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CRAC Analysis in Budding Yeast with HTP Tagged Proteins

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ABSTRACT

CRAC (Cross-linking and cDNA Analysis) is a method that uses UV-crosslinking to identify which RNAs bind to an RNA-Binding Protein of interest and the specific sites in the RNA where this binding occurs. CRAC is one of the family of UV crosslinking and immunoprecipitation (CLIP) protocols, reviewed by Lee and Ule (DOI: 0.1016/j.molcel.2018.01.005).The protein is tagged with with a tandem affinity tag: in this protocol we use an HTP tag (His_6-TEV-ProteinA) that allows immuno-affinity purification of the protein with its cross-linked RNAs by binding of the ProteinA moiety of the tag to IgG Sepharose columns. After washing, the protein is then released from the column by cleavage with TEV protease and subjected to nickel-affinity purification through the His_6 moiety under highly denaturing conditions. This allows accurate mapping of RNA binding sites (Bohnsack, *et al,* DOI: 10.1016/B978-0-12-396546-2.00013-9) ; Granneman, S. *et al* (DOI: 10.1073/pnas.0901997106). The protocol can also be adapted for use with HTF (His_6-TEV-$FLAG_3$) tags (Tree, J.J. *et al*, DOI: 10.1016/j.molcel.2014.05.006) or HF (His_8-Ala_4-FLAG) tags (Bresson, S., Shchepachev, V., Spanos, C., Turowski, T., Rappsilber, J. and Tollervey, D.(2020): DOI: /10.1101/2020.05.14.096354) by replacing the IgG sepharose column with anti-FLAG magnetic beads and elution with excess FLAG peptide, removing the need for TEV cleavage which can be inefficient. The cDNA libraries generated allow a transcriptome -wide analysis of the interactome of the protein.

DOI

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HTP tag, Cross-linking, budding yeast, CRAC

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MATERIALS

NAME	CATALOG #	VENDOR
T4 RNA Ligase 1 (ssRNA Ligase) - 5,000 units	M0204L	New England Biolabs
RNase H - 250 units	M0297S	New England Biolabs
MinElute PCR Purification Kit	28004	Qiagen
rATP (100mM), 400ul	E6011	Promega
HaloTEV Protease, 200 ul	G6601	Promega
TSAP Thermosensitive Alkaline Phosphatase, 100u	M9910	Promega
RNasin(R) RNase Inhibitor 10,000u	N2115	Promega
Recombinant RNasin(R) RNase Inhibitor, 2,500u	N2511	Promega
NUPAGE LDS sample buffer (4x)	NP0007	Thermo Fisher Scientific
1x NUPAGE MOPS SDS running buffer (20x)	NP0001	Thermo Fisher Scientific
T4 Polynucleotide Kinase - 2,500 units	M0201L	New England Biolabs
GeneRuler 50 bp DNA Ladder	SM0371	Thermo Fisher Scientific
YNB - w/o amino acids	CYN0405	Formedium
CSM-TRP	DCS0149	Formedium
Millipore MF-Membrane Filters 0.45um HA	HAWP09000	Merck Millipore Sigma
0.5mm Zirconia Beads	11079105Z	Thistle Scientific
complete ULTRA Mini EDTA-free Protease Inhibitor Cocktail Tablets in blister packs	5892791001	Sigma Aldrich
1.5ml Safe-lock tubes	0030120086	Eppendorf
IgG Sepharose 6 Fast Flow GE Healthcare	17-0969-01	Merck Millipore Sigma
Pierce™ Spin Columns - Snap Cap	69725	Thermo Fisher Scientific
Guanidine hydrochloride	G4505	Sigma Aldrich
RNace-It Ribonuclease Cocktail	400720	Agilent Technologies
Imidazole anhydrous	792527	Sigma Aldrich
Ni-NTA Superflow Beads (25 ml)	30410	Qiagen
TSAP Thermo-sensitive Alkaline Phosphatase	M9910	Promega
T4 RNA Ligase 1	M0204L	New England Biolabs
T4 RNA Ligase II truncated K227Q	M0351S/ M0351L	New England Biolabs
T4 Poynucleotide Kinase	M0201L	New England Biolabs
32P-γATP	NEG502Z-250/ NEG502Z-500	Perkin Elmer
rATP 100mM	E6011	Promega
Nuclease-free water	AM9937	
GlycoBlue Coprecipitant	AM9516	Life Technologies
NuPAGE LDS sample buffer 4×	NP0007	Life Technologies
NuPAGE 4–12% (wt/vol) polyacrylamide Bis-Tris gels	NP0335	Life Technologies
NuPAGE SDS-MOPS running buffer	NP0001	Life Technologies
SeeBlue Plus2 pre-stained standard	LC5925	Life Technologies
Prestained Protein Ladder – Broad molecular weight (10-245 kDa)	ab116028	Abcam
Hybond-C Extra membrane	10564755	Fisher Scientific
NuPage transfer buffer	NP00061	Life Technologies
Rabbit anti-TAP Tag Polyclonal Ab	CAB1001	Thermofisher
Kodak BioMax MS autoradiography film	8222648	Sigma Aldrich
Donkey anti-Rabbit IgG Dylight680 Antibody	SA5-10042	Thermofisher
Proteinase K	03115836001	Roche
Deoxynucleoside Triphosphate Set (lithium salt)	11277049001	Roche
Superscript IV Reverse Transcriptase	18090050	Life Technologies
RNase H	M0297L	New England Biolabs

