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REPORT

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

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Purely *in vitro* ribosome synthesis could provide a critical step towards unraveling the systems biology of ribosome biogenesis, constructing minimal cells from defined components, and engineering ribosomes with new functions. Here, as an initial step towards this goal, we report a method for constructing *Escherichia coli* ribosomes in crude S150 *E. coli* extracts. While conventional methods for *E. coli* ribosome reconstitution are non-physiological, our approach attempts to mimic chemical conditions in the cytoplasm, thus permitting several biological processes to occur simultaneously. Specifically, our integrated synthesis, assembly, and translation (iSAT) technology enables one-step co-activation of rRNA transcription, assembly of transcribed rRNA with native ribosomal proteins into functional ribosomes, and synthesis of active protein by these ribosomes in the same compartment. We show that iSAT makes possible the *in vitro* construction of modified ribosomes by introducing a 23S rRNA mutation that mediates resistance against clindamycin. We anticipate that iSAT will aid studies of ribosome assembly and open new avenues for making ribosomes with altered properties.

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Introduction

The *in vitro* construction of ribosomes is a topic of rapidly growing interest in systems and synthetic biology. These interests aim to elucidate broad principles that underlie the operation and assembly of the translation apparatus (Nierhaus, 1990; Erlacher *et al*, 2011; Polacek, 2011), design and build minimal cells to understand origins of life (Forster and Church, 2006; Jewett and Forster, 2010), and enable *in vitro* evolution to select for ribosomes that have enhanced functions or altered chemical properties (Cochella and Green, 2004; Wang *et al*, 2007; Neumann *et al*, 2010). To realize these goals, methods for *in vitro* ribosome synthesis are needed.

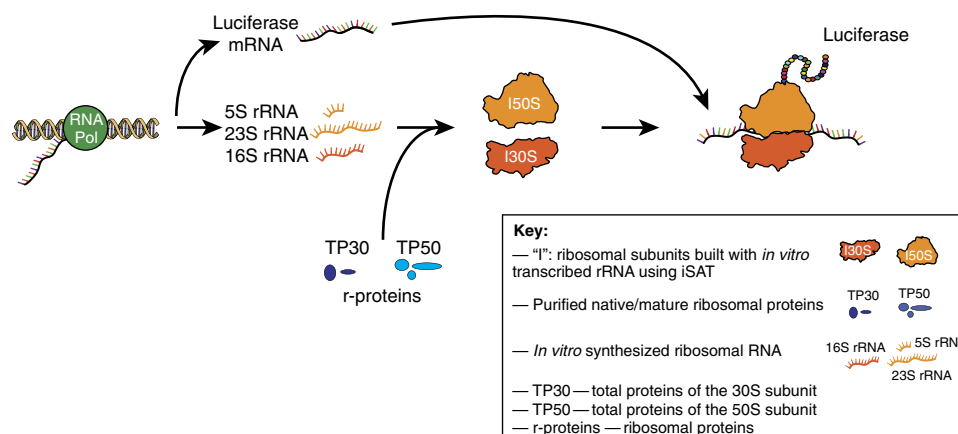
In vitro assembly, or reconstitution, of *Escherichia coli* ribosomes from purified native ribosomal components into functionally active small (30S) and large (50S) ribosomal subunits was first achieved in pioneering works ~40 years ago. The conventional 30S subunit reconstitution protocol involves a one-step incubation at 20 mM Mg²⁺ and 40°C (Traub and Nomura, 1968), and can be facilitated at lower temperatures by chaperones (Maki and Culver, 2005). The conventional 50S subunit reconstitution protocol involves a non-physiological two-step high-temperature incubation, first

at 4 mM Mg²⁺ and 44°C, then at 20 mM Mg²⁺ and 50°C (Nierhaus and Dohme, 1974).

While studies using the conventional reconstitution approach have revealed many important insights into ribosome assembly (Nierhaus, 1990), inefficiencies in reconstitution make the construction and analysis of engineered variants difficult (Semrad and Green, 2002). For example, conventionally reconstituted 50S subunits made with *in vitro*-transcribed 23S rRNA (lacking the naturally occurring post-transcriptional modifications) are up to 10 000 times less efficient in reconstitution than those using mature 23S rRNA as measured by the fragment reaction, where single peptide bonds are formed on isolated 50S subunits (Semrad and Green, 2002). Furthermore, the non-physiological two-step conditions for 50S assembly preclude coupling of ribosome synthesis and assembly in a single, integrated system.

In contrast to previous schemes, we aimed to develop an integrated method for the physiological assembly of *E. coli* ribosomes, in which ribosomes assemble from *in vitro*-transcribed rRNA and then conduct protein synthesis in the same compartment (Box 1). This approach mimics co-transcription of rRNA and ribosome assembly as it occurs *in vivo* (Talkington *et al*, 2005; Mulder *et al*, 2010). Moreover, it

Box 1 Integrated rRNA synthesis, ribosome assembly, and translation (iSAT)



iSAT: an integrated method for the assembly of ribosomes from *in vitro*-transcribed rRNA and subsequent translation by these ribosomes in the same compartment. In a single reaction, mRNA and rRNA are synthesized from plasmids by RNA polymerase (RNA Pol). rRNA assembles into ribosomal subunits with total ribosomal proteins purified from the subunits of tightly coupled ribosomes. mRNA is translated by newly assembled ribosomes and the resulting protein yield is measured to assess ribosome activity. Mutant genes encoding rRNA enable simple and efficient generation of modified ribosomes.

is aligned with a general guiding principle in cell-free synthetic biology; namely, that cytoplasmic mimicry provides advantages for reproducing cell-like behavior (Jewett *et al*, 2008; Hodgman and Jewett, 2012). Here we demonstrate our new method for integrated rRNA synthesis, ribosome assembly, and translation, termed iSAT (Box 1). In addition, we show how iSAT can be utilized to efficiently make a modified ribosome that is highly resistant to the antibiotic clindamycin in a single step. Although *Bacillus stearothermophilus* (Green and Noller, 1999) and *Thermus aquaticus* (Khaitovich *et al*, 1999; Erlacher *et al*, 2011) have quite active ribosomes reconstituted from *in vitro*-transcribed 23S rRNA lacking modifications, we focus on *E. coli* ribosomes because the translation apparatus of *E. coli* is the best understood and most characterized both biochemically and genetically (Forster and Church, 2006; Jewett and Forster, 2010).

