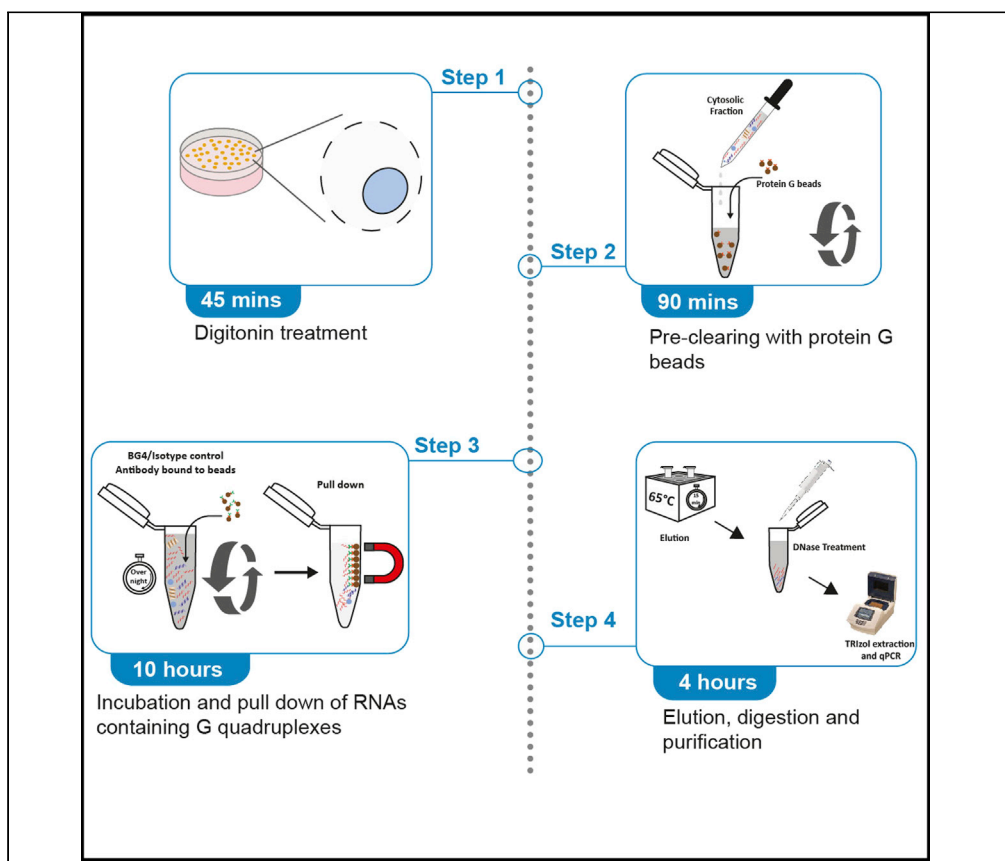


## Protocol

# Native RNA G quadruplex immunoprecipitation (rG4IP) from mammalian cells



G quadruplex structures play an important role in regulating DNA replication and transcription and mRNA translation. Although there are several techniques that can determine its formation *in vitro*, the study of RNA G quadruplexes *in vivo* is not simple. In the current protocol, we describe an optimized technique (RNA G quadruplex immunoprecipitation [rG4IP]) to selectively pull down native cytoplasmic RNAs containing G quadruplex structures in mammalian cells. We also use a bicistronic plasmid to confirm and pinpoint the structure location.

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### Highlights

Immunoprecipitation of RNAs with G quadruplex structures without using fixation

Specific lysis and DNase steps to selectively enrich cytosolic RNAs

*NRAS* and *MMP16* mRNAs used as positive controls and *TBP* mRNA as a negative control

Cloning into a bicistronic plasmid used as confirmatory step

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## Protocol

## Native RNA G quadruplex immunoprecipitation (rG4IP) from mammalian cells

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## SUMMARY

G quadruplex structures play an important role in regulating DNA replication and transcription and mRNA translation. Although there are several techniques that can determine its formation *in vitro*, the study of RNA G quadruplexes *in vivo* is not simple. In the current protocol, we describe an optimized technique (RNA G quadruplex immunoprecipitation [rG4IP]) to selectively pull down native cytoplasmic RNAs containing G quadruplex structures in mammalian cells. We also use a bicistronic plasmid to confirm and pinpoint the structure location.

For complete details on the use and execution of this protocol, please refer to Surani et al. (2022).

