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Quality control of purified proteins involved in homologous recombination

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Abstract

Biochemical reconstitution using purified proteins and defined DNA substrates is a key approach to develop a mechanistic understanding of homologous recombination. The introduction of sophisticated purification tags has greatly simplified the difficult task of purifying individual proteins or protein complexes, generating a wealth of mechanistic information. Using purified proteins in reconstituted recombination assays necessitates strict quality control to eliminate the possibility that relevant protein or nucleic acid contaminations lead to misinterpretation of experimental data. Here we provide simple protocols that describe how to detect in purified protein preparations contaminating nucleic acids and relevant enzymatic activities that may interfere with *in vitro* recombination assays. These activities include ATPases, indicating the potential presence of helicases or translocases, endo- and exonucleases, phosphatases, and type I or type II topoisomerases.

Keywords

ATPase; DNA helicase; DNA translocase; endonuclease; exonuclease; phosphatase; topoisomerase; in vitro recombination assays; protein purification

1. Introduction

Nearly two hundred genes have been identified to be involved in DNA repair processes in human cells (1). Homologs for most of these genes have also been found in other organisms, including the budding yeast *Saccharomyces cerevisiae*, underlining the value of model organisms (2). To understand the mechanisms of maintaining genomic integrity, the encoded proteins are usually overexpressed and purified to apparent homogeneity to identify their individual activities, reconstitute more complex *in vitro* recombination reactions, and determine their structure.

Obtaining high quality purified proteins is a prerequisite for these structural and mechanistic studies.

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Reconstitution of biological processes using purified proteins and model substrates has been a spectacularly successful approach in elucidating the mechanism of DNA replication, and has been canonized by the late Arthur Kornberg as the first commandment of enzymology: "Rely on enzymology to resolve and reconstitute biologic events" (3). Two additional commandments are of particular relevance for the present discussion: "Not waste clean thinking on dirty enzymes"(IV) and "Not waste clean enzymes on dirty substrates" (V). Here we provide simple protocols for quality control of purified proteins that are used in reconstituted *in vitro* recombination reactions. The presence of many proteins in a single reconstituted system requires that every component is scrutinized for potential contaminations. The first concern is the presence of nucleic acids (RNA/DNA) in the protein preparation, because such nucleic acids contaminate the designed substrates and compete for binding and activity by the proteins under study, leading to potential misinterpretations. During protein purification, nucleic acids are typically removed by various methods (see **Note 1**). However, residual nucleic acid contaminations may persist and confirming the absence of nucleic acids in purified recombination proteins is an important quality control step. The second concern is the presence of contamination by enzymatic activities that interfere with the intended assay by acting on the designed substrates, potential reaction intermediates, or reaction products (see commandment IV of (3)). Such activities include ATPases, indicating the potential presence of nucleic acid-based motor proteins such as helicases or translocases, nucleases (endo- or exonucleases), phosphatases, and topoisomerases (type I and II). Many of these activities are active at concentrations that cannot be visualized by standard techniques such as Coomassie staining of protein gels. Hence, even a protein that is apparently pure may be contaminated by relevant interfering activities. Here, we will provide simple protocols to test for such contaminating activities. It is more challenging to identify a potential contamination with the same type of activity as the intended purification target (*e.g.* a contaminating ATPase in a preparation of a protein that is an ATPase), but such a possibility should be taken into consideration.

2. Materials

2.1. Detection of nucleic acid contaminations

2.1.1. Agarose gel electrophoresis

1. Agarose, LE (low electroendosmosis).
2. Agarose gel running buffer: Tris-acetate-EDTA (TAE): 40 mM Tris-acetate, final pH 8.5, 2 mM EDTA, or Tris-borate-EDTA (TBE): 89 mM Tris base, 89 mM borate and 2 mM EDTA, final pH 8.0.

