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Detection of DNA-RNA hybrids *in vivo*

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Running title: R loop detection

i. Abstract

DNA-RNA hybrids form naturally during essential cellular functions such as transcription and replication. However, they may be an important source of genome instability, a hallmark of cancer and genetic diseases. Detection of DNA-RNA hybrids in cells is becoming crucial to understand an increasing number of molecular biology processes in genome dynamics and function and to identify new factors and mechanisms responsible for disease in biomedical research. Here we describe two different procedures for the reliable detection of DNA-RNA hybrids in the yeast *Saccharomyces cerevisiae* and in human cells: DNA-RNA Immunoprecipitation (DRIP) and Immunofluorescence.

ii. Key Words

DNA-RNA hybrids, R loop, genome instability, S9.6 antibody

1. Introduction

DNA-RNA hybrids are highly stable structures formed during essential cellular functions such as transcription and replication. Hybrids covering few nucleotides (around 10) are essential for Okazaki fragment synthesis during DNA replication as well as for RNA synthesis by RNA polymerases. In a different context, however, an RNA molecule may invade a double-stranded DNA (dsDNA) to form a DNA-RNA hybrid and the displaced DNA strand. These structures are known as R loops.

R loops have been shown to have a physiological role in initiation of replication of mitochondrial DNA or some bacterial plasmids, as well as in Immunoglobulin class switch recombination, a vital process for the generation of the genetic diversity that the defense systems of vertebrates requires, reviewed in [1]. In these cases, DNA-RNA hybrids may expand from tens of nucleotides to few hundreds, the S regions involved in Ig class switching having been reported to form hybrids that can exceed even 1 kb in size [2]. However, since a first report showing that yeast cells lacking the RNA biogenesis and export factor THO accumulate R loops responsible for high levels of transcription-dependent genome instability [3], different contributions have provided evidence that DNA-RNA hybrids or R loops can form at higher levels than previously foreseen, becoming an important threat to genome integrity [1]. The increasing abundance of factors identified to be involved in prevention or resolution of DNA-RNA hybrids have been shown to map all over the genome with particular hotspot regions, and different reports have demonstrated that they may play positive and negative roles in transcription. Importantly, however, it is becoming evident that cells have a large number of proteins involve in the prevention or resolution of R loops.

In most physiological cases, so far, it has not been either reported or explored whether DNA-RNA hybrids require in addition the participation of specific proteins for their formation or stability. In this sense, we know that the action of the CRISPR-Cas9 system of genome editing is based on the formation of a DNA-RNA hybrid indeed [4]. Thus the possibility that R loops may be facilitated or stabilized by specific proteins [5] needs to be further explore in the future. However, any *in vivo* study on R loops, whether naturally formed or artificially induced, requires to be completed with the *in vivo* detection of DNA-RNA hybrids in cells. This is so far a bottle neck step in all studies. Even though there have been several methods used in different studies, the most extended ones are based on the use of the monoclonal anti-DNA-RNA hybrid antibody of the S9.6 hybridoma [6,3,7-12,2,13]. Here we provide the step-by-step protocol of the two more-extended techniques used with this antibody, DNA-RNA immunoprecipitation (DRIP) [14] and immunofluoresecence (IF) analyses to detect hybrids both in yeast and human cells [15,16]. As can be seen it is essential in any DNA-RNA hybrid detection techniques to validate that the signals detected correspond unequivocally to DNA-RNA hybrids by eliminating them by treatment with RNase H *in vitro* or by overexpressing RNase H *in vivo*.

2. Materials

2.1 DNA-RNA Inmunoprecipiation (DRIP) in human cells

1. Cell cultures.
2. 10 cm plates.
3. PBS Tablets (GIBCO).
4. 1x TE buffer: 10 mM Tris-HCL pH 7.6, EDTA 1 mM pH 8.0.

5. 20 % SDS.
6. Proteinase K, recombinant (20 mg/ml, Roche).
7. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 0.1 mM EDTA.
8. Phase Lock Gel Heavy 2 ml tubes (% Prime GmbH).
9. 3 M Sodium Acetate.
10. Isopropanol.
11. Glass rod.
12. 70% Ethanol.
13. Restriction enzymes (*Hin*dIII 2.5 µl, *Eco*RI 2.5 µl, *Xba*I 2.5 µl, *Ssp*I 10 µl, *Bsr*GI 5 µl), BSA 2.5 µl and NEB Buffer 2 (New England BioLabs).
14. StrataClean Resin (Stratagene).
15. 50T5E buffer: 50 mM Tris-HCL pH 7.5, 5 mM EDTA pH 8.0.
16. 50% v/v slurry of Sephadex G-50 (Sigma) in 50T5E buffer (kept at 4ºC).
17. Empty micro Bio-spin chromatography columns (Biorad).
18. RNase H and appropriate 10x buffer (New England BioLabs).
19. Fluorometer apparatus and appropriate cuvettes.
20. Appropriate DNA fragments.
21. anti-DNA-RNA hybrid antibody (S9.6)
22. 1 M Na-Phosphate pH 7.0 (39 ml 2 M monobasic sodium phosphate NaH_2PO_4, 61 ml 2 M dibasic sodium phosphate Na_2HPO_4, 100 ml H_2O).
23. 10x Binding Buffer (100 mM $NaPO_4$ pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100, stored at room temperature).
24. Elution Buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5 % SDS)
25. Protein A-coated magnetic beads (Dynabeads, Novex by Life Technologies).

