DNA profiling of microdissected spermatozoa

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ABSTRACT

In sexual assault cases, DNA profiling for perpetrator identification requires isolation of pure spermatozoa because evidences are often mixture between the sperm cells and the victim's cells. Laser microdissection is a powerful tool that directly separates and collects the sperm cells without contamination from another cell. The objectives of this report are to study DNA profiling of laser microdissected spermatozoa for personal identification and to study different factors that may affect the interpretation of DNA profiles from laser microdissected spermatozoa. First, we demonstrated the ability of laser microdissection to identify and genotype spermatozoa. Second, three staining reagents; modified Hematoxylin & Eosin (H&E), eosin and sperm HY-LITER®, were evaluated on quality of DNA profiles that derived from laser microdissected spermatozoa. Finally, STR analysis was performed on laser microdissected cell from stained slides that stored in various time. The results showed that at least 150 sperm cells were required to obtain a complete DNA profile, whereas, a partial DNA profile was obtained from 25-75 haploid sperm cells. In staining comparison result, modified H&E provided the best result of DNA profiles and suitable to apply for laser microdissection. In addition, we obtained complete DNA profiles from stained slides stored for 1 month whereas the stained slides stored for 12 months yielded poor DNA profiles. It showed that storage time of the stained slides had an effect to DNA quality and DNA analysis. In conclusion, laser microdissection is a useful tool to isolate sperm cells and makes a chance to generate genetic profile from low number of sperm cells. However, number of starting sperm cells, staining and duration of storage time are factors that affect the quality of the DNA profile derived from laser microdissected spermatozoa. In addition, to obtain reliable DNA profiles, it is recommended to avoid the factors that may have negative effect on DNA typing process.

Keywords: DNA profile; laser microdissection; spermatozoa; sexual assault

INTRODUCTION

Sexual assault case investigation is one of the most difficult scenarios that forensic laboratories work with. DNA analysis has become a powerful tool for perpetrator identification involved in sexual assault crimes. Several factors impact the achievement of DNA results that either implicates or exonerates a suspect. Vaginal smear samples from victims are common evidence from sexual assault crimes. Therefore, identification of sperm cells in evidence is important to confirm the presumption of sexual assault cases. Moreover, sperm cells can lead to vital information in the process of identifying the assailant. To obtain independent DNA profiles of the victims and the perpetrators, the victim's epithelial cells and the male suspect's sperm cells should be separated for subsequent DNA extraction step. Sometimes the separation of these two cell types is unsuccessful, it would result in an uninterpretable mixed DNA profile. In 1985, the technique to separate sperm nuclei from vaginal cellular debris of semen-contaminated vaginal swabs was developed and led to the identification of the male DNA profile (Gill *et al*., 1985). This procedure is commonly referred to differential extraction (DE). The principle of DE involves differential stability of the cell type nuclear membrane through the preferential lysis of vaginal epithelial cell DNA. Sperm cell DNA was then separated from epithelial cell DNA and independent DNA profiles of male and female DNA were analyzed (Norris *et al*., 2009). The limitations of DE are time-consuming, requires extensive sample handling, difficult to automate, and

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inefficient separation of female DNA from the male fraction (Wiegand *et al*., 1992; Schoell *et al*., 1999; Norris *et al*., 2009).

Successfulness of the DNA analysis depends on the ability to obtain interpretable DNA profiles and also requires an abundance of sperm cells in order to generate complete DNA profiles. However, there are many factors may affect the interpretation of DNA profile such as low DNA yields because of DNA damage and degradation. Therefore, understanding of factors that affect the DNA profiling is necessary. Laser capture microdissection (LCM) is a technology that combines amplification function of a microscope with precisely cutting ability of a laser beam to isolate a single cell or interested cell populations from complex tissue samples. LCM was reported as a practical method for the rapid and efficient isolation of target cells and subsequently performed a variety of downstream analyses (Curran *et al*., 2000; Espina *et al*., 2006). The application of LCM is widely used in several fields including DNA genotyping and loss-of-heterozygosity (LOH) analysis, RNA transcript profiling, cDNA library generation, proteomics discovery and signal-pathway profiling (Espina *et al*., 2006). In 2003, LCM was first demonstrated that spermatozoa were isolated from microscope slides containing mixed cells of sperms and vaginal cells (Elliott *et al*., 2003; 2004). Short tandem repeat (STR) typing was obtained from microdissected sperm cells and single hair follicles (Martino *et al*., 2004a; 2004b). LCM has many potential benefits for application in forensic laboratories, especially in sexual assault cases. In addition, staining is an important step that enhances clear visualization and provides good discrimination of sperm cells. It is worth noting that appropriate staining methods for laser microdissection play important role to downstream analysis. Here, we report validated results on STR typing of laser microdissected spermatozoa for personal identification.

