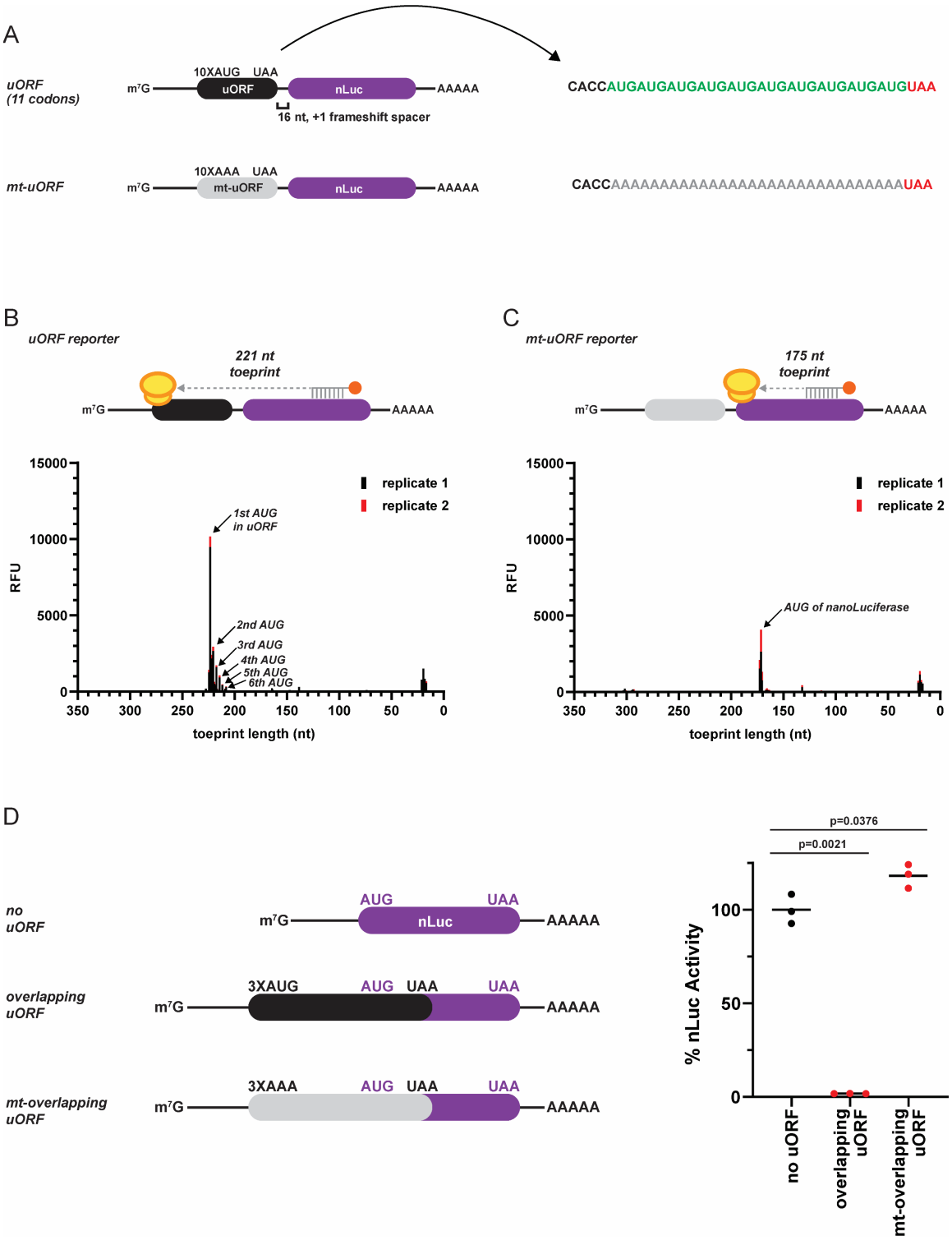
