Advanced ImmunoChemical Inc.
Providing for the Advancement of Medical Science

ASSAY NOTES
Protocol

Courtesy of HyTest, Ltd

GAPDH Western blot set

Direct MAb 6C5-HRP-conjugate instead of secondary antibodies!

5-minute GAPDH immunodetection in Western blotting (see protocol on page 3).

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is one of the key enzymes involved in glycolysis, catalyzing the reversible oxidative phos- phorylation of glyceraldehyde-3-phosphate. It has very high and stable expression in almost all cells and tissues. That is why GAPDH is widely used as loading control protein for Western blots and pro- tein normalization.

Monoclonal antibody 6C5 produced became the gold standard for the selective determina- tion of GAPDH in most of experimental applications. MAb 6C5 perfectly works in direct ELISA, sandwich immunoassay, Western blot, Dot-blot and immuno- cytochemistry. Below is a new fast and sensitive method for GAPDH immunodetection in Western blot.

Applications

1. Protein loading control/ Internal Standard / Calibrator

5-minute 6C5-HRP protocol shows good linearity and provides quantitative evaluation of loaded protein (Figs. 1 and 2).

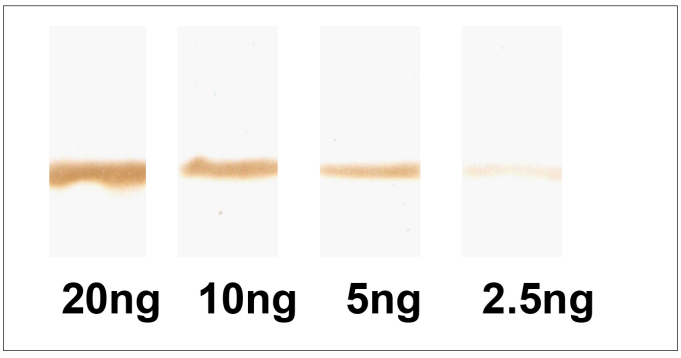

Figure 1: GAPDH calibrators in Western blot. *5 minute incubation with 6C5-HRP conjugate. 3,3`-diaminobenzidine was used as a HRP substrate. Loaded amounts of rabbit GAPDH* per track are marked under lanes.*

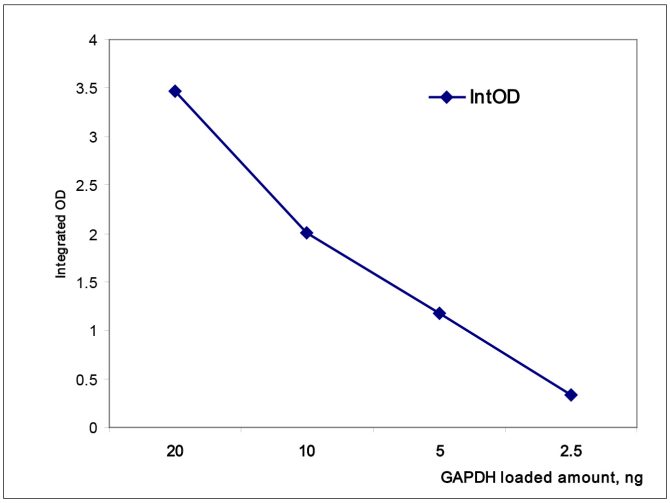

Figure 2: Calibration curve. *Integrated optical density (OD) of the bands (Fig. 1) was determined by One Dscan software.*

2. Normalizing Western Blot for cell number estimation without cell counting

Since GAPDH is referred to the group of housekeeping proteins, GAPDH is useful to standardize and compare levels of protein in cell lysates and tissue extracts. Having standard samples you can easily es- timate cell numbers in you samples without counting.

