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A hands-on overview of tissue preservation methods for molecular genetic analyses

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Abstract DNA studies have overwhelming importance in biological science. The aim of this paper is to present a compact and hands-on summary of widely available tissue preservation methods by listing dry, fluid/buffered and freezing techniques. Thereby, practical aspects, advantages and disadvantages, safety and feasibility issues of each method are discussed and compared.

Keywords Review · Tissue preservation · Desiccation · Buffers · Freezing

Introduction

Nowadays, DNA studies have overwhelming importance in many biological sciences. Although there is plenty of publications and reviews on DNA isolation protocols for various tissue types (for an excellent overview with detailed protocols, see Nishiguchi et al. 2002), the essential prerequisite for successful extraction is the presence of (ideally good-quality) DNA. Hence, appropriate preservation of the tissue samples is indispensable. In this field, there is still a certain lack of studies summarizing applicable methods for specific tissue storage—with the admitted exception of medical sciences, where strict, extensively tested and proven protocols exist. The most complete general summary so far was given by Prendini et al. (2002), presenting a major advance from earlier reviews (e.g. Dessauer et al. 1990, 1996). Although all research institutions and laboratories working with biological material are faced with this issue, either no special attention is given to that and long-established methods are used irrespective of specific needs or different types of biological materials, or specific preservation methods are actually being tested and optimized without publication of experiences and results. Here I present a practial overview of available storage techniques with comments on their application range in zoological and botanical studies using DNA. The majority of these methods were listed by Prendini et al. (2002). In the current paper, I present an update by citing some further techniques and a couple of recent developments, while focusing on the possible (dis) advantages, practical considerations and feasibility issues for collecting and archiving tissue samples.

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General considerations for establishing archival tissue collections

When molecular genetic investigations are foreseen based on tissue samples, several important issues should be considered prior to collecting and sampling efforts in order to optimize future storage conditions. These include—among others—the type of material that will be collected, the exact aim of the analysis, and certainly feasibility issues.

Establishing tissue and/or DNA sample collections can serve different aims. In most cases, tissue samples are collected related to projects with restricted, well defined aims (e.g. population genetic, phylogeographic or phylogenetic analysis of a given taxon), but also an increasing number of systematic collections is carried out for certain geographic

regions (e.g. representing the 'whole' biota of an island) or taxa (e.g. mosquitoes, birds, fish worldwide)—thanks to the global DNA barcoding initiative.

During the last years, large tissue and/or DNA collections were created in many natural history collections dedicated to long-term (i.e. archival) storage of genetic material. Unfortunately, and with a few exceptions only, serious and systematic investigations on the capability and performance of the different preservation methods were and still are partly neglected. A new science branch, biobanking, is emerging, but priorities will tend to be given to bioinformatic (e.g. databasing) and accessibility issues, etc. Protocols are mostly based on long-established, proven techniques, and most biobanks have biomedical relevance (for a global list of resources, check http://www.isber.org/links.html; for a European list, http://www.biobanks.eu/partners.html). These initiatives, however, also provide useful guidelines, 'best practices' to be followed (e.g. ISBER 2008).

Whenever possible, separate collections of specimens (i.e. for taxonomic studies) and tissues (for genetic analyses) are recommended, as in most cases they require different preparation methods, treatments (e.g. fixation), and conditions for archival preservation. Best practice of specimen mounting techniques in museum collections does not always overlap with the requisites for DNA preservation; see some examples in Mandrioli et al. (2006), Martinkova and Searle (2006). Ideally, this separation could be done while collecting, i.e. during fieldwork or expedition. Concerning larger organisms, usually a tissue sample will be taken, the removal of which at most insignificantly affects further identification and investigation of the specimen. For small-sized organisms, from which no tissue samples can be removed, the well-identified collection should be divided in two parts: one for genetic analyses, the other for taxonomic studies. However, alternatives such as 'DNA-friendly' methods for fixation and/or archival storage have to be considered and tested. On the other hand, killing methods may also affect DNA quality (e.g. Dean and Ballard 2001).

Special attention is required for type specimens, where the top priority is to minimize damage to the specimen, and therefore less invasive methods of tissue sampling should be favoured and applied: biopsy-like methods, removal of parts not used for taxonomic diagnosis, or minimally invasive sampling and DNA extraction techniques (e.g. Gilbert et al. 2007; Hunter et al. 2008; Phillips and Simon 1995; Pichler et al. 2001; Rohland et al. 2004; Rowley et al. 2007; Wisely et al. 2004).

With live animals, further minimally invasive methods are applicable: for example in vertebrates, blood withdrawal can be performed easily. For larger animals, from which sufficient amounts of sample can be taken, buccal swab methods might be good choices; protocols exist even for fish (Campanella and Smalley 2006). Furthermore, there are several additional possible sources of animal DNA that allow non-destructive sampling: feathers, hair, exuviae (shed skin), faeces and other excrements (e.g. owl pellets; Taberlet and Fumagalli 1996) can be sampled without significant damage to any part of the specimen. Even more peculiar sources of DNA have been identified: e.g., beach-washed shells of squids (Strugnell et al. 2006), scent marks of hyaenas (Malherbe et al. 2009), the foot mucus of snails (Armbruster et al. 2005), and the body mucus of fish (Lucentini et al. 2006) all have been used successfully for molecular genetic investigations. Moreover, Dalén et al. (2007) were able to recover fox DNA from fresh footprints in snow.

In general, more or less different preparation techniques and preservation methods are available and have been tested for major groups. Reviews and overviews exist for microorganisms (e.g. Mitchell and Takacs-Vesbach 2008; Morgan et al. 2006), plants (e.g. Flournoy et al. 1996; Pyle and Adams 1989), herbaria and seed collections (see the overview on plant biobanking by Hodkinson et al. 2007), invertebrates excluding insects (Dawson et al. 1998), insects (Dillon et al. 1996; Post et al. 1993; Quicke et al. 1999; Reiss et al. 1995), and vertebrates (Kilpatrick 2002; Seutin et al. 1991).