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NAME	CATALOG #	VENDOR
TaKaRa long and accurate (LA) Taq	RR002M	Takarabio
Metaphor Agarose	LZ50181	Lonza
SYBR™ Safe DNA Gel Stain	S33102	Thermo Fisher Scientific
New 6x Purple Loading Dye	B7024S	New England Biolabs
50bp DNA Ladder	N3236S	New England Biolabs
MinElute Gel extraction kit	28604	Qiagen
Qubit™ dsDNA HS Assay Kit	Q32851	
Illumina MiniSeq High Output Reagent Kit	FC-420-1001	Illumina, Inc.
Monoclonal M2 antibody (anti-FLAG)	F1804-200UG	Sigma Aldrich

SAFETY WARNINGS

A number of the Buffers used in this protocol include 2-mercaptoethanol which is toxic. Always dispense from the stock bottle in the fume hood.

This protocol uses a radio-active substance and some stages must be performed in a room suitable for such work according to Local and State Health and Safety Regulations by a person suitably trained for such purposes.

DISCLAIMER:

BEFORE STARTING

Check you have all of the reagents and buffers you will need. Be prepared for some long days.

Yeast Culture Day 1 1h

1 Autoclave **2 L** milliQ water per CRAC sample in 5 litre flasks. For each flask prepare **286 mL** each of sterile 10x Yeast Nitrogen Base without Amino Acids (YNB-aas; Formedium), 10x Complete Synthetic Medium - Tryptophan (CSM - TRP; Formedium) and 20% glucose. These will be combined at Step 3 to make Synthetic Minimal Medium - Tryptophan (SMM - TRP).

SMM - TRP is used because Tryptophan absorbs UV light at 280nm and so reduces the efficiency of UV cross-linking.

Pre-warm all to **30 °C** **Overnight** .

2 Grow yeast strains in 50 ml pre-made SMM-TRP at **30 °C** **Overnight** . Set-up up to 3 yeast cultures, each in 50 ml SMM-TRP or 100ml for 2 technical replicates (for control and +heatshock samples).

Yeast Culture Day 2, and Heat-shock 9h

3 Mix the large volume pre-warmed SMM media components from Step1 with the water in each flask and inoculate with

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yeast from the starter cultures to give a starting density of ~0.070 x10^7 cells/ml (OD600 ~0.05 for the first culture, OD600 ~0.035 for the 2nd and OD600 ~0.025 for the third to allow time to process each culture and cross-link). Shake each at **30 °C** until OD600 ~ 0.5 (~ **08:00:00**) - there should be around 45 mins between each culture being ready. Expect to get ~ 1g of cells/L of culture. Prechill as many tubes containing 50ml of PBS to allow 1 tube per culture plus another 2 empty tubes per culture and pre-chill **On ice** .

Cultures to be exposed to heat shock are rapidly collected onto 0.45µm MF-membrane filters (Millipore; or 0.8µm to reduce clogging) by vacuum filtration and the filter + cells transferred into a fresh 2.858ml of SMM-TRP medium pre-warmed to 42°C and incubated for exactly **00:16:00** at **42 °C** , followed immediately by cross-linking. Control samples not subjected to heatshock can be cross-linked immediately.

UV Crosslinking (per sample) 30m

4 UV crosslinking using the Megatron (UVO3: see Bohnsack Tollervey & Granneman (2012) Methods in Enzymology 511, 275-288)): Wash the Megatron tube once with de-ionised water, being careful to avoid spilling anything on the UV lamp. On the second wash with water, close the tap and screw on the the lid on the Megatron tube. Turn on the middle lamp first and then the one directly underneath the tube. The lid should begin to slowly turn green. You should be able to see blue light if you look through the window on the Megatron to the lamp. Continue until the lamp has gone past 100%. Switch off the lamps (middle one first). Set timer for 100s and pour in the culture. Turn on middle lamp, lower lamp, and immediately start timer. Tip the apparatus back and forth to mix cells across the light. Turn lamps off once time has expired. Open the tap on the Megatron and collect the irradiated culture into a large beaker. Collect the irradiated cells onto 0.45µm or 0.8µm MF-membrane filters by filtration. Transfer the cells + filter to a 50ml tube of pre-chilled PBS on ice, close the lid, tip the tube back and forth to release the cells and then remove the filter. Transfer 1/3 of the cells into each of the 2 further pre-chilled Falcon tubes and spin at **3000 x g, 4°C, 00:05:00** in a benchtop centrifuge. Pour off the supernatent, drain residual liquid from the tubes by decapping and briefly turning upside down on paper towels. Replace the caps on the tubes of cell pellets and then freeze them on dry ice before storing at -80°C. Meanwhile, wash the Megatron with water between samples and do a final rinse with 70% ethanol and allow to evaporate after the last sample.

Lysis 1h 25m

5 20m

Add **1V** (~ 1.0 ml/pellet from 1L of cells) of **TN150plus** (50mM Tris.HCl, pH7.8/ 150mM NaCl/ 0.1% NP-40 /5 mM 2-Mercaptoethanol (2-ME) + 1 small Roche complete EDTA-free protease inhibitor tablet for 10 ml, or 1 large tablet for 50 ml added fresh before use - see note below) to each cell pellet.

