International Journal of Legal Medicine (2023) 137:511–518
https://doi.org/10.1007/s00414-022-02917-8

ORIGINAL ARTICLE

Magnetic bead-based separation of sperm cells from semen-vaginal fluid mixed stains using an anti-ACRBP antibody

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Received: 23 August 2021 / Accepted: 15 November 2022 / Published online: 23 November 2022

Abstract

Forensic DNA analysis of semen-vaginal fluid mixed stains is essential and necessary in sexual assault cases. Here, we used a magnetic bead conjugated acrosin binding protein (ACRBP) antibody to separate and enrich sperm cells from mixed stains. Previously, western blotting indicated that ACRBP was specifically expressed in sperm cells, but not in female blood and epithelial cells, while immunofluorescence data showed ACRBP was localized to the acrosome in sperm cells. In our study, sperm were separated from mixed samples at three sperm cell/female buccal epithelial cell ratios (10^3:10^3; 10^3:10^4; and 10^3:10^5) using a magnetic bead conjugated ACRBP antibody. Subsequently, 23 autosomal short tandem repeat (STR) loci were amplified using the Huaxia™ Platinum PCR Amplification System and genotyped using capillary electrophoresis. The genotyping success rate for STR loci was 90% when the sperm to female buccal epithelial cell ratio was > 1:100 in mixed samples. Our results suggest that the magnetic bead conjugated ACRBP antibody is effective for isolating sperm cells in sexual assault cases.

Keywords Mixed stain · Acrosin binding protein · Sperm · Magnetic beads · Forensic science

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Introduction

In sexual assault cases, mixed stains comprising male sperm cells and female epithelial cells are often observed by forensic scientists. Typically, mixtures come from two individuals, which comprises the victim's and perpetrator's DNA, with the victim's DNA representing a major mixture component. These unbalanced two-individual DNA mixtures are complex and difficult when interpreting the DNA typing of the minor component [1]. Such difficulties include the following: cases involving low quantity or degraded samples causing allele dropout, and alleles shared by contributors leading to allele stacking and issues differentiating polymerase chain reaction (PCR) stutter artifacts from true alleles. To some degree, female component results can obscure male component results when autosomal short tandem repeat (STR) loci are genotyped by PCR amplification and capillary electrophoresis [2]. Therefore, it is important to interpret minor component genotyping without interference from the major component.

Statistical strategies can be used for interpreting DNA mixtures [3]. Moreover, a likelihood ratio can be calculated, which considers different propositions to include and/or exclude an individual by comparing a person of interest's reference DNA profile with an evidence DNA profile [4]. Additionally, several probabilistic genotyping software models are available to assist with mixture interpretations [5, 6]. However, they are restricted as they cannot analyze multi-source low-level DNA profiles and utilize peak height information. Based on the physical and chemical characteristics of sperm cell membranes, several methods have been developed to identify profiles in sperm cells from mixed samples containing vaginal epithelial cells; these include the differential lysis method, fluorescence activated cell sorting (FACS), laser capture microdissection (LCM), and Y chromosome short tandem repeat (Y-STR) analysis. Although simple modifications can be applied to reduce female DNA levels, operational processes in the differential lysis method are cumbersome, time-consuming, and poorly automated, and extracted DNA is easily mixed with female remnants [7]. Although FACS improves this issue to a certain extent, it does not effectively solve the issue due to limited enrichment rates for male samples when limited male sample quantities are present [8, 9]. Although LCM is accurate and displays good capture effects, it is limited by high equipment costs and high-level operational requirements, which are not conducive to mainstream public security agencies [10]. Usually, Y-STR profiling is advantageous in detecting male components in mixed stains when male contributor DNA is present only in very small amounts, such that the genetic profile of autosomal STRs cannot be detected [11]. However, based on a simulation model and software to approximate the distribution of the number of males with a matching Y profile, a simple solution was proposed to different values for the variance in reproductive success and the population growth rate [12]. Thus, Y profile values are highly comprehensible and verifiable; thus, more measures are required to improve autosomal typing detection.

