

**Supplementary table 1:** Plasmid sequences employed for **figure 1** (The importance of aligned spacing for initiation efficiency)

| Plasmid name | Note                           | D <sub>toStart</sub> | TIR (20 nt 5' UTR + 15 nt CDS)        |
|--------------|--------------------------------|----------------------|---------------------------------------|
| ME_in2       | distinct AUG<br>fLUC reference | 14                   | gtttataaggagggaacaatatggaagacgccaaa   |
| MK_in110     | AUG fLUC                       | 10                   | taaagttttataaggaggaaatggaagacgccaaa   |
| MK_in109     | AUG fLUC                       | 11                   | aaagttttataaggaggaaaatggaagacgccaaa   |
| ME_in5       | AUG fLUC                       | 12                   | aagttttataaggagggaacaatggaagacgccaaa  |
| ME_in4       | AUG fLUC                       | 13                   | agttttataaggagggaacaaaatggaagacgccaaa |
| MK_in43      | AUG fLUC                       | 14                   | gtttataaggagggaacaaaatggaagacgccaaa   |
| MK_in27      | AUG fLUC                       | 15                   | ttttataaggagggaacaataatggaagacgccaaa  |
| MK_in28      | AUG fLUC                       | 16                   | tttataaggagggaacaataaatggaagacgccaaa  |
| MK_in112     | GUG fLUC                       | 10                   | taaagttttataaggagggaagtggaagacgccaaa  |
| MK_in111     | GUG fLUC                       | 11                   | aaagttttataaggagggaagtggaagacgccaaa   |
| LM_05        | GUG fLUC                       | 12                   | aagttttataaggagggaacagtggaagacgccaaa  |
| LM_04        | GUG fLUC                       | 13                   | agttttataaggagggaacaagtggaagacgccaaa  |
| MK_in44      | GUG fLUC                       | 14                   | gtttataaggagggaacaaaagtggaagacgccaaa  |
| MK_in29      | GUG fLUC                       | 15                   | ttttataaggagggaacaataagtggaagacgccaaa |
| MK_in30      | GUG fLUC                       | 16                   | tttataaggagggaacaataagtggaagacgccaaa  |

**Supplementary table 2:** Plasmid sequences employed for **figure 2** (Open reading frame selection at AUGUG and GUGUG initiation sites)

| Plasmid name | Note       | D <sub>toStart</sub> | TIR (20 nt 5' UTR + 15 nt CDS)        |
|--------------|------------|----------------------|---------------------------------------|
| MK_in91      | AUGug fLUC | 10/12                | taaagttttataaggaggaaatgtgcgaagacgcc   |
| MK_in92      | AUGug fLUC | 11/13                | aaagttttataaggaggaacatgtgcgaagacgcc   |
| MK_in93      | AUGug fLUC | 12/14                | aagttttataaggaggaaacaatgtgcgaagacgcc  |
| MK_in94      | AUGug fLUC | 13/15                | agttttataaggaggaaacaaatgtgcgaagacgcc  |
| MK_in95      | AUGug fLUC | 14/16                | gttttataaggaggaaacaaaatgtgcgaagacgcc  |
| MK_in04      | auGUG fLUC | 10/12                | taaagttttataaggaggaaatgtggaagacgccca  |
| MK_in03      | auGUG fLUC | 11/13                | aaagttttataaggaggaacatgtggaagacgccca  |
| ME_in3       | auGUG fLUC | 12/14                | aagttttataaggaggaaacaatgtggaagacgccca |
| MK_in31      | auGUG fLUC | 13/15                | agttttataaggaggaaacaaatgtggaagacgccca |
| MK_in32      | auGUG fLUC | 14/16                | gttttataaggaggaaacaaaatgtggaagacgccca |
| MK_in167     | GUGug fLUC | 10/12                | taaagttttataaggaggaaatgtgcgaagacgcc   |
| MK_in168     | GUGug fLUC | 11/13                | aaagttttataaggaggaacgtgtgcgaagacgcc   |
| MK_in156     | GUGug fLUC | 12/14                | aagttttataaggaggaaacagtgtgcgaagacgcc  |
| MK_in157     | GUGug fLUC | 13/15                | agttttataaggaggaaacaagtgtgcgaagacgcc  |
| MK_in158     | GUGug fLUC | 14/16                | gttttataaggaggaaacaaagtgtgcgaagacgcc  |
| MK_in165     | guGUG fLUC | 10/12                | aagttttataaggaggaaagtgtggaagacgcccaaa |
| MK_in166     | guGUG fLUC | 11/13                | agttttataaggaggaaacgtgtggaagacgcccaaa |
| MK_in159     | guGUG fLUC | 12/14                | gttttataaggaggaaacagtgtggaagacgcccaaa |
| MK_in160     | guGUG fLUC | 13/15                | ttttataaggaggaaacaagtgtggaagacgcccaaa |
| MK_in161     | guGUG fLUC | 14/16                | tttataaggaggaaacaaagtgtggaagacgcccaaa |
| pRXG_MK16Y   | AUGug GFP  | 10/12                | taaagttttataaggaggaaatgtgcatccaag     |
| pRXG_MK16    | AUGug GFP  | 11/13                | aaagttttataaggaggaaaaatgtgcatccaag    |
| pRXG_MK4     | AUGug GFP  | 12/14                | aagttttataaggaggaaacaatgtgcatccaag    |
| pRXG_MK22    | AUGug GFP  | 13/15                | ttttataaggaggaaacaaatgtgcatccaaggg    |
| pRXG_MK17Y   | auGUG GFP  | 10/12                | aagttttataaggaggaaatgtgcatccaagggc    |

