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Hypoxyprobe™-1 HRP Kit

(HPI Catalog # HP16-XXX)

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)

Anti-pimonidazole mouse IgG1 monoclonal antibody (MAb1) HRP (60 micrograms/ml)

DAB HRP chromogen

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

Quantities:

- Hypoxyprobe™-1 Kit contains 100 mg, 200 mg or 1000 mg of pimonidazole HCl. Typically a dosage of 60mg/kg body weight is used for animal studies.
- 1 or 2 200uL units of exhausted supernatant from hybridoma clone 4.3.11.3 containing 60 micrograms/ml of an IgG1 mouse monoclonal antibody conjugated to horseradish peroxidase. Optimal dilution of MAb1-hrp is to be determined by investigators but a 1/50 dilution has been found to give strong immunostaining in mouse tumor tissue. Note that a chromogen is included in the kit to activate the horseradish peroxidase to produce a brown stain.

Storage:

- Store pimonidazole HCl solid at room temperature in the dark.
- Store the mouse monoclonal antibody (MAb1-hrp) and chromogen at 2-8 degrees Celcius.

Detailed Description of Hypoxyprobe™-1 HRP 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 hrp is a mouse IgG1 monoclonal antibody (MAb) hrp clone 4.3.11.3 that is supplied as a filter

sterilized, exhausted supernatant from hybridoma clone 4.3.11.3 containing 60 microgram/ml of MAb1-hrp. 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-hrp 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-hrp 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 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 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°C. 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 harvested by cytopspin, fixed and immunostained with MAb1-hrp 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	Reagent	Note
1	Soften paraffin	20	40°C	None	
2	Dewax, Dip and Blot x10	2	RT	Clear Rite 3	1
3	Rehydrate, Dip and Blot x10	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% H ₂ O ₂ in distilled water	4

9	Wash with rinse buffer	2	RT	PBS+ 0.2% Brij 35	
10	Antigen Retrieval	20	90°C	Target retrieval reagent	5
11	Cool to RT	20	RT	None	
12	Wash with rinse buffer	2	RT	PBS + 0.2% Brij 35	
13	Block non-specific binding	10	RT	Protein blocking agent	6
14	Apply primary MAb	60	RT	Pacific Blue dye-MAb1 (1:50-100)	7
15	Wash with rinse buffer	2	RT	PBS+ 0.2% Brij 35	
16	Counterstain	25s	RT	Hematoxylin	8
17	Cover tissue section	45	45°C	CC/Mount	9

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.

- Brij 35 is enzyme grade polyoxyethylene(23)lauryl ether available from Fisher Scientific (Cat# BP345-500). Alternatives to Brij35 such as Tris buffered saline (TBS) available from Chemicon International, Temecula, CA (Cat# 20845) can be used as the rinse solution. It is recommended that Tween 20® be added to the TBS rinse buffer at a final concentration of 0.1%.
- PBS is 10 mM phosphate buffered saline prepared from tablets available from Sigma (Cat# P-4417), for example.
- 3% H₂O₂ is diluted Analytical Reagent grade 31.3% H₂O₂ available from Malinckrodt Baker, Paris, KT (Cat# 5240). Commercial peroxidase blocking agents can be used.
- Antigen retrieval agents such as AbD Serotec Cat# BUF025B; Chemicon International Cat# 21545; or, DAKO Cat# S2369; can be used. 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.
- Any serum free protein blocker such as that from DAKO Corp. (Carpinteria, CA)(Cat# X0909) can be used. Note that the protein block is not washed from the section but flicked off to leave residual protein block on the section.
- The reagent is a CY 7 dye conjugated, mouse anti-pimonidazole monoclonal antibody CY 7 dye -Mab1. The concentration of the CY 7 dye-conjugated monoclonal antibody is 0.5 mg/ml and the CY 7 dye to protein molecular ratio is typically 4:1. The investigator will determine the fluorescence strength as it relates to the original reduced pimonidazole concentration bound to hypoxic tissue.
- Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International Cat# 20844.
- CC/Mount (Sigma; Cat# C9368), a direct replacement for Biomed's Crystal Mount, is an aqueous based permanent mount for immunostained sections. Alternatives include cover slipping with Permound (Fisher Scientific; Cat# SP15-500).

Procedure for Frozen Tissue Sections:

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.