

TREVIGEN[®] Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Anti-PBR Polyclonal Antibody

Polyclonal Antibody for the Detection of the Mouse and Rat
Peripheral-type Benzodiazepine Receptor (PBR)

Cat #: 6362-PC-100

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I. Introduction

The peripheral-type benzodiazepine receptor (PBR), named for its affinity for the benzodiazepine, diazepam (Valium), is a ubiquitous 18 KDa protein found in most steroidogenic tissues. PBR is located primarily in the outer mitochondrial membrane of these tissues and is involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane. PBR expression is correlated with high metastatic potential regarding human breast cancer lines, astrocytomas and other brain tumors. In addition, antitumor drugs that act via PBR (e.g., PBR ligand-drug conjugates) may be useful in the treatment of pancreatic and brain tumors. Pharmacological concentrations of high-affinity PBR drug ligands can induce apoptosis in various cancer cell lines, and PBR ligands may act as chemosensitizing agents for the treatment of human neoplasms. Moreover, PBR overexpression contributes to hormone-induced tumor cell proliferation, an important contributor to morbidity in cancer.¹

Trevigen's Anti-PBR Polyclonal Antibody (cat# 6362-PC-100) was raised against a peptide (VGLTLVPSLGGFMGAYFVR) found within the mouse and rat PBR proteins, and is affinity purified. It is stable for at least 3 years when stored at -20 °C. For long term storage, store aliquots at -20 °C to avoid freeze/thaw cycles. This PBR polyclonal antibody cross-reacts with mouse and rat samples.²

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures or patients.
2. The physical, chemical, and toxicological properties of the products to be used for the recommended procedures (below) may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. DAB is a known carcinogen and should be disposed of according to local and state laws.

III. Materials Supplied

Component	Amount Provided	Storage	Cat#
Anti-PBR antibody	100 µl	- 20 °C	6362-PC-100

IV. Materials/Equipment Required but Not Supplied

Equipment:

1. 1-20 µl pipettor
2. 20-200 µl pipettor
3. 200-1000 µl pipettor
4. microcentrifuge
5. vortex
6. cryostat
7. slide warmer
8. UV spectrophotometer
9. -20 °C and 4 °C storage
10. SDS-PAGE and Western blotting apparatus

Reagents (immunohistochemistry):

1. liquid nitrogen
2. dry ice
3. 70% ethanol
4. isopentane
5. methanol
6. 30 % H₂O₂
7. Fetal Bovine Serum (FBS)
8. 10X PBS (cat# 4870-500)
9. Poly-L -lysine (cat# 3438-100-01)
10. Glass coverslips (cat# 4862-1)
11. Cytonin™ IHC (cat# 4878-05-02)

Reagents (Western Blotting):

1. Recombinant PBR protein (cat# 6360-025-01)
2. Nitrocellulose membrane
3. Non-fat dry milk
4. TBS
5. TBST
6. HRP-conjugated Anti-Rabbit antibody
7. PeroxyGlow™ A: (cat# 4855-20-13); B: (cat# 4855-20-14)
8. Plastic wrap (e.g. Saran Wrap)
9. X-ray film
10. TE buffer

V. Assay Protocol

A. Immunocytochemistry

a) Sample Preparation

Cells grown in suspension can be fixed in solution and spotted onto pretreated glass microscope slides for processing. This method does not require any special equipment.

Method:

1. Harvest cell suspension by centrifugation at 500 x g for 5 minutes at room temperature.
2. Discard media and resuspend cells in 5 ml of 70% ethanol pre-chilled to -20 °C. Let stand for 10 minutes.
3. Spot 1×10^5 cells onto a clean glass microscope slide and air dry for 10 minutes.
Note: Glass slides pretreated for electrostatic adherence are recommended (cat# 4861-100). Other pretreatments (e.g. gelatin) can cause increased background staining.
4. Immerse in room temperature 70% ethanol for 10 minutes and air dry overnight. Alternatively, dry at 45 °C for 2 hours.
5. Immerse in 1X PBS and proceed to section V. d. Immunoperoxidase Detection.

b) Monolayer Cells

Sterile Chamber Slides

Cells should be grown on a surface that allows for both fixation and direct labeling for optimal outcomes. When using chamber slides remove the chamber walls and gasket after fixation.

Sterile Slides

The slides must be sterile and, if necessary, pretreated to ensure cell adhesion. Sterilize microscope slides by autoclaving in a large glass Petri dish. If needed, coat slides with sterile poly-L-lysine or collagen. Place sterile microscope slides in culture vessel directly before plating cells.

