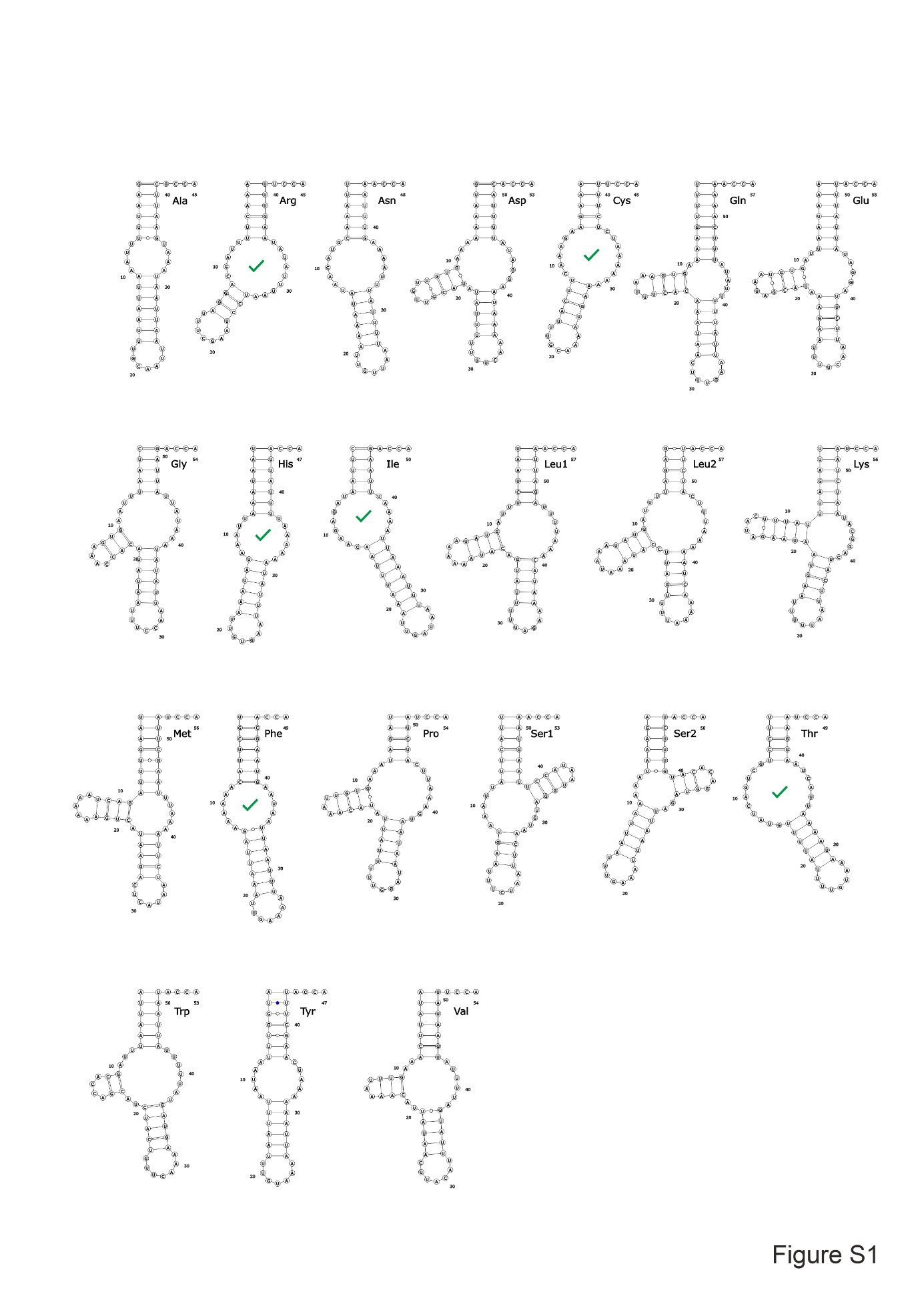
