Human mGluR3 Cell Line (HEK293/EAAC1/mGluR3/G16)

Technical Manual No. **Version 20080606**

I. Introduction

Glutamate, a major excitatory neurotransmitter, plays an important role in neuronal plasticity and neurotoxicity and is thought to be involved in many neuronal functions including memory acquisition, learning, and some neurodegenerative disorders, such as epilepsy and stroke. Glutamate acid activates ionotropic and metabotropic (G protein-coupled) receptors. The G-protein coupled metabotropic glutamate receptors (mGluR) are often not directly involved in fast synaptic transmission, but modulate the efficacy of glutamic acid GABAergic synapses. These receptors therefore constitute good targets for drugs modulating glutamatic acid function in the brain. Eight mGlu receptors have been characterized and classified into three groups based on sequence homology, pharmacology, and transduction mechanisms.

Group II mGlu receptors contain mGlu2 and mGlu3 receptors expressed mainly in the brain and spinal cord. Both of them inhibit adenylyl cyclase. The activation of these receptors has been shown to decrease synaptic transmission and glutamate release in the hippocampus. The distribution and prominent functions of group II mGlu receptors in neuronal excitability and synaptic transmission suggests that modulation of these GPCRs is a promising strategy for the treatment of neurological and neuropsychiatric disorders, such as anxiety, schizophrenia, and neuronal pain.

Gα15 and Gα16, which activate PLC (phosphoinositide-specific phospholipase C), have been reported to be activated by many different GPCRs, including those recognized as not stimulating PLC. They have recently been shown to couple many receptor types to PLC, even receptors that usually stimulate or inhibit adenylate cyclase. Using HDB's assay development technologies, mGluR3 cDNA was cloned into an expression vector and stably transfected into HEK/293 cells with stable expression of promiscuous G-protein (Gα15 and 16), with or without glutamate transporter gene EAAC1, allowing a quick detection of coupling mGluR3 through calcium mobilization pathways. The established cell line was pharmacologically validated for mGluR3 responses to corresponding agonists and antagonists. Our results indicate that the cell line remains stable for at least 25 passages with a good response window. The assay developed with the cell line is ready for use in high-throughput screening (either primary or secondary screening). It can also be used to identify agonists, antagonists, and allosteric modulators for mGluR3.

II. Cell Line Information

- Description:

  The HUMAN H3/HRH3 is amplified by PCR using a high-fidelity enzyme and subcloned into the pcDNA3.1/(+) mammalian expression vector. The full-length ORF has been confirmed by sequencing. The H3R reporter cell line is created by transfection of pcDNA3.1/H3R in an HDB parental cell line, HEK293/Gα15. The transfected cells are stably selected by 600 µg/ml G418. Single cell clones with high H3R inducibility

and low background are isolated using ring cloning and serial dilution. The clones with the largest dynamic ranges in calcium assay are chosen for pharmacological and stability studies.

III. Cell Culture Conditions

Complete Culture Medium:

DMEM: 90%

FBS: 10%

Supplements:

L-glutamine 2.0 mM

Amp 100 μg/ml

Strep 100 μg/ml

Medium for Stable Line Propagation:

Add 600 μg/ml G418 in complete culture medium.

Freezing Medium:

20-90% of fetal bovine serum and 10% dimethyl sulphoxide (DMSO)

Thawing Cells:

1. Quickly thaw frozen cells in a 37°C water bath, agitating continuously.
2. Using a 1 ml pipette, slowly pipet the cells up and down five times and add, drop by drop, to a 15 ml centrifuge tube containing 5 ml of fresh prewarmed complete DMEM medium. Then centrifuge at 1,000 rpm for five minutes.
3. Discard the supernatant medium and resuspend the cell pellet in 5 ml of fresh prewarmed complete DMEM medium. Transfer cells to a T25 flask and incubate at 37°C with 5% CO_2 until the cells reach >90% confluence. The recovery rate for frozen cells is usually 90% or above.

