In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation

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Supplementary Information

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Supplementary Table 1

Reagent	$[Reagent]_{final}$ (this work)	$[Reagent]_{final}$ (Nierhaus, 1990)*
Magnesium	16.4 mM	10.3 mM
Ammonium	10 mM	169 mM
Potassium	130 mM	
Glutamate	154 mM	
Chloride		170 mM
Acetate	2.4 mM	9.1 mM
ATP	1.2 mM	1.50 mM
GTP**	0.86 mM	0.05 mM
UTP**	0.86 mM	
CTP**	0.86 mM	
Folinic acid	34 μg/mL	
tRNA	171 μg/mL	333 μg/mL
Amino acids***	2 mM	0.08 mM
PEP	33 mM	5.00 mM
Pyruvate Kinase	1.63 μg/mL	25 μg/mL
NAD	0.33 mM	
CoA	0.27 mM	
HEPES-KOH, pH 7.6 (4C)	57 mM	5.83 mM
Tris-HCL, pH 8.0 (4C)		17.08 mM
Tris-OAc, pH 7.5 (4C)	2.4 mM	
Oxalic acid	4 mM	
Putrescine	1 mM	
Spermidine	1.5 mM	0.58 mM
Spermine		0.06 mM
EDTA		0.33 mM
S150 Extract	0.24 vol/vol	0.21 vol/vol
T7 RNA polymerase**	100 μg/mL	
Template (DNA or RNA)	13 μg/mL	830 μg/mL

* Note: The ionic milieu was calculated by summing the moles of salt from the energy mix, buffer 3 carry over (which contains the S-150 enzymes), and the reconstitution mixture.

**Because this work uses a combined transcription and translation system, GTP, UTP, CTP, and T7 RNA polymerase are present and lacking in the original poly(Phe) system.

***In this work, 20 amino acids are used. In the original poly(Phe) system, only phenylalanine is added.

****In this work, 2.4mM Tris-OAc and 2.4mM magnesium acetate carry over into the protein synthesis reaction from the S150 extract.

Supplementary Table 1 | Comparison of final reagent concentrations in the extract based transcription and translation system from this work and from the classic poly (Phe) system (Nierhaus, 1990).

Supplementary Table 2

Ionic solute/Additive	Optimization profile (mM/fraction of maximum yield)					
Potassium Glutamate	100/0.90	130/1.00	190/0.95	260/0.73	320/0.17	380/0.05
Putrescine	1/1.00	2/1.00	4/0.89	6/0.70	8/0.63	10/0.29
Spermidine	0.5/0.47	1/0.91	1.5/1.00	2/0.95	3/0.60	
Spermine	0/1.00	0.25/0.74	0.5/0.42	1.0/0.10		
r-protein/rRNA ratio *	0.9/0.83	1.1/1.00	1.3/0.61	1.5/0.16		
Chaperones #	-/1.00	+/0.98				

* In this case mM is replaced by the r-protein/rRNA ratio
# In this case mM is replaced with "+" or "-" to indicate presence or absence of additional chaperones

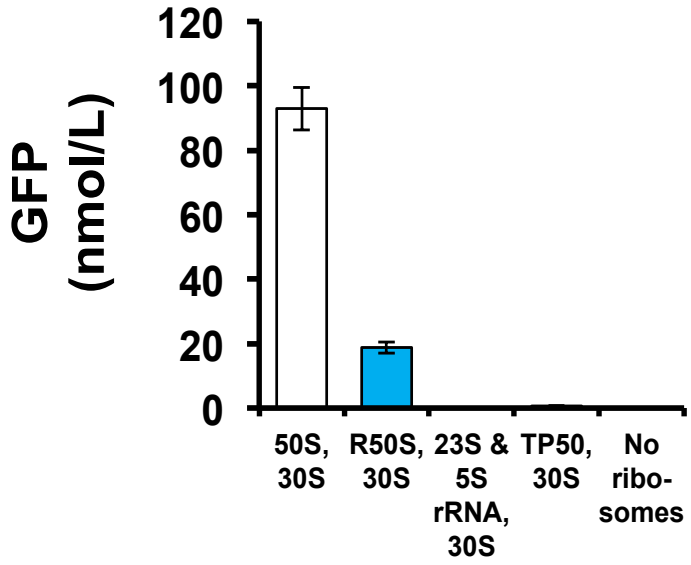
Supplementary Table 2 | Optimization studies in 50S subunit assembly and translation reactions aided by S150 extracts based on relative active Fluc expression. The trends for potassium glutamate, putrescine, spermidine, spermine, r-protein/rRNA ratio, and chaperone addition are shown. The values given as “100/ 0.90” indicate that a 100mM concentration of that ionic solute was added to the reaction and that 90% of the maximum expression of Fluc was achieved at that particular concentration. Only one solute was varied at a time. Fifteen microliter reactions were incubated at 37ºC for 4h. Reference conditions, which also turned out to be optimum, were 130mM potassium glutamate, 1mM putrescine, 1.5mM spermidine, and 0mM spermine, as well as a TP50/rRNA ratio of 1.1. For chaperone addition, 1.5pmol DnaK, 1.5pmol DnaJ, 3pmol GrpE, 1.5pmol GroEL, and 1.5pmol GroES were added for 0.75pmol 23S rRNA, along with an additional 1mM ATP. Chaperone addition did not provide a benefit.

