



Review

Biological sample collection and processing for molecular epidemiological studies

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Abstract

Molecular epidemiology uses biomarkers and advanced technology to refine the investigation of the relationship between environmental exposures and diseases in humans. It requires careful handling and storage of precious biological samples with the goals of obtaining a large amount of information from limited samples, and minimizing future research costs by use of banked samples. Many factors, such as tissue type, time of collection, containers used, preservatives and other additives, transport means and length of transit time, affect the quality of the samples and the stability of biomarkers and must be considered at the initial collection stage. An efficient study design includes provisions for further processing of the original samples, such as cryopreservation of isolated cells, purification of DNA and RNA, and preparation of specimens for cytogenetic, immunological and biochemical analyses. Given the multiple uses of the samples in molecular epidemiology studies, appropriate informed consent must be obtained from the study subjects prior to sample collection. Use of barcoding and electronic databases allow more efficient management of large sample banks. Development of standard operating procedures and quality control plans is a safeguard of the samples' quality and of the validity of the analyses results. Finally, specific state, federal and international regulations are in place regarding research with human samples, governing areas including custody, safety of handling, and transport of human samples, as well as communication of study results.

Here, we focus on the factors affecting the quality and the potential future use of biological samples and some of the provisions that must be made during collection, processing, and storage of samples, based on our experience in the Superfund Basic Research Program and Children's Environmental Health Center, at the University of California, Berkeley.

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1. Introduction

In recent years, epidemiology has been enriched tremendously with tools from molecular biology. It has branched into a complex field, named molecular epidemiology, incorporating the principles and methods of traditional epidemiology and the new,

expanding knowledge of molecular events that lead to disease [1–6]. The concept of biomarkers has been introduced to describe the molecular events characteristic for various stages between exposure and disease [7–9]. Molecular epidemiology offers insights into specific mechanisms underlying the causation of disease, including the interaction of genetic and environmental factors, which may determine individual susceptibility to toxic exposures [10]. Thus, developments in molecular epidemiology allow researchers to better understand mechanisms of toxicity, evaluate

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whether there is a causal relationship between specific hazards and biological effects, more accurately assess the risk from exposures to certain hazards, differentiate between groups of higher or lower susceptibility, and provide solid scientific support to policy makers toward intervention strategies [11].

Knowledge and appreciation of the continually developing biological/biochemical tools must be incorporated into study designs and procedures. In this light, certain provisions must be made for the preparation, preservation, and storage of biological samples collected for epidemiological, and other monitoring, studies. Hundreds of thousands of samples are currently being collected in many ongoing studies. New proposed projects will result in even more collected and banked biological specimens [12]. Despite the importance of biological sample collection and banking, very little has been published on selection and validation of these procedures and how they can affect the outcome of molecular epidemiology studies [13,14].

The purpose of this paper is to discuss the challenges and potential pitfalls of sample collection, processing, and banking, based on our experience with large epidemiological studies of genetic endpoints, in the Superfund Basic Research Program and Children's Environmental Health Center, at the University of California, Berkeley. The factors affecting the quality and the potential future use of biological samples are discussed and some of the provisions that must be made during collection, processing, and storage of samples are also addressed.

2. Challenges of molecular epidemiology

Fig. 1 presents the components of a molecular epidemiologic study that includes collection of biological samples for future analysis of various biomarkers. This review focuses on the components of the figure highlighted in bold, specifically the sample collection, processing and banking. The other components are mentioned briefly.

The main challenges that molecular epidemiologists face are: (a) obtaining a large amount of information from limited samples; (b) making provisions for evaluation of future biomarkers; and, (c) maximizing the information that can be obtained from banked samples

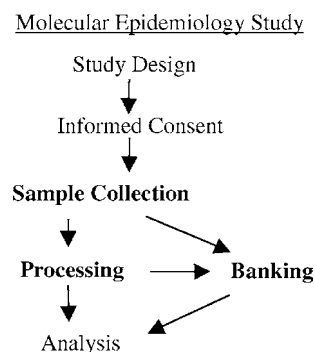


Fig. 1. Schematic structure of molecular epidemiologic or biomarker studies, two terms often used as synonymous. Items shown in bold are the focus of this paper.

in order to minimize research costs. In many research situations there is only a small window of opportunity after which the study subject may no longer be available or conditions may have changed. Thus, these precious samples must be handled and stored carefully, using procedures and protocols that have been validated in pilot studies. The handling includes the collection process, the transport, if necessary, and the initial processing before storage. Storage or banking of the samples is, in itself, a central issue especially for long-term studies and for sample use in new studies in the future. Storage can affect the quality of the samples and determines whether their future use is possible. Sophisticated sample processing is required in order to take advantage of high-throughput technology, such as real-time or TaqMan[®] PCR and DNA microarrays [15–17].

3. Study design

In many epidemiologic studies, the collection of biological samples has been often limited to serum and urine [18]. However, many of the new molecular tools use other types of biological samples and, thus, require different more extensive and careful collection and processing procedures [19]. For example, in studies of environmental epidemiology, the target tissue of an exposure and its metabolic route are important considerations. As shown in cases of exposure to formaldehyde via inhalation, the cytogenetic damage could be

detected in exfoliated cells of the nose and mouth, the main target tissues, while analysis of peripheral blood lymphocytes yielded negative results [20,21].

The potential biomarkers of interest need to be defined prior to data collection so that the appropriate collection and processing protocols can be designed. For example, procedures would be different if the ultimate goal is to store DNA only, live cells, or just serum. When the appropriate sample type is specified, the particular biomarker may need to be validated in a pilot study. For example, in an ongoing collaborative project on biomarkers in workers occupationally exposed to benzene (the National Cancer Institute (NCI), the Chinese Academy of Preventive Medicine, and the University of California at Berkeley), more than two dozen potential biomarkers were evaluated in the first stage of this study. This resulted in selection of the most informative biomarkers of genetic damage and genetic susceptibility, which are currently being applied in a larger cohort [22–24].

