Preparation of extracts from nocodazole-arrested HeLa cells.

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Hela S3 cells are grown in spinner culture (2-L spinner flasks) in DMEM supplemented with 10% fetal calf serum. To logarithmically growing cells (5-6x10^5 cells/ml) thymidine is added to a final concentration of 2 mM. After 24 hours, thymidine is removed by washing with prewarmed medium and cells are resuspended in 2L of medium. Following incubation for 3 hours, nocodazole is added to a final concentration of 0.1 μg/ml. Following further incubation for 11 hours, cells are collected by centrifugation (1,000 rpm, 10 min in 500-ml Sorvall flasks). From this stage, all operations are at 0-4°C, except where noted otherwise. The cells are washed twice with ice-cold PBS (500ml [for all 4 pellets] first time, 50 ml second time), transferred to a preweighed 15-ml tube and centrifuged again. The volume of cell pellet is estimated by its weight, and cells are suspended in 75% of pellet volume of hypotonic lysis buffer (20 mM HEPES-NaoH, pH 7.6, 5 mM KCl, 1 mM DTT, 10 μg/ml leupeptin and 10 μg/ml chymostatin). The cells are allowed to swell on ice for 30 min, and then the sample is frozen in liquid nitrogen, followed by rapid thawing in a 30°C water bath. The freeze-thawing procedure is repeated for a second time, and then the sample is gently passed (no foaming!) through a 21.5 G needle 10 times. A small sample is withdrawn for the estimation of total protein concentration and the rest is centrifuged in a refrigerated Eppendorf centrifuge at 5,000 rpm for 5 minutes. The supernatant is centrifuged again at 14,000 rpm for 60 minutes. To the final supernatant glycerol is added to a final concentration of 10%. The extract is divided to small samples in prechilled tubes, quick-frozen in liquid nitrogen and stored at -70°C.

Notes:

1. Thymidine is dissolved in DMEM at a 100 mM stock solution, sterilized by filtration and stored at -20°C.
2. Nocodazole is dissolved in DMSO at 1 mg/ml, and stored in small samples at -20°C in the dark. Each sample is used only once.

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3. The protease inhibitors are stored in x1000 stocks at -20°C, and added to lysis buffer prior to use. Chymostatin is dissolved in DMSO, and leupeptin is dissolved in water.
4. Samples of cells are taken for FACS analysis at before the addition of thymidine (asynchronous), after thymidine treatment, and after nocodazole treatment. The percentage of G2/M cells in nocodazole-arrested cells should be at least 80%.
5. The efficiency of cell lysis should be at least 50%, as estimated by protein concentration of cell lysates before and after centrifugation. The final protein concentration is usually in the range of 15-20 mg/ml.