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TITLE:

In Vitro Biochemical Assays using Biotin Labels to Study Protein-Nucleic Acid Interactions

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SUMMARY:

Presented here are protocols for in vitro biochemical assays using biotin labels that may be widely applicable for studying protein-nucleic acid interactions.

ABSTRACT:

Protein-nucleic acid interactions play important roles in biological processes such as transcription, recombination, and RNA metabolism. Experimental methods to study protein-nucleic acid interactions require the use of fluorescent tags, radioactive isotopes, or other labels to detect and analyze specific target molecules. Biotin, a non-radioactive nucleic acid label, is commonly used in electrophoretic mobility shift assays (EMSA) but has not been regularly employed to monitor protein activity during nucleic acid processes. This protocol

illustrates the utility of biotin labeling during in vitro enzymatic reactions, demonstrating that this label works well with a range of different biochemical assays. Specifically, in alignment with previous findings using radioisotope ^{32}P-labeled substrates, it is confirmed via biotin-labeled EMSA that MEIOB (a protein specifically involved in the meiotic recombination) is a DNA-binding protein, that MOV10 (an RNA helicase) resolves biotin-labeled RNA duplex structures, and that MEIOB cleaves biotin-labeled single-stranded DNA. This study demonstrates that biotin is capable of substituting ^{32}P in various nucleic acid-related biochemical assays in vitro.

INTRODUCTION:

Protein-nucleic acid interactions are involved in many essential cellular processes such as DNA repair, replication, transcription, RNA processing, and translation. Protein interactions with specific DNA sequences within the chromatin are required for the tight control of gene expression at the transcriptional level[1]. Precise posttranscriptional regulation of numerous coding and noncoding RNAs necessitates extensive and complicated interactions between any protein and RNA[2]. These layers of gene expression regulatory mechanism comprise a cascade of dynamic intermolecular events, which are coordinated by interactions of transcription/epigenetic factors or RNA-binding proteins with their nucleic acid targets, as well as protein-protein interactions. To dissect whether proteins in vivo are directly or indirectly associated with nucleic acids and how such associations occur and culminate, in vitro biochemical assays are conducted to examine the binding affinity or enzymatic activity of proteins of interest on designed substrates of DNA and/or RNA.

Many techniques have been developed to detect and characterize nucleic acid-protein complexes, including the electrophoretic mobility shift assay (EMSA), also termed gel retardation assay or band shift assay[3-5]. EMSA is a versatile and sensitive biochemical method that is widely used for studying the direct binding of proteins with nucleic acids. EMSA relies on gel electrophoretic shift in bands, which are routinely visualized using chemiluminescence to detect biotin labels[6,7], the fluorescence of fluorophore labels[8,9], or by autoradiography of radioactive ^{32}P labels[10,11]. Other purposes of biochemical studies are the identification and characterization of nucleic acid-processing activity of proteins, such as nuclease-based reactions to assess the cleavage products from nucleic acid substrates[12-14] and DNA/RNA structure-unwinding assays to evaluate helicase activities[15-17].

In such enzymatic activity assays, the radioisotope-labeled or fluorophore-labeled nucleic acids are often used as substrates due to their high sensitivity. Analysis of radiographs of enzymatic reactions involving ^{32}P labeled radiotracers has been found to be sensitive and reproducible[18]. Yet, in an increasing number of laboratories in the world, the usage of radioisotopes is restricted or even prohibited due to the health risks associated with potential exposure. In addition to biosafety concerns, other drawbacks are the required necessary equipment to conduct work with radioisotopes, required radioactivity license, short half-life of ^{32}P (about 14 days), and gradual deterioration of the probe quality due to radiolysis. Thus, alternative non-isotopic methods have been developed (i.e., labeling the

probe with fluorophores enables detection by fluorescent imaging[19]). However, a high-resolution imaging system is needed when using fluorescently labeled probes. Biotin, a commonly used label, readily binds to biological macromolecules such as proteins and nucleic acids. Biotin-streptavidin system operates efficiently and improves detection sensitivity without increasing non-specific background[20,21]. Besides EMSA, biotin is widely used for protein purification and RNA pull-down, among others[22-24].

This protocol successfully uses biotin-labeled nucleic acids as substrates for in vitro biochemical assays that include EMSA, in addition to enzymatic reactions in which biotin has not been commonly used. The MEIOB OB domain is constructed and two mutants (truncation and point mutation) are expressed as GST fusion proteins[25-27], as well as mouse MOV10 recombinant FLAG fusion protein[16]. This report highlights the effectiveness of this combined protocol for protein purification and biotin-labeled assays for miscellaneous experimental purposes.

PROTOCOL:

1. Protein preparation

1.1. MEIOB and MOV10 expression constructs

1.1.1. Generate cDNA expression constructs encoding mouse MEIOB-A, C, and E (**Figure 1A**) and MOV10.

1.1.1.1. Set up the polymerase chain reaction (PCR) reactions for each fragment. Mix 1 µL of mouse cDNA (from C57BL/6 mouse testis), 1 µL of dNTP, 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer, 1 µL of DNA polymerase, 25 µL of 2x PCR buffer, and 18 µL of double distilled H_2O (ddH_2O) in a final volume of 50 µL.

NOTE: The primers for the amplification of *Meiob* and *Mov10* gene fragments are listed in **Table 1.**

1.1.1.2. Perform PCR reactions using the following programs: 95 °C for 5 min, 35 cycles of heating at 95 °C for 15 s, annealing at 64 °C for 15 s, extension at 72 °C for 20 s (2 min for extending full length MOV10), and final extension at 68 °C for 7 min.

NOTE: Use primer pair MEIOB-E (forward) and MEIOB-E-mut (reverse) for amplifying MEIOB E and primer pair MEIOB-E-mut (forward) and MEIOB-E (reverse) to amplify two segments that contain the mutant sequence within an overlapping sequence at the 3’ and 5’ ends, respectively, to generate a mutant PCR template.

1.1.2. Analyze the amplified PCR DNA by gel electrophoresis, cut the band of required size from the gel quickly under a UV lamp, and place into a centrifuge tube.

NOTE: The expected product sizes visible on the agarose gel for MEIOB-A is 536 bp, MEIOB-C is 296 bp, MEIOB-E are 312 bp and 229 bp, and MOV10 is 3015bp.

1.1.3. Purify the PCR DNA with a gel extraction kit following the manufacturer's protocol.

1.1.3.1. Add an equal volume of dissolving buffer into the centrifuge tube from step 1.1.2 and melt gel in a 50–55 °C water bath for 5–10 min, ensuring that the gel pieces melt completely. Centrifuge briefly to collect any droplets from the wall of the tube.

NOTE: The mass/volume concentration of the gel and the dissolving buffer is 1 mg/μL.

1.1.3.2. Place the adsorption column in the collection tube, transfer the solution containing the dissolved gel fragment to the adsorption column, and centrifuge at 12,000 *x g* for 2 min.

1.1.3.3. Discard the filtrate at the bottom of the collection tubes. Add 600 μL of the wash buffer to the column, centrifuge at 12,000 *x g* for 1 min, and discard the filtrate.