Results and discussion

With technological applications of iSAT in mind, we chose to evaluate the activity of *in vitro* assembled ribosomes by quantifying their ability to synthesize active proteins. However, reconstituted *E. coli* ribosomes have, to our knowledge, not been tested for their ability to synthesize proteins. Activity is typically assessed by testing for the ability of ribosomal particles to bind tRNA (Maki and Culver, 2005), to form peptide bonds (Semrad and Green, 2002), or to synthesize poly(Phe) from UUU-templates (Nierhaus, 1990). Thus, to determine benchmark protein synthesis activity, we first assembled ribosomes by conventional reconstitution from purified components (e.g., mature 16S rRNA and the total proteins of the 30S subunit (TP30)) (Figure 1A). Then, we measured overall protein synthesis activity by directly adding the reconstitution reaction to a crude S150 *E. coli* extract-based transcription and translation assay (ETTA) (Figure 1A). The assay was programmed to synthesize firefly luciferase (Fluc). We observed that

the final Fluc yield after a 2-h incubation at 30°C from 30S and 50S reconstitution reactions (denoted 'R') was ~85 and ~50%, respectively, of intact native subunits (Figures 1B and C). These data are consistent with earlier results for poly(Phe) synthesis (Nierhaus, 1990). Protein synthesis reactions with excess 30S subunits performed better (see native ribosome control reactions in Figure 1C relative to Figure 1B), which we hypothesize is because initiation is limiting. Synthesis of green fluorescent protein was also possible (Supplementary Figure 1), but Fluc was chosen because of its sensitivity.

Based on our hypothesis that cytoplasmic mimicry would promote iSAT, we next redesigned the reconstitution process. We targeted changes in the ionic composition, because the conventional method uses 400 mM ammonium chloride, which is non-physiological, inhibits *in vitro* translation, and may interfere with favorable non-covalent interactions (Jewett and Swartz, 2004a). Instead of ammonium and chloride, we chose to use potassium and glutamate, because these are the most abundant ions in the *E. coli* cytoplasm and they have previously made possible the *in vitro* co-activation of central metabolism, oxidative phosphorylation, and protein synthesis (Jewett *et al*, 2008). We observed that 50S reconstitution reactions with potassium glutamate were equivalent to those with the conventional salts in our ETTA system (Figure 1D; Supplementary Figures 2 and 3). Functional 50S subunits, as well as 70S ribosomes, could also be assembled at constant 37°C and a single magnesium concentration when reconstituted in potassium glutamate (Figure 1D; Supplementary Figure 4). These results were unexpected because previous reports using ammonium chloride salts have not shown assembly of ribosomes at a single magnesium concentration and constant temperature to be possible (Nierhaus, 1990). In follow-up experiments, we provided evidence that our observation was due to the chemical composition of both our reconstitution and ETTA methods (Supplementary Table 1; Supplementary Figure 5).

