1. Immunostaining by Western Q Circulated Pulse Method (Standard Protocol)

The following data are immunostaining of western blotting using various antibodies as primary antibodies. Two times dilution series of total protein from 10μg to 0.625μg derived from IGF-1 stimulated (50ng/ml, 5minutes) mouse cultured cell, P19, was blotted on PVDF membrane. Shaking in antibody solution was conducted for traditional method, and Circulated Pulse Method was conducted for Western Q. Same concentrations of antibodies (recommended by manufacturers) were used for traditional shaking method and for Western Q Circulated Pulse Method.

A) β-Actin (C4) (Santa Cruz) 1/3000 dilution

Traditional Method: 4 hours exposure: 40 seconds

Western Q Circulated Pulse Method: 40minutes exposure: 40 seconds

Traditional Method: Blocking 1hour (5% skim milk), Primary antibody 1hour, Secondary antibody 1hour, Washing total 60 minutes, Total 4 hours
Western Q Standard Protocol: Blocking 5 minutes (0.5% skim milk), Primary antibody 15minutes, Secondary antibody 15minutes, Washing 5 minutes, Total 40minutes
Secondary antibody: Polyclonal goat anti-mouse immunoglobulins/HRP (Dako), 1/7500 dilution
ECL (GE Healthcare) was used for chemiluminescence reaction. Exposed on X ray film

B) Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) Mouse mAb（Cell Signaling） 1/2000 dilution

4℃ overnight reaction is recommended by the manufacturer

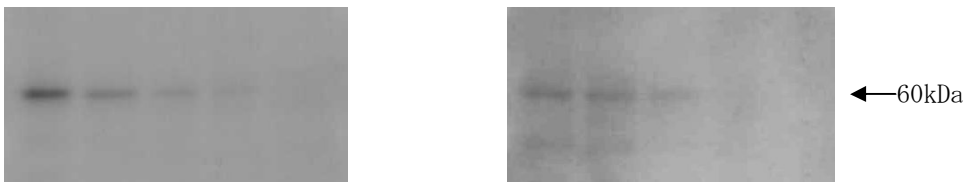
Traditional Method: Primary antibody 16 hours exposure 2.5 minutes

Western Q Circulated Pulse Method: 40 minutes exposure 10 minutes

Traditional Method: Blocking 1hour (5% skim milk), Primary antibody 16 hours, Secondary antibody 1hour, Washing total 60 minutes, Total 18 hours
Western Q Standard Protocol: Blocking 5 minutes (0.5% skim milk), Primary antibody 15minutes, Secondary antibody 15minutes, Washing 5 minutes, Total 40minutes
Secondary antibody: Polyclonal goat anti-mouse immunoglobulins/HRP (Dako), 1/7500dilution
ECL Plus (GE Healthcare) was used for chemiluminescence reaction. Exposed on X ray film

C) Phospho-Akt (Ser473) (Cell Signaling) 1/1000 dilution

4℃ overnight reaction is recommended by the manufacturer



Traditional Method: Primary antibody 16 hours exposure: 5 minutes

Western Q Circulated Pulse Method: 85 minutes exposure: 15 minutes

Traditional Method: Blocking 1hour (5% skim milk), Primary antibody 16 hours (4℃), Secondary antibody 1hour, Washing total 60 minutes, Total 18 hours
Western Q modified protocol: Blocking 5 minutes (0.5% skim milk), Primary antibody 60 minutes, Secondary antibody 15minutes, Washing 5 minutes, Total 85minutes
Secondary antibody: Polyclonal swine anti-rabbit IgG/HRP (Dako), 1/7500dilution
ECL Plus (GE Healthcare) was used for chemiluminescence reaction. Exposed on X ray film

Reaction speed by Western Q (Circulated Pulse Method) is several to dozen times higher than one by traditional shaking method.

☞ Protocol adjustment such as increasing time or raising concentration may be necessary for immunostaining which requires overnight reaction by traditional method because of antibodies or samples.

2. Use of Western Q stacking membrane in pairs

Immunostaining by Western Q (Circulated Pulse Method) was conducted stacking membrane in pairs using same antibody.

