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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Protocol to evaluate cell lineage stability of mouse natural and induced regulatory T cells using bisulfite sequencing

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SUMMARY

The establishment of regulatory T cells (Treg)-specific demethylation regions (TSDRs) is essential for the Treg-lineage stability. Here, we present a protocol using bisulfite sequencing to assess Treg-lineage stability. The protocol describes the isolation of lymphocytes and DNA extraction, followed by bisulfite conversion in unmethylated CpG DNA, bisulfite PCR and cloning, and sequencing to define the TSDR methylation. This protocol uses lymph nodes and spleen tissues and can be adapted to assess the methylation status of Tregs in other tissue types.

BEFORE YOU BEGIN

The protocol below describes the specific steps to investigate the methylation status in TSDRs by bisulfite sequencing, and focuses on the isolation of T cells from lymph nodes and spleen. If it is necessary to assess the methylation status in other tissue-derived Tregs, the same procedure from DNA extraction to the final sequencing can be used.

Institutional permissions

All animals used in this protocol were maintained in specific pathogen-free condition. All animal studies were performed following the guidelines on animal welfare of Osaka University.

Mice

Mouse strain selection will depend on the experiment. In this protocol, we have analyzed 8–12 weeks old BALB/c background mice, as well as various transgenic strains.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-mouse CD4-BV650 (RM4-5) (dilution used 1:200) | BD Biosciences | Cat#563747; RRID: AB_2716859 |
| Purified anti-mouse CD16/32 (93) (dilution used 1:200) | BioLegend | Cat#101302; RRID: AB_312801 |
| Anti-mouse CD25-BV421 (PC61) (dilution used 1:200) | BioLegend | Cat#102034; RRID: AB_11203373 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-mouse CD25-PE (PC61) (dilution used 1:200) | BD Pharmingen | Cat#553866; RRID: AB_395101 |
| Anti-mouse CD44-APC (IM7) (dilution used 1:200) | Invitrogen | Cat#17-0441-83; RRID: AB_469390 |
| Anti-mouse CD62L-BV421 (MEL-14) (dilution used 1:200) | BD | Cat#562910; RRID: AB_273785 |
| Anti-mouse CD62L-PerCP-Cy5.5 (MEL-14) (dilution used 1:200) | BD Pharmingen | Cat#560513; RRID: AB_10611578 |
| Anti-mouse Foxp3-Alexa Fluor 488 (FJK-16s) (dilution used 1:200) | Invitrogen | Cat#53-5773-82; RRID: AB_763537 |

**Bacterial and virus strains**

| E. coli DH5α Competent Cells | Takara | Cat#9057 |

**Chemicals, peptides, and recombinant proteins**

| Chemical/Peptide/Protein | Cat Number | Source |
|-------------------------|------------|--------|
| D-PBS (–) (1x) | Cat#14249-24 | Nacalai Tesque |
| RPMI 1640 | Cat#30264-85 | Nacalai Tesque |
| 0.5 M EDTA | Cat#06894-14 | Nacalai Tesque |
| Penicillin-streptomycin | Cat#26233-84 | Nacalai Tesque |
| Fetal Bovine Serum (FBS) | Cat#10437028 | Gibco |
| 2-(β-Mercaptoethanol (2-ME) | Cat#21985023 | Gibco |
| 2-Phospho-L-ascorbic acid trisodium salt ≥95.0% (A2P) | Cat#49752-10G | Sigma-Aldrich |
| Temizbarone | Cat#54260-50mg | Selleck |
| UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 | Cat#15567027 | Invitrogen |
| Sodium Chloride | Cat#31319-45 | Nacalai Tesque |
| Sodium Lauryl Sulfate (SDS) | Cat#31606-62 | Nacalai Tesque |
| Proteinase K Solution (20 mg/mL), RNA grade | Cat#25530049 | Invitrogen |
| Phenol:Chloroform:Isomylalkohol (PCI) | Cat#26058-96 | Nacalai Tesque |
| Chloroform:Isomylalkohol (CIA) | Cat#CO549-1PT | Sigma-Aldrich |
| Sodium Acetate | Cat#31137-25 | Nacalai Tesque |
| Ethachinmate | Cat#318-01793 | NIPPON GENE |
| Ethanol (95%) (use it as 100% EtOH) | Cat#14710-25 | Nacalai Tesque |
| NaOH | Cat#198-13765 | Wako |
| Sodium bisulfite | Cat#243973 | Sigma-Aldrich |
| Sodium disulfite | Cat#106528 | Merck |
| Buffer PB (500 mL) | Cat#19066 | OIAGEN |
| Buffer PE (concentrate, 100 mL) | Cat#19065 | OIAGEN |
| CAPS | Cat#347-00482 | Dojindo |
| 50 x TAE | Cat#313-90035 | NIPPON GENE |
| Agarose S (500g) | Cat#318-01195 | NIPPON GENE |
| Isopropyl-β-D-thiogalactopyranoside (IPTG) | Cat#19742-94 | Nacalai Tesque |
| 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) | Cat#06280-31 | Nacalai Tesque |
| Bacto™ Tryptone | Cat#211705 | BD Biosciences |
| Bacto™ Yeast Extract | Cat#212750 | BD Biosciences |
| Bacto™ Agar | Cat#214010 | BD Biosciences |
| LB Broth, Miller | Cat#20068-75 | Nacalai Tesque |
| KCI | Cat#28514-75 | Nacalai Tesque |
| MgSO4 | Cat#21003-75 | Nacalai Tesque |
| MgCl2 | Cat#20909-55 | Nacalai Tesque |
| Glucose | Cat#049-31165 | Wako |
| Kanamycin Sulfate | Cat#19860-44 | Nacalai Tesque |
| Water | Cat#06442-95 | Nacalai Tesque |

