**Cloning LSSmOrange from pLSSmOrange-N1 into pMiniT 2.0**

**pLSSmOrange-N1 4723bp**

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGAATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCTTTCAGACCGTTAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCTTGTCCCCTCAGTTCACCTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACCTCAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACTCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCATGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGGACAAGCTCAGGCTGAAGCTGAAGGACGGCGGCCACTACACCTCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGTTGCCCGGCGCCTACATCGTCGACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCGTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCATGCAT

**pLSSmOrange-N1 upstream region-LSSmOrange-downstream region template**

ATCCCAGTTGGAATCCTGGGAGATCGCCACCATGGTGAGC

CGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGAATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCTTTCAGACCGTTAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCTTGTCCCCTCAGTTCACCTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACCTCAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACTCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCATGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGGACAAGCTCAGGCTGAAGCTGAAGGACGGCGGCCACTACACCTCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGTTGCCCGGCGCCTACATCGTCGACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAAGCGGCCGCGACTCTAGATCATAATCAGCCATA

Complement to Reverse primer **GACGAGCTGTACAAGTAAGC**CAGCAACGCCGAGACACCGACTAT

**Virtual construct pMiniT2-LSSmOrange 3356bp; empty pMiniT2 2588bp**

AGGAGGTAAAAACCATGATATCCCAGTTGGAATCCTGGGAGATCGCCACCATGGTGAGCAAGGGCGAGGAGAATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCTTTCAGACCGTTAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCTTGTCCCCTCAGTTCACCTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACCTCAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACTCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCATGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGGACAAGCTCAGGCTGAAGCTGAAGGACGGCGGCCACTACACCTCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGTTGCCCGGCGCCTACATCGTCGACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAAGCCAGCAACGCCGAGACACCGACTAT

**NEB\_PCRclon\_primer\_FOR\_LSSmOrange\_donor** (40-mer):

ATCCCAGTTGGAATCCTGGGAGATCGCCACCATGGTGAGC 69/Q5/64 60.2/DreamTaq 65.2/Phusion/61

CCGCGGGCCCGGGATCCACCGG**TCGCCACCATGGTGAGC**

**NEB\_PCRclone\_primer\_REV\_LSSmOrange\_donor** (44-mer):

ATAGTCGGTGTCTCGGCGTTGCTGGCTTACTTGTACAGCTCGTC 63/Q5/64 54.6/DreamTaq 61.2/Phusion/61

**GACGAGCTGTACAAGTAAGC**CAGCAACGCCGAGACACCGACTAT

**PCR product 768bp**

**2023-11-06 Pilot PCR to synthesize LSSmOrange by Q5 HF polymerase SP; PCR cycler 312 room, B sector**

|  |  |  |  |
| --- | --- | --- | --- |
| **V=50ul** Q5 Polymerase | **“KRUPA3”-“prkn”** | No photo for PCR product |  |
| 22.5ul H2O | 98oC 30 sec |  |
| 10ul 5x NEB buffer SP | 98oC 10 sec |  |
| 1.25ul 10mM dNTP (0.25mM f.c.) SP from OG | 64oC 25 sec |  |
| 1ul/5ng pLSSmOrange-N1 template | 72oC 30 sec |  |
| 2.5ul 10uM LSSforw(0.5uM f.c.) | 72oC 5 min |  |
| 2.5ul 10uM LSSReverse (0.5uM f.c.) | 4oC hold |  |
| 10ul 5x Q5 High GC Enhancer | **35 cycles** |  |
| 0.5ul HF Q5 Polymerase SP | **PCR product - ok** |  |

[**https://www.neb.com/en/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491**](https://www.neb.com/en/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491)

**2023-11-07 Ligation reaction**

|  |  |
| --- | --- |
| 1ul/25ng Linearized pMiniT 2.0 Vector (25 ng/µl) |  |
| 0.7ul/60ng LSSmOrange PCR product elution1 85ng/ul |  |
| 3.3ul H2O |  |
| 4ul Mix1 |  |
| 1ul Mix2 |  |
| **10ul total** |  |

**Incubation 25°C for 15 min, ice 3-5 min, 4ul from 10ul for transformation 48ul XL, incubation on ice 45 min, heat shock 42°C/80sec, add 450ul LB, incubation 37°C/1hr, spread 2drops on left side/5drops (together 100ul) on right side of Carbenicillin plate, incubation o/n 37°C**

