Sample Collection, Storage, and Characterization

Steps Involved

DNA typing, since it was introduced in the mid-1980s, has revolutionized forensic science and the ability of law enforcement to match perpetrators with crime scenes. Each year, thousands of cases around the world are closed with guilty suspects punished and innocent ones freed because of the power of a silent biological witness at the crime scene. This book explores the science behind DNA typing and the biology, technology, and genetics that make DNA typing the most useful investigative tool to law enforcement since the development of fingerprinting over 100 years ago. As noted in the Introduction, this volume is intended primarily for DNA analysts or advanced students with a more in-depth look into subjects than its companion volume *Fundamentals of Forensic DNA Typing*.

**STEPS IN DNA TESTING PROCESS**

A summary of the steps involved in processing forensic DNA samples is illustrated in Figure 1.1. Following collection of biological material (Chapter 1) from a crime scene or paternity investigation, DNA is extracted from its biological source material (Chapter 2) and then measured to evaluate the quantity of DNA recovered (Chapter 3). Specific regions of the DNA are targeted and copied with the polymerase chain reaction, or PCR (Chapter 4). Commercial kits are commonly used to enable simultaneous PCR of 13 to 15 short tandem repeat (STR) markers (Chapter 5). STR alleles are interpreted relative to PCR amplification artifacts following separation by size using capillary electrophoresis (Chapter 6) and data analysis software. A statistical interpretation assesses the rarity of the alleles from the resulting DNA profile, which can be single-source or a mixture depending on the sample origin.
Ideally, the parameters and protocols for each step in this process are established through laboratory validation with quality assurance measures in place to aid in obtaining the highest quality data (Chapter 7). Following the DNA testing, a written report is created summarizing the work conducted and results obtained. If the case goes to court, expert witness testimony may be required of the laboratory report’s author (Chapter 18).

DNA analysis always requires that a comparison be made between two samples: (1) a questioned sample, commonly referred to as a “Q”, and (2) a known sample, referred to as a “K” (Figure 1.1). In forensic cases, crime scene evidence (Q) is always compared to a single suspect (K) or multiple suspects (K₁, K₂, K₃, etc.). In a case without a suspect, the evidence DNA profile may be compared to a computer database (Chapter 8) containing DNA profiles from previous offenders (K₁ … Kᵣ).

Note that in Figure 1.1 under the reference sample steps, no characterization of the sample is performed nor is there a statistical interpretation given of the rarity of the DNA profile. Since sample K is from a known source, there is no need to determine its origin (e.g., bloodstain vs. saliva stain) or to calculate a random match probability because through accurate chain-of-custody records the DNA analyst should truly know the source of the sample.

Other applications of DNA testing involve direct or biological kinship comparisons. With paternity testing, an alleged father (Q) or fathers (Q₁, Q₂, …) are compared to a child (K). The victim’s remains (Q) in missing persons or mass disaster cases (Chapter 9) are identified...

FIGURE 1.1 Overview of steps involved in DNA testing.
SAMPLE COLLECTION

Before a DNA test can be performed on a sample, it must be collected and the DNA isolated and put in the proper format for further characterization. This chapter covers the important topics of sample collection, characterization, and preservation. These steps are vital to obtaining a successful result regardless of the DNA typing procedure used. If the samples are not handled properly in the initial stages of an investigation, then no amount of hard work in the final analytical or data interpretation steps can compensate.

DNA Sample Sources

DNA is present in every nucleated cell and is therefore present in biological materials left at crime scenes. DNA has been successfully isolated and analyzed from a variety of biological materials. Introduction of the polymerase chain reaction (PCR), which is described in Chapter 4, has extended the range of possible DNA samples that can be successfully analyzed because PCR enables many copies to be made of the DNA markers to be examined. While the most common materials tested in forensic laboratories are typically bloodstains and semen stains, Table 1.1 includes a listing from one laboratory of over 100 DNA Sample Sources

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TABLE 1.1 Some Sources of Biological Materials Used for PCR-Based DNA Typing. This Listing of Exhibits Produced Successful DNA Profiles in the Canadian RCMP Forensic Biology Laboratories. Adapted from Kuperus et al. (2003).

<table>
<thead>
<tr>
<th>DNA SOURCE: HANDS</th>
<th>DNA SOURCE: MOUTH AND NOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arm-rest (automobile)</td>
<td>Salami (bite mark)</td>
</tr>
<tr>
<td>Baseball cap (brim)</td>
<td>Stamps (including self-adhesive)</td>
</tr>
<tr>
<td>Binder twine</td>
<td>Straw (from drinking glass)</td>
</tr>
<tr>
<td>Bottle cap</td>
<td>Telephone receiver</td>
</tr>
<tr>
<td>Chocolate bar (handled end)</td>
<td>Thermos (cup attached)</td>
</tr>
<tr>
<td>Cigarette lighter</td>
<td>Tooth</td>
</tr>
<tr>
<td>Cigarette paper</td>
<td>Toothbrush</td>
</tr>
<tr>
<td>Signal light control lever (automobile)</td>
<td>Toothpick</td>
</tr>
<tr>
<td>Credit card (ATM card)</td>
<td>Utensils (fork, spoon, etc.)</td>
</tr>
<tr>
<td>Detachable box magazine (pistol)</td>
<td>Vomit (bile-like sputum/liquid)</td>
</tr>
<tr>
<td>Dime</td>
<td>焊接 (焊缝)</td>
</tr>
<tr>
<td>Door bell</td>
<td>Sandblasting nozzle (face, hand, and body)</td>
</tr>
<tr>
<td>Door pull</td>
<td>Welding goggles (rim of eye/nose)</td>
</tr>
<tr>
<td>Drug syringe barrel exterior</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
unusual casework exhibit materials that yielded successful DNA profiles (Kuperus et al. 2003). Even a few cells left with latent fingerprint residue can serve as effective sources of DNA (Schulz & Reichert 2002, Balogh et al. 2003). DNA molecules are amazingly durable and in many cases can yield DNA typing results even when subjected to extreme conditions such as irradiation (Castle et al. 2003, Withrow et al. 2003) or explosive blasts (Esslinger et al. 2004).

