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ORIGINAL ARTICLE

Improving body fluid identification in forensic trace evidence—construction of an immunochromatographic test array to rapidly detect up to five body fluids simultaneously

Hannah Holtkötter[1] · **Kristina Schwender**[1] · **Peter Wiegand**[2] · **Heidi Peiffer**[1] · **Marielle Vennemann**[1]

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Abstract Body fluid identification is a substantial part of forensic trace analyses. The correct determination of the origin of a biological stain may give valuable information regarding the circumstances of a crime. A simple way to detect a body fluid in a stain is the use of immunochromatographic strip tests. They are easy to use, user-independent, quick, and cheap. Currently, however, it is only possible to analyze one body fluid at a time, requiring the analyst to make previous, possibly subjective, assumptions on the body fluid at hand. Also, identification of mixed body fluids requires the use of several tests, which results in additional sample and time consumption. To combine a simple approach with the possibility to simultaneously detect several body fluids, we constructed a combined immunochromatographic strip test array based on commercially available tests. The array rapidly detects up to five body fluids with a single analysis, and allowing for subsequent DNA extraction from the same material. With this test it was possible to identify the components of a mixture, the test was easily incorporated into standard laboratory work, and its sensitivity and specificity were shown to be comparable to those of conventional strip tests.

Keywords Body fluid identification · Immunochromatographic assay

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✉ Hannah Holtkötter
hannah.holtkoetter@ukmuenster.de

Kristina Schwender
kristina.schwender@ukmuenster.de

Peter Wiegand
peter.wiegand@uniklinik-ulm.de

Heidi Peiffer
heidi.pfeiffer@ukmuenster.de

Marielle Vennemann
marielle.vennemann@ukmuenster.de

[1] Institute of Legal Medicine, University of Münster, Röntgenstraße 23, 48149 Münster, Germany

[2] Institute of Legal Medicine, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

Introduction

In forensic laboratories or police work the identification of biological stains is daily fare. Not only is knowing the origin of a bodily fluid important for the correct handling of the evidence in the laboratory, but may also provide investigators with information on the course of the crime. Body fluids commonly found at crime scenes are blood, vaginal secretions, semen, urine, and saliva. The presence of a certain body fluid may specify the nature of the crime—blood presence points towards a physical fight or assault, while semen or vaginal secretions indicate sexual encounters [1]. To detect potential body fluids, i.e., protein catalytic activity [1] may be measured, chemical [2] and immunological tests [3, 4], spectroscopy [5–7] or microscopy [8, 9] can be applied. For the past decade, extensive research has gone into the development and improvement of methods for body fluid identification with the analysis of RNA expression [10–16] and DNA methylation patterns [17–22] being the focus of numerous recent studies. While these studies have provided very promising results, including the possibility of a multiplex approach to simultaneously detect several body fluids, they also highlighted the difficulties researchers face.

When applying RNA expression or DNA methylation analyses a considerable amount of training and experience is required, results are not always reproducible between different laboratories and may be influenced by inter- and intra-individual differences between donors [22, 23]. An easy-to-use alternative are immunochromatographic assays for the identification of body fluids. Various assays exist for the identification of venous blood [24–26], menstrual fluid [3, 4, 25], saliva [27, 28], urine [29], and semen [30, 31]. These assays have been shown to be highly sensitive, specific, and user-independent. They only require minimal training and have been validated for forensic samples. Yet, they only target one specific body fluid per test, requiring the analyst to make previous, possibly subjective, assumptions on the body fluid at hand. Furthermore, if a mixture of body fluids is expected in a stain, several tests need to be performed to identify every body fluid possibly present, resulting in additional sample and time consumption.

To overcome this issue our group created a combined immunochromatographic multiplex array from commercially available immunochromatographic kits to identify body fluids in a mixture simultaneously. This reduces the amount of sample and time compared to single strip analyses. To achieve successful application of the array with similar sensitivity and specificity as each individual test, buffer conditions had to be adjusted. The following kits were chosen for the detection of semen, saliva, urine, menstrual fluid, and blood:

For the detection of semen, the RSID™-Semen kit by Galantos (Mainz, Germany) was chosen for its high sensitivity and specificity [30] compared to other immunochromatographic tests. Tests targeting prostate-specific antigen (PSA) were rejected due to possible PSA presence in other body fluids such as female urine [35].

For the detection of saliva, the RSID™-Saliva kit by Galantos (Mainz, Germany) was chosen, again due to its high sensitivity and specificity [27].

