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In vitro DNA Protection Assay Using Oxidative Stress

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[Abstract] A wide range of stresses such as oxidative stress, acid, alkaline, UV, and metal can damage DNA. Here, we describe a protocol to measure the DNA nicking damage by Fenton reaction-mediated oxidative stress. Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) produces the highly deleterious hydroxyl radicals that damage the cellular components such as DNA, lipid and proteins.

Material and Reagents

1. 300 ng/µl plasmid DNA
   We used 7.2 kbp pMK3 plasmid purified from *E. coli* JM109 by QIA filter™ (Plasmid Midi kit). The high quality plasmid abundant in supercoiled form is required to monitor the reduction of the supercoiled form.
2. Proteins to be tested on their DNA protection ability
   a. BSA (Wako Pure Chemical Industries, catalog number: 01317843)
   b. Lysozyme (Wako Pure Chemical Industries, catalog number: 12202673)
3. Agarose
4. Ethidium bromide (EtBr) (Invitrogen, catalog number: 15585011)
   Caution: Ethidium bromide is toxic and strong mutagen. Use appropriate gloves, safety goggles and lab coat.
5. Ferrous ammonium sulfate (1.5 mM) (Wako Pure Chemical Industries, catalog number: 01412172)
   Note: The solution must be prepared just prior to the experiment.
6. 200 mM hydrogen peroxide (Wako Pure Chemical Industries, catalog number: 08104215)
7. NaCl (Wako Pure Chemical Industries, catalog number: 19101665)
8. Sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, catalog number: 08104215)
9. Tris (Nacalai tesque, catalog number: 3543421)
10. Acetic acid (Nacalai tesque, catalog number: 0021243)
11. EDTA (Nacalai tesque, catalog number: 15111)
12. CIA (chloroform:isoamyl alcohol = 24:1) (Nacalai tesque, catalog number: 0840255)
    Caution: Chloroform is suspect mutagen and harmful if inhaled. Avoid breathing

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vapor and prolonged contact with skin, using fume food and safety gloves. Follow the safety rule in your institute.

13. 300 ng/µl plasmid DNA (pMK3) (see Recipes)
14. 1 µg/µl bovine serum albumin (BSA) (see Recipes)
15. 1 µg/µl lysozyme (see Recipes)
16. Protein/binding buffer (see Recipes)
17. 1.5 mM ferrous ammonium sulfate (see Recipes)
18. 200 mM hydrogen peroxide (see Recipes)
19. 10% sodium dodecyl sulfate (SDS) (see Recipes)
20. 1x TAE buffer (see Recipes)
21. 50x TAE buffer (see Recipes)

Equipment

1. Eppendorf tubes (1.5 ml)
2. Eppendorf tubes (2.0 ml)
3. Tips
4. Thermostat bath
5. Centrifuge machine
6. Electrophoresis apparatus
7. UV Trans illuminator and recording system (Nippon Genetics, model: *e.g.* FAS III system)

Software

1. Image J (NIH) (http://imagej.nih.gov/ij/)

Procedure

1. Pre-incubate plasmid DNA (300 ng pMK3) with or without protein sample: MrgA, MrgA*, BSA, or Lysozyme (10 and 20 µg) in 30 µl of protein-binding buffer: For 1 h at 37 °C.
2. Add 8 µl of 1.5 mM fresh ferrous ammonium sulfate solution, gently mix the samples by pipetting, and then incubate for 5 min at room temperature (25 °C).
   Note: Ferroxidation of ferrous iron by oxygen does not proceed significantly before the addition of hydrogen peroxide at this time scale. This 5-min incubation in our experiment is to allow MrgA to sequestrate ferrous iron in its core.
3. Add 2 µl of 200 mM hydrogen peroxide (final conc. 10 mM) and gently mix the samples by pipetting, and then incubate for 5 min at room temperature (25 °C).
4. Add 10 µl of 10% SDS and 50 µl of CIA, and vortex. This is to denature and remove

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the proteins from DNA, and to recover DNA in aqueous phase in the next step.

5. Recover supernatant by centrifugation at ≥13,000 *x g* for 5 min at 4 °C.
6. Separate 20 µl of the supernatant on 1% agarose gel in TAE buffer (without EtBr) and electrophorese at 100 V for 40 min at room temperature. Following the electrophoresis, soak the gel in TAE buffer containing EtBr to visualize DNA.
7. Measure the signal intensities of supercoiled DNA using NIH image J software. In detail:
   a. Take agarose gel images by UV-trans illuminator and CCD camera (FASIII system). Scan the photo copy by conventional scanner (if you can get high quality digital image from CCD camera, it can be directly used for the measurement. We scanned the photocopy because the resolution of the digital image is low in case of FASIII system). Measure the signal intensities of supercoiled DNA were using NIH image J software. Measure each signal intensity by subtracting the background.
   b. Calculate relative signal intensities using the signal intensity of the supercoiled plasmid DNA without oxidative stress.
   c. Calculate the mean and standard deviation of the relative intensities from multiple independent experiments. Evaluate the statistical significance by Student's t-test.

