



# A Systemic Review On Advanced Techniques For Blood, Saliva, Semen, And Vaginal Fluid Identification

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## Abstract:

Although, DNA typing plays a decisive role in the identification of persons from blood and body fluid stains in criminal investigations, clarifying the origin of extracted DNA has also been considered an essential task in proving a criminal act. This review introduces the importance of developing precise methods for body fluid identification. Body fluid identification has long relied on enzymatic methods as a presumptive assay and histological or serological methods as a confirmatory assay. However, because the latest DNA typing methods can rapidly obtain results from very small and even old, poorly preserved samples, the development of a novel corresponding body fluid identification method is required. In particular, an immunochromatographic method has been introduced to identify saliva and semen from sexual crimes. In addition, for vaginal fluid identification, attempts have been made in the past decade to introduce a method relying on body fluid-specific mRNA expression levels. At present, the development of molecular biological methods involving microRNA, DNA methylation, and resident bacterial DNA is ongoing. Therefore, in criminal investigations, body fluid identification is an essential task for correctly applying the results of DNA typing, although further research and development are required.

**Keywords:** Body fluid identification, Immunochromatography, DNA, RNA, Sexual assault

## 1. Introduction

DNA analysis is used in most countries around the world to obtain individual genetic profiles and identify persons from biological samples linked to crimes for use in criminal investigations. At present, the short tandem repeat (STR) approach the main method used, which involves determining the number of repeats of several bases in multiple loci contained in the nuclear DNA of cells. In recent years, along with growth in the number of loci registered in the Combined DNA Index System (CODIS) DNA database [1], reagents with a large number of target loci have become widely used, such as the PowerPlex<sup>®</sup> fusion 5C and 6C (Promega,

USA) and the GlobalFiler™ PCR amplification kit (ThermoFisher Scientific, USA). In particular, the GlobalFiler™ PCR amplification kit amplifies 21 autosomal STR loci, DYS391 locus, Y indel locus, and amelogenin locus. Fujii et al. [2] reported that the probability of identity of these 21 autosomal loci obtained using GlobalFiler™ PCR amplification kit was  $1.84 \times 10^{-25}$  in the population, which was remarkably higher than that ( $1.8 \times 10^{-17}$ ) of the 15 autosomal loci obtained using AmpF/STR Identifier kit (ThermoFisher Scientific, USA) [3].

However, it is often difficult to extract nuclear DNA from hair or from degraded samples. An alternative is mitochondrial DNA, which is present in large quantities in cells. The individual identification level of mitochondrial DNA is far below the STR approach [4], but effectively augments the value of other information used for investigations. In addition, DNA methodologies, such as Y-STR typing, single nucleotide polymorphisms, and next-generation sequencing technology have also been applied to various practical tasks [5–7]. Due to its reliability, DNA is widely recognized, not only by investigators, but also by the general public, as essential in criminal investigations.

The number of cells constituting the human body had previously been estimated to be about 60 trillion. However, the latest report by Sender et al. [8] of the Weizmann Institute of Science in Israel obtained a total of about 30 trillion, almost half of the previous estimate, and it is considered that the estimated number of cells will one day be further revised downward. In any case, we can safely say that the body is composed of a huge number of cells. As all of these cells have the same DNA, when body fluid is the residual crime-related sample used, the DNA origin is essential in proving the criminal act. In particular, in sexual crimes, the identification of saliva, semen, and vaginal fluid is often required, and biochemical, histological, or instrumental analysis methods that focus on the characteristic proteins and cells in each type of body fluid have long been used. However, detection sensitivity and specificity are greatly affected by the type of body fluid, methodological differences, and differences in the environment in which the sample is left. To keep pace with recent improvements in the discriminating power of DNA typing, precise body fluid identification is required now more than ever.

In this paper, we review the current methods for identifying body fluids related to sexual crime, and we discuss both conventional methods and the latest serological and molecular biological methods, focusing on saliva, semen, and vaginal fluid.

## 2. Flow of Forensic Examinations Using Biological Samples

The basic and conventional workflow for forensic examinations using biological samples involves the following: Visual examination, presumptive assay, confirmatory assay, blood typing, and DNA typing. The procedure and the examination name vary slightly depending on the type of body fluid to be examined and the condition of the sample. We will briefly describe the workflow for bloodstains as they are the most frequently examined samples. First, the visual condition, which includes color tone, size, and age, is examined. This is followed by the bloodstain presumptive assay, which is a chemical method that exploits the peroxidase-like activity of heme in hemoglobin. The leucomalachite green assay [9], the luminol assay [10], and the tetramethylbenzidine assay [11] are also widely used. The BLUESTAR®

FORENSIC kit (BLUESTAR<sup>®</sup> Forensic, Monte Carlo, Monaco), which contains the luminol assay, is also available. The luminescence of this kit lasts longer than that of the normal luminol method and it does not require complete darkness.