## BEFORE YOU BEGIN

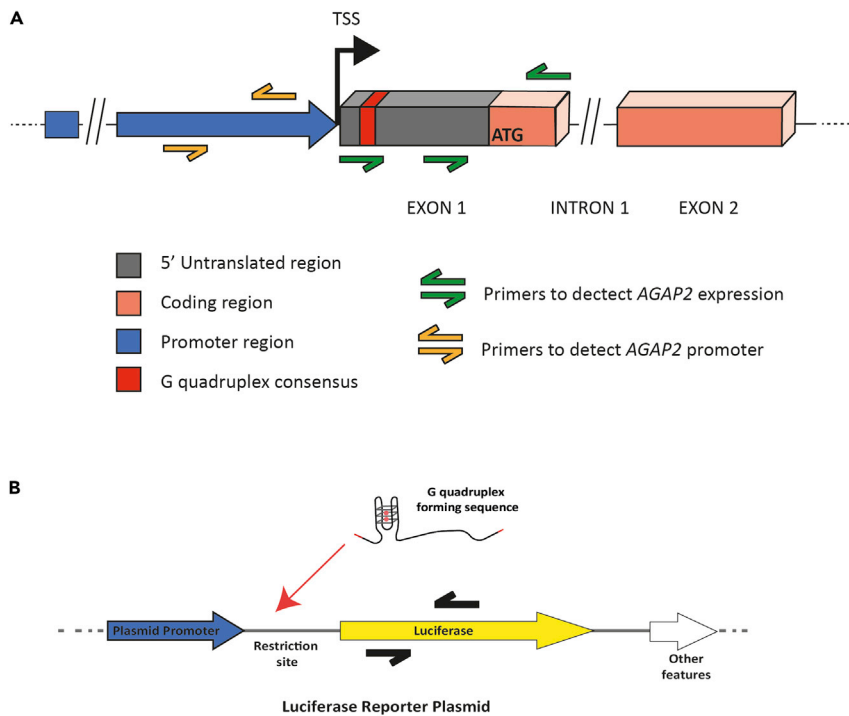
## Experimental considerations

This protocol is used to enrich RNA with G quadruplexes using a structure-specific antibody. The antibody (BG4) was generated by Balasubramanian's group (Biffi et al., 2013) to selectively bind DNA G quadruplexes and can also recognize RNA G quadruplexes (Biffi et al., 2014). Although another group has used this antibody to pull down RNA containing G quadruplexes (Maltby et al., 2020), the novelty of our protocol resides on its focus on cytoplasmic RNA and its avoidance of DNA contamination that could lead to false positive results. The protocol is designed to isolate the cytosolic fraction, remove contaminating genomic DNA, and enrich a population of RNAs with native G quadruplex structures. We also recommend to carefully select appropriate positive and negative controls to confirm the validity of the results. In this protocol, we have demonstrated successful pulldown of AGAP2 (Arf GAP with GTP-binding protein-like domain, Ankyrin repeat and PH domain 2) mRNA containing G quadruplex structure in the 5' untranslated region (UTR) (a gene of interest in our group (Doush et al., 2019)) and we have used the detection of *NRAS* and *MMP16* mRNAs as positive controls and the detection of *TBP* mRNA as a negative control.

## Primer design

1. Primers for the target mRNA should be designed to amplify a segment 50–200 bp downstream of the putative G quadruplex consensus region (see Figure 1).
2. To check the level of genomic DNA, primers should be designed to amplify a neighboring intron or, in those cases where the 5' UTR is analyzed, the promoter region within 500 bp of the G quadruplex forming sequence.





**Figure 1. Primer design considerations**

(A) Primers for the target mRNA should be designed to amplify a region 50–200 bp downstream of the G quadruplex consensus. The primers to detect genomic DNA should be designed in the intronic or promoter region within 500 bp of the potential G quadruplex forming sequence.

(B) When the potential G quadruplex forming sequence is inserted into a reporter vector, the primers should be designed to amplify a region of the adjacent luciferase gene within 200 bp of the G quadruplex fragment. Figure not drawn to scale.

3. The concentration and annealing temperature of all primer pairs should be optimized, and the primer efficiency calculated as per the MIQE guidelines (Bustin et al., 2009).
4. Appropriate primers for the positive controls should be also designed for mRNAs in which the formation of a G quadruplex structure has been previously established. For negative controls, mRNA which lacks G quadruplex consensus in their entire length should be used.

**Note:** The pqsfinder web application (Labudová et al., 2020) predicts potential G quadruplexes in mRNA sequences and can be used to select appropriate negative controls. In our protocol, *NRAS* and *MMP16* mRNAs were used as a positive control for the detection of G quadruplex structures in the 5' UTR (Kumari et al., 2007; Morris and Basu, 2009). *TBP* mRNA was used as a negative control as it lacks G quadruplex consensus sequences along the entire length of its mRNA.

5. The enrichment was defined relative to the signal obtained with the negative control antibody (an antibody from the same species and same isotype).
6. Complementary strategy (also see step 8):
  - a. The putative G quadruplex forming sequence can be cloned into a reporter vector and primers designed to amplify an adjacent reporter gene region within 200 bp of the G quadruplex fragment.

