DEVELOPMENTAL VALIDATION OF
SPERM HY-LITER™ *EXPRESS*

Jennifer Old, Chris Martersteck, Anna Kalinina,
Independent Forensics, Lombard IL

Background and Introduction

SPERM HY-LITER™ is the first immunofluorescent detection kit for the screening and/or identification of sperm from sexual assault evidence. The method has proved robust, specific and reliable. Like all immunofluorescent staining protocols, the method requires several sequential incubation steps; the length of these incubation steps (currently 30 minutes each, total staining time ~100 minutes) allows an analyst to process numerous slides in 'batch' mode and still have time to multitask before the next step in the procedure. Despite this advantage, we have received numerous requests for a version of **SPERM HY-LITER™** with reduced staining time; **SPERM HY-LITER™** ***EXPRESS*** has been developed in response to these comments.

The basic steps, methods and reagents used in **SPERM HY-LITER™** ***EXPRESS*** are identical to the staining protocol, methods and reagents used in **SPERM HY-LITER™** except that the incubation times have been cut in half such that slides may be stained, mounted and visualized in just one hour, start to finish. Staining may still be performed in batch mode; however the analyst will likely be working at the bench more or less continuously, adding solution, rinsing with wash buffer and preparing for the next addition of staining solution as incubation times are now just 15 minutes.

Analysts may observe even stronger sperm head staining with **SPERM HY-LITER™** ***EXPRESS*** than with **SPERM HY-LITER™** as the sperm-head specific monoclonal antibody that provides the specificity to this method has been labeled with an even more intense fluorophore than the original Alexa488. All other solutions in the kit are essentially identical: we have been able to keep the same shelf life (6 months) and the same staining protocol as the original **SPERM HY-LITER™,** but have been able to reduce incubation times (and thus total staining time) with no increase in background fluorescence, no change in the specificity of the method, and with increased signal to noise, *i.e.,* better staining.

Validation Requirements

Current forensic laboratory audit guidelines require that DNA laboratories demonstrate that protocols, kits and reagents perform to laboratory standards; so called critical reagents undergo even more stringent scrutiny. As the identical specificity

reagent (*i.e.,* the sperm head monoclonal antibody) is used in both **SPERM HY-LITER™ *EXPRESS*** and **SPERM HY-LITER™**, laboratories may convert to the new kit after performing an equivalence study demonstrating that **SPERM HY-LITER™ *EXPRESS*** has the same sensitivity as **SPERM HY-LITER™**. This can usually be accomplished by staining a defined series of slides containing different ratios of epithelial and sperm cells.

Laboratories may use their existing **SPERM HY-LITER™** SOP after making a single edit: dividing all incubation times in half (*i.e.*, change 10 minutes to 5 minutes for Step 1 and change 30 minutes to 15 minutes for Steps 2, 3 and 4). Storage conditions, QA/QC protocols, specificity, sensitivity, shelf life, MSDS information will all be identical for the new kit. Independent Forensics will continue to manufacture and support the original **SPERM HY-LITER™** kit for laboratories that prefer the multitasking option. ***SPERM STAINER™*** our soon to be released automated robotic slide stainer for **SPERM HY-LITER™**, will of course incorporate either protocol.

Here we detail the developmental validation of **SPERM HY-LITER™ *EXPRESS*** a new version of a previously released immunofluorescent staining kit for human sperm from sexual assault evidence. This new kit has improved staining signal and decreased overall staining time.

SPERM HY-LITER™ has been extensively studied for specificity (see the **SPERM HY-LITER™** developmental validation). The antibody in **SPERM HY-LITER™** is absolutely specific for human sperm heads and does not cross-react with other human body fluids. **SPERM HY-LITER™ *EXPRESS*** has an improved fluorophore to achieve brighter sperm head staining with shorter incubation times. Analysts may find that **SPERM HY-LITER™ *EXPRESS*** will identify more sperm from more challenging samples due to the increased signal.