More specifically, various types of tissues have been conserved according to different needs. Solid tissues (both soft and hard tissues; typically muscle, liver, bone, feather, egg shell membrane, etc.), fluids (blood or blood clots, saliva, sperm, smears), excrements (urine, faeces), and cell cultures each need specific treatment to achieve optimal storage environment and conditions; some examples are listed below. The choice of optimal preservation technique also depends on the aim of the analysis: Priorities have to be defined regarding the duration of storage (transport, short-term to very long-term), the targeted quality (e.g. whether mtDNA, nuclear DNA, RNA or protein based analyses are foreseen), and the importance of non-destructive or little-invasive sampling.

Furthermore, a feasibility analysis should be carried out prior to collecting. One has to plan carefully with the expected volume of samples. This issue is coupled with budget allowance: While a case of few, important samples allows the use of expensive and/or complex methods, large series of samples usually must be treated at low costs per specimen. In search of an appropriate method, one has to plan in advance with the time needed for the preparation of collecting equipment, storage media, etc., and has to keep in mind the tasks that have to be carried out during or immediately after fieldwork. Some other important questions have to be answered before fieldwork: Is cooling/freezing/buffering/drying possible during the field expedition? Which

are the possibilities and costs for transportation, and are they coupled with special permit procedures and/or safety risks? Is it possible to prepare a working stock of tissues and a backup stock (for cases of emergency)? Is it planned to isolate DNA from fresh material just after collecting?

Tissue preservation methods in overview

The main goals of tissue preservation are to avoid any enzymatic (especially nuclease) activity leading to the damage and degradation of nucleic acids, and to avoid oxidative degradation (Adams et al. 1999; Prendini et al. 2002; Yagi et al. 1996). As enzymes are active in the presence of water, the elimination of water from tissues is the central step in the vast majority of preservation techniques. Alternatively, irreversible denaturation of enzymes could be targeted, but aggressive chemical or physical treatments may also lead to serious damage to DNA and other macromolecules. In many cases, chaotropic agents are used which disrupt the spatial structure of macromolecules, and therefore denature them. Moreover, several factors such as temperature, salt concentration, presence of different chemical agents, etc., may affect and alter enzymatic activity.

In the following presentation of various techniques, methods, chemicals, equipments and kits, a simple scheme is followed. The method or agent is introduced briefly, then the main advantages and/or disadvantages are listed. Notes on specific application ranges and obvious safety aspects conclude the description.

All main preservation techniques and relevant usage aspects are summarized in Table 1.

Desiccation methods

Desiccation methods use simple physical processes or chemical agents. Therefore, methods ensuring quick and complete desiccation of the samples should be regarded as the ideal ones. Beyond extrinsic factors (most notably environmental temperature and humidity), the process of desiccation largely depends on the size and type of the tissue (e.g. chitinized or soft tissues, bones and other hard tissues, plant or animal tissues). Dividing, crushing or squeezing the samples into small pieces generally facilitates the progress of desiccation. One of the main (weak) points of desiccation methods is that the samples should remain completely dry, ideally without any traces of water, therefore environmental humidity must be eliminated over long periods. This can be done effectively by adding silica beads/gel to the samples in most cases, but regular control of humidity and change or desiccation of silica is inevitable for long-term/archival storage. In addition, desiccation methods might well be combined with freezing techniques (see corresponding section below).

The chemical agents listed in the "Chemical desiccation" section below are used for desiccation only, not for archival preservation of the samples (in contrast to the substances listed in the chapter on "Fluids for general tissue preservation"). A performance test of some chemical desiccating agents on biological materials (wasps) has been published by Austin and Dillon (1997).

Dry preservation can be used for a broad variety of samples, especially if combined with silica gel. However, it is generally recommended for bones, teeth, hairs, feathers, fin clips (these should be dried accurately prior to long-term preservation), egg shell membranes, exuviae, and faecal samples. A further advantage of desiccation is that usually no specific treatment is needed prior to DNA extraction; the samples can be used directly.

Physical desiccation

Sun-drying or air-drying in the field The method is very easy to use, does not cost anything, and no additional material is required. Of course, it is not applicable under humid environmental conditions, as drying must happen as quickly as possible. There is a risk of possible contamination from other organisms (e.g. flies depositing their eggs, worms burrowing into tissues), but using mosquito nets or repellents can help to avoid part of the problem. Air-drying is definitely not recommended in humid tropical environments, and most aquatic organisms need to be removed from their habitat immediately, otherwise fast and efficient desiccation cannot be achieved. The application of this method should be restricted to initial desiccation, if no other methods are available.

Simple drying under controlled lab conditions Desiccation under controlled laboratory conditions definitely has to be favoured over air-drying or sun-drying. In remote areas, however, controlled lab conditions are not at the researcher's disposal, thus alternative methods should be used. There is a wide selection of lab drying methods and equipment: laminar flows, walk-in chambers, flash drying or oven-drying are a few examples. In the latter case, the temperature should not exceed c. 60°C to avoid serious damage to the sample. The progress of drying can be followed and controlled using a hygrometer, weight measurements or other indicators. This method can be used effectively for desiccation of various tissue types, but archival storage of samples certainly is not possible in ovens. Therefore it is only suitable for initial desiccation. As laboratory equipment, electricity, etc., are needed, their costs have to be included in budgetary estimations.

Table 1 Summary of the main tissue preservation techniques; the appropriate method has to be selected after careful prioritization of potentially relevant aspects