Samples of the different study groups, e.g. cases and controls, need to be collected concurrently and treated in an identical manner, particularly if the study extends over a long period of time. The length of storage may affect the levels of a biomarker even under ideal storage conditions, which in turn will affect the observed differences if controls and case samples were collected or analyzed in distinctly different time frames.

Given the variety of factors affecting the quality of the samples, running preliminary pilot tests is an important step to assess the best collection conditions and factors that may affect the stability of a particular biomarker. Such pilot tests are an integral part of a good quality design in epidemiological studies, and must be carried out prior to the actual sample collection, so that the latter is planned accordingly. As an example, in a study of the ovarian cancer biomarker OVX1 [25] several parameters, including the immediate separation of serum, the temperature and length of storage prior to processing, the transit time of shipping, etc. were shown to affect the levels of this biomarker. The authors concluded that samples must be collected in plain EDTA tubes and not in heparin-containing tubes, and that serum must be separated immediately or the samples must be stored at 4 °C. Without such preliminary tests prior to sample collection, biomarker analysis may have been compromised.

4. Informed consent

The rapidly expanding potential of biotechnology and the growing public concern about undisclosed use of participants' biologic materials has led to complex ethical issues, and the issue of informed consent acquires a new dimension. Ethical concerns arise that challenge the traditional process of obtaining informed consent [26]. Until recently, participants were asked to give consent in order to inform them of immediate potential risks inherent in the study. Now, study scientists need to obtain consent so that participants are also informed of the current, future, and sometimes unforeseen uses of their samples. This is essential since scientists may want to perform additional analyses, unforeseen at time of collection, as new biomarkers become available.

A large volume of literature exists that addresses this very complex issue and it is not our intention to attempt a thorough review in this paper on this subject [27–43]. Additional information on bioethics can be found at NIH-sponsored sites such as:

- Bioethics Resources on the web: <http://nih.gov/sigs/bioethics>;
- NHGRI Ethical Legal and Social Issues Program: <http://www.nhgri.nih.gov/ELSI>;
- National Bioethics Advisory Commission: http://bioethics.gov/cgi-bin/bioeth_counter.pl.

In addition, the Office for Human Subject Research of NIH provides computer-based-training and certification on human subject research at <http://ohsr.od.nih.gov>.

5. Sample collection

5.1. Interaction between study subjects, field personnel and researchers

For a reliable and consistent sample collection, it is essential to establish clear communication between scientists, staff, and study subjects. The collection process depends as much on the nurse or technical staff who collect the specimens, as on the study subject. Special collection procedures may be necessary if collecting specimens from a special population, such as children. For example, blood collection from children

often requires a pediatric phlebotomist. Whenever possible, collection of blood from children should be concurrent with blood collection required by the doctor for clinical evaluation, so as to minimize additional inconvenience and discomfort to the child. In addition, some practical difficulties that can reduce participation of study subjects in the sample donation can be avoided with an extra effort on the part of the study team. For example, lead tests are required for impoverished children in State funded programs in California. However, compliance in many areas is very low. In a study of Latino farmworker children (CHAMACOS) (<http://ehs.sph.berkeley.edu/chamacos>), blood collection for the study was “piggybacked” onto the blood collection for lead analysis and a pediatric phlebotomist was employed, thereby benefiting the clinical evaluation by increasing compliance to nearly 80%.

The study scientists must deliver clear instructions to staff, including the timing of collection, fasting instructions, the volumes required, specific containers to be used, and even the size of the needle for venipuncture [13]. Detailed instructions are reinforced by written protocols and frequent communication between the study scientists and staff, and between staff and study subjects. If the processing laboratory is different from the location of sample collection, it is important to assure that the processing laboratory handles the specimens properly in order to avoid loss or damage from prolonged transport or storage, or unsuccessful delivery attempts. Clear instructions should also be provided to the study subjects. These instructions may be oral and/or written and provide information about fasting, timing of collection of urine or other samples at home, and storage and transport of specimens to the laboratory. For example, it may be important to place the urine sample promptly in the refrigerator or on ice until it is transferred to the study personnel, so as not to affect the level of metabolites and/or the integrity of the urothelial cells for cytogenetic analysis. It may be necessary to communicate to the participant the importance of precisely following sample collection protocols. Community outreach programs, including meetings with local groups, and information provided through local radio, TV and newspapers, may make participants more engaged in the success of the projects and thus more likely to follow protocols.

5.2. Non-invasive methods of sample collection

Invasive sample collection is sometimes necessary for specific analyses. Blood collection is most often used to obtain biological samples, as it is certainly less invasive than, e.g. biopsies. However, even less invasive methods, such as exfoliated cells collection from the mouth (buccal) or urine (urothelial), can be adequate for some purposes (genotyping, cytogenetic damage). These can minimize the use of valuable blood samples and reduce the blood volume needed from each study subject. In addition, use of such methods can increase the sample size of the study population significantly, because many participants may be more willing to provide a buccal swab or urine sample than donate blood [44] (also, in our childhood leukemia project only 50% of participants gave blood while >95% gave buccal cell samples; unpublished data). The collection of exfoliated cells is logistically less difficult (see Section 5.5) and does not require highly trained personnel such as a phlebotomist; thus, it may be more feasible than blood collection when the personnel are not on site, or the population chosen for a study is geographically dispersed. Le Marchand et al. recently reported successful collection of buccal cells for genotyping by mail from remote field sites [45]. Mailing specimens keeps the collection costs low and provides an acceptable DNA source from a large number of participants in a relatively short time. However, recent postal security practices including irradiation of mail, may affect the quality of samples irreversibly [46–48]. A pilot study has been conducted by CDC to examine the effects of postal service radiation on dried blood spots, as part of the newborn screening program (<http://www.cdc.gov>). Additional information can also be found at the Smithsonian Institution web page: http://www.si.edu/scmre/about/mail_irradiation.htm.

