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Discovery of Bacterial sRNAs by High-Throughput Sequencing

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Abstract

sRNA-Seq is an unbiased method that allows for the discovery of small noncoding RNAs in bacterial transcriptomes through direct cloning and massively parallel sequencing by synthesis. Small bacterial transcripts are enriched from a total RNA preparation and modified with 5' and 3' linkers that allow for downstream amplification and sequencing. This protocol includes a treatment that depletes small RNA fractions of tRNAs and 5S rRNA, thereby enriching the starting pool for non-tRNA/rRNA sequences. This protocol can be readily modified to target different RNA species for depletion or to change the size range of RNAs to be sequenced. Thus, sRNA-Seq represents a comprehensive, versatile cloning protocol that may be applicable to the cloning of small RNAs of any size range from any organisms.

Keywords

sRNAs; Transcriptome; Massively parallel sequencing; tRNA/rRNA depletion; Direct cloning

1. Introduction

Regulatory RNAs are ubiquitous in nature – they have now been found in all branches of life and in all organisms investigated. In bacteria, small (~30–500 nt) noncoding RNAs (sRNAs) are members of regulatory circuits involved in diverse processes including quorum sensing, carbon metabolism, stress responses, and virulence (1–3). Many of the best-characterized sRNAs base pair with mRNAs transcribed from distinct loci and affect the translation and/or stability of the targeted mRNA (4). Alternatively, several sRNAs affect downstream gene expression by binding protein transcription factors directly and inhibiting their activity (5, 6). Although most sRNAs characterized to date fall into one of these two categories, ones that are transcribed antisense to, or even within, annotated protein-encoding genes have also been described (7–10).

Both experimental and computational approaches have proven useful in identifying sRNAs in diverse species. These methods often focus on transcripts within intergenic regions (IGRs), where many sRNAs have been identified, or they focus on sRNAs of a specific size range. A more general method, sRNA-Seq, involves direct cloning and massively parallel sequencing by synthesis (MPSS), providing an unbiased approach that allows for interrogation of the entire sRNA repertoire in any bacterium. Using sRNA-Seq, we have recently reported the discovery of over 2,000 sRNAs in the *Vibrio cholerae* transcriptome (9). These results strongly suggest that there are many sRNAs remaining to be discovered in bacteria, even in *Escherichia coli*, where the majority of the sRNA discoveries have been made. This method includes a treatment that depletes total RNA fractions of highly abundant tRNAs and small subunit rRNA, thereby enriching the sample for sRNA transcripts with novel functionality. Similar methods of direct cloning and MPSS have been extremely powerful in the microRNA field (11), and we anticipate that sRNA-Seq will likewise allow for comprehensive sRNA identification in many bacteria.

2. Materials

2.1. General Materials

1. Disposable, RNase-free pipette tips, polypropylene 1.5-mL nonstick microcentrifuge tubes and thin-wall microfuge tubes for PCR.
2. RNase-Zap for preparing materials to be RNase-free (Ambion, Austin, TX).
3. Mini-PROTEAN 3 Electrophoresis System for vertical gel electrophoresis (Bio-Rad, Hercules, CA). The glass plates, combs, and chambers should be treated with RNase-Zap prior to use.
4. 10× TBE: 108 g Tris base, 55 g boric acid, 40 mL 0.5 M EDTA pH 8.0, 960 mL ultrapure H₂O. Filter-sterilize and store at room temperature. From this stock, prepare 1 L, filter-sterilized 1× TBE, as needed.
5. Polyacrylamide gels: 30% w/v acrylamide, 0.8% w/v bisacrylamide solution (37.5:1) (this is a neurotoxin when unpolymerized), urea, *N,N,N,N'*-tetramethylethylenediamine (TEMED).
6. Ammonium persulfate: prepare 1.6 or 10% (w/v) solutions in ultrapure water and store at 4°C. Use within 1 month of solution preparation.
7. Century and Decade Markers (with 10× PNK Buffer and Cleavage Reagent) (Ambion, Austin, TX). Store Century Markers in 5-μL aliquots and all RNA markers at −80°C.
8. 100-bp quick-load DNA ladder (NEB, Ipswich, MA). Store at 4°C.
9. Loading Buffer II (Ambion, Austin, TX). Store at −20°C and then thaw on ice prior to use.
10. SYBR Gold (Invitrogen, Carlsbad, CA). Store at −20°C. Prior to use, allow the dye to thaw completely at room temperature. This dye is light sensitive.
11. Sodium chloride (NaCl): prepare a 0.4 M solution from a room-temperature 5 M solution and ultrapure water. Filter-sterilize the 0.4 M solution immediately before use.
12. Nanosep tubes: either 100K or 0.2 μm size is appropriate (VWR, West Chester, PA).
13. Glycogen (5 mg/mL) (Ambion, Austin, TX). Store at −20°C in 100-μL aliquots.
14. 100 and 70% ethanol are prepared with highest-grade ethanol and ultrapure water. Store at −20°C.

