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## Identification of RNA–DNA Hybrids Associated with R-Loops at the *IgH* Switch Sequence in Activated B Cells

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### Abstract

During transcription and replication, R-loops that contain RNA–DNA hybrids are generated across numerous genomic loci and contribute to many biological events. Using S9.6, a monoclonal antibody against RNA–DNA hybrids, accelerated the study of R-loop biology. An outpouring of recent studies has implicated various contributions of R-loop in physiological cellular functions. Earlier studies using nondenaturing sodium bisulfite probing also supported existence of DNA–RNA hybrids formation in mammalian cells. In activated B cells, RNA–DNA hybrids formation at *IgH* gene locus of B cells is crucial for class switch recombination that ensure the proper effector function of the antibody. Here, we describe the identification of R-loops associated with the *IgH* locus using RNA–DNA hybrid immunoprecipitation sequencing and nondenaturing sodium bisulfite probing. This will be helpful for future studies of R-loops status on whole genome as well as on *IgH* locus in B cells.

### Keywords

Class switch recombination; B cells; DRIP-seq; Nondenaturing sodium bisulfite probing

## 1 Introduction

R-loop is a triple stranded nucleic acid structure composed of double stranded RNA–DNA hybrid and a displaced single-stranded DNA. RNA–DNA hybrids are known to be more stable than double strand DNA [1, 2] of the same sequences. R-loops are usually

generated during transcription, and distributed widely across the genome. R-loop formation and stability depend on negative supercoiling [3–5], skewed GC content between the two DNA strands [6, 7], G-quadruplex formation potential of the displaced strand [8, 9], and other factors that are still poorly characterized. Since in vitro R-loop structure forming under the 70% formamide condition was first reported in 1976 [10], many studies have detected R-loops formation in vivo from different organisms. R-loops have been regarded as byproducts during transcription in the early days, however, later studies have uncovered their diverse influence on physiological cellular functions. Programmed R-loop formation is involved in initiation of mitochondrial DNA replication [11, 12], DNA repair [13, 14], transcription initiation/elongation/termination [15–17], epigenetic regulation [18, 19], and immunoglobulin heavy chain class switch recombination (as in Fig. 1) [20–23]. Accumulation of R-loop structures can induce replication stress, chromatin alteration and genomic instability by unexpected double strand DNA breaks [18, 20, 24–27]. These imply that R-loops should be associated with various diseases including cancer. The level of R-loop formation at all specific regions on the genome are tightly regulated under physiological conditions. Recent studies have reported that alterations of RNA–DNA hybrids homeostasis are associated with human diseases, and identified many factors for balancing R-loop levels to maintain beneficial R-loops and/or to remove harmful R-loops (reviewed in [28, 29]). A large number of studies provided genome-wide mapping of R-loops using DNA–RNA hybrids precipitation (DRIP) with the S9.6 monoclonal antibody [30] and high throughput sequencing. In the past decade, methods for mapping of R-loops based on DRIP and NGS have been developed continuously for improved accuracy and resolution, and have helped a deeper understanding of R-loop functions in the nuclear processes. Here, we describe a basic method to extract genomic DNA and isolate fragments containing R-loops, as well as nondenaturing bisulfite probing following by DNA sequencing that provides near nucleotide resolution of the R-loop structure.

## 2 Materials

### 2.1 DRIP-Seq

1. Lipopolysaccharides from *Escherichia coli* O111:B4 (LPS): 20 mg/mL solution in water.
2. Recombinant murine Interleukin-4 (IL-4): 20 µg/mL solution in phosphate buffered saline.
3. Activated B cells (~70% confluent): cultured with 20 µg/mL of LPS and 20 ng/mL of IL-4.
4. Phosphate buffered saline (PBS): 150 mM sodium phosphate and 150 mM NaCl [pH 7.2].
5. 1× TE buffer: 10 mM Tris-Cl (pH 8.0), 1 mM EDTA.
6. 20% sodium dodecyl sulfate (SDS) solution in water.
7. Proteinase K.
8. Phenol–chloroform–isoamyl alcohol (25:24:1) (pH 8.0).

