Journal of Visualized Experiments

# Dual DNA rulers to study the mechanism of ribosome translocation with single-nucleotide resolution

--Manuscript Draft--

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|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE59918R2 |
| **Full Title:** | Dual DNA rulers to study the mechanism of ribosome translocation with single-nucleotide resolution |
| **Keywords:** | DNA rulers; ribosome translocation; frameshifting; force spectroscopy; magnetic labeling; force-induced remnant magnetization spectroscopy; mRNA looping; Antibiotics; atomic magnetometry |
| **Corresponding Author:** | Shoujun Xu<br>University of Houston<br>Houston, TX UNITED STATES |
| **Corresponding Author's Institution:** | University of Houston |
| **Corresponding Author E-Mail:** | sxu7@central.uh.edu |
| **Order of Authors:** | Shoujun Xu |
| | Heng Yin |
| | Yuhong Wang |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
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**TITLE:**

# Dual DNA Rulers to Study the Mechanism of Ribosome Translocation with Single-Nucleotide Resolution

**AUTHORS:**

Heng Yin[1], Shoujun Xu[1], Yuhong Wang[2]

[1]Department of Chemistry, University of Houston, Houston, TX, USA
[2]Department of Biology and Biochemistry, University of Houston, Houston, TX, USA

**Corresponding author:**

Shoujun Xu (sxu7@uh.edu)
Yuhong Wang (ywang60@uh.edu)

**Email Addresses of Co-authors:**

Heng Yin (hyin3@uh.edu)

**KEYWORDS:**

DNA rulers, ribosome translocation, frameshifting, force spectroscopy, magnetic labeling, force-induced remnant magnetization spectroscopy, mRNA looping, antibiotics, atomic magnetometry

**SUMMARY:**

Dual DNA ruler assay is developed to determine the mRNA position during ribosome translocation, which relies on the dissociation forces of the formed DNA-mRNA duplexes. With single-nucleotide resolution and capability of reaching both ends of mRNA, it can provide mechanistic insights for ribosome translocation and probe other nucleic acid displacements.

**ABSTRACT:**

The ribosome translocation refers to the ribosomal movement on the mRNA by exactly three nucleotides (nt), which is the central step in protein synthesis. To investigate its mechanism, there are two essential technical requirements. First is single-nt resolution that can resolve normal translocation from frameshifting, during which the ribosome moves by other than 3 nt. The second is the capability to probe both the entrance and exit sides of mRNA in order to elucidate the whole picture of translocation. We report the dual DNA ruler assay that is based on the critical dissociation forces of DNA-mRNA duplexes, obtained by force-induced remnant magnetization spectroscopy (FIRMS). With 2–4 pN force resolution, the dual ruler assay is sufficient to distinguish different translocation steps. By implementing a long linker on the probing DNAs, they can reach the mRNA on the opposite side of the ribosome, so that the mRNA position can be determined for both sides. Therefore, the dual ruler assay is uniquely suited to investigate the ribosome translocation, and nucleic acid motion in general. We show representative results which indicated a looped mRNA conformation and resolved normal translocation from frameshifting.

**INTRODUCTION:**

Biomolecular displacement is a fundamental parameter in studying the mechanism of the related biological functions. One particular example is the ribosome translocation[1,2], during which the ribosome moves by exactly three nucleotides (nt) on the messenger RNA (mRNA) normally, and by one, two, or other numbers of nt except three in the case of frameshifting. Therefore, a molecular ruler system single-nt resolution is required to distinguish the different step sizes. A greater challenge is to probe the ribosome movement on both the entrance and exit sides. In other words, only with a dual ruler system will we be able to reveal whether the mRNA is smoothly threaded through the ribosome, or there are intermediate steps in which the two sides have different step sizes leading to a kinked or looped mRNA conformation inside the ribosome.

Several methods have been developed to address the first challenge of resolving different steps on the exit side of the ribosome (the 3' end of the mRNA). The dual luciferase assay resolves the different reading frames by measuring the ratios of the resulting different proteins[3,4]. It is only applicable for the 3' end of the mRNA and thus insufficient to provide a complete picture of translocation. Mass spectrometry can analyze the different peptide fragments as the consequence of the corresponding code rearrangements[5]. But it cannot pinpoint to how many nt the ribosome moves on the mRNA. The toe-printing assay is another common method that uses a reverse transcriptase primed at the 3'-distal end to transcribe the mRNA toward the ribosome[6]. However, it is not applicable for the 5' end of mRNA that is entering the ribosome. Other techniques, including single molecule approaches and fluorescence methods[7], are difficult to achieve single-nt resolution.

We have developed the dual DNA ruler assay that can uniquely determine both the entrance and exit positions of the uncovered mRNA in ribosome-mRNA complexes. The ruler DNAs are DNA oligomers that form duplexes of certain numbers of basepairs (bp) with the mRNA uncovered by the ribosome, regardless of which end of the mRNA. The bp numbers then precisely reveal the ribosome position on the mRNA during translocation. The bp numbers of the duplexes are determined by their critical dissociation forces obtained from force-induced remnant magnetization spectroscopy (FIRMS)[8]. With 2–3 pN force uncertainty, the critical forces are sufficient to offer single-nt resolution. By implementing a linker molecule on the DNA rulers, the sterically hindered side of the mRNA by the ribosome can be probed. Different ribosomal displacements can thus be accurately resolved. We have successfully revealed a unique looped conformation of mRNA trapped by antibiotics during translocation[9], and resolved different reading frames that coexisted on a slippery mRNA sequence[10]. This article describes the details of the dual ruler assay, which include preparation of the ribosome complexes, surface functionalization of the glass slides, immobilization of the ribosome complexes and their hybridization with magnetically labeled DNA ruler molecules, magnetic detection, and force spectrum analysis by FIRMS.

**PROTOCOL:**

**1. Preparation of the ribosome complexes**

1.1. Make 1000 mL of TAM10 buffer, which consists of 20 mM tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 30 mM $NH_4Cl$, 70 mM KCl, 5 mM EDTA, 7 mM BME (2-mercaptoethanol), and 0.05% Tween20.

