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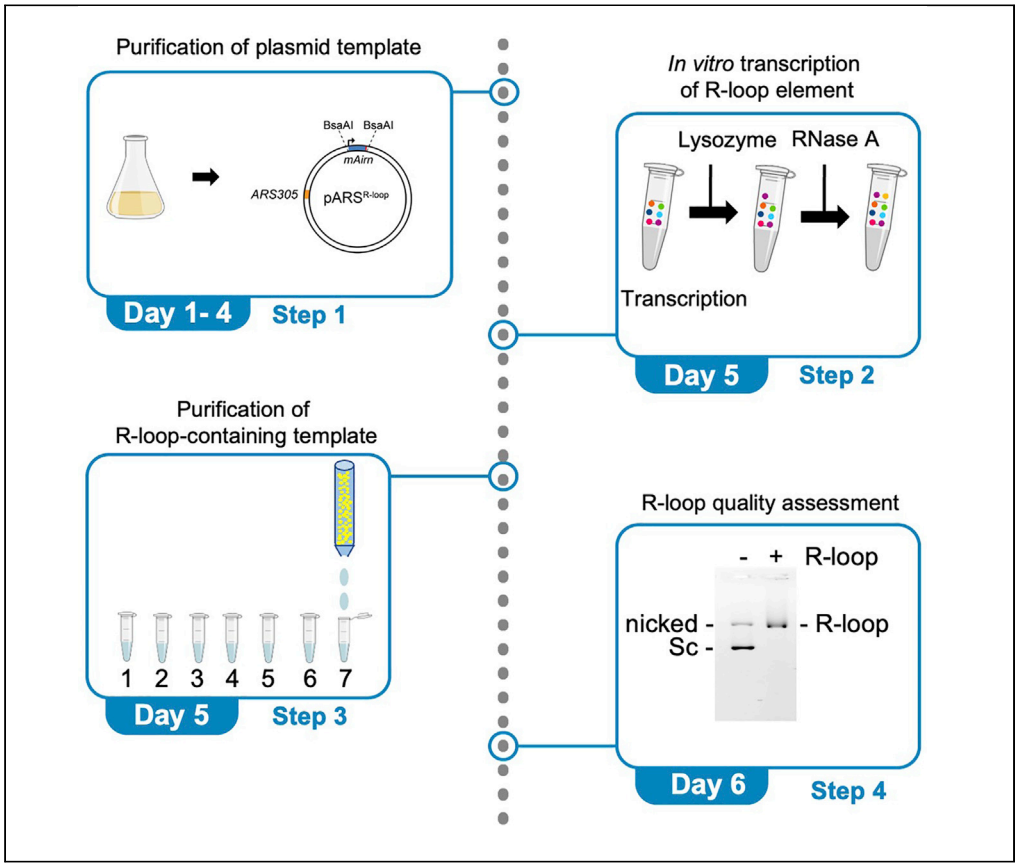
A transcription-based approach to purify R-loop-containing plasmid DNA templates *in vitro*



To study the direct effects of R-loops on DNA replication and other DNA-templated processes *in vitro*, R-loop-containing DNA templates need to be prepared efficiently and to near homogeneity. Here, we describe a simple transcription-based approach to form R-loops on plasmid DNA templates *in vitro*. We detail steps to transcribe a DNA sequence element with a high propensity to form co-transcriptional R-loops using T7 RNA polymerase. We describe nucleolytic digestion of free RNA, deproteinization, and repurification of R-loop-containing templates *via* gel filtration.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Transcription of R-loop element under the control of T7 promoter and terminator

T7 lysozyme treatment to prevent re-initiation of transcription

Removal of excess unannealed RNA by digestion with RNase A at high salt concentration

R-loop-containing template DNA isolated from reaction mixture by gel filtration

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A transcription-based approach to purify R-loop-containing plasmid DNA templates *in vitro*

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SUMMARY

To study the direct effects of R-loops on DNA replication and other DNA-templated processes *in vitro*, R-loop-containing DNA templates need to be prepared efficiently and to near homogeneity. Here, we describe a simple transcription-based approach to form R-loops on plasmid DNA templates *in vitro*. We detail steps to transcribe a DNA sequence element with a high propensity to form co-transcriptional R-loops using T7 RNA polymerase. We describe nucleolytic digestion of free RNA, deproteinization, and repurification of R-loop-containing templates *via* gel filtration.

