



15th International Forensic Science Symposium

Interpol – Lyon

23 - 26 October 2007

Review Papers

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Preface

Interpol recognises advances in scientific methods, and their application for forensic investigation, are occurring on an on going basis. Given the wide scope of scientific disciplines applied to forensic work, it is a challenge for the senior managers of forensic laboratories and forensic services to maintain current knowledge of the research and development which underpin these advances. The purpose of the Interpol International Forensic Science Symposium (**IIFSS**) is to bring together senior managers from member states, and to provide a forum to facilitate:

- a) the presentation of advances made in scientific methods over the previous three (3) years, and to provide a look into future forensic needs and advances;
- b) the exchange of information which will enhance scientific methods in criminal investigation and the administration of justice;
- c) the discussion of problem areas encountered by member states and the possible provision of solutions; and
- d) the exchange and pooling of ideas for future progress.

The symposium *Proceedings* on this CD concentrate on the Review Papers prepared by the Coordinating Laboratories, which highlight and summarise advances in the various evidence types.

The various evidence areas are grouped into five major areas:

❖ Chemical Criminalistics

- ◆ Fibres;
- ◆ Firearms;
- ◆ Toolmarks;
- ◆ Forensic geology; and
- ◆ Paint and glass;

❖ Drugs and Toxicology

- ◆ Drugs; and
- ◆ Toxicology;

❖ **Electronic Evidence**

- ◆ Audio Visual; and
- ◆ Computer forensics;

❖ **Fire, Explosives and Hazardous Materials**

- ◆ Hazardous materials;
- ◆ Environmental forensics
- ◆ Explosives; and
- ◆ Fire;

❖ **Identification Sciences**

- ◆ Biological evidence;
- ◆ Document examination; and
- ◆ Fingerprints.

As well as these main contributions, three thematic panels will be held during this 15th meeting on the topics of *'Back to Basics'*, *'International Assistance and Cooperation'*, and *'DVI and Mass Casualties'*.

IIFSS is only possible with the support of Interpol and the General Secretary, Ronald Noble. Interpol staff coordinated all aspects of Interpol's involvement from the meeting announcements to registration, the meeting venue and publication of the proceedings of the meeting.

To make IIFSS 2007 possible it took significant effort by the Organising Committee, each Coordinating Laboratory and the review paper authors. The Organising Committee especially thanks Mr Simon Dzidrovski from Interpol who acts as the principle contact person for the Organising Committee.

This CD would also not be possible without the work of Dr Niamh Nic Daéid who edits the submitted papers and prepares the proceedings.

Dr James Robertson

Chair

IIFSS Organising Committee

**RECENT PROGRESS IN PROCESSING
BIOLOGICAL EVIDENCE
AND
FORENSIC DNA PROFILING**

- A Review: 2004 to 2007-

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Introduction:

This review focuses on biology evidence screening and advances in DNA profiling technology during the past three years. The principal topics covered are divided into eleven sections: Biology Evidence Screening, Short Tandem Repeats (STR) and Supporting Technologies, MiniSTRs, Y-STRs (Reduced-size STR Amplicons), Mitochondrial DNA (mtDNA), SNPs (Single Nucleotide Polymorphisms), Automated DNA Extraction of Biological Evidence, Low Copy Number (LCN) and Sensitive DNA Detection, Software Assistance for DNA Analysis and Interpretation of STR DNA Evidence, DNA in Mass Disasters and Mass Screening/Kinship Analysis, and New Technologies for DNA and Biology Evidence Processing.

The selection of specific sections to be reviewed was based on a broad literature review of approximately 3,900 references which was further refined into eight categories (Figure 1). Some sections such as STRs were subdivided to cover the prevalence in the literature of specific approaches (low copy number or sensitive detection) or to address the refined use of the technology for a defined purpose (mass disaster and mass screening/kinship analysis). Categories such as biology evidence screening or new technology developments reflect the overall progress that has been made over the past 80 years or the need for better and more refined forensic processes. The lack of published papers on biology screening procedures (less than 5 in the last year) compared to 2,218 papers on some aspect of STR DNA profiling, strongly suggests that advances in basic evidence screening may be lacking or at least in need of refinement. If you cannot find or identify body fluids on evidence it may be difficult to ensure the efficiency of subsequent DNA profiling procedures or to derive a complete understanding of the probative nature of the evidence.

As in any review, bias is a challenge and the selection of papers to be noted will be based on the expertise and the interest of the authors. Other topics that potentially should be covered in future reviews should include X-chromosome markers, animal and plant biological evidence, and treatment of biological evidence following a terrorist event. It is also noted that hair evidence was not assigned a specific section in this review although there have been approximately 53 published articles relating to hair evidence over the past three years. Discussion regarding hair evidence has been noted, where appropriate, in the sections involving Y-STRs, SNPs, miniSTRs and mitochondrial DNA analysis. It is also our understanding based on past reviews for this symposium, that a more detailed review of hair evidence will be addressed as a separate topic.

Biology Evidence Screening

The past 20 years have witnessed tremendous leaps in the scientific advancement of the DNA typing field. The advent of modern DNA technology has resulted in the increased ability to perform human identity testing. Forensic laboratories worldwide have been continually investing significant time and resources to develop an automated DNA processing approach to increase the efficiency of extracting DNA

from a wide variety of evidence (see Automated DNA Extraction of Biological Evidence). With an increase in the automated extraction of DNA, the rate limiting step becomes the front end or identifying and preparing potential biological evidence for extraction, as well as the back end involving the interpretation and reporting of the results. The technological advancements in the field of forensic body fluid identification have not experienced the same priority or enhancements as that noted for the development of DNA profiling technology. While progress has been made in some aspects of biological evidence screening, many of the procedures were initially discovered over two centuries ago and were implemented in the past half century in the clinical field prior to adoption for forensic use.

Biological evidence screening uses either presumptive or confirmatory tests. The most desirable presumptive test is one that is specific, sensitive and will not hamper the extraction or subsequent amplification of DNA. To date, most biological evidence screening methods (previously considered serological tests) are cost effective and rapid but unfortunately the tests are often inefficient and non-specific. This has been an acceptable trade-off until recently, when improvements in the speed and sensitivity of DNA analysis have made this technique amenable to many forensic investigations, and better means for screening exhibits are required to find the potential evidence. In particular many stains that were once too dilute to render a prescreen test result can be processed successfully for a complete DNA STR profile.

It is important for forensic scientists to use the most effective biology screening processes. This will ensure that timely answers can be derived for biological evidence, and if DNA processing is required, triage can occur to ensure the best evidence is processed first. In the course of writing this paper, it became obvious that unlike many disciplines in forensic science that have experienced change over time, very few changes in protocol or advances in technology have been reported for biological evidence screening. To address this lack of published information, a world-wide survey was undertaken to determine what identification methods are currently being used for commonly encountered biological trace evidence types in forensic laboratories. At the time this report was written, responses had been received from 42 laboratories from across Canada, the United States, Australia, the Netherlands, Sweden, the United Kingdom, France, Finland, Singapore, and Japan (Table 1). Some of the findings of our survey will be reported in this paper as well as portions of the literature review conducted on the history of these techniques.

Screening for Blood

The first presumptive catalytic test for blood was successfully developed in 1863 when Christian Freidrich Schonbein carried out an enzyme-reactive test which made use of an erythrocyte catalase that released oxygen from a peroxide solution (1). The mechanism of reaction for all presumptive catalytic tests for blood is based on the peroxidase-like activity of hemoglobin and a number of its derivatives. Depending on the choice of suitable substrate, either a change of colour (i.e. phenolphthalien) or chemoluminescence that is visible in the dark (i.e. Luminol) develops (2, 3). Peroxide mediated oxidation reactions include Tetramethylbenzidine (Hemastix®, Bayer

Corporation, Elkhart IN, USA), Phenolphthalein (Kastle-Meyer), Leucomalachite Green, MacPhail's Reagent (Hemident™, Lightning Powder Company Inc., Jacksonville FL, USA), O-tolidine, Benzidine, Fluorescein, Luminol, Bluestar® (Bluestar, Monte-Carlo Monaco). All of the tests listed are highly sensitive to a minute trace of hemoglobin and its derivatives, but all suffer interference by other materials such as substrates found in fruits and vegetables that perform a similar function (peroxidase-like activity). In other words, these tests are not specific, as other substances may cause a positive reaction to occur. Therefore, a positive test can only lend inference that there is evidence of blood, and hence indicate areas that need further testing (Figure 2).

In a recent study conducted by Tobe, Watson, and NicDaeid, (4) six presumptive tests for blood (Luminol, Leucomalachite Green, Kastle-Meyer, Hemastix®, Hemident™, and Bluestar®) were evaluated for their specificity, sensitivity, and effect on high molecular weight DNA and resultant profile generation. Leucomalachite Green was found to be 10 times less sensitive ($1:10^3$ dilution) than all other tests and it destroyed the DNA resulting in no amplification product. The Kastle-Meyer test was determined to be extremely non-specific and it was noted that the amount of recoverable DNA was greatly reduced after application of the reagent. Hemastix® was non-specific showing cross-reactivity with a number of substrates while Hemident^J was reportedly specific but destroyed DNA evidence and therefore cannot be used directly on samples going forward for subsequent DNA analysis. Bluestar® was determined to be not specific as it displayed cross-reactivity with substances tested (4).

It was ascertained that Luminol was the best overall presumptive test. It had the greatest sensitivity ($1:10^4$ dilution), blood specificity and it did not destroy the DNA. Luminol also offers the advantage of being able to be reapplied provided this is done in complete darkness. The sensitivity of Luminol compared to other procedures was also reported by Webb, Creamer, and Quickenden in 2005 (5). The Kastle-Meyer test, Leucomalachite Green, Luminol, Hemastix®, and a forensic light source (Polilight®, Rofin Australia Pty Ltd., Dingley Victoria, Australia) were again compared for their sensitivity, ease of use and safety.

Since the early 20th century, crystal tests have been considered the only unequivocal method for the determination of blood. The crystal test derives its name from the positive reaction that manifests as a precipitation of a characteristic crystal form or habit which can be recognized under the microscope. Crystal tests are based primarily on the detection of heme and its derivatives, and the most commonly used are the Teichmann and Takayama test. The Teichmann test, developed in 1853, is based on the hematin hydrochloride derivative, and pyridine (or any other similar nitrogenous base) is the basis of the Takayama test first reported in 1912 (2, 6, 7). Crystal tests, while specific for blood, provide no information regarding its species of origin, and it can often be difficult to generate the formation of crystals with old blood samples. Therefore it is important to note that the failure to obtain a positive result with crystal tests does not necessarily indicate the absence of blood.

In 1901, a German hygienist named Uhlenhuth successfully differentiated between animal and human blood using an immunological procedure (1). Prior to this, a stain recognized as blood could not be identified by its specific species. The binding of an antibody to an antigen is the fundamental reaction of immunology which forms complexes that become insoluble and precipitate from solution. The serological techniques available for species identification of bloodstains and tissue all rely on the specificity of the antiserum. In other words, they rely on the ability of an antibody to distinguish between the serum proteins (or antigens) of different species.

In our survey, all of the forty-two laboratories employed some form of a presumptive screening test for blood and the Kastle-Meyer was the most commonly used. Over half (59%) of the laboratories that responded to the survey do not employ a test to confirm the presence of blood and of the laboratories that do, it was determined that the Hemochromogen or the Takayama test was the most commonly used. Of the labs that responded to the survey, 86% have a species identification method in place and Hematrace was the most frequently used (Table 2).

Screening for Semen

In 1826 and 1827, d'Angers, Barruel, and Orfila were the first scientists to make a systematic effort in the use of chemical tests for the presumptive identification of seminal fluid (3). Historically, the tests devised for identifying semen were based on the appearance of the stains, changes in colour and consistency upon heating and immersion in water, odour emitted by the moistened stain, and the behaviour of the aqueous extract toward a number of reagents and treatments (Figure 3).

Analysis of suspected seminal stains is usually initiated by carrying out presumptive biochemical tests for substances characteristically, but not uniquely, found in this body fluid. The acid phosphatase enzyme is present in high concentrations in semen, and it chemically reacts to cleave a variety of organic phosphates including p-nitrophenyl-, alpha-naphthyl-, and thymolphthalein monophosphates (7, 8). An important consideration is the usefulness of this test for cases involving oligospermic, azoospermic, and vasectomized individuals (9). In 1945, Lundquist first developed a method to identify acid phosphatase which involved the use of alpha-naphthylphosphate substrates (9). In the presence of acid phosphatase, the substrate releases the naphthol which, when liberated, couples with a diazonium compound to form an insoluble coloured product. This test (i.e. Fast Blue) is simple with a sensitivity documented as 1:100 dilution of semen stains (3, 9, 10). As with all presumptive tests, it demonstrates cross-reactivity with other substrates. This test is driven by an enzyme which under certain environmental conditions (exposure to mold, putrefaction, chemicals, or heat) can cause inactivation and may subsequently prevent detection.

The prostate-specific antigen (PSA) was first reported in the early 1970's but not characterized until 1978 by Sensabaugh *et al* when they determined the molecular weight of the protein to be 30,000 daltons hence coining the name p30 (9). Graves and Sensabaugh were able to demonstrate the prostate antigen's reliability as a forensic marker and developed the first immunometric assay for p30 (9, 11). Similar to

acid phosphatase, histological studies have shown that p30 in semen is produced in the epithelial cells that line the ductal elements of the prostate and therefore the presence of oligospermic or azospermic individuals as well as those who have had vasectomy will have no effect on the detection of seminal fluid (which contains both AP and p30) (9).

Clinical scientists found that elevated levels of p30 in blood could be used as a marker for prostate cancer, and more recently it has been identified as a marker for breast cancer. The Immunchromatographic PSA or p30 membrane test kits were originally developed for clinical use as an aid in the diagnosis of prostate cancer and through many evaluations the test kits have been found to be well suited for forensic application (11, 12). Two commercially produced kits are currently available to the forensic and clinical community, the One-Step ABACard® p30 Test (Abacus Diagnostics, West Hills, CA, USA) and the BioSign™ p30 Membrane Test kits (Princeton BioMeditech Corp, Princeton, NJ, USA). Both kits render sensitive detection with documented detection limits of 4ng/mL or 1:10⁵ dilutions of p30 (11-13). The main advantage noted over other conventional methods of p30 detection (i.e. counterimmunoelectrophoresis and ELISA) is that the membrane tests are less cumbersome and less time consuming. However, a noted limitation with the membrane tests is that, due to their high sensitivity, cross-reactivity can occur with samples containing male urine (11). The BioSigns membrane kit also appears to cross-react with condoms containing nonoxynol-9 (12).

Although, spermatozoa were first described by Ham in 1677, it was not until many years later that spermatozoa were associated to seminal fluid (3). While identification of sperm cells in a stain is not the oldest method for the medico-legal identification of seminal stains, many still believe it is the most reliable. Over the last half century, numerous microscopic techniques have been developed. As well, a large variety of biological stains have also been recommended to facilitate detection of sperm cells during microscopic examinations.

The Christmas Tree stain is one of the most commonly employed staining techniques in forensic laboratories and provides excellent morphological detail and good discrimination between sperm cells and epithelial cells. This is particularly important when determining cell types in challenging or dirty samples. Gaensslen recommended the Christmas Tree stain for forensic screening due to its ease of use, excellent cellular detection, and lack of interference with DNA typing post-staining (9, 14).

Many recent advances in microscopic instrumentation have allowed new methods of spermatozoa detection and separation to be introduced. Laser microdissection (LMD) enables the direct physical selection and separation of the target cells (namely spermatozoa) from a complex mixture by way of microscopic detection and excision with a laser. As noted by Sanders, Sanchez, Ballantyne, and Peterson (15) the microscope slide may be stained using a histological stain for increased discrimination between spermatozoa and epithelial cells. In this study, it was also determined that the LMD technique is able to collect pure populations of sperm with no apparent cross-contamination, therefore simplifying the extraction of DNA from sperm. A

minimum of 30-50 spermatozoa could be captured to yield a discrete, highly polymorphic DNA profile.

Sanders, Sanchez, Ballantyne, and Peterson (15) also evaluated five different histological stains for practical use in conjunction with the LMD for ease of visualization. The histological stains tested in this study were the Christmas Tree, Hematoxylin & Eosin, Methyl Green, Wright's, and acridine orange staining techniques. All stained specimens showed a significant decrease in total Relative Fluorescent Unit (RFU) values as compared to the unstained specimens. Though the Christmas Tree stain provided excellent discrimination, the resulting DNA profiles demonstrated poor signal intensity and a high signal to noise ratio. It was hypothesized that the loss of signal intensity was due to picric acid depurination damage to the DNA. In addition, indigo carmine (used as the counterstain) commonly used in the textile industry for dyeing of denim, is a known PCR inhibitor. It was determined that the Hematoxylin & Eosin stain was the best overall histological stain evaluated in this study (15).

In our survey, all of the forty-two laboratories employed some form of a presumptive screening test for semen and the alpha-naphthoylphosphate (i.e. Fast Blue) technique was the most commonly used. All of the laboratories confirm the presence of semen in some way, with the microscopic examination coupled with Christmas Tree staining for the presence of spermatozoa as the most commonly used technique. Interestingly, while a few laboratories (19%) employ PSA testing as a screening method, others (71%) consider a positive PSA result as a confirmatory method of detection. (Table 3)

Other Body Fluids Testing Techniques

The importance of studying other sources of biological evidence including hair, saliva, urine and feces, has been recognized by forensic scientists for many years (Figure 4). In 1928, Mueller first suggested the detection of amylase as a basis for identifying salivary stains (3). Saliva contains a high level of alpha-amylase, the enzyme responsible for the digestion of starch and the main salivary marker in forensics. In humans, there are two types of alpha-amylase: salivary and pancreatic. Nickolls was the first to publish a protocol for the analysis of saliva stains using the classic starch iodine reaction (3, 9).

Saliva testing was further advanced by Willot (16) when they first used starch substrates for the amylase assay and the identification of saliva. Phadebas⁷ Amylase Test Tablets (Pharmacia Diagnostics, Uppsala, Sweden) was first designed for a clinical setting to identify and quantitate elevated levels of amylase found in such instances as cases of mumps, hepatic disorders, peritonitis, and pancreatic carcinomas (3, 17). The principle matrix for the Phadebas[®] test tablet is a substrate which is a water-insoluble cross-linked starch polymer carrying a blue dye. If amylase is present in a sample, the substrate will be hydrolysed to a water-soluble starch carrying the blue dye in solution. A stain containing less than 0.02U/L amylase will not be detected by this test (16, 18).

Recently, Barni *et al* (19) reported on the usefulness of a technique known as the Amylase test which is specific for the detection of alpha-amylase (the main salivary marker). The Amylase test is a colorimetric assay based on the absorbance of 2-chloro-nitrophenol that can be measured at 450 nm. The rate of its release is kinetically measured by an increase in colour and is directly proportional to the alpha-amylase activity in the sample. The test can be performed directly on a biological fluid or on a stain to ascertain the presence of saliva in mixtures with perspiration or urine. Strong differences in alpha-amylase activity, notably an increase in the speed of the reaction, were reported depending on the presence or absence of saliva. It was thought that the alpha-amylase kinetic differences among biological fluids was due to the considerably higher concentration of alpha-amylase in saliva compared to the isoforms found in other tissues such as the pancreas (which also has different kinetic properties).

In our survey, 83% of the laboratories that responded indicated that they employ a technique to screen for saliva, with the Phadebas⁷ being the most commonly employed method. Of the laboratories that presumptively test for saliva, 20% employ a confirmatory method with the indication of the presence of high levels of amylase being the most common (Table 4).

For many years, the reaction between Lugol's iodine, developed in 1924, and vaginal epithelial cells was considered specific (20). The nucleated squamous epithelial cells of vaginal origin contain glycogen which will be stained brown by the iodine, making them easily viewed microscopically (9). However, one of the limitations of this technique is the fact that the nucleated squamous epithelial cells lining the urethral opening of the penis, the mouth, as well as the anus also contain glycogen in lower levels which can be detected with Lugol's solution (9). Interpretation of Lugol-positive samples must be done with caution. When searching for potential vaginal fluid on an exhibit, in the instances of sexual assault cases, the exhibits submitted or swabbed have the potential to be contaminated with nucleated squamous epithelial cells from the mouth or penis (i.e. penile swab) and therefore may be misleading if a positive result is viewed (9).

Paterson *et al* (21) determined the potential usefulness of a specific immunohistochemical technique for staining vaginal epithelial cells. Immunohistochemistry (IHC) is a technique that can be used to detect differential antigen expression between epithelial cells from different body sites/sources. Paterson *et al* (21) used this technique to investigate several proteins that may identify a marker unique for vaginal epithelial cells as well as further examined the expression of several proteins that may be suitable for distinguishing between buccal, and skin epithelial cells for forensic casework. However, they were not able to identify antibodies which could uniquely identify vaginal cells.

In our survey, 50% of the laboratories that responded do not test for vaginal fluid. Of the 50% that do screen for vaginal fluid, the use of an alternate light source to identify areas of interest was the most commonly employed method, followed by staining with Lugol's Iodine (Table 5).

In 1948 Cook suggested the first test for urine which detects urea using the enzyme urease (3). In the presence of urease, urea breaks down to produce ammonia which can be detected using the acid-base indicator bromothymol blue. The test is conducted with test paper impregnated with urease and bromothymol blue or in an agar gel in which the reagents have been incorporated. A bright blue colour is indicative of a positive reaction (22).

In 1886 German Biochemist Jaffe developed a presumptive test for the detection of creatinine in urine (3). The test is based on the fact that picric acid will react with creatinine in the presence of a weak base to form a deep orange-red colour. This method is not specific for creatinine as it will detect other chromogens (7, 23). In the late 1960's and early 70's, another presumptive test strip called Azostix® (Bayer Corporation, Elkhart IN, USA) for urea nitrogen in whole blood was developed (24). The test strip was originally developed for clinical use but functioned on the same principle as suggested by Cook and therefore was later successfully incorporated into forensic testing for urine.

In our survey, 40% of the laboratories that responded do not have a method in place to test for urine. Of the 60% of laboratories that do, the majority screen for the presence of urine using either the Jaffe Colour Test for Creatine, or the urease test (i.e. Azostix®) for urea. Of interest, one laboratory indicated the use of the Body Fluids Identification Services - Urine kit (BFID-Urine, Independent Forensics, Hillside, IL, USA) as a means to confirm the presence of urine (Table 5).

