Electronic Supplementary Material (ESI) for Chemical Communications.

Materials & Methods

Chemicals

All chemicals not indicated were purchased from Nacalai Tesque (Tokyo, Japan). GluK and GluMg were purchased from Sigma-Aldrich (St. Louis, MO, USA). CTP, Lys, and Folinic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Creatine kinase and tRNA were purchased from Roche (Basel, Switzerland). ATP, GTP, and Trp were purchased from Wako Pure Chemical Industries (Osaka, Japan). UTP was purchased from Affymetrix (Santa Clara, CA, USA).

Preparation of cell extract for iGeTT

General protocol is a modified version of LoFT method we previously reported[1]. *E.coli* BL21-CodonPlus(DE3)-RIPL (Agilent Technologies, Santa Clara, CA, USA) was cultivated in 1 L of LB medium, T7 RNA polymerase was induced by 0.1 mM IPTG at OD_{600} = 0.1, and harvested by centrifugation at OD_{600} = 0.8. Cell pellet was washed twice with 10 mL of 400 mM sucrose. Lysozyme chloride was added in the suspension as 400 μg/ml at final and incubated on ice for 30 minutes. After the incubation, cells were washed with 20 mL of 400 mM sucrose twice. The cell collected by 20,000 x g centrifugation for 3 min was resuspended in 1.5 times volume of S30A buffer (10 mM Tris-HCl [pH7.6], 60 mM GluK, 14 mM GluMg) to wet cell wight. The suspension was frozen by immersing in liquid nitrogen for 15 minutes and was thawed in ice water. Supernatant after centrifugation at 30,000 x g for 1 hour was obtained as S30 extract. To remove genomic DNA and RNA from the S30 extract, run-off reaction was performed as follows. S30 extract was incubated at 37°C for 70 minutes and supernatant after 10,000 x g centrifugation at 37°C for 20 minutes was collected. S30 extract was washed with 10 times volume of S30B buffer B (5 mM Tris-HCl [pH7.6], 60 mM GluK, 14 mM GluMg) by using Amicon Ultra-15 Centrifugal Filter Units-10k (Merck Millipore, Billerica, MA, USA). After the buffer exchange, S30 extract was stored in -30°C prior to use.

Purification of genomic DNA

The protocol for genome purification using agarose plugs was according to our previous study[2]. Cells at stationary phase grown in 50mL LB medium were collected by 20,000 x g centrifugation for 3 min. The pellet (0.2 g wet cell each) was dispensed into tubes and the cells in a tube were washed with 10 mL NT buffer (10 mM Tris-HCl [pH7.6], 1M NaCl) and resuspended with 0.2 mL NT buffer. The solution was incubated at 4°C for 1 hour after the addition of 0.3 mL of 1% low melting agarose (BIORAD). The agarose plug was immersed in EC buffer (10 mM Tris-HCl [pH7.6], 1 M NaCl, 0,1 M EDTA, 0.5% Brij-58, 0.2% deoxycholate,

0.5% SDS) containing 1 mg/mL Lysozyme chloride, and incubated at 37°C under 80 rpm shaking for overnight. Then, the agarose plug was transferred into ES buffer (0.5 M EDTA, 1% SDS) containing 0.1 mg/mL Proteinase K (Sigma Aldrich), and incubated at 37°C under 80 rpm shaking for overnight. The agarose plug was washed with TE buffer (10 mM Tris-HCl [pH7.6], 1 mM EDTA), and then transferred into TE buffer containing 1 mM PMSF and incubated at 43°C for 2 hours under 80 rpm shaking. After 2 sets of this step, the agarose plug treated was transferred to TE buffer and incubated at 25°C for 2 hours under 80 rpm shaking. After 4 sets of this washing treatments, agarose gel plug in TE was stored at 4°C.

iGeTT reaction

S30 extract was mixed with 2.0 mM ATP, 2.0 mM GTP, 1.3 mM UTP, 1.3 mM CTP, 0.6 mM 20 amino acids (0.5 mM each), 1 mM spermidine, 0.2 mg/mL tRNA, 100 mM GluK, 50 mM Hepes-KOH (pH 8.0), 67.8 μM folinic acid, 80 mM creatine phosphate, 250 μg/mL creatine kinase, and 0.1 nM genome. Final concentration of GluMg was adjusted to 15 mM by taking into account the concentration derived from the buffer of S30. Concentrations of GluMg and spermidine were varied if indicated. Reaction was proceeded at 25°C for 3 hours.