### Biological Evidence at Crime Scenes

Different types of biological evidence collected at a crime scene can be used to associate or to exclude an individual from involvement with a crime. In particular, the direct transfer of DNA from one individual to another individual or to an object can be used to link a suspect to a crime scene. As noted by Dr. Henry Lee, formerly of the Connecticut State Forensic Laboratory, this direct transfer could involve (Lee 1996):

1. The suspect’s DNA deposited on the victim’s body or clothing;
2. The suspect’s DNA deposited on an object;
3. The suspect’s DNA deposited at a location;
4. The victim’s DNA deposited on the suspect’s body or clothing;
5. The victim’s DNA deposited on an object;
6. The victim’s DNA deposited at a location;
7. The witness’s DNA deposited on victim or suspect; or
8. The witness’s DNA deposited on an object or at a location.

As Dr. Paul Kirk noted in his 1953 book Crime Investigation: “The blood or semen that [the perpetrator of a crime] deposits or collects—all these and more bear mute witness against him. This is evidence that does not forget... Physical evidence cannot be wrong; it cannot
perjure itself; it cannot be wholly absent… Only human failure to find, study and understand it can diminish its value” (Kirk 1953).

DNA evidence collection from a crime scene must be performed carefully and a chain of custody established in order to produce DNA profiles that are meaningful and legally accepted in court. DNA testing techniques have become so sensitive that biological evidence too small to be easily seen with the naked eye can be used to link suspects to crime scenes. The evidence must be carefully collected, preserved, stored, and transported prior to any analysis conducted in a forensic DNA laboratory. The National Institute of Justice has produced a brochure entitled “What Every Law Enforcement Officer Should Know About DNA Evidence” (now available as online training as well, see http://www.dna.gov) that contains helpful hints for law enforcement personnel who are the first to arrive at a crime scene.

One crime scene investigator (Blozis 2010) categorized three types of DNA samples: (1) unknown samples recovered from crime scenes, (2) elimination samples from individuals such as the victim(s) or family members who had prior legitimate access to the crime scene, and (3) biological material abandoned by an individual known to law enforcement. The last category might include a cigarette butt discarded in a public place.

It can be pointless to collect samples for DNA testing in many cases. For example, swabbing a car steering wheel to pick up touch DNA will likely reveal the owner(s) or legitimate drivers of the car rather than the perpetrator in a car theft situation. In many situations, an uninformative, complicated mixture may be created from the legitimate drivers rather than a clean, clear-cut DNA profile that can unambiguously be linked to a suspect. Likewise, just because human DNA was successfully isolated from a mosquito and helped solve a crime (Spitaleri et al. 2006) does not mean that mosquitoes are optimal evidence to collect for every case! Thus, thought and judgment are required by the crime scene investigators to collect optimal samples for DNA testing.

Unfortunately, the “CSI effect” (see Chapter 18) in some situations has spread to detectives and crime scene investigators who try to collect and submit as many samples as possible to the crime laboratory. The watching of “forensic” television shows has created unrealistic expectations in the general public and even some law enforcement officials in terms of both the speed and probability of success with DNA results obtained. The submission of excessive numbers and sometimes unnecessary samples can bog down the laboratory, which then limits the ability to process legitimate samples in a timely manner.

Evidence Collection and Preservation

The importance of proper DNA evidence collection cannot be overemphasized. If the DNA sample is contaminated from the start, obtaining unambiguous information becomes a challenge at best.

Samples for collection should be carefully chosen as well to prevent needless redundancy in the evidence for a case. The following suggestions are helpful during evidence collection to preserve it properly:

- Avoid contaminating the area where DNA might be present by not touching it with your bare hands, or sneezing or coughing over the evidence.
- Use clean latex gloves for collecting each item of evidence. Gloves and/or tweezers should be changed between handling of different items of evidence.
• Package each item of evidence separately to prevent potential transfer and cross-contamination between different items.
• Air-dry bloodstains, semen stains, and other types of liquid stain prior to sealing the package.
• Package samples in paper envelopes or paper bags after drying. Plastic bags should be avoided because water condenses in them, especially in areas of high humidity, and moisture can speed the degradation of DNA molecules. Packages should be clearly marked with case number, item number, collection date, and initialed across the package seal in order to maintain a proper chain of custody.
• Transfer stains on unmovable surfaces (such as a table or floor) with sterile cotton swabs and distilled water. Rub the stained area with the moist swab until the stain is transferred to the swab. Allow the swab to air dry without touching any others. Store each swab in a separate paper envelope.