The identification of fecal matter was first performed in 1860 (9). In this technique, a fecal sample was viewed with a bright field microscope and a histological stain such as the Christmas Tree stain. While microscopic examinations are still carried out, most laboratories employ the Edelman's test which was first devised for the clinical examination of both urine and fecal material, but was adapted for forensic use in the detection of fecal stains where only a small amount of material may be present (25). The Edelman Test relies on the formation of a green fluorescent zinc-urobilin complex. The resultant apple-green fluorescent supernatant is indicative of the presence of feces (viewed under UV light). It has been documented that the estimated limit of visual detection of urobilinoid fluorescence is in the range of 10 to 100 mg (24, 25).

In a study conducted by Lloyd and Weston (25), the fluorescent characteristics of fecal material are problematic and may be obscured by other bilirubin derivatives and extraneous substances. The Edelman's test may cross-react with substances such as urine, vomit and feces from various animals. A negative result or an absence of fluorescence may also occur with infants under the age of six months as well as individuals with abnormal liver function (24, 26).

In our survey, 52% of the laboratories that responded do not test for the presence of fecal material. Of the 48% that do screen for fecal material, the Urobilinogen Test or Edelman's Test was the most commonly employed. No confirmatory test was identified in this study (Table 5).

Since the 1600's, extensive microscopic hair examinations have been carried out with detailed descriptions of morphology and structure (27). Variation can be expected to be found between populations, between two individuals, as well as morphological variation within one individual. It was not until the 20th century that forensic scientists applied microscopic techniques and comparisons for the identification and association of hair evidence (27). Typically, today's forensic laboratory conducts hair examinations to determine the suitability of the hair for DNA analysis and whether the origin is human or another species. If the hair is determined to be of human origin, it may also be important to determine the body area of origin as well as racial origin of the hair.

In our survey, 95% of the laboratories that responded do have a hair assessment technique in place. Of the laboratories conducting hair comparisons, approximately 85% do a microscopic examination to screen for species and suitability while 35% of the laboratories that responded perform hair comparisons (Table 5).

DNA typing technology has become more sensitive, and yet the testing techniques used for screening and identification of biological fluids have not experienced the same increase in the levels of detection. Many dilute stains may yield a DNA result using today's technology but fail to react with a confirmatory or even presumptive body fluids test result. Many of the laboratories which responded to the survey indicate that they rely on DNA techniques for confirmation of a specific body fluid type as well as species identification as the DNA typing procedure is higher primate specific and sensitive to trace amounts of DNA. Essentially, the laboratories demonstrate a greater reliance on the more sensitive, less specific screening tests without confirmation prior to recommending further DNA testing. This essentially leaves the specificity, and in some instances, the sensitivity, up to the DNA typing process.

New techniques have been proposed which could positively identify human body fluids prior to DNA analysis. Juusola and Ballantyne reported in 2003 (28) and in 2005 (29) on a method to replace current conventional methodologies of body fluids identification. The authors developed a multiplex reverse transcription-polymerase chain reaction (RT-PCR) method for the definitive identification of body fluids commonly encountered in forensic casework such as blood, semen, saliva, and vaginal fluid. While DNA of all tissues in an individual are essentially identical, each tissue type is comprised of unique messenger RNA (mRNA) or gene expression. The methodology is based upon gene-expression profiling analysis in which body fluid specific genes are identified by detecting the presence of appropriate mRNA species through an mRNA-based RT-PCR multiplex followed by capillary electrophoresis approach. Therefore, analysis of the RNA profile in a sample may uniquely identify the fluids of interest. Finally, Juusola and Ballantyne (28, 29) stated that this approach could, in the future, allow for facilitated identification of unique tissue components in body fluid stains and essentially replace the technologically diverse serological and biochemical testing techniques used today.

Recent research conducted in biological markers for time of death as well as physical traits have opened up an entirely new area of forensic science that only a few researchers had worked on in the past. With the advent of rapid nanotechnology-based multiplex analysis or “biochips”, the potential for screening many more DNA or RNA markers following a single tissue extraction opens up the realm of forensic investigative leads with minimal additional laboratory work. Heinrich and coworkers (30) have attempted to use the relative quantitation of mRNA with real-time reverse transcription (RT-PCR) to measure gene expression in ubiquitously expressed genes from postmortem tissues. The concept was to understand the stability of tissues at different postmortem intervals in order to potentially estimate time of death. Unfortunately the authors noted that the five genes studied had a complex mRNA degradation process such that a single gene expression measurement for each tissue would lead to erroneous interpretation regarding time of death. Similar results were also reported in earlier studies by Bauer (31, 32) which suggested that additional work will be required but the potential determination of time interval since death may be part of future forensic investigative technologies. More importantly these and other studies by Ballantyne have reversed much of our understanding regarding the rapid degradation of RNA and the longevity of RNA left at the crime scene. Research into physical trait markers such as human pigmentation, hair colour, eye colour and other “Ancestry Informative Markers” (AIMs) will open up potential forensic investigative leads (33, see also SNPs in this paper) but also presents potential ethical dilemma and individual privacy concerns.

After several decades of using older evidence screening procedures to identify biology tissues from the crime scene, recent advances appear to have finally evolved to the point of offering promising new approaches to solve old questions, as well as enabling the rapid identification and staging of evidence for more complex and costly analysis.

STRs and Supporting Technologies

Most forensic laboratories have been using STRs and multiplex systems for over a decade and the literature has documented many case studies, population frequency reports, protocols to affect more sensitive detection of degraded DNA, and interpretation recommendations for mixed DNA profiles. In the past four years there have been more than 2,200 publications involving some aspect of forensic STR analysis. Three areas of considerable interest have been the review, concordance and evaluation studies of new commercial STR multiplex systems, development of better quantitation protocols to estimate recovery of DNA from biological exhibits, and papers documenting the validation undertaken to replace gel based fluorescent platforms by the automated capillary electrophoresis sequencers. Although a broad review on all STR related DNA articles over the past three years would be beyond the scope of our report, a concise history of STR analysis has been written by Butler (34) which supplements his excellent reference text (35).

In 2004, both commercially available STR kits, Powerplex® (Promega) and AmpF/STR® Identifiler® (Applied Biosystems), were validated according to the Scientific Working Group on DNA Analysis Methods (SWGDM) guidelines by

Greenspoon (36) and Collins (37) respectively. Both systems contain 15 STR loci plus amelogenin and in general provide concordant results between systems and with previous results derived from older multiplex kits run on different platforms. Some minor variation has been noted in allele detection and sensitivity but this is considered acceptable to the forensic community. Efforts to enhance the success of PCR multiplex systems with challenging samples have involved both the addition of new STR loci (35, also see miniplex STRs in this paper) as well as recommendations for optimizing all PCR parameters. A collaborative exercise involving degraded DNA was carried out with 38 forensic laboratories from 17 European countries (38) and identified common problems such as allele dropout, artefact signals and peak imbalance. Recommendations regarding the amount of template DNA, PCR cycles and Taq polymerase concentration were derived from this study. We are reminded by McNevin and colleagues (39) that new technologies and scientific enhancements can achieve much better success with a good basic understanding of the biology and chemical characteristics of challenging samples such as keratinised hair.

The main intention of forensic DNA quantitation is to determine the amount of extracted DNA recovered from evidence in order to make key decisions for subsequent multiplex STR analysis or the potential use of more specialized DNA technologies such as mtDNA or Y-STRs. Quantitation enables the best use of evidence and helps ensure that the optimal amount of DNA is amplified in order to achieve interpretable DNA results based on the validation and accepted protocols developed by each specific laboratory. Original quantitation methods such as slot blot hybridization using a human/primate specific DNA probe were labour intensive, time-consuming, could potentially lead to an underestimation of nuclear DNA in degraded samples, and provides no predictive information on potential PCR inhibitors. In recent years the application of real-time quantitative PCR (qPCR) has become the method of choice for quantitation because it allows for reproducible quantitation of DNA that is rapid and sensitive. In the past three years several quantitation methods have been published (Table 6) primarily based on qPCR (TaqMan[®] or Molecular Beacon probes) using commercially available kits or internally validated quantitation DNA markers. The original quantitation assays used single markers to measure either total genomic DNA, male or mtDNA (40, 41). Significant efforts have been made to develop multiplex quantitation assays that can measure male DNA and total genomic DNA or even allow for mtDNA quantitation at the same time. This approach saves time plus conserves potentially limiting biological evidence (Table 6). In addition, specialized quantitation assays have been developed based on smaller amplicons that should enable the forensic scientist to estimate the amount of nuclear DNA from degraded samples (42). Alonso (43) and von Wurmb-Schwark (44) have developed specific assays to estimate nuclear and mtDNA from forensic samples as well as ancient remains and anthropological samples.

The traditional slab gel electrophoresis platforms used to separate the different STR fragment lengths, have been gradually replaced by capillary electrophoresis (CE) systems such as the Applied Biosystems Prism 310, 3100 and 3700 genetic analyzers and the Amersham MegaBACE 1000 series instrument. Effort has been made by many forensic laboratories to ensure quality and accuracy of STR results, and many

validations, as well as concordance studies, have been recently published. The Forensic Science Services (45) have described an extensive series of validation experiments that should be carried out prior to introduction of a CE technology or even a specific model of an instrument. The three capillary array instruments tested, had specific characteristics with respect to resolution, cross talk between lanes, precision, as well as time required for analysis and maintenance. The absence of errors in the extensive concordance studies carried out with the AB 3100 and AB3700 has allowed the FSS to introduce these instruments into their system. Butler et al. (46) in a comprehensive review have also described sample preparation, injection, separation, detection and interpretation of STR results on CE instruments and the importance of run time balanced with temperature control. Both of these forensic CE validation studies are an interesting contrast to the different characteristics and parameters evaluated for clinical diagnostic laboratories (47). It is important to note that many of the variations that may be attributable to CE instrumentation may in fact be intrinsic to the specific STR kit being run. Duewer (48) and coworkers demonstrated from data received from more than 60 forensics laboratories, that seven out of eight commercially available STR multiplex kits had similar patterns of intensity differences using a variety of measurement platforms. Although all STR multiplex kits provided fit-for-purpose-results, minimum and maximum absolute signal intensities for specific STR loci varied and demonstrated a systematic decline in signal height with increasing allele size. Understanding these potential differences is important when comparing intra-laboratory results.

MiniSTRs

Although STRs are highly polymorphic and capable of generating typing results from very small amounts of samples through a multiplex reaction, highly degraded samples that have been significantly impacted by the environment remain a challenge. A direct relationship between amplification efficiency and amplicon size has been clearly demonstrated by Bender (49) and Chung (50). Specific interest has been focused on difficult and challenging samples such as telogen hairs found at the crime scene from either the putative offender or the victim or samples exposed to heat, physical or chemical trauma (post-terrorist events, mass disasters man-made and otherwise). Current commercial kits experience signal loss due to lack of amplification of the larger STR fragments. The large amplicons often have lower sensitivity and fall below the detection threshold and this phenomenon has been referred to by some authors as a “decay curve” of sensitivity (51-53) and presumably was not taken into account in the optimal primer selection for such polymorphic and important loci such as FGA and D21S11.

Original work of Brinkman and coworkers (54) demonstrated that STR results can be obtained with relatively short fragments of template DNA provided that amplification systems are optimized and in some instances on a single locus amplified one at a time. The basic primer criterion of avoiding secondary structures such as hairpin loops and selection of primers with nearly identical T_m values was explored in the creation of an early miniplex system that amplified both autosomal as well as Y-STR loci (55).

A newly designed primer set for shorter amplicon sizes in which the primers are positioned as close as possible to the repeat structure will have significant amplification advantages. The utility for amplification success on degraded forensic samples was described either one locus at a time (56, 57) or in small multiplexes comprising several loci together (53, 55). It is also important to recognize that new protocols which generate results from smaller versions of the current commercial megaplex STR systems should be compatible with the DNA profiling results contained in large criminal offender databases as well as potential crime scene evidence developed. Any future development and implementation of new STR systems must involve compatibility, and in order to demonstrate this prime characteristic, concordance studies must be carried out. In 2006, a report by the European Network of Forensic Science Institutes (ENFSI) and European DNA Profiling group (EDNAP) (58) recommended that standardization within Europe should take into account the use of miniSTR systems and their utility for obtaining results from badly degraded samples. Further they suggest that, current multiplex STR systems in current use should be re-engineered to enable small amplicon detection.

Size reduction of STR markers such as the recently released AMPF/STR® MiniFiler™ polymerase chain reaction kit developed by Applied Biosystems enables size reduction on eight of the larger STR loci amplified in the commercially available Identifiler® kit (Applied Biosystems). The new minifiler kit amplifies CSFIPO, FGA, D2S1338, D7S820, D13S317, D16S539, D18S51, and D21S11 as well as the sex typing locus, amelogenin. The new commercial miniplex also includes the three loci D2S441, D10S1248 and D22S1045 selected by ENFSI and EDNAP working groups for further STR standardization (58). Excellent concordance (99.7%) was demonstrated by Hill and coworkers (59) between the typing results developed from Identifiler® and the MiniFiler™ for 1308 samples. The 27 differences noted in the results from both kits were confirmed by re-amplification and were either attributed to primer differences that revealed genetic variations which caused allele dropout or an apparent repeat unit shift.

The concept of miniSTRs or “Miniplexes” was discussed in original studies by Butler and coworkers (53) and the primer sequences were designed to be compatible with Laser-Ablation-Inductively-Coupled-Plasma Mass Spectrometry (LA-ICP-MS) (60, 61) but subsequently modified to run on standard commercial capillary electrophoresis sequencers. The concept of amplification efficiency by targeting smaller amplicons must be balanced with template concentration in order to achieve optimal signal intensity and peak balance ratios for all the loci found in a multiplex system. The same holds true for miniplex systems and a comprehensive study comparing different sets of miniplexes (five sets with 3 loci per set) demonstrated concordance with commercial STR kits and had favorable peak balance and sensitivity on artificially degraded DNA samples as well as two human skeletal remain samples (50). It was noted, that in comparison studies with standard STR kits, signal intensity could be compensated for a certain extent with larger alleles in some loci that dropped out (FGA) by increasing template concentration. This is not always possible when samples are limited and DNA template recovery can be very low. Sensitivity varied

between 125-250 pg to achieve good peak balance between the different STR Miniplexes tested and overall demonstrated the need to ensure that the heterozygous peak balance should not be sacrificed in favor of sensitivity by the addition in the number of cycles in the amplification protocol (50) which primarily affects the smaller amplicons. Additional studies were conducted on these miniplex systems and reported by Coble and Butler (62). Corrections for the reported nomenclature and allele range reported in subsequent reports (63) for some loci are not entirely unexpected considering the significant numbers of laboratories currently involved in the exploration and implementation of miniplex systems.

Initially, several forensic groups explored options of reduced primer size to favorably influence amplification efficiency using conventional PCR systems (64-66). More recently, Meissner and coworkers (67) specifically targeted four STRs that are not included in the commercially released AmpFISTR® MiniFiler™ kit, and developed a miniplex which also contained the gender discrimination locus, amelogenin. The Tho1, vWA, D3S1358 and D8S1179 primer sets were constructed with a 10bp gap between the smallest common allele of one locus and the largest of another locus. The authors compared their miniplex to commercially available Powerplex 16™ and SGMplu™ and found concordance with 200 typed individuals, and overall the most problematic samples such as telogen shed hairs, formalin-embedded tissues, and bone buried for prolonged periods of time yielded full profiles when conventional STR systems failed. It is noted that many labs will be developing specific sets of multiplex systems for degraded and environmentally compromised samples. Although discrimination potential is low ($1:3.16 \times 10^4$) (67), the ability to obtain a result when other systems fail could be the difference between excluding or confirming a match based on the reality of the circumstances. What presumably could be more problematic in the long run is lack of standardization amongst different laboratories and the potential for developing systems which may never be commercially available due to competitive development of other markers, or patent safeguards and legal ramifications.

Grubwieser and coworkers (68) have also developed and reported success on a miniSTR-multiplex and their system and three of the five loci which are included in the new commercially available miniplex system from Applied Biosystems. Bender and Schneider (69) reported on the development of an 11-plex miniplex system with amplicon sizes smaller than 270 bp and its application for casework. It was noted that this laboratory used a novel approach for detecting their results by separating the miniplex amplicons into two categories through the use of biotin labeled primers (for one set) and streptavidin-coated Sepharose beads to capture 5 of the 11 STR systems and then running both assays separately on a capillary electrophoresis unit. Although considered more time consuming than one single run, the rationale was to conserve extremely limited amounts of forensic evidence and maximize the STR results. This 11-plex system did experience a low level of allele dropout (0.4%) when less than 100 pg of template was amplified. In most instances, significant loss of alleles occurred with 6.25-12.5 pg total DNA template.

A very balanced review of the advantages and disadvantages of using miniplex system is discussed in the context of high volume casework on degraded skeletal remains (70). Parsons and co-workers (70) have evaluated three short amplicon STR multiplexes (7-plex, 6-plex, and 5-plex) which average a 144 bp decrease in target loci, and proved useful for a more rapid resolution of co-mingled remains and partial skeletal identifications from mass graves. The authors identify inconsistencies in D18S51 and D8S1179 between profiles developed from family reference samples using commercially available STR kits and their miniplex systems results from degraded bone samples. In most circumstances the miniplex systems yielded better results and more complete profiles in 15-20% of the difficult cases, but many factors affect the utility of these systems. Factors such as the number of loci included in a kit is severely limited because some amplicons cannot be easily separated in the size range required and the sequence complexity of bacterial and fungal DNA may reduce PCR efficiency and give rise to artifactual results. Their conclusion notes "on balance, in our experience the circumstances under which the miniplexes provide substantial additional capability in recovering genetic data is rather narrow. Our success rate for typical samples is high enough that the advantages of standard commercial kits rule the day". They also point out that better extraction procedures optimized for difficult samples such as bone, would lead to significant improvements in amplification and subsequent DNA typing.

A comprehensive review of miniplex systems which will be updated in the early summer of 2007 can be found at: www.cstl.nist.gov/biotech/strbase/miniSTR.htm.

Y-STRs

Although autosomal STR loci are the most widely used DNA markers for human genetic testing and forensic DNA analysis, polymorphic STR regions located on the Y chromosome (Y-STRs) have become widely used for tracing historical human migration patterns (71) and genetic genealogy (72, 73). Y-STRs have also found specific applications for human identification and forensic investigations (74, 75). Unlike autosomal STR profiles that attain variability by either independent chromosomal assortment, recombination and mutation, only mutation plays a role in deriving human variation with Y-STR markers (76). Y-STR loci have ancient roots and demonstrate strong Y-linked conservation between species as noted from comparison studies of humans and higher primates (77). However, no primate biological samples have derived a complete Y-STR profile using any of the commercially available forensic amplification kits. (78).

Since the Y-STRs are found exclusively on the male chromosome, they have become very important in deriving a male specific DNA profile from complex sexual assault cases involving male-female body fluid mixtures. This is particularly important when attempts to separate the male and female components fail using the conventional differential DNA extraction (79). In addition, Y-chromosome markers have proven useful in missing person investigations when family reference samples are limited and patrilineal Y-STR analysis may be the only remaining option. Grandfather, father and son should have the same Y-STR profile provided no mutation has occurred between

generations (80). Recently, Y-STR analysis has also been used to narrow the list of potential suspects in mass screenings or aid in the identification of victims following a mass disaster by using kinship analysis and linkage to members of the same family (see Use of DNA in Mass Disasters and Mass Screenings/Kinship Analysis).

Due to the lack of independent segregation on the non recombining portion of the Y chromosome, Y-STR frequency estimations for individual loci cannot be combined through the use of the product rule to estimate discrimination potential (76). Multiple Y-STR profiles or “haplotype” frequencies can be determined by counting or through the use of more sophisticated Bayesian methods (81). In the same manner as autosomal STR multiplex systems, Y-STRs markers can be amplified simultaneously and the individual locus and alleles distinguished using conventional DNA fluorescent detection sequencers (82). It is important to note that due to the patrilineal relationship (brothers, sons, fathers, etc.) de Knijff (83) reported the importance of assessing access to the crime scene by relatives each time a potential match using Y-STR profiles is considered.

Geographical studies documenting human demography are often complex and poorly understood but are of great interest to anthropologists as well as medical or forensic geneticists. Typically, mtDNA analysis has been used to determine the extent of gene flow and population origins but more recently, Y-STRs have been examined in various populations resulting in discovery of significant Y-STR associations. Y-STR allele frequency studies have been reported for Brazilian populations using 17 loci (84), 14 loci study with father-son haplotypes (85), and for the US populations using 27 loci (86). More recently, autosomal as well as Y chromosome data revealed that a specific subset of the Chinese population has a distinct origin in the ancient Xibe group (3,000 year old ancestral group) from the northeastern part of China that can be traced back to 1764 (when 5,000 individuals migrated ~3,500 km to defend the northwestern borders (87). Although Y-STR diversity is significantly lower in Finnish populations compared to different European groups, significant inter-regional differences amongst western, middle and eastern parts of Finland have been noted, suggesting that the use of these markers for forensic casework may be challenging within Finland (129). Studies conducted with 1136 unrelated males from 10 provinces and three aboriginal tribes in Argentina demonstrated remarkable haplotype diversity in all the urban populations as well as the geographical regions to which they belong with the most reduced haplotype noted amongst the Amerindian populations (88). This finding is consistent with the impact of European male migration, genetic drift, endogamy and the founding effect of Native American populations (89, 90). More recent migration studies (79) highlighted by Y-STR analysis demonstrated that massive migrations in Poland after the Second World War had little effect in the Y chromosome polymorphisms. Studies conducted on 226 Polish male citizens indicated that the population of Southern Poland was most likely homogenous before the war and closely related to other Polish and Slavic populations.

Jobling and Tyler-Smith (73, 131) have reported that population structure does occur based on the Y-chromosome. This suggests the need for grouping of haplotypes based on the geographical origin of the donors. Other studies have also reported that

clusters of regional groups could be identified in Europe and the potential for identification of haplotype-based population sub-structure does exist (91). Estimation of population variation encountered in Y-STR studies will undoubtedly be of interest in the forensic interpretation of profiles found at the crime scene.

