Enzymatic Activity Assays for Base Excision Repair Enzymes in Cell Extracts from Vertebrate Cells

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[Abstract] We previously reported enzymatic activity assays for the base excision repair (BER) enzymes DNA polymerase β (pol β), aprataxin (APTX), and flap endonuclease 1 (FEN1) in cell extracts from *Saccharomyces cerevisiae* (Çağlayan and Wilson, 2014). Here, we describe a method to prepare cell extracts from vertebrate cells to investigate these enzymatic activities for the processing of the 5´-adenylated-sugar phosphate-containing BER intermediate. This new protocol complements our previous publication. The cell lines used are wild-type and APTX-deficient human lymphoblast cells from an Ataxia with Oculomotor Apraxia Type 1 (AOA1) disease patient, wild-type and APTX-null DT40 chicken B cells, and mouse embryonic fibroblast (MEF) cells. This protocol is a quick and efficient way to make vertebrate cell extracts without using commercial kits.

Materials and Reagents

1. Cell lines used in this study
   a. The human cell lines used are the wild-type C2ABR and AOA1 L938 (Harris *et al*., 2009). The AOA1 cell line was derived from the peripheral blood of a Japanese AOA1 patient and has a point mutation within the HIT domain of APTX involving substitution of proline for leucine at position 206.
   b. The DT40 cell lines are the wild-type and APTX null. The APTX null cell line has the *aptx* gene deletion from valine 78 onwards that inactivates APTX (Ahel *et al*., 2006).
   c. The mouse embryonic fibroblast cell lines are pol $\beta^{-/-}$ and pol $\beta^{+/+}$ MEFs previously developed in our laboratory (Sobol *et al*., 1996).
2. RPMI 1640 medium with glutamine (Gibco, catalog number: 11875-093)
3. DMEM high-glucose medium (HyClone, catalog number: SH30081)

4. Chicken serum (Life Technologies, catalog number 16110-082)
5. Glutamax-1 (Gibco, catalog number: 35050-061)
6. Fetal bovine serum-FBS (HyClone, catalog number: SH30910).
7. EDTA-free protease inhibitor cocktail tablet (Roche Applied Science, catalog number: 11836170001)
8. Bio-Rad Protein Dye Reagent Concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
9. EDTA (Sigma-Aldrich, catalog number: 93283)
10. Potassium chloride-KCl (Sigma-Aldrich, catalog number: P9333)
11. Sodium chloride-NaCl (Sigma-Aldrich, catalog number: S7653)
12. Glycerol (Sigma-Aldrich, catalog number: G9012)
13. Tissue-culture grade 2-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
14. Nonidet P 40-NP40 (Sigma-Aldrich, catalog number: 74385)
15. HEPES (Sigma-Aldrich, catalog number: H3375)
16. Magnesium chloride-$MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
17. Formamide (Sigma-Aldrich, catalog number: F9037)
18. Bromophenol blue (Sigma-Aldrich, catalog number: B0126)
19. Xylene cyanol (Sigma-Aldrich, catalog number: X4126)
20. Dithiothreitol-DTT (Sigma-Aldrich, catalog number: D0632)
21. Sodium borohydride-$NaBH_4$ (Sigma-Aldrich, catalog number: 247677)
22. Urea (National Diagnostic, catalog number: EC-605)
23. Trizma-base (Sigma-Aldrich, catalog number: T4661)
24. Boric Acid (Promega Corporation, catalog number: H5003)
25. AccuGel (40%) 19:1 Acrylamide to Bisacrylamide Stabilized Solution (National Diagnostic, catalog number: EC-850)
26. Ammonium persulfate (Sigma-Aldrich, catalog number: A3678-25G)
27. Tetramethylethylenediamine (Sigma-Aldrich, catalog number: T9281-25ML)
28. Sterile water
29. Purified enzymes: Recombinant human DNA polymerase β [purified as described Çağlayan *et al.* (2014)], recombinant human APTX (Fitzgerald catalog number: 80R-1256), and recombinant human FEN1 [purified as described Çağlayan *et al.* (2014)].
30. DNA substrate: The gapped DNA substrate with a uracil base at position 17 at the 5'-end of the 3'-end FAM-labeled oligonucleotides. The sequence information for the upstream, downstream and template oligonucleotides were previously published Çağlayan *et al.* (2015).
31. Lysis buffer (see Recipes)
32. 10x reaction buffer (see Recipes)
33. Gel-loading buffer (see Recipes)
34. 10x TBE solution and 1x TBE solution as PAGE running buffer (see Recipes)

