Supplementary Information for:

Ribosome biogenesis *in vitro*

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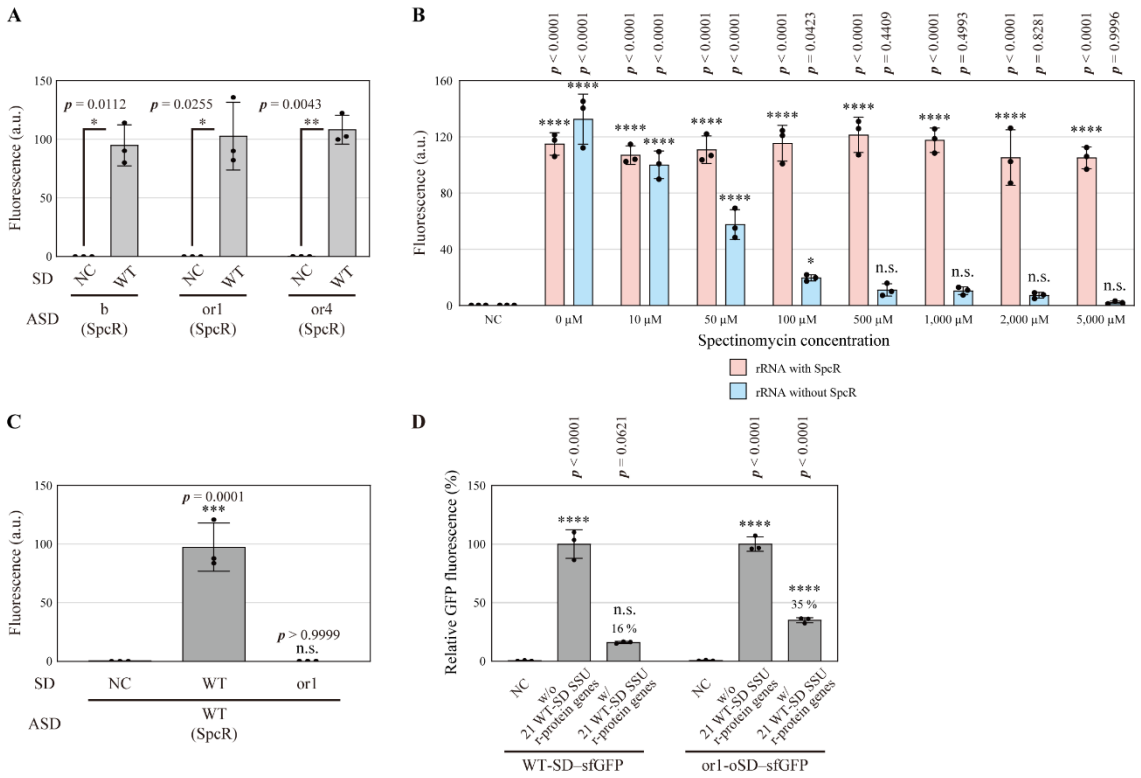
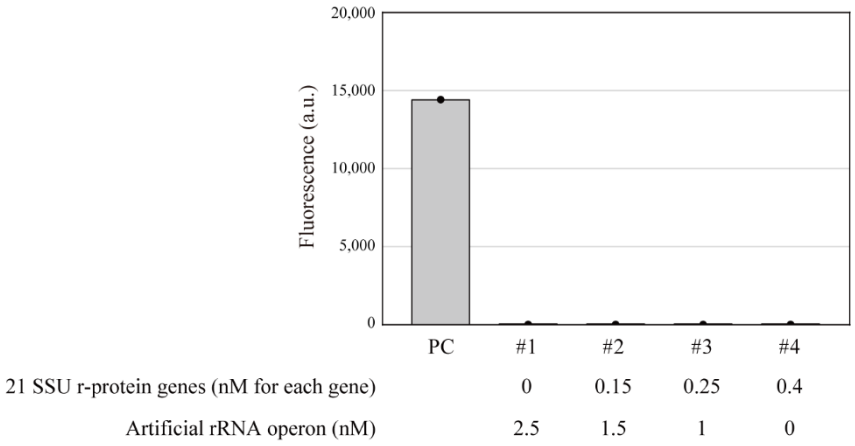


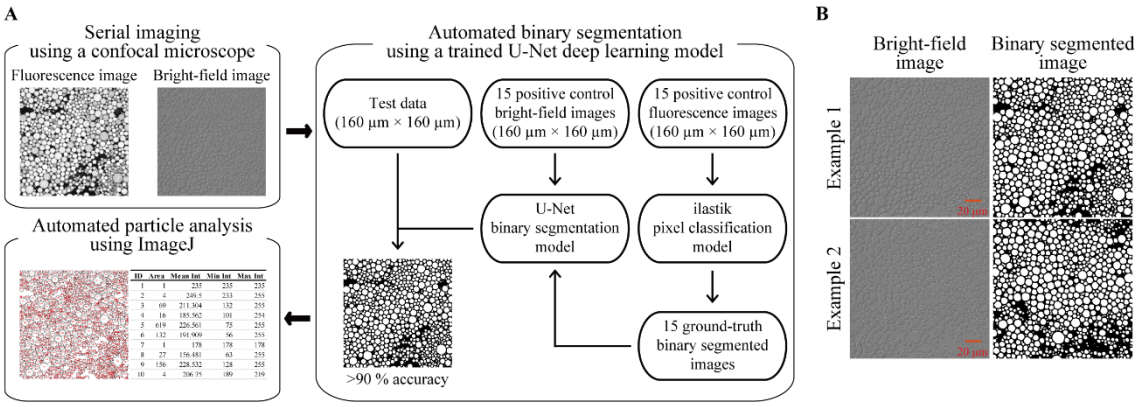
Supplementary Fig. 1. Supporting experiments for screening oSD·oASD pairs with two-sided orthogonality.

