WSE-7010

EzLabel FluoroNeo Manual

1. Caution for safety use of this product

For safety use of this product, please read this instruction manual thoroughly. Please do not use this products until fully understand the contents of this instruction manual adequately before using. Please note that this instruction manual provides only the methods to apply this product to the intended purpose. Please do not use this product for purposes/by methods not described in this instruction manual. If it is used for a purpose/by a method not described in this instruction manual, the user shall assume all responsibilities for taking necessary safety measures and dealing with unexpected circumstances. In addition, please read instruction manuals of devices used together thoroughly and understand them well.

2. Purpose of Use

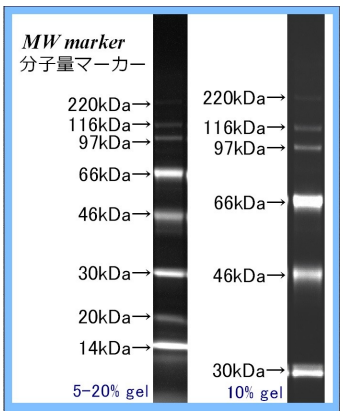
EzLabel FluoroNeo is a kit for SDS protein sample preparation for electrophoresis, and for simultaneously fluorescent protein labeling. By using it in place of conventional SDS sample buffer, amino groups are labeled with fluorescent reagent at the same time as SDS processing of proteins (λex=330, 470nm, λem=530nm).
The fluorescence of EzLabel FluoroNeo is quenched and it comes to emit fluorescence (540-580nm) after binding to amino group of protein. Therefore, there is no need to remove unincorporated fluorescent dyes. Labeled proteins can be separated by a standard electrophoresis method. In addition, the gel after electrophoresis is not required staining, it enables to immediate observe (520-560nm LP filters is suitable) gels by excitation with UV (312-354nm) or blue LED etc. (450-480nm).
General gel documentation system to observe nucleic acids such as DNA stained with fluorescent dye (EtBr, SYBR, etc.) can be used.
Gel staining is not required after electrophoresis,
and the observed gel can be used for other staining method (silver staining, CBB staining, etc.) or western blotting.

3. Product Components

Name	Volume	Qnt	Storages
Sample buffer (5x conc.)	12 mL	1 pc	-20℃
RIPA Lysis buffer	10 mL	1 pc	-20℃
Reducing agent (DTT)	300 mg	1 pc	-20℃
Labeling reagent	10 mg	1 pc	-20℃
MW marker	0.2 mL	1 pc	-20℃

*The MW marker is not labeled; label it by treatment in the same way as the protein sample. It is also a control for the labeling reaction.

*The figure on the right shows the result of electrophoresis of MW marker with 5μL/lane.



4. Composition

Name	Major Constituents
Sample buffer (5x conc.)	Surfactant(SDS）、buffer
RIPA Lysis buffer	Surfactant、buffer
Reducing agent (DTT)	DTT、bromophenol blue
Labeling reagent	Fluorescent reagent
MW marker	Protein、Glycerol

EzLabel FluoroNeo does not contain any poisonous and deleterious substance Please download MSDS of this product from ATTO homepage (http://www.atto.co.jp/) for more details.

5. Storage Method

- Keep the reagents included in EzLabel FluoroNeo frozen (-20°C). This product is stable as far as it is not expired nor opened. Expiration dates are printed on outer boxes/reagent bottles.
- Keep Labeling reagent frozen (-20°C) also after it is dissolved in ethanol or acetonitrile. After dissolution, the shelf life is about 6 months under a light-shielded, frozen (-20°C) condition.
- Keep the Reducing agent (DTT) frozen (-20°C) also after it is dissolved in distilled water (the color changes to brownish-red to blue-green after dissolution). After dissolution, DTT solution should be stored at -20°C in appropriate aliquots. Please avoid freeze-thaw cycles.

6. Disposal Method

- Disposal of each reagent should be done in accordance with the disposal procedure of your organization.

