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A Geno Technology, Inc. (USA) brand name

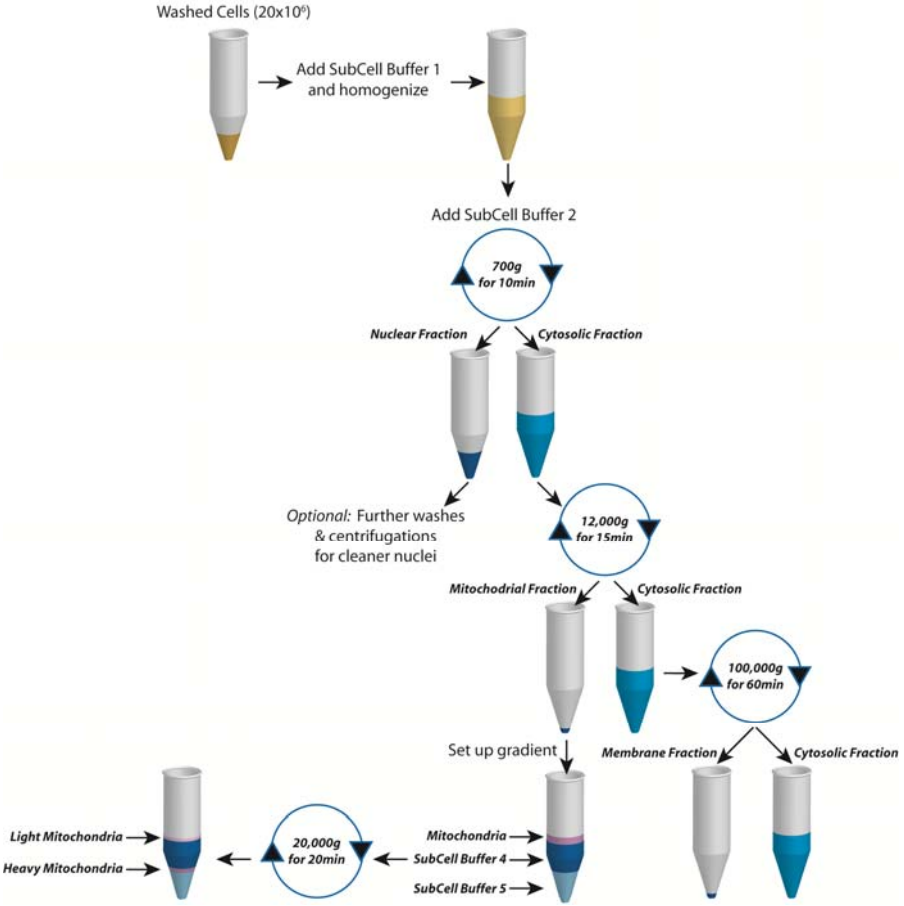
FOCUS™ SubCell

For the Enrichment of Subcellular Fractions

(Cat. # 786-260)

INTRODUCTION

FOCUS™ SubCell kit enables the fast and easy enrichment of nuclear, mitochondrial, membrane and cytosolic fractions from animal cells. The mitochondrial fraction can be subsequently separated into heavy and light fractions by gradient centrifugation. An additional step is included to minimize contaminations of the nuclear fraction by cytoplasmic elements (see schematic on the right). The majority of mitochondria, isolated with this kit, contain intact inner and outer membranes. FOCUS™ SubCell is suitable for cultured animal cells and can be adapted for animal tissues.



ITEM(S) SUPPLIED (Cat. # 786-260)

Description	Size
SubCell Buffer-I	60ml
SubCell Buffer-II [3X]	30ml
SubCell Buffer-III	25ml
SubCell Buffer-IV	25ml
SubCell Buffer-V	15ml
Mitochondria Storage Buffer	10ml
Mitochondria Storage Component	1 vial

STORAGE CONDITION

The kit is shipped at ambient temperature. After receiving store all the kit components at 4°C except store Mitochondria Storage Component at -20°C. The kit is stable for one year when stored unopened. Use aseptic techniques when handling the reagent solutions.

ITEMS NEEDED BUT NOT SUPPLIED

Syringes and 20 gauge needles or Wheaton Dounce homogenizer, centrifuge and centrifuge tubes. Optional reagents: Delipidated BSA, Trypsin, PBS and protease inhibitor cocktail.