Supplementary Table 3

Function	Products	54 Genes	Base Pairs	Peptide Bonds
Ribosomal Protein Large subunit	RL1	rplA	705	233
Ribosomal Protein Large subunit	RL2	rplB	822	272
Ribosomal Protein Large subunit	RL3	rplC	630	208
Ribosomal Protein Large subunit	RL4	rplD	606	200
Ribosomal Protein Large subunit	RL5	rplE	540	178
Ribosomal Protein Large subunit	RL6	rplF	534	176
Ribosomal Protein Large subunit	RL7/RL12 (4 copies)	rplL	1464	480
Ribosomal Protein Large subunit	RL9	rplI	450	148
Ribosomal Protein Large subunit	RL10	rplJ	498	164
Ribosomal Protein Large subunit	RL11	rplK	429	141
Ribosomal Protein Large subunit	RL13	rplM	429	141
Ribosomal Protein Large subunit	RL14	rplN	372	122
Ribosomal Protein Large subunit	RL15	rplO	435	143
Ribosomal Protein Large subunit	RL16	rplP	411	135
Ribosomal Protein Large subunit	RL17	rplQ	384	126
Ribosomal Protein Large subunit	RL18	rplR	354	116
Ribosomal Protein Large subunit	RL19	rplS	348	114
Ribosomal Protein Large subunit	RL20	rplT	357	117
Ribosomal Protein Large subunit	RL21	rplU	312	102
Ribosomal Protein Large subunit	RL22	rplV	333	109
Ribosomal Protein Large subunit	RL23	rplW	303	99
Ribosomal Protein Large subunit	RL24	rplX	315	103
Ribosomal Protein Large subunit	RL25	rplY	285	93
Ribosomal Protein Large subunit	RL27	rpmA	258	84
Ribosomal Protein Large subunit	RL28	rpmB	237	77
Ribosomal Protein Large subunit	RL29	rpmC	192	62
Ribosomal Protein Large subunit	RL30	rpmD	180	58
Ribosomal Protein Large subunit	RL31	rpmE	213	69
Ribosomal Protein Large subunit	RL32	rpmF	174	56
Ribosomal Protein Large subunit	RL33	rpmG	168	54
Ribosomal Protein Large subunit	RL34	rpmH	141	45
Ribosomal Protein Large subunit	RL35	rpmI	198	64
Ribosomal Protein Large subunit	RL36	rpmJ	117	37
Ribosomal Protein Small subunit	RS1	rpsA	1674	556
Ribosomal Protein Small subunit	RS2	rpsB	726	240
Ribosomal Protein Small subunit	RS3	rpsC	702	232
Ribosomal Protein Small subunit	RS4	rpsD	621	205
Ribosomal Protein Small subunit	RS5	rpsE	504	166
Ribosomal Protein Small subunit	RS6	rpsF	396	130
Ribosomal Protein Small subunit	RS7	rpsG	540	178
Ribosomal Protein Small subunit	RS8	rpsH	393	129
Ribosomal Protein Small subunit	RS9	rpsI	393	129
Ribosomal Protein Small subunit	RS10	rpsJ	312	102
Ribosomal Protein Small subunit	RS11	rpsK	390	128
Ribosomal Protein Small subunit	RS12	rpsL	375	123
Ribosomal Protein Small subunit	RS13	rpsM	357	117
Ribosomal Protein Small subunit	RS14	rpsN	306	100
Ribosomal Protein Small subunit	RS15	rpsO	270	88
Ribosomal Protein Small subunit	RS16	rpsP	249	81
Ribosomal Protein Small subunit	RS17	rpsQ	255	83
Ribosomal Protein Small subunit	RS18	rpsR	228	74
Ribosomal Protein Small subunit	RS19	rpsS	279	91
Ribosomal Protein Small subunit	RS20	rpsT	264	86
Ribosomal Protein Small subunit	RS21	rpsU	216	70
		Total peptide bonds to make all ribosomal proteins =		7434

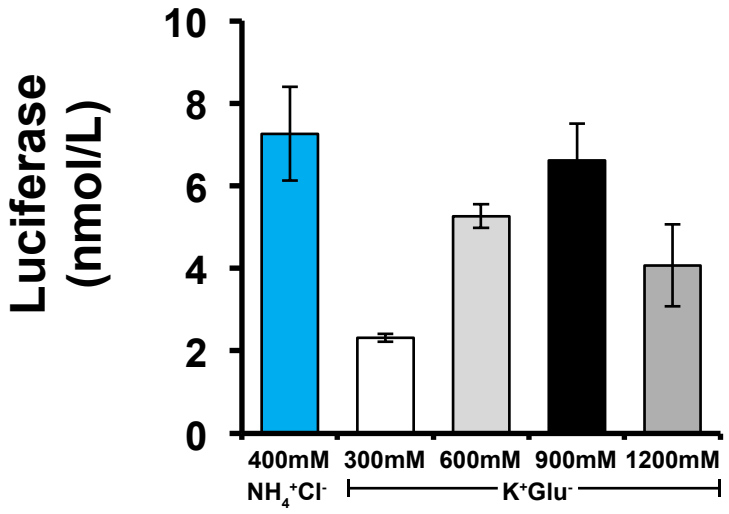
Supplementary Table 3 | Calculation of peptide bonds necessary to make a ribosome. Moving across each row is a single ribosomal protein, its product and gene name, the number of base pairs per gene (bp), and the number of peptide bonds necessary to make that gene product. For example, the rplA gene has 705 bp including the UAA stop codon. Thus it has 235 codons, 234 amino acids, and 233 peptide bonds. The sum of all peptide bonds, 7434, is shown. Table adopted from Forster and Church (Forster & Church, 2006).

Supplementary Figure 1



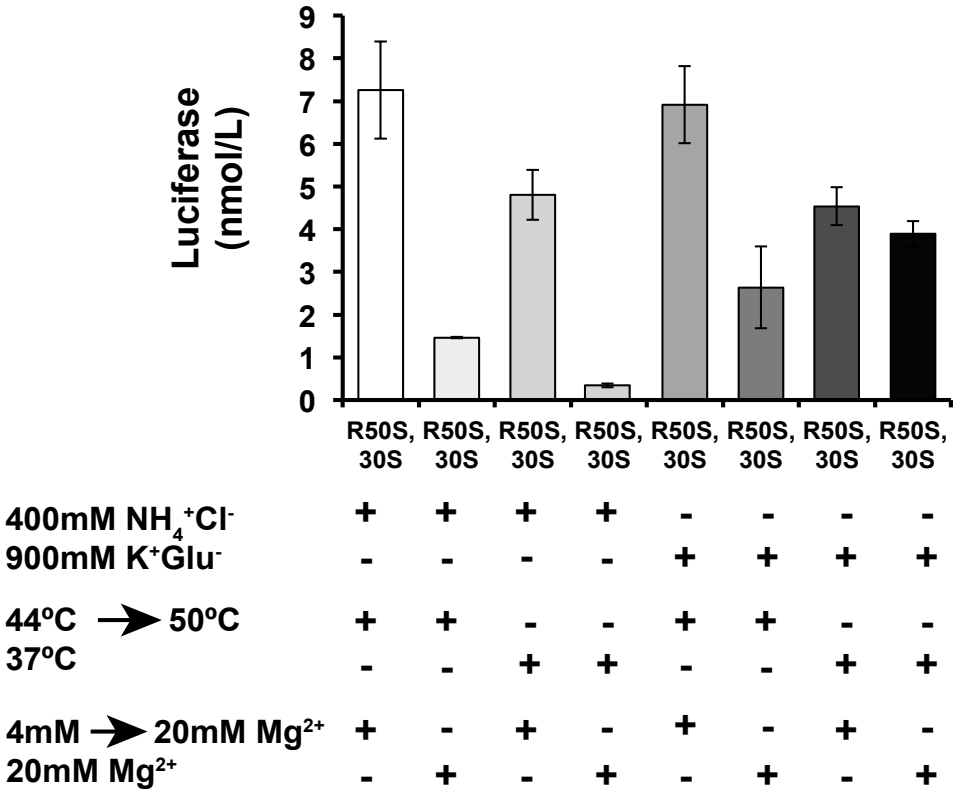
Supplementary Figure 1 | 50S ribosomal subunits reconstituted using the conventional method synthesize green fluorescent protein (GFP). GFP production in 15μL ETTA reactions where either 0.1μM native 50S subunits or 0.1μM 50S reconstitution reactions (R50S) were paired with 0.2μM native 30S subunits. Fluorescence was measured following 2h incubation at 30°C and 8h maturation at 4°C. Fluorescence was converted to an estimate of protein concentration using a standard curve created from dilutions of purified GFP (4000 Fluorescence units (arbitrary) ~ 1μg GFP). Reconstitutions in the absence of 23S and 5S rRNA or TP50 did not yield active particles. Control transcription and translation reactions without ribosomes or without GFP template DNA had immeasurable fluorescence. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments. While reconstitution reactions were competent in GFP synthesis, we chose to complete the study using luciferase because the detection assay was more sensitive.

