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# **Simultaneous profiling of transcripts and genomic regions associated with nuclear bodies using the RNA-DNA High-salt Recovered Sequence (RD-HRS) method**

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## **Summary**

Regulation of many nuclear functions in eukaryotic cells, such as transcription and splicing, relies on the association of specific genomic loci and/or transcripts with nuclear microenvironments having high molecular crowding, such as nuclear bodies. These membrane-less organelles are typically self-assembled through mechanisms of phase separation and, because of their liquid-like nature, analyzing their composition remains challenging. Here we present a detailed protocol of the RNA-DNA High-salt Recovered Sequence (RD-HRS) method, an extended version of the High-salt Recovered Sequence-sequencing (HRS-seq). Using high-salt treatments to make nuclear bodies insoluble, this extended version allows the profiling of both their RNA and genomic DNA contents, simultaneously, from the same biological sample.

**Key words:** Nuclear bodies RNAs, High-order chromatin organization, Phase separation

## 1. Introduction

Nuclear bodies are membrane-less cellular organelles typically self-assembled and disassembled through mechanisms of phase separation. They play a crucial role in 3D genome organization, promoting interactions between distant chromatin regions located in distinct Topologically Associating Domains (TADs) and sometimes even on different chromosomes [1]. They appear to be crucial for the regulation of gene expression [2], in particular transcription and splicing. However, because of their liquid-like nature, it remains challenging to purify them in order to examine their composition.

Based on experimental approaches developed in our laboratory [3,4], we have developed a genome-wide experimental method, High-salt Recovered Sequences sequencing (HRS-seq), which allows the identification of chromatin regions associated with nuclear microenvironment with high molecular crowding, like nuclear bodies [5]. Here we describe the detailed protocol of an extended version of this method, named the RNA-DNA High-salt Recovered Sequence (RD-HRS) method, that allows the simultaneous profiling of both the RNA and genomic DNA associated with nuclear-bodies from the same biological sample (**Figure 1**).

Using Triton X-100 permeabilization of purified cell nuclei on a filtration unit, we first extract nucleoplasmic RNAs (soluble RNA fraction) (**Figure 1**). We then use high-salt treatment in order to make nuclear bodies insoluble, resulting in the formation of the so-called nuclear halos. As previously demonstrated [5], a key point of the method is that the trapping of DNA and RNA inside the insoluble nuclear halos (inset in Figure 1) accurately reflects their *in vivo* location in nuclear micro-environments with high molecular crowding, like nuclear bodies or other large ribonucleoprotein complexes.

To analyze their DNA and RNA contents, these nuclear halos are incubated with a restriction enzyme (Styl) to obtain a soluble DNA fraction that passes through the filter while the RNA and DNA trapped in insoluble nuclear bodies remain on its surface (insoluble RNA and DNA fractions) (**Figure 1**). Unbiased high-throughput sequencing or locus-specific quantitative PCR / RT-PCR, can then be used to identify the RNAs and DNA regions associated with nuclear bodies as being those enriched in the insoluble fraction compared with their respective soluble RNA/DNA fraction.

## 2. Materials

1. Cell culture medium (appropriate for cells of interest) and trypsin-EDTA (for adherent cells).
2. Phosphate-buffered saline (PBS).
3. Buffer 1: 20 mM HEPES pH7.9; 10 mM KCl; 0.1% Triton X-100; Glycerol 20%; 1 mM MnCl<sub>2</sub>; 0.5 mM Spermidine; Protease Inhibitor 1X (e.g. Roche ref. 11697498001) (see **Note 1**).
4. Triton buffer: 10mM Tris-HCl pH 7.5, 100mM NaCl, 0.3M sucrose, 3mM MgCl<sub>2</sub>, 0.5% Triton-X100.
5. 2 mL centrifugal filter units with 0.22µm pores and 12 mL collection tubes (e.g. Millipore Ultrafree<sup>®</sup>-CL GV 0.22µm).
6. Home-made pistons: pistons of 2 mL syringe modified by adding a seal of interior diameter = 6 mm/exterior = 9 mm or home-made apparatus (HMA) for pressurizing filtration units (see **Note 2**).