1.2. Prepare the five mixtures listed in **Table 1**.

NOTE: The ribosome was from the MRE600 strain[11]. EF-Tu: elongation factor thermo unstable. EF-Ts: elongation factor thermo stable. GTP: guanosine triphosphate. PEP: phospho(enol)pyruvate.

1.3. Incubate the five mixes separately at 37 °C for 25 min before making the ribosome complexes. Prepare the five ribosome complexes as per **Table 2**.

NOTE: Post: post-translocation; Pre: pre-translocation.

1.4. Add each of the five ribosome complexes onto a 1.1 M sucrose cushion separately, with volume ratio 1:1. Purify each with 450,000 × *g* for 2 h in an ultra-centrifuge. Use a pipet to remove the supernatant and restore the ribosome complexes at -80 °C after resuspension of the pellet with TAM10 buffer.

**2. Preparation of biotin-coated glass slides**

2.1. Preliminary cleaning of the glass slides

2.1.1. Place 12 glass slides with dimensions of 60.0 × 4.0 × 0.3 $mm^3$ (L × W × T) in a short and wide glass dish.

2.1.2. Fill the glass dish with acetone and sonicate for 5 min. Then wash the slides with ultrapure water 5 times and fill ¾ of the dish with water.

2.1.3. Add 10 M KOH to fill the dish and sonicate the glass slides for 20 min. Wash the slides with water 5 times.

2.1.4. Add ethanol and sonicate for 5 min, pour out the ethanol and then dry them separately at 300 °C for 3 h.

2.2. Aminosilane coating

2.2.1. Place the 12 cleaned slides back into the glass dish containing methanol. Clean a PEGylation flask with methanol by sonicating for 5 min, then fill it with 25 mL methanol, 1.25 mL water, 0.125 mL HAc, 0.25 mL 3-aminopropyltriethoxysilane (AMEO).

2.2.2. Immediately replace the methanol in the glass dish with the prepared AMEO solution. Incubate at room temperature for 30 min.

2.2.3. Rinse the slides with water several times, then dry them by nitrogen purge. Place the dried slides in clean glass dishes.

2.3. PEGlation

2.3.1. Prepare $NaHCO_3$ solution (8.4 mg/mL) and PEGylation buffer (37.5 mg PEG, 6 mg biotinylated PEG, 150 µL $NaHCO_3$ solution). Mix them well by spinning at 6000 rpm for 1 min.

2.3.2. Place 25 µL of PEG solution onto each slide. Cover it with the other slide on top. Make sure that there are no bubbles in between the two slides. Place the slides in an empty pipet tip box. Be sure that the box is leveled and place it in a dark drawer for about 3 h.

2.3.3. Rinse the slides with water and dry again. Store the dried slides at room temperature under vacuum for up to 2 weeks.

**3. Sample preparation prior to magnetic and force measurements**

3.1. Machine a plastic sample well with dimensions 4 × 3 × 2 $mm^3$ (L × W × D). Glue a piece of biotin-coated glass (approximately 5 mm long, cut from the 60 mm long slides prepared in section 2) on the bottom surface using epoxy.

3.2. Add 20 µL of 0.25 mg/mL streptavidin aqueous solution into the sample well and incubate at room temperature for 40 min. Then rinse the sample well twice with $TAM_{10}$ buffer.

3.3. Immobilize the ribosome complexes.

3.3.1. Without antibiotics: Use a pipette to remove buffer from the sample well, then add 20 µL of 0.1 µM ribosome complex (MF-Pre or MF-Post) into the sample well. The ribosome complex will bind with the streptavidin on the surface via the 5'-end biotin on the mRNA. Incubate at 37 °C for 1 h and then rinse once with $TAM_{10}$ buffer.

3.3.2. For the experiment using both neomycin and fusidic acid: incubate the MF-Pre complex with neomycin at 37 °C for 10 min; incubate EF-G with fusidic acid at 37 °C for 20 min. The concentrations are as follows: 0.1 µM ribosome complex, 2 µM EF-G, 4 mM GTP, 4 mM PEP, 0.02 mg/mL pyruvate kinase, 0.2 mM neomycin, and 0.25 mM fusidic acid.

3.3.3. Carry out the other antibiotics experiments similarly. The concentrations are as follows: 0.2 mM viomycin, 0.4 mM hygromycin B, and 0.25 mM fusidic acid.

3.3.4. For frameshifting study, repeat the above steps for the MFNF-Pre and MFNF-Post complexes involving the slippery motif $U_6A$. Use antibiotics fusidic acid plus neomycin, and fusidic acid alone, respectively.

3.4. Remove buffer from the sample well, then add 20 µL of 1 µM biotinylated probing DNA strand and incubate at room temperature overnight. Rinse the formed DNA-mRNA duplex once with $TAM_{10}$ buffer.

3.5. Remove the buffer from the sample well. Then add 20 µL of 0.5 mg/mL streptavidin-coated magnetic beads into the sample well and incubate at room temperature for 2 h.

3.6. Carefully insert the sample well into a holder and place it in a centrifuge. Remove the free magnetic particles from the surface by centrifuging at 84 x *g* for 5 min.

**4. Magnetic and force measurements**

4.1. Turning on the laser

4.1.1. Turn on the laser using the key. Then press the power button.

4.1.2. Adjust the sensitivity of lock-in amplifier 1 (LIA1) to 500 mV and wait for about 2 h to warm up and stabilize the atomic magnetometer.

4.2. Setting up the atomic magnetometer

4.2.1. Run the instrument control software and set up the measurement parameters. Some parameters may slightly vary in each measurement.

NOTE: The atomic magnetometer comprises a laser (described above), an SR830 lock-in amplifier (referred to as LIA1), an SR530 lock-in amplifier (referred to as LIA2), DS345 (referred to as FG1) and ATF20B (referred to as FG2) function generators, a high-resolution motor and a computer. All of these should be turned on at this step of the protocol.

