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Confirmatory detection of sperm and semen *Via* proximity ligation real-time PCR

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ABSTRACT

Semen and sperm identification play a critical role in forensic investigation for understanding the circumstances surrounding a crime and determining whether a sexual act occurred or not. Yet current methods for sperm detection vary widely in speed, sensitivity, and specificity. Also, the only undisputable confirmatory test for the presence of semen is the microscopic observation of spermatozoa. This process can be extremely time consuming and labor intensive, as each sample must be processed and examined individually. Particularly in samples with low levels or no spermatozoa, analysts may spend hours searching a slide. Also, failure to identify sperms by microscopic examination is not conclusive for their absence. Here, we demonstrate the development of a confirmatory method employing Proximity Ligation Real Time PCR (PLiRT-PCR) for the identification of semen and sperm from sexual assault evidence. PLiRT-PCR is designed to detect and quantitate the expression of protein markers through an antibody-protein binding reaction followed by qPCR. Two protein targets have been chosen as candidates for the assay: cysteine-rich secretor protein 2 (CRISP-2) and prostate-specific antigen (PSA). CRISP-2 is specifically expressed in the male reproductive tract and localized inside the acrosome of spermatozoa. The acrosomal location protects CRISP-2 from environmental damage until lysis, allowing for successful detection even on aged forensic samples. Also, its location on the surface of the acrosome makes it likely for epitopes to be accessible to antibody probes. PSA was selected as a second candidate due to its high concentration in semen and its useful role when processing samples from azoospermic or vasectomized perpetrators. This study discusses the identification, specificity, and the limit of detection of Crisp2 and PSA markers in samples containing body fluid mixtures. Data demonstrate the potential for PLiRT-PCR as a confirmatory test for semen. The approach allows high throughput semen and sperm detection to be processed in parallel on a 96 well plate, enabling analysts to make the most informed decisions on the best analytical path forward in each case, preserve valuable evidence, overcome the drawbacks associated with the microscopic observation of spermatozoa, and save analyst time.

1. Introduction

Due to the large number of sexual assault cases that are submitted to forensic labs, time consuming microscopic identification of semen significantly affects case backlog. Therefore, an alternative microscope-free detection method that is capable of processing multiple samples at a time with high sensitivity, and that utilizes instruments commonly present in forensic laboratories, could significantly affect the throughput of evidence processing. A potential candidate to address this issue is proximity ligation real-time PCR, or PLiRT-PCR, which is designed to detect and quantitate the expression of protein markers through an antibody-protein binding reaction followed by PCR. In fact the assay combines the specificity of an immunological reaction with the sensitivity of qPCR. PLiRT-PCR has been used as a tool for medical diagnostics and molecular biology studies for identifying down to zeptomole amounts of disease markers and different forms of other proteins [1–4].

2. Materials and methods

2.1. Sample preparation

To mimic sexual assault evidence that are usually submitted to forensic labs in form of swabs, different combination of body fluid mixtures were prepared. Fifty microliter of vaginal secretions, neat

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male saliva, and 1:50 male blood were spotted onto either cotton or aged semen swabs. The aged semen swabs were prepared by spiking cotton swabs with 50 μL of neat semen. Swabs were then air dried and preserved in envelopes four months prior to the experiment. The purpose of using aged semen swabs was to simulate delays between collection and processing of evidence. Swabs were processed by excising 1/5th of the tip of the no protein control (NPC) or body fluid swabs with razor blades and eluting the cut tips in 100 μL 1x PBS. Then, samples as well as NPCs were lysed in ice-cold lysis buffer combined with Protease and Phosphatase inhibitors to maintain epitope integrety. A lysis step was necessay to expose Crisp2 becuse it is located within the acrosome of spermatozoa.

2.2. Proximity ligation assay

To generate assay proximity probes (antibodies combined with oligonucleotides), biotinylated Crisp2 and PSA polyclonal antibodies were conjugated separately to 5′ and 3′ streptavidin-linked single-stranded prox-oligonucleotides (supplied in the TaqMan Protein Expression Assay kit from Life Technologies). Three main steps of the PLiRT-PCR process were performed on a 96 well plate: binding, ligation, and RT-PCR (or qPCR). To allow the binding of assay probes to their protein targets, equal volumes of the generated 5′ and 3′ probes were gently mixed, and 2 μL of the mixture was incubated with either 2 μL of NPCs (total of six reactions per each probe assay) or 2 μL of body fluid samples (total of two reactions per each sample). Samples were incubated at 37 °C for 60 min to allow proximity probes to bind to their targets. Using the TaqMan® Protein Assays Core Reagent Kit (Life Technologies) the ligation reaction was prepared according to the manufacturer's instructions [5], added into each well, and incubated at 37 °C for another 15 min. During the ligation step, the DNA ligase joined the oligo free ends that were bound into close proximity and formed an amplifiable product. The newly generated DNA ligated fragment was subsequently amplified, quantified, and detected by TaqMan® real-time PCR assay.

2.3. Data analysis

To analyze data and determine whether a sample is positive for the presence of the Crisp2 or PSA, the average and the standard deviation of the NPC C_t values were calculated. Then a C_t threshold was determined by subtracting three times the calculated NPC standard deviation from the average NPC C_t value. The amount of target protein was proportional to the ΔC_t, which was the difference between the NPC C_t threshold and the average sample C_t value. Samples with C_t values below the threshold were positive while samples with C_t values above or at the threshold were negative. This corresponds to a 99% confidence that samples below the threshold were a result of true amplification of ligated proximity products rather than random background noise [5].