26. Magnetic rack (Life Technologies).
27. Proteinase K, recombinant (20mg/ml, Roche).
28. Rotator mixer.
29. Thermomixer.
30. PCR Purification Kit (Quiagen).
31. 1x TE buffer: 10 mM Tris-HCL pH 7.6, EDTA 1 mM pH 8.0.
32. Power SYBR green PCR master mix (Applied Biosystems).
33. Appropriate oligonucleotides (*see* **Note 10**)
34. Fast Real-Time PCR System.

2.2 DNA-RNA Immunoprecipiation in yeast cultures

1. Cell cultures.
2. Zymolyase 20T (USB)
3. Spheroplasting Buffer: 1 M sorbitol, 2 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 0.1 % v/v beta-mercapto-ethanol.
4. Solution I: 0.8 mM GuHCl, 30 mM Tris-HCl pH 8.0, 30 mM EDTA pH 8.0, 5% Tween 20, 0.5 % Triton X-100.
5. RNase A stock solution: 10 mg/ml (Roche; *see* **Note 11).**
6. Proteinase K, recombinant (20 mg/ml, Roche).
7. Chloroform-Isoamyl alcohol (24:1).
8. Isopropanol (at room temperature).
9. 70% Ethanol (at room temperature).
10. Restriction enzymes (*Hin*dIII 2.5 µl, *Eco*RI 2.5 µl, *Xba*I 2.5 µl, *Ssp*I 10 µl, *Bsr*GI 5 µl), BSA 2.5 µl and NEB Buffer 2 (New England BioLabs).
11. StrataClean Resin (Stratagene).

12. 50T5E buffer: 50 mM Tris-HCL pH 7.5, 5 mM EDTA pH 8.0.
13. 50 % v/v slurry of Sephadex G-50 (Sigma) in 50T5E buffer (kept at 4ºC).
14. Empty micro Bio-spin chromatography columns (Biorad).
15. RNase H and appropriate 10x buffer (New England BioLabs).
16. Fluorometer apparatus and appropriate cuvettes.
17. Appropriate DNA fragments.
18. anti-DNA-RNA hybrid antibody (S9.6)
19. 1 M Na-Phosphate pH 7.0 (39 ml 2 M monobasic sodium phosphate NaH_2PO_4, 61 ml 2 M dibasic sodium phosphate Na_2HPO_4, 100 ml H_2O).
20. 10x Binding Buffer (100 mM $NaPO_4$ pH 7.0, 1.4 M NaCl, 0.5 % Triton X-100, stored at room temperature).
21. Elution Buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5 % SDS).
22. Protein A-coated magnetic beads (Dynabeads, Novex by Life Technologies).
23. Magnetic rack (Life Technologies).
24. Proteinase K, recombinant (20 mg/ml, Roche).
25. Rotator mixer.
26. Thermomixer.
27. PCR Purification Kit (Quiagen).
28. 1x TE buffer: 10 mM Tris-HCL pH 7.6, EDTA 1 mM pH 8.0.
29. Power SYBR green PCR master mix (Applied Biosystems).
30. Appropriate oligonucleotides (*see* **Note 10**)
31. Fast Real-Time PCR System.

2.3 S9.6 immunofluorescence in mammalian cells

1. DMEM-Dulbecco's Modified Eagle Medium (GIBCO).
2. 24-well plates.

3. Tweezers.

4. Round Coverslips.

5. Bovine serum albumin (BSA).

6. PBS Tablets (GIBCO).

7. Blocking Solution: 2 % BSA in PBS (Phosphate-Buffered Saline).

8. anti-DNA-RNA hybrid antibody (S9.6).