**Note:** In the current protocol, the G quadruplex consensus region in the 5' untranslated region of *AGAP2* (GeneBank: NM\_014770.4) has been inserted upstream of the *Renilla* luciferase at the *NheI* restriction site in the pcDNA3 RLUC POLIRES FLUC plasmid created by Sonenberg's

group (Poulin et al., 1998). A primer pair targeting the *Renilla* luciferase was used to evaluate the enrichment of G quadruplex-containing mRNAs.

### Cell culture

⌚ Timing: 1 week

7. Prepare the required cell culture medium and grow the cells in the recommended culture conditions. The cells should be in culture for at least a week before starting the procedure.

**Note:** In the current protocol, the chronic myeloid leukemia TCC-S cell line (Van et al., 2005) and the prostate cancer cell line DU145 (RRID:CVCL\_0105) were used. TCC-S cells were cultured in RPMI supplemented with 2 mM L-Glutamine and 10% FBS, and DU145 cells were cultured in DMEM GlutaMAX supplemented with 10% FBS. Both cell lines were maintained at 37°C in a 5% CO<sub>2</sub> incubator.

⚠ **CRITICAL:** Determine the expression of the gene of interest in the cell line under study to ensure that sufficient levels (Ct values below 30) are present before carrying out the pulldown using this G quadruplex RNA immunoprecipitation (rG4IP) method. As a reference, the Ct values for the mRNAs used in this study were: *AGAP2 20*, *MMP16 27*, *TBP 22* and *NRAS 19*.

### Construction and transfection of reporter plasmid (complementary step)

⌚ Timing: 1 week

8. Prepare the reporter construct:
  - a. Design or amplify the potential G quadruplex sequence using relevant primers that contain in their 5' end the desired restriction site.
  - b. Digest the reporter vector with a restriction enzyme, treat with alkaline phosphatase to prevent religation, and separate the linearized vector in a 1% agarose gel. Cut the band and purify the DNA.

**Note:** In the current protocol, the dual-luciferase reporter plasmid pcDNA3 RLUC POLIRES FLUC (Poulin et al., 1998) and the *NheI* restriction enzyme were employed for fragment insertion (Figure 1B).

- c. Digest the G quadruplex containing fragment with the relevant restriction enzyme to produce ends complementary to the vector.
  - d. Ligate the purified digested vector and the G quadruplex containing insert using DNA ligase (bear in mind that ligation often fails due to the degradation of the ATP in the ligation buffer).
  - e. Transform the ligated plasmid into competent cells and culture in selective medium.
  - f. Choose positive clones, purify the plasmid, and verify the correct insertion by Sanger sequencing or PCR.
9. Transfect the reporter plasmid into the cell line of interest. In our study, the constructed vector or the empty plasmid was transfected using JetPRIME transfection reagent (Polyplus).
    - a.  $1 \times 10^6$  DU145 cells were seeded in a 100 mm culture dish 24 h before transfection.
    - b. 10  $\mu$ g of respective plasmids were diluted in the jetPRIME buffer and mixed by vortexing. JetPRIME reagent was then added, and the mixture was incubated at room temperature for 10 min.
    - c. The mixture was added dropwise to the cells and cells were incubated with the transfection mix at 37°C for 4 h followed by replacement with fresh growth medium.
    - d. The cells were collected after 48 h (see [troubleshooting 1](#)).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Mouse monoclonal anti-DNA/RNA G-quadruplex (clone BG4) – 3 µg used per IP	Absolute Antibody	Cat#Ab00174-1.1
Mouse IgG Isotype Control antibody – 3 µg used per IP	ThermoFisher	Cat#31903; RRID: AB_10959891
<b>Bacterial and virus strains</b>		
Subcloning Efficiency DH5α Competent Cells	Thermo-Fisher	Cat#18265017
<b>Chemicals, peptides, and recombinant proteins</b>		
Potassium chloride	Sigma-Aldrich	Cat#P9333; CAS:7447-40-7
HEPES	Sigma-Aldrich	Cat#H3375; CAS:7365-45-9
*Digitonin	Abcam	Cat#ab141501; CAS:11024-24-1
*Absolute Ethanol for molecular biology	Fischer Scientific	Cat#10644795
*2-Propanol for molecular biology	Sigma-Aldrich	Cat#278475
TWEEN 20	Sigma-Aldrich	Cat#P1379
Nuclease free water	Promega	Cat#P1193
DNase I (RNase-free)	ThermoFisher	Cat#AM2222
SureBeads Protein G	Biorad	Cat#161-4023
Glycogen	ThermoFisher	Cat#AM9510
NheI restriction endonuclease	Promega	Cat#R6501
*TRIzol Reagent	ThermoFisher	Cat#15596026
*Chloroform	Sigma-Aldrich	Cat#C2432
Alkaline Calf Intestinal Phosphatase	Promega	Cat# M1821
Ampicillin sodium salt	Sigma-Aldrich	Cat#A9518
T4 DNA Ligase	Promega	Cat#M1801
RNasin Ribonuclease Inhibitor	Promega	Cat#N2511
<b>Critical commercial assays</b>		
M-MLV Reverse Transcriptase	Promega	Cat#M1701
GoTaq® qPCR SYBR master mix	Promega	Cat#A6001
jetPRIME DNA/siRNA transfection reagent	Polyplus	Cat#114-01
NucleoSpin Plasmid Columns	Fischer Scientific	Cat#11932392
<b>Experimental models: Cell lines</b>		
Human: TCC-S (chronic myeloid leukaemia)	( <a href="#">Van et al., 2005</a> )	N/A
Human: DU145 (prostate cancer)	ATCC	Cat#HTB-81; RRID:CVCL_0105
<b>Oligonucleotides 5' → 3'</b>		
AGAP2 Forward Primer: CCAGAGGTGGTTGTTAGCCTG	This Study	N/A
AGAP2 Reverse Primer: GCGGCTCAAAGTCCATTCT	This Study	N/A
AGAP2 genomic (-425) Forward: GTGTAGAGAGGGCAATGGGTAC	This Study	N/A
AGAP2 genomic (-218) Reverse: CAAGCTAGGTCCGAGGTGC	This Study	N/A
Renilla Luciferase Forward: ATAAGTGGTCCGAGTGGTG	This Study	N/A
Renilla Luciferase Reverse: TAAGAAGAGGCCGCTTACC	This Study	N/A
G quadruplex forming sequence in AGAP2 5' UTR: AAAAGCTAGCAAGGGCGGGCAGGGCGGGGA GTTCTGGGCACAGCAGAAGGGCCTTCTGAGG TTTGGGGCTGTAGGGCCATGGGCCTCAGGGC CAGAGGTGGTTGTAGCCTGGCAAGACAGGTC TGGGCAACGCTAGCAAAA	This Study	N/A
Random Primers	Promega	Cat#C1181
<b>Recombinant DNA</b>		
pcDNA3 RLUC POLIRES FLUC	Addgene, ( <a href="#">Poulin et al., 1998</a> )	Cat#45642; RRID:Addgene_45642
<b>Other</b>		
RPMI 1640 cell culture Media	Gibco	Cat#52400025
Trypsin-EDTA (0.25%)	ThermoFisher	Cat#25200056

