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I. Introduction

LR White Resin is a very low viscosity resin, non-toxic and suitable for various applications in both light and Electron Microscopy.
Major advantage of LR White sections is that they show minimal non-specific staining. Sections of polymerized resin are hydrophilic, thus immunocytochemistry reagents easily penetrate into the section. Its low viscosity makes LR White an ideal tool for infiltrating plant tissues as well as decalcified bone and teeth.

II. Applications

1. LR White for Electron Microscopy

When using *LR White* embedding resin for electron microscopy, very few changes need to be made to the regime used for epoxy resin embedding. Every laboratory has its own individual embedding methods and we have described here a typical method for *LR White* as guidance for its use.

1.1 Fixation

No change from normal fixation should be made, if only EM is required from the final blocks.
If, however, good ultrastructure and a wide range of LM staining is required then we have found that the use of freshly depolymerised paraformaldehyde (3-4%) in a phosphate buffer pH 7,2 with 2,5% w/v sucrose (Product 840999) is the best compromise. Glutaraldehyde (Product 49627) alone and Karnovsky's glutaraldehyde-formaldehyde mixtures may lead to patchiness. LM staining and some stains which are not functioning or giving "false positives" (e.g. PAS) whereas normal formalin fixation yields unacceptable EM ultrastructure.
For the dual LM/EM role osmium tetroxide should be avoided due to its effect on many LM stains but 1% (w/v) phosphotungstic acid (Product 79690) in the first absolute alcohol step of dehydration improves electron contrast without adversely affecting most LM stains. Osmium tetroxide (Product 75633) may be used if the blocks are required for EM only.

1.2 Dehydration

A graded ethanol series is the method of choice when embedding in *LR White*. Acetone acts as a radical scavenger in the resin system and therefore traces of acetone left in the tissue during curing can interfere with polymerisation. For this reason the use of a graded acetone series and 2,2-dimethoxypropane, which generates acetone, are best avoided. If the use of 2,2-dimethoxypropane (Product 00660) is considered vital we recommend either a protracted resin infiltration or washing the tissue with dry ethanol prior to infiltration in order to minimize the risk of acetone contamination of the final resin.

1.3 Infiltration

The extreme low viscosity of *LR White* may be exploited by allowing the use of short infiltration times or large specimens BUT NOT BOTH! A 1mm cube of animal tissue will be adequately infiltrated in about 3 hours if 4-6 changes of *LR White* at 60^0C are employed during this period. An overnight infiltration at room temperature, followed by two short changes of resin will often be more convenient, however. The long shelf life and low extraction rate of *LR White* allows specimens (perhaps reserve tissue) to be stored safely in resin for many weeks at 4°C if required.
Larger blocks do require significantly longer infiltration times than small ones.

1.4 Prepolymerisation

The *LR White* catalyst is a form of benzoyl peroxide in a solid solution to render it safe for transport. One 500 ml bottle of *LR White* resin requires 9,9 g of catalyst to be added. The catalyst should be added to the resin at room temperature and the resin must be shaken thoroughly immediately after addition of catalyst. The catalyst will take a full 24 hours at room temperature to dissolve completely and during this time it is most helpful if the bottle can be shaken from time to time. Do not attempt to heat the resin in order to speed the dissolution of the catalyst.
Once mixed and fully dissolved the shelf life of the catalyzed resin stored at 4°C is at least 12 months.

1.5 Polymerisation

Osmium tetroxide (Product 75632 or 75633) reacted tissue should not be "cold-cured" with the accelerator. This process is strongly exothermic and the dark colour of the tissue leads to a focal heat accumulation which can cause local problems in and around the tissue.

