Capture Hybridization Analysis of RNA Targets (CHART)

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ABSTRACT

The genome is regulated by *trans*-acting factors that bind to specific loci in chromatin. In addition to protein factors, it has become clear that large non-coding RNAs can also act on chromatin at sites distant from where they are transcribed. This unit describes a means of identifying the genomic targets of those large non-coding RNAs. To accomplish this, the endogenous RNA of interest (here *Drosophila roX2* is used as an example) is enriched from cross-linked chromatin extracts using short biotinylated complementary oligodeoxyribonucleotides. The targets of the RNA can be determined by examining the proteins and DNA that are enriched under these conditions. This analysis can be extended genome-wide by subjecting the enriched DNA to deep sequencing. *Curr. Protoc. Mol. Biol.* 101:21.25.1-21.25.16. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit describes CHART (Capture Hybridization Analysis of RNA Targets), an approach used to analyze RNA targets that is analogous to chromatin immunoprecipitation (ChIP, *UNIT 21.19*) for proteins. Similar to a ChIP experiment, the factor of interest is enriched from cross-linked chromatin extracts. Whereas ChIP employs antibodies that recognize an accessible region on the protein of interest, CHART employs capture oligonucleotides that are designed to specifically hybridize to the RNA of interest. Using these capture oligonucleotides, the RNA is enriched together with its targets. Similar to a ChIP experiment, the CHART-enriched DNA can be analyzed to determine where the RNA was bound in the genome.

While the principles that underlie CHART are general for large non-coding RNAs (lncRNAs), for clarity, the protocol is presented here for purifying a specific RNA, *roX2*, from *Drosophila* cell extracts. Basic Protocol 1 describes the isolation of nuclei from *Drosophila* S2 cells, but can also be applied to mammalian cell lines. Basic Protocol 2 describes the use of these nuclei to map the accessible regions of the RNA for the design of capture oligonucleotides. Basic Protocol 3 describes the use of these capture oligonucleotides to enrich *roX2* along with its associated targets. To identify these targets, Basic Protocol 4 describes the analysis of CHART-enriched DNA and proteins.

PREPARING CROSS-LINKED NUCLEI

BASIC PROTOCOL 1

CHART enrichment is performed using formaldehyde-cross-linked chromatin extracts. Formaldehyde serves to covalently connect the RNA to its biological targets at the time of cross-linking, while the cells are still intact. As the chromatin-bound RNAs are found in the nucleus, it is beneficial (although not strictly necessary) to purify the nuclei from the cells prior to CHART analysis. The nuclei are later subjected to further cross-linking with higher levels of formaldehyde (see Basic Protocol 3). If this higher

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degree of cross-linking were to be performed initially, it could interfere with isolation of nuclei. Therefore, the first steps described in this protocol are to perform low levels of formaldehyde cross-linking and to enrich the cell nuclei.

Materials

Drosophila S2 cells (ATCC, cat. no. CRL-1963)
CCM3 medium (Hyclone, cat. no. SH30065.02)
Penicillin-streptomycin mixture (e.g., Lonza, cat. no. 12001-350)
Phosphate-buffered saline (PBS; *APPENDIX 2*)
16% (w/v) formaldehyde (10 ml ampules, Thermo Scientific, cat. no. 28908)
Liquid N_2
Sucrose buffer (see recipe), ice cold
Glycerol buffer (see recipe)

500-ml shaker flasks
27°C incubator without added CO_2: e.g., New Brunswick Scientific Excella E24 Incubator Shaker
End-over-end rotator
Refrigerated centrifuge
Glass Dounce homogenizer with tight pestle (15 ml)
15-ml conical tubes (e.g., BD Falcon)

1. Inoculate 50 ml of a 10^7 cell/ml S2 culture into a 500-ml shaker flask containing 200 ml of CCM3 medium (which can be supplemented with 1× penicillin-streptomycin) and shake at 27°C in a shaking incubator (without additional CO_2, e.g., New Brunswick Scientific Excella E24 Incubator Shaker). Shake until cells reach a density of 10^7 to 10^8 cells/ml.

   CHART experiments require similar quantities of starting material as ChIP experiments. Whether using insect cells such as the S2 cells described here, or mammalian cells, it is convenient to grow sufficient material to generate several cell pellets of 10^8 cells/aliquot for mammalian cell lines, or 10^9 cells/aliquot of insect cell lines. The minimum material required for a successful CHART experiment is around 2.5×10^6 cells, but using 10^7 to 10^8 cells per CHART enrichment is preferable, especially for deep sequencing of the enriched DNA.

2. Harvest 10^{10} cells by centrifugation for 15 min at 500 × *g*, room temperature, rinse once with PBS, and resuspend in 200 ml PBS.

   For mammalian cell lines, it is convenient to cross-link 10^8 to 10^9 cells.

3. Add formaldehyde to 1% (v/v) final concentration and allow the suspension to rotate end-over-end for 10 min at room temperature.

   Other cross-linking protocols, including those that involve the addition of formaldehyde directly to the medium of a mammalian cell culture dish, have also proven successful for CHART.

4. Capture the cells by centrifugation for 15 min at 500 × *g*, 4°C, rinse three times with cold PBS, centrifuging again in-between rinses, and use immediately or aliquot (1×10^9 cells/aliquot). Prior to freezing, decant the PBS, flash freeze the pellet, with liquid nitrogen, and store at −80°C.