4.2.2. Set up motor moving mode to **Noise** and default position to **0**. Press **Lock** on front panel, adjust the sensitivity of LIA1 back to 200 mV.

4.2.3. Adjust the current and voltage of the laser and find the proper resonance peak and signal-to-noise level. Press **Sweep** on the front panel. Note the amplitude/width ratio should be above 0.5 and phase value should be less than 5 degree. If not, re-do the sweep step.

4.2.4. Plug-in the output of LIA2 to the feedback of the laser to lock its frequency. This amplifier measures the optical rotation of an auxiliary cesium cell to maintain the laser frequency on resonance[12]. The state remains until the end of the measurement.

4.2.5. Plug-in function generator FG2 to input a square wave (500 mVpp, 100 mHz) as the reference signal. The square wave corresponds to 100 pT and is used to convert the current output of the amplifier to magnetic signal. Unplug the function generator.

4.2.6. Set up motor moving mode to **Two-way** and default position to 260 mm. Check the status of the temperature controller to ensure the proper temperature (~37 °C) of the atomic sensor.

4.2.7. Check the stability of the whole system by measuring the signals of the empty sample holder twice. Evaluate stability and noise level after subtracting the two traces. Typical noise level and fluctuation should be ±2 pT at 30 ms integration time.

4.3. Magnetizing the sample

4.3.1. Gently place the sample on the magnetization station and let it stay for 2 min.

NOTE: The magnetization station consists of a permanent magnet (~0.5 T) and a plastic spacer.

4.3.2. Put the sample back into the sample holder. Use 335.4 × *g* centrifugal force to remove the nonspecifically bound magnetic particles.

4.4. Magnetic measurements after applying forces

4.4.1. Use tweezers to load the sample onto the motor. Click **Lock** on the front panel to run the program. Meanwhile, use tweezers to load the other sample in the holder and place it in the centrifuge. Note that the coated glass side should face the center of the centrifuge.

NOTE: The technique of force-induced remnant magnetization spectroscopy (FIRMS) is used, which uses an atomic magnetometer to measure the magnetic signal of the sample after applying mechanical force on the molecular interactions in the sample. Here, the molecular interactions are between the DNA ruler molecules and the mRNA in the ribosome complex. The force is increased stepwise by increasing the centrifugal speed. After applying each force, the motor translates the sample to the atomic sensor and then moves back. Hence, two magnetic field profiles are obtained, one during the forward scan and the other during backward scan[13]. We only use the latter to extract the peak height due to its better signal-to-noise ratio. The peak height in current (nA) is converted to magnetic signal amplitude (pT) based on the calibration square wave.

4.4.2. Every measurement lasts approximately 5 min. When motor comes back to 0, click **Save** on the front panel. Carefully use tweezers to take samples from motor and centrifuge. Use initial speed corresponding to 335.4 × *g* (2000 rpm, revolution per minute for the centrifuge listed in the **Table of Materials**).

4.4.3. Exchange the two samples and apply a stronger force by increasing the centrifugal speed by 100 rpm or similar step size. Process alternately to gradually increase the force; a complete force spectrum is obtained after 10–12 data points.

4.5. Finishing the experiment

4.5.1. When all planned experiments are finished, turn off the equipment, proceeding in the opposite order as it was turned on.

4.5.2. Remove the samples from the holder and immerse them in ethanol for cleaning and future use. Clean up the sample holder with acetone in case of magnetic beads contamination.

4.6. Data analysis

4.6.1. Open the Python analysis script and input all the experimental data. Click **Load square** to input the square wave as the reference. Then click **Load baseline** to input residual magnetic signal as background.

4.6.2. Define the overall magnetic signal decrease as $B_0$. Normalize each magnetic signal decrease $B$ to $B_0$ and express it as a percentage. Plot the percentage ($B/B_0$) versus centrifugal force to obtain the FIRMS spectrum.

NOTE: The centrifugal force $F$ is calculated from the buoyant mass of the magnetic beads $m$ ($4.6 \times 10^{-15}$ kg), centrifugal speed $\omega$, and radius of the centrifuge $r$ (7.5 cm here) via equation $F = m\omega^2 r$. The typical force resolution is 2–4 pN and force range is 15–95 pN in this work.

**REPRESENTATIVE RESULTS:**

**Figure 1** shows the detection scheme and photographs of the major components. Magnetic detection is achieved by an atomic magnetometer using the scanning scheme (**Figure 1A**)[13]. The sample is placed on a rod mounted on a linear motor. The motor transports the sample to the atomic sensor inside a magnetic shield, then back to the original site for unloading. The atomic magnetometer detects the magnetic signal during the sample scan and produced a signal trace, with the maximum signal when the sample is the closest to the sensor. **Figure 1B** shows the photo of the overall instrument. **Figures 1C,D** show the photos of the magnetization station and the centrifuge used for force application, respectively.

Principle of the dual DNA ruler assay is shown in **Figure 2**. The ribosome complex is immobilized on the surface via the 5' end of the mRNA. Two DNA rulers are designed to probe the exact position of the ribosome, one for each side of the mRNA. The current immobilization scheme makes the 3' side easily accessible for the probing DNA rulers. Therefore, DNA oligomers conjugated with magnetic beads can form duplexes with the uncovered mRNA. The 5' side, however, is sterically hindered by the ribosome and the surface. A linker molecule is thus needed for the DNA to reach the uncovered mRNA on this side. By varying the linker length, we have determined that when the linker is longer than 50 T, we can detect a strong magnetic signal which indicates successful formation of DNA-mRNA duplexes (**Figure 2B**). The correlation of dissociation force to duplex length is achieved by varying the number of nt on the DNA that are complementary to the mRNA. **Figures 2C**,**D** show the correlation for the 3'- and 5'- sides, respectively. Because the force difference between duplexes of consecutive lengths is typically 12–20 pN and the force resolution is typically 2–4 pN, we routinely achieve single-nt resolution for the length of DNA-mRNA duplexes based upon their dissociation forces.