1.1.3.4. Repeat step 1.1.3.3 once.

1.1.3.5. Place the column back into the collection tube, and centrifuge at 12,000 *x g* for 2 min to remove all the remaining wash buffer.

1.1.3.6. Place the adsorption column in a 1.5 mL sterilized centrifuge tube, add 50 μL of ddH_2O to the center of the adsorption column and centrifuge at 12,000 *x g* for 1 min. Measure the DNA concentration of the eluate using spectrophotometer.

1.1.4. Restriction digestion of plasmids

1.1.4.1. Digest pGEX-4T-1 vector with BamHI and NotI. To do so, mix 4 μg of pGEX-4T-1 vector, 5 μL of 10x digest buffer, 1 μL of BamHI, and 1 μL of NotI and ddH_2O to a final reaction volume of 50 μL. Incubate at 37 °C for 2 h.

1.1.4.2. Digest pRK5 vector with BamHI and XhoI by mixing 4 μg of pRK5 vector, 5 μL of 10x digest buffer, 1 μL of BamHI, and 1 μL of XhoI and ddH_2O to a final reaction volume of 50 μL. Incubate at 37 °C for 2 h.

1.1.5. Analyze the vector DNA by gel electrophoresis, cut the desired size band from the gel quickly with a scalpel under a UV lamp and place it into a centrifuge tube.

1.1.6. Purify the vector DNA with a gel extraction kit as 1.1.3 following the manufacturer's instruction.

NOTE: The length of linearized plasmids: pGEX-4T-1, 4.4kb; pRK5, 4.7 kb.

1.1.7. Set up a standard recombinant ligation reaction by combining 0.03 pmol of linearized vector, 0.06 pmol of cDNA fragment, 2 µL of ligase, and 4 µL of 5x ligase buffer and ddH_2O in a final reaction volume of 10 µL.

NOTE: Clone MEIOB-A, C, and E into a pGEX-4T-1 vector and MOV10 into a pRK5 vector.

1.1.8. Incubate the mixture at 37 °C for 30 min, and then cool the reaction immediately for 5 min on ice. Transform MEIOB recombinant plasmids into BL21 bacteria and MOV10 recombinant plasmids into DH5α bacteria.

NOTE: Verify all recombinant constructs by Sanger sequencing.

1.1.9. Prepare glycerol stocks of bacterial cultures containing verified recombinant plasmids by adding an equal volume of 50% glycerol to liquid cultures, and store at -80 °C.

NOTE: For each subsequent experiment, streak out bacteria from glycerol stocks onto a fresh agar plate and use a single colony for the expansion as described in step 1.2.

1.2. MEIOB protein extracts from bacteria

1.2.1. Pick one colony of each BL21 strain transfected with the empty or recombinant pGEX-4T-1 plasmid verified by sequencing and inoculate in 3 mL LB containing 100 µg/mL ampicillin. Grow overnight at 37 °C with shaking at 220 rpm.

1.2.2. Inoculate 300 mL LB containing 100 µg/mL ampicillin from 3 mL overnight culture (from step 1.2.1). Grow the cultures with shaking at 37 °C for 2 h till OD_{600} reaches 0.5-1.0.

1.2.3. Induce the protein expression by adding isopropyl beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. Incubate the cultures for an additional 3 h with shaking at 180 rpm and 18 °C.

1.2.4. Centrifuge the bacterial culture at 3,500 *x g* and 4 °C for 20 min.

1.2.5. Resuspend the pellet in 20 mL of ice-cold Dulbecco's phosphate buffered saline (DPBS) buffer. Sonicate the bacterial suspension on ice for 25 cycles in short 10 s bursts (output power 20%) followed by 2–3 s resting on ice.

1.2.6. Centrifuge the lysate at 12,000 *x g* and 4 °C for 15 min. Transfer all the supernatant to a fresh tube.

1.2.7. Pre-wash beads.

1.2.7.1. Add 300 µL of glutathione-sepharose beads to a fresh 50 mL tube and wash the beads with 10 mL of ice-cold PBS buffer.

1.2.7.2. Centrifuge at 750 *x g* and 4 °C for 1 min to pellet the beads and discard the wash solution.

1.2.8. Add the lysate to the washed beads and incubate at 4 °C for 2 h. Centrifuge at 750 *x g* and 4 °C for 1 min to pellet the beads. Rinse the beads in 10 mL of ice-cold PBS 8x.

1.2.9. Elute the beads with 1 mL of the elution buffer (10 mM glutathione in 50 mM Tris-HCl at pH 8.0) 6x, incubating at 4 °C for 10 min prior to each elution step. Collect and pool the 6 fractions.

1.2.10. Transfer the eluted proteins into a centrifugal filter and concentrate by centrifugation at 7,500 *x g* to obtain a final volume of 100–200 µL.

1.3. MOV10 protein extracts from HEK293T cells

1.3.1. Transiently express the MOV10 proteins in cultured HEK293T cells.

1.3.1.1. Prepare MOV10-pRK5 plasmid at a concentration >500 ng/µL.

1.3.1.2. Seed HEK293T cells in 15 cm dishes. When the cell density reaches ~70%–90%, replace the cell culture medium with fresh Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

1.3.1.3. For one transfection, dilute 60 µg of MOV10-pRK5 plasmid DNA in 3 mL of the reduced serum medium, then add 120 µL of the transfection enhancer reagent, and mix well.

1.3.1.4. In a separate tube dilute 90 µL of the transfection reagent with 3 mL of reduced serum medium (without penicillin-streptomycin) and mix well.

1.3.1.5. Add the diluted DNA to each tube of diluted transfection reagent. Incubate at room temperature for 15 min.

1.3.1.6. Add the transfection mixture to the cell culture, and culture cells for ~36–48 h.

1.3.2. After 36-48 h, collect cells from each plate in a 50 mL tube. Centrifuge at 500 *x g* for 5 min at 4 °C. Wash each pellet with 10 mL of ice-cold PBS, and collect cells by centrifugation at 500 *x g* for 5 min at 4 °C.

1.3.3. Resuspend the pellet in 3 mL of cell lysis buffer containing complete ehylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail. Incubate for 30 min on ice. Centrifuge the lysate at 20,000 *x g* and 4 °C for 20 min.

1.3.4. Add 100 µL of anti-FLAG magnetic beads per dish of cells in a 1.5 mL tube.

1.3.5. Wash the magnetic beads 2x with K150 buffer (50 mM HEPES at pH 7.5, 150 mM KoAc, 1 mM DTT, 0.1% NP-40).

1.3.5.1 Resuspend the magnetic beads in 1 mL of ice-cold K150 buffer.

1.3.5.2. Incubate the magnetic beads for 2 min at 4 °C with gentle rotation and pellet the magnetic beads with the help of a magnet. Remove and discard the supernatant.

1.3.6. Add the magnetic beads to the cell lysate supernatant from step 1.3.3 and incubate at 4 °C for 2 h.

1.3.7. Wash the protein bound magnetic beads 3x with K150 buffer, then 2x with K150 containing 250 mM NaCl, then 3x with K150 buffer as 1.3.5.

