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In vitro diagnostic medical devices — Information supplied by the manufacturer with in vitro diagnostic reagents for staining in biology

Dispositifs médicaux de diagnostic in vitro — Informations fournies par le fabricant avec les réactifs de coloration de diagnostic in vitro utilisés en biologie

Reference number
ISO 19001:2002(E)

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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 19001 was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

Annex A of this International Standard is for information only.

Introduction

This International Standard relates to EN 375 and EN 376 and should be used in conjunction with these.

The use of reagents required for staining in biology as well as the specific examples of information supplied by the manufacturer for four staining procedures as provided in annex A are based on a European consensus; they constitute the scientific justification for the requirements listed in clause 4. This information is to assist manufacturers, suppliers and vendors of dyes, stains, chromogenic reagents and other reagents used for staining in biology in complying with the required specific product data.

In vitro diagnostic medical devices — Information supplied by the manufacturer with in vitro diagnostic reagents for staining in biology

1 Scope

This International Standard specifies requirements for information supplied by the manufacturer with reagents used in staining in biology. It applies to producers, suppliers and vendors of dyes, stains, chromogenic reagents and other reagents used for staining in biology. The requirements for information supplied by the manufacturer specified in this International Standard are a prerequisite for achieving comparable and reproducible results in all fields of staining in biology.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 31-8, *Quantities and units — Part 8: Physical chemistry and molecular physics*

ISO 1000, *SI units and recommendations for the use of their multiples and of certain other units*

EN 375, *Information supplied by the manufacturer with in vitro diagnostic reagents for professional use*

EN 376, *Information supplied by the manufacturer with in vitro diagnostic reagents for self-testing*

3 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply.

3.1
information supplied by the manufacturer
all printed, written, graphic or other information annexed to, or accompanying an in vitro diagnostic reagent

3.2
label
any printed, written or graphic information placed on a container

[EN 375]

3.3
in vitro diagnostic reagent
reagent that, used alone or in combination with other in vitro diagnostic medical devices, is intended by the manufacturer to be used in vitro for examination of substances derived from human, animal or plant sources, for providing information relevant to the detection, diagnosis, monitoring or treatment of physiological states, states of health or disease, or congenital abnormality

3.4
staining
impartment of colour to a material by means of reaction with a stain or chromogenic reagent

3.5
dye
coloured organic compound that, when dissolved in a suitable solvent, can impart colour to a material

NOTE The physical origin of colour is the selective absorbance (and/or emission) in the visible region of the electromagnetic spectrum between 400 nm and 800 nm. Dyes are molecules with large systems of delocalized electrons (conjugated π-electronic system). The light absorbance characteristics of dyes are displayed by absorbance spectra, resulting from plotting absorbance of light against wavelength. The shape of the spectra and the wavelength at maximum absorbance depend on the chemical structure of the dye, the solvent and on the conditions of the spectral measurements.

3.6
stain
solution of one or more dyes at defined concentrations in a defined solvent used for staining

NOTE The stain can be prepared by directly dissolving the dye in the solvent or by dilution of a stock solution with suitable agents.

3.6.1
stock solution of stain
stable defined solution of one or more dyes at a higher concentration than that used for staining

NOTE Stability refers to constant properties of the dye even in the presence of other dyes.

3.7
chromogenic reagent
reagent that reacts with certain chemical groups present or induced in cells and tissues with the formation of a coloured compound *in situ*

EXAMPLE A typical chromogenic reagent is:

a) diazonium salt;

b) Schiff's reagent.

3.8
fluorochrome
reagent that emits visible light when irradiated with excitation light of a shorter wavelength

3.9
antibody
specific immunoglobulin formed by B-lymphocytes in response to exposure to an immunogenic substance and able to bind to this

NOTE The molecule of an immunogenic substance contains one or more parts with a characteristic chemical composition, an epitope.

