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Discovering CsgD regulatory targets in Salmonella biofilm cells using chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq)

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Editorial Team,
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Dear Editors,

We are submitting our revised manuscript (60736_R2), "**Discovering CsgD regulatory targets in *Salmonella* biofilm cells using chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq)**", for publication as a Video article in JoVE. In total, there are 9 Figures and 1 Table, in addition to the required "Materials" Table.

For this revision, we edited the Main text to better match the video and made the two requested changes to the video. We have responded to each of these comments in the "Response to Editor" file.

Hopefully this addresses all the required revisions and we look forward to publishing in JoVE. Please contact me if you need any more information.

Sincerely,

Aaron White

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TITLE:

Discovering CsgD Regulatory Targets in *Salmonella* Biofilm Cells Using Chromatin Immunoprecipitation and High-Throughput Sequencing (ChIP-seq)

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KEYWORDS:
Chromatin immunoprecipitation, ChIP-seq, next generation sequencing, biofilm, bacteria, Salmonella

SUMMARY:

Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) is a method used to establish interactions between transcription factors and the genomic sequences they control. This protocol outlines techniques for performing ChIP-seq with bacterial biofilms, using *Salmonella enterica* serovar Typhimurium bacterial biofilm as an example.

ABSTRACT:

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a technique that can be used to discover the regulatory targets of transcription factors, histone modifications, and other DNA-associated proteins. ChIP-seq data can also be used to find differential binding of transcription factors in different environmental conditions or cell types. Initially, ChIP was performed through hybridization on a microarray (ChIP-chip); however, ChIP-seq has become the preferred method through technological advancements, decreasing financial barriers to sequencing, and massive amounts of high-quality data output. Techniques of performing ChIP-seq with bacterial biofilms, a major source of persistent and chronic infections, are described in this protocol. ChIP-seq is performed on *Salmonella enterica* serovar Typhimurium biofilm and planktonic cells, targeting the master biofilm regulator, CsgD, to determine differential binding in the two cell types. Here, we demonstrate the appropriate amount of biofilm to harvest, normalizing to a planktonic control sample, homogenizing biofilm for cross-linker access, and performing routine ChIP-seq steps to obtain high quality sequencing results.

INTRODUCTION:

Bacterial biofilms, structural aggregates of cells embedded in a self-produced matrix, are resistant to desiccation, antibiotics, and host immune responses[1]. As such, they cause persistent and chronic infections and present unique challenges to researchers due to their solutions to survival and transmission. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has been found to establish phenotypically heterogeneous populations of biofilm aggregates that are resistant to desiccation and antibiotics, and planktonic cells that express virulence factors in pure culture when exposed to environmental stress (28 °C, limiting nutrients)[2]. These two cell types arise due to bistable expression of the master biofilm regulator, CsgD[3]. We have hypothesized that this may be a bet-hedging strategy for *Salmonella*, where the planktonic cells participate in short-term transmission and biofilm cells are able to persist long-term in the environment until an opportunity to infect a new host arises[2,4]. Many of the regulatory elements that control *csg* operon expression are well characterized[5]. However, apart from *adrA* and the *csg* operon[6], few regulatory targets of CsgD are known. Identifying the CsgD regulatory network would help us to better understand the Salmonella lifecycle and the role that biofilms play in transmission.

Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) is a powerful in vivo technique for genome-wide identification of protein-DNA interactions and provides information on regulatory processes involving transcription factors, histone modifications, or nucleosomes[7]. Newer studies have used this technique to assess differential protein binding between growth conditions and cell types[8]. In chromatin immunoprecipitation (ChIP), DNA fragments with interacting proteins are enriched through antibody selection of the target proteins. Cells are harvested and DNA-binding proteins are crosslinked to DNA in vivo through formaldehyde cross-linker treatment. Cells are lysed, releasing the cell contents, and chromatin is sheared into approximately 200–600 bp fragments[7]. DNA fragments bound to the target protein are immunoprecipitated using antibody, and isolated by de-crosslinking followed by protein digestion[9]. These DNA fragments represent protein binding sites matched by hybridization to a microarray (ChIP-chip) or by deep sequencing and mapping to the genome sequence[10,11]. Although ChIP-chip experiments yield adequate regulatory data, they are limited due to the use of expensive probes, as well as hybridization and array bias[12]. ChIP-seq has a larger dynamic range with increased nucleotide resolution and coverage, improved signal-to-noise ratio, and fewer artifacts when compared to ChIP-chip[7].

ChIP has been used to analyze Sfh and OmpR regulation in *Salmonella* serovar Typhimurium[13,14], quorum-sensing AphA regulation in *Vibrio alginolyticus*[15], RpoS regulation in *Escherichia coli*[16], virulence regulator EspR regulation in *Mycobacterium tuberculosis*[17], potential diguanylate cyclases through AmrZ regulation in *Pseudomonas aeruginosa*[18], as well as VraSR regulation in *Staphylococcus aureus*[19]. The bacterial ChIP-seq experiments that have been described begin with growing cells in liquid media and harvesting in single cell form. However, analysis of biofilms and corresponding gene regulation represents a new set of challenges to generate a successful ChIP-seq protocol. In this protocol, ChIP-seq is used to find differential regulatory targets of CsgD, the *S.* Typhimurium master biofilm regulator in biofilm and planktonic cell types. This protocol describes techniques used to determine the appropriate amounts of biofilm for ChIP-seq, harvest biofilm from flask culture, normalize biofilm and single cell control samples, homogenize biofilm, and complete immunoprecipitation. This will be useful for studying the regulatory targets in bacterial biofilms and can be adapted for many species

PROTOCOL:

1. Flask culture cell growth

1.1. From frozen stocks, streak *Salmonella* serovar Typhimurium strains on LB agar (20 mL solid media in 100 mm Petri plate) with the appropriate antibiotic and incubate at 37 °C for 16–20 h to obtain isolated colonies.

1.2. Inoculate 5 mL LB broth containing the appropriate antibiotic in a 25 mm x 150 mm borosilicate culture tube with 1–3 colonies from streak plates from step 1.1. Incubate at 37 °C with shaking at 200 rpm for 7 h.

1.3. Measure OD_{600} of broth culture using a spectrophotometer. Dilute an aliquot of culture 1 in 4 in PBS in a 2 mL cuvette and measure the absorbance at 600 nm. We have found the most critical factor at this step to be the time of growth. The OD values are used to normalize the cultures to deliver the desired number of cells in step 1.4.

1.4. Add 1.0 OD_{600} volume equivalent of cell culture (i.e. ~10^9 cells) to an Erlenmeyer flask containing 100 mL of 1% tryptone with the appropriate antibiotic. Incubate at 28 °C with shaking at 200 rpm for 13 h.

Note: Biofilm cells will appear as aggregated flakes and single planktonic cells are in the cloudy media.

2. Collecting cell-free conditioned 1% tryptone

2.1. Collect flask culture for cell-free conditioned 1% tryptone. Pipette flask culture several times to mix before adding to a centrifuge tube.

2.2. Centrifuge at 12,000 x *g* for 10 min at 10 °C to pellet all cells.

2.3. Decant supernatant into a 0.2 µm filter unit, vacuum filter, and dispense into a new tube.

Note: Biofilm aggregates are adherent in conventional solutions (e.g. PBS) and will stick to the sides of tubes and pipette tips. Since it allows for easier manipulation, we use conditioned media (1% tryptone) to move biofilm initially and use warm PBS immediately before cross-linking (step 4.5) to remove any protein crosslinking substrates that could be present in the media.

3. Separating biofilm and planktonic cells from flask cultures[2]

3.1. Using a sterile 25 mL pipette, aliquot flask culture into 15 mL tubes. Centrifuge at slow speed (210 x *g*) for 2 min, to separate the two cell types.

Note: Biofilm cells should form a loose pellet at the bottom of the tube

3.2. Pipette the supernatant containing planktonic cells into two 40 mL centrifuge tubes for later (step 5). Remove all the remaining liquid, being careful not to disturb the pellet. Repeat until the cell types have been separated from all of the flask culture media.

4. Preparing biofilm aggregates

Note: Biofilm is harvested by wet weight to yield 25 µg of DNA, the recommended minimum starting material for ChIP. The Biofilm Conversion Factor (BCF) of *S.* Typhimurium is 1.73×10^8 CFU/mg[2]. Researchers preparing other biofilm types will need to empirically define the number of cells per unit weight of biofilm, which is used to find the weight of biofilm required for 25 µg of DNA starting material using this equation:

$$25\ \mu\text{g DNA}\ x\,\frac{1\text{ g}}{1\times 10^{6}\ \mu\text{g}}\times\frac{6.022\times 10^{23}\text{ bp}}{1\text{ mole}}\times\frac{1\text{ mole}}{650\text{ g}}\times\frac{1\text{ CFU}}{Genome\ size\ (\text{bp})}\times\frac{1}{BCF}=biofilm\ (\text{mg})$$

4.1. Weigh a 2 mL snap cap or screw cap tube accurately.

4.2. Resuspend the biofilm pellet in 1 mL of conditioned tryptone. Move the resuspended biofilm into a pre-weighed 2 mL snap cap or screw cap tube.

4.3. Centrifuge for 1 min at 11 000 x *g* at 20 °C

4.4. Carefully remove all supernatant from the tube and weigh the tube accurately. Subtract the tube weight from the weight of the tube with biofilm to find the weight of biofilm. It should be ±10% of the target biofilm weight.

4.5. Wash cells to remove remaining conditioned tryptone. Add 1 mL of warm PBS to the tube and vortex gently to resuspend the pellet. Centrifuge for 3 min at 8 000 x *g* to pellet biofilm and remove the supernatant. Add 1 mL of PBS to the tube and vortex to resuspend the pellet.

