

Supplementary Methods

Prevention of ribozyme catalysis through cDNA synthesis enables accurate RT-qPCR measurements of context-dependent ribozyme activity

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1 PCR amplification of DNA

Materials

- Bio-Rad T-100 Thermal cycler (BioRad, 1861096)
- DeNovix D-11 Series Spectrophotometer
- 200 μ L PCR tubes (USA Scientific, 1402-4700)
- Nuclease-Free Water (Ambion AM9938)
- 2x Phusion High-Fidelity Master Mix (ThermoFisher, F531L)
- QIAquick spin PCR Purification Kit (Qiagen, 28104)
- DNA gene fragments (ordered as eBlocks at 10 ng/ μ L in IDTE buffer from IDT)
- DNA oligonucleotides (ordered as standard desalted from IDT and prepared at 100 μ mol/L stocks in water)

Protocol

- 1) Prepare the master mix below and then add to the 200 μ L PCR tube containing the eBlock DNA
 - a. For IVT reactions, use T7fwd / T7rev as the two primers. For preparation of inserts for plasmids, use ga4_fwd / pET_ds_rev as the two primers (Supporting File S3)

Component	Final volume or concentration
2x Phusion MM	37.5 μ L
Fwd primer	0.5 μ mol/L
Rev primer	0.5 μ mol/L
Ambion water	X μ L to 74.85 μ L total

- 2) Add 0.15 μ L of 10 ng/ μ L eBlock DNA into each PCR tube containing master mix, pipette mix
- 3) Add samples to a thermocycler and execute the following protocol with the lid temperature set to 105 $^{\circ}$ C

Temp	Time	Repeat
98 $^{\circ}$ C	5 min	1x
98 $^{\circ}$ C	30 s	30x
60 $^{\circ}$ C	30 s	30x
72 $^{\circ}$ C	30 s	30x
72 $^{\circ}$ C	3 min	1x
4 $^{\circ}$ C	hold	

- 4) Run a PCR clean up with a QIAquick Spin PCR Purification Kit
 - a. Add 375 μ L of Buffer PB to a spin column
 - b. Add all 75 μ L of the PCR sample to the spin column containing Buffer PB and pipette mix thoroughly (10 to 20 times)
 - c. Spin column at 13,000 rpm for 1 min, discard flow through
 - d. Add 750 μ L of Buffer PE to the spin column
 - e. Spin column at 13,000 rpm for 1 min, discard flow through

- f. Spin column at 13,000 rpm for 1 min to remove any last Buffer PE, discard flow through
 - g. Place spin column insert into a fresh collection tube
 - h. To elute the PCR product, add 50 μ L of Buffer EB to the center of the spin column
 - i. Let the column sit for 1 min to 2 min at room temperature
 - j. Spin column at 13,000 rpm for 1 min, discard spin column
- 5) Measure the concentration of the purified PCR products with A260 of undiluted samples on a DeNovix D-11 Series Spectrophotometer

2 RNA production, purification, and protection from IVT

Materials

- Bio-Rad T-100 Thermal cycler (BioRad, 1861096)
- Qubit-4, Fluorometer
- 200 μ L PCR tubes (USA Scientific, 1402-4700)
- T7 RNA polymerase (RNAP) and associated 5x transcription buffer (ThermoFisher, EP0113)
- Ribonucleotide triphosphates (NTPs) (ThermoFisher, R0481)
 - These come as separate tubes at 100 mmol/L each
 - For IVT reactions, equal volumes of each NTP is mixed to produced a solution with 25 mmol/L of each of the four NTP types
- 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Invitrogen, AM9260G)
- THE RNA Storage Solution, (ThermoFisher, AM7001)
- RNase Away Decontamination Reagent, 250 mL (ThermoFisher, 10328011)
- Nuclease-Free Water (Ambion, AM9938)
- TE, pH 8.0, RNase-Free (ThermoFisher, AM9849)
- DNase I HC (50 U/ μ L), RNase-Free and associated 10x DNA Digestion Buffer (ThermoFisher, EN0523)
- RNA Binding Buffer (ZymoResearch, R1013)
- RNA Wash Buffer (ZymoResearch, R1003)
- DNA templates for transcription (prepared by PCR in Section 1)
- Blocking oligonucleotide (prepared at a stock concentration of 1000 μ mol/L in water)
- Zymo-Spin IC columns (ZymoResearch, C1004-50)
- Zymo collection Tubes, (ZymoResearch, C1001-50)
- Qubit RNA HS Buffer Kit (ThermoFisher, Q32852)
- Formamide (Millipore Sigma, S4117)