3.9.1
polyclonal antibody
mixture of antibodies capable of reacting specifically with a certain immunogenic substance

3.9.2
monoclonal antibody
antibody capable of reacting specifically with a single epitope of a certain immunogenic substance

3.10
nucleic acid probe
single stranded oligonucleotide or polynucleotide of defined length complementary to specific sequences of nucleotides in nucleic acids

3.11
lectin
protein of non-immunogenic origin with two or more binding sites that recognize and bind to specific saccharide residues

4 Requirements for information supplied by the manufacturer

4.1 General requirements

4.1.1 Information supplied by the manufacturer with reagents used for staining in biology

Information supplied by the manufacturer with reagents used for staining in biology shall be in accordance with ISO 31-8, ISO 1000, EN 375 and EN 376. Special attention shall be given to cautionary statements as given in EN 375. Furthermore, where relevant, the requirements as specified in 4.1.2, 4.1.3 and 4.1.4 shall be met for the various reagents used for staining in biology.

4.1.2 Product name

The product name shall include CAS-registry number and Colour Index name and number, where applicable.

NOTE 1 CAS-registry numbers are the Chemical Abstracts Service registry numbers. These are unique numerical code numbers assigned to chemical substances indexed by Chemical Abstracts.

NOTE 2 The Colour Index gives a 5-digit number, the C.I. number and a specially constructed name to most dyes.

4.1.3 Description of reagent

The description of the reagent shall include appropriate physico-chemical data accompanied by relevant data sheets for each batch. The data shall contain at least the following information:

a) the molecular formula including counter-ion;

b) the molar mass (g/mol) clearly stating whether this is with or without counter-ion;

c) the permissible limits of interfering substances.

For coloured organic compounds, the data shall also contain:

d) the molar absorbance (this can be substituted by content of the pure dye molecule but not by content of total dye);

e) the wavelength or wave number at maximum absorbance;

f) thin layer chromatographic, high performance liquid chromatographic, or high performance thin layer chromatographic data.

4.1.4 Intended use

A description shall be provided giving guidelines for staining in biology and for qualitative and quantitative procedures (if applicable). This shall include information on:

a) type(s) of biological material and handling and treatment before staining, e.g.:

   1) whether cell or tissue samples or both can be used;

   2) whether frozen or chemically-fixed material or both can be used;

   3) protocol for tissue processing;

   4) which embedding media can be used;

b) details of a suitable reaction procedure used by the manufacturer for testing the reactivity of the dye, stain, chromogenic reagent, fluorochrome, antibody, nucleic acid probe or lectin used for staining in biology;

c) result(s) expected when using the reaction procedure on the suggested type(s) of material in the way outlined by the manufacturer;

d) notes on suitable positive and negative control tissue and on interpretation of the result(s);

e) references to published results obtained using the product in the way suggested by the manufacturer.

4.2 Additional requirements for specific kinds of reagent

4.2.1 Fluorochromes

Independent of the type of application, fluorochromes offered for staining in biology shall be accompanied by the following information:

a) selectivity, i.e., a description of the target(s) which may be demonstrated using the conditions specified;

b) excitation and emission wavelengths;

c) for fluorochromes conjugated to antibodies, the ratio of fluorochrome/protein (F/P).

4.2.2 Metal salts

When offering metal compounds for use in metal uptake procedures in staining in biology, the following additional information shall be included:

a) the systematic name;

b) purity.

4.2.3 Antibodies

Antibodies offered for staining in biology shall be accompanied by the following information:

a) a description of the antigen (immunogenic substance) against which the antibody was raised, and if the antigen is defined by the cluster of differentiation systems, a CD number. This description shall contain, as appropriate, the type of (macro)molecule detected, which part of the molecule has been detected, its cellular localization, and in which cells and/or tissues it is found, and any cross reactivity with other epitopes;

b) for monoclonal antibodies, clone, method of production (tissue culture supernatant or ascitic fluid), immunoglobulin subclass and light chain identity;

c) for polyclonal antibodies, animal host and whether whole serum or gammaglobulin fraction is used;

d) a description of form (solution or lyophilized powder), amount of total protein and specific antibody and, if in solution, the nature and concentration of diluent or medium;

e) if applicable, a description of any molecular linkers or extenders added to the antibody;

f) a declaration of purity, purification techniques and detection methods for impurity (e.g. Western blotting, immunohistochemistry);

g) appropriate references to publications dealing with application of the antibody.

