Global mapping of RNA G-quadruplexes (G4-RNAs) using G4RP-seq

Sunny Y. Yang¹, David Monchaud², & Judy M.Y. Wong^{1,*}

¹*Faculty of Pharmaceutical Sciences, University of British Columbia, Pharmaceutical Sciences Building,
2405 Wesbrook Mall, V6T 1Z3, Vancouver, Canada*

²*Institut de Chimie Moléculaire, ICMUB CNRS UMR 6302, UBFC Dijon, France*

*correspondence: Dr. Judy Wong; Tel: 604-827-3314; Fax: 604-822-3035; Email: judy.wong@ubc.ca

EDITORIAL SUMMARY Guanine-rich RNAs folded into G-quadruplex structures (G4-RNAs) are captured from crosslinked RNA using a G4-specific chemical probe, BioTASQ, and mapped globally by sequencing.

PROPOSED TWEET #NewNProt for capturing RNA G-quadruplexes (G4-RNAs) using a G4-specific probe. @dmonchaud

PROPOSED TEASER Global mapping of G4 RNAs

Related links

Key reference(s) using this protocol

Yang, S. et al. Nat Commun 9, 4730 (2018): <https://doi.org/10.1038/s41467-018-07224-8>

Renard, I. et al. Nucleic Acids Research 47, 5502–5510 (2019): <https://doi.org/10.1093/nar/gkz215>

1 **ABSTRACT**

2 Guanine-rich RNAs can fold into four-stranded structures, termed G-quadruplexes (G4-RNAs), and
3 participate in a wide range of biological processes. Here, we describe in detail the G4-RNA-specific
4 precipitation (G4RP) protocol, which allows the transcriptomic profiling of G4-RNAs. The G4RP protocol
5 consists of a chemical crosslinking step, followed by affinity capture with the G4-specific probe,
6 BioTASQ. G4RP can be coupled with sequencing to capture a comprehensive global snapshot of folded
7 G4-RNAs. This method can also be used to profile induced changes (i.e. through G4 ligand treatments)
8 within the G4-RNA transcriptome. The entire protocol can be completed in 1-2 weeks and can be scaled
9 up or down depending on the specific experimental goals. In addition to the protocol details, we also
10 provide here a guide for optimization in different laboratory setups.

12 **INTRODUCTION**

13 G-quadruplexes (G4s) are non-canonical nucleic acid structures formed by Hoogsteen bonding of four
14 guanines to form planar guanine quartet (G-quartet) units, which π -stack on each other to form the
15 overall columnar structure¹⁻². G4s forming in DNAs (G4-DNAs)³ and RNAs (G4-RNAs)⁴ can affect the
16 corresponding biological processes. G4-forming sequences are found within the genome and
17 transcriptome of several prokaryotes, eukaryotes and viruses⁵. Importantly, G4-forming sequences are
18 highly saturated at sites of disease-relevance such as oncogenes, telomere, and repeat expansion
19 regions⁶. Biochemical⁷, bioinformatics⁸, optical imaging⁹ and genomic techniques^{7,10} have been used to
20 show that G4 formation is widespread in both the human genome and the transcriptome. However,
21 accurately quantifying and mapping by sequencing the G4-DNA¹¹⁻¹⁴ and G4-RNA sites^{15,16} formed within
22 living cells continues to be an ongoing challenge. It is important to note that G4s formation is often
23 outcompeted by Watson-crick duplex formation, protein-binding, and G4-destabilizing helicase (G4-

helicase)¹⁷ activities; thus, the G4 folding process requires windows of opportunities when the polynucleotides are single-stranded and in more openly accessible conformations. In support of this notion, it has been observed that open chromatin states are more favourable for the formation of G4-DNAs¹³. The high prevalence of potential G4-forming sequences in the human genome⁸ suggests that formation of G4s likely permeates throughout most, if not all, important biological events in the flow of genetic information. G4-DNAs may play roles in various biological functions, including telomere maintenance, transcription regulation, epigenetic regulation, replication and recombination¹⁸. Despite the increasing volume of research in the G4 field, much of the previous focus has been on G4-DNA rather than G4-RNA. Given the established ability of RNA strands to fold into intricate structures, which govern their functions¹⁹, and the superior thermostability of G4-RNA⁴, it is anticipated that G4-RNAs constitute a significant proportion of all folded G4s within living cells. G4-RNA has also been implicated in a wide range of biological processes, including translation, RNA processing, splicing, and ribonucleoprotein (RNP) formation⁴. Here, we discuss some of the current techniques used to study G4s and highlight our recently developed affinity-capture technique, called the G4-RNA-specific precipitation (G4RP) with sequencing (G4RP-seq), for studying G4-RNAs on a transcriptome-wide level.

Current methods for studying genomic and transcriptomic G4s in cells

While interest in G4s has been multiplying, there is still a limited number of techniques for studying G4s on a genome- and transcriptome-wide scale. For genome-wide studies, G4-seq¹⁵ combines the polymerase-stop assay with next-generation sequencing under conditions that favour (using K⁺ cations or G4-stabilizing ligand pyridostatin (PDS)²⁰) or disfavour G4 formation (using Li⁺ cations) in purified, protein-free DNA. As folded G4s can cause polymerase-stalling, quantification of these polymerization stop sites can infer G4 folding state and generate a genomic G4 map. While the resolution is high, a

major limitation of G4-seq is the in vitro experimental setting, which is not representative of the native chromatin state. Genome-wide G4 mapping by performing chromatin-immunoprecipitation sequencing (G4-ChIP-seq)¹³ using the G4-specific antibody BG4¹¹, a single-chain variable fragment antibody specific for G4, allows approximation of a more biologically relevant (i.e. “in vivo”) setting. In comparison, G4-seq observed over 750,000 potential G4 sites¹², whereas G4-ChIP-seq reported only 10,000 enriched sites¹³. This large discrepancy between the two techniques emphasizes the importance of the local chromatin environment in the formation of G4-DNAs.

A recently developed sequencing technique, rG4-seq¹⁵, which is conceptually similar to G4-seq, has been implemented for studying transcriptome-wide G4-RNAs. This technique exploits the effect of G4-RNAs on the processivity of reverse-transcriptase (RT) in complementary DNA synthesis. By mapping and comparing RT polymerization stop sites under G4-favourable and unfavourable ionic conditions in vitro, thousands of potential G4-RNA sites were discovered. As the query of G4 formation using purified RNA will not recapitulate the contributions of other biological factors (e.g. RNA-binding proteins, other nucleic acid structures.), rG4-seq possesses much of the same limitations as G4-seq. To that end, the RT stop-profiling method has been adapted to study G4-RNAs in vivo with an additional step of treating cells with dimethyl sulfate (DMS) before purification and RT. DMS-modified guanine bases are unable to fold into G4s in vitro and thus can be used to infer folding state in vivo indirectly²¹. Surprisingly, DMS/RT-stop profiling showed that at equilibrium, G4-RNAs are nearly all unfolded in vivo, a significant departure from observations made using live-cell imaging^{9,22} and in vitro quantification¹⁵ methods. This apparent discrepancy is likely due to the nature of the DMS/RT-stop profiling assay, which may be more suitable for establishing the shift of G4 equilibrium rather than capturing a snapshot of the folded G4 transcriptome. Drawing inspirations from G4-ChIP-seq, we developed the G4RP-seq protocol¹⁶, which allows a broad-level snapshot view of the folded G4 transcriptome. Using G4RP-seq, we showed that G4-RNAs fold in a sequence-dependent manner and that treatment with G4-stabilizing ligands enhances

these G4 folded states. Thus, the current model depicts G4-RNAs as short-lived structures which are continuously being folded and unfolded by various regulatory mechanisms inside living human cells. These findings present us with a new perspective in which G4-RNAs are portrayed more as events rather than simple physical structures.