For complete details on the use and execution of this protocol, please refer to Kumar et al.[1]

BEFORE YOU BEGIN

R-loops are three-stranded nucleic acid structures that result from the co-transcriptional re-annealing of the nascent transcript to the DNA to form an RNA:DNA hybrid on the template strand, resulting in the displacement of the non-template strand in the form of single-stranded DNA (ssDNA). Importantly, R-loops are a major source of genome instability associated with transcription-replication conflict.[2] However, due to the complexity of cellular R-loop structures, which may be modulated by R-loop-associated stalled RNA polymerases,[3] a condensed chromatin structure[4,5] or specific RNA:DNA hybrid-binding proteins,[6] the direct impact of the three-stranded R-loop structure itself on replication fork progression is difficult to interrogate *in vivo*. We, therefore, examined the consequences of R-loop-replisome collisions using the reconstituted budding yeast DNA replication system in conjunction with purified R-loop templates.[1] We found that both co-directional (CD; RNA:DNA hybrid on the leading strand template, displaced ssDNA forming the lagging strand template) and head-on (HO; RNA:DNA hybrid on the lagging strand template, displaced ssDNA forming the leading strand template) R-loops can adversely impact fork progression with a diversity in outcomes. Moreover, we found that both the RNA:DNA hybrid and G-quadruplexes (G4s) formed on the displaced single strand DNA of R-loops can impact fork progression.

To study the direct effects of R-loops on replisome progression, it was imperative to obtain pure R-loop templates. In our hands, protocols involving the annealing of synthetic RNA to double-stranded DNA (dsDNA) did not yield R-loops at the desired scale, purity, and efficiency.[7] We, therefore, opted for an approach involving the transcription of a ~ 1.4 kbp R-loop-forming sequence derived from the *mAirn* locus by T7 RNA polymerase (T7 RNAP) *in vitro*. Our method is an extension of an original earlier approach in which R-loop-containing plasmid DNAs were generated by transcription of the *mAirn* element *in vitro* using T3 RNA polymerase.[8] However, T7 RNA polymerase system is preferred here because of the availability of simple and well-defined terminator sequences. Importantly, we had

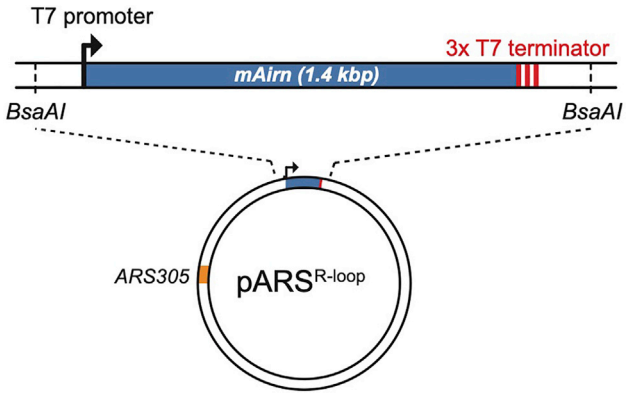

Figure 1. Schematic of plasmid used to generate R-loops by *in vitro* transcription

A 1.4 kbp fragment derived from the R-loop-forming *mAirn* locus is under the control of a T7 promoter. A tandem array of three terminator sequences is included downstream of the transcribed R-loop element. ARS: Autonomously Replicating Sequence (yeast replication origin).

previously found that transcription termination occurs inefficiently at a single terminator site.[9] Therefore, we routinely place a tandem repeat of three terminator sequences downstream of the R-loop element to constrain the region of transcription (Figure 1).

