Detailed protocol — Chromatin Integration labeling

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Method Article

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Abstract

Chromatin plays a crucial role in gene regulation, and chromatin immunoprecipitation followed by sequencing \(ChIP-seq) has been the standard technique for examining protein–DNA interactions across the whole genome. However, it is difficult to obtain epigenomic information from limited numbers of cells by ChIP-seq because of sample loss during chromatin preparation and inefficient immunoprecipitation. In this study, we established an immunoprecipitation-free epigenomic profiling method named Chromatin Integration Labeling \(ChIL), which enables the amplification of genomic sequences closely associated with the target molecules, before cell lysis. Using ChIL followed by sequencing \(ChIL-seq), we reliably detected the distributions of histone modifications and DNA-binding factors in 100 to 1,000 cells. In addition, ChIL-seq successfully detected genomic regions associated with histone marks at the single-cell level. ChIL-seq thus offers an alternative method to ChIP-seq for epigenomic profiling using small numbers of cells, in particular those attached to culture plates and after immunofluorescence.

Introduction

For identifying protein–DNA interactions across the genome, ChIP-seq has been used as the standard technique. The original ChIP-seq method required typically a million of \(at least 10^4) cells, because samples can be lost during chromatin preparation and immunoprecipitation, particularly when using crosslinked chromatin[1-4]. To start with smaller numbers of cells, several low-input methods have been developed[5-19], e.g. by using improved micrococcal nuclease treatment[7], transposase tagging[11], T7 RNA polymerase-based linear amplification[14], adaptor ligation[15, 16, 18], droplet technologies[10], and a special microfluidic device[8]. Recently, an immunoprecipitation-free CUT&RUN technique, based on the chromatin immunocleavage \(ChIC)20, has been reported as a convenient and efficient low-input method[17, 19, 20]. While an ultimate goal of low-input approach is to establish single-cell epigenome/protein-binding profiling, even the method thus far closest to this goal still suffers from low genome coverage[10]. Here we show ChIL \(Chromatin Integration Labeling) technique, which combines immunostaining, transposase tagging, and linear amplification, can be useful for low-input epigenome/protein-binding profiling even from single cells. ChIL also opens a way to link immunofluorescence and epigenome information in the same cells.

Reagents

- Cell lines of interest - DMEM \(Nacalai Tesque, 08458-16) - L-Glutamine–Penicillin–Streptomycin solution \(Sigma Aldrich, G6784) - FBS \(Gibco, 10270-106) - 2.5g/l-Trypsin/1mmol/l-EDTA solution \(Nacalai Tesque, 3555464) - 10x D-PBS\(-) \(Wako, 048-29805) - 16% Paraformaldehyde \(Electron Microscopy Sciences, 15710-S) - Polyethylene Glycol Mono-p-isooctylphenyl Ether \(TritonX-100) \(Nacalai Tesque, 2598785) - Blocking One-P \(Nacalai Tescue, 05999-84) - DBCO-PEG5-NHS Ester \(Click Chemistry Tools, A102P-2) - 2-Amino-2-hydroxymethyl-1,3-propanediol \(Tris) \(Wako, 204-07885) - N-\(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic Acid \(HEPES) \(Nacalai Tesque, 1751415) - N-Tris\