2.2. Total RNA Preparation

1. GS-3 tubes and Nalgene Oak Ridge phenol-resistant SS34 tubes (VWR, West Chester, PA). The SS34 tubes should be treated with RNase-Zap prior to use.
2. AE Buffer: 50 mM NaOAc pH 5.2, 10 mM EDTA pH 8.0, prepared with ultrapure water and filter-sterilized.
3. SDS: prepare a 20% w/v solution with ultrapure water and filter-sterilize; store at room temperature.
4. Acid phenol:chloroform (5:1) pH 4.5 (Ambion, Austin, TX). Store at 4°C.
5. Phase Lock Gel tubes (50 mL size) (Eppendorf, Hamburg, Germany).

6. Chloroform. Store at room temperature.
7. Sodium acetate (NaOAc): a 3 M solution is made with ultrapure water and adjusted to pH 5.2 with acetic acid. Filter-sterilize the final solution and store at room temperature.
8. Isopropanol. Store at room temperature.
9. DEPC-H₂O (Ambion, Austin, TX). Store at room temperature.

2.3. Addition of 3' Linkers

1. miRNA cloning Linker 1 (5'-rAppCTGTAGGCACCATCAAT/3ddC/-3') and 2 (5'-rAppCACTCGGGCACCAAGGA/3ddC/-3') (IDT, Coralville, IA). They are resuspended at 100 μ M concentration in IDTE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0) and stored at -20°C.
2. T4 RNA Ligase (10 U/ μ L) (Promega, Madison, WI) and diluted tenfold in 1 $\times$ Ligase Buffer immediately prior to use.
3. 5 $\times$ Ligase Buffer: 250 mM HEPES pH 8.3, 50 mM MgCl₂, 16.5 mM DTT, 50 μ g/mL BSA, 41.5% glycerol. Store buffer at -20°C in 100- μ L aliquots.

2.4. Depletion of tRNAs and rRNAs

1. Single-stranded DNA oligonucleotides to deplete tRNAs are ~30 nt long and complementary to the 3'-ends of the tRNAs. The tRNA sequences for many organisms can be found using the Genomic tRNA Database (<http://gtrnadb.ucsc.edu/>). Our lab has written a script that designs a set of oligonucleotides complementary to a given sequence set, allowing mismatches at the user's discretion (<https://sciviz.tufts.edu/confluence/display/CamillLaboratory/Camilli+Lab+Supplementary+Data+Site;jsessionid=9785E20CFBEA6DDCF7EB3DC51A7D73BE>). Two to three mismatches allow for one oligonucleotide sequence to potentially base-pair with several different tRNAs, ultimately reducing the number of oligonucleotide sequences that need to be designed and synthesized. Alternatively, by eye, one can align the 3' ends of tRNAs and design by hand oligonucleotides that are complementary to the tRNAs. Because the 3' ends of many tRNAs are identical, or highly similar, fewer oligonucleotides than tRNAs need to be designed. We designed 25 oligonucleotides that are sufficient for depleting all 98 tRNAs in the *V. cholerae* transcriptome. Depletion oligonucleotides are kept as 100 μ M solutions in 1 mM Tris-HCl pH 8.0 at -20°C.
2. Oligonucleotides to deplete the 5S rRNA are ~30 nt long and complementary to the 3' end of the 5S rRNA and additional regions of the transcript. For *V. cholerae*, we designed four oligonucleotides to deplete the 5S rRNA. These oligonucleotides were kept as 100 μ M solutions in 1 mM Tris-HCl pH 8.0 at -20°C.
3. Oligo Mix: as a starting point, mix all depletion oligonucleotides 1:1, to create a final 100 μ M solution. Store the Oligo Mix at -20°C in 100- μ L aliquots.
4. 2 $\times$ Depletion Buffer: 100 mM Tris-HCl pH 7.8, 600 mM KCl, 20 mM MgCl₂, 20 mM DTT.
5. RNase H (NEB, Ipswich, MA).

