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Immunoblot based assay for simultaneous densitometric determination of ubiquitin forms in Drosophila melanogaster

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Ágota Nagy[1], Levente Kovács[1], Zoltán Lipinszki[2], Margit Pál[2], Péter Deák[1]

[1]Department of Genetics, University of Szeged, Szeged, Hungary;
[2]Institute of Biochemistry, Biological Research Centre, Szeged, Hungary

Ágota Nagy

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We use this protocol and it's working

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Abstract

This protocol describes an immunoassay, originally developed to quantitate ubiquitin in mouse tissues (Oh et al., Anal. Biochem. 443, 153–155), which we adapted to *Drosophila melanogaster*. The method is suitable for the simultaneous determination of total, free and conjugated ubiquitin forms from whole protein extracts by densitometric analysis of Western blots. In this assay, endogenous deubiquitylating enzymes, DUBs, present in the lysates process all conjugated ubiquitins to monoubiquitins, therefore the total ubiquitin content of cell lysates can be determined in the form of monoubiquitins. The free monoubiquitin fraction in turn is determined from similar lysates, but supplemented with a potent DUB inhibitor, NEM. Appropriate samples of these lysates are immunoblotted together with ubiquitin standards that allow the quantification of the different ubiquitin forms by densitometric analysis of the 8,5 kDa monoubiquitin band.

Guidelines

Buffers and reagents
Tris base
NaCl
Glycine
Tween-20
EDTA
N-Ethylmaleimide (NEM, Sigma-Aldrich)
MG132 (Calbiochem)
EDTA-Free Complete Protease Inhibitor Cocktail (Roche)
DTT
Glycerol
Bromophenol blue
2-mercaptoethanol
Purified ubiquitin (Sigma-Aldrich)
PVDF membrane (Merck Immobilon-P)
Tris-Glycine SDS-PAGE gel
30-40% Acrylamid solution (29:1 acrylamide/bis-acrylamid)
Non-fat milk powder
anti-Ub antibody (rabbit, Dako)
Bovine serum albumin
HRP conjugated goat anti-rabbit secondary antibody
Immobilon Western Chemiluminescent HRP Substrate (Merck)
X-ray films (Fujifilm)
Tetenal X-ray film processing developer and fixative solutions

Troubleshooting

Before start

Precool the centrifuge to 4 ^{o}C. Buffers F and T should be prepared prior to use and kept on ice.

Buffer F:
100 mM Tris, pH 7.6
150 mM NaCl
1 mM EDTA
10 mM N-Ethylmaleimide (NEM)
20 µM MG132
1× EDTA-Free Complete Protease Inhibitor Cocktail

Buffer T:
100 mM Tris, pH 7.6
150 mM NaCl
20 µM MG132 (Calbiochem)
1× EDTA-Free Complete Protease Inhibitor Cocktail (Roche)
2 mM DTT

4 x Laemmli sample buffer:
40% glycerol
240 mM Tris-Hcl pH=6.8
8% SDS
0.04% Bromophenol blue
5 % 2-mercaptoethanol

TBS:
10 mM Tris pH 8.0
150 mM NaCl

Sample preparation

1 Collect 5-6 mg of synchronized animal or tissue samples from *Drosophila melanogaster* in pre-chilled 1.5 ml microfuge tubes.
5 mg

2 Add 100 µl buffer F (in which conjugated ubiquitins remain intact) and 100 µl buffer T (in which all conjugated ubiquitins are converted to monomers) to the samples and homogenize them by plastic tissue grinders.
100 µL

3 Centrifuge the samples at 4 ^{O}C, 13000 RCF.
00:10:00

4 Collect supernatants to new 1.5 ml microfuge tubes.
70 µL

5 Centrifuge the collected samples again at 4 ^{O}C, 13000 RCF.
00:10:00

6 Collect supernatants into new 1.5 ml microfuge tubes.
61 µL

7 Use 1 µl extract to determine the total protein content of the extracts in buffer F.

8 Add 4 x Laemmli sample buffer to the protein extracts in buffer F and boil.
20 µL
00:05:00

9 Incubate the protein extracts in buffer T at 25 ^{o}
03:00:00

10 Use 1 µl extract to determine the total protein content of the extracts in buffer T.

11 Add 4 x Laemmli sample buffer to the protein extracts in buffer T and boil.
20 µL
00:05:00

Western blot

12 Load 10 µl of appropriately diluted total protein extract onto 14 %, 1 mm thick Tris-Glycine SDS-PAGE gel, together with ubiquitin standards of 0.5, 1, 2 and 3 pmols.

> Note
>
> The appropriate loading concentration (determined in Western blot test runs) is the one in which the band intensity of the monoubiquitin appears to be within the band intensity range of the ubiquitin standards.

13 Perform the SDS-PAGE in a buffer containing 25 mM Tris pH 8.3, 192 mM Glycine and 0.1% SDS.

14 Perform standard wet transfer of the proteins onto a PVDF membrane in a blotting apparatus (300 mA constant current, in transfer buffer containing 20 mM Tris pH 8.0, 150 mM Glycine and 20 % Methanol).

02:30:00

15 Block the membrane by rinsing it in 5% non-fat milk in TBS at room temperature.

00:30:00

16 Incubate the membrane in anti-ubiquitin antibody, diluted 1:1000 in TBST-B (TBS supplemented with 0.05 % Tween-20 and 1 % bovine serum albumin) at room temperature.

01:00:00

17 Wash the membrane three times by vigorous shaking in TBS-T.

00:10:00 3x

18 Incubate the membrane with the secondary antibody, a horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody diluted 1:30000 in TBST-B at room temperature.

01:00:00

19 Wash the membrane three times by vigorous shaking in TBS-T.

00:10:00 3x

20 Incubate the membrane in Immobilon Western Chemiluminescent HRP Substrate for 3 minutes, then cover the membrane with Saran Wrap™.

21 In a dark room position a sheet of X-ray film over the membrane for the appropriate exposure time (20-30 seconds), then develop the film immediately using Tetenal X-ray film processing developer and fixative solutions.

Data analysis

22 Digitalize the developed X-ray films by using a digital camera or a high resolution gel documentation system (such as BioDoc-It^{TM} 220 Imaging System).

23 Use the Gel Analysis tool of the ImageJ 1.49v software (NIH, Bethesda, Maryland) to determine the density of the bands corresponding to the monoubiquitin and ubiquitin standards. Copy the band intensity data to an excel table for subsequent statistical analysis.

https://imagej.nih.gov/ij/index.html

24 Use the band intensity data of the ubiquitin standards (y) and their concentration (x) to generate a calibration curve in MS Excel with the XLSTAT, a statistical software and data analysis add-on for Excel. Create a regression line equation by applying the four parameter curve fit model (which can be found at the dose toolbar), then use it to calculate the ubiquitin concentration of the sample of interest.

https://www.xlstat.com/en/