PROTOCOL

Western Blot Protocol for PAb to PARP (509-524) (Prod. No. BML-SA253)

50 µl of PAb to PARP (509-524) is sufficient for 25 Western blots (minigels), without considering that the diluted antibody can be re-used many times*.

Conditions for detection of PARP or apoptosis-cleaved PARP by Western blot from cell culture: The number of cells detectable by PAb to PARP (509-524) during Western blot experiments depends on the cell line; 50'000 to 100'000 cells/well are generally sufficient.

After treatment of the cells (2 plates), determination of the number of cells is one in one plate by classical trypsination procedure; in the second plate, cells are scraped with a rubber policeman in sterile cold PBS, pH 7.4, or cold isotone (or spinned in case of work on suspended cells such as HL-60), then spinned in cold PBS, pH 7.4 or cold isotone 10 min. at 1'000 rpm at 4°C.

The supernatant is removed and the pellet is resuspended in a defined volume of reducing loading buffer (62.5 mM TRIS, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol (freshly added, i.e. 50 µl of stock solution (14.3 mol/l) in 1ml loading buffer)). Resuspension of cells in loading buffer can be done with a P1000 pipetman because the solution will be very viscous due to lysis of cells and hence presence of DNA. To break DNA, a sonication on ice is done for 20 seconds (microtips at limit, 40% duty cycle, Sonicator Vibracell, Sonics and Materials, CT) [Note: Omission of sonication and urea in sample buffer will result in incomplete PARP extraction and solubilization]. Samples are now ready and either kept at -20°C before analysis or directly incubated for 15 min. at 65°C before loading on a SDS-PAGE [Note: It is better to heat just the necessary aliquot at 65°C for 15 min. and keep the rest of the sample at -20°C].

Electrophoresis and transfer:

Samples are loaded on a SDS polyacrylamide gel (usually 8% or 10%) in an electrophoresis apparatus Bio-Rad containing running buffer (25 mM TRIS, 192 mM glycine, 0.1% SDS) at 100 V (for 75 min.) or 200 V (for 45 min.).
Proteins are transferred onto nitrocellulose membrane (Hybond C from Amersham) in a transblot cell (Bio-Rad) at +4°C under stirring. The transfer buffer is 25 mM TRIS, 192 mM glycine, 20% methanol and electrotransfer is done either at ~100 V for 1 hour or at 35 V overnight.

After transfer, nitrocellulose membrane is stained with Ponceau S (0.1% Ponceau S (w/v) in 5% acetic acid (v/v)) for 1 min. then washed with deionized water to see molecular weight markers and check protein profile of the samples. The nitrocellulose membrane is then washed with PBSMT solution (1x PBS, pH 7.4, 5% non-fat powdered milk, 0.1% Tween 20) for 5 min to remove Ponceau S staining and is submitted to Western blot analysis.

Western blotting (all steps are done at room temperature under gentle shaking):

- Nitrocellulose membrane is saturated in PBSMT solution for 1 hour.
- For one minigel, 2 µl of the primary antibody PAb to PARP (509-524) is diluted in 10 ml of PBSMT [final dilution: 1:5'000]
- After overnight incubation in 1 mM sodium azide with PAb to PARP (509-524), nitrocellulose membrane is washed in 1 x PBS containing 2M deionized urea and 0.05% Tween 20 (3 x 5-10 min.), then 10 min. in 1 x PBS containing 0.1% Tween 20. [Note: Incubation with PAb to PARP (509-524) can also be carried out for 2 hours (minimum)].
- Before hybridization, the blot is washed 6 x 30 min. with PBSMT.
- The secondary antibody (**anti-rabbit IgG** conjugated to peroxidase, from Jackson Lab.) is diluted 1:2'500 in PBSMT and incubated 30 min. The blot is washed again in PBSMT for 2 x 10 min and 3 x 5 min.
- The nitrocellulose membrane is washed 30 min with PBS before using the chemiluminescence detection system ("Renaissance" or "Renaissance Plus" from Dupont.

Erasure of the blot and reprobing:

- After detection step by chemiluminescence, blot can be stored at 4°C for a long time in a Saran Wrap or erased and reprobed with the same first antibody (more diluted or more concentrated) or with another antibody as follows:
- Nitrocellulose membrane is washed for 4 x 5 min. with 1 x PBS, pH 7.4, containing 0.1% Tween 20, then incubated in stripping buffer (62.5 mM TRIS-HCl, pH 6.8, 2% SDS, 100 mM fresh β-mercaptoethanol) for 30 min. at 65-70°C. The membrane is washed again in PBS containing 0.1% Tween 20 (6 x 5 min.).
- After this step, it is possible to check the absence of chemiluminescent signal (i.e. good stripping of antibodies from the membrane), by a new revelation with chemiluminescence reagents. An exposure of 5 min. or more should not reveal any signal.
- The membrane can be washed again for 4 x 5 min. with 1 x PBS, pH 7.4, containing 0.1% Tween 20, then incubated in PBSMT solution for 1 hour and is then ready for a new Western blot analysis.

**) The diluted primary antibody (PAb to PARP, 1:5'000) can be kept in PBSMT solution (1x PBS, 5% milk and 0.1% Tween 20) and is stable for many days (even a few weeks) stored at +4°C when containing 1 mM sodium azide, 1 U/ml penicillin and 1 µg/ml streptomycin.*

By reusing the diluted antibody, the background is further reduced (due to the progressive reduction of the antibody adsorption on the non-specific sites on the various blots).

For more details, please refer to:

Characterization of anti-peptide antibodies directed towards the automodification domain and apoptotic fragment of poly(ADP-ribose) polymerase: P.J. Duriez, et al.; Biochim. Biophys. Acta **1334,** 65 (1997)