Proximal Biotinylation By Episomal Recruitment (PROBER)

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Method Article

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Abstract

Precise identification of proteins binding to DNA-elements has been challenging due to high noise in LC-MS/MS runs. Here we present Proximal Biotinylation by Episomal Recruitment or PROBER, a tool to identify sequence-specific DNA-bound proteins from live cells that uses high-copy episomes to amplify signal-to-noise ratio (SNR). PROBER detects association of transcription factors (TFs) and corresponding chromatin regulators to short DNA sequences as well as differential protein binding at single-nucleotide variants (SNVs), thereby facilitating study of binding quantitative trait loci (bQTLs) and regulatory mutations that alter TF recruitment. PROBER functions in a variety of cell types and primary cells, thus offering a readily adaptable approach to rapidly identify sequence-specific DNA-protein interactions.

Introduction

Despite the rapid advancement of high throughput sequencing techniques that detect genomic binding sites of a given protein, unbiased detection of proteins binding to a given DNA sequence remains challenging. In vitro methods such as DNA pulldown[1, 2] lacks sensitivity and has the limitation of not being able to capture information from living cells. In vivo chromatin isolation-based techniques[3-7] and proximity-dependent proteomic mapping techniques[8-10] suffer from lower signal to noise ratio because they address only two targets per cell. PROBER utilizes DNA targets embedded in high-copy episomes, thereby elevating the functional number of targets more than 500 copies per cell.

PROBER is a two-component system that includes a BASU[11] promiscuous biotin sprayer and a cis-acting DNA bait. The sprayer comprises of Gal4 DNA-binding domain fused to BASU at its N-terminal, which is constitutively expressed from a small plasmid (pSprayer). The fusion protein also features a HA-tag and two nuclear localization signal (NLS). The DNA sequence of interest is cloned between three tandem repeats of drosophila upstream regulatory elements (UAS) on another small plasmid (pBait). A third plasmid expresses the SV40 large T-antigen that enables high-copy episomal replication of pBait, pSprayer, and pDriver plasmids through their SV40 origin of replication. All plasmids are simultaneously transfected in cells leading to recruitment of the BASU-Gal4 at UAS elements in close proximity of the DNA sequence of interest, which selectively biotinylates DNA-bound proteins when the media is supplemented with biotin. Use of high copy episomes allows PROBER to exceedingly elevate signal-to-noise ratio by increasing the functional number of DNA targets >500 copies per cell, which can be easily detected by western blot (WB) or LC-MS/MS.

Reagents

Reagents

Plasmid DNA: pBait with target sequence, pBait-YY1 (positive control), pBait-Scramble (negative control), pSprayer (for HEK293T cells), pSprayer-HE (for all cell types other than HEK293T), pDriver. Plasmids are available from Addgene.

Enzymes: *Sap*I (NEB), T4 DNA Ligase (NEB).

Poly-L-lysine (Sigma-Aldrich, P4707).

Gelatin, 2% solution, tissue culture grade (Sigma-Aldrich, G1393).

Cells: HEK293T.

Media: DMEM (Gibco), supplemented with 10% FBS and 1% penicillin–streptomycin.

Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific).

Lipofectamine 3000 (Thermo Fisher Scientific).

Biotin (Sigma-Aldrich, B4639).

Dulbecco's phosphate-buffered saline or DPBS (Gibco).

Tris-HCl, pH-7.5 (Invitrogen).

NaCl, 5M solution (Invitrogen).

UltraPure™ SDS, 10% solution (Invitrogen).

DTT (MP Bio).

cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche).

Dynabeads™ MyOne™ Streptavidin C1 (Thermo Fisher Scientific).

HEPES, 0.5 M solution, pH-7.5 (Alfa Aesar).

Sodium deoxycholate (Sigma-Aldrich, D6750).

Triton X-100 (Sigma-Aldrich, T8787).

UltraPure™ EDTA, 0.5 M solution, pH-8.0 (Invitrogen).

