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Obtaining, Storing and Archiving Specimens and Tissue Samples for Use in Molecular Studies

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Collection, storage and archiving of specimens and tissue samples are prerequisites for the successful acquisition of molecular data for any systematics or population genetics study. Field collections represent the most obvious source for tissues, but there are many ways in which tissues can be obtained without ever going into the field. Stock centers and culture collections, commercial supply companies, seed and spore banks, botanical gardens and zoological

parks provide an array of live organisms, fresh tissues, cultures or cell lines, while vast quantities of frozen, desiccated or preserved tissues are stored in the collections of frozen tissue facilities, natural history museums and herbaria, among others. This chapter reviews the more important practical aspects of the selection of appropriate tissues for protein or nucleotide extraction, the preservation and temporary storage of freshly collected tissues in the field and the acquisition of specimens and tissue samples from alternative sources. Practical guidelines for the transportation, long-term storage and archiving of tissue samples are presented, emphasising the importance of identification, documentation and voucher specimens to scientifically validate the results of a study. Finally, legal and ethical issues concerning the collection, transportation and storage of specimens and tissue samples, the loan and deposition of samples in collections and the publication of results obtained from their analysis, are addressed from international and U.S. perspectives.

1 Introduction

The obvious first step in any systematics or population genetics study is to focus on a group of organisms or a level of interest. We assume that these issues are self-explanatory and the reader is referred to the accompanying volume (EXS 92) for examples of the application of molecular techniques to a wide range of questions and a summary of the problems that can arise during the course of such studies. Equally important in the initial stages of the study are sampling strategy, collection, storage, vouchering and archiving. These last two points are especially critical since no standard protocol currently exists for the disposition of tissue or DNA vouchers to scientifically validate the results of a study. Fortunately, this situation is changing as various museums and research universities establish biorepositories for the long-term storage of genetic resources.

There are several ways in which tissues can be obtained for analysis, each with its own peculiarities, nuances and requirements. The purpose of this chapter is not to give an exhaustive account of collecting techniques, but rather to focus on the more important aspects of preservation and storage for successful isolation of the molecules of interest. Other publications have reviewed the plethora of field collection methods that exist for organisms as diverse as plants, fungi, vertebrates and invertebrates and the reader is advised to consult literature relevant to the taxa of interest for specific details about collecting these organisms. In this chapter we focus on five aspects of sampling and storage: 1) selection of appropriate tissues for protein or nucleotide extraction 2) storage of freshly collected tissues in the field 3) obtaining tissues from other sources, e.g., museum collections, stock centers and commercial supply companies 4) transportation, long-term storage and archiving of tissue samples and voucher specimens and 5) legal and ethical issues involved in the collection, transportation and storage of tissues.

2 Selection of Appropriate Tissues for Molecular Studies

Before embarking on a field trip to collect fresh samples or requesting samples from an official repository, commercial company or informal source, the researcher must decide what tissues are required for the study. Depending on the size of the organism or the kind of material available, DNA or proteins can be extracted from tissue samples or from a homogenate prepared from the whole organism or "clonal" collections of organisms, such as isofemale lines [1]. Tissue selection for molecular isolation depends on the organism to be studied and on the type of molecular work to be conducted. The aim is to use parts of the organism that are relatively free of compounds potentially damaging to the protein or nucleic acid of interest or that may interfere with cloning, PCR, sequencing or restriction digestion. Using specific tissues has the added benefit of reducing the risk of contamination with host or parasite tissues, gut flora or recently ingested prey. For example, DNA extraction from parasitic *Cuscuta* required using only internode tissue to prevent DNA contamination from its host plant species [2].

Young, actively growing leaves or shoots are the best tissues for DNA extraction from plants, but seeds, roots, flowers, stems, pollen, spores and gametophytes have all been used successfully [2–4]. Brown [5] reported successful amplification of DNA from the charred remains of wheat seeds.