If the tissue is not osmium tetroxide post-fixed, curing with *LR White* accelerator may be employed. As with curing blocks for LM we recommend cooling the moulds during polymerisation, but there is no need to exclude oxygen from the surface of the curing block.
Thermal curing should be used for osmium treated specimens and may be used for all specimens.. Here it is important to limit the contact of oxygen with the resin while polymerisation occurs. The most convenient way of achieving this with capsule-type embedding is to use gelatin capsules, fill up to the brim and slide the other half of the capsule on.
If flat embedding is required for cutting orientation then the surface of the resin must be covered to prevent contact with oxygen. One convenient method is to utilize the JB-4-type moulds (Sigma-Aldrich-Techware E 4390 and E 4140) and chucks, useful for LM. After polymerisation the block can be sawed off the stub and mould re-used.
Polymerisation time and temperature are fundamental to the physical characteristics of the final block, to a much higher degree than with undercured epoxy systems. We strongly recommend a temperature of $60^{\circ} \pm 2^{\circ}C$ for a period of 20-24 hours.
Some ovens are not capable of controlling polymerisation temperature so closely, and if faced with over brittle blocks, this is the first parameter to check.
LR White has extremely good powers of penetration and can penetrate and soften some low-density polyethylene capsules. This causes them to distort and collapse. Also polyethylene is not impermeable to oxygen and may allow enough contact with atmospheric oxygen to give the blocks an inhibited "tacky" surface. Both these problems may be overcome by the use of gelatin capsules (size 00 is similar to the popular polyethylene capsule size) and these are much cheaper and easier to seal during polymerisation.
The Resin may be used straight from the refrigerator and has a very low toxicity both in the monomeric and polymerized state, unlike epoxies (see Proc. Roy. Mic. Soc. 16, Pt. 4 p. 265-271). The cold cure accelerator does have some toxic risk and contact with skin and eyes should be avoided.
For cold curing the accelerator should be used at one drop per 10 ml of resin and this should cause polymerisation within 10 and 20 minutes. If polymerisation occurs faster than this we recommend either more careful metering of the one drop of accelerator or a higher volume of resin per drop of accelerator.

1.6 Trimming and Cutting
Trimming the block may be done with jewellers saw, razor blade or a with glass knife on the ultramicrotome, as with epoxy blocks.
Cutting, too, may be performed in the same way as for epoxy resin with glass or diamond knives. A typical cutting speed of about 1 mm per second is suitable.

1.7 Section Staining
All the common section stains give good results with tissue embedded in *LR White* resin. Stains made up in ethanol or methanol should be avoided as these solvents soften the resin and may remove sections from grids. As a alternative to uranyl acetate (Product 94260), 1% phosphotungstic acid (Product 79690) has proved to be a good general purpose stain, both as a block stain, as mentioned earlier, and as a section stain.

1.8 In the Electron Microscope
An initial reduction in electron density may accompany the initial exposure of the resin to the beam. This is thought to represent a loss of water, from knife-boat or staining solution. Thinning as such does not occur and specimens have been kept stationar under a 120 kV electron beam for 3 hours with no obvious sign of damage.

2. LR White for Electron Microscopic Immunocytochemistry

2.1 Introduction

LR White resin has five advantages which can be exploited for the localisation of antigens in sections of fixed and embedded tissue under the electron microscope.

1. It is a hydrophilic embedding agent, which means that ultra-thin sections allow the passage of aqueous solutions even at neutral pH, as opposed to the epoxides and polyesters, ultra-thin sections of which are much less permeable.
2. Its lipid solvency is apparently low for a plastic embedding agent and therefore membrane and cytosol structures can be observed under the electron microscope even when osmium has not been used to stabilize lipids. No low temperature methods are required although tissue structure is much improved by perfusion fixation methods.
3. It does not, prevent the demonstration of antigen by immunochemical techniques. No “etching“ or protease digestion has so far been necessary.
4. It is beam-stable, standing up well to even quite low kV’s thus representing a considerable advance over the more commonly used methacrylates.
5. It tolerates rapid, partial dehydration, accepting tissue from 70% ethanol. Such tissue has an improved antigenicity over tissue which has been fully dehydrated.