The use of surnames as markers for inbreeding and migration was described by Crow in 1983 (92), but the correlation of surnames and Y-STRs has only recently gained scientific relevance (93). Immel et al. (94) reported recently the Y-STR typing of 419 German males with the last name Halle. These males could be categorized into two distinct groups, suggesting that the major migration from Slavic countries in the 19th century was a recent rather than early event. The potential use of last name and Y-STR association for forensic investigative purposes has also been reported previously for the English population (80). A study involving two deep-rooted paternal genealogies in Central Germany demonstrated that a 13-generation and a second 9-generation family with the same last name could be potentially linked even if these individuals lived several hundred years ago (95). These and other studies suggest that the predictive Y-STR value for forensic investigations may warrant additional population studies.

A key characteristic of Y-STR analysis is the demonstrated male specificity and sensitivity to produce reliable results in male/female mixtures containing significant concentrations of DNA from the female donor, although reports vary from successful male DNA typing in mixtures representative of a 4,000 fold excess (81) to 1000-fold excess of female DNA (100). As noted by Mulero (97), optimal amplification conditions are paramount to obtaining both sensitive and specific Y-STR results. The alteration of a single PCR amplification condition such as a one degree change in annealing temperature above 63°C can cause peak imbalance or locus dropout in the common markers DYS456, DYS390, DYS389II, DYS19, DYS439 and YGATA H4 and an inverse relationship between an increase in annealing temperature and cross-reactivity with excess female DNA (97). In autosomal markers, allelic drop-out can be problematic for misinterpretation at heterozygous loci (98) but Y-STR analysis is not affected by stochastic effects since drop out for Y-STR loci will generally produce no information except in male/male mixtures and occasionally with the DYS385a/b marker. Some laboratories have reported excellent results with as little as 30-62 pg of template DNA if they alter the loading/injection times and use a larger volume of amplified sample to maximize the signal detection. However, an excess of target DNA should be avoided to minimize stutter, signal saturation, spectral bleed-through and cross-over artifacts (96).

Although it is difficult to estimate the number of potentially useful STRs on the Y chromosome, more than 200 have been reported by Kayser et al. (99,100). Specific assessment of Y variation from geographical locations have been carried out by many forensic laboratories including one large study reported by Budowle et al (75) involving five North American populations with 2443 individuals. Kayser, et al. (99) has also reported comprehensive screening of genomic information on the Y chromosome markers for forensic applications.

In recent years, the selection of a consensus-derived set of core Y-STRs for use by the forensic community has proven to be a significant challenge. The initially selected “minimal haplotype loci” or MHL (100-102) included DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393 and the polymorphic DYS385. In an effort to standardize the loci, the Scientific Working Group on DNA Analysis Methods (SWGDAM) accepted the core loci and added two additional Y-STRs, DYS438 and DYS439 for inclusion in the U.S. minimal haplotype (103). Useful comparison tables of allele sizes, chromosome location and mutation rates for the two commercial Y-STR kits have been reported in the comprehensive STR review article by Butler (34).

The DNA Commission of the International Society of Forensic Genetics (ISFG), which regularly publishes guidelines and recommendations concerning human DNA polymorphisms, released an update on its recommendations for the use of Y-STRs in forensic science (104). These guidelines served to clarify loci and allele definitions and provided nomenclature solutions as well as more comprehensive reporting methods to ensure uniformity in recording population data. These should be considered an augmentation to the first set of recommendations published in 2001 (105). Y-STR nomenclature related questions have been raised concerning variation in results due to primer pairs annealing at different sites (more common with Y-STRs than autosomal STRs) or the presence of two separate Y-STR loci lying between a pair of primers. There have been few studies on Y-STR mutation rates which have been mostly restricted to only a few sets of markers. However, such studies are very important considering that, based on current estimations, one would expect (106, 107) at least one allele mismatch will occur between father and son in about one out of 40 pairs analyzed. This mismatch could potentially increase to one out of 20 pairs if the father/son pairs are two generations apart and 18 to 19 Y-STR loci are analyzed. Mutation rates in Y-STRs can be studied by using well documented multi-generational pedigrees (108) or by male germ line transmission studies from confirmed father/son pairs (106, 85). Dupuy (106) noted that in studies involving either locus or allele-specific events (mutations), the formation of new Y-STR alleles are more common than allele losses. It is noted that the haplotype diversity cannot be predicted by combining the average diversity at each single locus but requires an estimate of the frequency distribution from the population of the whole haplotype and the variation accumulated within each lineage by mutation. Consequently, further studies involving larger databases and studies involving well documented mutation events from father/son pairs will continue to be important contributions to further the use of forensic Y-STRs analysis.

A comprehensive annotated STR physical map of the human Y chromosome has been described for forensic applications (109). In addition, the same authors have reported population studies for a novel highly discriminating Y-STR locus (DYS503) with a >0.92 genotype diversity in Caucasian and African American samples (110).

The search for additional Y-STR markers to add to the discriminatory potential of Y-STR systems has been reported by many authors over the past three years. Ballantyne and coworkers (111) initially reported a 19-locus Y-STR system with excellent sensitivity and proven results with highly compromised casework samples.

Later on, the same group described a 21 locus Y-STR megaplex designed specifically to augment the minimal haplotype loci for forensic casework (112). This megaplex identifies 25 sites of potential allelic variation and has shown a high level of sensitivity (down to 50 pg or ~8-9 diploid cells), a high degree of specificity (lack of derived female artifacts up to 300 ng of female DNA and in mixtures with a 100 fold excess of female to male contribution) and discriminatory capability on both test samples and non-probative casework. Three additional “noncore” Y-marker multiplex sets were described by Hanson, Berdos and Ballantyne (113). These contained either 8 Y-STRs (Multiplex III or MPIII) or 13 Y-STR loci (MPV) and consistently derived male DNA profiles with as little as 25-50 pg of male DNA even after samples were exposed to environmentally compromising conditions for up to one year. This study augmented the previous validation studies of the 21Y-STRs (MPIV) described by Hanson and Ballantyne (112).

After Y chromosome analysis gained public attention, when the two patrilineal Y chromosome haplotypes of Thomas Jefferson and Genghis Khan were analyzed (75, 114, 130) commercial paternity laboratories also considered the prospect of using Y-STR markers for either forensic paternity investigations or historical lineage reviews. Johnson and coworkers (115) reported a full validation and population study of a 10-plex multiplex system with an overall haplotype diversity of 0.996 for use in their commercial paternity and forensic casework laboratory.

Initial exploration of commercial Y-STRs kit development for forensic practitioners was led by ReliaGene with the release of Y-PLEX 6 (116), Y-PLEX 5 (117) and Y-PLEX 12 (78). Although the Y-PLEX 6 and Y-PLEX 5 kits are no longer available, scientists from ReliaGene demonstrated their usefulness on 188 forensic samples (118) and their work proved important in establishing the future use and acceptance of these markers for forensic applications. Currently, two main commercial Y-STR kits are available: 1) PowerPlex[®] Y System from Promega Corporation released in September 2003 contains 11 Y-STR markers and 2) AmpF/STR[®] Yfiler[™] PCR Amplification Kit from Applied Biosystems released in December 2004 has 17 Y-STRs. Over the past few years, many additional Y-STR loci have been evaluated for forensic applications and described in the literature (86, 113).

Although tedious to perform and laborious to review, it is becoming very important to carry out concordance analysis using the different developmental or commercially available Y-STR systems. Studies such as reported by Gross and coworkers (119) are invaluable in establishing a clear understanding on how the final results obtained for Y-STRs can be compared between different systems and various laboratories. The authors reported that in 11,600 allele calls from 200 different individuals, only five minor variations in specific loci occurred using different Y-STR systems. The lack of concordance was noted in four samples containing the same one bp deletion as previously reported by Schoske and coworkers (86) and a three allele variation (considered rare) was identified and further verified to account for a two allele determination from one kit and a three allele call with another kit. Overall, this and other studies have provided confidence in the determination of Y-STR profiles developed from different amplification kits and also serve to reinforce the importance

of establishing a database of allele results (designation, mutation and minor primer-induced variations) in order to ensure that haplotype variations are documented and understood should a less than perfect match occur between different forensic laboratories working with the same donor samples.

As noted by Krenke et al. (96) Y-STR international collaborative validation studies conform to both the European minimal haplotype loci recommended by the International Y-STR User Group and the 11-locus set recommended by SWGDAM. Accepting basic guidelines and conformance to standard loci have aided inter-laboratory comparisons. Krenke (96) described the inter-laboratory results from 17 different developmental validation studies using the Promega PowerPlex® Y System and included findings on amplification and detection with different numbers of cycles (24-32 cycles), amplification volumes (6.25 to 50 µL), various enzyme concentrations, primer volumes (0.5x to 2x), magnesium concentration (1.0 and 1.5 mM), alteration in annealing temperatures as well as concentration of target DNA. Full profiles were obtained by all laboratories with ≥ 125 pg of DNA. A similar study was conducted on Y-STR markers in North America populations (75). Both of these large studies demonstrated the sensitivity, specificity and reliability of the PowerPlex® system for forensic applications. Ampf/STR® Yfiler™ PCR amplification kit by Mulero describes (97) that 17 completed Y-STR profiles were also obtained with ≥ 125 pg of DNA using the manufacturer's optimized conditions. No detectable cross-reactive amplified products were obtained on human female DNA, bacteria and most common animal species (97).

Y-STRs have provided several important advantages for challenging forensic casework. Work originally reported by Sibille (120) and further advanced by Hall and Ballantyne (121) conclusively demonstrated the ability to obtain Y-STR results from donors who are aspermatic or from samples collected after a significant post-coital interval. More recent studies (112, 122) indicate that full Y-STR profiles can be routinely obtained in multiplex reactions optimized for forensic applications (21 locus megaplex) or with current commercially available kits even after the cervicovaginal samples were recovered 3-4 days following intercourse. In some instances, full and partial profiles were recovered from samples 5-6 days post-coitus if an additional purification process was used to further purify the extracted DNA. It is also noted that multiple DNA profiles may be developed from a sample containing several male donors but the interpretation of such a complex genetic set of profiles may be problematic depending on the number of contributors and the condition of the original sample to yield full and complete profiles (76). A practical consideration for the use of Y-STR analysis would be its use as a pre-screening tool to assist in familial identification (by descent) from a large number of victims following a mass disaster (123) or in a large case involving many potential suspects. In such events, it has been proposed that familial searching using moderate matches across shared autosomal loci in closely related relatives could be an invaluable forensic investigative tool (124, 76). If large numbers of samples can be categorized quickly using broad discrimination technologies such as mt-DNA or Y-STRs, small subsets of probative samples could be developed for further investigation using more discriminating autosomal STR typing.

Y-STRs continue to attract the attention of researchers in clinical and forensic laboratories world wide. Large Y-STR databases have been developed to assist frequency calculations for forensic purposes as well as family history studies for cultural or medical purposes. The Y Chromosome Haplotype Reference Database (YHRD) (www.yhrd.org) is the largest Y-STR reference database and has replaced three earlier versions collecting European, Asian and American Y data chromosome with a single freely accessible interactive database. This collaborative exercise with multiple laboratories using both SWGDAM-and ISFG-endorsed diagnostic Y-STR kits, has reviewed 9-locus profiles (minimal haplotype loci) from more than 51,253 individuals from more than 447 populations (125, 126). The YHRD release 21, April 2007 (91, 126) has included information on 49,396 haplotypes for 9 core loci and 23,075 for 11 loci as well as common Y mutation rates (i.e. deletions, flanking site mutations, duplications, repeat base mutations and Y deletions/amelogenin mutations) and continues to update a searchable interactive Y-STR haplotype database within specified metapopulations. The extremely large sample sets within genetically defined populations allow for biostatistical frequency estimates of unobserved or rare haplotypes using a metapopulation paradigm of discrete populations that interact through migration and gene flow (127). YHRD also contains basic population analysis functions for plotting the percentage of the 20 most frequent haplotypes within a given population sample with both numerical and graphical interfaces. This online resource with a worldwide commitment for maintenance and updates, will continue to be a key decision making tool supporting forensic investigations regardless of national affiliations (126, 128).

In addition, a summary of Y-STR databases and downloadable presentations have been made freely available from Butler and co-workers at http://www.cstl.nist.gov/biotech/strbase/y_strs.htm, <http://reliagene.com>. (4 populations, 4,623 individuals across 7 loci or 3,406 individuals across 11 loci) www.promega.com/techserv/tools/pplexy/ (5 populations, size of database not reported) and <http://www.appliedbiosystems.com/yfilerdatabase> (3,561 individuals in 8 different databases).

Mitochondrial DNA (mtDNA)

Although written in 2003, the review article by Budowle and coworkers (132) that describes mtDNA forensic analysis, application and challenges is very much relevant and should be principal reading for all interested practitioners. Many reports over the past few years have built on past experiences, increased our knowledge of specialized applications for difficult samples or were focused on increasing the size of reference databases. Significant advances in SNP based mtDNA analysis and interpretation, quality assurance and data reviews have also been reported. Issues still remain for heteroplasmy population variations, quantitation and the recent quest to automate with high levels of quality assurance, contamination controls in place and data integrity verification.

Building from past work, Parsons and coworkers (136), identification of DNA from human origins was also described using a simplified amplification and restriction digest of the mitochondrial cytochrome B region which was capable of separating humans from higher primates and domestic animal species. Variation of this method was reported by Matsuda (137) and a SWGDAM-validated study was also conducted by Branicki (138).

A unique study was carried out by Branicki (138) using common single copy nuclear gene (myelin basic protein gene) and higher copy mtDNA genes (cytochrome B and 28S ribosomal gene) isolated from degraded tissues in order to assess the impact that specific tissue type and the cellular environment may have in recovering forensically relevant DNA information. Overall results indicated that DNA degradation is influenced by a number of different factors including cellular location, chromatin structure and transcriptional activity which must be taken into account in order to find DNA loci robust enough to survive the initial degradation events.

Hair analysis has also taken new approaches combining both surface organic component analysis and mtDNA sequencing. Benner and coworkers (178) conducted a feasibility study and demonstrated that 1.5 cm segments of human hair can be subjected to on-line supercritical fluid extraction using gas chromatography and mass spectrometry and collectively this data interpreted with mtDNA results provided complementary information that defined the four different family units and corroborated the maternal inheritance of the DNA profiles.

Mitochondrial DNA offers significant advantages in processing biological evidence that is badly degraded or extremely limited in quantity such as hair shafts. Mitochondrial heteroplasmy is defined as the occurrence of two or more populations of mtDNA in the same individual, the same tissue, the same cell or the same mitochondrion and can be manifested as either a specific point mutation or a length mutation (173,174).

Heteroplasmy still remains an interesting phenomenon with different implications for forensic analysis. In a detailed but small sample set, Lee and coworkers (142) studied the levels of length heteroplasmy within the C-tract of the mitochondrial HV2 in 25 individuals. They found that samples of blood, brain, heart, liver, skeletal muscle and hair shaft samples collected during autopsy, showed different heteroplasmic distributions. Although the frequency of heteroplasmy did not differ significantly with age, its distribution patterns in the blood and organ tissues were prevalent in individuals over the age of 28.

Collaborative exercises continue to be a prominent component of mtDNA analysis and in the last three years several groups have reported studies primarily on hair analysis and issues associated with heteroplasmy. In 2003, the Spanish and Portuguese working group (GEP) of the International Society of Forensic Genetics (ISFG) conducted a study with 64 laboratories as a quality control exercise consisting of four bloodstains and two hair shaft fragments from the same donor (144). The laboratories were successful in identifying the one non-human bloodstain using either immuno-diffusion techniques or species-specific mtDNA markers (Cyt-b, 12S and 16S genes).

Although blood sample mtDNA analysis appeared to have a greater consistency amongst the majority of laboratories reporting, the same could not be said for the hair sample analysis and the lack of concordance was not due to the presence of inhibitors but primarily low quality and quantity of recovered DNA. This study highlighted two major points: 1) there is a need for external proficiency in order to achieve an acceptable standard and 2) laboratories engaged in non-forensic studies such as paternity analysis may use different parameters for extraction and starting amounts of target DNA which could lead to contamination and interpretation challenges for smaller samples (single hairs) and samples that are environmentally insulted. Interestingly, this working group persevered to write a second paper which described in more detail the mtDNA errors which were attributed primarily to documentation mistakes and documentation deficiencies with some contamination and sample mix-up also being noted (145). In 2006, a more detailed evaluation of the mixed semen-saliva stain sample of a further study from the original Spanish-Portuguese working group of ISFG involving mtDNA, Y-STR and autosomal analysis (146). This group noted that nuclear results were different than expected in attributing donor identifications to those described for mtDNA analysis. In fact a main conclusion of the study was that problematic extraction of DNA that failed to segregate the male/female donor cellular components in a mixed semen/saliva stain strongly influenced the interpretation of mtDNA evidence in unbalanced mixtures, which could lead to false exclusions. It was also considered remarkable that a significant number of practicing mtDNA laboratories did not follow international nomenclature which led to confusion and compounded difficulties in comparing results. Further mtDNA validation studies are planned on mixed samples although the strong conclusion was to use standard STR analysis for any mixed samples.

A similar collaborative exercise carried out by the EDNAP was also conducted to evaluate the distribution of mtDNA heteroplasmy amongst hairs of 10 individuals (55 hairs in total) who were also well documented for point heteroplasmy in their blood and buccal cells (147). This study also documented the reproducibility of mtDNA sequencing of hairs from ten participating laboratories using different chemistries and protocols. Overall, 9 of the 10 laboratories reported good results with the majority of the samples and that C/T point heteroplasmy observed in blood and buccal cells segregated differently between hairs within the individual and also varied between different laboratories. This represents a complex set of results and suggests that the potential link between pigmentation and degree of heteroplasmy may explain some of the different findings (148), although the issue of hair heteroplasmy in general, remains controversial. Although heteroplasmy has been observed in multiple positions within a single individual in previous studies (149-152) others including the members of the EDNAP working group have suggested that these findings may also be due to problems in mtDNA protocol such that contamination and amplification of nuclear pseudogenes could be a factor (152, 153). The overall conclusion of the collaborative exercise (146) is that rare mutational events will continue to challenge heteroplasmy interpretation in mtDNA analysis but that hair patterns for the majority of casework follow predictable patterns of heteroplasmic and homoplasmic differences that if interpreted according to acceptable guidelines(151, 154) should enable reliable interpretation to assist forensic investigations.

Inter-generational heteroplasmy study between 26 mother-child pairs using thermal temperature gradient electrophoresis in hair roots (155) noted that in three of the four families evaluated in depth, the variation in terms of single nucleotide heteroplasmic proportions between generations was the same variation as that found among several individual hairs of the same mother. In one family the opposite was found, in that intra-individual differences exceeded the maternal/child intra-individual differences. Interestingly, the conclusion from the authors was that the use of first-degree matrilineal relative's samples as the known sequence is unlikely to pose substantial additional complexity to the mtDNA interpretation and that the one disparity noted may have led to an inclusive result. However, based on standard interpretation guidelines this would not exclude the child as being related to their mother.

It is also noted that our understanding of mtDNA mutations as it applies to disease will also enhance our knowledge of fundamental evolutionary changes such as those noted in heteroplasmy, both point and length variations encountered in forensics. Towards this end, the enhanced MITOMAP (<http://www.mitomap.org>) has constructed the entire mutational history of the human mtDNA, defining haplo groups and differentiating ancient from recent mutations (158).

The establishment of a forensic mtDNA database is not a trivial exercise since it forms the basis of frequency estimations for mtDNA casework analysis and could be heavily criticized should errors be discovered. The recent development of the EDNAP mtDNA population database in October 2006 uses the highest standards of quality. A quasi-median network analysis that assesses interpretation and sequence information was designed to catch transcriptional errors. To date this database has 5172 individual mtDNA sequences and can be queried at <http://www.empop.org>.

Equally as important to forensic practitioners, are faster and more economical ways to obtain quality results. Towards this end, Gaffy and Foran (139) have described a simple alkaline extraction process for obtaining DNA from head hair shafts which can decrease the processing time from 22-24 hr to 6-7 hours total digestion and extraction.

Extraction and sample preparation is often paramount for successful typing using either nuclear or mtDNA procedures. Recent work by Nelson and Melton (140) described the modest use of bleach on compromised samples as often the best approach. The authors documented 116 DNA extractions and compare their 86% success rate in obtaining full profile or partial mtDNA results to the average age and condition of the samples. Law enforcement submitted remains are typically less challenging. Infant/fetal remains were uniformly successful and femurs and teeth were the most common samples submitted with a potential of 90% and 92% typing success. This study provides good advice on the submission of evidentiary and reference samples with applicability for historical or forensic casework as well as potential mass disasters.

Another practical study involving mtDNA and hair attempted to characterize the morphological features of human head hair in order to further understand the factors

that could influence the recovery of DNA and its subsequent amplification success rate (141). Using a Linear Array J duplex PCR system 2554 head hairs from 132 individuals were characterized into 1251 telogen hairs and 1303 hairs without roots. Controlling for telogen hairs, the findings suggested that the amplification success is independent of cosmetic hair treatment, medulla structure, shaft length, diameter, volume and scalp origin, however age, race and hair pigmentation all contribute to amplification success rate. Interesting and somewhat difficult to understand findings were noted with this study such as the success rate of telogen hair from decedents (83.3%) being higher than telogen hairs collected from living subjects (71.9%). In addition, other results from Roberts and Calloway (141) were in direct conflict with Melton's study using 691 casework hairs (143). Melton (143) reported obtaining full and partial mtDNA profiles on >92% of the casework hairs analyzed (82.5% full profiles). Melton also noted that amplification success increased with colour pigmentation and diameter which is in direct contrast to the Roberts and Calloway findings that light coloured hair yielded the best amplification and diameter played no role in the results obtained (141). Although age of collected hair, sample size, and hair preparation for extraction may have been contributors to this disparity, to the non-expert in this field many questions still remain.

Just and co-workers (159) describe the significant utility of using four different automated systems in tandem to determine variation differences in 11 different SNPs in the control region outside of the hyper-variable regions (HVI and HVII) as well as the coding region of the mtDNA. The intent of this laboratory is to increase the discrimination in mtDNA testing and follow the general scheme of other larger studies involving 59 SNP sites currently undertaken by many European laboratories (160). The Coble study (160) has organized the 59 different SNP sites into eight different multiplex panels targeting 18 specific common HVI/HVII types. A major concern of all forensic laboratories engaged in developing large scale databases, is safeguarding the accuracy and integrity of the processed data. In the past, several large databases have been called into question regarding their reliability (161-165, for a more detailed description of this issue). The AFDIL laboratory (159), which plans to add 5000 control region sequences per year, has begun to field test and validate a new set of electronic data review guidelines to safeguard the integrity of large DNA databases.

