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# **The High-salt Recovered Sequence-sequencing (HRS-seq) method: exploring genome association with nuclear bodies**

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*Running head: HRS-seq*

## **Summary**

Recent works indicate that, at specific loci, interactions of chromatin with membrane-less organelles self-assembled through mechanisms of phase separation, like nuclear bodies, are crucial to regulate genome functions, and in particular transcription. Here we describe the protocol of the High-salt Recovered Sequence sequencing method whose principle relies on high-throughput sequencing of genomic DNA trapped into large RNP complexes that are made insoluble by high-salt treatments.

**Key words:** High-order chromatin organization, nuclear bodies, Quantitative PCR

## 1. Introduction

The importance of genome organization at the supranucleosomal scale in the control of gene expression is increasingly recognized today (1, 2). In mammals, Topologically Associating Domains (TADs) and the active/inactive chromosomal compartments are two of the main nuclear structures that contribute to this organization level. However, recent works indicate that, at specific loci, chromatin interactions with nuclear bodies could also be crucial to regulate genome functions, in particular transcription (3). They moreover suggest that these nuclear bodies are membrane-less organelles dynamically self-assembled and disassembled through mechanisms of phase separation. Based on previous experimental approaches developed in our laboratory (4, 5), we have recently developed a novel genome-wide experimental method, High-salt Recovered Sequences sequencing (HRS-seq), which allows the identification of chromatin regions associated with large ribonucleoprotein (RNP) complexes and nuclear bodies (6). . Transcriptionally active nuclei preparations are treated at a high salt concentration and the insoluble fraction is purified and sequenced (Figure 1). We argue that the physical nature of such RNP complexes and nuclear bodies appears to be central in their ability to promote efficient interactions between distant genomic regions. The development of novel experimental approaches, including our HRS-seq method described in the protocol below, is opening new avenues to understand how self-assembly of phase separated nuclear bodies contributes to mammalian genome organization and gene expression.

## 2. Materials

1. Buffer 1: 0.3 M sucrose; 60 mM KCl; 15 mM NaCl; 5 mM MgCl<sub>2</sub>; 0.1 mM EGTA; 15 mM Tris-HCl pH7.5; 0.5 mM DTT; 0.1 mM PMSF; 3.6 ng/ml Aprotinin; 5 mM Na-Butyrate (see **Note 1**).
2. Buffer 2: buffer 1 supplemented by 0.8% v/v NP40.
3. Buffer 3: 1.2 M sucrose; 60 mM KCl; 15 mM NaCl; 5 mM MgCl<sub>2</sub>; 0.1 mM EGTA; 15 mM Tris-HCl pH7.5; 0.5 mM DTT; 0.1 mM PMSF; 3.6 ng/ml Aprotinin; 5 mM Na-Butyrate (see **Note 1**).
4. Glycerol buffer: 40% v/v Glycerol; 50 mM Tris-HCl pH 8.3; 5 mM MgCl<sub>2</sub>; 0.1 mM EDTA.
5. Triton buffer: 10mM Tris-HCl pH 7.5, 100mM NaCl, 0.3M sucrose, 3mM MgCl<sub>2</sub>, 0.5% Triton-X100.
6. Sterile 2ml centrifugal filter units with 0.22µm pore and 12ml collection tubes (e.g. Millipore Ultrafree-CL GV 0.22µm)..
7. Home-made pistons: pistons of 2ml 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**).
8. Extraction buffer: 20mM Tris-HCl pH 7.5, 2M NaCl, 10mM EDTA, 0.125mM spermidine.
9. 1X Styl enzyme buffer: 50mM Tris-HCl pH 7.5, 100mM NaCl, 10mM MgCl<sub>2</sub>, 0.2mM spermidine (see **Note 3**).
10. 10 U/µl Styl enzyme.