5. Preparing planktonic cells

5.1. Record the volume and the OD_{600} of the planktonic supernatant from slow speed centrifugation (step 3.2) using a spectrophotometer

5.2. Calculate the required volume for a final OD_{600} of 6.0:

$$Final\ volume\ (\text{mL}) = Volume\ planktonic\ supernatant\ (\text{mL}) \times \frac{Supernatant\ OD_{600}}{6.0\ OD_{600}}$$

5.3. Cool the centrifuge to 10 °C and sediment planktonic cells by centrifugation (10,000 x *g*, 10 min at 10 °C)

5.4. Remove the supernatant and resuspend the cell pellet in the final volume of PBS calculated in step 5.2.

5.5. Re-measure OD_{600} of the planktonic cells using a spectrophotometer and dispense the volume of 6.0 OD_{600} planktonic cells into a 2 mL snap cap or screw cap tube.

5.6. Bring volume to 1 mL with PBS.

6. Homogenizing cells

6.1. Add one sterilized 5 mm stainless steel bead to each of the tubes.

6.2. Homogenize using a mixer mill for 5 min at 30 Hz. Observe aggregate tubes to confirm that biofilm has been broken apart.

6.3. Transfer the homogenized cells to a new 1.5 mL tube, avoiding the metal bead. Bring the volume to 1 mL with PBS.

Note: Perform serial dilutions to enumerate input cells and check that the final cell number is close to the desired or predicted number.

7. Cross-linking of proteins to DNA

7.1. Dispense fresh formaldehyde into sample tubes to a final concentration of 1%. Incubate for 30 min at room temperature on a rotating wheel.

Caution: Formaldehyde is corrosive, a skin, eye, and respiratory irritant, and flammable. Dispense in a fume hood.

7.2. Add 1 M glycine to a final concentration of 125 mM to stop crosslinking. Incubate for 5 min at room temperature on a rotating wheel.

8. Washing cells to remove excess crosslinker

8.1. Sediment the cells at 8,000 x *g* for 3 min and remove the supernatant

8.2. Resuspend the pellet in 20 µL of 25x protease inhibitors and 500 µL of filter sterilized PBS.

8.3. Centrifuge tube for 3 min at 8000 x *g* and remove the supernatant.

9. Lysing cells

9.1. Resuspend the pellet in 600 µL Lysis buffer (refer to **Table 1**) and incubate on ice for 10 min.

9.2. Add 1.4 mL of IP dilution buffer (refer to **Table 1**) to a sterile 15 mL tube and move lysed cells to the 15 mL tube. Keep the tube on ice for 1.5–2 h, and vortex gently and occasionally.

Note: Some resistant cell material may remain in tubes. A long incubation period on ice with occasional vortexing will break apart material. The remainder will be broken up through sonication.

10. Fragmenting DNA by sonication

10.1. Place the 15 mL tube in a beaker of ice and place the 3 mm probe inside the tube.

10.2. Perform 5 sonication rounds of 30 s on at 20–40% of 400 W with 2 min cooling on ice between bursts.

10.3. Sediment precipitated material by centrifugation (8000 x *g*, 10 min at 4 °C). Transfer the supernatant to a new tube.

10.4. Optionally, perform decrosslinking at 65 °C for 30–60 min and digest RNA and protein at 45 °C for 30-60 min before running on a 2% agarose gel to check for proper size fragments.

Note: Immerse the probe into the cell lysate. Keep the solution on ice while sonicating and resting. Do not remove the tube while the sonicator probe is pulsing. The solution should appear cloudy pre-sonication and clear post-sonication.

11. Immunoprecipitating DNA-protein-antibody complexes

11.1. Preparation

11.1.1. Dispense one 1.35 mL aliquot for immunoprecipitation and one 200 µL aliquot as an input control. Store the input control at -80 °C until decrosslinking and digesting steps are performed.

11.2. Immunoprecipitation with primary antibody

11.2.1. Add 10 µg of purified protein-specific primary antibody to the 1.35 µL aliquot. Incubate overnight at 4 °C on a rotating wheel.

Note: The primary antibody can be a monoclonal antibody, high-quality polyclonal serum or commercial epitope antibody (i.e., anti-FLAG).

11.2.2. Add 50 µL of Protein G magnetic beads to the tubes from step 11.2.1 and incubate at 4 °C for 3 h on a rotating wheel.

11.2.3. Place IP wash buffer 1, IP wash buffer 2, and TE pH 8.0 (refer to **Table 1**) in an ice bucket. Warm elution buffer to 65 °C.

Note: Do not put solution containing elution buffer on ice.

11.2.4. Bind the Protein G magnetic beads to the side of tubes using a magnetic stand

11.2.5. Perform washes: wash 2x with 750 µL cold IP wash buffer 1, wash 1x with 750 µL cold IP wash buffer 2, and wash 2x with 750 µL cold TE at pH 8.0. Keep the tubes on the magnetic stand during washes.

11.2.6. Add 450 µL of IP Elution buffer (refer to **Table 1**) to each tube and incubate at 65 °C for 30 min with gentle vortexing every 5 min.

11.2.7. Bind beads to the side of tubes using a magnetic stand. Wait at least 2 min until the solution is clear.

11.2.8. Dispense the cleared supernatant to a new 1.5 mL tube, being careful to not disturb the magnetic bead pellet.

12. **Decrosslinking DNA-protein complexes and digesting co-purifying RNA and protein**

12.1. Add 2 µg RNase A and NaCl to a final concentration of 0.3 M to each tube.

12.2. Incubate at 65 °C, for ≥6 h, or overnight.

12.3. Complete protein digestion by adding 180 µg Proteinase K to each tube, then incubating at 45 °C for 3–5 h.

13. Library preparation and sequencing

13.1. Purify DNA using magnetic beads

Note: Magnetic beads are preferred for isolating the small amounts of DNA usually recovered during ChIP with bacterial cells.

13.2. Prepare libraries using a kit that is compatible with the selected sequencing platform.

13.3. Check library concentration with a fluorometer and a broad range dsDNA kit or a qRT-PCR library quantification kit.

Note: In our experience, fluorometer measurements may overestimate DNA concentration.

13.4. Pool libraries, accounting for adequate coverage for each library sample. For Illumina sequencing with *Salmonella*, we pooled 10–12 libraries. Add Tris-HCl pH 8.0 to a final concentration of 1 mM.

13.5. Sequence according to the selected platform’s specifications.

REPRESENTATIVE RESULTS:

Preparation of chromatin immunoprecipitation samples from bacterial biofilm involves several steps, as shown in **Figure 1**. These include growing biofilm in flask culture, collecting conditioned media, collecting an adequate amount of biofilm and planktonic cells as control, washing cells in PBS, homogenization, crosslinking proteins to DNA, lysing cells, fragmenting DNA by sonication, immunoprecipitating DNA with the appropriate antibodies and protein G beads, washing and eluting immunoprecipitated DNA, and purifying DNA for library preparation and sequencing.

S. Typhimurium cells grown in liquid culture under biofilm-inducing conditions undergo phenotype switching to form multicellular biofilm aggregates and planktonic cells. This population will contain approximately 40% biofilm aggregates, which express higher levels of diguanylate cyclases (DGCs) and biofilm regulators, and 60% planktonic cells, which express higher levels of virulence-associated genes[2,4] (**Figure 2A**). In this protocol, we describe a method to harvest both cell types to compare transcription sites through ChIP-seq; however, this protocol can be adapted for other types of biofilms formed by other bacterial species. Biofilm aggregates and planktonic cells are separated by slow speed centrifugation, which pellets biofilm and leaves planktonic cells in the supernatant[2] (**Figure 2B**). The cell types are then treated separately for subsequent steps in the protocol.

The recommended amount of DNA input for ChIP-seq is 10-25 µg[20]. In *S.* Typhimurium flask culture, 30 mg of biofilm yields approximately 25 µg DNA. Since biofilm aggregates have an abundance of proteinaceous extracellular material, we hypothesized that this would interfere with the efficiency of cross-linking. If we were simply to normalize the different cell types by cell number, treatment of planktonic cells may result in an unequal amount of crosslinked product presented for immunoprecipitation. A protein assay was performed to determine the equivalent amount of planktonic cell material to match 30 mg of biofilm (**Figure 3**). 6.0 OD_{600} of planktonic cells were harvested to match 30 mg biofilm.

Biofilm aggregates must first be broken apart to allow equal access of cross-linker to all cells. We found that the most uniform and high-throughput way to homogenize the cells was using a mixer mill and 5 mm stainless steel bead (**Figure 4A)**. Planktonic cells are treated the same way to reduce variables in the ChIP-seq experiment (**Figure 4B)**.

Once the DNA has been fragmented by sonication, crosslinked, and treated with RNase and Proteinase K, it can be visualized on a 2% agarose gel. Sonicated DNA is broken down into a collection of fragments that appear as a smear on the agarose gel. The average fragment size decreases with an increasing number of sonication bursts (**Figure 5**). Energy transfer will vary for different sonicator units, so a sonication assay is recommended to determine the number of sonication bursts required to fragment DNA to an average of less than 1 kb. For our ChIP-seq experiment, we chose 5 bursts.

ENCODE guidelines recommend a confirmation of antibody target-binding activity with an immunoblot[21]. In this case we measured our antibody's specificity and strength of binding by immunoblot on whole cell lysates prepared for ChIP (**Figure 6**). We confirmed that the antibody binds to CsgD-3xFLAG; anti-FLAG and anti-CsgD signals colocalize at the expected molecular weight (28 kDa) [22].

ChIP procedures often yield low concentrations of DNA. Therefore, a purification method that removes contaminants and retains a high proportion of the DNA was chosen. Magnetic bead purification was chosen over column-based purification because it retained low concentrations of input ChIP DNA better (**Figure 7**) and removed contaminants (data not shown).

Due to reduced starting amounts of DNA, library preparation of ChIP DNA may require diluted adapters and PCR enrichment. Prior to sequencing, libraries can be visualized by Bioanalyzer trace to confirm correct size distribution before and after PCR enrichment (**Figure 8**). Additionally, if there is a known regulatory target of the transcription factor, qPCR should be used to confirm enrichment of binding regions by comparing fold change (ΔΔCt) between known target and reference gene sequence abundance in immunoprecipitated and sonicated input DNA.