In our method, transcription of the R-loop element is initially carried out on negatively supercoiled plasmid templates to promote R-loop formation.[10] Moreover, R-loop-containing plasmid DNA is also stored in negatively-supercoiled form until further use to stabilize the R-loop structure. However, for subsequent applications, the template DNA may be linearized as needed prior to the experiment. To avoid the generation of partial transcripts and eliminate potential T7 RNAP elongation complexes, re-initiation of transcription is inhibited prior to the end of the transcription reaction by addition of the transcription initiation inhibitor T7 lysozyme.[11] A titration experiment in which T7 lysozyme is added prior to the initiation of transcription, like the one shown in Figure 2, can be utilized to determine the concentration of T7 lysozyme required for efficient inhibition of T7 RNAP. Excess RNA not associated with R-loops is subsequently digested with RNase A, followed by the elimination of both T7 RNAP and RNase A from the reaction by addition of proteinase K. Finally, R-loop-containing template DNA is isolated from the reaction mixture by gel-filtration through Sephacryl™ S–1000 resin. Our approach avoids the use of organic solvents, such as phenol, which may have unwanted effects on R-loop structure and stability due to intercalation into the DNA. While the protocol described here employs ~ 8.5 kbp DNA plasmid templates harboring a yeast origin sequence upstream of the R-loop-element specifically for *in vitro* DNA replication studies (pARS$^{R\text{-}loop}$, Figure 1), we note that the method is generally applicable to any R-loop element-containing plasmid template.

Purify closed circular plasmid DNA using CsCl-ethidium bromide gradients

Timing: 1 week

1. Maxi prep plasmids containing R-loop forming element.
   a. Culture *E. coli* strains carrying p1214 (harboring the *mAirn* element in the CD orientation) or p1215 (harboring the *mAirn* element in the HO orientation).
   b. Purify 1 mg of plasmid DNA by following the instructions of the Maxi Prep kit's manufacturer.
   c. Resuspend the final pellet in 4 mL of 10 mM Tris-HCl pH 8 / 1 mM EDTA (1x TE).
2. CsCl-Ethidium Bromide gradient (for more details refer to Molecular Cloning|Sambrook and Russell).
   a. Prepare DNA solution by mixing 4 mL of plasmid solution with 4.4 *g* of CsCl. Mix gently until the salt is dissolved.
   b. Add 100 μL of a 10 mg/mL ethidium bromide stock solution and mix gently.

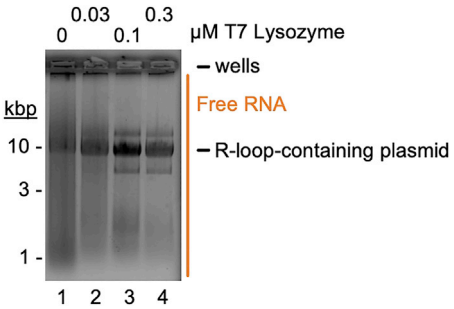

Figure 2. T7 lysozyme titration to determine effective concentration

50 μL transcription reactions were set up as described in step 1. Indicated concentration of T7 lysozyme was added before T7 RNAP to each reaction. After 20 min, the reactions were stopped by the addition of 20 nM EDTA and 0.8 U proteinase K. 5 μL of each reaction were separated on a 0.8% native agarose gel and subsequently stained with ethidium bromide.

c. Use a Pasteur pipette to transfer the CsCl-DNA solution into 6 mL ultracrimp PA tubes.
d. Fill to the brim using equivalent CsCl solution without DNA and balance the two tubes.
e. Insert plastic plugs and seal tubes with metal caps.
f. Centrifuge the gradients at 21 °C using Beckman VTi65 rotor at 45,000 rpm (193168 *g*) for 16 h.
g. At the end of the run, gently remove the tubes from the rotor and place them in racks covered in aluminum foil. In a dark room mount one tube into a clamp attached to a ring stand.
h. Two bands should be visible in ordinary light but if not, illuminate the tube with a handheld long-wavelength (300 nm) UV lamp.

⚠ CRITICAL: Act quickly to protect DNA from UV damage. In addition, wear safety glasses to protect the eyes.

i. Using a 21-gauge hypodermic needle, make a small hole at the top of the tube.
j. The lower band corresponds to the closed circular form of the plasmid. Attach a 3 mL syringe to an 18-gauge hypodermic needle and insert the needle into the tube such that the open side of the needle is positioned just under the band.
k. Slowly draw the plasmid DNA, taking care not to disturb the rest of the gradient.
l. Remove the ethidium bromide by extraction with water-saturated *n*-butanol.
    i. Add an equal volume of *n*-butanol to the DNA solution.
    ii. Mix by vortexing and allow the solution to stand at room temperature until the aqueous layer separates from the organic phase.
    iii. Use a Pasteur pipette to transfer the upper deep pink organic layer to an appropriate waste container.
    iv. Repeat until DNA solution is clear.
m. Dialyze the DNA solution against 1 x TE pH 8.0 for 12 h at 4°C using Slide-A-Lyzer™ Dialysis Cassette 10,000 MWCO.
n. Measure the concentration of the DNA using a NanoDrop spectrophotometer and store the DNA in small aliquots at −20 °C.

