Journal of Visualized Experiments

R-loop analysis by Dot-Blot

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62069R1
Full Title:	R-loop analysis by Dot-Blot
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Additional Information:	
Question	**Response**
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TITLE:

R-loop analysis by Dot-Blot

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KEYWORDS:

R-loop, RNA-DNA hybrid, RNase H, Senataxin, Dot-blot, S9.6 antibody

SUMMARY:

This protocol details a simple method that quantifies R-loop, a three-stranded nucleic acid structure that comprises of an RNA-DNA hybrid and a displaced DNA strand.

ABSTRACT:

The three-stranded nucleic acid structure, R-loop, is increasingly recognized for its role in gene regulation. Initially, R-loops were thought to be the by-products of transcription; but recent findings of fewer R-loops in diseased cells made it clear that R-loops have functional roles in a variety of human cells. Next, it is critical to understand the roles of R-loops and how cells balance their abundance. A challenge in the field is the quantitation of R-loops since much of the work relies on the S9.6 monoclonal antibody whose specificity for RNA-DNA hybrids has been questioned. Here, we use dot-blots with the S9.6 antibody to quantify R-loops and show the sensitivity and specificity of this assay with RNase H, RNase T1, and RNase III that cleave RNA-DNA hybrids, single-stranded RNA, and double-stranded RNA, respectively. This method is highly reproducible, uses general laboratory equipment and reagents, and provides results within two days. This assay can be used in research and clinical settings to quantify R-loops and assess the effect of mutations in genes such as senataxin on R-loop abundance.

INTRODUCTION:

This protocol provides a step-by-step guide to a dot-blot assay that allows a quick comparative assessment of the abundance of R-loop, a three-stranded nucleic acid structure. R-loop forms when RNA invades a double-stranded DNA to generate an RNA-DNA hybrid and displaces the other DNA-strand. R-loops are found in different stages of the lifecycle of RNA. In the transcriptional complex, the nascent RNA is synthesized complementary to the template DNA, and the non-template strand is displaced. The short RNA-DNA hybrid (<10 bp) is resolved to free the nascent RNA so it can leave the RNA polymerase complex through the exit channel[1,2]. Outside of the transcriptional complex, the nascent RNA is close to its DNA template, which is still slightly unwound from being copied, thus the RNA can rehybridize with its template DNA forming R-loops[3]. Additionally, R-loops can form when replication and transcription complexes collide[4], and in antisense transcription[5]. Given the many opportunities for their formation, R-loops are not rare, and can be found in 3-5% of the human genome[6], depending on the cell's transcription status. R-loops are found in gene promoters[7] and termination[5] sites in mRNA, and along ribosomal RNA[8] as well as transfer RNA[9]. R-loops are also in telomeric regions of chromosomes.

R-loops play a regulatory role. They regulate gene expression by affecting transcription at promoters[10,11], mediating class-switch recombination[12], and facilitating CRISPR-based genome editing[13-15]. Like many cellular events, R-loop abundance is tightly titrated; too many or too few R-loops impact normal cell function[16,17]. R-loops are regulated by a variety of proteins including RNase H, senataxin, and other helicases that unwind the RNA-DNA hybrids[18-22].

To monitor the abundance of R-loops, genome-wide methods first enrich for R-loops with the antibody S9.6[8,23,24] or with other nucleases[25] including RNase H[10,26,7], and then assess the number of enriched R-loops by sequencing. Early versions of these sequencing-based methods did not achieve adequate sequence coverage to allow precise quantitation but rapid improvement in sequencing technologies now allows locus-by-locus R-loop analysis. Immunofluorescence techniques have also been used to quantify and localize R-loops[10,17]. These methods are comprehensive, but they are not practical in many clinical settings or as initial assessments since they require expensive equipment and specialized analysis.

A procedure that can be done uniformly across laboratories in clinical settings is needed. Dot-blots provide such an option since they can be carried out without any specific equipment or computational analysis. As a preliminary step or in clinical settings to evaluate the effects of mutations on R-loops, these dot-blots must provide sensitive and specific results. Here, we describe our assay that identifies R-loops specifically; it excludes signals from double-stranded (ds) DNA, double-stranded RNA, and single-stranded RNA. Our protocol uses the S9.6 antibody[27] to identify RNA-DNA hybrids in R-loops and incorporates RNase H, an endoribonuclease that cleaves and therefore leads to the degradation of the RNA in an RNA-DNA hybrid[20,28], to ensure that the detected signals are those of hybrids. We also incorporated RNase T1 that cleaves single-stranded RNA at guanine[29,30], and RNase III that cleaves double-stranded RNA including stem-loops[31,32] to check for nonspecific signals. The S9.6 antibody recognizes RNA-DNA hybrids of varying lengths, even those that are only 8 nucleotides long[33].

Here, we present the protocol that begins with nucleic acid isolation followed by dot-blot preparation, and R-loop detection with S9.6 antibody. Our protocol includes steps to ensure that equal amounts of samples are loaded, and the signals are specific. It provides oligonucleotides to serve as positive and negative controls. This is a quick, easy, and user-friendly method to assess R-loop abundance.

PROTOCOL:

1. Cell lysis for nuclear fractionation

1.1. Wash cells with 1x phosphate-buffered saline (PBS) twice. Remove cells from tissue culture dishes using standard cell dissociation techniques such as trypsin. Count cells using a hemocytometer.

NOTE: The steps described below were used for the analysis of primary human skin fibroblasts, although an array of cell types can be assayed. Fibroblasts were grown in basal media containing 10% fetal bovine serum. Alternatively, cell lysis buffer (**Table 1**) can be added directly to the cell-culture after washing.

1.2. Transfer the cell suspension to a 1.5 mL tube to pellet the cells.

1.3. Centrifuge the sample at 300 x *g* for 5 min at 4 ˚C. Aspirate the media.

1.4. Wash twice with ice-cold 1x PBS using centrifugation settings in step 1.3.

1.5. Add cold cell lysis buffer (**Table 1**) to the cell pellet (300 µL per 2 x 10^6 cells). Pipette up and down to resuspend the pellet.

1.6. Incubate on ice for 10 min.

1.7. Spin at 500 x *g* for 5 min to pellet the nuclei.

1.8. Discard supernatant and re-suspend the nuclear pellet in 400 µL of cold nuclear lysis buffer (**Table 1**).

1.9 Incubate on ice for 10 min.

NOTE: Fractionation of the nuclear and cytoplasmic compartments of the cells ensures signal specificity. The quality of nuclear and cytoplasmic separation can be evaluated before proceeding (**Table 2**).

1.10. Add 3 µL of 20 mg/mL proteinase K and incubate for 3-5 h at 55 ˚C.

NOTE: Volumes indicated are for 2 x 10^6 cells, scale up or down as necessary.

2. Purification of genomic DNA (which includes RNA-DNA hybrids)

2.1. If DNA is viscous, perform sonication to reduce viscosity (e.g., sonication at high power output, 30 s ON/ 30 s OFF, for 2 min using a 4 ˚C water bath).

2.2. Add 400 µL of elution buffer (**Table 1**) and 400 µL of phenol:chloroform:isoamyl alcohol (25:25:1 pH 8.0).

2.3. Vortex for 10 s.

2.4. Spin down at 12,000 x *g* for 5 min at 4 ˚C.

2.5. Transfer the aqueous phase (approximately 350 µL) to a new tube.

2.6. Extract once using 1 volume of chloroform, vortex for 10 s, then spin down at 12,000 x *g* for 5 min at 4 ˚C. Transfer the aqueous phase to a new tube (approximately 300 µL).

