Enrichment of Nuclear, Mitochondrial, Cytosolic and Membrane Proteins with FOCUS™ SubCell

Researchers are often focused on particular subcompartments of a cell, rather than the whole cell. For example, in recent years there has been an increase in mitochondrial and nuclear research, particularly with their involvement in cell death or apoptosis. In addition, the large number of proteins in animal tissue often causes problems in proteomic analysis due to a saturation of proteomic analysis tools, such as 2D electrophoresis. A key technique used in these cases is subcellular fractionation, the ability to separate the cellular proteins into defined, enriched subcellular fractions.

FOCUS™ SubCell *kit enables the fast and easy isolation of nuclear, enriched mitochondrial, membrane and cytosolic fractions from animal cells and tissue.* FOCUS™ SubCell *is suitable for cultured animal cells and is adaptable for animal tissues.*

AIM

To evaluate FOCUS™ SubCell kit in the fractionation of NIH3T3 cells into fractions enriched in nuclear, mitochondria, cytosolic membrane or soluble proteins. After separation, the fractions were probed by Western blot with fraction specific antibodies and the mitochondrial fractions were analyzed for intact outer and inner membrane and mitochondrial functionality.

METHOD

The general method is represented by the scheme shown in figure 1.

NIH3T3 ($14x10^6$) cells were harvested and washed in 1ml ice-cold SubCell Wash Buffer. The washed cellular pellet was resuspended in 500µl ice cold FOCUS™ SubCell Buffer-I and the cells were then mechanically lysed. 250µl 3X FOCUS™ SubCell Buffer-II was added to give a final 1X concentration and the lysed cells were centrifuged at 700*g* for 10 minutes to pellet the nuclei. The cytosolic supernatant was transferred to a fresh tube.

The nuclear pellet was further enriched by gradient centrifugation. The nuclei pellet was resuspended in 100µl FOCUS™ SubCell Buffer-III and overlaid onto 300µl FOCUS SubCell Buffer-IV in a microcentrifuge tube and then centrifuged at 1,000*g* for 10 minutes at 4°C. The supernatant was removed from the enriched nuclear pellet.

For the enrichment of mitochondria, the cytosolic supernatant was centrifuged at 12,000*g* for 15 minutes at 4°C to pellet the mitochondria. The cytosolic supernatant was removed to a fresh tube.

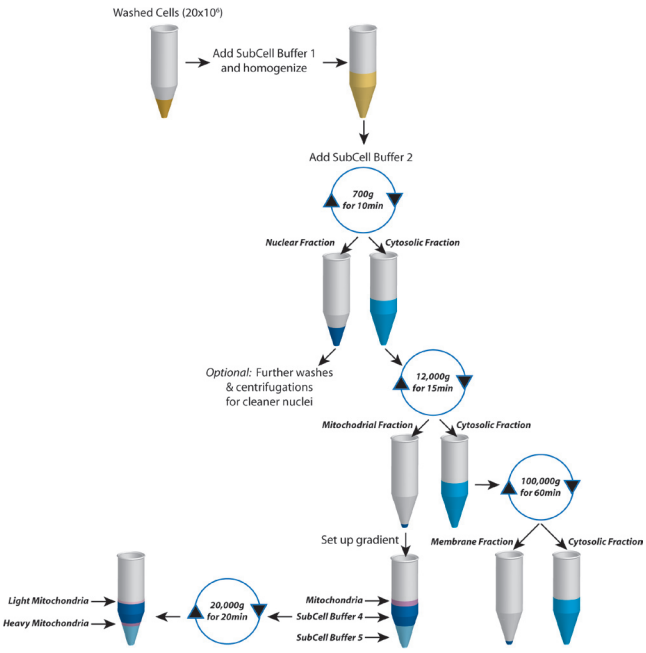

Figure 1:* FOCUS™ SubCell *fractionation scheme.

The mitochondria were further fractionated into enriched heavy and light mitochondria. To accomplish this, the mitochondria were resuspended in 100µl 1X FOCUS™ SubCell Buffer-II and floated on a gradient consisting of 200µl FOCUS™ SubCell Buffer-V under 200µl FOCUS™ SubCell Buffer-IV. The gradient was centrifuged at 20,000*g* for 20 minutes at 4°C. Two clear white bands were seen and the band at the interface was the heavy mitochondria and the upper band was the light mitochondria. The mitochondria were carefully removed to fresh tubes, washed with 1X FOCUS™ SubCell Buffer-II and resuspended in FOCUS™ Mitochondria Storage (FMS) Buffer.

Finally, the cytosolic membranes were removed from the soluble proteins by centrifuging the above supernatant at 100,000*g* for 1 hour at 4°C. The resulting pellet was the enriched membrane fraction.

4% total lysate and the enriched nuclear fraction, 15% of the mitochondrial (heavy and light) and cytosolic (membrane and soluble) fractions were resolved on a 4-20% SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane, which was probed with antibodies specific for: caveolin, a membrane associated protein; Oct-1, a nuclear transcription factor; Prohibitin, a mitochondrial marker; and β-Tubulin, a cytosolic protein.

To further analyze the mitochondria, a cytochrome C oxidase assay was used to determine the percentage of intact mitochondria, i.e. those with undamaged outer membranes (1). A second assay, the JC-1 (a cationic carbocyanine dye) uptake assay, was used to determine the integrity of the inner mitochondrial membrane and therefore the activity of the mitochondria (2).