MATERIALS AND METHODS

Sample collection

Liquid semen samples were left-over specimens and obtained from Department of Obstetrics and Gynecology, Faculty of Medicine of Ramathibodi hospital. The document for sample collection was approved by Ramathibodi Hospital Ethic Committee (ID 07-54-55, No.2011/370). Ten microliters of the semen sample were prepared to 1:1 dilution in sterile water and then 10µL of mixtures were smeared on a conventional glass slide, air dried at room temperature for 5 min and stained with one of the following methods.

Modified Hematoxylin and eosin (H&E) staining method

This H&E staining method was slightly modified from standard protocol. Soak slides in 75% ethanol for 1 min and rinsed with sterile water. Then stain in hematoxylin for 1 min and rinse with sterile water. Next, stain with eosin for 30 sec and rinse with sterile water again. Following air drying at room temperature for 10 min, the slides were ready to use.

Eosin staining method

Dip slides in 75% ethanol for 1 min and rinsed with sterile water. Then stain in eosin for 1 min and rinse with sterile water. Following air drying at room temperature for 10 min, the slides were ready to use.

Sperm HY-LITER™ staining method

According to the manufacturer's recommendations (Independent Forensics, Hillside, IL, USA).

Laser microdissection (LMD)

A PALM® MicroBeam instrument (Carl Zeiss Ltd.) fitted with a 355 nm UV-laser was used for laser microdissection of spermatozoa (400 x magnifications). The sperms were isolated, cut and catapulted against gravity into the caps of 0.5 mL reaction tube containing 60 µL of sperm cell lysis [10 µL of Proteinase K (QIAGEN, USA), 10 µL of 1mM DTT and 40µL of ATL (QIAGEN, USA)].

DNA extraction and STR typing

DNA extraction was performed followed Qiagen® (QIAGEN, USA) protocol supplied by manufacturer with some modifications. DNA amplification was performed using the AmpFlSTR® Identifiler kit™ (Applied Biosystems, Foster City, CA). Thermal Cycling was performed by modified conditions; incubate at 95 ºC for 11 min (polymerase activation); 94 ºC for 1 min (denaturation), 59 ºC for 2 min (annealing), 72 ºC for 1 min (extension) for 31 cycles; and then 60 ºC for 60 min (final extension). AmpFlSTR® Control DNA 9947A (Applied Biosystems, 0.1ng/ µl) and sterile water were used as positive and negative controls, respectively. Electrophoresis was performed on a 3130 genetic

analyzer (Applied Biosystems, USA), followed by data analysis using GeneMapper ID Software (Applied Biosystems, USA).

Validation of sperm cell numbers

Modified H&E stained slides were prepared. Cell identification was performed at a magnification under the x40 objective lens to collect 150, 75, 50 and 25 sperm cells using bright field on the PALM MicroBeam laser microdissection microscope. Cells were extracted using the Qiagen QIAamp® Micro kit, and followed by STR analysis. Quality of DNA profile and numbers of detected loci from each sample were compared.

Slide staining

Each smeared semen slide was stained with modified H&E, eosin and Sperm HY-LITER™ kit including an unstained control slide. Sperm cells were identified and cut using PALM® MicroBeam laser microdissection microscope under 400x magnifications. Collection of 150 sperm cells were recovered from each slide in duplicate, then cells were extracted using QIAamp® Micro kit, and followed by STR analysis. Quality of DNA profile and numbers of recovery loci from each sample were compared.

Stained slide storage

One hundred and fifty sperm cells were collected from various aging of modified H&E stained slides. All slides were stored at ambient temperature from 1 to 12 months. Collected sperm cells were extracted and amplified using QIAamp® and AmpFlSTR® kit, respectively. Finally, the STR profiles, the numbers of detected loci and quality of DNA profiles from each sample were compared.

RESULTS AND DISCUSSION

Validation of sperm cell numbers

Laser microdissection is a powerful tool to isolate specific cells and can apply to work with many fields of researches including forensic science. This technology can be appropriately used by researchers with expertise in morphological assessment of tissues to apply for several fields of interest (Emmert-Buck *et al*., 1996; Murray 2007). Use of laser microdissection has been evaluated for isolation of spermatozoa in forensic laboratories, especially in crime of sexual assault cases (Elliott *et al*., 2003; Di Martino *et al*., 2004; Sanders *et al*., 2006). It is reported that the mean DNA content for cell populations varies from 1.8 to 68.6 pg across cell types ranging from haploid sperm to polyploid megakaryocytes (Gillooly *et al*., 2015). Principally, it is a variable range to amplify DNA from a single haploid or diploid cell containing 3 - 6 pg but practically it may prove more difficult to obtain a reliable DNA profile (Lucy *et al*., 2007).

Here, we decided to apply laser microdissection to collect sperm cells from modified H&E stained slides in order to generate genetic profiles. Laser microdissection was performed to work and adjust many parameters for cutting and catapulting cells through PALM RoboSoftware (P.A.L.M. Microlaser Technologies AG, Germany). Lucy, et al. studied the relationship between the number of cells with the probability to obtain the DNA profile and found that it is necessary to use 15 to 20 haploid cells to generate complete DNA profiles (Lucy *et al*., 2007). The optimal number of sperm cells to obtain complete STR profiles was analyzed by decreasing numbers of microdissected sperm cells repeatedly. For our preliminary experiment in H&E staining, the slides were stained with standard protocol of H&E staining for 5 and 2 min, respectively. The results showed incomplete DNA profiles from 500, 200 and 150 microdissected sperm cells which detectable STR loci were 16, 9 and 0 loci, respectively (data not shown). Then, we slightly modified staining time of H&E staining to be 1 min and 30 sec, respectively and complete STR results were obtained from 150 microdissected sperm cells. The validation study of STR profiles generated from 150, 75, 50 and 25 sperm cells was performed by the Identifiler® kit which amplified 16 STR loci in duplicate experiment resulted in 32 expected STR loci. Complete DNA profiles of all 32 loci generated were obtained when the assay was performed on 150 sperm cells and the numbers of detected loci were decreased to 31, 28 and 23 when the numbers of sperm cells were reduced to 75, 50 and 25 cells, respectively (Table 1). The relative fluorescent unit (RFU) amount of each allele from all loci were considered. Scatter plot between various starting sperm cells and RFU amount showed positive correlation; DNA profile derived from 150 sperm cells represent the highest RFU amount and gradually decreased when derived from 75, 50 and 25 sperm cells (Figure 1).

Table 1 Detected STR loci of 25-150 spermatozoa microdissected from modified H&E stained slides in duplicate experiments.

Numbers of spermatozoa	Numbers of detected STR loci		Total number of detectable STR loci	Expected number of detected STR Loci
	1st experiment	2nd experiment		
150	16	16	32	32
75	15	16	31	32
50	15	13	28	32
25	10	13	23	32

