Small-scale extracts for the study of nucleotide excision repair and non-homologous end joining

Michael B. Smeaton¹, Paul S. Miller¹, Gary Ketner² and Les A. Hanakahi¹,*

¹Department of Biochemistry and Molecular Biology and ²Department of Molecular Microbiology and Immunology, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, MD, 21205, USA

Received August 28, 2007; Revised and Accepted October 18, 2007

ABSTRACT
The repair of DNA by nucleotide excision repair (NER) and non-homologous end joining (NHEJ) is essential for maintenance of genomic integrity and cell viability. Examination of NHEJ and NER in vitro using cell-free extracts has led to a deeper understanding of the biochemical mechanisms that underlie these processes. Current methods for production of whole-cell extracts (WCEs) to investigate NER and NHEJ start with one or more liters of culture containing 1–5 × 10⁹ cells. Here, we describe a small-scale method for production of WCE that can be used to study NER. We also describe a rapid, small-scale method for the preparation of WCE that can be used in the study of NHEJ. These methods require less time, 20- to 1000-fold fewer cells than large-scale extracts, facilitate examination of numerous samples and are ideal for such applications as the study of host–virus interactions and analysis of mutant cell lines.

INTRODUCTION
The chemistry of DNA makes it highly resistant, but not impervious, to damage. Environmental and intracellular agents can cause lesions that can lead to misinformation or break the flow of genetic information. In addition, DNA replication and somatic cell recombination introduce double-strand breaks (DSBs) in DNA that challenge genomic continuity. To maintain the proper flow of correct genetic information DNA repair mechanisms have evolved to identify and repair base lesions and strand breaks.

The nucleotide excision repair (NER) pathway repairs bulky DNA adducts or intrastand cross-links caused by exposure to UV light or alkylating agents. This process encompasses two sub-pathways that survey the entire genome (global genome repair, GGR) and the transcriptionally active part of the genome (transcription coupled repair, TCR) for base lesions that block the elongating RNA polymerase (1,2).

The importance of DSB repair is highlighted by the observation that failure to repair even a single DSB can result in the loss of genetic information, chromosomal translocation and even cell death (3). Non-homologous end joining (NHEJ) is an important pathway employed by mammalian cells in the repair of adventitious DSBs and also for the repair of programmed DSBs made during somatic cell recombination (2–4). Defects in NHEJ can result in gross chromosomal aberrations such as translocations and studies in mouse models have shown that such defects can lead to events that initiate or propagate tumorigenesis (3,4).

Investigation of the molecular mechanisms that underlie NER and NHEJ was stimulated by the development of cell-free extracts that supported these processes in vitro (5–8). Unfortunately, the requirement of 1–5 × 10⁹ cells for these methods limits the utility of standard protocols. Here, we report the development of small-scale, cell-free extract protocols to study NER and NHEJ in vitro.

MATERIALS AND METHODS
Cell culture
HeLa cells (Biovest International Inc., National Cell Culture Center) were grown in suspension to 10⁶ cells/ml in Joklik’s MEM, 5% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (PenStrep). 293 cells were cultured as monolayers in EMEM, 10% fetal bovine serum (FBS) and PenStrep in 25 cm² flasks. CHO AA8 (wild type), CHO UV5 (XPD) (gifts from Dr Michael Seidman) and CHO UV41 (XPF) (ATCC) cells were grown in minimal essential medium alpha modification, 10% FBS and PenStrep in 25 cm² flasks. CHO AA8 cells were grown to large scale using CellSTACK culture chambers (Corning); a 10-layer chamber yields ~10⁹ cells.
Viral infections

293 monolayers were grown in 25 cm² flasks; the medium was removed and was replaced with 1 ml fresh media with virus (MOI = 10 p.f.u./cell). Virus was allowed to adsorb (2 h, 37°C, with gentle agitation) after which the inoculum was aspirated away and replaced with fresh medium. Infected cells were cultured for times indicated.

