DATA SHEET

PACAP (1-38), human, ovine, rat - Product info

CATALOG # SP2830a

SIZE 0.5 mg

Calculated MW: 4534.27 Da

Peptides are lyophilized in a solid powder format. Peptides can be reconstituted in solution using the appropriate buffer as needed.

STORAGE

Maintain refrigerated at 2-8°C for up to 6 months. For long term storage store at -20°C.

PRECAUTIONS

This product is for research use only. Not for use in diagnostic or therapeutic procedures.

Provided below are standard protocols that you may find useful for product applications.

- **Western Blot**
- **Blocking Peptides**
- **Dot Blot**
- **Immunohistochemistry**
- **Immunofluorescence**
- **Immunoprecipitation**
- **Flow Cytomety**
- **Cell Culture**

Western Blotting Protocol

A. **Preparation of cell lysates**
1. Collect cells (confluent T-25) by trypsinization and spin.
2. Lyze the pellet with 100 ul lysis buffer on ice for 10 min. For 500,000 cells, lyze with 20 ul.
3. Spin at 14,000 rpm (16,000 g) in an Eppendorf microfuge for 10 min at 4°C.
4. Transfer the supernatant to a new tube and discard the pellet.
5. Determine the protein concentration (Bradford assay, A280, or BCA) (We use the Bradford assay from Bio-Rad.)
6. Take x ul (= y ug protein) and mix with x ul of 2x sample buffer.
7. Boil for 5 min and cool at RT for 5 min.
8. Flash spin to bring down condensation prior to loading gel.

B. **Polyacrylamide gel** (14.5 cm × 16.5 cm)

1. **Agarose plug**:

1% agarose dissolved in 1 ×Resolving gel buffer. (I make 50 ml, keep melting it as I need it, and re-adding water to maintain agarose conc.)

2. **Resolving gel**: 24 ml of a 9% gel

5.4 ml 40% acrylamide/bisacrylamide (29:1 mix)
3 ml 8x Resolving gel buffer
15.6 ml water
12 ul TEMED
60 ul 20% ammonium persulfate

3. **Stacking gel**: 8 ml

1 ml 40% acrylamide/bisacrylamide (29:1mix)
2 ml 4 ×Stacking gel buffer
5 ml water
8 ul TEMED
21.6 ul 20% ammonium persulfate

C. **Preparation of gel**

1. Assemble the glass plates and spacers (1.5 mm thick).
2. Pour an agarose plug (1-2 mm).
3. Pour the running gel to about 1 cm below the wells of the comb (~20 ml).
4. Seal with 1 ml water-saturated 1-butanol. (Can stop here and leave gel as is overnight if you want.)
5. When gel has set, pour off the butanol and rinse with deionized water.
6. Pour the stacking gel (~5 ml) and insert the comb immediately.
7. When the stacking gel has set, place in gel rig and immerse in buffer.
8. Prior to running the gel, flush the wells out thoroughly with running buffer.

D. **Running the gel**

1. After flash spinning the samples, load into the wells.
2. Be sure to use markers. We use 15 ul Bio-Rad Kaleidoscope Prestained Standards #161-0324 directly.
3. Run with constant current (35-37 mA with voltage set at >150 V).
4. Usual running time is about 1.3 hr.

E. **Using precast gels**

1. Assemble gel in gel rig.
2. Prepare protein samples (10 ug will suffice).
3. Use 5 ul of Kaleidoscope standard.
4. Run at 200 V (constant voltage) for 30 min.

F. **Preparation of membrane**

1. Cut a piece of PVDF membrane (Millipore Immobilon-P #IPVH 000 10).
2. Wet in methanol on a rocker at RT for 5 mins. Remove methanol and add 1x Transfer buffer until ready to use.

G. **Membrane transfer**

1. Assemble "sandwich" for Bio-Rad's Transblot.
2. Prewet the sponges, filter papers (slightly bigger than gel) in 1 ×Blotting buffer. Sponge - filter paper - gel - membrane - filter paper - sponge

3. Transfer for 1 hr at 15 volts at 4°C on a stir plate. Bigger proteins might take longer to transfer. For the Mini Transblot, it's 100 V for 1 hr with the cold pack and prechilled buffer.
4. Immerse membrane in Amido-Black stain 5 mins.
5. Destain 4 × 5 mins with destaining buffer.
6. When finished, immerse membrane in Blocking buffer and block for one hour at room temperature. H.

