



FOCUS™ Global Fractionation

INTRODUCTION

FOCUS™ Global Fractionation kit is specifically designed for a simple, rapid and highly reproducible method for fractionation of various classes of cellular proteins. This kit allows fractionation of soluble and insoluble proteins. The insoluble protein fraction may be further fractionated into various classes of membrane proteins, such as mildly hydrophobic membrane proteins, highly hydrophobic trans-membrane proteins, and membrane proteins rich in cholesterol, glycolipids, and glycosyl-phosphatidylinositol (GPI).

Two separate protocols are offered, one for the extraction of mildly hydrophobic membrane proteins and highly hydrophobic trans-membrane proteins using temperature dependent phase partition [1, 2]. The insoluble protein fraction is mixed, homogenized or suspended in the “Membrane Extraction Buffer”. After a brief incubation at 35-37°C, the sample is centrifuged resulting in the separation of detergent rich and detergent poor layers. Proteins anchored to the membrane or proteins containing one or two trans-membrane regions are extracted into the detergent rich layer with the efficiency higher than 50%. Lower efficiency may be obtained with more complex membranes. Mildly hydrophobic proteins are extracted into the detergent poor upper phase.

The second protocol is specifically designed for the extraction of membrane proteins concentrated in caveolin rich membranes and lipid rafts, membrane regions enriched in cholesterol, glycolipids, and glycosyl-phosphatidylinositol (GPI), which are generally not soluble in a wide range of non-ionic detergents. Those proteins are believed to be involved in directing intracellular membrane traffic [3-5] and hence termed “Membrane Signal” proteins. The extraction method involves solubilization of the insoluble protein fraction in a proprietary formulation of non-ionic detergents, Membrane Signal Protein Extraction Buffer” (MSE Buffer). The MSE Buffer solubilizes and extracts the hydrophobic (membrane) proteins, leaving “Membrane Signal” proteins as detergent insoluble fraction (pellet).

It is important to appreciate that membrane proteins extracted in detergent rich phase (using temperature dependent phase partition) may also contain “membrane signal” proteins and vice versa. After fractionation, the protein fractions are suitable for further downstream applications. These fractions may be solubilized in FOCUS™ Protein Solubilization Buffer (supplied with the kit) for 2D gel analysis.

ITEMS SUPPLIED

Cat # 786-018

Soluble Protein Extraction Buffer	[SPE Buffer]	50ml
Membrane Protein Extraction Buffer	[MPE Buffer]	50ml
Membrane Signal Protein Extraction Buffer	[MSE Buffer]	50ml
FOCUS™ Protein Solubilization Buffer	[FPS Buffer]	25g
FOCUS™ Extraction Buffer	DILUENT-III	30ml

STORAGE CONDITION

Shipped at ambient temperature. After first use, store the kit components as specified on the labels. The kit components are stable for 12 months.

ITEMS NEEDED BUT NOT SUPPLIED

Centrifuge, centrifuge tubes, reducing agent, alkylation agents, carrier ampholytes, and protease inhibitor cocktail.

PREPARATION BEFORE USE

The kit is supplied with a FPS Buffer and an appropriate diluent. Allow the FPS Buffer to warm to room temperature before opening the bottle. Read the instruction on the bottle labels carefully before use. Just before use, hydrate an appropriate amount of the FPS Buffer. Add needed agents such as reducing agent, carrier ampholyte, and if necessary, an appropriate protease cocktail.



Protease Inhibition- if the inhibition of protease activity is required, add a cocktail of protease inhibitors in SPE Buffer, MPE Buffer or MSE Buffer to prevent protease activities during extraction procedure (see Related Products for protease inhibitor Protease Arrest™) MPE Buffer - 30 minutes before use, chill the MPE Buffer in ice bucket. Shake the bottle before use.

PROTOCOLS

A. Extraction of soluble and insoluble proteins:

- 1 For each 100mg of tissue, use approximately 0.4-0.5ml SPE Buffer.
For each 0.05mg of wet animal cell pellet, use approximately 0.4-0.5ml SPE Buffer.
Yeast - for 0.05ml wet yeast pellet, use 0.4ml SPE Buffer.
Bacteria- for 0.05ml wet *E. coli* pellet, use 0.4ml SPE Buffer.
Plant - use 2ml SPE Buffer for each 1gram plant tissue.

The sample to buffer volume ratio specified above is only a guide and may be adjusted depending on the scale of preparation.

2. Sonicate the suspension with an ultrasonic probe to break the cells and break down the genomic DNA. Sonication should be performed in cold (ice cold bath) and during sonication, care must be taken to prevent heating. Sonication should be performed with bursts of 20-30 seconds and chill the suspension between ultrasonic bursts.

Disruption of cells depends upon the nature of cells. *E. coli* cells require longer sonication than animal cells and tissues. Yeast cells require even much vigorous sonication. Addition of glass beads in the yeast cell suspension greatly facilitates disruption of yeast cells.

3. Centrifuge the homogenate at 20,000xg for 30 minutes at 4-5°C.
4. Use a pipettor to transfer the clear supernatant (without disturbing the pellet) to a new tube.
5. Suspend the pellet in 1/4 the volume of SPE Buffer used in the previous Step-1. Sonicate the pellet once briefly (30 seconds). Repeat Steps 3-4. Collect the clear supernatant and pool with the first supernatant.
6. Wash the pellet with 0.5ml SPE Buffer - Suspend the pellet in SPE Buffer, vortex for 60 seconds, and centrifuge at 20,000xg for 15 minutes at 4-5°C. Remove and discard the wash, the clear supernatant (OPTIONAL- wash may be saved or pooled).

The insoluble pellet containing membrane proteins can be processed by either one of the following protocols (B or C) of your choice.