11. 10X PK buffer: 100mM Tris-HCl pH 7.5, 50mM EDTA, 5% SDS. Also diluted to a 2X stock.
12. 20 mg/ml proteinase K.
13. NucleoSpin Gel and PCR Clean-up kit compatible with SDS-containing samples (e.g. Macherey-Nagel, with buffer NTB).
14. Vacuum manifold.
15. Biotinylated adaptor 1 (15 $\mu$ M) with complementarity for Styl restriction sites:  
1R : 5'-P-CWWGTCGGACTGTAGAACTCTGAACCTGTCCAAGGTGTGA-Biotin-3' and  
1F : 3'-AGCCTGACATCTTGAGACTTGGACA-5 (see **Note 4**).
16. Quick Ligation Kit (NEB), containing Quick DNA ligase and 10X buffer.
17. 2X "Binding and Washing" BW buffer: 10mM Tris-HCl pH 7.5, 1mM EDTA, 2M NaCl. Also diluted to a 1X stock.
18. Dynabeads MyOne<sup>TM</sup> Streptavidin C1.
19. Magnetic support for Eppendorf tubes.
20. Rotating wheel.
21. TE buffer: 10mM Tris-HCl pH 7.5, 1mM EDTA.
22. Mmel (2  $\mu$ l) and 10X buffer provided by the enzyme supplier.
23. 10X SAM: 5 $\mu$ l of 32mM S-adenosyl-methionine diluted in water to a final volume of 325 $\mu$ l (see **Note 5**).
24. Thermomixer with programmable agitation.
25. Second adaptor ligation mix (per 50 $\mu$ l reaction): 1 $\mu$ l of 400U T4 DNA ligase, 5 $\mu$ l of 10X T4 DNA ligase buffer, 42 $\mu$ l of milli-Q water and 2 $\mu$ l of 15 $\mu$ M GEX adaptor 2 (600 nM final):  
2F : 5'-CAAGCAGAAGACGGCATAACGANN-3'  
2R : 3'-GTTCGTC TTCTGCC GTATGCT-P5' (see **Note 4**).

26. PCR master mix (48  $\mu$ l per reaction, final concentrations given for 1X mixture, after addition of 2 $\mu$ l template in step 58): 0.5 $\mu$ l of 2U/ $\mu$ l Phusion High-Fidelity DNA polymerase (1U final), 36 $\mu$ l of milli-Q water, 10 $\mu$ l of 5X HF Phusion buffer, 0.5 $\mu$ l of 25 $\mu$ M GEX PCR primer 1 (250nM final) (5'-CAAGCAGAAGACGGCATAACGA-3'), 0.5 $\mu$ l of 25 $\mu$ M GEX PCR primer 2 (250nM final) (5'-AATGATACGGCGACCACCGACAGGTTTCAGAGTTCTACAGTCCGA-3'), 250 $\mu$ M dNTPs.
27. PCR cycler.
28. Electrophoresis equipment and UV reader or DarkRider.
29. 1X NEBuffer 2 (10X stock from NEB): 10mM Tris-HCl pH 7.9, 50mM NaCl, 10mM MgCl<sub>2</sub>.
30. Costar Spin-X centrifuge tube filters (45 $\mu$ m).
31. 20 mg/ml Glycogen.
32. 3M Sodium acetate pH 5.2.
33. Absolute ethanol and 70% ethanol.
34. 2100 BioAnalyzer (Agilent) with high sensitivity DNA Chips.
35. Next-generation sequencing machine (e.g. Illumina Hi-Seq 2000).

### **3. Methods**

#### **3.1 Cell nuclei preparation (see Note 6)**

1. - If working with non-adherent cultured cells, proceed with step 2 below.  
- If working with adherent cell cultures, trypsinize, wash and filter through 40 $\mu$ m cell strainer to make a single-cell suspension before proceeding with step 2 below.

2. Wash cells in PBS.
3. Resuspend cells in 1.5 ml buffer 1.
4. Add 0.5 ml buffer 2 and mix slightly.
5. Put on ice 2 or 3 minutes.
6. Put 1 ml in 14 ml tubes (x2 tubes) containing 4 ml of buffer 3.
7. Centrifuge 20 minutes at 11300 g, 4°C.
8. Remove supernatant (use several tips to avoid NP40 contamination of the nucleus pellet).
9. Resuspend pellets in 50 µl of glycerol buffer and take a 4 µl aliquot to count nuclei in a Thoma cell. Adjust to a final concentration of about 20 million nuclei in 100 µl and, from this stock, make 100µl aliquots containing 150,000 nuclei diluted in glycerol buffer. Freeze into liquid nitrogen and store at -80°C.

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

10. Thaw on ice an aliquot containing 150,000 nuclei in a 1.5ml Eppendorf tube (important: do not increase the number of nuclei treated during these steps *i.e.* 150,000 nuclei, see **Note 7**).
11. For 100µl of aliquot, add 900µl of Triton buffer on the nuclei and homogenize by pipetting.
12. Incubate 15 minutes on ice (mix gently by pipetting 3 times during incubation).
13. Incubate 20 minutes at 37°C in a water bath or equivalent apparatus.
14. Transfer the Eppendorf content onto the filter of a centrifugal filtration unit equipped with a 12-ml collector tube below.

