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

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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.

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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

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

Protocol

Sample preparation

Step 1.

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

AMOUNT
5 mg :

Sample preparation

Step 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.

AMOUNT
100 µl :

Sample preparation

Step 3.

Centrifuge the samples at 4 °C, 13000 RCF.

DURATION
00:10:00 :

Sample preparation

Step 4.

Collect supernatants to new 1.5 ml microfuge tubes.

AMOUNT
70 µl :

Sample preparation

Step 5.

Centrifuge the collected samples again at 4 °C, 13000 RCF.

DURATION
00:10:00 :

Sample preparation

Step 6.

Collect supernatants into new 1.5 ml microfuge tubes.

AMOUNT
61 µl :

Sample preparation

Step 7.

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

Sample preparation

Step 8.

Add 4 x Laemmli sample buffer to the protein extracts in buffer F and boil.

AMOUNT
20 µl :

DURATION
00:05:00 :

Sample preparation

Step 9.

Incubate the protein extracts in buffer T at 25 °

DURATION
03:00:00 :

Sample preparation

Step 10.

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

Sample preparation

Step 11.

Add 4 x Laemmli sample buffer to the protein extracts in buffer T and boil.

AMOUNT
20 µl :
DURATION
00:05:00 :

Western blot

Step 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.

NOTES

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.

Western blot

Step 13.

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

Western blot

Step 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).

DURATION
02:30:00 :

Western blot

Step 15.

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

DURATION
00:30:00 :

Western blot

Step 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.

DURATION
01:00:00 :

Western blot

Step 17.

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

DURATION
00:10:00 : 3x

Western blot

Step 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.

DURATION
01:00:00 :

Western blot

Step 19.

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

DURATION
00:10:00 : 3x

Western blot

Step 20.

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

Western blot

Step 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

Step 22.

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

Data analysis

Step 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.

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

Data analysis

Step 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.

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