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1 Chapter 16. Chromatin pull-down methodology based on DNA triple helix formation

2

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4

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10

11 Running Head: Triplex-mediated nucleoprotein capture

12

13 Summary

14 Identification of the protein complexes associated with defined DNA sequence elements is essential to
15 understand the numerous transactions in which DNA is involved, such as replication, repair,
16 transcription, and chromatin dynamics. Here we describe two protocols, IDAP (Isolation of DNA
17 Associated Proteins) and CoIFI (Chromatin-of-Interest Fragment Isolation), that allow to isolate
18 DNA/protein complexes (i.e., nucleoprotein elements) by means of a DNA capture tool based on DNA
19 triple helix (triplex) formation. Typically, IDAP is used to capture proteins that bind to a given DNA
20 element-of-interest (e.g., a specific DNA sequence, an unusual DNA structure, a DNA lesion) that can
21 be introduced at will into plasmids. The plasmids are immobilized by means of a triplex forming probe
22 on magnetic beads and incubated in nuclear extracts; by using in parallel a control plasmid (that lacks
23 the DNA element-of-interest), proteins that preferentially bind to the DNA element-of-interest are
24 captured and identified by Mass Spectrometry. Similarly, CoIFI also uses a triplex forming probe to
25 capture a specific chromatin fragment from a cultured cell line that has been engineered to contain
26 multiple copies of the DNA element-of-interest.

27

1 Key words: DNA-based chromatin capture, Reverse-ChIP, Replication, Repair, Transcription,
2 Proteomics, Chromatin, Epigenetics, Drug screening

3

4 **1 Introduction**

5 Characterization of proteins involved in various DNA transactions is needed to understand functional
6 control of protein networks during all DNA transactions. For example, when taking into account
7 numerous studies, the control of transcription *in vivo* is best described as a complex protein network [1].
8 A straightforward way to delineate a protein network on DNA is to isolate and characterize nucleoprotein
9 fragments of interest from living cells. In these years, such approaches have been successfully applied
10 in various studies [2-6].

11 The formation of DNA triple helices has been well characterized and implemented in numerous
12 applications including therapeutic purposes [7, 8]. The principle of triplex formation is that a third strand
13 (i.e., Triplex Forming Oligonucleotide (TFO)) consisting of either a pyrimidine-rich or a purine-rich
14 sequence, forms Hoogsteen (or reverse Hoogsteen) base pairs with a complementary purine-rich strand
15 in the major groove of double-stranded DNA (dsDNA) without disrupting its canonical Watson-Crick
16 base pairs. In order to practically utilize triplex formation in applications, numerous efforts were
17 implemented to increase stability of triplexes. As a consequence, the stability of triplexes was largely
18 improved by using modified TFO probes composed of chemically altered nucleotides (e.g., peptide
19 nucleic acid (PNA), locked nucleic acid (LNA)) [9, 10] and by the introduction of a DNA intercalator
20 (e.g., acridine, psoralen) to one of the extremities of the probe [11-13].

21 By utilizing the unique feature of triplex formation, we developed a robust experimental
22 workflow in order to capture, identify and characterize proteins assembled, *in vivo* (CoIFI) or *in vitro*
23 (IDAP), on a given DNA sequence-of-interest [14]. Here, we introduce some applications of IDAP and
24 CoIFI as model cases.

25

26 **2 Materials**

27 All buffers are freshly prepared unless otherwise stated. When using H₂O, it is ultrapure water.

28

1 **2.1 TFO-conjugated plasmid for IDAP**

- 2 1. TE: 10 mM Tris-Cl (pH 7.5), 1 mM EDTA. Store at room temperature (RT).
3 2. TFO probes: Dissolve the probes in TE (Fig. 1) (*see Note 1*). Store at -20°C .
4 3. Plasmids: pAS114.1 γ and its linearized form in TE (Fig. 2) (*see Note 2*). Store at -20°C .
5 4. TF2: 10 mM Tris-Cl (7.5), 70 mM NaCl, 0.02% NP40.
6 5. A UVA lamp.

7
8 **2.2 Human nuclear extracts for IDAP**

- 9 1. Cells: Cultivate human HEK293 cells on plates.
10 2. CM-Lysis buffer: 10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.3 M Sucrose, 1%
11 NP40, 0.1 mM EDTA, Protease inhibitor cocktail.
12 3. SG buffer: 10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 1.5 M Sucrose, 0.1 mM
13 EDTA, Protease inhibitor cocktail.
14 4. NW buffer: 10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, Protease
15 inhibitor cocktail.
16 5. Extraction buffer: 20 mM HEPES (7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 1 mM DTT, 0.2 mM EDTA,
17 25% (v/v) Glycerol, Protease inhibitor cocktail.
18 6. Streptavidin magnetic beads (10 mg/ml).
19 7. A magnetic stand.
20 8. Liquid nitrogen.

21
22 **2.3 Nucleoprotein isolation via IDAP**

- 23 1. Streptavidin magnetic beads (10 mg/ml).
24 2. BSA.
25 3. NCL: 20 mM Tris-Cl (7.5), 4% glycerol, 8 mM DTT, 4 mM MgCl₂, 0.5 mM EDTA, 0.1% NP40.
26 4. NCL250: NCL with 250 mM NaCl.
27 5. NCL50: NCL with 50 mM NaCl.
28 6. 2 x PM2: 40 mM Tris-Cl (7.5), 8% glycerol, 16 mM DTT, 8 mM MgCl₂, 5 mM ATP, 0.04% NP40.

1 7. CB3: 15 mM Tris-Cl (7.9), 70 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% Sarkosyl, 0.2% SDS.

2

3 **2.4 IDAP under cross-linking conditions**

4 1. CLB1: 20 mM HEPES (7.9), 4 mM MgCl₂, 50 mM NaCl, 0.02% NP40.

5 2. Formaldehyde (HCHO): 1% HCHO in CLB1.

6 3. CQ3: 1.4 M Tris-Cl (7.4), 250 mM NaCl.

7 4. DTT.