9. Chloroform.
10. 3 M NaOAc (pH 5.2).
11. Isopropanol.
12. 100% ethanol, 80% ethanol.
13. DNase/RNase-free water.
14. Restriction enzymes: BamHI, EcoRI, HindIII, NcoI, SpeI, XbaI.
15. RNase H.
16. RNase III.
17. 10× Antibody binding buffer: 100 mM NaPO<sub>4</sub> (pH 7.0), 1.4 M NaCl, 0.5% Triton X-100.
18. S9.6 antibody.
19. Protein A/G magnetic beads.
20. Glycogen.
21. Qubit dsDNA HS Assay Kit.
22. PCR primers for validation of IP quality.  
S<sub>μ</sub>\_P1\_Fwd (GAGTACCGTTGTCTGGGTCACCTCAC).  
S<sub>μ</sub>\_P1\_Rev (CTGAAGTAGAGACAGCATCAGTACCTC).  
S<sub>μ</sub>\_P2\_Fwd (ATCAGTAAGGAGGGACCCAGGCT).  
S<sub>μ</sub>\_P2\_Rev (CTCGGATCTAAGCACTGTCCTTTGA).
23. PCR primers for spike-in control.  
Segmented Filamentous Bacteria\_Fwd (GACGCTGAGGCATGAGAGCAT).  
Segmented Filamentous Bacteria\_Rev (TGAATAACAATCCGTGCCGTC).  
*Helicobacter Pylori*\_Fwd (CTTAACCATAGA AACTGCATTTGA).  
*Helicobacter Pylori*\_Rev (ATACTCATTGCGAAGGCGACC).  
Universal Bacterial Primers\_Fwd (ACTCCTACGGGAGGCAGCAGT).  
Universal Bacterial Primers\_Rev (GCCAGCAGCCGCGGTAAT).  
*Lactobacillus* on gut bacterial DNA \_Fwd (AGCAGTA GGAATCTTCCA).  
*Lactobacillus* on gut bacterial DNA \_Rev (CTCCATGTG-TAGCGGTG).
24. QIAquick Gel Extraction Kit.
25. Bioruptor Plus sonication device (Diagenode).
26. NEBNext Ultra II DNA Library Prep Kit (NEB).
27. NEB next adaptor for Illumina (NEB).

28. AMPure XP Beads (Beckman Coulter).
29. 2100 Bioanalyzer instrument (Agilent).

## 2.2 Nondenaturing Sodium Bisulfite Probing

1. Denature solution 0.2 N NaOH (add 20  $\mu$ L of 1 N NaOH into 80  $\mu$ L of water).
2. Neutralizing solution (200 mM Tris-HCl, pH 7).
3. Sodium hydroxide.
4. Hydroquinone.
5. Sodium bisulfite.
6. DNA purification resin (Promega), or DNA spin columns (Zymo Research).
7. Microcentrifuge.
8. Rotate mixer.
9. Fresh 2 M NaOH (1.25 mL): Dissolve 0.1 g of NaOH in 1.2 mL of MilliQ water (or weigh a pellet of NaOH and dissolve it in an appropriate amount of water).
10. Fresh 20 mM hydroquinone (1 mL). Dissolve 2.2 mg hydroquinone per mL of water.
11. Fresh 2.5 M Sodium Bisulfite, pH 5.2 (1 mL). Weigh 0.476 g of sodium bisulfite. Add 250  $\mu$ L of 2 M NaOH. Add water to 1 mL. Vortex vigorously for ~1 min (*see* Note 1).

## 3 Methods

### 3.1 DNA–RNA Hybrid Immunoprecipitation and Sequencing

#### 3.1.1 Nucleic Acids Extraction from Activated B Cells

1. Transfer  $5 \times 10^6$  cells (from 70% confluent cultured cells) to 15 mL conical tube.
2. Pellet cells at  $300 \times g$  for 5 min at room temperature (RT) and remove supernatant.
3. Resuspend cells with 10 mL  $1 \times$  PBS and pellet cells again. Repeat **step 2**.
4. Resuspend cells completely with 1.6 mL  $1 \times$  TE and split cells into two 2 mL tubes containing 20  $\mu$ L of 20% SDS and add 5  $\mu$ L of proteinase K. Invert immediately each tubes several times and incubate them at 37 °C for overnight (*see* Note 2).
5. Add 800  $\mu$ L (1 vol) of phenol–chloroform–isoamyl alcohol (PCI) to incubated cell lysate tubes and invert them carefully several times (*see* Note 3).
6. Centrifuge at  $13,000 \times g$  for 5 min and transfer nucleic acids on aqueous upper phase to new 2 mL tubes (*see* Note 4).