One of the most common methods for optimally collecting cellular material is the so-called “double swab technique” where a moist swab is followed by a dry one (Sweet et al. 1997, Pang & Cheung 2007). The wet swab, which has been moistened by dipping it in sterile, distilled water, is first brushed over a surface to loosen any cells present and to rehydrate them. The second swab, which is initially dry, then helps collect additional cells from the surface. It is thought that the rehydrated cells adhere more easily to the second swab. Since both swabs are collected from the same sample, they are usually combined to maximize the yield of collected cellular material. Unfortunately, as will be discussed briefly in Chapter 2, poor extraction efficiencies from the swab can sometimes limit the amount of recovered DNA.

One of the challenges with collecting sexual assault evidence from vaginal samples using cotton swabs is that the sperm cells can stick to the cotton fibers and not be easily released during DNA extraction. Digestion of the cotton swab with cellulase, an enzyme that breaks down the cellulose fibers in cotton, was found to improve DNA recovery (Voorhees et al. 2006, Norris et al. 2007). In another approach, a nylon flocked swab was found to promote cell release during the extraction steps and produce a higher yield of DNA when compared with cotton swabs (Benschop et al. 2010). Another effective technique for recovering cellular material from clothing or other evidentiary items is the use of an adhesive tape attached to a plastic or acetate support (Hall & Fairley 2004, Hansson et al. 2009, Barash et al. 2010). The tape is pressed multiple times over the area where cellular material may be present. The tape is then placed directly into the DNA extraction tube and dissolved to enable optimal recovery (May & Thomson 2009). Tape lifting enables samples to be examined for gunshot residue or other trace evidence prior to being extracted for DNA.

Collection of Reference DNA Samples
To perform comparative DNA testing with evidence collected from a crime scene, biological samples must also be obtained from suspects or evidentiary DNA profiles searched against a database of potential suspects (Chapter 8). Family reference samples may be used in paternity testing, missing persons investigations, and mass disaster victim identifications (Chapter 9).
It is advantageous to obtain these reference DNA samples as rapidly and painlessly as possible. Thus, many laboratories often use buccal cell collection rather than drawing blood. Buccal cell collection involves wiping a cotton swab similar to a Q-tip against the inside cheek of an individual’s mouth to collect some skin cells. The swab is then dried or can be pressed against a treated collection card to transfer epithelial cells for storage purposes. Adhesive tapes may also be used for collecting reference DNA samples (Zamir et al. 2004).

Bode Technology Group (Lorton, VA) has produced a simple Buccal DNA Collector (Fox et al. 2002, Schumm et al. 2004, Burger et al. 2005) that is widely used for direct collection of buccal cell samples. This collection system also comes with a transport pouch containing a desiccant to keep the sample dry and has a unique bar code on each DNA collector to enable automated sample tracking. Several types of buccal collectors are shown in Figure 1.2.

A disposable toothbrush can be used for collecting buccal cells in a non-threatening manner (Burgoyne 1997, Tanaka et al. 2000). This method can be very helpful when samples need to be collected from children. After the buccal cells have been collected by gently rubbing a wet toothbrush across the inner cheek, the brush can be tapped onto the surface of treated collection paper for sample storage and preservation. Saliva collection also works and can be a useful method to obtain reference samples for human population genetic studies (Quinque et al. 2006).

If a liquid blood sample is collected, then typically a few drops of blood are spotted onto a piece of treated or untreated filter paper. Blood samples are advantageous in that it is easy to see that a sample has been collected (as opposed to a colorless swab from a saliva sample).

Regardless of the method of collecting a DNA sample from a reference or crime scene source, it is imperative that the collection material be DNA-free prior to use. For over 15 years investigators in Europe chased what was popularly referred to as the “German phantom,” a supposed serial offender whose DNA profile was continually appearing in a variety of crimes (Himmelreich 2009, Neuhuber et al. 2009). In 2008, the “offender” was discovered to be an elderly lady who worked for a manufacturer packaging DNA collection swabs. In placing the swabs in their packages, she had inadvertently contaminated some of them with her own DNA, which when used for the purpose of crime scene investigation revealed her DNA.
profile rather than biological material from the crime scene. The important issue of potential reagent and consumable contamination will be covered in greater detail in Chapter 4.

SAMPLE STORAGE AND TRANSPORT OF DNA EVIDENCE

Carelessness or ignorance of proper handling procedures during storage and transport of DNA from the crime scene to the laboratory can result in a specimen unfit for analysis. For example, bloodstains should be thoroughly dried prior to transport and storage to prevent mold growth. A recovered bloodstain on a cotton swab should be air-dried in an open envelope before being sealed for transport. DNA can be stored long-term as non-extracted tissue or as fully extracted DNA. DNA samples are, however, not normally extracted until they reach the laboratory.

Most biological evidence is best preserved when stored dry and cold (Baust 2008). These conditions reduce the rate of bacterial growth and degradation of DNA. Samples should be packaged carefully and hand-carried or shipped using overnight delivery to the forensic laboratory conducting the DNA testing. Evidence collection cardboard boxes have been designed for shipping and handling bloodstains and other crime scene evidence (Hochmeister et al. 1998). Inside the laboratory, DNA samples are either stored in a refrigerator at 4°C or a freezer at −20°C. For long periods of time, extracted DNA samples may be stored at −80°C.