The sperm head-staining antibody used in **SPERM HY-LITER™ *EXPRESS*** is identical to the antibody used in **SPERM HY-LITER™**, however the **SPERM HY-LITER™ *EXPRESS*** antibody has been covalently bound to a different fluorophore, CF488. Additional specificity studies were not performed as only a minor modification of the original protocol was required to validate this new version of the kit; all other reagents used in the protocol are unchanged.

Methods

Buccal/semen cells and post-coital extracts were deposited onto IFI 2 x 11 mm microscope slides; these slides have been pre-cleaned to remove machining oil residue from the manufacturing of the slide, coated with poly-L-lysine to promote better

adherence of biological materials, and hydrophobic masked to make two eleven millimeter 'wells' on the slide. Unless noted, all slides were prepared as follows: epithelial cells from buccal swabs were extracted in 1 mL PBS for 1 hour at room temperature. Cells were harvested by centrifugation at 13,000 RPM for 5 min., resuspended in 100 µL PBS, and 10 µL of cell suspension was deposited into slide well 1.

Sperm cells extracted from semen swabs, were extracted in 1 mL of PBS for at least 1 hour. Sperm cells were harvested by centrifugation at 13,000 RPM for 5 min., resuspended in 200 µL of PBS; and 2 µL of sperm cell suspension was mixed with buccal cell extract in slide area 1. Post-coital swabs were also extracted in PBS for at least 1 hour at room temperature. Cells were harvested by centrifugation at 13,000 RPM, resuspended in 100 µL of PBS, and 10 µL of this cell suspension was plated into slide area 2. Samples on fabric and challenge samples were prepared as described in the appropriate section. 1 M DTT was used at the '10X concentration' (5 µL of 1 M DTT solution per drop of Sample Preparation buffer) or at '15X concentration' (7.5 µL of 1 M DTT solution per drop of Sample Preparation buffer).

All slides were prepared in duplicate and stained with **SPERM HY-LITER™ EXPRESS** as per the manufacturer's instructions. CF488 labeled antibody was diluted in **SPERM HY-LITER™** antibody buffer to the indicated concentration. Incubation times for **SPERM HY-LITER™** were as follows: 10 minutes for fixation, and 30 minutes each for sample preparation, blocking, and sperm head staining. The total measured time for the **SPERM HY-LITER™** protocol, including washing steps, is 2 hours and 10 minutes. The **SPERM HY-LITER™** ***EXPRESS*** protocol cuts each incubation time in half: the overall measured staining time is 1 hour and 5 minutes.

Slides were examined on a properly configured fluorescent microscope using a 20X objective, and DAPI and FITC filters. Fluorescence intensity was graded from 0 – 5+, with 0 indicating no staining, and 5+ indicating the brightest staining intensity. Images were captured with a Canon PowerShot A640 camera a C-mount adapter. Shutter speeds were 1/10 sec except for DAPI which used a 1/100 shutter speed. Reported experimental results reflect the outcomes of several identical experiments.

Results

The question(s) addressed, a summary of the results, and the conclusion(s) reached are included for each experiment performed.

1. Comparison of **SPERM HY-LITER™** *antibody staining to* **SPERM HY-LITER™** ***EXPRESS*** *antibody staining (i.e., Alexa488 vs. CF488 – labeled antibody).*