2.5. Reverse Transcription

1. dNTPs (100 mM) (NEB, Ipswich, MA). Mix 1:1:1:1 and dilute tenfold with ultrapure water. The solution is stored at -20°C .
2. RT/REV Primer (5'-GATTGATGGTGCCTACAG) is resuspended in 1 mM Tris-HCl pH 8.0 to make a 100 μM solution and stored at -20°C . A working solution of 10 μM is made as needed.
3. SuperScript III Reverse Transcriptase (with 10 $\times$ buffer and 0.1 M DTT) (Invitrogen, Carlsbad, CA).
4. RNase Inhibitor (Ambion, Austin, TX).
5. ExoSAP-IT (USB, Cleveland, OH).
6. Phenol:chloroform:IAA (25:24:1). Store at 4°C .

2.6. PCR

1. *Taq* DNA Polymerase (with 10 $\times$ Buffer) (NEB, Ipswich, MA).
2. SIII forward primer (5'-GCCTCCCTCGCGCCATCAGGAT TGATGGTGCCTACAG-3') and SIII reverse primer (5'GCC TTGCCAGCCCCGCTCAGGTCCTTGGTGCCCGAG TG-3') are resuspended in 1 mM Tris-HCl pH 8.0 to make a 100 μM solution and stored at -20°C . A working solution of 10 μM is made, as needed. Underlined sequences are regions used by 454 to prime sequencing reactions.
3. 6 $\times$ DNA Loading Dye: 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10 mM Tris-HCl pH 7.5, and 50 mM EDTA pH 8.0.
4. *n*-Butanol. Store at room temperature.
5. Micro Bio-Spin 30 (Bio-Rad, Hercules, CA) columns may be used for buffer exchange. These should be stored at 4°C .
6. TOPO TA Cloning kit for Sequencing (Invitrogen, Carlsbad, CA).
7. QIAprep 96 Turbo Miniprep Kit (Qiagen, Valencia, CA).

3. Methods

The sRNA-Seq method is illustrated in Fig. 1. Representative gels from the various steps in the method are shown in Fig. 2. All the typical precautions against RNase contamination should be observed throughout the sRNA-Seq cloning protocol. Gloves should be worn at all times and changed frequently. Materials and solutions should be kept protected from dust. Any solid materials not supplied as RNase-free should be treated so that they are free from nucleases.

RNA transcripts in a size range predetermined by the investigator are gel-purified, enriched, and cloned through the addition of oligonucleotide linkers to both the 5' and 3' ends of the transcript. The depletion step takes place after addition of a 3' linker and effectively removes tRNAs and 5S rRNA from downstream analysis by separating these transcripts from the linker that is necessary for reverse transcription, amplification, and sequencing. Readout of each individual clone is accomplished through high-throughput 454 pyrosequencing (a robust means of MPSS). The direct cloning portion of the sRNA-Seq protocol, once optimized by the individual, takes less than 1 week to complete. Initially, we suggest running through the method 1–2 times to become familiar with the steps and using small-scale sequencing (96-well format) to optimize the protocol for the user's needs.

In the following sections, we provide an example of cloning 80–200 nt sRNAs and the use of 454 (Roche) sequencing. The long read lengths (~300 nt) of 454 sequencing render it highly applicable for sequencing sRNAs without the need for paired-end MPSS. Nevertheless, this method should be easily adaptable to other size ranges of sRNAs, as well as other methods of MPSS, such as Solexa (Illumina) or SOLiD (ABI) paired-end sequencing.