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[1]Several methods have been developed to remove nucleic acids during the early stages of a protein purification protocol. Nucleic acids can be removed by binding to a positively charged substance, such as polyethyleneimine (PEI) (8). If the target protein is acidic, elevated salt concentrations avoid precipitation of the target protein (9). Nucleic acids can also be eliminated by enzymatic digestion using DNase I (10) or Benzonase, a nuclease available from many commercial sources (*e.g.* Merck) which degrades linear or circular DNA and RNA in their single-stranded or double-stranded form, producing 5'-monophosphate terminating oligonucleotides of 2-5 bases. Alternatively, nucleic acids can be removed from protein samples by chromatography on strong anion exchange resins such as DEAE cellulose or Q-Sepharose (11, 12) or DNA binding proteins can be selectively captured by affinity chromatography including media such as DNA cellulose (13), Affi-Gel Blue (Bio-Rad) (14), or Fast Flow Cibacron Blue 3GA (Sigma) (15). A caveat for using DNA cellulose is the observation that DNA slowly leaches off the column, introducing contaminating DNA.

3. 10× nucleic acid agarose gel loading buffer: 20% Ficoll 400, 0.1 M EDTA, 1% SDS, 0.25% bromphenolblue, 0.25% xylencyanol.
4. Agarose gel apparatus (Owl Easy-Cast model B1A, tray size 7 cm × 8 cm, gel volume ~60 ml. or Owl Easy-Cast model B2, tray size 12 cm × 14 cm, gel volume ~100 ml).
5. Power supply.
6. Microwave oven.
7. 10 mg/ml ethidium bromide stock solution in ddH_2O or alternative dyes, for example SYBR dyes (Molecular Probes) (see **Note 2**).
8. Molecular size marker (EZ load 1 kb molecular ruler, Bio-Rad, #170-8355).
9. Transilluminator UV light and gel documentation system.

2.1.2. Spectrophotometry

1. Nano-Drop ND-1000 (NanoDrop Technologies, Inc.).
2. A micropipette that can measure a sample of 2 μl accurately.
3. Buffer used for protein storage.

2.1.3. 5’- [^{32}P]-labeling of nucleic acids

1. Equipment and safety measures for working with radioactivity (see **Note 3**).
2. CIA: 24:1 (v/v) chloroform/isoamyl alcohol.
3. PCIA: 1:1 (v/v) phenol/CIA, made with buffered phenol (25:24:1 (v/v/v) Phenol/chloroform/isoamyl alcohol) (see **Note 4**).
4. 100% ethanol.
5. 70% ethanol.
6. 3 M sodium acetate (NaOAc).
7. Antarctic phosphatase (New England Biolabs [NEB] M0289S, 5,000 units/ml).
8. 10× Antarctic phosphatase buffer (NEB, pH 6.0): 500 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 6.0.

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[2]The detection limit for ethidium bromide staining is ~ 20 ng per band of dsDNA, while the method is less sensitive for RNA or single-stranded DNA. Ethidium bromide is a known mutagen and appropriate caution should be taken for handling and disposal. More sensitive alternatives to ethidium bromide have been developed, for example SYBR dyes (Molecular Probes, Invitrogen) increase the sensitivity to ~1 ng per band, but are significantly more expensive. Ethidium bromide can be added directly into the gel solution before casting or the gel can be stained after electrophoresis. The latter staining method gives clearer background and limits the liquid waste volume.

[3]Basic equipment includes a Geiger counter, shields, mask or safety glasses, shielded liquid and solid waste containers. A user should be trained and follow the common and local rules for isotope usage.

[4]Phenol extraction is used to remove protein from the sample, which may interfere with the labeling process. The concentration of the nucleic acid contamination in the sample is likely to be low. Hence, the DNA pellet may be invisible. Mark the expected position of the pellet on the wall of the tube. Make sure that the pellet will not be lost. The minimal start volume of phenol extraction should be 0.2 ml to ease handling. Antarctic phosphatase (NEB) is used to dephosphorylate the 5’ ends of nucleic acids. This enzyme can be inactivated by incubation at 65 °C for 5 min. Labeling may also be performed directly without PCIA extraction.

