7. Rapidly thaw frozen cell suspension. Add 5 mg/ml lysozyme to 0.5 mg/ml final. Incubate 15 min on ice (cell suspension will be viscous). Add 4 M NaCl to 1 M final and mix thoroughly. Incubate on rotator for 15 min at 4°C.
8. Microcentrifuge 30 min at 4°C. Remove supernatants carefully and dialyze on 0.025-μm filter disks against 100 ml extract buffer 60 min at 4°C (*APPENDIX 3*). Freeze in dry ice/ethanol bath and store at –70°C.

References: Singh et al., 1989; Vinson et al., 1988.

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Rapid Separation of Protein-Bound DNA from Free DNA Using Nitrocellulose Filters

UNIT 12.8

QUANTITATION OF DNA BOUND TO PROTEIN

BASIC PROTOCOL

This protocol relies on the ability of nitrocellulose to bind proteins but not double-stranded DNA. Use of radioactively labeled DNA fragments allows quantitation of DNA bound to protein at various times and conditions and permits kinetic and equilibrium studies of DNA-binding interactions.

Materials (see APPENDIX 1 for items with ✓)

✓ 2× DNA-binding buffer
Double-stranded, ^{32}P-labeled DNA
Protein sample
0.45-μm nitrocellulose filter disks

1. Prepare 30 ml of 2× DNA-binding buffer and set aside 0.5 ml for use in step 3. Dilute remaining 29.5 ml to 1× with distilled water.
2. Soak filters in ~30 ml of 1× DNA-binding buffer ≥30 min. Retain ≥10 ml of 1× buffer for filtration step (step 6).
3. Set up a microcentrifuge tube for each sample as follows: Add ≥10,000 cpm double-stranded, ^{32}P-labeled DNA (2 to 10 ng) to 25 μl of 2× DNA-binding buffer and add water to 50 μl (accounting for protein volume to be added in step 4). Vortex and microcentrifuge 1 to 2 sec.
4. Add protein solution and *gently* invert to mix. Microcentrifuge 1 to 2 sec and incubate 25 min at 30°C.

   Time and temperature parameters will vary for different proteins. Add ~100 mg/ml BSA to stabilize low concentrations of protein.

5. Set up scintillation vials in vacuum apparatus and 5 min prior to incubation, place filters in apparatus with weights on top. At end of incubation in step 4, turn on vacuum source, apply contents of each tube gently but quickly to each filter, and filter at ~1 ml/min.
6. Wash each filter twice with 0.5 ml of 1× DNA-binding buffer.
7. Count each filter. Percent cpm retained = cpm on filter/total cpm (counts in filtrate and filter) × 100.

In some cases quantitation of DNA retained is insufficient information. The support protocol below describes how DNA can be recovered from the filters for further analysis by gel electrophoresis or amplification and cloning.

ALTERNATE PROTOCOL

DETECTION OF SPECIFICITY IN DNA BINDING

This alternate protocol creates conditions that disrupt weaker, presumably nonspecific binding interactions. Differences from the basic protocol include using more labeled DNA (representing a mixture of fragments) and more extensive washing. The goal is to recover enough of a single input fragment to visualize by subsequent autoradiography.

1. Prepare 40 ml 2× DNA-binding buffer. Set aside 3 ml and dilute remaining 37 ml to 1× with distilled water. Soak filters in ~10 ml of 1× binding buffer. Retain 60 ml of 1× binding buffer for filtration step.
2. Add ~100,000 cpm of double-stranded, ^{32}P-labeled DNA (20 to 100 ng of a mixture of labeled fragments) to 250 µl of 2× DNA binding buffer. Add water to 500 µl (accounting for the protein volume to be added in step 3), vortex, and microcentrifuge 1 to 2 sec.
3. Add protein solution and follow steps 4 and 5 in basic protocol.
4. Wash each filter three times with 2 ml of 1× binding buffer.

SUPPORT PROTOCOL

ELUTION OF BOUND DNA

Additional Materials (see APPENDIX 1 for items with ✓)

✓ Filter elution buffer
Carrier nucleic acid (e.g., tRNA)
100% ethanol

1. Remove filters and place face down in scintillation vials containing 450 µl filter elution buffer. Incubate 2 hr at 30°C with periodic swirling.
2. Transfer liquid to microcentrifuge tubes, add 1 µg carrier nucleic acid per tube, and mix. Add 1 ml of 100% ethanol and precipitate overnight at –20°C. Electrophorese (*UNIT 2.5*) and autoradiograph (*APPENDIX 3*) to determine which subset of input fragments was retained.

References: Ptashne, 1987; Riggs et al., 1970.

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