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ER-Protein Capture Kit

Catalog NO. FDV-0039

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Product Background

Endoplasmic reticulum (ER) is the largest organelle in the cell and has unique and dynamic tubular or sheet structures. ER plays essential roles in biosynthesis, precise folding and quality check of proteins and is a traffic origin of secreted pathway proteins including the Golgi apparatus, exocytosis, plasma membrane, and extracellular proteins. The major functions of ER are not only protein synthesis, but also carbohydrate metabolism, calcium storage, lipid metabolism, and lipid droplet synthesis. Although important roles of ER, biochemical isolation methods are highly limited presently because of its complicated structures. Some biochemical approaches with ultracentrifugation are utilized to roughly isolate ER membrane fractions, but conventional methods require time-consuming procedures with specialized equipment and show frequent contamination of other organelles such as endosomes, etc. To access the function of ER proteins, an isolation method for ER-associated proteins specifically with an easy procedure should be expected.

Our **"ER-Protein Capture Kit"** is based on the **E**R-localizable **R**eactive **M**olecule (ERM) technology originally developed by Dr. Itaru Hamachi and Dr. Tomonori Tamura, Kyoto University (Ref. 1). In this kit, there are two components, ERM (component A) and anti-rhodol antibody (component B). An overview of the kit principle is shown in Figure 1. ERM is a small compound which has two units, a rhodol-type green fluorescent dye, and a thioester-type protein labeling group (Figure 1). The rhodol-type dye has a high affinity to ER membranes and specifically and quickly accumulates into ER (Step-1 left). ER-specificity of the rhodol-type dye is comparable to a conventional ER staining reagent, fluorescent-labeled Glibenclamide. Immediately after the addition and specific accumulation of ERM into the ER, the labeling group of ERM reacts with nucleophilic amino acids in ER-proteins forming a covalent bond to proteins to label the rhodol-tag (Step-1 right). Subsequently, cells are lysed by cell lysis buffers (Step-2), and rhodol-tagged proteins are selectively purified by immunoaffinity purification with anti-rhodol antibody (Step-3). Purified rhodol-tagged proteins are separated by SDS-PAGE (Step-4) and analyzed by LS/MS-based proteomics or western blot method (Step-5). This kit enables to purify ER-associated proteins with easy procedures and no special equipment such as ultracentrifuge machine. Ref.1 shows proteomic analysis of ER-associated proteins using ERM. ERM-based proteomics shows that ERM enables to identify not only ER-resident proteins but also secreted pathway proteins including Golgi apparatus, plasma membrane and extracellular protein which are recruited to ER before their final destination. Ref.1 also shows a quantitative analysis of ER-associated proteins during tunicamycin-induced ER-stress and revealed some proteins clearly increased or decreased under the ER-stress condition. **"ER-Protein Capture Kit"** is a powerful tool to selectively purify ER-associated proteins to evaluate various ER roles.