5.3. Timing

Biomarker levels may have a minute-to-minute, or hour-to-hour, or metabolic variation. For example, there is a difference in hormone and various metabolite levels detected in the first morning urine in comparison to subsequent collections [13,49,50]. Multiple time-points of sample collection are often necessary in order to obtain the true time course of the relationship of the exposure and development of

the outcome, and to establish causal associations [49]. For example, levels of mercury in women's hair and blood samples before and during pregnancy may be more informative about the exposure to the embryo than samples collected from the mother after the baby is born [51–53].

The effect of pre-clinical disease on levels of biomarkers has been a debated issue, especially for biomarkers measured during the short period before the onset of disease. If the time of sample collection is within the period of the onset of the disease, but before it was clinically manifested, there is a chance that some of the biological parameters measured are the result of the disease, itself, and not of predictive value for the disease. Samples collected a long time before the onset of the disease may be more informative and better associated with the cause of the disease [49]. One example is the correlation of leukemia with the detection of cytogenetic damage in peripheral lymphocytes. If the collection of lymphocytes took place in a relatively short time before the onset of leukemia, it may not be clear whether the cytogenetic damage preceded the disease or it is one of the resulting abnormalities caused by the leukemia [23].

5.4. Stability of samples

Factors that affect the stability of biological samples include: (1) anticoagulants; (2) stabilizing agents; (3) temperature; (4) timing before initial processing; (5) sterility; (6) endogenous degrading properties (enzymes, cell death); (7) etc.

5.4.1. Anticoagulants

In their discussion of the collection requirements and factors that affect the quality of biomarkers, Landi and Caporaso [13] stressed the importance of careful selection of anticoagulants and preservatives in blood collection tubes. While certain anticoagulants are better or even required for analytical purposes, others may be contraindicated. For example, citrate-stabilized blood may afford better quality of RNA and DNA than other anticoagulants would, and produces a higher yield of lymphocytes for culture, whereas heparin-stabilized blood affects T-cell proliferation and heparin binds to many proteins. Also, EDTA is good for DNA-based assays, but it will influence Mg^{2+} concentration, and poses problems for

cytogenetic analysis (increases sister chromatid exchanges, decreases mitotic index, etc.). The collection of whole blood in any type of anticoagulant-containing tubes may cause the induction of cytokine production in vitro, and likely result in artificially elevated concentrations [54].

5.4.2. Stabilizing agents

Many components of blood that are potential biomarkers are labile and need to be preserved using stabilizing agents. For example, EDTA and ascorbic acid are stabilizing agents for folate in blood, and should be added as soon as possible after blood collection to assure the quality of the analysis [55–57]. Metaphosphoric acid or reduced glutathione are used to preserve ascorbic acid [13,58,59]. There are also special considerations for measuring volatile compounds as biomarkers, or the effect of hemolysis on the levels of electrolytes [13]. These factors of biomarker stability have to be explored and validated in a pilot study before large-scale collection takes place.

5.4.3. Timing before initial processing

The allowable time between collection and processing of biological samples depends on the component(s) of interest and their stability. If high cell viability is desired, processing of blood, buccal swabs or urine samples would need to take place within 24–48 h. For example, pilot studies have been done to address concerns about stability of specific sample components [60,61]. Based on our own experience, cell viability decreases rapidly after 48 h and exfoliated cells degenerate, resulting in poor cell structure preservation on slide preparations, or degradation of proteins and nucleic acids (unpublished data). Similarly, for many biomarkers the time between collection and processing affects the stability despite the presence of stabilizing agents. One example is the diminution of folic acid stability over time (at room temperature) even when a preservative, such as ascorbic acid, is added (our unpublished data) (some reports offer different estimates of stability [62,63]). Delays between collection and processing will affect the estimate of folate levels. Similarly, if the endogenous antioxidant activity is the focus of analysis, addition of exogenous antioxidant agents as preservatives is impossible, as they would directly interfere with the results of the assays that measure antioxidant activity. Therefore,

the longer the time between collection and analysis the less accurate is the estimate of the endogenous antioxidant activity [58] (our unpublished data). For cytokine analysis, cells must be separated from serum immediately after blood collection (see “Processing” below), again because delays between collection and processing will affect the results [54]. These considerations would determine when and how the collection and processing take place. For example, if the physical distance between the collection and processing facilities involves mailing or transportation delays, unstable biomarkers should be excluded. Alternatively, at least minimal initial steps have to be conducted before sample transfer to assure its integrity.

5.4.4. Temperature

Temperature may affect sample stability in two stages: during the time between sample collection and sample processing (if the samples are not processed immediately after collection) and during short and long-term storage. Ideally, the sample is separated into different components (plasma, cells) immediately after collection and each component is kept at the appropriate temperature. Generally, isolated DNA is stored at 4 °C for several weeks, at –20 °C for several months, at –80 °C for several years [64]. Isolated RNA must be stored at –80 °C. Live cells are stable at room temperature for up to 48 h but must be either cultured or cryopreserved in liquid nitrogen at –150 °C in order to remain alive (see Section 6.1). Serum and plasma contain a large number of soluble molecules and most require very low temperature to remain intact (–80 °C). Immunoglobulins in plasma are considered more stable, instead, even at room temperature for up to few days.

Temperature control during the time between collection and final sample processing and storage is essential, especially when this time involves several hours. The appropriate temperature depends on the biomarker(s) of interest and the researchers need to take into account the temperature requirements for the stability of each biomarker during the study design. If a very labile biochemical biomarker is the main focus of the study (e.g. cytokines) and the samples are not going to be analyzed immediately, they must be frozen to –80 °C, and repeated freeze-thaw cycles must be avoided. Freezing the collected sample as is (i.e. without separation), however, is incompatible

with maintaining viable cells for isolation (cells will rupture if frozen without DMSO, see Section 6.1), therefore the researcher must choose between immediate separation of the sample components so that each one can be preserved accordingly, or selection of one sample component to preserve immediately (e.g. cytokines), sacrificing the other components that would require different conditions (e.g. live cells). If instead one wishes to maintain cell viability for several hours or days, this can be achieved by keeping the sample at room temperature (for up to 48 h). This is, however, incompatible with preservation of labile biomarkers of protein nature (e.g. cytokines), antioxidants (ascorbic acid, uric acid, α -tocopherol), and others, such as folate and vitamin B12 [63]. Low temperature (4 °C) is often a good compromise between the two extremes of freezing or room temperature: cells can remain viable (reduced viability compared to room temperature) and it also protects, at least to some extent, against enzymatic degradation of sensitive protein biomarkers (see below).