(hydroxymethyl)methyl-3-aminopropanesulfonic Acid \(TAPS) \(Nacalai Tesque, 0894182) - 0.5 M Ethylenediamine-N,N,N',N'-tetraacetic Acid \(EDTA) solution \(Dojindo, MB01) - Sodium Chloride \(NaCl) \(Ncalai Tesque, 3132005) - Sodium Hydrogen Carbonate \(NaHCO3) \(Nacali Tesque, 3121315) - Sodium Dodecyl Sulfate \(SDS) \(Wako, 191-07145) - Dithiothreitol \(DTT) \(Wako, 042-29222) - Dimethyl Sulfoxide \(DMSO) \(Wako, 041-29351) - N,N-dimethylformamide \(DMF) \(Nacalai Tesque, 1301694) - 1 M Magnesium chloride \(MgCl2) solution \(Nacalai Tesque, 2094234) - Glycerol \(Nacalai Tesuque, 1703865) - Spermidine \(Wako, 195-09821) - Mouse anti-H3K4me3 \(CMA304 16H10)[21] - Mouse anti-H3K27me3 \(CMA323 1E7)[22] - Mouse anti-H3K27ac \(CMA309 9E2H10)[21] - Rabbit anti-CTCF \(Abcam, ab70303) - Rabbit anti-MyoD \(Santa Cruz ,sc-760, M-318) - AffiniPure Donkey Anti-Mouse IgG \(H+L) \(Jackson Immuno Research, 715-005-150) - AffiniPure Donkey Anti-Rabbit IgG \(H+L) \(Jackson Immuno Research, 711-005-152) - Tn5 \(in house)[23] - T4 DNA Ligase \(NEB, M0202L) - T4 DNA Polymerase \(NEB, M0203L) - T7 RNA Polymerase \(Thermo Fisher Scientific, AM2085) - Ribonucleoside 5'-Triphosphates \(NTP) \(Takara, 4041, 4042, 4043, 4044) - Recombinant RNase Inhibitor \(Takara, 2313A) - Recombinant DNase I \(RNase-free) \(Takara, 2270A) - SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing \(Takara, 634891) - Agencourt AMPure XP beads \(Beckman Coulter, A63881) - E-Gel Size Select II Agarose Gels, 2% \(Thermo Fisher Scientific, G661012) - DNA Standards for Library Quantification \(Takara, 638325) - Oligo DNAs \(Table 1) - PD MiniTrap G-25 \(GE Healthcare, 28918007) - Amicon Ultra-0.5 ml Centrifugal Filters 10k-off \(Merck Millipore, UFC501096) - Amicon Ultra-0.5 ml Centrifugal Filters 100k-off \(Merck Millipore, UFC510096) - Microcon Ultracel DNA Fast Flow Membrane \(Merck Millipore, MRCF0R100) - Magnetic rack for AMPure XP beads - MinElute PCR Purification Kit \(Qiagen, 28004) - RNeasy MinElute Cleanup Kit \(Qiagen, 74204) - Agilent High Sensitivity DNA Kit \(Agilent, 5067-4626) - Library Quantification Kit \(Takara, 638324)

Equipment

Equipment - Cell culture standard consumables - µ-Plate 96-well TC \(ibiTreat) \(ibidi, ib89626) - Protein LoBind 1.5 ml tube \(Eppendorf, 0030108116) - 0.2 ml 8-strip PCR tube \(Thermo Fisher Scientific, N8010580, N8010535) - Fluorescence \(confocal) Microscope - E-Gel Electrophoresis system \(Thermo Fisher Scientific, G6512ST) - Nanodrop \(Thermo Fisher Scientific) - qPCR \(Agilent, MX300p) - Bioanalyzer 2100 \(Agilent) - Centrifuge \(Eppendorf) - Thermal cycler \(Eppendorf) - Rotator \(Taitec) - Heat block incubator \(Taitec)

Procedure

Reagents Setup • 2x Tn5 dialysis buffer 100 mM HEPES-KOH \(pH7.2) 200 mM NaCl 0.2 mM EDTA 20% Glycerol 0.2% TritonX-100 2 mM DTT 1 M 20 µl Aliquot 1-2 ml each, store at -30° C • 5x TAPS-DMF buffer 50 mM TAPS-NaOH \(pH8.5) 25 mM MgCl2 50% DMF Aliquot 1-2 ml each, store at -30° C • 10x T7 RNA Polymerase buffer 400 mM Tris-HCl \(pH8.0) 80 mM $MgCl_2$ 20 mM Spermidine 50 mM DTT Aliquot 1-2 ml each, store at -30° C • DBCO-PEG5-NHS Ester Suspend 2 mg with 100 µl of DMSO \(28.8 mM), aliquot 20 µl each and store at -30° C • 10x annealing buffer 100 mM Tris-HCl \(pH7.4) 10 mM EDTA 1 M