7. Repeat **steps 5** and **6**, and repeat **steps 5** and **6** with 800  $\mu\text{L}$  (1 vol) chloroform instead of PCI.
8. Add 80  $\mu\text{L}$  of 3 M NaOAc (pH 5.2) and 800  $\mu\text{L}$  of isopropanol to each tube. Invert two tubes until forming white colored DNA clumps.
9. Take nucleic acids clumps by using cut tip from two tubes and combine them into a new 2 mL tube. Add 1.5 mL of fresh 70% EtOH and invert the tube gently several times. Incubate tube at RT for 5 min. Repeat this **step 3** times.
10. Transfer nucleic acids clump into a new tube and allow to air-dry. Add 100  $\mu\text{L}$  of 1 $\times$  TE and allow to dissolve at RT for 30 min (*see Note 5*).
11. Add 100  $\mu\text{L}$  of 2 $\times$  restriction enzyme cocktail and invert tube several times.
12. Incubate nucleic acids extract at 37  $^{\circ}\text{C}$  for 4 h and add 200  $\mu\text{L}$  of 1 $\times$  restriction enzyme cocktail.
13. Invert tube several times and incubate at 37  $^{\circ}\text{C}$  for overnight.
14. Add 1 vol PCI and invert tube several times and centrifuge at 13,000  $\times g$  for 5 min.
15. Transfer nucleic acids on aqueous phase into a new 1.5 mL tube and add 1 vol chloroform. Invert tube several times followed by centrifuge at 13,000  $\times g$  for 5 min.
16. Transfer nucleic acids on aqueous phase into a new 1.5 mL tube and add 0.1 vol of 3 M NaOAc (pH 5.2) and 2 vol of 100% ethanol. After at least 2 h incubation at  $-20^{\circ}\text{C}$ , centrifuge at 4  $^{\circ}\text{C}$ , 13,000  $\times g$  for 20 min.
17. Wash pellet with 70% ice-cold EtOH by centrifuge 13,000  $\times g$  at 4  $^{\circ}\text{C}$  for 5 min. Air-dry pellet.
18. Resuspend nucleic acids pellet with 200  $\mu\text{L}$  of 1 $\times$  TE and split into two new 1.5 mL tubes (*see Note 6*).
19. Add 5  $\mu\text{L}$  RNase H into one aliquot tube and 1  $\mu\text{L}$  of RNase III (to remove dsRNAs) into both aliquot tubes in 1 $\times$  RNase H buffer condition. Incubate at 37  $^{\circ}\text{C}$  overnight (*see Note 7*).
20. Repeat **steps 14–17**.
21. Resuspend nucleic acids pellet with 100  $\mu\text{L}$  of 1 $\times$  TE and measure nucleic acids concentration of each RNase H treated or untreated nucleic acids.

### 3.1.2 DNA–RNA Hybrids Immunoprecipitation

1. Take 5  $\mu\text{g}$  each of RNase H treated or untreated nucleic acids and dilute with 1 $\times$  TE up to 440  $\mu\text{L}$  vol (*see Note 8*).
2. Add 50  $\mu\text{L}$  of 10 $\times$  Ab binding buffer and 10  $\mu\text{g}$  of S9.6 antibody or 10  $\mu\text{g}$  of control normal IgG.
3. Rotate tubes at 4  $^{\circ}\text{C}$  overnight.