2.7. Add 35 µL of 3 M sodium acetate (pH 5.2), 1 µL glycogen and 700 µL of ice-cold 100% ethanol.

2.8. Vortex for 10 s and spin down at 12,000 x *g* for 30 min at 4 ˚C.

2.9. Wash the pellet with 1 mL of 70% ethanol.

2.10. Vortex for 10 s and spin down at 12,000 x *g* for 15 min at 4 ˚C.

2.11. Discard the supernatant and let the pellet air dry.

2.12. Add 12 µL of elution buffer and vortex for 10 s to resuspend. Incubate the sample for 30 min at 37 ˚C with agitation or at 4 ˚C overnight to re-suspend the pellet.

2.13. Measure the DNA concentration using standard spectrophotometry.

NOTE: Volumes indicated are for 2 x 10^6 cells, scale up or down as necessary. DNA (with RNA-DNA hybrids) may be stored at -20 ˚C, if needed.

3. Blotting DNA samples (which include RNA-DNA hybrids) onto nylon membranes

3.1. Prepare dilutions of nucleic acids to desired concentrations in elution buffer (i.e., 50 ng/µL, 25 ng/µL, or 12.5 ng/µL). These samples with a range of concentrations (200, 100, 50, 25, 12.5 ng) ensure that there will be signals within the linear range.

NOTE: Be sure to prepare enough sample for technical and biological replicates, and for the various RNase treatments, see Step 5.

3.2. Cut a positively charged nylon membrane to the appropriate size (0.5 cm x 0.5 cm) area on the grid for each 2 µL sample loaded).

3.3. Spot 2 µL of each sample onto 2 membranes. One for the S9.6 antibody and the other for dsDNA. Alternatively, a dot-blot or slot-blot apparatus which allows the loading of samples with larger volumes can be used.

3.4. Allow the samples to saturate into the membrane. Wait at least 2 min before crosslinking the membrane with UV light.

3.5. Place the membrane into the center of the UV device and crosslink the membrane using a UV crosslinker using the "**Auto Crosslink**" setting (1,200 µJ x 100).

4. RNA-DNA hybrid detection with S9.6 antibody

4.1. Incubate the membrane in blocking solution (5% milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 h at room temperature on a shaker.

NOTE: There should enough blocking solution to cover the membrane.

4.2. Incubate the membranes overnight in primary antibody (in 5% milk in TBST) at 4 ˚C with shaking. Add anti-dsDNA antibody (1:10,000 dilution) to one membrane. Add 1µg/mL S9.6 antibody to the second membrane (1:1,000 dilution).

NOTE: S9.6 antibody is available commercially or from Dr. S. Leppla, NIAID, National Institutes of Health.

4.3. Remove primary antibody and wash 3x with TBST. Perform each wash for 5-10 min with shaking at room temperature.

4.4. Incubate with horseradish peroxidase (HRP) conjugated secondary antibody (anti-mouse, 1:5,000 dilution) in 5% milk in TBST with shaking at room temperature.

NOTE: Anti-dsDNA and anti-RNA-DNA hybrid are both mouse antibodies.

4.5. Remove the secondary antibody and wash 3x with TBST for 5-10 min with shaking at room temperature.

4.6. Develop with enhanced-chemiluminescence (ECL) reagents to acquire signals for imaging.

4.7. Quantify signal intensity using standard image processing tools such as ImageJ.

NOTE: Troubleshooting is detailed in **Table 2.**

5. Ribonuclease treatments to evaluate signal specificity

NOTE: RNase treatment should be performed on the nucleic acid samples to demonstrate the specificity of S9.6 binding. Treatment with RNase H, but not RNase T1 or RNase III should result in a reduction in S9.6 immuno-staining.

5.1. Digest the samples containing RNA-DNA hybrids by preparing them in four separate tubes. Treat each of the 4 samples with either 5 U RNase H, 1000 U RNase T1, 0.5 U RNase III, or mock. Incubate samples at 37 ˚C for 15 min in 20 µL volumes.

5.2. Load 2 µL of each sample on a membrane as described in section 3.

6. Preparation of oligonucleotide controls to evaluate signal specificity

NOTE: Oligonucleotide controls can be used to demonstrate the specificity of S9.6 binding. S9.6 recognizes RNA-DNA hybrids, but not dsDNA or dsRNA controls, as has been previously reported[34].

6.1. Dissolve oligonucleotides (**Table 3**) in annealing buffer (10 mM Tris, pH 8.0; 50 mM NaCl, 1 mM EDTA) to 100 µM.

6.2. Prepare 4 reaction tubes for

6.2.1. RNA-DNA hybrid #1: Mix 10 µL of ssRNA top strand with 10 µL of ssDNA bottom strand and 80 µL of annealing buffer.

6.2.2. RNA-DNA hybrid #2: Mix 10 µL of ssDNA top strand with 10 µL of ssRNA bottom strand and 80 µL of annealing buffer.

6.2.3. dsRNA: Mix 10 µL of ssRNA top strand with 10 µL of ssRNA bottom strand and 80 µL of annealing buffer.

6.2.4. dsDNA: Mix 10 µL of ssDNA top strand with 10 µL of ssDNA bottom strand and 80 µL of annealing buffer.

6.3. Heat the 4 mixtures from step 6.2 at 95 ˚C for 10 min.

6.4. Allow tubes to cool slowly to room temperature to allow reannealing of the strands. Annealed standards can be stored at -30 ˚C for later use.

NOTE: Annealing efficiency should be checked by non-denaturing gel electrophoresis. Duplexes migrate more slowly than the unannealed oligonucleotides (**Table 2**).

6.5. Load 2 µL of each sample on 2 membranes, one for S9.6 antibody and one for dsDNA antibody, as described in section 3.

6.6. Perform steps described in section 4.

7 Quantification and normalization of S9.6 R-loop signal intensity using ImageJ.

7.1. Save images of S9.6, dsDNA staining in TIF format, and analyze them using the ImageJ software (https://imagej.nih.gov/ij/).

7.2. Select the image invert option (**Edit** | **Invert**). After inversion, each dot will be visible as white against a dark background.

7.3. Use the oval image selection tool to select an oval that is large enough to surround the largest dot on the image.

7.4. Use the ROI manager to add the selected area for quantification. Ensure that the "**Show All**" and "**Labels**" options are selected so that the regions of interest can be visualized.

7.5. Use the same oval selection area used during step 7.3 to add additional regions of interest around each dot to be quantified. Use **Command + Shift + E** shortcut to copy the selected area from step 7.3 to each of the subsequent dots.

7.6. Measure the integrated density of each of the regions of interest.

7.7. Divide the S9.6 signal intensity for each sample by the measurement of dsDNA to obtain the S9.6/dsDNA signal ratio. Verify the results by repeating the experiments (at least triplicates for both S9.6 and dsDNA signal acquisition). Calculate standard error of the mean can be calculated from the S9.6/dsDNA signal ratios.

REPRESENTATIVE RESULTS

Enzymatic treatment to evaluate the specificity of S9.6 (RNA-DNA) antibody.

Primary human skin fibroblasts were grown[17]. DNA with RNA-DNA hybrids was isolated and quantified. Two µg of the samples were digested with RNase T1, RNase H, or RNase III for 15 min at 37 ˚C. A mock sample was also analyzed for comparison to the RNase-treated samples. Samples (200, 100, 50, 25, 12.5, or 6.25 ng) were blotted onto two different membranes as described in section 3. The membranes were crosslinked, blocked and one of them was probed with S9.6 antibody (**Figure 1A**).

