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Hypoxyprobe™-1 Kit

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)
Mouse IgG₁ monoclonal antibody (MAb1)

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence; immunoperoxidase; western blotting; or, flow cytometry.

Quantities:

- Hypoxyprobe™-1 Plus Kits contain 100 mg, 200 mg or 1000 mg of pimonidazole HCl. Typically a dosage of 60mg/kg body weight is used for animal studies.
- 1.0 or 2.0 ml of an exhausted supernatant from hybridoma clone 4.3.11.3 containing 70 micrograms/ml of an IgG₁ mouse monoclonal antibody and 0.15% sodium azide. Optimal dilution of MAb1 is to be determined by investigators but a 1/50 dilution has been found to give strong immunostaining in mouse tumor tissue when combined with a secondary strategy comprised of biotin-conjugated F(ab')₂ antimouse secondary reagent, streptavidin peroxidase and peroxidase substrate. Alternative secondary strategies can be used.

Not supplied: Secondary reagents such as biotin conjugated F(ab')₂ antimouse secondary reagent, streptavidin peroxidase or standard IHC reagents including buffers, peroxidase substrate, etc.

Storage:

- Store Hypoxyprobe™-1 solid at room temperature or 2-8 degrees C in the dark.
- Store the mouse monoclonal antibody (MAb1) frozen.

Detailed Description of Hypoxyprobe™-1 Kit components

1) Hypoxyprobe™-1 is a substituted 2-nitroimidazole whose chemical name and only ingredient is pimonidazole hydrochloride. Hypoxyprobe™-1 has a molecular weight of 290.8; a water solubility of 400 millimolar equivalent to 116 mg/ml; and, ultraviolet absorbance at 324 nm with an extinction coefficient of 7020 in 0.9% saline. The free base, pimonidazole, has a molecular weight of 254.3, a pKa of 8.7 and an octanol water partition coefficient of 8.5. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications of Hypoxyprobe™-1 Kits.

Hypoxyprobe™-1 is chemically stable in both solid form and aqueous solution. For example, solid Hypoxyprobe-1 has been stored for two years at room temperature in subdued light without detectable degradation as assessed by UV and HPLC analyses. Hypoxyprobe™-1 solutions in 0.9% saline have been stored at a concentration of 100 gms/liter at 4°C for 4.5 years without detectable degradation (UV and HPLC analyses).

Pimonidazole is reductively activated in hypoxic cells. The activated intermediate forms stable covalent adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. The antibody reagent MAb1 binds to these adducts allowing their detection by immunochemical means. See www.hypoxyprobe.com for mechanism of action, frequently asked questions (FAQ) and applications for Hypoxyprobe™-1 kits.

2) MAb1 is a mouse IgG₁ monoclonal antibody (MAb) clone 4.3.11.3 that is supplied as a filter sterilized, exhausted supernatant from hybridoma clone 4.3.11.3 containing 70 microgram/ml of MAb1 and 0.15% sodium azide for added stability. Tissues of interest can be studied by immunohistochemistry on frozen fixed sections or formalin fixed paraffin embedded sections; by flow cytometry following tissue disaggregation; or, Western blotting. Typically, 100 microliters of a 1:50 dilution of MAb1 is added to tissue sections. A suitable secondary strategy is then applied to reveal pimonidazole adducts. Two examples of suitable protocols are provided below.

Note: MAb1 can bind to protein, peptide and amino acid adducts of pimonidazole in hypoxic cells but tissue processing for immunohistochemical assay washes out small molecule peptide and amino acid adducts so that immunohistochemical detection of hypoxia relies on protein adducts of pimonidazole in hypoxic tissue.