5. Resuspend one pellet (1×10^9 S2 cells or 1×10^8 mammalian cells) in 4 ml ice-cold sucrose buffer.

6. Transfer the suspension to an ice-cold Dounce homogenizer. Homogenize ten times with a tight pestle. Wait 5 min, then homogenize ten more times.

7. Add 4 ml of glycerol buffer to a 15-ml conical tube. Add 4 ml of glycerol buffer to the mixture in the Dounce homogenizer and mix by pipetting up and down several times. Carefully layer this solution of cell debris on top of the glycerol buffer in the 15-ml conical tube.
8. Centrifuge the tube 10 min at 1000 × *g*, 4°C, to pellet the nuclei.
9. Remove the supernatant using a pipet, taking care to remove the upper layer with minimal mixing.
10. Repeat steps 5 to 9 one additional time.

This pellet of enriched nuclei can either be carried directly into the RNase H mapping protocol (Basic Protocol 2), or further cross-linked and used for CHART enrichment (Basic Protocol 3).

DESIGN OF CAPTURE OLIGONULEOTIDES THAT TARGET ACCESSIBLE REGIONS OF THE RNA

BASIC PROTOCOL 2

The objective of this protocol is to design capture oligonucleotides that can hybridize specifically to the desired RNA, in this case *roX2*. In the context of cross-linked chromatin extracts, it is expected that some regions of the RNA will be more accessible for hybridization than others due to either secondary structure or steric occlusion by proteins. The purpose of this protocol is to identify the regions that are accessible for hybridization and design capture oligonucleotides that target these regions. To accomplish this, a chromatin extract is made from the nuclei generated in Basic Protocol 1. Candidate 20-mer synthetic DNA oligonucleotides are then mixed one at a time with this chromatin extract in the presence RNase H, which hydrolyzes RNA at sites of RNA-DNA hybrids. Oligonucleotides that hybridize to accessible sites in the RNA produce RNA-DNA hybrids and lead to enzymatic cleavage of the RNA. The degree of this RNase H sensitivity can be determined using RT-qPCR. Oligonucleotide sequences that lead to high RNase H sensitivity are used to design biotinylated capture oligonucleotides for CHART enrichment (Basic Protocol 3).

Materials

Nuclei pellet (10^9 S2 nuclei or 10^8 mammalian nuclei from Basic Protocol 1)
Nuclei wash buffer (see recipe)
Sonication buffer (see recipe)
Liquid N_2
1 M $MgCl_2$
1 M dithiothreitol (DTT)
5 U/μl RNase H (NEB, cat. no. M0297L)
20 U/μl SUPERasIN (Ambion, cat. no. AM2696)
20-mer oligonucleotides (IDT; for more information see Step 11.
RQ1 DNase (Promega, cat. no. M6101)
1 M $CaCl_2$
Quenching buffer (see recipe)
PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, cat. no, 12183-018)
SuperScript VILO cDNA Synthesis Kit (Invitrogen, cat. no. 11754-050)
iTaq UniverSYBR Green (Bio-Rad, cat. no. 172-5120)
Appropriate qPCR primer sets

Refrigerated centrifuge
Covaris S2 instrument (or other similar means of shearing DNA)
1.7-ml microcentrifuge tubes
PCR tubes in strips of 8

Thermal cycler
ABI 7500 RT-PCR instrument or equivalent
NanoDrop spectrophotometer (Thermo Scientific; also see *APPENDIX 3J*)

Additional reagents and equipment for microvolume quantitation of nucleic acids using a NanoDrop spectrophotometer (*APPENDIX 3J*)

1. Resuspend the nuclei in 5 ml ice-cold nuclei wash buffer.
2. Centrifuge the tube 10 min at 1000 × *g*, 4°C, to pellet the nuclei.
3. Repeat steps 1 to 2 one additional time (two rinses total).
4. Resuspend pellet in 3 ml of sonication buffer and centrifuge as in step 2.
5. Resuspend the pellet to 3 ml final volume (~1.5 ml added buffer) with sonication buffer.
6. Process the nuclei using a Covaris instrument (30 min program, 10% duty cycle, intensity of 5, 4°C) to make the chromatin soluble through fragmentation.

   This assay has been successful using extract solubilized by different means, including Branson sonication, and with average shear sizes ranging from 200 bp to 5 kb. It is likely that any instrument successfully used for ChIP experiments can be applied successfully so long as it does not lead to RNase contamination of the extract, which is one advantage of using a noninvasive instrument like Covaris.

7. Separate the extract into four 1.7-ml tubes and clear the extract by microcentrifugation for 10 min at 16,100 × *g*, room temperature.
8. Separate the cleared extract into aliquots of 250 µl. Proceed to step 9 immediately or flash freeze (N_2) and store at −80°C.
9. Set up a master mix (e.g., 36× master mix) of the following reagents:

   10 µl cleared extract (e.g., 360 µl)
   0.03 µ1 1 M $MgCl_2$ (e.g., 1.1 µl)
   0.1 µl 1 M DTT (e.g., 3.6 µl)
   1 µl 5 U/µl RNase H (e.g., 36 µl)
   0.5 µl 20 U/µl SUPERasIN (e.g., 18 µl).

10. In 8-strips of PCR tubes, add 10 µl master mix from step 9 to each tube.
11. Add 1 µl of DNA oligo (100 pmol/µl stock) to each tube except for two controls, where water should be used instead of a DNA oligonucleotide.

