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NP-40 Fractionation and Nucleic Acid Extraction in Mammalian Cells

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[Abstract] This technique allows for efficient, highly purified cytoplasmic and nuclear-associated compartment fractionation utilizing NP-40 detergent in mammalian cells. The nuclear membrane is not disturbed during the fractionation thus leaving all nuclear and perinuclear associated components in the nuclear fraction. This protocol has been modified from Sambrook and Russell (2001) in order to downscale the amount of cells needed. To determine the efficiency of fractionation, we recommend using qPCR to compare the subcellular compartments that have been purified with equivalent amount of control whole cell extracts.

Keywords: Cell fractionation, Cytoplasmic and nuclear extractions, Nucleic acid purification, Quantitative PCR

[Background] To fully obtain an understanding of cellular processes, fractionation of nuclear and cytoplasmic compartments are needed. There are many protocols and even some commercial kits available to help separate the two compartments. However, most require high centrifugation speeds and there is a high discrepancy in the yield and even the methods to verify the amount of contamination in the final products. Our protocol utilizes a small benchtop centrifuge at low speeds to obtain highly pure extractions for the cytoplasmic and a combined nuclear/perinuclear associated compartments as well as the data analysis to verify the percentage of contamination. To date, the cells lines that have been tested are 293 T, HeLa and GHOST cell lines. (Galvis, 2014; Galvis *et al*., 2014).

Materials and Reagents

1. BD 21 G needles (Fisher Scientific, catalog number: 14-823-55)
   Manufacturer: BD, catalog number: 305274.
2. BD tuberculin syringes (Fisher Scientific, catalog number: 14-823-2F)
   Manufacturer: BD, catalog number: 309602. This product has been discontinued.
3. TipOne 10 µl pipet tips (USA Scientific, catalog number: 1111-3200)
4. TipOne 1-200 µl natural pipet tips (USA Scientific, catalog number: 1111-1800)
5. TipOne 1,000 µl natural pipet tips (USA Scientific, catalog number: 1111-2020)

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6. Costar 6-well cell culture plates (Fisher Scientific, catalog number: 07-200-80)
   Manufacturer: Corning, catalog number: 3506.
7. 1.5 ml microcentrifuge tubes (Fisher Scientific, catalog number: 14-666-318)
8. Iscove's modification of DMEM (Mediatech, catalog number: 10-016-CV)
9. Tween 40 (CHEM-IMPEX INTERNATIONAL, catalog number: 01513)
10. 1x phosphate buffered saline (PBS) (MP Biomedicals, catalog number: 091860454)
11. 1x trypsin-EDTA (Mediatech, catalog number: 25-051-CI)
12. 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Fisher Scientific, catalog number: BP2482-500)
13. 10% sodium dodecyl sulfate (SDS) (Mediatech, catalog number: 46-040-CI)
14. Proteinase K (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EO0491)
15. Ribonuclease A, DNase free (Thermo Fisher Scientific, Thermo Scientific™, catalog number: EN0531)
16. Potassium acetate (Fisher Scientific, catalog number: P171-500)
17. Isopropanol (Fisher Scientific, catalog number: A416-500)
18. Ethanol 70% (Fisher Scientific, catalog number: BP82011)
19. Sodium hydroxide (NaOH) (Fisher Scientific, catalog: S318-500)
20. SYBR Green PCR Master mix (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4309155)
21. Magnesium chloride ($MgCl_2$) (Fisher Scientific, catalog number: BP214-500)
22. Sucrose (Fisher Scientific, catalog number: S3-500)
23. Tris-HCl buffer pH 7.4 (Lonza, catalog number: 51237)
24. Potassium chloride (KCl) (Fisher Scientific, catalog number: P330-500)
25. NP-40 10% solution (Thermo Fisher Scientific, catalog number: 28324)
26. 1 M $MgCl_2$ (see Recipes)
27. 2.5 M sucrose (see Recipes)
28. TMK (see Recipes)
29. TMK + 1% NP-40 (see Recipes)
30. S1 buffer (see Recipes)
31. S2 buffer (see Recipes)
32. Cell lysis buffer (see Recipes)

Equipment

1. Ventilated microcentrifuge (Fisher Scientific, model: accuSpin™ Micro 17R, catalog number: 13-100-676)
2. Gilson PIPETMAN Classic pipets (Gilson, model: P1000, catalog number: F123602)
3. Gilson PIPETMAN Classic pipets (Gilson, model: P20, catalog number: F123600)
4. Gilson PIPETMAN Classic pipets (Gilson, model: P200, catalog number: F123601)

