Published in final edited form as:

Methods Mol Biol. 2011 ; 759: 41–60. doi:10.1007/978-1-61779-173-4_3.

Genome-wide measurement of histone H3 replacement dynamics in yeast

Oliver J Rando [Prof.]

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abstract

Chromatin plays critical roles in processes governed in different time scales – responses to environmental changes require rapid plasticity, while long term stability through multiple cell generations requires epigenetically heritable chromatin. Understanding the dynamic behavior of chromatin is of great interest for fields ranging from transcriptional regulation through meiosis and gametogenesis. Here, we describe a protocol for measuring histone replacement rates genome-wide in the budding yeast *Saccharomyces cerevisiae*. With suitable modifications this protocol could be applied to other organisms, or to replacement dynamics of other DNA-associated proteins.

Keywords

Histone H3 replacement; *Saccharomyces cerevisiae*; budding yeast; epigenome; epigenetics; chromatin

1. Introduction

Chromatin plays critical roles in processes governed in different time scales – responses to environmental changes require rapid plasticity, while long term stability through multiple cell generations requires epigenetically heritable chromatin. It is of great interest, therefore, to understand the nature of dynamic chromatin states in living cells.

In bulk studies, histones are among the most stably bound DNA-associated proteins. Nonetheless, a number of recent experiments have identified loci where histones are evicted and replaced by different histone molecules in the absence of genomic replication. Furthermore, during DNA replication, histones from the mother chromosome transiently dissociate from the chromosome, raising the question of how chromatin states are stably maintained in the face of the perturbations encountered during genomic replication. The converse question may also be asked – even in the absence of genomic replication, how stable are chromatin states? While most studies paint a picture of stable chromatin states, is this a consequence of a static system in which the same molecules persist, or a consequence of active maintenance by dynamic processes? This question is central to understanding the processes that modify chromatin state, and to understand the molecular nature of stably heritable chromatin states.

Histones provide a formidable barrier to the elongation of RNA polymerase (1,2). In yeast, passage of RNA polymerase displaces nucleosomes (assessed by H3 occupancy), which are

rapidly reassembled after transcription is shut down (3). In *Drosophila*, RNA polymerase causes exchange of the canonical histone H3 (H3.1) for the “replication-independent” H3 variant H3.3, and H3.3 continues to turn over as long as a coding region is being actively transcribed (4-6). Single-gene and whole-genome studies in yeast have shown that H3 is removed from promoters upon gene activation (7-11,12). Reassembly of histones at the *PHO5* promoter after shutdown of transcription occurs *in trans*, in the sense that the histones that are reassembled on the promoter are not the same histones that were removed during gene activation (13). Intriguingly, H3 replacement is slow or unmeasurable over silenced coding regions (14-17). H2A/H2B exchange is globally much more widespread than H3 exchange, though it too is diminished over silenced loci.

Detailed understanding of high-resolution chromatin dynamics requires a rapid assay for the determination of chromatin structure over genomic scales. Using genome-wide tiling microarrays coupled with inducible expression of epitope-tagged histones, the dynamics of replication-independent H3 exchange across the yeast genome we and others has been characterized (17, 18). These methods enable the rapid assay of global histone dynamics at single-nucleosome resolution. Below, a protocol for the genome-wide measurement of H3 replacement rates in budding yeast is presented.

2. Materials

2.1. Combined Micrococcal Nuclease and ChIP (MNase-ChIP)

1. YPRaffinose medium (YPRaff): yeast extract (1% w/v)/peptone (1% w/v)/raffinose (2% w/v) medium.
2. PBS buffer: phosphate-buffered saline pH 7.4.
3. Buffer L: 50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X-100, 0.1% (w/v) sodium deoxycholate.
4. NP Buffer: 0.5 mM spermidine, 1 mM β -mercaptoethanol (β -ME), 0.075% (w/v) Tergitol-type NP-40 detergent (NP-40), 50 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$. Preparation of 5 mL of NP Buffer (made fresh on the day of the experiment): 10 μ L 250 mM spermidine, 3.5 μ L of 1:10 (v/v) (diluted in water) β -ME, 37.5 μ L 10% (w/v) NP-40. Bring up to 5 mL with NP buffer.
5. Buffer W1: Buffer L with 500 mM NaCl.
6. Buffer W2: 10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA.
7. Buffer Z: 1 M sorbitol, 50 mM Tris-HCl pH 7.4. Preparation of 1 L of buffer Z: 500 mL 2 M sorbitol, 50 mL 1 M Tris-HCl pH 7.4, 450 mL dH_2O .
8. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
9. (2 $\times$) Proteinase K solution: TE with 0.8 mg/mL glycogen, 2 mg/mL proteinase K.
10. Elution buffer: TE pH 8.0 with 1% (w/v) SDS, 150 mM NaCl, and 5 mM DTT (Note: do not add the DTT until just before use).
11. Zymolyase solution (10 mg/mL in Buffer Z; lasts up to 2 weeks at 4°C).
12. Micrococcal Nuclease (Worthington Biochem): Resuspended from lyophilized powder at 20 U/ μ L in Tris-HCl pH 7.4. Aliquot into tubes upon first use and store at -80°C.
13. Phenol/chloroform/isoamyl alcohol (PCI) (24:25:1) (v/v).

