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Dear Editor,

Thank you for considering our manuscript describing the kinetic CRAC method for JoVE. Our impression of the comments was generally very positive, and we were pleased with the fair and constructive feedback we received. In response to the comments, we have made a number of changes to the manuscript and included some new experimental data. We hope you agree that these new results have strengthened the manuscript. As many changes were made to the text, we uploaded not only the revised of the manuscript but also a copy with all the tracked changes. We have also uploaded a rebuttal where we provide a detailed response to the points raised by yourself and the reviewers.

We hope that you agree our revised manuscript has significantly improved and that it is now suitable for publication in JoVE.

On behalf of all the authors,

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TITLE:

Monitoring Protein-RNA Interaction Dynamics in vivo at High Temporal Resolution Using χCRAC

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SUMMARY:

Kinetic cross-linking and analysis of cDNA is a method that allows investigation of the dynamics of protein-RNA interactions in living cells at high temporal resolution. Here the protocol is described in detail, including the growth of yeast cells, UV cross-linking, harvesting, protein purification, and next generation sequencing library preparation steps.

ABSTRACT:

The interaction between RNA-binding proteins (RBPs) and their RNA substrates exhibits fluidity and complexity. Within its lifespan, a single RNA can be bound by many different RBPs that will regulate its production, stability, activity, and degradation. As such, much has been done to understand the dynamics that exist between these two types of molecules. A particularly important breakthrough came with the emergence of 'cross-linking and immunoprecipitation' (CLIP). This technique allowed stringent investigation into which RNAs are bound by a particular RBP. In short, the protein of interest is UV cross-linked to its RNA substrates in vivo, purified under highly stringent conditions, and then the RNAs covalently cross-linked to the protein are

converted into cDNA libraries and sequenced. Since its conception, many derivative techniques have been developed in order to make CLIP amenable to particular fields of study. However, one of the main limitations of CLIP-based techniques is their reliance on ultraviolet light for cross-linking, because it is notoriously inefficient. For some uses, this irradiation step can require extended exposure times that make temporal study of RBP-RNA interactions very challenging. To overcome this issue, we recently designed and built a much-improved UV irradiation and cell harvesting device. Using these new tools, we developed a protocol for time-resolved analyses of RBP-RNA interactions in living cells at high temporal resolution: Kinetic CRoss-linking and Analysis of cDNAs (χCRAC). We recently used this technique to study the role of yeast RBPs in nutrient stress adaptation. This manuscript provides a detailed overview of the χCRAC method and presents recent results obtained with the Nrd1 RBP.

INTRODUCTION:

RNAs often rely on RBPs to exert their function, which has led to great interest in understanding the dynamics between these molecules. Many RBPs have been identified in a wide variety of organisms. However, it has always been notoriously difficult to study RBP-RNA interactions in vivo. A major breakthrough in studying such interactions came with the emergence of CLIP[1]. This method utilizes ultraviolet (UV, 254 nm) irradiation to induce covalent bonds between RBPs and their directly bound RNAs (i.e., zero-distance cross-linking). Subsequently, the RBP of interest is immunopurified under stringent conditions to ensure that only RNAs covalently cross-linked to the proteins are identified. Bound RNAs are then partially digested with RNases and subsequently converted into cDNA libraries for sequencing. The high purification stringency is important, because it greatly increases the specificity of protein and RNA recovery, which is also further enhanced through SDS-PAGE purification of the cross-linked ribonucleoprotein (RNP) complex. CLIP and related methods also provide nucleotide resolution insight into the protein binding site, because during the preparation of the sequencing library, amino acids that cross-linked to the RNA frequently terminate the reverse transcriptase or cause the enzyme to introduce mutations at this site[1–3].

Since its introduction, the original CLIP protocol has produced a remarkable variety of derivative methodologies. A particularly important breakthrough came with the development of HITS-CLIP (or CLIP-seq), which merges high-throughput sequencing with the CLIP approach[3]. This has since been adopted by all CLIP-based methodologies. iCLIP introduced improvements in the RNase-mediated trimming and adaptor ligation techniques that facilitate more accurate mapping of the RBP binding sites[4]. PAR-CLIP combined 4thio-uridine/uracil labeling with cross-linking at 365 nm, making it possible to map cross-linking sites by analyzing T-C substitutions[5]. CRAC, urea-iCLIP, dCLIP, and uvCLAP introduced denaturing conditions and dual affinity purification steps that further reduce background binding to the affinity resin and further increase the specificity of protein capture[2,6–9]. Additionally, CRAC, uvCLAP, and dCLIP introduced tagging the RBP of interest with an affinity tag, thus overcoming the need to generate specific antibodies.

Several optimizations have also been made to expedite the CLIP methodology. The original CLIP protocol utilized radiolabeling of the captured RNAs in order to visualize the RBP-RNA complexes after SDS-PAGE. However, the use of radioactivity can be problematic for laboratories not set up

for such work. irCLIP incorporates a fluorophore-coupled adaptor that facilitates visualization through infrared imaging[10] and sCLIP utilizes biotinylation of captured RNAs in order to visualize them through streptavidin-conjugated HRP[11]. Furthermore, eCLIP completely forgoes RNA labeling; instead the protein is excised based solely on its known size[12]. Streptavidin-based purification has also been used to speed up the process of library preparation in FAST-iCLIP, where a biotinylated 3' adaptor is ligated onto the RNAs and used to enable purification after reverse transcription and circularization[13]. Additional enhancements to the iCLIP protocol also greatly increased the complexity of the libraries[4].

Finally, CLIP has been modified to enable capture of RBPs from different cellular subcompartments[14,15], to visualize newly transcribed RNAs using pulsed induction of photoactivable ribonucleosides[5,16,17], to capture methylated RNAs[18–20], to examine RNA-RNA interactions[21,22], and to map 3' ends[23,24].

Despite the great contributions of CLIP-based techniques in aiding our understanding of the interactions between RBPs and RNAs, it has been limited by the inefficiency of UV cross-linking. Although culture cells grown in a monolayer are generally relatively easy to cross-link, this is significantly more challenging in tissues or cells in solution. Tissues can require multiple rounds of UV exposure in order to penetrate to the required cell layers, while microbial cells are often grown in rich mediums that contain aromatic, UV-absorbing compounds[25]. Indeed, cross-linking times of up to 30 min have been used to generate sufficient bonding between RBPs and their bound RNAs for such samples[26–28]. This extended UV exposure induces stress responses within the cell, such as that from UV-induced DNA damage, which can contaminate the final data in some applications.

The majority of CLIP studies have focused on generating single "snapshots" of specific protein-RNA interactions in a cell. However, protein-RNA interactions are inherently dynamic, particularly when cells are subject to changes in their environment. This can include a sudden reduction in the availability of essential nutrients or rapid changes in temperature. As such, to truly understand the role of an RBP during stress, it is best to perform time-resolved analyses because they can capture the full spectrum of RBP targets during stress and determine at what stage of the stress response the chosen RBP is active. In particular, studies in yeast showed that the first few minutes of adaptation are absolutely crucial for survival and RNA half-lives in bacteria can vary from minutes to seconds[29–33]. Therefore, such time-resolved analyses should ideally be performed at high temporal resolution. However, the long cross-linking times make the study of early stage adaptive responses particularly challenging.

In order to overcome these issues, we recently developed an improved method that is capable of cross-linking and harvesting cells on minute-long timescales. Our χCRAC method allows quantitative measurement of dynamic changes in RBP-RNA interactions at previously unwitnessed resolution. Crucial to this method was the development of a novel UV irradiation device[32] that reduces the required cross-linking time in yeast and bacteria in solution around 10x, effectively freezing RBP-RNA interactions instantaneously. In addition, in order to rapidly harvest the cells after UV irradiation, we developed a vacuum filtration device that can harvest

exponentially growing yeast in a 0.5 L culture in ~30 s[32]. These technological innovations allow the study of RBP-RNA dynamics at minute-scale resolution. Additionally, we also introduced several optimizations to the original CRAC protocol[2] in order to increase its practicality.

Using χCRAC, we recently studied the targetome of a yeast nuclear RBP, Nab3, in response to glucose deprivation. In *Saccharomyces cerevisiae*, Nab3 can form a complex with Nrd1, an RBP, and the RNA helicase Sen1 to form the NNS complex. NNS binding to the RNA polymerase and the nascent transcript can trigger transcriptional termination[34]. This complex is mostly involved in removing cryptic noncoding RNA transcripts but has also been shown to regulate expression of protein-coding genes. The study showed differential targeting of Nab3 to noncoding and coding transcripts after only a minute of stress[32]. These results suggest that co-transcriptional termination by Nab3 results in a very transient, pulse-like expression of retrotransposon genes, which would have be difficult to detect using traditional CLIP-based approaches. Additionally, the short UV irradiation times in our UV cross-linker also significantly increased the recovery of short-lived noncoding RNAs[32]. χCRAC will likely be a crucial tool in elucidating not only how RBPs shape the response to stress on immediate timescales but also their changing roles during the whole lifecycle of a response. This manuscript provides a detailed overview of all the steps in the χCRAC protocol. For illustrative purposes, the method was used to study the yeast Nrd1 protein, which is involved in transcriptional termination and RNA decay[35, 36], and its RNA targetome in response to glucose deprivation across a multitude of timepoints. Finally, we also demonstrate that our UV irradiation unit can rapidly cross-link RBPs to RNA in HeLa cells, making it possible to also perform high-resolution time-resolved analyses in adherent cells.

PROTOCOL:

NOTE: The composition of all the buffers used is provided in **Table 1**.

[Place **Table** 1 here]

1. UV cross-linking and lysate production

1.1. Microorganisms in solution

1.1.1. Inoculate 3.5 L of the desired medium with yeast to a starting OD_{600} of 0.05. Grow overnight at 30 °C with continuous shaking at 180 rpm.

1.1.2. During the growth, prepare other necessary materials.

1.1.2.1. Prepare a container of liquid nitrogen.

1.1.2.2. Prepare 3 L of stress-inducing medium and warm to 30 °C in a water bath.

1.1.2.3. Set up the filter apparatus, turn on the cross-linker (**Figure 2A**) and label 50 mL conical tubes, one for each timepoint.

1.1.3. Once the cells reach your desired OD_{600}, pour 500 mL of cells straight into the cross-linker and UV irradiate with 250 mJ of 254 nm UV. See **Figure 2A** and **Figure 3A** for details on using the crosslinker.

NOTE: The UV irradiation energy must be carefully optimized for each protein of interest. See the Discussion for further details.

1.1.4. After cross-linking, filter the cells using one of the vacuum filtration devices (**Figure 2B**,**C**). Roll up the membrane with the filtered cells, place in the t = 0 (time zero) 50 mL conical tube, and flash-freeze in liquid nitrogen.

1.1.5. Filter the remaining cells over six different filters. Resuspend the collected cells in the 3 L of previously warmed stress-inducing medium by dropping the membranes in the medium and mixing vigorously with a stripette for 50 s. After the 50 s, prepare for taking the t = 1 sample.

1.1.6. After 1 min, crosslink 500 mL of cells and harvest through filtration as in steps 1.1.3–1.1.4. Repeat after 2, 4, 8, 14, and 20 min, or different timepoints as needed.

1.1.7. Store the conical tubes containing the cells at -80 °C. Set phosphate buffered saline (PBS) at 4 °C overnight.

1.1.8. The next day, take each conical tube containing a crosslinked sample and resuspend the cells in 25 mL of cold PBS by shaking vigorously.

1.1.9. Transfer the cell suspensions to new conical tubes and spin at 4,600 x *g*, 5 min at 4 °C.

1.1.10. Pour off the PBS, quickly spin again to collect residual PBS and then decant the remaining liquid with a pipette.

1.1.11. Calculate the weight of the pellet in the tube by comparing it to an empty tube.

1.1.12. Add two pellet volumes of ice-cold TN150, 60 µL of DNase 1, and 10 µL of RNase inhibitor. Incubate on ice for 30 min.

1.1.12.1. For example, for 400 mg of cells, add 800 µL of ice-cold TN150.

1.1.12.2. The addition of the DNase is not essential for most soluble proteins but is very important when studying chromatin-bound proteins such as RNA polymerase. Additionally, it reduces the viscosity of bacterial lysates. It is very important to use exactly two pellet volumes of the lysis buffer, or the lysis efficiency can decrease.

1.1.13. Add three pellet volumes (in mL) of zirconia beads to the cell suspension. For yeast, use 0.5 mm diameter beads and for bacteria use 0.1 mm.

1.1.13.1. For example, for 400 mg of cells, measure out 1.2 mL of zirconia beads in a 1.5 mL tube and add them to the cells resuspended in lysis buffer.