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's Modified Eagle Medium with GlutaMAX	Gibco	Cat#10566016
Fetal Bovine Serum	Biosera	Cat#FB1090/500
L-Glutamine (200 mM)	ThermoFisher	Cat#25030149
Phosphate Buffered Saline, pH 7.4	ThermoFisher	Cat#10010023
Countess 3 Automated Cell Counter	Fischer Scientific	Cat#15397802
Tube Rotator	VWR	Cat#10136-084

△ **CRITICAL:** An asterisk (\*) marks chemicals or reagents that are harmful or toxic. User must comply with COSHH risk assessments and follow institutional safety guidelines.

**Ethanol:** highly flammable, potential carcinogen, store larger volumes in fire-proof cabinets.

**Isopropanol:** Highly flammable. Irritating to eyes. Vapors may cause drowsiness and dizziness. Ensure there is sufficient ventilation of the area, store larger volumes in fire-proof cabinets.

**TRIzol:** Acute oral, dermal, and Inhalation toxicity; skin corrosion/irritation, specific target organ systemic toxicity, and mutagenicity. Use in a chemical fume hood.

**Digitonin:** Carcinogen; acute oral, dermal, and inhalational toxicity. Store aliquots of stock solution at  $-20^{\circ}\text{C}$  and use within 1 month.

**Chloroform:** Acute oral and inhalation toxicity; skin corrosion/irritation, serious eye damage, specific target organ systemic toxicity, carcinogenicity, and reproductive toxicity. Use only under a chemical fume hood.

## MATERIALS AND EQUIPMENT

### rG4IP lysis buffer:

Reagent	Final concentration	Amount
Digitonin (1 mg/mL)	25 $\mu\text{g/mL}$	25 $\mu\text{L}$
HEPES (1 M)	50 mM	50 $\mu\text{L}$
KCL (1 M)	150 mM	150 $\mu\text{L}$
RNasin Ribonuclease Inhibitor (40 U/ $\mu\text{L}$ )	100 U	2.5 $\mu\text{L}$
Nuclease free water	–	772.5 $\mu\text{L}$
<b>Total</b>	<b>n/a</b>	<b>1 mL</b>

Note on storage conditions: Prepare the buffer fresh and keep it on ice.

△ **CRITICAL:** The final concentration of digitonin in the rG4IP lysis buffer needs optimization for different cell types (see [troubleshooting 2](#)).

