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Production of large amounts of recombinant RNAs on a circular scaffold using a viroid-derived system in Escherichia coli

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Valencia, 30 July 2018

Dear Dr. Cao:

We are submitting a new revised version of our manuscript “Production of large amounts of recombinant RNAs on a circular scaffold using a viroid-derived system in Escherichia coli” (JoVE58472) in which we have addressed all your editorial comments.

Sincerely,

José-Antonio Daròs

TITLE:

Large-Scale Production of Recombinant RNAs on a Circular Scaffold Using a Viroid-Derived System in *Escherichia Coli*

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KEYWORDS:

Recombinant RNA, circular RNA, viroid, tRNA ligase, RNA aptamer, *Escherichia coli*

SUMMARY:

Here, we present a protocol to produce large amounts of recombinant RNA in *Escherichia coli* by co-expressing a chimeric RNA that contains the RNA of interest in a viroid scaffold and a plant tRNA ligase. The main product is a circular molecule that facilitates purification to homogeneity.

ABSTRACT:

With increasing interest in RNA biology and the use of RNA molecules in sophisticated biotechnological applications, the methods to produce large amounts of recombinant RNAs are limited. Here, we describe a protocol to produce large amounts of recombinant RNA in *Escherichia coli* based on co-expression of a chimeric molecule that contains the RNA of interest in a viroid scaffold and a plant tRNA ligase. Viroids are relatively small, non-coding, highly base-paired circular RNAs that are infectious to higher plants. The host plant tRNA ligase is an enzyme recruited by viroids that belong to the family *Avsunviroidae*, such as *Eggplant latent viroid* (ELVd), to mediate RNA circularization during viroid replication. Although ELVd does not replicate in *E. coli*, an ELVd precursor is efficiently transcribed by the *E. coli* RNA polymerase and processed by the embedded hammerhead ribozymes in bacterial cells, and the resulting monomers are circularized by the co-expressed tRNA ligase reaching a remarkable concentration. The insertion of an RNA of interest into the ELVd scaffold enables the production of tens of milligrams of the recombinant RNA per liter of bacterial culture in regular laboratory conditions. A main fraction of the RNA product is circular, a feature that facilitates the purification of the recombinant RNA to virtual homogeneity. In this protocol, a complementary DNA (cDNA) corresponding to the RNA of interest is inserted in a particular position of the ELVd cDNA in an expression plasmid that is used, along the plasmid to co-express eggplant tRNA

ligase, to transform *E. coli*. Co-expression of both molecules under the control of strong constitutive promoters leads to production of large amounts of the recombinant RNA. The recombinant RNA can be extracted from the bacterial cells and separated from the bulk of bacterial RNAs taking advantage of its circularity.

INTRODUCTION:

In contrast to DNA and proteins, protocols for easy, efficient and cost-effective production of large amounts of RNA are not abundant. However, research and industry demand increasing amounts of these biomolecules to investigate their unique biological properties[1], or to be employed in sophisticated biotechnological applications, including their use as highly specific aptamers[2], therapeutic agents[3], or selective pesticides[4]. *In vitro* transcription and chemical synthesis are commonly used in research to produce RNA. However, these methods entail important limitations when large amounts of the products are required. The logical alternative is to use the endogenous transcription machinery of living cells, followed by a purification process to separate the RNAs of interest from the cellular companions. Following this strategy, methods have been developed to produce recombinant RNAs in bacterial cells, such as the lab-friendly *Escherichia coli*[5] or the marine purple phototrophic alpha-proteobacterium *Rhodovolum sulfidophilum*[6]. Most methods to produce recombinant RNA in bacteria rely on the expression of a native highly stable RNA scaffold, such as a tRNA or an rRNA, in which the RNA of interest is inserted[7]. This imposes the necessity of releasing the RNA of interest out of the chimeric molecule, if the presence of extra RNA is a problem for the downstream applications[8]. Another concept in recombinant RNA biotechnology is the production of recombinant ribonucleoprotein complexes that may be the desired product *per se* or used as a protective strategy to increase the stability of the RNA of interest[9,10]. Similarly, the production of circular RNAs has also been suggested as a strategy to generate more stable products[11].

We have recently developed a new method to produce large amounts of recombinant RNA in *E. coli* that participates in three of the above concepts: the insertion of the RNA of interest in a highly stable circular RNA scaffold and the co-expression of the recombinant RNA with an interacting protein to likely produce a stable ribonucleoprotein complex that accumulates in remarkable amounts in bacterial cells[12]. In contrast to previous developments, we used an RNA scaffold completely alien to *E. coli*, namely a viroid. Viroids are a very particular type of infectious agents of higher plants that are exclusively constituted by a relatively small (246-401 nt) highly base-paired circular RNA[13]. Interestingly, viroids are non-coding RNAs and, with no help from their own proteins, they are able to complete complex infectious cycles in the infected hosts[14]. These cycles include the RNA-to-RNA replication in the nuclei or chloroplasts, depending on the viroid family –*Pospiviroidae* or *Avsunviroidae*, respectively–, movement through the infected plant and evasion of the host defensive response. Viroids must be ranked among the most stable RNAs in nature, as a consequence of being a naked circular RNA and having to survive in the hostile environment of plant infected cells. This property may make viroids particularly suitable as scaffolds to stabilize recombinant RNA in biotechnological approaches. In addition, the new method is based on co-expression of the viroid scaffold with an interacting plant protein. Viroids replicate through a rolling-circle mechanism in which host enzymes are recruited to catalyze the different steps of the process. Notably some viroids,

more specifically those that belong to the family *Avsunviroidae*[15], contain ribozymes that are also involved in replication. Depending on the viroid species, transcription of viroid RNAs is mediated by the host RNA polymerase II or the chloroplastic nuclear-encoded RNA polymerase (NEP). Viroid RNA processing seems to be catalyzed by a host type-III RNase, although in viroids with ribozymes oligomeric RNA intermediates self-cleave during replication. Finally, the resulting viroid monomers are circularized, depending on the viroid family, by the host DNA ligase 1 or the chloroplastic isoform of tRNA ligase[16,17]. This last enzyme is involved in ligation of the monomeric forms of the viroids in the family *Avsunviroidae*, such as *Eggplant latent viroid* (ELVd)[18].

In the course of a work to analyze the sequence and structural requirements of ELVd that determine recognition by the eggplant (*Solanum melongena* L.) tRNA ligase, we set up an experimental system based on co-expression of both molecules in *E. coli*[19]. We noticed that longer-than-unit ELVd transcripts self-cleave efficiently in *E. coli* cells through the embedded hammerhead ribozymes and that the resulting viroid monomers with 5'-hydroxyl and 2',3'-phosphodiester termini were efficiently circularized by the co-expressed eggplant tRNA ligase. Even more, the resulting circular viroid RNA reached an unexpected high concentration in *E. coli*, exceeding those of the endogenous rRNAs[12]. Absence of replication intermediates indicated lack of ELVd RNA-to-RNA amplification in these bacterial cells. Interestingly, insertion of heterologous RNAs in a particular position of the viroid molecule had a moderate effect on accumulation of the circular viroid-derived RNAs[12]. These observations made us envision a method to produce large amounts of recombinant RNAs in bacteria. In this method, the cDNAs corresponding to the RNAs of interest are inserted in the ELVd cDNA and the resulting chimeric RNA is expressed in *E. coli* through a strong constitutive promoter. For the system to work, *E. coli* must be co-transformed with a plasmid to express the eggplant tRNA ligase. The ELVd-derived longer-than-unit transcript is processed by the embedded hammerhead ribozymes and the resulting monomers with the appropriate termini are recognized and circularized by the co-expressed tRNA ligase. This way, the RNA of interest is inserted into a very stable circular scaffold consisting of the viroid circular molecule. This recombinant chimeric RNA is most probably further stabilized inside the *E. coli* cells by formation of a ribonucleoprotein complex through interaction with tRNA ligase. Using this method (see protocol below), RNA aptamers, hairpin RNAs and other structured RNAs have been easily produced in amounts of tens of milligrams per liter of *E. coli* culture in regular laboratory conditions and purified to homogeneity taking advantage of circularity[12].