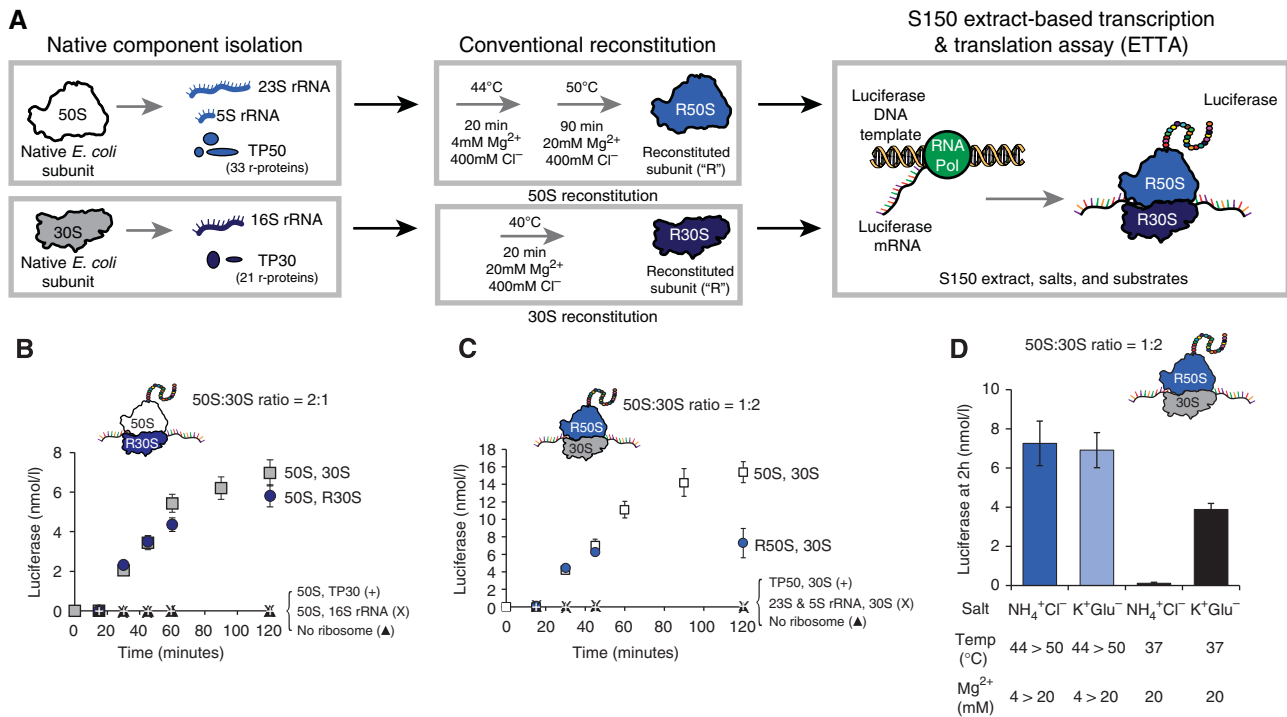


Figure 1 Reconstituted ribosomes synthesize active firefly luciferase (Fluc). **(A)** Basic steps of conventional ribosome reconstitution methods are illustrated (Nierhaus, 1990). Ribosomal components (e.g., 23S rRNA, 5S rRNA, and TP50) are first prepared from the native subunits of tightly coupled 70S ribosomes. Then, ribosomal subunits are reconstituted in defined conditions from mature rRNA and proteins. Finally, in a separate reaction, the activity of assembled subunits (denoted with 'R') is assessed by directly adding the reconstitution reaction to our ETTA system. **(B)** The protein synthesis activities of 0.1 μ M native 30S subunits or conventional 30S reconstitution reactions having 0.1 μ M ribosomal components (R30S) with 0.2 μ M native 50S subunits in an ETTA reaction. Batch reactions were carried out for 2 h at 30°C, where Fluc synthesis saturates. Reconstitutions in the absence of 16S rRNA or TP30 did not yield active particles. **(C)** The protein synthesis activities of 0.1 μ M native 50S subunits or conventional 50S reconstitution reactions having 0.1 μ M ribosomal components (R50S) with 0.2 μ M native 30S subunits in an ETTA reaction. Batch reactions were carried out for 2 h at 30°C. Reconstitutions in the absence of 23S rRNA and 5S rRNA or TP50 did not yield active particles. Yield differences between the control reactions in **B** and **C** are not due to run-to-run variability, which is shown by the standard deviation bars and is low. Rather, the changes are caused by using different ratios of 30S to 50S subunits to ensure an excess of the non-assembled ribosomal subunit. **(D)** The activities of 50S reconstitution reactions having 0.1 μ M ribosomal components (R50S) with 0.2 μ M native 30S subunits were compared for different salt, temperature, and magnesium conditions. Angle brackets indicate two-step reactions. The final yield of Fluc after 2h batch incubations in an ETTA reaction is shown. Negative control reactions (e.g., TP50, 30S subunit) gave immeasurable results overall conditions (not shown). Values in all panels show means with error bars representing standard deviations (s.d.) of at least eight independent experiments.

Following demonstration of one-step *in vitro* ribosome reconstitution, we next aimed to integrate ribosome assembly and translation *in vitro*. This is a distinct break from previous works, in which reconstitution must be carried out under conditions that do not support protein synthesis. The key point was to add ribosomal components directly to our ETTA system. Indeed, we observed that functionally active *E. coli* ribosomal subunits assembled from their individual rRNA and r-protein components and synthesized protein in a single reaction (denoted 'A' for ribosomes assembled in S150 extracts). When we used 0.2 μ M 30S or 50S ribosomal components (i.e., mature 23S rRNA, 5S rRNA, and TP50 for 50S subunits), we observed the synthesis of 1.02 ± 0.04 nmol/l (Figures 2A and B) and 3.27 ± 0.46 nmol/l Fluc (Figures 2C and D), respectively, after a 4-h reaction at 37°C. When we simultaneously assembled both subunits, we observed 1.10 ± 0.31 nmol/l Fluc (Supplementary Figure 6).

Our integrated approach synthesizes about 50% as much active Fluc based on the total number of picomoles of natural 23S rRNA as compared to our earlier results with separate ribosome assembly and translation-only reactions (see Figure 1, Supplementary Figures 3 and 4 for comparison).

However, it is difficult to put these numbers into context because of the fundamental differences between these reactions (e.g., temperature optimums, length of reaction) and the fact that S150 extract based cell-free translation may have substrate instabilities that plague extended reaction durations (Jewett and Swartz, 2004b; Carlson *et al*, 2012).

We subsequently carried out a series of optimization experiments to try to increase combined ribosome assembly and protein synthesis activity. We explored the effects of reaction temperature, Mg²⁺ concentration, polyamine concentrations, r-protein/rRNA ratios, and buffer components including the use of classical 'polymix' and 'HiFi' buffers (Supplementary Table 2; Supplementary Figures 6 and 7). We did not observe improvements despite a rigorous search, suggesting that ribosome assembly and translation work best in similar physicochemical conditions as captured in our original ETTA system.