The results showed that the S9.6 signal correlates with the abundance of the loaded sample. Treatment with RNase H, but not RNase T1 or RNase III results in a reduction in S9.6 staining.

A second membrane was probed with a dsDNA antibody (**Figure 1B**) for the normalization. Image J was used to analyze the signal intensities. The 50 ng samples were selected for quantification

as the signal intensities from the S9.6 and dsDNA antibodies were within the dynamic range. Signal intensities were normalized to those in mock samples. Data are shown in **Figure 2**.

S9.6 antibody dot-blot using synthetic nucleotide controls.

To evaluate the specificity of the S9.6 antibody, we used oligonucleotides corresponding to dsRNA, dsDNA, and RNA-DNA as described in section 6. A dilution series of RNA-DNA, dsRNA, and dsDNA nucleotides were prepared and blotted onto the nylon membrane as described in section 3. The membrane was probed with the S9.6 antibody (**Figure 3**). Results showed that the S9.6 antibody binds specifically to RNA-DNA hybrids in dose-dependent manner and showed minimal cross-reactivity to dsRNAs and dsDNAs.

FIGURE LEGENDS:

Figure 1: Specificity of S9.6 as shown by dot-blot loaded with nucleic acids from human fibroblasts. Nucleic acid samples from human fibroblasts were either mock or treated with RNase T1, RNase H, or RNase III and then loaded onto nylon membranes in a dilution series of 200, 100, 50, 25, 12.5, and 6.25 ng per 2 µL dot. Membranes were then probed with S9.6 antibody (**A**), or dsDNA antibody (**B**).

Figure 2: Quantification of S9.6 R-loop staining. 50 ng samples from **Figure 1** were selected for quantification with ImageJ. S9.6 signal was divided by dsDNA signal intensity, then normalized to the mock sample following the steps outlined in section 7.

Figure 3: S9.6 dot-blot using oligonucleotide controls. S9.6 antibody dot-blot against a dilution series of synthetic oligonucleotides as dsRNA, dsDNA, or RNA-DNA hybrid. S9.6 binds specifically to RNA-DNA hybrids in a dose-dependent manner.

DISCUSSION:

The 3-stranded nucleic acids, R-loops, form in different stages during the lifecycle of RNA and are increasingly found to regulate cellular processes. To fully understand R-loops, reliable techniques for R-loop detection are necessary. Here, we describe an approach to interrogate the abundance of R-loops using S9.6 antibody[8,23,24]. This method allows for a quick assessment of R-loop abundance from cells and tissue-culture samples. It does not require special equipment, or a large quantity of starting material. It ensures specific and reproducible results using a combination of RNase treatments.

Some have reported concerns about the specificity of the S9.6 antibody. As with any reagent, there may be batch to batch variability with the S9.6 antibody. Our protocol includes RNase H, RNase T1 and RNase III to check signal specificity. In addition, we use synthetic oligonucleotides to ensure the specificity of each batch of S9.6 antibody.

R-loop biology is a growing field; the development of reliable detection and quantification methods, such as the one presented here, will facilitate mechanistic studies to elucidate when R-loops form, how they are regulated, and what they regulate. With appropriate controls, this dot-blot assay is a simple method to screen for R-loop abundance in clinical and research settings.

ACKNOWLEDGMENTS:

This work was supported by the Howard Hughes Medical Institute and Intramural Research at the National Institute of Neurological Disorders and Stroke.

We thank Dr. Stephen Leppla for providing batches of S9.6 antibody for analysis. We also thank Dr. Dongjun Li for his assistance with ribonuclease treatments.

DISCLOSURES:

The authors have nothing to disclose.

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442 binding of *Escherichia coli* ribonuclease H1 to RNA-DNA hybrids using surface plasmon resonance.
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444