### PBS-T

Reagent	Final concentration	Amount
PBS (Phosphate Buffered Saline, pH 7.4)	–	50 mL
TWEEN 20	0.1%	50 $\mu\text{L}$

Note on storage conditions: Store at  $20^{\circ}\text{C}$ – $25^{\circ}\text{C}$  for up to a month. If filtered, it should last up to a year.

## STEP-BY-STEP METHOD DETAILS

### Digitonin treatment

⌚ Timing: 45 min

We used TCC-S cells to pull down native RNAs with G quadruplex sequences and we transfected DU145 cells with a plasmid that contains AGAP25' UTR – the sequence we expected to form G quadruplexes *in vivo* – as mentioned above.

1. Use  $10\text{--}15 \times 10^6$  cells for each condition to achieve around 10  $\mu\text{g}$  of total RNA/condition.
  - a. TCC-S: cell suspension is centrifuged at  $200 \times g$  for 5 min and the cells are resuspended in 3 mL of fresh medium and counted using an automatic cell counter (Fischer Scientific).
  - b. DU145: 48 h post-transfection, cells are trypsinized by adding 5 mL of trypsin-EDTA (Thermo Fisher Scientific) and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator for 5 min. After incubation, 10 mL of complete medium is added to neutralize the trypsin-EDTA followed by the collection of the detached cells. The cells are centrifuged at  $300 \times g$  for 5 min and resuspended in fresh medium.
2. Centrifuge cells at  $200 \times g$  for 5 min at  $4^\circ\text{C}$ .
3. Resuspend and wash the pellet with ice-cold PBS followed by centrifugation, repeat PBS wash.
4. Resuspend the pellet in 400  $\mu\text{L}$  of ice-cold rG4IP lysis buffer (see [troubleshooting 2](#)).
5. Incubate cells with lysis buffer in an end over end rotator at  $4^\circ\text{C}$  for 10 min.
6. After incubation, centrifuge the cells at  $2,000 \times g$  for 5 min at  $4^\circ\text{C}$  to pellet the nuclei.
7. Pipette out and save the supernatant: this is the cytosolic fraction (lysate).

⚠ **CRITICAL:** Save 10% of the lysate (40  $\mu\text{L}$ ) as an Input control which will be used for normalization.

### Pre-clearing

⌚ Timing: 90 min

A pre-clearing step helps reducing unspecific binding to the beads and contributes to lower background signal.

8. Prepare Protein G magnetic beads for pre-clearing by thoroughly resuspending the magnetic beads in their solution and transferring 100  $\mu\text{L}$  (1 mg, as stock 10 mg/mL) to a 1.5 mL Eppendorf tube.
9. Magnetize beads and remove the supernatant.
10. Wash the beads thrice with PBS-T, resuspend the beads thoroughly, magnetize and discard the supernatant.
11. Add the lysate to the washed beads and incubate for 1 h at  $4^\circ\text{C}$  in an end over end rotator.
12. After incubation, briefly centrifuge and magnetize the beads and pipette out the lysate for incubation with the bound antibody in step 19.

### Incubation of pre-cleared lysate with beads bound to antibody

⌚ Timing: 10 h

This section includes all necessary steps to bind the BG4 antibody to the magnetic beads and collect RNAs containing G quadruplex structures.

13. Prepare Protein G magnetic beads for antibody binding by thoroughly resuspending the magnetic beads in their solution and transferring 100  $\mu\text{L}$  (1 mg as 10 mg/mL) to a 1.5 mL Eppendorf tube.

14. Magnetize beads and remove the supernatant.
15. Wash the beads thrice with PBS-T, resuspend the beads thoroughly, magnetize and discard the supernatant.
16. Incubate the beads with 3  $\mu\text{g}$  of BG4 antibody or equivalent isotype-matched negative antibody control.
17. Incubate in an end over end rotator for 1 h at 4°C.
18. Following incubation, briefly centrifuge the beads and wash by resuspending in PBS followed by magnetizing the beads and discarding the supernatant. Repeat three times.
19. Add the pre-cleared lysate to the beads with antibody and incubate between 6 to 10 h at 4°C in a rotator.

⚠ **CRITICAL:** Do not incubate for more than 12 h as it would increase background signal (see [troubleshooting 3](#)).

### Washing and elution

⌚ **Timing:** 30 min

Necessary steps to remove unspecific binding and to collect bound RNAs.

20. After overnight incubation, magnetize the beads and save the flow-through lysate.

**Note:** If there are issues with antibody binding efficiency, the flow-through can be reused.

21. Wash the beads by resuspending in the lysis buffer and incubate in an end over end rotator for 5 min at 4°C. Repeat the washes three times.

**Note:** The magnetic rack should be placed on ice during washing. The number of washes could be increased to five times to reduce background signals (see [troubleshooting 3](#)).