Supplementary Figure 2



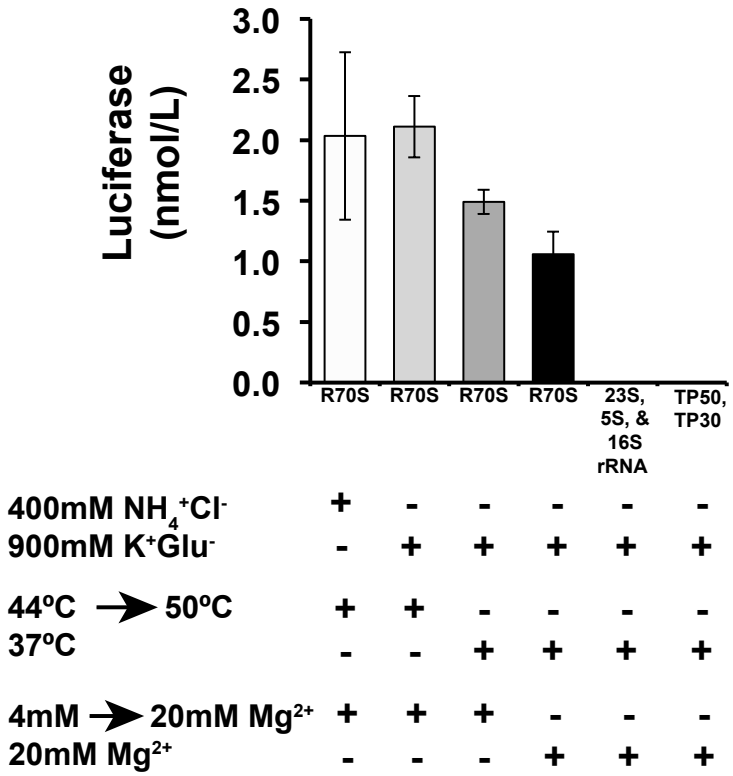
Supplementary Figure 2 | The optimization of potassium glutamate concentration as a substitute for ammonium chloride in conventional 50S reconstitution. The activity of 0.1μM 50S reconstitution reactions paired with 0.2μM native 30S subunits was compared for different salt conditions as indicated. The final yield of Fluc following a 2h ETTA reaction at 30°C is shown. Substituting potassium glutamate for ammonium chloride did not impact protein synthesis activity of reconstitution reactions at 900mM potassium glutamate concentration. Reconstitutions in the absence of 23S rRNA and 5S rRNA or TP50 did not yield active particles. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 3



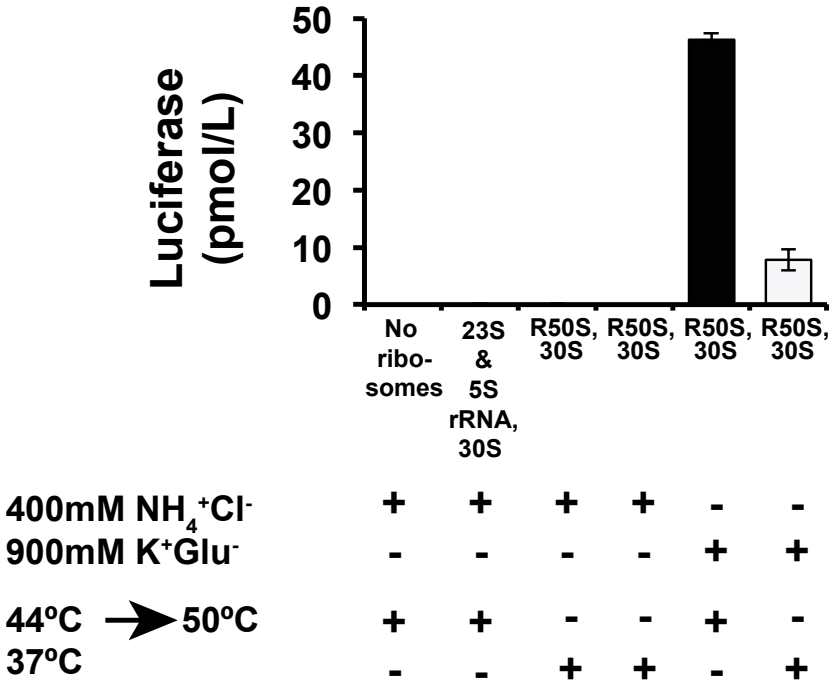
Supplementary Figure 3 | *E. coli* 50S ribosomal subunits reconstituted using the conventional approach under near-physiological conditions synthesize active Fluc. Expanded from **Figure 2d**, the dependence of protein synthesis activity on 50S reconstitution is shown for three factors: (1) the concentration of the major ionic component, (2) temperature, and (3) magnesium concentration. "+" or "-" indicate the presence or absence, respectively, of the specified factor in the reconstitution reaction. For the two-step reconstitution procedures (which are indicated with arrows such as 44ºC → 50ºC), 20-min incubation was followed by 90-min incubation. For the one-step procedure, reconstitution was carried out for 120-min. Following reconstitution, ETTA reactions with 0.1μM 50S reconstitution reactions (R50S) from specified conditions paired with 0.2μM native 30S subunits were compared. The final yield of Fluc following a 2h incubation at 30°C is shown. One-step reconstitution is achieved under near-physiological conditions (see black bar far right). Reconstitutions in the absence of 23S rRNA and 5S rRNA or TP50 did not yield active particles (not shown). Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 4



Supplementary Figure 4 | *E. coli* 70S ribosomes reconstituted using the conventional approach under near-physiological conditions synthesize active Fluc. The dependence of protein synthesis activity on 70S reconstitution is shown for three factors: (1) the concentration of the major ionic component, (2) temperature, and (3) magnesium concentration. "+" or "-" indicate the presence or absence, respectively, of the specified factor in the reconstitution reaction. For the two-step reconstitution procedures (which are indicated with arrows such as 44ºC → 50ºC), 20-min incubation was followed by 90-min incubation. For the one-step procedure, reconstitution was carried out for 120-min. Following reconstitution, protein yields synthesized in the ETTA system with 0.1μM 70S reconstitution reactions (R70S) from specified conditions were compared. The final yield of Fluc following a 2h incubation at 30°C is shown. One-step reconstitution was achieved under near physiological conditions (see black bar). Reconstitutions in the absence of ribosomal proteins or rRNA did not yield active particles (far right). Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