Among animals, successful amplifications have been achieved from bone, feathers, scales, hair, muscle, skin, whole blood (liquid and dried), serum, stomach contents and feces, among others. The following tissues, listed in order of desirability, are recommended for DNA extraction from vertebrates: brain, testes or ovary, liver, kidney, heart and skeletal muscle [1]. The nucleated erythrocytes of nonmammalian vertebrates provide a convenient source of DNA because they lack tough connective tissue [6], while milk provides an alternative source of DNA from mammals [7]. Toe clips are a common source of DNA for lizards and anurans [8] and scute notches provide a nondestructive alternative for chelonians [9]. Fecal and hair samples similarly offer noninvasive options for genetic sampling of mammals, especially endangered species [10–12].

Embryos are an exceptional source of insect mtDNA [1]. However, gonad or muscle tissues are generally suitable for both nuclear DNA and mtDNA isolation from arthropods and must be dissected out, avoiding the exoskeletal material if possible, since the presence of lipids and subcutaneous pigments may inhibit PCR. If specimens can be induced to spawn (for taxon-specific methods, see Strathman [13]), gametes may be obtained without the need for dissection. However, PCR may be inhibited if eggs or sperm are used, because of DNA viscosity [14]. Eggs are preferable to sperm for mtDNA isolation.

If gamete or muscle tissues are difficult to obtain, body tissues *sans* gut provide an alternative. For example, Koch et al. [15] extracted DNA from the heads of blackflies (*Simulium vittatum*) to avoid contamination by parasitic nematodes. Animals too small to be dissected should be starved for at least two days and

homogenised whole. Even when all the above procedures are impractical, it is still possible to obtain good results with whole organisms, especially if PCR primers or DNA probes are sufficiently taxon-specific to circumvent possible contamination. For example, Dorn et al. [16] successfully preserved and amplified the DNA of *Trypanosoma cruzi* from human blood samples.

Irrespective of which tissues are selected, they should be used, frozen or preserved immediately, before they begin to degrade. Fresh tissue is preferable if purified mtDNA is required, as freezing may break mitochondrial membranes, reducing yield at the step in a protocol where mitochondria are pelleted. However, if genomic DNA or total cellular DNA with a mtDNA fraction is desired, frozen tissue can provide high yield and quality.

High molecular weight DNA is required in applications such as cloning and RFLPs. In handling tissues intended for such studies, care should be exercised not to allow warming of the tissue after initial storage. Similarly, if protein analysis is intended, care should be taken to maintain specimens at temperatures that will prevent protein denaturation.

Although high molecular weight DNA is preferred for PCR, degraded DNAs will often amplify if the target fragment is small enough and the primers specific enough. DNA intended for PCR is routinely used from both desiccated and ethanol-preserved museum specimens, which may yield fairly degraded DNA. As progress continues with protocols developed for ancient samples, such issues may become *passé*. Interested readers should consult the *Ancient DNA Newsletter*, *DNA Amplifications* (the newsletter of Perkin-Elmer Inc., Wellesley, MA) and the journal *BioTechniques* (Eaton Publishing Co., Natick, MA) for updates of such technological advances. The website of the Molecular Biology Techniques Forum (Appendix 1) also provides a useful platform for questions.

3 Storage of Freshly Collected Specimens and Tissue Samples in the Field

Field collections represent the most important source for tissues, because the origin of the material (including habitat or provenance data) is fully documented. Additional advantages include the ability to obtain rare or poorly collected species (which can seldom be obtained from other sources), to collect within and among populations, and to reduce the time prior to DNA or isozyme extraction. Disadvantages include the time and expense associated with expeditions to remote areas, together with problems of transportation and permits (addressed below). Nevertheless, many unusual and important molecular systematics and population genetics studies, particularly those addressing biogeographic issues and rare, range-restricted or endangered species, cannot be conducted without field collections.

