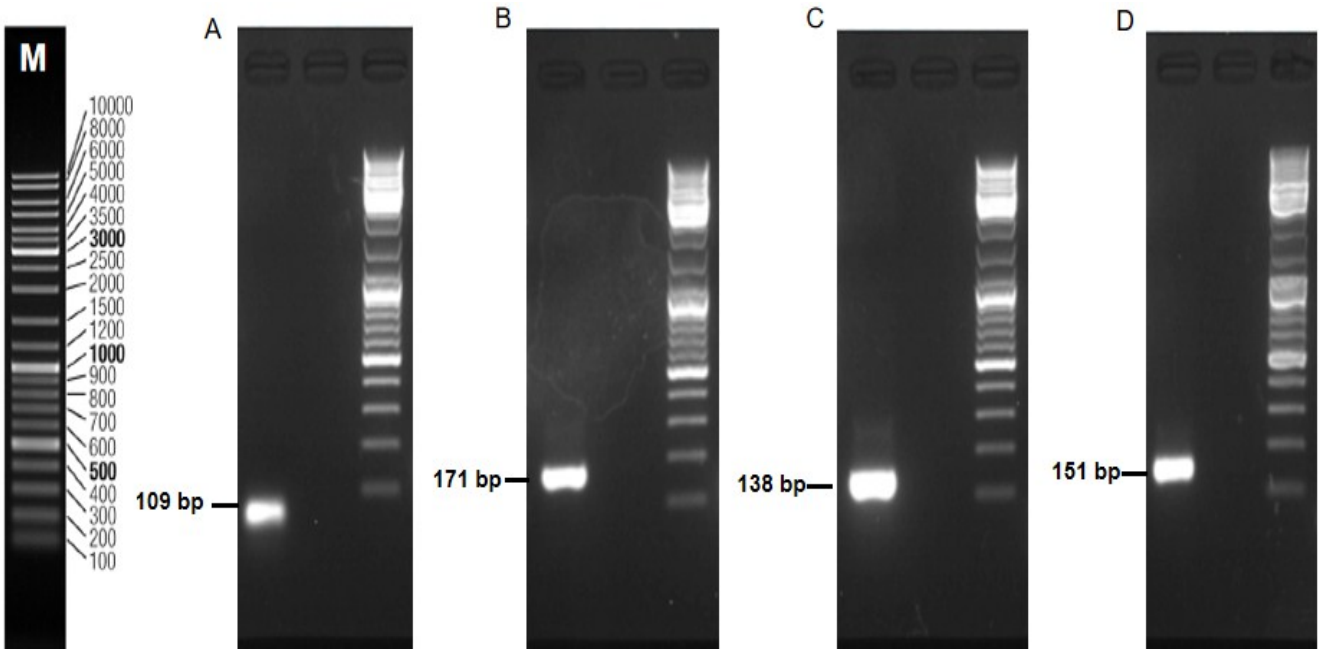


Figure S1. Agarose gel electrophoresis analysis for total RNA extracts of HeLa cells after RT-PCR analysis using primers specific to Dicer substrate site (A), GFP mRNA (B), RNA aptamer (C) and β-actin mRNA (D).

Note: Although the Dicer substrate site would be cleaved by Dicer enzyme to separate the RNA aptamer from the GFP mRNA in cells, it was still found to give a band in gel electrophoresis analysis after RT-PCR of the cell extracts. Dicer enzyme is predominantly present in cytosol, which is only highly efficient for RNA transcripts exported into the cytosol. On the other hand, RNA transcripts not exported from the nuclei could not be cleaved by Dicer enzyme, hence contributing to a band for the Dicer substrate site in gel electrophoresis analysis after RT-PCR of the cell extracts.