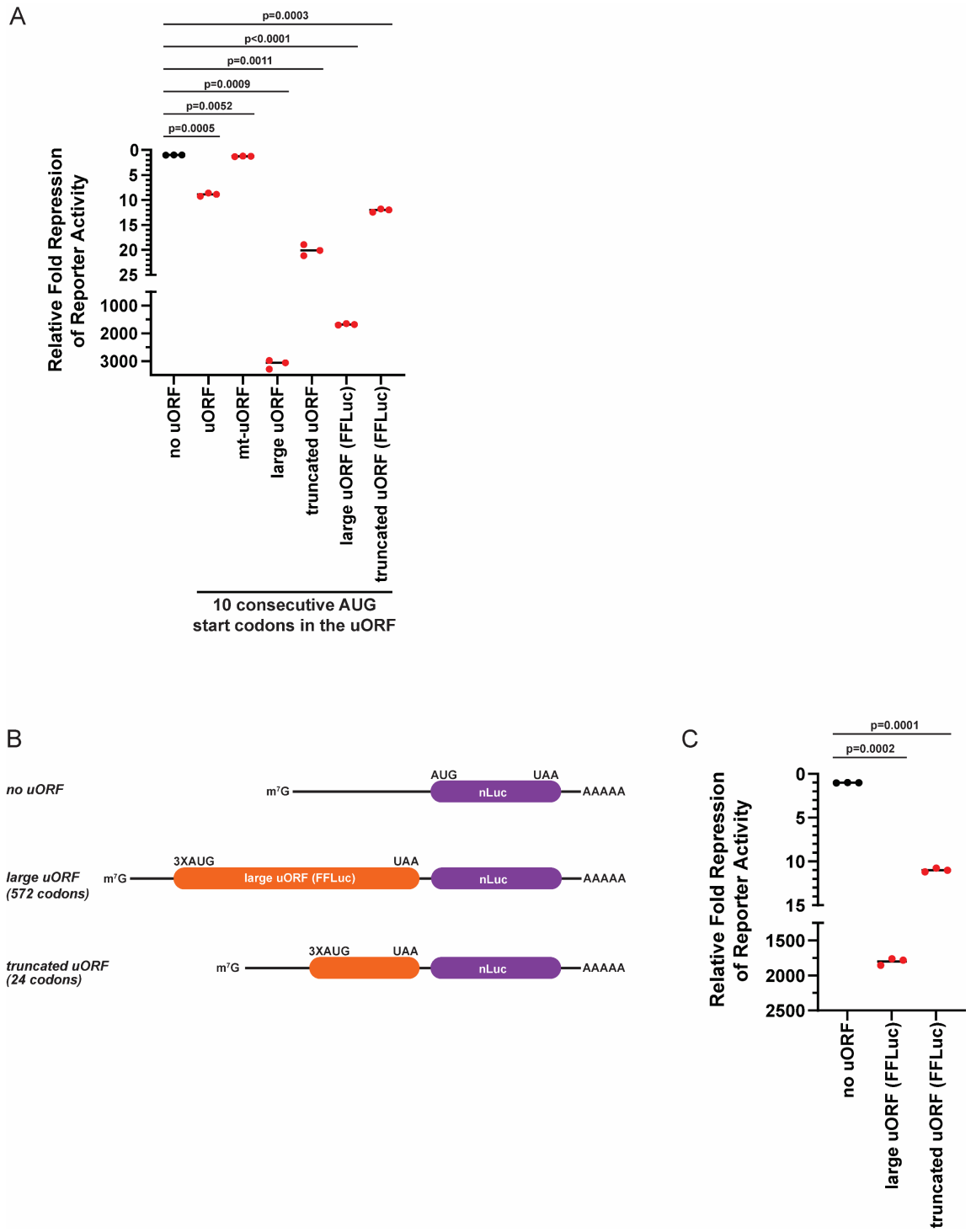
SUPPLEMENTAL MATERIAL