14. Yellow (heavy) Phase Lock Gel (PLG) tubes (Manual Phase Lock Gel™, PLG, 5Prime GmbH).
15. Protein A beads (Sigma).
16. Isopropanol.
17. Sigma protease inhibitor cocktail
18. NEB Buffer 3.
19. Calf intestinal phosphatase (CIP), New England Biolabs.
20. Qiagen Min Elute Enzyme Cleanup Kit.

2.2. DNA linear amplification

1. Calf Intestinal Phosphatase (CIP) – New England Biolabs
2. 10× NEB Buffer 3 (New England Biolabs)
3. TdT (NEB #M0252S or #M0252L)
4. 5× TdT Buffer: 1 M Potassium cacodylatec, 125 mM Tris-HCl pH 6.6, 1.25 mg/ml BSA (Roche #11 243 276 103)
5. 8% dideoxynucleotide tailing solution: 92 μM dTTP, 8 μM ddCTP (Invitrogen)
6. Ambion T7 Megascript Kit, #1334
7. DNA polymerase I Klenow fragment (New England Biolabs)
8. NEB Buffer 2 (New England Biolabs)
9. Qiagen RNeasy Mini Kit.
10. β-Mercaptoethanol (14.2 M).

2.3. Amplified antisense RNA (aRNA) labeling and cleanup

- 1 5 μg/μL anchored oligodT (22mer, Integrated DNA Technology, IDT).
- 2 **Cy3 and Cy5 dyes (Amersham):** Dye comes dried and should be resuspended in 11 μL of DMSO. This amount of material is sufficient for three labeling runs. If entire amount will not be used, make 3 μL aliquots. Dry down in SpeedVac vacuum concentrator and store at −20°C.
- 2 (50×) aa-dUTP mixture: Dissolve 1 mg amino-allyl dUTP (Sigma) with: 32.0 μL 100 mM dATP (12.5 mM, final), 32.0 μL 100 mM dGTP (12.5 mM, final), 32.0 μL 100 mM dCTP (12.5 mM, final), 12.7 μL 100 mM dTTP (5 mM, final), 19.3 μL dH₂O.
- 3 Superscript II (Invitrogen), with SSII buffer and DTT.
- 4 RNasin (Promega).
- 5 1M NaOH
- 6 0.5M EDTA
- 7 50 μL 1M Hepes, pH 7.5.
- 8 50 mM sodium bicarbonate, pH 9.0.
- 9 Qiagen Min Elute Enzyme Cleanup Kit.

2.4. Microarray Hybridization

1. Cot-1 DNA. Commercially available at 1 mg/mL. Prior to hybridization, concentrate Cot-1 DNA from 1 μ g/ μ L to 10 μ g/ μ L in a SpeedVac vacuum concentrator.
2. Yeast tRNA 10 μ g/ μ L.
3. PolyA RNA 10 μ g/ μ L.
4. (20 $\times$) SSC
5. 10% SDS
6. 1M Hepes-KOH (pH 7.0)
7. Microarrays and hybridization chambers – obtained from microarray suppliers such as Agilent, Nimblegen, etc.
8. Axon Genepix scanner.

2.5. Yeast strains and cultivation

The yeast strain required for this experiment carries an inducible copy of an epitope-tagged histone H3. Specifically, we utilize a strain carrying a plasmid with the *GALI-10* promoter driving expression of both untagged H4 and an N-terminally Flag-tagged H3. In our original published work (17), we used a strain that also carried a constitutively-expressed Myc-tagged H3, but in subsequent work we found the Myc tag to be unnecessary (19). We also find it useful to delete the gene encoding the Bar1 protease that degrades the alpha factor from the yeast strain to be used, thus enabling G1 arrest by alpha factor treatment with much lower concentrations of the factor.

A typical experiment involves growth of the yeast strain (carrying a deletion in a chromatin regulator of interest if desired) to mid exponential phase in YPRaffinose medium. Yeast are arrested with alpha factor (we recommend carrying out a titration in your strain background of interest) for 3 h (more for slow-growing strains). Galactose is added to the medium to a final concentration of 1% (w/v), and at varying times after galactose addition (15, 30, 45, 60, 90, 120, 150 min) mononucleosome-resolution ChIP is carried out as described below. After amplification, Flag ChIP is competitively hybridized against mononucleosomal “input” on whole-genome tiling microarrays.

Below, we provide detailed protocols for 4 steps:

1. Nucleosome-resolution ChIP (section 3.1).
2. Linear amplification (section 3.2).
3. Labeling for microarrays (section 3.3)
4. Hybridization to microarrays (section 3.4).