Next, human blood identification is performed as a confirmatory assay. Previously, a serological assay using anti-human hemoglobin precipitin was used, but at present, the main method is a simple and rapid immunochromatography assay using anti-human HbA<sub>0</sub> mouse antibody. BLUESTAR<sup>®</sup> OBTI (BLUESTAR<sup>®</sup> Forensic) (Fig. 1) is commercially sold and widely used as a forensic kit. However, OC-Hemocatch S (Eiken, Tokyo, Japan), a clinical reagent that is commercially available as a kit for detecting human HbA<sub>0</sub> in fecal matter, has been introduced in Japan. The forensic utility of this reagent has been fully verified [12], and its cost performance is also favorable. Accordingly, it is actively used in criminal investigations. Clarification of whether the sample concerns menstrual blood [13–17], maternal blood [18–20], fetal blood [21,22], or postmortem blood [15,23] is also often required, and this identification is performed through assays that target proteins or expressed genes that are characteristic of each blood type.

After both the presumptive assay and the confirmatory assay show positive results, ABO blood grouping and finally DNA typing are performed. Various blood grouping methods, including that of the ABO blood type, were used for person identification when DNA typing was not available. However, ABO blood grouping is now often omitted, and it sometimes provides valuable information for narrowing down the subjects for DNA typing because the ABO blood group can be determined, not only from bloodstains, but also from saliva, semen, and hair [24–26]. As mentioned above, DNA typing is based on the STR type and sex determination on the amelogenin gene, and there is no doubt about their personal identification power.

### 3. Saliva Identification

For saliva identification, the amylase assay, which combines a presumptive and confirmatory assay, is the most important test. Although,  $\alpha$ -amylase is present in all body fluids, the  $\alpha$ -amylase activity of saliva is much higher than that of other body fluids [27,28] and it has, thus, been regarded as essential for saliva identification. Many animals also have  $\alpha$ -amylase activity [27,29]: Mice, rats, guinea pigs, and squirrels have relatively high activity, whereas dogs, cats, cows, goats, sheep, and horses have little to no activity. Currently, a method using a reagent called blue starch, which is a blue dye chemically bound to starch, is used for the amylase assay. Many countries use the Phadebas method [30], based on the Phadebas<sup>®</sup> Amylase Test (Magle Life Sciences, Cambridge, MA, USA). This method uses a spectrophotometer to measure and quantify the blue pigment dissolved from blue starch by the  $\alpha$ -amylase in saliva.

In contrast, a unique method called the blue starch agarose plate method [31], has been used for many years by investigators in Japan. In this method, powdered blue starch is mixed with a hot agarose solution to form a flat gel. A saliva stain sample cut to a few millimeters or less is directly placed on the flat gel, and the dissolution of the blue pigment is visually observed. The important point is that the assessment needs to be made within a reaction time of approximately 30 min to 1 h at 37 °C, which is the optimum temperature of

the enzyme, and within a maximum of 2 h when the amount of saliva attached is extremely low [32]. In addition, the reagent SALIgAE<sup>®</sup> (Abacus Diagnostics, West Hills, CA, USA), which turns yellow when the substrate is decomposed by  $\alpha$ -amylase in saliva, is also effective in saliva identification [33].

Furthermore, identification with a more specific method may be required. The serological methods that have been so far used for this purpose, include immunodiffusion and immunoelectrophoresis using polyclonal antibodies, such as anti-human salivary precipitin, anti-human salivary  $\alpha$ -amylase precipitin, or anti-human sIgA precipitin [34]. However, it has become difficult to obtain high-titer polyclonal antibodies in recent years, and the frequency of their use is now extremely low. We have developed an enzyme-linked immunosorbent assay (ELISA) targeting statherin as a new marker in saliva and attempted to introduce it into practice [28]. Although, statherin is slightly less sensitive than  $\alpha$ -amylase in ELISA, it is highly specific and effective for saliva identification. Notably, statherin is found at high concentrations in nasal secretions [35]. Another saliva confirmatory test is the RSID<sup>™</sup>-Saliva (Independent Forensics, Lombard, IL, USA) kit for forensic science, which involves immunochromatography for human salivary  $\alpha$ -amylase. It is widely used because it can be tested quickly and easily [36].

Research on saliva identification using genes has also been actively conducted since the beginning of the 2000s. In particular, real-time RT-PCR using the mRNA expression levels of salivary-specific proteins has been reported [37–39]. We have also confirmed the usefulness of the *STATH* and *HTN3* genes [40,41] and performed various studies to introduce the method into practice [42–44]. In addition, methods focusing on microRNA [45–50] and DNA methylation [51–56] have been actively explored for saliva identification. At present, it would seem that further studies are necessary before microRNA and DNA methylation methods can be applied to practical work. Meanwhile, studies focusing on bacterial DNA such as *Streptococcus salivarius*, which parasitizes in the oral cavity, have also increased [57–60]. Such methods include the development of a simple and rapid method involving the loop-mediated isothermal amplification (LAMP) technique, as well as a useful method for deteriorated salivary stain samples.