Note: Freeze thaw cycles may nick the DNA. So, it is good practice to maintain small aliquots of this purified DNA for future applications.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
p1214	[1]	R-loop plasmid (CD)
p1215	[1]	R-loop plasmid (HO)
Chemicals and recombinant proteins		
T7 lysozyme-6x-His	[1]	Recombinant protein
CsCl	Sigma	7647-17-8

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
T7 RNA polymerase (20 U/μL)	Thermo Scientific	EP0111
RNase A (10 mg/mL)	Thermo Scientific	EN0531
Proteinase K (800 U/mL)	NEB	P8107S
RNase H (5000 units/mL)	NEB	M0297S
Other		
2 mL Stripette	Costar/Corning	4486
Sephacryl™ S-1000 Superfine	GE Healthcare	17-0476-01
Ultracrimp PA 6 mL tubes	Thermo Scientific	03945
Spare plugs/caps	Thermo Scientific	03999
Amicon® Ultra – 0.5 mL, 30K	Millipore	UFC503096
Column frits	Millipore/Sigma	58255
Maxi prep kit	Qiagen	12163
Ribonucleotide solution set	NEB	N0450S
Flow control adaptor	Fisher	31-500-557
Slide-A-Lyzer™ Dialysis Cassette	Thermo Scientific	66453

MATERIALS AND EQUIPMENT

1x TE		
Reagent	Final concentration	Amount
1 M Tris HCl pH 8.0	10 mM	0.5 mL
0.5 M EDTA	1 mM	0.1 mL
diH_2O		**49.4 mL**
Total	**N/A**	**50 mL**
Store at 21 °C, 1 year.		

T7 lysozyme storage buffer		
Reagent	Final concentration	Amount
50% glycerol	10%	10 mL
1 M Tris HCl pH 8.0	20 mM	1 mL
5 M NaCl	100 mM	1 mL
1 M DTT	1 mM	0.05 mL
diH_2O		**28.95 mL**
Total	**N/A**	**50 mL**

0.5 mM ribonucleotide mix		
Reagent	Final concentration	Amount
100 mM ATP	10 mM	10 μL
100 mM UTP	10 mM	10 μL
100 mM GTP	10 mM	10 μL
100 mM CTP	10 mM	10 μL
diH_2O		60 μL
Total	**N/A**	**100 μL**
Store at −20 °C, 1 month.		

STEP-BY-STEP METHOD DETAILS

In vitro transcription

Timing: 3 h

Transcription of the R-loop forming element is under the control of T7 promoter and terminator sequences. In this step the element will be transcribed and excess unannealed RNA will subsequently be digested with RNaseA.

1. Set up a 250 µL transcription reaction as follows:

Component	Final concentration	
Purified plasmid	20 µg	
ATP/GTP/UTP/CTP mix at 10 mM each	0.5 mM	12.5 µL
5× transcription buffer	1 x	50 µL
1 mM DTT freshly dissolved in water	20 mM	5 µL
T7 RNA polymerase (20 U/µL)	50 U	2.5 µL
2.5% Tween	0.05 %	5 µL
ddH_2O		Make up to **250** µL

5x transcription buffer contains 200 mM Tris-HCl pH 8.0, 30 mM $MgCl_2$, 50 mM NaCl and 10 mM spermidine, and is supplied with the enzyme.

2. Incubate at 37 °C for 30 min.
3. Prevent re-initiation events by adding 0.3 µM T7 lysozyme (20 mM Tris-HCl pH 7.9, 10% glycerol, 125 mM NaCl, 1 mM DTT). Incubate at 37 °C for 10 min.

⚠ CRITICAL: The effective concentration of T7 lysozyme can be determined by titrating T7 lysozyme into transcription reaction, as described in Figure 2.