3.1. General Protocols

1. To prepare a 1-mm thick, 10% polyacrylamide TBE-Urea (denaturing) minigel, wear personal protective equipment, then mix 1 mL 10× TBE with 3.33 mL 30% w/v acrylamide:0.8% w/v bis-acrylamide (37.5:1), 2.14 mL H₂O, 4.2 g urea, 330 μL 1.6% ammonium persulfate, and 5 μL TEMED. Pour the gel and add a ten-well comb. The gel should polymerize in about 30 min (see Note 1). Before loading gels with samples, prerun the gel for 30 min at 300 V in 1× TBE. Run all samples at 200 V.
2. To prepare a 1-mm thick, 12% TBE (native) minigel, mix 1 mL 10× TBE with 4 mL 30% w/v acrylamide:0.8% w/v bis-acrylamide (37.5:1), 4.93 mL H₂O, 70 μL 10% ammonium persulfate, and 5 μL TEMED. Pour the gel and add a ten-well comb. The gel should polymerize in about 30 min (see Note 1). Before loading gels with samples, prerun the gel for 30 min at 300 V in 1× TBE. Run all samples at 200 V.
3. The Decade and Century Markers can be used to estimate sizing of RNA (see Note 2).
 - (a) Prepare Decade Marker by mixing 1 μL of Decade Marker with 1 μL 10× PNK buffer, 7 μL DEPC-H₂O, and 1 μL Cleavage Reagent. Let the reaction proceed for 5 min at room temperature before adding 10 μL Loading Buffer II.
 - (b) Prepare Century Marker by adding 1 μL Century Marker to 4 μL DEPC-H₂O and 10 μL Loading Buffer II.
4. To stain a gel for nucleic acids, soak gel in 40 mL 1× TBE with 4 μL SYBR Gold for 30 min at room temperature, protected from the light. Nucleic acids can then be visualized with the following filter set: Ex 465 nm, Em 535 nm.
5. To elute and precipitate nucleic acids from a polyacrylamide gel, use a clean razor blade to cut out gel slices (~10 × 5 mm each) containing RNA/DNA of desired size. Use forceps to place gel slices into 1.5-mL tubes.
 - (a) For each tube, crush the gel slice and then add 400 μL filter-sterilized 0.4 M NaCl. Place the tube into well-crushed dry ice for ~30 min. Allow the tube to thaw, at room temperature, rotating, overnight.
 - (b) The following morning, transfer the gel slurry to a Nanosep tube and spin at 16,000 × *g* for 3 min. Remove and keep the flow-through. If there is any residual liquid remaining with the gel pieces, repeat the spin. Ethanol-precipitate the RNA by adding 8 μL glycogen and 1 mL ice-cold 100% ethanol, and placing the tube on well-crushed dry ice for ~30 min. Spin the tube at 16,000 × *g*, for 30 min at 4°C. (For PCR products, wash pellet with 500 μL ice-cold 70% ethanol and spin for 5 min at 16,000 × *g*.) Decant the liquid carefully and use a “Kimwipe” to wick off any excess liquid around the rim of the tube. Air-dry the tube, inverted, for 5–10 min at room temperature.

3.2. Prepare Total RNA

1. Grow up 300 mL of bacteria in desired medium to desired OD₆₀₀. Spin down the bacteria in a GS-3 tube at $6,000 \times g$ for 10 min at 4°C. Decant the supernatant. Add 12 mL AE Buffer to the tube and resuspend the pellet by vortexing and/or pipetting up and down. Transfer the mixture to an SS34 tube.
2. Add 1 mL 20% SDS, 12 mL acid phenol:chloroform (5:1). Vortex to mix. Incubate the tube for 10 min in 65°C water bath, vortexing for 10 s every minute. Incubate the tube for 5 min on ice. Spin the tube for 15 min at $12,000 \times g$ in an SS34 rotor. In the meantime, prespin a 50-mL Phase Lock Gel tube, for 5 min at $1500 \times g$.
3. After the 15-min spin, the aqueous and organic layer should have separated, with a white precipitate in the middle layer. Carefully remove the top (aqueous) layer with a glass pipette and transfer to the prespun Phase Lock Gel tube (see Note 3).
4. Add 13 mL chloroform to the Phase Lock Gel tube. Cap the tube and invert the tube several times to mix. Spin the tube for 10 min at $1500 \times g$ in a tabletop centrifuge at room temperature. Pour the supernatant (on top of the white gel) into a new SS34 tube.
5. Add 1 mL 3 M NaOAc (pH 5.2), 50 µL glycogen, and 10 mL room-temperature isopropanol. Spin the tube for 40 min at $15,000 \times g$ in an SS34 rotor.
6. Wash the pellet with 4 mL ice-cold 70% ethanol and spin again for 20 min at $15,000 \times g$. Pour off the ethanol and shake to remove residual liquid. There should be a clear or milky-white pellet that is the RNA. Place the tube, without the cap, in a speedvac with the rotor off on low heat for 15 min to remove residual ethanol.
7. Resuspend the pellet in DEPC-H₂O (see Note 4).
8. Quantitate the RNA in a spectrophotometer at OD₂₆₀. Pure RNA has an OD₂₆₀/OD₂₈₀ ratio around 2.0 (see Note 5).
9. Run 1 µg of the RNA on a 1% agarose gel (a native gel is fine here). The gel should show three distinct bands for the 23S rRNA, 16S rRNA, and the 5S rRNA and tRNAs (which will run together). The 23S rRNA band should be approximately twice as bright as the 16S rRNA band.
10. Store the RNA in 500-µg aliquots at -80°C until ready to use for the sRNA cloning steps (see Note 6).

3.3. Enrich for Transcripts 80–200 nt Long

1. Prepare a 10% TBE-Urea minigel.
2. Mix 500 µg total RNA with 1–2 volumes of Loading Buffer II. Heat the RNA and the markers at 65°C for 5 min just prior to loading onto the gel.
3. Load the markers into the leftmost lanes of the prerun 10% TBE-Urea gel. Split the RNA equally into the remaining lanes, leaving the lane next to the markers empty, if possible. Run the gel for 35 min at 200 V (see Note 7).
4. Stain the gel with SYBR Gold.
5. Visualize the RNA and cut out the RNA between 80 and 200 nt (Fig. 2a) (see Note 8).
6. Elute the RNA overnight and precipitate the RNA with glycogen and ethanol. Resuspend the RNA with DEPC-H₂O (combining all the tubes into one) into a final volume of 20 µL.

