Electronic Supplementary Material (ESI) for ChemComm.

Supporting Material for

Temperature-sensitive protein expression in protocells

Haiyang Jia[a], Michael Heymann[a], Tobias Härtel[a], Lei Kai[a], Petra Schwille[a*]

[a.] Dept. Cellular and Molecular Biophysics, Max Planck Institute of Biochemistry, Am Klopferspitz 18, D - 82152 Martinsried, Germany

***Corresponding author: Petra Schwille, schwille@biochem.mpg.de**

Methods

Plasmid construction

Plasmid constructions and DNA manipulations were performed as the standard molecular biology techniques. RNAT3-E1010 (E1010: http://partsregistry.org) was cloned from the plasmid (pSB1K3-J23119-RNAT3-E1010[1]) and was inserted into pCoofy1 vectors (Life Technologies) with Xba-I at the 5'end of the coding sequence (CDS) and a Xho-I restriction site at the 3'end of the CDS. The mutants of RNA Thermometers were constructed with the whole plasmid mutagenesis method[2]. All the plasmid used in the cell free reaction were extracted from *E. coli* (TOP10) with the QIGEN medium plasmid extract kit. The investigation of the RNATs *in vivo* were performed in the *E. coli*(BL21DE3) strain as the reported method[1].

In vitro transcription/translation mixture.

PURExpress® In Vitro Protein Synthesis Kit (NEB) was used in all the cell free reactions. The reactions were performed as 10μl Buffer A, 7.5μl Buffer B, 300ng DNA and the ddH_2O, then incubated at 30°C, 35°C, 37°C, 40°C, 42°C for 2 hours using incubators or Thermomixer C(Eppendorf) and stopped by placing the tube(s) on ice. Then the fluorescence of the reactions were tested by the Infinite M200 PRO plate reader (TECAN). The controls used in the experiment were the black control and the positive control(Pcoofy1-T7-RBS-E1010) All experiments were performed in triplicate, and differences between the mean values were considered as significant at $p<0.05$ with T-test.

Droplet generation

Droplet produced with 'shaken-not-stirred' method

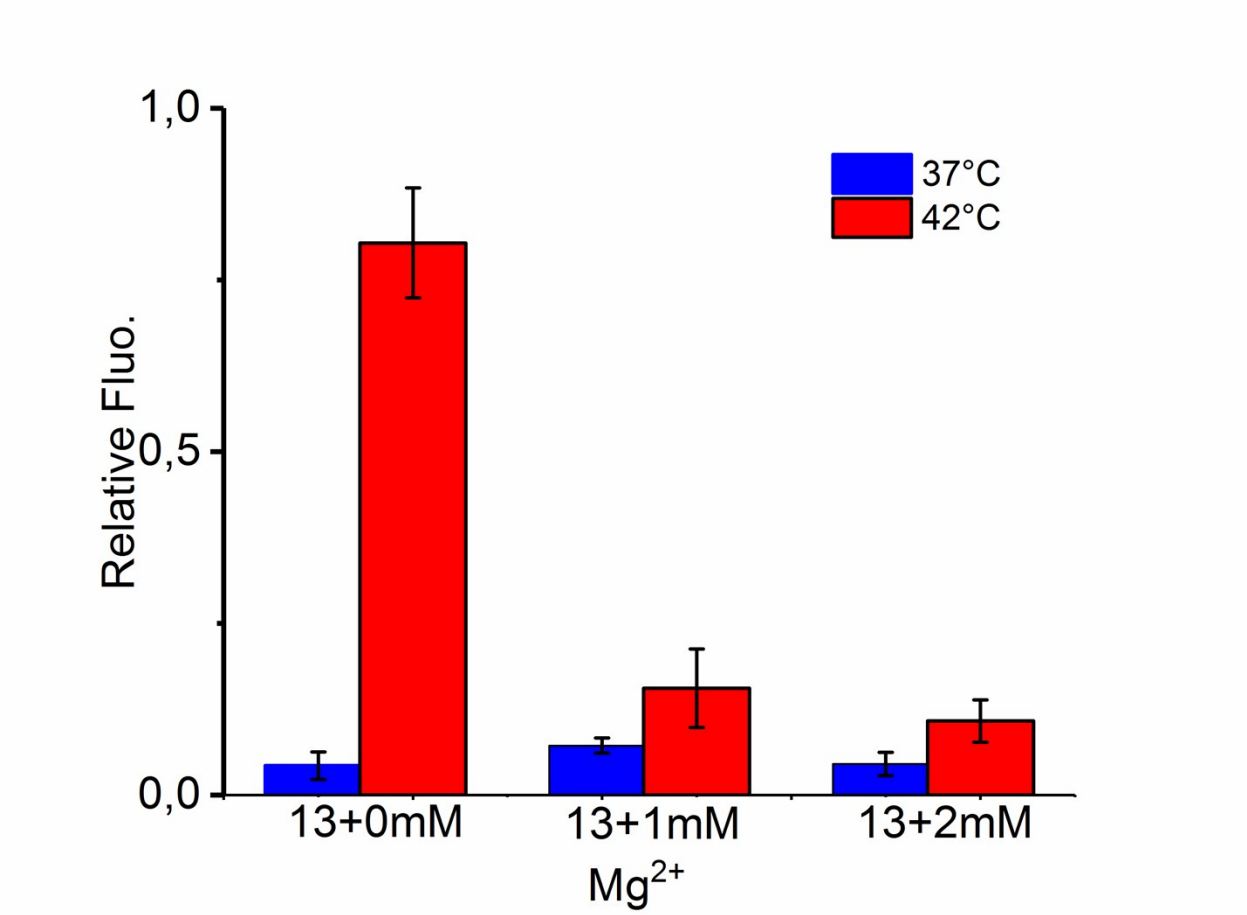
Different radii droplets were generated with the 'shaken-not-stirred' method. We mixed 5 μL cell free transcription/translation reaction mix with 25μL HFE7500 fluorinated oil with 1.8 % (w/w) PFPE-PEG-PFE biocompatible surfactant in PCR tubes (Eppendorf) using a benchtop vortex mixer for 60 s at maximum speed. Then the top droplets were absorbed in the 1.1*0.5mm glass vocabulary and the vocabulary was immobilized onto the glass slide for microscope measurements. This technique offers the possibility to generate a big range of different sized compartments in a very short time.