**Critical commercial assays**

| Red Blood Cell Lysing Buffer Hybri-Max™ | Cat#R7757 | Sigma-Aldrich |
| LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit | Cat#L34975 | Thermo Fisher Scientific |
## MATERIALS AND EQUIPMENT

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse CD4 T Lymphocyte Enrichment Cocktail | BD | Cat#51-9000633 |
| Streptavidin Particles Plus | BD | Cat#51-9000810 |
| eBioscience™ Fixation/Permeabilization Concentrate | Invitrogen | Cat#00-5123-43 |
| eBioscience™ Fixation/Permeabilization Diluent | Invitrogen | Cat#00-5223-56 |
| eBioscience™ Permeabilization Buffer (10X) | Invitrogen | Cat#00-8333-56 |
| Methyl Easy Xceed kit (Human Genetic Signatures) | Takara | Cat#ME002 |
| UltraPure™ Salmon Sperm DNA Solution | Invitrogen | Cat#15632011 |
| Zymo-Spin IC Columns | Zymo Research | Cat#C1004-50 |
| Collection Tubes | Zymo Research | Cat#C1001-50 |
| TaKaRa Ex Taq™ Hot Start Version | Takara | Cat#RR006A |
| QIAEX II Gel Extraction Kit (150) | QIAGEN | Cat#20021 |
| DynaExpress TA PCR Cloning Kit | BioDynamics | Cat#DS130L |
| illustra TemplPlfi DNA Amplification Kit | GE Healthcare | Cat#28964286 |
| BigDye™ Terminator v3.1 Cycle Sequencing Kit | Applied Biosystems | Cat#4337454 |
| Agencourt CleanSEQ Dye-Terminator Removal Kit | Beckman Coulter | Cat#A29154 |

### Experimental models: Organisms/strains

- **Mice:** BALB/c (male 8–12 weeks old) CLEA Japan N/A
- **Mice:** Foxp3-eGFP (male 8–12 weeks old) Sakaguchi Lab N/A

### Oligonucleotides

- Primers for bisulfite PCR (see Table 4) Ohkura et al. (2012) See Table 4
- Primers for sequencing (see Table 8) N/A See Table 8

### Software and algorithms

- **FlowJo_v10** FlowJo https://www.flowjo.com/
- **MethPrimer 2.0** Li and Dahiya (2002) http://www.urogene.org/methprimer2/
- **Sequencing Analysis Software Version v6.0** Applied Biosystems N/A

### Other

- **Millex-GV Syringe Filter Unit, 0.22 μm, PVDF, 33 mm, gamma sterilized** Millipore Cat#SLGVR33RS
- **60 mm / Non-treated Dish** Iwaki Cat#1010-60
- **40 or 70 μm Cell Strainer** Corning Cat#352340 Cat#352350
- **3 mL Syringes** Nipro Cat#08-470
- **BD FACSAria II cell sorter** BD N/A
- **BD IMag™ Cell Separation Magnet** BD Cat#552311
- **Eppendorf Thermomixer® C** Eppendorf Cat#5382000023
- **Eppendorf SmartBlock™ 1.5 mL** Eppendorf Cat#5360000038
- **Block incubator** Astec Co., Ltd. Cat#83165
- **Veriti™ 96-Well Thermal Cycler** Applied Biosystems Cat#43-757-86
- **EYELA Water bath** Tokyo Rikakikai Co., Ltd. Cat#NNT-2200
- **96S Super Magnet Plate** Alpaca Cat#A001322
- **3500XL Genetic Analyzer** Applied Biosystems N/A

### FACS buffer

| Reagent       | Final concentration | Amount  |
|---------------|---------------------|---------|
| D-PBS (-)     | N/A                 | 489.5 mL|
| 0.5 M EDTA    | 1 mM                | 500 μL  |
| FBS           | 2% (v/v)            | 10 mL   |
| **Total**     | N/A                 | 500 mL  |

Store at 4°C for up to 3 months.
### Lysis buffer

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| 5 M NaCl         | 100 mM              | 1 mL   |
| 1 M TrisHCl      | 10 mM               | 500 µL |
| 0.5 M EDTA       | 50 mM               | 5 mL   |
| 10% SDS          | 0.5% (v/v)          | 2.5 mL |
| Water            | N/A                 | Up to 50 mL |
| **Total**        | N/A                 | 50 mL  |

Store at room temperature (18°C–25°C) for up to 12 months.