1.1.14. Vortex the cell suspensions for 1 min, then place on ice for 1 min. Repeat for a total of 5x.

1.1.15. Add two pellet volumes of TN150 buffer and vortex vigorously to mix.

1.1.16. Centrifuge the suspension in the conical tube at 4,600 g for 20 min at 4 °C in a benchtop centrifuge.

1.1.16.1. After centrifuging, take a 50 µL sample of the supernatant for future Western blot analysis to examine the whole cell protein expression.

1.1.17. Transfer the supernatants into 1.5 mL tubes and spin the lysate for 20 min at 20,000 x *g* at 4 °C, in a microfuge.

1.1.17.1. Alternatively, if using 5 mL tubes, centrifuge at 13,000 x *g* for 20 min.

1.1.17.2. Following centrifugation, take a 50 µL sample of the supernatant for future Western blot analysis to examine the soluble expression of the protein.

1.1.18. Proceed to RBP capture (section 2).

1.2. Cultured adherent cells

1.2.1. Seed enough adherent cells in a Petri dish 24 h prior to UV cross-linking so they that reach 80% confluency the next day. Grow overnight in the desired medium in a cell culture incubator at 37 °C, 5% CO_2.

NOTE: If using quartz Petri dishes, it is beneficial to promote cell adhesion through the treatment of the cultureware with poly-D-lysine (70,000–140,000 wt) and fetal calf serum (FCS) 2.5 h before seeding. Add enough poly-D-lysine to cover the entire growth surface and incubate at room temperature (RT) for 5 min. Next, the quartz Petri dish should be rinsed thoroughly with water and dried in the cell culture incubator for 2 h or until completely dry. Afterwards, add enough FCS to completely cover the growth surface and place in the incubator for at least 30 min. The FCS should be completely removed before seeding cells.

1.2.2. Once the cells have reach 80% confluency, remove the media and wash with 15 mL of ice-cold PBS. Next, completely remove all remaining liquid and proceed immediately to the next step.

1.2.3. Transfer the Petri dish to the tray for adherent cells (**Figure 3B**) and UV irradiate with 300 mJ of 254 nm UV. See **Figure 2A** and **Figure 3B** for details on using the crosslinker.

NOTE: The UV irradiation energy must be carefully optimized for each protein of interest. See the Discussion for further details.

1.2.4. Immediately after cross-linking, place the Petri dish on ice and add 10 mL of ice-cold PBS. Collect cells by scraping and transfer to a 15 mL conical tube. Pellet through centrifugation at 300 x *g* for 5 min at 4 °C.

1.2.5. Remove the PBS and resuspend the cell pellet in 1 mL of ice-cold PBS, and transfer to a 1.5 mL microcentrifuge tube. Pellet cells again by centrifugation for 5 min at 300 x *g* at 4 °C.

1.2.6. Remove the PBS and snap-freeze the cell pellets on dry ice. Store the cell pellets at -80 °C until required.

1.2.7. Repeat steps 1.2.3–1.2.6 for each timepoint.

1.2.8. Resuspend the cell pellets in 1 mL of lysis buffer and transfer to a 15 mL conical tube. Afterwards, add 1 mL of lysis buffer for a total of 2 mL.

1.2.9. Add 5 µL of mammalian RNase inhibitor.

1.2.10. Sonicate 5x for 10 s on ice at 10 amp. Wait 30 s between sonication rounds.

1.2.11. Calculate the protein concentration of each sample and normalize to the lowest concentration.

1.2.12. Transfer 1.98 mL of lysate to a 2 mL tube.

1.2.13. Add 10 µL of DNase I and incubate at 37 °C for 5 min with shaking at 1,200 rpm.

1.2.14. Centrifuge the lysate at 16,000 x *g* for 20 min at 4 °C.

1.2.14.1. After centrifugation, take a 50 µL sample of the supernatant for future Western blot analysis to examine soluble expression of the protein.

1.2.15. Proceed to RBP capture (section 2).

2. RBP capture

2.1. Wash the magnetic anti-FLAG (75 µL of slurry per sample) or IgG agarose (500 µL of slurry per sample) beads 3x with 5 mL of TN150. Resuspend in a final volume of 700 µL of TN150 and add 100 µL of washed beads to seven 15 mL conical tubes.

2.1.1. Store on ice until required.

2.2. Once the lysates have been clarified, add the supernatant to the tube containing the anti-FLAG/IgG beads.

2.3. Nutate at 4 °C for 2 h.

NOTE: Some protocols describe overnight incubations with the beads, but this is not recommended, because long incubation times can dramatically reduce the recovery of cross-linked RNAs.

3. Washing the beads and TEV cleavage of the tags

3.1. Harvest the beads and remove the lysate.

3.1.1. Take a 50 µL of sample of the supernatant for future Western blot analysis to examine the uncaptured protein.

3.2. Resuspend the beads in ice-cold TN1000 and transfer to a 1.5 mL tube. Wash for 10 min, 4 °C, with nutation. Repeat for a total of three washes.

3.2.1. If using IgG agarose beads, wash with 5 mL of TN1000. If using anti-FLAG beads, use 2 mL.

3.3. Next, wash the beads 3x with TN150, with the same volume as above.

3.4. After the third wash, resuspend the beads in 600 µL of TN150.

3.5. Add 30 U of homemade GST-TEV protease to the bead suspension and rotate for 2 h at RT.

NOTE: Recombinant GST-TEV protease is now also commercially available, but it has not been tested with this protocol.

3.5.1. During the digestion, prepare for the next steps by setting up columns of three 1.5 mL tubes for each sample (i.e. for seven samples, have three rows of seven columns).

3.5.2. To the final row of tubes, add 0.4 g of guanidium hydrochloride, 27 µL of 5M sodium chloride and 3 µL of 2.5 M imidazole (pH = 8). Note that the pH of the imidazole must be 8. This is critical to maintain RNA integrity.

3.5.3. Additionally, wash the required volume of nickel beads in WB I 3x. Use 100 µL of slurry per sample. After the final wash, resuspend the beads in the same original volume of WB I and store on ice.

3.6. Once the TEV digestion is complete, collect the supernatant using a magnetic rack for anti-FLAG beads or centrifugation for IgG beads, and transfer to the first row of the tubes previously set up.

3.6.1. Take a 50 µL of sample of the TEV eluate for Western blot analysis.

3.7. Set a thermoblock incubator to 37 °C. To the second row of tubes, add 1 µL of RNase cocktail (1:50 dilution).

3.8. Take 550 µL of TEV eluate from the first row of tubes and add to the second row (containing the RNase cocktail). Pipette vigorously to ensure mixing.

3.9. After completing this for the first sample, immediately place the tube into the thermoblock and start a timer. Move on to the subsequent samples, such that each is staggered.

3.10. Incubate for exactly 5 min. Once completed, remove the first sample from the thermoblock and transfer the solution to the third row of tubes (containing the guanidium hydrochloride powder).

NOTE: A 5 min incubation with a 1:50 dilution of the RNase cocktail is usually suitable for most proteins, but this step will need to be carefully optimized with different incubation times or concentrations for each protein to make sure that the cross-linked RNAs are of the correct size (30–100 nt).

3.11. Immediately vortex for a couple of seconds at full speed to dissolve the guanidium powder and then move on to the next sample.

3.12. After all samples have been transferred into the guanidium powder, vortex again to ensure all the powder is fully dissolved.

3.13. Add 100 µL of washed nickel beads and rotate overnight at 4 °C. This incubation can be shortened to 2 h.

4. On-bead alkaline phosphatase treatment

4.1. Set a thermoblock to 37 °C.

4.2. Place a purification spin column in a 2 mL tube, one for each sample. Transfer the nickel beads to the columns and allow the supernatant to drain through. Afterwards, ensure that all the nickel beads were removed from the 1.5 mL tube by rinsing with WB I and applying to the column.

4.3. Set up 2 mL tubes, six per sample (one to collect each wash). Keep the outside of the columns dry to maintain flow. Wash the beads 3x with 500 µL of WB I and then 3x with 500 µL of NP-PNK.

4.4. Close the lid of the spin column and briefly spin beads to remove excess buffer.

4.5. Put the stopper on the column, put columns into 1.5 mL tubes and add 60 µL of the reaction mixture seen in **Table 2**.

[Place **Table 2** here]

4.6. Incubate the beads for 1 h at 37 °C.

4.7. Wash the beads 1x with 500 µL of WB I to inactivate the alkaline phosphatase and then 3x with 500 µL of NP-PNK buffer. Make sure to thoroughly rinse the inside of the column with the NP-PNK buffer to remove any traces of guanidium.

5. On-bead ligation of the App-PE linker to the 3' end of the RNA

5.1. Spin out the remaining buffer and add 60 µL of the mixture specified in **Table 3** (see **Table 4** for the App-PE sequence) to the columns. Incubate the reaction for 6 h at 25 °C.

[Place **Table** 3 here]

[Place **Table** 4 here]

5.2. Wash beads 1x with 500 µL of WB I and 3x with 500 µL of NP-PNK buffer. Put the column in a new tube and spin out the remaining buffer.

6. On-bead phosphorylation of the 5’ ends of the RNA

6.1. Add 80 µL of the mixture specified in **Table 5** to the columns. Incubate the reaction for 40 min at 37 °C.

[Place **Table 5** here]

NOTE: The samples will now be highly radioactive. Thus, all subsequent work should be performed behind a protective screen and waste should be disposed of according to local health and safety rules.

6.2. Add 1 µL of 100 mM ATP and let the reaction proceed for another 20 min. This will make sure that almost all of the 5’ ends have phosphates to facilitate ligation of the 5’ linker.

6.3. Set up 2 mL tubes, five per sample.

6.4. Wash beads 1x with 500 µL of WB I and 3x with 500 µL of NP-PNK buffer. Note that these elutions will be very radioactive and therefore should be disposed of appropriately.

6.5. Move the column to the final tube and spin out the remaining buffer.

7. On-bead ligation of the 5' linker

NOTE: The 5' linkers contain an RNA barcode that is used for identification of each sample after sequencing. Thus, it is absolutely crucial to note which linker is used for which sample.

7.1. Add 78 µL of the mixture described in **Table 6** to the columns. Add 2 µL of 5' adapter (100 µM; see **Table 4**) to each tube and incubate overnight at 18 °C.

[Place **Table** 6 here]

7.2. The following day, wash beads 1x with 500 µL of WB I and 3x with 500 µL of WB II and transfer the columns to a new 2 mL tube.

8. Elution, SDS-PAGE, and RNA extraction

8.1. Set the centrifuge to 4 °C. Prepare two rows of 1.5 mL tubes per sample for elution.

8.2. Spin out the void volume of the columns with nickel beads with a quick spin. Place the columns in the first row of elution tubes and add 200 µL of elution buffer. Wait 2 min, then force the buffer through the column with a quick spin.

8.3. Move the columns to the second row of tubes and repeat step 4. Each sample will now have 400 µL of eluate in total, split over two 1.5 mL tubes.

8.3.1. Take all the eluates and transfer them together to a 5 mL tube. Add 2 µL of 20 mg/mL of glycogen. Thus, if using seven samples, there will now be 2.8 mL of pooled eluate in the 5 mL tube.

8.4. Add 100 µL of trichloroacetic acid (TCA) per sample [e.g, 700 µL of TCA for 7 samples (2.8 mL of pooled eluate)] to the 5 mL tube, and vortex well for 30 s.

8.5. Incubate on ice for 20 min.

8.6. Centrifuge for 30 min at 17,000 x *g*, 4 °C, in a benchtop centrifuge.

8.7. Carefully remove the supernatant from the conical tube, checking the pipette with a Geiger counter to ensure the pellet has not been removed accidentally. If it has, return the supernatant to the tube and centrifuge for another 10 min.

NOTE: The supernatant might still be highly radioactive. Make sure to use proper shielding.

8.8. Fully resuspend the pellet in 2 mL of ice-cold acetone.

8.9. Centrifuge for 15 min at 17,000 x *g*, 4 °C.

8.10. Remove as much of the acetone with a P1000 pipette as is possible. Afterwards, briefly spin the tube to gather small droplets of acetone, and then remove with a P10 pipette. Dry for 2 min in a fume hood.

NOTE: The acetone supernatant can still be radioactive. Make sure to use proper shielding.

8.11. Resuspend the sample in 30 µL of 1x protein loading buffer. To ensure that the pellet is properly resuspended, check that the vast majority of the radioactivity is now present in the loading buffer and not left in the 1.5 mL tube by removing the solution in a P200 pipette and measuring the activity left in the 1.5 mL tube using a Geiger counter.

8.12. Heat the sample for 10 min at 65 °C. Load on a 1 mm, 4–12% precast Bis-Tris gel and run for 1.5 h at 125 V in MOPS buffer.

8.13. After the gel has finished running, open the gel cassette. The gel should be retained on the bottom plate. Dispose of the top.

8.14. Wrap the gel in cling film and then secure it using tape to the inside of a light-tight cassette. Ensure that the cassette has an amplifying screen to improve the signal.