PROTOCOL:

1. Plasmid Construction

1.1. Amplify by PCR (or by reverse transcription PCR if starting from an RNA template) the cDNA corresponding to the RNA of interest using primers with 5' extension to allow assembly into the expression plasmid. Use a commercial kit. To avoid undesired mutations, use a high-fidelity DNA polymerase.

1.1.1. To insert the cDNA in the expression plasmid by Gibson assembly[20], add the following 5' extensions to the PCR primers: forward, 5'-TCTCCCCCTCCCAGGTACTATCCCCTTXXXXXXXXXXXXXXXXXXXX-3'; reverse, 5'-CCCTCCTAGGGAACACATCCTTGAXXXXXXXXXXXXXXXXXXXX-3'; X represents nucleotides homologous to the terminal ends of the RNA of interest.

1.1.2. Incubate for 30 s at 98 °C, followed by 30 cycles of 10 s at 98 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension of 10 min at 72 °C.

1.2. Digest 100 ng of plasmid pLELVd-BZB with 10 U of the type-IIS restriction enzyme *Bpi* I for 1 h at 37 °C in a 20-µL reaction in a 0.5-mL tube in buffer G (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mg/mL BSA).

Note: All plasmids are available on request to the corresponding author. *Bpi* I is equivalent to *Bbs* I.

1.3. Separate the PCR and digestion products by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 7.2). Stain the gel for 15 min by shaking in 200-mL of 0.5 µg/mL ethidium bromide. Visualize the DNA using a UV transilluminator and cut the bands corresponding to the amplified cDNA and the *Bpi* I-digested plasmid (2046 bp) using a scalpel blade.

Note: *Bpi* I digestion of pLELVd-BZB also releases a 528-bp product corresponding to the LacZ blue-white reporter.

1.4. Elute the DNAs from the gel fragments using silica gel columns (gel DNA recovery kit in **Table of Materials**) and quantify the DNA concentration by spectrophotometric analysis.

1.5. Set up a Gibson assembly reaction using the amplified cDNA and the digested plasmid. Use a 3-fold molar excess of insert *versus* vector[20]. Incubate for 1 h at 50 °C and purify the reaction products using a silica gel column (DNA clean & concentrator kit in the **Table of Materials**).

1.6. Use the purified products of the Gibson assembly reaction to electroporate competent *E. coli* DH5α cells. Using 1-mm electroporation cuvettes, apply the following settings: 1500 V and 5 ms. Incubate for 1 h at 37 °C in super optimized broth with catabolite repression (SOC; 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0) liquid medium and then spread onto Luria-Bertani (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) agar (1.5%) plates containing 50 µg/mL ampicillin.

1.6.1. To screen for colonies corresponding to transformed *E. coli* clones that likely incorporated the insert, 15 minutes before plating the electroporated cells, spread 30 µL of 50 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide. Incubate plates overnight at 37 °C.

1.7. Pick several white colonies and grow overnight at 37 °C in liquid LB media. Purify plasmids using a miniprep kit (see **Table of Materials**) and analyze their sizes by electrophoresis in a 1% agarose gel in TAE buffer.

1.8. Select the most likely recombinant plasmid based on electrophoretic migration compared to the pLELVd-BZB control. Confirm the sequence of the selected plasmid by sequencing using primers 5'-CCTTTTTCAATATTATTGAAGC-3' and 5'-GATGCTCGTCAGGGGGGCGGAG-3' that flank the whole expression cassette in pLELVd-BZB.

Note: Remember that this plasmid contains a 528-bp polylinker with the LacZ marker that is replaced by the cDNA of interest. Restriction mapping may help to select the right recombinant plasmids.

2. RNA Expression

2.1. Co-electroporate (see conditions in 1.6) the selected *E. coli* strain (*E. coli* BL21 or a BL21 derivative) with the pLELVd-BZB-derivative that contains the cDNA corresponding to the RNA of interest and plasmid p15LtRnlSm to co-express the eggplant tRNA ligase. Use the *E. coli* HT115(DE3), which lacks RNase III[21], to express RNAs with long double-stranded regions.

Note: Both the RNA of interest and the tRNA ligase mRNA are transcribed by the *E. coli* RNA polymerase. No DE3 lysogen to express T7 RNA polymerase is required in the *E. coli* strain, although the presence of this lysogen has no deleterious effects.

2.2. After 1 h incubation at 37 °C in SOC liquid medium, plate electroporated bacteria in LB solid medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. Incubate overnight at 37 °C.

2.3. Pick a colony and inoculate a 1-L baffled Erlenmeyer flask with 250 mL of liquid Terrific Broth (TB) medium (12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4), containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. Incubate at 37 °C with vigorous shaking at 180 revolutions per minute (rpm). Harvest bacteria between 12 and 16 h after culture inoculation.

3. RNA Extraction and Purification

3.1. For analytical purposes, take 2-mL aliquots of the culture at the desired time points and centrifuge at 14,000 x g for 2 min. Discard the supernatant and resuspend the cells in 50 µL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) by vortexing.

3.1.1. Add one volume (50 µL) of a 1:1 (v/v) mix of phenol (saturated with water and equilibrated at pH 8.0 with Tris-HCl, pH 8.0) and chloroform. Break the cells by vigorous vortexing and separate the aqueous and organic phases by centrifugation for 5 min at 14,000 x g.

3.1.2. Carefully recover the aqueous phases (upper) that contains total bacterial RNA.

Note: Preparations can be stored at -20 °C for subsequent analysis.

3.2. For preparative purposes, pour culture into a 250-mL centrifuge bottle and spin down cells at 14,000 x g for 10 min. Discard supernatant. Wash the cells by resuspending in 30 mL of water. Transfer to a centrifuge tube and spin down cells again under the same conditions.

3.3. Discard the supernatant and resuspend the cells in 10 mL of chromatography buffer (50 mM Tris-HCl, pH 6.5, 150 mM NaCl, 0.2 mM EDTA) by vortexing.

Note: At this time, cells can be frozen at -20 °C to proceed with purification at any other moment.

3.4. Using a fresh or thawed bacterial preparation, break cells by adding 1 volume (10 mL) of phenol:chloroform (see step 3.2) and vortexing vigorously.

3.5. Centrifuge for 10 min at 12,000 x g, recover the aqueous phase and re-extract with 1 volume (10 mL) of chloroform under the same conditions.

Note: The RNA preparation can be stored at -20 °C at this point.

3.6. Further purify total bacterial RNA by anion-exchange chromatography. Filter the RNA preparation through a 45-µm syringe filter and load on a 1-mL diethylethanolamine (DEAE) column.

3.6.1. For chromatography purification, use a liquid chromatography system at a flow rate of 1 mL/min. Before sample loading, equilibrate the column with 10 mL of chromatography buffer (see step 3.3 for composition).

3.6.2. Load the sample and wash the column with 10 mL of chromatography buffer. Elute RNA with 20 mL of elution buffer (50 mM Tris-HCl, pH 6.5, 1 M NaCl, 0.2 mM EDTA) and collect 1-mL aliquots.

Note: RNA quickly elutes in the initial fractions. The column can be re-used for further purifications. For this, wash the column with 10 mL of water and store at 4 °C in 20% ethanol.

3.7. Since a major part of the recombinant RNA accumulates in *E. coli* in a circular form, this property can be profited for purification to homogeneity[12]. Separate circular RNAs from the linear counterparts by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) combining non-denaturing and denaturing (8 M urea) conditions[12,22].

4. RNA Analysis

4.1. Prepare a 5% polyacrylamide gel (37.5:1 acrylamyde:*N,N'*-methylenebisacrylamide, mass ratio) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 8 M urea.

4.2. Mix 20 µL of RNA preparations with 1 volume (20 µL) of loading buffer (98% formamide, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.0025% bromophenol blue, and 0.0025% xylene cyanol), incubate for 1.5 min at 95 °C in a heating block, and snap cool on ice.

4.3. Load the samples in the polyacrylamide gel and run the electrophoresis at appropriate conditions depending on the gel dimensions (*e.g.*, run 140 × 130 × 2 mm gels for 2.5 h at 200 V). Stain the gel for 15 min in 1 µg/mL ethidium bromide, wash with water, and visualize RNA under UV light.