**Note:** FBS should be sterile filtered through a 0.22 mm filter.

### T cell culture medium

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| RPMI 1640        | N/A                 | 444.5 mL |
| Penicillin-streptomycin | 1% (v/v) | 5 mL |
| FBS              | 10% (v/v)           | 50 mL  |
| 2-ME             | 0.1% (v/v)          | 500 µL |
| **Total**        | N/A                 | 500 mL |

Store at 4°C for up to 3 months.

### TE buffer

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| 1 M TrisHCl      | 10 mM               | 500 µL |
| 0.5 M EDTA       | 1 mM                | 100 µL |
| Water            | N/A                 | Up to 50 mL |
| **Total**        | N/A                 | 50 mL  |

Store at room temperature for up to 12 months.

### TAE buffer

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| 50 x TAE         | 1 x                 | 980 mL |
| Milli-Q water    | N/A                 | 20 mL  |
| **Total**        | N/A                 | 1 L    |

Store at room temperature for up to 3 months.

### 2% agarose gel

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| Agarose S        | 2% (w/v)            | 8 g    |
| TAE buffer       | N/A                 | 400 mL |
| **Total**        | N/A                 | 400 mL |

Solidify agarose gel and store in TAE buffer at room temperature for up to 6 months.

### LB medium

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| BactoTm Tryptone | 2% (w/v)            | 10 g   |
| Bacto Yeast Extract | 0.5% (w/v) | 2.5 g |
| 5 M NaCl         | 10 mM               | 1 mL   |

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**STEP-BY-STEP METHOD DETAILS**

**Sampling and cell isolation**

© Timing: 1–2 h

This section describes sampling lymph nodes from mice and preparation of cell suspension.

1. Set the 40–70 \( \mu m \) cell strainer on the 6 cm dish, and add 3 mL FACS buffer into them (A) (Figure 1).
2. Collect peripheral lymph nodes and spleen, transfer them to (A). Mash them using 3 mL syringe plunger.
3. Transfer cell suspension into 15 mL tube. Centrifuge the sample at 500 \( g \) for 5 min at 4°C.
4. Discard the supernatant and resuspend the sample with 1 mL Red Blood Cell Lysing Buffer Hybri-Max. Set the sample for 15 min on ice.
5. Add 9 mL FACS buffer. Centrifuge the sample at 500 \( g \) for 5 min at 4°C.
6. Discard the supernatant and resuspend the pellet with 100 \( \mu L \) Mouse CD4 T Lymphocyte Enrichment Cocktail and 200 \( \mu L \) FACS buffer. Set the sample for 15 min on ice.
7. Centrifuge the sample at 500 \( g \) for 5 min at 4°C.
8. Discard the supernatant and resuspend the pellet with 100 \( \mu L \) Streptavidin Particles Plus and 200 \( \mu L \) FACS buffer. Set the sample for 15 min on ice.
9. Add 2 mL FACS buffer, transfer the sample to 5 mL tube. Set this tube on Cell Separation Magnet for 6 min at room temperature.
10. Transfer the supernatant to 15 mL tube. Centrifuge the sample at 500 \( g \) for 5 min at 4°C.
11. Discard the supernatant and resuspend the pellet with 1 mL FACS buffer.
This section describes cell staining by antibodies and cell sorting by FACS.

12. Prepare the antibody cocktail below (Table 1).

   **Note:** We prepare 5 times dilution Dead Cell Stain Kit freshly diluted by FACS buffer.

13. Stain the cells with the antibody cocktail. Set the sample for 15 min on ice.
14. Wash the cells with FACS buffer. Centrifuge the sample at 500 $\times$ g for 5 min at 4°C.
15. Repeat step 14 (if using intracellular staining, move to step a below).

   **Note:** If you use mice without Foxp3 reporter (ex. GFP etc.), you should stain the cells along the protocol below.

### Table 1. Antibody cocktail for staining lymphocytes

| Reagent                                         | Final concentration | Amount |
|------------------------------------------------|---------------------|--------|
| Purified anti-mouse CD16/32 (93)                | 1:200               | 1 μL   |
| Anti-mouse CD4-BV650 (RM4-5)                    | 1:200               | 1 μL   |
| Anti-mouse CD25-PE (PC61)                       | 1:200               | 1 μL   |
| Anti-mouse CD44-APC (IM7)                       | 1:200               | 1 μL   |
| Anti-mouse CD62L-BV421 (MEL-14)                 | 1:200               | 1 μL   |
| LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (About dilution, see the Note below) | 1:1000              | 1 μL   |
| FACS buffer                                     |                     | 200 μL |

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**Figure 1. Cell isolation from mouse lymph nodes and spleen**

Placing lymph nodes and spleen into a cell strainer (A in Step 1) and mashing them using 3 mL syringe plunger in the 60 mm dish.