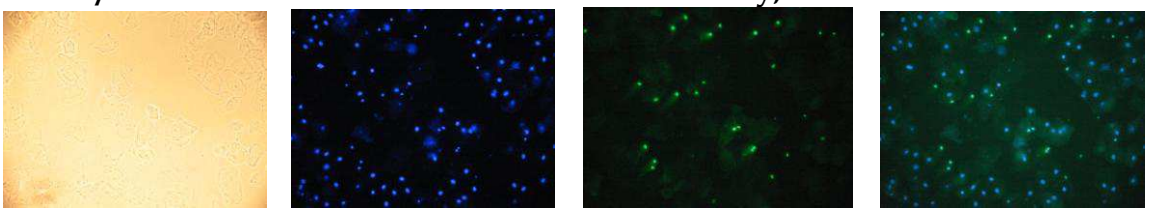
The purpose of this experiment was to compare CF488 antibody staining to Alexa488 antibody staining at either 15 min. or 30 min. incubation times. All other staining variables were unchanged for tested slides. Preliminary testing had indicated that a 15 min. incubation time and 15X DTT Sample Preparation buffer was comparable to the 30 min Alexa488 positive control result. Here we tested various concentrations of CF488 labeled antibody.

Slides Tested

Type	Sample Prep.	Blocking	Antibody
1. Negative Control	15 min (15X DTT)	15 min	15 min
2. Positive Control	15 min (15X DTT)	15 min	30 min
3. Test	15 min (15X DTT)	15 min	15 min (Conc 1X)
4. Test	15 min (15X DTT)	15 min	30 min (Conc 1X)
5. Test	15 min (15X DTT)	15 min	15 (Conc 2X)
6. Test	15 min (15X DTT)	15 min	30 (Conc 2X)

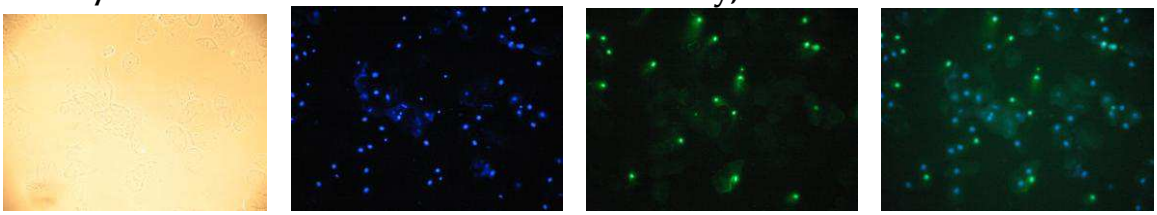
Results: The CF488 antibody staining was more intense (5-6+) than Alexa488 antibody staining for both the 1X and 2X Concentrations. There was no visible difference between CF488 stained slides that were incubated for 15 minutes or 30 minutes.

Buccal/Semen Cells Stained with Alexa488 Antibody, 30 min.



Phase DAPI FITC DAPI/FITC

Buccal/Semen Cells Stained with CF488 Antibody, 15 min.



Phase DAPI FITC DAPI/FITC

Conclusions: CF488 antibody had more intense staining than Alexa488 antibody at either incubation times (15 min. or 30 min.). CF488 antibody incubated for 15 minutes

provided as intense or better staining than the Alexa488 antibody incubated for 30 minutes.

2. How does CF488 antibody staining at 1X Concentration compare to Alexa488 staining?
The purpose of this experiment was to determine if CF488 staining at 1X Concentration for 15 minutes would be comparable to Alexa488 staining for 30 minutes.

Slides Tested

Type	Sample Prep.	Blocking	Antibody
1. Negative Control	15 min (15X DTT)	15 min	15 min (Alexa488)
2. Positive Control	15 min (15X DTT)	15 min	30 min (Alexa 488)
3. Test	15 min (15X DTT)	15 min	15 min(1X Conc. CF488)
4. Test	15 min (1X DTT)	15 min	30 min (1X Conc CF488)

Results: The positive control slide sperm staining intensity was 4+. Sperm staining intensity for slide 3 was also 4+. Slide 4 also had 4+ sperm staining intensity, however slightly increased background was observed in the buccal/semen cells for this slide.
Conclusions: The 15 minute incubation with CF488 is preferable to a 30 minute incubation. CF488 sperm staining at 1X Concentration was as intense as Alexa488 staining.