2.2 Fixation

To preserve antigenicity post-fixation in osmium tetroxide (Product 75632 and 75633) is best avoided but then a variety of possibilities is available depending on the requirements of the investigator. If tissue blocks are kept small (1-3 mm^3) four to six hours fixation in freshly depolymerized and purified 4% paraformaldehyde in a 0,1 M phosphate buffer pH 7,3 (Sigma HT 50-1, HT 50-1-1 or HT 50-1-2), is recommended for the preservation of maximum tissue antigenicity. Tissue is then washed overnight in buffer solution. Picric acid (Product 80450), included with formaldehyde, will improve tissue structure slightly and Zamboni’s fixative (Stephanini et al. Nature 216, 1967; 173-174) which is 4% phosphate-buffered paraformaldehyde with picric acid can be used. Glutaraldehyde, with its greather power to cross-link proteins, undoubtedly stabilises tissue structure to a greather degree than does formaldehyde, but in turn, tissue thus fixed demonstrates a reduced antigenicity. It is strongly recommended that the vacuum-distilled, purified form of monomeric or trimeric glutaraldehyde is used, to increase method reproducibility and avoid deleterious fixative impurities. Three to four hours in 1-2% glutaraldehyde in 0,1 M phosphate buffer pH 7,3, is perfectly adequate, remembering that the lower the concentration of glutaraldehyde the higher the antigenicity yield. Tissue is then washed overnight in buffer.
The effect of the avoidance of osmium tetroxide can be partially compensated, without detriment to tissue antigenicity, by the inclusion of picric acid in the glutaraldehyde solution. The suggested solution is as follows: 2 ml 50% purified glutaraldehyde + 15 ml picric acid (sat.aq.) + 83 ml 0,1 M phosphate buffer pH 7,3 giving a 1% glutaraldehyde, 0,25% picric acid solution. The percentage of glutaraldehyde and picric acid can be increased to give a more stable ultrastructure but this may be at the expense of antigenicity. Two to four hours for the fixation of 1-3 mm^3 blocks is recommended. More membrane structure is seen with this fixative than when glutaraldehyde is used alone,especially if it is perfused into animal tissue, when the molarity of the buffer should be decreased (0,05-0,08 M).
Further steps, such as post-osmication or “block-staining“ should not be carried out, because they may reduce immunocytochemical sensitivity – either through reduced antigenicity or enhanced background.

2.3 Dehydration

Tissue fixed in aldehyde alone, after buffer rinses, is dehydrated in a graded ethanol series. Tissue fixed in aldehyde/picric acid is placed directly into 70% ethanol where some of the picric acid can be washed out. Reducing the time in ethanol, and the concentration of ethanol used in dehydration will often increase antigenic yields. *LR White* will accept tissue from 70% ethanol so that after two washes of 30-60 minutes each, blocks may be transfered into *LR White*. When osmium is avoided, tissue shrinkage can be a problem and tissue taken from fixative in 70% ethanol and then straight into *LR White* may show shrinkage artefacts. This can be lessened by introducing an intermediate step of diluted *LR White* which is 2:1 *LR White* to 70% ethanol. Be careful not to carry over large amounts of 70% ethanol when transferring tissue.

2.4 Infiltration
Even though the tissue may be taken from 70% ethanol into *LR White* no special procedures are necessary. One Change for an hour, followed by an overnight change (preferably on a “rotamix“) and then a final change the following morning before embedding, using gelatine capsules, is usually sufficient unless the blocks of tissue are particularly large (i.e. in excess of 3 mm^3). Blocks may be stored in unpolymerised resin at 4 °C for weeks if necessary.

2.5 Prepolymerisation
The *LR White* catalyst is a form of benzoyl peroxide in a solid solution to render it safe for transport. One 500 ml bottle of *LR White* resin requires 9,9 g of catalyst to be added. The catalyst should be added to the resin at room temperature and the resin must be shaken thoroughly immediately after addition of catalyst. The catalyst will take a full 24 hours at room temperature to dissolve completely and during this time it is most helpful if the bottle can be shaken from time to time. Do not attempt to heat the resin in order to speed the dissolution of the catalyst.
Once mixed and fully dissolved the shelf life of the catalyzed resin stored at 4°C is at least 12 months.

2.6 Polymerisation
Some special care must be taken to ensure that tissue undergoing embedding is not exposed to temperature in excess of 55^0C if antigenicity is not be impaired. The “cold-cure“ procedure (see *LR White* for Electron Microscopy), should not be used (the exothermic reaction may exceed 60^0C) even though the tissue is not ossmium treated, instead an accurate oven or incubator set at 50^0C is preferred and a 24 hour polymerisation time is given. Although anaerobic polymerisation is advised for Beem capsules, in fact *LR White* polymerises well in a 50^0C oven without further precautions when contained in gelatin capsules, fully filled and thightly capped.
Trimming, cutting and use in the electron microscope are as for the application sheet for electron microscopy.