Development of the Biotinylated Template-Assembled Synthetic G-quartet (BioTASQ) and G4RP Protocol

A common recurring challenge in designing G4 ligands has been the lack of specificity due to unintended off-target binding. A class of biomimetic probes, called the Template-Assembled Synthetic G-quartet (TASQ)²³, was developed to mitigate these off-target binding events. The chemical design of TASQ, inspired by the natural formation of G-quartets in G4s, consists of a central core with four arms, each attached to a guanine (Fig. 1a). These guanines self-assemble into a synthetic G-quartet upon association with a G4 target through end-quartet stacking. The use of the synthetic G-quartet itself as the recognition molecule provides high specificity and defined binding properties. A caveat of TASQ is its preference for targeting G4s with a parallel conformation due to the accessible end-quartets for TASQ binding without steric hindrance from the loops. This binding preference is especially useful for targeting G4-RNAs due to their parallel-conformational constraints. The extra utility of TASQ molecules is demonstrated by swapping the central core with different application-specific moieties²³⁻²⁶. The newest generation of TASQ molecules is a biotinylated version of TASQ (BioTASQ)^{16,27}, allowing for affinity capture of G4 targets using streptavidin-biotin interactions. G4RP is derived from a standard RNA immunoprecipitation (RIP) protocol²⁸; however, instead of antibody-protein recognition in RIP, G4RP uses a ligand-nucleic acid structure recognition with BioTASQ. Like RIP and other similar methods, the G4RP protocol can be coupled with high-throughput sequencing to profile transcriptome-wide G4 distribution.

Advantages and Limitations of G4RP

Using G4 ligands as baits for G4 targets has previously been explored²⁹; however, specificity for the intended G4 targets has been an ongoing challenge for most, if not all, G4 ligands³⁰. BioTASQ was designed with these limitations in mind by providing higher specificity due to the strictly defined binding mechanism. The use of a synthetic G-quartet as the bait provides the best-predicted interactions given that G-quartet stacking interaction is obligatory and exclusive for all G4 formations and stabilization.

Some of the key advantages and limitations of G4-RNA quantification assays stem from whether the approach is direct or indirect. Direct and indirect G4-RNA identification approaches provide different perspectives due to the dynamics of G4-RNA formation, and thus the appropriate method of choice depends on the specific scientific question. Table 1 outlines some of the key differences between the two types of methods. We consider G4RP a direct approach to detect G4s due to the probe's direct association with the G4 target. However, direct approaches do have the potential pitfall of probe-assisted stabilization of the G4 targets. Since BioTASQ has properties of a G4 ligand, it conceivably can stabilize G4 targets, resulting in quantification biases in capturing G4-RNAs that are not representative of their true biological states. Therefore, an *in vivo* crosslinking step is added prior to cell lysis and BioTASQ-assisted pulldown in G4RP, which is crucial in minimizing this bias by preventing G4-folding *in vitro*. In contrast, indirect approaches, including rG4-seq¹⁵ and DMS/RT-stop profiling²¹, involve nucleic acid modifications prior to subsequent screening using reverse transcriptase. Indirect approaches heavily rely on the following two assumptions: first, modifying chemicals are assumed to access all cellular RNA and modify unprotected sites uniformly within the transcriptome of living cells, which is unlikely; second, *in vitro* folded G4s are presumed to be the sole cause of all RT stop sites, which may lead to overestimation of the actual number of G4 sites due to the inclusion of potential polymerase-stalling effects by other RNA structures¹⁹. Indirect approaches are likely biased against highly dynamic RNA structures as these readouts can only be estimated from highly stable structured RNAs under the

set of experimental conditions³¹. Depending on the experimental conditions, when the labelling chemicals are usually not the limiting reagent, chemical modification in live cells can often lead to overwhelmingly modified RNAs. Thus, G4RP is considered a more appropriate method for providing a less-biased view of transiently formed G4 structures. Finally, the currently reported indirect methods do not consider the formation of intermolecular G4-RNA since the principle of RT-stop profiling is based on the effect of the cis-acting intramolecular G4s. In this sense, G4RP provides measurements that are more representative of the biologically relevant interactions due to its ability to reporting on both intra- and inter-molecular folded G4-RNAs.

As indirect methods often provide high resolution data at near single base levels (e.g. chemical probing techniques, RT-stop profiling), a major limitation of G4RP is the lack of mapping resolution (>200 bases) due to the sonication step. The main challenge lies in striking a balance between over-sonication and under-sonication. Under-sonication prevents the proper release of cellular content, resulting in a reduction in resolution and lower pulldown efficiency. In contrast, over-sonication may increase mapping resolution but may also lead to disruption of the binding structure (G4 end stack) necessary for BioTASQ binding. Optimization to reach the specific goals of the experiment is highly recommended. While sequence analysis of the BioTASQ-enriched RNA fraction in G4RP shows robust enrichment of G-rich sequences, it should be noted that a minor portion of indirect interactions could be crosslinked and captured.

While G4RP does offer several advantages over the indirect polymerase-dependent methods, the choice of G4-RNA quantification method depends on the biological questions of interest. It is important to emphasize that no individual method is without limitations and caveats, and that necessary future steps should be invested in combining complementary methods in order to mitigate some of their individual limitations, thus providing a more unbiased view of the G4-RNA transcriptome.

Table 1. Indirect vs direct methods in studying transcriptomic G4-RNAs		
	Indirect method (rG4-seq, DMS/RT-profiling)	Direct method (G4RP-seq)
Readout method	Reverse-transcription stalling + sequencing	Probe-binding/extraction + sequencing
Methods to capture biological state	Modification of nucleic acids in live cells	Chemical crosslinking
Resolution	High	Low
Targets	-Intramolecular G4s	-Intramolecular G4s -Intermolecular G4s
Enrichment method	Poly (A) enrichment	Ribo-depletion
Biases/ Limitations	-in vitro biases -DMS modification could lead to underestimation of dynamic structures -Overestimation of G4 structures by counting stops induced by other structures -Not able to read 5' G4 if there is a strong RT stop caused by 3' G4 signal	-Probe-assisted stabilization could lead to overestimation of G4 structures -a small portion of crosslinked indirect interactions could be captured

142 Future applications

143 Theoretically, G4RP could be combined with chemical modification and in vitro RT-stop profiling to
 144 generate a high-resolution map of transcriptome-wide G4-RNA sites; however, sample processing
 145 compatibilities and assay optimization between these methods have not yet been tested. RT-stop
 146 profiling could be performed in parallel with G4RP on the same set of samples and subsequently
 147 analyzed and compared. Alternatively, RT-stop and G4RP could be performed sequentially by profiling
 148 BioTASQ-captured RNA to improve resolution. It is also yet to be determined how chemical modification
 149 (e.g. through DMS), if incorporated, could affect G4RP. While G4RP-seq could be used to infer in vivo
 150 folded state, rG4-seq (or equivalent RT-profiling methods) could be used to determine at high resolution

the positions in which intramolecular G4s are more likely to form in vitro. Further optimization of the sonication step (i.e. increasing sonication duration) and/or adding ribonucleases could potentially improve resolutions of G4RP-seq by decreasing fragment sizes. However, these optimization strategies are limited to improving the resolution of intramolecular G4s. Intermolecular G4s remain a challenge to map with the currently known techniques. The use of different crosslinking agents (e.g. psoralen) with different effective distances could also be informative to elucidating a complete picture of the G4-RNA interactions. Integration of G4RP-seq with other methods for identifying RNA-RNA interactions, such as LIGR-seq³², MARIO³³, SPLASH³⁴, and PARIS³⁵, may aid in identifying long-range interactions through G4s. G4RP may also be potentially useful, when coupled with proteomics, in identifying protein partners that may interact with G4-RNAs. While the G4RP protocol has initially been optimized for human cells, it can conceivably be adapted and optimized for other model organisms, including other eukaryotes, prokaryotes and even viruses^{5,36}. Given the unique G4 profiles of different organisms and species, G4RP-seq may be valuable for studying the evolution of G4-interactome and potential inter-organism interactions (e.g. host-pathogen)³⁷.