The method of tissue storage or preservation in the field is determined primarily by the nature of the study – the hierarchical level (population, species, higher taxon) focused on, the molecules (DNA, RNA or isozymes) to be exam-

ined, what protocol will be effective for extraction of the molecules, and how pure or intact they must be. Ultimately, the methods of choice will be constrained by logistical factors, most importantly the duration of the field trip, the facilities available and the transportation options. Readers interested in pursuing molecular studies requiring visitation and collection in foreign countries with even limited logistical support are advised to consult the recommendations of Mori and Holm-Nielsen [17] for a botanical perspective. Additional useful addresses are contained in Davis et al. [18].

3.1 Aspects of preservation and molecular degradation

When obtaining tissue samples, regardless of the study or molecule of interest, the goal should be to acquire the freshest, or best-preserved, samples possible. Denatured proteins and degraded nucleic acids present the single greatest obstacle to a successful molecular research programme. For example, PCR amplification of DNA from old or poorly preserved tissues can be hindered because various forms of damage reduce the average length of intact template molecules for the polymerase enzyme [19, 20]. Damage to the templates may cause the polymerase to stall, thereby retarding the initial rounds of amplification [21]. The average length of the DNA from ancient soft tissue is less than 200 base-pairs (bp) [22]. This reduction in length is partially attributable to strand breakage caused by autolytic processes (e. g., DNAse activity) that occur rapidly after death [19, 23, 24]. However, equally important sources of damage are subsequent oxidative and hydrolytic effects that either break, or labilise, phosphodiester and carbon-nitrogen (sugar-base) bonds [21, 25, 26]. Although desiccation appears to place a limit on endogenous hydrolytic damage, oxidative attack continues with time.

In an effort to minimise the processes of denaturation and degradation, preservation methods must aim to maintain the tissue samples at low temperature, exclude light and other forms of radiation, remove water and oxygen and sterilise against micro-organisms. These objectives are accomplished by freezing, desiccation or the addition of preservative fluids, and by storing samples in the dark, at constant, low temperature.

3.2 Field storage of tissue samples for molecular studies

Live animal specimens

Fresh material consistently provides the highest yield and quality of DNA for amplification, restriction digestion and isozyme analysis. Field trips of short duration are amenable to the collection of live invertebrates and small vertebrates (e. g., herpetofauna), which can usually survive for several days if kept

cool, well ventilated and sufficiently humid (dehydration is the primary cause of death in terrestrial taxa). Containers should be kept away from bright sunlight, inside a Styrofoam box and specimens checked twice daily (morning and evening) throughout the duration of the trip. At this time, moisture sources should be replenished, excreta removed and individuals in poor condition preserved immediately in ethanol. Rare specimens should be preserved on collection, rather than kept alive, to safeguard against death going unnoticed during the course of the trip, resulting in inadequate preservation.

Fresh plant samples

Contrary to earlier reports (e.g., [3, 4]), most fresh plant tissue need not be immediately placed on ice or frozen. A diverse array of plant tissues can be kept in a stable condition in Ziploc bags, stored away from light and fluctuating temperatures, inside a Styrofoam box [2]. If collected as whole plants, leaf or shoot cuttings, and placed in bags, leaf tissue is maintained the longest, provides an immediate voucher when some of the leaf tissue is separated (see below) and allows for subsequent propogation in the greenhouse.

Ziploc bags are ideal for maintaining leaf or shoot tissues in good condition without the addition of moisture (since the tissues will transpire naturally). The addition of a damp paper towel to increase the moisture content of the atmosphere is not recommended, as it can promote waterlogging and rotting of delicate leaves [2].

A small amount of wet ice, placed inside the Styrofoam box containing the bagged samples, is recommended if the trip is of short duration. Once a continuous source of refrigeration is available, the tissue samples should be kept in the refrigerator until processed or placed in long-term ultracold storage (see below). To prevent DNA degradation of fresh tissues, it is important to avoid fluctuating heat exposure or warm up from cold temperatures. The sensitive nature of protein activity in isozyme analysis usually requires that fresh tissues be rapidly exposed to cooler temperatures. However, adequate isozyme activity and high molecular weight DNA have been obtained from samples collected 8 days before being placed in ultracold storage [27]. In some taxa, senescence occurs after collection, during which many proteins disappear from leaves with seasonal aging [28]. It is therefore important to record the age of the leaves at harvest. Seeds, pollen and fern spores should be harvested only when mature. The collector should also be aware that hot and dry weather prior to harvest might cause synthesis of "storage proteins" in seeds and tubers to cease prematurely.