Magnetic activated cell sorting (MACS) is used to capture and separate sperm cells using an antibody against specific sperm surface antigens. The sperm-antibody-biotin complex is combined with avidin-magnetic beads which directionally move in an external magnetic field [13, 14]. This sorting provides for fast and efficient separation without complex processes and expensive equipment. In recent years, antibodies against tACE, MOSPD3 (motile sperm domain containing protein 3), AKAP3 (A kinase anchor protein 3), and PH-20 (also known as sperm adhesion molecule 1 (SPAM1)) proteins, typically associated with sperm fertilization and movement, were reported as effective in separating sperm cells [8, 15, 16]. However, because some sperm antigens are localized to specific sperm compartments (neck, midsection, or flagella), incomplete sperm cannot be captured due to target antigen loss in old and degraded samples. Therefore, to ensure the highest collection efficiency, selecting a suitable sperm surface antigen is critical for successful outcomes. A suitable sperm surface antigen should have the following characteristics: (1) The antigen should only be expressed in sperm and testis, not in epithelial, blood, and other cells. (2) The antigen should be highly expressed in the head of the sperm cell. (3) The antigen should exhibit no changes in structure and properties before and after sperm capacitation.

Acrosome binding protein (ACRBP) is specifically expressed in the testis and is located in the sperm acrosome; it binds to the pro-acrosome and packages and concentrates the pro-acrosome in the acrosome matrix [17]. Therefore, ACRBP is protected during capacitation. Immunoassays have previously indicated that almost all spermatozoa express ACRBP in the head of the sperm surface [18]. Additionally, specific ACRBP expression was confirmed by western blotting and immunostaining in our study (supplementary materials). Therefore, based on good ACRBP specificity and distribution, and no significant changes in structural properties and levels before and after sperm capacitation, ACRBP has potential applications in MACS technology. In this study, we used an ACRBP antibody to separate sperm cells from different donors in mixed stains and established a fast, convenient, and efficient detection and identification method for mixed stains.

Materials and methods

Samples

This study was approved by the Ethics Committee of China Medical University. Written informed consent was obtained from all participants. Samples (peripheral venous blood, buccal epithelial cells, and sperm cells) were collected from ten males. Buccal epithelial cells were also collected from ten females. All cell types were washed three times in phosphate buffered saline (PBS) to prepare single cell suspensions. Sperm cell suspensions (10^3 cells/mL) were quantified using a cell counter (Countess 3, Thermo Fisher Scientific, Waltham, MA, USA). Female buccal epithelial cell suspensions were similarly prepared at 10^3, 10^4, and 10^5 cells/mL. Mixed samples comprising three ratios were prepared in a 100 µL sperm cell suspension (10^3 cells/mL) and a 100 µL female buccal epithelial cell suspension (10^3, 10^4, or 10^5 cells/mL). Finally, 30 mixed samples were generated using sperm cell and buccal epithelial cell suspensions.

Additionally, we collected five dried vaginal swabs from rape cases. All were obtained by forensic experts within 24 h of a sexual assault and stored at room temperature in a dry environment for > 6 months. In each case, a single man was suspected and autosomal STR genotyping had been performed.

Sperm cell capture and isolation

The ACRBP antibody was labeled using EZ-Link Sulfo-NHS-LC-LC-biotin according to the manufacturer's recommendations (Cat. No. 21338, Thermo Scientific, MA, USA). Then, 5 µL biotin-labeled ACRBP antibody was added to 100 µL mixed sample and incubated at 4 ℃ for 2 h at 60 rpm. After centrifuging at 350 × *g* for 10 min, the supernatant was discarded, and the precipitate washed three times in 500 µL PBS. Then, 25 µL dynabeads (Dynabeads™ FlowComp™ Flexi Kit, Cat. No. 11061D, Thermo Scientific) were added to the PBS, incubated at 4 ℃ for 15 min at 60 rpm, and then biomagnetically separated. The sample was placed in a magnetic frame for 5 min. The supernatant was discarded and 200 µL PBS added to rinse cells. The procedure was repeated four times. Finally, 200 µL release buffer (Dynabeads™ FlowComp™ Flexi Kit) was added and incubated with the sample at 4 °C for 10 min at 60 rpm. The supernatant containing bead-free cells was transferred to a new tube in a magnetic frame.

