- Final Summary Overview -

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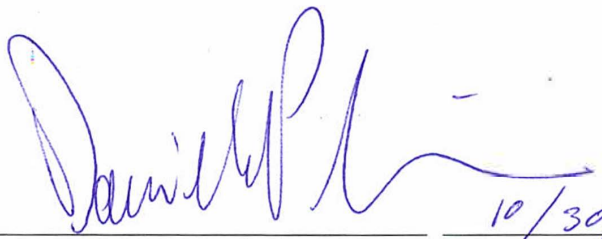
PI signature  10/30/2015

Daniele Podini date

Purpose

In sexual assault cases the identification of spermatozoa often plays a critical role in determining whether or not a sexual act occurred. In most crime labs the detection of semen begins with the seminal acid phosphatase presumptive test followed by the significantly more specific prostate specific antigen (PSA or p30) test [1,2]. The latter cannot be considered confirmatory for semen as PSA can be detected in other body fluids such as male and female urine, and breast milk [3].

The only undisputed method to confirm the presence of spermatozoa is to visualize sperm cells microscopically [1,3-5]. This is a time consuming task, as each sample must be processed and examined individually. Due to the large number of sexual assault cases that are submitted to forensic laboratories, the detection of semen significantly affects case backlog [3,6]. Automated fluorescence-based microscopic detection can decrease the time spent per sample, but still processes one sample at a time and requires expensive equipment [6].

Presence of semen can also be confirmed through the detection of sperm specific proteins. The proximity ligation real-time PCR, or PLiRT-PCR, designed for the detection of proteins, combines the specificity of an immunological reaction with the sensitivity of PCR and is used for medical diagnostics as well as molecular biology studies, such as, for example, the detection of growth factors [7-10]. The method has successfully detected proteins down to zeptomolar (10^{-21}) amounts [7,8].

In the assay preparation phase two sets of assay probes are created. This is achieved by separating polyclonal antibodies specific to the target protein into two groups and attaching DNA oligos with either a 3' free end or a 5' free end. The attachment takes

places using a biotin-streptavidin linkage; therefore biotin-conjugated antibodies must be used. Figure 1A shows a diagram of probe preparation.

Three main steps are involved in the PLiRT-PCR assay: binding, ligation, and RT-PCR (or qPCR) [7-10]. The first step is the binding reaction. In the binding reaction, the probes are added to the sample containing the target protein to which they will bind (Figure 2A). When two antibodies of opposite sets bind in proximity, the oligo free ends are brought close together. The next step is the ligation reaction; a connector oligo, complementary to the terminating sequences of the oligos, is added. The connector will hybridizes to the ends of both oligo strands stabilizing them so that they can then be ligated together by an added DNA ligase. The final step is to perform a TaqMan® assay to detect the newly generated DNA strand. The cycle threshold (C_t), is inversely proportional to the original amount of amplicon, which in turn is directly proportional to the amount of protein in the sample [7-10]. The PLiRT-PCR technique only requires a thermal cycler and a RT-PCR instrument, both commonly used in forensic labs.

The PLiRT-PCR method is sensitive, specific, uses technology commonly used by forensic practitioners, and is amenable to automation. The purpose of this study was to develop a microscope-free PLiRT-PCR based assay to identify and confirm the presence of semen from sexual assault evidence. Such a test has the potential to eliminate the time-consuming microscopic detection process, which will help reduce the backlog in processing sexual assault cases.

Design and methods.

Target. The first step in the development of the assay was the selection of sperm specific target protein. Several proteins were considered but the four most promising were SP-10, CRISP-2, PH-20, and SAMP32.

SP-10, or acrosomal vesicle protein 1 (ACRV1), is a testis specific, intra-acrosomal protein, the acrosomal location protects the protein from environmental elements, and its location on the surface of the acrosome makes it likely that epitopes will be accessible to the antibody probes [11]. CRISP-2 (also known as testicular protein TPX1) is an intra-acrosomal protein specific to the male reproductive tract and is likely to play a role in human gamete interaction. It is localized on the acrosomal membrane, specifically on the equatorial segment of the sperm head [12]. PH-20, also known as sperm adhesion molecule 1 (SPAM1), is a sperm specific plasma-membrane protein that has hyaluronidase activity crucial to the reproductive process [13]. SAMP32 is associated with the inner acrosomal membrane in the principal and the equatorial segments of the sperm acrosome [14]. The latter two failed to generate positive results.