PREPARATION BEFORE USE

- All buffers should be kept ice cold.
- Dilute appropriate volume of 3X SubCell Buffer-II to 1X with SubCell Buffer-I as needed (e.g. mix 2ml SubCell Buffer-I with 1ml SubCell Buffer-II).
  NOTE: *Do not dilute all 3X SubCell Buffer-II as some steps require the 3X concentrated SubCell Buffer II.*
- All centrifugation steps should be performed at 4°C.
- **Preparation of Working Mitochondria Storage Buffer**: Pipette 0.5ml Mitochondria Storage Buffer to Mitochondria Storage Component vial. Pipette up and down a few times to dissolve all components completely. Transfer the solution of Mitochondria Storage Component to Mitochondria Storage Buffer bottle and mix well. The Working Mitochondria Storage Buffer should be kept frozen for long-term use.

PROTOCOLS

A. Subcellular Fractionation from Animal Cells

This protocol is for processing 20x10^6 cells (or ~100µl wet cell pellet). It can be scaled up and down accordingly.

OPTIONAL: *Add appropriate protease inhibitor cocktail (e.g. G-Biosciences' ProteaseArrest, Cat. # 786-108) to SubCell Buffer-I immediately prior to use.*

1. Use fresh cells only. Pellet the harvested cells by centrifugation at ~800 x *g* for 1 minute. Carefully remove and discard the supernatant.

   OPTIONAL: *Wash the cell pellet with 1ml ice cold PBS, centrifuge it as above and discard the supernatant*.
2. Add 500µl of ice cold SubCell Buffer-I. Gently vortex to suspend the cells and incubate on ice for 10 minutes.
3. Perform this lysis step on ice. Using a narrow opening (20 gauge) syringe needle, gently pull the suspension up and down 10-30 times. Alternatively, transfer cell suspension to ice cold Dounce homogenizer. Homogenize the cells on ice using tight pestle. Perform 5 to 20 strokes to lyse the cells effectively. Transfer the lysate to a microcentrifuge tube. Rinse Dounce homogenizer with 200µl of SubCell Buffer-I and pool together. Invert the tube several times to mix.

   NOTE: *To check the cell lysis efficiency, spot 5µl of cell lysate onto a glass slide, add cover slip and view under a phase-contrast microscope. Pulling times or strokes in the above lysis step are only guidelines. Mechanical force needed to lyse cells depends on cell types, the total number of the cells and hands on experience. It is also dependent on the ultimate goal of lysis. Insufficient force will not lyse all the cells, but will achieve cleaner mitochondrial fractions with less nuclear contamination. Excess force may damage some nuclei, but a cleaner nuclear fraction will be obtained.*
4. Add 250µl 3X SubCell Buffer-II (350µl if Dounce homogenizer is used) and mix by inverting. This generates a 1X final concentration of SubCell Buffer-II.
5. Centrifuge the tube at 700x *g* for 10 minutes to pellet the nuclei. Transfer the supernatant to a new tube.

   NOTE: *For further cleaning the nuclear fraction, see Section B.*
6. Centrifuge supernatant at 12,000x *g* for 15 minutes. Transfer the supernatant (post mitochondria) to a new tube for further processing. The pellet contains mitochondria.

   NOTE: *To fractionate light and heavy mitochondria, and obtain more purified mitochondrial fractions, see Section C. For a crude mitochondrial fraction, continue with step 7.*
7. Add 500µl 1X SubCell Buffer-II to the pellet, and centrifuge again at 12,000 x *g* for 5 minutes. Discard the supernatant.
8. Resuspend the mitochondrial pellet with 50-100µl Working Mitochondria Storage Buffer and keep the suspension on ice before downstream processing. The

9. **Enrichment of other cell organelles:** The post mitochondria supernatant from step 6 can be further fractionated using a variety of gradient and differential centrifugations. For example, centrifugations of the post mitochondrial supernatant at 100,000x g for 60 minutes will sediment cellular membranes. The resulting pellet is an enriched cytosolic membrane fraction and the supernatant is soluble cytosolic fraction. This cytosolic fraction may be used for further fractionation.

