THE EXTRACTION, PURIFICATION AND QUANTIFICATION OF DNA

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The intent of this document is to give the reader an overview of the processes of recovery, cleanup and quantification of DNA as they are applied to body fluid stains analyzed in the crime laboratory.

QUANTITY OF DNA IN HUMAN CELLS

Diploid human cells each contain about 6 pg of DNA, contained almost totally in the 46 chromosomes. About 1% of the total cellular DNA is located in the mitochondria. Haploid cells (spermatozoa and ova) contain one half the diploid quantity of DNA, or about 3 pg/cell. For human somatic cells, only the erythrocytes are devoid of chromosomal DNA.

Since crime scene evidentiary materials most often are shed body fluids, it is useful to estimate the quantities of DNA expected to be in common fluids. For example, the normal number of leukocytes in human peripheral blood generally ranges between 5 and 10 million cells per ml of blood. This equates to DNA concentrations that can vary between 30 μg/ml and 60 μg/ml of peripheral blood. The average number of spermatozoa per ml of semen is 150 x 10^6/ml (Mann and Lutwak-Mann 1981). Thus the DNA concentration in semen is considerably higher than in blood and averages about 450 μg/ml. Each ml of semen can contain about 5 x 10^6 leukocytes which contribute another 30 μg DNA per ml. Thus semen from an average male possesses approximately 480 μg DNA per ml.

Considering that a satisfactory autoradiogram image of a restriction fragment linked polymorphism (RFLP) pattern can be obtained from 20–50 ng of human genomic DNA, one can calculate that, with a 100% yield, the DNA would have to be recovered from 4,000–10,000 nucleated cells at a minimum. Since the yield of DNA from stain material routinely is less than 100%, more than the minimum number of nucleated cells is required to give a satisfactory RFLP pattern.

GOALS OF THE DNA RECOVERY PROCESS

There are three major goals that must be met during the DNA recovery processes if the RFLP typing analysis is to be successful. The recovered DNA must be of high molecular weight; the DNA must be free of substances that would compromise restriction endonuclease activity; and the quantity of DNA retrieved must be sufficient to meet the analytical requirements of the test procedure.

Factors Affecting the Size of Recovered DNA

There are several factors that can affect the molecular size of the DNA recovered from biological evidentiary specimens. One of the most common causes of DNA destruction is the action of the endo- and exonucleases that are ubiquitous in nature. While the analyst can do nothing to prevent the destructive action of nucleases prior to specimen collection, there are several steps that can be taken to abrogate their activity once the specimen environment can be controlled. Specimens should be maintained cool and dry prior to the commencement of DNA recovery procedures. The extraction solution should contain a metal chelator, such as ethylenediaminetetraacetic acid (EDTA), to prevent the activity of DNases. Moreover, pipette tips, glassware, and appropriate reagent solutions should be autoclaved before use to inactivate any nuclease activity that might be present.

A common abuse inflicted on DNA in the laboratory during recovery is excessive shear force, such as prolonged agitation on a vortex mixer. Such mishandling can result in DNA fragmentation to an extent that the molecular weights of the fragments are too low for the remaining steps in the RFLP procedure to be carried out.

The molecular weight of intact DNA in the cell nucleus is about 10^{11} daltons. Careful recovery of DNA that has not been severely affected by nuclease activity or shear forces should be of molecular weight about 10^7 daltons (Gross-Bellard *et al.* 1973).

Factors Affecting the Susceptibility of DNA to Restriction Digestion

The recovery process must not only avoid destruction of the DNA, but it must render the DNA suitable for digestion by restriction endonuclease (RE) activity. Two factors can reduce the ability of RE to fully digest DNA: the presence of histone proteins that remain attached to the DNA and pre-

vent access of RE to all possible restriction sites; and the presence of adventitious substances that are inhibitory to the catalytic activity of the RE.

Histones are removed from the DNA and denatured by the action of detergents. This is usually done at moderately elevated temperature (for example, 56° C). The denatured histones then are hydrolyzed to peptides and amino acids by the action of a proteolytic enzyme incorporated into the extraction solution.

Adventitious substances that are present in the DNA preparation can inhibit the catalytic activity of the RE. Such adventitious substances either were present on the surface upon which the body fluid was deposited (for example, dirt, salt, acids) or were introduced during the DNA recovery (for example, phenol, chloroform). Regardless of their source, contaminants can be removed by precipitation of the DNA from solution with ethyl alcohol, leaving the contaminants in solution; by dialysis; or by filtration.