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9. T4 polynucleotide kinase (PNK) (NEB, M0201S, 10,000 units/ml).
10. 10× PNK buffer (NEB): 700 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT.
11. [γ-^{32}P]-ATP (Perkin Elmer, 10 μCi/μl).
12. Proteinase K solution: 0.71 % SDS, 0.357 M EDTA and 4.2 mg/ml Proteinase K (Roche #03115801001, PCR grade).
13. 0.8% agarose gel (see **2.1.1.**).
14. Phosphorimager (Storm, Molecular Dynamics) and ImageQuant software.
15. 30 °C and 37 °C water baths, 70°C heat block.
16. Gel dryer.

2.2. Detection of ATPase contaminations

2.2.1. Charcoal assay

1. Equipment and safety measures for working with radioactivity (see **Note 3**).
2. Activated charcoal (Sigma, C3345, 100-400 mesh) solution: 5% charcoal, 0.25 N HCl, 50 mM KH_2PO_4.
3. [γ-^{32}P]-ATP (Perkin Elmer, 10 μCi/μl), dilute 100× with 10 mM Tris-HCl, pH 7.0.
4. 2× ATPase assay buffer: 66 mM Tris-HCl, pH 7.5, 26 mM $MgCl_2$, 3.6 mM DTT, 2 mM ATP, 180 μg/ml BSA.
5. Commercial Form I ΦX174 (NEB, N3021L) and virion DNA (circular ssDNA, NEB, N3023L).
6. RecA protein (NEB, M0249S) as positive control.
7. 30 °C water bath.
8. 4 °C bench top centrifuge.
9. Liquid scintillation vials (volume ~10 ml), liquid scintillation cocktail (EcoLume, MP Biologicals Inc., #882470), and scintillation counter (Beckman LS6500 multipurpose scintillation counter).

2.2.2. Thin-layer chromatography (TLC) method

1. Equipment and safety measures for working with radioactivity (see **Note 3**).
2. Cellulose PEI-F (J. T. Baker, 4474-00), 5×20 cm sheets. The plates should be pre-run in 95% Ethanol, air-dried and then pre-run in ddH_2O and air-dried.
3. Glass thin-layer chromatography tank, 25 cm wide by 20 cm high.
4. Hair dryer (optional).
5. [γ-^{32}P]-ATP mixture: 10 mM Tris-HCl, pH 7.5, 9.6 mM unlabeled ATP and 0.385 μCi/μl [γ -^{32}P]-ATP.

6. Commercial Form I ΦX174 (NEB, N3021L) and virion DNA (circular ssDNA, NEB, N3023L).
7. RecA protein (NEB, M0249S) as positive control.
8. TLC running buffer: 1 M formic acid and 0.5 M LiCl.
9. Reaction stop solution: 6.7 mM ATP, 6.7 mM ADP and 33.3 mM EDTA.
10. 30 °C water bath.
11. Phosphorimager (Storm, Molecular Dynamics) and ImageQuant software.

2.3. Detection of nuclease and phosphatase contaminations

2.3.1 Endonuclease

1. Commercial RFI ΦX174 (NEB, N3021L) and virion DNA (circular ssDNA, NEB, N3023L) (see **Note 5**).
2. DNase I (NEB, M0303S).
3. 37 °C and 30 °C water baths, heat block.
4. 10× reaction buffer: 200 mM Tris-Acetate, pH 7.9, 100 mM $MgCl_2$, 500 mM KCl and 10 mM DTT. (see **Note 6**).
5. BSA, 10 mg/ml in 20 mM KPO_4, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol.
6. 0.8% agarose gel (see **2.1.1.**).
7. Transilluminator UV light and gel documentation system.