**Figure 3** presents the results of normal translocation in the absence and presence of various antibiotics. The translocation is from MF-Pre to MF-Post (**Figure 3A**). The inset indicates that MF-Pre carries vacant $tRNA^{fMet}$ and MF-$tRNA^{Phe}$ at the P- and A-sites, respectively. MF-Post carries $tRNA^{fMet}$ and MF-$tRNA^{Phe}$ at the E- and P-sites, respectively, with a vacant A-site. **Figure 3B** shows that without antibiotics, the ribosome moves by 3 nt on both sides (moving toward the 3'-end). This is because of the following reasons: (i) The DNA-mRNA duplexes at the 5' side exhibit 12 bp and 15 bp binding forces in MF-Pre and MF-Post, respectively. (ii) Duplexes at the 3' end exhibit a reversed change from 15 to 12 bp (**Figure 3C**). However, when both fusidic acid and neomycin are present, the force spectra show that the ribosome moves only by 1 nt at the 5' side but 2 nt at the 3' side (**Figure 3D,E**). This result indicates that the ribosome translocated via a stepwise mechanism, resulting a looped mRNA conformation that has an extra nt inside the ribosome, which has not been experimentally revealed before. This is consistent with reported theoretical simulation[14]. Alternatively, the ribosome may be stretched to cover 28 nt of mRNA, instead of the usual 27 nt[15]. After washing away the antibiotics, normal translocation occurs, as evidenced by 3 nt movements from both the 5' and 3' sides (purple trace in **Figure 3D, E**). The looped conformation does not form when only fusidic acid is used. The force spectra are consistent with the ribosome movement of 3 nt at both sides, similar to the situation for normal translocation (**Figure 3F,G**). The dual ruler assay also reveals that viomycin completely inhibited translocation, as shown by 0 nt movement on both sides (**Figure 3H,I**).

We have also investigated whether this looped conformation can form on "-1" frameshifting motifs. Here, the translocation from MFNF-Pre and MFNF-Post complexes takes place on the '$U_6$A' motif, which has been found to be part of the '-1' frameshifting motif in HIV[16]. **Figures 4A,B** show that in the MFNF-Post, the DNA-mRNA duplex is shortened by either 2 or 3 nt at the 3'-end; the duplex length increases by either 2 or 3 nt at the 5'-ends. The results suggest the coexistence of both normal translocation and "-1" frameshifting, with the former correlating with the 3-nt movement and the latter correlating with the 2-nt movement. The dual ruler assay is able to clearly resolve the two populations. With both neomycin and fusidic acid, we observe that the ribosome moves by 2 nt at the 3'-end but only 1 nt at the 5'-end, respectively (**Figure 4C,D**). Therefore, the looped mRNA conformation also takes place on the slippery sequence. When only fusidic acid is present for the MFNF-Pre (**Figure 4E,F**), the result is the same as that for MFNF-Post in panels A and B (blue traces), indicating that fusidic acid alone is not sufficient to pause translocation or frameshift. Therefore, it confirms that both fusidic acid and neomycin are required for the unequal displacements for the two sides of mRNA.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Instruments for the FIRMS-based dual DNA ruler assay.** (**A**) Schematic of the magnetic detection. 1: sample and its mount; 2: motor; 3: atomic sensor and magnetic shield. (**B**) Photo of the atomic magnetometer and sample handling system. The numbers indicate the actual corresponding components shown in the schematic. Note the magnetic shield is inside the cardboard box for improved thermal stability. (**C**) Magnetization station. (**D**) Centrifuge used for force application.

**Figure 2: Dual ruler assay with single-nt resolution for studying ribosome translocation.** (**A**) Schematic of the dual ruler assay, in which two DNA rulers are designed to respectively probe the 5'- and 3'- ends. The red line indicates the polyT linker. (**B**) Optimization of the linker length for Ruler-In. (**C**) FIRMS results to determine the dissociation forces of the duplexes between Ruler-Ins and mRNA. (**D**) Dissociation forces of the duplexes between Ruler-Outs and mRNA. The error bar is defined as the ratio between the instrument noise and the overall magnetic signal decrease ($B_0$), with typical value of ±3–5%. Figure has been reproduced with permission[9].

**Figure 3: Probing translocation steps under the influence of various antibiotics.** (**A**) Probing scheme for the MF-Pre and MF-Post complexes. Inset indicates the schematic ribosomes in Pre and Post, which correspond to the solid and dash-lined ovals, respectively. (**B, C**) FIRMS results with no antibiotics. (**D, E**) Results with both fusidic acid and neomycin. (**F, G**) Results with fusidic acid only. (**H, I**) Results with viomycin only. Left panels: using Ruler-In to probe the 5' side; right panels: using Ruler-Out to probe the 3' side. The error bars are calculated the same way as those in the previous figure. Figure has been reproduced with permission[9].

**Figure 4: FIRMS results of using dual rulers to probe frameshifting.** (**A, B**) MFNF-Pre and MFNF-Post without antibiotics. (**C, D**) Results with both fusidic acid and neomycin. (**E, F**) Results with only fusidic acid. Left panels: using Ruler-In to probe the 5' side; right panels: using Ruler-Out to probe the 3' side. The error bars are calculated the same way as those in the previous figure. Figure has been reproduced with permission[9].

**DISCUSSION:**

In our dual ruler assay, the magnetic beads play two essential roles. First, they serve as the force transducers because the centrifugal force is proportional to their buoyant mass. Second, the beads are signal carriers detected by an atomic magnetometer, which is currently the most accurate magnetic sensor. Combining mechanical manipulation and magnetic detection, the FIRMS technique is able to resolve a large number of molecular interactions based on their critical dissociation forces, which is the basis of the DNA rulers. The dual ruler assay is uniquely suited to probe both sides of mRNA during translocation. Because it is a physical approach that only relies on the formation of the duplexes, it is not limited by the mRNA sides or other biological constrains. This is an advantage compared to other techniques that are based on biochemical reactions. We have shown that by using a long linker molecule, the rulers can reach the targeted binding site to determine the mRNA position. Specifically, for the ribosome, the linker length is 50 T, which corresponds to 17 nm in length. This length is very close to the size of the ribosome[17]. The concentrations of the antibiotics are typical values from the literature. It is also possible to use our method to reveal the onset values for their functions.

