Chapter 21

Single Cell Gel Electrophoresis for the Detection of Genomic Ribonucleotides

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Abstract

Single cell gel electrophoresis or comet assay enables the quantification of DNA damage such as single-strand or double-strand breaks on a single cell level. Here, we describe a variant of this method for the detection of ribonucleotides embedded in genomic DNA. Briefly, cells are embedded in agarose on a microscopic slide, lysed under high salt and alkaline conditions and then subjected to in situ treatment with *E. coli* RNase HII which nicks 5′ to a ribonucleotide within the context of a DNA duplex thereby converting genomic ribonucleotides into strand breaks. After unwinding of genomic DNA using a highly alkaline buffer, electrophoresis under mild alkaline conditions is performed resulting in formation of comets due to migration of fragmented DNA toward the anode. Following SYBR Gold staining comets can be visualized by fluorescence microscopy. In this setting, the length and the intensity of comets formed reflect the level of genomic ribonucleotides present in a given cell.

Key words Comet assay, Single cell electrophoresis, Alkaline lysis, RNase H2, Ribonucleotides, Genomic DNA

1 Introduction

Ribonucleotides misincorporated into genomic DNA by replicative polymerases represent the most frequent DNA base lesion in replicating mammalian cells [1]. In vivo, genomic ribonucleotides are removed by ribonucleotide excision repair, which is initiated by ribonuclease H2 (RNase H2) through cleavage 5′ of a ribonucleotide followed by strand displacement synthesis by Pol δ, flap removal by FEN1, and ligation by DNA ligase 1 [2]. If left unrepaired, ribonucleotides render the DNA backbone susceptible to DNA strand breaks leading to genome instability [1, 3–5]. Thus, RNase H2 plays an essential role in the maintenance of genome integrity. In humans, mutations in the genes encoding the three

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RNase H2 subunits (*RNASEH2A*, *RNASEH2B*, *RNASEH2C*) cause the neuroinflammatory disorder Aicardi-Goutières syndrome [6]. While complete RNase H2 deficiency in mice is embryonic lethal due to massive DNA damage [1, 5], RNase H2 mutations found in patients with Aicardi-Goutières syndrome were shown to be hypomorphic [7, 8]. In patient cells, increased levels of genomic ribonucleotides cause chronic low level DNA damage leading to the activation of the antiviral type I interferon axis [8–10].

Here, we describe a method for the detection and quantification of ribonucleotides embedded in genomic DNA of human fibroblasts based on single cell gel electrophoresis or comet assay [8]. The basic principle of the comet assay rests on the migration of negatively charged DNA fragments out of agarose-embedded nuclei toward the anode during electrophoresis resulting in the formation of comet tails [11–13]. While electrophoresis under neutral pH conditions predominantly exposes DNA double-strand breaks, electrophoresis under highly alkaline denaturing conditions (pH >13) further exposes single-strand breaks in DNA [12]. Furthermore, the use of specific endonucleases that convert base lesions into single-strand breaks enables detection of lesion-specific DNA damage. For example, treatment of cells with 8-oxoguanine DNA glycosylase prior to electrophoresis allows detection of oxidized bases [14, 15]. In our protocol *E. coli* RNase HII is used to nick genomic DNA at sites of embedded ribonucleotides to create single-strand breaks. The steps involved in the comet assay for the detection of genomic ribonucleotides are depicted in Fig. 1. Cells are first embedded in agarose on a microscopic slide and lysed to remove membranes, cytoplasm, and nucleoplasm including nucleosomes. The remaining nucleoids are subjected to RNase HII treatment followed by electrophoresis using a mildly alkaline buffer. Migration of fragmented DNA leads to the formation of comets, the sizes of which reflect the extent of DNA fragmentation generated by RNase HII treatment. The representative images of comets obtained with this method in human fibroblasts deficient in RNase H2 are shown in Fig. 2.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ × cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise.

1. Low melting Agarose (1%): Dissolve 1 g agarose (type VII, low gelling temperature; Sigma Aldrich) in 100 ml 1× PBS. Make aliquots and store at −20 °C.