We next asked whether functional ribosomes could assemble from rRNA transcribed by RNA polymerase concurrently with translation in the same *in vitro* reaction (Box 1). We chose to focus first on 50S subunits, because the use of *in vitro*-transcribed 23S rRNA has represented the most serious

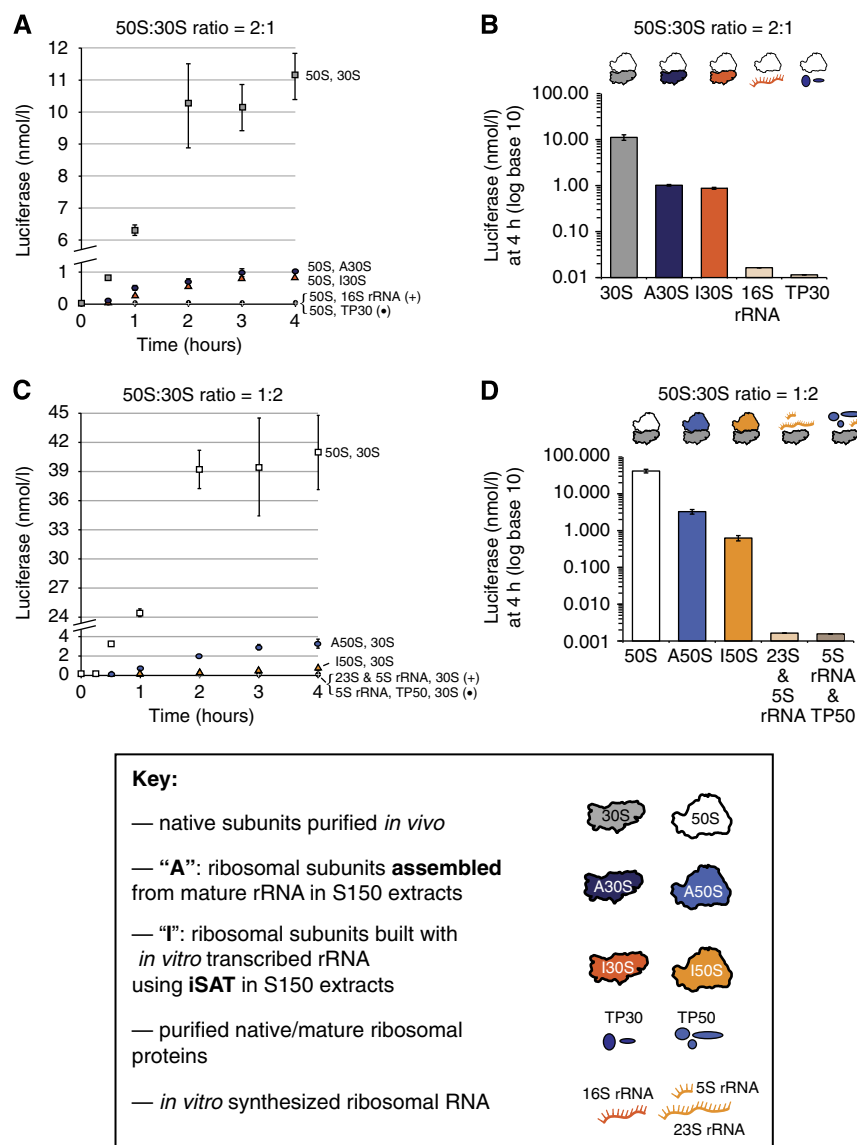


Figure 2 Integrated rRNA synthesis, ribosome assembly, and translation. (A) The protein synthesis activities of 0.2 μ M native or assembled 30S subunits with 0.4 μ M native 50S subunits in an ETNA reaction over time. Subunits were either assembled in S150 extracts from mature ribosomal parts (A30S) or built using the iSAT approach (I30S). In iSAT, the mature 16S rRNA was replaced by the 16S rRNA gene behind the T7 promoter. Reactions in the absence of 16S rRNA or TP30 did not yield active particles. Values are plotted with an axis break. (B) Final Fluc synthesized in nmoles/l after 4 h at 37°C on a log scale (base 10) for the data shown in ‘A’. (C) The protein synthesis activities of 0.2 μ M native or assembled 50S subunits with 0.4 μ M native 30S subunits in an ETNA reaction over time. Subunits were either assembled in S150 extracts from mature ribosomal parts (A50S) or built using the iSAT approach (I50S). In iSAT, the mature 23S rRNA was replaced by the 23S rRNA gene behind the T7 promoter. Reactions in the absence of 23S rRNA or TP50 did not yield active particles. Values are plotted with an axis break. (D) Final Fluc synthesized in nmoles/l after 4 h at 37°C on a log scale (base 10) for the data shown in ‘C’. Values in all panels show means with error bars representing the s.d. of at least four independent experiments.

bottleneck to *E. coli* ribosome construction *in vitro* (Semrad and Green, 2002). When we added 30S subunits, total proteins of the 50S subunit (TP50), and 5S rRNA directly to our ETNA system containing a plasmid encoding the 23S rRNA gene, iSAT was enabled at 37°C (denoted ‘I’ for iSAT). In these reactions, bacteriophage T7 RNAP was used to transcribe both the rRNA and the luciferase mRNA. Figure 2C shows Fluc accumulation over time for the optimized iSAT system, noting that there is a lag prior to the start of luciferase synthesis. The final yield of Fluc after the reaction saturated at 4 h was 0.63 ± 0.10 nmol/l (Figures 2C and D), while reactions without

the 23S rRNA gene showed no detectable luciferase synthesis (Figures 2C and D). After demonstrating iSAT, we subsequently confirmed that the activity of our *in vitro*-transcribed rRNA was not the result of unmasking some minor level of contaminating native rRNA from purified TP50 (Supplementary Figure 9). iSAT assembled 50S ribosomal subunits have ~20% the activity of ribosomes assembled in our ETNA system from mature rRNA (0.63 nmol/l versus 3.2 nmol/l). For comparison, in conventional reconstitution systems, 50S subunits assembled from *in vitro* transcripts of *E. coli* 23S rRNA have about 3% the activity of those assembled

from mature rRNA when stimulated by the antibiotic telithromycin and trimethylamine-oxide, or about 0.01% when these osmolytes are not added (Green and Noller, 1996, 1999; Semrad and Green, 2002). However, these studies utilized the fragment reaction, a measure of the reaction rate of single peptide bond formation on isolated 50S subunits. While it is difficult to compare the fragment reaction to the synthesis of an active 550-amino acid, two-domain eukaryotic Fluc protein, our results suggest that the iSAT approach may have efficiency advantages in ribosome assembly as compared to the conventional approach.