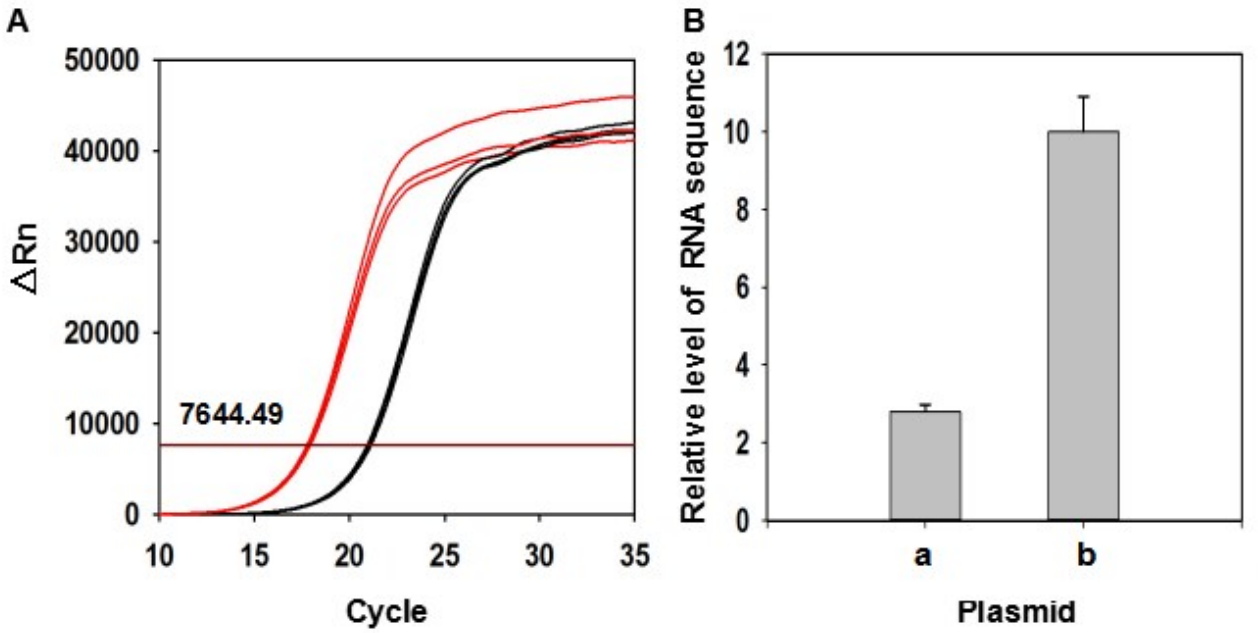


Figure S2. (A) qRT-PCR analysis for RNA extracts of HeLa cells transfected using single-promoter plasmid with (a) and without (b) a Dicer substrate site for co-expression of GFP mRNA and RNA aptamer for 24 h. Red: without a Dicer substrate site. Black: with a Dicer substrate site (B) Relative concentrations of the fusion mRNA.

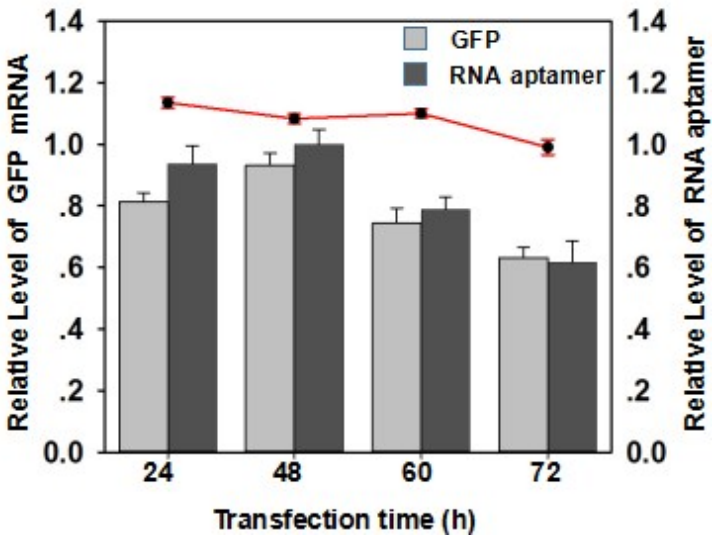


Figure S3. Relative concentrations of GFP mRNA and RNA aptamer in HeLa cells transfected using single-promoter plasmid for splittable fusion expression at different times, as determined by qRT-PCR.