7. Additional Equipment

- Block incubator (heating system)
- Micro centrifuge tube

8. Precautions for Use

- This product is delivered by refrigerated transportation. **Please store it in a freezer (-20°C) immediately after receipt**.
- Dissolve Labeling reagent in ethanol or acetonitrile and keep it frozen (-20°C). After dissolution, use it within 6 months. If the reagent turns to yellow, do not use it as it is highly likely that the reagent is deteriorated.
- Dissolve Reducing agent (DTT) in distilled water (blue-green color after dissolution) and keep it frozen (-20°C). After dissolution, DTT solution should be stored at -20°C in appropriate aliquots. Make sure to avoid freeze-thaw as far as possible (5 times max).
- **Sample buffer is not storable after adding Labeling reagent.** Mix the amount of reagents needed for your experiment before use. In addition, please use the mixture promptly (within 30 minutes after mixing).
- Sample buffer is 5x concentration. Sample buffer is crystalized and cannot be dissolved completely at room temperature. Dissolve it completely by heating it to 37-60°C before using. It can be used/ stored after diluting with distilled water to 2x concentration. The diluted Sample buffer can be dissolved at room temperature.
- Since this reagent labels amino groups, use of buffer containing amino groups may interfere protein labeling. Please refer to 9-VII. for more details.
- If protein is dissolved in a solution containing highly-concentrated

reducing agent, it may interfere protein labeling. Please refer to 9-VII. for more details.

9. Direction of Use

9-I. Preparation for Experiment

1. **Dissolve Labeling reagent in 550µL of acetonitrile or ethanol. After dissolution, avoid light and place on ice.** The dissolved Labeling reagent solution can be kept in freezer (-20°C).
2. **Dissolve Reducing agent (DTT) in 2ml of distilled water (the color changes to brownish-red to blue-green). After dissolution, place on ice.** If it is not well dissolved, heat it to 40-60°C. The dissolved DTT solution can be kept in freezer (-20°C).
3. **Dissolve the Sample buffer (5x conc.) completely at 40-60°C.** Note that it cannot be dissolve at room temperature due to its high SDS concentration.

※The Sample buffer (5x conc.) can be melted by a microwave oven. Heat it for 5-10 seconds by 500w (repeat it for several times watching its condition). At that time, be careful NOT to bring it to boil.

※Sample buffer (5x conc.) can be diluted to 2x concentration by adding distilled water to use/store. The diluted Sample buffer can be dissolved at room temperature.

※In case of using under a native electrophoresis condition, use a native protein sample buffer without detergents, reducing agents, and amino groups in place of Sample buffer (5x conc.) of this product.

9-II. Labeling Reaction (1)

*Following paragraphs explain how to: prepare master mix and react with a sample (labeling reaction (1)); react each reagent directly with a sample (labeling reaction (2)); and dilute the Sample buffer to 2x concentration before use (labeling reaction (3)).

<Reacting sample with pre-prepared Master Mix>

1. Dissolve the Sample buffer (5x conc.) completely at 40-60°C (or 5-10 seconds in a microwave oven).
2. Mix 100µL of Sample buffer (5x conc.) with 5µL of Labeling reagent to prepare master mix.

*The master mix is unstable. Make sure to use the prepared master mix immediately. Avoid light if it is left for more than 10 minutes.

3. Add 5µL of the master mix to 20µL of your protein sample.

*The MW marker is also treated in the same way as the protein sample. The labeled marker can be stored at -20°C.

4. Incubate the reaction mixture at 95°C for 3 minutes.

*The labeled protein sample turns yellow.

*Be aware that reacting at 95°C for more than 10 minutes may result in protein degradation.

*The reaction temperature may be 37 to 95°C. React for more than 30 minutes in case of 37°C, and for 5-30 minutes in case of 65°C.

5. Add 1µL of Reducing agent (DTT) to the reaction mixture in step 4. and mix.

*Omit this step in case of non-reduced protein sample.