3. Methods

3.1. Combined Micrococcal Nuclease and ChIP protocol (MNase-ChIP)

Before doing MNase-ChIP, a MNase titration series is needed to determine the proper amount of MNase enzyme to be used. For this purpose follow the protocol below as follows (starting from Day 1) stopping at the end of the MNase digestion and following these steps:

1. Add 150 μ L of STOP buffer (0.25 M EDTA, 5% (w/v) SDS).

2. Proteinase K treat overnight at 65°C. You may use extra-high concentrated proteinase K (20 mg/mL) with no glycogen.
3. Extract with Phenol/chloroform/isoamyl alcohol (PCI) reagent once using yellow (heavy) Phase Lock Gel (PLG) tubes.
4. Precipitate with (0.1×) volume 3M sodium acetate and 1 volume isopropanol
5. Spin immediately for 10 min at maximum speed (>12,000g).
6. Wash pellet in 70% (v/v) ethanol.
7. Resuspend in NEB Buffer 3.
8. RNase treatment for 1 h at 37°C.
9. Run gel to determine proper titration point.

3.1.1. Day 1 – Yeast culture—

1. Inoculate starter culture with the cells of interest (full size colony). Do this 1-2 days in advance.
2. Inoculate 400-550 mL of YPRaffinose medium in a 2 L baffled flask and grow culture shaking at 220 rpm at desired temperature. Target concentration is $8\text{--}9 \times 10^6$ cells/mL. This yields sufficient material for 4-6 immunoprecipitations (IPs).

3.1.2. Day 2 – Crosslinking and MNase Digest—

1. Early afternoon: Grow culture to the desired cell density, arrest with alpha factor and add galactose to a final concentration of 1% (w/v) for the desired amount of time. Add formaldehyde to 1% (w/v) final concentration (see volumes below) and shake (~200 rpm) for 15 min.

Cell culture volume (mL)	Formaldehyde (mL)	Glycine (mL)
400	10.7	20.5
450	12.0	23.0
500	13.2	25.3
550	14.8	27.5

2. To quench the formaldehyde, add 2.5 M glycine according to the table above, shake while adding, then shake or let stand at room temperature for 5 min. Cells can now be left on ice as long as necessary (for example, to collect more samples from a time course experiment).
3. Transfer to 0.5 or 1 L centrifuge bottle and spin down at >3,000g for 5 min at 4°C.
4. Wash once in a 50 mL volume of MilliQ water (ddH₂O).
5. Resuspend the cell pellet (from the 400-550 mL culture) in 39 mL Buffer Z.
6. Add 28 µL of β-ME 14.3 M (final concentration 10 mM) and vortex cells to resuspend.
7. Add 1 mL of zymolyase solution and incubate at 28-30°C, shaking, on tilted tube rack for 35-40 min (time will depend on assessment of spheroplasting efficiency – see below).

8. Spin zymolyase-treated yeast (now spheroplasts) at 5,000g, 10 min, 4°C.
 - 8a During spin, if doing a titration, aliquot the MNase to 4–6 eppendorf tubes per set. Pipette a dot of MNase on the side of the tube (directly under lid hinge). Suggested concentration range:
 - 8ai BY4741: 1–10 µL for 4 aliquots. Alternatively, increase number of aliquots with less amount of enzyme.
 - 8b MNase can be subject to 3 freeze-thaw cycles (–20°C). Store at –20°C after first freeze-thaw.
9. Aspirate supernatant and resuspend each aliquot in a total of (600 µL × # aliquots + 50 µL) NP buffer by pipetting up and down several times. Cells will be sticky, viscous and thick. If cells don't stick together in chunks but dissociate easily from each other spheroplasting may be incomplete.
10. Add 600 µL of cells to each eppendorf with MNase. Add the cells to the tubes directly on the spot of nuclease, and try to be even and quick between tubes. Start with the most diluted sample and use the same 1000 µL tip to add to each successive tube to minimize tip changing times.
11. As soon as all the cells have been added, start the timer, close the tubes, invert them once to mix, and incubate at 37°C (in water bath) for 20 min.
12. Stop the reaction after 20 min by transferring the tubes to an ice bath (4°C). This will halt the enzyme activity. Finally, add 12 µL of 0.5M EDTA (10 mM end concentration) per 600 µL aliquot to inactivate the enzyme.

3.1.3. Day 2 – Immunoprecipitation—

1. Prepare Protein A beads. Each immunoprecipitation (IP) aliquot takes two protein A bead aliquots, but one of the aliquots won't be used until the following morning. If preparing from a new vial, the beads are 5 mL of slurry in a total volume of 6.3 mL, with the supernatant consisting of 20% (v/v) ethanol. Add 3.7 mL of 20% ethanol to bring up to 10 mL of 50% slurry. This is equivalent to adding 3 mL of water and 750 µL of 100% ethanol.
 - 1a Pipette 700 µL of slurry per 8 IP aliquots into a 1.5 mL tube (enough for the preclear; you will need another 700 µL for 8 IP aliquots for the pulldown the following day)
 - 1b . Spin down at 3,000g in microcentrifuge for 1 min.
 - 1c Return supernatant to stock buffer if slurry is thick, otherwise discard.
 - 1d Wash beads twice in Buffer L.
 - 1e Distribute the Protein A beads equally to eight 1.5 mL tubes (truncate a pipette tip with a razor blade and use that for easier pipetting, though you may need to dial down the volume, since the pipetman will now draw up slightly more volume).
2. Pool the digested material and add the following to the digestion products to simulate Buffer L conditions. Important: Add the salts before the detergents. Amounts below are per a 600 µL digestion aliquot; scale accordingly. One IP aliquot will be ca. 800 µL afterwards.