22. After the washes, resuspend the beads in 100  $\mu\text{L}$  of nuclease-free water.
23. Incubate at 65°C for 15 min to unfold and release the nucleic acid bound to the antibody.

⚠ **CRITICAL:** Do not incubate at 65°C for more than 15 min.

24. Magnetize the beads and transfer the supernatant containing eluted nucleic acids into a new Eppendorf tube.

### DNase treatment

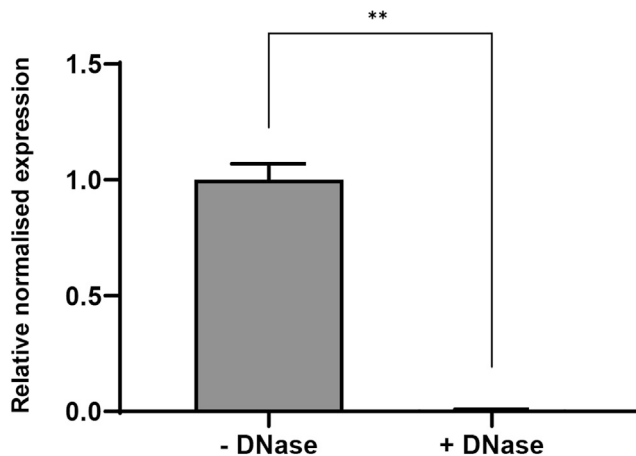
⌚ **Timing:** 15 min

25. Add 10  $\mu\text{L}$  of 10 $\times$  DNase I Buffer and 1  $\mu\text{L}$  of DNase I (2 U/ $\mu\text{L}$ ) to the eluted sample and incubate for 15 min at 37°C.

**Note:** Incubation time could be increased to 30 min to enhance the degradation of genomic DNA [see [troubleshooting 4](#)].

⚠ **CRITICAL:** The DNase treatment step is essential to prevent contamination and detection of DNA with G quadruplex structures ([Figure 2](#)). To ensure this step is effective we highly recommend using Ambion DNase I (see [key resources table](#) for catalog number).





**Figure 2. Genomic DNA contamination in the G quadruplex enriched rG4IP sample**

In the absence of DNase treatment, AGAP2 promoter region was detected in the eluant after immunoprecipitation with the BG4 antibody. The samples were processed identically except that in the [- DNase] sample the DNase treatment was omitted. The expression levels were normalized by the input control and presented relative to the [- DNase] condition. Data shown are the mean  $\pm$  SD of two independent experiments performed at least in duplicate. \*\* $p < 0.01$ .

### RNA extraction

⌚ Timing: 2 h

⚠ **CRITICAL:** Perform all procedures under RNase-free conditions using RNase-free buffer and consumables.

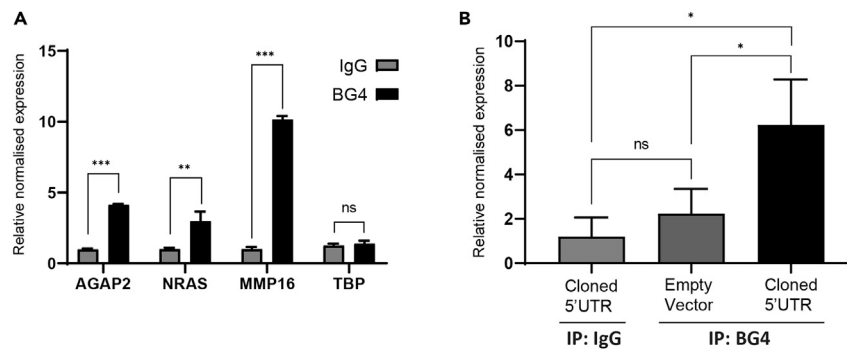
26. Add 1 mL TRIzol reagent to the DNase treated sample and 500  $\mu$ L TRIzol to the input controls which were saved after digitonin treatment.
27. Invert the tubes 5 times and incubate at room temperature for 5 min.
28. Add 200  $\mu$ L of chloroform per 1 mL of TRIzol used and shake the tube vigorously for 15 s and leave at room temperature for 5 min.
29. Centrifuge the tube at 12,000  $\times$  g for 15 min at 4°C to separate the solution into 3 layers: clear aqueous layer containing RNA, middle interphase layer containing mostly DNA and lower pink organic phase containing protein.
30. Carefully remove the top aqueous phase (60% of the volume of TRIzol Reagent used) without disturbing the interphase and transfer to a fresh tube.

### RNA precipitation and washing

31. Add 20  $\mu$ g glycogen and vortex briefly.
32. Add 500  $\mu$ L of 100% room temperature isopropanol per 1 mL TRIzol reagent used.
33. Invert the tubes 5 times and incubate at room temperature for 10–15 min.

**Note:** It is not recommended to incubate with isopropanol for more than 15 min or overnight at  $-20^{\circ}\text{C}$ .