4. Adjust the salt concentration to 0.4 M NaCl from 5 M NaCl stock and add RNase A at a final concentration of 10 µg/mL. Incubate at 37 °C for 20 min.

⚠ CRITICAL: The increased salt concentration will restrict the activity of RNase A towards RNA:DNA hybrids and promote the targeting of free RNA (Figure 3).

5. Add 4 U of Proteinase K (NEB) to deproteinize the sample. Incubate at 37 °C for 30 min.

Purification of R-loop templates

Timing: 3 h

This step involves fractionation of the transcription reaction through a homemade size exclusion column packed with Sephacryl™ S–1000 Superfine resin. In principle, any gel filtration resin that has a similar pore size can be used to separate R-loop containing plasmid template from free digested RNA.

⚠ CRITICAL: Because the size exclusion columns are prepared under non-sterile conditions, we prefer to pack the columns with resin immediately before use.

⚠ CRITICAL: The resin can be regenerated by treatment with NaOH and isopropanol as described below to avoid contamination of subsequent preparations with proteinase K.

6. Column preparation (Figure 4):
   a. To regenerate resin prior to use, wash the resin with 0.5 column volumes (CVs) of 0.2 M NaOH and 0.5 CV 30% isopropanol.

Note: The resin is typically regenerated after it is packed in the column.

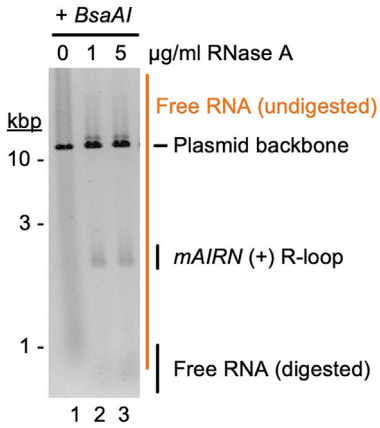

Figure 3. RNase A titration

50 µL transcription reactions were set up as in Step 1. After the NaCl salt concentration was increased to 0.4 M, RNase A was added at the indicated concentrations and incubation continued at 37 °C for 20 min. The reactions were stopped by addition of 0.8 U proteinase K and incubation for 30 min at 37 °C. Reactions were extracted with phenol:chloroform and 5 µL of aqueous phase of each reaction was digested with *BsaAI* and separated on a 0.8% native agarose gel and stained with ethidium bromide. RNase A treatment digests the free RNA into small fragments that can be separated from the R-loop template by gel-filtration.

b. Wash with 2 CV de-ionized water. Equilibrate the column with 10 mM Tris-HCl pH 7.5.
c. Cut a disposable column frit to fit in the 2 mL pipette and place frit at bottom of pipette for resin support.
d. Using a Pasteur pipette, pack the column with 1.8 mL (bed volume) S-1000 resin.

⚠ CRITICAL: While pouring the column, be careful not to include any air bubbles.

7. Purification:
   a. Concentrate the transcription reaction to ~100 µL using an Amicon spin concentrator.

⚠ CRITICAL: To ensure R-loops are not excluded during the concentration step, we use centrifugal concentrators with 3K cutoff.

   b. Fill the reservoir and the attached tubing with 10 mM Tris-HCl pH 7.5.
   c. Gently apply the concentrated reaction mix to the top of the column using a Pasteur pipette.

⚠ CRITICAL: Ensure not to introduce air bubbles.

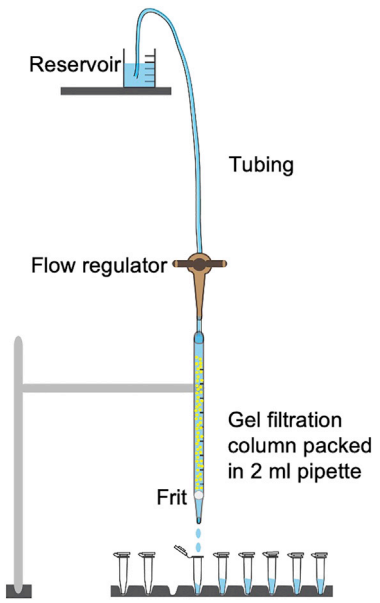

Figure 4. Schematic of gel filtration setup for purification of large R-loop-containing plasmid templates

A reservoir filled with 10 mM Tris-HCl pH7.5 is placed at a higher altitude compared to the column to apply pressure flow on the column. Tubing connects the reservoir to the flow regulator. A smaller piece of tubing seals the connection between the flow regulator and the column. S-1000 resin is packed in Costar® 2 mL pipettes. A frit cut to the size of the pipette is used for resin support. 200 µL fractions are manually collected.