LiCl (Sigma-Aldrich, L9650).

NP-40 / IGEPAL® CA-630 (Sigma-Aldrich, I8896).

NuPAGE™ 4X LDS Sample Buffer (Novex).

4-12% gradient Bis-Tris gel (Novex).

Mouse anti-HA (Abcam, ab130275).

Rabbit anti-YY1 (Abcam, ab109228).

Buffers and Solutions

Lysis buffer: 50 mM Tris, pH-7.4, 500 mM NaCl, 0.2% SDS, 1 mM DTT, protease inhibitors.

Wash buffer 1: 2% SDS.

Wash buffer 2: 50 mM HEPES, pH-7.5, 0.1% Sodium deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA.

Wash buffer 3: 10 mM Tris, pH-7.5, 250 mM LiCl, 1 mM EDTA, 0.5% Sodium deoxycholate, 0.5% NP-40.

50 mM Tris, pH-7.5.

Elution buffer: 1X LDS sample buffer, 4 mM biotin, 20 mM DTT.

Coating buffer: 0.002% Poly-L-lysine, 0.08% gelatin in DPBS.

1 mM Biotin in FBS-free DMEM (for labeling).

10 mM Biotin in 50 mM phosphate buffer, pH-7.0 (for elution buffer).

Equipment

- Temperature controlled centrifuge.
- 3K Omega™ Microsep® centrifugal devices (Pall Corporation).
- Magnetic bead separator (DynaMag-2 or similar).
- KingFisher FLEX instrument (Thermo Fisher).
- Revolver™ Adjustable Rotator (The Lab Depot) or similar.
- Thermal mixer (Eppendorf ThermoMixer C or similar).

Procedure

Cloning target sequence in pBait plasmid

A. Purification and Digestion of pBait

1. Transfect pBait empty plasmid in a *ccdB* resistant *E. coli* strain, such as One Shot™ ccdB Survival™ 2 T1R Competent Cells (Invitrogen).

2. Miniprep pBait using QIAprep® Spin Miniprep Kit (Qiagen) or similar method.

3. Digest pBait with *Sap*I (NEB) restriction enzyme according to supplier's protocol.

4. Optional: gel purify the 4770 bp backbone using QIAquick® Gel Extraction Kit (Qiagen) or similar method.

B. Design and Preparation of Insert

PROBER use 3 concatemers of the target DNA or motif by default, but single copy, duplicate, and 4 or more concatemers can also be used with variable efficiency. PROBER signal will be fade for inserts size longer than than ~80 bp. Inserts <80 bp can be generated by annealing two complementary single stranded oligos, and adding complementary sticky ends to the ends. SapI digestion of pBait generates 5'GAG overhang within the upstream UAS site and 5'CGG overhang just ahead of the downstream UAS site, so add 5'CTCCG to the first oligo and 5'CCG/3'CG to the second complementary oligo to resore the UAS sites and generate stocky ends. This will place the insert immediately next to the nearest UAS sites on both ends. An example is provided below:

5' CTCCG---------- 3'

3' GC----------GCC 5'

C. Annealing oligos

1. Combine both oligos in the following 10 µl reaction: oligo 1 & 2 (20 uM each), 1X NEB restriction enzyme buffer (e.g., NEB CutSmart buffer), water.

2. Incubate the tube at 95^0C for 5 minutes in a thermal cycler or heatblock.

3. Slowly bring down temperature to RT by cooling down 0.1°C/sec in thermal cycler, or leave the tube at room temperature for 10 minutes.

4. Dilute the annealed oligos 50-fold with water.

5. Use 1-2 µl of the diluted annealed oligos directly in ligation reaction.

D. Ligation and transformation

1. Set up the following ligation reaction: SapI digested pBait (50-100 ng), annealed oligos (1-2 µl), 10X NEB T4 Ligase Buffer (2 µl), NEB T4 Ligase (1 µl), nuclease-free water (adjust to 20 µl final).