Aspect / Preservation	Target organism/ tissue	Adequacy for fieldwork	Optimal storage period	Handling time, ease of use	Health hazards	Cost and supply
Physical desiccation	solid tissues and small organisms, incl. plant material	for some methods only	desiccation, transport, short- to long-term	usually simple, straightforward	none	usually inexpensive
Chemical desiccation	various, incl. plant material	generally yes	desiccation only or transport	variable	significant	usually inexpensive
Ethanol	various, but no plants	yes, but difficulties with transport	short-term to long-term, very long-term with freezing	simple, straightforward	low toxicity (only when ingested); flammability	relatively expensive
Other alcohols, buffers with alcohols	various, usually no plants	yes, but difficulties with transport	short-term to long-term	relatively simple	low to significant; some flammability	inexpensive to expensive
Di- and triols	various (further tests needed)	yes	transport to mid-term; long-term with freezing	relatively simple (transfer to ethanol may be necessary)	usually low toxicity	relatively expensive
Acetone, hexane	various, mainly insects	yes, but difficulties with transport	short-term to long-term	simple, straightforward	health damage; flammability	expensive
DMSO buffers	various	yes	short-term to long-term	requires advance preparation	usually modest	relatively expensive
CTAB buffers	various, preferred for plants	yes	short-term to long-term	requires advance preparation	health damage	relatively expensive
EDTA buffers	various	yes	transport to mid-term; long-term with freezing	requires advance preparation	usually low toxicity, depending on further substances	relatively inexpensive
Urea buffers	various, mainly animals	yes	short-term to long-term	requires advance preparation	health damage	relatively inexpensive
GITC buffers	various	yes	short-term to long-term	requires advance preparation	health damage	relatively expensive
Freezing at −20°C	various	no	short- to mid-term	simple	none	electricity needed
Freezing at −80°C	various	in form of dry ice	long-term to very long-term	simple	low (risk of cold injury)	electricity or source of dry ice needed
Freezing at −150°C	various (incl. living cells)	no	very long-term	simple	low (risk of cold injury)	electricity needed
Liquid nitrogen	various (incl. living cells)	difficult (liquid nitrogen tank)	very long-term	simple, but requires training	low (risk of cold injury)	source of liquid nitrogen needed
RNAlater, Allprotect	various	yes	transport or short-term; long-term with freezing	simple, straightforward	none or low	(very) expensive
FTA paper, Guthrie card, etc.	mainly fluid samples (e.g. blood, saliva, mucus) or small tissues	partly yes	short-term to long-term	usually simple, release of samples may be more difficult	none or low	relatively expensive
Household methods	various	yes	transport or short-term	simple, straightforward	usually none	usually inexpensive

Vacuum-drying Vacuum-drying is an efficient method to remove water or moisture from tissues. It is another lab-based method, and material should be prepared according to the requirements of the equipment. Vacuum pumps are usually diaphragm pumps, and a wide spectrum of capacities and pressures is commercially available. This method is only applicable for drying; another method has to be used for archival storage. It can be an alternative to the

previous method if rapid desiccation at room temperature is necessary.

Freeze-drying (lyophilization or cryodesiccation) Freeze-drying is a process by which the material is frozen, and frozen water is subsequently sublimated by pressure reduction and slight heating. The technique involves complex protocols (also including various dehydrating agents) that have to be optimized for the type of material. Similar to cryoprotectants (see the corresponding section below), so-called lyoprotectants aid in preserving macromolecules. One of the standard works on lyophilization was written by Jennings (1999). Although the technique is used extensively in pharmacology and the food industry, lyophilization is not sufficiently tested for archival storage of tissue samples, and due to its high costs (equipment prices and energy demands), it can only be considered as a real option when a large amount of (similar) samples has to be processed.

Silica gel beads Silica gel beads made from sodium silicate are very easy to use (Chase and Hills 1991), offering an inexpensive and relatively effective way to desiccate and store tissue samples. The major advantage of the technique is its wide range of application, including various size scales, kinds of tissues, etc. It is very straightforward to put silica beads as a desiccant into small tubes or vials; therefore it is a recommended dry preservation for field sampling as well. Desiccated samples can be stored at room temperature for longer terms, although the material has to be checked for dryness regularly. Combining silica and freezing is not recommended. Self-indicating silica gel (blue or orange) changes color when adsorbing a certain amount of water. It can be reused after desiccation in an oven. For optimal use, the ratio of silica to sample has to exceed 10:1, and ideally the tissue material should be dissected in small pieces. Generally, the method is applicable to various tissues for initial and/or archival storage, and also performs well on aquatic samples. For example, it is recommended for amphibian and fish tissues. Furthermore, using silica gel beads is a good option for transport: Dry material weighs less than buffered material, and no special regulations (e.g. on airplanes) are applicable. If one checks for dryness regularly, the method can be better, at the very least less complicated, than preservation in ethanol. Dry (i.e. white) silica gel is non-toxic and non-flammable, but it may cause skin irritation or allergic reactions, therefore some precautions are required. For faeces samples, a short ethanol treatment followed by silica gel desiccation can be favoured (Nsubuga et al. 2004; Roeder et al. 2004).

Rice Using rice as a desiccant is a very cheap, environment-friendly and easy-to-use alternative to silica gel, though its efficiency does not reach that of silica gel. Rice is widely available in most parts of the world, and after desiccation can be reused several time. However, as rice itself is a biological material, there is a chance of contamination (e.g. it can be infected by fungi). The use of rice as a desiccant is therefore only recommended in 'emergency' cases, when no other methods are available.

Sodium chloride (NaCl, table salt) crystals Similar to rice, crystallized sodium chloride is a very cheap and easy-to-use alternative to silica. Moreover, in sufficiently high concentration and in wet environments, it also denatures proteins, and therefore decreases protein activity. Unfortunately, it adsorbs significantly less moisture than silica gel, therefore cannot be seen as a satisfactory alternative. The use of salt is therefore not recommended for initial or provisional storage, even though it is a non-hazardous substance.

Calcium sulphate ($CaSO_4$, dierite) Dierite is a relatively widely used and inexpensive desiccating agent, also used at industrial scales for drying ("drierite"). It is successfully applicable to the transport or short-term preservation of various plant tissues, especially leaves (Liston et al. 1990). It performs relatively well in the short term, and can be regenerated, but has severe drawbacks for archival storage as it adsorbs less moisture than silica gel. As with other salt crystals, problems emerge in humid conditions. Calcium sulphate is definitely not recommended for animal tissues, even though it is non-hazardous.

Anhydrous calcium chloride ($CaCl_2$) In its anhydrous form, calcium chloride is a strongly hygroscopic material. It can be produced from lime (CaO, see "Chemical desiccation" below), and for laboratory application it is commercially available in pellet form. The use of calcium chloride as a desiccating agent is similar to that of silica gel, although it might not perform as effectively as silica. It can be a good and inexpensive alternative for desiccating solid tissues, leaves or seeds (Armitage et al. 1989). Anhydrous calcium chloride is non-hazardous; it is a permitted food additive.