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5. Gilson PIPETMAN Classic pipets (Gilson, model: P2, catalog number: F144801)
6. Water bath (Fisher Scientific, model: FS-14)
7. Vortexer (Fisher Scientific, catalog number: 02-216-108)
8. ABI Prism 7900HT sequence detection system (PE-Applied Biosystems) (Thermo Fisher Scientific, Applied Biosystems™, model: ABI PRISM™ 7900HT)

Procedure

A. NP-40 fractionation
1. Wash plate 2 x with 2 ml PBS per well. Plate should have 5-10 x 10^6 cells at 90-95% confluence.
2. Trypsinize cells (500 µl/well) and add 1 ml/well of media to neutralize. Combine the 6 wells of each plate. Can use a single 10 cm dish at the same confluence of 90-95% with 3-6 x10^7 cells.
3. Spin cells at 4 °C, 100 *x g* for 5 min.
4. Resuspend cells with 1 ml PBS.
5. Pellet cells at 100 *x g* for 4 min at 4 °C.
6. Resuspend cells in 200 µl TMK.
   Note: For qPCR analysis of whole cell extraction, you can take out 50 µl and extract the DNA before proceeding to Procedure B.
7. Add 150 µl of TMK +1% NP-40.
8. Incubate on ice for 5 min with occasional gentle vortexing.
9. Pellet at 228 *x g* for 5 min at 4 °C.
10. Remove the supernatant without disturbing the pellet–This is the Cytoplasmic fraction.
11. Suspend the stripped nuclear pellet in 500 ml of S1 buffer.
12. Layer the S1 suspended pellets over 500 ml of S2 buffer.
13. Centrifuge at 1,430 *x g* for 5 min at 4 °C.
14. Aspirate supernatant off.
15. Resuspend the nuclei in 300 µl PBS.
    Note: At this point, all samples can be stored at 4 °C overnight before proceeding to Procedure B.

B. DNA extractions
1. Add 600 µl ice-cold cell lysis buffer to the cell extracts. Expect SDS precipitation with most extractions; it will not affect the end result. Vortex thoroughly for 30 sec.
2. Add 3 µl of Proteinase K solution (20 mg/ml). Incubate DNA sample at 55 °C for 3-16 h. The time is dependent on how much SDS precipitation is noticed on the samples and cell line used. A rule of thumb is the more SDS precipitation and larger cells tend to require more incubation time.
3. Shear DNA by drawing repeatedly (15 x) through a 21 G needle, avoiding excessive foaming and loss by aerosolization.

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4. Allow cooling to RT, then add 3 µl of 4 mg/ml DNAse-free Ribonuclease A. Digest for 15-60 min at 37 °C.
   Note: At this point, all samples can be stored at 4 °C overnight before proceeding to step B5.
5. Cool to RT. Add 200 µl 5 M potassium acetate; vortex for 20 sec.
6. Pellet precipitated protein/SDS by centrifugation at 4 °C for 3 min at maximum RPM.
7. Transfer solution to a fresh tube with 600 µl isopropanol. Mix by inversion. Spin at maximum RPM for 3 min at RT.
8. Remove supernatant and wash DNA with 600 µl 70% EtOH. Spin for 3 min at maximum RPM to sediment the pellet. Aspirate the ethanol. Repeat with a 1-min spin and aspirate residual EtOH.
9. Air-dry for 15 min.
10. Redissolve the pellet in 100 µl 8 mM NaOH or 10 mM Tris. This may be slow–an incubation at 37 °C for 15 min is recommended.
11. The DNA may now be stored at -20 °C without incident.

C. Assessing efficiency of the NP-40 fractionation

To assess the accuracy of the nucleo-cytoplasmic separation, qPCR can be utilized using nuclear, mitochondrial and whole cell extract samples such as example below.

1. DNA samples were diluted 1 to 10 in dH_2O and incubated at 98 °C for 20 min followed by immediate cooling on ice.
2. Each PCR reaction contained 7.5 µl SYBR Green PCR Master mix (Fermentas), 2 µl of template, and 1 µM of each primer (Table 1) in a 15 µl reaction volume.