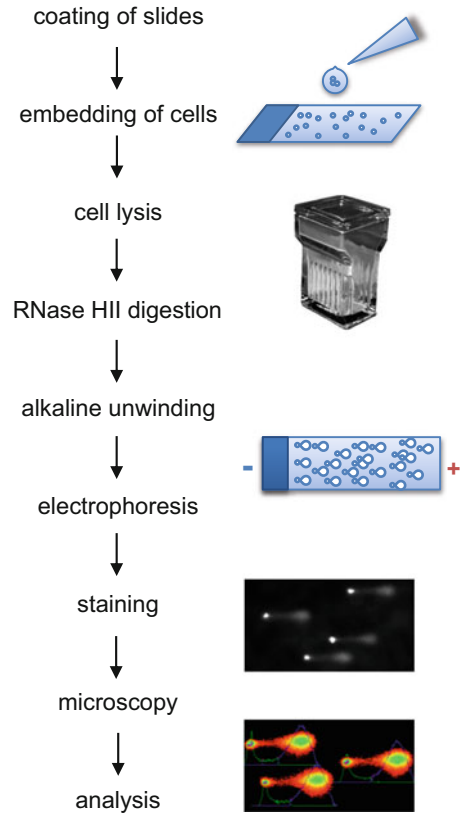

Fig. 1 Flow chart depicting steps involved in the modified comet assay for the detection of genomic ribonucleotides.

2. Normal melting Agarose (0.5%): Dissolve 0.5 g agarose (type II; Sigma Aldrich) in 100 ml 1× PBS. Make aliquots and store at −20 °C.
3. Normal melting Agarose (1%): Dissolve 1 g agarose (type II; Sigma Aldrich) in 100 ml 1× PBS. Make aliquots and store at −20 °C.
4. Antifade solution with SYBR Gold: Dissolve 2.33 g DABCO (1,4-diazabicyclo[2.2.2]octane; 20 mM) in 2 ml 1 M Tris–HCl (pH 8.0) and 80 ml glycerol by heating to 70 °C. Add distilled water to a volume of 100 ml. Add 1 μl of SYBR Gold stock solution (Thermo Fisher Scientific) to 10 ml Antifade solution and store in a dark vial at −20 °C.
5. Slides (Dakin Fully Frosted Slides, 71876-01; Electron Microscopy Sciences).
6. Phosphate-buffered saline (1× PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml distilled H_2O. Adjust pH to 7.4 with HCl. Add distilled water to a volume of 1 L.