Note: TN150 (50mM Tris.HCl pH7.8 / 150mM NaCl / 0.1% NP-40) should be made in advance as a stock solution in milliQ water and filter sterilised.

TN150plus (TN150 + 5 mM 2-Mercaptoethanol (2-ME) + 1X Roche complete EDTA-free protease inhibitor cocktail tablet of the appropriate size), to be made from TN150 shortly before use as 2-ME is unstable in water.

NB. 2- ME is toxic and should be added in a fume hood.

6 20m

Add **2.5V** of **Zirconia beads** (0.5mm, Thistle Scientific: ~ 2.5 ml, measure in 15 ml Falcon tube) to each tube and

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thaw the pellets rapidly by vortexing each tube for **00:01:00** (one vortex for each hand), then **00:01:00** **On ice** . Repeat the bead-bashing another 5x.

7 Add another **3V TN150plus** (~ 3 ml), shake vigorously, and centrifuge the suspension in the falcon tube for **00:20:00** at 4600*g* at **4 °C** 20m

8 Transfer the supernatant (~ 4.5 ml) to 3x 1.5 ml **eppendorf safe-lock tubes** and spin lysate again for **00:20:00** at 20,000*g* in a microcentrifuge at **4 °C** . 25m

Protein A:IgG purification 4h

9 Meanwhile, transfer **125 µl** of **IgG Sepharose** beads per sample (approx 75:25 beads:buffer so **167 µl** of slurry) to a 15 ml falcon tube & wash 2x with 5 ml of **TN150plus** . (Resuspend by gently swirling beads. To pellet, pulse to 1000 rpm.) Divide beads between the same number of 15 ml falcon tubes as samples to be processed and remove the buffer. Resuspend in an equal volume of **TN150plus**. 25m

Note: This bead selection method is for is for HTP tags. For HTF or HF tags, alternative selection methods using anti-FLAG beads are necessary, but not covered in this protocol.

10 Take a **5 µl** aliquot from each **crude extract** from step **8** to be used for western blotting and store at **-80 °C** . 10m

11 Mix the rest of each crude lysate with the IgG Sepharose beads prepared at step 9 and nutate for **02:00:00** at **4 °C** (can be left a bit longer). 2h 20m

NB: Be careful to avoid taking the lipid that floats at the top – this will cling to the pipette tip, and can sometimes be seen as white wisps when you pipette up the lysate.

12 Spin down IgG Sepharose beads (pulse in centrifuge to 1000*g* at **4 °C**) and remove most of the supernatant. 15m

13 Wash the beads 2x with **10 ml TN1000plus** (50mM Tris.HCl, pH7.8/ 1M NaCl/ 0.1% NP-40 with 5 mM 2-ME added fresh before use - see note below) then 2x with **10 ml TN150 (+ 2-ME but NO protease inhibitors!)** (for each wash, gently agitate in the cold room for **00:05:00**). Decant liquid between each wash. 40m

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Note: TN1000 (50mM Tris.HCl pH7.8 / 1M NaCl / 0.1% NP-40) should be made in advance as a stock solution in milliQ water and filter sterilised.

TN1000plus (TN1000 + 5 mM 2-Mercaptoethanol (2-ME) + 1X Roche complete EDTA-free protease inhibitor cocktail tablet of the appropriate size), to be made from TN1000 shortly before use as 2-ME is unstable in water.

NB. 2- ME is toxic and should be added in a fume hood.

NO Protease inhibitors should be added to the TN150 + 2-ME at this stage as they would inhibit the TEV protease required at step 16 below.

14 After the last wash step, using a 1ml micropipette, resuspend the beads in a small volume of **TN150 (+ 2-ME but NO protease inhibitors!)**, transfer to an eppendorf tube and remove the remaining buffer. 15m

TEV cleavage 2h 45m

15 Add **600 µl of TN150** (**+ 2-ME but NO protease inhibitors!**) to the beads to resuspend them 10m

16 Add **7 µl of HALO-TEV protease** (5u/µl, Promega) and mix by inverting tube. 5m

Note: The TEV cleavage step is required for HTP or HTF tags that incorporate a TEV protease cleavage step. For HF tags, this is replaced by a FLAG-peptide elution step.

17 Incubate **02:00:00** at **18 °C** on a rotating wheel (make sure beads remain in suspension). 2h

18 Spin down mixture (1000*g*) to capture all liquid stuck in the lid. Transfer everything to a SnapCap column (Pierce) in an eppendorf tube and force the eluate through by opening and closing the cap. Spin the column in a second tube up to 1000*g* and pool the eluates to get ~ 600 µl of eluate). 30m

KEEP THE ELUATE - BUT DO NOT PUT IT ON ICE (or it will not warm up sufficiently for the RNace-IT step below).