2.7 Staining
The choice of immunolocalisation technique is entirely up to the user, and PAP, hapten-anti-hapten, avidin-biotin or gold-colloid methods may all be adapted.
Of cource, from time to time, ultra-thin sections of tissue fixed, processed and embedded as described above should still be stained with uranyl acetate (15 minutes)(Product 94260) followed by lead citrate (3 minutes)(Product 15319) on the grids (Product-Aldrich Techware G 1403 and further) as for routine electron microscopy. The appearance of such tissues is different from that of post-osmicated tissue and it is important that the observer understands the material with which he is working.
Immunostained sections can be counterstained with lead citrate but if peroxidase techniques are in use this may confuse the picture.

3. LR White for Light Microscopy

3.1 Introduction
Resin embedding for light microscopy provides greatly improved cellular definition compared to paraffin embedding, and for this reason is now widely used in diagnoses particularly of renal disease, lymphomas and bone marrow trephines, as well as in research.
The acrylic resins currently used however are not suitable for electron microscopy (EM) and the epoxy resins used for EM are not easily stained for light microscopy (LM). *LR White* however can be used for both purposes and a lymph node for example (12x10x3mm) can be processed, cut and stained for LM, then the same block trimmed down, cut and stained for EM.
LR White can also be used for the histochemical demonstration of some of the more resistant enzymes, and for the immunocytochemical demonstration of intracellular immunoglobulins.
For those laboratories already using an acrylic resin e.g. HEMA or GMA, no alteration need be made to the current processing method, but we have proposed a "typical" method for *LR White* as guidance for its use.

3.2 Fixation
No change from normal fixation need be made if LM only is required from the final blocks (neutral buffered formalin recommended, see Sigma HT50-1-1 and so one). If however EM is required subsequent to LM we have found the use of freshly depolymerised paraformaldehyde (3-4%) in a phosphate buffer pH 7,2 with 2,5% w/v sucrose is the best compromise. Glutaraldehyde-formaldehyde-mixtures may lead to very pale staining with haematoxylin and patchy eosin, whereas normal formalin fixation gives unacceptable EM structure. For the dual LM/EM role osmium tetroxide should be avoided due to its effect on many LM stains but 1% phosphotungstic acid (w/v) (Product 79690) in the first absolute ethanol step of dehydration improves electron contrast without adversely affecting most LM stains. If this does not provide adequate electron density, then "staining" of ultrathin sections can be carried out with osmium (a brief exposure to 1% aqueous osmium tetroxide (Product 75633) or osmium tetroxide (Product 75631) vapour on a copper grid) or lead citrate (Product 15326).

3.3 Dehydration
A graded ethanol series is the method of choice when using *LR White*. Acetone acts as a radical scavenger in the resin system and traces of acetone left in the tissue during curing can interfere with polymerisation.

3.4 Infiltration
The extreme low viscosity of *LR White* allows the use of short infiltration times, but these will obviously depend on the size of the tissue. Infiltrated tissue will become translucent and sink to the bottom of the container.
A typical dehydration and infiltration schedule for a block (12x10x3mm) on a shaker would be:

- Two changes 70% alcohol, 30 mins. each.
- Two changes absolute alcohol, 30 mins. each.
- Infiltrate with LR White at RT, 2-3 changes 60 mins. each or leave overnight.

3.5 Prepolymerisation
The *LR White* catalyst is a form of benzoyl peroxide in a solid solution to render it safe for transport. One 500 ml bottle of *LR White* resin requires 9,9 g of catalyst to be added. The catalyst should be added to the resin at room temperature and the resin must be shaken thoroughly immediately after addition of catalyst. The catalyst will take a full 24 hours at room temperature to dissolve completely and during this time it is most helpful if the bottle can be shaken from time to time. Do not attempt to heat the resin in order to speed the dissolution of the catalyst.
Once mixed and fully dissolved the shelf life of the catalyzed resin stored at 40C is at least 12 months.

3.6 Polymerisation
Either heat or cold curing can be used for LM, cold curing gives slightly better cutting and staining qualities. When cold curing it is important to cool the moulds in a bath of cold water during polymerisation, to disperse the heat produced by the exothermic reaction, but it is not necessary to exclude oxygen from the surface of the curing block. Some polymerisation problems have been experienced when embedding very flat pieces of tissue which stick to the base of the embedding mould. The way to avoid this is to smear the base of the mould with accelerator before adding mixed resin, and allow the tissue to sink to the base of the mould rather than applying pressure.