Factors Affecting the Quantity of DNA Recovered

The quantity of DNA recoverable from forensic specimens is influenced by a number of factors. Obviously, the size of the stain or tissue sample affects the potential quantity of DNA that is available for recovery. In addition, the type of surface onto which the fluid has been deposited can dramatically affect the level of DNA recovery for body fluid stains.

GENERAL METHODS FOR RECOVERY AND EVALUATION OF DNA

The process of recovering DNA from forensic specimens can be broken into five steps: (1) Rehydration of the stain and solubilization of the stain components; (2) denaturation and hydrolysis of proteins; (3) removal of denatured proteins; (4) purification of the DNA; and (5) quantification of DNA and assessment of its quality for RFLP analysis. Each of these steps will be considered further.

Solubilization of Stain Components

Dried stains can lose considerable water in the course of drying. This water must be replaced and the stain components resolubilized for the recovery procedures to succeed. During this phase of the recovery process, the DNA must be protected from unnecessary degradation. Stains are cut and solubilization is accomplished by soaking the stain in buffer, usually at temperatures from 37° C to 56° C. A chelator of magnesium, such as EDTA, is included to prevent the action of the nucleases that would destroy the DNA. Many procedures call for an overnight extraction period (Kanter *et al.* 1986; Gill *et al.* 1987).

Denaturation/Hydrolysis of Proteins

The presence of detergents in the stain extraction buffer is responsible for the lysis of cellular membranes and for the dissociation and denaturation of the histone proteins that are tightly attached to the DNA strands. Detergents destroy the secondary and tertiary structures of proteins which leads to their decreased solubility in aqueous solution and increased susceptibility to the hydrolytic activity of proteolytic enzymes. A commonly employed detergent is sodium dodecylsulfate (SDS).

Proteinase K (Ebeling *et al.* 1974) is widely used in procedures for the isolation of DNA as an effective tool for the hydrolysis of histone proteins. This enzyme is active across a wide range of pH, is active in the presence of SDS (in fact, its activity is enhanced), and it is unaffected by metal chelators such as EDTA.

Removal of Denaturation Products

Denatured proteins can be removed effectively from the extraction solution by treatment with phenol and chloroform. Phenol, and to some extent chloroform, is an effective protein denaturant. Moreover, the products of denaturation and proteolysis are soluble in phenol (Kirby 1957). Some recovery procedures call for the use of phenol and chloroform mixtures, while others use phenol first followed by one or more treatments with chloroform to ensure complete removal of phenol. Isoamyl alcohol is included in mixtures of phenol and chloroform to reduce the tendency of proteins to foam when they are denatured during shaking with the organic solvents (Marmur 1961).

Purification of DNA

Additional cleansing of the DNA is necessary before restriction digestion is attempted. The purpose of this step in the procedure is to remove small molecules that are potential inhibitors of RE catalytic activity. Three approaches to additional DNA cleanup that have been used are: (1) Precipitation of the DNA from solution by ethanol; (2) dialysis of the DNA solution against large volumes of buffer (Kanter *et al.* 1986); and (3) ultrafiltration through selectively permeable membranes (Marashi *et al.* 1985).

Assessment of Quantity and Quality of DNA

There are four approaches that can be used to determine the quantity or quality of DNA. While all four permit quantification of DNA, not all enable a comprehensive assessment of DNA quality.

Ultraviolet Absorption

The purine and pyrimidine bases in DNA absorb light in the ultraviolet (UV) region of the light spectrum. While each of the four bases that occur in DNA has its own specific absorption maximum, the composite maximum occurs at 260 nm. A DNA solution of concentration 50 μg/ml will yield an absorbency at 260 nm that is equal to 1.0. The lower limit of detection for DNA by UV is about 0.5 μg DNA/ml.

Ultraviolet measurements are useful also for determining if proteins and/or phenol remain with the DNA after isolation and purification. If proteins have been effectively removed from the DNA, the ratio of absorbencies determined at 260 nm and 280 nm will equal about 1.8. Likewise, when the ratio of absorbencies 260 nm/270 nm equals 1.2, the preparation can be considered to be free of phenol. If either protein or phenol remains in the preparation, the respective ratio of absorbencies will fall. However, UV determinations do not enable an estimate of the extent of DNA degradation.