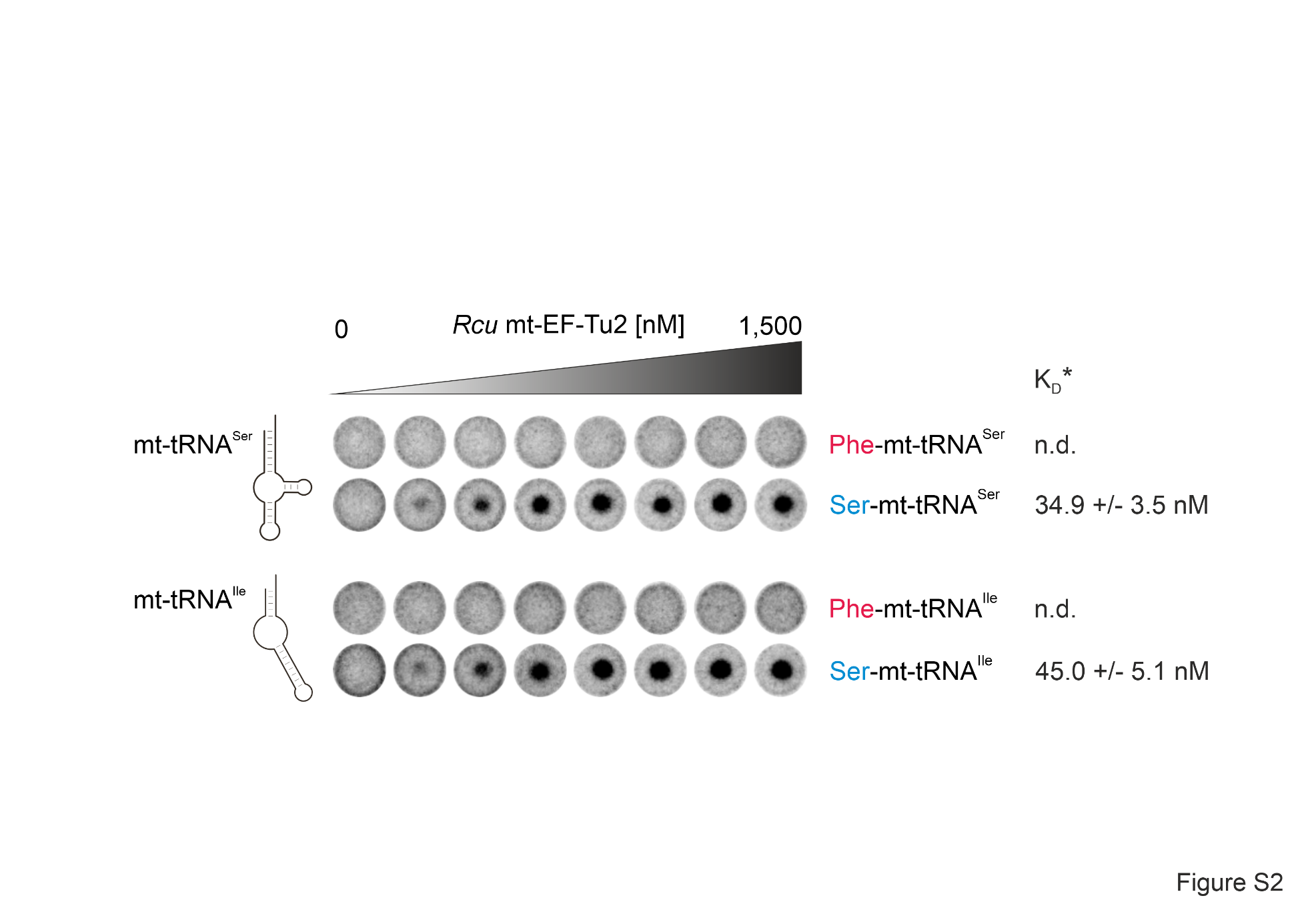
**Supporting Information**



