$KMnO_4$ Footprinting

Ümit Pul, Reinhild Wurm and Rolf Wagner*

Molecular Biology of Bacteria, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
*For correspondence: r.wagner@rz.uni-duesseldorf.de

[Abstract] The $KMnO_4$ footprinting method offers a rapid and easy way to detect and localize single-stranded regions within a duplex DNA molecule, such as it occurs for instance within an actively transcribing RNA polymerase-DNA complex or during R-loop formation in DNA-RNA hybrid structures. The method is based on the selective oxidation of single-stranded thymines in DNA. The modified nucleotides react with strong bases by ring opening and subsequent phosphodiester cleavage. Because the modified nucleotides will not be recognized by DNA polymerase sites of modification can also be analyzed by primer extension with Klenow DNA polymerase, which stops elongation one residue before the modification. Hence, localization of the modified base positions can be performed on denaturing polyacrylamide gels either after piperidine catalyzed phosphodiester cleavage of 3'- or 5'-^{32}P-end-labeled DNA or by primer extension with non-labeled DNA employing ^{32}P-labeled oligonucleotide primers. Due to the fact that $KMnO_4$ can penetrate through membranes the footprinting method can also be used for footprint analyses within living cells.

Materials and Reagents

1. Radiolabeled DNA fragment of interest
2. 14.3 M β-mercaptoethanol
3. 500 mM EDTA
4. Phenol
5. Bromophenol blue (Sigma-Aldrich, catalog number: BO126)
6. Xylene cyanol (Sigma-Aldrich, catalog number: X4126)
7. Formamide deionized (Panreac Applichem, catalog number: A2156)
8. Cholorophorm
9. Piperidine, purity grade: pro analysis (p.a.) (*e.g.* Sigma-Aldrich, catalog number: 411027)
10. Ethanol, purity grade: pro analysis (p.a.)
11. X-ray films
12. Glycogen (1 µg/µl) (*e.g.* Roche, catalog number: 10901393001)
13. NaOAc (300 mM pH = 5.5)

    Optional for Procedure B (primer extension analysis)

14. Non-radio labeled DNA fragment of interest
15. 5'-^{32}P-labeled desoxyoligonucleotide primer
16. NaOH (10 mM)
17. Tris
18. $MgSO_4$
19. DTT
20. Klenow fragment of DNA polymerase I (*e.g.* Biolabs, catalog number: MO210S)
21. NH_4OAc
22. 370 mM $KMnO_4$ stock solution (a 1:1 dilution with H_2O is used for the reaction) (see Recipes)
23. Phenol/Chloroform (see Recipes)
24. Neutralization solution (see Recipes)
25. dNTP mix (see Recipes)
26. Stop mix (see Recipes)
27. Electrophoresis loading buffer (see Recipes)

Equipment

1. Table-top centrifuge
2. Vortex shaker
3. Incubator
4. Polyacrylamide gel electrophoresis system
5. Vacuum concentrator

Procedure

A. Footprinting with 3'- or 5'-end-labeled DNA analyzed by piperidine-catalyzed strand scission.
   1. Add 4 µl of 160 mM $KMnO_4$ solution to radiolabeled DNA fragments of interest (40 ng; 5,000 to 10,000 cpm) (in the presence or absence of a binding partner) in a total volume of 40 µl.
   2. Incubate samples for 2 min at 30 °C; mix gently.
   3. Stop reaction by the addition of 4.8 µl β-mercaptoethanol; mix again.
   4. Put samples on ice.
   5. Add 5.3 µl 500 mM EDTA.
   6. Extract samples three times with 100 µl phenol/chloroform by vigorous shaking on a vortex for 1 min each time.
   7. Centrifuge samples (5 min, 3,000 rpm) and take aqueous layer.