1.3.8. Resuspend the beads in 300 µL of FLAG elution buffer (100 mM NaCl, 20 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 10% glycerol), add FLAG peptide to a final concentration of 0.5 µg/µL, and incubate with beads on a rotator at 4 °C for 1 h, then pellet the magnetic beads with the help of a magnet.

1.3.9. Collect the supernatant which contains the eluted MOV10 proteins, determine the concentration, and store at -80 °C for future use.

2. Nucleic acid preparation

2.1. Purchase DNA and RNA oligonucleotides (oligos) with or without biotin labels from a suitable source. Dilute each oligo in RNase-free ddH_2O to 20 µM and keep it at -80 °C for the future use.

NOTE: The oligo sequences of DNA/RNA substrates used in this study are listed in **Table 2**.

2.2. Prepare the following mixture for the double-stranded RNA (dsRNA) annealing reaction for MOV10 helicase activity assay: mix 60 mM N-2-hydroxyethylpiperazine-N-ethane-sulphonic acid (HEPES) at pH 7.5, 6 mM KCl, 0.2 mM $MgCl_2$ and RNase-free ddH_2O in a final reaction volume of 20 µL.

2.3. Anneal RNA oligos to form RNA duplex by heating a mixture of the biotin-labeled top strand (2 µM, final concentration) and a 1.5-fold of its unlabeled complementary bottom strand in the annealing buffer (step 2.2) at 95 °C for 5 min, and then slowly cool it to room temperature (RT).

3. In vitro biochemical assays

3.1. EMSA and enzymatic reactions

3.1.1. For the MEIOB EMSA assay, mix 50 mM Tris HCl at pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 10 mM EDTA, 2 mM dithiothreitol (DTT), 0.01% NP-40, 0.8 mM (or other relevant concentrations as shown in **Figure 2** and **Figure 3**) MEIOB protein, and 10 nM biotin-labeled oligonucleotides and ddH_2O in a final reaction volume of 20 µL. Incubate at RT for 30 min, and add 5x stop buffer (125 mM EDTA, 50% glycerol) to stop the reaction.

3.1.2. Set up MEIOB nuclease activity reactions as described in step 3.1.1 but without the addition of 10 mM EDTA.

3.1.3. For the MOV10 helicase activity assay, mix 50 mM Tris-HCl at pH 7.5, 20 mM KoAc, 2 mM $MgCl_2$, 0.01% NP-40, 1 mM DTT, 2 U/µL RNase inhibitor, 10 nM biotin-labeled RNA substrate, 2 mM adenosine triphosphate (ATP), 100 nM RNA trap, and 20 ng of MOV10 protein and ddH_2O in a final reaction volume of 20 µL. Incubate the reaction mixture at 37 °C for 10 min, 30 min, and 60 min. Add the 5x stop buffer to stop the reaction.

NOTE: RNA trap, a biotin-unlabeled oligo with sequence, complementarity to the labeled oligo, which prevents the unwound dsRNA from annealing again.

3.2. Polyacrylamide gels

3.2.1. Wash the gel plates (16 cm x 16 cm) and 1.5 mm combs. Assemble the gel electrophoresis units.

3.2.2. To prepare a 10% native polyacrylamide gel, mix 14 mL of ddH_2O, 1.25 mL of 10x Tris-boric acid-EDTA (TBE), 8.3 mL of 30% acrylamide, 1.25 mL of 50% glycerol, 187.5 µL of 10% freshly prepared ammonium persulfate (APS), and 12.5 µL of tetramethylethylenediamine (TEMED).

3.2.3. To prepare a 20% native polyacrylamide gel, mix 5.5 mL of ddH_2O, 1.25 mL of 10x TBE, 1.25 mL of 50% glycerol, 16.7 mL of 30% acrylamide, 187.5 µL of 10% APS, and 12.5 µL of TEMED.

3.2.4. Pour the acrylamide solution immediately to the gel and insert the comb. Let the mixture polymerize for approximately 30 min.

3.3. Gel running

3.3.1. Remove the comb, fill the tanks with the electrophoresis running buffer (0.5x TBE).

3.3.2. Rinse the sample wells with 0.5x TBE buffer, then pre-run the gel at 100 V on ice for 30 min. Replace the running buffer with fresh 0.5x TBE.

3.3.3. Load 20–25 µL samples into each well.

3.3.4. Use a 10% native acrylamide gel for the EMSA assay and a 20% native acrylamide gel for the enzymatic assays. Run electrophoresis at 100 V on the ice bath until the bromophenol blue marker has migrated to the bottom quarter of the gel.

3.4. Disassemble the gel plates, trim the gel by removing loading wells and unused lanes. Place the gel in 0.5x TBE buffer.

3.5. Cut the filter paper and the nylon membrane to the size of the gel. Pre-wet the clean filter paper and the nylon membrane.

3.6. Assemble the stack for transfer.

3.6.1. Place the pre-wet membrane onto the pre-wet filter paper.

3.6.2 Place the gel on the membrane.

3.6.3. Cover the gel with another layer of pre-wet filter paper.

3.6.4. Remove all air bubbles by rolling a clean pipette from center to edge.

3.7. Transfer the samples from the gel to the membrane in a semi-dry electrophoretic apparatus at 90 mA for 20 min.

3.8. Stop the transfer, and then dry the membrane on a new filter paper for 1 min.

3.9. Crosslink the samples by irradiating the membrane at 120 mJ/cm^2 for 45–60 s in a UV-light crosslinker equipped with 254 nm bulbs (auto crosslink function). Air dry the membrane at RT for 30 min.

3.10. Chemiluminescence detection

3.10.1. Protocol 1: Use a standard volume of commercial chemiluminescent nucleic acid detection kit.

3.10.1.1. Add 20 mL of blocking buffer to the membrane and incubate for 15–30 min with gentle shaking on a rotator at 20–25 rpm.

3.10.1.2. Prepare conjugate/blocking buffer solution by adding 66.7 µL stabilized streptavidin-horseradish peroxidase conjugate to 20 mL of blocking buffer.

3.10.1.3. Gently remove the blocking buffer and replace it with conjugate/blocking buffer. Incubate for 15 min on a rotator at 20–25 rpm.

3.10.1.4. Wash the membrane 4x with shaking at 40–45 rpm for 5 min each.

3.10.1.5. Add 30 mL of substrate equilibration buffer to the membrane. Incubate the membrane for 5 min with shaking at 20–25 rpm.

3.10.1.6. Prepare substrate working solution by adding 6 mL of luminol/enhancer solution to 6 mL of stable peroxide solution. Avoid light.

3.10.1.7. Cover the entire surface of the membrane with substrate working solution and incubate for 5 min.

3.10.1.8. Scan the membrane in a chemiluminescent imaging system for 1–3 s.

3.10.2. Protocol 2: Use 2x diluted commercial chemiluminescent nucleic acid detection kit and follow the steps 3.10.1.1–3.10.1.8.