To assess if combining rRNA transcription and ribosome assembly was beneficial, we substituted the 23S rRNA gene with *in vitro*-transcribed and purified 23S rRNA. We observed that final Fluc yields were ~200-fold lower (Supplementary Figure 8), demonstrating that combining transcription and assembly was advantageous. As we did not analyze post-transcriptional modifications, it is unclear if the benefit of iSAT arises from the possibility of RNA modification by the presence of RNA modification enzymes or some other factor (e.g., assembly-helper proteins) in the extract. However, we observed in a follow-up experiment that supplementation of 10–100 μ M S-adenosyl-methionine, a potential methyl donor for nucleoside modification, did not stimulate the combined ribosome synthesis and assembly reaction. Further investigation is needed to fully characterize the ribosomes assembled in iSAT.

The iSAT approach was then extended to the assembly of functional 30S ribosomal particles. Based on the final yield of Fluc after a 4-h reaction at 37°C, 30S subunits assembled with *in vitro*-transcribed 16S rRNA had ~85% the activity of those assembled with mature 16S rRNA (Figures 2A and B). This is consistent with previous results showing that *in vitro*-transcribed 16S rRNA has a lower activity than mature 16S rRNA (Cunningham *et al*, 1991). As with 50S iSAT reactions, rRNA gene swapping experiments demonstrated no contaminating species (Supplementary Figure 9). We also constructed functional 70S ribosomes by the iSAT method (Supplementary Figure 10).

Any technological application of iSAT will require increased efficiency. To this end, we altered the S150 extract preparation procedure in search of parameters that would increase the level of Fluc synthesized in a 70S ribosome iSAT reaction, while maintaining low or negligible background levels of native ribosomes. Several alterations resulted in an increase in the overall translation activity of ribosomes made by the iSAT method. Specifically, harvesting the cells at OD₆₀₀ = 3 instead of OD₆₀₀ = 0.5, altering the dialysis buffer to increase salt concentrations and add polyamines, and increasing the total extract protein concentration from ~4 mg/ml to ~10 mg/ml resulted in an increase in Fluc synthesis (Supplementary Figure 11). Combined, these changes improved iSAT activity approximately 300-fold (Figure 3). iSAT assembled 70S ribosomes in the improved extracts have ~12% the activity

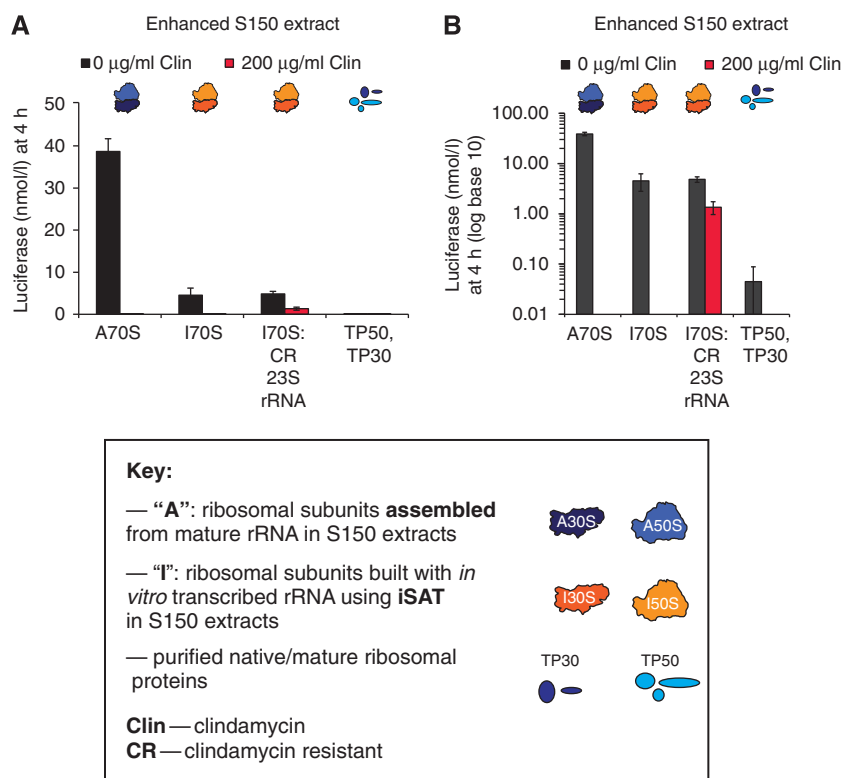


Figure 3 iSAT enabled construction of modified ribosomes. (A) The protein synthesis activities of wild-type and clindamycin-resistant (CR) ribosomes in a modified, enhanced S150 extract ETTA reaction with or without 200 μ g/ml clindamycin (Clin). 70S ribosomes were assembled from mature 16S and 23S rRNA (A70S), as well as *in vitro*-transcribed 16S and 23S rRNA with (I70S: CR 23S rRNA) and without (I70S) the CR mutation. Reactions in the absence of 23S and 16S rRNA did not yield active particles. (B) Final Fluc synthesized in nmoles/l after 4 h at 37°C on a log scale (base 10) for data shown in ‘A’. Values show means with error bars representing the s.d. of at least four independent experiments.

of ribosomes assembled from mature rRNA as measured by luciferase synthesis (4.8 nmol/l versus 38 nmol/l). We estimate that the number of peptide bonds synthesized per 70S ribosome assembled from *in vitro*-transcribed 16S and 23S rRNA or mature 16S and 23S rRNA to be 260 ± 60 or 2120 ± 160 , respectively (~ 0.5 or 4 Fluc proteins per ribosome, see Supplementary Figure 12).