8 5. 4 x LDS buffer: 0.8 M Triethanolamine-Cl (7.6), 4% Lithium dodecyl sulfate, 4% Ficoll 400, 0.025%

9 Phenol red, 0.025% Coomassie G250, 2 mM EDTA, 40% Glycerol.

10

11 **2.5 A specific DNA substrate for IDAP**

12 1. Plasmids: pAS200.2 and pAS203 in TE (*see Note 3*). Store at -20°C.

13 2. Nickases: Nb.BsrD1 and Nt.BspQ1.

14 3. Nick buffer: 50 mM Tris-Cl (7.9), 100 mM NaCl, 10 mM MgCl₂, 0.1 mg/ml BSA.

15 4. 6 x BPB buffer: 0.01% Bromophenol blue, 30% Glycerol, 1 mM EDTA.

16 5. A gel extraction kit.

17 6. Annealing buffer: 20 mM Tris-Cl (7.5), 250 mM NaCl.

18 7. A 12-mer oligo (*see Note 4*).

19 8. Ligase buffer: 50 mM Tris-Cl (7.5), 10 mM DTT, 10 mM MgCl₂, 1 mM ATP.

20 9. T4 DNA Ligase.

21

22 **2.6 Specific capture of the constructed plasmids in IDAP**

23 1. Plasmids: pAS200.2, pAS203, pAS203(rcc), pAS203(gap), pAS203(cSL) in TE (*see Note 5*).

24 2. Streptavidin magnetic beads (10 mg/ml).

25 3. TS1: 10 mM Tris-Cl (7.4), 250 mM NaCl, 0.5 mM EDTA, 0.1% NP40.

26

27 **2.7 Cross-linking conditions of a human cell line for CoIFI**

28 1. A human cell line, clone H62 (*see Note 6*).

- 1 2. An aspirator.
- 2 3. PBS: 3 mM Na₂HPO₄, 1 mM KH₂PO₄, 155 mM NaCl.
- 3 4. Formaldehyde (HCHO): 3% HCHO in PBS.
- 4 5. CQ2: 1.4 M Tris-Cl (7.4), 150 mM NaCl.

- 5 6. A cell scraper.

6

7 **2.8 Input sample preparation for CoIFI**

- 8 1. pre-Lysis buffer: 10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl₂, 1% Triton X-100, 0.3 M Sucrose.
- 9 2. Lysis2 buffer: 10 mM HEPES (7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.5
- 10 mM EGTA, 0.6% NP40, 0.25% Triton X-100, 0.3 M Sucrose, Protease inhibitor cocktail.
- 11 3. A dounce homogenizer with a tight pestle.
- 12 4. PBST: PBS with 0.25% Triton X-100.
- 13 5. RNaseA.
- 14 6. CB1.3: 15 mM Tris-Cl (7.9), 1 mM EDTA, 0.5 mM EGTA, 0.1% Sarkosyl, 0.2% SDS, Protease
- 15 inhibitor cocktail.
- 16 7. A sonicator.
- 17 8. Streptavidin polyacrylamide resin (50% slurry).

18

19 **2.9 Nucleoprotein isolation via CoIFI**

- 20 1. TFO probes: TFO-1, TFO-3, Scr-1, and Scr-2 (*see Note 1*). Store at -20°C.
- 21 2. CB3.1: 15 mM Tris-Cl (7.9), 50 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% Sarkosyl, 0.2%
- 22 SDS.
- 23 3. Streptavidin magnetic beads (10 mg/ml).
- 24 4. CB4: 15 mM Tris-Cl (7.9), 70 mM NaCl, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% Sarkosyl, 1% SDS.
- 25 5. DIT4: 10 mM Tris-Cl (7.4), 30 mM NaCl, 0.2% SDS.
- 26 6. ER2: 10 mM Tris-Cl (7.4), 250 mM NaCl, 0.5 mM EDTA, 0.2% SDS, 20 mM D-biotin.

27

28 **3 Methods**

1 All procedures are implemented at RT or in an incubator set at 25°C unless otherwise stated. When using
2 H₂O, it is ultrapure water.

3

4 **3.1 TFO-conjugated plasmid for IDAP**

- 5 1. 600 ng of plasmids (pAS114.1γ and its linearized form) are adjusted to 15 μl by TF2 containing 10
- 6 mM MgCl₂ and 3 pmol TFO-1.
- 7 2. Incubation with mixing for ≈17-18 hr.
- 8 3. Transfer the mixture onto a plastic support (*see Note 7*).
- 9 4. Irradiate the mixture with UVA (365 nm) for 0.2 J/cm² (Fig. 3) (*see Note 8*).
- 10 5. Store the sample at –20°C until use.

11

12 **3.2 Human nuclear extracts for IDAP**

- 13 1. Prepare 4 x 10⁷ cells of human HEK293 as a pellet.
- 14 2. Dissolve the pellet thoroughly with 400 μl of CM-Lysis buffer through vigorous pipetting.
- 15 3. Overlay each half of the suspension onto 1 ml of SG buffer in tubes (total two tubes).
- 16 4. Centrifugation at 13.4 kg for 1 min at 4°C, followed continuously by at 1 kg for 8 min at 4°C. Discard
- 17 supernatant.
- 18 5. Resuspend all pellets of the two tubes with 1 ml of NW buffer (total one tube).
- 19 6. Centrifugation at 13.4 kg for 30 sec at 4°C. Discard supernatant.
- 20 7. Mix gently the pellet with 20 μl of Extraction buffer.
- 21 8. Incubation on ice for 1 hr.
- 22 9. Centrifugation at 20.4 kg for 5 min at 4°C. Recover supernatant into a tube.
- 23 10. Rinse the pellet with 4 μl of Extraction buffer. Recover supernatant into the above tube.
- 24 11. The recovered supernatant is mixed with 10 μl of streptavidin magnetic beads (*see Note 9*).
- 25 12. Incubation with mixing for 10 min. Recover supernatant via a magnetic stand.
- 26 13. Repeat the **steps 11 and 12**.
- 27 14. Freeze the recovered supernatant by liquid nitrogen. Store the sample at –70°C until use.