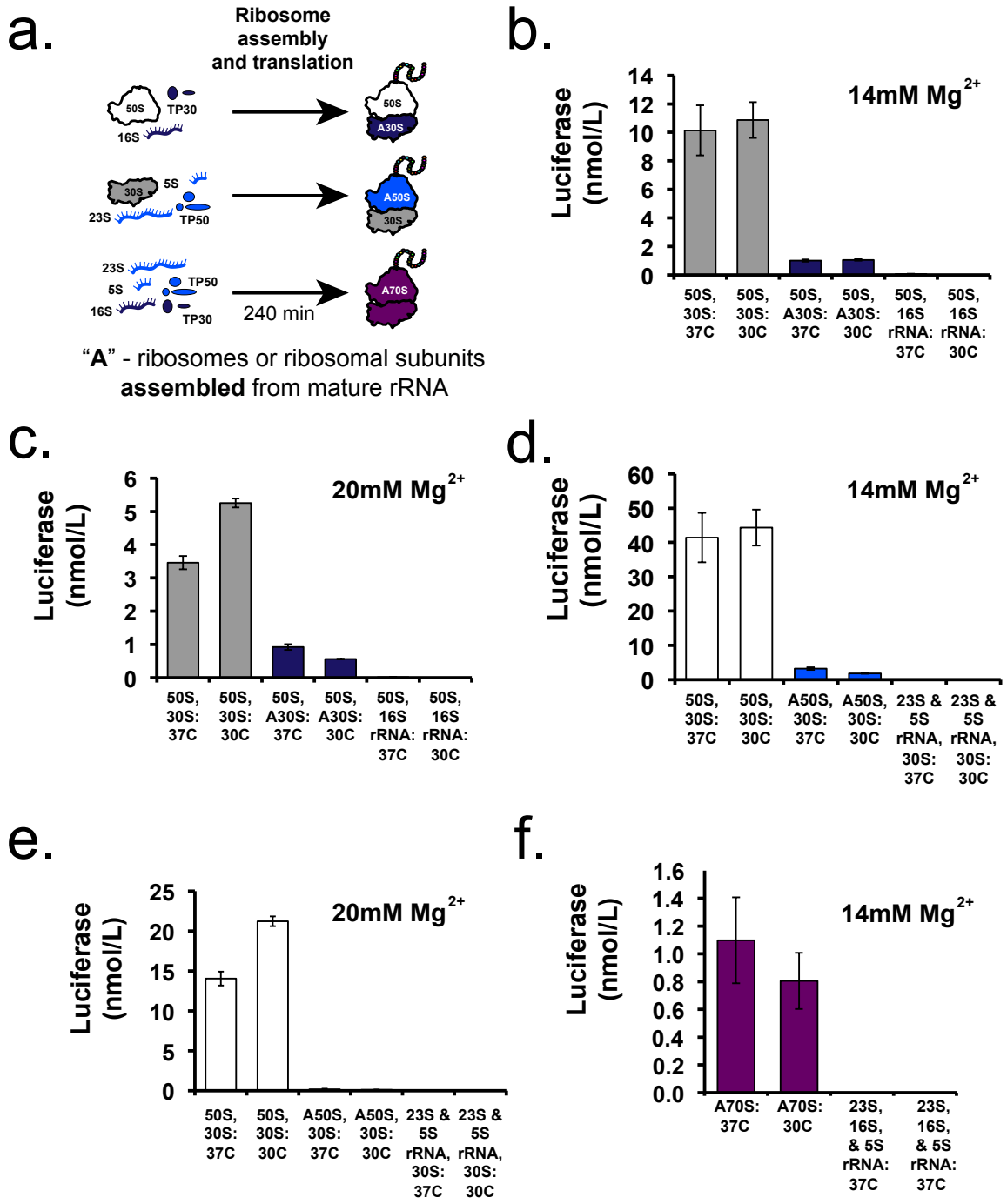
Supplementary Figure 5



Supplementary Figure 5 | Fluc production from ribosomes reconstituted using the conventional approach is markedly decreased in ETTA reactions when the chemical composition of the classical poly (Phe) translation system is used instead of the Cytomim system from this study. Although our study primarily focuses on ribosome reconstitution protocols, major differences exist between the ETTA system used in this study and that used in previous works (see **Supplementary Table 1**). Here, we test protein synthesis activity of 50S reconstitution reactions (R50S) derived from different protocols in the chemical composition of the classical poly (Phe) translation system reported previously (Nierhaus, 1990). Each reconstitution included a two-step [Mg^{2+}] change from 4mM → 20mM. "+" or "-" indicate the presence or absence, respectively, of the specified factor in the reconstitution reaction. Following reconstitution, ETTA reactions with 0.1μM 50S reconstitution reactions from specified conditions paired with 0.2μM native 30S subunits were compared. The final yield of Fluc following a 2h incubation at 30°C is shown. Fluc synthesis using ribosomes reconstituted with 400mM ammonium chloride was immeasurable. In contrast, 50S ribosomes reconstituted in glutamate salts synthesized active protein in an ETTA reaction, suggesting advantages for our new protocol. Based on final Fluc titers, our ETTA system designed to mimic the cytoplasm was ~150-fold more productive in the two-step temperature protocol and ~650-fold more productive

in the isothermal protocol than when the ionic environment of the classic poly (Phe) system was used (See **Figure 2** for comparison). These results demonstrate the importance of developing a coordinated ribosome assembly and translation platform. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 6

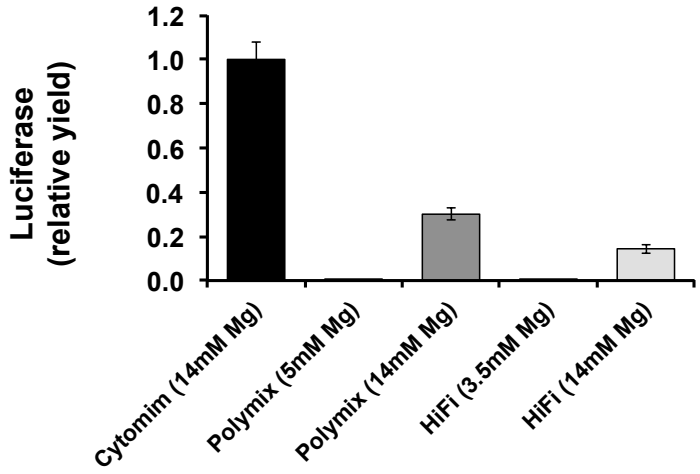


Supplementary Figure 6 | The impact of temperature and magnesium concentration on combined ribosome assembly and translation in an S150 extract based ETTA system. a,