9. Nucleolin antibody (Abcam).

10. Ice-cold methanol: -20ºC 100 % methanol (see **Note 16**).

11. Alexa Fluor 488 goat anti-rabbit (Invitrogen).

12. Alexa Fluor 594 goat anti-mouse (Invitrogen).

13. Vacuum line.

14. Immu-Mount Mounting Medium (Thermo Scientific).

15. Microscope Slides.

16. Confocal microscope.

17. DAPI solution (1 μg/ml in PBS).

2.4 S9.6 immunofluorescence in yeast

1. Yeast cultures.
2. Formaldehyde 37 %.
3. Phosphate buffer 0.1 M pH 6.4.
4. Sorbitol-citrate 1.2 M: 218.6 g/l sorbitol, 7 g/l citric acid, 17.418 g/l K_2HPO_4.
5. Poly L Lysine Treated Slides.
6. Glusulase (Perkin Elmer).
7. Zymoliase 20T (USBiological): 50 mg/ml in sorbitol 1 M.
8. SDS.

9. PBS-BSA Solution: 1 % BSA, 0.04 M K2HPO4, 0.01 M KH2PO4, 0.15 M NaCl, 0.1 % NaN3. Filter sterilized and stored at 4ºC.
10. Digestion Mix: 200 μl of 1.2 M sorbitol-citrate, 20 μl of glusulase, 2 μl of zymolyase 20T.
11. Alexa Fluor 594 goat anti-mouse (Invitrogen).
12. anti-DNA-RNA hybrid antibody (S9.6).
13. PBS Tablets (GIBCO).
14. DAPI
15. Fluorescence microscope

3. Methods

3.1 DNA-RNA immunoprecipitation in human cells

1. Transfer the content of a 10 cm plate of 90 % confluent cells ($3x10^6$ cells) to a 15 ml falcon tube and pellet them at 400 x*g*, 4ºC in a swing rotor for 5 min. Remove supernatant.
2. Wash and resuspend cells with 10 ml 1x cold PBS and pellet them again. Remove supernatant.
3. Resuspend cells in 1.6 ml of TE 1x and split in two tubes with a cut-off pipette tip (*see* **Note 1**). Add 21 μl of 20 % SDS, and 2.5 μl of Proteinase K to each sample (0.8 ml) and invert the tube a few times. Incubate overnight at 37ºC. (*see* **Note 2**)
4. Pour the DNA from previous step into a phase lock 2 ml tube (*see* **Note 3**). Add 1 volume (0.8 ml) of Phenol:Chloroform:Isoamyl Alcohol, invert gently a few times, spin down 5 min at 13,000 x*g*. (*see* **Note 4**)
5. Pour the DNA (top aqueous phase) in a new phase lock tube and repeat step 4.

6. Add 1/10 volume 3 M NaOAc (160 μl) and 1 volume (1.6 ml) isopropanol to a 15 ml tube and pour in the DNA (top aqueous phase, rejoin the samples in one tube) from two phase lock tubes.
7. Invert gently until the DNA begins to precipitate. Spool DNA on a glass rod. (*see* **Note 5**)
8. Wash DNA with 70 % Ethanol by allowing the EtOH run down glass rod.
9. Allow to air dry. Break off the tip with DNA and put it in a 1.5 ml tube with 150 μl TE 1x. Do not resuspend the DNA by over-pipeting/vortexing. Mix several times by gentle shaking. (*see* **Note 6**)
10. Digest the DNA overnight using cocktail restriction enzymes according to suppliers instructions (add BSA 1x, final volume is 250 μl). (*see* **Note 7**).
11. Add 3 μl StrataClean Resin, pipet with cut tips and spin down at full speed in a microcentrifuge for 1 min. (*see* **Note 8**)
12. Purify over Sephadex g-50 column (in 50T5E buffer). (*see* **Note 9**)
13. Split the flow-through containing DNA into two new tubes (125 μl).
14. Treat the half of the DNA (one tube) with 5 μl RNase H (NEB) overnight at 37ºC. In the meantime keep the other half at 4ºC.
15. Repeat step 12.
16. Check DNA concentration (samples treated and untreated with RNase H)
17. Take 1 μg of digested DNA (treated and untreated with RNase H) (INPUT) and process immediately as described in steps 25 and 26.
18. Dilute 5 μg of digested DNA (treated and untreated with RNase H) in 450 μl TE1x.
19. Add 51 μl of 10x Binding Buffer.
20. Add 10 μl of anti-DNA-RNA hybrid antibody (Stock 1 mg/ml).
21. Incubate overnight at 4ºC on a rotator mixer.

22. Wash 40 μl of Protein A magnetic beads per DRIP sample, twice with 1ml of 1x Binding Buffer (10x diluted in TE1x). Resuspend in the original volume (40 μl).
23. Add the prepared magnetic beads to the extracts (40 μl per reaction) and incubate 2 hours at 4ºC on a rotator mixer.
24. Place the precooled magnetic rack on ice, insert the tubes, aspirate all liquid off and resuspend the beads in 1 ml of 1x Binding Buffer. Repeat the same procedure to achieve 3 washes with 1x Binding Buffer.
25. Add 120 μl Elution Buffer and 7 μl Proteinase K and incubate at 55ºC 45 min in a shaker. Dilute 1μg of INPUT samples (step 17) in 47 μl of TE1x and add 3 μl of Proteinase K, incubate at 55ºC 45 min.
26. Purify (INPUT and PRECIPITATE) over PCR purification columns (e.g., from Quiagen) according to manufacturer protocol and elute in 150 μl TE1x. Store samples at -20ºC.
27. Quantify the enriched DNA fragments in the PRECIPITATE by real time quantitative PCR (qPCR). We perform qPCR using Fast SYBR Green dye and the absolute quantification protocol in the 7500 Fast Real-Time PCR System (Applied Biosystems). Standard curves for all pairs of primers are performed for each analysis. All PCR reactions are perfomed in triplicate. The enrichment for each PCR of interest is normalized with respect to the corresponding ratios of the INPUT. (*see* Fig. 1)

3.2 DNA-RNA immunoprecipitation in yeast cultures

1. Grow 100 ml yeast cultures in the appropriate medium and temperature to an absorbance at 600 nm of about 0.8.
2. Harvest cells by centrifugation at 4000 x*g,* 4ºC for 5 min.
3. Wash cells twice with 20 ml cold H_2O.

4. Harvest cells by centrifugation at 4000 x*g,* 4ºC for 5 min.
5. Resuspend the cell pellet in 2.4 ml Spheroplasting Buffer (freshly made) (add 2 mg Zymolyase to 2.4 Spheroplasting Buffer). Split the sample in two tubes. (*see* **Note 1**).
6. Incubate at 30ºC for 30 min with rotation.
7. Spin down for 5 min at 4500 x*g* in a microcentrifuge and discard the supernatant. Remove all the supernatant.
8. Carefully wash the spheroplast pellet by adding 500 µl of cold H_2O without resuspending.
9. Spin briefly and discard the water.
10. Break the spheroplasts by resuspending in 1.125 ml Solution I (565 µl per tube, rejoin the samples) and transferring in 2 ml eppendorf tubes. (*see* **Note 12**)
11. Add 20 µl RNase.
12. Incubate for 30 min at 37ºC.
13. Add 75 µl Proteinase K (20 mg/ml) and incubate at 50ºC for 60 min. (*see* **Note 13**)
14. Spin down for 5 min at 4500 x*g* and transfer supernatant to a new tube.
15. Add 800 µl Chloroform-Isoamyl alcohol.
16. Spin down for 5 min at 4500 x*g*.
17. Carefully collect the upper phase with 1 ml cut tips and transfer into a 2 ml tube.
18. Add 800 µl isopropanol to the aqueous phase and invert several times until genomic DNA precipitates. Spool DNA on a glass rod. (*see* **Note 5**)
19. Wash DNA with 70% Ethanol by allowing the EtOH run down glass rod.
20. Allow to air dry, break off the tip with DNA and put it in a 1.5 ml tube with 150 µl TE 1x. Do not resuspend the DNA by over-pipetting/vortexing. Mix several times by gentle shaking. (*see* **Note 6**)

21. Digest the DNA overnight using cocktail restriction enzymes according to suppliers instructions (add 1x BSA, final volume is 250 μl). (*see* **Note 7**)
22. Add 3 μl StrataClean Resin, pipet with cut tips and spin down at full speed in a microcentrifuge for 1 min. (*see* **Note 8**)
23. Purify over Sephadex g-50 column (in 50T5E buffer). (*see* **Note 9**)
24. Split the flow-through containing DNA into two new tubes (125 μl).
25. Treat half of the DNA (one tube) with 8 μl RNase H (NEB) overnight at 37ºC. In the meantime keep the other half at 4ºC.
26. Repeat step 12.
27. Take 20 μl of digested DNA (treated and untreated with RNase H) (INPUT) and process immediately as described in step 35.
28. Dilute digested DNA (treated and untreated with RNase H) in 450 μl TE1x.
29. Add 51 μl of 10x Binding Buffer.
30. Add 10 μl of anti-DNA-RNA hybrid antibody (Stock 1 mg/ml).
31. Incubate overnight at 4ºC on a rotator mixer.
32. Wash 40 μl of Protein A magnetic beads per DRIP sample, twice with 1 ml of 1x Binding Buffer (10x diluted in TE1x). Resuspend in the original volume (40 μl).
33. Add the prepared magnetic beads to the extracts (40 μl per reaction) and incubate 2 hours at 4ºC on a rotator mixer.
34. Place the precooled magnetic rack on ice, insert the tubes, aspirate all liquid off and resuspend the beads in 1 ml of 1x Binding Buffer. Repeat the same procedure to complete 3 washes with 1x Binding Buffer.
35. Add 120 μl Elution Buffer and 7 μl Proteinase K and incubate at 55ºC 45 min in a shaker. Add 27 μl TE1x to INPUT samples (step 27), 3 μl Proteinase K and incubate at 55ºC 45 min.