3.4. Add on 3' Linker 1

1. Prepare a 10% TBE-Urea minigel.
2. Using the RNA from Subheading 3.3, set up three ligation reactions, one of which serves as a negative control. Save the remaining unused RNA at -80°C for future cloning, if needed.
 - (a) Set up two of the same reactions: to 5 μL of RNA, add 1 μL Linker 1, 2 μL 5 $\times$ Ligase Buffer, 1 μL DEPC- H_2O , and 1 μL T4 RNA Ligase (1 U/ μL ; see Note 9).
 - (b) For the negative control, to 1 μL RNA, add 2 μL 5 $\times$ Ligase Buffer and 7 μL DEPC- H_2O .
 - (c) Let reactions proceed for 2 h at room temperature.
3. Add 20 μL Loading Buffer II to each +Ligase reaction; add 10 μL Loading Buffer II to the -Ligase reaction. Heat the sample and the markers at 65°C for 5 min. Load the +Ligase reactions across the four rightmost lanes of the pre-run 10% TBE-Urea gel. Load the markers and the -Ligase sample in lanes on the left. Run the samples at 200 V for 45 min.
4. Stain the gel with SYBR Gold, visualize the RNA, and cut out the RNA between 98 and 218 nt (Fig. 2b).
5. Elute the linkered-RNA overnight.

3.5. Deplete Linkered-RNA of tRNAs and 5S rRNA

1. Prepare a 10% TBE-Urea minigel.
2. Take the overnight elution and ethanol-coprecipitate the RNA and depletion oligonucleotides by adding 15 μL Oligo Mix, 8 μL glycogen, 1 mL ice-cold 100% ethanol, and placing the tube on well-crushed dry ice for ~ 30 min (see Note 10). Finish the ethanol precipitation as usual.
3. Resuspend the dry pellets of linkered-RNA with 1 $\times$ Depletion Buffer and combine the samples into one tube with a final volume of 10 μL . Transfer the sample to a PCR tube.
4. Using a thermocycler, heat up the sample at 65°C for 5 min, then cool to 37°C , $0.1^{\circ}\text{C}/\text{s}$. When the sample reaches 37°C , add 0.5 μL RNase H and incubate the reaction at 37°C for 30 min. Repeat this step once.
5. Add 20 μL Loading Buffer II to the sample. Heat the sample and markers at 65°C for 5 min. Load the sample across the four rightmost lanes of the prerun 10% TBE-Urea gel. Load the markers in lanes on the left. Run the gel at 200 V for 45 min.
6. Stain the gel with SYBR Gold, visualize the RNA, and cut out the RNA between 98 and 218 nt (Fig. 2c) (see Note 11).
7. Elute the RNA overnight and precipitate the RNA with glycogen and ethanol. Resuspend the RNA with DEPC- H_2O , combining all tubes, to a final volume of 10 μL .

3.6. Reverse Transcription of sRNA-Enriched Pool

1. In a PCR tube, to the 10 μL of RNA, add 1 μL 10 mM dNTPs, 10 μL RT/REV primer (10 μM), and 1 μL DEPC- H_2O . Heat the mixture at 65°C for 5 min; transfer the tube to ice.

2. To the sample, add 4 μL 5 $\times$ RT Buffer, 1 μL 0.1 M DTT, 1 μL RNase Inhibitor, and 1 μL Reverse Transcriptase. Incubate the reaction at 50°C for 1 h; This will denature the partially RNA:cDNA duplex that forms at the 3' end of the cDNA (RT leaves ~20 bp of RNA:cDNA duplex), and make the cDNA susceptible to ExoSAP-IT in the next step. Do not heat inactivate!
3. Add 8 μL ExoSAP-IT to the sample and incubate the reaction at 37°C for 30 min.
4. Transfer the reaction to a new 1.5-mL tube. Clean up the reaction by adding 25 μL phenol:chloroform:IAA (25:24:1) to the sample. Vortex the sample for 30 s and spin the tube for 3 min at $16,000 \times g$ at room temperature.
5. Remove the aqueous layer (20 μL) and ethanol precipitate the cDNA by adding 2 μL 3 M NaOAc (pH 5.2), 8 μL glycogen, and 90 μL ice-cold ethanol. Place the tube into well-crushed dry ice for ~30 min and then spin down the sample for 30 min, at $16,000 \times g$, at 4°C. Decant the liquid from the tube and air-dry the sample for 10 min before resuspending the pellet in 11 μL DEPC-H₂O.