Schematic of combined ribosome assembly and translation. Temperature and magnesium concentration were varied to identify parameters leading to the highest level of luciferase synthesis. Temperatures shown include 30°C (the optimum temperature for active Fluc synthesis) and 37°C (the physiological temperature for optimum *E. coli* growth and the temperature used in one-step reconstitution experiments). Magnesium concentrations shown include 20mM Mg^{2+} (the experimentally determined optimum for conventional 30S and 50S reconstitution) and 14mM Mg^{2+} (the experimentally determined optimum for the extract based transcription and translation system). The experimentally determined magnesium concentration optimum is higher than those typically used for *in vitro* translation systems composed of purified components (~5 mM) (Jelenc & Kurland, 1979; Pavlov & Ehrenberg, 1996), although somewhat similar to the poly (Phe) system of Nierhaus (Nierhaus, 1990) (**Supplementary Table 1**) and to the PURExpress® translation system (Shimizu et al, 2001; Shimizu et al, 2005). This discrepancy likely results from our use of an extract based protein synthesis system designed to mimic the intracellular physicochemical environment (Jewett et al, 2008; Jewett & Swartz, 2004). The concentration of glutamate in the extract based system used here is ~150 mM, which is significantly higher than *in vitro* translation systems that have magnesium concentration optimums around 5mM (Jelenc & Kurland, 1979; Pavlov & Ehrenberg, 1996). Since the reported log stability constant for $[MgGlu^-]$ /[Mg][Glu] is 1.9 (Dawson, 1990), it seems reasonable that 150mM glutamate would have a strong buffering effect on the concentration of free Mg^{2+} and would therefore impact the observed optimum for total $[Mg^{2+}]$. **b,c,** The protein synthesis activities of either 0.2μM native 30S subunits or 0.2μM 30S ribosomal subunit components (*e.g.*, mature 16S rRNA and TP30: A30S) paired with 0.4μM native 50S subunits were compared at different temperature and magnesium concentrations. The final yield of Fluc following a 4h ETTA reaction is shown. Reactions in the absence of TP30 did not yield active particles. **d,e,** The protein synthesis activities of either 0.2μM native 50S subunits or 0.2μM 50S ribosomal subunit components (*e.g.*, mature 23S rRNA, 5S rRNA and TP50: A50S) paired with 0.4μM native 30S subunits were compared at different temperature and magnesium concentrations. The final yield of Fluc following a 4h ETTA reaction is shown. Reactions in the absence of TP50 did not yield active particles. **f,** The protein synthesis activities of 0.2μM native 70S ribosome components (A70S) were compared at different temperature and magnesium concentrations. The final yield of Fluc following a 4h ETTA reaction is shown. Reactions in the absence of TP70 did not yield active particles. Protein synthesis from assembly of 70S ribosomes at 20mM Mg^{2+} was not measurable. In all cases, combined ribosome assembly and translation activity was

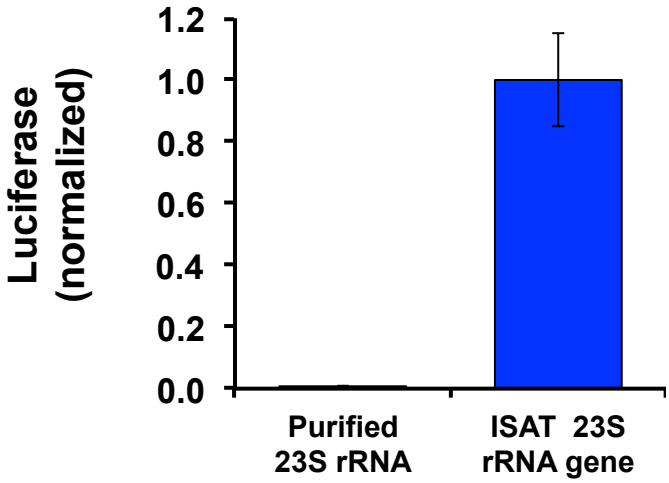
higher at 37°C and 14mM Mg^{2+}. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 7



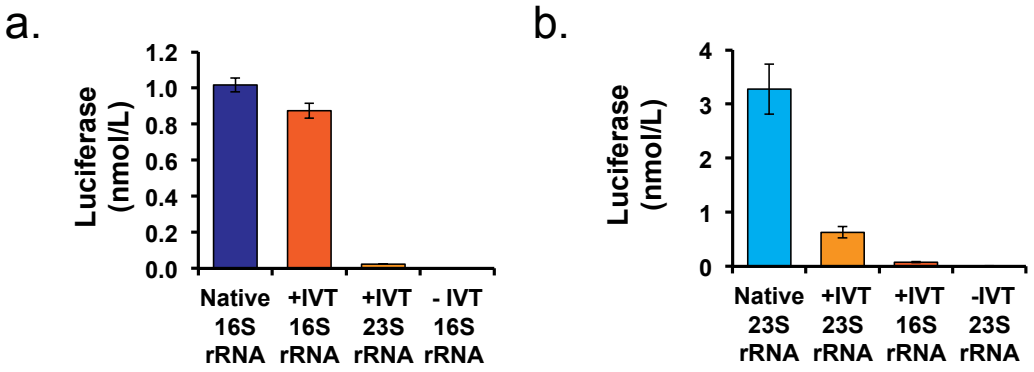
Supplementary Figure 7 | The impact of changing the Cytomim buffer used in this study with conventional polymix (Jelenc & Kurland, 1979) and HiFi (Gromadski & Rodnina, 2004) translation buffers on combined 50S ribosome assembly and translation reactions. Reactions were carried out in S150 extracts for 4h at 37°C. Three buffers were used (i) Cytomim buffer (this study) - 14mM Mg $(Glu)_2$, 130mM K (Glu), 10mM NH_4 (Glu), 1mM putrescine, 1.5mM spermidine; (ii) Polymix buffer - 5mM Mg $(OAc)_2$ or 14mM Mg $(OAc)_2$ and 95mM KCl, 5mM NH_4Cl, 8mM putrescine,1 mM spermidine, 1mM DTT, 5mM K_2HPO_4, and 0.5mM $CaCl_2$ (magnesium concentration is indicated above); and (iii) HiFi buffer - 3.5mM $MgCl_2$ or 14mM $MgCl_2$ and 30mM KCl, 70mM NH_4Cl, 8mM putrescine, 0.5mM spermidine, 1mM DTT, and 50mM Tris HCl, pH 7.5 (magnesium concentration is indicated above). The glutamate buffer used in this study enables at least 4-fold higher levels of luciferase synthesis at the same magnesium concentration. The use of lower magnesium concentration yielded no active particles. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 8



Supplementary Figure 8 | The impact of substituting *in vitro* transcribed and purified 23S rRNA for the 23S rRNA gene in an iSAT reaction making 50S subunits. The final yield of Fluc after a 4h reaction at 37ºC from 0.2μM 50S ribosomal components having purified *in vitro* transcribed 23S rRNA added to a ribosome assembly and translation assay (Purified 23S rRNA) as compared to *in vitro* transcribed 23S rRNA from the 23S rRNA gene (iSAT) paired with 0.4μM native 30S subunits. Reactions in the absence of 23S rRNA did not yield active particles (not shown). The ~200-fold greater luciferase synthesis capability of the iSAT approach suggests benefits for co-rRNA transcription and ribosome assembly in the presence of S150 extract. Values shown indicate the mean with error bars representing the s.d. of 3 independent experiments.