6. Incubate the reaction mixture in step 5. at 95°C for 3 minutes.

*Omit this step in case of non-reduced protein sample.

7. Labeling reaction is completed. Place samples with avoiding light until electrophoresis.

*Store the labeled protein sample at -20°C and with avoiding light (the shelf life is about 1-3 month).

9-III. Labeling Reaction (2)

<< Reacting sample with each reagent directly >>

1. Dissolve the Sample buffer (5x conc.) completely at 40-60°C (or 5-10 seconds in a microwave oven).
2. Mix 20µL of protein sample with 5µL of Sample buffer (5x conc.).

*The MW marker is processed in the same way as the protein sample. The labeled marker can be stored at -20°C.

3. Add 0.25µL of Labeling reagent to the reaction mixture in step 2. and mix.

*For easier handling, prepare the diluted Labeling reagent by adding acetonitrile or ethanol.

e.g.) Dilution Method for Labeling reagent

Add 15µL of acetonitrile or ethanol to Labeling reagent prepared in I-1 to obtain 4-fold dilution. Add 1µL of the diluted Labeling reagent to the reaction mixture in step 2.

*The diluted Labeling reagent can be stored at -20°C and with avoiding light.

4. Incubate the reaction mixture in step 3. at 95°C for 3 minutes.

*The labeled protein sample turns yellow.

*Be aware that reacting at 95°C for more than 10 minutes may result in protein degradation.

*The reaction temperature can be 37 to 95°C. React for more than 30 minutes in case of 37°C, and for 5-30 minutes in case of 65°C.

5. Add 1µL of Reducing agent (DTT) to the reaction mixture in step 4. and mix.
6. Incubate the reaction mixture in step 5. at 95°C for 3 minutes.

*Omit this step in case of non-reduced protein sample.

7. Labeling reaction is completed. Place samples with avoiding light until electrophoresis.

*The labeled protein sample can be stored at -20°C with avoiding light (the shelf life is about 1-3 month).

9-IV. Labeling Reaction (3)

<Reacting sample with pre-prepared 2x Sample Buffer>

1. Dissolve Sample buffer (5x conc.) completely at 40-60°C (or 5-10 seconds in a microwave oven).
2. Add 60µL of distilled water to 40µL of Sample buffer (5x conc.) to prepare 2x Sample buffer (2x conc.).
3. Mix 100µL of 2x Sample buffer with 2µL of Labeling reagent to prepare a master mix.

*The master mix is not storable. Make sure to use the prepared master mix immediately. Avoid light if it is left for more than 10 minutes.

*In case of not using the mater mix, mix 20µL of protein sample and 20µL of 2x Sample buffer, and add and mix 0.4µL of Labeling reagent to the mixture.

4. Mix 20µL of protein sample and 20µL of master mix.

*The MW marker should be treated in the same way as the protein sample. The labeled marker can be stored at -20°C.

5. Incubate the reaction mixture in step 4. at 95°C for 3 minutes.

*The labeled protein sample turns yellow.

*Be aware that reacting at 95°C for more than 10 minutes may result in degradation of protein.

*The reaction temperature can be 37 to 95°C. For instance, react for more than 30 minutes in case of 37°C, and for 5-30 minutes in case of 65°C.

6. Add 1µL of Reducing agent (DTT) and mix.

*Omit this step in case of non-reduced protein sample.

7. Incubate the reaction mixture in step 6. at 95°C for 3 minutes.

*Omit this step in case of non-reduced protein sample.

8. Labeling reaction is completed. Place samples with avoiding light until electrophoresis.

*The labeled protein sample can be stored at -20°C and with light-shielded (the shelf life is about 1-3 month).

9-V. Gel Electrophoresis documentation

1. The fluorescent labeled protein sample is separated by electrophoresis.

* General electrophoresis system and method are available. If electrophoresis is completed within 2-3 hours, light-shielding is not required. If it takes more hours, fading may be caused due to the effect of fluorescent light.