DNA molecules survive best if they are dry (to prevent base hydrolysis) and protected from DNA digesting enzymes called DNases. A common method of storing DNA reference samples is on bloodstain cards (Kline et al. 2002, Sjöholm et al. 2007, Coble et al. 2008). This method involves adding a few drops of liquid blood to a cellulose-based filter paper and then air-drying the bloodstain before storing it. Some bloodstain cards have been treated with chemicals to enhance DNA longevity. Buccal (cheek) cells can also be transferred to treated paper for storage (Sigurdson et al. 2006). The dried bloodstain card can also be vacuum sealed with a desiccant to prevent humidity from breaking the stored DNA molecules into smaller pieces and destroying the ability to recover a full DNA profile.

Many police evidence lockers and storage vaults that hold crime scene evidence have freezers to enable storage of rape kits or other material containing biological evidence. Storage and availability of this evidence after many years, in some cases, has enabled post-conviction DNA testing of individuals incarcerated prior to the availability of DNA testing (see Butler 2010, Fundamentals D.N.A. Box 1.1). Large-scale DNA reference sample collection has been performed by the U.S. military since the early 1990s in an effort to be able to identify all recovered remains of military casualties and thus prevent there ever being another “unknown soldier” (see Butler 2010, Fundamentals D.N.A. Box 4.3).

While large freezers work well for preserving evidence by keeping it cold, these freezers are expensive to power and to maintain. Freezers generate a lot of heat and take up considerable space. Recent room temperature storage approaches through chemically treating DNA samples to protect them from degradation have been developed by several companies including Biomatrica (San Diego, CA) and GenVault (Carlsbad, CA).

In the summer and autumn months of 2007, a set of DNA samples stored at ambient temperatures in Biomatrica’s SampleMatrix were shipped back and forth across the United States...
with no insulation or refrigeration (Lee et al. 2010). These samples, which were dried down aliquots of 1 ng/µL, 0.25 ng/µL, and 0.05 ng/µL pristine genomic DNA, were compared at various time points over a 208-day window against equivalent samples stored in the laboratory. While the shipped samples experienced extreme temperature ranges of almost 45°C and relative humidity differences of almost 60%, full Identifiler DNA profiles were obtained with all of the tested samples (Lee et al. 2010). These data suggest that the SampleMatrix material, now marketed by QIAGEN (Valencia, CA) as QiaSafe, will help preserve DNA outside of a stable, cold environment enabling cost savings for storing biological samples.

Studies have shown that bloodstain samples which are stored dry (through vacuum sealing with a desiccant) can be successfully stored for over 20 years at ambient temperatures and still yield full DNA profiles (Coble et al. 2008, Kline 2010). Furthermore, an examination of bloodstains on four different filter papers found that keeping the sample dry through desiccation was more important than the type of paper (treated or untreated) that the sample was stored on (Kline et al. 2002). Likewise, appropriate room temperature storage of soft tissue samples, which may be recovered during disaster victim identification (Chapter 9) has been successful (Graham et al. 2008).

Every effort should be made to avoid completely consuming or destroying evidence so that a portion is available for future testing if needed. As the 1996 National Research Council’s *The Evaluation of Forensic DNA Evidence* states: “The ultimate safeguard against error due to sample mixup is to provide an opportunity for retesting” (NRCII, p. 81).

**SAMPLE CHARACTERIZATION**

When crime scene evidence is first received into a laboratory, it is usually evaluated to see if any biological material is present. Some laboratories perform both preliminary tests and confirmatory tests prior to sending a cutting or swab for DNA testing in an effort to develop a DNA profile. A presumptive test, which really serves as a preliminary evaluation or examination, may be followed by a confirmatory test to verify the results of the first test.

In a 2007 survey of 42 laboratories from 10 different countries, Ron Fourney and colleagues at the Royal Canadian Mounted Police found that most of the surveyed laboratories perform some form of either presumptive or confirmatory tests for biological screening (Fourney et al. 2007). A summary of their results is found in Table 1.2.

**Forensic Serology: Presumptive and Confirmatory Tests**

Forensic evidence from crime scenes comes in many forms. For example, a bed sheet may be collected from the scene of a sexual assault. This sheet will have to be carefully examined in the forensic laboratory before selecting the area to sample for further testing. Prior to making the effort to extract DNA from a sample, presumptive tests are often performed to indicate whether or not biological fluids such as blood or semen are present on an item of evidence (e.g., a pair of pants). Locating a blood or semen stain on a soiled undergarment can be a trying task. Primary stains of forensic interest come from blood, semen, and saliva. Identification of vaginal secretions, urine, and feces can also be important to an investigation.
Serology is the term used to describe a broad range of laboratory tests that utilize antigen and serum antibody reactions (Ballantyne 2000). For example, the ABO blood group types are determined using anti-A and anti-B serums and examining agglutination when mixed with a blood sample (Li 2008). One of the principle tools of forensic science in the past, serology still plays an important role in modern forensic biology but has taken a backseat to DNA since presumptive tests do not have the ability to individualize a sample like a DNA profile can.

Presumptive tests should be simple, inexpensive, safe, and easy to perform (Shaler 2002). They should use only a small amount of material and have no adverse effect on any downstream DNA testing that might be conducted on the evidentiary material (Tobe et al. 2007, Virkler & Lednev 2009). Besides helping to locate the appropriate material for DNA analysis, stain characterization can in some cases provide probative value to a case (e.g., semen in a victim’s mouth as evidence of an oral sexual assault).