|           |           |       |                                      |
|-----------|-----------|-------|--------------------------------------|
| pRXG_MK17 | auGUG GFP | 11/13 | agttttataaggaggaaaatgtggcatccaagggc  |
| pRXG_MK3  | auGUG GFP | 12/14 | gttttataaggaggaacaatgtggcatccaagggc  |
| pRXG_MK23 | auGUG GFP | 13/15 | ttttataaggaggaacaaatgtggcatccaagggc  |
| pRXG_MK25 | GUGug GFP | 11/13 | aaagttttataaggaggaaaagtgtgcatccaag   |
| pRXG_MK19 | GUGug GFP | 12/14 | aagttttataaggaggaacagtgtgcatccaag    |
| pRXG_MK21 | GUGug GFP | 13/15 | agttttataaggaggaacaagtgtgcatccaag    |
| pRXG_MK27 | GUGug GFP | 14/16 | gttttataaggaggaacaaagtgtgcatccaag    |
| pRXG_MK24 | guGUG GFP | 11/13 | agttttataaggaggaaaagtgtggcatccaagggc |
| pRXG_MK12 | guGUG GFP | 12/14 | gttttataaggaggaacagtgtggcatccaagggc  |
| pRXG_MK20 | guGUG GFP | 13/15 | ttttataaggaggaacaagtgtggcatccaagggc  |
| pRXG_MK6  | guGUG GFP | 14/16 | tttataaggaggaacaaagtgtggcatccaagggc  |

**Supplementary table 3:** Plasmid sequences employed for **figure 3** (Detection of distinct peptide products by translation of two ORFs from an AUGUG ambiguous start site)

| Plasmid name | Note                        | Dt <sub>oStart</sub> | TIR                                   |
|--------------|-----------------------------|----------------------|---------------------------------------|
| MK_in136     | 3.8 kDa AUG ORF             | 12                   | aagttttataaggaggaacaatgaagaagacgcca   |
| MK_in137     | 6.3 kDa GUG ORF             | 14                   | aagttttataaggaggaacaaagtgaagaagacgcca |
| MK_in126     | 3.8 & 6.3 kDa<br>AUGUG ORFs | 12/14                | aagttttataaggaggaacaatgtgaagaagacgcca |

**Supplementary table 4:** Plasmid sequences employed for **figure 5** (Luciferase reporter assays of native initiation sequences harboring potentially ambiguous start sites)