2.3.2 Exonuclease/phosphatase

1. Equipment and safety measures for working with radioactivity (see **Note 3**).
2. Commercial RFI ΦX174 (NEB, N3021L).
3. *Xho*I (NEB, R0146S, 20,000 Units/ml) and 10× buffer supplied by manufacturer.
4. T7 Exonuclease (NEB, M0263S, 10,000 units/ml), a 5' → 3' exonuclease. Control enzyme.
5. Exonuclease III (NEB, M0206S, 100,000 units/ml), a 3' → 5' exonuclease. Control enzyme.

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[5]Commercial DNA is adequate for this purpose, although the quality of the batches varies in the proportion of Form I supercoiled DNA for dsDNA (as opposed to Form II nicked and Form III linear DNA) and the proportion of circular DNA for ssDNA (as opposed to linear ssDNA). However, sufficient circular DNA for endonuclease assays and supercoiled DNA for topoisomerase assays must be present. Be aware that frequent freeze/thaw cycles generate nicks in DNA.

[6]The buffer should provide conditions under which most nucleases exhibit at least some activity. If a nuclease contamination is suspected, the important parameters (Mg^{2+} concentration, buffer type, salt type and concentration) can be varied to enhance sensitivity. The standard buffers provided with restriction enzymes provide a useful range of reaction conditions. These are 10× concentrations.

NEBuffer 1: 100 mM Bis-Tris-Propane-HCl, pH 7.0, 100 mM $MgCl_2$, 10 mM DTT.

NEBuffer 2: 100 mM Tris-HCl , pH 7.9 , 100 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT.

NEBuffer 3: 500 mM Tris-HCl, pH 7.9, 100 mM $MgCl_2$, 1000 mM NaCl, 10 mM DTT.

NEBuffer 4: 200 mM Tris-acetate, pH 7.9, 100 mM Magnesium Acetate, 500 mM potassium acetate, 10 mM DTT.

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6. [γ-^{32}P]-ATP (Perkin Elmer, 10 μCi/μl).
7. T4 polynucleotide kinase (PNK) and 10× PNK buffer (see **2.1.3**.).
8. 37 °C and 30 °C water baths.
9. Boiling water bath or heat block.
10. CIA and PCIA (see **2.1.3.**).
11. 10× reaction buffer: 200 mM Tris-Acetate, pH 7.9, 100 mM $MgCl_2$, 500 mM KCl and 10 mM DTT (see **Note 6**).
12. BSA stock solution: 10 mg/ml BSA, 20 mM KPO_4, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 5 % glycerol.
13. 0.8 % agarose gel (see **2.1.1.**)
14. Transilluminator UV light and gel documentation system.
15. Liquid scintillation vials (volume ~10 ml), liquid scintillation cocktail (EcoLume, MP Biologicals, Inc.), scintillation counter (Beckman LS6500 multipurpose scintillation counter).

2.4. Detection of topoisomerase contaminations

2.4.1. Topoisomerase I

1. Commercial RFI ΦX174 (NEB, N3021L) (see **Note 5**).
2. *E. coli* Topoisomerase I (NEB, M0301S) as control enzyme.
3. 2× topoisomerase I reaction buffer: 50 mM Tris-acetate pH 7.9, 20 mM $MgCl_2$, 100 mM potassium acetate, 200 mM NaCl, 2 mM DTT, 1 mM EDTA.
4. Proteinase K solution: 0.71% SDS, 0.357 M EDTA and 4.2 mg/ml Proteinase K.
5. BSA stock solution: 10 mg/ml BSA, 20 mM KPO_4, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol.
6. 30 °C water bath.
7. 0.8% agarose gel made with TAE, TAE running buffer and staining buffer (see **2.1.1.)**
8. 10× nucleic acid agarose gel loading buffer (see **2.1.1)**.
9. Transilluminator UV light and gel documentation system.