NaCl Store at room temperature • Antibody labeling with DBCO-PEG5-NHS Ester Mix antibody with DBCO-PEG5-NHS Ester in a Protein LoBind tube * conjugation is performed with a molar ratio of about 1:10 IgG 100 µg $NaHCO_3$ \(1 M, pH8.3) 10 µl DBCO-PEG5-NHS Ester \(28.8 mM) 0.2 µl PBS upto 100 µl ↓ Incubate the sample at room temperature for 1 h by rotating the tube During this step, equilibrate the a PD MiniTrap G-25 desalting column with 2.5 ml of PBS, three times ↓ Apply the sample into the column Add 500 µl of PBS and discard the flow through ↓ Place the column onto a new 1.5 ml tube Add 500 µl of PBS and recover the flow through ↓ Transfer the sample into an Amicon Ultra-0.5 NMWL 10kDa centrifugal filter Centrifuge at 14,000 xg for 20 min at 4° C ↓ Recover the sample \(~25 µl) into a new 1.5 ml tube Add ~50 µl of PBS ↓ Measure the concentration of IgG and DBCO using Nanodrop * molar extinction coefficiency of DBCO = 12,000 M^{-1} cm^{-1} at 309 nm Typically approximately three DBCO per antibody ↓ Dilute to 1 mg/ml IgG with PBS Store at 4 ° C • ChILT DNA Mix oligo DNAs in a 0.2 ml PCR tube ChILT primer_Fw-azido \(100 µM) 10 µl ChILT primer_Rv-Ph,TMR \(100 µM) 10 µl 10x annealing buffer 10 µl Nuclease-free water 70 µl Total 100 µl ↓ Thermal Cycler: incubate 95° C 5 min → lowering the temperature by 0.1oC/s to 20oC Store at -30° C • Oligonucleotide conjugation with antibody Mix DBCO-PEG5 labeled-IgG with ChILT DNA in a Protein LoBind tube * conjugation is performed with a molar ratio of 1:2 DBCO-PEG5 labeled-IgG \(1 mg/ml) 75 µl ChILT DNA \(annealed, 10 µM) 100 µl ↓ Incubate the sample at 4° C for 1 week by rotating the tube * Cu-free click reaction with azido-DNA is much slower than classical click reaction ↓ Apply the sample into a Microcon Ultracel DNA Fast Flow Membrane Add 400 µl of PBS Centrifuge at 500 xg for 20 min at 4° C ↓ After discarding the flow through, add 400 L of PBS Centrifuge at 500 xg for 20 min at 4° C Repeat this step 4 times ↓ Transfer the sample \(~100 µl) into an Amicon Ultra-0.5 NMWL 100kDa centrifugal filter Centrifuge at 14,000 xg for 20 min at 4° C ↓ Recover the sample \(~25 µl) into a new 1.5 ml tube Adjust the volume to 85 µl \(two-fold concentrated) with PBS Store at 4° C **ChILT work flow** **\(Day 1)** Cell sorting, plating Plate cells on a 96-well ibidi plate Single cell by cell sorter ≥100 cells by manual ↓ Incubate at 37° C in a humidified atmosphere of 5% CO_2 overnight ↓ **\(Day 2)** immunostaining After removing the medium, add 100 µl of 1% formaldehyde/DMEM Incubate at room temperature for 5 min, without shaking ↓ After removing the formaldehyde, wash the cells with 200 µl of PBS ↓ After removing PBS, add 150 µl of 1% Triton/PBS Incubate at room temperature for 20 min with gentle shaking ↓ After removing Triton, wash the cells with 200 µl of PBS ↓ After removing PBS, add 100 µl of Blocking One-P Incubate at room temperature for 20 min with gentle shaking ↓ After removing Blocking One-P, wash the cells with 200 µl of PBS ↓ After removing PBS, add 100 µl of primary antibody \(2 µg/ml) in 0.1x Blocking One-P/PBS Incubate at room temperature for 6 h with gentle shaking ↓ After removing the primary antibody, wash the cells with 200 µl of PBS Gently shaking at room temperature for 5 min, three times ↓ Plate on ice ↓ After removing PBS, add 100 µl of ChILT probe \(1 µg/ml) in 0.1x Blocking One-P, 0.5 M NaCl/PBS \(ice-cold) Incubate at 4° C overnight with gentle shaking ↓ **\(Day 3)** ChILT reaction After removing the ChILT probe, wash the cells with 200 µl of PBS \(ice-cold) Gently shaking at 4° C for 20 min, three times ↓ After removing PBS, add 50 µl of Tn5 \(90 ng/well; 0.883 mg/ml 0.1 µl/well) in 1x Tn5 dialysis buffer Incubate at room temperature for 10 min with gentle shaking ↓ Without removing Tn5, add 50 µl of MEDS-B \(10 µM, 0.1 µl/well) in 1x Tn5 dialysis buffer Incubate at room temperature for 1 h with gentle shaking ↓ After removing the supernatant, wash the cells with 100 µl of PBS Gently shaking at room temperature for 5