Subculturing:

When the cells reach confluence, they need split. This cell line is normally split twice weekly at 1:4 to 1:8 dilutions.

1. Carefully aspirate all the media. Gently rinse the cell layer with appropriate amount of 0.2% trypsin-EDTA, and aspirate it off.
2. Wait for about one minute. Then dislodge the cells by gently tapping the sides of flask or dish.
3. Resuspend cells with appropriate amount of complete DMEM medium, and split cells as desired.

Changing Medium:

This is normally done every other day.

1. Gently aspirate off medium.
2. Transfer fresh warm complete DMEM medium (37°C) into a flask (5 ml for T25 and 10 ml for T75).

Freezing Cells:

1. Repeat the steps 1-3 of subculturing section.
2. Centrifuge down the cells at 1,000 rpm for five minutes.

3. Aspirate off the supernatant and resuspend the cells in fresh freezing medium at a density of 2-3 × 10^6 cells/ml. Add 1 ml cells per cryogenic vial.
4. Put the cryogenic vial of cells into cryo freezing container. Then transfer the container to a -80°C environment and leave it there overnight.
5. Transfer cryogenic vial into liquid nitrogen (-196°C).

IV. Assay Procedure

Calcium Assay of HEK293/EAAC1/ mGluR3/G16 Cells

Plate coating with Matrigel:

1. Add about 30 μl BD Matrigel® Matrix in serum free DMEM solution into Corning Blkl/Clr 96-well plate, and incubate the plate in 37°C for 60 minutes.
2. Seal and transfer the plate into a 4°C refrigerator until use. A coated plate should be used within 48 hours.

Standard protocol for Calcium Assay with calcium assay kit from HDB:

PART 1: Calcium Assay of HEK293/EAAC1/mGluR3/G16 Cells with agonists

1) Cell Plating: Seed cells at a density of 2x10^4/well in a Matrigel® coated 96-well black plate and incubated in CO_2 incubator.
2) 12-24 hours later, discard the growth media. Add 100 μl/well of Glutamine-free medium (5% dFBS) and incubate overnight (12-24 hrs) at 37°C.
3) The day of the assay, remove cell plates from incubator. Remove 50 μl of the medium, and add 2X dye-loading solution into wells (50 μl per well for 96-well plates).
4) Incubate the plate at 37°C in dark for one hour before calcium signal readout.
5) Prepare agonists addition plates in advance of assay. To a 96-well plate, add 5X work concentration of agonist/antagonist compound in HDB Calcium Assay solution A (add 25 μl/well to a 96-well cell plate).
6) Read with FlexStation or FLIPR at room temperature using the specified settings and save data. Usually collecting data for two minutes is enough.

Parameters	
Excitation wavelength (nm)	485
Emission wavelength (nm)	525
Emission cut-off (nm)	515

PART 2: Calcium Assay of HEK293/EAAC1/mGluR3/G16 Cells with Antagonist (LY341495)

1) Cell Plating: Cells are seeded at a density of 2x10^4/well in a Matrigel® coated 96-well black plate, and incubated in CO_2 incubator.
2) 12-24 hours later, discard the growth media. Add 100 μl/well of Glutamine-free medium (5% dFBS) and incubate overnight (12-24 hrs) at 37°C.
3) The day of the assay, remove cell plates from incubator. Remove 60 μl of the medium, and add 2X dye-loading solution into wells (50 μl per well for 96-well plates).
4) Add 10X working concentration of LY341495 in HDB Calcium Assay solution A (10 μl/well) to cell plate.
5) Incubate the plate at 37°C in dark for one hour before calcium signal readout.
6) Prepare agonists addition plates in advance of assay. To a 96-well plate, add 5X working concentration of agonist/antagonist compound in HDB Calcium Assay solution A (add 25 μl/well to a 96-well cell plate).
7) Read with FlexStation or FLIPR at room temperature using the specified settings and save data. Usually

collecting data for two minutes is enough.