Primary providers for presumptive forensic serology tests have been Abacus Diagnostics (West Hills, CA) and Seratec (Goettingen, Germany). Their in-vitro diagnostic tests, which appear very similar to home pregnancy tests, involve applying a small aliquot of a sample to a cartridge with a membrane containing specific antibodies. The presence of the appropriate molecules (e.g., hemoglobin with a blood test) on this immuno-chromatographic strip test will be detected as a colored line. Internal standards are run to verify that the test is working properly.
Independent Forensics (Hillside, IL) has released lateral flow strip tests for detecting the presence of blood, saliva, semen, and urine from forensic evidence. The RSID (Rapid Stain Identification) tests are confirmatory for blood (Schweers et al. 2008) and semen and presumptive for saliva and urine. These tests use different markers from the commonly used lateral flow strip tests (i.e., they do not use hemoglobin, PSA/p30, urea, or enzymatic activity for the detection of blood, semen, urine, or saliva, respectively) and are therefore more specific with fewer false positives and false negatives.

Independent Forensics also has developed a forensic-specific fluorescence kit for staining microscope slides used to scan sexual assault evidence for sperm called “SPERM HY-LITER.” This test is confirmatory for human sperm heads. The RSID Blood and RSID Semen tests are confirmatory and designed to not cross-react with other human body fluids or body fluids of other animals like some of the presumptive tests do. Information on the RSID products is available at http://www.ifi-test.com/rsid.php.

Edwin Jones in his review of methods for identification of semen and other body fluids points out that the fastest way to locate a body fluid stain is by visual examination (Jones 2004). Dried semen stains as well as saliva, urine, and vaginal fluid stains contain substances that when irradiated with a handheld UV lamp or argon laser can fluoresce, or emit light, in the visible-light region. A high-intensity light source with appropriate excitation and emission filters is known as an alternate light source, or ALS. ALS is an effective screening tool in the initial examination of forensic evidence (Vandenberg & van Oorschot 2006).

Bloodstains

Blood is composed of liquid plasma and serum with solid components consisting of red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes). Most presumptive tests for blood focus on detecting the presence of hemoglobin molecules, which are found in the red blood cells and used for transport of oxygen and carbon dioxide.

A simple immuno-chromatographic test for identification of human blood is available from Abacus Diagnostics (West Hills, CA) as the ABAcard HemaTrace kit. This test has a hemoglobin limit of detection of 0.07 μg/mL and shows specificity for human blood along with higher primate and ferret blood (Johnston et al. 2003). On the other hand, the RSID Blood test from Independent Forensics utilizes monoclonal antibodies to the red blood cell membrane specific protein glycophorin A rather than hemoglobin and does not cross-react with ferret, skunk, or primate blood (Schweers et al. 2008).

Luminol is another presumptive test for identification of blood that has been popularized by the TV series CSI: Crime Scene Investigation. The luminol reagent is prepared by mixing 0.1 g 3-amino-phthalhydrazide and 5.0 g sodium carbonate in 100 mL of distilled water. Before use, 0.7 g of sodium perborate is added to the solution (Saferstein 2001). Large areas can be rapidly evaluated for the presence of bloodstains by spraying the luminol reagent onto the item under investigation. Objects that have been sprayed need to be located in a darkened area so that the luminescence can be more easily viewed. Luminol can be used to locate traces of blood that have been diluted up to 10 million times (Saferstein 2001). The use of luminol has been shown to not inhibit DNA testing of STRs that may need to be performed on evidence recovered from a crime scene (Gross et al. 1999).
Demonstration that presumptive tests do not interfere with subsequent DNA testing can be important when making decisions on how biological evidence is processed in a forensic laboratory (Hochmeister et al. 1991, Budowle et al. 2000). Unfortunately the use of Hemastix, a screening test for bloodstains, has been shown to introduce problems with downstream processing involving magnetic-bead DNA extraction (Poon et al. 2009). This problem was solved by first transferring a portion of the bloodstain under investigation to a separate piece of filter paper for the presumptive test. The remaining portion of the original sample could then be processed for DNA extraction without coming in contact with the interfering chemicals.

Saliva Stains

A presumptive test for amylase is used for indicating the presence of saliva (Whitehead & Kipps 1975, Auvdel 1986), which is especially difficult to see since saliva stains are nearly invisible to the naked eye. Two common methods for estimating amylase levels in forensic samples include the Phadebas test and the starch iodine radial diffusion test (Shaler 2002, Myers & Adkins 2008). The presence of saliva in a stain has also been verified through detecting oral bacterial DNA (Nakanishi et al. 2009, Donaldson et al. 2010).

Saliva stains may be found on bite-marks, cigarette butts, and drinking vessels (Abaz et al. 2002, Shaler 2002). As will be described later in this chapter, a molecular biology approach using messenger RNA profiling is being developed to enable sensitive and specific tests for various body fluids including saliva (Juusola & Ballantyne 2003, Hanson & Ballantyne 2010). This approach holds promise to permit simultaneous tests for blood, semen, and saliva with great specificity and sensitivity.