min, three times ↓ After removing PBS, wash the cells with 100 µl of 1x Tn5 dialysis buffer ↓ After removing the buffer, add 100 µl of 1x TAPS-DMF buffer Incubate at 37° C for 1 h with gentle shaking ↓ After removing the buffer, add 100 µl of 0.2% SDS Incubate at room temperature for 10 min, without shaking ↓ After removing SDS, wash the cells with 100 µl of PBS, three times, without interval ↓ After removing PBS, wash the cells with 100 µl of 1x T4 DNA Ligase buffer ↓ Prepare fill-in solution \(according to the number) Nuclease-free water 88.5 µl 10x T4 DNA Ligase buffer 10 µl dNTP mix \(10 mM) 0.5 µl T4 DNA ligase 0.5 µl T4 DNA Polymerase 0.5 µl Total 100 µl After removing the buffer, add 100 µl of fill-in solution Incubate at room temperature for 30 min with gentle shaking ↓ After removing the supernatant, add 100 µl of 0.2% SDS Incubate at room temperature for 10 min, without shaking ↓ After removing SDS, wash the cells with 100 µl of PBS, three times, without interval ↓ After removing PBS, wash the cells with 100 µl of 1x T7 RNA Polymerase buffer ↓ Prepare in situ transcription solution \(according to the number) Nuclease-free water 80 µl 10x T7 RNA Polymerase buffer 10 µl 25 mM each NTP 8 µl RNase Inhibitor 1 µl T7 RNA Polymerase 1 µl Total 100 µl After removing the buffer, add 100 µl of in situ transcription solution Seal the wells with parafilm Incubate at 37° C overnight with gentle shaking ↓ **\(Day 4)** library preparation: SMART-seq v4 Add 1 µl of DNaseI Incubate at 37° C for 30 min with gentle shaking ↓ Purify the ChILT RNA by RNeasy MinElute Cleanup Kit

_____ Recover the supernatant into a 1.5 ml tube ↓ Add 350 µl of Buffer RLT into the well and transfer this rinsed fraction to the same tube ↓ Add 250 µl of EtOH and mix well by pipetting up and down ↓ Transfer the sample into RNeasy MinElute Spin Column Centrifuge at 13,000 rpm for 1 min ↓ Place the column in a new collection tube Add 500 µl of Buffer RPE Centrifuge at 13,000 rpm for 1 min ↓ After discarding the flow through, add 500 µl of 80% EtOH Centrifuge at 13,000 rpm for 2 min ↓ After discarding the flow through, centrifuge again at 13,000 rpm for 1 min with the lid opened ↓ Place the column on a new 1.5 ml tube and add 10 µl of Nuclease-free water directly to the membrane After waiting for 1 min, centrifuge at 13,000 rpm for 1 min ↓ Recover the flow through and add again to the membrane After waiting for 1 min, centrifuge again at 13,000 rpm for 1 min _____ ↓ SMAT-seq v4 using custom primers