5.4.5. Sterility

The requirement for aseptic conditions during the collection process is essential if the intention is to isolate RNA or to culture cells from the sample. Bacteria or fungal contamination can be detrimental for the quality of the biomarkers, can introduce new products and metabolites, and can render the sample unreliable.

5.4.6. Degradation

Enzymatic degradation may affect many biochemical biomarkers. Proteins are sensitive to degradation by proteases, particularly if cell integrity has been compromised. Protein integrity is protected by addition of commercially available protease inhibitors (aprotinin 100 KIU/ml, pepstatin 1 μ g/ml, antipain 5 μ g/ml, leupeptin 5 μ g/ml, benzamidine 1 mM, and PMSF 1 mM, in final volume) to the sample immediately after collection [65]. It must be mentioned here that protease inhibitors are toxic to live cells, and therefore must not be added to whole blood if cell viability is desired. Furthermore, all steps during protein handling must take place on ice. RNA is also particularly sensitive to degradation by abundant and ubiquitous RNases. RNA integrity is secured with RNase-free handling and addition of commercially available RNase inhibitors. Commercial reagents are

easily available from many companies. Unlike proteins, however, RNA is not protected at low temperatures. In contrast, DNA is the most stable component in biological samples, including blood, exfoliated cells, and other tissues. There are reports showing that DNA from exfoliated cell specimens was stable, for up to a week at room temperature. In fact, exposure to 37 °C for a week also does not affect the DNA yield [44]. The stability of DNA allows it to be retrieved and analyzed from dried bodily fluids, clotted blood, from Guthrie cards, dried blood smears on slides, or from clothing, as is often the case in forensic investigations.

5.5. Containers/equipment

The choice of the size and characteristics of tubes, bottles or other containers for sample collection and transportation, depends on the sample volume, means of transfer to the laboratory, their cost, storage efficiency, and the type of intended analyses. Several types of containers for blood collection are available [13]. Small blood samples can be collected by finger prick on commercially available cards pretreated to prevent sample degradation and contamination. Buccal cells are commonly collected with a small cyto-brush or tongue depressor, which is then rinsed in conical centrifuge tubes containing stabilizing buffer [66]. Use of commercial mouthwash and simple mouth rinse for buccal cell collection has recently gained popularity [44,67,68]. Collection of buccal cells can also be done on pretreated cards [69,70]. In their publications, Harty et al. also address issues of stability during transport, volume and type of containers, as they affect the cost of collection procedures along with the practicalities of sample handling [69,70]. Dry, compact vehicles of buccal cell samples are particularly useful if processing is not possible at the site of collection and long transport to the processing facility is required. However, dried vehicles for collection of biological samples limit their usefulness to fewer applications, such as DNA isolation, inorganic compound detection (e.g. Hg, As), and possibly a few others.

Besides the primary container used for the sample collection, subsequent containers in the process may also affect the sample quality. Certified RNase free containers must be used for all steps of handling RNA

samples. Single-use, sterile laboratory tubes are sufficient for this purpose, provided that all associated handling does not introduce contamination sources (e.g. gloves and an RNA-clean work area are required). Sterile single-use containers must also be exclusively used when cells are isolated for culture and/or cryopreservation.

5.6. Safety

Several issues of safety arise when handling human biological materials, and precautions must be taken at all stages of work. Human tissues are potentially infectious and detailed tests for pathogen profiles are typically not done unless they are part of the study, e.g. HIV, hepatitis, or other transmissible or parasitic disease. Personnel must be trained to handle human materials with the necessary safety precautions for their own protection and for the protection of others involved in the whole process (e.g. transportation personnel). In general, in epidemiologic cohorts there is a significant risk of infection if subjects are a random sample of the population or an occupational cohort about whom there is no health information related to infectious diseases. There is a particularly high safety concern about cohorts from countries with acknowledged high rates in infectious diseases, such as hepatitis in China, tuberculosis in East European countries, HIV in Africa, etc. In most epidemiologic studies involving “healthy” volunteers or children there is relatively little safety concern. However (unless the study subjects have been screened for transmissible diseases and diagnosed negative) the risk is unknown, and therefore the same precautions must be taken as if the samples were infectious. Sharp items, such as needles, pose a particularly high risk for personnel contamination and must be contained at all times. Good sources for safety regulations and guidelines related to handling of human tissues and body fluids in research are available through the Occupational Safety and Health Administration (OSHA) regulations “29 CFR, Part 1910.1030” (<http://www.osha.gov>), the National Institutes of Health (NIH) Health and Safety Manual, chapter 7 (<http://www.niehs.nih.gov/odhsb/manual/home.htm>), and the NIH Office of Research Services, Division of Safety (<http://www.nih.gov/od/ors/ds/index.html>).

5.7. Shipment

The awareness of potential risks during transport of biologic materials has increased, not only among scientists, but also among the public. Biologic materials potentially pose a very high risk of infectious disease transmittance. International [International Civil Aviation Organization (ICAO) and International Air Transport Association (IATA)] laws and Federal regulations [Department of Transportation (DOT)] govern the transport of potentially infectious and other hazardous materials, and specific regulations are in place regarding the packaging, labeling and documentation of shipped goods according to their classification. Therefore, any study director engaged in shipping of such materials is responsible for providing training to their employees in order to ensure conformance to regulations. It is also required that training be repeated every 2 years. The regulations do not allow any room for error, and improperly packaged or labeled goods will be refused for transport by airlines or be delayed at customs. Hence, it is in the study director's best interest to follow the regulations precisely so that damage to valuable samples, and potentially to the entire

study, is avoided. Training and certification is available through Saf-T-Pak® (<http://www.saftpak.com>).