2. Incubate ligation reaction 10 minutes at RT, following transformation into *E. coli* DH5α or similar chemically competent cells and plating on LB plates containing 30 µg/mL kanamycin.

Cell culture and transfection

1. A night before transfection, plate HEK293T cells in 10 cm (for PROBER-WB) or 15 cm (for PROBER-MS) cell culture dishes with DMEM supplemented with 10% FBS and 1% penicillin–streptomycin (optional: plates can be coated with coating buffer containing Poly-L-lysine for better cell attachment).

2. Next day when cells are ~90% confluent, perform transfection (scale up 2X for 15 cm dishes).

a) Make transfection premix 1: 500 µl Opti-MEM, 32 µl P3000 enhancer, 10 ug pBait-YY1, 3 ug pSprayer, 3 ug pDriver, incubate 5 minutes in RT.

b) Make transfection premix 2: 500 µl Opti-MEM, 32 µl Lipofectamine L3000 reagent.

c) Combine transfection premixes 1 and 2, mix well.

d) Incubate at RT for 15-20 minutes.

e) Gently add 1 ml transfection mix to the plate containing cells, return plate to incubator.

3. Optional: Media chan be changed after 4-6 hours of transfection.

4. After 20 hours (at least 18 hours, at most 24 hours) of transfection, replace media with DMEM (with 10% FBS and 1% penicillin–streptomycin) containing 50 mM Biotin, return plates to incubator.

5. After 5 hours of adding biotin, remove plates from incubator.

6. Wash plates with 5 ml cold DPBS, aspirate DPBS.

7. Harvest cells with a cell scraper in 1 ml cold PBS and transfer cells to 1.7 ml centrifuge tube (cell dissociation reagents such as trypsin can be used without affecting PROBER results).

8. Spin down cells in 4^0C at 3000 rpm (~850 rcf), discard DPBS.

9. Proceed immediately with pulldown or freeze pellets in -80^0C for indefinite period.

Cell lysis (scale up 2X for 15 cm dishes)

1. Thaw cell pellets on ice if cells were frozen after harvesting.

2. Resuspend cells in 500 µl Lysis Buffer by pipetting up and down 10 times (note: the pellet may not resuspend fully).

3. Add 40 µl 25% Triton X-100, mix by brief vortexing.

4. Sonicate samples for 10-20 seconds at 10% amplitude in a Branson Digital Sonifier (or similar sonicator) using a 2.4 mm microtip, repeat post-cooling if lysate is not clear.

5. Spin lysate at maximum speed for 10 minutes at 4^oC to remove cell debris.

6. Transfer clear lysate to a fresh tube avoiding the slimy pellet.

7. Add 500 µl-1 ml lysis buffer (or 50 mM Tris, pH-7.5, for less stringent condition) to dilute lysate.

8. Load lysate to 3K Omega™ Microsep® centrifugal devices (or similar tubes), spin at 4^oC according to manufacturer's protocol until the volume reduces to ~500 µl.

9. Pipette 2-3 times and collect the lysate from top chamber.

10. Optional: perform protein assay using Pierce™ Reducing Agent Compatible BCA Protein Assay Kit (or similar reducing agent compatible reagent), normalize concentration across samples using lysis buffer.

11. Save 10-25 µl lysate for analysis by western blot.

Binding

1. Prepare Streptavidin MyOne C1 dynabeads (50 µl beads for <2 mg lysate, 100 µl beads for 2 mg and above).

a. Aliquot appropriate volume in a 1.7 ml microcentrifuge tube.

b. Magnetize beads using a DynaMag-2 magnetic bead-separator (or similar instrument), aspirate the supernatant.

c. Wash with 50 mM Tris, pH-7.5: remove tube from magnet and resuspend beads in 1 ml buffer by piperring up and down 10 times. Magnetize and aspirate buffer.

d. Wash 1-2 times with 1 ml lysis buffer following the same method, remove buffer.

e. Resuspend beads in initial volume of lysis buffer (i.e., 50 µl lysis buffer for 50 µl beads).

