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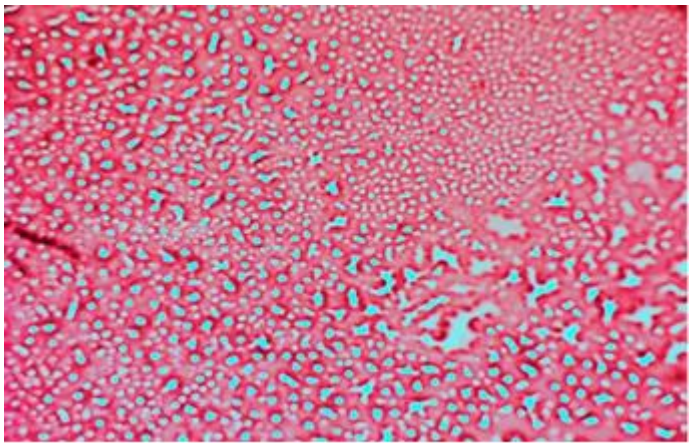

RED 594 dye linked anti pimonidazole mAb on hypoxic mouse lung tissue from a mouse treated with hypoxia marker pimonidazole HCl.

Hypoxyprobe™-1 ATTO RED 594 Kit

(HPI Catalog # HP13-XXX)

Kit contents:

Solid pimonidazole HCl (Hypoxyprobe™-1)

RED 594 dye conjugated to mouse IgG1 monoclonal anti pimonidazole antibody (RED 594-MAb1)

Fluorescence: Bright far red fluorescence with very high quantum yield. Fluorescence lasts several nanoseconds. pH stable and no degradation of dye over several hours. Excitation maximum around 600nm and emission maximum around 628nm.

Applications: Designed for super resolution fluorescence microscopy, ELISA, flow cytometry, Western Blotting, Micro Array Assays, IF. Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immunoperoxidase or flow cytometry. This Hypoxyprobe™-1 RED 594 Kit provides strong immunostaining with very low background in formalin fixed, paraffin embedded mouse tumor tissue.

Quantities: a. Hypoxyprobe™-1 RED 594 Kits contain 100 mg, 200 mg or 1000 mg of Hypoxyprobe™-1 (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 RED 594 dye-conjugated IgG1 mouse monoclonal antipimonidazole antibody (clone 4.3.11.3). Each vial contains 200 microliters of a 0.50 mg/ml solution of RED 594 dye -MAb1 in PBS containing 1 % BSA and 0.09% sodium azide. Optimal dilution of RED 594 dye -MAb1 is to be determined by the investigator but a 1:50-100 dilution has been found to give strong immunofluorescence staining in mouse tumor tissue. Designed specifically for immunofluorescence, RED 594 dye -MAb1 can visualized under a fluorescence microscope or in Flow cytometry and Western Blotting applications. Antibody dilution is typically lower for immunofluorescence than used in immunoperoxidase detection systems.

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 °C in the dark).

b. Store RED 594 dye -MAb at 2-8 degrees C in the dark. **Do not freeze.**

Detailed Description of Hypoxyprobe™-1 RED 594 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 under Knowledge Center 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 g/liter) at 4oC 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. RED 594 dye -MAb1 binds to these adducts allowing their detection by immunochemical means.

2) RED 594 dye -MAb1, a dye 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: RED 594 dye -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 pO2 = 10 mm Hg at 37oC. 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, frequently asked questions, link at www.hypoxprobe.com under Knowledge Center for references). For comparison, plasma halflives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Mouse tissues of interest may be harvested 60 minutes, 3 half lifes, or longer 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 60 minute or longer experiment so that any non- specific binding due to residual Hypoxyprobe™-1 is undetectable.

Spheroid and Cell Culture Method:

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 RED 594 MAb-1.

Suggested protocol for immunostaining pimonidazole adducts in formalin-fixed, paraffin-embedded

tissue sections using RED 594-conjugated anti-pimonidazole mouse monoclonal antibody.

Step	Procedure	Time, Min	Temp	Reagent	Note
1	Soften paraffin	20	40ºC	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	90ºC	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	RED 594 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	45ºC	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 RED 594 dye conjugated, mouse anti-pimonidazole monoclonal antibody RED ATTO dye -Mab1. The concentration of the RED 594 dye-conjugated monoclonal antibody is 0.5 mg/ml and the RED ATTO 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.
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 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 -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.