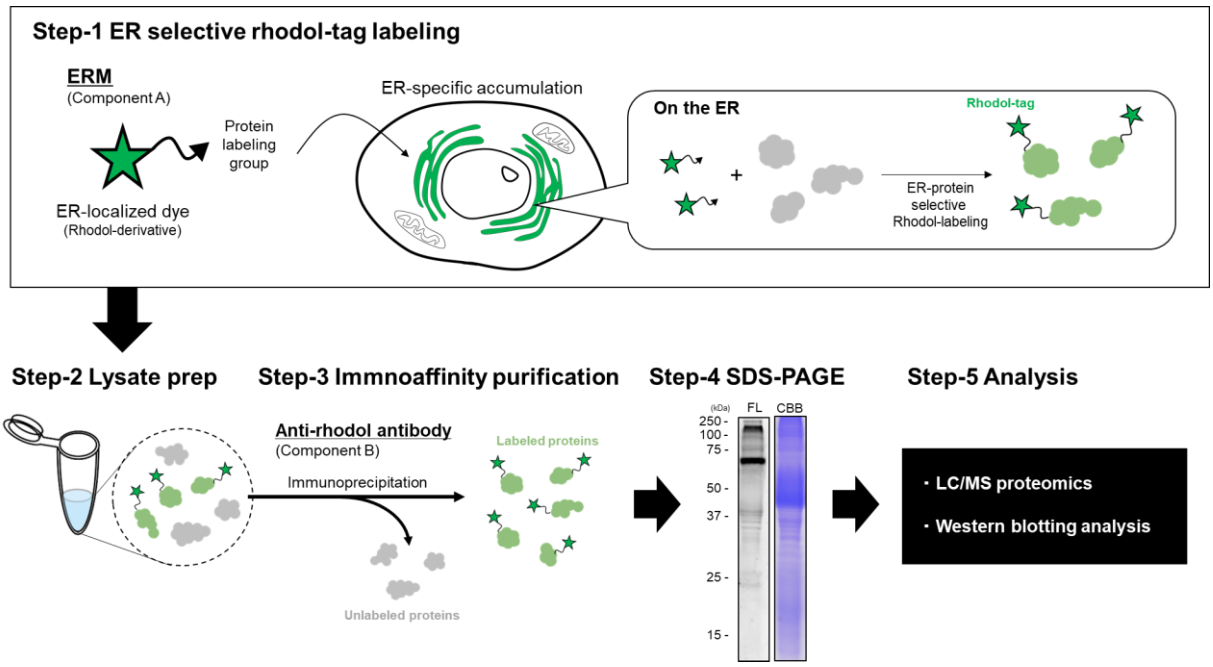


Figure 1. Overview of "ER-Protein Capture Kit"

Description

Catalog Number: FDV-0039

Kit component:

A: ERM

Size: 10 nmol

Formulation: $C_{37}H_{28}F_2N_2O_4S$

Molecular weight: 634.6g/mol

Solubility: Soluble in DMSO

Ex/Em: 509/524 nm

* Commercial FITC filter sets are available

Storage: -20ºC

Reconstitution: Stock solution recommended concentration 0.1mM to 1 mM in 100% DMSO

Note:

After reconstitution in DMSO, aliquot and store at -20 °C. Avoid repeated freeze-thaw cycles. Protect from light.

B: anti-Rhodol antibody

Size: 200 µg

Conc. 1 mg/ml

Formulation: 1 x PBS containing 50% glycerol

Host/Clonality: Rabbit polyclonal

Purification : Protein G purified

Lot Number: See vial label

Storage : -20ºC

<Note>

ERM and anti-Rhodol antibody can be purchased separately as Catalog code #FDV-0038 (product name ERseeing) and Catalog code #FDV-0039B, respectively.

Application

- ER-associated proteins specific fluorescent labeling
- ER-associated proteins selective purification
- Comprehensive identification of ER-associated proteins by LC/MS proteomics
- Quantitative analysis of ER-associated proteins by quantitative MS
- Individual detection of ER-associated proteins by western blotting

How to use

Materials in kit

- ERM (component A)
- Anti-Rhodol antibody (component B)

Materials additionally required

- Cell culture dish, medium
- PBS
- Cell lysis buffer (RIPA buffer, etc.)
- Protein A- or Protein G-conjugated beads for immunoaffinity purification
- Wash buffer: Cell lysis buffer (RIPA buffer etc.) or buffers including any detergent such as tween20 etc.
  NOTE: High concentration of SDS or detergents may disrupt antibody-antigen interaction.
- Equipment and reagents for general SDS-PAGE
- Optional: Fluorescent microscopy
- Optional: Fluorescent gel imager
- Optional: BCA assay kit
- Optional: In gel protease-digestion reagents and the reagents/equipment for general LC/MS proteomics
- Optional: Equipment and reagents for general western blotting analysis