2. Remove the gel after electrophoresis from the gel plate, and rinse it lightly with distilled water.

*In case of excitating with blue LED, it is not necessary to remove the gel from the plate. Rinse the surface of the plate holding the gel lightly with water, and after cleaning the electrophoresis buffer, wipe the plate with a paper towels etc.

3. Place the gel (or the plate holding gel) onto the gel documentation system and acquire the image.

<Conditions for Documentation>	
UV Excitation: 312nm/365nm	Filter: 520-560LP
Blue LED Excitation: 440-500nm	Filter: 520-560LP

*Gel documentation can be performed after fixing. Fluorescence is stable even after fixing.

9-VI. Option

<Not Performing Fluorescent Labeling>

Without adding Labeling reagent to Sample buffer (5x conc.), follow the procedures described from 9-I to 9-IV.

<Lysis of Cells/Tissues>

*RIPA Lysis buffer provided in this kit does not contain amino groups. It can be used for fluorescent labeling reaction directly after cell lysis.

1. Wash the cell and tissue sample with ice-cooled PBS.
2. Add proper quantity of RIPA Lysis buffer and homogenize.

*The quantity of RIPA Lysis buffer required to enable cell lysis is about 1mL for a cell of $5x10^6$-$2x10^7$ size.

3. Incubate on ice for 15 minutes.
4. Centrifuge at 14,000 x g for 5-15 minutes.
5. Collect the supernatant in a new micro centrifuge tube.
6. Perform fluorescent labeling according with the procedure described in the above II.-IV.

<Under Non-denaturing/Non-reduced Condition>

Use a sample buffer not containing detergent, reducing agent, and amino group in place of sample Buffer (5x conc.) provided in this product. Refer to II.-IV. for the usage.

<For Western Blotting>

1. Acquire the image of the gel after electrophoresis of the labeled sample in accordance with the method described above V.
2. Then perform western blotting in a general method with the gel after acquiring image. After blotting, transferred proteins on membrane can also be detected by excitation light.

*Note that since *EzLabel FluoroNeo* labels amino group, in some cases the titer of antibodies may possibly be affected.

<For agarose gel 2D electrophoresis >

*Aagarose gel 2D electrophoresis, labeling can be done by the following procedures.

1. Perform isoelectric focusing electrophoresis in the first dimension by a general method.

*After isoelectric focusing electrophoresis, fix the gel in the first dimension with TCA and rinse it with distilled water.

2. Dilute Sample buffer (5x conc.) to 1x concentration.

*For instance, add 4mL of distilled water to 1mL of Sample buffer (5x conc.) and mix.

3. Add 1/100 volume of Labeling reagent to the diluted sample buffer in step 2.

*For instance, add 50µL of Labeling agent to 5mL of Sample buffer (1x conc.) in step 2. and mix (= SDS equilibration buffer).

4. Soak the TCA-fixed, rinsed first-dimension gel in SDS equilibration buffer in step 3.
5. Avoid light and incubate for 30 minutes with gentle shaking.
6. Add Reducing agent by 1/20 amount of the SDS equilibration buffer in step 3. and mix.

*Omit this step in case of non-reduced protein sample.

7. Avoid light and incubate for 10-30 minutes with gentle shaking.
8. Perform electrophoresis in the second dimension by a general method.

9-VII. Notes on Inhibition of Labeling Reaction

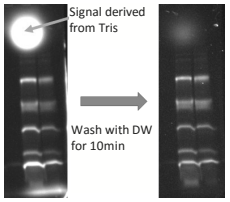
EzLabel FluoroNeo is a fluorescently-labeling reagent to amino groups. If your protein is solubilized in a solution containing amino groups, labeling reaction may be inhibited. Amino groups included in electrophoresis gel and buffer (such as Tris) would not affect to labeling reaction. Also, if any reducing agent is contained in the solvent, the fluorescence of the fluorescent reagent may be interfered. Followings are the description of how to prepare the sample against possible labeling reaction inhibitors.