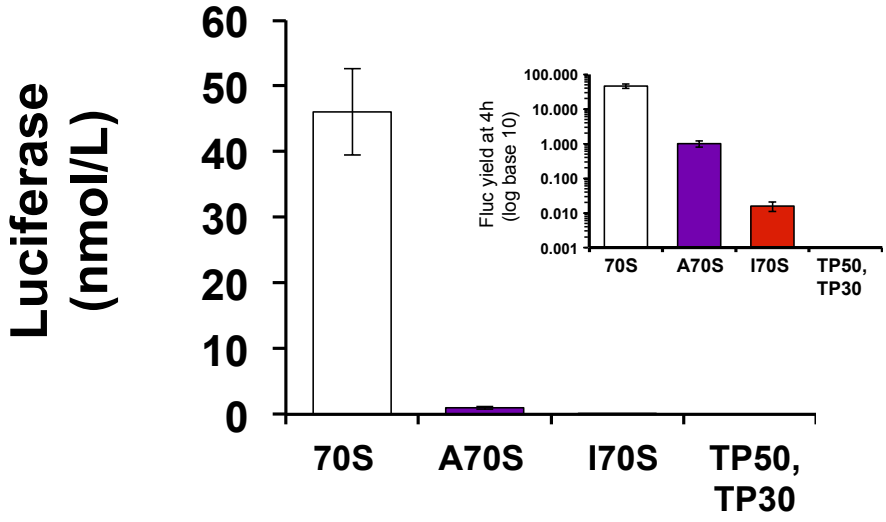
Supplementary Figure 9



Supplementary Figure 9 | Synthesis of non-specific rRNA in reactions integrating rRNA synthesis, ribosome assembly, and translation (iSAT) demonstrates that possible contaminating native rRNA is not masked by excess ribosomal proteins. To test that the activity of our *in vitro* transcribed rRNA was not the result of unmasking some minor level of contaminating native rRNA from the purified TP50 or TP30 mixtures (as previously observed in the RNAse P field (Gold & Altman, 1986)), we carried out rRNA swapping experiments as inbuilt controls. Since unmasking may conceivably have some sequence specificity, it should also be noted that there is an excess of tRNA and luciferase mRNA in the reactions. Moreover, we also point out that unmasking has not previously been reported when using *in vitro* transcribed rRNA to assemble ribosomes (Green & Noller, 1996; Semrad & Green, 2002). **a,** 23S rRNA was transcribed in 30S subunit iSAT reactions by substituting plasmid pCW1 (encoding the gene for 23S rRNA) for pWK1 (encoding the gene for 16S rRNA). The final yields of Fluc after a 4h reaction at 37ºC from 0.2μM 30S ribosomal parts having mature 16S rRNA (native), *in vitro* transcribed 16S rRNA (+IVT), *in vitro* transcribed 23S rRNA (+IVT), and no 16S rRNA (-IVT) paired with 0.4μM native 50S subunits were compared. Reactions in the absence of 16S rRNA did not yield active particles. Doping in purified naturally derived 23S rRNA into 30S assembly reactions with *in vitro* transcribed 16S rRNA had no effect (not shown). **b,** 16S rRNA was transcribed in 50S subunit iSAT reactions by substituting plasmid pWK1 for pCW1. The final yields of Fluc after a 4h reaction at 37ºC from 0.2μM 50S ribosomal parts having mature 23S rRNA (native), *in vitro* transcribed 23S rRNA (+IVT), *in vitro* transcribed 16S rRNA (+IVT), and no 23S rRNA (-IVT) paired with 0.4μM native 30S subunits were compared. Reactions in the absence of 23S rRNA did not yield active particles. Doping in purified naturally derived 16S rRNA into 50S assembly reactions with *in vitro* transcribed 23S rRNA had no effect (not shown).

Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

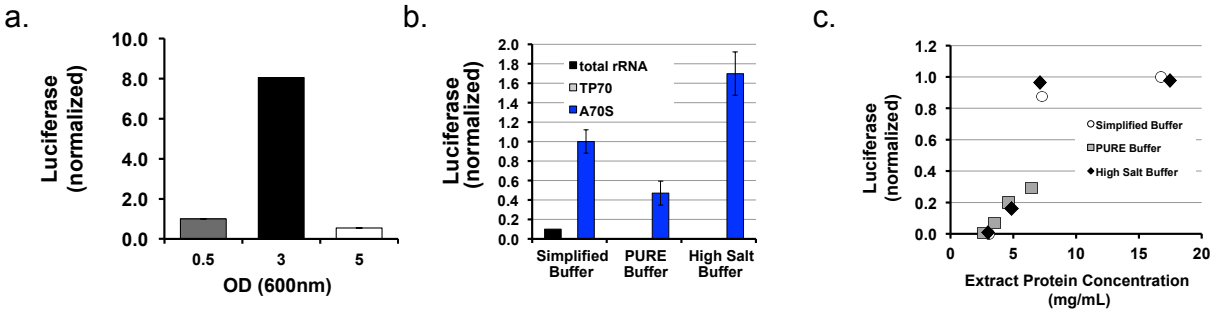
Supplementary Figure 10



Key:
"**A**" - ribosomes or ribosomal subunits **assembled** from mature rRNA
"**I**" - ribosomes or ribosomal subunits built using **iSAT**

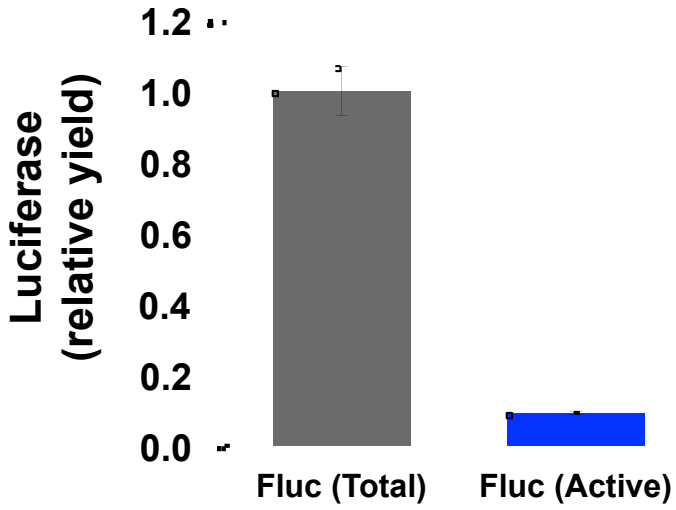
Supplementary Figure 10 | Translation activity of 70S ribosomes assembled from *in vitro* synthesized 23S and 16S rRNA in an S150 extract-based ETTA system. The protein synthesis activities of tightly coupled 70S ribosomes as well as 70S ribosomes assembled from mature 16S and 23S rRNA (A70S) or with *in vitro* transcribed 16S and 23S rRNA (I70S) were assessed following a 4h incubation at 37°C. The final yield of Fluc following 4h ETTA reactions at 37ºC is shown. Inset shows Fluc yield values at 4h on a log scale (base 10). Reactions without rRNA (TP50, TP30) did not yield active particles. Values shown indicate the mean with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 11