(A) Functional cell extract preparation. S12 cell extracts were prepared using *E. coli* expressing an artificial rRNA operon with oASD (b, or1, or or4) and C1192U spectinomycin resistance (SpcR). The cell extracts were mixed with the WT-SD–LacZ reporter. The experiment was conducted without spectinomycin to assess the functionality of the cell extracts. a.u., arbitrary unit; NC, negative control without the reporter. Mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; two-tailed Welch's *t*-test. (B) Optimizing spectinomycin concentrations. S12 cell extracts were prepared using BL21 Star™ (DE3) *lacZ::frt* expressing an rRNA operon with WT-ASD and with or without SpcR. The cell extracts were mixed with the WT-SD–LacZ reporter at various spectinomycin concentrations. We used spectinomycin at 5 mM in the following experiments if not specified. Mean ± SD ($n = 3$). ****, $p < 0.0001$; n.s., not significant; one-way ANOVA with Dunnett's test against NC. (C) A follow-up control experiment to verify two-sided orthogonality of the or1-oSD·oASD pair. A cell extract was prepared using *E. coli* expressing an artificial rRNA operon with WT-ASD and SpcR. The extract was mixed with the WT-SD–LacZ or the or1-oSD–LacZ reporter in the presence of spectinomycin. Mean ± SD ($n = 3$). ***, $p < 0.001$; one-way ANOVA with Dunnett's test

against NC. (D) The translational activities of native and artificial ribosomes in the presence of mRNAs with WT-SD. S12 cell extracts were prepared using *E. coli* expressing an artificial rRNA operon with or1-oASD and SpcR. The cell extracts were mixed with a WT-SD–sfGFP reporter or an or1-oSD–sfGFP reporter with or without 21 SSU r-protein genes with WT-SD (0.15 nM each). The artificial ribosomes showed decreased, but significant residual translational activity in the presence of 21 SSU r-protein genes. Mean ± SD ($n = 3$). One-way ANOVA with Dunnett's test against NC. Three biological replicates were used in all experiments. Source data are provided as a Source Data file.

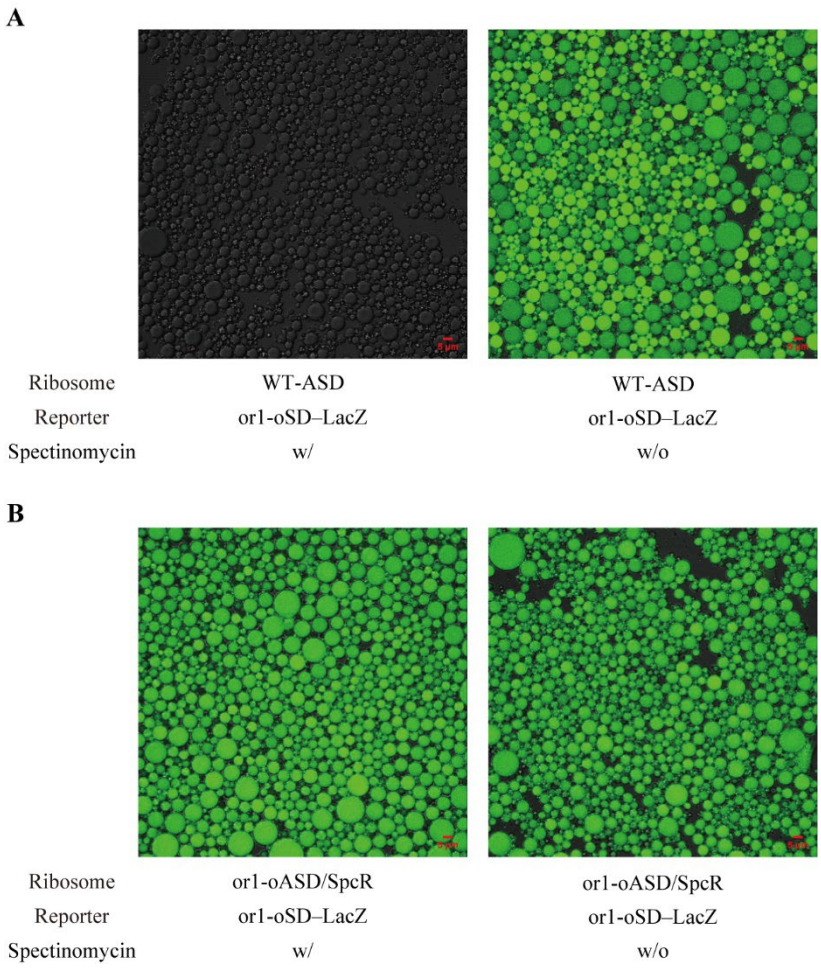


Supplementary Fig. 2. An initial trial to realize SSU biogenesis *in vitro*.
Experimental scheme for SSU biogenesis *in vitr*o is shown in Fig. 3A. Briefly, the first reaction is designed for coactivating the transcription of the artificial rRNA operon with or1-oASD and SpcR, the transcription and translation of 21 SSU r-protein genes, and the assembly in an optimized S150 cell extract. The second reaction aimed at detecting the translational activity of nascent artificial SSU using the or1-oSD–LacZ reporter. Exploring several reaction conditions did not generate any fluorescence signals derived from nascent SSU. In the first reaction, the concentrations of the native ribosomes, the artificial rRNA operon, and 21 SSU r-protein genes were 20, 0–2.5, and 0–0.4 nM each, respectively. The second reaction was conducted in the presence of the or1-oSD–LacZ reporter and spectinomycin. a.u., arbitrary unit; PC, positive control using native ribosomes and the WT-SD–LacZ reporter. Source data are provided as a Source Data file.



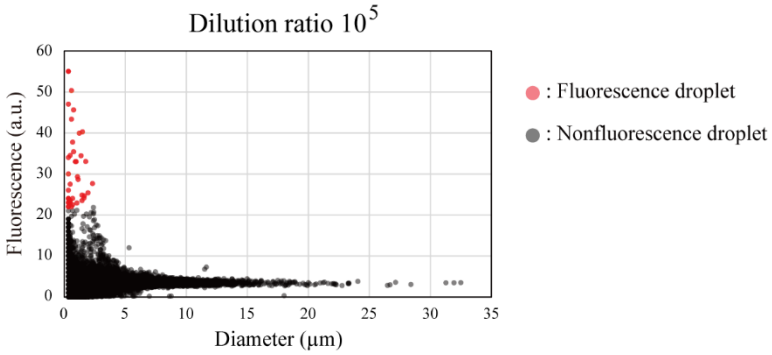
Supplementary Fig. 3. Development of a deep-learning-assisted automated femtoliter droplet assay.