8.15. Expose an autoradiographic film to the gel and store the cassette at -80 °C during the exposure. The exposure time will vary between proteins with different cross-linking efficiencies.

8.15.1. When placing the film, there must be a way of realigning it to the cassette in order to cut out the band of interest in the subsequent step. To ensure this, use a fluorescent ruler and also ensure that the gel is at a corner of the cassette, which is then covered by the film also placed in the utmost corner.

NOTE: As a rule of thumb, eluates in loading buffer that give a reading of at least ~250 cps when displayed to a Geiger counter give sufficient signal for an exposure of 3 h. Otherwise, overnight exposure is performed.

8.16. Develop the film. Cut away the cling film covering the gel but do not move the gel. Otherwise, the image will be offset from the gel.

NOTE: The gel will likely be highly radioactive. Make sure to use proper shielding when cutting out the gel slice.

8.17. Place the film over the gel and excise the band of interest. Put the gel slice into a 2 mL tube.

8.18. Smash the gel slice using a P1000 pipette tip and add 600 µL of proteinase K buffer plus 200 µg of proteinase K (this protocol uses 10 µL of a 20 mg/mL proteinase K solution). Incubate for 2 h at 55 °C with vigorous shaking.

8.19. Afterwards, cut the end of a P1000 tip off with a clean scalpel and transfer the supernatant and gel pieces to a spin column placed in a 2 mL tube.

8.20. Spin the column for 1 min at 17,000 x *g* at RT. Collect the flow-through, which contains the radioactive, isolated RNAs.

8.21. Perform a phenol:chloroform extraction.

8.21.1. Add 50 µL of 3 M sodium acetate (pH = 5.2) and 500 µL of phenol:chloroform and vortex well. Spin for 5 min at 17,000 x *g*. Remove the aqueous top layer and place in a new 1.5 mL tube.

8.21.2. Add 500 µL of chloroform and vortex vigorously for 10–15 s. Spin for 5 min at 17,000 x *g* at RT. Remove the aqueous layer and place in a new 1.5 mL tube.

8.21.3. Add 1 µL of 20 mg/mL glycogen and 1 mL of ice-cold, 96% ethanol. Precipitate for 30 min at -80 °C or overnight at -20 °C.

8.21.4. Centrifuge for 30 min at 4 °C, 17,000 x *g*. Remove the supernatant, add 500 µL of 70% ethanol and centrifuge for 5 min, 4 °C at 17,000 x *g*. Remove all the ethanol, perform a quick spin to gather residue and remove excess with a P10 pipette.

8.21.5. Dry the pellet for ~3 min in a fume hood. Resuspend in 20 µL of DEPC-treated water.

8.22. Store the RNA at -80 °C overnight or proceed immediately to the reverse transcription step.

9. Reverse transcription

9.1. Add 2 µL of 10 µM RT oligo (PE_reverse; see **Table** *7*) and 4 µL of 5 mM dNTPs to the 20 µL of RNA.

[Place **Table** 7 here]

9.2. Transfer to a preheated thermoblock at 85 °C for 3 min, then snap-chill on ice for 5 min. Collect the contents of the tube by brief centrifugation and then add 8 µL of 5x reverse transcriptase buffer, 2 µL of 100 mM DTT, and 2 µL of RNase inhibitor.

9.3. Incubate the mixture at 50 °C for 3 min and then add 2 µL of reverse transcriptase and incubate for 1 h at 50 °C.

9.4. Inactivate the reverse transcriptase by incubation at 65 °C for 15 min.

9.5. Transfer the tubes to a preheated thermoblock at 37 °C and leave for 3 min to acclimatize.

9.6. Add 2 µL of RNase H and incubate for 30 min at 37 °C.

9.7. Isolate the cDNA using SPRI beads.

9.7.1. Add two volumes of 84 µL of beads. Incubate for 15 min. Put the beads on a magnetic rack and leave for 1 min to harvest the beads.

9.7.2. Remove and dispose of the supernatant and add 200 µL of 70% ethanol. Do not remove the beads from the magnetic rack. Incubate the beads with the ethanol for 30 s.

9.7.3. Remove the ethanol and repeat the wash step. Remove all the residual ethanol using a P10 tip.

9.7.4. Put the beads in a fume hood for 2 min to dry them. Remove the beads from the rack, resuspend them in 12 µL of water, and then put the beads back on the rack. Remove 11 µL of supernatant.

9.8. Freeze the cDNA at -20 °C or proceed immediately to the PCR step.

10. qPCR reaction

10.1. Prior to the final PCR for amplification of the cDNAs, a quantitative polymerase chain reaction (qPCR) is performed to identify the optimal number of cycles for amplifying the cDNAs to prevent overamplification of the library.

10.2. Set up a qPCR reaction on ice according to **Table 8**. See **Table 7** for all primers.

[Place **Table** 8 here]

10.3. For proper quantification of the cycles needed for amplification, use three technical replicates for the cDNA and three negative (i.e., water) controls.

10.4. Seal the plates with optically transparent film and run the qPCR according to the kit manufacturer's instructions.

10.5. Analyze the samples through an absolute quantification method to identify the number of cycles at which the knee of exponential growth is reached (see **Figure 4C** for an example). This number of cycles is then used for the final amplification of the rest of the cDNA.

11. PCR reaction and gel extraction

11.1. Set up the PCR reaction on ice according to **Table 9**. See **Table 7** for all primers.

[Place **Table** 9 here]

NOTE: Only 5 µL of the cDNA library is used.

11.2. Run PCR as follows: 95 °C for 2 min; n cycles of 98 °C for 20 s, 52 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. The number (n) of cycles for amplifying the χCRAC library is determined by the qPCR described in section 10.

11.3. Add 1 µL of exonuclease I and incubate at 37 °C for 60 min.

11.4. Clean up the amplified cDNA using SPRI beads as described above using two volumes of beads (i.e., 100 µL). Elute in 11 µL.

11.5. Add 3 µL of 6x loading dye and run on a precast 6% TBE gel at 100 V for 1 h in 1x TBE buffer. Use a ladder appropriate for quantification of short DNA fragments.

11.6. Once finished, remove the gel from the cassette and place in a suitable, liquid-tight container with enough 1x TBE to cover the gel (e.g., ~50 mL). Add an appropriate amount of SYBR safe dye (e.g., for 50 mL, use 5 µL of a 10,000x dye)

11.7. Allow the gel to stain through gentle mixing for 15 min at RT. Drain the SYBR-containing 1x TBE and replace with clean 1x TBE. Wash the gel for 10 min with gentle shaking at RT.

11.8. Drain the 1x TBE and place the gel in a transparent folder. Cut the folder to an appropriate size.

11.9. Image the gel through an appropriate means such as a phosphorimager. Excise DNA fragments between ~175 bp and ~400 bp. Put the gel slice in a 1.5 mL tube.

11.10. Thoroughly smash the gel slice using a P1000 tip and add 400 µL of H_2O. Incubate at 37 °C with shaking for 1 h in a thermoblock.

11.11. Freeze the sample on dry ice for 10 min, then place back in the thermoblock at 37 °C with shaking for 1 h.

11.12. Create a filter unit by taking a filter column and inserting two glass microfiber filters inside. Place the unit in a 1.5 mL tube.

11.13. Cut off the end of a P1000 tip with a clean scalpel and uptake the smashed TBE gel suspension, then dispense into the filter unit created in step 11.12. Spin at 17,000 x *g* for 30 s.

11.14. Add 1 µL of glycogen to the supernatant, along with 40 µL of sodium acetate (pH = 5.2) and 1 mL of 96% ethanol. Incubate at -80 °C for 30 min.

11.15. Centrifuge for 30 min at 17,000 x *g*, 4 °C. Discard the supernatant and wash with 500 µL of 70% ethanol.

11.16. Spin for 5 min, remove the ethanol entirely and then dry the pellet in a fume hood for 3 min.

11.17. Resuspend in 10 µL of H_2O and measure the DNA concentration.

REPRESENTATIVE RESULTS:

To demonstrate the efficacy of the χCRAC method, a time-course experiment with yeast strains expressing an HTP-tagged Nrd1 protein was performed. A detailed schematic representation describing how the method works is provided in **Figure 1**. Like Nab3, Nrd1 is involved in nuclear RNA decay of a variety of RNA transcripts[37]. Previous work from the Corden lab suggested that Nrd1 binding to its RNA targets changes significantly when cells are subjected to glucose starvation[28,38]. In this case, cells growing exponentially in medium containing glucose (SD-TRP) were shifted to the same medium without glucose (S-TRP) over a time-course to monitor dynamic changes in Nrd1-RNA interactions. Samples were taken and cross-linked in the Vari-X-linker chamber (**Figure 3A**) before the shift and then after 1, 2, 4, 8, 14, and 20 min. The medium used for cell growth was deliberately deficient in tryptophan to reduce UV absorption by this aromatic amino acid. Note that it is best to use synthetic medium that is filter sterilized, because autoclaving the medium can lead to caramelization of the sugars, which can reduce the cross-linking efficiency.

Figure 4A shows a representative autoradiograph from a χCRAC experiment. Note that in this example, the samples were not pooled together. Instead, each was run individually on the gel. This is recommended for initial experimental tests to show that the protein cross-links effectively to RNA at all of the tested timepoints. A particularly intense signal was observed at the expected molecular weight of the RBP, representing the protein bound to very short, radiolabeled RNAs not amenable for sequencing. Therefore, the smeary signal above this band, which is the protein crosslinked to longer RNA fragments, was isolated. The fragment was cut from just above the protein band plus around 30 kDa. **Figure 4B** shows an autoradiogram following excision, with the protein cross-linked to short RNAs left in the gel and the previously smeary signal now excised.

After reverse transcription, the cDNA library must be amplified using PCR. However, it is not ideal to overamplify the library, because this can introduce bias towards sequences preferentially amplified by the polymerase as well as the appearance of long PCR products that are the result of amplification artifacts. Overamplified libraries also contain a large number of duplicate sequences that waste reads on the sequencer. qPCR was used to calculate the ideal number of PCR cycles for amplification of the final library: An aliquot of the cDNA library was analyzed through qPCR using the P5 and BC oligonucleotides, and the first cycle at which the library reached peak fluorescence was used as the PCR cycle count. **Figure 4C** gives an example of a qPCR from a typical cDNA library, which yielded a peak cycle count of 16. This value was then used for the final χCRAC PCR. In order to process the sequenced data, we used software previously developed in our lab (pyCRAC) and the corresponding pipeline for analysis of the kinetic CRAC

data (Nues et al., 2017; https://git.ecdf.ed.ac.uk/sgrannem/pycrac, https://bitbucket.org/sgrann/kinetic_crac_pipeline/src/default/). These open source software tools enable demultiplexing and trimming of the data, removal of PCR duplicates, identification of statistically significant peaks, cluster reads into contiguous sequences, and identify binding motifs[39]. Further details of how these tools operate are found on their respective webpages.

We also started to develop a χCRAC protocol for mammalian cells. The majority of mammalian cell lines are grown as a monolayer and the tray in our crosslinker with the UV-permeable bag is not suitable for experiments with adherent cells. To overcome this problem, we developed a stage where users can UV irradiate 1–2 Petri dishes (150 mm diameter and 25 mm in depth) with adherent cells (**Figure 3B**). As a first test, the efficiency of the cross-linker for mammalian cells was measured through cross-linking and capture of stably tagged GFP-RBM7 using anti-GFP antibodies and a traditional CLIP-based purification. As shown in **Figure 5A**, the cross-linker was able to recover protein-RNA complexes from mammalian cells grown as a monolayer using 254 nm UV irradiation at efficiencies comparable to a widely used UV irradiation device. However, standard cell culture plasticware normally used for UV cross-linking experiments is impenetrable to 254 nm UV. Therefore, in our cross-linker the cells would only receive irradiation from the upper bank of UV lamps. To overcome this, we developed a UV-permeable quartz Petri dish for cell growth and cross-linking. Use of the quartz cultureware displayed robust recovery of protein-RNA complexes with as few as 2 s of UV irradiation (**Figure 5B**). When combined with RBP capture methods for mammalian cells such as CLIP technologies, these short cross-linking times are amenable with timecourses to recover spatiotemporal RNA-binding profiles of RBPs in response to genotoxic stresses or rapid depletions of protein factors, or in parallel with transcriptional or cell cycle synchronization.