Volume (μL)	Component
80	0.5 M Hepes-KOH pH 7.5
22.4	5 M NaCl
6.4	12.5% (w/v) sodium deoxycholate
80	10% (w/v) Triton X-100
8	Sigma protease inhibitors

3. Set aside at least 80 μL (~5%) of pool as non-IP “input”; needed also for gel verification of the MNase digest. This should yield about 0.75-1 μg of DNA in the end.
4. Add adjusted digest product (~800 μL per IP aliquot) to one protein A bead aliquot (pre-equilibrated in buffer L) and rotate at 4°C on tube rotisserie for 1 h to remove proteins that bind nonspecifically to the beads.
5. Spin for 30s at 3,000g at 4°C (to avoid MNase reactivation), and transfer supernatant to another tube containing the appropriate amount of antibody.
6. Incubate with rotation at 4°C for 4 h to overnight (up to 16 h).
7. Spin for 30 s at 3,000g at 4°C and transfer to a tube containing another protein A bead aliquot.
8. Incubate with rotation at 4°C for 1 h (longer is allowable but not necessary).
9. Spin for 30 s at 3,000g at 4°C and for subsequent pelleting steps in washes.
10. Wash beads successively with 1 mL of the following buffers, for 5 min (on rotisserie at 4°C) each, in the following order: Buffer L (twice), W1 (twice), W2 (twice), and TE pH 8.0 (twice).

3.1.4. Day 2 – Elution—

1. Incubate the beads in 125 μL of elution buffer at 65°C for 10 min with frequent mixing (vortex for a few seconds every 3 min). Be sure to add the DTT in the elution buffer (to 5 mM final concentration) beforehand.
2. Pellet the beads by centrifugation at 10,000 g for 2 min and keep the supernatant.
3. Repeat steps 25 and 26 and discard the protein A beads when done.

3.1.5. Day 2 – Reverse crosslinks—

1. Add 0.5 volume of (2×) Proteinase K solution to both the ChIP input and the eluted samples. (ChIP input samples can receive 10 μL of 20 mg/mL Proteinase K instead).
2. Overlay input and eluted samples with mineral oil (only if volume is <150 μL) and incubate at 65°C overnight.

3.1.6. Day 3 – Protein Degradation and DNA Purification—

1. Cool the samples to room temperature and spin down (> 12,000g).
2. Extract with one volume phenol (once), then with one volume chloroform/isoamyl alcohol (25:1) (once) using chloroform only (24:25:1 PCI is also acceptable). For the first extraction (all at room temperature) vortex for 10 s, transfer to phase lock gel tube, then spin 5 min at maximum speed (>12,000g). For subsequent

extractions, pipette volume up and down to mix it. The volume will be approximately 400 μL at this stage. Use light (green) phase lock gel tubes for phenol and heavy (yellow) or light gel tubes for chloroform/isoamyl alcohol (extracting twice with 24:25:1 PCI is also acceptable).

3. Add 0.1 volume of 3.0 M sodium acetate pH 5.3 and 2.5 volumes of ice cold 100% ethanol.
4. Allow DNA to precipitate overnight at -20°C (to maximize yield).

3.1.7. Day 4 – DNA Purification—

1. Pellet the DNA by centrifugation at 14,000g for 15 min at 4°C .
2. Wash once with cold 70% (v/v) ethanol, and spin at 14,000g for 5 min at 4°C .
3. Aspirate and allow pellet to dry.
4. Resuspend pellet in 29 μL of NEB Buffer 3 with 0.5 μg of DNase-free RNase A (0.5 mg/mL).
5. Incubate at 37°C for 1 h. (can freeze at -20°C at this point)
6. Add 0.75 μL of CIP and leave at 37°C for 1 h. Scale up volume (and enzyme) if more than 50 ng/ μL is expected.
7. Clean up reaction with Qiagen MinElute Enzyme Cleanup Kit, with elution volume of 20 μL .

3.2. DNA Linear Amplification

While many amplification protocols have been successfully used for ChIP material in genomic studies, it has been shown that for material <500 bp many extant protocols introduce biased genomic representation. The protocol here is superior to other tested protocols for material of this size, which of course includes mononucleosomal DNA.