Similar computer assisted analysis is also undergoing evaluation for monitoring the inheritance of inter-generational heteroplasmy in blood samples. Brandstatter and co-workers (172) screened 135 different families and discovered that 20 mother offspring pairs demonstrated more than 1 mtDNA haplotype, 3 point mutations and 8 length mutations. In more than 90% of the cases reviewed the search algorithms provided by the software analysis detected the subtle variations in sequence discordance and suggested that automated reviews for large sequence databases could be universally applied if the overall quality of the results is ensured.

An intriguing and challenging study compared the quantity of recovered DNA from forensically relevant samples (plucked, shed, eyebrow and body hairs, fingerprints deposited on paper and detected with fingerprint enhancement techniques, and a variety of watches, rings, glasses, earrings and bracelets) for nuclear and mtDNA

analysis (166). Andréasson found that there was a wide variation in recovered DNA from the various samples using a real-time PCR quantitation, recovered DNA from hair (different donors or different samples from the same donor) proved to be extremely variable and within the first centimeter of hair varied as much as 77-fold between plucked and shed hair and is most likely a consequence of different growth phases of hair. The yield of DNA in both plucked and shed hairs was noted in more distal hair segments. Other DNA extraction yields from the various samples evaluated appeared to be related to the epithelial sloughing of cells in each individual and underlined the importance of quantitation analysis prior to determination of either nuclear or mtDNA analysis on touched or body associated objects.

Length heteroplasmy was also studied (167) between three monozygotic triplets and showed the intriguing result that although no statistical differences were detected between blood and buccal cells of the same individuals or their brothers, the frequencies of the length variation differed significantly within the six hairs evaluated in each of the brothers. The authors suggest that in keeping with the findings of Calloway et al. (149) levels of sequence variations may increase in with age in different tissues suggesting that mtDNA hair matrix cells may be more prone to rapid aging compared to other tissues due to their high energy demand during the growth phase. The discrepancy between finding heteroplasmy and not finding heteroplasmy in healthy individuals is yet to be resolved.

Technology advances for a rapid screening of mtDNA relevant sequence variation using commercially available simplified HVI/HVII mtDNA linear arrays was described by Divne et al (169). Their system proved to be easy to perform in three hours, and was capable of detecting 10 polymorphic regions using a series of immobilized sequence specific probes arranged in 31 locations on assay strips. The utility of this pre-screening technology was demonstrated by excluding 56% of the samples from 16 different cases involving 90 samples (57 evidence samples and 33 reference samples) and compared favorably to a retrospective analysis of the same samples excluded by conventional mtDNA sequencing (the rapid mito strip technology could exclude 79% of the samples excluded by sequencing). The introduction of a rapid pre-screening technology for routine mtDNA analysis offers different advantages and disadvantages but has not been without controversy as aptly noted in a series of letters to the editor of Journal of Forensic Science (170, 171).

Real time PCR estimation of nuclear and mtDNA copy number has been applied for several years and will undoubtedly be the standard practice in forensic science. Various commercial applications have been introduced and in other laboratories specific fluorogenic probes have been designed to monitor both nuclear and mtDNA content plus the integrity of the copies for further DNA analysis. Alonso (43) describes a process that uses two separate fragments 174 bp apart in the hyper-variable region HVI to determine the copy number in each size category thereby also developing a method to determine quality for further studies since the ratio of small to large fragment concentration would imply the degree of potential degradation. A separate real time PCR based test for a 112 bp Y-specific fragment and an X-specific 106 bp fragment was used to assess content of male and female nuclear DNA. As expected,

current forensic samples (4-5 years old) yielded more success than ancient remains (500-1500 years old) with either system.

Several recent papers have contributed to our knowledge of initial sample or skeletal preparation for anthropological investigation and the subsequent recovery of amplifiable DNA. The main point of these studies was to determine whether common skeletal preparation maceration methods (bacterial, cold and warm water, chemical, enzymatic and invertebrate) are inadvertently compromising DNA integrity and thereby destroying potential physical evidence. The results from several laboratories appear to be conflicting and overall suggest that further studies need to be performed. Steadman (175) demonstrated that nuclear and mtDNA could be amplified from all experiments using cleaning preparations involving mild detergents, hot water, and boiling or microwaving, but lengthy procedures at room temperature using hydrogen peroxide, bleach and EDTA/papain resulted in low recovery of DNA and lack of amplification. This was in contrast to the previous work (176) that suggested that heat methods are deleterious to DNA recovery. Other investigators have noted that age of the exhibits as well as the specific source of the bone could have significant implications on the recovery of DNA using the various maceration methods (177).

Over the past three years significant mtDNA work has been carried out on non-human species both for wildlife conservation genetics as well as for potential forensic casework involving domestic animals. In a very recent study, Gundry and co-workers (133) sequenced the 1270 bp control region from the domestic dog (*Canis familiaris* - 16,727 bp genome) and described 45 haplotypes from a total of 125 dogs representing 43 breeds. Overall the analysis of dog hairs found at the crime scene could prove invaluable trace evidence and the availability of a validated mtDNA domestic dog data base adds to our forensic capability. A total of 26 informative SNPs were chosen to identify the major clusters in the dog data set and these changes as well as the fewer unique informative sequences compared to the human genome (humans have three times as many informative sites and twice as many haplotypes) are consistent with previous published reports (134, 135). Work has been conducted in wildlife forensics to aid conservation and protection of endangered species as noted by Malisa et al. (156) who demonstrated that a simple cost effective PCR/RFLP analysis of the mtDNA marker "mt700" could discriminate 13 species of cervidae (9 wild) and could be augmented with sex determination using gender-specific SRY and ZFY/X chromosome domains. A review of wildlife mtDNA technologies by Linacre (157) covering several different approaches but primarily focusing on the cytochrome B gene found in the mitochondrial genome, advocates the compilation of databases for endangered species and standardization of technologies and reporting in order to aid the Convention on the International Trade in Endangered Species (CITES) and Wild Flora and Fauna. Forensic approaches in mtDNA analysis has also found utility in non-forensic applications as indicated by Hutter et al. (168) who used standard polymorphisms in the non-coding regions of the mtDNA genome to authenticate and identify potential contamination of human leukemia-lymphoma cell lines.

Single Nucleotide Polymorphisms (SNPs)

The most widespread and diverse polymorphisms encountered in the human genome are single nucleotide polymorphisms or “SNPs” resulting from base substitutions, insertions or deletions. They are considered evolutionarily stable with easily interpretable inheritance. It is estimated that 85-90% of all human sequence variation can be attributed to SNPs, making it a largely untapped resource for human identity testing (179-187). In order for a single base change to be considered a SNP, the least abundant allele must be greater than or equal to 1% and an abundant allele would be encountered with a frequency of 10% or greater in a given population (188). Since most SNPs are biallelic, they are considered less informative for identity testing compared to STR analysis. However, specialized applications such as typing mtDNA, Y chromosome lineage analysis, characterization of highly degraded DNA samples, migration and lineage biographical ancestry analysis, high throughput rapid screening of DNA samples prior to conventional STR analysis, as well as potential determination of physical traits may be developed through the use of SNP technologies and have been well described in a detailed review article by Daniel and Walsh (213).

Current STR markers (34) have relatively large amplicons (between 150- 450 bp), and it has been demonstrated that smaller amplicons are more likely to be amplified from biological samples that are environmentally challenged or badly degraded (189). In order to reduce amplicon size, the flanking regions to tandem repeats can be shortened to derive “mini STRs” (see elsewhere in this report and 190), or strategies for detecting specific regions of single nucleotide polymorphism can be derived (SNP technology). Consequently, SNPs are beneficial for badly degraded or compromised samples when STR typing fails to yield a result or when a partial profile is obtained in challenging investigations involving mass disasters or fire and explosive trauma (123). However, it is fully recognized that any biallelic system including SNPs, will be problematic in situations involving biological mixtures containing multiple donors. Statistical characteristics are not amenable to the interpretation of complex mixtures and a robust highly quantitative assay would be theoretically required before any consideration of heterozygous and homozygous balance and thresholds could be taken into account.

Additional markers for more discrete human identification have always been a quest harbored by forensic scientists. The use of PCR-based systems for SNP analysis have been described for more than 17 years (191) but only recently been validated as a multiplex system with enough combined discrimination and robustness for forensic applications. In a jointly issued assessment of SNPs, the working groups of the ENFSI and the US SWGDAM indicated that SNPs are unlikely to replace STRs as the predominant method of testing for forensic casework and criminal databases (192). To obtain the same power of discrimination as existing STR multiplex systems, a panel of at least 50-100 SNP autosomal loci would be required (193, 194). It has been aptly demonstrated by a number of laboratories that creating large multiplexes that are validated for forensic analysis, is an extremely significant challenge (195, 189). To multiplex more than 30 SNP loci, the required amount of target DNA may

exceed 500 ng which would preclude its use for many forensic investigations (189, 196).

A principal use for SNP analysis may be the enhancement of mtDNA analysis by enabling a process for rapid screening as well as adding to the discrimination potential by including single base differences outside the two hyper variable regions (HVI and HVII) that are normally sequenced in mtDNA analysis (74, 197, 220). Kline and co-workers, (198) described the utility of two Linear Array mtDNA HVI/HVII Region-Sequencing Typing Kits (Roche Applied Science, Indianapolis, IN) that were used to rapid type seven nucleotide polymorphisms in three populations using an automated workstation. It was noted that although the utility of using a broad coding region identified by SNP specificity can subdivide many individuals, the ability to detect and measure multiple SNP signals could be challenging. In addition, sequence heteroplasmy and signal loss due to failed hybridization because of destabilization polymorphisms would be highly problematic. This is a particular issue which would make the process difficult to automate for some screening applications. A second non-autosomal SNP analysis application has been focused on the rapid detection of Y chromosome polymorphisms. This process has been used for lineage-based studies (199). In a large study involving 1126 unrelated males distributed among 12 worldwide populations the 29 SNPs were only able to discriminate between the major human groups present in the world (200). Although Brion and co-workers (200) noted that specific geographic distribution of the Y-chromosome haplotypes exhibits extensive genetic differentiation, the selection of SNPs is complex and critical. In order to provide discrimination between more closely related populations, many new SNP markers will be required.

To date, a small number of validated SNP assays have been used for casework. These include mini-sequencing assays for mtDNA (159, 201, 202), Y chromosome (203), a red hair assay (204), and one autosomal 21-multiplex (189). The validated autosomal 21-locus SNP assay was originally developed by the Forensic Science Services (205), for specific challenging forensic samples. More than 81% complete SNP profiles were derived from degraded saliva stains (after 147 days of exposure) and full SNP profiles were recovered from blood samples left to the elements for 243 days. In addition, Dixon and co-workers (189) developed a computer program to aid in the interpretation of SNP profile results from the severely compromised samples. As noted in the previous report to the Interpol Symposium, Gill and coworkers intended to fully review the SNP performance of their 21 locus system (Foren-SNPs™) compared to mini-STRs and STRs in a collaborative exercise involving 9 different forensic laboratories (189) through the auspices of the EDNAP and the ENFSI. The analysis of artificially degraded DNA using both STRs and SNPs proved to be a significant challenge to most laboratories (189) as indicated in the inconsistent results across laboratories. The SNP assay had less detected genotypes and was the only multiplex to show allele drop out in control samples. The performance of the mini-STRs assay kits (provided by NIST) gave the best overall results when compared to standard STR assays (SGM+™) and the Foren-SNPs™ kit (189). It was determined that re-engineering STR amplicons such that primers lie closer to the repeat region of

interest (i.e. mini-STRs) and the optimization of the cycle and amplification conditions, were potentially the most effective process to be used for compromised samples.

Although many different approaches have been used for SNP analysis, the majority of SNP genotyping assays utilize one of four basic processes: allele specific hybridization, primer extension, oligo nucleotide ligation and invasive cleavage. Each assay process has its benefits and challenges and a complete review of the SNP processes has been evaluated in the review paper by Sobrino and co-workers (185). Strategies involving SNaPshot[™], SNPstream[®], Ultra High Throughput System and electro spray ionization mass spectrometry have been discussed in the comprehensive article by Budowle (74). In addition, SNP assays have been carried out on DNA micro arrays, FRET analysis by Light Cycler, MALDI-TOF spectrometry, Pyrosequencing, TaqMan probes and Molecular Beacons (207-210). Although mass spectrometry analysis has the potential advantage of speed, sensitivity, the ability to interrogate mixtures (74, 206) and is more amenable to automation, the single nucleotide allele-specific primer extension using the SNaPshot[™] assay is simple and cost effective. The SNaPshot[™] reagents and targets are amplified and resultant products detected with equipment used for conventional STR assays that are commonly found in current forensic laboratories. Quintans and co-workers (211) have written a clearly delineated paper that addresses the SNaPshot[™] process and the selection of target DNA regions, development and optimization of the assay, and its implementation for anthropological and forensic interests. Although the multiplex capability of the SNaPshot[™] process is impressive, it has minimal quantitation capabilities which are required to interpret most forensic casework. Other technologies for SNP analysis that may be practical for the smaller forensic laboratory include the Amplification Refractory Mutation System (ARMS) and PCR combined with Universal Reporter Primers (URP). Such technologies have enabled development of a 20 autosomal SNP plus Amelogenin (gender discrimination). This system (189) has been preferred since it allows for quantitative assessment of the profiles using similar guidelines designed for STR multiplex systems and with the same standard laboratory equipment. Older technologies such as TaqMan[®] assay (Applied Biosystems, Foster City, CA) rely on 5' nuclease activity of Taq polymerase and requires two specific oligonucleotide probes which bind to the SNP region of interest (185) and although more time consuming and labour intensive, could be amenable to most forensic laboratories currently using automated quantitation assays such as QuantiFiler[™] and Y-Filter[®] for routine STR casework.

With the capability of rapid biallelic analysis using automated technologies, the lack of mutation at most SNP loci, the selection from both coding and non-coding regions of the genome and the foundation for enhancing SNP genotype differences among populations, the potential for intelligence mining through DNA analysis has become a potential reality. Recently Kidd and co-workers (212) have described the selection process for development and testing of a SNP panel designed to investigate man=s ancestral origins as well as providing forensically relevant information. Important criteria such as: 1) high locus diversity (heterozygosity >0.45), 2) independent chromosome location or minimal distances from other informative forensic STR loci (>

1.0 Mb between loci), and 3) low association with populations ($F_{st} < 0.01$) were used to screen more than 90,000 candidate loci to derive the best 19 SNPs which were assembled into a multiplex system. Current studies are being conducted on numerous populations to determine variation and potential individual differences in the SNP genotypes which are expected to be much greater than the current STR loci used in forensic investigations.

In addition to geographical population variation, specific SNP markers have been used to determine specific physical traits such as red hair (213-215) skin pigmentation (216) and eye colour (217) and have been extensively discussed in Daniel and Walsh's review article (213) and more recently from a technical as well as practical consideration by Tully (33). Using a SNP panel for the melanocortin 1 receptor gene (MC1R), Tully (33) has been quick to point out that although SNP typing for physical pigmentation genes are potentially useful for investigative purposes, often such traits are very complex and pleiotropic such that appearance represents a continuous spectrum based on self-descriptions and how others may describe the same person. In addition, self-descriptions and eyewitness accounts have always been subject to personal disguise, age, environmental conditions (poor lighting, night etc.) as well as difficulty in standard recording of attributes.

Frudakis et al., (218,221) described the first commercially available screening process for physical trait detection using a panel of 745 SNPs, of which 335 were found in the eye iris pigmentation genes. Further development by Strum and co-workers (221) noted that COA2 gene on chromosome 15 is the major determinant of brown or blue eye colour although other genes could play a role. This led to the first high throughput genetic test for eye colour (RETINOME^J) which has been based on thousands of Europeans and in blind trials has been reported by the authors to be 97% accurate in prediction of ancestry. Candidate loci picked for geographical profiling have been referred to as AIMS (222) and significant correlations have been made between estimates of individual ancestry and skin pigmentation gradients (216). A further extension of Frudakis' work (223) involving 211 human pigmentation and xenobiotic metabolism genes led to the development of 56 informative SNP loci that have been reported by the authors to accurately classify European, African, and Asian donors with 99%, 98%, and 100% accuracy, respectively. Using a subset of this panel composed of 15 loci the accuracy drops to 98%, 91%, and 97% respectively. This research has currently been made into a commercially available service called DNAWitness[™] for inference of bio-geographical ancestry for both genealogical studies as well as a potential investigative tool. There is little doubt that our knowledge of other SNP systems as well as STR and mtDNA profiling will add to the potential AIMS knowledge base in the near future. Although the AIMS tests have achieved some success with police investigations, there are still many questions to be addressed on the science regarding the application of this procedure and the ethical as well as the privacy issues associated with tracking and databasing known human physical trait markers (33, 219).

Automated DNA Extraction of Biological Evidence

Automated processing for forensic DNA analysis is considered essential for high throughput as well as a quality assurance measure which could greatly reduce error in routine sample preparations. Many consider the most critical phase of forensic DNA analysis is the initial sample preparation and extraction of as much highly purified DNA as possible from an exhibit while preventing and minimizing any potential contamination. A forensic laboratory's first experience with DNA automation often comes through work first performed under a controlled sample collection process using a fixed substrate or oral swab for high criminal offender databases (240, 241). The primary route chosen to achieve automated processing for operational forensic casework laboratories required to process many different substrates and biological tissues has been the optimization and validation of silica-coated magnetic bead purification, and what was initially exploratory from a research perspective has now been made commercially available from several vendors (i.e. Qiagen, Tecan, Beckman, Promega). In the last three years considerable effort has been expended to validate DNA automated extraction protocols at the small laboratory as well as larger higher throughput processing centres.

Typically the key to automated DNA extraction using silicon based capture methods involves pre-treatment of stains and samples to promote cell lysis. Laboratories have used different pre-incubation extraction procedures to prepare samples prior to a more rigorous lysis treatment in the presence of a chaotropic agent. This step is followed by binding the DNA to silica-coated magnetic beads and isolating the DNA fraction through magnetic force or a combination of centrifugation and magnetic isolation followed by elution of the DNA from the isolated concentrated DNA-bound beads. This theme has been repeated by numerous laboratories usually in a manual process first, followed by its adaptation on a robotic platform.

Nagy and coworkers (224) have optimized sample preparation using an M-48 BioRobot⁷ from Qiagen (GenoM™ M-48 Robotic Workstation Genovision, Wien, Hilden, Germany) for more than 20,000 routine forensic laboratory samples without the use of centrifugation and solvent extraction protocols through a completely closed extraction robot. Through careful control of contamination between runs using both a specialized drip tray and UV sterilization, blood and other forensic samples were extracted using the DNA Kit GenoPrep^J and the Mat Attract DNA Blood M96 Kit (both from Qiagen). The authors defined their pre-extraction procedures for each commonly encountered forensic sample, using both an enzymatic lysis buffer (Proteinase K addition) and detergent based buffers. In general a Proteinase K detergent buffer was reported to promote effective cell lysis through the use of extended incubation times at 90°C, however, special modifications for telogen hair, sperm and bone (crushed and decalcified using ethylenediamine tetraacetic acid (EDTA) were also described using an automated protocol. The authors reported that a complete ProfilerPlus^J STR profile could be generated from as few as 3°C cells with some drop off of alleles and compared favorably to the 15 -18 cell minimum required to obtain results using an

organic based manual extraction process. The authors also confirmed the usefulness of a TN_{CA} lysis buffer described originally by Hellmann et al. (64) for automated DNA extraction of hair samples yielding a 60% full profile success rate on more than 90 hairs and a 21% partial STR profile for the remaining hair samples. A similar finding was reported for the TN_{CA} lysis process by the German Federal Police Office from hair analysis over the past four years (225). Overall, the study clearly demonstrated that the automated silica-based DNA capture procedure was more successful in obtaining high quality DNA from forensic exhibits than the traditional phenol/chloroform extraction procedure. In cases which failed to yield an automated DNA result, the phenol/chloroform procedure conducted on the remaining lysis sample (approximately one third of the total extracted DNA from the lysis step was retained prior to further DNA processing) also failed to yield any DNA results using the ProfilerPlus™ STR assay. No contamination was reported from the automated extraction component which used a drop catcher, UV sterilization, and disposable aerosol prevention tips. Mixed STR profiles were encountered with bone DNA processing which suggested the contamination was most likely attributable to the preparation of the bone prior to the automated extraction step. The authors did not advocate the use of quantitation and went directly to STR analysis based on what the expected DNA recovery would be from a variety of samples previously evaluated.

Other laboratories followed similar approaches to validate automated extraction procedures using a variety of incubation lysis protocols prior to DNA isolation with magnetic beads. Komonski and coworkers (226) used the commercially available DNA IQ™ (Promega, Madison Wisconsin) for rapid processing of DNA from a range of forensic samples including blood, gum, dried nasal secretions, cigarette butts and swabbed drink containers. The authors noted that the manual extraction procedure advocated by the manufacturer was primarily successful for blood, but a pre-incubation process involving both lower temperatures and enzymatic cell lysis and a single resin wash, promoted better recovery of DNA from the more challenging forensic samples. Preliminary work using the DNA IQ™ protocol on the automated liquid handling platform MultiPROBE® System from Perkin Elmer Corporation, proved to be very successful with recovery of high quality DNA yields.

Extensive validation and operational casework has also been performed on Tecan robotic workstations using DNAIQ™ and a combination of fixed tips and disposable tips (242, 243). A critical issue for fixed tip manipulation of liquids with robotic systems is the development of processes that eliminate crossover contamination through an extensive series of on-board cleaning protocols. The balance of cost-effective elimination of disposable tips must be carefully considered with the amount of time required to purge and clean all tips that contacted DNA-containing solutions so that the end state is a rapid processing of large numbers of samples (242).

