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A Geno Technology, Inc. (USA) brand name

FOCUS™ Global Fractionation

(Cat. # 786-018)

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-phosphitidylinositol (GPI).

Two separate protocols are offered, one for the extraction of mildly hydrophobic membrane proteins and highly hydrophobic transmembrane 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 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 transmembrane 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-phosphitidylinositol (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 for downstream analysis, including 2D gel analysis.

ITEM(S) SUPPLIED (Cat. # 786-018)

Description	Size
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
DILUENT-III	30ml

STORAGE CONDITION

Shipped at ambient temperature. Store the kit components as specified on the labels. If stored correctly, the kit is stable for 12 months.

ADDITIONAL ITEMS REQUIRED

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

PREPARATION BEFORE USE

FOCUS™ Protein Solubilization Buffer

The kit is supplied with FPS Buffer and DILUENT-III. 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 to the SPE Buffer**,** MPE Buffer or MSE Buffer to prevent protease activities during extraction procedure.

MPE Buffer

30 minutes before use, chill the MPE Buffer in ice bucket. Shake the bottle before use.

PROTOCOLS

A. Extraction of Soluble & Insoluble Proteins

1. For each 100mg tissue, use approximately 0.4-0.5ml SPE Buffer
   For each 50µ wet animal cell pellet, use approximately 0.4-0.5ml SPE Buffer
   For each 50µl wet yeast pellet, use 0.4ml SPE Buffer
   For each 50µl wet *E. coli* pellet, use 0.4ml SPE Buffer
   For each 1g plant tissue, use 2ml SPE Buffer
   NOTE: *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.
3. 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.
4. Centrifuge the homogenate at 20,000 x g for 30 minutes at 4°C.
5. Use a pipette to transfer the clear supernatant (without disturbing the pellet) to a new tube.
6. Suspend the pellet in ¼ the volume of SPE Buffer used in Step 1. Sonicate the pellet once briefly (30 seconds). Repeat Steps 4-5. Collect the clear supernatant and pool with the first supernatant.
7. 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°C. Remove and discard the wash, the clear supernatant
   OPTIONAL: *Wash may be saved or pooled.*
8. 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 & Highly Hydrophobic Transmembrane Proteins

1. Suspend the insoluble pellet in 0.3-0.5ml of prechilled 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 37°C heating block or incubator. Incubate for 30 minutes. Vortex the suspension periodically, 3-4 times for 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 interphase and the pellet at -70°C until the analysis is complete.
6. Mark the Tubes as follows:
   - **Top Layer:** Mildly-hydrophobic Membrane Protein Fraction
   - **Bottom Layer**: Hydrophobic Trans-Membrane Protein Fraction
7. Determine protein concentration of the membrane protein fraction. We recommend using Non-Interfering Protein Assay (Cat. # 786-005).
8. See appendix for preparing fractions for 2D electrophoresis.

C. Extraction of Membrane Signal Proteins

1. Suspend the pellet (from part A) in 0.3-0.5ml of prechilled Membrane Signal Protein Extraction Buffer (MSE Buffer). Vortex the suspension 4-5 times, 60 seconds each time. You may need to grind or sonicate briefly to break the pellet. Hold the suspension in ice-cold bath between vortexing.
2. Incubate the suspension in ice-cold bath for 15 minutes.
3. 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.
4. Collect the pellet, which contains detergent insoluble "Membrane Signal" protein and suspend the pellet in 0.1-0.3ml FPS Buffer to solubilize the insoluble Signal Protein Fraction.
5. Vortex the suspension 4-5 times, 60 seconds each. Incubate for 10-15 minutes at room temperature, vortexing the suspension periodically. Centrifuge 18,000xg for 10 minutes at 20-25°C and collect the clear supernatant.
6. Re-extract any residual pellet with 1/3 the volume of FPS Buffer used in the step 4. Pool the supernatant with the previous supernatant.
7. Determine protein concentration of the membrane protein fraction. We recommend using Non-Interfering Protein Assay (Cat. # 786-005).
8. See appendix for preparing fractions for 2D electrophoresis.
   NOTE: *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.*

APPENDIX: PROCESSING FRACTIONS FOR IEF/2D ANALYSIS

The following procedure uses G-Biosciences Perfect-FOCUS kit (Cat. # 786-124). A different method of your choice to remove salts and detergent may be used.
For IEF/2D gel analysis, use an appropriate amount of the membrane Protein Fraction, process only as much protein as you need (i.e. 50-200µg protein /run).