To evaluate expression levels of the large fragment of nanoluciferase (LgBiT), purified HiBiT protein (His-HiBiT-SUMO, Table S1) was added to the iGeTT mixture. To evaluate protein expression by HiBIT tag, His-SUMO-LgBiT was added to the iGeTT mixture after the reaction. Both His-HiBiT-SUMO and His-SUMO-LgBiT were expressed in *E. coli* BL21-CodonPlus(DE3)-RIPL, purified by HisTrap HP, and their buffer was exchanged with S30B buffer by AmiconUltraFilter-10k. After iGeTT reaction, nLuc activity was measured by using Nano-Glo Luciferase Assay System (Promega, Madison, WI, USA) and ChemiDoc Touch MP (BIORAD, Hercules, CA, USA) or GloMax Explorer (Promega).

Insertion of a reporter gene cassette into genomic DNA by λ-red recombinase

The insertion cassette including T7 promoter, LgBiT gene, T7 terminator, and a gene encoding chloramphenicol acetyltransferase was constructed by using PCR fragments and iVEC3 method[3]. *E. coli* iVEC3 strain was distributed from NBRC (Tokyo, Japan). The final cassette sequence is shown in Table S1. The insertion cassette into *lacZ* gene was amplified by PCR using PrimeSTAR Max DNA polymerase (TAKARA, Kyoto, Japan), purified genome of *E.coli* K-12 MG1655, and a primer pair (CATCTACACCAACGTGACCTAT/ CGCCATTTGACCACTACCAT). The PCR fragment dissolved in sterilized water was added to 100 μL competent cell for electroporation of *E.coli* MG1655 harboring pKD46 [4] and mixed gently by a few times of pipetting. The suspension was transferred into sufficiently cooled electroporation cuvette (0.2 cm, BIORAD). After pulsing at 2.5 kV and 4.8 msec, the

suspension was added to 1 mL SOC medium incubated at 37°C and was cultured for 1 hour. Suspension of cells was spread on LB plate with 50 μg/mL kanamycin and cells were cultivated overnight. Insertion of the cassette into genomic DNA was confirmed by PCR targeting outside the cassette inserted and DNA sequencing.

Insertion of a reporter gene cassette into genomic DNA by transposon system

The insertion cassette including ThrABCL terminator [5], PJ23119 promoter [6], SUMO-msfGFP-HiBiT gene (constructed in this study, Table S1), and T500 terminator [7] was cloned with pET15 vector by using PCR fragments and the iVEC3 method. The final cassette sequence is shown in Table S1. The cassette amplified by PCR using a primer pair (TCTAGAACTAGTG GATCCGGCGCCAGCAACCGCAC / GACGGTATCGATAAGCTTCCGGATATAGTTCCTCC) was digested with SpeI/Hind III (NEB, Ipswich, MA, USA), and the digested fragment was cloned into the SpeI/HindIII site, which locates in the internal site of the two S1 sites of pBSL181 [8], using SY327 strain (*λpir F- araD Δ[lac – pro] argE rif nalA recA56*) [9] as the host. The resultant plasmid (pBSL181-ThrLt-GFP-HiBiT) was introduced into chemical competence cells of *E. coli* MG1655. For induction of Tn10 encoded in the pBSL181 vector, LB agar plate containing 0.1 mM IPTG and 30 μg/mL chloramphenicol was used. The genome locus of the cassette inserted was confirmed by PCR using random primers and DNA sequencing as described elsewhere[10]. In the strain used for genome preparation in this study, the cassette was inserted around 350~500 bp from the start codon of yiaU gene in the reverse direction.