Figure 1 shows the result of recombinant MrgA and MrgA*. MrgA and MrgA* were prepared as described previously (Ushijima *et al.*, 2014), and their purities were higher than 95% in SDS-PAGE stained with Coomassie brilliant blue. MrgA has both ferroxidase activity and DNA binding activity, while MrgA* is impaired in its ferroxidase activity. As a negative control, either 1 µg/µl bovine serum albumin (BSA) or 1 µg/µl lysozyme can be used (see Recipes).

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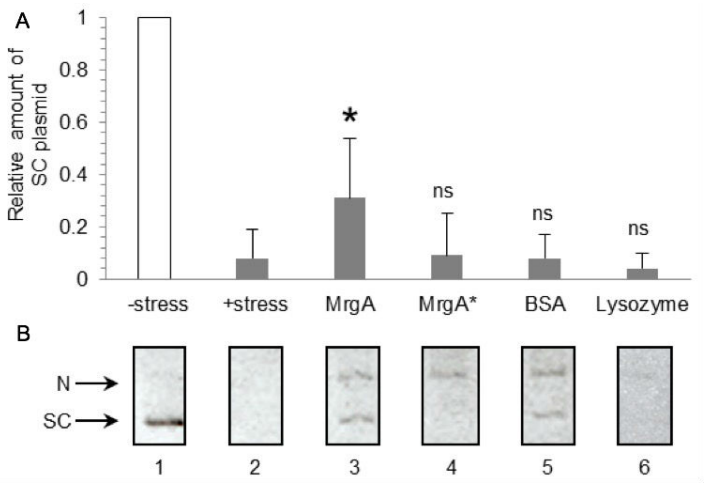

Figure 1. Representative data. A. Relative amount of supercoiled plasmid DNA. Mean and SD values of no protein (n=9), MrgA (n=4), MrgA* (n=3), BSA (n=6), and lysozyme (n=4) are shown. All proteins are 10 µg. *: Significant difference compared to oxidative stress exposure (lane 2) in t-test, $p < 0.05$. ns: Not significant, $p > 0.25$. B. Representative gel image of DNA protection assay. Lanes 1: Plasmid DNA without oxidative stress. Lanes 2: Plasmid DNA treated with oxidative stress (Fe^{2+}/H_2O_2). Lanes 3~6: Plasmid DNA was pre-incubated with 10 µg of MrgA (lane 3), MrgA* (lane 4), BSA (lane 5) or lysozyme (lane 6) prior to the Fe^{2+} addition. SC: Supercoil, N: Nicked. DNA protection by MrgA was dependent on the intact ferroxidase activity, and DNA binding by MrgA* didn't contribute to DNA protection (Ushijima *et al.*, 2014).

Notes

1. We confirmed that at least Tris or HEPES based buffer can be used for the DNA nicking damage by Fenton reaction-mediated oxidative stress.
2. 1.5 mM ferrous ammonium sulfate should be freshly prepared and the dissolved solution should not exceed pH 7.0 (pH of MilliQ water is 5.50-6.86). The solution with pH values over 7.0 oxidizes ferrous iron to ferric iron immediately (Morgan and Lahav, 2007).
3. Note that signal intensities can vary depending on experiments: some of the signal intensities in Figure 1B representative image are different from the mean intensities in Figure A. At least three independent experiments and the statistical evaluation are recommended. To compare distinct gel images, put reference samples that can be used to normalize the signal intensities, *e.g.* marker DNAs with known quantities. We employed one-side Student's t-test.
4. EDTA can be used to stop the Fenton reaction. However, when we tried to add EDTA before purification, it did not increase the DNA signal intensity, but rather generated smear signal.

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Recipes

1. 300 ng/µl plasmid DNA (pMK3)
   Extract pMK3 purified from *E. coli* JM109 by Plasmid Midi Kit
   Adjust concentration with MilliQ water
2. 1 µg/µl bovine serum albumin (BSA)
   10 mg BSA
   Adjust total volume to 10 ml with protein/binding buffer
3. 1 µg/µl lysozyme
   10 mg lysozyme
   Adjust total volume to 10 ml with protein/binding buffer
4. Protein/binding buffer
   20 mM Tris-HCl (pH 8.0)
   200 mM NaCl
5. 1.5 mM ferrous ammonium sulfate
   1.17 mg Ferrous ammonium sulfate
   2 ml MilliQ water
6. 200 mM hydrogen peroxide
   10.2 µl Hydrogen peroxide (30% w/v)
   439.8 µl MilliQ water
7. 10% sodium dodecyl sulfate (SDS)
   10 g sodium dodecyl sulfate
   Adjust total volume to 100 ml with MilliQ water
8. 1x TAE buffer
   20 ml 50x TAE
   980 ml MilliQ water
9. 50x TAE buffer
   242 g Tris
   57.1 ml acetic acid
   100 ml 0.5 M EDTA
   Adjust total volume to 1 L with MilliQ water

Acknowledgements

This protocol was modified from *in vitro* DNA damage assay described previously (Martinez and Kolter, 1997).

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