Large-scale extracts for NER

Large-scale preparations of NER-competent whole-cell extracts (WCE) were carried out essentially as previously described (8–10). Briefly, cells were harvested and rinsed with phosphate-buffered saline (PBS). Subsequent steps were carried out at 4°C. Cells were resuspended in four packed cell volumes (PCV) of hypotonic lysis buffer (HLB: 10 mM Tris–HCl pH 8.0, 1 mM EDTA) with 5 mM DTT and incubated on ice for 20 min. Protease inhibitors were added to 1 mM PMSF, 5 μg/ml leupeptin, and 100 μg/ml soybean trypsin inhibitor and cells were homogenized [20 strokes, Potter-Elvejem] 10 ml glass tube with a Teflon pestle (Kontes)]. The homogenate was transferred to a beaker in an ice bath. Using a magnetic stirrer, 4 PCV of sucrose-glycerol buffer [50 mM Tris–HCl pH 8, 10 mM MgCl₂, 2 mM DTT, 25% sucrose (w/v), 50% glycerol (v/v)] and 1 PCV of saturated (NH₄)₂SO₄ (pH 7.0) were added and the solution was mixed slowly for 30 min. Ultracentrifugation (SW 41, 3 h, 125 000 g [microcentrifugation, 20 min, 11 500 g]) was used to remove solid material. A total of 0.33 g per ml finely ground solid (NH₄)₂SO₄ was added to the supernatant with 10 μl of 1 M NaOH/g of (NH₄)₂SO₄ to maintain neutral pH and the extract was allowed to stir for 30 min. Precipitated proteins were collected (centrifugation, 45 min, 12 000 g, 4°C) and the pellet was resuspended in a minimal volume of NER-dialysis buffer (25 mM HEPES pH 7.9, 100 mM KCl, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT, 12% glycerol) and dialyzed overnight against NER-dialysis buffer and insoluble material was removed [microcentrifugation, 10 min, 11 337 g (13 000 r.p.m.), 4°C]. The resulting WCE was aliquoted, frozen on liquid nitrogen and stored at −80°C. Protein concentration: 15–20 mg/ml by Bradford assay.

Large-scale extracts for NHEJ

HeLa WCE was prepared essentially as previously described (5). Briefly, 5 x 10⁸ cells were harvested, washed twice with PBS, resuspended in two PCV of HLB with 1 mM DTT and held on ice for 20 min. Cells were opened by dounce homogenization in the presence of protease inhibitors (1 mM PMSF, 2.2 ng/ml aprotinin, 1 ng/ml leupeptin, 1 ng/ml pepstatin A, and 1 ng/ml chymostatin). High salt buffer (HSB: 83.5 mM Tris pH 7.5, 1.65 M KCl, 3.3 mM EDTA, 1 mM DTT) was added to adjust the final salt concentration to 0.33 M KCl and the sample was held on ice for 20 min. The sample was subject to ultracentrifugation (170 000g, 3 h, 4°C, Beckman SW41 Ti rotor), the supernatant was collected and dialyzed against 20 mM Tris pH 8.0, 0.1 M KOAc, 20% glycerol, 0.5 mM EDTA and 1 mM DTT for 2 h. The resulting WCE was snap frozen on liquid nitrogen, and stored at −80°C. Protein concentration: 15–20 mg/ml by Bradford assay.

Mini-NER–WCE (mNER–WCE)

Extracts were prepared from 5 x 10⁷ CHO AA8, XPD or XPG cells (175 cm² culture area) using a modified Manley procedure (8). Cells were harvested at 90–95% confluency by addition of trypsin to cover the plate surface and removal of the excess trypsin. The cells were resuspended in 40 ml of medium and pelleted (1000 g, 10 min). The pellet was washed with 1 ml cold PBS, transferred to a silanized Eppendorf tube, pelleted [microcentrifugation, 200 g (1600 r.p.m.), 5 min, Baxter Heraeus Biofuge 13] and lyzed by freezing on liquid nitrogen and thawing rapidly at 37°C. Cell pellets can also be stored at −80°C until extract preparation is convenient. Subsequent steps were carried out at 4°C and DTT was added fresh to each buffer on the day of use. The cell pellet (100 μl) was resuspended with 10–20 strokes of a P-1000 pipette in 4 PCV (400 μl) of HLB with 5 mM DTT, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 100 μg/ml soybean trypsin inhibitor and lysis was measured by trypsin blue dye exclusion. Four PCV of sucrose–glycerol buffer were added and mixed slowly using a wide-bore pipette tip. One PCV (100 μl) of saturated (NH₄)₂SO₄ pH 7.0 was added slowly and mixed by rotation (30 min, 4°C). Insoluble material was removed [microcentrifugation, 20 min, 11 500 g (13 000 r.p.m.), 4°C] and the supernatant (~800 μl) was transferred to a fresh silanized Eppendorf tube. Solid (NH₄)₂SO₄ (finely ground) was added at 0.33 g of ammonium sulfate per milliliter of solution (0.264 g) with 1 μl of 0.1 M NaOH per 10 mg of (NH₄)₂SO₄ to maintain pH 7.0 and the solution was mixed by rotation (20 min, 4°C). Precipitated proteins were collected [microcentrifugation, 20 min, 11 500 g (13 000 r.p.m.), 4°C] and resuspended in 10 μl of NER dialysis buffer and dialyzed against 100 ml of NER dialysis buffer (6 h to overnight, 4°C) using Slide-A-Lyzer mini dialysis units 10 000 MWCO (Pierce, presoaked 20 min in deionized water to remove glycerol) to yield 80–100 μl of mNER-WCE per 100 μl PCV. Extracts may be concentrated by placing the dialysis unit on a bed of dry CM-Sephadex (Pharmacia) until the volume is ~2/3–1/2 (~15–30 min). We consistently observed increased excision activity using this concentration method, however the protein concentration does not increase as much as would be expected for a given volume reduction, presumably due to loss of protein on the dialysis membrane. mNER-WCE was aliquoted and frozen on liquid nitrogen and stored at −80°C. Protein concentration: 13–14 mg/ml by Bradford assay.