Experimental Design

Selection of cell models and treatment endpoints. G4RP is expected to be compatible with most mammalian cell lines. The samples should be chosen with appropriate controls to ensure an unbiased comparison (e.g. vector controls for overexpression experiments). Comparisons between cell lines should include ones with similar genomic backgrounds. Typically, for drug treatments (e.g. small-molecule compounds such as G4 ligands: BRACO-19 and RHPS4^{16,27}), a dose-response profile for an observable endpoint should be generated to assess the appropriate doses for subsequent experiments. This would ensure that the intracellular concentration of the ligand is sufficient to cause a biologically observable change in an endpoint (e.g. cytotoxicity) without leading to excessive cell death and

subsequently the loss of sample. We suggest using subtoxic or low toxic dose (LD5-25; dose producing 5-25% of cell deaths). For classical G4 ligands, such as BRACO-19 and RHPS4, we have observed optimal concentrations ranging from 0.5 to 20 μ M depending on the cell line. For treatments, the vehicle (i.e. solvent for compound) should be used as the control. Another factor of consideration is the duration of the treatment. Typical treatment time points include 6h, 12h, 24h, 48h and 72h. A different endpoint measure, other than cytotoxicity, may be chosen depending on the experimental goals.

Chemical crosslinking. Crosslinking could also impact sonication efficiency; however, we advise against deviating from our recommended crosslinking duration of 5 to 10 min. Most formaldehyde formulations, including those with methanol additive, are effective for crosslinking. Methanol increases cell permeabilization, which may increase crosslinking efficiency. Since formaldehyde is sensitive to light and air, we suggest storing it in aliquots in a dark and sealed environment. Fresh formaldehyde without methanol, sold in ampules, can also be considered to decrease potential variability.

Sonication and fragmentation. Fragmentation of the RNA material is required for the G4RP. Typically, the range of fragments should fall between 200-800 nt with centering around 400-500 nt. Gel electrophoresis coupled with RNA-staining dye is a simple method for test. Other techniques requiring more advanced equipment, such as a Bioanalyzer, can also be used. We suggest using mechanical sonication as it is more consistent and sonication devices are commonly available in most institutions. However, different sonicators (e.g. Covaris ultrasonicator, Diagenode Bioruptor, Branson Sonifier, or other equivalent equipment) will require additional optimization. We recommend testing a range of sonication durations, with the optimal total sonication duration likely to be between 2 to 4 minutes. Different sonicators may have a recommended range in terms of the input number of cells. Please check with the manufacturer-supplied manual or recommended protocols as a guide for the initial optimization. Sonicators used for G4RP should also be equipped with a temperature control system to

prevent heating and premature crosslink reversal. Mechanical sonication serves two purposes: lysing the cell and fragmenting the nucleic acids. Use of any other non-mechanical shearing alternatives (e.g. detergent-based lysis and nuclease-mediated fragmentation) will require a different set of optimization and additional considerations of potential biases.

Bead binding. Our protocol is optimized using magnetic streptavidin-coupled beads, specifically the Promega Streptavidin MagneSphere. Other brands or types of streptavidin-coupled beads (agarose) are likely to be compatible with BioTASQ as well; however, additional optimization and validation may be necessary. BioTASQ concentration should greatly exceed the binding capacity to ensure the capture is driven by BioTASQ, not by non-specific binding to the beads. Typically, each G4RP capture uses 10-20 nmol of BioTASQ with 50-100 µg of beads. Increasing the amount of beads or switching to high-capacity beads can increase the capture yield. When altering the amounts of beads used, negative controls must be tested in parallel.

Negative control and normalization. There are two types of controls used in G4RP. For the negative control, G4RP is performed in the same manner with biotin instead of BioTASQ. The yield of purified RNA from the negative G4RP control sample is typically much lower and serves as a control for non-specific bindings to the beads. The negative control is necessary for early optimization but is optional after the protocol has been optimized and only negligible background is detected. The input control, a small aliquot of the material collected prior to BioTASQ binding, is critical and required for all experiments. In contrast with genomic DNA, cellular RNA content can vary widely, and this input control serves as an internal control for each G4RP experiment during the normalization step of the analyses.

PCR Primer selection. We have included a list of recommended targets for checking the efficiency of G4RP (Table 2). However, if there are other targets of interest, additional sets of primers may be used. These primers should undergo standard validation for specificity to the target region and PCR efficiency.

Typically, at least three biological replicates should be used to quantify each locus. For sequencing, we recommend having multiple replicates (at least 4-6). Based on the results of qPCR screening, the best 2-3 replicates can be used for library preparation. Any newly discovered region of interest from sequencing analyses should also be validated using qPCR and appropriate primers.

Library preparation and sequencing depth. For library preparation of the G4RP samples, we highly recommend the use of ribodepletion over poly-A enrichment. Due to the fragmentation step in G4RP-seq, poly-A selection will likely result in loss of signal and a significant 3' bias. Always use the minimal number of PCR cycles for the library generation. In the case of low amount of input, consider pooling multiple parallel-processed samples to increase concentration. Sequencing depth will depend on the experimental question. We recommend 15-20 million uniquely mapped reads; however, this could be scaled up to 50 million reads to detect rarer events or changes. Typically, sequencing 2-3 replicates for each BioTASQ-enriched sample is sufficient for cost-effective data analysis. More replicates would improve the robustness of the statistical analysis but are unlikely to change the overall conclusions of the experiment. Sequencing of the negative control is optional and is unlikely to result in any useful data due to the low levels of the transcriptome of interest and significant rRNA background. Each condition must be accompanied by the sequencing of at least one input control. Ideally, for the most robust normalization, each BioTASQ-enriched sample can be accompanied by its respective input control.

MATERIALS

Biological materials

Cell lines of interest. We have used G4RP successfully in variety of transformed or cancer-derived human cell lines of different types, including MCF7 (https://scicrunch.org/resolver/CVCL_0031), MDA MB-231

(https://scicrunch.org/resolver/CVCL_0062), HELA (https://scicrunch.org/resolver/CVCL_0030), HEK293 (https://scicrunch.org/resolver/CVCL_0045), and HT29 (https://scicrunch.org/resolver/CVCL_0320).

With proper optimization of later steps, we expect G4RP to be compatible with most organisms. Here, our protocol focuses on cultured cell lines. The use of tissue may also be compatible but will require additional optimization of tissue homogenization, crosslinking and sonication steps.

CAUTION: The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

CRITICAL Most standard molecular and cell biology grade reagents can be obtained from various vendors and do not need to be from the ones specified here.

Cell culturing

- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, cat. no. LS11965092)
- Fetal bovine serum (FBS) (GE Lifescience, cat. no. SH3039602)
- Penicillin-streptomycin (P/S) (Gibco, cat. no. 151140148)

Standard buffer components in the G4RP protocol

- Nuclease-free water (Thermos, cat. no. AM9922 or vendors of choice or made in-house using DEPC treatment)
- Diethyl pyrocarbonate (DEPC) (Sigma, cat. no. D5758)
- Formaldehyde (37% wt/vol) (Sigma, cat. no. 252549)

CRITICAL: Keep away from light and air; aliquot before use.