4.2.4 Nucleic acid probes

Nucleic acid probes offered for staining in biology shall be accompanied by the following information:

a) the base sequence and whether the probe is double- or single-stranded;

b) the molar mass of the probe or the number of bases and, if applicable, the number fraction (in percent) of guanine-cytosine base pairs;

c) marker used (radioactive isotope or non-radioactive molecule); for non-radioactive markers, point(s) of attachment to the probe (3' and/or 5') and substance fraction in percent of probe marked;

d) the target gene (DNA or RNA sequence) detected;

e) a description of form (lyophilized powder or solution) and amount (pg or pmol) or concentration (pg/ml or pmol/ml) as appropriate and if in solution, the nature and concentration of diluent or medium;

f) a declaration of purity, purification techniques and detection methods for impurity, e.g. HPLC (high performance liquid chromatography);

g) appropriate references to publications dealing with the source description of DNA sequence, existence of any known patents and information on application of the nucleic acid probe.

Annex A
(informative)

Examples of information supplied by the manufacturer with reagents commonly used in biological staining procedures

A.1 General

The following information is provided as examples of procedures and should not be considered as the only way the procedures described can be carried out. These procedures could be used by the manufacturer to test the reactivity of the dyes, and illustrates how the manufacturer may present the information in order to conform to this International Standard.

A.2 Methyl green-pyronin Y stain

A.2.1 Methyl green dye

Information concerning methyl green dye is the following.

a) Product identity:

- methyl green (synonyms: double green SF, light green);
- CAS-registry number: 22383-16-0;
- Colour Index name and number: basic blue 20, 42585.

b) Composition:

- molecular formula including counter ion: $C_{26}H_{33}N_3^{2+}\,2BF_4^-$;
- molar mass with (and without) counter ion: 561,17 g mol^{-1}, (387,56 g mol^{-1});
- mass fraction (content) of methyl green cation: 85 %, determined by absorption spectrometry;
- permissible limits of interfering substances all given as mass fractions:
  1) water: less than 1 %;
  2) inorganic salts: less than 0,1 %;
  3) detergents: not present;
  4) coloured impurities including crystal violet: not detectable by thin layer chromatography;
  5) indifferent compounds: 14 % soluble starch.

c) Wavelength of maximum absorbance of dye solution: 633 nm.

d) Thin layer chromatography: only one major component present consistent with methyl green.

e) Handling and storage: stable when kept in a tightly stoppered brown glass bottle at room temperature (18 °C to 28 °C).

A.2.2 Ethyl green dye

Information concerning ethyl green dye is the following.

a) Product identity:

   1) ethyl green (synonym: methyl green).

   2) CAS-registry number: 7114-03-6.

   3) Colour Index name and number: no Colour Index name, 42590.

b) Composition:

   1) molecular formula including counter ion: $C_{27}H_{35}N_3^{2+}$ 2 BF_4^-;

   2) molar mass with (and without) counter ion: 575,19 g mol^{-1} (401,58 g mol^{-1});

   3) mass fraction of ethyl green cation: 85 %, determined by absorption spectrometry;

   4) permissible limits of interfering substances all given as mass fractions:

      — water: less than 1 %;

      — inorganic salts: less than 0,1 %;

      — detergents: not present;

      — coloured impurities including crystal violet: not detectable by thin layer chromatography;

      — indifferent compounds: 14 % soluble starch.

c) Wavelength of maximum absorbance of dye solution: 633 nm.

d) Thin layer chromatography: only one major component present consistent with ethyl green.

e) Handling and storage: stable when kept in a tightly stoppered brown glass bottle at room temperature (18 °C to 28 °C).