When thermal curing, it is important to limit the contact of oxygen with resin while polymerisation occurs. The most convenient way of achieving this is to use gelatine capsules for small pieces of tissue. Fill up to the brim and slide the other half of the capsule on. For larger specimens, the surface of the resin must be covered and one convenient method is to utilise the JB-4 type moulds, one being used as a lid for another, or to polymerise in a nitrogen environment.
Polymerisation time and temperature are fundamental to the physical character of the final block, to a much greater extent than with undercured epoxy systems.
We strongly recommend a temperature of 60°C ± 2°C for a period of 20-24 hours. Some ovens are not capable of controlling temperature so closely and if faced with overbrittle blocks this is the first parameter to check.
Resin may be used straight from the refrigerator and has a very low toxicity in both monomeric and polymerised states unlike epoxies (see Proc.Roy.Mic.Soc. 1981, 16, Pt.4, p 265-271). The cold cure accelerator does have some toxic risk and contact with skin and eyes should be avoided.
For cold curing the accelerator should be used at one drop per 10 ml of resin and this should cause polymerisation in 10-20 minutes. If polymerisation occurs faster than this we recommend either more careful metering of the one drop of accelerator or a higher volume of resin per drop of accelerator.

3.7 Cutting and Mounting

Although it is possible to cut *LR White* on a standard microtome with a steel knife the method of choice would be to use a heavy duty motorised microtome, and glass (Ralph type) knife.
LR White can be cut as thin as 0,25 µm on some microtomes, but it is very difficult to obtain a satisfactory stain intensity with anything other than toluidine blue (Sigma T 0394) at this thickness, simply because there is so little tissue in the section.
For haematoxylin (Sigma HHS-16 etc) and eosin (Sigma HT110-1-16 etc) staining as well as most other routine stains we recommend sections of 2-3 µm. It is of course possible to cut thicker (up to 15 or 20 µm) if required.
Blocks can be cut dry, the sections picked up and floated out on 30-40% acetone on a hot plate (approx. 60-70°C), and then allow to dry at this temperature. For hard tissues, and blocks which contain a combination of hard and soft tissues, such as marrow trephines, the following floating out fluid is recommended, again on a hot plate (60-70°C).
To 20 ml acetone add 0,5 ml benzyl alhohol (Product 13160) mix then make up to 50 ml with distilled water. A section adhesive such as egg albumin, can be added to this if required.

3.8 Section Staining

Most routine stains give good results on tissue embedded in *LR White* resin using standard times and temperatures although it may occasionally be necessary to extend some staining times e.g. methyl green/pyronin (Sigma HT70-1-16). Stains made up in ethanol (EtOH) or methanol (MeOH) should be avoided as these solvents soften the resin and may remove sections from the slide. Dehydration of solution through graded alcohols after staining should also be avoided. Sections should be blotted, air dried and then mounted in a resinous mounting medium (Sigma M 9668 and 1000-4)

4. LR White for Immunohistochemistry

4.1 Introduction

Sections from *LR White* embedded tissue have been used successfully for immunocytochemistry at both the light microscope and electron microscope levels. This demonstrates quite clearly that the visualization steps of the immunocytochemical procedure will penetrate the resin and react with tissue antigens if they have been preserved in the tissue.

As with all immuno-localisations the key factor is whether or not the tissue antigen has survived fixation, processing and embedding in such a form as to be recognisable to the specific antibody. This is difficult to predict with certainty, but some antigens have been shown to be highly resistant whilst others are fickle even in unfixed frozen sections. Much interest has centred on using immunocytochemistry to detect protein hormones and the various classes of immunoglobulins and generally these classes of antigens have proved resistant to alteration both in processing to paraffin-wax and to *LR White* resin.

It is the special hydrophilic nature of *LR White* which allows immunochemicals to permeate the suppporting resin and reach its sites of binding and no resin pretreatment is necessary, or indeed possible, to facilitate this penetration.

We have been successful with *LR White* blocks only when they have been thermally cured, probably because when accelerator-curing the resin, the heat produced is sufficient to damage the integrity of the tissue antigen. Some workers have also reported that a slight under-cure of *LR White*, say at 55^{0}C for 20-24 hours aids subsequent penetration of antisera, but we have obtained good results without deviating from the standard polymerisation method.