Protocol

RNA production from IVT:

- 1) For IVT reactions, prepare the following reaction mix as below in 200 μ L PCR tubes
 - a. For most experiments, a master mix was prepared with all components other than the DNA templates and DNA templates were added to tubes after distributing the master mix
 - b. For U2h and G2h, which had low transcription efficiency due to only a single G at the 5' end of the transcripts, the DNA template concentration was increased to 100 nmol/L and T7 RNAP concentration was increased to 10 U/ μ L

Component	Final volume or concentration
Ambion water	X μ L to 25 μ L total
5x txn buffer	1x (5 μ L for 25 μ L reaction)
NTPs (25 mmol/L each)	2 mmol/L each (2 μ L for 25 μ L reaction)
DNA template (PCR product)	25 nmol/L
T7 RNAP (200 U/ μ L)	1 U/ μ L (0.13 μ L for 25 μ L reaction)

- 2) Add samples to a thermocycler and incubate at 37 °C with the lid temperature set to 105 °C for 1 h

RNA purification from IVT:

- 3) In RNase free PCR tubes, add 2 µL DNase I HC (50 U/µL) to 3 µL 10x DNA Digestion Buffer and mix by gentle inversion
- 4) Add all 5 µL of this DNase I mixture to 25 µL of IVT RNA samples
- 5) Incubate at 37°C for 20 m
- 6) Add 60 µL RNA Binding Buffer supplemented with EDTA (final concentration 36 mmol/L)
- 7) Add 90 µL of 100% ethanol and mix pipette mix immediately
- 8) Transfer sample to the Zymo-Spin IC column and centrifuge for 30 s at 7,000 x g
- 9) Transfer the column into a new Zymo collection tube
- 10) Add 700 µL RNA Wash Buffer to the column and centrifuge for 30 sat 7,000 x g
- 11) Discard the supernatant
- 12) Add 400 µL RNA Wash Buffer to the column and centrifuge for 1.5 min at 12,000 x g
- 13) Transfer the column carefully into an RNase free tube
- 14) Add 20 µL of sterile TE buffer directly to the column matrix, incubate for 5 min at room temperature and centrifuge for 2 min at 12,000 x g
- 15) Transfer to the fresh tube with 20 µL of THE RNA Storage Solution for prolonged storage
- 16) Dilute total RNA 1:5 by volume with THE RNA Storage Solution before quantitation with Qubit
- 17) Verify RNA concentration using Qubit with Qubit RNA HS Buffer Kit
- 18) The protocol can be paused at this stage and the RNA stored at -80 °C

RNA protection with blocking oligonucleotide:

- 19) Transfer 120 ng of purified IVT RNA into a fresh PCR tube (calculate necessary volume according to Qubit quantitation)
- 20) Add 2.2 µL of blocking oligonucleotide (stock concentration 1000 µmol/L) and add RNA Storage Solution to reach a total volume of 14 µL
- 21) Transfer the sample to a thermocycler and run the following annealing protocol:

Temp (ramp)	Time	Repeat
90 °C	5 m	1x
90 °C (-0.1 °C)	6 s	99x
80 °C (-0.1 °C)	6 s	99x
70 °C (-0.1 °C)	6 s	99x
60 °C (-0.1 °C)	6 s	99x
50 °C (-0.1 °C)	6 s	99x
40 °C (-0.1 °C)	6 s	99x
30 °C (-0.1 °C)	6 s	99x
20 °C	hold	

