WSE-7422 EzSubcell Fraction

November 9, 2012: The 1st version

1. Instructions for ensuring the safe use of this Product

Please read this Instruction Manual thoroughly to ensure the safe use of this Product. Do not use the Product unless you fully understand the contents of this Instruction Manual. This Instruction Manual describes the method and purpose of use intended for the Product. Please refrain from using the Product for the purpose and method not described in this Instruction Manual. If the Product should be used for the purpose and method not described in this Instruction Manual, the user will be responsible for any necessary safety measures and any unpredictable consequences.
Also, please read thoroughly the instruction manuals for the devices to be used with the Product.

2. Purpose of use

The EzSubcell Fraction is a kit to isolate and fractionate mitochondria, nucleus and cytoplasm (including other organelles) from mammalian cells. This kit can be used either with detergent or without detergent according to the purpose. With the detergent, cell breakage is easy. Fractions can be used for electrophoresis, immunoprecipitation, ELISA, and other biochemical/immunological analysis. On the other hand, fractions prepared without using detergent can be used for enzyme activity experiment, bioactive experiment, etc.

3. Product configuration

Name	Content	Piece(s)	Storage
Fraction buffer 1	50 mL	1	2 -8 °C
Fraction buffer 2	50 mL	1	2 -8 °C
RIPA Lysis buffer	20 mL	1	2 -8 °C
Detergent mix	1 mL	1	- 20 °C
Protease Inhibitor	700 µL	1	- 20 °C

4. Composition

Name	Principal elements
Fraction buffer 1	Detergent, buffer solution
Fraction buffer 2	Detergent, buffer solution
RIPA Lysis buffer	Detergent, buffer solution
Detergent mix	50 × concentration, Detergent, buffer solution
Protease Inhibitor	100 × concentration, aprotinin, pepstatin A, leupeptin, DMSO

The Product does not contain any poisonous and deleterious substances under the Poisonous and Deleterious Substances Control Act or any substances that are to be notified and exceed the exemption amount under the Industrial Safety and Health Act. Matters that are to be notified and exceed the exemption amount for the specified chemicals under the PRTR Act are partially included. Please download the MSDS of the Product from our website (http://www.atto.co.jp/) to check details.

5. Storage

- Fraction buffer 1, 2 and RIPA Lysis buffer (**R**adio-**I**mmuno**p**recipitation **a**ssay Lysis buffer) shall be kept in cold storage (2 - 8 °C). Unless it is opened, it will stay stable until the expiration date.
- Detergent mix and Protease Inhibitor shall be kept in frozen storage (-20 °C). Unless it is opened, it will stay stable until the expiration date.

6. Disposal

Please comply with the disposal method of your organization when disposing reagents.

7. Required items other than the Product

- Ice cold PBS buffer
- 2mL micro-centrifuge tube
- Vortex mixer
- Microscope: to check the cell breakage state
- 0.5 - 1.0% Trypan Blue / PBS
- Pipetman, tip
- Cooled centrifuge (Micro-centrifuge tube)
- Syringe with a 25G needle: This is necessary when using the product without detergent. This can be substituted by a Dounce-type homogenizer.

8. Precaution on use

- The Product is delivered by refrigerated goods transportation. Please store the product under the temperature suitable for each reagent after receiving.
- Please ice-cool all the reagents before you start the experiment. Also, please perform the experiment and operation on the ice or at a low temperature.
- Some types of cells are easily broken and others are not. The breakage condition varies depending on cell types. Please check whether the cell is sufficiently broken in the cell breakage step by using a microscope or other appropriate tool during the experiment.
- As the Protease Inhibitor includes DMSO, it may be frozen at a low temperature. Please thaw it completely at room temperature before use.
- Please increase or decrease the amount for adding the Protease Inhibitor or add AEBSF, Bestatin or other inhibitors as needed.

9. How to use

I. Preparation of cells

1. Prepare cell suspension collected by the trypsin treatment or other appropriate method.

*For Hela, the number of cells per dish of 10cm is 5 - 12 × 10^6. Prepare the number of cells within the range of 1 - 2 × 10^7 cells.