3.10.3. Protocol 3: Use self-made reagents[6].

3.10.3.1. Prepare blocking buffer: mix 0.1 M Tris-HCl at pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, and 3% bovine serum albumin Fraction V. AP 7.5 buffer: mix 0.1 M Tris-HCl at pH 7.5, 0.1 M NaCl, and 2 mM $MgCl_2$. AP 9.5 buffer: mix 0.1 M Tris-HCl at pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$. TE buffer: mix 10 mM Tris-HCl at pH 8.0, 1 mM EDTA at pH 8.0.

3.10.3.2. Soak the membrane in blocking buffer at 30 °C for 1 h.

3.10.3.3. Add 8.5 µL of streptavidin alkaline phosphatase to 10 mL of AP 7.5 Buffer. Shake the membrane gently in this solution at RT for 10 min. Then, wash the membrane 2x in 15 mL of AP 7.5 buffer for 10 min.

3.10.3.4. Wash the membrane one more time in 20 mL of AP 9.5 buffer for 10 min. Add 20 mL of TE buffer to stop the reaction.

3.10.3.5. Add 7.5 mL of development solution onto the membrane, and scan the membrane in a chemiluminescent imaging system for 1–3 s.

REPRESENTATIVE RESULTS:

The protein structure of MEIOB and the expression constructs used in this study are illustrated in **Figure 1A**. OB folds in MEIOB are compact barrel-like structures that can recognize and interact with single-stranded nucleic acids. One of the OB domains (aa 136–307, construct A) binds single stranded DNA (ssDNA), the truncated protein (aa 136–178 truncations, construct C) and the point mutant form (R235A mutation, construct E) of MEIOB do not have DNA-binding activity[26]. The GST-MEIOB fusion proteins were overexpressed in

BL21 bacteria, with subsequent isolation steps resulting in purified proteins shown by Coomassie blue staining and western blot analysis **(Figure 1B).** Nucleic acid substrates at different concentrations illustrate the high sensitivity of the biotin signal, with a detectable signal of 1 nM oligo after a relatively short exposure time for 1-3 s **(Figure 2A)**. The wild-type MEIOB-A protein, but not the mutant MEIOB-E and MEIOB-C proteins, bind strongly to 36 nt biotin-labeled ssDNA substrates (the same length and sequence as used previously[26]) **(Figure 2B)** and cleave the substrates into ladders **(Figure 2C)**.

The in vitro assay of MEIOB proteins with RNA oligos of the same sequence as ssDNA substrates used in **Figure 2B,C** illustrates binding capacity and exonuclease activity of MEIOB on 36 nt single-stranded RNA (ssRNA) **(Figure 3A,B)**. Binding activity of MEIOB with DNA and RNA was further quantitatively analyzed **(Figure 3C)**. Additionally, FLAG-tagged MOV10 proteins were purified from HEK293T cells **(Figure 4A)**. To measure the helicase activity of MOV10, a duplex RNA was designed (same length but different sequence than used previously[16]) bearing an 18 nt 5' overhang **(Figure 4B)**. When the biotin-labeled RNA duplex was incubated with MOV10 in the presence of ATP, a lower band corresponding to the released single-stranded biotin-labeled RNA appeared with increasing time, reflective of the MOV10's function as an RNA helicase. Lastly, to reduce costs, it was attempted to optimize the usage of reagents for chemiluminescence detection of the biotin label. It was found that a two-fold dilution of the chemiluminescent nucleic acid detection kit did not negatively affect the chromogenic sensitivity of the biotin-streptavidin system, and excitingly, the self-made reagents worked almost equally well **(Figure 5)**.

FIGURE LEGENDS:

Figure 1: Purification of MEIOB proteins. (**A**) Schematic representation of the MEIOB constructs used in this study[26]. MEIOB contains an OB domain. All MEIOB constructs (A, C, E) were expressed as GST fusion proteins. (**B**) Coomassie blue staining and western blot analysis of the MEIOB proteins purified using GST-bacteria system. The red arrows indicate the positions of purified MEIOB proteins. Bands at approximately 26 KDa correspond to glutathione. For western blot, anti-GST antibody was used with 1:6000 dilution.

Figure 2: In vitro assays of MEIOB-ssDNA interactions. (**A**) Signal strength test of different concentrations of 36 nt biotin-labeled ssDNA. (**B**) EMSA result of MEIOB protein binding to biotin 5' end-labeled DNA substrates (10% native gel). (**C**) MEIOB-mediated cleavage of biotin 5' end-labeled DNA substrates (20% native gel).

Figure 3: In vitro assays of MEIOB-ssRNA interactions. (**A**) EMSA result of MEIOB protein binding to biotin 5' end-labeled RNA substrates (10% native gel). (**B**) MEIOB-mediated cleavage of biotin 5' end-labeled RNA substrates (20% native gel). (**C**) Plot of percentage of DNA/RNA-bound versus MEIOB-A concentration.

Figure 4: Purified MOV10 protein and its unwinding of 5' tailed dsRNA in vitro. (**A**) Coomassie blue staining of MOV10 protein purified using the FLAG-HEK293T system. The red

arrows indicate the positions of purified MOV10 protein. Bands on the Coomassie gel with a molecular weight of approximately 55 kDa correspond to the heavy immunoglobulin chain (IgG) from the FLAG antibody. (**B**) MOV10 unwinds 5' tailed dsRNA with increasing time (10, 30, 60 min) at 37 °C. ssRNA = 18 nt single-stranded RNA, dsRNA = 54 nt double-stranded RNA with an 18 nt 5' tail (20% native gel).

Figure 5: Alternative methods of using biotin chromogenic reagents on MEIOB assay. Commercial standard volume: instructed by chemiluminescent nucleic acid detection kit; 2x diluted commercial volume: two-fold dilution of each buffer in chemiluminescent nucleic acid detection kit, self-made reagents: see details in step 3.7.3.

Table 1: Primers used to PCR amplify the gene fragments of Meiob and Mov10. The bold letters in forward and reverse primers are BamHI and NotI cutting sites; the italic bold letters in a reverse primer are XhoI cutting sites; boxes indicate the nucleotides corresponding to the point mutation R235A.

Table 2: Sequences of DNA/RNA substrates used in this work.

DISCUSSION:

Investigating protein-nucleic acid interactions is critical to our understanding of molecular mechanisms underlying diverse biological processes. For example, MEIOB is a testis-specific protein essential for meiosis and fertility in mammals[25-27]. MEIOB contains an OB domain that binds to single-stranded DNA and exhibits 3' to 5' exonuclease activity[26], which directly relates to its physiological relevance during meiotic recombination. As another example, MOV10 is an RNA helicase with ubiquitous function that may associate with RNA secondary structures[16]. Accordingly, MOV10 displays broad RNA-binding properties and 5' to 3' RNA duplex unwinding activity[16]. The studies reporting the above-mentioned biochemical activities of these proteins relied on the use of ^{32}P isotope to label nucleic acids for in vitro assays. In the present study, we have established protocols for a series of biotin-labeled *in vitro* experiments of MEIOB and MOV10 function. These protocols begin with the preparation of active proteins and ended with imaging of biotin signals.