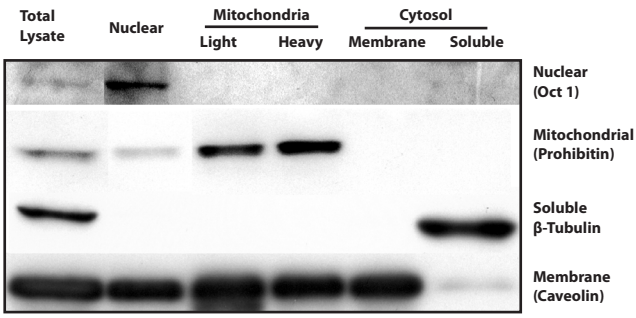

***Figure 2:* FOCUS™ SubCell fractionation of NIH3T3 cells.** 4% total lysate and nuclear fraction and 15% heavy and light mitochondria and cytosolic membrane and soluble protein fractions were resolved on a 4-20% SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane, which was probed with antibodies specific for Oct-1, a nuclear transcription factor; Prohibitin, a mitochondrial marker; β-tubulin, a cytosolic protein and caveolin, a membrane associated protein.

RESULTS AND DISCUSSION

Figure 2 depicts the result of the immunoblot, using antibodies for specific cellular compartments. Oct-1, a nuclear transcription factor, was detected as a major band in the enriched nuclear fraction and a weaker band of the same molecular weight in the total lysate. The nuclear protein was not visualized in the mitochondrial of cytosolic fractions. Prohibitin is an evolutionarily conserved protein located in the inner membrane of mitochondria and was strongly localized to both the heavy and light mitochondrial fractions. A small amount of prohibitin localized to the nuclear fraction, however several researchers have reported that prohibitin can have a nuclear location (3, 4). The distribution of soluble cytosolic proteins was determined with antibodies against β-tubulin and this was localized solely to the total lysate and the soluble cytosolic fraction. Finally, the distribution of the cellular membranes was detected by caveolin. Caveolin was found in all fractions except the soluble cytosolic fraction. Recent literature suggests that the location of caveolin is not restricted to the caveolae on cytosolic membranes, but can be found involved with the secretory pathway and mitochondria and can be localized to all intracellular membranes (5, 6). This data combined with the other immunoblot data demonstrates that the FOCUS™ SubCell kit can successfully fractionate cells into enriched nuclear, light and heavy mitochondrial and cytosolic membrane and soluble protein fractions. The cytochrome C oxidase assay was used to determine the percentage of undamaged mitochondria in the enriched heavy and light mitochondrial fractions. The heavy fraction consisted of 85% and the light fraction 79% undamaged, intact mitochondria. To ensure the enriched mitochondrial fractions were active, the JC-1 assay was used (figure 3). Figure 3 clearly demonstrates that the enriched heavy and light mitochondrial fraction retain the integrity of their inner membranes and their activity.

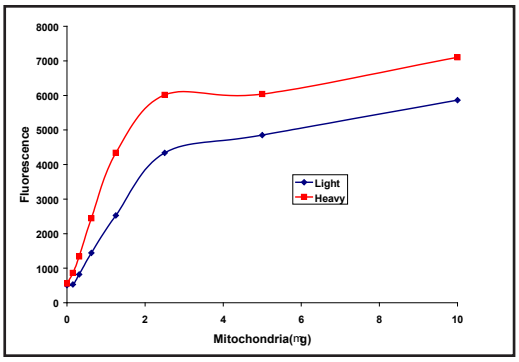

Figure 3: Activity of Mitochondria as determined with a JC-1 Assay.

The FOCUS™ SubCell kit is fast and convenient for the generation of enriched nuclear, mitochondrial (heavy and light), membrane and soluble cytosolic fractions. The enriched mitochondrial fraction contains a high percentage of intact mitochondria, which retain their activity.

ORDERING INFORMATION

Cat. #	Description/ Size
786-260	FOCUS™ SubCell Kit/ 1kit

REFERENCES

1. Hovius, R., Lambrechts, H., Nicolay, K. and de Kruijff, B. (1990) Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane. Biochim Biophys Acta. 1021:217-26.
2. Reers, M., Smith, T.W., Chen, L.B. (1991) J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry. 30:4480-6.
3. Fusaro, G., Dasgupta, P., Rastogi, S., Joshi, B. and Chellappan, S. (2003) Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem. 278:47853-61.
4. Witzmann, F.A., Li, J., Strother, W.N., McBride, W.J., Hunter, L., Crabb, D.W., Lumeng, L. and Li, T.K. (2003) Innate differences in protein expression in the nucleus accumbens and hippocampus of inbred alcohol-preferring and -nonpreferring rats. Proteomics. 3:1335-44.
5. Robenek, M.J., Schlattmann, K., Zimmer, K.P., Plenz, G., Troyer, D. and Robenek, H. (2003) Cholesterol transporter caveolin-1 transits the lipid bilayer during intracellular cycling. FASEB J. 17:1940-2.
6. Li, W.P., Liu, P., Pilcher, B.K. and Anderson, R.G. (2001) Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria. J Cell Sci. 114:1397-408.
7. Marjatta Son, Krishna Puttaparthi, Hibiki Kawamata, Bhagya Rejendran, Philip J. Boyer, Giovanni Manfredi, Jeffrey L. Eliott. Overexpression of CCS in G93A-S0D1 mice leads to accelerated neurological deficits with severe mitochondrial pathology. PNAS 2007 104: 6072

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