Figure 6 shows several examples of the Nrd1 data processed by the χCRAC pipeline. This figure was prepared using the bedgraph files generated by the pipeline and the python GenomeBrowser package (https://pypi.org/project/GenomeBrowser/1.6.3/), which we designed to simplify making publication-quality genome browser images of the data. The grey rectangles represent genomic regions that expressed noncoding RNAs, such as the cryptic unstable transcript (CUTs), stable uncharacterized transcripts (SUTs)[40], and Xrn1-sensitive unstable transcripts (XUTs)[41]. The data in **Figure 6** show that Nrd1 binds to many of these noncoding RNA transcripts, consistent with the idea that this protein is involved in degradation of this class of transcripts[42]. **Figure 6A** shows a ~15 kb region on chromosome IV. Here there was a significant increase in binding of Nrd1 to transcripts encoding the high-affinity glucose transporters HXT6 and HXT7, both of which are upregulated during glucose starvation. It is likely that transcription termination by the NNS complex may influence the induction kinetics of these genes during glucose starvation. **Figure 6B** shows an example of Nrd1 cross-linking to the Imd3 transcript, which is known to be regulated by Nab3[43]. In this case the data demonstrated a significant reduction in binding upon glucose starvation. Previous work shows decreased binding of Nab3 to the Tye7 transcript during glucose starvation[44]. Consistent with this observation, the Nrd1 χCRAC data with Nrd1 suggest that binding of NNS factors indeed decreased during glucose starvation and Nrd1 cross-linking to Tye7 was at its lowest after 8 min of stress (**Figure 4C**). However, it appears that this effect was only transient, because after 14 min of glucose starvation, Nrd1 binding went back to starting levels.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the χCRAC protocol. Tagged strains were grown until the desired density. RBP indicates RNA-binding protein. Afterwards, a reference sample was taken and cross-linked with 254 nm UV light. The remaining cells were harvested by filtration and then rapidly shifted to the stress-inducing medium. For the χCRAC experiment described here, samples were taken and cross-linked 1, 2, 4, 8, 14, and 20 min after the shift (1). The RBP of interest was then purified using a highly stringent two-step affinity purification (2). Next, the captured cross-linked RNAs were partially digested with RNases, radiolabeled at the 5' end and adapters were ligated onto them (3). The 5' adapters contained unique "in-read" barcode sequences so that the individual samples could be separated bioinformatically after sequencing. The RBP-RNA complexes were then eluted, pooled, and precipitated together (4), resolved by SDS-PAGE and visualized through autoradiography (5). Subsequently, a single gel slice containing the radioactive signal just above the main band, illustrated with dashed red box in the autoradiography image, was cut from the gel (5). The gel slices were treated with protease K and the RNA was subsequently extracted (6), converted into cDNAs, and amplified through PCR (7). The PCR step introduced additional barcodes (yellow block introduced by P7 oligo) so that many libraries could be multiplexed into a single lane.

Figure 2: Cross-linking and vacuum filtration. (**A**) The cross-linker. The cell suspension is poured into a funnel located in the top right of the machine (also see **Figure 3A** for a close-up) and held in a UV-transparent bag located in the middle tray. This bag is flanked by two shutters that remain closed until the user instructs the machine to start the irradiation step. The cells are irradiated with UV light from the trays both above and below. The machine comes supplied with 254 and 365 nm UV lamps, with the latter being applicable for PAR-CLIP experiments. The machine is operated through a touchscreen panel located on the top right which allows one to control UV dosage or exposure time. (**B**) Following cross-linking, the cells are drained from the left-hand side of the machine. Cell suspensions are recovered through vacuum and drained into a glass flask where they can be subsequently poured into a vacuum filtration device for harvesting. (**C**) Vacuum filtration devices. These are opened and closed via a clip and a filter is inserted between it. Four filtration devices were used in parallel for very short time series to not lose any time as a result of changing filters. (**D**) Following filtration, the media supernatant was drained into flasks for subsequent disposal. Valves were installed below the vacuum filtration devices to maintain the vacuum in the system when the filter is removed.

Figure 3: Cross-linking suspended vs. adherent cells. (**A**) The cross-linker with the Vari-X-linker chamber for suspension cells. The cell culture is poured into the sample inlet (funnel) located in the top right of the tray. (**B**) Tray that can hold plastic or quartz Petri dishes for cross-linking adherent cells or small volumes of suspension cells.

Figure 4: Library preparation. (**A**) Example of an autoradiogram from a Nrd1-HTP χCRAC experiment. The strong, concentrated signal represents the protein crosslinked to very short RNAs, while the smear above represents the protein cross-linked to RNAs of sufficient length for sequencing. (**B**) The smear was excised as shown in an autoradiogram taken after gel excision.

(**C**) A representative qPCR from a χCRAC cDNA library. In this example, maximum amplification of the cDNA was reached at 16 cycles. Thus, 16 cycles were used for the final amplification. The error bar represents the standard deviation of three technical qPCR replicates. (**D**) Example of a phosphorimage from a cDNA library on a 6% TBE gel. (**E**) cDNA length and quality analysis from a chip-based capillary electrophoresis.

Figure 5: High RNase test iCLIP experiment to test crosslinking in mammalian cells. Shown are autoradiograms from GFP-RBM7 iCLIP experiments which tested the efficiency of RNP recovery across various cross-linking energies. Immunoprecipitations were performed using anti-GFP antibodies coupled to magnetic beads on cross-linked cells which stably expressed GFP-RBM7. Immunoprecipitates were incubated with high concentrations of RNase I in order to trim associated RNAs to short, uniform lengths. RNPs were visualized by ^{32}P labelling and SDS-PAGE and migrate as a defined band, close to the migration of the non-cross-linked protein. Quantification indicates the results of densitometric analyses of radiolabeled RBM7-RNA signal normalized to the anti-GFP western blot signal. (**A**) Cross-linking time-course of the commonly used UVP cross-linker versus our cross-linker (Vari-X-linker; VxL). (**B**) Cross-linking time-course of our cross-linker on quartz (left) and plastic (right) cultureware.

Figure 6: **Example genome browser plots showing the power of χCRAC to show differential, temporal binding of Nrd1 to its targets.** Each box shows plots for individual genomic regions. The arrows indicate on which strand the genes are encoded (left pointing arrow = minus strand; right pointing arrow = plus strand). The timepoints (min) are indicated by t0, t1, t2, etc on the y-axes of each subplot. Roman numerals indicating the chromosomes and the coordinates are shown. (**A**) Upon glucose deprivation, Nrd1 binds two high-affinity glucose transporters, HXT6 and HXT7, which are both upregulated in this condition. (**B**) Nrd1 is observed to bind to Imd3, an already validated target of Nab3 [44], with reducing intensity following glucose starvation. (**C**) Nrd1 binding of Tye7 exhibits a dynamic and transient nature, decreasing after glucose starvation to a minimum after 8 min of stress. However, binding subsequently returns to basal levels after 14 min. Reads were normalized to “reads per million” (RPM; y-axis). Grey boxes indicate regions encoding noncoding RNAs.

Table 1: The buffers required for χCRAC and their compositions.

Table 2: Alkaline phosphatase reaction mixture.

Table 3: App-PE linker ligation reaction mixture.

Table 4: The sequences of the DNA and RNA adaptors required for ligation onto the 5’ and 3’ ends of captured RNAs. These were purified through RNase-free HPLC.

Table 5: Phosphorylation reaction mixture.

Table 6: 5’ linker ligation reaction mixture.

Table 7: The PCR primers (including the barcode sequences) and the reverse transcription primer.

Table 8: qPCR reaction mixture.

Table 9: PCR reaction mixture.

DISCUSSION:

The χCRAC method, combined with the new cross-linking and cell harvesting devices, has great potential because it is applicable to a wide range of model organisms and therefore should be of general interest to the RNA field. There are many areas in which χCRAC can be utilized. For example, the method could be used to measure the hierarchical assembly of proteins into large macromolecular complexes, such as the spliceosome and the ribosome, which often involves dynamic interactions between proteins and RNA molecules. We also now routinely use it to monitor interactions between RNA decay factors and their substrates when cells are subjected to diverse kind of stresses. This enables us to determine at what stage of the adaptive response these factors are most active, what substrates they bind to, and how dynamic these interactions are. Such data should enable researchers to determine the relative contribution of each factor in adaptation to environmental changes.

χCRAC uses dual affinity purification tags (HTF or HTP) to purify the protein under highly stringent and denaturing conditions. This ensures that the copurified RNA is highly enriched for RNAs that were covalently cross-linked to the protein of interest. However, relying on affinity tags has disadvantages. For example, the tag could interfere with protein function, which could give a distorted readout of its RNA-binding interactome. Additionally, for some model organisms it may not always be possible to utilize tags because the genetic tools to integrate DNA fragments into the genome or to transform expression plasmids are not yet available. However, it is straightforward to alter some parts of the χCRAC protocol to make it compatible with CLIP-based protocols that rely on antibodies for purification of the RBP. Indeed, this study showed that it is possible to combine iCLIP-based purifications with our crosslinker. We are now in the process of developing CLIP protocols to study the temporal association of human RNA-binding proteins with nascent RNA transcripts.

When performing χCRAC on a new protein, the UV exposure must be optimized in order to induce maximal cross-linking. This is important, because high UV exposures can reduce the recovery of the bait protein during the purification step. Cells expressing the recombinant RBP were exposed to various UV doses, 100 mJ/cm^2, 250 mJ/cm^2, 500 mJ/cm^2, and 1 J/cm^2. The RNPs were then captured and the RNAs were fragmented and radiolabeled. Afterwards, the RNPs were resolved by SDS-PAGE and an autoradiogram was taken in order to deduce which exposure gave the most intense signal (i.e. the maximal cross-linking).

Once the experimental conditions are optimized, several control experiments are recommended when performing χCRAC. First, a UV irradiated, untagged sample can be used to monitor

background binding to the purification beads. Second, when applying χCRAC during a shift experiment, a second time series where the cells are shifted back into the original medium enables investigation into whether the filtration of the cells itself induces changes in RNA levels or protein-RNA interactions.

As mentioned in the Introduction, numerous recently published papers suggest a number of optimizations to the CLIP protocol. This includes the use of fluorescently labeled adapters for detecting the protein-RNA complex through infrared scanning[10] as well as optimizations to various nucleic acid purification and size selection steps shown to increase the complexity of the resulting libraries[12,45]. We are currently implementing some of these improvements to further refine the χCRAC protocol. The protocol presented here already contains a number of improvements to the original CRAC and χCRAC protocols that increase the complexity of the data. For example, previously, after resolving the cross-linked, radioactive protein-RNA complexes on SDS-PAGE gels, they were transferred to a nitrocellulose membrane and the cross-linked RNA was isolated from the blot. However, the transfer of the RNP and subsequent RNA extraction can be very inefficient, particularly when dealing with large RBPs such as RNA polymerase subunits. This can result in a significant reduction in the recovery of the cross-linked RNA. In the current protocol, the cross-linked RNA is extracted directly from SDS-PAGE gel slices as illustrated in **Figure 1**. This increased the recovery of cross-linked RNAs. Additionally, after PCR amplification of the cDNAs the product was originally resolved on 3%, low melting temperature agarose gels, and then 175–300 bp PCR products were extracted from the gel. However, these gels can be easily overloaded, resulting in very poor separation of the DNA. Replacing agarose gels with precast TBE gels resulted in more consistent size separation and better recovery of PCR products.

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A. Langford and W. Worboys are affiliated with UVO3, a commercial company. They had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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45. Buchbender, A. et al. Improved library preparation with the new iCLIP2 protocol. *Methods*. In Press (2019).