3. How do slides treated with 10X 1M DTT compare to those treated with 15X 1M DTT?
All slides were stained with CF488 at 1X Concentration for 15 minutes. Only the DTT concentration in the sample preparation buffer was tested. The **SPERM HY-LITER™** protocol uses 10X DTT in the Sample Preparation buffer with an incubation time of 30 minutes. Here we test if 10X DTT Sample Preparation buffer will provide sufficient staining with **SPERM HY-LITER™ *EXPRESS*** .

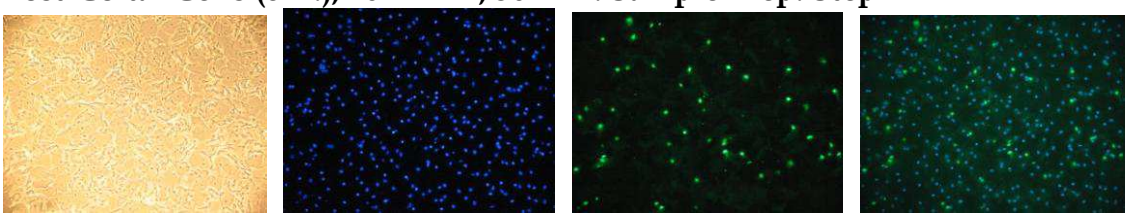
Slides Tested

Slide #	Sample Prep.	Blocking	Antibody (1X Conc CF488)
1	30 min (10X DTT)	15 min	15 min
2	15 min (10X DTT)	15 min	15 min
3	15 min (15X DTT)	15 min	15 min

Results: Sperm head staining with **SPERM HY-LITER™ *EXPRESS*** and 15 minute incubation time and 10X DTT Sample Preparation buffer was identical to the staining observed with 15X DTT for 15 minutes and essentially similar to the staining observed with 10X

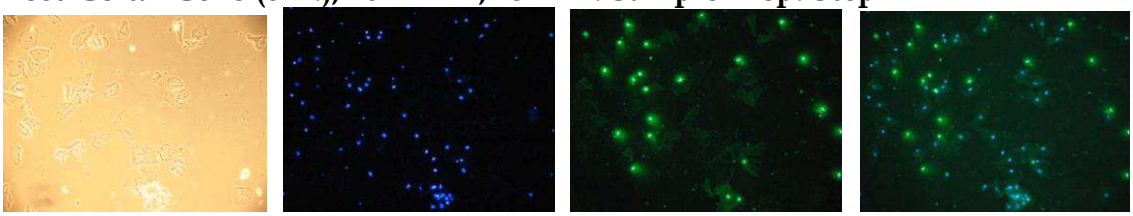
DTT for 30 minutes. Sperm head staining intensity was recorded as 4-5+, and more 5+ cells were observed in post-coital cell preparations.

Post-Coital Cells (8hr.), 10X DTT, 30 min. Sample Prep. Step



Phase DAPI FITC DAPI/FITC

Post-Coital Cells (8hr.), 10X DTT, 15 min. Sample Prep. Step



Phase DAPI FITC DAPI/FITC

Conclusions: The **SPERM HY-LITER™ *EXPRESS*** protocol can use 10X 1M DTT in the sample preparation step for 15 minutes with no loss of signal.

4. What is the quality of staining with 1X DTT in the sample preparation buffer, using the ***SPERM HY-LITER™ EXPRESS*** *protocol?*

All slides were stained with CF488 at 1X Concentration for 15 minutes. Only the DTT concentration in the sample preparation buffer was reduced to 1X DTT concentration. 'Heavy' and 'light' buccal/semen cell and post-coital cell sample concentrations were compared. Heavy cell concentrations were used with 10X DTT as a positive control.

Results: Sperm heads were not properly prepared with the 1X DTT, such that sperm head staining was not optimal (3-4+). Incomplete and 'ring' staining was observed with the 1X DTT concentration and the 15 min incubation. Sperm head staining was identical in the heavy and light cell preparations. Slides treated with 10X DTT exhibited brighter sperm head staining when examined with both the FITC and DAPI/FITC filters.