Step-1 Labeling of ER-associated proteins

1. Culture cells of interest in 10 cm dish (around x10^6~10^7 cells/dish) for over 24 hours
2. Prepare 0.1 μM ERM in serum-free fresh medium (5 mL medium / 1 dish)
   NOTE: Highly recommend starting with 0.1 μM concentration, higher concentration shows non-specific labeling to mitochondrial and nucleolus proteins, etc.
3. Remove culture medium and wash cells by PBS or serum-free media several times
4. Add ERM-containing medium prepared in step-2 to the cells.
5. Incubate cells at 37°C for over 1 hour
   NOTE: The reaction rate of rhodol-tag labeling to proteins by ERM is dependent on incubation time. To efficiently label proteins at least 1-hour incubation is recommended. To increase the labeling efficiency, incubation time may be increased.
6. Wash the cells by PBS several times to remove unreacted ERM reagent.

Optional: To confirm the ERM labeling, the cells can be observed by fluorescent microscopy (Ex / Em =509/524 nm, commercial FITC conditions are compatible).

Step-2 Preparation of cell lysate

Precaution: Cell lysis procedure in this chapter is only a reference, empirical optimization may be required for your experiment.

1. Lyse the labeled cells with any cell lysis buffer such as RIPA buffer, etc.

Optional: Proteins are precipitated by chilled acetone, washed several times and re-solubilized by cell lysis buffer to concentrate proteins and eliminate non-protein components.

Optional: Protein quantification by BCA assay to check the total protein amount.

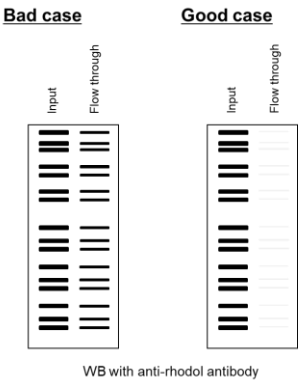
Step-3 Immunoaffinity purification (immunoprecipitation)

Precaution: Immunoaffinity purification procedure in this chapter is only a reference, empirical optimization may be required for your experiment.

0. Reserve a small portion of the lysate as “Input fraction”
1. Add 10 μg of anti-rhodol antibody per one sample prepared from one 10 cm dish to protein lysate.
   NOTE: The amount of anti-rhodol antibody is only a reference and highly depends on the amount of total protein, you may need to empirically optimize for your experiment.

2. Incubate for over 1 hour (or overnight) at 4°C with rotation or shake.
3. Add the appropriate volume of Protein A or G-beads into the lysate.
   NOTE: As anti-rhodol antibody is a purified rabbit polyclonal IgG antibody, both Protein A and Protein G are compatible with immunoprecipitation. This kit does not contain Protein A or G-beads, the user must provide. The volume of purification-beads highly depends on its product specification. The researcher should empirically optimize the proper amount of Protein A or G-beads required.
4. Incubate for over 1 hour at 4°C with rotation or shake
5. After spin-down of Protein A or G-beads, the supernatant is transferred to a new tube label as "Flow-through fraction".
6. Wash the beads with any wash buffers containing detergents such as tween 20 etc. several times.
7. Add appropriate volume (cf. 2-3 vol of beads) of SDS-sample buffer to the beads and heat beads for 5 min at 95°C
8. Collect SDS-sample buffer to a new tube label as "Purified fraction"

Option: The efficiency of immunoaffinity purification can be estimated by SDS-PAGE with fluorescence gel imager or western blotting with anti-rhodol antibody using "Input fraction", "Flow-through fraction. If "Flow-through fraction" still has a large amount of rhodol-tagged proteins (left), an increase of anti-rhodol antibody or decrease the input sample is highly recommended. A small amount of the labeled signal in "Flow-through fraction" (right), indicates the immunoaffinity purification is going well.



Step-4 SDS-PAGE

Purified proteins can be separated by conventional SDS-PAGE and detected by total protein staining (CBB, silver staining, etc.) or fluorescence gel imager (FITC filter set compatible).