With these improvements in hand, we then set out to demonstrate the utility of iSAT by assembling modified ribosomes. Specifically, we used iSAT to generate 70S ribosomes with a point mutation in their 23S rRNA (A2058U) that is resistant to the antibiotic clindamycin (Cochella and Green, 2004). Without clindamycin, the mutation was silent when compared to the wild-type sequence (Figures 3A and B). However, with 200 μ g/ml clindamycin, only the ribosomes with the A2058U 23S rRNA mutation synthesized 1.3 ± 0.4 nmol/l luciferase after a 4-h reaction at 37°C, whereas wild-type ribosomes did not make protein (Figures 3A and B).

Although the conventional reconstitution of ribosomes *in vitro* is well established (Nierhaus, 1990), the iSAT method offers a powerful new tool for observation and modification of the system under more physiological conditions. Furthermore, it was previously necessary to construct *E. coli* ribosomes *in vitro* using individually purified rRNAs (Green and Noller, 1996, 1999; Semrad and Green, 2002). Using iSAT, mutant ribosomes can be readily generated directly from plasmid DNA in a one-step reaction, streamlining the process for *in vitro* ribosome construction and study. iSAT can also accelerate our ability to assess the importance of assembly RNases, modifying enzymes, and assembly factors *in vitro*. Moreover, our integrated platform could be used in a high-throughput screen to identify novel antibiotics targeting ribosome assembly.

Beyond ribosome biogenesis studies, we expect that iSAT will also benefit efforts that seek to completely synthesize ribosomes *in vitro*, such as minimal cell projects (Forster and Church, 2006; Jewett and Forster, 2010; Stano and Luisi, 2013). Follow-up experiments showed that we could extend the iSAT method to the synthesis of ribosomal proteins (Supplementary Figure 13). However, improvements are needed to be able to synthesize the number of peptide bonds necessary for making one ribosome equivalent (7434 peptide bonds in total, Supplementary Table 3). Potential steps in this direction could include tuning rRNA-expression levels and rates, assessing and removing possible substrate limitations (e.g., ATP instabilities), and increasing ribosomal part concentrations, which are currently 10-fold lower than typical S30 crude extract systems. A further challenge is building a ribosome in a completely defined translation system. Although we have shown it possible to couple ribosome assembly and protein synthesis in the PURExpress[®] translation system lacking ribosomes (Supplementary Figure 14), 50S synthesis from *in vitro*-transcribed rRNA in this purified system is still a hurdle. Differences between the S150 extract based and PURE systems will enable eventual dissection of the responsible components. Looking forward, we anticipate that iSAT could also enable *in vitro* evolution to select for modified ribosomes that have altered chemical properties (Cochella and Green, 2004; Wang *et al*, 2007; Neumann *et al*, 2010).

Materials and methods

Isolation of tightly coupled 70S ribosomes and ribosomal subunits

Tightly coupled 70S ribosomes were obtained from strain MRE600 as reported by Blanchard *et al*, 2004. Notably, per this protocol, acetate salts were used in the sucrose gradients and final resuspension (acetate buffer: 20 mM TrisOAc (pH = 7.5 at 4°C), 60 mM NH₄Cl, 7.5 mM Mg(OAc)₂, 0.5 mM EDTA, and 2 mM dithiothreitol). 30S and 50S subunits were derived from ribosomes dialyzed against acetate buffer with only 1 mM Mg(OAc)₂ followed by three rounds of sucrose density ultracentrifugation in the same buffer. Results from five independent ribosome preparations and subsequent rRNA and total protein preparations were used and averaged to generate the final results shown in the manuscript.

S150 crude extract preparation

S150 extract was derived from the supernatant of the tightly coupled 70S ribosome pellet (see above). Specifically, S30 lysate generated from cells harvested at OD₆₀₀ of 0.5 was layered in a 1:1 volumetric ratio on a high-salt sucrose cushion containing 20 mM Tris-HCl pH = 7.2 at 4°C, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA, 6 mM beta-mercaptoethanol, and 37.7% sucrose in a Type Ti70 ultracentrifuge tube. Samples were subsequently centrifuged at 90 000 g overnight. Then, the supernatant was collected and centrifuged again at 150 000 g for three hours. The top two-thirds of this supernatant was collected, concentrated three-fold using Sartorius Vivaspin20 concentration modules (3000 MWCO), and dialyzed overnight against four exchanges of buffer containing 10 mM Tris-OAc pH = 7.5 at 4°C and 10 mM Mg(OAc)₂ for use as S150 extract. The final total *E. coli* protein concentration in the S150 extract was ~ 4 mg/ml, as determined by a Bradford assay using a commercially available assay reagent (Bio-Rad: Hercules, CA). Bovine serum albumin was used as a protein standard.

Isolation of mature rRNA and r-proteins

Total proteins of the 50S subunit (TP50), total proteins of the 30S subunit (TP30), mature 23S rRNA, mature 16S rRNA, and mature 5S rRNA were prepared as described by Nierhaus (Nierhaus, 1990). TP30 and TP50 were purified using acetone precipitation.