Sterile Glass Cover Slips

Cells can be cultured directly on sterile cover slips that are placed into a 12-well tissue culture plate. Sterilize cover slips by autoclaving in a large glass Petri dish. If needed, coat cover slips with sterile poly-L-lysine or collagen. Place sterile glass cover slips in wells of tissue culture dishes (12 mm cover slips fit into 24 well tissue culture plates) using fine tipped sterile forceps. Handle only at edges prior to cell plating.

Method:

1. Remove media from cells and rinse once with 1X PBS at room temperature.
2. Fix cells for 10 min on ice with 70% ethanol, prechilled at -20 °C.
3. Rinse cells with 1X PBS, for 2 minutes each wash.
4. Immerse in 1X PBS and proceed to V. d. Immunoperoxidase Detection

c) Preparation of tissues

Fresh frozen tissue should be used for immunohistochemistry. Quick-freeze tissue or biopsy immediately after removal by immersing in liquid nitrogen or in a bath of ethanol/dry ice. Store frozen tissue below -70 °C.

Samples may be embedded in a cutting matrix prior to freezing. Position the sample within cutting matrix in a suitable container. Immerse embedded tissue in isopentane chilled on dry ice. Using the cutting matrix, attach the sample to cutting block. Equilibrate the temperature of the cryostat before sectioning. Collect sections (10-20 µm thickness) on glass slides pretreated for electrostatic adherence.

Method:

1. Dry overnight at room temperature, or for at least 2 hours at 45 °C on a slide warmer.
2. Immerse for 10 minutes in -20 °C prechilled 70% ethanol.
3. Wash two times in 1X PBS, 2 minutes each wash.
4. Proceed with labeling.

Many samples are routinely fixed by the immersion or perfusion methods, however, the utility of this antibody with tissues fixed in cross-linking fixatives and embedded in paraffin has not yet been determined.

d) Immunoperoxidase Detection

Method:

1. Wash fixed samples twice in 1X PBS, 5 minutes each.
2. Incubate with 3% hydrogen peroxide solution in methanol for 5 minutes to quench endogenous peroxidases (prepare immediately before use).
3. Wash sample in 1X PBS, 2 minutes.
4. Incubate sample in 1X PBS, 10% FBS for 1 hour.
5. Dilute PBR antibody 1:250 to 1:500 in Cytonin IHC™ (Cat# 4878-05-02) or in PBS, 10% FBS, and cover samples with diluted antibody. Incubate overnight at 4 °C. Wash samples three times with 1X PBS.

6. Incubate with a goat anti-rabbit IgG-HRP diluted in 1X PBS-10% FBS or Cytonin IHC™. Dilute to concentration recommended by the manufacturer and incubate as directed. Wash three times with 1X PBS, 2 minutes each.

Two detection options are suggested:

i. DAB staining

1. Incubate in 0.5 µg/ml diaminobenzamide tetrahydrochloride (DAB) 0.03% hydrogen peroxide in 1X PBS for 10 minutes at room temperature. When using Trevigen's DAB (Cat# 4800-30-07), thaw reagent at 37 °C.

Immediately before use, combine:

- 250 µl of DAB (Cat# 4800-30-07)
- 50 µl of 30% hydrogen peroxide
- 50 ml of 1X PBS Buffer

Dispose of DAB according to local, state and federal regulations.

2. Wash twice in deionized water, 2 minutes each.
3. Counterstain with 1% Methyl Green (Cat# 4800-30-18). Incubate for 30 seconds. Incubations up to 5 minutes may be required depending on the section.
4. Wash and dehydrate the samples using either option (1) or (2).

(1) Ethanol option

1. Wash in deionized water at least 10 times until run off is clear.
2. Dip ten times in 95% ethanol.
3. Dip ten times in 100% ethanol.
4. Go to step 5; clarification in Xylenes

(2) Butanol option:

1. Dip in 1-butanol until sample turns from blue to green (5-10 sec).
2. Dip in fresh 1-butanol until excess counterstain is washed away (5-10 sec).

5. Clarify sample by dipping 10 times in 2 changes of xylenes.
6. Mount with appropriate mounting media e.g. Trevigen mounting media (cat# 4865-25) or PermOUNT®.

ii. AEC staining

1. Incubate samples in hydrogen peroxide diluted 1:1000 in AEC, for 1 hour at 37 °C.
2. Counterstain with Trevigen's Blue Counterstain (cat# 4820-30-13) or hematoxylin
3. Immerse samples in deionized H₂O for 2 minutes.
4. Immerse samples for 5 seconds to 5 minutes in Blue Counterstain.
5. Wash slides sequentially by dipping ten times each in:
 1. Tap water
 2. Ammonium Water (to prepare mix):
0.25 ml of 30% ammonium hydroxide in 500 ml tap water
 3. Tap water

The Ammonium Water is used as a blueing agent during counterstaining. Stain should change from purple to blue.