36. Purify INPUT and PRECIPITATE over PCR purification columns according to manufacturer protocol and elute in 150 μl TE1x. Store samples at -20ºC.
37. Quantify the enriched DNA fragments in the PRECIPITATE by real time quantitative PCR (qPCR). We perform qPCR using Fast SYBR Green dye and the absolute quantification protocol in the 7500 Fast Real-Time PCR system (Applied Biosystems). Standard curves for all pairs of primers are performed for each analysis. All PCR reactions are performed in triplicated. The enrichment for each PCR of interest is normalized with respect to the corresponding ratios of the INPUT.

3.3 S9.6 Immunofluorescence in mammalian cells

1. HeLa cells are cultured on coverslips (*see* **Note 14**) at a concentration of $2x10^5$ cells/well in a 6 well plate.
2. After 24 h, coverslips are transferred to a 24-well plate well with cold PBS. (*see* **Note 15**)
3. Aspirate the PBS with the vacuum system and add 1 ml of 100% ice-cold methanol (*see* **Note 16**). Incubate for 7 min at -20ºC.
4. Remove methanol (*see* **Note 16**) and wash twice with 1 ml/well of PBS.
5. Incubate the cells with Blocking Solution (1 ml/well) and incubate overnight at 4ºC.
6. Remove Blocking Solution and add anti-DNA-RNA hybrid (1:500 in Blocking Solution) and Nucleolin (1:1000 in Blocking Solution) antibodies in a total volume of 250 μl/well. Incubate overnight at 4ºC.
7. Wash three times in PBS (5 min each) and incubate with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse secondary antibodies (1:1000 in Blocking Solution) 1h at room temperature.

8. Wash twice for 5 min each in PBS. Incubate with DAPI solution for 5 min at room temperature and wash twice more for 5 min each in PBS. Wash once in distilled H_2O.
8. Coverslips are removed using tweezers and placed on a microscope slide with Immumount mounting medium. (*see* **Note 17**)
9. The slides are placed flat at room temperature for 24h and then stored at 4ºC. (*see* Fig. 2)

3.4 S9.6 Immunofluorescence in yeast

1. Collect at least 1 ml of mitotic culture.
2. Add 100 µl of 37 % formaldehyde to the media and vortex.
3. Spin the culture for 1 min at 900 x*g* and resuspend in 3.7 % formaldehyde (*see* **Note 18**). Fix 10 min at room temperature.
4. Wash twice in 1 ml of 0.1 M Phosphate Buffer pH 6.4.
5. Resuspend in 1 ml of 1.2 M sorbitol-citrate (*see* **Note 19**)
6. Spin down and resuspend in digestion mix. Rotate on rack at 30ºC for 5 min (*see* **Note 20**)
7. Spin down at 900 x*g* for 2 min. Remove supernatant and resuspend in 1 ml of sorbitol by inversion (*see* **Note 21**)
8. Spin down at 900 x*g* for 3 min. Remove supernatant and resuspend in sorbitol by inversion (*see* **Note 21**).
9. Clean the slide with a scrubber under distilled water (*see* **Note 22**). Let it dry completely.
10. Place 5 µl of 0.1 % Poly-L-lysine on each well and let it sit for 5 min (*see* **Note 23**)
11. Wash the slide under distilled water and let it dry completely.

12. Prepare a humid chamber by wrapping a petri dish in aluminum foil and put a wet paper towel inside.
13. In order to make the cells burst, drop 5 μl of the cells onto the slide from a height of 30-40 cm (*see* **Note 24**). Let cells sit for 10 min.
14. Remove supernatant by placing vacuum tip to the side of the well (*see* **Note 23**)
15. Put slide in 4ºC methanol (*see* **Note 16**) for 3 min followed by 10 seconds in 4ºC acetone. Let it dry completely.
16. Add 5 μl of primary antibody (anti-DNA-RNA hybrid 1:200 in PBS/BSA). Incubate in a wet chamber for 2h at room temperature.
17. Remove supernatant and wash five times in PBS/BSA (*see* **Note 25**).
18. Add 5 μl of secondary antibody (anti-mouse Alexa Fluor 546). Incubate 2 h at room temperature.
19. Remove supernatant and wash five times in PBS/BSA.
20. Add 3 μl of mounting medium with DAPI to each sample. Put on a coverslip. Paint with nail polish around the edges of the coverslip and let it dry for 10 min.