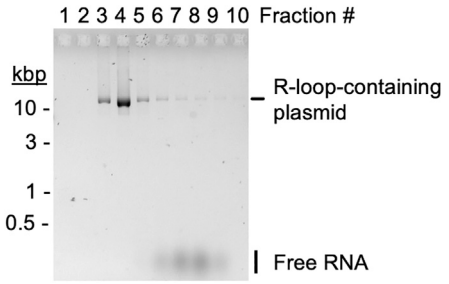

Figure 5. Elution profile of R-loop containing plasmid template

5 μL of each fraction from a gel filtration run were separated on 0.8% native agarose gel and stained with ethidium bromide. R-loop containing plasmids elute early, in fractions 3–5, while free RNA fragments elute later, in fractions 7–9. Fractions containing the R-loop-containing template are pooled, concentrated and stored in small aliquots at −20 °C.

d. Fill the top of the column with 10 mM Tris-HCl pH 7.5 and gently connect the tubing from the reservoir, making sure not to introduce any air bubbles.
e. Open the flow regulator and initiate fractionation. Manually collect ~200 μL fractions (8–12 drops).
f. Run 5 μL of each fraction on a 0.8% agarose gel.
g. Stain with ethidium bromide to visualize the elution profile (Figure 5). R-loop-containing plasmid templates are typically found in fractions 3–5.
h. Pool the fractions containing the R-loop-containing plasmid and concentrate to ~100 μL using a spin concentrator. Store at −20°C in small aliquots.

Optional: Inclusion of 60 μCi [α-^{32}P]-GTP in the transcription reaction can be used to radio-label the RNA in R-loop templates to facilitate probing of the RNA moiety. In this case, the gel-filtration step will also help eliminate unincorporated radio-nucleotides.

Note: To maintain the stability of the R-loops, we prefer to avoid freeze-thawing of the final sample and store it in small aliquots at −20°C. Purified R-loop need not be to flash frozen with liquid nitrogen.

Pause point: The proteinase K-treated transcription reaction can be stored at −20 °C until further processing. The packed and washed column can be stored at 4 °C for 24 h. Cover with parafilm to ensure the column does not dry and crack.

EXPECTED OUTCOMES

The final yield of R-loop-containing plasmid DNA is usually between 10–12 μg when starting out with 20 μg of plasmid DNA, with most of the final plasmid template containing an R-loop. As the presence of R-loops can be difficult to assess by gel-shift in the context of larger plasmid backbones, determination of the efficiency of R-loop formation can be facilitated by excising the R-loop-forming element from the plasmid backbone prior to gel analysis. This has the additional advantage of ensuring that R-loop formation has specifically occurred at the R-loop element. For example, in the case of p1214/p1215, the *mAirn* expression cassette is flanked on either side by *BsaAI* restriction sites. Digestion of the p1214/p1215 plasmid templates with *BsaAI* will thus release a 1.5 kbp band comprising the *mAirn* locus that will exhibit an RNase H-sensitive upshift if R-loops have been formed on the fragment (Figure 6).

LIMITATIONS

Our analyses indicate that R-loops generated by transcription of the *mAirn* element are inherently heterogenous.[1] This heterogeneity derives from differences both in the length and position of the R-loops in the *mAirn* locus, which also determines the G4 content of the R-loops, respectively.

The *mAirn* sequence has a strong propensity to form G4s.[1,8] On the one hand this feature facilitates the formation of co-transcriptional R-loops,[12] on the other hand this results in the specific production of G4-rich R-loops, which may not be representative of all R-loops.