Ribosome reconstitution

Conventional reconstitution reactions were performed as previously described (Nierhaus, 1990). In brief, the conventional 30S reconstitution was a one-step procedure (Nierhaus, 1990). In a total volume of 15 μ l, 0.5A₂₆₀ units of 16S rRNA were incubated with 1.2 equivalents of TP30 in 20 mM Tris-OAc (pH 7.4 at 37°C), 20 mM Mg(OAc)₂, 400 mM NH₄Cl, 0.2 mM EDTA, 5 mM 2-mercaptoethanol at 40°C for 20 min. The conventional 50S reconstitution was a two-step procedure (Nierhaus, 1990). In a total volume of 15 μ l, 0.5A₂₆₀ units of 23S rRNA, 0.02A₂₆₀ units of 5S rRNA were incubated with 1.2 equivalents of TP50 in 20 mM Tris-OAc (pH 7.4 at 37°C), 4 mM Mg(OAc)₂, 400 mM NH₄Cl, 0.2 mM EDTA, 5 mM 2-mercaptoethanol at 44°C for 20 min. Then, the Mg(OAc)₂ concentration was raised to 20 mM and the temperature was raised to 50°C for 90 min. The redesigned, more physiological, reconstitution reactions described in this work substituted potassium glutamate for NH₄Cl and altered the temperature and Mg(OAc)₂ concentration profiles as described in the text. Glutamate salts were selected to mimic the *in vivo* state *in vitro* (Record *et al*, 1998). Following reconstitution, subunit concentrations were calculated from A₂₆₀ measurements (1A₂₆₀ unit of 50S = 36 pmol, 1A₂₆₀ unit of 30S = 72 pmol (Nierhaus, 1990)).

Extract-based transcription and translation assay (ETTA)

The activity of 50S and 30S ribosomal subunits, as well as 70S ribosomes, was assessed in an S150 ETTA. ETTA reactions were

programmed to synthesize a reporter gene (either Fluc or enhanced green fluorescent protein (GFP)) and were carried out as 15 µl batch reactions in 1.5 ml Eppendorf tubes for 2 h at 30°C (the observed optimum temperature for maximum active Fluc and GFP synthesis). To exclude the possibility of contaminating ribosomal subunits in the S150 extract, we performed the ET TA assay with reconstitution reactions containing only 16S rRNA or TP30, or only 23S rRNA and 5S rRNA or TP50. In these experiments, no protein synthesis was observed. The standard reaction mixture contained the following components that were taken from the Cytomim *in vitro* transcription and translation system (Jewett and Swartz, 2004a; Jewett et al, 2008): 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 34 µg/ml folinic acid, 170.6 µg/ml *E. coli* tRNA mixture (Roche, Indianapolis, IN), 16.6 µg/ml plasmid DNA (pk7LUC or pk7GFP, see below), 100 µg/ml T7 RNA polymerase, 2 mM each of 20 unlabeled amino-acids, 33 mM phosphoenolpyruvate (Roche), 0.33 mM NAD, 0.26 mM CoA, 130 mM potassium glutamate, 10 mM ammonium glutamate, 14 mM magnesium glutamate, 1.5 mM spermidine, 1 mM putrescine, 4 mM sodium oxalate, and 0.24 volume of S150 extract. Except where specified, reagents were from Sigma (St Louis, MO). T7 RNA polymerase was prepared as described earlier (Jewett and Swartz, 2004a). In the case of 50S or 30S assembly, background protein production levels with 30S or 50S subunits only, respectively, were subtracted from measured values. Comparison of luciferase production from an independent set of highly active ribosomes was used to confirm ribosome quality (Supplementary Figure 15). Subunit molar ratios for the various reconstitution experiments were 50S:R30S = 2:1 and R50S:30S = 1:2. Plasmids pk7LUC (encoding the gene for Fluc) and pk7GFP (encoding the gene for eGFP) were constructed for this study. The Fluc gene (from pZE21-luc (Lutz and Bujard, 1997)) and the GFP gene (from pZE21G (Isaacs et al, 2004)) were amplified by PCR and subcloned into the expression plasmid pk7 after removing the CAT-encoding sequence from pk7CAT (Jewett et al, 2008) to yield pk7LUC and pk7GFP. The following primer sequences were used: 5' primer for Fluc, 5'-GGT-GGT-CAT-ATG-GAA-GAC-GCC-AAA-AA-C-AT-3'; 3' primer for Fluc, 5'-GGT-GGT-GTC-GAC-TTA-CAA-TTT-GGA-C TT-TCC-GC-3'; 5' primer for GFP, 5'-GGT-GGT-TCT-AGA-AAT-AAT-TTT-G TT-TAA-CTT-TAA-GAA-GGA-GAT-ATA-CAT-ATG-CGT-AAA-GGA-GAA-GA-A-CTT-T-3'; 3' primer for GFP, 5'-GGT-GGT-GTC-GAC-TTA-TTT-GTA-TAG-TTC-ATC-CAT-GCC-A-3'. Nucleotide sequences of recombinant genes were verified by DNA sequencing (Agencourt Bioscience).

Reporter protein quantification

Bioluminescence of active Fluc was measured using the SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Forty microliters of ONE-Glo™ luciferase assay reagent was combined with the entire 15 µl reaction, and luminescence was measured immediately thereafter. Relative light units were converted to an estimate of protein concentration using a standard curve created from dilutions of purified recombinant Fluc (1RLU ~ 15 fg). The active amount of GFP synthesized in each ET TA reaction was calculated by measuring the fluorescence after a maturation period of 8 h at 4°C. Longer maturation times did not increase fluorescence. By calculating the signal-to-background ratio (S-B)/B for multiple wavelength pairs, the optimum excitation and emission spectra were determined to be 490 nm/520 nm. Fluorescence was measured using the SpectraMax M5 plate reader (Molecular Devices). Fluorescence was converted to an estimate of protein concentration using a standard curve created from dilutions of purified recombinant GFP (Roche Diagnostics Corporation, Indianapolis, IL) (excitation/emission = 395 nm/510 nm). To quantify the amount of synthesized protein using radioactivity, reaction samples were analyzed by incorporation of ¹⁴C-leucine into trichloroacetic acid-precipitable radioactivity using a liquid scintillation counter (MicroBeta2, PerkinElmer, Waltham, MA) following treatment with 0.1 N NaOH to stop translation as previously described (Jewett et al, 2008).