_____ • Primer annealing Prepare the reaction buffer \(according to the number, for 10 samples below) 10x Lysis buffer 4.75 µl Read2 primer \(12 µM) 5 µl RNase Inhibitor 0.25 µl Total 10 µl Mix ChILT RNA and the reaction buffer in a 0.2 ml 8-strip PCR tube RNA Sample 5.25 µl Reaction buffer 1 µl Total 6.25 µl ↓ Thermal Cycler: incubate 72° C 3 min → on ice ↓ • First-strand cDNA Synthesis Prepare the master mix \(according to the number) 5x Ultra Low First-Strand Buffer 2 µl SMART-Seq v4 Oligonucleotide \(48 µM) 0.5 µl RNase Inhibitor 0.25 µl SMART Scribe Reverse Transcriptase 1 µl Total 3.75 µl Add the master mix into the RNA sample RNA sample 6.25 µl Master mix 3.75 µl Total 10 µl ↓ Thermal Cycler: incubate 42° C 1.5 h → 70° C 10 min → 4° C • cDNA Amplification Prepare the master mix \(according to the number) 2x SeqAmp PCR Buffer 12.5 µl Ad1 primer \(12 µM) 0.5 µl SeqAmp DNA Polymerase 0.5 µl Nuclease-free water 1.0 µl Total 14.5 µl Mix ChILT cDNA, Ad2 primer and the master mix in a new 0.2 ml 8-strip PCR tube First-strand cDNA 10 µl Ad2 primer \(12 µM) 0.5 µl Master mix 14.5 µl Total 25 µl ↓ Thermal Cycler: 95° C 1 min → \(98° C 10 sec → 65° C 30 sec → 68° C 3 min) →72° C 10 min → 4° C

_____ ↓ **\(Day 5)** library purification Incubate the AMPure XP beads at room temperature and mix well by vortexing before use _____ Add 10x Lysis buffer and AMPure XP beads to the amplified ChILT DNA Amplified DNA Sample 25 µl 10x Lysis buffer 0.5 µl AMPure XP beads 25 µl Total 50 µl Mix well by pipetting up and down and incubate for 5 min ↓ Place on a magnetic rack and wait for 5 min ↓ Discard the supernatant \(45 µl) ↓ Wash the beads with 200 µl of 80% EtOH, twice After incubating for 30 sec, discard the supernatant ↓ Wait for ≦5 min. Take care not to over dry ↓ Outside the rack, Add 27 µl of EB buffer \(Qiagen) and mix well by pipetting up and down Incubate for 2 min ↓ Place on the rack and wait for 5 min ↓ Recover the supernatant \(25 µl) into a new 0.2-ml PCR tube ↓ Repeat again the purification with 25 µl of AMPure XP beads * 10x Lysis buffer is not required in second purification ↓ Recover the elute \(25 µl) into a new tube _____ ↓ * option Pool and purify the samples by MinElute PCR Purification Kit _____ Mix the samples and adjust the volume to 100 µl with Nuclease-free water ↓ Add 500 µl of Buffer PB and mix well by pipetting up and down ↓ Transfer the sample into a MinElute column Centrifuge at 13,000 rpm for 1 min ↓ After discarding the flow through, add 700 µl of Buffer PE Centrifuge at 13,000 rpm for 1 min ↓ After discarding the flow through, centrifuge again at 13,000 rpm for 1 min with the lid opened ↓ Place the column on a new 1.5 ml tube and add 25 µl of Buffer EB After waiting for 1 min, centrifuge at 13,000 rpm for 1 min _____ ↓ • Size selection using E-Gel Electrophoresis system with E-Gel Size Select II agarose gel Recover 250-350 bp fragments ↓ Purify the size selected samples by MinElute PCR Purification Kit _____ Add 5 volumes of Buffer PB to 1 volume of the sample and mix well ↓ Transfer the sample into MinElute column Centrifuge at 13,000 rpm for 1 min ↓ After discarding the flow through, add 700 µl of Buffer PE Centrifuge at 13,000 rpm for 1 min ↓ After discarding the flow through, centrifuge again at 13,000 rpm for 1 min with the lid opened ↓ Place the column on a new 1.5 ml tube and add 10 µl of Buffer EB After waiting for 1 min, centrifuge at 13,000 rpm for 1 min _____ ↓ • Quantification of the ChILT library Bioanalyzer \(DNA high sensitivity kit) qPCR \(TaKaRa Library Quantification Kit)