RNAse treatment and Nickel purification 17h 5m

19 Meanwhile, for each sample, you should have an eppendorf tube prepared with 0.4 g of **Guanidine-HCl** in it. 10m

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20 **Partial RNase digestion with RNace-IT**: Put **550 µl** of the TEV eluate from step **18** in a new tube (also take 25 µl aliquots for western blot samples (store at -80^{o}C – labelled "**TEV eluate**"). Preincubate the remaining TEV eluates at **37 °C** then add **1 µl of diluted RNace-IT** and incubate the mixture for exactly **00:04:00** minutes at **37 °C** , staggering the samples by 30 seconds, so that each one gets precisely the same time of RNase treatment. When the time is up, immediately add **500 µl** to one of the tubes of **0.4 g Guanidine-HCl** from step 19, cap and immediately invert. 30m

Note: **the amount of RNace-IT required to partially digest the RNA needs to be empirically determined for each RNA & protein**: 1:10/1:20 give shorter fragments, 1:50/1:100 longer. For Ssd1 CRAC we used 1 µl of a 1:100 stock, diluted in water).

21 Vortex the samples well to dissolve the Guanidine HCl. Final volume should be around 700 µl. 5m

Note: the Guanidine will increase the pH to ~8.2. **Make sure that the pH of your Tris buffer stock is exactly 7.8**.

22 Now put the samples back on ice and to each tube, add **27 µl 5 M NaCl** (final conc 300 mM) and **3 µl 2.5 M Imidazole (pH 8.0)** (final conc 10 mM). 10m

23 Mix the solution and add all to **50 µl** of **Nickel beads** per reaction, aliquoted into eppendorf tubes (from 100 µl of 1:1 beads:slurry; pre-equilibrated 2x in **10V Wash Buffer I** and spun up to 1000*g* before removing the buffer). 10m

Note: **Wash Buffer I** (50mM Tris.HCl pH7.8 / 300mM NaCl / 0.1% NP-40/6M Guanidine HCl (28.66g/50ml)/10mM Imidazole/5mM 2-ME) should be made fresh shortly before use.

NB. 2- ME is toxic and should be added in a fume hood .

24 Nutate **Overnight** at **4 °C** . 16h

Nickel purification (cont'd) 50m

25 Transfer the beads/buffer mixture to a **SnapCap column** in an eppendorf tube **On ice** . 10m

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26 Wash beads **3x** in **400 µl cold Wash Buffer I plus** by gravity flow. 40m

For the first wash with a new buffer, **wash around the internal rim** of the column.

Close and open the cap to get the flow started, and to hurry things along if the column is running slowly.

27 If this is a test purification, for **ONE SAMPLE** go straight to **Elution/PAGE (Step 42** - to check protein purification), for **SECOND SAMPLE** wash 3x with **1xPNK buffer + 2-ME** then go to **"Phosphorylating the 5' ends of the RNA"** (**Step 36** -to check RNA crosslinking). If this is NOT a test purification, continue to **Step 28**.

TSAP Alkaline phosphatase treatment of precipitated RNAs 1h 45m

28 Wash beads 3x with 600 µl cold **1x PNK buffer + 2-ME** - **rinse inner wall of column with first wash**. 20m

Note: **1x PNK buffer** (50mM Tris.HCl, pH7.8/ 10mM $MgCl_2$/ 0.5% NP-40) can be made in advance.
For **1x PNK buffer + 2-ME** , add 2-ME, which is unstable, to 5mM shortly before use.

NB. 2- ME is toxic and should be added in a fume hood.

29 Spin out the remaining buffer at **1000 x g, 00:00:10** , put a plug (supplied with the columns) on the bottom of the SnapCap column to retain the reaction buffer, and place the column in a clean Eppendorf tube. Then, **add 80 µl** of the following mix (**set up at room temp.**): 16 µl **5x PNK Buffer** + 54µl **MilliQ water** + 2µl **RNasin** + 8µl **TSAP** (Thermo-sensitive Alkaline Phosphatase) to each sample. 20m

For **5x PNK buffer** (250mM Tris.HCl, pH7.8/50mM $MgCl_2$/ 25mM 2-ME) - make up 10ml stock in advance and aliquot into **200 µl** aliquots and store at **-20 °C** .)

30 Mix by stirring with pipette tip then flicking column gently. Close the lid. 5m

31 Incubate the beads for **00:30:00** at **37 °C** . 30m

ALWAYS open lid **BEFORE** removing plug, check occasionally for leakage from plug.

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32 On ice, wash the beads once with 400 µl **Wash Buffer I** (to make sure that the TSAP is inactivated; first wash should be round rim) and then three times with 400 µl **1x PNK buffer** (to get rid of the guanidine). 30m

On-bead ligation of miRCat-33 DNA linker (activated 5', blocked 3'; aliquoted on arrival and thawed only once) 6h 5m

33 Spin out the remaining buffer and add **80 µl** of a Master Mix composed of: 16 µl **5x PNK Buffer** + 50µl **MQ water** + 2µl **RNasin** + 8µl **3' linker** (10µM) + 2µl **T4 RNA Ligase I** (NEB) + 2µl **T4 RNA Ligase II K227A** per sample . 20m

Note: prepare a MasterMix with enough for 1 more sample than you are working with to ensure you have enough.