Schiffner and coworkers (227) reviewed commercially available extraction procedures (DNA IQ™ (Promega, Madison Wisconsin, USA and QiaAMP, Qiagen, Valencis, CA, USA) and compared yields and quality of DNA to results obtained from simple extraction procedures using a single buffer. It was found that reducing the

manipulation to fewer steps and using Chelex-100 or a 0.01% SDS lysis buffer containing Proteinase K followed by ultra-filtration concentration enhanced the recovery of DNA and STR profiling compared to results obtained from samples processed with commercial extraction products. A key aspect to their optimized yield recovery was using PolyA RNA as both carrier and matrix binding agent to prevent DNA from binding preferentially to plastic and membrane surfaces. This study demonstrated that recovery of low copy number DNA from challenging samples could be enhanced through the use of fewer handling and transferring steps. This makes the entire process extremely cost effective and amenable to automation simply by using the robot as a liquid handling manipulator. Subsequent large scale multi-sample recovery on a single plate equipped with 96 filtration wells (Microcon-96 Retentate Assembly Plate, Millipore, Bedford, MA, USA) was also evaluated. It is noted that Schiffner (227) focused primarily on recovery of low copy number of DNA ($\leq 200\text{pg}$) and it is unknown how well their simplified protocol would work on evidence with larger amounts of DNA (i.e. $\geq 200\text{ pg}$) or more challenging samples (other than blood and buccal). Other approaches for rapid DNA processing from biological exhibits have included the use of novel enzymes that are controlled by temperature shift and enable a simple one tube closed DNA extraction system (244). Beyond some exploratory manual casework analysis, there is no indication from the scientific literature that this approach has been implemented on automated platforms.

The application of automation and its potential for a small forensic laboratory was demonstrated by Crouse et al (228), using a BioMek2000 (Beckman Instruments, Fullerton, CA, USA) and a modified DNA IQ™ process. An extraction and quantitation process for challenging exhibits such as blood, tissue, bone, hair, epithelial cells (touched evidence) and mixed stains such as semen was developed with an end result that increased the number of evidence samples tested and decreased the number of negative samples amplified. The automated procedure based on disposable fixed tips proved to be highly effective and enabled 96 samples to be processed after cell lysis in approximately 20 minutes without any contamination or carryover. Equally important was the demonstration that real time PCR quantitation provided a key efficiency step such that any sample that was truly negative (no amplifiable template) would be immediately identified and no further work involving STR analysis was required. The validation of Quantifiler Human and Y Quantitation kits (Applied Biosystems, Foster City, CA, USA) used 36 cycles and the absence of DNA results as the negative threshold. Interestingly, this laboratory went further and evaluated the feasibility of using a micro fabricated 96-channel radial capillary electrophoresis array prototype (229). The validation outline the authors developed, as well as the threshold sensitivity studies performed for real-time quantitation, is useful additions to any laboratory planning to carry out automation work.

A key factor for achieving an efficient automated processing data bank system relies on using strict guidelines and a sensitive standard collection protocol. This ensures uniformity in samples which promotes optimal process efficiencies. However, most forensic laboratories process very challenging samples and unlike clinical processes in data banks, hospital or research settings, the quantity or the quality of starting material is highly variable and often degraded or contaminated with a variety of

substances. Consequently, any robotic system that functions in an operational forensic laboratory must be robust enough to address known and unknown variations in sample evidence. The most difficult sample recovery is arguably from sexual assault samples which also represents the majority of serious cases encountered in a forensic program. By the very nature of the offence, a sexual assault sample typically represents a mixture of two donors, usually composed of female epithelial cells and male spermatozoa. To fully promote automation in the forensic setting one must address automated differential extraction of sexual assault samples.

The Greenspoon laboratory (234-236) has been a leading proponent of high throughput automated DNA extraction. This laboratory has reduced the manual organic extraction of sexual assault samples from a 5 hour manual procedure to a 1 hour and 50 minute semi-automated process capable of extracting 88 samples plus controls using a 96 well plate format on a BioMek2000. High quality DNA was recovered from a wide variety of forensic samples on different substrates (235). Several optimized pre-extraction incubation steps for cell lysis pre-treated a variety of biological samples collected on different substrates. The lysed DNA- containing buffer was applied to a single standard automated paramagnetic bead recovery system using an open well, fully-automated extraction process based on the DNA IQ^J system. A complete validation study that looked at mixtures, sensitivity, environmental insults, inhibition by substrates, and contamination demonstrated minimal cross-contamination. The detection sensitivity (down to 1:1000 blood dilutions) typically outperformed manually organic extracted samples in three different blood dilution tests. Interestingly, some STR alleles were detected at 1:10,000 dilution of blood even though the standard presumptive test using phenolphthalein-tetramethylbenzidine failed to indicate blood was present. It was noted that sexual assault samples proved challenging beyond a 1:100 female to male ratio, and better performance using the automated process over manual organic extraction was reported.

An unknown crossover contamination was noted (235) with the automated protocol in some sample wells but was absent in the control negative sample wells. The authors attributed these unusual results to potential reuse of training samples and the introduction of contamination during the initial sample preparation. It was also noted that biological fluids applied on some oily substrates yielded DNA that only partially amplified suggesting that a contaminant may have co-extracted along with the DNA which could interfere with recovery of DNA as well as the quality of the final extracted product.

Additional studies were reported in a different paper by the same authors (236) on enhancement of the process through prototype software which controls the robotic interface program (Normalization Wizard, Beckman, Fullerton CA) and enables rapid and accurate dilution of all test samples. PCR products using the automated dilution software and the robotics platform were comparable to more time-consuming labour-intensive manual processes. The authors used a disposable tip robotic platform and kept all amplification tubes and PCR master mix tubes closed and off the robotic deck at all times. An impressive cross-laboratory validation comparison involving 1,018

samples processed on five independent BioMek2000 automated workstations by seven scientists demonstrated the enhanced capacity of automated processing. Although eight very weak STR profiles (drop-in alleles) were detected in 509 blank negative controls, these profiles were only evident in extreme situations where adjacent blank wells were surrounded by artificially spiked wells containing high concentrations of biological sample (DNA in checkerboard pattern). Other test samples taken through the entire STR typing process showed no extraneous unaccounted alleles. It should be noted that the STR detection assay (FMBio Fluorimager - STR fluorescent system, Hitachi, San Francisco, CA, USA) and the DNA quantitation system (AluQuant® Human DNA Quantitation System, Promega, Madison WI, USA) used to assess the results of their automated process have different detection sensitivities and operating parameters than those of other forensic laboratories employing Applied Biosystem technologies.

Several robotic platforms have been introduced to the forensic laboratory over the past decade, primarily for processing samples collected for criminal offender databases. The majority of these robots are large format multi-functional platforms designed to process multiple 96- or 384- well plates. The size and cost of such robots may be prohibitive for the smaller forensic laboratory required to process biological evidence from fewer numbers of cases. Two robots have been introduced that are primarily designed for the smaller laboratory or for special purpose use such as mass disaster triage centres or mobile labs that process samples close to the scene of the incident.

The Qiagen BioRobot EZ1 workstation is a small, rapid and reliable extraction instrument that functions using pre-programmed extraction protocol cards and a single use cartridge system. Six or fewer samples are initially incubated under different optimized cell lysis conditions depending on the nature of the biological evidence, and then placed on the robotic platform for automated extraction and recovery of DNA in approximately 20 minutes. The self-contained cartridges use a strong chaotropic agent (guanidine thiocyanate and guanidine hydrochloride) to fully lyse the cells, denature all remaining proteins and inhibit nucleases, as well as promote the subsequent binding of DNA to paramagnetic silica beads. On the BioRobot EZ1 the binding of DNA to the silica beads as well as the washing steps occur within a barrier pipette tip before release of purified DNA by elution in a solution of low ionic strength.

Several validation studies using the Qiagen EZ1 robot have been published. Initial work by Montpetit (237) for the San Diego Police Department included validation of a wide variety of biological samples on different substrates as well as over 1,000 forensic casework samples. In general the robot-extracted DNA was comparable in both quality and yield to DNA recovered using the more labor-intensive phenol-chloroform manual extraction process. DNA recovery, elution optimization, cross-contamination, bloodstain, saliva and mock sexual assault samples were evaluated as part of the validation process. Some loss of DNA was reported (60-70%) compared to the maximum yield expected, and considering the nature of the exhibits and the elution volume used. No cross contamination was reported in over 400 test samples evaluated and the magnetic bead process in general was considered an effective

means to eliminate dyes and PCR inhibitors that sometimes co-purify or become trapped in the aqueous phase of an organic extraction. Montpetit (237) did set a cut-off threshold of 300 spermatozoa for their automated extraction. Factors such as stain size, age and abundance of sperm and epithelial cells should be assessed in deciding on a suitable extraction strategy.

Anslinger et al. (238) also published a validation report for the Qiagen EZ1 also in 2005. Multiple samples from a variety of biological origins including blood and semen, cigarette butts, blood contact stains collected on cotton swabs soaked in ethanol, tooth pulp and paraffin embedded fixed tissues yielded excellent results with the automated process and surpassed their comparison results from chelex extracted casework. The key to their success was preparing the stain or biological evidence in a buffer that ensured full lysis of the cells and solubilization of the DNA. This laboratory did report some variability in the reagent extraction cartridges provided as a commercial products from the kit manufacturer, which they attribute as the cause of some of the variation noted in reproducibility studies conducted on the same samples. It should be realized that their work was done early in the stages of EZ1 forensic implementation.

A very comprehensive validation was performed on the Qiagen system (BioRobot® EZ1 up to 6 samples at a time and BioRobot® M48 6-48 samples) by Kishore and co-workers (239) prior to its robotic implementation in casework. The M48 consistently produced lower recoveries of DNA compared to standard organic extraction and the EZ1 also underperformed using the manufacturer's standard recommendations. However, through a careful series of experiments involving serially diluted blood and semen it became apparent that the automated process was experiencing a loss of a fixed amount of DNA due to nonspecific adhesion to sites on the silica beads and/or the walls of the container. This was especially prevalent for samples containing low amounts of DNA. By adding RNA as a carrier to the cell lysates, a significant increase in DNA yield was achieved, as much as 40-fold in some cases compared to samples processed without a carrier according to the manufacturing instructions for the QiAMP Micro Kit, MagAttract DNA Mini M-48 Kit or the EZ1 DNA Tissue Kit (Qiagen). Overall with this simple addition of a carrier, the robot's performance was as good as or better than organic manual procedures and proved to be most effective in the elimination of PCR inhibitors and promoting rapid DNA forensic casework processing. These studies prompted Qiagen to change the kit protocols and currently the addition of carrier RNA is an option provided with their reagent cartridges and has also prompted a change to many in-house procedures in different laboratories which experienced DNA loss in forensic exhibits containing less than 500 pg of DNA.

The second small service function robot recently released by Promega Corporation - Maxwell¹ 16 Instrument (250) - allows efficient, automated purification from a wide range of sample types using preprogrammed purification protocols and pre-dispensed reagent cartridges (249). The instrument processes up to 16 samples in approximately 30 minutes using a proprietary magnetic-silica-bead device that captures the DNA-coated magnetic beads and transports the beads through a series of washing and elution steps to achieve purified DNA. The MaxwellTM 16 Instrument

has only been recently released and there are no independent published reports at the time that this paper was written.

Extensive automation validation studies have also been reported for robotic systems using the Tecan Genesis RSP 150/8 workstation (243, 247) as well as the Hamilton Star robot systems (248). Frégeau and co-workers (247) report on a stepwise evaluation of the DNAIQ™ system using liquid handling with fixed tips on high volume casework as well as differential extraction for sexual assault processing. Considerable effort was expended to ensure no contamination occurred between adjacent wells using a 96 well plate through a series of specialized washes and redesigned tips and fluid trays. The end result was a rapid casework processing system that incorporated the extraction as well as quantitation of DNA from a wide variety of forensic exhibits within a rigorous documented sample tracking and control system. A large-scale single processing system involving 8 Tecan robotic platforms (EVO) set up in series that is capable of processing and quantifying 800 samples per day was also implemented by the South African Police Services (243). Although there was no published literature regarding the validation studies and final results of this project at the time this report was written, there is no doubt that lessons learned and best practices for such a major project will enhance our knowledge of automated DNA implementation in single large centres versus multiple smaller DNA processing centres. Hancock and Schumm (248) also reported validation studies, the first operational experiences using a Hamilton Star robotic platform and the development of adaptable software that provides significant flexibility in a commercial environment. It was clearly noted that an operational commercial laboratory that provides client-based services, will require different sample handling parameters as well as adaptability to use several different STR kits and extraction chemistries on a single robotic platform.

The forensic community is also looking to clinical diagnostic fields involved in high-throughput genotyping for human blood cells, platelets and leukocyte antigens to develop best practices and potential forensic applications. Platforms that combine amplification and detection will undoubtedly usher in a new era of clinical diagnostics and potentially hold promise for rapid screening of forensic exhibits (230). Real-time PCR using the LightCycler (Roche) has been evaluated for genotyping of blood groups Duffy, RhD and RhCE, and the Taqman (Applied Biosystem) PCR methodology has been used to conduct Kidd genotyping and fetal RhD results. Blood group genotyping has shown excellent concordance between the new pyrosequencing assay and traditional genotyping using allele specific primer amplification. Pushing classical serotyping to its limits has been demonstrated with the GenomeLab SNP-stream system which has simultaneously processed more than 20 blood and platelet antigen groups at a rate of 4,600 > 800,000 SNP genotypes in a single day (231, 232). The process design involves PCR amplification, amplicon hybridization with SNP primers, capture of extended primers on an array plate and detection of fluorescence. A parallel system using BeadChip technology has also been used for genotyping a variety of blood groups with full concordance (233). It is highly likely that the larger market for clinical diagnostic tests and the prospect of commercial development will advance the innovation and implementation of rapid screening technologies much

faster than what would be expected for similar tests developed exclusively for forensic applications. Consequently, although there may be a slight delay in the implementation of a clinical diagnostic tool for forensic use, the similar validation, rigorous quality control, ease of use as well as low cost and high benefit characteristics of such tests should enable the rapid adoption and implementation of clinical diagnostic technologies for forensic purposes.

A detailed and comprehensive review of automated forensic sample processing has been published by Leclair and Scholl (245). This report leveraged the authors' extensive experience in criminal and clinical genotyping as well as mass disaster victim identification which allowed them to develop effective best practices guidelines for the creation and implementation of a casework sample processing system. The authors reinforced the primary expectations of increased sample flow, creation of robust and computer-based chain of custody, improvement of the timeliness of processing and cost reduction. The attributes of automated fluid handling with different pipette tips (fixed and disposable) were illustrated by numerous examples and clearly demonstrated the validation and implementation steps required to achieve a successful automated extraction and processing system. The basic automated liquid class protocols were also discussed with respect to integrating volume verification steps (246) to improve data quality by capitalizing on the precision and accuracy afforded by automation. This review also discussed the need for continued development and improvement of new technologies to safeguard against over-capacity and under-utilization, such that the maximum efficiency and best performance is gained through high-utilization. This single paper presents the process and strategic considerations of forensic automation along with advantages and disadvantages in a manner that will ensure its place as a key reference for many years to come.

Low Copy Number and Sensitive DNA Detection

In the field of DNA analysis the expectation of obtaining more probative information from smaller amounts of evidence (251) must be balanced with valid and reliable processes, not exceeding the limits of technology. This is particularly relevant in obtaining DNA profiles from trace evidence transferred by touch and casual contact which contains low amounts of template DNA or what many refer to as using "low copy number" (LCN) analysis (259, 263). LCN DNA typing has been defined as "the analysis of any results below the stochastic threshold for normal interpretation". Samples containing < 100 pg DNA fall into this category and require specialized techniques and rules of interpretation (259). Many aspects of LCN enhancements have been reported in the past few years, most of which fall into the categories of: 1) raising the number of amplification cycles (254, 268) 2) using whole genome amplification (265,266,269,270) 3) developing special guidelines for the interpretation and statistical analysis of data (258, 259, 267) and 4) more recently, enhancements of extraction procedures to recover better quality DNA (253, 264). LCN no doubt may be the last recourse to solving a challenging older case (252) but strategies dealing with allele dropout and drop-in, higher stutter peaks and sporadic contamination must be fully appreciated. From a more practical consideration, knowledge of shed evidence

and understanding the crime scene may have as much relevance to using LCN as any technical or scientific consideration.

How much DNA is enough and how did it get there in the first place? The forensic scientist has faced the same problems as anthropologists regarding authentication and plausibility of results (255). In some instances guidelines for the interpretation of STR profiles derived from less than 100 pg have contributed to better use of evidence but have also raised cautionary issues (258, 259), and in the end, guidelines alone may not distinguish true identity from contamination. Several recent studies highlight our lack of understanding regarding the potential for casual contact DNA being present at the crime scene. Although experiments on LCN represent difficult control challenges (absence of DNA), Port and coworkers (260) have demonstrated that a person speaking less than 30 seconds from a distance ranging between 69-115 cm can deposit sufficient amounts of DNA that the speaker can be identified from objects or persons directly in front of them. Our basic understanding of casual contact and transfer of DNA biological material has also been investigated using experimental designs originally described by Lowe et al (262). Phipps and Petricevic (261) attempted to characterize the variables associated with “shedding” (loss or shedding of DNA containing cellular material from individuals) with 60 volunteers tested under different conditions of hand washing and time that elapsed since touching. Their findings proved to be more complex than expected and the authors conclude that they do not know what makes a good or bad “shedder” but such knowledge is very important prior to the interpretation of trace DNA evidence.

In reviewing LCN results it is important to recognize the difference between contamination and biological evidence that may contain a mixed DNA profile. Contamination or the introduction of new material (i.e. biological material) that was not originally at the crime scene may be caused by the investigator or laboratory personnel. Elimination databases of DNA profiles composed of profiles from police and laboratory members who collect and process evidence, is a key strategy to interpret LCN or any DNA results. One recognizes the potential for LCN contamination concerns after recent studies conducted by van Oorschot (256) which report the transfer of DNA from fingerprint brushes that were used to powder over biological stains (blood or saliva). In contrast to other studies, the authors noted (256) that some fingerprint powders can inhibit PCR amplification. In other experiments, material accumulated on one brush after powdering a biological stain was enough to transfer DNA to other brushes stored together, and derive STR identification. This study concluded that fingerprint brushes should be a consideration in the interpretation of unknown profiles derived from evidence and the authors made a detailed 15 point set of recommendations regarding prevention.

Although the prospect of truly “negative controls” or the complete absence of any DNA in the laboratory has been discussed (275), contamination from plastic ware and water as an exogenous source of DNA remains concerning. Tamariz (257) reported on successful STR amplification results following contamination studies with limited amounts of DNA. The longevity and accumulation of DNA that was found on “mocked-up” tubes following routine laboratory extraction and PCR practices was

evaluated. They found that very close exposure to ultraviolet irradiation from a commercial UV cross linker (Stratagene, La Jolla, CA) for 30 minutes, could greatly diminish the carryover of DNA from lab ware and water used for subsequent PCR amplification. Unfortunately, such a prolonged exposure and the potential loss of intensity over time of many UV irradiation devices (UV tube decay) makes this a challenging solution to prevent contamination for many routine DNA analysis procedures.

One approach not always taken into consideration for processing limited amounts of DNA evidence is better extraction and use of amplified products. Smith and Ballantyne (253) compared several commercially available nucleic acid ultra filtration devices as well as enzyme hydrolysis mediated purification procedures to concentrate the post PCR fluorescently labeled STR product. They found that 28 cycle amplified products purified by the Qiagen MiniElute silica column resulted in a fourfold increase in fluorescent signal intensity over unpurified product. The net result from this and other studies by Hutchinson (264) clearly demonstrate that a simple procedure which enhances signal intensity of STR amplified products obtained from standard amplification protocols, may provide equivalent or better results than LCN analysis.

Interpretation of the results and presentation in court would follow the precedent set for standard STR DNA evidence practices.

Software Assistance for DNA Analysis and Interpretation of STR DNA Evidence

An expert system is any type of artificial intelligence software that uses a pre-programmed set of rules to provide interpretation or conclusions and tries to parallel the human logic decision making process derived through experience and expertise (293). One of the more challenging aspects of forensic science is the interpretation of DNA evidence from a crime scene which can be compounded in complexity by multiple donors. The use of expert computer systems has become more prevalent as high-throughput analytical systems generate large amounts of data which are time-consuming to process (273, 278, 281). This has been particularly noted in mass disaster victim identification as well as a fundamental addition for growing criminal offender databases (294, 279). There are two major approaches taken in expert systems: 1) the probabilistic approach which uses information contained in large databases to formulate the probabilities of alleles being present or not present based on prior knowledge and 2) the graphical simulation model that is designed to replicate all aspects of the DNA process and can be used to predict the outcome of specific scenarios based on the quality of the DNA data sets.

Typically, any number of alleles may be observed in a sample of unknown origin (i.e. crime scene) but if only one or two alleles per locus are observed, then the sample is considered to come from a single donor. Degradation of the evidence which could lead to an incomplete DNA profile (i.e. fewer loci giving results), a mixture with one donor representing less than ten percent of the amplified product or the overall recovery of low amounts of DNA could lead to masking of a second or multiple donor(s) making it difficult to determine the number of overall potential contributors

(271). Recent studies by Buckleton and coworkers (272) have assessed the risk associated with current practices of using likelihood ratios to interpret mixed DNA profiles and reject the Paoletti et al. (271) argument that no assessment of such evidence can be made. The effect of masking on the number of alleles presented in a mixture is evaluated with simulation studies performed on actual data sets composed of the Profiler Plus set of loci from Australian forensic laboratories and SGM+™ from New Zealand and the United Kingdom. The true risk of the incorrect assignment of numbers of contributors was evaluated with both STR systems and specific recommendations are made based on past work conducted on STR interpretation guidelines (273). Considerations for mixture interpretation have posed an interesting challenge for low levels of DNA detection (259), and indicate that repeat measurements of DNA evidence for multiple donors and population substructure can be invaluable to address concerns (274). A further extension of interpretation of low copy number (LCN) STR profiles was described by Gill (258) with the development and testing of an expert interpretation system (*LoComotionN*) which uses probabilistic theory to calculate numerous alternative explanations for the likelihood of origin of specific STR profiles. Unlike the consensus method which requires allele concordance demonstration from separate PCR analyses, *LoComotionN* is intended to maximize the full potential of limited DNA evidence which is particularly relevant to enhanced or sensitive detection of STR data from touched evidence or environmentally challenged biological exhibits. The mixture complexity issue will undoubtedly be a contentious topic of future discussions and has also been addressed as a priority for review by a working subgroup of the SWGDAM (July 2007).