**2023-11-08 raised one colony with appropriate size from 100ul cells. Spread rest (400ul) on new Carbenicillin plate, incubation o/n 37°C.**

**2023-11-09 1 colony hand-made miniprep, precipitation by isopropanol; dissolved in 50ul H2O**

**1 2 3 4 5 6**

|  |  |
| --- | --- |
|  | 1-pX333-gRNA1 cl1 |
| 2-pX333-gRNA1 cl2 |
| 3-pX333-gRNA1 cl3 |
| 4-pX333-gRNA1 cl4 |
| 5-pMiniT2-LSSmOrange cl1 |
| 6-1kb marker |

**Restriction pMiniT2-LSSmOrange cl1 by BbsI (Borys) to check LSSmOrange insertion**

**1 2 3**

|  |  |  |  |
| --- | --- | --- | --- |
| 5.5ul H2O |  | 1-pMiniT2-LSSmOrange cl1 uncut | 7-pX333-gRNA1 cl4 PCRtest |
| 1ul 10x rCutSmart Buffer Borys | 2-pMiniT2-LSSmOrange cl1+BbsI | 8-pX333-empty PCRtest |
| 3ul pMiniT2-LSSmOrange clone1 | 3-1kb marker |  |
| BbsI 0.4ul Borys | 4-pX333-gRNA1 cl1 PCRtest | Empty pMiniT2 2588bp |
| **10ul total** | 5-pX333-gRNA1 cl2 PCRtest | LSSmOrange 768bp |
| **Incubation 37°C for 2hrs, store at - 20°C** | 6-pX333-gRNA1 cl3 PCRtest | pMiniT2-LSSmOrange 2588+768=3356bp |

**2023-11-14 Restriction pMiniT2-LSSmOrange clone1 by EcoRV-GATATC (SP) and BamHI-GGATCC/SalI-GTCGAC to check the orientation of LSSmOrange insertion**

**1 2 3**

|  |  |  |  |
| --- | --- | --- | --- |
| 6.5ul H2O |  | 4ul H2O | 1-1kb marker |
| 1ul 10xFD Buffer SP | 1ul 10xFD Buffer SP | 2-pMiniT2-LSSmOrange cl1+BamHI/SalI |
| 2ul pMiniT2-LSSmOrange cl1 hand-made | 4ul pMiniT2-LSSmOrange cl1 hand-made | 3-pMiniT2-LSSmOrange cl1+EcoRV |
| 0.5ul EcoRV FD SP | 0.5ul BamHI FD/0.5ul SalI FD SP | 4-pX333-gRNA1-gRNA2 cl1 |
| **10ul total** | **10ul total** | 5-pX333-gRNA1-gRNA2 cl2 |
| **Incubation 37°C for 60min** | **Incubation 37°C for 60min** | 6- |

**Summary: wrong orientation for LSSmOrange**

**Transformation XL cells by rest of pMiniT2-LSSmOrange ligation mix from 2023-11-07 (store at -20°C/3days, transfer to +4°C/to date)**

**3ul (from 6ul) for transformation 50ul XL, incubation on ice 30 min, heat shock 42°C/90sec, add 450ul LB, incubation 37°C/1hr, spread 9drops (together 100ul) of Carbenicillin plate, incubation o/n 37°C**

**2023-11-15 miniprep Fermentas, pMiniT2-LSSmOrange clone1 elution 65ul 115ng/ul.**

**Several colonies with extremely high background raised on Carbenicillin plate, hard to pick up separate colony. Inoculate 2 colonies in 4ml LB-Carbenicillin each.**

**To check the orientation of LSSmOrange insertion**

**TKIT\_pMiniT2\_LSSmOrange\_donor 3356bp; for** good orientation - restriction byBamHI GGATCC-SalI gtcgac 659+2697bp; restriction by EcoRV GATatc just one restrictase cut