Figure 1

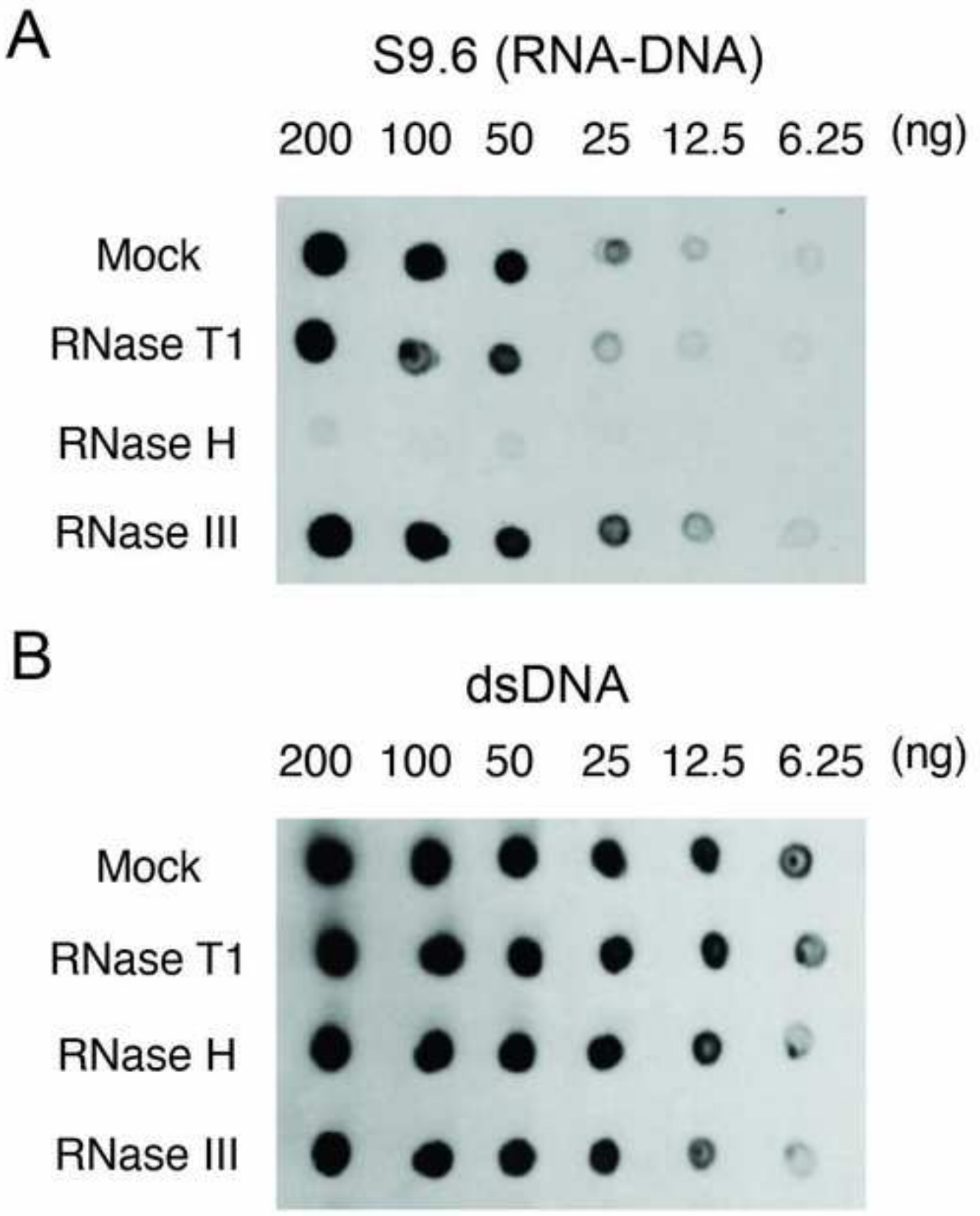

Figure 2

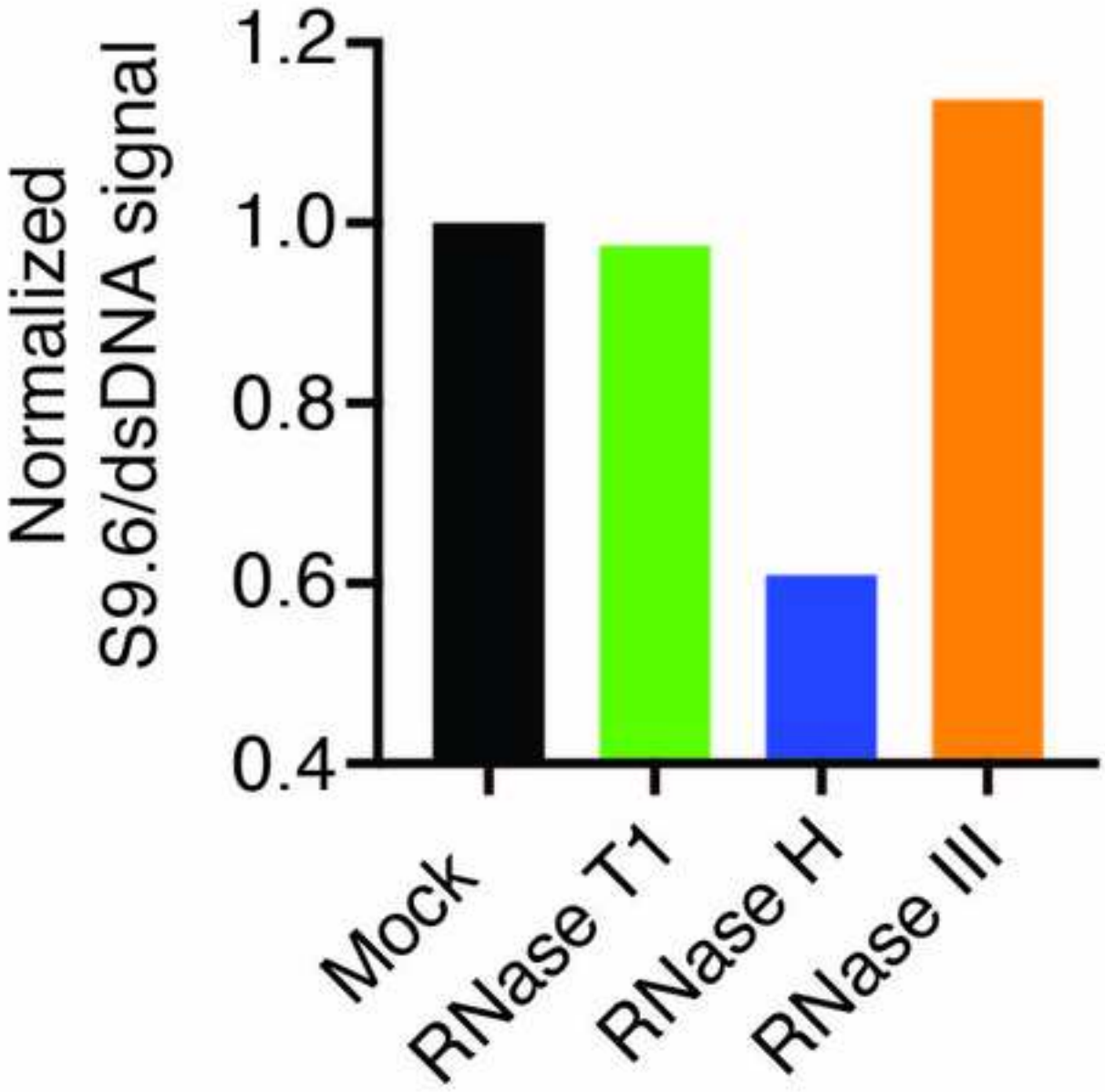

Figure 3

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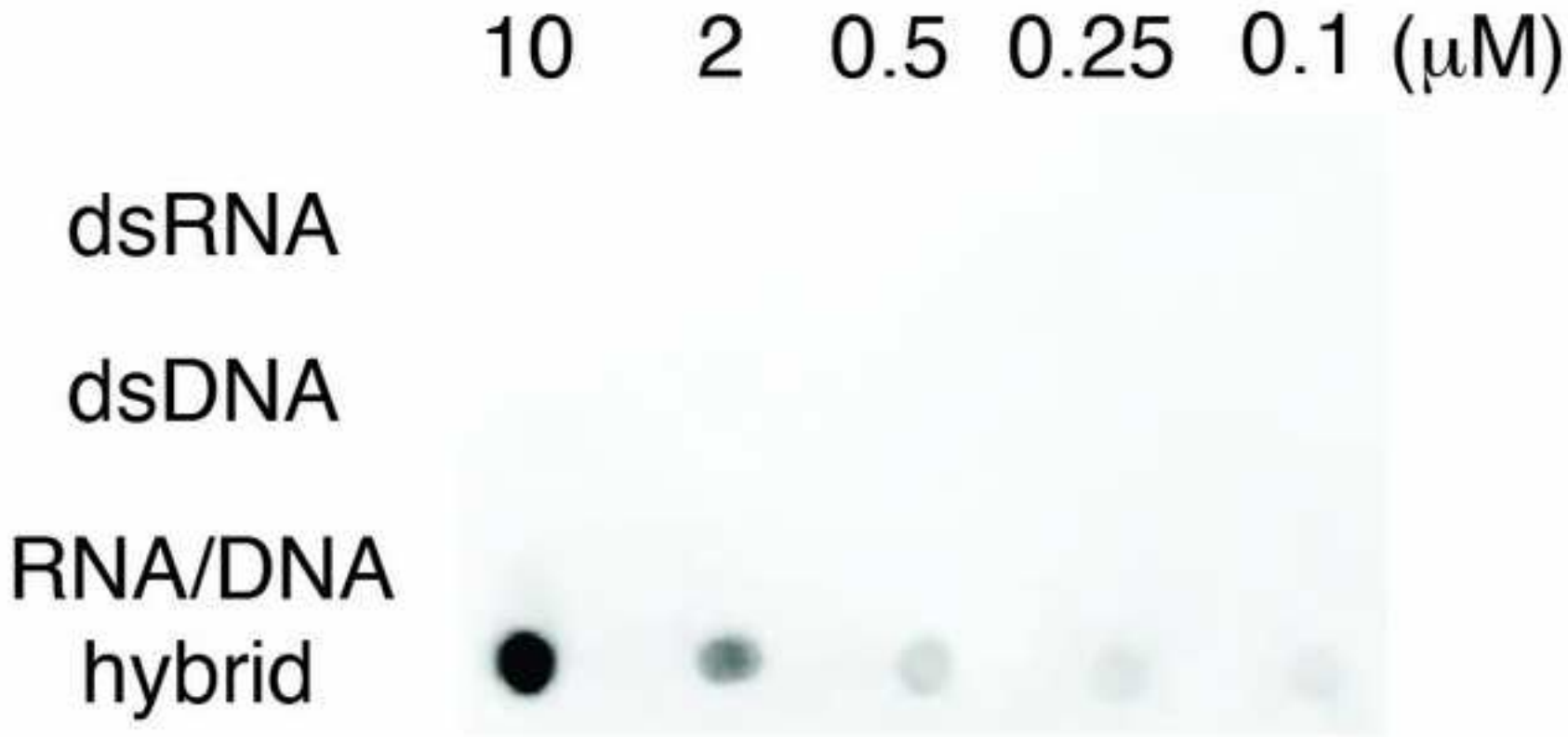

