abcam

# DNA-RNA immunoprecipitation (DRIP) protocol

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# DRIP protocol

A step-by-step DRIP protocol, including R-loop preparation and associated reagents.
DNA-RNA immunoprecipitation (DRIP) uses the S9.6 anti-RNA-DNA hybrid antibody to capture RNA-DNA hybrids along chromosomes. DRIP is typically followed by mapping DNA fragments on a few loci or even across the whole genome with qPCR, microarray hybridization, or deep sequencing.

Thanks to Professor Frédéric Chédin's lab at UC Davis for providing us with this protocol.

# R-Loop preparation

## Reagents

25 mM rNTP stock (NEB N0466S) - dilute to 2.5 mM rNTP for experiment

T3 RNA Polymerase, 50 U/µL (NEB M0378S)

10 x RNAPol Reaction Buffer (NEB M0378S)

1M DTT (from frozen stock, made in house)

2.5% Tween-20 (diluted in water, made in house)

pCALM3_2 plasmid (pCALM3_2 carries an R-loop forming portion of the human *CALM3* gene)

RNase A, 10 mg/mL (DNase free) – dilute to 1.0 mg/mL for experiment

RNase H, 5 U/µL (NEB M0297S)

Proteinase K, 10 mg/mL (Sigma P2308)

DuRed (nucleic acid dye), 1000X (Brigen D009)

ApaLI, 2500 units (NEB R0507S)

## Protocol

1. Mix:

   pCALM3_2 2 µg
   10x buffer 10 µL
   1M DTT 2 µL
   2.5% Tween-20 2 µL
   2.5 mM rNTP 10 µL
   $H_20$ to 99.4 µL total

2. Initiate reaction by adding 0.6 µL of T3 RNA Polymerase, mix gently, and split into two reactions (50 µL each). Incubate at 37°C for 10 minutes.

3. Inactivate enzyme by adding 1 µL of 10 mM EDTA.

4. To one sample, add 10 µL of 0.1 mg/mL RNase A, and the other (negative control) add 10 µL 0.1 mg/mL RNase A and 4 µL RNase H. Incubate for 30 minutes at 37°C.

5. Add 4 µL of Proteinase K and incubate for 30 minutes at 37°C.

6. Cleaned up on Axygen PCR purification kit and eluted in 50 µL $ddH_2O$, separately.

7. Each sample (2 samples totally) split into two tubes, put one tube on ice, the other one was used for the following digestion.

8. Digest:

   25 µL DNA
   5 µL 10X Cutsmart buffer NEB
   1 µL LI ApaLI NEB
   19 µL $ddH_2O$
   50 µL total

9. 37°C 2 hours.

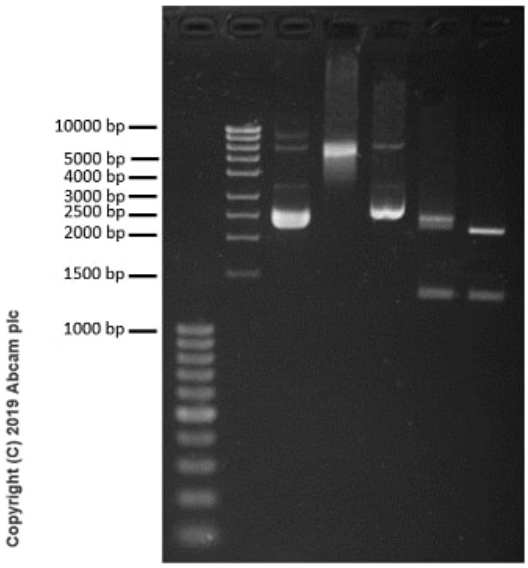
10. Cleaned up on Axygen PCR purification kit and eluted in 25 µL $ddH_2O$. Four samples (2–5) plus one pCALM3_2 plasmid (1) were obtained for DRIP experiment.

11. In order to verify that R-loop formation did occur, load ~200 ng on a 0.9% 1x TBE gel **WITHOUT** nucleic acid dye and run at 90V for 60 min.

    Use Glycerol at 10% final as a loading dye. Post-stain with DuRed (nucleic acid dye).

    R-loop formation causes a characteristic shift in mobility compared to un-transcribed or RNase H-treated samples. Results are shown below (Figure 1).

| | M1 | M2 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| pCALM3_2 plasmid | - | - | + | + | + | + | + |
| T3 transcribed | - | - | - | + | + | + | + |
| RNase A | - | - | - | + | + | + | + |
| RNase H | - | - | - | - | + | - | + |
| ApaLI digest | - | - | - | - | - | + | + |




**M1** : GeneRuler 100bp DNA ladder (Thermo FisherSM0242)

**M2** : MassRuler High Range DNA Ladder (Thermo FisherSM0393)

**Figure 1.** Transcription from pCALM3_2 to generate R-loops. Each digestion reaction was run on an agarose gel. pCALM3_2 carries a portion of the human *CALM3* gene that forms R-loops when transcribed with the T3 RNA polymerase. Treatment with RNase A (digests single-stranded RNA) does not affect the R-loops structure (lane 2) whereas treatment with RNase H (digests RNA in DNA-RNA hybrids) destroy R-loops structure (lane 3). pCALM3_2 plasmid can be digested by ApaL restriction enzyme without affecting the R-loop structures.