2. Centrifuge the cell suspension at 200 × g for 3 - 5 minutes.
3. Carefully remove and dispose of the centrifugal supernatant. Then, add 10 mL of ice cold PBS to the cells (centrifugal sediment) and suspend them well. Collect a part of the cell suspension and count the number of cells.
4. Centrifuge the cell suspension at 200 × g for 3 - 5 minutes.
5. Carefully remove and dispose of the centrifugal supernatant. Then, add the appropriate amount of ice cold PBS to the cells (centrifuge sediment) until the number of cells becomes 1 - 2 × 10^7 cells/mL.
6. Collect 1mL of the cell suspension and transfer it into the 2mL micro-centrifuge tube.
7. Centrifuge the cell suspension at 200 × g for 3 - 5 minutes.
8. Remove and dispose of the centrifugal supernatant, and the cell preparation will be completed. Until the next procedure starts, place them on the ice at rest.

*Perform the cell fraction and the extraction operation promptly after the cell preparation.

II. With detergent

(1) Preparation of reagents

1. Mix the necessary Protease Inhibitor and Detergent mix in accordance with the table below. Place the solution after mixing on the ice at rest until it is used.

	Amount required for 1 sample	Protease Inhibitor (blue lid)	Detergent mix (red lid)
With detergent			
Fraction buffer 1	0.8 mL	8 μL	16 μL
Fraction buffer 2	0.8 mL	-	-
RIPA Lysis buffer	0.4 mL	5 μL	-

2. Mix 200 μL of Fraction buffer I and II respectively to prepare the Fraction buffer mix. Place the solution after mixing on the ice at rest until it is used.

*Fraction buffer mix is used for washing or suspension of isolated nuclear fractions and mitochondria fractions. Fraction buffer mix is not necessary when performing washing or suspension with the solution suitable for the purpose of experiment to be conducted after the fraction.

*In the case that the number of cells is small (1 × 10^7cells per sample or less), please decrease the amount of Fraction buffer 1 and 2 as needed before the cell fraction and extraction operation.

(2) Cell fraction and extraction operation

1. Add 0.8 mL of ice cold fraction buffer 1 (containing Protease Inhibitor and Detergent mix) to the cells (centrifugal sediment) in the 2mL micro-centrifuge tube.
2. Mix them with a vortex mixer for 5 seconds.
3. Incubate them on the ice for 10 minutes.

*Please flop them upside down two or three times every one - two minutes during incubation.

4. Set the scale mark of Pipetman (for 1000 μL) to 1mL. Then, with the tip end slightly touching the bottom of the tube, aspirate the entire amount of cell homogenate and dispense it at once. By using this method, perform pipetting of cell homogenate 20 - 30 times.

* Please perform the pipetting operation on the ice.

*Vortex does not break cells. Cells are broken by the pressure generated when the cells go through the tip end by the pipetting operation.

*Avoid bubbling as much as possible and perform pipetting as strongly as possible.

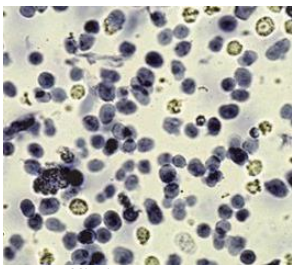
5. Collect a part of homogenate and stain with the Trypan Blue. Then, check with a microscope whether almost 100% of the cells are broken.

*Please check whether almost all the cells are stained with the Trypan Blue.

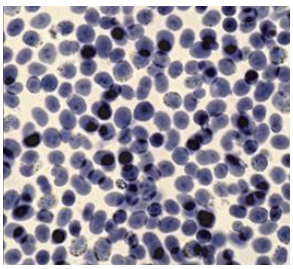
*Please check if individual cells have no membrane and they have the nucleus exposed.

*In the case that the cell breakage is not sufficient, perform additional pipetting 5 times or more. In the case that the cells are not broken by pipetting, homogenize the homogenate 10 - 20 times with a syringe with a 25G needle. During homogenizing, repeat the following operation: suck the entire amount of cell fluid and immediately discharge.

*Syringe can be substituted by a Dounce-type homogenizer.



Insufficient breakage



Good breakage

6. Add 0.8 mL of ice cold Fraction buffer 2 and flop them upside down.
7. Centrifuge the homogenate at 700 × g for 10 minutes.
8. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal sediment is equivalent to the nuclear fractions. Place the centrifugal sediment on the ice at rest until it is used.