DNA extraction and STR genotyping

Genomic DNA was extracted using the chelex-100 method [19] and autosomal STR (23) genotyping performed using the VeriFiler™ Plus PCR Amplification System (Applied Biosystems®, Life Technologies™, USA) in a GeneAmp® PCR 9700 (Thermo-Fisher Scientific) thermal cycler, according to the manufacturer's recommendations [20]. PCR products were detected and separated using the Applied Biosystems™ 3500 Series Genetic Analyzer™ (Thermo-Fisher). Raw data were analyzed using GeneMapper *ID-X* 1.4 software (Thermo-Fisher). Allelic nomenclatures were determined using an allelic ladder provided by the Huaxia™ Platinum PCR Amplification System.

DNA quantification

DNA from different samples was quantified using an ABI 7500 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). Quantification was performed for both *GAPDH* and *SRY* loci [21, 22]. The *GAPDH* primer was used to confirm DNA presence and quality in samples. The *SRY* primer was used to measure male DNA quantity. Female DNA quantification was calculated by subtracting male DNA from total DNA. The following primers were used:

GAPDH forward primer: 5′-CCC CAC ACA CAT GCA CTT ACC-3′

GAPDH reverse primer: 5′-CCT AGT CCC AGG GCT TTG ATT-3′

SRY forward primer: 5'-TCT TCC AGG CAC AGA AAT T-3'.

SRY reverse primer: 5'-CTT CCG ACG AGG TCG ATA CTT ATA A-3'.

Reaction conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s.

Processing vaginal swabs

A vaginal swab was cut into 6–10 pieces and soaked in distilled water for 2 h with gentle shaking. This increased mechanical shear forces during initial thermo-mixer incubation steps and facilitated biological material release from swabs [23]. After centrifugation at 1000 × *g* for 10 min, the supernatant was removed. The remaining sample was processed using the differential lysis method [24] or MACS using the ACRBP antibody. Subsequently, DNA was extracted using the chelex-100 method and amplified using the Huaxia™ Platinum PCR Amplification System.

Results

Sperm cells captured using the anti-ACRBP MACS approach

Sperm successfully bound to the anti-ACRBP antibody. The biotin-labeled ACRBP antibody bound to magnetic beads via biotin-avidin interactions. Finally, magnetic beads captured sperm cells via the biotin-labeled ACRBP antibody. Under 400 × microscopy, sperm cells were bound by one or more magnetic beads, with beads mainly located to the acrosome (Fig. 1). Sperm cell morphology was intact.

For dried vaginal swabs in the rape case, almost no integral sperm with intact tails were identified (Fig. 2). However, magnetic beads captured sperm cells via the ACRBP antibody which bound to the acrosome.

STR genotyping

Mixed samples (three ratios) were prepared using a 100 µL sperm cell suspension (10^3 cells/mL) and 100 µL female buccal epithelial cell suspensions (10^3, 10^4, or 10^5 cells/mL). Finally, 30 mixed samples were generated using sperm cell and female buccal epithelial cell mixed suspensions.