8. Precipitate samples with 2.5 volumes absolute ethanol (-20 °C).
9. Centrifuge tubes (10 min, 10,000 rpm, 4 °C).
10. Dissolve wet ethanol pellets in 70 µl of 10% (v/v) piperidine.
11. Incubate samples at 90 °C for 30 min.
12. Lyophilize samples in a vacuum concentrator until dry.
13. Add 30 µl distilled water and lyophilize again.
14. Repeat step 9.
15. Dissolve final pellets in 50 µl distilled water.
16. Add 4 µl 300 mM NaOAc (pH 5.5).
17. Add 1 µl glycogen (1 µg/µl) to aid precipitation.
18. Precipitate samples with 2.5 volumes absolute ethanol (-20 °C).
19. Wash pellets with 150 µl 70% ethanol.
20. Dry samples briefly in a vacuum concentrator to get rid of excess ethanol and dissolve in 5 µl electrophoresis loading buffer.
21. Separate on a 10% or 15 % denaturing polyacrylamide gel, respectively, depending on the size of the DNA fragment.
22. Visualize radioactive bands by autoradiography (over-night X-ray exposure).

B. Footprinting with non-labeled DNA analyzed by primer extension

1. Add 4 µl of 160 mM $KMnO_4$ solution to 40 µl non-labeled DNA fragments (~100 ng) in presence or absence of a binding partner.
2. Follow steps 2 to 8 of procedure A.
3. Dissolve pellet in 35 µl distilled water.
4. Add 1 µl of an appropriate 15 to 20^{mer} 5'-^{32}P-labeled desoxyoligonucleotide primer complementary to the downstream sequence of interest (~ 5 x 10^5 cpm). For convenient resolution the primer should not bind farther than 50 to 80 nucleotides downstream to the region of interest.
5. Add 4 µl 10 mM NaOH.
6. Incubate for 2 min at 80 °C.
7. Put samples on ice for 5 min.
8. Add 4.5 µl of neutralization solution and mix samples.
9. Incubate for 3 min at 68 °C for hybridization.
10. Put samples on ice and add 5 µl of all four dNTPs (5 mM each), 1 unit Klenow fragment of DNA polymerase I and mix gently.
11. Extension reaction is carried out for 10 min at 50 °C.
12. Put samples on ice and terminate the reaction by the addition of 17 µl stop mix.
13. Samples are precipitated with 2.5 volumes ethanol (-20 °C).

14. Wash pellets with 150 µl 70% ethanol.
15. Dry samples briefly in a vacuum concentrator to get rid of excess ethanol and dissolve in 5 µl electrophoresis loading buffer.
16. Separate on a 10% or 15% denaturing polyacrylamide gel, respectively, depending on the size of the cDNA fragments expected.
17. Visualize radioactive bands by autoradiography (over-night X-ray exposure).

Recipes

1. $KMnO_4$ stock solution (370 mM)
   $KMnO_4$ (MW = 158.04) has a solubility limit of ~60 g/L (corresponding to 0.37 M).
   A 200 ml stock solution is prepared by adding 12 g $KMnO_4$ to 220 ml distilled water. The solution is boiled until it reaches a final volume of 200 ml. This stock solution can be kept in a dark bottle for several months.
2. Phenol/Chloroform
   A 1:1 mixture (v/v) of phenol and chloroform, purity grade: pro analysis (p.a.) is used for extraction. The phenol has been saturated with 1 M Tris-HCl (pH 7.9) before and 0.1% (final concentration) 8-Hydroxychinolin should be added to the phenol to avoid oxidation.
3. Neutralization solution
   0.5 M Tris-HCl (pH 7.2)
   0.1 M $MgSO_4$
   2 mM DTT (optional for procedure B)
4. dNTP mix
   5 mM each, dATP, dCTP, dGTP, dTTP (optional for procedure B)
5. Stop mix
   4 M NH_4OAc
   20 mM EDTA (optional for procedure B)
6. Electrophoresis loading buffer
   0.1% (w/v) bromophenol blue
   0.1% xylene cyanol
   95% formamide deionized
   25 mM EDTA (pH 8.0)

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