(A) Scheme of the droplet assay. After cell-free transcription and translation in femtoliter droplets, bright-field and fluorescence images of the droplets were obtained using a confocal fluorescence microscope. The bright-field images were binary segmented (droplet or background) using a trained U-Net deep-learning model and used for particle analysis with ImageJ. The results are redirected to corresponding fluorescence images. Finally, the features of each droplet, including the area and fluorescence (arbitrary unit), were obtained. (B) Bright-field images of the droplets (left panels) were processed into binary segmented images (right panels; droplet or background) with >90 % accuracy (number of correctly classified pixels/total number of pixels) using the trained U-Net deep-learning model. In the right panels, white and black regions indicate the droplets and background, respectively. Scale bars = 20 μm.



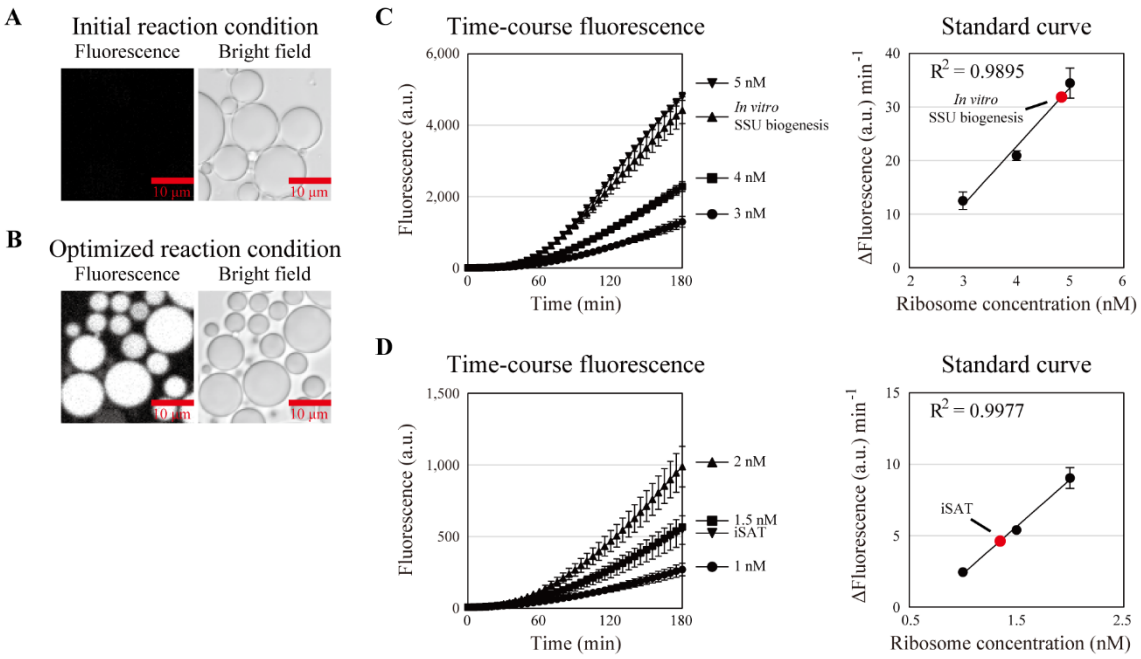
Supplementary Fig. 4. Combining the orthogonal reporter and spectinomycin to specifically detect the artificial ribosome translational activity in the droplet assay. (A) Representative micrographs of droplets after the droplet assay using control S12 cell extracts containing only native ribosomes. The droplets emitted fluorescence without spectinomycin as the droplet assay is so sensitive that a very weak interaction between the native ribosomes and the or1-oSD–LacZ reporter could be detected. The combined use of the orthogonal reporter with 100 μM spectinomycin, which produced background signals when used alone (right panel and Fig. S1B), could eliminate them, even in the droplet assay (left panel). Scale bars = 5 μm. (B) Representative micrographs of droplets after the droplet assay using S12 cell extracts containing artificial ribosomes with or1-oASD and C1192U spectinomycin resistance (SpcR). The droplets emitted fluorescence with or without 100 μM spectinomycin. Signals derived from the translation of the or1-oSD–LacZ reporter by the native ribosomes and the artificial ribosomes with or1-oASD showed no major differences (Supplementary Fig. 4A right panel and Supplementary Fig.

4B) owing to signal saturation. We used spectinomycin at 100 μM in the following experiments if not specified. Representative images from three biological replicates



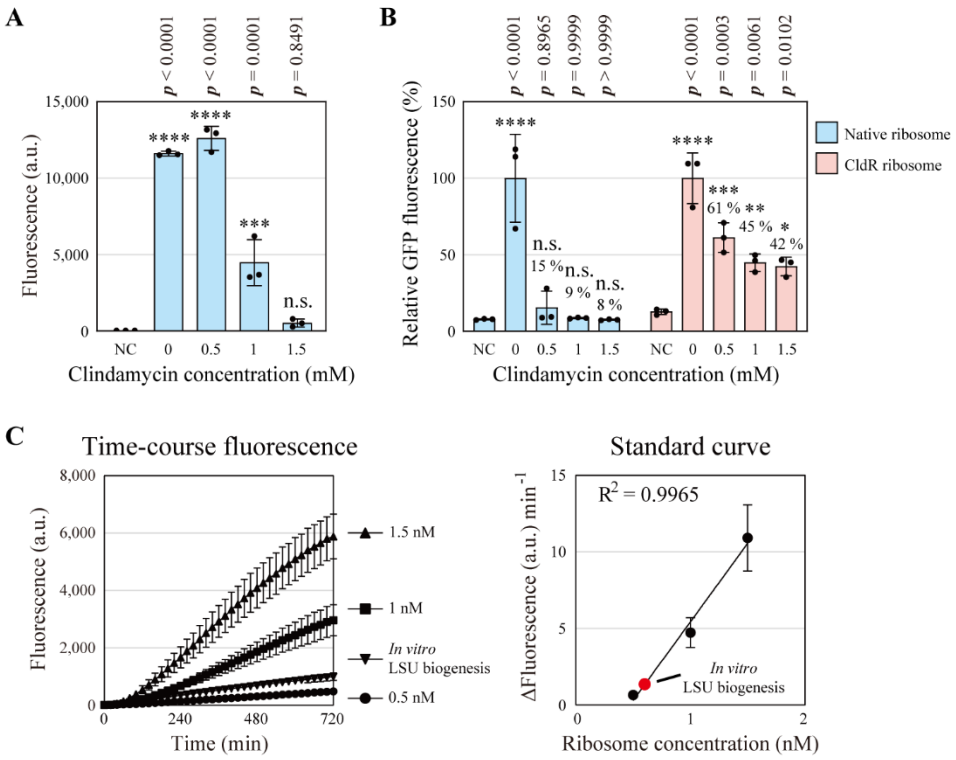
Supplementary Fig. 5. Single-ribosome-level detection of the artificial ribosome translational activity.