2. Add pre-washed beads to lysates 1.5 ml in Eppendorf Protein LoBind tubes (50 µl beads for <2 mg lysate, 100 µl beads for 2 mg and above).

3. Rotate for 2 hours at room temperature or overnight at 4^0C in a Revolver™ Adjustable Rotator (The Lab Depot) or similar instrument.

Washing

1. Remove tubes from rotator and manetize beads, remove the lysate

(optional: save the lysate as “unbound fraction” for western blot).

2. Wash beads with Wash Buffer 1: resuspend beads in 1 ml buffer and mix by pipetting up and down. Rotate tubes in rotator for 10 minutes at room temperature. Magnetize and and aspirate supernatant.

3. Repeat washing with 1 ml Wash Buffer 1 (rotate 10 minutes).

4. Wash with 1 ml Wash Buffer 2 (rotate 10 minutes), remove supernatant.

5. Wash with 1 ml Wash Buffer 3 (rotate 10 minutes), remove supernatant.

6. Wash with 1 ml 50 mM Tris, pH-7.5 (rotate 10 minutes) by pipetting up and down for 10 times.

7. Optional: repeat Tris wash twice more times (specially for on-bead mass spectrometry sample processing).

8. Remove buffer and spin tubes briefly at low speed (< 5000 rpm) to collect all liquid at the bottom.

9. Magnetize and remove all residual buffer using a P20 (2-20 µl) pipette tip.

Elution

1. Add 50 µl freshly prepared elution buffer to each tube.

2. Place tubes in an eppendorf thermomixer (or similar instrument) and shake at 95^{o}C at 1000 RPM for 15 minutes (place thermomixer lid or use tube caps to prevent tubes from popping).

3. Spin tubes briefly at low speed to collect all buffer at the bottom.

4. Magnetize tubes and transfer eluate to fresh tubes.

5. The eluate can be used for analysis immediately or stored at -20^{o}C for indefinite period.

KinFisher FLEX Protocol (after lysis step)

1. Load 1 ml Wash Buffer 1 (2 plates), Wash Buffer 2, Wash Buffer 3, and 50 mM Tris, pH-7.5 in 96-well Kingfisher FLEX deep well plates.

2. Load appropriate volume of lysate from lysis step in a 96-well Kingfisher FLEX deep well plate.

3. In the same plate, add approipriate volume of pre-washed beads to the lysates.

4. Load comb, tip plate, and all deep well plates in Kingfisher FLEX instrument.

5. Perform binding and washes using the following program:

a) Binding: 2 hours (Bottom mix - 30 sec, Half mix - 1 min, Slow - 18 min 30 sec, repeat 6 cycles).

b) Wash with Wash Buffer 1: Release beads, Half mix - 2 min, Medium - 8 min.

c) Wash with Wash Buffer 2: Release beads, Half mix - 2 min, Medium - 8 min.

d) Wash with Wash Buffer 3: Release beads, Half mix - 2 min, Medium - 8 min.

e) Wash with 50 mM Tris, pH-7.5: Release beads, Half mix - 2 min, Medium - 8 min (leave beads in Tris buffer).

6. Collect beads from Tris plate and perform elution as mentioned before.

Quality check by WB

1. Run 10 µl eluate alongside saved lysates as input (5 µl of 1/5 diluted lysate) and a molecular weight marker in a NuPAGE™ 4-12% Bis-Tris gel.

2. Transfer to nitrocellulose (or PVDF) membrane and probe with appropriate primary antibodiy together with anti-YY1 and anti-HA antibodies.