REPRESENTATIVE RESULTS:

To produce recombinant RNA in *E. coli* using the ELVd-derived system[12], the RNA of interest is grafted into an ELVd RNA scaffold. This chimeric RNA is co-expressed along the eggplant tRNA ligase in *E. coli*. Once processed, cleaved and circularized, the chimeric circular RNA, from which the RNA of interest protrudes, likely forms a ribonucleoprotein complex with the co-expressed eggplant enzyme that reaches remarkable concentration in the bacterial cells (**Figure 1**). Consequently, the first step to produce recombinant RNA using this system consists of inserting the cDNA corresponding to the RNA of interest into a particular position in the ELVd cDNA, T245-T246 in the ELVd sequence variant AJ536613. Plasmid pLELVd-BZB, which contains a polylinker on this position with two *Bpi* I recognition sites and a LacZ blue/white marker gene, facilitates this insertion by the very efficient Gibson assembly method[20]. Selection of transformed *E. coli* clones containing the desired recombinant plasmids is facilitated by the blue/white screening of LacZ marker. Plasmids in which the double *Bpi* I digestion was incomplete and could not incorporate the insert likely produce blue colonies in the presence of X-gal. **Figure 2** shows the electrophoretic analysis of several recombinant plasmids in which different cDNAs were inserted. These plasmids show different migrations when compared to pLELVd-BZB. Note that pLELVd-BZB contains the LacZ marker (528 bp) that is replaced by the cDNA corresponding to the RNA of interest. So, although this depends on the size of the inserted cDNA, the recombinant plasmids usually migrate faster than the control plasmid (**Figure 2**).

The pLELVd-BZB-derivatives that contain the cDNAs corresponding to the RNAs of interest are used along p15LtRnlSm to co-electroporate *E. coli*. Co-transformed bacteria are selected on plates containing ampicillin and chloramphenicol. Then, selected *E. coli* clones are grown at 37 °C with strong agitation in the rich TB medium. Under this culture conditions, production of the recombinant RNA is maximized[12]. The presence of the recombinant RNA in the bacteria can be easily monitored by breaking the cells with a mix of phenol and chloroform in the presence of a buffer and analyzing the RNA, which partitions in the aqueous buffer, by denaturing PAGE. **Figure 3** show ethidium bromide stained polyacrylamide gels in which total RNAs from different *E. coli* clones were separated. The pictures show strong bands that correspond to empty ELVd and chimeric ELVd forms in which different RNAs of interest were inserted. Interestingly we find

a major fraction of the recombinant RNA as circular form. Using a combination of two PAGEs under denaturing conditions at high and low ionic strength, the circularity of the main fraction of the recombinant RNA can be easily observed (**Figure 4**).

Depending on the downstream application, the total *E. coli* RNA preparation that contains the chimeric ELVd-RNA of interest may be the goal of the current protocol. However, when needed, the RNA preparation can be further purified by anion-exchange chromatography. Chromatogram in **Figure 5** shows the efficient retention of *E. coli* RNA on the column at low ionic strength (150 mM NaCl) and subsequent elution at high ionic strength (1 M NaCl). Most of the RNA is collected in fractions 2 and 3. To purify the recombinant RNA to homogeneity, advantage of circularity can be taken. Circular RNAs are delayed with respect to their linear counterparts in denaturing conditions[22]. These RNAs can be eluted from the gel after ethidium bromide staining. The use of a solubilizable polyacrylamide gel, such as those cross-linked with *N,N'*-bis(acryloyl)cystamine[23], facilitates purification of large amounts of recombinant RNAs (**Figure 6**).

Figure Legends:

Figure 1: Schematic representation of the viroid-based system to produce recombinant RNA in *E. coli*. The cDNA corresponding to the RNA of interest (the 98-nt-long RNA aptamer Spinach in the scheme) is inserted into plasmid pLELVd-BZB. *E. coli* is co-transformed with the pLELVd-BZB-derivative and plasmid p15LtRnlSm to co-express eggplant tRNA ligase. *In E. coli*, the chimeric ELVd-Spinach RNA transcript is self-cleaved (red arrowheads) by the viroid hammerhead ribozymes. The resulting monomer is recognized by the co-expressed tRNA ligase and circularized. The recombinant RNA consists of a circular viroid scaffold from which the RNA of interest (in green) protrudes. Accumulation of the recombinant RNA to large amounts in *E. coli* likely results from stabilization of the ribonucleoprotein complex with tRNA ligase. In addition to the split ELVd cDNA, plasmid pLELVd-BZB contains a pUC replication origin (pUC ori), an ampicillin selection gene (Amp^R), the *E. coli* murine lipoprotein (*lpp*) promoter and rRNA (*rrnC*) terminator, and the LacZ marker. Plasmid p15LtRnlSm contains a p15A replication origin (p15A ori), a chloramphenicol resistance gene (Cm^R), the *E. coli lpp* promoter and a phage T7 transcription terminator, in addition to the eggplant tRNA ligase cDNA.

Figure 2: Electrophoretic analysis of pLELVd-BZB-derived expression plasmids that contain different cDNAs of interest. Plasmids were separated by electrophoresis in a 1% agarose gel that was stained with ethidium bromide. Lane 1, DNA marker with sizes in kbp of some of the components on the left; lane 2, pLELVd-BZB; lane 3, pLELVd-BZB derivative to express an empty ELVd; lanes 4 to 7, pLELVd-BZB derivatives to express the RNA aptamer Spinach (lane 4), the streptavidin binding aptamer (lane 5), an RNA hairpin with a contiguous 42 bp double-stranded region (lane 6), and the 3' cap-independent translation enhancer (CITE) of a plant virus (lane 7).

Figure 3: Recombinant RNA produced in *E. coli* using the viroid-based system. Total RNAs from different *E. coli* clones were extracted by treatment with phenol:chloroform and aliquots separated by denaturing PAGE. Gels stained with ethidium bromide are shown. Recombinant RNAs were produced in *E. coli* (**A**) BL21(DE3) and (**B**) HT115(DE3). (**A** and **B**) Lanes 1, RNA

marker with sizes in nt on the left; lanes 2, RNAs from *E. coli* clones to express an empty ELVd (333 nt). (**A**) Lanes 3 and 4, RNAs from *E. coli* clones to express a chimeric ELVd form containing the aptamer Spinach (98 nt) and the streptavidin binding aptamer (42 nt), respectively. (**B**) Lane 3, RNAs from an *E. coli* clone to express a chimeric ELVd form containing a 42-bp-long double stranded RNA. Red arrows point to the circular recombinant RNAs.

Figure 4: Circularity of the recombinant RNA produced in *E. coli* using the viroid-based system. Total RNAs from an *E. coli* clone to express a chimeric ELVd that contains the aptamer Spinach were separated by 2D denaturing PAGE first under high and then under low ionic strength. The gels were stained with ethidium bromide. The blue arrows indicate directions of electrophoretic migration. The red arrow points to the circular ELVd-Spinach that is selectively delayed from the linear counterparts of the same size during the two separations.

Figure 5: Chromatographic purification of a recombinant RNA produced in *E. coli* using the viroid-based system. Total RNAs from an *E. coli* clone that produces a chimeric ELVd-3' CITE (55 nt) were loaded on a DEAE chromatography column that was washed in the presence of 150 mM NaCl. RNA was eluted in the presence of 1 M NaCl. The chromatogram shows absorbance at 254 nm (blue line) and conductivity in mS/cm (orange line) *versus* volume. The different steps of the chromatographic separation (equilibration, injection, wash, elution and column regeneration) are indicated. Collected fractions are also indicated.

Figure 6: Purification to homogeneity of a recombinant RNA produced in *E. coli* using the viroid-based system. Total RNAs from an *E. coli* clone that produces a chimeric ELVd-3' CITE were first purified by chromatography using a DEAE-Sepharose column and then separated by 2D PAGE. First gel was run under denaturing conditions (8 M urea, buffer TBE). After staining with ethidium bromide, the gel band that contains the circular form of the recombinant RNA was transferred to the top of a second gel that was run under non-denaturing condition (buffer TAE). The acrylamide of the second gel was cross-linked with *N,N'*-bis(acryloyl)cystamine, which allowed solubilization of the gel fragment that contained the pure recombinant RNA.