Semen Stains

Prior to the expanded use of DNA testing for high-volume crimes such as burglary, roughly two-thirds of cases pursued with DNA analysis involved sexual assault evidence. Hundreds of millions of sperm are typically ejaculated in several milliliters of seminal fluid. Semen stains can be characterized with visualization of sperm cells, or acid phosphatase (AP) or prostate specific antigen (PSA or p30) tests (Jones 2004).

A microscopic examination to look for the presence of spermatozoa is performed in some laboratories on sexual assault evidence. However, aspermic or oligospermic males have either no sperm or a low sperm count in their seminal fluid ejaculate. In addition, vasectomized males will not release sperm. Therefore tests that can identify semen-specific enzymes are helpful in verifying the presence of semen in sexual assault cases.

Acid phosphatase (AP) is an enzyme secreted by the prostate gland into seminal fluid and is found in concentrations up to 400 times greater in semen than in other body fluids (Sensabaugh 1979, Saferstein 2001). A purple color with the addition of a few drops of sodium alpha naphthylphosphate and Fast Blue B solution or the fluorescence of 4-methyl umbelliferyl phosphate under a UV light indicates the presence of AP. Large areas of fabric can be screened by pressing the garment or bed sheet against an equal sized piece of moistened filter paper and then subjecting the filter paper to the presumptive tests. Systematic
searches may also be performed by carefully examining sections of the garment or bed sheet. Each successive test can then help narrow the precise location of the semen stain (Saferstein 2001).

Prostate specific antigen (PSA) was discovered in the 1970s and shown to have forensic value with the identity of a protein named p30 due to its apparent 30000 molecular weight (Sensabaugh 1978). p30 was initially thought to be unique to seminal fluid although it has been reported at lower levels in breast milk (Yu & Diamandis 1995) and other fluids (Diamandis & Yu 1995). PSA varies in concentration from approximately 300 ng/mL to 4200 ng/mL in semen (Shaler 2002). Seratec (Goettingen, Germany) and Abacus Diagnostics (West Hills, CA) market PSA/p30 test kits that are similar to home-pregnancy tests and which may be used for the forensic identification of semen stains (Hochmeister et al. 1999, Simich et al. 1999).

Laboratory reports where presumptive tests for semen were performed may indicate that an item was found to be “AP positive” or “p30 positive”—in other words, semen was detected implying some form of sexual contact on the evidentiary item.

**Direct Observation of Sperm**

Most forensic laboratories like to observe spermatozoa as part of confirming the presence of semen in an evidentiary sample (note that in Table 1.2, 42 out of 42 labs confirm semen). A common method of doing this is to recover dried semen evidence from fabric or on human skin with a deionized water-moistened swab. A portion of the recovered cells are then placed onto a microscope slide and fixed to the slide with heat. The immobilized cells are stained with a “Christmas Tree” stain consisting of aluminum sulfate, nuclear Fast Red, picric acid, and indigo carmine (Shaler 2002). The stained slide is then examined under a light microscope for sperm cells with their characteristic head and long tail. The Christmas Tree stain marks the anterior sperm heads light red or pink, the posterior heads dark red, the spermatozoa’s mid-piece blue, and the tails stain yellowish green (Shaler 2002).

Professor John Herr at the University of Virginia developed several “sperm paints” to fluorescently label the head and tail portions of spermatozoa with antibodies specific to sperm and thus make it easier to observe sperm cells in the presence of excess female epithelial cells (Herr 2007).

Independent Forensics’ SPERM HY-LITER PLUS kit enables detection of even a single human sperm head in the presence of an overwhelming amount of epithelial cells. Development of sample characterization tools that utilize fluorescently tagged monoclonal antibodies, such as the SPERM HY-LITER kit, represents a major advancement and should enable much faster and accurate processing of sexual assault evidence.

**Body Fluid Identification with RNA Testing**

Another method for body fluid identification that has seen recent research activity is the monitoring of cell-specific gene expression through the analysis of ribonucleic acid (RNA). Erin Hanson and Jack Ballantyne from the University of Central Florida published a thorough review of RNA profiling efforts for body fluid identification (Hanson & Ballantyne 2005).
They note that conventional methods for body fluid identification often involve labor-intensive, diverse approaches that are performed sequentially rather than simultaneously. Both time and sample are lost when many of these older characterization assays are performed.

As can be seen by reviewing the previous sections and Table 1.2, there are different presumptive and confirmatory tests for each type of body fluid. Some of the current tests have cross-reactivity with other species and most are not specific to a tissue or fluid. In addition, because these traditional tests can only be performed one at a time, precious evidentiary sample is often consumed and time expended in trying to identify a stain. A method that was both sensitive and specific to multiple body fluids would be a major advance over the conventional approaches now in use.

Different types of cells in our body, such as skin versus semen, contain different collections of mRNA (messenger ribonucleic acid) that are unique to that cell type. Therefore, mRNA profiling offers an opportunity to develop fluid- or tissue-specific assays provided that unique target genes can be found.

Research on RNA techniques has shown that, although less stable than DNA (due to its single-stranded structure and often rapid destruction from digesting enzymes), RNA is useful for stain identification (Juusola & Ballantyne 2003). Work with RNA requires modified extraction protocols in order to co-extract RNA and DNA (Alvarez et al. 2004). The RNA can then be used for body fluid ID while the DNA can be PCR-amplified for DNA typing purposes (Figure 1.3).