3.2.1. Calf Intestinal Phosphatase (CIP) Treatment of samples with terminal 3' phosphate groups—

Reagent (for every 10 μL volume)	Volume	Final concentration
2.5 U enzyme (NEB #M0290S)	0.25 μL	0.25 U/ μL
(10 $\times$) NEB Buffer 3	1 μL	(1 $\times$)
Template DNA (max. 500 ng per 10 μL)	8.75 μL	max 50 ng/ μL
Total	10 μL	

(Each reaction can be scaled up to 100 μL per tube)

1. Incubate at 37°C for 1 h.
2. Clean up reaction with the Qiagen MinElute kit (# 28204; see end of protocol). Elute in 20 μL .

3.2.2. Tailing Reaction with TdT (NEB #M0252S or #M0252L)—

Reagent	Volume	Final conc.
(5×) TdT buffer (see Note 1)	2 μ L	(1×)
8% dideoxynucleotide tailing solution (see Note 2)	0.5 μ L	5 μ M dTTP (8% ddCTP)
5 mM CoCl ₂	1.5 μ L	0.75 mM
Template DNA (max 75 ng) (see Note 3)	5 μ L	max 7.5 ng/ μ L
20U TdT enzyme (add this at the end) (see Note 4)	1 μ L	2 U/ μ L
Total	10 μ L	

1. Add 1-2 drops of mineral oil to the top of the mixture, to prevent evaporation loss during incubation.
2. Incubate at 37°C for 20 min.
3. Stop reaction by adding 2 μ L (per 10 μ L reaction volume) of 0.5M EDTA pH 8.0.
4. Clean up reaction with the Qiagen MinElute kit. If started with 10 μ L, it may be necessary to add 10 μ L of ddH₂O to bring the volume up to 20 μ L. Elute in 20 μ L volume.

3.2.3. Second Strand Synthesis with Klenow Fragment Polymerase—

Reagent*	Volume	Final concentration.
25 μ M T7-A ₁₈ B primer (see Note 5)	0.3 μ L	300 nM
(10×) NEB Buffer 2 (see Note 6)	2.5 μ L	(1×)
5.0 mM dNTP mix (see Note 7)	1 μ L	200 μ M
ddH ₂ O	0.2 μ L	
T-Tailed DNA	20 μ L	
Total	24 μ L	

Important: Do not use mineral oil. Trace amounts of mineral oil appears to interfere with cleanup and in vitro transcription (IVT).

Use the following program in a thermal cycler:

Start: 94°C, 2 min to melt; ramp –1°C per second to 35°C, then hold for 2 min to anneal; ramp –0.5°C per second to 25°C; hold for 45 s (pause here for as long as needed, up to 6 min); add 1 μ L of (5U) Klenow DNA polymerase (NEB# M0210S) during this time and spin down condensation on tube if necessary; 37°C, 90 min to extend; Keep at 4°C to halt enzyme activity until removal of reaction tubes out of cycler.

Stop reaction by adding 2.5 μ L of 0.5M EDTA, pH 8.0 (end concentration, 50 mM). Clean up reaction with the Qiagen MinElute kit and elute in 20 μ L.

3.2.4. In Vitro Transcription (IVT)—Double strand DNA (dsDNA) preparation: The in vitro transcription (IVT) requires that the dsDNA be in 8 μ L volume. Dry down the eluate from 20 to 8 μ L in a SpeedVac vacuum concentrator for 10-12 min (drying rate approximately 1 μ L/min).

Reagent (from Ambion T7 Megascript Kit, #1334)	Volume
75 mM NTP mix (A, G, C, and UTP) (see Note 8)	8 μ L
Reaction buffer (warm to RT first) (see Note 9)	2 μ L
Enzyme mix (RNase inhibitor and T7 RNA Pol)	2 μ L
Template dsDNA	8 μ L
Total	20 μ L

Incubate at 37°C overnight (5-20 h). Incubate in thermal cycler with heated lid, or in air incubator, and in 0.2 mL RNase-free PCR tubes to minimize vapor volume.

3.2.5. Amplified antisense RNA (aRNA) Purification Using Qiagen RNeasy Columns—Use the Qiagen RNeasy Mini kit (#74104) for this procedure. You may eventually have to order additional Buffer RPE if you use the entire kit for this purpose.

Preparation of Master Mix:

Reagent	Volume
β -ME (14.2M stock solution)	3.5 μ L
RNase-free water	80 μ L
Buffer RLT (from Qiagen RNeasy Mini kit)	350 μ L
Total	433.5 μ L

1. Pre-aliquot the mix to 1.5 mL RNase-free tubes.
2. Transfer contents of the IVT mix to the RNase/DNase-free tube and vortex gently and briefly.
3. Add 250 μ L of ethanol (95-100% v/v) and mix well by pipetting. Do not spin here.
If you have a Qiagen vacuum manifold, skip to step 8.
Without vacuum manifold:
4. Apply sample (700 μ L) to RNeasy mini spin column sitting in a collection tube. Centrifuge 15 s at 8,000g. Discard flow-through.
5. Transfer RNeasy column to a new 2 mL collection tube (supplied). Add 500 μ L of Buffer RPE (which must contain ethanol) and centrifuge 15 s at 8,000g. Discard flow-through but reuse tube.
6. Pipette 500 μ L buffer RPE (included with RNeasy kit) onto RNeasy column and centrifuge for 2 min at >12,000g.
7. Remove flow trough and pipette another 500 μ L Buffer RPE onto column. Centrifuge for 2 min at >12,000g (this is an additional wash that is not in the

Qiagen protocol which we have found necessary because of guanidine isothiocyanate (GITC) contamination in the eluted RNA). Skip to step 12.