| Plasmid name | Note                         | D <sub>toStart</sub> | Native TIR - fLUC fusion                      |
|--------------|------------------------------|----------------------|---|
| MK_in117     | <i>hdhA</i> auGUG (wt) fLUC  | 11/13                | gaagttcacaggaggtttatgtgttaattctgacgaagac      |
| MK_in119     | <i>hdhA</i> AUGug fLUC       | 11/13                | gaagttcacaggaggtttatgtgtttaattctgacgaagac     |
| MK_in118     | <i>hdhA</i> GUG control fLUC | 13                   | gaagttcacaggaggtttacgtgttaattctgacgaagac      |
| MK_in176     | <i>secF</i> auGUG (wt) fLUC  | 11/13                | ctgtcaatctgaggagtgcatgtggcacaggaatatgaagac    |
| MK_in177     | <i>secF</i> AUGug fLUC       | 11/13                | ctgtcaatctgaggagtgcatgtggcacaggaatgaagac      |
| MK_in178     | <i>secF</i> GUG control fLUC | 13                   | ctgtcaatctgaggagtgcaagtggcacaggaatatgaagac    |
| MK_in107     | <i>pqiA</i> AUGug (wt) fLUC  | 12/14                | cgcacattaaggagttaccaatgtgcaacatcatgaagac      |
| MK_in108     | <i>pqiA</i> auGUG fLUC       | 12/14                | cgcacattaaggagttaccaatgtgcaacatcatca gaagac   |
| MK_in130     | <i>pqiA</i> AUG control fLUC | 12                   | cgcacattaaggagttaccaatgagcgaacatcatgaagac     |
| MK_in179     | <i>tamA</i> auGUG (wt) fLUC  | 19/21;<br>10/12      | aaaaaggatattcaggagaaaaatgtgcgctatatccgaagac   |
| MK_in180     | <i>tamA</i> AUGug fLUC       | 19/21;<br>10/12      | aaaaaggatattcaggagaaaaatgtgcgctatatccgaagac   |
| MK_in181     | <i>tamA</i> GUG control fLUC | 21; 12               | aaaaaggatattcaggagaaaaatgtgcgctatatccgaagac   |
| MK_in185     | <i>asnB</i> AUGug (wt) fLUC  | 13/15                | accaggtaacggagaaggttatgtgttcaatgttgaagac      |
| MK_in186     | <i>asnB</i> auGUG fLUC       | 13/15                | accaggtaacggagaaggttatgtgttcaatgttgaagac      |
| MK_in187     | <i>asnB</i> AUG control fLUC | 13                   | accaggtaacggagaaggttatgagttcaatgttgaagac      |
| MK_in182     | <i>nagZ</i> guGUG (wt) fLUC  | 11/13                | aataaaacaataaggagagcagtggtggtccagtaatgaagac   |
| MK_in183     | <i>nagZ</i> GUGug fLUC       | 11/13                | aataaaacaataaggagagcagtggtggtccagtaacgaagac   |
| MK_in184     | <i>nagZ</i> GUG control fLUC | 13                   | aataaaacaataaggagagcaaaatggtggtccagtaatgaagac |

**Supplementary table 5:** Plasmid sequences employed for **figure 6** (Mutational study of the tamA RBS to analyze the contribution of the two SD motifs to start codon selection)

| Plasmid name | Note                       | D <sub>toStart</sub> | Native TIR - fLUC fusion                    |
|--------------|----------------------------|----------------------|---|
| MK_in179     | tamA (SD1&2)<br>auGUG fLUC | 19/21;<br>10/12      | aaaaaggatattcaggagaaaatgtgcgctatatccgaagac  |
| MK_in180     | tamA (SD1&2)<br>AUGug fLUC | 19/21;<br>10/12      | aaaaaggatattcaggagaaaatgtgcgctatatccgaagac  |
| MK_in210     | tamA (SD1)<br>auGUG fLUC   | 19/21                | aaaaaggatattcaaaaaaaaaatgtgcgctatatccgaagac |
| MK_in213     | tamA (SD1)<br>AUGug fLUC   | 19/21                | aaaaaggatattcaaaaaaaaaatgtgcgctatatccgaagac |
| MK_in209     | tamA (SD2)<br>auGUG fLUC   | 10/12                | aaaaaaaaatattcaggagaaaatgtgcgctatatccgaagac |
| MK_in212     | tamA (SD2)<br>AUGug fLUC   | 10/12                | aaaaaaaaatattcaggagaaaatgtgcgctatatccgaagac |

**Supplementary table 6:** Plasmid sequences employed for **figure 7** (Impact of A4 on start codon selection at AUGUG and GUGUG ambiguous sites)