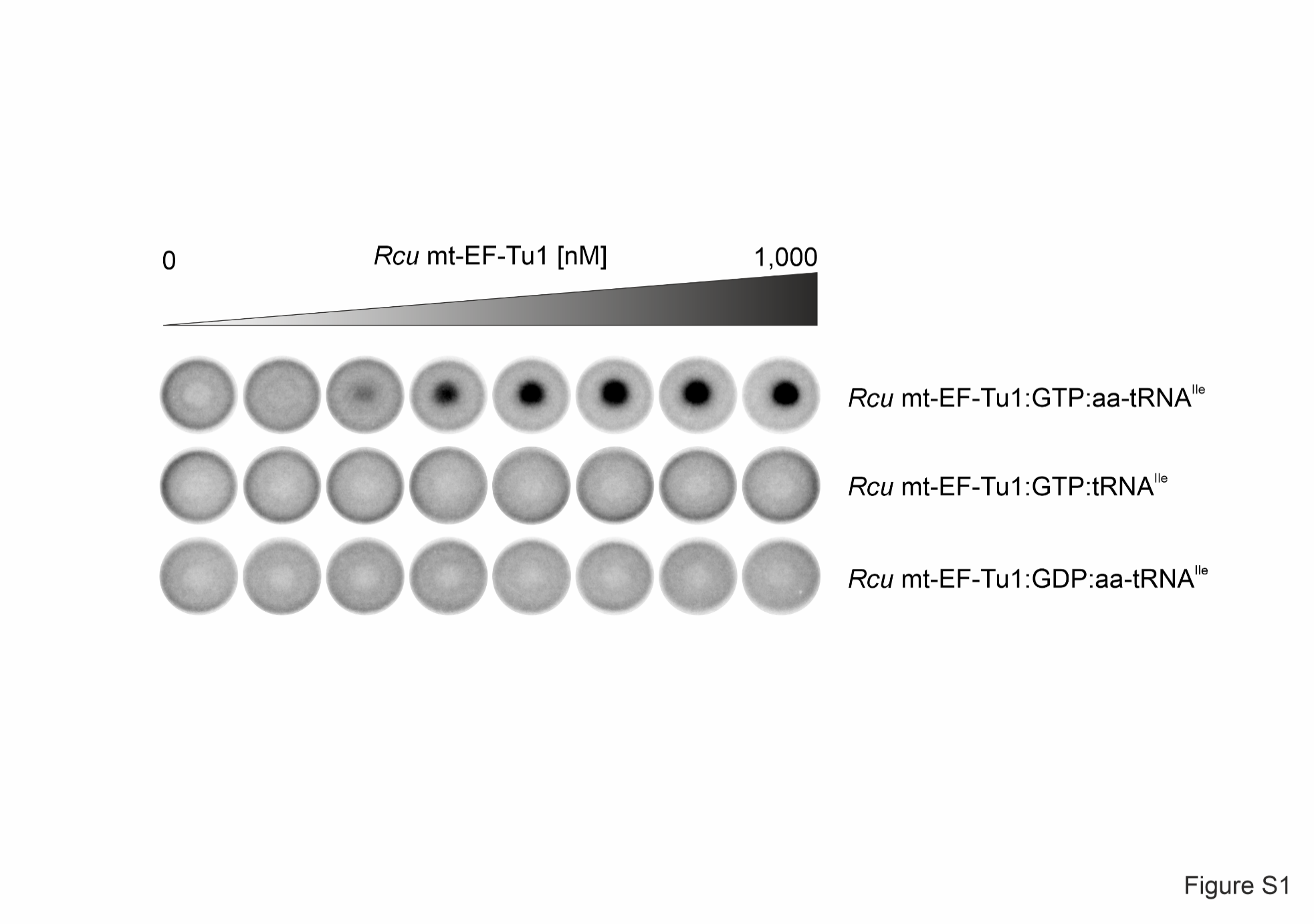
**Figure S1. Predicted mitochondrial tRNAs from *R. culicivorax*.**