7. Extraction buffer: 20mM Tris-HCl pH 7.5, 2M NaCl, 10mM EDTA, 0.125mM spermidine.
8. 1X Styl enzyme buffer: 50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM MgCl<sub>2</sub>, 0.2mM spermidine (see **Note 3**).
9. 10 U/μL Styl enzyme.
10. 10X PK buffer: 100mM Tris-HCl pH 7.5, 50mM EDTA, 5% SDS. Also diluted to a 2X stock.
11. 20 mg/mL proteinase K.
12. NucleoSpin<sup>®</sup> Gel and PCR Clean-up<sup>®</sup> kit compatible with SDS-containing samples (e.g. Macherey-Nagel<sup>®</sup>, ref.740609, with NTB, NT3 and NE buffers).
13. Vacuum manifold.
14. Qubit fluorimeter and dsDNA (ref. Q32850) / RNA HS (ref. Q32852) assays, or equivalent.
15. 5M NaCl.
16. 20 mg/mL glycogen.
17. 100% ethanol and 70% ethanol.

### 3. Methods

#### 3.1 Cell nuclei preparation (see Note 4)

1. If working with non-adherent cultured cells, proceed with **step 2** below. If working with adherent cell cultures, trypsinize cells (**Note 5**), before proceeding with **step 2** below.
2. Wash cells in PBS.
3. Resuspend cells in 100 μL of ice-cold buffer 1 per 500,000 cells.

4. Incubate 10 minutes on ice.
5. Centrifuge 3 minutes at  $600 \times g$ ,  $4^{\circ}\text{C}$ .
6. Remove supernatant.
7. Resuspend pellets in the same volume of ice-cold buffer 1 as in **step 3** and take a 10  $\mu\text{L}$  aliquot to count nuclei in a Thoma cell, or any other cell counting device, after trypan blue staining.
8. Adjust nuclei preparation to a final concentration of about 20 million nuclei in 100  $\mu\text{L}$  and, from this stock, make 100  $\mu\text{L}$  aliquots containing 100,000 nuclei diluted in buffer 1. Slowly freeze at  $-80^{\circ}\text{C}$  in a room temperature isopropanol-filled chiller.

### **3.2 Generation of nuclear halos by high-salt treatment**

9. Thaw on ice an aliquot containing 100,000 nuclei in a 1.5 mL Eppendorf tube (important: do not increase the number of nuclei treated during these steps *i.e.* 100,000 nuclei, see **Note 6**).
10. For 100  $\mu\text{L}$  of aliquot, add 900  $\mu\text{L}$  of PBS on the nuclei and homogenize by pipetting.
11. Transfer the Eppendorf content onto the filter of a centrifugal filtration unit equipped with a 12-mL collector tube below.
12. Apply a mild pressure with the help of the piston or Home-Made Apparatus (HMA) (see **Note 2**) to push the liquid through the filter at a drop-by-drop rhythm (no more than 1 drop per second) until as little liquid remains as possible above the filter (do not dry the filter).
13. Add a band of parafilm under the filter part to obstruct the filtration unit and place it back on the collector tube.
14. Add 1 mL of Triton buffer and homogenize by gently pipetting.

15. Incubate 15 minutes on ice (mix gently by pipetting 3 times during incubation).
16. Incubate 20 minutes at 37°C (**Note 7**).
17. Remove the parafilm and put the filtration unit on a 12-mL collector tube.
18. Use the piston (or HMA) to pass the entire volume as described in **step 12**. This fraction is the **permeabilization fraction** containing soluble RNAs (**Figure 1**).
19. Add 100  $\mu$ L of 10X Proteinase K (PK) buffer as well as 3  $\mu$ L of 20mg/mL PK to the permeabilization fraction, and incubate 30 minutes at 50°C. Proceed to **subheading 3.6** to extract total RNA from this fraction.
20. Add 1 mL of extraction buffer, onto the filter of the filtration unit and homogenize gently by pipetting. Push 6 drops out (dead volume of the column).
21. Incubate 5 minutes at room temperature.
22. Use the piston (or HMA) to pass the entire volume of extraction buffer through the filter at a drop-by-drop rhythm.
23. Add 1.9 mL of enzyme buffer. Pass all the liquid through the filter at a drop-by-drop rhythm. Repeat one more time, passing all the liquid through the filter and emptying the collection tube to the waste (these washings allow removal of high salt concentration present in the extraction buffer).