CAUTION: Formaldehyde is toxic through ingestion, inhalation and skin contact. Always wear appropriate personal protective equipment and operate only inside a fume hood. All waste material should be collected separately and disposed according to institutional procedures.

- 266 • Glycine (Fisher, cat. no. G45-212)
- 267 • 10X PBS (Fisher, cat no. BP39920)
- 268 • HEPES (Sigma, cat. no. H3375)
- 269 • NaCl (Fisher, cat. no. S271-10)
- 270 • EDTA (Sigma, cat. no. E5134)
- 271 • EGTA (Sigma, cat. no. E3889)
- 272 • KCl (Fisher, cat. no. P330)
- 273 • DTT (Fisher, cat. no. R0861)
- 274 • IPEGAL (NP40) (Sigma, cat. no. I3021)
- 275 • Tris (Fisher, cat. no. BP152)
- 276 • Chloroform (Sigma, cat. no. C2432)
- 277 CAUTION: Chloroform is toxic if inhaled or swallowed. Always wear appropriate personal
- 278 protective equipment and operate only inside a fume hood. All waste material should be
- 279 collected separately and disposed according to institutional procedures.
- 280 • Isopropyl alcohol (Sigma, cat. no. I9516)
- 281 • Ethyl alcohol
- 282 • RiboLock RNA inhibitor (ThermoFisher, cat. no. EO0381)
- 283 • Protease-inhibitor cocktail (PIC) (optional; usually supplied as 100X; NEB #5871S)
- 284 • TRIZOL (Invitrogen, cat. no. 15596026)
- 285 CAUTION: TRIZOL is a corrosive material and is toxic through ingestion, inhalation and skin
- 286 contact. Always wear appropriate personal protective equipment and operate only inside a
- 287 fume hood. All waste material should be collected separately and disposed according to
- 288 institutional procedures.
- 289 G4RP-specific reagents

- 290 • Biotin (Sigma, cat. no. 2031)
- 291 • BioTASQ (available soon through Merck/MilliporeSigma)
- 292 • Promega MagneSphere Paramagnetic (Streptavidin beads) (Promega, cat. no. Z5481)

293 Reverse Transcription and PCR

- 294 • Superscript III (Invitrogen, cat. no. 18080093)
- 295 • Random hexamer pd(N)6 (Invitrogen, cat no. N8080127)
- 296 • dNTP mix (NEB, cat no. N0447L)
- 297 • SYBR green PCR-mix (BioRad, cat. no. 1725120)

298 Agarose Gel reagents

- 299 • UltraPure Agarose (ThermoFisher, cat. no. 16500100)
- 300 • Bleach (Clorox)
- 301 • SYBR Green II RNA Gel Stain (ThermoFisher, S7564)

302 Sequencing kits

- 303 • RNEasy Plus RNA purification kit (Qiagen, cat. no. #74134)
- 304 CRITICAL: Illumina Ribo-Zero has been discontinued and incorporated into the TruSeq Stranded
- 305 Total RNA kit. Use either the old Illumina Ribo-Zero + TruSeq RNA library kit or the new Illumina
- 306 Stranded Total RNA library prep kit.
- 307
- 308 • Old version: Illumina Ribo-Zero + TruSeq RNA library prep kit (Illumina, cat. no. RS-122-2001)
- 309 • New version: TruSeq Stranded Total RNA library prep (Illumina, cat. no. 20020596)

310 **Equipment**

311 Lab equipment

- 312 • Standard cell culture incubator (e.g. HERACell, Thermos)
- 313 • Standard Benchtop centrifuge (e.g. Eppendorf)
- 314 • Temperature controlled centrifuge (or a standard unit in a refrigerated environment)
- 315 • Standard laboratory rocker

- 316 • Standard benchtop aspirator
- 317 • Cell sonicator (e.g. Covaris Ultrasonicator, Diagenode Bioruptor, Branson Sonifier)
- 318 • Tube rotator
- 319 • Nanodrop spectrophotometer (Thermos)
- 320 • Thermal cycler (Bio-Rad)
- 321 • Real-time PCR detection system (Bio-Rad)
- 322 • Gel imager (Alpha Innotech)
- 323 • Illumina HiSeq, NextSeq or NovaSeq sequencer

324 Other consumables

- 325 • Cell and tissue culture dishes (vendor of choice)
- 326 • Cell culture treated flasks (vendor of choice)
- 327 • Cell scrapers (Corning, cat. no. 3011)
- 328 • Wide-mouth tips (VWR, cat. no. 89049)
- 329 • Covaris microtube AFA Fiber Pre-Slit Snap-Cap (Covaris, cat. no. 520045)
- 330 • PCR 8-tube strip and caps (Bio-Rad, TLS0851)
- 331 • Hard-shell 96-well PCR plate (Bio-Rad, HSP9601)

332

333 Bioinformatics Tools

- 334 • Trimmomatic³⁸ - <https://github.com/timflutre/trimmomatic>
- 335 • HISAT2³⁹ (https://scicrunch.org/resolver/SCR_015530) - <http://daehwankimlab.github.io/hisat2/>
- 336 • Integrative Genomics Viewer⁴⁰ (https://scicrunch.org/resolver/SCR_011793) -
- 337 <http://software.broadinstitute.org/software/igv/>
- 338 • HTSeq-count⁴¹ (https://scicrunch.org/resolver/SCR_005514) -
- 339 <https://htseq.readthedocs.io/en/master/>

- DESeq2⁴² (https://scicrunch.org/resolver/SCR_015687) -

<https://doi.org/doi:10.18129/B9.bioc.DESeq2>

Reagent Setup

CRITICAL: All buffers should be sterile and carefully prepared using RNase-free water.

DEPC H₂O

0.1% vol/vol DEPC-treated water can be made by adding 1 mL DEPC to 1000 mL of ddH₂O. Mix and incubate at room temperature (20-22°C) for 1h before autoclaving.

DEPC-PBS

Dilute 10X PBS (RNase-free) to 1X in DEPC H₂O or nuclease-free water from a vendor. Store at room temperature.

1X Fixing buffer (50 mM HEPES KOH pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0 in DEPC H₂O)

Prepare a 5X stock solution and store at room temperature for at least 1 year.

1M Glycine

Prepare in DEPC H₂O and filter sterilize. Store at 4°C for up to 2 months.

G4RP Buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% vol/vol NP40 in DEPC H₂O or nuclease-free water)

Prepare the stock buffer and store at 4°C for at least 1 year. A working stock should be made for every new experiment by transferring the required amount for the experiment to a conical tube and adding fresh RNase inhibitor. Use the manufacturer-recommended amount of RNAase (typically ~100 U/mL of buffer). Optionally, fresh 1X PIC may be added, especially if the downstream application involves protein.

1 mM Biotin

Make 1 mM stock using pure ddH₂O. Can be stored at 4°C for at least 3 months.

1 mM BioTASQ

Make 1 mM stock using pure ddH₂O. Can be stored at 4°C for at least 6 months.

Streptavidin MagneSphere Paramagnetic particles (Promega)

Always mix well before transferring beads with a pipette using wide-mouth pipette tips. The magnetic beads are stored in a stabilizing buffer (with PBS/ 0.02% wt/vol sodium azide/ 1mg/mL BSA). You may gently mix well with tapping and aliquot to small volumes (e.g. 30 µL) for later use. For a new experiment, prepare the beads by washing in 1 mL of DEPC-PBS 3 times with gentle tapping; use a magnetic rack, aspirate the buffer and resuspend in fresh DEPC-PBS to make a working stock (for one supplied 0.6 mL tube of beads at 1 mg/mL, resuspend in 100 µL of DEPC-PBS to make 6 mg/mL). While prepared beads may be stored short term (<1 week) at 4°C, it is highly recommended that fresh beads are prepared for each experiment for optimal results.