Scatter plot of the mean fluorescence intensity against the diameter of each droplet was generated using the dataset of Fig. 2B. Droplets exceeding the threshold (mean fluorescence intensity ≥ 22) are shown in red. The threshold was set at a value that none of the droplets in the negative control exceeded, as presented in Fig. 2B. From the Poisson distribution formula, most of the fluorescent droplets (99 %) were estimated to contain only one artificial ribosome (refer to Sensitivity calculation of the droplet assay in Materials and Methods). Source data are provided as a Source Data file.



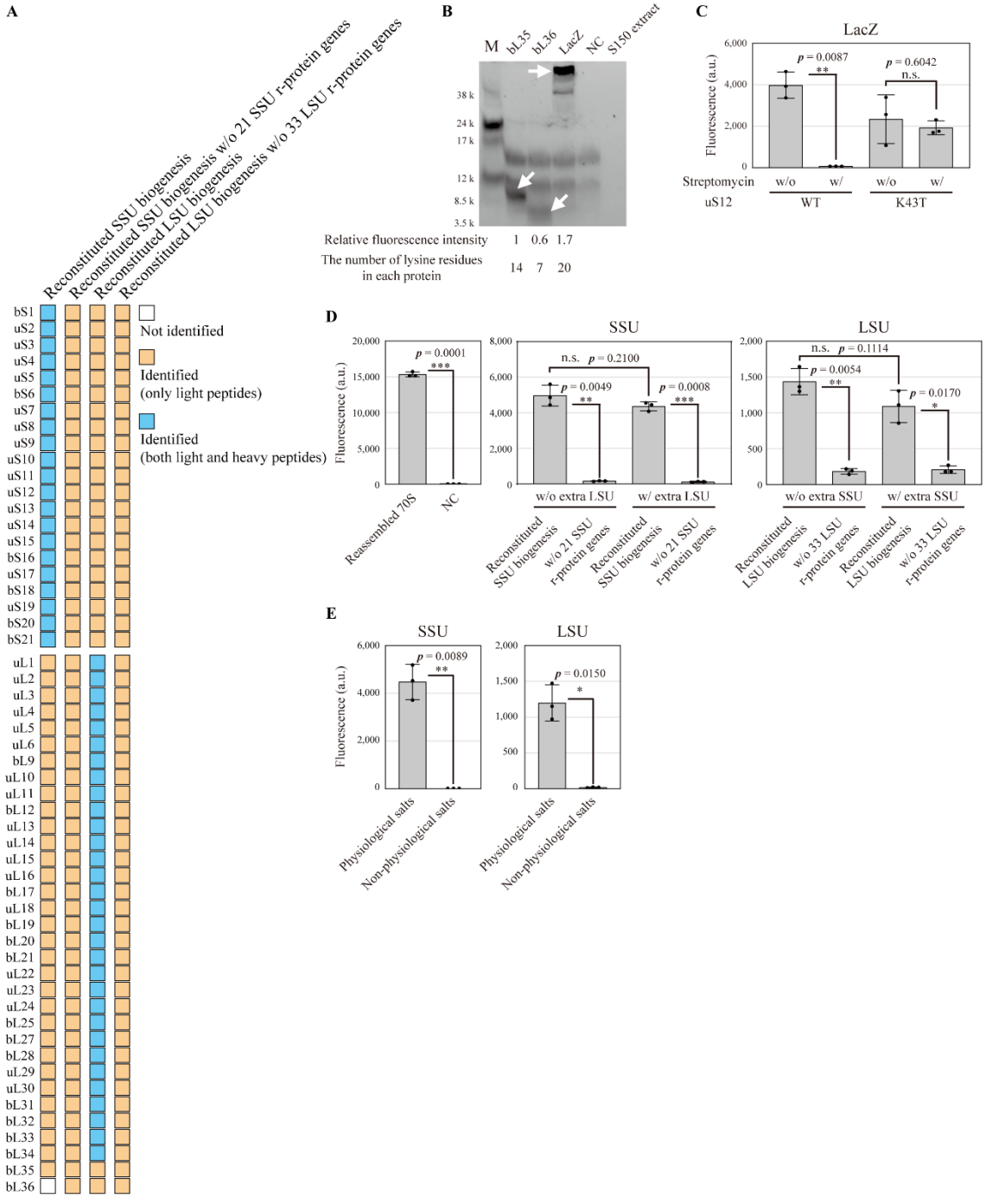
Supplementary Fig. 6. Supporting experiments for the *in vitro* SSU biogenesis. (A) A representative micrograph of droplets in the initial trial. In the first reaction, the concentrations of the native ribosomes, the artificial rRNA operon with or1-oASD and C1192U spectinomycin resistance (SpcR), and 21 SSU r-protein genes were 20, 1, and 0.25 nM each, respectively. The second reaction was conducted with the or1-oSD–LacZ reporter and spectinomycin using the droplet assay. (B) A representative micrograph of droplets in the optimized reaction condition. In the first reaction, the concentrations of the native ribosomes, the artificial rRNA operon, and 21 SSU r-protein genes were 80, 0.3, and 0.05 nM each, respectively. The second reaction was conducted with the or1-oSD–LacZ reporter and spectinomycin using the droplet assay. (C) Standard curve for translational activity during *in vitro* SSU biogenesis using the bulk assay. The standard curve was generated based on the experimental procedure applied for *in vitro* SSU biogenesis. Left graph illustrates the time-course fluorescence derived from standard native ribosomes. Right graph displays the standard curve for translational activity. The translational activity for the *in vitro* SSU biogenesis shown in Fig. 3D is superimposed on the graphs, indicating a value of 32 ΔFluorescence (a.u.) min^{-1}. Given that the second reaction solution was diluted by a factor of 2 from the first reaction solution (refer to Cell-free transcription and translation (CF-TXTL) in Materials and Methods), the estimated concentration of the nascent artificial SSU is 9.8 nM. a.u., arbitrary unit. Mean ± SD (n = 3, biological replicates). (D) Standard curve for translational activity during the iSAT reaction using the bulk assay. The standard curve was generated based on the