For the detection of blood, the SERATEC® PMB Test (Göttingen, Germany) was chosen. The main advantage of this test compared to other immunochromatographic blood tests is the simultaneous detection of blood and menstrual fluid: The test identifies samples containing human blood by detecting the presence of human hemoglobin and identifies samples containing menstrual fluid by detecting the presence of D-dimers [25].

The RSID™-Urine kit by Galantos (Mainz, Germany) was chosen for the detection of urine. The test strip is designed to detect the presence of Tamm-Horsfall glycoprotein (THP), the most abundant protein present in urine [29].

We report a simple proof-of-principle study for forensic samples including specificity and sensitivity studies, mixture analyses, analysis of aged samples, and DNA extraction directly from the sample pad with subsequent short tandem repeat (STR) profiling.

Materials and methods

Multiplex array setup

All of the immunochromatographic test strips have the first antibody deposited on a conjugate pad beneath the sample window. On the opposite end of the sample window a wick absorbs the remaining test fluid to avoid backflow of the sample.

For the construction of the multiplex array, the strips from the four kits were removed from their plastic cassettes and placed on a glass surface in a cruciform arrangement. They were interconnected using a piece of round filter paper that acts as sample pad, overlapping approximately 0.4 mm of all four strips (Fig. 1). DNA-free gloves were worn at all times. This system allows for adding as many strips as needed to analyze the body fluids that are expected to be present in the mixture. The array design is flexible and test strips can be replaced by others at any time.

Overall, three different buffers are necessary for the multiplex array: the RSID™-Universal buffer (for semen and saliva), the RSID™-Urine buffer (for urine), and the SERATEC® PMB buffer (for menstrual fluid and blood). Although it is advised to only use the buffers specifically developed for each kit, it was obviously necessary to develop a universal buffer system for the array. Therefore, the three buffers were combined for a customized buffer system. Initial experiments showed that the following quantities per sample yielded high quality and unambiguous results for all tests (data not shown): 200 μl of RSID™-Universal buffer, 100 μl of RSID™-Urine buffer, and 100 μl SERATEC® PMB buffer. The total amount of extraction buffer, 400 μl, therefore lies within the range of each test strip needed for sample extraction. The complete extract is then applied to the sample pad of the multiplex array. This ensures that each strip is sufficiently supplied with the amount of fluid necessary to run correctly. If only certain body fluids are expected in the sample and less than four test strips are used, the amount of buffer can be reduced accordingly. For this study, a master mix of the customized buffer was prepared to use for the following tests.

Sample set

Body fluid samples were collected with informed consent using procedures approved by the local ethical committee (Ethik-Kommission der Ärztekammer Westfalen-Lippe

Table 1 Immunochromatographic kits chosen for the multiplex array. Data extracted from each tests' user manual or validation study

Kit	Body fluid	Antibody specific for	Sensitivity	Human specific
RSID™-Semen	Semen	Semenogelin	10 nl of semen	Yes
RSID™-Saliva	Saliva	α-Amylase	10 nl of saliva	Yes
SERATEC® PMB	Blood	Hemoglobin	0.002 nl of blood	Yes
	Menstrual fluid	D-Dimer	0.24 μl of menstrual fluid	Yes
RSID™-Urine	Urine	Tamm-Horsfall (THP) glycoprotein	10 μl of urine	No

und der Westfälischen Wilhelms-Universität Münster). Liquid menstrual fluid was collected using a menstrual cup (Mooncup Ltd., Brighton, UK), a soft silicone cup used as an alternative to sanitary towels or tampons. Blood was collected by venipuncture, and saliva and semen were collected in plastic cups. All samples were stored frozen in 200 μl aliquots.

As negative control 400 μl of pure customized buffer was used and added to the sample pad.

Sensitivity

To ensure that extracting the samples in the customized buffer does not result in a decrease in sensitivity of each single test, a sensitivity study was performed. Samples were diluted down to the detection limit given in each test strip's individual manual [25, 32–34]:

- For semen and saliva, 0.1 μl of sample was extracted in 1000 μl of customized buffer.
- For urine, 50 μl of sample was extracted in 1000 μl of customized buffer.
- For menstrual fluid, 3 μl of sample was extracted in 1500 μl of customized buffer.
- For blood, 0.02 nl of sample was extracted in 1000 μl of customized buffer.