| Plasmid name | Note                           | D <sub>toStart</sub> | TIR (20 nt 5' UTR + 15 nt CDS)      |
|--------------|--------------------------------|----------------------|-------------------------------------|
| ME_in2       | distinct AUG fLUC<br>reference | 14                   | gtttataaaggaggaacaatatggaagacgcaaaa |
| ME_in3       | auGUG fLUC                     | 12/14                | gtttataaaggaggaacaatgtggaagacgcaaaa |
| MK_in06      | auGUGA fLUC                    | 12/14                | gtttataaaggaggaacaatgtgaaagacgcaaaa |
| MK_in32      | auGUG fLUC                     | 14/16                | ttataaaggaggaacaaaatgtggaagacgcaaaa |
| MK_in34      | auGUGA fLUC                    | 14/16                | ttataaaggaggaacaaaatgtgaaagacgcaaaa |
| MK_in159     | guGUG fLUC                     | 12/14                | gtttataaaggaggaacagtgtggaagacgcaaaa |
| MK_in162     | guGUGA fLUC                    | 12/14                | gtttataaaggaggaacagtgtgaaagacgcaaaa |
| MK_in161     | guGUG fLUC                     | 14/16                | ttataaaggaggaacaaagtgtggaagacgcaaaa |
| MK_in164     | guGUGA fLUC                    | 14/16                | ttataaaggaggaacaaagtgtgaaagacgcaaaa |
| pRXG_MK1     | distinct AUG GFP<br>reference  | 14                   | gtttataaaggaggaacaatatggcatccaagggc |
| pRXG_MK3     | auGUG GFP                      | 12/14                | gtttataaaggaggaacaatgtggcatccaagggc |
| pRXG_MK5     | auGUGA GFP                     | 12/14                | gtttataaaggaggaacaatgtgacatccaagggc |
| pRXG_MK6     | auGUG GFP                      | 14/16                | ttataaaggaggaacaaaatgtggcatccaagggc |
| pRXG_MK7     | auGUGA GFP                     | 14/16                | ttataaaggaggaacaaaatgtgacatccaagggc |
| pRXG_MK12    | guGUG GFP                      | 12/14                | gtttataaaggaggaacagtgtggcatccaagggc |
| pRXG_MK14    | guGUGA GFP                     | 12/14                | gtttataaaggaggaacagtgtgacatccaagggc |
| pRXG_MK13    | guGUG GFP                      | 14/16                | ttataaaggaggaacaaagtgtggcatccaagggc |
| pRXG_MK15    | guGUGA GFP                     | 14/16                | ttataaaggaggaacaaagtgtgacatccaagggc |

**Supplementary table 7:** Plasmid sequences employed for **figure 9** (RF2 dependent activity of luciferase constructs harboring AUGUGA or GUGUGA ambiguous initiation sites)

| Plasmid name | Note                             | D <sub>toStart</sub> | RBS and leading CDS with alternative frame termination site    |
|--------------|----------------------------------|----------------------|--|
| MK_in06      | auGUGA fLUC                      | 12/14                | gtttataaaggaggaacaatgTG <b>Aaagacgccaaa</b>                    |
| MK_in34      | auGUGA fLUC                      | 14/16                | tttataaaggaggaacaaaatgTG <b>Aaagacgccaaa</b>                   |
| MK_in162     | guGUGA fLUC                      | 12/14                | gtttataaaggaggaacagtgTG <b>Aaagacgccaaa</b>                    |
| MK_in164     | guGUGA fLUC                      | 14/16                | tttataaaggaggaacaaagtgTG <b>Aaagacgccaaa</b>                   |
| MK_in34      | auGUGA (2nd codon stop) fLUC     | 14/16                | <b>taaggagga</b> aacaaaatgTG <b>Aaagacgccccaaaacataaagaaa</b>  |
| MK_in68      | auGUG (4th codon stop CUGA) fLUC | 14/16                | <b>taaggagga</b> aacaAAatgtg <b>gaacTGA</b> ccccaaaacataaagaaa |
| MK_in97      | auGUG (8th codon stop CUGA) fLUC | 14/16                | <b>taaggagga</b> aacaaaatgtg <b>gaagacgccccaaaaccTGA</b> agaaa |
| MK_in164     | guGUGA (2nd codon stop) fLUC     | 14/16                | <b>taaggagga</b> aacaaagtgTG <b>Aaagacgccccaaaacataaagaaa</b>  |
| MK_in171     | guGUG (4th codon stop CUGA) fLUC | 14/16                | <b>taaggagga</b> aacaaagtgtg <b>gaacTGA</b> ccccaaaacataaagaaa |
| MK_in172     | guGUG (8th codon stop CUGA) fLUC | 14/16                | <b>taaggagga</b> aacaaagtgtg <b>gaagacgccccaaaaccTGA</b> agaaa |

**Supplementary table 8:** Plasmid sequences employed for **figure 10** (Impact of A4 on narQ expression)

| Plasmid name | Note                          | D <sub>toStart</sub> | <i>narQ</i> native TIR - fLUC fusion                   |
|--------------|-------------------------------|----------------------|--|
| MK_in202     | <i>narQ</i> +A4 (wt) fLUC     | 14/16                | atTTTTgt <b>ggaga</b> aagacgcgtgtg <b>aaagacgccaaa</b> |
| MK_in203     | <i>narQ</i> -A4 fLUC          | 14/16                | atTTTTgt <b>ggaga</b> aagacgcgtgtg <b>gaagacgccaaa</b> |
| MK_in204     | <i>narQ</i> alt frame fLUC    | 14/16                | atTTTTgt <b>ggaga</b> aagacgcgtgtg <b>caagacgccaaa</b> |
| MK_in205     | <i>narQ</i> distinct GUG fLUC | 14/16                | atTTTTgt <b>ggaga</b> aagacgcaagtg <b>aaagacgccaaa</b> |