Assay Instructions

1. Investigations of normal or tumor tissue hypoxia begin with the intravenous infusion, intraperitoneal injection or oral ingestion of a HypoxyprobeTM-1 solution at a dosage of 60 mg/kg body weight. For a 25 gram mouse this amounts to 1.5 mg/mouse. (Dosages up to 400 mg/kg have been used in mice without detectable toxicity or change in tissue hypoxia but 60 mg/kg gives good immunostaining at minimum cost.) The solubility of HypoxyprobeTM-1 in saline is 116 mg/ml so that very small volumes can be used to administer HypoxyprobeTM-1.

Following injection or ingestion, HypoxyprobeTM-1 distributes to all tissues including brain but it forms adducts with thiol containing proteins only in those cells that have a oxygen concentration less than 14 micromolar -- equivalent to a partial pressure $pO_2 = 10$ mm Hg at 37°C. In addition to tumors, normal tissues such as liver, kidney and skin possess cells at, or below, a pO_2 of 10 mmHg. These normal tissues bind HypoxyprobeTM-1.

The plasma half-life of HypoxyprobeTM-1 in mice is approximately 25 minutes (see the FAQ link at www.hypoxprobe.com for references). For comparison, plasma half-lives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Mouse tissues of interest may be harvested 15 to 90 minutes after HypoxyprobeTM-1 administration. HypoxyprobeTM-1 residing in tissues at the time of harvest will be bound when dissected tissues go anoxic. However, the amount of residual HypoxyprobeTM-1 is very small compared to the amount that tissues are exposed to during a 15 to 90 minute experiment so that any non-specific binding due to residual HypoxyprobeTM-1 is undetectable.

In addition to animal studies, HypoxyprobeTM kits can be used for cells in tissue culture (see Applications link at www.hypoxprobe.com). Typically, cell suspensions are incubated under hypoxia for 1 to 2 hours in the presence of 100 to 200 micromolar HypoxyprobeTM-1. The cells are harvested by cytopspin, fixed and immunostained with MAb1 and a chromogenic or fluorescent secondary reagent.

Suggested procedure for immunostaining Hypoxyprobe™-1 adducts in formalin-fixed, paraffin-embedded tissues using a F(ab')₂ secondary strategy.

Raleigh, Chou et al., Int. J. Radiat. Oncol. Biol. Phys. 42: 727-730, 1998

Hypoxyprobe™ technology is robust and investigator-initiated modifications are encouraged.

Step	Procedure	Time, min.	Temp.	Reagents	Notes
1	Warm paraffin tissue section	20	40°C	None	
2	Dip and blot 10 times		RT	Clear-Rite 3	1
3	"		RT	100% Ethanol	
4	"		RT	95% Aqueous ethanol	
5	"		RT	80% Aqueous ethanol	
6	"		RT	0.2% Brij 35 in distilled water	2
7	"		RT	PBS+ 0.2% Brij 35	3
8	Quench tissue peroxidase	5	RT	3% H ₂ O ₂ in distilled water	4
9	Antigen retrieval	20	90°	Target retrieval agent	5,6
10	Wash	2	0°	PBS + 0.2% Brij 35	7
11	Block non-specific binding	5	RT	Protein blocking agent	8,9
12	1° MAb1, slides horizontal	40	RT	Hypoxyprobe-1 MAb1 (1/50)	10
13	Wash	2	0°	PBS + 0.2% Brij 35	7
14	2° antibody	10	RT	Biotin-conjugated F(ab') ₂ (1/500)	11
15	Wash	2	0°	PBS + 0.2% Brij 35	7
16	Peroxidase chromogen	10	RT	Streptavidin peroxidase	12
17	Wash	2	0°	PBS + 0.2% Brij 35	7
18	Peroxidase substrate	10	RT	DAB	13
19	Wash	2	RT	Distilled water	14
20	Counterstaining	0.5	RT	Hematoxylin	15
21	Wash	2	RT	Distilled water	14
22	Mount & dry slides	20	RT	Crystal Mount	16

Technical Notes

1. For deparaffinization (step 1), hydration (steps 2-7) and peroxidase quenching (step 8), slides are held vertically. ProbeOn plus slides (cat# 15-188-52) and a MicroProbe Staining Station, both from Fisher Scientific, are suitable for these steps. Clear-Rite 3, a less toxic alternative to xylene, is available from Richard-Allan Scientific (Kalamazoo, MI)(cat# 6901).

