Detection of Protein Oxidative Activity Using Reduced RNase A

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[Abstract] This assay allows to determine whether proteins possess oxidative activity-the ability to introduce disulfide bond *in vitro*. The substrate for potential oxidases is a ribonuclease A which, for its activity, needs 4 properly formed disulfide bonds (Raines, 1998).

RNase A activity can be detected by:

1. Monitoring the digestion of RNA (Lambert and Freedman, 1983);
2. Methylene Blue assay (Greiner-Stoeffele *et al.*, 1996);
3. Analyzing the cleavage of the cyclic CMP (Lyles and Gilbert, 1991; Lyles and Gilbert, 1991).

We here describe method for measurements of oxidative activity, based on the cleavage of cCMP.

Oxidative activity will be tested by measuring spectrophotometrically RNase A cleavage of cyclic-2', 3'-cytidinemonophosphate (cCMP) to 3'-cytidinemonophosphate (3' CMP), which results in an increase in absorption at 296 nm.

The reaction equation: RNase A +2' 3'-cCMP→RNase A + 3' CMP.

Materials and Reagents

1. Flat-bottomed clear 96-well microplates (Optimum Line, catalog number: GP700)
2. Desalting columns-Biorad Econo-Pac 10DG Desalting Columns, 30 units (Bio-Rad Laboratories, catalog number: 7322010)
3. Bio-Scale Mini Profinity IMAC Cartridges (Bio-Rad Laboratories, catalog number: 7324614)
4. ENrich™ SEC 70 size exclusion columns (Bio-Rad Laboratories, catalog number: 7801070)
5. Pierce™ Protein Concentrators, 9K MWCO (7 ml) (Thermo Fisher Scientific, catalog number: 89884A) or Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (EMD Millipore Corporation, catalog number: UFC801008)
6. Proteins of interest (purified to homogeneity proteins, concentration approx 7-8 mg/ml)
7. Phosphate buffered saline (PBS) (Sigma-Aldrich, catalog number: P4417-50TAB)
8. Ribonuclease A from bovine pancreas (RNase A) (store -20 °C) (Sigma-Aldrich, catalog number: R6513-10 mg)

9. L-Glutathione oxidized disodium salt (GSSG) (Sigma-Aldrich, catalog number: G4626-100 mg)
10. L-Glutathione reduced (GSH) (Sigma-Aldrich, catalog number: G6529-1 g)
11. DL-Dithiothreitol (DTT) (AppliChem GmbH, catalog number: 3483-12-3; Sigma-Aldrich, catalog number: 43815-1 G)
12. Guanidine hydrochloride (Gdn-HCl) (AppliChem GmbH, catalog number: A11061000)
13. 1 M Tris (pH 8.0) (Eurx, catalog number: E0273-01)
14. 0.5 M EDTA (pH 8.0) (Eurx, catalog number: E240-01)
15. DTNB (Ellman's Reagent) (5, 5-dithio-bis-(2-nitrobenzoic acid) (Thermo Fisher Scientific, catalog number: 22582)
16. Sodium phosphate dibasic (pH 8.0) (Sigma-Aldrich, catalog number: S3264-250 G)
17. Cytidine 2': 3'-cyclic monophosphate monosodium salt (cCMP) (store -20 °C) (Sigma-Aldrich, catalog number: C9630-100 mg)
18. 2x Reaction buffer (see Recipes)
19. Reduction buffer (see Recipes)

Equipment

1. Plate reader (Tecan, Infinite®, model: 200 PRO series)

Procedure

A. Preparation of proteins for assay (approx 1 week) (Chim *et al.*, 2013)
   1. Purify proteins.
      Note: We overexpressed proteins by autoinduction (Studier, 2005) and then purified by affinity chromatography using the NGC chromatography system (see Optional materials).
   2. To obtain higher purity, load your proteins onto size exclusion columns and elute with PBS.
   3. Determine the amount of protein by nanodrop.
   4. Oxidize proteins with 50 mM oxidized glutathione (GSSG) and incubate for 1 h at RT (5-7 mg in 1 ml).
   5. Exchange buffer on desalting columns according to manufacturer's guidelines.
   6. Determine the amount of protein by nanodrop.
   7. Follow the Ellman assay to confirm proper redox state.
   8. Concentrate if necessary (using protein concentration columns). For this assay you need approx 3 mg/ml.
   9. Use fresh or in the next couple of days.

B. Reduction and denaturation of RNase A (2 days) (Daniels *et al.*, 2010)

1. Resuspend 10 mg of RNase A in 1 ml of reduction buffer (in 1.5 ml tube).
   Note: 10 mg of RNase A permit to perform approx. 20 assays.
2. Incubate overnight at RT without shaking.
3. Desalinate on columns and elute with PBS (according to manufacturer's guidelines).
4. Determine the amount of protein by nanodrop.
5. If necessary, concentrate (for Ellman assay you need 2.5 mg/ml of RNase A).
6. Follow the Ellman assay to confirm proper redox state.
7. Use the same day.

C. Oxidative folding of reduced RNase A (1 day) (Daniels *et al.*, 2010)
1. Prepare 2x reaction buffer.
2. Prepare mixture of protein and reduced RNase A in a volume of 100 µl (add PBS if necessary) in a 96-well plate (40 µM protein and 20 µM RNase A).
   Note: With concentration of 2.5 mg/ml you need to add 11 µl to the well to achieve 20 µM of RNase A.
3. Add 100 µl of 2x reaction buffer to the mixture of protein and RNase A; mix gently by pipetting up and down.
4. Insert plate into the plate reader and start program.

Representative data

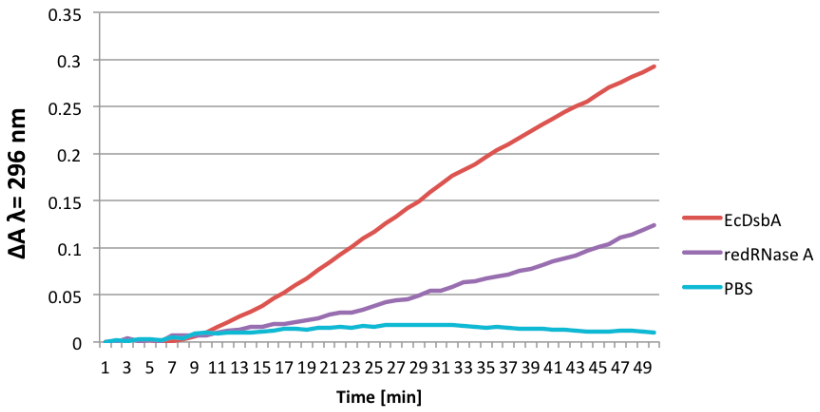

Figure 1. The results from one representative experiment. EcDsbA stands for main oxidase in *E. coli*.

Notes

1. Notes about microplate reader settings (Table 1).

Table 1. Universal settings for microplate reader

Temperature	27 °C
Kinetic cycle	
Cycles	Duration 1 h
Kinetic	Interval time 1 min
Absorbance	
Wavelength	Measurement 296 nm
Read	Number of flashes 10 Settle time 2

Recipes

1. 2x reaction buffer
   200 mM Tris/HCl (pH 8.0)
   4 mM EDTA
   0.4 mM GSSG
   2 mM GSH
   9 mM cCMP
2. Reduction buffer
   100 mM Tris/HCl (pH 8.0)
   6 M Gdn-HCl
   140 mM DTT

Acknowledgements

The work was supported by the National Science Centre (grant no. 2012/05/B/NZ1/00039).

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