A.2.3 Pyronin Y dye

Information concerning pyronin Y dye is the following.

a) Product identity:

   1) pyronin Y (synonyms: pyronine Y, pyronin G, pyronine G);

   2) CAS-registry number: 92-32-0;

   3) Colour Index name and number: no Colour Index name, 45005.

b) Composition:

   1) molecular formula including counter ion: $C_{17}H_{19}N_2O^+$ Cl^-;

   2) molar mass with (and without) counter ion: 302,75 g mol^{-1} (267,30 g mol^{-1});

   3) mass fraction of pyronin Y cation: 80 %, determined by absorption spectrometry;

4) permissible limits of interfering substances all given as mass fractions:

   — water: less than 1 %;

   — inorganic salts: less than 0,1 %;

   — detergents: not present;

   — coloured impurities: not detectable by thin layer chromatography;

   — indifferent compounds: 19 % soluble starch.

c) Wavelength of maximum absorbance of dye solution: 550 nm.

d) Thin layer chromatography: only one major component present consistent with pyronin Y.

e) Handling and storage: stable when kept in a tightly stoppered brown glass bottle at room temperature (18 °C to 28 °C).

A.2.4 Intended use of the methyl green-pyronin Y staining method

A.2.4.1 Type(s) of material

Methyl green-pyronin Y stain is used for fresh frozen cryostat, paraffin or plastic sections of various kinds of tissue.

A.2.4.2 Handling and treatment before staining

Suggested fixatives include:

— Carnoy's fluid [ethanol (volume fraction 99 %) + chloroform + acetic acid (mass fraction of 99 %) mixed as volumes (60 + 30 + 10) ml]; or

— formaldehyde (mass fraction 3,6 %) buffered with phosphate (pH = 7,0); routine dehydration, clearing, paraffin infiltration and embedding; routine preparation of microtome sections.

A.2.4.3 Working solution

Dissolve ethyl green or methyl green solution in an amount corresponding to a mass of 0,15 g pure dye, calculated as the coloured cation (in the above examples 0,176 g in either case), in 90 ml warm (50 °C) distilled water.

Dissolve an amount corresponding to a mass of 0,03 g pyronin Y calculated as the coloured cation (in the above example 0,038 g) in 10 ml 0,1 mol/l phthalate buffer, (pH = 4,0). Mix the latter solution with the ethyl green or methyl green solution.

A.2.4.4 Stability

The working solution is stable for at least one week when kept in a tightly stoppered brown glass bottle at room temperature (18 °C to 28 °C).

A.2.4.5 Staining procedure

A.2.4.5.1 Dewax paraffin sections.

A.2.4.5.2 Hydrate sections.

A.2.4.5.3 Stain for 5 min at room temperature (about 22 °C) in working solution.

A.2.4.5.4 Rinse in two changes of distilled water, 2 s to 3 s in each.

A.2.4.5.5 Shake off surplus water.

A.2.4.5.6 Agitate in three changes of 1-butanol.

A.2.4.5.7 Mount directly from 1-butanol in a hydrophobic synthetic resin.

A.2.4.6 Result(s) expected

The following results would be expected with the types of materials listed in A.2.4.1:

a) for nuclear chromatin: green (Carnoy fixed) or blue (formaldehyde fixed);

b) for nucleoli and ribosome rich cytoplasm: red (Carnoy fixed) or (lilac-) red (formaldehyde fixed);

c) for cartilage matrix and mast cell granules: orange;

d) for muscle, collagen and red cells: unstained.

A.3 Feulgen-Schiff reaction

A.3.1 Pararosaniline dye

CAUTION — For R 40: possible risk of irreversible effects;

For S 36/37: wear suitable protective clothing and gloves.