35. 15% Denaturing Polyacrlamide Gel or PAGE solution (see Recipes)

Equipment

1. Eppendorf tubes
2. Screw cap conical tube (15 ml)
3. Refrigerated table-top centrifuge
4. Refrigerated Eppendorf centrifuge
5. Table-top heat block
6. Tissue culture CO_2 incubators set at 34, 37, and 39.5 °C
7. Cell scraper
8. Polyacrylamide gel electrophoresis (PAGE) apparatus

Procedure

A. Cell growth

1. DT40 cells (wild-type and APTX-null) are maintained in RPMI 1640 medium supplemented with 10% FBS, 1% chicken serum and 2 mM L-glutamine. 2-mercaptoethanol (50 μM) is added fresh to the medium at the time of use and cells are grown in a 5% CO_2 incubator at 39.5°C (Okamoto *et al*., 2014).
2. The human lymphoblastoid cells (wild-type and APTX-deficient AOA1) are maintained in RPMI 1640 medium containing FBS and grown in a 5% CO_2 incubator at 37°C (Harris *et al*., 2009).
2. MEF cells (wild-type and pol β-deficient) are maintained in DMEM medium containing 10% FBS and 4 mM glutamax and grown in a 10% CO_2 incubator at 34°C (Sobol *et al*., 1996).
3. 1-2 x 150 mm dishes of MEF cells are washed twice in 10 ml cold PBS, then harvested by scraping. Cells are collected in 15 ml PBS in a 15 ml tube, then centrifuged at 1600 rpm at 4 °C for 5 min.
4. The required volume of suspension cells (DT40 and human lymphoblasts) is centrifuged at 1600 rpm at 4 °C for 5 min, and the cell pellet are transferred to a 15 ml tube and washed twice with 10 ml PBS.
5. Cell pellets are transferred to Eppendorf tubes in 1 ml PBS and briefly spun down. Supernatants are discarded and pellets are frozen immediately in dry ice and stored at -80 °C until use.
6. Pellets of 20-50 million cells are used for cell extract preparation.

B. Preparation of cell extracts

7. The cell extracts from vertebrate cells are prepared as reported (Biade *et al.*, 1998) and summarized below.
8. Resuspend the cell pellet in 400 µl of ice-cold lysis buffer.
9. Rotate the resuspended cell pellets for 1 h at 4 °C.
10. Centrifuge the mixture at 14,000 rpm at 4 °C for 10 min to remove cell debris.
11. Carefully transfer the supernatant fraction to a fresh Eppendorf tube. Be careful not to touch the pellet.
12. Determine the protein concentration of the extract using Bradford assay dye reagent with BSA as standard (Bradford, 1976).