Note: *These steps were designed for the MicroProbe Staining Station but other routine IHC procedures can be used.*

2. Brij 35 is enzyme grade polyoxyethylene(23)lauryl ether available from Fisher Scientific (cat# BP345-500). Alternatives to Brij35 can be used including Tris buffered saline (TBS) available from Chemicon International, Temecula, CA (Cat# 20845) containing a final concentration of 0.1% Tween 20®.

3. PBS is 10 mM phosphate buffered saline prepared from tablets available from Sigma (Cat# P-4417).

4. 3% H₂O₂ is diluted Analytical Reagent grade 31.3% H₂O₂ available from Malinckrodt Baker (Paris, KT)(Cat# 5240). Commercially available peroxidase inhibitors may also be used.
5. Antigen retrieval agents include AbD Serotec Cat# BUF024C (pH8); Chemicon International Cat# 21545 or DAKO Cat# S2369. For antigen retrieval, slides are submerged in retrieval reagent in a slide holder and heated for 5-20 minutes at 90°C.
6. Slides held vertically in slide incubator.
7. Slides washed with magnetically stirred PBS + 0.2% Brij 35 in a Coplin jar held on ice.
8. For example, serum free protein blocker from DAKO Corp. (Carpinteria, CA)(Cat# X0909).
9. Slides held horizontally for steps 11-18 so as to limit non-specific, edge staining of the sections.
10. Exhausted supernatant containing MAb1 diluted 1/50 in 10 mM PBS containing 0.2% Brij 35 and 1 drop of DAKO protein blocker/ml. Commercially available antibody diluents may also be used. Typically, 100 uL of diluted MAb1 solution is applied to each tissue section.
11. For example, Biotin-conjugated rabbit F(ab')₂ fragment that binds to mouse IgG diluted 1/500 in 10 mM PBS containing 0.2% Brij 35 and 1 drop of DAKO protein blocker/mL

Secondary strategies other than the F(ab')₂ approach can be used. For example, DAKO Catalyzed Signal Amplification for mouse antibodies (Kit# K1500) is used routinely in our laboratories for clinical samples.

12. Peroxidase conjugated streptavidin from DAKO (Cat# K1016).
13. Liquid 3,3'-diaminobenzidine reagent (DAB) from DAKO (cat# K3465).
14. Slides washed with magnetically stirred distilled water at RT in a Coplin jar.
15. Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International (Cat# 20844).
16. Biomedica Crystal Mount from Fisher Scientific (Cat# BM-M03).

Procedure for immunostaining Hypoxyprobe-1 adducts in frozen, fixed tissues.

Most of the published work reporting fluorescence immunohistochemical detection of pimonidazole adducts is based on frozen sections and much of the data comes from Dr. A. J. van der Kogel's laboratory in Nijmegen. The tumor or tissue specimen is collected and directly frozen in liquid nitrogen until cryosectioned into 4 um sections. Consecutive sections are cut at the largest circumference of the tissue. The sections are then stored at -80°C until stained. After thawing, the sections are fixed in cold acetone (4°C) for 10 min. The sections are rinsed and incubated overnight at 4°C with mouse monoclonal anti-pimonidazole antibody (clone 4.3.11.3)(MAb1) diluted in PBS containing 0.1% bovine serum albumin c and 0.1% Tween 20 – the extent of dilution determined by investigator. The sections are then incubated for 90 min with Cy-3-conjugated goat anti-mouse antibody 1:150 (Jackson Immuno Research Laboratories). Between all steps of the staining procedure, the sections are rinsed three times with for 2 minutes in PBS.