Purification of yeast RPA from bacteria

This method yields approximately 1 mg/liter of culture. Keys to a good yield include (1) inducing cells immediately after transformation, (2) avoiding precipitation following the ssDNA column by using short dialysis times and (3) avoiding the use of the pLysS strain of BL21(DE3).

BL21(DE3) cells are transformed with pJM126. The next morning many colonies are innoculated directly into LB containing 0.1 mg/ml ampicillin at 37°C and grown to OD=0.25. The culture is shifted to 17°C for about an hour after which the OD = 0.5 and temperature = 30°C. Add IPTG to 0.4 mM and continue induction at 30°C for 4-5 h.

Cells are harvested in a centrifuge (e.g., RC3B at 4k/10 min). The pellet is resuspended in 1/20 volume of TED (Tris/EDTA/Dextrose) and 0.2 mg/ml lysozyme is added. Following a 15 min incubation on ice, NP-40 is added to a final concentration of 0.1% and cells are frozen at -80 for purification the next day.

Upon thawing, an equal vol 2X A buffer (7.5) is added. Cells are lysed and chromosomal DNA is dispersed by sonication (4 min total at 2” on 2” off; moderate power for 2L prep) on ice. The extract is clarified by centrifugation at 20,000 x *g* for 20 min at 4 °C, and adjusted to conductivity equal to A50 (A buffer with 50 mM NaCl).

The extract produced from 2 liter of culture is loaded on a 100-ml phosphocellulose column equilibrated in buffer A with 50 mM NaCl, and the column washed with 500 ml of buffer A with 50 mM NaCl. *Escherichia coli* SSB is found in the flow-through of this column. RPA is eluted with a 600-ml gradient from 50 to 800 mM NaCl in buffer A. RPA elutes at 200 mM NaCl. Peak fractions are identified by 17% SDS-PAGE and Coomassie Blue staining and pooled. (There is A LOT of 70 and 34K protein that does not bind PC, and some 70K and its breakdown products elute shortly after RPA heterotrimer at ~300 mM). It appears to be fortuitous that the trimer elutes at a unique salt concentration. Any contaminating 70K in the RPA pool flows through the next step.

NaCl is added to 500 mM, and the sample loaded slowly onto a 5 ml ssDNA-cellulose (Affymetrix Cat #14395) column equilibrated in buffer A with 500 mM NaCl. This column is washed with 10 CV of buffer A containing 500 mM NaCl, 5 CV of buffer A containing 800 mM NaCl, and 5 CV of buffer A containing 1.5 M NaCl and 40% ethylene glycol. The protein peak from this last step contains approximately 1 mg of recombinant RPA from a 1-liter culture that appears 95% pure but dilute.

This pool is dialyzed against Buffer A (7.5) containing 500 mM NaCl for 3-4 hours. The goal is to get the salt down to 100 mM but this method avoids lengthy dialysis against A100 that causes RPA to precipitate. The sample (now at 500 mM) is diluted 5-fold with A0 (pH 8.3) and loaded onto 1 ml Q HiTrap column at 0.3 ml/min. This requires 5 hours for a 2L culture but at this protein concentration the RPA does not ppt. The Q column is washed with 3CV A100 (8.3) and eluted with a 5CV gradient from 100 – 500 mM NaCl in A (8.3). The higher pH aids in binding Q. RPA elutes at 240 mM NaCl. Run gel to check purity, dial to A200, and store at -80°C.

1X A Buffer: (Make a 2X Stock for convenience)
25 mM Tris (7.5)
1 mM EDTA
0.01% NP-40
10 % Glycerol
1 mM DTT (added freshly, as always)
0.1 mM PMSF (added freshly, as always)