Figure 1

Click here to access/download;Figure;Figure_1.ai

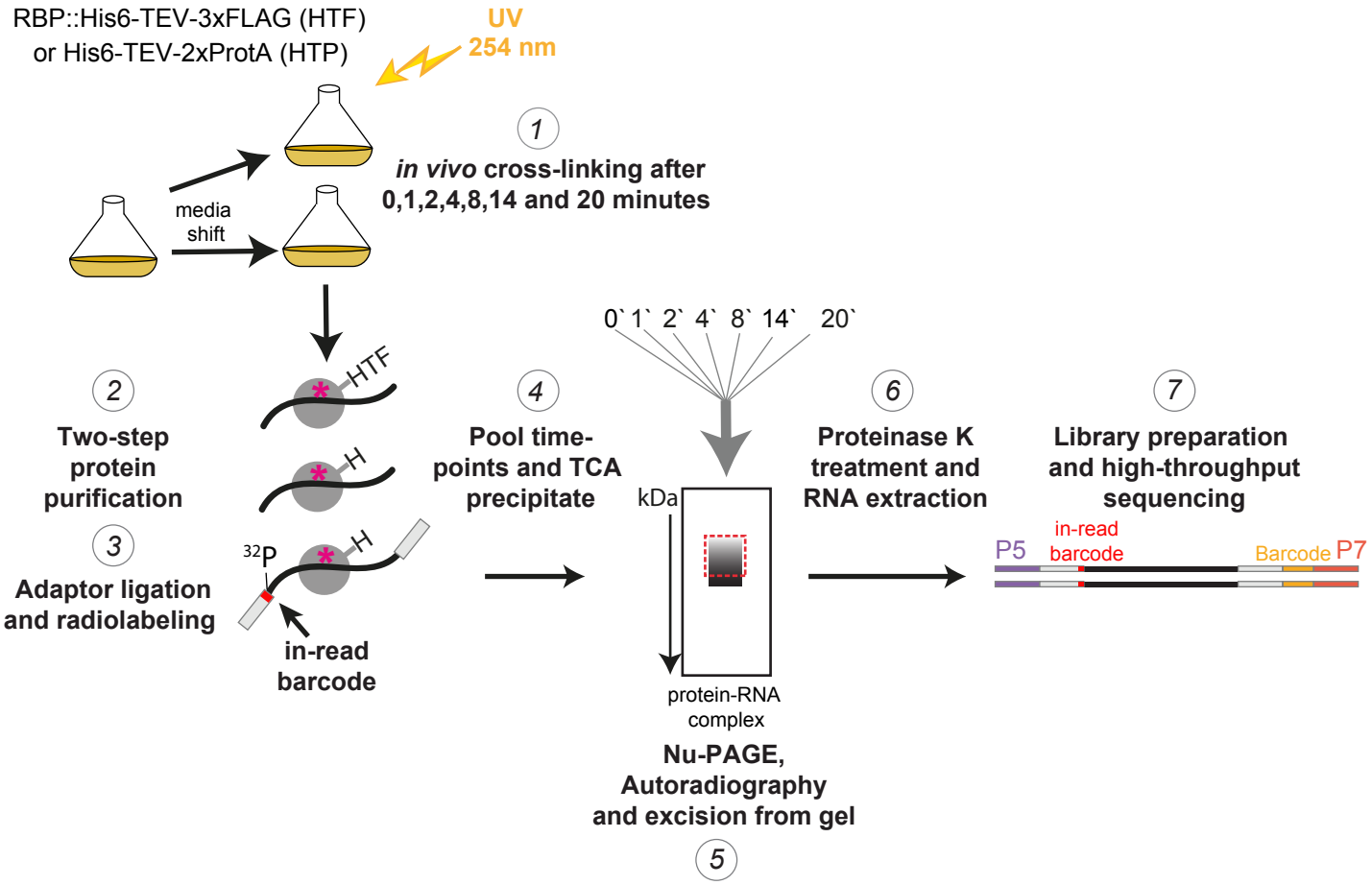


Figure 2

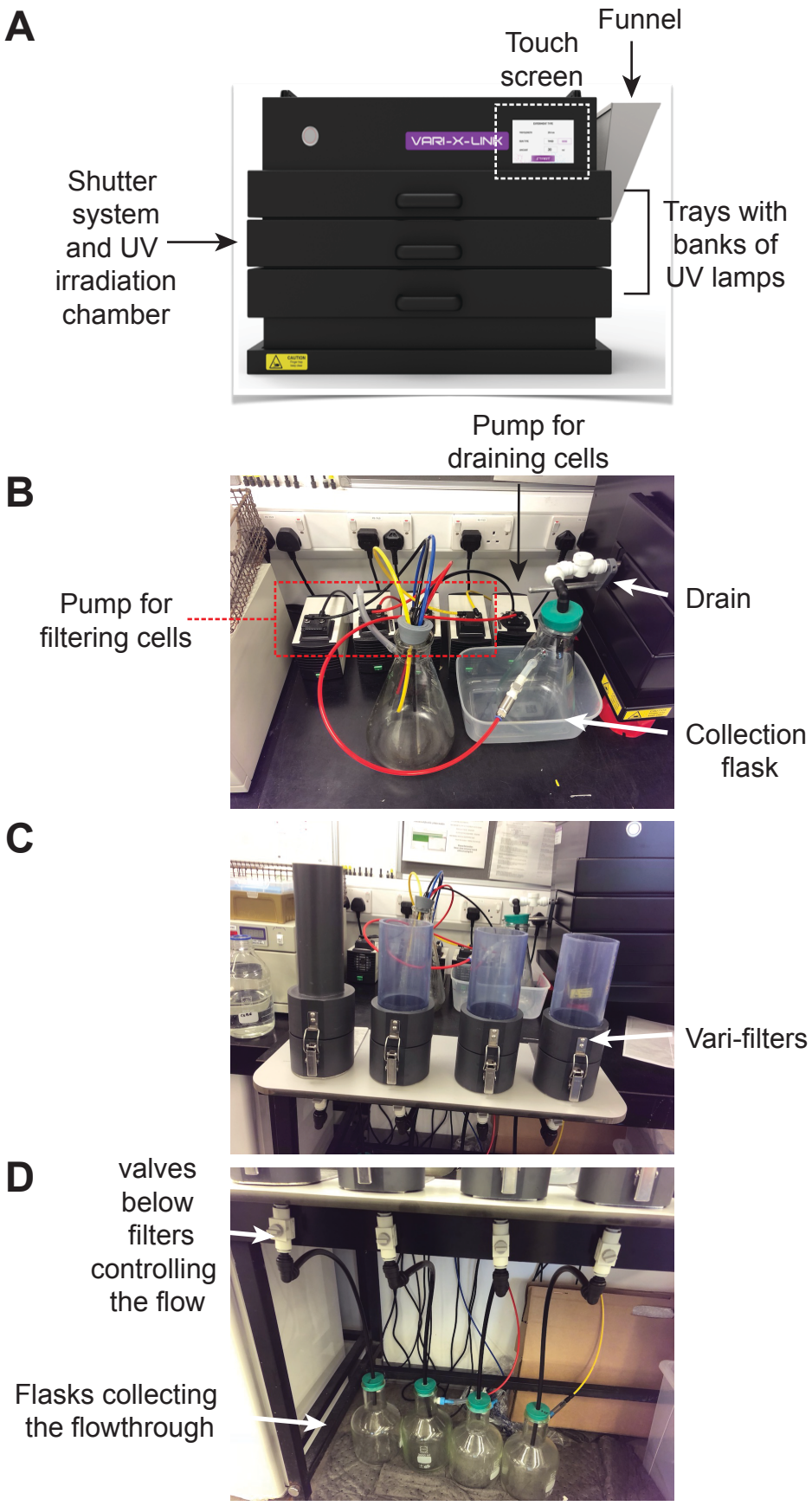
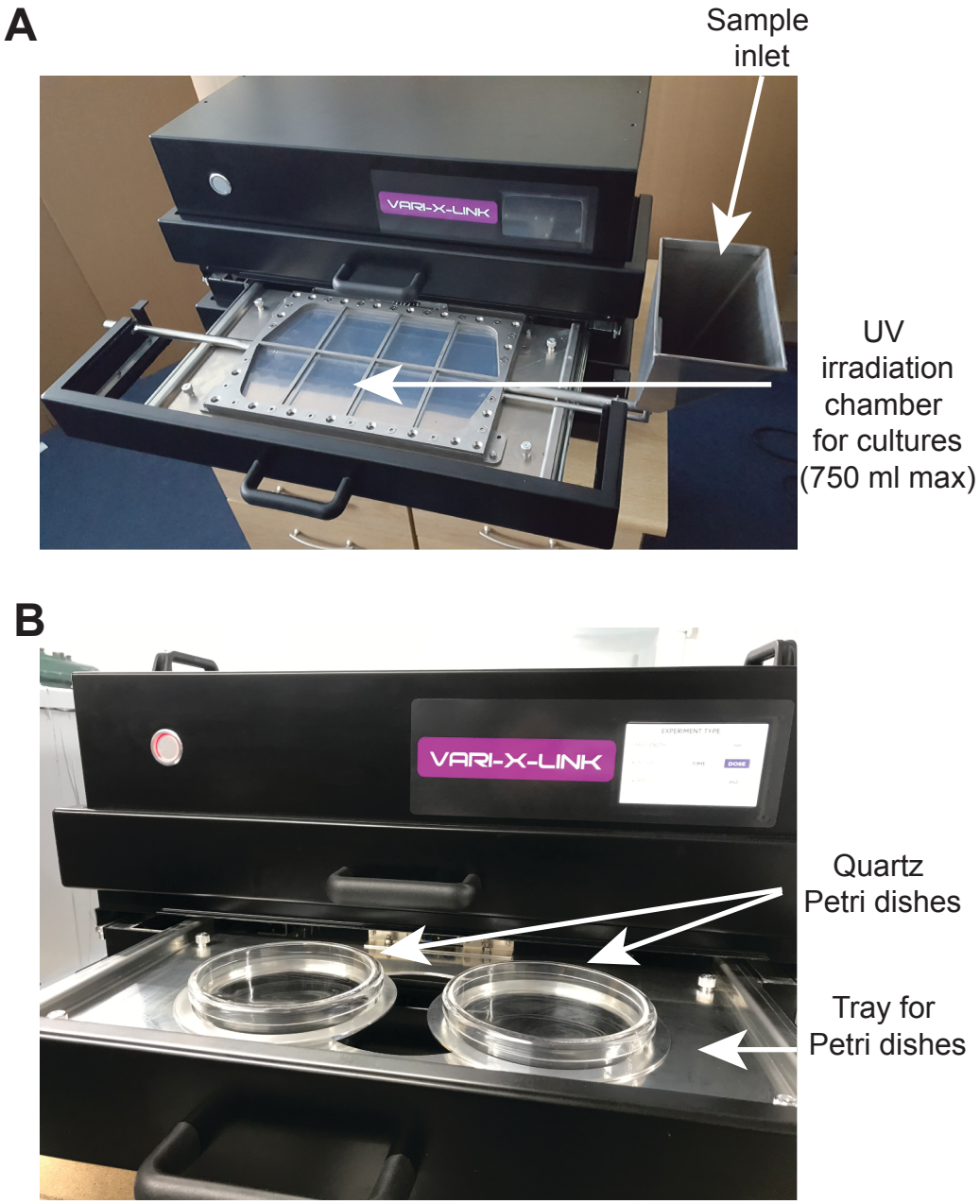