The **3' linker** DNA oligo has a blocked 3' end and an activated adenosine at the 5' end (5'-rAppTGGAATTCTCGGGTGCCAAGG/ddC/-3'). The blocked 3' end prevents self-ligation. Activated adenosine is like the intermediate in T4 ligase reactions and helps efficiency of this step (3' linker ligation is least efficient step). T4 ligase doesn't "like" ligating RNA and DNA. This linker can be ligated to the RNA substrate in the absence of ATP (so any residual phosphorylated RNA isn't self-ligated into a circle). After this step it is OK to have ATP there because transcripts with the 3' linker attached will be blocked at the 3' end). In this case, the RNAs should not ligate to themselves but only to the linker. Produces 5'OH-------ddddddd-/ddC/-3'

34 Incubate the reaction for at least **05:00:00** at **25 °C** 5h

35 Wash beads once with 400 µl **wash buffer I** (freshly made; this ensures that the ligase is inactivated; first wash should be round rim) and then **3x** with 400 µl **1x PNK buffer** (to get rid of guanidine) as before. 45m

Phosphorylating the 5' ends of the RNA (radiolabelling) 2h 40m

36 Spin out the remaining buffer, plug columns and transfer to fresh 1.5ml tubes and add in the following order: 16µl **5x PNK Buffer** + 56µl **MilliQ water**, then 4µl **T4 PNK** (Sigma, 5U/µl) and then 4µl **^{32}P-γATP** (10µCi/µl) per sample (make Master Mix without ^{32}P-γATP added and then add the radiolabel). 30m

37 Incubate the reaction for **00:40:00** at **37 °C** . 40m

38 Add 1 µl 100 mM ATP and let the reaction proceed for another 20 minutes. 30m

This step ensures that almost all of the 5' ends have phosphates.

39 At room temperature, wash beads 3x with 400 µl fresh **wash buffer I** then 3-4x times with **1x PNK buffer** (until ~20-50 cps in flow through, as measured with a GM monitor). 1h <

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Here, we typically place a large number of open eppendorf tubes into a rack. We put the column into the first tube, let the wash flow through, then transfer the column to the next tube for the next wash. This avoids replacing the column into a tube that has contained radioactive washes, which can get messy.

On-bead ligation of the 5' SOLEXA linker 16h 30m

40 30m

Spin out the remaining buffer from Step **39** and add 80 µl of the following mix: 16µl **5x PNK Buffer** + 55.2µl **MilliQ water** + 0.8µl **100mM ATP** + 2µl **100µM 5'-linker** (*a unique linker for each sample*) + 2µl **RNasin** + 4µl **T4 RNA ligase**.

The 5' linkers came from IDT. They are mixed DNA:RNA oligos with inverted ddT at the 5' end to prevent degradation; at the 3' end is a barcode sequence, a random 3-mer to reveal pcr duplicates, and a constant 3 nucleotide 3' terminus to ensure all linkers ligate with equal efficiency. Again, these are aliquotted into 5 µl aliquots, which are only thawed once or twice.

41 Incubate at **16 °C** **Overnight** . 16h

Elution and PAGE (Day 1) 8h 30m

42 Wash the beads 3x with 400 µl of **Wash Buffer II**. 30m

Wash Buffer II (50mM Tris.HCl,pH7.8/ 50mM NaCl/ 0.1% NP-40/ 5mM 2-ME - made fresh)

Wash Buffer II plus (To 5ml **Wash Buffer II**, add 280µl of 140mM Imidazole stock just before use).

43 Spin out the void volume and elute RNP complexes twice with **wash buffer II plus.** For each elution, put plug on column, incubate beads with 62.5 µl of elution buffer (for -/+ heatshock samples) or 125µl (for single control samples) for10 minutes, spin into RNase-free eppendorf, repeat); pool eluates. 30m

44 Add 40µg (2µL) of **Glycoblue** to each sample and **5V** of acetone (1.25 mL) and leave at -20ºC for at least **02:00:00** before spinning at **16000 x g, 4°C, 00:05:00** , removing the liquid, spinning again briefly to remove residual acetone and resuspending the pellets in 25µl of 1x NuPAGE Sample Buffer (with 2-ME added to 6%). Heat samples at **65 °C** for **00:10:00** mins and then spin briefly. Pipette up and down to check fully resuspended using the Geiger counter. (At the same time, the crude lysate and TEV eluate samples collected earlier can 3h

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be mixed with 4x NuPAGE Sample Buffer + 24% 2-ME, heated at **95 °C** for **00:05:00** , ready to run on a second NuPAGE gel for Western Blot analysis.

45 Load radioactive samples in alternate wells of a 1.5 mm thick 10 well **NuPage 4-12% gradient gel** in **1x NuPAGE MOPS** running buffer (500 ml). Ensure samples and ladder (SeeBlue Plus 2 or similar) are well separated, and use a standard 1.0mm thick12-well NuPAGE 4-12% gradient gel with Abcam protein markers (or similar) for the Western Analysis. Run gels at 150V for **01:00:00** - **01:30:00** until the blue dye reaches the bottom. 1h 45m

NB. this gel system is **absolutely essential** since the pH remains roughly **pH7** during the run. You cannot use the "normal" SDS-PAGE gels because the pH can go up to **pH9** leading to hydrolysis of your RNA.