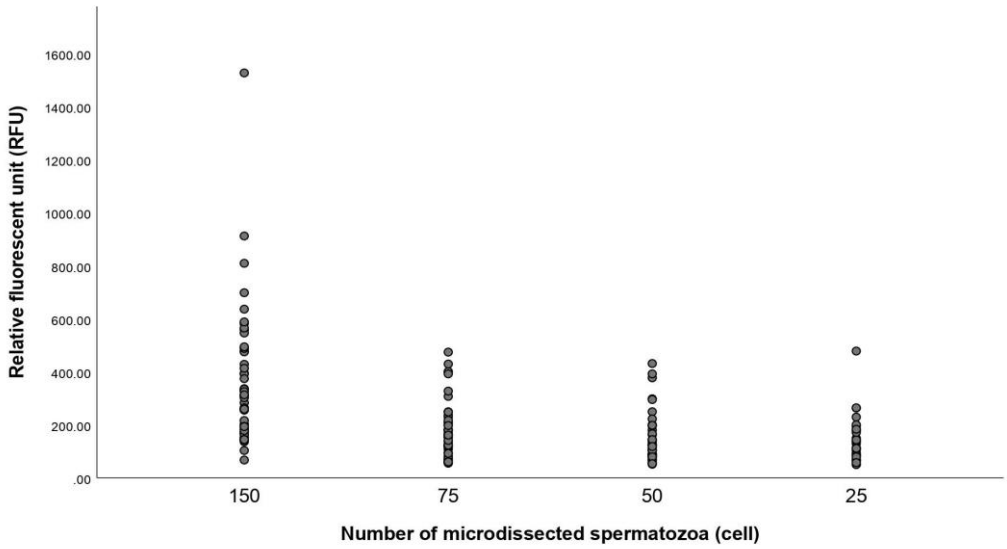

Figure 1 Relative fluorescent unit (RFU) of each allele from every locus were plotted in scatter plot to considered correlation between various starting number of microdissected sperm cells and RFU.

Quality of each DNA profile were considered. In fact, imbalance peak height of heterozygote alleles can be observed in a case of low DNA template which can resulted in failure to amplify one or both alleles of heterozygous alleles leading to allele dropout and locus dropout events (Benschop *et al*., 2011). The percentage of locus dropout obtained from starting 75, 50 and 25 sperm cells were 3.13, 12.50 and 18.75 %, respectively. Whereas DNA profile derived from 150 sperm cells were not observed locus dropout event (Table 2). Similarly, the percentage of allele dropout were increased when decreasing number of sperm cells were amplified; 1.85%, 11.11%, 20.37% and 44.44% from 150, 75, 50 and 25 sperm cells, respectively (Table 3). Peak height ratio (PHR) of heterozygote alleles were calculated to considered the balance of alleles by dividing peak height of lower RFU amount allele with peak height of higher RFU amount allele in the same loci then converted to a percentage whereas null or silent alleles due to allele drop out were counted as 0. The expected percentage of PHR for heterozygote alleles should be greater than 60% (Butler, 2014). We founded that the number of heterozygous loci that reached the acceptance criteria were mostly observed in DNA profile obtained from 150 sperm cells and gradually decreased correlating with decreasing number of microdissected sperm cells (Figure 2). Previous study showed that laser microdissection of 10, 20 and 30 sperm cells from a semen smear resulted in useful DNA profiles with a few typical allelic drop-outs (Di Martino *et al*., 2004). These authors demonstrated that although the sperm heads were morphologically normal but DNA inside may be degraded because of the fixation and staining procedure. Laser microdissection coupled with on-chip low volume PCR for the isolation and genotyping of as few as 15 sperm cells demonstrated that 13–16 loci of 16 complete loci were obtained, whereas at least 40 sperm cells were required to obtain 13–16 loci by standard PCR protocol (Li *et al*., 2011). For single spermatozoa STR typing with 3% diluted primer protocol, 6-12 of 16 loci (8.7 ± 2.8 loci) were reported (Miyazaki *et al*., 2008).

Table 2 Numbers of observed locus and the percentage of locus dropout for the assay performed on 150, 75, 50 and 25 sperm cells.

Number of sperm cells	Total locus	Observed locus	Missed locus	% Locus dropout
150 cells	32	32	0	0.00%
75 cells	32	31	1	3.13%
50 cells	32	28	4	12.50%
25 cells	32	23	6	18.75%

Table 3 Numbers of observed alleles and the percentage of allele dropout for the assay performed on 150, 75, 50 and 25 sperm cells. Reference profile had 27 alleles in duplicate experiment resulted in 54 total alleles.

Number of sperm cells	Total alleles	Observed alleles	Missed alleles	% Allele dropout
150 cells	54	53	1	1.85%
75 cells	54	48	6	11.11%
50 cells	54	43	11	20.37%
25 cells	54	30	24	44.44%