DISCUSSION:

While researching the ELVd sequence and structure requirements involved in the recognition by the eggplant tRNA ligase, we noticed that co-expression of both molecules in the non-host *E. coli* led to an unexpected large accumulation of viroid circular forms in bacterial cells[19]. We understood that the large accumulation of viroid RNA in *E. coli* most probably was the consequence of co-expressing a highly stable RNA molecule, such as the relatively small (333 nt), highly based-paired, circular viroid, and a protein for which this particular viroid displays high affinity. ELVd RNA must recruit the host tRNA ligase to mediate its circularization during replication in the infected plant[17]. Although we do not have experimental confirmation, we anticipate that both molecules form a ribonucleoprotein complex in *E. coli* that further stabilizes the viroid RNA and allows reaching the remarkable concentration of 150 mg per liter of bacterial culture that highly exceeds those of endogenous RNAs, such as rRNAs[12]. Since ELVd does not replicate in *E. coli*, we reasoned that insertion of a heterologous RNA was not going to dramatically affect accumulation of the viroid-derived RNA and, in fact, that was the case. This

observation is the basis of the method to produce large amounts of recombinant RNA in *E. coli* that we describe here. The method uses the circular RNA molecule of ELVd as a scaffold on which the RNA of interest is presented. To produce a circular ELVd scaffold in *E. coli*, we need to express a precursor RNA with the duplicated domain of the viroid hammerhead ribozyme. ELVd ribozymes self-cleave very efficiently in *E. coli* and the resulting viroid monomers are recognized and circularized by the co-expressed tRNA ligase. Co-expression of the tRNA ligase is a key feature of the system. ELVd RNA is hardly detected in *E. coli* unless this particular enzyme is co-expressed[12].

In our protocol, plasmid pLELVd-BZB allows to insert the cDNA corresponding to the RNA of interest by simple and efficient Gibson assembly between positions U245 and U246 of ELVd. This site corresponds to the terminal loop of a long hairpin present in the most likely ELVd conformation[24,25]. Insertion in this particular position must promote that the RNA of interest protrudes from the very stable scaffold formed by ELVd and must avoid intramolecular interaction between the RNA of interest and ELVd (**Figure 1**). We have not explored the effect of inserting the RNA of interest in alternative positions of the ELVd molecule. Plasmid p15LtRnlSm allows co-expression of the eggplant tRNA ligase. RNAs are transcribed in both plasmids under the control of a constitutive strong *E. coli* promoter, namely murein lipoprotein (*lpp*). This makes the system constitutive with no need of induction. However, we also build a similar plasmid (p15tRnlSm) to express the tRNA ligase under the control of the phage T7 RNA polymerase in an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible system. Using this plasmid, accumulation of the recombinant RNA only occurs after induction of tRNA ligase expression by adding IPTG[12]. In the current system, the RNA of interest is expressed from a high copy number plasmid with pUC replication origin, while the tRNA ligase is expressed from a moderate copy number plasmid with p15A replication origin. Whether altering the copy number of both plasmids involved in the system improves accumulation of the recombinant RNA has not been explored so far. The size range of the RNA of interest admitted by the system has not been systematically investigated either, although small RNAs are logically expected to accumulate to higher concentrations than larger ones.

One of the reasons why production of recombinant RNA *in vivo* is not easy is most probably due to the intrinsic low half-life of RNA molecules. Our system is not alien to this problem. In fact, at a late *E. coli* growing phase, accumulation of recombinant RNA starts to decrease to virtually disappear[12]. This forces one to find the right window to harvest the cells. We observed, though, that this window is large enough to make the system friendly. However, we also observed that the optimum window depends on many factors including the particular *E. coli* strain, culture medium, growing conditions (agitation, volume of the flask, airing, *etc.*) and the physiological stage of the bacteria used to start the liquid culture. We recommend starting recombinant RNA production using fresh *E. coli* colonies from a plate inoculated the previous day and grown at 37 °C. Storing plates in the fridge makes the harvest window more unpredictable. We obtained the most consistent results by starting liquid cultures with bacteria picked from a plate with a toothpick. The use of a liquid pre-culture, particularly if saturated, also drives to variability in the production protocol.

Regarding purification, treatment of bacterial cells with phenol:chloroform is a very effective manner to break the cells and allows for the quantitative recovery of total bacterial RNA in the aqueous phase. Depending on the downstream application of the recombinant RNA, this aqueous phase or the preparation that results from precipitating the RNA from this aqueous phase with an alcohol may be enough. If further purification is needed, we recommend anion-exchange chromatography using a DEAE anion exchange column (**Figure 5**). This purification step allows efficient removal of DNA and bacterial metabolites that also partitioned in the aqueous phase. If the downstream application of the recombinant RNA requires purification to homogeneity, the viroid-derived system offers a clear advantage when compared to other methods. Since a main fraction of the recombinant RNA is produced as a circular form, advantage of this property can be taken to separate it from the bulk of bacterial RNAs. Circular RNAs experience a delay in electrophoretic migration in denaturing conditions with respect to the linear counterparts of the same size[22]. This delay is more pronounced when the RNA is electrophoresed under lower ionic strength. In practice, circular RNAs can also been separated by two-dimensional denaturing PAGE under high and low ionic strengths (**Figure 4**). After electrophoretic separation, the recombinant RNA must be eluted from the gel. The use of a reversible cross-linker of acrylamide, such as *N,N'*-bis(acryloyl)cystamine[23], facilitates recovery, particularly when purifying large amounts of RNA (**Figure 6**). Finally, if downstream application of the produced RNA demands separation of the RNA of interest from the viroid scaffold, our protocol does not offer any particular advantage to previous methods. The RNA of interest must be excised from the chimeric molecule using some of the previously described strategies, such as the use of ribozymes, DNAzymes or, possibly most efficient, the use of RNase H guided by two DNA oligonucleotides[7]. Trying to avoid this last cumbersome purification step, we have worked on trying to reduce the size of the viroid scaffold, although with partial success. We were able to produce notable amounts of recombinant RNA using viroid deleted forms (175, 215 and 246 nt) but increasing deletions of the viroid scaffold correlated with decreasing accumulation[12].

In sum, here a protocol is described to produce large amounts of recombinant RNA in *E. coli* that, to our knowledge, improves the yield of previously published methods. The protocol is based of co-expression in *E. coli* of the RNA of interest inserted into a viroid (ELVd) scaffold and the eggplant tRNA ligase, the enzyme that circularizes this viroid in infected plants. A distinctive feature of our protocol is that recombinant RNA is mainly produced as a circular form, a property that facilitates purification to homogeneity for downstream applications. Using this protocol tens of milligrams of recombinant RNA can be easily produced per liter of *E. coli* culture in regular laboratory conditions.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that the technology described in this protocol has been patented (US Patent No. EP14382177.5, PCT/EP2015/060912).