Step-5 Analysis of purified proteins

LC/MS-based proteomics: Proteins on SDS-PAGE gel are digested by proteases and peptides are purified according to the general proteomics procedure.

Western blotting: Purified proteins can be analyzed by a specific antibody of interest.

Note: Because our capture antibody (anti-rhodol antibody) is rabbit polyclonal IgG antibody, the first choice of WB-detection antibody is mouse/rat monoclonal antibodies to avoid non-specific detection of heavy or light chain fragments of the capture antibody. If there is no desirable mouse/rat antibody of interest, the alternative choice is using rabbit antibodies as primary antibody and non-denatured IgG-specific (conformation-specific) anti-Rabbit antibody as secondary antibody.

Troubleshooting

Q. Low labeling efficiency.

A. Labeling efficiency depends on the labeling time of ERM, prolong the labeling incubation time. The increase of ERM concentration may increase non-specific labeling to non-ER proteins, changing the concentration of ERM is not recommended. Serum proteins may inhibit specific labeling of ERM, carefully check labeling media. Serum-free buffers are highly recommended for ERM labeling media.

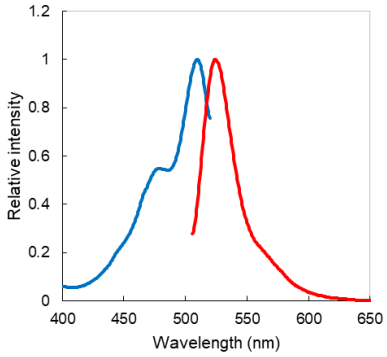
Q. Low purified proteins.

A. There are some suggestions to increase purified protein. 1) Increase the starting cell number to increase the total protein amount. 2) Increase the amount of antibody in the immunoaffinity purification step to increase purification efficiency.

Reference data

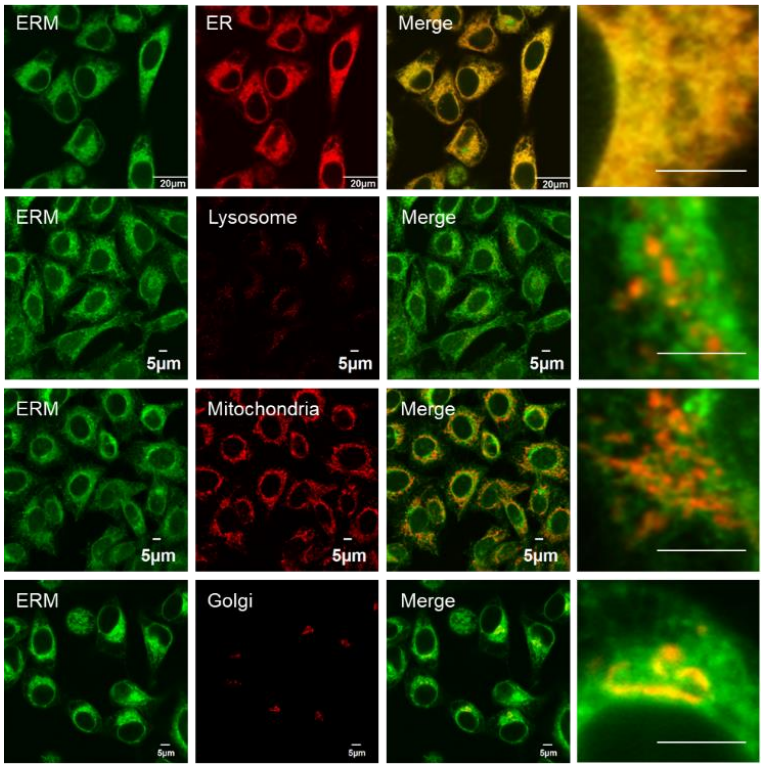
Absorption and fluorescent spectrum of ERM

Excitation (blue) and fluorescent (red) spectrum. Ex_{max}/Em_{max} = 509/524 nm. Commercial FITC filter sets are compatible.