15. Push 2 drops out with the help of the piston or Home-Made Apparatus (HMA) (see **Note 2**).
16. Apply again a pressure with the help of the piston or HMA 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).
17. Add 1ml of extraction buffer, homogenize gently by pipetting and push 6 drops out (dead volume of the column).
18. Incubate 5 minutes at room temperature.
19. Use the piston (or HMA) to pass the entire volume of extraction buffer through the filter at a drop-by-drop rhythm.
20. Add 950  $\mu$ l of enzyme buffer. Pass all the liquid through the filter at a drop-by-drop rhythm. Repeat three more times, passing all the liquid through the filter and emptying the collection tube to the waste (these washings allow removal of high salt concentration presents in the extraction buffer).

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

21. For each filtration unit, 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**).
22. Add a band of parafilm under the filter part to obstruct the filtration unit and place it back on the collector tube.
23. 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).



24. Collect evaporation drops in the tube cap and around tube walls and place them back in the liquid onto the filter. Add 20 $\mu$ l of 10U/ $\mu$ l Styl, mix gently by pipetting and incubate 30 minutes more.

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

25. 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.
26. 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.
27. Add 1ml of enzyme buffer to rinse the nuclear halos and pass it all through the filter in the same collector tube as step 26. Save this eluate, corresponding to the **soluble fraction** (2ml).
28. Add 500 $\mu$ l of enzyme buffer to the filter, place a parafilm to obstruct the filtration unit and put the filtration unit on a clean collector tube (12 ml).
29. Add 500 $\mu$ l of 2X PK buffer and 10 $\mu$ l of 20 mg/ml proteinase K to the filter. Homogenize gently by pipetting.
30. Add 200 $\mu$ L of 10X PK buffer and 10 $\mu$ l of 20 mg/ml proteinase K to the **soluble fraction** from step 27. Homogenize gently by pipetting.
31. 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).
32. Pass all the liquid through the filter of the filtration unit. Save this eluate in a clean collection tube, corresponding to the **insoluble fraction** (1ml).

Steps 33-38 refer to the Macherey-Nagel® NucleoSpin Clean-up® protocol.

33. Add 5ml of NTB buffer to the insoluble fraction and 11ml to the soluble fraction (5 volumes). The soluble and insoluble fractions are then processed in parallel.
34. Add 700µl of one fraction to a NucleoSpin column and pass all the liquid by aspiration on a vacuum manifold (or by centrifugation 30 seconds at 11,000g, room temperature). Repeat these 700µl loadings until all of one fraction has passed through the same column.
35. Pass 700µl of NT3 buffer through the column using the vacuum manifold (or centrifuge 30 seconds at 11,000g, room temperature) and empty collector tube in the waste.
36. Pass again 700µl of NT3 buffer through the column as in step 35.
37. Place the column on a clean Eppendorf tube properly labelled.
38. Add 50µl of NE buffer. Incubate 1 minute minimum at room temperature.  
Centrifuge 1 minute at 11,000g, room temperature.

For each sample, repeat steps 10-38 until a total of at least 150 ng of DNA is obtained for each fraction (DNA quantification by Qubit or an equivalent apparatus).

Add milli-Q water to each fraction to a final volume of 500µl (see **note 9**).

### **3.5 Re-digestion of genomic DNA (see Note 10)**

39. Add 55µl of 10X digestion buffer (see **Note 3**) and 1µl of 10U/µl Styl to each fraction from step 38. Incubate 30 minutes at 37°C.
40. Purify the DNA with NucleoSpin columns as in steps 33-38, except that 2.5ml NTB buffer (5 volumes) is first added.

41. Check DNA concentration as in step 38. If required, concentrate each fraction by ethanol precipitation and resuspend the DNA to reach a final concentration of at least 10ng/μl (see **Note 11**).

### **3.6 Sequencing library preparation**

42. To 150ng of Styl-digested genomic DNA (soluble or insoluble fraction), add 2μl of 15μM biotinylated adaptor 1, 25μl of 2X Quick Ligation Reaction Buffer, 6.66μl of 400U/μl Quick DNA Ligase (see **Note 12**).
43. Add milli-Q water to a final reaction volume of 50μl and incubate for 15 minutes at room temperature.
44. Resuspend the stock of Dynabeads MyOne™ Streptavidin C1 beads by vortexing, then transfer 10μl beads to a 1.5ml Eppendorf tube.
45. Add 50μl of 1X BW buffer to the beads, vortex and briefly centrifuge the tube, then place the beads on a magnetic support for 2 minutes until the solution becomes clear.
46. Remove the supernatant by pipetting, remove the tube from the magnetic support.
47. Repeat steps 45-46 two more times.
48. Remove the supernatant and add 50μl of 2X BW buffer to the beads.
49. Add 50μl of DNA ligated with the first adaptor (from step 43). Incubate 15 minutes at room temperature on a rotator wheel.
50. Place the tubes on a magnetic support for 2 minutes, until the solution becomes clear.