(*see* Fig. 3)

4 Notes

1. Always pipet DNA with cut tips to preserve DNA-RNA hybrids. Do not attempt to resuspend DNA by over-pipetting/vortexing.
2. The solution becomes viscous.
3. This step is extremely important. Phase lock gel heavy 2 ml tubes (5 PRIME GmbH, Hamburg, Deutschland) can be used to facilitate sample phenol treatment.
4. Wear gloves and a lab coat while working with phenol, because it is a very dangerous compound. Do it in the hood. Normally, the aqueous phase forms the

upper phase. You should always dispose of phenol waste in a specially sealed container and ensure that it is eliminated according to your institution's policies for dangerous waste.

5. Use a glass Pasteur pipette to spool the DNA. Do not spin to precipitate the DNA. Centrifugation results in high loads of RNA contamination.
6. Do not attempt to resuspend the DNA by vortexing, eventually the DNA could be resuspended at 30ºC (1-2 hours). Invert the tube occasionally.
7. Use Buffer 2.1 (NEB) for the restriction. You do not need to remove the cut glass rod. We often run 4 μl on gel the next day to ensure the digestion is complete and the samples are free of RNA (*see* Fig. 3)
8. Better processing groups of two or three samples.
9. To prepare Sephadex G-50 columns, place empty micro Biospin chromatography columns in 2 ml eppendorf tubes, add 800 μl of Sephadex G-50 slurry and centrifuge at 2000 x*g* for 2 min in a microcentrifuge. The Sephadex G-50 columns are then placed in new 1.5 ml tubes, which serve to collect the purified DNA after centrifugation at 2000 x*g* for 2 min in a microcentrifuge.
10. We use the Primer Express application software (Applied Biosystems) to design the oligonucleotides.
11. The RNase A should be carefully boiled to eliminate residual DNase activity.
12. Spheroplasts are very difficult to resuspend at this step. Use 1 ml cut tips.
13. At this point if there are not many clamps in the samples you can proceed further with the extraction. If the preps are "dirty" you can incubate overnight at 30ºC.
14. Use tweezers to place two to three round coverslips on a well before seeding the cells.

15. Fill the 24-well plate with 1 ml of PBS per well and place one coverslip per well with the cells on the upper side.
16. Methanol is a hazardous chemical with significant toxic, flammable, and reactive properties that can produce deleterious impacts on human health and the environment when not properly handled. Wear lab coat and gloves when working with methanol and manipulate it always in a chemical fume hood.
17. Place a drop of about 30 µl of mounting medium on a slide and place the coverslip with the cells facing the mounting medium.
18. Formaldehyde is a sensitizing agent and a cancer hazard. Wear gloves and lab coat and work always in a chemical fume hood.
19. At this step, samples can be stored at -20ºC overnight.
20. Do not vortex.
21. Check digestion by mixing equal amounts of cells and 1% SDS. You should see crystals at the microscope.
22. It is very important for cells to burst properly that the slides are really clean.
23. Do not touch the well with the tip.
24. You can stand on a chair to get a good height to drop the cells.
25. Lay down a drop and remove it with a vacuum line.

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5 References

1. Aguilera A, Garcia-Muse T (2012) R loops: from transcription byproducts to threats to genome stability. Mol Cell 46 (2):115-124.

2. Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR (2003) R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nat Immunol 4 (5):442-451.

3. Huertas P, Aguilera A (2003) Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. MolCell 12 (3):711-721

4. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337 (6096):816-821.

5. Gavalda S, Santos-Pereira JM, Garcia-Rubio ML, Luna R, Aguilera A (2016) Excess of Yra1 RNA-Binding Factor Causes Transcription-Dependent Genome Instability, Replication Impairment and Telomere Shortening. PLoS Genet 12 (4):e1005966.

6. Boguslawski SJ, Smith DE, Michalak MA, Mickelson KE, Yehle CO, Patterson WL, Carrico RJ (1986) Characterization of monoclonal antibody to DNA.RNA and its application to immunodetection of hybrids. J Immunol Meth 89 (1):123-130

7. El Hage A, French SL, Beyer AL, Tollervey D (2010) Loss of Topoisomerase I leads to R-loop-mediated transcriptional blocks during ribosomal RNA synthesis. Genes & Dev 24 (14):1546-1558.

8. Pohjoismäki JLO, Holmes JB, Wood SR, Yang M-Y, Yasukawa T, Reyes A, Bailey LJ, Cluett TJ, Goffart S, Willcox S, Rigby RE, Jackson AP, Spelbrink JN, Griffith JD, Crouch RJ, Jacobs HT, Holt IJ (2010) Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid. J Mol Biol 397 (5):1144-1155.

9. Mischo HE, Gómez-González B, Grzechnik P, Rondón AG, Wei W, Steinmetz L, Aguilera A, Proudfoot NJ (2011) Yeast Sen1 helicase protects the genome from transcription-associated instability. Mol Cell 41 (1):21-32.

10. Wahba L, Amon JD, Koshland D, Vuica-Ross M (2011) RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Mol Cell 44 (6):978-988.

