WSE-7421 EzSubcell Extract

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 must 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 Extract is a kit to prepare extracts of cytoplasm, membrane, nuclear compartment, etc. from mammalian cell culture. The Product can also separate/extract organelle compartments from cryopreserved cells. The organelle compartment extracts can be used for electrophoresis, immunoprecipitation, ELISA, enzyme activity experiment and other biochemical/immunological analysis.

3. Product configuration

Name	Content	Piece(s)	Storage
Extraction buffer 1	50 mL	1	2 -8 °C
Extraction buffer 2	50 mL	1	2 -8 °C
Extraction buffer 3	25 mL	1	2 -8 °C
Extraction buffer 4	50 mL	1	room temperature
Protease Inhibitor	1.25 mL	1	- 20 °C
DNase I (Deoxtribonuclease)	250 µL	1	- 20 °C

4. Composition

Name	Principal elements
Extraction buffer 1	Surfactant, buffer solution
Extraction buffer 2	Surfactant, buffer solution
Extraction buffer 3	Surfactant, buffer solution
Extraction buffer 4	Surfactant, buffer solution
Protease Inhibitor	100 × concentration, aprotinin, pepstatin A, leupeptin, DMSO
DNase I (Deoxyribonuclease)	10kU/m+ Deoxyribonuclease I

The Product does not contain any substances that are to be notified and exceed the exemption amount under the PRTR Act or the Industrial Safety and Health Act.

5. Storage

- Extraction buffer 1 - 3 should be kept in cold storage (2 - 8 °C). Unless it is opened, it will be stable until the expiration date.
- Extraction buffer 4 should be kept at room temperature (20 - 30 °C). If it is stored at a low temperature, the elements may be precipitated although there is no quality problem. In such a case, the buffer can be used after it is completely thawed in the hot bath around 30 °C. Unless it is opened, it will be stable until the expiration date.
- Protease Inhibitor and DNase I (**D**eoxyribo**n**ucle**ase**) should be kept in frozen storage (-20 °C). Unless it is opened, it will be 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
- Micro-centrifuge tube
- Vortex mixer
- Seesaw shaker or rotator
- Cooled centrifuge (Micro-centrifuge tube)

8. Precaution on use

- The Product is delivered by refrigerated goods transportation. Please open the package immediately after receiving and store the product under the temperature suitable for each reagent.
- Please ice-cool the reagents excluding Extraction buffer 4 before you start the experiment. Also, please process the experiment and operation on the ice or at a low temperature.
- 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.
- When using any cryopreserved cells, please be sure to wash the cells with PBS before freezing them and store the cells at – 80 °C after removing the PBS by centrifugation (centrifugal sediment). (Please refer to II. Preparation of cells in 9. How to use.)

9. How to use

I. Preparation of the Extraction buffer

1. Mix the necessary Protease Inhibitor or the DNase I in accordance with the table below. Place the Extraction buffer 1 - 3 after mixing on the ice at rest until it is used.

Place the Extraction buffer 4 at rest at room temperature as it is precipitated at a low temperature.

	Amount required for 1 sample	Protease Inhibitor (blue lid)	DNase I (opaque)
Extraction buffer 1	1 mL	10 µL	-
Extraction buffer 2	1 mL	10 µL	-
Extraction buffer 3	0.5 mL	5 µL	5 µL
Extraction buffer 4	0.5 mL	-	-

II. 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 5 - 20 × 10^6 cells.

2. Centrifuge the cell suspension at 200 × g for 3 - 5 minutes.
3. Carefully remove and dispose 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 the centrifugal supernatant. Then, add the appropriate amount of ice cold PBS to the cells (centrifuge sediment) until the number of cells becomes 5 - 20 × 10^6 cells/mL.
6. Collect 1mL of the cell suspension and transfer it into the 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.

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

*The centrifugal sediment (cells) described in Item 8 above can be stored at – 80 °C until it is used. When using cryopreserved cells, thaw the cells on the ice before performing III (Cell fraction and extraction operation).

III. Cell fraction and extraction operation

1. Gently loosen the cells (centrifugal sediment) by tapping and add 1mL of ice cold Extraction buffer 1.
2. Mix them with a vortex mixer for 5 seconds.

*Suspend them sufficiently until the clumps of cells disappear.

3. Incubate with a seesaw shaker or a rotator at 4 °C for 10 minutes.

*If a seesaw shaker or a rotator is not available, place them on the ice and flop them upside down two or three times every one - two minutes.

4. Centrifuge the cell solution at 700 × g for 5 minutes (4 °C).
5. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube (mainly for the fraction containing cytoplasm protein). Remove the remaining solution by absorbing it with paper towel or other appropriate cloth to prevent it from being mixed with the next treatment solution. Place the centrifugal supernatant on the ice after being transferred.

[Option]

Wash the centrifugal sediment to improve the refining level for the extracts. Add 1 mL of ice cold PBS to the centrifugal sediment and mix them with a vortex or pipetting. Then, centrifuge at 700 × g for 1 minute and collect the centrifugal sediment.

6. Add 1mL of ice cold Extraction buffer 2.
7. Mix them with a vortex mixer for 5 seconds.

*Suspend them sufficiently until the clumps of cells disappear.

8. Incubate with a seesaw shaker or a rotator at 4 °C for 30 minutes.

*If a seesaw shaker or a rotator is not available, place them on the ice at rest and flop them upside down two or three times every five - ten minutes.