Chemical desiccation

Amyl acetate ($CH_3COO(CH_2)_4CH_3$) Amyl acetate is an organic substance (ester) with a banana-like aroma. It is a solvent commonly used in industry to remove water. Amyl acetate has been used in one of the traditional techniques for the preservation of anatomical dissections (Saunders and Rice 1944), but not extensively for tissue preservation in molecular biology. It is a flammable liquid, and can be harmful (especially in higher concentrations). It can cause

skin irritation as well. Therefore, it may not be a good and proven alternative to other agents.

Hexamethyldisilazane ($[(CH_3)_2Si]_2NH$, HMDS) Hexamethyldisilazane is a clear, moisture-sensitive liquid. It makes a good alternative to critical-point drying, when no dryer or pump is available. Due to its generally rapid infiltration, it can be used well for insect tissues (e.g. Heraty and Hawks 1998). The main application for HMDS has been in microscopy, but it was also recommended for pollen-drying (Chissoe et al. 1994). For optimal results, it can be combined with vacuum-drying. It is flammable, can be harmful if swallowed, inhaled or absorbed through the skin, and can cause severe irritations or burns to skin, eyes and the respiratory tract, therefore requires special precautions.

Xylene (Dimethylbenzene) Xylene is a colorless, sweet-smelling liquid. It is commonly used as a solvent in printing, the rubber and leather industries, but also applied in paints. In biology, xylene is used as a fixation agent. Furthermore, it is an effective desiccating agent. Austin and Dillon (1997) used a 50:50 (*v*/*v*) mixture of 95% ethanol and xylene degreasing followed by a treatment of 100% xylene and air-drying. Mainly due to safety reasons, it cannot be recommended for tissue samples. Xylene evaporates quickly and can enter the body rapidly when its vapor is inhaled. It can also be absorbed through the skin, particularly during prolonged contact. Overexposure to xylene affects the nervous and respiratory systems, and the skin. Furthermore, the substance is very flammable, thus has to be dismissed as a practical desiccating agent.

Methyl cellosolve/cellusolve (Ethylene glycol monomethyl ether) Methyl cellosolve is a clear, colorless liquid with an ether-like odor. It is a widely used industrial solvent for paints, inks, and also applied as an additive for deicing airplanes. In biology, it is used to desiccate various plant materials, e.g. for microscopic analyses. It has been tested extensively for the desiccation of protoplasts (O'Brien et al. 1997) and found to be the best technique because, unlike alcohols, it dehydrates the protoplast gradually (Weir 2001). For this reason it is not recommended for tissue desiccation where quick drying and the preservation of DNA (rather than of the tissue fine structure) are targeted. If it enters the human body, methyl cellusolve exerts its effect primarly on the hematopoietic and central nervous systems. It evaporates easily, and the vapor is a mild irritant.

Calcium oxide (CaO, lime) Calcium oxide is a white crystalline solid; it is usually made from limestone containing calcium carbonate. It is an effective desiccant, easily and vigorously reacts with water, and this reaction is exothermic (i.e. produces heat). There is a reversible reaction between calcium hydroxide and calcium oxide, therefore if the hydrated lime is heated to redness, the calcium oxide will be regenerated. Although it is inexpensive, calcium oxide can be recommended only for special cases (e.g. for desiccating seeds or solid tissues), because it can cause severe irritations and burns. It is certainly harmful when swallowed.

Sulphuric acid (H_2SO_4) Sulphuric acid is a strong mineral acid, and in its concentrated form a colorless oily liquid. It acts as a potent dehydrating agent, and is widely used to produce dried fruits. Similarly to CaO, it is only recommended in special cases (e.g. seeds, solid tissues), and has to be handled with serious caution, as sulphuric acid is a very aggressive substance. It is corrosive, causes eye and skin burns, and can cause severe respiratory and digestive tract irritations with possible burns. In the worst case, it can be fatal when inhaled. Therefore, it should be used only where controlled, safe laboratory conditions are available.

Use of additives for dry storage

In contrast to freezing methods (see the corresponding chapter below), there is no clear indication that the use of additives significantly improves the persistence of ideal storage conditions and the state of the samples. Ambient temperature is one of the main factors influencing the condition of dry samples. Smith and Morin (2005) reported significant quality loss in dry samples stored over longer terms between temperatures of −20°C and 4°C. The best results were found at −80°C, regardless of storage additives. In another test, almost similar quality was measured when using dried samples in the presence of trehalose (room temperature or −80°C). The use of 15% trehalose was also tested. Some other additives, such as TE buffer or Hind III digested lambda-DNA, can be used.

Fluids for general tissue preservation

Environmental humidity is omnipresent, thus maintaining controlled and 'completely' dry conditions for archival storage is an unexpectedly difficult task. Therefore, other methods using liquids have been established and tested for archival tissue storage. The aim remains the same, namely to decrease or eliminate enzymatic activity while at the same time preserving DNA. The fluids used for sample preservation usually contain alcohols, one or more specific substances inhibiting enzymatic activity, chaotropic agents, or are combinations of those. The major drawbacks of liquids are that wet samples weigh more than dry ones, the time needed for buffer preparation, and the hazards and safety aspects in many cases. Furthermore, many of these

liquids (especially the ones containing alcohols) have to be removed prior to DNA extraction. On the other hand, some buffers are also used in extraction protocols.

Alcohols (especially ethanol) and specific tissue preservation buffers are applicable to a wide range of samples, including whole animals (mainly invertebrates or small-sized vertebrates), muscle samples, any kind of inner organs, blood, fin clips, tail tips, scat, etc. In contrast, alcohol preservation is not commonly applied to plant samples.