    For a relatively short (~600 nt) RNA such as roX2, the majority of the RNA is tiled (meaning that overlapping oligonucleotides spanning the entirety of the region of the RNA are designed as described below). However, for longer RNAs, comprehensive tiling would be very resource-intensive, and instead candidate regions are chosen based on the following criteria when information is available: (1) regions near conserved elements within the target RNA, (2) regions near known sites of protein interactions, and (3) regions that have low repeat density. The tiled nucleotides are 20-mers that are complementary to the target RNA and overlap each other by 10 nt (e.g., Oligo 1 targets nucleotides 1 to 20; Oligo 2 targets nucleotides 10 to 30; Oligo 3 targets nucleotides 20 to 40; etc.). The DNA oligonucleotides used do not need to be purified beyond standard desalting.

12. Mix by pipetting up and down 20 times. Bring the liquid to the bottoms of the tubes by quick (~5 sec) microcentrifugation.
13. Incubate in a thermal cycler at 30°C for 30 min.

    A range of temperatures (30° to 37°C) and times (30 min to 1 hr) has been successfully employed.

14. Quick-spin the tubes as in step 12 and add 1 µl of the following freshly premixed reagents cocktail (with the example of a 40-reaction experiment):

    1 µl per reaction RQ1 DNase (e.g., 40 µl)
    0.1 µl per reaction of 60 mM $CaCl_2$ (made by diluting 6 µl of 1 M stock into 94 µl distilled, deionized water).

15. Incubate at 30°C for 10 min.

16. Quick-spin (see step 12) to capture all of the liquid. To quench, add 2 µl of freshly made quenching buffer into the cap, close, quick-spin, mix by gentle flicking, and quick-spin once more.

17. Incubate in a PCR thermal cycler for 60 min at 55°C; then 30 min at 65°C.

    This short cross-link reversal protocol saves time and works nearly as well as longer protocols. For modest increases in reversal efficiency, extend the 65°C incubation 1 to 2 hr.

18. Quick-spin (see step 12) to capture the liquid. Purify RNA using a PureLink RNA isolation kit (or similar product) according to the manufacturer's directions. Include an extra on-column DNase step. Elute the RNA into 30 µl distilled, deionized water.

    Other RNA isolation products have also been successfully used. Also note that the earlier DNase treatment (step 14) is prior to cross-link reversal (step 17), and therefore a second DNase treatment is included to remove DNA that was protected by cross-linking.

19. Determine the approximate concentration of RNA, e.g., using a NanoDrop spectrophotometer (*APPENDIX 3J*).

    This step is for quality control to ensure the RNA was not lost during handling. Usually the yield is between ~100 and 200 ng/µl.

20. Set up reverse transcription (RT) reactions as follows:

    2 µl 5× VILO master mix
    7 µl RNA solution from step 18
    1 µl VILO RT enzyme (include one RT-minus control).

21. Incubate as follows:

    10 min at 25°C
    60 min at 42°C
    5 min 85°C
    hold at 4°C.

22. Dilute each RT reaction with 10 µl distilled deionized water.

23. Prepare iTaq UniverSYBR Green as follows:

    10 µl Supermix (need about 1.00 ml/plate)
    5 µl of primer mix (6 pmol of each primer)
    5 µl of RT reaction (use multichannel pipettor)

24. Cycle as follows:

1 cycle:	5 min	94°C (initial denaturation)
40 cycles:	30 sec	94°C (denaturation)
	30 sec	52°C (annealing)
	1 min	72°C (extension).

For each RNase H reaction, analyze using at least three primer sets: (1) a primer set that amplifies a region of the target cDNA that includes the oligo probe, (2) a control primer set for an unrelated RNA (e.g., Act-5C transcript) to normalize input levels, and (3) a control primer set outside the putative region of sensitivity but part of the target cDNA.

25. Analyze results with the following formula:

$$\text{RNase H sensitivity} = \left(\frac{\text{efficiency}_{\text{TARGET PRIMERS}}^{C_{T,\,\text{oligo}} - C_{T,\,\text{no oligo}}}}{\text{efficiency}_{\text{CONTROL PRIMERS}}^{C_{T,\,\text{oligo}} - C_{T,\,\text{no oligo}}}} \right)$$

Equation 21.25.1

The locations of the peaks in sensitivity are robust, but the numerical sensitivities vary. This is acceptable because only the relative (and not the absolute) sensitivity is important. The efficiencies for each primer set can either be determined experimentally [as in Simon et al. (2011), figure S1A] or estimated as ∼2.

26. Analyze the peaks from RNase H mapping, focusing on regions where two or more consecutive oligonucleotides induce sensitivity. Optimize 24- to 25-nt sequences by BLAST for specificity in the genome and against off-target RNAs.

Generally, calculated melting temperatures between 58°C and 65°C are optimal.

Determination of the relative importance of various capture oligonucleotide design parameters is ongoing; the optimization of these parameters will be established as CHART is applied to more RNAs.

27. Use these sequences to synthesize oligonucleotides of the form: [OLIGO SEQ]-L-BIO, where L represents a C18-spacer, and BIO is 3′-biotin TEG.

These oligonucleotides can be ordered commercially (e.g., http://www.idtdna.com).

Using 3′-modified oligonucleotides (as opposed to 5′-modified oligonucleotides) is preferable because the modifications will block the capture oligonucleotides from unwanted participation in downstream library preparation steps.

