Hypoxyprobe™-1 Kit for the Detection of Tissue Hypoxia

100mg Pimonidazole
1mL Hypoxyprobe™-1Mab1

Cat. No. HP1-100

Sufficient for the analysis of 5 sections each from 67 mice.

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures
Introduction

Oxygen gradients exist in normal and tumor tissue. These gradients affect gene expression and are important in normal and pathological conditions. Until recently it was difficult to measure these gradients at the cell; however, with the development of 2-nitroimidazole hypoxia markers, it is now possible to accurately measure oxygen gradient at the cellular level. There are a number of ways that the hypoxia markers can be detected. The immunohistochemical technique is particularly attractive because gradients of hypoxia can be visualized and compared with underlying hierarchical structures in tissues and with gene expression on a cell-by-cell basis. This insert describes the Hypoxyprobe™ system of 2-nitroimidazole hypoxia markers and its application to studies of tissue hypoxia under both normal and pathological conditions. The hypoxia marker that has received most attention in the Hypoxyprobe™ system of markers is Hypoxyprobe™-1, which is also known as pimonidazole hydrochloride.

Hypoxyprobe™-1 (pimonidazole hydrochloride)

Background

In 1976, Varghese et al. reported that $^{14}$C-labelled misonidazole formed adducts in hypoxic cells in vitro and in vivo (1). It was subsequently found that adducts form with thiol groups in proteins, peptides and amino acids in a way that all atoms of the ring and side-chain of the 2-nitroimidazole are retained (2-5). Hypoxia (pO$_2$ < 10 mmHg) is required for binding but binding is not dependent on the presence of specialized redox enzymes such as P450 nitroreductases. Furthermore, wide variations in NADH and NADPH levels do not change the oxygen dependence of binding (6, 7). Chapman et al. showed that the oxygen dependence of binding was fortuitously close to that for radiation resistance and suggested that misonidazole binding
might be used as a hypoxia marker in solid tumors (8). The clinical feasibility of the hypoxia marker idea was demonstrated by means of autoradiographic analyses of 3H-misonidazole binding in a variety of human tumors (9). While the 3H-misonidazole approach had limited clinical utility, it spurred the development of a variety of non-invasive assays for tissue hypoxia based on 2-nitroimidazoles. These included single photon electron capture tomography, positron emission tomography, nuclear medicine analysis and magnetic resonance spectroscopy of suitably labeled 2-nitroimidazole analogues (for review see ref. (10)).

During 19F MRS investigations of tumor hypoxia with the hexafluorinated 2-nitroimidazole, CCI-103F, it became clear that an histological assessment of hypoxia would be a useful complement to non-invasive assays (11-13). This led to the invention of the immunochemical hypoxia marker technique based on monoclonal and polyclonal antibodies raised against protein adducts of reductively activated 2-nitroimidazoles (14, 15). Preclinical testing of immunochemical reagents in spontaneous canine tumors showed that immunochemical hypoxia markers would be useful in their own right (16-21). In addition to providing a quantitative measure of hypoxia, immunohistochemical markers provide insights into microregional relationships between hypoxia and factors such as necrosis, proliferation, differentiation, apoptosis, and oxygen regulated protein expression. A variety of immunochemical hypoxia markers has now been used in clinical (22-27) and preclinical studies (16, 28-33) of such relationships. An example of the unique value of the immunohistochemical marker approach is the observation that neither metallothionein nor vascular endothelial growth factor are expressed in the majority of hypoxic cells in human squamous cell carcinomas (24, 25) even though in vitro studies would have predicted otherwise (34, 35).

With respect to quantifying hypoxia by the immunochemical technique, image analysis (24-27) or flow cytometric analysis (36, 37) appear most promising. Preclinical studies of sampling error showed that stratification of patients is feasible with the immunohistochemical approach if 3-4 biopsies are obtained from geographically separate regions of each tumor. Precision can be increased by increasing the number of sections analyzed per biopsy from 1 to 3 but analysis of multiple biopsies is the most important factor. Interestingly, the accuracy of the immunohistochemical analysis increases as the amount of hypoxia decreases (19, 21).