PLiRT-PCR. The mechanics of PLiRT-PCR are summarized above, the kit used in this project is the TaqMan® Protein Assay commercialized by Life Technologies™(Foster City, CA).

Antibodies. Several antibodies were selected and tested for the four targets selected and are listed in table 1A.

Probe preparation. Polyclonal antibodies, when available, were purchased labeled with biotin, when not available biotinylation was performed. Antibody quantitation was performed since titer values provided by the manufacturers have been unreliable. Antibody/biotin ratio is important for a successful labeling reaction thus knowing the correct antibody concentration is paramount. Quantitation was performed using Qbit® 2.0 Fluorimeter (Life Technologies) following manufacturer recommendations. Several labeling approaches were evaluated in this project and are listed n table 1. The most efficient methods were FastLink and Mix-n-Stain. The

manufacturer protocol was followed when using FastLink. When labeling with Mix-n-Stain, the manufacturer's protocol was followed until the quenching step: rather than adding the biotinylated antibody into the quenching solution excess biotin was removed by spinning the sample through an ultrafiltration tube MWCO - 10k (also manufactured by Biotium) and washing it twice with 1xPBS. To collect the cleaned antibodies 1xPBS was added onto the membrane of the tube and then pipetted out. Once the antibodies were labeled, to evaluate the appropriate efficiency of the reaction, the forced proximity reaction was performed following the recommendations outlined by the manufacturer [15]. Biotinylated antibodies that passed the forced proximity test were then divided in two aliquots and separately labeled with 5' (Probe A) and 3' (Probe B) oligonucleotides respectively [15].

Sample preparation. The location of the target protein determines whether a cell lysis step is necessary. For example when targeting prostate specific antigen (PSA), which is secreted in the seminal fluid, sample lysis is not necessary but if the targets are acrosomal proteins, such as SP-10 or CRISP-2, lysis is necessary. The cell lysis buffer consisted of cell lysis reagent (Life Technologies™) and Calbiochem® protease inhibitor cocktail set 'I' and phosphatase inhibitor cocktail set 'II' from EMD Millipore (Billerica, MA), these are necessary to maintain antigen integrity. Each cocktail set is added to obtain a final concentration of 1x in the solution. Samples were mixed with cell lysis buffer in a 1:1 ratio and placed on ice for at least 10 minutes.

Binding, ligation, and amplification. After cell lysis, or if targeting non membrane proteins simply after sample elution, 2 µL of sample was mixed with 2 µL probe mixture containing both probes A and B and incubated one hour at 37°C for the binding reaction to occur. After incubation 96 µL of ligation reaction mix (90.8 µL water, 5 µL ligation

reaction buffer 20x, 0.2 µL 5x ligase) were added to the tube and incubated for 10 minutes at 37°C followed by 10 minutes at 4°C. Once the ligation reaction was completed, if not proceeding immediately to amplification, a protease step was necessary to prevent further ligation of the probes. Binding and ligation both were performed on a GeneAmp® PCR System 9700 from Applied Biosystems™. The TaqMan assay was then performed either on the SmartCycler® by Cepheid® (Sunnyvale, CA) or on the 7500 Real Time PCR system (Life Technologies). When using the former 11.25µL of ligated product to 13.75 µL of PCR mix (12.5 µL ExTaq 2x, 1.25 µL universal PCR assay 20x), when using the latter 10 µL ExTaq 2x, 1 µL universal PCR assay 20x, 5 µL water, and 4 µL ligated product, were used. The PCR mix was also changed from the premix used with the TaqMan® Protein Assays kit to Takara Premix Ex Taq from Clontech Laboratories, Inc. (Mountain View, CA).