ER specificity

Hela cells were treated with ERM (100 nM) and organelle markers, Glibenclamide-type ER staining, lysosomal staining, mitochondrial staining and Golgi apparatus staining dyes. ERM was highly overlapped with ER (Piason coefficiency >0.9) but not correlated with the lysosome marker or mitochondria marker. Only a small portion of staining by ERM was overlapped with the Golgi apparatus. It was considered that this is attributed to the vesicle transport of ERM itself or rhodol-labeled proteins from ER to the Golgi apparatus. The ER-to-Golgi traffic inhibitor decreased overlap between ERM-staining and Golgi apparatus-staining (Detail information is described in Ref. 1).



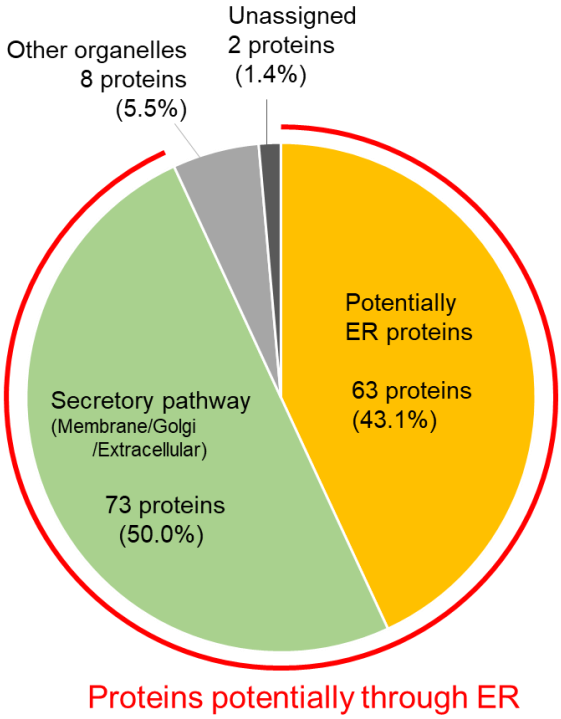
Application data

Comprehensive identification of ER-associated proteins by LC/MS-proteomics

HeLa cells in 10 cm dish were treated with 100 nM ERM for 1 hour and lysed by cell lysis buffer. After total protein precipitation by chilled acetone, proteins were resolved in 4% SDS lysis buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 4% SDS, 1% sodium deoxycholate) with sonication, and then diluted 4-fold with RIPA buffer to ~1% SDS concentration. Total protein concentration was measured with BCA assay and 2.5 mg of total proteins were applied for immunoaffinity purification. Protein A-beads and anti-rhodol antibody complex was added to total protein solution and incubate for overnight at 4°C. After incubation, the beads were washed and purified proteins were eluted with SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and the gel was fixed. After slicing the gel, sliced gels were subjected to in-gel digestion using trypsin/Lys-C. The eluted peptides derived from the gel were purified and subjected to nanoLC-MS/MS analysis.