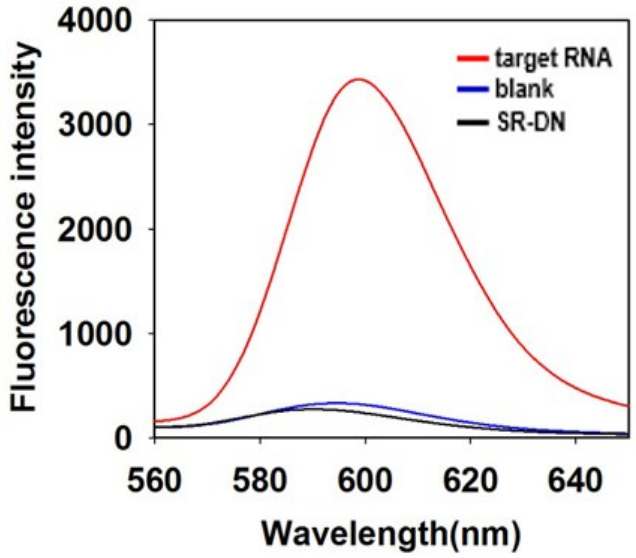


Figure S4. Fluorescence spectral responses from 1 μM SR-DN conjugate, RNA sensor and RNA sensor plus 5 μM *survivin* mRNA.

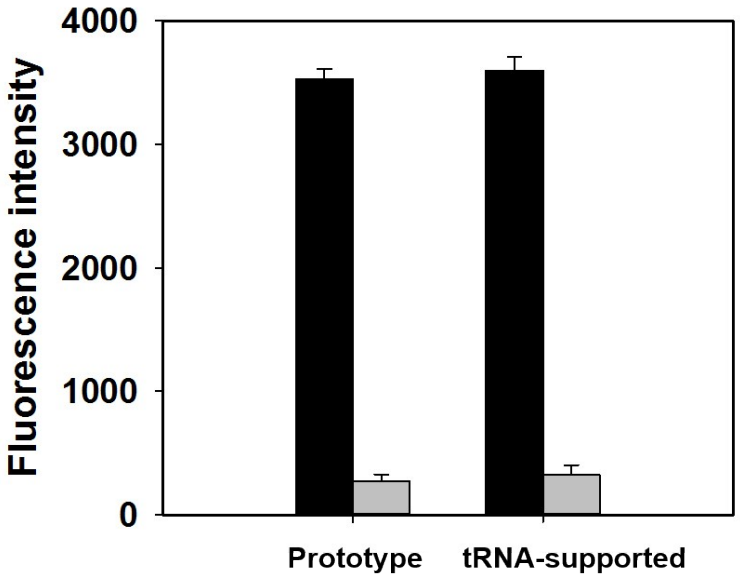


Figure S5. Fluorescence intensities of prototype RNA sensor and the tRNA-supported sensor plus 5 μM *survivin* mRNA, respectively. Black column: plus *survivin* mRNA, gray column: blank.

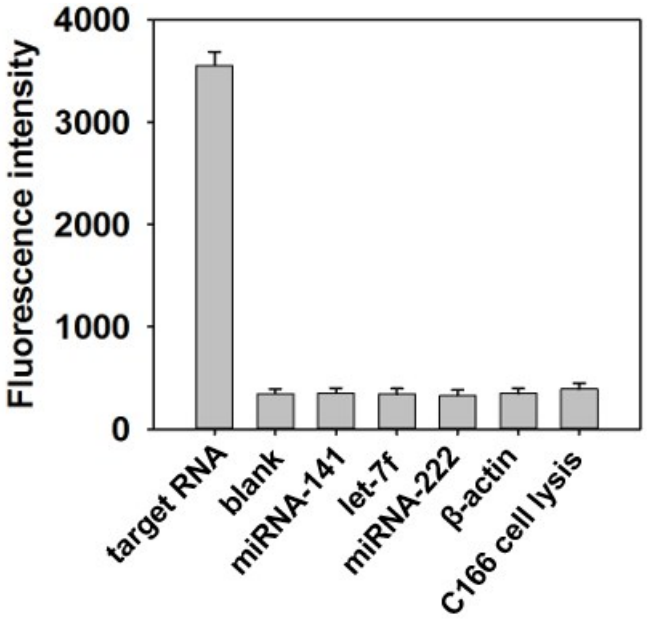


Figure S6. Fluorescence intensities of RNA sensor at 598 nm in response to *survivin* mRNA and other cellular components.