2.4.2. Topoisomerase II—The same as **2.4.1.** except:

1. Human Topoisomerase II (Inspiralis, HT201, 10 units/μl) as control enzyme.
2. 2× Topoisomerase II reaction buffer: 40 mM Tris-HCl, pH 7.9, 20 mM $MgCl_2$, 2 mM DTT, 100 mM KCl, 100 mM NaCl, 1 mM EDTA, 2 mM ATP.

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3. Methods

3.1. Detection of nucleic acid contaminations

3.1.1. Agarose gel electrophoresis—Agarose gel electrophoresis is a robust method to visualize and separate nucleic acids based on their size and topology. Staining by ethidium bromide or alternative dyes provides a simple way to detect nucleic acid contaminations in protein preparations. (see **Note 2**).

1. Set up the gel tray and make sure the surface is level. Prepare 0.8% agarose LE solution with TAE or TBE. Carefully heat the solution with microwave until the agarose is completely dissolved. Pour the gel.
2. Add 1/10 volume of 10× nuclei acid gel loading buffer to each sample (final volume up to 30 μl). Load the sample on the gel. A DNA ladder is loaded as a size standard.
3. Run gel at 90 V (~ 5 V/cm) for 1.5-2 h.
4. Stain the gel in 1 μg/ml ethidium bromide. Destain the gel in ddH_2O for 10-20 min.
5. Visualize nucleic acids with UV transilluminator and document.

3.1.2. Spectrophotometry with NanoDrop—Protein and nucleic acids show UV absorption maxima at 280 nm and 260 nm, respectively. The OD_{260}/OD_{280} ratio is typically used to evaluate the purity of nucleic acids preparations, ensuring the absence of protein. Likewise, nucleic acid contamination in protein preparations can be detected the same way. The NanoDrop is ideal for this purpose for its sparing use of sample (as little as 2 μl).

1. Start the Nanodrop application on your computer. Follow the screen prompt to initialize the machine.
2. Blank the machine using 2 μl storage buffer used for storing the protein sample. Wipe the buffer from the upper and low pedestals
3. Establish a UV spectrum (220-350 nm) of the sample by applying a 2 μl volume. A peak or shoulder at 260 nm and a ratio of A_{260}/A_{280} greater than 1 indicates nucleic acid contamination in the protein sample.

3.1.3. DNA phosphorylation methods (^{32}P)—During protein extraction it is likely that all nucleic acids have been sheared to their linear form. This facilitates their detection by 5'-end-labeling with polynucleotide kinase after removing any potential phosphates using a phosphatase. This method is significantly more sensitive than the electrophoretic or spectrophotometric approaches and can be performed directly with a sample of the purified protein (start at step 10) or after extraction of the nucleic acid (steps 1-9), as tight binding to the protein may prevent access by the phosphatase/kinase. We recommend doing both (see **Note 4**).

1. Start with a sample 0.2 ml in a 1.5 ml Eppendorf tube. Add an equal volume of PCIA.

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2. Vortex vigorously for about 10 sec. Spin 1 min at 16,000 g in a table-top centrifuge.
3. Carefully transfer the top aqueous phase to a new 1.5 ml Eppendorf tube. Repeat the PCIA extraction process 1-2 times.
4. Extract the aqueous phase 1x with CIA to eliminate residual phenol.
5. Add 1/10 volume of 3 M sodium acetate, pH 5.2 to the aqueous solution, mix well.
6. Add 2.5 volumes ice-cold 100% ethanol. Vortex. Keep the sample at –80 °C for 30 min or –20 °C overnight.
7. Spin the tube at 4 °C (16,000 g) for 20 min. Carefully remove the supernatant.
8. Wash the pellet in 0.5 ml ice cold 70% ethanol. Remove the supernatant completely after centrifugation. Dry the nucleic acid pellet by leaving the tube open on bench for at least 30 min or use a Speedvac (see **Note 4**).
9. Resuspend nucleic acids in 12.5 μl ddH_2O.
10. Add 1.5 μl 10x Antarctic phosphatase reaction buffer and 1 μl of Antarctic phosphatase. Incubate the mixture at 37 °C for 1 h.
11. Incubate the mixture at 70 °C for 10 min to inactivate Antarctic phosphatase.
12. Add 3 μl 10×PNK buffer, and 10 Units (1 μl) T4 polynucleotide kinase. 3 μl [γ-^{32}P]-ATP. Adjust final volume to 30 μl by adding 8 μl ddH_2O. Incubate the reaction at 37°C for 1 h.
13. Add 5 μl proteinase K solution, incubate at 30°C for 20 min.
14. Load 20-30 μl of the mixture into one well of an 0.8% agarose gel and run gel with 5 V/cm, for 2 h.
15. Dry gel and expose to a phosphorimager and quantify with ImageQuant.