22) Verify RNA concentration using Qubit with Qubit RNA HS Buffer Kit

- a. We found that the presence of the blocking oligonucleotide would typically result in an increased concentration compared to our measurements of the RNA prior to blocking oligonucleotide addition. This could be up to four times higher than the nominal concentration of RNA we added to the blocking oligonucleotide reaction

3 Gel electrophoresis

Materials

- Bio-Rad T-100 Thermal cycler (BioRad, 1861096)
- E-Gel Power Snap-Electrophoresis Device (Invitrogen, G8100)
- Gel Documentation System: FastGene FAST-Digi PRO (NIPPON EUROPE Genetics, GP-07LED)
- DNase I (2 U/ μ L), RNase-Free (New England Biolabs, M0303S)
- 50 mmol/L CaCl_2 (prepared from CaCl_2 salt in DI water)
- Formamide (Millipore Sigma, S4117)
- 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Invitrogen, AM9260G)

Protocol

For denaturing gel electrophoresis of IVT RNA without blocking oligonucleotide:

- 1) Mix 1 μ L of DNase I (stock at 2 U/ μ L) and 1 μ L of 50 mmol/L CaCl_2 with 10 μ L of the IVT samples described in Section 2 after the 1 h incubation 37 °C, pipette mix
- 2) Allow samples to incubate for 20 min at 37 °C in the thermocycler
- 3) Add 10 μ L of ~100 % formamide supplemented with 36 mmol/L of EDTA, pipette mix
- 4) Increase the temperature of the thermocycler to 85 °C and incubate the samples for 5 min at this temperature
- 5) Immediately load 20 μ L of sample to a well in a 4% Agarose E-gel EX pre-stained with SYBR Gold
- 6) Run gel for 30 min on an E-Gel Power Snap-Electrophoresis Device
 - a. Select the 4% agarose gel setting in the device when setting up protocol
- 7) Allow gel to cool to room temperature and then image on a gel documentation system
 - a. We found that gels taken for imaging right after electrophoresis were hot to the touch and did not image well until allowed to cool down

For denaturing gel electrophoresis of IVT RNA with blocking oligonucleotide:

- 1) Starting with RNA that has been annealed with blocking oligonucleotide (taken through Step 21 of Section 2), make the following mixture:

Component	Final volume
SuperScriptR III RT/PlatinumR Taq Mix	0.2 μ L
2X SYBR Green Reaction Mix	5 μ L
Purified and blocked RNA	4.8 μ L

- 2) Incubate samples for 10 min in thermocycler at desired temperature (37 °C or 50 °C)
- 3) Add 10 μ L of ~100 % formamide supplemented with 36 mmol/L of EDTA, pipette mix
- 4) Increase the temperature of the thermocycler to 85 °C and incubate the samples for 5 min at this temperature
- 5) Immediately load 20 μ L of sample to a well in a 4% Agarose E-gel EX prestained with SYBR Gold

- 6) Run gel for 30 min on an E-Gel Power Snap-Electrophoresis Device
 - a. Select the 4% agarose gel setting in the device when setting up protocol
- 7) Allow gel to cool to room temperature and then image on a gel documentation system

4 Plasmid assembly and cloning

Materials

- Bio-Rad T-100 Thermal cycler (BioRad, 1861096)
- Eppendorf Eporator (Eppendorf, 4309000027)
- pETDuet-1 plasmid DNA (Millipore Sigma, 71146-3)
- Falcon 14 mL Round Bottom High Clarity PP Test Tube (Corning, 352059)
- 2x Phusion High-Fidelity Master Mix (ThermoFisher, F531L)
- SOC outgrowth medium (New England Biolabs, B9020S)
- QIAquick spin PCR Purification Kit (Qiagen, 28104)
- DPNI (ThermoFisher, FD1703)
- Qiagen Spin Miniprep Kit (Qiagen, 27104)
- 2x Gibson Assembly Master Mix (New England Biolabs, E2611L)
- Electrocompetent cells derived from DH5 α Cells (ThermoFisher, 18265017).
- Electrocompetent cells derived from One Shot BL21 Star (DE3) Chemically Competent *E. coli* (Invitrogen, C601003)
- LB, Miller (ThermoFisher, BP1426)
- 2% Agarose E-gels, SYBR Safe (Invitrogen, A42135)
- LB agar plates with ampicillin (100 μ g/mL)
- MicroPulser Electroporation Cuvettes, 0.2 cm gap (BioRad, 1652086)