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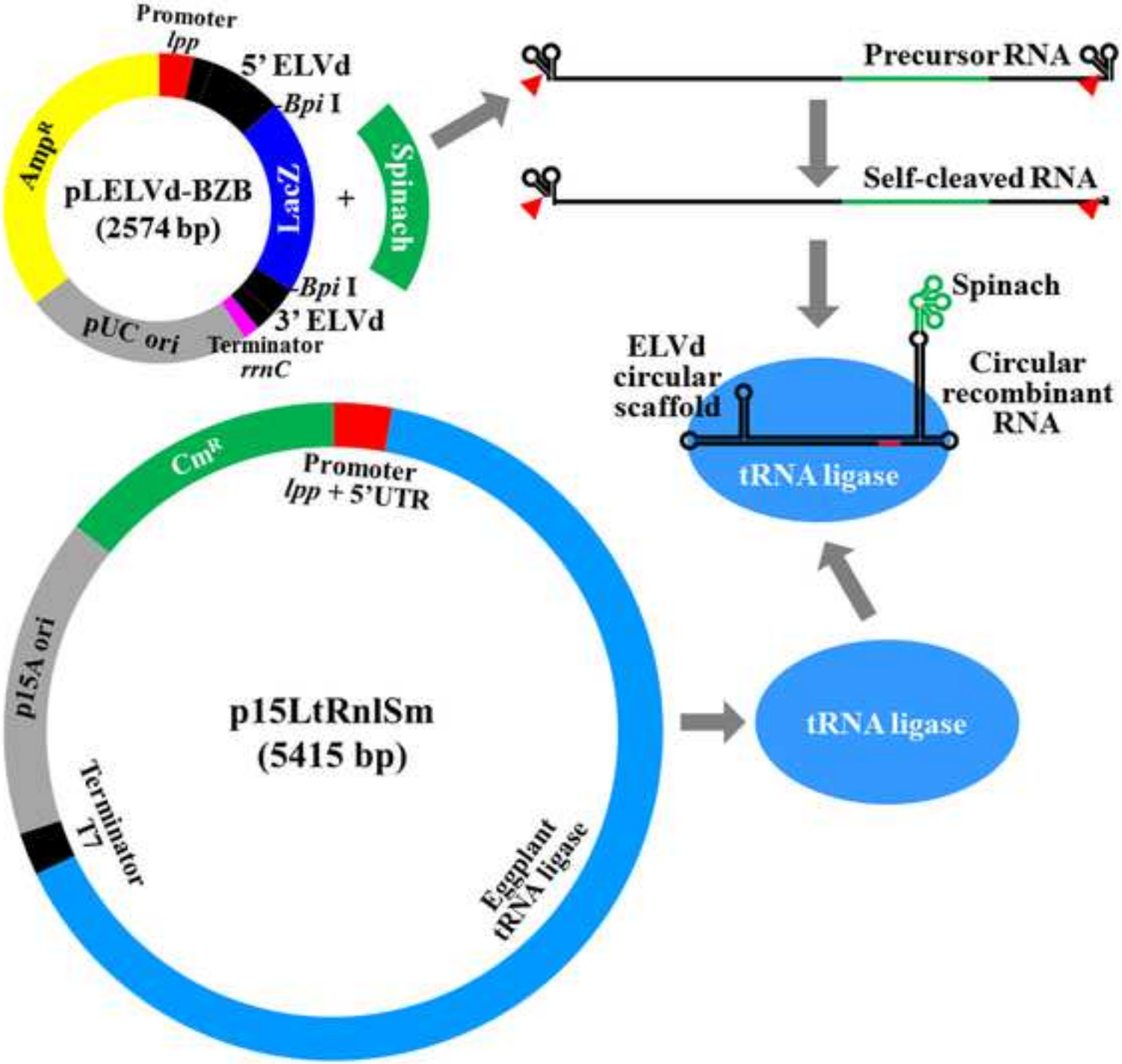
Figure 1



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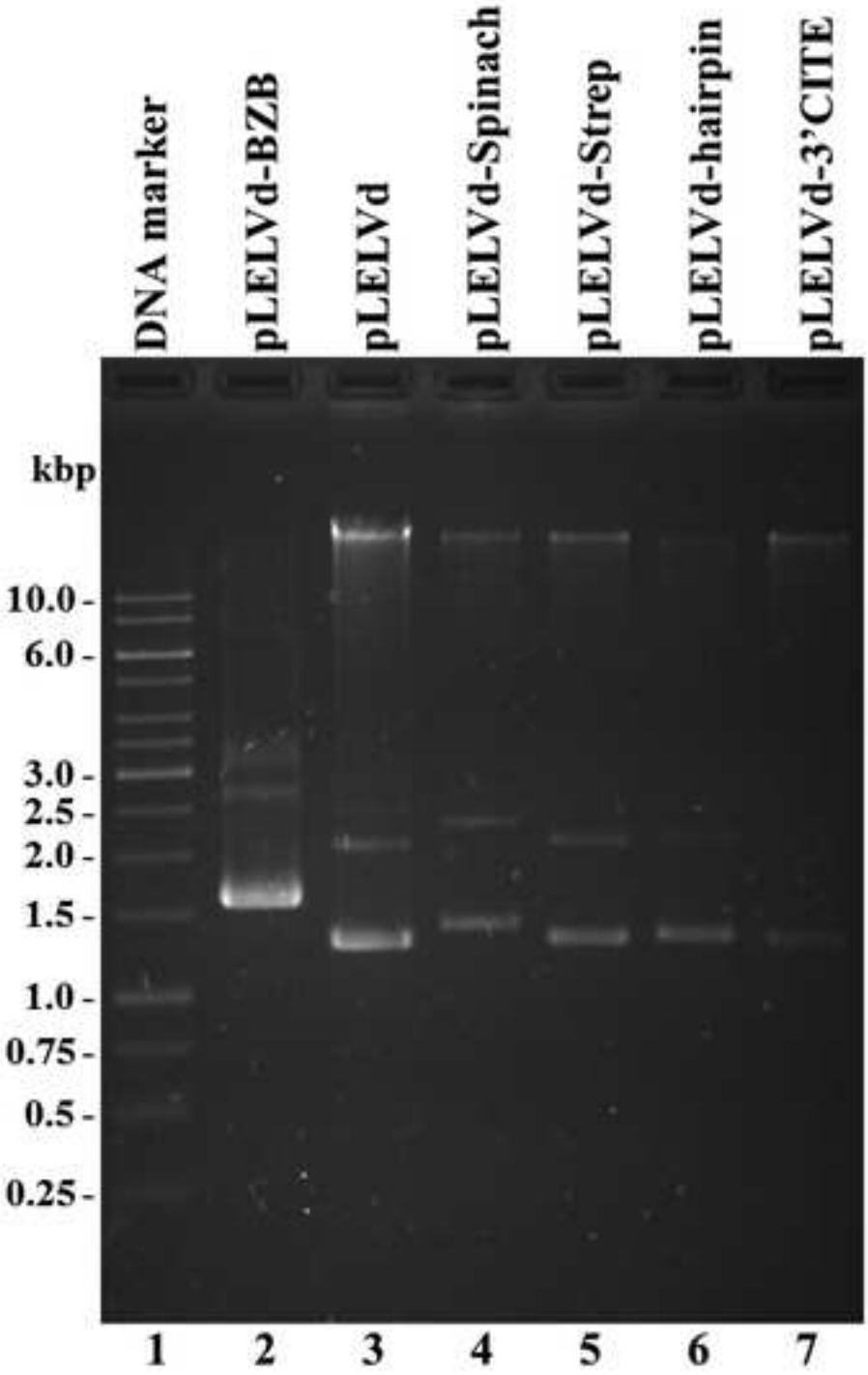

Figure 3

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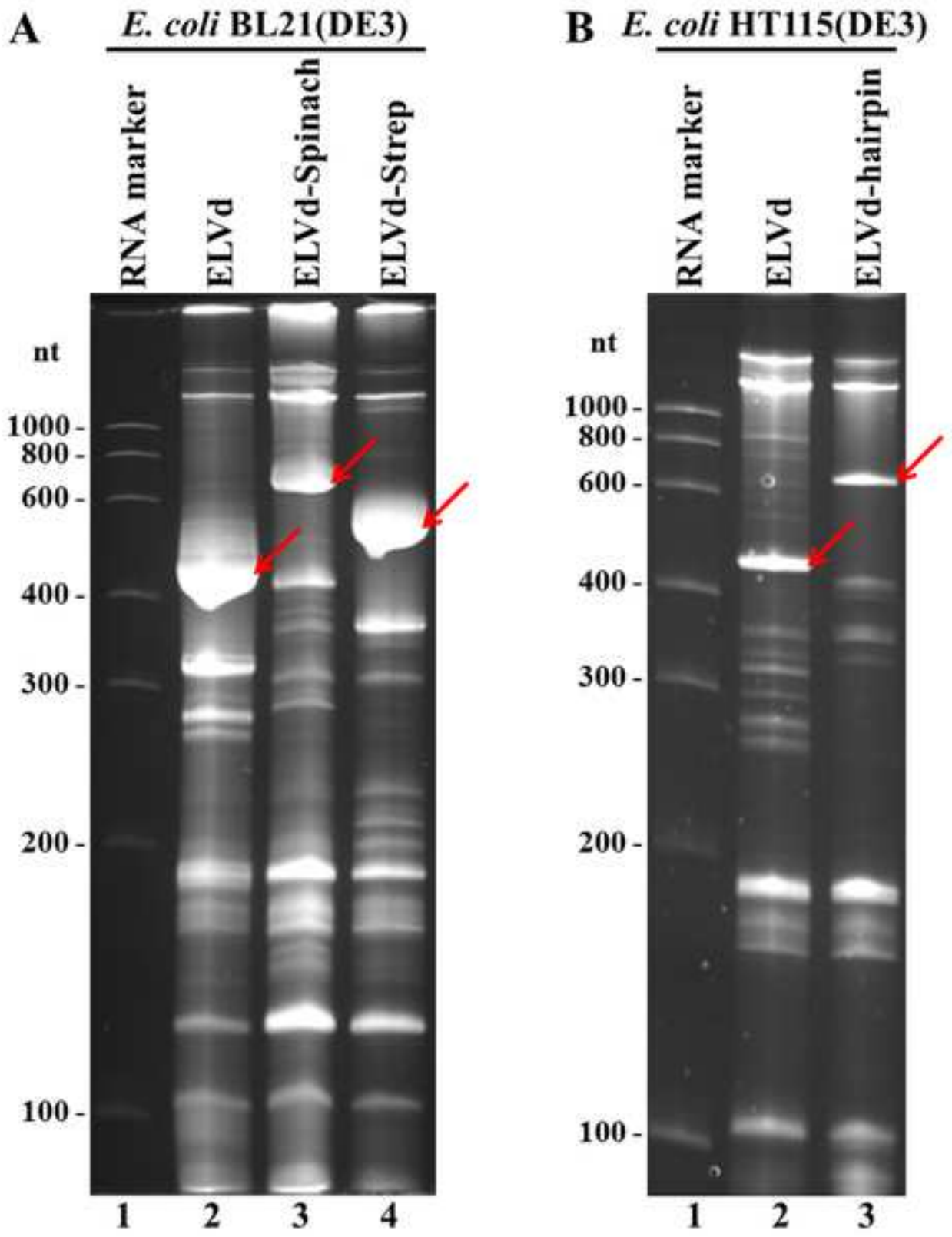