Supplementary Figure 11 | Alterations to the S150 extract preparation protocol enhance integrated ribosome assembly and translation. The protein synthesis activities of 70S ribosomal components (*i.e.*, mature 23S, 16S and 5S rRNA (total rRNA), and total protein of the 70S ribosome (TP70)) were compared in an S150 assembly and translation reaction following a 4h incubation at 37°C. **a,** The impact of harvest OD_{600} on ribosome assembly and translation. Specifically, we explored whether harvesting cells for S150 extract preparation in mid-exponential phase (rather than early log phase OD_{600} = 0.5) would provide benefits. Accordingly, we grew MRE600 cells to 0.5, 3, and 5 OD_{600}. The most active extracts were from cultures harvested at OD_{600} = 3.0. Negative control reactions (*e.g.*, total rRNA or TP70 alone) gave immeasurable results over all conditions (data not shown). **b,** The impact of extract buffer on S150 extract activity. Three extracts were prepared as originally described, except the cells were grown in a 10L fermentor to OD_{600} = 3.0, and one of three dialysis buffers was used: Simplified Buffer (10 mM TrisOAc, pH 7.5 at 4°C, 10 mM Mg $(OAc)_2$, 2 mM DTT), PURE Buffer (50 mM HEPES-KOH pH 7.6, 100 mM KGlu, 13 mM Mg $(OAc)_2$, 2 mM spermidine, 1 mM DTT), or High Salt Buffer (10 mM TrisOAc, pH 7.5 at 4°C, 10 mM Mg $(OAc)_2$, 20 mM NH_4OAc, 30 mM KOAc, 200 mM KGlu, 1 mM spermidine, 1 mM putrescine, 1 mM DTT). The High Salt Buffer enabled the highest yields of luciferase following an assembly and translation reaction. Reactions in the absence of total rRNA or TP70 are also shown. **c,** The impact of condensation on extract activity. The protein synthesis activities from extracts condensed to different concentrations, and dialyzed with different buffers as indicated, were assessed. We found extract protein concentration of about 10mg/mL to have maximum luciferase synthesis. Note that this is not the total protein concentration of the ETTA reaction, which is 4-fold lower. Values shown indicate means with error bars representing the s.d. of 3 independent experiments.

Supplementary Figure 12

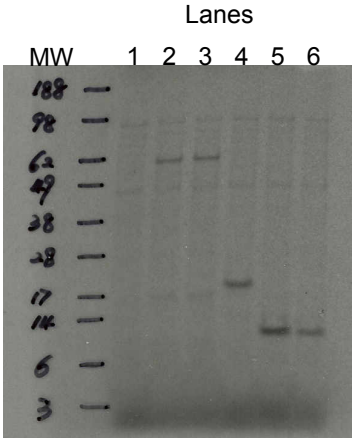


Supplementary Figure 12 | Comparison of total and active luciferase production. Here, Fluc synthesis is carried out at 37ºC for 4h in S150 ETTA reactions. The normalized protein synthesis activity from 0.2μM native 50S subunits paired with 0.4μM native 30S subunits, 0.2μM 50S ribosomal parts (*e.g.*, mature 23S rRNA, 5S rRNA and TP50) paired with 0.4μM native 30S subunits, and 0.2μM 30S ribosomal parts (*e.g.*, mature 16S rRNA and TP30) paired with 0.4μM native 50S subunits are averaged in the above data (n=2 for each case). For both native and assembled ribosomes, ~10% of luciferase synthesized is active. Total yield of Fluc as determined by ^{14}C-leucine incorporation by TCA precipitable counts using a filter paper assay and liquid scintillation counting (grey bar). Active yield of Fluc as determined by enzymatic assay (blue bar).

If one were to assume 100% yield of active ribosomes (*i.e.,* all ribosomal components assemble into functional translating ribosomes) and 100% active luciferase, approximately 13 ± 3 and 106 ± 8 peptide bonds per 70S ribosome assembled from *in vitro* transcribed or mature 23S and 16S rRNA, respectively, were synthesized in the enhanced extracts (**Figure 4a**). However, we measured that only about 10% of the luciferase produced in our system is active, with either native or assembled ribosomes (see above). If we estimate that the assembly efficiency in our extract based system is as good as the traditional method (~50%), then our data suggest that the system is synthesizing about 260 ± 60 or 2120 ± 160 peptide bonds per ribosome (*i.e.,* 13 ± 3 /(10%*50%)) from *in vitro* transcribed or mature 23S and 16S rRNA, respectively. For