3.7. Add on 3' Linker 2

1. The addition of the second linker proceeds just as with the addition of the first linker. Prepare a 10% TBE-Urea minigel.
2. Using the cDNA from Subheading 3.6, set up three ligation reactions, one of which serves as a negative control.
 - (a) Set up two of the same reactions: to 5 μL of cDNA, add 1 μL Linker 2, 2 μL 5 $\times$ Ligase Buffer, 1 μL DEPC-H₂O, and 1 μL T4 RNA Ligase (1 U/ μL) (see Note 9).
 - (b) For the negative control, to 1 μL cDNA, add 2 μL 5 $\times$ Ligase Buffer and 7 μL DEPC-H₂O.
 - (c) Let reactions proceed for 2 h at room temperature.
3. Add 20 μL Loading Buffer II to the +Ligase reactions; add 10 μL Loading Buffer II to the -Ligase reaction. Heat the sample and the markers at 65°C for 5 min. Load the +Ligase reactions across the four rightmost lanes of the prerun 10% TBE-Urea gel. Load the markers and the -Ligase sample in lanes on the left. Run the samples at 200 V for 50 min.
4. Stain the gel with SYBR Gold, visualize the linkered-cDNA, and cut out the cDNA between 116 and 236 nt (Fig. 2d) (see Note 12).
5. Elute the cDNA overnight and precipitate the cDNA with glycogen and ethanol. Resuspend the cDNA with DEPC-H₂O, combining all tubes, to a final volume of 10 μL .

3.8. Prepare the Samples for Sequencing

1. Prepare a 12% TBE gel.
2. For the first PCR (PCR-I), mix 3 μL of the linkered-cDNA with 38.5 μL dH₂O, 5 μL 10 $\times$ Standard Taq Buffer, 1 μL 10 mM dNTPs, 1 μL 10 μM SIII forward primer (10 μM), 1 μL SIII reverse primer (10 μM), and 0.5 μL Taq. Incubate the reaction at 95°C for 10 min, followed by 25 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s. After a final extension at 72°C for 5 min, add 30 μL 6 $\times$ DNA Loading Dye and run the sample across four lanes in a prerun 12% TBE gel at 200 V for 47 min. Use a 100-bp ladder (5 μL) for a marker.

3. Stain the DNA with SYBR Gold, visualize the DNA, and excise the region between 154 and 274 bp (see Note 13).
4. Elute the DNA overnight and precipitate the DNA with glycogen and ethanol. Resuspend the pellet in 30 μ L IDTE pH 7.5.
5. For the second PCR (PCR-II), set up 8 $\times$ 50 μ L reactions. For each reaction, mix 1 μ L of the PCR-I product with 40.5 μ L dH₂O, 5 μ L 10 $\times$ Standard Taq Buffer, 1 μ L 10 mM dNTPs, 1 μ L 10 μ M SIII forward primer (10 μ M), 1 μ L SIII reverse primer (10 μ M), and 0.5 μ L Taq. Incubate the reaction at 95°C for 10 min, followed by 15 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s (see Note 14). After a final extension at 72°C for 5 min, combine all eight samples and concentrate the sample with *n*-butanol, followed by buffer exchange (see Note 15); the final volume should be 80–100 μ L. Add an equal volume of 6 $\times$ DNA Loading Dye and run the sample across six lanes in a prerun 12% TBE gel at 200 V for 47 min. Use a 100-bp ladder (5 μ L) for a marker.
6. Stain the DNA with SYBR Gold, visualize the DNA, and excise the region between 154 and 274 bp (Fig. 2e) (see Note 13).
7. Elute the DNA and precipitate the DNA with glycogen and ethanol. Resuspend the pellet in 20 μ L IDTE pH 7.5 (see Note 16).
8. The sample is now ready to be quantified and then sequenced.
 - (a) If available, run some of the sample (1 μ L) on a bioanalyzer (see Note 17).
 - (b) TOPO clone 2 μ L of the sample and sequence 48–96 clones to check that transcripts are of appropriate size, and tRNA/rRNA depletion is adequate (see Note 18).
 - (c) If depletion of tRNA/rRNA is adequate, sequence sample by 454 with Primer B (see example of reads in Fig. 3).