Alcohols

Ethanol No doubt, ethanol is the most frequently used medium for tissue preservation in zoology, but there is significant variation in the details of its application. It is easy to use, and a good method for tissue preservation. On the other hand, it is a relatively expensive, flammable liquid that also evaporates quickly. If possible, the use of non-denaturated ethanol is favoured, but traces of denaturating agents (e.g. isopropanol, acetone, methyl ethyl ketone, also gasoline) usually do not significantly alter the performance. However, it is important to know the denaturating agent and its concentration to evaluate potential risks. According to plenty of test, the optimal concentration is about 95–99% (King and Porter 2004, who also compared with isopropanol). EDTA (ethylenediaminetetraacetic acid) can be added to 95% ethanol (Dessauer et al. 1996). With 70% ethanol, a high degree of degradation has been observed, even after one year. An alternative is 70% ethanol diluted with 1xTE buffer (instead of water), which yields good results (B. S. Hedges pers. comm.). Another alternative is to use 70–80% ethanol with 1–3% glycerine, which keeps the specimens moist if the alcohol evaporates. However, in the latter case similar quality loss can be expected as with slightly diluted ethanol. In contrast, diluted ethanol (over 70%) can be recommended as one of the optimal storage media for faecal samples; e.g., 90% ethanol was favoured for brown bear faeces (Murphy et al. 2002). Interestingly, absolute ethanol may not be the optimal medium as it is most expensive and can contain traces of benzene that seriously affect DNA preservation (Ito 1992). The ethanol-tissue ratio should exceed 5:1, although some authors recommend much higher ratios (e.g. Martin 1977). With large pieces of tissue or whole animals, injecting ethanol can/should be carried out. Especially during the first days of preservation, replacing and controlling ethanol is important, as tissues still contain plenty of water. For samples to be stored in ethanol, the use of tubes and vials with caps that close perfectly are self-evident (e.g. high-quality microtubes, screw-cap tubes with rubber rings, or additional external sealing with Parafilm). Inscriptions on the containers should be made alcohol-resistent (using ethanol-proof inks or pencils). If necessary, dry and/or frozen samples can be transferred to ethanol without any problems. The removal of ethanol prior to DNA extraction is of central importance; one of the easiest ways is to use a thermoshaker or oven for drying at a temperature below 60°C (a few minutes are sufficient in most cases).

Other alcohols with one hydroxyl group Other simple alcohols—most notably methanol, propanol and isopropanol—are similar to ethanol in that they are easy to use but flammable, therefore similar precautions are needed. They can be significantly cheaper than ethanol. However, industrial-grade alcohols are often contaminated (e.g. with metals), which affects quality. Using isopropanol (Rake 1972) or absolute methanol and proteinase (pronase E) can be favoured if ethanol is unavailable, although methanol is toxic. EDTA can be added to isopropanol as well; it represents a good and safe alternative for preserving blood samples. In many natural history collections, isopropanol is used for archival storage of specimens due to budgetary constraints.

Buffers containing alcohols Several buffers containing alcohols have been developed in order to optimize storage conditions of specimens or tissue samples. One of the major drawbacks of absolute or nearly absolute ethanol is that it makes tissues rigid, inelastic and shrunken, so further positioning of specimens is not easy. In contrast, water-diluted alcohol leads to DNA degradation. Two buffers containing alcohols which are also widely used as tissue fixatives are listed by Srinivasan et al. (2002). They only differ in the type of alcohol: Carnoy's solution is made of 60% ethanol, 30% chloroform and 10% glacial acetic acid, whereas in methacarn ethanol is replaced by methanol. A slightly similar solution, "MA80" is one of the standard preservatives for water mites (Saito and Osakabe 1992); it is a 2:2:1 mixture of methanol, glacial acetic acid and distilled water.

Di- and triols and their buffers

(A) Glycerin/glycerol (propane-1,2,3-triol). Glycerin is a color- and odorless, viscous liquid which is widely used in the food industry. It is relatively non-toxic, and (similar to ethanol) easy to use for tissue preservation. It is significantly less flammable than ethanol; therefore no special handling precautions are required. It also acts as a cryoprotectant, and therefore makes a recommended agent for freezing methods. In its pure form it can be applied for tissue preservation, but in this case it is relatively expensive, and its quality may not reach that of ethanol. Glycerin can also be combined with glacial acetic acid. Buffered glycerin (50%) is a good alternative for transporting

tissues when refrigeration is not available (Munson 2000); the buffer is composed of (A) 21 g citric acid mixed in 1,000 g water, and (B) 28.4 g anhydrous sodium phosphate in 1,000 g distilled water; the final buffer is a mixture of 9.15 ml of A and 90.85 ml of B (A+B together 100 ml) with 100 ml of glycerin. Another buffer containing glycerin is Koenike's fluid, a smelly but often-used preservative for water mites (Mitchell and Cook 1952); it is a 5:4:1 mix of glycerin, water and glacial acetic acid. It should be noted, however, that according to Rey et al. (2002), a further buffer, Angelier's fluid (Valdecasas and Baltanás 1989) performs even better for preserving water mites than 70% ethanol or Koenike's fluid, even though it only contains water, chromic acid and acetic acid (i.e. no alcohol). Especially for blood samples, 2% 2-phenoxyethanol can be added to glycerol.

(B) Propylene glycol (propane-1,2-diol). Propylene glycol is a sweet, odorless liquid. It is a permitted food additive, also acts as a cryoprotectant, and is easy to use for tissue preservation. In contrast to ethylene glycol, which is one of the most common antifreeze agents and also used in insect traps, propylene glycol is regarded as safe due to its (relative) non-toxicity and non-flammability, thus is allowed to be carried on airplanes without safety restrictions. Similar to ethanol, it is relatively expensive. In general, using pure propylene glycol seems to be a good alternative to ethanol (e.g. Vink et al. 2005); it is recommended for small parts of tissues. Most notably, transfer of samples to ethanol is required (for c. 1 day) prior to DNA extraction.

(C) Polyethylene glycol (PEG). The term polyethylene glycol refers to a group of oligo- or polymers of ethylene oxide. The types that can also be applied to tissue conservation are viscous, non-hazardous and non-flammable liquids. Pure polyethylene glycols are not well tested for tissue preservation. Instead, they are usually used as embedding media in microscopy (Wolosewick 1980; see also Bosman and Go 1981 for tests) or as additives in buffers/alcohols or in cryopreservation techniques in lower concentrations (even for ovarian tissues in reproduction medicine; see Isachenko et al. 2008), and may further increase the performance of these methods. Moreover, PEG is also used for purification of DNA from plant samples (Rowland and Nguyen 1993). For routine preservation of field samples, further tests are needed to evaluate the potential of PEG.