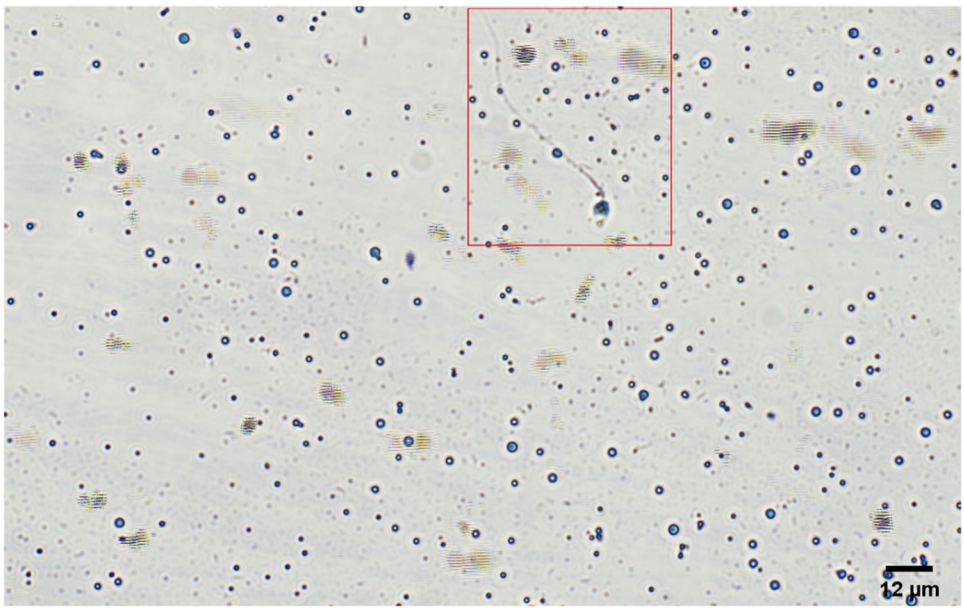

Fig. 1 Sperm cells captured by the anti-ACRBP MACS method using microscopy. Note: The red box shows an intact sperm cell captured by MACS

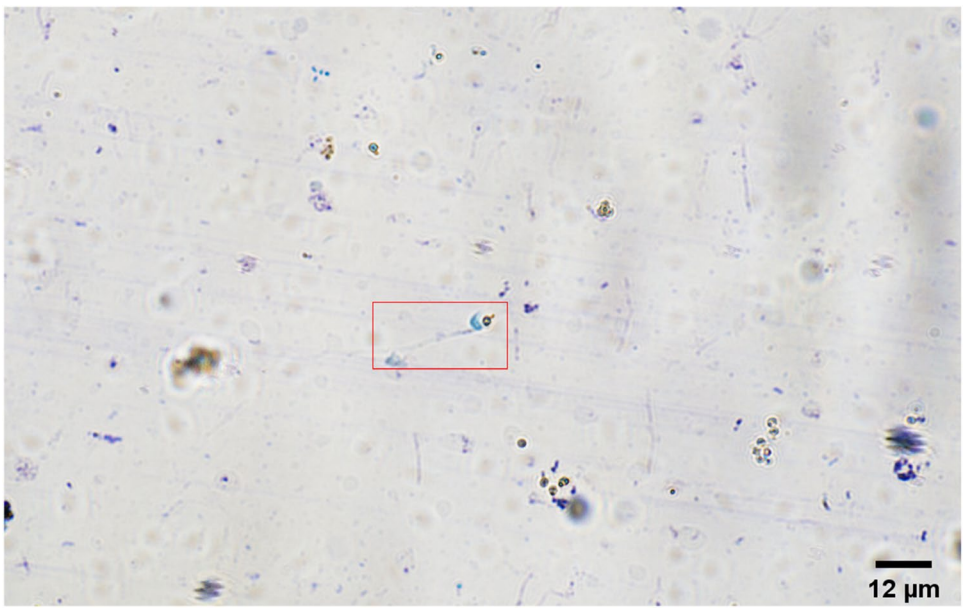

Fig. 2 Sperm cells captured by the anti-ACRBP MACS method in a vaginal swab from a rape case. Note: The red box shows an intact sperm cell captured by MACS