5.8. Paper trail

An appropriate paper trail includes collection details, including date; sample number, types and volumes; shipping information, such as FedEx electronic receipt; and chain of custody forms. Personal information about the participants is encoded, in compliance with the privacy protection regulations. Paper forms can be replaced with more versatile electronic database systems, and barcodes are increasingly used to effectively encode samples and enable electronic verification and processing. Additionally, all protocols of sample collection and processing in addition to electronic logs are stored in secured locations.

It is also worth noting that in many studies there are documents in several languages as a result of multinational projects. While this presents a challenge in communication, it is particularly important that all groups maintain a common paper trail, even if that would require professional translations. General requirements

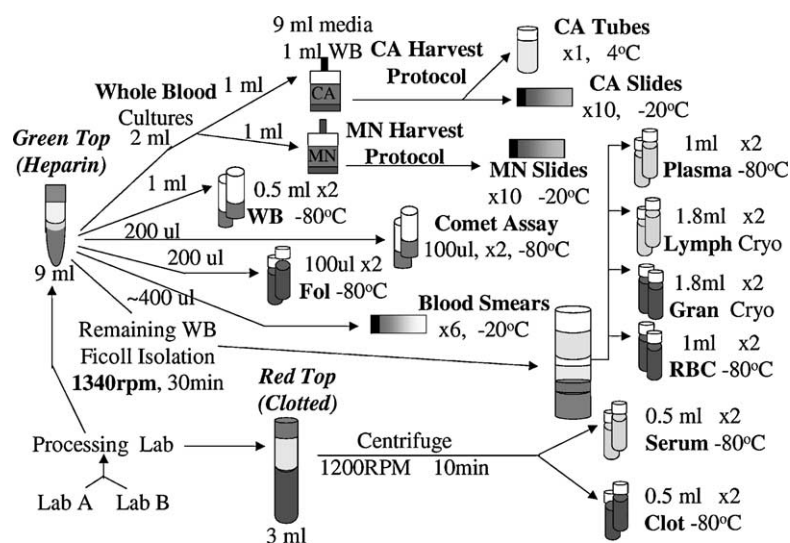


Fig. 2. Schematic representation of clotted and heparinized blood sample processing. The original sample is divided into multiple aliquots according to the final purpose of the analysis. Part of the original blood is separated into plasma, lymphocyte, granulocyte and red blood cell fractions through a Ficoll® gradient. Another part is used to set up whole blood cultures for different cytogenetic assays. Storage conditions are noted on the flow chart, including storage at 4°C, and in -20 and -80°C freezers, and cryopreservation in liquid N₂. More information can be found at our website: <http://ehs.sph.berkeley.edu/holland/protocollibrary.html> (CA: chromatid exchange; Fol: folate; Gran: granulocytes; Lymph: lymphocytes; MN: micronucleus; RBC: red blood cells; WB: whole blood).

for all study procedures should be reflected in the quality assurance/quality control (QA/QC) plan.

5.9. Strict adherence to protocols

The larger the study the greater is the challenge for consistency in handling all the biological specimens. It is inevitable that several individuals, and possibly

several laboratories, will handle the samples, either at the collection or at the processing stages. The challenge here is not only to produce clear and explicit protocols or standard operating procedures (SOPs) and train all individuals for all steps to be followed, but also to ensure that everyone adheres strictly to the SOPs. We have found that easy-to-follow protocols and graphic representations (flow-charts) of the SOPs

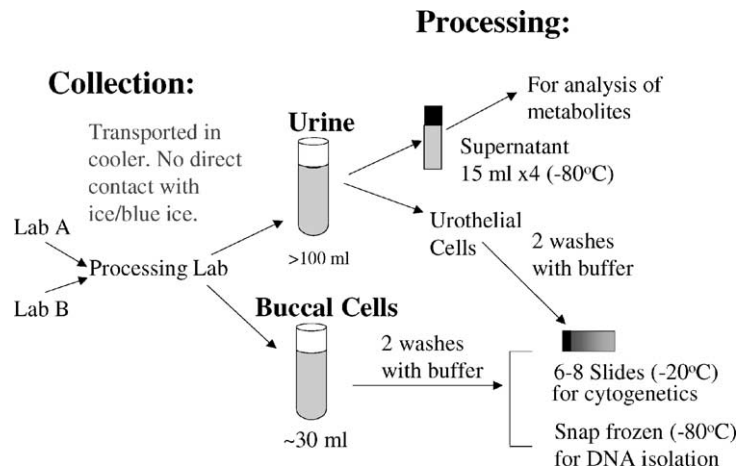


Fig. 3. Exfoliated cells from urine and mouth epithelium are processed to prepare histology slides for cytogenetic analysis, cell pellets for DNA/RNA isolation, and urine supernatant for analysis of metabolites. More information can be found at our Superfund Laboratory Protocols website: <http://ehs.sph.berkeley.edu/holland/protocollibrary.html>.