Troubleshooting

Low transfection efficiency

1. Check and optimize transfection efficiency using a control GFP plasmid (such as Amaxa pmaxGFP™ plasmid). Alternative transfection methods should be tested if low (<30%) transfection efficiency or high cell-death post transfection is observed. PROBER has been successfully performed using the following transfection methods:

· Lipofectamine 2000 (Thermo Fisher Scientific)

· FuGENE® 6 (Promega)

· FuGENE® HD (Promega)

· ViaFect™ (Promega)

· TransIT-X2® (Mirus)

· Polyethylenimine or PEI (Invitrogen)

· Cell Line Nucleofector™ Kits (Lonza)

2. Check the quality of the purified plasmid. Plasmid preps containing RNA, salt and other impurities result in decreased PROBER efficiency and increased cell death after transfection. Plasmid purified with Qiagen miniprep (QIAprep® Spin Miniprep) and maxiprep (HiSpeed® Plasmid Maxi, QIAGEN® Plasmid Plus Maxi) kits works well in HEK293T cells. Use of endonuclease-free plasmid prep is recommended for sensitive and primary cells (using Qiagen EndoFree® Plasmid Maxi or similar kits).

No or suboptimal YY1 enrichment at YY1 control

1. Check the ability of the anti-YY1 antibody used by running lysate on WB.

2. Check YY1 expression in your cell type by WB. If YY1 exporession is low, an alternative control (like NF-κB or c-Jun motif) can also be used.

3. Check BASU-Gal4 expression on WB by probing with anti-HA antibody. BASU-Gal4 is designed to express at low level to minimize noise and may not be visible in the lysate, but a clear band near 50 kDa should be visible in the pulldown. Optimize pBait : pSprayer : pDriver ratio if required. pSprayer-HE should be used for all cell types other than HEK293T.

4. Check biotinylation efficiency by running lysate and pulldown fractions in WB and probing with probe with IRDye® 680RD Streptavidin (Licor P/N: 926-68079) or similar. A strong smear should be visible in the pulldown fraction. Optimize the biotin labeling concentration and/or labeling time if required. PROBER has been successfully reproduced with overnight (16-18 hours) labeling, however, it may result in higher noise.

5. Check efficiency of the streptavidin beads used by running lysate, unbound fraction, and pulldown on WB and probing with probe with IRDye® 680RD Streptavidin. A good binding will result in >90% biotinylated proteins bound to the beads. If significant loss is observed, try to optimize binding condition and/or use more beads.

6. Check the cells for mycoplasma contamination. Suboptimal PROBER enrichment has been observed with mycoplasma infected and/or old cells.

High background (scramble control has high YY1 signal)

1. Check expression of BASU-Gal4 by WB of lysate and pulldown. A strong HA band in lysate indicates higher than normal expression, optimize pBait : pSprayer : pDriver ratio if required. Use of pSprayer-HE in HEK293T will also result in high background.

2. Optimize labeling time and biotin concentration. PROBER signal has been detected with labeling time as short as 30 minutes, however, it may result in weak enrichment.

3. Check biotin concentration of the media used. Some media contains high biotin, which may result in higher background. Use of an alternative biotin-free media post transfection is recommended in such cases. If an alternative biotin-free media is unavailable or not possible, excess biotin can can be removed by adding a biotin blocker, such as BioLock Biotin blocking solution (IBA Life Sciences, Cat. no. 2-0205-050) to the media post transfection.

Good YY1 enrichment on WB but no enrichment of target protein at test motif or sequence

1. Check expression of the target protein (if known) by WB.

2. Check the primary antibody for target protein by running lysate on WB. For PROBER-MS (mass spec) samples, its recommended to proceed for MS even when no WB enrichment of target protein (if any) is visible at the test sequence.

3. The target protein doesn't not bind to the test sequence, which can be orthogonally validated by EMSA, MST, ChIP-qPCR or other methods.

Time Taken

Anticipated Results

In a quality check western blot, a successful PROBER will result in minimal or no YY1 enrichment at scramble sequence, and a 50 to100-fold YY1 enrichment at YY1 motif over scramble. Fold cange is calculated by normalizing the YY1 bands with of BASU-Gal4 band, which is visualized by HA antibody at ~50 kDa. Note that BASU-Gal4 is optimized to express at very low level to minimize noise, thus it is below WB detectable range in lysates.

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