Figure 3



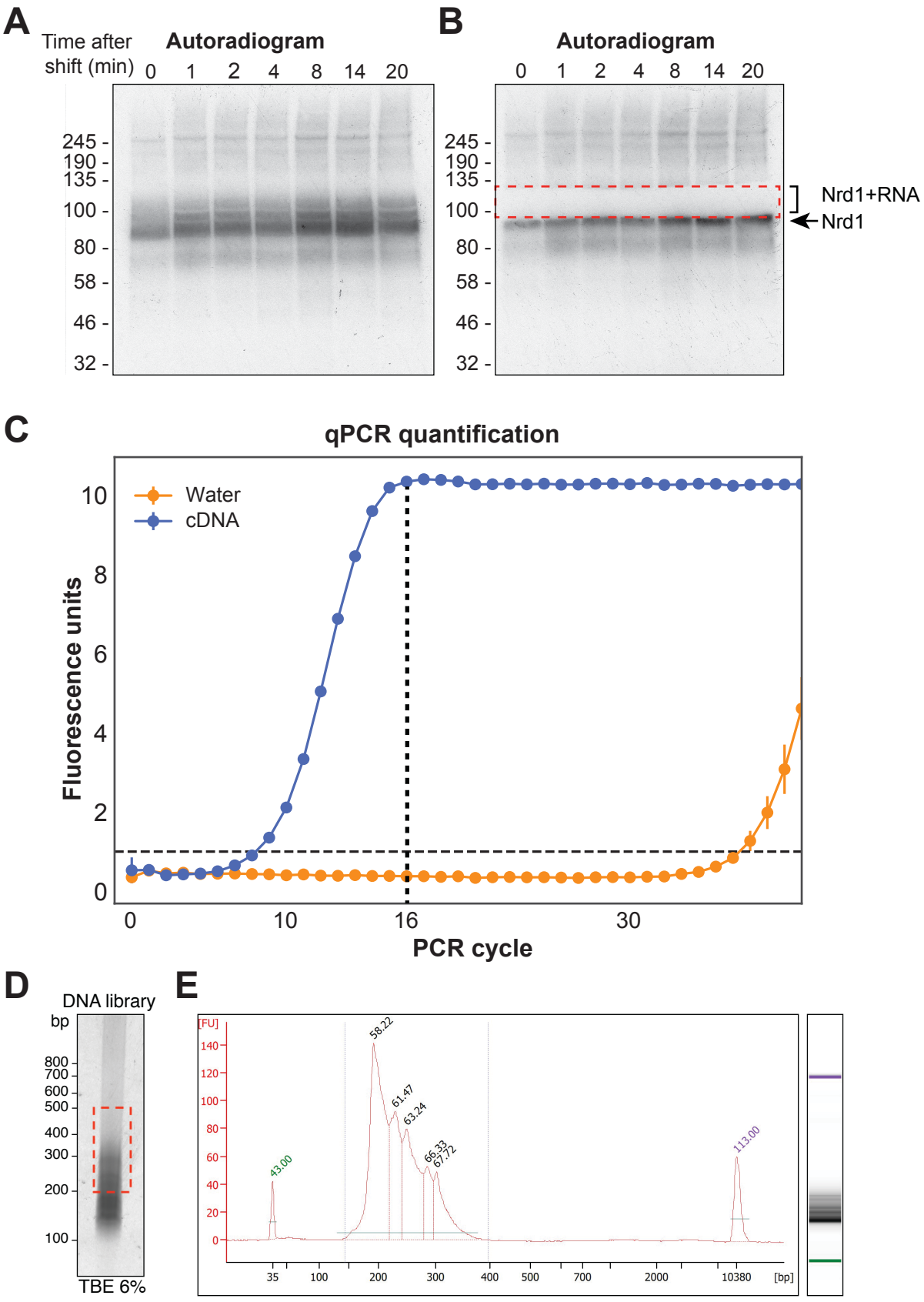


Figure 5

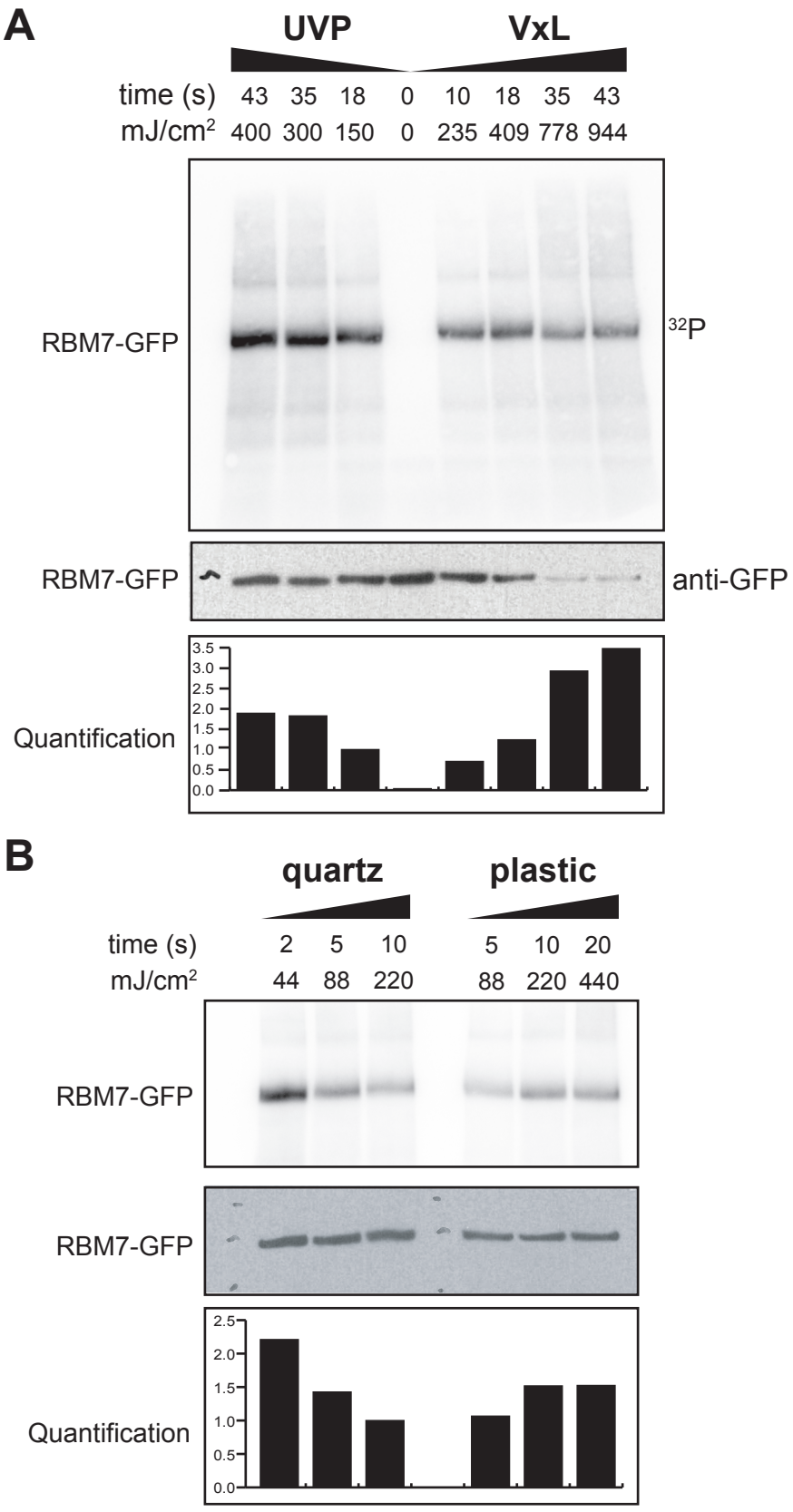
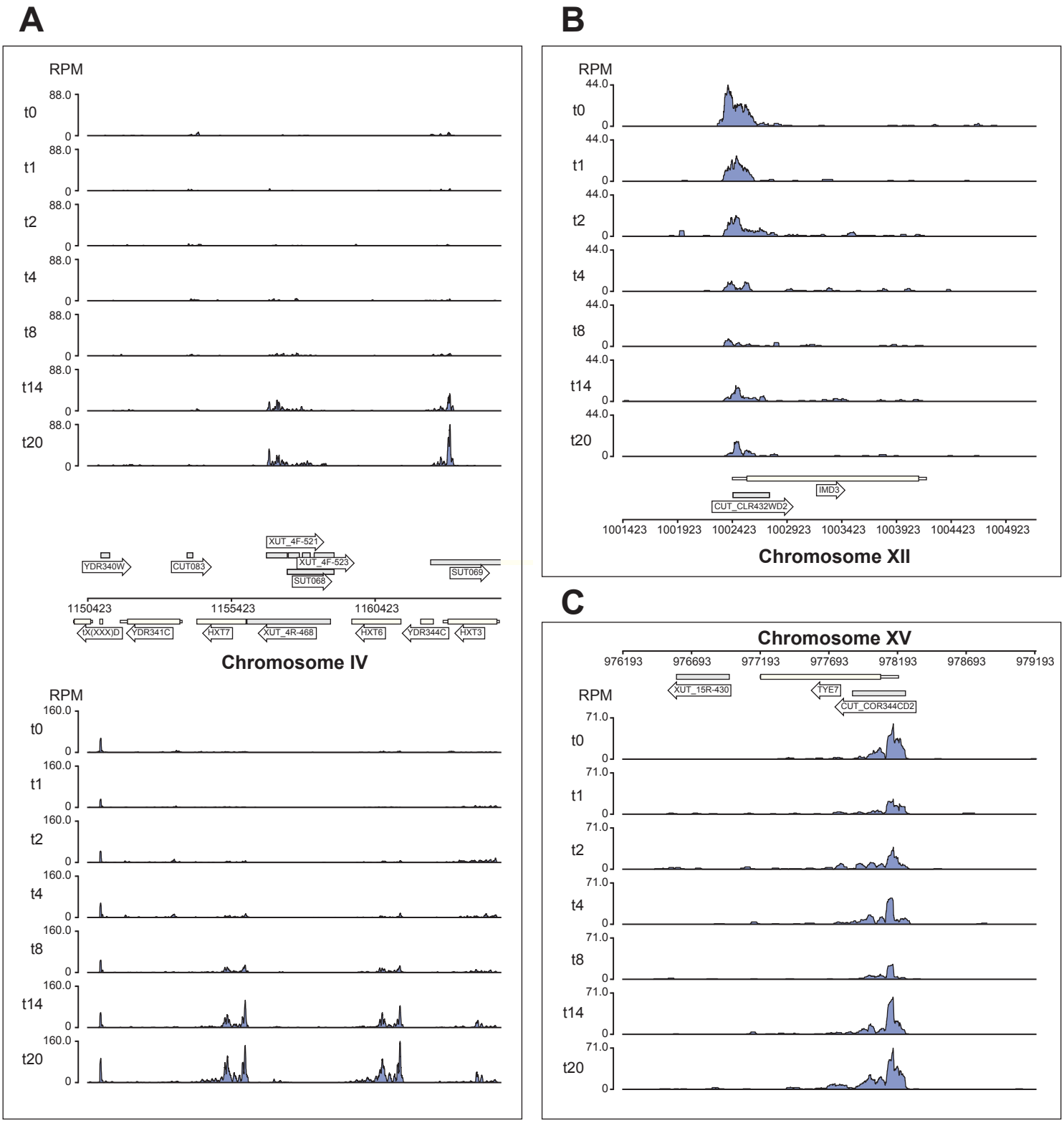


Figure 6



TN150
50 mM Tris pH 7.8
150 mM NaCl
0.1% NP-40
1X protease inhibitor

TN1000
50 mM Tris pH 7.8
1M NaCl
0.1% NP-40

NP-PNK
50 mM Tris-HCl pH 7.8
10 mM $MgCl_2$
0.1% NP-40
5 mM beta-mercaptoethanol

5 x PNK
250 mM Tris-HCl pH 7.8
50 mM $MgCl_2$
50 mM beta-mercaptoethanol

WB I
50 mM Tris-HCl pH 7.8
300 mM NaCl
10 mM imidazole
6M guanidine-HCl
0.1% NP-40
5 mM beta-mercaptoethanol

WB II
50 mM Tris-HCl pH 7.8
50 mM NaCl
10 mM imidazole
0.1% NP-40
5 mM beta-mercaptoethanol

Elution buffer
50 mM Tris pH 7.8
50 mM NaCl
250 mM imidazole
0.1% NP-40
5 mM beta-mercaptoethanol

Protease K buffer
50 mM Tris
0.1% NP-40
5 mM β-mercaptoethanol
1% SDS
5 mM EDTA
50 mM $NaCl_2$

Mammalian lysis buffer
50 mM Tris-HCl pH 8
100 mM NaCl
0.5% v/v Triton X-100
0.25% w/v Na-deoxycholate
0.1% w/v SDS
5 mM EDTA
1 mM DTT (added fresh)
1X protease inhibitor

Component	**1x**	**7.5x**
5 x PNK buffer	12	90
Alkaline phosphatase	4	30
RNase inhibitor	2	15
H_2O	42	315
Final volume	**60 µL**	**450 µL**

Table 3

Component	**1x**	**7.5x**
5 x PNK buffer	12	90
App-PE adapter (100 µM)	0.6	4.5
T4 RNA ligase 2 truncated K227Q	3	22.5
RNase inhibitor	1.5	11.25
50% PEG 8000	12	90
H_2O	30.9	231.75
Final volume	**60 µL**	**450 µL**

Table 4

Oligonucleotide name
L5Aa
L5Ab
L5Ac
L5Ad
L5Ba
L5Bb
L5Bc
L5Bd
L5Ca
L5Cb
L5Cc
L5Cd
L5Da
L5Db
L5Dc
L5Dd
L5Ea
L5Eb
L5Ec
L5Ed
App_PE

Sequence (5'-3')

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrArArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArUrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrCrGrCrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrGrCrUrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArGrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrUrGrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrArCrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrCrUrCrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrGrGrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArCrUrCrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrArCrUrUrArGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrGrUrGrArUrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrCrArCrUrArN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrArGrUrGrCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArUrCrArCrGrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrArCrUrGrUrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrUrGrArCrArN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrGrUrCrArCrN-OH

invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArCrArGrUrGrN-OH

App-NAGATCGGAAGAGCACACGTCTG-ddC

Table 5

Component	**1x**	**7.5x**
5 x PNK buffer	16	120
^{32}P-ɣATP (10 µCi/µL)	3	22.5
T4 PNK	3	22.5
H_2O	58	435
Final volume	**80 µL**	**600 µL**

Component	**1x**	**7.5x**
5 x PNK buffer	16	120
ATP (10 mM)	8	60
RNase inhibitor	2	15
T4 RNA ligase	4	30
H_2O	48	360
Final volume	**78 µL**	**585 µL**

Oligonucleotide name
P5 forward
BC1
BC3
BC4
BC5
BC7
BC8
BC9
BC10
PE_reverse

Sequence (5'-3')

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATCAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATTAGCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATGATCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

CAGACGTGTGCTCTTCCGATCT

Table 8

Component	**1x**
2x qPCR reaction mastermix	5
0.1 µM P5 primer (forward)	0.8
0.1 µM BC primer (reverse)	0.8
cDNA (or water as a negative control)	1
H_2O	2.4
Final volume	**10 µL**

Component	**1x**
10 x proof-reading polymerase buffer	5
10 µM P5 primer (forward)	1
10 µM BC primer (reverse)	1
5 mM dNTPs	2.5
Proof reading polymerase enzyme	1
cDNA	5
H_2O	34.5
Final volume	**50 µL**