1X TAE (40 mM Tris, 20 mM acetic acid, 1mM EDTA, pH 8)

Make 50X stock and store at room temperature.

PROCEDURE

CRITICAL The G4RP protocol itself can be completed in two days. Prior to G4RP, cell culture and live-cell chemical treatment steps can take a few days to a week to set up. It is recommended that the weekly schedule is planned to avoid any interruption in the experimental pipeline. In case there are time constraints or other factors that cause anticipated interruptions, we have included a recommended pause point in which the experiments can be temporarily halted and resumed later. Please refer to Fig. 1b for an overview of the protocol.

Seeding cells and treatment with G4 ligands TIMING: 2 days to 1 week

- 1) Seed and treat cells as necessary in DMEM culture medium supplemented with FBS and P/S (or with the culture medium suitable for your cell line).

Generally, for most cell lines, 250,000-1,000,000 cells (minimum 150,000 cells) are seeded per 10 cm dish for a 3-4 days experiment (including seeding, cell growth and 2-3 day treatments). Seeding density can be scaled for a shorter or longer experimental period. The exact timeframes need to be optimized for different cells and treatments. Typically, the cells are harvested while in the log-phase growth with confluence at around 60-90%.

CRITICAL STEP: If G4 ligand treatment is used, make sure a suitable dose is chosen. Cell lines can respond differently to different G4 ligands. A dose-finding experiment should be performed, and a low-toxic (LD15 to LD25) dose should be chosen for subsequent experiments.

Crosslinking TIMING: 1-2 h

CAUTION: Formaldehyde is highly toxic. Always handle high concentrations (>4% formaldehyde) of formaldehyde inside the chemical fume hood. Diluted (1-4%) formaldehyde may be used outside of hood briefly (<10 min). After the crosslinking step, all formaldehyde waste should be placed in a separate waste container in the fume hood to be disposed of appropriately according to institutional procedures.

- 2) Make fresh 1% vol/vol formaldehyde in 1X Fixing Buffer before a new experiment.
- 3) Aspirate media and wash once with 10 mL of DEPC-PBS
- 4) Aspirate PBS and add 10 mL of 1% formaldehyde/1X Fixing Buffer to a 10 cm dish for 5 min on a rocker at room temperature.
- 5) Quench crosslinking by adding glycine to a final concentration of 125 mM and incubate 5 min on a rocker at room temp.

CRITICAL STEP: The duration of the formaldehyde crosslinking step is important. Try to keep it as consistent as possible between samples. Stagger the addition of formaldehyde and quenching if processing several samples at the same time. You may want to optimize the duration of crosslinking.

410 It should generally be between 5 to 10 minutes. Typically, crosslinking over 10 minutes is not
411 recommended as over-crosslinking may limit the accessibility of epitopes.

412 CRITICAL STEP: After the crosslinking step, keep the sample at 4°C as much as possible.
413 Centrifugation should be done at 4°C as well. All buffers from this point on need to be RNase-free.

414 6) Remove the fixing solution, add 0.8 mL of DEPC-PBS (pre-chilled) and scrape cells into an Eppendorf
415 tube. Keep on ice.

416 7) Spin tubes at 1500 rpm (200 x g) for 3 min at 4°C.

417 8) Aspirate supernatant (avoid disturbing the cell pellet) and wash again with 1 mL of DEPC-PBS.
418 Aspirate the supernatant and discard.

419 PAUSE POINT: You may freeze down the cell pellets at this stage and store them at -20°C to be
420 processed later. The pellets can be stored for at least 2 months. However, for optimal results, we
421 recommend that you do not pause at this point as processing fresh samples generally gives a better and
422 more consistent yield for the final RNA targets from G4RP.

423 **Sonication (TIMING: 1-2.5 h)**

424 9) Resuspend the pellet in 400 µL G4RP buffer (supplemented with 100U/mL RNase inhibitor; it is
425 optional to add 1XPIC). If cell pellets were frozen, thaw on ice for 30 min before resuspending in
426 G4RP buffer.

427 ? TROUBLESHOOTING

428 10) Sonicate on a Covaris m220 focused ultrasonicator using 10% duty factor “Chromatin Shearing”
429 preset for 2 min at 7°C temperature setpoint. The Covaris AFA Fiber microtube-130 is used for the
430 sonication. Mix well before adding the resuspended cells to the tube. Make sure there are no
431 bubbles when adding the sample to the AFA microtubes prior to sonication. Each sonication tube

should contain exactly 130 μ L corresponding to approximately 2-3 million cells. Repeat until all the resuspended cells are sonicated.

CRITICAL STEP: Optimization with specific sonicators (Covaris, Diagenode, and other brands) is required. The sonication protocol should be similar to that recommended for ChIP according to sonicator-specific published protocols. We recommend that different durations of sonication be tested. Generally, the sonication duration should not exceed that recommended for ChIP, and a good starting point for optimizing the duration is to use half of what is recommended for ChIP. Make sure the temperature is kept around 2-7°C. Sonication can be checked by proceeding to Steps 22-36 and running a 1% bleach gel⁴³ as outlined in Mini-procedure Box (Fig. 2a).

Mini-procedure Box: RNA bleach gel

1. Make a gel mix with a final concentration of 1X TAE buffer and 1.5% Agarose in ddH₂O.
 2. Microwave until boiling then swirl to mix
 3. Add bleach to final concentration of 1%, microwave to boil again. Swirl to mix.
 4. Pour gel mix into gel cast. Let gel cool.
 5. Load RNA samples with 1X loading dye. Run at 100V for 45 min.
 6. Post-stain using SYBR Green dye diluted 1:5000 in 1X TAE buffer for 30 min away from light.
- Image gel on a gel imager.

? TROUBLESHOOTING

- 11) Spin sonicated lysate at 13200 rpm (16100 x g) for 10 min at 4°C. Move the supernatant to new tubes. The supernatant (sonicate), containing the cellular materials that are successfully released from sheared cells, will be used for the subsequent steps.

PAUSE POINT: It is possible to freeze down the sonicate for later processing. Consider using this pause point rather than the previous one if the issue with the clumpy frozen cell pellets arises. However, for optimal results, it is recommended to continue directly to the next step.

Binding and washes TIMING: O/N + 4 h

- 12) Each sonicate of ~390 μ L can be aliquoted into two tubes (each with 180 μ L): one for the biotin control and the other for BioTASQ. If the volume is insufficient (<360 μ L), the volume can be adjusted by adding the small amount of the remaining G4RP buffer, prepared previously, to a final volume of 390 μ L. Save 8.75 μ L of the remaining sonicate as a 5% input control. The input control can be stored temporarily at 4°C then processed later with the other samples.
- 13) Add 20 μ L of 1 mM stock biotin (20 nmol) to a final concentration of 100 μ M to the control tube. Add 20 μ L of 1 mM stock BioTASQ (20 nmol) to a final concentration of 100 μ M to the G4RP tube.
- 14) Incubate overnight at 4°C on a rotator.
- 15) Next day, use wide-mouth tips to add 10 μ L of MagneSphere magnetics beads (60 μ g) to each tube. CRITICAL STEP: The amount of magnetic bead added can be optimized. The recommended amount is between 20-100 μ g, depending on the brand and make of the magnetic beads. An optimization experiment using different amounts of beads is highly recommended. Generally, a higher amount increases yield but also increases non-specific background binding. Always mix well before transferring the beads to another tube.
- 16) Incubate for 2 h at 4°C on a rotator.
- 17) Use the magnet to pull beads to the side by leaving tubes on the magnet for 2 min. Shift the tubes slightly to pull beads closer together. Aspirate carefully without removing the beads.
- 18) Wash beads 3 times using 1 mL of G4RP buffer. Put on rotator at room temp for 5 min for each wash.
- 19) Wash 2 times in 1 mL of DEPC-PBS for 2 min each.