One hundred and twenty-minute mini-NHEJ–WCE (mNHEJ–WCE)

Adherent cells were harvested at 70–80% confluency using a cell scraper, collected (centrifugation, 5 min, 800 g, 4°C), washed twice in PBS, frozen on dry ice and stored at −80°C. Frozen cell pellets were resuspended in 2 PCV of HLB, incubated on ice for 20 min and lyzed by vortexing...
for 30 s. Nuclei were collected (microcentrifugation, 800g, 2 min, RT) and the supernatant was retained. Nuclei were resuspended in 1 PCV of nuclear extract buffer (25mM Tris pH 8.0, 0.33 M KCl, 1.5 mM EDTA) and incubated on ice for 20 min. The retained supernatant was added back to the nuclei and cell debris was removed (microcentrifugation, 10 min, 16 500g, 4°C). The resulting supernatant was collected as mNHEJ–WCE and used without dialysis. Protein concentration: 5–10 mg/ml by Bradford assay. MNHEJ–WCE was aliquoted and frozen on liquid nitrogen and stored at −80°C up to 3 months without significant loss of activity.

**Nucleotide excision assay**

Repair substrate: oligonucleotides were synthesized using standard phosphoramidite chemistry on an Applied Biosystems Model 3400 DNA/RNA synthesizer, purified by SAX HPLC and desalted on a C-18 SEP PAK cartridge (Waters, Inc.). To prepare the platinated oligonucleotide: standard phosphoramidite chemistry on an Applied Bio-Repairs substrate: oligonucleotides were synthesized using Nucleotide excision assay significant loss of activity.

For the study of NER, which reduces the number of cells required for analysis by 20-fold. As shown in Figure 1, when a DNA substrate that carries a site-specific 1,3 platinum lesion is treated with large-scale extracts of the large-scale extract. Because the mNER–WCE is less concentrated than large-scale extracts the observed excision activity was lower than that observed with the large-scale extracts. Figure 1 shows that concentration of the mNER–WCE increased the excision activity and made it comparable to the large-scale extract.

To test the dependence of the mNER–WCE-catalyzed excision events on known excision repair factors, we prepared mNER–WCE from cells deficient in XPD or XPG.

**RESULTS**

**Mini-WCEs for the study of NER in vitro: mini-NER–WCE**

We have developed a mini-extract protocol that can be used in the study of NER, which reduces the number of cells required for analysis by 20-fold. As shown in Figure 1, when a DNA substrate that carries a site-specific 1,3 platinum lesion is treated with large-scale extracts prepared in the traditional manner from 10⁹ cells we observe the expected cleavage events 5′ and 3′ of the platinum lesion to produce 25–30 nt long excision products. When the same substrate is treated with mini-NER–WCE (mNER–WCE) prepared from 5 × 10⁹ cells we observed the same cleavage near the platinum modification, which indicates that the mNER–WCE contains all of the factors necessary for recognition and excision of a platinum lesion.

Because the mNER–WCE is less concentrated than large-scale extracts the observed excision activity was lower than that observed with the large-scale extracts. Figure 1 shows that concentration of the mNER–WCE increased the excision activity and made it comparable to the large-scale extract.

To test the dependence of the mNER–WCE-catalyzed excision events on known excision repair factors, we prepared mNER–WCE from cells deficient in XPD or XPG.