11. Stirling PC, Chan YA, Minaker SW, Aristizabal MJ, Barrett I, Sipahimalani P, Kobor MS, Hieter P (2012) R-loop-mediated genome instability in mRNA cleavage and polyadenylation mutants. Genes & Dev26 (2):163-175.

12. Gómez-González B, Aguilera A (2007) Activation-induced cytidine deaminase action is strongly stimulated by mutations of the THO complex. Proc Natl Acad Sci USA 104 (20):8409-8414.

13. Wahba L, Costantino L, Tan FJ, Zimmer A, Koshland D (2016) S1-DRIP-seq identifies high expression and polyA tracts as major contributors to R-loop formation. Genes & Dev 30 (11):1327-1338.

14. Ginno PA, Lott PL, Christensen HC, Korf I, Chédin F (2012) R-loop formation is a distinctive characteristic of unmethylated human CpG island promoters. Molecular Cell 45 (6):814-825.

15. Sollier J, Stork CT, García-Rubio ML, Paulsen RD, Aguilera A, Cimprich KA (2014) Transcription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. MolCell 56 (6):777-785.

16. Garcia-Rubio ML, Perez-Calero C, Barroso SI, Tumini E, Herrera-Moyano E, Rosado IV, Aguilera A (2015) The Fanconi Anemia Pathway Protects Genome Integrity from R-loops. PLoS Genet11 (11):e1005674.

Figure legends

Fig. 1. Relative amount of R loops in the patient FANCD2-/- human PD20 cell line and the corrected PD20 FANCD2+/+ control at 4 different genes. Results shown correspond to DRIP-qPCR assays using the S9.6 monoclonal antibody with and without RNase H (RNH) treatment. Signal values of DNA-RNA hybrids immunoprecipitated in each region are normalized to input values. A.U., Arbitrary Units. (Reproduced from [16] with modifications).

Fig. 2. Representative image of HeLa cells stained with DAPI (DNA), S9.6 (DNA-RNA hybrids) and anti-nucleolin (nucleoli) antibodies. DNA-RNA hybrids can be observed at mitochondria, nucleoli and nuclear DNA.

Fig. 3. Genomic DNA digested with a mix of restriction enzymes for DRIP analysis. 4 μl of each digestion reaction were run on agarose gel to confirm that they are free of RNA.

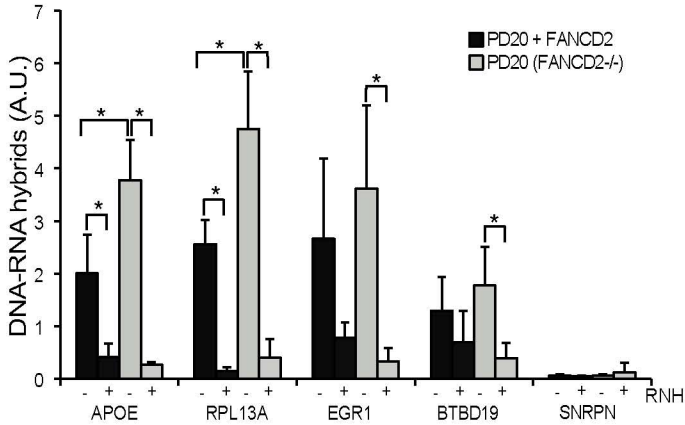
DNA-RNA hybrids (A.U.)
PD20 + FANCD2
PD20 (FANCD2-/-)
APOE
RPL13A
EGR1
BTBD19
SNRPN
RNH