Fluorescence Measurements

Without modification, DNA does not have the ability to fluoresce. Fluorescence measurements can be made only after a suitable dye has been allowed to interact with the DNA. Two dyes that have been used for this purpose are Hoechst 33258 (Brunk *et al.* 1979) and ethidium bromide. The excitation and emission maxima for both dyes shift when they are bound to DNA. Hoechst 33258 appears to bind preferentially to regions of DNA that are rich in A–T base pairs; whereas ethidium bromide intercalates between the stacked bases (Watson *et al.* 1987). Quantification of DNA by fluorescence is about 10 times more sensitive than by UV measurement. Fluorescence measurements only permit quantification of DNA, nothing can be learned about the molecular weight of DNA the level of contamination by protein or by phenol.

Yield Gel Measurements

The approximate concentration and molecular weight of DNA obtained from specimens can be readily determined by yield gel procedures. Yield gels are small agarose gels that contain ethidium bromide. A fraction of each DNA specimen along with DNA calibration standards are subjected to a brief electrophoretic separation at relatively high voltage. The standards cover the range from 15 ng DNA up to about 300 ng DNA, generally in doubling steps. After the electrophoretic run is complete, the gel is placed under UV light and a photograph is taken. For analyst safety, test gels should not be evaluated while the gel is irradiated with UV light. The intensity of fluorescence of the DNA test specimen(s) in the photograph is compared with the intensity of the standards and an estimate is made of the DNA concentration in the test specimen(s). This method of determining DNA concentration in test specimens is semi-quantitative and is the least accurate of the available quantitative methods.

Yield gel measurement is the most rapid method of assessing the molecular weight of the DNA in a test specimen. High molecular weight DNA remains as a compact band that does not migrate far from the origin during the brief electrophoretic period. In contrast, DNA that has been degraded will migrate more rapidly than high molecular weight DNA and if DNA has been degraded completely, the fluorescing band will migrate with or ahead of the bromophenol blue tracking dye that is added to each sample. Partially degraded DNA will be seen as a smear of fluorescing material that runs from the high to the low molecular weight regions of the gel. It is recommended that standards of RE digested viral DNA be included on each yield gel as comparative size standards. For example, RE digestion of lambda phage DNA by HindIII, yields six DNA fragments that range in size from 2027 base pairs (bp) up to 23130 bp. Yield gels do not enable estimates of protein or phenol contamination.

None of the quantitative/qualitative methods described to this point permit estimates of the human DNA that is present in a specimen. Since DNA other than human can potentially be present in a forensic specimen, these methods can result in an overestimate of the quantity of human DNA.

Slot/dot Blot and Human DNA Probe

This method, unlike those previously described, permits the specific estimation of the amount of human DNA in a specimen. To carry out the slot/dot blot test, DNA recovered from the specimen is denatured and bound to a nylon membrane. Generally, the membrane is held in an appa-

ratus that enables the application of multiple specimens on one membrane. The sample application area can have either the configuration of a dot or a slot, hence the term, slot blot or dot blot. The membrane-bound DNA specimens are then hybridized with a radioactively-labeled DNA probe that is complementary to a highly repetitive sequence found only in human DNA. An example of such a DNA probe is p17H8 (Waye *et al.* 1989) that is complementary to a highly repetitive sequence located in human chromosome 17. When this probe is used under low stringency conditions, it will hybridize with closely related sequences found in the DNA of other chromosomes. Because the concentration of the target sequences is high, many molecules of probe are bound and an intense autoradiographic signal can be obtained in just a few hours. Standards of DNA are placed onto the membrane along with the test specimens as quantitative references. This technique, although semi-quantitative, is extremely sensitive, enabling the detection of human DNA in the pg range. Dot/slot blot techniques do not inform the analyst about protein or phenol levels in the specimen, nor do they reveal anything about the intactness of the specimen DNA, unless the target DNA is so thoroughly degraded that the probe will not hybridize.

CONCLUSIONS

The isolation of DNA from forensic specimens is not a difficult procedure. As with any technique however, care must be exercised to insure that the limited quantities of DNA that are available in such specimens are not squandered by poor or sloppy analytical technique. It has been the experience of many individuals that improved yields of DNA are seen as analysts gain experience with, and confidence in, the procedures.

Newer methods for the recovery of DNA from forensic specimens may be on the horizon. Procedures have been published which show that DNA can be recovered effectively while avoiding the use of hazardous reagents such as phenol. These procedures utilize high NaCl concentrations (Miller *et al.* 1988), or chaotropic agents such as guanidinium hydrochloride or urea (Jeanpierre 1987; Lindblom and Holmlund 1988) to effect the removal of proteins from DNA.

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