There are two critical aspects for the dual ruler assay. One is the delicate functionalized surface for molecular immobilization and subsequent biological reactions. Every loading and washing step should be performed with minimum disturbance to the surface, so that the molecules immobilized on the surface will remain intact. For the DNA-mRNA hybridization process, sufficient time is necessary to ensure the completion of the process. It is also advised to use

$TAM_{10}$ buffer with extra 1 M NaCl to maintain a homeostatic system. The other critical aspect is the magnetization of the beads. Since excessive beads are used, there are plenty of free beads not immobilized on the surface. Therefore, when magnetizing the sample, the coated surface should approach and leave the magnet vertically. Any minor swing of the sample can possibly cause scratching damage on the surface.

Our dual DNA ruler assay is currently the only method that can objectively probe both the entrance and exit sides of mRNA in the ribosome complexes with single-nt resolution. Novel mechanistic information regarding ribosome translocation has been obtained. The method is generally applicable for molecular displacement of nucleic acids, which is widely encountered in molecular biology.

For future development and applications, we have shown that using acoustic radiation force to replace the centrifugal force will further improve the force resolution, reaching sub-nt regime[18]. We are also investigating multiplexed detection using atomic magnetometers to improve the detection efficiency. With these improvements, we expect our method detailed in this work will find broad applications in biological research.

**ACKNOWLEDGMENTS:**

This work is supported by the US National Institutes of Health (R01GM111452, Y.W., S.X.). Y. W. acknowledges support from the Welch Foundation (E-1721).

**DISCLOSURES:**

No potential conflict of interest was reported by authors.