### ***3.3 Digestion of nuclear halos***

24. For each filtration unit, add a band of parafilm under the filter part to obstruct the filtration unit and place it back on the collector tube.
25. Add a mix containing 960  $\mu$ L of enzyme buffer and 20  $\mu$ L 10U/ $\mu$ L Styl, and homogenize gently by pipetting (see **Note 8**).

26. Incubate the filtration unit for 30 minutes at 37°C (take care that all the liquid in the filtration unit should be immersed into the water bath and check regularly that there is no leakage due to a lack of parafilm obstruction).
27. Collect evaporation drops in the tube cap and around tube walls and place them back in the liquid onto the filter. Add 20 µL of 10U/µL Styl, mix gently by pipetting and incubate 30 minutes more.

### ***3.4 DNA purification of Styl-digested DNA from soluble and insoluble fractions***

28. Remove tubes from the water bath. Collect evaporation drops in the tube cap and around the tube walls, and place them back with the remaining liquid on the filter. Homogenize gently by pipetting.
29. Remove the parafilm, collect residual drops on the parafilm and place them back in the liquid on the filter. Place the filter part on a clean collector tube and pass all the liquid through the filter.
30. Add 1 mL of enzyme buffer to rinse the nuclear halos and pass it all through the filter in the same collector tube as **step 29**. Save this eluate, corresponding to the **soluble fraction** (2 mL) containing the soluble DNA (**Figure 1**). Proceed to **step 34** to extract DNA from this fraction.
31. Place a parafilm to obstruct the filtration unit and put the filtration unit on a clean collector tube (12-mL).
32. Add 500 µL of enzyme buffer to the filter,
33. Add 500 µL of 2X PK buffer and 10 µL of 20 mg/mL proteinase K to the filter. Homogenize gently by pipetting.



34. Add 200  $\mu\text{L}$  of 10X PK buffer and 10  $\mu\text{L}$  of 20 mg/mL proteinase K to the **soluble fraction** from **step 30**. Homogenize gently by pipetting.
35. Incubate the filtration unit and the tube containing the soluble fraction for 30 minutes at 50°C in a water bath (take care to keep them well immersed).
36. Pass all the liquid through the filter of the filtration unit. Save this eluate in a clean collection tube. It corresponds to the **insoluble fraction** (1 mL) containing the biological material made insoluble by the high-salt treatment (**Figure 1**).
37. Split the 1 mL of insoluble fraction in two 500  $\mu\text{L}$  aliquots. Proceed to **Subheading 3.6** to extract RNA from the first aliquot and to **step 38** to extract DNA from the second aliquot.

**Steps 38-43** refer to the Macherey-Nagel® NucleoSpin® Clean-up® protocol.

38. Add 2.5 mL of NTB buffer to the insoluble fraction and 11 mL to the soluble fraction (5 volumes). The soluble and insoluble fractions are incubated for 5 minutes at 50°C in a water-bath (**Note 9**) and then processed in parallel.
39. Add 700  $\mu\text{L}$  of one fraction to a NucleoSpin® column and pass all the liquid by aspiration on a vacuum manifold (or by centrifugation 30 seconds at 11,000  $\times g$ , room temperature). Repeat these 700  $\mu\text{L}$  loadings until all of one fraction has passed through the same column.
40. Pass 700  $\mu\text{L}$  of NT3 buffer through the column using the vacuum manifold (or centrifuge 1 minute at 11,000  $\times g$ , room temperature) and empty collector tube in the waste.
41. Pass again 700  $\mu\text{L}$  of NT3 buffer through the column as in **step 40**.
42. Place the column on a clean Eppendorf tube properly labelled.