Parameters	
Excitation wavelength (nm)	485
Emission wavelength (nm)	525
Emission cut-off (nm)	515

Note: The calcium assay can also be performed with FLIPR Calcium 3 or Calcium 4 Assay Kit from Molecular Devices, which should give out comparable results.

V. Results

L-Glutamate Dose Response of HEK293/EAAC1/mGluR3/G16 Cell Line

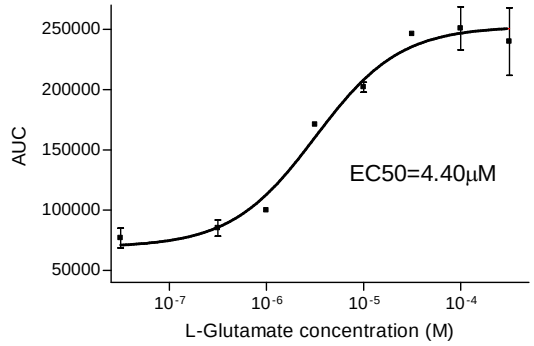


Fig 1: Dose response of calcium activity was monitored with FlexStation plate reader upon treatment with L-Glutamate. Assay was performed in accordance with the procedure described above. Data represent means ± SEM for duplicate samples. EC50 value for L-Glutamate dose response was determined using GraphPad Prism 4 software.

LY341495 Dose Response of HEK293/EAAC1/mGluR3/G16 Cell Line

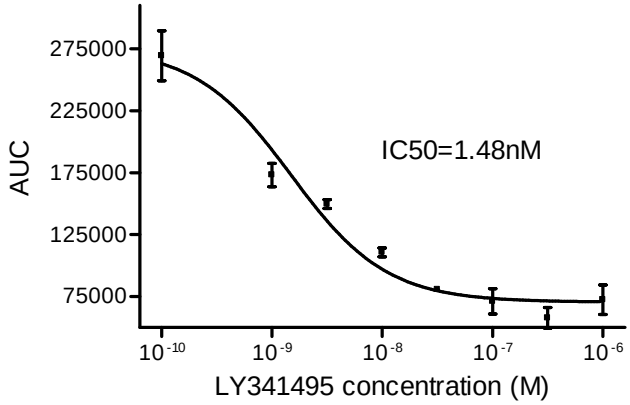


Fig 2: Dose response of calcium activity was monitored with FlexStation plate reader upon treatment with different concentrations of LY341495 at 10 M L-Glutamate. Assay was performed in accordance with the procedure described above. Data represent means ± SEM for duplicate samples. IC50 value for LY341495 dose response was determined using GraphPad Prism 4 software.

HEK293/EAAC1/mGluR3/G16 Cell Line Mycoplasma Detection

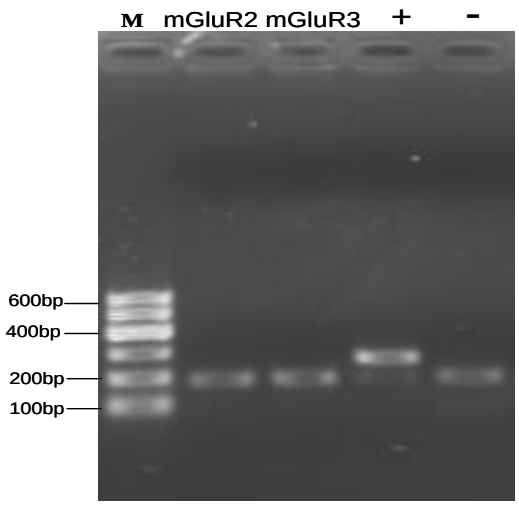


Fig 3: Mycoplasma detection. Assay was done using Venor@GeM Mycoplasma Detection kit. M, 100 bp DNA ladder marker; mGluR2: HEK293/mGluR2/Gα16 cell line; mGluR3: HEK293/EAAC1/mGluR3/Gα16 cell line; +: Positive Control; -: Negative control.