Data analysis. To determine whether a sample is positive for the presence of the target protein the cycle threshold (C_t) value of the sample is compared to the average C_t value obtained from the non-protein controls (NPC). Background ligation will always occur even if the targeted antigen is not present due to freely diffusing probes hybridizing to the connecting oligo and being ligated. The average C_t value of the NPCs is calculated together with the standard deviation. The C_t threshold below which a sample is considered positive is then determined by subtracting form the average NPC C_t value three times the standard deviation (Figure 3A). This corresponds to a 99% confidence [16]. The amount of target protein is proportional to the ΔC_t, which is the difference between the average NPC C_t and the average sample C_t value (also samples are run in replica).

Probe dilution test. Prior to proceeding with the actual testing the ideal probe concentration must be determined. This is done empirically by testing a known positive with varying probe dilutions (1:2, 1:4, etc.) of the preparation concentration, to determine the highest ΔC_t value. Optimizing the probe concentration will maximize the overall sensitivity of the assay.

Testing. Several experiments were performed and significant efforts were invested in optimizing the process. The following describes the optimized assay for SP-10, CRISP-2 and a "Combo-assay". Neat semen was purchased from the Serological Research Institute (Richmond, CA).

SP-10 sensitivity. The best performing antibody for SP-10 was purchased from Protein Technologies (Tucson, AZ). Liquid semen dilutions were prepared using phosphate buffered saline PBS 1:10, 1:50, 1:100, 1:500, 1:1,000, 1:5,000, and 1:10,000. Each semen dilution and NPC were lysed as described above.

SP-10 Specificity - forensic-type samples on swabs. Four male body fluid samples were prepared on swabs including neat blood, saliva, urine, and a 1:100 dilution of semen, as well as a NPC swab. Each sample was prepared by pipetting 50 µL of the respective liquid on a cotton swab, and all swabs were dried in a hood. A small portion (about 1/4) of each swab was cut off and eluted in 50 µL of PBS. Then, elutions were lysed and run in triplicate.

Dilutions of semen on swabs. Two dilutions of semen were prepared on swabs, 1:1,000 and 1:5,000 along with a NPC swab. This was achieved by pipetting 50 µL of each liquid onto separate cotton swabs, and drying them in a hood. A small portion (about 1/4) of each swab was cut off and eluted in 50 µL of PBS.

Forensic-type mixture samples on swabs. Five samples were prepared: a cheek swab with 50 µL of 1:10 diluted semen, a cheek swab alone, a swab with 10 µL of blood with 50 µL of 1:10 diluted semen, a swab with 10 µL of blood alone, and a NPC (PBS) swab. The swabs were dried in a hood for three days. A small portion (about 1/4) of each swab was cut off and eluted in 50 µL of PBS.

CRISP-2 Sensitivity. The CRISP-2 biotinylated polyclonal antibody was acquired from R&D Systems® (Minneapolis, MN). Four liquid semen dilutions were prepared using PBS: 1:10, 1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000. Each test was run in triplicate.

CRISP-2 Specificity -Forensic-type samples on swabs. Four bodily fluid swabs were obtained: two semen swabs, a male saliva swab, and a vaginal swab. The semen swabs were prepared with a 1:10 dilution of semen pipetted on each. The first swab was stored for 24 months, and the second swab was stored for 9 months, both at room temperature in paper envelopes. The male saliva swab and the vaginal swabs were 24 months old. Samples were tested as described above.

Combo-assay. Neat male blood and semen were spotted on swabs, also a buccal and vaginal swabs were collected from donors. The tip of each swab was cut and eluted in 100 µL of PBS. Multiple dilutions of the eluted body fluids were then tested with probes prepared with antibodies against CRISP-2, PSA (R&D Systems), and Amylase I (LifeSpan Biosciences Inc).

Data analysis.

SP-10. Semen dilutions down to 1:5,000 were positive. Semen dilution on swabs 1:1,000 and 1:5,000 were also positive. In the specificity test the only sample that tested positive was the semen 1:100 swab and in the mixture study the only samples that tested positive

were the ones containing semen (Figure 1). Once the first polyclonal antibody for SP-10 was consumed results were not replicated with newly purchased polyclonal antibodies. This highlights the main concern with this technology i.e. the fact that different batches of polyclonal antibodies will have different affinity for the antigen. This issue will be discussed below.