Microfluidic method

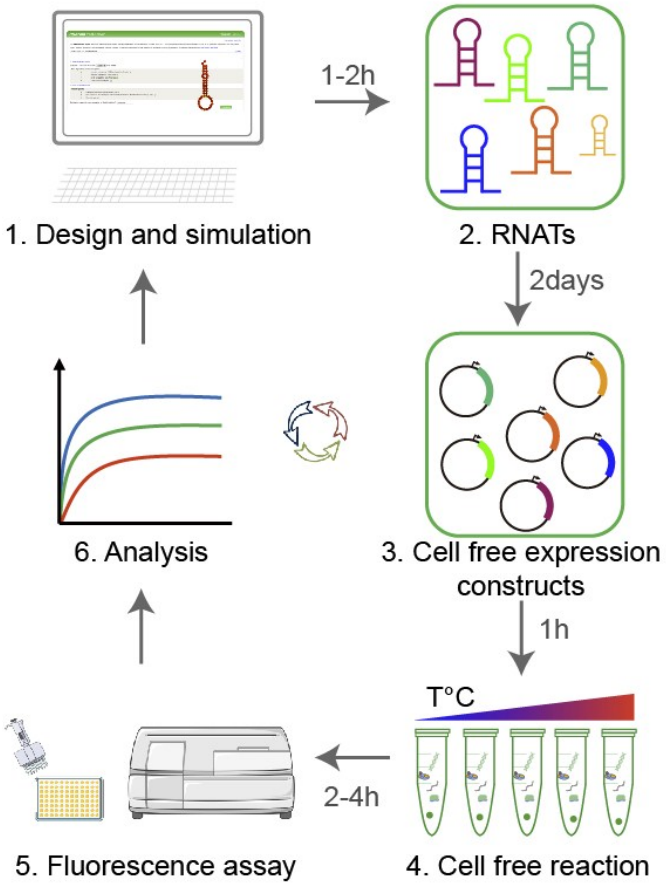
Microfluidic chip based droplets were generated according to the published protocol[3]. Briefly, all fluids were loaded into syringes (BD Luer-Lock™ 1-mL syringe) mounted onto high precision syringe pumps (neMESYS base 120 with neMESYS 290N). We formed micro-droplets by injecting PURE system as the inner phase and HFE7500 fluorinated oil with 1.8 % (w/w) PFPE-PEG-PFE biocompatible surfactant as the outer phase.