C. Enzymatic activity assays in cell extracts
1. Prepare 10 µl of reaction mixture (final volume) including 1x reaction buffer and 100 nM DNA substrate. The DNA substrate used includes an adenylated uracil base at the 5′ end of the 3′-FAM–labeled oligonucleotide (Çağlayan *et al.*, 2014). For the reference reactions including purified proteins, pol β, APTX, and FEN1, the reaction mixture included a gapped DNA substrate that was pre-incubated with UDG as described (Çağlayan *et al.*, 2014). For the reactions including cell extracts, the DNA substrate was included in the reaction mixture without UDG pretreatment.
2. Start the reaction by adding cell extract, prepared as above, to the reaction mixture. For the reference reactions, start each reaction by adding the purified protein to the reaction mixture in final concentrations as follows: pol β (500 nM), APTX (100 nM), and FEN1 (100 nM) (Çağlayan *et al*., 2014 and Çağlayan *et al*., 2015).
3. Incubate the reaction mixture at 37 °C for 15 min.
4. Stabilize the reaction products by addition of 1 M freshly prepared and ice-cold $NaBH_4$ to a final concentration of 100 mM.
5. Incubate the reaction samples on ice for 30 min.
6. Mix the reaction products with 10 µl of gel-loading dye.
7. The reaction products in cell extracts from vertebrate cells are separated on a 15% polyacrylamide gel. The gel is scanned, and the data analyzed as reported (Çağlayan *et al*., 2014 and Çağlayan *et al*., 2015).

Representative data

A sample gel image of products for the pol β dRP lyase, FEN1 excision and APTX DNA deadenylation enzymatic activities in the extracts from DT40 cells is presented below (Figure 1). Migration positions of DNA substrate (line 1) and the reaction products after pol β lyase removal of the 5’-AMP-dRP group (indicated as 17-mer and shown at line 2 in reference reaction and lines 5-6 in cell extracts), APTX 5’-AMP removal (indicated as 17^{dRP}, and shown at line 3 in reference reaction and lines 6 in cell extract), and FEN1 excision products (indicated as 15-mer and 16-mer and shown at line 4 in reference reaction and

lines 5-6 in cell extracts) are presented. The original results showing enzymatic activities in the cell extracts from the vertebrate cell lines listed below were published in Çağlayan *et al*. (2015).

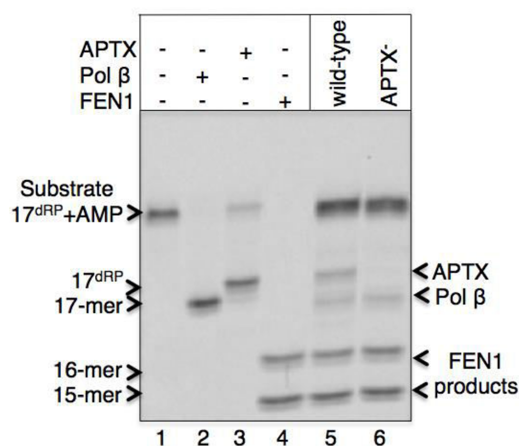

Figure 1. Pol β, APTX, and FEN1 activities in cell extracts from DT40 wild-type and APTX-null cell lines

Notes

After addition of lysis buffer to the cell pellet, the Eppendorf tube including resuspended cells should be gently mixed by tapping. Enzymatic activity in vertebrate cell extracts may be lost if samples are mixed by vortex instead of rotating at 4 °C. Typically, the enzymatic activities are determined under steady-state conditions with the substrate in excess over enzyme and under conditions of a linear time-course. In addition, the enzymatic activity should be under conditions of linearity between activity and amount of extract added to the reaction mixture.

Recipes

1. Lysis buffer
   10 mM Tris–HCl (pH 7.8 in the final buffer solution)
   200 mM KCl
   1 mM EDTA
   20% glycerol
   0.1% NP-40
   1 mM DTT (add fresh)
   Protease inhibitor tablet
2. 10x reaction buffer
   500 mM HEPES (pH 7.5 in the final buffer solution)

100 mM $MgCl_2$
200 mM KCl
5 mM EDTA
20 mM DTT

3. Gel-loading buffer
95% formamide
20 mM EDTA
0.02% bromophenol blue
0.02% xylene cyanol
4. 10x TBE solution
108 g Tris-base
55 g Boric Acid
40 ml 0.5 M EDTA
$H_2$0 up to 1 L
5. Denaturing PAGE solution (15%)
40 g Urea
30 ml 19:1 AccuGel (40%)
8 ml 10% TBE solution
10 ml $H_2$0
250 μl 10% APS
35 μl TEMED

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