B. Extraction of mildly hydrophobic and highly hydrophobic trans-membrane proteins:

1. Suspend the insoluble pellet in 0.3-0.5ml of pre-chilled Membrane Protein Extraction Buffer (MPE Buffer). Vortex the suspension 4-5 times, 60 seconds each. You may need to grind or sonicate briefly to break the pellet. Hold the suspension in ice-cold bath between vortexing. Incubate the suspension in ice-cold bath for 10 minutes.
2. Transfer the suspension to a 35-37°C heating block or incubator. Incubate for 30 minutes. Vortex the suspension periodically, 3-4 times 30-40 seconds each.
3. Centrifuge the tube at 18,000 x g for 5 minutes at room temperature.
4. Examine the tube carefully. You will notice two visible phases. Remove the top layer and transfer to a clean tube.
5. Collect the bottom detergent rich phase. Save and store the inter-phase and the pellet at -70°C until the analysis is complete.

Mark the Tubes (Fractions) as follows:

Top Layer	Mildly-hydrophobic Membrane Protein Fraction
Bottom Layer	Hydrophobic Trans-Membrane Protein Fraction

Processing “Membrane Protein Fraction” for IEF/2D Analysis - Determine protein concentration of the membrane protein fractions (use Non-Interfering Protein Assay, *G-Biosciences* Cat # 786-005). For IEF/2D gel analysis, use an appropriate amount of the Membrane Protein Fraction and process with Perfect-FOCUS kit (Cat# 786-124) or the method of your choice to remove salts and detergent. Process only as much protein as you need (i.e. 50-200µg protein /run). If using

Perfect-FOCUS kit, you will collect a protein pellet at the end of the protocol, suspend the pellet in FPS Buffer and run IEF/2D gel analysis.

NOTE- The Membrane Protein Fraction may be directly mixed with FPS Buffer for running IEF/2D analysis. If the Membrane Protein Fraction is sufficiently concentrated, you may mix 1 part Membrane Protein Fraction with >20 parts FPS Buffer without seriously diluting the FPS Buffer.

NOTE - *Hydrophilic proteins may also be processed for IEF/2D analysis using Perfect-FOCUS kit as described above for the membrane protein fraction.*

C. Extraction of Membrane Signal proteins:

1. Suspend the pellet (from part B) in 0.3-0.5ml of pre-chilled Membrane Signal Protein Extraction Buffer (MSE Buffer). Vortex the suspension 4-5 times, 60 seconds each. You may need to grind or sonicate briefly to break the pellet. Hold the suspension in ice-cold bath between vortexing. Incubate the suspension in ice-cold bath for 15 minutes.
2. Centrifuge the suspension at 20,000xg for 15 minutes at 4-5°C. Remove the clear supernatant, which is the detergent soluble membrane protein fraction.
3. Collect the pellet, which contains detergent insoluble “Membrane Signal” proteins. Suspend the pellet in 0.1-0.3ml FPS Buffer to solubilize the insoluble Signal Protein Fraction. Vortex the suspension 4-5 times, 60 seconds each. Incubate for 10-15 minutes at room temperature, vortex the suspension periodically. Centrifuge 18,000xg for 10 minutes at 20-25°C and collect the clear supernatant.
4. Re-extract any residual pellet with 1/3 the volume of FPS Buffer used in the previous step. Pool the supernatant with the previous supernatant.

Determine protein concentration (use Non-Interfering Protein Assay, Cat. # 786-005).

Make appropriate dilution in the FPS buffer before running IEF/2D gels.

Debris: Depending on the source and the nature of the sample, some insoluble materials (debris) may be recovered after the pellet solubilization steps. For solubilization of difficult-to-extract proteins, you may try the range of specialized FOCUS-Extraction Buffers we offer. Visit www.GBiosciences.com for more information or contact our Tech Support.

REFERENCES

1. *Towards the recovery of hydrophobic proteins on two-dimensional electrophoresis gels.* Santoni. V., Rabilloud. T., Dumas. P., Rouquie. D., Manbsion. M., Kieffer. S., Garin. J., and Rossignol. M. (1999) *Electrophoresis*, 20, 705-711.
2. *Preparation of mammalian plasma membranes by aqueous two-phase partition.* Morre, J.D., and Morre, D. M. (1989). *BioTechniques* 7(9), 946-958.
3. *The glycosyl-phosphatidylinositol anchor of membrane proteins,* Low. M.G. (1989) *Biochemica et Biophysica Acta*, 988, 427-454.
4. *Caveolin, a protein component of caveolae membrane coats.* Rothberg. K.G., Heuser. J.E., Donzell. W. C., Ying. Y., Glenney. J. R., and Anderson. R.G. W. (1992) *Cell*, 68, 673-682.
5. *Potocytosis: Sequestration and Transport of Small Molecules by Caveolae.* Anderson, R.G. W., Kamen. B. A., Rothberg. K. G., and Lacey. S. W. (1992) *Science*. 255,410-411.

RELATED PRODUCTS

1. **FOCUS Protease Arrest (Cat # 786-108F):** A protease cocktail specifically developed for sample preparation for 2D-studies and provides 95-98% inhibition of protease activity.
2. **FOCUS-Fast Silver (Cat # 786-240):** Silver staining for protein gels, compatible for MassSpec analysis.
3. **NI Protein Assay Kit (Cat # 786-005):** A protein assay that is free from interference of common laboratory agents including reducing agents, detergents, dyes, EDTA etc.
4. **RAPID-Stain (Cat # 786-31):** For staining protein in gels. RAPID-Stain only stains proteins, leaving clear background with high band visibility. Generally does not require de-staining.

LU 11.04.08-SA/MM/IA