[Option (1) (Washing of nucleus)]
Gently loosen the nuclear fractions (centrifugal sediment) described in Item 8 above, by tapping. Add 200 μL of ice cold Fraction buffer mix and suspend them. Centrifuge the suspension at 700 × g for 5 minutes. Remove and dispose of the centrifugal supernatant and collect the nuclear fractions (centrifugal sediment).
[Option (2) (Purification of nucleus)]
Gently loosen the nuclear fractions (centrifugal sediment) described in Item 8 above, by tapping. Add 500 μL of ice cold Fraction buffer mix (separate from the specified amount) and suspend them.

Transfer the nuclear suspension to the 2mL micro-centrifuge tube. Add 1 mL of PBS containing 0.88M Sucrose from under the nuclear suspension by using the Pipetman to make double layer. With the edge of Pipetman tip slightly (0.5 - 1 mm) above the bottom of the tube, gently add the PBS containing 0.88M Sucrose to make the double layer. Please be careful not to put air in the tube when making the double layer. Centrifuge at 1,200 × g for 10 minutes. Remove and dispose the centrifugal supernatant and collect the nuclear fractions (centrifugal sediment).

9. Centrifuge the centrifugal supernatant described in Item 8 above, at 12,000 × g for 10 minutes.
10. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal supernatant is equivalent to the cytoplasm fractions. The centrifugal sediment is equivalent to the mitochondria fractions.

[Option (1) (Washing of mitochondria)]
Gently loosen the mitochondria fractions (centrifugal sediment) described in Item 10 above, by tapping. Add 200 μL of ice cold Fraction buffer mix and suspend them. Centrifuge the suspension at 12,000 × g for 5 minutes. Remove and dispose of the centrifugal supernatant, and collect the mitochondria fractions.
[Option (2) (Purification of microsome)]
Centrifuge the cytoplasm fractions (centrifugal supernatant) described in Item 10 above, at 100,000 × g for 60 minutes. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal sediment is equivalent to the microsomal fractions and the centrifugal supernatant is equivalent to the cytoplasm fractions.

III. Without detergent
(1) Preparation of reagents
1. Mix the necessary Protease Inhibitor in accordance with the table below. Place the solution after mixing on the ice at rest until it is used.

	Amount required for 1 sample	Protease Inhibitor (blue lid)	Detergent mix (red lid)
Without detergent			
Fraction buffer 1	0.8 mL	8 μL	-
Fraction buffer 2	0.8 mL	-	-
RIPA Lysis buffer	0.4 mL	4 μL	-

2. Mix 200 μL of Fraction buffer 1 and 2 respectively to prepare the Fraction buffer mix. Place the solution after mixing on the ice at rest until it is used.

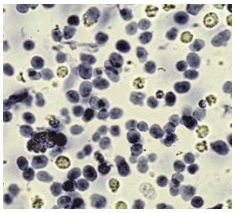
*Fraction buffer mix is used for washing or suspension of isolated nuclear fractions and mitochondria fractions. Fraction buffer mix is not necessary when performing washing or suspension with the solution suitable for the purpose of experiment to be conducted after the fraction.
*In the case that the number of cells is small (1 × 10^7cells per sample or less), please decrease the amount of Fraction buffer 1 and 2 as needed before the cell fraction and extraction operation.

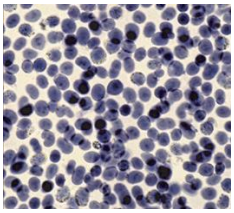
(2) Cell fraction and extraction operation
1. Add the Fraction buffer 1, which is added 0.8 mL of ice cold Protease Inhibitor, to the cells (centrifugal sediment) in the 2mL micro-centrifuge tube.
2. Mix them with a vortex mixer for 5 seconds.
3. Incubate them on the ice for 10 minutes.

*Please flop them upside down two or three times every one - two minutes during incubation.
4. Homogenize the homogenate 15 - 30 times by using a syringe with a 25G needle. During homogenizing, repeat the following operation: suck the entire amount of cell fluid and immediately discharge.

*Avoid bubbling as much as possible and perform pipetting as strongly as possible.
*Syringe can be substituted by a Dounce-type homogenizer.
5. Collect a part of homogenate and stain with the Trypan Blue. Then, check with a microscope if almost 100% of the cells are broken.