The presented tRNA secondary structures are derived from Jühling et al: (1). Structure prediction was carried out using the Vienna RNA package (2) and fine-tuned by manual adjustments. Visualization was done using VARNA software (3). Green check marks indicate structures verified by sequence analysis of *in vivo* mt tRNAs and in-line probing of *in vitro* transcripts (4, 5).



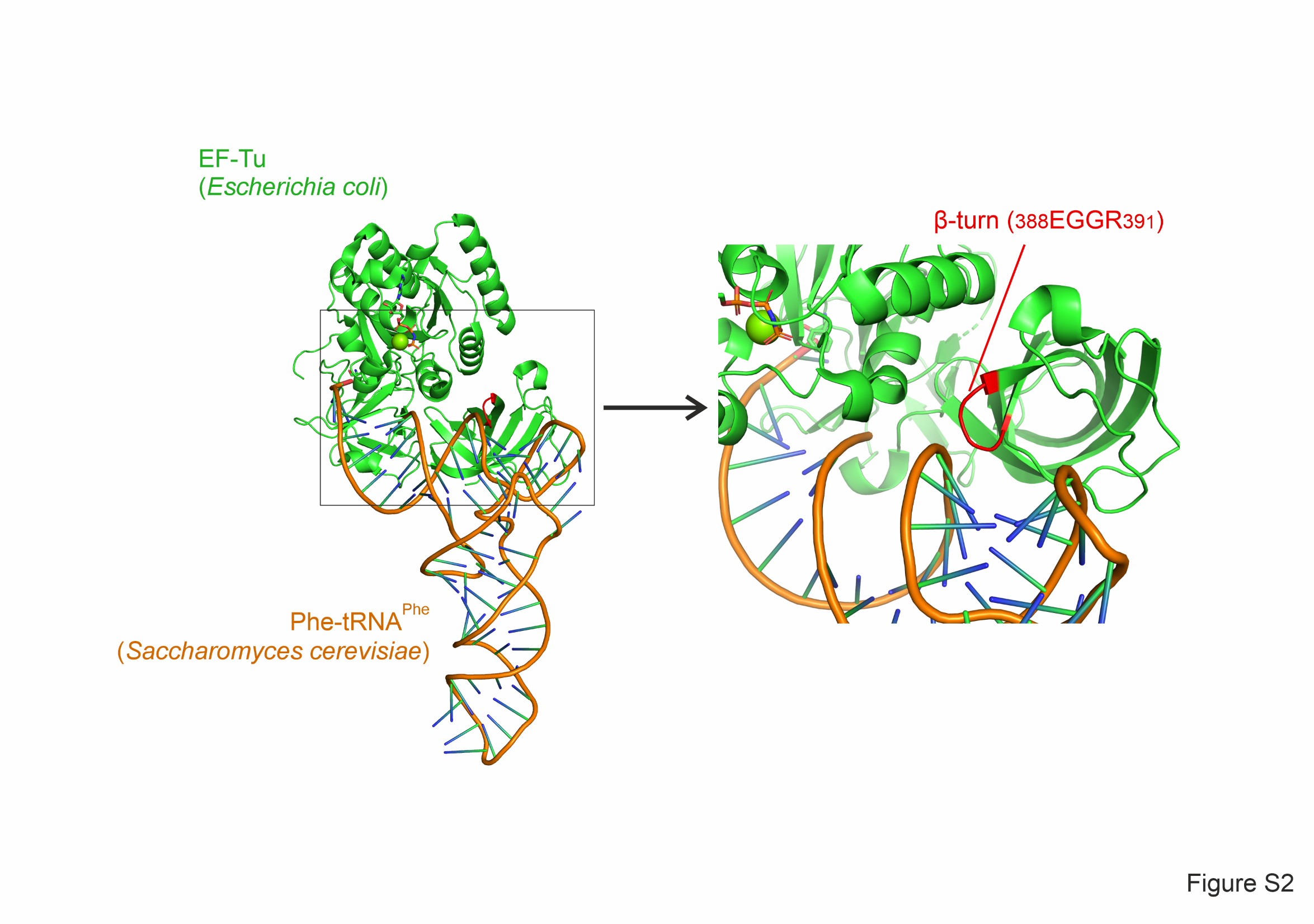
**Figure S2. Mitochondrial EF‑Tu2 from *R. culicivorax* is serine-specific.**

As its counterparts in *C. elegans* and *T. britovi*, this protein specifically recognizes the serine moiety (blue) (6, 7), regardless of whether the tRNA body is represented by the D-arm-lacking tRNASer (upper panel) or the completely armless tRNAIle (lower panel) In contrast, the tRNAs charged with phenylalanine (red) are not recognized by *Rcu* mt‑EF‑Tu2. KD values represent apparent values (KD\*), as the active fraction of mt‑EF‑Tu2 was not determined.