3.2. Detection of ATPase contamination—Hundreds of cellular enzymes hydrolyze ATP to generate energy in support of different functions. Indeed many proteins involved in homologous recombination display or are predicted to exhibit ATPase activity, including homologs or paralogs of RecA, DNA helicases, and DNA translocases. Of practical importance are also molecular chaperones that use the energy of ATP hydrolysis to support folding and often co-purify with overexpressed recombinant proteins. Contaminations by DNA helicases or DNA translocases can lead to changes in the designed substrates, potential intermediates, or reaction products in reconstituted recombination reactions, severely compromising potential interpretations. These enzymes often display DNA-dependent ATPase activity (ssDNA or dsDNA) and require Mg^{2+}. Hence, these co-factors must be provided to detect such contaminations. Here, we provide protocols for two simple ATPase assays (see **Note 7**).

[7]The charcoal and TLC assays have similar sensitivities. The volumes in the TLC assay can be reduced for more sparing use of protein sample. In addition, the TLC assays provide information on the reaction products of the ATPase activity.

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3.2.1. Charcoal assay—Activated charcoal specifically binds nucleotides but not phosphate, allowing to monitor the hydrolysis of ATP using [γ-^{32}P]-ATP by measuring the accumulation of [^{32}P] in solution (4). Any radioactivity above background indicates ATPase activity.

1. Add 25 μl 2× ATPase buffer, 1 μg ssDNA and 1μg dsDNA in a 1.5 ml Eppendorf tube.
2. Add an aliquot of protein preparation (up to 24 μl) or 1 μg RecA as positive control.
3. Add 1 μl [γ-^{32}P]-ATP solution (containing 0.1 μCi ^{32}P).
4. Adjust the final volume to 50 μl with ddH_2O.
5. Two control reactions are needed, background and input. Both of them contain buffer and [γ-^{32}P]-ATP only.
6. Incubate at 30 °C for 30 min.
7. Add 0.5 ml charcoal solution (agitate during adding), vortex, incubate on ice for 10 minutes for reaction and background control. For input control, add the same volume of ddH_2O instead. Vortex once in the middle of incubation. Spin at 16,000 g at 4 °C for 10 min.
8. Transfer 0.4 ml of supernatant to a scintillation vial containing 4 ml liquid scintillation cocktail. Mix well.
9. Determine radioactivity in supernatant by scintillation.

3.2.2. Thin-layer chromatography assay—Thin-layer chromatography on polyethyleneimine plates allows the separation of ATP, ADP, AMP, and phosphate. The protocol provided here is simpler than the original version (5), as the labeled phosphate is separated more easily from the [γ-^{32}P]-ATP than [α-^{32}P]-ADP from [α-^{32}P]-ATP.