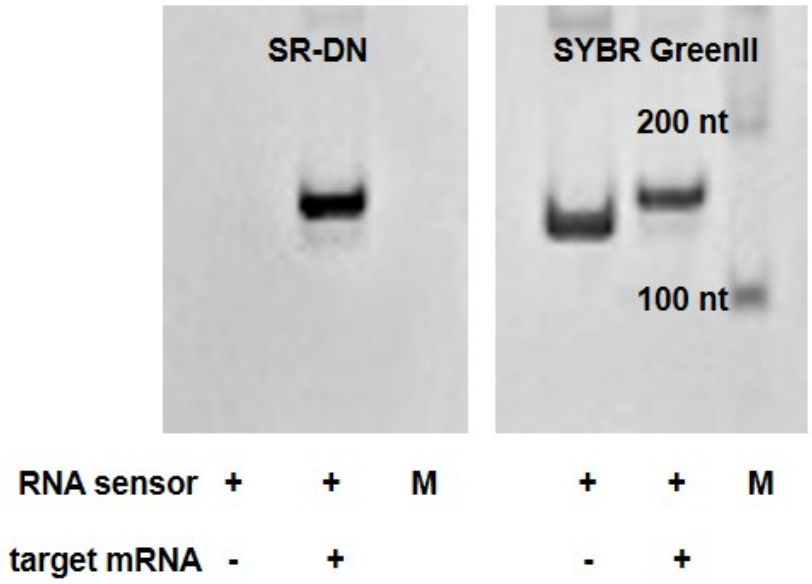


Figure S7. Gel electrophoresis image for RNA sensor with or without *survivin* mRNA stained using SR−DN conjugate or SYBR Green II. M denotes molecular weight markers.