Antibodies and detection

1. Incubate with primary antibody diluted to 2 ug/ml in total volume of 3 mL in Blocking buffer for one hour at room temperature.
2. Wash 4 × 5 min with 0.05% Tween 20 in TBS.
3. Incubate with secondary antibody diluted 1:10,000 (HRPOanti-rabbit) in Blocking buffer for 1 hour at room temp.
4. Wash 4 × 5 min with 0.05% Tween 20 in TBS.
5. Detect with Pierce Chemiluminescent kit (Prod # 34080).

I. **Stripping blot**

1. Rinse blot off with 0.05% Tween 20 in PBS.
2. Put blot into Kapak bag cut to slightly bigger size than blot.
3. Add about 5 to 10 ml Stripping buffer.
4. Remove as much air as possible and seal bag.
5. Immerse into 80°C water bath and incubate for 20 min.
6. Rinse blot off with 0.05% Tween 20 in PBS.
7. Block for about 1 hr with 5% BSA/Tween 20, or overnight with 3% BSA/Tween20.

Buffers for Westerns

Lysis buffer:
0.15 M NaCl
5 mM EDTA, pH 8
1% Triton X100
10 mM Tris-Cl, pH 7.4
Just before using add:
1:1000 5 M DTT
1:1000 100 mM PMSF in isopropanol
1:1000 5 M ε–aminocaproic acid

2x sample buffer:
130 mM Tris-Cl, pH8.0
20% (v/v) Glycerol
4.6% (w/v) SDS
0.02% Bromophenol blue
2% DTT

8x Resolving gel buffer: 100 ml
0.8 g SDS (add last)
36.3 g Trizma base (= 3 M)
Adjust pH to 8.8 with concentrated HCl
4x Stacking gel buffer: 100 ml
0.4 g SDS (add last)
6.05 g Trizma base (= 0.5 M)
Adjust pH to 6.8
10x Running buffer: 1 L
30.3 g Trizma base (= 0.25 M)

144 g Glycine (= 1.92 M)
10 g SDS (= 1%)--add lastDo not adjust the pH!!
10x Blotting buffer: 1 L
30.3 g Trizma base (= 0.25 M)
144 g Glycine (= 1.92 M)
pH should be 8.3; do not adjust

To make 2 L of **1x Blotting buffer**:
400 ml Methanol
200 ml 10 ×Blotting buffer
1400 ml water

Blocking buffer: 0.5 L
3% Bovine serum albumin (Fraction V)
Make up in PBS and sterile filter.
Then add 0.05% Tween 20.
Keep at 4°C to prevent bacterial contamination.

Stripping buffer:
0.5 L (sterile filter solution and keep at 4°C)
0.2 M Glycine, pH 2.5
0.05% Tween 20

Blocking Peptide Protocol

We describe below general recommendations for using blocking peptides in western blot and immunostaining techniques. The precise conditions should be optimized for a particular assay.
Protocol:

Peptides are used to block antibody binding to its target. In order to visualize the inhibitory effect of the peptide, they are usually used at 10 ×to 100 ×excess compared to antibody molarity. We recommend using a 1 ×, 10 ×and 100 ×excess of peptide first, and then to narrow this range if a more accurate study is required.

Calculation

Antibodies are manufactured at 0.25 mg/ml. Using the antibody at 1:100 dilution as recommended corresponds to 2.5 ug/ml. Estimating the MW of an antibody at 150,000 Da, the final antibody concentration is ca. 17 nM. A peptide of 15 residues long has an average MW of 1650 Da (110 Da multiplied by 15). For an excess of 100 ×of peptide over the antibody used at 17 nM, a concentration of 1.7 uM or 1.7 uM ×1650 = 2.8 ug/ml, is needed. Since one antibody binds two peptides, 5.6 ug/ml is 100 ×excess.

Important Note
It is very important to mix the antibody with the peptide before incubation with the cell lysate or onto slide. Otherwise, you may not be able to disrupt antibody binding from native target.

DOT Blot Protocol

Cut membrane, loading sample, blocking

1. According to the amount of sample to cut 8 cm × 6 cm nitrocellulose membrane. Gently draw on rectilinear reference lines per 1cm to separate the membrane into 48 grids, then marked with numbers.

2. Dilute the peptide into 5 ug/mL by 2 mL PBS (pH 7.4). Drop 2 uL the dilution slowly to the centre of the grid. Adding sample order: the upper row is NP-pep, the nether row is P-pep. Each antigen should be loaded two grids. Place 30 min to dry.

3. According to the number to cut the membrane as a unit with two grids. Incubate the membrane in blocking buffer (5% non-fat milk in TBST (m/v)) for 1 hour at room temperature.