Specifically, in line with previous studies[25,26], MEIOB proteins were overexpressed in bacteria with and purification yielded one single band with strong Coomassie staining signal after gel electrophoresis. However, purification of full-length MOV10 protein was more effective when overexpressed as FLAG-tag-fused protein in HEK293T cells than as a GST-fused protein in bacteria (data not shown). To obtain sufficient amounts of protein at adequate purity for subsequent reactions, these two systems of protein purification need to be compared to determine the most suitable method for proteins with different sizes and/or properties. Nucleic acids were then labeled using biotin instead of ^{32}P as substrate and obtained robust signal when examining the nucleic acid-binding affinity or nucleic acid-processing activities of both proteins. However, as proteins purified from bacteria are frequently contaminated with RNase, it is difficult to rule out the possibility that the cleavage activity seen during the

in vitro reaction may in part result from contaminating RNase. In vitro assays with MEIOB mutants with reduced catalytic activity (truncated and point mutant) showed substantial impairment of RNA substrate processing, but possible RNase contamination cannot be excluded. The results obtained with each of MEIOB constructs acting on ssDNA and MOV10 unwinding dsRNA are similar to those obtained in previous study[16,26]. However, MEIOB processes DNA to generate a smear, while a more discrete band is seen with RNA according to the experimental results (**Figure 2C** and **Figure 3B**). Possibly, MEIOB has differential binding abilities to DNA and RNA substrate (**Figure 2B, Figure 3A,C**), which leads to the difference in their cleavage products. It may also be possible that MEIOB cleaves DNA and RNA in a distinct manner. The exact role of MEIOB in RNA processing remains to be further investigated (for example, using FLAG-tag-fused MEIOB protein expressed in HEK293T cells).

Biotin-labeled nucleic acid probes are advantageous over ^{32}P-labeled probes in that they do not require specific protection and waste disposal. Secondly, biotin-labeled probes can be stably preserved for at least 1 year at -20 °C, whereas ^{32}P-labeled probes last only for 2 weeks. Hence, the same batch of the biotin-labeled nucleic acids can be used over a long period of time, maintaining reproducibility of experiments. Finally, rapid autoradiography of radioactive probes may depend on expensive instruments such as phosphor screen. In contrast, all biotin-labeled assays described here can be performed within a day and do not require special equipment. The drawbacks of biotin labeling encompass mainly additional experimental steps including gel transfer and chemiluminescence that are necessary to detect biotin-labeled substrates but may additionally require optimization or troubleshooting. Another general weakness is the relatively low sensitivity of biotin-labeling compared with that of radioisotope-labeling. In these assays, nonetheless, well-visible detection of very low concentration of nucleic acids was achieved (**Figure 2A**).

In addition, semi-dry gel transfer apparatus is suitable for transferring longer-than-regular gels to membranes. Compared with wet transfer, semi-dry transfer is faster especially for nucleic acids, and yields a low background signal. Furthermore, costs of the chromogenic reaction of the biotin-streptavidin system were cut by either diluting the commercial reagents or making our own, both of which achieved similar signals. The detection sensitivity of the self-made reagents may not seem that high, albeit sufficient herein (**Figure 5C**), but it can be enhanced by extending the blocking time (unpublished data). Also, the signals can be enhanced with an increased concentration of the nucleic acid probe used for the assays. Given the above experimental evidence, the biotin label may be an advantageous substitute for ^{32}P in multiple in vitro biochemical assays.

Collectively, this protocol offers a biotin-labeled platform for the study of protein-nucleic acid interactions that proves to be robust, reliable, efficient, and affordable.

DISCLOSURES:

No conflicts of interest are declared.