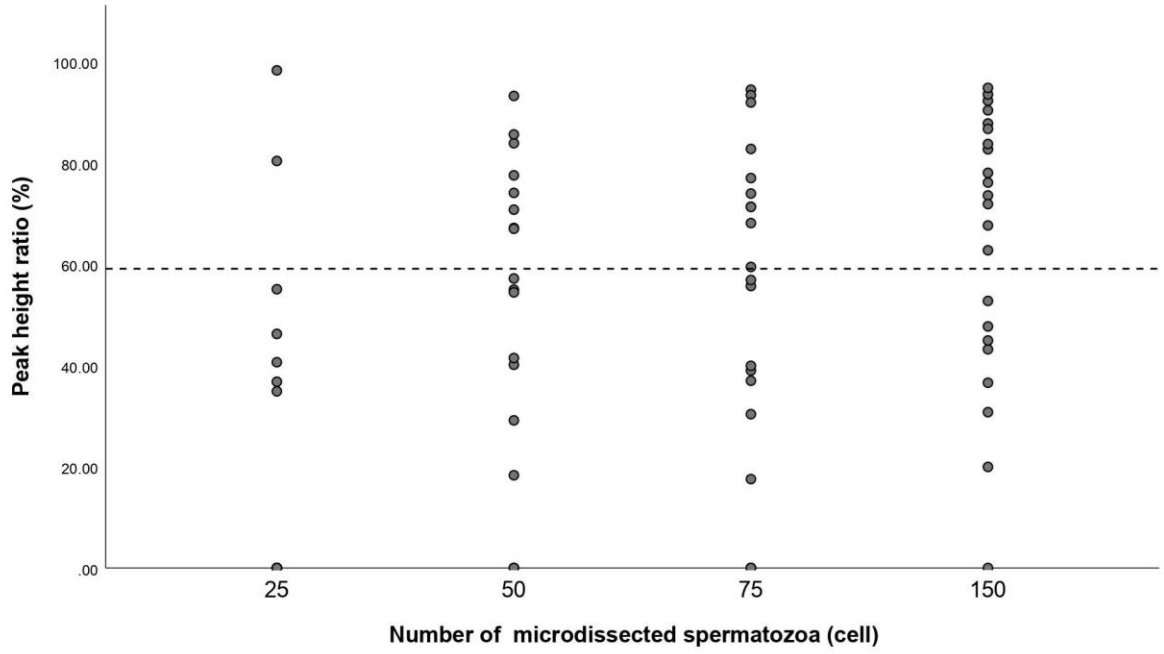

Figure 2 A scatter plot demonstrated the percentage of peak height ratio (%PHR) from heterozygote alleles with varying number of microdissected sperm cell at 150, 75, 50 and 25 cells. A discontinuous line mark the 60% peak height ratio threshold.

Slide staining and storage

Staining is an important process that enhances clear visualization and provides good discrimination of sperm cells. Moreover, stained slide increases speed of detection and isolation whereas identification of unstained slides is slow and laborious. Despite its advantage, it may have negative effect on the DNA quality. Some staining methods may have an influence on DNA recovery because DNA from sperm cells may be degraded as a result of

the fixation and staining procedure (Di Martino *et al*., 2004; Sanders *et al*., 2006). Therefore, it is important that appropriate staining methods for laser microdissection must have minimal impact on downstream DNA analysis. We performed DNA typing from microdissected sperms using 16 STR specific primers. In the preliminary experiment, we always succeeded in obtaining complete DNA profiles from 150 stained sperm cells. Three different staining methods were examined in duplicate experiments to obtain successful STR typing results from 150 sperm cells. The STR profiles from modified H&E, Eosin and sperm HY-LITER™ stained slides including unstained control slide were shown (Table 4). Thirty two loci were detected from unstained and modified H&E stained slides compared to 31 loci detected from Eosin and sperm HY-LITER™ stained slides. For staining method in our system, good results of complete STR profiles were obtained from modified H&E stained slides of 150 sperm cells. Another report supported our result that full STR profiles were produced from the DNA extracted from the unstained positive control and H&E stained slides (Simons and Vintiner, 2011). There was no significant difference in the amount of DNA recovered from stained slides treated with the H&E and Christmas Tree stains (Simons and Vintiner, 2011). In addition, the alternate light source to visualize semen stains present on surfaces, acid phosphatase test and presumptive tests are generally suitable in detecting semen prior to DNA profiling (Rodriguez *et al*., 2019).

Table 4 Detected STR loci of 150 spermatozoa microdissected from unstained, modified H&E, Eosin and Sperm HY-LITER™ stained slides in duplicate experiments.

Types of slides	Numbers of detected STR loci		total number of detectable STR loci	Expected number of detected STR Loci
	1st experiment	2nd experiment		
unstained	16	16	32	32
modified H&E	16	16	32	32
Eosin	16	15	31	32
Sperm HY-LITER™	15	16	31	32

Table 5 Numbers of detectable STR loci from 150 spermatozoa of modified H&E stained slides in duplicate experiments with various storage time.

