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Hypoxyprobe™ Kit

(HPI Catalog # HP1-XXX)

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)
Anti-pimonidazole mouse IgG_1 monoclonal antibody (MAb1) (60 micrograms/ml)

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

Quantities: a. Hypoxyprobe™ Kit contains 100 mg, 200 mg or 1000 mg of pimonidazole HCl. Typical doses are 60mg/kg body weight for small animal studies and 14mg/kg body weight for human studies

b. 1.0 or 2.0 ml of exhausted supernatant from hybridoma clone 4.3.11.3 containing 60 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. Note that 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: a. Store pimonidazole HCl solid at room temperature in the dark.

b. Store the mouse monoclonal antibody (MAb1) frozen. Avoid repeated freeze thaw cycles by aliquoting antibody into small volumes for storage at -20 degrees C.

Detailed Description of Hypoxyprobe™ 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 solution solution. 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™ 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 solution 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™ kits.

2) MAb1 is a mouse IgG1 monoclonal antibody (MAb) clone 4.3.11.3 that is supplied as a filtersterilized, exhausted supernatant from hybridoma clone 4.3.11.3 containing 60 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 by 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

Investigations of normal or tumor tissue hypoxia begin with the intravenous infusion, intraperitoneal injection or oral ingestion of a Hypoxyprobe™-1 solution at a recommended 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 Hypoxyprobe™-1 in saline solution is 116 mg/ml so that very small volumes can be used to administer Hypoxyprobe™-1.

Following injection or ingestion, Hypoxyprobe™-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 pO2 = 10 mm Hg at 37 ºC. In addition to tumors, normal tissues such as liver, kidney and skin possess cells at, or below, a pO2 of 10 mmHg. These normal tissues can bind Hypoxyprobe™-1.

The plasma half-life of Hypoxyprobe™-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 are typically harvested 60 to 90 minutes after Hypoxyprobe™-1 administration. Hypoxyprobe™-1 residing in tissues at the time of harvest will be bound when dissected tissues go anoxic. However, the amount of residual Hypoxyprobe™-1 is very small compared to the amount that tissues are exposed to during a 60 to 90 minute experiment so that any non-specific binding due to residual Hypoxyprobe™-1 is undetectable.

In addition to animal studies, Hypoxyprobe™ 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 Hypoxyprobe™-1. The cells are then harvested by cytospin, fixed and immunostained with MAb1 and a chromogenic or fluorescent secondary reagent.

Suggested procedure for immunostaining pimonidazole 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	Dewax, Dip and Blot x 10	2	RT	Clear-Rite 3	1
3	Rehydrate, Dip and Blot x 10	2	RT	100% Ethanol	
4	"	2	RT	95% Aqueous ethanol	
5	"	2	RT	80% Aqueous ethanol	
6	"	2	RT	0.2% Brij 35 in distilled water	2
7	"	2	RT	PBS+ 0.2% Brij 35	3
8	Quench tissue peroxidase	5	RT	3% H2O2 in distilled water	4
9	Antigen retrieval	20	90 °C	Target retrieval agent	5,6
10	Cool to RT	20	RT	None	
11	Wash	2	RT	PBS + 0.2% Brij 35	7
12	Block non-specific binding	5	RT	Protein blocking agent	8,9
13	1° MAb1, slides horizontal	60	RT	Hyproxyprobe-1 MAb1 (1/50)	10
14	Wash	2	0 °C	PBS + 0.2% Brij 35	7
15	2° Reagent	10	RT	Biotin-conjugated F(ab')2 (1/500)	11
16	Wash	2	0 °C	PBS + 0.2% Brij 35	7
17	3° Reagent	10	RT	Streptavidin peroxidase	12
18	Wash	2	0 °C	PBS + 0.2% Brij 35	7
19	Peroxidase substrate	10	RT	DAB	13
20	Wash	2	RT	Distilled water	
21	Counterstaining	0.5	RT	Hematoxylin	14
22	Wash	2	RT	Running tap water	
23	Cover tissue sections	45	45 °C	Aqueous CC/Mount	15

Technical Notes

1. Clear-Rite 3 -- a less toxic alternative to xylene -- is available from VWR (Cat# 84000-052). For dewaxing (step 2) and tissue rehydration (steps 3-7), ProbeOn Plus slides (Fisher Scientific; Cat# 15-188-52) are held vertically in a MicroProbe Staining Station (Fisher Scientific) in pairs with tissue sections facing each other. The paired slides are dipped in solvent allowing capillary action to carry solvents over the tissue sections. Solvent is removed by blotting the lower end of the slide pair on adsorbent filter paper. The dip and blot procedure is repeated a total of 10 times for each of steps 2-7.

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 solution (TBS) available from Chemicon International, Temecula, CA (Cat# 20845) containing a final concentration of 0.1% Tween 20®.
3. PBS = 10 mM phosphate buffered saline solution that can be 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# BUF025B; Chemicon International Cat# 21545 or DAKO Cat# S2369. For antigen retrieval, slides are submerged in retrieval reagent in a slide holder and heated for 20 minutes at 90°C. Pimonidazole protein adducts are very robust so that the antigen retrieval reagent can be chosen on the basis of requirements for other factors of interest in tissue sections.

6. Slides held vertically in slide incubator.
7. Slides washed with magnetically stirred PBS + 0.2% Brij 35 in a rectangular staining jar.
8. For example, serum free protein blocker from DAKO Corp. (Carpinteria, CA; Cat# X0909). Protein blocker not washed from slide but flicked off so that residual blocker remains on tissue section.
9. Slides held horizontally for steps 13-20 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, although smaller volumes can be used if the tissue sections are "dammed" with a Pap pen.

Note: Incubation overnight at 4 degrees C can increase sensitivity in some cases.

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

Note: 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. For example, peroxidase conjugated streptavidin from DAKO (Cat# K1016).
13. Liquid 3,3'-diaminobenzidine reagent (DAB) from DAKO (cat# K3465).
14. Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International (Cat# 20844).
15. CC/Mount (Sigma; Cat# C9368), a direct replacement for Biomeda's Crystal Mount, is an aqueous based permanent mount for immunostained sections. Alternatives include cover slipping with Permount (Fisher Scientific; Cat# SP15-500).

Procedure for immunostaining pimonidazole 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 -80ºC until stained. After thawing, the sections are fixed in cold acetone (4ºC) for 10 min. The sections are rinsed and incubated overnight at 4ºC 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.