Imaging and image analysis

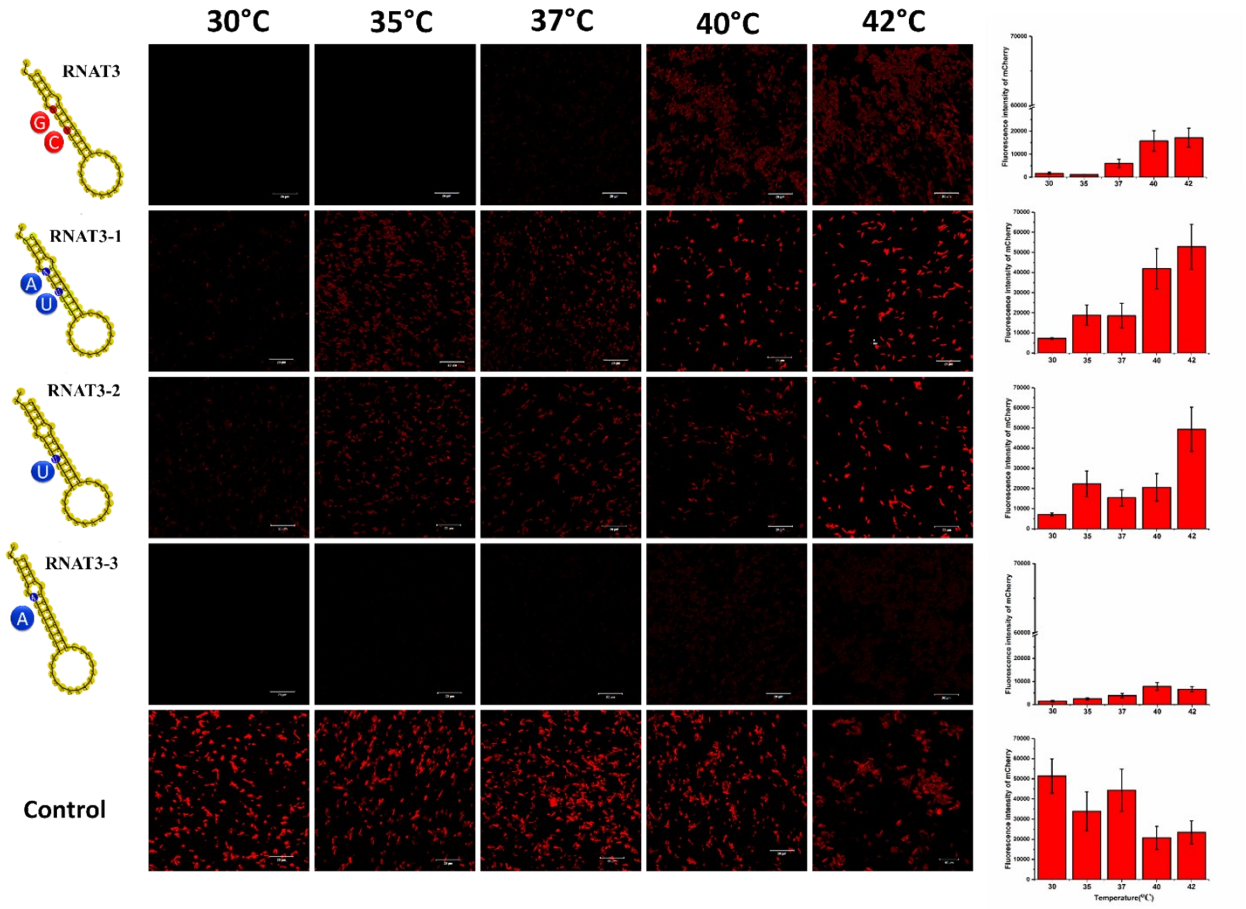
Imaging was taken by confocal microscopy(LSM780) equipped with an argon laser, C-Apochromat 40x/1.20 water-immersion objective and a incubation chamber. Laser lines 488nm, and 561nm lasers were used for fluorescence imaging. Image analysis and processing was carried out with Fiji[4]. The frames of time-lapse movies were normalized to have a constant overall intensity. Representative micrographs and intensity curves correspond to at least three successfully repeated experiments. Fluorescene intensities of droplets were quantified by Analyze Particles Plugins.