28. Prepare working dilutions of the capture oligonucleotide cocktails at 300 pmol/µl of each oligo.

BASIC PROTOCOL 3

PERFORMING CHART ENRICHMENT

The capture oligonucleotides from Basic Protocol 2 can be used to enrich the target RNA from cross-linked chromatin extracts. For optimal enrichment, the chromatin extract is made using nuclei that are cross-linked to a greater extent than in traditional ChIP protocols. Therefore, the first part of this protocol is formaldehyde treatment of the nuclei. The chromatin is then sheared into smaller fragments, and the capture oligonucleotides are added under hybridization conditions. These conditions are optimized to maintain high solubility of the chromatin extract and balance high yields of the desired RNA with the necessary stringency to avoid hybridization-induced artifacts. After capturing and rinsing the desired RNA with its targets, the bound material is eluted enzymatically.

Materials

Nuclei pellet from 1×10^9 cells (Basic Protocol 1)
Phosphate-buffered saline (PBS, *APPENDIX 2*), pH 7.4
16% (w/v) formaldehyde (10-ml ampules, Thermo Scientific, cat. no. 28908)
Wash Buffer 100 (WB100; see recipe)
20 U/µl SUPERasIN (Ambion, cat. no. AM2696)

Roche complete EDTA-free protease inhibitor cocktail (resuspend each tablet in 500 µl H_2O to provide a 100× working solution)
Liquid N_2
1 M dithiothreitol (DTT)
Denaturant buffer (see recipe)
2× hybridization buffer (see recipe)
20 µM CHART capture oligo cocktail (Basic Protocol 2)
MyOne Dynabeads C1 (Invitrogen, cat. no. 650.02)
Wash buffer 250 (WB250): see recipe for WB100, but use 250 mM NaCl
RNase H elution buffer (HEB; see recipe)
5 U/µl RNase H (NEB, cat. no. M0297L)

End-over-end or spinning rotator
15- and 50-ml conical centrifuge tubes (BD Falcon)
Sonicator (micro-tip type, e.g., Misonix 3000, *http://www.misonix.com/*)
Dynal magnets for 1.7-ml tubes
Dynal magnetic stand

1. Thaw a pellet of nuclei from Basic Protocol 1 on ice.
2. Rinse the pellet twice, each time with 10 ml PBS using centrifugation for 10 min at 1000 × *g*, 4°C, to capture the nuclei between each rinse. Use the rinses to transfer the nuclei to a 50-ml conical centrifuge tube.
3. Resuspend pellet in 40 ml PBS and add an entire 10-ml ampule of 16% formaldehyde. Rotate the tube for 30 min at room temperature on an end-over-end or spinning rotator.
4. Centrifuge 10 min at 1000 × *g*, 4°C, to collect the nuclei, remove the supernatant, and resuspend to 50 ml with PBS.
5. Centrifuge 5 min at 1000 × *g*, 4°C, to collect the nuclei, remove the supernatant, and transfer the pellet to a 15-ml conical tube using two rinses of 5 ml PBS (10 ml total).
6. Centrifuge 5 min at 1000 × *g*, 4°C, to collect the nuclei, and rinse pellet twice with WB100, centrifuging again between rinses.
7. Resuspend the nuclei to 3 ml final volume with WB100 supplemented with SUPERasIN (20 U/ml final) and 1× protease inhibitors.
8. Sonicate the nuclei at power level 5 to 6 (holding the output between 30 to 40 W) for 10 min total process time (15 min on, 45 min off) in an ice bath.

   We have also had success using Covaris to fragment the chromatin. These conditions should be determined empirically.

9. Separate into six 1.7-ml tubes and clear the extract by centrifugation for 20 min at 16,100 × *g*, 4°C.
10. Aliquot the cleared extract (250 to 500 µl aliquots) and either proceed directly to step 11 or flash freeze (N_2) and store at −80°C.
11. Take 500 µl of extract from step 10.
12. Supplement the extract with:

    10 µl 20 U/µl SUPERasIN
    5 µl 1 M DTT
    5 µl 100× protease inhibitors.

13. Add 250 µl of denaturant buffer.

14. Add 750 µl of 2× hybridization buffer.

15. For each CHART experiment, use ~400 µl, which leaves enough for the *roX2* CHART, the sense control, and a no-oligo control (from which the supernatant can act as an input control). Add 54 pmol (2.7 µl of 20 µM CHART capture oligo cocktail prepared in Basic Protocol 2) for every 100 µl of extract (i.e., 10.8 µl/400 µl extract). Mix thoroughly with a pipet.

    Depending on the oligo cocktail, there is room for optimization of the concentrations of individual capture oligonucleotides in the cocktail, and also the total concentration of capture oligonucleotides (ranging from 10 to 50 µM stocks at the volumes listed above).

    A good control for CHART experiments is to perform the experiment using the sense oligo control, in which the sequence of the oligonucleotides is of the wrong strand to hybridize to the target RNA. Other possible controls include using scrambled oligo controls, or using oligos directed against an unrelated RNA. Using a sense oligo control has the advantage that any artifactual signal caused by direct interactions between the capture oligos and the DNA will also be detected in the sense oligo control, and can therefore be subtracted bioinformatically.

16. Incubate at room temperature for 6 to 12 hr.

17. Centrifuge 10 min at 16,100 × *g*, room temperature, to clear hybridization reaction. Transfer the supernatant to a fresh tube.