RT-qPCR

In case of living cells, *E. coli* MG1655 cells at OD_{600}=0.4 or at stationary phase grown in 2xYTPG medium was harvested by centrifugation. The pellet was suspended with 100 μL of TE buffer containing 1 mg/mL Lysozyme chloride by vortex. After 30 minutes incubation at 37°C, 1 mL TRIzol Regent (Invitrogen, Waltham, MA, USA) was mixed with the solution and incubated at 25°C for 5 minutes. 0.2 mL Chloroform was added into the suspension and mixed by upside down shaking for 15 seconds. After incubation at 25°C for 5 minutes, the suspension was centrifuged at 4°C, 12,000 x g for 15 minutes. After collection of the upper layer, an equal volume of isopropanol was added and incubated at 25°C for 10 minutes. After centrifugation at 4°C, 12,000 x g for 10 minutes, supernatant was discarded, and then, 1 mL of 75 %(v/v) EtOH was added and mixed by vortex. After 7,500 x g centrifugation at 4°C for 5 minutes, supernatant was discarded and the RNA pellet was air-dried at 25°C for 30-60 minutes with covering the lid with a lap. 30 μL of RNase-free water was added to the pellet and incubated at 60°C for 10 minutes. The remaining DNA in the total RNA was further

removed with RQ1 RNase-free DNase (Promega) at 37°C for 30 minutes. 3 μL of 3 M Sodium Acetate (pH 4.3) and 67 μL of phenol: chloroform (1:1) were mixed with the DNase-treated total RNA by upside down shaking for 15 seconds. After incubation at 25°C for 2 minutes, the suspension was centrifuged at 4°C, 12,000 x g for 2 minutes. After collection of the upper layer, an equal volume of isopropanol was added and incubated at 25°C for 10 minutes. After centrifugation at 4°C, 12, 000 x g for 10 minutes, the supernatant was discarded, and then, 100 μL of 75%(v/v) EtOH was added and mixed by vortex. After centrifugation at 4°C, 7,500 x g, for 5 minutes, the supernatant was discarded and the RNA pellet was air-dried at 25°C for 30-60 minutes with covering the lid with a lap. 30 μL of RNase-free water was added to the pellet and incubated at 60°C for 10 minutes, and then, the RNA solution was stored at -80°C prior to use. In case of cell-free translation-transcription, total RNA after 3-hour iGeTT reactions at 25°C was purified by using TRIzol LS Reagent or RNeasy kit. RT-qPCR was performed with One Step TBGreen PrimeScript RT-PCR kit II (TAKARA) and Light cycler (Roche). All primers used for RT-qPCR are listed in Table S2. Ct values were calculated by second derivative maximum method as following manufactures instruction. mRNA transcribed by T7 RiboMAX Express Large Scale RNA Production System (Promega) was used as the standards for the absolute quantity. Template DNA for mRNA synthesis was prepared by PCR using MG1655 genome and the primer set in Table S2.

Genome encapsulation in W/O emulsion and iGeTT reaction in liposomes

One mg of POPC dissolved in chloroform was thoroughly dried using argon gas. Then, 500 μL of mineral oil was added to the dried film. Lipids were dispersed in oil by ultrasonication (Bransonic, Branson, Chatham, MA, USA) at 60°C for 90 minutes. The mixture was thoroughly mixed with vortex, and the resultant solution was used as lipid solutions. For emulsification, iGeTT solution with or without 1/10000 diluted SYBR™ Green I Nucleic Acid Gel Stain (TAKARA) was mixed with the lipid solution at a ratio of 10:100 and tapped 30 times. Genome was replaced with ultrapure water or a plasmid (pOR2OR1-sfGFP)[1] if indicated. Liposomes were prepared by the droplet-transfer method after preparing the emulsion. As an outer solution, a reaction mixture, in which genome DNA and S30 were replaced with equivalent volume of TE buffer or S30B buffer, were used. For microscopic observation, a double-sided tape sandwiched between a slide glass and a cover glass [11] was used. 10 μL of emulsion solution or liposome solution was supplied to the cover slip and were observed by Axio Observer.Z1 (Zeiss, Berlin, Germany) with ORCA FLASH 4.0 v2 (Hamamasu Photonics, Sizuoka, Japan), confocal microscopy (FV1000, Olympus, Tokyo, Japan), or a luminescent microscope (LV200, Olympus). In the case of iGeTT reaction in liposome, Nano-Glo Live Cell Assay System (Promega) was used to detect luminescence.

