PARTE 1:
His-tagged IF2 was overexpressed using the T7 expression system (modified pET30a to include a TEV cleavage site) by inducing BL21(DE3) with 1mM IPTG cells for 4 hr. The cells were collected and resuspended in lysis buffer (0.1MTris pH 8, 500mMKCl, 5mM BME and Roche protease inhibitor tablet) and cell lysis was carried out by sonication. The sonicated cell lysate was incubated for 30 min at 65ºC. Most of the endogenous proteins precipitated and were removed by centrifugation at 10,000 rpm for 25 min. Imidazole was added to supernatant (containing IF2) to 20 mM and pH was adjusted to 7.5. It was then loaded onto a Ni-NTA column pre-equilibrated with Ni-NTA loading buffer (50 mM HEPES pH 7.6, 20 mM imidazole, 500 mM KCl and 5 mM BME). The protein was eluted by imidazole gradient and fractions containing IF2 were pooled. The TEV protease was added to remove the N-terminal tag and dialyzed overnight into 50mMHEPES-KOH pH 7.5, 5mMBME without KCl. Next, it was loaded onto HiTrap Q column pre-equilibrated with 50 mM HEPES-KOH pH 7.5, 25 mM KCl and 1 mM DTT. IF2 was eluted with a KCl gradient and the fractions containing IF2 were pooled and buffer exchanged to storage buffer (30 mM HEPES-KOH pH 7.5, 30 mM NH4Cl, 5 mM Mg(OAc)2, and 1 mM DTT). It was frozen as small aliquots in liquid nitrogen and stored _80_C till further use.

PARTE 2:
Large-Scale Lysis of *S. cerevisiae* Cells

Reconstitution of translation initiation requires the synthesis and purification of a large number of components on a relatively large scale. The only way to increase the yield of some components is to increase the culture size, often to >12 liters. This creates a problem in the speed and efficiency with which the yeast cultures can be lysed. Goode has described a protocol for large-scale lysis of *Saccharomyces cerevisiae* using liquid nitrogen and a Waring blender. This method allows relatively rapid and efficient lysis of large amounts of yeast cells, while at the same time minimizing sample degradation by stabilizing the lysate as a frozen powder.

Here, we summarize this protocol, which is used in a number of purification schemes presented in this chapter.

Pellet the washed cells in a single bottle and determine the pellet weight. Using a vol equal to 0.33 the weight of the pellet (1 ml . 1 g) resuspend the pellet in the appropriate lysis buffer or ddH2O. Slowly drip the cell suspension into a bucket of liquid N_2 using a 25 ml pipette to create frozen cell droplets. Carefully scoop the frozen droplets into a plastic bottle, cap loosely, and store at -80º until all the liquid N_2 has evaporated, then cap tightly.

Dry a Waring blender canister overnight in an oven or the fume hood. Any residual water in the turning mechanism will freeze upon addition of liquid N_2, causing the propeller to lock in place, which can burn out the motor. For safety, set up the blender apparatus in the fume hood or behind a radiation shield to deflect liquid N2 spray, and wear safety goggles and cryo gloves. Briefly cool the canister by blending a small amount of liquid N_2. Fill the canister no more than half-full with frozen cell pellets (30 to 150 g). Overfilling can cause yeast lysate to spray from the canister. Add liquidN_2 to the level of the cell pellets and, holding

the canister lid in place, turn the blender on high and blend until the liquid N_2 evaporates, about 15 to 30 s. This will be marked by a change in the pitch of the blender sound. (Note: If yeast powder is expelled from the lid vents, stop and wait until the liquid N2 level has decreased to the level of the pellets and begin again.) Tap down the powder and again add liquid N2 to the canister to just above the level of the powder. Repeat the process at least 4 times for the best lysis. Transfer the powder into a 1 liter bottle using a funnel and a spatula. Add lysis buffer to the desired final volume. Shake the bottle vigorously until all the cells have been completely resuspended. You can run warm water over the bottle to help thaw the cells. (Important: Remember to open the cap occasionally until all the N_2 gas has escaped).

PARTE 3:

Negative staining is an easy, rapid, qualitative method for examining the structure of isolated organelles, individual macromolecules and viruses at the EM level. However, the method does not allow the high resolution examination of samples – for this more technically demanding methods, using rapid freezing and sample vitrification are required. Also, because negative staining involves deposition of heavy atom stains, structural artefacts such as flattening of spherical or cylindrical structures are common. Nevertheless, negative staining is a very useful technique because of its ease and rapidity, and also because it requires no specialized equipment other than that found in a regular EM laboratory.

Samples should be suspended in a suitable buffer (e.g. 10 mM HEPES or PIPES), in 1% ammonium acetate, or in distilled water. It is best not to use phosphate buffer or PBS as they may contaminate the grid with salt residues that have to be washed off after staining resulting in a loss of contrast. Uranyl salts, in particular, react with phosphate ions to produce a fine crystalline precipitate that obscures the specimen. [The precipitation of uranyl ions by phosphate ions is also a potential problem when using uranyl acetate as a third fixative/en bloc stain during processing of specimens for TEM].