**Cell staining and sorting**

**Ø** Timing: 1–2 h

This section describes cell staining by antibodies and cell sorting by FACS.
a. Discard the supernatant and resuspend the pellet with 200 μL fixation buffer (Fixation/Permeabilization Concentrate: Fixation/Permeabilization Diluent = 1 : 3). Set the sample for 30 min on ice.
b. Wash the cells with permeabilization buffer. Centrifuge the sample at 600 × g for 5 min at 4°C.
c. Repeat step b.
d. Discard the supernatant and resuspend the pellet with the antibody cocktail below (Table 2).
e. Wash the cells with FACS buffer. Centrifuge the sample at 600 × g for 5 min at 4°C.
f. Repeat step e.
g. Discard the supernatant and resuspend the pellet with 2 mL FACS buffer. Now, it is ready to sort the cells.

16. Discard the supernatant and resuspend the pellet with 2 mL FACS buffer. Now, it is ready to sort the cells.

17. Sort the cells (up to 100,000 cells each) into 1.5 mL tube with T cell culture medium using FACSAria II cell sorter with the gating strategy below (Figure 2).

**Note:** For *in vitro* induced Treg (iTreg) cells induction, we referred Mikami et al., (2020), induced and sorted iTreg cells in the same way above.

### Cell lysis

**Timing:** Overnight (12–24 h)

This section describes cell lysis and protein digestion.

18. Centrifuge the sample tube at 500 × g for 5 min at 4°C.
19. Discard the supernatant carefully and resuspend the pellet with 400 μL Lysis buffer.
20. Add 2 μL Proteinase K solution to the tube.
21. Shake and incubate the tube at 1,300 rpm for overnight at 55°C.

**Note:** If you sort the fixed cells, please shake and incubate the tube at 65°C for removing crosslink.

**Pause point:** If you need the stopping at step 19, store the sample at −30°C (also available from −20°C to −80°C) after resuspension.

### DNA extraction

**Timing:** 1 h

This section describes DNA extraction from cell lysed buffer.

22. Add 400 μL PCI to the sample tube and vortex well. Centrifuge it at 15,000 × g for 5 min at room temperature.
23. Transfer the upper layer (400 μL aqueous layer) to the new 1.5 mL tube.
24. Repeat steps 22 and 23.

| Table 2. Antibody cocktail for intracellular staining lymphocytes |
|---------------------------------------------------------------|
| **Reagent**          | **Final concentration** | **Amount** |
| Anti-mouse Foxp3-Alexa Fluor 488 (FJK-16s) | 1:200 | 1 μL |
| Permeabilization buffer | 200 μL | |

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**Note:** For *in vitro* induced Treg (iTreg) cells induction, we referred Mikami et al., (2020), induced and sorted iTreg cells in the same way above.
25. Add 400 μL CIA to the sample tube and vortex well. Centrifuge it at 15,000 × g for 5 min at room temperature.

26. Repeat step 23. Add 1 μL Ethachinmate, 40 μL 3 M sodium acetate and 1 mL 100% EtOH to the sample tube. Mix thoroughly by gentle inversion. Centrifuge it at 15,000 × g for 20 min at 15°C.

27. Discard the supernatant carefully and add 500 μL 80% EtOH to the sample tube. Centrifuge it at 15,000 × g for 15 min at 4°C.

28. Discard the supernatant carefully and dry up pellet for 5 min.

29. Elute the pellet with 20 μL TE buffer.

Note: If you use the fixed cells, please elute the pellet with 400 μL TE buffer, repeat step 21 at 65°C and repeat DNA extraction step again.

**Bisulfite conversion**

© Timing: 2 h

This section describes bisulfite conversion in unmethylated CpG DNA.

The bisulfite base conversion was carried out using the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures) by following the manufacturer’s protocol. If not using
the kit, please perform the protocol below, we added minor modifications to previous reports (Frommer et al., 1992; Olek et al., 1996; Rakyan et al., 2004; Darst et al., 2010; Harrison and Parle-McDermott, 2011).

**Note:** We used Zymo-Spin IC Columns as the collecting columns in the alternative protocol.

**Note:** We set heat blocks at 80°C, 37°C, and 95°C.

30. Prepare the bisulfite solution (Table 3, for 4 samples). Set this solution at 80°C.

**Note:** 3.5 mM sodium disulfite in 0.45 M NaOH solution is the substitute as the bisulfite solution.

31. Add 2.2 μL 3 M NaOH to 20 μL sample. Set the sample tube for 15 min at 37°C.
32. Add 220 μL bisulfite solution to the sample tube. Set the sample tube for 1 h at 80°C.
33. Add 500 ng salmon sperm DNA solution and 240 μL Buffer PB to the sample tube.
34. Transfer the sample to the column and centrifuge it at 15,000 × g for 30 s at room temperature.
35. Discard the flowthrough. Add 200 μL Buffer PE to the column and centrifuge it at 15,000 × g for 30 s at room temperature.
36. Repeat step 35.
37. Discard the flowthrough. Set the column on the new 1.5 mL tube and add 50 μL 50 mM NaOH to the column.
38. Set the column for 1 min at room temperature. Centrifuge it at 15,000 × g for 30 s at room temperature.
39. Set the sample for 30 min at 95°C for the desulfonation. Add 5 μL 1 M TrisHCl (pH 7.5) to the sample.
40. Set the sample on ice. Now, it is ready to use the sample for bisulfite PCR.