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Figure 1



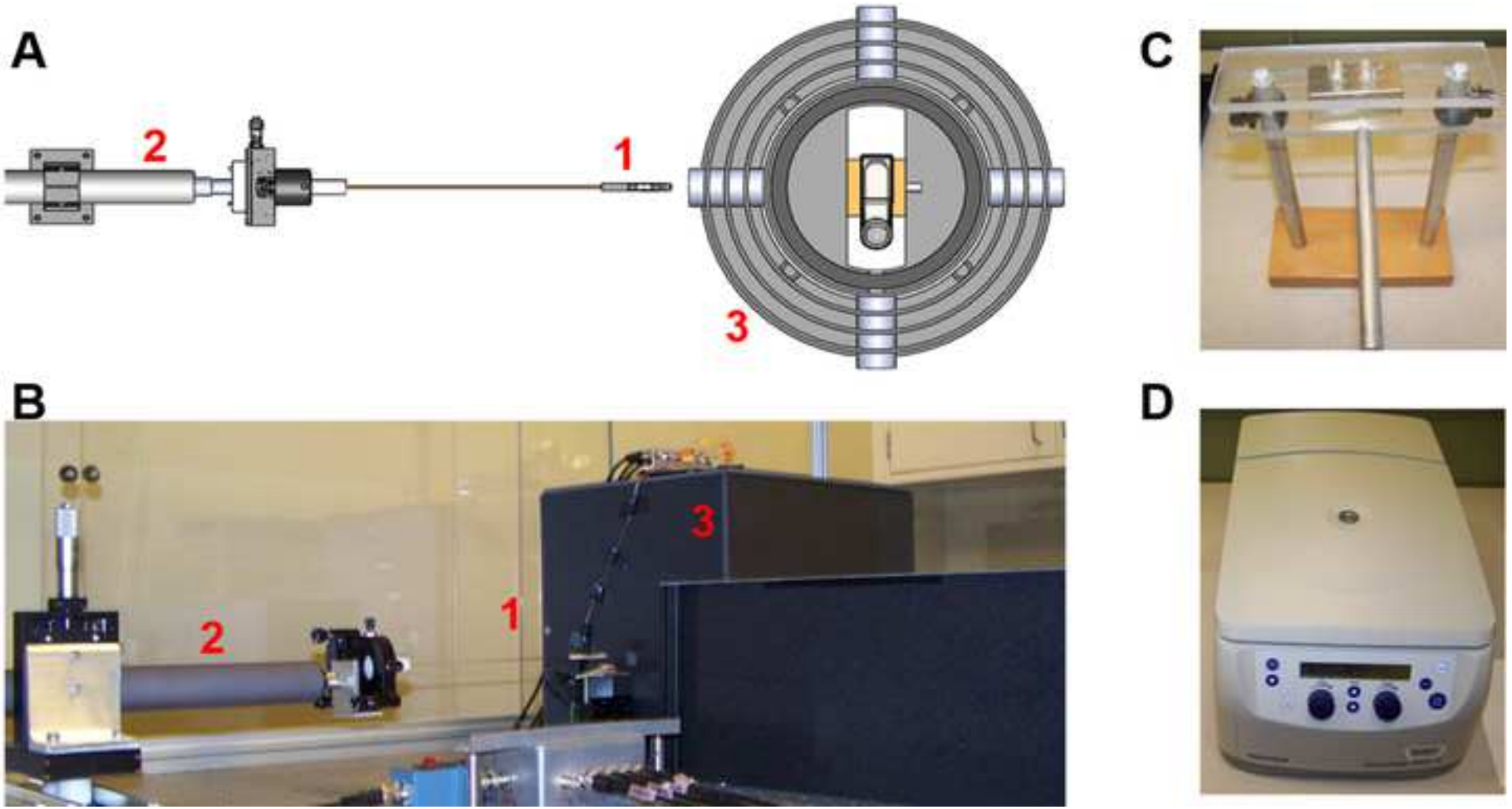

Figure 2



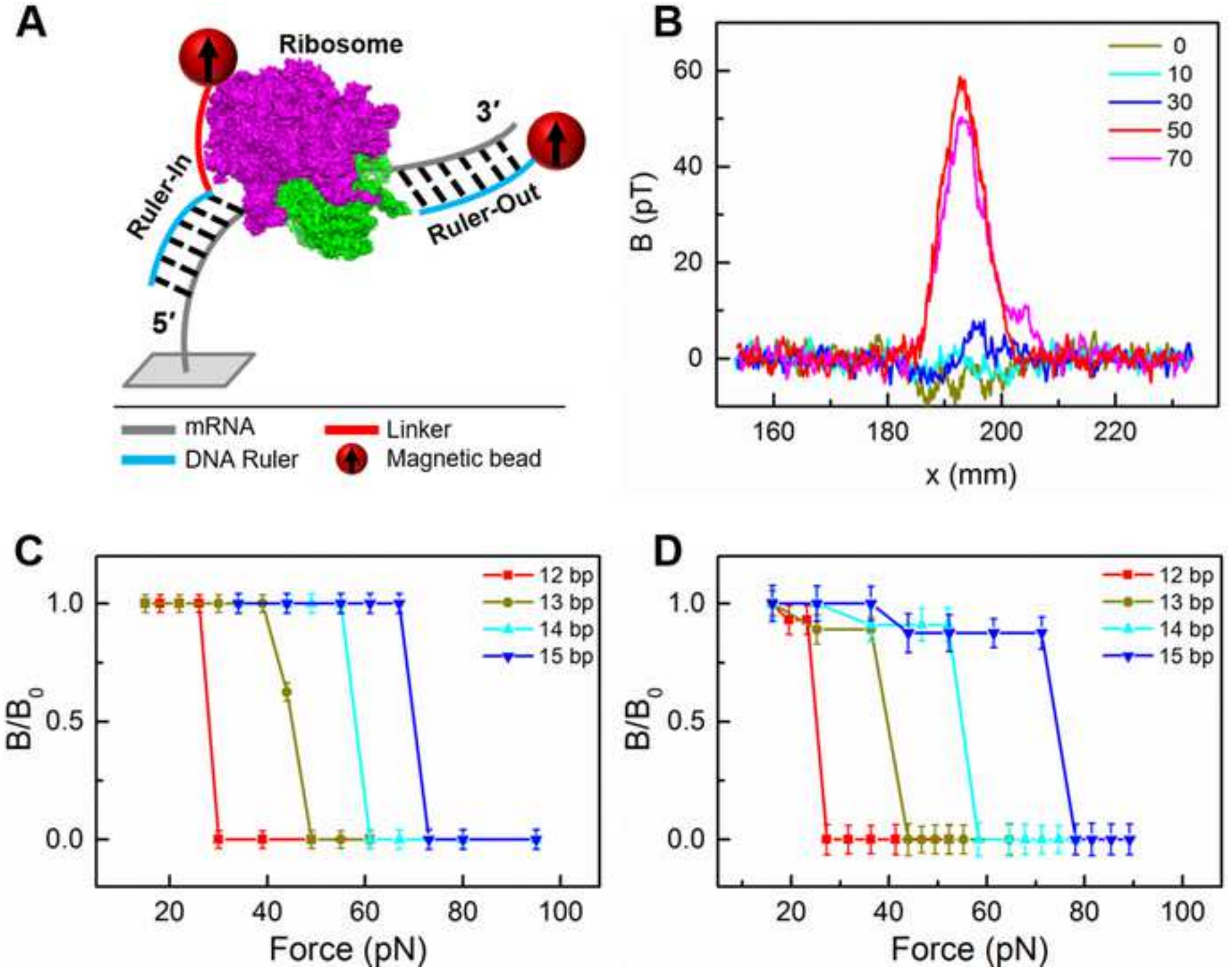

Figure 3

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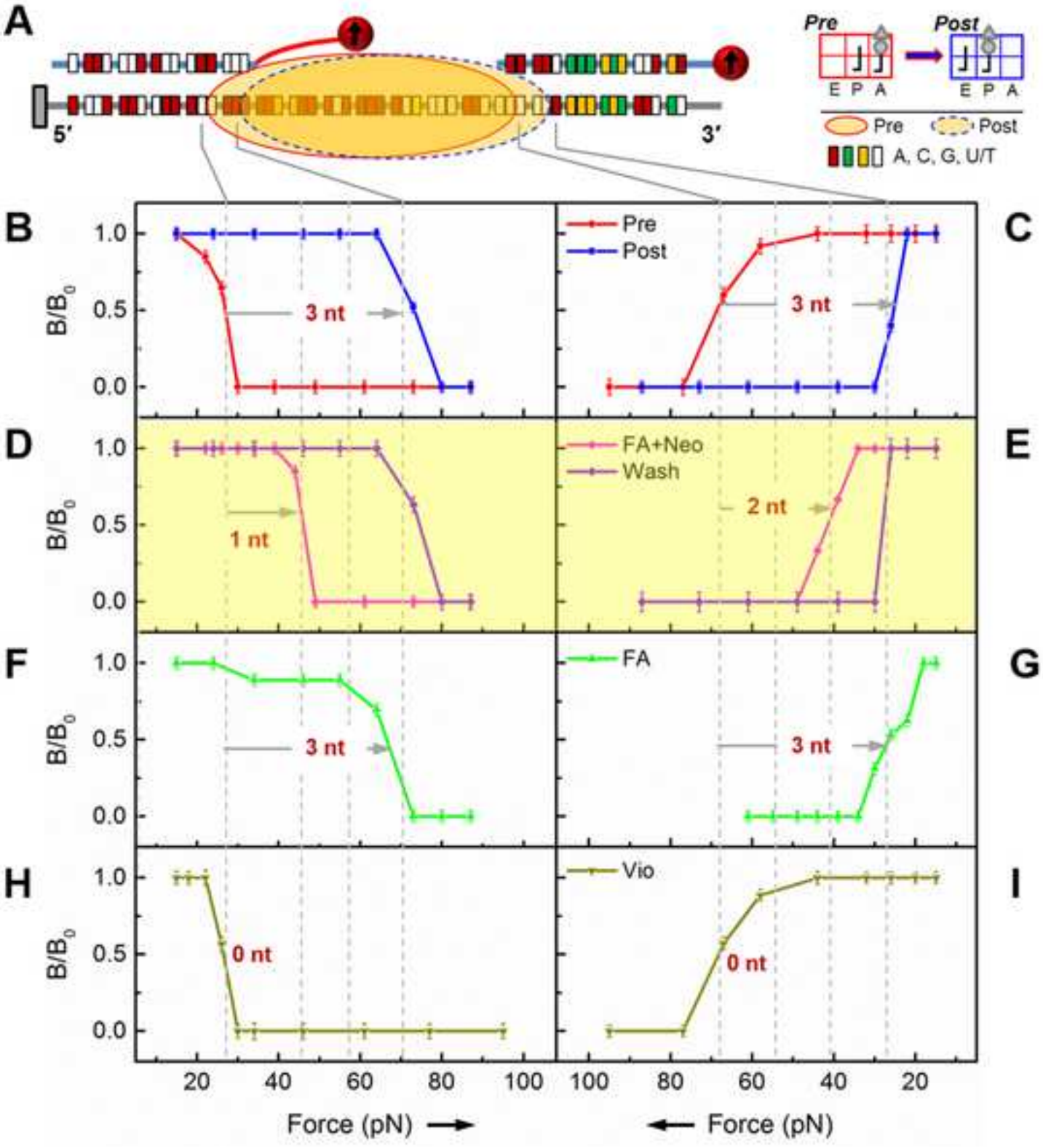