477 20) Resuspend beads in 100 μ L of DEPC-PBS (supplemented with RNase inhibitor). Take out the 5%
478 input saved from Step 12 and add 100 μ L DEPC-PBS (with RNase inhibitor) to it as well.

479 21) Incubate at 70°C for 1 h to reverse crosslinks.

480 **RNA purification TIMING: 2 h**

481 CAUTION: TRIZOL and chloroform should be handled in the chemical fume hood. Waste should be
482 disposed of appropriately according to institutional procedures.

483 22) Add 1 mL of TRIZOL to each tube directly to the beads and mix gently by flicking the tubes 10-15
484 times.

485 23) Incubate the sample in TRIZOL for 5-10 min at room temp to dissociate any ribonucleoproteins
486 (RNPs) and other structures.

487 24) Add 0.2 mL of chloroform. Make sure the cap is sealed and vigorously shake for 15 s. Leave at room
488 temp for 3 min.

489 25) Centrifuge at 12000 x g for 15 min at 4°C. The mixture will separate into the top aqueous phase, the
490 middle interphase and the lower pink-coloured organic phase. The aqueous phase contains the RNA.

491 26) Transfer the aqueous phase to a new tube by tilting the tube at 45° and slow pipetting. Avoid
492 disrupting the interphase/organic phase.

493 27) Add 0.5 mL of fresh isopropanol to the tube with the aqueous phase. Incubate at room temp for 10
494 min.

495 28) Centrifuge for 10 min at 12000 x g at 4°C.

496 CRITICAL STEP: The RNA pellet will be very small and unlikely to be seen by naked eye. Do not touch
497 the bottom of the tube with the pipette. Pipette out slowly by drawing against the side of the tube.

498 29) Carefully and slowly discard the supernatant but leave 10-15 μ L of liquid at the bottom.

499 30) Add 1 mL of fresh 75% (vol/vol) ethanol.

500 PAUSE POINT: You may store pellets in ethanol at 4°C for up to 3 days or -20°C for months.

501 31) Vortex briefly and centrifuge at 7500 x g for 5 min at 4°C.

502 CRITICAL STEP: It is unlikely that you will see a pellet here, as the amount of RNA is very low. Avoid

503 touching the pipette tip to the bottom or creating any motion that could disturb the pellet. The

504 addition of 25 µg of linear acrylamide during isopropanol precipitation step may also be considered

505 to assist with recovery and visualization.

506 32) Slowly remove the ethanol leaving approximately 10 µL of liquid at the bottom of the tube.

507 33) Keep the lids of the tubes open and air dry for 10 min at room temp.

508 34) Add 30 µL of RNase-free water and pipette up and down.

509 35) Incubate at 55°C for 15 min.

510 36) Check RNA concentration and quality using Nanodrop.

511 ? TROUBLESHOOTING

512 PAUSE POINT: RNA samples can be stored long term at -70°C. Freezing and thawing should be

513 minimized to avoid RNA degradation. We recommend to immediately process the reverse

514 transcription-qPCR to check for G4RP efficiency. The remaining stored samples may be used to

515 process for library preparation after successful validation through RT-qPCR.

516 **Reverse transcription and qPCR quantification TIMING: 4-5 h**

517 CRITICAL: After adding different components, always mix by gently flicking the tube, followed by a

518 quick pulse with a centrifuge. Do not vortex.

519 37) Thaw purified RNA (including all G4RP, biotin control, and input control samples) from Step 36 on

520 ice.

521 38) A master mix can be made with pdN6 and dNTP. Add 2 µL to each tube containing RNA and mix:

Component (1 st part)	Amount (μL)	Final concentration in RT reaction
Purified RNA sample from Step 36	12.5	varies
50 μM random hexamer (pdN6)	1	2.5 μM
10 mM dNTP mix	1	500 μM

522

523 39) Incubate at 65°C for 5 min.

524 40) Place on ice for 5 min.

525 41) Make a master mix of the following components and gently mix by flicking. Add 20 μL to each tube:

Component (2 nd part)	Amount (μL)	Final concentration in RT reaction
5X first strand buffer	4	1X
0.1 M DTT	1	5 mM
RNAse inhibitor (40U/μL)	0.25	10U
Superscript III (200U/μL)	0.25	50U
Total	20	

526

527 42) Gently mix by flicking the RT reaction tube and pulse spin by brief centrifugation.

528 43) Incubate at room temp for 5 min.

529 44) Then, incubate at 55°C for 45 min. These incubations (steps 43-45) can be performed on a

530 thermocycler.

531 45) Inactivate reaction by incubating at 70°C for 15 min and cool down to room temp. Proceed to real-

532 time qPCR with the appropriate primer set and SYBR mix of your choice. Table 2 includes primer sets

533 for several genes that have been tested in MCF7, MDA MB-231 and HEK293 cell lines. However,

534 depending on your experimental objectives and models, you may require a different set of controls.

535 PAUSE POINT: The cDNA samples can be stored at -20°C long term.

536 46) Make a master mix with the appropriate primer set. Typically, we suggest the use of two to three

537 technical replicates. Use qPCR plates or strip tubes compatible with the real-time qPCR machine. An

538 example of a single qPCR reaction is as follows:

539

Component	Amount (μL)	Final concentration in RT reaction
forward primer (10 μM)	0.5	500 nM
reverse primer (10 μM)	0.5	500 nM
2X SYBR mix	5	1X
PCR-grade H ₂ O	3	-
G4RP RT sample (add last)	1	varies
Total	10	

- 47) Run the real-time qPCR using the default amplification protocol for your instrument. It should typically take around 20-30 cycles to reach exponential phase (~2-3 hours).
- 48) Normalization is performed by calculating the percentage of G4RP or biotin control sample against its respective input control using the following formula:

$$G4RP \text{ qPCR signal} = 5 * (2^{\text{Average}(Cq_{\text{input}}) - Cq_{G4RP \text{ or biotin}}})$$

The G4RP qPCR signal is expressed as % of total amount of input going through G4RP that is effectively captured by BioTASQ. For each technical replicate, find the difference between the average of the Cq input and the individual technical replicate. Then, multiply by two to power of the previously calculated value (difference in the Cq values) by 5 (due to using 5% of the total input for normalization purposes).

? TROUBLESHOOTING

Table 2. PCR primer sequences for G4RP-qPCR		
Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
NRAS	ATGACTGAGTACAACTGGTGGT	CATGTATTGGTCTCTCATGGCAC
VEGFA	CCTTGCCTTGCTGCTCTACC	AGATGTCCACCAGGGTCTCG
MALAT1	AAAGCAAGGTCTCCCCACAAG	GGTCTGTGCTAGATCAAAAGGCA

Ribo-depletion and Library Preparation (TIMING: 6-8 h)

- 49) Thaw the remaining TRIZOL-purified RNA samples (biotin-control, G4RP, and input; from step 36) that have been validated successfully with RT-qPCR (steps 37-48) on ice.

