**Hypoxyprobe, Inc**
**121 Middlesex Turnpike**
**Burlington, MA 01803 USA**
**www.hypoxyprobe.com**

# Hypoxyprobe™-1 Plus Kit

**(HPI Part # HP2-XXX)**

## Kit contents:

**Solid pimonidazole HCl (Hypoxyprobe™-1)**

**FITC conjugated to rat $IgG_1$ monoclonal antibody (FITC-MAb1) *and***

**Rabbit anti-FITC anti dye conjugated with horseradish peroxidase as a secondary reagent.**

Applications: Immunochemical detection of cell and tissue hypoxia including immunofluorescence, immuno-peroxidases or flow cytometry. There is very low background signal from naturally green fluorescing proteins (that would usually interfere with the hypoxic signal) in rat tissues when the Hypoxyprobe™-1 Plus Kit is combined with an anti-FITC secondary reagent.

Quantities: a. The 3 different size Hypoxyprobe™-1 Plus Kits contain either 100 mg, 200 mg or 1000 mg of pimonidazole HCl. A dosage of 60mg/kg body weight is sufficient for animal studies.

b. The 3 different size Hypoxyprobe™-1 Plus Kits also contain One vial (100 and 200 mg Kits) or two vials (1000 mg Kit) containing 200 uL of an anti-pimonidazole, FITC-conjugated $IgG_1$ rat monoclonal antibody (clone 11.23.22.R). Each vial contains 200 microliters of a 0.50 mg/ml solution of the FITC-MAb1 in PBS containing 1 % BSA and 0.09% sodium azide. Optimal dilution of the FITC-MAb1 is to be determined by the investigator but a 1:50-100 dilution has been found to give strong immunostaining in rat 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 in the dark (can also be stored at 2-8 degrees C).

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, anti-pimonidazole, rat $IgG_1$ monoclonal antibody (MAb clone 11.23.22.R), is supplied at a concentration of 0.5 mg/ml in PBS containing 0.09% proclin as a stabilizer. 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 anti dye protein 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 signal from any naturally green fluorescing proteins present in formalin fixed, paraffin embedded rat 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 25g rat this amounts to 1.5 mg/rat. 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, pimonidazole 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.hypoxyprobe.com for references). For comparison, plasma half-lives for rats is 45 minutes, dogs 90 minutes and humans 300 minutes. Rat 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 Knowledge Center (hypoxyprobe.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 FITC-MAb1 and the anti-FITC chromogenic secondary reagent.

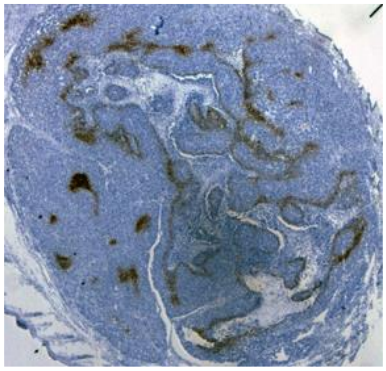

**Figure**. Immunoperoxidase staining for pimonidazole binding in a formalin-fixed paraffin embedded tissue section from a rodent tumor using 1:100 dilution of FITC-MAb1 and 1:100 dilution of horseradish peroxidase conjugated rabbit anti-FITC IgG.

**Suggested protocol for immunostaining pimonidazole adducts in formalin-fixed, paraffin-embedded tissue sections using FITC-conjugated anti-pimonidazole rat monoclonal antibody and peroxidase-conjugated anti-FITC rabbit secondary 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% $H_2O_2$ 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 | FITC-MAb1 (1:50-100) | 7 |
| 15 | Wash with rinse buffer | 2 | RT | PBS+ 0.2% Brij 35 | |
| 16 | Apply anti-FITC secondary | 30 | RT | HRP linked to rabbit anti-FITC (1:50-100) | 8 |
| 17 | Wash with rinse buffer | 2 | RT | PBS+ 0.2% Brij 35 | |
| 18 | Peroxidase chromogen | 10 | RT | DAB | 9 |
| 19 | Stop DAB reaction | 1 | RT | Distilled water flush | |
| 20 | Counterstain | 0.5 | RT | Hematoxylin | 10 |
| 21 | Cover tissue section | 45 | 45ºC | CC/Mount | 11 |

**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% $H_2O_2$ is diluted Analytical Reagent grade 31.3% $H_2O_2$ available from Malinckrodt Baker, Paris, KT (Cat# 5240). Commercial peroxidase blocking agents can be used.

5. Antigen retrieval agents such as BIORAD 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 primary reagent is a FITC conjugated, rat anti-pimonidazole monoclonal antibody (FITC-Mab1). The concentration of the FITC-conjugated monoclonal antibody is 0.5 mg/ml and the FITC 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 FITC-MAb1 is applied to each tissue section. FITC-MAb1 can be used on tissue sections from all species including mice.

8. 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.

9. 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).

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

11. 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).