**Pause point:** Able to store the sample at 4°C until next step.

**Note:** 3 M NaOH will be expired in 4 weeks. Please prepare the solution freshly if possible.

**Note:** 10 mM CAPS buffer is the substitute as 50 mM NaOH in step 37. In this case, adding 1 M TrisHCl is not necessary.

**Note:** Insufficient desulfonation leads to failed bisulfite PCR. Desulfonation for 30 min or more is better.

### Bisulfite PCR

**Timing:** 2.5 h

This section describes targeted bisulfite PCR in TSDRs.

We used the primers for the bisulfite PCR (Table 4) from Ohkura et al. (2012).
We used TaKaRa Ex Taq® Hot Start Version (Takara) for bisulfite PCR.

41. Prepare the bisulfite PCR mix (Table 5). Set this mix on ice until PCR.
42. Run the PCR program below (Table 6).
43. Electrophoresis of the PCR products using 2% agarose gel (Figure 3).
44. Cut the aimed band in the gel and transfer the gel pieces to 1.5 mL tube.

Pause point: Able to store the sample at –30°C until next step for 1 month.

### Gel extraction

© Timing: 30 min

This section describes extraction of bisulfite PCR products from gel pieces.

The DNA extraction from the gel pieces was carried out using QIAEX II Gel Extraction Kit (QIAGEN) by following the manufacturer’s protocol.

45. After dissolving and removing the gel and washing with the silica beads (QIAEX II Suspension), elute the pellet with 8 µL TE buffer.

### Ligation of PCR products for TA cloning

© Timing: 30 min–2 h

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### Table 4. Primers for the bisulfite PCR

| Primer name            | Sequence (5'–3')                      |
|------------------------|---------------------------------------|
| mFoxp3_CNS2_Fwd        | ATT TGA ATT GGA TAT GGT TTG T         |
| mFoxp3_CNS2_Rev        | AAC CTT AAA CCC CTC TAA CAT C         |
| mIkzf2_intron3a_Fwd    | AGG ATG GTT TTT ATT GAA GGT GAT       |
| mIkzf2_intron3a_Rev    | ATA CAC ACC AAA CAA ACA CTA CAC C     |
| mIkzf4_int-last2_Fwd   | TAA GAA ATT GGG TGT GAT ATA TGT A     |
| mIkzf4_int-last2_Rev   | TTT CCC CTA CTA AAA CTC CTT AAA C     |
| mCIta4_exon2_Fwd       | TGG TGT TGG TTA GTA GTT ATG GT T      |
| mCIta4_exon2_Rev       | AAA TTC CAC CTT ACA AAA ATA CAA TC    |
| mIl2ra_int1(2500)_Fwd  | TTT TAG AGT TAG AAG ATA GAA GGT ATG GAA |
| mIl2ra_int1(2500)_Rev  | TCC CAA TAC TTA ACA AAA CCA CAT AT    |
| mTnfrsf18_exon5_Fwd    | GAG GTG TAG TGG TTA GTT GAG GAT GT    |
| mTnfrsf18_exon5_Rev    | AAC CCC TAC TCT CAC CAA AAA TAT AA    |

### Table 5. Components of the bisulfite PCR mix

| Reagent                | Amount |
|------------------------|--------|
| 10× ExTaq buffer       | 2.0 µL |
| dNTP mix (2.5 mM each) | 1.6 µL |
| Primer mix (10 µM each)| 1.6 µL |
| ExTaq HS (5 U/µL)      | 0.5 µL |
| Water                  | 10.3 µL|
| Sample                 | 4.0 µL |
| Total                  | 20 µL  |
This section describes ligation of extracted PCR products into the vector.

The TA cloning was carried out using DynaExpress TA PCR Cloning Kit (BioDynamics). We used the water baths at 42°C and 37°C.

46. Prepare the vector ligation mix (Table 7) in 1.5 mL tube. Set the tube on ice until a ligation step.
47. Set the tube onto a heat block at 16°C for 30 min-2 h.

**DH5α transformation**

© Timing: 1 h

This section describes transformation of competent cells.

*Note:* Add 50 μL 0.1 M IPTG and 50 μL 2%(w/v) X-gal to the Kanamycin-added LB plate and spread these solutions well.