4. Notes

1. To ensure complete polymerization of the gels, pour the gels the day before and after 30 min of polymerization, wrap the gel (with its comb intact) in a paper towel (wet with 1 $\times$ TBE) and plastic wrap. Store the gel at 4°C overnight.
2. The Decade Marker can be used to set the lower boundary for RNA sizing, while the Century Marker can be used to set the upper boundary. We also found it useful to add 1 pmol of an oligonucleotide of known size (we used a 37-mer) to the Century Marker to help calibrate the sizing of the Decade Marker bands on the gel.
3. When separating the aqueous layer (with the RNA) from the organic (phenol:chloroform) layer, you do not need to remove the entire aqueous layer. Leaving behind some of the aqueous layer will ensure that you do not accidentally carry over the white precipitate into the Phase Lock Gel tube.
4. To resuspend the pellet of RNA, begin by adding approximately 500 μ L and gently pipetting up and down. You may need to heat the pellet at 65°C, briefly, to help dissolve the pellet. The RNA may be very viscous, which will make quantification difficult. Add more DEPC-H₂O, if necessary, to reduce viscosity and to dissolve the pellet completely. At this point, keeping a high concentration of RNA will reduce the volume needed to load onto the gel for sRNA enrichment. However, if the RNA is too viscous, then it is difficult to accurately quantitate the sample.

5. For the RNA, an OD₂₆₀/OD₂₈₀ ratio between 1.8 and 2.0 is fine. Using this protocol, we generally have RNA preps that are between 5 and 10 µg/mL; you will likely need to make a 1:10 dilution to get an accurate OD₂₆₀ reading.
6. We have successfully used this total RNA preparation protocol to isolate RNA (including sRNAs) from *E. coli*, *V. cholerae*, and *Streptococcus pneumoniae*. This protocol was originally designed for use with *Saccharomyces cerevisiae* (<http://derisilab.ucsf.edu>).
7. The exact timing of how long to run a gel will need to be determined empirically. When samples are run on a gel for longer periods of time, a higher degree of resolution is achieved. However, this increases the size of the gel that must be cut out, which makes the downstream gel elution and RNA precipitation steps more laborious. Also, when we have set out to clone sRNAs 20–200 nt long, we split the samples into two sets of gels. We used a 10% gel to clone 80–200 nt sRNAs and a 15% gel to clone 20–80 nt sRNAs.
8. We visualize the gel with a fluorescent ruler and then, at the bench, we use the ruler to guide our cutting of the gel pieces. To account for both minor errors in how the samples may run and experimental error during cutting, we tend to cut a slightly broader range (50–250 nt) during the sRNA enrichment step to ensure that the desired window is obtained.
9. T4 RNA Ligase generally has some trace ATP contamination that is carried over during purification of the protein. This ATP can charge the 5' end of phosphorylated RNA/DNA, which may result in undesired RNA-RNA ligations or circularization of RNA. To avoid these contaminating products, a 1:10 dilution of the T4 RNA Ligase (in 1× Ligase Buffer) is used to dilute out the ATP. Linker 1 and 2 are both 5'-preadenylated and 3'-modified such that the only ligations that should occur within the +Ligase reactions should be between the 5' end of the linker and a 3'-OH. Any sRNAs that have a 3'-modification will, therefore, not be cloned.
10. We find that it is important that the Oligo Mix is added to the linkered-RNA prior to ethanol precipitation. The amount of Oligo Mix to use is something that should be tested empirically by the researcher; we have had success cloning *V. cholerae* sRNAs using 1.5 nmol of a mixture containing 29 different oligonucleotides. Most of the oligonucleotides are mixed in a 1:1 ratio. With *V. cholerae*, we observed through pilot sequencing (see Note 18) that several tRNAs were overrepresented in the final cDNA library and that tRNA Ser(GCT) was highly abundant. Oligonucleotides complementary to the overrepresented tRNAs were twice as abundant in the final Oligo Mix used (2:1 ratio). For the highly abundant Ser(GCT), we added 0.5 nmol of the anti-Ser(GCT) oligonucleotide directly to the linkered-RNA, prior to ethanol precipitation, in addition to the 1.5 nmol Oligo Mix, and successfully depleted the Ser(GCT) and all other tRNAs to ~25% of the final pool of cDNAs.
11. After the first ligation reaction, when running the linkered-RNA on a polyacrylamide gel, several sharp bands should be visible after staining (Fig. 2b): bands representing the 5S rRNA and linkered-5S rRNA, as well as bands for the tRNAs and linkered-tRNA. After depletion, most of these sharp bands should be replaced instead by diffuse smears (Fig. 2c). The presence of sharp bands may indicate that the depletion step did not work completely, although this should be confirmed with pilot sequencing (see Note 18).