CTGATAATAATTAATTAAGACGTCAGAATTCTCGAGGCGGCCGCATGTGCGTCTCCCTATAGTGAGTCGTATTAATTTCGCGGGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAATGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCCGGTTCAGACAGGATAAAGAGGAACGCAGAATGTTAGACAACACCCGCTTACGCATAGCTATTCAGAAATCAGGCCGTTTAAGCGATGATTCACGAGAATTGCTGGCCCGCTGCGGCATAAAAATTAATTTACACACTCAGCGCTGATGAATCCCCTAATGATTTTGGTAAAAATCATTAAGTTAAGGTGGACACACATCTTGTCATATGATTAAATGGTTTCGCGAAAAATCAATAATCAGACAACAAGATGTGCGAACTCGATATTTTACACGACTCTCTTTACCAATTCTGCCCCGAATTACACTTAAAACGACTCAACAGCTTAACGTTGGCTTGCCACGCATTACTTGACTGTAAAACTCTCACTCTTACCGAACTTGGCCGTAACCTGCCAACCAAAGCGAGAACAAAACATAACATCAAACGAATCGACCGATTGTTAGGTAATCGTCACCTGCAGGAAGGTTTAAACGCATTTAGGTGACACTATAGAAGTGTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATatcccagttggaatcctgggagatcgccaccatggtgagcaagggcgaggagaataacatggccatcatcaaggagttcatgcgcttcaaggtgcgcatggagggctccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagggctttcagaccgttaagctgaaggtgaccaagggtggccccctgcccttcgcctgggacatcttgtcccctcagttcacctacggctccaaggcctacgtgaagcaccccgccgacatccccgactacctcaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgactcaggactcctccctgcaggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagaccatgggcatggaggcctcctccgagcggatgtaccccgaggacggcgccctgaagggcgaggacaagctcaggctgaagctgaaggacggcggccactacacctccgaggtcaagaccacctacaaggccaagaagcccgtgcagttgcccggcgcctacatcgtcgacatcaagttggacatcacctcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccactccaccggcggcatggacgagctgtacaagtaagccagcaacgccgagacaccgactat

For BAD orientation – restricition byBamHI GGATCC-SalI gtcgac 165+3191bp; no site for EcoRV

AAGCGAGAACAAAACATAACATCAAACGAATCGACCGATTGTTAGGTAATCGTCACCTGCAGGAAGGTTTAAACGCATTTAGGTGACACTATAGAAGTGTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATatagtcggtgtctcggcgttgctggcttacttgtacagctcgtccatgccgccggtggagtggcggccctcggcgcgttcgtactgttccacgatggtgtagtcctcgttgtgggaggtgatgtccaacttgatgtcgacgatgtaggcgccgggcaactgcacgggcttcttggccttgtaggtggtcttgacctcggaggtgtagtggccgccgtccttcagcttcagcctgagcttgtcctcgcccttcagggcgccgtcctcggggtacatccgctcggaggaggcctccatgcccatggtcttcttctgcattacggggccgtcggaggggaagttggtgccgcgcagcttcaccttgtagatgaactcgccgtcctgcagggaggagtcctgagtcacggtcaccacgccgccgtcctcgaagttcatcacgcgctcccacttgaagccctcggggaaggacagcttgaggtagtcggggatgtcggcggggtgcttcacgtaggccttggagccgtaggtgaactgaggggacaagatgtcccaggcgaagggcagggggccacccttggtcaccttcagcttaacggtctgaaagccctcgtaggggcggccctcgccctcgccctcgatctcgaactcgtggccgttcacggagccctccatgcgcaccttgaagcgcatgaactccttgatgatggccatgttattctcctcgcccttgctcaccatggtggcgatctcccaggattccaactgggat

**Cloning TWO gRNAs into pX333 by BbsI AGATCT (first cloning), by BsaI CTCGAG (second cloning) to cut Hs-HPCA full-length from neurons (knock-in)**

**Protocol from** [**https://www.nature.com/articles/nprot.2013.143#Sec13**](https://www.nature.com/articles/nprot.2013.143#Sec13)

**2023-11-06 Digestion pX333 plasmid by BbsI from Borys**

|  |  |  |
| --- | --- | --- |
| **V=50ul** | 1-1kb marker |  |
| 40.25ul H2O free | 2-LSSmOrange PCR product |  |
| 5ul 10x rCutSmart Buffer Borys | 3- LSSmOrange PCR product |
| 3.75ul/0.5mkg pX333 134ng/ul Borys | 4-blank |
| 1ul BbsI Borys | 5-1kb marker |
| Incubation **37°C** 40min | 6-pX333+BbSI ok |
| Thermoinactivation at **65°C** 20min | 7-pX333+BbSI ok |
|  | 8 |