43. Add 100  $\mu\text{L}$  of NE buffer to the column corresponding to the soluble fraction and 50  $\mu\text{L}$  to the column corresponding to the insoluble fraction. Incubate 5 minutes minimum at room temperature. Centrifuge 1 minute at  $11,000 \times g$ , room temperature.
44. Quantify the DNA within each fraction with a Qubit fluorometric DNA assay or equivalent (see **Note 10**).
45. Repeat **subheadings 3.2–3.4** on further aliquots until at least 150 ng DNA and 150 ng total RNA is obtained for each fraction. Add milli-Q water to each fraction to make a final volume of 500  $\mu\text{L}$ .

### **3.5 Re-digestion of genomic DNA (see Note 11)**

46. Add 55  $\mu\text{L}$  of 10X digestion buffer (see **Note 3**) and 1  $\mu\text{L}$  of 10U/ $\mu\text{L}$  Styl to each fraction from **step 45**. Incubate 30 minutes at 37°C.
47. Purify the DNA with NucleoSpin® columns as in **steps 38-43**, except that 2.5 mL NTB buffer (5 volumes) is first added.
48. Quantify the DNA within the fractions with a Qubit fluorometric assay or equivalent. If required, concentrate each fraction by ethanol precipitation and resuspend the DNA to reach a final concentration of at least 10ng/ $\mu\text{L}$  (see **Note 12**).
49. HRS-seq DNA libraries are then made and analyzed as previously described [6]. Each library requires 150ng of Styl-digested genomic DNA (soluble or insoluble fraction) as starting materials (see **Note 13**).

### **3.6 RNA purification from permeabilization and insoluble fractions**

50. Add 2 volumes of PCA to each fraction ( $\frac{1}{2}$  Phenol;  $\frac{24}{25}$  Chloroform;  $\frac{1}{50}$  Isoamyl alcohol) (*i.e.* 2 mL for the permeabilization fraction from **step 19** and 1 mL to the first aliquot of the insoluble fraction from **step 37**). Shake vigorously for 15 seconds.
51. Centrifuge for 15 minutes at  $13,000 \times g$ ,  $4^{\circ}\text{C}$ . Save the supernatants into clean tubes.
52. Add 2 volumes of SEVAG ( $\frac{24}{25}$  Chloroform;  $\frac{1}{25}$  Isoamyl alcohol) to each fraction (*i.e.* 2 mL of SEVAG to the permeabilization fraction and 1 mL to the insoluble fraction aliquot).
53. Centrifuge for 15 minutes at  $13,000 \times g$ ,  $4^{\circ}\text{C}$ . Save the supernatants into clean tubes.
54. Add 0.05 volume of 5M NaCl to each fraction (*i.e.* 50  $\mu\text{L}$  to the permeabilization fraction and 25  $\mu\text{L}$  to the insoluble fraction), as well as 1  $\mu\text{L}$  of glycogen to each fraction.
55. Add 2.5 volumes of cold Ethanol to each fraction (*i.e.* 1.25 mL to the permeabilization fraction and 2.5 mL to the insoluble fraction). Incubate 1 hour at  $-80^{\circ}\text{C}$  (or overnight at  $-20^{\circ}\text{C}$ ).
56. Centrifuge for 20 minutes at  $17,000 \times g$ ,  $4^{\circ}\text{C}$  and remove ethanol.
57. Add 1 mL of cold 70% ethanol on the pellets and centrifuge for 10 minutes at  $17,000 \times g$ ,  $4^{\circ}\text{C}$ .
58. Add 30  $\mu\text{L}$  of RNase-free water to the dried pellets and shake carefully for 5 minutes.
59. To each resuspended pellet, add 1  $\mu\text{L}$  of DNase, 25  $\mu\text{L}$  of 10X DNase buffer and 194  $\mu\text{L}$  of RNase-free water (*i.e.* 250  $\mu\text{L}$  final volume).
60. Incubate 20 minutes at  $34^{\circ}\text{C}$  and repeat **steps 50 to 58** (adapt the volumes of reactants to a sample volume of 250  $\mu\text{L}$ ).
61. Quantify the RNA within the permeabilization and insoluble fractions with a Qubit fluorometric RNA assay or equivalent.