    It is important that the centrifuge not heat the samples. Therefore a temperature-controlled centrifuge should be used.

18. Repeat step 17 one more time.

    It is important to remove small amounts of precipitation that form during the hybridization step as this precipitate can dramatically increase background in the CHART experiment.

19. Pre-rinse 150 µl of MyOne Dynabeads twice with 500 µl distilled, deionized water, each time using the magnetic stand to capture the beads in between rinses.

20. Resuspend beads in 100 µl distilled, deionized water, and then add 50 µl denaturant buffer.

21. Add the cleared extract from step 14 to the bead mixture and incubate overnight, rotating gently end over end.

22. Capture the beads using a magnet and save the supernatant from the no-oligo control for later analysis.

    The supernatant from a no-oligo control makes for a good control, since it takes into account any composition changes during handling of the samples.

23. Quick-spin the bead suspension (5 sec) and then resuspend the beads completely by pipetting.

24. Transfer 150 µl of the bead solution into three fresh tubes, each containing 750 µl of WB250.

25. Capture the beads with a Dynal magnet and wash three times with 750 µl WB250, completely resuspending the beads with gentle inversion between each mix.

26. Use three rinses with 200 µl of HEB to transfer the combined bead mixtures to a fresh 1.7-ml tube.

27. Capture the beads, remove the majority of the supernatant, centrifuge the tubes briefly (1000 × *g*, 5 sec), replace the tubes in the magnet, and remove the residual liquid.

28. Remove the tubes from the magnet and add 100 µl fresh HEB, resuspending the beads gently by pipetting.

29. To elute the CHART-enriched material, add 2 µl of 5 U/µl RNase H, flick gently, and incubate at room temperature for 10 min at room temperature.

    Make sure the RNase H is highly active (i.e., relatively new). The enzyme can lose activity upon handling; if the enzyme is insufficiently active, this will prevent elution and dramatically reduce CHART yields.

30. Centrifuge the tubes briefly (1000 × *g*, 5 sec), and capture the beads.

31. Transfer the supernatant to a fresh tube and either process immediately (see Basic Protocol 4) or flash freeze in liquid nitrogen and store at −80°C.

PREPARATION OF TARGET DNA, RNA, AND PROTEINS FROM CHART ENRICHMENT

BASIC PROTOCOL 4

The material resulting from CHART enrichment (Basic Protocol 3) is a cross-linked mixture of biomolecules consisting of the RNA of interest and its interacting partners, including its DNA and protein targets. Depending on the purpose of the experiment, the eluted material may be used for analysis of the enriched DNA, RNA, or proteins. This protocol describes the handling of CHART-enriched material to prepare it for standard analyses such as quantitative PCR or western blot analysis. This protocol also describes how to prepare the DNA for analysis by deep sequencing.

Materials

CHART-enriched eluate (Basic Protocol 3)
Nucleic Acid XLR Buffer (see recipe)
20 mg/ml proteinase K (Ambion, AM2548)
Phenol:$CHCl_3$:isoamyl alcohol, 25:24:1, saturated with 10 mM Tris·Cl, pH 8.0/1 mM EDTA (Sigma, cat. no. P3803)
$CHCl_3$ (Fluka, cat. no. 25668)
3 M sodium acetate, pH 5.5 (*APPENDIX 2*)
100% and 70% ethanol
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, cat. no. 12183-018)
VILO reverse-transcription cDNA synthesis kit (Invitrogen, cat. no. 11754-050)
iTaq UniverSYBR Green (Bio-Rad, cat. no. 172-5120)
Appropriate primer sets: these primers should include at least one set that are specific to the target RNA, but do not contain the sequence targeted by the capture oligo; the region of the RNA targeted by the capture oligo will be digested by RNase H during CHART enrichment—also include at least one primer set for an abundant but unrelated RNA
55° and 65°C water baths or heat blocks
Phase-Lock tubes (5Prime, cat. no. 2302800)
Centrifuge
MicroTube (6 × 16 mm), AFA fiber with snap-cap, round bottom, glass (Covaris, cat. no. 520045)
Covaris S2 instrument
ABI 7500 qPCR Instrument

Prepare CHART-enriched nucleic acids

1. To remove proteins and cross-links for analysis of the CHART-enriched nucleic acids, use 100 µl of the eluate and add 25 µl Nucleic Acid XLR Buffer. Include an additional tube for analysis of the input.

   Note that the cross-link reversal for the purposes of analyzing the enriched proteins is described in the Support Protocol 1, below, and requires a different cross-link reversal solution.

   Generally it is convenient to use an input sample that represents 10% of the total material that was used in each CHART enrichment. To ensure similar processing between the input and the experimental samples, dilute this sample using HEB (see recipe in Reagents and Solutions).

2. Heat to 55°C for 1 hr and then to 65°C for 1 hr.

   For genome-wide mapping experiments, it is convenient to use 100 µl for analysis of DNA (step 3a to 13a) and the remaining 25 µl for analysis of the RNA (step 3b to 8b).

To prepare DNA for deep sequencing

3a. Dilute 100 µl of material from step 2 into 100 µl of distilled, deionized water in a 1.5-ml phase-lock tube and 200 µl of phenol:$CHCl_3$:isoamyl alcohol.

4a. Shake vigorously and microcentrifuge 5 min at 12,000 × *g*, room temperature.