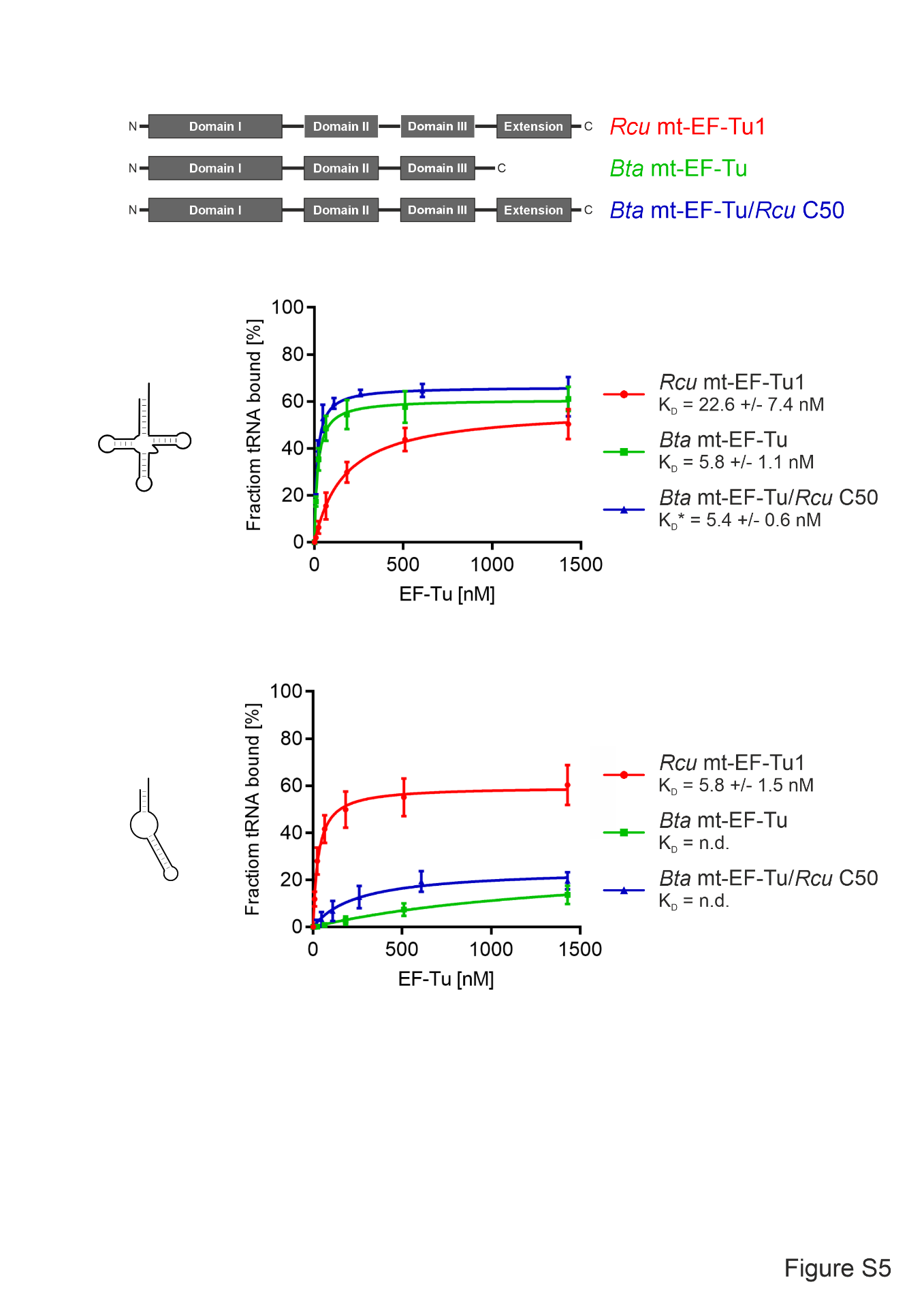
**Figure S3. A ternary complex is only formed with mt-EF-Tu1, GTP, and aminoacylated tRNA.**

As an example, the interaction of *Rcu* mt‑EF‑Tu1 with GTP and aminoacylated tRNAIle is shown in a DRaCALA experiment (upper row). Middle row: If the tRNA is not aminoacylated, no ternary complex is formed. Lower row: If GDP instead of GTP is offered, no ternary complex is formed either. These data indicate that mt‑EF‑Tu1 selectively interacts with aa-tRNA and GTP.

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**Figure S4. Crystal structure of *Eco* EF‑Tu complexed with the GTP analog GDPNP and Phe-tRNAPhe (pdb data base entry 1OB2).**

For reasons of clarity, the antibiotic kirromycin that is also bound in this complex is not shown. A beta-turn element (red) of EF‑Tu (green) is in close contact to the T‑arm of the bound tRNA (orange), representing a possible site of interaction with armless tRNAs in *Rcu* mt‑EF‑Tu1.