Fig. 1

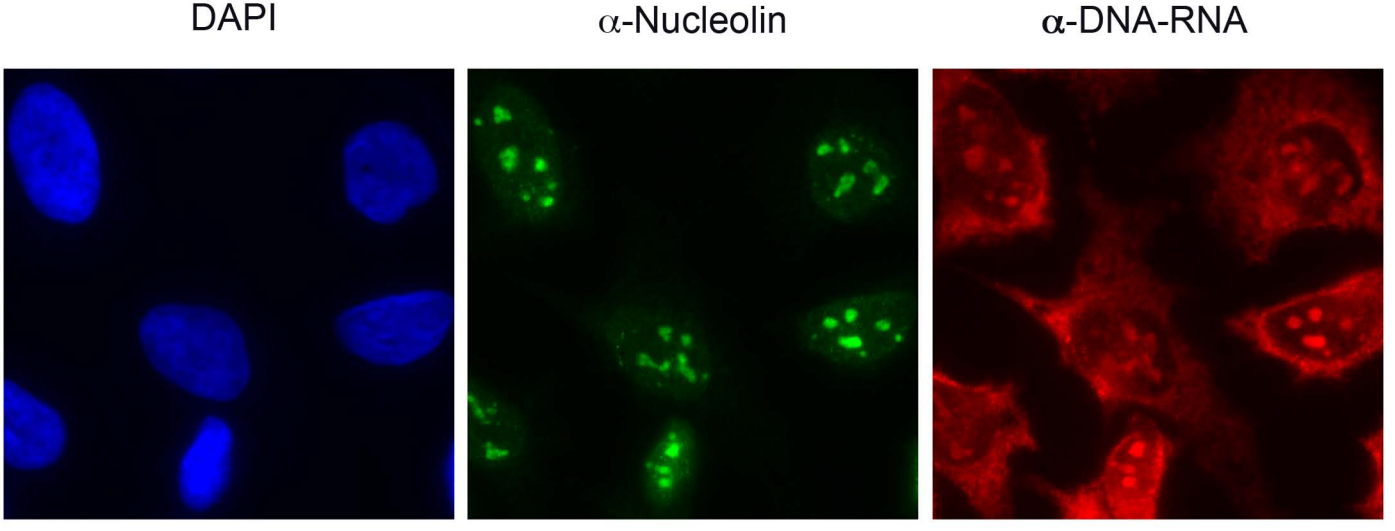

Fig. 2

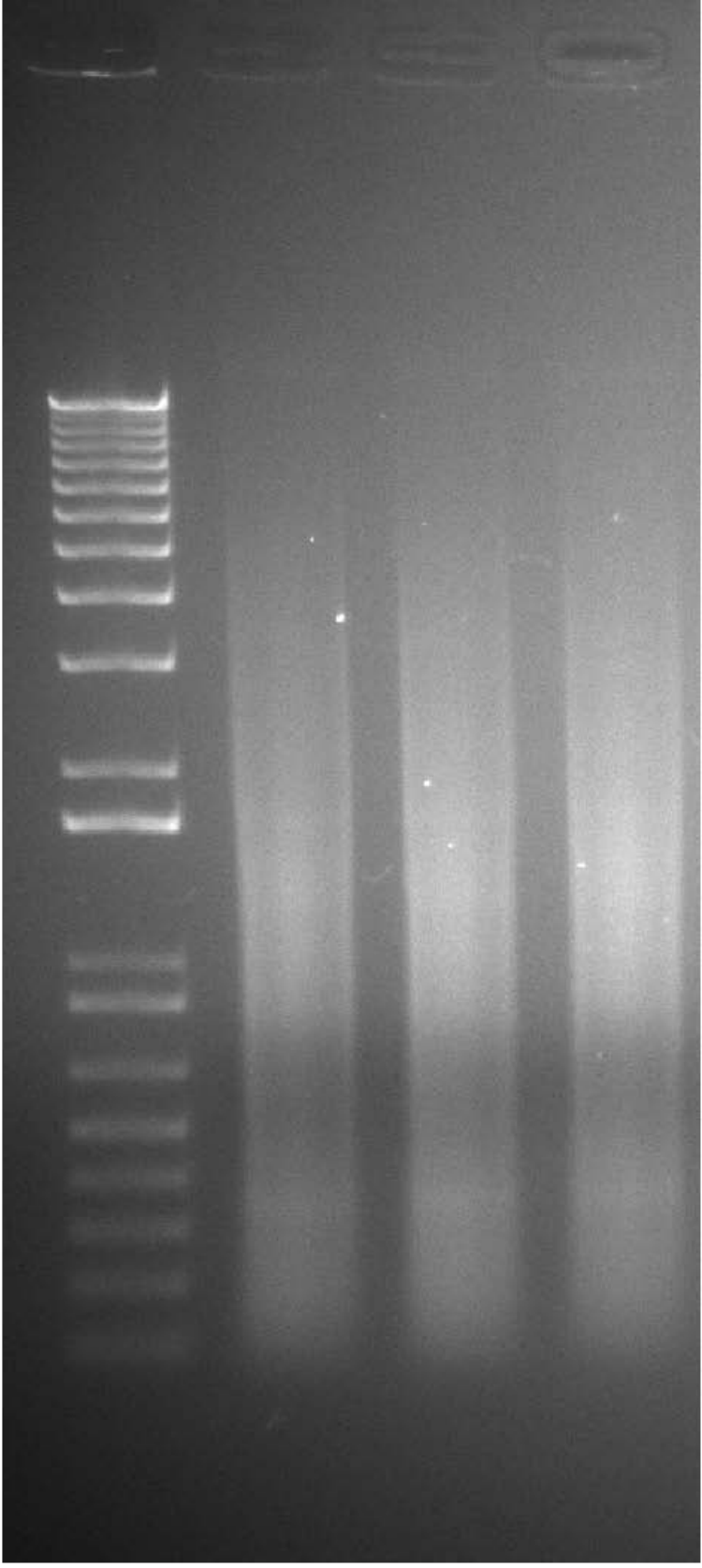

Fig. 3

Figure legends

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