B. Cleaning of the Nuclear Fraction

1. Resuspend the nuclear pellet in 300µl SubCell Buffer-III. Using a sharp pipette tip, remove the sticky lump if any. The lump is formed from dead cells and some lysed nuclei.
2. Centrifuge the tube at 700x *g* for 5 minutes and discard the supernatant. The pellet containing nuclei is clean enough for most purposes. If further cleaning required, go to next step.
3. Pipette 300µl SubCell Buffer-IV to a 1.5ml centrifuge tube. Resuspend the nuclear pellet in 100µl SubCell Buffer-III. Carefully overlay the nuclear suspension on the surface of SubCell Buffer-IV.
4. Centrifuge the tube at 1,000x *g* for 10 minutes. Remove the supernatant and collect the very clean nuclear pellet in the tube.

C. Fractionation of light and heavy mitochondria by gradient cushion

1. Suspend the mitochondrial pellet in 100µl 1X SubCell Buffer-II.
2. Make a step gradient by adding 200µl SubCell Buffer-V to a centrifuge tube and then overlaying with 200µl SubCell Buffer-IV. Gently float the mitochondrial suspension on the surface of the step gradient.
3. Centrifuge the gradient at 20,000x g for 20 minutes. Observe the two white bands. The band at the interface of SubCell Buffer-IV and V is the heavy mitochondria fraction. The band above the heavy mitochondria band is the light mitochondria fraction.
4. Carefully remove each band to a separate tube. Dilute the mitochondrial suspensions with equal volume of 1X SubCell Buffer-II. Centrifuge the tubes at 12,000x g for 15 minutes and discard the supernatant.
5. Suspend the mitochondrial pellets with 30-50µl Working Mitochondria Storage Buffer and keep the suspensions on ice before downstream processing. The suspensions may be stored on ice up to 48 hours. Freezing and thawing may compromise mitochondria integrity.

D. Subcellular Fractionation from Soft Tissues (liver or brain)

OPTIONAL: *Delipidated BSA can be added to 1X SubCell Buffer-II to the concentration of 2mg/ml for removing free fatty acids prior to processing. An appropriate amount of protease inhibitor cocktail can be added to the 1X SubCell Buffer-II just before use.*

1. Use a fresh tissue sample (obtained within one hour of sacrifice) kept on ice. Do not freeze.
2. Weigh approximately 50-100mg tissue. On a cooled glass plate, with the aid of a scalpel, mince the tissue into very small pieces.
3. Perform this step on ice. Transfer the minced tissue to an ice-cold Dounce tissue homogenizer. Add 10 volumes of 1X SubCell Buffer-II and using a loose-fitting pestle, disaggregate the tissue with 5-10 strokes or until the tissue sample is completely homogenized. Using a tight-fitting pestle, release the nuclei with 8-10 strokes. Do not twist the pestle as nuclei shearing may occur.
4. Stand on ice for 2 minutes. Transfer the homogenate to a centrifuge tube and leave large chunks of tissue fragments in the homogenizer to be discarded. Centrifuge the sample at 700x *g* for 5 minutes to pellet the nuclei.
   NOTE: *For further cleaning the nuclear fraction, see Section B.*
5. Carefully transfer the supernatant into a new tube. Centrifuge supernatant at 12,000x*g* for 10 minutes.
6. Transfer the supernatant (post mitochondria) to a new tube and resuspend the pellet in 10 volumes of 1X SubCell Buffer II without BSA.
7. Centrifuge as in step 4. Repeat step 5 and remove the supernatant. The pellet contains mitochondria.
   NOTE: *To fractionate light and heavy mitochondria, and obtain more purified mitochondrial fractions, see Section C. For a crude mitochondrial fraction, continue with step 8.*
8. Suspend the mitochondrial pellet in Working Mitochondria Storage Buffer (approximately 50µl for pellet from ~100mg tissue) and keep the suspension on ice before downstream processing. The suspension may be stored on ice for up to 48 hours. Freezing and thawing may compromise mitochondria integrity.
9. **Enrichment of other cell organelles:** The post mitochondria supernatant from step 6 can be further fractionated using a variety of gradient and differential centrifugations. For example, centrifugations of the post mitochondrial supernatant at 100,000xg for 60 minutes will sediment cellular membranes. The resulting pellet is an enriched cytosolic membrane fraction and the supernatant is soluble cytosolic fraction. This cytosolic fraction may be used for further fractionation.