Information concerning pararosaniline dye is the following.

a) Product identity:

1) pararosaniline (synonyms: basic rubin, parafuchsin, paramagenta, magenta 0);

2) CAS-registry number: 569-61-9;

3) Colour Index name and number: basic red 9, 42500;

b) Composition:

1) molecular formula including counter ion: $C_{19}H_{18}N_3^+ Cl^-$;

2) molar mass with (and without) counter ion: 323,73 g mol^{-1} (288,28 g mol^{-1});

3) mass fraction of pararosaniline cation: 85 %, determined by absorption spectrometry;

4) permissible limits of interfering substances all given as mass fractions:

— water: less than 1 %;

— inorganic salts: less than 0,1 %;

— detergents: not present;

— coloured impurities: the methylated homologues of pararosaniline may be present in trace amounts as detected by thin layer chromatography but acridine shall not be present;

— indifferent compounds: 14 % soluble starch.

c) Wavelength of maximum absorbance of dye solution: 542 nm.

d) Thin layer chromatography: one major component consistent with pararosaniline; methylated homologues in trace amounts.

e) Handling and storage: stable when kept in a tightly stoppered brown glass bottle at room temperature (18 °C to 28 °C).

A.3.2 Intended use of the Feulgen-Schiff reaction

A.3.2.1 Type(s) of material

The Feulgen-Schiff reaction is used for paraffin or plastic sections of various kinds of tissue or cytological material (smear, tissue imprint, cell culture, monolayer).

A.3.2.2 Handling and treatment before staining

A.3.2.2.1 Suggested fixatives

Suggested fixatives include:

a) histology: formaldehyde (mass fraction of 3,6 %) buffered with phosphate, (pH = 7,0);

b) cytology:

1) wet fixed material: ethanol (volume fraction of 96 %);

2) air dried material:

— formaldehyde (mass fraction of 3,6 %) buffered with phosphate;

— methanol + formaldehyde (mass fraction of 37 %) + acetic acid (mass fraction of 100 %) mixed as volumes (85 + 10 + 5) ml.

Material fixed in Bouin's fixative is not suitable for this reaction.

Details of procedure used by the manufacturer to test the reactivity of the chromogenic reagent are as follows.

A.3.2.2.2 Pararosaniline-Schiff reagent

Dissolve 0,5 g pararosaniline chloride in 15 ml of 1 mol/l hydrochloric acid. Add 85 ml of an aqueous solution of $K_2S_2O_5$ (mass fraction of 0,5 %). Wait 24 h. Shake 100 ml of this solution with 0,3 g charcoal for 2 min and filter. Store the colourless solution at a temperature no lower than 5 °C. The solution is stable for at least 12 months in a tightly closed container.

A.3.2.2.3 Rinsing solution

Dissolve 0,5 g $K_2S_2O_5$ in 85 ml distilled water. Add 15 ml of 1 mol/l hydrochloric acid. The solution is immediately ready for use and can be used for up to 12 h.

A.3.2.3 Staining procedure

A.3.2.3.1 Deparaffinize paraffin sections in xylene for 5 min, followed by a 2 min rinse, firstly in ethanol of volume fraction 99 % and then in ethanol of volume fraction 50 %.

A.3.2.3.2 Hydrate plastic sections, deparaffinized paraffin sections and cytological material in distilled water for 2 min.

A.3.2.3.3 Hydrolyze the material in 5 mol/l hydrochloric acid at 22 °C for 30 min to 60 min (the exact time for hydrolysis depends on type of material).

A.3.2.3.4 Rinse in distilled water for 2 min.

A.3.2.3.5 Stain in pararosaniline-Schiff reagent for 1 h.

A.3.2.3.6 Wash in three subsequent changes of rinsing solution, 5 min each.

A.3.2.3.7 Wash in two rinses of distilled water, 5 min each.

A.3.2.3.8 Dehydrate in ethanol of volume fraction of 50 %, then of 70 % and finally of 99 %, 3 min each.

A.3.2.3.9 Rinse in two changes of xylene, 5 min each.

A.3.2.3.10 Mount in a synthetic hydrophobic resin.

A.3.2.4 Results expected

The following results would be expected with the types of materials listed in A.3.2.1:

for cell nuclei (DNA): red.

A.4 Immunohistochemical demonstration of oestrogen receptors

CAUTION — The reagent contains sodium azide (15 mmol/l). NaN_3 can react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water.

A.4.1 Monoclonal mouse antihuman oestrogen receptor

Information concerning the monoclonal mouse antihuman oestrogen receptor is the following.

a) Product identity: monoclonal mouse antihuman oestrogen receptor, clone 1D5.

b) Clone: 1D5.

c) Immunogen: recombinant human oestrogen receptor protein.

d) Source of antibody: monoclonal mouse antibody supplied in liquid form as tissue culture supernatant.

e) Specificity: the antibody reacts with the *N*-terminal domain (A/B region) of the receptor. In immunoblotting it reacts with the 67 kDa polypeptide chain obtained by transformation of *Escherichia coli* and transfection of COS cells with plasmid vectors expressing oestrogen receptor. Further, the antibody reacts with the cytosolic extracts of luteal endometrium and the human breast cancer cell line MCF-7.

f) Cross reactivity: the antibody reacts with the oestrogen receptor from rats.

g) Composition: tissue culture supernatant (RPMI 1640 medium containing foetal calf serum) dialysed against 0,05 mmol/l Tris/HCl, pH = 7,2, containing 15 mmol/l NaN_3.

   — Ig concentration: 245 mg/l;

   — Ig isotype: IgG1;

   — light chain identity: kappa;

   — total protein concentration: 14,9 g/l.

h) Handling and storage: stable for three years from release when kept at a storage temperature of 2 °C to 8 °C.

A.4.2 Intended use

A.4.2.1 General

The antibody is used for qualitative and semiquantitative detection of oestrogen receptor expression (e.g. breast carcinoma).

A.4.2.2 Type(s) of material

The antibody can be used on formalin-fixed, paraffin-embedded tissue sections, acetone-fixed cryostat sections and on cell smears. Furthermore, the antibody can be used as a detection antibody for ELISA (Enzyme Linked Immunosorbent Assay).

A.4.2.3 Staining procedures for immunohistochemistry

A.4.2.3.1 General

For formalin-fixed, paraffin-embedded tissue sections, a variety of sensitive staining techniques are suitable, including immunoperoxidase procedures, the APAAP (Alkaline Phosphatase Anti-Alkaline Phosphatase) technique and avidin-biotin methods such as LSAB (Labelled StreptAvidin Biotin) methods. Antigen retrieval, such as heating in 10 mmol/l citrate buffer, pH = 6,0, is mandatory. The slides should not dry out during this treatment or during the following immunohistochemical staining procedure. For staining cell smears, the APAAP method is suggested.

Details of the procedure used by the manufacturer on formalin-fixed paraffin-embedded tissue sections to test the reactivity of the antibody for immunohistochemistry are given in A.4.2.3.2 to A.4.2.3.4.

A.4.2.3.2 Reagents

A.4.2.3.2.1 **Hydrogen peroxide**, of a mass fraction of 3 % in distilled water.

A.4.2.3.2.2 **Tris-buffered saline** (TBS), consisting of 0,05 mol/l Tris/HCl and 0,15 mol/l NaCl at pH = 7,6.

A.4.2.3.2.3 **Primary antibody**, consisting of monoclonal mouse antihuman oestrogen receptor diluted optimally in TBS (see A.4.2.3.4).

A.4.2.3.2.4 **Biotinylated goat antibody to mouse/rabbit immunoglobulins**, working solution.

Prepare this solution at least 30 min, but no longer than 12 h, before use, as follows:

— 5 ml TBS, pH = 7,6;

— 50 µl biotinylated, affinity-isolated goat antibody to mouse/rabbit immunoglobulins in 0,01 mol/l phosphate buffered saline, 15 mmol/l NaN_3, in an amount sufficient to achieve a final concentration of 10 mg/ml to 20 mg/ml.