After incubating the samples for 1 h at room temperature (RT), 400 μl of each solution was applied to the sample pad of the multiplex array. Results were read after 10 min (15 min for urine).

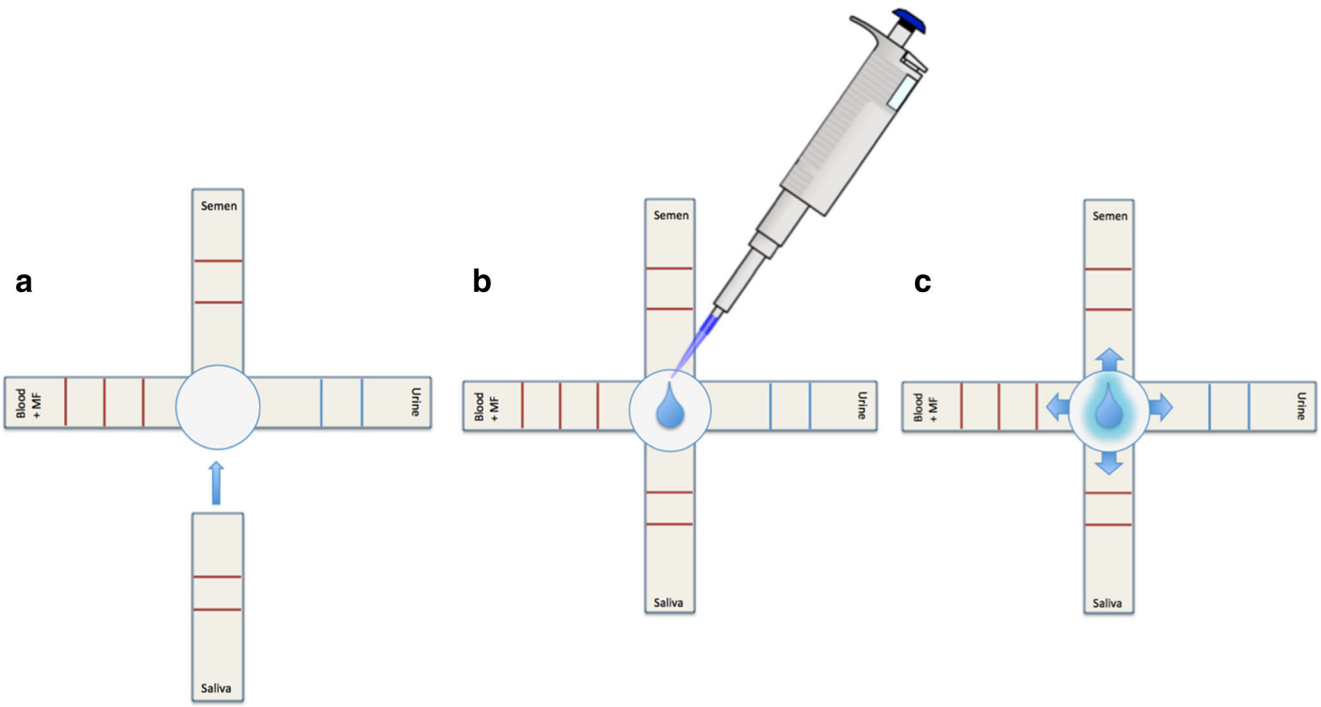

Fig. 1 Setup of the multiplex array constructed from single immunochromatographic test strips. Each test strip is added to the array individually and connected by a round filter paper that acts as sample pad (**a**). Four hundred microliters of buffer is added to the filter paper (**b**) that ensures equal distribution of the solution onto all strip tests (**c**). MF menstrual fluid

Specificity

Cross-reactivity of each test strip with other body fluids was assessed by adding each body fluid separately to the multiplex array. Samples were prepared to reach the same concentrations as suggested for positive controls by each test's manual using the customized buffer instead the individual buffers:

- For semen, 50 μl was deposited on a cotton swab, which was extracted in 1000 μl of buffer for 5 min at RT. Then, 20 μl of this extract was diluted in 380 μl of buffer to a total volume of 400 μl.
- For saliva, 50 μl was deposited on a cotton swab, which was extracted in 1000 μl of buffer for 5 min at RT. Then, 80 μl of this extract was diluted in 320 μl of buffer to a total volume of 400 μl.
- For menstrual fluid and blood, 3 μl was extracted in 1500 μl of SERATEC's PMB buffer for 5 min at RT.
- For urine, 100 μl of urine was deposited on a cotton swab, which was extracted in 300 μl of buffer for 1 h at RT.