If the particular antigen under consideration has already been localised in paraffin-wax sections then a trusted fixation regime will be established and should be adhered to. For those approaching the problem for the first time there is an extensive bibliography available regarding fixation for immunocytochemistry, much of it contradictory, and a reference list is provided as some guidance. Rules of thumb seem to be to avoid glutaraldehyde and perhaps use an acid rather than a neutral fixative, but there are many conflicting and strongly held views on these topics.

Similarly, the need to enzyme digest sections prior to reaction is fraught with controversy and may indeed be linked to the fixation regime chosen. We have used both protease type VII (Sigma P 0384) and trypsin type II (Sigma T 7168) to good effect on our neutral buffered formalin fixed material.

If frozen, dewaxed or etched epoxy resin sections are used for immunostaining, the tissue is not surrounded by a supporting matrix when they are being reacted. When using *LR White* sections the resin is still intact and therefore diffusion to the sites of reaction must occur prior to reaction of antisera with antigen. For this reason we have found it necessary to use antisera at approximately ten times the concentration that would work on dewaxed sections. The exact titre of each antibody will depend upon its source and how well it has been stored, but we have used many commercial anti-immunoglobulins at about a 1 in 10 dilution.

For the same reason the antibody stages of the reaction often benefit from a longer incubation time. Up to 2 hours at room temperature or overnight at 4^{0}C in a moist chamber may be used.

Various immunoperoxidase techniques have given results on *LR White* tissue sections including the peroxidase-antiperoxidase complex method (PAP)(Sternberger, 1970), the hapten sandwich technique (Jasani et al., 1981), and the indirect peroxidase method. The avidin-biotin-peroxidase complex method of Hsu has not been successful in our hands with *LR White* embedded material, probably due to the molecular size of avidin. As fairly strong antibody concentrations are required, a highly sensitive method of detection is to be prefered and for this reason the PAP or hapten sandwich techniques are more suitable than a two layer indirect peroxidase reaction.

Visualisation of the bound peroxidase is achieved with the diaminobenzidine-peroxidase reaction as described by Graham and Karnovsky (1966)

Any technique where the sections are subjected to hydrogen peroxide solutions twice during staining is likely to tend to lift sections from the slides. We have found that poly-L-lysine (MW 350'000), (Sigma P 8920) is an excellent adhesive for immunocytochemical work, and also care should be taken to dry sections onto slides very thoroughly in an oven rather than a hot-plate at 60^{0}C for two hours. This step should not have any effects on the antigencity of the tissue as it will already have spent 20-24 hours at 60^{0}C during polymerisation.

It is clear that no “standard“ immunohistochemical staining regime can be cited as there are so many variables, but a “typical“ regime is described below for general guidance

4.2 PAP Procedure for LR White Sections (3μm)

- Block endogenous peroxidase with 1% phenylhydrazine hydrochloride (Product 78690) in P.B.S (optional) 30 minutes.
- Wash in P.B.S. 1 x 5 minutes, and 2 x 5 minutes at 37^0C.
- 0,1% trypsin (Sigma T 7168) in 0,1% aqueous $CaCl_2$ (Product 21110) 20 minutes
- Wash in ice cold distilled water. 10 minutes
- 2% Goat serum (Sigma S 6898) in P.B.S. 20 minutes
- First antibody (approx. 1:10 dilution) 2 hours at 37^0C or overnight at 4^0C (preferably use Sigma antibodies).
- Wash in P.B.S. 10 minutes
- Goat anti-rabbit antibody (Sigma R 2004)(approx. 1:20 dilution) 3 hours at 37^0C or overnight at 4^0C.
- Wash in P.B.S. 10 minutes
- PAP (Sigma P 1901or P 2026) at 1:200 dilution 2 hours at 37^0C or overnight at 4^0C.
- Wash in P.B.S. 10 minutes.
- Wash in TRIS HCl pH 7,6 10 minutes.
- DAB/H_2O (Sigma D 4168 or D 4293, or D 4418 or D 0426) 15 minutes.
- Wash in distilled water 10 minutes.
- Counterstain as required.