*Please check if almost all the cells are stained with the Trypan Blue.
*Please check if individual cells have no membrane or if they have nucleus exposed.
*In the case that the cell breakage is not sufficient, perform additional homogenate 5 times or more.


Insufficient breakage


Good breakage

6. Add 0.8 mL of ice cold Fraction buffer 2 and flop them upside down.
7. Centrifuge the homogenate at 700 × g for 10 minutes.
8. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal sediment is equivalent to the nuclear fractions. Place the centrifugal sediment on the ice at rest until it is used.

[Option (1) (Washing of nucleus)]
Gently loosen the nuclear fractions (centrifugal sediment) described in Item 8 above, by tapping. Add 200 μL of ice cold Fraction buffer mix and suspend them. Centrifuge the suspension at 700 × g for 5 minutes. Remove and dispose of the centrifugal supernatant and collect the nuclear fractions (centrifugal sediment).
[Option (2) (Purification of nucleus)]
Gently loosen the nuclear fractions (centrifugal sediment) by tapping. Add 200 μL of ice cold Fraction buffer mix and suspend them. Transfer the nuclear suspension in the 2mL micro-centrifuge tube. Add 1 mL of PBS containing 0.88M Sucrose from under the nuclear suspension by using the Pipetman to make double layer. With the edge of Pipetman tip slightly (0.5 - 1 mm) above the bottom of the tube, gently add the PBS containing 0.88M Sucrose to make the double layer. Centrifuge at 1,200 × g for 10 minutes. Remove and dispose the centrifugal supernatant and collect the nuclear fractions (centrifugal sediment).

9. Centrifuge the centrifugal supernatant described in Item 8 above, at 12,000 × g for 10 minutes.
10. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal supernatant is equivalent to the cytoplasm fractions and the centrifugal sediment is equivalent to the mitochondria fractions.

[Option (1) (Washing of mitochondria)]
Gently loosen the mitochondria fractions (centrifugal sediment) described in Item 10 above, by tapping. Add 200 μL of ice cold Fraction buffer mix and suspend them. Centrifuge the suspension at 12,000 × g for 5 minutes. Remove and dispose of the centrifugal supernatant and collect the mitochondria fractions.

[Option (2) (Purification of microsome)]
Centrifuge the cytoplasm fractions (centrifugal supernatant) described in Item 10 above, at 100,000 × g for 60 minutes. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube. The centrifugal sediment is equivalent to the microsomal fractions and the centrifugal supernatant is equivalent to the cytoplasm fractions.

IV. Preparation of organelle fraction extracts
Please suspend or replace the organelle fractions with the solution suitable for the subsequent experiments. This Instruction Manual describes the preparation of extracts by using the RIPA Lysis buffer.

(1) Preparation of the extracts
1. Add the Protease Inhibitor of 1/100 of the specified amount to the RIPA Lysis buffer. Place the solution after adding on the ice at rest until it is used.
2. Add appropriate amount of RIPA Lysis buffer to cytoplasm, nuclear, and mitochondria fractions and suspend them by pipetting.

*Add the RIPA Lysis buffer 5 - 10 times as much as the sediment amount.
* Efficiency of nuclear protein extraction will increase by adding SDS to obtain the final concentration of 0.5%. As the viscosity increases due to DNA elution, perform ultrasonic fragmentation or treatment with DNase I (100 - 500U/mL).

3. Mix them with a vortex mixer for 5 seconds.
4. Incubate them on the ice for 10 minutes.

*Please flop them upside down two or three times every one - two minutes during incubation.

5. Centrifuge the homogenate at 14,000 × g for 10 minutes.
6. Carefully collect and transfer the centrifugal supernatant to a new micro-centrifuge tube.
7. The extract can be used for immunoprecipitation, ELISA, and other protein interaction experiments.

(2) Sample preparation for SDS-PAGE
1. Add the EzApply (AE-1430) of the same amount as that of the extract or add 2 × SDS sample treatment liquids to the cytoplasm fraction fluid and to the extract described in (1) above, and mix them well. Or, add appropriate amount of 1 × EzApply (AE-1430) or 1 × SDS sample treatment liquids directly to the organelle fractions and mix them well.
2. Perform heating of the mixed solution at 100 °C for 10 minutes.
3. Centrifuge at 14,000 × g for 10 minutes.
4. The centrifugal supernatant can be used for the sample for SDS-PAGE.