---

**Table 1. Oligonucleotide duplexes used in this study**

| Duplex | Sequence |
|--------|----------|
| A      | 5′-TGATCTCCTAACGTTAGGCTTC 3′-CTGATGATTTGCAAATCAGAGGACTG |
| B      | 5′-CTGACTCACTGTTAAGGCTTTCAGCC 3′-AGTCCACTTCGAATGTCAGAG |
| C      | 5′-CTTCTACTTCTGCAATGAGAAA 3′-GATTAGACGGTAC |
| D      | 5′-TTCATCTTATATCTGCAATGAGAAA 3′-GATTAGACGGTAC |
| E      | 5′-TTCATCTTATATCTGCAATGAGAAA 3′-GATTAGACGGTAC |
| F      | 5′-TTCATCTTATATCTGCAATGAGAAA 3′-GATTAGACGGTAC |

Ligation of these duplexes mediated by complementary overhangs (bold) resulted in ordering of the fragments as A-B-C-X-D-E-F.
Mini-NER–WCE prepared from XPD or XPG deficient cells is unable to excise the platinum lesion. Combining the XPD and XPG mini-extracts restores excision activity. These data show that extracts prepared using our small-scale mNER–WCE method are catalytically active for NER and that this method is applicable to a variety of cell lines.

Mini-WCE for the study of NHEJ in vitro: 120-min mini-NHEJ–WCE

Establishment of an in vitro DNA ligase IV-dependent NHEJ assay enabled examination of the molecular mechanism of the ligation step of NHEJ. As shown in Figure 2A, NHEJ–WCE prepared using the conventional large-scale method catalyze end joining, which is sensitive to the PI3-kinase inhibitors LY294002 and wortmannin. End joining catalyzed by the large-scale NHEJ–WCE is also sensitive to treatment with neutralizing anti-XRCC4 antibodies (lane 3) but not to treatment with control anti-GST antibodies (lane 4), which demonstrates a specific requirement for the XRCC4 protein. The data presented in Figure 2 are consistent with the original description of this method (5).

To facilitate our investigation of the molecular mechanism of NHEJ we developed a mini-extract protocol that reduces the number of cells required for analysis from 5×10⁶ to 5×10⁵ cells. Mini–NHEJ–WCEs were prepared from 293 cells and end joining was assayed for DNA end joining. As shown in Figure 2B, treatment with either anti-XRCC4 antibodies, LY294002 or wortmannin resulted in a significant decrease in ligation efficiency (Figure 2B), which indicated a requirement for both XRCC4 and DNA-PKcs and identified the DNA end joining catalyzed by mNHEJ-WCE as bona fide mammalian NHEJ. Treatment with control anti-GST antibodies had no effect and similar results were obtained with mini-extracts prepared from HeLa cells (data not shown).

Inhibition of NHEJ by human adenovirus (Ad5)

Studies have shown that infection with human Ad5 results in inhibition of NHEJ in vivo (12–16). The utility of the
DISCUSSION

Here we report the development of a small-scale cell extract protocol that can be used to prepare extracts for the study of NER \textit{in vitro}. This method uses 20-fold fewer cells than previously published methods (8–10). We show that mNER–WCEs produce plantinated lesion-directed cleavages that are characteristic of the NER pathway and require participation of known NER factors XPD and XPG. Importantly, this method can be used for complementation analysis of NER-deficient cell lines or to screen the participation of NER factors in the repair of different DNA lesions.

We also describe a rapid, small-scale method for the production of NHEJ-competent WCEs. This method reduces the number of cells used to prepare extracts for NHEJ analysis from $5 \times 10^{10}$ cells used in the large-scale method of Baumann and West (5) to as few as $5 \times 10^6$ cells. While this is not the first report of a small-scale NHEJ assay, the method described by Diggle \textit{et al.} (17) requires $0.5 - 1 \times 10^5$ cells and requires ultracentrifugation and dialysis. The method described here requires 10- to 20-fold fewer cells than the Diggle method, takes ~120 min and does not require ultracentrifugation or dialysis.