A novel forensic approach for evaluating expert systems for STR interpretation was developed by Gill et al. (281, 276) and uses a graphical model to simulate stochastic variation associated with the entire DNA process starting from extraction of the sample followed by PCR preparation and the STR results following amplification. The authors have derived specific input efficiency parameters for each step of the DNA process and the expected output parameters of a quality STR profile (heterozygote balance, PCR slippage mutations or stutter and allelic drop-out) are evaluated from a perspective of improving the overall DNA profile results and the performance of the process. Several statistical methods have been introduced in the past to aid interpretation (273, 277, 278) but the models developed are primarily based on STR results typically derived from an optimal DNA process. The Gill model (276) based on a Bayes Net specifically focuses on predictive scenarios that allow for data input for less than optimal DNA processing and allows input from conditions involving low copy number DNA amplification, minor contributions and highly variable stutter with significant heterozygote imbalance. The overall concept of this innovative approach was to derive a formal statistical model with a computer application called PCRSIM that enables test data sets to be developed from allele frequency databases and that takes into account the most difficult DNA processing parameters. In theory, this should enable the faster validation of new methods and better optimization of multiple DNA processing parameters. How this approach will be incorporated into current forensic research and development with actual test scenarios across different laboratory systems, should prove to be both an exciting challenge as well as providing

a new way of evaluating future techniques and critiquing past experiences to achieve better optimization.

The availability of computer software to carry out systematic mathematical analysis using available STR profile characteristics (peak heights, heterozygous ratios, presence and absence of alleles etc.) is expected to reduce the time required for DNA profile interpretation and improve its consistency. Studies that have been presented in recent years involve the interpretation of mixtures based on quantitative allele peak data and recommendations are based on observation and experience from operational casework. A group at Forensic Science Services (FSS) have developed PENDULUM (273) which is marketed as *i*-Stream, part of their i^3 software package which is based on the previous guidelines established by Clayton (280). Perlin and Szabady (278) have reported on the linear mixture analysis method (LMA) in actual forensic scenarios depending on what is known about the genotype of the potential contributors. A probabilistic expert system developed in conjunction with quantitative peak data was also discussed by Mortera (282) that also takes into account the number of known genotype donors. Wang et al. (283) have also developed a framework for interpretation (Least-squares Devolution -LSD) that is guided by the least square analysis of results of the quantitative peak data of either peak area or height of the STR profile results and a set of heuristic rules for best fit mass proportion ratios of genotype contributors. All of the methods make assumptions. For example, the LSD method (283) assumes correct allele calls, no artifacts (no stutter or pull up) and no peak overloading or saturation and all peak proportionality is based on alleles amplified to the same consistency.

The natural evolution of interpretation software development is validation and concordance studies performed by end users of the software. Ryan and coworkers (284) have developed and used a concordance analysis system (CompareCallsSM) to specifically compare STR databases in order to assist in meeting the US Department of Justice's (CODIS - Combined DNA Index System) public compliance for "100% technical review" of vendor generated STR data. CompareCallsSM was applied to 290,676 CODIS STR markers generated from unreviewed data from GenotyperTM (Applied Biosystems, Foster City, CA) with human-reviewed data from SurelockIDSM (Myriad Genetics Laboratories, Salt Lake City, UT). The automated allele concordance system simplified the comparison and flagged any potential questionable allele calls demonstrating an efficient means to review high-quality data for CODIS. The validation conducted by the New York State Convicted Offender DNA Databank Group (285) was the first US laboratory validation of TrueAllele[®] and clearly demonstrated that human intervention and expert review was only required for low-quality STR data.

Consequently, laboratories engaged in adopting some form of artificial intelligence or software- driven guidelines will need to assess the best approach based on their specific needs and the quality of the data sets produced in their own laboratory. This will involve a benchmark set of acceptable assumptions. At the very least, software assistance could be used as an evaluation tool for casework, with a level of objectivity based on mathematics and accumulated simulations from large data sets to achieve

some form of consistency in interpretation and the potential for an unbiased review. How this will unfold in years to come will depend on rigorous evaluations and acceptance within international working groups. It is interesting to note that the sub-committee SWGDAM (July 2007 Quantico VI), has recently recommended that forensic interpretation software could be used for the stand-alone interpretation of STR profiles from single source donors such as samples collected for criminal offender databases but was not acceptable at this time for samples of unknown origin that could be composed of multiple donors and the use of these types of software systems should be used only as a data evaluation tool.

Unrelated to statistical interpretation of STR profiles but equally important is the development and documentation of validation studies conducted on forensic DNA processes. Although larger laboratories may have the benefit of specifically trained project managers, the average forensic laboratory must assign this responsibility to select operational scientists. Recently, Applied Biosystems has introduced a software package (VALID™) which is specifically designed to assist in validation studies required by SWGDAM. The software is based on a traditional project management approach which allows the end-user to design, implement, document/archive all findings and data and write up the final study results. This software should reduce the amount of time and labour required to validate new technologies. The software was recently introduced and there are no current reports on its functionality in an operational laboratory at this time.

Further interpretation challenges in STR DNA casework have also been addressed in potential laboratory contamination simulations by Gill and Kirkham (275). In particular the issue of negative controls for monitoring tube-specific contamination was determined to be an inadequate assurance that an associated batch of extracted casework material is contaminant-free. The authors discuss in detail the different types of contamination, detection and the potential impact by empirical review of interpretation guidelines for STR analysis and real data sets taken from the operations of the Forensic Science Services. An estimation of expected contamination of false positive results can be established and mitigation of risk was discussed with respect to regular and low level DNA evidence and the use of expert systems to analyze negative controls.

DNA in Mass Disasters and Mass Screening/Kinship Analysis

Primarily due to tragic circumstances on a very large scale (9/11 World Trade Center Attack and 2004 Tsunami disaster in South East Asia), considerations over the processing and use of DNA evidence, biological collection, kinship comparisons and the overall logistics of processing large complex data sets have recently been a major endeavour of many forensic laboratories. Numerous detailed reference guides concerning all aspects of evidence collection and incident-specific mass disaster management or mass screening have been written. The principal rationale for forensic scientists engaged in DNA analysis following a mass disaster or large scale criminal investigation is to derive DNA evidence and affect individualization of biological material for missing persons and unsolved casework. This task is complex

and challenging from a scientific as well as from a ethical perspective raising privacy and security issues.

Budowle, Bieber and Eisenberg (295) have noted that law enforcement/and or public safety and health officials often have a primary responsibility for identifying human remains found at the scene so the missing individuals can be identified and returned to their families and loved ones. Although DNA has been used in the past to identify victims of aircraft or natural disasters, the large scale utility for developing reference databases and associating many separated remains or body parts has been unprecedented. These authors derive a set of general guidelines from a very logical step-by-step action plan that incorporates all aspects of sample collection (preservation, shipping and storage), documentation/tracking of samples, laboratory facilities, quality assurance practices and controls, work packages, laboratory processes and use of automation, bioinformatics, governance and advisory panels, education as well as communication messages, and privacy and security issues. A particularly relevant comment made by the authors is the reminder that any information that supports a correct identification is invaluable, which is sometimes forgotten by specialists who are primarily trained in a single forensic discipline.

Alonso et al (296) have also identified the main challenges of identifying victims in several large incidents such as the South Asian Tsunami disaster, Madrid bombings of March 11, 2004 and an aircraft accident. A practical description of the logistics and management of an incident are noted in an expanded discussion on database searching and concordance kinship analysis with respect to match significance and the need for interpretation criteria for biostatistical evaluation of joint autosomal and haploid DNA data. The DNA Commission of the ISFG published a set of 12 recommendations which cover in detail the main considerations for forensic genetics and mass victim identification (297). The US Department of Justice also published a mass fatality incident guideline for forensic identification which is written from the perspective of a first responder and was co-authored by numerous experts specifically experienced in mass disasters (298).

Although many presentations at professional conferences have been made on the forensic work involved in the 9/11 World Trade Center Attack, publications have only recently become available, with undoubtedly many more to come. The lessons learned from such a tragic event will have a major impact on all future responses to man-made or natural tragedies. To date, three articles stand out. The US Department of Justice Lessons Learned from the 9/11: DNA Identification in Mass Fatality Incident is written directly from the experience of those involved (279). The information is factual and covers the scope from before the incident to the completion of the project, captured in 14 chapters with major points highlighted in bullet fashion. Key to future success is the lessons learned which this publication highlights with real data, practical comparisons such as types of samples collected from the World Trade Center Response and the resulting profiles derived from each type of sample. It is important to note that this report builds from past experience and publications (298), to derive a scalable response and disaster planning guide, culminating in the actual approach formulated and implemented during the aftermath of the World Trade Center

Attack. It is anticipated that a similar guide may evolve from the South Asian Tsunami disaster with additional lessons associated on an even larger scale with respect to numbers of victims and countries involved.

Significant DNA bioinformatics and sample continuity challenges occurred with victim identification of the World Trade Center terrorist attack. To mitigate the risk associated with rapid and continuous software development throughout the life of this large complex identification task or initiative, three different software packages were used (299). Early reports by Cash and coworkers (299) describe the challenges facing the development of the Mass Fatality Identification System (M-FYSis) during the time the identifications were being made. STRs, mtDNA, miniSTRs and SNPs were all used to assist in the identification and software cross-comparison tools did not exist prior to their immediate need. Large scale likelihood cluster probability estimates were produced by Brenner (300) using DNAView, a specialized software that assisted in the identification of the victims. Leclair et al. in a recent report (301) describe the continuity cross-checks, sample documentation and large scale genotyping kinship analysis challenges imposed by the thousands of samples processed. Leclair et al. also describe in detail the logic behind management of kinship relationships through detailed sample tracking and frequent comparisons made during the course of the investigation. The large numbers of samples demonstrated a real concern regarding the discrimination of the data sets as aptly demonstrated by a 13 STR loci profile making a fortuitous trio match involving non-related individuals amongst the victims. The large scale of the identification task which involved significant numbers of comparisons clearly presents the issue of having sufficient numbers of STR loci to reduce the risk of identification errors and also confirmed the usefulness of rapid screening using less discriminating marker sets such as mtDNA and Y-STRs. These DNA analytical techniques have specific characteristics useful to an investigation such as sensitivity or the availability of reference samples from blood relatives.

At the time of the terrorist attack on the World Trade Center, there was no infrastructure for rapid and effective victim identification in large mass disasters. One of the key strategies developed by the US National Institute of Justice for the identification project was the formation of a specialized group of scientific and medical experts called the Kinship and Data Analysis Panel (KADAP- 123). This group played an advisory role for all the DNA identification efforts (123). Experts in forensics, bioinformatics, molecular and medical statistics and population genetics recognized the “open” fatality list of an estimated 3,000 victims which was in marked contrast to a “closed” or “fixed” number of victims that would have an access to a specific area where the disaster occurred (e.g. passenger manifest on a specific aircraft). The *Science* article summarized the use of STRs, followed by mtDNA and miniSTRs and SNP technologies. The rationale regarding the minimum random match probability is briefly discussed along with issues regarding software and collection strategies (~ 1/6 of the initial reference data sets had to be re-sampled due to poorly thought out design parameters and lack of assistance). Considering that 52,000 STR, 44,000 mtDNA and 17,000 SNPs were developed more than 850 of the 1,594 victim identifications established for the 2,749 victims were made by DNA alone. In spite of the intense heat and significant co-mingling of the human remains, STRs proved to be the key

protocol followed by miniSTRs and SNPs. No direct mtDNA-based identifications were acknowledged. There is little doubt that better preparedness will enhance the rapid and effective identification of future victims of future incidents.

Although the direct comparison of DNA profiles from known individuals to crime scenes is the most common use of forensic analysis, indirect analyses using the DNA from biological relatives are often used for humanitarian identification. In the past few years, the Forensic Science Services and other police investigations (302) have offered moderate matches or less stringent comparisons between potential relatives and DNA profiles developed from evidence. This has also led to mass screenings of potential candidate volunteers for similar DNA profiles that could suggest a close relative was involved with a case (124, 304, 303). Simulation studies have indicated that kinship analysis would reveal genetic similarities and provide an invaluable tool for detecting potential suspects who are parents, children or siblings of persons whose profiles are found in national forensic databases. This raises compelling questions about the rights of the individual with respect to security and individual privacy. Bieber et al (124) highlight the improved match rate as a result of familial searching (estimated from the CODIS database, the match rate would increase by 40 %). In addition, the issues and assumptions involving kinship matching from simulations are discussed as well as specific questions about genetic surveillance and the shift in interest from an individual to a family unit. North Americans concerned over civil liberties and kinship searching of forensic databases have been very vocal and presented in a number media accounts. A detailed and informative discussion regarding the practical use of and ethical concerns regarding missing persons DNA registries, kinship matching and mass screening for casework was summarized in a special project and conference hosted by the American Society of Law, Medicine and Ethics (304). A symposium collection of 25 key articles on how to treat DNA forensic information by leading experts in the fields of science, ethics and law represents the most informative single publication on this topic and sets the foundation for all future discussions regarding society's drift towards genetic surveillance and the individual's rights to privacy and security.

Budowle (295) discussed in detail sample collection, particularly at the scene of an incident and family reference topics as well as considerations over best process techniques initially starting with STR analysis followed by miniSTRs or SNP-based technologies where warranted. The primary method of analysis will be the technology predominantly used in the forensic laboratory at the time of the mass disaster and routine standard operating procedures should be attempted first. Any new technology requires additional resources for evaluation and a laboratory engaged in a mass disaster response may not have the ability to assess the efficacy of the new technologies. In the end, the question of technology and the use of DNA must also be balanced with a careful consideration of the utility of familial searching and use of criminal as well as humanitarian DNA databases such that policy development takes into account the legal and ethical implications of privacy and security.

New Technologies for DNA and Biology Evidence Screening

For the past decade, the quest for faster and more efficient biology evidence processing has been focused primarily on DNA analysis. The advancements made in the micro fabrication field, developed as an offshoot of the electronics industry, have promised more sensitive detection of forensic DNA profiles in a shorter period of time and at a significantly reduced cost. A summary article published in 1999 (305) on the miniaturization of chemical analysis and synthesis on microchips, reported the additional advantages of maximizing genetic information from smaller amounts of biological sample with minimal sample handling which would reduce the potential for contamination. In retrospect, it is interesting to note that many of the principal investigators in the 1999 article reported that progress had been good, but eight years later are still challenged by the quality and variety of crime scene samples and only recently started to publish encouraging reports on adapting the “lab-on-a-chip” (LOC) or micro-total analysis systems (μ TAS) principles for forensic science needs. There is no doubt that significant advancements have been made in this field. However, compared to the larger and more lucrative economic incentives offered by the clinical diagnostic markets, forensic applications involving DNA, RNA or proteomics are often a secondary goal or an application that has not yet attracted the research and funding required making this technology fully functional for forensic science needs (317, 319, 312).

Hair examiners and other trace experts have taught us over many years that one of the most powerful methods in forensic investigation is the visual analysis of a sample. Finding biological evidence, and in particular spermatozoa, from sexual assault samples is time-consuming, difficult to quantitate and is subjective, based on the expertise of the examiner. Recently Buel and coworkers (321) have begun preliminary studies to develop expert system software that can self-direct the stage of a microscope to scan and record potential hits for different cell types, in particular, female epithelial cells and male spermatozoa from slides differentially stained and prepared from sexual assault evidence. Although the system does not replace the forensic trace expert, faster quantitative measurement of the many features of the image could potentially assist in automating preliminary screening of sexual assault evidence to find candidate matches. This should enable the forensic analyst to rapidly confirm the majority of matches while allowing more time for the review of challenging evidential results. It is noted that automated identification and measurement of a variety of microscopic images has been carried out for many years in the clinical diagnostic field. Automated screening has been developed for yeast colony counting and classifying, cell micro array annotation, tumour cell quantitation, wound healing assays and tissue topology measurements (306). Some may consider that a more automated process for the rapid detection and quantitation of spermatozoa could be a potential stop-gap before automated extraction and separation can be accomplished by micromachine devices. However, it is important to remember that the visual identification of cell and tissue types has a long legal precedence that is long-lasting and often requested by the investigator and expected by the courts.

The potential to incorporate sample processing steps (DNA extraction, quantitation, amplification, separation and detection of labeled products) is the ultimate goal of many forensic investigators (312). A key decision in developing microdevices for any application, including those for forensic investigations, is choosing the overall design principle. There are currently two basic categories: 1) modular design - which uses different chips for specific steps in a process or for different sample preparations or 2) integrated design - a seamless step-wise process such that all DNA sample preparation and recovery processes as well as STR analysis would be carried out on a single chip. The integrated design principle promotes the concept of “sample-in-answers-out” capability (i.e. LOC, μ TAS). To date a fully validated forensic chip based on either design principle is still remote, but several promising prototype chips that carry out some steps in the forensic process have been reported (307,308,309,310,313,317,320).

Although some laboratories have reported separation of complex human DNA mixtures using high performance liquid chromatography (311), until recently this has not been successfully performed on a micromachine device. Bienvenue (307) and coworkers have reported microchip-based cell lysis and DNA extraction from sperm cells using glass micromachined slides containing silica beads and a fluorescent based monitoring system to follow the process. This lysis chip has the potential to integrate with another chip developed by the same laboratory (320) which can separate sperm and epithelial cells in mocked up sexual assault samples. Other investigators have concentrated on rapid detection of labeled fluorescent STR products using a variety of miniaturized electrophoresis and/or electrochemical separation processes to track and identify the size of amplified products. Goedecke (308) has used a 16-lane micromachined 10-15 cm glass micromachined device that enabled rapid single base resolution of amplified products using standard commercial STR amplification kits and linear polyacrylamide matrices. The compact “uni-block” nature of the device has excellent thermal/mechanical stability and is ideal for rapid sensitive detection of STR profiles developed from low copy DNA samples or from samples composed of multiple donors (308). The device is completely mobile and is not prone to vibration interference, but it is large and does not fit the concept of a micromachine. A commercial version of the device called the GeneBench (312) conducts 16 tests simultaneously and can be scaled up to 96 and potentially 384 well formats.

In contrast, the Mathies group (309) has developed a 96-channel micro fluidic device that has integrated amplification and detection. Amplification is performed in a 160-nL chamber and the labeled STR fragments are separated in 7-cm long microcapillary array electrophoresis (μ CAE) wells etched on a circular 15 cm wafer containing 96 wells in prototype versions of the same device. STR fragments labeled with commercially available kits, have been evaluated and the researchers report excellent resolution and the ability to detect simple two donor mixtures. Yeung and coworkers (310) originally reported full STR multiplex detection with 0.17 ng of amplified target DNA and minor allele mixture detection in 3:1 female to male mixtures. A year later with slight modifications to their procedures, Liu reported (309) similar results but with a resolution improvement that enabled detection of minor STR profiles amplified from

10:1 female to male, DNA mixtures. Although sensitivity was excellent, it was noted that below 100 pg or 33 copies of DNA, the stochastic effects of low target thresholds affected reproducibility. This finding is not surprising, and is similar to results obtained from current STR mixture analysis using conventional STR protocols. A series of non-probative casework samples were evaluated with the device and found to be concordant with results developed with conventional STR amplification and electrophoresis fluorescent based detection systems (228, 309).

Microarrays from commercial manufacturers such as Affymetrics (Santa Clara, CA, USA) may be the first exposure many forensic laboratories have to this new technology (317). The GeneChip Human Mitochondrial Resequencing Array contains 5 arrays, and has a sequence capacity of 16kb and delivers a complete sequence in 48 hours. The advent of miniaturization and refinements for rapid HVI and HVII sequencing and SNP variant detection in multiple redundant processing steps on a single chip is presumably within current manufacturing capabilities. The 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) microchip electrophoresis systems were evaluated by Alonso and coworkers (316). Forensic and ancient DNA samples were interrogated with assays developed for detection of the mitochondrial hyper variable regions as well as cytochrome b and 16S ribosomal genes. The Alonso laboratory reported that they achieved fast and sensitive detection of human and animal DNA amplified fragments derived from hair and bone samples and amplicon sizes down to 100 bp. The combination of rapid analysis and sensitive detection for smaller sized STR fragments proved to be an excellent quantitative tool and was used to validate different DNA extraction strategies.

The prospect of faster DNA analysis for forensic samples and a more rapid biological detection process may receive significant support from work ongoing in microbial forensics (315, 318). There is little doubt that recent terrorist events have acted as a catalyst to derive rapid detection processes for potential bioweapons. Initial work to collect, isolate and decontaminate forensic evidence has been described (322). Investigations conducted in specialized laboratories designed to isolate and protect the investigator as well as the public, will undoubtedly create special conditions of use. However, as described by Ecker and coworkers (314), mass spectrometry derived composition signatures from PCR amplified regions of pathogen genomes can be automated to rapidly identify organisms and potential subspecies of the same bacteria and virus that contain engineered DNA components. This approach plus its miniaturization using micromachined devices for detection and identification for microbial forensics will create new expertise and experiences which benefit the forensic community as a whole.

Summary

The past three years have demonstrated progress in the field of forensic DNA typing and most recently, advances in enhanced biological evidence screening protocols. If the probative biological evidence can be found, it should be possible to integrate the laboratory process to ensure the best use of the evidence in subsequent DNA analysis. The capacity to process more samples using automated procedures should

enable DNA backlogs to be diminished and provide information in real-time for complex and high profile casework. With automated rapid processing of significant amounts of evidence, the complexity of the data and interpretation of results has grown exponentially. Fortunately, expert systems and highly refined documentation of results should provide some assistance in the final interpretation of the forensic biological evidence.

Validity and reliability of new technology or equipment rests significantly with peer review and collaborative research evaluations as noted in the numerous studies documented over the past three years. In many ways, DNA analysis is pushing the limits of detection and the ability to derive more probative data from smaller or more degraded samples must be balanced with the realistic interpretation of the information. As forensic scientists, we are also balancing significant investigative information such as biological characteristics and physical markers or kinship family relationships with an individual's rights for privacy and security of personal information. For the first time, the heightened expectations promised by micromachine technology has been partially realized with the demonstration of cell separation and cell lysis, STR amplification and detection of fluorescent tagged alleles from non-probative casework samples. As always, there will be more advances and future developments to enhance our abilities to process biological evidence, but the last three years has been very exciting with a promise of much more to come.

Acknowledgment

We thank Louise Richer and Nerine Waldron for assistance in preparation of this manuscript and Brian Yamashita, Chantal Fréreau, Ted Lukaszewski, Hiron Poon and Barbara Oattes for their many helpful comments. We are also indebted to the many laboratories who participated in our biology evidence survey.