Name of Material/Equipment	Company	Catalog Number
1,4-dithioreitol	Merck	10708984001
1.5 mL tubes	Eppendorf	0030 120.086
2 mL tubes	Eppendorf	0030 123.344
32P-yATP	Perkin Elmer	NEG502Z-250
4-12% Bis-Tris gel	Invitrogen	NP0321BOX
4X loading buffer	Novex	NP0008
50 bp ladder	New England Biolabs	N3236
50% PEG	NEB	B100045
6% TBE gel	Invitrogen	EC6265BOX
Acetone	ACROS Organics	423245000
anti-FLAG beads	Sigma Aldrich	M8823-1ML
ATP (100 mM)	Thermo Fisher Scientific	R0441
Beta-mercaptoethanol	Sigma Aldrich	M3148-100ML
Biomax MS intensifying screen	Sigma Aldrich	Z363162-1EA
Chloroform	Thermo Fisher Scientific	1010219
cOmplete EDTA-free protease inhibitor cocktail	Roche	11873580001
Complete supplement mixture - TRP	Formedium	DCS0149
Costar Spin-X 0.22 µm filters	Sigma Aldrich	CLS8160
DNase RQ1	Promega	M6101
dNTPs (10 mM)	Sigma Aldrich	4638956001
Ethanol	Thermo Fisher Scientific	10041814
Ethylenediaminetetraacetic acid	Invitrogen	AM9261

Exonuclease I	New England Biolabs	M0293
Glass microfiber filters	Whatman	1823-010
Glucose	Formedium	GLU03
Glycogen (20 mg/mL)	Roche	10901393001
GST-TEV	Homemade	
Guanidium hydrochloroide	Thermo Fisher Scientific	10071503
IgG beads	GE Healthcare	17-0969-01
Imidazole	Sigma Aldrich	I2399-100G
Isoamyl alcohol	Thermo Fisher Scientific	A393-500
Luna Universal One-Step RT-qPCR	NEB	E3005S
Magnesium chloride	Fluka Analytical	63020-1L
Membrane filters	Millipore	AAWP09000 for yeast or HAWP09000 for bacteria
Micro bio-spin columns	Biorad	732-6204
Ni-NTA beads	Qiagen	30210
NP-40	Sigma Aldrich	I8896-100ML
Pfu polymerase	Promega	M7741
Phenol	Sigma Aldrich	P4682-400ML
Pierce spin columns	Thermo Fisher Scientific	69725
Protease K	Roche	3115887001
Quartz Petri dish	UVO3	N/A
Radiography films	Amersham	28906843
RNAClean XP beads	Beckmann	A63987
RNase H	New England Biolabs	M0297
RNase-It	Agilent	400720
rRNasin	Promega	N2511

Sodium acetate	Sigma Aldrich	S2889-1KG
Sodium chloride	Thermo Fisher Scientific	7647-14-5
Sodium deoxycholate	Sigma Aldrich	D6750-100G
Sodium dodecylsulfate	Sigma Aldrich	L3771-1KG
SUPERase-In	Invitrogen	AM2694
SuperScript IV	Thermo Fisher Scientific	18090010
T4 PNK	New England Biolabs	M0201
T4 RNA ligase 1	New England Biolabs	M0204
T4 RNase ligase 2, truncated K222Q	NEB	M0351S
TBE buffer (10X)	Invitrogen	15581-028
TEV protease	Homemade	
Thermosensitive alkaline phosphatase	Promega	M9910
Trichloroacetic acid (100%)	Sigma Aldrich	T0699-100ML
Tris hydrochloride	Invitrogen	15504-020
Triton X-100	Sigma Aldrich	T8787-100ML
Vari Filter	UVO3	N/A
Vari-X-Linker	UVO3	N/A
Yeast nitrogen base	Formedium	CYN0410
Zirconia beads	Thistle	11079105Z for y

Comments/Description

Buffer component in mammalian cell lysis
General reaction tube
For holding columns and collection of waste

For radiolabelling the 5' end of the RNA
SDS-PAGE gel
Protein loading dye concentrate

Reference ladder for excising region of interest from the amplified cDNA library
For the L5 linker ligation
For separation and purification of the cDNA library

Washing of TCA-precipitated proteins

For purifcation of FLAG-tagged RBPs

For ligation of the L5 linker onto the 5' end of captured RNAs

Buffer component

For intensifying the autoradiogram signal

For phenol-chloroform extraction following RNA purification

For inhibition of cellular proteases after lysis

For preparation of synthetic defined medium

For isolating the excised cDNAs following gel extraction
For DNA digest following cell lysis

For reverse transcription and PCR

For phenol-chloroform extraction following RNA purification and DNA precipitation

For protease K buffer

For degradation of primers following PCR
For isolating the excised cDNAs following gel extraction
For preparation of glucose-containing, synthetic defined medium
Precipitation of proteins, RNA and DNA
Construct and purification protocol is available upon request

Required for pulldown denaturing conditions and washing buffer

For purification of protein A-tagged RBPs

For elution of captured proteins from Nickel beads

For phenol-chloroform extraction following RNA purification

For qPCR of the cDNA in order to calculate required number of PCR cycles

For PNK buffer

For vacuum filtration of cells
For collecting eluate after gel extraction
For secondary protein capture

Buffer component
For amplification of the cDNA library

For phenol-chloroform extraction following RNA purification

For on-column enzymatic reactions
For degradation of the RBP following gel extraction
For cross-linking of adherent cells. Available from https://www.vari-x-link.com for 400 GBP
For autoradiography visualisation
SPRI beads for clean up of RNAs and cDNAs

For degradation of RNAs following reverse transcription
For RNA digestion
For inhibition of any contaminating RNases during enzymatic reaction

For phenol-chloroform extraction following RNA purification and DNA precipitation

Buffer component

Buffer component in mammalian cell lysis

For protease K buffer
For inhibition of cellular RNases after lysis

For reverse transcription

For radiolabelling the 5' end of the RNA

For ligation of the L5 adaptor onto the RNA 5' end

For ligation of the App_PE linker onto the 3' end of captured RNAs
For running TBE gels
For eluting captured proteins following FLAG capture

For 5' and 3' dephosphorylation of RNAs

For precipitation of RBP-RNA complexes
Buffer component

Buffer component in mammalian cell lysis
Device for vacuum harvesting cells. Available from https://www.vari-x-link.com for 100 GBP
Cross-linker for cross-linking cells. Available from https://www.vari-x-link.com for 16,000 GBP
For preparation of synthetic defined medium
For cell lysis via bead beating

Editorial comments

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have now more thoroughly proofread the manuscript and corrected all noticed errors.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

The manuscript has now been formatted appropriately for JoVE.

3. Please reduce the number of personal pronouns (we, you, your).

This stylistic recommendation has now been addressed. We have reduced the number of pronouns to a minimum. When their use is unavoidable, we have used the impersonal 'one' (lines 367, 420, 457, 484, 568, 647).

4. Please include email addresses for all authors in the manuscript itself.

The addresses have now been included on the first page of the revised manuscript.

5. Please provide 6-12 key words or phrases in the manuscript.

We now provide keywords on the second page of the revised manuscript

6. Please ensure all references are numbered superscripts.

The referencing style has now been updated.

7. Please define all abbreviations before use; e.g., CRAC.

Done. We have further checked the manuscript for abbreviation use and are confident that we have now defined them appropriately.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript (and figures) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Vari-X Linker, Vari-Filter, Pyrex, Falcon, Sepharose, Superase-In, Eppendorf, RNace-It, Pierce, Biorad, SuperScript IV, RNAClean, Novex,

We were originally unaware of this limitation but have now removed all commercial language from our manuscript. The Table of Materials and Reagents is now the only document containing such language. We do feel it is important to note that the company that developed the cross-linker is paying for a portion of the publication costs and that this is therefore a joint corporate publication. Please advise on whether in this case it is still necessary to remove Vari-X-linker and Vari-filter.

Protocol:
1. Please move the long paragraphs in the protocol to the introduction, results, or discussion, as appropriate; alternatively, split into numbered protocol steps.

All of these paragraphs have been relocated. For example, our original experimental description (found on page 6 on the original manuscript) has now been explained in the Introduction and Representative Results (found on pages 5 and 18 in the updated manuscript). We still include a small paragraph of three lines in the 5' linker ligation step (lines 366-368) as this contains a crucial notice to the reader.

2. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm) (see e.g. Elution… step 2).

All centrifuge speeds have been updated accordingly.

3. There is a (roughly) 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (formatted as detailed above; including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the sections that we think would be useful to film.

4. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We believe that we are explicit in describing how each step is performed. If there are any sections where the Editor feels we have not done this appropriately, please let us know and we would be happy to update the text accordingly.

Specific Protocol steps:
1. Oligonucleotides and Buffers sections: Please move the information in these sections to the steps where they are most relevant. Please reformat as notes, numbered protocol steps, or tables (which see below).

We have now moved the oligonucleotides and buffer information into dedicated tables. Please see attached Table 1.xlsx, Table 2.xlsx and Table 3.xlsx.

Figures and Tables:
1. Figure 1: Step 4 isn't detailed in the legend.

Apologies, we have now changed the figure legend accordingly.

2. Figure 3C: What are the error bars here?

Three technical replicates were included in the qPCR experiment and the error bars represent standard deviations. The solid line represents the mean values. We apologise for not explaining this in the text. This has now been included in the legend of Figure 4 (previous Figure 3)

3. Please remove the embedded tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

As requested, we have removed the embedded tables and submitted these separately as Excel spreadsheets. We have also included a brief description of each table after the Representative Results section.

References:
1. Please do not reference product websites or manuals (e.g., ref. 34); all relevant material should be in the Table of Materials.

We have now removed the reference to UVO3's website from the manuscript. However, we still include two in-text references to our pyCRAC software (lines 580-581) as this is an open-access package that is relevant to other groups performing similar type of analyses.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have now updated the Table of Materials with previously missed materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Granneman et al present a nice manuscript and protocol for the development and implementation of xCRAC. The importance of kinetic measurements is understudied across most fields, but especially in the space of RNA-protein interactions due to the lack of tools and methods. As such, I think xCRAC is positioned well to make an impact in this area.

We thank the reviewer for the very positive and constructive comments.

Major Concerns:
The only omission I think the authors have is the cost of the Vari-X-Linker. My understanding is that they are ~£16,000 which could be an important practical factor for labs considering establishing xCRAC.

We agree that this is an important point and as a result we have added the purchasing costs of the Vari-X-linker system and the quartz Petri dishes to the Material table. Unfortunately, JoVE does not allow us to put this information into the main text. We have also added the costs for the new qartz pertridishes.

Minor Concerns:

Lines 144-171: These are helpful comments regarding general considerations for the protocol, however there could be a bit more structure provided to the readers about the pointers. The first paragraph has information about the media preparation type, media composition, cross linking time, and cross-linking type. It could be broken into smaller sections with more context around why each of these parameters is important to consider.

We have now moved this large paragraph and described our experimental system in the Introduction and Representative Results. We hope that the flow has now improved.

Throughout the protocol you note often "in our example". It might be simpler to write the experimental choices you have up front in the introduction and call out when these can be changed in different contexts. This way the protocol will read more seamlessly.

We agree. In response to the editors comments we have moved some of the paragraphs where we detail specific optimization steps to the Discussion section (see page 23 of the revised manuscript) and we report our standard experimental settings in the Protocol itself but instructed the reader that these can be changed as appropriate (for example, see page 10, lines 313-316).

Line 182: Is there some obvious way to operate the Vari-X-llnker? This step simply says "UV irradiate" the cells. Are there multiple steps to performing the UV X-linking? Additional details here could be helpful.

We include a guide to operating the Vari-X-Linker in Figure 2 and the new Figure 3, where we describe how to add cells to the machine, how they are cross-linked and how to remove cells from the machine. The touch screen panel is obvious in its usage, where users simply define either a specific UV dose or an exposure time, and then press a 'start' button. However, to make this clearer, the operation of our cross-linker with both cell cultures and monolayers will be detailed in the recorded aspect of the publication.

Line 182: This notes that the amount of UV should be "carefully optimized for each protein of interest". Can the authors briefly explain to the readers what optimization should occur? What readout would a reader need to look at to perform this optimization?

Excellent point. We now include more details in a dedicated paragraph in the Discussion (728-734), where we describe how to perform such optimisation and how to deduce the optimal UV exposure.

Line 206 and 238: Why are the FLAG and IgG beads harvested differently? Are they on different resins? If so, this seems like it would introduce bias across the control vs experimental conditions. Is it possible to put the FLAG and IgG antibodies on the same resin?

Both beads are from commercial companies; we use Sigma's anti-FLAG beads and GE Healthcare's IgG beads. The reason for the different harvesting technique is that the anti-FLAG bead resin is magnetic, thus facilitating their harvest using a magnetic rack, while the IgG beads are simple agarose beads and thus must be harvested via centrifugation.