4.1 General Staining Procedure – Indirect Technique

- Block endogeneous peroxidase with 0,3% H_2O_2 in 50% methanol 20 minutes
- Block only when peroxidase conjugated secondary antibodies are used.
- Block with levamisole (Product 87963 or Sigma L 9756) when alkaline phosphatase conjugated secondary antibodies are used.
- Rinse in P.B.S. 2 x 5 minutes
- Treat sections according to the antigen retrieval technique indicated below.
- Rinse in P.B.S. 2 x 5 minutes
- Incubate with primary antibody, suitably diluted in P.B.S. (see product information sheet). Optimal dilution should be tested in combination with second antibody.
- Rinse in P.B.S. 2 x 5 minutes
- Incubate with conjugated second antibody. 30 minutes
- Dilute second antibody suitably with P.B.S. Second antibody must be directed against antibodies of the species in which the primary antibody was raised.
- After incubation with fluorochrome labeled antibodies, sections are rinsed in P.B.S. and mounted in a mounting media such as DPX (Product 44581), Gelvatec, Gelvastab, PVA-DAPCO or PVA-NDP.
- After incubation with enzyme conjugated antibodies (peroxidase, alkaline phosphatase) rinse in P.B.S.
- Apply appropriate chromogen substrate.
- Rinse and counterstain, mount sections.

4.2 Antigen Retrievals

- Proteolytic enzyme treatment
- Trypsin solution: 0,1% trypsin (Product 93640) + 0,1% $CaCl_2$ Dissolve in distilled water, adjust to pH 7,8.
- Pepsin solution: 0,4% pepsin (Product 77152). Dissolve in 0,01N HCl. Incubation time in pepsin solution up to 2 hours at RT.
- Pronase solution: 0,1% pronase (Product 81748) + 0,01% $CaCl_2$ Incubation time in pronase solution 10 minutes at 37^0C
- Microwave oven treatment

- Place slides in a coplin jar (Sigma C 8338) containing sodium citrate buffer pH 6,0. Incubate for 3 x 5 minutes in a microwave oven, 750 W, (Sigma A 9209) with 1 minutes intervals.
- Rinse for 30 minutes in P.B.S. at RT.

For immunocytochemistry *LR White* must be thermally cured and <u>not</u> accelerator cured!

5. LR White for Hard Tissue

5.1 Introduction
LR White can be used for the microtomy of decalcified bone and teeth and also for microtomy or "sawing and grinding" of non-decalcified tissues.

5.2 Decalcified Tissue
May be processed, cut and stained similarly to soft tissue (see *LR White* for Light Microscopy), except that dehydration and infiltration times may need to be extended depending on the size of tissue. It is also recommended that bone be "de-fatted" to improve the penetration of resin into marrow cavities.. This can be achieved by using chloroform (Product 25691) after dehydration, returning to absolute alcohol to remove the chloroform before infiltration with resin and polymerising.

5.3 Non-decalcified Tissue
Dehydration and infiltration times will vary depending on size and density of tissue. Those laboratories using methyl- or butylmethacrylate (Product 66200 and 64110 respectively) at present can use similar dehydration times, but infiltration will probably be shortened due to the low viscosity of the resin.

5.4 Dehydration
A graded series of alcohols should be used for dehydration of tissue, and when processing bone "de-fatting" is recommended to improve the penetration of resin into the marrow cavities. This can be done using chloroform (Product 25691), for the same length of time that would be necessary to clear the tissue. The bone should then be taken back to absolute alcohol and given sufficient changes to remove the chloroform before infiltration with *LR White*.

5.5 Infiltration
Several changes of resin will be necessary and impregnation under vacuum is recommended.

5.6 Prepolymerisation
The *LR White* catalyst is a form of benzoyl peroxide in a solid solution to render it safe for transport. One 500 ml bottle of *LR White* resin requires 9,9 g of catalyst to be added. The catalyst should be added to the resin at room temperature and the resin must be shaken thoroughly immediately after addition of catalyst. The catalyst will take a full 24 hours at room temperature to dissolve completely and during this time it is most helpful if the bottle can be shaken from time to time. Do not attempt to heat the resin in order to speed the dissolution of the catalyst.
Once mixed and fully dissolved the shelf life of the catalyzed resin stored at 4°C is at least 12 months.