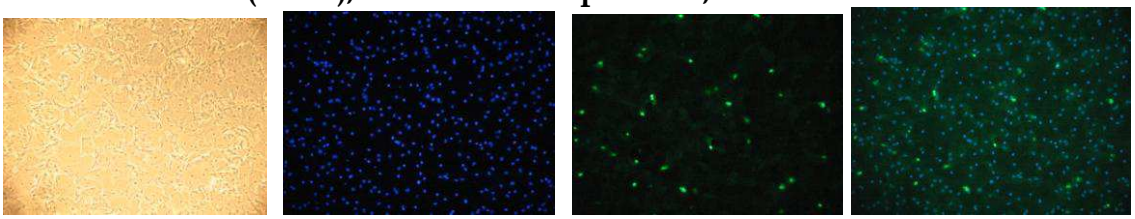
Conclusions: 1X DTT is not recommended for the **SPERM HY-LITER™ *EXPRESS*** protocol. 1M DTT should be used at 10X concentrations (5 µL per drop of sample preparation buffer).

5. What is the quality of staining with CF488 antibody at 1.5X Concentration and 1X Concentration and 0.5X Concentraion for both heavy and light cell preparations? Are sperm heads underneath epithelial cells readily identified?

The **SPERM HY-LITER™ *EXPRESS*** protocol was followed, with 10X 1M DTT and the two different concentrations of CF488 antibody. Heavy buccal/semen and post-coital cell preparations were made such that 90-100% of the slide was covered by cells while light cell preparations were made such that only 40-60% of the slide was covered by cells.

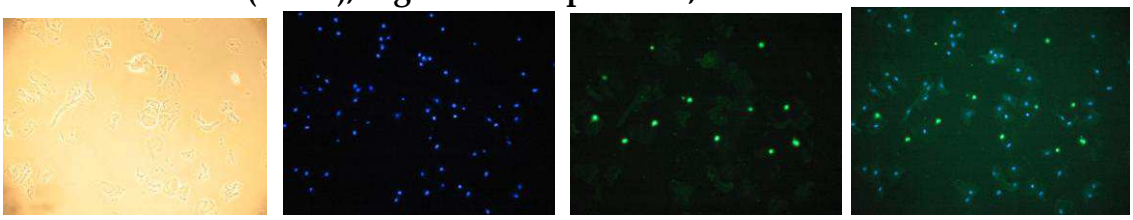
Results: Sperm head staining with CF488 antibody at 1.5X Concentration were the brightest (5+), followed by antibody at 1X Concentration (staining intensity judged as 4-5+) and followed by 0.5X with staining intensity at 3-4+. Sperm heads under epithelial cells were identified at 1.5X Concentration and 1X Concentration and though visible at 0.5X, this concentration was not judged sufficient as sperm cell under epithelial cells exhibited staining at the periphery of the sperm head (*i.e.,* ring staining)