28

1 **3.3 Nucleoprotein isolation via IDAP**

- 2 1. 40 ng of TFO-conjugated plasmids (pAS114.1 γ and its linearized form) prepared in **Subheading 3.1**
- 3 are mixed with 3 μ l of streptavidin magnetic beads (*see Note 10*). As a negative control, 40 ng of
- 4 pAS114.1 γ in absence of TFO are mixed with 3 μ l of streptavidin magnetic beads as well (*see Note 10*).
- 5 2. Incubation with mixing for 30 min. Discard supernatant via a magnetic stand (Fig. 3).
- 6 3. Wash the beads with 100 μ l of the following buffers: NCL250 x 3 times, then NCL50 x 2 times.
- 7 4. Resuspend the beads with 5 μ l of 2 x PM2 and 3.8 μ l of H₂O.
- 8 5. Add 1.2 μ l of the human nuclear extracts prepared in **Subheading 3.2**.
- 9 6. Incubation with mixing for 1 hr (*see Note 11*).
- 10 7. Discard supernatant via a magnetic stand.
- 11 8. Wash the beads with 100 μ l of NCL50 x 3 times.
- 12 9. Resuspend the beads with 10 μ l of CB3.
- 13 10. Incubation at 98°C for 1.5 min.
- 14 11. Transfer supernatant into a new tube via a magnetic stand.
- 15 12. Analyze the recovered supernatant (Fig. 4a).

16

17 **3.4 IDAP under cross-linking conditions**

- 18 1. Implement the same protocol until the **step 6** in **Subheading 3.3**.
- 19 2. Add 190 μ l of 1% HCHO in CLB1.
- 20 3. Incubation with mixing for 10 min.
- 21 4. Add 128 μ l of CQ3.
- 22 5. Incubation with mixing for 5 min. Discard supernatant via a magnetic stand.
- 23 6. Wash the beads with 100 μ l of NCL250 x 3 times.
- 24 7. Resuspend the beads with 6.5 μ l of CB3.
- 25 8. Add 1 μ l of 0.5 M DTT and 2.5 μ l of 4 x LDS buffer. Incubation at 99°C for 25 min.
- 26 9. Analyze the sample (Fig. 4b).

27

28 **3.5 A specific DNA substrate for IDAP**

- 1 1. Mix 18 µg of pAS203 with 180 units of Nb.BsrD1 and 100 units of Nt.BspQ1 in 360 µl of Nick buffer
- 2 (Fig. 5).
- 3 2. Incubation at 50°C for 2.5 hr.
- 4 3. Purify DNA by EtOH precipitation following phenol/CHCl₃ extraction.
- 5 4. Resuspend it with 180 µl of TE.
- 6 5. Incubation at 75°C for 10 min, followed by on ice for 10 min (*see Note 12*).
- 7 6. Add 36 µl of 6 x BPB buffer.
- 8 7. Separate a gapped pAS203 from a short DNA fragment (33-mer) detached from the region of CAG
- 9 triplet repeat through 0.7% agarose with 0.5 µg/ml ethidium bromide (EtBr) gel electrophoresis.
- 10 8. Cut out the separated gapped pAS203 from the agarose gel.
- 11 9. Extract DNA from the piece of agarose gel using a gel extraction kit. This extracted DNA is named
- 12 pAS203(gap) (Fig. 6a).
- 13 10. Mix 2 µg of pAS203(gap) with 10-fold relative molar excess of a 12-mer oligo to pAS203(gap) in
- 14 20 µl of annealing buffer (Fig. 6a).
- 15 11. Incubation at 75°C for 5 min, 65°C for 15 min, 45°C for 10 min, then keep it on bench for 1 hr (*see*
- 16 **Note 13**).
- 17 12. Add 2,000 units of T4 DNA ligase in 60 µl of ligase buffer. Incubate the mixture at 16°C for 2.5 hr.
- 18 13. Purify the ligated product by means of cut-out from an agarose gel as in **steps 6 to 9**. The extracted
- 19 DNA is named pAS203(cSL) (Fig. 6).
- 20 14. Analyze the sample (Fig. 7).

21

22 **3.6 Specific capture of the constructed plasmids in IDAP**

- 23 1. 50 ng of plasmids (pAS200.2, pAS203, pAS203(rcc), pAS203(gap), and pAS203(cSL)) are adjusted
- 24 to 15 µl by CB3 containing 10 mM MgCl₂ and 0.5 pmol TFO-1.
- 25 2. Incubation with mixing for ≈17-18 hr.
- 26 3. Mix with 5 µl of streptavidin magnetic beads (*see Note 14*).
- 27 4. Incubation with mixing for 1 hr. Discard supernatant via a magnetic stand.
- 28 5. Wash the beads with 50 µl of TS1 with 5 mM MgCl₂ x 3 times.

- 1 6. Resuspend the beads with 10 μ l of CB3.
- 2 7. Incubation at 98°C for 1.5 min.
- 3 8. Transfer supernatant into a new tube via a magnetic stand.
- 4 9. Analyze the recovered supernatant (Fig. 8).

5

6 **3.7 Cross-linking conditions of a human cell line for CoIFI**

- 7 1. Cultivate a human cell line, clone H62, until $\approx 7 \times 10^7$ cells on plastic dishes in order to prepare input
- 8 samples for CoIFI approach (Fig. 9).
- 9 2. Discard cell culture media in the dishes by an aspirator.
- 10 3. Cross-link the cells by 5 ml of 3% HCHO in PBS per dish for 30 min with mixing.
- 11 4. Add 5 ml of CQ2 per dish.
- 12 5. Incubation with mixing for 5 min.
- 13 6. Detach the attached cells on dishes by a cell scraper, and collect the cells from all dishes into a tube.
- 14 7. Centrifugation at 1 kg for 2 min at 4°C. Discard supernatant.
- 15 8. Wash the collected cells with 25 ml of PBS.
- 16 9. Centrifugation at 3 kg for 5 min at 4°C. Discard supernatant.
- 17 10. Repeat 2 times for the **steps 8 and 9**.
- 18 11. Store the sample at -70°C until use.