Protocol

- 1) Prepare the insert DNA with PCR as described in Section 1
 - a. Use ga4_fwd / pET_ds_rev as these will result in a PCR product that has the 30 base homology domains for Gibson Assembly
 - b. We ordered eBlocks with the 30 base homology domains flanking the T7 promoter and terminator domains, but extended T7fwd / T7rev primers containing the homology domains could also be used to prepare inserts for cloning (T7fwd_ga4 / T7rev_pET_ds in Supporting File S3)
- 2) Prepare the pET backbone with PCR:

Component	Final volume or concentration
2x Phusion MM	37.5 μ L
ga4_fwd primer	0.5 μ mol/L
pET_ds_rev primer	0.5 μ mol/L
Ambion water	X μ L to 74.85 μ L total

- 3) Add 0.15 μ L of 10 ng/ μ L pETDuet-1 plasmid DNA into each PCR tube containing master mix, pipette mix

- 4) Add samples to a thermocycler and execute the following protocol with the lid temperature set to 105 °C

Temp	Time	Repeat
98 °C	5 min	1x
98 °C	30 s	30x
62 °C	30 s	30x
72 °C	2 min	30x
72 °C	5 min	1x
4 °C	hold	

- 5) Run a PCR clean up with a QIAquick Spin PCR Purification Kit
- See Step 4 in Section 1
 - Elute in 30 µL Buffer EB
- 6) To the 35 µL purified pET backbone DNA, add 1.5 µL DPNI and 3.5 µL DPNI CutSmart Reaction Buffer
- 7) Incubate at 37 °C for 1 h
- 8) Run a PCR clean up with a QIAquick Spin PCR Purification Kit
- See Step 4 in Section 1
 - Elute in 30 µL Buffer EB
- 9) Measure the concentration of the purified PCR products with A260 of undiluted samples on a DeNovix D-11 Series Spectrophotometer
- 10) Using purified DNA insert and DNA backbone, prepare the following Gibson Assembly mix:

Component	Final volume or concentration
2x NEB GA mix	3.75 µL
DNA insert	15 ng/µL (final concentration)
DNA backbone	15 ng/µL (final concentration)
Ambion water	X µL to 7.5 µL total

- 11) Incubate Gibson Assembly mix at 50 °C for 1 h
- 12) Mix 1.5 µL of Gibson Assembly mixture with 50 µL of electrocompetent DH5α cells in MicroPulser Electroporation Cuvettes
- 13) Load cuvette into Eppendorf Eporator and run electroporation at 2500 V
- 14) Mix all 50 µL cells with 900 µL of SOC media in 1.7 mL microfuge tubes and grow with 1000 rpm shaking for 1 h time at 37 °C
- 15) Spin down the outgrowth cultures at 12800 rpm for 1 min
- 16) Remove 700 µL of supernatant and discard
- 17) Resuspend the cell pellet with the remaining media in the 1.7 mL microfuge tube and plate all of this resuspension (~200 µL) on LB agar plates supplemented with ampicillin
- 18) Incubate plates overnight at 37 °C

19) The next day, pick 2 to 5 colonies, mix in 50 μ L of water and conduct a colony PCR to verify insert size with gel electrophoresis

a. Use the following PCR protocol:

Component	Final volume or concentration
2x Phusion MM	27.5 μ L
pET seq fwd primer	0.5 μ mol/L
pET seq rev primer	0.5 μ mol/L
Colony in water	2 μ L
Ambion water	X μ L to 55 μ L total

b. Mix 2 μ L of PCR product with 18 μ L of water and load all 20 μ L on a 2 % Agarose E-gel to check insert size

20) For colonies that have the correct insert size, purify the PCR products with a QIAquick Spin PCR Purification Kit

a. See Step 4 in Section 1

21) Send purified PCR products for Sanger sequencing verification using the primers from the colony PCRs as the sequencing primers