VI. Appendix

Reagents & Consumables:

1. PfuUltra™ High-Fidelity DNA Polymerase, Stratagene, Cat # 600380.
2. QIAprep Spin Miniprep Kit, Qiagen, Cat # 27104.
3. QIAquick Gel Extraction Kit, Qiagen, Cat # 28706.
4. QIAquick PCR Purification Kit, Qiagen, Cat # 28106.
5. QuikChange Site-Directed Mutagenesis Kit, Stratagene, Cat # 200518.
6. DMEM: Dulbecco's Modified Eagle Medium powder, high glucose, Gibco BRL, Cat #12100-046.
7. DMEM, Glutamine-free: Dulbecco's Modified Eagle Medium, high glucose, without L-Glutamine, without Sodium Pyruvate, Gibco BRL, Cat #11960.
8. FBS: Fetal Bovine Serum, Hyclone, Cat #CH30160.03.
9. dFBS: Dialyzed Fetal Bovine Serum, Hyclone, Cat #SH30079.
10. L-Glutamine: 200 mM, Gibco BRL, Cat # 25030-081.
11. Zeocin: Invitrogen, Cat # R25001.
12. Trypsin: 1:250 rom Bovine Pancreas, Gibco BRL, Cat # 27250016.
13. Trypsin-EDTA: prepared in HD Biosciences.
14. PBS: prepared in HD Biosciences (see Media & Solution)
15. MATRIGEL: BD Bioscience Cat# 354230.
16. Lipofectamine™ 2000: Invitrogen Cat #11668-019.
17. DMSO: dimethyl sulphoxide, for molecular biology, Sigma, Cat #D8418.
18. Hepes: Sigma, Cat #H-3375.

19. Fluo-3, AM: Molecular Probes, Cat #F14242.
20. WASH Free Calcium Assay Kit: HD Biosciences, Cat # HD02-0010.
21. Probenecid: Sigma, Cat #P 8761.
22. L-Glutamic acid: Sigma, Cat #G1251.
23. L-CCG-I: Tocris, Cat # 333.
24. (1S, 3R)-ACPD: Tocris, Cat #0284.
25. LY 341495: Tocris, Cat #1209.
26. LY 487379: provided by Organon.
27. EGLU: Tocris, Cat #971.
28. MPPG: Tocris, Cat #853(S).
29. MCPG: Tocris, Cat # 337.
30. Venor@GeM Mycoplasma Detection kit: Minerva Biolabs Cat #11-1050.
31. T25 flask: 25 cm^2 cell culture flask, Corning, Cat #430639.
32. 6 cm dish: Orange Scientific, Cat # 2050200.
33. 10 cm dish: Corning, Cat # 430167.
34. 6-well plate: Corning, Cat #3516.
35. Cryogenic Vial: Corning, Cat #430289.
36. 96 Well Plate: Costar, Cat# 3603, BlackWall/Clear Bottom, Polystyrene, sterilized.
37. 384 Well Plate: Costar, Cat# 3709, BlackWall/Clear Bottom, Polystyrene, sterilized.

Media and Solutions:

1. PBS (for preparation of 500 ml)

1)	KCl:	0.1 g
2)	KH_2PO_4:	0.1 g
3)	NaCl:	4.0 g
4)	$Na_2HPO_4.12H_2O$:	1.4425 g

Dissolve the above components in double-distilled water (ddH_2O) and adjust pH to 7.4 with 0.1 N NaOH. Add ddH_2O to the final volume of 500 ml. Autoclave and store at 4°C.