(3) Sample preparation for 2D electrophoresis
1. Add appropriate amount of Solution 2 of EzApply 2D Kit (AE-1435) or 2D electrophoresis sample treatment liquids to the cells, nucleus, mitochondria, and cytoplasm fractions and mix them.
2. Centrifuge at 14,000 × g for 10 minutes.

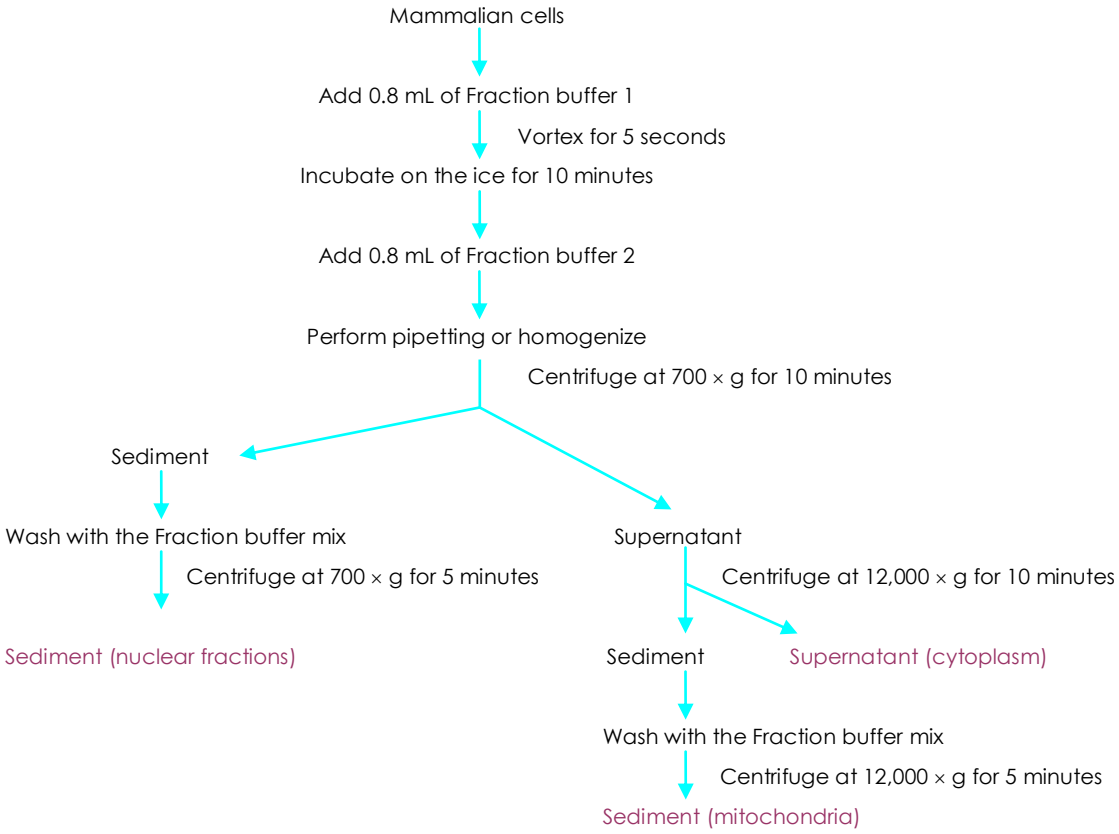
3. The centrifugal supernatant can be used for the sample for 2D electrophoresis.

10. Related ATTO products

SDS-PAGE sample preparation kit
AE-1430 : EzApply
Sample preparation kit for 2D electrophoresis
AE-1435 : EzApply 2D kit
Cell solubitization kit
WSE-7420 : EzRIPA Lysis buffer
Organelle extraction kit
WSE-7421 : EzSubcell Extract
Electrophoresis equipment with built-in power supply
WSE-1020 : Compact PAGE / Twin-R
WSE-1100 : PageRun-R

*The experiment operation may have a significant variance in the results due to a slight technical difference in the same protocol. It is important to know the "knack" to obtain optimal result.
As our website provides various "tips on experiment" and you can download the document, please visit our website below and read the article:
http://www.atto.co.jp/

Simplified diagram for the operation method



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