Multiple RNA transcripts have been detected with reverse-transcriptase-PCR followed by gel or capillary electrophoresis (Juusola & Ballantyne 2007, Haas et al. 2009, Fleming & Harbison 2010) or real-time PCR (Noreault-Conti & Buel 2007, Haas et al. 2009). Blood, semen, saliva, menstrual blood, and vaginal secretions have been simultaneously identified with some of these assays (Haas et al. 2009, Fleming & Harbison 2010). mRNA markers for blood and saliva have provided successful results on 16-year-old stains (Zubakov et al.
At least nine different research groups have identified candidate gene targets for the various body fluids of forensic interest (Table 1.3) and developed tissue-specific mRNA assays in order to create a comprehensive approach to body fluid identification. These groups include efforts at the University of Central Florida (Orlando, Florida), Erasmus University (Rotterdam, Holland), the University of Bonn (Bonn, Germany), the Institute of Environmental Science and Research (ESR, Auckland, New Zealand), the University of Zurich (Zurich, Switzerland), the Vermont Forensic Laboratory (Waterbury, Vermont), Applied Biosystems (Foster City, CA), Ingenetix Ltd. (Vienna, Austria), and the Japanese National Research Institute of Police Science (Chiba, Japan).

A number of so-called “housekeeping” genes which should be present in every sample, have been included in various assays to act as an RNA positive control. These include β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin conjugating enzyme (UCE), glucose-6-phosphate dehydrogenase (GGPDH), and transcription elongation factor 1α (TEF).

The European DNA Profiling Group (EDNAP) conducted a collaborative exercise with 16 labs examining HBB, SPTB, and PBGD target genes for the identification of blood (Haas et al. 2011). Despite most of the laboratories not having prior RNA experience, 15 of the 16 participating labs produced successful RNA profiles even with 6 different kits for RNA extraction, 7 different reverse transcription kits, 5 different PCR mixes, 7 different thermal cyclers, and 6 different Genetic Analyzers (Haas et al. 2011).

In an effort to find reliable tissue-specific signatures from potentially degraded stains, microRNA (miRNA) targets, which are typically less than 25 nucleotides in length, are being examined (Hanson et al. 2009, Zubakov et al. 2010). Only time will tell how successful research efforts are for identifying and applying RNA or miRNA targets to body fluid identification.

FIGURE 1.4 RNA profiling approaches. The reverse transcriptase step uses gene-specific primers to convert RNA into a double-stranded form (complementary DNA, cDNA) for stability. The PCR step performs exponential amplification of the cDNA targets and can be detected with either real-time PCR (see Chapter 3) or capillary electrophoresis (see Chapter 6).
Attempts to Determine Bloodstain Age

Many times in crime scene reconstruction it would be helpful to know how long a bloodstain has been on a surface. The quantitation of RNA degradation has been used in an effort to determine post-mortem intervals and to determine the age of blood stains (Anderson et al. 2005, Bauer et al. 2003a, Bauer et al. 2003b). A group from The Netherlands has shown that targeting T-cell rearrangements can be used to predict human age from blood with an accuracy of ±9 years (Zubakov et al. 2010). Application of this approach could enable the classification of a sample’s source to an appropriate generation (child vs. adult vs. elderly individual).

Species Identification

Samples being processed as biological evidence may come from non-human sources. For example, in some missing person or disaster victim identification situations, human bones may need to be sorted from non-human bones. As part of sample characterization, species identification may be performed or outsourced to a laboratory that specializes in animal DNA testing (Chapter 16).

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**TABLE 1.3** List of Abbreviations for mRNA Gene Markers Used in Body Fluid Identification. For More Information, See Hanson & Ballantyne (2010) and Associated References.

<table>
<thead>
<tr>
<th>Blood</th>
<th>Semen</th>
<th>Saliva</th>
<th>Vaginal Secretions</th>
<th>Menstrual Blood</th>
<th>Housekeeping</th>
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<tbody>
<tr>
<td>HBB</td>
<td>PRM1</td>
<td>HTN3</td>
<td>MUC4</td>
<td>MMP7</td>
<td>S15</td>
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<tr>
<td>HBA</td>
<td>PRM2</td>
<td>STATH</td>
<td>HBD1</td>
<td>MMP10</td>
<td>β-actin</td>
</tr>
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<td>ALAS2</td>
<td>SEGM1</td>
<td>PRB4</td>
<td>ESR1</td>
<td>MMP11</td>
<td>GAPDH</td>
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<tr>
<td>HMBS</td>
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<td>SPRR3</td>
<td></td>
<td>CK19</td>
<td>TEF</td>
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<tr>
<td>GYPA</td>
<td>TGM4</td>
<td>SPRR1A</td>
<td></td>
<td>PR</td>
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<td>PSA</td>
<td>KRT4</td>
<td></td>
<td></td>
<td>G6PDH</td>
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</tbody>
</table>
CONTAMINATION CONCERNS

Modern DNA testing methods are very sensitive and can be capable of generating DNA profiles from as little as a single cell (see D.N.A. Box 11.3). It is imperative that every precaution be taken to collect samples carefully with appropriate personal protective equipment (Blozis 2010). While a sample may be collected properly from a crime scene, there may be other opportunities for contamination either at the crime scene before the evidence was collected, while the evidentiary item (e.g., a gun) is being handled by examiners in other forensic disciplines—such as fingerprint or firearms examiners—as part of routine casework, or while the sample is being processed in the DNA lab. More on the issues surrounding potential consumable contamination in the DNA lab are covered in Chapter 4.