Antibody dilution

The antibody should be diluted into 0.5 ug/mL by the blocking buffer. Incubation primary antibody Put the blocking membrane into the diluted antibody solution for 1 hour at room temperature. Incubation secondary antibody (HRP labeled goat anti rabbit Ig antibody)

1. Washing the membrane with TBST (pH 7.4) for 3 ×5min.
2. Dilute the secondary antibody with the blocking buffer.
3. Incubation secondary antibody for 1 hour at room temperature. Coloration and developing
4. Washing the membrane with TBST (pH 7.4) for 3 ×5min.
5. Put the membrane into the substrate of HRP solution for coloration for 7 min.
6. Developing

Immunohistochemistry Protocol

Preparing Tissue Sections for Immunostaining:

- Fix the tissue in 10% formalin at 4°C overnight.
- Embed fixed tissue in paraffin.
- Mount tissue sections on slides.
- Clear the paraffin with xylene for ten minutes; move slides to a fresh dish of xylene for an additional ten minutes. NOTE: Perform all xylene washes in a fume hood!
- Rinse the slides twice for 2 minutes in 100% alcohols (18:1:1 100% ethanol: 100% methanol: 100% isopropanol).
- Rinse the slides twice for 2 minutes in a 95% solution of the 100% alcohols.
- Place slides in an 80% solution of the 100% alcohols for 2 minutes, followed by deionized water for 5 minutes.
- Rinse slides several times with fresh deionized water followed by another five minutes wash using fresh water.

Sodium Citrate Antigen Retrieval:

- Place slides in a glass slide holder and fill in the rest of the rack with blank slides (10 totals) to ensure even heating.
- Place rack in 600 ml of 10 mM sodium citrate, pH 6.0 in a glass 2 L-beaker. Mark a line at the top of the liquid on the beaker.
- Microwave for 20 min total, replacing evaporated water every 5 min.
- Cool slides for 20 min.
- Wash 4 ×3 min in ddH_2O, and 3 min in 1 ×PBS. Blocking
- Block endogenous peroxidases by soaking slides in a solution of 90% methanol/3% H_2O_2 for 15 minutes at room temperature. Wash 3 ×in PBS.
- Immerse slides in a dish containing blocking buffer (serum from host species of secondary antibody to be used, diluted 1:10 in TBS). Incubate at 37°C for one hour. Incubation with Primary Antibodies
- Cover the tissue sections with primary antibody diluted in blocking buffer. Antibody is diluted 1:50 and 1:100. Incubate for 1 hour at 37°C.
- Blot excess liquid from slides and rinse three times in PBS for five minutes each wash. Incubation with Secondary Antibodies

- Cover the tissue sections with secondary antibody diluted in blocking buffer according to manufacturer's instructions. We routinely use prediluted universal secondary antibody (Jackson ImmunoResearch Laboratories). Incubate at 37°C for30 min.
- Blot excess liquid and rinse twice in TBS for five minutes each wash. Counterstaining and Visualization
- Counterstain with Hematoxylin.
- Rinse several times in deionized water. Blot excess water around tissue, then apply one drop of mounting media to tissue and place coverslip over slide. Seal with nail polish.

Citrate Solutions:
Description: Formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection of certain proteins. The citrate based solution is designed to break the protein cross-links, thereby unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections and enhancing the staining intensity of antibodies.

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) --------- 2.94 g
Distilled water ---------- 1000 ml
Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.
Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0):
Citric acid (anhydrous) ---------- 1.92 g
Distilled water ---------- 1000 ml
Mix to dissolve. Adjust pH to 6.0 with 1N NaOH and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.

Washing Buffer:

1 ×PBS:
NaCl ---------- 8 g
KCl ---------- 0.2 g
Na2HPO4---------- 1.44 g
KH_2PO_4---------- 0.24 g
Distilled Water---------- 800 mL
Adjust pH to 7.2 with HCl.
Adjust volume to 1 L with additional H_2O

Normal Serum Blocking Buffer:

2% serum from host species of secondary antibody (blocking)
1% BSA (stabilizer)
0.1% cold fish skin gelatin (blocking)
0.1% Triton X-100 (penetration enhancer)
0.05% Tween 20 (detergent and surface tension reducer)
0.05% sodium azide (preservative)
Dissolve in 1 ×PBS
Mix well and store at 4°C.
Avidin/Biotin Block:
Avidin 0.001% in 1 ×PBS
Biotin 0.001% in 1 ×PBS

Store these blocking solution at 4°C. Primary Antibody Dilution Buffer:
1%BSA (stabilizer and blocking)
0.1% cold fish skin gelatin (blocking)

0.05% sodium azide (preservative)
0.01M PBS pH7.2

Note: 1) Antibodies diluted using this buffer can be stored at 4°C for 6 months without reducing binding activity. 2) This buffer cannot be used for diluting HRP conjugated antibodies since sodium azide is an inhibitor of HRP.