19

20 **3.8 Input sample preparation for CoIFI**

- 21 1. Place a tube containing the cross-linked cell pellet (H62: $\approx 7 \times 10^7$ cells) prepared in **Subheading 3.7**
- 22 on ice.
- 23 2. Resuspend the pellet with 582 μ l of pre-Lysis buffer.
- 24 3. Centrifugation at 1 kg for 5 min at 4°C. Discard supernatant.
- 25 4. Resuspend the pellet with 660 μ l of Lysis2 buffer.
- 26 5. Homogenize the mixture in a 1 ml dounce homogenizer with a tight pestle (20 strokes on ice).
- 27 6. Centrifugation at 1 kg for 5 min at 4°C. Discard supernatant.
- 28 7. Wash the pellet with 840 μ l of PBST.

- 1 8. Centrifugation at 1 kg for 5 min at 4°C. Discard supernatant.
- 2 9. Repeat the **steps 7 and 8**.
- 3 10. Resuspend the pellet with 840 µl of PBST containing a protease inhibitor cocktail and 1 mg/ml of
- 4 RNaseA.
- 5 11. Incubation with mixing for 1 hr at 37°C.
- 6 12. Centrifugation at 1 kg for 5 min at 4°C. Discard supernatant.
- 7 13. Wash the pellet with 1.4 ml of PBST.
- 8 14. Centrifugation at 1 kg for 5 min at 4°C. Discard supernatant.
- 9 15. Repeat 2 times for the **steps 13 and 14**.
- 10 16. Resuspend the pellet with 840 µl of CB1.3.
- 11 17. Centrifugation at 3 kg for 5 min at 4°C. Discard supernatant.
- 12 18. Resuspend the pellet with 690 µl of CB1.3 on ice.
- 13 19. Treat the mixture by a sonicator at 4°C (*see Note 15*).
- 14 20. Centrifugation at 16 kg for 15 min. Recover supernatant and measure its volume.
- 15 21. Add final 50 mM NaCl and final 1 mg/ml RNaseA, followed by incubation for 21 hr at 37°C.
- 16 22. The supernatant is mixed with 28 µl of streptavidin polyacrylamide resin for 1 hr (*see Note 16*).
- 17 23. Centrifugation at 16 kg for 15 min. Recover supernatant.
- 18 24. Freeze the recovered supernatant (i.e., input sample) by liquid nitrogen. Store the sample at -70°C
- 19 until use.

20

21 **3.9 Nucleoprotein isolation via CoIFI**

- 22 1. Prepare 20 µl of a mixture containing the input sample ($\approx 1 \times 10^6$ cells equivalent) prepared in
- 23 **Subheading 3.8**, 10 mM MgCl₂ and each 1.5 pmol of TFO-1 and TFO-3, adjusted the volume by CB3.1.
- 24 As a negative control, use Scr-1 and Scr-2 instead of the TFOs.
- 25 2. Incubation with mixing for ≈ 17 -18 hr.
- 26 3. Mix the sample with 4.8 µl of streptavidin magnetic beads (*see Note 17*).
- 27 4. Incubation with mixing for 2 hr. Discard supernatant via a magnetic stand.

- 1 5. Wash the beads with 100 μ l of the following buffers: CB3 with 7 mM MgCl₂, CB4 with 7 mM MgCl₂,
- 2 TS1, DIT4 x 2 times (incubation at 55°C for 5 min in each time), TS1 (discard supernatant after
- 3 transferring the mixture (the beads in TS1) into a new tube), then TS1.
- 4 6. Resuspend the beads with 10 μ l of ER2.
- 5 7. Incubation with mixing for 1 hr, followed by incubation at 98°C for 1.5 min.
- 6 8. Transfer supernatant into a new tube via a magnetic stand.
- 7 9. Analyze the recovered supernatant (Fig. 10).

8

9 **4 Notes**

10 1. Specific DNA isolation via triplex formation requires two core components, a TFO probe and a TFT
11 (Triplex Forming Tag) site. The TFO probe is composed of a psoralen residue, an LNA/DNA mixed
12 oligonucleotide, and a spacer arm conjugated with a desthiobiotin residue (Fig. 1). The TFT site is
13 consisted of a complementary dsDNA to form triplex with the TFO probe. We are using four different
14 22-mer TFO probes (TFO-1, TFO-3, Scr-1, Scr-2), two different 22 bp TFTs (TFT-1, TFT-2) and a TFT
15 cassette. TFO-1 and TFO-3 form triplexes with TFT-1 and TFT-2, respectively. Scr-1 and Scr-2 are
16 negative controls not form triplexes with the above TFTs because their sequences are not complementary.
17 The TFT cassette is a 61 bp dsDNA containing both TFT-1 and TFT-2. Detailed sequence compositions
18 of the TFO probes are described in [14].

19 2. In order to isolate proteins binding to a transcriptional regulatory region, pAS114.1 γ derived from
20 pGL4.12 (Promega) is constructed (Fig. 2). This plasmid is cloned a transcriptional regulatory region of
21 human *CDKN1A* gene encoding p21 [15, 16], whose cloning region is from -4554 to +45 bp around its
22 transcription start site at +1 bp. A region between -1181 and -792 bp of the transcriptional regulatory
23 region is replaced by a TFT site composed of 6 tandemly repeated TFT cassettes. A hygromycin resistant
24 gene is derived from a region between BamHI and Sall of pGL4.32 (Promega). A linearized form of
25 pAS114.1 γ is prepared by treatment of restriction enzymes at sites depicted as RE (2 sites) in Fig. 2. By
26 the restriction enzyme treatment, two DNA fragments (~3.9 kbp and ~6.8 kbp) is produced and the larger
27 fragment contains the TFT site.