5a. Rinse the aqueous layer twice with 100 µl $CHCl_3$.

6a. Transfer 200 µl of the aqueous solution to a fresh tube and add 10 µl of 3 M sodium acetate, pH 5.5, and 1 µl of GlycoBlue. Then add 500 µl of 100% ethanol, vortex, and incubate overnight at −20°C.

7a. Pellet the nucleic acids by microcentrifugation for 20 min at 16,000 × *g*, 4°C.

8a. Carefully remove the supernatant and rinse the pellet with 500 µl of 70% ethanol.

   For longer-term storage, keep pellet in the 70% ethanol at −80°C.

9a. Remove all of the liquid, then air dry for 5 min at room temperature with the tube covered by a Kimwipe.

10a. Resuspend the pellet in 100 µl of 10 mM Tris·Cl, pH 8.0.

11a. Transfer the liquid to a MicroTube for Covaris.

12a. To reduce the average fragment size to 200 to 500 bp, process the tube by Covaris in a Covaris S2 instrument under the following conditions.

   DUTY CYCLE: 5%
   INTENSITY: 5
   CYCLES/BURST: 200
   TIME: 60 sec (4-min program)
   BATH TEMP: 4°C.

13a. Use this sheared material directly for library construction (e.g., *UNIT 21.19*, Basic Protocol 2).

To prepare CHART-enriched RNA for RT-qPCR analysis

3b. Purify 25 µl of the CHART-enriched, cross-link-reversed RNA from step 2, and the input sample as a control, using a standard purification kit (e.g., PureLink, Invitrogen). Include an on-column DNase digestion step.

4b. Set up reverse transcription (RT) reactions as follows:

2 µl 5× VILO master mix from Vilo RT kit
7 µl RNA solution from step 3b
1 µl VILO RT enzyme (from Vilo RT kit; include one without enzyme as an RT-minus control).

5b. Incubate as instructed (25°C for 10 min.; 42°C for 60 min.; 85°C for 5 min.; 4°C hold).

6b. Dilute the reverse transcription reactions with 30 µl distilled, deionized water.

7b. Analyze by qPCR using a ABI 7500 RT-PCR instrument and Bio-Rad iTaq SYBR Green Supermix with ROX (the dye, unrelated to *roX2*). Prepare the following master mix:

10 µl Supermix (need about 1.00 ml/plate)
5 µl of primer mix (6 pmol of each primer)
5 µl of RT reaction (use multichannel pipettor to add and mix).

8b. Perform qPCR using the following thermal cycling program on the ABI 7500 RT-PCR instrument:

1 cycle:	5 min	94°C	(initial denaturation)
40 cycles:	30 sec	94°C	(denaturation)
	30 sec	52°C	(annealing)
	1 min	72°C	(extension).

9b. Calculate yields as follows:

$$\text{Yield} = \left(\frac{\text{input dilution factor}}{\text{efficiency}_{\text{PRIMERS}}^{C_{T,\,\text{CHART}} - C_{T,\,\text{INPUT}}}} \right)$$

Equation 21.25.2

For further discussion of qPCR, see UNIT 11.10. The efficiencies for each primer set can be determined experimentally (the values should be ~2). Given the high yields of roX2 recovered by CHART, it is convenient to use an input that is equivalent to 10% of the material used for each CHART enrichment.

ANALYSIS OF CHART-ENRICHED PROTEINS

SUPPORT PROTOCOL 1

In addition to using CHART to analyze nucleic acids that are associated with the RNA of interest, it is also possible to prepare the CHART-enriched protein to test for enrichment of candidate proteins by western blot (such as MSL3 for roX2).

Materials

CHART-enriched eluate (Basic Protocol 3)
Protein XLR Buffer (see recipe)
Lane Marker Non-Reducing Sample Buffer (Pierce, cat. no. 39001)
PCR tubes
PCR thermal cycler

Additional reagents and equipment for immunoblotting (western blotting; *UNIT 10.8*)

1. Transfer 20 µl of CHART-enriched material from Basic Protocol 3 into a PCR tube.
2. Add 5 µl of Protein XLR Buffer.
3. Heat to 95°C for 1 hr in a PCR thermal cycler block, then cool to room temperature.

   This step reverses the cross-links. Make sure to use a heated lid to avoid drying the samples.

4. Add 7.5 µl of sample loading buffer (e.g., Pierce Non-Reducing Sample Buffer) and perform western blot analysis under standard conditions (e.g., *UNIT 10.8*).

   Note that the final salt concentration is fairly high in these samples. Therefore, the input samples should be diluted in a buffer of similar salt to ensure that the lanes of the gel run evenly during PAGE analysis.

SUPPORT PROTOCOL 2

QUALITY CONTROL OF CHART DNA ENRICHMENT BY qPCR

Before sending samples for sequence analysis, it is good practice to validate the CHART enrichment by qPCR analysis, both before constructing a deep sequencing library, and after library construction. The libraries should show similar enrichment to that observed before library construction.