9. Centrifuge the cell solution at 4,000 × g for 5 minutes (4 °C).
10. Carefully feed the centrifugal supernatant with a decanter to a new micro-centrifuge tube (mainly for the fraction containing membrane protein). Remove the remaining solution by absorbing it with a paper towel or other appropriate cloth to prevent it from being mixed with the next treatment solution. Place the centrifugal supernatant on the ice after being transferred.

[Option]

Wash the centrifugal sediment to improve the refining level for the extracts. Add 1 mL of ice cold PBS to the centrifugal sediment and mix them with a vortex or pipetting. Then, centrifuge at 4,000 × g for one minute and collect the centrifugal sediment.

11. Gently loosen the centrifugal sediment by tapping and add 0.5mL of ice cold Extraction buffer 3.
12. Immediately perform pipetting and suspend the centrifugal sediment well.

*The sediment may become viscous. Slowly suspend the sediment until the clumps of centrifugal sediment disappear.

13. Incubate with a seesaw shaker or a rotator at 4 °C for 60 minutes or longer.

*If a seesaw shaker or a rotator is not available, place the sediment on the ice at rest and flop them upside down two or three times every ten - fifteen minutes.

*Perform incubation until the viscosity is eliminated. In the case that the viscosity remains, place the sediment at 4 °C for one night, increase the amount of DNase I to be added, or increase the amount of Extraction buffer 3.

14. Centrifuge the cell lysate at 9,000 × g for 5 minutes (4 °C).
15. Carefully collect and transfer the centrifugal supernatant with a decanter to a new micro-centrifuge tube (mainly for the fraction containing nuclear protein). Remove the remaining solution by absorbing it with a paper towel or other appropriate cloth to prevent it from being mixed with the next treatment solution. Place the centrifugal supernatant on the ice at rest after being transferred.

[Option]
Wash the centrifugal sediment to improve the refining level for the extracts. Add 1 mL of ice cold PBS to the centrifugal sediment and mix them with a vortex or pipetting. Then, centrifuge at 9,000 × g for 1 minute and collect the centrifugal sediment.

16. Add 0.5 mL of Extraction buffer 4 to the centrifugal sediment and dissolve them. The lysate is equivalent to insoluble protein mainly consisting of cytoskeleton protein.

10. Reference

(1) Sample preparation for SDS-PAGE

1. Add the EzApply (AE-1430) of the same amount as that of extracts or add 2 × SDS sample treatment liquids 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 °C).
4. The centrifugal supernatant is used for the sample for SDS-PAGE.

(2) Acetone precipitation (for 2D electrophoresis, etc.) of protein

1. Add ice cold acetone (100%) four times as much as the extract to each extract and mix them.
2. Incubate them on the ice for 15 minutes.
3. Centrifuge at 14,000 × g for 15 minutes (4 °C).
4. Remove and dispose of the supernatant. Then, add 80% acetone to the centrifugal sediment and suspend them.
5. Centrifuge at 14,000 × g for 5 minutes (4 °C).
6. Remove and dispose of the supernatant. Then, air-dry the centrifugal sediment.
7. The sediment can be used by suspending it with sample treatment solution for 2D electrophoresis, etc.

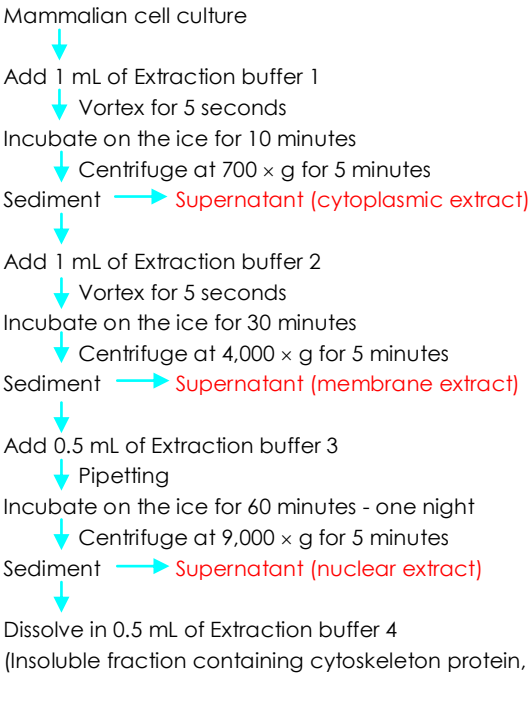
*Extraction buffer 3 and 4 contain low-level anionic surfactant which can block isoelectric focusing electrophoresis. When performing 2D electrophoresis of protein extracted with the Extraction buffer 3 or 4, be sure to perform solvent displacement with acetone sediment, etc.

11. Related ATTO products

SDS-PAGE sample preparation kit
AE-1430 : EzApply
Sample preparation kit for 2D electrophoresis
AE-1435 : EzApply 2D kit
Cell solubilization kit
WSE-7420 : EzRIPA Lysis buffer
Organelle fraction kit
WSE-7422 : EzSubcell Fraction
SDS removal reagent
AE-1390 : SDS-eliminant
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 "Tips" 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



ATTO CORPORATION
3-2-2 Motoasakusa, Tel +81 3 5827 4863
Taito-ku Fax +81 3 5827 6647
Tokyo 111-0041 E-mail: eig@atto.co.jp
Japan
http://www.atto.co.jp

Distributed by: