

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

Michael C Jewett, Brian R. Fritz, Laura E. Timmerman, George M. Church

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

18 March 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now finally heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, several concerns, which should be convincingly addressed in a revision of this work.

The major issues raised by the reviewers refer to the following points:

1. The implications of this work for synthetic and systems biology should be much better explained/demonstrated (Reviewer #1).
2. A direct comparison between conventional reconstitution and extract-mediated reconstitution should be provided (Reviewer #2).
3. The fate of the *in vitro* transcribed 23S rRNA should be clarified (Reviewer #3).

We note that one reviewer suggested to create a 'supplementary discussion' section. We would prefer *not* to have a separate discussion section in supplementary information and dedicate supplementary information exclusively to data and evidence that are formally required to demonstrate the conclusions of the paper.

REFeree REPORTS:

Reviewer #1 (Remarks to the Author):

In this manuscript entitled "*In vitro* integration of ribosomal RNA synthesis, ribosome assembly, and translation", the authors developed an integrated *in vitro* system that allows a single-step

assembly of ribosome subunits from in vitro transcribed rRNAs and natural ribosomal proteins. Moreover, the authors demonstrated that the assembled ribosome showed translation activity in the same reaction. This study provides a significant progress as compared with the previous ribosome reconstitution methods in the point that this method paves the way for the expression and assembly of all the ribosome subunits in a compartment, which might be one of the largest challenges in the field of in vitro synthetic biology.

This study is technically sound and represents a significant advance, but I have one major points to be clarified regarding the general interest of this study and two minor points as listed below.

Major point:

1. I agreed that the iSAT made a progress as compared to previous reconstitution methods of ribosome. But I am not sure whether this study is of interest to many audience of Molecular Systems Biology because it might attract only the researchers trying to reconstitute ribosome in vitro. I feel that the author should clarify more about the general importance of this study in the understanding of biological systems.

Minor points:

1. In page 6, the author described as "These data suggest that the extract based iSAT approach may have efficiency advantages for ribosome assembly as compared to the conventional approach", but Semrad & Green reported 3.3% 50S reconstitution with telithromycin and TMAO, the similar efficiency to this study (Figure 1B in Semrad and Green 2002 RNA).
2. The graphs in figure 3 are too complicated. How about moving the bar chars to separate panels?

Reviewer #2 (Remarks to the Author):

In the manuscript entitled "in vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation", Jewett and coworkers describe the in vitro assembly of bacterial ribosomes and their subunits from purified and, in the case of ribosomal RNAs, synthetic components. Their major findings are:

1. Ribosome reconstitution using a single concentration of magnesium at constant temperature can be achieved using a potassium glutamate-based buffering system, which is arguably more physiologically relevant than the ammonium chloride-based systems that have been employed previously.
2. Demonstration of the coupling of ribosomal assembly from purified components with translation in a single reaction using *E. coli* lysates.
3. Extension of point 2 above to include in vitro transcribed ribosomal RNA (and use this technique to assemble an antibiotic-resistant modified ribosome).

The studies described are scientifically rigorous, and the conclusions drawn are interesting and well supported by the data presented. This work will be of particular significance to future studies of ribosome assembly and efforts to construct a "minimal cell" from defined components. It may also facilitate ribosomal engineering studies.

Major criticism:

1. On page 5, the authors state "The key point was to add ribosomal components directly to our ETNA system, postulating that enzymes in the S150 extract could both help facilitate ribosome assembly and enable protein biosynthesis." However, it is unclear from the remainder of the manuscript if this postulate is upheld or not, with respect to facilitation of ribosome assembly. It appears that no experimental evidence directly comparing the activity of reconstituted ribosomes (or ribosomal subunits) to cell lysate assembled ribosomes (or ribosomal subunits) has been presented (comparing the data for 50S subunits presented in Supplementary Figure 3 with that presented in Figure 3, and the data for 70S ribosomes presented in Supplementary Figure 4 with that presented in Supplementary Figure 6f suggests that assembly in *E. coli* cell lysate does not improve translational activity). The manuscript would be improved by some discussion of this point and/or further data directly comparing these two conditions.

2. The data in Fig. 3, i.e. the establishment of iSAT, are the really interesting part and the main theme of this work. Unfortunately, the main text of the present manuscript consumed a large space for the discussion of the data in Fig. 2, and therefore this section can be shortened and move to the Supporting discussion. Instead, expand more detail description and discussion for the data in Fig. 3 in the main text.

Minor criticisms:

1. Color description is unclear. For instance, there are dark and light orange in Fig. 1 for the assignment of the respective components, but the description is available for only the dark orange in text. Similarly, Fig. 3, white and grey should indicate the native components, but description for only back was given.
2. Page 5 - The authors state that "previous reports have not shown [assembly of ribosomes at a single magnesium concentration and constant temperature] to be possible." It is not clear if this means not possible under the conditions that they have used themselves (with potassium glutamate) or using ammonium chloride reconstitution. This needs to be clarified.
3. In two places in Supplementary Table 1 the units in each column do not match (micrograms compared to milligrams).

Reviewer #3 (Remarks to the Author):

Reconstitution of the ribosome from ribosomal proteins and in vitro transcribed ribosomal RNA is one of the essential methodologies for the understanding or the engineering of the ribosome. However, it has been demonstrated that Escherichia coli 50S ribosomal subunit is difficult to be reconstituted from in vitro transcribed ribosomal RNA as designated by the authors in the manuscript.

In the manuscript, the authors developed a novel system for the reconstitution of both ribosomal subunits by integrating ribosomal RNA synthesis and ribosome assembly in the presence of E. coli cell-extracts. The development of the system is based on the extensive optimization of the reaction mixtures and the confirmation using luciferase mRNA translation is properly done with several control experiments.

The developed method may become important technique for the reconstitution of the mutant ribosome for understanding the mechanism of the ribosome as well as for engineering the ribosome to synthesize desired proteins in vitro. Thus, I consider that the paper is worthwhile for the publication. For the improvement of the manuscript, the following points should be improved.

1. P.6, lanes 22-23: 'combining transcription and assembly was advantageous' is misleading. Authors did not analyze the post-transcriptional modification in the transcribed 23S rRNA and there is a possibility that the modification by corresponding enzymes and residual substrates in the S150 fraction. They should describe this point.
2. Supplementary Figure 6, 'While glutamate is not generally used in purified translation systems' is misleading. The authors describe that PURE Buffer contains 100 mM KGlu in Supplementary Figure 11.
3. Supplementary Figure 12, Authors describe that total yield of Fluc as determined 14C-leucine incorporation. How did they determine? PAGE analysis? filter binding analysis? They should explain.

Point-by-point response to editor and reviewer comments:

Editor's comments:

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Thank you very much for the opportunity to submit a revised version of the manuscript. In our revised submission, we feel that we were able to directly and carefully address the concerns raised by the referees. This is delineated in our response below.

*We note that one reviewer suggested to create a 'supplementary discussion' section. We would prefer **not** to have a separate discussion section in supplementary information and dedicate supplementary information exclusively to data and evidence that are formally required to demonstrate the conclusions of the paper.*

*As requested, we did not create a 'supplementary discussion' section. This has resulted in the manuscript being a little longer than suggested for *Reports*, but we believe the value added significantly improves the manuscript.*

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

*We believe that we have dealt with these points and those listed by the referees in a satisfactory manner. These updates clarify and significantly improve our work, providing better context for our advances that we believe make a unique and essential contribution to *Molecular Systems Biology*.*

*Yours sincerely,
Thomas Lemberger, PhD
Chief Editor
Molecular Systems Biology*

Please note that all responses are indicated in “red” in the resubmission for ease of identification of the changes.

Reviewer #1 (Remarks to the Author):

In this manuscript entitled "In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation", the authors developed an integrated in vitro system that allows a single-step assembly of ribosome subunits from in vitro transcribed rRNAs and natural ribosomal proteins. Moreover, the authors demonstrated that the assembled ribosome showed translation activity in the same reaction. This study provides a significant progress as compared with the previous ribosome reconstitution methods in the point that this method paves the way for the expression and assembly of all the ribosome subunits in a compartment, which might be one of the largest challenges in the field of in vitro synthetic biology.

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Major point:

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Response: In the revised manuscript we clarify more about the general importance of this study in the understanding of biological systems. As R2 points out, “This work will be of particular significance to future studies of ribosome assembly and efforts to construct a ‘minimal cell’ from defined components. It may also facilitate ribosomal engineering studies.” We now highlight these points.

Specifically, we have made the following changes to the abstract:

“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 toward 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.”

We have also included the following paragraph to open the introduction section on page 3:

“The construction of ribosomes *in vitro* 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 (Erlacher et al, 2011; Nierhaus, 1990; Polacek, 2011), design and build minimal cells to understand origins of life (Forster & Church, 2006; Jewett & Forster, 2010), and enable *in vitro* evolution to select for ribosomes that have enhanced functions or altered chemical properties (Cochella & Green, 2004). To achieve these goals, methods for *in vitro* ribosome synthesis are needed.”

Lastly, we expanded the original final paragraph of the manuscript to read:

“Although the conventional reconstitution of ribosomes *in vitro* is well established (Nierhaus, 1990), the iSAT method solves a critical barrier to basic biological studies of ribosome biogenesis and activity, offering a powerful 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 & Noller, 1996; Green & Noller, 1999; Semrad & 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.”