Sperm cells were captured using anti-ACRBP MACS from all 30 mixed samples at three ratios (10^3:10^3; 10^3:10^4; and 10^3:10^5 cells/mL). The DNA from each sample after MACS separation was quantified using real-time PCR (Fig. 3). After one MACS separation, average recovery rates were 79%, 65%, and 31% in three mixed samples at 1:1, 1:10, and 1:100 male and female component ratios, respectively. MACS separation removed female components, but also caused a loss of sperm cells, especially at the high male: female ratio. Subsequently, 23 autosomal STR loci were genotyped after DNA extraction (Fig. 4). The average peak heights in STR profiles after separation are shown (Table 1). In mixed samples at three ratios, female buccal epithelial cells were completely removed after four separations. Recovery rates were as follows: 72% in the mixed 1:1 ratio sample, 68% in the mixed 1:10 ratio sample, and 26% in the mixed 1:100 ratio sample. Excessive female epithelial cells appeared to decrease sperm recovery rates. These results showed that a single male individual was detected and genotyped without female profile using MACS sperm cell capture using an anti-ACRBP antibody (Table 2). In the mixed 1:1 ratio sample, all ten samples were successfully genotyped in 23 STR loci. In the mixed 1:10 ratio sample, nine samples were successfully genotyped in 23 STR loci. In the mixed 1:100 ratio sample, only two samples were successfully genotyped in 23 STR loci.

For the five dried vaginal swabs in the rape case, three samples were successfully genotyped in 23 STR loci (Fig. 5). After four MACS separations, the female component was removed and a full male profile generated. When compared with the differential lysis method, MACS was more successful in effectively removing female cells. The success rate of the five dried vaginal swabs was 60% for dried vaginal swabs stored for > 6 months.

Discussion

In this study, the ACRBP antibody was used to specifically bind to sperm cells. The biotin-labeled antibody then bound with magnetic beads via biotin-avidin interactions. Thus, sperm cells were separated and enriched in the magnetic frame. Finally, female epithelial cells were removed by repeated elution, and only sperm cells were collected for genotyping in autosomal STR analysis.

ACRBP expression occurs in sperm cells, but not in blood or buccal epithelium cells. Immunofluorescence data previously suggested that ACRBP was distributed in the acrosome of sperm cells. Therefore, in old sperm cells where the tail is missing, the ACRBP antibody can be successfully used for sperm capture. In sperm cells, nuclear DNA is located inside the sperm head; therefore, the ACRBP antibody is ideal for capturing degraded sperm cells when compared with other proteins expressed in the midpiece or tail [25, 26]. The genotyping rate of our method was higher than that in magnetic beads coupled to the anti-hLCN6 monoclonal antibody and equivalent to the rate in magnetic beads coupled to the anti-PH-20 antibody [16, 27]. In the study by Chen et al., when sperm cell counts were 10^3/mL, 10^4/mL, and 10^5/mL in mixed stain samples, STR typing success rates were 40%, 90%, and 100%, respectively [27]. In the study by Zhao et al., the anti-PH-20 antibody-coupled to immunomagnetic beads successfully generated single-sourced DNA profiles at a successful rate of 90% in 20 cell mixtures, where epithelial cell and sperm concentrations were fixed at 10^5/mL and 10^3/mL, respectively [16]. However, our success rate decreased to 60% when we used the anti-ACRBP MACS approach in the five vaginal swabs from a rape case. This might be due to the storage condition of the sample. In magnetic bead-based separation using the anti-MOSPD3 antibody, the profile rate decreased with extended storage time. For dried vaginal swab specimens, the successful detection