46 Transfer the proteins to **Hybond-C Extra nitrocellulose membrane (Amersham;** or Similar**)** using the wet transfer system and **NuPAGE Transfer Buffer** (Life Technologies : 1 litre; **15 % MeOH** (although the methanol can be omitted for large proteins). Transfer the proteins for **01:30:00** at 100V. 1h 45m

Blot setup:

Hold membrane and 1 Whatman filter paper together and dunk in Transfer Buffer (TB). Smooth out bubbles. Place on top of gel and peel gel off onto membrane. Add 1 Whatman filter soaked in TB to top. Soak sponges, and assemble the final sandwich. Ensure gel to negative (black), membrane to positive. (Cut membrane and three whatmans to 8 x 6.5cm. Crack open with wedge and place membrane on top. Dip into the buffer. Place one Whatman filter on top of membrane. Flip over so that you can see the gel and dip in buffer. Pry apart with wedge. Place on white side of cassette (on top of the sponge). Place whatman on top and close. Place in apparatus. (Black to black and white to red.).

47 For Western analysis block **01:00:00** in 5% skimmed milk in TBS-Tween (TBS-T), and probe **Overnight** at 4°C with a 1:5000 dilution of rabbit anti-TAP antibody (Open Biosystems) in the blocking buffer. 1h

Note: Different antibodies will be needed to detect HF-tagged proteins.

48 For the radiolabelled blot (test crosslinking or full CRAC expt): briefly dry membrane, expose to film (place membrane in acetate sleeve with luminous ladder for orientation well away from the membrane then put into film cassette so that the film is next to the intensifying screen (white) then the membrane is on top of the film. **01:00:00** at -80°C with an intensifying screen is normally enough but sometimes **Overnight** is required (as was the case for Ssd1-HTP CRAC). Kodak MS film is most sensitive film, MP is less sensitive but sometimes adequate. 15m

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Elution and PAGE (Day 2) 6h 20m

49 Wash the western blot 3x in TBS-T for **00:10:00** each then probe **01:00:00** at RT with 1:10,000 IRDye680 conjugated Donkey anti-rabbit secondary antibody in TBS-T. Wash 3x **00:05:00** each in excess TBS-T then coolect image on the Licor Odyssey. 1h 45m

50 Develop film of radio-active blot and then take a photo or scan the film as it will be cut at the next step. 30m

51 Cut out the position of the bands corresponding to the size of your protein + the RNA from the autorad: cut from the middle of the tight band plus the smear above (to try and get mostly cross-linked species). Use the position of the luminous ladder to orientate the film over the membrane and cut out the region of the membrane within the cut-out region of the film, using a separate scalpel for each sample to prevent cross-contamination. Slice each piece of membrane into a few bits. (Can store membrane slices **Overnight** at **-80 °C**). 20m

52 Incubate the membrane slices with **400 µl of wash buffer II containing 1% SDS and 5 mM EDTA** and **add 100 µg (5 µl) of Proteinase K** (Roche; 20 mg/ml stock in water, stored at -20ºC). Shake at **500 rpm, 55°C, 02:00:00** . 2h

53 Transfer the supernatant to a fresh tube (to remove the membrane), and add **50 µl of 3M NaOAc, pH 5.2** (pH'd with acetic acid) and **500 µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1).** Vortex, **12000 x g, Room temperature** for **00:20:00** then take the top layer to a new tube. 30m

54 Precipitate the RNA with **1 ml of** absolute **Ethanol** and **2µl of Glyco-blue**. Incubate at **-80 °C** for **00:30:00** then spin at **16000 x g, 4°C, 00:20:00**) (or store at **-20 °C** **Overnight**). 1h

55 Wash pellet with **70% EtOH** (vortex), spin for **16000 x g, 4°C, 00:05:00** , remove liquid and air-dry. 15m

Reverse Transcription 2h 10m

56 Resuspend the RNA pellet in 13µl of the following mix (by pipetting): 11µl of MQ water + 1µl of 10µM **miRCat RT oligo** (CCTTGGCACCCGAGAATT) + 1µl of freshly mixed **10mM dNTPs** and heat at **80 °C** for **00:03:00** before snap-chilling on **On ice** for **00:05:00** . Collect the contents by brief centrifugation and add 6 µl of the following mix: 4µl of 5x First Strand Buffer (from Superscript IV kit) + 1µl of 100mM DTT (from kit) + 1µl RNasin (40u/µl) prepared as a 7x Mastermix.

57 Incubate the mixture at **50 °C** for **00:03:00** mins and then add 1 µl of **Superscipt IV** (kit) and incubate the reaction for **01:00:00** at **50 °C** . 1h 3m

58 Inactivate **Superscipt IV** by incubation at **65 °C** for **00:15:00** . 15m

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59 Add 2 µl of **RNase H** (NEB) and incubate for **00:30:00** at **37 °C** . 35m

PCR Amplification 3h 20m

60 Use 3x 4 µl of each RT reaction for PCR. For each RT reaction, make 3x 50µl reactions containing 5µl 10x LA Taq Buffer + 1µl each of 10µM PCRfwd (P5 PCR Forward Primer)and PCRrev (PE mircat Reverse Primer) primers + 1µl of 10mM dNTPS (fresh!) + 39.25µl of nuclease-free water + 0.5µl Takara LA Taq Polymerase + 4µl of RT reaction from previous step. (NB: the oligo sequences depend on your 5' linker). 50m

P5 PCR Forward Primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

PE mircat Reverse Primer:
CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGGCCTTGGCACCCGAGAATTCC

61 PCR amplify using the following Programme: (95ºC for 2 min; (98ºC for 20s/52ºC for 20s/ 68ºC for 20s) for 20-25 cycles(use the minimum number of cycles possible - try 23-24 for a first attempt, then reduce the number of cycles if possible to give a total yield of ~5-50 ng of DNA - used 22 cycles for Ssd1-HTP CRAC); 72ºC for 5 mins). 1h

62 Pool and precipitate products: add, on ice, 2.5V **EtOH** (375 µl) + 0.1V **3.0 M NaOAc** (15 µl) + 20 µg Glycoblue (1 µl of 20 µg/µl); incubate at **-80 °C** for **00:30:00** then spin at **16000 x g, 4°C, 00:20:00** , wash pellets in **70% EtOH**, spin again **16000 x g, 4°C, 00:20:00** , air dry (make sure all ethanol is evaporated, or your sample will float out of the wells when you run it on the gel) and resuspend in 15 µl nuclease-free water. 1h 30m

Gel Purification 5h 25m

63 Prepare a **3% Metaphore agarose** gel (75 ml in 1x TBE Buffer) while PCR is running, (takes a while): soak agarose in 1xTBE for 30 min; heat slowly in microwave (will make lots of foam); add 1:10,000 dilution of SYBR Safe ; pour carefully (use a pipette to eliminate bubbles) and remove bubbles (rake with comb); when gel is set, put it at 4°C until samples are ready (at least 30 min). 2h

64 Add 6x Purple Loading Dye (New England Biolabs) to samples (use 5 µl of loading dye for a 15 µl sample, to help ensure that the sample does not float out of the wells even if there is a little bit of residual ethanol). Run the gel with the kit mounted on a metal block in ice to prevent it melting! 10m

65 Load samples (rinse tip in beaker of 1xTBE after taking up sample, to ensure no sample is on the outside of the tip) and include 6 µl of a 50 bp DNA ladder (NEB). 15m

66 Run gel at 80V until bromphenol blue band to the bottom. Scan gel using the phosphorimager, and print at 100% magnification as a negative image. 2h 30m

67 To visualize bands, place the printed 100% sized gel image under a sheet of acetate. Cut bands from agarose gel using a scalpel. You should see a sharp band at ~120 bp from linker dimers... you do not want to cut this. Your library should migrate as a smear starting at ~130 bp and extending for 50 bp or more. Used scalpels (1 per lane) to cut out area of gel above primer dimers up to ~180bp. Re-scan gel after cutting out to ensure the correct region has been excised OK. 30m

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Gel Extraction 2h

68 Using the QIAquick Gel Purification KIt (Qiagen) or Zymo Gel Clean and Concentrate Kit with **mini elute columns**, follow the provided gel extraction protocol by melting the gel at **42 °C** , washing in Buffer QG (without isopropanol) and letting the column stand in an additional PE Buffer wash step for **00:10:00** (inverting the column: ensures all GHCl is removed). Elute with 20µl nuclease-free water: put water on membrane and let stand for 2-3 mins before spinning out. This is your sample for high throughput sequencing. We measure the DNA concentration using the QuBit with the dsDNA HS Assay Kit. 2h

Checking CRAC Library Concentrations 15m

69 The concentration of the CRAC Library generated above is checked on the Qubit Fluorometer (ThermoFisher Scientific), using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and manufacturer's protocol. 15m

Bring all solutions to room temperature.

69.1 Prepare Standards and Samples to be Tested: 30m

Set up required number of Qubit 0.5ml assay tubes for standards (x2) and the number of samples to be assayed and label the lids.

Prepare sufficient Qubit® Working Solution (to accommodate all standards and samples, allowing 200µl for each) by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer.

Add 190 µL of Qubit® working solution to each of the standard tubes and 198 µL to each sample tube.

Add 10 µL of each Qubit® standard or 2 µl of test sample to the appropriate tube, then mix by vortexing for 2–3 mins. (Be careful not to create bubbles!).

Incubate at room temp. for 2 mins.

69.2 Calibration of Fluorimeter: 10m

Plug in Qubit 2.0 Fluorimeter and switch on. On the Home screen, press 'DNA', then select 'dsDNA High Sensitivity' as the assay type. The 'Read standards' screen is displayed. If option to use previous calibration is offered, ignore it and set new standards. Press 'Read Standards' to proceed.

Insert Standard #1 tube into the sample chamber, close the lid, then press 'Read standard'. When the reading is complete (~3s), remove Standard #1. Repeat with Standard #2. Instrument will draw a Standard Curve.

69.3 Measuring Sample Concentrations: 40m

Press 'Run Samples'.

Add first sample tube and measure - this gives the QFvalue of the diluted sample in ng/ml. By diluting the sample 2 µL to 200µL for measurement this is the concentration of a 1/100 dilution.

Calculate stock concentration by multiplying by 100.

Conc. of library = QFvalue x 200/2 ng/ml

Convert to ng/µL = conc. in ng/ml/1000

Repeat for each sample.

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69.4 Prepare 10nM dilutions of each library: 30m

Average concentration of 150nt DNA fragments @ 1ng/µL = 10nM

Make 10nM dilutions of each library by diluting to 1ng/µL

(ie. if library conc. = y ng/µL, dilute 2µL of library to a total volume of (2 x y)µL.