With vacuum manifold:

8. Apply sample (700 μ L) to RNeasy mini spin column, attached to vacuum manifold. Apply vacuum to the mini spin column.
9. Remove vacuum and pipette 500 μ L Buffer RPE onto RNeasy column. Apply vacuum.
10. Repeat Step 9, and transfer columns to 2 mL collection tubes (supplied). Spin 1 min at $>12,000g$.
11. Return columns to vacuum manifold and apply 500 μ L Buffer RPE. Apply vacuum.
12. Transfer columns back to the 2 mL tubes. Spin at $>12,000g$ for 1 min to completely dry column.
13. Transfer RNeasy column into a new 1.5 mL collection tube (supplied) and add 30 μ L RNase-free water directly onto membrane. Centrifuge for 1 min at $8,000g$ to elute. Repeat if expected yield is $>30 \mu g$.
14. Check RNA concentration and quality by measuring A_{260} and A_{260}/A_{280} .

3.2.6. Qiagen MinElute Kit Protocol—The MinElute kit removes free nucleotides below 40 nucleotides (nt). Fragments between 40-70nt might be removed but with a lower efficiency.

1. In a sample of volume 20-100 μ L, add 300 μ L Buffer ERC and mix thoroughly. If the sample is in less than 20 μ L, bring up volume with ddH₂O. If the sample is in more than 100 μ L, split sample and do in parallel.
2. Add sodium acetate (pH 5.0) if the buffer color is orange or purple (i.e. pH > 7.5). If the buffer is yellow, no additional sodium acetate is necessary.
3. Apply sample to column. Spin 1 min at $>12,000g$ in microcentrifuge.
4. Discard flow-through and add 750 μ L buffer PE (which must contain ethanol). Spin 1 min. at $>12,000g$ in microcentrifuge.
5. Discard flow-through. Spin 1 min. at $>12,000g$ in microcentrifuge to dry column.
6. Transfer columns to fresh 1.5 mL tubes. Pipette 10-20 μ L Buffer EB or ddH₂O directly onto column membranes. Let stand for 1 min, then spin 1 min at $>12,000g$ in microcentrifuge to elute.
7. Note on elution volumes: When working with amounts less than 100ng of DNA, the 10 μ L elution volume in Qiagen's MinElute protocol may recover less than the 80% claimed by Qiagen. Increase elution volume to 15-20 μ L, and dry the volume down if necessary. At the post-Second Strand Synthesis step (3.2.3), an elution volume of 20 μ L increases yields by 30-40% for a 50 ng sample.
8. Note on Second Strand Synthesis with limiting primer amounts: Limiting amount of primer is highly advisable when amplifying from very small amounts of starting material. Not only will it decrease the amount of primer-dimer product, it may also increase the yield of the desired amplification product. The table below shows what single reaction volumes have to be used for a specific amount of starting material.

DNA (ng)	T7 primer (μL) [*]	NEB 2 Buffer (μL)	5 mM dNTPs (μL)	Water (μL)	Tailed DNA (μL)	Klenow (μL)	Total volume (μL)
>75	0.60 (25 μM)	5.0	2.0	20.4	20.0	2.0	50
50-75	0.30 (25 μM)	2.5	1.0	0.20	20.0	1.0	25
25	0.15 (25 μM)	2.5	1.0	0.35	20.0	1.0	25
10 [*]	1.50 (1 μM)	1.0	0.4	0.20	6.5	0.4	10
5 [*]	0.75 (1 μM)	1.0	0.4	0.95	6.5	0.4	10
2.5 [*]	0.38 (1 μM)	1.0	0.4	1.32	6.5	0.4	10

^{*} If a thermal cycler without a heated lid is used, spin down tubes every 30 min during the 37°C incubation step.

Tailed DNA will have to be dried down in a vacuum centrifuge to the indicated volume for reaction volumes with 10 μL total volume.

3.3. Amplified Antisense RNA (aRNA) Labeling and Cleanup

3.3.1. Reverse Transcription to make aa-dUTP labeled cDNAs—

1. Combine 2 μg RNA and dH₂O to a final volume of 14.5 μL.
2. Add 1 μL of 5 μg/μL anchored oligodT (22mer, Integrated DNA Technology, IDT). Mix.
3. Heat at 70°C for 10 min. Snap cool on ice 10 min.
4. Quick spin to bring down condensation.
5. At room temperature, add 14.5 μL of master mix, which has been assembled just before use, adding enzymes at the end.