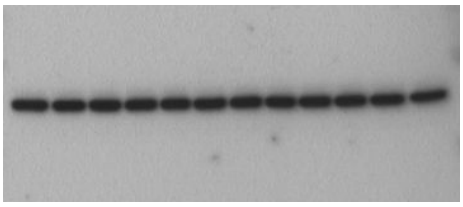
[Materials]

Sample: Total protein derived from human cultured cell (3.3 μg on each lane) was electrophoresed and blotted on PVDF membrane (80×80mm).
Blocking: 0.5% skim milk (filtrated)
Primary antibody: Mouse monoclonal β-Tublin (Sigma), 1/2000 dilution
Secondary antibody: Polyclonal goat anti-mouse immunoglobulins/HRP (Dako), 1/7500 dilution

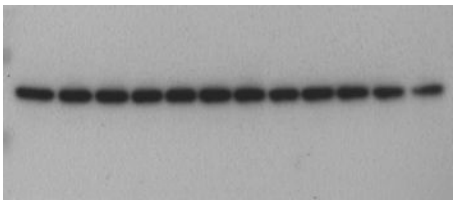
[Reaction condition]

Standard protocol (Circulated Pulse Method) of Western Q on the protocol book was adopted for reaction condition.
Blocking: 8ml, Circulated in Speed 2 for 5minutes
Primary antibody: 8ml, Circulated in Speed 2 by pulse flow for 15minutes
Secondary antibody: 8ml, Circulated in Speed 2 by pulse flow for 15minutes
Washing: 100ml, Discharged in Speed 3
ECL (GE Healthcare) was used for chemiluminescence reaction. Exposed on X ray film for 10 seconds.

[Result]



Upper membrane



Lower membrane

Almost equal reaction was observed on whole membrane.

3. Immunostaining by Western Q Incubation Method (Simplified Protocol)

Immunostaining by Western Q Incubation Method (Simplified Protocol) was conducted.

[Materials]

Sample: Two times dilution series of total protein from 10μg to 0.625μg derived from IGF-1 stimulated (50 ng/ml, 5minutes) mouse cultured cell, P19, was blotted on PVDF membrane (45×75mm).
Blocking: 0.5% skim milk (filtrated)
Primary antibody solution: β-Actin (C4) (Santa Cruz), 1/2000 dilution (×1) or 1/700 dilution (×3)
Secondary antibody solution: Polyclonal goat anti-mouse IgG/HRP (Dako), 1/7500 dilution (×1) or 1/2500 dilution (×3)

Two kinds of immunostaining by incubation method, x1 and x3, were conducted. Antibody concentration was x1 for both primary and secondary antibodies on x1 experiment, and x3 for both primary and secondary antibodies on x3 experiment.

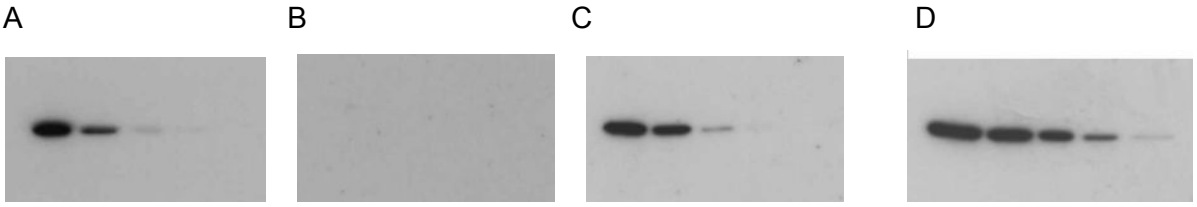
[Reaction condition]

Incubation method protocol of Western Q on the protocol book was adopted for reaction condition.

Blocking: 3ml, incubated for 5 minutes, and discharged in speed 2
Primary antibody: 3ml, incubated for 10 minutes, and discharged in speed 2
Secondary antibody: 3ml, incubated for 10 minutes, and discharged in speed 2
Washing: 100ml, discharged in Speed 3

ECL (GE Healthcare) was used for chemiluminescence reaction. Exposed on X ray film for 40 seconds.

[Result]



Traditional Method	Western Q Incubation Method	Western Q Incubation Method	Western Q (Ref.) Circulated Pulse Method
Antibody concentration ×1	Antibody concentration ×1	Antibody concentration ×3	Antibody concentration ×1
Exposed for 40 seconds each			

Similar result to traditional method was obtained using 3 times higher concentration on both primary and secondary antibodies on the β-Actin experiment. It is necessary to increase concentration of antibodies for sensitive result by Incubation Method.