For each body fluid, 400 μl of solution was applied to the filter paper serving as sample pad. Results were read after 10 min (15 min for urine).

Mixture analyses

Various mixtures of body fluids were prepared (Table 2). Body fluids were added to 400 μl of buffer with the following amounts: 20 μl of urine, 0.04 μl of semen, 0.04 μl of saliva, 0.008 nl of blood, and 0.8 μl of menstrual fluid. These volumes ensure that each body fluid lies within their detection limit. The mixtures were vortexed vigorously and extracted for 1 h at RT. The whole solution was applied to the sample window and results were read after 10 min (15 min for urine).

Aged samples

Four mixtures with each 20 μl of urine, 0.04 μl of semen, 0.04 μl of saliva, 0.008 nl of blood, and 0.8 μl of menstrual fluid were prepared in a reaction tube and vortexed. Each mixture, 21.5 μl in total, was added to a cotton swab. The swabs were stored at RT for 7, 14, and 21 days. They were extracted in 400 μl customized buffer for 1 h before applying the solution to the sample pad of the multiplex array.

STR profiling

DNA was extracted from the sample pad from three different samples (pure menstrual fluid, pure semen, and a mixture of the two) using the DNA IQ™ Casework Pro Kit on the Maxwell® 16 Forensic Instrument, quantified using the PowerQuant® System, and amplified using the PowerPlex® ESX 16 kit (Promgea, Mannheim, Germany) according to manufacturer's recommendations. The samples were analyzed using the 3130 Genetic Analyzer with the GeneMapper® ID software by Thermo Fisher Scientific. To investigate whether the sample pad was DNA-free before sample analysis, the filter paper was extracted and a DNA quantification was performed.

Results and discussion

Results are summarized in Table 2.

Selected tests

Table 1 gives an overview of the immunochromatographic kits chosen for the multiplex array. Information on the detected antibody as well as on sensitivity and specificity is given.

The RSID™-Semen test uses two mouse monoclonal antibodies specific for human semenogelin and is a confirmatory test for human semen. The kit was validated for forensic purposes by Old et al. in 2012. Mixtures with semen and an overshadowing presence of human saliva, blood, urine, breast milk, or vaginal secretions resulted in positive results for semenogelin presence. No cross-reactivity or inhibition with these body fluids or with semen from bull, cat, dog, goat, horse, mouse, pig, and sheep was observed. Moreover, it was shown that it is possible to use the remaining semen extract for DNA extraction and STR analyses [30]. According to the protocol for the RSID™-Semen kit, the test strips detect the presence of semen down to 10 nl [34].

Apart from detecting semenogelin for semen identification, other common methods include the detection of prostate-specific antigen (PSA) presence (e.g., SERATEC® PSA Semiquant (Göttingen, Germany)) or measuring acid phosphatase (AP) activity. Because both PSA and AP have been found in other body fluids including urine and vaginal secretions [30, 35–37], these methods were not considered for incorporation in the multiplex array.

The RSID™-Saliva test uses two monoclonal antibodies specific for human α-amylase, the most characteristic enzyme of saliva. The detection limit for RSID™-Saliva is 10 nl of saliva and the kit was validated for forensic samples in 2009 by Old et al. [27]. No cross-reactivity with blood, semen, and urine was observed. Mixtures of saliva and these body fluids resulted in clear positive signals for amylase presence. While breast milk reacted slightly positive, it was estimated that it is at least 20-fold less reactive on the kit than human saliva. The species specificity testing resulted in negative signals for a variety of both exotic and common animals, with the exception of saliva taken from gorilla that gave a positive signal. According to the protocol for the RSID™-Saliva kit, the test strips detect the presence of saliva down to 10 nl [32].

Table 2 Summary of the results using the combined immunochromatographic array. No false negative or false positive results were received. The components of mixtures were positively identified.