experimental procedure employed for the iSAT reaction. The translational activity of the iSAT reaction shown in Fig. 3D is superimposed on the graphs, showing a value of 4.8 ΔFluorescence (a.u.) min^{-1}. Given that the second reaction solution was diluted by a factor of 2 from the first reaction solution, this translational activity corresponds to 2.8 nM. Mean ± SD (n = 3, biological replicates). Source data are provided as a Source Data file.



Supplementary Fig. 7. Supporting experiments for the *in vitro* LSU biogenesis. (A) Optimization of the clindamycin concentrations using the bulk assay. S150 cell extracts containing 80 nM native ribosomes were mixed with the WT-SD–LacZ reporter at different concentrations of clindamycin. We used 1.5 mM clindamycin to inactivate the native ribosomes for all the experiments unless specified otherwise. a.u., arbitrary unit; NC, negative control without the reporter. Mean ± SD ($n = 3$). ***, $p < 0.001$; ****, $p < 0.0001$; n.s., not significant; one-way ANOVA with Dunnett's test against NC. (B) Determination of the residual translational activity of the artificial ribosomes with A2058U clindamycin resistance (CldR) in the presence of clindamycin using the bulk assay. Two types of S12 cell extracts were prepared using *E. coli* SQ171, which lacks all chromosomal *rRNA* alleles, expressing a plasmid-borne *rRNA* operon with or without CldR. In the presence of 1.5 mM clindamycin, the ribosomes without CldR showed no significant translational activity, whereas those with CldR showed 42 % residual translational activity. Mean ± SD ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; one-way ANOVA with Dunnett's test against NC. Relative GFP fluorescence (%) is shown with the translational activity at 0 mM clindamycin as 100. (C) The standard curve for the translational activity during the *in vitro* LSU biogenesis using the bulk assay. Left graph illustrates the time-course fluorescence derived from standard native ribosomes. Right graph displays the standard curve for translational activity. The translational activity for

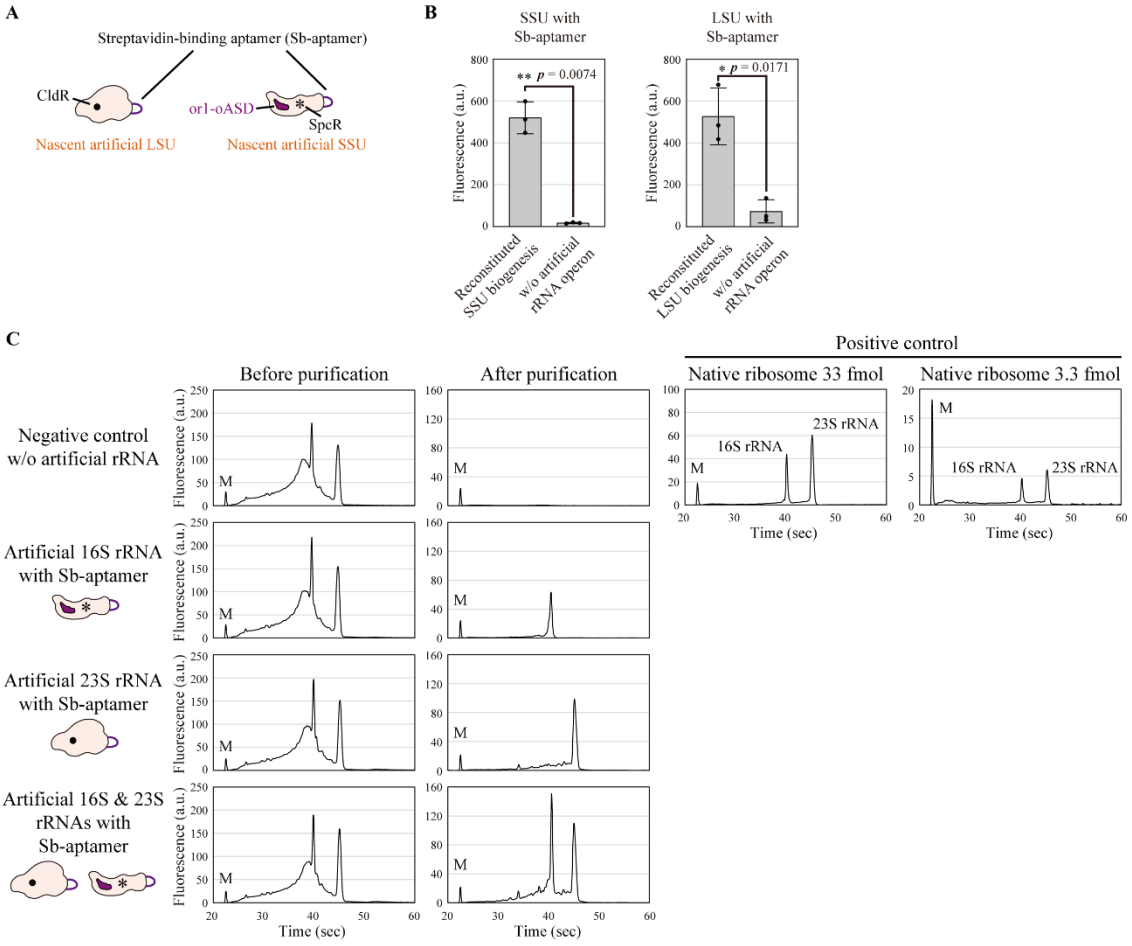
the *in vitro* LSU biogenesis shown in Fig. 4D is superimposed on the graphs, indicating a value of 1.43 ΔFluorescence (a.u.) min^{-1}. Given that the translational activity of the ribosomes with CldR was reduced to 42 % in the presence of 1.5 mM clindamycin, and that the second reaction solution was diluted by a factor of 2 from the first reaction solution, the estimated concentration of the nascent artificial LSU is 3 nM. Mean ± SD (n = 3). Three biological replicates were used in all experiments. Source data are provided as a Source Data file.