ChIP-sequencing data should be analyzed by assigning quality scores, trimming low quality bases, aligning forward and reverse reads (in paired end sequencing), assembling to a high-quality reference genome, finding peaks above background, and performing downstream analysis (**Figure 9A**). Downstream analysis should include visualization and annotation of peaks, consistency with biological replicate samples, and motif analysis, and could include differential binding analysis between conditions or cell types, and data integration with gene expression from known pathways. For ChIP-seq data such as ours, peaks should be identified as significantly above background (**Figure 9B,C**, red bars) with a *p* value determination, not simply by fold-change. In the final analysis, the mapped reads are visualized against the annotation of the reference genome (**Figure 9D)**. A poor ChIP-seq result would appear like input-seq without any significant peaks above background.

FIGURE AND TABLE LEGENDS:

Figure 1. Illustration of steps in ChIP-seq with bacterial biofilms. In the described procedure, *S.* Typhimurium cells are grown in 1% Tryptone flask culture at 28 °C with shaking (1), cell-free conditioned media is collected for handling biofilm cell types (2), which is used to collect an adequate amount of biofilm and planktonic cells (3). These cells are washed with PBS and homogenized to break apart biofilm (4). Proteins are crosslinked to DNA with a formaldehyde crosslinker, after which cells are lysed to release cell contents (5). DNA in the cell lysate is fragmented by sonication (6). DNA crosslinked to the target protein is selected through immunoprecipitation with an antibody that binds to the target protein and Protein G magnetic beads (7). Selected DNA is washed and eluted from Protein G magnetic beads (8) and purified for library preparation and sequencing (9).

Figure 2. In vitro flask model for studying *S.* Typhimurium biofilm development. (**A**) *S.* Typhimurium cells grown in 1% tryptone for 13 h at 28 °C undergo phenotype switching to form biofilm aggregates and planktonic cells in the liquid phase of the flask culture. (**B**) Biofilm aggregates and planktonic cells from the flask culture in (A) were separated through centrifugation at 210 x **g** for 2 min. The supernatant was harvested for planktonic cell preparations and the pellet was harvested for biofilm cell preparations.

Figure 3. Total protein concentrations for planktonic cells and biofilm cell samples harvested from *S.* Typhimurium flask culture. Colorimetric protein assays were performed and protein amounts relative to a BSA standard curve were measured at 750 nm. Protein content of lysed planktonic cell samples at 4.0, 6.0, and 8.0 OD_{600} were compared to 30 mg of biofilm aggregates.

Figure 4. Homogenization of cell samples from *S.* Typhimurium biofilm flask cultures. (**A**) Biofilm aggregates from flask culture resuspended in PBS **(left) were homogenized using a mixer mill at 30 Hz for 5 min (right**). (**B**) Planktonic cells from flask culture resuspended in PBS were processed by mixer mill at 30 Hz for 5 min to ensure consistency between cell type samples.

Figure 5. Sonication assay with pre-ChIP cell lysates. Biofilm aggregate and planktonic cell samples were separated, homogenized, crosslinked, and lysed. DNA-protein complexes were sonicated up to 8 times at 30 s on and 2 min off on ice to break DNA into smaller fragments. A portion of the sonicated lysate was separated on a 2% agarose gel.

Figure 6. Target-binding specificity of anti-FLAG antibody used in ChIP-seq. Antibody specificity was assessed by immunoblotting against whole cell lysates prepared from flask cultures of the *S.* Typhimurium strains as shown. Strain ΔcsgD + p3xFLAG/*csgD* and WT biofilm samples represent

biofilm aggregates harvested from flasks, whereas strain Δ*csgD* ± p3xFLAG and WT planktonic cells represent planktonic cells. Purified His-tagged CsgD was loaded as a control. Whole cell lysates were normalized so that 30 µg of total protein was analyzed in each lane. Numbers on the left refer to the size (in kDa) of the prestained molecular weight markers. Primary antibody used for the upper blot was rabbit-anti-FLAG polyclonal antibody (Sigma-Aldrich #F7425), whereas the lower blot was incubated with rabbit-anti-FLAG together with a CsgD-specific monoclonal antibody[2]. Goat anti-rabbit IgG (LiCor IRDye 680RD, 925-68071) and goat anti-mouse IgG (LiCor IRDye 800CW, 925-32210) were used as secondary antibodies. Fluorescent signals were visualized using the Odyssey CLx imaging system and Image Studio 4.0 software package (Li-Cor Biosciences). Representative images are shown.

Figure 7. Column- or magnetic bead-based purification strategies for small amounts of DNA resulting from ChIP-seq experiments. Samples of known quantities of DNA were purified using column-based kits or magnetic beads and the concentration of the eluate was measured post-purification. Magnetic beads showed better recovery at low DNA levels, similar to the low amounts of DNA typically encountered at the end of ChIP-seq protocol, but prior to NGS library preparation.

Figure 8. ChIP DNA preparations and subsequent libraries visualized by Bioanalyzer. (**A**) Average fragment size of genomic DNA after cell lysis and sonication was <1000 bp, with a majority of fragments in the 200–500 bp range. (**B**) Starting material for library preparation was below detection. (**C**) Adapter ligation and PCR enrichment allows for amplification of library fragments. (**D**) A poor library preparation has low DNA peaks, odd shaped or high molecular weight peaks, or abundant adapter peaks at approximately 100 bp.

Figure 9. ChIP-seq data is analyzed using bioinformatic tools and visualized on a genome browser. A. Flowchart for typical bioinformatic analysis of ChIP-seq data. Raw reads for biofilm (Δ*csgD* strain + p3xFLAG/*csgD*) and planktonic cells (Δ*csgD* + p3xFLAG) were cleaned for low-quality reads and adapters, and mapped to *S*. Typhimurium 14028s genomes (NC_016856.1 for the chromosome and NC_016855.1 for the plasmid) by Bowtie2 (v2.3.3.1) with default parameters[23]. MACS2 (v2.1.2) was used to call the peaks with parameters of '-q 0.01 -- nomodel', taking biofilm replicates as testing datasets and planktonic ones as control[24]. The MACS2 bdgcmp module was further used to generate fold-enrichment track with parameters of '-m FE'. The significant peak file with fold-enrichment track was transformed to a wig file using bedtools/bedClip/bedGraphToBigWig[25] and visualized on the reference genome using Integrative Genomics Viewer v2.5.1[26]. Representative peaks are shown (red bars). (**B**) Large region of ~40 kbp, which contains multiple peaks, is an atypical result but still potentially valuable. (**C**) This is a more typical result, showing two significant peak regions. (**D**) Zoom in on left peak in C, showing reads mapping to the region of the *S.* Typhimurium 14028s genome containing divergent promoters for *uspA* and *uspB*.

DISCUSSION:

This protocol outlines a technique for performing ChIP-seq with *Salmonella enterica* serovar Typhimurium biofilm and planktonic cells from pure culture, to find regulatory targets of the master biofilm regulator, CsgD. We expect differential binding information due to the bi-stability of CsgD in the two cell types, with high levels in biofilm aggregates and low levels in planktonic cells[2,3]. However, this technique can also be applied to other bacterial transcription factors, cell types, or growth conditions. In particular, this technique can be adapted to other bacterial biofilms using an experimentally determined conversion factor for CFU per unit weight of biofilm cells.

ChIP-seq can be prone to amplification of technical error through sequencing; however, confirmation at critical steps can prevent this[27]. Primary antibody specificity is important for selecting only the target transcription factor and the DNA it controls during immunoprecipitation[7]. In this experiment, *csgD* was fused to a plasmid-encoded 3xFLAG tag at the 3' end of the gene, corresponding to the C-terminus of CsgD[22]. The p3xFLAG plasmid without csgD was used as a "control" (*S.* Typhimurium 14028 ΔcsgD + p3xFLAG). ENCODE guidelines recommend performing immunoblots with primary antibody against the protein of interest, and lysates of ChIP strains and deletion strains[21]. It is important to ensure that growth conditions are consistent across replicates to reduce variability in transcription factor binding and downstream sequencing results. If one is careful to ensure inoculum sizes and pre-inoculum growth conditions are consistent, biofilm growth in flask culture is consistent[4]. It is important to confirm that all biofilm has been broken apart after homogenization, to ensure equal access of reagents, particularly formaldehyde cross-linker, to all cells.

Several modifications may enable researchers to use this protocol for other DNA-binding proteins, cell types, strains, growth conditions, or lab equipment. If it is used for biofilm-forming species other than *S.* Typhimurium, the conversion factor of colony forming units per unit weight of biofilm needs to be determined experimentally by harvesting different amounts of biofilm, homogenizing the biofilm thoroughly, and performing serial dilutions and plate counting to create a standard curve, which may not be accurate at lower biofilm weights[2]. Sonicator energy transfer and cell type resistance may differ; therefore, a sonication assay is recommended to find the number of sonication bursts that will produce the fragment size range required for downstream library preparation and sequencing[11]. Chromatin fragmentation by micrococcal nuclease is an alternative to sonication that produces high resolution of occupancy sites; however, this method may introduce fragmentation bias[7,28]. The DNA size range may be altered depending on downstream library preparation kits and sequencing platforms. Other methods of homogenization may be used in lieu of the mixer mill. For example, a glass tissue homogenizer may be substituted, but it may aerosolize or incompletely break up biofilm. In terms of controls, pre-immunoprecipitate DNA can be normalized in post-sequencing processing and analysis. A mock-IP control with species-matched normal antibody serum can be used as a control as well[13].

Researchers must consider the limitations to this method when considering a ChIP-seq experiment. Significant modifications may be required to adapt it for other biofilm types. For example, with *Salmonella* Typhimurium immediate resuspension of biofilm in PBS leads to measurable loss of biofilm aggregates. Immunoprecipitation targeting regulatory targets of transcription factors in bacteria may result in very small amounts of DNA, which is a challenge for purification and detection at later steps. Bias can be introduced during shearing and downstream manipulation during library detection[9]. In addition, this method does not reveal in vivo expression levels in response to transcription factor binding. Researchers who have little experience in bioinformatics may be challenged by the analysis pipeline. This method can be time-intensive and expensive; however, the cost of sequencing is often lower than a microarray and is decreasing over time as technological improvements continue to be made.