Peroxidase Blocking Solution (3% H_2O_2 in PBS):

30% H_2O_2 ---------- 2 ml
1 ×PBS ---------- 18 ml
Mix well and store at 4°C for up to 3 months. This solution is recommended for paraffin sections

References:

1. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem. 1993 Nov; 41(11):1599-604. PubMed Abstract
2. Kanai K, Nunoya T, Shibuya K, Nakamura T, Tajima M (1998) Variations in effectiveness of antigen retrieval pretreatments for diagnostic immunohistochemistry. Res Vet Sci. 64(1):57-61. PubMed Abstract
3. Brown RW, Chirala R (1995) Utility of microwave-citrate antigen retrieval in diagnostic immunohistochemistry. Mod Pathol. 8(5):515-20. PubMed Abstract
4. Morgan JM, Navabi H, Schmid KW, Jasani B. Possible role of tissue-bound calcium ions in citrate-mediated high-temperature antigen retrieval. J Pathol. 1994 Dec; 174(4):301-7. PubMed Abstract
5. Pellicer EM, Sundblad A (1994) Antigen retrieval by microwave oven with buffer of citric acid. Medicina (B Aires). 54(2):129-32. PubMed Abstract
6. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem. 41(11):1599-604. PubMed Abstract
7. Brown, D., et al. (1996) Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS).Histochem Cell Biol 105:261–267.

Immunofluorescence Protocol

Step-by-step procedure:

1. Add a coverslip into a 12-well plate and grow cells in culture media until they reach 50% confluence.
2. Aspirate media from plates and wash twice with PBS.
3. Fix cells with 4% paraformaldehyde solubilized in 0.1% Triton ×100-PBS for 20 min at room temperature (RT).
4. Block for 1 hr with 2 ml of 1% BSA-4% goat serum-PBS. (Note: always spin down any sera, antibodies, or antisera for 5 min at 10,000 g before use, to remove small aggregates)
5. Wash twice for 5 min with 2 ml of PBS.
6. Stain with primary antibody for 45 min at RT in 40 ml of 1%BSA-PBS by forming a drop on the coverslip.
7. Wash twice for 5 min with 0.2% BSA-PBS.
8. Stain with conjugated secondary antibody for 30 min at RT in 40 ml of 1% BSA-PBS.
9. Wash twice for 5 min with 2 ml of PBS.
10. Mount slide with anti-fading agent.

Reagents:

PBS（pH7.4）:
10 mM Na2HPO4, 1.8 mM KH2PO4, 50 mM NaCl, 2.7 mM KCl

Fixative preparation:

4% paraformaldehyde solubilized in PBS: Depolymerize paraformaldehyde by adding 1-2 drops of 10N NaOH/25 ml and warm the tube up to 65ºC to get a clear solution, put back on ice and check the pH.

Immunoprecipitation Protocol

Step-by-step procedure:

1. Harvest cells with PBS-EDTA or Trypsin, and count cells.
2. Lyse the cells in prechilled RIPA buffer (1 ml/10^7 cells) for 1 hr rocking at 4°C.
3. Centrifuge for 20 min at 14,000 g at 4°C.Transfer super natant to a new tube.
4. Prepare protein A/G agarose beads by washing twice with PBS and restoring to a 50% slurry bead suspension with RIPA buffer.
5. Pre-clear the cell lysate by adding 50 ml of bead slurry per ml of cell lysate and incubate at 4°C rocking for 10 min. Centrifuge for 10 min at 10,000 g at 4°C. Transfer supernatant to a new tube.
6. Prepare IP reaction by pipetting 0.5 ml pre-cleared cell lysate (corresponding to 5 ×10^6 cells) into a new tube. Add 8-15 ug antibody per reaction and incubate rockin g for 3 hrs on ice. The optimal amount of antibody required to immunoprecipitate the antigen from a given cell lysate should be empirically tested.
7. Add 50 ml of 50% slurry beads and rock for 1 hr at 4°C.
8. Centrifuge sample at 10,000 g for 15 sec in microcentrifuge. Carefully discard the supernatant.
9. Wash beads twice with 1ml RIPA buffer（to remove non specifically associated proteins） and then 3 times with 1ml PBS to remove detergents.
10. Finally, resuspend beads in 60 ul Sample buffer, and boil at 95°C for 5 min. Centrifuge sample at 10,000 g for 15 sec in microcentrifuge before loading on SDS-PAGE.