2. Trypsin-EDTA (for preparation of 100 ml)

1)	Trypsin:	0.25 g
2)	2%EDTA:	2 ml
3)	PBS:	98 ml

Dissolve trypsin in 2%EDTA and PBS completely. Sterilize the solution by passing through a 0.20 µm membrane filter. Store at 4°C.

3. Culture medium (for preparation of 1 L)

1) Measure out 950 ml distilled water to dissolve the media components, stirring gently until the solution becomes clear.
2) Add $NaHCO_3$ 3.7 g for high glucose DMEM.
3) Adjust pH of medium to 0.2-0.3 below the desired final working pH (using 1 N NaOH or 1 N HCL is recommended). Add slowly while stirring.
4) Dilute to 1 liter with ddH_2O.
5) Sterilize the medium immediately using the method of membrane filtration. Store at 4°C.

4. Complete DMEM medium

1) DMEM: 90%, FBS: 10%

2) Supplements: L-glutamine 2.0 mM

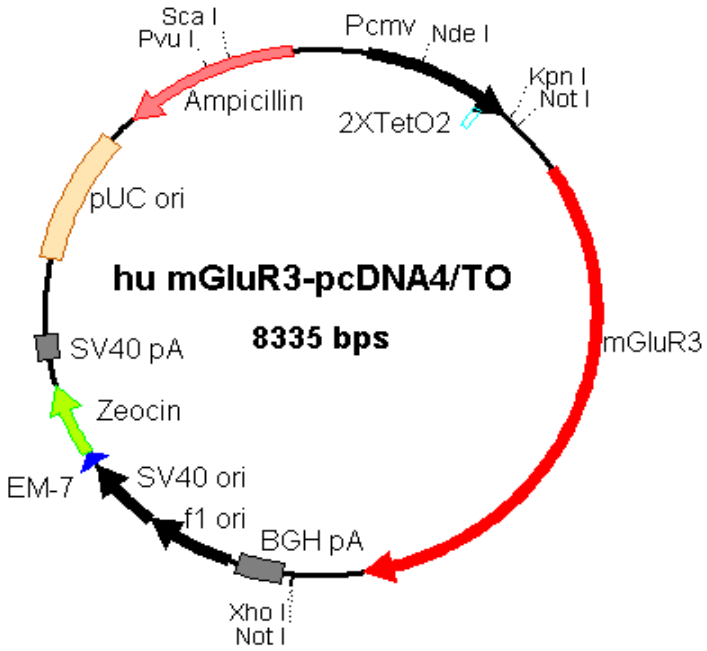
5. Freezing Medium

1) DMEM: 70%, FBS: 20%
2) Supplements: dimethyl sulphoxide (DMSO) 10%

Map of hu-mGluR3-pcDNA4/TO

Insert gene: *Homo sapiens* glutamate receptor, metabotropic 3, mGluR3
Length of insert: 3257bp Sequence reference: Gene Bank NM_000840
Vector: pcDNA4/TO (Invitrogen) Insert site: *Not* I

Plasmid Map:



Comments for hu-mGluR3-pcDNA4/TO 8335 nucleotides

CMV promoter: bases 232-819
TATA box: bases 804-810
Tetracycline operator (2X $TetO_2$) sequences: bases 820-859
hu mGluR3 gene: bases 1320-3959
BGH reverse priming site: bases 4346-4363
BGH polyadenylation sequence: bases 4352-4576
f1 origin: bases 4685-5113
SV40 promoter and origin: bases 5055-5424
EM-7 promoter: bases 5439-5506

Zeocin resistance gene (ORF): bases 5507-5881

SV40 early polyadenylation signal: bases 6011-6141

pUC origin: bases 6524-7194

Ampicillin (bla) resistance gene: bases 7339-8199 (complementary strand)

Nucleotide sequence of *Homo sapiens* glutamate receptor, metabotropic 3 (mGluR3) in pcDNA4/TO. The initiation codon and stop codon are in framed red text, and the restriction enzyme sites used for insertion are underlined.