Duration (month)	Numbers of detectable STR loci		total number of detectable STR loci	Expected number of detected STR Loci
	1st experiment	2nd experiment		
1	16	16	32	32
2	13	11	24	32
3	8	7	15	32
4	10	8	18	32
5	6	10	16	32
6	5	7	12	32
7	6	8	14	32
8	5	4	9	32
9	6	4	10	32
10	3	2	5	32
11	2	2	4	32
12	3	1	4	32

Table 6 The results of this current report were compared to previous studies.

Author	Slide preparation	LMD	DNA isolation	PCR	Results
T his report	- Standard glass slide - modified H&E staining; 75% ethanol 1 min HE 1 min Eosin 30 sec	- PALM microbeam LMD - 0.5 ml collection tube - 60µl of lysis buffer; 10 µL of Proteinase K 10 µL of 1mM DTT 40µL of ATL	- Qiagen QIAamp - Elute 30 µl	- AmpFlSTR® Identifiler 5 µl of DNA 5.25 µl of PCR reaction mix 2.75 µl of Primer mix 0.25 µl AmpliTaq Gold - 31 cycles	- 150 cells (16.0 ± 0.0) - 75 cells (15.0 ± 1.0) - 50 cells (14.0 ± 1.0) - 25 cells (11.5 ± 1.5)
Cai-xia Li, 2011	- PEN membrane slide - Hematoxylin; 75% ethanol 2 min HE 1 min	- PALM microbeam LMD - 0.2 ml collection tube - 4µl of lysis buffer; 0.1 mg/ml proteinase K 5 mM DTT	- 16,000 x g 5 min - incubate 56°C 40 min - boiled 10 min	- AmpFlSTR® Identifiler 3.4 µl of DNA 4.2 µl of PCR reaction mix 2.2 µl of Primer mix 1 U of AmpliTaq Gold - 28 cycles	- 50 cells (15.3 ± 1.2) - 40 cells (14.6 ± 1.3) - 30 cells (10.9 ± 2.8) - 20 cells (4.9 ± 2.8)
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Mado, 2009	- Standard glass slide - SPERM HY-LITER™ kit	-PALM microbeam LMD - 0.2 ml collection tube - 20µl of lysis buffer; 18 µl PicoPure DNA extraction buffer 2 µl of 1 M DTT	-PicoPure kit	- AmpFlSTR® Profiler Plus - 33 cycles	Full DNA profiles were recovered when 30 or more spermatozoa
Sander, 2006	- PEN membrane slide - H&E; HE 5 min Eosin 5 min	- Leica microsystems LMD - 0.2 ml collection tube - 20µl of TE buffer; 10 mM Tris-HCL 0.1 mM EDTA, pH8	- Qiagen QIAamp - Elute 20 - 25 µl	- AmpFlSTR® Profiler Plus - Standard method 28 cycles - Extended method 34 cycles	- Standard method 300 cells (100%) 150 cells (96 ± 3%) 75 cells (72 ± 12%) - Extended method 150 cells (100%) 75 cells (100%)

The current work focuses on the analysis of STR profiles from modified H&E stained sperm slides stored for varying lengths of time. These stained slides were stored at room temperature for a range of time periods from 1 to 12 months in duplicated experiments. The numbers of detectable STR loci from 150 sperm cells were shown in Table 5. There are variable storage times of stained sperm slides prior to obtain complete STR profiles reported as 72 hours, 10 weeks, 8 years and 32 years (Axler-DiPerte *et al*., 2011; Simons and Vintiner, 2011; Hara *et al*., 2013; Costa *et al*., 2017). We obtained complete DNA profiles from 1-month storage time of stained slides whereas 12-month storage time gave poor DNA profiles. Our current results were compared to previous studies (Table 6). Technical difficulty of laser microdissection included incomplete capture of target cells that caused by many factors: optimal laser settings, debris from vaginal swabs and semen stains, heat and chemical fixatives (Axler-DiPerte *et al*., 2011). For conclusion, optimal condition of sperm slide preparation for laser microdissection is a total number of 150 sperm cells stained with modified H&E. These slides can be stored for maximum of 1 month at room temperature prior to perform STR analysis that can obtain complete profiles for personal identification.

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