Minor points:

1. In page 6, the author described as "These data suggest that the extract based iSAT approach may have efficiency advantages for ribosome assembly as compared to the conventional approach", but Semrad & Green reported 3.3% 50S reconstitution with telithromycin and TMAO, the similar efficiency to this study (Figure 1B in Semrad and Green 2002 RNA).

Response: We thank the reviewer for pointing out the need for clarification. Semrad and Green reported ~3% 50S reconstitution efficiency with telithromycin and TMAO for peptide bond formation as measured by the fragment reaction for using *in vitro* transcribed rRNA as compared to natural mature 23S rRNA. iSAT assembled 50S ribosomal subunits have ~20% the activity of ribosomes assembled from mature rRNA. Thus, iSAT has a higher efficiency as compared to the conventional approach when looking at differences between *in vitro* transcribed and mature rRNA. We have removed our comparison in the text to “natural whole ribosomes” that have not been reconstituted, which we believe led to the confusion.

The specific changes to the text are here:

“iSAT assembled 50S ribosomal subunits have ~20% the activity of ribosomes assembled in our ETNA system from mature rRNA (0.63nmol/L vs. 3.2nmol/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 & Noller, 1996; Green & Noller, 1999; Semrad & 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.”

2. *The graphs in figure 3 are too complicated. How about moving the bar charts to separate panels?*

Response: To make the figure less complicated, the bar charts have been moved to separate panels as suggested. In addition, we also separated the original Figure 3 into a revised Figure 3 and Figure 4.

Reviewer #2 (Remarks to the Author):

In the manuscript entitled "in vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation", Jewett and coworkers describe the in vitro assembly of bacterial ribosomes and their subunits from purified and, in the case of ribosomal RNAs, synthetic components. Their major findings are:

- 1. Ribosome reconstitution using a single concentration of magnesium at constant temperature can be achieved using a potassium glutamate-based buffering system, which is arguably more physiologically relevant than the ammonium chloride-based systems that have been employed previously.*
- 2. Demonstration of the coupling of ribosomal assembly from purified components with translation in a single reaction using *E.coli* lysates.*
- 3. Extension of point 2 above to include in vitro transcribed ribosomal RNA (and use this technique to*

assemble an antibiotic-resistant modified ribosome).

The studies described are scientifically rigorous, and the conclusions drawn are interesting and well supported by the data presented. This work will be of particular significance to future studies of ribosome assembly and efforts to construct a "minimal cell" from defined components. It may also facilitate ribosomal engineering studies.

Major criticism:

1. On page 5, the authors state "The key point was to add ribosomal components directly to our ETTA system, postulating that enzymes in the S150 extract could both help facilitate ribosome assembly and enable protein biosynthesis." However, it is unclear from the remainder of the manuscript if this postulate is upheld or not, with respect to facilitation of ribosome assembly.

Response: As the reviewer correctly points out we do not explicitly support the postulate that "enzymes in the extract in the S150 extract could both help facilitate ribosome assembly and enable protein biosynthesis." We have therefore removed this postulate.

It appears that no experimental evidence directly comparing the activity of reconstituted ribosomes (or ribosomal subunits) to cell lysate assembled ribosomes (or ribosomal subunits) has been presented (comparing the data for 50S subunits presented in Supplementary Figure 3 with that presented in Figure 3, and the data for 70S ribosomes presented in Supplementary Figure 4 with that presented in Supplementary Figure 6f suggests that assembly in *E. coli* cell lysate does not improve translational activity). The manuscript would be improved by some discussion of this point and/or further data directly comparing these two conditions.

Response: Per the reviewer's request, we now provide some discussion of this point. Specifically we have added the following to the text on page 6:

"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 2, Supplementary Figures 3 & 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 extract based cell-free translation has substrate instabilities known to plague extended reaction durations (Carlson et al, 2012; Jewett & Swartz, 2004b)."

Notably, we do observe a significant improvement for *in vitro* transcribed rRNA in cell lysate assembled ribosomes as compared to *in vitro* transcribed rRNA in separate reconstitution and translation reactions (see R1, response to minor point 1). As mimicking co-transcription and ribosome assembly was the key objective of the current work, the iSAT approach provides an important advance for efforts to construct ribosomes from *in vitro* synthesized parts.

2. The data in Fig. 3, i.e. the establishment of iSAT, are the really interesting part and the main theme of this work. Unfortunately, the main text of the present manuscript consumed a large space for the discussion of the data in Fig. 2, and therefore this section can be shortened and move to the Supporting discussion. Instead, expand more detail description and discussion for the data in Fig. 3 in the main text.

Response: We have expanded the detail description and discussion for the data in the original Figure 3, including the expansion of the original Figure 3 to a new Figure 3 and Figure 4. However, per the editor's request, we have not made a separate discussion section in supplementary information. Although our expanded text (including changes to the introduction and conclusion) puts us over the character limit for a *Report*, now at ~29,500 characters rather than 22,000, we believe the changes make the paper stronger and thank the reviewer for raising this concern.

Beyond the changes described above (and the response to R1 minor point 1 and R2 major point 1), we also now added the following sentence to add quantitative metrics for iSAT synthesized 70S ribosomes in our improved extracts:

"iSAT assembled 70S ribosomes in the improved extracts have ~12% the activity of ribosomes assembled from mature rRNA as measured by luciferase synthesis (4.8nmol/L vs. 38nmol/L)."

Here is a further example of text added to provide a more detailed description.

"In these reactions, bacteriophage T7 RNAP was used to transcribe both the rRNA and the luciferase mRNA. Figure 3c shows Fluc accumulation over time for the optimized iSAT system, noting that there is a lag prior to the start of luciferase synthesis."

Minor criticisms:

1. *Color description is unclear. For instance, there are dark and light orange in Fig. 1 for the assignment of the respective components, but the description is available for only the dark orange in text. Similarly, Fig. 3, white and grey should indicate the native components, but description for only back was given.*

Response: The color descriptions have been clarified to be consistent in the text, figure legends, and key of each figure. In addition to changes to Figures 1 and 3, Supplementary Figure 14 was also changed to improve clarity.

2. Page 5 - The authors state that "previous reports have not shown [assembly of ribosomes at a single magnesium concentration and constant temperature] to be possible." It is not clear if this means not possible under the conditions that they have used themselves (with potassium glutamate) or using ammonium chloride reconstitution. This needs to be clarified.

Response: The text has been updated to clarify this statement. The previous report used ammonium chloride reconstitution. To our knowledge, 50S ribosome reconstitutions have not been previously reported with the use of potassium glutamate salts.

The updated text reads:

"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)."

3. *In two places in Supplementary Table 1 the units in each column do not match (micrograms compared to milligrams).*

Response: The units in Supplementary Table 1 have been changed to micrograms/mL in all cases for consistency.

Reviewer #3 (Remarks to the Author):

Reconstitution of the ribosome from ribosomal proteins and in vitro transcribed ribosomal RNA is one of the essential methodologies for the understanding or the engineering of the ribosome. However, it has been demonstrated that Escherichia coli 50S ribosomal subunit is difficult to be reconstituted from in vitro transcribed ribosomal RNA as designated by the authors in the manuscript.

In the manuscript, the authors developed a novel system for the reconstitution of both ribosomal subunits by integrating ribosomal RNA synthesis and ribosome assembly in the presence of E. coli cell-extracts. The development of the system is based on the extensive optimization of the reaction mixtures and the confirmation using luciferase mRNA translation is properly done with several control experiments.

The developed method may become important technique for the reconstituion of the mutant ribosome for understanding the mechanism of the ribosome as well as for engineering the ribosome to synthesize desired proteins in vitro. Thus, I consider that the paper is worthwhile for the publication. For the improvement of the manuscript, the following points should be improved.

1. P.6, lanes 22-23: 'combining transcription and assembly was advantageous' is misleading. Authors did not analyze the post-transcriptional modification in the transcribed 23S rRNA and there is a possibility that the modification by corresponding enzymes and residual substrates in the S150 fraction. They should describe this point.

Response: We thank the reviewer for this suggestion and now describe this point. As the reviewer points out, we did not analyze post-transcriptional modifications and there is this possibility. Follow-up experiments with supplementation of S-adenosyl-methionine, a potential methyl donor for nucleoside modification, did not stimulate the combined ribosome synthesis and assembly reaction. We now add the following text to the manuscript:

“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.”

2. Supplementary Figure 6, 'While glutamate is not generally used in purified translation systems' is misleading. The authors describe that PURE Buffer contains 100 mM KGlu in Supplementary Figure 11.

Response: We fully agree with this reviewer and have revised the text to avoid the confusion, now referencing directly the previous manuscripts we intended to compare.

The legend of Supplementary Figure 6 was revised to read:

*“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).”*

3. *Supplementary Figure 12, Authors describe that total yield of Fluc as determined ¹⁴C-leucine incorporation. How did they determine? PAGE analysis? filter binding analysis? They should explain.*

Response: We thank the reviewer for raising this point of clarification. We now explain our ¹⁴C-leucine incorporation in the methods "Reporter protein quantification" section and note them in the legend of Supplementary Figure 12.

"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)."

In the legend of Supplementary Figure 12, we also write:

"Total yield of Fluc as determined by ¹⁴C-leucine incorporation by TCA precipitable counts using a filter paper assay and liquid scintillation counting (grey bar)."