Frozen samples

In comparison with plants, field-collected tissue samples from animals, especially vertebrates, have traditionally been stored frozen, using combinations of wet ice, dry ice and liquid nitrogen. An extensive discussion of methods can be found in Dessauer et al. [3, 4, 28], Simione and Brown [29] and Simione [30]. Although cryopreservation remains important for studies involving proteins

and RNA (see below), researchers are increasingly adopting alternative strategies for field-storage of animal tissues.

There are several reasons for this shift in protocol. First, cryopreservation in the field involves considerable planning and logistical support, since sources of dry ice and liquid nitrogen are required. Although dry ice is generally available from airlines, whereas liquid nitrogen can be obtained from medical and veterinary clinics, universities and hospitals, among others (refer to the list provided by Dessauer and Hafner [31]), field trips must be planned to intercept such sources at regular intervals. This may be impractical when trips take place in remote locations or are prolonged in duration. Second, air transportation of dry ice and liquid nitrogen is strictly regulated (see below). Finally, many investigators have obtained suitable yields of high molecular weight DNA, nearly comparable with those obtained from frozen samples, using less elaborate protocols (e. g., preservation in 95–100% ethanol).

If frozen tissues are to be collected, they should be sampled while the specimen is alive or as soon after death as possible and rapidly placed in the cold and away from light. Tissues should be packed in plastic cryotubes (e. g., Nunc), Ziploc bags, or wrapped tightly in aluminium foil, excluding as much air as possible. Small blood samples can be collected in heparinised hematocrit or microcentrifuge tubes, whereas larger samples are most efficiently collected from heart or caudal vessels using a heparinised syringe [3, 4, 28]. Blood cells should be separated from plasma, prior to freezing, by using a commercial hand centrifuge or a lightweight, plastic centrifuge (e. g., [32]).

As soon as possible after collection and packaging, tissues should be quick (snap) frozen by dropping directly into liquid nitrogen or covering with dry ice. However, since quick freezing often shatters hematocrit and microtubes filled with liquids, such tubes should be frozen slowly in a freezer (if available) before subjection to ultracold temperatures.

For cryopreservation of vegetative plant tissues (mainly leaves), material should be washed in distilled water and quick frozen in liquid nitrogen for subsequent storage at ultracold temperatures. Dessauer et al. [28] provided the following protocol for cryopreservation of seeds: remove fleshy portion of seed; dry seed; place seed in vacuum-sealed container; store in the dark, below 0°C. Seeds stored in this manner have remained viable for up to 10 years. Pollen and fern spores may be similarly treated (but must first be cleaned of debris and treated with organic solvents to remove lipids) and have yielded stable proteins after 4 years' storage at ≤ –30°C.

Cell lines

Cryopreservation of living cells requires special collecting, freezing and storage procedures if cells are to survive the freezing and thawing process [33]. Few cells will survive freezing and thawing without a cryoprotectant, e. g., glycerol or dimethylsulphoxide (DMSO). Each species, tissue and freezing system has an optimum cryoprotectant concentration and freezing rate, which must be ascertained to recover the maximum number of viable cells from the sample [34].

Cryoprotectant concentration must be sufficient to protect the cells from freeze damage, yet dilute enough to avoid chemical injury to cells, while the rate of freezing should also be precisely controlled, depending on the species, size of sample, cryoprotectant and freezing system. Rapid freezing induces death by the formation of crystals within the cells, while slow freezing causes death from the chemical consequences of solute concentration. For further guidelines on the field preservation of cell lines, refer to Hay and Gee [33] and Dessauer et al. [3, 4].