**Purification pX333-BbsI through the gel; elution1 6.5ng/ul, elution2 2.5ng/ul (quite low concentration), Borys box**

**2023-11-07 Cloning first gRNA into pX333**

**Hpca\_5UTR\_gRNA\_1stick BbsI**

**CACC**AGTCGGTGTCTCGGCGTTGC

TCAGCCACAGAGCCGCAACG**CAAA**

1. Preparation 10uM gRNA1Fw/10uM gRNA1Rev (Borys did all dilutions)
2. Phosphorylate and anneal the oligos in a thermocycler by using the following parameters: 37 °C for 30 min; 95 °C for 5 min; ramp down to 25 °C at 5 °C min−1.

**Phosphorylation and annealing gRNA1 pair primers to clone into pX333-BbsI**

|  |  |  |
| --- | --- | --- |
| 2ul H2O | **Folder “krupa”, programme “ramping”** |  |
| 2ul 10xT4 DNA ligase Buffer SP (source of ATP) | **37°C** 30 min |  |
| 7.5ul 10uM gRNA1Fw Borys (f.c. 3.75uM; should be 10uM) | **95°C** 5 min and then ramp down to **25°C** at **5°C**/min | Should be 1ul 100uM oligo |
| 7.5ul 10uM gRNA1Rev Borys (f.c. 3.75uM; should be 10uM) | Actually, ramp down was 12 min instead 14 min | Should be 1ul 100uM oligo |
| 1ul T4 PNK SP | Rate was **0.1°C**/sec |  |
| **20ul total** |  |  |

1. From Protocol – “Dilute phosphorylated and annealed oligos 1:200 by adding 1 μl of oligo to 199 μl of room temperature ddH2O”. From 10uM to 50nM; f.c. in following ligation reaction is 5 nM by diluting 2ul phosphorylated and annealed oligoduplex in 20 ul total volume mix.

**Ligation reaction – use reagents from Borys Basic protocol Ligation reaction and incubation o/n at 4oC (without Quick Ligase)**

|  |  |
| --- | --- |
| 0ul H2O | ul H2O |
| 6ul 2xQuick Ligase reaction buffer Borys | 1ul 10xT4 DNA ligation buffer OG |
| 1ul phosphorylated and annealed oligoduplex (diluted 75x times, 2ul+148ul H2O) | 1ul phosphorylated and annealed oligoduplex (diluted 200x times, 1ul+199ul H2O) |
| 4ul/26ng BbsI digested plasmid pX333; use elution1 c=6.5ng/ul | ng BbsI digested plasmid pX333; use elution1 c=ng/ul |
| 1ul Quick Ligase Borys | 1ul T4 DNA ligase (Fermentas exp.2016) IK |
| **12ul total** | **10ul total** |

**Incubation 25°C for 15 min, ice 3-5 min, 4ul from 12ul for transformation 48ul XL, incubation on ice 30 min, heat shock 42°C/80sec, add 450ul LB, incubation 37°C/1hr, spread 2drops on left side/5drops on right side of Carbenicillin plate, incubation o/n 37°C**

[**https://www.neb.com/en/protocols/0001/01/01/quick-ligation-protocol**](https://www.neb.com/en/protocols/0001/01/01/quick-ligation-protocol)

2. Gently mix the reaction by pipetting up and down and microfuge briefly.  
3. Incubate at room temperature (25°C) for 5 minutes.  
4. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells. Alternatively, Store at -20°C.  
5. Do not heat inactivate – heat inactivation dramatically reduces transformation efficiency.

**2023-11-08 Raised tens colonies with appropriate colony size (from 100ul cells after transformation). Inoculate 4 colonies in 4ml LB-Carbenicillin each.**

**2023-11-09 4 colonies hand-made miniprep, precipitation by isopropanol; dissolved in 50+150ul H2O**

**1 2 3 4 5 6**

|  |  |
| --- | --- |
|  | 1-pX333-gRNA1 cl1 |
| 2-pX333-gRNA1 cl2 |
| 3-pX333-gRNA1 cl3 |
| 4-pX333-gRNA1 cl4 |
| 5-pMiniT2-LSSmOrange cl1 |
| 6-1kb marker |