3. Results and discussion

To assess the performance of the assay, we analyzed different body fluid concentrations and combinations against both CRISP-2 and PSA. Semen was present in the first six mixture samples and Sample 7 through 10 included either individual or mixture body fluids but lacked semen. PSA was detected in all samples that contained semen (Samples 1–6) and the assay gave a $\Delta C_t \geq 5$. CRISP-2 PLiRT-PCR assay gave positive ΔC_t with Samples 1–5 but the assay barely detected the presence of semen in Sample 6. This

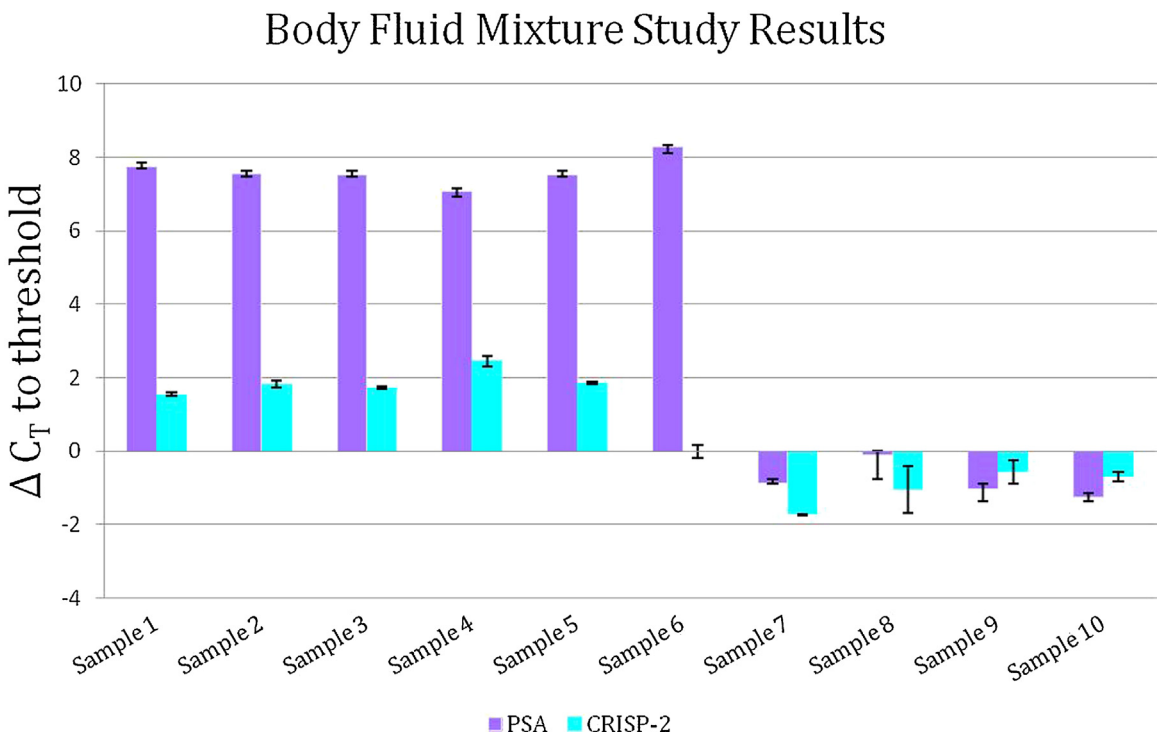

Fig. 1. Detection of PSA and Crisp2 in body fluid mixture mock samples. *Y* axis displays the ΔC_t, which was the difference between the NPC C_t threshold and the average sample C_t value; *X* axis display samples used in the study. Positive ΔC_t indicates positive detection of PSA and CRISP-2. Sample 1 (1:50 male blood + neat semen swab 4 months old); Sample 2 (neat male saliva + neat semen swab 4 months old); Sample 3 (vaginal secretion + neat semen swab 4 months old); Sample 4 (1:50 male blood + vaginal secretion + neat semen swab 4 months old); Sample 5 (neat saliva + vaginal secretion + neat semen swab 4 months old); Sample 6 (vaginal secretion + 1:50 male blood + neat saliva + neat semen swab 4 months old); Sample 7 (neat saliva + 1:50 male blood); Sample 8 (vaginal secretion + neat male saliva); Sample 9 (neat male saliva); Sample 10 (1:50 male blood).

demonstrates that PSA assay had higher sensitivity compared with the CRISP-2 assay. The result was not surprising considering each assay's limit of detection and, more importantly, the expected abundance of the particular analyte. PSA is present at much higher concentrations compared to sperm cells, thus providing more targets for proximity probes to bind to. Also, it might be possible that the combination of 4 different body fluids spotted on the swab from which Sample 6 originated had further diluted the sample, decreased the amounts of sperms, and diminished the limit of Crisp2 detection. However, regardless of the combinations of body fluids used, no false positive results were recorded with PSA and CRISP-2 probes in this experiment (Fig. 1 Samples 7–10), indicating that PLiRT-PCR assays had high specificity. Also, although 4-month old semen swabs were used to prepare Samples 1–6, as opposed to fresh semen, the performance of the assays was not affected, indicating that the relevant protein targets and epitopes were well-preserved.

4. Conclusion

Results show that PLiRT-PCR can specifically detect PSA (semen) and CRISP-2 (sperm cells) with the detection of PSA exhibiting higher sensitivity than that of CRISP-2. The necessary instrumentaition for the assay is already present in practically every forensic laboratory. Furthermore the small sample size, together with the use of the 96-well format, make it amenable to automation thus increasing sample throughput. These features make PLiRT-PCR an attractive candidate for the development of a robust and sensitive assay for the confirmatory detection of semen and sperm from sexual assault evidence.

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Conflict of interest

None.

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