1 3. A plasmid pAS200.2 contains the tetracycline resistance gene from pBR322, the pUC origin from
2 plasmid pAS03 [14], and a TFT site composed of 4 tandemly repeated TFT-1 (Fig. 5). In order to isolate
3 proteins binding to a DNA triplet repeat whose genetic instability causes neurological diseases [17], a
4 plasmid pAS203 is constructed through insertion of a 44 bp dsDNA cassette into the cloning site on
5 pAS200.2 depicted in Fig. 5. The dsDNA cassette contains 7 tandemly repeated CAG•CTG triplets and
6 two nickases recognition sites surrounding the triplet repeat (Fig. 5).

7 4. A 12-mer oligo is used as a scaffold to obtain pAS203 with a closed stem-loop. The oligo is annealed
8 on both 6 nucleotides flanking the CAG triplet repeat (Fig. 6a).

9 5. pAS203(rcc) is a relaxed closed circular form of pAS203 and is constructed as follows: Introduce a
10 nick on pAS203 by a nickase. Self-ligate the nicked pAS203. Cut out the ligated DNA from an agarose
11 gel. The extracted DNA is named pAS203(rcc).

12 6. A human cell line, 293 F cells (Invitrogen) are transfected with pAS114.1 γ linearized by SalI (Fig. 9).
13 Monoclonal cell lines that have stably integrated the plasmid are selected by hygromycin. One of stably
14 integrated cell lines (named clone H62) is used in the CoIFI approach. Its integrated plasmid copy
15 number is \approx 688 deduced from a semi-quantitative PCR analysis whose protocol is described in [14].
16 Biological functionality of the cloned CDKN1A regulatory region is confirmed by a luciferase reporter
17 assay using a Luciferase assay system in presence of trichostatin A (TSA).

18 7. We use a plastic cap of a plastic centrifugation tube.

19 8. By UVA irradiation, the psoralen on TFO probe intercalated in dsDNA as depicted in Fig. 1 is
20 covalently cross-linked with adjacent thymine(s) [14].

21 9. Before using the beads, they are washed two times with Extraction buffer.

22 10. Before mixing a plasmid, the beads are washed two times with NCL250. Then, the beads are
23 resuspended in 10 μ l of NCL250 with 1.25 mg/ml BSA. The indicated amount of plasmid is added into
24 the prepared beads solution.

25 11. Depending upon experimental purposes, the incubation time is flexibly varied.

26 12. A short DNA fragment (33-mer) generated by the double nicks on the plasmid is released via this
27 heat step.

1 13. During this heat step, it is expected that the CAG triplet repeat forms a stem-loop as drawn in Fig.
2 6a. Owing to the stem-loop formation, 6 nt ssDNA regions flanking the CAG triplet repeat are closely
3 placed. Subsequently, the 12-mer oligo is annealed to the proximate 6 nt ssDNA regions.

4 14. Before using the beads, they are washed two times with CB3.

5 15. Outcomes of sonication (i.e., patterns of genomic DNA fragmentation) are largely influenced by
6 buffer components, types of cell lines, cell density, cross-link conditions, and so on. The sonication
7 condition needs to be optimized.

8 16. Before using the resin, it is washed two times with CB1.3 with 50 mM NaCl.

9 17. Before mixing the sample, the beads are washed two times with CB3.1. Then, the beads are
10 resuspended in 10 μ l of CB3.1 with 1.25 mg/ml BSA.

11

12

13 **Figure legends**

14 **Fig. 1** A schematic view of triplex formation. A TFT region on DNA is shown in blue. A red line in the
15 TFO probe represents an oligonucleotide that can form a triplex with the TFT.

16

17 **Fig. 2** Construction of pAS114.1 γ . ori: high copy number pUC origin in *E. coli*. Ap: ampicillin resistance
18 gene. TFT: Triplex Forming Tag. SV40: SV40 early enhancer/promoter. Hyg: hygromycin resistance
19 gene. RE: restriction enzyme cut site. CDKN1A regulatory region: a transcriptional regulatory region
20 of human *CDKN1A* gene. Luc: Luciferase gene for a reporter assay, whose expression is controlled by
21 the CDKN1A regulatory region.

22

23 **Fig. 3** A schematic view of IDAP approach.

24

25 **Fig. 4** Nucleoprotein isolation in IDAP. (a) 40% of the elution products are analyzed via silver staining
26 following SDS-PAGE. Lane 1 is pAS114.1 γ in absence of TFO (as a negative control). Lanes 2 and 3
27 are pAS114.1 γ with TFO and its linearized form with TFO, respectively. Input (nuclear extracts) from
28 $\approx 2.5 \times 10^4$ cells is loaded. MW: molecular weight marker. Non-CL: non cross-link. *: streptavidin.

1 Samples corresponding to lanes 1 to 3 are also analyzed for presence of plasmid DNA by PCR and
2 histone H3 by western blotting (WB). In PCR, 0.01% of the elution products are used as a template. In
3 WB, 50% of the elution products are used. (b) 21% of the elution products are analyzed via silver
4 staining following SDS-PAGE. Lane 1 is pAS114.1 γ in absence of TFO. Lanes 2 and 3 are pAS114.1 γ
5 with TFO and its linearized form with TFO, respectively. CL: cross-link. *: streptavidin. All of data
6 clearly show high specificity for isolation of nucleoprotein complexes in IDAP. It notes that band
7 patterns on silver staining are somewhat different depending upon experimental setups (i.e., circular
8 DNA vs linear DNA; Non-CL vs CL).

9

10 **Fig. 5** Construction of pAS200.2 and pAS203. pUC ori: high copy number pUC origin in *E. coli*. Tet:
11 tetracycline resistance gene. TFT: Triplex Forming Tag. CAG on pAS203: it contains 7 tandemly
12 repeated CAG·CTG triplets and two nickases recognition sites surrounding the triplet repeat.