It would be possible to covalently couple IgG beads to the same type of magnetic beads as the anti-FLAG beads and some labs prefer to do this. However, since we switched from the HTP to the smaller HTF tag we decided not to pursue this further. The larger HTP tag can in some cases impair protein function, which is why we switched to the smaller HTF. But the handling steps for the IgG-coupled magnetic beads are identical as for the anti-FLAG beads.

Line 215: It could read "Add 2 *pellet-*volumes of ice-cold.." To be more explicit

We have now updated this line, and those similar, with Reviewer 1's suggestion (lines 200, 206, 208 and 214).

Line 223: Since the beads are difference sizes for each organism, I might write "To homogenize the cells, first add zirconia beads. For yeast cells, use 0.5mm diameter, for bacterial cells, us 0.1mm diameter."

Good point. We have restructured this sentence to make it clearer (line 208)

Line 262: Since most labs will not have your "home made" GST-TEV enzyme, it is important to include a commercial vendor with units of that enzyme required for cleavage. Otherwise the protocol is difficult to generalize across laboratories.

We appreciate this comment and understand the concern. However, the issue here is that one must use a TEV enzyme that does not utilise a histidine tag, otherwise it will bind to the nickel beads. When the original CRAC protocol was being developed, we contacted several commercial companies in to ask if they offer a GST-TEV protease that does not have a HIS6 tag as the enzyme would otherwise be co-purified during the Ni-NTA step. Unfortunately, all the commercially available TEV proteases had HIS6 tags and were therefore not useful. As such, we produced our own TEV protease that has a GST tag for glutathione-based purification and a 6x arginine tag to enable efficient capture with cation exchange chromatography. We recently discovered that Creative Diagnostics in the US offers a GST-tagged TEV protease, however, since we have enough GST-TEV to last us for several years, we have not tested it. Therefore, we do not know how many units of any commercial enzyme would be required. We now state on page 9 (lines 290-291) of the revised manuscript that GST-TEV can also be purchased from this company.

In the past we have always sent our GST-TEV-ARG6 construct as well as the purification protocol to interested labs. We now state in the Materials sheet that the reagents for producing GST-TEV are available upon request. Should JoVE wish that we include these protocols as well, we would be happy to do so, but the purification procedure is fairly straightforward.

Line 276: I think the authors mean "RNase-It"? The RNase "One" ? If so, there is a type here and on subsequent lines.

We have corrected this error to ‘RNase cocktail’ in order to avoid commercial language (lines 304, 307, 313). RNase-It is the RNase T1/A cocktail from Agilent that we routinely use for CRAC.

Line 312: I am unfamiliar with the notation "Ve". Should this more simply read "final volume" ?

That is correct. We have now updated our notation with this suggestion.

Line 332: The authors should include notes about handling radiation safely as well as disposal of waste subsequent to this step since the material and elations can be radioactive.

We have included comments to alert the user to the fact that the experiment is now radioactive and appropriate measures should now be taken for protection and waste disposal (lines 355, 362, 399, 405, 431).

Line 520: Can the authors provide any example data or images of the PAGE gel results from a successful library amplification?

We have now included a Bioanalyzer trace and a gel image of a successful library in Figure 4D.

Line 545: Standard desalting or are other purifications needed?

We now state in the legend of Table 2 that we use RNase-free HPLC purification for our DNA and RNA adapter oligonucleotides.

Reviewer #2:

Manuscript Summary:
Granneman and colleagues present a very detailed experimental plan for conducting xCRAC from yeast cells. The description of the protocol and the strategy are well laid out and easy to follow as is the library preparation. This very nicely written and constructed manuscript will be a real asset for the CRAC and RNA-binding protein research community.

Major Concerns:
None

We thank the reviewer for the positive and constructive comments.

Minor Concerns:

1) A clearer description of how the Var-X-linker works and how this improves the cross linking efficiency would improve the readers understanding of the pros and cons of this approach.

We apologize for not making this sufficiently clear. The previous iteration of the Vari-X-linker was discussed in significant detail in our Nature Communications paper (van Nues et al, 2017) where data describing the improvement of our cross-linker over existing ones can be found. We have summarized the advantages of the Vari-X-linker on pages 4 and 5 and refer to the van Nues paper.

2) I realize that this protocol focuses on XCRAC however some discussion of the bioinformatics processing of the reads and links to example datasets would be very useful.

We agree with this reviewer and initially we included more details on the bioinformatic processing of our data. However, we soon discovered that providing a proper description of our pipelines and data analysis procedures would require a significant increase in the length of the current manuscript, and indeed could form an independent manuscript in itself. As such, we have referenced our Nature Communications paper when discussing χCRAC data processing (lines 579-581) which includes a full description of our data normalization and data processing steps. Additionally, we have included weblinks to our Github and Bitbucket pages which guide the reader to our pyCRAC software (lines 580-581). This software includes a detailed manual on how to use pyCRAC and contains its own example datasets. We are also still in the process of improving the data analysis pipelines and hope to publish a small paper that provides more details of the data analysis steps in the next year or so. We hope that this is an acceptable compromise.

3) Insertion of the "x" symbol from XCRAC has not always been successful.

We thank the Reviewer for spotting these and have now corrected all errors.

4) Some variation in tense throughout.

We have further proof-read the manuscript and believe that we have corrected all grammatical issues.

Overall, this is an excellent manuscript that with the addition of these very minor points will be used widely

We thank the reviewer for the positive comments!

Reviewer #3:

Manuscript Summary:
This manuscript describes a method of derivative of CLIP-seq for living cells grown in suspension. They built in-house devices for improved UV irradiation and cell harvesting to investigate time-resolved analysis of dynamic RBP-RNA interactions. Overall, the method procedure is well described. Below are my comments that need to be addressed for understanding this method clearly.

We thank the reviewer for their positive and constructive comments.

Minor Concerns:

1) Can this method be applied for living cells grown on monolayer? If yes, use notes to help to change parameters if monolayer cells might be used. If not, it should be clear mentioned that this method is only applicable for suspension culture in the abstract and the introduction.

Yes, it can. In collaboration with Torben Heick Jensen's lab we are currently in the process of adapting the method to perform kinetic analyses in mammalian cells. The protocol is still under heavy development and therefore will be published elsewhere. To make these analyses possible with monolayers, we have developed a new tray for the Vari-X-linker that can hold both standard commercially available plastic Petri dishes as well as our new quartz Petri dishes. The Vari-X-Linker utilises UV lamps both above and below the input in order to maximise cross-linking. However, standard lab plasticware is UV-absorbent and thus will severely hinder illumination from the bottom banks of UV lights. As such, we developed a UV-transparent, quartz Petri dish that enables both the bottom and top lamps to irradiate the sample and this should enable much shorter cross-linking times in the Vari-X-Linker (see new Figure 3B).

We have not had time to extensively test the new Petri dish setup yet but in the revised manuscript we have included some recent data that were generated by Ross Cordiner, a post-doc from Torben Heick Jensen's group when he visited the Granneman lab (see Figure 5). These results show that with the Vari-X-linker and the plastic Petri dish, we get cross-linking yields comparable to the commercially available UVP box that is commonly used by other labs. The main difference here is that cross-linking in the UVP has to be done on ice without any liquid because the cells are only ~1.5 cm away from the lamps and the box can get very hot. In the Vari-X-linker, however, we cross-link the cells at the operating temperature of the machine (set at 37°C), which remains fairly constant during UV irradiation. Thus, we suggest that the Vari-X-linker reduces the thermal shock incurred during cross-linking.

Strikingly, with the quartz Petri dish we already saturate the cross-linking after 2 seconds of exposure. We believe that with the current setup we will be able to could cross-link monolayer cells in growth medium in less than 10 seconds. This could be a huge advantage as this would make it possible to cross-link monolayers as well as small volumes of suspension cells in a very short time without changing their environment.

2) Suppliers for some of reagents such anti-flag, IgG beads, DNase RQ1, SYBR safe dye, etc. are missing in materials. Suppliers not needed for routine reagents are needed in the materials section.

We have further updated our Table of Materials to include all of the necessary information.

3) Need a specific volume of a buffer used is missing, for example, in line 258 how much ice-cold TN1000 is needed?

We have now updated the wash step with the required volumes (line 269).

4) It appears that alkaline phosphatase treatment and subsequence procedure for linker ligation and 5' end phosphorylation were done in RNA associated with a target protein. In line 296 the subtitel should indicate this point for readers not familiar with any CLIP-seq based methods.

We have now updated all of the necessary subtitles with an 'On-bead' description (lines 343, 351, 365)

5) In figure 3, the different condition between A and B should be indicated. And it looks that they are not smeared bands above Nrd1. Is that possible to replace figure 3B showing smeared bands indicating for Nrd1+RNA complex?

We apologize for not making this sufficiently clear. Panels A and B display the same autoradiograph, but before and after excision of the radioactive signal above the main Nrd1 band. This is explained in the legend for Figure 4 (previously Figure 3) (lines 664-665). We have now also made it clearer in the Representative Results section (lines 563-565). This smear rises from the ~90 kDa range (representing Nrd1 bound to very short RNA fragments) to ~110 kDa, representing Nrd1 bound to longer RNA fragments. This is the region that is excised and contains cross-linked RNAs which we subsequently detected through next generation sequencing.

Reviewer #4:

Manuscript Summary:
The authors describe here a detailed protocol of their recently developed variant of the CRAC method termed kinetic CRAC. This method is based on an ultrafast crosslinking device (Vari-X-Linker) and a fast harvesting system (Vari-Filter) that allows cross-linking and harvesting cells on minute timescales. As the method seem to work very reproducibly, it allows quantitative measurement of dynamic changes in RBP-RNA interactions. The authors also optimized the CRAC protocol. Kinetic CRAC promises to be a very useful tool to study RBP-RNA interactions during cellular stress or the cell cycle in yeast and bacteria.

We thank the reviewer for their positive comments on our manuscript.

Major Concerns:
One has to purchase the Vari-X-Linker and the filtration device to perform this method.

This is correct. The dependence of the χCRAC method (at least on suspension cells) relies heavily on our cross-linker and cell harvesting devices as these currently are the only commercially available systems that enable cross-linking at such short intervals. To make groups aware of associated costs we have included the list price of the machines and associated materials in the Materials list.

Minor Concerns:
It remains unclear whether this method is usable for mammalian tissue culture or other tissues. Even if one would purchase a Vari-X-linker, adherently grown cells and intact tissues cannot be harvested by filtering. But one could certainly use a similar system for cells grown in suspension or dissociated tissue. The authors mentioned in the discussion that their system has been coupled to iCLIP.

Yes, it can. In collaboration with Torben Heick Jensen's lab we are currently in the process of adapting the method to perform kinetic analyses in mammalian cells. The protocol is still under heavy development and therefore will be published in the future. To make these analyses possible with monolayers, we have developed a new tray for the Vari-X-linker that can hold both standard commercially available plastic Petri dishes as well as our new quartz Petri dishes. The Vari-X-Linker utilises UV lamps both above and below the input in order to maximise cross-linking. However, standard lab plasticware is UV-absorbent and thus will severely hinder illumination from the bottom banks of UV lights. As such, we developed a UV-transparent, quartz Petri dish that enables both the bottom and top lamps to irradiate the sample and this should enable much shorter cross-linking times in the Vari-X-Linker (see new Figure 3B).

We have not had time to extensively test the new Petri dish setup yet but in the revised manuscript we have included some recent data that were generated by Ross Cordiner, a post-doc from Torben Heick Jensen's group when he visited the Granneman lab (see Figure 5). These results show that with the Vari-X-linker and the plastic Petri dish, we get cross-linking yields comparable to the commercially available UVP box that is commonly used by other labs. The main difference here is that cross-linking in the UVP has to be done on ice

without any liquid because the cells are only ~1.5 cm away from the lamps and the box can get very hot. In the Vari-X-linker, however, we cross-link the cells at the operating temperature of the machine (set at 37°C), which remains fairly constant during UV irradiation. Thus, we suggest that the Vari-X-linker reduces the thermal shock incurred during cross-linking.

The Vari-X-linker also seems to be more efficient at longer exposure times. Strikingly, with the quartz Petri dish we already saturate the cross-linking after 2 seconds of exposure. We believe that with the current setup we will be able to could cross-link monolayer cells in growth medium in less than 10 seconds. This could be a huge advantage as this would make it possible to cross-link monolayers as well as small volumes of suspension cells in a very short time without changing their environment.

It would have been interesting to know how the cells were harvested.

We have now added further details on the mammalian cell cross-linking and harvesting (lines 227-265). Briefly, following cross-linking, ice-cold PBS was added and the cells were then scraped and transferred to a 15 mL tube. Afterwards, the cells were pelleted, the supernatant decanted, and then the cells resuspended in 1 mL of ice-cold PBS and transferred to a 1.5 mL tube. Finally, the cells were pelleted once more, the supernatant was decanted and the pellets were frozen on dry ice.