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Figure 1

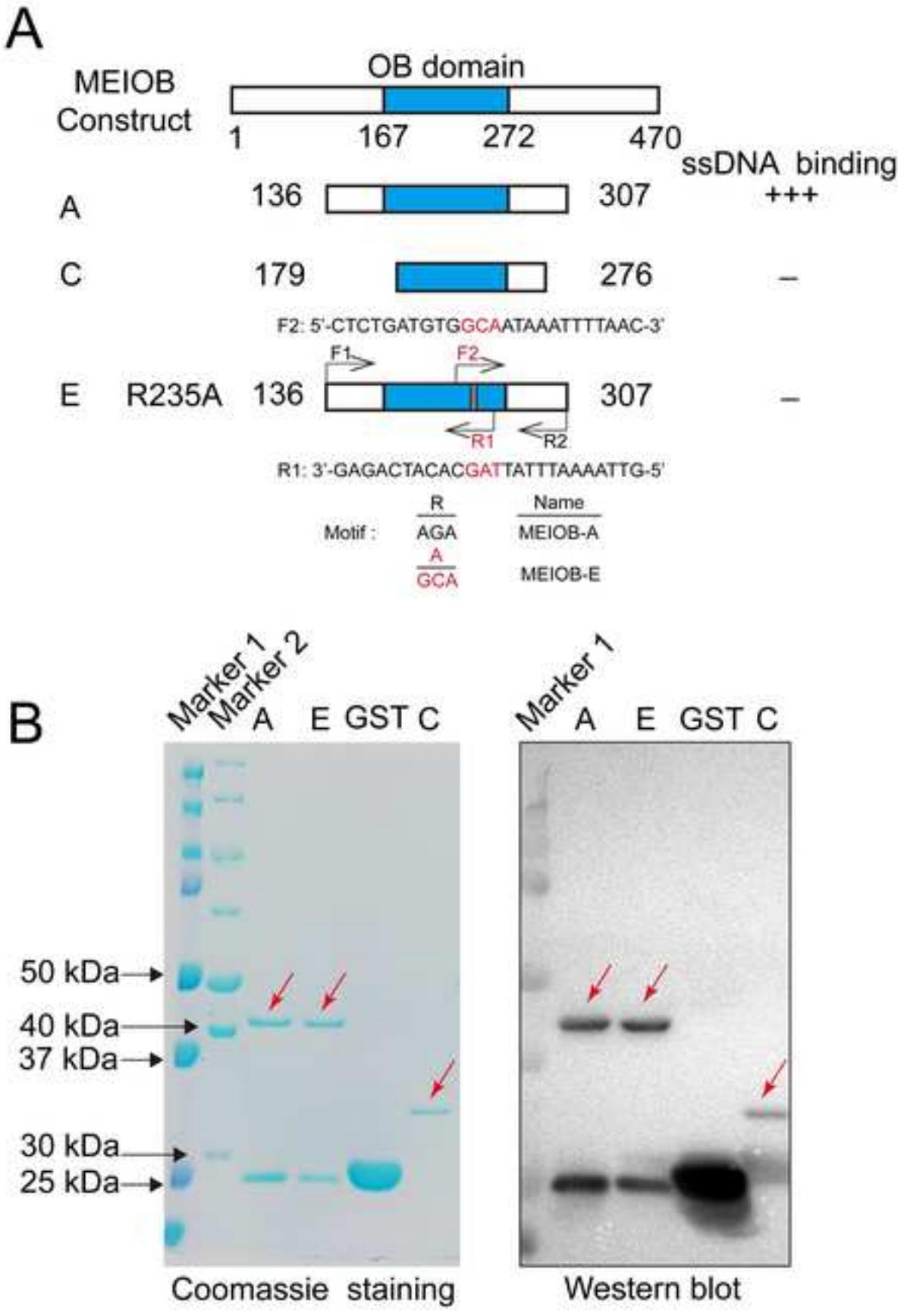
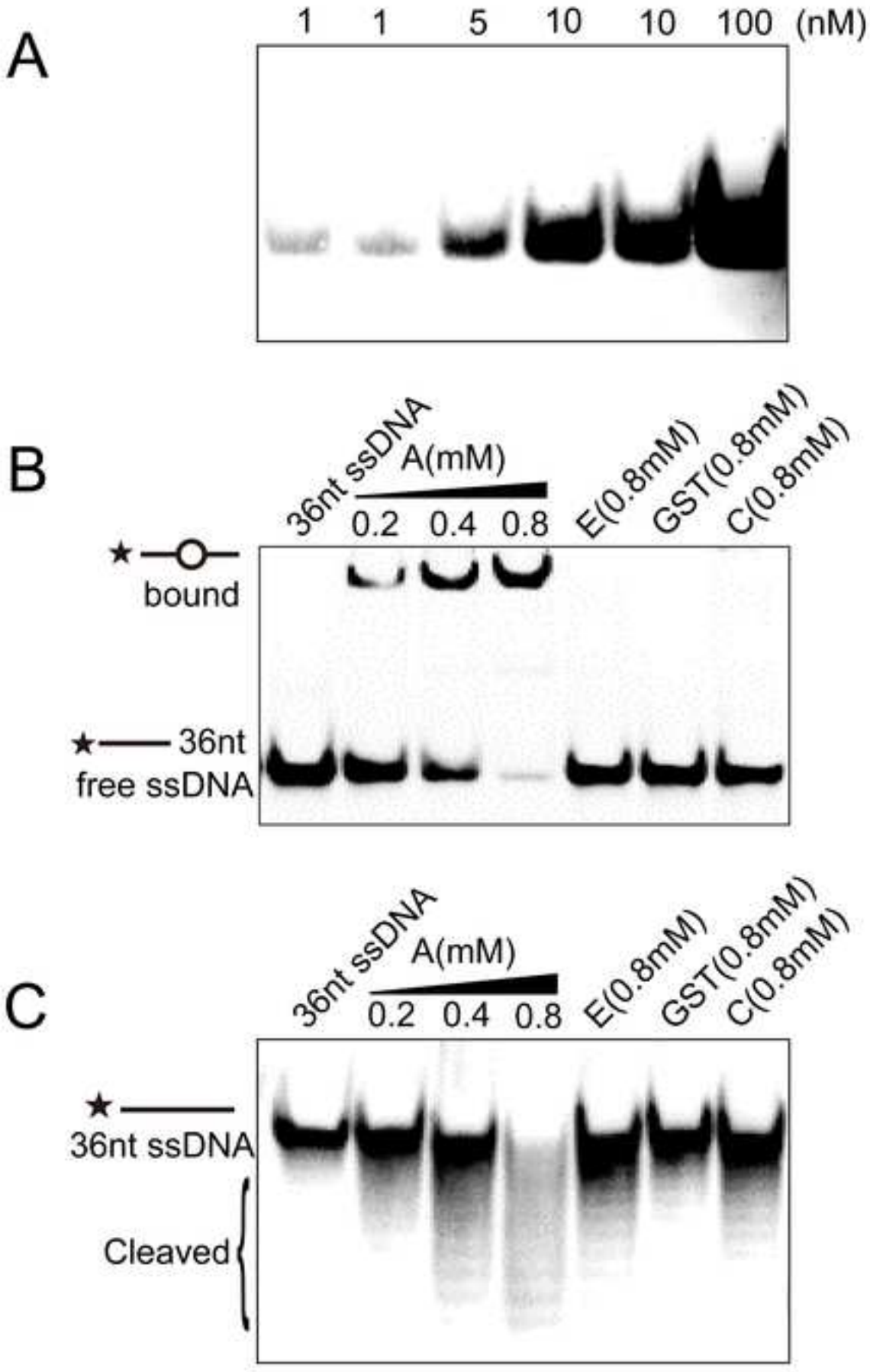