1. Assemble the reactions as in the charcoal assay (**3.2.1.**).
2. Add 2.5 μl [γ-^{32}P]-ATP mixture to a final volume of 50 μl.
3. Incubate the reaction at 30 °C.
4. Withdraw 2.5 μl from the reaction at 15, 30, and 60 min and mix with 1.25 μl stop solution.
5. 1 μl of the mixture from step 4 is spotted on the cellulose PEI-F plate close to the bottom edge.
6. Run the PEI-F plate in the TLC running solution in a TLC tank until the front of the solution almost reaches the top of the plate.
7. Remove the plate from the tank and let it dry in fume hood or using a hair dryer.
8. Expose overnight.
9. Scan and quantify with ImageQuant software. Calculate the free phosphate increase above background.

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3.3. Detection of nuclease/phosphatase contaminations

Nuclease contaminations significantly compromise the integrity of the substrates in reconstituted recombination reactions, and it is important to confirm the absence of ssDNA and dsDNA endoand exonuclease activities. Using 5’-end-labeled substrates for exonuclease assays concomitantly monitors also for contaminations by phosphatases.

3.3.1. Endonuclease—The conversion of circular to linear DNA provides a simple reaction to detect endonuclease activity by electrophoretic mobility differences on agarose gels and commercially available circular ssDNA and dsDNA provides easy access to substrates.

1. Incubate 20 μl reaction containing 2 μl 10x buffer, 2 μl BSA stock solution, 1-5 μg purified protein with 0.5 μg of form I dsDNA or circular ssDNA for 2 h at 30 °C.
2. Use DNase I (NEB, M0303S) as positive control, adding 2 units per reaction, and no protein added as negative control.
3. Add 2.5 μl proteinase K solution in each reaction tube, incubate at 30 °C for 20 min.
4. Add 1/10 volume 10× nucleic acid agarose gel loading buffer to each sample.
5. Run the sample on a 0.8% agarose gel with TAE buffer system, 5V/cm, 2 h.
6. Stain the gel with 1 μg/ml ethidium bromide and visualize with a transilluminator UV light. Any change in band intensity and migration position of the Form I or the circular ssDNA bands compared to the negative control signals potential endonuclease activity (see also **3.4.**).

3.3.2. Exonuclease/phosphatase—Exonucleases require a terminus of a poly-nucleotide chain to hydrolyze dsDNA and/or ssDNA. Using 5’-[^{32}P]-labeled linearized ΦX174 dsDNA as a substrate provides sensitive assays to detect contaminating exonuclease and phosphatase activities.

A. Linearization of circular dsDNA and 5’-end labeling

1. 30 μg of ΦX174 RFI dsDNA in 100 μl volume with 10 μl 10× NEBuffer #4, 10 μl BSA stock solution, and 50 units *Xho*I at 37 °C for 1 h. Check for completion of linearization by agarose gel electrophoresis.
2. Remove restriction enzyme by PCIA extraction (see **3.1.3.**), precipitate with ethanol plus NaOAc, and dissolve in 80 μl ddH_2O.
3. Take 15 μl the linearized dsDNA from step 2, add 5 μl 10× Antarctic phosphatase buffer, 28 μl ddH_2O and 1.5 μl (7.5 units) Antarctic phosphatase. Incubate at 37°C for 1 h.
4. Inactivate the Antarctic phosphatase by heating the sample at 70°C for 10 min.
5. Add ddH_2O to 200 μl. Remove the phosphatase by PCIA extraction and ethanol precipitation (steps 1-9 of **3.1.3.**). Dissolve the DNA in 40 μl ddH_2O.

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6. Add 5 μl 10× PNK buffer, and 10 units (1 μl) T4 polynucleotide kinase. 4 μl [γ-^{32}P]-ATP (10 μCi/μl). Incubate the reaction at 37 °C for 1 h.
7. Add ddH_2O to 200 μl. Remove T4 polynucleotide kinase by PCIA extraction and enthanol precipitation (steps 1-9 of **3.1.3.**). Dissolve the labeled DNA in 50 μl TE.
8. Analyze 1 μl of the labeled DNA on an agarose gel to monitor the efficiency of labeling, stain with ethidium bromide and visualize under UV, then dry gel and analyze using a phosphorimager. Determine radioactivity of 1 μl of the labeled DNA by liquid scintillation.