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Figures

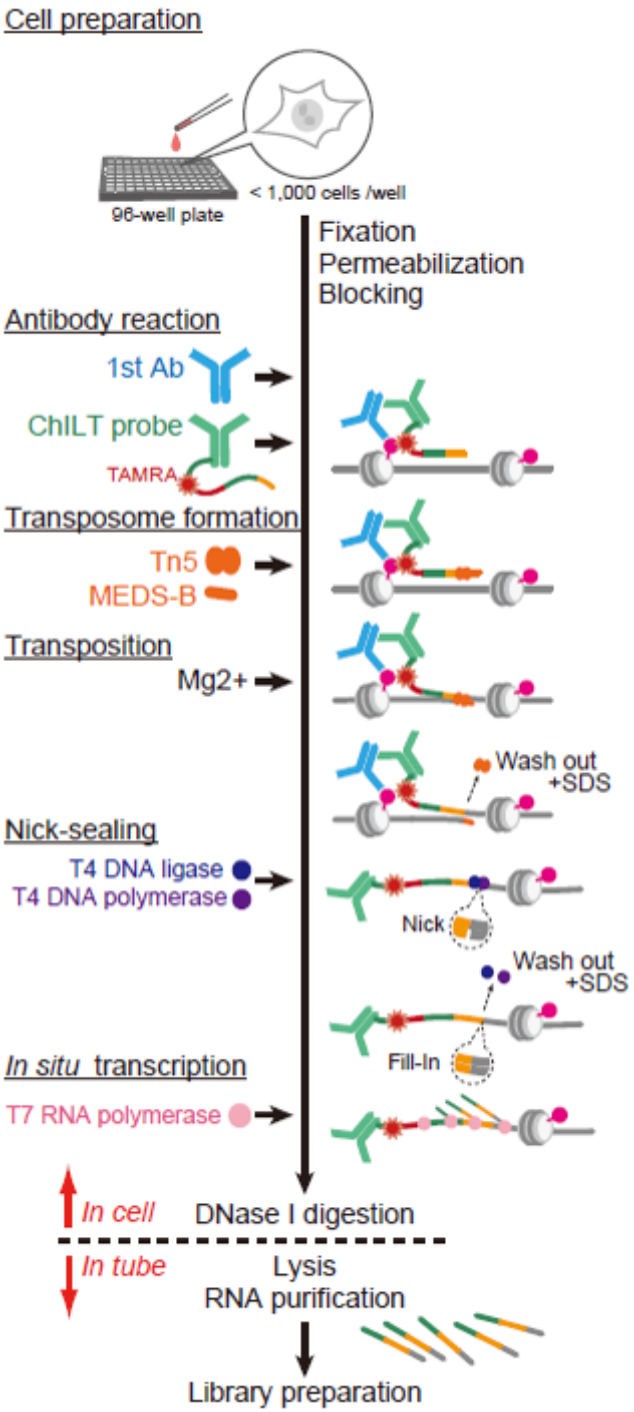

Figure 1

Fig.1 ChIL workflow Fig.1 ChIL workflow

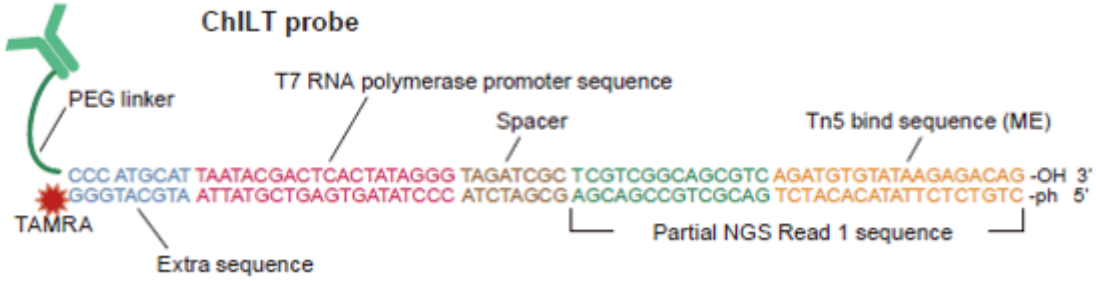

Figure 2