In the final part of this chapter, we briefly explore two potential concerns with “contamination” before the evidence is collected. The first is commonly referred to as secondary transfer, which is the possibility of individual #1 handling an item and transferring his/her DNA to the item that is then transferred to individual #2 upon their touching the item at a later time. The second concern is the potential for “fake DNA” to be planted at a crime scene to possibly implicate someone else or to confuse investigators.

Impacts of Other Forensic Examinations

Brushes used to dust for fingerprints can cross-contaminate samples if precautions are not taken by the crime scene investigator (van Oorschot et al. 2005). One study found that out of 51 used latent fingerprint brushes tested a full or partial DNA profile was obtained 86% of the time (Proff et al. 2006). Some secondary transfer with contaminated fingerprint brushes was also demonstrated. Thus, changing brushes after investigating crime scenes or a thorough decontamination procedure for brushes after use is recommended (Proff et al. 2006).

Another illustration of potential contamination during other forensic examinations is with firearms examinations, which should be conducted after DNA collection if possible. Alternatively, the firearm examiners need to wear gloves, masks, and other personal protective equipment in order to protect the evidence from contamination. In all cases, it is beneficial to have DNA profiles from latent print and firearms examiners on file as part of the staff elimination database (see Chapter 4). In addition, prior to utilizing chemicals for presumptive testing, it is best to evaluate potential impact on obtaining successful results with downstream DNA testing.

Secondary Transfer Studies

Since DNA results can be successfully obtained from only a few cells (Chapter 11), concerns exist regarding the potential for transfer of cells between an individual and an object or another person, which is commonly referred to as primary transfer. When DNA that has been deposited on an item or a person is, in turn, transferred to another item or person or onto a different place on the same item or person, this is referred to as secondary transfer (Goray et al. 2010a). In other words, can a DNA profile be obtained from collected cells that were transferred through a second contact rather than the primary contact from the original source? As noted by Mariya Goray and colleagues, a biological sample that has been transferred
multiple times, if it can even be detected, will most often appear as a component in a DNA mixture (Goray et al. 2010a).

Studies have shown that the amount of secondary transfer is highly dependent on the surface texture and sample moisture. Porous substances and/or dry samples provided on average less than 0.36% of the original biological material being transferred (Goray et al. 2010a). In this same study, moist samples and non-absorbent surfaces, such as plastic, produced transfer rates of 50% to 95% while moist samples with absorbent surfaces, such as cotton or wool, transferred on average 2%—and only 5% when friction was applied (Goray et al. 2010a). While it has been demonstrated that secondary transfer of DNA can occur, whether or not it is plausible in a particular case will be dependent on a variety of factors including the surface texture and sample moisture (e.g., a fresh bloodstain versus an older bloodstain).

Fake DNA and Sample Authentication

In August 2009, the New York Times and several other news sources ran a story on the potential of DNA evidence being manufactured and planted at crime scenes (Pollack 2009). This story arose because scientists at a company named Nucleix (Tel Aviv, Israel) published an article in Forensic Science International: Genetics where they artificially created a biological sample with a manufactured DNA profile (Frumkin et al. 2010). This work was done under the guise of trying to create a unique service for authenticating natural versus artificial DNA samples via a methylation detection test. Several letters to the editor following this article expressed concern over why this work was performed (Morling et al. 2011, Barash 2011).

As the International Society of Forensic Genetics board members note in their letter to the editor: “In itself, the problem of possible manipulation and questioning the integrity of any forensic evidence is not new, and has always been a consideration both during the investigation and the trial phase” (Morling et al. 2011). They go on to note: “Forensic experts and perpetrators committing crimes are in what could be considered an evolutionary race where criminal minds react to advances in forensic science with remediation such as gloves to not leave fingerprints, condoms to prevent semen evidence, and putting on their victims’ clothes as to not deposit any fibers. Fortunately for all law abiding citizens, not all perpetrators are that organized and of course impulse crimes are not planned at all, so that standard forensic techniques are still a useful tool for processing a crime scene. Scientists need to stay ahead in this race” (Morling et al. 2011). A primary purpose in writing this book is to help forensic DNA scientists do their best in staying ahead of criminals with the new technologies available.

**READING LIST AND INTERNET RESOURCES**

**Overall DNA Testing Process**


**Sample Collection**

1. SAMPLE COLLECTION, STORAGE, AND CHARACTERIZATION


**DNA Sample Sources**


**Biological Evidence at Crime Scenes**


Evidence Collection


Reference Sample Collection


ADVANCED TOPICS IN FORENSIC DNA TYPING: METHODOLOGY

**Sample Storage**


**Sample Characterization**


**Bloodstains**


**Saliva Stains**


1. SAMPLE COLLECTION, STORAGE, AND CHARACTERIZATION


Semen Stains


Sperm and Sperm Detection


Body Fluid Identification with RNA Testing


ADVANCED TOPICS IN FORENSIC DNA TYPING: METHODOLOGY


**miRNA**


**Attempts to Determine Bloodstain Age**


ADVANCED TOPICS IN FORENSIC DNA TYPING: METHODOLOGY


**Potential Impact of Other Forensic Tests**


**Transfer Studies**


**Fake DNA and Sample Authentication**


ADVANCED TOPICS IN FORENSIC DNA TYPING: METHODOLOGY