Desiccated samples

Rapid desiccation is one good alternative to cryopreservation for storing tissue samples in the field and may be preferred because it requires neither refrigeration nor flammable substances. Many proteins in carefully dried tissues are stable for short duration at ambient temperature and for longer periods in refrigerators or freezers. For example, acetone powders, solids precipitated from tissues with cold acetone, retain sufficient enzymatic activity for use in endocrinological studies [28]. Desiccated tissues are also suitable for subsequent extraction and PCR amplification of high molecular weight DNA. Specific protocols for the storage of desiccated samples are provided in Protocol 1.

Protocol 1 Methods of desiccation

1. *Insects.* Air-dried, pinned insect samples are convenient, require low maintenance and are lighter than fluid-preserved samples. Insects may also be preserved in ethanol or acetone and later desiccated using a critical point drier [35–37]. Both techniques are suitable for DNA extraction, but marked degradation may occur if tissue dehydration is prolonged (e.g., in humid climates or with large specimens). Placing samples in contact with silica gel for at least 12 hours (determined by the size of the specimen) is recommended in this case [38]. Removing the head, legs, wings, etc. enhances desiccation of large specimens.

Various methods of chemical desiccation, e.g., hexamethyldisilazane (HMDS), amyl acetate, xylene, methyl cellusolve and acetone vapour, provide an alternative to air desiccation, especially in humid climates, yielding high molecular weight DNA amenable to PCR [36]. Specimens can also be dried from ethanol using chemical techniques. Phillips and Simon [39] presented a protocol for the isolation of DNA from pinned insects without destruction of the exoskeleton.

2. *Parasites.* Toe et al. [40] evaluated methods for the field preservation of parasite and vector samples (e.g., *Onchocerca volvulus* and *Simulium damnosum*) for PCR. Preservation of desiccated tissues from parasites and their associated vectors on microscope slides yielded the greatest quantity of high molecular weight DNA.

3. *Vertebrates.* Blood samples may be spotted on Guthrie cards or on Whatman paper (3 mm) and left to dry in bright sunlight for 30 minutes. Makowski et al. [41] recommended usage of guanidium thiocyanate-impregnated filter paper

(GT-903), which binds PCR inhibitors and preserves DNA in an aqueous extractable form. If GT-903 is unavailable, consult Ostrander et al. [42] and McCabe et al. [43] on methods for extracting DNA from regular paper. Weisberg et al. [44] found that DNA extracted from lyophilised (freeze-dried) blood was similar in length to that extracted from fresh or frozen blood and suitable for PCR.

Sodium chloride (NaCl) can be used as a desiccant for solid vertebrate tissues, e. g., muscle. Approximately 1 g of tissue should be sliced off and centered in a 15 ml or 45 ml conical test tube containing NaCl. Upon return to the laboratory, the tissues need only be rinsed under distilled water before commencing with DNA extraction. PCR from such samples has resulted in mtDNA fragments up to 300 bp in length [45]. However, the success of this method for isozyme analysis has not been established and is probably unreliable.

4. *Plants.* Plant tissues are often difficult to preserve for molecular studies, due to the presence of phenols, polysaccharides and lipids. Methods of dehydrating plant tissue prior to transport include lyophilisation, dehydration with a food drier, air-drying and herbarium specimen (forced air or heat) drying [46–54]. However, these techniques cannot be universally applied to plants and should be tested before embarking on a field trip [55]. For example, lyophilisation often leads to decreased protein activity, although animal and fungal tissues are usually unaffected [56]. DNA extracted from dried plant tissues is often too degraded for use in restriction site analysis, although successful amplification may be achieved with PCR.

Desiccation of plant tissue in Dierite ($CaSO_4$) or silica gel offers the best alternative to collection of fresh or frozen plant tissue [55, 57, 58], having been successfully tested by extraction and sequencing of DNA, and by digestion with restriction enzymes, over a diversity of angiosperm taxa [2, 53]. It also appears to be less likely to damage proteins than other methods, e. g., lyophilisation [59]. Since the method is simple, the chemicals readily available and easy to transport, it is ideal for obtaining species that exist in remote areas or are to be collected by colleagues in other countries.