CRISP-2. Semen dilutions down to 1:5000 tested positive while in the specificity test

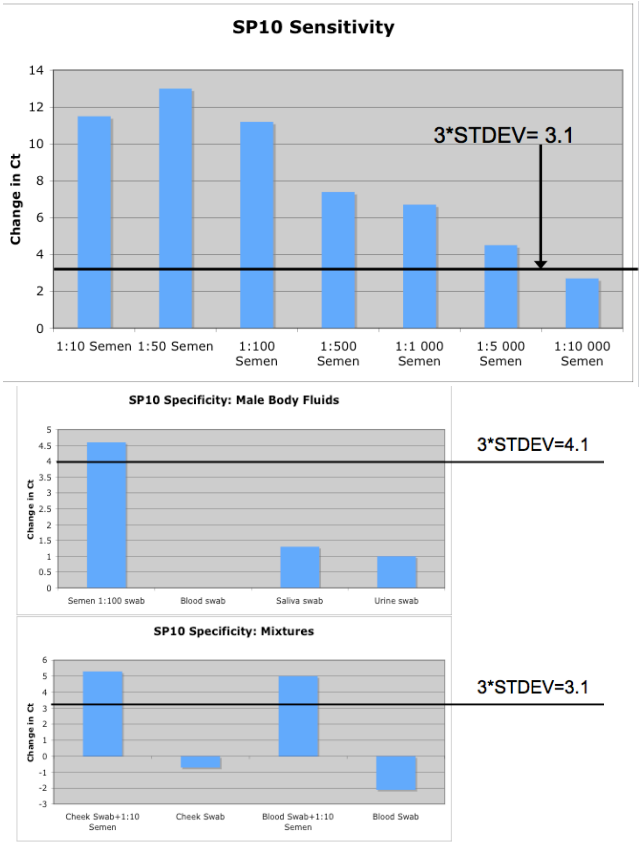


Figure 1. Graphical representation of PLiRT-PCR results. On the X axis are the different body fluid tested and on the Y axis is the ΔC_t value. The black line represents the threshold above which the sample can be considered positive.

only the samples containing semen were positive although the saliva sample generated a ΔC_t value close to the threshold (Figures 2). Further testing (data not shown) demonstrated a low level of cross-reactivity of the CRISP-2 assay with saliva possibly

due to the presence of CRISP-3 in saliva secretions given the similarities between the two proteins.

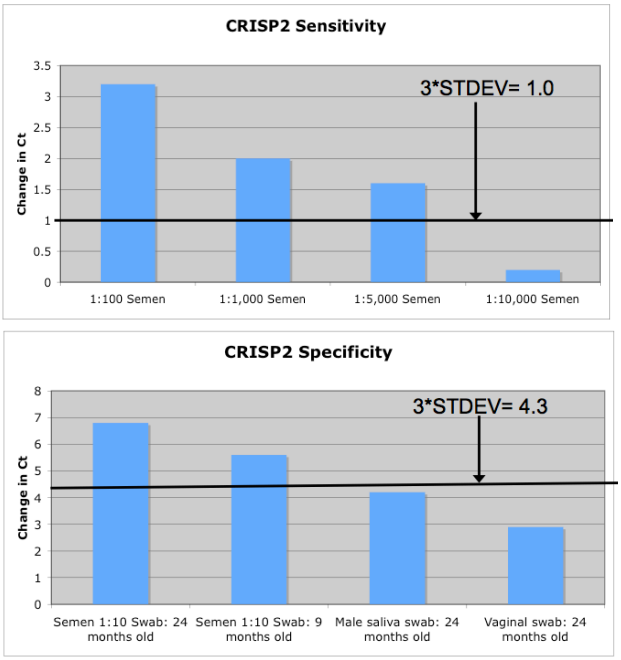


Figure 2. Graphical representation of PLiRT-PCR results. On the X axis are the different body fluid tested and on the Y axis is the ΔC_t value. The black line represents the threshold above which the sample can be considered positive.

Combo assay. All samples when testing the neat dilution of each body fluid gave positive results. This is probably a consequence of an excessive amount of protein in the ligation reaction that acts as a crowding agent increasing the amount of free probes binding to the connecting oligo and being ligated. However the highest ΔC_t value was always that of the correct body fluid. Further dilutions (1:100 and lower) of the eluted samples only tested positive with the expected probes.