Post-coital Cells (10 hr.), Dense Cell Preparation, CF488 1X Concentration



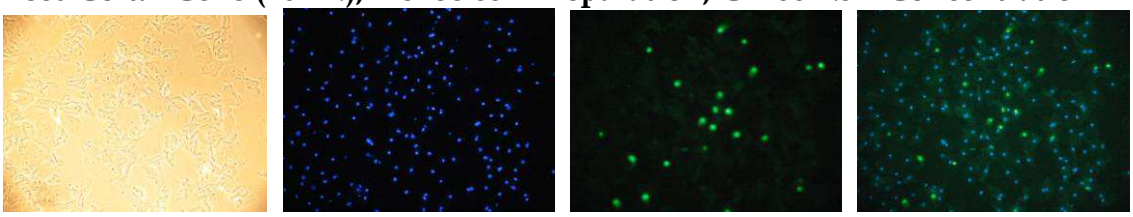
Phase DAPI FITC DAPI/FITC

Post-coital Cells (10 hr.), Light Cell Preparation, CF488 1X Concentration



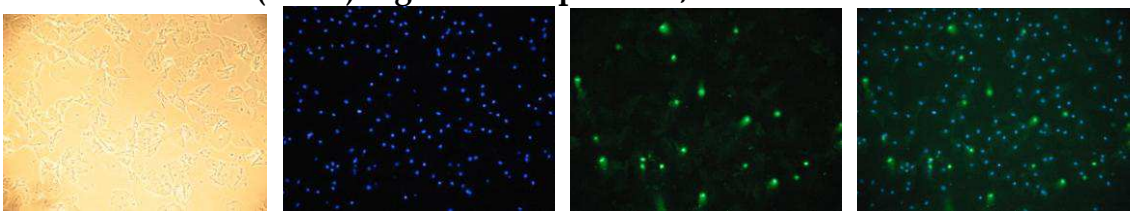
Phase DAPI FITC DAPI/FITC

Post-Coital Cells (10 hr.), Dense cell Preparation, CF488 1.5X Concentration



Phase DAPI FITC DAPI/FITC

Post-Coital Cells (10 hr.) Light cell Preparation, CF488 1.5X Concentration



Phase DAPI FITC DAPI/FITC

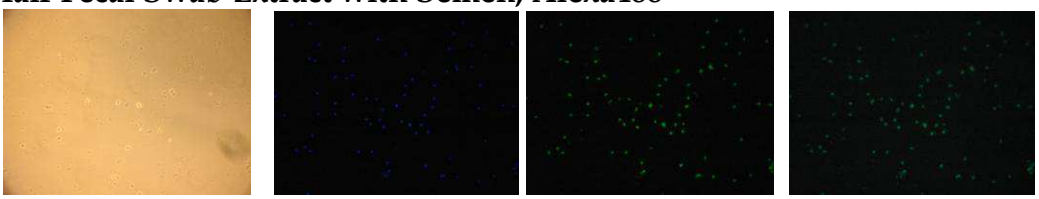
Conclusions: CF488 antibody at 1X and 1.5X Concentrations was effective for visualization of sperm heads next to and below epithelial cells. CF488 at 1.5X demonstrated slightly higher fluorescence intensity, and is recommended for kit formulation.

6. How does **SPERM HY-LITER™ *EXPRESS*** *perform on challenging, forensic-type samples?*
Neat semen in 50 µl aliquots was deposited on different fabrics, with or without a mixture of other body fluids including saliva, urine, and blood. The stains were extracted in 200 µL of 1X PBS, and cells were pelleted by centrifugation at 13,000 rpm for 5 minutes, resuspended in 40 µL and plated onto IFI 2-position slides.
Additional challenge samples were prepared by centrifuging 4 day old menstrual blood. This 30 µL pellet was resuspended in 50 µL of 1x PBS, and 10 µL of the menstrual blood suspension was mixed with 2 µL of neat semen, and the mixture was then plated onto IFI 2-position slides.
Fecal and semen mixture samples were prepared by extracting a whole or half cotton swab tip containing fecal matter. Extracts were centrifuged as above, 2 µl of semen suspension was added to the extract, and the mixture was plated onto IFI 2-position slides.
Control slides contained buccal and semen cells prepared as described (see Methods). All slides were prepared in duplicate. One slide was stained with the standard **SPERM HY-LITER™** kit while the other slide was stained with **SPERM HY-LITER™ *EXPRESS***.
Results:
Staining with **SPERM HY-LITER™ *EXPRESS*** resulted in slightly higher immunofluorescence intensity, compared to Alexa488 staining. Immunofluorescence intensity was graded from 1-5+. Isolated sperm and sperm in samples with heavy debris were more readily observed using CF488. Staining intensity was higher in samples with less fecal debris.