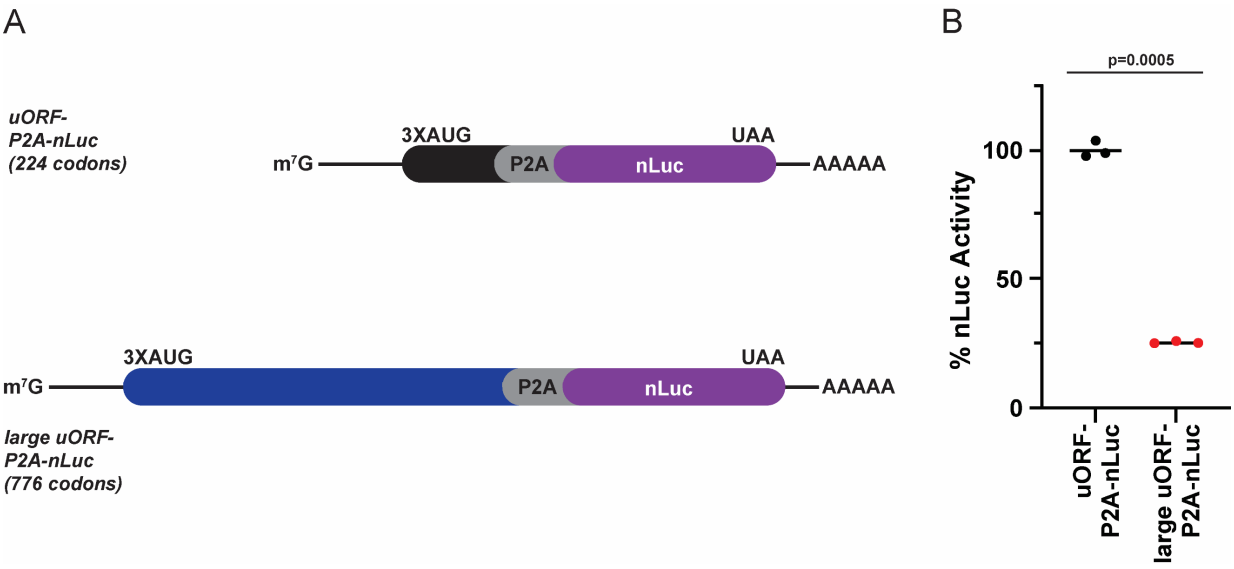
Supplemental Figure 1. Confirming the efficacy of designed uORFs to sequester all scanning initiation complexes *in vitro* and in HeLa cells. A) Design of re-initiation specific nanoLuciferase (nLuc) reporters harboring an uORF encoded by 10 consecutive AUG start