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Appendix 1.

Recent Progress in Processing Biological Evidence and Forensic DNA Profiling A Review: 2004 to 2007 Fournery, RM.

Tables:

Table 1. Biological Evidence Survey Laboratory Participants

Table 2 Biological Evidence Screening for Blood - Survey Results of the Participating Laboratories

Table 3 Biological Evidence Screening for Semen - Survey Results of the Participating Laboratories

Table 4. Biological Evidence Screening for Saliva - Survey Results of the Participating Laboratories

Table 5. Biological Evidence Screening for Vaginal Fluid, Urine, Fecal Material and Hair Examinations - Survey Results of the Participating Laboratories

Table 6 Recent Forensic DNA Quantitation Assays

Table 7. Additional References on Statistical and Interpretation of STR Evidence

Figures:

Figure 1. Literature Survey of Biological Evidence and DNA Profiling using Medline and Embase Search Engines for the period of 2004-June 21, 2007.

Figure 2. Forensic Timeline For Biological Evidence Screening
Blood Identification

Figure 3. Forensic Timeline for Biological Evidence Screening
Semen Identification

Figure 4. Forensic Timeline for Biological Evidence Screening
Hair and other Evidence

Table 1. Biological Evidence Survey Laboratory Participants

Country	Participating Laboratory
Canada	Center of Forensic Science (CFS), Toronto, Ontario
	Laboratoire de Sciences Judiciaires et de Medicine Legal, Montreal, Quebec
	Royal Canadian Mounted Police Forensic Science and Identification Services
United States of America	Maine State Police Crime Laboratory, Augusta
	Broward County Sheriff's Office, Florida
	Orchid Cellmark, Gaithersburg, Texas
	CA Department of Justice, Central Valley Laboratory, California
	Georgia Bureau of Investigation
	Vermont Forensic Laboratory
	Arizona Department of Public Safety Crime Laboratory, Arizona
	The Florida Department of Law Enforcement, Florida
	Federal Bureau of Investigation (DNAUI)
	Palm Beach County Sheriff's Office Crime Laboratory, Florida
	PA State Police Crime Lab, Greensburg, Pennsylvania
	Office of the Chief Medical Examiner, New York City, New York
	Office of the Chief Medical Examiner, Albany, New York
	Washington State Patrol, Crime Lab Division
	Washoe County Sheriff's Office, Nevada
	Virginia Department of Forensic Science
	Texas Department of Public Safety, Lubbock
	Boston Police Department Crime Laboratory
	UNT System Center for Human Identification
	Minnesota Bureau of Criminal Apprehension/FBI Regional MtDNA Program
	Metropolitan Forensic Science Center Albuquerque, New Mexico
	Alabama Department of Forensic Sciences
	Illinois State Police
	Orange County Sherrif-Coroner Department, California
	US Army Criminal Investigation Lab (USACIL)
	Montana Department of Justice
	State of Connecticut Department of Public Safety, Meriden
Australia	Victoria Police Forensic Services Department, Macleod Victoria
	Forensic Science Services Branch, NSW Police Department, Parramatta, NSW
	Path West Laboratory Medicine, Perth, Western Australia
	Forensic Science, South Australia
	Queensland Health Scientific Services, Coopers Plains
Finland	National Bureau of Investigation, Crime Laboratory DNA Department
France	Institut National de Police Scientifique (INPS)
Japan	National Research Institute of Police Science
Netherlands	Netherlands Forensic Institute (NFI)
Singapore	Center of Forensic Science, Health Sciences Authority
Sweden	SKL – Swedish National Laboratory of Forensic Science
United Kingdom	Forensic Science Services Limited (FSS)

Table 2. Biological Evidence Screening for Blood - Survey Results of the Participating Laboratories

Test	Number	%
Blood Presumptive Testing		
Phenolphthalein (Kastle-Meyer)	27 / 42	64
Tetramethylbenzidine (ie Hemastix [®])	12 / 42	28
Luminol or Bluestar [®]	16 / 42	38
Leucomalachite Green	10 / 42	24
O-tolidine	7 / 42	17
Fluorescin	2 / 42	5
Benzidine	1 / 42	2
Tetabase/Barium Peroxide reagent	1 / 42	2
Combination of Tetramethylbenzidine and Kastle-Meyer (PTMB)	1 / 42	2
Blood Confirmation Testing		
Do not confirm for blood	25 / 42	59
Takayama (Hemochromogen)	8 / 17	47
Immunological technique (ie HemaTrace [®])	7 / 17	41
Precipitin technique (ie Ouchterlony)	2 / 17	12
Rapid Stain Identification–Blood kit (RSID-Blood)	1 / 17	6
Counter current electrophoresis	1 / 17	6
Macroscopic visual identification	1 / 17	6
Blood Species Identification Testing		
Do not have a species identification method	6 / 42	14
Immunological technique (ie HemaTrace [®])	25 / 36	69
Precipitin technique (ie Ouchterlony)	14 / 36	39
Anti-Human Globulin Test	4 / 36	11
Rocket-Laurel Technique	1 / 36	3
Crossover electrophoresis	1 / 36	3
Counterimmunoelectrophoresis	1 / 36	3

Table 3. Biological Evidence Screening for Semen - Survey Results of the Participating Laboratories

Test	Number	%
Semen Presumptive Testing		
Alpha-naphthylphosphate (ie Fast Blue)	39 / 42	93
Alternative Light Sources	36 / 42	86
Immunological technique (ie Abacus <i>One-Step</i> ABACard® for p30)	8 / 42	19
Sodium Thymolphthalein Monophosphate (STMP) Test	3 / 42	7
5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) Test	3 / 42	7
Laser	2 / 42	5
p-Nitrophenyl Phosphate	1 / 42	2
Choline Microcrystal Test	1 / 42	2
Phenolphthalein diphosphate Test	1 / 42	2
ELISA for p30	1 / 42	2
Semen Confirmatory Testing		
Microscopic examination with Christmas Tree staining	34 / 42	81
Immunological technique (ie Abacus <i>One-Step</i> ABACard® for p30)	30 / 42	71
Phase Contrast Microscopy	15 / 42	36
Microscopic examination after Proteinase K treatment	10 / 42	24
Microscopic examination with Hematoxylin & Eosin staining	4 / 42	9
Microscopic examination with Baecchi staining	1 / 42	2
Microscopic examination with Corin-Stockis staining	1 / 42	2
Differential Interference Contrast Microscopy	1 / 42	2
Microscopic examination with Acid Fuchsine staining	1 / 42	2
Microscopic examination with Fluorberberine Sulfate, Isaac and Wurch and/or Schorr staining	1 / 42	2
Laser Dissection Microscopy	1 / 42	2
Rapid Stain Identification – Semen Kit (RSID-Semen)	1 / 42	2
Crossover electrophoresis for p30	1 / 42	2
Nano-Sg counter current electrophoresis	1 / 42	2
ELISA for p30	1 / 42	2

Table 4. Biological Evidence Screening for Saliva - Survey Results of the Participating Laboratories

Test	Number	%
Saliva Presumptive Testing		
Do not test for saliva	7 / 42	17
Alternate Light Sources	29 / 35	83
Phadebas [®]	21 / 35	63
Amylase Diffusion	10 / 35	28
Microscopic examination for epithelial cells	9 / 35	26
Rapid Stain Identification – Saliva Kit (RSID-Saliva)	4 / 35	11
Laser	2 / 35	6
Procion Red Amylopectin Paper	1 / 35	3
SALigAE [®]	1 / 35	3
Saliva Confirmatory Testing		
Do not use a confirmatory test for saliva	28 / 35	80
High levels of amylase determined by various testing techniques	5 / 7	71
Counterimmunoelectrophoresis	1 / 7	14
Amylase Diffusion	1 / 7	14

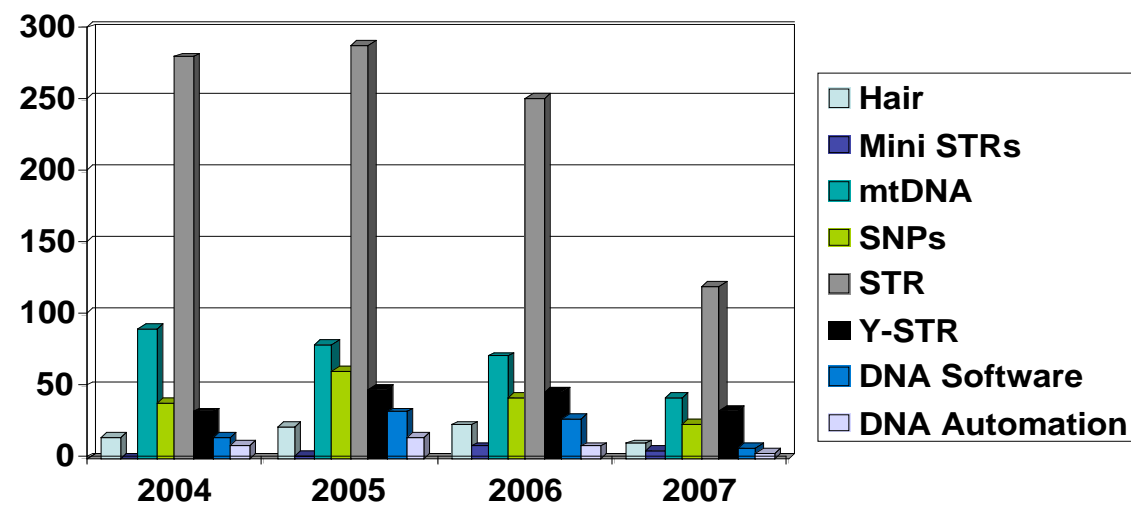
Table 5. Biological Evidence Screening for Vaginal Fluid, Urine, Fecal Material and Hair Examinations - Survey Results of the Participating Laboratories

Body Fluid	Test	Number	%
Vaginal Fluid	Presumptive Testing		
	Do not test for vaginal fluid	21 / 42	50
	Alternate Light Sources	16 / 21	76
	Lugol's Iodine	3 / 21	14
	Alpha-naphthylphosphate (ie Fast Blue)	2 / 21	9
	Microscopic examination for nucleated squamous epithelial cells	1 / 21	5
	Confirmatory Testing		
	Do not perform confirmatory testing for vaginal fluid	20/21	95
	Fast Blue testing coupled with PSA and microscopic examination	1	
Urine	Presumptive Testing		
	Do not test for urine	17 / 42	40
	Alternate Light Sources	19 / 25	76
	Jaffe Colour Test for Creatinine	12 / 25	48
	Urease and bromothymol blue (Azostix®) test for urea nitrogen	11 / 25	44
	Para-dimethylamioncinnamaldehyde (DMAC) test for urea	6 / 25	24
	Uricase Test	1 / 25	4
	DGT Test	1 / 25	4
	Confirmatory Testing		
	Do not perform confirmatory testing for urine	24 / 25	96
	Body Fluids Identification – Urine Kit (BFID-Urine)	1	
Feces	Presumptive Testing		
	Do not test for fecal material	22 / 42	52
	Urobilinogen (Edelman's Test)	16 / 20	80
	Microscopic examination for fecal material	9 / 20	45
	Olfactory Test	1 / 20	5
	Do not confirm the presence of fecal material	42 / 42	100
	Species Identification Testing		
	Do not determine the species of fecal material	17 / 20	85
	Precipitin Test	2 / 3	67
	Microscopic examination	1 / 3	33
Hair	Do not examine/assess hair evidence	2 / 42	5
	Microscopic examination for suitability/species ident/origin	34 / 40	85
	Hair comparison	14 / 40	35
	Sent away for analysis to another laboratory	3 / 40	7

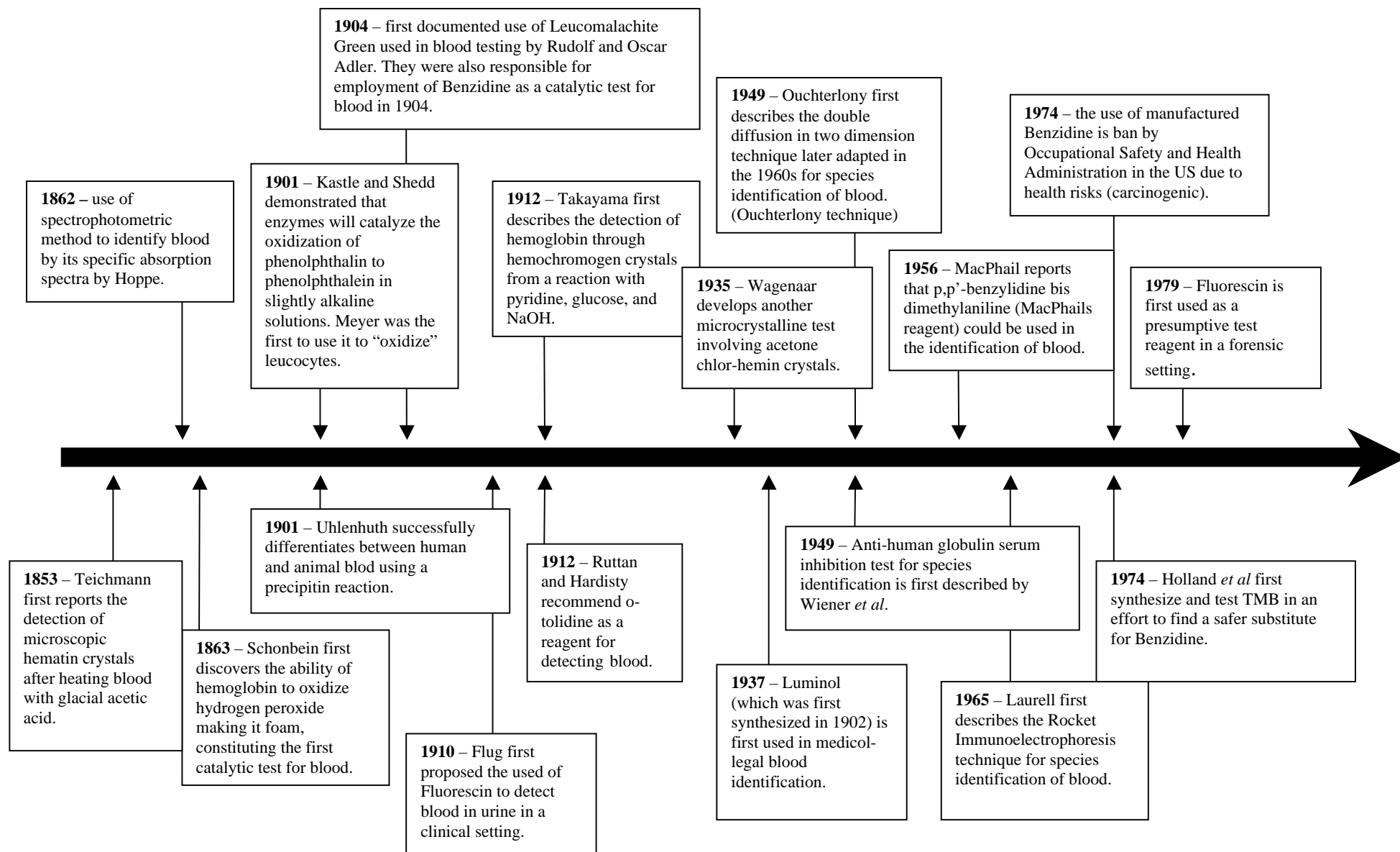
Table 7. Additional References on Statistical and Interpretation of STR Evidence

Lack of direct concordance in electropherogram peak heights using two versions of GeneScan7 Software indicates that peak height variation could occur with the same data and should be taken into consideration for consistency when comparing data or developing new interpretation guidelines	Gilder et al. (2004)	(287)
Specific in-house system management, for tracking of samples and mitochondrial DNA protocols used to identify skeletal remains by the Armed Forces Institute of Pathology: A detailed review of sample collection, data management, mobile field identification for collection and documentation of 1000 DNA specimens, mtDNA protocols, instrumentation, personnel management, selection of sample types, contamination safeguards and population resolution.	Edson et al. (2004)	(291)
Current thinking on interpretation of forensic evidence with chapters on validation, sampling, mixtures, low copy number, disaster victim identification, intelligence databases, parentage testing, relatedness, population genetics and principles of biological relatedness. A text for both novice and senior caseworker.	Buckleton et al. (2005)	(292)
Recommendations are made for discrimination between half-siblings with maternal genotypes from unrelated fathers and findings indicate that mother=s genotypes at a locus provides more information for discrimination on average than adding more loci (ie > 10-25) in the queried individuals themselves.	Mayor and Balding (2006)	(288)
Alternative options to using the 2p rule for the conservative interpretation of allele dropout for low copy number and partial STR profiles is proposed when replication of the PCR analysis is not possible.	Buckleton and Triggs (2006)	(289)
EasyDNA Program- general statistical principles for forensic DNA analysis for statistical assessment of kinship and mixture evaluation	Fung et al. (2006)	(286)
Recommendations of the ISFG on the interpretation of mixtures with special emphasis of low copy number reporting. The use of likelihood ratio calculations and the “random man not excluded” probability are compared. This should be considered the initial guideline for discussion purposes to be followed by more detailed discussion and data review.	Gill et al. (2006)	(290)

Figure 1 - Forensic Biology/DNA Papers
Searches of the PubMed* and EMBASE databases**



* PubMed – 1990 – June 2007
 ** EMBASE – 1993 – June 2007



**Figure 2. Forensic Timeline For Biological Evidence Screening
Blood Identification**

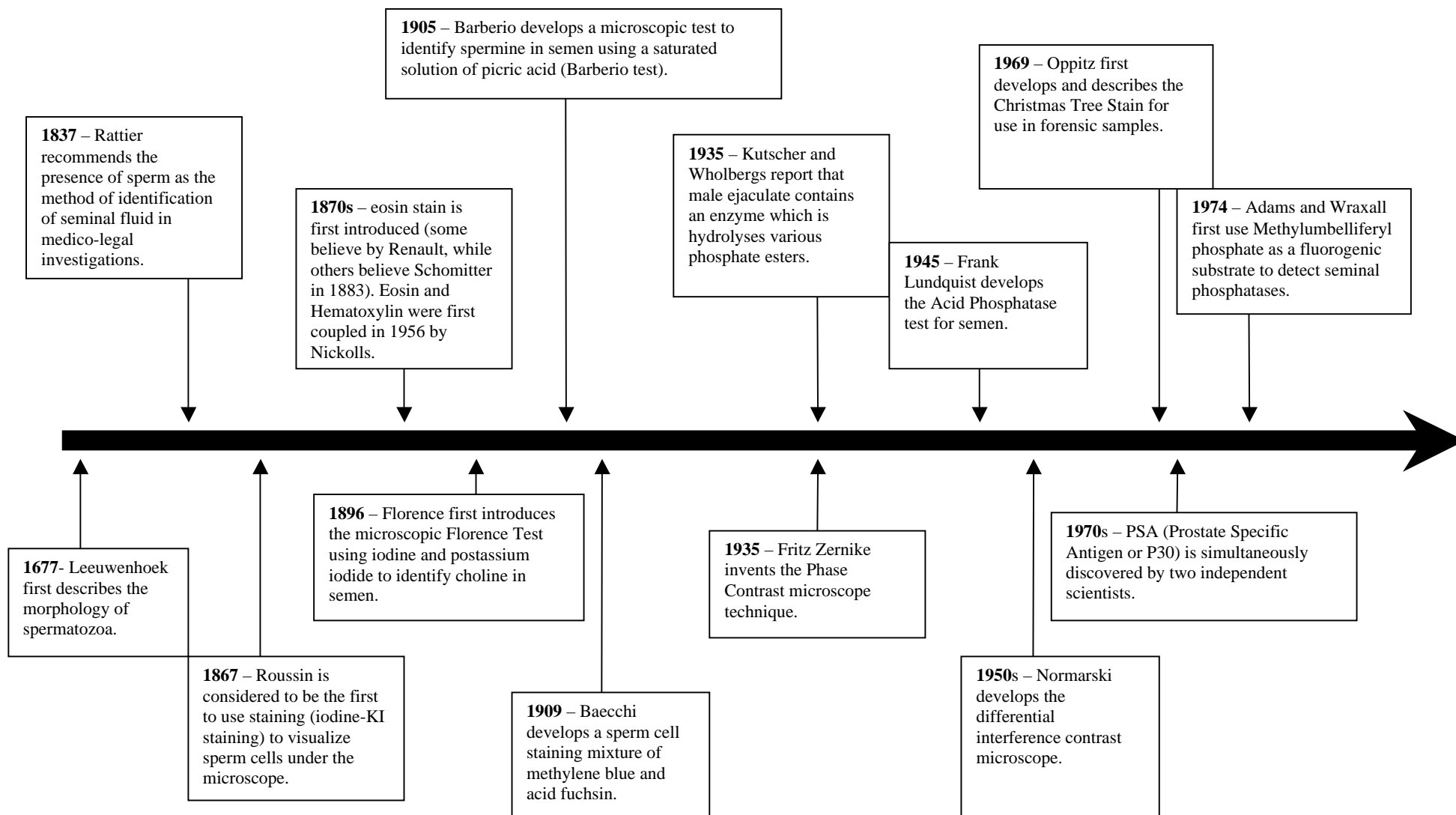


Figure 3. Forensic Timeline for Biological Evidence Screening Semen Identification

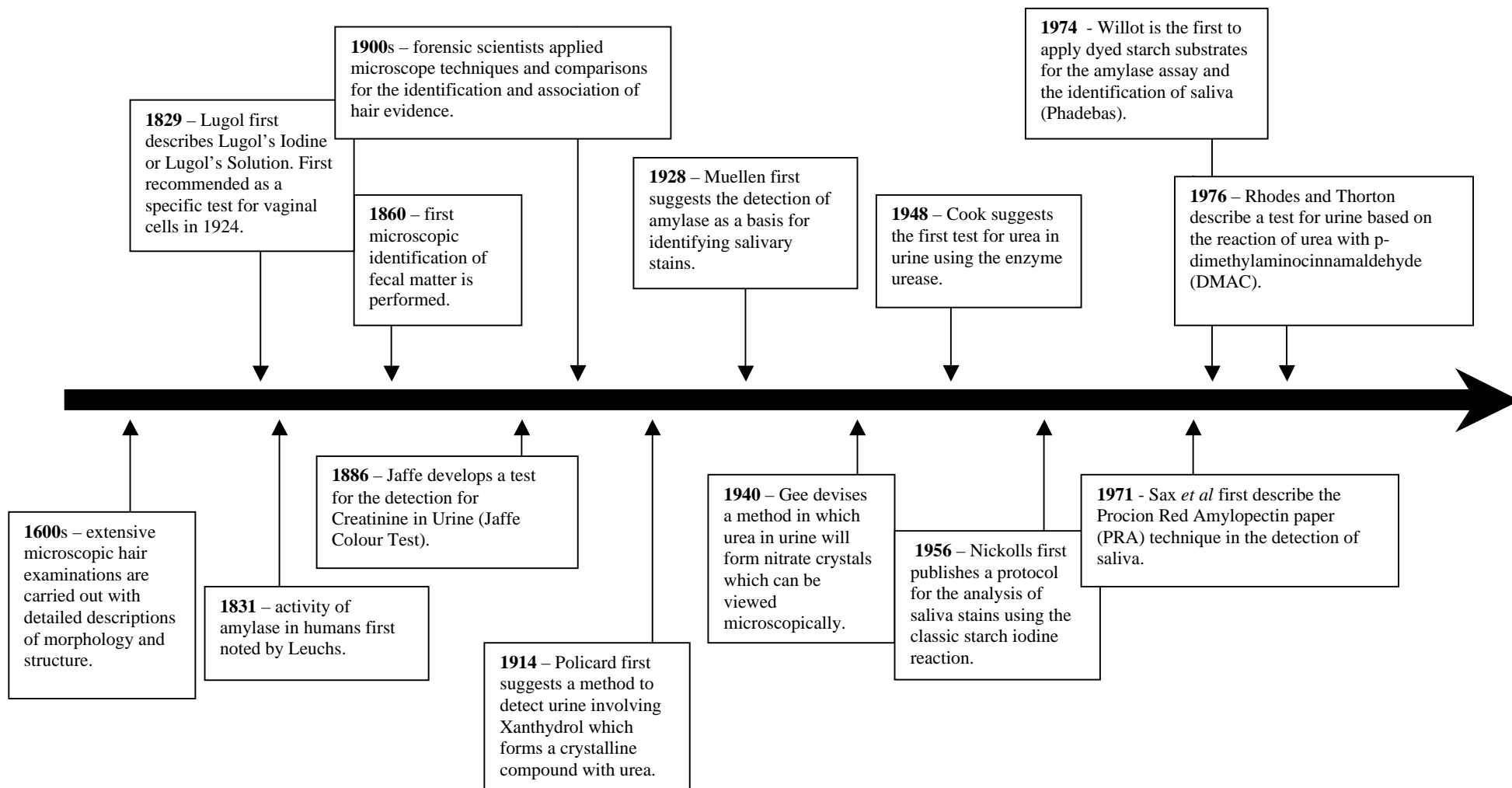


Figure 4. Forensic Timeline for Biological Evidence Screening Hair and other Evidence

Recent Progress in Processing Biological Evidence and Forensic DNA Profiling
A Review: 2004 to 2007
Fourney, RM.