Supplement Figure S1. Mg^{2+} concentration influences the functionality of RNAT3 in PURE**.** Compared to the 13 mM Mg^{2+} reference concentration, adding more Mg^{2+} (Magnesium acetate, ≤ 1–2mM (inhibition concentration)) drastically inhibited the switching-on behavior of RNAT3 at 42°C. The possible reason could be that high concentrations of magnesium ions mediate the RNA secondary structure stabilization [5].

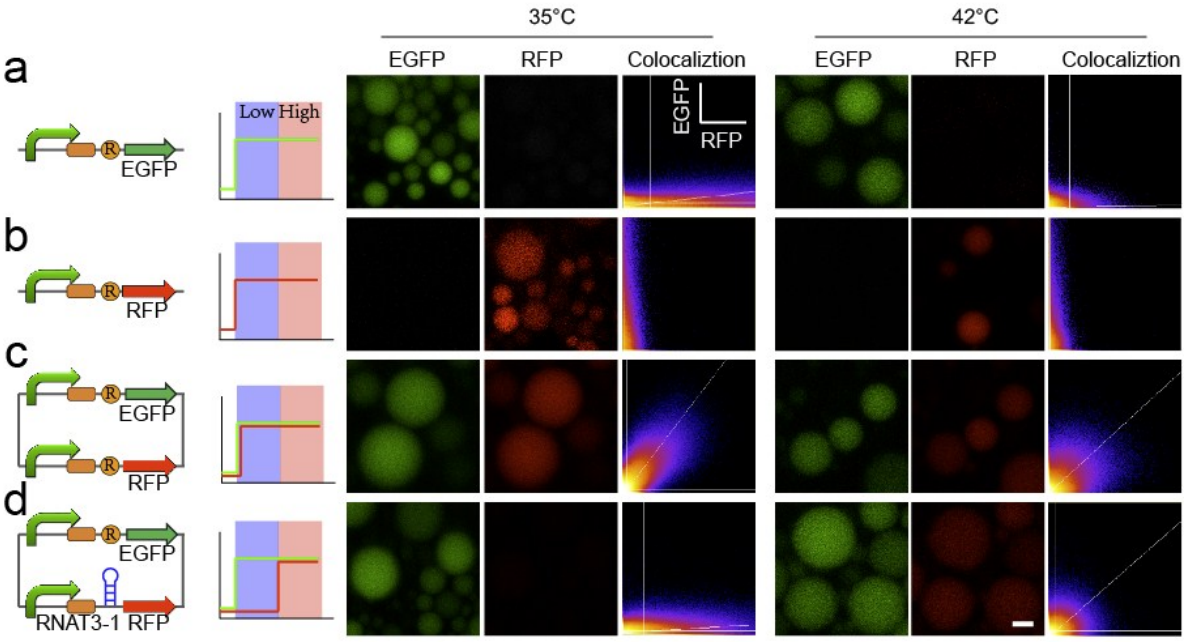


Supplement Figure S2. Tuning RNA thermometers in cell free system. Illustration of *in vitro* design and investigation process: (1-2) RNA thermometers sequence design and simulation with online tool; (3) Plasmid construction with site mutation or fragment changing. (4) Cell free reaction setup at different temperature; (5) Fast fluorescence measurement by plate reader. Then according to the results, the RNATs will be kept as candidates or redesigned for next round