The usefulness of both of these methods comes primarily from a reduction in the number of cells required to produce the mini-extracts. Elimination of ultracentrifugation steps reduces the time required for extract preparation and relaxes the need for specialized equipment. In the 120-min mNHEJ–WCE protocol, elimination of the dialysis step saves time and reduces sample manipulation. All of these improvements make the mini-extract protocols more amenable to multiple sample preparation and examination of numerous variables. As we show in Figure 3, the mNHEJ–WCE protocol is particularly useful in the examination of host–virus interactions where large-scale infections would be cumbersome.
ACKNOWLEDGEMENTS

We thank Joyce Cheung, Timra Gilson, Brenda Salerno, Sumithra Jayaram, B.T. Rantipole and Bill Russ for many thoughtful discussions. We thank Drs Joyce Reardon and Aziz Sancar for helpful advice on preparing NER extracts; Ms Maggie Wear for assistance in oligonucleotide synthesis; and Dr Michael Seidman for generously providing CHO AA8 and CHO UV5 cell lines. UV41 cells were obtained from American type culture collection under grant number CA082785. HeLa cells were obtained from the National Cell Culture Center (Minneapolis, MN, USA), supported under grant number U42 RR05991 from the National Center for Research Resources, NIH. This work was supported by the National Institutes of Health (NIH) grants GM070639-1 (to L.A.H.), 5R01CA082127 (to G.K.) and CA082785 (to P.S.M.), and by the Johns Hopkins University Bloomberg School of Public Health Faculty Research Initiatives Fund (to L.A.H. and G.K.). Funding to pay the Open Access publication charges for this article was provided by National Institutes of Health (NIH) grants GM070639-1.

Conflict of interest statement. None declared.

REFERENCES

1. Yang,W. (2006) Poor base stacking at DNA lesions may initiate recognition by many repair proteins. DNA Repair, 5, 654–666.
2. Hoeijmakers,J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366–374.
3. Ferguson,D.O. and Alt,F.W. (2001) DNA double strand break repair and chromosomal translocation: lessons from animal models. Oncogene, 20, 5572–5579.
4. Burma,S., Chen,B.P. and Chen,D.J. (2006) Role of non-homologous end joining (NHEJ) in maintaining genomic integrity, DNA Repair, 5, 1042–1048.
5. Baumann,P. and West,S.C. (1998) DNA end-joining catalyzed by human cell-free extracts. Proc. Natl Acad. Sci. USA, 95, 14066–14070.
6. Wood,R.D. (1989) Repair of pyrimidine dimer ultraviolet light photoproducts by human cell extracts. Biochemistry, 28, 8287–8292.
7. Wood,R.D., Robins,P. and Lindahl,T. (1988) Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts. Cell, 53, 97–106.
8. Manley,J.L., Fire,A., Cano,A., Sharp,P.A. and Gefter,M.L. (1980) DNA-dependent transcription of adenosine genes in a soluble whole-cell extract. Proc. Natl Acad. Sci. USA, 77, 3855–3859.
9. Wood,R., Biggerstaff,M. and Shivji,M.K.K. (1995) Detection and measurement of nucleotide excision repair synthesis by mammalian cell extracts in vitro. Compani ion Methods Enzymol., 7, 163–175.
10. Reardon,J.T. and Sancar,A. (2006) Purification and characterization of Escherichia coli and human nucleotide excision repair enzyme systems. Methods Enzymol., 408, 189–213.
11. Hanakahi,L.A., Bartlet-Jones,M., Chappell,C., Pappin,D. and West,S.C. (2000) Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. Cell, 102, 721–729.
12. Carson,C.T., Schwartz,R.A., Stracker,T.H., Lilley,C.E., Lec,D.V. and Weitzman,M.D. (2003) The Mre11 complex is required for ATM activation and the G2/M checkpoint. EMBO J., 22, 6610–6620.
13. Stracker,T.H., Carson,C.T. and Weitzman,M.D. (2002) Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature, 418, 348–352.
14. Boyer,J., Rohleder,K. and Ketner,G. (1999) Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology, 263, 307–312.
15. Nicolas,A.L., Munz,P.L., Falck-Pedersen,E. and Young,C.S. (2000) Creation and repair of specific DNA double-strand breaks in vivo following infection with adenovirus vectors expressing Saccharomyces cerevisiae HO endonuclease. Virology, 266, 211–224.
16. Baker,A., Rohleder,K.J., Hanakahi,L.A. and Ketner,G. (2007) Adenovirus E4 34k and Elb 55k oncoproteins target host DNA ligase IV for proteasomal degradation. J. Virol., 81, 7034–7040.
17. Diggle,C.P., Bentley,J., Knowles,M.A. and Killie,A.E. (2005) Inhibition of double-strand break non-homologous end-joining by cisplatin adducts in human cell extracts. Nucleic Acids Res., 33, 2531–2539.