51. Wash 3 times with 1 volume of 1X BW buffer, then two times with 1 volume of TE buffer (as described in steps 45-46).
52. Remove the supernatant, add 10µl of NEBuffer 4, 10µl of 10X SAM and 76µl of milli-Q water to the beads (see **Note 4**).
53. Add 4µl of 2U/µl Mmel and incubate for 90 minutes on a thermomixer at 37°C, 1400 rpm.
54. Remove supernatant and wash beads as in step 51.
55. Remove supernatant and add 50µl of second adapter ligation mix to the beads. Incubate 2h at 20°C in a thermomixer with 15 seconds of agitation every 2 minutes.
56. Remove supernatant and wash beads as in step 51.
57. Remove the supernatant and resuspend beads in 10µl milli-Q water.
58. Transfer 2µl of beads with the ligated DNA (from step 57) to PCR tubes with 48µl PCR master mix per tube.
59. Perform a PCR according to the following program:

Cycle step	Temperature	Time	Cycles
Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	15
Annealing	60°C	30 seconds	
Extension	72°C	15 seconds	
Extension	72°C	10 minutes	1

60. Load PCR the PCR reaction on to a 6% 1X TBE acrylamide gel and perform electrophoresis.

61. Visualize the gel with ethidium bromide or equivalent and UV, and excise the main band (expected size 95-97bp), placing into a 0.5ml tube that has been perforated 5 times with a needle and itself placed in a 2ml Eppendorf tube.
62. Centrifuge 2 minutes at 20,000g, room temperature and discard the 0.5 ml tube.
63. Add 100µl of 1X NEBuffer2 on acrylamide which is in the 2ml tube. Incubate overnight at room temperature on the rotator wheel.
64. Transfer the buffer/acrylamide mix to a Spin-X-filter column and centrifuge for 2 minutes at 20,000g, room temperature.
65. Transfer the 100µl of buffer containing the DNA into a 1.5ml tube. Add 1µl of glycogen, 10µl of 3M sodium acetate pH 5.2 and mix by shaking.
66. Add 325µl of absolute ethanol and shake. Centrifuge for 30 min at 19,000g, 4°C.
67. Remove the supernatant and wash the pellet with 500µl of 70% ethanol.
68. Centrifuge for 15 minutes at 14,000g, 4°C.
69. Remove the supernatant, dry the pellet at 37°C, then add 10µl of milli-Q water dissolve the DNA for 15 minutes at 37°C.
70. Verify on Agilent Bioanalyzer that the samples provide a single DNA band at the expected size (95-97 bp).
71. Load sequencing flow cells according to the recommendation of the supplier (e.g. for Illumina HiSeq 2000 apparatus, use 50-nt single reads, and load 6 pM of DNA library per flow cell and use the following primer for cluster generation: 5'-CCACCGACAGGTTTCAGAGTTCTACAGTCCGAC-3').

### **3.12 Bioinformatic analyses of HRS-seq data (see Note 13)**

72. HRS-seq raw data are trimmed for primer sequences.
73. Reads are aligned on the reference genome and those mapping to multiple positions or with more than 2 mismatches are removed.
74. Reads shorter than 18 nt or longer than 20 nt are removed (see **Note 14**).
75. Reads that do not end by the sequence WWGG are removed (see **Note 15**).
76. Count the total number of reads obtained for each Styl restriction fraction in the reference genome.
77. Use appropriate statistical tools (*e.g.* DEseq or edgeR) to determine which Styl restriction fragments display a statistically significant overrepresentation of reads counts in the insoluble fraction compared with the soluble fraction. These fragments that are those used for further bioinformatic analyses (*e.g.* comparison between different experimental conditions or cell-types...).