34. Centrifuge at 12,000  $\times$  g for 30 min at 4°C to precipitate the RNA.
35. Remove the supernatant carefully and wash the pellet in 1 mL 75% ice-cold ethanol per 1 mL TRIzol reagent, briefly vortex to get the pellet off the side of the tube.
36. Centrifuge at no more than 7,500  $\times$  g for 5 min at 4°C.



**Figure 3. In vivo rG4IP**

(A) G quadruplex RNA immunoprecipitation (rG4IP) was performed in the TCC-S cells and the immunoprecipitated samples were normalized by their input controls. *NRAS* and *MMP16* mRNAs were used as a positive control for the presence and detection of G quadruplex structures, as documented in the literature. *TBP* mRNA was used as a negative control as it lacks G quadruplex consensus sequences in its entire mRNA.

(B) DU145 cells were transfected with either the pcDNA3 RLUC POLIRES FLUC empty vector or the vector with a potential G quadruplex region found in *AGAP2* 5'UTR cloned in front of the *Renilla* luciferase gene. After the rG4IP assay, the levels of *Renilla* luciferase mRNA in the immunoprecipitated samples were normalized by their input controls. An unspecific isotype antibody (IgG) was used as a negative control. Data shown correspond to three independent immunoprecipitations and the error bars denote standard deviation. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

37. Repeat the washing step with 75% ice-cold ethanol followed by centrifugation.
38. Note the position of the pellet and carefully remove the ethanol using a pipette. Then centrifuge again to collect the remaining ethanol.

### RNA solubilization

39. Carefully remove the remaining ethanol and air dry the pellet for 2–3 min at room temperature.

△ **CRITICAL:** Do not over-dry the pellet as it may be difficult to redissolve.

40. Resuspend the pellet in 15  $\mu\text{L}$  of nuclease-free water (we expect between 100–150 ng RNA/ $\mu\text{L}$  after rG4IP) and use this whole volume in the reverse transcription, using random primers.
41. Use 1  $\mu\text{L}$  of cDNA to perform quantitative real-time PCR to analyze enrichment relative to the negative control antibody. Validate findings using other relevant positive and negative controls.

### EXPECTED OUTCOMES

We have successfully demonstrated that, following this protocol, we can detect a significant enrichment for the positive target controls *NRAS* and *MMP16* mRNAs in the BG4-immunoprecipitated fraction, whilst no statistically significant differences were noted for the negative target control *TBP* mRNA (Figure 3A). Furthermore, we have also detected the enrichment of *AGAP2* mRNA in the BG4 antibody fraction, supporting our hypothesis that this mRNA does contain G quadruplex structures *in vivo*. And by using our plasmid transfection approach, we have confirmed those structures' location in the 5'UTR and revalidated the efficiency of our technique to pull down mRNAs containing G quadruplex structures (Figure 3B and see also troubleshooting 5) (Surani et al., 2022). Our technique, which involves no fixation steps, validates the formation of these structures inside the cell and, if coupled with RNA sequencing, can be used to identify mRNAs with G quadruplex structures, and expand our knowledge of these structures on key cellular processes and pathophysiological conditions.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software (version 8). Normality was evaluated using the Shapiro-Wilk test. For experiments where two groups were compared (Figure 2),

a Mann-Whitney U-test was used for the analysis. Comparisons between three or more groups were performed either with a Kruskal-Wallis test followed by an uncorrected Dunn's test (non-parametric data), or with a one-way ANOVA followed by post-hoc Sidak multiple comparisons test.

## LIMITATIONS

The rG4IP technique is based on an antibody-mediated enrichment and requires enough starting material to detect the RNA population containing G quadruplex structures. Consequently, it is important to determine the level of mRNA of interest in the cell line under study to ascertain a decent starting concentration. Alternatively, the construction of a plasmid vector containing the potential G quadruplex forming sequence could be also used as demonstrated here.

The BG4 antibody used in the technique also detects G quadruplexes structures in genomic DNA (Javadekar et al., 2020). It is therefore vital to avoid genomic contamination of the cytosolic fraction to prevent false positives. However, we have found that not all DNases are equally efficient and, being this step crucial, we recommend using the Ambion DNase I to ensure detection of only specifically enriched RNAs.

We also need to highlight that the BG4 antibody is biased toward G quadruplexes with parallel orientation and although till recently these were considered the only type of RNA G quadruplex structures, now we know this is not the case (Xiao et al., 2018) and our method might not work for RNAs with antiparallel orientation.

And, as a final comment, the formation of the G quadruplex structure is dependent on a variety of factors such as availability of monovalent cations to stabilize these structures, level of helicases that unwind these high order structures, and G quadruplex binding proteins (Fay et al., 2017; Mendoza et al., 2016). Depending on the stability of these complexes, some of these proteins could interfere with the antibody binding under our gentle extraction conditions, which might underrepresent the total RNAs with G quadruplexes present in the cell.