6. Mount with aqueous mounting media (e.g. Crystal/Mount, <http://www.genetex.com>)

B. Western Blotting

The antibody can be used to assess the PBR in cell extracts or in mitochondrial fractions. The following procedures are suggested:

a) Preparation of cell extracts

1. Pellet 2×10^6 to 1×10^7 cells by centrifugation at 250 x g, for 10 minutes at 4 °C and discard supernatant.
2. Loosen pellet by tapping tube and add cold 1X PBS to wash. Centrifuge at 250 x g, 10 minutes at 4 °C.
3. Resuspend cells in 1 ml of hot 2X SDS gel sample buffer (250 mM Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, and 0.2% bromophenol blue, 20 mM dithiothreitol*).

*Since DTT is not stable, omit it from the initial preparation and add in the buffer immediately before use.

4. Heat extracts in a boiling water bath for 10 minutes and place on ice. (Extracts can be sonicated at this step to reduce viscosity.)
5. Load desired amount of cell extract on a SDS-PAGE gel.

b) Preparation of mitochondrial fraction

Whenever possible keep glassware, solutions and biological samples on ice. Perform the experiment in a short period of time to avoid unnecessary degradation of sample. Centrifugation steps should be performed at 4 °C. Consult section V for suggested controls.

The following protocol is suggested for rat liver samples:

1. Euthanize the animal by cervical dislocation and guillotine.
2. Quickly remove the liver and rinse in cold HMS (220 mM D-Mannitol, 70 mM sucrose, 2 mM K-HEPES pH 7.4, prepared fresh weekly).
3. Blot dry the tissue with filter paper and weigh (weight required for later steps).
4. In a small beaker, add 10 ml of HMS to the tissue and quickly mince thoroughly (2 mm cubes) using scissors. Add ~20 ml HMS to the minced tissue and swirl to mix.
5. Discard supernatant. Transfer the minced tissue to a Potter-Elvehjem Teflon/glass homogenizer (~50 ml size) and complete volume to ~40 ml with 4 °C HMS.
6. Homogenize the sample on ice using approximately 20 strokes.
7. Dilute the tissue with 4 °C HMS to make a 15% homogenate (i.e. 15 g liver in 100 ml HMS).
8. Centrifuge at 1,000 x g for 1 minute to remove debris.
9. Decant supernatant into a clean tube and re-centrifuge at 10,000 x g for 10 minutes.

10. Carefully decant and discard supernatant.
11. Gently resuspend pellet in 1 volume HMS (1 ml/g liver) on ice. Vortex, with no added buffer first, then add small amounts of buffer and mix until a thick, uniform suspension is obtained.
12. Add one volume of chilled digitonin solution (1 ml/g liver) and gently swirl on ice for 2 minutes. To prepare digitonin: dissolve 35 mg digitonin in 19 ml of HMS with heat and stirring. Do not boil. Chill, add 1.0 ml of 10 mg/ml BSA. Make fresh every time.
13. Dilute sample with HMS to 5 volumes, and centrifuge at 20,000 x g for 5 minutes. Decant and discard most of the supernatant. Very gently disturb the loose (fluffy) layer on top of the pellet by swirling and pour it off.
15. Resuspend the pellet in 2 volumes of HMS and centrifuge at 20,000 x g for 5 minutes.
16. Repeat steps 12 and 13 (there will be little fluffy material after the first high-speed centrifugation). Decant the supernatant. The pellet primarily contains mitochondria, usually free of lysosomes and other cellular components. The expected yield should be 150 mg mitochondrial protein/g liver.

c) Western blotting and Detection

1. Process sample by SDS-PAGE electrophoresis and transfer to a nitrocellulose or PVDF membrane according to in-house procedures for western blotting.
2. Wash membrane with TBS for 5 minutes (20 mM Tris-Cl pH 7.5, 0.5 M NaCl).
3. Block the membrane in TBST (20 mM Tris-Cl pH 7.5, 0.5 M NaCl, 0.05% Tween[®] 20) with 5-10% non-fat dry milk for 1 hour.
4. Wash the membrane with TBS.
5. Dilute primary antibody 1:1000 in TTBS and incubate at room temperature for 2 hours or at 4 °C for about 16 hours. This dilution is the recommended starting point. Titration of the antibody is recommended to optimize the results.
6. Wash membrane in TTBS, 3 times for 10 minutes each.
7. Incubate with conjugated anti-rabbit antibody (diluted according to manufacturers instructions) for one hour at room temperature.
8. Quickly rinse the membrane with dH₂O. Wash membrane in TTBS, 3 times for 10 minutes each.
9. Proceed with detection method of choice depending on secondary antibody used.