22) For colonies with sequence verified inserts, start overnight cultures in 5 mL of LB+Amp media in 14 mL Falcon tubes

a. Grow at 37 $^{\circ}$ C with 300 rpm shaking for at least 16 hr

23) From the overnight cultures, extract plasmids with a Qiagen Spin Miniprep Kit according to manufacture protocol

24) Transform purified plasmids into electrocompetent BL21 Star (DE3) *E. coli*

a. Mix 0.5 μ L to 2 μ L of purified plasmid with 50 μ L of electrocompetent cells

i. Typically adding ~50 ng total plasmid DNA

ii. Do not exceed 2 μ L of total plasmid DNA volume

b. Repeat steps 13 through 18

25) Prepare glycerol stocks from transformants and store at -80 $^{\circ}$ C for future use

a. Prepare 3 mL overnight liquid cultures (LB+Amp)

b. Mix 500 μ L of 40 % by volume glycerol with 500 μ L of overnight culture

5 RNA production, extraction, and protection from *E. coli*

Materials

- Bio-Rad T-100 Thermal cycler (BioRad, 1861096)
- Qubit-4 Fluorometer (ThermoFisher)
- Genesys 30 visible spectrophotometer (ThermoFisher, 840-277000).
- Falcon 14 mL Round Bottom High Clarity PP Test Tube (Corning, 352059)
- Falcon 50 mL High Clarity Conical Centrifuge Tubes (Corning, 352098)
- Falcon 15 mL Conical Centrifuge Tubes (Corning, 352196)
- 0.5 mol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Invitrogen, AM9260G)
- THE RNA Storage Solution (ThermoFisher, AM7001)
- RNAProtect Bacteria Reagent (Qiagen, 1018380)
- RNase Away Decontamination Reagent, 250 mL (ThermoFisher, 10328011)
- Nuclease-Free Water (Ambion, AM9938)
- TE, pH 8.0, RNase-Free (ThermoFisher, AM9849)
- Ethanol, 200 PROOF, (Decon Labs, DSP-AZ-1)
- LB media, Miller (ThermoFisher, BP1426)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG) (Millipore Sigma, I5502)
- DNase I HC (50 U/ μ L), RNase-Free and associated 10x DNA Digestion Buffer (ThermoFisher, EN0523)
- TRI Reagent (ZymoResearch, R2050)
- Chloroform, Molecular Biology Reagent (ThermoFisher, J67241-AP)
- RNA Binding Buffer (ZymoResearch, R1013)
- RNA Wash Buffer (ZymoResearch, R1003)
- Zymo-SpinTM IICG Column (ZymoResearch, C1006-50-G)
- Collection Tubes (ZymoResearch, C1001-50)
- Qubit RNA HS Buffer Kit (ThermoFisher, Q32852)

Protocol

RNA production in E. coli:

- 1) Start overnight culture in 14 mL Falcon tubes from glycerol stock stab in 3 mL LB+Amp (100 μ g/mL) media and grow at 37 °C and 300 rpm shaking for 16 hours
- 2) Dilute overnight cultures 1:100 into 10 mL of LB+Amp (100 μ g/mL) with 100 μ mol/L IPTG and grow in 50 mL Falcon tubes at 37 °C and 300 rpm shaking until the culture reaches an optical density at 600 nm of 0.5 to 0.8
 - a. Optical density measurements were conducted on a Genesys 30 visible spectrophotometer

RNA extraction from E. coli:

- 3) Transfer 1.5 mL of culture to 15 mL Falcon centrifuge tube
- 4) Add 3 mL of RNA protect Bacteria Reagent incubate 5 min at room temperature
- 5) Centrifuge for 10 min at 5000 x g at 4 °C