**Checking of insertion gRNA1 into pX333 by PCR (U6 forward+gRNA1 reverse;** U6For GAGGGCCTATTTCCCATGATTCC, **gRNA\_1stick Antisense** TCAGCCACAGAGCCGCAACG**CAAA?)**

**3 4 5 6 7 8**

|  |  |  |  |
| --- | --- | --- | --- |
| **V=100ul=5x20ul** | **“KRUPA3”-“Cas9”** |  | 1-pMiniT2-LSSmOrange cl1 uncut |
| 71.5ul H2O SP | 95oC 3 min | 2-pMiniT2-LSSmOrange cl1+BbsI |
| 10ul 10xDream Taq Greeen buffer SP | 95oC 30 sec | 3-1kb marker |
| 2.5ul 10mM dNTP (0.25mM f.c.) SP | 58oC 30 sec | 4-pX333-gRNA1 cl1 PCRtest PCR product should be 250-260bp |
| 5ul=5x1ul pX333-gRNA1 cl1-4+empty plasmid | 72oC 15 sec | 5-pX333-gRNA1 cl2 PCRtest |
| 5ul 10uM **U6Fw** (0.5uM f.c.) SP | 72oC 5 min | 6-pX333-gRNA1 cl3 PCRtest |
| 5ul 10uM gRNA1rev (0.5uM f.c.) Borys | 4oC hold | 7-pX333-gRNA1 cl4 PCRtest |
| 1ul Dream taq N2 exp.2014 SP | **35 cycles** | 8-pX333-empty PCRtest control |

**2023-11-10 minipreps Zymokit pX333-gRNA1 clone1 160ng/ul; pX333-gRNA1 clone2 1125ng/ul**

**1 2 3**

|  |  |
| --- | --- |
|  | 1-1kb marker |
| 2-pX333-gRNA1 **clone1 160ng/ul** |
| 3-pX333-gRNA1 **clone2 1125ng/ul** |
|  |
|  |
|  |

**2023-11-12 Cloning second gRNA into pX333-gRNA1 clone1 Digestion pX333-gRNA1 clone1 160ng/ul by BsaI-HFv2 from Borys**

|  |  |  |
| --- | --- | --- |
| **V=50ul** | 1-1kb marker |  |
| 37.75ul H2O free | 2-pX333-gRNA1 clone1-BsaI-HFv2 |
| 5ul 10x rCutSmart Buffer Borys | 3-pX333-gRNA1 clone1-BsaI-HFv2 |
| 6.25ul/1mkg pX333-gRNA1 clone1 160ng/ul SP |  |
| 1ul **BsaI-HFv2** Borys |  |
| Incubation **37°C** 48min |  |
| Thermoinactivation at 80**°C** 20min |  |

**Purification pX333-gRNA1 clone1-BsaI-HFv2 through the gel; elution1 30ul 13ng/ul, elution2 25ul 6ng/ul (quite low concentration), Borys box**

**Hpca\_3UTR\_gRNA\_152rev**

**CACC**GATCTCCCAGGATTCCAACT

CTAGAGGGTCCTAAGGTTGA**CAAA**

1. Preparation 10uM gRNA2Fw/10uM gRNA2Rev (Borys did all dilutions)
2. Phosphorylate and anneal the oligos in a thermocycler by using the following parameters: 37 °C for 30 min; 95 °C for 5 min; ramp down to 25 °C at 5 °C min−1.

**Phosphorylation and annealing gRNA2 pair primers to clone into pX333-gRNA1-BsaI-HFv2**

|  |  |  |
| --- | --- | --- |
| 2ul H2O | **Folder “krupa”, programme “ramping”** |  |
| 2ul 10xT4 DNA ligase Buffer SP (source of ATP) | **37°C** 30 min |  |
| 7.5ul 10uM gRNA1Fw Borys (f.c. 3.75uM; should be 10uM) | **95°C** 5 min and then ramp down to **25°C** at **5°C**/min | Should be 1ul 100uM oligo |
| 7.5ul 10uM gRNA1Rev Borys (f.c. 3.75uM; should be 10uM) | Actually, ramp down was 12 min instead 14 min | Should be 1ul 100uM oligo |
| 1ul T4 PNK SP | Rate was **0.1°C**/sec |  |
| **20ul total** |  |  |

1. From Protocol – “Dilute phosphorylated and annealed oligos 1:200 by adding 1 μl of oligo to 199 μl of room temperature ddH2O”. From 10uM to 50nM; f.c. in following ligation reaction is 5 nM by diluting 2ul phosphorylated and annealed oligoduplex in 20 ul total volume mix.