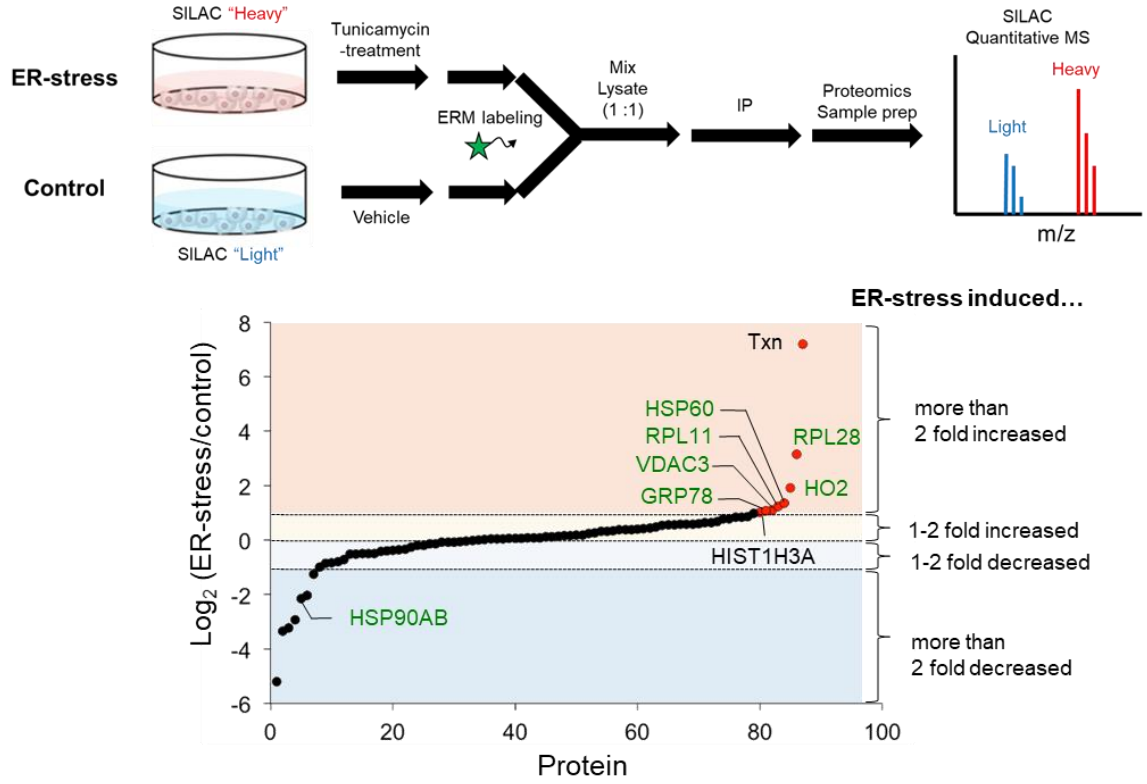
Total of 146 proteins were identified in this experiment. 63 proteins were categorized in ER-resident proteins and 73 proteins were categorized in the secretory pathways such as membrane, Golgi apparatus, and extracellular proteins. As secretory pathway proteins basically move to the final destination via ER, ERM-based assay could identify ER-associated proteins with around 93% probability.

Note: ER proteins (63 proteins) were assigned by two methods. One is based on database analysis (28 proteins) and another is manually assigned with a literature survey (35 proteins). The information for ER-associated proteins in the commercially available database is still incomplete. Please note data analysis of identified proteins should be carefully performed. The detail annotation method was described in Ref.1.



Quantitative profiling of ER-associated proteins during ER stress by SILAC assay

HeLa cells were continuously grown in “light” SILAC media or “heavy” SILAC media. The “heavy” isotope-labeled cells and the “light” isotope-labeled cells were treated with tunicamycin (2.5 μg/ml), a chemical inducer of ER-stress, and DMSO as vehicle control for 4 hours, respectively. After tunicamycin or DMSO treatment, both cells were incubated with 100 nM ERM for 1 hour and lysed by cell lysis buffer. Equal amounts of “heavy” isotope-labeled proteins and “light” isotope-labeled proteins were mixed in a 1:1 ratio and rhodol-labeled proteins were purified with anti-rhodol antibody/protein A-beads and analyzed described above. A total of 87 proteins were identified and quantified. Among 87 proteins, 39 proteins (45%) were classified as ER proteins and 84 proteins (97%) were assigned with ER-associated proteins including secretory pathway proteins. SILAC analysis indicates 6 proteins were upregulated by more than 2-fold in tunicamycin-treated cells. On the other hand, 7 proteins were downregulated by more than 2-fold in tunicamycin-treated cells.



Reference

1. Fujisawa *et al., J. Am. Chem. Soc.*, **140**, 17060-17070 (2018) Chemical Profiling of the Endoplasmic Reticulum Proteome Using Designer Labeling Reagents.

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