codons (uORF) or AAA codons (mt-uORF). A 16 nt spacer between the uORF and nLuc ORF allows specific detection of re-initiation. B-C) Ribosome toeprinting of lactimidomycin inhibited 80S ribosomes after start codon recognition on (B) small uORF nLuc reporter mRNA and (C) mutated uORF nLuc reporter mRNA from *in vitro* translation. Signal from duplicate samples is shown in black and red. D) Response of nLuc reporters that harbor an overlapping 3XAUG start codon uORF in HeLa cells. Here, the stop codon of the uORF (black) is downstream and out-of-frame of the start codon of nLuc (purple). Thus, active nLuc is only produced if a 43S PIC scans through the overlapping uORF ("leaky scanning") and initiates at the start codon of nLuc. n=3 biological replicates. Bar represents the mean. Comparisons were made using a two-tailed unpaired *t*-test with Welch's correction.

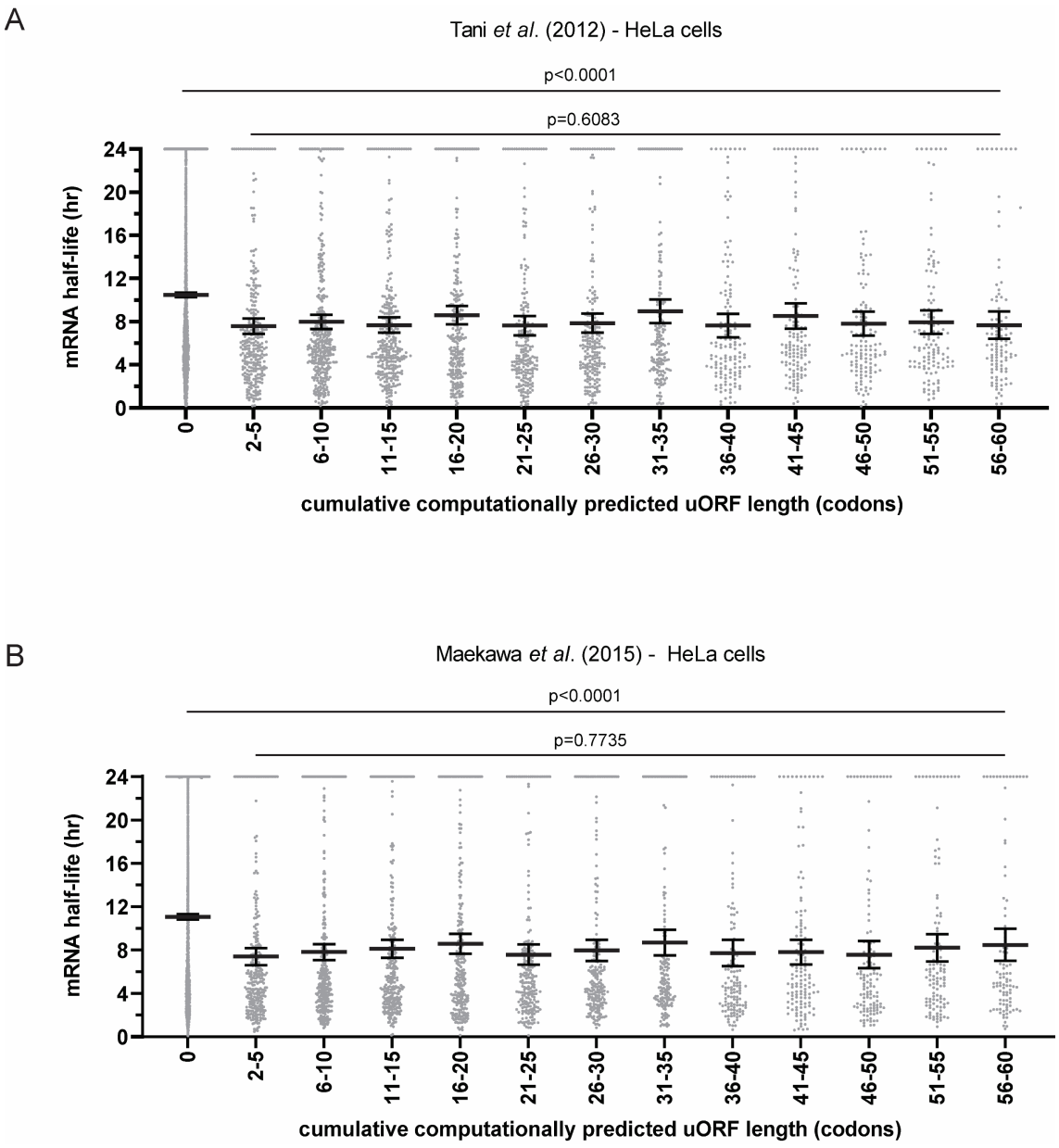


Supplemental Figure 2. Less translation re-initiation after large uORFs is not strictly uORF sequence specific. A) Response of nLuc reporters that harbor a small, mutant, large, or truncated uORF from *in vitro* translation. All uORFs had 10 consecutive AUG start codons as described in **Supplemental Figure 1A** (instead of the three AUG start codons in perfect Kozak

context in **Fig. 1**). Large and truncated uORFs using the FFLuc sequence are clearly labeled from those that used the original HT-GFP sequence. n=3 biological replicates. Bar represents the mean. Comparisons were made using a two-tailed unpaired *t*-test with Welch's correction. B) The large uORF sequence described in **Fig. 1** was switched from HT-GFP to Firefly Luciferase (FFLuc). Three AUG start codons in perfect Kozak context were used to trap all scanning initiation complexes at the uORF. C) Response of nLuc reporters that harbor a large (FFLuc) or truncated uORF (FFLuc) from *in vitro* translation. Similar repression and rescue were seen here with FFLuc as the large uORF as observed with HT-GFP sequence in **Fig. 2**. n=3 biological replicates. Bar represents the mean. Comparisons were made using a two-tailed unpaired *t*-test with Welch's correction.



Supplemental Figure 3. Small and large uORF P2A fusion reporters to test translation efficiency *in vitro*. A) Insertion of the P2A "ribosome skipping motif" (gray) was used to assess the relative translation efficiency of reporters that harbored a 3XAUG start codon sequence without and with the large HT-GFP (blue) sequence upstream of the nLuc coding sequence (purple). B) Relative nLuc activity of the small and larger uORF P2A fusion reporters from *in vitro* translation. n=3 biological replicates. Bar represents the mean. Comparisons were made using a two-tailed unpaired *t*-test with Welch's correction.



Supplemental Figure 4. uORF length has minimal effect on mRNA stability in cells. A-B) Transcriptome-wide comparison of cumulative computationally predicted uORF length and mRNA half-life from Tani *et al*. (2012) (A) and Maekawa *et al*. (2015) (B). Bar represents the mean ± 95% confidence interval. One-way Welch's ANOVA was used to compare between cumulative uORF length bins.