Protein adducts of reductively-activated pimonidazole are effective immunogens for the production of both polyclonal and monoclonal antibodies. The antibodies have been used for immunoperoxidase analysis of formalin fixed, paraffin embedded sections (6, 16, 23-26, 32, 40); for immunofluorescence analysis of frozen fixed sections (27, 41-43); and, for flow cytometry with directly labeled or secondary fluorescent antibodies (36). The antibodies have also been used in
enzyme linked immunosorbent assays (6, 16, 40). As is the case for Hypoxyprobe™-1 itself, the antibodies to pimonidazole adducts are very robust. For example, aqueous solutions of the IgG1 monoclonal antibody against pimonidazole adducts (clone 4.3.11.3) is stable indefinitely when stored at -20°C and is stable for at least 4 months at 4°C when supplemented with 10 mg/mL of bovine serum albumin and 10 millimolar sodium azide. One final attractive feature of pimonidazole is the fact that pimonidazole adducts in vivo are long-lived (16). This provides flexibility in the timing of biopsy taking which is an advantage in a clinical setting. In summary Hypoxyprobe™-1 and associated antibodies form a very attractive basis on which to develop a low tech, low cost kit for measuring normal and tumor tissue hypoxia.

**Application**

The rationale for developing pimonidazole hydrochloride (Hypoxyprobe™-1) as a hypoxia marker for experimental and clinical use was based on its chemical stability, water solubility, wide tissue distribution and, in the case of clinical studies, the fact that human toxicity data were available from earlier radiosensitizer trials. This facilitated early clinical application and Hypoxyprobe™ kits have now been used in many experimental studies and clinical trials worldwide. Solid Hypoxyprobe™-1 is very stable being unchanged after storage for 2 years at room temperature in subdued light. Saline solutions of Hypoxyprobe™-1 used for clinical studies (34 millimolar in 0.9% saline, pH 3.9 ± 0.1) are extremely stable being unchanged after 1.5 years at 4°C in subdued light as determined by high performance liquid chromatography and ultraviolet spectroscopy. In addition to chemical stability, Hypoxyprobe™-1 has high water solubility (400 millimolar; 116 grams per 1000 mL) that facilitates intravenous marker infusion and produces a short plasma half-life of 5.1 ± 0.8 hours. In spite of the water solubility of its hydrochloride salt (pKa 8.7), pimonidazole itself has an octanol-water partition coefficient of 8.5 (38) and diffuses readily into tumors and normal tissues including brain (39). Consistent with a large, 155 liter volume of distribution, pimonidazole concentrates approximately 3 fold above plasma levels in tumors and normal tissues (39) thereby increasing the sensitivity of hypoxia marking. At the Hypoxyprobe™-1 dose of 0.5 g/m² used in hypoxia marking, pimonidazole causes neither central nervous system toxicity nor sensation (e.g., flushing) (39). Central nervous system toxicity was of particular interest because this was the dose limiting toxicity for Hypoxyprobe™-1 at the higher, multiple doses used in radiosensitizer trials. In addition to the absence of central nervous system effects, the overall procedure from Hypoxyprobe™-1 infusion to tumor biopsy is well-tolerated in both inpatient and outpatient settings.

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Kit Components

1. **Hypoxyprobe-1**: (Part No.: 90203) 100mg Pimonidazole
2. **Hypoxyprobe-1Mab1**: (Part No.: 90204) 1mL Exhausted supernatant from the hybridoma clone 4.3.11.3.

Storage

**Hypoxyprobe 1**: Hypoxyprobe™-1 (Pimonidazole Hydrochloride) has great chemical stability in solid form and in aqueous solutions and requires no stabilizer. 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 10 gms/liter (34 millimolar) at 4°C in subdued light for 1.5 years without detectable degradation (UV and HPLC analyses). When exposed to laboratory light, Hypoxyprobe™-1 slowly turns yellow.

**Hypoxyprobe 1-Mab1**: Exhausted hybridoma supernatant containing Hypoxyprobe™-1MAb1 has been stored at -20°C for 6 years without detectable loss of activity. For short term storage, undiluted Hypoxyprobe-1MAb1 supernatant containing 1 drop/mL of protein block (DAKO) or 10 mg/mL of bovine serum albumin and 10 millimolar sodium azide can be stored at 4°C for up to 4 months without detectable loss of activity.

Assay Instructions

1. Although doses of Hypoxyprobe™-1 up to 400 mg/kg have been used without measurable toxicity in mice (36), a dose of 60 mg/kg body weight is routinely used in studies of tissue hypoxia in rodents. The high water solubility of Hypoxyprobe™-1 permits small volume injections to be made which is convenient for studies with small animals. Intravenous injection or intraperitoneal injection can be used. The plasma half-life of Hypoxyprobe™-1 is 0.5 hours in C3H/He mice. Hypoxyprobe™-1 is distributed to all tissues in the body including brain but binds only to cells that have oxygen concentrations less than 14 micromolar, which is equivalent to a pO₂ of 10 mm Hg at 37°C. Tumors and normal liver, kidney and skin have cells at, or below, this pO₂. For dogs, whole body doses of 0.28 gm/m² are recommended (16). The plasma half-life for Hypoxyprobe™-1 in dogs is 1.5 ± 1.0 hours.