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Table 1. Preparation of buffers

Cell lysis buffer	For 10mL	For 200mL	Final Conc.
Water, nuclease-free	9mL	180mL	-
10% NP-40	0.5mL	10mL	0.50%
2M KCl	0.4mL	8mL	40mM
0.5M PIPES (pH 8.0)	100uL	2mL	5mM

Nuclear lysis buffer	For 10mL	For 200mL	Final Conc.
Water, nuclease-free	8.65mL	173mL	-
10% SDS	1mL	20mL	1%
1M Tris-HCl (pH 8.0)	0.25mL	5mL	5 mM
0.5M EDTA	100uL	2mL	5 mM

Elution Buffer	For 10mL	For 200mL	Final Conc.
1M Tris-Cl, pH 8.5	0.1 mL	2 mL	10 mM
Water, nuclease-free	9.9 mL	198 mL	-

Table 2: Troubleshooting

Step	Problem	Possible reason
1.9	N/A	Verify cell fractionation
2.1	Sample is too viscous	Cell number is too high.
2.12	No visible pellet	
2.13	Low DNA concentration	
3.1	Not enough DNA for dilutions	Insufficient starting material or loss during extraction.
3.4	Sample won't saturate into the membrane	
4.6	Patchy or speckled pattern is detected	
4.6	The dots have a "coffee ring" appearance	
5.2	The RNaseH control shows no decrease in signal	The ribonuclease digestion is incomplete.
6.5	No signal for the oligo controls	Duplex wasn't formed. Oligonucleotides weren't properly annealed.
6.5	S9.6 signal isn't specific to hybrids	S9.6 antibody batch has non-specific binding

Solution

The quality of nuclear and cytoplasmic separation can be evaluated by adding standard protease inhibitor cocktails to the cell and nuclear lysis buffers (Table 1). The cytoplasmic and nuclear fractions can be evaluated by western blotting analysis to confirm adequate restriction of labeling with cytoplasmic markers (for example, GAPDH or HSP90) in cytoplasmic fractions and labeling with nuclear markers (for example, HDAC1 or Histone H3) in nuclear fractions. The contribution of mitochondrial contamination in the nuclear fraction can be evaluated by qPCR analysis with probes specific for mitochondrial DNA.
Reduce the DNA by half and continue with the sonication step 2.1

Start from the beginning using more cells.

Soak the membrane in 1x TBST. Allow excess buffer to dry and start at step 3.4

Add 0.1% Sodium dodecyl sulfate (SDS) to the sample and continue at step 3.1

Add 0.01% Sarkosyl to the sample and continue at step 3.1

Increase incubation or increase enzyme concentration.

Verify the ratios of oligonucleotides and annealing buffer.
Validate the sensitivity and specificity of new batches of S9.6 antibody with the use of either RNase enzyme treatments or synthetic oligonucleotide analysis.

Table 3. Co

ssRNA, top strand
ssDNA, top strand
ssRNA, bottom strand
ssDNA, bottom strand

ntrol oligonucleotide sequences

5’-UGGGGGCUCGUCCGGGAUAUGGGAACCACUGAUCCC-3’
5’-TGGGGGCTCGTCCGGGATATGGGAACCACTGATCCC-3’
5’-GGGAUCAGUGGUUCCCAUAUCCCGGACGAGCCCCCA-3’
5’-GGGATCAGTGGTTCCCATATCCCG GACGAGCCCCCA-3’

Name of material/equipment	Company	Catalog #
Anti-dsDNA antibody	Abcam	ab27156
Anti-RNA-DNA hybrid antibody (S9.6)	Kerafast	ENH001
Biorupter sonicator	Diagenode	UCD-200
EB Buffer	Qiagen	19086
EDTA (0.5M)	Invitrogen	AM9261
Hybond N+ nylon membrane	GE healthcare Life Sciences	RPN203B
KCl (2M)	Invitrogen	AM9640G
NP-40 (Igepal CA-630)	Sigma	I8896
PBS	Invitrogen	10010-023
Phenol:chloroform:isoamyl alcohol	Invitrogen	15593031
PIPES (0.5M, pH 8.0)	VWR	AAJ61406-AE
Proteinase K	Qiagen	19131
Rnase III	Invitrogen	AM2290
Rnase H	New England Biolabs	M0297
Rnase T1	ThermoFisher Sci.	EN0541
SDS (10%)	Invitrogen	15553027
sodium acetate (3M, pH 5.2)	Invitrogen	AM9740
Tris-buffered saline (10X)	Corning	46-012-CM
Tris-HCl (1M, pH 8.0)	KD Medical	RGF-3360
TrypLE	Invitrogen	12605010
Tween-20	Sigma	P9416
UV Stratalinker 2400	Stratagene	Stratalinker 2400
Whatman marking pen	Sigma	WHA10499001

Comments/Description

Response to reviewers;

We thank the reviewers for their review of our work and feel that the recommended changes have helped to improve the quality of the manuscript. We have addressed each of the reviewers' concerns as follows and highlighted the corresponding changes within the manuscript in blue.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have reviewed the manuscript thoroughly to check spelling and grammar. Abbreviations have been defined at first use.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Response: We have edited the reference numbers as requested.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: TrypLE; Eppendorf tube; Biorupter; Qiagen; NanoDrop; GE Healthcare Life Sciences cat#RPN203B; Whatman membrane; UV Stratalinker 2400 (Stratagene); Abcam cat#ab27156; Kerafast cat# ENH001; Sigma cat#03978; New England Biolabs cat# M0297; ThermoFisher Scientific cat# EN0541; Invitrogen cat# AM2290;

Response: Commercial language has been removed from the manuscript.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: Additional information has been added throughout the protocol to add details to the protocol steps. We have added additional information to specify how the procedures are performed in sections 1.1, 3.1, 4.1, 4.3, 4.4, 6.4, and 7.7. We have also added table 2 with instructions on troubleshooting.

5. 1.1: Which cells are you culturing? In what medium? Please move the information in the representative results to the beginning of the protocol.

Response: The cells and culture conditions have been added to section 1.1 of the protocol.

We added the following note to 1.1:

NOTE: The steps described below were used for the analysis of primary human skin fibroblasts, although an array of cell types can be assayed. Fibroblasts were grown in basal media containing 10% fetal bovine serum. Alternatively, cell lysis buffer (Table 1) can be added directly to the cell-culture after washing.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: The essential steps of the protocol (sections 3 and 4) have been highlighted on pgs. 6 and 7.

7. As we are a methods journal, please add any modifications and troubleshooting of the technique to the Discussion.

Response: Additional troubleshooting information was added to the second paragraph of the discussion section on pg. 11. We have added table 2 with specific instructions on troubleshooting.

We have added the following information into the discussion on pg.11:

Some have reported concerns about the specificity of the S9.6 antibody. As with any reagent, there may be batch to batch variability with the S9.6 antibody. Our protocol includes RNase H, RNase T1 and RNase III to check signal specificity. In addition, we use synthetic oligonucleotides to ensure the specificity of each batch of S9.6 antibody.

8. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Wherever possible, please include volume and issue numbers for all references. Do not abbreviate journal names and capitalize the first letters of each word in the journal name.

Response: References were edited as requested.

9. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.). All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Figure legends and table captions should be included in the figure and table legends section after the representative results. Please remove embedded figures and tables from the manuscript.

Response: Embedded figures and tables have been removed and submitted in the requested formats.

10. Please sort the Materials Table alphabetically by the name of the material.

Response: The materials table has been ordered alphabetically.

Reviewer #1:

Manuscript Summary:

This is very timely protocol to get a general idea for changes in genomic R loops and is simpler and far less costly than DRIP-seq or related assays. Indeed, it is very useful starting step to assess whether or not to proceed towards more elaborate (and expensive) NGS-based approaches. The authors are experts in this field and their protocol is detailed and well-written so it should be easily understandable even to newcomers. My only request would be the add a note in the Introduction about the size of RNA-DNA hybrids that the S9.6 Ab can detect. The control substrates used in this protocol are 36 nt but I imagine many R loops are much smaller such as those near TSSs that are frequently detected by DRIP assays. Since replication origins also harbor short RNA-DNA hybrids due to RNA priming of DNA polymerases, does the S9.6 Ab detect these too? It is important to know since many origins of replication are very close to or overlapping TSSs and so many of the TSS-associate R loops may be arising from replication origins. It is a general question unrelated to the protocol here, but it would be good to address it since I do not find this being addressed in the literature (although, admittedly, I may have missed this).

Response: *We thank the reviewer for their comments, and we have now included a reference (33) in the introduction which reports the detection of RNA-DNA hybrids down to 8bp in length at the bottom of pg. 3.*

The specific reference that has been added is:

Phillips, D.D., Garboczi, D.N., Singh, K., Hu, Z., Leppla, S.H., Leysath, C.E. The sub-nanomolar binding of DNA-RNA hybrids by the single chain Fv fragment of antibody S9.6. *J Mol Recognit*. **26** (8), 376-381 (2013).

Major Concerns:

None

Minor Concerns:

None

Reviewer #2:

Manuscript Summary:

This manuscript describes a method to detect mainly cytoplasmic RNA-DNA hybrids (also known as R-loops) by dot blot in mammalian cells. The manuscript includes a useful protocol to detect R-loops in genomic DNA.

Major Concerns:

This method has been previously described in Wahba et al., eLIfe 2013; Stork et al. eLife 2016 (for example). Hence it seems to me that it is somehow too minimal. It would have been nice to show how R-loops accumulate in cells treated with DNA damaging agents such as campthotecin for instance previously shown to induce R-loops accumulation (Hodroj et al., 2017 EMBO J) and also include some cell fractionation experiments to detect cytoplasmic and nuclear R-loops.

Response: We do not intend to say that we have developed a new method for assessing R-loops. Like many other papers in JOVE, this is a step-by-step guide, and in this case to quantify R-loops without special equipment. We receive requests from researchers and clinical laboratories for this protocol as an initial study of R-loops in their samples. This manuscript is intended for them, and by no means is a substitute of the many excellent papers on R-loops that show dot-blots.

Minor Concerns:

1. In the introduction, more recent reviews describing R-loops resolving enzymes must be included

Response: We have included two references (21 and 22) in the second paragraph of the introduction on pg. 3 which provides a recent review of R-loop resolving enzymes.

The specific references added are:

21. Hyjek, M., Figiel, M., Nowotny, M. RNases H: Structure and mechanism. DNA Repair. **84**, 102672 (2019).

22. Pohl, T.J., Zakian, V.A. Pif1 family DNA helicases: A helpmate to RNase H? DNA Repair. **84**, 102633 (2019).

2. In the method section, as it is described, it appears that the DNA is spotted on the membrane without the use of a dot-blot apparatus, why? It seems to me that using a dot-blot apparatus makes experiments more reproducible and allows less loss of material.

Response: We thank the reviewer for this recommendation. The blotting we have described works well without a dot-blot apparatus, however the use of such an

apparatus can be considered if the reader would prefer. We have edited the manuscript section 3.3 which reads

3.3 Spot 2μL of each sample onto 2 membranes. One for the S9.6 antibody and the other for dsDNA. Alternatively, a dot-blot or slot-blot apparatus which allows the loading of samples with larger volumes can be used.

3. Further, the isolation of genomic DNA includes a sonication step. This procedure may disturb R-loops stability, and might reduce the amount of those R-loops that are not very abundant (as it is the case for nuclear versus cytoplasmic R-loops).

Response: We appreciate the concern the reviewer has regarding sonication. We have performed dot-blot assays with and without sonication, and have observed that there is no reduction in signal intensity with sonication. In samples with increased DNA viscosity, sonication allows for more even distribution of S9.6 and dsDNA signal intensity.

4. When using oligonucleotides it is important to assess their degree of annealing, and better purify the annealed nucleic acids to make sure that what it is spotted represent an homogenous population of nucleic acids species.

Response: We recognize that the purification and validation of a homogenous population of nucleic acids is important. To ensure high-quality oligonucleotide controls, we have provided a detailed protocol in section 6 using oligonucleotide sequences that have been previously reported to serve as appropriate positive and negative controls for S9.6 signal interpretation. We added a note under step 6.4 on pg. 8 for annealing efficiency of oligonucleotides to be checked by non-denaturing gel electrophoresis. It reads:

NOTE: Annealing efficiency should be checked by non-denaturing gel electrophoresis. Duplexes migrate more slowly than the unannealed oligonucleotides (Table 2).

5. Details of how quantification of R-loops shown in Figure 2 are missing and/or unclear. Authors state that these are representative results, but no errors bars are displayed on the graphic.

Response: We thank the reviewer for this request. We have clarified in figure legend 2 on pg. 10 that the steps needed for signal quantification are specified in section 7. It reads:

S9.6 signal was divided by dsDNA signal intensity, then normalized to the mock sample following the steps outlined in section 7.

We have also added information in step 7.7 on pg. 9 to discuss how variation within a sample can be assessed by loading triplicates of the sample for S9.6 and dsDNA analysis. It reads:

7.7 The S9.6 signal intensity for each sample should be divided by the measurement of dsDNA to obtain the S9.6/dsDNA signal ratio. The results should be verified by repeating the experiments (we suggest at least triplicates for both S9.6 and dsDNA signal acquisition). Standard error of the mean can be calculated from the S9.6/dsDNA signal ratios.

The graph in Figure 2 is intended to show the quantitation of the S9.6 signals and loading controls shown in Figure 1, and not averages from replicates therefore no error bars were shown.

Reviewer #3:

Manuscript Summary:

This work provides a detailed easy method to quantify, a three-stranded nucleic acid structure R-loop. The authors have attempted to address a long-lasting challenge in the quantitation of R-loops based on the S9.6 monoclonal antibody. They combined dot-blots with the S9.6 antibody to quantify R-lo. They examined sensitivity and specificity of this dot blot assay with various RNA structure sensitive enzymes RNase H, RNase T1, and RNase III. They made this assay reproducible and easy to perform. This assay will be applied in research and clinical settings to quantify R-loops abundance. I recommend its publication.

Response: We thank the reviewer for their support of our manuscript.