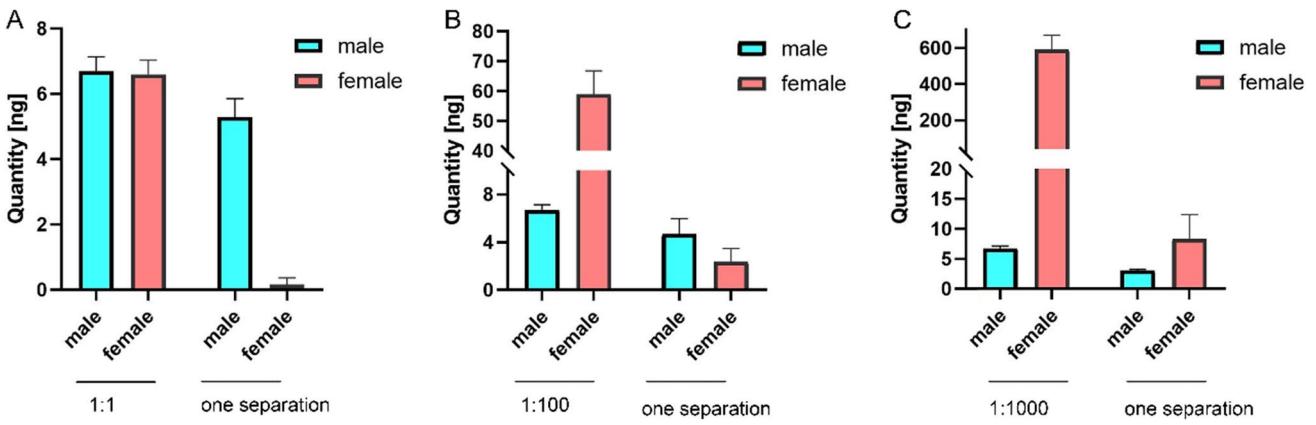

Fig. 3 Extracted DNA quantification after MACS separation using real-time PCR

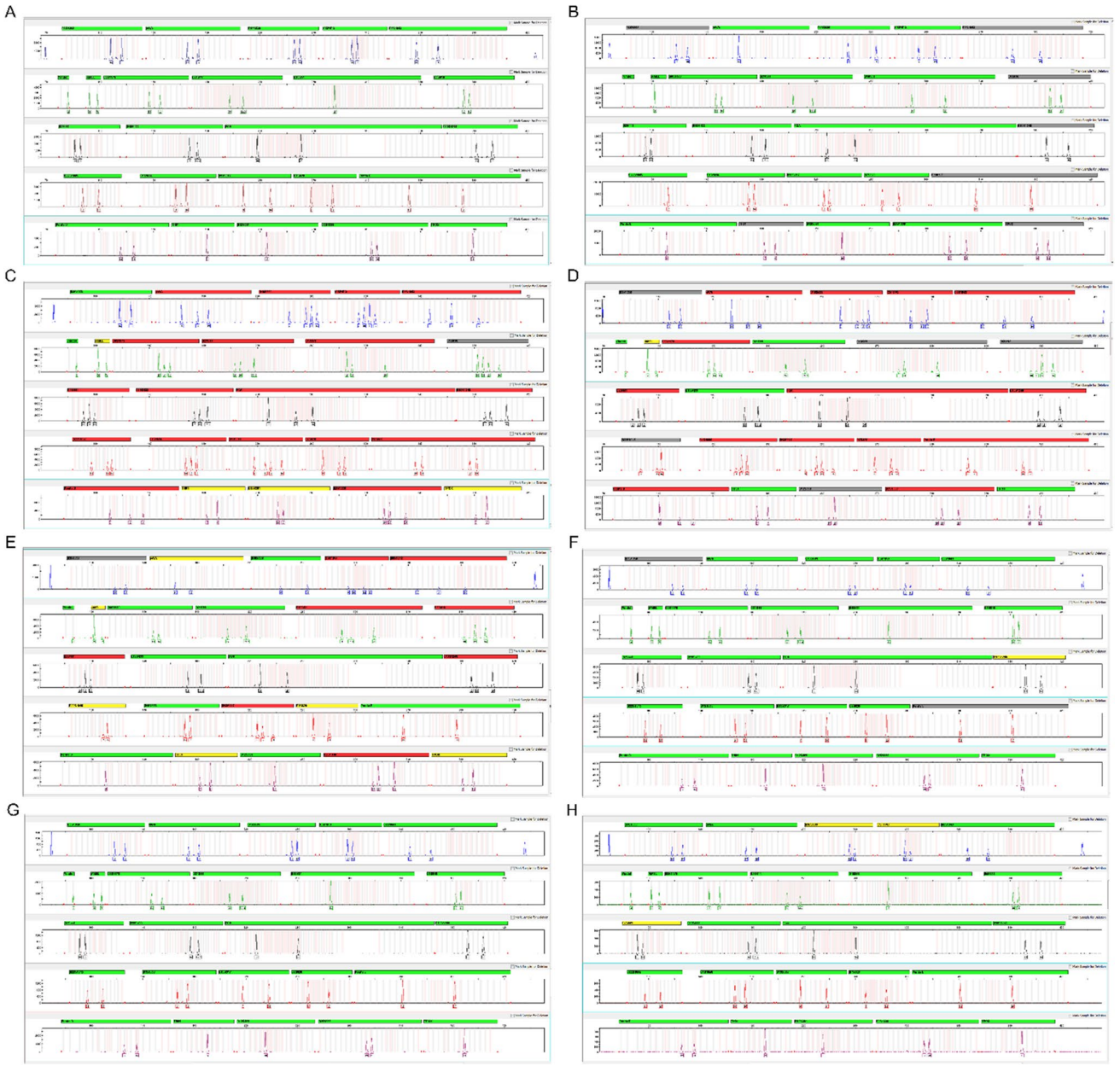

Fig. 4 STR loci profiles genotyped in mixed samples. **A** Genotyping in sperm cells; **B** genotyping in female buccal epithelial cells; **C** genotyping in mixed male and female cells at a 1:1 ratio; **D** genotyping in mixed male and female cells at a 1:10 ratio; **E** genotyping in mixed male and female cells at a 1:100 ratio; **F** genotyping in mixed samples at a 1:1 ratio after MACS separation; **G** genotyping in mixed samples at a 1:10 ratio after MACS separation; **H** genotyping in mixed samples at a 1:100 ratio after MACS separation

Table 1 Average peak heights of STR profiles genotyped in 30 mixed samples using MACS separation

Sperm cell to female buccal epithelial cell ratio	Average peak height (male:female)				
	Without separation	One separation	Two separations	Three separations	Four separations
1:1	3942:3751	3578:590	3260:183	3179: < 50	2850: < 50
1:10	1670:6726	1427:734	1342:155	1263: < 50	1142: < 50
1:100	690:7029	389:2471	296:1276	224:348	182: < 50

Table 2 Number of STR loci successfully genotyped in 30 mixed samples

Sperm cell:female buccal epithelial cell (cells/mL)	STR loci successfully amplified			
	23	21 ~ 22	19 ~ 20	< 19
10^3:10^3	10	0	0	0
10^3:10^4	9	1	0	0
10^3:10^5	2	1	5	2

rate was 40% in flocked swabs and 16.67% in cotton swabs when both of the sample were preserved for 10 days [15]. Additionally, for undiluted sperm samples, an average recovery rate of 58% was observed when the MACSprep™ Forensic Sperm MicroBead Kit was used and 43% for the Erase Sperm Isolation Kit [23].