Table 1. Primers for DNA amplification

Gene	Primers name	Primer sequence	Amplicon
β-globin	LA1	ACACAACTGTGTTCACTAGC	110 bp
	LA2	CAACTTCATCCACGTTCACC	
mitochondria	MitouaF	GATCACAGGTCTATCACCCT	151 bp
	MitouaR	GGATGAGGCAGGAATCAAAG	

3. QPCR was performed in triplicate for each sample using an ABI Prism 7900HT sequence detection system (PE-Applied Biosystems) for amplification and detection. PCR conditions were as follows:

95 °C	10 min	1 cycle
95 °C	15 sec	40 cycles
60 °C	60 sec	
60 °C	5 min	1 cycle
12 °C	forever	1 cycle

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Data analysis

1. The accuracy of the NP-40 nucleo-cytoplasmic fraction can be verified by comparing the amount of mitochondrial DNA copies detected in the nuclear fraction and the amount of β-globin DNA copies in the cytoplasmic fraction with the respective copies found in the total cell lysate. The data is analyzed by delta method which requires an average threshold cycle and standard deviation. Hence,
   Ct = threshold cycle, a = nuclear or cytoplasmic sample, b = whole cell lysate sample:

   Ratio = $2^{Ct\ (a)\ -\ Ct\ (b)}$

   Error (a + b) =$\sqrt{\mathrm{Error(a)\hat{}2\ +\ Error(b)\hat{}2}}$

2. The yield recovered is compared to cytoplasmic contamination in nuclear fractions. Figure 1 adapted from Galvis (2014) is an example of the data analysis graphs that can be composed with the obtained data. The ratio of mitochondrial DNA copies in the nuclear fractions to mitochondrial DNA copies in the whole cell lysate. The example demonstrates a successful fractionation. The nuclear fraction has a 1:1 ratio of nuclear DNA with the whole cell extract; while at the same time there is minimal mitochondrial DNA in comparison to the whole cell extract. The cytoplasmic fraction contains minimal nuclear DNA in comparison to the whole cell extract while it contains an almost 1:1 ratio of mitochondrial DNA with the whole cell extract.
3. Note that the recommended inclusion data is that all triplicates must be within one cycle apart from each other and all obtained cycles must be smaller than the negative control that need to be included in the qPCR runs.

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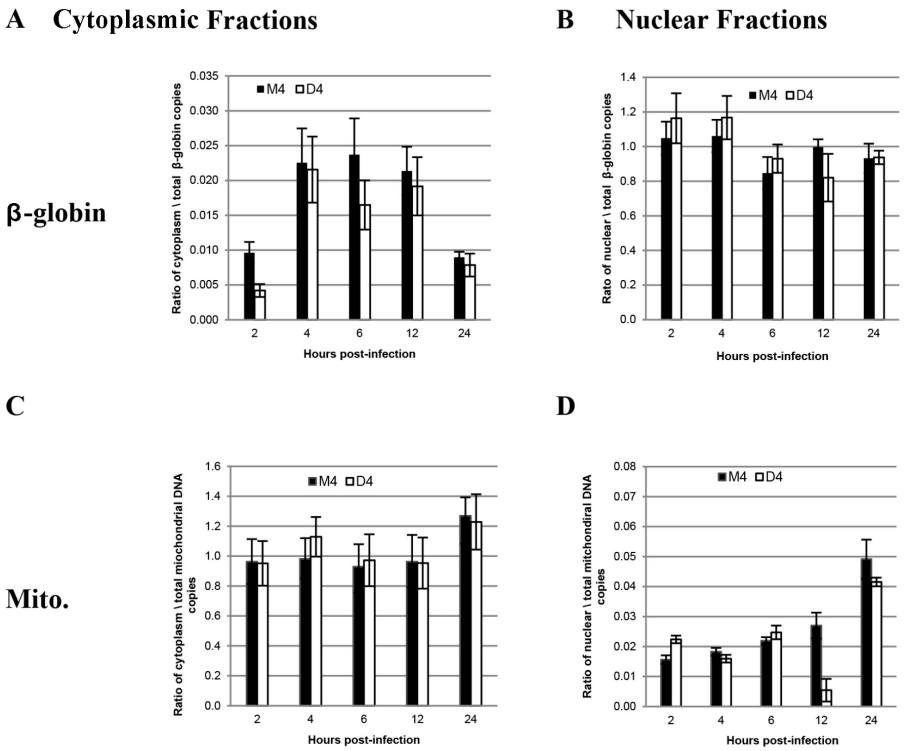