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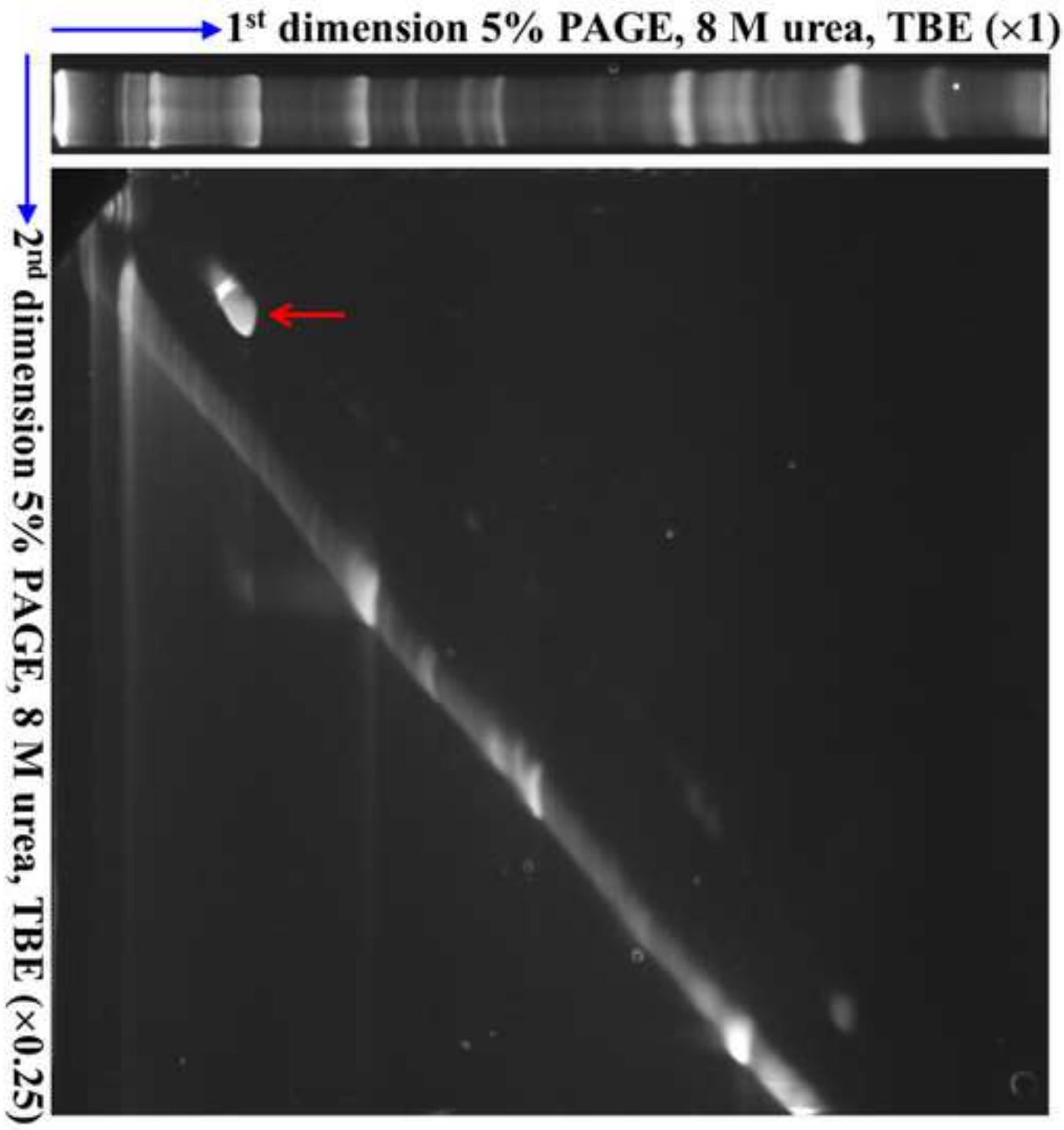

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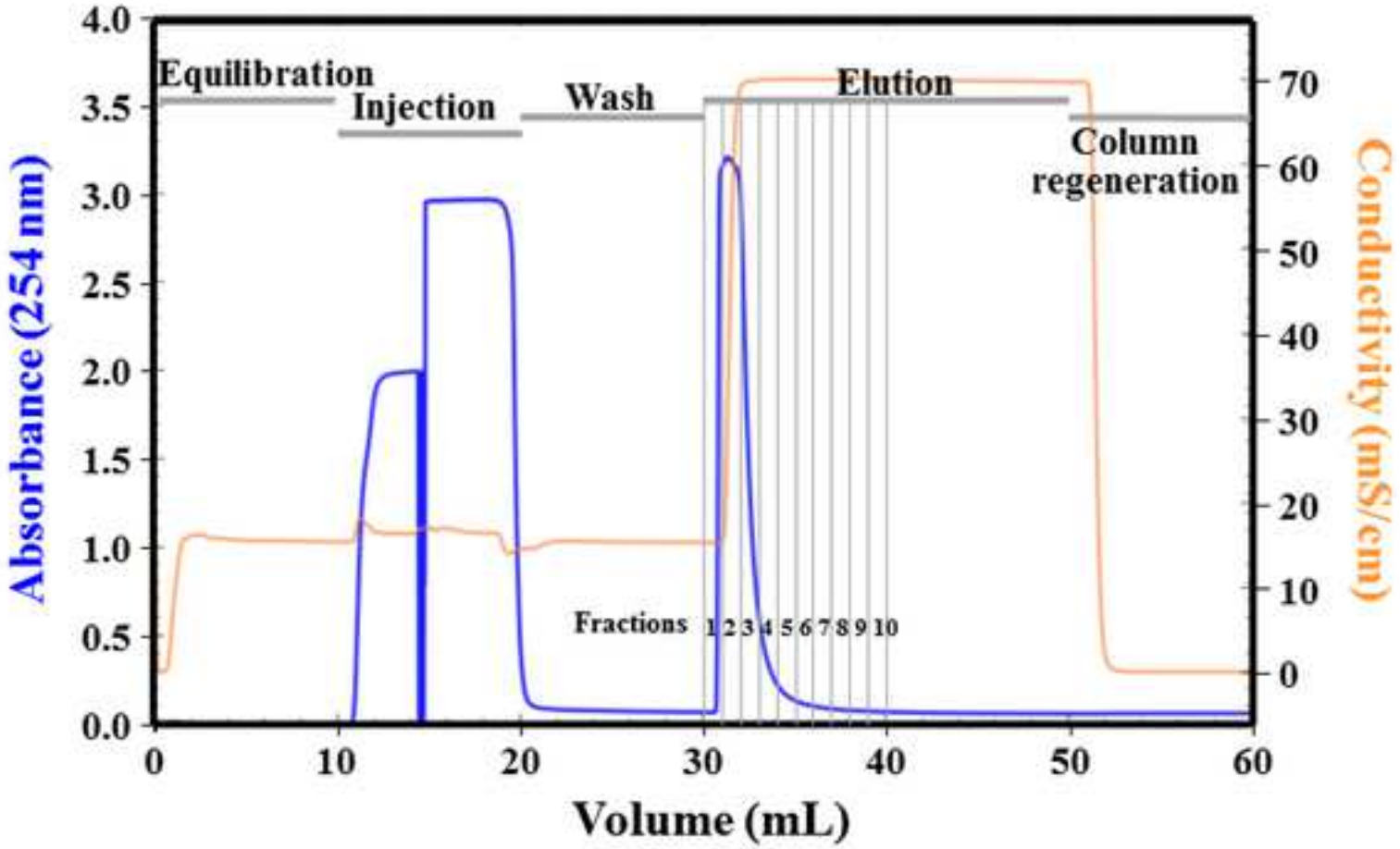