Fig.2 ChIL probe Fig.2 ChIL probe

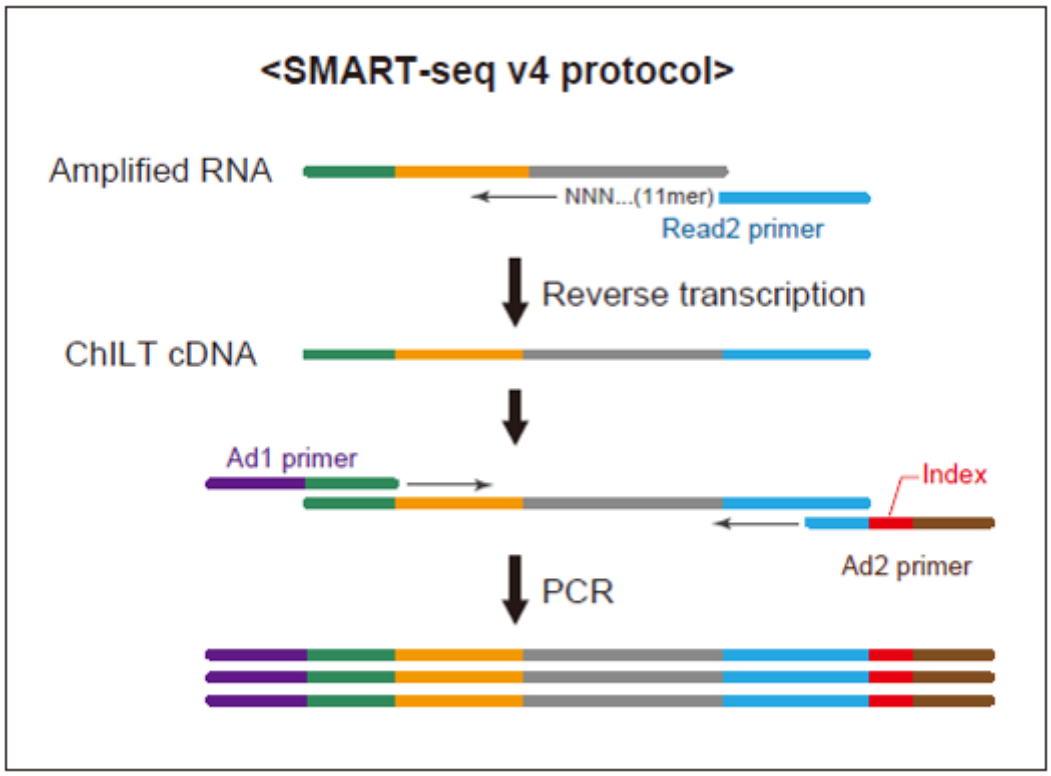

Figure 3

Fig.3 ChIL library Fig.3 ChIL library

Supplementary Files

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- supplement0.xlsx