Reviewer #4:

Manuscript Summary:

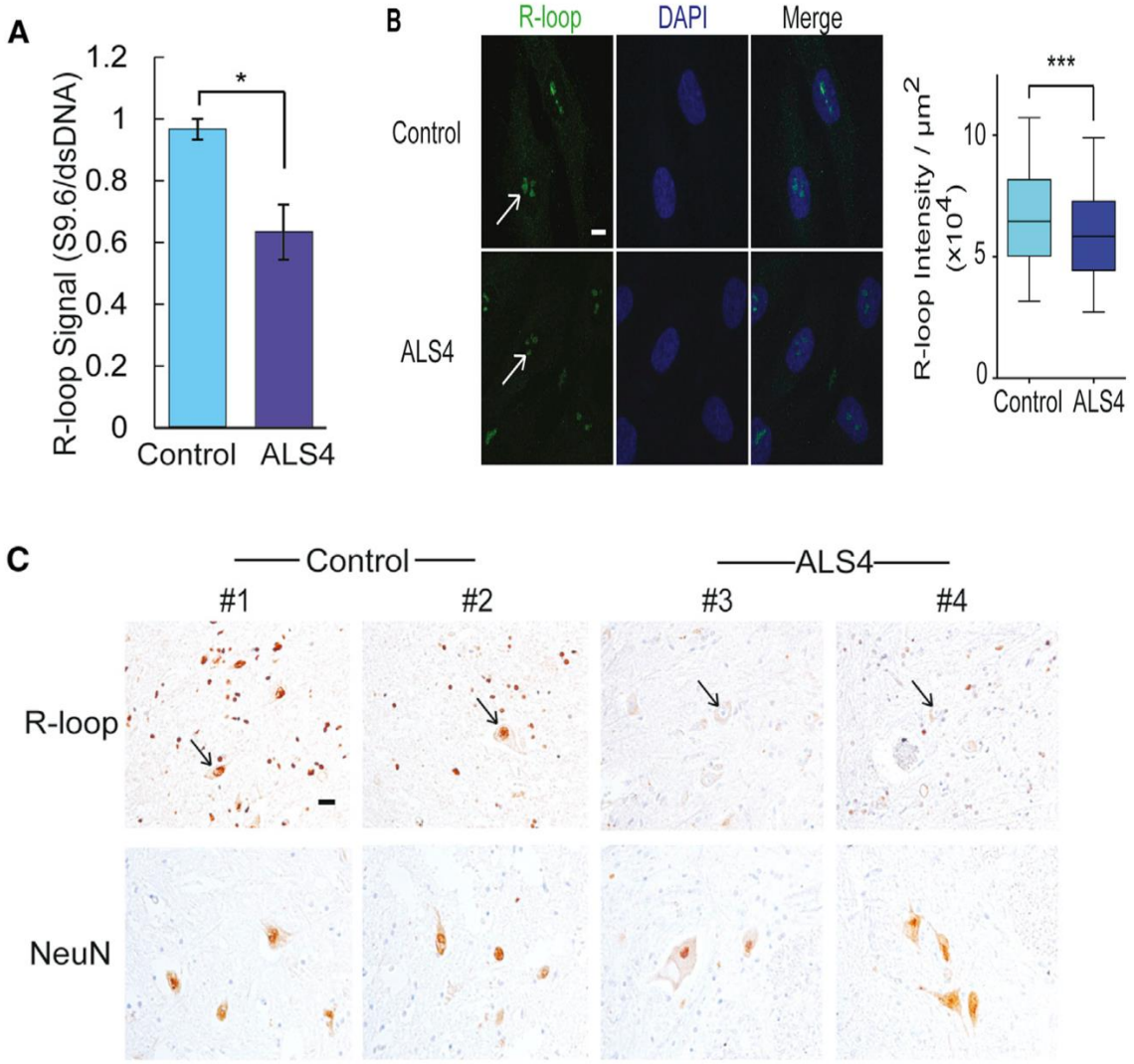
In this manuscript, Ramirez and colleagues propose a method to quantify RNA:DNA hybrids by dot blotting using the well-characterized monoclonal antibody S9.6. This protocol is not novel, but to my knowledge, there is currently no published method on the subject. It should therefore be very useful to an ever-growing community of scientists interested in these structures. Indeed, R-loops have attracted a lot of attention during the past decade. Although dot blots are frequently used to measure R-loop levels in a population of cells, people also often use immunofluorescence (IF), which generates images with a low signal-to-noise ratio and are often very difficult to quantify in a reliable manner. This protocol could therefore be very important for the community by promoting the use of dot blot instead of IF. Overall, this protocol is straightforward and all the major steps are clearly described. With the addition of video, it could become a reference in the field. However, several important issues need to be addressed to improve the manuscript.

Major Concerns:

1. Several general statements on R-loops in the abstract and the introduction are incorrect or misleading. For instance, the sentence "…recent findings of fewer R-loops in diseased cells made it clear that R-loops have functional roles in a variety of human cells (line 33)" is confusing. A large body of evidence indicates that an excess of R-loops correlates with diseases, but I am not aware of any disease caused by a deficit in R-loops. Unless if the authors can refer to specific references, this statement should be removed. Along the same line, the authors indicate that the physiological roles of R-loops were discovered after their pathological consequences (lines 64-68), which is incorrect. They also refer to different types of R-loops (line 49) but it is unclear to which types they refer to. It would be more useful to mention RNA:DNA hybrids that do not necessarily correspond to R-loops and that could also be detected by the S9.6 antibody. Finally, they incorrectly quote an article from the Chedin lab by stating that "5% or more of RNA can be found in R-loops". What the paper shows is that R-loops may cover up to 5% of single-copy loci in mammalian genomes, which is quite different. I would recommend that a senior author of the manuscript carefully double checks the introduction section and the references before publication.

Response: We thank the reviewer for the opportunity to clarify why discussion of R-loop deficits and disease were included in the manuscript.

In the referenced manuscript Grunseich et al., Mol Cell 2018, the authors show that ALS4 patients with mutation in senataxin have fewer R-loops. This reduction in R-loops was measured by dot-blot (A) and S9.6 immunofluorescence (B) in patient and control fibroblast cells and S9.6 immunohistochemistry staining in patient and control spinal cord tissues (C).



We have edited the second paragraph of the introduction on pg.3 so that the normal physiologic functions of R-loops are introduced first. It now reads:

R-loops play a regulatory role. They regulate gene expression by affecting transcription at promoters[10,11], mediating class-switch recombination[12,] and facilitating CRISPR-based genome editing[13,14,15].

The beginning of the introduction on pg.2 was edited to clarify that R-loop formation occurs for many different reasons. It now reads:

R-loop forms when RNA invades a double-stranded DNA to generate an RNA-DNA hybrid and displaces the other DNA-strand. R-loops are found in different stages of the lifecycle of RNA.

We have corrected the referenced information in the Sanz et al. manuscript to indicate that R-loops can be found in 3-5% of the human genome in the first paragraph of the introduction on pgs. 2-3. It now reads:

Given the many opportunities for their formation, R-loops are not rare, and can be found in 3-5% of the human genome[6], depending on the cell's transcription status.

2. The authors use sonication to reduce the viscosity of genomic DNA. Since it has been reported by different labs that sonication could destabilize at least a subset of R-loops, it would be important to compare the intensity of the S9.6 signal before and after sonication. If needed, DNA could be fragmented with a cocktail of restriction enzymes, which is the procedure most commonly used in DRIP-seq protocols.