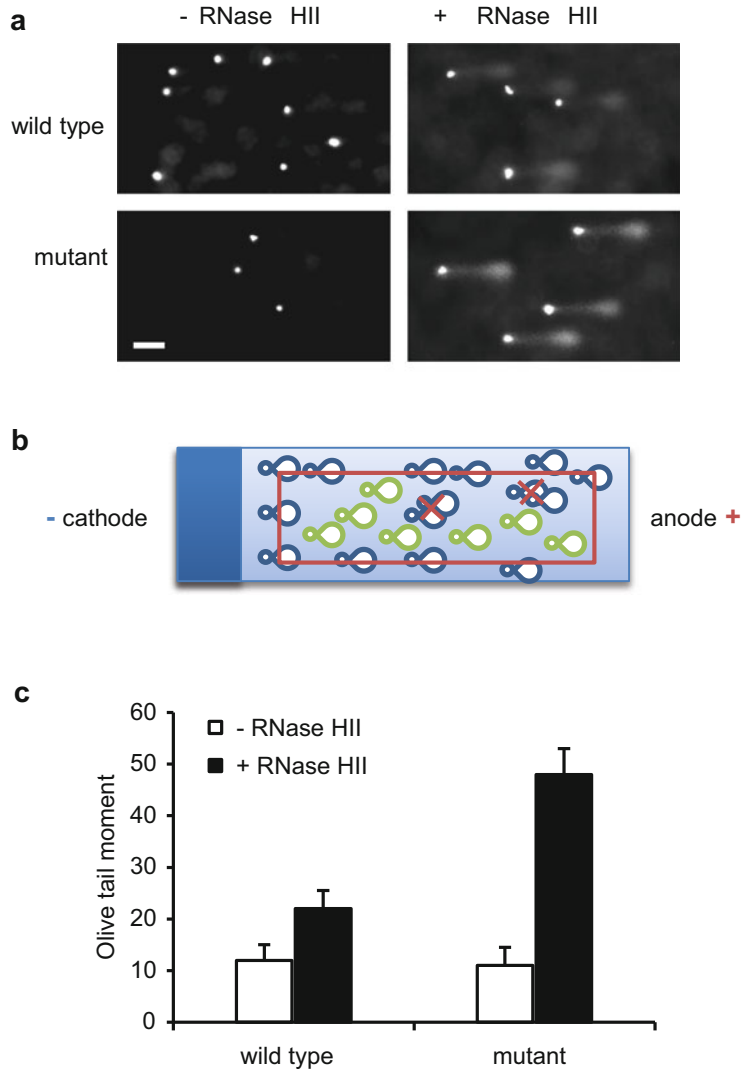

Fig. 2 Modified comet assay of RNase H2-deficient human fibroblasts. (**a**) Images of comets obtained from primary human fibroblasts of a healthy control (wild type) and a patient with RNase H2 deficiency (mutant) with and without RNase HII (−/+ RNase HII) treatment. Scale bar 20 μm. (**b**) Schematic of slide after electrophoresis and SYBR Gold staining. The red box indicates the area from which comets should be sampled for analysis. Superimposed comets should be excluded from analysis. (**c**) Olive tail moments as determined with CASP software. At least 50 comets were analyzed per slide. Shown are the means and SD.

7. Tris-borate-EDTA (10× TBE): 0.9 M Tris base, 0.9 M boric acid, 20 mM EDTA): Dissolve 108 g Tris, 55 g boric acid and 9.3 g Na_2H_2EDTA in 800 ml distilled water. Add distilled water to a volume of 1 L.

8. Alkaline lysis buffer: 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris. Dissolve 73.05 g NaCl, 18.61 g Na_2H_2EDTA and 0.606 g Tris in 400 ml distilled water. Adjust to pH 10 with 1 N NaOH. Adjust volume to 500 ml with distilled water. Add 1 ml Triton X-100 per 100 ml buffer (1%) prior to lysis.
9. RNase H2 buffer: 20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$. Mix 20 ml 1 M Tris–HCl (pH 8.0), 10 ml 1 M $(NH_4)_2SO_4$, 10 ml 1 M KCl and 2 ml 1 M $MgSO_4$. Adjust to pH 8.8 with 1 N NaOH. Add distilled water to a volume to 1 L. Add 1 ml Triton X-100 per 1 L buffer (0.1%) prior to use.
10. *E. coli* RNase HII (M0288; NEB).
11. 0.1% SDS: Dissolve 1 g SDS in 1 L distilled water.
12. Alkaline unwinding buffer: 0.3 M NaOH, 1 mM Na_2H_2EDTA. Mix 60 ml 5 N NaOH and 2 ml 0.5 M Na_2H_2EDTA with 800 ml distilled water. Adjust pH to 13.1 with 10 N NaOH and add distilled water to a volume of 1 L.
13. Neutral electrophoresis buffer (0.5× TBE).

3 Methods

Carry out all the procedures at room temperature unless otherwise specified.

3.1 Coating of Slides

Pre-warm slides on a hot plate (IKAMAG Rec-G) at 50 °C. First layer: Disperse 50 µl of warm (50 °C) 0.5% normal melting agarose per slide using a second slide to spread the agarose. Let agarose solidify. Second layer: Disperse 400 µl of warm (50 °C) 1% normal melting agarose per slide. Quickly cover the agarose with a 24 × 60 mm coverslip to smoothen the surface (*see* **Note 1**). Agarose-coated slides can be stored in a moist chamber at 4 °C for up to 1 week.