			RSID™-Semen	RSID™-Saliva	SERATEC® PMB		RSID™-Urine
					Blood	Menstrual fluid	
Negative control	Customized buffer		–	–	–	–	–
Cross-reactivity	Semen		+	–	–	–	–
	Saliva		–	+	–	–	–
	Blood		–	–	+	–	–
	Menstrual fluid		–	–	+	+	–
	Urine		–	–	–	–	+
Mixtures (liquid)	Semen +	Saliva	+	+	–	–	–
		Blood	+	–	+	–	–
		Menstrual fluid	+	–	+	+	–
		Urine	+	–	–	–	+
	Saliva +	Semen	+	+	–	–	–
		Blood	–	+	+	–	–
		Menstrual fluid	–	+	+	+	–
		Urine	–	+	–	–	+
	Blood +	Semen	+	–	+	–	–
		Saliva	–	+	+	–	–
		Menstrual fluid	–	–	+	+	–
		Urine	–	–	+	–	+
	Menstrual fluid +	Semen	+	–	+	+	–
		Saliva	–	+	+	+	–
		Blood	–	–	+	+	–
		Urine	–	–	+	+	+
	All 5 body fluids		+	+	+	+	+
Aged samples	All 5 body fluids on one swab	For 7 days	+	+	+	+	+
		For 14 days	+	+	+	+	+
		For 21 days	+	+	+	+	+

The SERATEC® PMB test for blood and menstrual fluid is based on a previous test strip for blood only [26] in combination with a method for detecting menstrual blood recently validated for forensic purposes [3]. The new duplex test was validated for forensic samples by our group [38] testing dilutions of liquid menstrual blood, cross-reactivity with vaginal fluid, urine, semen, or saliva as well as dog, cat, horse, and goat blood, by analyzing mixtures of menstrual blood with vaginal fluid, urine, semen, or saliva, and by performing an STR analysis. Clear positive results for D-dimer and hemoglobin presence were received with concentrations of 20 and 0.002 nl, respectively. No cross-reactivity with other body fluids or blood from other species was observed. Mixtures of menstrual fluid with other body fluids did not hamper the test results, and it was possible to receive a full STR profile from the remaining extract and sample pad.

An alternative for the detection of hemoglobin for blood identification is the detection of the protein glycophorin A (e.g., Galantos' RSID™-Blood test). For the development of the immunochromatographic multiplex assay, the SERATEC® PMB test was chosen over RSID™-Blood test as it incorporates not only the detection of peripheral but also of menstrual blood into the array.

The RSID™-Urine test has a detection limit of 10 μl of human urine [33]; however, a definite limit of detection cannot be established due to quite high variation in THP levels between samples and individuals as shown in the validation study [29]. For cross-reactivity testing, extracts from semen, saliva, blood, menstrual blood, and vaginal fluid were tested

on the cassettes, which all resulted in negative signals for THP presence. When mixtures of urine and these body fluids were tested, however, it was shown that the presence of blood inhibits the signal from urine, with a decreasing signal with increasing amounts of blood extract in a dose-dependent manner. This has to be considered if the presence of blood is suspected on a piece of evidence. Even though this test is not human specific and does show problem in potential mixtures, it was chosen for incorporation into the array because it is currently the only immunochromatographic test for urine available. Due to the highly flexible design of the array, this test can be replaced by a new and improved urine test at any time.

Multiplex array

Removing test strips from their original cassette and arrangement in a multiplex array did not disturb the test performance. Using the customized buffer also did not hamper the tests. All of the tests worked correctly as shown by the internal positive control line (C). Negative controls reacted negative for all test strips.

Sensitivity

The sensitivity testing showed that the customized buffer did not impair the tests' sensitivity and the same detection limits were reached (see figure S1): Semen samples gave clear positive signals for semenogelin presence with 10 nl of semen, saliva samples gave a clear positive signal for α-amylase presence with 10 nl of saliva, blood samples gave a clear positive signal for hemoglobin presence with 0.002 nl of blood, menstrual fluid samples gave a clear positive signal for D-dimer presence with 10 nl of menstrual fluid, and urine samples gave a clear positive signal for THP presence down to 10 μl of urine. It should be noted that the signal for hemoglobin was slightly weaker, however, it was clearly visible.

Specificity

No false positive results were obtained meaning none of the tests reacted with body fluids other than those they are specific for (see Table 2).

Mixture analyses

It was possible to correctly analyze mixtures with the constructed multiplex array: Semen-containing mixtures only reacted positive for semenogelin presence, saliva-containing samples only reacted positive for α-amylase presence, blood-containing samples only reacted positive for hemoglobin presence, menstrual fluid-containing samples reacted positive for hemoglobin and D-dimer presence, and urine samples only gave a signal for THP presence. When all five body fluids were combined, each test strip gave the correct positive signal in all tests of the array (Fig. 2).