## TROUBLESHOOTING

### Problem 1

When to collect cells for lysis after transfection (refers to step 9d).

#### Potential solution

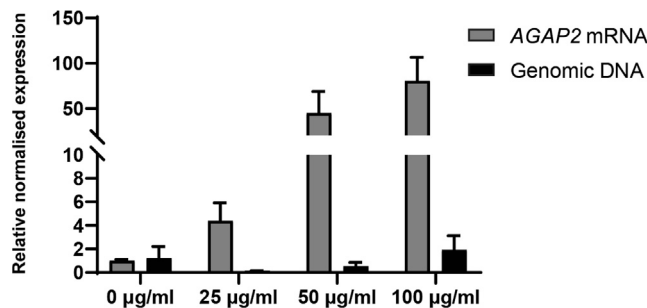
There are different methods to transfect cells and the transfection conditions and consequent expression should be tested. It is common to use a GFP plasmid for this, checking GFP expression under a fluorescence microscope at different times after transfection. However, transfection conditions for plasmids of different sizes are not always the same. Therefore, we suggest using the *Renilla* luciferase primers provided on the [key resources table](#) to test the expression of the pcDNA3 RLUC POLIRES FLUC plasmid at different times after transfection. And select the time when the expression is highest to collect the samples for rG4IP.

### Problem 2

Isolation of cytoplasmic RNAs using a mild detergent (digitonin) to break the cell membrane, leaving the nuclear membrane intact, needs some optimization (see step 4 in [step-by-step method details](#)).

#### Potential solution

Perform RNA extraction from cells lysed with rG4IP lysis buffers that contain a range of digitonin concentrations and follow with RT-qPCR. The lowest concentration of digitonin yielding satisfactory levels of the mRNA of interest and low levels of genomic contamination should be selected (click [Figure 4](#) to see a useful example).



**Figure 4. Selection of digitonin concentration**

TCC-S cells were lysed with rG4IP lysis buffer containing varying concentrations of digitonin. After lysis and extraction of the cytosolic fraction, the RNA was isolated and analyzed using qRT-PCR. The expression level of AGAP2 mRNA and its genomic DNA (primers amplifying the promoter region) were normalized using the housekeeping gene *HPRT* and presented relative to the no digitonin (0 µg/mL) condition. Data shown are the mean  $\pm$  SD of two independent experiments performed at least in duplicate.

### Problem 3

Prolonged incubation with BG4 antibody might yield unwanted background (step 19 in [step-by-step method details](#)).

### Potential solution

It is not recommended to incubate the lysate with BG4 antibody bound to beads for longer than 12 h as it would result in non-specific binding of the RNA to protein G beads. If the nonspecific background is noted in the BG4 lysate at incubation times less than 12 h, it is suggested to increase the washing step to five times (step 21 in [step-by-step method details](#)).

### Problem 4

Genomic contamination noted in BG4 eluant even after DNase treatment (step 25 in [step-by-step method details](#)).

### Potential solution

It is important to assess the level of genomic contamination in the final eluant to avoid enrichment of G quadruplex structures in genomic DNA. It is therefore recommended to perform DNase treatment of the eluant. If there is a significant amplification of genomic DNA after DNase treatment, it is suggested to increase DNase incubation time to 30 min.

### Problem 5

Unexpected result: negative result in native rG4IP and positive for plasmid rG4IP (see [expected outcomes](#) section).

### Potential solution

The first step to address this outcome would be to ensure that the note in step 7 has been taken into consideration and there was sufficient RNA to detect the target RNA with a Ct value  $<$  30 in the qPCR before performing the rG4IP. It is also necessary to confirm that positive controls are detected in the native immunoprecipitated sample. Once this is checked, if the outcome is still the same, it could be possible that the folding of the RNA sequence into the G quadruplex might be affected by the rest of the sequence. We have used the plasmid approach here to confirm the presence of the G quadruplex within a limited sequence (a 5'UTR fragment) and we consider these two approaches complementary: one to demonstrate presence *in vivo*/native conditions, the other one to narrow the localization of the quadruplex within the RNA sequence. We would recommend expanding the length of the RNA sequence transfected, having constructs with different lengths and checking for the presence of the G quadruplex. But ultimately, we would give priority to the results from the native rG4IP.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cristina Montiel-Duarte ([cristina.montielduarte@ntu.ac.uk](mailto:cristina.montielduarte@ntu.ac.uk)).

### Materials availability

Plasmids generated in this study are in the process of been deposited to Addgene.

### Data and code availability

This study did not generate/analyze datasets nor code.

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## AUTHOR CONTRIBUTIONS

C.M.D. conceptualized and designed the protocol. A.A.S. performed the experiments and optimized the steps in the protocol. A.A.S. prepared the first draft, and C.M.D. reviewed it.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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