E. Subcellular Fractionation from hard tissues (skeletal or heart muscle)

NOTE: *For facilitating homogenization of the hard tissue, 0.25mg/ml Trypsin should be added to 1X SubCell Buffer-II. A concentrated BSA solution is needed to quench the proteolytic reaction after Trypsin treatment.*

1. Use a fresh tissue sample (obtained within one hour of sacrifice) kept on ice. Do not freeze.
2. Weigh approximately 50-100mg tissue. On a cooled glass plate, with the aid of a scalpel, mince the tissue into very small pieces.
3. Suspend the sample with 8 volumes of 1X SubCell Buffer-II containing 0.25mg/ml trypsin in a 2ml centrifuge tube.
4. Incubate on ice for 3 minutes and then spin down the tissue for a few seconds in the centrifuge.
5. Remove the supernatant by aspiration and add 8 volumes of 1X SubCell Buffer-II containing 0.25mg/ml Trypsin. Incubate on ice for 20 minutes.
6. Add BSA Solution to a final concentration of 10mg/ml and mix. Spin down the tissue at 1,000 x g for 5-10 seconds in the centrifuge.
7. Remove the supernatant by aspiration. Wash the pellet with 8 volumes of 1X SubCell Buffer-II without Trypsin, and spin down the tissue for a few seconds in the centrifuge.
8. Remove the supernatant by aspiration and add 8 volumes of the 1X SubCell Buffer-II without Trypsin.
9. Transfer the suspension to an ice-cold Dounce tissue homogenizer and using a loose-fitting pestle, disaggregate the tissue with 5-15 strokes or until the tissue sample is completely homogenized. Using a tight-fitting pestle, release the nuclei with 8-10 strokes. Do not twist the pestle as nuclei shearing may occur.
10. Stand on ice for 2 minutes. Transfer the homogenate to a centrifuge tube and leave large chunks of tissue fragments in the homogenizer to be discarded. Centrifuge the lysate at 700x *g* for 5 minutes to pellet the nuclei.
    NOTE: *For further cleaning the nuclear fraction, see Section B.*
11. Transfer the supernatant to a new tube. Centrifuge it at 12,000x*g* for 10 minutes. Transfer the supernatant (post mitochondria) to a new tube. The pellet contains mitochondria.
    NOTE: *To fractionate light and heavy mitochondria, and obtain more purified mitochondrial fractions, see Section C. For a crude mitochondrial fraction, continue with step 12.*
12. Suspend the mitochondrial pellet in Working Mitochondria Storage Buffer (approximately 50µl for pellet from ~100mg tissue) and keep the suspension on ice before downstream processing. The suspension may be stored on ice for up to 48 hours. Freezing and thawing may compromise mitochondria integrity.
13. **Enrichment of other cell organelles:** The post mitochondria supernatant from step 11 can be further fractionated using a variety of gradient and differential centrifugations. For example, centrifugations of the post mitochondrial

supernatant at 100,000x g for 60 minutes will sediment cellular membranes. The resulting pellet is an enriched cytosolic membrane fraction and the supernatant is soluble cytosolic fraction. This cytosolic fraction may be used for further fractionation.

F. Solubilization of the sub-cell fractions:

The fractionated cell organelles (nuclei or mitochondria) may be solubilized in any suitable buffer consistent with downstream procedures. For IEF/2D gel electrophoresis, the enriched fractions may be solubilized in a chaotropic extraction buffers. G-Biosciences offers a wide selection of buffers and reagents for IEF/2D gel electrophoresis. FOCUS/Extraction Buffer-VI (Cat # 786-233) is suitable for solubilization of all pellet fractions. The soluble cytosolic fraction can be concentrated using *Perfect*-FOCUS™ kit (Cat# 786-124). For more information visit our website at www.GBiosciences.com

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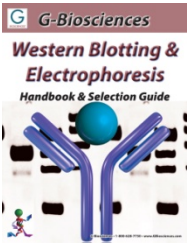

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