13

14 **Fig. 6** Construction of pAS203 with a closed stem-loop. (a) pAS203 is introduced double nicks by using
15 two nickases indicated in Fig. 5. A short DNA fragment produced by the double nicks is released by a
16 heat treatment in order to generate a gapped pAS203 (named pAS203(gap)). pAS203(gap) is treated by
17 heat for a stem-loop formation composed of the CAG triplet repeat. Subsequently, a 12-mer oligo serves
18 as a molecular splint to fix the extruded stem-loop through ligation reaction. (b) The resultant ligation
19 products using various amounts (0 to 50-fold) of the 12-mer oligo are analyzed on 2% agarose gel
20 containing 0.5 μ g/ml EtBr. The gel clearly shows that formation of pAS203 with a closed stem-loop
21 (named pAS203(cSL)) depends upon presence of the 12-mer oligo. Lane Gap is pAS203(gap) as a
22 control. The oc and cc labelled on right side of the gel indicate positions of open circular and closed
23 circular DNA, respectively. The positions of oc and cc reflect positions of pAS203(gap) and
24 pAS203(cSL), respectively.

25

26 **Fig. 7** Confirmation of characteristic properties of pAS203 derivatives. (a) Lane sc is a super coiled
27 form of plasmid, which is the natural form prepared from *E. coli*. Lane rcc is a relaxed closed circular
28 form of plasmid (*see Note 5*). On the upper gel, plasmids are analyzed on 2% agarose gel containing

1 0.5 $\mu\text{g/ml}$ EtBr. On the lower gel, plasmids are analyzed on 2% agarose gel in absence of EtBr. As
2 expected, pAS203(rcc) and pAS203(cSL) show representative patterns of relaxed forms of ccDNA. (b)
3 Plasmids are treated by RsaI that cuts two sites on plasmids as drawn. By this treatment, a short DNA
4 fragment, 181 bp from pAS200.2 and 225 bp from pAS203, is produced. The treated plasmids are
5 analyzed via 8% native polyacrylamide gel electrophoresis. As shown on the gel, all short DNA
6 fragments from pAS203 derivatives are differently migrated. It reveals all fragments are consisted of
7 different structures.

8

9 **Fig. 8** The constructed plasmids capture via TFO-mediated triplex formation. 40% of the elution
10 products are analyzed on 2% agarose gel containing 0.5 $\mu\text{g/ml}$ EtBr. As a control, 13 ng of each input
11 (pAS200.2, pAS203, pAS203(rcc), pAS203(gap), and pAS203(cSL)) are loaded on the same gel.
12 Recovery yields of all plasmids through the TFO-mediated plasmid capture are nearly same ($\approx 65\%$ of
13 input). Thus, the constructed plasmids are suitable to use as DNA substrates in the IDAP approach.

14

15 **Fig. 9** Schematic views of construction of a human stable cell line and isolation of nucleoproteins in
16 CoIFI approach.

17

18 **Fig. 10** Nucleoprotein isolation in CoIFI. (a) Genomic DNA is purified from the input sample for CoIFI
19 through deproteinization and reverse cross-link. The sample is analyzed on 0.7% agarose gel. (b)
20 Plasmid detection via PCR. 0.1% of the elution products are used as a template to amplify a DNA
21 segment in the CDKN1A regulatory region. Lane sTFO corresponds to the elution product in CoIFI
22 using Scr-1 and Scr-2 as a negative control. Lane tTFO corresponds to the elution product in CoIFI
23 using TFO-1 and TFO-3. An estimated recovery yield in CoIFI for the tTFO sample is $\approx 30\%$ of input.
24 The way how to determine recovery yields based upon PCR is described in [14]. (c) Histone H3
25 detection via WB. $\approx 79\%$ of the elution products and input from ≈ 40 cells equivalent is loaded. Both
26 PCR and WB clearly show high specificity for isolation of the specific nucleoprotein complexes in
27 CoIFI.

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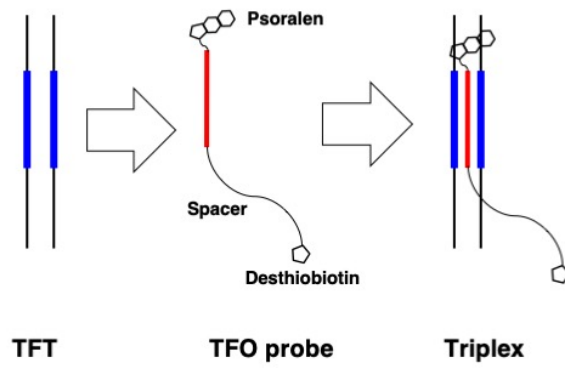
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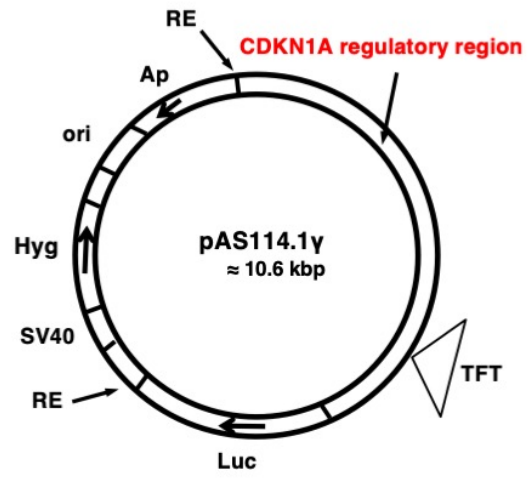
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4 **Fig. 1**