Integrated ribosome assembly and translation

For the 50S subunit, 0.2 µM (or as specified) mature 23S rRNA, mature 5S rRNA, TP50, and 0.4 µM (or as specified) native 30S subunits were added directly to S150 extract on ice. Next, a pre-chilled reagent mixture

at 4°C comprising the necessary salts, energy substrates, T7 RNA polymerase, and pk7LUC for the ET TA reaction was added. The reaction was mixed to homogeneity by pipetting and then incubated for up to 4 h at 37°C. For the 30S subunit, 0.2 µM (or as specified) mature 16S rRNA and TP30, and 0.4 µM (or as specified) native 50S subunits were added directly to an S150 ET TA reaction. For the 70S ribosome, 0.2 µM (or as specified) mature 23S rRNA, 16S rRNA, 5S rRNA, TP50, and TP30 were added directly to an S150 extract ET TA reaction. In some experiments, polymix (Jelenc and Kurland, 1979) and HiFi (Gromadski and Rodnina, 2004) buffers were used in place of the glutamate Cytomim system. Polymix buffer contained 5 mM Mg(OAc)₂ or 14 mM Mg(OAc)₂ and 95 mM KCl, 5 mM NH₄Cl, 8 mM putrescine, 1 mM spermidine, 1 mM DTT, 5 mM K₂HPO₄, and 0.5 mM CaCl₂. HiFi buffer contained 3.5 mM MgCl₂ or 14 mM MgCl₂ and 30 mM KCl, 70 mM NH₄Cl, 8 mM putrescine, 0.5 mM spermidine, 1 mM DTT, and 50 mM Tris-HCl pH 7.5. When indicated, the PURExpress translation system lacking ribosomes was substituted for the S150 extract-based system following the manufacturer's recommendations for transcription and translation. For clarity, these reactions are not conventional reconstitutions because the biochemical processes are coupled.

Integrated rRNA synthesis, ribosome assembly, and translation

Combined assembly and translation reactions were set-up as described above. However, the *in vivo* derived rRNA to be synthesized was not added. Rather, a plasmid encoding the target rRNA gene behind the T7 promoter was added (pCW1 (Weitzmann et al, 1990) for full length 23S rRNA and pWK1 (Krzyzosiak et al, 1987) for full length 16S rRNA). The final concentration of pCW1 was 16.6 µg/ml and pWK1 was 13.3 µg/ml. Competent ribosomes that assemble from *in vitro*-transcribed rRNA engage on Fluc mRNA to synthesize active Fluc. Linearized plasmids of pCW1 (digested with Afl II) and pWK1 (digested with BSU361) were 80 ± 6% and 60 ± 7% less active in Fluc synthesis relative to adding the entire plasmid, respectively. We hypothesize this to be a result of nucleases in the extract. When indicated, the PURExpress® translation system lacking ribosomes was substituted for the S150 extract.

Modified S150 extract preparation procedure

As described in the text, we optimized the S150 extract preparation procedure, which is described here. First, instead of growing cells in rich 2xYT media containing 16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, and buffered to pH 7.2 with NaOH, we grew cells in 2xYTPG media containing the above components plus 22 mM KH₂PO₄, 40 mM K₂HPO₄, and an additional 100 mM glucose. Second, instead of growing cells in a shaking incubator at 280 r.p.m. and 37°C, we grew the cells in a Sartorius C-10 10 l fermentor containing 10 l of 2xYTPG growth media and 1 ml antifoam A (Sigma). The following parameters were used for the fermentation: 37°C, 10 standard liters per minute (SLPM) of air, and 750 r.p.m. agitation speed. Potassium hydroxide (1 M) was used to maintain a pH of 7.2. Third, instead of harvesting the cells at OD₆₀₀ of 0.5, we harvested at OD₆₀₀ of 3 (see Supplementary Figure 11). Fourth, instead of using the simple dialysis buffer described above (10 mM Tris-OAc, pH 7.5 at 4°C, 10 mM Mg(OAc)₂, 2 mM DTT), we used a high-salt buffer (10 mM Tris-OAc, pH 7.5 at 4°C, 10 mM Mg(OAc)₂, 20 mM NH₄OAc, 30 mM KOAc, 200 mM Kglu, 1 mM spermidine, 1 mM putrescine, 1 mM DTT) (see Supplementary Figure 11). Fifth, the extract was concentrated to approximately 10 mg total extract protein/mL as determined by Bradford assay with BSA as a standard. This concentration was chosen because we found that it provides the best signal-to-background levels, as higher protein concentrations increase background translation activity while essentially providing the same iSAT activity (see Supplementary Figure 11).

Assembly of clindamycin-resistant ribosomes

The point mutation A2058U, which conveys ribosomal resistance to clindamycin (Cochella and Green, 2004), was introduced into the 23S

rRNA gene of the pCW1 plasmid through inverse PCR and blunt-end ligation using the following primers: 5'-AAGACCCGTGAACCTTTAC-3', 5'-ACCGTCTTGCCTGCGG-3'. The plasmid (pCW1-CR) was used in iSAT to assemble ribosomes as described above and the resulting ribosomes were assessed for clindamycin resistance in the ETTA system. Clindamycin was added to S150 extract for a final reaction concentration of 200 µg/ml prior to mixing in other translation components, and Fluc yield was measured after 4-h incubation at 37°C.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Author contributions: MCJ and GMC conceived the study; MCJ, BRF, and LET designed and performed experiments; GMC and MCJ provided a supervisory role. All authors wrote and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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