Materials

iTaq UniverSYBR Green (Bio-Rad, cat. no. 172-5120)
iTaq SYBR Green Supermix with ROX (Bio-Rad, cat. no. 172-5850)

ABI 7500 qPCR Instrument
Appropriate primer sets

CHART enrichment should be analyzed both before and after library construction by qPCR. Before library construction, the data is analyzed as yield relative to input according to Equation 21.25.2 (see Basic Protocol 4). After library construction, the diluted libraries should be assayed in triplicate using primers that will amplify known or expected binding sites (e.g., the endogenous *roX2* locus and CES-5C2), and negative controls (e.g., Pka and Act-5C). Include a library constructed from the input. Note that the C_T values for the input with different primers should be very similar to each other (within 1 to 2 C_T values). Normalize the signal to input and to one of the negative controls (e.g., Act-5C, which was used because amplification of the PKA was undetected for all three replicates in the *roX2* CHART-enriched samples):

$$\text{Fold enrichment} = \left(\frac{\text{efficiency}^{C_{T,\,\text{CHART}} - C_{T,\,\text{INPUT}}}_{\text{TARGET PRIMERS}}}{\text{efficiency}^{C_{T,\,\text{CHART}} - C_{T,\,\text{INPUT}}}_{\text{ACT-5C PRIMERS}}} \right)$$

Equation 21.25.3

It is not rare that the CHART-enriched library has undetectable levels of one of the negative controls. A conservative estimate of the enrichment can be made by entering C_T values of 40 in cases where no amplification is observed after 45 cycles.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Denaturant buffer

8 M urea
200 mM NaCl
100 mM HEPES, pH 7.5
2% (w/v) SDS
Store up to 1 month at room temperature

Glycerol buffer

125 ml glycerol (25% final)
5 ml of 1 M HEPES (10 mM final)
1 ml of 0.5 M EDTA (*APPENDIX 2*; 1 mM final)
50 µl of 1 M EGTA (0.1 mM final)
16.7 ml of 3 M potassium acetate (100 mM final)
Distilled, deionized H_2O up to 500 ml
Store up to 6 months at 4°C
Immediately before use, take 40 ml of the above solution and add:
200 µl of 0.1 M spermidine (store stock in aliquots at –80°C; 0.5 mM final)
60 µl of 0.1 M spermine (store stock in aliquots at –80°C; 0.15 mM final)
400 µl of 100× Complete EDTA-free Protease Inhibitor (Roche)
40 µl of 1 M dithiothreitol (DTT; 1 mM final)
20 µl of 20 U/µl SUPERasIN (10 U/ml final)

Hybridization buffer, 2×

1.5 M NaCl
1.12 M urea
10× Denhardt solution (see *APPENDIX 2*)
10 mM EDTA, pH 8 (*APPENDIX 2*)
Prepare fresh

Nuclei rinse buffer

5 ml of 1 M HEPES, pH 7.5 (50 mM final)
1.5 ml of 5 M NaCl (75 mM final)
20 µl of 0.5 M EGTA (0.1 mM final)
Distilled, deionized H_2O to 100 ml
Store up to 6 months at 4°C
Immediately before use, add to 5 ml of the above:
5 µl of 20 U/µl SUPERasIN (1 U/ml final)
5 µl of 1 M dithiothreitol (1 mM final)
50 µl of 100× Complete EDTA-free Protease Inhibitor (Roche)

Nucleic acid XLR buffer

100 µl of 1 M Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 µl of 10% (w/v) SDS (*APPENDIX 2*)
200 µl of 20 mg/ml proteinase K (Ambion, cat. no. AM2548)
Prepare immediately before use

Protein XLR buffer

67 µl of 1.5 M Tris·Cl, pH 8.8 (*APPENDIX 2*)
100 µl 10% (v/v) SDS (*APPENDIX 2*)
33 µl 2-mercaptoethanol
Prepare immediately before use

Quenching buffer

20 µl of 0.5 M EDTA (*APPENDIX 2*)
20 µl of 1 M Tris·Cl, pH 7.2 (*APPENDIX 2*)
20 µl of 10% (w/v) SDS (*APPENDIX 2*)
20 µl of 20 mg/ml proteinase K (Ambion, cat. no. AM2548; add immediately before use)
Prepare fresh

RNase H elution buffer (HEB)

100 µl of 1 M HEPES pH 7.5 (50 mM final)
30 µl of 5 M NaCl (75 mM final)
0.1 ml of 5% (w/v) *N*-lauroylsarcosine (0.125% final)
4 µl of 10% (w/v) sodium deoxycholate (0.025% final)
6 µl of 1 M $MgCl_2$ (3 mM final)
2 µl of 20 U/µl SUPERasIN (20 U/ml final)
20 µl of 1 M DTT (10 mM final)
1738 µl H_2O (2 ml total volume, final)
Prepare fresh

Sonication buffer

500 µl of 1 M HEPES pH 7.5 (50 mM final)
150 µl of 5 M NaCl (75 mM final)
2 µl of 0.5 M EGTA (0.1 mM final)
1 ml of 5% (w/v) *N*-lauroylsarcosine (0.5% final)
100 µl of 10% (w/v) sodium deoxycholate (0.1% final)
Store up to 6 months at 4°C
Immediately before use take 5 ml of the above solution and add:
5 µl of 20 U/µl SUPERasIN (10 U/µl)
25 µl of 1 M dithiothreitol (DTT; 5 mM final)