Figure 4

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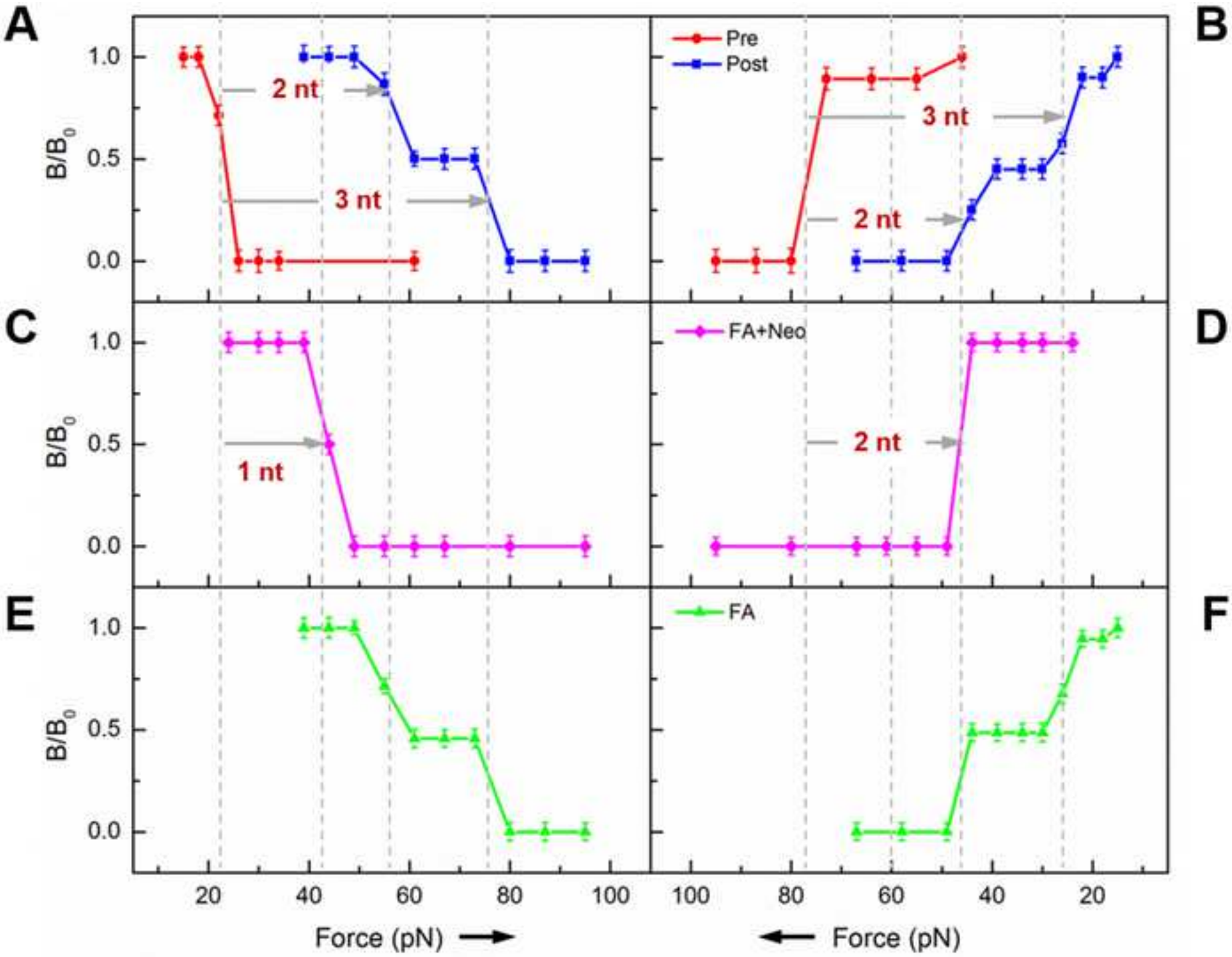