Silica gel is a more efficient desiccant than Dierite as it can be obtained in smaller mesh size (28–200, grade 12 from Fisher Scientific, Chicago, IL), allowing for greater surface coverage of leaf tissue. Usually 4–6 g of fresh leaves are placed in small Ziploc bags. Leaf tissue dries faster and thus with less DNA degradation, if first torn into smaller pieces. Subsequently, 50–60 g of silica gel (minimum 10:1 gram ratio of silica gel to leaf tissue) is added to the bag. Drying should take place within 12 hours, determined if tissue snaps cleanly when bent. Longer exposures to silica gel may be required, e. g., in monocots, but will usually result in DNA degradation. The dried tissue in Ziploc bags should be stored with trace amounts of indicator silica gel (6–16 mesh, grade 42), which changes colour from violet-blue to whitish pink when hydrated, to verify that rehydration has not occurred. The Ziploc bags should, in turn, be kept in tightly sealed plastic boxes. Schierenbeck [60] provided a modified polyethylene-glycol DNA extraction protocol for silica gel-dried plants.

5. *Marine algae.* Holzmann and Pawlowski [61] obtained high molecular weight DNA from air-dried samples of foraminifera stored at ambient temperature for up to 11 weeks. Positive results were also obtained with foraminifera stored for 2–3 years, although amplification products were < 500 bp long.
6. *Marine invertebrates.* Desiccation is generally not viable for marine invertebrates (e.g., Scyphozoa, Polychaeta) because their high water content is incompatible with the requirement that samples be dried within 12 hours to prevent DNA degradation [55].

Fluid-preserved samples

Tissue samples from invertebrates, intended for molecular studies, are routinely stored in 95–100% ethanol at ambient temperature (e.g., [62]). Preservation in methanol and propanol is contraindicated [38], although tissue samples preserved in 100% methanol will yield higher molecular weight DNA than samples preserved in formalin [63].

Greer et al. [64] demonstrated that only storage in 95–100% ethanol (v/v) results in PCR products of 1–2 kb after 30 days. Adequate preservation in 70–80% ethanol may occur if specimens or tissue samples are small and, in the case of arthropods, weakly sclerotised, but DNA isolated from larger specimens preserved in 70% ethanol is usually highly degraded. For large or heavily sclerotised samples, even 95–100% ethanol is no guarantee against degradation, because saturation of the tissues may be delayed by the size or impermeable nature of the tissues [65]. Such samples should be injected with ethanol, dissected into smaller pieces or, in the case of arthropods, cut in several places along the exoskeleton, to allow the ethanol to diffuse directly into the internal tissues. Excess ethanol (i.e., a high ratio of ethanol to tissue sample volume) should always be used when preserving samples for molecular studies, to minimise dilution that occurs with addition of the sample [66]. Ethanol should be replaced after the initial fixation and periodically thereafter. Initial fixation of fresh or frozen tissues at –20°C has been found to contribute significantly to the quality of tissue preservation in arthropod samples [67], but refrigeration is seldom available in the field.

The method of euthanasia may also affect the preservation of tissues, especially if whole organisms are placed directly in ethanol. Animals as diverse as nematodes and scorpions are prone to close their oral, anal and respiratory openings on placement in ethanol, thus further hindering ethanol diffusion into the internal tissues after death. Such animals should be frozen alive, placed in warm ethanol (unless being used for isozyme analysis), very dilute (e.g., 10%) ethanol, or dissected/cut (after euthanasia with ethyl acetate, chloroform or cyanide), prior to placement in 95–100% ethanol. Alternatively, lysis buffers may be used for their preservation (see below).

Ethanol is suited to the storage of vertebrate tissues and has been used successfully in DNA hybridisation and sequencing. Solid tissues, e.g., muscle, should be cut into pieces approximately 10 mm in diameter to allow rapid penetration of ethanol. After saturation in ethanol for at least two

days, moist tissues may be transferred to plastic bags for storage or shipment [28].