Project findings

Results from this project demonstrate that the PLiRT-PCR method has the potential for high throughput confirmatory testing for the presence of spermatozoa and other body fluids contingently to the antibody quality. During this project significant

efforts were made with antibodies that never resulted effective. One of the issues was that often the antibody titer provided by the manufacturer was inconsistent with that measured in our laboratory. Antibody concentration is very important in the biotinylation step thus it is recommended that laboratories don't trust what reported by the manufacturer and measure in house the titer prior to the labeling step. Furthermore several antibodies were never able to detect the presence of the antigen. This could be a consequence of the fact that the PLiRT-PCR method is a relatively new approach for protein detection and manufactures tailor their product to more conventional methods such as ELISA, Wstern Blot, etc.

Another source of variability registered in some of the experiments was a significant difference in NPCs C_t values. This variability caused a high standard deviation, which results in increased ΔC_t thresholds above which a sample can be considered positive for the presence of the targeted protein. Increasing the amount of ligase in the ligation step, adding vortexing steps, and carful pipetting stabilizes the assay significantly reduced the variability between replica.

The ideal approach would be to develop well-characterized monoclonal antibodies that target separate specific antigens of the body fluid-specific protein and optimize the assay with a combination of these. In this manner the consistency of the antibodies is guaranteed by the fact that immortal cell lines are producing them. An assay with these antibodies can then be validated for forensic casework since it will be consistent in time.

The approach of testing multiple targets evaluated in the 'combo-assay' is also very promising. By testing multiple targets on the same sample and by generating a PLiRT-PCR based "serological profile", it is possible to confirm the body fluid type.

Further testing in this direction needs to be performed but results obtained thus far suggest the possibility of developing a multiplex assay simultaneously targeting different body fluid-specific proteins.

Results of this project also showed that the PLRT-PCR process is amenable to automation and sample batching resulting in high throughput. Furthermore the software used for 7500 Real Time PCR system data analysis has a tools where thresholds for presence/absence of the target can be set minimizing analysts' efforts for data interpretation (Figure 4A).

Implication for the criminal justice policy and practice

A manuscript detailing the experiments and results summarized in this report is being drafted and will be submitted to either FSI: Genetics or PLOS-ONE. The manuscript, if published, will demonstrate to the forensic science community that the PLiRT-PCR methodology has the potential for high throughput microscope-free confirmatory sperm detection.

Life Technologies provided a 7500 Real Time PCR system that was used during part of this project. Further efforts will be made to test older semen stains and more forensic like samples possibly collaborating with practitioners. It is the intention of the PI to seek further collaborations/funding for purchasing monoclonal antibodies for proteins specific to semen, saliva, and human blood and work on the development of a multiplex assay that would enable simultaneous confirmatory detection of the three body fluids from a single sample elution.

Results form this project have been presented at several forensic science meetings, listed in the appendix, and two manuscripts are in progress and will be submitted to FSI: Genetics and/or Journal of Forensic Sciences.

Appendix

References

1. Baechtel SF. The identification and individualization of semen stains. In: Saferstein R, editor. Forensic science handbook, Volume II. Englewood Cliffs: Prentice Hall, 1988; 347.
2. Hochmeister MN, Budlowe B, Rudin O, Gehrig C, Borer U, Thali M, et al. Evaluation of prostate-specific antigen (PSA) membrane antigen test assays for the forensic identification of seminal fluid. J Forensic Sci 1999; 44: 1057-60.
3. Old J, Schweers BA, Boonlayangoor PW, Fischer B, Miller KWP, Reich K. Developmental validation of RSID™ semen: A lateral flow immunochromatographic strip. J Forensic Sci 2012; 57(2): 489-99.
4. Noppinger K, Morrison R, Jones NH, Hopkins H. An evaluation of an enzymatic choline determination for the identification of semen in casework samples. J Forensic Sci 1987; 32(4): 1069-74.
5. Allery JP, Telmon N, Mieusset R, Blanc A, Rouge D. Cytological detection of spermatozoa: Comparison of three staining methods. J Forensic Sci 2001; 46(2): 349-51.
6. Christoffersen S. Immunohistochemical staining of human sperm cells in smears from sexual assault cases. Int J Legal Med 2011; 125: 887-90.
7. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gustafsdottir SM, et al. Protein detection using proximity-dependent DNA ligation assays. Nat Biotechnol 2002; 20: 473-