4. Add 30  $\mu\text{L}$  of prewashed Protein A/G magnetic bead (*see* Note 9).
5. Rotate tubes at 4  $^{\circ}\text{C}$  for 2 h.
6. Place tubes on the magnetic rack and remove binding buffer after all magnetic beads bind to the tube's wall (*see* Note 10).
7. Remove tubes from magnetic rack and resuspend magnetic beads with 1 mL of 1 $\times$  Ab binding buffer. Rotate tubes at RT for 5 min. Go to **step 6**.
8. Repeat **steps 6** and **7** four times. Remove tubes from the magnetic rack.
9. Add 200  $\mu\text{L}$  of elution buffer with 5  $\mu\text{L}$  of proteinase K and incubate at 50  $^{\circ}\text{C}$  for 1 h on a rotator.
10. Add 1 vol PCI and invert tube several times and centrifuge at 13,000  $\times g$  for 5 min.
11. Transfer RNA–DNA hybrids from aqueous phase into a new 1.5 mL tube and add 1 vol chloroform. Invert tube several times followed by centrifuge at 13,000  $\times g$  for 5 min.
12. Transfer RNA–DNA hybrids from aqueous phase into a new 1.5 mL tube and add 20 ng of glycogen, 0.1 vol NaOAc (pH 5.2) and 2.5 vol 100% ethanol. After 2 h incubation at  $-80^{\circ}\text{C}$ , centrifuge at 4  $^{\circ}\text{C}$ , 13,000  $\times g$  for 10 min (*see* Note 11).
13. Wash pellet with 80% ice-cold EtOH and air-dry.
14. Resuspend pellet with 50  $\mu\text{L}$  1 $\times$  TE.
15. Store tubes at  $-20^{\circ}\text{C}$  before qPCR or library preparation for NGS (*see* Note 12).

### 3.1.3 Library Preparation

1. Quantify the total amount of immunoprecipitated DNA using the Qubit 1 $\times$  dsDNA HS Assay Kit, and use 30  $\mu\text{L}$  of each IP product for the library preparation.
2. Optional (**steps 2–4**): add internal controls (“spike-in”) to normalize the number of reads after NGS. We recommend to use DNA from different species for spike-in (e.g., four different species of bacteria are used in this protocol).
3. Amplify specific targets by PCR for spike-in controls. Purify each PCR products using the QIAquick Gel Extraction Kit, quantify and make an equimolar mix of these PCR products using 1 $\times$  TE for dilution.
4. Mix the IP products with the spike-in controls (*see* Note 13).
5. Sonicate the IP product (with or without the spike-in controls) using BioRuptor Plus sonication system (program set: three pulses at low intensity for 30 s).
6. Add 10  $\mu\text{L}$  (if using spike-in) or 20  $\mu\text{L}$  of 1 $\times$  TE to complete the sheared DNA volume up to 50  $\mu\text{L}$ . Use the NEB Next Ultra II DNA Library Prep Kit (NEB) for DNA end processing and adaptor ligation (follow exactly the kit protocol). Add 3  $\mu\text{L}$  of NEB next ultra II end prep enzyme mix and 7  $\mu\text{L}$  of NEB next ultra

II end prep reaction buffer, incubate 30 min at 20 °C follow by 30 min at 65 °C on a thermocycler.

7. For adaptor ligation (NEB next adaptor for Illumina), first prepare a dilution of this adaptor in Tris/NaCl, pH 7.5–8.0 (typically 1:10 dilution when your input DNA is between 5 to 100 ng). Then add 2.5 µL of NEB next adaptor for Illumina (diluted if needed), 30 µL of NEB next ultra II ligation master mix, and 1 µL of NEB next ligation enhancer mix to the end processed DNA mixture (60 µL reaction product of **step 6**) and mix well. Incubate at 20 °C for 15 min in a thermocycler. Add 3 µL of USER® enzyme to the ligation mixture and incubate at 37 °C for 15 min.
8. Clean up the adaptor-ligated IP DNA fragments using AMPure XP Beads.
9. Mix the 15 µL of the adaptor-ligated DNA fragments, 25 µL of NEB next ultra II Q5 2× master mix, 5 µL of i7 primer and 5 µL of i5 primer and amplify DRIP DNA library (*see* Note 14).
10. Clean up the PCR reaction using AMPure XP Beads.
11. Quantify DNA concentration of each DRIP libraries using Qubit dsDNA HS Assay Kit and perform a Bioanalyzer to see the size distribution. Calculate the library concentrations in nM (regarding to the mass concentration and the average size) and prepare a working solution with all the combined libraries at 4 nM final into 5 µL using dilution buffer (10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20).