Fungi and marine algae may also be stored in ethanol. Ethanol-preservation was widely considered ineffective for maintaining adequate yields of high molecular weight DNA from land plants [51] until Flournoy et al. [68] obtained excellent yields from samples stored in 95–100% ethanol or 100% methanol by addition of proteinase (Pronase E). Vacuum infiltration of ethanol resulted in better DNA preservation than passive infiltration [68].

In the absence of ethanol, most samples may be stored in saturated salt or buffer solutions until transported to laboratories equipped with appropriate resources. Even laundry detergent has proven to be a rapid and uncomplicated temporary storage solution for recovering high yields of DNA [69, 70].

Many of these buffer solutions are also used for DNA isolation and may be advantageous for the preservation of highly sclerotised organisms. For example, Sansinforiano et al. [71] used lysis buffer with a high urea concentration for preserving the impermeable mucopolysaccharide capsules of *Cryptococcus neoformans* and other pathogenic yeasts, and acquired high molecular weight DNA after storage at ambient temperature for up to 6 months. Details of these methods are listed in Protocol 2.

Protocol 2 Preservation in buffer solutions

1. *Blood.* Preservation of vertebrate blood samples in the field has traditionally employed buffer solutions, e.g., 2% 2-phenoxyethanol with glycerol or DMSO, 2-propanol with ethylene-diamine-tetraacetate (EDTA) or sodium dodecyl sulphate (SDS). Such buffers can preserve blood proteins (e.g., plasma albumin) and mRNA for up to three weeks without refrigeration [72, 73], but must usually be frozen within 24 hours of collection [28].

Quinn and White [74] recommended that blood samples from birds, injected into 5 ml vacutainer/EDTA tubes, should be frozen within 10 hours of collection to prevent significant DNA degradation. However, Cann et al. [45] obtained high molecular weight DNA from unrefrigerated blood samples of birds, bats and wallabies, using a scaled-down version of Quinn and White's protocol.

Cann et al. [45] reported that microhematocrit tubes of blood blown into 500 µl of TNE_2 (10 mM tris-hydroxymethyl amino methane, 10 mM NaCl and 2 mM EDTA, pH 8.0) are stable for months if refrigeration is impossible and still provide high molecular weight DNA. The use of mannitol-sucrose buffer in mtDNA studies is also reported to be adequate for storing samples up to 2 weeks without refrigeration [75]. Gelhaus et al. [76] provided protocols for the isolation of DNA from urea-preserved blood. For practical guidelines on the extraction of blood samples from vertebrates, refer to Dessauer et al. [3, 4].

2. *Vertebrates.* The lenses of vertebrate eyes, collected for sequence studies of alphacrystallin, have been preserved in saturated guanidine hydrochloride [28] and this solution is also purported to be effective for the preservation of proteins in other vertebrate tissues [45].
High molecular weight DNA has been extracted from vertebrate solid tissue samples, stored at ambient temperature in 4 M guanidium isothiocyanate (GITC) from 41 days [77] to 3 months [78]. Vertebrate tissue samples may be kept up to three years without refrigeration in TNES-urea (6 or 8 M urea; 10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS) and still yield high molecular weight DNA [79].
3. *Invertebrates.* Sperm has been stored in 0.01–0.02% sodium azide solution [14]. Laulier et al. [77] extracted high molecular weight DNA from field collected samples of viruses, bacteria, yeasts and invertebrates stored up to 41 days at ambient temperature, in 4 M GITC.
Dawson et al. [80] assessed the effects of five buffer solutions (70% ethanol, Queen's lysis buffer [0.01 M Tris, 0.01 M NaCl, 0.01 M disodium-EDTA and 1.0% n-lauroylsarcosine, pH 8.0], DMSO-NaCl solution, CTAB-NaCI solution, and a urea extraction buffer) on the preservation of marine invertebrate samples for DNA isolation. In accord with Seutin et al. [81], these authors concluded that dimethylsulphoxide and sodium chloride (DMSO-NaCl) was the best solution in which to store marine tissue samples. Reiss et al. [65] recommended that insect samples be thoroughly homogenised in order to achieve adequate DNA preservation in buffer solutions.
4. *Plants.* Samples of plant tissue preserved using cetyltrimethylammonium bromide (CTAB) yield high molecular weight DNA, thereby providing an alternative to standard desiccation methods [82].