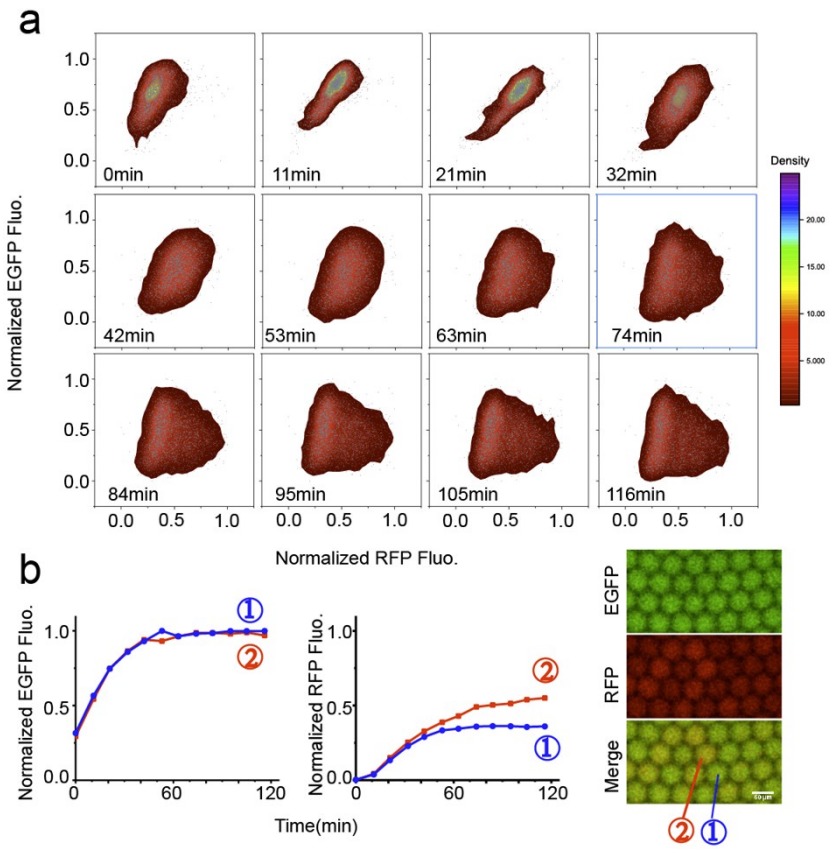


Supplement Figure S3. *In vitro* screened RNA thermometers function *in vivo*. The represent fluorescence images show RFP expression controlled with RNATs at different temperature. Scale bars, 20 μm. The right column charts show the quantified fluorescence intensity of the whole population of *E. coli* at different temperature with image J. The sensing temperature slightly shifted to lower temperature in vivo. The temperature threshold of RNAT3-1 and RNAT3-2 *in vivo* were 35°C and 30°C (*in vitro* 37°C and 35°C), respectively. It seems that their secondary structures *in vitro* were more stable than *in vivo*. Intriguingly, RNAT3 and RNAT3-3 remained comparable *in vitro* and *in vivo*. Compared the sequence between RNAT3 and RNAT3-2, only one site is different, but their switching manners showed significant different both *in vitro* and *in vivo*. This variability may arise from mRNA secondary structure. Several factors *in vitro* may be more susceptible to effect the secondary structure compared with the natural biological system. Firstly, the PURE system lacks RNA helicases that utilize the energy from ATP hydrolysis to unwind RNA[6]. Secondly, Magnesium ions play an important role in maintaining the secondary structure

during translation. The concentration of Mg^{2+} in PURE system is 13mM[6], which is much higher than in *E. coli* (5 mM). High concentration of magnesium ions will stabilize the RNA secondary structure [7, 8].



Supplement Figure S4. Thermal controlled multiplexer. a.-d.) The diagrams show the design of thermos-gene circuits and the multiplexed thermal activation with different temperature. T7-RBS-EGFP, T7-RBS- RFP and the T7-RBS-EGFP-T7-RBS- RFP (on two plasmids) were regarded as the control constructions. In the multiplexer, RFP is gated by RNA thermometer (RNAT3-1) and T7 promoter. EGFP is gated by T7 promoter and an unblocked ribosome binding site on another plasmid. Right: represent images of thermal-multiplexer controlled protocell at 35 and 42 °C. The images were analysed by colocalization for demonstrating the two-colour fluorescence proteins expression in the droplets. Scale bar, 20μm.



Supplement Figure S5. a.)Normalized EGFP versus normalized RFP intensities of the whole population of droplets after the start of expression. b.) Fluorescence time courses of EGFP and RFP in a single droplet. Scale bar, 50 μm. ① and ② are two respective droplets that showed different expression ability of RFP.

Supplement TableS1

Name	Sequence
RNAT3	TACTAGAGCTCTTTAAAAAAAAAAAGTACTAAGGAGTACTAG
RNAT3-1	TACTAGAACTTTTTAAAAAAAAAAAGTACTAAGGAGTACTAG
RNAT3-2	TACTAGAGCTTTTTAAAAAAAAAAAGTACTAAGGAGTACTAG
RNAT3-3	TACTAGAACTCTTTAAAAAAAAAAAGTACTAAGGAGTACTAG