3.2 Embedding of Cells

Melt aliquots of 1% low melting agarose in a water bath at 42 °C. For cooling of slides, prepare ice box and place metal plate on ice (*see* **Note 2**). Prepare alkaline lysis buffer with 1% Triton X and cool to 4 °C. Trypsinize fibroblasts and centrifuge at 200 × *g* for 5 min and 4 °C. Discard the supernatant, resuspend cells in 1 ml 1× PBS. Dilute cell suspension to 1×10^6 cells per ml in 1× PBS. Pre-warm agarose-coated slides to 42 °C on a hot plate and remove coverslip shortly prior to adding the agarose-cell suspension. Add 150 µl of cell suspension to 600 µl of 1% low melting agarose and mix carefully using a pipette with a cut tip to prevent shearing of cells. Pipette 150 µl of agarose-cell suspension per precoated slide and quickly cover with a 24 × 60 mm coverslip. Transfer the slide immediately onto a cold plate on ice.

3.3 Cell Lysis

Allow the agarose-cell suspension to solidify for 5 min, carefully remove the coverslip, and let slides air-dry for a few minutes. Incubate slides in a glass staining jar filled with precooled alkaline lysis solution at 4 °C overnight (*see* **Note 3**). Wash slides 10× for 10 min each with 1× PBS.

3.4 RNase HII Digestion

Equilibrate slides 3× for 20 min each in RNase H2 buffer, drain excess buffer by placing slides upright on a paper towel. Dilute *E. coli* RNase HII in RNase H2 buffer at 100 U per 50 µl. Add 50 µl of RNase HII solution per slide. Add 50 µl RNase H2 buffer without enzyme per no enzyme control slide. Cover slides with a 24 × 60 mm coverslip and incubate them in a wet chamber at 37 °C overnight. Stop RNase HII digestion by washing the slides in 0.1% SDS for 10 min. Wash slides 10× for 10 min each with 1× PBS.

3.5 Alkaline Unwinding

Wash slides 2× using alkaline unwinding buffer for a few seconds. Equilibrate cells for 20 min in alkaline unwinding buffer. Wash slides 10× for 10 min each in 0.5× TBE.

3.6 Electrophoresis Under Neutral Conditions

Precool neutral electrophoresis buffer to 4 °C and pour into an electrophoresis chamber (Owl Scientific, model 5) just to cover the tray holder. Place slides next to each other onto try holder with the short edges facing the electrodes. Fill up electrophoresis chamber with buffer. Conduct electrophoresis at 1 V/cm and 15 mA for 25 min (*see* **Note 4**).

3.7 Staining of Comets

Place slides upright on paper towel for 10 min to drain buffer. Place slides horizontally on a paper towel, add 200 µl Antifade solution with SYBR Gold per slide and cover with a coverslip. Store slides in a dark wet chamber at 4 °C for at least 30 min before microscopic analysis.

3.8 Microscopic Analysis

Use a fluorescence microscope with an excitation filter at 495 nm and an emission filter at 537 nm and a 10× objective. Image 50–100 randomly selected comets per slide using identical imaging settings. The fluorescence intensity of the comet tail relative to the head reflects the number of DNA breaks. Quantify the degree of DNA migration by assessing the Olive tail moment using comet assay analysis software such as CASP (CaspLab) [16] (*see* **Note 5**). The Olive tail moment is defined as the product of the tail length and the fraction of total DNA in the tail [11].

4 Notes

1. Avoid formation of bubbles in agarose, which could interfere with electrophoresis. Per cell line to be analyzed prepare three slides with RNase HII digestion and three slides without RNase HII as no enzyme control.