Master mix:

6.0 μL	(5×) SS II RT buffer
3.0 μL	0.1 M DTT
0.6 μL	(50×) aa-dUTP mix ** (see below)
2.0 μL	dH ₂ O
1.9 μL	SS II RT
1.0 μL	RNasin

6. Pipette to mix.
7. Incubate at 42°C for 2 h.
8. Incubate at 95°C for 5 min. Snap cool on ice.
9. Add 13 μL 1M NaOH and 1 μL 0.5M EDTA to hydrolyze RNA. Mix, quick spin.
10. Incubate at 67°C for 15 min.
11. Neutralize reaction with 50 μL 1M Hepes, pH 7.5. Vortex. Quick spin.
12. Purify reaction over Zymo column to remove unincorporated nucleotides as follows:

13. Add 1 mL of binding buffer to reaction. Mix. Load half of this material onto the column. Spin 10 s at >12,000g. Discard flow-through.
14. Add remainder of reaction. Spin again, 10 s at >12,000g. Discard flow-through.
15. Add 200 μ L of wash buffer to each column. Spin 30 s at >12,000g.
16. Add another 200 μ L wash buffer to each column. Spin 1 min at >12,000g. Discard flow-through and spin 1 min at >12,000g.
17. Add 10 μ L of 50 mM sodium bicarbonate, pH 9.0 to the filter. Incubate for 5 min at room temp. Spin 30 s at >12,000g to elute cDNA.
18. Couple aa-dUTP incorporated cDNAs to dye by adding the eluted material directly to 3 μ L Cy5 dye (Amersham) resuspended in DMSO. Incubate at room temp for 1h to overnight shielded from light.

3.3.2. Cleanup—

1. Keep Cy5 and Cy3 separate for MinElute cleanup (QIAGEN Cat. #28004) to measure CyDye incorporation, if desired. If using thin coverslips with small probe volume, you may need to purify Cy3 and Cy5 together or use a SpeedVac vacuum concentrator to reduce volume after elution.
2. Add 600 μ L of Buffer PB (Binding Buffer) to each sample.
3. Assemble the MinElute column on the provided 2 mL collection tubes.
4. Add the entire 720 μ L to a MinElute column.
5. Centrifuge for 1 min at 10,000g. Discard flow-through and reuse 2 mL tube.
6. Add 750 μ L of WASH Buffer PE to the column.
7. Centrifuge at 10,000g for 1 min. Discard flow-through and reuse 2 mL tube.
8. Centrifuge again at >12,000g for 1 min to remove residual ethanol.
9. Place column in a fresh 1.5 mL tube. Add 10 μ L of dH₂O to elute.
10. Allow elution buffer to stand for at least 2 min before spinning.
11. Centrifuge at >12,000g for 1 min. Add 10 μ L of H₂O to elute.
12. Allow elution buffer to stand for at least 2 min before spinning.
13. Centrifuge at >12,000g for 1 min.
14. Measure how many μ L eluted for each sample (still keep Cy5 and Cy3 separate) – should be around 18 μ L for each column.
15. Proceed to hybridization (below).

3.4. Microarray Hybridization

3.4.1. Preparation of hybridization mix—

1. Make Cot, Poly A and yeast tRNA mix:

10 μ g/ μ L Cot1 human DNA (Gibco-BRL)	2 μ L
10 μ g/ μ L polyA RNA (Sigma, #P9403)	2 μ L

10 µg/µL tRNA (Gibco-BRL, #15401-011) 2 µL

2. Combine Cy5 and Cy3-labeled material from labeling step.
3. Add Cot, Poly A and yeast tRNA mix, (20×) SSC, 10% SDS to the combined Cy5 and Cy3 probe, as below:

Cover Slip Size	22×60 regular thin coverslips	22×60 Erie M-series Lifter Slips
Total Hyb Volume (µl)	35	55
Probe Vol (µl)	28	36
Cot, PolyA, tRNA Mix	6	6
(20×) SSC (µl)	5.95	9.35
10% SDS (µl)	1.05	1.65
1M (pH 7.0) Hepes-KOH	0.84	1.32

For other coverslip sizes, volumes can be adjusted accordingly to maintain the same SSC, SDS, and Hepes concentrations. However, the blocking mix (Cot, PolyA, and tRNA) should only be 6 µL in all cases.

4. Denature probe by heating at 95-100°C for 2 min. Typically, this is done using a heat block set at 100°C with distilled water in the tube holders.
5. Let probe sit in the dark, at room temperature, for 10 min.
6. Spin probe at >12,000g for 5 min at room temperature.

Notes: • Avoid introducing bubbles. Do not vortex after adding SDS. Hepes is recommended for all probes.

3.4.2. Apply Hybridization Mixture to Microarray—

1. While probe is sitting at room temperature, set up hybridization chamber. Many different types of chamber are available commercially. Open chamber on clean flat surface, and place microarray slide in the chamber with the array side facing up.
2. Optional: We have found that for some home-printed oligo microarrays (which can often be dim), precipitation of labeled probe can occur at room temperature, which leads to a “pin-prick” morphology with precipitated probe filling in the depressions created during the printing process. To help mitigate this issue, it helps to place the hybridization chamber on a heat block at 55-60°C prior to addition of probe solution.
3. Once probe is spun down, carefully pipette probe solution onto the microarray surface in one drop towards one end of the array. Make sure not to create any bubbles during pipetting, and being careful not to touch microarray surface with pipette tip. Leave ~2 µL of probe in the tube.
4. Carefully apply a coverslip by placing one edge of the coverslip on the slide near the probe and slowly lowering the other edge, using another coverslip as a lever and wedge to lower it.
5. Close chamber and immediately submerge in 65°C water bath. Be careful not to tilt the chamber. For safety purposes, metal tongs may be used to place the hybridization chamber in the water bath.