Important Notes

- Perform the entire procedure at 4-5° C (ice bucket) unless specified otherwise. Various incubation conditions must be strictly followed. Use 1.5ml microfuge tubes for processing protein samples. 0.5ml microfuge tubes are not recommended.
- Always position the microfuge-tubes in the centrifuge in the same orientation, i.e. cap-hinge facing outward. This will allow the pellet to remain glued to the same side of the tube during centrifugation and washing steps and minimize the loss of the protein pellets.
- Chill OrgoSol Buffer at –20°C for ~1hr or more before use

Protocol

1. Transfer 1-100µl protein solution (containing 1-100µg protein per sample) into a 1.5ml microfuge tube.
2. Add 300µl UPPA-I and mix well. Incubate at 4-5°C (ice-bucket) for 15 minutes.
3. Add 300µl UPPA-II in to the mixture of protein and UPPA-I, then vortex the tube.
   NOTE*: For larger sample size, use 3 volumes each of UPPA-I and UPPA-II for each volume of sample. See Appendix: Processing Large Samples.*
4. Centrifuge the tube at 15,000x g for 5 minutes to form a tight protein pellet.
5. As soon as the centrifuge stops, remove the tube from the centrifuge.
   NOTE*: Pellets should not be allowed to diffuse after centrifugation is complete.*
6. Carefully, without disturbing the pellet, use a pipette tip to remove & discard the entire supernatant.
7. Carefully reposition the tube in the centrifuge as before, i.e. cap-hinge facing out-ward. Centrifuge the tube again for 30 seconds. Use a pipette tip to remove the remaining supernatant.
8. Add 40µl of FOCUS-Wash on top of the pellet. Carefully reposition the tube in the centrifuge as before, i.e. cap-hinge facing out-ward.
   NOTE: *For larger sample size, add Wash 3-4 x times the size of the pellet.*
9. Centrifuge the tube again for 5 minutes. Use a pipette tip to remove and discard the Wash.
10. Add 25µl of pure water on top of the pellet.
    NOTE: *For large sample size, add water just enough to cover the pellet, i.e. a volume equal to the size of the pellet.*
11. Vortex the tube.
    NOTE: *Pellets do not dissolve in water*.

12. Add 1ml OrgoSol Buffer, pre-chilled at –20°C, and 5µl SEED.
    NOTE: *For large samples size, for each 0.1-0.3ml protein solution add 1ml OrgoSol Buffer. In addition, OrgoSol Buffer must be at least 10 fold in excess of the water added in Step 10.*
13. Vortex to suspend the pellet. It is important that the pellet is fully suspended in OrgoSol Buffer.
    NOTE: *Pellets do not dissolve in OrgoSol Buffer.*
14. Incubate the tube at –20°C for 30 minutes. Periodically vortex the tube, 20-30 seconds vortex each burst.
15. Centrifuge at 15,000xg for 5 minutes to form a tight pellet.
16. Remove and discard the supernatant. You will notice a white pellet in the tube. Air-dry the pellet. On drying, the white pellet will turn translucent.
    NOTE*: Do not over dry the pellets - parched dry pellets may be difficult to dissolve*.
17. Add an appropriate volume of hydrated FPS Buffer to suspend the pellet. Vortex the tube for 30 seconds. Incubate and vortex periodically until pellet is dissolved. Centrifuge and collect a clear protein solution and load on IEF gel.
    NOTE*: The Membrane Protein Fraction may be directly mixed with hydrated 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 hydrated FPS Buffer without seriously diluting the FPS Buffer.*
    NOTE*: Hydrophilic proteins may also be processed for IEF/2D analysis using PrefectFOCUS kit as described above for the membrane protein fraction.*

REFERENCES

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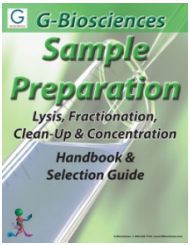

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