12. After the second 3' linker ligation reaction, the sample will appear very faint when run on a gel (Fig. 2d). You may see hardly any cDNA on the gel after staining. This makes the use of markers particularly important as they will guide you to cut out the correct region of the gel.
13. The PCR products, when run out on a gel, may contain some sharp bands (Fig. 2e). These bands should be avoided whenever possible when cutting the gel to recover DNA. They represent primer-dimer contaminants, linkers with only 1–2 bases in between them, or may indicate that the depletion step did not work well.
14. Two rounds of PCR are used to prevent any one (or set of) sequence to take over the entire reaction. The number of cycles for each round of PCR may need to be determined empirically; in our sequencing reactions with *V. cholerae*, we had success with 25 cycles followed by a 1/30 dilution of the DNA and another 15 cycles.
15. To concentrate a sample using *n*-butanol, add 1–3 volumes of *n*-butanol to an aqueous sample. Vortex the sample and then spin it at $16,000 \times g$ for 3–5 min. Remove and discard the top organic layer. Add another 1–3 volumes of *n*-butanol and repeat the spin. Through repeated addition and removal of *n*-butanol, the aqueous layer, with the cDNA, should decrease in volume. After concentration, the aqueous layer should be passed through a buffer-exchange column (e.g., Micro Bio-Spin 30) to remove salts and any residual *n*-butanol.
16. IDTE buffer is used when submitting samples for 454 sequencing.
17. Running the samples out on a bioanalyzer will provide more accurate information about the concentration of your sample than using either a Nanodrop or other UV spectrophotometer. You will expect to see a broad curve over the range of desired transcript size and not sharp peaks.
18. The best way to optimize your Oligo Mix (see Note 10) is to TOPO-clone the final cDNA library and sequence at least 48 (96, if possible) clones and confirm that tRNAs and rRNA are being depleted. A QIAprep 96 Turbo Miniprep Kit is very useful for preparing the clones for sequencing. If depletion is not adequate, then the oligonucleotide ratios can be altered or additional depletion oligonucleotides can be added, and the process repeated iteratively. The pilot sequencing experiments may even point out abundant sRNAs, providing some experimental data prior to the large-scale sequencing.

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Fig. 1.

Flowchart depicting the sRNA-Seq method. First-time users are strongly encouraged to run through the procedure several times to become familiar with the methodology. PCR products from these pilot experiments should be TOPO-cloned, and clones may be sequenced in 96-well format, providing important information that can be used to optimize the procedure, particularly the depletion step.

Fig. 2.

Examples of gels run out for direct cloning of sRNAs in *V. cholerae*. All gels were stained with SYBR Gold for 30 min. Regions in *dotted boxes* were gel-purified. In gels (a–d), Decade Marker is loaded in the first lane on the *left* and Century Marker, plus 1 pmol of a 37-mer, is loaded in the second lane. In gel (e), 100 bp DNA ladder is loaded; a control (Ø) consisting of PCR-amplified (Linker 2–TA–Linker 1) is loaded next to the ladder.

GTCCTTGGTGCCCGAGTGGGGGGCCCTGGTCCTCCCGCAACACTAGTTCGTGAACCTGGTC
 AGATCCGGAAGGAAGCAGCCACAGCGGATGATGTGTGTGCCGGGATGTGGCTGGGGTCTCC
 GCAAAACTGTAGGCACCATCAATCCTGATGGCGCGAGGGAGGC
 110 nt; BLASTN: IGR 1067/8, 4.5S RNA

GTCCTTGGTGCCCGAGTGTAAAGATGTTCTATCTTTCAGACCTTTTGTTCACGTTATTGG
 ATTAGGCTGATTAGCGCCCGCCAGTCACCATTTGACTGGGGCGTTTTTAACTGTAGGCA
CCATCAATCCTGATGGCGCGAGGGAGGC
 93 nt; BLASTN: ORF VC0110

GTCCTTGGTGCCCGAGTGGCAGATGTTTTGTGGAGCCTCAACTCCAATACAGAACATTCA
 GGGGGAGTAGTGCCGAGGTGAATCAAAGTTGTGGCTTTGGTTTATCGGTTGAACGGGCTGAA
 TCCCTTCAACTGTCATCAGCTCGAATCTGATGAAGAGCTTCTGAGGGAATCTTTCAC
ACTGTAGGCACCATCAATCCTGATGGCGCGAGGGAGGC
 163 nt; BLASTN: IGR VC0391/2

Fig. 3.

Example sequences (read 5' to 3') from sRNA-Seq, sequenced by 454 using Primer B. *Underlined sequences* correspond to Linkers 2 and 1. Sequence in *italics* is part of SIII forward primer and allows for 454 sequencing using Primer A. Sequences between *underlined* linkers were used for BLASTN analysis, the results of which are listed below each sequence.