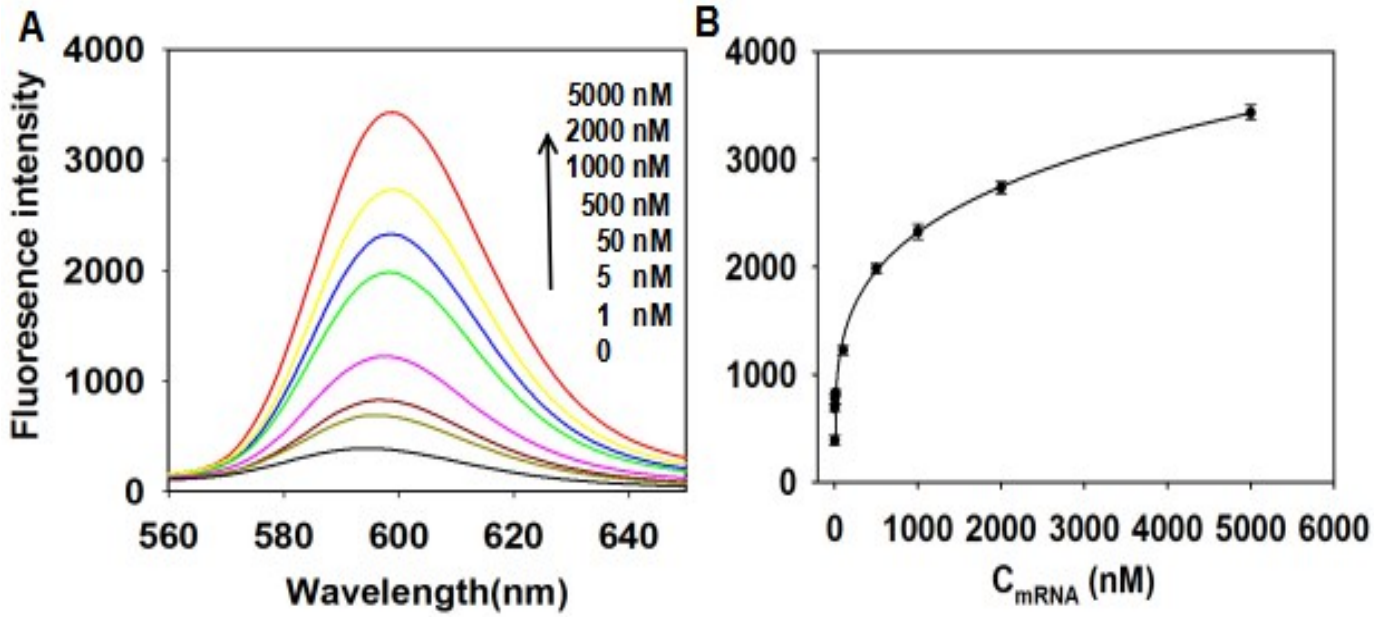


Figure S8. (A) Fluorescence spectra of RNA sensor to *survivin* mRNA of varying concentration. (B) Plot of fluorescence intensities (ex/em at 560/590 nm) of RNA sensor (5 μM) versus *survivin* concentrations.

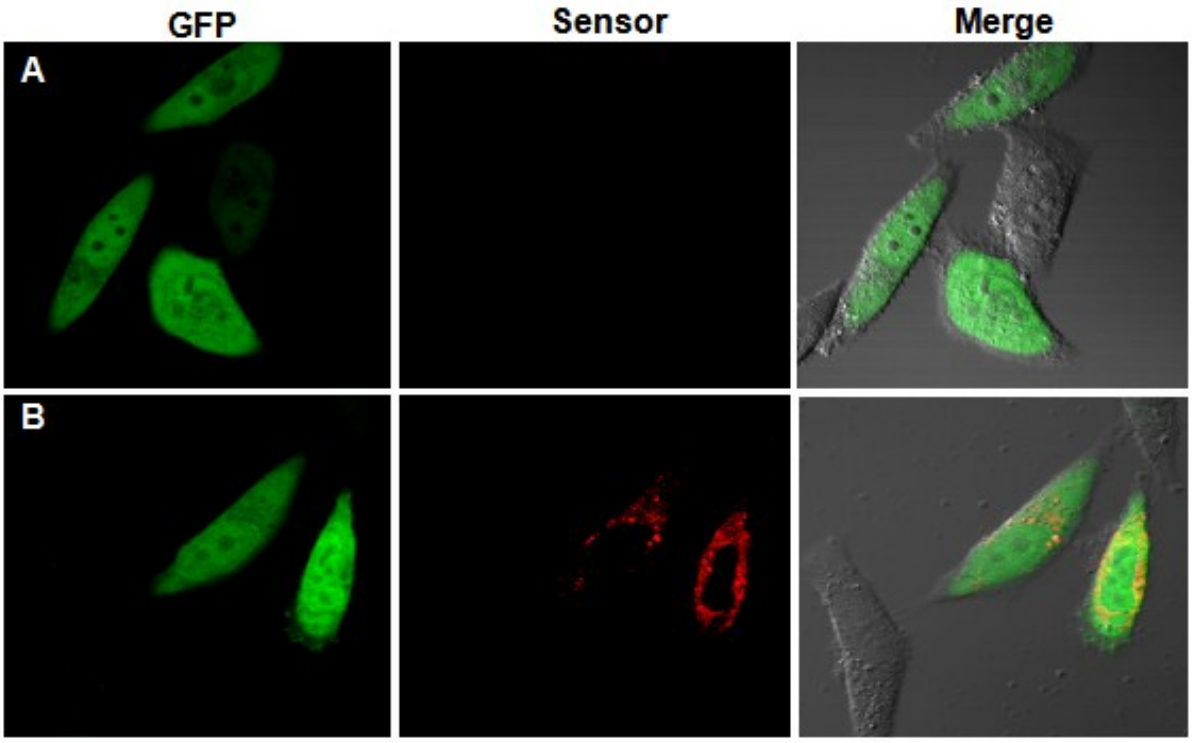


Figure S9. Confocal microscopy images for HeLa cells transfected using single-promoter plasmid with no Dicer substrate site (A) and a Dicer substrate site (B) for co-expression of SR aptamer and GFP mRNA in the presence of SR−DN.

