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Hypoxyprobe™-1 Plus Kit

Kit contents:

**Solid pimonidazole HCl (Hypoxyprobe™-1)
FITC conjugated to mouse IgG₁ monoclonal antibody (FITC-MAb1) and
Rabbit anti-FITC conjugated with horseradish peroxidase as a secondary reagent.**

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immunoperoxidase or flow cytometry. Very low background in mouse tissues when the Hypoxyprobe™-1 Plus Kit is combined with an anti-FITC secondary reagent.

Quantities: a. 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.

b. One vial (100 and 200 mg Kits) or two vials (1000 mg Kit) containing 200 uL of a diluted FITC-conjugated IgG₁ mouse monoclonal antibody (clone 4.3.11.3)(FITC-MAb1). Each vial contains 200 microliters of a 0.50 mg/ml solution of FITC-MAb1 in PBS containing 1 % BSA and 0.09% sodium azide. Optimal dilution of FITC-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-FITC secondary reagent. While not designed specifically for immunofluorescence, FITC-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 or 2-8 ° C in the dark.
b. Store FITC-MAb1 and HRP conjugated rabbit anti-FITC at 2-8 degrees C in the dark.
Do not freeze.

Detailed Description of Hypoxyprobe™-1 Plus 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. FITC-MAb1 binds to these adducts allowing their detection by immunochemical means.

2) FITC-MAb1, a fluorescein-conjugated mouse IgG₁ 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: FITC-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.

3) The chromogenic anti-FITC secondary reagent is an affinity-purified rabbit IgG polyclonal conjugated to horseradish peroxidase that has been prepared for Hypoxyprobe, Inc. The peroxidase conjugated anti-FITC secondary protocol provides strong immunostaining with very low background in formalin fixed, paraffin embedded mouse tumor 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 cytopsin, fixed and immunostained with FITC-MAb1 and the anti-FITC chromogenic secondary reagent.

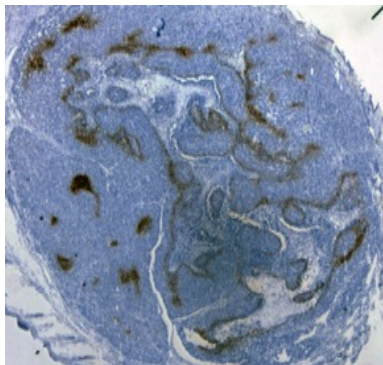


Figure. Immunoperoxidase staining for Hypoxyprobe binding in a formalin-fixed paraffin embedded tissue section from a rodent tumor using 1:100 dilution of FITC-MAb1 and 1:100 dilution of horseradish peroxidase conjugated rabbit anti-FITC IgG.

**Suggested protocol for immunostaining Hypoxyprobe™-1 adducts in formalin-fixed, paraffin-embedded tissue sections using a peroxidase conjugated anti-FITC secondary
(Samoszuk et al., Histochem Cytochem 52(6):837-839, 2004).**

Step	Procedure	Time, Min	Temp	Reagent	Note
1	Soften paraffin	20	45°C	None	
2	Deparaffinize	3	RT	Xylene	1
3	Deparaffinize	1	RT	Xylene	
4	Deparaffinize	1	RT	Xylene	
5	Clear xylene	1	RT	100% Ethanol	
6	Clear xylene	1	RT	100% Ethanol	
7	Hydrate tissue section	1	RT	95% Aqueous ethanol	
8	Hydrate tissue section	1	RT	70% Aqueous ethanol	
9	Hydrate tissue section	1	RT	Distilled water	
10	Hydrate tissue section	1	RT	Distilled water	
11	Transfer to rinse buffer	2	RT	1 x TBS + 0.1% Tween 20	2
12	Quench tissue peroxidase	5	RT	3% H ₂ O ₂ in distilled water	3
13	Antigen Retrieval	20	90°C	10 mM Citrate, pH 6	4
14	Wash in rinse buffer	2	RT	1 x TBS + 0.1% Tween 20	2
15	Block non specific binding	5	RT	Protein blocking agent	5
16	Wash in rinse buffer	2	RT	1 x TBS + 0.1% Tween 20	2
17	Apply primary MAb	30	RT	FITC-MAb1 (1:50-100)	6
18	Wash in rinse buffer	2	RT	1 x TBS + 0.1% Tween 20	2
19	Apply anti-FITC secondary	30	RT	HRP linked to rabbit anti-FITC (1:50-100)	7
20	Wash in rinse buffer	2	RT	1 x TBS + 0.1% Tween 20	2
21	Peroxidase chromogen	10	RT	DAB	8
22	Stop DAB reaction	1	RT	Distilled water	
23	Wash in rinse buffer	1	RT	1 x TBS + 0.1% Tween 20	2
24	Counterstain	1	RT	Hematoxylin	9
25	Wash in rinse buffer	1	RT	1 x TBS + 0.1% Tween 20	2
26	Dehydrate and clear	Note	RT	100% Ethanol and xylene	10
27	Coverslip	N/A	RT	Permunt	11

Technical Notes

1. To remove paraffin use clean xylene or Clear-Rite 3, a less toxic alternative to xylene that is available from Richard Allen Scientific, Kalamazoo, MI (Cat# 6901).
2. For example, stock (x20) Tris buffered saline available from Chemicon International, Temecula, CA (Cat# 20845). It is recommended that Tween 20® be added to the TBS rinse buffer at a final concentration of 0.1% (1.0 mL of Tween 20® for each 1 liter of 1 x TBS rinse buffer) and mixed thoroughly.
3. Rinse off the 3% H₂O₂ thoroughly as it can interfere with subsequent steps.

4. Antigen retrieval solution such as the citrate buffer available from Chemicon International (Cat# 21545) diluted 10 fold as a working solution.
5. For example, Chemicon International blocking agent (Cat# 20773). 1% BSA or 1% normal mouse serum in TBS can also be used.
6. The primary reagent is a FITC conjugated, mouse anti-hypoxypore monoclonal antibody (FITC-Mab1). The concentration of the FITC-conjugated monoclonal antibody is 0.5 mg/ml and the FITC to protein molecular ratio ca 4:1. The investigator will determine the optimum dilution for his or her application 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, mouse tumor sections. Typically, 100 microliters of diluted FITC-MAb1 is applied to each tissue section.
7. A rabbit, horseradish peroxidase-conjugated, secondary reagent is included in the Hypoxyprobe Plus Kit but any suitably labeled, secondary anti-FITC antibody can be used. A 1:50-100 dilution of the secondary rabbit anti-FITC horseradish peroxidase conjugated antibody in the Hypoxyprobe-1 Plus Kit is typically used.
8. Any commercially available liquid 3,3'-diaminobenzidine reagent (DAB) is suitable including DAB A and B reagents from Chemicon International (Cat# 71895 and 71896, respectively).
9. Any commercially available hematoxylin counterstaining reagent is suitable including Chemicon International Cat# 20844.
10. Use clean, fresh solutions to incubate slides for 1 minute each in 4 changes of 100% ethanol and then for 1 minute each in 3 changes of xylene.
11. Permount solution is available from Fisher Scientific (Cat# SP15-500). Other mounting procedures such as water-based Crystal Mount can be used.