Sequences of reporters used in this study

The plasmid backbone, reporter name, and restriction sites are given for each construct. For reference, nanoLuciferase (nLuc) is always highlighted in pink. The multiple start sites for the uORF and single stop codon are highlighted in yellow and red, respectively. The HT-GFP sequence, FFLuc sequence and IRES sequences are highlighted in green, blue, and turquoise, respectively. The unstructured CAA repeats are in lowercase. The P2A sequence is highlighted in gray. The strong hairpins used upstream of the IRES sequences is in **bold**. For the pTet-Off All-In-One (pTet-Off AIO) plasmids, the tight TRE promotor is shown in purple and the intron is underlined.

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pcDNA3.1+/uORF nLuc (SacI/XbaI)

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pcDNA3.1+/mt-uORF nLuc (SacI/XbaI)

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pcDNA3.1+/10AUG uORF nLuc (SacI/XbaI)

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pcDNA3.1+/10AAA mt-uORF nLuc (SacI/XbaI)

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pcDNA3.1+/large uORF nLuc (SacI/XbaI)

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pcDNA3.1+/uORF nLuc (SacI/XbaI)_115 nt spacer

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pcDNA3.1+/large uORF nLuc (SacI/XbaI)_115 nt spacer

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pcDNA3.1+/truncated uORF nLuc (SacI/XbaI)

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pcDNA3.1+/10AUG large uORF nLuc (SacI/XbaI)

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pcDNA3.1+/10AUG truncated uORF nLuc (SacI/XbaI)
GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
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CTGTACAAGtaaacaacaacaacaacaaATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGC
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TTCAGAATCTCGGGGTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATG
GGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGGCGACCAAATGG
GCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGTGATC

CTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTATTTCGGA
CGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTG
TGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTC
CGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGGACTA
CAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG
TAAGGCCGCGACTCTAGA

pcDNA3.1+/large uORF (FFLuc) nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
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CTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTACGCC
GAGTACTTCGAGATGAGCGTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACA
AACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTTGGGT
GCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAGCTG
CTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAA
AAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAGCA
AGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTTGCCACCCGG
CTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACCATCGCCCTGAT
CATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCG
CTTGTGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCCGACA
CCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCTTCGGCATGTTCACCACGCTGGGCT
ACTTGATCTGCGGCTTTCGGGTCGTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGC
GCAGCTTGCAAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGCTTCTTC
GCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCGCCAGCGGC
GGGGCGCCGCTCAGCAAGGAGGTAGGTGAGGCCGTGGCCAAACGCTTCCACCTACCAGG
CATCCGCCAGGGCTACGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAAGG
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ACTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCGGCGAGCTGTGCGTCCGTGGC
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CGTGGACCGGCTGAAGAGCCTGATCAAATACAAGGGCTACCAGGTAGCCCCAGCCGAACT
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ACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCG
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GTGCGAACGCATTCTGGCGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATC
GATTACAAGGATGACGATGACAAGTAAGGCCGCGACTCTAGA

pcDNA3.1+/truncated uORF (FFluc) nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
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GACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTTCA
GAATCTCGGGGTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCT
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ACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAG
TAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGGACTACAAAG
ACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTAAGG
CCGCGACTCTAGA

pcDNA3.1+/10AUG large uORF (FFLuc) nLuc (SacI/XbaI)
GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
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CGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTA
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CGCTTGTGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCCGA
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CGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCGCCAGCGG
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TCGATTACAAGGATGACGATGACAAGTAAGGCCGCGACTCTAGA

pcDNA3.1+/overlapping uORF nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
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TGTGTCCAGTTTGTTTCAGAATCTCGGGGTGTCCGTAACTCCGATCCAAAGGATTGTCCTG
AGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGAGC
GGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATC
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CGACTATTTCGGACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGATCACTGT
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TCTGGCGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGAT
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pcDNA3.1+/mt-overlapping uORF nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
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GGTGTGTCCAGTTTGTTTCAGAATCTCGGGGTGTCCGTAACTCCGATCCAAAGGATTGTCC
TGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGA
GCGGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCA
TCACTTTAAGGTGATCCTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATG
ATCGACTATTTCGGACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGATCACT
GTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGAC
GGCTCCCTGCTGTTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACG
CATTCTGGCGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAG
GATGACGATGACAAGTAAGGCCGCGACTCTAGA

pcDNA3.1+/10AUG truncated uORF (FFLuc) nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
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GACAGACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGT
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GGCTGAAGATCGACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGGCGACCAAATGG
GCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGTGATC
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TGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTC
CGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGGACTA

CAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG
TAAGGCCGCGACTCTAGA

pcDNA3.1+/uORF-P2A-nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
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TGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGT
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TGGGCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGT
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GGACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACC
CTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTG
TTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGGA
CTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGAC
AAGTAAGGCCGCGACTCTAGA

pcDNA3.1+/large uORF-P2A-nLuc (SacI/XbaI)

GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
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GTGCTGTTCCTGCACGGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCAT
GTTGCACCGACCCATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAATCCGACAAA
CCAGACCTGGGTTATTTCTTCGACGACCACGTCCGCTTCATGGATGCCTTCATCGAAGCCC
TGGGTCTGGAAGAGGTCGTCCTGGTCATTCACGACTGGGGCTCCGCTCTGGGTTTCCACT
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TCCCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCAGGCCTTCCGCACCA
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ATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCAATCGCCGGTGAGC
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GTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGTAAGGCCGCGACTC
TAGA

pcDNA3.1+/PV IRES no uORF nLuc (SacI/XbaI)
GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGGCTCGAG**TTGGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCCGG**
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CAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG
TAAGGCCGCGACTCTAGA

pcDNA3.1+/PV IRES uORF nLuc (SacI/XbaI)_16 nt spacer
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CATGACATCGATTACAAGGATGACGATGACAAGTAAGGCCGCGACTCTAGA

pcDNA3.1+/PV IRES uORF nLuc (SacI/XbaI)_115 nt spacer
GAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCA
CTATAGGGCTCGAG**TTGGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCCGG**
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CGAACAAGCACTTCTGTTCCCCCGGTGAGGCTGTATAGGCTGTTTCCACGGCTAAAAGCG
GCTGATCCGTTATCCGCTCATGTACTTCGAGAAGCCTAGTATCACCTTGGAATCTTCGATG
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pcDNA3.1-D/EMCV IRES no uORF nLuc (SacI/XbaI)
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pcDNA3.1-D/EMCV IRES uORF nLuc (SacI/XbaI)_16 nt spacer
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pcDNA3.1-D/EMCV IRES uORF nLuc (SacI/XbaI)_115 nt spacer
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pcDNA3.1-D/HCV no uORF nLuc (SacI/XbaI)
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pcDNA3.1-D/HCV IRES uORF nLuc (SacI/XbaI)_16 nt spacer
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CTATAGGGAGACCCAAGCTGG**TTGGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGC**
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pcDNA3.1-D/HCV IRES uORF nLuc (SacI/XbaI)_115 nt spacer
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pcDNA3.1-D/CrPV IGR IRES no uORF nLuc (SacI/XbaI)
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pcDNA3.1-D/CrPV IGR IRES uORF nLuc (SacI/XbaI)_16 nt spacer

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pcDNA3.1-D/CrPV IGR IRES uORF nLuc (SacI/XbaI)_115 nt spacer

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pTet-Off AIO/no uORF nLuc (MluI/XbaI)

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pTet-Off AIO/no uORF nLuc + 3' UTR intron (MluI/PflMI)

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pTet-Off AIO/uORF nLuc (MluI/XbaI)

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pTet-Off AIO/large uORF nLuc (MluI/XbaI)

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pTet-Off AIO/HP + no uORF nLuc (MluI/XbaI)

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pTet-Off AIO/HP + no uORF nLuc + 3' UTR intron (MluI/PflMI)

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pTet-Off AIO/HP + uORF nLuc (MluI/XbaI)

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pTet-Off AIO/HP + large uORF nLuc (MluI/XbaI)

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pTet-Off AIO/no uORF nLuc + 3' UTR intron (MluI/PflMI)_stop mutated

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pTet-Off AIO/uORF nLuc (MluI/XbaI)_stop mutated

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pTet-Off AIO/large uORF nLuc (MluI/XbaI)_stop mutated

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