8. Gullberg M, Gustafsdottir SM, Schallmeiner E, Jarvius J, Bjarnegard M, Betsholtz C, et al. Cytokine detection by antibody-based proximity ligation. Proc Natl Acad Sci 2004; 101(22): 8420-4.
9. Gustafsdottir SM, Schallmeiner E, Fredriksson S, Gullberg M, Soderberg O, Jarvius J, et al. Proximity ligation assays for sensitive and specific protein analyses. Anal Biochem 2005; 345: 2-9.
10. Gustafsdottir SM, Nordengrahn A, Fredriksson S, Wallgren P, Rivera E, Schallmeiner E, et al. Detection of individual microbial pathogens by proximity ligation. Clin Chem 2006; 52(6): 1152-60.
11. Freemerman AJ, Wright RM, Flickinger CJ, Herr JC. Tissue Specificity of the Acrosomal Protein SP-10: A Contraceptive Vaccine Candidate Molecule. Biology of Reproduction 1994; 50: 615-621.
12. Busso D, Cohen DJ, Hayashi M, Kasahara M, Cuasnicu PS. Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. Molecular Human Reproduction 2005; 11(4): 299-305.
13. Sabeur K, Cherr GN, Yudin AI, Primakoff P, Li MW, Overstreet JW. The PH-20 Protein in Human Spermatozoa. Journal of Andrology 1997; 18(2): 151-158.
14. Hao Z1, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, Westbrook VA, Coonrod S, Flickinger CJ, Herr JC. SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. Biol Reprod. 2002 Mar;66(3):735-44.

15. Applied Biosystems. TaqMan® Protein Assays: Probe Development Protocol. Life Technologies Corporation 2010; 1-61.

16. Applied Biosystems. TaqMan® Protein Assays: Assay Chemistry Manual. Life Technologies Corporation 2010; 1-71.

Figures and Tables

Figures

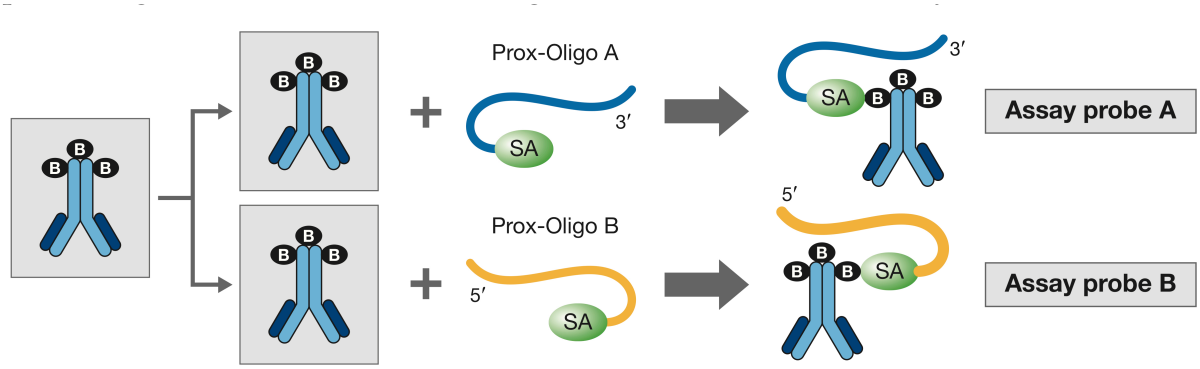


Figure 1A. Schematic of the preparation of assay probes, image from the Applied Biosystems. TaqMan® Protein Assays: Assay Chemistry Manual.