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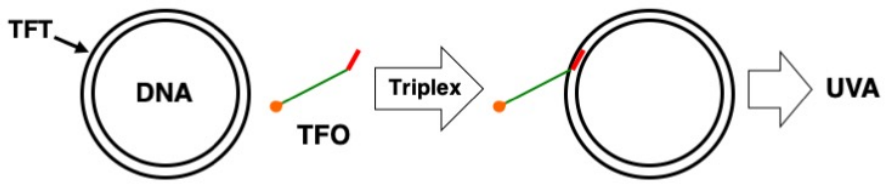
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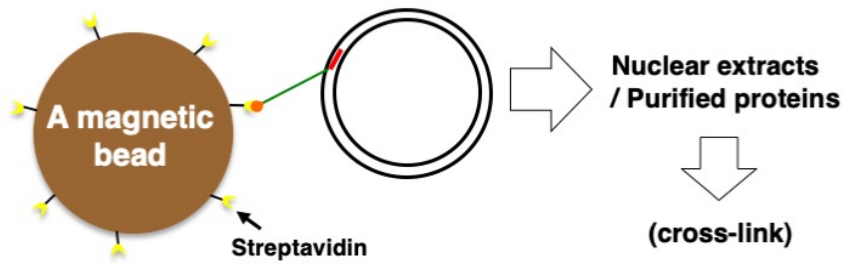
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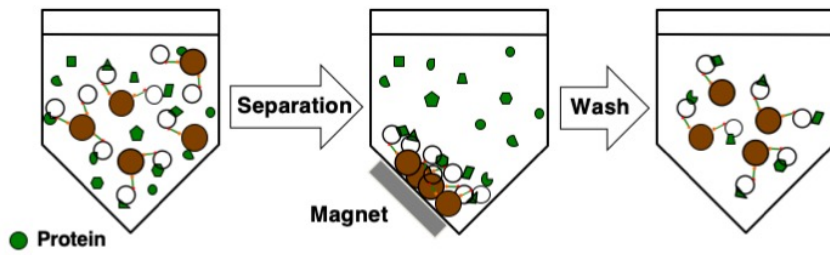
TFO-conjugated DNA



Reaction



Protein Isolation



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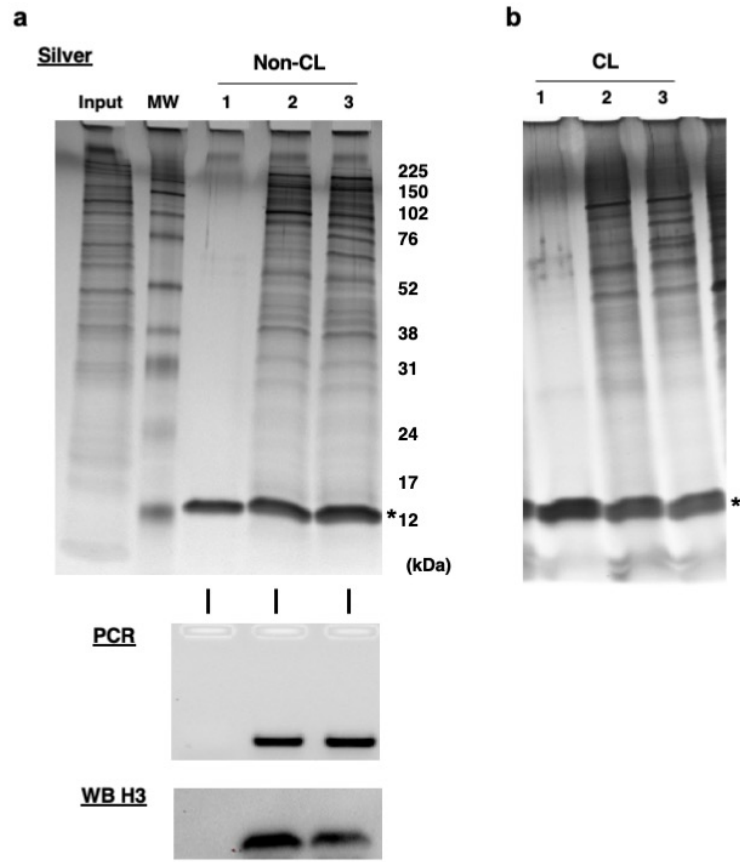
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4 Fig. 3

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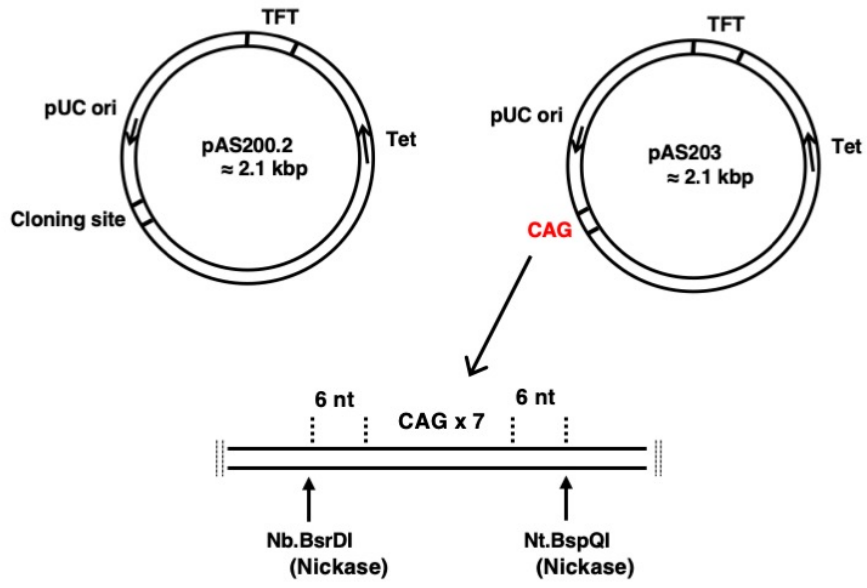
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4 **Fig. 4**

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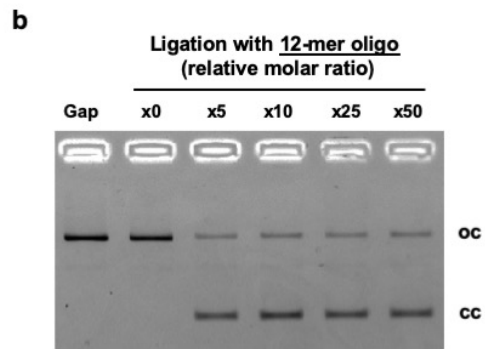
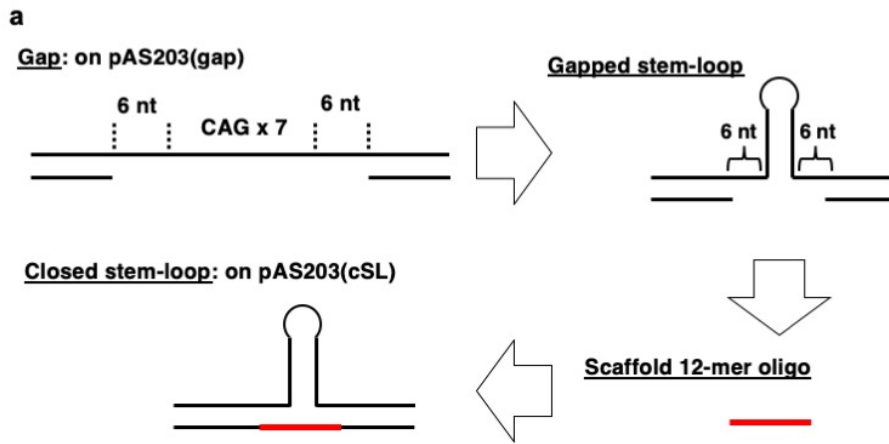
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4 Fig. 5