Response: We appreciate the concern regarding sonication. We have performed dot-blot assays with and without sonication, and have observed that there is no reduction in signal intensity. In samples with increased DNA viscosity, sonication allows for more even distribution of S9.6 and dsDNA signal intensity.

3. Line 73: It would be worth mentioning that the earliest versions of DRIP-seq protocols are not quantitative. Most genome-wide approaches provide therefore qualitative and positional information rather than quantitative results, which further justifies the use of dot blot. The authors should also mention IF in this paragraph and recommend the use of dot blot instead of IF because of the many issues with IF mentioned above.

Response: We have added additional language to the third paragraph of the introduction on pg.3 to indicate that early versions of the DRIP-seq protocols did not achieve sequence coverage to allow precise quantification. We have also mentioned the option of immunofluorescence, and the limitations with this technique in this paragraph. It now reads:

Early versions of these sequencing-based methods did not achieve adequate sequence coverage to allow precise quantitation but rapid improvement in sequencing technologies now allows locus-by-locus R-loop analysis. Immunofluorescence techniques have also been used to quantify and localize R-loops[10,17]. These methods are comprehensive, but they are not practical in many clinical settings or as initial assessments since they require expensive equipment and specialized analysis.

4. Line 79: Many people use slot blot as an alternative to dot blot. The difference between slot blot and dot blot should be discussed.

Response: We thank the reviewer for this comment. The blotting we have described works well without a dot-blot apparatus, however the use of such an apparatus can be considered depending on the preference of the reader. We have edited the manuscript section 3.3 on pg.6 to include mention of a dot-blot apparatus. It now reads:

3.3 Spot 2μL of each sample onto 2 membranes. One for the S9.6 antibody and the other for dsDNA. Alternatively, a dot-blot or slot-blot apparatus which allows the loading of samples with larger volumes can be used.

5. RNase H degrades RNA, rather than cleave it.

Response: We have included a reference from Nowotny et al., Cell 2005 which reports on the cleavage of RNA strands in RNA/DNA hybrids. We recognize that RNase H activity on RNA-DNA hybrids involves both its cleavage and subsequent degradation. We have edited the fourth paragraph of the introduction on pg. 3 to indicate that the enzyme activity involves both cleavage and degradation. It now reads:

Our protocol uses the S9.6 antibody[27] to identify RNA-DNA hybrids in R-loops and incorporates RNase H, an endoribonuclease that cleaves and therefore leads to the degradation of the RNA in an RNA-DNA hybrid[20,28], to ensure that the detected signals are those of hybrids.

6. The concentration of S9.6 antibody should be indicated, not only the dilution.

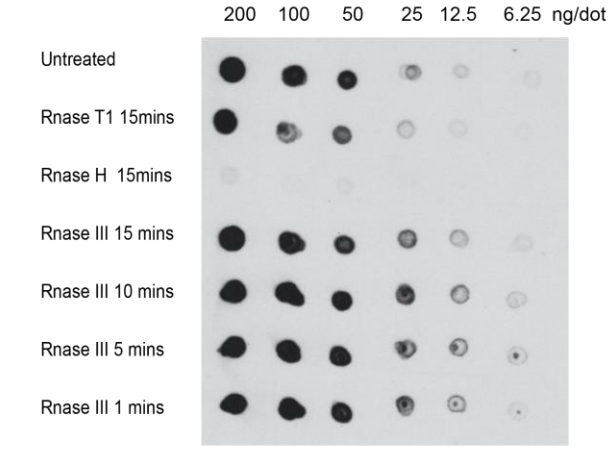
Response: Thank you. We have provided the concentration in section 4.2 of the protocol on pg.7. It now reads:

Add the S9.6 antibody to the second membrane (1:1000 [1μg/mL] dilution).

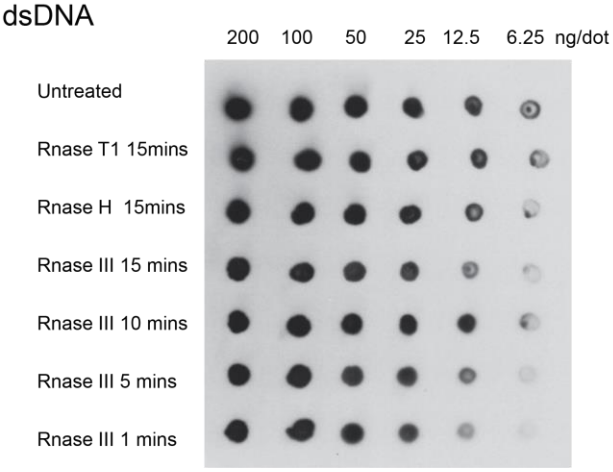
7. The authors treated DNA samples with various RNases at 37°C for only 15 minutes, the kinetic of enzyme digestion can be included to determine whether longer period of RNase treatment might alter the abundance of RNA:DNA hybrids.

Response: The RNases used have been demonstrated to have substrate specificity in previous studies (Wang IX et al., Genome Research 28:1405-1414, 2018, and Hartono et al., J Mol Biol 430:272-284, 2017). We are attaching data below showing stability of S9.6 and dsDNA signals following a time course of RNaseIII treatment.

S9.6



dsDNA



8. The authors used lysis buffer and low-speed centrifugation to separate nuclear and cytoplasmic fraction before isolating genomic DNA. To exclude a possible contamination of genomic DNA with R-loops from mitochondrial DNA origin, the authors could quantify the relative amount of mitochondrial DNA in total DNA using qPCR.

Response: We thank the reviewer for this comment. We have added language concerning signal specificity to section 1.9 of the protocol on pg. 5. It reads:

NOTE: Fractionation of the nuclear and cytoplasmic compartments of the cells ensures signal specificity. The quality of nuclear and cytoplasmic separation can be evaluated before proceeding (Table 2).

We have provided information in table 2 which provides a method for verifying cell fractionation. This recommendation includes a suggestion to assess for mitochondira contamination in the nuclear fraction by qPCR. It reads:

The quality of nuclear and cytoplasmic separation can be evaluated by adding standard protease inhibitor cocktails to the cell and nuclear lysis buffers (Table 1). The cytoplasmic and nuclear fractions can be evaluated by western blotting analysis to confirm adequate restriction of labeling with cytoplasmic markers (for example, GAPDH or HSP90) in cytoplasmic fractions and labeling with nuclear markers (for example, HDAC1 or Histone H3) in nuclear fractions. The contribution of mitochondrial contamination in the nuclear fraction can be evaluated by qPCR analysis with probes specific for mitochondrial DNA.

9. The use of methylene blue to quantify total DNA is not very convincing. Considering the importance of normalization, I would rather omit this part and only recommend the use of anti-DNA antibodies.

Response: We agree and have removed the methylene blue quantification from the manuscript.

10. The authors should provide additional guidelines on how to ensure that the signal measured is in the linear range of the assay and how many biological replicates should be performed to generate reliable results.

Response: Additional guidance on sample preparation to ensure a linear range of detection has been added to section 3.1 on pg. 6 of the manuscript. Discussion of biological replicates has also been added to this section. It reads:

3.1 Prepare dilutions of nucleic acids to desired concentrations in elution buffer (ie. 50ng/μL, 25ng/μL, or 12.5ng/μL). These samples with a range of concentrations (200, 100, 50, 25, 12.5ng) ensure that there will be signals within the linear range.

Note: Be sure to prepare enough sample for technical and biological replicates, and for the various RNase treatments, see Step 5.