#### 3.1.4 Sequencing of DRIP Libraries

1. Prepare a 0.2 N NaOH denature solution (*see* Note 15).
2. Denature the DNA by combining 5 µL of the 4 nM library mix with 5 µL of 0.2 N NaOH, vortex briefly, spin down at  $280 \times g$  for 1 min, and incubate for 5 min at RT.
3. Neutralize denatured DNA by adding 5 µL of neutralizing solution, and vortex gently and spin down at  $280 \times g$  for 1 min.
4. Dilute the libraries to 20 pM by adding 985 µL of HT1 buffer (*see* Note 16).
5. Prepare the sequencing tube to load on NextSeq cartridge (follow kit instructions) and add PhIX positive control for sequencing quality test (*see* Note 17).
6. Thaw a NextSeq reagent cartridge, load the denatured DNA libraries with the PhIX, and run the sequencing.

### 3.2 Nondenaturing Sodium Bisulfite Probing

1. Digest 5–10 µg of purified genomic DNA with restriction enzyme(s) to generate fragments 10 kb without disrupting of the amplicon (the region to be PCR amplified for sequencing analysis).

2. Purify the digested DNA by phenol–chloroform extraction followed by ethanol precipitation. Use sodium acetate instead of glycogen during the ethanol precipitation step. Resuspend in 30  $\mu$ L of 1 $\times$  TE (pH 8.0).
3. Combine 457.5  $\mu$ L of 2.5 M sodium bisulfite, 12.5  $\mu$ L of 20 mM hydroquinone and 30  $\mu$ L DNA sample in a microfuge tube (total volume = 500  $\mu$ L).
4. Add mineral oil to seal the top to prevent evaporation.
5. Incubate the microfuge tube in the dark at 37  $^{\circ}$ C for 12–16 h.
6. Purify bisulfite converted DNA using 1 mL of Wizard resin (Promega) or a DNA spin column (Zymo Research) according to the manufacturer's instructions.
7. Adjust the volume of the eluted DNA to 96  $\mu$ L with 1 $\times$  TE, [pH 8.0].
8. Add 24  $\mu$ L of 2 M NaOH.
9. Incubate at 37  $^{\circ}$ C for 15 min (desulfonation).
10. Purify desulfonated DNA by ethanol precipitation, or, Wizard resin (Promega) or DNA spin column (Zymo Research).
11. Recover DNA in 30  $\mu$ L of 1 $\times$  TE, pH 8.0 and use 1–2  $\mu$ L as PCR template to amplify regions of interest (*see* Notes 18–23).
12. Store the rest of DNA in  $-20^{\circ}$ C.

#### 4 Notes

1. A very small amount of sodium bisulfite crystals may remain undissolved; this will not affect the usage of the sodium bisulfite solution.
2. Do not use a pipette to avoid unexpected gDNA breaks. DNase and RNase free reagents and plastic wares were used for all step in DRIP-Seq experiment.
3. Do not vortex.
4. Use wide-orifice P1000 tips.
5. Add 100  $\mu$ L 1 $\times$  TE immediately after air drying. Do not overdry. Efficiency of DNA digestion can be checked before and after restriction enzyme treatment (Subheading 3.1.1, **steps 10 and 18**).
6. Do not overpipette while resuspending nucleic acids.
7. RNase H treatment is essential in this method because RNA–DNA hybrids that are sensitive to RNase H treatment should only be accounted as potential R-loops.
8. Use 5  $\mu$ g nucleic acids for S9.6 antibody and control IgG each. To avoid DNA loss, we used the low binding 1.5 mL tubes from Subheading 3.1.2, **step 1** to Subheading 3.1.2, **step 6**.
9. Wash beads with 1 $\times$  Ab binding buffer two times.