Few methods in the literature describe ChIP with bacteria, and most bacterial experiments begin with a simple growth condition[13-15,17,18]. ChIP sample preparation with single cells is straightforward and presents fewer challenges than ChIP sample preparation with biofilm aggregates. As for ChIP-seq, previous methods of studying cis-regulatory circuitry, including systematic evolution of ligands by exponential enrichment (SELEX), electrophoretic mobility shift assays (EMSA), ChIP-chip, promoter deletion and reporter analysis have been complemented or replaced by ChIP-seq[29]. ChIP-seq is replacing ChIP-chip due to advancing technologies and availability of sequencing. Deep sequencing provides researchers with massive amounts of data that can identify sites with lower binding affinity and higher base pair resolution with less starting material than ChIP-chip[11,30]. Analysis of ChIP data from organisms with high quality reference genomes is generally fast and specific. Additionally, ChIP-seq is a thorough method for discovering in silico predicted binding sites that may not be occupied in all cell types, growth phase, or environmental conditions[31].

In the future, this protocol can be used to facilitate the understanding of bacterial pathogenesis, particularly biofilm-forming organisms. Broad adoption of this technique and availability of new datasets can lead to data integration to identify groups of proteins that co-localize to control cellular processes in biofilm-forming microorganisms[32]. In addition, ChIP-seq and RNA-seq data can be integrated to build regulatory system models[33].

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DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1. Costerton, J. W., Stewart, P. S., Greenberg, E. P. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science.* **284** (5418), 1318–1322, doi:10.1126/science.284.5418.1318 (1999).
2. MacKenzie, K. D. et al. Bistable Expression of CsgD in Salmonella enterica Serovar Typhimurium Connects Virulence to Persistence. *Infection and Immunity*. **83** (6), 2312–2326, doi:10.1128/IAI.00137-15 (2015).
3. Grantcharova, N., Peters, V., Monteiro, C., Zakikhany, K., Römling, U. Bistable expression of CsgD in biofilm development of Salmonella enterica serovar typhimurium. *Journal of Bacteriology.* **192** (2), 456–466, doi:10.1128/JB.01826-08 (2010).
4. MacKenzie, K. D., Palmer, M. B., Köster, W. L., White, A. P. Examining the Link between Biofilm Formation and the Ability of Pathogenic Salmonella Strains to Colonize Multiple Host Species. *Frontiers in Veterinary Science.* **4**, 307, doi:10.3389/fvets.2017.00138 (2017).
5. Steenackers, H., Hermans, K., Vanderleyden, J. Salmonella biofilms: an overview on occurrence, structure, regulation and eradication. *Food Research International.* **45** (2), 502–531, doi:10.1016/j.foodres.2011.01.038 (2012).
6. Brombacher, E., Dorel, C., Zehnder, A. J. B., Landini, P. The curli biosynthesis regulator CsgD co-ordinates the expression of both positive and negative determinants for biofilm formation in Escherichia coli. *Microbiology.* **149** (Pt 10), 2847–2857, doi:10.1099/mic.0.26306-0 (2003).
7. Park, P. J. ChIP-seq: advantages and challenges of a maturing technology. *Nature Reviews Genetics.* **10** (10), 669–680, doi:10.1038/nrg2641 (2009).
8. Wu, D.-Y., Bittencourt, D., Stallcup, M. R., Siegmund, K. D. Identifying differential transcription factor binding in ChIP-seq. *Frontiers in Genetics.* **6**, 169, doi:10.3389/fgene.2015.00169 (2015).
9. Valouev, A. et al. Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. *Nature Methods.* **5** (9), 829–834, doi:10.1038/nmeth.1246 (2008).
10. Lieb, J. D. Genome-wide mapping of protein-DNA interactions by chromatin immunoprecipitation and DNA microarray hybridization. *Methods in Molecular Biology (Clifton, N.J.).* **224**, 99–109, doi:10.1385/1-59259-364-X:99 (2003).
11. Robertson, G. et al. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nature Methods.* **4** (8), 651–657, doi:10.1038/nmeth1068 (2007).
12. Kim, T. H. et al. A high-resolution map of active promoters in the human genome. *Nature.* **436** (7052), 876–880, doi:10.1038/nature03877 (2005).
13. Dillon, S. C. et al. Genome-wide analysis of the H-NS and Sfh regulatory networks in Salmonella Typhimurium identifies a plasmid-encoded transcription silencing mechanism. *Molecular Microbiology.* **76** (5), 1250–1265, doi:10.1111/j.1365-2958.2010.07173.x (2010).
14. Perkins, T. T. et al. ChIP-seq and transcriptome analysis of the OmpR regulon of Salmonella enterica serovars Typhi and Typhimurium reveals accessory genes implicated in host colonization. *Molecular Microbiology.* **87** (3), 526–538, doi:10.1111/mmi.12111 (2013).

15. Gu, D., Liu, H., Yang, Z., Zhang, Y., Wang, Q. Chromatin Immunoprecipitation Sequencing Technology Reveals Global Regulatory Roles of Low-Cell-Density Quorum-Sensing Regulator AphA in the Pathogen Vibrio alginolyticus. *Journal of Bacteriology.* **198** (21), 2985–2999, doi:10.1128/JB.00520-16 (2016).
16. Peano, C. et al. Characterization of the Escherichia coli σ(S) core regulon by Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis. *Scientific Reports.* **5** (1), 10469, doi:10.1038/srep10469 (2015).
17. Blasco, B. et al. Virulence regulator EspR of Mycobacterium tuberculosis is a nucleoid-associated protein. *PLoS Pathogens.* **8** (3), e1002621, doi:10.1371/journal.ppat.1002621 (2012).
18. Jones, C. J. et al. ChIP-Seq and RNA-Seq reveal an AmrZ-mediated mechanism for cyclic di-GMP synthesis and biofilm development by Pseudomonas aeruginosa. *PLoS pathogens* **10** (3), e1003984, doi:10.1371/journal.ppat.1003984 (2014).
19. Sengupta, M., Jain, V., Wilkinson, B. J., Jayaswal, R. K. Chromatin immunoprecipitation identifies genes under direct VraSR regulation in Staphylococcus aureus. *Canadian Journal of Microbiology* **58** (6), 703–708, doi:10.1139/w2012-043 (2012).
20. Gill, A. Cross-linking chromatin immunoprecipitation (X-ChIP) protocol. at <http://docs.abcam.com/pdf/protocols/X-ChIP_protocol.pdf> (2017).
21. Landt, S. G. et al. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Research.* **22** (9), 1813–1831, doi:10.1101/gr.136184.111 (2012).
22. MacKenzie, K. D. et al. Parallel evolution leading to impaired biofilm formation in invasive Salmonella strains. *PLoS Genetics.* **15** (6), e1008233, doi:10.1371/journal.pgen.1008233 (2019).
23. Langmead, B., Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nature Methods.* **9** (4), 357–359, doi:10.1038/nmeth.1923 (2012).
24. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biology.* **9** (9), R137–9, doi:10.1186/gb-2008-9-9-r137 (2008).
25. Quinlan, A. R., Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics.* **26** (6), 841–842, doi:10.1093/bioinformatics/btq033 (2010).
26. Thorvaldsdóttir, H., Robinson, J. T., Mesirov, J. P. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Briefings in Bioinformatics.* **14** (2), 178–192, doi:10.1093/bib/bbs017 (2013).
27. Schoppee Bortz, P. D., Wamhoff, B. R. Chromatin immunoprecipitation (ChIP): revisiting the efficacy of sample preparation, sonication, quantification of sheared DNA, and analysis via PCR. *PloS One.* **6** (10), e26015, doi:10.1371/journal.pone.0026015 (2011).
28. Tolstorukov, M. Y., Kharchenko, P. V., Goldman, J. A., Kingston, R. E., Park, P. J. Comparative analysis of H2A.Z nucleosome organization in the human and yeast genomes. *Genome Research.* **19** (6), 967–977, doi:10.1101/gr.084830.108 (2009).
29. Geertz, M., Maerkl, S. J. Experimental strategies for studying transcription factor-DNA binding specificities. *Briefings in Functional Genomics* **9** (5-6), 362–373, doi:10.1093/bfgp/elq023 (2010).
30. Mardis, E. R. ChIP-seq: welcome to the new frontier. *Nature Methods* **4** (8), 613–614,

doi:10.1038/nmeth0807-613 (2007).

31. Lee, T. I. et al. Transcriptional regulatory networks in Saccharomyces cerevisiae. *Science.* **298** (5594), 799–804, doi:10.1126/science.1075090 (2002).

32. Wade, J. T., Struhl, K., Busby, S. J. W., Grainger, D. C. Genomic analysis of protein-DNA interactions in bacteria: insights into transcription and chromosome organization. *Molecular Microbiology.* **65** (1), 21–26, doi:10.1111/j.1365-2958.2007.05781.x (2007).

33. Myers, K. S., Park, D. M., Beauchene, N. A., Kiley, P. J. Defining bacterial regulons using ChIP-seq. *Methods (San Diego, Calif.).* **86**, 80–88, doi:10.1016/j.ymeth.2015.05.022 (2015).