In addition to animal studies, Hypoxyprobe™ kits can be used for cells in tissue culture (6, 44). 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 an antibody for pimonidazole adducts. Sufficient
concentrations of pimonidazole adducts are formed on the surface of cells to elicit a response to complement or activated cytotoxic lymphocytes (44).

2. Hypoxyprobe™-1MAb1 is a monoclonal antibody IgG1 (MAb) that detects protein adducts of Hypoxyprobe™-1 in hypoxic cells. Hypoxyprobe™-1MAb1 is provided as an untreated exhausted supernatant from the hybridoma clone 4.3.11.3. Fifteen to ninety minutes after the injection of Hypoxyprobe™-1, tumor or normal tissue is excised or biopsied. The tissue of interest is then studied as frozen sections, formalin-fixed paraffin-embedded sections, or as disaggregated tissues in flow cytometry assays. In the case of formalin-fixed, paraffin-embedded tissue sections, 150 µL of a 1:50 dilution of Hypoxyprobe™-1MAb1 is added to each tissue section. Chromogenic or fluorescent secondary antibody reagents are then used to reveal Hypoxyprobe™-1 adducts in the hypoxic tissue. For immunoperoxidase studies, we strongly recommend the use of the F(ab')2 secondary protocol described below because it can be used for a variety of animal species and tissues; it minimizes non-specific immunostaining; and, it is rapid. Hypoxyprobe™ kit 100MAb1 has enough hypoxia marker and antibody to analyze 5 sections from 67 (25 gram) mice.

Peroxidase Immunohistochemistry Technique

Recommended procedure for immunostaining Hypoxyprobe-1 adducts in formalin-fixed, paraffin-embedded tissues from a variety of animal species including mice.


<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Time, min.</th>
<th>Temp.</th>
<th>Reagents</th>
<th>Notes</th>
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<tr>
<td>1</td>
<td>Warm paraffinized tissue section</td>
<td>20</td>
<td>40°C</td>
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<td>2</td>
<td>Dip and blot 10 times</td>
<td>2</td>
<td>RT</td>
<td>Clear-Rite 3</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
<td>Dip and blot 10 times</td>
<td>2</td>
<td>RT</td>
<td>100% Ethanol</td>
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<tr>
<td>4</td>
<td>Dip and blot 10 times</td>
<td>2</td>
<td>RT</td>
<td>95% Aqueous ethanol</td>
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<tr>
<td>5</td>
<td>Dip and blot 10 times</td>
<td>2</td>
<td>RT</td>
<td>80% Aqueous ethanol</td>
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<tr>
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<td>Procedure</td>
<td>Time, min.</td>
<td>Temp.</td>
<td>Reagents</td>
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<tr>
<td>6</td>
<td>Dip and blot 10 times</td>
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<td>RT</td>
<td>Deionized water + 0.2% Brij 35</td>
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<tr>
<td>7</td>
<td>Dip and blot 10 times</td>
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<td>RT</td>
<td>PBS + 0.2% Brij 35</td>
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<tr>
<td>8</td>
<td>Quench tissue peroxidase</td>
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<td>RT</td>
<td>3% H₂O₂ in distilled water</td>
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<td>9</td>
<td>Antigen retrieval</td>
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<td>40°</td>
<td>0.01% Pronase</td>
<td>5,6</td>
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<tr>
<td>10</td>
<td>Wash</td>
<td>2</td>
<td>0°</td>
<td>PBS + 0.2% Brij 35</td>
<td>7</td>
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<tr>
<td>11</td>
<td>Block non-specific binding</td>
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<td>RT</td>
<td>DAKO blocking solution</td>
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<tr>
<td>12</td>
<td>1° MAb, slides horizontal</td>
<td>40</td>
<td>RT</td>
<td>Hypoxyprobe-1 MAb1 (1/50)</td>
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<td>13</td>
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<tr>
<td>14</td>
<td>2° antibody</td>
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<td>Biotin-conjugated F(ab')2 (1/500)</td>
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<tr>
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<tr>
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<td>2</td>
<td>RT</td>
<td>Distilled water</td>
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</tr>
<tr>
<td>22</td>
<td>Mount &amp; dry slides</td>
<td>20</td>
<td>RT</td>
<td>Crystal/Mount</td>
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</table>
Technical Notes

1. For deparaffinization, hydration and peroxidase quenching, steps 2-8, slides are held vertically. ProbeOn plus slides (cat# 15-188-52) and a MicroProbe Staining Station, both from Fisher Scientific, are suitable for these steps. Clear-Rite 3 is a non-toxic alternative to xylene and is available from Richard-Allan Scientific (Kalamazoo, MI)(cat.# 6901).