Acetone (propanone)

Acetone is an extremely flammable liquid, and an effective chemical desiccating agent. It is applicable for tissue preservation and for sample preparation in electron microscopy (Walpole et al. 1988; Ware and Cross 1989), either in its pure form or with additives. It is used and recommended as one of the high-quality archival methods for insects (Fukatsu 1999; Trumen 1968), but may not have significant advantages over ethanol. Instead, it is often used to dry ethanol-preserved specimens, especially insects (van Noort 1995). In addition to its fire safety risks, acetone is harmful when swallowed or inhaled, causes irritation to skin, eyes and the respiratory tract, and also affects the central nervous system. Acetone has been used as a fixative in the acetonemethylbenzoate-xylene (AMeX) technique (Sato et al. 1990). This method involves overnight fixation of tissues in acetone at −20°C, then clearing in ethylbenzoate and xylene before embedding in paraffin. Regarding DNA preservation issues, this method has to be favoured in comparison to fixation techniques using formaldehyde. Prior to DNA extraction, the removal of acetone is necessary.

Dimethylsulfoxide (DMSO) buffers

Buffers containing dimethylsulfoxide (DMSO) provide a good-quality and cost-efficient method for tissue preservation. DMSO is mainly used in aqueous solutions. Therefore, these buffers are not flammable (unlike pure DMSO) but they should be prepared in advance. According to tests, about 20–25% DMSO in saturated NaCl provides (very) good results, also at room temperature. Similarly to ethanol preservation, the ratio between DMSO buffer and sample should exceed 5:1 (at the very least equal 3:1). An improved protocol uses 20% DMSO, 0.25M sodium-EDTA and NaCl to saturation; pH=7.5. These types of DMSO buffers are especially recommended for marine samples (e.g. Dawson et al. 1998; Häussermann 2004; Maiers et al. 1998). According to Dawson et al. (1998), the combination of DMSO and NaCl was the best-performing storage method for various marine invertebrate samples such as scyphozoans, anthozoans, polychaetes and gastropods. Häussermann (2004) corroborates this finding, and also refers to methods for relaxation of anemones before sampling/killing (e.g. using menthol crystals). Another type of DMSO buffers contains SDS (sodium dodecyl sulphate); the DMSO 0.5% SDS lysis buffer is one of the standard buffers for shipments at the United States Department of Agriculture (USDA); it is easy to prepare from 97.5 ml of DMSO buffer solution and 2.5 ml of 20% SDS (both components can also be used separately). Furthermore, DMSO can be combined with citrate buffers which are often used in microscopy; this method is also applicable to archival storage (Vindeløv et al. 1983). Finally, DMSO can be combined with EDTA in buffers; this DMSO/EDTA/Tris/salt (DETs) method seems to be one of the most

effective preservation techniques for some peculiar biological materials such as faeces (Frantzen et al. 1998).

CTAB (hexadecyl-trimethyl-ammonium bromide, cetrimonium bromide) buffers

CTAB is a detergent commonly used for DNA extraction (e.g. Doyle and Doyle 1987). It is mainly used for plant samples, but also performs well on animal tissues or protozoan cells (e.g. Shahjahan et al. 1995; Winnepenninckx et al. 1993). CTAB in saturated NaCl was originally applied for the preservation of leaves (Rogstad 1992). Since then, some modifications of the method have been tested and published. According to Thomson (2002), the addition of 200 mM sodium ascorbate acting as an antioxidant improves the quality. Storchová et al. (2000) suggested to homogenize the samples in a sorbitol wash buffer before CTAB extraction, as this eliminates polyphenolics, polysaccharides and other substances without significant loss of DNA. Similar advice was given by Krizman et al. (2006).

EDTA (ethylenediaminetetraacetic acid) buffers

Longmire's lysis buffer Longmire's lysis buffer (Longmire 1997) is an inexpensive, non-flammable liquid that is relatively widely used for tissue preservation, with a quality close to that of DMSO/salt buffers. It contains 2M Tris-HCl, 0.5M EDTA, 5M NaCl and 20% SDS (at pH=8.0). There are several slight modifications to the concentrations of the ingredients (e.g. "easy blood" from Munson 2000). It is also called TNES buffer and represents a good-quality buffer for transporting biological (mainly blood) samples without the necessity of cooling or freezing, and it is recommended for short-term preservation. Also, 20% SDS solution can be used alone at room temperature for similar purposes. It should be noted that SDS is flammable in its pure (crystal) form, and a respiratory, skin and eye irritant. When making a solution, SDS has to be added to (plenty of) water. On the other hand, the aqueous solution of Tris, EDTA and NaCl (without additional substances) is also used as storage medium, although it may not represent an optimal solution. This buffer is called TNE or TNE2, and usually has a concentration of 10 mM TrisHCl, 0.01 mM NaCl, and 0.2 mM EDTA at pH=8.0. Especially for blood samples, a modified form of TNE is applicable for short- or mid-term storage; it contains 10 mM Tris-hydroxymethyl-amino-methane, 10 mM NaCl, and 2 mM EDTA at pH=8.0.

EDTA-thymol buffers EDTA buffers with added thymol and NaF represent relatively inexpensive, non-flammable storage media which yield good results for many types of tissues. They are good and safe alternatives to ethanol, and also suitable for longer-term preservation when frozen, although they have to be prepared prior to sampling. Usually, they contain around 10% EDTA, 0.5% NaF, 0.5% thymol, and 1% Tris at pH=7.5, but concentrations vary (Arctander 1988). Also, sodium in the EDTA salt can be substituted by potassium, but the latter is less soluble in water. A similar solution containing EDTA and thymol has been patented in Europe; it contains 0.39–0.56 M EDTA in its trisodium form, and 0.33–0.48 M sodium fluoride (NaF). This solution is saturated with thymol (extracted from *Thymus vulgaris*; approximately 0.0001 M), and the pH is set to 8.0 with HCl.

Queen's lysis buffer Queen's lysis buffer (Seutin et al. 1991) is a typical storage medium mainly used for invertebrate or especially blood samples. It contains 10 mM Tris, 10 mM NaCl, 10 mM disodium-EDTA, and 1% n-lauroylsarcosine (pH=7.5–8.0). Similar to other buffers, disodium-EDTA can be replaced by dipotassium-EDTA. Although it is widely used (e.g. by ornithologists), it may not yield optimal results, as several quality problems have been reported (e.g. by Conrad et al. 2000).