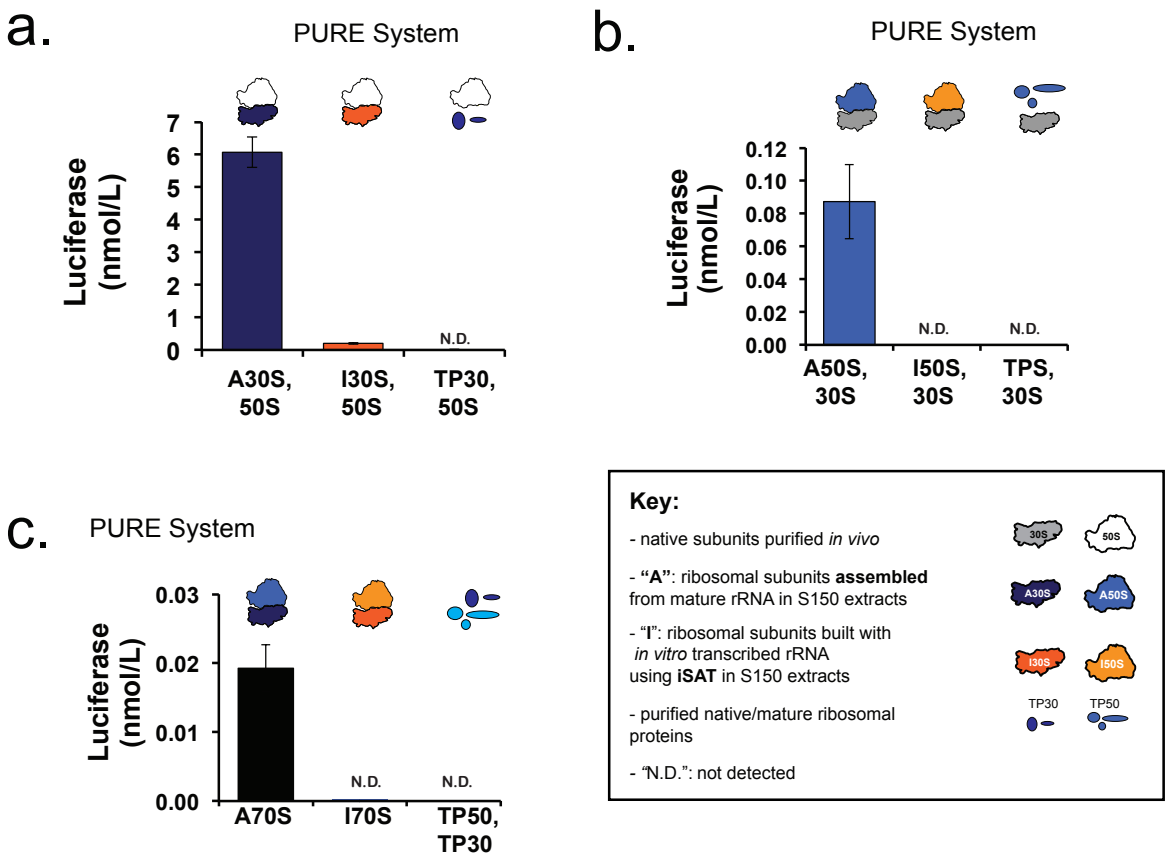
comparison, the best batch S30 crude extract cell-free protein synthesis systems (~10g *E. coli* protein/L with ribosomes) synthesize ~10,000-15,000 peptide bonds per ribosome (Jewett & Forster, 2010). *In vivo* (~200g *E. coli* protein/L), we estimate that about 45,000 ± 10,000 peptide bonds per ribosome are made. This estimate for *in vivo* capability was calculated using two different approaches. In one approach, we use the rRNA to protein ratio (Neidhardt, 1987). For example, if the mass ratio is 15%/6% and there are 4566 rNMPs per ribosome and the monomer mass ratio is 330/110 then (15/6)*(330/110)*4566 ~ 35,000 peptide bonds per ribosome. Using an alternative approach, one estimates ~ 55,000 peptide bonds per ribosome. If the cellular concentration is ~200g protein/L (Record et al, 1998a; Record et al, 1998b), then to double the cell needs to remake it's protein complement. Using an average amino acid MW of 110g/mol, a ribosome concentration of 42μM and estimating 80% active ribosomes in translation (Underwood et al, 2005), then (200g/L)*(mol/110g)*(10^6 μM/M)* (1/ (42 μM*0.8)) ~ 55,000 peptide bonds per ribosome.

Supplementary Figure 13



Supplementary Figure 13 | Autoradiogram showing ^{35}S-methionine incorporation into proteins synthesized by ribosomes assembled using the iSAT method. Combined 23S rRNA transcription, 50S subunit assembly, and translation reactions (15μL) with 0.2μM 30S subunits and 0.1μM 50S components (5S rRNA and TP50) and the plasmid encoding 23S rRNA. Reactions were carried out for 4h at 37°C. Lane 1: negative control – no 23S rRNA with luciferase plasmid. Lane 2: luciferase plasmid, expected size 61kDa. Lane 3: luciferase plasmid, expected size 61kDa. Lane 4: dihydrofolate reductase (DHFR) plasmid, expected size 18kDa. Lane 5: rpsJ plasmid, expected size 12kDa. Lane 6: rpsJ plasmid, expected size 12kDa. Lanes 5 and 6 show that ribosomes assembled from *in vitro* transcribed 23S rRNA can synthesize r-proteins, suggesting the potential for making all r-proteins.

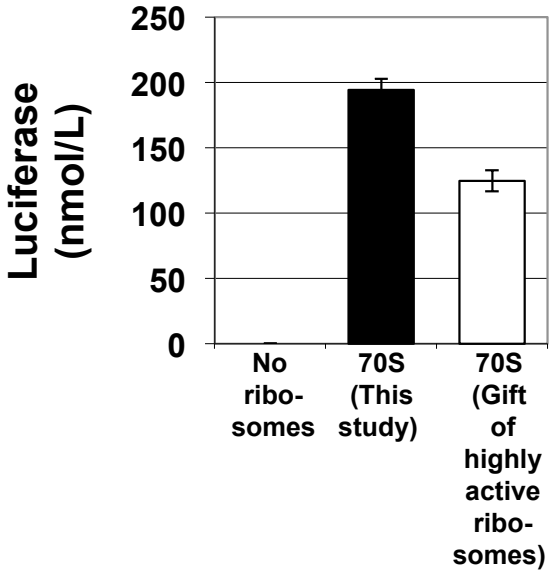
Supplementary Figure 14



Supplementary Figure 14 | Integrated ribosome assembly and translation in the PURExpress® system lacking ribosomes. a, The protein synthesis activities of 0.4μM assembled 30S subunits paired with 0.8μM native 50S subunits in the PURExpress® translation system lacking ribosomes. Subunits were either assembled from mature ribosomal parts (A30S) or built using the iSAT approach (I30S), where the mature 16S rRNA was replaced by the 16S rRNA gene behind the T7 promoter. Reactions in the absence of 16S rRNA (negative control) did not yield active particles. **b,** The protein synthesis activities of 0.4μM assembled 50S subunits paired with 0.8μM native 30S subunits were assessed following a 4h incubation at 37°C. 50S subunits were assembled from mature ribosomal components (A50S). However, the iSAT approach (I50S), where the mature 23S rRNA was replaced by the 23S rRNA gene behind the T7 promoter, did not yield active ribosomes. This defines the current block to fully characterized ribosome synthesis. Reactions in the absence of 23S rRNA (negative control) did not yield active particles. **c,** The protein synthesis activities of 0.4μM 70S ribosomes were assessed following a 4h incubation at 37°C. 70S subunits were assembled from mature

ribosomal components (A70S). However, the iSAT approach (I70S), where mature 23S and 16S rRNAs were replaced by the 23S and 16S rRNA genes behind T7 promoters, did not yield active ribosomes. Reactions in the absence of rRNA (negative control) did not yield active particles. Values shown indicate means with error bars representing the s.d. of 4 independent experiments.

Supplementary Figure 15



Supplementary Figure 15 | Comparison of Fluc production between our control 70S ribosomes (this study) and an independent set of 70S ribosomes (gift) demonstrates high ribosome quality. We tested our ribosome quality in head-to-head ETTA reactions against an independent set of highly active coupled 70S ribosomes that were given to us as a generous gift and had been purified according to Rodnina and Wintermeyer (Rodnina & Wintermeyer, 1995). ETTA reactions with 0.5μM 70S ribosomes were carried out at 37ºC for 4h. Synthesis of active luciferase was determined by enzymatic assay. Values shown indicate means with error bars representing the s.d. of 4 independent experiments.

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