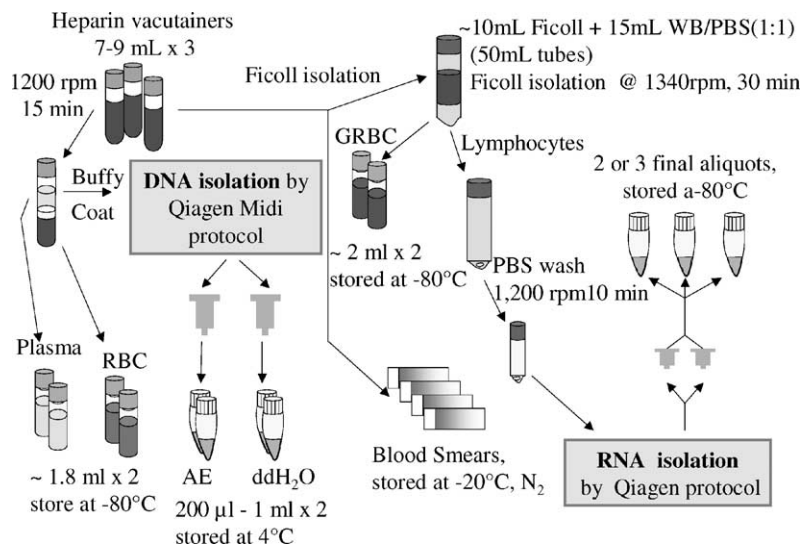


Fig. 4. Blood sample processing for DNA/RNA isolation. In this example DNA is prepared from buffy coat and RNA from isolated lymphocytes using Qiagen® kits. More information can be found at our website: <http://ehs.sph.berkeley.edu/holland/ProtocolLibrary.html> (GRBC: granulocytes; RBC: red blood cells; WB: whole blood).

Table 1
QA/QC procedures

Record keeping	Sample integrity	Storage	Equipment
Sample labeling	Replicates	Back-up system	Maintenance records
Chain of custody form	Internal standards	Temperature monitoring	Calibration
Laboratory log	Pilot analysis	Retrieval	
Database data entry			
Problem resolution			

help the technical staff to avoid confusion and misunderstanding (e.g. Figs. 2–4).

An appreciation of the challenge for clear protocols and raw data maintenance is reflected in the good laboratory practice (GLP) standards, regulations introduced by FDA and EPA regarding all health-effects studies, including environmental and toxicology studies that will be used in support of regulatory decisions [71,72]. Research laboratories need to be aware of, and in compliance with these regulations, and to develop detailed GLP programs. Quality assurance is part of the GLP requirements. Although not all laboratories are required to comply with these regulations, many laboratories understand the benefit of GLP procedures and elect to develop similar standards and record-keeping procedures so as to achieve a self-imposed high standard of scientific work. Detailed guidelines for the contents and level of detail required for GLP-QA/QC documents are provided by EPA: <http://www.epa.gov/quality/qatools.html> and <http://www.epa.gov/quality/exmural.html>.

Additional standards include good manufacturing practice (GMP) [73] and ISO 9001 [74].

Some of the issues addressed in QA/QC documents are summarized in Table 1.

6. Sample processing

Whether the original biological sample is whole blood, urine, buccal cells, bronchial lavage, or other tissue (e.g. biopsies), the processing can produce a variety of banked specimens for future purposes. The sooner the samples are processed the better the quality of the extracted components of interest. Processing may be extremely simple, for example, aliquoting and freezing, or separation of blood into clot and serum. Efficient and effective processing ensures that

the appropriate components of the samples can be retrieved after storage, and that the highest yield of those components is achieved. More effective sample processing includes provisions for: (1) isolating large quantities of DNA; (2) storing high-quality RNA; (3) using buccal cell DNA, blood clot (or blood smears) for genotyping; (4) separating lymphocyte from granulocyte DNA/RNA; (5) making metaphase spreads (useful for many years); (6) cryopreserving freshly isolated lymphocytes or whole blood to be recultured, and (7) preparing slides of exfoliated cells from mouth and urine.

In order to achieve the above, the original sample may be divided into separate aliquots appropriate for different purposes. In this situation, different buffer conditions and storage conditions have to be used. For example, aliquots prepared for RNA analysis are usually mixed with RNA stabilizing buffer containing β -mercaptoethanol (commercially available buffer is commonly used). Aliquots prepared for analysis of folate must contain antioxidant agents (ascorbic acid) and EDTA [56,57,75] (see Section 5.4); immunological biomarkers are stabilized in the presence of DMSO, which is also required for samples used in the Comet assay [76].

Our approach with sample processing is depicted in Fig. 2. Other aids can be in the form of time schedules and pre-made tables for recording the number and volumes of samples produced in the processing. Importantly, dividing each of the various sample forms that emerge from initial processing into multiple aliquots (e.g. multiple aliquots of serum, aliquots for RNA isolation, multiple slides for cytogenetic analysis and blood smears, etc.) preserves the integrity of the components by avoiding the damage inflicted by repeated freeze/thaw cycles, and allow each component to be appropriately stored or distributed to collaborators.

6.1. Cryopreservation of freshly isolated cells

Most studies require, or can benefit from, storage of viable cells that can be recultured in the future. For example, some assays using immunological biomarkers or molecular analyses may require a high number of cells. This can be achieved by culturing the original sample to expand the population; also, culture is required for possible immortalization of certain lineages of blood cells (e.g. CD34+ lymphocytes) [77]. Whole blood can be cryopreserved in equal volume of a mix of fetal bovine serum (FBS) and DMSO (10% final concentration of DMSO) (our protocol) or DMSO is added directly to whole blood without FBS [78]. More typically, cells are separated from whole blood before storage and are cryopreserved in FBS/DMSO for future use [78]. Lymphocytes can be isolated from whole blood by density centrifugation through gradients, such as Ficoll®, or by using Lymphoprep™ or similar tubes (<http://www.axis-shield-poc.com/optiprep/C04.pdf>; <http://www.progen.de>). Satisfactory results in lymphocyte isolation have also been reported using specialized commercial vacutainers or “cell preparation tubes” (CPT) [79–81]. Differential density properties of the blood components also allow adequate separation by a simple centrifugation step without a density gradient. Lymphocytes and monocytes form a layer (buffy coat) just above the granulocytes and red blood cell pellet. Buffy coat collection is simpler and cheaper than Ficoll® or Percoll® isolation, and can be readily performed in the field with the simplest centrifuge. However, Ficoll® isolation is more complete and allows separation of granulocytes from lymphocytes. It assures more efficiency and enables more possibilities for reculture and use of the cryopreserved cells.