48. Add optimized DH5α competent cells solution to the tube. Set the tube on ice for 10 min.

Table 6. Bisulfite PCR program using Ex Taq Hot Start Version

| Steps                | Temperature | Time  | Cycles |
|----------------------|-------------|-------|--------|
| Initial denature     | 98°C        | 1 min | 1 cycle|
| Denature             | 98°C        | 10 s  | 40 cycles|
| Annealing            | 55°C        | 30 s  |        |
| Extension            | 72°C        | 45 s  |        |
| Final extension      | 72°C        | 2 min | 1 cycle|
| Hold                 | 18°C        | ∞     |        |

Figure 3. Electrophoresis of bisulfite PCR products in Foxp3-CNS2 region of naïve Tconv and nTreg cells

We used the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit as a commercial bisulfite kit, and the original protocol using two different elution buffers.
49. Set the tube in the water bath for 45 s at 42°C.
50. Transfer the tube on ice. Add SOC solution (10 times volume of the sample) to the tube.
51. Set the tube in the water bath for 30–45 min at 37°C.
52. Centrifuge the tube at 2,200 × g for 3 min at room temperature.
53. Discard the supernatant until 100 μL. Spread the cell suspension gently and well.
54. Put the plate in the incubator (5% CO₂ 37°C) for 12–16 h.

Pause point: The colony formed plate can be stored at 4°C until you move to the next step.

**Colony PCR**

© Timing: 4.5 h

This section describes colony PCR after DH5α transformation.

*Note:* We used illustra TempliPhi DNA Amplification Kit for Rolling Circle Amplification. In addition, we modified the protocol from the manufacturer.

55. Aliquot 2.5 μL Denature buffer into the PCR tube or 96 well PCR plate.
56. Pick up a white colony by the toothpick, then dip the colony into the Denature buffer.
57. Set the sample on the thermal cycler for 3 min at 95°C.
58. Remove the sample from the thermal cycler and add 2.5 μL Premix into it.
59. Set the sample on the thermal cycler for 4 h at 30°C, 10 min at 65°C, and keep it at 25°C.
60. Remove the sample from the thermal cycler.

Pause point: Able to store the sample at 4°C until next step for 24 h.

**Big dye**

© Timing: 2 h

This section describes Big Dye PCR.

*Note:* We used BigDye™ Terminator v3.1 Cycle Sequencing Kit for analyzing the PCR product-inserted plasmid vector. In addition, we modified the protocol from the manufacturer.

61. Prepare the BigDye mix below (Table 8), and put the mix on ice in dark until the reaction.
62. Run the program below (Table 9) by the thermal cycler.
63. Remove the sample from the thermal cycler.

Pause point: Able to store the sample at 4°C in dark until next step for 24 h.

*Note:* We performed our original modified protocol here. Please check each setting in your lab.

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Table 7. Component of the ligation mix

| Reagent                      | Amount |
|------------------------------|--------|
| 2× Ligation buffer           | 1.2 μL |
| pTAKN-2 vector (50 ng/μL)    | 0.2 μL |
| Ligation mixture             | 0.2 μL |
| Sample                       | 0.8 μL |

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Note: We performed our original modified protocol here. Please check each setting in your lab.
Purification of the sequencing reactions

© Timing: 10 min

This section describes purification of Big Dye PCR products before the sequencing.

64. Add 1.5 µL CleanSEQ beads and 40 µL 80% ethanol into each well.
65. Vortex and spin down, and set the PCR tube or plate on the magnetic rack for 5 min.
66. Aspirate the supernatant with taking care of the beads.
67. Add 100 µL 80% ethanol into each well.
68. Repeat steps 65 and 66.
69. Dry the beads well. Add 18 µL water into each well.
70. Repeat step 65.
71. Transfer the sample-eluted water to the sequencing plate.

Note: Do not transfer the beads to the sequencing plate for avoiding a sequencing error.

Sequencing

© Timing: 1 h

This section describes definition of the TSDR methylation by Sanger sequencing.

Note: We used 3500×L Genetic Analyzer for Sanger sequencing and performed “RapidSeq” mode.

72. Prepare the sample sheet and modify the sequencing setting.
73. Sequence the sample.
74. Analyze the base called sequence data by base analyzing tools.
75. Check the methylation status according to the reference sequence. We show the Foxp3 CNS2 region as an example below (Table 10).

