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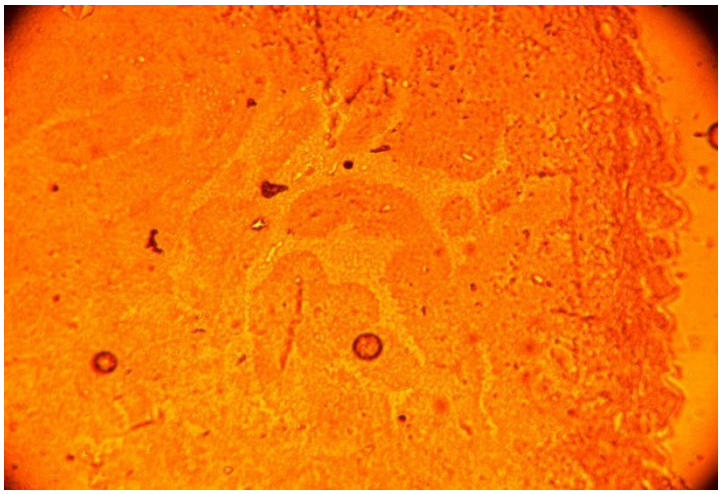

Hypoxyprobe™-1 TRITC-MAb Kit

HPI Catalog # HP9-XXX

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)
TRITC conjugated to mouse IgG_1 monoclonal antibody (TRITC-MAb1).

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immunoperoxidase or flow cytometry. This kit provides strong immunostaining with very low background in formalin fixed, paraffin embedded mouse tumor tissue.

Quantities: a. Hypoxyprobe™-1 TRITC Kits contain100 mg, 200 mg or 1000 mg of pimonidazole HCl. Typically a dosage of 60mg/kg body weight is used for animal studies.

b. One vial (100 and 200 mg Kits) or two vials (1000 mg Kit) containing 200 uL of a diluted TRITC-conjugated IgG_1 mouse monoclonal antibody (clone 4.3.11.3). Each vial contains 200 microliters of a 0.50 mg/ml solution of TRITC-MAb1 in PBS containing 1 % BSA and 0.09% sodium azide. Optimal dilution of TRITC-MAb1 is to be determined by the investigator but a 1:50-100 dilution has been found to give strong immunostaining in mouse tumor tissue when combined with a 1:50-100 dilution of the peroxidase

conjugated anti-TRITC secondary reagent. While not designed specifically for immunofluorescence, TRITC-MAb1 can be used for this purpose. Antibody dilution is typically lower for immunofluorescence than for immunoperoxidase detection.

Not supplied: Standard reagents used for immunohistochemical analyses.

Storage:
a. Store Hypoxyprobe™-1 solid at room temperature in the dark (can also be stored at 2-8 degrees C)
b. Store TRITC-MAb at 2-8 degrees C in the dark. **Do not freeze.**

Detailed Description of Hypoxyprobe™-1 TRITC Kit components

1) Hypoxyprobe™-1 is a substituted 2-nitroimidazole whose chemical name is pimonidazole hydrochloride with a molecular weight of 290.8; a water solubility of 400 millimolar (116 mg/ml); and, ultraviolet absorbance at 324 nm (extinction coefficient 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 for Hypoxyprobe™-1 kits.

Solid Hypoxyprobe™-1 has been stored for two years at room temperature in subdued light and stored in 0.9% saline (100 gms/liter) at 4°C for 4.5 years without detectable degradation (UV and HPLC analyses).

Pimonidazole is reductively activated in hypoxic cells and forms stable adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. TRITC-MAb1 binds to these adducts allowing their detection by immunochemical means.

2) TRITC-MAb1, a Rhodamine-conjugated mouse IgG_1 monoclonal antibody (MAb clone 4.3.11.3), is supplied at a concentration of 0.5 mg/ml in PBS containing 1 % BSA and 0.09% sodium azide as stabilizers. Tissues of interest can be studied by immunohistochemistry on frozen fixed sections or formalin fixed paraffin embedded sections or by flow cytometry following tissue disaggregation.

Note: TRITC-MAb1 binds to protein, peptide and amino acid adducts of pimonidazole but tissue processing during immunohistochemistry washes away peptide and amino acid adducts so that immunohistochemical hypoxia detection 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 (pimonidazole HCl) 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 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 is distributed 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 = 10 mmHg. These normal tissues 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 may be harvested 15 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 but the amount of residual Hypoxyprobe™-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 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 cytospin, fixed and immunostained with TRITC-MAb1.

Updated 2018

Suggested protocol for immunostaining pimonidazole adducts in formalin-fixed, paraffin-embedded tissue sections using TRITC-conjugated anti-pimonidazole mouse monoclonal antibody.

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% H2O2 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	TRITC-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.

2. 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%.

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

4. 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.

5. 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.

6. 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.

7. The reagent is a TRITC conjugated, mouse anti-pimonidazole monoclonal antibody (TRITC-Mab1). The concentration of the TRITC-conjugated monoclonal antibody is 0.5 mg/ml and the TRITC to protein molecular ratio is typically 4:1. The investigator will determine the optimum dilution but a 1:50-100 dilution in antibody diluent (e.g., Chemicon International, Cat# 21544) gives strong immunostaining with low background for formalin fixed, paraffin embedded, tissue sections. Typically, 100 microliters of diluted TRITC-MAb1 is applied to each tissue section. TRITC-MAb1 can be used on tissue sections from all species including mice.

8. Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International Cat# 20844.

9. 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.