Specific storage conditions are required for successful cryopreservation of cells. Liquid nitrogen in specialized containers (dewars) is used to achieve very low temperatures. In the past, the vials containing the cells used to be stored in the liquid phase of nitrogen (−196 °C). This caused the seals to break or the plastic vial to crack and, as a consequence, resulted in damage or complete loss of the sample. Storing the vials in the vapor phase of the liquid nitrogen (−150 °C) has become common practice to avoid such consequences.

Cell viability tests are performed on cryopreserved cells in pilot trials as part of the quality control prac-

tices in order to verify that the conditions of cryopreservation are effective, i.e. all or nearly all the cells are viable upon thawing [82]. The main goal of such tests is to ensure that cell loss is minimized. Loss of cell viability is observed when cells are warmed up to −132 °C or higher, even temporarily; a situation that occurs each time a rack of boxes containing vials is taken out of the dewar for addition or removal of a sample [83]. Extensive loss of cell viability may affect the results of intended studies: if only a subpopulation is viable it may not be representative of the whole, or rare genetic events (e.g. translocations, mutations) may be missed or underestimated. In addition, cryopreservation may affect cell characteristics and studies have been carried out to address such concerns [84–88].

In a recent report it was shown that successfully cryopreserved lymphocytes can be used as an alternative to repeated venipuncture and resampling to establish transformed cultures [77]. The recovery of the cryopreserved cells was comparable to freshly isolated lymphocyte cultures. In addition, the storage of multiple aliquots made it possible to achieve successful viable cell lines with 100% efficiency, i.e. all of the cryopreserved samples were successful [77].

6.2. Preparation of cells for cytogenetic analysis

Interphase cells may be sufficient for some types of cytogenetic analysis, such as micronucleus (MN) analysis, but metaphase cells are frequently needed. Metaphases are necessary for visualizing chromosomal aberrations (CA), sister chromatid exchanges (SCE), and for comparative genome hybridization assays [89]. Lymphocytes from blood are the most common cell type used for these kinds of analyses. Cells in culture can be stimulated to divide by various mitogens including PHA, concanavalin, interleukin, and pokeweed. To obtain sufficient cells in metaphase, inhibitors of mitosis, such as colcemid, are added 2–4 h before the cell harvest. The cell cultures can be started either from whole blood or from isolated lymphocytes. If whole blood is used to start the cultures, the lymphocytes must be isolated at the time of the harvest using Ficoll® gradient, as described above [90]. The slides are prepared with an optimum density of isolated cells. Micronucleus (MN) analysis does not require metaphase preparation but, if culture is

possible (lymphocytes), treatment with cell-division inhibitors (cytochalasin B) is preferred [20]. Slides for MN analysis are prepared most often from peripheral blood lymphocytes and epithelial exfoliated cells. All slides can be used immediately or stored for future use. Storage of slides at -20°C in N_2 gas is essential for successful fluorescent in situ hybridization (FISH) [91].

6.3. Preparation of exfoliated cells from mouth and urine

Exfoliated cells from urine (urothelial) or mouth (buccal) are easy to obtain (see “Collection”) [44,67,69]. Unlike blood cells, exfoliated cells cannot be as easily grown in culture. Preparation of the exfoliated cells is simple and is depicted in Fig. 3. The cells are washed in Tris–HCl/EDTA-buffer and then spread on histology slides for use in cytogenetic analysis. Alternatively, they can be frozen at -80°C and serve as a source of DNA in future studies. Urine supernatant is stored for analysis of biomarkers of exposure, such as benzene and its metabolites [92,93], biomarkers of oxidative damage, such as 8-oxo-2'-deoxyguanosine, etc. [94].

6.4. High-quality DNA and RNA

The quality of nucleic acids obtained from biological samples depends on several factors: (a) the quality of the original sample (handling); (b) the quality of extraction and the method used; (c) the storage conditions. A very recent review addresses preservation of DNA quality in more detail [95].

Compromised cell integrity and rupture of intracellular compartments membranes release an abundance of degrading enzymes that cause nucleic acid damage or significant degradation. Apoptosis is a controlled cell disintegration process that leads to extensive nucleic acid degradation. Therefore, for best results the original sample needs to be handled carefully and under conditions that ensure the cells remain intact. An important observation is that whole blood contains inhibitors of PCR reaction if dry blood spots are used to obtain template DNA. Inactivation of these inhibitors is achieved by an overnight methanol fixation step or use of pretreated cards for specimen collection [96].

High-quality RNA is harder to achieve than of DNA, mostly because of instability (see Section 5.4). RNA can be extracted effectively from isolated lymphocytes after Ficoll[®] gradient separation of whole blood (Fig. 4). This approach is more cost effective and produces better RNA yield than direct isolation from whole blood.

RNA and DNA purification kits are commercially available and easy to use, and include all the necessary buffers and detailed instructions. Qiagen[®] and Gentra[®] kits are among the most widely used. The original method of phenol/chloroform extraction is still one of the most efficient in DNA yield, as shown in studies comparing the DNA yield of different methods [67]. It may be necessary before the beginning of the study to compare different DNA extraction methods for their relative efficiency and quality of DNA yield and compatibility with the purpose of the study [67,97]. Importantly, a large percent of DNA isolated from buccal cells is non-human DNA, and only 11–49% is actually human DNA [67]. Therefore, human specific sequences must be used to quantify the yield.

Finally, long-term storage (years) may affect the integrity of nucleic acids, if it is not done under the right conditions. Both DNA and RNA must be stored at -80°C , although -20°C maybe adequate for DNA for shorter periods (months) [64]. Multiple aliquots are necessary in order to avoid repeated freezing and thawing and to prevent loss of the entire sample due to cross-contamination. It is important to note, here, that frost-free freezers must be avoided since they cause small volumes of aliquots to dry, even in capped tubes.

7. Sample banking

Large epidemiological studies produce tens of thousands of valuable samples that may be stored for years. It becomes apparent from the descriptions above that making provisions for future studies can lead to a wide variety of processed samples originating from a single tube of blood. Adequate physical storage, and an effective labeling and inventory management system are essential.