Supplement TableS2 list of vectors genes

Pcoofy1-RNAT3-E1010
TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCTACTAGAGCTCTTTAAAAAAAAAAAGTACTAAGGAGTAC TAGATGGCTTCCTCCGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGG TCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTT ACCAAAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGT TAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGA ACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTT AAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGAAGCTTCCA CCGAACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAAGACGGTG GTCACTACGACGCTGAAGTTAAAACCACCTACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTTACAAAACC GACATCAAACTGGACATCACCTCCCACAACGAAGACTACACCATCGTTGAACAGTACGAACGTGCTGAA.GGTC GTCACTCCACCGGTGCTTAATAACGCTGATAGTGCTAGTGTAGATCGC
LacI-RBS-T7-T7-lacO-RNAT3-1-E1010
tcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgccag ggtggtttttctttcaccagtgagacgggcaacagctgattgcccttcaccgcctggccctgagagagttgcagcaagcggtccacgctggtttgc cccagcaggcgaaaatcctgtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgtatcccactaccgagatatccgcac caacgcgcagcccggactcggtaatggcgcgcattgcgcccagcgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattc agcatttgcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgctatcggctgaatttgattgcgagtgagatatttatgcca gccagccagacgcagacgcgccgagacagaacttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagatgctccacgccc agtcgcgtaccgtcttcatgggagaaaataatactgttgatgggtgtctggtcagagacatcaagaaataacgccggaacattagtgcaggcagc ttccacagcaatggcatcctggtcatccagcggatagttaatgatcagcccactgacgcgttgcgcgagaagattgtgcaccgccgctttacaggct tcgacgccgcttcgttctaccatcgacaccaccacgctggcacccagttgatcggcgcgagatttaatcgccgcgacaatttgcgacggcgcgtgc agggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtgccacgcggttgggaatgtaattcagctccgccatc gccgcttccactttttcccgcgttttcgcagaaacgtggctggcctggttcaccacgcgggaaacggtctgataagagacaccggcatactctgcga catcgtataacgttactggtttcacggtatatctccttcttaaagttaaacaaaattattcctatagtgagtcgtattacgggatctcgacgctctccct tatgcgactcctgcattaggaagcagcccagtagtaggttgaggccgttgagcaccgccgccgcaaggaatggtgcatgcaaggagatggcgcc caacagtcccccggccacggggcctgccaccatacccacgccgaaacaagcgctcatgagcccgaagtggcgagcccgatcttccccatcggtg atgtcggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacgatgcgtccggcgtagaggatcgagatctcgatcccgcg aaattaatacgactcactataggggaattgtgagcggataacaattcccctctagaTACTAGAACTTTTTAAAAAAAAAAAGTACTA AGGAGTACTAGATGGCTTCCTCCGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCC GTTAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAAC TGAAAGTTACCAAAGGTGGTCCGCTGCCGTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAA GCTTACGTTAAACACCCGGCTGACATCCCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACG TGTTATGAACTTCGAAGACGGTGGTGTTGTTACCGTTACCCAGGACTCCTCCCTGCAAGACGGTGAGTTCATCT ACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCGGTTATGCAGAAAAAAACCATGGGTTGGGA AGCTTCCACCGAACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGAAATCAAAATGCGTCTGAAACTGAAA

GACGGTGGTCACTACGACGCTGAAGTTAAAACCACCTACATGGCTAAAAAACCGGTTCAGCTGCCGGGTGCTT
ACAAAACCGACATCAAACTGGACATCACCTCCCACAACGAAGACTACACCATCGTTGAACAGTACGAACGTGCT
GAAGGTCGTCACTCCACCGGTGCTTAAgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaacaaagcccg
aaaggaagctgagttggctgctgccaccgctgagcaataactagcataaccccttggggcctctaaacgggtcttgaggggttttttg

References

1. H. Jia, X. Sun, H. Sun, C. Li, Y. Wang, X. Feng and C. Li, *ACS Synth. Biol.*, 2016, **5**, 312-320.
2. C. Papworth, J. Bauer, J. Braman and D. Wright, *Strategies*, 1996, **9**.
3. O. Wagner, J. Thiele, M. Weinhart, L. Mazutis, D. A. Weitz, W. T. Huck and R. Haag, *Lab Chip*, 2016, **16**, 65-69.
4. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld and B. Schmid, *Nat. methods*, 2012, **9**, 676-682.
5. D. E. Draper, *Rna*, 2004, **10**, 335-343.
6. Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa and T. Ueda, *Nat. biotechnol*, 2001, **19**, 751-755.
7. Y. Xue, B. Gracia, D. Herschlag, R. Russell and H. M. Al-Hashimi, *Nat. Commun*, 2016, **7**.
8. D. Thirumalai and N. A. Denesyuk, *bioRxiv*, 2016, 037895.