#### 4. Semen Identification

Semen is the most frequently examined body fluid in sexual crimes. Semen gives off a unique odor when it is fresh, whereas dried semen stains are pale yellow to gray in color and sometimes stiff and lustrous. However, when the amount and site of semen deposition is unclear, it is helpful to use a variable wavelength light source device such as a Polilight (Rofin Australia, Victoria, Australia).

Semen consists of sperm and seminal plasma. First, a presumptive assay is performed to detect acid phosphatase [61], which is found at high concentrations in seminal plasma. The acid phosphatase test has long been performed via colorimetric tests, such as a method using  $\alpha$ -naphthyl phosphate and Fast Blue B and a method using phenolphthalein diphosphate and ammonia [62]. When the test is positive, the former develops a purple color that is stable for a long time, whereas the latter develops a reddish pink color that fades in a relatively short time. SM test reagent (Wako, Osaka, Japan), a kit that applies the former method, is commercially available and widely used in sexual crime investigations. If the presumptive assay is positive, a sperm test is performed as the confirmatory assay.



Microscopic examination involving tissue staining is generally performed. Various staining methods are used, such as Baecchi, Corin-Stockis, Christmas tree (Oppitz), and hematoxylin and eosin staining [63–66]. In particular, in Christmas tree staining, sperm show a red head and a green tail that are readily visualized [62]. A useful approach when the sample is old or difficult to distinguish from bacteria and dust is SPERM HY-LITER™ (Independent Forensics, Lombard, IL, USA), which is a commercially available kit involving a fluorescent label [67].

At the same time, serological methods using antibodies, can be applied to provide proof of seminal plasma. In particular, in the case of azoospermia or oligospermia, proof of seminal plasma is essential. Immunodiffusion and immunoelectrophoresis using high-titer anti-human semen precipitin [34] have long been used, but now an immunochromatography kit is the technique of choice. Prostate-specific antigen (PSA) is the oldest marker of seminal plasma [68], and SERATEC® PSA Semiquant (Seratec, Gottingen, Germany), which reacts to 1 million-fold diluted semen, has been frequently used as a highly sensitive kit [69]. However, PSA is also expressed in women's periurethral glands [70–72], especially when they are on contraceptives, and it is highly possible to be tested PSA-positive without intercourse [73], indicating that caution is required when PSA is being evaluated alone without sperm. In contrast, seminogelin, derived from seminal vesicles, is a semen-specific antigen that is not detected in females [74], making it a reliable option for determining the presence of seminal plasma. RSID™-SEMEN (Independent Forensics, Lombard, IL, USA) is also commercially available as a kit containing anti-seminogelin antibody [75] (Fig. 2).

Many studies of semen identification using real-time RT-PCR have been reported [37–39]. In particular, SEMG1 and PRM2 are effective target genes [40] with high specificity. As PRM2 is expressed in the sperm itself, it will not be expressed in the semen of azoospermic individuals. In addition, as for saliva, there are many reports on the use of microRNA [45,46,48–50] and DNA methylation [51–56,76,77] in semen identification.

## 5. Vaginal Fluid Identification

Unlike other body fluids, vaginal fluid does not have characteristic proteins for identification, making it difficult to find proof of its presence. Examinations center on tissue stains, such as Papanicolaou (Pap) staining [78] and Lugol's staining. In particular, the Lugol's staining method has been considered effective because the inner surface of the vagina is covered with stratified squamous epithelial cells and contains abundant glycogen [79,80]. However, staining may also be seen in cells of the oral cavity [81,82], and it is now considered that careful evaluation is required. As serological methods, immunodiffusion and immunoelectrophoresis were previously performed using anti-human vaginal fluid precipitin [34]. However, the serological method is rarely performed given the high-titer antibody is not available.

In this review of the current methods for identifying body fluids related to sexual crime, we have discussed various methods currently in use, as well as the latest reports regarding the development of new methods, focusing on saliva, semen, and vaginal secretions. In criminal investigations, body fluid identification is an

essential task in correctly applying the obtained results of DNA typing, and thus further research and development are essential.

## 6. Conclusion:

This systemic review reveals the possible analytical methods and their sensitivity, specificity, time and cost effective as well as their simplicity of use. This paper served the basic conventional and recent advanced methods of Blood, Saliva, Semen and Vaginal Fluid identification

## 7. Declarations:

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Legends to figures:



Fig. 1: BLUESTAR® OBTI (BLUESTAR® Forensic) kit



Fig. 2: Rapid Stain Identification of Semen (RSID™-Semen)