50) It is recommended to have an additional column purification step before RNA-seq library preparation to remove any potential residual contamination (e.g. phenol, guanidium salt, etc.) and DNA contaminant. We recommend using the Qiagen RNeasy Plus Mini Kit and following the manufacturer's protocol. Elute at the low volume (<25 µL) in RNase-free water. Quantify RNA for concentration using Qubit fluorometer.

51) For library preparation, use the Illumina TruSeq RNA Library prep kit (with Ribo-Zero) following manufacturer's protocol. We recommend a minimum RNA input of >50 ng for the library preparation process. To minimize PCR bias, higher concentration (>200 ng) is preferred if sufficient input material is available. Briefly, the steps are as follows:

- Deplete rRNA using Ribo-Zero oligos and beads.
- Thermally fragment RNA briefly at 4 min.
- Synthesize the first-strand of cDNA followed by the second cDNA strand.
- Adenylate 3' ends and ligate adapters.
- Enrich DNA fragments by PCR amplification. Use the minimum number of PCR cycles if possible (5-7 cycles).
- Quality-check and quantify library using the BioAnalyzer or equivalent methods.

CRITICAL STEP: If possible, use a higher amount (200-1000 ng) of input to improve library quality and reduce PCR cycles. G4 sequences may be sensitive to PCR by inhibiting polymerase processivity and could be underrepresented after a higher number of cycles. If the input is too low for individual samples, it is recommended to pool multiple replicate samples together.

CRITICAL STEP: The fragmentation step can be modified depending on the fragment size. Due to the sonication step earlier, the average length of RNA fragments will be reduced. To avoid over-

fragmentation, the duration of the thermal fragmentation should be reduced. We recommend 2-4 min of thermal fragmentation.

52) Pool sequencing libraries at equivalent proportion and sequence on a HiSeq, NextSeq or NovaSeq platform using pair-end and read length of 75bp or higher. We recommend the sequencing depth of each sample to be 15-20 million reads.

Sequencing Bioinformatics analyses TIMING: varies

CRITICAL An overview of the G4RP-seq analysis steps is depicted in Fig. 2b. For investigators who are less experienced with bioinformatics or lack a local workstation computer (minimum requirement: >8 core processor, >32 GB memory, >3 TB hard drive) performing data analysis through a remote Galaxy server⁴⁴ (usegalaxy.org), which contains all the required bioinformatics packages, is highly recommended.

53) Quality-check raw FASTQ file using FASTQC and trim reads using Trimmomatic (38) (or equivalent read-trimming tool) with default settings.

54) Align reads to the target genome (e.g. human genome hg19 or hg38 assembly) using HISAT2 (39) using default settings. The output file should be formatted as the compressed binary version of sequence alignment files (BAM format). Integrative Genome Viewer (IGV) (40) can then be used to visualize these alignments. To load in IGV, index files will need to be generated from igvtools for each BAM file.

55) Prepare and download a gene annotation file (in GTF format) from UCSC. This can be accessed at <http://genome.ucsc.edu/cgi-bin/hgTables> . Make sure to choose the right genome and assembly version. Choose “Genes and Gene Predictions” for the group and “NCBI RefSeq” in the track. To use gene symbols as an attribute of interest, choose “refFlat” for the table. The parameters may be changed depending on what attributes you want to be listed. Select “genome” for region and GTF as output format. The resulting GTF file can be either downloaded directly or sent as output to Galaxy.

600 56) Count features and annotate the aligned reads using HTSeq-count (41) with the prepared GTF file.
601 Use “Union” mode and set values as follows: feature type=exon, ID attribute=gene_id. Depending
602 on the library prep kit used, you can set the strandedness mode either “stranded” or “non-
603 stranded.” The “stranded” setting would keep data on the directionality of the transcript.
604 57) Use the raw count output from HTSeq-count as input for DESeq2 (42) for normalization and
605 differential expression analysis.
606 58) Run DESeq2 for the BioTASQ sample vs input control for each treatment condition to generate
607 relative BioTASQ enrichment scores (ES), which are log fold change readout between the test
608 conditions for each transcript. The gene transcripts can be ranked according to their enrichment
609 score. It is important to note that all subsequent comparisons are relative measures.
610 59) Compare the enrichment scores between conditions to quantify ligand-induced changes in G4 RNA
611 structures. The enrichment score change (Δ ES) is calculated as follows: ES (treated) - ES (untreated).
612 60) Using Excel, filter transcripts by counts (as a measure of RNA abundance) and fold change. The filter
613 threshold can differ depending on the quality and depth of sequencing.

614 **TIMING**

615 Step 1, Cell culture phase (including seeding and treatment): 2 days to 1 week (depending on cell
616 culturing experimental setup)
617 Steps 2-8, Crosslink: 1-2 hours (depending on the number of samples)
618 Steps 9-11, Sonication: 1-2.5 hours (depending on the number of samples)
619 Steps 12-21, Binding and washing: overnight + 4 hours
620 Step 22-36, RNA purification: 2 hours

- 621 Step 37-48, Reverse transcription and qPCR quantification: 4-5 hours
- 622 Step 49-51, Ribodepletion and library preparation: 6-8 hours
- 623 Step 52-60, Sequencing and bioinformatics – varies (depending on sequencing queues and
- 624 computational power)
- 625
- 626
- 627 **TROUBLESHOOTING**
- 628 Please refer to Table 3 for advice on troubleshooting.

Table 3. Troubleshooting table			
Step	Problem	Possible Reason	Solution
9	Cells are too clumpy and difficult to resuspend	Freezing after formaldehyde may cause clumping	Do not freeze down pellet and directly proceed to the sonication step.
10	No bands corresponding to purified RNA from sonication check fraction visible on gel	Crosslink reversal is insufficient	Increase duration of the crosslinking reversal step to 2h.
	Either faint bands or bands with apparently high molecular weight observed in purified RNA of sonication check fraction	Cells not sonicated properly.	Reoptimize the sonication step. Increase sonication duration.
		Cellular components not released into the supernatant	Add 0.1% (wt/vol) SDS to resuspended cells before sonication
36	Low A260/280 ratio for RNA	Contamination from TRIZOL components (e.g. Phenol), protein or DNA.	Wash twice, instead of once, with ethanol during the TRIZOL extraction. This will normally not impact qPCR quantification. Perform an additional column purification on RNA before sequencing library preparation.

	Low RNA yield of all extracted samples including input control	Cells not sonicated properly; cellular components not released into the supernatant.	Reoptimize the sonication step. Increase sonication duration. Adding 0.1% SDS to resuspended cells before sonication may also help.
		RNAse contamination	Remake new buffers and ensure no RNAse contamination is present. Add in fresh RNAse inhibitors in every new experiment.
		Problem with TRIZOL RNA purification	Check reagents used for RNA purification (i.e. TRIZOL, chloroform, isopropanol, ethanol). Change to fresh reagents.
		Insufficient reversal of crosslinking leading to loss of RNA to RNPs during TRIZOL step	Increase duration of the crosslinking reversal step to 2h.
48	High signal in biotin control	Concentration of streptavidin beads too high	Add lower amounts of beads Optimize pull-down by testing different amounts of beads at the lower range (20-60 µg)
		Input too high due to too many cells being used which reduces signal-to-background ratio	Decrease number of cells for sonication or dilute cells in higher volume of shearing buffer prior to sonication
	Lower enrichment of BioTASQ sample as detected by RT-qPCR (G4RP qPCR signal is <0.5%)	Incorrect concentration of BioTASQ and beads are used	Confirm that the stock concentrations are correct. Remake stock concentration if uncertain.
		Insufficient amounts of beads	Increase the amounts of beads; optimize pull-down using different amount of beads at the higher range (50-250 µg)
		Over crosslinking leading to loss of accessibility to the G4 epitopes	Reduce crosslinking duration; it should never exceed 10 min. Some formaldehyde contains additives (e.g. methanol) may increase cell membrane permeability and crosslinking efficiency, consider using fresh formaldehyde without additives. Make fresh glycine to quench effectively. Slightly increase glycine concentration.