EXPECTED OUTCOMES

For representative gating strategy of flow cytometry data, see Figure 2. As previous several reports, naturally occurring Treg (nTreg) cells would establish the Treg-specific demethylation in Foxp3 conserved non-coding sequence (CNS) 2 region as well as other TSDRs such as Tnfrsf18,
Ctla4, and Iкzf4 (Polansky et al., 2008; Ohkura et al., 2012; Sakaguchi et al., 2020). On the other hand, naïve T conventional (Tconv) cells showed almost completely methylated Foxp3-CNS2 region (Figure 4A). Thus, naïve Tconv cells would be a precise control sample for the basal methylation status. Effector Tconv cells maintained fundamentally similar methylation status as naïve Tconv cells (data not shown). In female cells, X-chromosome inactivation controls the activation status on X-chromosomes functionally and structurally (Bacher et al., 2006; Loda et al., 2022). Foxp3 is an X-linked gene, and regulated by the same mechanism (Nunzio et al., 2009). Analyzing Foxp3 methylation status, female nTreg cells have theoretically 50% methylated Foxp3-CNS2 region (Figure 4B). In the past decade, iTreg cells had been thought as quite unstable in not only Foxp3 expression level but also Foxp3-CNS2 demethylation status (Ohkura et al., 2013). However, Mikami et al. developed the procedure to produce stable iTreg cells, based on the Foxp3-CNS2 demethylation status (Mikami et al., 2020). In addition, Foxp3 expression can be enhanced by supplementing retinoic acids into culture medium (Mucida et al., 2007). In this paper, we also induced stable iTreg cells following the previous several works using ascorbic acid and Tamibarotene as synthetic retinoic acid (Nair et al., 2016; Yue et al., 2016). Adding ascorbic acid trisodium salt into the culture medium, we confirmed the capacity to confer the Foxp3-CNS2 demethylation upon the induced iTreg cells while increased Foxp3 expression by retinoid didn’t establish stable Foxp3-CNS2 epigenome (Figure 5).

**LIMITATIONS**

This protocol meets the basic needs for analyzing the potential stability of nTreg and iTreg cells, as well as other Tconv cells in most organs, but there are the small number of Treg cells and CD4+ T cells in particular tissue such as large intestine or adipose tissue. If necessary to analyze such tissue-derived Treg cells, further optimization or pooling cells will be needed to acquire the unbiased results. We confirmed the isolation of genomic DNA and bisulfite conversion at least 1,500 cells in optimized conditions. However, small input of initial cell number sometimes leads PCR bias, so we should take the results carefully. Several reports suggest various Foxp3-CNS regions regulates stable Foxp3 expression and functional stability of naturally occurring Treg cells (Zheng et al., 2010; Josefowicz et al., 2012; Kawakami et al., 2021). In this protocol, we showed only Foxp3-CNS2 methylation, however it is available to analyze the methylation status in other Foxp3 genomic regions according to the same procedure.

### Table 10. CpG position of Foxp3-CNS2 region before/after bisulfite conversion (the bold regions are primer-binding regions and under bars show CpG)

| Region name                        | Amplified sequence                                      |
|------------------------------------|----------------------------------------------------------|
| Foxp3-CNS2                         | AACCTTGGGCCCCTCTGGCATTCAAGAAAGACAGATTCAATA               |
|                                    | GAACCTTGGGCTTGGATGGAGCAGTGTCACACACTACAGCAGAAAAAGATGAGAT|
|                                    | ATGGATGACTAGCCTTCATTTCTGGGAAAGACCTGATGGGTAGA            |
|                                    | TTATCTGCCCCTTCTTCCTCTCTGGGCTGAGAAGGCGCAA               |
|                                    | TGGATCTGGGCCCCTGAGTGAAATGGCAGTCAATGGGACAAAAATCTGGGCG   |
|                                    | AAGTTCAACGGTTGACACAGAAGGCCGAGATGAACACAGCTAG           |
|                                    | TACCACTAGAGACAGCTAACATCCAGTACG                        |
| Bisulfite-converted Foxp3-CNS2      | AACCTTAAACCCCTCTCAATCACAAAAAACAAAAATCATAAA             |
| (complete demethylation)           | AACCTTAAATTTTACATAAAACAAAAAACAAAAATCCTACCTACCA        |
|                                    | TCCACTAACAACCACCATACCTACCTACAACTTATCCACTAC           |
|                                    | AAAATAAAAATACACATTCACCATCAAACAAAAACCTATATAAAAAT       |
|                                    | TTATCTACCCCTTCTTCCTCTCTCTTATTTAAATAAAAACCCCAA         |
|                                    | TACATTACCAACCATCAATCAAAAGAAAAAACATTCAA                |
|                                    | ATCCAAAAATATCAAACAAAAACAAAAATAAAAAACCCCAATATAAA      |
|                                    | AAAACATATCTATATACAAAAAAAACACTCCATACCAACTTCT          |
|                                    | AAAAAACATACAAAAAAAACAAAAAAAABAAACACAAAAATAAAAAAATCAA |
|                                    | AACTAACAACCAACACACCTCAGAATTTATACACCAC                 |
|                                    | TACAAACAAACACACACACACACACACACACACACACACACACAC       |