Sucrose buffer

51.3 g sucrose (0.3 M final)
50 ml of 10% (v/v) Triton X-100 (1% final)
5 ml of 1 M HEPES, pH 7.5 (10 mM final)
16.7 ml of 3 M potassium acetate (100 mM final)
50 µl of 1 M EGTA (0.1 mM)
Distilled, deionized H_2O to 500 ml
Store up to 6 months at 4°C
Immediately before use, take 20 ml of the above solution and add:
100 µl of 0.1 M spermidine (store stock in aliquots at −80°C; 0.5 mM final)
30 µl of 0.1 M spermine (store stock in aliquots at −80°C; 0.15 mM final)
200 µl of 100× Complete EDTA-free Protease Inhibitor (Roche)
20 µl of 1M dithiothreitol (DTT; 1 mM final)
200 U SUPERasIN (10 µl of 20 U/µl)

Wash buffer 100 (WB100)

1 ml of 5 M NaCl (1.00 mM final)
500 µl of 1 M HEPES, pH 7.5 (10 mM final)
200 µl of 0.5 M EDTA (2 mM final)
100 µl of 0.5 M EGTA (1 mM final)
1 ml of 10% (w/v) SDS (0.2% w/v final)

continued

1 ml of 5% (w/v) *N*-lauroylsarcosine (0.1% final)
Distilled, deionized H_2O to 50 ml
Prepare fresh
Immediately before use, add 100 μl of 0.4 mM phenylmethylsulfonyl fluoride (PMSF) to the above solution, then filter using a 0.22-μm filter

COMMENTARY

Background Information

There are a growing number of large noncoding RNAs (lncRNAs) that have been implicated in the regulation of chromatin (Koziol and Rinn, 2010). One important goal is to determine the targets of these RNAs, including where they directly act in the genome. To this end, there has been substantial interest in using hybridization-based approaches to map the targets of RNAs (Carter et al., 2002; Mariner et al., 2008; Chu et al., 2011; Simon et al., 2011). The advantage to the CHART protocol described here is the minimization of hybridization-induced artifacts by (1) targeting accessible regions of the RNA, and (2) avoiding extensive denaturation of the DNA. The conditions described here allow the isolation of both protein and DNA targets of an RNA, and can be extended to genome-wide mapping of the binding sites of a lncRNA (Simon et al., 2011).

Critical Parameters and Troubleshooting

The CHART reaction conditions have been carefully optimized to provide high yields of the desired RNA with its targets. Important parameters include the concentration of the extract, the level of cross-linking, the ionic strength, and the concentration of urea. Using concentrated extracts improves CHART yield. Lower levels of cross-linking (such as those used in ChIP) lead to low yields of DNA. The high ionic strength of the CHART conditions is critical for high yields, but higher ionic strength leads to precipitation of the chromatin. The high concentration of urea in the hybridization conditions is critical to maintain chromatin solubility and to provide the necessary stringency. The resolution of the experiment is determined by the shear size of the input chromatin. However, since the target RNA can also be sheared, a balance needs to be maintained between high levels of shearing of the chromatin that increases resolution but that might decrease CHART yield, and lower levels of shearing that may increase CHART yield but decrease the resolution of the experiment.

While CHART is optimized to avoid hybridization-induced artifacts, care still should be taken at each step to minimize likely artifacts. For example, it is important to use algorithms such as BLAST to avoid capture oligonucleotides that have the potential to base pair with off-target RNAs (i.e., avoid sequences with >14 nt matches to other expressed RNAs). One effective strategy to control for off-target effects has been to use independent cocktails of capture oligonucleotides (Chu et al., 2011). In genome-wide data, artifacts tend to have sharp peaks and occur at genomic sites with high homology to either the capture oligonucleotide or the target RNA. Therefore, care must be taken when interpreting peaks that meet these criteria.

Anticipated Results

In a successful CHART experiment, target RNA yields range from 5% to 50%. The corresponding DNA yields range from 0.1% to 2%, which is also similar to the yields of tightly bound proteins. The enrichment values determined by comparing enriched loci with control loci are similar to ChIP, ranging up to thousands-fold. As the yields and enrichment are similar to ChIP, it is unsurprising that successful CHART experiments require a similar scale (10^7 to 10^8 cells/experiment).

Time Considerations

Starting from a cell pellet, the capture oligonucleotides can be designed within approximately 2 days of work: 1 day for extract preparation and 1 day for RNase H mapping and oligonucleotide design. Once the capture oligonucleotides are obtained, CHART enrichment can be performed in 3 partial days of work: 1 day for extract preparation and initiation of the hybridization reactions, 1 day for the addition of the beads, and 1 day for washing the beads, elution, cross-link reversal, and DNA analysis.

Literature Cited

Carter, D., Chakalova, L., Osborne, C.S., Dai, Y.F., and Fraser, P. 2002. Long-range chromatin regulatory interactions in vivo. *Nat. Genet.* 32:623-626.

Chu, C., Qu, K., Zhong, F.L., Artandi, S.E., and Chang, H.Y. 2011. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol. Cell* 44:667-678.

Koziol, M.J. and Rinn, J.L. 2010. RNA traffic control of chromatin complexes. *Curr. Opin. Genet. Dev.* 20:142-148.

Mariner, P.D., Walters, R.D., Espinoza, C.A., Drullinger, L.F., Wagner, S.D., Kugel, J.F., and Goodrich, J.A. 2008. Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. *Mol. Cell* 29:499-509.

Simon, M.D., Wang, C.I., Kharchenko, P.V., West, J.A., Chapman, B.A., Alekseyenko, A.A., Borowsky, M.L., Kuroda, M.I., and Kingston, R.E. 2011. The genomic binding sites of a noncoding RNA. *Proc. Natl. Acad. Sci. U.S.A.* 108:20497-20502.