Table of Samples Tested

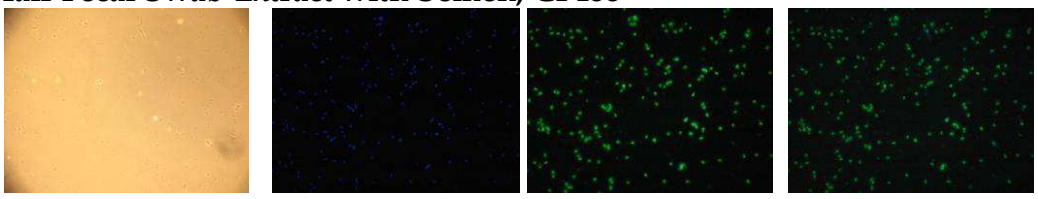
Sample Type	Substrate	Alexa488	CF488 (Express)
Buccal/Semen (Control)	Cotton swab	4+ sperm	4-5+ sperm
Semen, saliva	White cotton	4+ sperm	4-5+ sperm
Semen	Black jeans	3+ sperm	3-4+ sperm
Semen	Blue jeans	3-4+ sperm	4+ sperm
Semen	Lace	4+ sperm	4-5+ sperm
Semen	Blue t-shirt	4+ sperm	4-5+ sperm
Semen	White cotton	2-3+ sperm	3+ sperm
Semen, urine, saliva, blood	White cotton	1-2+ sperm, only 1 sperm found	2+ sperm, 10-20 sperm found
Semen, urine, saliva, blood	Black jeans	1-2+ sperm, heavy debris	2-3+ sperm, heavy debris
Semen, urine, saliva, blood	Blue t-shirt	2+ sperm, only 1 sperm found	2-3+ sperm, 10-20 sperm found
Menstrual blood, semen	Cotton swab	2+ sperm, heavy epithelial cells	2+ sperm also visible under epi cells
Fecal, semen	Whole cotton swab	1+ sperm, heavy debris	2-3+ sperm, heavy debris
Fecal, semen	Half cotton swab	2+ sperm, low debris	3+ sperm, low debris

Half Fecal Swab Extract with Semen, Alexa488



Phase DAPI FITC DAPI/FITC

Half Fecal Swab Extract with Semen, CF488



Phase DAPI FITC DAPI/FITC

Conclusions: CF488 antibody and the **SPERM HY-LITER™ *EXPRESS*** is effective for visualization of sperm extracted from fabrics and sperm mixed with other body fluids such as urine, saliva, blood, menstrual blood, and fecal debris.

Summary

SPERM HY-LITER™ *EXPRESS* works as expected for sperm head staining from mock sexual assault samples. The above experiments describe the appropriate concentrations of antibody (1.5 X Concentration), DTT (10X) and incubation times (15 min) required to obtain optimal staining of sperm.

The CF488 fluorophore is more intense than the Alexa488 fluorophore and thus **SPERM HY-LITER™ *EXPRESS*** can achieve high quality staining with better staining intensity and shorter incubation times.

Using **SPERM HY-LITER™ *EXPRESS*** it is now possible to detect more sperm under microscopic examination in shorter times. Given the increased intensity of **SPERM HY-LITER™ *EXPRESS*** debris filled microscope fields may be easy to scan at lower magnification.

Laboratories wishing to convert to **SPERM HY-LITER™ *EXPRESS*** can do so with confidence: an equivalence study and a minor modification of existing SOPs and protocols are all that is required to implement this new version of the kit.

SPERM HY-LITER™ *EXPRESS* stained slides can only be visualized with a properly configured fluorescence microscope. All Independent Forensics' **SPERM HY-LITER™** microscope systems including the newest **EZ Viewers** are fully compatible and optimized for this application

Current **SPERM HY-LITER™** users will not require any additional hardware or software to use **SPERM HY-LITER™ *EXPRESS***.

CF488 Dye Characteristics: Excitation Max: 490nm; Emission Max: 515nm

For additional details please contact Ms Dina Mattest at Dina@ifi-test.com