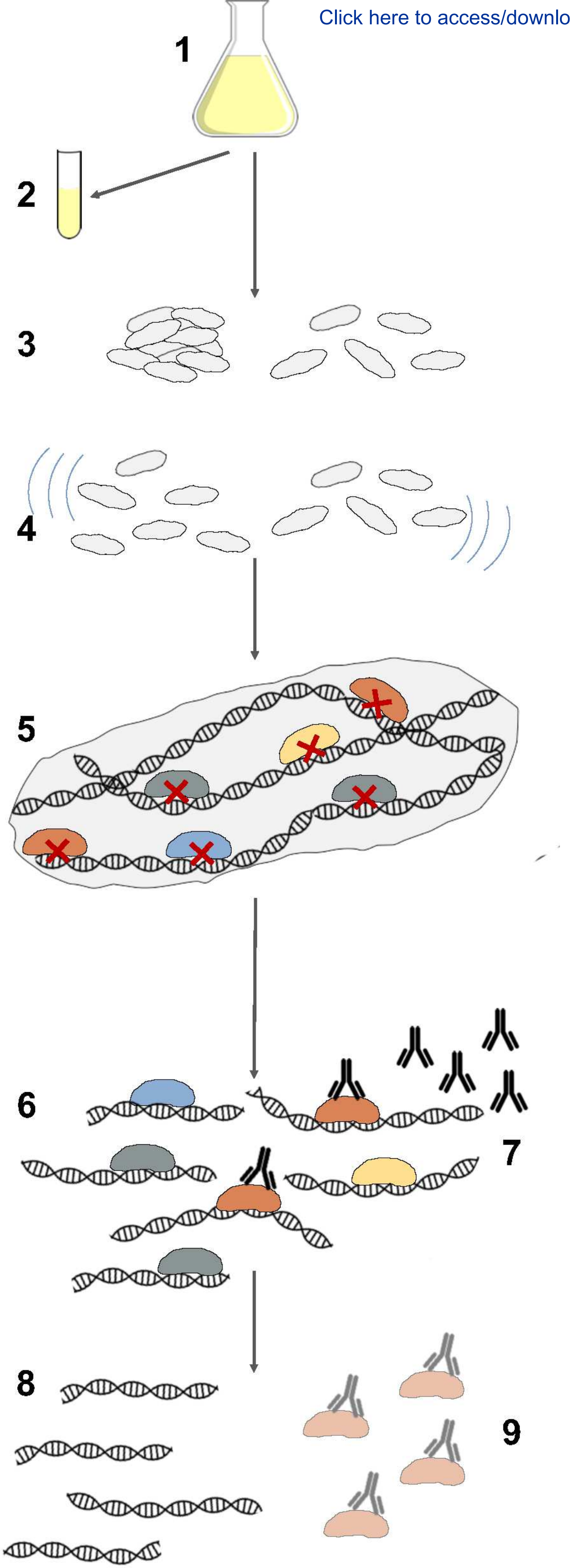
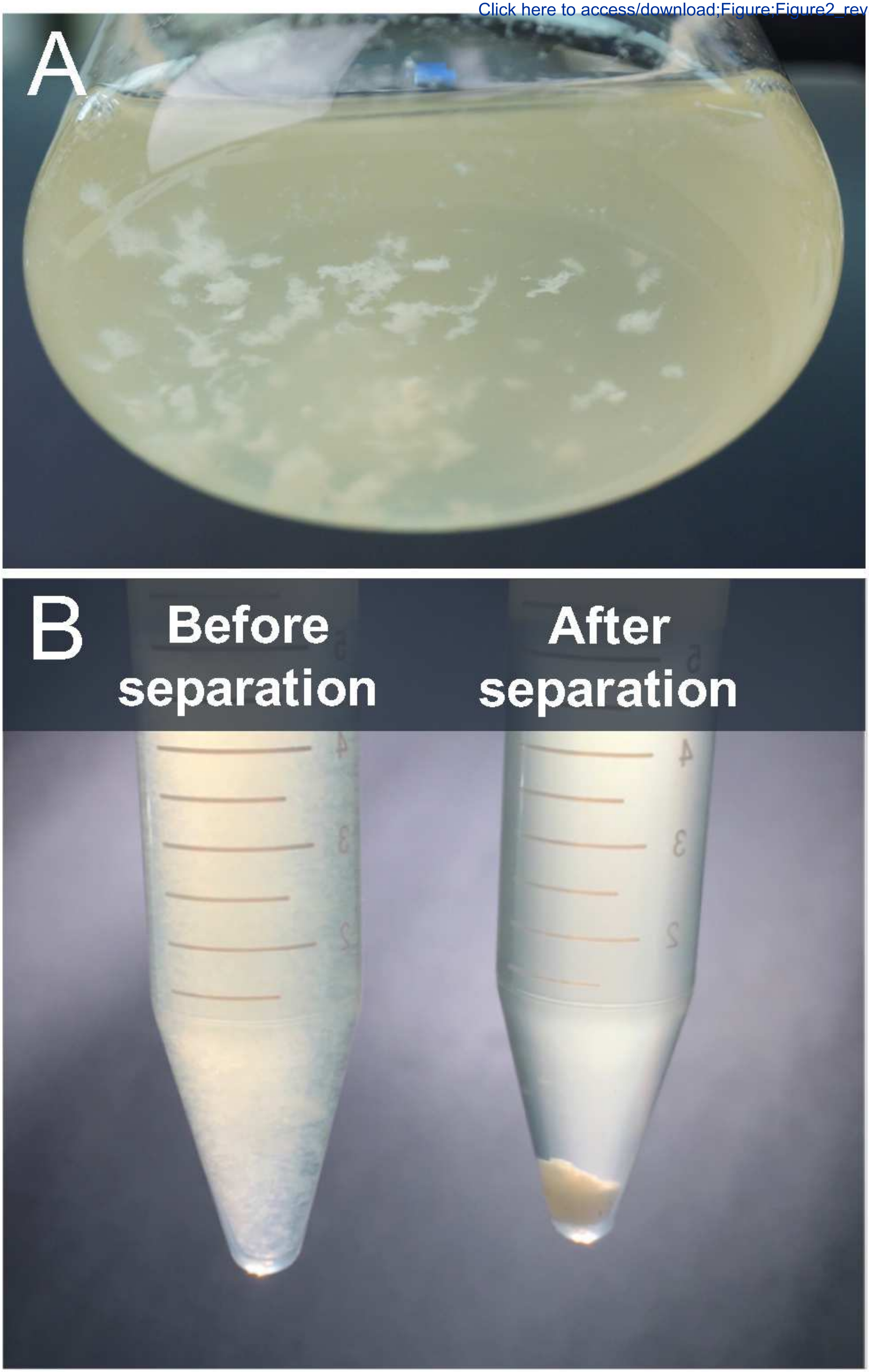

1
2
3
4
5
6
7
8
9

Figure 2



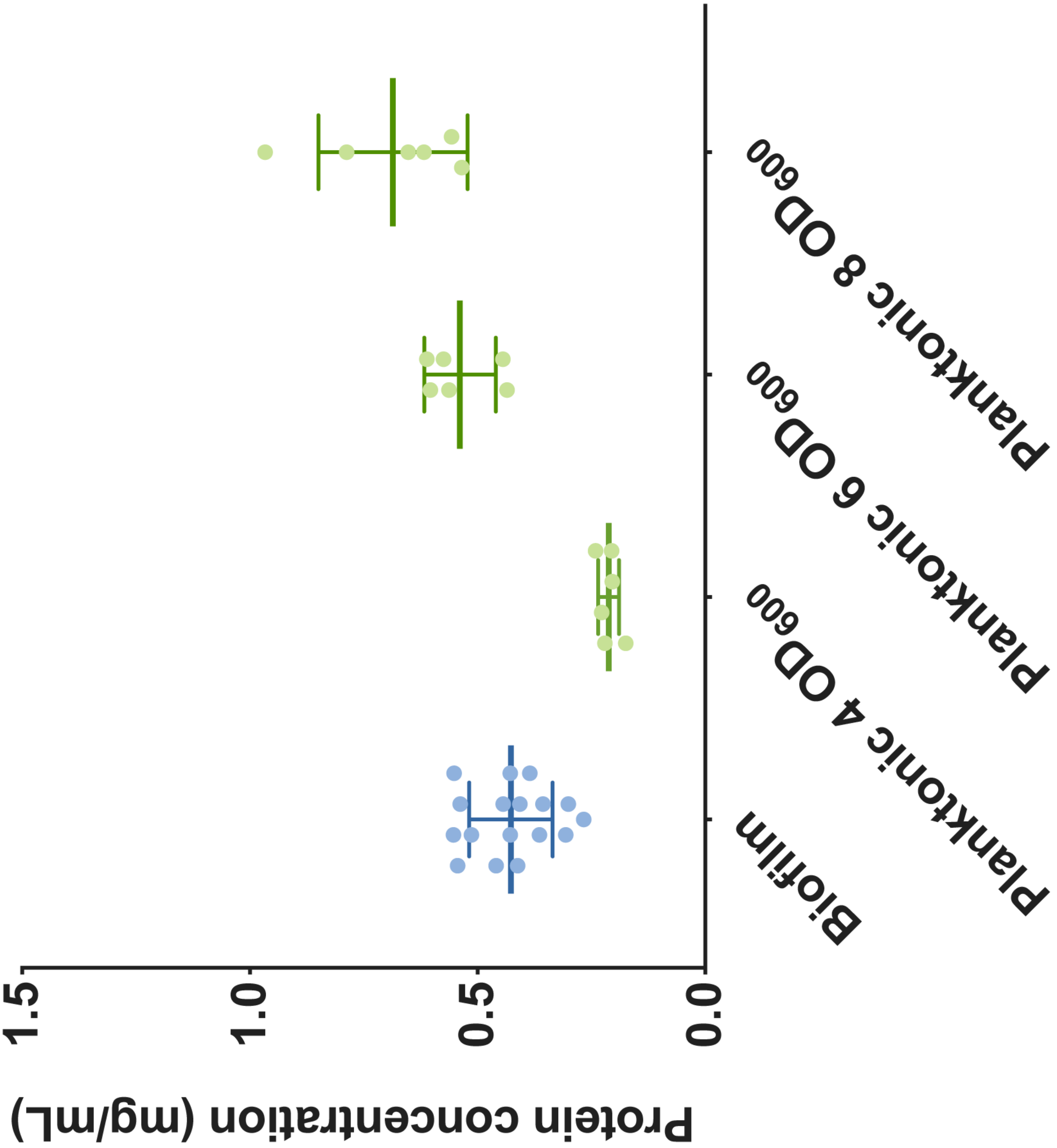

Protein concentration (mg/mL)
1.5
1.0
0.5
0.0
Biofilm
Planktonic 4 OD_{600}
Planktonic 6 OD_{600}
Planktonic 8 OD_{600}