Supplementary Fig. 8. Characterizing properties of the *in vitro* SSU and LSU biogenesis.

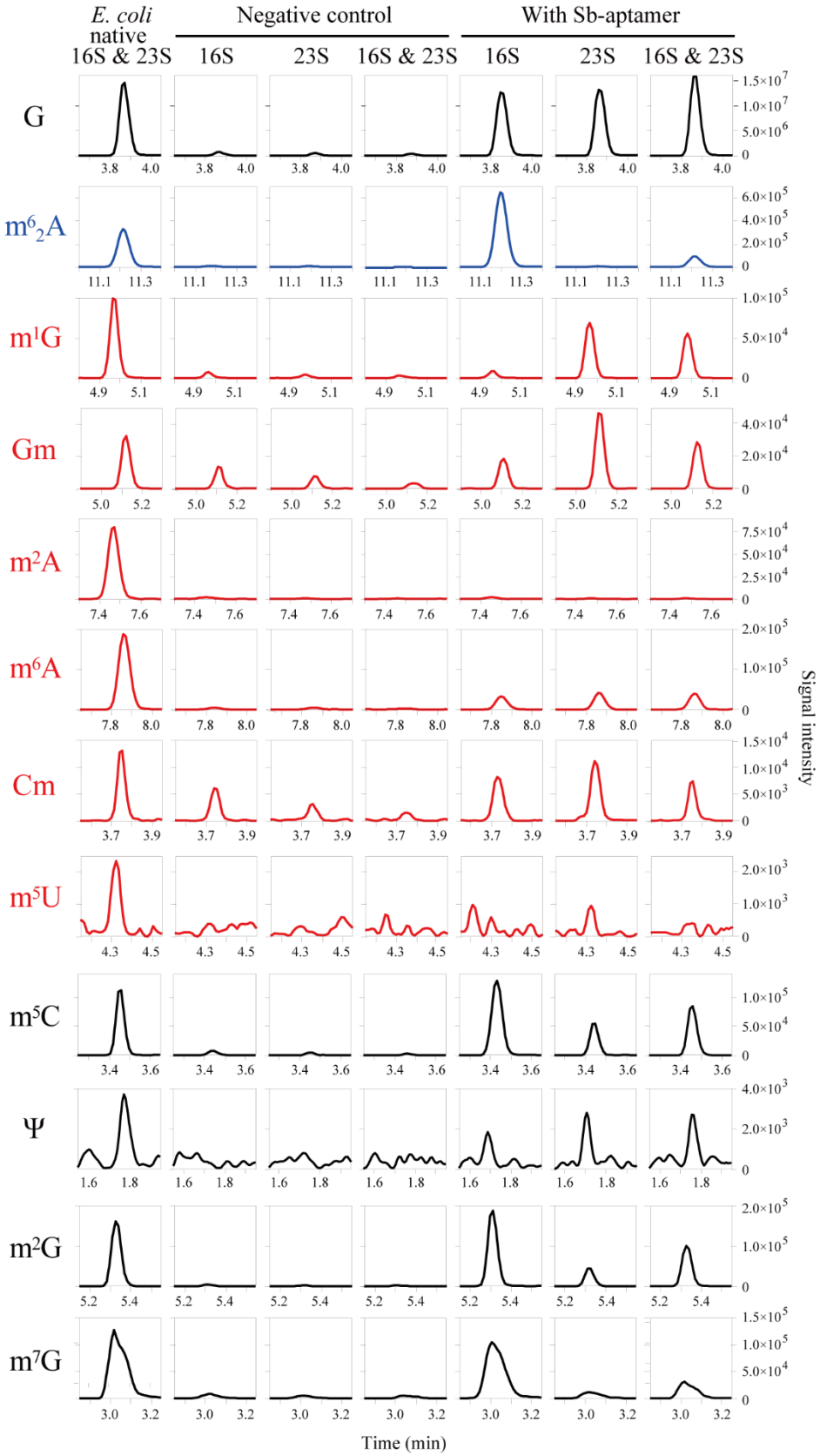
(A) Detection of preexisting and nascent r-proteins. We used stable isotope-labeled (heavy) L-arginine ($^{13}C_6$, $^{15}N_4$) and L-lysine ($^{13}C_6$, $^{15}N_2$) in the reaction solutions during SSU or LSU biogenesis *in vitro*. Representative data from two biological replicates. (B) Detection of nascent bL35 and bL36 in the S150 cell extract. Genes encoding bL35, bL36,