# DNA-RNA hybrid Immunoprecipitation by using antibodies pre-immobilized on beads

## Reagents

PBS (phosphate buffer)

Triton X100

ssDNA (Salmon sperm single strand DNA)

Recombinant Anti-DNA:RNA hybrid antibody [S9.6] (ab234957)

Isotype antibody (Mouse (G3A1) mAb IgG1 Isotype Control #5415, 2.5 mg/ml, Mouse IgG1, kappa)

EDTA (Ethylene Diamine Tetraacetic Acid)

2.5% Tween-20 (diluted in water, made in house)

ApaLI (NEB R0507S)

RNase A, 10 mg/mL (DNase free) – dilute to 1.0 mg/mL for experiment

RNase H, 5 U/µL (NEB M0297S)

Proteinase K, 10 mg/mL (Sigma P2308)

DuRed (nucleic acid dye), 1000X (Brigen D009)

Qiagen PCR purification kit

## Protocol

Antibodies pre-immobilized on beads

1. Prepare eight tubes of Protein A beads.

2. 100 µL protein A beads each tube washed twice in 1 mL of 1X PBS, 0.1% Triton X100, centrifuge 1 min at 3,600 rpm 4°C, carefully aspirate the supernatant.

3. Each tube resuspended with 1 mL 1X PBS, 0.1% Triton X100 and 7.5 µg ssDNA (Salmon sperm single strand DNA/20 µL beads), shake gently 10 mins at room temperature.

   Then centrifuge 1 min at 3,600 rpm at 4°C, aspirate the supernatant. Wash once in 1 mL of 1X PBS, 0.1% Triton X100, centrifuge 1 min at 3,600 rpm 4°C, carefully aspirate the supernatant.

4. 5 µL S9.6 antibody (1mg/mL) (test antibody 5 µg, add to protein A) added to four tubes as positive control and 5 µL isotype antibody (5 µg) added to rest of four tubes as a negative control, make up the samples to 1 mL with 1X PBS, 0.1% Triton X100.

   Shake gently 10 mins at room temperature.

5. Wash twice with 1 mL 1X PBS, 0.1% Triton X100, centrifuge 1min at 3,600 rpm 4°C, aspirate the supernatant.

6. Resuspend each tube in 100 µL PBS, 0.1% Triton X100 and add 1 µL 0.5M EDTA.

Add DNA

7. Remove 5 µL of each input R-loop (RNase A treated), R-loop (RNase A+H treated), ApaLI digested R-loop (RNase A treated), ApaLI digested R-loop (RNase A+H treated) for gel analysis as control.

8. Add 35 µL of R-loop (RNase A treated), R-loop (RNase A+H treated), ApaLI digested R-loop (RNase A treated), ApaLI digested R-loop (RNase A+H treated) to two tubes (S9.6, isotype), respectively.

9. Rotate gently for 2 hours at 4°C.

10. Centrifuge 1 min at 3,600 rpm 4°C, remove all depleted supernatants and retain for electrophoresis.

11. Wash three times in 1X PBS, 0.1% Triton X100, centrifuge 1 min at 3,600 rpm 4°C and aspirate the supernatant.

12. Add 50 µL elution buffer + 5 µL proteinase K, then shake in 1,400 rpm 30 mins 50°C, centrifuge 1 min at 13,000 rpm at room temperature. Collect supernatant.

13. Clean up by Qiagen PCR purification kit and eluted in 30 µL.

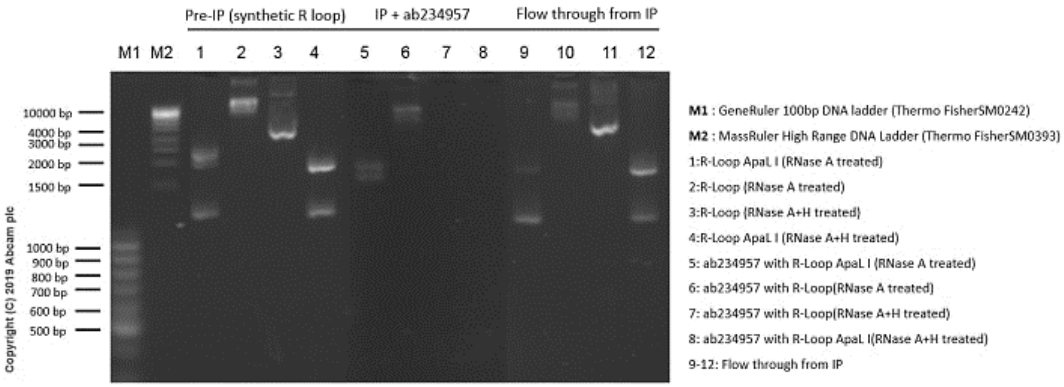



**Figure 2.** DNA-RNA hybrid Immunoprecipitation (DRIP) data. pCALM3_2 was used to generate R-loops. S9.6 (ab234957) immunoprecipitates R-loops in the presence or absence of prior digestion by ApaLI, which does not affect R-loop structure. Prior treatment with RNase A (digests single-stranded RNA) does not affect the IP signal whereas prior treatment with RNase H (digests RNA in DNA-RNA hybrids) eliminates the signal.