5.7 Polymerisation
The tissue can be heat or accelerator cured after embedding in strong plastic moulds, such as the JB-4 or Peel-a-way type, or in aluminium foil dishes (Sigma-Aldrich-Techware E 4390 and E 4140).
When heat curing, the moulds should first be filled with resin then the tissue added and orientated. Polymerisation will occur in 18-24 hours at 60^0C $\pm$ 2^0C. The surface of the block exposed to oxygen may remain slightly sticky, but this will not affect the cutting quality of the face of the block. Some ovens are not capable of controlling temperature so closely and if faced with overbrittle blocks this is the first parameter to check.
When accelerator or "cold" curing, the moulds should be placed in a bath of ice-cold water to disperse the heat produced during the exothermic polymerisation. The base of the moulds should first be smeared with accelerator using a cotton-wool bud or swab, the accelerator is then added to the resin, one drop per 10 ml resin, and thoroughly mixed before pouring into the mould, the tissue is then placed into the mould and orientated. Polymerisation should occur in 10-20 minutes, if its occurs faster than this we recommend either more careful metering of the one drop of accelerator or a higher volume of resin per drop of accelerator.. N.B. The accelerator is toxic and contact with skin and eyes should be avoided.

5.8 Cutting and Mounting

Bone marrow trephines and small pieces of cancellous bone, cortical bone and teeth offer too much resistance to the microtome knife and preparations of this material must be prepared by sawing and grinding.

5.9 Microtomy

Sections can be cut, using Ralph type glass knifes for trephines or a tungsten carbide knife for larger pieces of cancellous bone, from 2-10 μm. Blocks can be cut dry, the sections picked up and floated out on a hot plate at 60-70^{0}C using the following solution:
20 ml acetone (Product 00580) add 0,5 ml benzyl alcohol (Product 13160) mix then make up to 50 ml with distilled water. A section adhesive such as egg albumin can be added to this if required. Sections should be allowed to dry on a hot plate for at least 30 minutes before staining.

5.10 Sawing and Grinding

Thick slices 150-200 μm can be cut using a milling machine and then ground to the required thickness, usually 20 μm for staining or 70 μm for microradiography; the section is inclined to fragment if grinding is continued much below 20 μm.
Using the newer types of saw microtome, such as the Leitz 1600 which has a diamond-coated-internal-hole-saw, sections can actually be cut at 20 μm and no further grinding is necessary.

5.11 Section Staining

Sections of material embedded in *LR White* are stained “free floating”, times of staining are usually longer than those for paraffin sections, and dehydration through alcohols should be avoided. A recommended metod for haematoxylin and eosin staining is as follows:

- Remove calcium deposits, which would otherwise interfere with the staining, from the sections by treating with Kristensen’s decalcifying solution or Accumate RDO™ rapid decalcifying solution (Sigma D 5551 or D 6057)for about 15 minutes.

- Wash in running tap water for a few minutes to remove the formic acid from the tissue.

- Transfer the section to several changes of distilled water, a few minutes each and then into a 0,5% w/v solution of periodic acid in distilled water (Sigma 395-1-32, 1+1 diluted) where it should be left for 5 minutes.

- Wash the section with several changes of distilled water and stain with Harris haematoxylin (Sigma HHS-16) for about 1 hour.

- Transfer the section, after a short time in distilled water, into running tap water to “blue” the haematoxylin stained tissue.

- Check the haematoxylin stain; if the tissue should be overstained or the surface of the resin has become stained with haematoxylin, this may be corrected by a short differentiation in acid alcohol (0,5% HCl in 70% alcohol) and “reblueing” of the stained tissue.

- Rinse the section in distilled water and counterstain, using a 5% solution of eosin Y (Product 45240) in distilled water; leave the section in the eosin stain for 30 minutes to 1 hour, wash briefly in running tap water and check the staining of the tissue. Nuclei and haematoxophilic elements should be bright blue, cytoplastic structures in various shades of red-pink.

- Rinse the section in distilled water, blot dry with filter paper and either clear briefly in xylene (Product 95692) or xylene alternative (Sigma X 2752) and mount in a mounting media such as DPX (Product 44581), Gelvatec , Gelvastab, PVA-DAPCO or PVA-NPD, or directly in *LR White* resin by adding a drop of accelerator to 1 ml of resin.

III. Ordering information

Cat.No.: 62662	*LR White* Embedding Kit	
Cat.No.: 62661	*LR White* Resin	500 ml
Cat.No.: 62660	*LR White* Accelerator	10 ml
Cat.No.: 62663	LR White Catalyst	10 g

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Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

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