Table 1. The five mixtures for preparing ribosome complexes

| Mix | Components |
|---|---|
| ribosome mix | 1 µM ribosome, 1.5 µM each of IF1, IF2, IF3, 2 µM of mRNA, 4 µM of charged fMet-tRNA$^{fMet}$, 4 mM of GTP. |
| TuWG mix | 4 µM EF-Tu, 0.4 µM EF-Ts, 2 µM EF-G, 4 mM GTP, 4 mM PEP, and 0.02 mg/mL pyruvate kinase |
| Tu0G mix | 4 µM EF-Tu, 0.4 µM EF-Ts, 4 mM GTP, 4 mM PEP, and 0.02 mg/mL pyruvate kinase |
| Phe mix | 100 mM Tris (pH 7.8), 20 mM $MgAc_2$, 1 mM EDTA, 4 mM ATP, 7 mM BME, 0.1 mg/mL total synthetase, 50 $A_{260}$/mL total tRNA, and 0.25 mM phenylalanine |
| Asn mix | 100 mM Tris (pH 7.8), 20 mM $MgAc_2$, 1 mM EDTA, 4 mM ATP, 7 mM BME, 0.1 mg/mL total synthetase, 50 $A_{260}$/mL total tRNA, and 0.25 mM asparagine |

Table 2. The five ribosome complexes

| Complex | Components | Incubation Condition |
|---|---|---|
| MF-Post | ribosome mix, TuWG mix, Phe mix (1:2:2) | 37 °C for 15 min |
| MF-Pre | ribosome mix, Tu0G mix, A mix (1:2:2) | 37 °C for 2 min |
| MFN-Post | MF-Post (1μM), TuWG mix, Asn mix (1:2:2) | 37 °C for 15 min |
| MFNF-Post | MFN-Post (1μM), TuWG mix, Phe mix (1:2:2) | 37 °C for 15 min |
| MFNF-Pre | MFN-Post (1μM), Tu0G mix, Phe mix (1:2:2) | 37 °C for 2 min |

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Xu.xlsx

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| Styrene Strip | City of Industry | MS-861 |
| Glass slides | Evaporated Coatings | |
| Acetic acid | Millipore Sigma | A6283-500ML |
| 3-Aminopropyltriethoxysilane | UCT specialties | 21400088 |
| mPEG-SVA | Laysan Bio | 154-82 |
| Biotin-PEG-SVA | Laysan Bio | 152-84 |
| Sodium bicarbonate | Millipore Sigma | S5761-500G |
| Epoxy glue | Devcon | 31345 |
| Streptavidin | ThermoFisher | 434301 |
| Fusidic Acid | Millipore Sigma | F0756-1G |
| Neomycin Sulfate | Millipore Sigma | 1458009 |
| Viomycin Sulfate | Millipore Sigma | 1715000 |
| Hygromycin | invitrogen | 10687-010 |
| Tris-HCl | Millipore Sigma | T5941-100G |
| Magnesium acetate | Millipore Sigma | M5661-50G |
| Ammonium chloride | Millipore Sigma | A9434-500G |
| Potassium chloride | Millipore Sigma | P9333-500G |
| EDTA | GIBCO | 774750 |
| 2-mercaptoethanol | Millipore Sigma | M6250-500ML |
| Tween20 | Millipore Sigma | P1379-250ML |
| GTP | Millipore Sigma | G8877-100MG |
| PEP | Millipore Sigma | P7127-100MG |
| Pyruvate Kinase | Millipore Sigma | P1506-5KU |
| Sucrose | Millipore Sigma | S7903-5KG |
| Dynabeads M-280 Streptavidin | ThermoFisher | 11205D |
| mRNA Oligo | Integrated DNA Technologies | 133899727 |
| DNA Oligo | Integrated DNA Technologies | 157468630 |
| DNA Oligo | Integrated DNA Technologies | 164845370 |
| DNA Oligo | Integrated DNA Technologies | 157468628 |
| DNA Oligo | Integrated DNA Technologies | 163472705 |
| DNA Oligo | Integrated DNA Technologies | 138678130 |

| | | |
|---|---|---|
| DNA Oligo | Integrated DNA Technologies | 138678131 |
| DNA Oligo | Integrated DNA Technologies | 138678132 |
| DNA Oligo | Integrated DNA Technologies | 138678133 |
| Centrifuge | Eppendorf | 5427R |
| Micro Ultracentrifuge | Hitachi | CS150FNX |
| Vortex mixer | VWR | VM-3000 |
| Lock-in Amplifier | Stanford Research Systems | SR530 |
| Lock-in Amplifier | Stanford Research Systems | SR830 |
| Laser | Newport | TLB-6918-D |
| Function generator | Stanford Research Systems | DS345 |
| Photo detectors | Thorlabs | DET36A |

**Comments/Description**

$60.0 \times 4.0 \times 0.3\ mm^3$

5’-Bio- CAA CUG UUA AUU AAA UUA AAU UAA AAA GGA AAU AAAA AUG UUU AAU UUU UUA GGG CGC AAU CUA CUG CUG AAC UC-3’
3’- TAA TTT AAT TTA ATT TTT CGA AAU AT50/TEGBio/-5’
3’-AAT TTA ATT TTT CCT TTA AAA AT50/TEGBio/-5’
3’-AAA ATC CCG CGT TAG AAC UGG GG/TEGBio/-5’
3’-CCG CGT TAG ATG ACG AGA ACG GG/TEGBio/-5’
3’-AGA TGA CGA CTT CTC GGG/TEGBio/-5’

3’-T AGA TGA CGA CTT CTC GGG/TEGBio/-5’
3’-TT AGA TGA CGA CTT CTC GGG/TEGBio/-5’
3’-GTT AGA TGA CGA CTT CTC GGG/TEGBio/-5’

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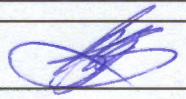
| | |
|---|---|
| Name: | Shoujun Xu |
| Department: | Chemistry |
| Institution: | University of Houston |
| Title: | Associate Professor |
| Signature: |  |
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## Response to Editorial Comments

*Manuscript JoVE59918_R1, by Heng Yin, Shoujun Xu, and Yuhong Wang*

We have carefully re-written all the sections that were marked red by the Editor. In addition, we followed the Editor's suggestion by organizing sections 1.2.1-1.2.5 into Table 1, and sections 1.4-1.8 into Table 2. Furthermore, we have addressed all the comments. All the changes are marked in the revised manuscript.

We appreciate the Editor's outstanding effort in making our work more suitable for JoVE.

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