<Tris and Other Amino Groups>

A background signal derived from amino groups of Tris is observed at the upper part (200kDa) of the gel after electrophoresis. The signal can be eliminated by rinsing gel with distilled water or fixing solution (left lane). Alternatively, the sample should be treated TCA/acetone precipitation and exchange to another buffer without inhibitors before labeling reaction (right lane).

<DTT and Other Reducing Agent>

- Reducing agent up to 1mM is available for labeling reaction although the labeling efficiency would be slightly inferior.
- If the sample contains 1-10mM of reducing agent, before labeling reaction, treat samples with 10mM of hydrogen peroxide for 5 minutes (at room temperature). Labeling can be performed although the labeling efficiency would be slightly inferior.
- If the sample contains 10mM or more reducing agent, treat the sample by TCA or acetone precipitation and exchange appropriate buffer before labeling reaction.



<Other Inhibitors>

- Imidazole or urea affects the labeling efficiency. If the concentration is high, labeling reaction is inhibited. Therefore, treat the sample by TCA or acetone precipitation and exchange appropriate buffer before labeling reaction.
- Labeling reaction is not affected by buffering agents such as phosphoric acid/carbonic acid/boric acid/acetic acid. Also, salt concentration/EDTA/metal ions such as Mg^{2+} and Ca^{2+}.

<TCA Precipitation of Protein>

1. Add TCA to the protein solution to obtain the final concentration of 10-20% and mix.
2. Incubate for 10-30 minutes on ice.
3. Centrifuge at 12,000 x g for 5 minutes.
4. Rinse the precipitate with ice-cooled acetone or ethanol.
5. Centrifuge at 12,000 x g for 5 minutes.
6. Dry the precipitate.
7. Suspend the precipitate in proper amount of distilled water and perform labeling reaction.

10. Related Products by ATTO

Gel Documentation System

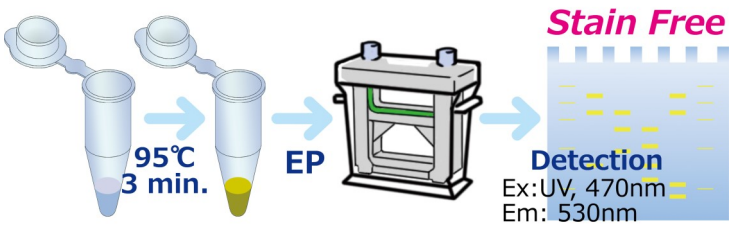
WSE-5200	*Printgraph 2M*
AE-6932/33	*Printgraph*
AE-6935B	*VISILAYS-B*
AE-9160/9300	*Ez Capture ST/MG*

*The result of experimental operation may be significantly varied due to the technique even the protocol is same. To obtain the optimum result, it is important to have appropriate skills too. Please download and utilize the material containing various "tips of experiment" available on ATTO's homepage.

Outline of Method of Experiment

Preparation of Experiment

- Labeling reagent: dissolve in 550µL EtOH or acetonitrile (store at -20°C)
- Reducing agent (DTT): dissolve in 2mL of distilled water (store at -20°C)
- Sample buffer: dissolve at 40-60°C or microwave for 10 seconds (store at -20°C)



Labeling Reaction (1)

Method with Master Mix

Make master mix

Sample buffer: 100µL
Labeling reagent: 5µL

Mix with protein

Protein sample: 20µL
Master Mix: 5µL

3 minutes at 95°C

Add DTT

Reducing agent (DTT): 1µL

3 minutes at 95°C

End of labeling

Store at -20°C, light-shielded

Labeling Reaction (2)

Method without Master Mix

Mix with protein

Protein sample: 20µL
Sample buffer: 5µL
Labeling reagent: 0.25µL
* Labeling reagent diluted with EtOH/acetonitrile is acceptable.

3 minutes at 95°C

Add DTT

Reducing agent (DTT): 1µL

3 minutes at 95°C

End of labeling

Store at -20°C, light-shielded

For more information, please contact us:

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