Figure 6

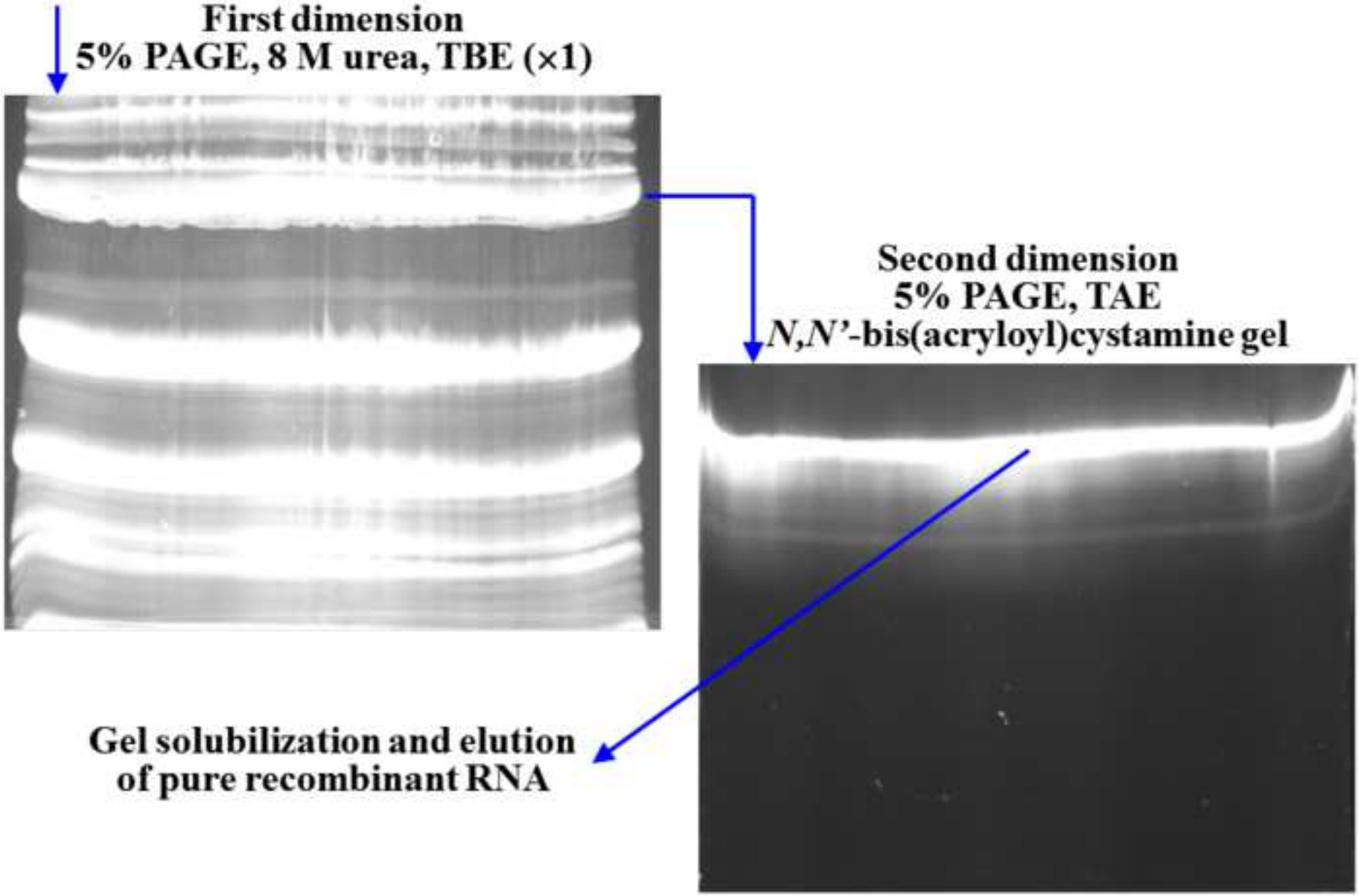

Table of Materials

Click here to download Table of Materials 2018-07-25 Recombinant circular RNAs.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
Phusion High-Fidelity DNA polymerase	Thermo Scientific	F530S
Bpi I	Thermo Scientific	ER1011
Agarose	Conda	8010
Tris	PanReac AppliChem	A1086,1000
Acetic acid	PanReac AppliChem	131008.1214
EDTA	Sigma-Aldrich	E5134-500G
Ethidium bromide	PanReac AppliChem	A1152,0025
Zymoclean Gel DNA Recovery	Zymo Research	D4001
NanoDrop	ThermoFisher Scientific	ND-3300
NEBuilder HiFi DNA Assembly Master Mix	New England BioLabs	E2621S
DNA Clean & Concentrator	Zymo Research	D4003
Eporator	Eppendorf	4309000019
Escherichia coli DH5α	Invitrogen	18265-017
Tryptone	Intron Biotechnology	Ba2014
Yeast extract	Intron Biotechnology	48045
NaCl	PanReac AppliChem	131659.1211
Agar	Intron Biotechnology	25999
Ampicillin	PanReac AppliChem	A0839,0010
X-gal	Duchefa	X1402.1000
N,N-Dimethylformamide	PanReac AppliChem	131785.1611
NucloSpin Plasmid	Macherey-Nagel	22740588.250
Escherichia coli BL21(DE3)	Novagen	69387-3
Escherichia coli HT115(DE3)		
Chloramphenicol	Duchefa	C 0113.0025
Glycerol	PanReac AppliChem	122329.1211
KH_2PO_4	PanReac AppliChem	131509.1210
K_2HPO_4	PanReac AppliChem	122333.1211
HCl	PanReac AppliChem	131020.1211
Phenol	Scharlau	FE04791000
Chloroform	PanReac AppliChem	A3691,1000
Filtropur S 0.2	Sarstedt	83.1826.001

HiTrap DEAE Sepharose FF column	GE Healthcare Life Sciences	17-5055-01
ÄKTAprime plus liquid chromatography system	GE Healthcare Life Sciences	11001313
Acrylamyde	PanReac AppliChem	A1089,1000
N,N' -methylenebisacrylamide	Sigma-Aldrich	M7279-100G
Urea	PanReac AppliChem	146392.1211
Boric acid	PanReac AppliChem	A2940,1000
Formamide	PanReac AppliChem	A0937,2500
Bromophenol blue	Sigma-Aldrich	B8026-5G
Xylene cyanol	Sigma-Aldrich	X4126-10G
N,N'-Bis(acryloyl)cystamine	Sigma-Aldrich	A4929-5G

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D1 low EEO

1%

Ref. Timmons et al., 2001

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Valencia, 26 July 2018

Dear Dr. Cao:

We are submitting a revised version of our manuscript "Production of large amounts of recombinant RNAs on a circular scaffold using a viroid-derived system in Escherichia coli" (JoVE58472) in which we have addressed all editorial comments as well as concerns and comments raised by the three reviewers. We have used the MS Word revision tools to highlight changes in our manuscript. In addition, below you can find a detailed point-by-point response to reviewers.