10. Use pipette and remove buffer carefully. Do not touch magnetic beads.
11. Incubate at least at  $-80^{\circ}\text{C}$  for 2 h or at  $-20^{\circ}\text{C}$  overnight. We used precipitated DNA for sequencing because reverse transcription-based strand specific DRIP-seq is challenging to perform in cells that have RNA processing/decay defects due to enrichment of dsRNAs or at GC-rich switch sequences.
12. IP quality has to be validated by qPCR using  $\text{S}\mu$  specific primers before the next steps. Fold enrichment compared to the Input should be clearly superior to the negative control (RNase H treatment and control IgG antibody).
13. We recommend a 1:10,000 ratio, with excess of IP product. For each condition add exactly the same amount of spike-in mix (e.g., 10  $\mu\text{L}$  of diluted PCR products in 30  $\mu\text{L}$  of DRIP IP product, use large volume to avoid pipetting error).
14. Please follow the kit instruction to set up best PCR cycles and temperatures. This step will tag your samples with specific barcodes from NEB Next Multiplex Oligos from Illumina (NEB), choose an appropriate combination of barcodes.
15. We follow instructions from Illumina NextSeq system to denature and to dilute the libraries.
16. HT1 buffer is contained in NEXTSeq reagent kit.
17. DRIP DNA libraries have high diversity, so only 1% of PhIX is enough.
18. Sodium bisulfite treatment severely damages DNA, possibly through the actions of free radicals [31]. Typically, up to 90% of the input DNA are damaged such that the recovered DNA are not suitable for subcloning, transforming *E.coli*. or being used directly for library preparation for Illumina or PacBio sequencing.
19. PCR of bisulfite-treated DNA is usually done with a set of primers flanking the region of interest. Taq DNA polymerase or other thermal stable polymerases that are compatible with uracil containing template (e.g., Q5U from New England Biolabs) should be used for PCR. The PCR product is subsequently Topo-cloned (ThermoFisher) and sequenced (typically  $>30$  clones) to identify patches of continuous C-to-U conversions (readout as C-to-T transition) in some of the clones. Alternatively, PCR amplicon can be analyzed by high-throughput long-read sequencing such as PacBio sequencing.
20. Native murine switch regions are long, GC-rich, repetitive, and difficult to PCR across the entire length. Therefore, the strategy described above is only applicable to engineered shorter switch regions or genomic loci that are amenable to PCR amplification.
21. A primer that is complementary to the anticipated bisulfite converted sequences can be used to selectively amplify alleles that adopt R-loop conformation [21]. Only R-loops covering the priming site will be amplified. Such primer is termed a “converted” primer. The usage of a converted primer only allows analysis of part of an R-loop (up to the priming site of the converted primer) and does not allow quantitation of the frequency of R-looped alleles unless sophisticated

scheme is designed to control for the difference of PCR efficiency between converted and nonconverted primers.

22. Native bisulfite probing can be applied to S9.6-enriched nucleic acid to greatly facilitate R-loop analysis as most non-R-looped DNA fragments are excluded.
23. RNase H treatment prior to bisulfite conversion should be included as a negative control for all R-loop analyses.

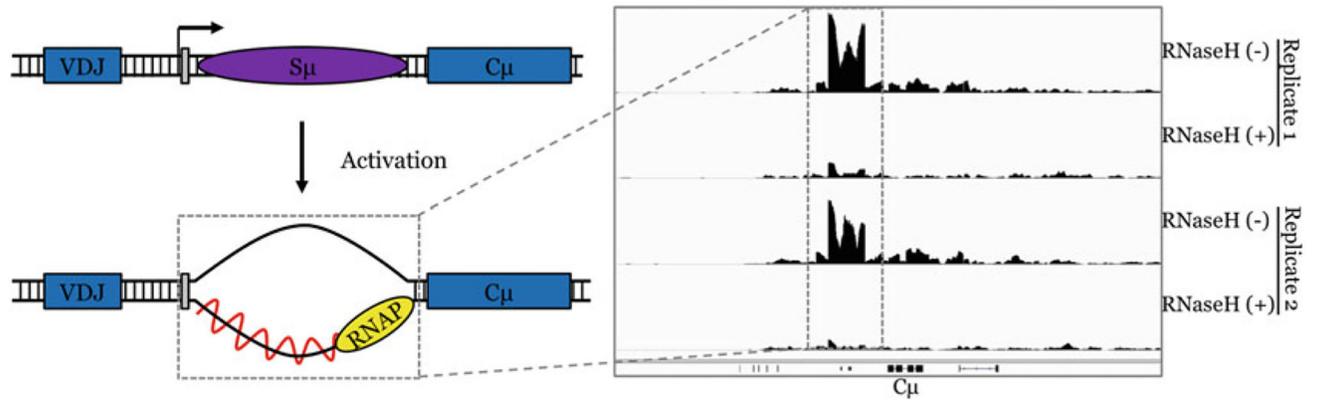
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**Fig. 1.** RNA–DNA hybrids at Ig  $S_{\mu}$  switch region. RNA–DNA hybrid peaks on IGV track are modified from DRIP-seq data [20]