Figure 4

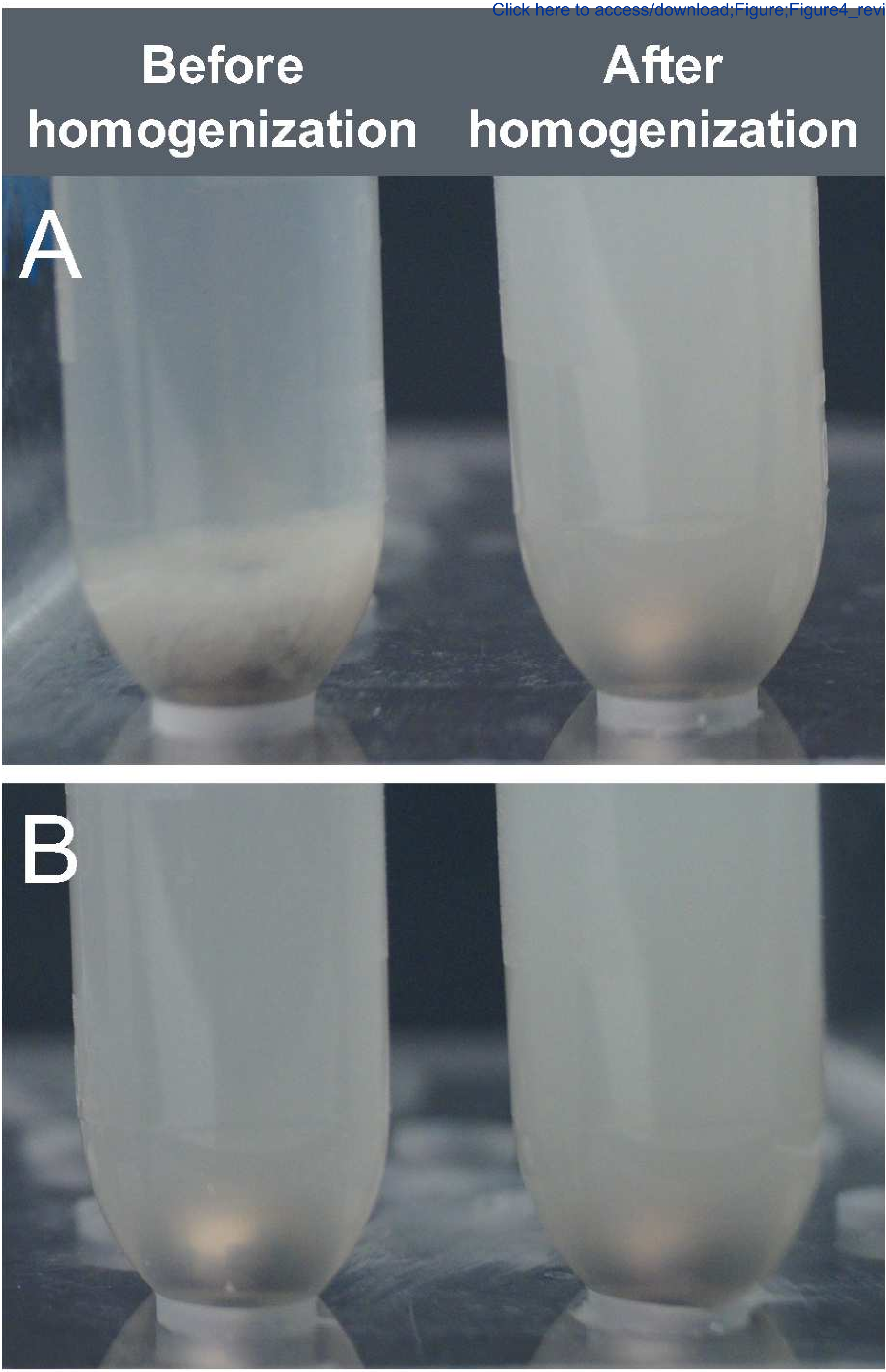


Figure 5

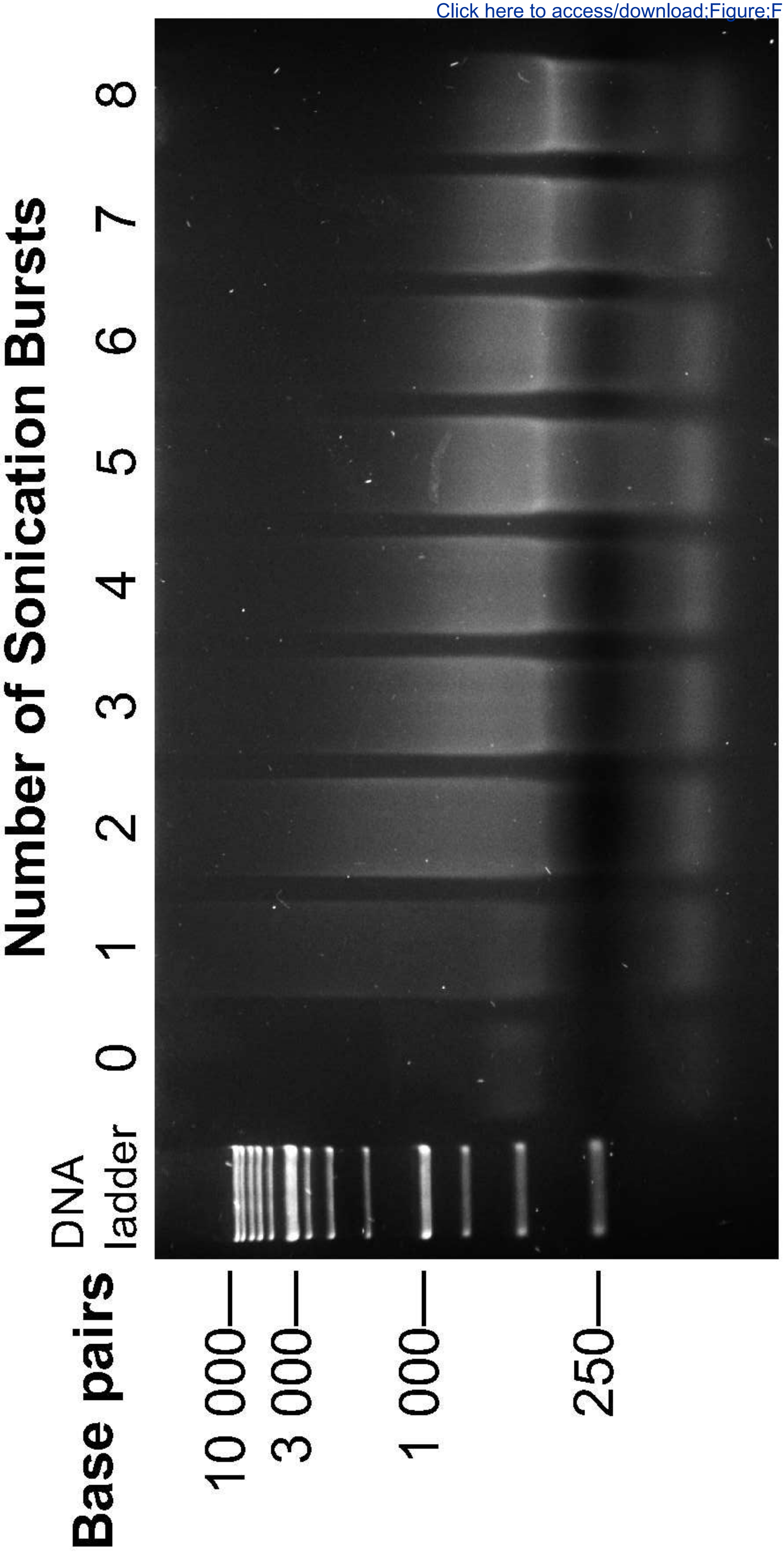

Number of Sonication Bursts
DNA ladder
0
1
2
3
4
5
6
7
8
Base pairs
10 000
3 000
1 000
250