or LacZ were expressed in the S150 cell extract containing lysine-charged tRNA labeled with the fluorophore BODIPY. M, molecular-weight size marker. NC, negative control without gene addition. Representative image from three biological replicates. (C) Direct evidence for the incorporation of newly synthesized r-proteins into nascent ribosomes. In the first reaction, the concentrations of the native ribosomes, the artificial rRNA operon with or1-oASD and C1192U spectinomycin resistance (SpcR), and 21 SSU r-protein genes were 80, 0.3, and 0.05 nM each, respectively. A mutant r-protein gene encoding streptomycin-resistant uS12 K43T was used instead of an r-protein gene encoding native uS12. The second reaction was conducted with the or1-oSD–LacZ reporter and spectinomycin with or without streptomycin using the bulk assay. a.u., arbitrary unit. Mean ± SD ($n = 3$, biological replicates). **, $p < 0.01$; n.s., not significant; two-tailed Welch's *t*-test. (D) Effects of adding extra ribosomal subunits. Native SSU and LSU were purified from native ribosomes, and their functionality was verified by examining the translational activity of ribosomes reassembled from 30 nM native SSU and LSU using the improved LacZ reporter (left graph). The *in vitro* SSU and LSU biogenesis were performed using the optimized reaction conditions after adding an extra 5 nM of LSU and SSU, respectively. NC, negative control without the purified subunits. Mean ± SD ($n = 3$, biological replicates). *, $p < 0.05$; ***, $p < 0.001$; two-tailed Welch's *t*-test. (E) Effects of physiological salts on the *in vitro* SSU and LSU biogenesis. S150 extracts with physiological salts (potassium and glutamate) and non-physiological salts (ammonium and chloride) were used. Mean ± SD ($n = 3$, biological replicates). two-tailed Welch's *t*-test. Source data are provided as a Source Data file.



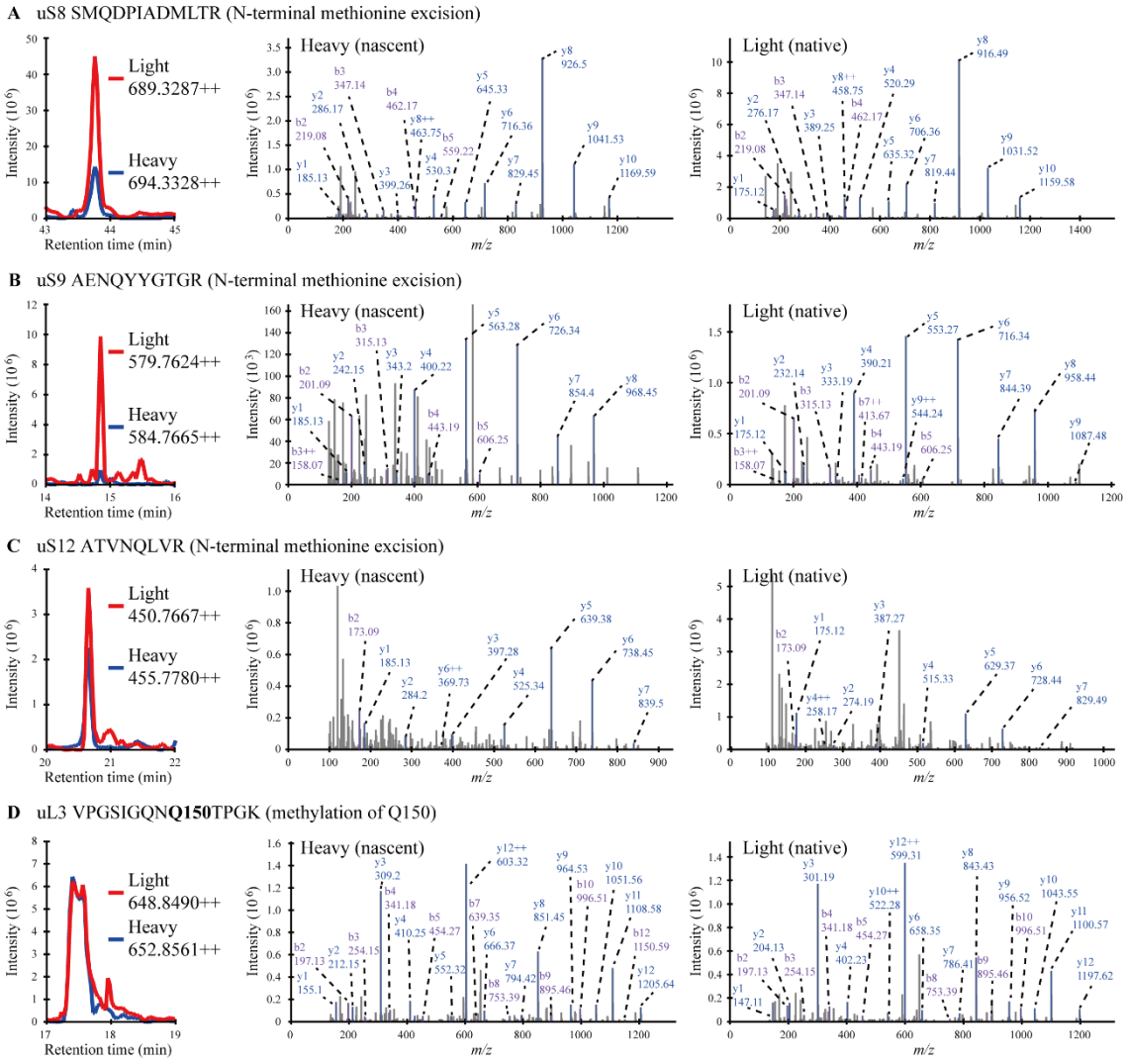
Supplementary Fig. 9. Purification of nascent rRNAs.

(A) Illustration of the nascent artificial ribosomes with streptavidin-binding aptamer (Sb-aptamer). (B) Successful detection of translational activity of nascent artificial SSU and LSU with Sb-aptamer. The experimental conditions were identical to those of the optimized conditions using the bulk assay described in Fig. 3 and Fig. 4, except that the plasmids encoding rRNAs with Sb-aptamer were used. a.u., arbitrary unit. Mean ± SD ($n = 3$, biological replicates). *, $p < 0.05$; **, $p < 0.01$; two-tailed Welch's t-test. (C) Purification of nascent artificial SSU and/or LSU. Nascent Artificial SSU and/or LSU with Sb-aptamer were purified using streptavidin agarose under a subunit dissociation condition (1 mM Mg^{2+}). rRNAs were analyzed using Bioanalyzer. M, electrophoresis marker. Representative gel electrograms from three biological replicates. Source data are provided as a Source Data file.