Reagents:

RIPA buffer:

50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton×100, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF, 1 ug/mL aprotinin, 1 ug/mL leupeptin.

PBS (pH7.4):

10 mM Na2HPO4, 1.8 mM KH2PO4, 50 mM NaCl, 2.7 mM KCl.
1 ×Sample buffer:
65 mM Tris-Cl (pH8.0), 10% (v/v) glycerol, 2.3% (w/v) SDS, 0.01% bromophenol blue, 1% DTT.

Flow Cytometry Protocol

Preparation of Buffer:
PBS buffer: PH=7.4
Blocking Buffer: 0.5% BSA-PBS
Fix Buffer: 2% paraformaldehyde
Penetrating Buffer: 90% methanol

Step-by-step procedure:

1. Cell Collection: Collect the cell suspension, adjust the cell concentration into 1-5 x 10^6 cells/ml.
2. Wash and Centrifuge: Add 2 ml blocking buffer, then shake slightly and centrifuge at 1500-2000 rpm for 5 min.
3. Cell Fixation: Drop the supernatant, then fix cells with 1 ml fix buffer and incubate at room temperature for 10 min.
4. Wash and Centrifuge: Centrifuge at 1500-2000 rpm for 5 min, then wash cells once with 1 ml blocking buffer and centrifuge again at the same condition.

5. Cell penetration: Drop the supernatant, add 1 ml precool penetrating buffer and incubate at room temperature for 10 min. (If it is the extrazellular stain, just skip this step)
6. Wash and Centrifuge: Centrifuge at 1500-2000 rpm for 5 min, then wash cells once with 2 ml blocking buffer and centrifuge again at the same condition.
7. Blocking: Incubate cells in blocking buffer for 30 min at room temperature.
8. Incubate Primary Antibody: Add primary antibody at 0.025 mg/ml and incubate for 90 min at room temperature.
9. Wash and Centrifuge: Centrifuge at 1500-2000 rpm for 5 min, then wash cells once with 2 ml blocking buffer and centrifuge again at the same condition. Repeat again.
10. Incubate Secondary Antibody: Incubate with FITC-conjugated secondary antibodies for 40 min at room temperature (Keep in dark place).
11. Wash and Centrifuge: Centrifuge at 1500-2000 rpm for 5 min, then wash cells once with 2 ml blocking buffer and centrifuge again at the same condition.
12. FC analysis: Re-suspend cells in 1 x PBS and analyze on flow cytometry.

Cell Culture Protocol

Thawing Cells

1. Place frozen cells in 37 °C water bath for approximately 2 minutes or until cells are thawed.
2. Place cells into MEM media (which should be at room temperature) + 10% FBS for 10 minutes.
3. Centrifuge the cells at 1200 rpm for 5 minutes at room temperature.
4. Resuspend the cells to a final concentration of 10^5 cells/mL.

Culturing the Cells

1. Place ~2.5 $\times 10^6$ cells per culture flask (25 mL of 1 $\times$ 10^5 resuspended cells).
2. Close the flask lid, but not too tightly.
3. Place the flask with cells in the incubator at 37 °C with 5% CO2.
4. Check the flasks daily for changes in media color and/or monolayer.

Changing the Cell Culture Media

1. Always leave one flask alone and feed the following day for fear of contamination problems.
2. Cultures should be fed every 2/3 days – once the media starts to change color.
3. Take the culture flasks out of the incubator and place in the laminar flow hood.
4. Remove 50% to 75% of current media from the flask. Replace the amount taken with room temperature MEM media (10% FBS).
5. Place the flask back into the incubator and record the information on the data sheet.

Subculturing Cells

1. Remove present culture media.
2. Add 10 mL of 0.025%-0.25% trypsin, and let the cells sit for 10 minutes at room temperature. It may be necessary to bang the culture flasks on the hood counter to remove any “sticky” cells from the flask surface.
3. Immediately after the 10 minutes have passed – add room temp MEM media (10%FBS) to inactivate the trypsin.
4. Adjust the cell concentration to 1 x 10^5 cells per mL.
5. Add 2.5 $\times 10^6$ cells per culture flask (25 mL of 1 $\times 10^5$ resuspended cells).
6. Close the flask lid, but leave slightly lose.
7. Place culture flasks back into the incubator and check daily for media changes/monolayer formation.
8. Record information on data sheet.