Figure 6

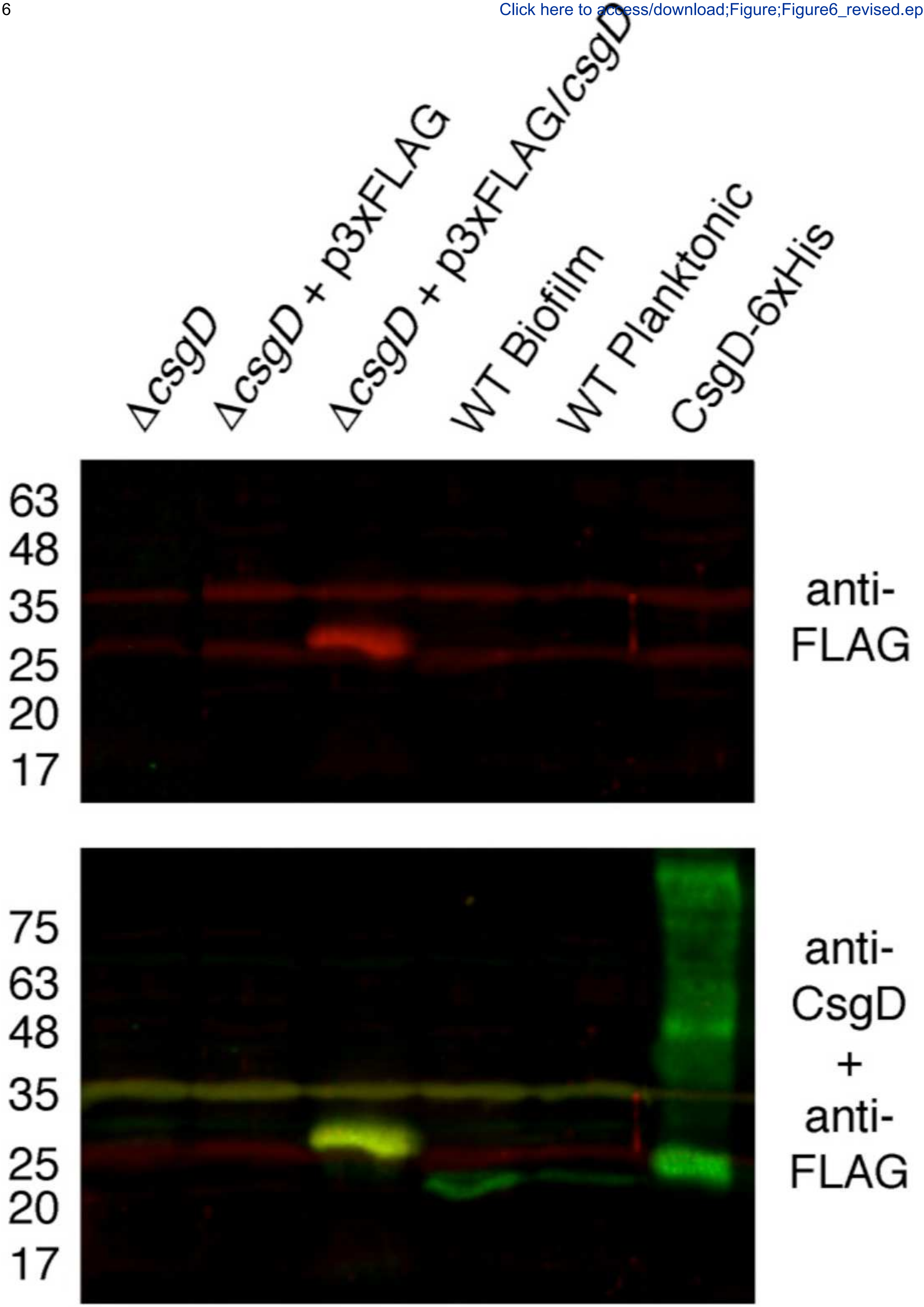

ΔcsgD
ΔcsgD + p3xFLAG
ΔcsgD + p3xFLAG/csgD
WT Biofilm
WT Planktonic
CsgD-6xHis
63
48
35
25
20
17
anti-FLAG
75
63
48
35
25
20
17
anti-CsgD + anti-FLAG

Figure 7

Click here to access/download;Figure;Figure7_revised.eps

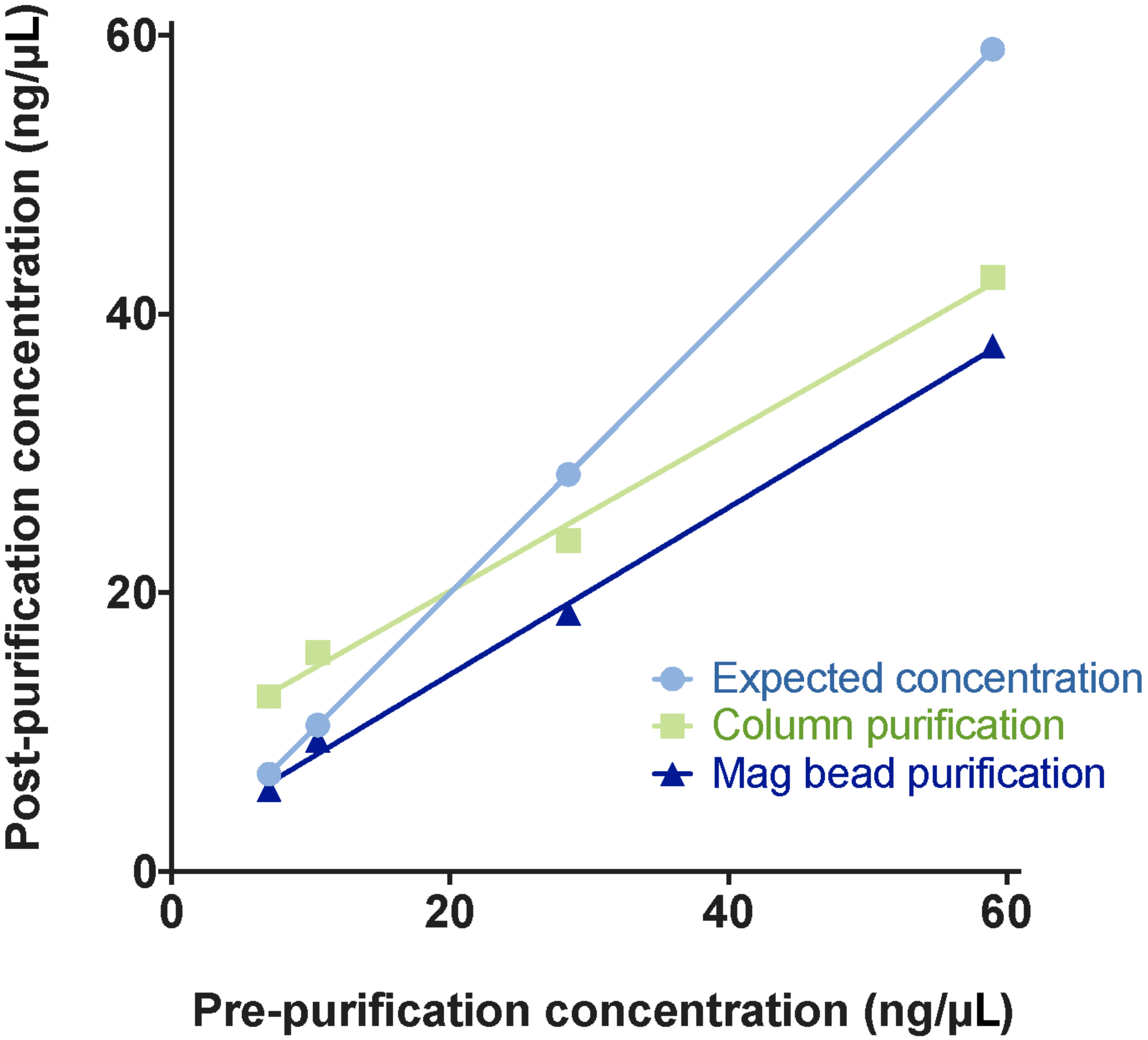


Figure 8

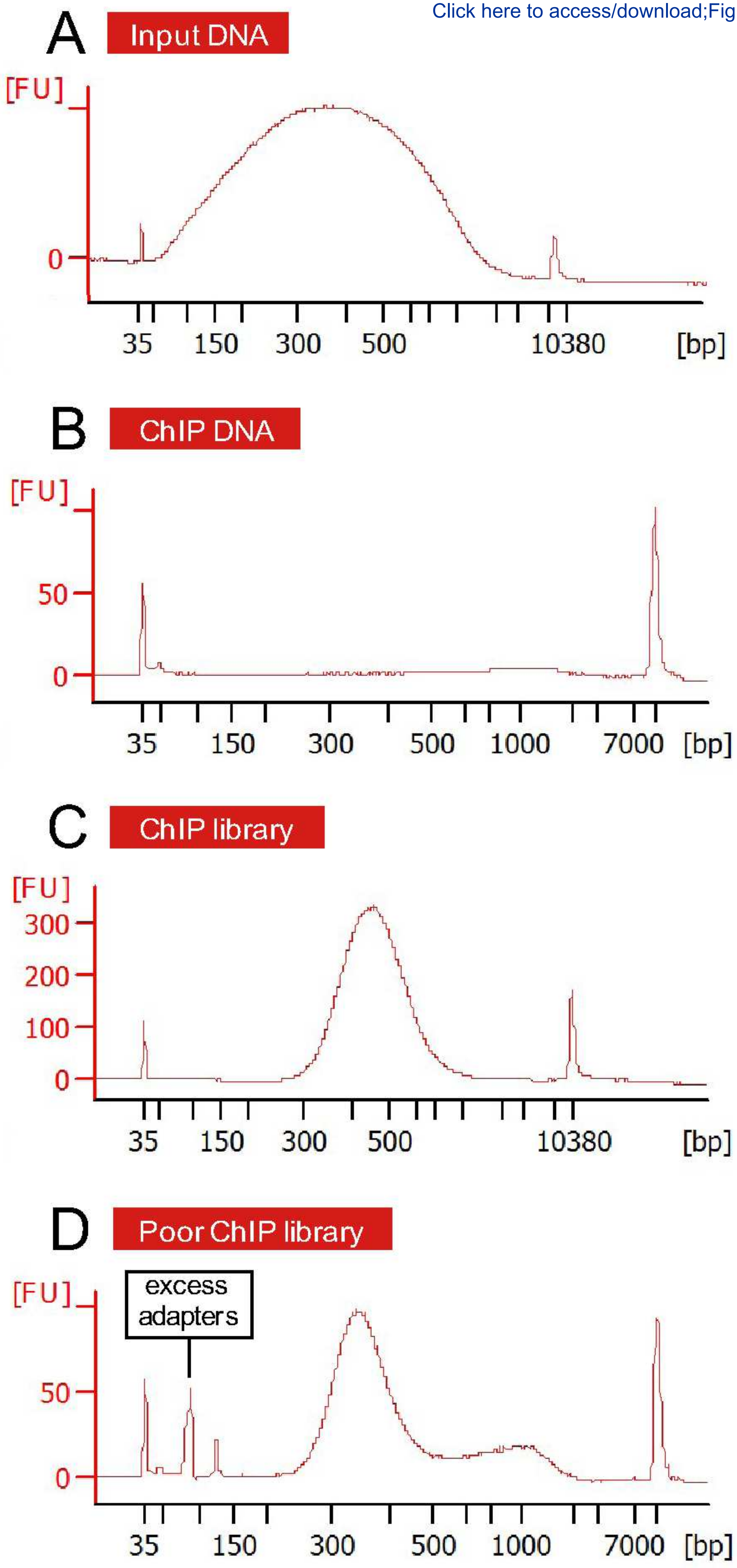

A
Input DNA
[FU]
0
35
150
300
500
10380
[bp]
B
ChIP DNA
[FU]
50
0
35
150
300
500
1000
7000
[bp]
C
ChIP library
[FU]
300
200
100
0
35
150
300
500
10380
[bp]
D
Poor ChIP library
excess adapters
[FU]
50
0
35
150
300
500
1000
7000
[bp]