MSB-EDTA buffer A further type of EDTA buffers contains sugars as additional agents. MSB-EDTA buffer (Lansman et al. 1981) is made of 0.21 M mannitol, 0.07 M sucrose, 0.01 M EDTA, and 0.05 M Tris-HCl. This buffer is also used in protocols for separate isolation of mitochondrial DNA. Furthermore, mannitol is known to be a scavenger of oxygen radicals. It may be an option for preserving blood samples or for short-term preservation in general, but certainly many other methods exist which may perform better.

Hexane

Hexane is an alkane, a colorless, flammable liquid with low toxicity. In biology, it is often used as a preservation agent for materials intended for various hydrocarbon analyses (French and Jefferies 1971); it can also be combined well with acetone. Moreover, hexane is compatible with subsequent DNA or isozyme studies (Narang and Seawright 1990; Narang et al. 1993). Therefore, it represents a preservative for specific (mainly insect) samples which are also intended to be used for other analyses.

Buffers containing urea (carbamid, diaminomethanal)

Urea is a nitrogen-containing organic compound, an important product mainly used as a fertilizer in agriculture. In its pure form, urea is a white solid that irritates skin and eye, and which is hazardous when inhaled or ingested.

Highly concentrated aqueous solutions of urea effectively denature proteins and DNA, and urea is one of the main ingredients in denaturing gels (e.g. polyacrylamide gels) used in molecular biology. Similarly, urea can be used for preservation purposes for different solid vertebrate tissues (Asahida et al. 1996, for example, suggested it for fish samples), as well as for small invertebrates or blood samples. The TNES-urea buffer used by Asahida et al. (1996) contains 6–8 M urea, 10 mM Tris-HCl, 125 mM NaCl, 10 mM EDTA, and 1% SDS (pH=7.5). Dawson et al. (1998) confirmed the good performance of urea in long-term preservation.

Guanidinium isothyiocyanate (GITC) buffers

Guanidinium (or guanidine) isothyocyanate is a powerful chaotropic agent; it effectively denatures macromolecules such as RNA, DNA and proteins. It is used in some DNA extraction protocols (e.g. Chirgwin et al. 1979; Chomczynski and Sacchi 1987), and is particularly suitable for 'difficult' samples (Konomi et al. 2002). It performs well in preserving solid tissues: a 4 M solution of GITC can be used as storage medium. GITC buffers are harmful; especially the contact with acids has to be avoided as the reaction can release highly toxic hydrogen cyanide. An alternative (for specific cases) is saturated guanidine hydrochloride; this agent is also used for DNA extraction.

Fixatives and DNA preservation

The application of 'DNA-friendly' fixatives is particularly important when the same sample has to be used for several different purposes, i.e. for microscopic, morphological and molecular genetic investigations. The problem is that several cytological fixatives affect DNA integrity and quality, and can lead to severe damage of the macromolecules (e.g. Douglas and Rogers 1998). This is often the case with small invertebrates where dissection can be impossible. Here, specific fixation techniques should be selected which are compatible with subsequent DNA or RNA studies. Recently, good progress has been achieved concerning the replacement of formalin, a widely used fixative agent. One of the alternatives using acetonemethylbenzoate-xylene (AmeX) was already presented above (Sato et al. 1990).

Another method, the HOPE technique (Hepes glutamic acid buffer mediated organic solvent protection effect; Olert et al. 2001) is based on an incubation of tissues in a special mixture containing amino acids (pH=5.8–6.4). This treatment is followed by a dehydration step in cold acetone. According to tests, a good-quality DNA or RNA yield can be achieved this way. UMFIX is another recently developed fixative that protects macromolecules (Vincek et al. 2003).

Freezing methods

Beside desiccation and buffering, freezing represents the third alternative for archival storage of tissue samples, because low temperature effectively inhibits enzymatic activity and generic DNA degradation. In general, however, buffered samples perform far better after thawing and refreezing, because they are (partly) protected against degradation occurring during temperature changes. Furthermore, desiccation can be combined with freezing (in optimal cases it is lyophilization).

Freezing at −20°C

Freezing tissue samples at −20°C is a convenient and simple way for DNA preservation. Only a household freezer is needed; in the simplest case no additional supplies other than electricity are required. Moreover, simple freezing can be combined well with other methods in order to reach optimal storage conditions. Using additional techniques (most notably ethanol or buffers as listed in the preceding chapters) provides a safeguard for cases of power failure. At this temperature, however, there still is enzymatic activity and degradation; therefore freezing alone is not suitable for long-term storage. Moreover, freezing at −20°C is not well suitable to preserve living cells; even mutagenic effects of freezing-thawing events were reported in yeasts (Stoycheva et al. 2007).

Ultra low-temperature freezing at −80°C and at −150°C

Ultra low-temperature freezing at −80°C yields good and stable preservation quality. There are two ways to reach this temperature. (1) The use of dry ice (frozen CO_2) is well applicable under field conditions or for sample transport; all that is needed is a well-isolating (e.g. styrofoam- or vacuum-insulated) box. However, it represents a good option only for relatively small amounts of sample. Due to the volume consumed by the dry ice, packing and insulating material, the stored items easily become bulky. Moreover, special regulations apply for the transportation of dry ice on airplanes. (2) For archival storage, ultra low-temperature freezers are available, but they are expensive and their power consumption is high. Coupled with CO_2 or N_2 backup and/or automated alarm systems they present a relatively safe option even in cases of brief power loss. The method can be combined well with some preservation media to reach optimal storage conditions. Using ethanol or 30% glycerol and ultra low-temperature freezing is optimal for (very) long-term storage. However, near the upper end of the ultra-low temperature range, there still is DNA degradation as a result of weak enzymatic activity. Significant progress in ultra low-temperature freezing has

been achieved with freezers operating at around −150°C. This temperature lies below the recrystallization point of water (around −130°C), therefore offers an excellent storage environment for any tissue sample. For long-term or 'permanent' storage, it represents the recommended—though expensive—way. The power consumption of such freezers is very high, but maintenance of the equipment and samples is far less complicated and corresponding costs are lower than in the case of liquid nitrogen preservation.