Labeling of samples so that they are efficiently tracked and retrieved can be done with electronic data management programs. Barcoding of biological

specimens allows automation of the banking system and error-proof operation. A unique barcode ID is given to each sample, generating a system of easily tracked specimens. The characteristics of each sample and related epidemiological information are linked to the barcode ID in the database system.

Electronic databases are far more efficient in the management of large sample banks than older paper-based versions of storage records. Depending on the size of the biorepository and the size of the project, a number of electronic database options are available. The Center for Disease Control (CDC) Repository has a capacity of 6 million banked specimens. The sophistication of both the physical and the electronic structure for supporting such a system is very high [14]. Similarly the Biological Specimen Inventory System servicing the NCI at NIH provides extensive network support to over 175 users (<http://bsi-ii.com>). These larger capacity systems run on structured query language (SQL) servers. Smaller organizations may develop less complex systems according to their needs. Some commercially available versions of software can handle a relatively small number of samples (e.g. <http://www.freezerworks.com/>). Other software development companies can design custom-made versions based on existing versatile programs, such as Microsoft Access, Oracle or SQL server (<http://www.inputautomation.com>; <http://www.fei.com>; <http://www.computype.com>; <http://www.brady.com>). All of the database management systems mentioned above utilize a barcode-based tracking system.

Our own experience draws from the increasing number of samples we collect and process for several projects within the Superfund Center and Center for Children's Environmental Research, at UC Berkeley. The equally increasing demand for effectively tracking our samples prompted us to have a customized database system developed, based on Microsoft Access software, with the help of a local software programming company. While our sample bank is still relatively modest compared to large centers, it is rapidly expanding and we found the barcoding and database system extremely helpful for keeping track of old and new samples from multiple projects. The features of our database system include: (a) flexibility with different processing protocols; (b) support for many users; (c) user-friendly design of the interface

with multiple check-points; (d) use of barcodes for automatic scanning; (e) rapid and error-proof data entry and automated label printing (essential for integrating the record entry and label printing with the sample processing routine in the lab); and (f) daily back-up on the server and monthly back-up on CD. A good resource for information related to sample handling with emphasis on banking is the International Society for Biological and Environmental Repositories (ISBER) at <http://www.isber.org/>.

8. Sample analysis

A broad spectrum of analytical methods with equally broad applications is available today: simple toxic substance detection (e.g. lead, mercury, benzene metabolites); immunological methods (ELISA, RIA, flow-cytometry) to detect levels of antibodies, cytokines, protein or DNA adducts, etc.; classical cytogenetic methods that detect chromosomal aberrations, SCE, MN formation; or more sophisticated multicolor FISH; or finally, advanced genetic analysis, based on the TaqMan[®], or microarray technologies. The challenge of analysis relies on the need to combine several analytical methods for each sample so that a better, more complete, picture can emerge about, for example, the exposure, the genetic predisposition, and the effect that is measured. A number of assays that we have performed on different biological specimens are listed in Table 2.

Table 2
Various components of biological samples can be useful for a number of assays

Specimen	Assays
Whole blood	Lead, folate, cholinesterase, ferritin, DNA/RNA extraction
Clot	HLA phenotype, genetic markers
Serum	Organochlorine and organophosphate pesticides, total IgE
Plasma	Cytokines, paraoxonase
Buffy coat	HLA phenotype, DNA/RNA extraction
RBC	Hb adducts
Blood smears	Molecular cytogenetics, DNA extraction
Buccal cells	Molecular cytogenetics, DNA extraction
Urothelial cells	Molecular cytogenetics
Urine	Benzene metabolites, cotinine, 8-oxo-2'-deoxyguanosine

8.1. Maximum use of available samples

Because the collected samples are valuable, (either limited in volume or difficult to obtain, or both), laboratory scientists need to make extra efforts to minimize the volumes used to perform assays, sometimes by re-adjusting existing protocols, [49,98]. Another approach is collaborating with investigators who can measure a range of biomarkers using multiplex methods [49]. An important consideration is to retain a minimum volume of control samples for the possibility that a study subject in the control group develops the disease later on and becomes part of the cases group.

9. Laboratory core

Our experience in meeting needs of sample collection and processing has led to the formation of Laboratory Cores responsible for several large epidemiological projects for the Superfund Basic Research Program and for the Center for Children's Environmental Health Research at UC Berkeley. The Specific goals of the Laboratory Cores include: (a) development of sample collection and processing protocols (SOPs) and QA/QC procedures; (b) coordination of sample handling with the field offices and/or hospital; (c) initial sample processing; (d) banking of biological and environmental samples; and (e) coordination and collaboration of sample analysis. Expertise of the researchers and technical assistants, space in the nitrogen tanks and freezers, and monitoring and processing equipment can be used more effectively, resulting in the improvement of the quality of the overall project as well as significant savings in expenses.

10. Summary

We have presented a series of issues related to sample collection, processing, and banking. The advances in molecular genetics can only be taken advantage of if sample quality is assured for already available and future biomarkers. High-throughput technology offers enormous power to the analysis of large number of samples in a time efficient manner, along with increased sensitivity, accuracy and reproducibility. Proper handling of biological samples from the time

of collection to the analysis protects the quality of the specimens and the validity of the results. Actions to be taken include: (a) identify the appropriate tissue with preference to non-invasive approaches; (b) determine the timing of collection and examine the biomarker stability requirements; (c) obtain the necessary equipment for the processing facilities, develop the detailed protocols and flow charts, train the employees, carry out pilot studies on the efficiency of cryopreservation or DNA/RNA purification; (d) organize the physical storage facilities and equipment and set up the barcoding and the electronic database management systems; (e) review all the legal requirements, including compliance with safety in handling human tissues, shipping of potentially infectious materials, human subject research approval and informed consent from the study subjects, laboratory and field QA/QC procedures, and possible collaborations in the analysis. All these issues have to be addressed in the standard operating procedures and carried out through Quality Assurance Program as an integral part of the successful molecular epidemiologic study.

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