If concentration of the untagged negative control sample is below 1ng/µL, dilute by the same amount as for the lowest library concentration.

Preparing CRAC Libraries for Sequencing 2h

70 Prepare for MiniSeq Run:

Note: this library could now be submitted to (any) Illumina sequencer. The Miniseq is only one option.

70.1 Remove Reagent Cartridge from -20°C Freezer and thaw gently in a tray of warm tap water for at least **00:30:00** . 1h

Thaw Hybridisation Buffer from -20°C at **Room temperature** and then store **On ice** , vortex briefly before use.

Bring Flow Cell to **Room temperature** for at least **00:30:00** in its foil wrapper.

Make a fresh dilution of 0.1N NaOH from stock and mix well.

Make a 200mM dilution of Tris.HCl, pH7.0 and mix well.

Prepare RSB Buffer: 10mM Tris.HCl, pH8.5 + 0.1% Tween-20.

70.2 Dilute and Denature Libraries: 30m

Prepare 10µL of 1nM pooled libraries by mixing 1µL of each 10nM library and making it up to 10µL with RSB Buffer, vortex briefly and spin at **1000 rpm, Room temperature , 00:01:00** .

Denature 5µL of this pool by adding 5µL of 0.1N NaOH and incubate at **Room temperature** for **00:05:00** .

Add 5µL of 200mM Tris.Hcl, pH7.0, vortex briefly and spin **280 x g, 00:01:00** .

Add 985µL of pre-chilled Hybridisation Buffer from Kit to denatured library (Total vol. = 1ml of 5pM). Vortex briefly and spin at **280 x g, 00:01:00** .

Transfer 135µL diluted library to a new microcentrifuge tube and add 365µL of pre-chilled

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Hybridisation Buffer. Total vol. is 500µL at 1.8pM.

Vortex briefly and spin at **280 x g, 00:01:00** .

70.3 Set up MiniSeq System: 30m

Switch Instrument ON and after automated check, select START.

From Home Screen, select **Sequence**. This releases consumables from previous run and opens a series of run setup screens.

Open reagent door by gently pulling forward on the side edges and remove formamide waste bottle and transfer waste to bottle in fumehood, replace waste bottle.

Prepare flow cell: remove from foil with clean gloves, unscrew container and carefully remove flow cell from holder by the plastic cartridge. Clean glass surface of flow cell, avoiding the black flow cell gasket, with an alcohol-soaked lens tissue and dry with a dry lens tissue. Hold up to light and check for any smears. Dry flow cell with spray air and recheck. Load into Flow Cell Compartment after removing old one by pressing the release button. Close latch and compartment door.

Dry thawed Reagent Cartridge with paper towel, invert 5x to ensure reagents within are mixed and then check for no bubbles or ice crystals. Tap gently on bench to disperse any bubbles

Load denatured library into well 16 of Reagent Cartridge by piercing the foil with a clean pipette tip and then loading with a micropipette. Check for bubbles at bottom.

Remove old spent cartridge if present. Slide cartridge loaded with library into slot on sequencer until cartridge stops.

70.4 Enter Run Parameters: 15m

1. Enter a run name of your preference.
2. [Optional] Enter a library ID of your preference.
3. From the Recipe drop-down list, select a recipe. Only compatible recipes are listed (For CRAC use 'TruSeqSmallRNA', sequence length = '75')
4. Select a read type, either Single read or Paired end (we do Single End Reads so select "Single").
5. Enter the number of cycles for each read in the sequencing run.

****NOTE: Do not open the reagent compartment door or the flow cell compartment door during the automated check or during the sequencing run.****

71 Collect Sequencing Data from the machine and Analyse. The structure of a sequencing read prepared from the library is shown below. We use Sander Granneman's CRAC pipelines (available from https://git.ecdf.ed.ac.uk/sgrannem/crac_pipelines), built on the pyCRAC software, to analyse the data. However, the choice of analysis steps depends on your protein's binding patterns and on your scientific goals.

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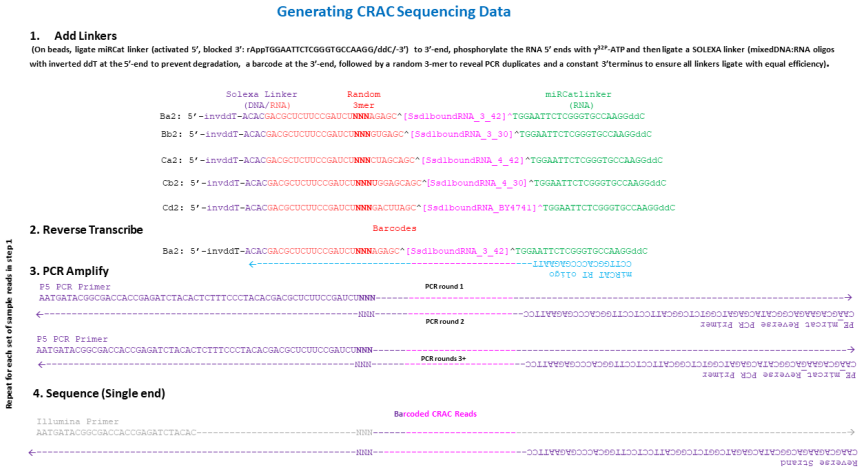

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