#### 4. Notes

1. DTT, PMSF and Aprotinin should be added extemporaneously to this buffer.
2. Home Made Apparatus (HMA) for pressurizing filtration units. A home-made apparatus was developed for pressurizing filtration units. Such apparatus is making use of a hydrostatic pump. It allows 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 (see **Figure 2**).
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 two primers used to make the adaptors are mixed in equimolar amount and annealed together on a PCR bloc by heating them at 100°C and then decreasing the temperature to 25°C by 0.1°C steps thanks to 750 cycles of 1 minute each.
5. Some enzyme suppliers provide Mmel directly supplemented with SAM (e.g. NEB). In that case SAM is replaced by 10 µl of milli-Q water (Step 52).
6. The present protocol is intended for cultured cells as previously described (9). If working with tissues, prepare nuclei as previously described in references (7, 8) and proceed with step 2.
7. **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 (150,000) 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 pressures on the liquids using a piston or HMA (10).
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. Quality controls can be made at this stage by qPCR to check for enrichment of specific sequences (4, 6).
10. Partial digestion is a technical concern; steps 39-41 ensure full digestion before sequencing library preparation.
11. The minimal concentration for DNA samples after step 40 is 10ng/µl. At this concentration, one requires to add 15µl (150ng) at step 42, which is the maximum volume allowed to reach 50µl of final volume at step 43. Therefore,

if less than 500ng have been obtained after step 40 (final volume of 50µl *i.e.* 10ng/µl), 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, 1µl of 20mg/ml glycogen and 150µl 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 without the concentration step 41.

12. Critical step: 150ng corresponds to the amount recommended to ensure a good quality of the final libraries. Add the biotinylated adaptor and the other reagents to the genomic DNA as specified. Do not make a “pre-mix” of the reagents with the ligase as this may lead to the formation of dimers of adaptors and would reduce the quality of the sequencing library.
13. This section provide guidelines to data processing as used in our previous work (6). The choice of software used to perform these tasks are largely depended on laboratory facilities and experimental conditions.
14. According to our protocol, Mme I digestion provides reads of 18 to 20 nucleotides.
15. According to our protocol, the reads should have one of their extremity next to a Styl restriction site.

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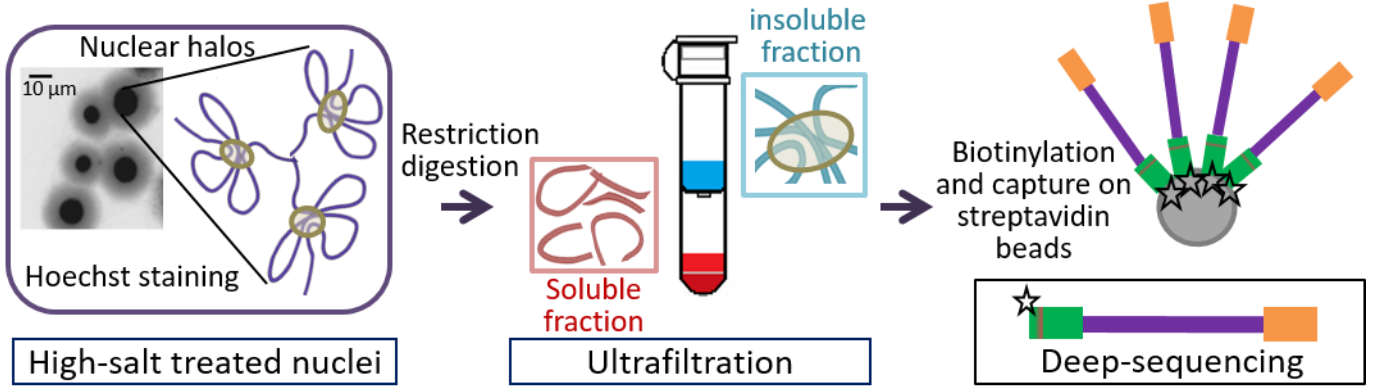
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**Figure caption:**

Figure 1: Principle of the High-salt Recovered Sequence (HRS) assay. The principle of the HRS technique relies on purification of large RNP complexes that are made insoluble by high-salt treatments. Accurate quantifications of target sequences in the insoluble vs soluble fractions by quantitative PCR (HRS-assay) or by high-throughput sequencing (HRS-seq method) are then performed. Significant overrepresentation of a restriction fragment in the insoluble fraction reflects its retention into large RNP complexes.

Figure 2: View of the Home-Made Apparatus (HMA) mounted with ultrafiltration units:  
(a) electrical plug, (b) switch, (c) pressure setting, (d) pressure display, (e) racks  
mounted with filtration units, (f) air pipes, (g) tap to close air pipe when not used.

**FIGURE 1**



**FIGURE 2**