The following protocol is for peroxidase detection following use of a peroxidase conjugated secondary antibody in step 7:

- i) Combine equal volumes of Trevigen's Peroxyglow Reagents A and B (cat#s 4855-20-13 and 4855-20-14) and cover membrane for 5 minutes.
- ii) Remove excess buffer from membrane, and place face down on plastic wrap. Turn the plastic covered membrane face-up and expose to a photographic film for 20 seconds (starting point). Results obtained can then be used as reference to determine the optimal exposure time.

C. Controls

- a) For Western blotting, purified recombinant PBR protein is available from Trevigen (cat# 6320-025-01). Load 1 µg of PBR protein (5 µl) per lane. Heat denature the control protein for 5 minutes before loading. The recombinant PBR is a fusion protein with a MW of 20.4 KDa, and is provided in 20 mM Tris pH 7.9, 0.5 M NaCl, 40 mM EDTA, 1% SDS . The size of the native protein is 18 kDa.

The following negative controls may be included in the experimental procedure:

- b) Omission of primary antibody
- c) Omission of secondary antibody
- d) A positive control for the antibody may be included. The MDA-231 breast cancer cell line has been shown to express high levels of PBR (1).

VI. References

1. Li W, Hardwick MJ, Rosenthal D, Culty M, Papadopoulos V. 2007. Peripheral-type benzodiazepine receptor overexpression and knockdown in human breast cancer cells indicate its prominent role in tumor cell proliferation. *Biochem Pharmacol* **73**:491-503.
2. Maeda J, Higuchi M, Inaji M, Ji B, Haneda E, Okauchi T, Zhang MR, Suzuki K, Suhara T. 2007. Phase-dependent roles of reactive microglia and astrocytes in nervous system injury as delineated by imaging of peripheral benzodiazepine receptor. *Brain Res* **1157**:100-11.

VII. Related Products

Apoptosis Kits

Catalog #	Description	Size
4817-60-K	FlowTACS™ Apoptosis Detection Kit	60 samples
4815-30-K	TumorTACS™ <i>In Situ</i> Apoptosis Detection Kit	30 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 tests
4830-01-K	TACS® Annexin V FITC Kit	100 samples
4835-01-K	TACS® Annexin V Biotin Kit	100 samples
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 tests
6305-100-K	MitoShift™ Mitochondrial Potential Assay Kit	100 tests
4823-30-K	NeuroTACS™ II <i>In Situ</i> Apoptosis Detection Kit	30 samples
4827-30-K	CardioTACS™ <i>In Situ</i> Apoptosis Detection Kit	30 samples
4829-30-K	DermaTACS™ <i>In Situ</i> Apoptosis Detection Kit	30 samples
4826-30-K	VasoTACS™ <i>In Situ</i> Apoptosis Detection Kit	30 samples
4828-30-DK	TACS•XL® DAB <i>In Situ</i> Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label <i>In Situ</i> Apoptosis Detection Kit	30 samples
4810-30-K	TACS® 2 TdT DAB <i>In Situ</i> Apoptosis Detection Kit	30 samples
4811-30-K	TACS® 2 TdT Blue Label <i>In Situ</i> Apoptosis Detection Kit	30 samples
4812-30-K	TACS® 2 TdT Fluorescein Apoptosis Detection Kit	30 samples
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	20 samples
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests
4684-096-K	HT Colorimetric PARP Apoptosis Assay	96 samples
4685-096-K	HT Chemiluminescent PARP Apoptosis Assay	96 samples

Antibodies

Catalog #	Description	Size
2281-MC-100	Anti-Bax Monoclonal Antibody (Clone YTH-6A7)	100 µg
2291-MC-100	Anti-Bcl-2 Monoclonal Antibody (Clone YTH-8C8)	100 µg
4411-PC-100	Anti-Phosphorylated Histone-γ-H2AX polyclonal	100 µl
6361-PC-100	Anti-human/mouse-PBR polyclonal	100 µl
6370-MC-100	Anti-human/murine-Cytochrome C	100 µg
6380-MC-100	Anti-human/murine-Holocytochrome C	100 µg
4335-MC-100	Anti-PAR polymer mAb (10HA)	100 µl
4336-BPC-100	Anti- PAR polymer polyclonal	100 µl
4338-MC-50	Anti-human/murine-PARP mAb (clone C2-10)	50 µg

Reagents

Catalog #	Description	Size
6360-025-01	Recombinant PBR Protein	25 µl
4878-05-02	Cytonin IHC™	2 x 5 ml
4870-500	10X PBS	500 ml
4865-25	Mounting Medium	25 ml
4866-20	Fluorescence Mounting Medium	20 ml
4855-20-13	PeroxyGlow™ A	20 ml
4855-20-14	PeroxyGlow™ B	20 ml
4861-100	Treated Glass Microscope Slides	100

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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