**Ligation reaction – use reagents from Borys**

|  |  |
| --- | --- |
| 0ul H2O | **Basic protocol Ligation reaction and incubation o/n at 4oC (without Quick Ligase)** |
| 5ul 2xQuick Ligase reaction buffer Borys |  |
| 1ul phosphorylated and annealed oligoduplex (diluted 75x times, 2ul+148ul H2O) |  |
| 3ul/40ng BsaI-HFv2 digested plasmid pX333-gRNA1; use elution1 c=13ng/ul |  |
| 1ul Quick Ligase Borys |  |
| **10ul total** |  |

**Incubation 25°C for 18 min, 5ul from 10ul for transformation 50ul XL, incubation on ice 20 min, heat shock 42°C/80sec, add 450ul LB, incubation 37°C/15 min, spread 2drops on left side/6drops on right side of Carbenicillin plate, incubation o/n 37°C**

[**https://www.neb.com/en/protocols/0001/01/01/quick-ligation-protocol**](https://www.neb.com/en/protocols/0001/01/01/quick-ligation-protocol)

2. Gently mix the reaction by pipetting up and down and microfuge briefly.  
3. Incubate at room temperature (25°C) for 5 minutes.  
4. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells. Alternatively, Store at -20°C.  
5. Do not heat inactivate – heat inactivation dramatically reduces transformation efficiency.

**2023-11-13 Raised tens colonies with appropriate colony size (from 100ul cells after transformation). Inoculate 4 colonies in 4ml LB-Carbenicillin each.**

**2023-11-14 2 colonies miniprep Fermentas, elution1 50ul 13ng/ul**

**1 2 3 4 5**

|  |  |
| --- | --- |
|  | 1-1kb marker |
| 2-pMiniT2-LSSmOrange cl1+BamHI/SalI |
| 3-pMiniT2-LSSmOrange cl1+EcoRV |
| 4-pX333-gRNA1-gRNA2 cl1 c=260ng/ul 45ul |
| 5-pX333-gRNA1-gRNA2 cl2 c=260ng/ul 45ul |
|  |

**Checking of insertion gRNA2 into pX333-gRNA1 by PCR (U6 for+gRNA2 reverse;** U6For GAGGGCCTATTTCCCATGATTCC, gRNA\_2 Antisense CTAGAGGGTCCTAAGGTTGACAAA)

**1 2 3 4 5 6**

|  |  |  |  |
| --- | --- | --- | --- |
| **V=60ul=3x20ul** | **“KRUPA3”-“Cas9”** |  | 1-1kb marker |
| 43ul H2O SP | 95oC 3 min | 2-pX333-gRNA1-gRNA2 cl1 c=260ng/ul 45ul |
| 6ul 10xDream Taq Greeen buffer SP | 95oC 30 sec | 3-pX333-gRNA1-gRNA2 cl2 c=260ng/ul 45ul |
| 1.5ul 10mM dNTP (0.25mM f.c.) SP | 58oC 30 sec | 4-pX333-gRNA1-2 cl1 PCRtest PCR product should be 250-260bp |
| 3ul=3x1ul pX333-gRNA1-2 cl1,2+empty plasmid | 72oC 15 sec | 5-pX333-gRNA1-2 cl2 PCRtest PCR product should be 250-260bp |
| 3ul 10uM **U6Fw** (0.5uM f.c.) SP | 72oC 5 min | 6-pX333-empty PCRtest control |
| 3ul 10uM gRNA2rev (0.5uM f.c.) Borys | 4oC hold |  |
| 1ul Dream taq N2 exp.2014 SP | **35 cycles** |  |