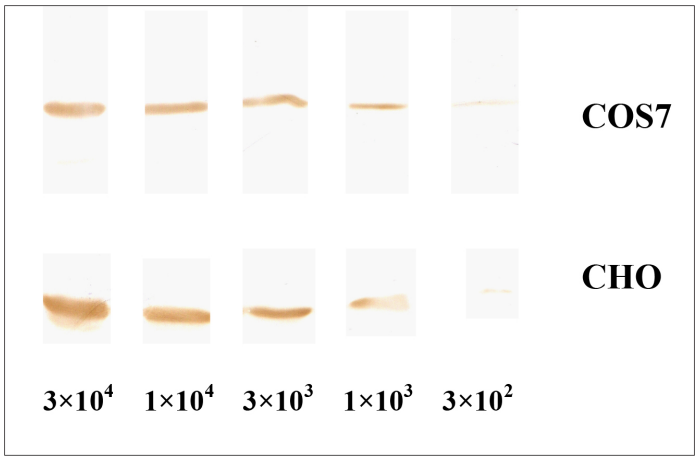

Figure 3: GAPDH determination in cell lysates by 5-minutes 6C5-HRP protocol. *COS7 (monkey kidney fibroblast-like) and CHO (Chinese hamster ovary) cell cultures were taken. Numbers of cells loaded per track are pointed underneath.*

3. Species reactivity

MAb 6C5 has been shown to react with GAPDH in human, rabbit, monkey, porcine, canine, cat, rat, hamster, mouse, duck and fish (Fig. 4). Since GAPDH is highly conserved across species, MAb 6C5 can probably be applied for many other species not tested yet.
MAb 6C5 doesn't react with GAPDH in cow, goat, *Drosophila and Saccharomyces cerevisiae.*

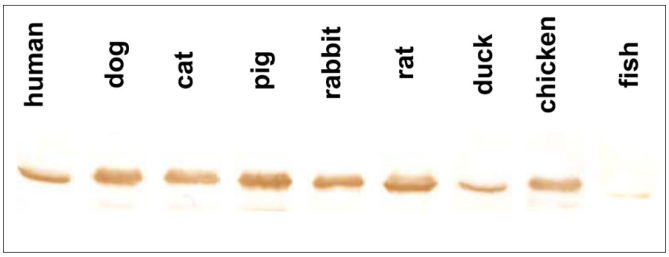

Figure 4. GAPDH determination in heart tissue extracts of different ani- mal spieces by 5-minutes 6C5-HRP protocol. *Each band corresponds to about 0.25 mg of tissue.*

5-minute protocol for Western blotting of GAPDH with 6C5-HRP

1. Sample preparation

• Prepare your cell lysates or tissue extracts sam- ples in SDS sample buffer.
• We don't recommend using lysates with cell concentration higher than 106 cell/ml and tissue extracts containing more than 50 mg tissue per milliliter.
• Boil samples for 5 minutes.
• Store samples at -80oC until use.

2. SDS PAGE and transfer

• Run samples on 15% acrylamide SDS-PAGE gel using standard protocol.
• Transfer proteins from gel to Nitrocellulose or PVDF membrane. We recommend electric trans- fer at constant 100 V for 1 hour in Transfer buffer: 25 mM Tris, 192 mM Glycine, 20% Ethanol. Stain membrane with Ponceau Red to evaluate the transfer.

3. Immunostaining

3.1. Immunostaing with following ECL signal de-tection

• Block the membrane in 5% skimmed milk in PBST (PBS + 0.1% Tween-20) for 5 minutes at room temperature with shaking.
• Add 6C5-HRP diluted 1:5000 in PBST, incubate for 5 minutes at room temperature with shaking. • Wash 4 times with PBST.
• Detect immunoreactivity using standard protocol of utilized ECL Western blotting detection kit.

3.2. Immunostaing with following DAB substrate signal detection.

• Block the membrane in 5% skimmed milk in PBST for 1 minute at room temperature with shaking. • Add 6C5-HRP diluted 1:500 in PBST, incubate for 5 minutes at room temperature with shaking.
• Wash 4 times with PBST.
• Prepare HRP-substrate solution: 50 mM Tris-HCl, pH 7.5, 0.1% (m/v) 3,3`-diaminobenzidine (DAB). Add 10 µl of 30% H2O2 per 10 ml of the solution. • Incubate the membrane with substrate solution for 5 minutes, wash with water and dry.

Figure 3. Interaction of anti-GAPDH antibodies 9B3, 10B8 and 4G5 with GAPDH, isolated from human heart tissue in direct fluoroimmunoassay. *100 ng of human antigen per well for plate coating was used.*

Ordering Information:

ANTIGEN

Product Name	Cat #	Remarks
GAPDH Western blot set	2-RGM2-set	Contains anti-GAPDH MAb 6C5 (HRP-conjugated) and human GAPDH antigen. For GAPDH immunodetection in Western blotting and DOT blot, using GAPDH sample as internal standard or calibrator.