To prevent back ground reactions outside liposomes due to leakage, 0.1 mg/mL Proteinase K (Sigma) was added to the outer solution. The substrate and buffer were mixed at 1:19, one quarter amount of the mixture was added to the liposome solution, and luminescence was observed with LV200.

Table S1. Insertion cassettes for the reporter systems in this study

T7 promoter – SUMO-LgBit - T7term (to *lacZ* by λ RED system)

(lacZ) CATCTACACCAACGTGACCTATCCCATTACGGTCAATCC

GCCGTTTGTTCCCACGGAGAATCCGACGGGT (lacZ366)

<T7 promoter with a strong SD sequence>

GATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATC

CCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAG

GAGATATACCATG

<HisSUMO>

ATGGGTCATCACCATCATCATCACGGGTCCCTGCAGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTC

AAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAG

GCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGAT

CAGGCCCCTGAAGATTTGGACATGGAGGATAACGATATTATTGAGGCTCACCGCGAACAGATTGGAGGTGGATCC

<LgBiT>

GTTTTCACCCTGGAAGATTTTGTTGGTGATTGGGAACAGACCGCAGCATATAATCTGGATCAGGTTCTGGAACAAGGTGGTGTTA

GCAGCCTGCTGCAGAATCTGGCAGTTAGCGTTACCCCGATTCAGCGTATTGTTCGTAGCGGTGAAAATGCCCTGAAAATTGATAT

TCATGTGATCATCCCGTATGAAGGTCTGAGCGCAGATCAGATGGCACAGATTGAAGAAGTGTTCAAAGTTGTTTATCCGGTGGAT

GACCACCATTTTAAAGTTATTCTGCCGTATGGCACCCTGGTTATTGATGGTGTGACCCCGAATATGCTGAATTATTTCGGTCGTCC

TTATGAAGGTATTGCCGTTTTTGATGGCAAAAAAATCACCGTTACCGGTACACTGTGGAACGGTAACAAAATTATCGATGAACGTC

TGATTACACCGGATGGTAGCATGCTGTTTCGTGTTACCATTAATAGCTAA

<T7 terminator>

CTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGA

GCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

<CmR cassette>

GACCGGGTCGAATTTGCTTTCGAATTTCTGCCATTCATCCGCTTATTATCACTTATTCAGGCGTAGCAACCAGGCGTTTAAGGGCA

CCAATAACTGCCTTAAAAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGA

AGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGCCCATGGTGAA

AACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAA

CATATTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCC

GGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATC

CCATATCACCAGCTCACCGTCTTTCATTGCCATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCC

GGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGC

AACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTT

AGCTTCCTTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTTATTTCATTATGGTGAAAGTTGGAACCTC

TTACGTGCCGATCAACGTCTCAAGGAGGAACTATATCCGGA

(lacZ2529) GCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGTAGTGGTCAAATGGCG

ThrABCL term- σ70 promoter - SUMO-msGFP-HiBiT - T500term (by Tn10)

< thrABCL terminator>

GGCGCCAGCAACCGCACGGTACCggaaacacagAAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTTcgaccaaagg

< PJ23119 promoter with a strong SD sequence>

AGATCTCGATCCCGCGAAATTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCCCTCTAGAAATAATTTTGTTTAACTTTAAGA

AGGAGATATACC

< SUMO-msGFP-HiBiT >

ATGGGTCATCACCATCATCATCACGGGTCCCTGCAGGACTCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTC

AAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAG

GCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGAT

CAGGCCCCTGAAGATTTGGACATGGAGGATAACGATATTATTGAGGCTCACCGCGAACAGATTGGAGGTGGATCC

AGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCCG

TGGAGAGGGTGAAGGTGATGCAACAAACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCA

ACACTTGTCACTACTTTAACTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCACATGAAACGGCATGACTTTTTCAAGAGTGC

CATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAAGTCAAGTTTGAA

GGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACTCGAGTACAA

CTTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACGTTGAAG

ATGGATCTGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTG

TCGACACAATCTAAACTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTAC

ACATGGCATGGATGAGCTCTACAAA

GGAACCTCTGGTGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCTAA

<T500 terminator>

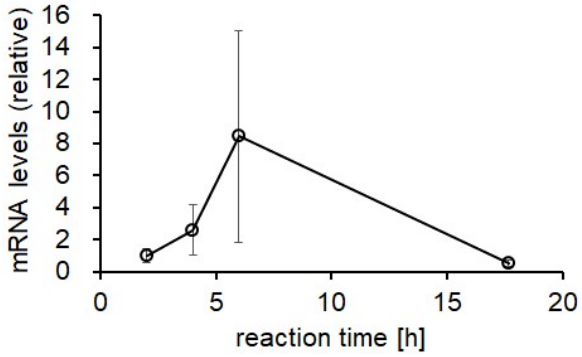
CTCGAGGATCCGGCTGCTCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAGGCTGAGTTGGTGCTGCCACCAAAGCCCGCCG