B. Detection of exonuclease/phosphatase

1. Set up four parallel sets of reactions, two for dsDNA and two for ssDNA to be analyzed either by scintillation or by gel electrophoresis. The dsDNA can be used directly from protocol

3.3.2 A for ssDNA, heat denature the linear dsDNA by boiling in a heat block for 1 min and placing the tube directly on ice

1. Each 20 μl reaction contains 0-10 μg purified protein, 2 μl 10× reaction buffer, 0.2 μl BSA stock solution, and 0.5 – 1 μl of 5'-[^{32}P] labeled linearized ΦX174 dsDNA or ssDNA.
2. Incubate the reaction at 30 °C for 2 h.
3. Add 4 μl proteinase K solution. Continue the incubation at 30 °C for 20 min.
4. In the set for scintillation detection, add 36 μl ddH_2O and 12 μl 30% TCA to a final concentration of 5%. Incubate the tubes on ice for 30 min. Then spin the samples at 16,000 g for 10 min at 4 °C. Transfer the supernatant into 2 ml EcoLume. Mix well and determine radioactivity in scintillation counter. Any increase in radioactivity in the supernatant above the no protein control signals potential exonuclease or phosphatase activity.
5. In the set for gel electrophoresis, add 2.2 μl agarose gel loading buffer to each tube. Run the sample on 0.8% agarose gel with TAE at 5 V/cm. Visualize the DNA under a transilluminator UV light.
6. Dry the gel and exposure the gel to a phosphorimager screen for 12 h. Scan and quantify the gel.

Loss of label compared to the no protein control indicates exonuclease or phosphatase activity. Shortening of the labeled DNA indicates 3'-5' exonuclease activity or endonuclease activity, depending on the results with protocol **3.3.1.**

3.4. DNA topoisomerase

Relaxation of naturally negatively supercoiled DNA into a ladder of more relaxed topological isomers provides a simple assay to detect type I and type II DNA topoisomerases. While type I enzymes catalyze the reaction independent of ATP, type II enzymes require ATP, making for easy distinction. The activity of bacterial gyrase is not

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detected in this assay, as it only relaxes positive but not negative supercoils. Gyrase, however, is an ATPase and should be identified as a contamination by protocol **3.2.** More detail is found in the following sources (6, 7).

3.4.1. Topoisomerase I

1. 30 μl reaction mixture contains 15 μl 2× topoisomerase I buffer, 0.5 μg Form I dsDNA, 100 μg/ml BSA, 2.5 units of *E. coli* Topo I or 0-10 μg purified protein. Adjust the final volume to 30 μl with ddH_2O.
2. Incubate at 30 °C for 1-2 h.
3. Stop the reaction by adding 6 μl Protease K solution. Incubate at 30 °C for 20 min.
4. Add 4 μl 10× DNA gel loading buffer, mix. Load on 0.8% agarose gel made with 1× TAE buffer. Run the gel at 5 V/cm for 2 h in 1x TAE.
5. Stain the gel with 1 μg/ml ethidium bromide solution for 30 min and destain the gel with ddH_2O for 20 min with agitation, and visualize bands with gel documentation system.

3.4.2. Topoisomerase II

1. Set up 30 μl reactions including 15 μl 2× topoisomerase II reaction buffer containing ATP, 0.5 μg Form I dsDNA, 100 μg/ml BSA, 2 units human topoisomerase II or 0-10 μg purified protein.
2. Incubate the reactions at 30 °C for 1-2 h and proceed as described for topoisomerase I (**3.4.1.**).

In both assays, topoisomerase contamination is signaled by the appearance of a ladder of more relaxed topological isoforms running above the negatively supercoiled input DNA.

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