A.4.2.3.2.5 **StreptABComplex/HRP** (StreptAvidin-biotin complex/horseradish peroxidase), working solution.

Prepare this solution as follows:

— 5 ml TBS, pH = 7,6;

— 50 µl StreptAvidin (1 mg/l) in 0,01 mol/l phosphate buffered saline, 15 mmol/l NaN_3;

— 50 µl biotinylated horseradish peroxidase (0,25 mg/l) in 0,01 mol/l phosphate buffered saline, 15 mmol/l NaN_3.

A.4.2.3.2.6 **Diaminobenzidine substrate solution (DAB)**.

Dissolve 6 mg of 3,3'-diaminobenzidine tetrahydrochloride in 10 ml of 0,05 mol/l TBS, pH = 7,6. Add 0,1 ml hydrogen peroxide mass fraction of 3 % in distilled water. Filter if precipitation occurs.

A.4.2.3.2.7 Haematoxylin.

Dissolve 1 g haematoxylin, 50 g aluminium potassium sulphate, 0,1 g sodium iodate and 1,0 g citric acid in 750 ml of distilled water. Make up to 1 000 ml with distilled water.

A.4.2.3.3 Staining procedure

A.4.2.3.3.1 Deparaffinize and rehydrate tissue sections; perform antigen retrieval (see staining procedures above).

A.4.2.3.3.2 Incubate with hydrogen peroxide mass fraction of 3 % in distilled water for 5 min.

A.4.2.3.3.3 Rinse with distilled water and place in TBS for 5 min.

A.4.2.3.3.4 Incubate with monoclonal mouse antihuman oestrogen receptor diluted optimally in TBS (see A.4.2.3) for 20 min to 30 min.

A.4.2.3.3.5 Rinse with TBS and place in TBS bath for 5 min.

A.4.2.3.3.6 Incubate with biotinylated goat antibody to mouse/rabbit immunoglobulins working solution, for 20 min to 30 min.

A.4.2.3.3.7 Rinse with TBS and place in TBS bath for 5 min.

A.4.2.3.3.8 Incubate with streptABComplex/HRP (StreptAvidin-biotin complex/horseradish peroxidase) working solution, for 20 min to 30 min.

A.4.2.3.3.9 Rinse with TBS and place in TBS bath for 5 min.

A.4.2.3.3.10 Incubate with DAB solution for 5 min to 15 min (when handling DAB, use gloves).

A.4.2.3.3.11 Rinse with distilled water.

A.4.2.3.3.12 Counterstain with haematoxylin solution for 30 s.

A.4.2.3.3.13 Rinse in running tap water for 3 min.

A.4.2.3.3.14 Rinse with distilled water for 5 min.

A.4.2.3.3.15 Dehydrate in ethanol of volume fraction 50 % for 3 min, then for 3 min at 70 % and finally for 3 min at 99 %.

A.4.2.3.3.16 Rinse in two changes of xylene, 5 min each.

A.4.2.3.3.17 Mount in a synthetic hydrophobic resin.

A.4.2.3.4 Suggested dilution

Optimal staining can be obtained by diluting the antibody with TBS of pH = 7,6, mixed as volumes (1 + 50) µl to 1 + 75) µl when tested on formalin-fixed, paraffin-embedded sections of human, breast carcinoma. The antibody can be diluted with TBS, mixed as volumes (1 + 50) µl to (1 + 100) µl, for use in the APAAP technique and avidin-biotin methods, when tested on acetone-fixed cryostat sections of breast carcinoma.

A.4.2.3.5 Results expected

The antibody strongly labels the nuclei of cells known to contain abundant amounts of oestrogen receptor, e.g. epithelial and myometrial cells of the uterus, and normal and hyperplastic epithelial cells in mammary glands. The staining is predominantly localized to the nuclei with no cytoplasmic staining. However, on cryostat sections a

positive staining of oestrogen receptor in the nucleus as well as in the cytoplasm can be seen. Tissues known to contain small or non-detectable amounts of oestrogen receptor (e.g. colonic epithelium, cardiac muscle cells, brain- and connective-tissue cells) are consistently negative with the antibody. The antibody labels epithelial cells of breast carcinoma, which express the oestrogen receptor.