Figure 2



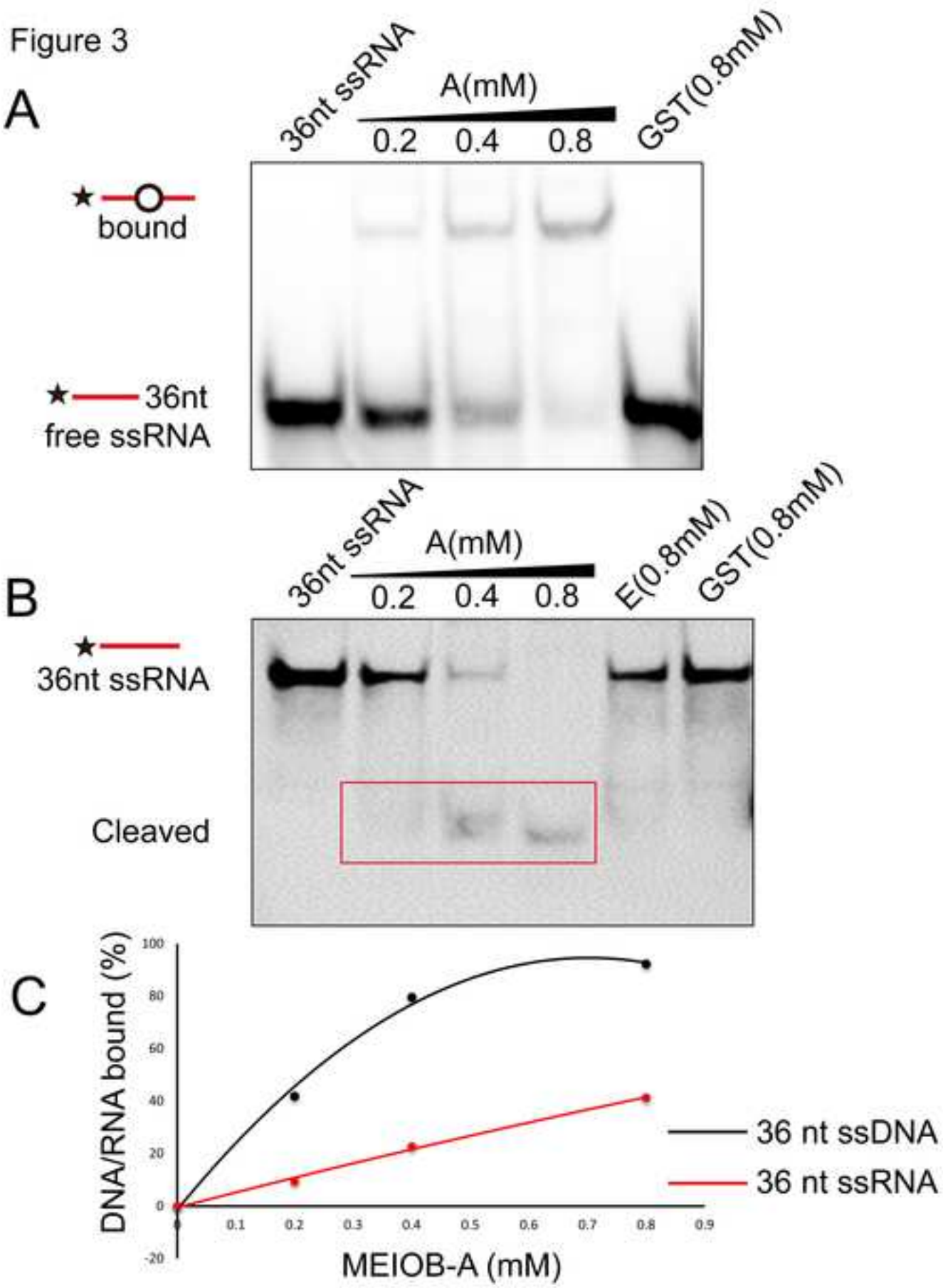


Figure 4

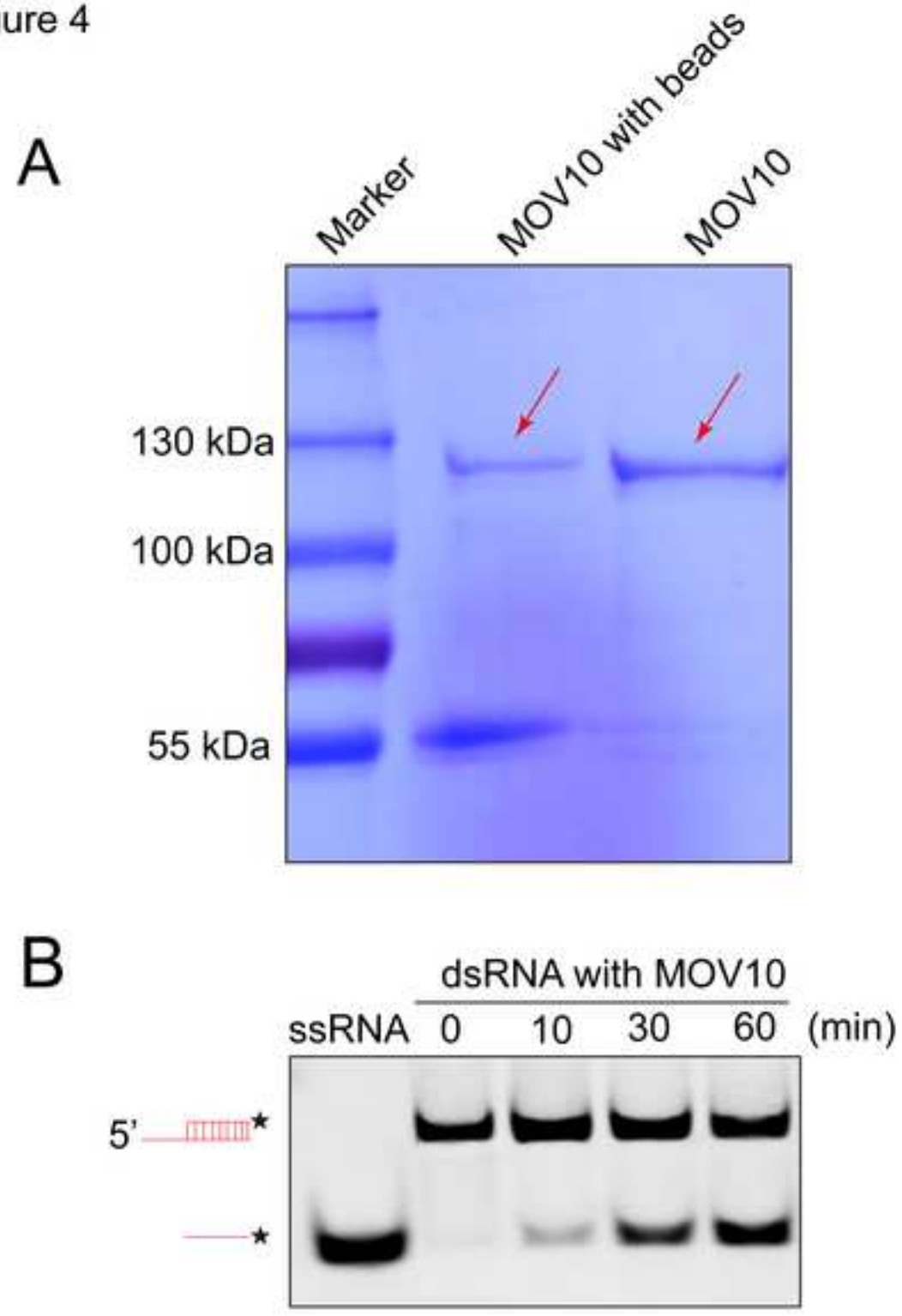


Figure 5

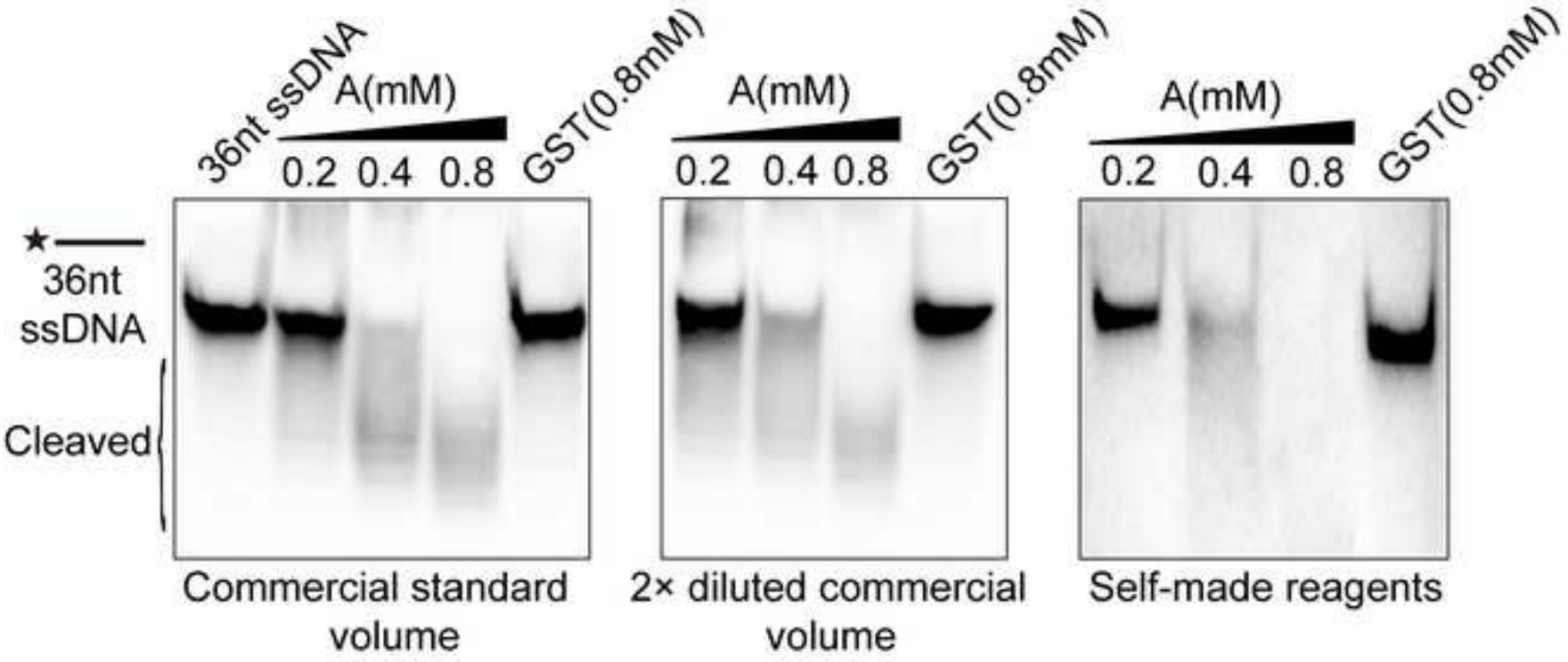


Table 1

Fragments	Primers	Sequences (from 5’ to 3’)
MEIOB-A	forward	CG**GGATCC**ATGTTACTTTCTTTGATACACTTGCC
	reverse	AT**GCGGCCGC**TACTTTTAGCTGTTCCACTG
MEIOB-C	forward	CCGCGT**GGATCC**ATGGAACCAAAATACTTTACAACTTCA
	reverse	CGAT**GCGGCCGC**CTCTTTATTTTCTCTTATATAATTCAGTAG
MEIOB-E	forward	CCGCGT**GGATCC**ATGTTACTTTCTTTGATACACTTGCC
	reverse	CGAT**GCGGCCGC**TACTTTTAGCTGTTCCACTG
MEIOB-E-mut	forward	CTCTGATGTGGCAATAAATTTTAAC
	reverse	GTTAAAATTTATTGCCACATCAGAG
MOV10	forward	GACGACGATGACAAG**GGATCC**ATGCCTAGCAAGTTCAGCTGCC
	reverse	GCTTACTCAGCTAAG***CTCGAG*** TCAGAGCTCATTTCTCCACTCTG

For figures	Names
Figure 2, 5	36 nt ssDNA
Figure 3	36 nt ssRNA
Figure 4	54 nt 5’ tailed dsRNA

Sequences (From 5' to 3')
5’-Biotin-GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT-3’ (DNA)
5’-Biotin-GUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU-3’ (RNA)
5’-Biotin-ACCGCUGCCGUCGCUCCG-3’ (RNA) 5’-ACGAGGGAGACGAGGAGACGGAGCGACGGCAGCGGU-3’ (RNA)

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Equipment			
Centrifuge	Eppendorf, Germany	5242R	
Chemiluminescent Imaging System	Tanon, China	5200	
Digital sonifer	Branson, USA	BBV12081048A	450 Watts; 50/60 HZ
Semi-dry electrophoretic blotter	Hoefer, USA	TE77XP	
Tube Revolver	Crystal, USA	3406051	
UV-light cross-linker	UVP, USA	CL-1000	
Materials			
Amicon Ultra-4 Centrifugal Filter	Milipore, USA	UFC801096	4 ml/10 K
Nylon membrane	Thermo Scientific, USA	TG263940A	
TC-treated Culture Dish	Corning, USA	430167	100 mm
TC-treated Culture Dish	Corning, USA	430597	150 mm
Microtubes tubes	AXYGEN, USA	MCT-150-C	1.5 mL
Tubes	Corning, USA	430791	15 mL
Reagents			
Ampicillin	SunShine Bio, China	8h288h28	
Anti-FLAG M2 magnetic beads	Sigma, USA	M8823	
ATP	Thermo Scientific, USA	591136	
BCIP/NBT Alkaline Phosphatase Color Development Kit	Beyotime, China	C3206	
CelLytic™ M Cell Lysis Reagent	Sigma, USA	107M4071V	
ClonExpress II one step cloning kit	Vazyme, China	C112	
Chemiluminescent Nucleic Acid Detection Kit	Thermo Scientific, USA	T1269950	
dNTP	Sigma-Aldrich, USA	DNTP100-1KT	