**Ctla4, and Iкzf4** (Polansky et al., 2008; Ohkura et al., 2012; Sakaguchi et al., 2020). On the other hand, naïve T conventional (Tconv) cells showed almost completely methylated Foxp3-CNS2 region (Figure 4A). Thus, naïve Tconv cells would be a precise control sample for the basal methylation status. Effector Tconv cells maintained fundamentally similar methylation status as naïve Tconv cells (data not shown). In female cells, X-chromosome inactivation controls the activation status on X-chromosomes functionally and structurally (Bacher et al., 2006; Loda et al., 2022). Foxp3 is an X-linked gene, and regulated by the same mechanism (Nunzio et al., 2009). Analyzing Foxp3 methylation status, female nTreg cells have theoretically 50% methylated Foxp3-CNS2 region (Figure 4B). In the past decade, iTreg cells had been thought as quite unstable in not only Foxp3 expression level but also Foxp3-CNS2 demethylation status (Ohkura et al., 2013). However, Mikami et al. developed the procedure to produce stable iTreg cells, based on the Foxp3-CNS2 demethylation status (Mikami et al., 2020). In addition, Foxp3 expression can be enhanced by supplementing retinoic acids into culture medium (Mucida et al., 2007). In this paper, we also induced stable iTreg cells following the previous several works using ascorbic acid and Tamibarotene as synthetic retinoic acid (Nair et al., 2016; Yue et al., 2016). Adding ascorbic acid trisodium salt into the culture medium, we confirmed the capacity to confer the Foxp3-CNS2 demethylation upon the induced iTreg cells while increased Foxp3 expression by retinoid didn’t establish stable Foxp3-CNS2 epigenome (Figure 5).
Problem 1
Seeing less pellet of fixed cells. (Identified at step 15b of “cell staining and sorting”).

Potential solution
Fixed cells are also permeabilized by step a. Permeabilized cells reduced their density and weight, so we can easily lose the cell in steps 15b–15g. Please use centrifuge with high gravity for longer time and wash the cells carefully.

Problem 2
Seeing non-specific stained cell leaking to FITC (eGFP) range. (Identified at step 17 of “cell staining and sorting”).

Potential solution
Granulocytes or red blood cells would cause this problem. Generally, granulocytes such as eosinophils contain a bunch of intracellular granules to emit non-specific fluorescence and have large cell bodies. As known, red blood cell sneak into various detector of fluorescence. These cells could be removed by Mouse CD4 T Lymphocyte Enrichment Cocktail (containing clone M1/40 for

Figure 4. Methylation assay for ex vivo naïve Tconv and nTreg cells
(A) Methylation status in Foxp3-CNS2 region of naïve Tconv and nTreg cells derived from male Foxp3-eGFP mice. White circles indicate demethylated CpGs and black circles indicate methylated CpGs.
(B) Data derived from female Foxp3-eGFP mice. Barplots and dotplots are represented as mean ± SEM.
granulocytes and Ter119 for red blood cells) in this protocol. Please reduce the size of P1 gating or add increased amount of this cocktail for effective negative selection.

**Problem 3**
Weak or no band in electrophoresis. (Identified at step 43 of “bisulfite PCR”).

**Potential solution**
There are various possibilities. If you start this procedure with more than 10,000 cells, you can see the pellets after step 18. If not, you should increase input of initial cell number, check the step in gDNA extraction, or increase input of sample into bisulfite PCR. Insufficient bisulfite conversion or desulfonation would lead incomplete annealing of bisulfite PCR primers. Please inspect bisulfite conversion steps. For example, you should treat sample with bisulfite solution for longer time in step 32.

**Problem 4**
Few or no white colony in TA cloning. (Identified at step 56 of “colony PCR”).

**Potential solution**
Insufficient PCR products or ligation are supposed. Please check the procedure in gel extraction and ligating time. If impossible to see any colony, please check steps in DH5α transformation and optimize preculture and overnight culture, for instance, to do preculture for longer time to enable DH5α to recover from heat shock.

**Problem 5**
Seeing a lot of guanines in the analyzing step. (Identified at step 75 of “sequencing”).

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**Figure 5. Methylation assay for iTreg cells**
(A) Representative data of FACS plots of day 3 induced iTreg cells and Foxp3-CNS2 methylation status with adding A2P as ascorbic acid or Tamibarotene as synthetic retinoid to iTreg inducing culture medium. White circles indicate demethylated CpGs and black circles indicate methylated CpGs.

(B) iTreg induction rate in each condition adding A2P or Tamibarotene.

(C) Foxp3-CNS2 methylation status in each condition. Barplots and dotplots are represented as mean ± SEM.
Potential solution
If impossible to get bisulfite-converted data like Table 10, insufficient bisulfite conversion is possible. Please check bisulfite conversion steps. For example, you should treat sample with bisulfite solutio for longer time in step 32.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masaya Arai (marai@ifrec.osaka-u.ac.jp).

Materials availability
This study did not generate any unique reagents.

Data and code availability
This protocol did not in itself generate any data or code availability.

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AUTHOR CONTRIBUTIONS
M.A. outlined and optimized protocol methods, designed the project, performed the experiments, and wrote the manuscript with contributions from all authors. A.F., R.M., Y.N., and Z.C. tested protocol and generated data for write-up. M.A. and S.S. supervised this protocol paper. All authors were involved in manuscript writing and generation.

DECLARATION OF INTERESTS
The authors declare no competing interests associated with this manuscript.

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