2. Brij 35 is enzyme grade polyoxyethylene(23)lauryl ether available from Fisher Scientific (cat.# BP345-500).

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

4. 3% H₂O₂ is diluted Analytical Reagent 31.3% H₂O₂ available from Malinckrodt Baker (Paris, KT)(cat.# 5240).


6. Slides held vertically in slide incubator.

7. Slides washed with magnetically stirred PBS + 0.2% Brij 35 in a Coplin jar held on ice.

8. Serum free protein blocker from DAKO Corp. (Carpinteria, CA)(cat. #X0909).

9. Slides held horizontally for steps 11-18 so as to limit non-specific, edge staining of the sections.

10. Exhausted supernatant containing 1°MAb diluted 1/50 in 10 mM PBS containing 0.2% Brij 35 and 1 drop of DAKO protein blocker/mL. Typically, 150 µL of diluted 1° MAb solution is applied to each tissue section.

11. Biotin-SP-conjugated F(ab')₂ fragment of a rabbit anti-mouse IgG from Accurate Chemical Scientific Corp. (Westbury, NY)(cat.#JZM066045) diluted 1/500 in 10 mM PBS containing 0.2% Brij 35 and 1 drop of DAKO protein blocker/mL. Secondary strategies other than the F(ab')₂ approach also can be used.

12. Peroxidase conjugated streptavidin from DAKO (cat.# K1016).

13. Liquid 3,3'-diaminobenzidine reagent (DAB) from DAKO (cat.#K3465).


15. Aqua Hematoxylin from Innovex Science (Richmond, CA)(cat.#NB305).

Frequently Asked Questions

Q: What is the best solvent for Hypoxyprobe™-1?

A: Hypoxyprobe™-1 is the hydrochloride salt of a weak base and, as such, is very soluble in aqueous solutions including neutral buffered saline (116 mg/mL or 400 millimolar).

Q. What dose of Hypoxyprobe™-1 should be used for hypoxia marking experiments?

A: For small animals of uniform size such as laboratory rats and mice, a dose of Hypoxyprobe™-1 of 60 mg/kg body weight is recommended. Doses ranging from 30 mg/kg (47) to 400 mg/kg (36) have been used in mice and rats without toxicity or altered oxygen levels due to blood flow effects. Nevertheless, significant effects on blood flow at doses above 100 mg/kg of Hypoxyprobe™-1 have been observed in tumors implanted in the hind legs of mice. Caution must be taken, therefore, when doses > 100 mg/kg are used.

For larger animals with non-uniform body size, the dose is calculated on the basis of surface area. For humans, the recommended dose is 0.5 gm/m2 (23) while for dogs a dose of 0.28 gm/m2 has been used (16).

Q. Does Hypoxyprobe™-1 penetrate hypoxic brain and brain tumor tissue?

A: Although Hypoxyprobe™-1 is water soluble, its corresponding free base has an octanol water coefficient of 8.5 and, as a result, the marker freely penetrates into both brain and brain tumor tissue (48, 49).
Q. Is Hypoxyprobe™-1 the best probe for detecting hypoxia in vivo?

A: Although other markers are available for detecting hypoxia in vivo, Hypoxyprobe™-1 has some real advantages. Foremost is its high solubility in aqueous solution (400 millimolar; 116 mg/mL of saline), which allows the marker to be administered to rodents as small volume (= 0.1 mL) injections of saline either intraperitoneally or intravenously. Other markers such as the hexafluorinated CCI-103F have aqueous solubilities of 10 millimolar or less.

Although Hypoxyprobe™-1, the hydrochloride salt of pimonidazole, is very water soluble, pimonidazole itself has an octanol-water partition coefficient of 8.5 and penetrates all tissues including brain.

Another advantage is that pimonidazole binding can be detected by immunofluorescence in frozen fixed tissue sections; by immunoperoxidase in formalin fixed paraffin embedded tissue sections; by ELISA or by flow cytometry.

Q. Can the monoclonal antibody to Hypoxyprobe™-1 adducts be used on mouse tissue?

A: Yes. For formalin fixed paraffin embedded tissues we recommend a peroxidase F(ab)2 secondary antibody strategy (32). This gives a very clean background and is applicable to a variety of animal species.

References


34. Leith, J. T. and Michelson, S. Secretion rates and levels of vascular endothelial growth factor in clone A or HCT-8 human colon tumour cells as a function of oxygen concentration, Cell Prolif. 28: 415-430, 1995.


Warranty

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