2. We use the stainless-steel cover of an instrument tray, which is large enough to fit the number of slides needed for the experiment. One may also use a glass plate.
3. For a Coplin staining jar (Thermo Fisher Scientific) holding eight slides, 70 ml lysis buffer are needed. Remove and add washing buffers very slowly to avoid agarose from detaching off the slides. We use a glass funnel pointed toward the wall of the staining jar when adding buffer.
4. To eliminate effects of electric field inhomogeneity during electrophoresis, place replicate sample slides and controls in alternating positions into the electrophoresis chamber avoiding the outer rims of the tray holder. Make sure to run enzyme treated and no enzyme controls in the same electrophoresis run. Two to three rows of slides can be placed into the electrophoresis chamber we use. Run electrophoresis in a 4 °C cold room.
5. Quantification of comets can be done either by visual scoring or by using image analysis software tools available as commercial packages or freeware (for an overview *see* www.cometassay.com).

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References

1. Reijns MA, Rabe B, Rigby RE, Mill P, Astell KR, Lettice LA, Boyle S, Leitch A, Keighren M, Kilanowski F et al (2012) Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell 149:1008–1022
2. Sparks JL, Chon H, Cerritelli SM, Kunkel TA, Johansson E, Crouch RJ, Burgers PM (2012) RNase H2-initiated ribonucleotide excision repair. Mol Cell 47:980–986
3. Nick McElhinny SA, Kumar D, Clark AB, Watt DL, Watts BE, Lundstrom EB, Johansson E, Chabes A, Kunkel TA (2010) Genome instability due to ribonucleotide incorporation into DNA. Nat Chem Biol 6:774–781
4. Kim N, Huang SN, Williams JS, Li YC, Clark AB, Cho JE, Kunkel TA, Pommier Y, Jinks-Robertson S (2011) Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science 332:1561–1564
5. Hiller B, Achleitner M, Glage S, Naumann R, Behrendt R, Roers A (2012) Mammalian RNase H2 removes ribonucleotides from DNA to maintain genome integrity. J Exp Med 209:1419–1426
6. Crow YJ, Leitch A, Hayward BE, Garner A, Parmar R, Griffith E, Ali M, Semple C, Aicardi J, Babul-Hirji R et al (2006) Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutieres syndrome and mimic

congenital viral brain infection. Nat Genet 38:910–916

7. Kind B, Muster B, Staroske W, Herce HD, Sachse R, Rapp A, Schmidt F, Koss S, Cardoso MC, Lee-Kirsch MA (2014) Altered spatio-temporal dynamics of RNase H2 complex assembly at replication and repair sites in Aicardi-Goutieres syndrome. Hum Mol Genet 23:5950–5960
8. Gunther C, Kind B, Reijns MA, Berndt N, Martinez-Bueno M, Wolf C, Tungler V, Chara O, Lee YA, Hubner N et al (2015) Defective removal of ribonucleotides from DNA promotes systemic autoimmunity. J Clin Invest 125:413–424
9. Mackenzie KJ, Carroll P, Lettice L, Tarnauskaitė Ž, Reddy K, Dix F, Revuelta A, Abbondati E, Rigby RE, Rabe B et al (2016) Ribonuclease H2 mutations induce a cGAS/STING-dependent innate immune response. EMBO J 35:831–844. doi:10.15252/embj.201593339
10. Pokatayev V, Hasin N, Chon H, Cerritelli SM, Sakhuja K, Ward JM, Morris HD, Yan N, Crouch RJ (2016) RNase H2 catalytic core Aicardi-Goutières syndrome-related mutant invokes cGAS-STING innate immune-sensing pathway in mice. J Exp Med 213:329–336. doi:10.1084/jem.20151464
11. Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298
12. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191
13. Olive PL, Banath JP (2006) The comet assay: a method to measure DNA damage in individual cells. Nat Protoc 1:23–29
14. Collins AR, Duthie SJ, Dobson VL (1993) Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis 14:1733–1735
15. Kushwaha S, Vikram A, Trivedi PP, Jena GB (2011) Alkaline, Endo III and FPG modified comet assay as biomarkers for the detection of oxidative DNA damage in rats with experimentally induced diabetes. Mutat Res 726:242–250. doi:10.1016/j.mrgentox.2011.10.004
16. Końca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wojcik A (2003) A cross-platform public domain PC image-analysis program for the comet assay. Mutat Res 534:15–20