We hope our work is now acceptable for JoVE. We trust publication in your innovative journal will highly increase the visibility of our method.

Sincerely,

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--- Ok.

2. Figure 3: Please line up the panels better. Please apply the same vertical dimension for A and B if possible.

--- Done.

3. Figure 5: Change “ml” to “mL”.

--- Changed.

4. Keywords: Please provide at least 6 keywords or phrases.

--- We added one more.

5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

--- Rephrased.

6. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

--- After a first introductory sentence, the goal of the protocol is clearly stated in the second sentence of Long Abstract.

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

--- The overall goal of the method is stated at the end of Introduction.

8. Please define all abbreviations before use (SOC, etc.).

--- Done.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

--- Done.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

--- Converted.

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Thermo Scientific, Zymoclean Gel DNA Recovery, NanoDrop, New England BioLabs, DNA Clean & Concentrator, Zymo Research, Eporator, Eppendorf, NucloSpin Plasmid, Macherey-Nagel, Sarstedt, GE Healthcare Life Sciences, etc.

--- Removed.

12. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

--- Done.

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

--- Done.

14. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

--- Done.

15. 1.1: Please list the PCR conditions.

--- Listed.

16. 1.2: Please provide the composition of buffer G. What volume of buffer G is used, and what container is used in this step?

--- Provided.

17. 1.3: Please break up into sub-steps and add more details. For instance, what wavelength is measured? What is the elution solution? Please specify throughout.

--- Done.

18. 1.5: What volume of liquid medium is used?

--- Kit instructions must be followed.

19. 2.3: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

--- Done.

20. 3.3: After centrifugation, is supernatant discarded? Please specify.

--- Specified now.

21. 3.7: Please break up into sub-steps.

--- Done.

22. 3.8: Please write the text in the imperative tense.

--- Done.

23. 4.2: What is used to heat?

--- Clarified now.

24. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

--- Done.

25. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

--- Done.

26. Please discuss critical steps within the protocol and any limitations of the technique in the Discussion.

--- Done.

27. JoVE article does not have a Conclusion section. Please move information in the Conclusion section to Results or Discussion section.

--- Removed.

28. Please disclose your patent for the method in the Disclosure section.

--- Disclosed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Manuscript describes a good method for mass-production of RNA through a circular viroid. Embedded RNA will be a part of circular RNA.

Major Concerns:

Major question is that the self-splicing ribozyme is known to cut and ligate RNA (reversible reaction) at the specific site. Only magnesium ion is required. Did the authors test their system without the tRNA ligase? If this is the case, the technique becomes much simpler and more useful. Authors mentioned that for "the system to work", the tRNA ligase is required. No proof.

--- Ribozyme-mediated self-ligation is extremely inefficient. Substantial ligation in *E. coli* only occurs in the presence of co-expressed eggplant tRNA ligase. This has been largely demonstrated in our previous works (see references 12 and 19).

Minor Concerns:

Line 282: recombinant plasmids usually migrate "faster" (it should be slower). Fig. 3: I do not see the cellular tRNAs (about 80-100nt). There is no evidence for the comment (lines 400-401). Pointing to Fig. 1 does not make it real!

--- The reason why recombinant plasmids usually migrate faster is because the polylinker that is released during the cloning process is usually larger than the cDNAs corresponding to the RNAs of interest. In Fig. 3, E. coli tRNAs run out of the gel (note the position of the 100 nt RNA marker). In discussion, we speculate that RNA of interest must protrude from the viroid scaffold and point to the schematic representation in Fig. 1 for the readers to understand what we mean (not to demonstrate the speculation). However, in our opinion this speculation is rather likely based on the extremely stable secondary structure of the viroid scaffold and the experimental observation with the Spinach aptamer that folds properly and efficiently emits green fluorescence (see Reference 12).

Reviewer #2:

Manuscript Summary:

The authors describe a method for RNA production in large amounts. It is based on two E. coli plasmids: one carries a copy of a viroid (longer-than unit-length including two hammerhead ribozyme motifs) and the second a tRNA ligase. The viroid transcript is cleaved by the internal ribozymes into a unit-length molecule that is circularized by the co-expressed ligase. The viroid circles are produced in impressive amounts mainly due to their resistance against degradation. In addition the circularity of the RNA allows for easy purification. The viroid sequence (335 nt) is used as a scaffold containing an insert for the RNA of interest. The method is described for four different inserts: the aptamer Spinach (with 84 nt length, mimicking GFP), a Streptavidin-binding aptamer (45 nt?), an RNA hairpin (with 42 basepair double-stranded region), and a 3' cap-independent translation enhancer element. Features of these inserts and their relative yields are not described; such short additional descriptions might be helpful for the reader's judgment on possible further inserts. Fragmentation of the circular RNA and isolation of the insert are not described. The manuscript is based on a prior publication (Daros, Aragones & Cordero (2018) A viroid-derived system to produce large amounts of recombinant RNA in Escherichia coli. Sci. Rep. 8, 1904).

Minor Concerns:

I would change three minor things in the ``Long Abstract'':

1. ``... an ELVd precursor is efficiently transcribed and processed by the embedded hammerhead ribozymes ..." => ``... an ELVd precursor is efficiently transcribed by the E. coli RNA polymerase and processed by the embedded hammerhead ribozymes ..." (line 43--44)
This small addition avoids the impression that the precursor is transcribed by the RZ (or needs an additional polymerase).
2. ``, most probably based on formation of a stable ribonucleoprotein complex." (line 53)
I would delete this part of the sentence because the manuscript does not show any experimental hints for this hypothesis.
3. ``... facilitates the easy purification ..." => ``... facilitates the purification ..."(line 48)
IMHO, the `easy' is superfluous.
--- Changed.
Line 89: ``Viroids must account between the most stable RNAs ..." => ``Viroids must be ranked among the most stable RNAs ..." [Note that I am not a native speaker.]
--- Corrected.
Line 118: ``promote." => ``promoter."
--- Corrected.
Line 173: ``interst." => ``interest."
--- Corrected.
Line 318: ``... ribozymes and. "
Either the ``and" is unnecessary or some additional text is missing.
--- Corrected.
At several places the greek letter `micro' seems to be missing; for examples see lines 166, 189 (2X), 209, 210, 255 (2X), 261, etc.
--- This must be a problem with pdf conversion. It is okay in the original MS Word document.
The last three pages of the PDF show some random(?) text.
--- Same for this.

Reviewer #3:

Manuscript Summary:
The manuscript by Cordero et al. describes the production of recombinant RNA using a viroid-derived system. This manuscript follows up upon their detailed publication in Scientific Reports (2018), so it is not surprising that this work is detailed, well-written and almost ready for publication.
My comments are about the writing, since the scientific part is sound and well established.

Minor Concerns:

1) Given that this paper is an addition to the Sci Rep publication, I recommend trying to reduce the length of it, if possible.

--- We did our best to condense information.

2) The word amazing appears in six instances of the text. Please, reword the sentences or find suitable synonyms.

--- Good advice! We replaced all but one.

3) The sentence starting in line 112 (to 113) sounds odd to me. Please reword.

--- Reworded.

4) Line 118: "promoter".

--- Done.

5) Line 171: Consider advising the reader to use restriction mapping if electrophoretic migration of intact plasmids does not give clear results.

--- Done.

6) Line 219: Speeds are given in rpm. The g force exerted is dependent on the centrifuge used. Please change rpm to g in all instances.

--- Done.

7) Line 270, add "the" before first.

--- Done.

8) Line 286: have the authors had any problems with co-electroporation? In my hands, sometimes that did not work, so I had to transform in sequential order.

--- With the plasmids in this system, we have excellent results co-electroporating and we definitively recommend this strategy to save time.

9) Line 296: "dimensional" seems to be missing before PAGE.

--- Corrected.

10) Line 308: facilities?

--- Corrected to facilitates.

11) Line 318: revise the point in that sentence.

--- Revised.

12) Line 322: "to form" seems out of place.

--- Corrected.

13) Line 415: ...than large"r" "ones".?

--- Corrected.

14) Line 423: use etc. instead of "..."

--- Done.

15) Please revise in the Materials table, cell A71.

--- Thanks! This was a left over.