AAAGGCGGGCTTTTCTGTGA

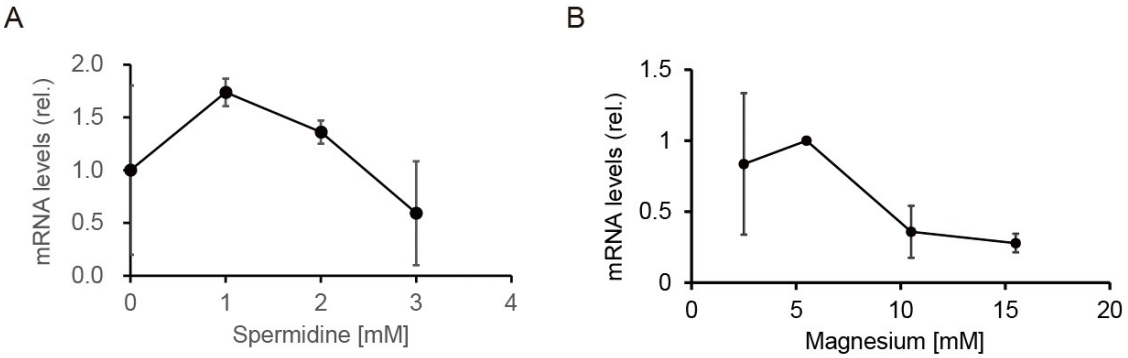
TCTGGAGGAACTATATCCGGA

Table S2. Primers for RT-qPCR

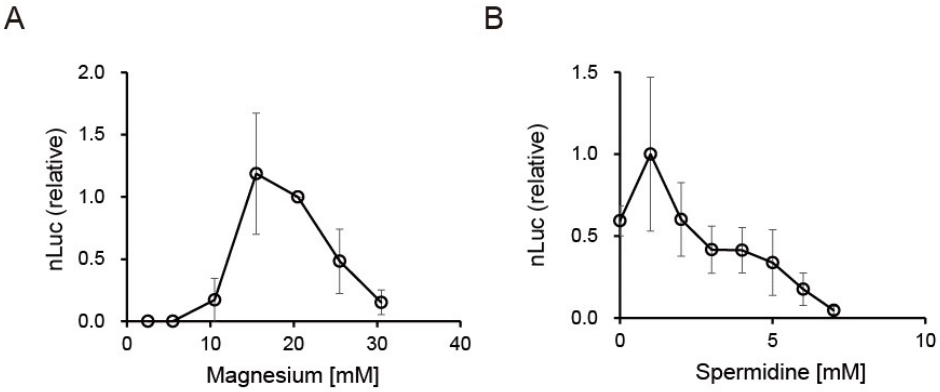
Primer name	Sequence (5'→3')
eno-F (*E.coli*)	TAATACGACTCACTATAGGGAGACTGCTGGTTATGAACTGGGC
eno-R (*E.coli*)	AGTCAGATTCGTCCAGACCG
fbaA-F (*E.coli*)	TAATACGACTCACTATAGGGAGAAAAACACTTCGCAGCTACCG
fbaA-R (*E.coli*)	GCAACCCAGTTCGATTTCCA
fbaB-F (*E.coli*)	TAATACGACTCACTATAGGGAGAGCCGACAACCTTTTACAGCA
fbaB-R (*E.coli*)	AAGATATCCTGTGCCAGCCA
gapA-F (*E.coli*)	TAATACGACTCACTATAGGGAGACACGCTACTACCGCTACTCA
gapA-R (*E.coli*)	GCCATACCAGTCAGTTTGCC
gpmA-F (*E.coli*)	TAATACGACTCACTATAGGGAGAGAATGTGCTGGACGAACTGG
gpmA-R (*E.coli*)	AGTCACTGCAAAACCACGAC
pfkA-F (*E.coli*)	TAATACGACTCACTATAGGGAGACTACATGGGTGCAATGCGTC
pfkA-R (*E.coli*)	AAGAAGAGGTGTCACGCAGA
pfkB-F (*E.coli*)	TAATACGACTCACTATAGGGAGATTGGCGGATGAAAATGTCCC
pfkB-R (*E.coli*)	CTTCAAGCTGGCGAAACTCA
pgi-F (*E.coli*)	TAATACGACTCACTATAGGGAGAGCATCACTGAAGAGACGCTG
pgi-R (*E.coli*)	TGCCATCAACCAAAATCGGG
pgk-F (*E.coli*)	TAATACGACTCACTATAGGGAGACTGGATCTTGCTGGGAAACG
pgk-R (*E.coli*)	CTTCGTTGTACTCGCCTTCG
pykA-F (*E.coli*)	TAATACGACTCACTATAGGGAGAGTACTGACGCTGTGATGCTG
pykA-R (*E.coli*)	TGGTTAGCTGCGTACATTGC
pykF-F (*E.coli*)	TAATACGACTCACTATAGGGAGAGTAAAACCGCCGCTATCCTG
pykF-R (*E.coli*)	GTTGCCAACAGACAGGTCAG
tpiA-F (*E.coli*)	TAATACGACTCACTATAGGGAGAAGCGGGCAAAACTGAAGAAG
tpiA-R (*E.coli*)	CTTTAGCGATGTGGTCACGG



Supplementary Figure S1. Time course of transcription levels by iGeTT reaction. Levels of enolase mRNA during iGeTT reaction were tracked. Values were normalized the average RNA level at 2h iGeTT reaction. Error bars indicate standard deviation (n=3).



Supplementary Figure S2. Concentration dependence of transcription in iGeTT on spermidine and magnesium. A, Spermidine dependence. B, Magnesium dependence. mRNA levels were determined by quantifying mRNA levels of enolase (eno) by RT-qPCR. The levels were normalized by the value of 0 mM spermidine or 5.5 mM magnesium, respectively. Error bars indicate standard deviation (n=3).



Supplementary Figure S3. Concentration dependence of transcription-translation in iGeTT on spermidine and magnesium. Concentration dependence of magnesium (A) or spermidine (B) on protein expression by iGeTT using the T7 reporter system. Error bars indicate standard deviation (n=3). The levels were normalized by the value of 20 mM magnesium or 1 mM spermidine, respectively.

Supplementary references

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