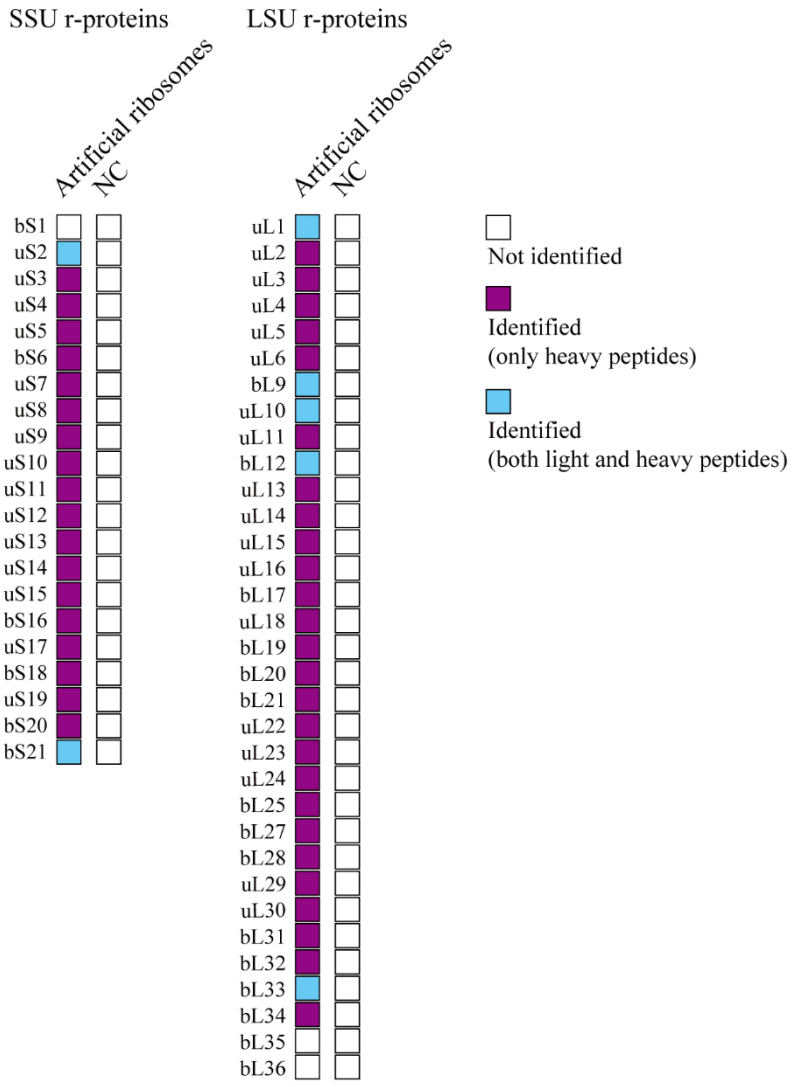

E. coli native 16S & 23S
Negative control
16S
23S
16S & 23S
With Sb-aptamer
16S
23S
16S & 23S
G
m62A
m1G
Gm
m2A
m6A
Cm
m5U
m5C
Ψ
m2G
m7G
Signal intensity
Time (min)

228
229

Supplementary Fig. 10. Characterizing the modifications of nascent rRNAs.
Purified nascent rRNAs were digested into nucleosides. The digested samples were separated on an Inertsil ODS-3 column, followed by electrospray ionization and triple quadrupole mass spectrometry in the multiple reaction monitoring mode. In this mode, 11 of 17 modification types in *E. coli* rRNAs are detectable. The other six modification types (m^3U, $m^3\Psi$, m^4Cm, ho^5C, D, and Um) are not detectable because of the lack of authentic standards, the lack of available multiple reaction monitoring methods, or sensitivity limits. The equivalent of 1.8 ng of nucleosides was analyzed for each sample. Negative controls were prepared using the same production, purification, and digestion procedures except that the plasmids encoding artificial rRNAs with Sb-aptamer were not used. For the negative controls, the same volume as the corresponding samples was subjected to nucleoside digestion. Guanosine (G) was used as a loading control. Modifications presented in blue and red are 16S and 23S rRNA-specific modifications in the native *E. coli* rRNAs, respectively. Modifications presented in black are present in the 16S and 23S rRNAs in the native *E. coli* rRNAs. Representative mass chromatograms from two biological replicates.



Supplementary Fig. 11. Characterizing the modifications of the nascent r-proteins. Nascent r-proteins were labeled with heavy L-arginine ($^{13}C_6$, $^{15}N_4$) and L-lysine ($^{13}C_6$, $^{15}N_2$) for the *in vitro* SSU and LSU biogenesis. They were then extracted, reduced, alkylated, digested, and cleaned up. The resultant peptides were analyzed by Orbitrap Exploris™ 240. The analysis identified some post-translational modifications observed in native ribosomes, such as N-terminal methionine excision of (A) uS8, (B) uS9, (C) uS12 and glutamine methylation of (D) uL3. Peptide identification is based on retention times of peptides in liquid chromatography and fragmentation patterns in mass spectrometry. The peptides derived from native r-proteins (light) and nascent r-proteins (heavy) showed the same retention time as expected. They showed the expected fragmentation patterns with a series of b- and y-ions in their MS/MS spectra. Representative mass chromatograms from two biological replicates.



Supplementary Fig. 12. Analyzing the r-protein composition of nascent ribosomes. Nascent artificial SSU and LSU with streptavidin-binding aptamer (Sb-aptamer) were synthesized by the *in vitro* SSU and LSU biogenesis using stable isotope-labeled (heavy) L-arginine ($^{13}C_6$, $^{15}N_4$) and L-lysine ($^{13}C_6$, $^{15}N_2$) to label nascent r-proteins. These subunits were mixed and purified using streptavidin resin under the subunit dissociation condition (1 mM Mg^{2+}), as described in Supplementary Fig. 9C. As a negative control (NC), we performed the same production and purification procedure without expressing the artificial rRNA operon with Sb-aptamer during the *in vitro* SSU and LSU biogenesis. The samples were then extracted, reduced, alkylated, digested, and cleaned up. The resultant peptides were analyzed by Orbitrap Exploris™ 240. The number of biological replicates is one.