**Store at -80°C transformed XL cells with pX333-gRNA1-gRNA2 cl1/pX333-gRNA1-gRNA2 cl2.**

pX333

**gagggcctatttcccatgattcc**ttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccgggtcttcgagaagacctgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttttgttttagagctagaaatagcaagttaaaataaggctagtccgtttttagcgcgtgcgccaattctgcagacaaatggctctaga**gagggcctatttcccatgattcc**ttcatatttgcatatacgatacaaggctgttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccggagacctattcgccttaaggtctcggttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttttgttttagagctagaaatagcaagttaaaataaggctagtccgtttttagcgcgtgcgccaattctgcagacaaatggggtacccgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattgtgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtcgaggtgagccccacgttctgcttcactctccccatctcccccccctccccacccccaattttgtatttatttattttttaattattttgtgcagcgatgggggcggggggggggggggggcgcgcgccaggcggggcggggcggggcgaggggcggggcggggcgaggcggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttccttttatggcgaggcggcggcggcggcggccctataaaaagcgaagcgcgcggcgggcgggagtcgctgcgcgctgccttcgccccgtgccccgctccgccgccgcctcgcgccgcccgccccggctctgactgaccgcgttactcccacaggtgagcgggcgggacggcccttctcctccgggctgtaattagctgagcaagaggtaagggtttaagggatggttggttggtggggtattaatgtttaattacctggagcacctgcctgaaatcactttttttcaggttggaccggtgccaccatggactataaggaccacgacggagactacaaggatcatgatattgattacaaagacgatgacgataagatggccccaaagaagaagcggaaggtcggtatccacggagtcccagcagccgacaagaagtacagcatcggcctggacatcggcaccaactctgtgggctgggccgtgatcaccgacgagtacaaggtgcccagcaagaaattcaaggtgctgggcaacaccgaccggcacagcatcaagaagaacctgatcggagccctgctgttcgacagcggcgaaacagccgaggccacccggctgaagagaaccgccagaagaagatacaccagacggaagaaccggatctgctatctgcaagagatcttcagcaacgagatggccaaggtggacgacagcttcttccacagactggaagagtccttcctggtggaagaggataagaagcacgagcggcaccccatcttcggcaacatcgtggacgaggtggcctaccacgagaagtaccccaccatctaccacctgagaaagaaactggtggacagcaccgacaaggccgacctgcggctgatctatctggccctggcccacatgatcaagttccggggccacttcctgatcgagggcgacctgaaccccgacaacagcgacgtggacaagctgttcatccagctggtgcagacctacaaccagctgttcgaggaaaaccccatcaacgccagcggcgtggacgccaaggccatcctgtctgccagactgagcaagagcagacggctggaaaatctgatcgcccagctgcccggcgagaagaagaatggcctgttcggaaacctgattgccctgagcctgggcctgacccccaacttcaagagcaacttcgacctggccgaggatgccaaactgcagctgagcaaggacacctacgacgacgacctggacaacctgctggcccagatcggcgaccagtacgccgacctgtttctggccgccaagaacctgtccgacgccatcctgctgagcgacatcctgagagtgaacaccgagatcaccaaggcccccctgagcgcctctatgatcaagagatacgacgagcaccaccaggacctgaccctgctgaaagctctcgtgcggcagcagctgcctgagaagtacaaagagattttcttcgaccagagcaagaacggctacgccggctacattgacggcggagccagccaggaagagttctacaagttcatcaagcccatcctggaaaagatggacggcaccgaggaactgctcgtgaagctgaacagagaggacctgctgcggaagcagcggaccttcgacaacggcagcatcccccaccagatccacctgggagagctgcacgccattctgcggcggcaggaagatttttacccattcctgaaggacaaccgggaaaagatcgagaagatcctgaccttccgcatcccctactacgtgggccctctggccaggggaaacagcagattcgcctggatgaccagaaagagcgaggaaaccatcaccccctggaacttcgaggaagtggtggacaagggcgcttccgcccagagcttcatcgagcggatgaccaacttcgataagaacctgcccaacgagaaggtgctgcccaagcacagcctgctgtacgagtacttcaccgtgtataacgagctgaccaaagtgaaatacgtgaccgagggaatgagaaagcccgccttcctgagcggcgagcagaaaaaggccatcgtggacctgctgttcaagaccaaccggaaagtgaccgtgaagcagctgaaagaggactacttcaagaaaatcgagtgcttcgactccgtggaaatctccggcgtggaagatcggttcaacgcctccctgggcacataccacgatctgctgaaaattatcaaggacaaggacttcctggacaatgaggaaaacgaggacattctggaagatatcgtgctgaccctgacactgtttgaggacagagagatgatcgaggaacggctgaaaacctatgcccacctgttcgacgacaaagtgatgaagcagctgaagcggcggagatacaccggctggggcaggctgagccggaagctgatcaacggcatccgggacaagcagtccggcaagacaatcctggatttcctgaagtccgacggcttcgccaacagaaacttcatgcagctgatccacgacgacagcctgacctttaaagaggacatccagaaagcccaggtgtccggccagggcgatagcctgcacgagcacattgccaatctggccggcagccccgccattaagaagggcatcctgcagacagtgaaggtggtggacgagctcgtgaaagtgatgggccggcacaagcccgagaacatcgtgatcgaaatggccagagagaaccagaccacccagaagggacagaagaacagccgcgagagaatgaagcggatcgaagagggcatcaaagagctgggcagccagatcctgaaagaacaccccgtggaaaacacccagctgcagaacgagaagctgtacctgtactacctgcagaatgggcgggatatgtacgtggaccaggaactggacatcaaccggctgtccgactacgatgtggaccatatcgtgcctcagagctttctgaaggacgactccatcgacaacaaggtgctgaccagaagcgacaagaaccggggcaagagcgacaacgtgccctccgaagaggtcgtgaagaagatgaagaactactggcggcagctgctgaacgccaagctgattacccagagaaagttcgacaatctgaccaaggccgagagaggcggcctgagcgaactggataaggccggcttcatcaagagacagctggtggaaacccggcagatcacaaagcacgtggcacagatcctggactcccggatgaacactaagtacgacgagaatgacaagctgatccgggaagtgaaagtgatcaccctgaagtccaagctggtgtccgatttccggaaggatttccagttttacaaagtgcgcgagatcaacaactaccaccacgcccacgacgcctacctgaacgccgtcgtgggaaccgccctgatcaaaaagtaccctaagctggaaagcgagttcgtgtacggcgactacaaggtgtacgacgtgcggaagatgatcgccaagagcgagcaggaaatcggcaaggctaccgccaagtacttcttctacagcaacatcatgaactttttcaagaccgagattaccctggccaacggcgagatccggaagcggcctctgatcgagacaaacggcgaaaccggggagatcgtgtgggataagggccgggattttgccaccgtgcggaaagtgctgagcatgccccaagtgaatatcgtgaaaaagaccgaggtgcagacaggcggcttcagcaaagagtctatcctgcccaagaggaacagcgataagctgatcgccagaaagaaggactgggaccctaagaagtacggcggcttcgacagccccaccgtggcctattctgtgctggtggtggccaaagtggaaaagggcaagtccaagaaactgaagagtgtgaaagagctgctggggatcaccatcatggaaagaagcagcttcgagaagaatcccatcgactttctggaagccaagggctacaaagaagtgaaaaaggacctgatcatcaagctgcctaagtactccctgttcgagctggaaaacggccggaagagaatgctggcctctgccggcgaactgcagaagggaaacgaactggccctgccctccaaatatgtgaacttcctgtacctggccagccactatgagaagctgaagggctcccccgaggataatgagcagaaacagctgtttgtggaacagcacaagcactacctggacgagatcatcgagcagatcagcgagttctccaagagagtgatcctggccgacgctaatctggacaaagtgctgtccgcctacaacaagcaccgggataagcccatcagagagcaggccgagaatatcatccacctgtttaccctgaccaatctgggagcccctgccgccttcaagtactttgacaccaccatcgaccggaagaggtacaccagcaccaaagaggtgctggacgccaccctgatccaccagagcatcaccggcctgtacgagacacggatcgacctgtctcagctgggaggcgacaaaaggccggcggccacgaaaaaggccggccaggcaaaaaagaaaaagtaagaattcctagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctggggggtggggtggggcaggacagcaagggggaggattgggaagagaatagcaggcatgctggggagcggccgcaggaacccctagtgatggagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccgacgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgcagctgcctgcaggggcgcctgatgcggtattttctccttacgcatctgtgcggtatttcacaccgcatacgtcaaagcaaccatagtacgcgccctgtagcggcgcattaagcgcggcgggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccttagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgatttgggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaactctatctcgggctattcttttgatttataagggattttgccgatttcggtctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaaaatattaacgtttacaattttatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagccccgacacccgccaacacccgctgacgcgccctgacgggcttgtctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgagacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtggaagccgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatgcgccattctcgagcatgcgccattctcgagcatgctcgagaatggcgcatgt