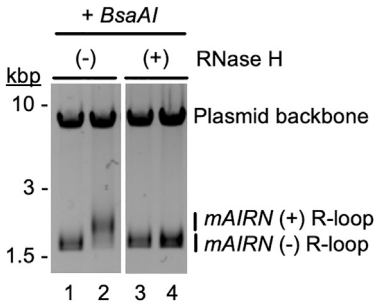

Figure 6. Determination of R-loop formation efficiency

Purified R-loop templates are digested with *BsaAI*. The digestion reactions are separated on native agarose gels and stained with ethidium bromide. The 1.5 kbp fragment harboring the *mAirn* R-loop forming element is shifted up and appears smeary when an R-loop is formed. Treatment with RNase H restores the mobility of the fragment to that of double-stranded DNA.

Scaling up the yield is challenging because the amount of DNA that can be purified through the column is limited to 20 μg. Overloading the column results in inefficient separation of R-loop templates and impurities.

The use of RNase A is important to eliminate free RNA from the reaction which would otherwise be difficult to separate from the R-loop template by gel-filtration. While the use of elevated salt concentrations during the RNase A treatment restricts the activity of RNase A on RNA:DNA hybrids, we note that RNase A may still target RNA flaps in addition to free RNA under these conditions, which may affect the structure of the R-loop. Moreover, 3′ RNA flap cleavage by RNase A will result in the loss of the RNA 3′-OH as RNase A cleavage products feature 5′-OH and 3′-PO_4 ends, which may affect subsequent processing/extension of the RNA.[1] However, the 3′-OH may be restored by T4-PNK treatment of the R-loops.[1]

TROUBLESHOOTING

Problem 1

RNase A may target the RNA moiety in the R-loop. (step 4).

Potential solution

RNase A may digest both free RNA and the RNA in RNA:DNA hybrids. To circumvent this issue, we exploit the fact that RNase A specificity towards RNA:DNA hybrids is significantly more salt sensitive than its specificity towards free RNA. Accordingly, high salt concentrations suppress the activity of RNase A on RNA:DNA hybrids. It is, therefore, important to increase the salt concentration to 0.4 M NaCl during the RNase A treatment to avoid digestion of the RNA:DNA hybrid.

Problem 2

R-loop-containing plasmid template is contaminated with Proteinase K. (step 7).

Potential solution

Proteinase K sticks non-specifically to the S-1000 resin. Contaminating traces of proteinase K may impact downstream biochemical applications. Washing the resin with 0.5 CV of 0.5 M NaOH followed by 0.5 CV of 30% isopropanol before equilibration with running buffer eliminates potential proteinase K contamination.

Problem 3

Insufficient separation of R-loop-containing plasmid template from free RNA. (step 7).

Potential solution

In gel-filtration, column resolution tracks with column height. We, therefore, prefer the small diameter of 2 mL Costar® serological pipettes to prepare 2 mL gel-filtration columns.

The resolution also decreases if the column is overloaded. Hence, the input is limited to 20 μg of plasmid DNA.

Problem 4
Flow of the gel-filtration column is restricted. (step 7).

Potential solution
Fractionation through the gel-filtration column may be performed under gravity flow (step 7). However, resupplying buffer to the top of the column from a sealed reservoir significantly accelerates the fractionation.

As in any column chromatography procedure, but particularly in a setup that does not involve pumps, air bubbles in the column need to be strictly avoided to prevent a restriction to the flow.

Problem 5
R-loop formation appears to be inefficient (expected outcomes).

Potential solution
Inefficient R-loop formation may just be an artifact of the method used to analyze the final R-loop-containing templates. For example, R-loops may be destabilized by gel-electrophoresis in the presence of ethidium bromide. Therefore, it is important that R-loop formation is assessed by native agarose gel-electrophoresis in the absence of ethidium bromide.

RNase A is a robust enzyme. Adding excessive amounts of this enzyme may thus result in the RNA:DNA hybrid being digested, despite the salt restriction. We, therefore, recommend titrating the concentration of RNase A in order to determine the minimum RNase A concentration required for efficient digestion of the free RNA.

When working with RNA, it is good practice to take precautions to avoid RNase contamination, e.g., wipe bench with RNaseZap™ before the procedure. The use of certified RNase free reagents and water is also recommended; however, we do not commonly find the use of those to be critical.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dirk Remus (remusd@mskcc.org).

Materials availability
Plasmids used in this study are available on request.

Data and code availability
This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS
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AUTHOR CONTRIBUTIONS
C.K. and D.R. conceptualized, wrote, and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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