In our study, the successful genotyping rate for all 23 autosomal STR loci was 90% when the sperm cell count was 10^4/mL in mixed samples and 100% when the count was 10^5/mL. According to a 2010 WHO (World Health Organization) report (WHO laboratory manual for the examination and processing of human semen), lower reference limits for semen characteristics were as follows: Total sperm count is 39×10^6/ejaculate, and the sperm concentration is 15×10^6/mL. Plausibly, sperm cells from sexual assault cases can meet magnetic bead-based sperm isolation requirements [23]. Nevertheless, not only are total sperm counts in mixed samples important, but also sperm to epithelial cell ratios will affect separation efficiencies. In rape cases, it is difficult to completely genotype minor male DNA profiles under interference from major female components. In our method, sperm cells were successfully captured by the anti-ACRBP MACS method, and the genotyping rate reached 90% in the presence of 90% female components.

The MACS system is advantageous in terms of its simple operation, fast separation, and relatively inexpensive experimental instruments; therefore, it can be used in identification agencies and public security facilities. Additionally, the method rarely damages sperm cells and can be used to simultaneously separate, purify, and enrich sperm cells, with future cope for automated detection. However, the MACS method has some limitations. Firstly, antigens on sperm cell membranes may be damaged or lost, which may decrease capture capability. Plus, this capturing ability will decrease in degraded samples. Secondly, underlying inhibitors at crime scenes may affect the binding strength of the antigen–antibody [28]. Finally, magnetic bead characteristics, such as size, shape, and material, must be optimized in the future [29].