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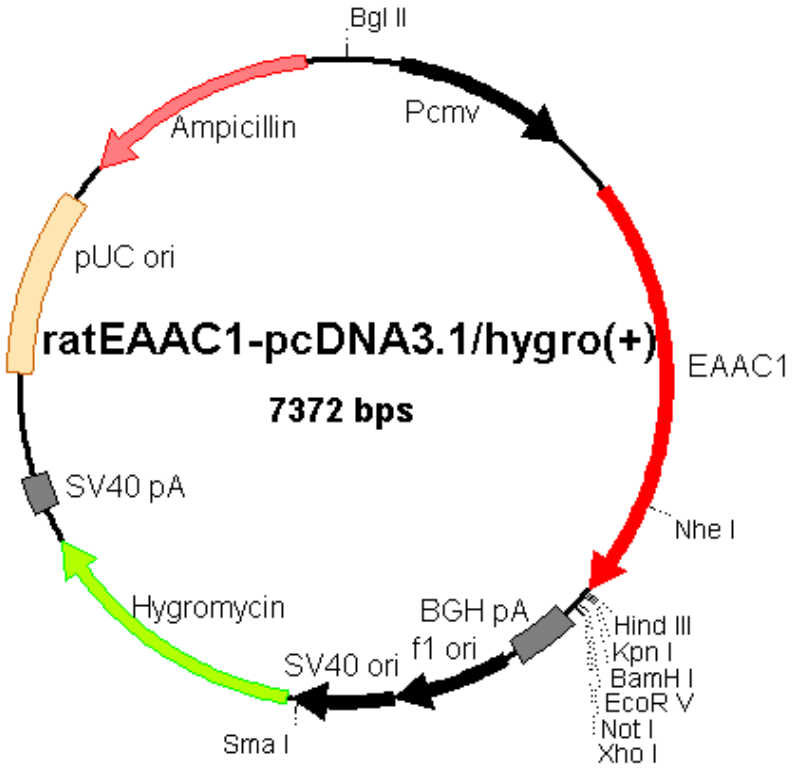
CGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCG
TTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCG
TAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCC
CGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGA
AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTA
CTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGT
TGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATG
TATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

Map of Rat-EAAC1-pcDNA3.1/Hygro(+)

Insert gene: *Rattus norvegicus* high-affinity glutamate transporter, EAAC1
Length of insert: 1783 bp Sequence reference: Gene Bank RNU39555
Vector: pcDNA3.1/Hygro(+)(Invitrogen) Insert site: *Hind* III

Plasmid Map:



Comments for rat-EAAC1-pcDNA3.1/Hygro(+) 7372 nucleotides

CMV promoter: bases 232-819

T7 promoter/priming site: bases 863-882

Rat EAAC1 gene: bases 1097-2668

BGH reverse priming site: bases 2793-2810

BGH polyadenylation sequence: bases 2799-3023

f1 origin: bases 3069-3497

SV40 promoter and origin: bases 3502-3872

Hygromycin(R) resistance gene (ORF): bases 3890-4915

SV40 early polyadenylation signal: bases 5045-5175

pUC origin: bases 5558-6231

Ampicillin(R) resistance gene: bases 6376-7236 (complementary strand)

Nucleotide sequence of .*Rattus norvegicus* high-affinity glutamate transporter in pcDNA3.1/Hygro(+).The initiation codon and stop codon are in framed red text, and the restriction enzyme sites used for insertion are underlined.

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATC
TGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGAC
AATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATTGA
TTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTAC
GGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGC
CAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT

GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTT
TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGA
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TCTGACACCATCTCGTTCACTCAGACCTCGCAGTTCTAGAGTCGACCTGCAGAAGCTTGGTACCGAGCTCGGATCCACTAG
TCCAGTGTGGTGGAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATC
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GCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTG
GTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACG
TTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGCATCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCC
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