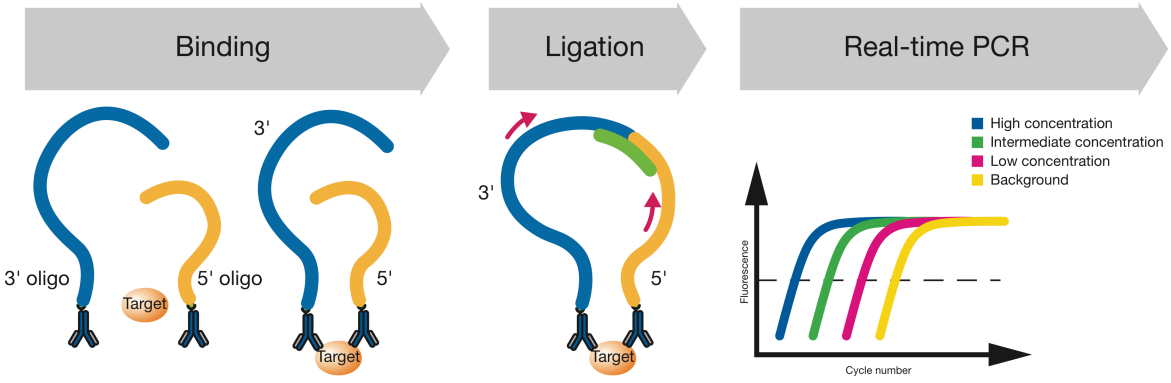


Figure 2A. Schematic of the PLiRT-PCR assay, including the three main steps: binding, ligation, and real-time PCR image from the Applied Biosystems. TaqMan® Protein Assays: Assay Chemistry Manual.

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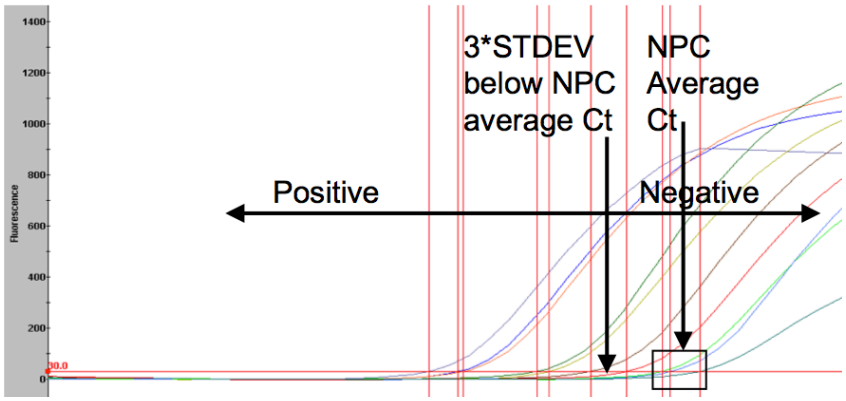

Figure 3A. Graphical representation of the process of determining whether a sample is positive for the presence of the antigen. Background ligation will always occur even if the targeted antigen is not present due to freely diffusing probes hybridizing to the connecting oligo and are ligated. The average Ct value of the NPCs is calculated together with the standard deviation, the Ct threshold below which a sample is considered positive is then determined by subtracting form the average NPC Ct value three times the standard deviation. This corresponds to a 99% confidence.

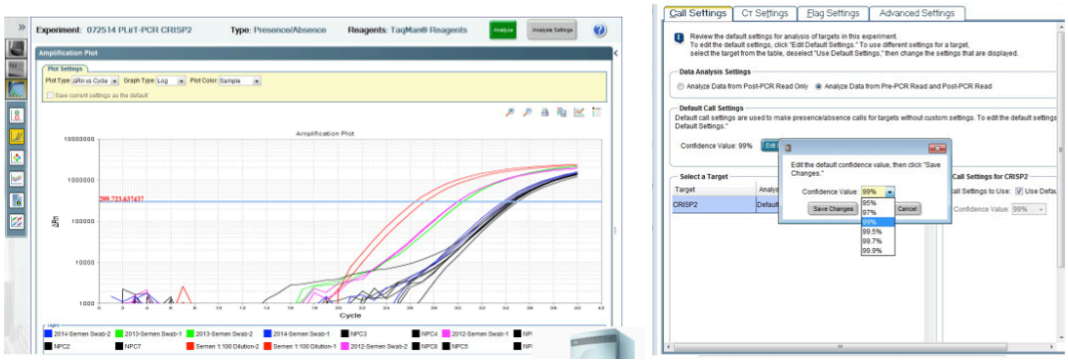

Figure 4A. Screenshots from 7500 Real Time PCR System PLiRT PCR assays, on the left CRISP-2 assay on different semen concentrations. The figure on the right shows a screenshot of the Presence/Absence threshold setting function.