6. If hybridizing multiple arrays, quickly move on to the next one, as the probes should not sit at room temperature for widely-varying times (we try to get all probes onto slides within ~10-15 min of each other).
7. Incubate chambers at 65°C for 16-20 h.

3.4.3. Array washing—

1. Prepare dishes with the following solutions from stocks of 20× SSC, 10% SDS, and dH₂O:

Wash 1 – (1×) SSC + 0.03% SDS

Wash 2 – (0.2×) SSC

Wash 3 – (0.05×) SSC

	Wash 1A	Wash 1B	Wash 2	Wash 3
(20×) SSC	20 mL	20 mL	4 mL	1 mL
10% SDS	1.2 mL	1.2 mL	-----	-----
dH ₂ O	379 mL	379 mL	396 mL	399 mL
Temperature	RT	RT	37°C	RT

2. Turn on Axon scanner from the connected black box, located behind the monitor. Let the scanner warm up for at least 15 min to allow the lasers' output time to stabilize.
3. Launch the GenePix Pro software and let it connect to the scanner and the network hardware key.
4. Remove one hybridization chamber from water bath. As quickly as possible, wipe chamber dry with paper towel, open chamber, remove slide and using your gloved hand, tap the slide against the bottom of the Wash 1A until coverslip gently slides off slide. Then transfer the slide to a pre-submerged rack in Wash 1B. Maintain slides in Wash 1B until all arrays have been transferred.
5. Quickly move the rack from Wash 1B to Wash 2 (tilting the rack back and further a couple of times to remove excess wash solution). Plunge up and down several times in Wash 2. Incubate 5 min with occasional plunging up and down.
6. Quickly move the rack from Wash 2 to Wash 3 (tilting the rack back and further a couple of times to remove excess wash solution). Plunge up and down several times in Wash 3. Incubate 5 min with occasional plunging up and down.
7. As quickly as possible, move rack of slides from Wash 3 to place in Beckman tabletop centrifuge, with paper towels under the rack and spin at 500-600 rpm for 5 min. Place microarrays in an opaque box, and begin scanning immediately. Typically, 4-5 arrays are washed at a time, and if more than 5 were hybridized then the next set of 4-5 should be washed as the last array is being scanned from the first batch.

4. Notes

1. Do not use the NEB Buffer 4 supplied with the NEB enzyme: use the cacodylate buffer (1M potassium cacodylate, 125mM Tris-HCl, and 1.25 mg/mL BSA, pH 6.6) supplied with the Roche enzyme.

2. Ensure that the dNTP mixes do not go through more than 3 freeze-thaw cycles. Additional freeze-thaw cycles will further degrade the dNTPs and will reduce the efficiency of the reaction.
3. Aim for ~1 pmol of template molecules. Tested range is 2.5-75 ng DNA per 10 mL reaction volumes. Scale up the reaction volume accordingly for higher starting amounts.
4. The use the NEB enzyme, as indicated, is strongly recommended. TdT enzyme from other sources may not perform optimally. If using the Roche recombinant TdT, use double volume of enzyme.
5. T7-A₁₈B primer: (5'-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAG(A)₁₈[B], where B refers to C, G or T). This should be obtained with HPLC, PAGE, or equivalent purification. If production of template-independent product is a significant problem, scale down the reaction volume, while keeping the reagent concentrations (except for the T-Tailed DNA) constant. See end of protocol for an example.
6. New England Biolabs (NEB) in early 2004 switched the supplied buffer for Klenow enzyme from EcoPol Buffer to NEB Buffer 2. This buffer should provide at least comparable yields to the old buffer, and may actually increase yields up to ~14%.
7. Ensure that the dNTP mixes do not go through more than 3 freeze-thaw cycles. Additional freeze-thaw cycles will degrade the dNTPs and will reduce the efficiency of the reaction.
8. If new kit, combine NTPs into one tube, then aliquot back out into the four tubes. In the first three freeze-thaw cycles, yields drop approximately 10-15% after each cycle. If the NTPs go through more than 3 freeze-thaw cycles, each subsequent freeze-thaw cycle may drop the yield by as much as 50%.
9. If you add cold buffer and dsDNA, you may risk precipitation of your DNA. Also, if there is precipitate present, warm buffer to 37°C until the precipitate dissolves.

Acknowledgments

These protocols were largely developed by CL Liu, and have been modified to meet the formatting requirements of this volume. This work was supported by grant GM079205 from National Institute of General Medical Sciences (NIGMS).

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