Aged samples

Aging the samples at RT for up to 21 days did not impair the test results. The mixtures, containing semen, saliva, blood, menstrual fluid, and urine, extracted after 7, 14, and 21 days all gave clear positive signals for semenogelin, α-amylase, hemoglobin, D-dimer, and THP presence.

Figure 2 gives an example of the test's performance. It shows the negative control using only buffer on the left, and

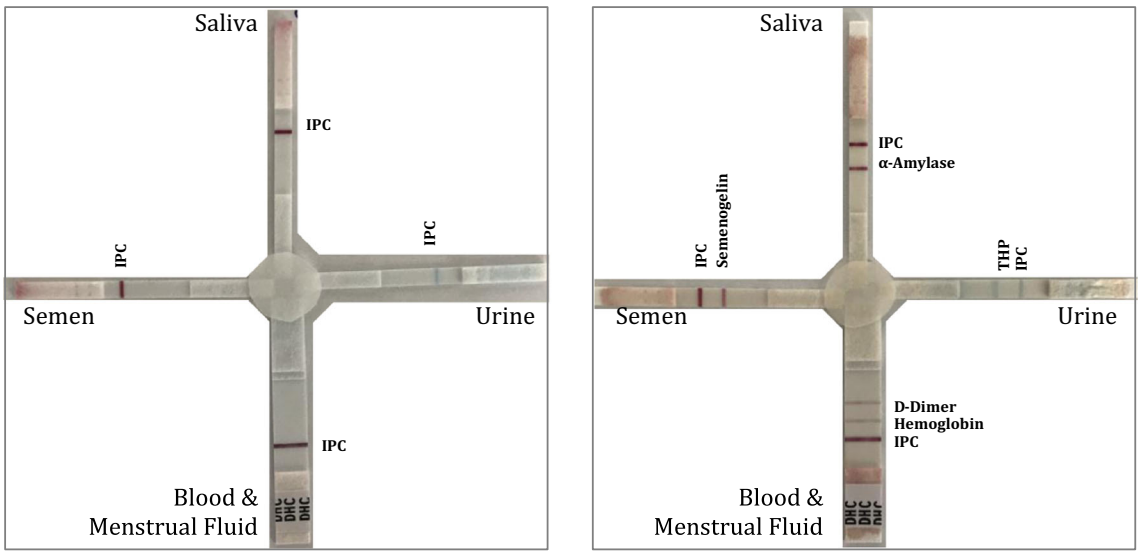

Fig. 2 When using buffer only as negative control (left) only the internal positive controls (IPC) of each test give a positive signal. and menstrual fluid (right) shows positive signals for each body fluid present

the mixture of all five body fluids on the right. With the negative control, only the internal positive controls (IPC) of the tests give a signal and no false positives are received. When the mixture is applied, all tests give a line for the IPC as well as positive reactions for each body fluid present in the mixture.

STR profiling

It was possible to generate full STR profiles from the DNA extracted from the sample pads. The sample pad containing menstrual fluid produced a full STR profile with a single female component; the sample pad containing semen produced a full STR profile with a single male component. It was shown that the mixture of semen and menstrual blood clearly stemmed from two donors, male and female (see figure S2). The DNA quantification of the filter paper without sample revealed that the filter paper used is DNA-free and does not lead to contamination of the sample.

Conclusion

The novel multiplex immunochromatographic strip test array is a reliable method that expresses high sensitivity and specificity similar to the original single strip kits by Galantos and SERATEC®. The combined strip test requires minimal training of the analyst and is a fast method to detect up to five body fluids in a mixture simultaneously. The strip test is very easy to use and shows immediate results. No special instrumentation is needed and tests can be performed in the forensic laboratory and also directly at the crime scene. Moreover, the strip test can be customized to meet the needs of the analysis: The strips can be added or removed individually according to the expected mixture in a stain. Lastly, the strip test can simply be incorporated into routine forensic laboratory work as STR profiles can be produced from the filter paper serving as sample pad saving additional sample for analysis.

Combining individual tests into an immunochromatographic array allows the analyst to blind test trace evidence because no pre-analysis decision on a certain body fluid test is needed. This limits the risk of missing one component in a body fluid mixture. Furthermore, the novel immunochromatographic array allows testing for the presence of several body fluids with only minimal sample loss, which is further reduced by successful DNA extraction from the sample pad.

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Compliance with ethical standards Body fluid samples were collected with informed consent using procedures approved by the local ethical committee (Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Westfälischen Wilhelms-Universität Münster).

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