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Tables

SP10 (ACRV1)		
Origin	**Target region**	**Antibody Type**
ProteinTech Group, Inc.	Whole	Rabbit polyclonal IgG
Abnova Inc.	Whole	Mouse polyclonal
Atlas Antibodies	Two separate epitopes	Rabbit polyclonal IgG
Fitzgerald Industries Intl.	Partial, N-terminus	Rabbit polyclonal IgG
BIOSS Inc.	Partial, C-terminus	Rabbit Polyclonal IgG
University of Virginia	Two separate epitopes	Mouse monoclonal IgG
CRISP-2		
Company Name	**Target region**	**Antibody Type**
R&D Systems	Whole	Goat polyclonal IgG
SAMP32 (SPACA1, SACA1)		
Company Name	**Target region**	**Antibody Type**
US Biological Inc.	Partial, 28a.a.	Rabbit polyclonal IgG
ProteinTech Group, Inc.	Whole	Rabbit polyclonal IgG
PH20 (SPAM1)		
Company Name	**Target region**	**Antibody Type**
Santa Cruz Biotech Inc.	Two separate epitopes	Goat/Rabbit polyclonal IgG
Alpha Amylase		
Company Name	**Target region**	**Antibody Type**
LifeSpan Biosciences Inc.	Whole	Rabbit polyclonal IgG
AssayPro	Whole	Rabbit polyclonal IgG
PSA		
Company Name	**Target region**	**Antibody Type**
Molecular Innovations Inc.	Whole	Sheep polyclonal IgG
R&D Systems	Whole	Goat polyclonal IgG
Biotinylation Kits		
Life Technologies Inc.	Biotin-XX Microscale Protein Labeling Kit	
Life Technologies Inc.	APEX^{TM} Biotin-XX Labeling Kit	
Thermo Scientific Inc.	EZ-Link® Sulfo-NHS-LC-Biotinylation Kit	
Abnova Inc.	FastLink Biotin Labeling Kit	
Biotium Inc.	Mix-n-Stain Biotin Labeling Kit	

Table 1A. List of antibodies and biotin labeling kit evaluated during the course of this project.

Project dissemination

Scholarly Talks

- S. Riman,. D. Podini, Streamlining Sperm Cell Detection Via Proximity Ligation Real-Time PCR with Forensic DNA Analysis. Accepted for oral presentation and the American Academy of Forensic Sciences 2016 annual meeting, Las Vegas NV, February 2016.

- D. Podini, PLiRT PCR- Real Time assay for detecting Spermatozoa- Investigation of Cold Cases through DNA, Annual DNA & Investigators Workshop – Bode Mid-Atlantic November 2014, Arlington VA.

- D. Podini, Proximity Ligation Real Time PCR (PLiRT PCR) for the detection of spermatozoa. Green Mountain DNA Conference, Burlington, VT, July 2014.

- D. Podini, Detecting Spermatozoa using Proximity Ligation Real Time PCR). 13th Annual DNA Technical Workshop – Bode East, Orlando, FD, May 2014.

- J. Benjamin, L. Prugh, C. Ayoub, and D. Podini, Detecting Spermatozoa using Proximity Ligation Real Time PCR (PLiRT-PCR). American Academy of Forensic Science annual meeting in Seattle, WA, February 2014.

Poster presentations

- S. Riman, C.H. Shek, and D. Podini. Semen and sperm cell detection via proximity ligation real-time PCR. 25th International Symposium on Human Identification, Grapevine TX, October 2015.

- S. Riman, C. H. Shek, V. Clermont Beaudoin, M. Peck, J. Benjamin, C. Ayoub, L. Prugh, and D. Podini. Confirmatory Detection of Sperm and Semen Via Proximity Ligation Real-Time PCR. International Society of Forensic Genetics annual meeting, Krakow, Poland September 2015.

- CH. Shek, V.Clermont-Beaudoin, J. Benjamin, D. Podini, High throughput Spermatozoa Detection using the Proximity Ligation Real Time PCR (PLiRT-PCR) Method. American Academy of Forensic Sciences annual meeting, Orlando FL, February 2015.