- 6) Decant the supernatant, remove residual supernatant
- 7) Resuspend the cells pellet in 100 μ L 50 mmol/L EDTA solution
- 8) Add 1 mL of TRI Reagent
- 9) Homogenize for 5 min at room temperature
- 10) Add 200 μ L of chloroform and vortex for 15 s
- 11) Incubate the homogenate for 5 min at room temperature
- 12) Centrifuge at 12,000 x g for 10 min at 4°C for phase separation
- 13) Transfer 600 μ L of top aqueous phase to a new 1.5 mL microcentrifuge tube
- 14) Add 600 μ L of 100 % ethanol and mix immediately
- 15) Transfer 700 μ L of the sample to the Zymo-Spin IIICG Column in a Zymo Collection Tube
- 16) Centrifuge for 30 s at 7,000 x g at room temperature, remove the supernatant
- 17) Transfer the remaining 500 μ L of the sample to the same Zymo-Spin IIICG Column in a Zymo Collection Tube
- 18) Centrifuge for 30 s at 7,000 x g
- 19) Transfer the column into a new collection tube
- 20) Add 700 μ L of RNA Wash Buffer to the column and centrifuge for 30 s at 7,000 x g at room temperature, remove the supernatant
- 21) Add 400 μ L of RNA Wash Buffer to the column and centrifuge for 1.5 min at 12,000 x g
- 22) Transfer the column carefully into an RNase free tube
- 23) Add 50 μ L DNase/RNase-free water directly to the column matrix, incubate for 5 min at room temperature and centrifuge for 2 min at 12,000 x g
- 24) In an RNase-free PCR tube, add 5 μ L DNase I HC (50 U/ μ L) to 6 μ L 10x DNA Digestion Buffer and mix by gentle inversion
- 25) Add all 11 μ L of DNase I mixture to 50 μ L of extracted total RNA
- 26) Incubate at 37°C for 30 min
- 27) Add 120 μ L RNA Binding Buffer supplemented with EDTA (final concentration 36 mmol/L)
- 28) Add 180 μ L of 100% ethanol and mix immediately
- 29) Transfer sample to the Zymo-Spin IC column and centrifuge for 30 s at 7,000 x g
- 30) Transfer the column into a new Zymo collection tube
- 31) Add 700 μ L RNA Wash Buffer to the column and centrifuge for 30 sat 7,000 x g
- 32) Discard the supernatant
- 33) Add 400 μ L RNA Wash Buffer to the column and centrifuge for 1.5 min at 12,000 x g
- 34) Transfer the column carefully into an RNase free tube
- 35) Add 20 μ L of sterile TE buffer directly to the column matrix, incubate for 5 min at room temperature and centrifuge for 2 min at 12,000 x g
- 36) Transfer to the fresh tube with 20 μ L of THE RNA Storage Solution for prolonged storage
- 37) Dilute total RNA 1:5 by volume with THE RNA Storage Solution before quantitation with Qubit
- 38) Verify RNA concentration using Qubit with Qubit RNA HS Buffer Kit
- 39) The protocol can be paused at this stage and the RNA stored at -80 °C

Total RNA protection with blocking oligonucleotide:

- 40) Transfer 240 ng of purified total RNA from *E. coli* into a fresh PCR tube (calculate necessary volume according to Qubit quantitation)
- 41) Add 2.2 μL of blocking oligonucleotide (stock concentration 1000 $\mu\text{mol/L}$) and add RNA Storage Solution to reach a total volume of 14 μL
- 42) Transfer the sample to a thermocycler and run the following annealing protocol:

Temp (ramp)	Time	Repeat
90 °C	5 min	1x
90 °C (-0.1 °C)	6 s	99x
80 °C (-0.1 °C)	6 s	99x
70 °C (-0.1 °C)	6 s	99x
60 °C (-0.1 °C)	6 s	99x
50 °C (-0.1 °C)	6 s	99x
40 °C (-0.1 °C)	6 s	99x
30 °C (-0.1 °C)	6 s	99x
20 °C	hold	

- 43) Verify RNA concentration using Qubit with Qubit RNA HS Buffer Kit
 - a. We found that the presence of the blocking oligonucleotide would typically result in an increased concentration from our measurements of the RNA prior to blocking oligonucleotide addition