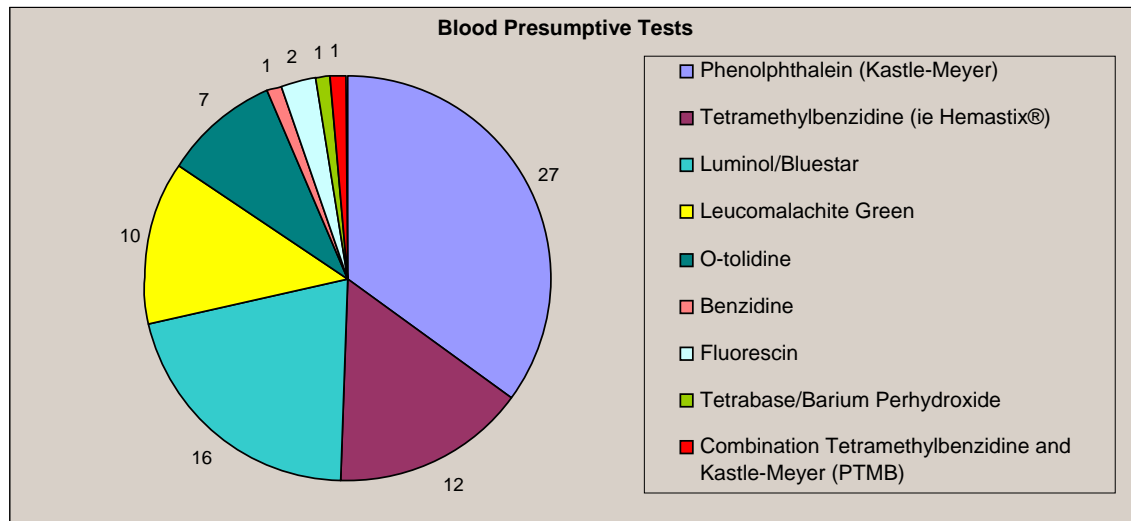
Appendix:

Appendix Addition to Table 2. Biological Evidence Screening for Blood - Survey Results of the Participating Laboratories

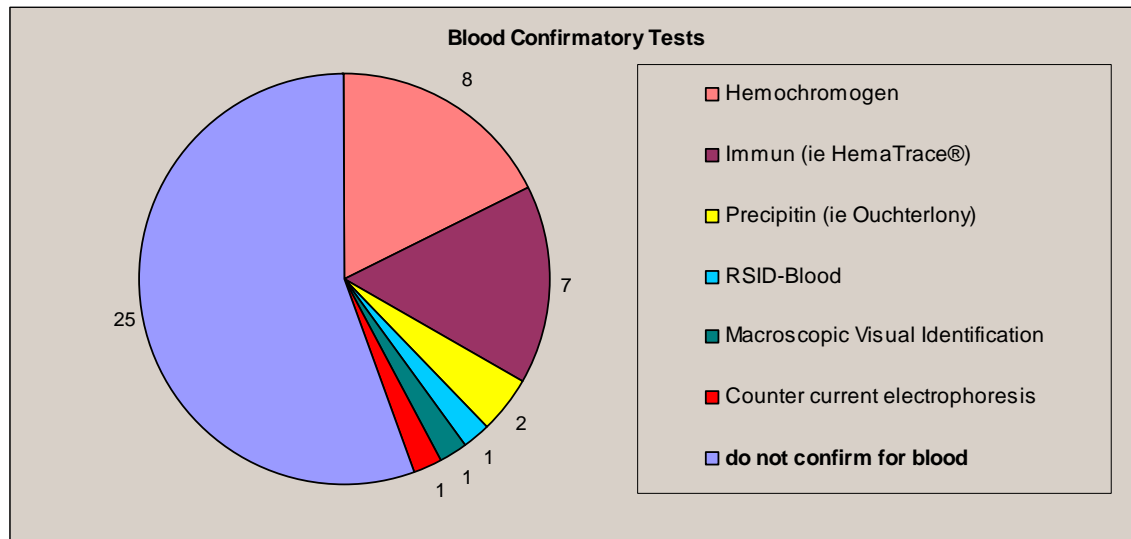
Appendix Addition to Table 3 Biological Evidence Screening for Semen - Survey Results of the Participating Laboratories

Appendix Addition to Table 4. Biological Evidence Screening for Saliva - Survey Results of the Participating Laboratories

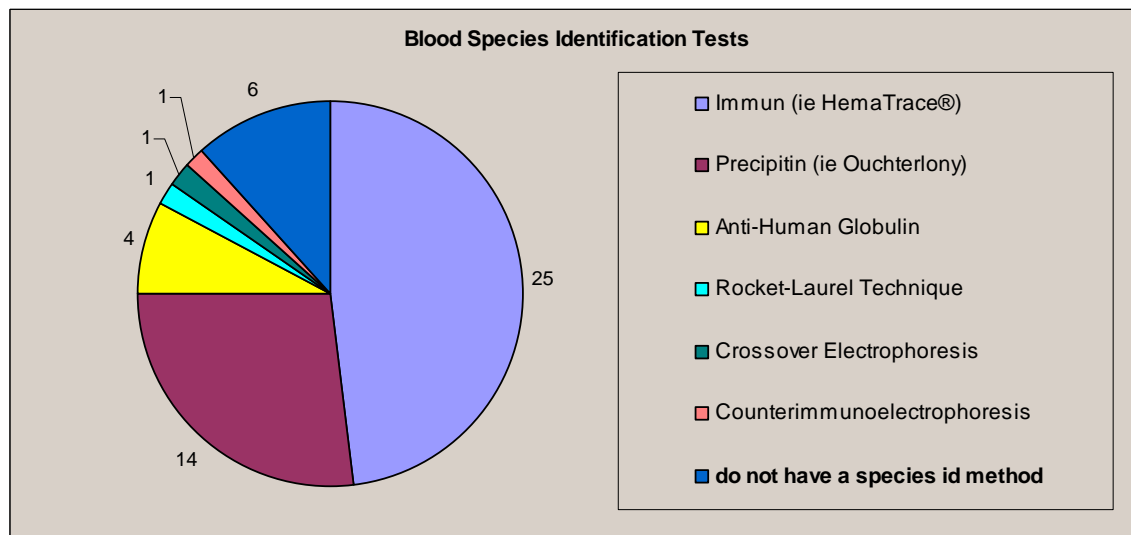
A



B

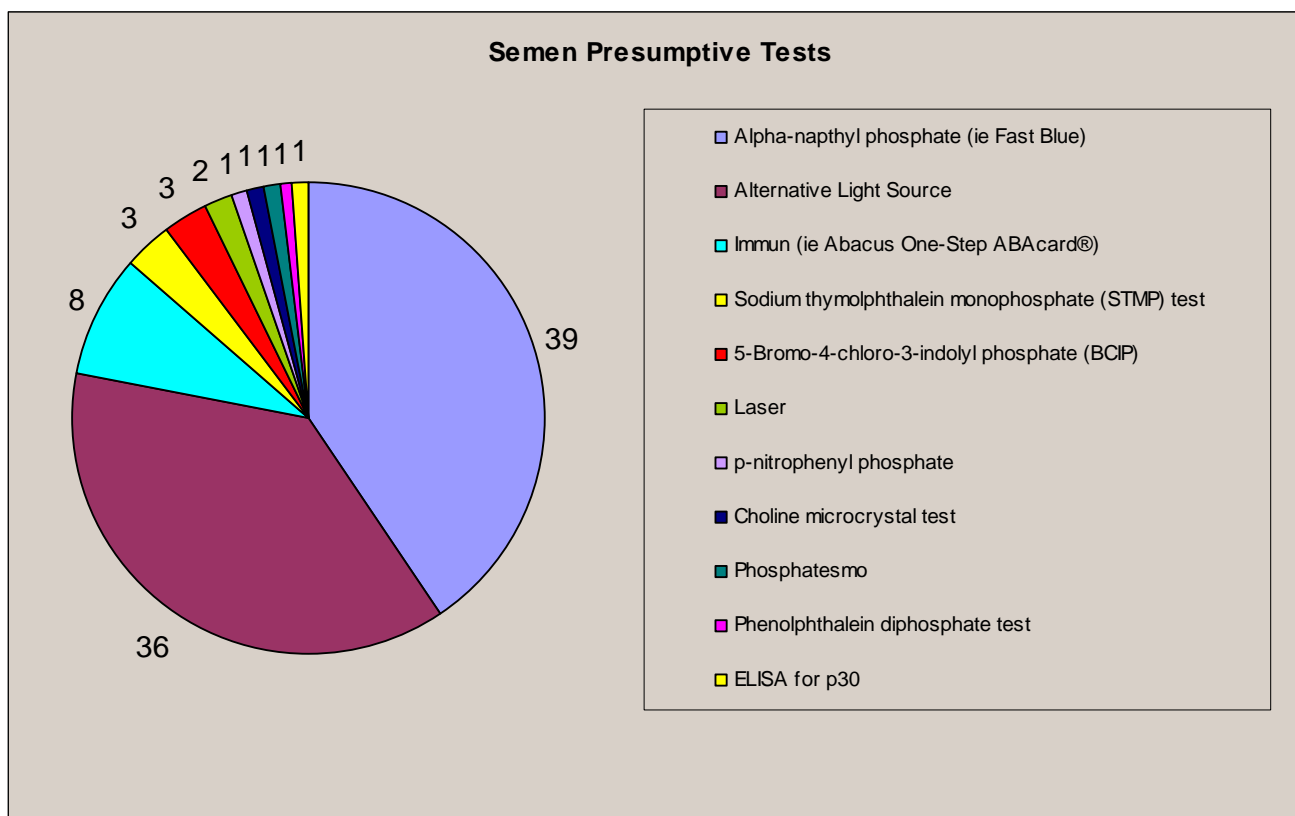


C

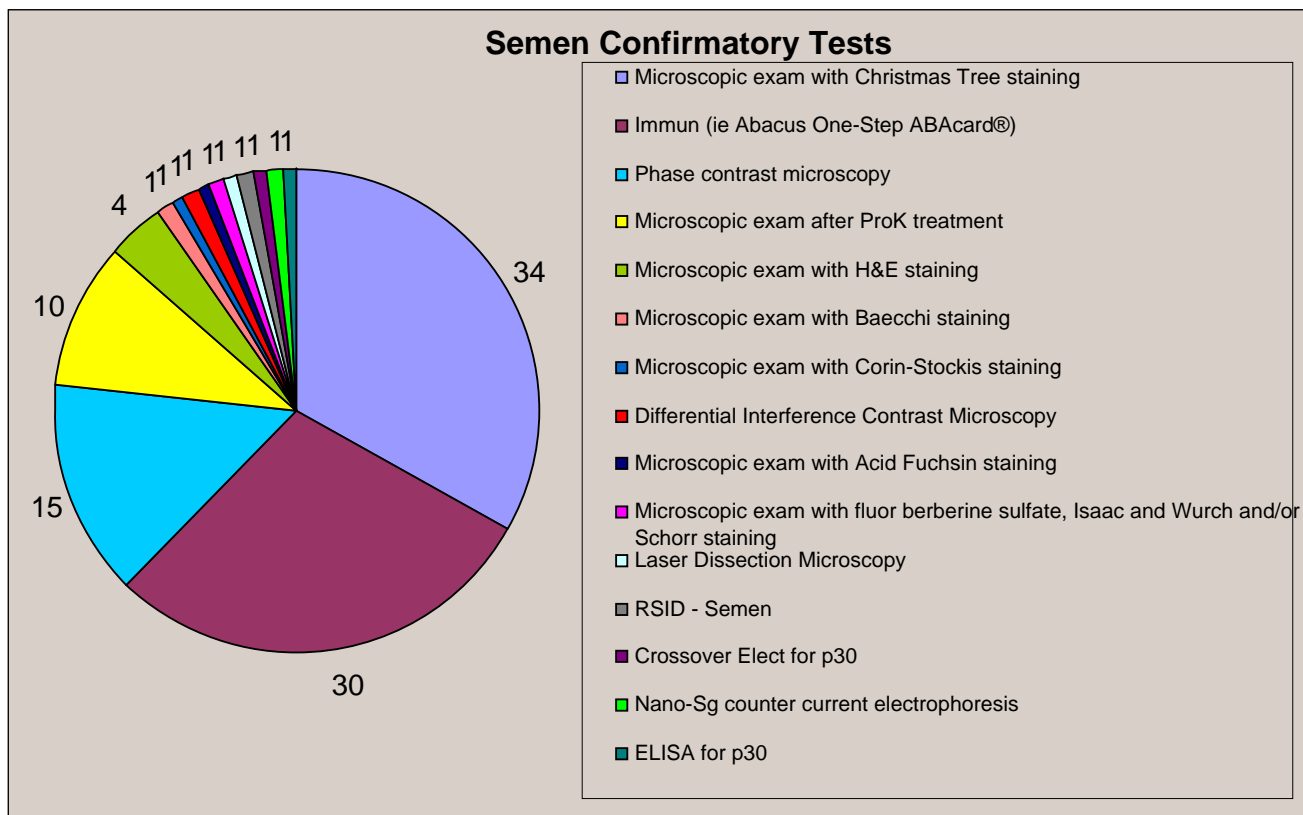


Appendix Addition to Table 2. Distribution of presumptive, confirmatory and species blood identification tests. A) distribution of presumptive tests used for blood identification, B) distribution of confirmatory tests used for blood identification, and C) distribution of tests used for species identification in blood stains.

A

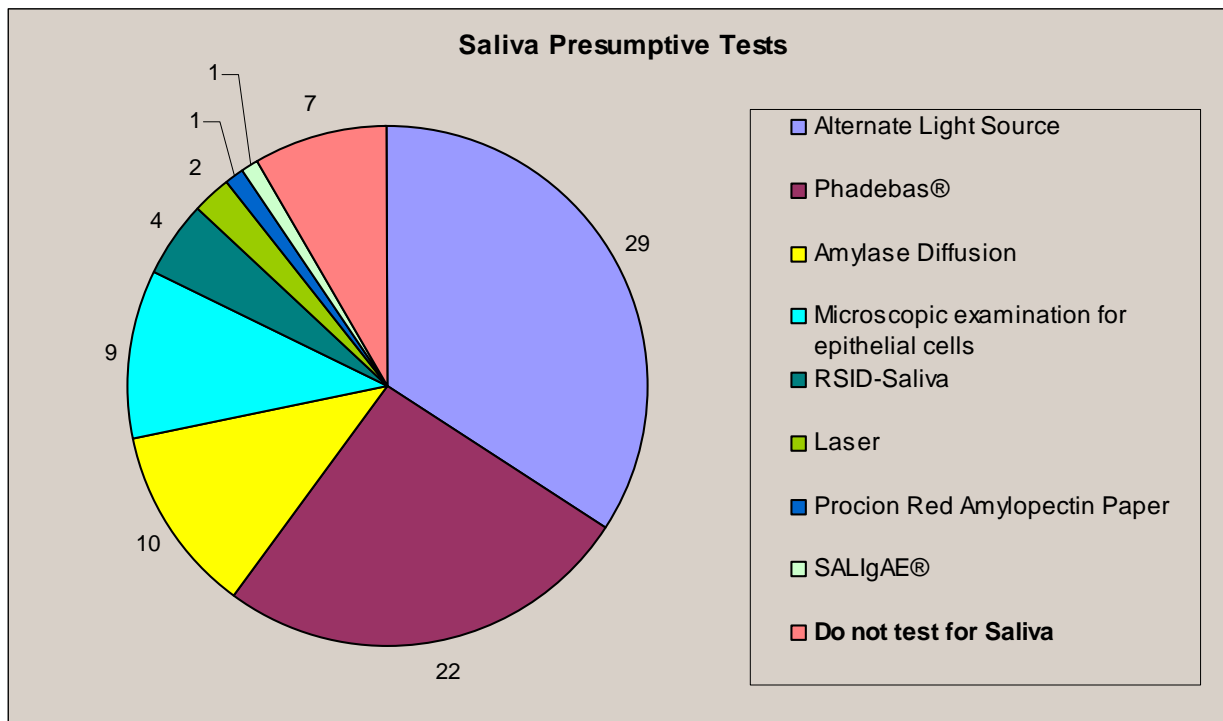


B

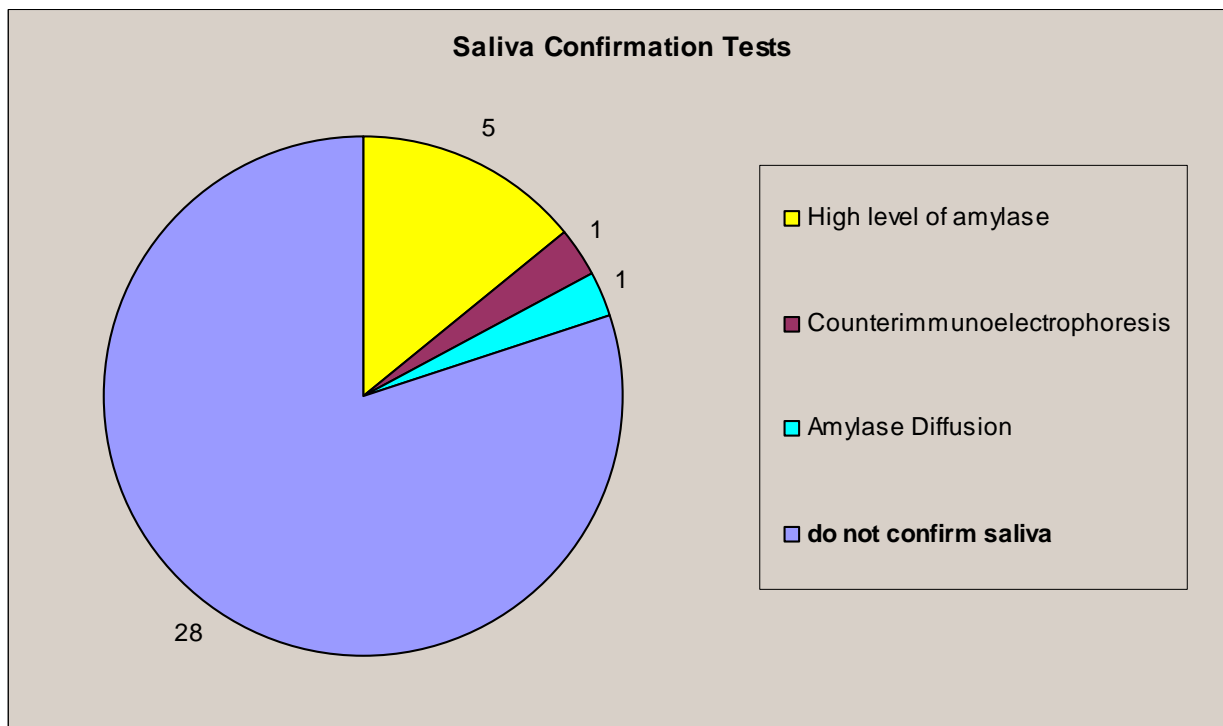


Appendix Addition to Table 3. Distribution of presumptive and confirmatory semen identification tests. A) distribution of presumptive tests used for semen identification and B) distribution of confirmatory tests used for semen identification.

A



B



Appendix Addition to Table 4. Distribution of presumptive and confirmatory saliva tests.
 A) distribution of presumptive tests used for saliva identification and B)
 distribution of confirmatory tests used for saliva identification.