**Figure S5. Binding behavior of *Bta* mt‑EF‑Tu carrying the C-terminal extension of *Rcu* mt‑EF‑Tu1 (*Bta* mt‑EF‑Tu/*Rcu* C50).**

**Upper diagram:** Like the wild type proteins *Bta* mt‑EF‑Tu and *Rcu* mt‑EF‑Tu1, the chimera efficiently recognizes a canonical tRNA substrate.

**Lower diagram:** In contrast, a noncanonical armless tRNA is only recognized by wt *Rcu* mt‑EF‑Tu1, but neither by wt *Bta* mt‑EF‑Tu nor by the chimeric bovine protein carrying the C-terminal extension of Rcu mt‑EF‑Tu1.

Error bars represent standard deviation (SD).

The asterisk indicates an apparent KD value, where the active fraction of the wt protein was used in the calculation.

**Table S1. Active fractions of EF-Tu preparations**

Active fractions were determined in 2-3 independent experiments and showed highly consistent values in the same range as published for other EF-Tu proteins (8–10).

|  |  |
| --- | --- |
| **EF-Tu version** | **active fraction (%)** |
| ***Rcu* mt-EF-Tu1** |  |
| wt | 31 |
| ΔC50 | 29 |
| K380D | 23 |
| K380R | 53 |
| K380A | 30 |
| K380E | 25 |
| ***Bta* mt-EF-Tu** |  |
| wt | 33 |
| D384A | 33 |
| ***Eco* EF-Tu** |  |
| wt | 27 |
| ***Tbr* mt-EF-Tu1** |  |
| wt | 3 |
| S381K | 2 |
| S381D | 3 |
| ***Cel* mt-EF-Tu1** |  |
| wt | 8 |
| K386D | 9 |
|  |  |

**Table S2. KD values of EF-Tu versions.**

Asterisks indicate apparent KD values, where the corresponding wt active fraction was used

for calculation.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **EF-Tu version** | **aa-tRNA** | | | |
| ***Rcu* mt‑EF‑Tu1** | **canonical**  **(tRNAPhe)** | **armless**  **(mt-tRNAIle)** | **T-armless**  **(mt-tRNALys)** | **D-armless**  **(mt-tRNASer)** |
| wt | 22.6 | 5.8 | 5.2 | 66.4 |
| ∆C3 | 23.6\* | 8.8 | - |  |
| ∆C6 | 16.2\* | 15.2\* | - |  |
| ∆C9 | 27.2\* | 26.1\* | - |  |
| ∆C15 | 22.3\* | 40.7\* | - |  |
| ∆C18 | 20.8\* | 50.4\* | - |  |
| ∆C41 | 31.7\* | 84.2\* | - |  |
| ∆C50 | 26.1 | 82.1 | - |  |
| chimera region A | - | 17.1\* | - |  |
| chimera region B | - | 18.6\* | - |  |
| chimera region C | - | 4.8\* | - |  |
| chimera region D | - | 14.6\* | - |  |
| chimera region E | - | 10.3\* | - |  |
| chimera region F | - | 221.7\* | - |  |
| K380D | 197.4 | 70.4 | - |  |
| K380A | 58.4 | 33.4 | - |  |
| K380E | 150.6 | 149.5 | - |  |
| K380R | 60.5 | 16.5 | - |  |
| D381G | - | 4.9\* | - |  |
| G382N | - | 7.8\* | - |  |
| K383R | - | 11.3\* | - |  |
| ∆C50/K380D | - | 349.9\* | - |  |
| ***Cel* mt‑EF‑Tu1** |  |  |  |  |
| Wt | - | 17.1 | - |  |
| K386D | - | 62.6 | - |  |
| ***Tbr* mt‑EF‑T1** |  |  |  |  |
| Wt | - | 4.4 | - |  |
| S381K | - | 3.0 | - |  |
| S381D | - | 7.1 | - |  |
| ***Bta* mt‑EF-Tu** |  |  |  |  |
| Wt | 5.8 | n.d. | - |  |
| D384K | 76.6\* | 96.8\* | - |  |
| D384A | 11.5 | 60.1 | - |  |

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