6 RT-qPCR experiments

Materials

- ViiA 7 Real-Time PCR System (Applied Biosystems)
- SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (ThermoFisher, 11736059)
- Platinum *Taq* DNA Polymerase (ThermoFisher, 10966018)
- Applied Biosystems MicroAmp Optical 96-Well Reaction Plate (ThermoFisher, N8010560)
- Applied Biosystems MicroAmp Optical Adhesive Film (ThermoFisher, 4360954)
- Eppendorf PCR-Cooler 0.2 mL for 96-well PCR plates (Eppendorf, 022510541)
- 8 channel VOYAGER Adjustable Tip Spacing Pipette, 12.5 μ L (INTEGRA Biosciences, 4721)
- 96-well PCR-Cooler rack (Eppendorf, 022510541)
- 12.5 μ L tips (INTEGRA Biosciences, 4405)
- VIAFLO, Single Channel Electronic Pipette, 300 μ L (INTEGRA Biosciences, 4013)
- 300 μ L tips (INTEGRA Biosciences, 4435)

Protocol

- 1) For all samples, normalize purified RNA that is annealed with blocking oligonucleotide to a final concentration of 1 ng/ μ L with THE RNA Storage Solution
 - a. Verify RNA concentrations after dilutions using a Qubit – 1 ng/ μ L is approximately the lowest concentration that can be reliably measured
- 2) Using THE RNA Storage Solution, dilute the normalized RNA to a final concentration of 50 pg/ μ L if from *E. coli* or a 1 pg/ μ L if from IVT
 - a. These dilutions should be done for both gate (G) RNA and uncleaved control (U) RNA, as well as cleaved control RNA (C) if included
- 3) Prepare aliquots in 1.5 mL microcentrifuge tubes, store in -80°C
 - a. Thaw before using, vortex and centrifuge well.
- 4) Set up RT-qPCR reactions on ice using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit. Below are the volumes for a single reaction, but these volumes were typically scaled up to make a master mix for multiple samples without the RNA added

Components (Single reaction)	Sample	NTC	NRTC
SuperScriptR III RT/PlatinumR Taq Mix	0.5 µL	0.5 µL	0.5 µL - Platinum Taq DNA Polymerase
2X SYBR Green Reaction Mix	12.5 µL	12.5 µL	12.5 µL
Forward primer (10 µmol/L stock)	0.5 µL	0.5 µL	0.5 µL
Reverse primer (10 µmol/L stock)	0.5 µL	0.5 µL	0.5 µL
ROX Reference Dye	0.05 µL	0.05 µL	0.05 µL
Purified and oligo blocked RNA (1 pg/µL or 50 pg/µL)	2 µL	No template	2 µL
RNase free water to 25 µL	9 µL	7 µL	9 µL

- 5) Add 23 µL of master mix to the wells of Applied Biosystems MicroAmp Optical 96-Well Reaction Plate using 300 µL single channel electronic for repeated dispensing
- 6) Add 2 µL of template from PCR strip tubes to the plate well using 12.5 µL 8 channel adjustable tip electronic pipette for repeated transfer and pipette mixing
- 7) Seal the plate with Applied Biosystems MicroAmp Optical Adhesive Film
- 8) Centrifuge the 96-well plate in PCR-Cooler rack at 1000g for 1 min at 4 °C
 - a. Keep plate in the PCR-Cooler rack until ready to load the plate into the instrument
- 9) **Supplementary Information Section 2.1 has a diagram of the plate layout we typically used for these experiments**
 - a. This section also describes how we prepared the dilution series for primer efficiency analysis using the normalized RNA samples
- 10) Set up ViiA 7 Real-Time PCR System experiment program:
 - a. Select the 96-Well Block type (0.2 mL) using to run the experiment
 - b. Select the Comparative $\Delta\Delta C_t$ experiment type
 - c. Select the SYBR Green reagent using to detect the target sequence
 - d. Select the Standard ramp speed for the experiment
 - e. Melting curve analysis is run by default for the SYBR Green reagent
 - f. Use the following cycling protocol:

Temp	Time	Repeat
37 °C or 50 °C	10 min	1x
95 °C	5 min	1x
95 °C	15 s	40x
60 °C	1 min	40x
60 °C to 95 °C		1x