Preservation in liquid nitrogen

Keeping biological samples in liquid nitrogen (temperature around −200°C) yields very good storage quality for all types of macromolecules, as virtually no chemical or biological processes are working at such extremely low temperatures. Moreover, keeping samples below −130°C also prevents recrystallization. The method is well applicable for long-term storage of DNA, RNA and protein samples, and also for living cells (e.g. reproductive cells). Unfortunately, long-term preservation in liquid nitrogen is fairly expensive, as continuous maintenance and a permanent source of liquid nitrogen are required. An alternative option is the use of public or commercial services for very long-term storage in liquid nitrogen (e.g. Ambrose Monell Cryo Collection, American Museum of Natural History, New York). A nice example of preserving "genetic voucher specimens" was published by Hanner and Webster (2002). It is fairly complex to use liquid nitrogen in field expeditions, but large, vacuum-insulated metal tanks and portable dry-shippers are available for this purpose as well.

Cryoprotectants

Related to freezing techniques, several chemical agents can be used to protect macromolecules and/or tissue integrity from freezing damage caused by ice formation and other crystallization events. Withers (1980) reviewed these substances and techniques in detail. The most commonly used cryoprotectants are various types of glycol (ethylene glycol, propylene glycol, glycerol), DMSO, sugars (most notably trehalose, glucose and sucrose), amino acids, methanol, polymers or colloids, and other specific mixtures. A critical review of cryopreservation and cryoprotectants was presented by Karlsson and Toner (1996).

Other special methods or kits

In this chapter, some further, partly recently developed methods are summarized that do not belong to the preceding categories. The majority of the preserving agents listed below can be purchased commercially. Manuals or protocols giving useful advice on the respective procedure are usually enclosed with corresponding ready-to-use kits.

RNAlater®, Allprotect Tissue Reagent

RNAlater® (a registered trademark of Ambion) is a recently developed, little to non-toxic, and non-flammable fluid that performs very well in DNA and RNA preservation, although the tests performed so far do not include data series over long periods. The substance is especially suitable for RNA preservation, for DNA/RNA microarray analyses, gene expression studies, and when complex genetic investigations require high-molecular RNA or DNA (Barrett et al. 1999; Mutter et al. 2004). Moreover, a broad scale of samples (microorganisms, plant and animal tissue samples, cell cultures) can be preserved in RNAlater at room temperature for short periods. For archival storage, freezing is definitely recommended. The product is (very) expensive compared to other methods, therefore recommended only for cases in which sample quality has the highest priority. A similar recent development intended for the stabilization of biomolecules is the Allprotect Tissue Reagent (Qiagen). According to the manufacturer, this substance is able to stabilize DNA, RNA and proteins simultaneously in various tissue samples. For processing of samples preserved in RNAlater or Allprotect Tissue Reagent, different, proven DNA/RNA or protein extraction kits of the same manufacturers are available.

FTA paper®

FTA paper® (patented by L. A. Burgoyne, Whatman) is a commercial product; it is an impregnated thick filter paper (i.e. a macroporous cellulose matrix) for tissue or DNA preservation (Smith and Burgoyne 2004). It is especially recommended for fluid samples, and usually yields good results with blood samples, blood clots, small tissue samples (max. 3–5 mm in diameter; also successfully tested for fin clips of fish), saliva, sperm or even cheek wipes. In particular for sperm samples, FTA paper represents a good alternative to the traditionally used 0.01–0.02% sodium azide solution. It represents a dry preservation method; samples have to be spotted on the filter and air-dried. Nonetheless, the technique is simple. The paper is impregnated with 50 µl of 2% SDS, 10 mM EDTA, and 60 mM Tris spotted on 1 cm^2 Whatman 3 mm paper, then dried. Smith and Burgoyne (2004) published four processing methods for different sample types; according to them the subsequent DNA extraction is fairly easy. For buccal samples collected on FTA paper, Johanson et al. (2009) recently published a DNA elution method using a modified methanol fixation. FTA paper is relatively expensive, but

this technique may be advantageous and should be preferred when the aim is to establish a systematic collection using standardized methods.

Guthrie cards, and other filter papers for dried blood spots (DBS)

Guthrie cards are routinely used for storing blood samples in medical practice. It was originally developed and used for phenylketonuria screening of newborns (Guthrie and Susi 1963). The performance of Guthrie cards is similar to that of FTA paper. DNA extraction from Guthrie cards is well possible (Schneeberger et al. 1992); an improved processing method for human blood samples was published by Makowski et al. (1995). Filter papers used to dry blood spots (such as GT-903) can be impregnated with GITC (see the section on that substance above), which can improve general preservation quality (Makowski et al. 1997). Furtermore, high-throughput DNA extraction methods are available for Guthrie cards (Hamvas et al. 2001). These cards or filter papers are easy to use and represent a proven method for blood samples, but they are relatively expensive.

Vacuum packing

Vacuum packing (i.e. preserving samples in a low-pressure/vacuum environment) recently became a widely used technique in the food industry. The major advantage of the method is that it does not require any additives or chemicals and therefore does not significantly alter the integrity and characteristics of the sample. However, it is not sufficiently tested for tissue samples or a broad range of biological materials. It may represent a good (though probably not very cheap) alternative to other methods, although its application may be restricted to laboratory use, as it requires equipment to create the vacuum and pack the sample immediately.

Household methods

Household methods are inexpensive, widely available for tissue preservation, and do not require special equipment. However, in general, the achievable quality is questionable. As these techniques do not follow exact protocols, they are not sufficiently tested and proven, thus can only serve as an 'emergency' option when no other alternatives are available. One of the simplest method for blood samples and other body fluids is the use of toilet paper. Also for other types of materials and for short periods, samples can be preserved using laundry detergents (e.g. Kuch et al. 1999). Moreover, laundry detergents can be applied for DNA extraction as well (Bahl and Pfenninger 1996; Brzuzan 1997). Instead of ethanol, extremely hard liquor (e.g. triple-distilled vodka) can be used (Oakenfull 1994).

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