Conclusions

The ACRBP antibody was successfully used to capture and separate sperm cells using magnetic beads in a magnetic frame via biotin-avidin interactions. After female epithelial cells were removed by repeated elution, male sperm cells were collected for genotyping using autosomal STR analysis. The genotyping rate of STR loci was 90% when

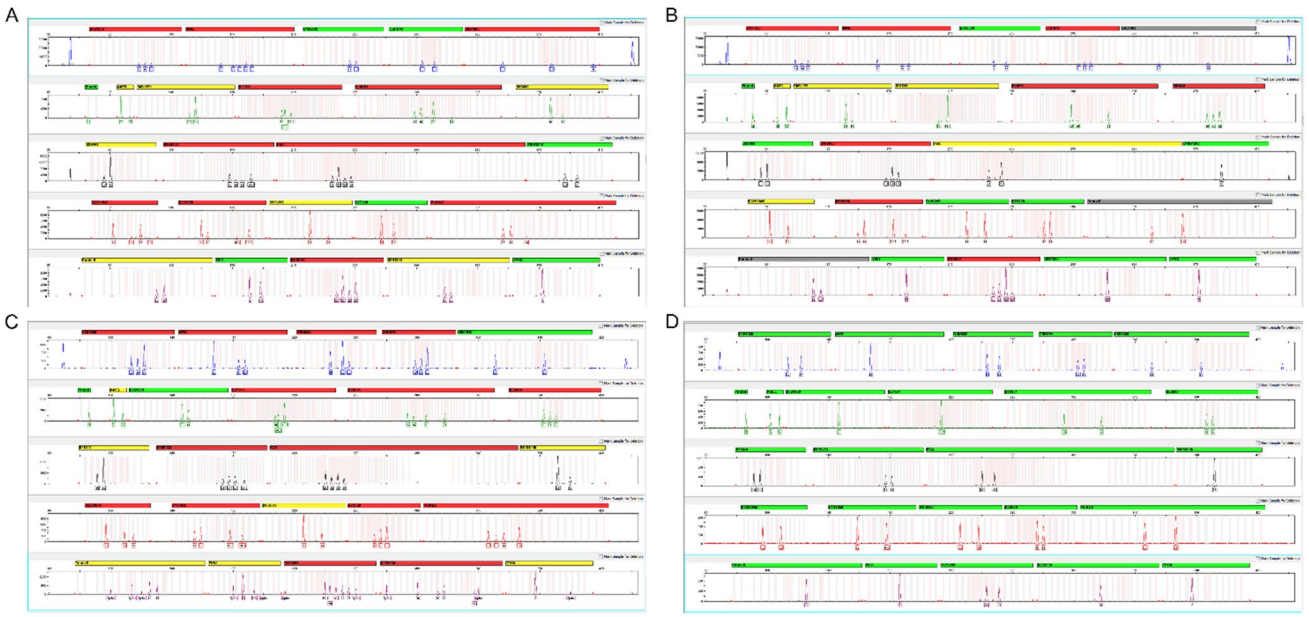

Fig. 5 Genotyping profiles in vaginal swab samples from a rape case. **A** The profile after soaking the vaginal swab; **B** the soaking profile after differential lysis; **C** the soaking profile after two MACS separations; **D** the soaking profile after four MACS separations

the sperm cell to female buccal epithelial cell ratio was more than 1:100 in mixed samples. Our results suggest that capturing sperm cells using the anti-ACRBP MACS method has promising applications for mixed samples in forensic medicine.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00414-022-02917-8.

Funding This study was supported by Open Project of Shanghai Key Laboratory of Forensic Medicine and Judicial Expertise by the Ministry of Justice Key Laboratory (No. KF202110) and Basic Scientific Research Project of the Ministry of Public Security (No. 3242020014).

This study was approved by the Ethics Committee of China Medical University. All the participants were included after providing the written informed consent.

Declarations

Conflict of interest The authors declare no competing interests.

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