62. Purified total RNA can then be analyzed either by standard RNA-seq procedures (**Note 14**) and/or by reverse transcription and quantitative PCR (RT-qPCR) targeting transcripts of interest.

#### 4. Notes

1. Spermidine and protease inhibitors should be added extemporaneously to this buffer.
2. A Home Made Apparatus (HMA) for pressurizing filtration units was developed. Such apparatus is making use of a hydrostatic pump. It enables to exert a fixed and constant pressure on the liquid above the filter which can be easily set-up by controlling the speed of the hydrostatic pump. This apparatus was previously described in a publication on the former HRS-seq protocol [6].
3. We recommend to use homemade restriction buffer since commercial buffers usually contain reducing agents like DTT that may alter the insoluble fraction thus affecting DNA retention.
4. The present protocol, adapted from the EpiCypher Cutana® kit (ref.14-1048), is intended for cultured cells as previously described [7] . If working with mammalian tissues, prepare nuclei as previously described in references [8,9] and proceed with **step 2**. The quality of nuclei preparation is a critical point of the procedure. This protocol is intended for metazoan cultured cells or tissues. If using organoids or plant cells, make sure to used adapted protocols for nuclei preparation.
5. e.g. incubate cells 5 minutes at 37°C with (trypsin 0.25%; EDTA 10mM; diluted into PBS), add culture medium containing 5% FCS, centrifuge 5 minutes at 290 × g, or use any other dissociation protocol adapted to the cell-type used.

6. **Important note:** do not increase the number of nuclei treated during **steps 10-38**. It is extremely important to keep a small number of nuclei (100,000) diluted in a big volume during these steps because nuclear halos tend to aggregate together and such aggregation could generate *in vitro* artefacts. For the same reason, do not pellet nuclear halos by centrifugation, but, as stated in this protocol, always use filtration units and apply gentle pressure on the liquids using a piston or HMA [10] .
7. Make sure that the liquid level in the column is fully immersed in the water bath
8. If using a different enzyme to Styl, the enzyme buffer and the Biotinylated adaptor 1 provided in this protocol will need to be modified accordingly.
9. Samples should be heated to 50°C to avoid clogging the purification columns.
10. Quality controls can be made at this stage by qPCR to check for enrichment of specific sequences [3,5] .
11. Partial digestion is a technical concern; **Subheading 3.5** ensures full digestion before DNA library preparation.
12. The minimal concentration for DNA samples after **Subheading 3.5** is 10ng/μL. If less than 500ng have been obtained, the sample will need to be concentrated to at least 10ng/μL by ethanol precipitation (to a 50 μL sample add 2,5 μL of 5M NaCl, 1 μL of 20mg/mL glycogen and 150 μL of ethanol, mix, keep 45 minutes at -80°C, and centrifuge 15 minutes at 15,700g, room temperature). If more than 500ng have been obtained, the sample can be used directly.
13. Critical step: 150ng corresponds to the amount recommended to ensure a good quality of the final libraries.
14. Use oriented RNA-seq after ribodepletion of total RNA if premature/nascent RNAs are to be investigated.

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**Figure caption:**

**Figure 1** Principle of the RNA-DNA high-salt recovered sequence (RD-HRS) assay. Starting from purified cell nuclei preparations, nucleoplasmic RNAs are first extracted by gently permeabilizing the nuclei with Triton-X100 on a filtration unit. The principle of the HRS technique then relies on purification of nuclear bodies that are made insoluble by high-salt treatments. The soluble DNA can be released after digestion with a restriction enzyme (StyI), while the insoluble RNA and DNA fractions, trapped into the nuclear bodies, can be purified from the biological material that remains on the filter. Accurate quantifications of RNAs and target sequences in the insoluble vs soluble fractions by quantitative PCR/RT-PCR or by high-throughput sequencing are

then performed. Significant overrepresentation of an RNA or a restriction fragment in the insoluble fraction reflects its retention into nuclear bodies.