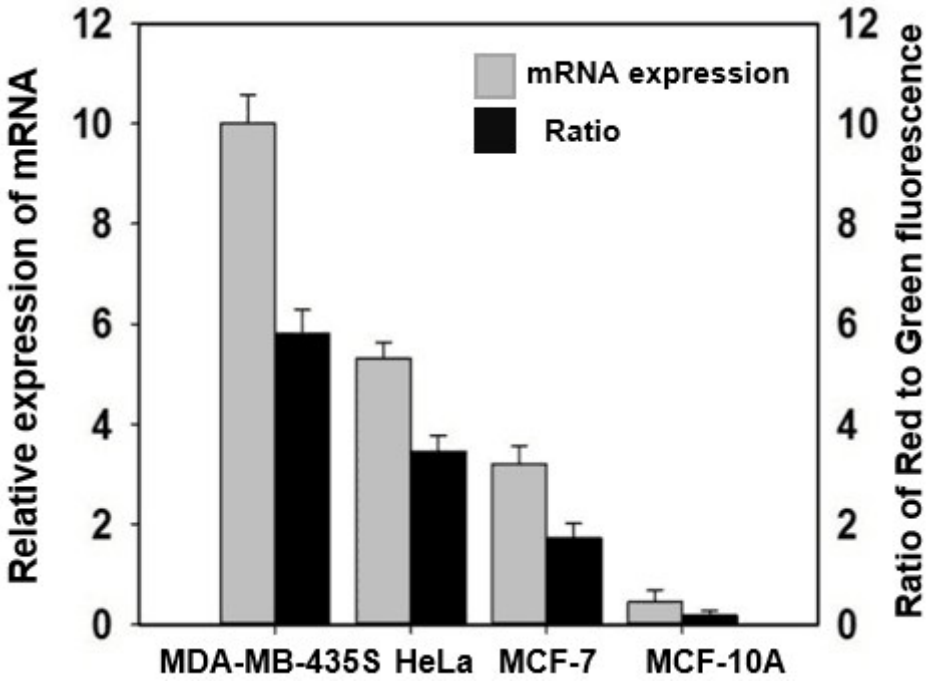


Figure S10. Average values of red-to-green fluorescence ratio in ROIs (ten ROIs in each image) compared with relative expression levels of *survivin* mRNA in different cells as determined by qRT-PCR.

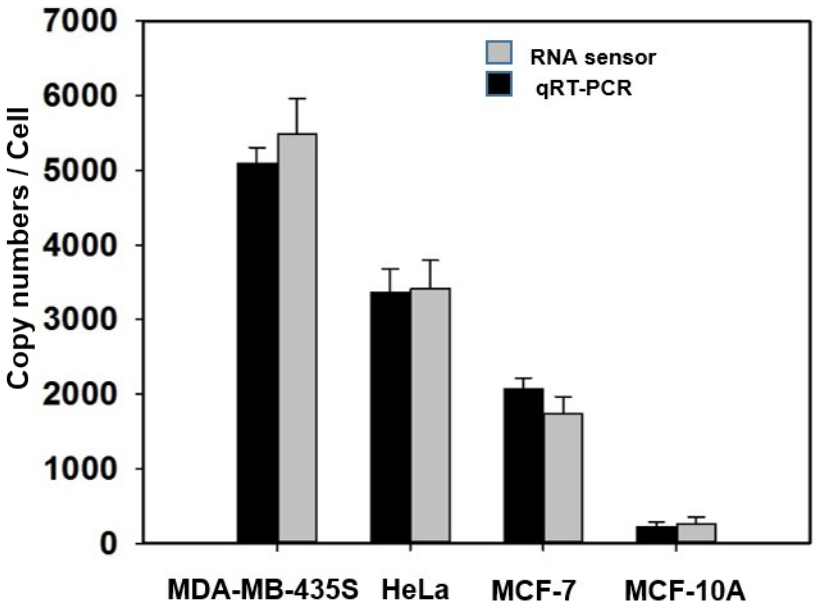


Figure S11. Copy numbers of *survivin* mRNA in different cells as determined by qRT-PCR or calculated according to the average values of red-to-green fluorescence ratio in ROIs (ten ROIs in each image).