Figure 9

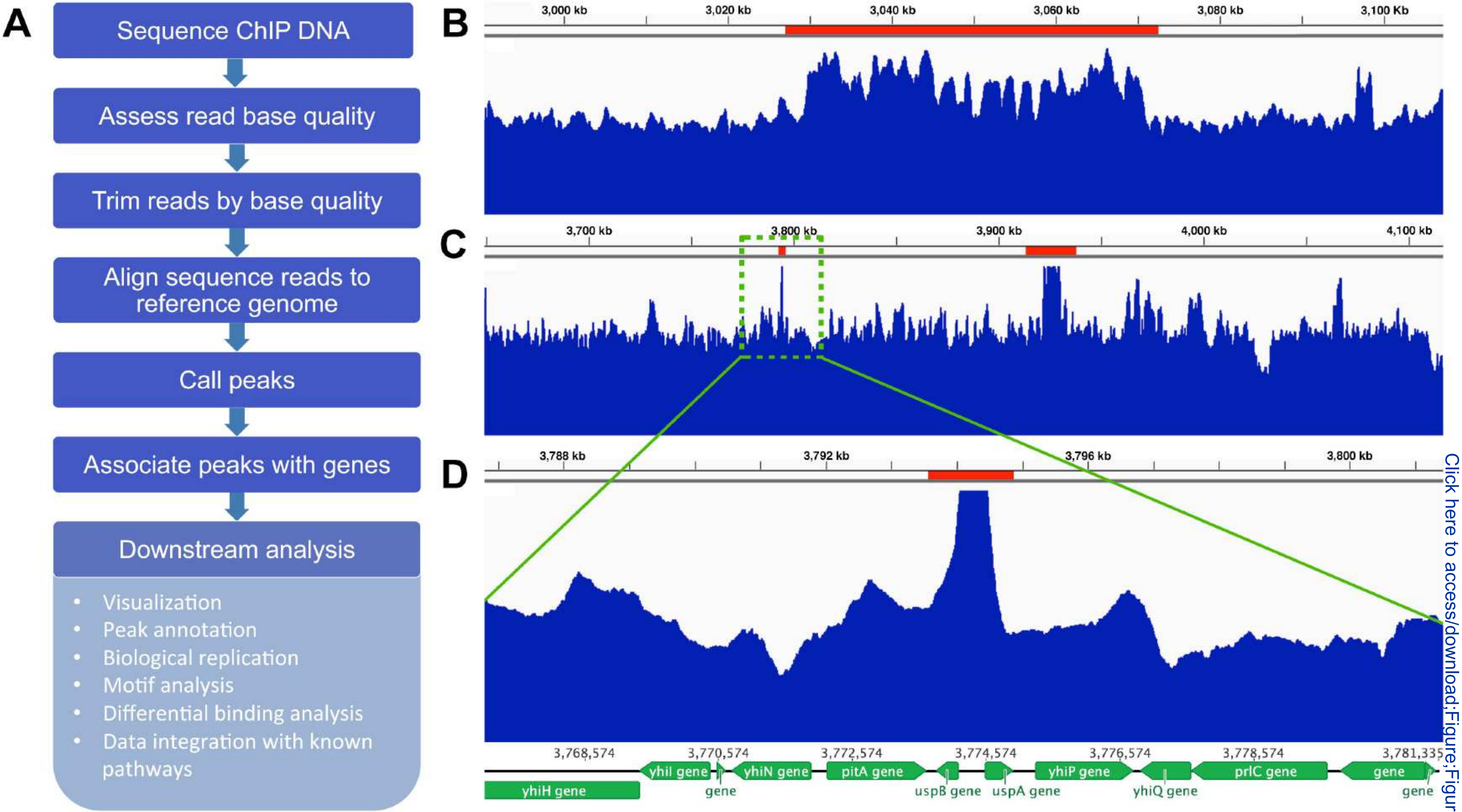

A
Sequence ChIP DNA
Assess read base quality
Trim reads by base quality
Align sequence reads to reference genome
Call peaks
Associate peaks with genes
Downstream analysis
Visualization
Peak annotation
Biological replication
Motif analysis
Differential binding analysis
Data integration with known pathways
B
3,000 kb
3,020 kb
3,040 kb
3,060 kb
3,080 kb
3,100 Kb
C
3,700 kb
3,800 kb
3,900 kb
4,000 kb
4,100 kb
D
3,788 kb
3,792 kb
3,796 kb
3,800 kb
3,768,574
3,770,574
3,772,574
3,774,574
3,776,574
3,778,574
3,781,335
yhiH gene
yhiI gene
gene
yhiN gene
pitA gene
uspB gene
uspA gene
yhiP gene
yhiQ gene
prlC gene
gene
gene

Table 1

Cell lysis buffers

Lysis buffer
50 mM Tris-HCl pH 8.1
10 mM EDTA
1% SDS
protease inhibitors

IP dilution buffer
20 mM Tris-HCl pH 8.1
2 mM EDTA
150 mM NaCl
1% Triton X-100
0.01% SDS

Immunoprecipitation buffers

IP wash buffer 1
20 mM Tris-HCl pH 8.1
2 mM EDTA
50 mM NaCl
1% Triton X-100
0.1% SDS

IP wash buffer 2
10 mM Tris-HCl pH 8.1
250 mM LiCl
1 mM EDTA
1% NP-40
1% deoxycholic acid

TE ($T_{10}E_1$) pH 8.0
10 mM Tris-HCl
1 mM EDTA

Elution buffer
1% SDS
100 mM NaHCO3

Name of Material/Equipment	Company	Catalog Number
Agarose powder	FroggaBio	A87-500G
Balance (Explorer pro)	Ohaus	EP214
Benchtop Centrifuge (8510R)	Eppendorf	022627023
Bioanalyzer 2100	Agilent	G2939BA
BR dsDNA kit	ThermoFisher Scientific	Q32850
ChIP-Grade Protein G Magnetic Beads	Cell Signaling Technology	9006
cOmplete, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	COEDTAF-RO ROCHE
Conical tube 15 mL	FroggaBio	TB15-500
Conical tube 50 mL	FroggaBio	TB50-500
DC Protein Assay Kit II	BioRad	5000112
Disposable Cuvette, 1.5 mL	Fisher Scientific	14-955-127
Electrophoresis equipment (mini-PROTEAN)	Bio-Rad	1658000
Floor centrifuge (Sorvall Evolution RC)	Thermo Scientific	728211
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific	Q33216
Formaldehyde	Sigma-Aldrich	252549
GelRed® Nucleic Acid Gel Stain 10 000x in water	Biotium	41003
High Sensitivity DNA kit	Agilent	5067-4626
Incubator (shaking)	VWR	1570-ZZMFG
Incubator (Water bath shaking)	New Brunswick	G76D
LB media	VWR	90000-808
Magnetic stand (DynaMag)	Fisher Scientific	12321D
Microcentrifuge (refrigerated)	Eppendorf	5415R
Microcentrifuge Tubes, 1.5mL	Fisher Scientific	05-408-129
MiSeq Reagent Kit v3 (150 cycle)	Illumina	MS-102-3001
Mixer mill	Retsch	MM400
Nalgene 115 mL Filter Units, Sterile, 0.2 µm pore size	Thermo Scientific	73520-980
NEBNext Ultra DNA Library Prep Kit for Illumina	New England BioLabs	E7370S
NGS Cleanup and Size Select magnetic beads	Machery-Nagel	744970.5
Normal mouse serum	AbCam	ab188776
Petri Dishes with Clear Lid (100 mm x 15 mm)	Fisher Scientific	FB0875712
Pipette controller	FroggaBio	MP001

Proteinase K	ThermoFisher Scientific	AM2542
QIAquick PCR Purification Kit	Qiagen	28104
Rabbit Anti-FLAG Polyclonal Antibody	Sigma-Aldrich	F7425
RNase A (10 mg/mL; DNase and protease-free)	ThermoFisher Scientific	EN0531
Rotating wheel	Crystal Technologies & Industries	TR 01A
Safe-Lock Tubes (2.0 mL, colorless)	Eppendorf	22363344
Serological pipette 25 mL	FroggaBio	94024
Spectrophotometer	Montreal Biotech Inc.	Libra S22
Stainless Steel Beads, 5 mm	Qiagen	69989
Tapered microtip 1/8" (3mm) Sonicator probe	Sonics & Materials, Inc.	630-0418
Tryptone media	VWR	90000-286
Ultrasonic Liquid Processor (Sonicator)	Sonics & Materials, Inc.	VC300
Vacuum equipment (Vacufuge plus)	Eppendorf	022820001
Water bath 1 (Lab-line aquabath)	Thermo Scientific	18000-1
Water bath 2 (Precision)	Thermo Scientific	51221044

JoVE manuscript # 60736_R2
Author Responses are listed in BLUE below each Editor Comment
Line numbers refer to the numbers in the "Clean Copy"

Editorial comments:
1. 3:00-3:20/note after Protocol section 2: There seems to be more information in the video narration (regarding cross-linking, etc.) than in the note; please clarify.
We have edited the section in the manuscript that corresponded to this part in the video. The proper protocol details were already included, but we had not spelled out certain aspects. We think it is better explained now. Lines 123-127

Line 153 – we added the word "warm" to match what was said in the video

2. 12:05 - For consistency with our other videos, this title card should read "Results" or "Representative Results".
We have added the title card "Representative Results" at 12:05 of the video

3. 12:38 - Again, for consistency with our other videos, the "Conclusions" title card should go here.
We have added the title card "Conclusions" at 12:38, where there was previously no heading displayed