		RNAse contamination	Remake new buffers and ensure no RNAse contamination is present. Add in fresh RNAse inhibitors in every new experiment.
	Low ligand-induced change detected	Under crosslinking leading to bias towards BioTASQ stabilization	Increase crosslinking duration in the range between 5 min to 10 min. Keep samples on ice or 4°C after crosslinking.
		Treatment with an insufficient dose of G4 ligand	Cell lines can exhibit varying degrees of sensitivity to G4 ligands. Perform a dose-response curve to profile the cytotoxicity of a specific G4 ligand in a specific cell line and chose a subtoxic dose. Reconsider the treatment duration. Typical timepoints include 24h and 48h. Depending on the stability of the ligand, new stocks may need to be produced or purchased.
	Low qPCR signal (high Ct values; >35 cycles) for all samples	Problems with reverse transcription and/or qPCR	Check reagents for RT-qPCR. Run a positive control with a different confirmed sample.

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631

632 ANTICIPATED RESULTS

633 Sonication check

634 Due to the different sonication techniques used by different laboratory groups, it is advised that a
635 sonication check be performed using different sonication conditions. For example, for the Covaris m220
636 Ultrasonicator, we divided a crosslinked sample and performed parallel comparisons between different
637 sonication conditions (sonication duration: 0 min, 2 min and 4 min) under the same settings. Sonicated
638 samples can be heated for 1h to reverse crosslinks, and the RNA can be extracted using TRIzol or RNA
639 column purifications. The purified RNA can then be observed on an RNA bleach gel stained with SYBR

green nucleic acid gel stain⁴³. As depicted in Fig. 2a, absence of sonication would result in an inefficient release of RNA from crosslinked material. Note that crosslink reversal is inefficient in unsonicated material and thus would result in a loss of extracted RNA, as shown by the lack of RNA band on the gel for the 0 min condition. Generally, a sonication duration of 2-4 min is sufficient, yielding a range of bands between 200-800 nt. Longer sonication duration leads to a shorter average length of RNA fragments, as seen by the shifting of the median band length between the 2 and 4 minutes of sonication conditions. It is important to note that, while longer sonication may help increase resolution, it may also disrupt some interactions. Thus, a balanced approach is required to prevent under- or over-sonication.

RNA yield from G4RP

After TRIZOL-purification, the purified RNA can be quantified using Nanodrop or other comparable methods. A lower A260/280 ratio (<1.8) is usually indicative of contamination from TRIZOL extraction. However, A260/280 value may not be reliable if the sample concentration is too low. In that case, a more sensitive method, such as Qubit, may be employed. Typically, the concentration of the 5% input sample is around 100 ng/μL (with a 30 μL yield = 3 μg). The concentration of the BioTASQ-enriched sample varies around 100-200 ng/μL (30 μL yield of 3-6 μg). The concentration of the biotin control sample is much lower at ~50 ng/μL (30 μL yield of 1.5 μg), as the biotin control consists mostly of non-specific rRNA binding to the beads. Regardless of purity or concentration, we highly recommend column purification before further processing (i.e. ribodepletion and library preparation) for RNA-seq.

G4RP-qPCR quantification

RT-qPCR quantification of the G4RP samples typically yields high signal-to-background ratios (>20 fold enrichment) when comparing % input yield from BioTASQ versus the biotin control sample (Fig. 2b). Ligand-induced change ranges from 2-fold to as high as 12-fold, depending on the RNA target and G4 ligand used.

G4RP-seq analysis

After processing the raw sequencing files through a series of bioinformatics tools (Fig. 3a), the differential expression values (output from DESeq2) derived from comparing the BioTASQ sample with its corresponding input control, are used to determine the BioTASQ enrichment score. A quick indicator of a successful G4RP-seq is to rank the transcripts by enrichment score and check for an increase in GC content. At baseline levels (derived from samples not treated with a G4 ligand), the enrichment score is expected to be positively correlated with the GC% of the gene transcript (Fig.3b). Signals of aligned reads can be visualized using IGV (Fig. 3c). The biotin control is expected to show negligible levels with reads primarily aligned to rRNA regions. By comparing the BioTASQ signal with that of the input control, the relative level of BioTASQ interaction with the target can be elucidated (Fig. 3c). An example of a high G4-ligand-inducible target is the long non-coding RNA, MALAT1. The raw signals show much higher alignments in the G4-ligand-treated samples compared to that of the untreated sample.

FIGURE LEGENDS

Figure 1. Overview of G4RP. **a**, Chemical structure of BioTASQ in its open (in absence of a G4 target) and closed conformations (in the presence of a G4 target). **b**, Schematics of the G4RP workflow. 1) Cells are cultured in 10 cm culture dishes. If required, cells may be treated with G4 ligands at this stage to increase global G4 levels. 2) Cells are crosslinked using formaldehyde. 3) Cells are harvested by scraping and sonicated using an ultrasonicator. 4) BioTASQ is added to the sonicate. 5) Streptavidin beads are added. 6) Beads are washed multiple times. 7) Beads are heated to reverse the crosslinks. 8) TRIZOL is added to the sample, and the RNA fraction is purified. 9) Reverse-transcription is performed on the RNA sample before quantification using real-time quantitative PCR.

Figure 2. Expected results from G4RP. **a**, An example of an bleach RNA gel (1.5% agarose w/v, 1% bleach, v/v, 1X TAE; post-staining with SYBR Green Gel Stain) for crosslinked samples that have undergone different durations of sonication (0, 2, 4 min). **b**, (Top) G4RP-qPCR signal of BioTASQ vs biotin control for RNA transcript targets, VEGFA and NRAS. Multiple t-test was performed. (Bottom) G4 ligand (BRACO-19⁴⁵ and RHPS4⁴⁶)-induced changes in the G4RP-qPCR signals for targets, VEGFA, NRAS and MALAT1. One-way ANOVA with Dunnett's multiple correction was performed for each gene to compare each condition against its respective non-treated control. Each point represents a biological replicate. Three biological replicates were used to quantify the means. Error bars represent SEM. These data are derived from a previous published work¹⁶.

Figure 3. Expected results from G4RP-seq. **a**, Schematic of the G4RP-seq bioinformatics analyses. **b**, Linear regression plot comparing the BioTASQ Enrichment Score with GC content of respective gene transcripts at baseline (derived from untreated sample). Each dot represents a gene transcript (n=5083). Analysis shows a significant non-zero relationship with a positive slope ($p < 0.0001$) and a moderate positive correlation ($R = 0.47$). **c**, Signal of aligned sequencing reads in the MALAT1 region for (Left) biotin control, BioTASQ in untreated sample, BioTASQ in BRACO-19-treated sample and BioTASQ in RHPS4-treated sample and (Right) the corresponding input controls. The G4RP-seq datasets used are available under the accession number GSE112898.

DATA AVAILABILITY

All relevant data are available upon request. The G4RP-qPCR dataset (Fig. 2) is publicly available on FigShare (<https://doi.org/10.6084/m9.figshare.16684870>). The raw sequencing data used in the examples shown in Fig. 3 in this protocol are publicly available and accessible at GSE112898.

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AUTHOR CONTRIBUTIONS

S.Y.Y., D.M. and J.M.Y.W designed the experiments. S.Y.Y. and J.M.Y.W. wrote the manuscript with inputs from D.M.

COMPETING INTERESTS

The authors declare no competing interests.

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