Figure 1. Sample data analysis of fractionation protocol. This is a ratio of mitochondrial and β-globin DNA in nuclear and cytoplasmic fractions vs. total cell lysates. GHOST-R5X4 cells were transfected either with DBR1 shRNA pHyper-D4 (D4) or DBR1 shRNA triple mismatch, pHyper-M4 (M4). Two to twenty-four hours later, the cells were either fractionated into nuclear and cytoplasmic fractions or lysed to prepare whole cell extracts. DNA was isolated for qPCR to evaluate the success of fractionation. A. β-Globin primers were utilized to assay the exclusion of nuclear contamination in the cytoplasmic fraction in contrast to whole cell extracts. The graph illustrates the ratio of β-globin copies in the cytoplasmic fractions to β-globin copies in the whole cell lysates. B. β-Globin primers were utilized to assay concentration of nuclear fractions compared to whole cell extracts. The graph illustrates the ratio of β-globin copies in the nuclear fractions to β-globin copies in the whole cell lysate. C. Mitochondrial primers were used to assay concentration of cytoplasmic fraction compared to whole cell extracts. The graph illustrates the ratio of mitochondrial DNA copies in the nuclear fractions to β-globin copies in the whole cell lysates. D. Mitochondrial primers were utilized to assay the exclusion of mitochondrial contamination in the nuclear extractions.

Notes

1. The data presented in Procedure C were the unpublished controls for experiments denoted in Galvis *et al.*, 2014. Debranching enzyme 1 (DBR1) in our experiments has been implicated in reverse transcription of the HIV-1 genome.
2. In Procedure A step 12, it is very important not to disturb the pellet and hence it is better to leave some of the supernatant behind. The purpose of the subsequent steps is to remove the

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remaining mitochondrial DNA.

3. In our experience, the resuspension of the DNA with 8 mM NaOH has given us better overall recovery yield for downstream applications in comparison to 10 mM Tris. However, if the experiments desired only need for qPCR purposes only and not sequencing then 10 mM Tris is sufficient for resuspension of the final pellet.

Recipes

1. 1 M $MgCl_2$ (500 ml)
   Add 101.65 g $MgCl_2 \cdot 6H_2O$ into 500 ml ddH_2O
   Store at room temperature
2. 2.5 M sucrose
   8.55 g of sucrose
   Up to 10 ml with ddH_2O
   Need to heat to 37 °C in order to have sucrose fully dissolve
   Store at room temperature
3. TMK (10 ml; store in 4 °C)

Stock volume	**(ml)**	**mM (final)**
1 M Tris-HCl (pH 7.4)	250 µl	25 mM
1 M KCl	50 µl	5 mM
2 M $MgCl_2$	5 µl	1 mM
ddH_2O	to 10 ml	

4. TMK + 1% NP-40 (2 ml)
   1.8 ml TMK
   200 µl 10% NP-40
   Store in 4 °C
5. S1 buffer
   0.25 M sucrose
   10 mM $MgCl_2$

Stock	**To make 20 ml**
2.5 M sucrose	1.96 ml
1 M $MgCl_2$	0.2 ml
dH_2O	up to 20 ml

6. S2 buffer
   0.35 M sucrose
   0.5 mM $MgCl_2$
   Store at 4 °C

Stock	**To make 40 ml**
2.5 M sucrose	5.5 ml
1 M $MgCl_2$	0.02 ml
dH_2O	up to 40 ml

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7. Cell lysis buffer

   10 mM Tris-HCl, pH 7.4

   0.1% SDS

   1 mM EDTA

   Store at 4 °C

Stock	To make 10 ml
1 M Tris-HCl (pH 7.4)	100 µl
10% SDS	100 µl
0.5 M EDTA	20 µl
H_2O	to 10 ml

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References

1. Galvis, A. E. (2014). An RNA lariat intermediate in HIV-1 cDNA synthesis. *ProQuest* 3615193.
2. Galvis, A. E., Fisher, H. E., Nitta, T., Fan, H. and Camerini, D. (2014). Impairment of HIV-1 cDNA synthesis by DBR1 knockdown. *J Virol* 88(12): 7054-7069.
3. Sambrook, J. and Russell, D. W. (2001). Molecular cloning: a laboratory manual. *CSHL Press*.