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids can produce artefacts or false negative results.

A.5 Flow cytometric demonstration of T-cells

CAUTION — The reagent contains sodium azide (15 mmol/l). NaN_3 may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water.

A.5.1 Monoclonal mouse antihuman T-cells

Information concerning the monoclonal mouse antihuman T-cells is the following.

a) Product identity: monoclonal mouse anti-human T-cell, CD3.

b) Clone: UCHT1.

c) Immunogen: human infant thymocytes and lymphocytes from a patient with Sézary disease.

d) Source of antibody: purified monoclonal mouse antibody.

e) Specificity: the antibody reacts with T-cells in thymus, bone marrow, peripheral lymphoid tissue and blood. The majority of T-cell neoplasms also express the CD3 antigen, but it is absent from non T-cell lymphoid malignancies. Consistent with the pattern of synthesis of the antigen in normal thymocytes, the earliest site detectable within neoplastic cells is the cell cytoplasm.

f) Composition:

— 0,05 mol/l Tris/HCl buffer, 15 mmol/l NaN_3, pH = 7,2, bovine serum albumin, mass fraction of 1 %;

— Ig isotype: IgG1;

— light chain identity: kappa;

— total protein concentration: 14,9 g/l;

— Ig purification: protein A sepharose column;

— purity: mass fraction approximately 95 %;

— conjugate molecule: fluorescein isothiocyanate isomer 1 (FITC);

— (F/P)-ratio: $E_{495nm}/E_{278nm} = 1,0 \pm 0,1$ corresponding to a molar FITC/protein ratio of approximately 5.

g) Handling and storage: stable for three years from release when kept at a temperature of 2 °C to 8 °C.

A.5.2 Intended use

A.5.2.1 General

The antibody is intended for use in flow cytometry. The antibody can be used for qualitative and quantitative detection of T-cells.

A.5.2.2 Type(s) of material

The antibody can be used on fresh and fixed cell suspensions, acetone-fixed cryostat sections and on cell smears.

A.5.2.3 Procedure to test the reactivity of the antibody for flow cytometry

The details of the procedure used by the manufacturer are as follows.

a) Collect venous blood into a test tube containing an anticoagulant.

b) Isolate mononuclear cells by centrifugation on a separation medium; alternatively, lyse the red cells after the incubation step specified in d).

c) Wash the mononuclear cells twice with RPMI 1640 or phosphate-buffered saline (PBS) (0,1 mol/l phosphate, 0,15 mol/l NaCl, pH = 7,4).

d) To 10 µl FITC conjugated monoclonal mouse anti-human T cell, CD3 reagent add cell suspension containing 1×10^6 cells (usually about 100 ml) and mix. Incubate in the dark at 4 °C for 30 min (for double-staining, the RPE- (R-phycoerythrin) conjugated antibody should be applied at the same time).

e) Wash twice with PBS + 2 % bovine serum albumin; resuspend the cells in an appropriate fluid for flow cytometry analysis.

f) Use an irrelevant FITC-(fluorescein isothiocyanate) conjugated monoclonal antibody of the same isotype as a negative control.

g) Fix by mixing the sedimented cells with 0,3 ml paraformaldehyde mass fraction of 1 % in PBS. If stored in the dark at 4 °C, the fixed cells can be kept for up to two weeks.

h) Analyse using a flow cytometer.

A.5.2.4 Suggested dilution

The antibody should be used in concentrated form for flow cytometry (10 µl/test). For use on cryostat sections and cell smears the antibody should be mixed with a suitable diluent as volumes (1 + 50) µl.

A.5.2.5 Results expected

The antibody detects the CD3 molecule on the surface of T-cells. When evaluating staining of cryostat sections and cell smears, the reaction product should be localized at the plasma membrane.

Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids can produce artefacts or false negative results.

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