DMEM	Gibco, USA	10569044	
DPBS buffer	Gibco, USA	14190-136	
EDTA	Invitrogen, USA	AM9260G	0.5 M
EDTA free protease inhibitor cocktail	Roche, USA	04693132001	
EndoFree Maxi Plasmid Kit	Vazyme, China	DC202	
FastPure Gel DNA Extraction Mini Kit	Vazyme, China	DC301-01	
FBS	Gibco, USA	10437028	

FLAG peptide	Sigma, USA	F4799	
Glycerol	Sigma, USA	SHBK3676	
GST Bulk Kit	GE Healthcare, USA	27-4570-01	
HEPES buffer	Sigma, USA	SLBZ2837	1 M
IPTG	Thermo Scientific, USA	34060	
KoAc	Sangon Biotech, China	127-08-02	
Lipofectamin 3000 Transfection Reagent	Thermo Scientific, USA	L3000001	
$MgCl_2$	Invitrogen, USA	AM9530G	1 M
NaCl	Invitrogen, USA	AM9759	5 M
NP-40	Amresco, USA	M158-500ML	
Opti-MEM medium	Gibco, USA	31985062	
PBS	Gibco, USA	10010023	PH 7.4
RNase Inhibitor	Promega, USA	N251B	
Streptavidin alkaline phosphatase	Promega, USA	V5591	
TBE	Invitrogen, USA	15581044	
Tris-HCI Buffer	Invitrogen, USA	15567027	1 M, PH 7.4
Tris-HCI Buffer	Invitrogen, USA	15568025	1 M, PH 8.0
Tween-20	Sangon Biotech, China	A600560	

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Institution: Nanjing Medical University

Article Title: Biotin-labeled In Vitro Biochemical Assays on Interaction of Protein with Nucleic Acids

Signature: ke zheng Date: Feb-13-2019

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Apr 09, 2019
Vineeta Bajaj, Ph.D.
Review Editor
JoVE
RE: JoVE59830

Dear editors,

Thanks a lot for your further editorial reviews. We have made essential edits accordingly. Here's my response.

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Response: We are very grateful to your modifications on the manuscript. We have retained the same.

2. Please address all the specific comments marked in the manuscript.

Response: We have addressed all the specific comments marked in the manuscript.

3. The manuscript needs thorough proofreading. Please employ professional copyediting services.

Response: We have asked a science editor to read our manuscript and made essential edits.

4. Once done, please ensure that the highlight is no more than 2.75 pages including the headings and spacings.

Response: We have highlighted about 2.75 page of the protocol for the video.