Embryonic tissues
Developmental data are becoming increasingly important in modern evolutionary and systematics studies [83, 84]. Developmental studies utilise embryonic tissues that hold expression data (transcribed mRNA for *in situ* studies and translated proteins for antibody staining approaches), the preservation of which is essential. Accordingly, embryonic expression work is best conducted with live material. This can be obtained from embryos collected in the field or from sources of live organisms listed in the next section.

Knowledge of embryogenesis in the organisms of interest can aid in the collection and treatment of embryos in the field. For instance, if an organism displays brooding behaviour, large numbers of embryos can be obtained by locating reproducing individuals [85, 86]. Such individuals can be brought alive to the lab and manipulated for fixation.

If live material cannot be transported to the laboratory, embryos may be preserved in the field, provided that the preservation of mRNA and proteins can be assured. Although embryos can be frozen at –80°C with liquid nitrogen, fixation methods are easier and more efficient for their preservation in the field. However, knowledge of the embryology of organisms is required, because

fixation of embryos without certain kinds of pretreatment may render them unusable. For example, *Drosophila* embryos require dechorionation prior to fixation. Such pre-fixation treatments may be simple to accomplish in the field. Alternatively, if embryonic tissues like imaginal discs in insects are the targets of molecular developmental research, whole larvae can be preserved in fixatives to allow later dissection of tissues for whole mount or sections. Specific protocols for preservation of embryos in the field are provided in Protocol 3.

Protocol 3 Storage and fixation of embryos

1. *Dechorionation.* This procedure is accomplished by treating embryos in sodium hypochlorite followed by thorough washing in water or 0.7% saline. This step can be accomplished in the field with test tubes and a small supply of Pasteur pipettes. Other pre-fixation treatments may be necessary to prepare the embryos or developing organisms for fixation or the researcher may determine that pre-fixation treatments are unnecessary and proceed directly to fixation of materials.
2. *Fixation.* Dechorionated embryos or untreated embryos can be fixed by several methods depending on whether antibody staining or mRNA *in situ* detection is the goal. Antibody staining requires that larvae be fixed in a solution of 4% formaldehyde in 50 mM cacodylic acid (pH 7.4) for 1 hour at 20°C, dehydrated in an ethanol series and stored in methanol at –20°C. *In situ* hybridization requires that larvae be fixed in a solution of 4% formaldehyde in PBS, dehydrated in an ethanol series and stored in methanol at –20°C.
3. *Storage.* Fixed embryos should be transferred as quickly as possible to a –20°C freezer, where they will remain for long-term storage.

Fecal samples

PCR amplification of DNA from fecal samples is dependent on preservation method, PCR-product length and whether nuclear or mtDNA is assayed. Storage in DMSO/EDTA/Tris/salt solution (DETs) is most effective for preserving nuclear DNA, but storage in 70% ethanol, freezing at –20°C, and desiccation using silica beads perform equally well for mtDNA and short (< 200 bp) nuclear DNA fragments [87, 88]. Protocols for the isolation of DNA from fecal samples stored by these methods are provided by Wasser et al. [87], Frantzen et al. [88], Shankaranarayanan and Singh [89] and Launhardt et al. [90]. Dowd et al. [91] described protocols for the extraction of DNA from formalin-fixed fecal samples.

3.3 Practical Considerations

Contamination

Throughout the duration of fieldwork, collectors should be aware of the importance of keeping their instruments, containers and reagents clean in order to prevent cross-contamination. Individual tissue samples should be