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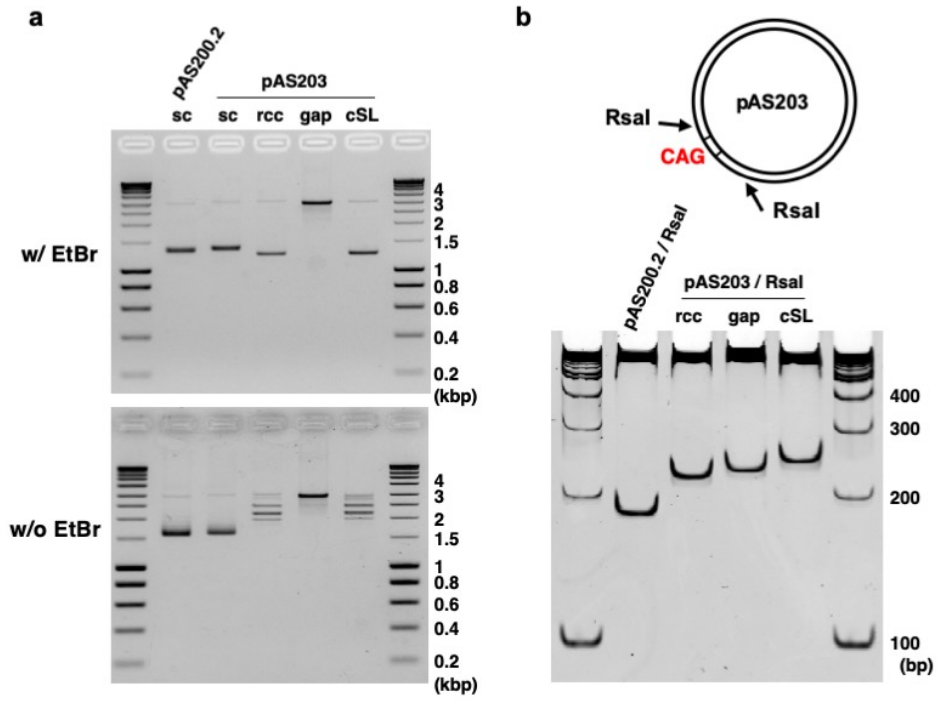
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4 Fig. 6

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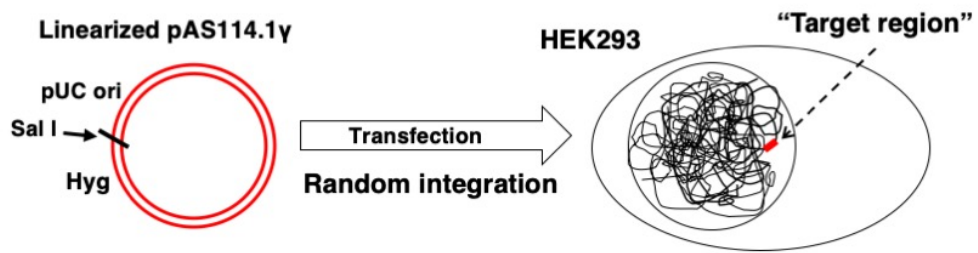
4 Fig. 7

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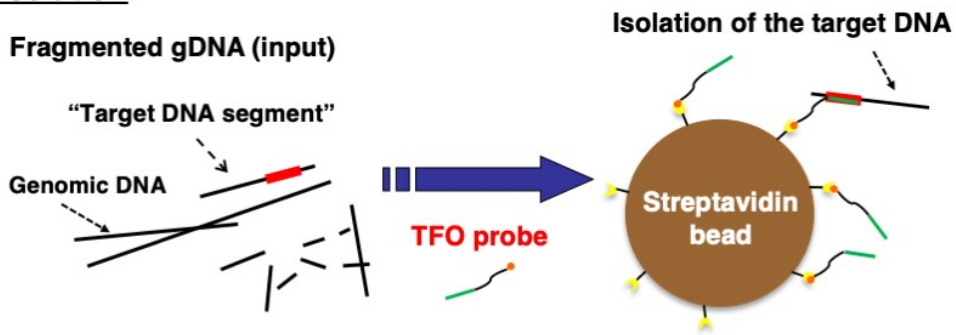
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Construction of a cell line



Isolation



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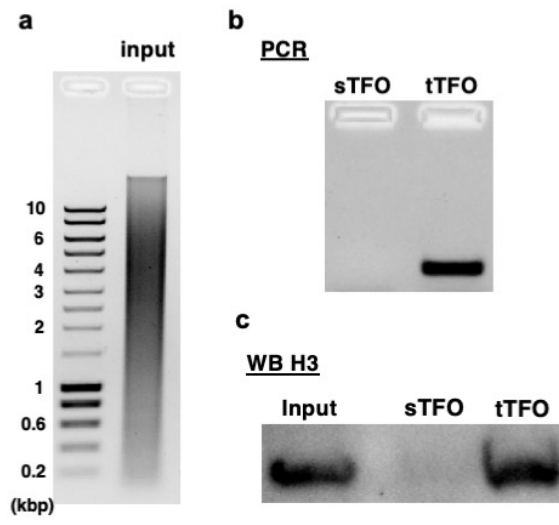
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4 Fig. 9

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4 **Fig. 10**